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Bioengineered Lysozyme Reduces Bacterial Burden and Inflammation in a Murine Model of Mucoid *Pseudomonas aeruginosa* Lung Infection

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The spread of drug-resistant bacterial pathogens is a growing global concern and has prompted an effort to explore potential adjuvant and alternative therapies derived from nature’s repertoire of bactericidal proteins and peptides. In humans, the airway surface liquid layer is a rich source of antibiotics, and lysozyme represents one of the most abundant and effective antimicrobial components of airway secretions. Human lysozyme is active against both Gram-positive and Gram-negative bacteria, acting through several mechanisms, including catalytic degradation of cell wall peptidoglycan and subsequent bacterial lysis. In the infected lung, however, lysozyme’s dense cationic character can result in sequestration and inhibition by polyanions associated with airway inflammation. As a result, the efficacy of the native enzyme may be compromised in the infected and inflamed lung. To address this limitation, we previously constructed a charge-engineered variant of human lysozyme that was less prone to electrostatic-mediated inhibition *in vitro*. Here, we employ a murine model to show that this engineered enzyme is superior to wild-type human lysozyme as a treatment for mucoid *Pseudomonas aeruginosa* lung infections. The engineered enzyme effectively decreases the bacterial burden and reduces markers of inflammation and lung injury. Importantly, we found no evidence of acute toxicity or allergic hypersensitivity upon repeated administration of the engineered biotherapeutic. Thus, the charge-engineered lysozyme represents an interesting therapeutic candidate for *P. aeruginosa* lung infections.

The spread of antibiotic resistance among bacterial pathogens represents a looming public health crisis (1). With a rise in multidrug-resistant bacteria and few new antimicrobials in the pipeline, there is a need to explore potential alternative and adjuvant therapies (1). Human lysozyme (hLYS) is a naturally occurring antimicrobial peptide found in a variety of tissues, cells, and secretions involved in the pathobiology of lung infection, e.g., the airway surface liquid and cytoplasmic granules of neutrophils (2). It plays a key role in the innate immune response to infection, with levels rising in response to microbial invaders (3, 4). hLYS exerts its antimicrobial effect through catalytic hydrolysis of cell wall peptidoglycan (5) and muramidase-independent processes that have yet to be fully elucidated (6, 7). It has been shown to be effective against both Gram-positive and Gram-negative organisms, including *Pseudomonas aeruginosa* (8–10).

Several studies have examined lysozyme’s potential as an exogenously administered biotherapeutic. Recently, Bhavsar et al. administered aerosolized recombinant hLYS as a treatment for *P. aeruginosa* lung infection in hamsters (11). They found that 2 h of treatment for 3 consecutive days decreased the bacterial burden in both bronchoalveolar lavage fluid (BALF) and lung homogenate. The enzyme treatment also decreased lung tissue inflammation, reduced BALF leukocytes and neutrophils, and decreased alveolar septal apoptosis (11). A follow-up study found that a single nebulized dose of coadministered hLYS and tobramycin decreased the lung and BALF bacterial burden and reduced markers of inflammation (12). They concluded that hLYS is an interesting therapeutic candidate for treatment of lung infections in humans.

While there is precedent for using inhaled lysozyme as an exogenously administered antibacterial, experimental evidence suggests that cationic antimicrobials such as lysozyme are sequestered by anionic biopolymers associated with inflammation. Moreover, it is thought that this electrostatic sequestration compromises antibacterial efficacy in the infected and inflamed lung (4, 13). We have previously shown that wild-type hLYS is inhibited *in vitro* by anionic biopolymers, and we have employed biomolecular engineering to remodel the enzyme’s electrostatic potential field to mitigate this limitation. In brief, a library of charge-altered lysozyme variants was constructed by combinatorial mutagenesis of eight basic residues that possessed low-level evolutionary conservation. The library was screened, under inhibitory conditions, for lytic activity against *Micrococcus luteus*. The enzyme variant 2-3-7 retained wild-type levels of bactericidal activity toward *M. luteus* and *P. aeruginosa* strain PA01, but it was found to exhibit far superior lytic activity in the presence of the inhibitory polyanions alginate, DNA, mucin, and F-actin. The details of the construction and *in vitro* characterization of 2-3-7 are reported elsewhere (14, 15).

In the current study, we employed a murine model of mucoid *P. aeruginosa* lung infection to assess the therapeutic potential of variant 2-3-7. The presence of alginate and extracellular DNA is a hallmark of chronic *P. aeruginosa* infection of the human airway (16, 17), and we have previously shown that these biopolymers do in fact accumulate in our mouse model of lung infection (14). Here, we describe a series of systematic studies that evaluate the in
were anesthetized briefly with isoflurane and inoculated with 40
C57BL/6J mice (age, 8 to 12 weeks; Jackson Laboratories, Detroit, MI)
agar (Difco), followed by incubation at 37°C for 24 h. Adult male
tobramycin were purchased from Sigma-Aldrich (St. Louis, MO), and the
genetically engineered enzyme 2-3-7 was produced and purified as previ-
ously described (14). We employed P. aeruginosa strain FRD1, a mucoid
clinical isolate (18) that exhibits antibiotic resistance under in vitro
conditions relevant to lung infections (19–21).

In vitro quantitative culture. For antipseudomonal assays, 25,000
CFU/ml of mid-log-phase P. aeruginosa strain FRD1 was mixed with 7.5
μg of purified enzyme in activity buffer (10% [vol/vol] Luria-Bertani
broth [LB]) in 10 mM potassium phosphate, pH 7.0) in a total reaction
volume of 115 μl. Dilutions were plated after a 60-min incubation at 37°C,
colonies were enumerated following overnight outgrowth, and results
were compared to those for dilution plates sampled at time zero. Assays
were performed in triplicate on each of two different days to yield biological
replicates.

Pulmonary infection model and treatment regimen. Overnight LB cultures of P. aeruginosa were pelleted, washed twice with phosphate-
buffed saline (PBS; 137 mM NaCl, 2.6 mM KCl, 10 mM Na₂HPO₄, 1.7
mM KH₂PO₄, pH 7.4), and resuspended to give 5 × 10⁷ viable P. aerugi-
 nosa bacteria in 40 μl of PBS. The actual inoculum was determined by
serial dilution of the input bacterial suspension on Pseudomonas isolation
agar (Difco), followed by incubation at 37°C for 24 h. Adult male
C57BL/6J mice (age, 8 to 12 weeks; Jackson Laboratories, Detroit, MI)
were anesthetized briefly with isoflurane and inoculated with 40 μl (5 ×
10⁷ CFU) of P. aeruginosa via oropharyngeal aspiration. At 1 h postinfection,
a second inoculation with either hLYS or 2-3-7 in 40 μl of PBS was
administered in the same fashion. Enzyme doses were typically 100 μg,
with the exception of the doses in the 2-3-7 dose-response study (1
μg, 10 μg, and 100 μg). Tobramycin, when applicable, was administered at
the time of the second inoculation as an intraperitoneal injection of 75 μg
in 200 μl PBS per mouse. Intraperitoneal injection is an accepted route for
systemic drug delivery in rodent models.

BALF collection, cell count, and cell differential. At 24 h postinfection,
mice were anesthetized with intraperitoneal sodium pentobarbital,
tracheas were cannulated, and BALF was collected using an instillation of
1 ml of cold PBS. The BALF was centrifuged, and mouse immune cells
were enumerated using an Advia automated cell counter (Siemens, Berlin,
Germany). The cell-free protein content of BALF was determined by
the Bradford assay with bovine serum albumin as a standard. Cytokine levels
in BALF were determined using Bio-Plex Pro assays according to the
manufacturer’s instructions (Bio-Rad, Hercules, CA).

Quantification of P. aeruginosa bacterial burden. Once the BALF
had been obtained as described above, lungs were excised and placed into
1 ml of cold PBS, followed by homogenization. Viable bacterial counts in
the lung homogenate were determined by plating serial dilutions (100 μl)
on Pseudomonas isolation agar, followed by incubation at 37°C for 24 h.

Liver histology. Livers were fixed in buffered formalin for 24 h, em-
bedded in paraffin, sectioned, and stained with hematoxylin-eosin. His-
tological sections were reviewed by two independent observers blinded to
treatment group. Representative images were obtained using an Olympus
BX50 light microscope with an Optronics MagnaFire digital camera.

Toxicology model. Adult male C57BL/6J mice (age, 8 to 12 weeks)
were given 100 μg of enzyme 2-3-7 by oropharyngeal aspiration on one,
two, or three consecutive days (for a total of one, two, or three doses).
Control animals were treated with PBS. Replicate groups were sacrificed
on day 4 and day 10. BALF was obtained as described above for determina-
tion of the white blood cell content. In addition, serum from anesthe-
tomized mice was collected by right heart puncture. On day 10, total IgG1
and IgE levels were determined by enzyme-linked immunosorbent assay using
capture and detection antibodies, according to the manufacturer’s in-
structions (BD Pharmingen, San Diego, CA).

Statistical analysis. Results were analyzed using one-way analysis of
variance (ANOVA) with Dunnett’s post hoc comparison (22) to the PBS
control. In all cases, statistical significance was assessed at an α level of
0.05.

RESULTS

Lysozyme in vitro activity toward P. aeruginosa strain FRD1. In
prior reports, we detailed the in vitro activity of engineered lysozyme
2-3-7, specifically highlighting its capacity to evade electrostatic inhibition by anionic biopolymers (14, 15). As a com-
ponent of that work, we showed that 2-3-7 was equivalent to wild-
type hLYS in quantitative culture experiments assessing bacteri-
al activity toward the nonmucoid P. aeruginosa strain PAO1. For the purpose of evaluating in vivo efficacy, we were primarily
interested in P. aeruginosa strain FRD1, as this bacterium’s mu-
coid phenotype has greater clinical relevance to chronic lung in-
fec tions (23). Surprisingly, quantitative culture experiments with
FRD1 showed the engineered enzyme to have reduced bactericidal
activity compared to that of wild-type hLYS (Fig. 1). Because,
however, the charge-engineered enzyme had been designed spec-
cifically for enhanced performance in the infected and inflamed
lung environment, we continued to pursue in vivo studies, despite
this unexpected preliminary result.

Repeated dosing of 2-3-7 is nontoxic and nonallergenic. To
assess the acute toxicity of the engineered enzyme 2-3-7, we examined
lung and liver inflammation after repeated dosing (100 μg once per day for one, two, or three consecutive days). Compared to
a PBS control, none of the dosing regimens caused significant
increases in BALF immune cells, as measured on day 4 or day 10
(Fig. 2A and B). Similarly, there was no evidence of liver toxicity,
as determined by histological sampling on day 4 or day 10 (Fig.
2C). To determine if 2-3-7 induced an allergic response, we quan-
tified serum immunoglobulins on day 10 of the repeat dosing
study. Again, none of the dosing regimens caused a significant
increase in serum IgE or IgG1 levels (Fig. 3). Thus, there is no
evidence that the engineered enzyme causes acute toxicity or allergic hypersensitivity during short-term repeated dosing.

2-3-7 decreases *P. aeruginosa* burden and airway inflammation. A dose-response study of enzyme 2-3-7 was conducted to assess the enzyme’s therapeutic performance. The lungs of mice were infected with $5 \times 10^7$ *P. aeruginosa* FRD1 cells by oropharyngeal aspiration, and at 1 h postinfection escalating doses of 2-3-7 were administered by the same route. The numbers of bacterial CFU were quantified at 23 h posttreatment, and a dose-response effect showed that peak efficacy was approached between 10 and 100 µg (Fig. 4A). It bears noting that preliminary studies with nonmucoid *P. aeruginosa* strain PAO1 found a similar dose-response trend at lower enzyme concentrations (100 ng to 1 µg), although the results were not statistically significant at these low doses (one-way ANOVA, $P = 0.188$; data not shown).

Following the same protocol, the efficacy of variant 2-3-7 was compared head-to-head with that of wild-type hLYS at a 100-µg dosage. Mice treated with either enzyme showed a statistically significant reduction in *P. aeruginosa* lung burden compared to that achieved with a PBS sham treatment (Fig. 4B). While not significant at $\alpha$ equal to 0.05, mice treated with 2-3-7 trended toward lower bacterial burdens than mice treated with wild-type hLYS ($P = 0.132$, two-tailed t test). We also sought to determine if enzyme therapy reduced infection-associated lung inflammation and injury. Compared to the results for a PBS control group, treatment with 2-3-7 showed a strong trend toward fewer airway immune cells (Fig. 5A) and significantly less leakage of protein into the airway (Fig. 5B), whereas treatment with hLYS yielded no significant difference. Additionally, we found that treatment with 2-3-7 resulted in significantly reduced BALF concentrations of tumor necrosis factor alpha (TNF-α) (Fig. 5C) and keratinocyte-derived cytokine (KC) (Fig. 5D). In contrast, wild-type hLYS did not reduce lung cytokine levels to a statistically significant degree. In aggregate, these results provide evidence that engineered variant 2-3-7 outperforms wild-type hLYS in combating airway infections by mucoid *P. aeruginosa*.

2-3-7 is as effective as tobramycin in treating mucoid *P. aeruginosa* lung infection. To determine if there was an added benefit to combining lysozyme treatment with standard antibacterial therapies, mice were infected with *P. aeruginosa* as described...
above and treated with either tobramycin, tobramycin combined with hLYS, or tobramycin combined with 2-3-7. A 75-μg intraperitoneal dose of tobramycin resulted in a significantly reduced P. aeruginosa burden compared to that achieved with a PBS sham treatment (ANOVA, \( P < 0.005 \)). Surprisingly, when tobramycin was coadministered with hLYS, the combination therapy resulted in slightly higher mean bacterial counts than the PBS sham treatment (Fig. 6A). In contrast, the combination of variant 2-3-7 and tobramycin yielded a slightly lower bacterial burden than that achieved with tobramycin alone (average numbers of lung CFU = 90,636 versus 116,615, respectively). A similar pattern emerged in quantitative measures of airway inflammatory cells (Fig. 6B). We conclude that while 2-3-7 combined with tobramycin does not completely eradicate the model infection, neither does the engineered enzyme severely antagonize the aminoglycoside, as observed with wild-type hLYS.

**DISCUSSION**

Colonization of the lower respiratory tract by various bacterial pathogens leads to a wide spectrum of pulmonary diseases, and as a whole, lung infections cause a greater global burden of disease than any other category, including HIV/AIDS, cancer, heart attacks, or malaria (24). Even in the United States, the evidence suggests that mortality rates from lung infections have failed to decline appreciably since the 1950s (25). One key contributor to this lack of progress is the emergence and rapid spread of antibiotic resistance among pathogenic bacteria (26, 27). Indeed, antibiotic resistance is a widespread health concern that often complicates ventilator-associated pneumonia (28, 29) and contributes to patient morbidity and mortality in cases of underlying chronic pulmonary disease, such as chronic obstructive pulmonary disease and cystic fibrosis (30–32). These observations are driving a medical imperative to develop new antimicrobial agents for bacterial infections of the lung.

Fueled in part by advances in recombinant protein production technologies (33, 34), endogenous antimicrobial proteins, such as lysozyme, have emerged as prospective therapeutic candidates. These agents are naturally occurring within humans, play important roles in innate immunity, exert broad-spectrum antimicrobial activity, and function via mechanisms distinct from those of traditional antibiotics. Additionally, relative to conventional chemotherapeutics, they are thought to have a lower propensity toward rapid induction of resistance phenotypes (35). While natural

![Figure 4](image-url)  
**FIG 4** Efficacy of lysozyme treatments (\( n = 5 \) mice per group). (A) Reduction in the numbers of P. aeruginosa CFU with escalating doses of charge-engineered lysozyme 2-3-7 (ANOVA, \( P = 0.005 \)); (B) numbers of lung P. aeruginosa CFU following treatment with PBS, 100 μg of wild-type hLYS, or 100 μg of variant 2-3-7 (ANOVA, \( P = 0.0002 \)). The means ± SEMs are indicated. *, \( P < 0.05 \) compared with the PBS control group; **, \( P < 0.01 \) compared with the PBS control group; ***, \( P < 0.001 \) compared with the PBS control group.

![Figure 5](image-url)  
**FIG 5** Assessment of lung inflammation and injury following P. aeruginosa infection and treatment with 100 μg wild-type or engineered lysozyme. (A) BALF immune cell concentration (\( n = 5 \) mice per group; ANOVA, \( P = 0.067 \)); (B) BALF protein concentration as a surrogate of lung injury (\( n = 5 \) mice per group; ANOVA, \( P = 0.008 \)); (C) BALF cytokine KC concentration as a marker of inflammation (\( n = 6 \) mice per group; ANOVA, \( P = 0.040 \)); (D) BALF TNF-α concentration as a marker of inflammation (\( n = 6 \) mice per group; ANOVA, \( P = 0.039 \)). The means ± SEMs are indicated. *, \( P < 0.05 \) compared with the PBS control group; **, \( P < 0.01 \) compared with the PBS control group.
lysozymes advantageously possess bactericidal activity against both Gram-positive and Gram-negative bacteria, there exists considerable evidence that their efficacy within the infected and inflamed lung may be compromised by electrostatic interactions with disease-associated, anionic biopolymers (4, 13).

We have successfully redesigned the electrostatic potential field of hLYS, creating a charge-engineered variant that is less prone to electrostatic inhibition (15). A previous in vitro analysis had shown that variant 2-3-7 exerted wild-type or better bactericidal activity toward both Micrococcus luteus and P. aeruginosa strain PAO1 (14), but here, we found it to exhibit 2.6-fold lower in vitro activity toward the P. aeruginosa clinical isolate FRD1. We emphasize, however, that variant 2-3-7 was designed specifically for the infected and inflamed lung environment, and consistent with this objective, it outperformed wild-type hLYS in our murine model of FRD1 lung infection. Compared to a PBS sham treatment, mice treated with the engineered enzyme showed a significant reduction in measures of lung damage and inflammation, whereas treatment with the wild-type enzyme did not yield statistically significant results. In head-to-head comparisons of the engineered and wild-type enzymes, the engineered variant showed a strong trend toward a greater reduction in bacterial burden. Toxicology studies with the engineered enzyme showed no evidence of acute toxicity or allergic hypersensitivity. These observations suggest that the enhanced in vivo performance of the variant stems from a direct effect of the enzyme itself rather than some secondary effect of an inflammatory response elicited by the exogenous protein.

We also analyzed combination treatments of lysozyme and tobramycin, a frontline chemotherapeutic for cystic fibrosis patients. During acute respiratory exacerbations, patients are commonly given systemic tobramycin via intravenous administration. During acute respiratory exacerbations, patients are commonly given systemic tobramycin via intravenous administration. Therapeutic utility.

In aggregate, we show here that our charge-engineered lysozyme variant decreases the infection-derived bacterial burden and lung inflammation without associated toxicity or hypersensitivity. These results suggest that the engineered enzyme is an interesting therapeutic candidate for treating P. aeruginosa infections of the airway, although studies with additional clinical isolates will be needed to rigorously assess the protein’s broader therapeutic utility.

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