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The Potential Role of Cathelicidin Peptides for the Treatment of Lower Respiratory Tract Infections

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Abstract

Bacterial pneumonia, one of the leading causes of hospitalization and mortality worldwide, is caused by the colonization of invasive bacteria in the airways, leading to pulmonary inflammation and lung dysfunction. The development of antibiotic resistant bacterial infections has limited the effectiveness of current therapeutics and is particularly concerning in the setting of chronic bacterial infections, such as observed in cystic fibrosis and ventilator-associated pneumonia. The development of novel therapeutics for the treatment of multi-drug resistant bacterial pneumonia is urgently needed.

The overall objective of this body of work was the development of a new therapeutic compound to treat multi-drug resistant bacterial infections in the lung. It was hypothesized that a cathelicidin/exogenous surfactant compound could be developed as a novel therapeutic agent for the treatment of bacterial pneumonia. To test this hypothesis, we first screened several cathelicidin peptides combined with a commercial exogenous surfactant, bovine lipid extract surfactant (BLES), and identified a lead compound (CATH-2) for further testing. Following this, we investigated three main outcomes: 1) the antimicrobial activity of CATH-2 and BLES+CATH-2 against multi-drug resistant, clinically isolated bacteria; 2) the immunomodulatory potential of both CATH-2 and BLES+CATH-2 *in vivo*; and 3) the bactericidal activity of BLES+CATH-2 treatment *in vivo*, using two models of bacterial pneumonia.

It was discovered that CATH-2 was able to kill bacteria *in vitro*. In addition, CATH-2-killed bacteria administered into the lungs of mice did not induce an inflammatory response *in vivo*, and the ability to prevent this inflammation was maintained by BLES+CATH-2. Finally, while BLES+CATH-2 is able to kill multi-drug resistant,

clinically derived bacteria *in vitro*, there is little bactericidal activity of BLES+CATH-2 in *in vivo* models of bacterial pneumonia.

Overall, we identified the therapeutic potential of BLES+CATH-2 for use as a therapeutic treatment for bacterial pneumonia. Despite promising *in vitro* activity, we were unable to show bactericidal activity for BLES+CATH-2 *in vivo*. Future directions will require the optimization of the surfactant-cathelicidin compound in order to develop a viable therapeutic for clinical practice.

Keywords

Cathelicidins; exogenous pulmonary surfactant; inflammation; cytokines; bacterial pneumonia; cystic fibrosis; ventilator-associated pneumonia; *Pseudomonas aeruginosa*

Co-authorship statement

Chapters 2, 3, 4, and 5 describe experimental studies performed by Brandon Banaschewski under the supervision of Dr. Ruud Veldhuizen and Dr. Cory Yamashita.

Drs. Ruud Veldhuizen, Cory Yamashita, and Edwin Veldhuizen provided intellectual contribution to all studies described herein, including data interpretation and manuscript review.

Mrs. Lynda McCaig provided instrumental assistance with all animal studies, including teaching, hands-on technical assistance, and animal protocol ethics development.

In Chapter 2, Dr. Elenora Keating performed sample collection and analysis of atomic force microscopy samples.

Ms. Shannon Seney provided assistance with Multiplex assays described in Chapter 3 and Chapter 4.

In Chapter 4, Dr. Johan Delport was responsible for the collection, isolation, and identification of all clinical samples obtained from adult cystic fibrosis patients.

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List of Abbreviations

AFM – Atomic force microscopy

ANOVA – Analysis of variance

AMP – Antimicrobial peptide

ASL – Airway surface liquid

BALF – Bronchoalveolar lavage fluid

BLES – Bovine lipid extract surfactant

BMAP-27 – Bovine myeloid antimicrobial peptide 27

BW - Bodyweight

CATH-1 – Chicken cathelicidin 1

CATH-2 – Chicken cathelicidin 2

CAP-18 – Cationic antimicrobial peptide of 18 kDa

CBA – Chocolate blood agar

CDS – Constrained sessile drop surfactometer

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CFU – Colony forming units

ddH_2O – Double distilled water

DNA – Deoxyribonucleic acid

DPPG – 1,2-Dipalmitoyl-sn-glycero-3-phosphatidyl glycerol

ELISA – Enzyme-linked immunosorbent assay

$FEV1_{PRED}$ – Forced expiratory volume in one second, percentage of predicted value

FiO_2 – Fraction of inspired oxygen

FPR2 – Formyl peptide receptor 2

FPRL1 – Formyl peptide-like receptor 1

G – Gauge

G-CSF – Granulocyte colony stimulating factor

GM-CSF – Granulocyte macrophage colony stimulating factor

IL-1α – Interleukin 1α

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-12p70 – Interleukin 12 subunit p70

IL-23p19 – Interleukin 23 subunit p19

IP – Intraperitoneal

KC – Keratinocyte chemoattractant

LBP – Lipopolysaccharide binding protein

LL-37 – Human cathelicidin peptide 37

LTA – Lipoteichoic acid

LPS – Lipopolysaccharide

MBC – Minimum bactericidal concentration

MCP-1 – Monocyte chemotactic protein 1

mCRAMP – Murine cathelicidin-related antimicrobial peptide

MDR – Multi-drug resistant

MgrX2 – Mas-related gene X2

MHB – Müeller-Hinton Broth

MIC – Minimum inhibitory concentration

MIP-2 – Macrophage inflammatory protein 2

MMP-9 – Matrix metalloproteinase 9

MST – Minimum surface tension/minimum achievable surface tension

MRSA – Methicillin-resistant *Staphylococcus aureus*

NaCl – Sodium chloride

NF-κB – Nuclear factor-κB

nRDS – Neonatal respiratory distress syndrome

$PaCO_2$ – Arterial partial pressure of carbon dioxide

PaO_2 – Arterial partial pressure of oxygen

PBS – Phosphate buffered saline

PC – Phosphatidylcholine

PG – Phosphatidylglycerol

PIP – Peak inspiratory pressure

PL - Phospholipid

PMAP-23 – Porcine myeloid antimicrobial peptide 23

RNA – Ribonucleic acid

SEM – Standard error of the mean

SMAP-29 – Sheep myeloid antimicrobial peptide 29

SP-A – Surfactant protein A

SP-B – Surfactant protein B

SP-C – Surfactant protein C

SP-D – Surfactant protein D

TLR 1 – Toll-like receptor 1

TLR 2 – Toll-like receptor 2

TLR 4 – Toll-like receptor 4

TLR 5 – Toll-like receptor 5

TLR 6 – Toll-like receptor 6

TLR 9 – Toll-like receptor 9

TNF-α – Tumor necrosis factor α

TSA – Tryptic soy agar

TSB – Tryptic soy broth

VAP – Ventilator-associated pneumonia

ZymA – Zymosan A

Chapter 1: General Introduction and Literature Review

Every day, approximately 10 000 L of air will flow into and out of the lungs, and bring with it foreign particles, such as bacteria, from either the surrounding environment or the nasopharynx. Due to the unique branching structure of the lung, some of this particulate-filled air will pass into the terminal bronchioles, respiratory bronchioles, and alveoli. Over the course of this steady siege, some pathogens are able to circumvent the innate immune system, leading to colonization and infection of the lung, known as pneumonia. Pneumonia is one of the most common presentations of bacterial infections, with millions of people being diagnosed annually (1). It is the leading cause of death due to infection; estimates by the World Health Organization suggest that bacterial pneumonia is responsible for 15% of all children deaths under the age of 5, and an estimated 3.2 million deaths annually (2–5).

Furthermore, the rate of drug-resistant infections is rapidly escalating, and is becoming a large global health concern. Multi-drug resistant infections are currently estimated to be responsible for 700 000 deaths per year, with that number expected to rise to 10 million per year by 2050 without immediate intervention (6). The high prevalence of pneumonia infection and increasing rates of antibiotic resistant infections are two factors culminating into a potential global health disaster.

The purpose of this thesis will be to determine the efficacy of a novel therapeutic for the treatment of multi-drug resistant bacterial pneumonia. Here, we investigate the development of a potential treatment using a cathelicidin peptide in conjunction with exogenous surfactant as a vehicle to improve delivery to the respiratory system. This chapter will provide an overview of homeostatic respiratory functions, pathologic processes that develop over the course of chronic bacterial respiratory infections in cystic

fibrosis or mechanical ventilation, and the morbidities of antibiotic resistance associated with these pulmonary infections. Finally, it will provide an overview of both cathelicidins and exogenous surfactant, and their therapeutic potential for the treatment of multi-drug resistant bacterial pneumonia.

1.1 Lung Structure and Function in Homeostatic Conditions

The primary function of the lungs is gas exchange. In the lungs, oxygen diffuses from the alveoli at the terminal ends of the airways, across epithelia and endothelia, and into the deoxygenated blood within the surrounding alveolar capillaries; mirroring this, carbon dioxide moves down its concentration gradient from the blood stream into the alveoli and is eventually expired out of the body (Reviewed in (7)).

The anatomy of the lungs involves a complex branching network, beginning in the nasopharynx, to the trachea, and then branching into two primary bronchi. From there, the bronchi will continue to divide into smaller and smaller airways, eventually forming terminal bronchioles. These structures are involved in air flow, but do not participate in gas exchange. Distal to the terminal bronchioles are respiratory bronchioles, which are structurally similar with the exception that they contain budding alveoli that will participate in gas exchange. Past these respiratory bronchioles are alveolar sacs, containing groups of alveoli, the primary regions of gas exchange. The complex branching structure allows for a large surface area at the alveolar level, allowing for rapidly efficient gas exchange with the surrounding alveolar capillaries (7).

The alveoli themselves are structured by a combination of thin, squamous Type I alveolar cells and a smaller number of cuboidal Type II alveolar cells. Type I cells are

essential for gas exchange, as their large surface area and thin structure allows for maximal diffusion. On the other hand, Type II cells are essential for numerous other functions, including replenishing Type I cell populations after injury, and producing pulmonary surfactant, a lipoprotein substance that contributes to breathing mechanics (8, 9). Other cells within the alveolar space include alveolar macrophages, essential phagocytes tasked with patrolling the alveoli, engulfing inhaled pathogens and debris particles, and regulating the innate and adaptive immune system (10–12).

1.1.1 Pulmonary mechanics

To facilitate gas exchange, air flows in and out of the lungs by a process known as ventilation. This process requires the coordinated contraction of both external intercostal muscles and the diaphragm to increase the volume of the thoracic cavity. This increased volume causes a decrease in pressure, as predicted by Boyle's Law, and causes the movement of a discrete volume of external air to enter into the lungs, referred to as the tidal volume. At rest, exhalation is due to the relaxation of these muscles; the decreased thoracic volume then creates a (relatively) high-pressure environment within the lungs, and air moves out to the external environment. During exertion, internal intercostals, abdominals, and oblique muscles will also contract to reduce thoracic cavity volume with greater force, increasing the volume and rate of exhalation (7).

In addition to the role of the respiratory muscles, there are numerous other processes within the lungs that affect ventilation. One specific measurement of these mechanics is lung compliance. Lung compliance refers to the amount of force required to inflate the lungs. This is calculated as the volume change per unit of pressure change (7). There are two major factors in the lung responsible for compliance – lung elastic tissue, and surface

tension within the small airways and alveoli (discussed in section 1.1.2). The elastance of the lung is proportional to the structural components of the lungs, specifically the amount of elastin and collagen content within the airway interstitium (7). Increased elastin and collagen content within the lung leads to reduced compliance, as it increases the force required to expand the lungs upon inhalation. The surface tension within the lungs has a major effect on lung function, as increased surface tension has a significant impact on lung compliance and alveolar stability upon exhalation. Pulmonary surfactant is directly responsible for reducing the surface tension within the lung.

1.1.2 The pulmonary surfactant system

As noted above, one of the forces responsible for determining lung compliance is surface tension. The surface tension within the lungs is caused by a thin liquid layer lining the entire respiratory tract, known as the airway surface liquid (ASL), that begins in the nasopharynx and extends down to the alveoli. Within the alveoli, the surface tension exerted by water molecules of the ASL produce an elastic force that reduces compliance. Pulmonary surfactant is responsible for reducing this force by adsorbing to the air-liquid interface of the ASL and acting to reduce surface tension during exhalation and inhalation (13, 14). During exhalation, due to the decreasing size of the alveoli, lateral compression of the pulmonary surfactant film reduces surface tension to minimal values; this minimizes any collapse force on the alveoli (14). By reducing surface tension, the collapse force is reduced, making the lungs easier to inflate. Therefore, reducing surface tension is directly associated with increased lung compliance.

Pulmonary surfactant is a lipoprotein complex produced and secreted by type II alveolar cells (9). It is found in all mammalian species and has a highly conserved

composition (15). It is made up of ~80% lipids by mass, such as phosphatidylcholine (PC, 80% of lipid subfraction) and phosphatidylglycerol (PG, 10-11% of lipid subfraction),~10% neutral lipids, such as cholesterol, and ~10% surfactant associated proteins, named SP-A, -B, -C, and -D (16). Upon synthesis of these lipid-protein mixtures, type II cells store surfactant in intracellular structures known as lamellar bodies until signalled to secrete into the airspace (17, 18). Once secreted, the surfactant will adsorb to the air-liquid interface to form a surface-active film, which is further discussed below (19, 20). The "functional" components of surfactant are experimentally known as the "large aggregate". During repeated cyclic expansions and compressions of the surface film (throughout the course of respiration), small surfactant vesicles are generated, and then recycled by resident alveolar macrophages and type II cells; this "inactive" surfactant component is experimentally referred to as "small aggregate" (21, 22).

While true *de novo* adsorption only occurs during the first breath at birth, surfactant secreted into the alveolar liquid lining spreads rapidly along the interface in order to form the surface-active film responsible for surface tension reduction upon compression. Surfactant-associated proteins SP-B and SP-C promote the breaking of stable phospholipid bilayers in the aqueous layer to induce adsorption of lipids to the air-liquid interface. The rapid diffusion and spreading of the surfactant film allows for the initial reduction of surface tension in the alveoli, improving lung compliance and alveolar stability (19). After this first breath, surfactant is cycled into the air-liquid interface over the course of respiration with the help of SP-B and SP-C, as inactivated surfactant lipids are recycled back to type II cells or degraded by alveolar macrophages (23).

Patients with a poorly functioning pulmonary surfactant system, or surfactant deficient states, typically exhibit a severe decrease in lung compliance, eventually leading to atelectasis (alveolar collapse) and decreased oxygenation. One such example is neonatal respiratory distress syndrome (nRDS), where premature infants are born without a fully developed pulmonary surfactant system (24). Administration of exogenous surfactant to neonates lacking sufficient pulmonary surfactant has been demonstrated to rapidly improve compliance, oxygenation, and patient outcomes (25–27). Since its identification as a viable therapeutic in the 1980's, exogenous surfactant administration has become a standard treatment for premature infants diagnosed with nRDS (25, 28). Due to its superior clinical efficacy and its innate ability to spread throughout the respiratory tract, surfactant has since been investigated for numerous other therapeutic functions, such as a vehicle for pulmonary therapy distribution (29, 30).

1.2 Chronic Bacterial Lung Infections

Bacterial pneumonia is an infection of the respiratory tract, and is one of the leading causes of hospitalization (4, 31–34). Over the course of prolonged infection, the continual inflammatory environment can precipitate structural changes to the respiratory tract, eventually resulting in severe decreases in oxygenation and potentially death (35, 36). Chronic infections associated with cystic fibrosis or mechanical ventilation are two major examples in which prolonged infections associated with these underlying conditions increase the complexity of the disease and increase the rate of mortality (4, 35, 37). Typical treatment strategies for these two diseases involve administration of antibiotics to target the infection; however, the bacteria are typically incompletely eradicated, and antibiotic use is

forced to continue over a prolonged period, or to treat acute exacerbations (38). As such, prolonged administration of antibiotics, without the effective clearance of the colonizing bacteria, can contribute directly to the high prevalence of antimicrobial resistant infections, further complicating treatment strategies.

1.2.1 Cystic fibrosis

Cystic fibrosis (CF) is an inherited disease of the cystic fibrosis transmembrane conductance regulator (CFTR). The development of this disease has been thoroughly investigated since the initial identification of the CFTR gene (39–41). In these patients, a non-functioning/hypofunctioning CFTR channel prevents the movement of chloride and bicarbonate ions into the air surface liquid. Due to the poor movement of ions across the luminal membrane, there is a decrease in osmotic forces into the lumen, and as a result the ASL becomes dehydrated and viscous. This highly viscous mucus is unable to properly detach from the secretory ducts of submucosal glands after secretion (42). To further compound this problem, the cilia on the apical surface of the airway epithelia become less-functional, meaning that any particles or pathogens trapped in the thickened mucus can no longer be cleared from the airways. These individual factors combine to elicit the bacteria-laden mucus plugs characteristic of cystic fibrosis pulmonary disease (43). Combined with the defect in the mucociliary escalator, studies have also shown that there is a severe detriment in the innate host defense of recruited cell types as well (44). Decreases in the pH of the ASL, likely due to the reduction of bicarbonate movement, has been demonstrated to be responsible for lack of bacterial killing induced by the innate factors present, including cathelicidins, defensins, and other antimicrobial peptides (44).

Interestingly, this acidified environment also appears to have an effect on the viscosity of the ASL itself (45).

The result of these deficiencies allows pathogens to colonize and proliferate, and without a functioning clearance system, CF patients develop frequent, spontaneous lung infections of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, and other pathogens from birth (46, 47). Failure to clear these pathogens eventually leads to a positive feedback loop of neutrophil recruitment, degranulation, and ineffective clearance. This can be observed in the bronchoalveolar lavage fluid (BALF), where there is a large percentage of neutrophils recovered from these patients, as well as increasing concentrations of the chemokine IL-8, correlating with bacterial infection (48). Progressive bronchiectasis as a result of this inflammatory cycle, and mucus plugging from the hyper-viscous submucosal gland secretions, significantly hampers lung function, as can be observed through $FEV1_{PRED}$ measurements (43, 49). This lung disease is the leading cause of morbidity and mortality in CF patient populations (42, 43, 47, 50, 51).

Often, bacteria in CF airways will undergo genetic changes in order to survive the harsh inflammatory environment. Some of the more pathogenic bacterial strategies involve the development of mucoid phenotypes in *P. aeruginosa* species, and the formation of biofilm production in *P. aeruginosa* and many other CF-derived species (52, 53). Antibiotic treatment strategies will select for mutants with high intrinsic antibiotic resistance, and as such, antibiotic resistance will rapidly spread throughout the patient (54). As expected, antibiotic resistance rates for CF patients are high, with 15% of patients colonized with multi-drug resistant *P. aeruginosa*, although rates have been reported as high as 30% of *P. aeruginosa* infected patients; drug-resistant *S. aureus* is observed in ~25% of patients (54–

56). As a result, some antibiotics have become completely ineffective for CF patient therapy, as it was observed in one study that over 70% of *P. aeruginosa* isolates were resistant to amikacin, gentamicin, and fosfomycin, while over 90% of methicillin-resistant *S. aureus* (MRSA) isolates were resistant to both clindamycin and erythromycin (57). Eventually, physicians and patients are burdened with severely limited antibiotic options, and the chronic lung infections caused by the multi-drug resistant bacteria result in hypoxia, respiratory failure, and death (43).

While the understanding of CF pathophysiology has been well documented through the use of various *in vitro* cell culture models and *in vivo* animal models that accurately model the human course of disease, the development of multi-drug resistant bacterial lung infections remains the most challenging clinical problem to manage. However, the ability to isolate and culture multi-drug resistant bacteria allows for multiple permutations of *in vitro* testing, including **investigating the bactericidal activity of novel antimicrobial agents against these clinically-isolated pathogens**.

1.2.2 Ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) is the development of lower respiratory tract infection >48 h after the initiation of treatment, and is one of the most common hospital-acquired infections (58). Similar to the complex pathology associated with CF and the development of CF-lung disease, the development of VAP is equally complex. The development of VAP has been attributed to multiple different factors, such as damage caused by the insertion of the tube leading to a breached epithelial defense layer, ineffective host defense systems, and the patients' impaired ability to swallow respiratory secretions, all resulting in spontaneous airway colonization (59). Consistent with this variable disease

etiology, the reported percentages of patients that develop VAP also vary widely, with reports anywhere between 5-72% of patients receiving mechanical ventilation (60, 61). The mortality rates for VAP patients is equally variable, with reports anywhere between 20-76%, depending on the study (60, 62). Bacterial species commonly associated with these infections include *S. aureus, P. aeruginosa, Klebsiella pneumonia, and Acinetobacter baumii* (63).

Regardless of incidence rates, the pathology of VAP is similar to community-acquired pneumonia, with the major complication of an endotracheal tube. To complicate the treatment of this pneumonia infection, the endotracheal tube acts as a reservoir for bacteria, preventing the ability of the host defense system to completely clear the infection (59). In addition, bacteria grown in this environment often form a biofilm, which makes treatment of the infection even more difficult, as the biofilm acts as a shield to protect the bacteria from host defense systems and antibiotic penetration (64).

Similar to CF patients, the prolonged bacterial colonization leads to rampant pulmonary inflammation, eventually resulting in respiratory failure and death. Although BALF collected from these patients is typically used to perform bacterial culture analysis, analysis of this fluid presents with high rates of neutrophil influx and cytokine production (58, 60, 61, 64, 65). Antibiotics are administered to manage these infections, but prevalence of antibiotic resistant bacteria is anywhere between 42-69% (63, 66–69). Left untreated, oxygenation becomes severely reduced and patients can develop acute respiratory distress syndrome, eventually leading to multi-organ failure and death (60).

Despite the high incidence of multi-drug resistant infections associated with VAP, the overall injury is severely understudied. However, there are multiple animal models

useful for investigation, and useful physiologic measurements to investigate, such as bacterial clearance, as well as oxygenation and inflammatory responses. These animal models allow for a controlled environment in order to investigate VAP infections (70). **Using these models, it is possible to investigate the therapeutic potential of antimicrobial/immunomodulatory agents that directly affect three clinically relevant outcomes – oxygenation, bacterial clearance, and inflammation.**

1.3 Antimicrobial Resistance and the need for novel therapeutics

As mentioned previously, the typical treatment for prolonged bacterial infection of the airways is the administration of antibiotics. However, improper antibiotic usage, or prolonged exposure to antibiotics without effective clearance of the bacteria, such as the situation observed in cystic fibrosis patients, can lead to the development of drug-resistance (71).

Increasing rates of multi-drug antibiotic resistance are quickly becoming a concern in clinical settings, which limits the therapeutic options available to both patients and physicians (6, 72–74). Antimicrobial resistance is one of the largest global threats currently being faced, which has been caused by 1) the natural selection of drug-resistant bacterial strains due to improper antibiotic use, as well as 2) poor stewardship of conventional antibiotics and improper administration (75). In addition to intrinsic resistance strategies found within the bacteria naturally, spontaneous mutations and horizontal gene transfer can cause rapid dissemination of drug resistance, making the antibiotics ineffective against the once-susceptible bacterial strains (52, 76). The increasing incidence of drug-resistant *P. aeruginosa*, MRSA, and a variety of other bacterial species, is threatening treatment

regimens of pneumonia patients, as therapeutic strategies become limited by the decreasing number of effective antibiotics (4). In case of CF and VAP, the situation is more dire, with drug-resistant bacteria being responsible for infection in between 9.2 – 26% and 42 - 69% of cases, respectively (63, 66–69, 77).

The rising prevalence of antimicrobial resistance requires investment into the development of new antibiotics. However, with fewer pharmaceutical companies investing in the discovery of new therapeutics, there is a reduced number of new therapeutics currently in development (75, 78). Consequently, a number of research groups have begun to investigate the potential of antimicrobial peptides (AMPs) as potential sources of new therapeutics (79–86).

1.4 Antimicrobial peptides

Antimicrobial peptides are a diverse group of endogenous peptides produced as part of the innate immune system. They have been identified in almost every plant and animal species investigated. A full list of the 2600+ peptides can be found here (http://aps.unmc.edu/AP) (87). These peptides can be subdivided into different groups, based on their tertiary structures, amino acid composition, or the ability to form disulphide bonds through specific cysteine residues (87).

The two largest families within the antimicrobial peptide class are cathelicidins and defensins. For the purpose of this literature review, we will focus solely on the role of cathelicidins, and their potential for the treatment of multi-drug resistant bacterial infections.

1.4.1 Cathelicidins: mechanisms of action and therapeutic potential

Cathelicidins are a diverse family of peptides found in multiple species, including (but not limited to) humans (88), rats (89), mice (90), and chickens (91). They are grouped together based on a common pro-domain, known as a "cathelin" domain (named due to its homology to the cathepsin-L-inhibitor), that is roughly 100 amino acids long (92, 93). The full-length, unprocessed peptides contain a short signalling domain on the N-terminus, the conserved cathelin domain, and the active cathelicidin peptide at the C terminus (94). The N-terminal signal domain is responsible for directing the cathelicidin into storage granules, at which point it is proteolytically removed (95–98). Once the secretory granules are released, or fused with phagosomes, the active C-terminus is cleaved from the cathelin portion by elastase (or proteinase 3, specifically for LL-37), releasing the active antimicrobial peptide (96–104). Cathelicidins were first identified in leukocytes and myeloid cells, but have since been identified in almost all epithelial and immune cells, adding evidence for their important role in innate immunity (94, 105–111). Recently, cathelicidin expression levels in circulating immune cells was investigated, which showed highest levels of cathelicidin expression in neutrophils, followed by monocytes, and then lymphocytes (112). Their expression and release can be both constitutive or upon stimulation, either through activation by microbial products such as lipopolysaccharide (LPS), or through stimulation by inflammatory cytokines, such as IL-1α (110, 113, 114). In humans and primates, induction of cathelicidin production is regulated by vitamin D receptor activation, not direct NF-κB regulation (115). Instead, toll-like receptor activation induces cathelicidin production indirectly, via upregulation of the vitamin D receptor (116).

1.4.2 Antibacterial activity

Initial interest in these peptides was due to their bactericidal properties (117). Despite being phylogenically ancient, these peptides have maintained potent antimicrobial activity with very few species of bacteria presenting with intrinsic resistance towards them. The efficacy of these evolutionarily old peptides is due in part to the multiple mechanisms of bactericidal activity (100, 118).

Upon their release from the secretory granules, the positively charged cathelicidins interact with the negatively charged phospholipids found on the bacterial plasma membrane, as well as components of the outer membrane, including lipoteichoic acid (LTA) or LPS (119). These electrostatic interactions account for the selection of bacterial cell membranes over the neutrally charged eukaryotic membranes (79). After the interaction with the cell membrane, cathelicidins have been shown to function through two methods (Figure 1.1). The first is solely through the cell membrane interactions, where the peptides will permeabilize bacterial membranes through either carpet-, barrel stave-, or toroidal pore-models, depending on the peptide in question (which varies due to each cathelicidin's structure, shape, and amino acid composition) leading to loss of membrane integrity and bacterial death (100, 120–122). While it is possible for bacteria to undergo major membrane neutralization to prevent cathelicidin binding, these changes often lead to an evolutionary disadvantage once the selective pressure is removed. Additionally, bacterial membrane neutralization appears to be limited, and the membrane changes are not enough to completely prevent cathelicidin activity (123, 124).

In the second method, after binding and insertion into the bacterial membrane, the peptide will move into the cell and interact with negatively charged molecules within

(including DNA and RNA, amoung others) to prevent transcription, translation, and other necessary bacterial survival activities (125). These multiple mechanisms of action make rapid development of resistance very difficult for bacteria, and explains why these phylogenetically ancient peptides still remain effective antimicrobials (124).

In multiple studies, cathelicidins have repeatedly been shown to effectively kill Gram-positive, Gram-negative bacteria (108, 126), mycobacteria (104), as well as fungi (127), and to neutralize membrane-enveloped viruses (128, 129), all of which are the sources of clinically relevant infections in the airways. However, while their bactericidal role has been well established *in vitro*, there are concerns about the activity of these peptides within physiologically relevant settings. Travis *et al*. showed that, while all cathelicidins investigated had potent bactericidal activity in the absence of salt, only two peptides (SMAP-29 from sheep and CAP-18 from rabbits) showed similar bactericidal activity at "high" salt concentrations (100mM NaCl) (130), which is still lower than physiologically relevant conditions found within the lung (131). Other peptides, however, have been shown to have salt-insensitive activity, such as CATH-2 (101, 132). However, various modifications can affect bactericidal activity in these high salt conditions, as Veldhuizen *et al*. demonstrated that shortening of cathelicidins, particularly through truncations to the C-terminal of the peptide, tends to lead to increased salt sensitivity with respect to antimicrobial activity. They postulated that α-helix stability was one of the factors behind the disparity in activity (133).

Regardless of the controversy surrounding their direct bactericidal role *in vivo*, cathelicidins have been repeatedly shown to have direct anti-infective functions in clinically relevant models of infection. They were first identified as key members of

immunity in mouse models of Group A *S. pyogenes* skin infection, where loss of mCRAMP expression via knockout led to reduced clearance of the bacteria and an increase in the necrotic tissue area at the site of infection (134). This was one of the earliest studies to provide direct evidence of cathelicidin function *in vivo*, which initiated the investigation of these peptides in other disease models, including respiratory infections.

In CF, despite the failed bacterial clearance by the immune system, there are significantly elevated levels of LL-37 within the sputum, which correlates with disease severity (135). However, sputum samples that were treated with lipopolysaccharide-binding protein (LBP) and DNase *in vitro* were able to reduce bacterial load, presumably by releasing the AMPs within the sputum from LPS and DNA/F-actin that may have been binding to and masking the peptide's direct bactericidal functions (136, 137). Loss of endogenous cathelicidin expression *in vivo* has been shown to significantly reduce bacterial clearance in infection models using *K. pneumoniae* and *P. aeruginosa*, again demonstrating the important role of cathelicidins in the innate immune response towards bacterial infections (138).

Despite the vast amount of research investigating the potential of cathelicidin peptides as exogenous therapies, administration of exogenous cathelicidins and cathelicidin-derivatives to directly treat bacterial lung infections *in vivo* have shown much more modest results to date. A recent study by Mardirossian *et al* showed that, despite efficacy *in vitro* and peptide tolerability in naïve animals, intratracheal instillation of BMAP-27 and its peptide derivatives was unable to significantly reduce *P. aeruginosa* bacterial load 24 hours after treatment (139). Using a similar model, Beaumont *et al* showed that intratracheal administration of LL-37 after *P. aeruginosa* infection aids in the bacterial

clearance, but via augmentation of the endogenous host defense system, not through direct bactericidal activity (140).

Taken together, these studies provide evidence of the disparity in cathelicidin activity between individual peptides with respect to direct bactericidal activity. The lack of translational activity between *in vitro* studies and actual effects observed after *in vivo* administration highlight the need for thorough investigation of various cathelicidins in order to identify an optimal peptide which maintains salt-insensitive, *in vivo* bactericidal activity, in order for further development of a clinical therapeutic agent.

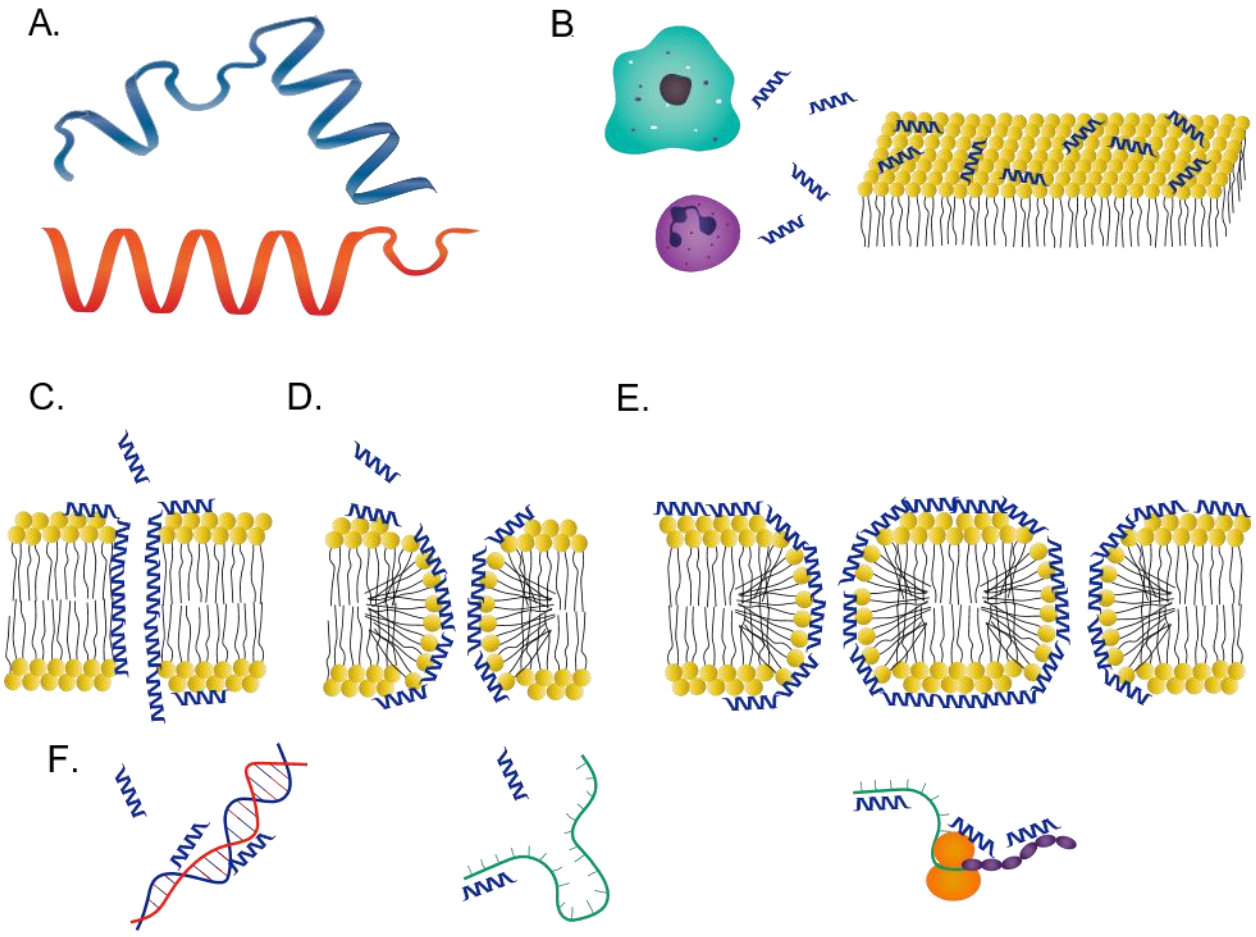

Figure 1.1. a) A cartoon ribbon model of two α-helix structured cathelicidin peptides; CATH-2 (blue), and LL-37 (red). b) Cathelicidins have been found to be endogenously released by both macrophages (light blue), neutrophils (purple), and epithelial cells (not shown) after induction of inflammatory signalling, at which point the peptide is now free to interact directly with the bacterial membrane to begin bactericidal activities. The three proposed mechanisms of bactericidal activity via pore formation are c) barrel-stave model, where the neutrally-charged portion of the cathelicidin interacts with the lipid tail groups of the bacterial cell membrane, eventually forming a pore; d) the toroidal pore model, in which the positively-charged portions of the peptide interact with the negatively-charged phospholipid head groups by inserting into the bacterial membrane, forcing the membrane to fold inwards as well; e) carpet model, in which the peptides orient in parallel to the bacterial membrane, and upon reaching a "critical" concentration, form micelle-like structures and lyse the cell membrane. f) Mechanisms of intracellular activity, which include inhibition of DNA, RNA, and protein synthesis.

1.4.3 Cathelicidin immunomodulatory activity

As a result of the unclear nature of cathelicidins' bactericidal activity *in vivo*, some research groups have begun testing cathelicidin immunomodulatory activity (83, 141–143). It has been well documented, both *in vitro* and *in vivo*, that cathelicidins are able to modulate inflammatory signalling, by either eliciting a pro-inflammatory or anti-inflammatory signal, which appears to be dependent upon microbial stimulation and microbial by-products initiating the signal (140, 144, 145).

The presence of the human cathelicidin LL-37 has been shown to skew differentiating monocytes towards M1 (pro-inflammatory) macrophages (146). However, LL-37 has been shown to be able to reduce pro-inflammatory cytokine production from these M1 macrophages if co-administered with LPS or LTA at physiologically relevant levels, without affecting macrophage phagocytic activities (82). There is evidence that LL-37 appears to inhibit cytokine expression of stimuli activating the toll-like receptors TLR 2 and TLR 4 (LTA and LPS, respectively) (144). However, this anti-inflammatory activity appears to be very specific, as cytokine production after stimulation by of TLR2/1 via the synthetic lipopeptide PAM_3, or TLR2/6 via ZymA is not affected by administration of LL-37 (82, 146).

Asides from LL-37, other cathelicidins have been shown to have similar activity across several distinct species. Murine CRAMP has been shown to act similarly to LL-37, inducing macrophage polarization and reducing cytokine production upon microbial stimulation by LPS, LTA, as well as bacterial flagellin (a TLR 5 agonist) (147). Chicken cathelicidins CATH-1, -2, and -3 have been shown to similarly block LPS-induced cytokine

production from mouse macrophage cell lines, which importantly indicates cross-species activity (101, 132, 148).

However, the anti-inflammatory properties of cathelicidins are not as straight-forward as initially suspected. Both LL-37 and CATH-2 have been shown to be involved in the association of DNA complexes that activate TLR 9 and increase inflammatory signalling (149, 150). Similarly, mCRAMP-knockout (*CAMP*$^{-/-}$) mice showed a reduced ability to respond after CpG DNA injection through a reduction in inflammatory cytokine production (TNF-α, IL-6), while it was shown at the same time that mCRAMP is responsible for complexing with CpG DNA and TLR 9 in the endolysosome of murine macrophages (151).

Adding to their immunomodulatory properties, many cathelicidins have also been documented to act as chemoattractant agents. LL-37 has been shown to induce chemotaxis in monocytes, neutrophils (152), eosinophils (153), and T-cells (154). Similar neutrophil chemotactic activities have been found for additional peptides, such as mCRAMP, PR-39 (155), and chicken CATH-1 (156), while mononuclear cell recruitment appears to be less universal. The chemotactic functions of these peptides appears to be regulated through the formyl peptide receptor 2/formyl peptide receptor-like 1 (FPR2/FPRL1) (152). Similar receptor interactions have been identified for other species, such as mCRAMP and the murine homologue of FPR2 (157). For mast cell specific mobilization and activation, LL-37 has been able to act through the MrgX2 receptor specific to mast cells (158). There is also some evidence of LL-37 acting as a direct ligand for the CXCR2 chemokine receptor found on neutrophils, where interaction induces calcium influx in a dose-dependent manner (159).

Independent of their direct chemotactic activity, cathelicidins are also able to upregulate the production of chemokines from other cell types. Macrophages stimulated with LL-37 produced significantly higher levels of MCP-1, a key chemokine for the recruitment of monocytes, natural-killer cells, and T-cells, however LL-37 incubation with LPS inhibited the release of MCP-1 from airway epithelia. Additionally, stimulation with LL-37 alone produced IL-8, a neutrophil chemokine, in a dose dependent manner (160). This IL-8 production appears to be through the interaction of these peptides with both the P2X7 receptor and epidermal growth factor receptor, and has been shown to be produced from epithelial cells, fibroblasts, and macrophages (161–163). LL-37 binding with GAPDH from monocytes also induces the production of CXCL1, another neutrophil chemokine (145).

Based on the studies of immunomodulatory activities described above, cathelicidins have become attractive therapeutic targets as immune system adjuvants. Summation of the data presented above suggests that cathelicidins have the ability to maintain a complex inflammatory balance within an infection environment. This capability to balance the immune system, ensuring a sufficient inflammatory response to eliminate invading pathogens while preventing excessive host responses, suggests that cathelicidins are extremely promising as therapeutics for many chronic lung diseases, and especially CF lung disease where excessive pro-inflammatory activation often leads to collateral tissue damage and remodelling. The use of a cathelicidin peptide that possesses antibacterial activity *in vivo*, while being able to modulate inflammatory responses, would be an ideal therapeutic for this type of disease.

1.4.4 Chicken cathelicidin CATH-2

CATH-2 is one of four cathelicidins found in chickens (91). Produced almost exclusively by heterophils (chicken homologue of human neutrophils), the active CATH-2 peptide is cleaved from its pro-piece by serine proteases and released upon immune system stimulation, such as LPS activation (164). Once released, the peptide can exert its potent broad-spectrum microbicidal activity (127, 132). CATH-2 shows antimicrobial activity against a wide variety of both chicken and human pathogens, generated minimum inhibitory concentration (MIC) values between 0.6-5.3 μM. Unlike other cathelicidin peptides, this antimicrobial activity appears to be unaffected by increasing salt concentrations (101, 132, 165). CATH-2 appears to kill through multiple mechanisms of action, depending on peptide concentration, including direct membrane permeabilization, as well as interacting with intracellular components, although the exact target is unknown (166).

In addition to this salt-insensitive broad-spectrum activity, CATH-2 has also been shown to have strong immunomodulatory activity. It has been shown to directly bind to multiple immunostimulatory products, such as LPS (132, 166, 167). Interactions of CATH-2 with LPS, as well as the gram-positive endotoxin LTA, are able to significantly reduce TNF-α production from macrophages *in vitro*, while interactions with CpG DNA increases TNF-α production, consistent with observations from other cathelicidin peptides (151, 167).

In vitro, CATH-2 has demonstrated the unique ability to kill a broad-spectrum of human pathogens, maintains bactericidal activity within physiologically relevant salt concentrations, and is able to modulate inflammatory responses *in vitro* across multiple

different stimulatory molecules, in both chickens, as well as mouse cell lines. **Based on both its bactericidal and immunomodulatory effects, CATH-2 is a highly promising cathelicidin of interest for further investigation.** However, the use of CATH-2 in *in vivo* models, and its bactericidal activity against multi-drug resistant human pathogens, is yet unknown.

1.5 Cathelicidins in clinical trials

Due to their multiple mechanisms of bactericidal activity, cathelicidins are ideal peptides for next generation antibiotics due to their low propensity for the development of bacterial resistance. Despite the bacterial development of protease secretion and membrane/exotoxin post-translational modifications as possible resistance mechanisms, the fact that these peptides retain broad-spectrum activity against a variety of human pathogens reinforces their clinical potential in future therapies (124).

According to ClinicalTrials.gov, there are currently approximately 80 trials involving cathelicidins, as well as other antimicrobial peptides, either through direct administration of exogenous antimicrobial peptides, through treatments aimed to increase endogenous cathelicidin pool sizes within patients (through the administration of butyrate, vitamin D, or their alternative forms), or through treatments aimed at the inhibition of cathelicidin production for diseases of peptide over expression. In addition, there is a large field of research involving pre-clinical studies investigating multiple cathelicidin peptides in many infection and disease states (141).

However, despite the therapeutic promise of cathelicidin peptides, very few have been developed through all stages of clinical trials. Obstacles to development often involve

either toxicity towards eukaryotic cells, which can be observed at high doses (101), or the poor stability of cathelicidin peptides to endogenous proteases found in the gastrointestinal tract, serum, and even bronchoalveolar lavage fluid (83, 139). Therefore, optimal cathelicidin therapy for pulmonary infections would require a) a peptide that is functional in the pulmonary, high salt, environment, b) a method to protect the peptide from degradation, limited toxicity, and c) a method to deliver it directly to the injured location.

1.6 Delivery of therapeutics to the airways

During treatment of lower respiratory tract infections, adequate antibiotic/antimicrobial site concentrations are imperative for the clearance of infection. This is made difficult by the unique branching structure of the lung (168). Despite this, the delivery of antibiotics directly to the site of infection within the lung, which is currently limited to aerosolized administration, has numerous benefits over current systemic administration routes, including increased local concentrations of antibiotics, and enhanced microbial clearance. Furthermore, there is a reduced risk of systemic toxicity which is common in numerous conventional antibiotics (29, 169–171). However, aerosolized administration of antibiotics is typically found to accumulate in the central airways, with large volumes of antibiotics required to reach adequate concentrations at the distal sites of infection (30, 172, 173). Therefore, to optimize delivery of therapeutics to the lungs, other drug delivery vehicles must be identified.

1.6.1 Exogenous pulmonary surfactant as a vehicle for therapeutic delivery

In order to overcome these delivery issues, many groups have investigated the potential of pulmonary surfactant as a delivery vehicle (29, 169, 170, 174–177). Pulmonary

surfactant is a lipoprotein mixture, endogenously produced by type II alveolar cells within the lung, composed of approximately 80-85% phospholipids, 5-10% neutral lipids, and 5-10% surfactant specific proteins (9, 13). Exogenous clinical surfactants, derived from animals including bovine and porcine sources, have been used over the past 35 years as a treatment for neonatal respiratory distress syndrome, which is characterized in premature infants as a lack of sufficient endogenous surfactant (19, 178, 179). Surfactant acts by adsorbing and spreading at the air-liquid interface within the alveoli and acts to reduce surface tension, thereby improving lung compliance and preventing alveolar collapse upon exhalation (14).

The spreading capabilities of surfactant has been investigated as a potential mechanism of distributing therapeutics to distal airways (29). The benefits of surfactant spreading were first demonstrated by Kharasch *et al.*, who showed that surfactant administration was superior to aqueous solution administration with regards to lung tissue distribution (30). They demonstrated that surfactant was able to disperse more uniformly throughout the lungs, in comparison to saline solution, which tended to localize to the central lung regions (30). Surfactant distribution rates are also far superior to current practices involving aerosolized administration, which only achieves ~10% deposition in small airways (180, 181).

Based on the promise of these initial distribution studies, several groups began investigating the potential of utilizing surfactant as a respiratory vehicle, with the purpose of increasing concentrations of therapeutics at the infection site and reducing toxicity risks of systemically administered antibiotics (29, 30, 170, 176). Van't Veen *et al.* were able to show that the combination of surfactant with the antibiotic tobramycin was able to

significantly improve survival in mice infected with *K. pneumoniae* compared to animals receiving surfactant alone, or tobramycin alone (176). The same group was then able to show that the *in vivo* administration of a surfactant preparation with cyclosporine A suspended in ethanol had no impact on surfactant function (177), and were able to reduce inflammation and acid sphingomyelinase activity after "fortification" of surfactant with a NF-κB inhibitor (182).

As mentioned previously, pulmonary surfactant has been used as a clinical therapy from 1980 for the treatment of neonatal respiratory distress syndrome (25). While the use of surfactant for the treatment of adult respiratory distress syndrome failed to show significant clinical improvement, it has repeatedly been demonstrated to be a safe therapeutic (183). However, more recent studies have begun investigating the potential of surfactant as a mucokinetic agent, which would act to improve clearance of mucus plugging (184). Studies by Al-Saiedy *et al*. demonstrated that the addition of the exogenous surfactant BLES to an *in vitro* mucus plug system was able to improve mucus "velocity" over the course of ventilation (185).

Along with its potential as a respiratory vehicle, the multifunctionality of a combined surfactant/antimicrobial peptide compound to distribute therapeutics throughout the airways, reduce mucus plugging, and eliminate multi-drug resistant bacteria would be of major therapeutic benefit as a treatment for many different respiratory diseases, including cystic fibrosis and ventilator-associated pneumonia. However, as demonstrated by van't Veen *et al*., this combination must be able to maintain the innate functions of each of its parts, as some antibiotics have demonstrated a loss of activity after instillation with surfactant, and surfactant inactivation would compromise lung function (175).

1.7 Summary, objectives, and hypothesis

Over the course of respiration, the lung is constantly bombarded with pathogenic bacteria. In some cases, these bacteria are able to circumvent the natural host defense systems and develop into an airway infection. Left untreated, these infections can lead to severe tissue damage in the lungs, resulting in inefficient gas exchange, poor oxygenation, respiratory failure, and eventually death. Normally, treatment of these infections involves a simple course of antibiotics, resulting in clearance of the pathogen. However, ineffective antibiotic stewardship combined with a lack of pharmaceutical investment has cumulated into a global healthcare "perfect storm" with the increasing emergence of antibiotic resistant bacteria. **There is a dire need for the development of novel therapeutics for the treatment of antibiotic resistant bacterial infections**.

To develop these new therapeutics, we have investigated the potential of cathelicidin peptides. Their direct antimicrobial activity, coupled with their immunomodulatory functions, are theoretically ideal for the treatment of infection, as they present the ability to directly kill colonized bacteria while downregulating the inflammatory response associated with bacterial death. In addition to cathelicidins, we have investigated an exogenous pulmonary surfactant, BLES, as a vehicle for these cathelicidin peptides, in order to improve therapeutic administration into the peripheral airways of the lung.

However, interaction between cathelicidin peptides and exogenous pulmonary surfactant is currently unknown. In **Chapter 2**, we investigated **the proof-of-principle of a cathelicidin/surfactant compound, and select an optimal cathelicidin for therapeutic use.** This investigation was performed by assessing the biophysical and antimicrobial

activities of multiple cathelicidin peptides in combination with exogenous surfactant in order to select an ideal peptide/surfactant combination for future therapeutic study. Once identified, we tested the safety of this therapeutic after intratracheal administration in naïve animals.

After identifying the optimal cathelicidin/surfactant combination, and performing safety studies, **Chapter 3** investigated the **immunomodulatory activity of the selected cathelicidin, CATH-2, using administration of killed *P. aeruginosa*.** Bacteria were killed via three separate mechanisms, and intratracheally instilled into mouse lungs in order to study the inflammatory response elicited. The ability of CATH-2 to kill bacteria in an immunogenically "silent" manner, as well as the ability of CATH-2 to down-regulate the inflammatory response of other killed-bacteria were investigated.

Following the investigation of CATH-2's immunomodulatory activity, **Chapter 4** focused on the **bactericidal and immunomodulatory properties of BLES+CATH-2 against multi-drug resistant clinical isolates obtained from CF patients**. Similar to previous methodology, we investigated the *in vitro* bactericidal activity of BLES+CATH-2, and investigated the immunomodulatory activity of BLES+CATH-2 upon instillation *in vivo* with heat-killed bacteria.

Finally, in **Chapter 5** we investigated the **bactericidal and immunomodulatory activity of BLES+CATH-2 in two distinct models of pneumonia.** To test this, two models - a mouse model of bacterial pneumonia, and a rat model of ventilator-associated pneumonia - were used to assess the bactericidal activity of BLES+CATH-2 treatment *in vivo* in clinically relevant models of respiratory infections.

The **overall objective** of this thesis was to develop an antibiotic-alternative, novel therapeutic for the treatment of multi-drug resistant respiratory infections. The **overall hypothesis** is that the administration of a cathelicidin/surfactant compound will improve clinically relevant outcomes in the treatment of bacterial pneumonia.

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Chapter 2: Surfactant supplemented with an antimicrobial peptide for the treatment of bacterial pneumonia: antimicrobial and biophysical properties

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2.1 Introduction

The emergence of highly resistant strains of bacteria currently represents a significant public health issue for patients due to the pervasive use of antibiotics on a global scale. Patients with acute or chronic pulmonary infections where antibiotic use is widespread, such as in case with cystic fibrosis and ventilator-associated pneumonia, antimicrobial resistance is particularly problematic and is a strong predictor of poor outcomes (1). Furthermore, the distinct structure of the lung, combined with the potential of inaccessible areas due to collapse and edema arising from infections, significantly impairs effective antimicrobial drug delivery in this organ. New, innovative therapeutic approaches to combat lung infections are desperately needed.

Antimicrobial peptides (AMPs) form part of the innate immune system and are evolutionarily conserved across a wide variety of organisms including humans (2). One class of antimicrobial peptides, cathelicidins, possess antimicrobial function through a variety of mechanisms including direct interaction with bacterial cell membranes and interference of intracellular bacterial targets (2). The net positive charge of these peptides ensures they are more likely to interact with the negative cell walls of bacteria than the neutral cellular membranes of eukaryotic cells. Importantly, it has been demonstrated that they remain functional against microbes with antibiotic resistance, and considering their mechanism of action, it is less likely that resistance will develop due to the changes in membrane structure that would be required for effective resistance (2). It has been shown *in vitro* that AMPs can induce transient resistance, but there is no evidence that this occurs *in vivo* (3). Based on these properties, antimicrobial peptides have received a lot of attention as alternatives to antibiotics, particularly for topical therapies (4). Utilization for pulmonary

infections has been investigated but is complicated by delivery issues and to date has not been successful in clinical trials (5–7).

To address the pulmonary delivery issue, exogenous surfactant has been proposed as carrier for a variety of biological agents (8). Exogenous surfactant is derived from endogenous pulmonary surfactant, a lipoprotein complex naturally produced by type II alveolar cells, that is made up of approximately 80-85% phospholipids, 5-10% neutral lipids (including cholesterol), and 10% proteins (9). The main function of surfactant is to reduce surface tension at the air-liquid interface of the alveoli. To accomplish this goal, surfactant adsorbs and spreads rapidly to form a surface film consisting or lipid-condensed and lipid-expanded regions at the air-liquid interface which upon lateral compression forms a stable multilayer structure capable of reducing surface tension values to near 0 mN/m (10, 11). Through its ability to spread throughout the lung and open up collapsed lung areas, exogenous surfactant therapy has been shown to reduce mortality of premature infant afflicted with neonatal respiratory distress syndrome (12).

The general characteristics of surfactant and positive findings of exogenous surfactant therapy led to a number of laboratory studies to investigate the possibility of exogenous surfactant as a pulmonary delivery vehicle for other drugs (13–17). The rationale for this approach is that the spreading properties of surfactant would improve therapeutic distribution throughout the lung, while opening of collapsed lung regions to improve drug availability directly at the site of an infection (8). Imperative for this approach is that the drug of choice does not impact surfactant's ability to spread throughout the lung and reduce surface tension, and, vice versa, that surfactant does not interfere with the therapeutic efficacy of the drug.

Based on this information our objective was to evaluate the spreading, biophysical capabilities, and bactericidal function of four cathelicidin peptides, CATH-1, CATH-2, mCRAMP, and LL-37, combined with a clinical exogenous surfactant, bovine lipid-extract surfactant (BLES). Maintenance of respective surfactant and cathelicidin functions when the two compounds are combined would provide a first proof-of-principle toward the hypothesis that a combination treatment of cathelicidins with an exogenous surfactant vehicle would be effective in treating antibiotic resistant lung infections.

2.2 Materials and Methods

Surfactant/Peptide Compounds

BLES (BLES Biochemicals, London, ON, Canada) is a commercially available clinical preparation, stored in 100 mM sodium chloride and 1.5 mM calcium chloride with a phospholipid concentration of 27 mg/ml. BLES contains natural phospholipids found in bovine surfactant, along with surfactant-specific proteins SP-B and SP-C and a small percentage of cholesterol.

Antimicrobial peptides were synthesized using Fmoc solid-phase synthesis as described previously (18). All peptides were purified to a minimum purity of 95% by reverse phase high-performance liquid chromatography prior to biological testing. The sequences of the peptides used in this study are shown in Table 2.1. Four AMPs were tested in this experiment: chicken cathelicidin-1 and 2 (CATH-1, CATH-2), mouse cathelicidin CRAMP, and human cathelicidin LL-37. All peptides were suspended in non-buffered sterile saline. BLES and peptide were mixed to concentrations of 10 mg/ml BLES, and 10/40/100/200 μM for the peptides.

Peptide	Amino Acid Sequence	Net Charge
CATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	+8
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF	+9
mCRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	+6
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6

Table 2.1 Amino acid sequence and charge of cathelicidins investigated.

Spreading

Adsorption of BLES and the various peptides were measured using the Wilhelmy probe and FilmWare 2.51 software of the Langmuir balance. A petri dish with a diameter of 100 mm, and surface area of 78.54 cm^2 was used. It was filled with 25 ml of 10% sucrose, with a 15 ml layer of ddH_2O layered on top. The probe of the Langmuir balance was placed on the surface of the ddH_2O sub-phase, and 50 µl of each peptide-surfactant sample was pipetted into the water phase. The surface tension was recorded over the subsequent 600 seconds.

Minimal surface tension over multiple compression-expansion cycles

To investigate the minimum achievable surface tension over ten compression-expansion cycles, a constrained sessile drop surfactometer (CDS) (BioSurface Instruments, HI) was used (19–21). A ten microliter drop of sample was placed upon the drop pedestal, and using an external stepper motor, the drop was cyclically compressed-expanded for 10 cycles at a rate of 5 seconds/cycle, and a compression ratio of approximately 27%. Images of the droplet were recorded at a rate of one image per second, and were analyzed. The image with the lowest surface tension throughout each cycle was determined to be the minimum surface tension (MST) for that cycle. Minimum surface tensions for all 10 cycles were measured.

AFM imaging of surface films

In order to examine the effect of peptides on the surfactant surface films, Langmuir-Blodgett films were prepared using a Kibron™ Trough SE (Helsinki, Finland). Briefly,

films were spread by depositing droplets of surfactant samples uniformly throughout the air–water interface. Deposits were taken at surface pressure of 30 mN/m on freshly-cleaved mica. Topographical atomic force microscope (AFM) images were obtained using a Nanoscope III scanning force multimode microscope (Digital Instruments, Santa Barbara, CA). Samples were scanned with a J-type scanner using contact mode in air. A silicon nitride cantilever with a spring constant of 0.12 N/m was used. Image analysis was performed using the Nanoscope III software (version 5.12r3). All AFM images were subjected to quantitative analysis using ImageJ Software (National Institute of Health) to determine the surface area of condensed domains. At least 9 AFM images were used for each sample and the results are shown as averages and standard error.

Antimicrobial assays

An overnight culture of either Methicillin-resistant *Staphylococcus aureus* WKZ-2, or *Pseudomonas aeruginosa* VW178 (isolated from a cystic fibrosis patient) were diluted 1/1000 in tryptic soy broth (TSB) and left to incubate for three hours at 37°C in order to reach exponential growth phase. The optical density was measured, and bacteria were further diluted in PBS to reach an initial concentration of approximately $2x10^6$ CFU/ml. From there, 25 µl of peptides at various concentrations, with or without BLES, were added to polypropylene coated round-bottom 96 well plates, along with 25 µl of bacteria, and incubated at 37°C for three hours with no shaking. Subsequently, 200 µl of minimal media was added, and the colonies were serially diluted 10-1000-fold. After the serial dilution, 100 µl of each dilution were plated on tryptic soy agar (TSA) plates and left to incubate overnight at 37°C. Following overnight incubation, the colonies on the plates were counted.

Minimum bactericidal concentrations (MBC) were defined for these experiments as the concentration of surfactant/peptide compound at which less than 10 colonies were counted at the highest dilution, corresponding to less than 100 CFU/ml.

In vivo analysis of safety and tolerability of BLES+CATH-2 treatment

Male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada), weighing 22-30 g, were used for this experiment. All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, and followed the approved guidelines described by the Canadian Council of Animal Care. Mice were anesthetized by intraperitoneal injection of a ketamine (100 - 125 mg/kg body weight) and dexmedetomidine (0.5 mg/kg BW) mixture, and then intubated using a 20 G catheter, with the aid of a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Texas, USA). Once intubated, the mice were instilled with 50 µl of one of five treatments: a) air bolus, b) sterile saline, c) BLES (10 mg phospholipid/ml), d) CATH-2 (100 µM, suspended in sterile saline), or e) BLES+CATH-2 (10 mg phospholipid/ml, 100 µM peptide). Mice were extubated following successful instillation and were subsequently injected with the reversal agent for dexmedetomidine, Antisedan, and allowed to breathe spontaneously for the following four hours. After four hours, the mice were euthanized by intraperitoneal (IP) injection of sodium pentobarbital (110 mg/kgBW) and dissection of the descending aorta. The animals were placed on a FlexiVent© in order to measure physiologic parameters, such as lung capacity, compliance, and airway resistance. Following these measurements, whole lung lavage was collected by 3 x 1 ml aliquots of sterile saline. The whole lung lavage was immediately centrifuged at 150 x g at 4°C, and

the pellet was collected for cell analysis, while the supernatant was used to measure protein and IL-6 content. Differential cell analysis of the cells obtained in the lavage was done as previously described (22). Protein content within the lavage fluid was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), according to manufacturer's instructions. IL-6 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, Calif., USA), according to manufacturer's instructions.

Statistical Analysis

The effect of various cathelicidin peptides on BLES' surface tension reducing capabilities, and adsorptive properties, were calculated by one-way measure analysis of variance (ANOVA) followed by a Dunnett post-hoc test using BLES as the control group. Analysis of *in vivo* assays were performed by one-way ANOVA followed by Tukey post-hoc test. Means are reported ± SEM and values were considered significantly different at a probability value < 0.05.

2.3 Results

Surface Tension after 600 seconds

Initial assessment involved an analysis of the ability of the surfactant plus peptides to form a surface film on a clean air-water interface. This data reflects the "spreadability" of the surfactant which, extrapolating to a clinical scenario, is an important indicator of the ability of this material to distribute throughout the lung and reach the distal airways and alveoli. Each sample was allowed to form a film for 600 seconds and surface tension reached was recorded. As shown in Figure 2.1, there were no significant differences in the end surface tensions between any of the surfactant/cathelicidin mixtures at 10 μM concentrations and BLES (control). However, at a cathelicidin concentration of 40 μM, a significant decrease in the surface tension at the end of 600 seconds of spreading was observed for BLES+CATH-1 ($p<0.01$) and BLES+CATH-2 ($p<0.05$) mixtures compared to BLES (control) indicating improved spreading characteristics. All four surfactant/cathelicidin mixtures tested resulted in significantly lower surface tension at the end of 600 seconds spreading at 100 μM concentrations compared to BLES controls.

Figure 2.1

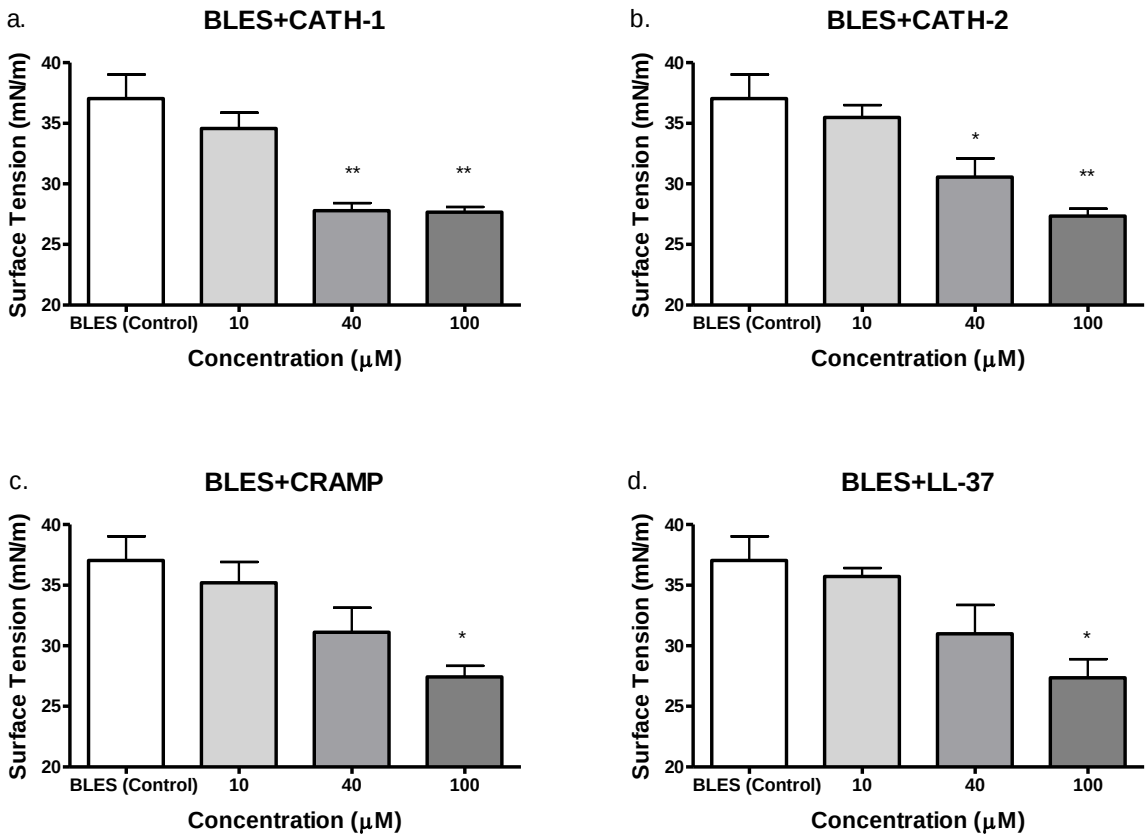

Figure 2.1. Adsorption assay of exogenous surfactant BLES in mixture with cathelicidin peptides. a) CATH-1, b) CATH-2, c) mCRAMP, and d) LL-37 at concentrations of 0, 10, 40, and 100 μM. Values are mean surface tension ± SEM after 600s. (n=3) *$p<0.05$ vs. BLES (Control), **$p<0.01$ vs. BLES (Control).

Minimum surface tension over multiple compression cycles

In order to examine the surface tension reducing function of the peptide/surfactant mixtures, the samples underwent ten cyclic compressions at a rate of five seconds/cycle via the CDS, as a physiologically relevant model of multiple expansion-contraction cycles during respiration. Minimum achievable surface tension (MST) values were recorded for ten cycles, as a marker of surfactant activity which, in the *in vivo* situation, would reflect the ability of this material to stabilize the alveoli and allow for ease of inflation. BLES alone was able to reach MST values of ~2.5 mN/m consistently over all ten cycles. It was observed that all concentrations of BLES+CATH-2 had a robust surface tension reducing activity, consistently reaching MST values below 4 mN/m. BLES+CATH-2 only showed significantly higher MST values at cycles 8, 9, and 10 at a concentration of 10 μM compared to BLES (control). BLES+CATH-1 mixtures at 40 and 100 μM concentrations showed significantly higher MST values compared to BLES (control) at cycles two through ten (Figure 2.2a), increasing slightly from 4 mN/m to MST of roughly 5 mN/m at the end of the ten compressions. BLES+mCRAMP mixtures had consistently higher MSTs of approximately 4.5 mN/m over all cycles at 40 and 100 μM compared to BLES alone (Figure 2.2c). BLES+LL-37 mixtures had significantly increased MSTs between 4.5-5.5 mN/m over all cycles versus BLES (control), but only significantly different at concentrations of 10 and 40 μM (Figure 2.2d). Minimum surface tensions of BLES+LL-37 mixtures at 100 μM were not statistically different from the BLES (control). Mixtures of BLES+CATH-2 were the only surfactant/cathelicidin mixture to show no significant difference versus BLES (control) at 40 and 100 μM concentrations.

Figure 2.2

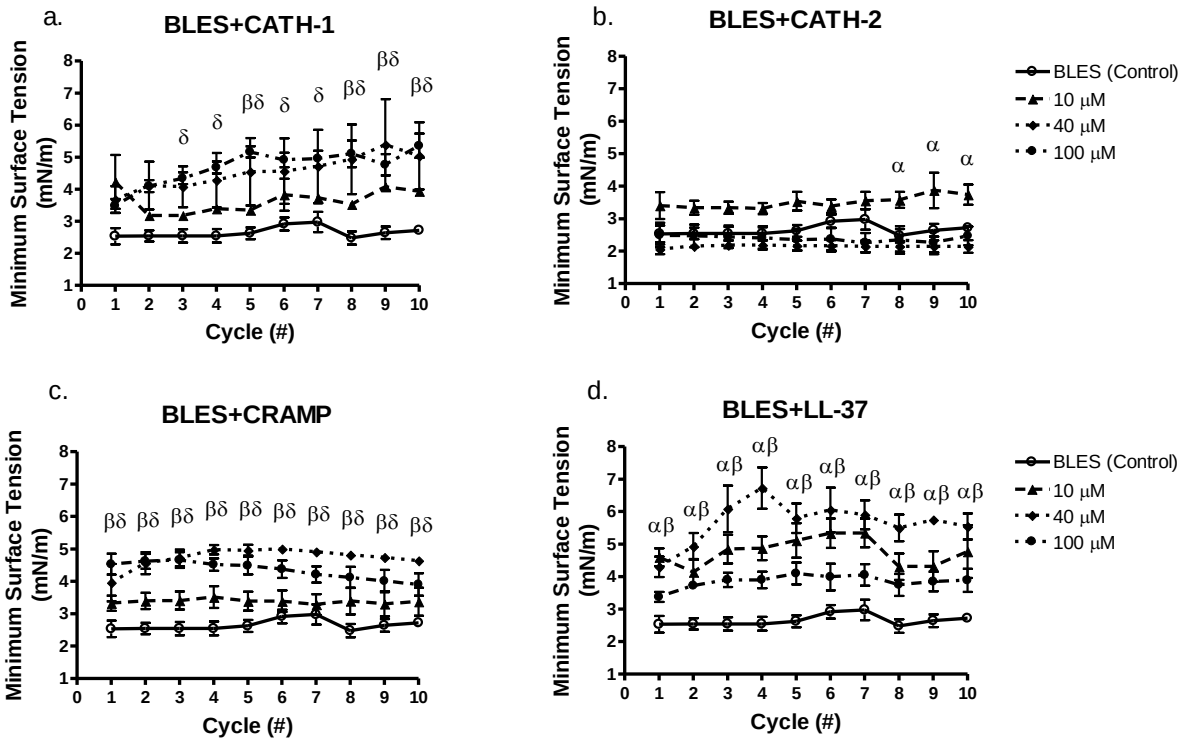

Figure 2.2. Measurement of minimum surface tension during cyclic compressions of exogenous surfactant BLES in mixture with cathelicidin peptides. a) CATH-1, b) CATH-2, c) mCRAMP, and d) LL-37 at concentrations of 0 (open circle), 10 (triangle), 40 (diamond), and 100 μM (circle). Values are mean minimal achievable surface tension ± SEM. (n=3-5) α = 10 μM <0.05 vs. BLES (Control), β = 40 μM < 0.05 vs. BLES (Control), δ = 100 μM <0.05 vs. BLES (Control).

Atomic force microscopy images

To assess the influence of peptides on surfactant film structure, AFM images were analyzed focusing specifically on the formation of liquid condensed domains, as these domains have been suggested to be important in surfactant function (11). Characteristic AFM topographic images of BLES films in the absence and presence of 100 μM peptides (CATH-2, CATH-1, LL-37 and mCRAMP) are shown in Figure 2.3. The brightness in these images is proportional to the height. Phase separation, as indicated by the presence of lighter, condensed domains, is evident in all samples at surface pressure of 30 mN/m (Figure 2.3 a-e). Quantitative analysis of these images shows that addition of CATH-1 and CATH-2 to BLES leads to a change in its lateral organization. More specifically, addition of CATH-1 and CATH-2 to BLES leads to an increase in the number of condensed domains and a decrease in the average surface area of the individual domains (Figure 2.4). Addition of LL-37 and mCRAMP to BLES has no significant effect on average surface area of condensed domains at surface pressure 30 mN/m (Figure 2.4).

Figure 2.3

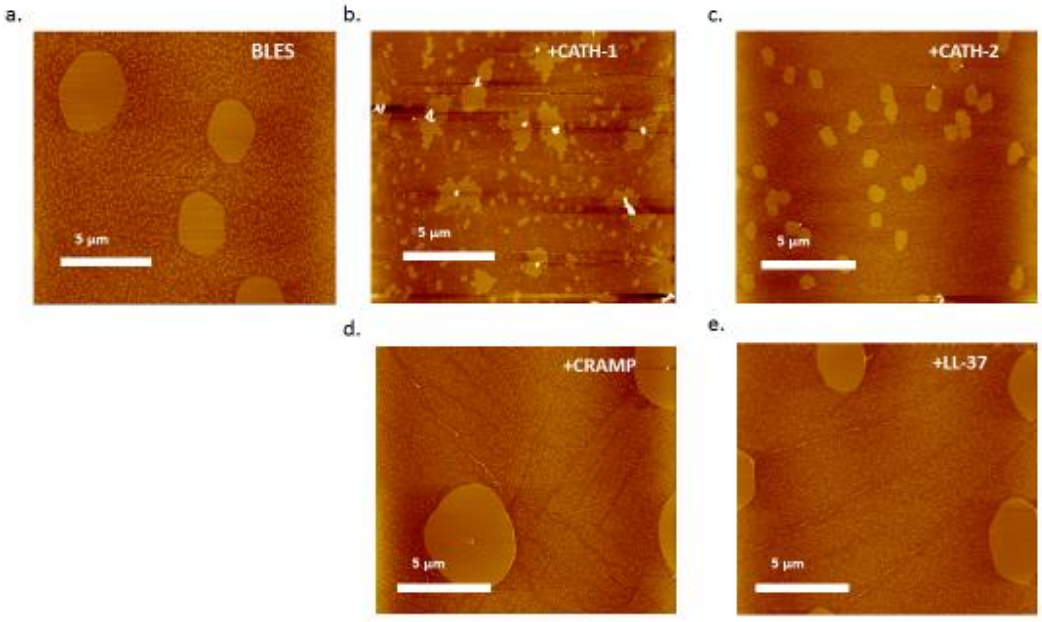

Figure 2.3. Characteristic AFM topographic images showing the effect of peptide addition on the lateral organization of BLES monolayers. The scan area is 20 x 20 µm^2. All films were deposited at a surface pressure of 30 mN/m. (a) BLES; (b) BLES + 100 µM CATH-1; (c) BLES + 100 µM CATH-2; (d) BLES + 100 µM mCRAMP and (e) BLES + 100 µM LL-37.

Figure 2.4

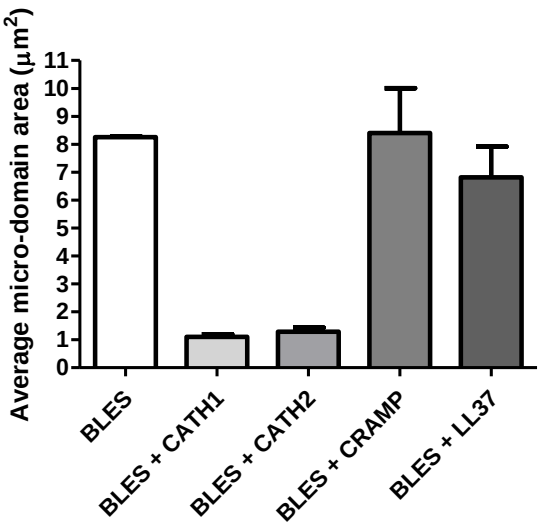

Figure 2.4. Quantification results showing the effect of peptide addition (CATH-1, CATH-2, mCRAMP, and LL-37) on the size (i.e. surface area) of the condensed domains in BLES monolayers at surface pressure 30 mN/m. These results were obtained from four unique AFM topographic images of different samples. (n=2-24)

Antimicrobial assays

In order to determine the bactericidal properties of the surfactant/cathelicidin mixtures, bactericidal assays of each peptide were performed in both the presence and absence of BLES. CATH-1, CATH-2, and LL-37 in PBS were found to have MBC values of 5 μM, reducing CFU/ml values of MRSA by more than four log units (Figure 2.5a,b,d), while mCRAMP reduced levels by three log units at 40 μM concentrations (Figure 2.5c). In the presence of BLES (Figure 2.5), CATH-2 had a MBC of 200 μM, but had considerable antimicrobial activity resulting in a two-log reduction of viability counts at 50 μM. CATH-1 reduced CFU/ml levels by two log units at 200 μM, while mCRAMP and LL-37 mixtures resulted in either a one log reduction, or no reduction at 200 μM concentrations in the presence of BLES.

Similar results were seen against *P. aeruginosa*, where CATH-1, CATH-2, and LL-37 peptides (in the absence of BLES) had MBC values from 5-10 μM (Figure 2.6), while mCRAMP reduced bacterial viability by three log units at 40 μM (Figure 2.6c). In the presence of BLES, CATH-2 again had a MBC of 200 μM, and a three-log reduction at 50 μM. CATH-1 reduced *P. aeruginosa* viability by three log units at 200 μM. mCRAMP and LL-37 showed negligible bactericidal activity in BLES up to 200 μM concentrations.

Figure 2.5

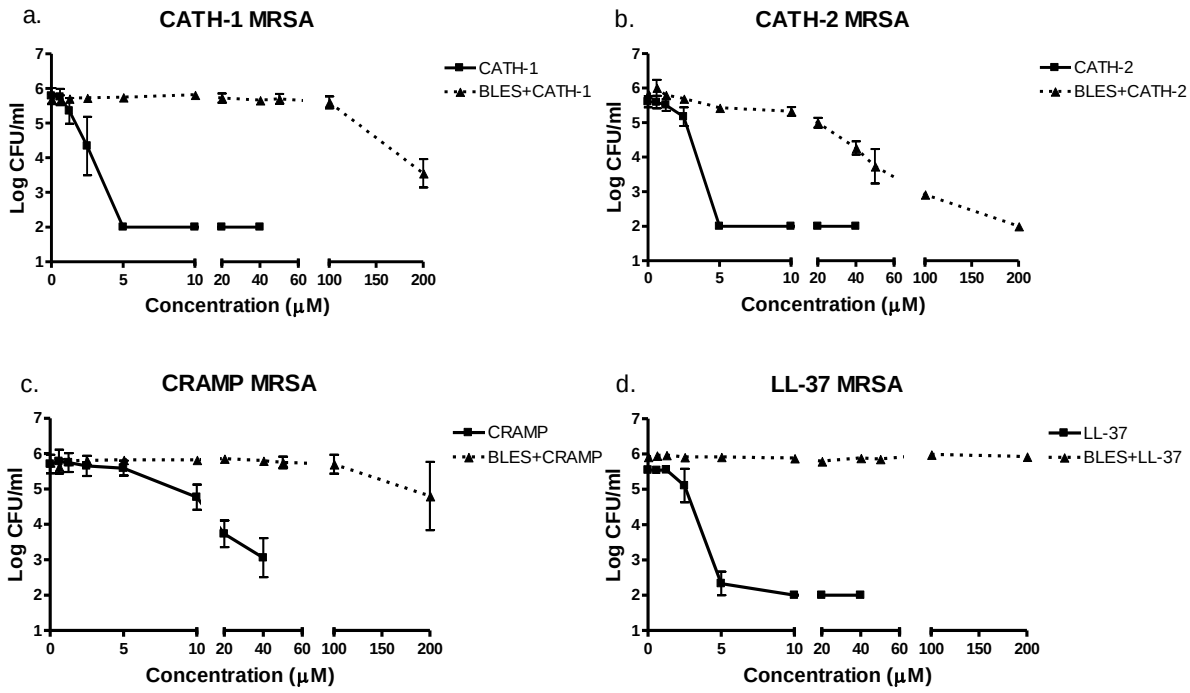

Figure 2.5. Antimicrobial activities of cathelicidins against MRSA. a) CATH-1, b) CATH-2, c) mCRAMP, and d) LL-37 in the absence (square, solid line) and presence (triangle, dotted line) of 10 mg/ml BLES against Methicillin-resistant *Staphylococcus aureus* WKZ-2 in PBS after 3h at 37°C. (n=3)

Figure 2.6

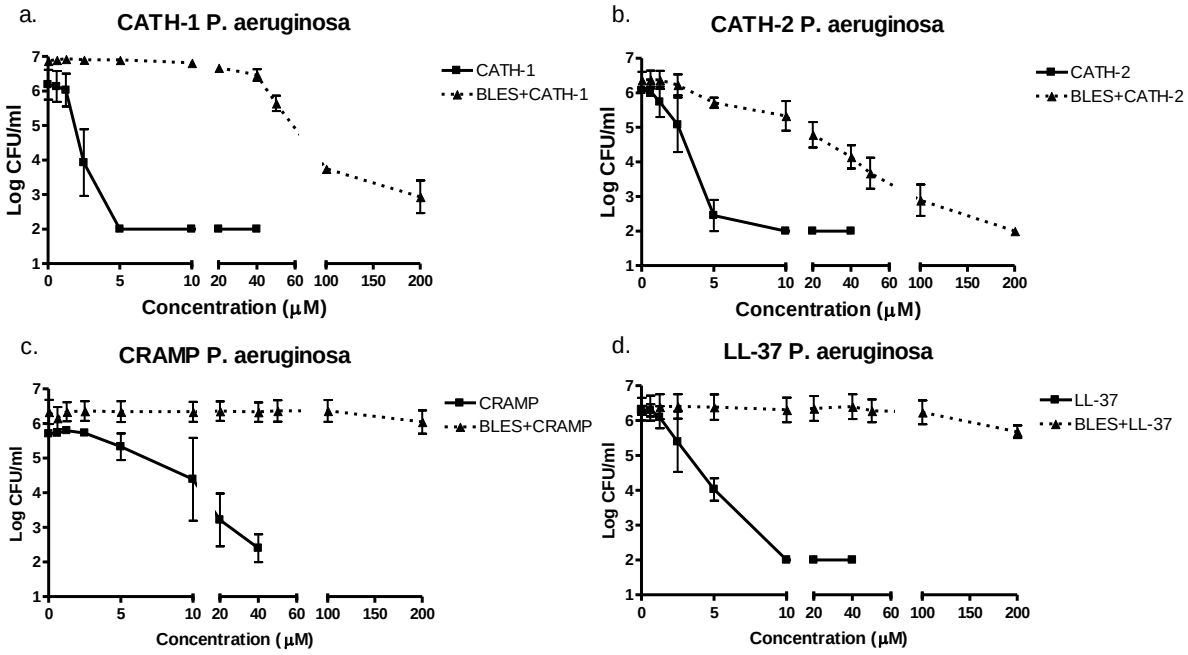

Figure 2.6. Antimicrobial assay of cathelicidin peptides against *P. aeruginosa*. a) CATH-1, b) CATH-2, c) mCRAMP, and d) LL-37 in the absence (square, solid line) and presence (triangle, dotted line) of 10 mg/ml BLES against *Pseudomonas aeruginosa* VW178 in PBS after 3 h at 37°C. (n=3)

In vivo analysis of BLES+CATH-2 treatment

The results of the experiment testing safety and tolerability are shown in Figure 2.7. The experiment focused on CATH-2 and included two control groups (air and saline instilled groups) and three experimental groups (CATH-2, BLES + CATH-2 and BLES instilled groups). Analysis of lung physiology included lung compliance and airway resistance (Figure 2.7a-b) which showed no significant difference among the groups. Mice that were instilled with CATH-2 alone had significantly higher protein content within the bronchoalveolar lavage fluid (BALF) versus animals in other treatment groups, and had significantly increased IL-6 levels versus animals treated with BLES alone (Figure 2.7c-d). BLES+CATH-2 treated animals showed no significant difference in protein or IL-6 levels when compared with air, saline, or BLES controls. Cell count analyses found no significant difference in total cell counts (Figure 2.7e) or neutrophil recruitment (Figure 2.7f) for any treatment group.

Figure 2.7

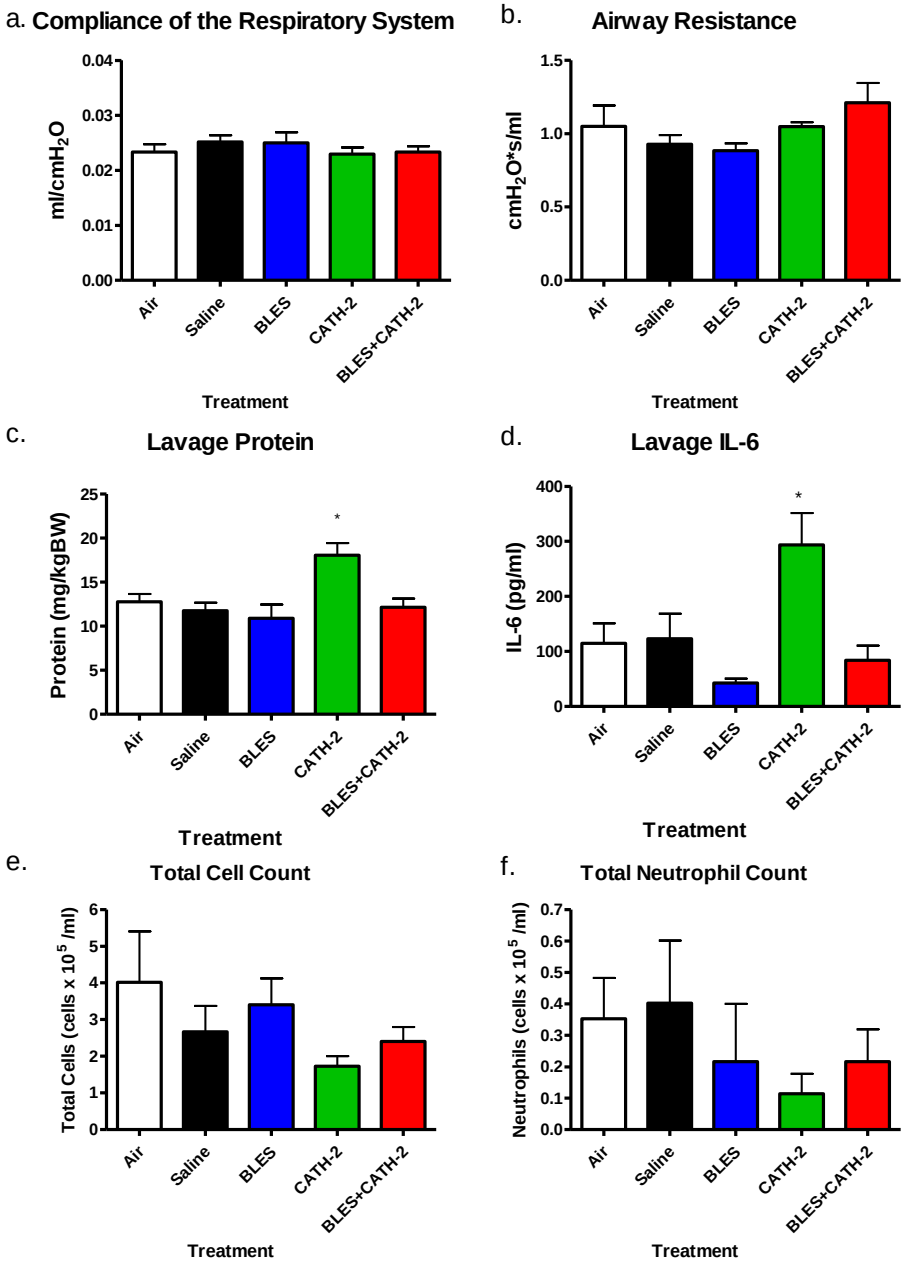

Figure 2.7. From *in vivo* tolerance model, a) compliance of the respiratory system, and b) airway resistance, as measured by FlexiVent; c) protein content, d) IL-6 content, e) total immune cells, and f) total number of recruited neutrophils obtained from animal BALF samples. (n=5-6) *$p < 0.05$.

2.4 Discussion

The goal of the current study was to investigate the biophysical and antimicrobial properties of four novel surfactant/cathelicidin mixtures and to determine whether combining such elements together impacts native function. Four cathelicidin peptides were tested in combination with an exogenous surfactant, BLES. The presence of all cathelicidin peptides accelerated surface film formation of BLES as indicated by the spreading capabilities of BLES plus peptides over 600 seconds. While all other cathelicidin peptides decreased the ability of BLES to achieve low surface tensions during cyclic compressions on the CDS, the addition of CATH-2 to BLES had a minimal effect on the biophysical function of the exogenous surfactant even at high concentrations. AFM images suggested that the peptides did incorporate into the film and were capable of altering the film structure. Although there was mitigation of the bactericidal capabilities of BLES+CATH-2 when compared to CATH-2 activity alone, the BLES+CATH-2 compound could consistently reduce bacterial CFUs to below detectable levels, unlike all other surfactant/cathelicidin mixtures, which showed either a large decrease or complete loss of bactericidal function in the presence of BLES. Lastly, when intratracheally instilled into naïve mice, BLES+CATH-2 showed no effect on animal physiology, lung edema (as measured by protein leak), or IL-6 inflammatory response. Taken together, we conclude that it would be feasible to develop a surfactant/cathelicidin compound that is capable of maintaining surfactant and AMP properties and that, among the current peptides tested, BLES+CATH-2 appears to be the most promising candidate for future studies to investigate the efficacy of this therapy as an antimicrobial treatment *in vivo*.

The overall rationale for our study was that surfactant can assist in the delivery of an antimicrobial peptide into the lung, thereby overcoming some of the limitations of local delivery of drugs in pulmonary bacterial infections. For such compounds to be effective, a direct interaction between the surfactant and the peptide would be essential to facilitate co-distribution of the two compounds when delivered to the lung. Our data, including the AFM images of surfactant films and experiments related to the spreading of the surfactant indicate that such direct interaction occurs, as observed by the definitive changes in microdomain size and formation, particularly for CATH-1 and CATH-2 addition. The peptide-surfactant interaction is likely related to the amphipathic, cationic nature of cathelicidins, and their ability to interact with negatively charged phospholipids. Pulmonary surfactant contains approximately 10-15% acidic phospholipids, mainly phosphatidylglycerol (PG), with which the cathelicidins could electrostatically interact (23). Although surfactant composition is more complex than simple lipid mixtures, the idea of the interaction with PG is supported by studies by Sevcsik et al (24). This group utilized binary lipid mixtures to investigate interactions between lipid mixtures and LL-37, and found that even in binary mixtures the interaction between the cathelicidin and lipid mixture is dictated by one of the lipids present. Although the lipid ratios in their experimental model and those found within pulmonary surfactant are different, it is still reasonable to conclude that the interaction observed is between the cathelicidin peptides and PG since it appears this interaction with negatively charged phospholipids contributes to its basic mechanism of antimicrobial activity (2). We suggest that the interaction occurring between the surfactant phospholipids and cathelicidin peptides are affecting the individual properties of

each component, such as the surface tension reducing functions of BLES, and bactericidal capabilities of the cathelicidin peptides.

Whereas the above discusses a general mechanism of interaction between cathelicidins and surfactant, we observed substantial variations among the peptides in terms of their behavior when mixed with surfactant. Specifically, marked differences were obtained when analyzing the antimicrobial activity when peptides were combined with BLES, with only CATH-2 maintaining a relatively robust activity. Differences were also observed in the microdomain shape and size via AFM when CATH-1 or CATH-2 were added to BLES, but this was not observed for mCRAMP or LL-37. Although those differences did not correlate with surfactant activity, these changes do provide evidence of direct interaction, and possibly incorporation, of the peptides in the surfactant film. Other studies have also found different lipid interactions among various AMPs. Studies by Neville et al (25) demonstrated that the addition of LL-37 to a monolayer of DPPG caused monolayer collapse at high surface pressures, but that addition of SMAP-29, a sheep cathelicidin, actually improved DPPG stability upon compression. This group suggested that this was due to the differences in peptide structures and hydrophobicity, and that SMAP-29 was more likely to interact with the head groups of the phospholipids, while LL-37 was more likely to insert into the fatty acid groups of the lipid monolayers. This may help explain the differences observed within our study, as the peptides tested here have different structures. It is possible that CATH-2 interacts with PG similarly to SMAP-29, allowing CATH-2 to maintain its activity as more cationic residues are exposed to the external environment and able to interact with bacterial membranes. Other peptides, where the residues may be shielded by the interaction with the monolayer, would be less readily

available to interact with the targeted bacteria, and therefore have reduced bactericidal capabilities. One interesting feature of CATH-2 that could be related to this difference in activity is the presence of a proline residue at amino acid position 14 that forms a kink region between the two alpha-helical segments of the CATH-2 peptide. This kink is not present in the other tested AMPs. It was shown previously that this hinge region is essential for antibacterial activity since substitution of the proline residue, straightening the peptide, resulted in highly reduced activity (26, 27). Whether this structural difference between CATH-2 and the other AMPs is the basis of the observed differences in activity would be an interesting future investigation, and could potentially lead to the development of improved designer peptides for use in surfactant mixtures.

It has been repeatedly shown that intratracheal surfactant administration results in better pulmonary distribution than aerosol and saline administration (28, 29). Exogenous surfactant has been shown to enhance the pulmonary delivery and bioavailability of other potential therapeutic agents such as anti-oxidants, antibiotics, corticosteroids and adenoviral vectors (8, 30). Similar to the current experiments, several studies have investigated the bactericidal and biophysical functions of antibiotics in the presence of exogenous surfactant. In general, it was found using *in vitro* techniques that some, but not all, of the antibiotics tested were inhibited in the presence of surfactant (31). A subsequent *in vivo* study in *Klebsiella pneumoniae* infected mice demonstrated that intratracheal administration surfactant with the antibiotic tobramycin had significantly reduced mortality compared to surfactant or tobramycin alone (32). These studies provide support for the concept of surfactant as a vehicle for drug delivery, including in the setting of bacterial infection. Our concept of utilizing AMP-based surfactant expands on these studies, as

cathelicidin peptides have the added advantage of lacking microbial resistance towards them, while maintaining broad-spectrum bactericidal functions against drug-resistant bacteria (2).

Whereas the main focus of our *in vitro* experiments was the maintenance of surfactant and antimicrobial activities of potential AMP-based surfactant preparations, it should be noted that it has also been reported that AMP can have cytotoxic effects towards mammalian cells (26, 33, 34). Such effects could potentially limit the therapeutic applicability of this approach. Our *in vivo* experiment tested this issue for the most promising AMP-surfactant preparation, BLES+CATH-2, by instilling a therapeutic dose in healthy mice and assessing lung physiology and inflammation. Consistent with the reported effects for AMP, instillation of CATH-2 by itself did result in slightly elevated lavage protein levels and IL-6 concentrations, indicative of mild pulmonary edema and inflammation. Importantly, no such deleterious effects were observed by administration of BLES+CATH-2. This data suggests that BLES+CATH-2 is well tolerated and safe when instilled in a healthy lung and that mild negative side-effects of the instillation of a high dose of CATH-2 by itself are mitigated by co-administration with surfactant.

Although our main interpretation of our spreading data was that peptides did not inhibit this property of surfactant, it was interesting to note that the addition of all cathelicidin peptides actually improved the adsorptive function of surfactant, as seen by a reduced surface tension after 600 seconds, representing more surface-active material available at the air-liquid interface. Addition of all four peptides to exogenous surfactant led to significantly lower surface tension values reached after 600 seconds. It is possible that these findings are related to the ability of cathelicidin peptides to penetrate bacterial

membranes. In the context of surfactant this would imply that the peptides act as “bilayer breakers” thereby enhancing the ability of surfactant lipids to adsorb to the interface. This is similar to the proposed mechanisms by which the two hydrophobic surfactant proteins, SP-B and SP-C, are thought to enhance surfactant adsorption (35, 36). Interestingly, isolated SP-B has been reported to have antimicrobial activity as well (37). Although this activity was completely mitigated by the interaction with surfactant lipids, the finding does support the concept that there may be shared biophysical properties between AMPs and surfactant properties in terms of lipid interaction (38).

In conclusion, our results support the proof of concept that AMP-based surfactant can be utilized for the therapy of bacterial infections. It appears that the interaction between cathelicidins and surfactant varies and among the four peptides tested, CATH-2 would be optimal for further testing. The current observations have been limited to *in vitro* studies, and *in vivo* safety studies. Future studies are required to test the efficacy of this compound, by itself as well as in combination with other drugs, in an animal model of bacterial pneumonia.

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Chapter 3: Administration of *Pseudomonas aeruginosa* killed by chicken cathelicidin-2 prevents lung inflammation *in vivo*

3.1 Introduction

Pseudomonas aeruginosa is an opportunistic gram-negative bacteria, which is commonly found in patients with cystic fibrosis (CF), non-CF bronchectasis, ventilator-associated pneumonia, or patients that are immunocompromised due to various underlying comorbidities (1–4). Treatment of *P. aeruginosa* airway infections, particularly in chronic disease states such as CF, are made difficult due to the emergence of antibiotic resistance (1, 5–7). Chronic infection by *P. aeruginosa* can lead to tissue injury, ultimately resulting in progressive lung dysfunction, respiratory failure, and death (8–12). Due to the increasing prevalence of antibiotic resistance, and the lack of new therapeutics being produced in the pharmaceutical pipeline (13), the development of novel antimicrobials to combat these infections are desperately needed.

To address this issue, cathelicidins, a family of host defense peptides with direct antimicrobial activity, are being explored as potential alternatives for antibiotics. These peptides, which are endogenously produced by multiple cell types, including epithelial cells, macrophages, monocytes, and neutrophils, have a broad spectrum bactericidal activity and are less susceptible to classical mechanisms of bacterial resistance (14). Furthermore, cathelicidins exhibit a variety of immunomodulatory functions, which may provide additional benefits in their use to combat pulmonary infections (15).

One specific cathelicidin, the chicken cathelicidin CATH-2, has previously been identified as a strong candidate for therapeutic use. This peptide has been demonstrated to kill *P. aeruginosa* in physiologically relevant conditions (16) and was shown to retain significant antimicrobial function when combined with a pulmonary surfactant vehicle for lung targeted delivery (17). Recent *in vitro* studies have demonstrated that the killing of

bacteria by CATH-2 can occur without inducing a marked inflammatory response (18, 19). This “silent killing” activity was demonstrated by incubating a macrophage cell-line with either CATH-2-, heat-, or antibiotic-killed *Escherichia coli*, or *Salmonella enteritidis* (19). Macrophages exposed to the bacterial products from either heat- or antibiotic-killed bacteria responded by producing high levels of inflammatory mediators such as IL-6 and TNF-α; as compared to the CATH-2-killed bacteria, which elicited minimal inflammatory cytokine production. Based on the clinical observations that chronic maladaptive inflammatory responses can lead to indiscriminate tissue damage and expedited lung injury, as observed in CF patients (19–22), this silent-killing property of CATH-2 may further extend its clinical potential.

To date, silent killing by CATH-2 has only been observed *in vitro*. The *in vivo* environment within the lung is more complex as inflammation will involve multiple cell-types, cell migration and activation, physiologic lung dysfunction, and a local inflammatory environment that may vary between different airways and alveoli. Therefore, the objective of the current study was to determine the role of CATH-2 in dampening the inflammatory response induced by killed bacteria *in vivo*. It is hypothesized that CATH-2-killed bacteria will elicit a reduced inflammatory response after *in vivo* intratracheal instillation as compared to heat and antibiotic killed bacteria.

3.2 Materials and Methods

Preparation of killed bacteria for in vivo analysis

An overnight culture of *P. aeruginosa* ATCC 27853 was diluted 1/10 in tryptic soy broth (TSB). The optical density was measured, and bacteria were further diluted in sterile saline to reach an initial concentration of approximately $2x10^6$ CFU/ml. Subsequently, the bacteria were killed by CATH-2 (20 µM at 37°C for one hour), heat (90°C for one hour), or gentamicin (1 mg/ml gentamicin at 37°C for one hour). In a subset of bacteria killed by heat or gentamicin, 20 µM CATH-2 was added to the bacterial mixture before instillation. After intratracheal instillation, part of the bacterial solutions was plated via spot plating on tryptic soy agar (TSA), and incubated overnight at 37°C. The plates were examined the following morning to ensure complete bacterial killing**.**

In vivo analysis following administration of killed bacteria

Male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada), weighing 23-32 g, were used for this experiment. All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, and followed the approved guidelines described by the Canadian Council of Animal Care. Mice were anesthetized by intraperitoneal (IP) injection of ketamine (100 – 125 mg/kg body weight) and dexmedetomidine (0.5 mg/kg BW), and then intubated using a 20G catheter with the aid of a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Texas, USA). Once intubated, mice were randomized into treatment groups and instilled with 50 µl of a) CATH-2-killed bacterial preparations, b) heat-killed bacteria, c) heat-killed bacteria supplemented with CATH-2, d) gentamicin-killed bacteria, e) gentamicin-killed

bacteria supplemented with CATH-2, or f) instilled with an air bolus (naïve controls). Mice were extubated following successful instillation and were allowed to breathe spontaneously for six hours. After six hours, the mice were euthanized by IP injection of sodium pentobarbital (110 mg/kgBW) and dissection of the descending aorta. The animals were placed on a FlexiVent© in order to measure lung compliance and elastance. Following these measurements, whole lung bronchoalveolar lavage fluid (BALF) was collected by 3 x 1 ml aliquots of sterile saline. The whole lung lavage was immediately centrifuged at 400 x g at 4°C for 10 minutes, and the pellet was collected for cell analysis, while the supernatant was collected and used to measure protein content, and cytokine analysis. A sample of the supernatant was centrifuged at 40 000 g at 4°C for 15 minutes, in order to separate pulmonary surfactant subfractions. Pulmonary surfactant concentrations were determined using a modified Duck-Chong assay as previously described (23). Total cell counts and differential cell analysis of the cells obtained in the lavage was done as previously described (24). Protein content of the lavage fluid was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), as per manufacturer's instructions. Concentrations of mouse cytokines were measured using multiplexed immunoassay kits according to the manufacturers' instructions (R&D Systems, Minneapolis, MN). A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/ml) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad).

Statistical Analysis

Analysis of the inflammatory response induced by killed bacteria were performed by one-way measure analysis of variance (ANOVA) followed by Tukey post-hoc test. Means were reported ± standard error of the mean. Values were considered statistically significant at a probability value < 0.05.

3.3 Results

Effect of killed bacteria administration on respiratory function

Six hours after receiving intratracheal instillation of killed bacteria, lung physiology was measured via FlexiVent. There was no significant difference between any of the treatment groups in any lung function measurements, including lung compliance, elastance, or total lung capacity (Table 3.1). Bronchoalveolar lavage fluid volumes were not significantly different between treatment groups (Table 3.2). Protein concentrations from lung lavage of animals receiving gentamicin-killed bacteria supplemented with CATH-2 were significantly increased compared to naïve controls, but there was no significant difference among the other treatment groups (Table 3.2).

Treatment Group	Weight (g)	Lung Function		
		Total Lung Capacity (ml)	Compliance (ml/cmH2O)	Elastance (cmH2O/ml)
Naïve	29.5 ± 0.66	0.794 ± 0.041	0.0578 ± 0.0029	17.54 ± 0.92
CATH-2-Killed	28.0 ± 1.40	0.752 ± 0.048	0.0529 ± 0.0041	19.41 ± 1.62
Heat Killed	27.8 ± 0.72	0.777 ± 0.056	0.0558 ± 0.0033	18.14 ± 1.18
Heat+CATH-2	27.8 ± 0.91	0.812 ± 0.034	0.0557 ± 0.0023	18.09 ± 0.73
Gentamicin Killed	28.9 ± 0.65	0.862 ± 0.068	0.0581 ± 0.0031	17.51 ± 0.93
Gent+CATH-2	27.8 ± 0.57	0.845 ± 0.031	0.0588 ± 0.0025	17.11 ± 0.71

Table 3.1 Weight and lung function measurements of mice that were intratracheally administered killed bacteria. Values are expressed as mean ± SEM. (n=5-6)

Treatment Group	Lavage Volume (ml)	Surfactant Analysis			Protein Content (mg/kgBW)
		Total Surfactant (mgPL/kg BW)	Small Aggregate (mgPL/kg BW)	Large Aggregate (mgPL/kg BW)	
Naïve	2.82 ± 0.07	8.82 ± 0.71	5.17 ± 0.45	3.23 ± 0.57	14.04 ± 0.72
CATH-2 Killed	2.75 ± 0.09	8.82 ± 0.82	5.65 ± 0.34	3.14 ± 0.49	21.60 ± 3.72
Heat Killed	2.88 ± 0.06	7.85 ± 0.22	3.87 ± 0.25	2.53 ± 0.35	14.44 ± 1.38
Heat+ CATH-2	2.66 ± 0.12	9.72 ± 1.04	5.17 ± 0.26	2.94 ± 0.77	16.63 ± 2.21
Gentamicin Killed	2.88 ± 0.07	9.12 ± 1.24	4.67 ± 1.04	3.24 ± 0.63	15.74 ± 2.53
Gent+ CATH-2	2.68 ± 0.14	7.94 ± 1.12	3.89 ± 0.58	2.73 ± 0.20	34.47 ± 9.56[a]

Table 3.2 Lavage volume, surfactant analysis, and protein content from BALF of mice instilled killed bacteria. Values are expressed as mean ± SEM. (n=5-6)

[a]$p < 0.05$ vs. naïve treatment group.

CATH-2-killed bacteria elicit reduced total cell counts and reduced neutrophil influx

Instillation of either heat-killed or gentamicin-killed bacteria caused a significant inflammatory response, with significant increases in total cells in the BALF compared to naïve animals (Figure 3.1). *Pseudomonas aeruginosa* killed by 20 μM of CATH-2 elicited a reduced inflammatory response. The number of cells recovered from the BALF of animals instilled with CATH-2-killed bacteria was a significantly reduced compared to gentamicin-killed bacteria. There was also a reduction in cell recruitment induced by CATH-2-killed bacteria compared to heat-killed bacteria that did not reach statistical significance.

In addition to reducing total cell influx, the percentage of neutrophils in the BALF was also significantly reduced in animals treated with CATH-2-killed bacteria compared to both heat-killed and gentamicin-killed bacterial treatments (Figure 3.2).

Addition of CATH-2 to heat-killed bacteria downregulates cell influx

Heat-killed *P. aeruginosa* that was supplemented with 20 μM CATH-2 after bacterial killing decreased the inflammatory response induced by heat-killed bacteria alone. There was a trend for reduced cellular influx between heat-killed and heat-killed+CATH-2 treated animals (Figure 3.1), and the total cell counts for animals administered heat-killed bacteria + CATH-2 were similar to naïve treatment groups. Supplementation of CATH-2 to gentamicin-killed bacteria did not have the same anti-inflammatory effect. There was no significant difference in total cell counts between gentamicin-killed bacteria and gentamicin-killed+CATH-2 treatment groups.

Similarly, the supplementation of CATH-2 to heat-killed bacteria induced a significant reduction in percentage of neutrophils in the BALF compared to heat-killed bacteria alone, reducing levels to the values observed in animals administered CATH-2-killed bacteria (Figure 3.2). The percent of neutrophils recovered from gentamicin-killed+CATH-2 treated animals were not reduced compared to gentamicin-killed bacteria (Figure 3.2).

Figure 3.1

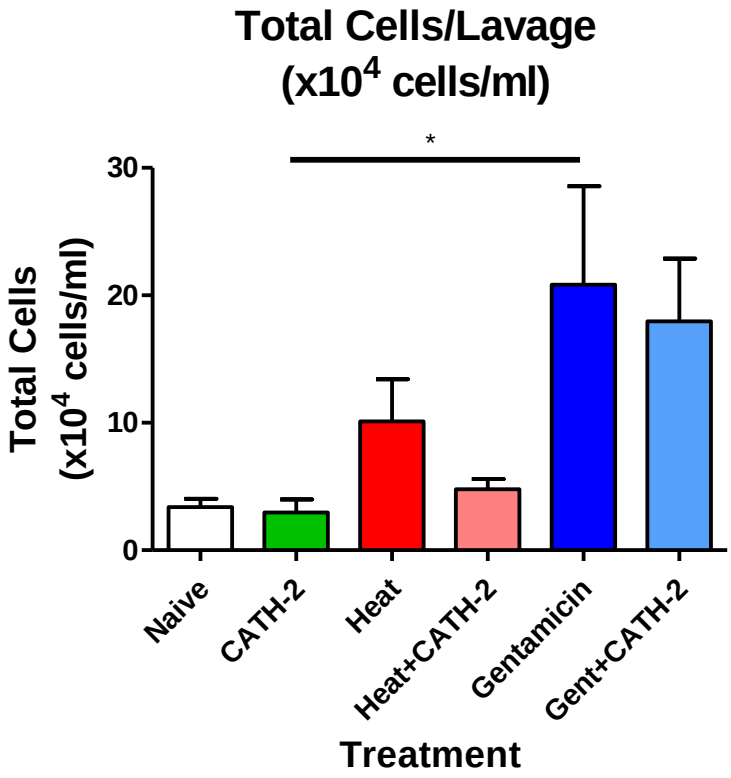

Figure 3.1. Total cells recovered from whole lung BALF of mice instilled killed bacteria. (n=5-6) *$p < 0.05$.

Figure 3.2

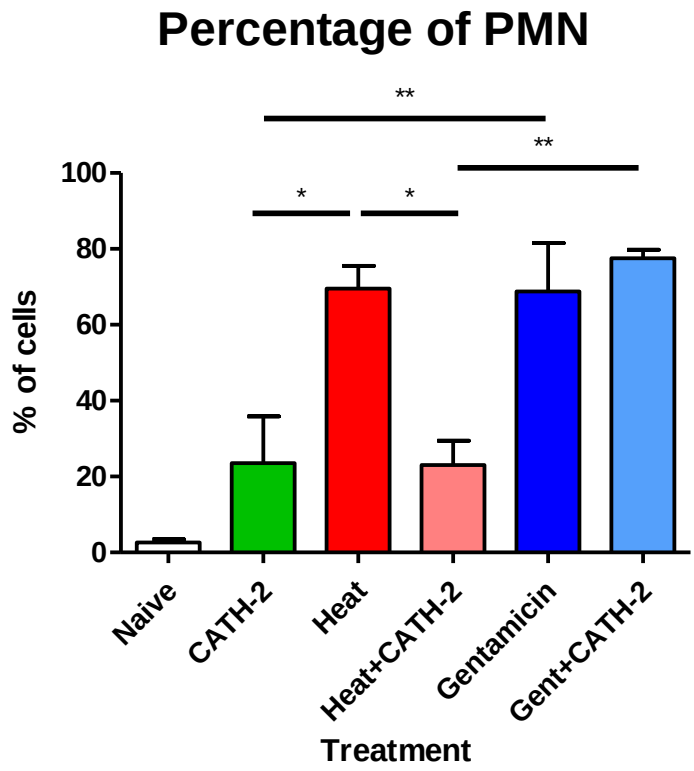

Figure 3.2. Percentage of neutrophil cells recovered from whole lung BALF of mice instilled killed bacteria. (n=5-6) *p<0.05; **p<0.01.

CATH-2-killed bacteria elicited reduced inflammatory cytokine release

Bacteria killed by CATH-2 induced significantly less cytokine release compared to both heat-killed and gentamicin-killed bacteria. Levels of TNF-α, IL-6, and KC were significantly reduced in the BALF of animals receiving CATH-2-killed bacteria compared to animals receiving either heat- or gentamicin-killed bacteria, and IL-12p70, G-CSF, MIP-2, IL-23p19, and MMP-9 were significantly reduced compared to animals administered gentamicin-killed bacteria alone (Figure 3.3).

Similar to cellular influx data, the addition of CATH-2 to heat-killed bacteria was able to reduce the inflammatory cytokine production induced by heat-killed bacteria. Levels of TNF-α and KC were significantly reduced compared to the heat-killed bacteria group alone, while reduction trends persisted for the majority of inflammatory cytokines. On the other hand, there was no significant difference between animals administered gentamicin-killed bacteria supplemented with CATH-2 and gentamicin-killed bacteria alone in many cytokines, including TNF-α, KC, IL-12p70, G-CSF, MIP-2, or MMP-9. In fact, IL-6 and IL-23p19 showed increases in release from animals administered gentamicin-killed+CATH-2 compared to gentamicin-killed bacteria alone (Figure 3.3).

Figure 3.3

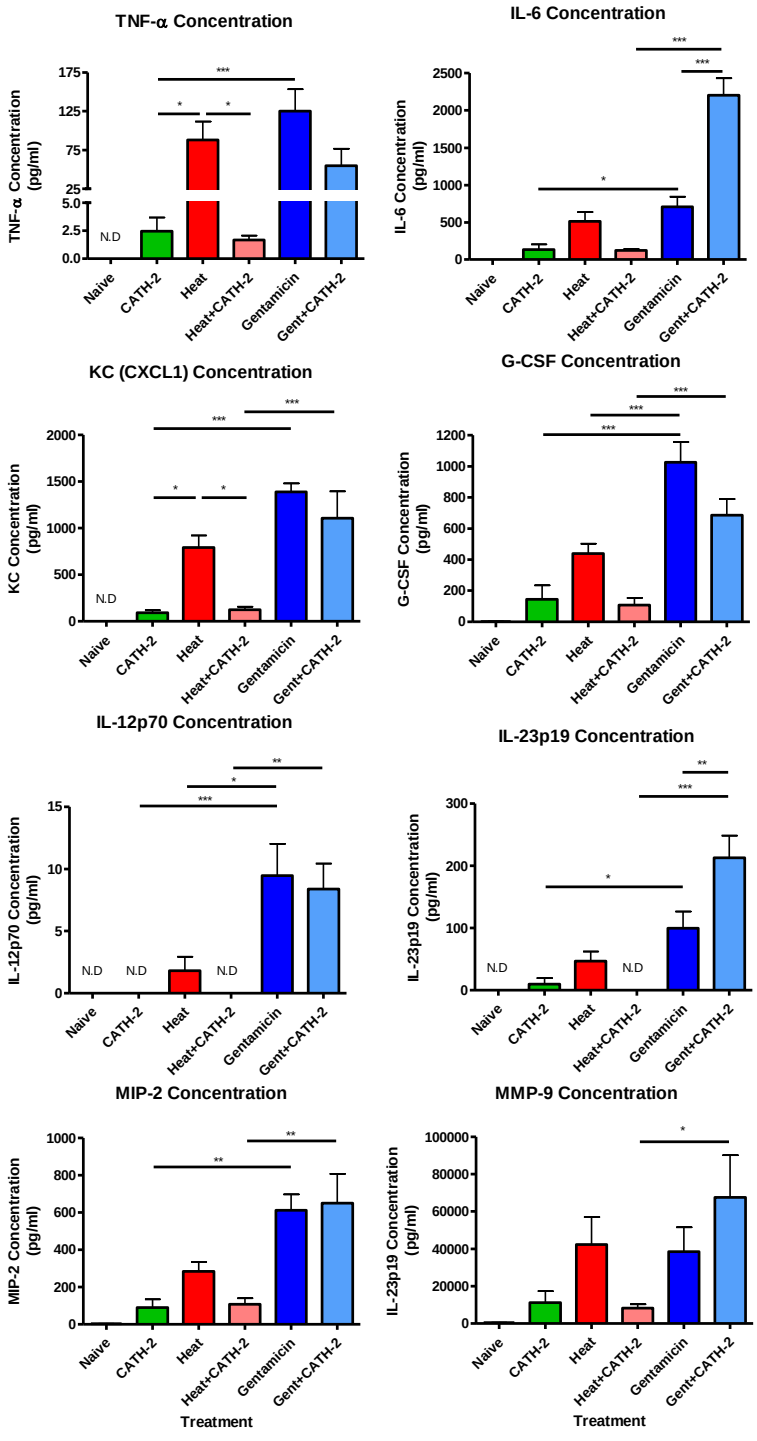

Figure 3.3. Cytokine and chemokine concentrations from whole lung BALF. (n=5-6) *p<0.05; **p<0.01; ***p<0.001.

3.4 Discussion

In this study, we have shown that CATH-2 is able to kill a clinically-relevant lung pathogen, *P. aeruginosa*, and intratracheal instillation of these killed bacteria does not induce a marked inflammatory response, as observed with heat or conventional antibiotic killed bacteria. Compared to other methods of bacterial killing, the bacteria killed by this cathelicidin peptide induced a significantly dampened inflammatory response after instillation, through the reduced recruitment of leukocytes and decreased concentrations of pro-inflammatory cytokines in the lavage fluid. Furthermore, the addition of CATH-2 to heat-killed bacteria eliminated the exacerbated inflammatory response induced by heat-killed bacteria, although this anti-inflammatory effect was not observed with gentamicin-killed bacteria.

To address our hypothesis that CATH-2-killed bacteria will elicit a reduced inflammatory response after *in vivo* intratracheal instillation, we utilized a model of intratracheal instillation of killed bacteria into healthy mice. None of the experimental groups showed any sign of lung dysfunction as indicated by measurements of compliance, elastance, surfactant composition, as well as total protein in the lavage. This data suggests that the inflammatory responses measured in this experiment were in response to the instilled bacterial products and peptide, and not an indirect effect due to tissue injury within the lung. The use of killed bacteria allowed for the investigation of CATH-2's ability to modulate the inflammatory responses induced by products released after bacterial death, without the confounding factors associated with toxin production or epithelial damage induced by live bacteria (3, 11). Therefore, this provides evidence for the role of cathelicidin peptides in modulating immunity and inflammatory responses after bacterial

clearance, as observed through the changes in cell recruitment and inflammatory cytokine production.

The main finding of this study is that the mechanism of bacterial death affected the inflammatory response *in vivo*. While heat-killed and gentamicin-killed bacteria were able to induce robust inflammatory responses, CATH-2-killed bacteria induced a significantly lower inflammatory response, as fewer neutrophils were recruited, and the concentration of inflammatory cytokines recovered from the BALF was significantly lower for multiple different markers. CATH-2's ability to kill bacteria without inducing an inflammatory response has previously only been documented *in vitro,* as it was shown that treatment of macrophages with peptide-killed *E. coli*, *S. enteritidis*, or *P. aeruginosa* exhibit a reduced inflammatory response after co-incubation (19). Our data provides clear evidence that this phenomenon also occurs in the multicellular *in vivo* environment within the lung. Taken together, these studies strongly support the conclusion that the cathelicidin peptide CATH-2 can not only kill bacteria directly, but is also able to dampen the immunogenic response induced by the bacterial components, such as endotoxins and other bacterial structures, released upon bacterial cell death.

A second important finding of this study was the ability of CATH-2 to down-regulate the inflammatory responses caused by heat-killed bacteria. The fact that CATH-2 supplementation was able to return inflammation to basal levels adds evidence of the ability of CATH-2 to directly modulate the inflammatory response induced by bacterial products, and that this effect is can be independent from its killing activity. However, this down regulation was not observed when CATH-2 was supplemented to gentamicin-killed bacteria, where in fact an exacerbated inflammatory response was observed. While

gentamicin has been mainly studied in nephrotoxicity and ototoxicity due to its common side effects, the mechanisms of action to induce both apoptosis and necrosis *in vivo* involve the accumulation of high levels of gentamicin intracellularly, causing non-specific activation of multiple apoptotic and necrotic pathways (25). It is possible that the high concentration of gentamicin (1 mg/ml) that was used to kill the bacteria induced some of these cytotoxic effects once administered intratracheally. Recent studies in our lab have demonstrated that bacteria killed with a lower dose of gentamicin induced inflammatory effects that were downregulated by co-administration of CATH-2, similar to heat-killed+CATH-2 observations (Brandon Baer, unpublished results). Overall, these results require further investigation into the mechanism of action, in order to understand the potential of cathelicidins as active therapeutics in co-treatment with conventional antibiotics.

Initial interest in cathelicidins for therapeutic development focused solely on their direct bactericidal functions (26, 27). CATH-2 has been repeatedly shown to possess bactericidal activity against multiple different bacterial strains under a wide variety of environmental conditions in *in vitro* settings (28–31). Unlike other cathelicidin peptides, such as human LL-37, which lose bactericidal activity under physiologic salt conditions (32), CATH-2 appears to be relatively insensitive to a variety of different conditions, including serum and high ionic environments. The ability of CATH-2 to maintain bactericidal activity provides evidence for its potential direct bactericidal role *in vivo* (16, 17). Regardless of their degree of bactericidal activity, it is abundantly clear that cathelicidin peptides can be directly responsible for the clearance of bacterial infection, and that a loss/decrease in peptide can lead to a strong reduction in immune response and

bacterial clearance (33, 34). From the data presented here, we suggest that CATH-2 has the ability to both kill bacteria, as well as dampen inflammatory responses after stimulation with the bacterial products. This direct bactericidal activity along with the ability to inhibit the inflammatory response induced by killed bacteria is key in regulating proper immune responses. These regulatory functions prevent excessive inflammatory responses, and indiscriminate cytotoxic damage caused by neutrophil migration and activation, commonly associated with chronic infection states (35–38).

One of the common immunomodulatory functions of various cathelicidin peptides is their (relatively) conserved ability to bind and neutralize bacterial endotoxins, such as LPS, thereby silencing the downstream inflammatory signalling process (39–41). In combination with these previous observations, our results suggest that, in addition to CATH-2 direct antimicrobial activity via bacterial membrane binding and cell lysing, there is a potential anti-inflammatory activity of CATH-2 via binding to the LPS released from the bacterial membrane, thereby inhibiting cellular activation through TLR 4. Additional studies have also shown that cathelicidins are able to down-regulate signalling through other TLR pathways such as TLR 5 (42). As *P. aeruginosa* contains flagellin, the main agonist of TLR 5, it is possible that CATH-2 has some immunomodulatory interaction with this pathway as well, similar to that observed with other cathelicidin peptides.

In a natural host immune response, activation of TLRs by bacterial by-products elicits a series of complex intracellular cascades, with the focus on translocation and activation of the nuclear factor-κB (NF-κB) (43). This transcription factor is found in all major cell types, and is one of the transcription factors responsible for this pro-inflammatory cytokine release (44, 45). The goal of most inflammatory cytokines released

is the recruitment of circulating neutrophils to aid in the removal of an invading pathogen (46). However, in patients with underlying diseases, such as cystic fibrosis, this recruitment can be detrimental as neutrophils release a vast array of toxins and oxidizers, which act to kill bacteria, but can damage host tissues in the process (47). The ability of CATH-2 to prevent this signalling caused by bacterial debris, which is found throughout the CF airways over the course of a patient's life, would therefore be able to protect tissue damage caused by the exacerbated inflammatory response often observed (47).

The ability to both kill clinically-relevant pathogens, as well as reduce the ensuing inflammatory environment, are essential properties for the development of CATH-2 as a therapeutic in cystic fibrosis. The CF microenvironment is incredibly complex, and is marred by the combination of a non-functional mucociliary elevator (22), incompetent innate immune responses (47), ineffective removal of apoptotic cells and debris (48), all cumulating in a tissue-damaging pro-inflammatory environment (47, 49, 50). Therefore, these studies provide evidence for the potential therapeutic use of CATH-2 in the treatment of *P. aeruginosa* infection in CF patients. Based on these experiments, CATH-2 may be developed in order to both reduce the bacterial load via direct bactericidal activity, as well as minimize the inflammatory impact of the bacterial debris after death. These two properties could make CATH-2 a powerful future therapeutic option in the treatment of CF-associated pneumonia.

In conclusion, CATH-2 is an effective bactericidal agent, and bacteria killed through this peptide result in a diminished inflammatory response *in vivo*, presumably enabling the host organism to prevent an inflammatory environment after the colonizing bacteria are cleared. This anti-inflammatory role adds merit to cathelicidins' potential as

therapeutic antimicrobial agents in the future. However, their pro-inflammatory role with bacteria and conventional antibiotics raises questions as to the mechanism of interaction between these compounds, and requires further investigation before the two can be used effectively together.

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Chapter 4: The antibacterial and anti-inflammatory activity of an exogenous surfactant preparation fortified with chicken cathelicidin-2 against cystic fibrosis associated pathogens

4.1 Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which leads to alterations in the respiratory epithelial cilia and increased viscosity of the airway-surface mucosal layer. As a consequence of the inability to efficiently clear airway secretions, chronic airway colonization of bacterial pathogens ensues, resulting in a vicious circle of repeated lower respiratory tract infections, inflammation and tissue remodelling (1, 2). The prolonged use of oral or systemic antibiotics used to treat infections in these patients frequently results in airway colonization of multi-drug resistant bacteria, which represents an independent predictor of adverse clinical outcomes, decline in lung function and death (2–4). In addition, the complex pro-inflammatory environment induced by chronic bacterial airway infections further promotes airway epithelial cell injury and airway remodelling (5–11). Over time, the development of antibiotic-resistant bacterial infections coupled with, chronic, maladaptive inflammation poses a significant therapeutic challenge. Novel therapies are urgently needed in order to improve the quality of life for this challenging patient population.

Cathelicidins are a class of host-defense peptides that have been investigated for their potential therapeutic use against antibiotic-resistant infections (12). Cathelicidins are essential components of the innate immune system that are highly conserved across multiple mammalian species and have been shown to possess both direct bactericidal activity and immunomodulatory activity including alterations of inflammatory cytokine production and immune cell migration (13–21). Due to their broad-spectrum antimicrobial and immunomodulatory effects, these peptides may represent an excellent alternative to

conventional antimicrobials and are currently being investigated for use in patients with CF (22–25).

In order to optimize the delivery of therapeutics throughout the airways, exogenous surfactant has been investigated as a potential delivery vehicle due to its ability to improve distribution of lung targeted therapeutics to peripheral lung regions (26–28). The use of exogenous surfactant has been thoroughly investigated and currently exists as the standard of care for neonates with surfactant deficiency in the setting of neonatal respiratory distress syndrome, and is therefore safe and well tolerated (26, 28, 29). Our group has previously investigated a combined approach using a chicken cathelicidin, CATH-2, together with a commercially available exogenous surfactant preparation, bovine lipid-extract surfactant (BLES), and has shown that BLES+CATH-2 maintains both excellent surfactant biophysical properties, antimicrobial activity, and was tolerated when administered intratracheally to naïve mice (30). Therefore, the objective of the current study was to assess the specific antimicrobial or anti-inflammatory properties of BLES+CATH-2 for the treatment of CF related lung infections. It was hypothesized that BLES+CATH-2 will exhibit bactericidal activity against multi-drug resistant bacteria obtained from CF patients and additionally, that BLES+CATH-2 will reduce the inflammatory response induced by bacterial products in an *in vivo* model of pulmonary inflammation.

4.2 Materials and Methods

Clinical isolates

Clinical bacterial isolates were obtained from nine adult CF patients attending the London Health Sciences Centre outpatient CF clinics and protocols were approved by the Human Ethics subcommittee at The University of Western Ontario. Isolated bacteria were stored in citrate glycerol solutions at -80°C, and were isolated, identified and sub-cultured onto chocolate blood agar (CBA) prior to use in study. The samples obtained included eight *P. aeruginosa,* one *A. xylosoxidans,* and three *S. aureus* bacterial strains isolated from CF sputum samples. Antibiotic sensitivities of the isolated bacteria were obtained according to Clinical Laboratory Standards Institute procedures.

Bactericidal activity against clinical bacteria

Bactericidal activity was measured using a spot plating assay. In brief, an overnight culture of bacteria grown in TSB diluted in Müeller-Hinton Broth (MHB). The turbidity was adjusted to 0.5 McFarland, followed by an additional 50x dilution in either MHB or saline. Subsequently, 50 µl of bacteria and 50 µl of either CATH-2 or BLES+CATH-2 (0-200 µM of CATH-2; 10 mg/ml phospholipid of BLES) was added to a polypropylene coated 96-well plate, and incubated at 37°C for three hours with no shaking. After incubation, the bacterial suspension was serially diluted 10-10 000-fold, and 10 µl of each dilution was spot plated in triplicate on CBA plates. These plates were incubated overnight, and colonies on the plates were counted the following morning. Minimum bactericidal concentration (MBC) was determined as the minimum CATH-2 concentration at which no

bacterial growth was observed, corresponding to a bacterial concentration of less than 100 CFU/ml.

In vivo antimicrobial-independent inflammation model

One mucoid strain of *P. aeruginosa* (Patient 6) and one *S. aureus* (Patient 4) (Table 4.1) species were selected for use in this study. An overnight culture of *P. aeruginosa* or *S. aureus* was diluted 1/10 in MHB. The optical density was measured using a spectrophotometer, and bacteria were further diluted in MHB to reach an initial concentration of approximately $2x10^6$ CFU/ml. Subsequently, the bacteria were killed by heat (90°C for one hour). In a subset of bacteria killed by heat, CATH-2 (20 µM) or BLES+CATH-2 (10 mg/ml phospholipid; 100 µM peptide) was added to the killed-bacteria mixture prior to instillation. The dead-bacteria solutions were plated via spot plating on chocolate agar, and incubated overnight at 37°C to confirm complete bacterial killing of instilled samples.

The killed bacteria were then instilled into male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada) as previously described in Chapter 3. Once intubated, mice were randomized to receive a 50 µl instillation of either heat-killed bacteria, heat-killed bacteria + CATH-2, heat-killed bacteria + BLES+CATH-2, or MHB without bacteria as a negative control. Mice were extubated following successful instillation, followed by a subcutaneous injection of 0.5 ml of saline and were allowed to breathe spontaneously for six hours. Following the 6-hour time period, the mice were euthanized by sodium pentobarbital overdose (110 mg/kgBW) and severance of the descending aorta, and were placed on a FlexiVent© to measure quasi-static lung compliance and elastance. Following Flexivent

measurements, whole lung bronchoalveolar lavage fluid (BALF) was collected by flushing the lungs with 3 aliquots of 1 ml sterile saline. The whole lung lavage was immediately centrifuged at 400 x g at 4°C, and the pellet was collected for cell analysis, while the supernatant was collected and used to measure protein content and cytokine quantification.

Differential cell analysis of the cell pellets obtained from the lavage was done as previously described (31). Protein content of the lavage fluid was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), according to the manufacturer's instructions. Concentrations of mouse cytokines were measured using multiplexed immunoassay kits according to the manufacturers' instructions (R&D Systems, Minneapolis, MN). A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/ml) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad).

Statistical Analysis

Analysis of the inflammatory response induced by heat-killed *P. aeruginosa* and *S. aureus* was performed by a one-way measure of variance (ANOVA) followed by Tukey post-hoc test. Means were reported ± standard error of the mean, and values were considered statistically significant at a probability value (p) < 0.05.

4.3 Results

Patient Data

In vitro bacterial-resistance patterns were obtained using protocols performed by the London Health Science Centre Clinical Microbiology Laboratory and clinical characteristics of cystic fibrosis patients are shown in Table 4.1. Patients ranged between 24 and 57 years of age. Severity of disease was variable between patient groups, with $FEV1_{PRED}$ between 28 and 87%. Two-thirds of patients were colonized with at least one strain of *P. aeruginosa*, 3 out of the 9 patients were infected with *S. aureus*, and one patient was infected with *A. xylosoxidans*. Of the eight *P. aeruginosa* isolates investigated, 50% presented with a mucoid phenotype. Of all bacteria investigated, 58% were resistant to at least two conventional antibiotics. Bacterial resistance did not correlate with age, or disease severity.

Patient No.	Sex (M/F)	Age (years)	Weight (kg)	BMI (kg/m^2)	FEV1 %Pred	Bacteria	Resistance Profiles
1	F	57	60.6	22.5	39	*Pseudomonas aeruginosa*	NR
2	M	24	72	25.3	50	*Staphylococcus aureus*	Clindamycin, Erythromycin R
3	M	50	57	19.3	43	*Achromobacter xylosoxidans*	Gentamicin, Meropenem, Tobramycin R; Imipenem, Ciprofloxacin I
4	F	35	62	22.4	87	*Staphylococcus aureus*	NR
						Pseudomonas aeruginosa (muc)	NR
						Pseudomonas aeruginosa	Tobramycin, Gentamicin R
5	F	44	59	22	73	*Pseudomonas aeruginosa* (muc)	Amikacin, Gentamicin, Meropenem, Ciprofloxacin, Tobramycin R
6	F	40	57	20.9	52	*Pseudomonas aeruginosa* (muc)	Gentamicin, Tobramycin R
7	M	37	52.5	19.3	28	*Pseudomonas aeruginosa* (muc)	NR
8	M	27	62	18.7	48	*Staphylococcus aureus*	Clindamycin, Erythromycin, Cloxacillin R
9	F	31	40.5	18.2	55	*Pseudomonas aeruginosa (muc)*	NR
						Pseudomonas aeruginosa	Meropenem R; Ciprofloxacin, Gentamicin I

Table 4.1. Resistance profiles of clinical samples. Resistance profiles were obtained by London Health Sciences Centre Clinical Microbiology Laboratory using clinical laboratory and standards institute protocols. Muc = mucoid phenotype. NR = no resistance identified, R = resistant, I = impaired activity.

Bactericidal activity of BLES+CATH-2 against bacteria from cystic fibrosis patients

After *in vitro* incubation of either CATH-2 or BLES+CATH-2 with the isolated bacteria, it was observed that CATH-2 alone exhibited potent bactericidal activity against all *P. aeruginosa* strains tested, regardless of antibiotic resistance profile of the bacteria (Figure 4.1). The MBC of CATH-2 against all *P. aeruginosa* strains was between 2.5 – 10 µM. BLES+CATH-2 bactericidal activity against the clinical *P. aeruginosa* was much more variable, with bacterial recovery varying between a 3-log reduction to complete bacterial killing. Overall, BLES+CATH-2 MBC values were typically 100 µM, and as low as 50 µM against select CF-derived isolates (Figure 4.1).

Similar to *P. aeruginosa*, CATH-2 exhibited potent antimicrobial activity against all strains of *S. aureus* tested. CATH-2 again showed bactericidal activity against all *S. aureus* isolates investigated, with MBC values between 5 – 10 µM for all strains investigated (Figure 4.2). BLES+CATH-2 showed consistent bactericidal activity, and achieved MBC values at 100 µM against all *S. aureus* isolates tested. Bactericidal activity of CATH-2 and BLES+CATH-2 against *A. xylosoxidans* was similar to activity observed against both *P. aeruginosa* and *S. aureus* (Figure 4.3).

Figure 4.1

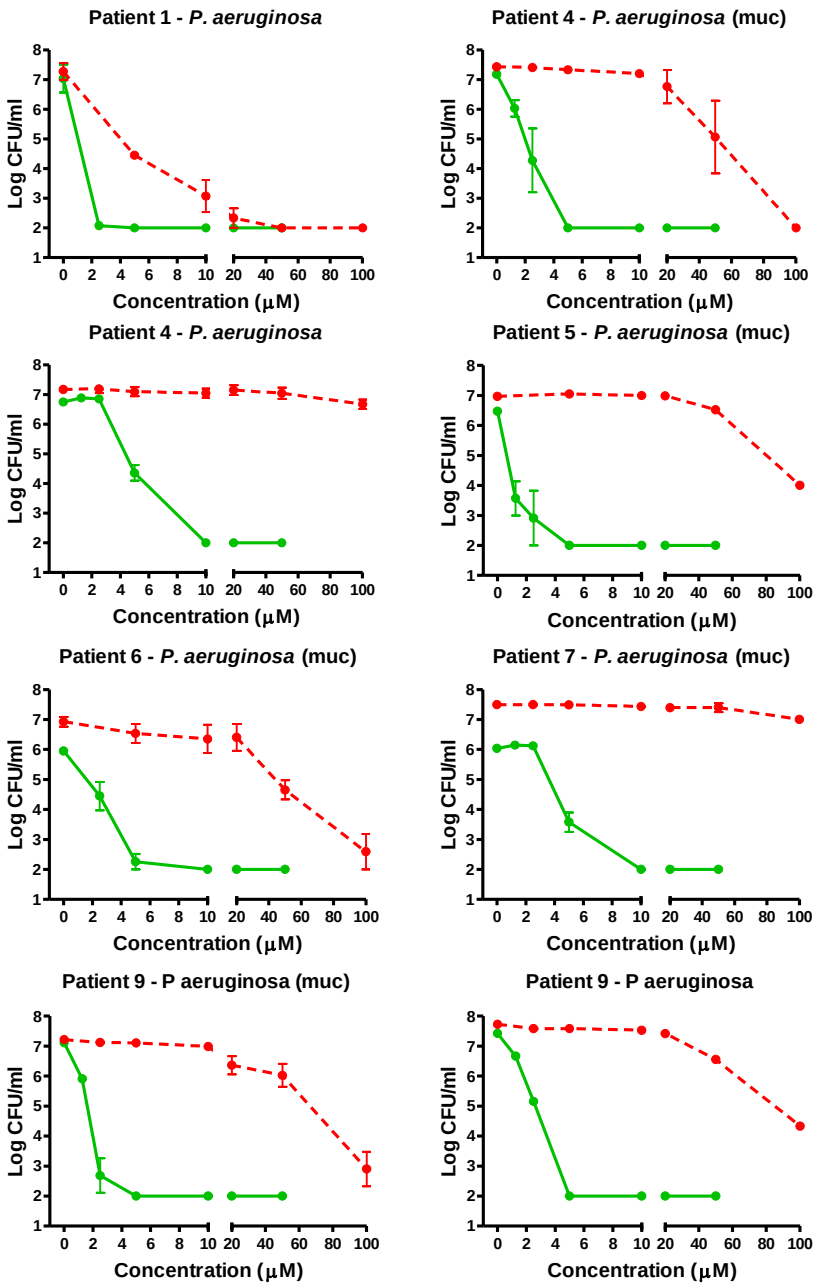


Figure 4.1. Antimicrobial assays of CATH-2 and BLES+CATH-2 against *P. aeruginosa* isolates. The bacteria were suspended in MHB, and then treated with various concentrations of CATH-2 (green, solid line) or CATH-2 suspended in 10 mg/ml phospholipid of BLES (red, dotted line). (n=3)

Figure 4.2

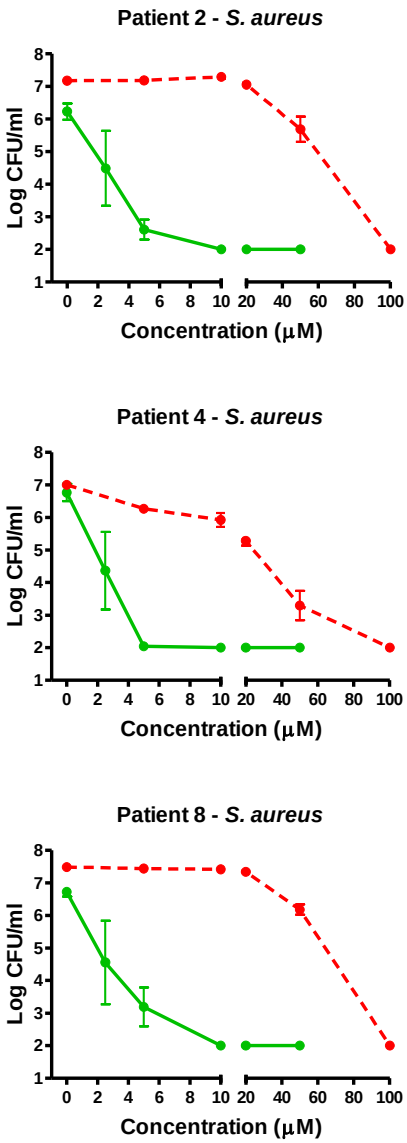


Figure 4.2. Antimicrobial assays of CATH-2 and BLES+CATH-2 against *S. aureus* isolates. The bacteria were suspended in MHB, and then treated with various concentrations of CATH-2 (green, solid line) or CATH-2 suspended in 10 mg/ml phospholipid of BLES (red, dotted line). (n=3)

Figure 4.3.

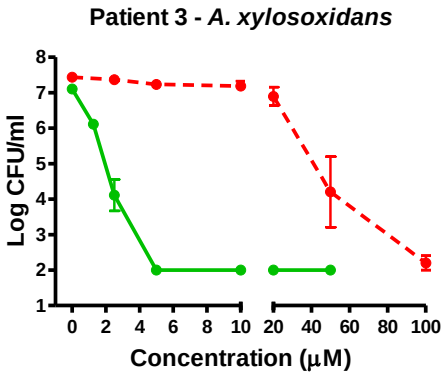


Figure 4.3. Antimicrobial assays of CATH-2 and BLES+CATH-2 against *A. xylosoxidans*. The bacteria were suspended in MHB, and then treated with various concentrations of CATH-2 (green, solid line) or CATH-2 suspended in 10 mg/ml phospholipid of BLES (red, dotted line). (n=3)

Bactericidal activity of CATH-2 and BLES+CATH-2 in saline

Figure 4.4 demonstrates the bactericidal activity of CATH-2 and BLES+CATH-2 in saline against the same CF-derived bacteria used above. Against *P. aeruginosa*, CATH-2 was an effective antimicrobial agent, and was able to reduce bacterial recovery to below detectable levels at concentrations between 5-10 μM. The addition of BLES severely inhibited bactericidal activity, as BLES+CATH-2 was unable to reduce any strain of bacteria to below detectable levels, even at 100 μM concentrations.

CATH-2 possessed minimal bactericidal activity against either *S. aureus* or *A. xylosoxidans*, as it was only able to reduce bacteria to below detectable levels in one strain (Patient 8 *S. aureus*) at a concentration of 50 μM. While increasing doses of CATH-2 were able to reduce some bacterial recovery (2-3 log reduction), BLES+CATH-2 exhibited no detectable bactericidal activity against any isolates of *S. aureus* or *A. xylosoxidans*.

Figure 4.4

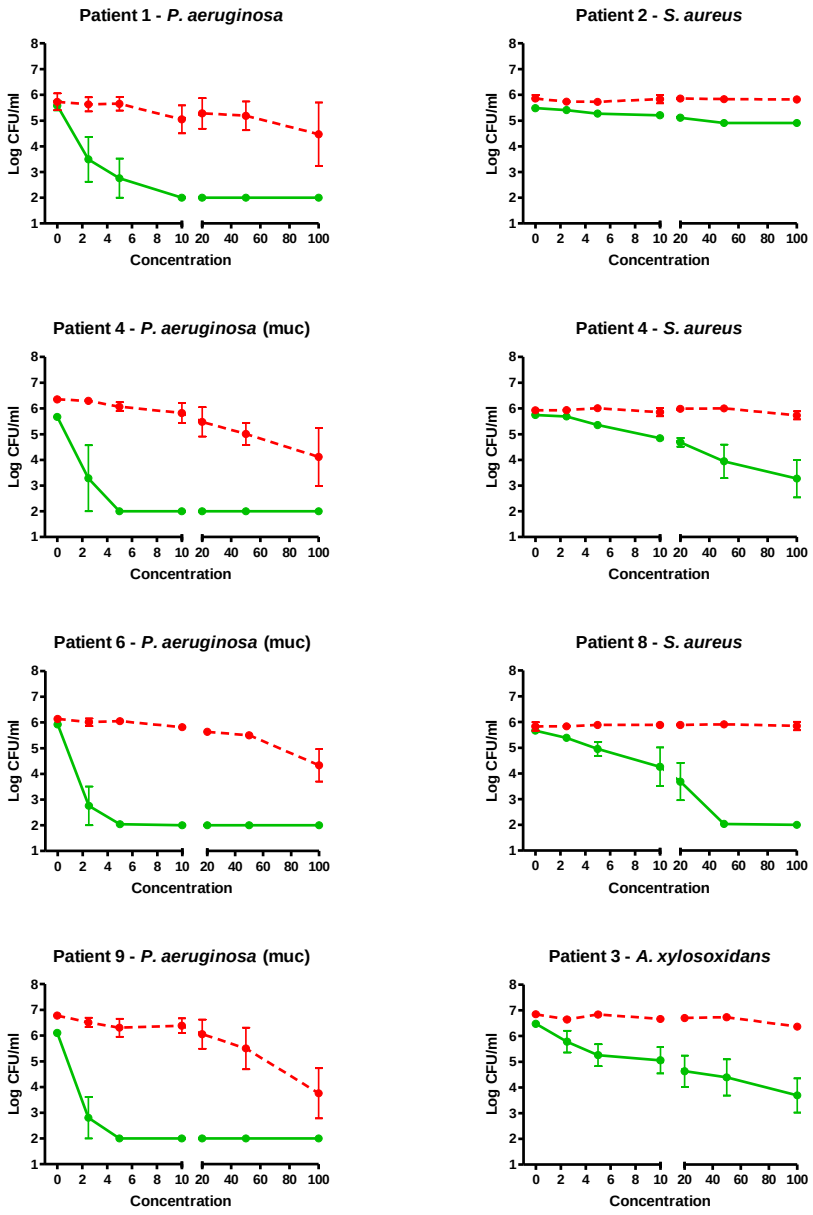


Figure 4.4. Representative antimicrobial assays of CATH-2 and BLES+CATH-2 against various bacterial species isolated from CF patient sputum in saline. The bacteria were suspended in saline, and then treated with various concentrations of CATH-2 (green, solid line) or CATH-2 suspended in 10 mg/ml phospholipid of BLES (red, dotted line). (n=3)

In vivo inflammation induced by killed cystic fibrosis derived bacteria

To investigate the potential anti-inflammatory effects of BLES+CATH-2, we used a previously reported model of inflammation induced by killed bacteria as described in Chapter 3. After instillation of the killed bacteria, there were no significant differences in lung compliance, elastance or protein content found within the lungs of instilled animals (Table 4.2). We observed a significant increase in total cell counts recovered in the BALF from animals that received intratracheally-administered heat-killed *P. aeruginosa* compared to control treatments (Figure 4.5a). We demonstrated that there was no increase in total cells recovered from animals administered CATH-2-killed *P. aeruginosa* compared to animals that did not receive killed bacteria. The differential cell counts demonstrated a significant increase in neutrophils from animals administered heat-killed *P. aeruginosa* compared to control animals, which was not observed in animals administered CATH-2-killed bacteria (Figure 4.5b). Supplementation of heat-killed *P. aeruginosa* with either CATH-2 or BLES+CATH-2 significantly reduced total cells in the BALF, including significantly lower number of neutrophils compared to heat-killed control groups (Figure 4.5a). These values for heat-killed bacteria supplemented with CATH-2 or with BLES+CATH-2 groups were not significantly different from the control group, or animals receiving CATH-2-killed bacteria alone (Figure 4.5b). Multiplex cytokine analysis showed that heat-killed *P. aeruginosa* elicited a significant increase in KC, MIP2, and GM-CSF compared to controls, while CATH-2-killed bacteria did not (Figure 4.6). Heat-killed *P. aeruginosa* supplemented with either CATH-2 or BLES+CATH-2 did not increase any of the inflammatory cytokines measured compared to negative controls, and significantly decreased KC concentrations compared to heat-killed bacteria. BLES+CATH-2

supplementation also significantly reduced MIP-2 levels compared to heat-killed *P. aeruginosa*.

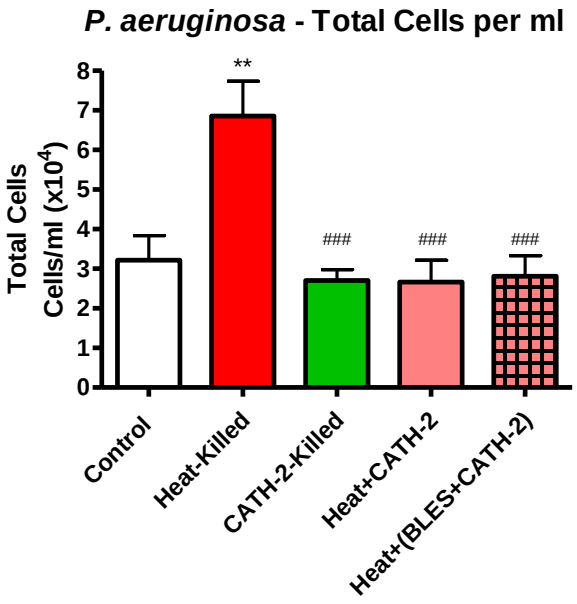
For *S. aureus*, cell counts recovered from mice administered heat-killed bacteria were significantly higher than the negative control group (Figure 4.7a). Cell counts from animals administered CATH-2-killed *S. aureus* were not statistically different from control animals, but was not statistically different compared to animals receiving heat-killed bacteria. Heat-killed bacteria supplemented with CATH-2 or BLES+CATH-2 were significantly reduced compared to heat-killed alone (Figure 4.7a). Heat-killed *S. aureus* supplemented with CATH-2 or BLES+CATH-2 also had a significantly lower recovery of neutrophils compared to heat-killed bacteria alone (Figure 4.7b). Despite the differences in cells recovered and cell differentials, there was no increase in any cytokine production after administration of heat-killed *S.* aureus compared to the control treatment, and no significant difference in any cytokine investigated for animals administered killed *S. aureus* (Figure 4.8).

Treatment	Protein Content (mg/kg BW)	Quasi-Static Compliance (ml/cmH_2O)	Quasi-Static Elastance (cmH_2O/ml)
Control	21.62 ± 3.0	0.0777 ± 0.016	13.28 ± 2.70
P. aeruginosa			
Heat-killed	20.56 ± 9.7	0.0718 ± 0.0080	14.08 ± 1.76
CATH-2-killed	24.80 ± 8.2	0.0676 ± 0.0071	14.92 ± 1.55
Heat+CATH-2	19.44 ± 5.9	0.0755 ± 0.0069	13.35 ± 1.34
Heat+(BLES+CATH-2)	17.58 ± 4.9	0.0801 ± 0.0064	12.56 ± 1.07
S. aureus			
Heat-killed	20.79 ± 5.2	0.0754 ± 0.0132	13.70 ± 3.02
CATH-2-killed	22.08 ± 4.4	0.0739 ± 0.0066	13.62 ± 1.24
Heat+CATH-2	20.98 ± 7.8	0.0736 ± 0.0080	13.74 ± 1.64
Heat+(BLES+CATH-2)	18.39 ± 8.0	0.0712 ± 0.0071	14.16 ± 1.46

Table 4.2. Protein content obtained from BALF after whole lung lavage, and quasi-static compliance and elastance as measured by FlexiVent. Values presented as mean ± SEM. (n=5-7)

Figure 4.5

a)



b)

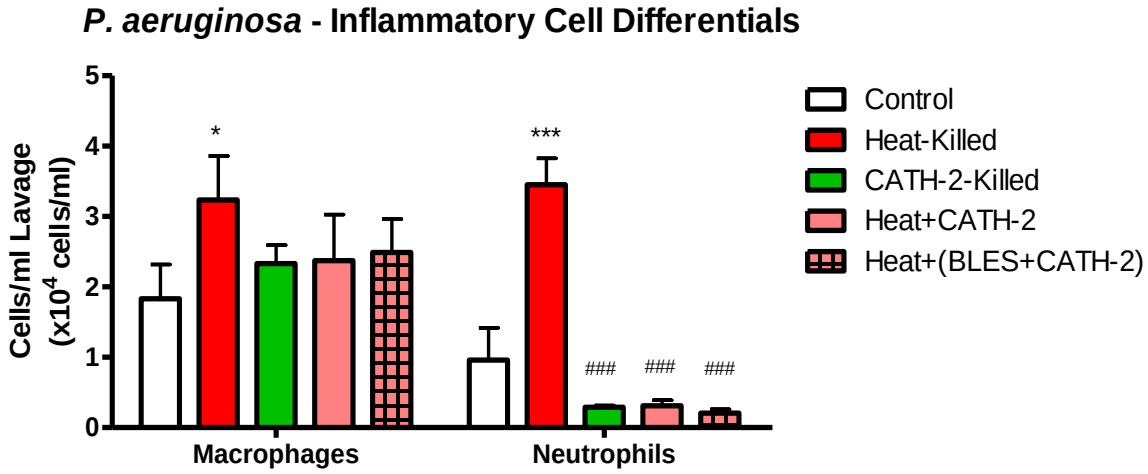


Figure 4.5. Cell counts and differentials cell counts of animals administered heat-killed *P. aeruginosa* obtained from Patient 6. a) Total cell counts obtained from BALF of animals administered killed bacteria. b) Macrophage and neutrophil concentrations obtained from BALF of animals administered killed bacteria. (n=4-7) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control; $^{###}p < 0.001$ vs. heat-killed bacteria.

Figure 4.6

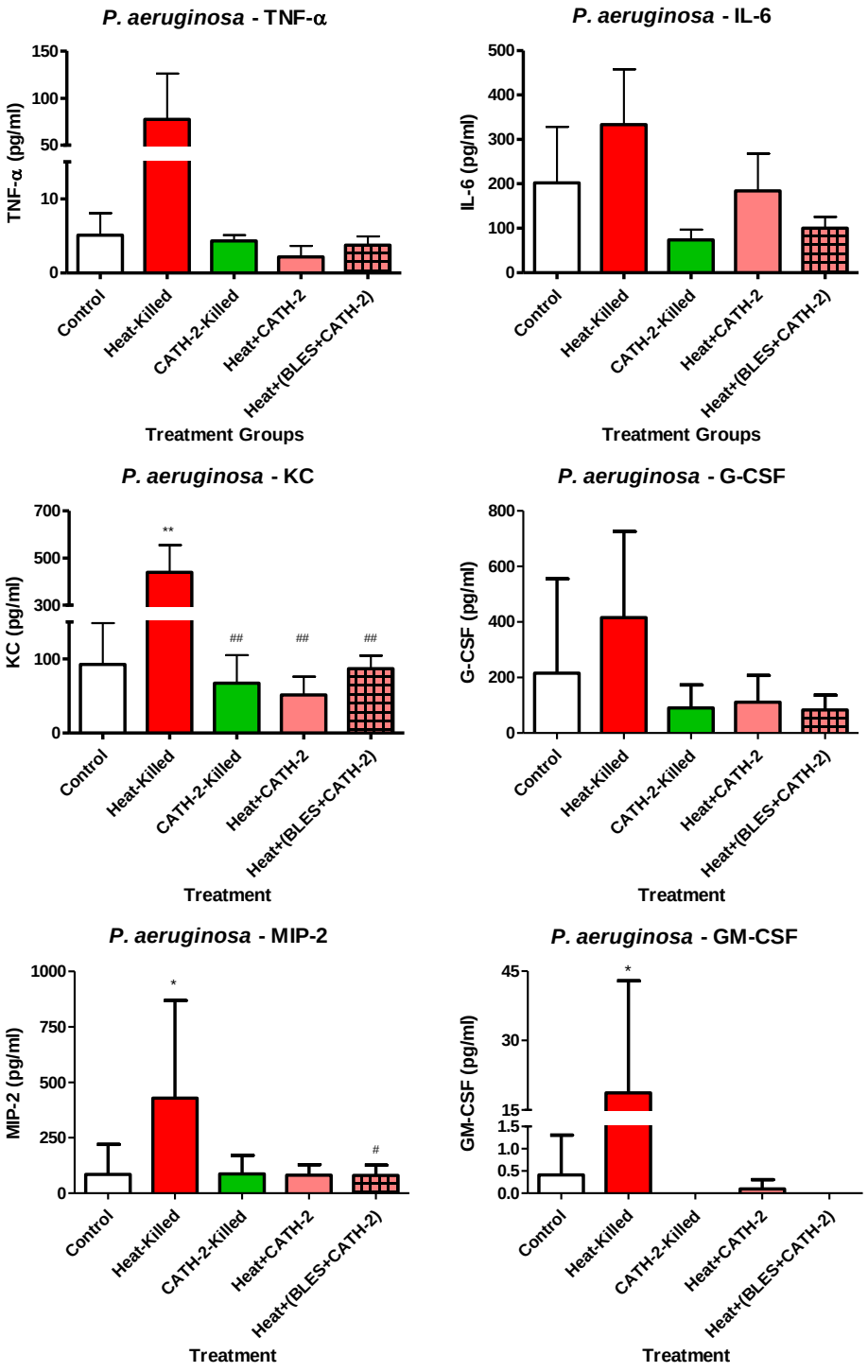
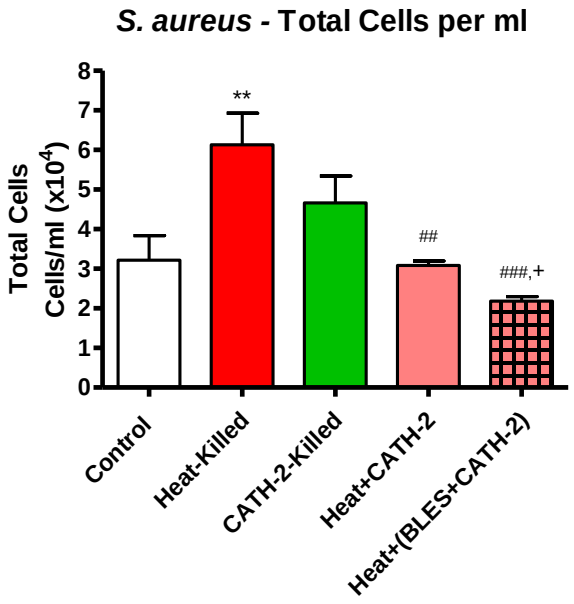


Figure 4.6. Cytokine content in the BALF obtained from animals administered killed *P. aeruginosa*. (n=5-7) *$p < 0.05$ vs. control; $^{\#\#}p < 0.01$ vs. heat-killed.

Figure 4.7

a)



b)

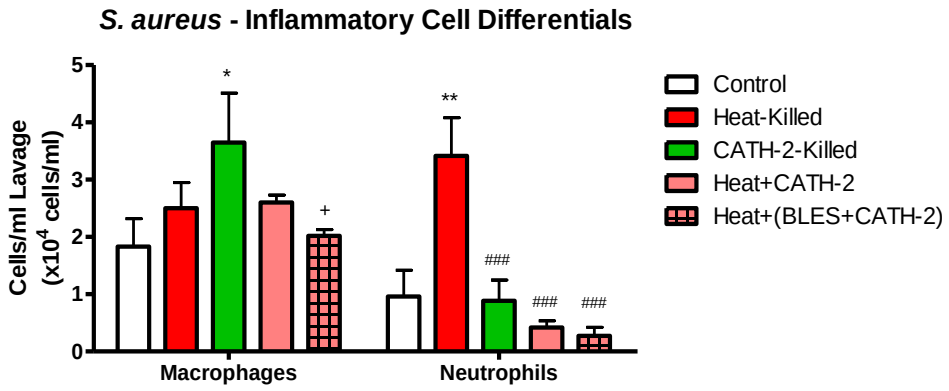


Figure 4.7. Cell counts and differentials of animals administered heat-killed Patient 4 *S. aureus*. a) Total cell counts obtained from BALF of animals administered killed bacteria. b) Macrophage and neutrophil concentrations obtained from BALF of animals administered killed bacteria. (n=5-6) *$p < 0.05$, **$p < 0.01$ vs. control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. heat-killed bacteria; $^{+}p < 0.05$ vs. CATH-2-killed bacteria.

Figure 4.8

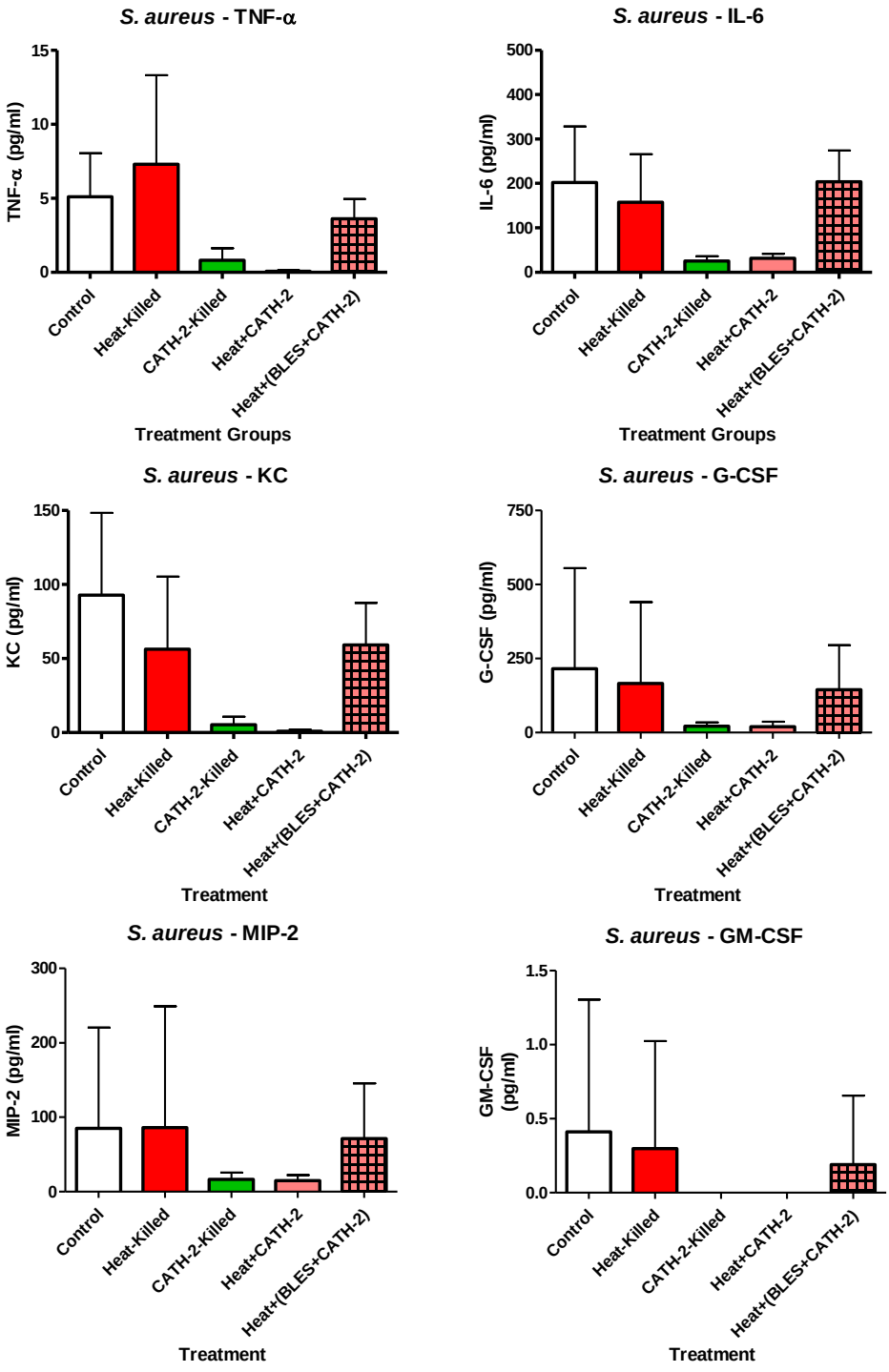


Figure 4.8. Cytokine content in the BALF obtained from animals administered killed *S. aureus*. (n=6-7)

4.4 Discussion

In the current study, the antimicrobial and anti-inflammatory effects of BLES+CATH-2, a chicken cathelicidin suspended in a clinically-used exogenous surfactant, were investigated as a potential therapeutic compound for the treatment of cystic fibrosis. Here, we provided evidence that BLES+CATH-2 exhibits bactericidal activity against an array of clinically derived pathogens from adult cystic fibrosis patients, as well as being able to reduce the recruitment of inflammatory cells and modulate proinflammatory cytokine production after heat-killed pathogens were instilled *in vivo*. Taken together, the bactericidal and immunomodulatory properties of BLES+CATH-2 represent a potential therapy that warrants further investigation for the treatment of cystic fibrosis lung infections.

The first goal of this study was to identify the bactericidal potential of BLES+CATH-2 against multiple cystic fibrosis-associated bacterial isolates, including multi-drug resistant isolates. *P. aeruginosa* represents one of the most prevalent bacteria found in CF patients, while *S. aureus* is the most common bacteria in patients under the age of 18 (8, 32–36). In addition to being the most common bacteria, these are also the most drug-resistant bacteria isolated from CF patients, with reports of resistance rates as high as 22.6% and 42.7% against select antibiotics for both *S. aureus* and *P. aeruginosa* respectively (37, 38). Consistent with this information, the multiple strains of *P. aeruginosa* (including both mucoid and non-mucoid phenotypes), *S. aureus,* and *A. xylosoxidans* isolated from CF patient sputum samples displayed a wide range of antibiotic resistance patterns. Although the antimicrobial activity of BLES+CATH-2 against these clinical strains was reduced compared to the CATH-2 peptide alone, BLES+CATH-2 exhibited

significant antimicrobial activity at higher CATH-2 concentrations. At 100 µM peptide concentrations, BLES+CATH-2 was able to reduce eight out of twelve bacterial isolates to below detectable levels after three-hour incubations. For the remaining bacteria tested, BLES+CATH-2 was still able to reduce bacteria by 3 log values for two of the four isolated strains. This data was similar to what was previously observed when BLES+CATH-2 was tested against other strains of *P. aeruginosa* or *S. aureus* (30). The concentration of 100 µM in the BLES+CATH-2 compound was the concentration previously used in safety and tolerability studies in **Chapter 2**, suggesting that this concentration would be effective as a therapeutic.

Interestingly, CATH-2 exhibited minimal bactericidal activity against *S. aureus* when suspended in saline, but was significantly more active when the bacteria were suspended in Müeller-Hinton broth. Previous groups have also shown this disparity in activity, albeit during investigations of different peptides and different bacteria (39). They determined that the key component of media that was responsible for these differences in activity was carbonate, which appeared to increase bacterial susceptibility via alterations in the membrane thickness. It is possible that similar changes occurred in this study, which would explain the increased sensitivity of *S. aureus* to CATH-2, and the enhanced antimicrobial activity of BLES+CATH-2 against both *S. aureus* and *P. aeruginosa* suspended in MHB as opposed to saline. Importantly, it is suggested that the MHB media conditions may be more accurate measures of antimicrobial activity. First, the Clinical and Laboratory Standards Institute guidelines for investigations of antibiotic efficacy are performed in MHB, and therefore our results presented here are directly applicable to clinical investigation guidelines (40). Secondly, due to the carbonate and bicarbonate

content found naturally within the lung, we believe that these media conditions are more relevant to the *in vivo* environment, supporting the potential *in vivo* antimicrobial activity of CATH-2 and BLES+CATH-2. Interestingly, as the CFTR is also a bicarbonate transporter, and its loss of function leads to reduced bicarbonate concentrations in the air-surface liquid, it may help explain the diminished activity of endogenous host defense systems observed in CF lung infections (41–43).

Following the bactericidal assays, we investigated whether the ability of CATH-2 to modulate inflammatory responses by killed bacteria was maintained after suspension in the exogenous surfactant BLES. Two of the CF-isolated bacteria were killed *ex vivo* by heat, before intratracheal administration. This specific experimental procedure was utilized to specifically focus on the immunomodulatory properties of BLES+CATH-2 in response to the bacterial products from clinical strains of bacteria. The data obtained provided evidence that BLES+CATH-2 was capable of down-regulating inflammatory responses induced by killed bacteria, and that the immunomodulatory activity occurred via an antimicrobial-independent mechanism. This data is consistent with previous observations obtained with CATH-2 alone utilizing a non-clinical strain of *P.* aeruginosa presented in **Chapter 3**. The induction of inflammatory responses by heat-killed *P. aeruginosa* was shown to be completely mitigated after co-treatment with BLES+CATH-2, as shown by significant reductions in neutrophil influx, as well as reductions in all inflammatory cytokines investigated, including KC (mouse analog of CXCL1) and MIP-2 (mouse analog of CXCL2). This ability to not only kill clinically isolated antibiotic resistant bacteria, but to also diminish the inflammatory response after killing provides a unique multi-functional

therapeutic that would target two disease processes that are detrimental to CF patients (11, 32, 41, 42, 44).

Despite the profound anti-inflammatory activity of BLES+CATH-2 against heat-killed *P. aeruginosa*, the administration of identical concentrations of heat-killed *S. aureus* produced a markedly different inflammatory response after intratracheal administration. Heat-killed *S. aureus* administration lead to a significant increase in inflammatory cells in the lavage, while co-treatment with both CATH-2 and BLES+CATH-2 significantly reduced inflammatory cell infiltration, similar to results observed after administration of heat-killed *P. aeruginosa*. Despite the increased neutrophil influx, no changes in cytokine production induced by heat-killed *S. aureus* stimulation were observed. It is possible that our method of heat-killing the bacteria induced significant changes to the immunostimulatory products of *S. aureus*, such as lipoteichoic acid or the peptidoglycan layer, as the lack of cytokines produced in this setting is inconsistent with previous studies investigating TNF-α production by alveolar macrophages *in vitro* (45). An alternative explanation for the lack of cytokine production observed in this study may be due to the downregulation of virulence genes, including toxins and cell surface adhesion molecules, which is commonly observed in CF patient-derived *S. aureus* isolates, therefore eliciting a diminished inflammatory response (46–48).

It should be noted that, while the heat-killed bacteria model of inflammation used is not entirely indicative of the clinical environment, we believe this to be a superior method compared to instillation of single bacterial endotoxins, such as isolated lipopolysaccharide, or other immune-stimulating bacterial toxins such as lipoteichoic acid, flagella, etc. This method of inflammatory stimulation can account for a more relevant immune response, as

it has previously been reported that the initial inflammatory response induced by heat-killed bacteria is qualitatively similar to the response induced by live bacteria (49). In addition, while many pathogen associated molecular patterns interact with specific pathogen-recognition receptors, whole bacteria will induce signalling and lead to changes in cytokine expression through multiple pathways simultaneously (50). In the context of the current experiment, this suggests that CATH-2, as well as BLES+CATH-2, has the ability to interfere with multiple mechanisms of immune stimulation by bacteria, rather than via blocking of one specific virulence factor (45, 49, 51, 52).

The use of exogenous surfactant as a vehicle to spread therapeutics throughout the lung has been explored extensively, including the delivery of conventional antibiotics (27–29, 53–56). It has the ability to increase therapeutic concentrations deposited in the airways compared to aerosolized administration, as there is more uniform distribution and less material lost in the central conducting airway regions during administration (26, 57). Importantly, it also has the ability to bypass systemic administration, and reduces side effects often associated with high circulating concentrations of antibiotics (58). For cathelicidin peptides, this route of administration is especially important because of the relatively short half life of most peptides (59, 60). While previous studies have demonstrated that CATH-2 retains the most antimicrobial activity after suspension in the exogenous surfactant BLES compared to other peptides (30), there are no studies investigating the immunomodulatory activity of other cathelicidin peptides in BLES. If the immunomodulatory activity is retained, despite antimicrobial activity loss, the cathelicidin peptide library may become a crucial source for developing anti-inflammatory therapies for respiratory disease, and warrants further investigation.

In conclusion, we showed that the chicken cathelicidin CATH-2, in combination with the exogenous surfactant BLES, possessed bactericidal activity against clinically-derived antibiotic-resistant CF pathogens *in vitro*. In addition to this, we showed that BLES+CATH-2 was able to downregulate inflammation through antimicrobial-independent mechanisms by silencing the inflammatory response induced by killed bacteria. Taken together, the ability to kill multi-drug resistant bacteria and while being able to modulate the hyper-inflammatory environment induced by these pathogens within the CF patient airways provides strong evidence for the potential of BLES+CATH-2 as a therapeutic for the management of respiratory infections associated with cystic fibrosis.

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Chapter 5: Efficacy of BLES+CATH-2 therapeutic administration in *in vivo* models of bacterial pneumonia

5.1 Introduction

Bacterial infection of the lungs, known as bacterial pneumonia, is the leading cause of mortality due to bacterial infection, accounting for 35% of all deaths due to infectious disease (1–3). While not always severe, approximately 24.8 cases per 10 000 adults with community acquired pneumonia will be placed in intensive care units, with 6% of those patients requiring mechanical ventilation (4). *Pseudomonas aeruginosa* is a common bacterial pathogen associated with respiratory tract infection in immunocompromised patients (5, 6). It is the second-leading cause of ventilator-associated pneumonia (VAP) worldwide, and is associated with mortality rates as high as 90% after infection of mechanically ventilated patients (7–10). Prevalence of antibiotic resistant infections has been steadily increasing, with between 40-82% of hospital-acquired bacterial pneumonia cases being caused by drug resistant infections (8). Indeed, *P. aeruginosa* resistance rates are not exempt from this trend. The prevalence of multi-drug resistant and extensively drug resistant isolates have steadily increased, and the rate of *P. aeruginosa* resistance towards carbapenems, a class of "last resort" drugs, has significantly increased over the past decade, with 34% of VAP isolates resistant to meropenem (10, 11). New treatment options are desperately needed.

To develop a new therapy for the treatment of *P. aeruginosa* pneumonia, previous work has identified a cathelicidin/surfactant combination that has shown effective combined activity - specifically, the chicken cathelicidin CATH-2 in conjunction with bovine lipid-extract surfactant (BLES). CATH-2 is one peptide that has been shown to possess potent antimicrobial activity, and unlike many cathelicidins, is able to maintain this activity in physiologically relevant salt conditions (12, 13). When suspended in BLES,

BLES+CATH-2 has shown enhanced adsorptive and spreading properties compared to the surfactant alone, whilst retaining the best bactericidal activity of all peptide/surfactant combinations investigated as demonstrated in **Chapter 2** (14). **Chapter 4** presents evidence of the therapeutic potential of this compound's ability to kill multi-drug resistant bacteria. However, BLES+CATH-2 activity *in vivo* has yet to be investigated. In this chapter, we hypothesize that treatment with BLES+CATH-2 in animal models of bacterial pneumonia will improve physiologic outcomes and increase bacterial clearance from the airways.

5.2 Materials and Methods

Bacterial Preparation

An overnight culture of *P. aeruginosa* ATCC 27853 was diluted 1/10 before optical density was measured. The bacteria were then further diluted in either sterile saline for mouse experimentation, or Müeller-Hinton Broth (MHB) for rat experiments, in order to reach the initial concentration required for each individual experiment.

In vivo treatment of acute bacterial pneumonia

Male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada), weighing 19-28 g, were used for this experiment. All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, and followed the approved guidelines described by the Canadian Council of Animal Care. Mice were anesthetized by intraperitoneal injection of ketamine (100-125 mg/kg body weight) and dexmedetomidine (0.5 mg/kg BW), and then intratracheally intubated using a 20 G catheter, with the aid of a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Texas, USA). Once intubated, mice were infected with 50 µl of bacteria at a concentration of $2x10^6$ CFU/ml. Five minutes after bacterial administration, the mice were administered 50 µl of one of the following five treatments: a) air bolus; b) saline; c) BLES (20 mg phospholipid/kgBW); d) CATH-2 (100 µM; 0.64 mg/kgBW); or e) BLES+CATH-2. Mice were extubated following successful instillation and were subsequently injected with the reversal agent for dexmedetomidine, Antisedan, and allowed to breathe spontaneously for either four or eighteen hours. After, the mice were euthanized by IP injection of sodium pentobarbital (110 mg/kgBW) and dissection of the descending aorta. The lungs were

removed *en bloc*, and placed in 3 ml of sterile saline before homogenization using a Potter-Elvehjam homogenizer. Aliquots from the homogenized tissue were plated on modified cetrimide agar plates, and colony enumeration was performed the following morning.

In vivo rat ventilator-associated pneumonia model

Sprague-Dawley rats (Charles River, Sherbrooke, QC, Canada) weighing between 330-435 g were used for this experiment. In brief, animals were anesthetized with a mixture of ketamine:dexmedetomidine (80 mg/kgBW; 0.5 mg/kgBW). Once no toe-pinch response was observed in the animals, they received a subcutaneous injection of the analgesic buprenorphine (0.05 mg/kgBW), followed by an additional subcutaneous injection of sensorcaine (bupivacaine HCL, 0.2 ml – 2.5%) in the ventral neck region at the site of incision. The right and left jugular veins were exposed and catheterized with PE-50 tubing in order to continuously infuse anesthetic (propofol, 5-20 mg/h), the neuromuscular blocker Nimbex (cisatracurium besilate, 0.4 mg/hr), and sterile fluids (heparinized saline 1000 IU/l). One carotid artery was also exposed and catheterized in order to monitor heart rate, blood pressure, and blood oxygenation levels, as well as allowing for additional sterile fluids infusion. The trachea was then exposed, and intubated using a 14 G endotracheal tube and secured using 2-0 ligatures. After recording initial hemodynamic measurements, the rats were administered a bolus of Nimbex (0.4 mg/kgBW) and immediately placed on a volume-cycled rodent ventilator (Harvard Instruments, St. Laurent, QC, Canada). Mechanical ventilation was performed using 100% oxygen and a ventilation strategy that was set at a tidal volume of 8 ml/kg BW, a respiratory rate of 54-60 bpm, and a positive-end expiratory pressure of 5 cmH_2O.

Throughout mechanical ventilation, airway pressures were monitored every 15 minutes using an airway pressure monitor (Sechrist Industries, Anaheim, USA), and blood gas measurements were performed by ABL800. Fifteen minutes after being placed on ventilation, a blood gas was collected as a baseline. Inclusion criteria required an arterial oxygenation of 400 mmHg partial pressure of oxygen/fraction of inspired oxygen (PaO_2/FiO_2).

After reaching inclusion criteria, the animals were infected intratracheally with *P. aeruginosa*. As noted above, bacteria were diluted in MHB to reach an initial concentration of ~$1x10^9$ CFU/ml, of which 500 µl was instilled intratracheally. After instillation, the animals received 3 ml air bolus followed by a single breath-hold in order to optimize bacterial distribution throughout the airways. The animals were then returned to mechanical ventilation for one hour, with monitoring of hemodynamics and arterial blood gases. At this time, the animals were briefly disconnected from the ventilator and randomized to receive one of the following treatments: a) saline; b) BLES (20 mg phospholipid/kgBW); c) CATH-2 (100 µM; 0.64 mg/kg BW); or d) BLES+CATH-2. After instillation, the animals received 3 ml air bolus followed by a breath hold, similar to post-bacterial instillation, and returned to the ventilator and monitored for three hours. At the end of ventilation, the animals were euthanized by administration of a bolus of sodium pentobarbital (110 mg/kgBW) via the catheterized jugular vein, followed by dissection of the descending aorta.

From one cohort, the lungs, spleen, liver, and blood were collected for homogenization. Tissues were suspended in various volumes of sterile saline (2 ml for spleen, 3 ml for lung, and 10 ml for liver tissue) and homogenized using a Potter-Elvehjem

homogenizing potter. The homogenate was then serially diluted and plated on cetrimide agar plates and incubated overnight before performing colony enumeration the following day.

In the second cohort, bronchoalveolar lavage fluid (BALF) was collected via whole lung lavage as previously described (15). A sample of the BALF was serially diluted and plated on cetrimide agar plates in order to perform colony enumeration. The remaining BALF was then centrifuged at 150 g for 10 minutes. Supernatant was aliquoted for protein content analysis and inflammatory cytokine concentrations. The remaining cell pellet was suspended in 1 ml of PlasmaLyte, and diluted 100x. An aliquot of the diluted cell pellet was mixed with an equal volume of trypan blue in order to perform total cell counts using a hemocytometer and light microscopy. In addition, a 10x dilution of the cell pellets were centrifuged onto cytospin slides at 1000 rpm for six minutes before staining with Hemacolor stain (Harleco, EMD Chemicals Inc., Gibbstown, NJ, USA). Cell differentials were then performed under light microscopy as previously described (16). Protein content within the lavage fluid was determined using a Micro-BCA protein kit (Pierce, Rockford, IL) according to the manufacturer's instructions. IL-6 and TNF-α levels were measured using respective enzyme-linked immunosorbent assay kits (ELISA kits; BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical significance for the bactericidal activity of the various treatments *in vivo* was determined using a one-way measure analysis of variance (ANOVA) followed by Tukey's post-hoc test. The effect of various treatments on peak inspiratory pressures and

arterial oxygenation was assessed using a two-way ANOVA followed by Tukey's post-hoc test. Total cell counts, cell differentials, and inflammatory cytokine concentrations were also analyzed by one-way ANOVA. Means are reported ± standard errors of the mean, and values were determined to be significantly different at a probability value (p) < 0.05.

5.3 Results

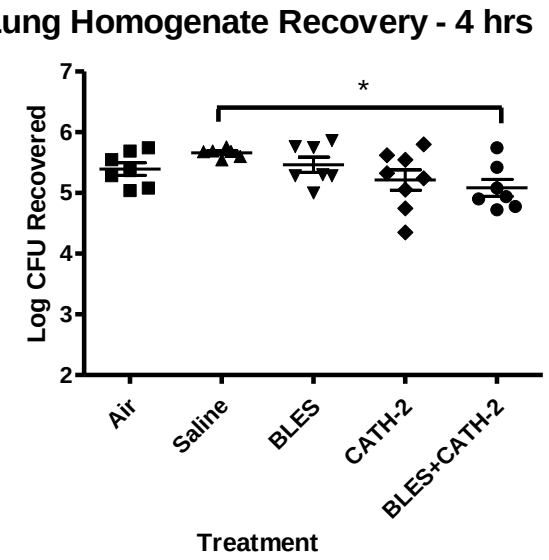
Bacterial recovery in mice

After intratracheal inoculation of ~$2x10^6$ CFU/ml of *P. aeruginosa* ATCC 27853, mice were treated with a 50 µl intratracheal instillation of either CATH-2 (100 µM) or BLES+CATH-2 (20 mg/kgBW phospholipid; 100 µM). Four hours after treatment, bacterial recovery was significantly reduced from lung homogenate of animals treated with BLES+CATH-2 compared to saline controls (Figure 5.1a). CATH-2 treatment alone showed no significant effect on bacterial load in lung homogenate.

After 18 hours, all control treatment groups had a reduced bacterial recovery from the lung homogenate, indicating bacterial clearance by the animals' innate immune defense system. The effect of BLES+CATH-2 administration was no longer observed 18 hours after instillation, as there was no significant difference in bacterial recovery from lung homogenate in animals treated with BLES+CATH-2 compared to any of the control treatment groups (Figure 5.1b). In contrast, lung homogenate from animals administered CATH-2 had a significantly increased bacterial load compared to all other treatment groups.

Figure 5.1

a)



b)

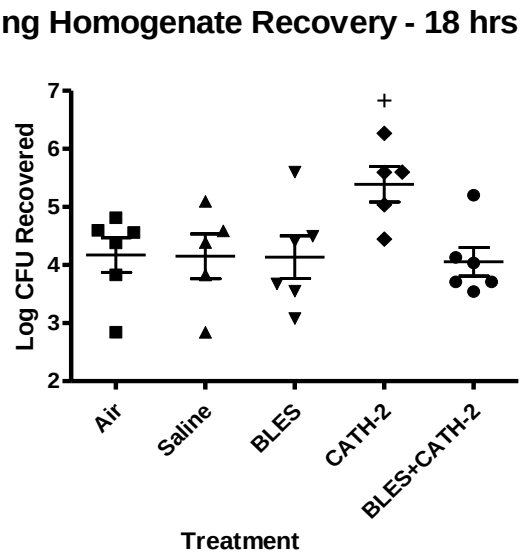


Figure 5.1. Bacterial recovery from lung tissue homogenate a) four hours, or b) eighteen hours after administration of *P. aeruginosa*. (n=5-8) *$p < 0.05$ vs. saline treatment; $^{+}p < 0.05$ vs. all treatment groups.

Physiologic outcomes in ventilator-associated pneumonia model

In the rat VAP model, the animals were placed on mechanical ventilation using a clinically relevant ventilation strategy (8 ml/kg BW tidal volume; 5 cmH_2O positive end expiratory pressure, 54-60 breaths per minute respiratory rate). After reaching inclusion criteria of PaO_2/FiO_2 > 400 mmHg, the animals were inoculated with ~ $5x10^8$ CFU of *P. aeruginosa* ATCC 27853. One hour following bacterial administration, the animals were administered treatments, and returned to the ventilator for three hours. Throughout ventilation, peak airway pressure was recorded. Saline-treated animals showed a significant increase in peak-inspiratory pressure (PIP) over the course of ventilation, indicative of decreasing lung compliance and development of lung injury (Figure 5.2). CATH-2 instillation elicited a significant increase in PIP 15 minutes after instillation compared to BLES alone, with no other significant differences observed. Similarly, CATH-2 treated animals had significantly higher PIP values from 180 – 225-minute ventilation time points compared to BLES+CATH-2.

In addition, arterial oxygenation was also measured over the course of ventilation. For saline control treated animals, arterial oxygenation at the end of ventilation was reduced compared to baseline measurements, with the mean oxygenation decreasing to 199 mmHg by the end of ventilation, again indicating respiratory dysfunction (Figure 5.3). Animals that received CATH-2 treatment presented a significant, severe drop in arterial oxygenation immediately after administration (t_{60mins} = 508.7 ± 15.12 mmHg; t_{75mins} = 112.0 ± 16.73 mmHg). Arterial oxygenation at this time-point was significantly different to every other treatment group, and CATH-2 treated animals showed significantly reduced oxygenation compared to BLES treated animals throughout the remaining three hours of ventilation.

After treatment, BLES+CATH-2-treated animals also showed a significant increase in arterial oxygenation compared to CATH-2 treated animals throughout the course of ventilation. However, there was no significant difference between BLES+CATH-2 treatment and either saline or BLES treatment (Figure 5.3). There was no significant difference in mean arterial pressure, heart rate, or the arterial partial pressure of carbon dioxide at any time point between any of the treatment groups. There was a significant reduction of pH in animals receiving CATH-2 alone compared to all other treatment groups at t = 75 mins (Table 5.1).

Figure 5.2

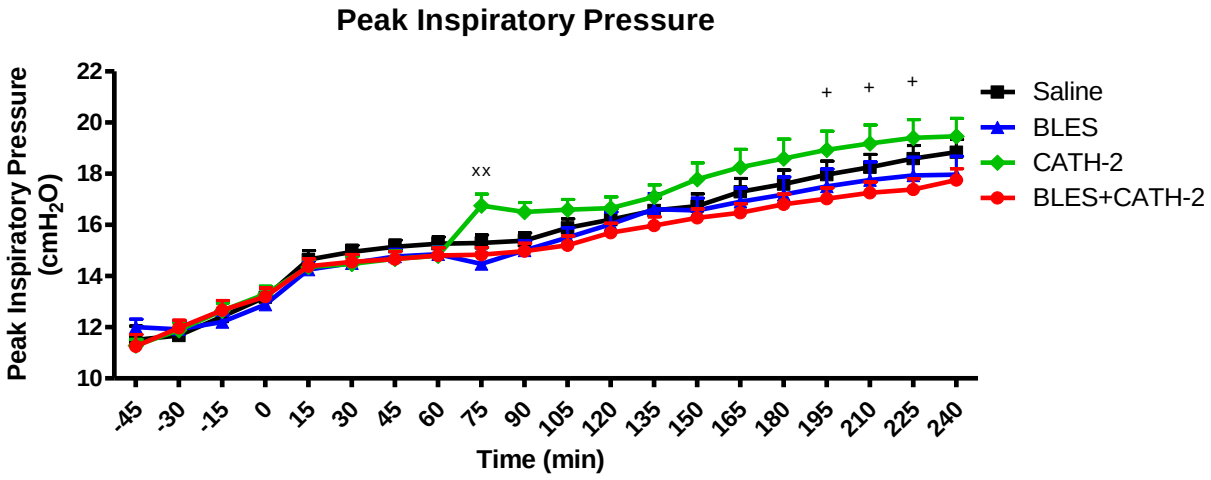


Figure 5.2. Peak inspiratory pressure over the duration of ventilation. (n=17-18) $^{xx}p < 0.01$ vs. BLES; $^{+}p < 0.05$ vs. BLES+CATH-2.

Figure 5.3

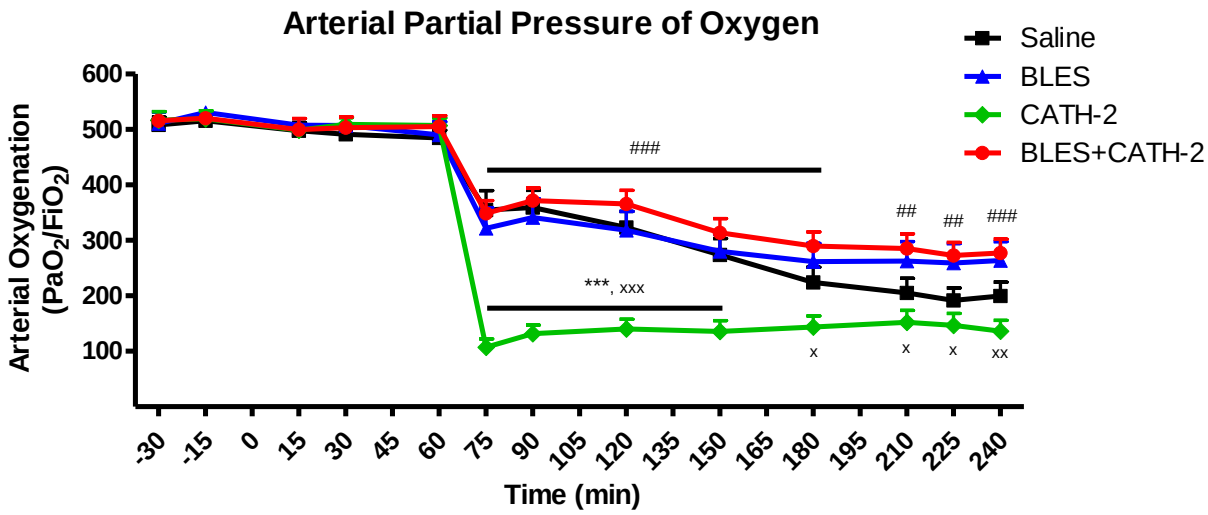


Figure 5.3. Arterial oxygenation during mechanical ventilation. Bacterial instillation at t = 0, treatment instillation at t = 60 min. (n=17-18) ***$p < 0.001$ vs. Saline; $^{x}p < 0.05$, $^{xx}p < 0.01$, $^{xxx}p < 0.001$ vs. BLES; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. CATH-2.

Treatment Group	Baseline	t=15	t=75	t=240
Mean Arterial Pressure (mmHg)				
Saline	168.9 ± 6.78	110 ± 5.60	90.8 ± 5.41	53.5 ± 2.60
BLES	161.9 ± 4.60	112.6 ± 6.39	83.7 ± 6.16	47.82 ± 3.74
CATH-2	161.2 ± 4.89	115.2 ± 3.63	73.4 ± 2.92	45.6 ± 3.74
BLES+CATH-2	168.8 ± 4.04	109.0 ± 5.41	81.78 ± 4.64	51.0 ± 3.08
Heart Rate (bpm)				
Saline	248.9 ± 5.13	253.0 ± 4.19	232.4 ± 5.13	252.5 ± 6.02
BLES	238.9 ± 5.58	252.4 ± 7.32	223.6 ± 7.32	252.2 ± 8.19
CATH-2	241.7 ± 6.75	256.3 ± 2.99	218.1 ± 6.86	255.2 ± 8.19
BLES+CATH-2	243.2 ± 5.23	250.8 ± 4.98	220.0 ± 5.38	249.9 ± 6.83
PaCO2 (mmHg)				
Saline	45.07 ± 1.52	49.78 ± 1.34	51.24 ± 1.79	62.40 ± 3.48
BLES	44.90 ± 2.08	48.39 ± 1.73	51.15 ± 2.26	58.09 ± 4.42
CATH-2	43.80 ± 1.75	48.94 ± 1.77	57.09 ± 2.50	65.33 ± 4.81
BLES+CATH-2	43.76 ± 1.50	49.22 ± 1.70	50.12 ± 1.30	56.66 ± 2.03
pH				
Saline	7.356 ± 0.001	7.312 ± 0.010	7.271 ± 0.009	7.122 ± 0.017
BLES	7.349 ± 0.012	7.314 ± 0.011	7.273 ± 0.012	7.136 ± 0.022
CATH-2	7.359 ± 0.011	7.313 ± 0.008	7.227 ± 0.014*	7.107 ± 0.018
BLES+CATH-2	7.350 ± 0.011	7.312 ± 0.011	7.276 ± 0.010	7.154 ± 0.012

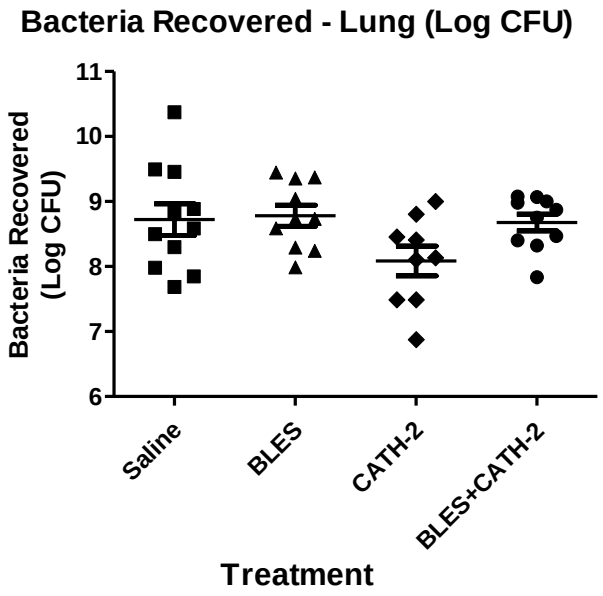
Table 5.1. Physiologic measurements of rats through duration of mechanical ventilation. (n=17-18) *$p < 0.05$ vs. all treatment groups.

Bacterial Clearance and Immune Response

There was no significant difference in bacterial recovery between any treatment group in both the whole lung homogenate (Figure 5.4a) or from the BALF (Figure 5.4b). No significant difference in bacterial recovery was observed for any other tissues investigated, such as spleen, liver, or serum (data not shown). Total cells recovered from the BALF were not significantly different between treatment groups (Figure 5.5a). From the total cells, the majority of cell types (>90%) recovered from all treatment groups were neutrophils, however there was no significant difference in percentage of neutrophils between any treatment groups (Figure 5.5b). There was no significant difference in total protein content recovered from the BALF of any treatment group (Figure 5.5c). Cytokine analysis from recovered BALF fluid also showed no significant difference in TNF-α (Figure 5.5d).

Figure 5.4

a)



b)

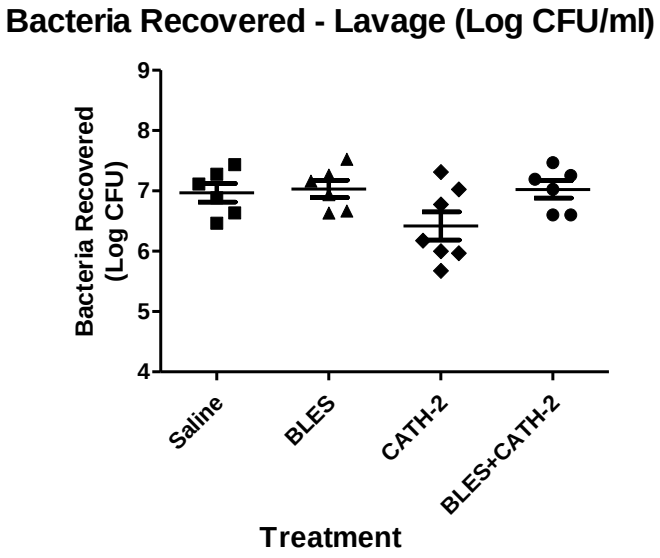
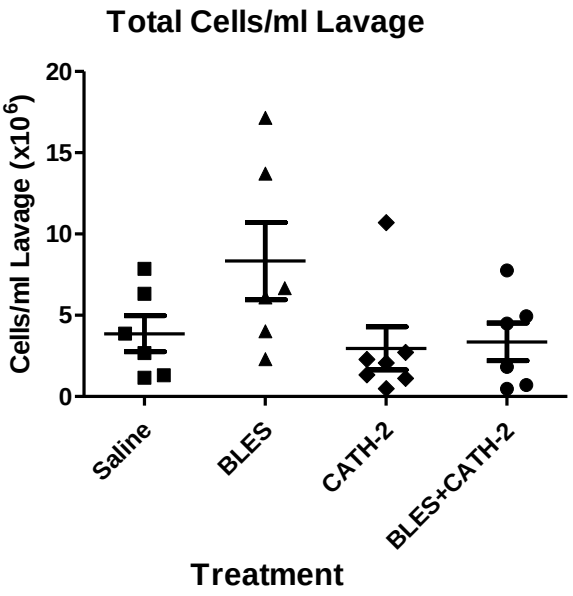


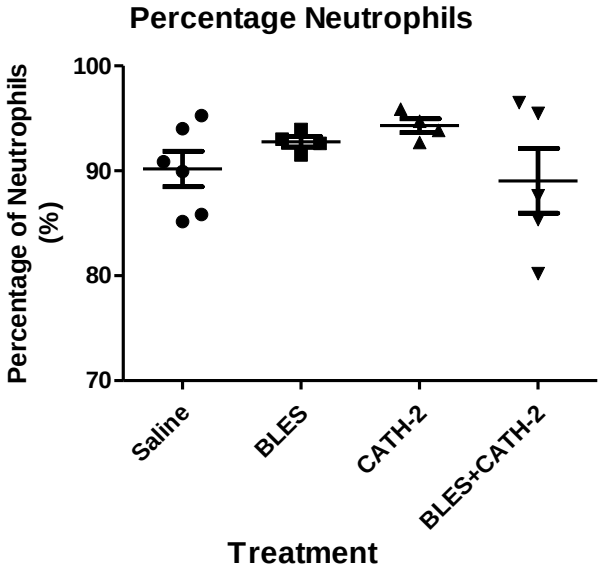
Figure 5.4. Bacterial recovery from a) lung tissue homogenate (n=9-11), or b) whole lung BALF after ventilation (n=6-7).

Figure 5.5.

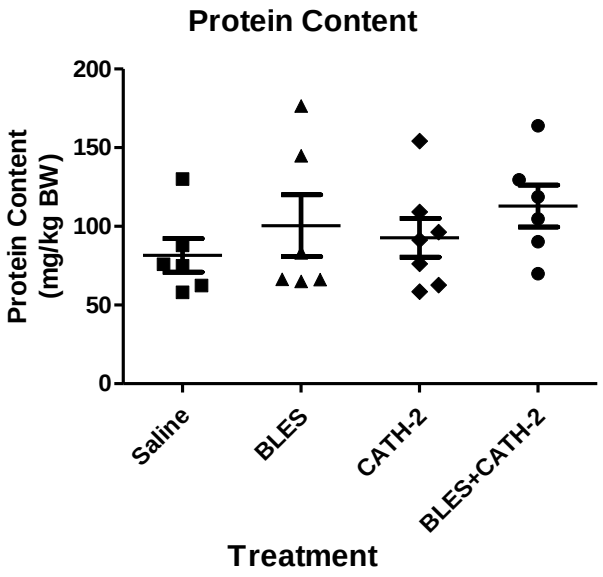
a)


Total Cells/ml Lavage
Cells/ml Lavage (x10^6)
20
15
10
5
0
Saline
BLES
CATH-2
BLES+CATH-2
Treatment

b)


Percentage Neutrophils
Percentage of Neutrophils (%)
100
90
80
70
Saline
BLES
CATH-2
BLES+CATH-2
Treatment

c)



d)

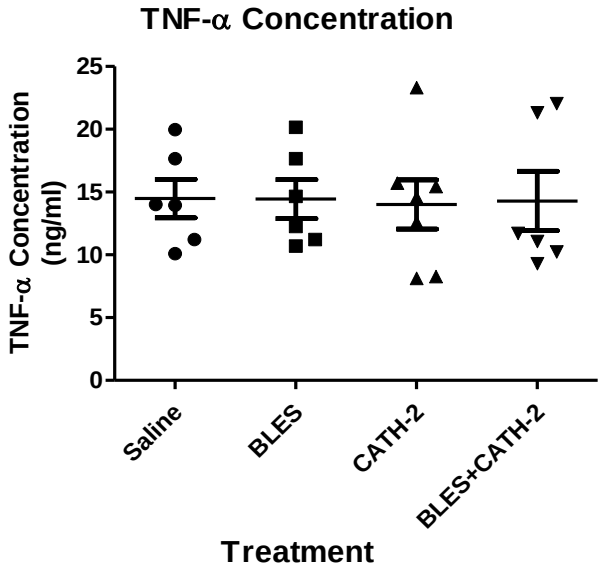


Figure 5.5. a) Total cell counts recovered from whole lung BALF, and b) percentage of neutrophils from recovered cells. c) Total protein content recovered from BALF. d) TNF-α content recovered from BALF. (n=4-7)

5.4 Discussion

This study investigated the *in vivo* efficacy of BLES+CATH-2 as a treatment for bacterial pneumonia. Two animal models were investigated in order to determine BLES+CATH-2 bactericidal function against the clinically relevant pathogen *P. aeruginosa*. In our spontaneously breathing mouse model, BLES+CATH-2 was able to significantly reduce bacterial load from lung tissue four hours after intervention compared to saline controls, although this significant difference was no longer observed 18 hours after treatment. However, this bactericidal activity was not observed in the ventilator-associated pneumonia rat model, as BLES+CATH-2 had no effect on bacterial recovery from the lung tissue or bronchoalveolar lavage fluid. In addition, BLES+CATH-2 showed no significant improvement in arterial oxygenation at the end of ventilation. Taken together, these results demonstrate limited efficacy of BLES+CATH-2 after *in vivo* administration.

The rationale for this study was based in part on work described in **Chapter 2**, which investigated the *in vitro* properties of the exogenous surfactant BLES with a panel of different antimicrobial peptides (14). Throughout *in vitro* assays, it was identified that the cathelicidin peptides interacted differently with BLES, and therefore activity was modified as well. The most striking differences between peptides was their bactericidal activity *in vitro,* where despite potent antibacterial activity of all four peptides, the addition of BLES to mCRAMP and LL-37 completely abrogated all bactericidal function. On the other hand, while CATH-2 activity was mitigated, it showed the greatest bactericidal activity after suspension in BLES, and was therefore the most promising candidate for investigating its *in vivo* bactericidal efficacy.

Despite a plethora of studies to suggest bactericidal efficacy of cathelicidins *in vitro*, data suggesting *in vivo* bactericidal activity is sparse (17, 18). Similar experimental models using different cathelicidin peptides have previously failed to show any therapeutic benefit (19). Other groups suggest that most cathelicidins, specifically LL-37 or mCRAMP, exert their therapeutic role through the recruitment of neutrophils, and that the direct bactericidal function of these peptides in negligible *in vivo* (20). It has been hypothesized that environmental conditions and/or the short half-life of cathelicidins are responsible for this lack of direct bactericidal activity *in vivo* (19, 21). In this study, we provide evidence that BLES+CATH-2 may be able to exert some bactericidal activity, as shown through the reduction in bacterial recovery from lung homogenate.

Previous studies performed in both **Chapter 2** and **Chapter 4** provided convincing evidence of CATH-2 bactericidal potential *in vitro*, and moreover, that the addition of BLES would actively reduce this antimicrobial activity. However, observations from this study suggest that the bactericidal activity of CATH-2 and BLES+CATH-2 are much less clear, as in our mouse pneumonia model, we observed a marginal decrease in bacterial recovery from animals administered BLES+CATH-2, that was not observed from CATH-2 treatment alone. This may in fact be due to the improved distribution and increase site concentrations in these infection models, as hypothesized by the use of an exogenous surfactant vehicle. The use of exogenous surfactant as a vehicle to spread therapeutics throughout the lung has been explored extensively using conventional antibiotics (22–28). Studies have shown that the use of exogenous surfactant as a vehicle has the potential to increase the delivery efficiency of therapeutics to the lung over parenteral or intravenous therapy (22, 26, 29–31). The four-hour acute pneumonia studies provide some evidence to

suggest that BLES may be increasing the distribution of CATH-2 to the infection sites. However, it appears that this increased spreading is not sufficient to significantly improve bactericidal activity, as both the 18-hour pneumonia model and the VAP model showed no reduction in bacterial recovery.

Expanding on the well-studied antimicrobial and immunomodulatory activity throughout this thesis, this chapter also investigated the ability of BLES+CATH-2 to improve arterial oxygenation, a clinically relevant outcome for patients placed on mechanical ventilation (32). This study provides evidence that BLES+CATH-2 can mediate a small improvement in arterial oxygenation, as animals administered BLES+CATH-2 had an average increase of 77 mmHg compared to saline treated animals, although this was not statistically significant. Additionally, this effect was also observed by BLES treatment alone, which also improved oxygenation an average of 64 mmHg by 240 minutes, but again was not statistically significant. This data lends some support to the hypothesis that the use of BLES can distribute throughout the lungs and into small peripheral airways and alveoli, as previous work has suggested that this exogenous surfactant administration is directly related to improved oxygenation (31, 33).

While both BLES and BLES+CATH-2 were able induce a mild improvement in arterial oxygenation, CATH-2 treatment alone showed a significant deleterious effect. This effect was most clearly observed in the VAP model, where despite a mild reduction in bacterial recovery, CATH-2 administration caused a rapid and severe decrease in oxygenation. This decrease was statistically lower than all other treatment groups between time point 75 mins (immediately after treatment instillation) until the end of the ventilation protocol. It is hypothesized that this drastic reduction is due to cytotoxic activity of CATH-

2, leading to diffuse airway damage and decreasing oxygenation. The cytotoxic activity of CATH-2 has been demonstrated *in vitro* via hemolytic assays, and we theorize that this activity is the cause of the rapid decline in oxygenation (13). We expect that this is also the reason behind the increased bacterial recovery observed at 18 hours in the mouse, where increased epithelial damage would improve bacterial colonization and proliferation. However, we could not detect any differences in protein content or inflammatory cytokines within the BALF, which would be expected in the case of severe epithelial cell death, and therefore further studies to confirm these observations are required. Importantly, BLES+CATH-2 administration did not induce any of these deleterious changes, indicating greater safety and tolerability of administration, even though data supporting any therapeutic benefit is currently lacking.

As mentioned, this chapter is the first investigation of direct bactericidal activity, and ability to affect clinically relevant outcomes, in two separate *in vivo* animal models. In this chapter, we utilized a well-described model of acute bacterial pneumonia in a C57Bl/6 mouse, and a rat VAP model using Sprague-Dawley rats. The mouse model investigated here has previously been used to study the effect of exogenous cathelicidin administration for the treatment of *P. aeruginosa* respiratory infection (20). However, whereas previous studies collected BALF and measured cell recruitment and cytokine production, our initial studies solely investigated bacterial recovery, and were therefore limited by the use of tissue homogenate instead of lavage fluid analysis. In order to expand on these initial studies, the VAP-rat was used to collect and analyze both tissue homogenate and BALF rather than the mouse pneumonia model. This second model was selected for two reasons: 1) a significantly greater volume of BALF is available in rats, and 2) we are able to measure

peak-inspiratory pressure, arterial oxygenation, and a number of other clinically-relevant parameters in the VAP model that were unavailable in the mouse pneumonia model. Thus, the inclusion of the VAP model permitted a greater range of outcome measurements. In addition, this study provided the opportunity to investigate the therapeutic potential of BLES+CATH-2 in severe lung dysfunction and inflammation settings, as noted by decreased oxygenation, and increased inflammatory cytokine production. Taken together, these two models were able to provide substantial data towards the efficacy of BLES+CATH-2 treatment in separate *in vivo* pneumonia models.

However, despite the large amount of data acquired from these models, there are many limitations of study. The first issue involves the use of *P. aeruginosa* as our bacterial model of infection. It has been well documented that, in healthy animal models, *P. aeruginosa* can be readily cleared by the host immune system (34). This is the likely explanation for why there was such a large reduction in bacterial recovery 18 hours after administration in the mouse pneumonia model regardless of treatment. Similar limitations were observed in the rat VAP model, as rats clear *P. aeruginosa* even more efficiently than mice (34). To this effect, preliminary studies performed in collaboration with Dr. Nicholson identified the bacterial load required to develop a respiratory infection that caused a significant reduction in arterial oxygenation over the course of ventilation, without the innate clearance of the bacteria. The resulting data suggested that the rat VAP model required a much larger bacterial load to develop signs of lung injury during infection than the acute pneumonia model used in mice. It is possible that this large dose was the reason why no bactericidal activity of BLES+CATH-2 was observed in this model, a phenomena known as the “inoculum effect”, in which antibiotics lose potency as bacterial load

increases (35). In future, animal models should utilize different strategies for infection, in order to more accurately model development of bacterial pneumonia. Use of different animal/bacteria combinations, would better describe the progression of a respiratory infection and development of chronic disease states, which more accurately represents the situation in clinical settings and would better predict the efficacy of therapeutic treatments against these infections (10, 36–40). Animal models that are administered multiple therapeutic doses would also be more viable for future testing, as again these would be more accurate of the treatment regimens currently prescribed (41–44).

In conclusion, this study presented evidence that BLES+CATH-2 can exhibit some bactericidal activity *in vivo* as observed in the mouse model of acute bacterial pneumonia, which showed a significant reduction in bacterial recovery four hours after instillation compared to control treatments, and lead to a mild improvement in oxygenation in VAP models compared to saline controls, although there was no statistically significant difference between these treatment groups. Moreover, while CATH-2 appeared to induce detrimental effects after administration, none of the negative outcomes were observed after BLES+CATH-2 treatment, suggesting safety and tolerability of this compound. While we were unable to identify any statistically significant improvement to clinical outcomes in both of our infection models, this was the first study to investigate the activity of BLES+CATH-2 *in vivo*, and supports the conclusions that BLES+CATH-2 does not induce harmful side effects after administration. Future studies will be required in order to improve BLES+CATH-2 therapeutic activity *in vivo*, via increased bacterial clearance and improved arterial oxygenation, without sacrificing compound tolerability.

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Chapter 6: Summary and discussion of major findings

The objective of this thesis was to develop and investigate the therapeutic potential of a cathelicidin peptide/exogenous surfactant compound mixture for the treatment of bacterial pneumonia. The high incidence of bacterial pneumonia, coupled with the increasing prevalence and global health crisis of multi-drug resistant infections, was the rationale for our pursuit of therapeutic development (1, 2).

Our hypothesis was that the use of cathelicidin peptides (multifunctional peptides with potent antimicrobial activity and immunomodulatory properties) in combination with exogenous pulmonary surfactant (which is able to spread rapidly throughout the airways, with improved deposition compared to aerosol administration) would improve outcomes in bacterial pneumonia. Throughout this thesis, we developed and tested a cathelicidin/surfactant compound for it's ability to a) maintain biophysical activity, b) provide antibacterial activity, c) modulate the inflammatory response during stimulation by dead bacteria, and finally d) investigating the antibacterial, immunomodulatory, and physiologic benefit of BLES+CATH-2 in *in vivo* models of pneumonia.

Initially, we investigated the properties of various cathelicidins with the exogenous surfactant BLES to select an optimal cathelicidin/surfactant combination in **Chapter 2**. By investigating the biophysical properties of the surfactant with various cathelicidin peptides, we observed that the four cathelicidin peptides tested had minimal effect on biophysical function, i.e. the ability to reduce surface tension upon lateral compression. When investigating the adsorptive properties of these cathelicidin/surfactant combinations, we observed that cathelicidins enhanced diffusion in a dose-dependent manner. We theorized that this increased adsorption was caused by the cathelicidins' ability to interact with and "break" the surfactant bilayers in the aqueous subphase, increasing surfactant material

released and available to move to the air-liquid interface. The surfactant protein SP-C, which is relatively similar to cathelicidins in structure, is crucial for surfactant adsorption (3, 4). It is possible that cathelicidins interact with the surfactant phospholipids through a similar mechanism, which would explain the phenomena observed in this thesis.

In addition to these findings, we also discovered that bactericidal activity of cathelicidins was severely impaired when mixed with surfactant. Due to the cationic nature of cathelicidins, and the presence of anionic phospholipids, such as PG, we theorize that this reduced activity is due to electrostatic interactions of the peptides with PG, "masking" the cationic charge of the peptide and preventing the interaction with bacterial membranes. These results were verified when investigating BLES+CATH-2 activity against MDR-isolates obtained from CF patients in **Chapter 4**. However, the degree of inhibition appears to be cathelicidin dependent; while CATH-2 and CATH-1 still maintained some bactericidal activity, LL-37 and mCRAMP completely lost antimicrobial functions. It is possible that, due to different peptide structures, different cathelicidin interactions/orientations within the phospholipid membranes may differ the charge masking, as previously observed (5–7). This would allow peptides such as CATH-2 (with two α-helical regions, both containing basic amino acid residues) to maintain some interaction with bacterial membranes, while other peptides (i.e. LL-37, with a single α-helical region that spans the majority of the peptide) insert deeper inside the membrane, and completely mask antimicrobial activity. In all, the key conclusions to be drawn from these results is that a) BLES+CATH-2 shows the most potent bactericidal properties of all cathelicidin/surfactant combinations tested to date, and b) that this compound retains bactericidal activity against MDR-clinical isolates.

While BLES+CATH-2 showed the most potent bactericidal activity, the immunomodulatory activity of CATH-2, as well as BLES+CATH-2, was relatively unknown. Unpublished results from our lab, as well as from our collaborator Dr. Coorens at Utrecht University demonstrated that, *in vitro*, CATH-2 and BLES+CATH-2 were able to prevent pro-inflammatory cytokine production from macrophages after treatment with either LPS or killed bacteria. These studies are supported by a wealth of data about other cathelicidin peptides, which suggest that one of the endogenous functions of cathelicidins is to downregulate inflammatory signalling by bacterial toxins, such as LPS (8–11).

To expand on this, **Chapter 3** and **Chapter 4** investigated whether CATH-2 and BLES+CATH-2 could provide this anti-inflammatory activity with killed bacteria *in vivo*. Instillation of heat-killed bacteria, which has previously been used to study inflammatory responses (12–14), were used to focus solely on CATH-2 immunomodulatory activity. The benefit of this model over instillation of a single bacterial PAMP (i.e. LPS or LTA) was the presence of multiple immunogenic compounds able to activate multiple different PAMP receptors and signalling pathways simultaneously. In these studies, the co-treatment of CATH-2 or BLES+CATH-2 with heat-killed *P. aeruginosa*, including both ATCC strains and MDR-clinical isolates, were able to reduce inflammatory cytokine production to concentrations equal to naïve and control treatment groups. These studies present evidence that CATH-2, and importantly BLES+CATH-2, can prevent and/or reduce the inflammation elicited by killed bacteria.

On top of the ability of CATH-2/BLES+CATH-2 to modulate inflammatory responses, **Chapter 3** demonstrated that bacteria killed by CATH-2 did not induce any inflammatory response. This activity, termed “silent killing”, was initially presented by Dr.

Coorens *in vitro*, and showed that the cathelicidin peptide CATH-2 was able to kill bacteria in co-cultures with macrophages without inducing an inflammatory response (15). These results were expanded to the *in vivo* setting, which demonstrated that CATH-2-killed bacteria did not induce an inflammatory response in the airways after intratracheal instillation. Animals that received CATH-2-killed bacteria, similar to heat-killed bacteria co-treated with CATH-2 or BLES+CATH-2, has less inflammatory cells recovered from the BALF, and reduced cytokine concentrations in BALF. This "silent" killing mechanism has vast therapeutic potential moving forward, as the ability to kill bacteria without eliciting an inflammatory response would greatly increase recovery time, and limit the collateral tissue damage caused by inflammatory cell migration and activation.

However, treatment of VAP with BLES+CATH-2 showed no beneficial effects in **Chapter 5**. Indeed, while treatment in the mouse acute pneumonia model showed a significant difference between saline-controls and BLES+CATH-2 treatment, this bactericidal activity was not observed in the rat VAP model. While treatment with BLES+CATH-2 significantly increased oxygenation in this model compared to CATH-2 alone, there was no significant improvement over saline or BLES controls. In all, it appears that BLES+CATH-2 was an ineffective therapeutic for *P. aeruginosa* induced VAP in our rat model.

Based on the data accumulated throughout this thesis project, we conclude that BLES+CATH-2 has some therapeutic potential for the treatment of multi-drug resistant bacterial pneumonia. However, the lack of antimicrobial activity demonstrated *in vivo* suggests that further research is needed in order to improve BLES+CATH-2 activity. The

purpose of this chapter is to discuss potential future directions in order to continue development and improve the efficacy of BLES+CATH-2 as a therapeutic compound.

6.1 Future Directions

6.1.1 Alterations to exogenous surfactant composition

As described in **Chapter 2**, we theorize that the cause of the mitigated bactericidal activity of BLES+CATH-2 involves the electrostatic interactions between the highly cationic CATH-2 and anionic phospholipid PG in BLES. As such, one method to improve the bactericidal activity of BLES+CATH-2 would be to reduce the content of anionic phospholipids in the surfactant, freeing more cathelicidin peptide to interact with bacterial membrane.

Previous work has gone into great detail over the orientation of cathelicidin peptides in lipid membranes with different phospholipid content (5–7). From this, and from our observations with cathelicidin peptides and the exogenous surfactant BLES, we hypothesize that a reduction in PG content would reduce any electrostatic activity with the cathelicidin peptide, without sacrificing surfactant biophysical properties. Indeed, many natural surfactants and synthetic surfactants have varying phospholipid concentrations, with PG content ranging anywhere between 20% (15) to as low as 7% found in endogenous rabbit surfactant (16). By reducing the content of the acidic phospholipids in BLES from ~10% to as low as 5-7%, we hypothesize that we would be able to increase natural cathelicidin activity (i.e. bactericidal function), without significant alterations to surfactant spreading or biophysical function. However, future testing is required to determine if this

hypothesis is correct, and if this would improve the therapeutic viability of a modified surfactant + CATH-2.

One experimental approach to investigate this would be to formulate a synthetic surfactant. With the addition of recombinant surfactant proteins, this synthetic surfactant could be produced with a specific phospholipid composition aimed at improving the interactions between the surfactant and the antimicrobial peptide to optimize antimicrobial and immunomodulatory functions *in vivo*. This new surfactant could then be re-investigated using the protocols described in this thesis to determine biophysical properties, antimicrobial activities, immunomodulatory functions, and *in vivo* efficacy. Using throughput screening of different surfactant compositions and cathelicidin peptides, we anticipate that a surfactant/cathelicidin compound could be identified and developed as a suitable therapeutic.

6.1.2 Focus on development of anti-inflammatory therapeutic

In lieu of the strong anti-inflammatory activity shown by BLES+CATH-2 in **Chapter 3** and **Chapter 4**, our lab has continued focus on investigating these properties of BLES+CATH-2. Unlike the antimicrobial activity, BLES+CATH-2 appears to exhibit much more consistent immunomodulatory activity. Work recently performed by an undergraduate student, Christina Arsenault, showed that both CATH-2 and BLES+CATH-2 was able to downregulate inflammatory responses after co-administration with heat-killed *P. aeruginosa*, similar to results observed in **Chapter 4**. However, in addition, Ms. Arsenault showed that after a 15-minute delay in administration (i.e. instillation of treatment 15 minutes after instillation of heat-killed bacteria), CATH-2 lost its anti-

inflammatory properties, while BLES+CATH-2 treated animals showed reduced inflammatory cytokine production and cell infiltration.

In addition to this, more work done by a graduate student, Brandon Baer, has continued these investigations by re-visiting the combined therapy of CATH-2 and conventional antibiotics. Mr. Baer has identified that bacteria killed by a lower, more clinically relevant dose of gentamicin than what was used in **Chapter 3** (8 μg/ml vs. 1 mg/ml in **Chapter 3**) still elicit an inflammatory response similar to what was described in this thesis. However, unlike previous results, the addition of CATH-2 did once again reduce the production of inflammatory cytokines. Adding to the potential anti-inflammatory activity of CATH-2, Mr. Baer has revisited other cathelicidin peptides, including LL-37 and PMAP-23, which showed that these anti-inflammatory properties appear to be relatively well conserved across multiple different peptides.

This data, combined with the work performed by Ms. Arsenault, provides a rationale for investigating the immunomodulatory activity of different cathelicidin peptides with the exogenous surfactant BLES. The anti-inflammatory activities of cathelicidin peptides, when administered with antibiotic-killed bacteria, presents a unique avenue for therapeutic development in order to manage inflammation post-infection. In addition, the supplementation of BLES appears to have a significant therapeutic benefit to this immunomodulatory activity, and if this beneficial effect is conserved with other peptides, it would vastly increase the library of therapeutic agents available for clinical development and use. Therefore, there is a strong rationale to continue investigations into the immunomodulatory activity of different cathelicidin peptides/exogenous surfactant is to increase understanding of the therapeutic benefit of these compounds. The use of a various

animal models to investigate multiple different mechanisms of lung inflammation, such as asthma or chronic obstructive pulmonary disease, would allow for a thorough, robust investigation to the limits and potential of BLES+CATH-2 as a respiratory anti-inflammatory treatment.

6.2 Concluding Remarks

The presence of multi-drug resistant infections is the largest global health concern facing this generation, and without the development of new therapies and practices, will become the leading cause of death worldwide by 2050 (2). The high prevalence of bacterial pneumonia, makes development of therapeutics specifically for respiratory tract infections especially important.

Cathelicidin peptides are a unique source of development for future therapeutics for respiratory infections. Their combined microbicidal activities and immunomodulatory properties give these peptides a vast diversity of potential uses. The combined treatment strategy of a cathelicidin peptide with exogenous surfactant, ideal to spread the peptides throughout the complex lung structure, was investigated in this thesis. While the proof-of-principle determined the validity of this approach *in vitro*, the lack of antimicrobial activity and physiologic improvements in *in vivo* models limits the potential of this compound. Instead, anti-inflammatory activity was much more well defined, and may represent another avenue for BLES+CATH-2 therapeutic development.

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Appendix 1: The University of Western Ontario animal use sub-committee protocol approval

2013-053::2:

AUP Number: 2013-053
AUP Title: Antimicrobial Peptide Fortified Surfactant for the Treatment of Bacterial Lung Infections
Yearly Renewal Date: 04/01/2016

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2013-053 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Appendix 2: Human Research Ethics approval

ROMEO - Researcher Portal

General Info

FileNo: 103931
Title: Antimicrobial Peptide-Fortified Surfactant for the Treatment of Cystic Fibrosis
Start Date: 05/06/2017
End Date: 05/06/2018
Keywords: Cystic Fibrosis,surfactant,antimicrobial peptides

Project Members

Principal Investigator

Prefix: Dr.
Last Name: Yamashita
First Name: Cory
Affiliation: Schulich School of Medicine and Dentistry\Medicine-Dept of
Rank: Assistant Professor

Institution: St. Joseph's Health Care London
Country:
Comments:

Description	File Name	Version Date
Approval Notice	DOC062413-06242013152954-0014.pdf	24/06/2013
	REB.docx	
	DOC080114-08012014141450-0001.pdf	
2017/05/25 - CER	DOC060517-0006.pdf	05/06/2017

Appendix 3: Information about copyright release for publication

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Curriculum Vitae

Brandon Banaschewski

Education

The University of Western Ontario, London ON

Doctorate of Medical Sciences; Physiology and Pharmacology, Currently Ongoing

The University of Western Ontario, London ON

Bachelor of Medical Sciences Honors (4 years); Honors Specialization in Physiology, June 2012

Research Experience

QrumPharma, Inc **2016-Present**

Scientific Consultant

Leading projects to raise capital, coordinating with collaborators worldwide, and implementing protocols for the testing and development of two novel clinical therapeutics for the treatment of nontuberculous mycobacterial infections in cystic fibrosis. Working in collaboration with PharmBioTech, Dr. Diane Ordway, and Dr. Veronique Dartois.

The University of Western Ontario, London ON **2012-2017**

Graduate Student

Supervisors: Ruud A. Veldhuizen, Cory Yamashita

Lead projects on developing a novel treatment of bacterial pneumonia through the fortification of pulmonary surfactant with cathelicidins. Working in collaboration with Dr. Edwin Veldhuizen and Dr. Henk Haagsman.

The University of Western Ontario, London ON **2011-2012**

Undergraduate Student, 4th year Seminar & Research Project

Supervisors: Ruud A. Veldhuizen, Cory Yamashita, Jim Lewis

Contributed to research programs investigating the susceptibility of adult Wistar rats to ventilator-induced lung injury through diet induced hypercholesterolemia. Worked in collaboration with Joshua Qua Hiansen.

Honours/Awards

Department of Medicine Research Day – Poster Award ($500)	2016
Ontario Graduate Scholarship ($15 000)	2016
Department of Medicine Research Day – Poster Award ($500)	2015
Ontario Graduate Scholarship ($15 000)	2015
OUA Academic All-Canadian	2014
Internal Research Fund, St. Joseph's Hospital, London, ON ($15,000)	2013
OUA Academic All-Canadian	2013
OUA Academic All-Canadian	2012
Dean's Honour List	2012
OUA Academic All-Canadian	2011
Dean's Honour List	2011
Dean's Honour List	2009
The Western Scholarship of Distinction ($2500)	2008

Teaching Experience

The University of Western Ontario, London ON

Teaching Assistant (2016)

Physiology 2130: Physiology tutorial (undergraduate); Anita Woods

- Nominated for Graduate Student Teaching Assistant Award

Teaching Assistant (2015)

Physiology 3140A: Physiology Assistant (undergraduate); Anita Woods, John Di Guglielmo

Teaching Assistant (2014-2015)

Physiology 2130: Physiology Tutorial (undergraduate); Anita Woods

Teaching Assistant (2013-2014)

Physiology 2130: Physiology Tutorial (undergraduate); Anita Woods

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Teaching Assistant (2012-2013)

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Publications

Brandon Banaschewski, Brandon Baer, Teah Jazey, Edwin Veldhuizen, Doug Fraser, Johan Delaport, Tracey Gooyers, James F Lewis, Henk Haagsman, Cory Yamashita, and Ruud Veldhuizen. The antibacterial and anti-inflammatory activity of chicken cathelicidin-2 against cystic fibrosis pathogens Pseudomonas aeruginosa and Staphylococcus aureus. In progress.

Maarten Coorens*, **Brandon Banaschewski***, Cory Yamashita, Henk Haagsman, Ruud Veldhuizen#, and Edwin Veldhuizen#. Killing of P. aeruginosa by chicken cathelicidin-2 prevents lung inflammation *in vivo*. Submitted to Antimicrobial Agents and Chemotherapy

* and # represent equal contributions to the manuscript

Scott Milos, Joshua Qua Hiansen, **Brandon Banaschewski,** Yi Zuo, Li-Juan Yao, Lynda McCaig, James Lewis, Cory Yamashita, and Ruud Veldhuizen. The effect of diet-induced serum hypercholesterolemia on the surfactant system and the development of lung injury. Biochemistry and Biophysics Reports. 2016; doi:10.1016/j.bbrep.2016.06.009

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Abstracts

Brandon Banaschewski, Stefan Ufer, Thomas Hofmann. The use of nebulized antimycobacterial antibiotics (QRM-003 and QRM-006) for the treatment of nontuberculous mycobacterial infections in cystic fibrosis patients. Presented at Cystic Fibrosis Foundation Research Conference, 2017

Brandon Banaschewski, Brandon Baer, Teah Jazey, Johan Delaport, Edwin Veldhuizen, Henk Haagsman, Cory Yamashita, Ruud Veldhuizen. The efficacy of BLES+CATH-2 against *Pseudomonas aeruginosa* in models of bacterial pneumonia. Presented at Department of Medicine Research Day, 2017

Brandon Banaschewski, Teah Jazey, Johan Delaport, Edwin Veldhuizen, Henk Haagsman, Cory Yamashita, Ruud Veldhuizen. Bactericidal activity of CATH-2 and BLES+CATH-2 against multi-drug resistant clinical bacteria. Presented at Department of Medicine Research Day, 2016

Brandon Banaschewski, Maarten Coorens, Edwin Veldhuizen, Henk Haagsman, Cory Yamashita, Ruud Veldhuizen. Bacteria killed by CATH-2 elicit reduced inflammatory signalling after in vivo intratracheal instillation. Presented at ALTANT: Innate host defense mechanisms and infections – From basic science to applications, 2016

Cory Yamashita, **Brandon Banaschewski**, Teah Jazey, Johan Delaport, Jim Lewis, Tracy Gooyers, Edwin Veldhuizen, Henk Haagsman, Ruud Veldhuizen. Bactericidal activity of a surfactant-based host-defense peptide against antibiotic-resistant CF bacteria. Presented at ALTANT: Innate host defense mechanisms and infections – From basic science to applications, 2016

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Brandon Banaschewski. A cathelicidin-fortified exogenous surfactant as a potential therapeutic for the treatment of bacterial pneumonia. Talks on Fridays, 2015

Brandon Banaschewski. The use of an exogenous surfactant/cathelicidin compound for the treatment of respiratory infections. London-Toronto Lung Symposium, 2015

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