The Antibacterial and Anti-inflammatory Activity of Chicken Cathelicidin-2 combined with Exogenous Surfactant for the Treatment of Cystic Fibrosis-Associated Pathogens

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Cystic fibrosis (CF) is characterized by recurrent airway infections with antibiotic-resistant bacteria and chronic inflammation. Chicken cathelicidin-2 (CATH-2) has been shown to exhibit antimicrobial activity against antibiotic-resistant bacteria and to reduce inflammation. In addition, exogenous pulmonary surfactant has been suggested to enhance pulmonary drug delivery. It was hypothesized that CATH-2 when combined with an exogenous surfactant delivery vehicle, bovine lipid extract surfactant (BLES), would exhibit antimicrobial activity against CF-derived bacteria and downregulate inflammation. Twelve strains of CF-pathogens were exposed to BLES + CATH-2 in vitro and killing curves were obtained to determine bactericidal activity. Secondly, heat-killed bacteria were administered in vivo to elicit a pro-inflammatory response with either a co-administration or delayed administration of BLES + CATH-2 to assess the antimicrobial-independent, anti-inflammatory properties of BLES + CATH-2. CATH-2 alone exhibited potent antimicrobial activity against all clinical strains of antibiotic-resistant bacteria, while BLES + CATH-2 demonstrated a reduction, but significant antimicrobial activity against bacterial isolates. Furthermore, BLES + CATH-2 reduced inflammation in vivo when either co-administered with killed bacteria or after delayed administration. The use of a host-defense peptide combined with an exogenous surfactant compound, BLES + CATH-2, is shown to exhibit antimicrobial activity against antibiotic-resistant CF bacterial isolates and reduce inflammation.

Cystic fibrosis (CF) is an autosomal recessive disease caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that result in decreased and/or dysfunction of the trans-epithelial protein responsible for chloride and bicarbonate ion transport leading to an increased viscosity of the airway-surface mucosal layer. This altered mucous layer results in chronic airway colonization with bacteria that ultimately leads to a vicious cycle of repeated lower respiratory tract infections, inflammation and tissue remodelling. Furthermore, the repeated use of oral, inhaled and systemic antibiotics eventually leads to the development of airway colonization with multi-drug resistant organisms, and represents an independent predictor of adverse clinical outcomes including a more rapid decline in lung function and premature death. Ultimately, this complex pro-inflammatory environment induced by chronic bacterial airway infections promotes airway epithelial...
cell injury and airway remodelling\textsuperscript{6–12} and results in greater challenge from a therapeutic standpoint. Therefore, novel therapies are urgently needed to improve clinical outcomes for patients with CF.

Exogenous cathelicidins are a class of innate host-defense peptides that are currently under investigation for their potential therapeutic use against antibiotic-resistant bacterial infections and have been shown to possess both direct antibacterial activity and immunomodulatory activity. Importantly, based on their multiple mechanism of action, these host defense peptides are notable for their antimicrobial activity against a wide spectrum of bacteria that exhibit resistance to conventional antibiotics and therefore represent an attractive target for therapeutic development\textsuperscript{13–16}. Furthermore, an additional capability for cathelicidins to modulate the host inflammatory responses has been identified including alterations of inflammatory cytokine production and immune cell migration\textsuperscript{17–25}. Currently, outside of use in topical skin infections, the widespread adoptions of HDPs to treat antibiotic resistant infections remains limited due to constraints surrounding optimization of systemic delivery methods and an ability to achieve sufficient concentrations of peptide at localized sites of infection.

In order to facilitate the pulmonary delivery of therapeutics, exogenous surfactant has been investigated as a potential delivery vehicle due to its ability to improve distribution of therapeutics to peripheral lung regions\textsuperscript{26–28}. The use of exogenous surfactant has been extensively investigated as a therapeutic option with acute respiratory distress syndrome and as the standard of care in preterm neonates with surfactant deficiency. Universally, exogenous surfactant administration has been demonstrated to be safe and well tolerated across a spectrum of lung disease, although its benefits as a therapeutic alone in adult populations with ARDS has not been consistently observed\textsuperscript{26,28,29}. Previous studies have investigated the use of exogenous pulmonary surfactant with cathelicidin peptides for the treatment of respiratory pathogens\textsuperscript{30}. Our group recently demonstrated in a series of ‘proof-of-principle’ experiments, that an approach which combines a chicken cathelicidin, CATH-2, with a commercially available exogenous surfactant preparation, bovine lipid-extract surfactant (BLES), maintains excellent surfactant spreading properties, antimicrobial activity, and is well tolerated when administered intratracheally to naïve mice\textsuperscript{31}.

The objective of the current study was to assess specific antimicrobial and/or anti-inflammatory properties of a BLES+CATH-2 preparation, as a novel therapeutic approach to treat CF-related lung infections. It was hypothesized that BLES+CATH-2 would exhibit potent bactericidal activity against bacterial isolates obtained from CF patients and, additionally, could reduce inflammation associated with bacterial killing \textit{in vivo}.

**Results**

**Resistances Patterns of Clinical CF Bacterial Isolates.** Clinical characteristics and \textit{in vitro} antibiotic-resistance patterns of bacterial isolates obtained from CF patients were obtained from the London Health Science Centre Clinical Microbiology Laboratory and are shown in Table 1. Patients ranged between 24 and 57 years of age. Disease severity as measured by lung function was variable among patients, with the predicted forced expiratory volume in one second (FEV1) ranging between 28% and 87% predicted. Six out of nine patients were colonized with at least one strain of \textit{P. aeruginosa}, 3 out of the 9 patients were infected with \textit{S. aureus}, and one patient was infected with \textit{A. xylosoxidans}. Of the eight strains of \textit{P. aeruginosa} isolated, half were identified as a ‘mucoid’

| Patient No. | Isolate No. | Sex (M/F) | Age (years) | Weight (kg) | BMI (kgm\textsuperscript{-2}) | FEV1 Pred | Bacteria | Resistance Profiles |
|-------------|-------------|-----------|-------------|-------------|-----------------------------|-----------|---------|---------------------|
| 1           | 1           | F         | 57          | 60.6        | 22.5                        | 39        | \textit{Pseudomonas aeruginosa} | NR       |
| 2           | 2           | M         | 24          | 72          | 25.3                        | 50        | \textit{Staphylococcus aureus} | Clindamycin, Erythromycin R |
| 3           | 3           | M         | 50          | 57          | 19.3                        | 43        | \textit{Achromobacter xylosoxidans} | Gentamicin, Meropenem, Tobramycin R, Imipenem, Ciprofloxacin I |
| 4           | 4A          | F         | 35          | 62          | 22.4                        | 87        | \textit{Staphylococcus aureus} | NR       |
| 4B          |             |           |             |             |                             |           | \textit{Pseudomonas aeruginosa} (muc) | NR       |
| 4C          |             |           |             |             |                             |           | \textit{Pseudomonas aeruginosa} | Tobramycin, Gentamicin R |
| 5           | 5           | F         | 44          | 59          | 22                          | 73        | \textit{Pseudomonas aeruginosa} (muc) | Gentamicin, Tobramycin R |
| 6           | 6           | F         | 40          | 57          | 20.9                        | 52        | \textit{Pseudomonas aeruginosa} (muc) | Gentamicin, Tobramycin R |
| 7           | 7           | M         | 37          | 52.5        | 19.3                        | 28        | \textit{Pseudomonas aeruginosa} (muc) | NR       |
| 8           | 8           | M         | 27          | 62          | 18.7                        | 48        | \textit{Staphylococcus aureus} | Clindamycin, Erythromycin, Ceftriaxone R |
| 9           | 9A          | F         | 31          | 40.5        | 18.2                        | 55        | \textit{Pseudomonas aeruginosa} (muc) | NR       |
| 9B          |             |           |             |             |                             |           | \textit{Pseudomonas aeruginosa} | Meropenem R, Ciprofloxacin, Gentamicin I |

Table 1. Demographics and resistance profiles of clinical isolates utilized in the study.
phenotype. Of all bacteria isolated, seven isolates were resistant to at least two conventional antibiotics while two strains were resistant to three or more antibiotics.

**Bactericidal Activity of BLES+CATH-2 Against Clinical Isolates.** After *in vitro* incubation of bacterial isolates with either CATH-2 or BLES+CATH-2, it was observed that CATH-2 alone exhibited potent bactericidal activity against all *P. aeruginosa* isolates, regardless of *in vitro* antibiotic-resistance profiles, with the Minimum bactericidal concentration (MBC) ranging between 2.5−10 µM of CATH-2 (Fig. 1). BLES+CATH-2 exhibited greater bactericidal variability against *P. aeruginosa* isolates, with bacterial activity ranging between a 3-log reduction in bacterial growth to complete bacterial killing. Overall, BLES+CATH-2 MBC values were typically 100 µM, and as low as 50 µM against selected CF-derived isolates (Fig. 1).

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

**Anti-inflammatory effects of BLES+CATH-2 in vivo.** To investigate the potential anti-inflammatory effects of BLES+CATH-2, we utilized a model whereby heat-killed bacteria were instilled intratracheally in mice to elicit a proinflammatory response. Through this technique, the antimicrobial-independent anti-inflammatory properties of BLES+CATH-2 could be more accurately assessed. One antibiotic-resistant strain of *P. aeruginosa* (obtained from Patient 6) and one antibiotic-resistant strain of *S. aureus* (obtained from Patient 4) was chosen randomly from CF patient samples (see Table 1). Heat-killed isolates were incubated with either CATH-2 or BLES+CATH-2 in a control, prior to intratracheal instillation, to specifically evaluate bactericidal independent anti-inflammatory effects. Initially, lung function parameters (as measured using a Flexivent®), and protein values in the lavage were analyzed to determine potential effects of BLES+CATH-2 on the development of lung injury. These results revealed no significant differences in lung compliance, elastance or protein values among the 5 experimental groups (Table 2).

A significant increase in total cell counts in the BALF was observed in animals that received intratracheally-administered heat-killed *P. aeruginosa* compared to the group of animals receiving saline only. On the other hand, incubation of heat-killed *P. aeruginosa* with either CATH-2 or BLES+CATH-2 prior to instillation reduced lavage cell counts similar to levels observed with saline control instillation (Fig. 3A). Differential cell counts demonstrated a significant increase in neutrophils in animals receiving heat-killed *P. aeruginosa* compared to saline control animals, whereas macrophages were not significantly different (Fig. 3B and C). Animals instilled with heat-killed bacteria supplemented with CATH-2 or BLES+CATH-2 had macrophage and neutrophil counts that were similar to saline control animals (p > 0.05). A similar pattern of change was observed for concentrations of BALF inflammatory cytokines with heat-killed *P. aeruginosa* eliciting a significant increase in KC, MIP2, and GM-CSF compared to saline controls, with no significant increases observed in TNF-α and IL-6 (Fig. 4). Likewise, animals receiving heat-killed bacteria supplemented with CATH-2, either alone or in combination with BLES, did not result in a significant increase of any of the BALF inflammatory cytokines measured and was not statistically different from saline controls.

The results for the inflammatory responses observed with a killed *S. aureus* isolate are shown in Figs 5 and 6. Similar to observations made for *P. aeruginosa*, incubation of *S. aureus* with CATH-2 or BLES+CATH-2 resulted in a significant reduction in the total cell counts recovered from the BALF compared to heat-killed bacteria alone (Fig. 5A). Differential cell counts revealed that heat-killed *S. aureus* resulted in significantly higher neutrophil counts compared to other groups (Fig. 5C). Despite the differences observed in total cell counts and differential cell counts, no effect on pro-inflammatory cytokines was observed for instillation of heat killed *S. aureus*, and furthermore, supplementation with CATH-2 or BLES+CATH-2 did also not lead to any significant differences in cytokine counts although concentrations were generally lower in the CATH-2 group (Fig. 6).

**Effect of delayed BLES+CATH-2 administration after induction of inflammation with dead bacteria.** The second *in vivo* experiment assessed whether BLES+CATH-2 could reduce the inflammatory response induced by heat-killed *P. aeruginosa*, if administered after a 15-minute delay subsequent to the intratracheal delivery of the dead bacteria. Similar to what was observed in the prior experiment, instillation of a heat-killed laboratory strain of *P. aeruginosa* resulted in an elevated number of inflammatory cells, the majority of which were neutrophils and this effect was mitigated by co-administration of CATH-2 or BLES+CATH-2 (Fig. 7A,B). Subsequently, the delayed administration of CATH-2 alone exhibited values of BAL neutrophil counts that were not different from the heat-killed *P. aeruginosa* group, however, the delayed administration of BLES+CATH-2 resulted in total cell count values that were significantly lower than that of the heat-killed *P. aeruginosa* group (Fig. 7B). Administration of BLES alone had no effect on inflammatory cell influx.

Concentrations of three inflammatory mediators in the eight experimental groups are shown in Fig. 8. Heat killed *P. aeruginosa* caused a significant increase in lavage concentrations of IL-6, TNF-α and KC compared to saline control. Co-administration of CATH-2 or BLES+CATH-2 resulted in a complete mitigation of the cytokine response resulting in concentrations not significantly different from saline control values. Delayed administration of CATH-2 resulted in values that were slightly higher than those of co-administration with the difference reaching statistical significance for IL-6 and KC. Lavage concentrations of the three cytokines after delayed administration of BLES+CATH-2 reached values in between those of the positive, *P. aeruginosa*, and negative, Saline, control groups with only KC being significantly different from co-administration values.
Discussion

In the current study, the antimicrobial and anti-inflammatory effects of BLES+CATH-2, a chicken cathelicidin suspended in a clinical exogenous surfactant preparation, BLES, were evaluated for therapeutic potential as a novel treatment for antibiotic-resistant bacterial lung infections. The data presented in the current manuscript demonstrate that BLES+CATH-2 exhibits bactericidal activity against an array of antibiotic-resistant bacterial isolates obtained from patients with cystic fibrosis. Furthermore, BLES+CATH-2 was demonstrated...
to mitigate the recruitment of inflammatory cells and modulate proinflammatory cytokine production when co-administered with heat-killed pathogens, and had the ability to mitigate inflammatory cell recruitment when administered after initiation of inflammation. This data provides evidence for a potential therapy targeting antibiotic-resistant infections and associated inflammation using a combined host defense peptide and exogenous surfactant delivery vehicle.

The first goal of this study was to identify the bactericidal properties of BLES+CATH-2 against multiple cystic fibrosis-associated antibiotic-resistant bacteria. 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 18.32–36. Due to the frequent use of conventional antibiotics used to treat these pathogens both on an acute and chronic basis,

Table 2. Protein content obtained from BALF after whole lung lavage, and quasi-static compliance and elastance as measured by FlexiVent.

| Treatment                  | Protein Content (mg/kg BW) | Quasi-Static Compliance (ml/cmH₂O) | Quasi-Static Elastance (cmH₂O/ml) |
|----------------------------|---------------------------|-----------------------------------|----------------------------------|
| Control                    | 21.60 ± 3.0               | 0.0777 ± 0.016                    | 13.278 ± 2.70                    |
| P. aeruginosa              |                           |                                   |                                  |
| Heat-killed                | 20.56 ± 9.7               | 0.0718 ± 0.0080                   | 14.077 ± 1.76                    |
| Heat+CATH-2                | 19.44 ± 5.9               | 0.0755 ± 0.0069                   | 13.347 ± 1.34                    |
| Heat+(BLES+CATH-2)         | 17.58 ± 4.9               | 0.0801 ± 0.0064                   | 12.558 ± 1.07                    |
| S. aureus                  |                           |                                   |                                  |
| Heat-killed                | 20.79 ± 5.2               | 0.0754 ± 0.0132                   | 13.700 ± 3.02                    |
| Heat+CATH-2                | 20.98 ± 7.8               | 0.0736 ± 0.0080                   | 13.742 ± 1.64                    |
| Heat+(BLES+CATH-2)         | 18.39 ± 8.0               | 0.0712 ± 0.0071                   | 14.156 ± 1.46                    |

Figure 2. Colony Count assays of CATH-2 and BLES+CATH-2 against three S. aureus isolates and one A. xylosoxidans isolate. The bacteria were suspended in MHB, and then treated with various concentrations of CATH-2 (open circles) or CATH-2 suspended in 10 mg/ml phospholipid of BLES (solid squares). A value of Log CFU/ml of 2 represents the detection limit of the analysis.
the prevalence of antibiotic resistance among these strains has been reported to be as high as 22.6% for *S. aureus* and 42.7% for *P. aeruginosa* among patients with CF. Consistent with this information, the specific strains of *P. aeruginosa*, *S. aureus*, and *A. xylosoxidans* isolated from CF patient sputum samples in our study, 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, with the compound able to reduce eight out of twelve

**Figure 3.** Inflammatory cells obtained from BALF of control animals and animals administered heat-killed *P. aeruginosa*, either alone, or co-administered with CATH-2 or with BLES+CATH-2, six hours after instillation. (A) Total cell counts, (B) Macrophage, and (C) neutrophil *p ≤ 0.05 vs. control, *p < 0.001 vs. heat-killed *P. aeruginosa*. 
bacterial isolates to below detectable levels after three-hour incubations. For the remaining four bacteria tested, BLES + CATH-2 was able to reduce bacteria by three log values for two of the isolated bacteria. This data expands upon work published previously by our research group which demonstrated the efficacy of BLES + CATH-2 against laboratory strains of \( P. \ aeruginosa \) or \( S. \ aureus \) \(^{31}\). Based on previous findings that a 100 \( \mu \)M concentration of BLES + CATH-2 was well tolerated in naïve mice, this concentration was chosen for subsequent in vivo studies to assess the specific antibiotic-independent anti-inflammatory properties of BLES + CATH-2.

To test the ability of CATH-2, and BLES + CATH-2 to modulate inflammatory responses induced by bacterial products we utilized a model in which killed CF isolates were administered intratracheally to naïve mice to induce an inflammatory response. This experimental technique was utilized to assess the immunomodulatory properties of CATH-2 and BLES + CATH-2. The data obtained provided evidence that CATH-2, and BLES + CATH-2, can down-regulate inflammatory responses induced by killed bacteria including reductions in proinflammatory mediators including IL-6, TNF-\( \alpha \) and KC. This data builds on previous observations utilizing \( E. \ coli \) or a laboratory strain of \( P. \ aeruginosa \) in which CATH-2 killed bacteria resulted in a downregulated inflammatory response as compared to antibiotic or heat killed bacteria \(^{39,40} \). Specifically, our data extended these observations by demonstrating this anti-inflammatory effect utilizing two clinical strains of bacteria, including a

**Figure 4.** Cytokine content in the BALF obtained from animals six hours after administration of killed \( P. \ aeruginosa \), either alone, or co-administered with CATH-2 or BLES + CATH-2. *p \leq 0.05\) vs. control, **p \leq 0.01\) vs. control, ##p \leq 0.01\) vs. heat-killed.
Gram-positive and a Gram-negative isolate. In addition, our data demonstrated that BLES + CATH-2 maintained this anti-inflammatory activity previously shown for just CATH-2 by itself. Overall, this ability of BLES + CATH-2 to not only kill clinically isolated antibiotic resistant bacteria, but to also diminish the inflammatory response induced by bacterial products after killing may provide a unique multi-functional therapeutic approach that would be of high therapeutic potential particularly in the context of CF.[12,32,41]

Interestingly, whereas heat-killed *P. aeruginosa* induced a significant pro-inflammatory cytokine response at our 6 hour timepoint following instillation, administration of heat-killed *S. aureus* did not lead to this increased inflammatory cytokine production, despite a significant increase in total cells and neutrophils in the lavage fluid. These results appear to contradict other studies which demonstrated a significant increase in TNF-α production.

Figure 5. Inflammatory cells obtained from BALF of control animals and animals administered heat-killed *S. aureus*, either alone, with CATH-2 or with BLES+CATH-2 six hours after instillation. (A) Total cell counts, (B) Macrophage, and (C) neutrophil *p* < 0.05 vs. control, *p* ≤ 0.001 vs. heat-killed *S. aureus*.
after exposure of alveolar macrophages to heat-killed *S. aureus* in vitro. It is possible that different methods of heat-killing may have altered the immunogenic by-products of *S. aureus*, which led to a reduction in cytokine induction. Alternatively, the levels of inflammatory cytokine in the BALF may exhibit temporal variation and therefore could have returned to baseline levels at the point of assessment (i.e. four hours after instillation), despite persistence of elevated cell counts.

The use of exogenous surfactant preparations as a vehicle to delivery therapeutics to the lung has been previously explored for a variety of therapies including conventional antibiotics. Advantages of this approach over other techniques include the ability to increase therapeutic concentrations deposited in the lung and, based on the innate spreading properties of surfactant, a more uniform distribution throughout the lung. Furthermore, based on its inherent biophysical properties, exogenous surfactant has the ability to open closed lung units and blocked airways which may be of particular relevance in the setting of CF and viscid airway secretions. Previous work published by our group demonstrated that the addition of BLES as a vehicle for host-defense peptides, to some extent, mitigated the antimicrobial activity of host-defense peptides compared to the innate antimicrobial activity of the peptide alone. Therefore, the ability of BLES+CATH-2 to reduce inflammation compared to CATH-2 alone after an established inflammatory insult, would provide significant rationale for this combined approach utilizing BLES as a drug delivery vehicle.

**Figure 6.** Cytokine content in the BALF obtained from animals six hours after administration of killed *S. aureus* either alone, with CATH-2 or with BLES+CATH-2.
Consistent with the above conclusion, the current study demonstrated that the addition of BLES to CATH-2, compared to the delivery of intratracheal CATH-2 alone demonstrated superior outcomes such as IL-6 concentrations in the BAL, when delivered after the administration of dead bacteria with respect to reduction in inflammation. We speculate that this effect is likely due to the enhanced distribution of CATH-2 obtained with BLES, as the administration of BLES alone failed to exhibit any independent anti-inflammatory properties. Another explanation might be that CATH-2, associated with surfactant, is released slowly resulting in a more sustained effect of the peptide. It is therefore notable that the suspension in surfactant is an important aspect of utilizing CATH-2 for pulmonary infections. In addition to the advantages listed above in terms of distribution and local concentrations, this method bypasses systemic administration and associated side effects. Further support of the importance of targeted delivery for CATH-2 comes from the clinical data that indicates that the most promising utilization of host defense peptides for infection comes from its use for skin wound healing in which a peptide containing cream can be directly applied to the infection. This localized administration appears especially important for antimicrobial purposes but may also be important for immunomodulation effects of the peptides.

The antimicrobial mechanisms of action for CATH-2 and other cathelicidins has been reported extensively. The pathways involved in the anti-inflammatory properties of these peptides requires further study, and broadly encompass a number of both pro- and anti-inflammatory processes, such as cytokine production, recruitment of immune cells and alterations to apoptotic pathways. It has been shown that CATH-2, and other cathelicidin peptides, have the ability to bind directly to LPS and LTA, thereby limiting downstream activation of inflammatory cytokines via toll-like receptor signalling cascades. The anti-inflammatory effects of cathelicidins appear to be independent on cell type, as they have been demonstrated in vitro to effect inflammatory cytokine production from multiple different cell lineages. In our study, the anti-inflammatory effects were also observed when BLES + CATH-2 was administered 15 minutes after killed bacteria were administered intratracheally, suggesting that preventing toll-like receptor activation may not be the sole mechanism, and that some other immunomodulatory activity of the cathelicidin, such as alterations of cellular apoptosis, is responsible for these observations. Further support for this comes from data showing that PMAP-23, a porcine cathelicidin with low LPS affinity, maintained some anti-inflammatory properties against heat-killed . Thus, although binding of LPS likely contributes to the anti-inflammatory properties of CATH-2, other properties certainly will contribute and require further study.

From a clinical perspective, the findings of the current study are of relevance not only to CF associated lung infections, but may also have an impact on other infections complicated by resistant bacteria or difficult to treat bacteria like tuberculosis, ventilator-associated pneumonia, and severe community-acquired pneumonia. In each of these circumstances, clinical outcomes can be directly linked to the presence or absence of antibiotic-resistant organisms. Despite a looming global pandemic stemming from antimicrobial resistance, the response on the part of the pharmaceutical industry to develop novel antibiotics has been underwhelming, and therefore a high premium has been placed on the development to novel, antimicrobial approaches. Although our pilot studies will require much more in-depth investigation prior to clinical application, our experiments will serve as a critical framework by which further host-defense peptides and exogenous surfactant preparations can be evaluated.

Figure 7. The total number of inflammatory cells (A) and Neutrophils (B) per mL of lung lavage samples collected from mice in the different experimental groups four hours after instillation of killed bacteria. The negative (Saline) and positive (P. aeruginosa) controls are represented as the two bars to the left of the dotted line. The bars to the right of the dotted line represent the data for the 6 groups that had their treatments either co-administered with the dead bacteria, or 15 minutes after the administration of dead bacteria, for three treatments: BLES, CATH-2, and BLES+CATH-2. *p ≤ 0.05 vs. Saline, #P < 0.05 vs. P. aeruginosa.
Unfortunately, at the current time, there are limited options in individuals with CF who harbour multidrug-resistant pathogens, however a novel therapy such as BLES+CATH-2 may provide an alternate treatment as a means of delaying disease progression and CF associated morbidity.

Despite the insight into the properties of BLES+CATH-2 that our study provides, there are several limitations that need to be considered. First, the dosing in our anti-inflammatory experiments was 20 µM of CATH-2 and 100 µM CATH-2 when suspended in BLES. This strategy was employed as BLES was shown to mitigate direct bacterial killing to an extent whereby these two concentrations are, approximately, similar in bactericidal efficacy; however, the dosing effect on anti-inflammatory properties could be different. A limitation of the second in vivo study was that only a 15-minute delay was assessed in treatment subsequent to the administration of killed bacteria, and therefore, future studies examining the specific anti-inflammatory effects of BLES+CATH-2 after a prolonged latency or in models of polymicrobial, chronic bacterial lung infections would be of high interest. Finally, our study investigated the independent bactericidal and anti-inflammatory effect of BLES+CATH-2 in separate experimental approaches. Future studies should investigate both phenomenon in in vivo models of bacterial infection.

In conclusion, it is demonstrated that the chicken cathelicidin, CATH-2, in combination with an exogenous surfactant, BLES, possesses both bactericidal activity against clinically-derived CF pathogens, and can furthermore 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 while being able to modulate the hyper-inflammatory environment induced by these pathogens provides strong evidence for the potential of BLES+CATH-2 as a therapeutic for the management of respiratory infections associated with CF and other pulmonary bacterial infection.

**Methods**

All experimental protocols were approved by the Lawson Health Research Institute (London, Ontario, Canada).

**Surfactant/Peptide Compounds.** Chicken cathelicidin CATH-2 was synthesized using Fmoc solid-phase synthesis as described previously. All peptides were purified to a minimum purity of 95% by reverse phase...
high-performance liquid chromatography prior to biological testing. The peptide was then suspended in non-buffered sterile saline. 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 and contains all-natural phospholipids found in bovine surfactant, along with surfactant-associated proteins SP-B and SP-C. BLES (re-suspended in sterile saline) and peptide were mixed to concentrations of 10 mg/ml phospholipid of BLES, and 0–200 µM CATH-2.

Clinical CF Sputum Isolates. Clinical isolates were obtained from ten adult CF patients attending the London Health Sciences Centre outpatient CF clinics and protocols were approved by the Human Ethics subcommittee at Western University, London, ON, Canada. Informed consent was obtained from all patients participating in the study. All methods were performed in accordance with the Canadian Institutes of Health Research Tri-council Policy Statement: Ethical Conduct for Research Involving Humans, December 2010. Isolated bacteria were stored in citrulline solution at ~80 °C, and were isolated, identified and sub-cultured onto chocolate blood agar (CBA) prior to use in study. The samples obtained included seven Pseudomonas aeruginosa, one Acinetobacter xylosidans, and three Staphylococcus aureus bacterial strains isolated from CF sputum samples. Antibiotic susceptibility profiles of the isolated bacteria were obtained using Vitek-2 ID/AST Instrument (bioMérieux, France).

Bactericidal activity against clinical isolates. Bactericidal activity was measured using a spot plating assay. In brief, an overnight culture of bacteria grown in TSB diluted in Mueller-Hinton Broth (MHB). The turbidity was adjusted to 0.5 McFarland, followed by an additional 50x dilution. Subsequently, 50 µL of bacteria and 50 µL of either CATH-2 or BLES + CATH-2 was added to a polypropylene coated 96-well plate, resulting in a concentration range of CATH-2 from 0–100 µM with or without 10 mg/ml BLES. These bacterial suspensions were incubated at 37 °C for three hours with no shaking after which they were serially diluted 10–1000-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 at the highest dilution, corresponding to a bacterial concentration of less than 100 CFU/ml.

Anti-inflammatory effects against clinical isolates. One P. aeruginosa and one S. aureus species were randomly selected for use in this experiment. 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 2 × 10^8 CFU/ml. Subsequently, the bacteria were killed by heat (90 °C for one hour) with complete bacterial killing confirmed via spot plating on chocolate agar and incubated overnight to assess for bacterial growth. In a subset of bacteria killed by heat, CATH-2 (20 µM) or BLES + CATH-2 (10 mg/ml phospholipid; 100 µM CATH-2) was added to the killed-bacteria. Thereafter, the dead-bacterial suspensions were instilled into male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada) as described. All methods were carried out in accordance with guidelines and regulations set forth by the Western University Council for Animal Care, the Canadian Council on Animal Care and Ontario Animals in Research Act. Briefly, once intubated, mice were randomized to one of 4 experimental groups with all receiving a 50 µL instillation of either: 1) heat-killed bacteria, 2) heat-killed bacteria supplemented with CATH-2, 3) heat-killed bacteria supplemented with BLES + CATH-2, or 4) control MHB without bacteria. Mice were subsequently extubated and allowed to recover and breathe spontaneously for six hours. Following the recovery period, mice were euthanized by sodium pentobarbital overdose and aortic transection, 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.

Cell counts and differential cell analysis of the cell pellets obtained from the lavage was done as previously described. After centrifugation, the cell pellet was resuspended in Plasma-Lyte A solution (Baxter Healthcare, Dearfield, IL), and were visualized with 1:1 v/v of 0.4% trypan blue for cell counts via a Bright-Line hemocytometer (Hauser Scientific, Horsham, PA). For cell differential analysis, the cell pellet was centrifuged at 1000 rpm using a Shandon Cytospin 4 (Thermo Scientific, Cheshire, UK) for six minutes, and were fixed using a Hemacolor Eosin kit according to manufacturer’s instructions (EM Science, Gibbstown, NJ). Protein content of the lavage fluid was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), per manufacturer’s instructions. Concentrations of mouse cytokines were measured using multiplexed immunoassay kits per manufacturers’ instructions (R&D Systems, Minneapolis, MN). A Bio-Plex 200 readout system was used to measure cytokine content following VMM software (v. 4.4.1.1, Bio-Rad).

To further explore the anti-inflammatory effects of BLES + CATH-2, a second experiment was performed with a laboratory strain of P. aeruginosa (ATCC 27853). The goal of this experiment was to test if co-administration of CATH-2 or BLES + CATH-2 was essential to provide its anti-inflammatory effects against heat-killed P. aeruginosa. Experimental procedures were similar as described above with the following modifications. Once intubated, animals received two intratracheal instillations of 50 µL. The first suspension was instilled into the lungs via the endotracheal tube. After 15 minutes, 50 µL of the second suspension was instilled, followed by extubation and subsequent intraperitoneal injection of atipamezole hydrochloride (1 mg/kg BW; Zoetis Canada), a dexmedetomidine-counteracting agent. The eight experimental groups, with in brackets the 1st and 2nd installation respectively, were: 1. Negative Control (1st: Saline, 2nd: Saline), 2. Positive Control (1st: Heat...
killed *P. aeruginosa*, 2nd: Saline), 3. Co-administered BLES (1st: Heat killed *P. aeruginosa* + BLES, 2nd: Saline). 4. Delayed-administered BLES (1st: Heat killed *P. aeruginosa*, 2nd: BLES). 5. Co-administered CATH-2 (1st: Heat killed *P. aeruginosa* + CATH-2, 2nd: Saline) 6. Delayed-administered CATH-2 (1st: Heat killed *P. aeruginosa*, 2nd: CATH-2). 7. Co-administered BLES + CATH-2 (1st: Heat killed *P. aeruginosa* BLES + CATH-2, 2nd: Saline). 8. Delayed-administered BLES + CATH-2 (1st: Heat killed *P. aeruginosa*, 2nd: BLES + CATH-2). The installation was followed by a 4-hour recovery period after which the mice were euthanized, the lungs were lavaged and the lavage fluid was analyzed for differential cell analysis and cytokine concentrations by ELISA. The TNF-α and IL-6 cytokines were analyzed using a BD OptEIA ELISA (BD Biosciences, San Diego, CA), while KC was analyzed using a Duoset ELISA (R&D Systems, Minneapolis, MN).

**Statistical Analysis.** All data generated during this study are included in this published article. Data from the *in vitro* killing assays represent the average of at least three independent repetitions. Results from the *in vivo* experiments were obtained with n-values of 6–8 mice per group. Statistical significance was determined by two way analysis of variance and one-way analysis of variance (ANOVA) followed by a Dunnett’s post hoc test to elucidate differences versus positive and negative control values and a Tukey’s post hoc test to determine differences among experimental groups. Results are presented as mean ± the standard error of the mean (SEM), and were considered statistically significant with a P-value of less than 0.05.

**Data Availability.** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

B.J.H.B. was involved in all aspects of the study and writing the manuscript, B.B. and C.A. performed/assisted with the two in vivo experiments, T.J. performed/assisted with the in vitro experiments, E.J.A.V. and H.P.H. were involved in study design, writing of the manuscript and peptide design, J.D. cultured clinical isolates and performed antibiotic sensitivity assays, T.G. obtained informed consent and clinical samples, J.F.L. was involved in study design and patient identification, R.A.W.V. and C.Y. were involved in all aspects of the study design and writing the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.
