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EXPERIMENTAL THERAPEUTICS

Preclinical Evaluation of Recombinant Microbial Glycoside Hydrolases as Antibiofilm Agents in Acute Pulmonary *Pseudomonas aeruginosa* Infection

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ABSTRACT The bacterium *Pseudomonas aeruginosa* can colonize the airways of patients with chronic lung disease. Within the lung, *P. aeruginosa* forms biofilms that can enhance resistance to antibiotics and immune defenses. *P. aeruginosa* biofilm formation is dependent on the secretion of matrix exopolysaccharides, including Pel and Psl. In this study, recombinant glycoside hydrolases (GHs) that degrade Pel and Psl were evaluated alone and in combination with antibiotics in a mouse model of *P. aeruginosa* infection. Intratracheal GH administration was well tolerated by mice. Pharmacokinetic analysis revealed that, although GHs have short half-lives, administration of two GHs in combination resulted in increased GH persistence. Combining GH prophylaxis and treatment with the antibiotic ciprofloxacin resulted in greater reduction in pulmonary bacterial burden than that with either agent alone. This study lays the foundation for further exploration of GH therapy in bacterial infections.

KEYWORDS *Pseudomonas aeruginosa*, antibiotic, antimicrobial combinations, biofilm, exopolysaccharide, bacteria, Pel, Psl, glycoside hydrolase (GH), acute pulmonary infection

The bacterium *Pseudomonas aeruginosa* is a Gram-negative organism and a common cause of respiratory infections in adult patients with chronic pulmonary disease, such as chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis, and asthma (1–4). *P. aeruginosa* infection accelerates the progressive destruction of the airways and consequent decline in lung function in these chronic pulmonary diseases (2). Despite early therapeutic intervention, *P. aeruginosa* remains a predominant and persistent airway microbial pathogen that is associated with increased morbidity and mortality rates for patients, which underscores the need for novel treatment strategies (5–7). One approach to improving patient outcomes is to target molecules involved in bacterial pathogenesis.

During infection, *P. aeruginosa* forms communities of aggregated bacterial cells embedded in an extracellular matrix of self-produced and host-derived components known as a biofilm (8–12). The bacterium-derived biofilm matrix is dependent on the production of macromolecules such as proteins (13, 14), extracellular DNA (15, 16), and exopolysaccharides (11, 17, 18). Of these matrix molecules, exopolysaccharides play a key role in biofilm formation (17, 19). During biofilm formation, the synthesis of three exopolysaccharides, namely, Pel, Psl, and alginate, can be upregulated depending on the *P. aeruginosa* strain and host-mediated selective pressures (8, 10, 19, 20). Pel is a cationic polymer composed predominantly of

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N-acetyl-D-galactosamine (GalNAc) and *N*-acetylated D-glucosamine (GalN) residues, one or both of which are partially de-*N*-acetylated (18). Psl is a branched neutral polymer composed of pentasaccharide repeats of D-mannose, L-rhamnose, and D-glucose (21). Alginate produced by mucoid *Pseudomonas* strains is an anionic exopolysaccharide composed of nonrepeating β-1,4-linked partially *O*-acetylated D-mannuronic acid and L-guluronic acid (22, 23). Pel and/or Psl production is essential for biofilm formation *in vitro*, as mutants lacking the ability to produce both polymers are impaired in biofilm formation (19, 24), while alginate is dispensable to biofilm architecture (24–29). Psl initiates bacterial surface attachment and maintains biofilm architecture in Psl-dominant strains, whereas Pel mediates cell-cell interactions for multicellular aggregate formation in Pel-dependent strains (30). Redundancy in the structural roles of these polymers has been demonstrated, and Pel and Psl were found to contribute interchangeably to the biofilm architecture (19, 24). The Pel/Psl matrix functions as a barrier to antimicrobial agents and host defenses, thereby enhancing the persistence of *P. aeruginosa*. Psl provides a barrier to penetration by antibiotics in newly formed biofilms, while protection mediated by Pel has been observed in more established mature biofilms (31). Pel enhances the resistance of Pel-dominant *P. aeruginosa* biofilms to killing by neutrophil-like leukocytes (32), whereas Psl enhances the resistance of Psl-dominant *P. aeruginosa* to opsonophagocytosis by neutrophils (33). Production of Psl and/or Pel enhances the fitness of *P. aeruginosa* in acute models of pulmonary infection (24, 33). The genetic capacity for *P. aeruginosa* to synthesize biofilm exopolysaccharides with redundant roles may allow bacterial cells to adapt to the selective pressures of chronically inflamed airways.

The dependence of *P. aeruginosa* on the structural and functional roles of Pel and Psl in bacterial pathogenesis led us to hypothesize that targeting Pel and Psl to disrupt the biofilm matrix encasing *P. aeruginosa* cells may be an effective therapeutic approach. Within the 7- and 12-gene operons that encode the Pel and Psl biosynthetic machinery, respectively, are genes that are predicted to encode glycoside hydrolases (GHs) (21, 29, 34, 35). The *pelA* gene within the Pel operon encodes the multidomain enzyme PelA, which contains a GH domain (34). The *pslG* gene in the Psl operon is predicted to encode the GH PslG (35). Mass spectrometry enzymatic fingerprinting and nuclear magnetic resonance (NMR) studies using the soluble recombinant GH domain from the PelA protein ($PelA_h$) revealed that $PelA_h$ is an endo-α-1-4-*N*-acetyl-D-galactosaminidase from the GH166 family that cleaves GalNAc-GalNAc linkages within acetylated and deacetylated regions of GalN-GalNAc oligomers (36). Although the enzymatic activity of PslG has not been elucidated in detail, structure-function studies with $PslG_h$ suggest that the enzyme contains a catalytic groove that resembles endo-acting GHs (35). In biofilm disruption studies performed *in vitro*, both soluble recombinant $PelA_h$ and $PslG_h$ hydrolyzed Pel and Psl within Pel- and Psl-dependent biofilms, respectively, and both inhibited biofilm formation and disrupted multicellular aggregates in mature biofilms (32). A combination of $PslG_h$ and $PelA_h$ ($PslG_h$-$PelA_h$) was more effective than either GH alone in disrupting biofilms formed by a matrix-hyperproducing isolate, demonstrating that these GHs are compatible in combination and effective against strains that are genetically capable of upregulating expression of both exopolysaccharide operons (19, 32, 37).

Early studies have suggested that combining $PslG_h$ and $PelA_h$ with antimicrobials is a promising approach to treating *P. aeruginosa* infections. Combining $PslG_h$ or $PelA_h$ with the antimicrobial peptide colistin reduced the viability of Pel- or Psl-dependent *P. aeruginosa* (32), and a combination of $PslG_h$ and $PelA_h$ enhanced the bactericidal activity of the aminoglycosides tobramycin and neomycin and the antimicrobial peptides polymyxin and colistin against Psl-dependent *P. aeruginosa* biofilms (38). $PelA_h$ disruption of mature Pel-dependent biofilms increased the level of killing of *P. aeruginosa* by leukocyte-like cells, suggesting that $PelA_h$ can potentiate the responses of host immune cells (32). In an acute wound model of *P. aeruginosa* infection, $PslG_h$-tobramycin prophylaxis enhanced bacterial clearance (38). Collectively, these studies demonstrate that GHs can potentiate antimicrobial activity and potentially enhance host cell responses against persistent *P. aeruginosa* infection (8, 18, 19, 32).

The biofilm-forming mold *Aspergillus fumigatus* secretes a Pel-like cationic matrix exopolysaccharide galactosaminogalactan (GAG) that is composed of α-1,4-linked D-galactose and partially deacetylated GalNAc residues (39). As with Pel, the GAG biosynthetic pathway

includes a GH enzyme, Ega3 (40–43). Ega3 is an endo-α-1-4-D-galactosaminidase with specificity for GalN-GalN linkages, and treatment with the recombinant hydrolase domain of Ega3 ($Ega3_h$) can disrupt both GAG-dependent fungal and Pel-dependent *P. aeruginosa* biofilms *in vitro* (42). Interestingly, $PelA_h$ also exhibits cross-kingdom antibiofilm activity and can disrupt GAG-dependent *A. fumigatus* biofilms, suggesting the presence of regions of similar composition in the two polymers (44).

Here, the tolerability and anti-*P. aeruginosa* activity of recombinant GH prophylaxis with the combination of $PslG_h$ and $PelA_h$ ($PslG_h$-$PelA_h$) or $PslG_h$ and $Ega3_h$ ($PslG_h$-$Ega3_h$), alone and with antibiotics, were evaluated *in vitro* and *in vivo*. Checkerboard studies were performed to test the activity of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ in combination with the fluoroquinolone ciprofloxacin or the cephalosporin ceftazidime against *P. aeruginosa* biofilms *in vitro*. GH combinations potentiated the antibacterial activity of ciprofloxacin and ceftazidime. Intratracheal administration of single-dose combination $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ was well tolerated by uninfected mice. In a mouse model of acute pulmonary *P. aeruginosa* infection, the administration of single-dose $PslG_h$-$PelA_h$ at the time of infection potentiated the antibacterial activity of ciprofloxacin but not that of ceftazidime, whereas $PslG_h$-$Ega3_h$ failed to potentiate either ciprofloxacin or ceftazidime activity. Administration of GH combinations without antibiotics did not reduce pulmonary bacterial burden and was associated with a trend toward increased hematogenous dissemination of *P. aeruginosa*. Collectively, these findings suggest that specific GH antibiotic combinations may have potential for the prevention and/or treatment of pulmonary of *P. aeruginosa* infections.

RESULTS

GHs enhance antibiotic activity against *P. aeruginosa* biofilms *in vitro*. Previous studies of $PslG_h$ and $PelA_h$ produced in *Escherichia coli* or $Ega3_h$ produced in *Pichia pastoris* ($Ega3_h$) demonstrated that these soluble recombinant GH domains could disrupt Psl-dependent or Pel-dependent *P. aeruginosa* biofilms by degrading Pel or Psl, respectively (32, 35, 42). To extend these findings, dose-response matrix analyses were performed to evaluate the antimicrobial activity of GH-antibiotic combinations against *P. aeruginosa* biofilms. Given the genetic capacity of the *P. aeruginosa* PAO1 strain to produce Psl and Pel, combinations of Psl-degrading $PslG_h$ and either the Pel-degrading $PelA_h$ or $Ega3_h$ were tested for their ability to augment the antimicrobial activity of ciprofloxacin and ceftazidime. In comparison to ciprofloxacin treatment alone, greater biofilm biomass reduction was observed with increasing doses of $PslG_h$-$PelA_h$ beginning at 0.625 μM combined with ciprofloxacin at $\geq$250 μg/mL (Fig. 1). In contrast, additive biomass reduction was observed with concentrations of $PslG_h$-$Ega3_h$ beginning at 0.625 μM combined with ciprofloxacin at concentrations of $\geq$15.65 μg/mL (Fig. 1). These results suggest that, while ciprofloxacin activity against biofilm biomass was potentiated by either $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$, $PslG_h$-$Ega3_h$ exhibited greater potency than $PslG_h$-$PelA_h$. In the case of ceftazidime, $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ combinations beginning at 0.625 μM enhanced the activity of ceftazidime at concentrations of $\geq$3.9 μg/mL (Fig. 1). Together, these observations suggest that $PslG_h$-$PelA_h$ and $PslG_h$-$Ega3_h$ had greater potentiation effects when used in combination with ceftazidime, compared with ciprofloxacin.

Intratracheal GH treatment is well tolerated by mice. Previously, we found that the intratracheal administration of a single dose of up to 500 μg $PelA_h$ or $Ega3_h$ alone was well tolerated by mice (45). To extend this work, the tolerability of intratracheal $PelA_h$ or $Ega3_h$ in combination with $PslG_h$ was tested. BALB/c mice were administered a single intratracheal dose of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ (up to 250/250 μg of each GH), monitored for changes in weight and temperature, and then euthanized 6 days later for measurement of pulmonary injury and inflammation. Treatment with a single dose of up to 250/250 μg of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ was well tolerated by mice, without signs of respiratory distress or death. GH-treated mice exhibited no difference in body weight and temperature, compared with mice treated with buffer alone (see Fig. S1 and S2 in the supplemental material).

To test whether intratracheal GH therapy induced pulmonary injury, pulmonary damage was assessed by measuring lactate dehydrogenase activity in bronchoalveolar lavage (BAL)

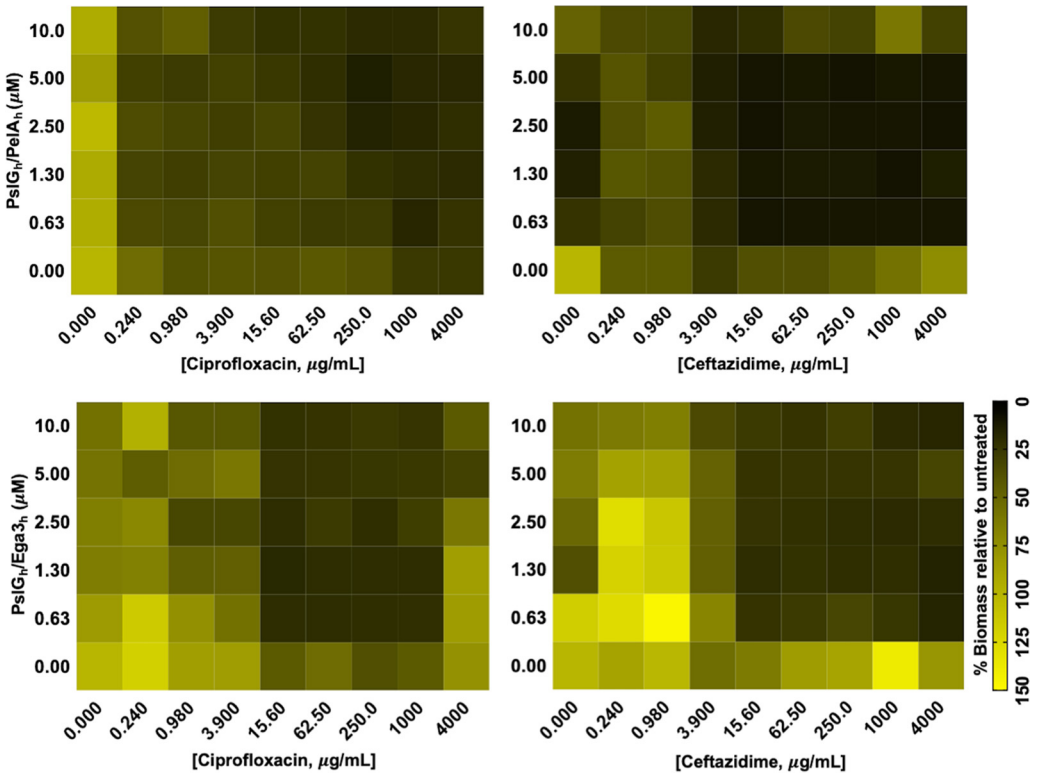

FIG 1 GHs potentiate antibiotic activity against *P. aeruginosa* biofilms *in vitro*. Dose-response matrix analyses of *P. aeruginosa* biofilms cotreated with 2-fold serial dilutions of either PslG$_h$-PelA$_h$ or PslG$_h$-Ega3$_h$ and 4-fold serial dilutions of ceftazidime or ciprofloxacin in a MBEC high-throughput assay as indicated are shown. Biofilm biomass was quantified with crystal violet staining. Colored squares represent the ratio of treated to untreated biofilms of ≥2 independent experiments. Lighter yellow boxes represent high biofilm biomass and darker green boxes represent low biofilm biomass, compared with untreated control wells.

fluid from mouse lungs. No significant increase in lactate dehydrogenase activity was detected in the BAL fluid from mice treated with a 250/250-μg dose of PslG$_h$-PelA$_h$ or PslG$_h$-Ega3$_h$, compared with mice treated with buffer alone (Fig. 2A), suggesting that single-dose GH treatment does not induce pulmonary injury in mice. Consistent with these findings, histological examination of pulmonary tissues did not reveal any differences between GH-treated and buffer-treated mice (see Fig. S3).

To further probe the host response to intratracheal GH treatment, pulmonary leukocytes from GH-treated mice were quantified by flow cytometry (Fig. 2B). PslG$_h$-PelA$_h$ administration to mice at a 250/250-μg dose had no effect on macrophage, eosinophil, and neutrophil numbers. A significant increase in pulmonary lymphocyte numbers was observed following treatment with 250/250 μg of PslG$_h$-PelA$_h$. Treatment with PslG$_h$-Ega3$_h$ was associated with a significant increase in pulmonary eosinophils and a small but significant increase in neutrophils in mice at the 250/250-μg dose. Treatment with PslG$_h$-Ega3$_h$ at the 250/250-μg dose resulted in effects on macrophage and lymphocyte populations similar to those observed with PslG$_h$-PelA$_h$ treatment. Taken together, these data suggest that, while 250/250-μg doses of PslG$_h$-PelA$_h$ and PslG$_h$-Ega3$_h$ are likely nearing the maximal tolerated intratracheal dose, the PslG$_h$-Ega3$_h$ combination induces a greater inflammatory response than PslG$_h$-PelA$_h$ and may be less well tolerated by mice.

Combined GHs have longer pulmonary half-lives than individual GHs alone. To inform the design of efficacy studies, the pulmonary pharmacokinetics of GHs alone and in combination were determined. Mice were given a single intratracheal dose of 500 μg PelA$_h$, PslG$_h$, or Ega3$_h$, and at select time points their lungs were harvested and homogenized in a cocktail of protease inhibitors to prevent degradation of GHs. Lung homogenates were assayed by Western blotting using specific rabbit anti-GH antibodies and quantified by densitometry. PslG$_h$ displayed the longest half-life, i.e., 18 h, compared to PelA$_h$ and Ega3$_h$

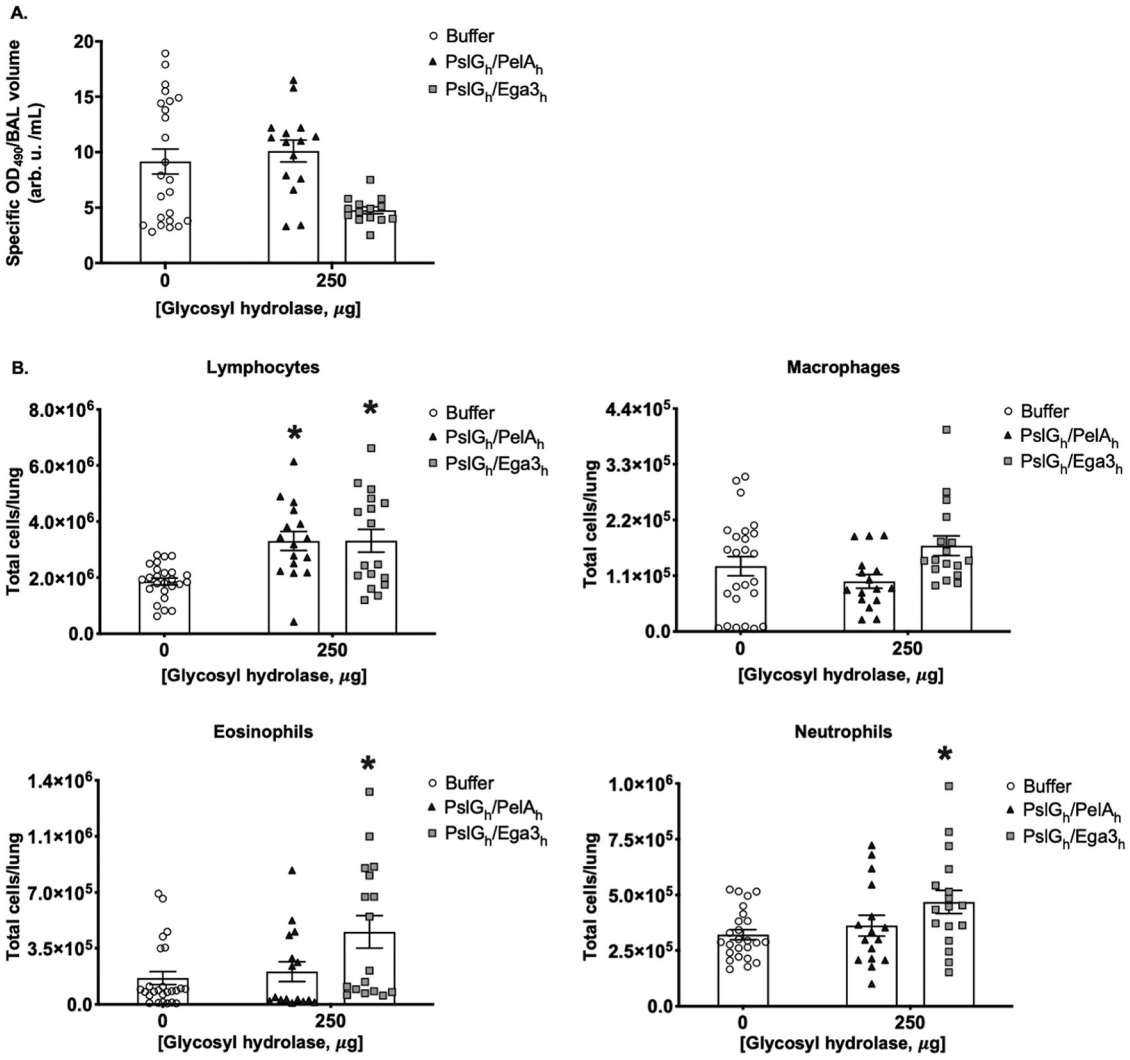

FIG 2 Intratracheal GH therapy does not induce pulmonary damage. Immunocompetent BALB/c mice were intratracheally administered a single dose of 250/250 μg $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$. (A) Lactate dehydrogenase activity was quantified in BAL fluid. (B) Pulmonary leukocyte populations, including lymphocytes, macrophages, eosinophils, and neutrophils, were quantified by flow cytometry 7 days after GH treatment. Bars represent the means $\pm$ standard errors of 4 independent experiments with $\geq$13 mice per group. Asterisks indicate significant differences ($P < 0.0020$), relative to the buffer-treated group and where there are no asterisks no significant differences were observed as compared with the buffer-treated group ($P > 0.7794$), as determined by two-way ANOVA with Dunnett's multiple-comparison test.

with half-lives of 3 h and 9 h, respectively (Fig. 3A). To evaluate the stability of each GH in GH combinations, mice were given a single 250/250-μg intratracheal dose of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$. The half-life of $PslG_h$ in combination therapy was lower than that when administered alone (16 h with $PelA_h$ and 12 h with $Ega3_h$) (Fig. 3B and C). In contrast, compared with GH monotherapy, the half-lives of $PelA_h$ and $Ega3_h$ in combination with $PslG_h$ increased to 5 h from 3 h and to 12 h from 9 h, respectively (Fig. 3B and C). These data suggest that, although the persistence of $PslG_h$ is marginally reduced in combination with either $PelA_h$ or $Ega3_h$, $PelA_h$ and $Ega3_h$ are more stable in combination with $PslG_h$ than when administered alone.

To determine whether the short half-lives of the GHs might be a consequence of proteolytic degradation, 10 μg of $PelA_h$ and/or $PslG_h$ was treated with 100 μg/mL neutrophil elastase *in vitro*. Degradation of $PelA_h$ and $PslG_h$ by neutrophil elastase was detectable as early as 1 h (see Fig. S4). Consistent with the shorter half-life of $PelA_h$ *in vivo*, $PelA_h$ exhibited greater sensitivity to elastase than $PslG_h$ and was no longer detectable after 24 h of coincubation.

Attenuation of bacterial virulence by prophylaxis with GHs in combination with antibiotic treatment is drug specific in an acute model of pulmonary *P. aeruginosa* infection. $PslG_h$-$Ega3_h$ induced a greater inflammatory response than $PslG_h$-$PelA_h$. In previous studies

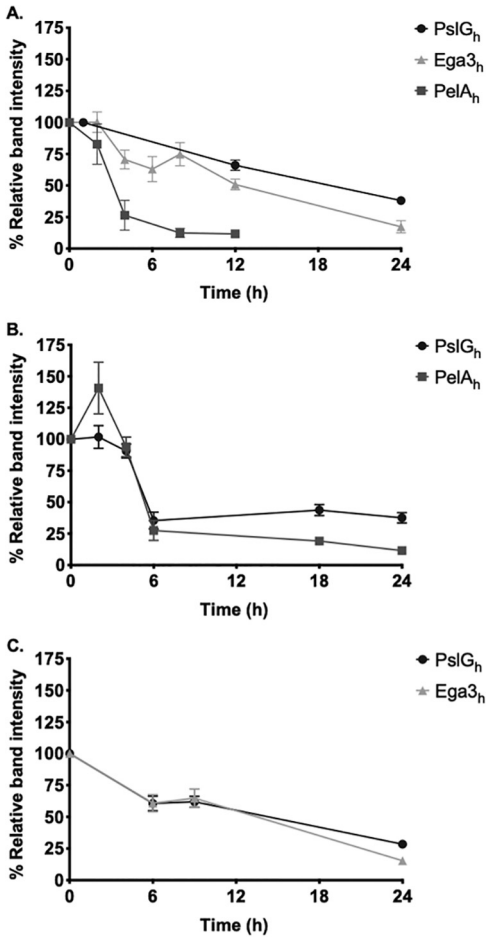

FIG 3 Pulmonary pharmacokinetics of intratracheally administered GHs. Mice were treated intratracheally with a single dose of 500 μg PelA$_h$, Ega3$_h$, or PslG$_h$ (A) or 250/250 μg each of a combination of PslG$_h$ and PelA$_h$ (B) or PslG$_h$ and Ega3$_h$ (C) and then sacrificed at the indicated time points. Lung homogenates were assessed by Western blotting. Dots represent the means ± standard errors of band intensities normalized to total band intensity at 0 h from 2 independent experiments with ≥5 mice per time point.

of Ega3 for therapy of invasive aspergillosis, we observed that administration of a soluble recombinant form of Ega3 produced in a human embryonic kidney (HEK) cell line (Ega3$_h$-HEK) was less inflammatory than Ega3$_h$ produced in yeast (45). Therefore, for subsequent *in vivo* efficacy studies, we used Ega3$_h$-HEK in combination with PslG$_h$ to reduce inflammatory responses that might have been generated against fungal glycosylation patterns or trace amounts of fungal β-glucan in *Pichia*-derived Ega3$_h$ preparations (46, 47).

The antibacterial effects of intratracheal GHs alone and in combination with antibiotics were assessed in a mouse model of acute *P. aeruginosa* infection. Mice were intratracheally infected with *P. aeruginosa* and administered a single dose of 250/250 μg PslG$_h$-PelA$_h$ or PslG$_h$-Ega3$_h$-HEK alone or in combination with a subtherapeutic dose of ciprofloxacin (10 mg/kg every 8 h). Twenty-four hours following infection, pulmonary and blood CFU were quantified as a measure of bacterial burden. Prophylaxis with PslG$_h$-PelA$_h$ or PslG$_h$-Ega3$_h$-HEK alone did not reduce pulmonary bacterial burden, compared with buffer-treated mice (Fig. 4A and B). Blood cultures of infected mice revealed a higher rate of bacteremia in animals that received PslG$_h$-PelA$_h$, in comparison to those that received PslG$_h$-Ega3$_h$-HEK or buffer (30% versus 10% versus 12.5%, respectively) (Tables 1 and 2). These observations are consistent with histological examinations of pulmonary tissues, which revealed increased inflammation in PslG$_h$-PelA$_h$-treated animals, compared with animals that received PslG$_h$-Ega3$_h$-HEK (see Fig. S5). These data suggest that GH therapy alone may increase bacterial dispersion and actually worsen outcomes for pulmonary *P. aeruginosa* infections. In contrast,

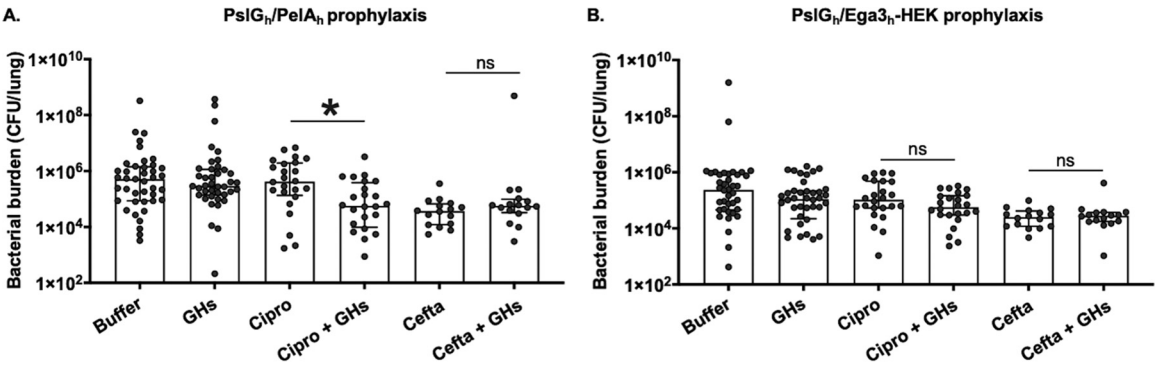

FIG 4 Specific GH-antibiotic combinations reduce bacterial burden in an acute mouse model of pulmonary *P. aeruginosa* infection. Mice were intratracheally infected with 3×10^7 *P. aeruginosa* CFU coadministered with or without PslG$_h$-PelA$_h$ (A) or PslG$_h$-Ega3$_h$-HEK (B) and then treated as indicated with 10 mg/kg ciprofloxacin or 25 mg/kg ceftazidime every 8 h for 1 day. Pulmonary bacterial burden was determined by CFU quantification. Bars represent the medians with interquartile ranges of at least 2 independent experiments with ≥16 mice per group. *, significant difference ($P = 0.0347$) between combinations of GHs and ciprofloxacin and ciprofloxacin alone; ns, no significant difference ($P > 0.9999$), as determined by the Kruskal-Wallis test with Dunn's multiple-comparison test. Cipro, ciprofloxacin; Cefta, ceftazidime.

a significant reduction in pulmonary bacterial burden was observed in infected mice that received PslG$_h$-PelA$_h$-ciprofloxacin in combination, compared to ciprofloxacin or PslG$_h$-PelA$_h$ alone (Fig. 4A). This observation suggests that GH pretreatment potentiated the antimicrobial activity of ciprofloxacin *in vivo* and that combination therapy might be more effective than ciprofloxacin therapy alone (Fig. 4A). Bacteremia was not observed in mice treated with the PslG$_h$-PelA$_h$-ciprofloxacin combination (Table 1), suggesting that coadministration of antibiotics can also protect against GH-mediated bacterial dissemination. The pulmonary bacterial burden of mice treated with PslG$_h$-Ega3$_h$-HEK-ciprofloxacin was not significantly different from that of mice treated with ciprofloxacin alone (Fig. 4B), suggesting that GH potentiation of ciprofloxacin activity is dependent on the combination of GHs.

To determine whether GHs could potentiate another class of *P. aeruginosa* antibiotics *in vivo*, these experiments were repeated with ceftazidime (25 mg/kg) in place of ciprofloxacin. While ceftazidime treatment alone reduced the pulmonary bacterial burden, compared with that of buffer-treated mice, neither PslG$_h$-PelA$_h$ nor PslG$_h$-Ega3$_h$-HEK in combination with ceftazidime exhibited increased antimicrobial activity, compared with ceftazidime alone (Fig. 4A and B). Collectively, these results suggest that the observed GH potentiation of antibiotic activity is dependent on the combination of GHs with antibiotic. Bacteremia was not observed in mice treated with PslG$_h$-Ega3$_h$-HEK-ceftazidime (Table 2), but a similar number of mice that received PslG$_h$-PelA$_h$-ceftazidime developed bacteremia, compared with buffer-treated mice (12.5% versus 12.2%) (Table 1).

DISCUSSION

In this study, GHs PslG$_h$-PelA$_h$ and PslG$_h$-Ega3$_h$ were found to potentiate the antimicrobial activity of the commonly used antibiotics ciprofloxacin and ceftazidime against *P. aeruginosa* biofilms *in vitro*. Single-dose pulmonary administration of PslG$_h$-PelA$_h$ or PslG$_h$-Ega3$_h$ was demonstrated to be well tolerated and induced minimal immune responses in uninfected

TABLE 1 Single-dose combination PslG$_h$-PelA$_h$ prophylaxis disseminates bacteria in an acute mouse model of pulmonary *P. aeruginosa* infection

Treatment	No. of mice with bacteremia	Total no. of mice	% of mice with bacteremia	Odds ratio (95% confidence interval)	Corrected *P*[a]
Buffer	5	41	12.2	3.09 (1.03–8.61)	0.18
PslG$_h$-PelA$_h$	12	40	30		
Ciprofloxacin	0	24	0	NA[b]	NA
Ciprofloxacin + PslG$_h$-PelA$_h$	0	24	0		
Ceftazidime	1	16	6.3	2.143 (0.22–32.86)	>0.99
Ceftazidime + PslG$_h$-PelA$_h$	2	16	12.5		

[a]*P* values were corrected by the Bonferroni method for three comparisons.
[b]NA, not applicable.

TABLE 2 Single-dose combination $PslG_h$-$Ega3_h$-HEK prophylaxis disseminates bacteria in an acute mouse model of pulmonary *P. aeruginosa* infection

Treatment	No. of mice with bacteremia	Total no. of mice	% of mice with bacteremia	Odds ratio (95% confidence interval)	Corrected *P*[a]
Buffer	5	40	12.5	0.78 (0.22–2.94)	>0.99
$PslG_h$-$Ega3_h$-HEK	4	40	10		
Ciprofloxacin	0	24	0	NA[b]	NA
Ciprofloxacin + $PslG_h$-$Ega3_h$-HEK	0	24	0		
Ceftazidime	2	16	12.5	0.000 (0.000–2.12)	>0.99
Ceftazidime + $PslG_h$-$Ega3_h$-HEK	0	16	0		

[a]*P* values were corrected by the Bonferroni method for three comparisons.
[b]NA, not applicable.

mice. While $PelA_h$ and $Ega3_h$ alone have relatively short half-lives *in vivo*, the stability of these GHs increased when coadministered with $PslG_h$. In a pulmonary model of acute *P. aeruginosa* infection, the GH combination $PslG_h$-$PelA_h$ potentiated the antibacterial activity of ciprofloxacin. *In vivo* augmentation of antibiotics was demonstrated to be dependent on GH-antibiotic combinations, as neither $PslG_h$-$PelA_h$-ceftazidime nor $PslG_h$-$Ega3_h$-HEK-ciprofloxacin or -ceftazidime combinations reduced pulmonary bacterial burden more than antibiotic therapy alone. GH prophylaxis alone did not lead to reduced pulmonary bacterial burden and was associated with increased rates of *P. aeruginosa* hematogenous dissemination.

Single-dose GH therapy was well tolerated and resulted in minimal changes in the pulmonary inflammatory response in the absence of infection. Although the $PelA_h$ and $Ega3_h$ GHs exhibited short half-lives when administered as monotherapy, combining these enzymes with $PslG_h$ prolonged their pulmonary half-lives. It is unlikely that the stability of the GHs is a reflection of quantitative effects, as equimolar amounts of the GHs were administered in these experiments. Previously, we demonstrated that, in a leukopenic mouse model, the half-life of $Ega3_h$ was similar to that in the current study; however, the half-life of $PelA_h$ was longer (45) than in immunocompetent mice. Together, these findings suggest that, while some GHs may be less stable in mice with an intact immune system, combining GHs increases GH stability, possibly through the formation of a complex in which the GHs are less susceptible to proteolytic degradation. While repeated topical $PslG_h$ dosing was previously reported to be well tolerated in a chronic wound model of *P. aeruginosa* infection in immunocompetent mice (38, 48), the effects of repeated pulmonary GH administrations remain to be evaluated, and more detailed immunotoxicity studies of multiple combined GH doses and anti-GH antibody responses are required to advance these agents toward use in clinical trials.

Different combinations of GH enzymes with antibiotics exhibited differences in efficacy. In this study, $PslG_h$-$PelA_h$ prophylaxis potentiated the activity of ciprofloxacin but not that of ceftazidime, and neither $PslG_h$-$Ega3_h$-HEK prophylaxis in combination with ciprofloxacin nor that with ceftazidime resulted in reduction of the pulmonary bacterial burden. These observations contrast with the *in vitro* checkerboard potentiation experiments, in which combinations of either $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$-HEK with either ciprofloxacin or ceftazidime resulted in biofilm biomass reduction. These observations suggest that *in vivo* bacterial susceptibility to the therapy and biofilm disruption by GHs may be influenced by host determinants (48, 49). While these observations suggest that specific GH-antibiotic combinations exhibit unique efficacy, the observation that $PslG_h$-$PelA_h$ monotherapy was associated with the highest rates of bacterial dissemination suggests that the specific ability of $PslG_h$-$PelA_h$ to enhance ciprofloxacin activity may simply reflect greater antibiofilm activity. In support of this hypothesis, pulmonary histopathology demonstrated that $PslG_h$-$PelA_h$ monotherapy was uniquely associated with an increased inflammatory response, consistent with augmented bacterial dispersion following exopolysaccharide degradation.

The mechanism by which $PslG_h$-$PelA_h$ potentiates the antibacterial activity of ciprofloxacin remains to be determined. Pharmacokinetic analyses revealed that $PslG_h$ and $PelA_h$ have relatively short half-lives *in vivo*. Previously, we demonstrated that single-dose administration of the recombinant form of the fungal GH Sph3, $Sph3_h$, potentiated the antifungal posaconazole in reducing fungal burden in a model of *A. fumigatus* pulmonary infection,

despite the fact that this enzyme exhibited a short pulmonary half-life relative to predicted timing of GAG exopolysaccharide expression *in vivo* (45, 50). Furthermore, a catalytically inactive variant of $Sph3_h$, $D166A_{AC}$, exhibited activity levels similar to those of enzymatically active $Sph3_h$, suggesting that cleavage of GAG was dispensable for the activity of this enzyme *in vivo*. Possible explanations for these observations include direct activation of pulmonary immune cells by GH enzymes prior to significant amounts of GAG production (45) or direct lectin-like interactions of catalytically inactive $Sph3_h$ with GAG that interfere with the virulence properties of this polymer (32). In contrast to the fungal system, Psl and Pel secretion by *P. aeruginosa* occurs well within the period during which $PslG_h$ and $PelA_h$ enzymes are present (11, 30). The higher rates of bacterial dissemination observed in mice that received $PslG_h$-$PelA_h$ alone suggest that at least one of the Pel or Psl polymers is present during GH exposure and is cleaved by these enzymes. However, the inability of GH therapy alone to decrease the pulmonary bacterial burden suggests that exopolysaccharide cleavage alone is insufficient to attenuate bacterial virulence during pulmonary infection. These findings are consistent with observations in previous studies demonstrating that treatment with $PslG_h$ or other GHs, such as α-amylase and β-cellulase, alone was insufficient to reduce the bacterial burden in a wound model of acute *P. aeruginosa* infection (38, 48).

Disruption of the extracellular matrix by biofilm-targeting therapies has the potential to lead to bacterial dissemination and worsening of clinical outcomes. Approximately one-third of mice that received $PslG_h$-$PelA_h$ exhibited bacteremia, although the addition of ciprofloxacin was effective in preventing dissemination. Bacterial dissemination has been reported *in vivo* with manipulation of *P. aeruginosa* biofilm regulatory pathways (51), as well as enzymatic treatment of *P. aeruginosa* biofilms with the GHs α-amylase and β-cellulase (48, 52). Interestingly, GH-mediated dissemination was not observed during treatment of experimental pulmonary *A. fumigatus* infection (45). These differences in outcomes between bacterial and mold infections may stem from their unique morphologies and capacity for motility. Molds grow as multicellular filamentous hyphae that intertwine and thus are unlikely to passively disperse and disseminate like unicellular bacteria (53). Furthermore, unlike *P. aeruginosa* and many other bacteria with motility appendages, including type IV pili or flagella, *A. fumigatus* hyphae are not motile and therefore are unable to actively disseminate from the site of infection (54–58). The results of a study using a nonmotile bacterial species, *Staphylococcus aureus*, support this hypothesis, as bacterial dissemination was not observed following α-amylase- and β-cellulase-mediated disruption of bacterial biofilms in a chronic wound model (52). In the study here, degradation of exopolysaccharides might have facilitated *P. aeruginosa* dissemination mediated by flagellar swarming (59) or possibly through intraleukocyte transport (the so-called Trojan horse mechanism) (60–62). Collectively, these data suggest that the result of biofilm-directed therapies may partly depend on the morphology and capacity for motility of an organism.

The results of this study demonstrate that intratracheal administration of $PslG_h$-$PelA_h$ in combination with ciprofloxacin can improve the outcomes of *P. aeruginosa* infection in a mouse model of acute bacterial infection. While this proof-of-concept preclinical study provides evidence for the efficacy of combination GH-antibiotic prophylaxis in acute *P. aeruginosa* pulmonary infection, further studies are required to understand the therapeutic utility of GH therapy. The results of these studies lay the foundation for future work to elucidate the molecular mechanisms by which combinations of antibiotics with these enzymes and other forms of recombinant microbial GHs limit bacterial growth *in vivo*. Developing easily administrable aerosolizable formulations of GH enzymes will be critical to advancing their clinical development, including expanded multidose immunotoxicity, GH efficacy in established infection models, and pharmacokinetic studies.

MATERIALS AND METHODS

Strain and growth conditions. The *P. aeruginosa* PAO1 strain was grown on Luria-Bertani (LB) agar plates overnight at 37°C, from −80°C stocks. For experiments, *P. aeruginosa* was cultured overnight and subcultured in LB broth (BD Difco Miller LB) to mid-log phase at 37°C. Bacteria were resuspended to 3×10^8 CFU/mL in phosphate-buffered saline (PBS) for intratracheal infections.

Recombinant GH expression and purification. His-tagged $PelA_h$ and $PslG_h$ were expressed in ClearColi cells grown in Terrific Broth (BioShop) or autoinduction medium with 50 μg/mL kanamycin (Bio Basic) as

described previously (32, 35, 41). Bacterial cultures in Terrific Broth were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Bio Basic) when the cells reached an optical density at 600 nm (OD_{600}) of 1.2 to 1.4. The cells were incubated postinduction overnight at 18°C with shaking at 200 rpm before being harvested by centrifugation at 5,000 $\times$ *g* for 30 min at 4°C. Both proteins were purified by fast protein liquid chromatography using Ni-nitrilotriacetic acid columns (GE Healthcare) followed by buffer exchange, and the purity of the protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (35).

Expression of $Ega3_h$ in the PichiaPink system was optimized as described previously (42). To generate a glycosylated form of Ega3 that would mimic mammalian glycosylation patterns (63), $Ega3_h$-HEK was expressed in a cell line of HEK cells (HEK293) as described previously (45). The culture supernatants containing the secreted proteins were harvested at 6 days (45). His-tagged protein was purified from the supernatant by affinity chromatography followed by gel filtration using a HiLoad 16/600 Superdex 200 preparation-grade column (GE Healthcare).

Checkerboard studies. Bacterial biofilm susceptibility to killing by antibiotics and GH potentiation of antibiotics were quantified in a minimum biofilm eradication concentration (MBEC) high-throughput assay, modified from that described previously (64). Bacterial biofilms were grown in MBEC/Calgary Biofilm Device plates (Innovotech) for 20 h at 37°C. The biofilm-coated peg lids were rinsed with water, placed in new base plates containing serial 2-fold and 4-fold dilutions of 4,000-μg/mL concentrations of the antibiotics ciprofloxacin or ceftazidime and 10 μM concentrations of the GH combinations $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$-Pp, respectively, in a two-dimensional checkerboard, and incubated overnight at 37°C. To quantify biofilm biomass, peg lids were separated from base plates and stained with 150 μL 0.1% crystal violet (BioShop) for 10 min. Crystal violet was solubilized with 150 μL ethanol (BioShop) for 10 min, and the absorbance of 150 μL of the solution was measured at 595 nm (SpectraMax M2).

Mice. Six- to 8-week-old BALB/c female mice (Charles River Laboratories Inc, Senneville, QC, Canada and Kingston, NY, USA) were used for animal studies. Mice were anaesthetized with 4% isoflurane prior to intratracheal infection and prophylaxis/treatment with GHs/drugs. Body weight was measured using a top-loading balance, and surface body temperature was measured on the abdomen using a digital infrared thermometer. Moribund animals were euthanized by isoflurane and CO_2 overdose.

Tolerability studies. Immunocompetent mice were treated intratracheally with a single dose of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ at 10/10 μg or 250/250 μg in 50 μL PBS or with PBS alone. Mice were monitored daily for 6 days for signs of illness, and body weights and temperatures were recorded. For histopathology studies, lungs from immunocompetent mice were inflated with 10% buffered formalin (Thermo Fisher Scientific) and fixed in formalin as described previously (65). Lungs were then embedded in paraffin, and 4-μm-thick sections were stained with hematoxylin and eosin. Scanned sections (Aperio scanner; Leica Biosystems) were analyzed with QuPath v0.1.2 software (66).

Pulmonary leukocyte quantification. Immunocompetent mice were treated intratracheally with a single dose of the GHs $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ at 10/10 μg or 250/250 μg in 50 μL PBS or with PBS alone. Seven days after treatment, mice were euthanized, and their lungs were washed in PBS, minced in RPMI 1640 medium (Wisent) containing 5% (vol/vol) fetal bovine serum (FBS) (Wisent), and then digested with 150 U/mL collagenase (Sigma) (65, 67). The resulting suspension was passed through a 70-μm cell strainer and treated with ammonium-chloride-potassium (ACK) buffer (Gibco). Approximately 1 $\times$ 10^6 leukocytes were resuspended in a fixable viability dye (eBioscience) and washed, and their Fc receptors were blocked by unlabeled anti-CD16/32 antibodies (FcBlock; BD Pharmingen) as described previously (45, 65). Cell surface components were then stained with fluorescently labeled antibodies (BD Biosciences) as described previously (45, 65). Leukocytes were washed, fixed with paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and then resuspended in PBS as described previously (45, 65). Data were acquired on an LSR Fortessa flow cytometer with FACSDiva software (BD Biosciences) and analyzed with FlowJo software v10 (FlowJo, LLC). Immune cell subsets were defined as described previously (45, 65). Total cell populations were calculated using CountBright absolute counting beads (Invitrogen).

Densitometry and antibody production. SDS-PAGE and Western blotting techniques were used to assess pulmonary GH pharmacokinetics. Rabbit polyclonal antibodies specific for each of the GHs were produced by Cedarlane (Burlington, Canada) as described previously (35). Mice were treated intratracheally with a single dose of 500 μg of each GH or 250/250 μg of each GH for a combination of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$, and then they were euthanized and their lungs were harvested at the indicated time points. Lungs were homogenized in a cocktail of protease inhibitors (Roche), and pulmonary GH concentrations were quantified by Western blotting with rabbit anti-GH antibodies. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad) binding was detected with a chemiluminescent substrate (Thermo Fisher Scientific). The half-lives of the GHs were determined by densitometric analysis using ImageJ software. Band intensity at each time point was normalized to the intensity at the zero-hour time point. The half-life was determined as the time to 50% relative intensity of the bands, compared to the zero-hour time point.

SDS-PAGE and Western blotting techniques were used to confirm the sensitivity of GHs to proteolytic degradation *in vitro*. $PelA_h$ or $PslG_h$ (10 μg) was treated with 100 μg/mL neutrophil elastase. Proteolysis was stopped with SDS loading buffer, and GHs were detected by Western blotting using rabbit anti-GH antibodies as described above.

Pulmonary damage. Mice were treated intratracheally with a single dose of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$ at 10/10 μg or 250/250 μg in 50 μL PBS or with PBS alone. Seven days after treatment, mice were euthanized and their lungs were lavaged twice with 1 mL PBS as described previously (45, 65). Lactate dehydrogenase activity was measured in the pooled BAL fluid by commercial assay (CytoTox 96 nonradioactive cytotoxicity assay; Promega), according to the manufacturer's instructions.

Mouse model of acute pulmonary *P. aeruginosa* infection. Mice were intratracheally infected with a 50-μL suspension of 1.5×10^7 *P. aeruginosa* CFU in PBS and treated with either a single dose of $PslG_h$-$PelA_h$ or $PslG_h$-$Ega3_h$-HEK at 250/250 μg or PBS alone. For antibiotic combination studies, mice were treated by intraperitoneal injection with 10 mg/kg ciprofloxacin or subcutaneous injection with 25 mg/kg ceftazidime or 0.1 N NaOH in PBS reconstitution solution every 8 h beginning 4 h after infection (see Fig. S6 in the supplemental material). One day after infection, mice were euthanized and their blood and lungs were harvested for bacterial burden assessment, modified from the method described previously (68).

(i) Pulmonary bacterial burden. Lungs were harvested and homogenized in 5 mL PBS with a Polytron tissue homogenizer. Homogenates were serially diluted at a 1:5 ratio and spot plated on *Pseudomonas* isolation agar plates (Sigma-Aldrich). Pulmonary bacterial burden was determined by colony quantification.

(ii) Blood bacterial burden. Blood was diluted in PBS at a 1:10 ratio, serially diluted at a 1:5 ratio, and spot plated on *Pseudomonas* isolation agar (Sigma-Aldrich). Blood bacterial burden was determined by colony quantification.

Statistical analysis. Data are presented and statistical significance was calculated as indicated. All graphs were generated and statistical analyses were performed with GraphPad Prism v9.0.0 software. Significant differences between values were compared by two-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test, the Kruskal-Wallis test with Dunn's multiple-comparison test, and Fischer's exact test with Bonferroni correction for multiple comparisons. Odds ratios were calculated by the Baptista-Pike method.

Ethics statement. All procedures involving mice were approved by the animal care committees of the Institutional Animal Care and Use Committee (IACUC) of the McGill University Health Centre (protocol number 2016-7808) and the Animal Care and Use Review Office (ACURO) of the U.S. Army Medical Research and Materiel Command (USAMRMC).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.4 MB.

ACKNOWLEDGMENTS

D.R., I.L., S.G., P.S., N.C.B., and P.B. designed plasmid constructs and synthesized and purified recombinant GHs. D.R. performed and analyzed checkerboard studies. M.L. performed *in vivo* assays for tolerability, processed samples, and acquired and analyzed monitoring and immunophenotyping data. R.C. performed *in vivo* assays for GH efficacy studies. R.C. processed samples and acquired bacterial burden data. D.R., I.L., S.G., and P.S. performed and acquired and analyzed data for pharmacokinetic studies. H.O., D.R., F.N.G., P.L.H., and D.C.S. interpreted the tolerability, pharmacokinetic, and efficacy data. H.O. assembled figures and performed statistical analyses on tolerability, pharmacokinetic, and GH efficacy data. H.O. wrote the original draft of the manuscript. H.O., D.R., P.L.H., and D.C.S. reviewed and edited the final draft of the manuscript. P.L.H. and D.C.S. conceptualized the study and acquired funding for the project.

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