

EFFECTS OF RHCC10 ON THE  
PRO/ANTI-INFLAMMATORY PROFILE  
OF THE IMMATURE LUNG

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A Dissertation

Submitted To

The Temple University Graduate Board

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In Partial Fulfillment Of  
The Requirements For The Degree of  
DOCTOR OF PHILOSOPHY

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By

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January, 2014

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## ABSTRACT

There is a gap in the treatment of preterm infants with respiratory distress syndrome. Despite addressing surfactant insufficiency and mechanical instability, currently available exogenous surfactant does not reduce the inflammation that results from aggressive ventilation and hyperoxia. Preterm infants are also deficient in anti-oxidant and anti-inflammatory defenses. All of these factors predispose the infant to bronchopulmonary dysplasia. Recombinant human Club Cell Secretory Protein 10 (rhCC10), known to inhibit sPLA2, has been used to reduce ventilator induced lung inflammation. The long-term goal of this study is to understand the impact of rhCC10 on the pro/anti-inflammatory balance during early development. We will interrogate the link between Toll-Like Receptors (TLR 4) and rhCC10 because TLRs are integral to lung inflammation. By measuring the mRNA expression, protein, and downstream signaling activity in rhCC10 treated preterm lamb lung and then in A549 cells, an alveolar epithelial cell-like system, the anti-inflammatory effect of rhCC10 will be differentiated. This research is significant because it will improve understanding of the effect of rhCC10 on pro/anti-inflammatory regulation and provide insight regarding potential co-therapies when treating with rhCC10.

## ACKNOWLEDGEMENTS

Marla R. Wolfson, MS, PhD: Thank you for the continual guidance, mentorship and education. The challenges you presented will only make me stronger.

Satoru Eguchi, MD: Thank you to you and your team, especially Keita Kimura. Our initial collaboration was the starting point of this project for me.

Madesh Muniswamy: Thank you to you and your team, especially Karthik Mallilankaraman. Without your team's assistance and teaching, I would not have been able to perform the cell culture work.

Michael Autieri, PhD: You were always helpful in pointing me in the right direction: Be it a technique/methods question, helpful questions during committee meetings, or some of the best lectures that I have attended. Thank you.

Stefania Gallucci, MD: You too were helpful in point me in the right direction and multiple times during a meeting or conference came to my defense. Thank you.

Aprile Pilon, PhD: Thank you for participating in my graduate project. You went above and beyond in helping me explain intricate issues in the dissertation, providing much needed reagents, and tackling paperwork issues.

Dr. Shaffer, MSE, PhD: Thank you for the numerous contributions you have made to this project and my graduate education. You always bring a unique and helpful perspective that helps solve whatever problem is at hand.

I could also not have done this project without the assistance of others. I want to recognize my fellow labmates and coworkers who provided much needed assistance throughout the project: Jichuan Wu, Sandy Baker, Mariola Marcinkiewicz, and Philip Berger. Thanks guys.

## **DEDICATION**

This work is dedicated to my patient wife, Alanna, for her loving support and to my daughter, Maeve, who helped provide the final push to finish.

**TABLE OF CONTENTS**

ABSTRACT .....	i
ACKNOWLEDGEMENTS .....	ii
DEDICATION .....	iv
LIST OF FIGURES .....	vi
INTRODUCTION.....	ix
CHAPTER 1: IMPACT OF RHCC10 ON ENDOGENOUS CC10 IN THE PRETERM LUNG ....	1
Introduction .....	1
Methods .....	3
Results .....	7
Discussion .....	21
CHAPTER 2: DOSE RESPONSE EFFECTS OF RHCC10 ON TLR 4 SIGNALING AND PRODUCTION .....	26
Introduction .....	26
Methods .....	28
Results .....	30
Discussion .....	45
CHAPTER 3: RHCC10 INDUCED MITIGATION OF TOLL-LIKE RECEPTOR INJURY IN AN ALVEOLAR-LIKE CELL LINE .....	47
Introduction .....	47
Methods .....	49
Results .....	52
Discussion .....	73
CONCLUSIONS AND FUTURE DIRECTIONS .....	75
REFERENCES .....	81

## LIST OF FIGURES

Figure 1-1: Lung Tissue rhCC10 as a Function of rhCC10 Dose.....	8
Figure 1-2: IL-8 as a Function of rhCC10 treatment .....	9
Figure 1-3: TNF- $\alpha$ Protein as a Function of rhCC10 treatment.....	10
Figure 1-4: IFN- $\gamma$ mRNA Expression as a Function of rhCC10 treatment .....	11
Figure 1-5: Schematic Illustration of Ovine CC10 Gene Isolation.....	12
Figure 1-6: Confirmation of Isolation of Ovine CC10 Protein.....	13
Figure 1-7: Cross Species Comparison of CC10 Gene.....	14
Figure 1-8: Cross Species Comparison of CC10 Protein.....	14
Figure 1-9: The Preterm Lamb is Deficient in rhCC10 .....	15
Figure 1-10: Dose Response Effect of rhCC10 on CC10 mRNA Expression.....	16
Figure 1-11: Representative Western Blots of the Dose Response Effect of rhCC10 on Endogenous CC10 Protein .....	17
Figure 1-12: Dose Response Effect of rhCC10 on Endogenous CC10 Protein Western Blot .....	18
Figure 1-13: Endogenous CC10 Protein as a function of lung tissue .....	19
Figure 1-14: Endogenous CC10 as a function of pro-inflammatory mediators.....	20
Figure 1-15: Combined Effect of IL-8 protein and Lung Tissue rhCC10 on Endogenous CC10 Protein.....	21
Figure 1-16: Working Model of Regulation of CC10 by rhCC10 Treatment .....	24
Figure 2-1: Comparison of Preterm and Adult Ovine Lung IL-6 and IL-8 Protein .....	31
Figure 2-2: Comparison of Preterm and Adult Ovine Lung TLR 4 Expression.....	32
Figure 2-3: Comparison of Preterm and Adult Ovine Lung TLR 4 Protein.....	33
Figure 2-4: Comparison of Preterm and Adult Ovine Lung TLR 4 Activity .....	33
Figure 2-5: Comparison of Preterm and Adult Ovine Lung NF-kB Activity.....	34
Figure 2-6: Representative Western Blots of the Developmental Differences on TLR Mediated Inflammation .....	35

Figure 2-7: Dose Response Effect of rhCC10 on IL-6 Protein.....	36
Figure 2-8: Dose Response Effect of rhCC10 on IL-8 Protein.....	37
Figure 2-9: Dose Response Effect of rhCC10 on IFN- $\gamma$ mRNA Expression .....	38
Figure 2-10: Dose Response Effect of rhCC10 on IL-10 mRNA Expression.....	39
Figure 2-11: Dose Response Effect of rhCC10 on TLR 4 Activity.....	40
Figure 2-12: Dose Response Effect of rhCC10 on NF-kB Activity .....	41
Figure 2-13: Representative Western Blots of the Dose Dependent Effects of rhCC10 on TLR Mediated Inflammation .....	42
Figure 2-14: Dose Response Effect of rhCC10 on TLR 4 mRNA Expression .....	43
Figure 2-15: Dose Response Effect of rhCC10 on TLR 4 Protein .....	44
Figure 2-16: Dose Response Effect of rhCC10 on SP-A mRNA Expression .....	45
Figure 3-1: Effect of rhCC10 on LPS induced IL-6 and IL-8 mRNA expression.....	53
Figure 3-2: Effect of rhCC10 on LPS induced IFN- $\gamma$ and IL-10 mRNA expression .....	54
Figure 3-3: Effect of rhCC10 on LPS induced TLR 4 Activity.....	55
Figure 3-4: Effect of rhCC10 on LPS induced NF-kB Activity .....	56
Figure 3-5: Representative Western Blots of the Effect of rhCC10 on LPS induced Inflammation .....	56
Figure 3-6: Effect of rhCC10 on LPS induced TLR 4 mRNA expression .....	57
Figure 3-7: Effect of rhCC10 on LPS induced TLR 4 Protein .....	58
Figure 3-8: Effect of rhCC10 on LPS induced mucin 1 mRNA expression.....	59
Figure 3-9: cPLA <sub>2</sub> mRNA Expression of Cells Transfected with shRNA constructs .....	60
Figure 3-10: sPLA <sub>2</sub> Activity Assay of Selected Transfected Cell Groups .....	61
Figure 3-11: sPLA <sub>2</sub> % Knockdown as Compared to Untransfected Wild Type.....	61
Figure 3-12: Effect of rhCC10 on IL-6 mRNA expression in the sPLA <sub>2</sub> KD Transfected Cells .....	63

Figure 3-13: Effect of rhCC10 on IL-8 mRNA expression in the sPLA2 KD Transfected Cells .....	64
Figure 3-14: Effect of rhCC10 on IFN- $\gamma$ mRNA expression in the sPLA2 KD Transfected Cells .....	65
Figure 3-15: Effect of rhCC10 on IL-10 mRNA expression in the sPLA2 KD Transfected Cells .....	66
Figure 3-16: Effect of rhCC10 on TLR 4 Activity in the sPLA <sub>2</sub> KD Transfected Cells .....	67
Figure 3-17: Effect of rhCC10 on NF- $\kappa$ B Activity in the sPLA <sub>2</sub> KD Transfected Cells .....	68
Figure 3-18: Representative Blots of the Effect of rhCC10 on TLR 4 Mediated Inflammation in the sPLA <sub>2</sub> KD Transfected Cells Exposed to LPS.....	69
Figure 3-19: Representative Blots of the Effect of rhCC10 on TLR 4 Mediated Inflammation in the sPLA2 KD Transfected Cells Exposed to Hyperoxia.....	70
Figure 3-20: Effect of rhCC10 on TLR 4 mRNA expression in the sPLA <sub>2</sub> KD Transfected Cells .....	71
Figure 3-21: Effect of rhCC10 on TLR 4 protein in the sPLA <sub>2</sub> KD Transfected Cells .....	72
Figure 3-22: Effect of rhCC10 on mucin 1 mRNA expression in the sPLA <sub>2</sub> KD Transfected Cells .....	73
Figure 4-1: Unified Model of rhCC10 Action .....	79

## INTRODUCTION

*Note to the Reader: This dissertation is organized with each chapter being its own individual manuscript. Therefore, certain methods and the rationale in each individual chapter may be restated. In addition, prior to Chapter 1, I provide an overall introduction and after Chapter 3, an integrated discussion.*

The lung is important in the maintenance of overall systemic homeostasis and normal physiologic function. Without proper gas exchange, many diseases and problems can develop to the detriment of the patient. Preterm infants are especially vulnerable due to underdevelopment and immature systems<sup>12, 18, 33, 37, 61, 62, 64, 88</sup>. The lungs of preterm infants are often surfactant insufficient predisposing these infants to respiratory distress syndrome (RDS)<sup>22, 22, 54, 75, 83</sup>. Dependent upon severity, RDS necessitates intervention with exogenous surfactant treatment, hyperoxia, and mechanical ventilatory support. The noxious stimuli associated with hyperoxia and ventilation puts the infant at risk for development of bronchopulmonary dysplasia (BPD) due to the inflammatory response to treatment<sup>4, 5, 26, 37, 66</sup>. For each day that an infant requires neonatal intensive care and associated respiratory care, there is an associated cost of approximately \$4000<sup>10</sup>. Mitigation of the inflammatory cascade that leads to BPD ostensibly could reduce the length of stay in the NICU. rhCC10, a recombinantly made Club Cell protein, offers a possible treatment to prevent the inflammatory response associated with treating RDS, thus decreasing pulmonary, cardiovascular, and developmental complications due to BPD. Multiple studies have shown that rhCC10 is an effective anti-inflammatory

treatment in the preterm lung. Our group has previously shown reductions in the pro-inflammatory biomarkers IL-6 and IL-8 protein, as well as increases in VEGF, and surfactant proteins in the lung, when rhCC10 is used in addition to surfactant treatment<sup>83</sup>,

<sup>96</sup>.

Despite this promise as a treatment, the mechanism(s) of rhCC10 action and its effect in an underdeveloped lung is unclear. rhCC10 is known to inhibit sPLA<sub>2</sub> and fibronectin but this effect does not account for all of its anti-inflammatory action<sup>2, 48</sup>. There is also no known receptor for rhCC10, adding a challenge to investigation the mechanism(s) of action. Because Toll-like receptors have been shown to be an integral part of lung inflammation, with TLR inhibition or knockout improving animal and cell model life span and lowering inflammatory markers when challenged to hyperoxia<sup>69</sup>, we used the toll-like receptor pathway as a model system to dissect the mechanisms of action by which rhCC10 may mitigate inflammation. We first evaluated the impact of rhCC10 on TLR expression in preterm lamb lung tissue. Expecting TLR expression to decrease with rhCC10 treatment in our preterm lamb lung tissue, we performed qPCR of TLR4 mRNA. Instead we observed an increase in mRNA expression and a possible conundrum: an unexpected uncoupling of NF-κB activity, as represented by decreased IL-6 and IL-8 protein, and increased TLR mRNA expression. This observation along with our data showing that rhCC10 treatment lowers Mucin 1 protein in the lung, a key regulator of TLR activity, led us to ask what is occurring. This led us to our central hypothesis that, **an exogenous anti-inflammatory agent will down-regulate endogenous anti-inflammatory mechanisms during early development.** If this is the case, modified

rhCC10 dosing strategies may need to be considered until anti-inflammatory defenses mature. We tested this hypothesis through the following aims:

Aim 1: Characterize the differences between the preterm lamb lung and adult sheep lung pro and anti-inflammatory profiles. **We hypothesized that the preterm lamb lung is deficient in anti-inflammatory protection and has increased inflammatory damage relative to the hyperoxic ventilated adult lung.** We used frozen-banked preterm lamb and adult sheep lung tissue to analyze TLR4 mRNA expression, protein, and signaling activity, NF- $\kappa$ B activation by I $\kappa$ B degradation, and downstream cytokine profile.

Aim 2: To determine the relationship between TLRs and CC10 signaling. **We hypothesized that rhCC10 will inhibit TLR mediated signaling, leading to a decrease in downstream signaling and NF- $\kappa$ B mediated inflammatory biomarkers.** We used frozen-banked preterm lamb lung tissue to analyze TLR 4 mRNA expression, protein, and signaling activity, Mucin 1 mRNA expression and protein, NF- $\kappa$ B activation by I $\kappa$ B degradation, and downstream cytokine profile.

Aim 3: To determine if the effect of rhCC10 on TLR signaling is independent of sPLA<sub>2</sub>. **We hypothesized that rhCC10 will inhibit TLR mediated signaling, leading to a decrease in downstream signaling and NF- $\kappa$ B mediated inflammatory biomarkers even when sPLA<sub>2</sub> is knocked down.** We used an A549 cell model system to test rhCC10 pre-treatment prior to challenge by TLR 4 specific agonists and by hyperoxia respectfully, in the presence and absence of sPLA<sub>2</sub>.

*Clinical Translation and Significance:* Preterm infants are vulnerable to RDS due to surfactant insufficiency. Premature infants develop high surface tension at the air-liquid interface of their alveoli, and a decrease in lung compliance, thereby leading to a ventilation and perfusion mismatch and impaired gas exchange<sup>32</sup>. Over the past three decades, care strategies including the use of exogenous surfactant and advanced ventilation strategies, have significantly improved clinical outcomes in preterm infants<sup>32</sup>. At the same time, due to inflammation from hyperoxia, innate limitations in anti-oxidant and anti-inflammatory defenses, aggressive ventilation, and increased survival of younger preterm infants, incidence of BPD has not decreased<sup>4, 5, 54</sup>. Further, safe anti-inflammatory treatment for the preterm infant is lacking, with steroid treatment having numerous side effects<sup>9</sup>. Overall, while extending the life of the infant with RDS by biomechanically protecting the lung, current animal derived surfactant treatment alone has not attenuated pulmonary inflammation and puts the patient at risk of the sequelae of this inflammation. This proposal will extend our previous work to address this gap in treatment options by exploring the mechanism of action of exogenous rhCC10 and the effects of rhCC10 on endogenous anti-inflammatory action.

*Club Cell Secretory Protein 10:* CC10 deficiency is associated with asthma<sup>30, 35</sup>, COPD<sup>74, 99</sup>, and cancer malignancy<sup>13, 53</sup>. Similar to surfactant insufficiency, preterm infants are also deficient in CC10<sup>50, 75, 83</sup>, suggesting that rhCC10 treatment could be highly effective. In fact, rhCC10 has been used successfully in preclinical<sup>65, 83</sup> and clinical<sup>50</sup> trials to decrease pulmonary inflammation. CC10 is known to bind to

fibronectin<sup>2, 2, 75, 75</sup> thus limiting cellular adhesion and scar deposition. Its is understood to competitively inhibit phospholipase-A<sub>2</sub> IIA<sup>48</sup>, and a reduction in NF-kB and IRF mediated pro-inflammatory cytokines<sup>50, 83</sup>. However, due to enzyme kinetics, the exponential decrease in phospholipase-A<sub>2</sub> activity suggests that CC10 is not only competitively inhibiting sPLA<sub>2</sub> but has other unknown anti-inflammatory effects<sup>48</sup>.

*Toll-like Receptor 4:* TLR 4 is in a family of dimerized transmembrane receptors classified as pathogen associated molecular pattern receptors<sup>7, 24, 63, 94</sup>. It is expressed on the surface membrane of immune cells and epithelial cells. The TLR family as a whole recognizes classes of molecules that are generally present on bacteria and viruses. However, certain TLRs have endogenous ligands as well<sup>16, 69</sup>, such as what would be produced in cell death. Activation of TLRs on pulmonary epithelial cells leads to activation of pro-inflammatory transcription factors and apoptosis through either the MyD88 or TRIF dependent pathways,<sup>7, 19</sup> both of which can activate NF-kB through IKK phosphorylation. Local intra-organ activation of TLRs causes increased chemokine production and thereby greater immune cell recruitment that further damage the lung epithelia and induce greater TLR activation. Currently, there is no FDA approved treatment to directly inhibit TLR signaling. The potential of mucin 1 to regulate TLR activity has also been recently demonstrated<sup>44, 90</sup>.

*Mucin 1:* Mucin 1 is a member of the mucin family of glycosylated proteins. Mucin 1 is a membrane bound protein, with two main domains: a heavily glycosylated extracellular domain and a cytoplasmic tail. The cytoplasmic tail is required for mucin 1 signaling

activity<sup>43, 52, 92, 93</sup>. The proposed model of mucin 1 activation is that when an inflammatory stimulus is present, TLRs are activated and immune cells are recruited<sup>44</sup>. This causes an up regulation in TNF- $\alpha$ , which induces mucin 1 expression<sup>14, 45</sup>. The cytoplasmic tail of mucin 1 and the cytosolic domain of TLRs then crosstalk to suppress TLR signaling activity.

## CHAPTER 1: IMPACT OF RHCC10 ON ENDOGENOUS CC10 IN THE PREMATURE LUNG

### Introduction

Surfactant replacement therapy, along with lung protective, low tidal volume ventilation, is the standard of care for preterm infants with respiratory distress syndrome (RDS) <sup>5, 32, 54</sup>. Despite advances in the development of novel surfactant treatments, dosing strategies, and delivery methods, long term morbidity associated with the progression to bronchopulmonary dysplasia has not been effectively mitigated <sup>9, 62</sup>. This is likely due to damage from inflammation caused by the initial lung injury in RDS, aggravated by aggressive, but necessary treatment such as hyperoxia and ventilation, coupled with developmental insufficiency of endogenous anti-inflammatory proteins <sup>4, 5, 37</sup>. Current therapies that can mitigate inflammatory induced lung injury in the developing lung, such as postnatal steroids, improve oxygenation and pulmonary mechanics but are contraindicated in critically ill preterm infants due to adverse side effects <sup>95</sup>. New anti-inflammatory therapies are needed that target the acute iatrogenic inflammation in the premature lung.

CC10, is a 10 kDa dimeric protein produced in the lung by airway non-ciliated bronchial epithelia cells. It has been demonstrated to confer protection against lung inflammation through competitive inhibition of sPLA<sub>2</sub>, inhibition of inflammatory cell chemotaxis, and by up regulating vascular endothelial growth factor (VEGF) and Surfactant Protein A (SP – A). However, these known actions of CC10 do not account for its total anti-inflammatory effect and it is likely other mechanisms contribute to the anti-inflammatory

action of CC10<sup>2, 34, 41, 48, 65, 66, 83, 96</sup>. It has been shown previously that expression of CC10 is up regulated by inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  and that rhCC10 lowers inflammatory cytokine production and is lung protective<sup>1, 11, 50, 56, 58, 65, 66, 75, 83, 97</sup>. Our group has previously demonstrated in preterm lambs with RDS that recombinant club cell secretory protein 10 (rhCC10) is an effective adjunct to surfactant treatment to attenuate hyperoxic ventilation induced lung inflammation and improved gas exchange at doses greater than 1.5 mg/kg<sup>83</sup>. A preliminary clinical trial in preterm infants demonstrated safety, pharmacokinetics and anti-inflammatory effects after intratracheal delivery of a single dose of rhCC10 in preterm infants with RDS<sup>50</sup>. However, the effect of rhCC10 dosing strategies on endogenous CC10 production and the relationship between inflammation and endogenous CC10 in the developing, CC10 deficient lung<sup>50, 55</sup> is unknown.

Since CC10 is regulated by inflammatory cytokines, we reasoned that exogenous rhCC10 treatment would accumulate in the lung tissue, lower inflammatory cytokine levels, and thereby lower the stimulus for endogenous CC10 production, in a dose-response manner. To test this hypothesis, we investigated the effect of intratracheally administered rhCC10 in the preterm lamb on endogenous CC10 in the lung and the relationship between lung tissue inflammatory mediators and endogenous CC10.

## Methods

### *Animal Protocol and Tissue Collection*

Frozen lung tissue used in this project was derived from a previous study<sup>83</sup>. In brief, lambs (gestational age of  $126 \pm 3$  days,  $n = 24$ ,  $n = 6$  per group) were anesthetized, instrumented, delivered by cesarean section, ventilated with 100% oxygen, treated with exogenous surfactant, the current standard of care, (Survanta<sup>®</sup>, Ross laboratories 4 mL/kg, 100 mg/kg) or surfactant followed by one of three doses of intratracheal rhCC10 (0.5 mg/kg, 1.5 mg/kg, or 5.0 mg/kg). In addition, adult sheep ( $n=6$ ) were anesthetized and instrumented in a similar manner and ventilated with 100% oxygen. All animals were followed for 4 hours, after which lungs were harvested for frozen and fixed tissue collection. The experimental protocols and procedures for this study were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine and conducted in accordance with NIH and AALC guidelines.

### *RNA Extraction and cDNA Synthesis*

Frozen ovine lung tissue was homogenized and processed using the Qiagen RNeasy miniprep kit (Qiagen, Valencia, CA). RNA concentration was determined by measuring absorbance at 260nm and then diluted to a concentration of 1 ug/mL. cDNA synthesis was then performed using the Revertaid<sup>™</sup> First Strand Synthesis Kit (Fermentas, Glen Burnie, MD).

### *Cloning and Sequencing*

Following cDNA synthesis, ovine cDNA was amplified by polymerase chain reaction (PCR) using designed bovine CC10 primers (GenBank AY994053.1; Primer3; EWG Operon). The amplified PCR product was then isolated by agarose gel electrophoresis. Following staining by mupid blue, the amplified sequence was isolated from the gel, ligated to an iEGFP vector and sequenced (NAPcore facility of the Children's Hospital of Philadelphia). The CC10 iEGFP vector was transfected into HEK293A cells. CC10 and green fluorescent protein expression in the transfected cell lysate were checked by western blot and compared to control vector transfected cell lysate.

### *Quantitative Real-Time PCR*

Based on the sequenced ovine CC10 gene, we designed and ordered ovine CC10 specific primers (Primer3; MWG Operon: Huntsville, AL). 18S and IFN- $\gamma$  primers were readily available and ordered (MWG Operon). Using SYBR Green technology, CC10 gene and IFN-  $\gamma$  expression was measured in duplicate using a Mastercycler<sup>®</sup> ep Realplex (Eppendorf, Hauppauge, NY) and normalized by 18S mRNA expression<sup>29</sup>.

### *Protein Extraction*

Frozen lung tissue was homogenized in cold RIPA solution using a tissue grinder. Following cold centrifugation for 10 min at 10,000 RPM, the supernatant was collected by aspiration. The protein concentration of the supernatant was measured using the Bradford method. These samples were used for ELISA and western blot.

### *ELISA*

A custom designed sandwich ELISA was used to measure TNF- $\alpha$  and IL-8 in lung tissue homogenate supernatant<sup>83</sup>. Protein was captured onto the plate using mouse anti-ovine capture antibody (IL-8: Serotec, Raleigh, NC ; TNF- $\alpha$ : CSIRO and CAB of University of Australia). IL-8 and TNF- $\alpha$  were detected using rabbit anti-ovine antibodies (IL-8: Serotec ; TNF- $\alpha$ : CSIRO and CAB of University of Australia). HRP-conjugated goat anti-rabbit (Cat. #AP132P, Millipore) was used as the secondary antibody. Following the reaction with the substrate, protein was detected using a plate reader (MRX Revelation ; Dynex, Chantilly, VA). RhCC10 was measured in lung tissue homogenate supernatant by using a human specific CC10 ELISA kit (APC Biomaterials, LLC, Rockville, MD). Signal was detected using a plate reader (MRX Revelation ; Dynex).

### *Western Blot of Endogenous CC10*

Aliquots of the supernatant were then diluted to a uniform concentration and then equal volumes of each sample were loaded onto the gel. Recombinant bovine and human CC10 were used as positive and negative controls respectively. SDS-PAGE was performed using a uniform volume of each sample. Following transfer, a western blot was performed (SNAP ID system; Millipore, Billerica, MA) using rabbit anti-bovine CC10 (Catalog # APC016, APC Biomaterials, LLC, Rockville, MD) and HRP conjugated goat-anti-rabbit (Cat. #AP132P, Millipore) antibodies for primary and secondary antibodies respectively. GAPDH was used for normalization with mouse anti-human GAPDH (Catalog # MCA4740, AbD Serotec, Raleigh, NC) and HRP conjugated anti-mouse (Catalog # NA931V, GE Healthcare, Pittsburgh, PA) as primary and

secondary antibodies) respectively. After reaction with substrate, images were taken (LAS-3000 ; Fujifilm, Valhalla, NY) and band intensity was measured (Multi Gauge Software; Fujifilm).

### *Data Analysis*

All data were imported into Microsoft Excel (Microsoft, Redmond, WA) and Prism 5.0 (Graphpad, La Jolla, Ca). Group comparisons of rhCC10 dose were analyzed by ANOVA vs control with individual group comparisons performed using the Dunn-Bonferroni method. Values are expressed as mean  $\pm$  SEM and significance was accepted at  $p < 0.05$ .

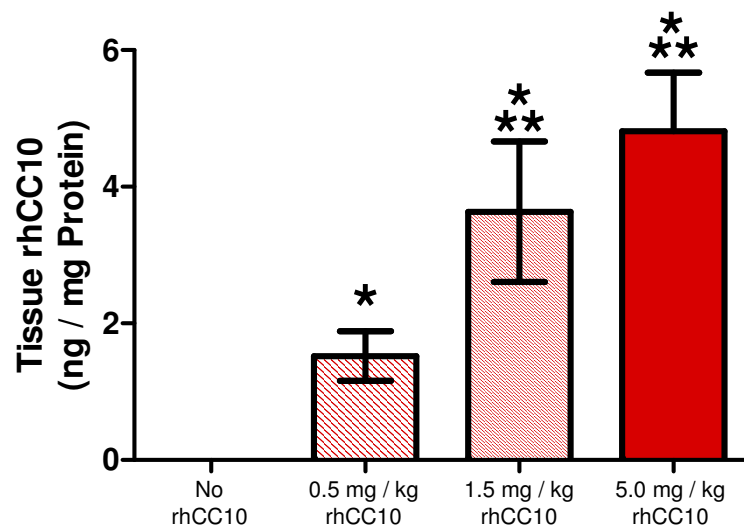
A non-linear fit regression of exponential decay was used to model the association between lung tissue rhCC10 and other measured biomarkers. So as to determine significance, lung tissue rhCC10 data was normalized by logarithmic transformation and then compared by linear regression analysis to other measured biomarkers. Association of endogenous rhCC10 protein with pro-inflammatory biomarkers was determined by linear regression respectfully. The p values, , coefficient of determination values, and slopes describing the relationship between endogenous rhCC10 vs IL-8, TNF- $\alpha$ , IFN- $\gamma$ , and tissue rhCC10 were respectfully compared so as to determine the variable that has the strongest effect on endogenous CC10 protein. Significance was accepted at  $p < 0.05$ . Sigma Plot 8.0 (Systat Software, Inc., San Jose, CA) was used for creation of the 3D diagram.

DNA and Protein Sequences were manually compared in Microsoft Word. Sequence analysis so as to determine secondary structure and disulfide bonds were determined using ExPASy.

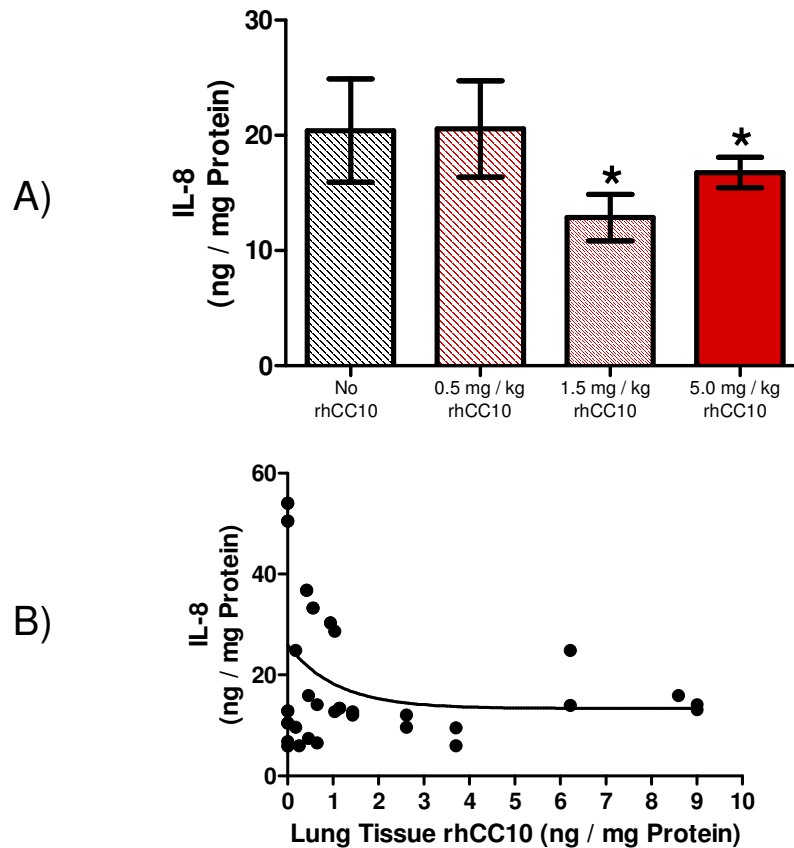
## **Results**

In mechanically ventilated, surfactant-treated preterm lambs with RDS, rhCC10 treatment increased (\* $p < 0.05$ ) the rhCC10 locally present in the lung tissue, with the two highest dose groups having the greatest increase (\*\* $p < 0.05$ ) in lung tissue rhCC10 (Figure 1-1). Lung IL-8 and TNF- $\alpha$  proteins and IFN- $\gamma$  mRNA expression were inversely ( $p < 0.05$ ) associated with rhCC10 dose (Figures 1-2A, 3A, and 4A). In addition, lung IL-8 and TNF- $\alpha$  proteins and IFN- $\gamma$  mRNA expression were inversely ( $p < 0.05$ ) associated with tissue rhCC10 (Figures 1-2B, 3B, 4B). Overall, these results demonstrate that rhCC10 accumulates in the lung tissue in a dose dependent manner, decreased pro-inflammatory cytokines in a dose dependent manner, and that increasing lung tissue rhCC10 is associated with a decrease in pro-inflammatory cytokines.

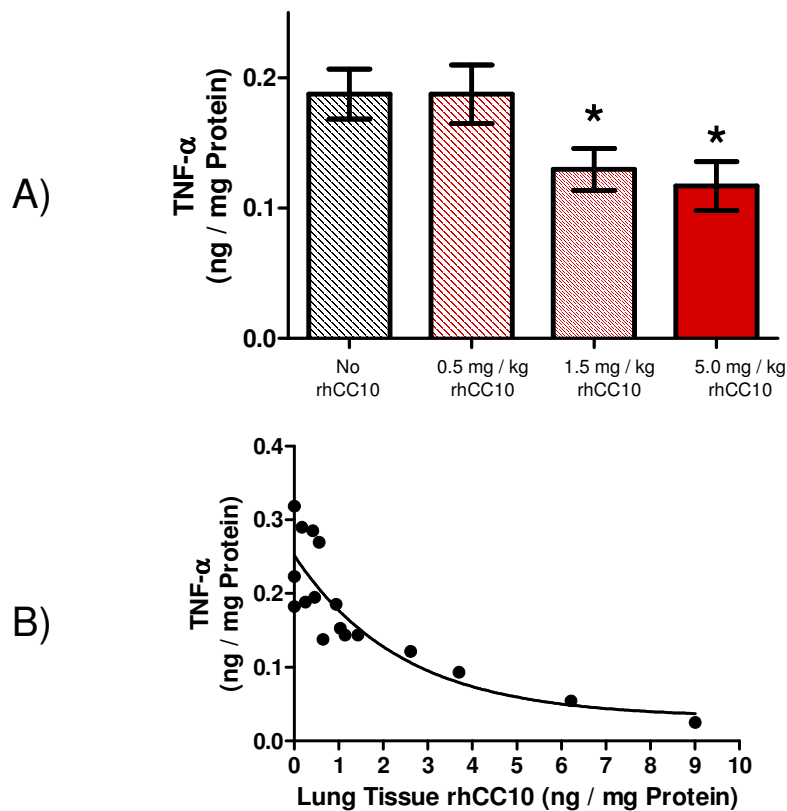
To analyze the effect of rhCC10 treatment on endogenous CC10, we needed to first sequence and clone the ovine CC10 gene (Genebank #FJ959385 ; May 10, 2010).



**Figure 1-1: Lung Tissue rhCC10 as a Function of rhCC10 Dose.** rhCC10 within the homogenized lung tissue was measured for each respective dosage. All three rhCC10 dosed groups had an increased rhCC10 ( $*p < 0.05$ ) over the control no rhCC10 group. The 1.5 mg and 5.0 mg had the highest ( $**p < 0.05$ ) lung tissue rhCC10, with no difference between the respective groups. (Mean  $\pm$  SE; n = 6 per group)



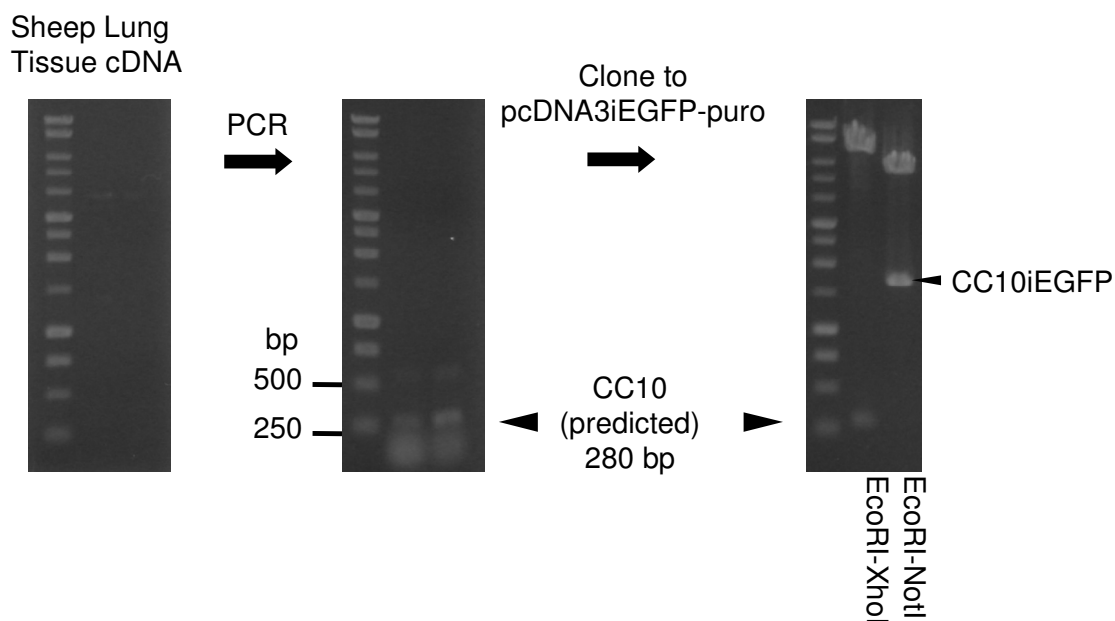
**Figure 1-2: IL-8 as a Function of rhCC10 treatment.** A) There was no difference in the IL-8 protein between the surfactant treated and the surfactant + 0.50 mg/kg rhCC10 groups and between the 1.5 and 5.0 mg/kg rhCC10 groups respectively. Both the medium and high dose rhCC10 groups had a lower (\* $p < 0.05$ ) IL-8 protein than either of the other groups. (Mean  $\pm$  SE;  $n = 6$  per group). B) Lung IL-8 was plotted as a function of lung tissue rhCC10. As the lung tissue rhCC10 increased ( $p = 0.02$ ,  $r^2 = 0.40$ ), IL-8 protein decreased.



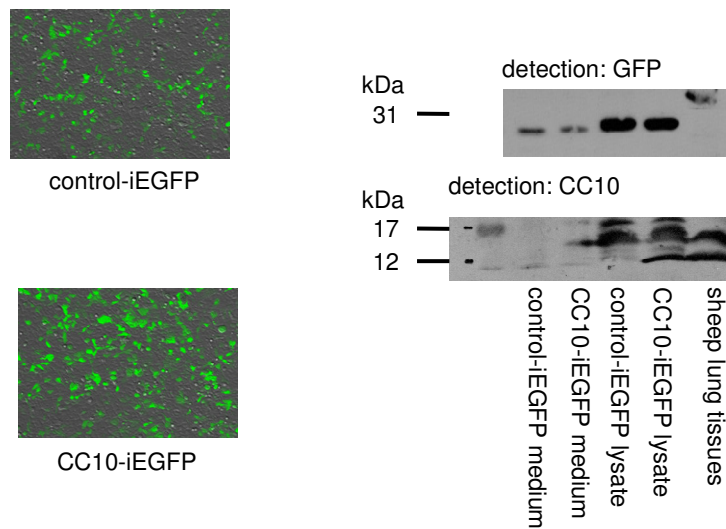
**Figure 1-3: TNF- $\alpha$  Protein as a Function of rhCC10 treatment.** A) There was no difference in the TNF- $\alpha$  protein between the surfactant treated and the surfactant + 0.50 mg/kg rhCC10 groups and between the 1.5 and 5.0 mg/kg rhCC10 groups respectively. Both the medium and high dose rhCC10 groups had lower (\* $p < 0.05$ ) TNF- $\alpha$  protein than either of the other groups. (Mean  $\pm$  SE;  $n = 6$  per group). B) Lung TNF- $\alpha$  was plotted as a function of lung tissue rhCC10. As the lung tissue rhCC10 increased ( $p < 0.001$ ,  $r^2 = 0.80$ ), TNF- $\alpha$  protein decreased.



the cellular lysate of the cells transfected with CC10 iEGFP vector produced a band at 10 kDa (Figure 1-6). The control transfected cell lysate and the medium of either cell lysate group did not produce a band at 10 kDa, indicating that the 10kDa protein represents ovine CC10 and that it was not secreted into the medium.



**Figure 1-5: Schematic Illustration of Ovine CC10 Gene Isolation.** Schematic illustrating how ovine lung tissue cDNA was amplified using PCR with primers for bovine CC10. Following electrophoresis of the PCR product the gel was stained with methylene blue and the resultant band was excised.



**Figure 1-6: Confirmation of Isolation of Ovine CC10 Protein.** Typical images of fluorescence microscopy and western blots of the cellular lysate against Green Fluorescent Protein (GFP) showed successful transfection of the HEK293A with the GFP containing vector by GFP protein expression. This result was further confirmed by western blot of the cell lysates. The vector with the incorporated sequence has protein recognized by anti-bovine CC10 antibody while the lysate of the control cells and the medium of either cell type does not produce a similar band. All samples were derived at the same time and processed in parallel.

To further verify the identification of the ovine CC10 gene, the sequence of the ovine CC10 cDNA (Genebank #FJ959385) was compared to the corresponding sequences of CC10 cDNA in other species (Figure 1-7). There is a greater than 60% sequence homology across species and a 90% homology between the ovine and bovine cDNA sequences. There is also a high protein residue similarity across species and identity at the critical cysteine residues, which is important for the disulfide linkage integral to the dimerized structure of CC10 (Figure 1-8)<sup>73, 85</sup>.

```

sheep      GGTGGAATCACCATGAAGCTCACCATCG
bovine    ATGAAGCTCACCATCGCCATCGTCTGGTACCCTGACTCTCTTCGAGACCTGCATCC
dog       ATGAAGCTCGCTGTATCCTTGCCTGGTACCCTGGCTCTCTACTGCAGCCTGCTTCT
human     ATGAAACTCGCTGTACCCCTACCCTGGTACACTGGCTCTCTGCTGCAGCTCCGCTTCT
rat       ATGAAGATCGCCATCACAATCACTGTGCTCATGCTGCCATCTGCTGCAGCTCAGCTCT
mouse     ATGAAGATCGCCATCACAATCACTGTGGTCTGCTGTCATCTGCTGCAGCTCAGCTTCT
rabbit    ATGAAGCTCGCCATCACCCTGCCTGGTACCCTGGCTCTCTCTGAGCCTGCATCT

sheep      ACAGAGTGTGCCCCAGCCTTCTGTATGCCCTGGGAAACCTCCTCATAGGCAGCCTTCC
bovine    ACAGAGTGTGCCCCAGCCTTCTGTATGCTTGGGAAACCTCATGGCCGAACACCTTCC
dog       GCAGAGATCTGCCAGAACTTCTAAATGTCTATCAAAGCCCTCTCTGGACACGCCTTCC
human     GCAGAGATCTGCCAGCCTTTCAGCGTGTCTATCAAAGCCCTCTCATGGACACACCTTCC
rat       TCGACATCTGCCCAGGATTTCTCAAGTCTTGAAGCCCTCTCTAGGCTCAGAGTCT
mouse     TCGACATCTGCCCAGGATTTCTCAAGTCTTGAAGCCCTCTCTATGGAATCAGAGTCT
rabbit    GCAGGATCTGCCGAGATTTGCACAGTCTATTGAAACCTCTCTCTGGCACGCCCTCC

sheep      AGTACGAGGCTGCCCTGGAACCTTTCAGCCCTGACGAAGACATGAAAGAACCAAGA
bovine    AGCTTTGAGGCTACCTTGAACCTTTCAGCCCTGACGAAGACATGAAAGAGCAACAAGT
dog       AGTTACCAGGCTGCACCTTGAATTTTCAACCCAGACGACATGAAAGATGCANTGATC
human     AGTTATGAGGCTGCCATGGAACCTTTCAGCCCTGATCAAGACATGAGGGAGCAGGGGCT
rat       AATTATGAGGCTGCCATGGAACCTTTCAGCCCTGATCAAGACATGAGGGAGCAGGGGCT
mouse     GGTATGAGGCTGCCATGGAACCTTTCAGCCCTGATCAAGACATGAGGGAGCAGGGGCT
rabbit    AGTTACGAGACATCCCTGGAAGAAATTTGAACCTGATGACACCATGAAAGATGACGGGATG

sheep      CAGCTGAAGAGCTGATAGACACCTCTCCCGAAGCCCAAGGACAGCTGTTACAGCTC
bovine    CAGCTGAAGACGCTGTTAGACACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC
dog       CAGCTGAAGAGCTGTTGGACACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC
human     CAGCTGAAGAGCTGTTGGACACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC
rat       CAGCTGAAGAGCTGTTGGATACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC
mouse     CAGCTGAAGAGCTGTTGGATACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC
rabbit    CAGATGAAGAGCTGTTGGATACCTCTCCCGAAGCCCAAGGACAGCATGTTAGAGCTC

sheep      ATGGGAAAAATAGTACAAAGCCAGAGTGTCTAGCTCAGCACC      (FJ959385.1)
bovine    ATGATGAAAAATAACAAAGCCAGAGTGTCTAG----- (NM_001076976.3)
dog       ACGGAGGACATCAAAAAGCCAGAGTGTCTAG----- (XM_005631626.1)
human     ATGGAAAAATAGCCCAAGCTCACTGTGTAATTAG----- (NM_003357.4)
rat       ACGGAGAAGATCCTAACAGTCTCTGTTGAGCAAGATTTAAGAGTCTGA (NM_013051.1)
mouse     ACGGAGAAAAATCCTAACAGTCTCTGTTGAGCAAGATTTAAGATCTGA (NM_011681.2)
rabbit    ACGGAAAAATAGTGAAGAGCCACTGTGT----- (K01657.1)

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**Figure 1-7: Cross Species Comparison of CC10 Gene.** There is over a 90% homology between the bovine and ovine gene sequence. Across all species shown there is a high homology in the genetic sequence of CC10. The Genbank accession number for each gene is located at the end of each respective sequence.

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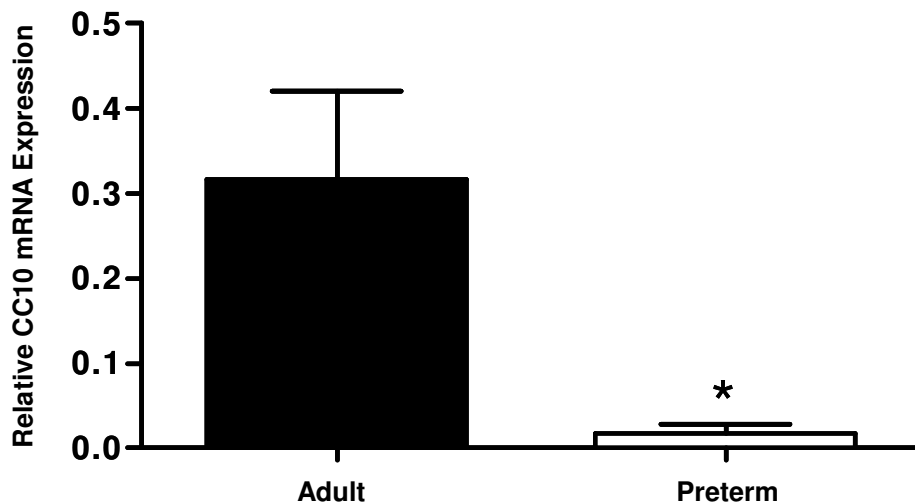
sheep      MKLTIAFALVTLTLF [C] RPASTEVE [C] PSLLYALGNLLIGTPSSYEAALEPFPSPDEDMKEATR
bovine    MKLTI AIVLVTTLTLF [C] RPASTEVE [C] PSLLYVLGNLIAGTPSSFEATLEPFPSPDEDMKEATS
dog       MKLAVL LALVTL LALY [C] SPASAEI [C] QNFLNVIKALFLDTPSSYQAALFEFFNPADMDKAMI
human     MKLAVT LTLVTL LALC [C] SSASAEI [C] PSFQRVIE TL LMDTPSSYEAAELFSPDQDMREAGA
rat       MKIAIT ITVMLS IC [C] SSASSDI [C] PGFLQVLEALLLGSSESNYEAA LKPFNPASDLQNA GT
mouse     MKIAIT ITVMLS IC [C] SSASSDI [C] PGFLQVLEALLLMESESGYVA SLKPFNPGSDLQNA GT
rabbit    MKLAIT LALVTL LALL [C] SPASAGI [C] PRFAHV IENLL LGTPSSYETSLKEFEPDDTMKDAGM

sheep      QLKELIDT LSPKAKDSVLQ LMGKIVQSPEC A-----
bovine    QLKTLVD T LSPKAKDSML ELMMKIIQSPEC A-----
dog       QLKSLVD T LPSNTTENI LKFTEAVIKSPEC A-----
human     QLKKLVD T LPPKPRE SI IKLMEKIAQSSLCN-----
rat       QLKRLVD T LPQETRINIV KLTEKILTSPLCEQDLRV
mouse     QLKRLVD T LPQETRINIM KLTEKILTSPLCKQDLRF
rabbit    QMKKVLD SLPQTTRENI MKLTEKIVKSPLC-----

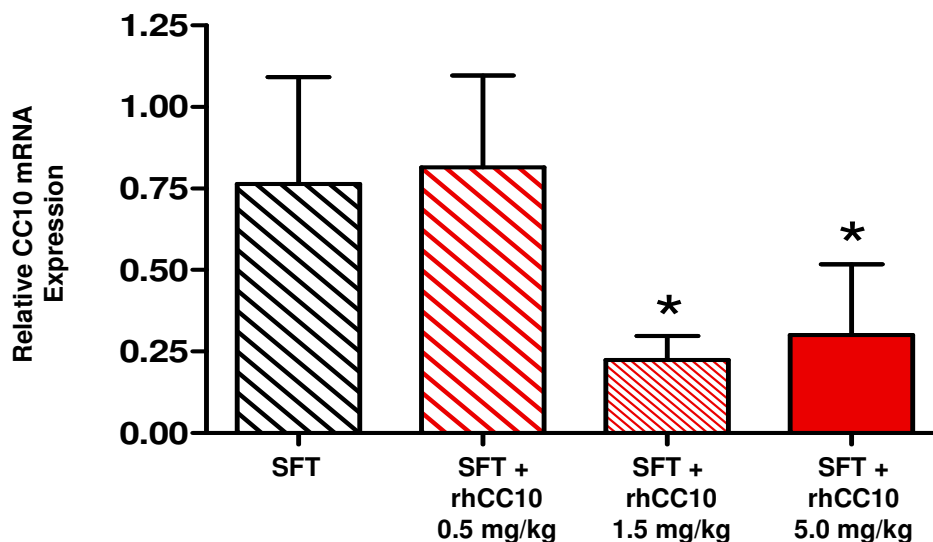
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**Figure 1-8: Cross Species Comparison of CC10 Protein.** In particular, the bracketed cysteine residues, that are required for the integral disulfide bond, align across species and an ExPASy analysis showed the typical alpha helical structures present in CC10. Note that the underline region corresponds to the primers used to amplify the gene.

Upon the verification of the ovine CC10 gene sequence, specific primers for qPCR analysis were designed to quantify ovine CC10 mRNA expression. As shown in Figure 4 and 5, the primers were used to evaluate CC10 mRNA expression. As has been demonstrated in the human lung, the preterm lamb as compared to the adult ovine lung, has decreased ( $p < 0.05$ ) CC10 mRNA Expression (Figure 1-9). Furthermore, when preterm lambs were treated with rhCC10, CC10 mRNA expression was down-regulated. Following surfactant treatment, the two highest doses of rhCC10, 1.5 mg/kg and 5.0 mg/kg, had the lowest ( $p < 0.05$ ) CC10 mRNA expression out of all groups, with no difference between the 1.5 mg/kg and 5.0 mg/kg groups respectively. There was also no difference between the surfactant only and the 0.5 mg/kg rhCC10 group (Figure 1-10).



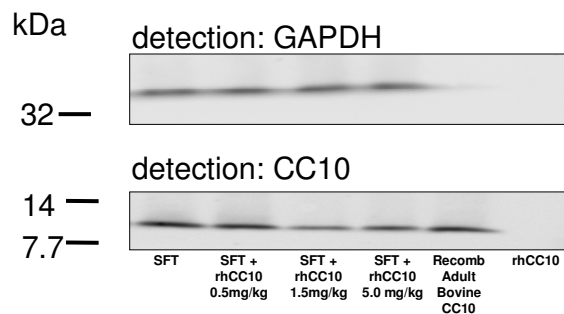
**Figure 1-9: The Preterm Lamb is Deficient in native endogenous CC10.** The preterm lamb has decreased ( $*p < 0.05$ ) rhCC10 mRNA expression as compared to the adult ovine lung. (Mean  $\pm$  SE;  $n = 6$  per group)



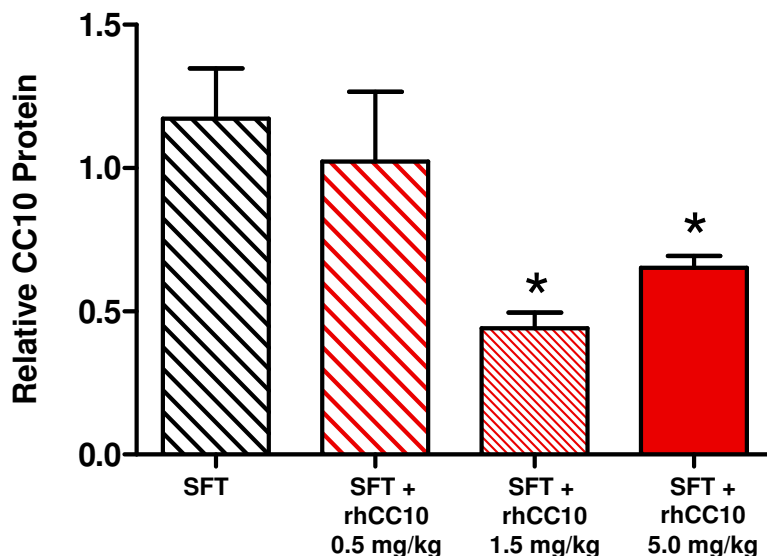
**Figure 1-10: Dose Response Effect of rhCC10 on CC10 mRNA Expression.** There was no difference between the CC10 mRNA expression of surfactant treated and surfactant + 0.5 mg/kg rhCC10 and the 1.5 and 5.0 mg/kg rhCC10 groups respectively. Both the medium and high dose rhCC10 groups demonstrated a greater decrease (\* $p < 0.05$ ) in CC10 mRNA expression than either of the other groups. (Mean  $\pm$  SE;  $n = 6$  per group)

After determining that the preterm lambs had decreased CC10 mRNA expression due to rhCC10 dosing, relative endogenous CC10 in the homogenized lung tissue was measured using western blot (Figure 1-11 and 1-12). The blots were performed with bovine anti-CC10 antibody, since the bovine and ovine protein have a high degree of identity and similarity across protein residues and the anti-bovine CC10 antibody does not detect human rhCC10 (Figure 1-11). The production of endogenous CC10 protein coincided with the mRNA expression results, with the 1.5 mg/kg and 5.0 mg/kg rhCC10 groups having the lowest amount of endogenous CC10 of all groups, with no difference between

the 1.5 mg/kg and 5.0 mg/kg groups respectively. There was also no difference between surfactant only and the 0.5 mg/kg rhCC10 group (Figure 1-12).

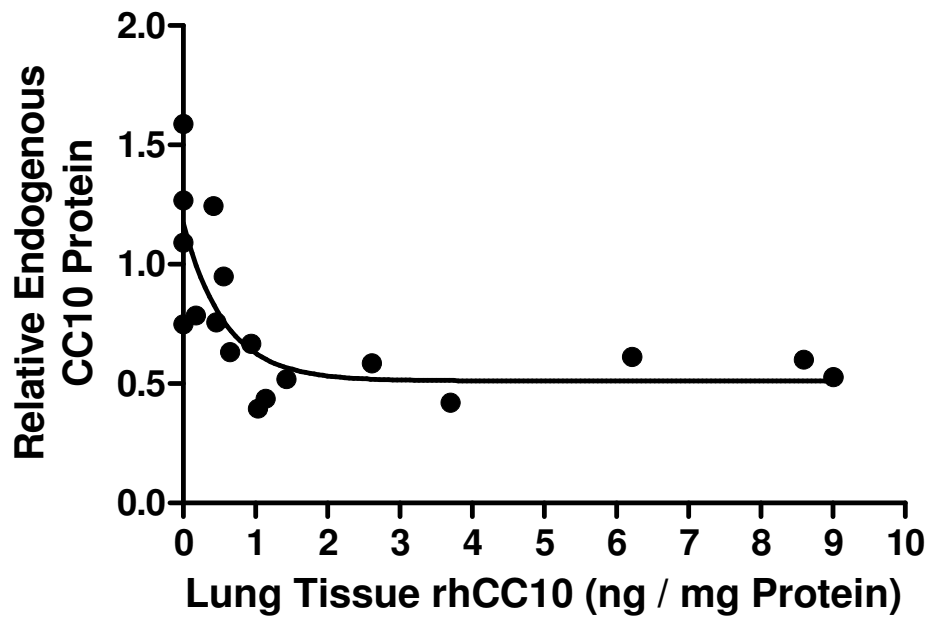


**Figure 1-11: Representative Western Blots of the Dose Response Effect of rhCC10 on Endogenous CC10 Protein.** CC10 bands were present at 10kDa in all groups except the rhCC10. The 1.5 mg/kg and 5.0 mg/kg rhCC10 groups showed a less intense CC10 band compared to the other lamb treated groups. GAPDH was used for normalization and was present in all samples derived from animal tissue. All samples were derived at the same time and processed in parallel (n = 6 per group).

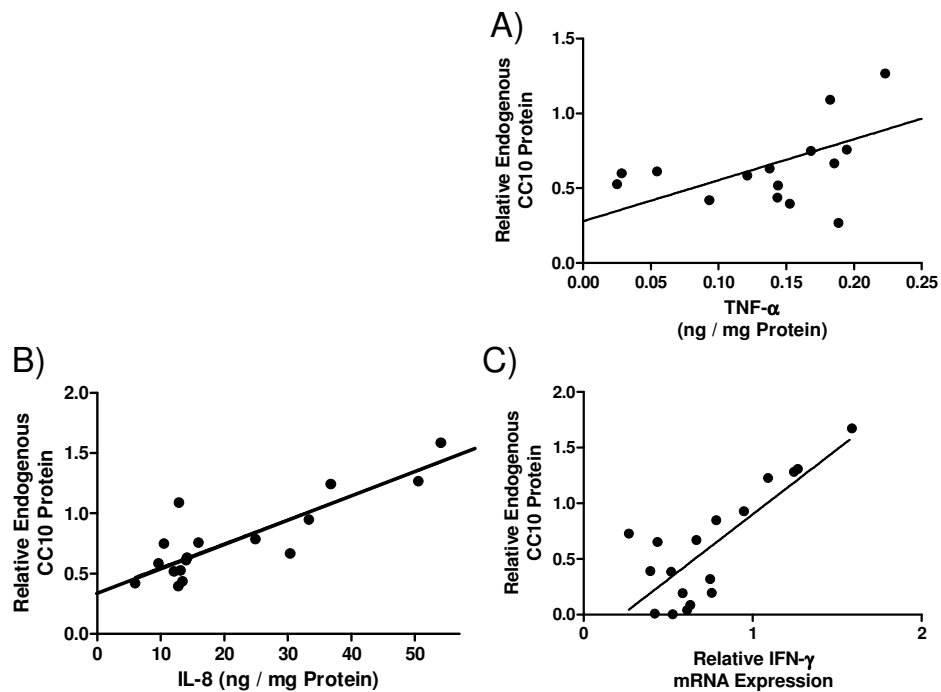


**Figure 1-12: Dose Response Effect of rhCC10 on Endogenous CC10 Protein Western Blot.** There was no difference in the CC10 protein between the surfactant treated and the surfactant + 0.50 mg/kg rhCC10 groups and between the 1.5 and 5.0 mg/kg rhCC10 groups respectively. Both the medium and high dose rhCC10 groups had lower (\* $p < 0.05$ ) CC10 protein than either of the other groups. (Mean  $\pm$  SE;  $n = 6$  per group)

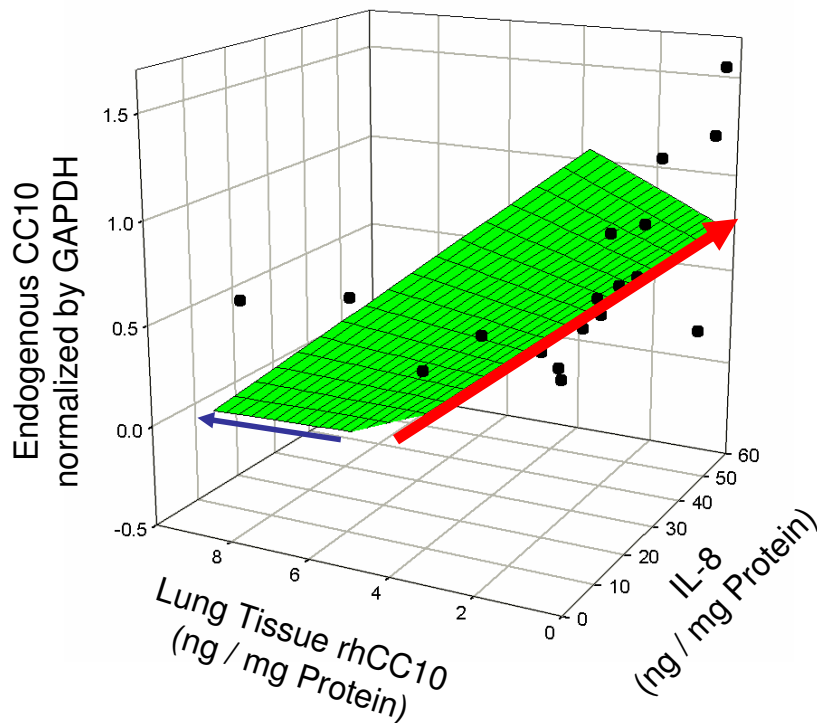
Endogenous CC10 protein was plotted as a function of, tissue rhCC10 and pro-inflammatory mediators (Figures 1-13:1-15). Endogenous CC10 protein decreased ( $p < 0.05$ ) as a function of tissue rhCC10. Endogenous CC10 protein increased ( $p < 0.05$ ) as a function of inflammatory mediators IL-8 and TNF- $\alpha$  proteins and IFN- $\gamma$  mRNA expression. Pro-inflammatory mediators IL-8 and IFN- $\gamma$  were determined as having a greater effect, as compared to lung tissue rhCC10, on endogenous CC10 protein by comparison of  $p$  values, the coefficient of determination, and slopes (Figure 1-13:15).



**Figure 1-13: Endogenous CC10 Protein as a function of lung tissue rhCC10.** Lung endogenous CC10 protein was plotted as a function of lung tissue rhCC10. As the lung tissue rhCC10 increased ( $p < 0.001$ ,  $r^2 = 0.60$ , slope =  $-0.01$ ), endogenous CC10 protein decreased



**Figure 1-14: Endogenous CC10 as a function of pro-inflammatory mediators.** A) Endogenous CC10 was plotted as a function of lung tissue TNF- $\alpha$  protein. As the TNF- $\alpha$  protein increased ( $p < 0.001$ ,  $r^2 = 0.46$ , slope = 2.74), endogenous CC10 protein increased. B) Endogenous CC10 was plotted as a function of lung tissue IL-8 protein. As the IL-8 protein increased ( $p < 0.001$ ,  $r^2 = 0.72$ , slope = 0.02), endogenous CC10 protein increased. C) Endogenous CC10 was plotted as a function of relative IFN- $\gamma$  mRNA expression. As the mRNA expression of IFN- $\gamma$  increased ( $p < 0.001$ ,  $r^2 = 0.64$ , slope = 1.17), endogenous CC10 protein increased.



**Figure 1-15: Combined Effect of IL-8 protein and Lung Tissue rhCC10 on Endogenous CC10 Protein.** 3D visual representation of the effect of lung tissue rhCC10 protein and IL-8 protein on relative endogenous CC10 Protein. IL-8 was selected as a representative pro-inflammatory mediator. As illustrated by the slope of superimposed grid, IL-8 protein had a greater effect on endogenous CC10 as compared to lung tissue rhCC10.

## Discussion

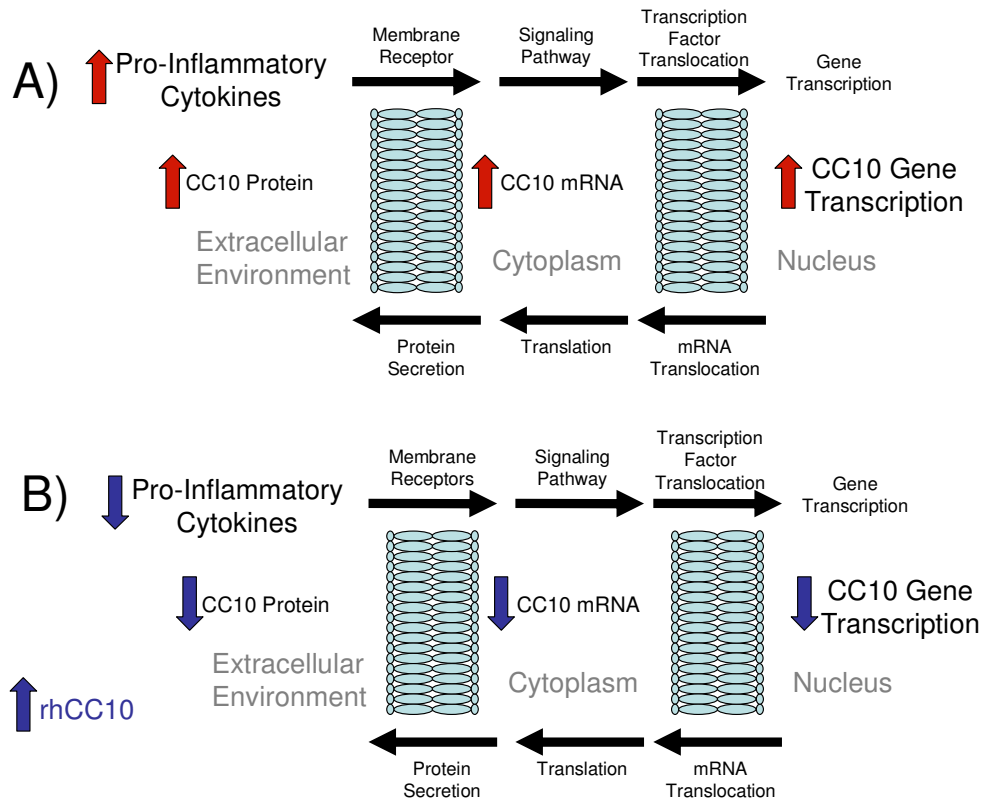
In this study, we successfully identified the ovine CC10 gene, confirmed it by using multiple methods, and used this new information as a tool to analyze the effect of exogenous rhCC10 on endogenous CC10 in the preterm lung. Upon ovine CC10 cloning and sequencing, we found that the cDNA and amino acid sequences of CC10 are highly conserved across various species with a high degree of homology between the ovine and bovine CC10 sequences. Our analysis of the protein sequence was even more conclusive with ExPASy predicting that the ovine CC10 sequence has a helical structure, similar to

the CC10 of other species<sup>73,85</sup>, and that it also contains the necessary cysteines for disulfide bridge supported dimerization.

To our knowledge, this is the first report of a western blot being used to selectively measure endogenous ovine CC10 protein. Based on these data, any future investigation into the effects of intervention on endogenous CC10 protein production, can be analyzed in this animal model. Using the appropriate respective antibodies and western blot, it is now possible to measure both exogenous rhCC10 protein and endogenous CC10 protein produced within the same tissue. In addition, using this approach we have demonstrated an age-dependent difference in endogenous CC10 in the preterm lamb that mirrors the human. This finding expands the clinical relevance of this animal preparation as a translational surrogate in lieu of the human preterm infant and adult.

While the discovery of the ovine CC10 gene and further development of CC10 western blot techniques are important findings, our primary goal in this work was to analyze the impact of rhCC10 on endogenous CC10. We demonstrated that rhCC10 treatment increases local lung tissue rhCC10 and that this is correlated with a decrease in inflammation, as represented by IL-8 and TNF- $\alpha$  proteins and IFN- $\gamma$  mRNA expression. This work demonstrates that exogenous rhCC10 accumulates in the lung tissue, that pro-inflammatory cytokines are reduced as a function of exogenous rhCC10 in the lung. Furthermore, endogenous CC10 production is decreased as a function of both exogenous rhCC10 and inflammation. While direct effects of rhCC10 on endogenous CC10 production can not be ruled, it is known that as production of CC10 is naturally

controlled by pro-inflammatory cytokine mediated promoters, including IFN- $\gamma$  and TNF- $\alpha$ ,<sup>56, 58, 97</sup> and should self-regulate wherein a decrease in inflammation will decrease endogenous CC10 production. It is therefore reasonable that the rhCC10 treatment is decreasing the stimulation for endogenous CC10 production through decreased cytokine production. We further demonstrate that this is the case through our comparison of the relationships between lung tissue exogenous rhCC10 protein, endogenous CC10 protein, and IL-8 protein wherein the relationship between endogenous CC10 and IL-8 is stronger than with tissue rhCC10 . Our data demonstrates that rhCC10 treatment mitigates lung inflammation and induces physiological regulatory feedback of endogenous CC10 in the immature, CC10 deficient lung (Figure 1-16). While it is important that the effect is to reduce lung inflammation, the impact on endogenous CC10 production may be an important biomarker to monitor to provide guidance for rhCC10 dosing strategies in lungs that are already CC10 deficient, such as in the preterm<sup>50, 55</sup> or in patients with COPD<sup>8, 84</sup>.



**Figure 1-16: Working Model of Regulation of CC10 by rhCC10 Treatment.** A) Pro-inflammatory cytokines bind to their respective membrane receptor, initiate their signaling pathway and transcription factors are translocated into the nucleus. CC10 gene transcription increases and mRNA is translocated out of the nucleus for translation into protein. CC10 protein is processed and secreted into the extracellular environment. B) With the addition of rhCC10 in the extracellular environment, there is a reduction of inflammation and a decreased levels of pro-inflammatory cytokines. This leads to a reduction in CC10 gene transcription, CC10 mRNA expression, and CC10 protein.

Overall, we have developed new approaches for identifying the effects of rhCC10 treatment on the immature lung in the preterm lamb. We have used these tools to analyze the dose response effect of rhCC10 on endogenous CC10 at the protein and mRNA level. We have shown that rhCC10 decreases endogenous CC10 in a dose dependent manner in the preterm lamb lung coinciding with previous data showing a dose dependent decrease in inflammation. Finally, we have provided supportive evidence that the impact of

rhCC10 on endogenous CC10 is more tightly coupled to the decrease in inflammation as opposed to local tissue rhCC10. Future work will expand the use of these newly developed tools to analyze developmental differences in endogenous CC10 production, to investigate other methods of action of rhCC10, and further effects of rhCC10 on the lung and other organ systems.

## CHAPTER 2: DOSE RESPONSE EFFECTS OF RHCC10 ON TLR 4 SIGNALING AND PRODUCTION

### Introduction

Despite advances in the standard of care for the treatment of Respiratory Distress Syndrome (RDS) that have lowered patient mortality, long term morbidity, such as the development of Bronchopulmonary Dysplasia, has not been successfully addressed<sup>4, 5</sup>. While surfactant therapy and lung protective ventilation strategies biomechanically improve lung function, they have not adequately decreased the inflammation seen in RDS<sup>5, 37, 83</sup>. Furthermore, use of supplemental oxygen increases the production of reactive oxygen species in the lung which will lead to greater inflammatory damage<sup>11, 69</sup>. Also, the use of prenatal and antenatal steroids is still debatable due to contraindicative effects on development of the infant<sup>26, 95</sup>. Overall, new treatments that augment surfactant therapy are required that will treat the acute iatrogenic inflammation seen in RDS and thereby prevent the digression into bronchopulmonary dysplasia .

Club Cell Secretory Protein 10 (rhCC10) is an anti-inflammatory protein produced by airway nonciliated epithelial cells. It is known to inhibit sPLA<sub>2</sub> and fibronectin and is associated with modulated NF-kB regulated inflammatory cytokines<sup>2, 47, 48, 50</sup>. In its recombinant form, it has been shown that in supplementation to surfactant treatment in clinical and preclinical trials and to decrease multiple inflammatory biomarkers, increase vascular endothelial growth factor and surfactant protein A mRNA expression, and modify metalloproteinase activity<sup>65, 66, 96</sup>.

Toll-Like Receptors (TLRs) are important regulators of immune system function. In the lung TLRs are expressed on the cell membrane surface of epithelial cells and leukocytes and in endosomes within leukocytes such as alveolar macrophages. There are 12 known TLRs and they recognize pathogen associated molecular patterns (PAMPs) such as Lipopolysacchride (LPS) by TLR 4<sup>7, 19, 57, 63, 67, 69, 94</sup>. Several of the TLRs also recognize similar structured endogenous damage associated molecular patterns (DAMPs)<sup>80</sup>.

DAMPs are produced in the inflamed lung, when non-programmed cell death causes the biomolecular compounds that are usually sequestered by the cellular membrane components to enter the extracellular space and interact with membrane receptors on other nearby cells<sup>95</sup>. Examples of DAMPs include mRNA and heat shock protein recognition by TLR 3 and 4 respectively.

Multiple groups have demonstrated that TLRs play an integral role in lung inflammation across a variety of disorders and that inhibiting TLR function causes a decrease in inflammation<sup>57, 63, 67, 69, 89, 90, 94</sup>. Currently, rhCC10 is known to have several anti-inflammatory effects through direct competitive inhibition but the sum of its known action does not account for the total anti-inflammatory action of rhCC10. We propose that this effect may be due in part to its effect on toll-like receptor function and activity. To address this issue, a preterm lamb lung injury model was first characterized vs injured adult sheep lung and then used to investigate the effect of rhCC10 on TLR activity and expression. Multiple biomarkers within the TLR signaling pathway as well as TLR expression were measured by qPCR of mRNA expression and western blot protein

analysis as appropriate. We hypothesize that rhCC10 will lower toll-like receptor activity and expression in a dose response manner.

## **Methods**

### *Animal Protocol and Tissue Collection*

Frozen lung tissue used in this project was derived from a previous study<sup>83</sup>. In brief, lambs (gestational age of  $126 \pm 3$  days,  $n = 24$ ,  $n = 6$  per group) were anesthetized, instrumented, delivered by cesarean section and ventilated with 100% oxygen. Lambs were randomized to receive no surfactant or rhCC10 treatment, surfactant treatment alone (Survanta<sup>®</sup>, Ross laboratories 4 mL/kg, 100 mg/kg) or surfactant followed by one of two doses of intratracheal rhCC10 (0.5 mg/kg or 1.5 mg/kg). In addition, adult sheep ( $n = 12$ ) were anesthetized and instrumented in a similar manner and ventilated with 100% oxygen or room air. All animals were followed for 4 hours, after which lungs were harvested for frozen and fixed tissue collection. Following the animal study, we used a custom designed ELISA to measure IL-6 and IL-8 of lung tissue homogenate after 4 hours of ventilation<sup>83</sup>. The experimental protocols and procedures for this study were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine and conducted in accordance with NIH and AALC guidelines.

### *RNA extraction and cDNA synthesis*

Frozen lung tissue was homogenized from previous studies<sup>83</sup> and processed using the Qiagen RNeasy miniprep kit (Qiagen, Valencia, CA). RNA concentration was determined by measuring absorbance at 260nm and then diluted to a concentration of 1

ug/mL. cDNA synthesis was then performed using the Revertaid™ First Strand Synthesis Kit (Fermentas, Glen Burnie, MD).

#### *Quantitative Real-Time PCR*

Primers were available for ovine TLR 4, IFN- $\gamma$  and IL-10 and purchased from Eurofins Operon (. Using Syber Green technology, CC10 gene expression was measured using a Mastercycler® ep Realplex (Eppendorf, Hauppauge, NY) and normalized by 18S mRNA expression <sup>29</sup>.

#### *Western Blot*

Frozen lung tissue was homogenized in RIPA solution and the protein concentration of the supernatant was measured using the Bradford method and aliquots of the supernatant were diluted to a uniform concentration. SDS-PAGE was performed using a uniform volume of each sample. Following transfer, a western blot was performed using rabbit anti-human IKK (L570, Cell Signaling), anti-pIKK (Ser 176/180) (16A6, Cell Signaling), anti-pI $\kappa$ B (Ser 32) (14D4, Cell Signaling), anti-TLR (H-80, Santa Cruz) and HRP conjugated goat-anti-rabbit (Millipore) antibodies for primary and secondary antibodies respectively. As reported by the manufacturer, there was no cross reactivity between phosphorylated and unphosphorylated analogs. GAPDH was used for normalization with mouse anti-human GAPDH (ABD Serotec) with HRP conjugated anti-mouse (Millipore) as primary and secondary antibodies respectively. I $\kappa$ B was measured with a mouse anti-human I $\kappa$ B (Cell Signaling) as the primary antibody. After reaction with substrate,

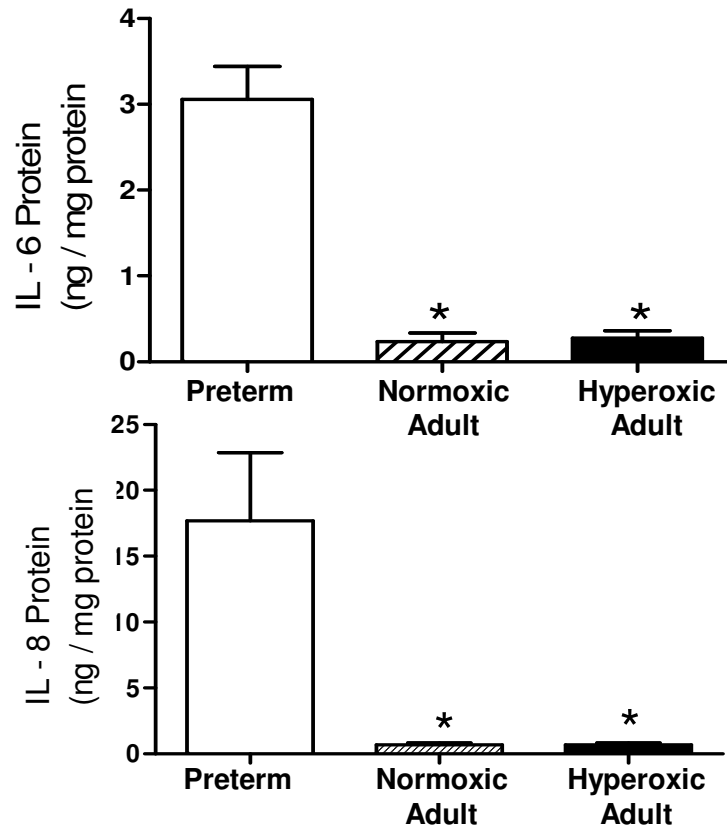
images were taken (LAS-3000 ; Fujifilm, Valhalla, NY) and band intensity was measured (Multi Gauge Software; Fujifilm).

### *Data Analysis*

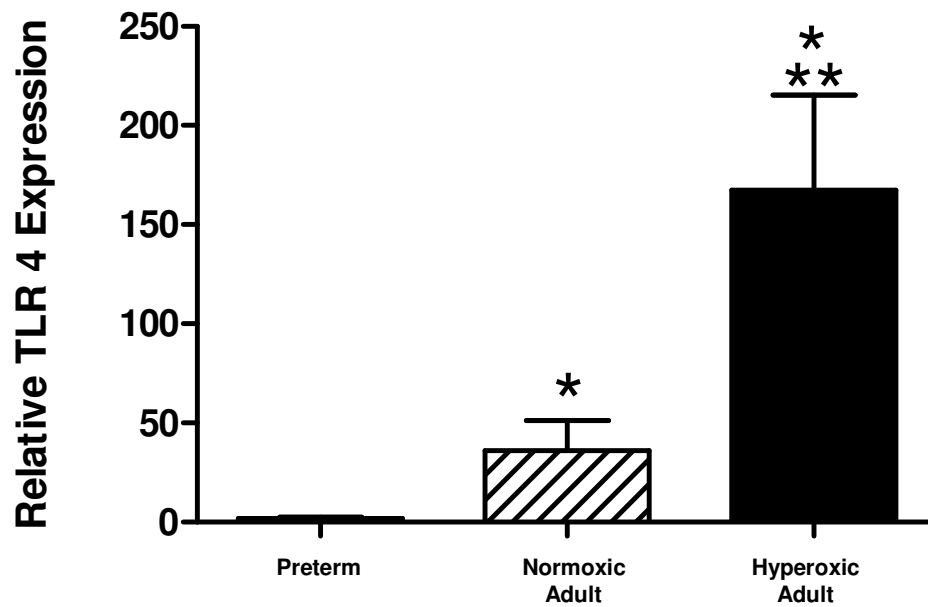
All data was imported into Microsoft Excel (Microsoft, Redmond, WA) and analyzed by ANOVA using Prism 5.0 (Graphpad, La Jolla, Ca). Values are expressed as mean  $\pm$  SEM and significance was accepted at  $p < 0.05$ .

### **Results**

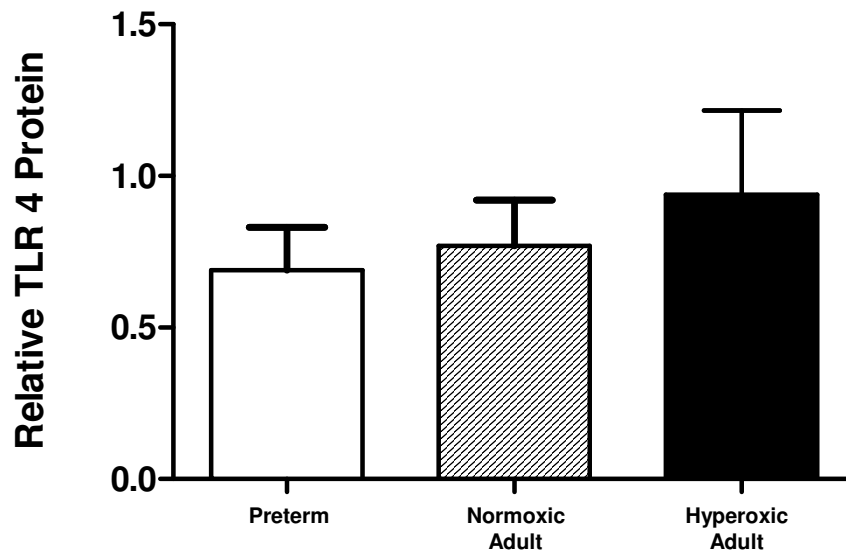
The untreated preterm lamb lung had increased ( $*p < 0.05$ ) IL-6 and IL-8 protein as compared to lungs from both hyperoxic and normoxic ventilated adult ovine lung (Figure 2-1). The hyperoxic adult ovine lung had the highest ( $**p < 0.05$ ) TLR 4 mRNA expression, but both adult groups had increased ( $*p < 0.05$ ) TLR 4 mRNA expression as compared to the preterm lamb (Figure 2-2). However, there was no difference in the TLR 4 protein between all three groups (Figure 2-3 and 2-6A). The preterm lamb lung and the hyperoxic adult ovine lung had a similar level of TLR activity, as indicated by IKK phosphorylation, and a similar level of NF-kB activity, as indicated by I $\kappa$ B phosphorylation. The normoxic ventilated adult ovine lung had the lowest ( $*p < 0.05$ ) IKK and I $\kappa$ B phosphorylation, respectively (Figures 2-4 : 2-6B & C).



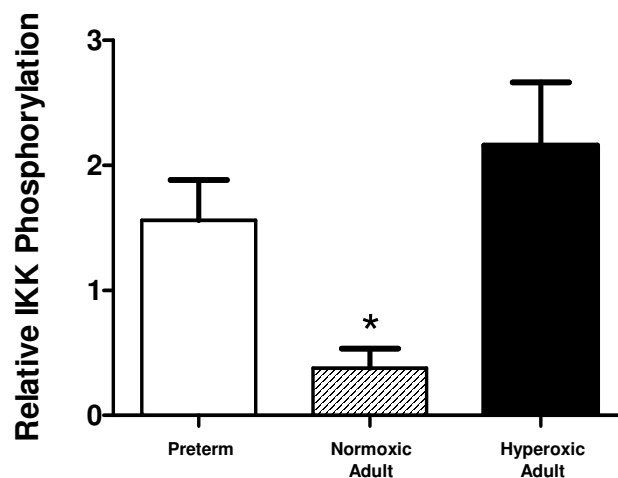
**Figure 2-1: Comparison of Preterm and Adult Ovine Lung IL-6 and IL-8 Protein.** The preterm lamb had increased IL-8 and IL-6 protein as compared to both adult sheep groups. There was no difference between the two adult sheep groups. (Mean  $\pm$  SE; n = 6 per group)



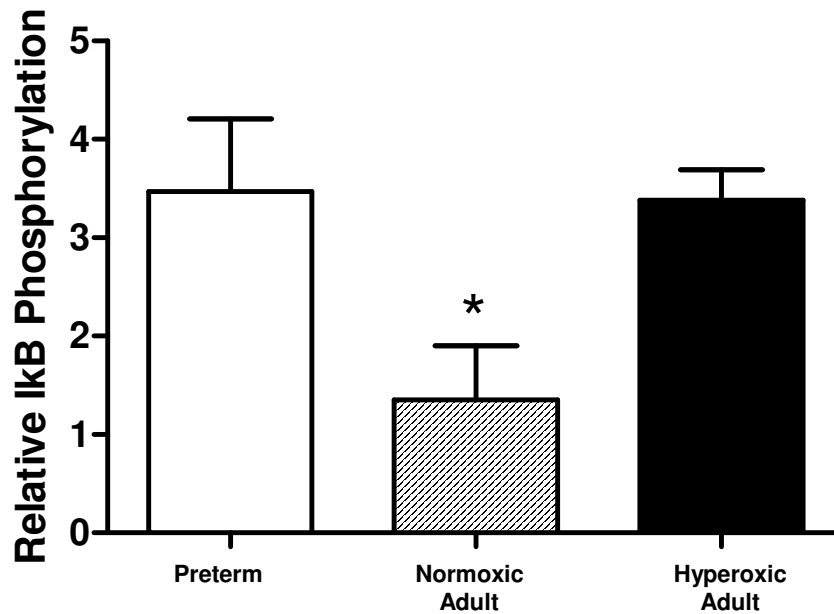
**Figure 2-2: Comparison of Preterm and Adult Ovine Lung TLR 4 Expression.** The hyperoxic adult sheep had the highest (\*\* $p < 0.05$ ) TLR 4 mRNA expression. Both adult sheep groups had a greater (\* $p < 0.05$ ) TLR 4 mRNA expression than the preterm lamb. (Mean  $\pm$  SE;  $n = 6$  per group)



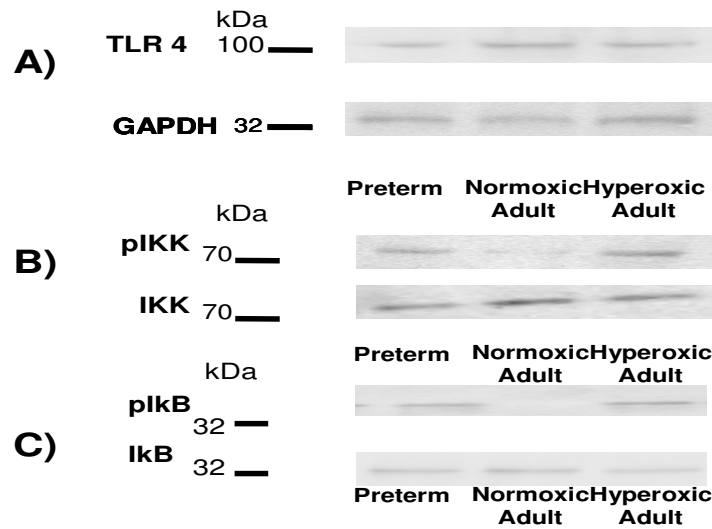
**Figure 2-3: Comparison of Preterm and Adult Ovine Lung TLR 4 Protein.** TLR 4 protein was normalized by GAPDH protein. There was no difference in the TLR 4 Protein between the three groups. (Mean  $\pm$  SE; n = 6 per group)



**Figure 2-4: Comparison of Preterm and Adult Ovine Lung TLR 4 Activity.** There was no difference in the relative phosphorylation of IKK between the preterm and hyperoxic adult sheep. The normoxic sheep had a decreased (\* $p < 0.05$ ) relative IKK phosphorylation. (Mean  $\pm$  SE; n = 6 per group)

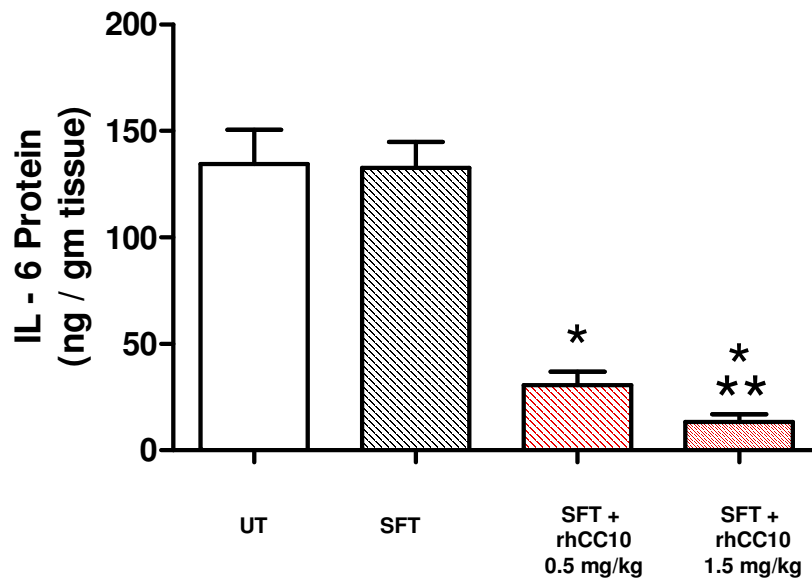


**Figure 2-5: Comparison of Preterm and Adult Ovine Lung NF-κB Activity.** There was no difference in the relative phosphorylation of IκB between the preterm and hyperoxic adult sheep. The normoxic sheep had decreased (\* $p < 0.05$ ) relative IκB phosphorylation compared to the other groups. (Mean  $\pm$  SE;  $n = 6$  per group)

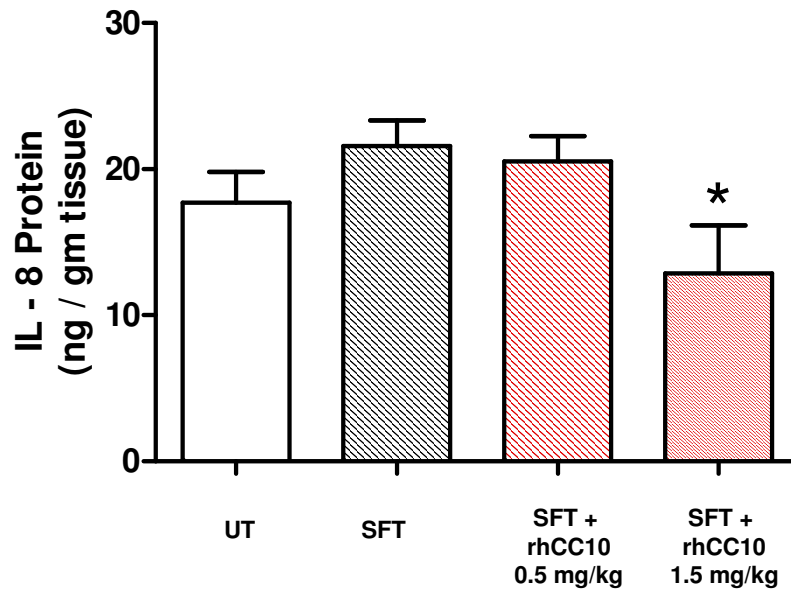


**Figure 2-6: Representative Western Blots of the Developmental Differences on TLR Mediated Inflammation.** Presented here are: A) Representative bands of TLR, normalized by GAPDH B) Representative bands of pIKK, normalized by IKK C) Representative bands of pIκB, normalized by IκB. The molecular weights of all bands coincide with the manufacturer specifications for the respective antibodies. All samples were derived at the same time and processed in parallel.

In mechanically ventilated, surfactant-treated preterm lambs with RDS, rhCC10 mitigated the pro-inflammatory profile in the lung<sup>83</sup>. Specifically, in a dose response manner, rhCC10 lowered IL-6 and IL-8 protein in homogenized lung tissue (Figure 2-7 and 2-8). The 1.5 mg/kg rhCC10 treated group had the lowest  $** (p < 0.05)$  lung IL-6 protein, with the 0.5 mg/kg rhCC10 group having decreased  $(*p < 0.05)$  IL-6 protein as compared to the surfactant only and untreated groups no significant differences in IL-6 protein between the other three groups. Animals treated with 1.5 mg/kg rhCC10 had decreased  $(*p < 0.05)$  IL-8 protein compared to the 0.5 mg/kg rhCC10, surfactant only, and untreated preterm lamb group.

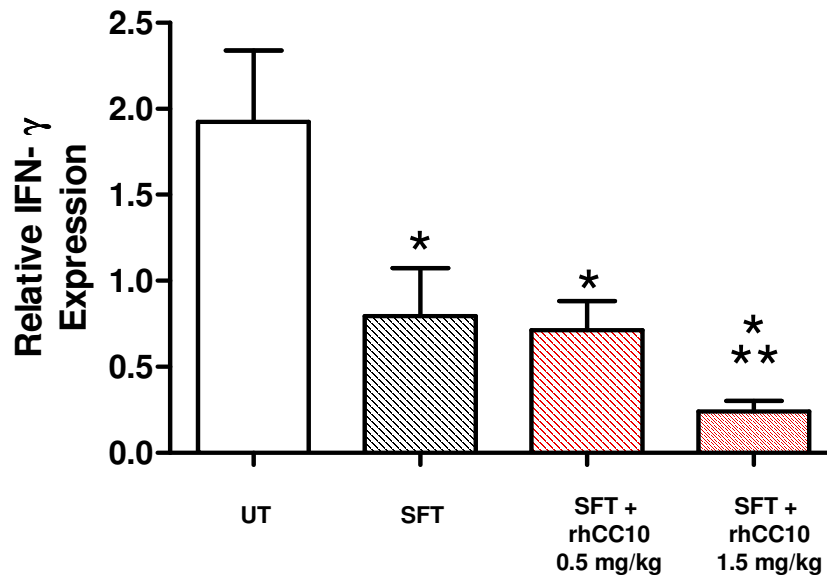


**Figure 2-7: Dose Response Effect of rhCC10 on IL-6 Protein.** There was no difference in the IL-6 protein between untreated and surfactant treated groups. The 0.50 mg/kg and 1.5 mg/kg rhCC10 groups had lower (\* $p < 0.05$ ) IL-6 protein than the untreated and surfactant treated groups. The 1.5 mg/kg rhCC10 treated group was lower (\*\* $p < 0.05$ ) than the 0.5 mg/kg rhCC10 treated group. (Mean  $\pm$  SE ;  $n = 6$  per group)

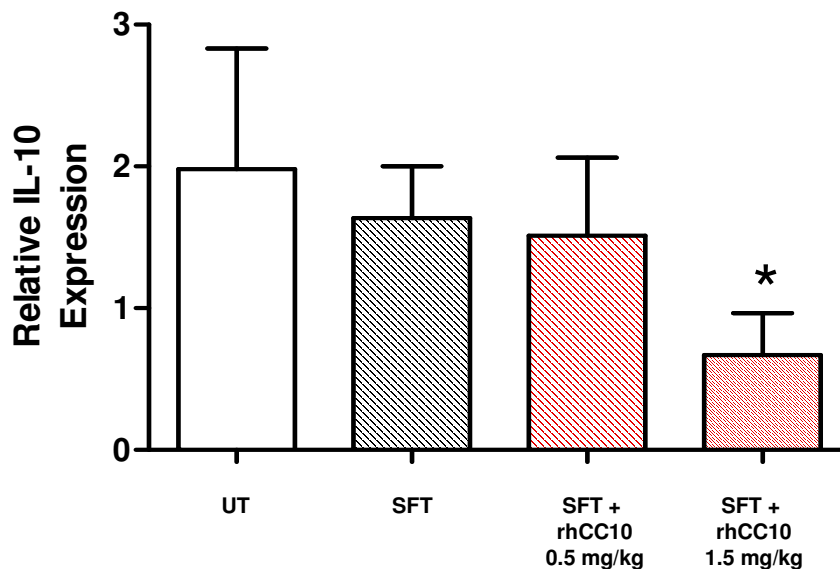


**Figure 2-8: Dose Response Effect of rhCC10 on IL-8 Protein.** There was no difference between the UT, SFT, and 0.50 mg/kg rhCC10 treated groups. The 1.5 rhCC10 group had lower (\* $p < 0.05$ ) IL - 8 than all other groups. (Mean  $\pm$  SE ; n = 6 per group)

There was also an rhCC10 mediated dose response effect in IFN- $\gamma$  and IL-10 mRNA expression (Figures 2-9 and 2-10). Compared to untreated control, surfactant alone and surfactant + 0.5 mg/kg rhCC10 both had decreased (\* $p < 0.05$ ) IFN- $\gamma$  mRNA expression. The 1.5 mg/kg rhCC10 group had the lowest (\* $p < 0.05$ ) IFN- $\gamma$  mRNA expression. The 1.5 mg/kg rhCC10 group had the lowest (\*\* $p < 0.05$ ) IL-10 mRNA expression, with no difference between the other three groups.

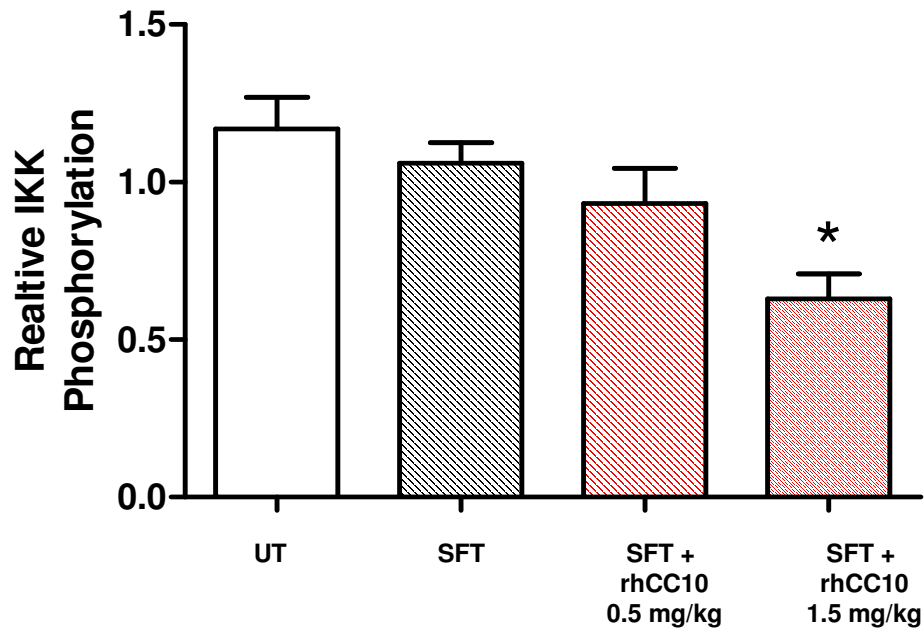


**Figure 2-9: Dose Response Effect of rhCC10 on IFN- $\gamma$  mRNA Expression.** The surfactant treated and both rhCC10 groups had a lower ( $*p < 0.05$ ) IFN- $\gamma$  mRNA expression than the untreated group. The 1.5 mg/kg rhCC10 treated group had the lowest ( $**p < 0.05$ ) IFN- $\gamma$  mRNA expression (Mean  $\pm$  SE ; n = 6 per group).

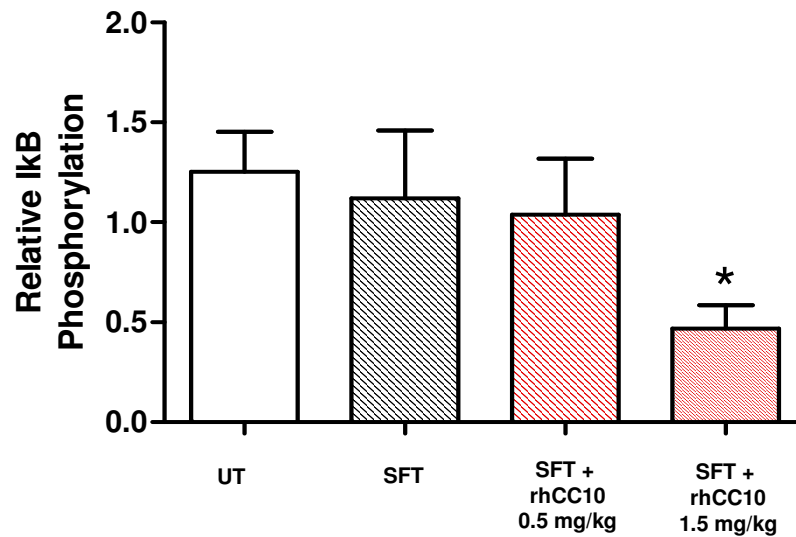


**Figure 2-10: Dose Response Effect of rhCC10 on IL-10 mRNA Expression.** There was no difference in the IL-10 mRNA expression between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a lower (\* $p < 0.05$ ) IL-10 mRNA expression than the other three groups. (Mean  $\pm$  SE ;  $n = 6$  per group)

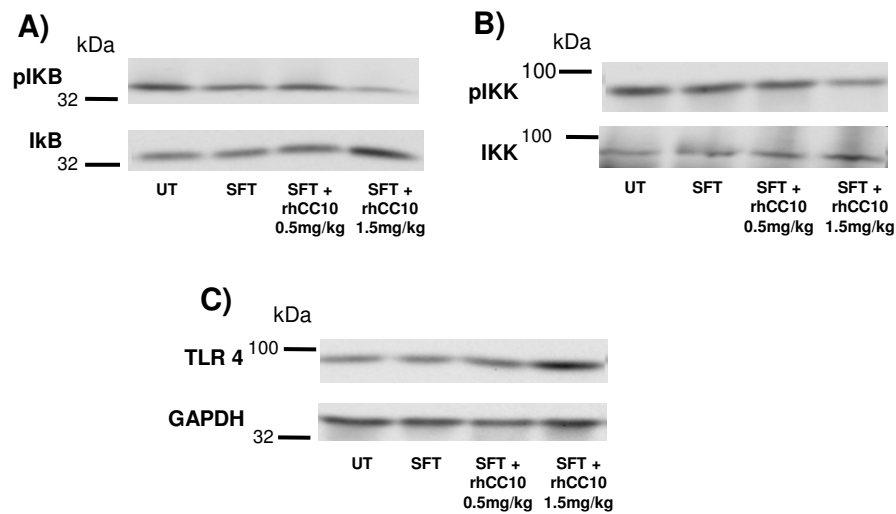
After determining, the effect of rhCC10 treatment on the cytokine profile of the preterm lung, we analyzed two key phosphorylation events in the TLR 4 signaling path. After activation of TLR 4, there are several secondary messenger steps that culminate in the phosphorylation of IKK which in turn activates NF- $\kappa$ B, a key pro-inflammatory transcription factor, by phosphorylation of I $\kappa$ B. Through our use of western blot, we measured the relative activity of IKK and NF- $\kappa$ B. In Figures 2-11, 2-12, and 2-13A & B, we show that the 1.5 mg/kg rhCC10 group had the lowest (\* $p < 0.05$ ) TLR Activity and NF- $\kappa$ B activity, respectively with no difference between the other three groups.



**Figure 2-11: Dose Response Effect of rhCC10 on Relative TLR 4 Activity.** There was no difference in the TLR activity, as represented by pIKK protein normalized by IKK protein, between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a lower (\* $p < 0.05$ ) TLR activity than the other three groups. (Mean  $\pm$  SE ;  $n = 6$  per group)

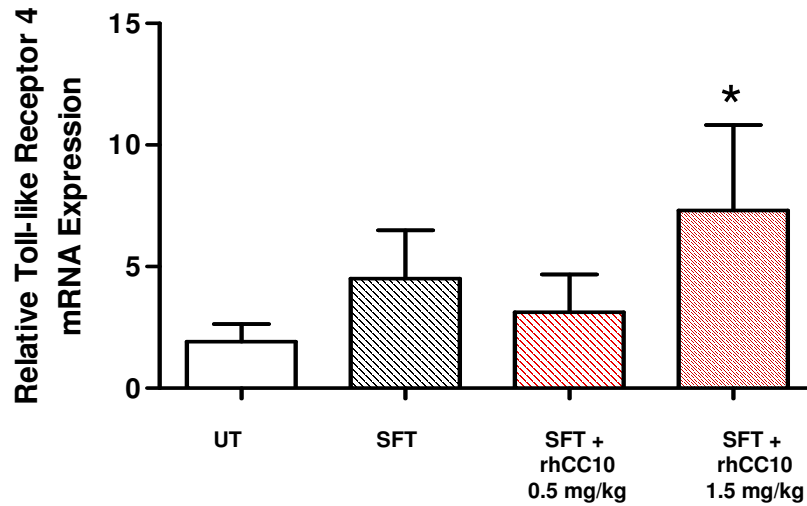


**Figure 2-12: Dose Response Effect of rhCC10 on NF-κB Activity.** There was no difference in the NF-κB activity, as represented by pIκB protein normalized by IκB protein, between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a lower (\* $p < 0.05$ ) NF-κB activity than the other three groups. (Mean  $\pm$  SE ; n = 6 per group)

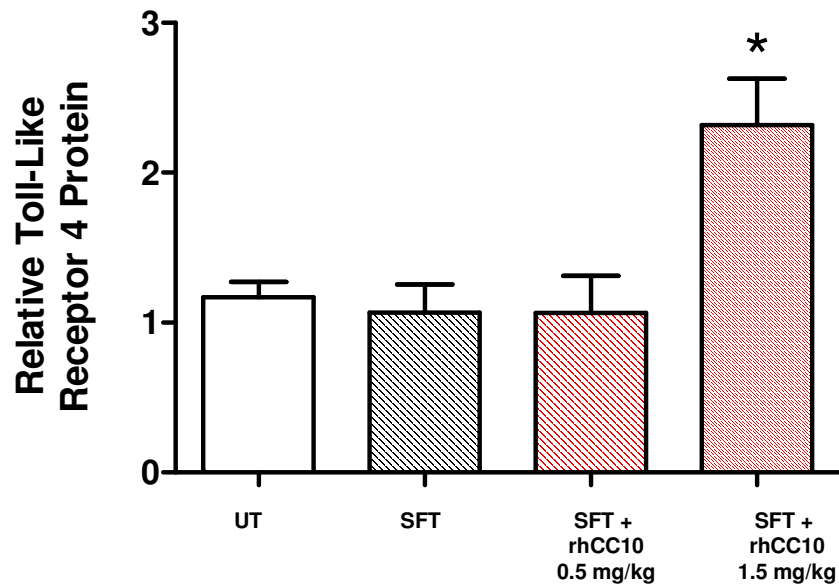


**Figure 2-13: Representative Western Blots of the Dose Dependent Effects of rhCC10 on TLR Mediated Inflammation.** Presented here are: A) Representative bands of TLR, normalized by GAPDH B) Representative bands of pIKK, normalized by IKK C) Representative bands of pIkB, normalized by IκB. The molecular weights of all bands coincide with the manufacturer specifications for the respective antibodies.

We also analyzed the dose dependent effect of rhCC10 on TLR 4 mRNA expression and protein. As presented in Figures 2-13C, 2-14, and 2-15, there is a rhCC10 dose dependent increase in TLR 4 mRNA expression and protein. Respectively, there was no difference in the TLR 4 mRNA expression and TLR 4 protein between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a higher ( $*p < 0.05$ ) TLR 4 mRNA expression and TLR 4 protein than the other three groups.

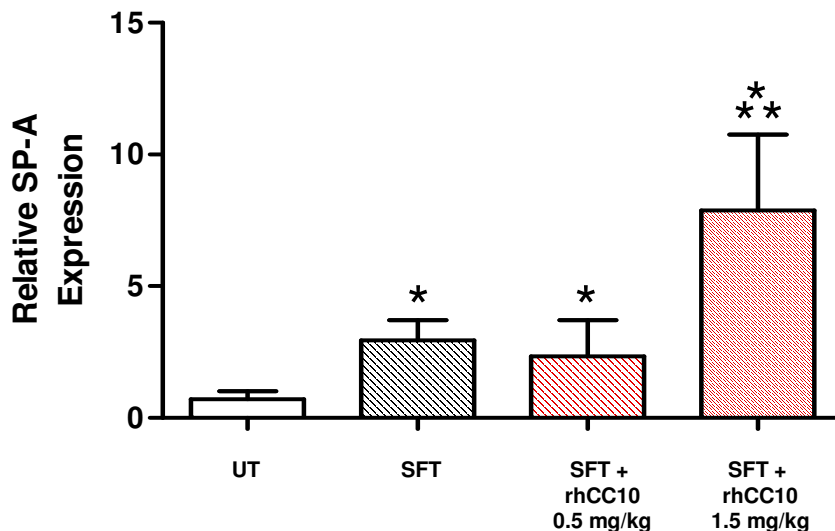


**Figure 2-14: Dose Response Effect of rhCC10 on TLR 4 mRNA Expression.** There was no difference in the TLR 4 mRNA expression between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a higher (\* $p < 0.05$ ) TLR 4 mRNA expression than the other three groups. (Mean  $\pm$  SE ;  $n = 6$  per group)



**Figure 2-15: Dose Response Effect of rhCC10 on TLR 4 Protein.** TLR 4 protein was normalized by GAPDH protein. There was no difference in the TLR 4 protein between untreated, surfactant treated, and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 group had a higher (\* $p < 0.05$ ) TLR 4 protein than the other three groups. (Mean  $\pm$  SE ;  $n = 6$  per group)

As previously described,, surfactant protein A decreases TLR activity while increasing expression<sup>36</sup>. In Figure 2-16, we demonstrate that there is a rhCC10 mediated dose dependent effect on SP-A mRNA expression. The surfactant treated and 0.5 mg/kg rhCC10 group had increased (\* $p < 0.05$ ) SP-A mRNA expression as compared to untreated control. The 1.5 mg/kg group had the highest (\*\* $p < 0.05$ ) level of mRNA expression of SP-A. This coincides with previous work published by our group<sup>96</sup>.



**Figure 2-16: Dose Response Effect of rhCC10 on SP-A mRNA Expression.** All treated groups had an increased (\* $p < 0.05$ ) SP-A mRNA expression compared to untreated. There was no difference between the surfactant treated and 0.5 mg/kg rhCC10 groups. The 1.5 mg/kg rhCC10 treated group had the highest (\*\* $p < 0.05$ ) SP-A mRNA expression. (Mean  $\pm$  SE)

## Discussion

In this study, we first demonstrated that the ventilated preterm lung is in an inflamed state that responds to anti-inflammatory intervention. Across multiple markers of inflammation, we have shown that the preterm lung either has increased, or is no different in, pro-inflammatory biomarkers as compared to the adult ovine lung. As has been reported in the literature, and as we demonstrated in Chapter 1, the preterm lung is known to be deficient in anti-inflammatory protection<sup>55</sup>. The combination of the preterm having increased pro-inflammatory biomarkers and decreased anti-inflammatory biomarkers as

compared to the adult demonstrates that the preterm lung has a greater relative level of inflammation and a greater propensity for injury.

In our evaluation of the effects of rhCC10, we have shown that rhCC10 alters the inflammatory profile in a dose dependent manner. It decreases endogenous IL-6 and IL-8 protein, IFN- $\gamma$  expression, IL-10 expression, I $\kappa$ B phosphorylation, and IKK phosphorylation. This demonstrates that rhCC10 is decreasing TLR 4 mediated inflammation. We have also demonstrated that this effect is independent of changes in IL-10 expression. Due to the importance of TLR mediated inflammation in multiple lung diseases, including Respiratory Distress Syndrome, rhCC10 offers a potential therapeutic to supplement the current standard of care.

Importantly, the decreased TLR 4 activity occurs in contrast to an increase in TLR 4 mRNA expression and protein. We also see an increase in SP-A which coincides with the increased TLR 4 expression and protein and decreased TLR 4 signaling activity. It has been shown in the literature that SP-A can decrease TLR activity while increasing TLR protein<sup>36</sup>. Due to the heterogenous nature of homogenized lung tissue, we cannot determine in this current model if the effect of rhCC10 on TLR 4 function is independent of the other known anti-inflammatory effects of rhCC10. We will further expand this investigation into the effect of rhCC10 on TLR 4 function and determine if it is independent of sPLA<sub>2</sub> using A549 cell culture.

## CHAPTER 3: RHCC10 INDUCED MITIGATION OF TOLL-LIKE RECEPTOR INJURY IN AN ALVEOLAR-LIKE CELL LINE

### Introduction

RhCC10 is a potential therapeutic to combat pulmonary inflammation in a variety of lung disorders<sup>1, 2, 8, 11, 13, 27, 50, 65, 66, 72, 75, 83, 99</sup>, including respiratory distress, as we previously demonstrated in a preterm lamb model. Toll-like receptor signaling, and specifically TLR 4, has been shown to be an integral DAMP receptor for inflammation in the lung<sup>23</sup>. There are currently a lack of known therapeutics to inhibit TLR mediated inflammation.

We have shown that rhCC10 decreases hyperoxia induced TLR 4 activity in the preterm lung. We believe this effect could be mediated by surfactant protein A (SP-A).

Previously, it has been demonstrated by our group that rhCC10 increases SP-A mRNA expression and protein in the premature lung<sup>96</sup>. rhCC10 has also been shown to protect against lung injury from sPLA<sub>2</sub> IIA and IB mediated surfactant degradation<sup>25, 60</sup>. It has also been shown that SP-A modulates TLR expression while mitigating TLR signaling<sup>36</sup>.

We will seek to further test this notion and also determine if the rhCC10 mitigation of TLR induced inflammation is independent of the known mechanisms of rhCC10 action, competitive inhibition of PLA<sub>2</sub> and inhibition of fibronectin<sup>2, 47-49, 98</sup>. We will also determine if rhCC10 is mitigating TLR activity and expression through muc-1 or IL-10 immunoregulation<sup>20, 42, 44, 70, 90, 93</sup>

The lung is a heterogenous environment with many cell types present which limits the usefulness of an *in vivo* model in isolating the mechanism of the effect of rhCC10 on

TLR 4. A homogenous culture model of lung-like cells offers a way to tackle this question. Specifically, this will eliminate any effect of fibronectin induced immune cell migration and will afford us the ability to minimize effects of sPLA<sub>2</sub> IIA while still measuring the effect of rhCC10 on TLR 4 expression and signaling. Using A549 cells, a pneumocyte type II like cell, we used two strategies to determine the effect of rhCC10 on TLR 4 activity. The first measured the inflammatory response to TLR 4 specific agonist, LipopolySaccharide (LPS) with and without rhCC10 treatment. The second was to knockdown sPLA<sub>2</sub> and then measure the effect of rhCC10 treatment in response to respective pro-inflammatory stimuli LPS and hyperoxia. The knockdown of sPLA<sub>2</sub> IIA, was performed using a lentivirus against cPLA<sub>2</sub> IVA, an upstream enzyme required for multiple types of sPLA<sub>2</sub> activation<sup>3</sup>. By knocking down a broader swath of sPLA<sub>2</sub> enzymes, we therefore minimized any compensation for loss of sPLA<sub>2</sub> IIA by other types of sPLA<sub>2</sub>. Overall, this dual prong stepwise approach, allowed us to determine if rhCC10 mediates toll-like receptor 4 and if this effect is independent of sPLA<sub>2</sub> and fibronectin.

This study expands upon previous work where rhCC10 increased surfactant protein A and TLR 4 expression and protein, while decreasing TLR 4 mediated inflammation in a surfactant treated preterm lamb lung. In the injured lung, sPLA<sub>2</sub> degrades surfactant protein A. In culture, surfactant protein A has been shown to increase TLR expression while decreasing activity<sup>36</sup>. It is therefore reasonable to question if the effect of rhCC10 on TLR 4 signaling is mediated through surfactant protein A. .

## Methods

### *Cell Culture*

A549 cells (Passage 80; ATCC) , were grown to 90% confluence, as determined by visual inspection, in F-12K culture medium (ATCC), supplemented with penicillin (100 units / mL ; ATCC) and streptomycin (100 ug / mL ; ATCC) and 10% fetal bovine serum (ATCC) and maintained at 37 °C, 95% humidity, and 21% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub><sup>21, 31, 69, 76</sup>

### *Transfection*

6 groups of plasmids containing puromycin resistance and shRNA targeting cPLA<sub>2</sub> Group IVA will be purified from qualified MISSION<sup>®</sup> bacterial stocks (Sigma-Aldrich, St. Louis, MO) using the PerfectPrep Endofree Maxi Kit (5Prime, Gaithersburg, MD). They, along with plasmids for lentivirus envelope and scaffold was transfected using TransIT\*-LT1 Transfection Reagent (Mirus Bio, Madison, WI) into HEK293 cells and cultured for 24-48 hours. Virus containing media was collected and used to expose wildtype A549 cells (Passage 83) to respective virus. Transfected A549 cells were selected by puromycin 48 hours later. After reaching 90% or greater confluence, cPLA<sub>2</sub> Group IVA mRNA expression of each respective group was then assessed by qPCR and sPLA<sub>2</sub> IIA activity was assessed by sPLA<sub>2</sub> activity assay (Cayman Chemical, Ann Arbor, MI) and compared to wildtype A549 cells respectively. The group with the lowest sPLA<sub>2</sub> activity was selected to be used as the sPLA<sub>2</sub> knockdown for this study. To further assure sPLA<sub>2</sub> knockdown, an shRNA resistant cPLA<sub>2</sub> rescue plasmid was constructed (Blue Heron Bio, Bothell, WA). The cell group selected by the sPLA<sub>2</sub> assay was then transfected with the shRNA resistant plasmid using TransIT\*-LT1 Transfection reagent.

The mRNA expression of cPLA<sub>2</sub> Group IVA and sPLA<sub>2</sub> activity was then measured to verify rescue of sPLA<sub>2</sub> function.

#### *Hyperoxia Exposure*

A549 cell culture plates (Passage 88) were divided into eight groups total, with at least a N of 6 culture plate wells per group. Cells were exposed to (5% CO<sub>2</sub>, balance O<sub>2</sub>) for 24 hours, at 37°C. Similarly, normoxic groups were kept at 37°C. rhCC10 treated groups were pre-treated with 1 mg (0.5 mg / mL) of rhCC10 (APC Biomaterials, LLC, Rockville, MD) in the media immediately prior to exposure<sup>21, 31, 69, 76</sup>. The media was not changed during the exposure period.

#### *LPS Exposure*

A549 cells culture plates (Passage 88) were divided into eight groups total, with at least a N of 6 culture plate wells per group. Cells were be exposed to 10 ug / mL LPS (Irradiated, Cell Culture Grade, Sigma-Aldrich) for 24 hours, at 37°C. Similarly, non-LPS exposed groups were kept at 37°C. rhCC10 treated groups were pre-treated with 1 mg (0.5 mg / mL) rhCC10 (APC Biomaterials) in the media immediately prior to exposure<sup>15, 28, 34, 46, 76</sup>. The media was not changed during the exposure period.

#### *Western Blot*

A549 cells were spun at 1,000 RPM for 3 minutes, the supernatant was aspirated, and the pellet was resuspended in sterile PBS. Following an additional spin, and then aspiration, the pellet was resuspended in sterile PBS. Cells from each sample were counted by hemocytometer in a 20uL aliquot using trypan blue staining. Cells from each sample

were then aliquoted into sterile saline so as to ensure a homogenous cell count (cells / mL) across all samples in all groups. Cells were then homogenized using a 25 gauge needle and sterile syringe. Following boiling, and addition of loading buffer, samples were run in 15% sds-polyacrylamide gels and transferred to a nitrocellulose membrane. Following transfer, a western blot was performed using rabbit anti-human IKK (L570, Cell Signaling), anti-pIKK (Ser 176/180) (16A6, Cell Signaling), anti-pI $\kappa$ B (Ser 32) (14D4, Cell Signaling), anti-TLR (H-80, Santa Cruz) and HRP conjugated goat-anti-rabbit (Millipore) antibodies for primary and secondary antibodies respectively. As reported by the manufacturer, there was no cross reactivity between phosphorylated and unphosphorylated analogs. GAPDH was used for normalization with mouse anti-human GAPDH (ABD Serotec) with HRP conjugated anti-mouse (Millipore) as primary and secondary antibodies respectively. I $\kappa$ B was measured with a mouse anti-human I $\kappa$ B (Cell Signaling) as the primary antibody. After reaction with substrate, images were taken (LAS-3000 ; Fujifilm, Valhalla, NY) and band intensity was measured (Multi Gauge Software; Fujifilm).

#### *Quantitative polymerase chain reaction (qPCR)*

RNA were extracted from A549 cells using the Qiagen (Valencia, CA) RNeasy mini prep kit. RNA concentration was determined by measuring the absorbance at 260nm and then diluting to a concentration of 1 ug/uL. cDNA synthesis was performed on samples using the Fermentas (Glen Burnie, MD) RevertAid cDNA synthesis kit.. The qPCR was performed in a Realplex thermocycler (Eppendorf) using SYBR green as the detection method. Primers for IL-6, IL-8, IFN- $\gamma$ , 18S, and IL-10 were commercially available and

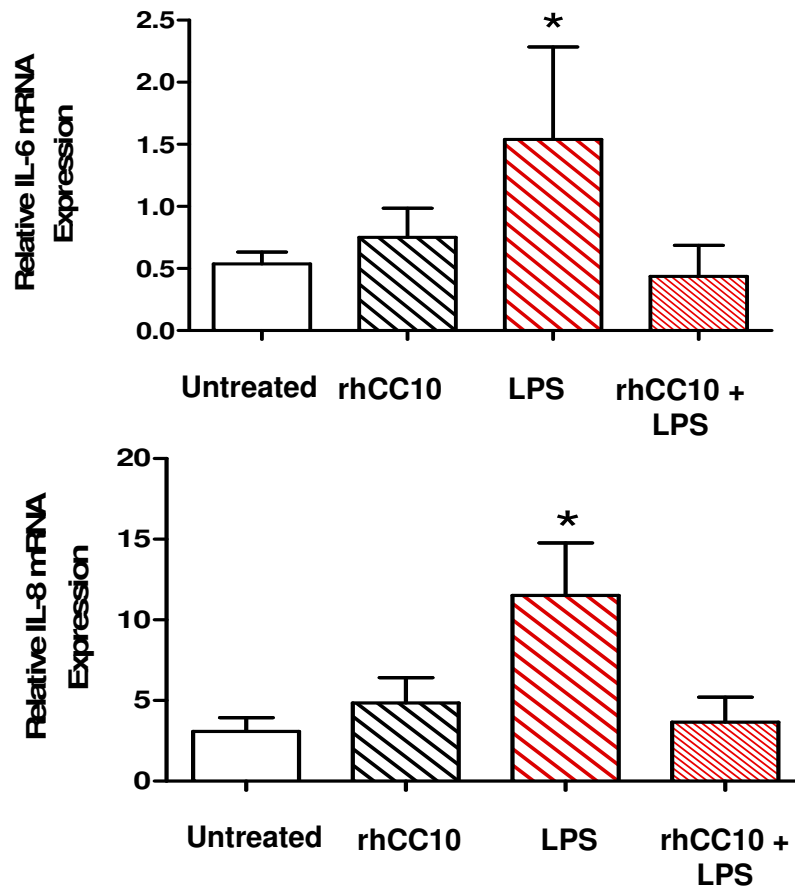
were purchased through Eurofins EWG Operon (Huntsville, AL) and the SYBR Green Master Mix was purchased from Fermentas. 18S mRNA expression was used for normalization.

### *Statistical Analysis*

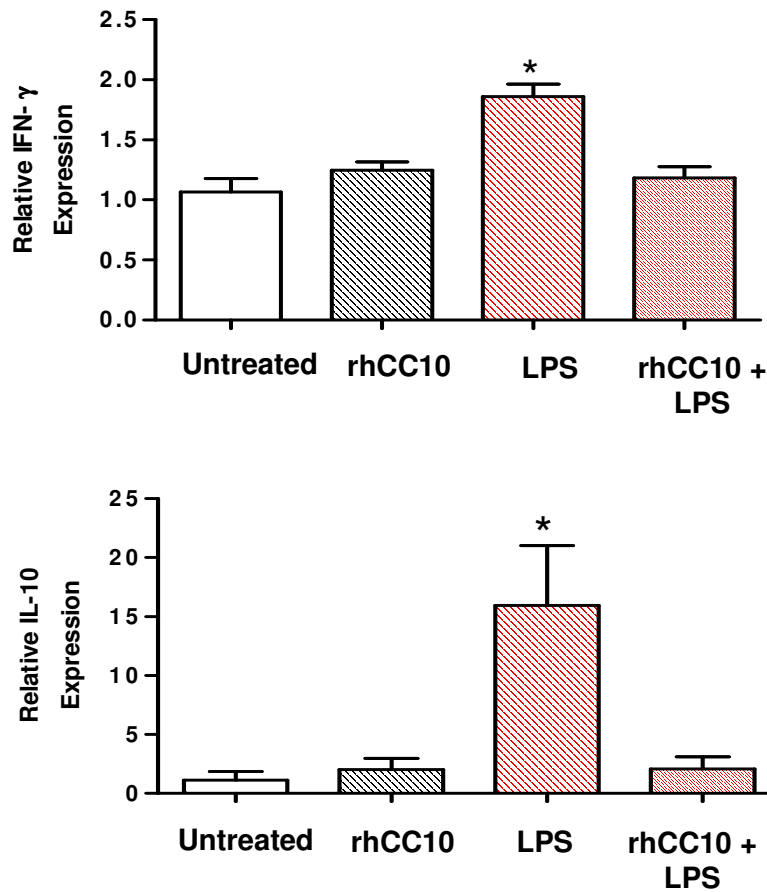
Data from cellular assays is presented as the mean  $\pm$  standard error. Data was analyzed using a multi-factorial analysis of variance for hyperoxia and rhCC10 treatments using Prism 5.0 (Graphpad Software, Inc., San Diego, CA) to determine differences between experimental conditions. Significance was accepted at  $p < 0.05$ .

### **Results**

Characterization of the cytokine profile demonstrated that LPS increased (\* $p < 0.05$ ) IL-6, IL-8, IFN- $\gamma$ , and IL-10 mRNA expression in wild type normoxic A549 cells. rhCC10 mitigated that increase and the respective cytokines responses were no different from unexposed controls. rhCC10 treatment itself did not affect the mRNA expression of the cytokine profile (Figures 3-1 and 3-2).

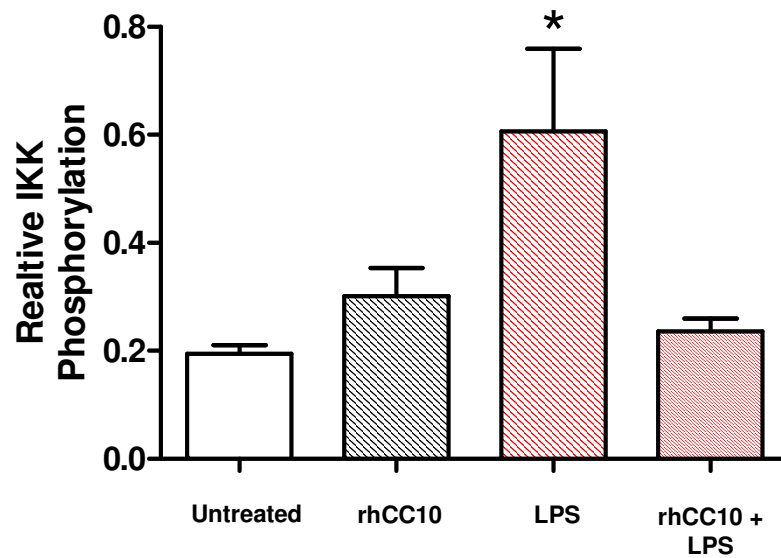


**Figure 3-1: Effect of rhCC10 on LPS induced IL-6 and IL-8 mRNA expression.** LPS exposure increased ( $*p < 0.05$ ) IL-6 and IL-8 mRNA expression. There was no difference in IL-6 and IL-8 mRNA expression between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)

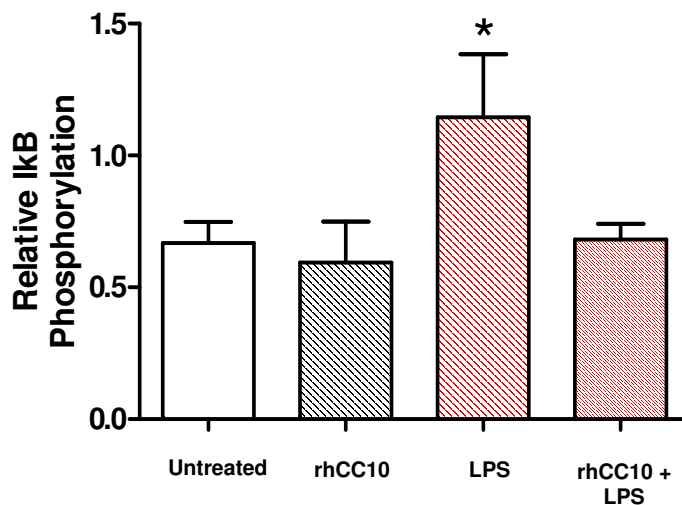


**Figure 3-2: Effect of rhCC10 on LPS induced IFN- $\gamma$  and IL-10 mRNA expression.** LPS exposure increased (\* $p < 0.05$ ) IFN- $\gamma$  and IL-10 mRNA expression. There was no difference in IFN- $\gamma$  and IL-10 mRNA expression between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)

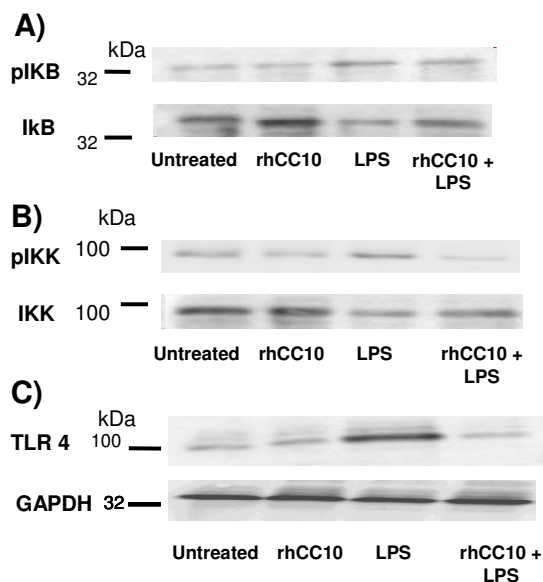
TLR activity and NF- $\kappa$ B activity were measured by the relative ratio of phosphorylated IKK and I $\kappa$ B to their unphosphorylated analogues, respectively. IKK was used an NF- $\kappa$ B independent marker of TLR signaling. As seen in Figures 3-3, 3-4, and 3-5A & B, LPS increases (\* $p < 0.05$ ) TLR and NF- $\kappa$ B activity as compared to unexposed controls. rhCC10 treatment mitigates this increase so as to be no different from unexposed controls.



**Figure 3-3: Effect of rhCC10 on LPS induced Relative TLR 4 Activity.** LPS exposure increased (\* $p < 0.05$ ) relative IKK phosphorylation. There was no difference in IKK phosphorylation between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)

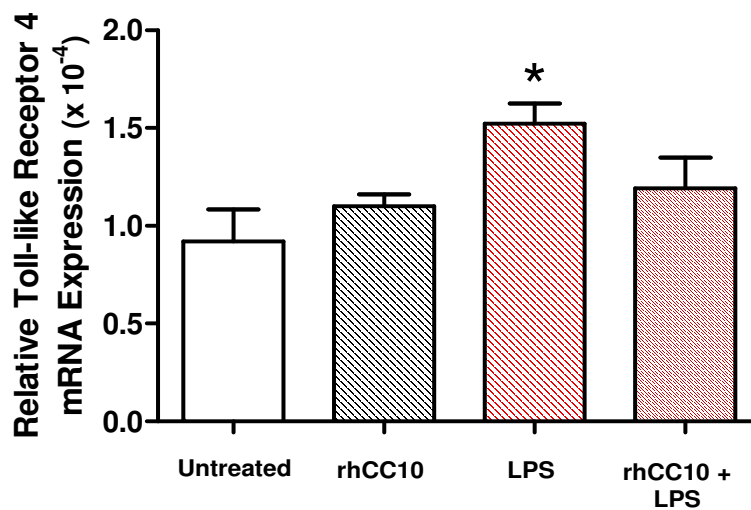


**Figure 3-4: Effect of rhCC10 on LPS induced NF-κB Activity.** LPS exposure increased (\* $p < 0.05$ ) relative IκB phosphorylation. There was no difference in IκB phosphorylation between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean ± SEM;  $n = 6$  per group)



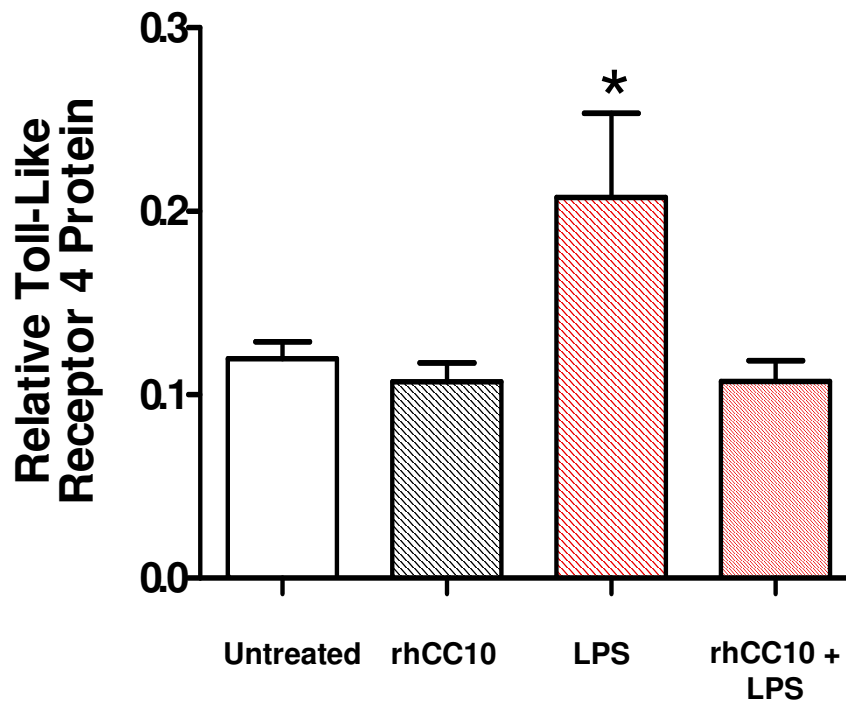
**Figure 3-5: Representative Western Blots of the Effect of rhCC10 on LPS induced Inflammation.** LPS exposure increased ( $p < 0.05$ ) NF-κB activity, as represented by IκB phosphorylation, TLR activity, as represented by IKK phosphorylation, and TLR 4 protein. rhCC10 mitigated the LPS induced inflammation.

LPS exposure increased (\* $p < 0.05$ ) TLR 4 mRNA expression and protein as compared to all other groups (Figures 3-5C, 3-6 and 3-7). rhCC10 treatment mitigated this effect, so as the rhCC10 treated LPS exposed group was no different from unexposed controls. There was not a rhCC10 associated increase in TLR mRNA expression and protein.



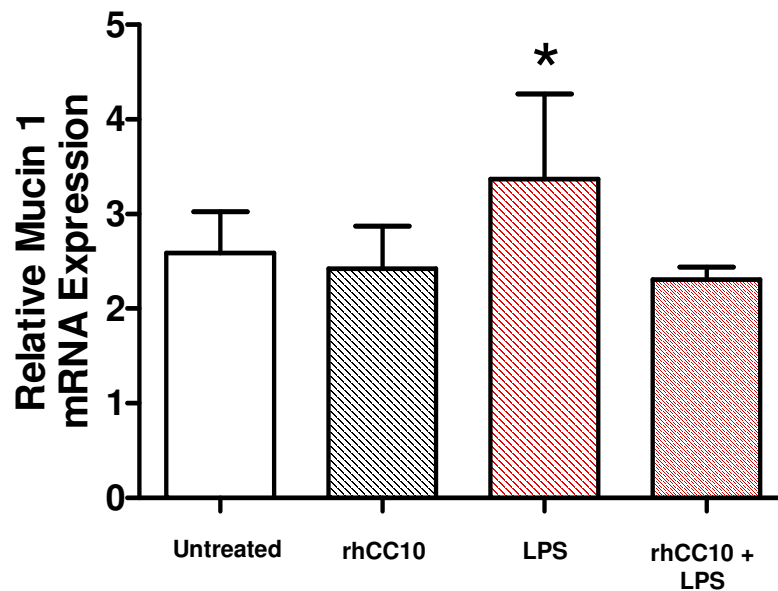
**Figure 3-6: Effect of rhCC10 on LPS induced TLR 4 mRNA expression.**

LPS exposure increased (\* $p < 0.05$ ) TLR 4 mRNA expression. There was no difference in TLR 4 mRNA expression between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)



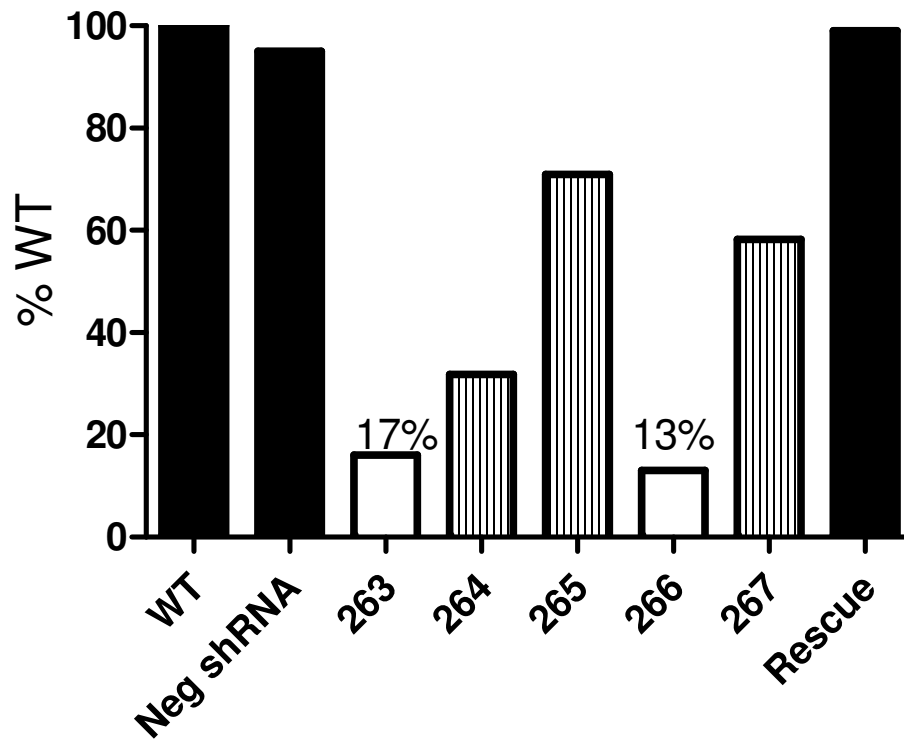
**Figure 3-7: Effect of rhCC10 on LPS induced TLR 4 Protein.** TLR 4 protein was normalized by GAPDH protein. LPS exposure increased (\* $p < 0.05$ ) TLR 4 protein. There was no difference in TLR 4 protein between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)

LPS exposure increased (\* $p < 0.05$ ) mucin 1 expression and rhCC10 treatment mitigated this effect (Figure 3-8). There was no difference in the mucin 1 expression between the other 3 groups. rhCC10 treatment alone did not increase mucin 1.

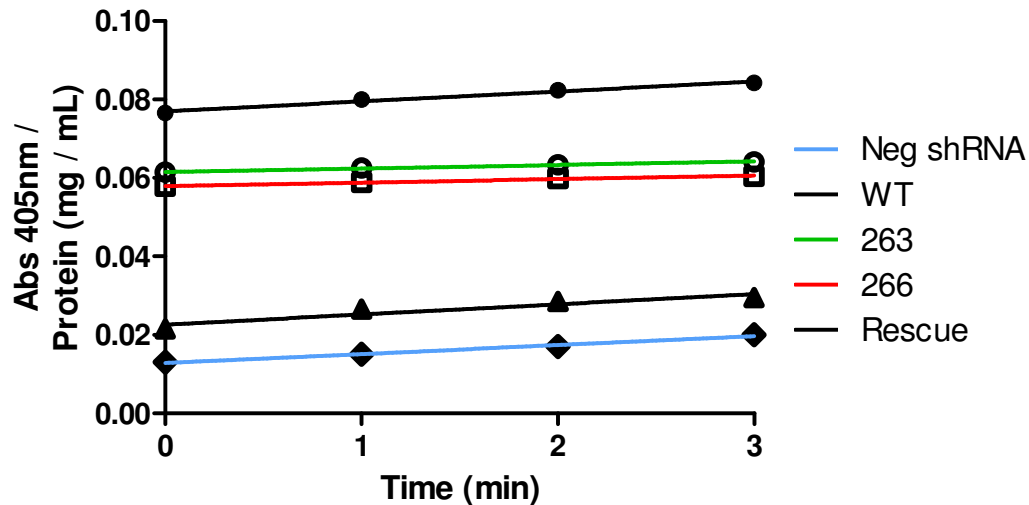


**Figure 3-8: Effect of rhCC10 on LPS induced mucin 1 mRNA expression.** LPS exposure increased (\* $p < 0.05$ ) mucin 1 mRNA expression. There was no difference in mucin 1 mRNA expression between untreated, rhCC10 treated, and rhCC10 treated LPS exposed cells, respectively. (Mean  $\pm$  SEM;  $n = 6$  per group)

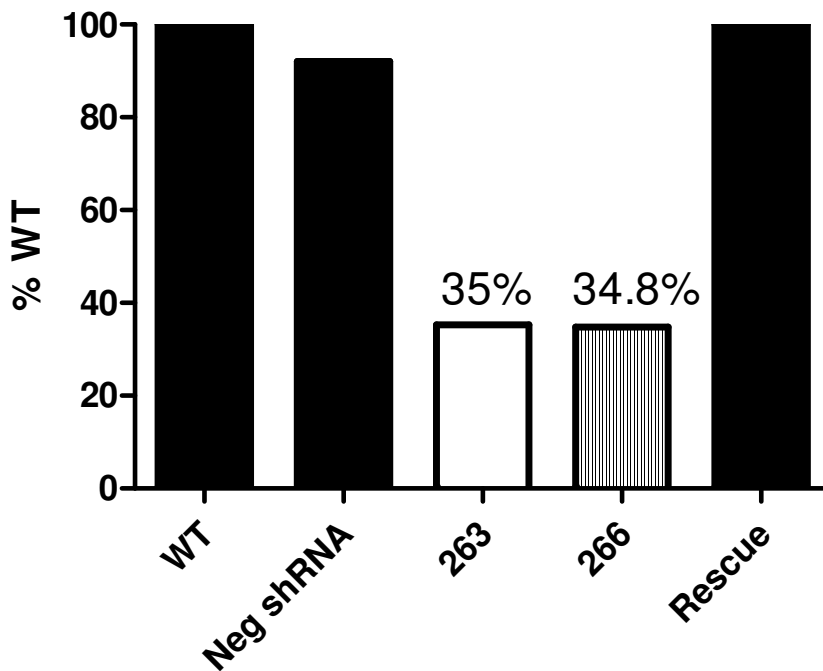
In Figures 3-9 to 3-11, we demonstrate development of a knockdown of sPLA<sub>2</sub> through inhibition of cPLA<sub>2</sub> Group IV. Figure 3-9 displays the relative knockdown of cPLA<sub>2</sub> in the multiple transfected groups as compared to wild type. The negative control shRNA and transfected rescue both are within 10% of wild type cPLA<sub>2</sub> mRNA expression. Two groups, 263 and 266 group have an mRNA below 20%. Upon further analysis using an sPLA<sub>2</sub> activity assay (Figures 33, the 263 and 266 groups are found to be roughly equivalent with an approximate 35% activity of wild type A549 cells. For use in our studies, we selected the 266 group due to its slightly lower sPLA<sub>2</sub> activity.



**Figure 3-9: cPLA<sub>2</sub> mRNA Expression of Cells Transfected with shRNA constructs.** cPLA<sub>2</sub> mRNA expression was measured in 5 groups of transfected cells. Groups transfected with the 263 or 266 had the lowest level of cPLA<sub>2</sub> expression. The negative control transfected group, Neg shRNA had a similar level of cPLA<sub>2</sub> expression to wildtype. Cells from group 266 that were transfected with the rescue cPLA<sub>2</sub> plasmid had a similar expression to wild type.

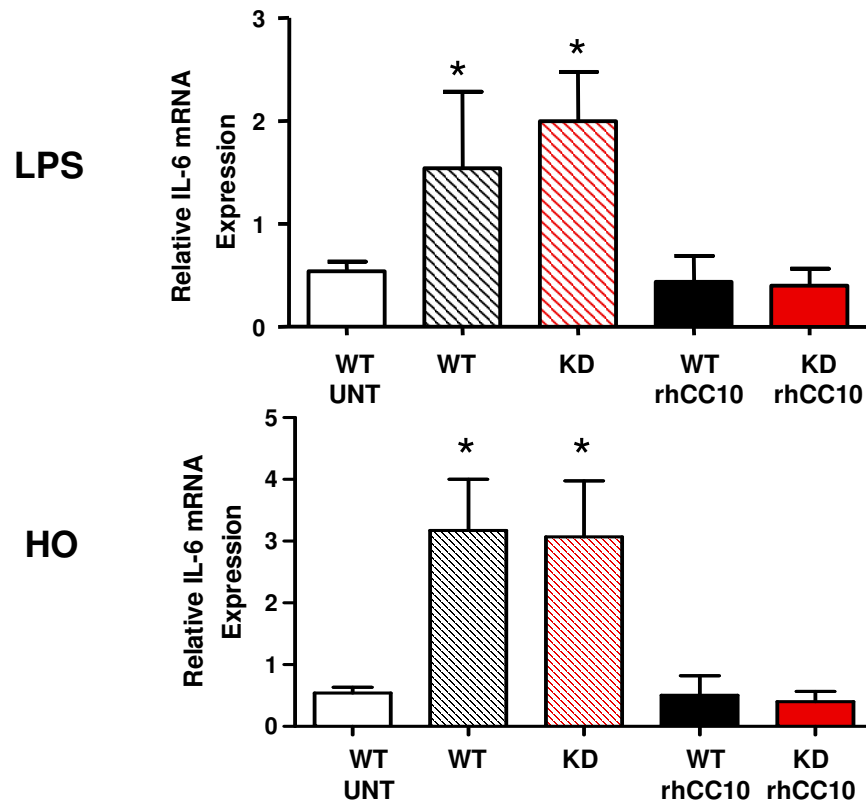


**Figure 3-10: sPLA<sub>2</sub> Activity Assay of Selected Transfected Cell Groups.** sPLA<sub>2</sub> activity, as represented by the slope of the line, was decreased ( $p < 0.05$ ) in the 2 groups of cPLA<sub>2</sub> knockdown transfected cells (263 and 266). There was no difference in sPLA<sub>2</sub> activity between wild type, negative control, and transfected cPLA<sub>2</sub> rescue cells.

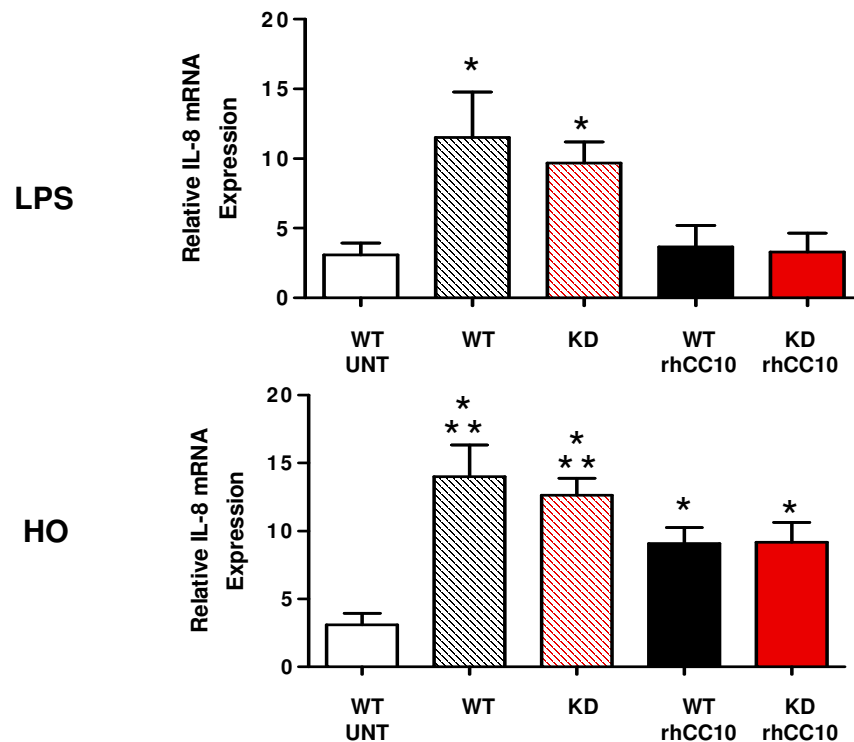


**Figure 3-11: sPLA<sub>2</sub> % Knockdown as Compared to Untransfected Wild Type.** Groups 263 and 266 had about 35% sPLA<sub>2</sub> activity of wild type cells. Negative control and rescue groups both had a percent activity over 90%.

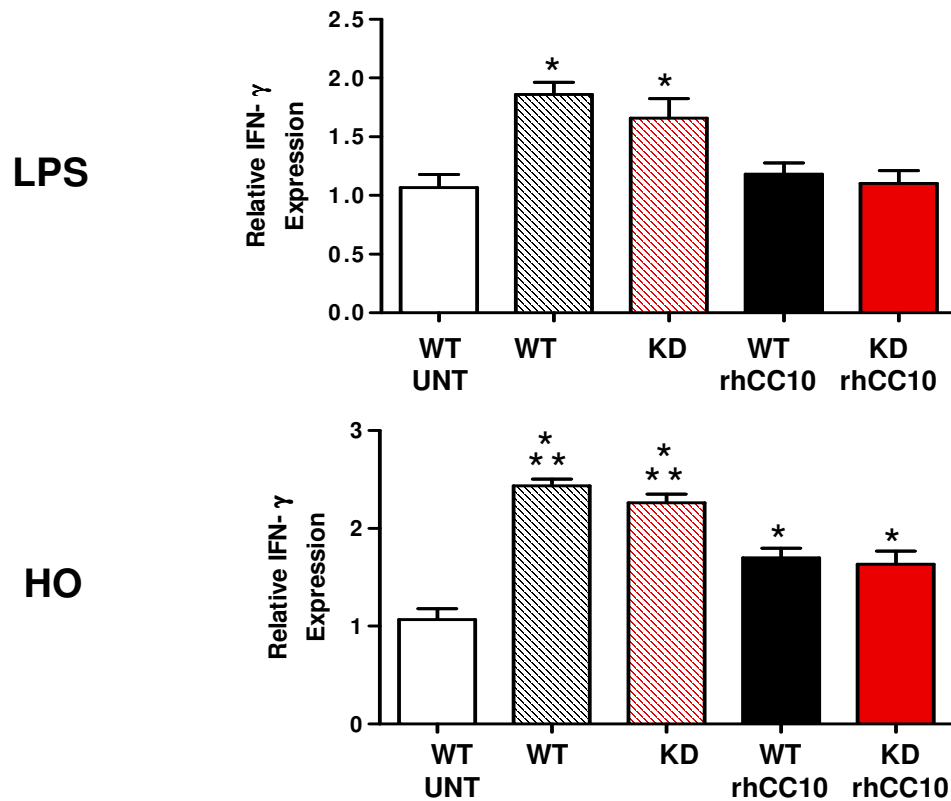
After creating the knockdown model, we measured the effect of rhCC10 on the inflammatory profile of A549 cells when exposed to hyperoxia or LPS in the absence and presence of sPLA<sub>2</sub>. Figures 3-12 to 3-15, show the cytokine profile, with measurements of the mRNA expression of IL-6, IL-8, IFN- $\gamma$ , and IL-10. Across all of these respective cytokines, there was no difference between wild type and knockdown cells. IL-6 and IL-8 mRNA expression was increased (\* $p < 0.05$ ) in both LPS and hyperoxia exposed cells with rhCC10 treatment decreasing the respective biomarkers so as to be no different from unexposed control. Following LPS or hyperoxia exposure IFN- $\gamma$  and IL-10 were increased (\* $p < 0.05$ ) with rhCC10 decreasing both of the respective biomarkers. IFN- $\gamma$  and IL-10 mRNA was increased ( $p < 0.05$ ) in rhCC10 treated, hyperoxia exposed cell, as compared to normoxic control.



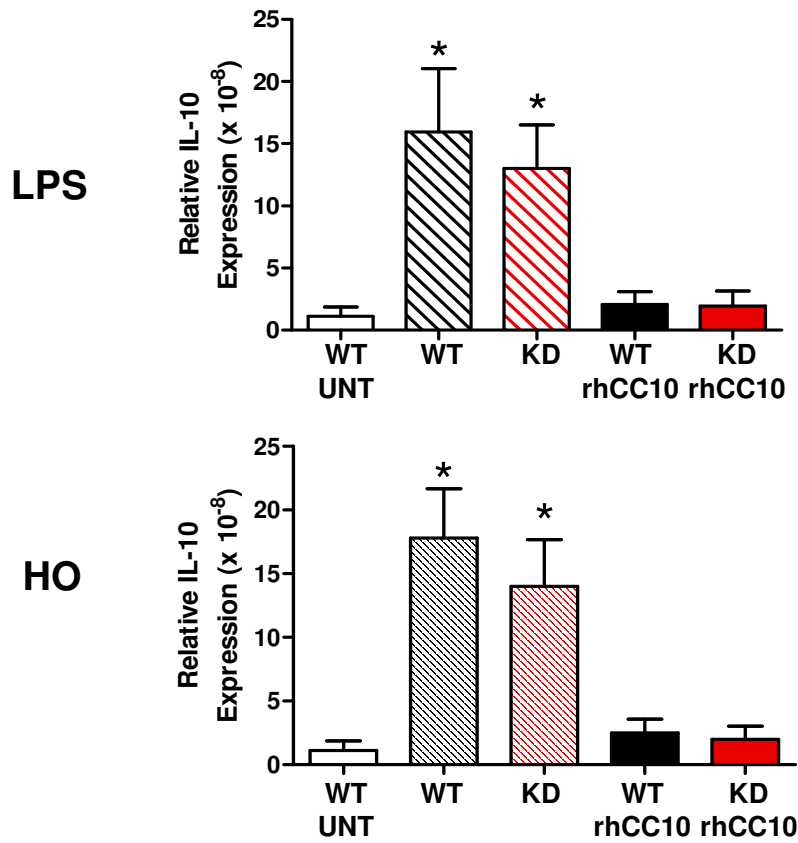
**Figure 3-12: Effect of rhCC10 on IL-6 mRNA expression in the sPLA<sub>2</sub> KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) IL-6 mRNA expression respectively as compared to wild type cells. rhCC10 treatment decreased IL-6 mRNA expression (\* $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)



**Figure 3-13: Effect of rhCC10 on IL-8 mRNA expression in the sPLA2 KD Transfected Cells.** LPS and HO exposure increased (\* < 0.05) IL-8 mRNA expression respectively as compared to wild type cells. During LPS exposure, rhCC10 treatment decreased IL-8 mRNA expression ( $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. During hyperoxia exposure, as compared to rhCC10 treated groups wild type and knockdown cells have an increased (\*\* $p < 0.05$ ) IL-8 mRNA expression (Mean  $\pm$  SEM;  $n = 6$  per group)



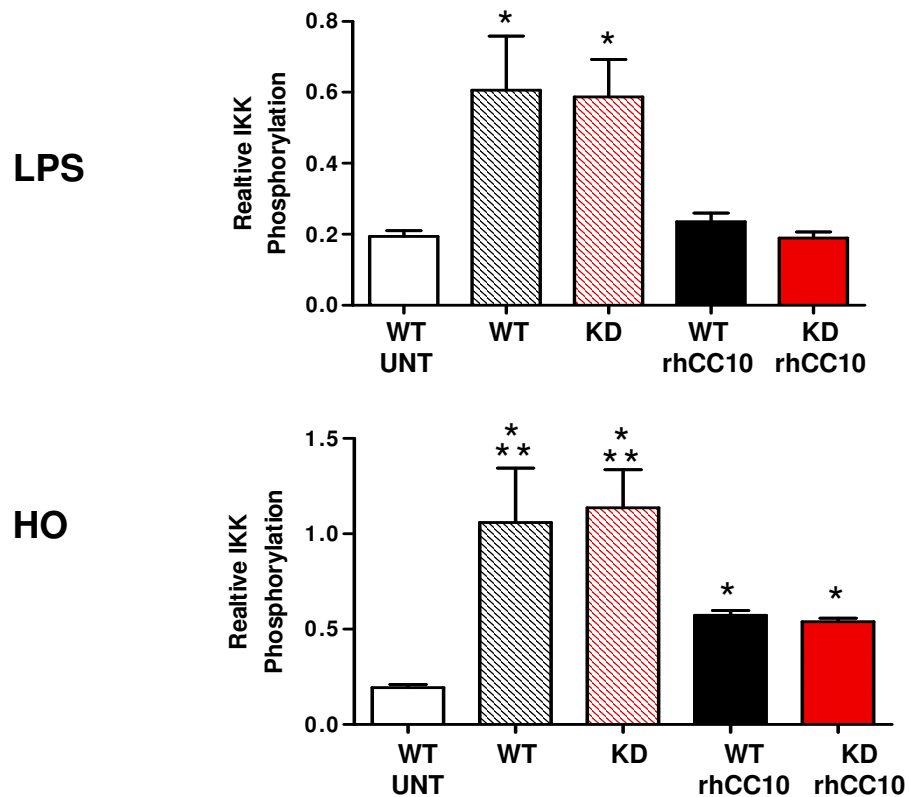
**Figure 3-14: Effect of rhCC10 on IFN- $\gamma$  mRNA expression in the sPLA2 KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) IFN- $\gamma$  mRNA expression respectively as compared to wild type cells. During LPS exposure, rhCC10 treatment decreased IFN- $\gamma$  mRNA expression (\* $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. During hyperoxia exposure, as compared to rhCC10 treated groups wild type and knockdown cells have an increased (\*\* $p < 0.05$ ) IFN- $\gamma$  mRNA expression. (Mean  $\pm$  SEM;  $n = 6$  per group)



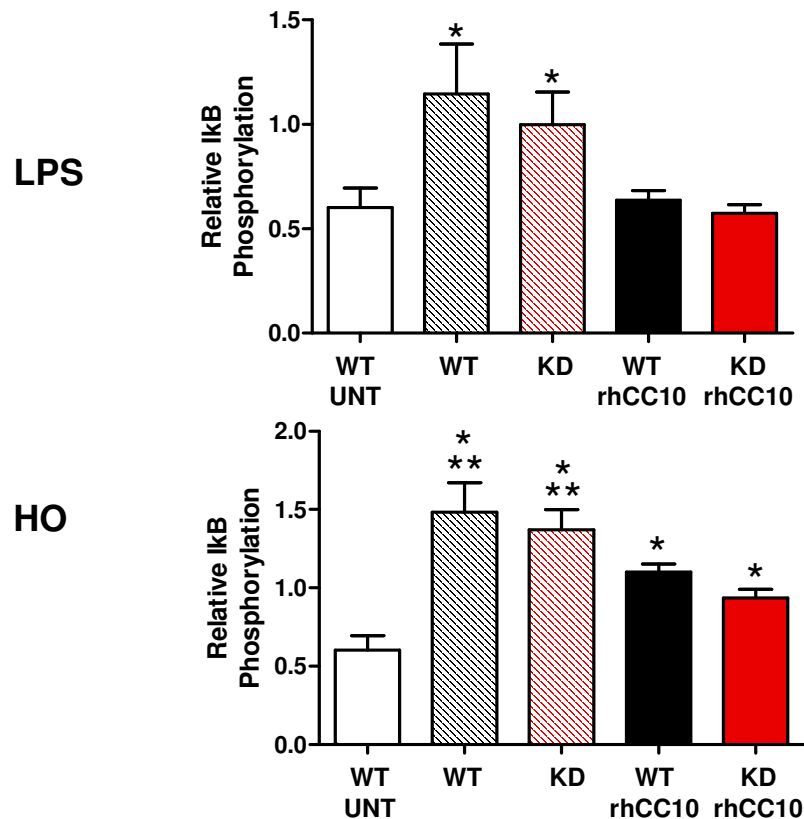
**Figure 3-15: Effect of rhCC10 on IL-10 mRNA expression in the sPLA2 KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) IL-10 mRNA expression respectively as compared to wild type cells. rhCC10 treatment decreased IL-10 mRNA expression (\* $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)

Following measurement of the cytokine profile, we determined the impact of rhCC10 on TLR and NF- $\kappa$ B activity in the presence and absence of sPLA<sub>2</sub>. Figures 3-16, 3-17, 3-18B & C, and 3-19B & C, show that when exposed to LPS, TLR and NF- $\kappa$ B activity is increased (\* $p < 0.05$ ) in both the wild type and knockdown cells versus control. rhCC10 mitigates LPS exposure, so as that exposed cells are no different from unexposed control. When exposed to hyperoxia, TLR and NF- $\kappa$ B activity are increased (\*\* $p < 0.05$ ) vs

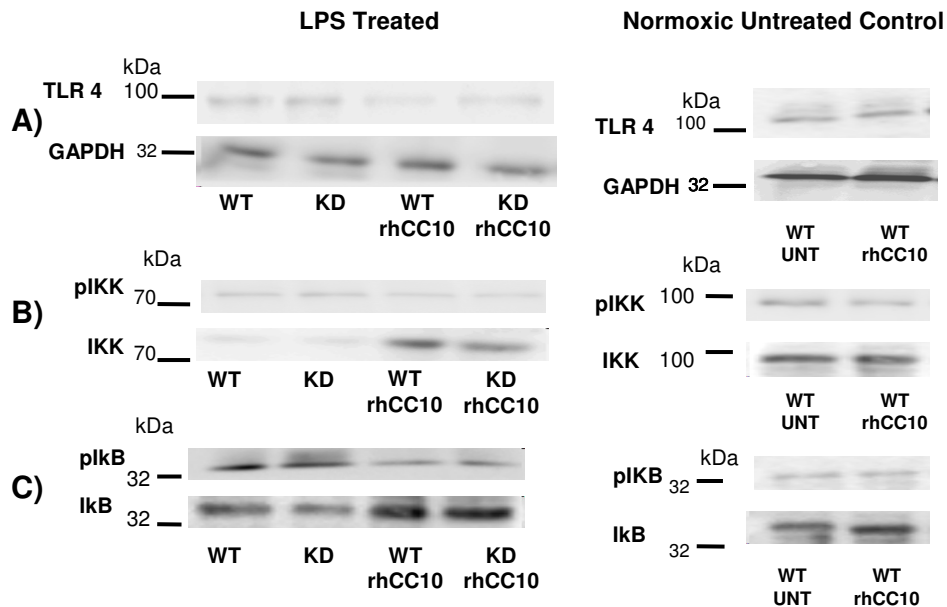
rhCC10 treated groups and all exposed groups have increased ( $*p < 0.05$ ) TLR and NF- $\kappa$ B activity vs normoxic control.



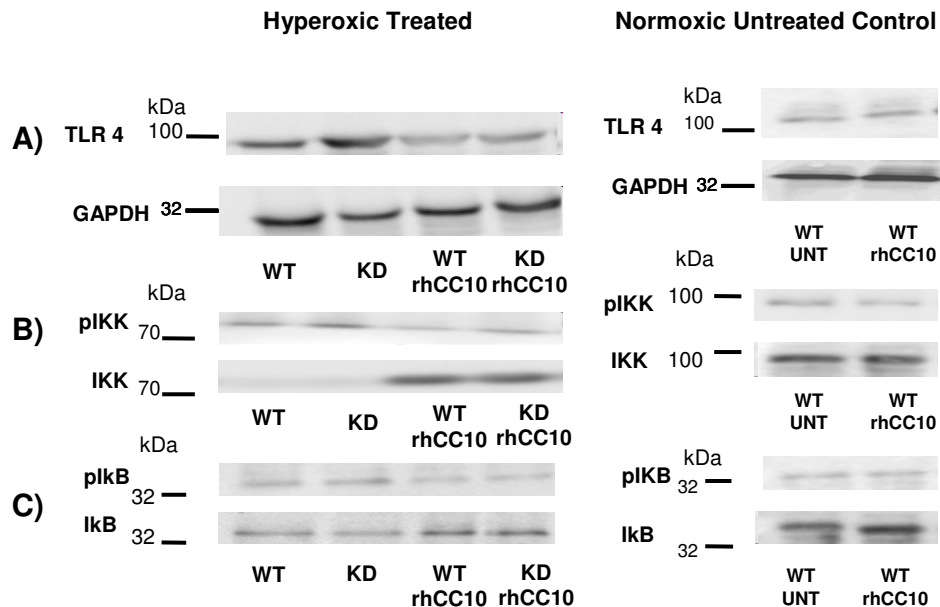
**Figure 3-16: Effect of rhCC10 on TLR 4 Activity in the sPLA<sub>2</sub> KD Transfected Cells.** LPS and HO exposure increased ( $*p < 0.05$ ) relative IKK phosphorylation respectively as compared to wild type cells. rhCC10 treatment decreased relative IKK phosphorylation ( $*p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)



**Figure 3-17: Effect of rhCC10 on NF-κB Activity in the sPLA<sub>2</sub> KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) relative IκB phosphorylation respectively as compared to wild type cells. rhCC10 treatment mitigated LPS exposure so relative IκB phosphorylation was no different from untreated control. During hyperoxia exposure, as compared to rhCC10 treated groups wild type and knockdown cells have an increased (\*\* $p < 0.05$ ) IκB phosphorylation. (Mean ± SEM;  $n = 6$  per group)

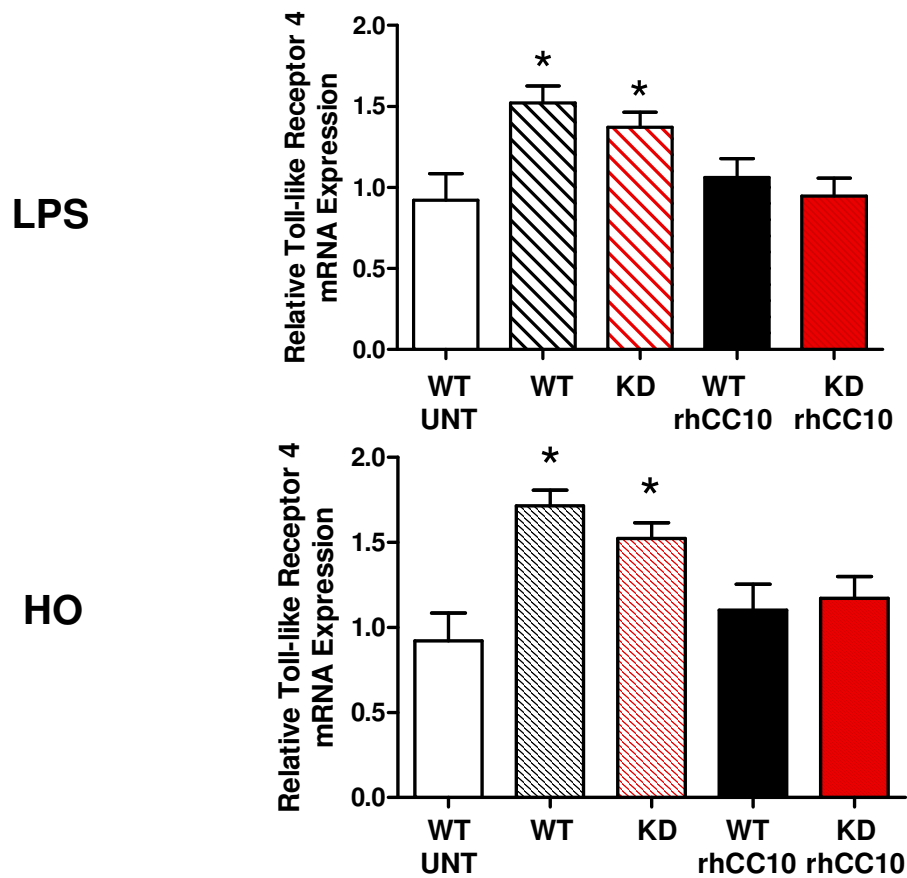


**Figure 3-18: Representative Blots of the Effect of rhCC10 on TLR 4 Mediated Inflammation in the sPLA<sub>2</sub> KD Transfected Cells Exposed to LPS.** rhCC10 decreased ( $p < 0.05$ ) the LPS induced inflammation. There was no difference between knockdown and wild type cells when under the same conditions. All samples were derived at the same time and processed in parallel.

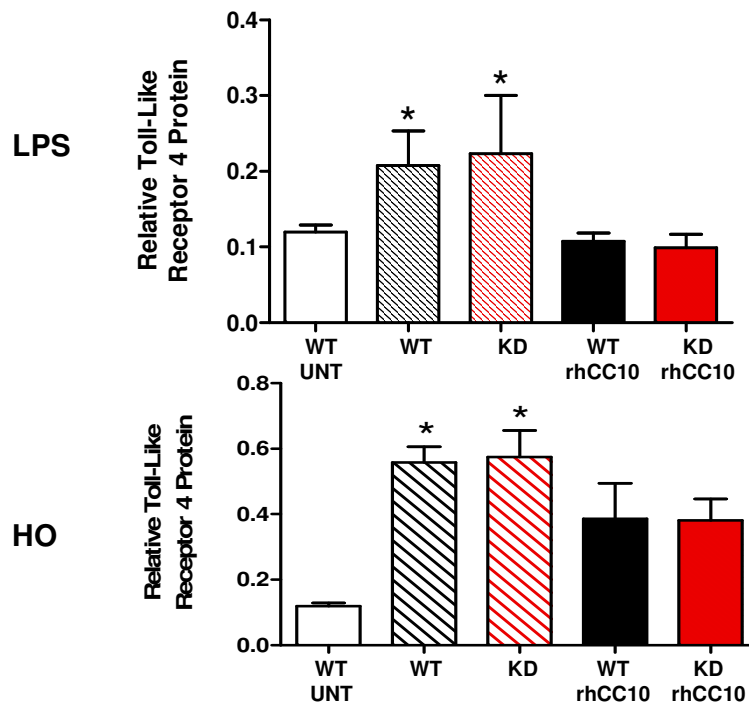


**Figure 3-19: Representative Blots of the Effect of rhCC10 on TLR 4 Mediated Inflammation in the sPLA2 KD Transfected Cells Exposed to Hyperoxia.** rhCC10 decreased ( $p < 0.05$ ) the hyperoxia induced inflammation. There was no difference between knockdown and wild type cells when under the same conditions. All samples were derived at the same time and processed in parallel.

The effect of rhCC10 in mitigating LPS and hyperoxia increases in TLR 4 mRNA expression and protein was also measured. In Figures 3-18A, 3-19A, 3-20, and 3-21, LPS and hyperoxia both increase ( $*p < 0.05$ ) TLR 4 mRNA expression and protein. rhCC10 treatment mitigates this increase so as to be no different from control unexposed cells.

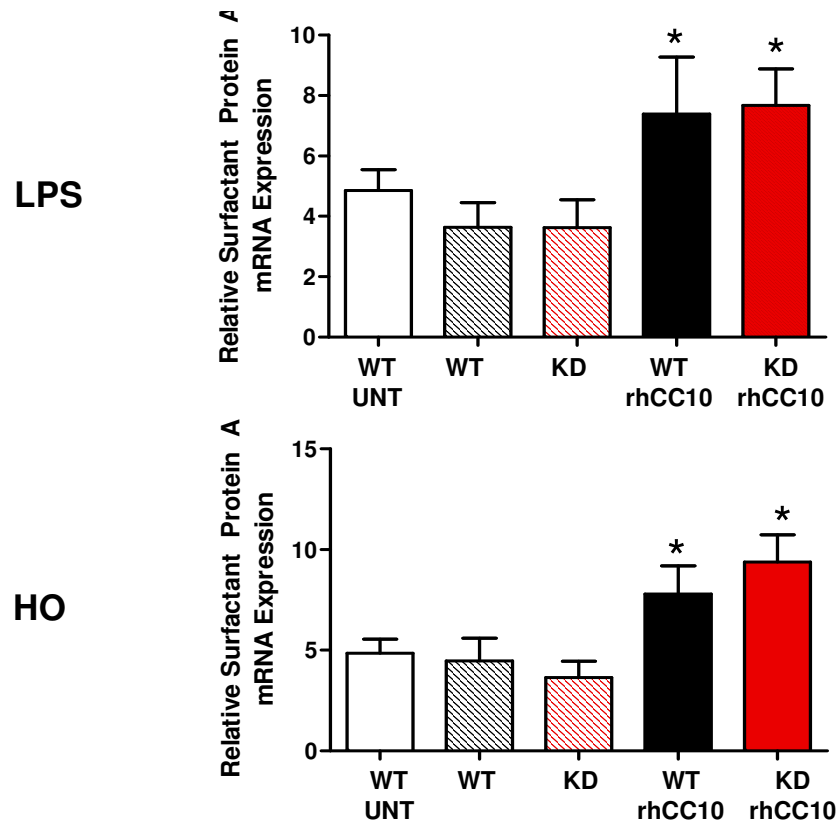


**Figure 3-20: Effect of rhCC10 on TLR 4 mRNA expression in the sPLA<sub>2</sub> KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) TLR 4 mRNA expression respectively as compared to wild type cells. rhCC10 treatment decreased TLR 4 mRNA expression ( $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)



**Figure 3-21: Effect of rhCC10 on TLR 4 protein in the sPLA<sub>2</sub> KD Transfected Cells.** TLR 4 protein was normalized by GAPDH protein. LPS and HO exposure increased ( $p < 0.05$ ) TLR 4 protein respectively as compared to wild type cells. rhCC10 treatment decreased TLR 4 protein ( $*p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)

Due to the rhCC10 mediated increase in SP-A mRNA expression in the preterm lung model, we measured the mRNA expression in this sPLA<sub>2</sub> knock down cell system (Figure 3-22). LPS and hyperoxia exposure did not change SP-A expression in the wild type or transfected groups. rhCC10 treatment increased ( $*p < 0.05$ ) SP-A mRNA expression in both the wild type and transfected cell groups.



**Figure 3-22: Effect of rhCC10 on SP-A mRNA expression in the sPLA<sub>2</sub> KD Transfected Cells.** LPS and HO exposure increased (\* $p < 0.05$ ) SP-A mRNA expression respectively as compared to wild type cells. rhCC10 treatment decreased SP-A expression ( $p < 0.05$ ) wild type and knockdown cells so as to be no different from untreated control. (Mean  $\pm$  SEM;  $n = 6$  per group)

## Discussion

In this work, we demonstrated that rhCC10 inhibits TLR mediated inflammation in the presence and absence of sPLA<sub>2</sub>. As we demonstrated, this response is not associated with increases in IL-10 or mucin 1. We did not observe an rhCC10 mediated increase in TLR 4 expression and protein, as demonstrated in the preterm lamb lung. Although we do see an effect of an rhCC10 mediated increase on SP-A expression, we do not see a corresponding impact on TLR expression and protein. This conflicts with our previous

reasoning and is likely to be due to innate differences between the animal and cell culture models. Possible explanations include, but are not limited to, modification of the rhCC10 by other inflammatory processes *in vivo*<sup>77</sup>, differences in overall pro-inflammatory exposure time between the *in vivo* and *in vitro* models, and a lack of low overall total content of surfactant proteins in the liquid, non-gas interface, submerged culture<sup>81, 82</sup>.

These results have led us to conclude that it is possible that the effect of rhCC10 on TLR signaling and expression are mediated by separate mechanisms. It is also doubtful that the effect of rhCC10 on TLR 4 activity is mediated through SP-A like we initially believed. RhCC10 could affect TLR 4 through direct binding or modification of the membrane lipid organization, as previously described with other agents<sup>86, 91</sup>. We will seek to initially test these hypotheses by measuring rhCC10 induced changes in membrane fluidity<sup>87, 91</sup>, rhCC10-TLR4 interaction using a binding assay, and measurement of the effect of rhCC10 on TLR 4 signaling and expression in an air-liquid interface culture.

Overall, this work provides further insight into potential mechanisms of rhCC10 activity and demonstrates that rhCC10 inhibits TLR 4 mediated inflammation. Although, study and analysis is required, due to the common structural and signaling components in the TLR family, rhCC10 likely modulates other TLRs. TLR mediated inflammation is important in a variety of pulmonary and non-pulmonary diseases<sup>16, 23, 39, 63, 68, 69</sup>. As a DAMP receptor, TLRs like TLR 4, amplify localized inflammation and thereby initiate greater cell damage and death. As an inhibitor of TLR activity, rhCC10 is a potential therapeutic to combat many DAMP mediated inflammatory processes such as in acute respiratory distress syndrome, sepsis, and multi-organ failure<sup>23, 38-40, 51, 68</sup>.

## CONCLUSIONS AND FUTURE DIRECTIONS

Maintenance of a physiologic lung environment is necessary to prevent a host of disease processes. In the preterm lung, multiple protective mechanisms are insufficient and thereby lead to development of respiratory distress syndrome and eventually bronchopulmonary dysplasia. Surfactant treatment will biomechanically protect the preterm lung but it has not been shown to decrease inflammation. Postnatal steroids are effective anti-inflammatory agents but they can have multiple negative side effects. Supplemental oxygen and ventilation, while often physiologically necessary for survival, potentially increase lung damage and the production of DAMP ligands in the lung.

To address the potential limitations in the current standard of care of the injured preterm lung and to further our understanding of CC10 biology, this thesis is a significant addition to the research field. In it, we have accomplished our aims as initially presented in a logical and stepwise manner. First, we demonstrated that rhCC10 modulates inflammation in the preterm lung. With our work in sequencing the ovine CC10 gene, we were able to measure the rhCC10 dose-dependent decrease in endogenous CC10 in the preterm lung. We were able to determine the association between the rhCC10 delivered to the tissue, inflammatory biomarkers, and endogenous CC10 protein. We furthermore demonstrated that the preterm lung is deficient in anti-inflammatory protection as compared to the adult lung.

Second, we analyzed the dose dependent effect of rhCC10 treatment on the toll-like receptor mediated signaling pathway in the preterm lung. We demonstrated a dose

dependent decrease in TLR 4 activity with rhCC10 but an unexpected rhCC10 dose dependent increase in TLR 4 expression and protein. It has been demonstrated that a similar effect on TLR function can be seen with SP-A treatment<sup>36</sup>. We have shown here an rhCC10 dose-dependent increase in SP-A mRNA expression, which is in agreement with previous work from our group that rhCC10 increases SP-A mRNA expression and protein<sup>96</sup>.

Third, we investigated the effect of rhCC10 on TLR 4 pathway in an alveolar lung cell model. This model offered us the ability to knockdown sPLA<sub>2</sub> and isolate the effect of rhCC10 on TLR 4 signaling and expression. We demonstrated that rhCC10 decreased TLR signaling but did not increase TLR 4 expression and protein of cells treated with either hyperoxia or LPS. This difference in the TLR 4 expression and protein between the *in vivo* and *in vitro* studies could be because rhCC10 is modified in lung and not in culture<sup>78</sup>. However, another explanation could be a lack of surfactant protein in the culture model. We did find an increase in SP-A expression, but any effect of SP-A is limited due to the minimal quantity of SP-A protein produced by submerged, non-air-liquid interface, A549 cells.

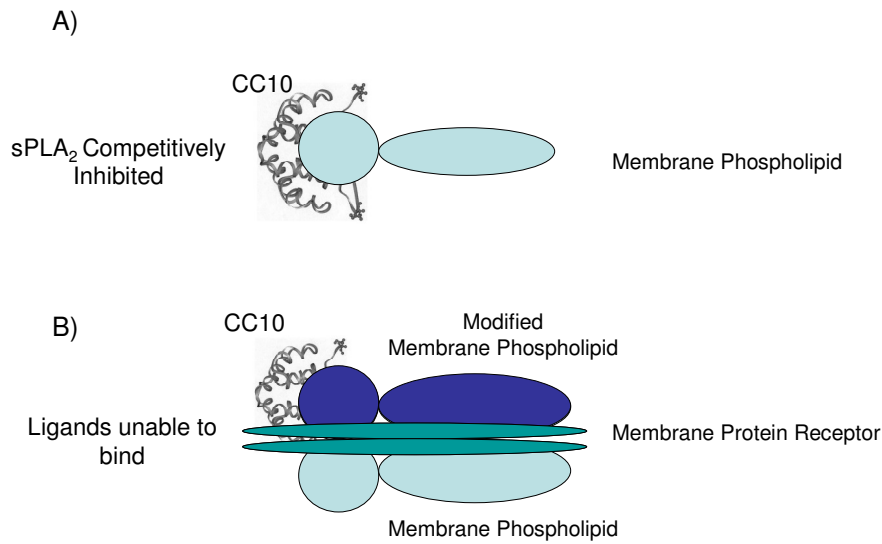
This work leads to further questions and inquiries into the nature of rhCC10 and how it is functioning in the normal and injured lung. We initially believed that rhCC10 decreased TLR signaling and increased TLR expression through an increase in SP-A. While a standard *in vitro* system to study disease processes in the lung, submerged A549 cells unfortunately did not produce significant quantities of surfactant proteins. However,

rhCC10 still did decrease TLR mediated inflammation due to hyperoxia and LPS treatment. As such, these data suggest that the effect of rhCC10 on TLR 4 signaling is not necessarily linked to SP-A.

While we have a greater understanding of the actions of rhCC10 treatment, we do not have a concrete mechanism of its action, only further clues as to how it works. One idea is that rhCC10 directly interacts with TLRs. To test this, a ligand binding assay could be used. TLR 4 would be over expressed in HEK293 cells. The transfected HEK293 cells and control untransfected HEK293 cells would both be plated in 96 well plates. Fluorescent tagged rhCC10 would then be applied to the plate. If fluorescent tagged rhCC10 is not available, then unmodified rhCC10 can be used instead with an additional tagged anti-CC10 antibody step. After washing, fluorescence would be measured using a spectrophotometer plate reader. If the over expressed cells have a higher fluorescence than control, then rhCC10 is directly interacting with TLR 4. Although, potential protein-protein interactions of rhCC10 and TLRs could explain the effect of rhCC10 on TLR signaling, it does not explain the other anti-inflammatory effects of rhCC10.

Another suggestion is that rhCC10 does not function through an actual receptor pathway, but instead associates with the cell membrane directly<sup>6,71</sup>. Structurally, the CC10 protein has a large hydrophobic pocket and that this pocket has been shown to bind to negatively charged heads of phospholipids in the presence of calcium. This has been theorized to be the mechanism of inhibition of sPLA<sub>2</sub> (Figure 4-1), where CC10, endogenous or rhCC10, will bind to membrane phospholipids and then block sPLA<sub>2</sub> from

releasing arachidonic acid. Similarly, binding of CC10 to the membrane phospholipid could theoretically alter membrane fluidity (Figure 4-1). Changes in phospholipid organization in the membrane have been previously demonstrated to modify multiple signaling pathways and vesicle<sup>59, 79, 86</sup>. To further analyze this concept, isolated vesicles *in vitro* could be treated with rhCC10 and changes in lipid fluidity measured by the laurden method and electron spin resonance<sup>87, 91</sup>. This concept could potentially explain the multiple anti-inflammatory effects of rhCC10. It is also possible that this method of action is not independent of direct protein-protein interactions of rhCC10 and cell membrane receptors.



**Figure 4-1: Unified Model of rhCC10 Action.** A) CC10 associates with the polar head group of membrane phospholipids and thereby blocking sPLA<sub>2</sub>. B) Binding of CC10 to the polar head group of membrane phospholipids modifies orientation of the phospholipid so as to affect membrane fluidity. This changes the conformation of the membrane protein receptor so as to limit its function, such as decreasing its ability to recognize ligands.

The translational significance of this work goes beyond the immediate impact of rhCC10 on TLR 4 biology. Toll-like receptors are a unique family of receptors in that, while each respective type of receptor recognizes a different ligand, they share multiple downstream pathway components<sup>7, 19, 57, 69</sup>. Other TLRs have been implicated in pulmonary inflammation across a variety of lung diseases<sup>16, 17, 57, 67, 69, 89, 94</sup>. While we did not investigate the effect on other members of the TLR family, it is not an unfair presumption to believe that rhCC10 will down regulate other TLRs.

In fact, due to the ubiquitous nature of TLRs across multiple organs systems, and their role in cell death and inflammation, they represent a therapeutic target for any number of disease, syndromes, and trauma.

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