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TITLE: Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens Pseudomonas aeruginosa and Aspergillus Fumigatus

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14. ABSTRACT
Major accomplishments this period:
1. All *in vitro* work (Major Task 1) was completed and demonstrated synergy between glycoside hydrolases (GHs) and a wide range of antimicrobials.
2. We completed studies of the tolerability and pharmacokinetics of GH enzymes (Major Task 2 and 3). GH variants (Major Task 4) were developed for Sph3, Ega3 and PelA, through chemical and genetic modifications to address issues of tolerability (Ega3) and short half-life *in vivo* (PelA, Sph3). Variants are currently being evaluated, but results to date indicate that these new variants exhibit similar enzymatic activity and pharmacokinetics but increased resistance to some commercial protease and decreased immunogenicity.
3. In an acute model of murine invasive aspergillosis, we completed studies of GH monotherapy and combination therapy, demonstrating that all GH treatment regimens resulted in significantly reduced pulmonary fungal burden (Major Task 5). Intra-tracheal Sph3 was also found to extend survival of immunosuppressed mice challenged with *Aspergillus* and further studies evaluating the effects of other GHs on survival are ongoing. Studies of GH therapy in the chronic model of invasive aspergillosis have been initiated (Major Task 6).
4. The effect of PslG and PslG/PelA on bacterial burden in presence or absence of antibiotics, have been performed in a model of *P. aeruginosa* pulmonary infection (Major Task 5). The results of these studies indicate that GH therapy alone may increase bacterial dissemination. However, GH therapy enhances the activity of the ciprofloxacin, and the combination protects against bacterial dissemination (Major Task 7, initiated ahead of schedule). Studies to probe the mechanisms by which GH therapy in the absence of antibiotics increases *Pseudomonas* dissemination are ongoing.

15. SUBJECT TERMS
Pseudomonas aeruginosa; *Aspergillus fumigatus*; virulence; biofilm; exopolysaccharide; glycoside hydrolase; antimicrobial potentiation.

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1. INTRODUCTION:

The bacteria *Pseudomonas aeruginosa* (PA) and fungus *Aspergillus fumigatus* (AF) are common causes of pulmonary disease in immunocompromised patients. These infections are associated with high morbidity and mortality, underscoring the urgent need for new effective therapies for these conditions. During pulmonary infection, both pathogens form biofilms, which enhance resistance to antimicrobials and immune defenses. Biofilm formation is dependent on the synthesis of matrix exopolysaccharides – Pel, Psl for PA, galactosaminogalactan (GAG) for AF. Exopolysaccharide-deficient mutants of PA and AF are less virulent in animal models, suggesting that these glycans are promising therapeutic targets. We have identified and produced recombinant versions of microbial glycoside hydrolase (GH) enzymes, PelA, and PslG from PA and Ega3, and Sph3 from AF, which degrade exopolysaccharides and disrupt biofilms *in vitro*. We hypothesize that treatment with these GHs alone or in combination with antimicrobials will be well tolerated and improve outcomes in experimental pulmonary infection with PA and AF. We therefore propose the following studies: (1) To characterize the ability of recombinant GH enzymes to enhance the activity of antimicrobial agents against PA and AF *in vitro* (2) Perform tolerability and pharmacokinetic studies of intratracheal therapy with recombinant GH in mice. (3) Evaluate the efficacy of GH therapy alone and in combination with antimicrobials for the treatment of acute and chronic PA and AF infection mouse models. In the short term these studies will provide solid preliminary data for the preclinical evaluation of pulmonary GH therapy against two of the most important opportunistic pulmonary pathogens. In the long-term, these results can also be extended to develop GH therapy pulmonary infections with other exopolysaccharide-producing pathogens such as *Staphylococcus*, *Acinetobacter* and *Mucor* species.

KEYWORDS:

Pseudomonas aeruginosa; *Aspergillus fumigatus*; virulence; biofilm; exopolysaccharide; glycoside hydrolase; antimicrobial potentiation.

2. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Please note this is partnered award with research being performed at McGill University (PI: Sheppard) and The Hospital for Sick Children (PI: Howell). The material presented herein pertains to both awards. Award numbers: W81XWH-16-1-0283 and W81XWH-16-1-0284

MAJOR GOALS FOR YEAR 2:

Specific Aim 1: To characterize the ability of the hydrolases to enhance the activity of antimicrobial agents in vitro.

Major Task 1: Identify antimicrobials that are potentiated in the presence of candidate hydrolases.

Subtask 3: Test PslG/PelA and PslG/Ega3 in checkerboard combinations with antibiotics against *P. aeruginosa* biofilms (Months 1-9). (PI: Howell)

Subtask 4: Test candidate hydrolase-antimicrobial combinations in an in vitro fluid biofilm culture model system (Months 6-12). (PI: Howell and Sheppard)

Milestone: *Identification of hydrolase-antimicrobial combinations that synergize against* A. fumigatus *and* P. aeruginosa*. These antimicrobials will be prioritized and used in Aim3.*

Specific Aim 2: To perform preliminary tolerability and pharmacokinetic studies of candidate hydrolases in vivo.

Major Task 2: Test candidate hydrolases for toxicity in vivo.

Milestone achieved*: Obtain animal use approvals.*

Milestone achieved*: Evaluation of pulmonary toxicity of candidate hydrolase regimens.*

Major Task 3: Pharmacokinetic studies of candidate hydrolases

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 - 5 (Months 6-12). (PI: Howell)

Subtask 2: Test pharmacokinetics of hydrolases (Sph3, Ega3, PelA and PslG/PelA and PslG / Ega3 combinations) in immunocompetent mice [25 mice per group (5 per time point) X 5 hydrolase therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice] (Months 6-12). (PI: Sheppard)

Subtask 3: Test pharmacokinetics of hydrolases (Sph3, Ega3, PelA and PslG/PelA and PslG/ Ega3 combinations) in immunocompromised mice [25 mice per group (5 per time point) X 5 hydrolase therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice (Months 6-12). (PI: Sheppard)

Subtask 4: Determine concentrations of candidate hydrolases and their combinations using animal tissue samples. (Months 6-12). (PI: Howell)

Milestone*: Evaluation of pharmacokinetics of candidate hydrolase regimens. (Predicted completion: month 12*)

Major Task 4 (as required): Development of candidate hydrolase variants

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 5 (Months 9-15). (PI: Howell)

Subtask 2: Test protease resistance of candidate hydrolases against *A. fumigatus* isolates in the epithelial cell damage assay using Western-blot analysis, and mass spectrometry (as required). Cell lines used: A549 epithelial cells [ATCC] (Months 9-15). (PI: Sheppard-Howell)

Subtask 3: Test protease resistance of candidate hydrolases against *P. aeruginosa* isolates in the epithelial cell damage assay using Western-blot analysis, and mass spectrometry (as required). Cell lines used: A549 epithelial cells [ATCC] (Months 9-15). (PI: Howell)

Subtask 4: Test chemical modification as a means to increase the stability of candidate hydrolases (as required). (Months 9-21). (PI: Sheppard-Howell)

Subtask 5: Test site-specific modification as a means to increase the stability of candidate hydrolases (as required). (Months 9-21). (PI: Sheppard-Howell)

Milestone*: Development of stable candidate hydrolases (Predicted completion: month 21).*

Specific Aim 3: To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections *in vivo*.

Major Task 5: Test hydrolases for activity in animal models of acute disease

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2 – 5 (Months 9-15). (PI: Howell)

Subtask 2: Determine the effects of hydrolases (Sph3, Ega3, PelA) on survival of immunosuppressed mice infected with *A. fumigatus* [10 mice per group X 8 experimental groups X 3 hydrolase regimens, AND 3 mice for histopathology all performed in duplicate = 624 mice] (Months 13-30). (PI: Sheppard)

Subtask 3: Determine the effects of hydrolases (Sph3, Ega3, PelA) on fungal burden of mice infected with *A. fumigatus*. [10 mice per group X 8 experimental groups X 3 hydrolase regimens, all performed in duplicate = 480 mice] (Months 13-30). (PI: Sheppard)

Subtask 4: Determine the effects of hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of mice infected with three strains of *P. aeruginosa* [10 mice per group X 7 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points AND 3 mice for histopathology X 7 groups X 2 hydrolase regimens X 3 strains at a single time point all performed in duplicate = 1932 mice] (Months 13-30) (PI: Sheppard).

***Milestone**: Efficacy of candidate hydrolase regimens in the treatment of acute infection with A. fumigatus and P. aeruginosa confirmed (Predicted completion: month 30).*

<u>Major Task 6</u>: Test hydrolases for activity in animal models of chronic disease

Subtask 1: Express and purify Ega3, PelA and PslG for subtasks 2 -3 (Months 13-30) (PI: Howell).

Subtask 2: Determine the effects of candidate hydrolases (Ega3) on fungal burden of immunocompetent mice chronically infected with *A. fumigatus* [10 mice per group X 6 experimental groups X 1 hydrolase regimens X 2 time points AND 3 mice for histopathology at a single time point X 6 groups X 1 hydrolase regimens performed in duplicate = 276 mice] (Months 13-30) (PI: Sheppard)

Subtask 3: Determine the effects of candidate hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of immunocompetent mice chronically infected with *P. aeruginosa*. [10 mice per group X 6 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points AND 3 mice for histopathology X 6 groups X 2 hydrolase regimens X 3 strains at a single time point all performed in duplicate = 1656 mice] (Months 18-30) (PI: Sheppard)

***Milestone**: Efficacy of candidate hydrolase regimens in the treatment of chronic infection with A. fumigatus and P. aeruginosa confirmed. (Predicted completion: month 30)*

<u>Major Task 7</u>: Test hydrolases for synergy with antimicrobials **(*studies initiated early)**

Subtask 1: Express and purify Sph3, Ega3, PelA and PslG for subtasks 2,3. (Months 24-36) (PI: Howell).

Subtask 2: Determine the effects of hydrolase (Sph3, Ega3, PelA)-antifungal combinations on fungal burden of mice infected with *A. fumigatus* [10 mice per group X 6 experimental groups X 1 hydrolase regimens X 2 time points X 2 antifungals AND 3 mice for histopathology X 6 groups X 1 hydrolase regimens X 2 antifungals at a single time point all performed in duplicate = 552 mice] (Months 24-36) (PI: Sheppard).

Subtask 3: Determine the effects of hydrolase (PslG/PelA and PslG/Ega3)-antibiotic combinations on burden of mice infected with *P. aeruginosa* [10 mice per group X 5 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points X 2 antibiotics AND 3 mice for histopathology X 5 groups X 2 hydrolase regimens X 2 antibiotics at a single time point all performed in duplicate = 460 mice] (Months 24-36) (PI: Sheppard).

***Milestone**: Shown proof-of-concept for candidate hydrolases for use in treatment of A. fumigatus and P. aeruginosa. Ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials (Predicted completion: month 36).*

What was accomplished under these goals?

Accomplishments for Year 2:

Specific Aim 1: To characterize the ability of the hydrolases to enhance the activity of antimicrobial agents *in vitro*.

MAJOR TASK 1 Identify antimicrobials that are potentiated in the presence of candidate hydrolases.

Previous accomplishments in the Major Task:
During Year 1, our teams built the molecular tools to allow glycoside hydrolase (GH) production in bacteria (ClearColi® *E. coli*) or in yeast (PichiaPink® *P. pastoris*). Then, we optimized the culture conditions. Finally, we were able to efficiently produce the toxin-free recombinant GHs that were required for both *in vivo* and *in vitro* experiments. (**Subtask 1**: completed)

Using these enzymes, we were able to demonstrate that GHs potentiate the activity of antifungal drugs of three classes (polyenes, azoles and echinocandins) against *A. fumigatus* biofilms. (**Subtask 2**: completed). The most effective GH/antifungal combinations tested were posaconazole/Sph3 and caspofungin/PelA. These combinations will be therefore prioritized for further *in vivo* studies (Major Task 7).

Subtask 3: **Test PslG/PelA and PslG/Ega3 in checkerboard combinations with antibiotics against** *P. aeruginosa* **biofilms**. Dr Howell's lab. SOW Time Period: Months 1-9. Completion level = 100%.

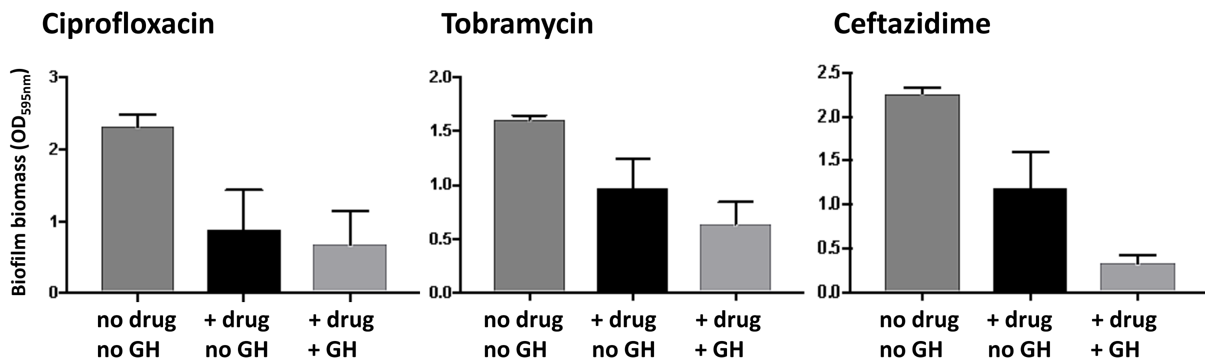
Previous accomplishments in the Subtask:
During Year 1, unexpected difficulties forced us to explore and optimize two different *in vitro* systems for the production of biofilms to enable us to test their susceptibility to antibiotics: the Calgary Biofilm Device (CBD) and a standing biofilm tube assay. With the help of our collaborator, Dr Parsek of University of Washington, and using a recently published protocol (Habash *et al*., Antimicrob. Agents Chemother. 2017 Oct 24), we successfully addressed these difficulties, and we were able to obtain reproducible results using the CBD.

During preliminary studies, using a 1 μM fixed concentration of a combination of PelA/PslG on 24 h grown biofilms of *P. aeruginosa* PA01, we were able to demonstrate a significant potentiation of four assayed antibiotics: tobramycin, ciprofloxacin, colistin and neomycin. although the effect of colistin was not statistically significant. This potentiation was seen using an increasing concentration of hydrolases combined with antibiotics as well as using a fixed concentration of hydrolases added to a gradient of antibiotics.

Accomplishments during Year 2:
Methodology: MBEC plates (CBD) were used for the assay at 37°C with the strain *P. aeruginosa* PA01. Biofilm biomass was evaluated using crystal violet staining. All checkerboard assays have been performed in triplicate. One-way ANOVA and multiple comparison test were used for statistical analyses on GraphPad Prism.

Results: As expected, we were able to reproduce the results observed with tobramycin (an antibiotic of the aminoglycoside class), with ciprofloxacin (of the fluoroquinolone class) and with ceftazidime (an antibiotic of the β-lactam class). Combining GH with antibiotics resulted in a greater reduction in biofilm biomass than when either the hydrolase or antibiotic were used separately. This potentiation was observed both when adding an increasing concentration of hydrolases to a fixed concentration of antibiotics and when adding a fixed concentration of hydrolases to a gradient of antibiotics. For simplicity, Figure 1 summarizes the optimal potentiation observed, at a single concentration of the antibiotic (15.6 μg/ml) as well as of the hydrolase combination (1.25 μM each).

PslG/PelA = 0 or 1.25 μM each + antibiotic = 15 μg/mL



PslG/Ega3 = 0 or 1.25 μM each + antibiotic = 15 μg/mL

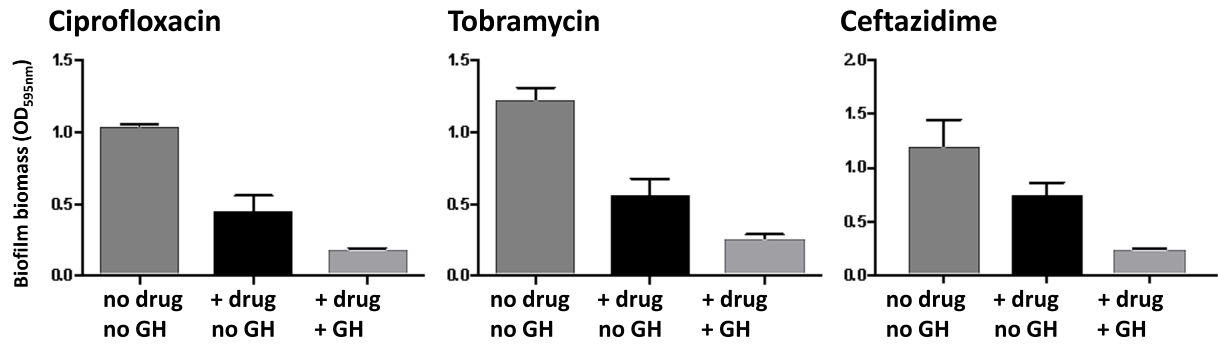


Figure 1. Potentiation of antibiotics efficiency against *P. aeruginosa* by addition of tobramycin, ciprofloxacin or ceftazidime at a concentration of 15 μg/mL with GH combinations PslG/PelA or PslG/Ega3 at a concentration of 1.25 μM each. Charts indicate the result of triplicated experiments.

Major Task 1 Conclusions: for Major Task 7, given these results and our experience with dosing ciprofloxacin in mice, this antibiotic was selected for use in our first combination studies *in vivo*. As ceftazidime showed the greatest degree of enhancement of activity, this agent will be used for our second antibiotic combination.

Subtask 4: Test candidate hydrolase-antimicrobial combinations in an *in vitro* fluid biofilm culture model system. Dr Howell and Sheppard's labs. SOW Time Period: Months 1-6. Completion level = will not be pursued further *A. fumigatus*as flow conditions are not present during airway infection, we will not pursue further fluid biofilm studies, as explained in section "5. CHANGES/PROBLEMS".

☑ **Milestone Achieved: Identification of hydrolase-antimicrobial combinations that synergize against** ***A. fumigatus*** **and** ***P. aeruginosa*****.**

Specific Aim 2: To perform preliminary tolerability and pharmacokinetic studies of candidate hydrolases *in vivo*.

MAJOR TASK 2: Test candidate hydrolases for toxicity *in vivo.*

Previous accomplishments in the Major Task:
IACUC protocol number #2016-7808 for animal use were submitted and approved by the McGill University IACUC on 01-JUN-2016, then by the USAMRMC Animal Care and Use Review Office (ACURO) on 18 Nov 2016. (**Subtask 1**: completed)
BALB/c mice have been used in all of the experiments described below.

☑ **Milestone Achieved: Obtain Animal use approval.**

Routine production of recombinant GHs in our labs has become efficient enough to meet our needs in quantity and quality for subsequent subtasks. (**Subtask 2**: completed.)

Subtask 3: **Test toxicity of pulmonary administration of GH combinations** (PslG/PelA and PslG/Ega3 combinations) **in immunocompetent mice**. Dr Sheppard's lab. SOW Time Period: Months 6-12. Completion level = 100%.

Previous accomplishments in the Subtask:
In the 2017 annual report, we presented the results of duplicate experiments for both monotherapy and GH combination therapy. Data showed no significant change in pulmonary injury or leukocyte recruitment during GH monotherapy or during GH combination therapy, with GH concentrations up to 500 μg / mouse. A single experiment with Ega3 demonstrated high levels of leukocyte recruitment at concentrations as low as 1 μg. Multiple repeat experiments with Ega3 have not reproduced this finding, and we believe this was due to a contaminated preparation. The data presented here excludes this outlier experiment.

Accomplishments during Year 2:
Although not directly outlined in the SOW, we have completed a full assessment of monotherapy with each of the GH enzymes. Compiled data for GH monotherapy are presented below. These findings suggest that the GHs, alone or in combination, are well tolerated by immunocompetent mice.

Methodology. Recombinant GH enzymes alone were administered intratracheally at doses ranging from 1-500 μg. Mice were monitored for 7 days, then sacrificed to investigate the sign of pulmonary injury or inflammation. Pulmonary leukocyte recruitment was assessed by flow-cytometry performed on tissue digests and by histopathology.

Results. Data presented here are the combined results of all GH monotherapy experiments (PslG, PelA, Sph3, Ega3). We observed:

- No significant lung injury was detected by measurement of lactate dehydrogenase (LDH) release in the bronchoalveolar lavage (BAL) fluid (Figure 2)
- Significant pulmonary leukocyte recruitment was only observed with intratracheal doses of all GHs at doses of 100 μg and above (Figure 3).
- Intratracheal GH therapy did not result in a significant increase in total serum IgE levels
- For Ega3 alone, a significant increase in pulmonary eosinophil recruitment was observed started at 10 μg (Figure 3), and lymphocyte recruitment was noted at higher doses of GHs at doses of 100 and 500 μg. Serum IgE levels were not significantly increased after Ega3 therapy (Figure 4).

Ega3 is the only GH that is produced in *Pichia pastoris* rather than *E. coli*. Since *P. pastoris* is a eukaryote yeast, we hypothesized that these findings may result from host reaction to fungal N-glycans. Therefore, we explored the effects of: (i) enzymatically deglycosylating Ega3 post-production; and (ii) expressing the protein in mammalian cells to alter glycosylation (task accomplished and described further, in section “Major Task 4”).

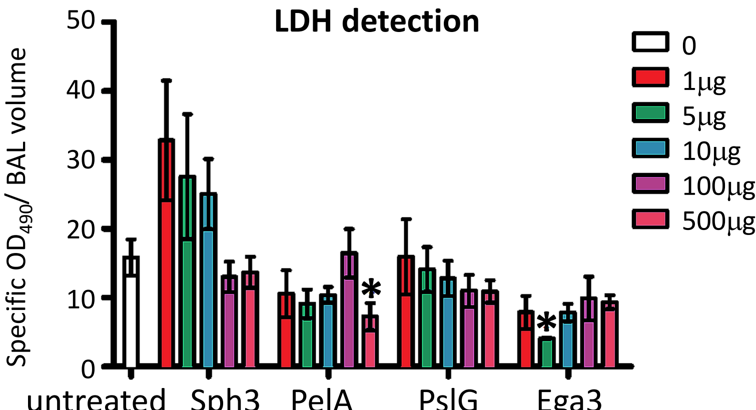


Figure 2. Quantification of lactate dehydrogenase (LDH) activity in the bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH. Two independent experiments were performed

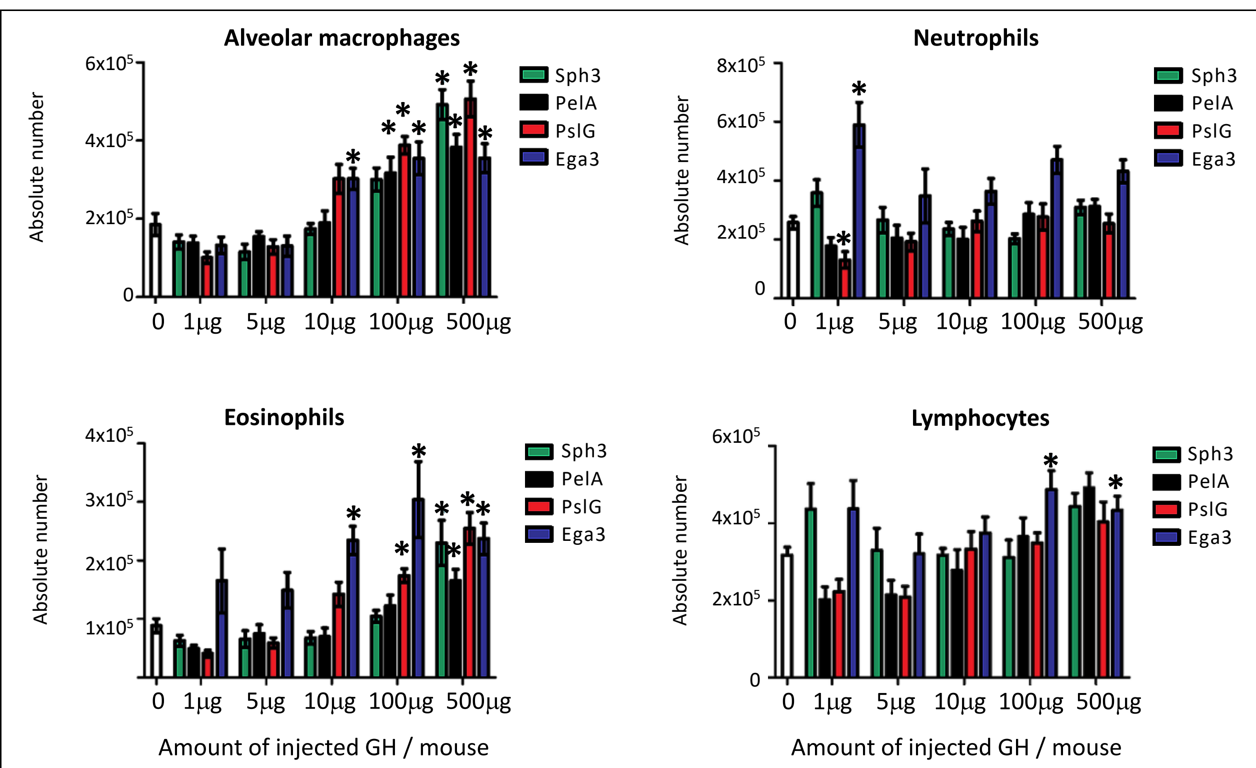


Figure 3. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung digests. Lungs were harvested 7 days following treatment with a single dose of the indicated GH. * indicates a significant difference with the untreated group with $p \leq 0.05$ in ANOVA test. Two independent experiments were performed.

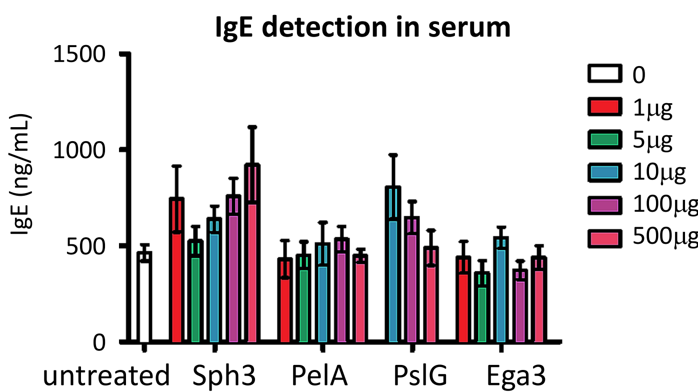


Figure 4. Absolute quantification of IgE in mouse sera as measured by commercial EIA (BD optEIA mouse IgE). Serum was collected 7 days following treatment the indicated GH. No significant difference with the untreated group with $p \leq 0.05$ in ANOVA test was observed. Two independent experiments were performed.

Subtask 4: **Test tolerability of pulmonary administration of hydrolase combinations** (PslG/PelA and PslG/Ega3) **in immunosuppressed mice**. Dr Sheppard's lab. SOW Time Period: Months 6-12. Completion level = 100%.

Previous accomplishments in the Subtask:
In the previous annual report, we presented the results of duplicate experiments with high dose of GH combination (250 μg of each GH / mouse). Data showed no significant change in injury or leukocyte recruitment.

Accomplishments during Year 2:
Replicates were performed with low doses of GH and compiled data are presented below

Methodology. Mice were rendered neutropenic by injection of 250 mg/kg cortisone subcutaneously and 250 mg/kg cyclophosphamide intraperitoneally 2 days prior to infection, followed by 250 mg/kg cortisone subcutaneously plus 200 mg/kg cyclophosphamide intraperitoneally 3 days post-infection. At day 0, combinations of recombinant GH enzymes were administered intratracheally at doses ranging from 1 to 250 μg of each GH as a mixture 1:1. Mice were monitored for 7 days, then sacrificed to investigate signs of pulmonary injury or inflammation. Pulmonary leukocyte recruitment was assessed by flow-cytometry performed on lung digests and by histopathology.

Results. Data presented hereafter confirmed that, similarly to what was observed in immunocompetent mice, no significant toxicity was detected in immunosuppressed mice. Injection of GHs up to 500 μg / mouse did not induce lung injury as per lactate dehydrogenase (LDH) release in the BAL fluid (Figure 5) or histological examination (data not shown). No significant leukoctyte recruitment was observed in response to combination GH therapy (Figure 6).

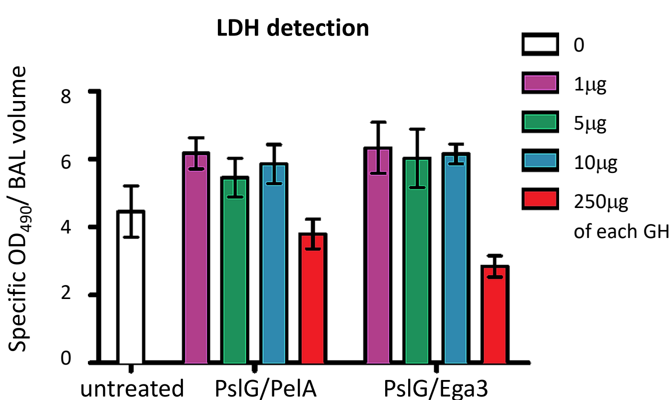


Figure 5. Quantification of lactate dehydrogenase (LDH) activity in the bronchoalveolar fluid harvested from mice 7 days following treatment with a single dose of the indicated GH combination. Two independent experiments were performed.

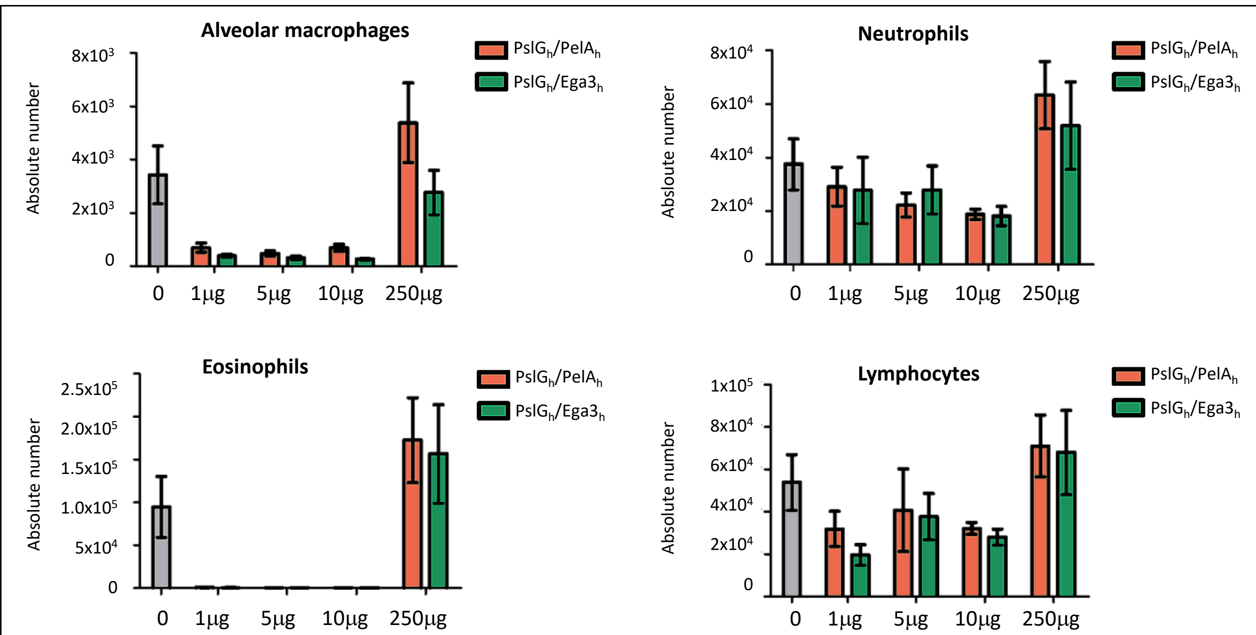


Figure 6. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung digests. Lungs were harvested 7 days following treatment with a single dose of the indicated GH combination. Two independent experiments were performed.

Major Task 2 Conclusions: All GH combinations tested were well tolerated for the host, both in immunocompetent or immunosuppressed mice with the exception of Ega3 produced in *Pichia*. We have successfully mitigated this issue by producing Ega3 in HEK cells as detailed in Major Task 4 below. As predicted, immunosuppression reduced the pulmonary recruitment of eosinophils and other leukocytes following GH therapy.

☑ **Milestone Achieved: Evaluation of pulmonary toxicity of candidate hydrolase regimens.**

MAJOR TASK 3: Pharmacokinetic studies of candidate hydrolases

Previous accomplishments in this Major Task:
GH production in our lab is now routine and we can produce sufficient protein, both in quantity and quality (endotoxin-free enzyme, verified activity) to meet all requirements for the other subtasks of Major Task 3. (**Subtask 1**: completed.)

Polyclonal antibodies that recognize Sph3, PelA or PslG were produced by our group prior to the start of this project. The necessity to change Ega3 production from *E. coli* to *P. pastoris* resulted in a delay in preparation of an anti-Ega3 antibody and the completion of following Subtasks 2, 3 and 4. A polyclonal anti-Ega3 antibody was successfully produced and characterized during the first year of this grant, allowing us to initiate these studies.

We validated the antibody specificity for detection of recombinant GHs in pulmonary tissue by intratracheal injection of each GH in mice followed by Western-blot analysis of lung homogenates samples.

Subtask 2: Test pharmacokinetics of GHs (Sph3, Ega3, PelA and PslG/PelA and PslG/Ega3 combinations) in immunocompetent mice [25 mice per group (5 per time point) X 5 GH therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice]. Dr Howell and Sheppard's labs. SOW Time Period: Months 6-12. Completion level = 85%. Combined with **Subtask 4**: Determine concentrations of candidate hydrolases and their combinations using animal tissue samples.

Previous accomplishments in this Subtask:
During Year 1, we were able to detect all GHs in immunocompetent mice lung homogenates by Western blot analysis and to quantify their half-life in single experiment. Replicates were scheduled for Year 2.

Accomplishments during Year 2:
Methodology: GHs were administered intratracheally to BALB/C female mice at a dose of 500 µg for single GHs or 250 µg of each GH if they were injected as a combination. At time points ranging from 1 to 48 h, the mice were sacrificed and their lungs harvested and homogenized in phosphate buffer enriched with a cocktail of protease inhibitors. Lung homogenates were analyzed by Western-blotting using rabbit anti-GH antibodies. A goat-anti-rabbit-HRP antibody was used as the secondary antibody; the HRP signal was detected and analyzed by densitometry when the percent band intensity was normalized to the total band intensity at the 0h time point for each mouse. For each time-point, a minimum of 4 mice was used in each experiment 4. Following the results of the first experiment, earlier or later time-points were added as required to encompass the half-life of each GH.

Results: All data were derived from densitometry analysis of Western-blots and are presented graphically in Figure 7. Year 2 data confirmed our initial study results, *i.e.* the half-life of the GHs ranged from less than 1h (Sph3) to more than 18h (PslG).

Surprisingly, when dosed in combination with PslG, the half-life of PelA was significantly increased from 3h to 16h, while half-life of PslG was not altered. However, these studies lacked time points between 4 and 24 hours, and thus the estimation of 16h is not precise. Should we find that this GH combination is particularly effective in our *in vivo* studies we will repeat these experiments with more time points to define the half-life of PelA more precisely.

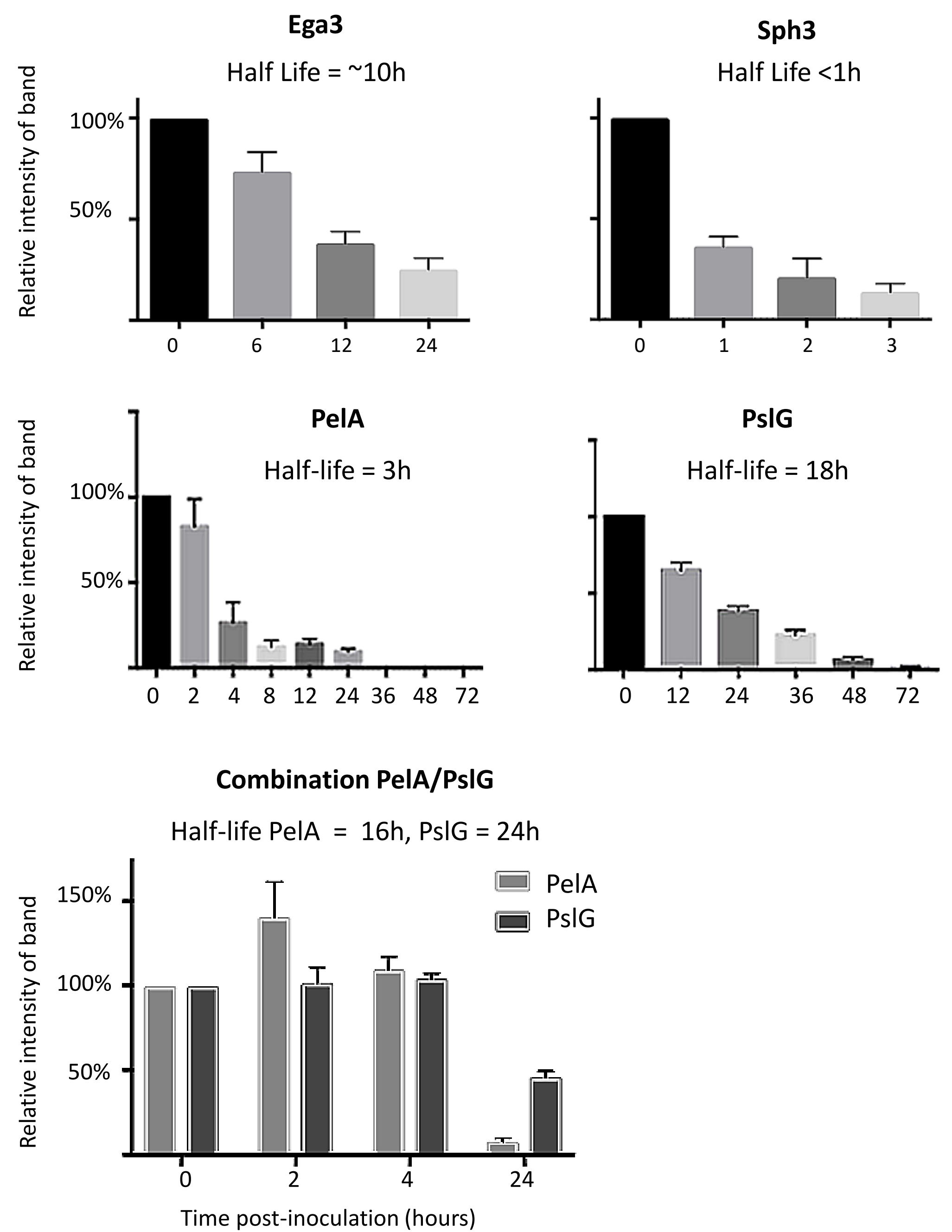


Figure 7. Determination of pulmonary GH pharmacokinetics by Western-blot analysis. GHs were intratracheally administered to immunocompetent mice as a single 500 μg dose of pure GH or as a mix of 250 μg of mixed GHs; each bar represents the average densitometry value form a minimum of 4 mice per experiment, with 2 independent experiments performed. Results are corrected for background signal obtained from untreated mouse lung samples and was normalized to the total band intensity at the 0h time point for each mouse.

Subtask 3: Test pharmacokinetics of hydrolases (Sph3, Ega3, PelA and PslG/PelA and PslG/Ega3 combinations) in immunocompromised mice [25 mice per group (5 per time point) X 5 hydrolase therapies; 1 group of 25 untreated mice. All performed in duplicate = 300 mice. Dr Howell and Sheppard's labs SOW Time Period: Months 6-12. Completion level = 85%. Combined with **Subtask 4**: Determine concentrations of candidate hydrolases and their combinations using animal tissue samples.

Previous accomplishments in this Subtask:
During Year 1, no pharmacokinetics were done in immunocompromised mice.

Accomplishments during Year 2:
Methodology: Two days prior to treatment, mice were rendered neutropenic by injection of 250 mg/kg cortisone subcutaneously and 250 mg/kg cyclophosphamide intraperitoneally. GH injection, lung treatment and pharmacokinetics were performed as described above (Subtask 2).

Results: All data were derived from densitometry analysis of Western-blots. For clarity we show only the graphic interpretation of these data (Figure 8). The half-life of PelA, PslG and Ega3, was extended in immunocompromised mice as compared to immunocompetent mice, suggesting leukocyte-dependent degradation of GHs. The half-life of Sph3 was less than 1h in both models. In this model, the increase in half-life of PelA when combined with PslG that was observed in immunocompetent mice was not observed.

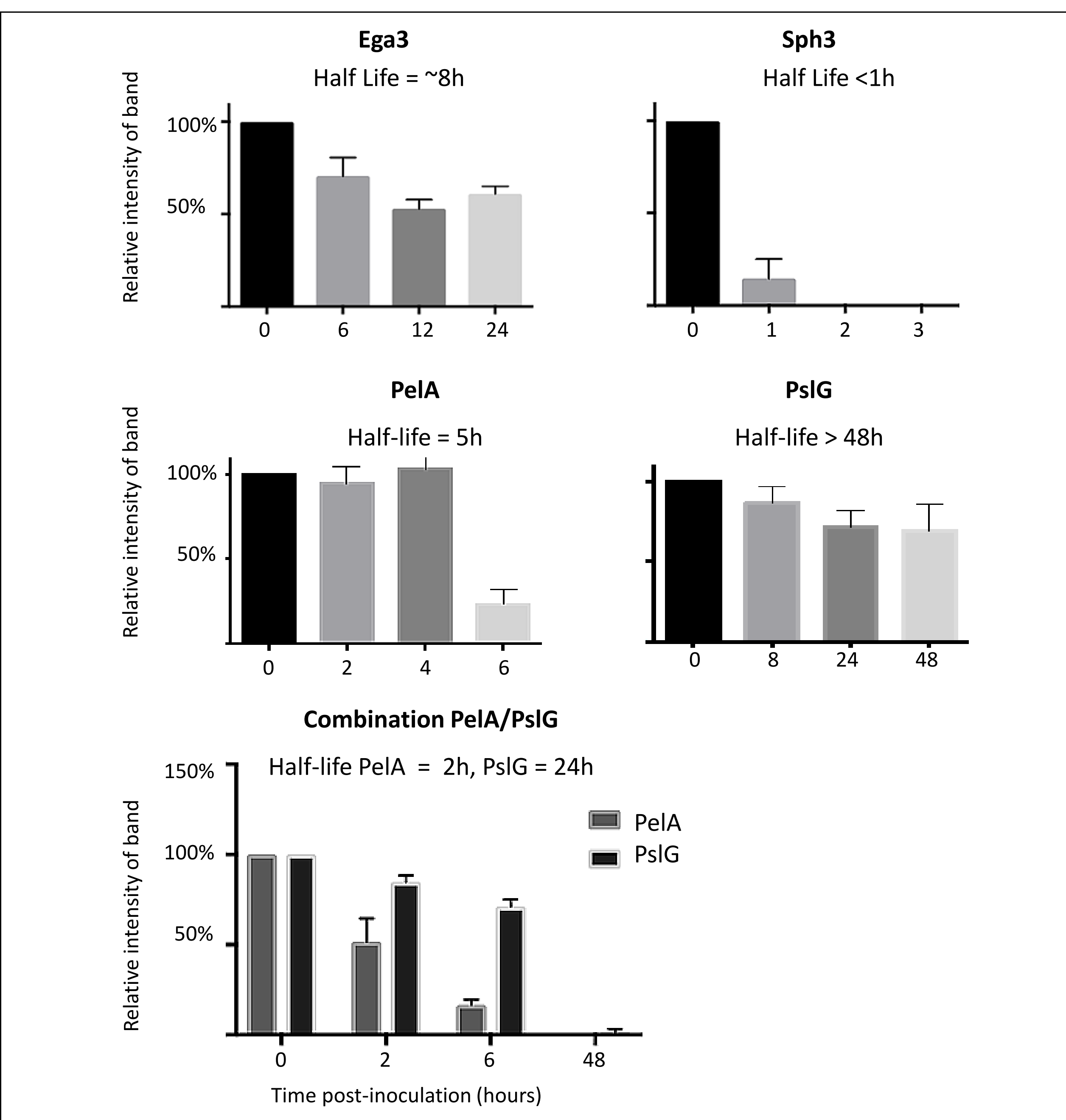


Figure 8. Determination of pulmonary GH pharmacokinetics by Western-blot analysis. GHs were intratracheally administered to immunosuppressed mice as a single 500 μg dose; each bar represents the average densitometry value form a minimum of 4 mice per experiment, with 2 independent experiments performed. Results are corrected for background signal obtained from untreated mouse lung samples.

The table below (Table 1) summarizes the pharmacokinetics experiments described in **Subtasks 2, 3 and 4**.

Hydrolase	Ega3	Sph3	PelA	PslG	PslG/PelA		PslG/Ega3	
					PelA	PslG	Ega3	PslG
Estimated half-life in immunocompetent mice (Subtask 2)	8h	<1h	3h	18h	16h	24h	in progress	in progress
Estimated half-life in immunocompromised mice (Subtask 3)	10h	<1h	5h	>48h	2h	24h	in progress	in progress

Table 1. Estimation of half-life of GHs in mouse lung after intratracheal injection of 500 µg of GH.

❑ **Milestone to achieve: to evaluate the pharmacokinetics of candidate hydrolase regimens.**

<u>**Major Task 4**</u> (as required): **Development of candidate hydrolase variants.**

<u>Background:</u>
Our ongoing pharmacokinetic studies suggest that modification of some of the GHs may be required to increase their half-life. For instance, Sph3 showed a half-life of less than 1h in both mouse models (Table 1). In contrast, the *in vivo* half-life of PslG is near 18 h in immunocompetent mice, and greater than 48 h in immunosuppressed mice; therefore modification of this GH to increase its pharmacokinetic profile seems not required at present.

Subtask 1: Production of required GHs - completed (see above)

Subtask 2 and 3: Test protease resistance of candidate hydrolases against *A. fumigatus* **and** *P. aeruginosa* **isolates** in the epithelial cell damage assay using Western-blot analysis, and mass spectrometry. Cell lines used: A549 epithelial cells [ATCC]. Dr Howell and Sheppard's labs. Months 9-15. Completion level = 30%

<u>Rationale</u>:
Chemical modifications of Sph3 and PelA, and modification of the Ega3 glycosylation and construct length are ongoing (see Subtask 4 and 5). We will wait for these modified GHs to be available before extensively testing protease resistance of candidate variants. In preparation for these experiments we have developed an *in vitro* protease resistance assay to test the susceptibility of the GH's to proteases from diverse sources: commercial (proteinase K) or biological (BAL fluids and lung homogenates lung homogenate from mice, or fungal culture supernatants).

<u>Accomplishments</u>:

Methodology: BAL fluid and lung homogenate were collected from uninfected unexposed mice, or from Sph3 exposed lungs. For Sph3 exposed mice, a single of dose of 500 µg of Sph3 was administered intratracheally, the mice were sacrificed after 1 day, and their lungs were harvested and resuspended in phosphate buffer saline (PBS) without protease inhibitors. To generate *A.*

fumigatus culture supernatants (Af293 culture supernatant) 10^6 conidia were grown for 3 days in 100 mL of Brian synthetic medium, then culture supernatants were harvested and filter sterilized.

For the protease resistance assay, 1 µg of GH was incubated at 37 °C with 60 µl of either BAL fluid, lung homogenate, fungal culture supernatant or PBS (control). Samples were collected at the indicated time points. GH degradation was stopped by adding SDS-PAGE buffer and samples were analyzed by SDS-PAGE and Western-blotting.

Results: In our first experiment, we observed that both Sph3 and PelA were resistant to the proteases present in BAL fluids and lung homogenates obtained from non-infected mice for up to 24 h (Figure 9). Surprisingly, GHs appeared to degrade faster in PBS buffer than in BAL fluids and lung homogenates.

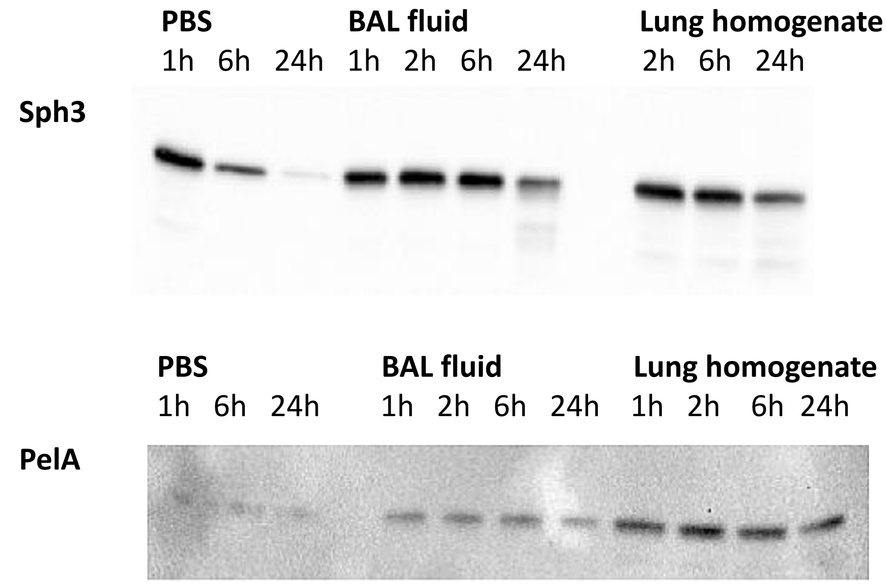


Figure 9: Western-blot monitoring of Sph3 and PelA persistence in PBS, BAL fluids and lung homogenates obtained from non-infected mice.

As these results did not reproduce our findings of short half-lives of these GHs, we hypothesized that GH therapy could induce the production of pulmonary proteases. We therefore tested the effects of lung homogenates and BAL fluid from mice pre-exposed to the GH Sph3 on the stability of Sph3 and PelA.

Results: Pre-exposing healthy mice to Sph3 did not increase the susceptibility of Sph3 and PelA to proteases present in the lung homogenate (Figure 10): GH degradation was observed only after 24h of incubation, similar to what was observed with non-GH exposed lungs (Figure 9). The proteins were sensitive to Af293 supernatant contained proteases, as well as commercial enzymes (proteinase K and elastase).

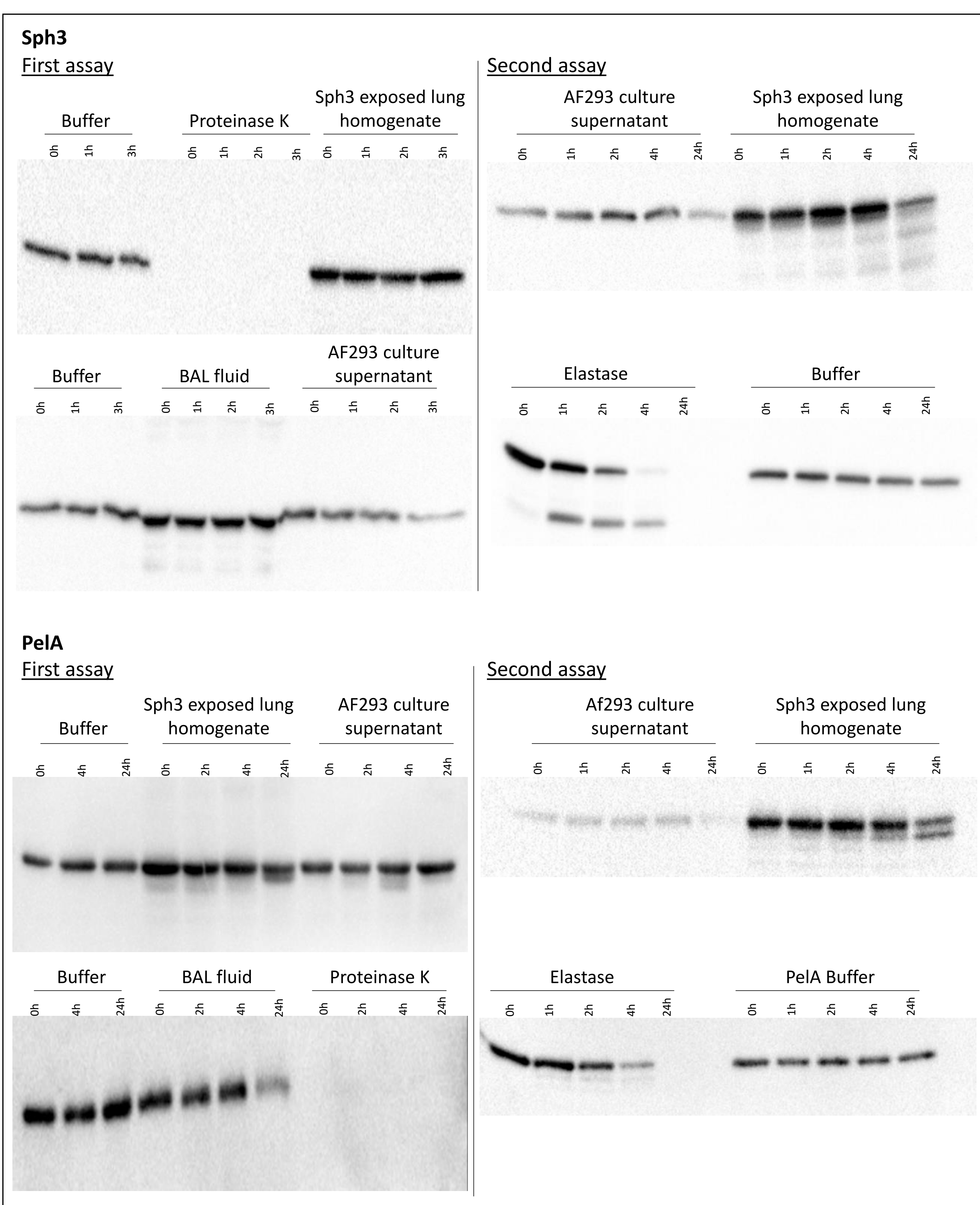


Figure 10: Western-blot monitoring of Sph3 and PelA persistence in BAL fluids and lung homogenates obtained from non-infected mice.

Subtask 4: Test chemical modification as a means to increase the stability of candidate hydrolases. Dr Howell's lab. Months 9-21. Completion level = 35%

Background: Our pharmacokinetic studies demonstrated that PelA and Sph3 have short half-lives (less than 6h for PelA, and less than 2h for Sph3), in both immunocompetent and immunocompromised mice. In an attempt to improve the stability of these proteins *in vivo*, our first strategy has been to chemically link polyethyleneglycol (PEG) to the protein. This is one of the most common techniques used to improve stability without affecting the activity of an enzyme.

Accomplishments in this subtask:
Methodology: A PEGylation commercial kit (EZ-Link™ NHS-PEG4 Biotinylation Kit) has allowed us to selectively PEGylate the lysine residues of the PelA and Sph3 GHs, following manufacturer's instructions. Then, we performed an *in vitro* protease assay to test the ability of different proteases (trypsin, chemotrypsin, elastase, and papain) to degrade the PEGylated protein compared to the native protein.

Results: Following PEGylation of both PelA and Sph3, we observed a molecular weight shift of the corresponding band in SDS-PAGE (Figure 11). This shift confirmed the chemical modification of the GHs. Biofilm disruption assays showed that PEGylated PelA conserved their enzymatic activity through the PEGylation process (Figure 12). Activity assays are in process for PEGylated Sph3. Although PEGylation process of PelA and Sph3 was successful, no protection of either modified GH was observed when exposed to trypsin or chymotrypsin (Figure 11). In contrast, PEGylation protected both GHs against elastase for up to 1h and papain at least 2h.

Further assays on PEGylated GHs will be placed on-hold until we have developed an assay with more relevant proteases than commercial ones, *i.e.* lung and culture supernatant proteases.

Figure 11. Coomassie stained SDS PAGE gel of native and PEGylated PelA and Sph3. When indicated, an incubation of 60 min or 120 min was performed with the appropriate protease.

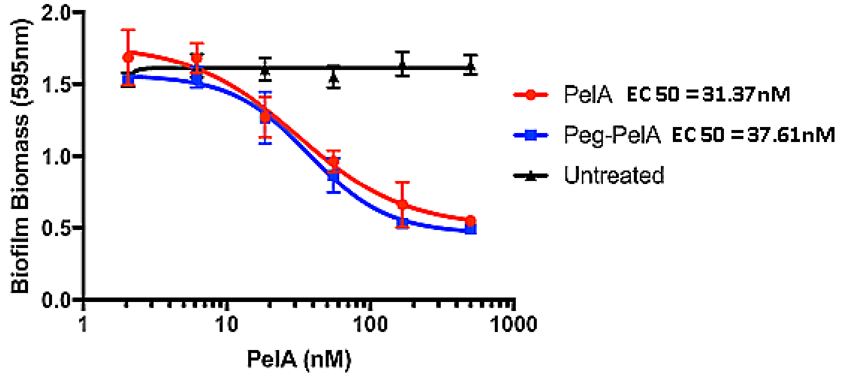


Figure 12: Biofilm disruption assay against *P. aeruginosa* PA14 biofilms was performed with native and PEGylated PelA to test the effects of PEGylation on GH activity.

Subtask 5: Test site-specific modification as a means to increase the stability of candidate hydrolases. Dr Howell's lab. Months 9-21. Completion level = 100%

Previous accomplishments in the Subtask:

Production of Ega3 had to be performed in an organism different from *E. coli* in order to meet our needs in terms of GH amount and purity (endotoxin-free especially) and because, unlike the other GHs, Ega3 was insoluble when produced by *E. coli*. We chose *P. pastoris*. The recombinant protein was soluble and endotoxin-free, but as *P. pastoris* is a eukaryotic organism, the resulting protein exhibited fungal-specific glycosylation. We hypothesized this glycosylation pattern was responsible for the higher levels pulmonary eosinophil recruitment seen with this protein. Therefore we have explored the possibility of expressing Ega3 in a human embryonic kidney cells line (HEK293) to generate a glycosylated GH which is soluble but which mimics mammalian-like glycosylation patterns.

Accomplishments during Year 2:

1- Production of Ega3 in a mammalian cell line and validation.

Methodology: The His-tagged *ega3* coding sequence was cloned into a pHL-sec vector under the control of a mammalian promoter. Two different constructs of Ega3 were cloned into the vector: $Ega3_{46\text{-}318}$ (referred to as Ega3-DSS) and $Ega3_{38\text{-}318}$ (referred to as Ega3-68) (Figure 13).

Figure 13: Predicted domain boundaries of *ega3* from *A. fumigatus*. Grey arrows indicate that two start sites for the constructs being expressed in Freestyle 293 F and Freestyle 293 S cells. (TM = transmembrane domain).

The plasmids were then transfected into mammalian cell lines (HEK-293 "Freestyle" F and HEK-293 "Freestyle" S) for expression trials using FectoPro transfection reagent. The culture supernatants containing the secreted proteins were harvested at 3 and 6 days to check for protein yield. It was established that incubation of the cells for 6 days post-transfection was essential for optimal protein expression. The cells were spun down and the secreted protein from the supernatant was purified by affinity chromatography, using a His-Trap Column.

Results: In the absence of glycosylation, the expected size for Ega3 and Ega3–DSS are 32 kDa, and for Ega3–68 (loss of the transmembrane domain) is 30 kDa. Analysis under denaturing conditions (SDS-PAGE) showed a shift in migration for all Ega3 proteins, confirming that Ega3 was glycosylated when produced in a yeast system (*P. pastoris*) as well as in a mammal system (HEK293) (Figure 14).

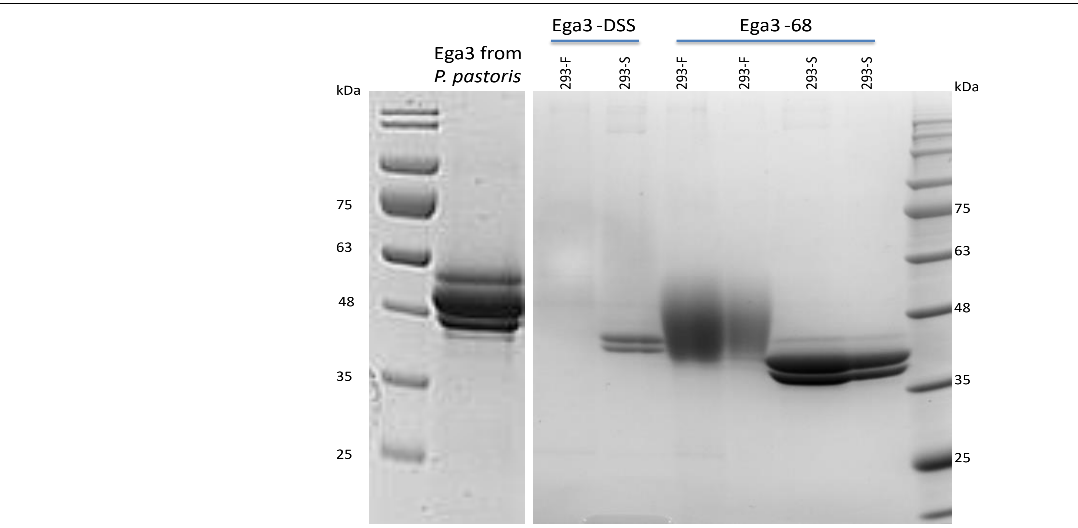


Figure 14. Coomassie stained SDS-PAGE of Ega3 produced in *P. pastoris* or in freestyle HEK expression systems (Ega3-DSS and Ega3-68), following purification.

Therefore, the protocol of expression of these proteins was optimized in the two cell lines to reach yields similar to the *P. pastoris* expression. Ega3-68, the GH without the transmembrane domain, showed better expression and yielded 7 mg GH / 200 ml of cell culture. Importantly, Ega3-68 demonstrated an enzymatic activity similar to the Ega3 enzyme produced in *P. pastoris* (Figure 15 and Table 2). This construct has hence been chosen for further functional studies.

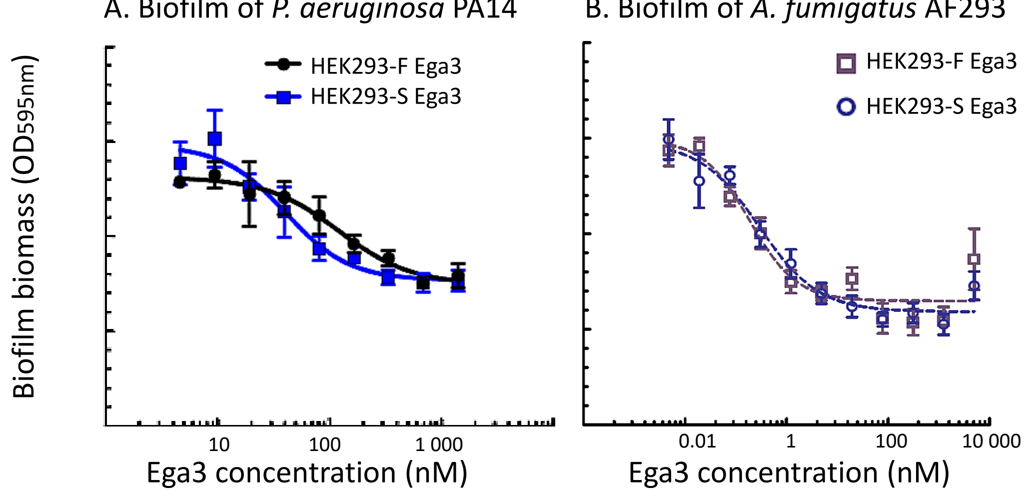


Figure 15: Biofilm disruption assay using Ega3 produced in HEK293-F or in HEK293-S cells against biofilm of *P. aeruginosa* PA14 strain and *A. fumigatus* Af293.

Producing organism or cell line	EC_{50} on *A. fumigatus* Af293 biofilm	EC_{50} on *P. aeruginosa* PA14 biofilm
Pichia pastoris	0.70 nM	94.7 nM
HEK293-S	0.19 nM	35.1 nM
HEK293-F	0.29 nM	86.5 nM

Table 2: Comparative table of Ega3 EC_{50} from different organisms or cell lines. Data were obtained using our standard crystal violet biofilm disruption assay on biofilm of *A. fumigatus* Af293 and *P. aeruginosa* PA14 strain.

2- Pulmonary tolerability of HEK293-produced Ega3

Tolerability studies of Ega3 as shown in Major Task 2 were performed with Ega3 from *P. pastoris*. As we anticipate moving to the use of HEK293-produced Ega3, it is necessary to reassess the tolerability of this GH.

Methodology: Recombinant GH enzymes alone were administered intratracheally at a single dose of 100 or 500 µg in immunocompetent mice. Mice were monitored for 7 days, then sacrificed to perform immunoprofiling and assess tissue injury. Tissue damage was assessed by lactate dehydrogenase (LDH) or total protein release (using a bicinchoninic acid (BCA) assay) in BAL fluid. Pulmonary leukocyte recruitment was assessed by flow-cytometry performed on tissue digests and by histopathology.

Results: Total protein levels and lactose dehydrogenase (LDH) activity in the BAL fluid of Ega3-treated mice were similar or lower to the untreated controls, independently of the origin of the GH (Figure 16) suggesting that all three protein preparations do not induce pulmonary injury. Additionally, no significant increase in leukocyte recruitment was observed with HEK293-produced Ega3 (Figure 17). The eosinophil recruitment that we had observed with Ega3 from *P. pastoris* was not observed. These results suggest that the new Ega3 preparations are non-toxic and do not induce allergic responses.

A direct head-to-head comparison of new Ega3 formulations with *P. pastoris*-produced Ega3 has been performed and data analysis is ongoing at the time of writing this report.

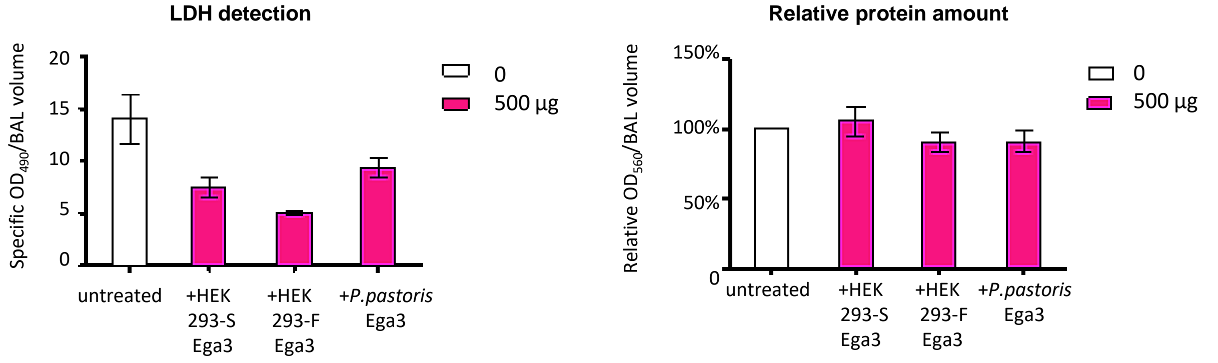


Figure 16: Lung damage as measured by LDH activity and total protein via BCA assay in the BAL fluid from mice 7 days post treatment with a single dose of Ega3 produced in HEK293-S, in HEK-293-F cells or in *P. pastoris*. Data presented here are the average of 2 independent experiments. Note: results *with P. pastoris* Ega3 are from previously reported studies with this protein.

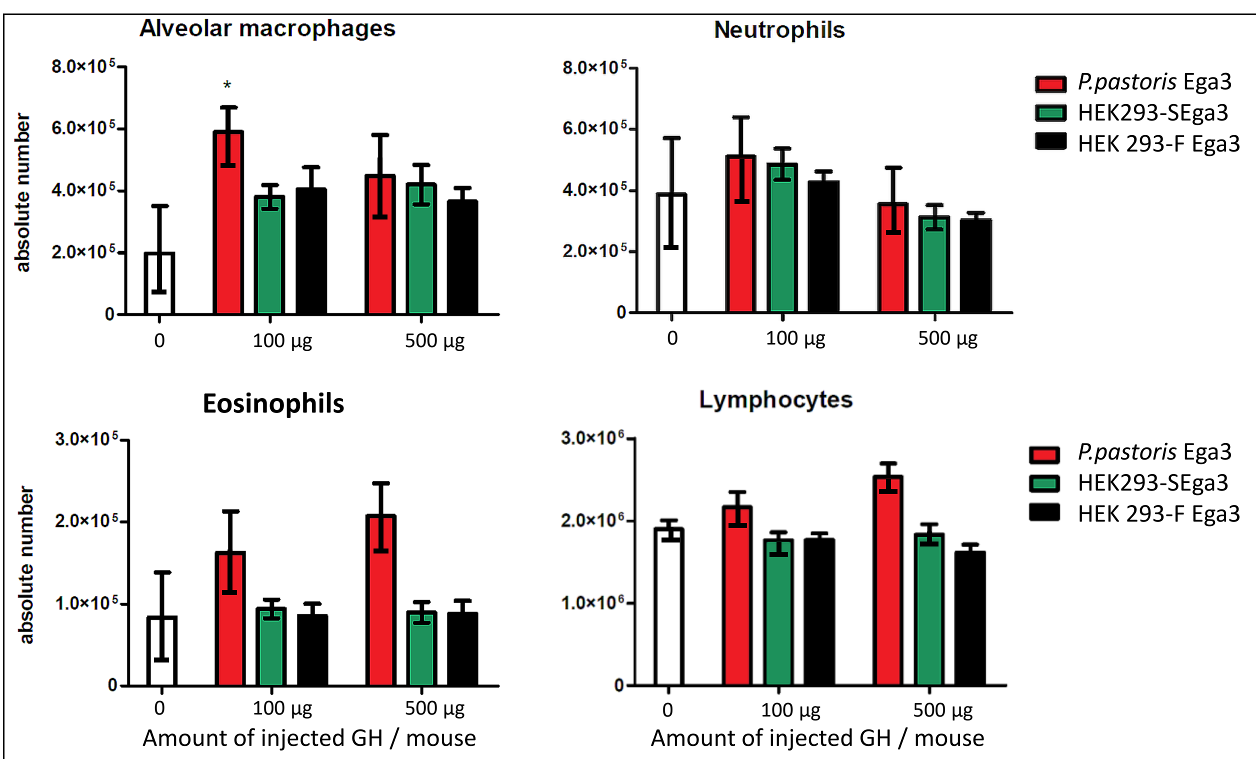


Figure 17: Absolute quantification of leukocytes populations, by flow cytometry, in mouse lung digest at 7 days post treatment with a single dose of Ega3 produced in HEK293-S, in HEK-293-F cells or in *P. pastoris*. Data presented here are the average of 2 independent experiments. Note: results with *P. pastoris* Ega3 are from previously reported studies with this protein.

3- <u>Pharmacokinetics of HEK293-produced Ega3</u>

Methodology: To determine if HEK293-produced Ega3 exhibited similar pulmonary pharmacokinetics to *P. pastoris*-produced Ega3, immunocompetent mice were treated with HEK293F-Ega3 and HEK293S-Ega3, then sacrificed at different time points for Western-blot analysis of lung homogenates using rabbit anti-GH antibodies. A goat-anti-rabbit-HRP antibody was used as the secondary antibody; the HRP signal was detected and analyzed by densitometry when the percent band intensity was normalized to the total band intensity at the 0h time point for each mouse. For each time-point, a minimum of 4 mice was used during the initial experiment. During the duplication, another minimum of 4 mice was used, and earlier or later time-points were added whenever needed.

Results: The half-life of mammalian-produced Ega3 was approximately 9h (Figure 18), which did not differ substantially from the results obtained from the Ega3 expressed in *P. pastoris* (8h as per Major Task 3 Subtask 2).

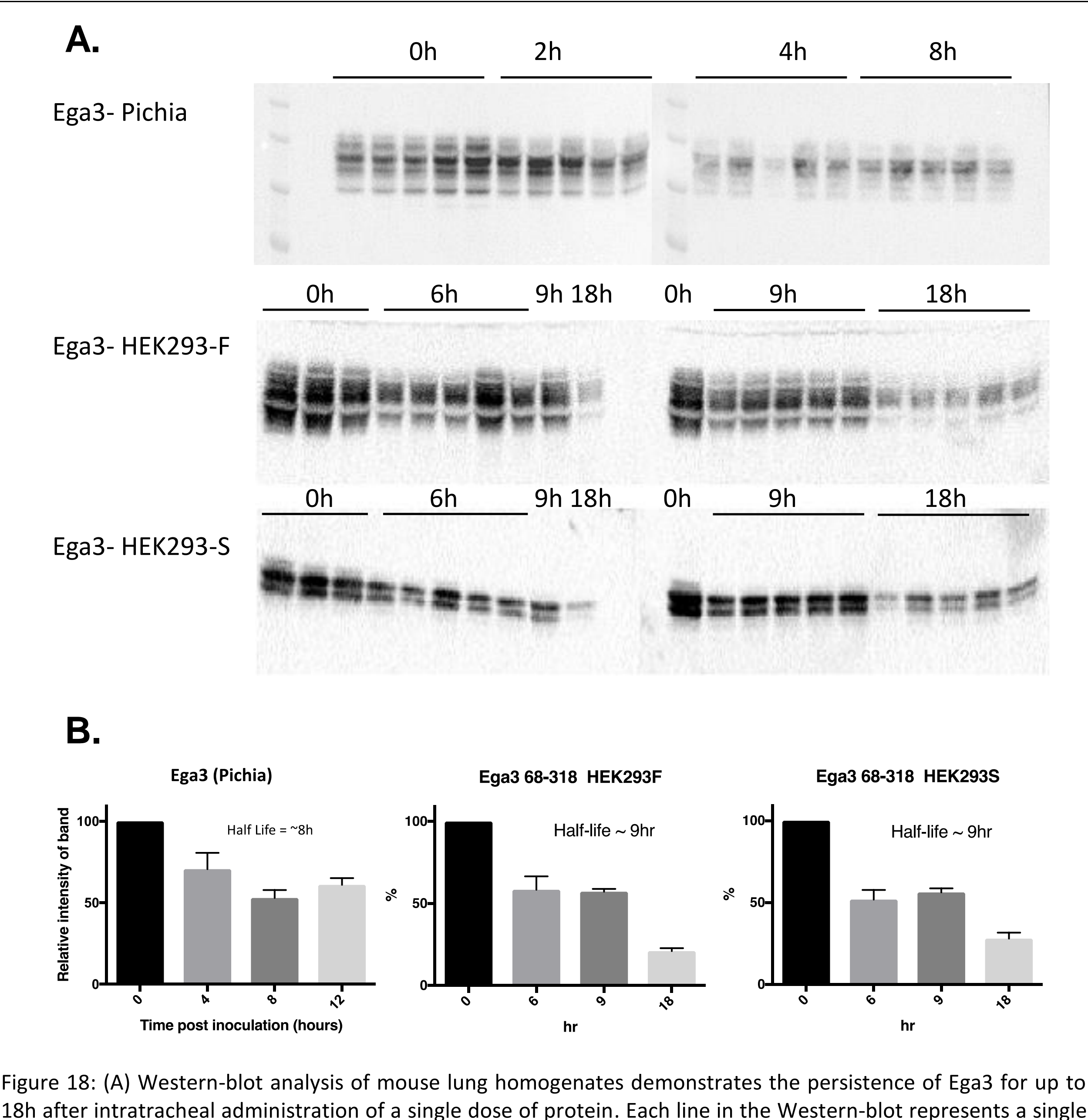


Figure 18: (A) Western-blot analysis of mouse lung homogenates demonstrates the persistence of Ega3 for up to 18h after intratracheal administration of a single dose of protein. Each line in the Western-blot represents a single mouse. (B) Densitometric analyses using the ImageJ software indicate a half-life of approximately 8h for Ega3 from *P. pastoris* vs. 9h for Ega3 from HEK293-F or HEK293-S.

❑ **Milestone to achieve: to develop stable candidate hydrolases.**

SPECIFIC AIM 3: To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections *in vivo*.

Major Task 5: Test hydrolases for activity in animal models of acute disease

Routine production of recombinant GHs in our labs has become efficient enough to meet the needs in quantity and quality required for the subtasks involving the study of GH treatment in acute disease (**Subtask 1: completed**).

Subtask 2: Determine the effects of hydrolases (Sph3, Ega3, PelA) **on survival of immunosuppressed mice** infected with *A. fumigatus*. [10 mice per group X 8 experimental groups X 3 hydrolase regimens, AND 3 mice for histopathology all performed in duplicate = 624 mice] Dr Sheppard's lab. SOW Time Period: Months 13-30. Completion level = 33%

Accomplishments:
Methodology: Mice were rendered neutropenic by subcutaneous injection of 250 mg/kg of cortisone plus intraperitoneal injection of 250 mg/kg of cyclophosphamide at day -2, followed by injection of 200 mg/kg cyclophosphamide intraperitoneally at day +3. At day 0, a 50 μL suspension containing $5x10^3$ conidia of the indicated fungal strain as well as 500 μg of Sph3 (or sterile GH buffer) in a ratio 1:1 was administered to mice. Mice were then monitored daily. For survival assay, mice were euthanized upon reaching clinical endpoints. For leukocyte recruitment, on days 1, 2 and 4 post-infection, airways were lavaged and the lungs were harvested and either digested with collagenase (for pulmonary leukocyte enumeration) or homogenized in PBS with a cocktail of protease inhibitors (for fungal burden). Leukocyte populations in the lavage fluids, lung digests and blood were quantified by flow cytometry, while fungal burden in lung homogenates were assayed by galactomannan assay using the Platelia® Aspergillus EIA (BioRad); the GM values were then normalized to a highly infected lung homogenate standard (our lab).

Results:
1) GH therapy in mice rendered neutropenic by cortisone + cyclophosphamide:
a) Mouse survival:
Sph3 treatment resulted in a trend to increased survival during *Aspergillus* infection (Figure 19) with an increase in median survival to increase from 7 days (Af293 alone) to 9 days (Af293 + Sph3). This increase failed to reach statistical significance in a Wilcoxon-rank test with this single replicate.

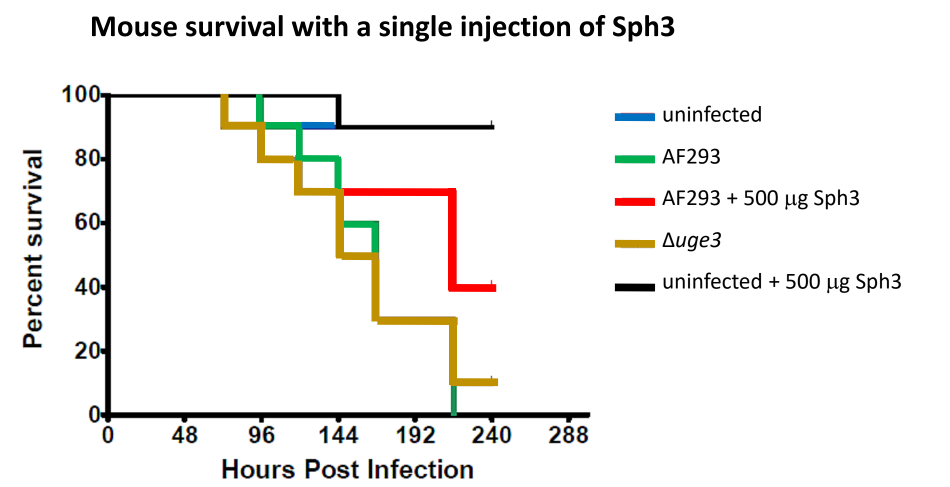


Figure 19. Survival of immunosuppressed mice intratracheally injected with a mix of conidia (5x10^3) and GH at 500 µg 1:1, or the corresponding sterile buffers. * indicates a significant change in survival with p<0.05 in Wilcoxon-rank test compared to mice infected with Af293 alone. 2 independent experiments were performed.

As shown above, in our usual model of immunosuppressed (cyclophosphamide + cortisone) a single dose of Sph3 at the time of infection resulted in only a modest trend towards increased survival during *Aspergillus* infection (7 days for Af293 alone *vs*. 9 days for Af293 + Sph3, but not statistically significant). These results are consistent with the short half-life for Sph3 (less than 1h) and suggest that the antifungal effects of Sph3 single dose therapy are likely lost after 4 days. Multi-dose treatment, or a less acute model of invasive aspergillosis will be likely required to study the effects of GH therapy on survival.

In light of this short half-life of Sph3, and our prior data demonstrating that *in vitro* GH treatment of *A. fumigatus* increases exposure of pro-inflammatory β-glucans, we elected to perform studies of the effects of Sph3 therapy on leukocyte recruitment at days 1, 2, 4 and fungal burden at days 1 and 2. In addition, we evaluated the effects of Sph3 therapy on survival in a less acute model of invasive aspergillosis – the neutrophil-depleted mouse model.

b) Leukocyte recruitment:

At these early time points, neutrophil recruitment in BAL fluid and total lung homogenates was observed in all experimental groups treated with Sph3, independent of infection with Af293 (Figure 20). Neutrophil accumulation was highest at days 1 and 2 post treatment, and declined by day 4. While Sph3 treatment alone was also associated with leukocyte recruitment to the lung, this was more marked in the presence of *A. fumigatus*. Interestingly, no increase in pulmonary neutrophil recruitment was seen during infection with the GAG-deficient *Aspergillus* Δ*uge3* strain despite the fact that, as with Sph3-treated mice, previous studies have found that these mice also exhibit lower fungal burden at day 4 of infection.

Collectively, these data suggest that the increased neutrophil recruitment seen in Sph3-treated mice may not contribute to the antifungal activity of GH therapy. This hypothesis is consistent with the antifungal effects of Sph3 seen in the highly neutrophil depleted mouse model described above, and may indicate that the primary function of Sph3 monotherapy is to attenuate adhesion of *A. fumigatus* to host tissues through degrading GAG.

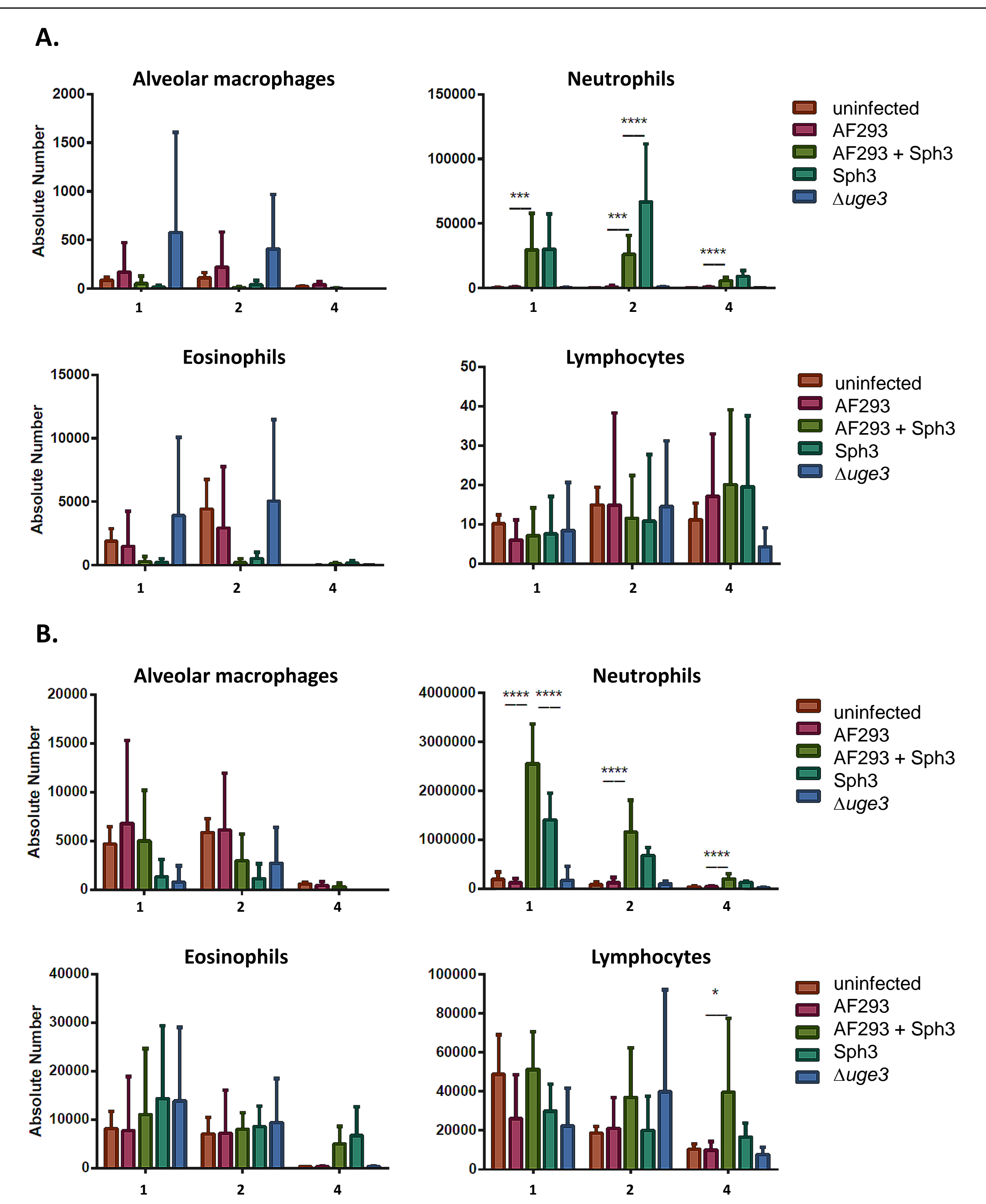


Figure 20. Absolute quantification of leukocyte populations in (A) BAL fluid or (B) lung digests of immunocompromised mice infected with a single dose of conidia ($5x10^3$) and Sph3 (500 µg) or the corresponding sterile buffers. Lungs were lavaged then harvested after 1, 2 or 4 days following intratracheal injection with Aspergillus conidia (Af293 or Δ*uge3*) and/or a single 500 µg dose of Sph3. Leukocyte populations were measured by flow-cytometry. These data are the average of three biological replicates. * and **** indicate a significant difference between the indicated conditions on the same day, with *p*<0.05 and <0.01 respectively, by ANOVA.

As expected, pulmonary fungal burden, as measured by galactomannan levels, was low at early time points and was not significantly different between experimental groups, although a trend to lower fungal burden in the Sph3-treated and Δ*uge3* mutant arms was beginning to emerge (Figure 21).

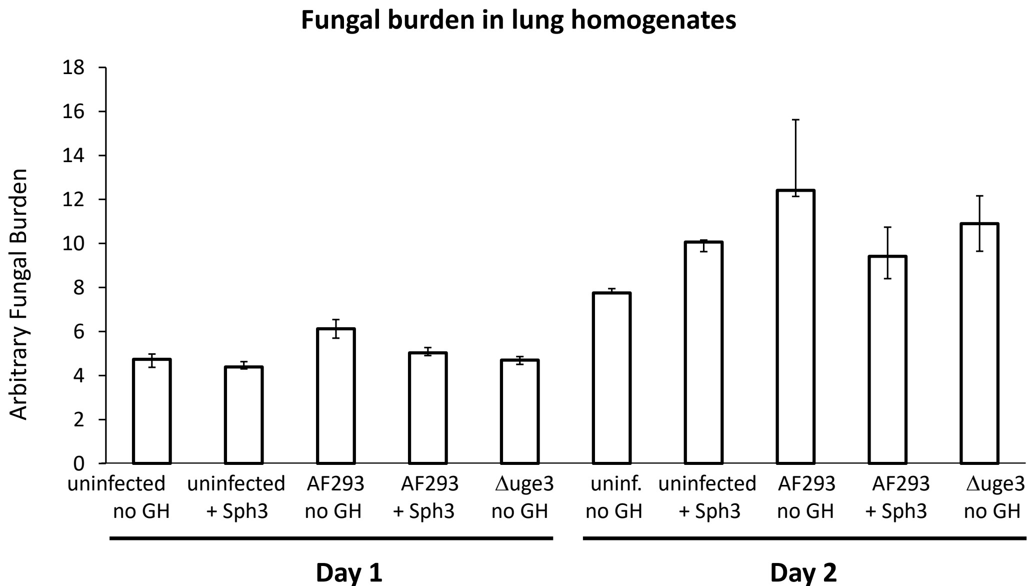


Figure 21. Relative fungal burden in lungs of immunosuppressed mice intratracheally injected with a single dose of conidia ($5x10^3$) and Sph3 (500 μg) or the corresponding sterile buffers. Pulmonary fungal burden was determined by galactomannan content (Platelia® Aspergillus EIA) in lung homogenates. These data are the average of three biological replicates.

d) Inflammation and lung damage:
No significant differences in inflammatation of lung damage were detectable between experimental groups by histology at these early time points.

2) <u>GH therapy in mice rendered neutropenic by anti-neutrophil antibody.</u>
Methodology: Mice were rendered neutropenic by treatment with anti-Ly6G antibody (200 μg intraperitoneally every 48 hours beginning 1 day prior to infection). Mice were then infected intratracheally with a 50 μL suspension of 10^7 Af293 conidia (or sterile conidial buffer) with or without 500 μg of Sph3. Mice were monitored daily and euthanized upon reaching clinical endpoints.

Results: Mice infected with Af293 conidia that did not receive Sph3 fully succumbed to infection by 6.5 days post infection (156h) (Figure 22). In contrast, infected mice that received a single dose of 500 μg Sph3 intratracheally at the time of infection exhibited a strong trend towards protection, with only 50% of the treated mice succumbed to the infection within the same period of time. Survival average was 114 hours after Af293 injection vs. 162 hours after injection Af293 + Sph3; yet, the difference failed to reach statistical difference in a Wilcoxon-rank test (*p*=0.07). We will repeat this experiment in the next quarter to ensure reproducibility.

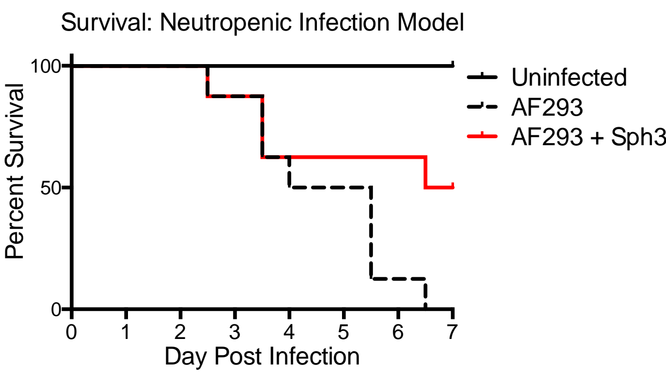


Figure 22. Survival of neutropenic mice infected with a single dose of *A. fumigatus* strain Af293 conidia (10^7) and Sph3 (500 μg) or the corresponding sterile buffer. 8 mice per group in a single experiment. * indicates a significant change in survival with p<0.05 by the Wilcoxon-rank test as compared to mice infected with Af293 alone.

Subtask 3: **Determine the effects of hydrolases** (Sph3, Ega3, PelA) **on fungal burden** of mice infected with *A. fumigatus*. [10 mice per group X 8 experimental groups X 3 hydrolase regimens, all performed in duplicate = 480 mice] Dr Sheppard's lab. Months 13-30. Completion level = 100%.

Accomplishments:

Methodology: Mice were rendered neutropenic by subcutaneous injection of 250 mg/kg of cortisone plus intraperitoneal injection of 250 mg/kg of cyclophosphamide at day -2, followed by 200 mg/kg cyclophosphamide at day +3. At day 0, a 50 μL suspension was administered intratracheally to each mouse. The administrated suspension contained 5×10^3 conidia of the appropriate fungal strain (or sterile conidial buffer) as well as 500 μg of the appropriate GH (or sterile GH buffer) in a ratio 1:1. At day 4, all mice were sacrificed and their lungs were harvested for fungal burden estimation by galactomannan (GM) quantification with the commercial kit "Platelia™ Aspergillus EIA" from BioRad. The GM reading was then normalized to the weight of the harvested lung and to a highly infected lung homogenate standard (our lab). This experiment has now been replicated four times (n=4) for Sph3 and twice for the other GHs and GH combinations.

Results: Treatment of mice with 500 μg of any of the GHs, as well as treatment with the indicated GH combinations (250 μg of each GH), resulted in a significant reduction in pulmonary fungal burden 4 days after *A. fumigatus* challenge (Figure 23). GH therapy was associated with fungal burden levels similar to that observed in mice infected with a GAG-deficient strain of *A. fumigatus* (Δ*uge3*), suggesting that these enzymes efficiently degrade GAG *in vivo* to attenuate virulence.

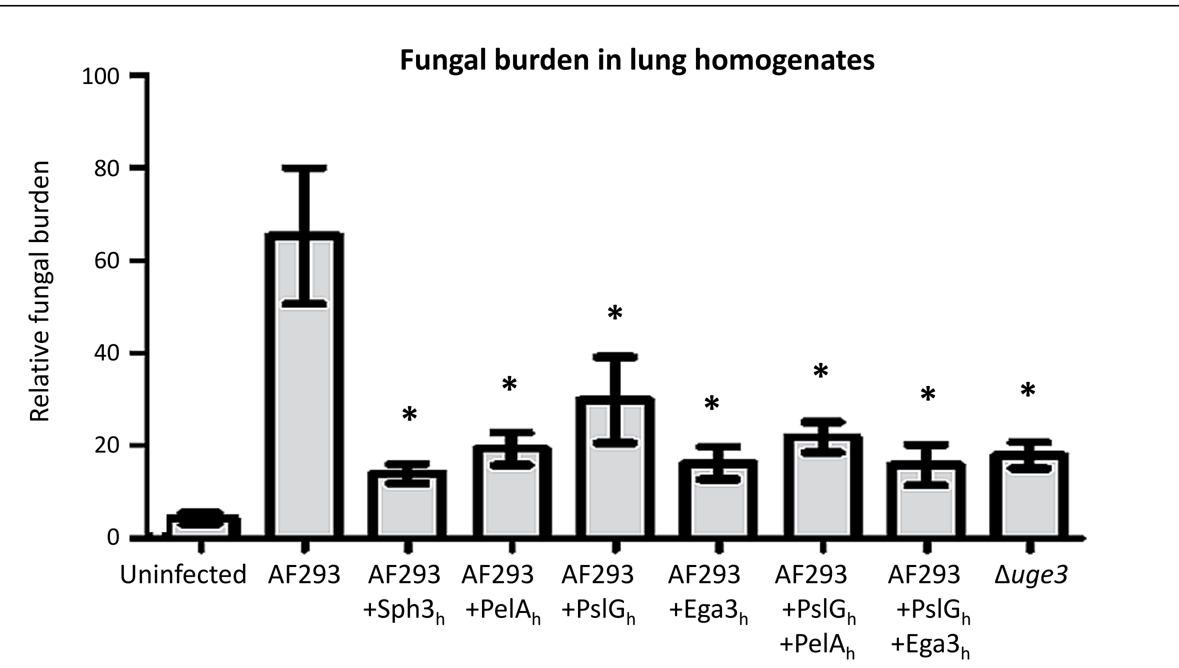


Figure 23. Fungal burden in lungs of immunosuppressed mice intratracheally infected with $5x10^3$ conidia of *A. fumigatus* and treated with the indicated GH (500 µg single dose for monotherapy or 250 µg of each GH for combination therapy), or the corresponding sterile buffer. * indicates a significant difference in GM content as compared with untreated, infected mice with $p<0.05$ by ANOVA. 4 independent experiments were performed (n=4)

Subtask 4: Determine the effects of hydrolases (PslG/PelA and PslG/Ega3 combinations) **on bacterial burden** of mice infected with three strains of *P. aeruginosa*. Dr Sheppard's lab. Months 13-30. Completion level = 20%. [10 mice per group X 7 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points AND 3 mice for histopathology X 7 groups X 2 hydrolase regimens X 3 strains at a single time point all performed in duplicate = 1932 mice]

Accomplishments

Methodology: Mice were treated intratracheally with a 50 µL suspension containing $1.5x10^7$ bacteria (*Pseudomonas aeruginosa* PAO1), combined with 500 µg PslG, or PelA, or PslG inactive form, or a 1:1 mix of PslG/PelA (250 µg each), or the sterile GH buffer, as indicated. At 48 hours post-infection, all mice were sacrificed, BALs were performed; blood and lungs were harvested and plated for colony forming unit (CFU) counting. The CFUs were normalized by lung weight. Leukocyte populations in BAL fluid were analyzed by flow cytometry.

Results:

1) Effects of PslG on mice infected with wild-type P. aeruginosa.

Surprisingly, mice treated with PslG exhibited a trend towards higher pulmonary bacterial 2 days post infection, compared to the mice that received PAO1 alone, although this was not statistically significant (Figure 24).

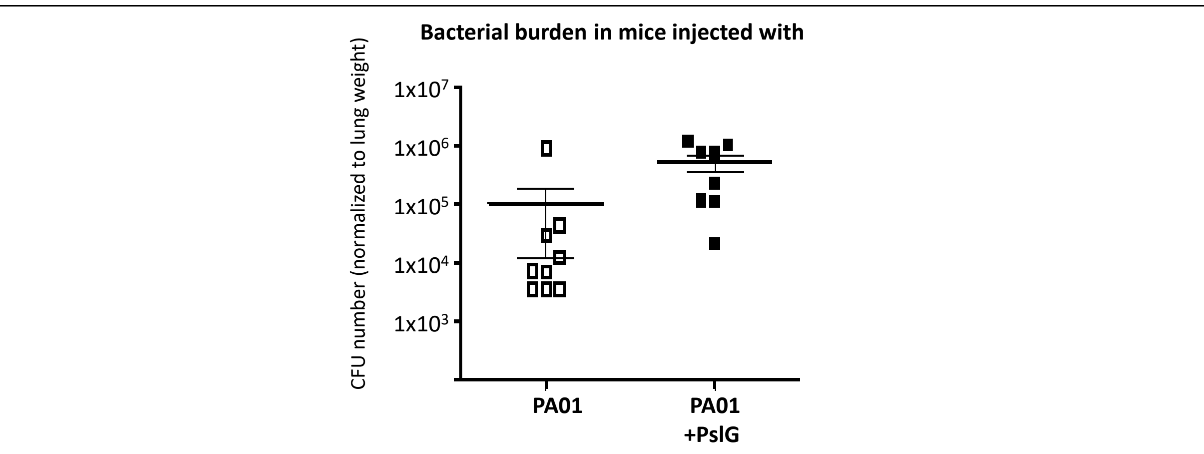


Figure 24. Bacterial burden in mice as determined by quantitative culture of lung homogenates or blood, as indicated. No significant difference between the groups was shown by ANOVA.

To further explore these unexpected results, a repeat experiment was performed with the addition of two additional control groups: along with *P. aeruginosa* PA01 infection we administered GH buffer, or a catalytically inactive variant of PslG. The blood of the mice was also cultured, to determine if pulmonary infection was associated with extra-pulmonary dissemination.

A trend towards elevated bacterial burden was again observed in the lungs of mice treated with either active or inactive PslG (Figure 25). Similarly, the bacterial burden in blood was significantly higher in the presence of either form of PslG compared to the buffer controls, in which very little bacterial dissemination was observed (Figure 25).

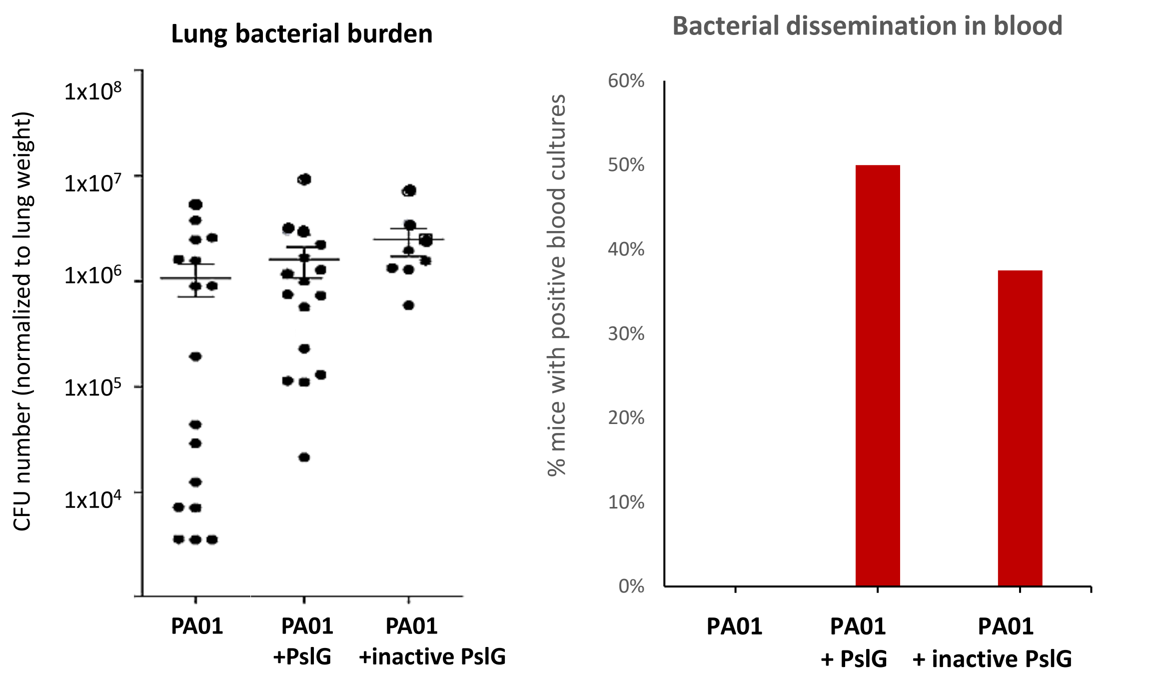


Figure 25. Effects of PslG and modified PslG (inactive PslG) on pulmonary bacterial burden and hematogenous dissemination of bacteria as determined by CFU counting of lung homogenate.

Flow cytometry analyses revealed a significant increase of eosinophil recruitment, and a significant decrease of neutrophil and lymphocyte recruitment to infected lungs in presence of PslG, as compared to untreated infected lungs (Figure 26). Treatment with inactive PslG gave similar results, but without any significant increase in eosinophil recruitment.

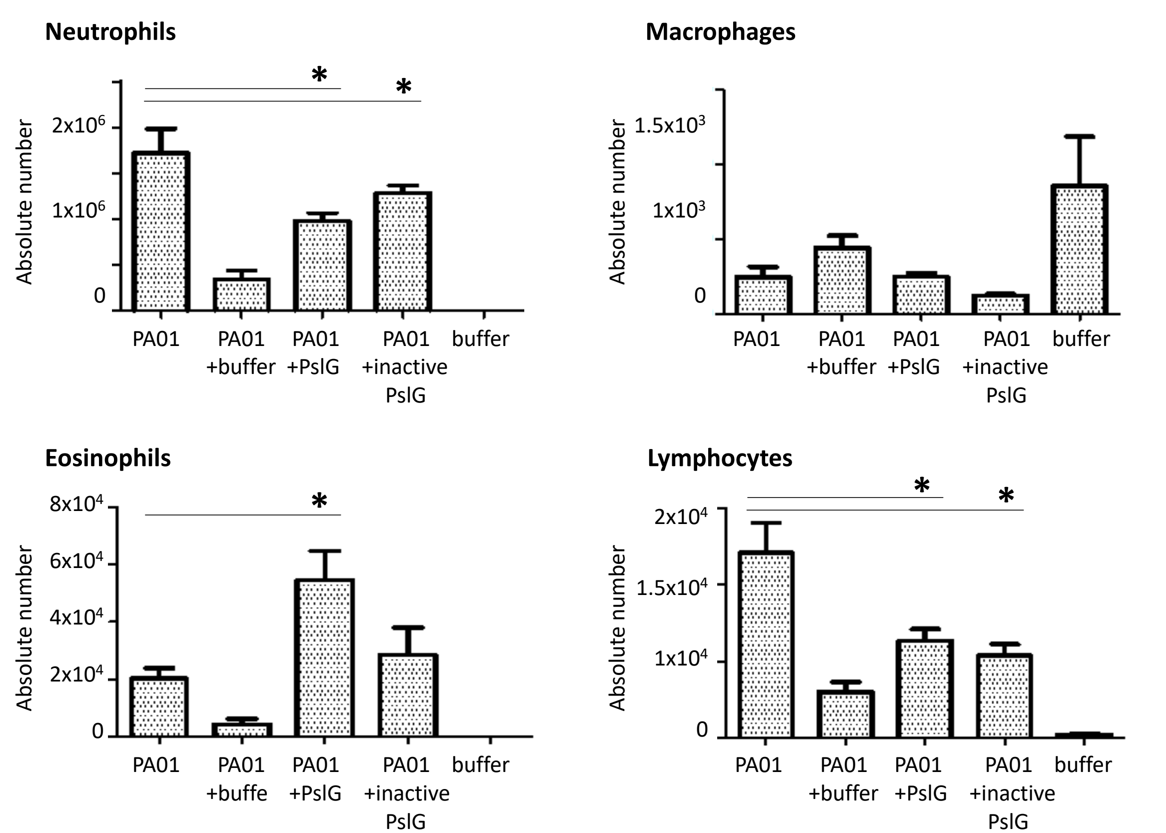


Figure 26. Absolute quantification of leukocyte populations in mouse lungs as measured by flow-cytometry performed on lung digests. Lungs were harvested 2 days following *P. aeruginosa* infection in combination with a single dose of the indicated GH. * indicates a significant difference with the untreated group with $p<0.05$ by ANOVA.

These data indicate that, in contrast to our findings with GH therapy for *Aspergillus*, PslG alone may not be not protective against *P. aeruginosa* infection, and could increase extra pulmonary dissemination. There are multiple potential explanations for this observation. For example, it is possible that PslG degradation of *P. aeruginosa* at the time of inoculation results in reduced adherence to pulmonary tissues and increased motility *P. aeruginosa* leading to enhanced tissue invasion. This hypothesis is consistent with a recent observation that PslG therapy can increase *P. aeruginosa* motility in vitro (Zhang *et al*., Appl. Environ. Microbiol. doi:10.1128/AEM.00219-18). Alternately, PslG may have a direct effect on host tissue glycans that enhances tissue invasion.

Therefore, in addition to testing the effects of combination GH therapy, we have initiated a series of experiments to probe the mechanisms underlying the effects of PslG therapy on *P. aeruginosa* virulence. First, we have tested the virulence of a Psl-deficient mutant strain (Δ*psl*) as compared to wild-type *P. aeruginosa*, and the effects of PslG treatment during infection with this mutant

strain to determine if the increased virulence we observe is dependent on the presence of Psl. In addition, we will attempt to verify the findings that PslG enhances *P. aeruginosa* motility and determine the effects of PslG treatment during infection with a motility-deficient mutant of *P. aeruginosa*.

2) Effects of PslG on mice infected with Psl deficient P. aeruginosa.

Methodology: Mice were intratracheally administered a 50 µL suspension containing 1.5×10^7 bacteria (*Pseudomonas aeruginosa* wild-type strain PAO1 or the Psl-deficient strain Δ*psl*), combined with 500 µg PslG or its sterile buffer. At 48 hours post infection, all mice were sacrificed, BALs were performed; blood and lungs were harvested and plated for quantitative culture. The resulting colony forming unit (CFU) numbers were normalized by lung weight. The absence of Psl with the strain Δ*psl* was validated in flow-cytometry, using the antibody Psl0096, a human monoclonal antibody conjugated to Alexa Fluor 647.

Results: Infection with the Psl-deficient *P. aeuruginosa* strain (Δ*psl*) was associated with lower pulmonary bacterial burden then with wild-type PA01 (Figure 27) suggesting that Psl may play a role in lung colonization. As expected from previous set of experiments (Figure 25), the addition of PslG resulted in a higher bacterial burden in PA01 infected mice but also in mice infected with the Δ*psl* strain. Similarly, PslG therapy was associated with higher levels of hematogenous dissemination during infection with both wild-type and Δ*psl P. aeruginosa,* while both strains exhibited similar degrees of dissemination in the absence of PslG therapy (Figure 27). These data suggest that although Psl plays a role in lung colonization by *P. aeruginosa*, the GH PslG may also act on the host tissues or on other bacteria present in the mouse lungs to enhance bacterial dissemination.

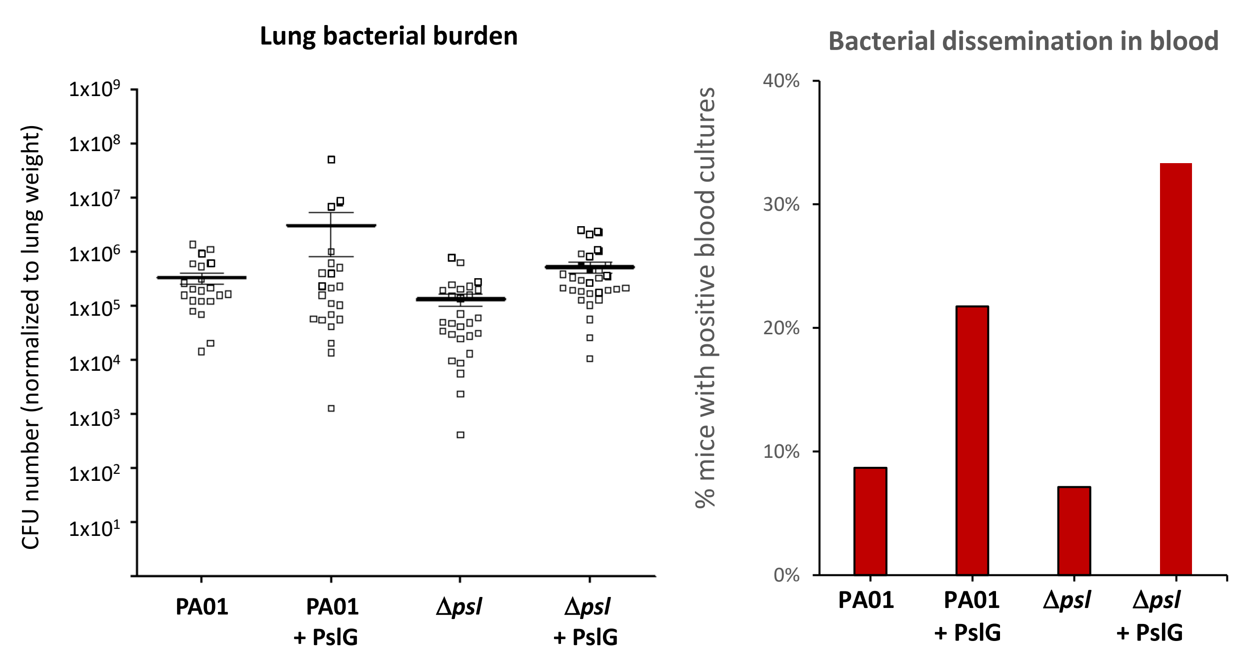


Figure 27. Effects of PslG on pulmonary bacterial burden and hematogenous dissemination of bacteria as determined by CFU counting of lung homogenate and blood, respectively. The strain *P. aeruginosa* Δ*psl* is a Psl-deficient mutant of the wild-type PA01 strain. Results shown are the aggregate of three independent experiments.

3) Effects of PslG on P. aeruginosa motility.

Methodology: 1% LB-agar with or without PslG (0.05 or 2 µM as final concentration) were stab inoculated with approximately $4x10^8$ of each bacterial strain. After 24 hours of incubation at 37°C, petri dishes were strained with crystal violet and washed. The diameter of the halos formed by bacteria was measured. Four bacterial strains were assayed: two wild-type: PA01, mPA01, and two motility deficient mutants: FRT, a strain containing a mutation in the *pilT* gene, and Tn5, a strain containing a mutation in the *pilA* gene.

Results: As expected, non-motile mutants exhibited reduced growth diameter as compared to motile strains (Figure 28). Exposure to PslG significantly increased the spreading of motile bacteria in a dose dependent manner, but had no effect on non-motile strains. These results suggest that PslG treatment increases the motility of *P. aeruginosa*.

***P. aeruginosa* twitching motility assay**

LB
LB + 0.05 µM PslG
LB + 2 µM PslG
Colony diameter (in cm)
WT PAO1
mPAO1
FRT (*pilT*)
Tn5 (*pilA*)
Bacterial strain

Figure 28. Effect of PslG on the motility of *P. aeruginosa.* Motile and non-motile bacterial strains were point-inoculated on a nutritive agar plate enriched or not with PslG at 0.05 or 2 µM. Bacterial spreading was measured as proportional to the diameter of the biofilm attached to the plastic of the plate. * indicates a significant difference with the untreated group with $p<0.05$ by ANOVA.

4. Effects of combination GH therapy on acute pulmonary infection with P. aeruginosa:

In addition to the experiments probing the mechanisms of PslG action in vivo, we have also performed experiments to study the role of PslG/PelA on bacterial burden during acute disease, as per SOW (PslG/Ega3 combination therapy, will be tested during next period).

An unusually high degree of hematogenous dissemination of *P. aeruginosa* was observed in this study in the mice receiving therapy with buffer alone. In addition, several mice succumbed to infection prior to the pre-determined sampling point. We suspect a contamination of one of our experimental reagents used in preparing the inoculum for these experiments. For completeness, the data are displayed in Figure 29, but we will repeat this experiment with fresh reagents in the coming period.

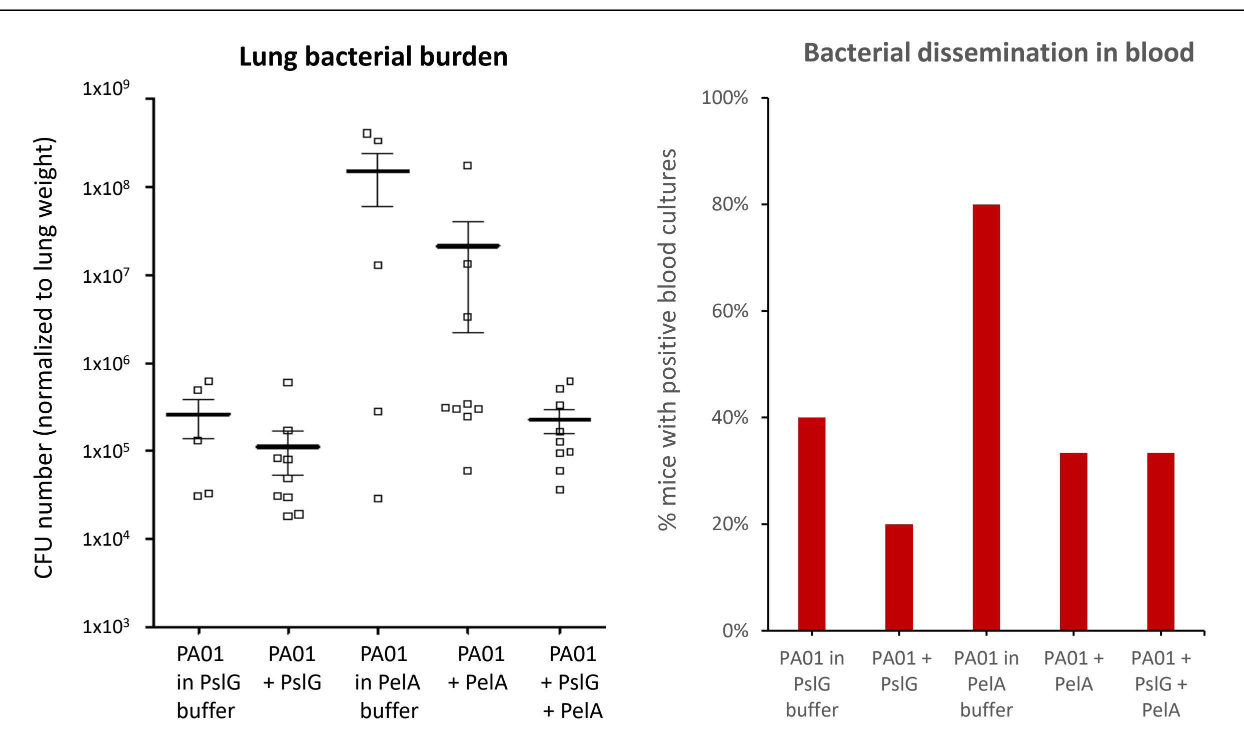


Figure 29. Effects of PslG, PelA and PslG/PelA combination on pulmonary bacterial burden and hematogenous dissemination of bacteria as determined by CFU counting of lung homogenate and blood, respectively. Results shown are from a single experiment.

❑ **Milestone to achieve: To confirm efficacy of candidate hydrolase regimens in the treatment of acute infection with *A. fumigatus* and *P. aeruginosa*.**

Major Task 6: Test hydrolases for activity in animal models of chronic disease

Background: Routine production of recombinant GHs in our labs has become efficient enough to meet our needs in quantity and quality for subsequent subtasks. (**Subtask 1**: completed)

Subtask 2: Determine the effects of candidate hydrolases (Ega3) on fungal burden of immunocompetent mice chronically infected with *A. fumigatus*. Dr Sheppard's lab. Months 13-30. Completion level = 25%.

Background:
As detailed above, *in vivo* studies using Ega3 were delayed as we optimized formulation of this enzyme. In the interim, we performed *in vivo* studies using Sph3 as a proof of principle study in chronic aspergillosis.

Accomplishments:
Methodology: A 50 μL suspension of agarose beads with or without 1.25×10^6 conidia of *A. fumigatus* strain Af293 embedded within them was administered intratracheally to immunocompetent female BALB/c mice. Beads were suspended in a solution containing 500 μg of GH, or sterile buffer. At days 2 and 7, mice were sacrificed and their lungs were harvested and homogenized to assay for fungal burden as assayed by galactomannan (GM) analysis with the commercial kit "Platelia™ Aspergillus EIA" from BioRad. GM readings were normalized to the weight of the harvested lung and to a highly infected lung homogenate standard. Lungs were also processed for histopathology examination to determine inflammatory responses and for immunostaining for exopolysaccharides.

Results:
In contrast to the results of experiments testing the use of Sph3 and Ega3 for acute invasive *Aspergillus* infection, no differences in pulmonary fungal burden were observed between GH-treated and untreated mice (Figure 30, 31). Interestingly, in these experiment, there was also no significant difference between the pulmonary fungal burden of mice infected with wild-type and the GAG-deficient Δ*uge3* mutant. While it is possible that the use of agar beads allows retention of fungi within the airways despite the loss of GAG, these experiments will need be repeated in the coming period to confirm these findings. In addition, we will examine tissue histology from these animals to ensure that the GM assay results are representative of total fungal burden and examine the distribution and morphology of fungal lesions in treated and untreated animals.

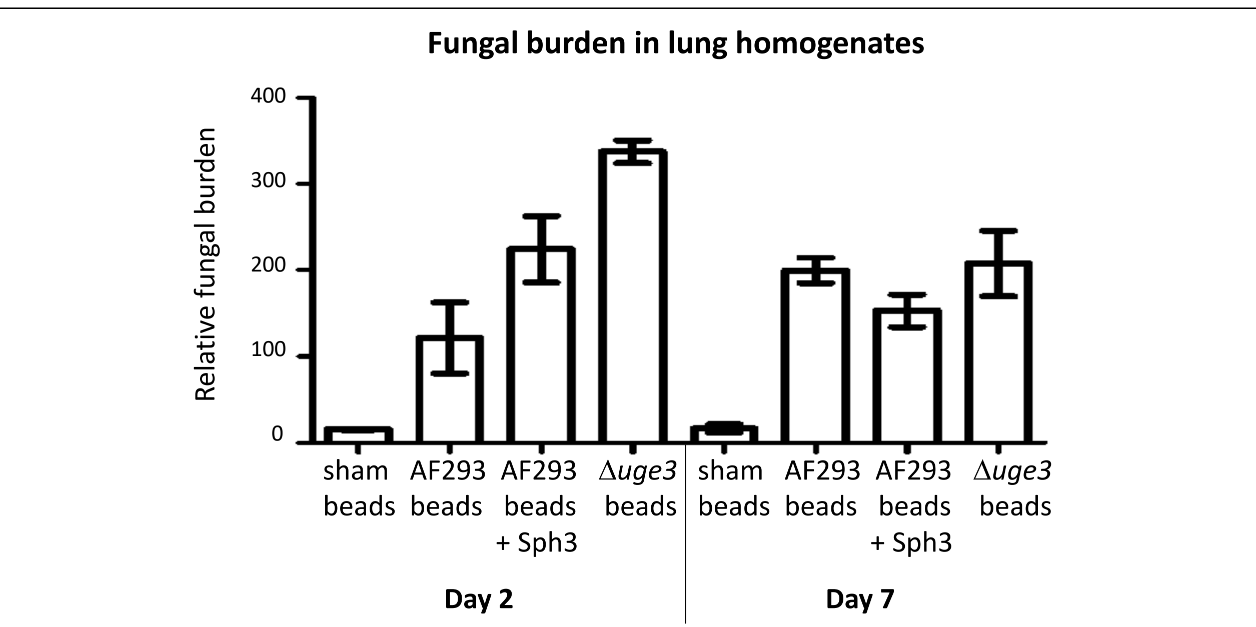


Figure 30: Relative fungal burden at Day 2 and Day 7 post-injection, in lungs of immunocompetent mice intratracheally infected with a single dose of agar beads containing buffer only (sham) or 1.25x10^6 conidia of Af293, suspended in Sph3 (500 μg) or the corresponding sterile buffer. Fungal burden was determined by galactomannan content (Platelia® Aspergillus EIA) of the lung homogenates at the indicated time points. Differences between all infected groups are not significant.

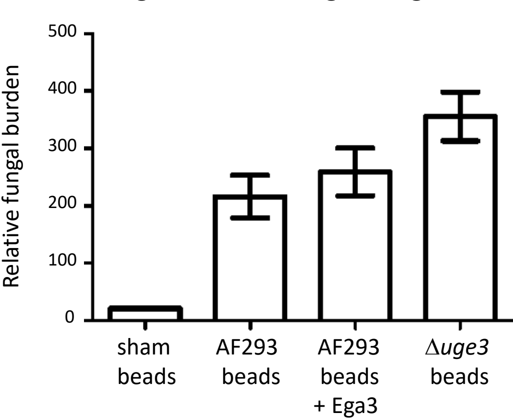


Figure 31: Relative fungal burden at Day 7 post-injection, in lungs of immunocompetent mice intratracheally infected with a single dose of agar beads containing buffer only (sham) or 1.25x10^6 conidia of Af293, suspended in HEK293-S produced Ega3 (500 μg) or the corresponding sterile buffer. Fungal burden was determined by galactomannan content (Platelia® Aspergillus EIA) of the lung homogenates.

Subtask 3: Determine the effects of candidate hydrolases (PslG/PelA and PslG/Ega3 combinations) on bacterial burden of immunocompetent mice chronically infected with *P. aeruginosa*. Dr Sheppard's lab. Months 18-30. Completion level = 0%.

There is currently no progress to report for this year.

❑ **Milestone to achieve: To confirm efficacy of candidate hydrolase regimens in the treatment of chronic infection with *A. fumigatus* and *P. aeruginosa*.**

Major Task 7: Test hydrolases for synergy with antimicrobials.

Background: Routine production of recombinant GHs in our labs has become efficient enough to meet our needs in quantity and quality for subsequent subtasks. (**Subtask 1**: completed)

Subtask 2: Determine the effects of hydrolase (Sph3, Ega3, PelA)-antifungal combinations on fungal burden of mice infected with *A. fumigatus*. Dr Sheppard's lab. Months 25-36. Completion level = 0%.

As per SOW, this Subtask will be initiated at month 25.

Subtask 3: Determine the effects of hydrolase (PslG/PelA and PslG/Ega3)-antibiotic combinations on bacterial burden of mice infected with *P. aeruginosa*. Dr Sheppard's lab. Months 25-36. Completion level = 0%. [10 mice per group X 5 experimental groups X 2 hydrolase regimens X 3 strains X 2 time points X 2 antibiotics AND 3 mice for histopathology X 5 groups X 2 hydrolase regimens X 2 antibiotics at a single time point all performed in duplicate = 460 mice]

Accomplishments:
We have begun to evaluate the effects of combining GH therapy with antibiotics *in vivo* using the combination of PslG or PelA and ciprofloxacin.

Methodology: Mice were intratracheally infected with a 50 µL suspension containing 1.5×10^7 bacteria (*P. aeruginosa* PAO1), in combination with 500 µg PslG or PelA or sterile GH buffer. Ciprofloxacin was administered intraperitoneally at 4h, 12h and 20h post-infection. At 48 hours post infection, all mice were sacrificed. Blood and lungs were harvested and plated for quantitative culture. The CFUs were normalized to the weight of the harvested lung.

Results:
1) PslG + ciprofloxacin therapy:
In the absence of PslG, ciprofloxacin had minimal effects on the pulmonary bacterial burden at doses as high as 25mg/kg, resulting in a log reduction of bacterial burden of only 0.5 (Figure 32). Therapy with PslG significantly potentiated the activity of ciprofloxacin at doses as low as 10 mg/kg, resulting in a 2.2 log reduction in pulmonary bacterial burden. The increase degree of extrapulmonary dissemination of *P. aeruginosa* seen with PslG monotherapy was reduced by ciprofloxacin therapy (Figure 33). These experiments have been performed in in triplicate, and support the conclusion that PslG potentiates the effect of ciprofloxacin *in vivo.*

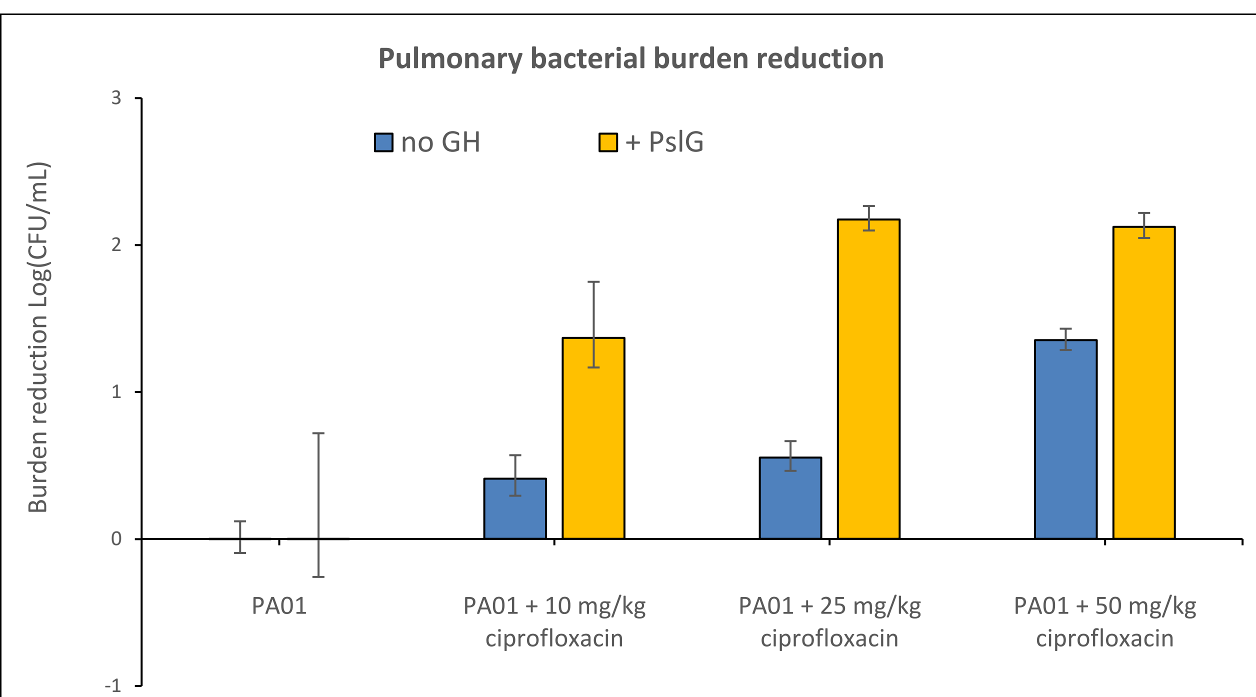


Figure 32. Effects of PslG and ciprofloxacin on pulmonary bacterial burden of bacteria as determined by CFU counting of lung homogenate CFU numbers were normalized to the weight of the harvested lungs, then expressed as log(CFU) reduction as compared to PA01 alone or PA01 + PslG without ciprofloxacin, respectively. Results shown are the aggregate of three independent experiments.

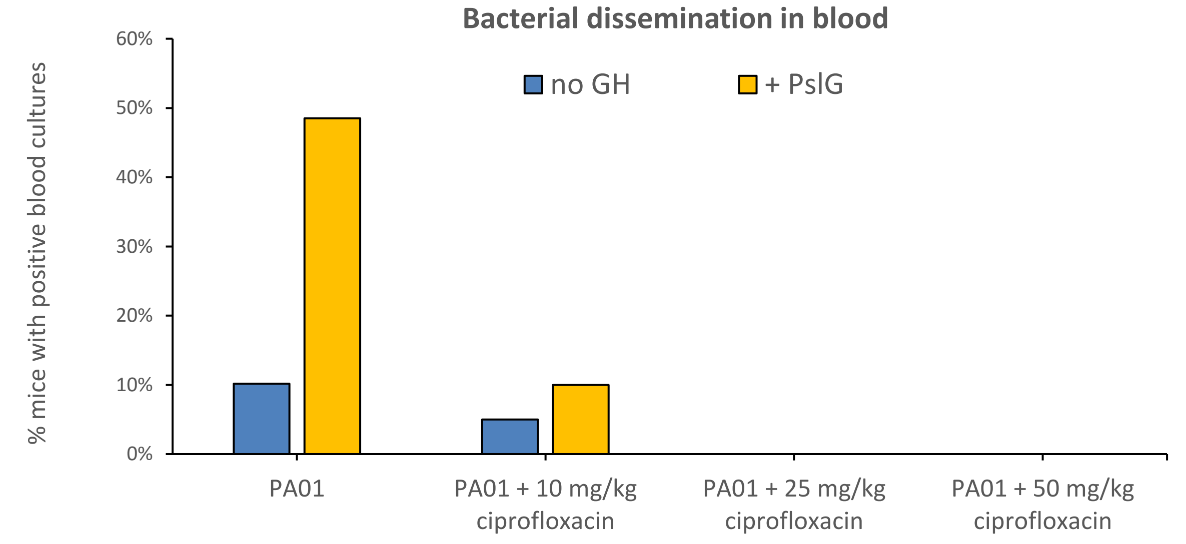


Figure 33. Effects of PslG on hematogenous dissemination of bacteria as determined by quantitative culture of blood. Results shown are the aggregate of three independent experiments.

2) PelA + ciprofloxacin therapy:

A single experiment with this GH antibiotic combination has been performed. Interestingly pulmonary bacterial burden in lungs was lower in mice receiving PelA therapy (Figure 34). However, a repeat of this experiment is required to confirm the reproducibility of this observation. As with PslG, the combination of PelA and ciprofloxacin was associated with a more

significant reduction in pulmonary bacterial burden (2.5 log) as compared with monotherapy with either agent alone (Figure 34) and with a lower rates of hematogenous dissemination (Figure 35).

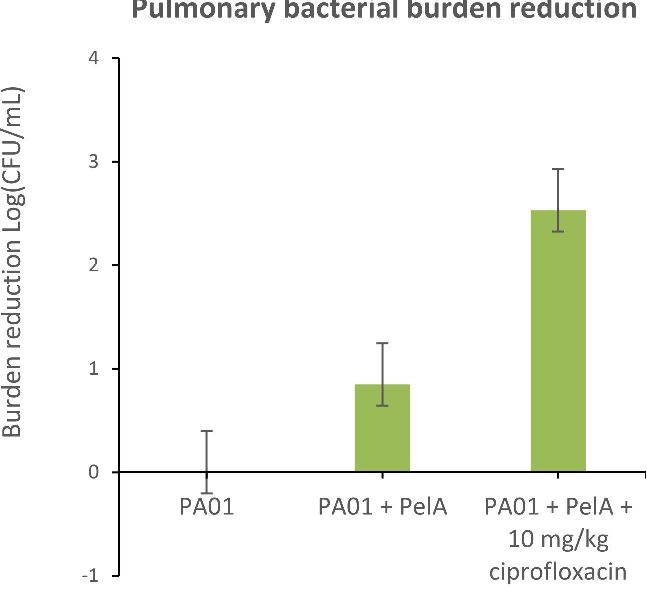


Figure 34. Effects of PelA as a single injection of 500 µg, and ciprofloxacin, at a dose of 10 mg/kg of tissue, on pulmonary bacterial burden of bacteria as determined by quantitative culture of lung homogenates. CFU numbers were normalized to the weight of the harvested lungs, then expressed as log(CFU) reduction as compared to PA01 alone resuspended in their respective sterile buffers. Results shown are from a single experiment.

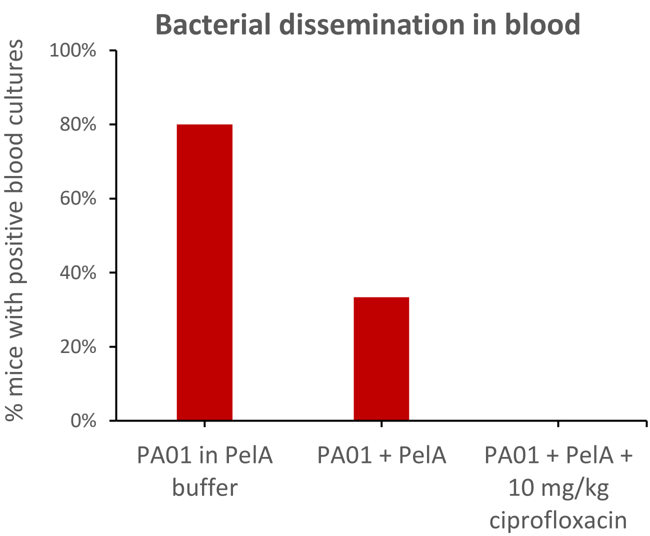


Figure 35. Effects of PelA as a single intratracheal dose of 500 µg, and ciprofloxacin, at a dose of 10 mg/kg, on hematogenous dissemination of bacteria as determined by CFU counting of blood. Results shown are from a single experiment.

❑ **Milestone to achieve: Show a proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa*. Make ready to initiate trials of delivery systems and detailed pharmacodynamics experiments as a prelude to Phase I clinical trials.**

What opportunities for training and professional development has the project provided?

Rachel Corsini and James Stewart were trained by Melanie Lehoux in regard of animal care. They learned to perform intratracheal injection of GHs in mice, as well as mouse infection with pathogens. They also learned to isolate *P. aeruginosa* from lung tissues and monitor this population.

Brian Hicks, an undergraduate research assistant, was trained by Ira Lacdao in modern biochemical techniques, specifically protein expression and purification.

Ira Lacdao, Natalie Bamford, Brendan Snarr, Caitlin Zacharias and François LeMauff presented their results in relation to this grant at several conferences, thus improving their presentation skills. Conferences were:

- Trends in Molecular Mycology, Belgrade, Serbia. October 6-9, 2017.
- 8th Advances Against Apergillosis Meeting, Lisbon, Portugal. Feb 1-3, 2018.
- Canadian Glycomics Symposium 2017, Banff, Alberta, Canada. May 9-11, 2018.
- McGill Department of Microbiology and Immunology Research Day, Montreal, Canada. June 08, 2018.

How were the results disseminated to communities of interest?

Results of our GH studies were chosen to be amongst the 10 scientific projects of the year in the review Quebec Science.

What do you plan to do during the next reporting period to accomplish the goals?

All Major Tasks listed in SOW have been either completed or initiated at this point. In Year 3, we will complete the unfinished subtasks on which work has been accomplished as described above. Briefly:

Major Task 1: complete.

Major Task 2: complete.

Major Task 3

Subtask 1: complete

Subtask 2, 3 and 4: pharmacokinetic studies are now complete with all single GHs and with the PslG/PelA3 combination as well. We experienced delays due to the need for production of the new HEK293 formulation of ga3; this issue is now resolved, and we will now move forward with the PslG/Ega3 combination pharmacokinetic studies to complete Major Task 3.

Major Task 4

Subtask 1: complete.

Subtasks 2 and 3: We will wait to have Ega3 with glycosylation modifications, as produced through the new HEK239 expression systems, to assay Ega3 in the same system as Sph3 and PelA. When exposed to broncho-alveolar lavage fluids and lung homogenates*,* Sph3 and PelA were minimally degraded by the resident proteases, even after 24h. This result was surprising since Sph3 and PelA showed a half-life of less than 6h when injected in mouse lungs (pharmacokinetics results of Major Task 3). This experiment will be repeated to ensure reproducibility. In parallel we will work to optimize our *in vitro* degradation assays. For instance, we will perform it using BAL fluid and lung homogenate from mice administered with a different GH, in order to determine if GH administration increases the lung's degradative capacity. Alternatively, we will develop an assay to test the resistance of GHs to neutrophils and neutrophil lysates since the dramatic difference in persistence of PslG in immunosuppressed mice (48h) vs immunocompetent mice (18h) suggests a role of PMNs in GH degradation.

Subtask 4: We have shown that commercial kits for PEGylation successfully modify the GHs Sph3 and PelA, but provide a limited protection against commercially available proteases. Since these proteases might not be relevant to mimic what happens in the lungs, before attempting to make more chemical modifications to the GHs, we will wait until the *in vitro* GH degradation assay using BAL fluid and lung homogenate (Subtasks 2 and 3) is optimized. Then we will determine if PEGylation provides protection to the GHs against degradation in the lungs, and at that point will decide whether to pursue further chemical modifications to the GHs based on these results.

Subtask 5: complete.

Major Task 5: Test GHs for activity in animal models of acute disease

Subtask 1: complete.

Subtask 2: Experiments will be repeated to complete our study of the effect of Sph3 on survival of immunosuppressed mice infected with *A. fumigatus*. Experiments with Ega3 and PelA will be initiated for completion by Month 30.

Subtask 3: complete.

Subtask 4: Two further experiments will be performed to elucidate the biological mechanism underlying these results and guide the further development of GH therapy. We will test the effects of Psl treatment on the motility of a Psl-deficient mutant of *P. aeruginosa* to confirm that this effect is a direct consequence of enzymatic degradation of Psl. This experiment will be critical given our findings that PslG enhanced virulence and dissemination of the Psl-deficient mutant, and will clarify if PslG directly affects motility independent of its enzymatic activity against Psl. Similarly, we will test the effects of PslG therapy during infection with non-motile *P. aeruginosa* strains to determine if the effects of PslG on motility are responsible for enhanced dissemination of *P. aeruginosa* during PslG treatment.

Major Task 6: Test GHs for activity in animal models of chronic disease

Subtask 1: complete.

Subtask 2: As detailed above, we will repeat experiments to verify the effects of Sph3 and Ega3 on fungal burden in chronic pulmonary aspergillus infection and examine pulmonary histopathology specimens.

Subtask 3: will be initiated for completion by month 30.

Major Task 7: Test hydrolases for synergy with antimicrobials.
Has been initiated for completion by month 36.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

Our demonstration that microbial GHs can disrupt biofilms has generated significant interest in the scientific community. Besides research manuscripts, we have published an editorial in Future Microbiology describing the potential of these therapeutics in the fight against antimicrobial resistant organisms. We also have been invited to submit a "Pearl manuscript" in PLOs Pathogens.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

The results of the studies described in this report add value to our existing intellectual property and patent describing the use of microbial GHs as anti-biofilm therapeutics.

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Major Task 1 Subtask 4:
During previous year, we have successfully developed a method to grow *A. fumigatus* and *P. aeruginosa* under flow biofilm conditions. Consistent with the anti-GAG activity of PslG and Sph3, GH-treated biofilms (at 2 µM for 2h) exhibited reduced SBA staining of *A. fumigatus* wild-type compared to the untreated sample. However, given that pulmonary biofilms are not subjected to flow conditions, and the activity of hydrolases has not proven different under flow and static conditions, we will not pursue these experiments further and are focusing our studies on static conditions and more relevant *in vivo* models of biofilm infection.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

No use of human subjects in this grant

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications

Zhang S, Chen Y, Ma Z, Chen Q, Ostapska H, Gravelat FN, Lu L, Sheppard DC. (2017) *PtaB, a lim-domain binding protein in* Aspergillus fumigatus *regulates biofilm formation and conidiation through distinct pathways*. Cell Microbiol. Nov 7. doi: 10.1111/cmi.12799.

Snarr BD, Howell PL, Sheppard DC. (2018) *Hoisted by their own petard: do microbial enzymes hold the solution to treating and preventing biofilm infections?* Future Microbiology 13:395-39. EPub: 0.2217/fmb-2017-0243, Feb 14, 2018.

D.J. Little, R. Pfoh, F. Le Mauff, N.C. Bamford, C. Notte, P. Baker, M. Guragain, H. Robinson, G.B. Pier, M. Nitz, R. Deora, D.C. Sheppard, P. L. Howell. (2018) *Molecular basis for the disruption of PNAG dependent biofilms by the periplasmic processing glycoside hydrolase PgaB*. Plos Pathogens 14(4) e1006998.

D. Asker, T.S. Awad, P. Baker, P. L. Howell, B.D. Hatton. (2018) *Non-eluting, surface bound enzymes disrupt surface attachment of bacteria by continuous biofilm polysaccharide degradation*. Biomaterials. 167, 168-176. (Co-SRA)

Kristin E. Low and P.L. Howell. *Carbohydrate active enzymes involved in exopolysaccharide biosynthesis in Gram-negative bacteria*. (2018) Curr Opin in Struct Biol. 53, 32-44.

Books or other non-periodical, one-time publications.

No publication in books or other non-periodical to be reported

Other publications, conference papers, and presentations.

Oral presentation at conferences:

Howell PL. *Microbial Biofilms: Molecular Mechanisms to Potential Therapeutics*. Genetech Inc, San Francisco, November 6-8, 2017.

Howell PL. *Microbial Biofilms: Mechanisms to potential therapeutics*. FASEB Meeting, Phoenix, USA. June 17-22, 2018.

Howell PL. *Microbial Biofilms: Mechanisms to potential therapeutics*. July 17-22, American Crystallographic Association Annual Meeting, Toronto, Canada. July 21-25, 2018.

Howell PL. *Microbiology: Microbes & the Body*. Youth Summer Program, Faculty of Medicine, University of Toronto, Canada. Aug 3, 2018

Sheppard DC. Keynote lecture at 16th Echinocandin Forum, Tokyo Japan. *Breaking the mold – from bench to bedside, novel therapeutics for* Aspergillus *infections*. March 10, 2018.

Sheppard DC. Invited Seminar at Faculty of Veterinary Medicine, University of Montreal, Canada. *Breaking the mold – from bench to bedside, novel therapeutics for* Aspergillus *infections*. Feb 21, 2018

Sheppard DC. Invited Speaker, US Department of Defense, JCP-2 Update, Fort Detrick, Maryland, USA. *Glycoside hydrolases as novel antibiofilm therapeutics*. May 20, 2018.

Sheppard DC. Invited Speaker, Banff Conference on Infectious Diseases, Alberta, Canada. *Breaking the mold – targeting biofilms of Aspergillus fumigatus.* May 25, 2018

Sheppard DC. Invited Speaker. Division of Infectious Diseases, Centre Hospitalier Universite de Montreal, Canada. *Breaking the mold: Development of new therapies to manage refractory fungal infections*. June 4, 2018.

Sheppard DC. Invited speaker, McGill Research Centre for Complex Traits Symposium, Montreal, Canada. *Breaking the mold:* Aspergillus *galactosaminogalactan*. June 11, 2018.

Zacharias C, Lehoux M, Bamford N, Sheppard DC. *The role of the immune response in hydrolase mediated prophylaxis of fungal infections*. McGill Department of Microbiology and Immunology Research Day, Montreal, Canada. June 08, 2018.

Posters at conference:

Snarr BD, Baker P, Bamford NC, Sato Y, Liu H, Lehoux M, Gravelat FN, Baistrocchi SR, Cerone RP, Filler SG, Howell PL, Sheppard DC. (2017) *Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity*. Trends in Molecular Mycology, Belgrade, Serbia. October 6-9, 2017.

Le Mauff F, Bamford N, Alnabelseya N, Howell PL, Sheppard DC. *Molecular mechanism of* Aspergillus *biofilm disruption by fungal and bacterial glycoside hydrolases*. 8th Advances Against Apergillosis Meeting, Lisbon, Portugal. Feb 1-3, 2018.

Lacdao I, Hicks B, Bamford N, Baker P, Sheppard DC, Howell PL. *Optimizing microbial glycoside hydrolase expression and purification for therapy development against bacterial and fungal biofilms*. Canadian Glycomics Symposium, Banff, Alberta, Canada. May 7-9th, 2018.

Pfoh R, Little DJ, Le Mauff F, Bamford NC, Notte C, Baker P, Guragain M, Robinson H, Pier GB, Nitz M, Deora R, Sheppard DC, Howell PL. *Molecular basis for the disruption of PNAG dependent biofilms by the dual-functional deacetylase and glycoside hydrolase PgaB*. American Crystallographic Association, Toronto, Canada. July 21-25, 2018.

- **Website(s) or other Internet site(s)**

No dissemination of the results through a website to be reported

- **Technologies or techniques**

No new technology to be reported.

- **Inventions, patent applications, and/or licenses**

<u>Patent</u>

1. Howell PL, Baker P, Alnabelseya N, Sheppard DC, Bamford N, Little D, Snarr B, United States Provisional Patent application (No. 62/008,836) entitled “Soluble Bacterial and Fungal Proteins and Methods and Uses Thereof in Inhibiting and Dispersing Biofilm”. National phase filing in US, Canada, Europe, Australia and Japan occurred between Dec 2016 – Jan 2017 (Actual date depends on jurisdiction).

- **Other Products**

No other product to be reported

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Na*me:* *P. Lynne Howell*
Project Role: PI
Research Identifier 0000-0002-2776-062X
Nearest person month worked 3
Contribution to Project: Responsible for the research performed at The Hospital for Sick Children.

Name: *Deepa Raju*
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 12
Contribution to Project: Dr Raju is co-responsible for protein expression and purification, as well as responsible for antibiotic potentiation and pharmacokinetic assays.

Name: *Ira Lacdao*
Project Role: Technician
Researcher Identifier (e.g. ORCID ID): Not available
Nearest person month worked: 12
Contribution to Project: Ms Lacdao is co-responsible for protein expression and purification.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: N/A

Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens *Pseudomonas aeruginosa* and *Aspergillus Fumigatus*

Grant Log #PR150786P1

Award #W81XWH-16-1-0284

PI: P. Lynne Howell **Org: The Hospital for Sick Children** **Award Amount:** $972,320

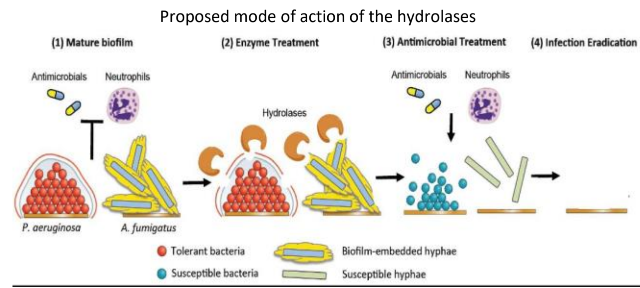
Study/Product Aim(s)

- Aim 1. To characterize the ability of microbial hydrolases to enhance the activity of antimicrobial agents in vitro.
- Aim 2. Perform preliminary tolerability and pharmacokinetic studies of candidate hydrolases in vivo.
- Aim 3. To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections in vivo.

Approach

A. fumigatus and *P. aeruginosa* are two lung opportunistic pathogens that embed themselves in a biofilm, becoming therefore more resistant to drugs and host defenses. We will test the use of four therapeutic enzymes, two hydrolases from fungal origin, two from bacterial origin, to render microorganisms more susceptible to antimicrobials *in vivo.* We will determine the concentration of hydrolases that are both efficient and well tolerated by the host. Our purpose is to conduct proof of concept studies to move these agents into early clinical trials.

Proposed mode of action of the hydrolases



Accomplishments in 2018: Demonstration of antibiotic potentiation by GHs on *P.aeruginosa* biofilm and with three classes of antibiotics. Determination of half-lives of GHs, alone and in one combination, in lung of immunocompetent and immunosuppressed mice. Development of GH variants for longer persistence in lung and lower immunogenicity. Study of GH impact on survival, microbial burden and host immune response in animal models of acute disease and chronic decrease. Test for synergy of GH and antibiotics in microbial burden of infected mice.

Timeline and Cost

Activities CY	16	17	18	19
Specific Aim 1 - Major task 1				
Specific Aim 2 - Major task 2				
Specific Aim 2 - Major task 3				
Specific Aim 2 - Major task 4				
Specific Aim 3 - Major task 5				
Specific Aim 3 - Major task 6				
Specific Aim 3 - Major task 7				
Estimated Budget ($K)	**$123**	**$327**	**$310**	**$212**

Updated: Sept.15, 2018

Goals/Milestones

CY16 Goal:

☑ Express and purify Sph3$_h$, Ega3$_h$, PelA$_h$, PslG$_h$ for Major Task 1

CY17 Goals/Milestones:

☑ Express and purify Sph3$_h$, Ega3$_h$, PelA$_h$, PslG$_h$ for Major Task 1 – 5

☑ Test hydrolase combinations in checkerboard assay with antibiotics

☑ Determine concentration of hydrolases and their combinations in animal tissue samples.

☑ Identification of hydrolase-antimicrobial combination that synergize against *A. fumigatus* and *P. aeruginosa*.

☑ Evaluation of pulmonary toxicity of candidate hydrolase regimens

❑ Evaluation of pharmacokinetics of candidate hydrolase regimens

CY18 Goals/Milestones:

☑ Express and purify Sph3$_h$, Ega3$_h$, PelA$_h$, PslG$_h$ for Major Task 5 - 7

❑ Develop variants of the candidate hydrolases (as required)

❑ Development of stable candidate hydrolases (as required)

CY19 Goals/Milestones:

☑ Express and purify Sph3$_h$, Ega3$_h$, PelA$_h$, PslG$_h$ for Major Task 5 – 7

❑ Confirm efficacy of candidate hydrolase regimens for the treatment of

- ❑ acute infection with *A. fumigatus* and *P. aeruginosa*
- ❑ chronic infection *with A. fumigatus* and *P. aeruginosa*

❑ Demonstrate proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa* infections

Budget Expenditure to Date:

Projected Expenditure: $ 675,222

Actual Expenditure: $ 701,714

Received: 29 June 2017 | Revised: 24 October 2017 | Accepted: 25 October 2017
DOI: 10.1111/cmi.12799

WILEY

RESEARCH ARTICLE

PtaB, a lim-domain binding protein in *Aspergillus fumigatus* regulates biofilm formation and conidiation through distinct pathways

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 31470193; Fonds de Recherche Quebec Santé; Canadian Institutes of Health Research, Grant/Award Numbers: 123306 and 81361; The priority academic program development (PAPD) of Jiangsu Higher Education Institutions

Abstract

The exopolysaccharide galactosaminogalactan (GAG) plays an important role in mediating adhesion, biofilm formation, and virulence in the pathogenic fungus *Aspergillus fumigatus*. The developmental modifiers MedA, StuA, and SomA regulate GAG biosynthesis, but the mechanisms underlying this regulation are poorly understood. PtaB is a lim-domain binding protein that interacts with the transcription factor SomA and is required for normal conidiation and biofilm formation. Disruption of *ptaB* resulted in impaired GAG production and conidiation in association with a markedly reduced expression of GAG biosynthetic genes (*uge3* and *agd3*), developmental regulators (*medA* and *stuA*), and genes involved in the core conidiation pathway. Overexpression of *medA* and dual overexpression of *uge3* and *agd3* in the Δ*ptaB* mutant increased biofilm formation but not conidiation, whereas overexpression of core conidiation genes rescued conidiation but not biofilm formation. Overexpression of *stuA* modestly increased both conidiation and biofilm formation. Analysis of *ptaB* truncation mutants revealed that overexpression of the lim-domain binding region restored conidiation but not biofilm formation, suggesting that *ptaB* may govern these processes by interacting with different partners. These studies establish that PtaB governs GAG biosynthesis at the level of substrate availability and polymer deacetylation and that PtaB-mediated biofilm formation and conidiation are largely independent pathways.

KEYWORDS

Aspergillus fumigatus, Biofilm, Conidiation, Galactosaminogalactan

1 | INTRODUCTION

Aspergillus fumigatus is an opportunistic mould that causes invasive pulmonary infections in immunosuppressed patients. Despite antifungal treatment with the currently available antifungal agents, the mortality of invasive aspergillosis (IA) remains between 50% and 95% (Abad et al., 2010). There is therefore a pressing need for novel therapeutic strategies to treat or prevent IA. A better understanding of the pathogenesis of IA is one approach that may inform the development of new therapeutic targets.

The ability of *A. fumigatus* to adhere to host cells is a key factor in the pathogenesis of IA and is required for host cell invasion and virulence (de Groot, Bader, de Boer, Weig, & Chauhan, 2010; Liu et al., 2016; Sheppard, 2011; Tronchin, Pihet, Lopes-Bezerra, & Bouchara, 2008). The adherence of *A. fumigatus* hyphae to host cells is mediated by production of the adhesive exopolysaccharide galactosaminogalactan (GAG; Briard, Muszkieta, Latge, & Fontaine, 2016; Gravelat et al., 2011). GAG is synthesised through the activity of the protein products of a five-gene cluster. These genes encode a glucose 4-epimerase (*uge3*) required for the production of UDP-

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Editorial

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Future MICROBIOLOGY

Hoisted by their own petard: do microbial enzymes hold the solution to treating and preventing biofilm infections?

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"The rising rates of antimicrobial resistance have raised the specter of a post-antibiotic era within our lifetime and highlighted the need for novel strategies to combat antimicrobial resistance."

First draft submitted: 20 October 2017; Accepted for publication: 30 October 2017; Published online: 14 February 2018

Keywords: biofilm • glycoside hydrolase • therapeutics

Biofilms are microbial communities that grow within a self-produced heterogeneous extracellular matrix (ECM). The production of ECM by pathogenic bacteria and fungi during biofilm growth confers a number of advantages during infection including mediating adherence to host tissues and biomedical devices as well as enhancing resistance to antimicrobial agents and host immune defenses. Exopolysaccharides are a key component of the ECM and have been directly implicated in mediating adhesion, immune evasion and antimicrobial resistance. Recent studies have suggested that microbial enzymes can be used to degrade the biofilm exopolysaccharides of several pathogens and increase their susceptibility to antimicrobials *in vitro* and *in vivo*. We will review current progress in this area, and highlight areas for future research and development.

Current lines of research

Bacterial biofilms

Staphylococcus species

The polysaccharide poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG) is an important biofilm component of many *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, as well as some Gram-negative pathogens such as *Acinetobacter baumannii, Klebsiella pneumonia, Yersinia pestis* and *Escherichia coli* [1]. A recombinant glycoside hydrolase (GH) known as DispersinB originating from *Actinobacillus actinomycetemcomitans* cleaves PNAG [2], and can disrupt PNAG-dependent biofilms of these organisms *in vitro*. DispersinB treatment enhances the antimicrobial activity of cefamandole nafate against both *S. aureus* and *S. epidermidis* biofilms *in vitro* [3]. Animal model studies have also demonstrated that DispersinB enhanced the antimicrobial effects of silver nanoparticles in a mouse methicillin-resistant *S. aureus* chronic wound model [4]. Systemic use of DispersinB in other models of Staphylococcal infection has not been reported.

Yersinia species

NghA is a GH active against PNAG produced by *Yersinia pseudotuberculosis*. The related pathogen *Yersinia pestis* lacks a functional *nghA* gene and, as a result, is able to form PNAG-based biofilms within its flea vector [5]. Heterologous expression of *nghA* in *Y. pestis* prevented biofilm formation in the flea gut, and biofilm formation by *Y. pestis* and *S. epidermidis in vitro* was inhibited by recombinant NghA [5]. The effects of NghA on the susceptibility of *Yersinia* sp, or on virulence in mammalian infection models have yet to be reported.

RESEARCH ARTICLE

PgaB orthologues contain a glycoside hydrolase domain that cleaves deacetylated poly-β(1,6)-N-acetylglucosamine and can disrupt bacterial biofilms

Dustin J. Little[1,2☯¤], Roland Pfoh[1☯], François Le Mauff[3,4], Natalie C. Bamford[1,2], Christina Notte[1], Perrin Baker[1], Manita Guragain[5,6], Howard Robinson[7], Gerald B. Pier[8], Mark Nitz[9], Rajendar Deora[5,6], Donald C. Sheppard[3,4], P. Lynne Howell[1,2*]

1 Program in Molecular Medicine, The Hospital for Sick Children, Toronto, ON, Canada, 2 Department of Biochemistry, University of Toronto, Toronto, ON, Canada, 3 Departments of Medicine and of Microbiology and Immunology, McGill University, Montréal, QC, Canada, 4 Infectious Diseases and Immunity in Global Health Program, Research Institute of the McGill University Health Centre, Montréal, QC, Canada, 5 Department of Microbiology and Immunology, Wake Forest School of Medicine, Winston-Salem, NC, United States of America, 6 Department of Microbial Infection and Immunity, The Ohio State University Wexner Medical Center, Columbus, OH, United States of America, 7 Photon Sciences Division, Brookhaven National Laboratory, Upton, NY, United States of America, 8 Division of Infectious Diseases, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, United States of America, 9 Department of Chemistry, University of Toronto, Toronto, ON, Canada

☯ These authors contributed equally to this work.
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* howell@sickkids.ca

OPEN ACCESS

Citation: Little DJ, Pfoh R, Le Mauff F, Bamford NC, Notte C, Baker P, et al. (2018) PgaB orthologues contain a glycoside hydrolase domain that cleaves deacetylated poly-β(1,6)-N-acetylglucosamine and can disrupt bacterial biofilms. PLoS Pathog 14(4): e1006998. https://doi.org/10.1371/journal.ppat.1006998

Editor: David Skurnik, Channing Laboratory, Brigham and Women's Hospital, UNITED STATES

Received: October 20, 2017

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Data Availability Statement: Coordinates of the structure have been deposited in the Protein data bank PDB code: 6AU1.

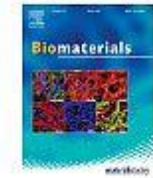
Funding: Research described in this paper is supported by operating grants from the Canadian Institutes of Health Research (CIHR) (#43998 to PLH, #89708 to MN, and #81361 to PLH and DCS) and with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health

Abstract

Poly-β(1,6)-N-acetyl-D-glucosamine (PNAG) is a major biofilm component of many pathogenic bacteria. The production, modification, and export of PNAG in Escherichia coli and Bordetella species require the protein products encoded by the pgaABCD operon. PgaB is a two-domain periplasmic protein that contains an N-terminal deacetylase domain and a C-terminal PNAG binding domain that is critical for export. However, the exact function of the PgaB C-terminal domain remains unclear. Herein, we show that the C-terminal domains of Bordetella bronchiseptica PgaB ($PgaB_{Bb}$) and E. coli PgaB ($PgaB_{Ec}$) function as glycoside hydrolases. These enzymes hydrolyze purified deacetylated PNAG (dPNAG) from Staphylococcus aureus, disrupt PNAG-dependent biofilms formed by Bordetella pertussis, Staphylococcus carnosus, Staphylococcus epidermidis, and E. coli, and potentiate bacterial killing by gentamicin. Furthermore, we found that $PgaB_{Bb}$ was only able to hydrolyze PNAG produced in situ by the E. coli PgaCD synthase complex when an active deacetylase domain was present. Mass spectrometry analysis of the PgaB-hydrolyzed dPNAG substrate showed a GlcN-GlcNAc-GlcNAc motif at the new reducing end of detected fragments. Our 1.76 Å structure of the C-terminal domain of $PgaB_{Bb}$ reveals a central cavity within an elongated surface groove that appears ideally suited to recognize the GlcN-GlcNAc-GlcNAc motif. The structure, in conjunction with molecular modeling and site directed mutagenesis led to the identification of the dPNAG binding subsites and D474 as the probable catalytic

Biomaterials

Volume 167, June 2018, Pages 168-176



Non-eluting, surface-bound enzymes disrupt surface attachment of bacteria by continuous biofilm polysaccharide degradation

Dalal Asker [a, b], Tarek S. Awad [a], Perrin Baker [c], P. Lynne Howell [c, d], Benjamin D. Hatton [a]

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Abstract

Bacterial colonization and biofilm formation on surfaces are typically mediated by the deposition of exopolysaccharides and conditioning protein layers. *Pseudomonas* aeruginosa is a nosocomial opportunistic pathogen that utilizes strain-specific exopolysaccharides such as Psl, Pel or alginate for both initial surface attachment and biofilm formation. To generate surfaces that resist *P. aeruginosa* colonization, we covalently bound a Psl-specific glycoside hydrolase ($PslG_h$) to several, chemically-distinct surfaces using amine functionalization (APTMS) and glutaraldehyde (GDA) linking. *In situ* quartz crystal microbalance (QCM) experiments and fluorescence microscopy demonstrated a complete lack of Psl adsorption on the $PslG_h$-bound surfaces. Covalently-bound $PslG_h$ was also found to significantly reduce *P. aeruginosa* surface attachment and biofilm formation over extended growth periods (8 days). The $PslG_h$ surfaces showed a ~99.9% (~3-log) reduction in surface associated bacteria compared to control (untreated) surfaces, or those treated with inactive enzyme. This work demonstrates a non-eluting 'bioactive' surface that specifically targets a mechanism of cell adhesion, and that surface-bound glycoside hydrolase can significantly reduce surface colonization of bacteria through local, continuous enzymatic degradation of exopolysaccharide (Psl). These results have significant implications for the surface design of medical devices to keep bacteria in a planktonic state, and therefore susceptible to antibiotics and antimicrobials.

Development of New Therapeutics Targeting Biofilm Formation by the Opportunistic Pulmonary Pathogens *Pseudomonas aeruginosa* and *Aspergillus Fumigatus*

Grant Log #PR150786P1
Award #W81XWH-16-1-0284

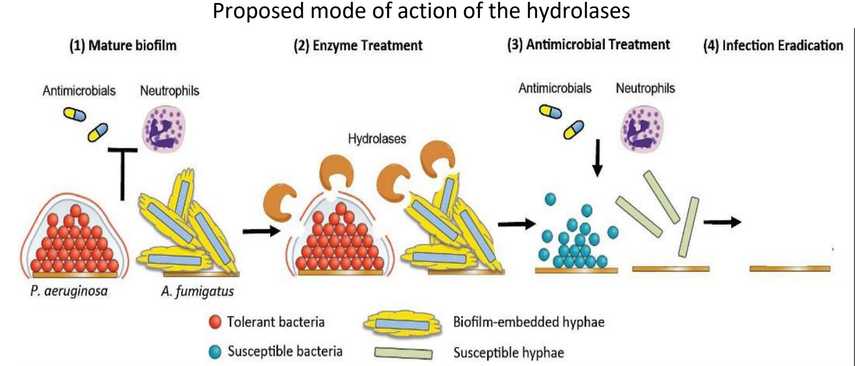
PI: P. Lynne Howell **Org: The Hospital for Sick Children** **Award Amount:** $972,320

Study/Product Aim(s)

- Aim 1. To characterize the ability of microbial hydrolases to enhance the activity of antimicrobial agents in vitro.
- Aim 2. Perform preliminary tolerability and pharmacokinetic studies of candidate hydrolases in vivo.
- Aim 3. To evaluate candidate hydrolases alone and in combination with antimicrobial agents in the treatment of experimental *A. fumigatus* and *P. aeruginosa* pulmonary infections in vivo.

Approach

A. fumigatus and *P. aeruginosa* are two lung opportunistic pathogens that embed themselves in a biofilm, becoming therefore more resistant to drugs and host defenses. We will test the use of four therapeutic enzymes, two hydrolases from fungal origin, two from bacterial origin, to render microorganisms more susceptible to antimicrobials *in vivo*. We will determine the concentration of hydrolases that are both efficient and well tolerated by the host. Our purpose is to conduct proof of concept studies to move these agents into early clinical trials.



Accomplishments in 2018: Demonstration of antibiotic potentiation by GHs on *P.aeruginosa* biofilm and with three classes of antibiotics. Determination of half-lives of GHs, alone and in one combination, in lung of immunocompetent and immunosuppressed mice. Development of GH variants for longer persistence in lung and lower immunogenicity. Study of GH impact on survival, microbial burden and host immune response in animal models of acute disease and chronic decrease. Test for synergy of GH and antibiotics in microbial burden of infected mice.

Timeline and Cost

Activities CY	16	17	18	19
Specific Aim 1 - Major task 1				
Specific Aim 2 - Major task 2				
Specific Aim 2 - Major task 3				
Specific Aim 2 - Major task 4				
Specific Aim 3 - Major task 5				
Specific Aim 3 - Major task 6				
Specific Aim 3 - Major task 7				
Estimated Budget ($K)	**$123**	**$327**	**$310**	**$212**

Updated: Sept.15, 2018

Goals/Milestones

CY16 Goal:

☑ Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$, $PslG_h$ for Major Task 1

CY17 Goals/Milestones:

☑ Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$, $PslG_h$ for Major Task 1 – 5
☑ Test hydrolase combinations in checkerboard assay with antibiotics
☑ Determine concentration of hydrolases and their combinations in animal tissue samples.
☑ Identification of hydrolase-antimicrobial combination that synergize against *A. fumigatus* and *P. aeruginosa*.
☑ Evaluation of pulmonary toxicity of candidate hydrolase regimens
❑ Evaluation of pharmacokinetics of candidate hydrolase regimens

CY18 Goals/Milestones:

☑ Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$, $PslG_h$ for Major Task 5 - 7
❑ Develop variants of the candidate hydrolases (as required)
❑ Development of stable candidate hydrolases (as required)

CY19 Goals/Milestones:

☑ Express and purify $Sph3_h$, $Ega3_h$, $PelA_h$, $PslG_h$ for Major Task 5 – 7
❑ Confirm efficacy of candidate hydrolase regimens for the treatment of
  ❑ acute infection with *A. fumigatus* and *P. aeruginosa*
  ❑ chronic infection *with A. fumigatus* and *P. aeruginosa*
❑ Demonstrate proof-of-concept for candidate hydrolases for use in treatment of *A. fumigatus* and *P. aeruginosa* infections

Budget Expenditure to Date:

Projected Expenditure: $ 675,222
Actual Expenditure: $ 701,714