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14. ABSTRACT

The purpose of this project is to demonstrate that MUC1-ED synthetic peptides protect against Pseudomonas aeruginosa lung infection using both in vitro and in vivo model systems. For months 1-12 of the project, studies were conducted demonstrating that MUC1-ED 20-, 40-, 60-, 80-, and 100-mer peptides 1) bound to P. aeruginosa and its flagella; 2) inhibited P. aeruginosa and flagella binding to human lung cells, and bacterial motility; 3) were not cytotoxic to lung cells; 4) did not affect lung cell barrier formation; 5) exhibited no damage to mouse lung, liver or kidney; and 6) displayed appropriate lung bioavailability in vivo. For months 13-42, studies were performed demonstrating that 1) all peptides exhibited appropriate pharmacokinetic parameters in vivo; and 2) the 80-mer and 100-mer MUC1-ED peptides reduced lung infection and inflammation, and improved survival, when administered to mice simultaneously with, prior to, or following sublethal or lethal infection with laboratory or clinical strains of P. aeruginosa, compared with scrambled control peptides.

15. SUBJECT TERMS
mucin-1; MUC1; synthetic peptide; Pseudomonas aeruginosa; flagella; lung; infection; antibiotic; epithelial; adhesion, motility

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1. INTRODUCTION: More than 250,000 *Pseudomonas aeruginosa* infections are estimated to occur annually in the U.S. with an associated healthcare cost greater than $2 billion. The widespread use of antimicrobial antibiotics has led to the emergence of difficult-to-treat, multidrug-resistant *Pseudomonas aeruginosa* strains, necessitating the use of less-desirable, alternative drugs that are severely limited by their decreased effectiveness and increased toxicity and costs. Therefore, alternative therapies to treat *Pseudomonas aeruginosa* infections are urgently needed. To address this unmet need, we propose that synthetic peptide drugs derived from a naturally-produced lung protein, MUC1, will offer new treatment options for patients with *Pseudomonas aeruginosa* lung infections. MUC1 peptide drugs will provide a novel class of *Pseudomonas aeruginosa* therapeutics with reduced off-target effects and enhanced effectiveness in the clinic. Because the mode of action of MUC1 peptide drugs in preventing *Pseudomonas aeruginosa* infection relies on inhibiting bacterial adhesion to human lung cells, rather than a direct bactericidal approach as with current antibiotics, MUC1 peptides are unlikely to spur the development of bacterial resistance. In this project, MUC1 peptides will be tested for their ability to inhibit *Pseudomonas aeruginosa* lung infection using both in vitro and in vivo techniques. We expect to identify at least 1 peptide with a high therapeutic index for treating an ongoing *Pseudomonas aeruginosa* infection, and that is readily bioavailable and nontoxic following aerosol delivery to mouse lungs in an experimental model of *Pseudomonas aeruginosa* pneumonia. We envision that, once commercialized, MUC1 peptide therapeutics will be used in combination with current standard of care antibiotics to increase patient survival beyond that achieved by antibiotics alone.

2. **Keywords:** mucin-1; MUC1; synthetic peptide; *Pseudomonas aeruginosa*; flagella; lung; infection; antibiotic; epithelial; adhesion, motility

3. **ACCOMPLISHMENTS**

3A. Major goals of the project (months 1-42) (Note: The original project goals were approved covering the time period 2/15/19-8/14/20. Due to University of Maryland, Baltimore COVID-19 research restrictions, a 12 month no-cost extension was approved on 7/7/20 covering the time period 8/15/20-8/14/21. Subsequently a second 12 month no-cost extension was approved on 7/7/21 covering the time period 8/15/2021-8/14/2022. Please see section 5 for more information.)

- 3A1. Major Task 1. Determine MUC1-ED synthetic peptide binding to *P. aeruginosa* and its flagella.
  - 3A1A. Subtask 1. Obtain all necessary materials and supplies, including MUC1-ED synthetic peptides, *P. aeruginosa* strains, and human airway epithelial cells (month 1).
  - 3A1B. Subtask 2. Test *P. aeruginosa* strains on motility agar (month 1).
  - 3A1C. Subtask 3. Purify *P. aeruginosa* flagellins and cross-link to agarose (month 2).
  - 3A1D. Subtask 4. Measure binding affinity of Alexa Fluor 488-labeled MUC1-ED peptides for *P. aeruginosa* and flagellin-agarose using fluorescence polarization assays (months 2-4).
- 3A2. Major Task 2. MUC1-ED peptide inhibition of *P. aeruginosa* and flagella binding to airway epithelial cells.
  - 3A2A. Subtask 1. Perform competitive binding assays with MUC1-ED peptides for *P. aeruginosa* and flagellin binding to human airway epithelial cells (months 5-7).
  - 3A2B. Subtask 2. Perform bacterial motility assays with MUC1 peptides. (months 8-9).
- 3A3. Major Task 3. Test Effects of MUC1-ED peptides on airway epithelial cell toxicity and monolayer integrity.
  - 3A3A. Subtask 1. Test MUC1-ED peptides for airway epithelial cell toxicity and monolayer integrity, and measure therapeutic index for the optimum MUC1-ED peptides (months 10-12).
- 3A4A. Major Task 4A (optional). If necessary, perform alternative protocols to resolve potential pitfalls with MUC1-ED synthetic peptides.

3A4A1. Subtask 1. MUC1-ED gene segments will be synthesized corresponding to 20-, 40-, 60-, 80-, and 100-amino acid regions of the VNTR, codon-optimized for expression in *E. coli*, and the corresponding recombinant MUC1-ED proteins purified (month 12). Major Task 4A, Subtask 1 was not performed

3A4B. Major Task 4B. Obtain IACUC approval.

3A5B1. Subtask 1. Obtain USAMRMC ORP and local IACUC approval (months 9-12).

3A5. Major Task 5. Purchase mice.

3A5A. Purchase C57BL/6 mice from a certified commercial vendor (n=100) (month 12).

3A6. Major Task 6. *P. aeruginosa* pretreatment with MUC1-ED peptides prior to infection.

3A6A. Subtask 1. Perform dose tolerance and pharmacokinetic studies of MUC1-ED peptides in mice (month 12).

3A6B. Subtask 2. Assess lung CFUs, BALF cytokines, lung histopathology, and survival following infection of mice with *P. aeruginosa* pretreated with the optimum MUC1-ED peptide (months 13-20).

3A7. Major Task 7. Pre-infection prophylaxis treatment study.

3A7A. Subtask 1. Assess lung CFUs, BALF cytokines, lung histopathology, and survival when the optimum MUC1-ED peptide is administered as aerosols before *P. aeruginosa* infection of mice (months 21-30).

3A8. Major Task 8. Post-infection therapeutic study.

3A7A. Subtask 1. Assess lung CFUs, BALF cytokines, lung histopathology, and survival when the optimum MUC1-ED peptide is administered as aerosols after *P. aeruginosa* infection of mice (months 31-42).

3B. Accomplishments under these goals (months 1-42)

3B1. Major Task 1. Determine MUC1-ED synthetic peptide binding to *P. aeruginosa* and its flagella.

3B1A. Subtask 1. Obtain all necessary materials and supplies, including MUC1-ED synthetic peptides, *P. aeruginosa* strains, and human airway epithelial cells (month 1). Materials and supplies were purchased from commercial vendors or obtained from in-house sources.

Table 1. Materials and Supplies

Item	Source
Blood agar plates	VWR
TSA agar plates	VWR
Ethanol, 4x4L	Sigma-Aldrich
PNA-agarose	Vector Labs
BSA, 30%	VWR
Crystal violet	Sigma-Aldrich
Acetic acid	Sigma-Aldrich
MUC1-ED Ab	LSBio
Tissue homogenizer	VWR
APS, TEMED	Sigma-Aldrich
Protein A/G agarose	Invitrogen
P. aeruginosa, PAK	In-house
P. aeruginosa, PA01	In-house
P. aeruginosa, clinical #1	In-house
P. aeruginosa, clinical #2	In-house
P. aeruginosa, clinical #3	In-house
P. aeruginosa, clinical #4	In-house
P. aeruginosa, clinical #5 (MDR)	In-house
MUC1-ED synthetic peptide, 20-mer	In-house
MUC1-ED synthetic peptide, 40-mer	In-house
MUC1-ED synthetic peptide, 60-mer	In-house

MUC1-ED synthetic peptide, 80-mer	In-house
MUC1-ED synthetic peptide, 100-mer	In-house
Normal human bronchial epithelial (NHBE) cells	In-house

3B1B. Subtask 2. Test *P. aeruginosa* strains on motility agar (month 1). *P. aeruginosa* laboratory strains (PAK, PAO1, PAK/ΔfliC), and isolates from patients with lung infections (clinical strains 50241, 50255, 50296, 50312, 50327), including multidrug-resistant (MDR) bacteria, were washed and resuspended in Luria-Bertani (LB) culture broth. The bacteria were stab-inoculated into 0.3% LB agar plates, incubated overnight, and colony diameters (length, width) measured as an indicator of bacterial motility. All bacteria exhibited motility, with the exception of the flagella-deficient PAK/ΔfliC negative control (Table 2).

Table 2. *Pseudomonas aeruginosa* Motility

P. aeruginosa	Diameter 1 (mm)	Diameter 2 (mm)	Average
PAK	11.0	11.5	11.3
PA01	14.5	14.5	14.5
Clinical strain 50241	12.0	13.0	12.5
Clinical strain 50255	10.5	10.0	10.3
Clinical strain 50296	11.5	15.5	13.5
Clinical strain 50312	12.5	12.5	12.5
Clinical strain 50327	10.5	10.0	10.3
PAK/ΔfliC	0.0	0.0	0.0

3B1C. Subtask 3. Purify *P. aeruginosa* flagellins and cross-link to agarose (month 2). An overnight culture of *P. aeruginosa* strains PA01, PAK, and the flagellin-deficient PAK/ΔfliC were centrifuged at 5,000xg for 30 min, resuspended in Krebs-Ringer buffer, and incubated for 1 h at 37°C. The bacteria were collected by centrifugation, and the flagella-containing supernatant filtered through a 0.22-µm pore membrane and the filtrate boiled for 20 min. The filtrate was concentrated by centrifugal ultrafiltration, adjusted to pH 6.0, and flagellin proteins were purified from the isolated flagella by sequential ion exchange chromatography using Macro-Prep High S and Macro-Prep HighQ supports. Aliquots of column fractions were resolved by SDS-PAGE and stained with Coomassie Blue to detect the 40-50 kiloDalton (kDa) flagellin protein bands (Figure 1). Other column aliquots were processed for flagellin immunoblotting and for *P. aeruginosa* pilin immunoblotting to confirm the absence of pilin contamination (Figure 2). Flagellin-containing fractions were incubated with polymyxin B-agarose to remove endotoxin, after which less than 0.1 endotoxin unit/µg of protein was detected by the *Limulus* amebocyte lysate assay. For flagellin chemical cross-linking to agarose, solutions of 1.0 mg/ml of the a-type and b-type flagellins were added to NHS-activated agarose in a spin column and mixed with end-over-end rotation for 1 h at 22°C. The agarose was collected by centrifugation at 1,000xg for 1 min , and washed once with PBS, pH 7.4. The agarose was incubated with 1.0 M Tris, pH 8.0 for 1 h at 22°C to block unreacted sites, washed twice with PBS, pH 7.4, and stored at 4°C until use.

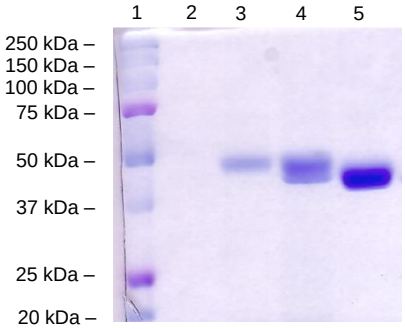


Figure 1. Purified flagellins from *P. aeruginosa.* Lane 1, prestained molecular weight marker proteins. Lane 2, flagellin-deficient strain PAK/ΔfliC. Lane 3, a-type flagellin strain PAK. Lane 4, b-type flagellin strain PA01. Coomassie blue stain.

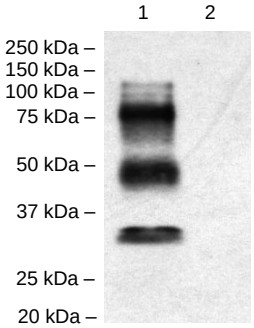


Figure 2. Pilin immunoblot to confirm the absence of pilin contamination. Lane 1, positive pilin control. Lane 2, a-type flagellin purified from strain PAK.

3B1D. <u>Subtask 4. Measure binding affinity of Alexa Fluor 488-labeled MUC1-ED peptides for *P. aeruginosa* and flagellin-agarose using fluorescence polarization assays (months 2-4).</u> The 20-mer monomer MUC1-ectodomain (MUC1-ED) synthetic peptide (GSTAPPAHGVTSAPDTRPAP) and head-to-tail 40-mer, 60-mer, 80-mer, and 100-mer peptides of the 20-mer were labeled with Alexa Fluor 488. $1x10^6$ colony forming units (CFUs)/ml of *P. aeruginosa* strains PAK or PA01, or an equivalent amount of a-type or b-type flagella-agarose, were incubated for 1 hr at 4°C with increasing concentrations of labeled peptides (0, 0.5, 1.0, 2.5, 5.0, 10, 20, 20 ng/well). Unbound peptide was removed by centrifugation at 5,000xg for 10 min at 4°C and the remaining bound fluorescence measured at 488 nm. Fluorescence polarization (Figure 3) and Scatchard analysis (Figure 4) of the binding data was performed, and the dissociation constant (Kd) of each peptide-flagellin interaction was determined in nM. The results demonstrated that the 20-mer, 40-mer, 60-mer, 80-mer, and 100-mer peptides bound to both the PAK and PA01 bacterial strains, and to both a-type and b-type flagella. Scatchard analysis of the peptide binding demonstrated that the peptides bound to both *Pseudomonas aeruginosa* and its flagella with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

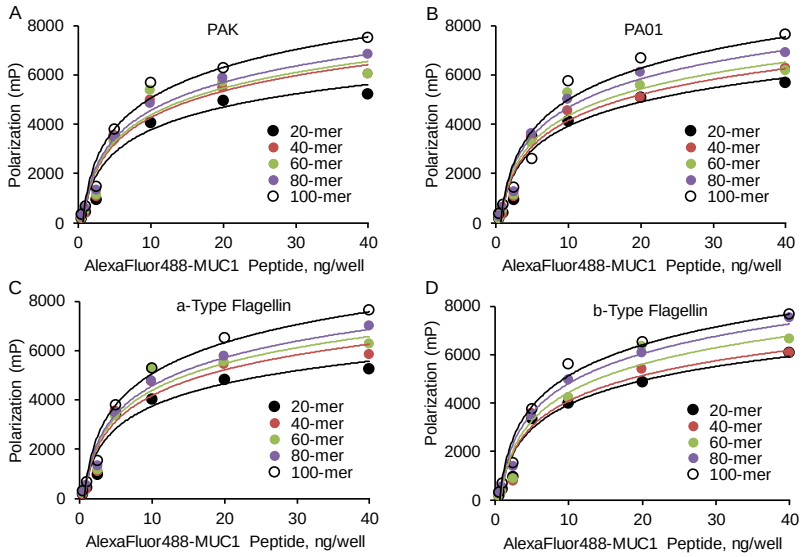


Figure 3. Fluorescence polarization analysis of AlexaFluor488 MUC1 peptides binding to (A) PAK bacteria, (B) PA01 Bacteria, (C) a-type flagellin, and (D) b-type flagellin.

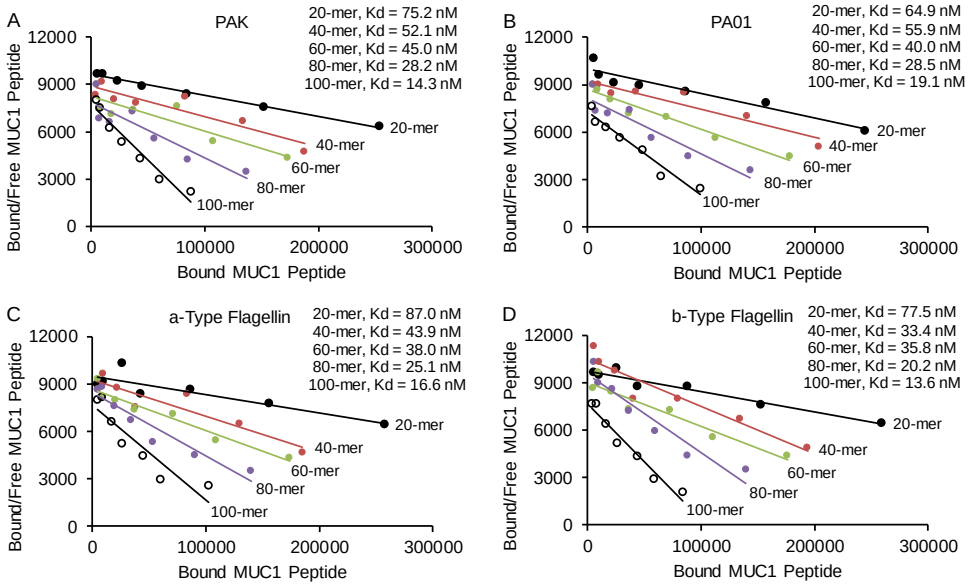


Figure 4. Scatchard analysis of AlexaFluor488 MUC1-ED peptides binding to (A) PAK bacteria, (B) PA01 Bacteria, (C) a-type flagellin, and (D) b-type flagellin. The affinity (Kd) for each binding interaction is indicated in the upper right of each panel.

3B2. Major Task 2. MUC1-ED peptide inhibition of *P. aeruginosa* and flagella binding to airway epithelial cells.

3B2A. <u>Subtask 1.</u> <u>Perform competitive binding assays with MUC1-ED peptides for *P. aeruginosa* and flagellin binding to human airway epithelial cells (months 5-7).</u> NHBE cells in 24-well plates ($2x10^5$ cells/well) were incubated with *P. aeruginosa* laboratory strains, PAK and PA01, and clinical strain, 50241 ($2x10^7$ CFUs/well) in the presence of increasing concentrations of MUC1-ED peptides or scrambled control peptides. The cells were washed and bacterial CFUs bound to the cells quantified on agar plates (Figure 5). Binding of Alexa Fluor 488-labeled a-type and b-type flagella to NHBE cells will be studied in the presence of increasing concentrations of MUC1-ED peptides or scrambled controls (Figure 6). Half-maximal inhibitory concentration (IC_{50}) values for the peptides were calculated based on the binding inhibition curves. The results demonstrated that all of the peptides dose-dependently inhibited *P. aeruginosa* and flagella binding to the cells with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

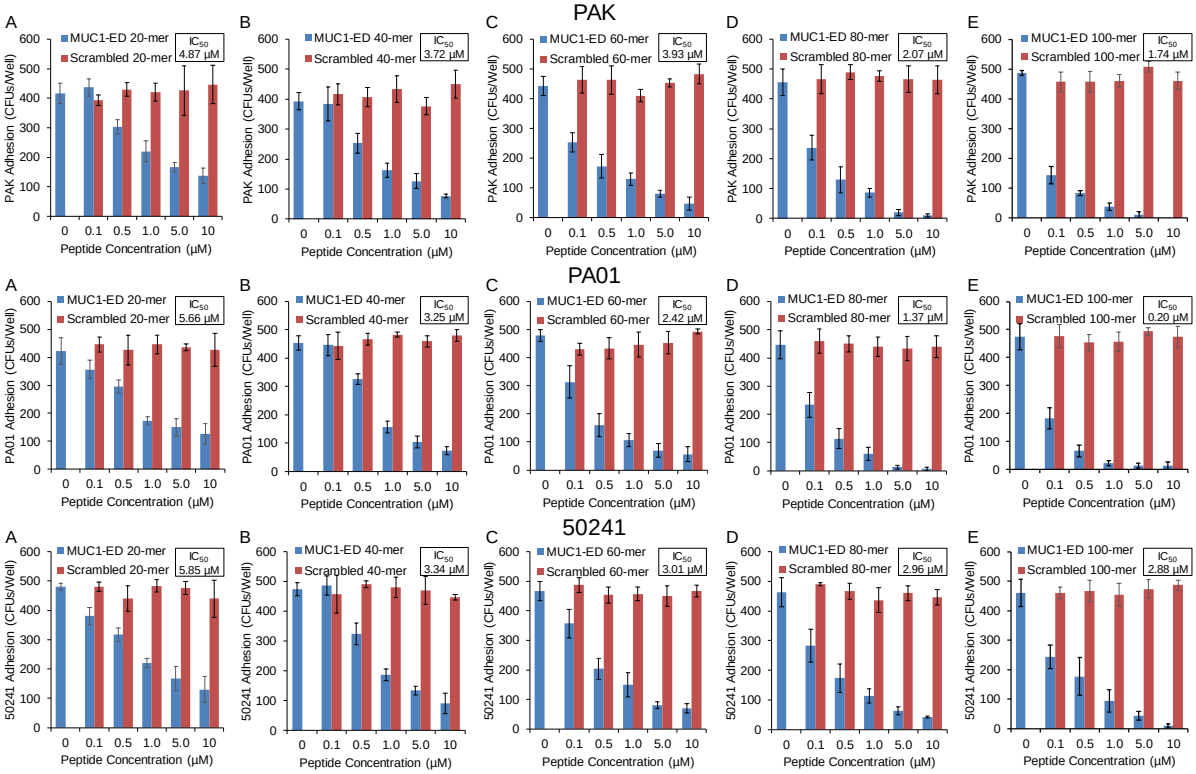


Figure 5. Inhibition of binding of *P. aeruginosa* strains PAK (upper), PA01 (middle), and 50241 (lower) to NHBE cells by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides. The IC_{50} value for each MUC1-ED peptide inhibition is indicated in the upper right of each panel.

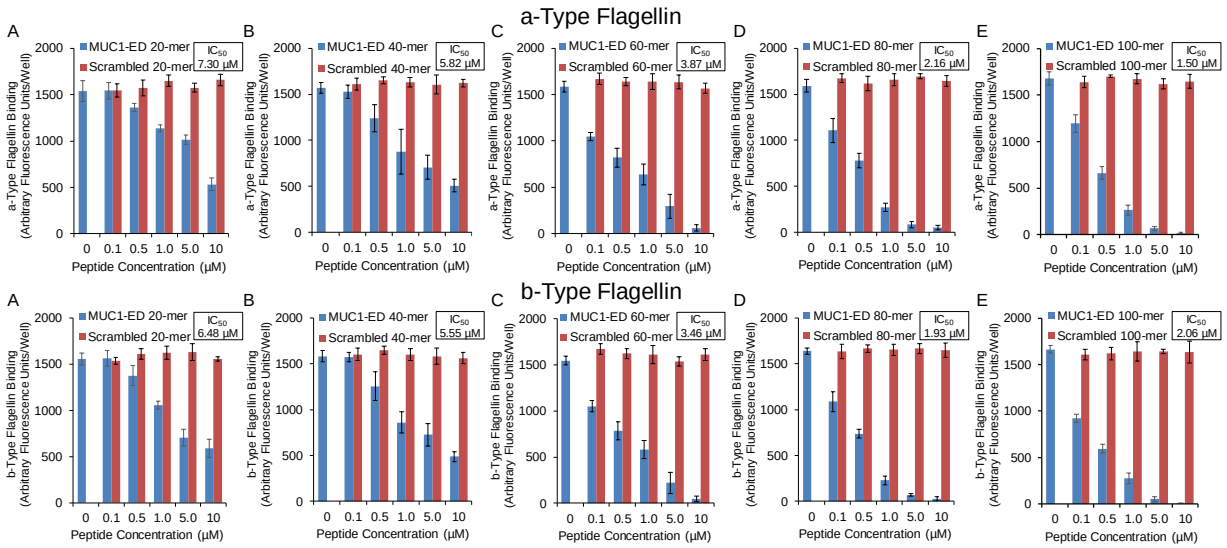


Figure 6. Inhibition of binding of *P. aeruginosa* a-type flagellin (upper) and b-type flagellin (lower) to NHBE cells by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides. The IC_{50} value for each MUC1-ED peptide inhibition is indicated in the upper right of each panel.

3B2B. <u>Subtask 2. Perform bacterial motility assays with MUC1 peptides. (months 8-9).</u> Increasing concentrations of the MUC1-ED peptides were incorporated into 0.3% motility agar. *P. aeruginosa* laboratory strains PAK and PA01, and clinical strain 50241 were stab-inoculated into the agar, incubated overnight, and colony diameters measured as an indicator of bacterial motility. As shown in Figure 7, the peptides dose-dependently inhibited motility of all bacterial strains with the rank order 100-mer > 80-mer > 60-mer > 40-mer > 20-mer. As a negative control, the flagella-deficient PAK/ΔfliC strain did not exhibit bacterial motility (Table 2).

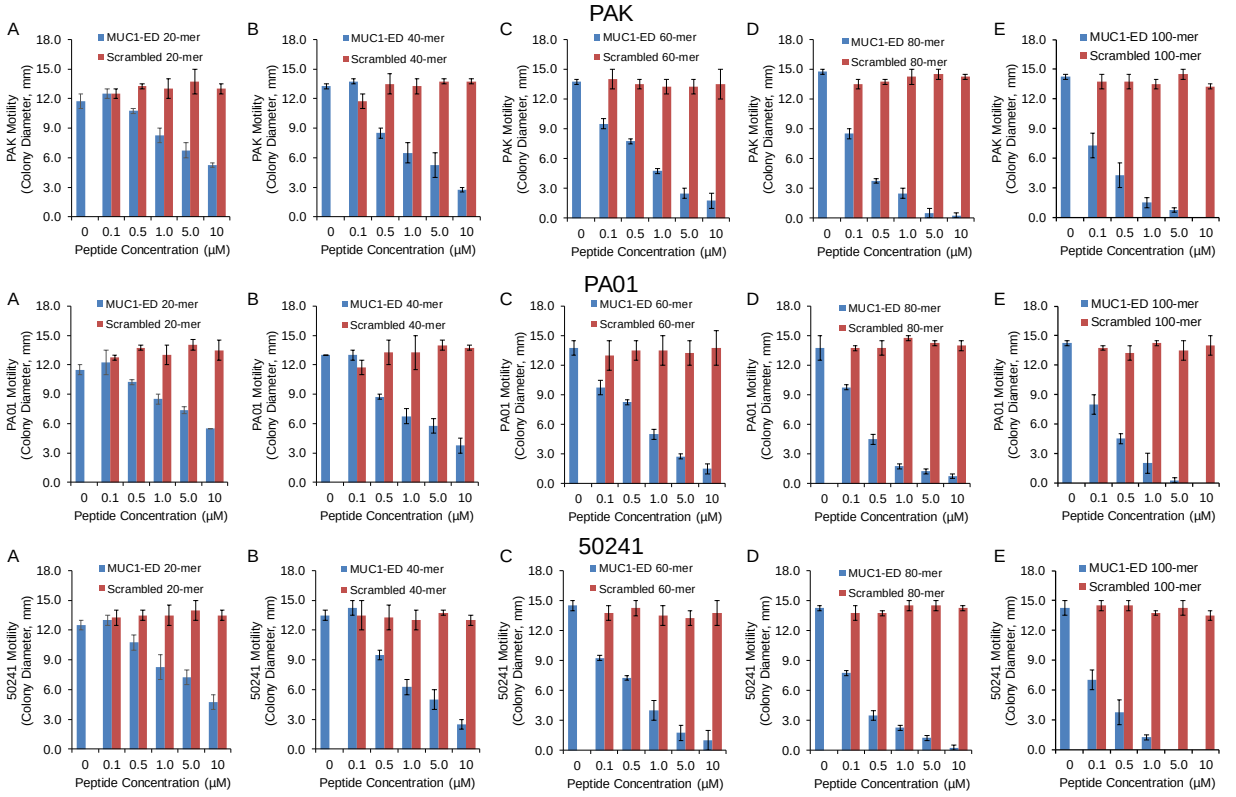


Figure 7. Inhibition of motility of *P. aeruginosa* strains PAK (upper), PA01 (middle), and 50241 (lower) by (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides.

3B3. Major Task 3. Test Effects of MUC1-ED peptides on airway epithelial cell toxicity and monolayer integrity.

3B3A. Subtask 1. Test MUC1-ED peptides for airway epithelial cell toxicity and monolayer integrity, and measure therapeutic index for the optimum MUC1-ED peptides (months 10-12). Starting at 5.0 mM, $\log_{10}$ dilutions of each peptide were incubated with NHBE cells for 24 hr and viability determined using 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. To assess the effects of MUC1-ED peptides on monolayer integrity, a nontoxic dose of peptides was added to the apical surface of NHBE cells and transepithelial electrical resistance (TEER) measured. None of the peptides at any tested concentrations affected cell viability (Figure 8A) or monolayer integrity (Figure 8B).

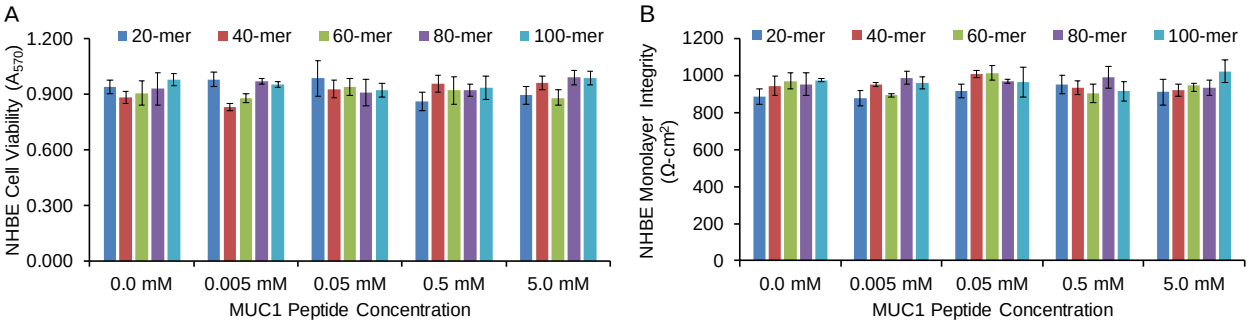


Figure 8. Effect of MUC1-ED peptides on NHBE (A) cell viability and (B) monolayer integrity.

3B4A. Major Task 4A (optional). If necessary, perform alternative protocols to resolve potential pitfalls with MUC1-ED synthetic peptides.

3B4A1. Subtask 1. MUC1-ED gene segments will be synthesized corresponding to 20-, 40-, 60-, 80-, and 100-amino acid regions of the VNTR, codon-optimized for expression in *E. coli*, and the corresponding recombinant MUC1-ED proteins purified (month 12). This Subtask was not performed since it was only proposed as a backup in the event that the 40-mer, 60-mer, 80-mer, and 100-mer peptides did not exhibit increased potency compared with the 20-mer. However, as shown in Figures 3-7, the MUC1-ED peptides exhibited a rank-order profile of potency of 100-mer > 80-mer > 60-mer > 40-mer > 20-mer.

3B4B. Major Task 4B. Obtain IACUC approval.

3A5B1. Subtask 1. Obtain USAMRMC ORP and local IACUC approval (months 9-12). USAMRMC ORP approval was obtained on 12 February 2019. University of Maryland Baltimore IACUC approval was obtained on 15 June 2018.

3B5. Major Task 5. Purchase mice.

3A5A. Subtask 1. Purchase C57BL/6 mice from a certified commercial vendor (n=100) (month 12). Mice were purchased from Jackson Labs and passed quarantine on 20 November 2019.

3B6. Major Task 6. *P. aeruginosa* pretreatment with MUC1-ED peptides prior to infection.

3A6A. Subtask 1. Perform dose tolerance and pharmacokinetic studies of MUC1-ED peptides in mice (month 12). Mice were administered intranasally with 5.0 mM/kg (10 mg/kg) of the MUC1-ED 80-mer and 100-mer peptides, the most efficient peptides for

inhibiting *P. aeruginosa* adhesion/flagella binding established in Major Tasks 1-3. At 24 hr post-administration, the mice were sacrificed and lungs, liver, and kidneys harvested and assessed for histological signs of damage by H&E staining. Neither of the peptides exhibited signs of organ damage at 10 mg/kg (Figure 9). MUC1-ED and scrambled control peptide levels in lung homogenates were measured at 0, 1, 2, 4, 8, and 24 hr post-administration by enzyme-linked immunosorbent assay (ELISA) to assess peptide half-life ($t_{1/2}$) values, and peptide c_{max}, t_{max}, and $AUC_{0-\infty}$ pharmacokinetic parameters. The results indicated that all peptides exhibited equivalent $t_{1/2}$ values (Figure 10), and that all peptides exhibited c_{max} values > 60 mg/L, t_{max} values < 4 hr, and $AUC_{0-\infty}$ values > 1,500 mg/Lxhr, the minimum values established in the Statement of Work (Table 3).

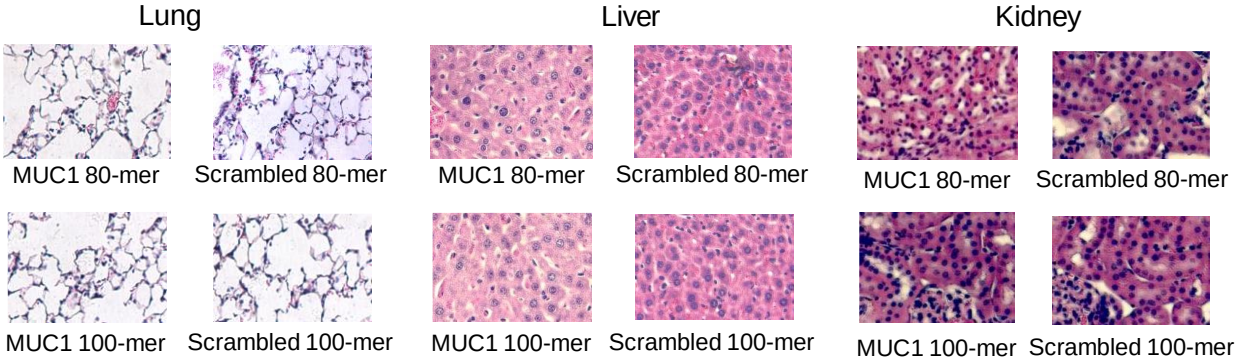


Figure 9. Dose tolerance effect of MUC1-ED 80-mer and 100-mer peptides on mouse organ damage as assessed by H&E staining.

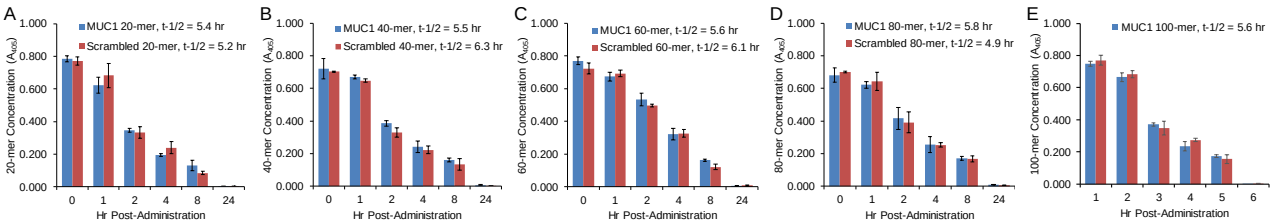


Figure 10. Lung pharmacokinetics of (A) 20-mer, (B) 40-mer, (C) 60-mer, (D) 80-mer, and (E) 100-mer MUC1-ED and scrambled control peptides. The $t_{1/2}$ values for each peptide are indicated in the upper right of each panel.

Table 3. Peptide Pharmacokinetic Parameters				
Peptide		c_{max} (mg/L)	t_{max} (hr)	$AUC_{0-\infty}$ (mg/Lxhr)
20-mer	Scrambled	62.5	1.14	10,925
	MUC1-ED	71.7	0.95	11,425
40-mer	Scrambled	121.4	1.23	20,400
	MUC1-ED	135.7	1.18	21,175
60-mer	Scrambled	145.0	1.10	21,225
	MUC1-ED	155.5	1.26	24,375
80-mer	Scrambled	241.8	0.99	35,675
	MUC1-ED	255.9	1.12	31,625
100-mer	Scrambled	325.6	1.18	44,025
	MUC1-ED	324.0	1.21	44,425

3A6B. <u>Subtask 2. Assess lung CFUs, BALF cytokines, lung histopathology, and survival following infection of mice with *P. aeruginosa* pretreated with the optimum MUC1-ED peptide (months 13-20).</u> A sublethal dose of *P.* aeruginosa laboratory strain PAK or clinical strain 50241 ($1x10^6$ CFU/mouse) equal to 10% of the half-maximal lethal dose ($0.1xLD_{50}$) was treated with 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the

corresponding scrambled control peptides. These peptides and concentrations were chosen based on the previous results showing that they were the most efficient for inhibition of *P. aeruginosa* adhesion and flagella binding to human lung cells *in vitro* (Figures 5, 6).The bacteria were washed to remove unbound peptide, and administered intranasally to mice. At 0, 4, 8, 12, 24, and 48 hr post-infection, bronchoalveolar lavage fluid (BALF) was collected to measure (i) *P. aeruginosa* colony forming units (CFUs) as a measure of bacterial lung burden, (ii) infiltrating leukocyte numbers (neutrophils, macrophages, lymphocytes) , (iii) levels of the cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), and keratinocyte-derived chemokine (KC), and (iv) lung sections were stained with hematoxylin and eosin (H&E) for inflammation. The results indicated that treatment of the mice with either the 80-mer or 100-mer MUC1-ED peptides simultaneous with sublethal infection with either *P. aeruginosa* strain PAK or 50241 reduced *P. aeruginosa* CFUs in the BALF (Figure 11), decreased leukocyte numbers (Figure 12), diminished TNF (Figure 13), IL-6 (Figure 14), and KC (Figure 15) levels in BALF, and reduced lung inflammation (Figure 16), each compared with the respective scrambled control peptides. Next, a lethal dose of *P. aeruginosa* laboratory strain PAK or clinical strain 50241 ($2x10^7$ CFU/mouse) equal to 2-times the half-maximal lethal dose ($2xLD_{50}$) was treated with 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides. At 0, 1, 2, 3, 4, 5, and 6 days post-infection, mouse survival was assessed by Kaplan-Meier analysis. The results indicated that treatment of the mice with either the 80-mer or 100-mer MUC1-ED peptides, simultaneous with lethal infection with either *P. aeruginosa* strain PAK or strain 50241, increased mouse survival compared with the respective scrambled control peptides (Figure 17).

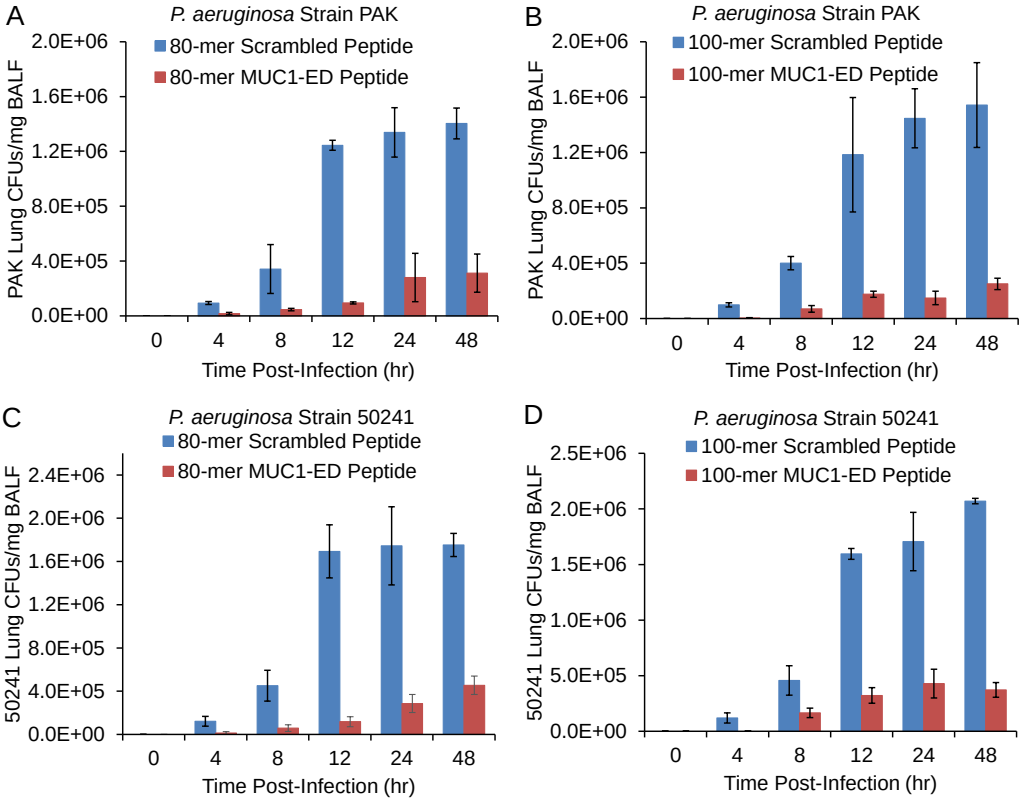


Figure 11. *P. aeruginosa* CFUs in BALF of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

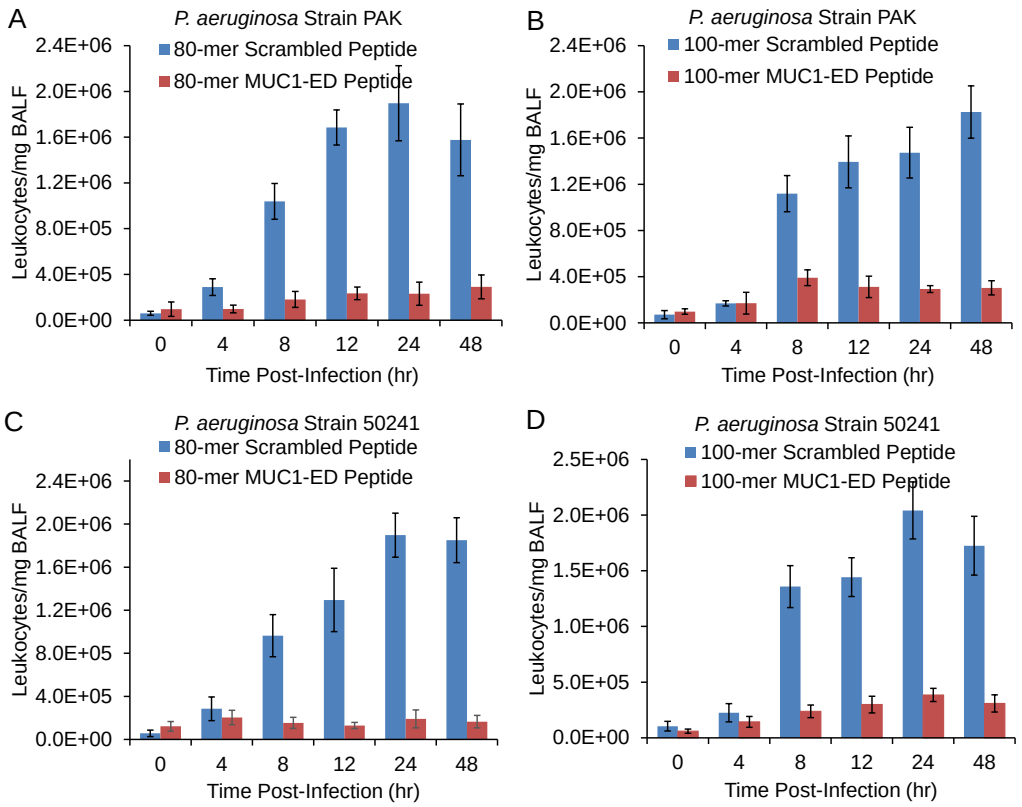


Figure 12. Leukocyte numbers in BALF of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

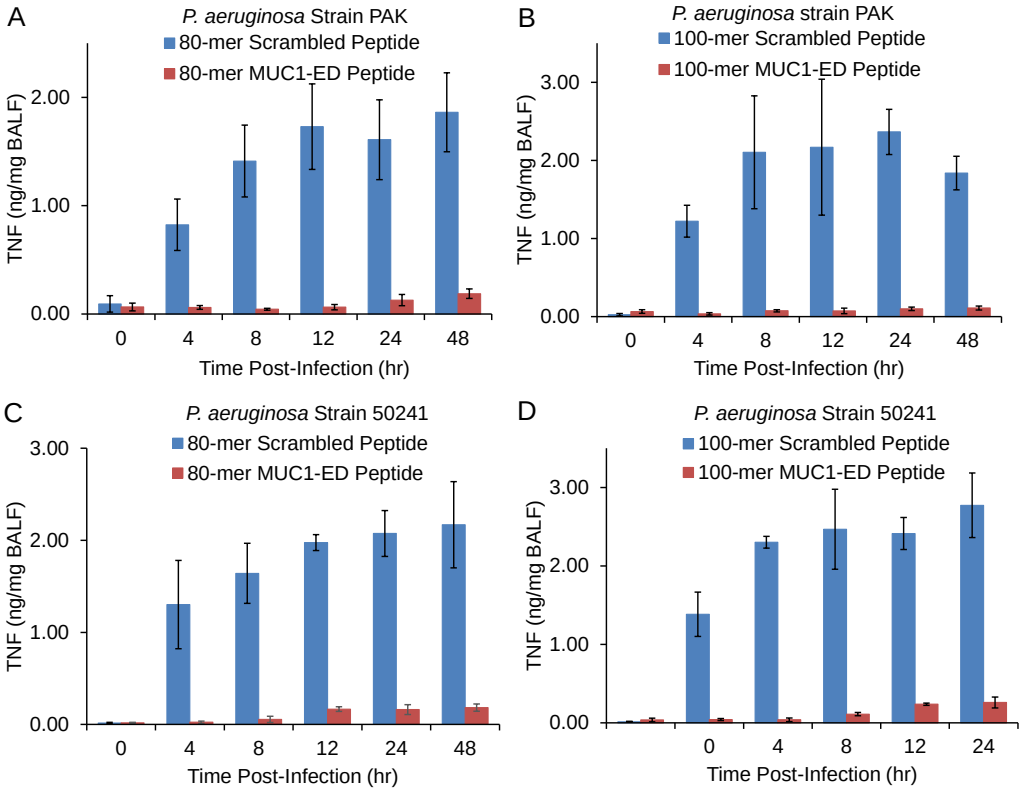


Figure 13. TNF cytokine levels in BALF of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

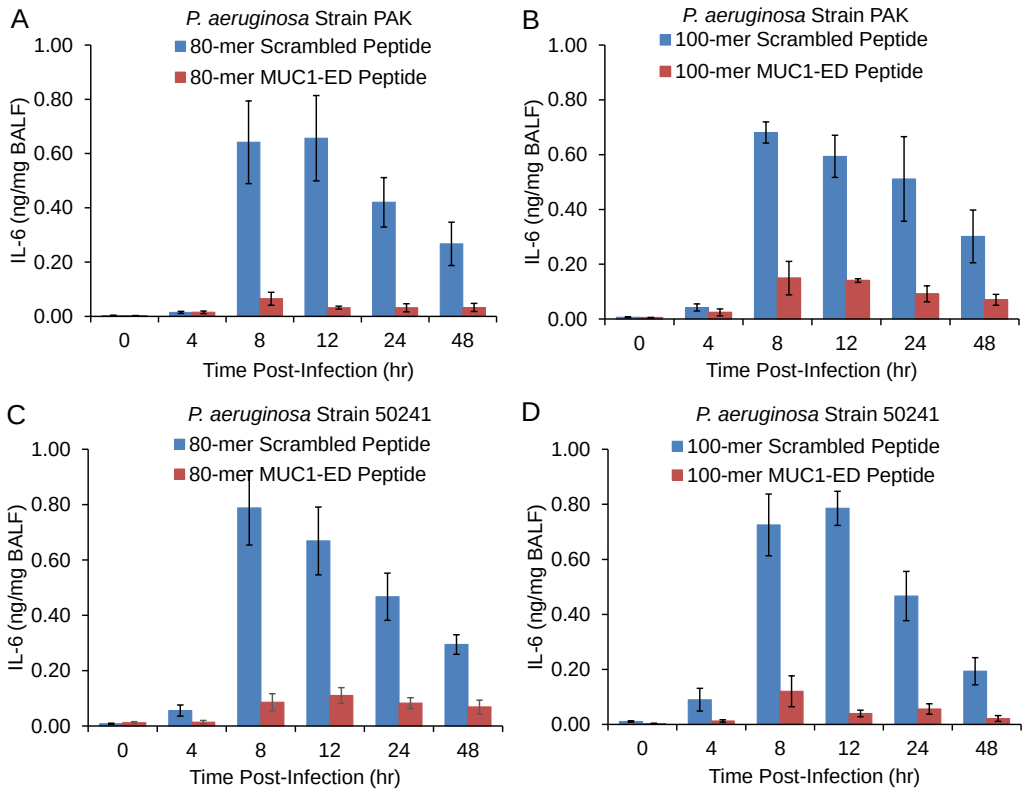


Figure 14. IL-6 cytokine levels in BALF of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

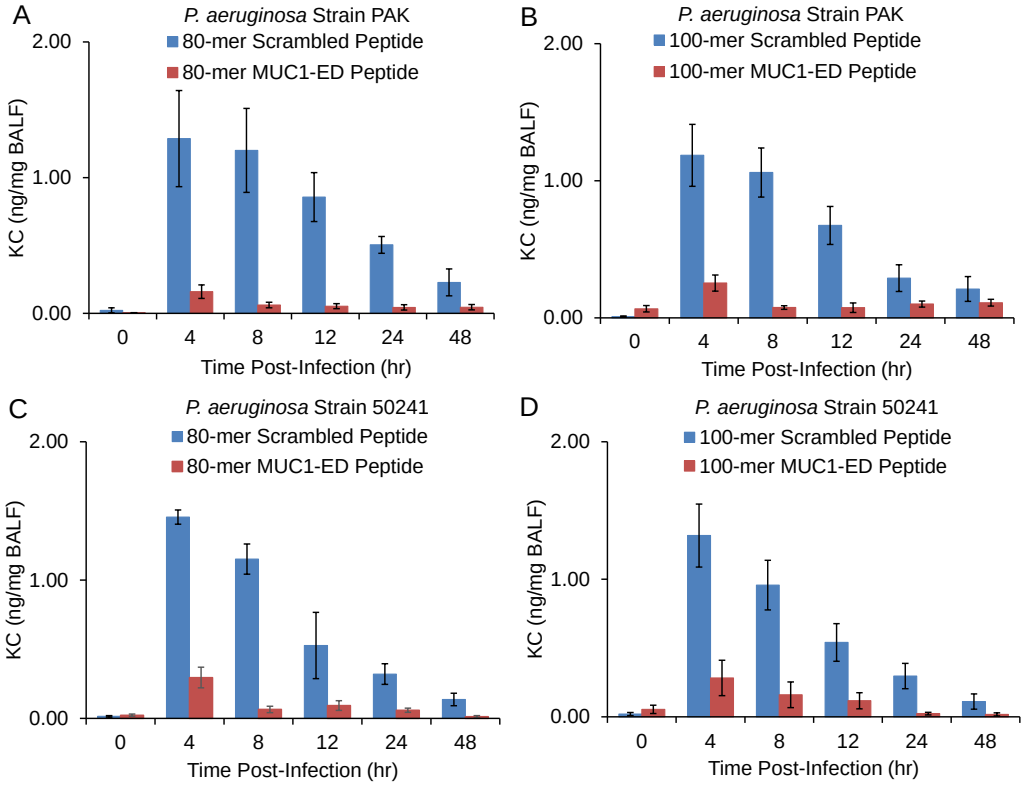
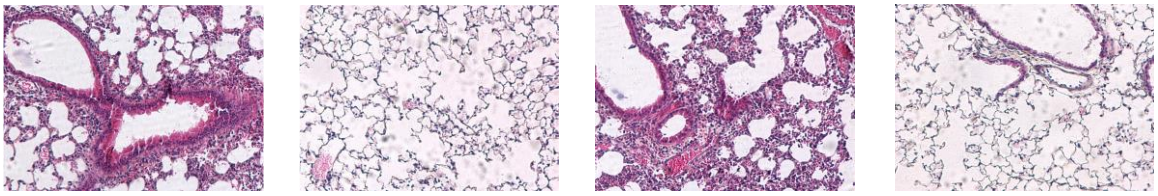


Figure 15. KC cytokine levels in BALF of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).



80-mer Scrambled Peptide | 80-mer MUC1-ED Peptide | 100-mer Scrambled Peptide | 100-mer MUC1-ED Peptide

Figure 16. Lung inflammation by H&E staining in mice treated with 10 µM of the 80-mer or 100-mer MUC1-ED or scrambled control peptides, and simultaneously infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK.

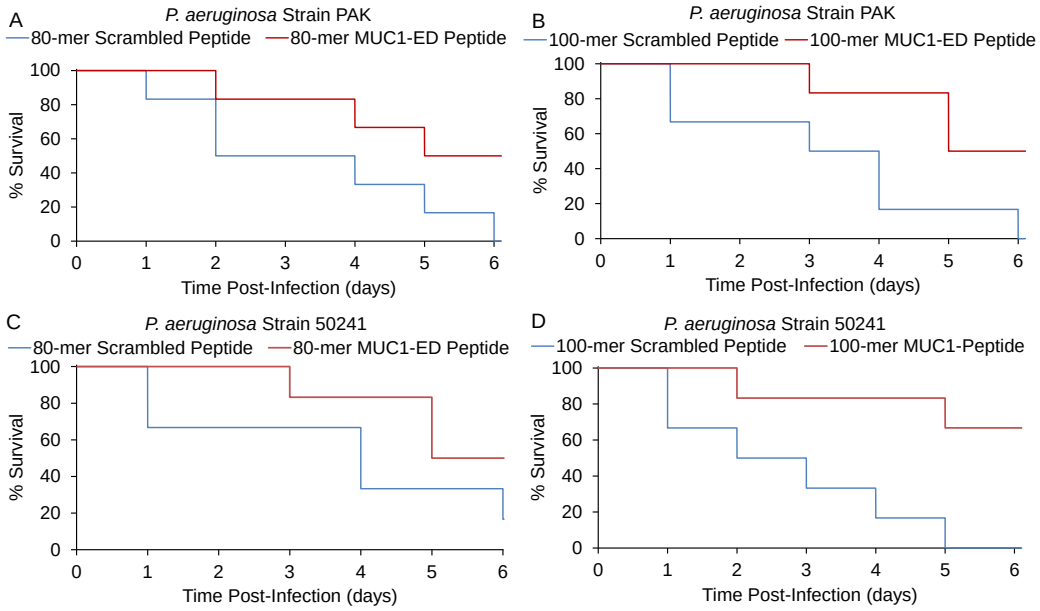


Figure 17. Survival of mice treated with 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides, and simultaneously infected with a lethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

3B7. Major Task 7. Pre-infection prophylaxis treatment study.

3B7A. <u>Subtask 1. Assess lung CFUs, BALF cytokines, lung histopathology, and survival when the optimum MUC1-ED peptide is administered as aerosols before *P. aeruginosa* infection of mice (months 21-30).</u> 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides, were administered to mice as aerosols. These peptides and concentrations were chosen based on the previous results showing that they were the most efficient for inhibition of *P. aeruginosa* adhesion and flagella binding to human lung cells *in vitro* (Figures 5, 6), and for their ability to reduce *P. aeruginosa* lung infection when administered simultaneously with the bacteria (Figures 11-17). At 4 hr following peptide administration, the mice were intranasally infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK or clinical strain 50241 ($1x10^6$ CFU/mouse) equal to 10% of the half-maximal lethal dose ($0.1xLD_{50}$). At 0, 4, 8, 12, 24, and 48 hr post-infection, bronchoalveolar lavage fluid (BALF) was collected to measure (i) *P. aeruginosa* colony forming units (CFUs) as a measure of bacterial lung burden, (ii) infiltrating leukocyte numbers (neutrophils, macrophages, lymphocytes) ,(iii) levels of the cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), and keratinocyte-derived chemokine (KC), and (iv) lung sections were stained with hematoxylin and eosin (H&E) for inflammation. The results indicated that pretreatment of the mice with both the 80-mer and 100-mer MUC1-ED peptides prior to sublethal infection with either *P. aeruginosa* strain PAK or 50241 reduced *P. aeruginosa* CFUs in the BALF (Figure 18), decreased leukocyte numbers (Figure 19), diminished TNF (Figure 20), IL-6 (Figure 21), and KC (Figure 22) levels in BALF, and reduced lung

inflammation (Figure 23), each compared with the respective scrambled control peptides. However, treatment of mice with the 80-mer or 100-mer MUC1-ED peptides prior to sublethal infection with *P. aeruginosa* strain PAK or 50241 (Figures 18-23) was not as effective for reducing BALF CFUs, leukocyte numbers, TNF, IL-6, or KC levels, or lung H&E staining, compared with treatment with the same MUC1-ED peptides simultaneous with sublethal *P. aeruginosa* infection (Figures 11-16). Next, 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides, were administered to mice as aerosols. At 4 hr following peptide administration, the mice were intranasally infected with a lethal dose of *P. aeruginosa* laboratory strain PAK or clinical strain 50241 ($2x10^7$ CFU/mouse) equal to 2-times the half-maximal lethal dose ($2xLD_{50}$). At 0, 1, 2, 3, 4, 5, and 6 days post-infection, mouse survival was assessed by Kaplan-Meier analysis. The results indicated that pretreatment of the mice with either the 80-mer or 100-mer MUC1-ED peptides, prior to lethal infection with either *P. aeruginosa* strain PAK or strain 50241, increased mouse survival compared with the respective scrambled control peptides (Figure 24). However, treatment of mice with the 80-mer or 100-mer MUC1-ED peptides prior to lethal infection with *P. aeruginosa* strain PAK or 50241 (Figure 24) was not as effective for increasing mouse survival, compared with treatment with the same MUC1-ED peptides simultaneous with lethal *P. aeruginosa* infection (Figure 17).

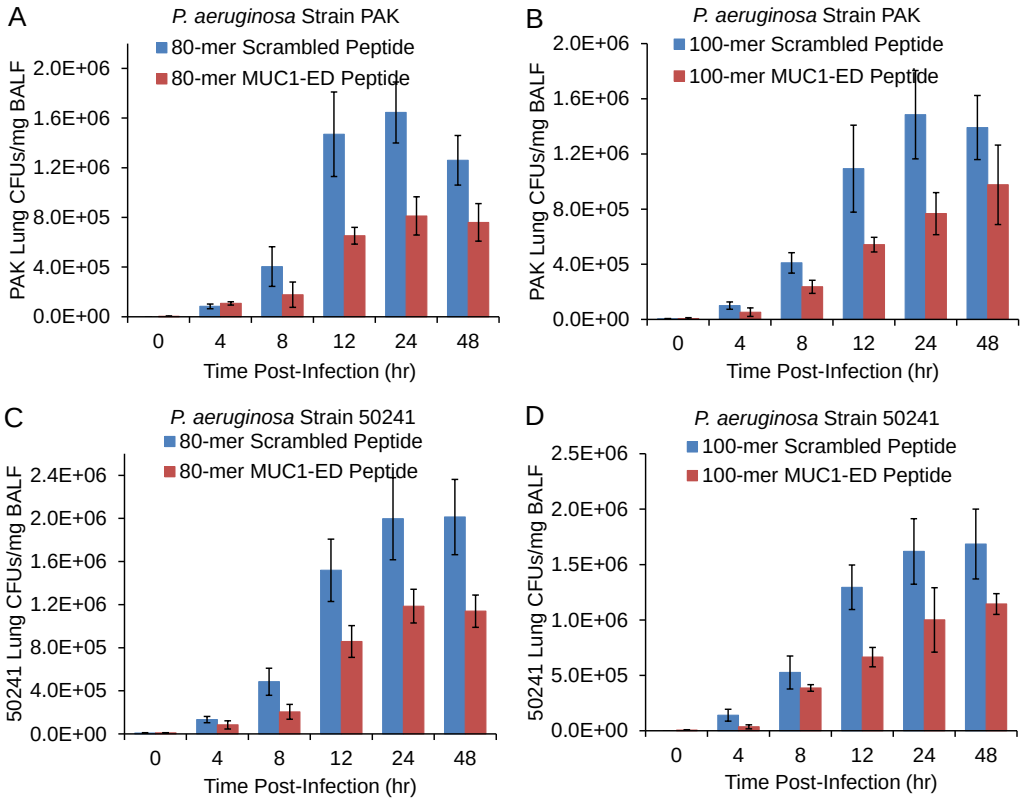


Figure 18. *P. aeruginosa* CFUs in BALF of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

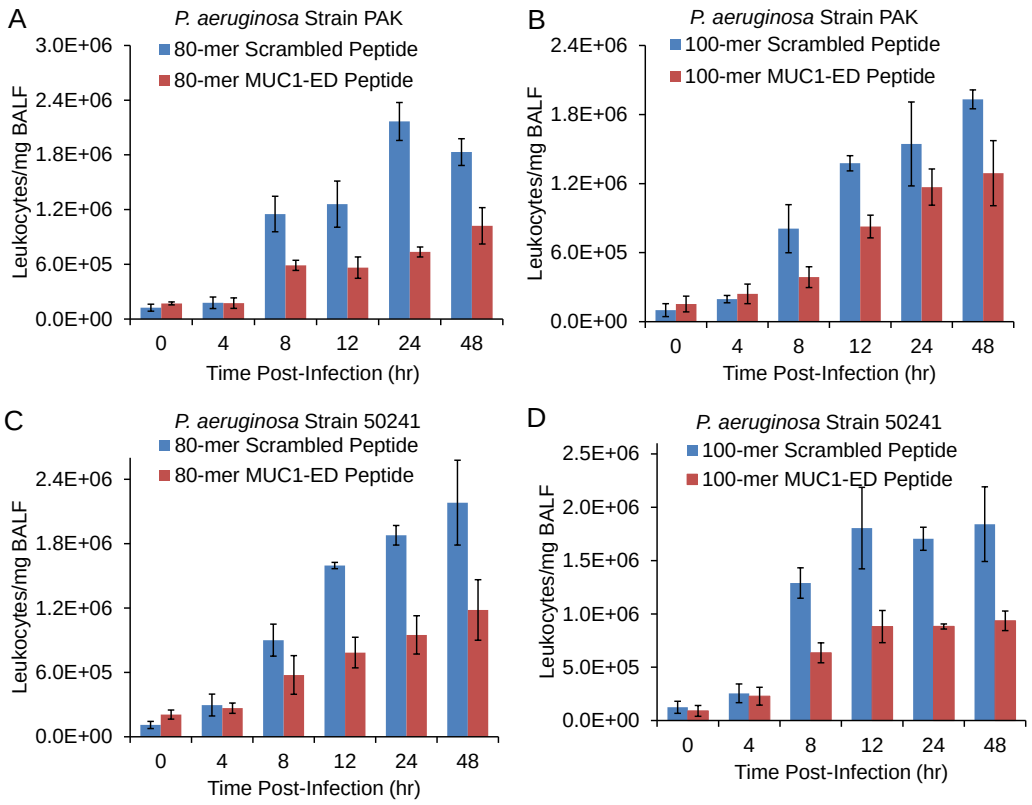


Figure 19. Leukocyte numbers in BALF of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

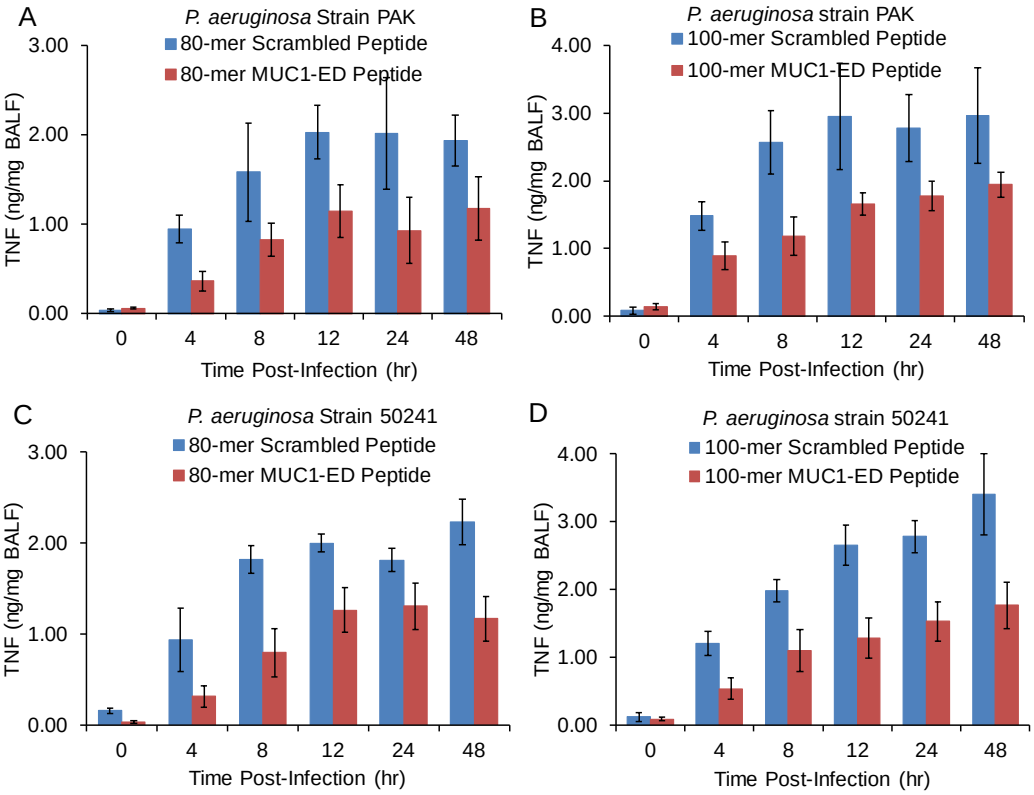


Figure 20. TNF cytokine levels in BALF of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

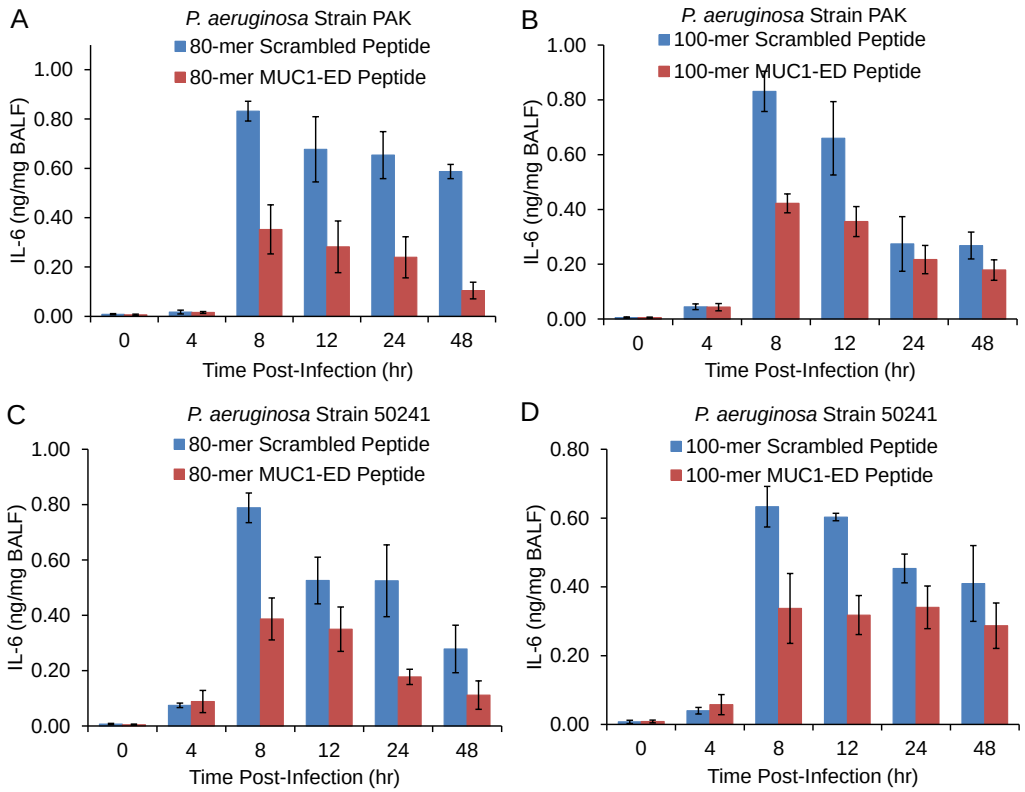


Figure 21. IL-6 cytokine levels in BALF of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

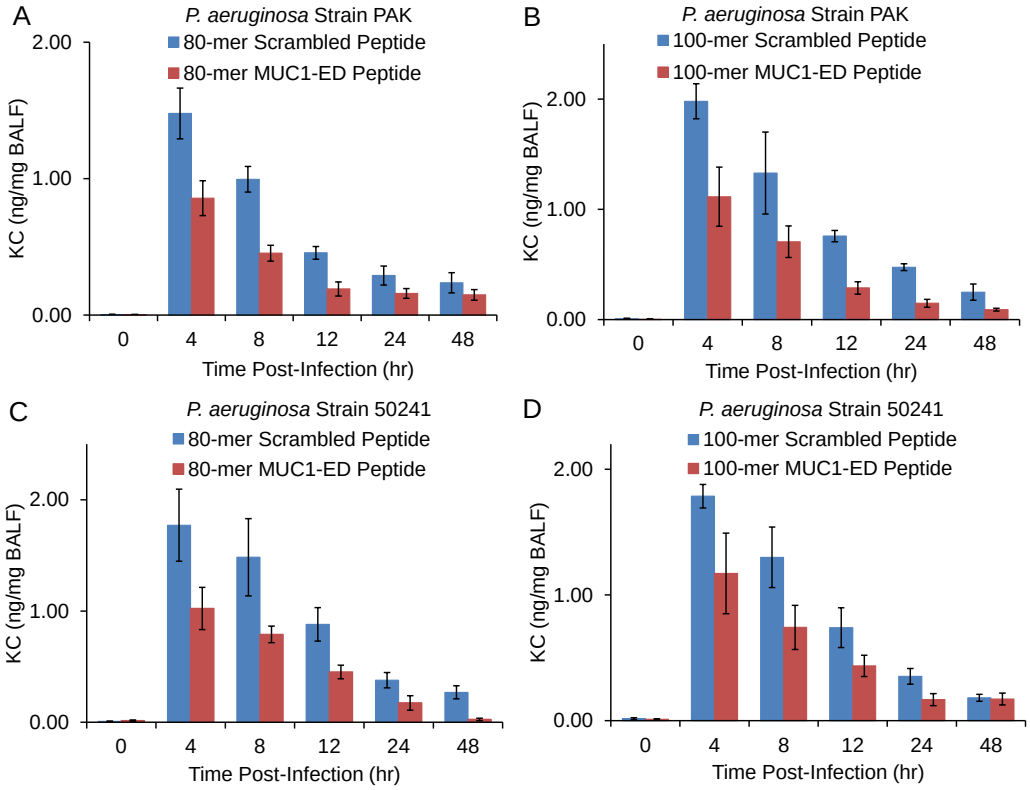
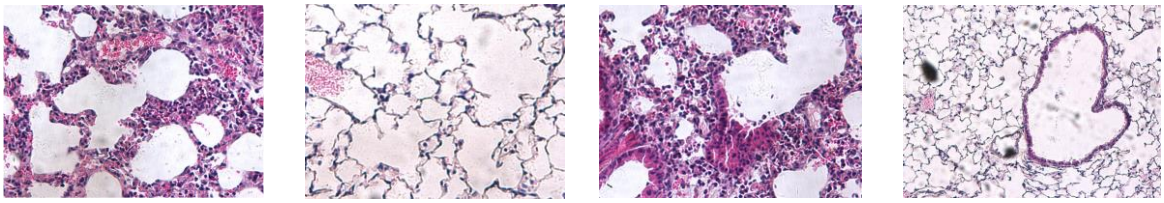


Figure 22. KC cytokine levels in BALF of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).



80-mer Scrambled Peptide | 80-mer MUC1-ED Peptide | 100-mer Scrambled Peptide | 100-mer MUC1-ED Peptide

Figure 23. Lung inflammation by H&E staining in mice pretreated with the 80-mer or 100-mer MUC1-ED or scrambled control peptides 4 hr prior to infection with a sublethal dose of *P. aeruginosa* laboratory strain PAK.

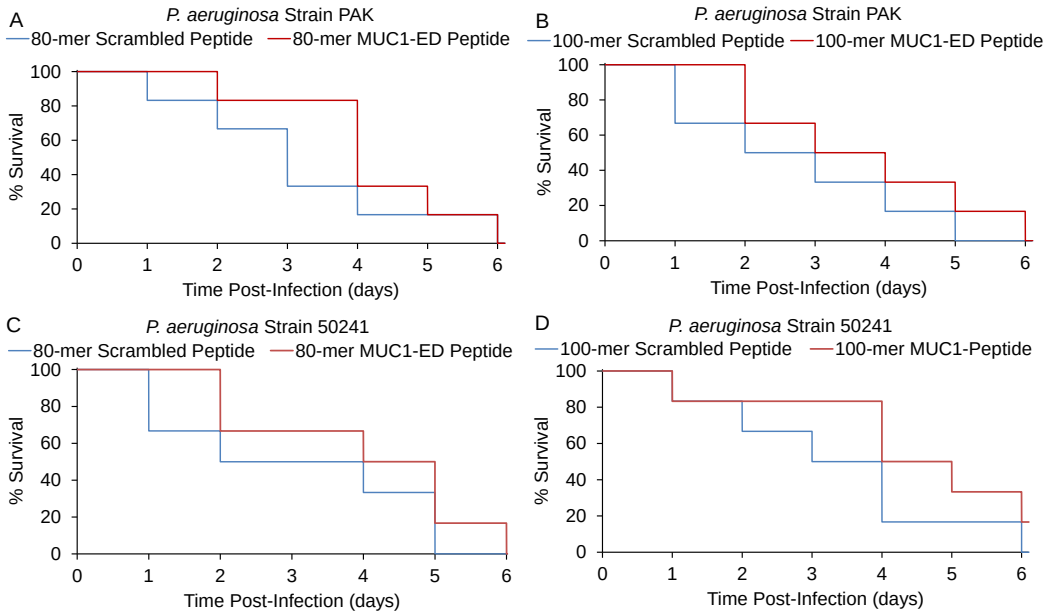


Figure 24. Survival of mice pretreated with the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides 4 hr prior to infection with a lethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D).

3B8. Major Task 8. Post-infection therapeutic study.

3B8A. <u>Subtask 1. Assess lung CFUs, BALF cytokines, lung histopathology, and survival when the optimum MUC1-ED peptide is administered as aerosols after *P. aeruginosa* infection of mice (months 31-36).</u> At 4 hr following peptide administration, the mice were intranasally infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK or clinical strain 50241 ($1x10^6$ CFU/mouse) equal to 10% of the half-maximal lethal dose ($0.1xLD_{50}$). At 4 hr post-infection, the mice were administered aerosols containing 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides. These peptides and concentrations were chosen based on the previous results showing that they were the most efficient for inhibition of *P. aeruginosa* adhesion and flagella binding to human lung cells *in vitro* (Figures 5, 6), and for their ability to reduce *P. aeruginosa* lung infection when administered simultaneously with (Figures 11-17) or prior to (Figures 18-24) the bacteria. At 0, 4, 8, 12, 24, and 48 hr following administration of the peptides, bronchoalveolar lavage fluid (BALF) was collected to measure (i) *P. aeruginosa* colony forming units (CFUs) as a measure of bacterial lung burden, (ii) infiltrating leukocyte numbers (neutrophils, macrophages, lymphocytes) ,(iii) levels of the cytokines tumor necrosis factor (TNF), interleukin-6 (IL-6), and keratinocyte-derived chemokine (KC), and (iv) lung sections were stained with hematoxylin and eosin (H&E) for inflammation. The results indicated that administration to mice of either the 80-mer and 100-mer MUC1-ED peptides following sublethal infection with either *P. aeruginosa* strain PAK or 50241 modestly reduced *P. aeruginosa* CFUs in the BALF (Figure 25), decreased leukocyte numbers (Figure 26), diminished

TNF (Figure 27), IL-6 (Figure 28), and KC (Figure 29) levels in BALF, but did not affect lung inflammation as assessed by H&E staining (Figure 30), each compared with the respective scrambled control peptides. Administration to mice of the 80-mer or 100-mer MUC1-ED peptides following sublethal infection with *P. aeruginosa* strain PAK or 50241 (Figures 25-30) was not as effective for reducing BALF CFUs, leukocyte numbers, TNF, IL-6, or KC levels, or lung H&E staining, compared with treatment with the same MUC1-ED peptides simultaneous with (Figures 11-16) or prior to (Figures 18-23) sublethal *P. aeruginosa* infection. Next, 10 µM of the 80-mer or 100-mer MUC1-ED peptides, or the corresponding scrambled control peptides, were administered to mice as aerosols at 4 hr following intranasal infection with a lethal dose of *P. aeruginosa* laboratory strain PAK or clinical strain 50241 ($2x10^7$ CFU/mouse), equal to 2-times the half-maximal lethal dose ($2xLD_{50}$). At 0, 1, 2, 3, 4, 5, and 6 days post-infection, mouse survival was assessed by Kaplan-Meier analysis. The results indicated that administration of either the 80-mer or 100-mer MUC1-ED peptides following lethal infection with either *P. aeruginosa* strain PAK or strain 50241 had no effect on mouse survival compared with the respective scrambled control peptides (Figure 31).

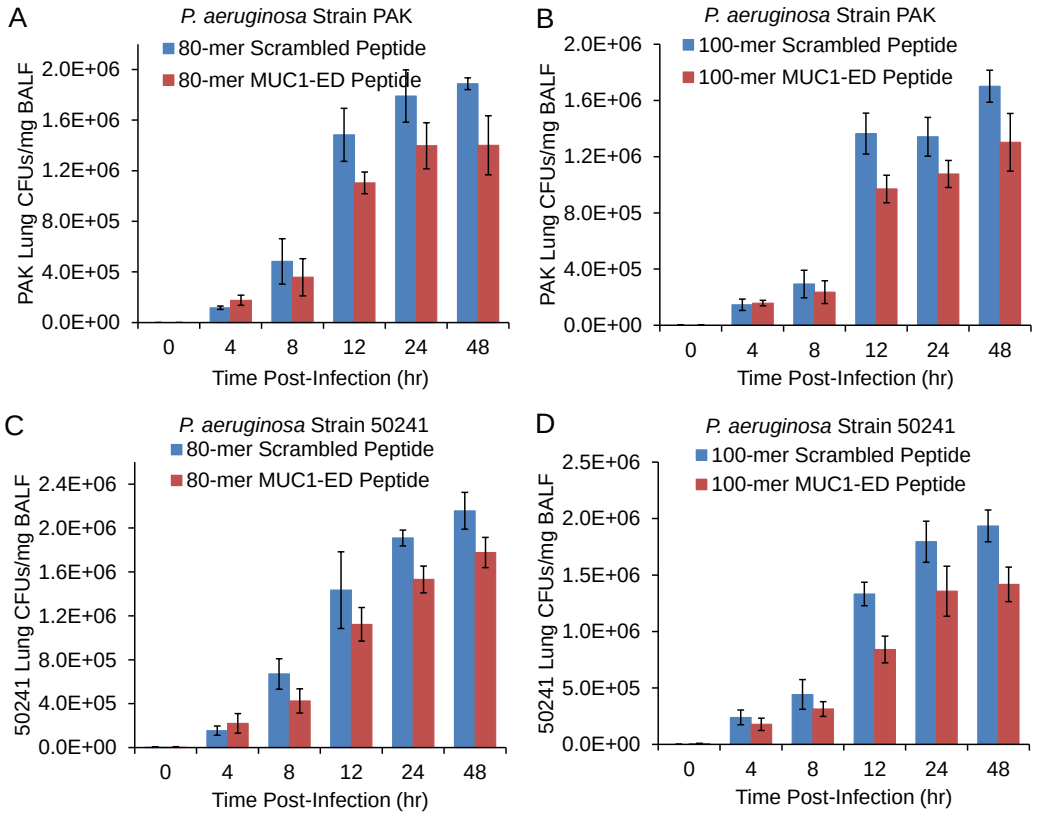


Figure 25. *P. aeruginosa* CFUs in BALF of mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

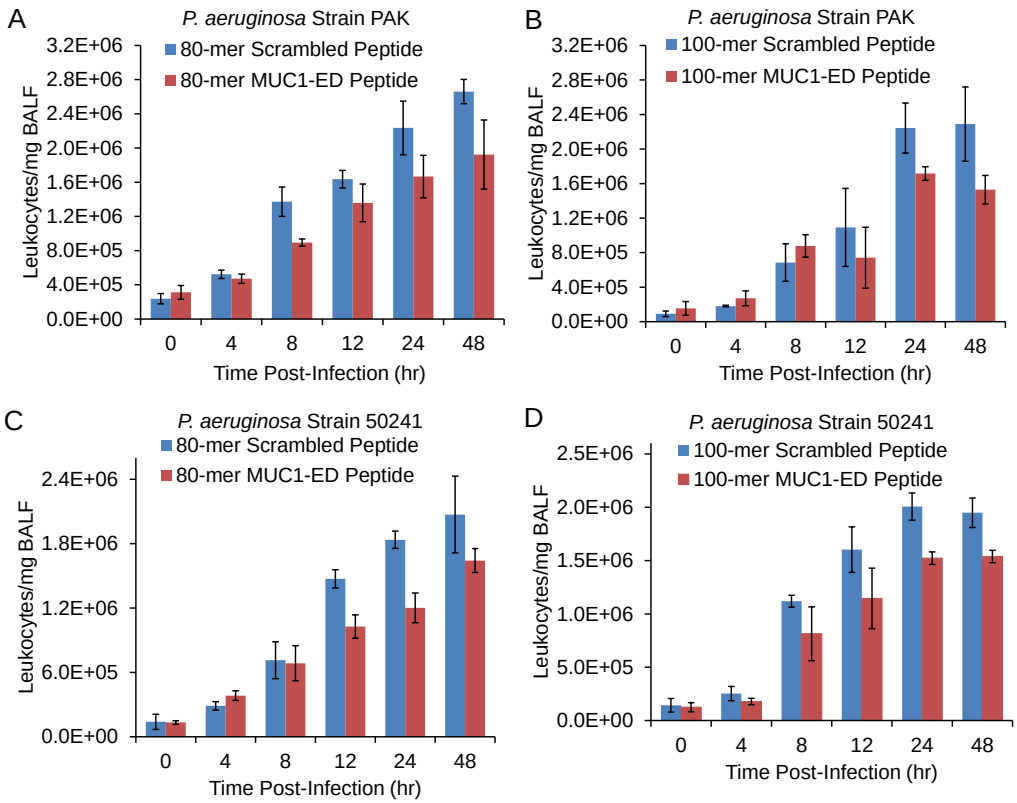


Figure 26. Leukocyte numbers in BALF of mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

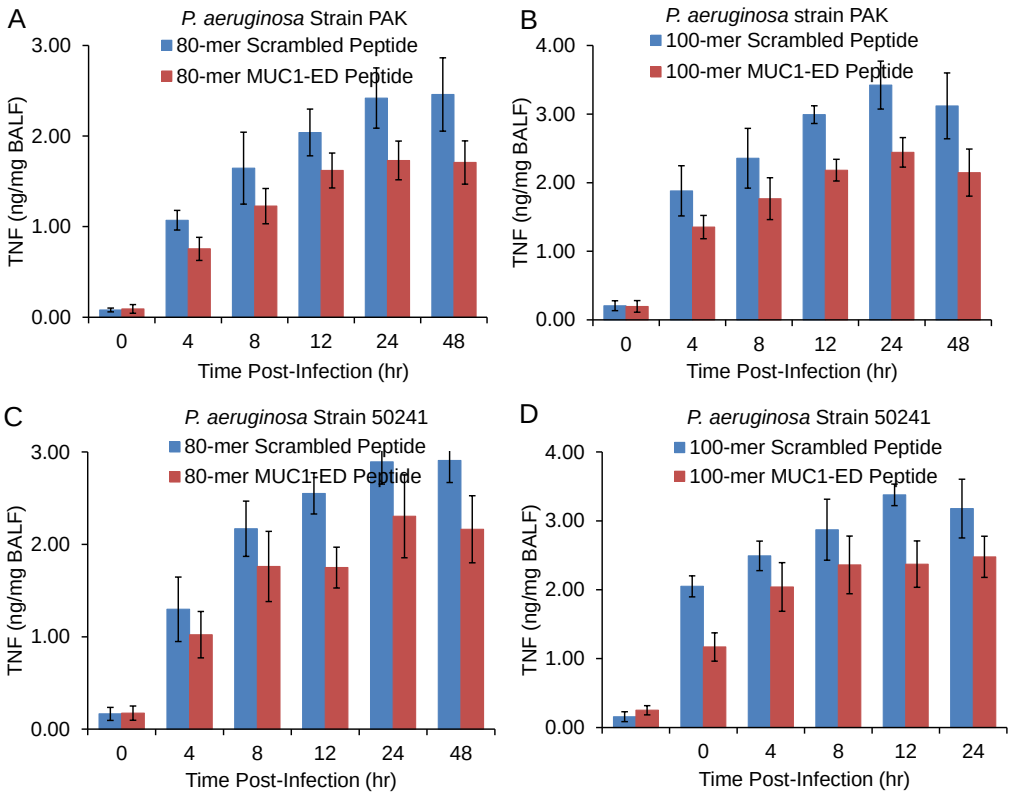


Figure 27. TNF cytokine levels in BALF of mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

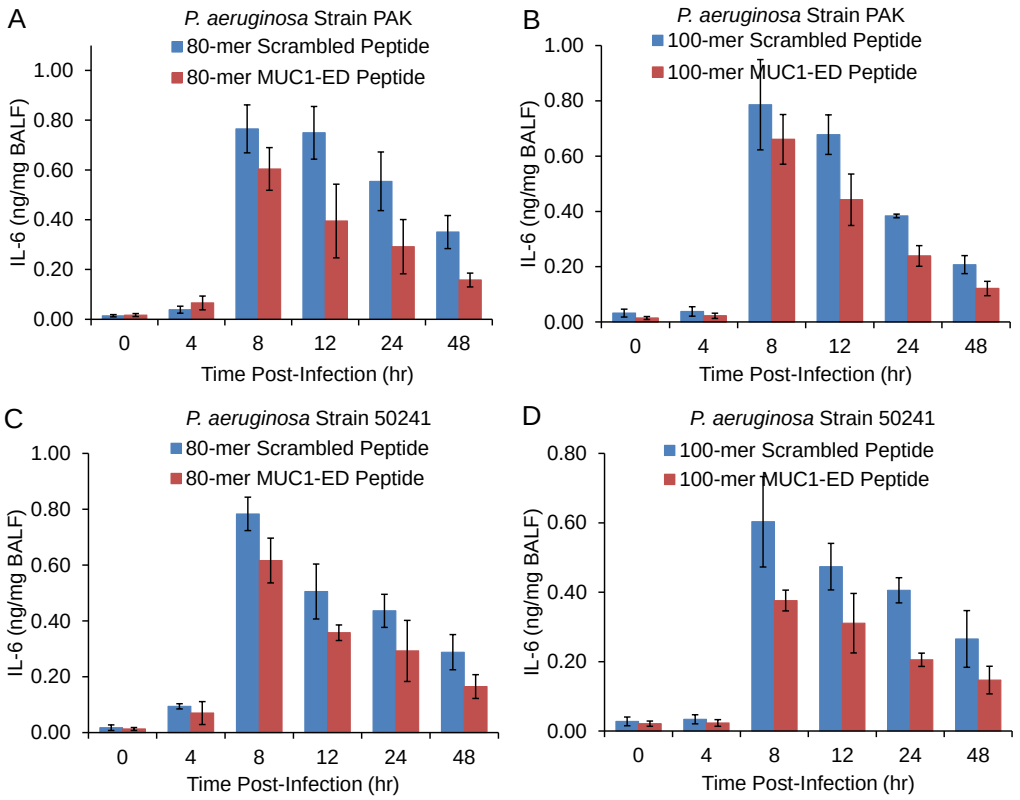


Figure 28. IL-6 cytokine levels in BALF of mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

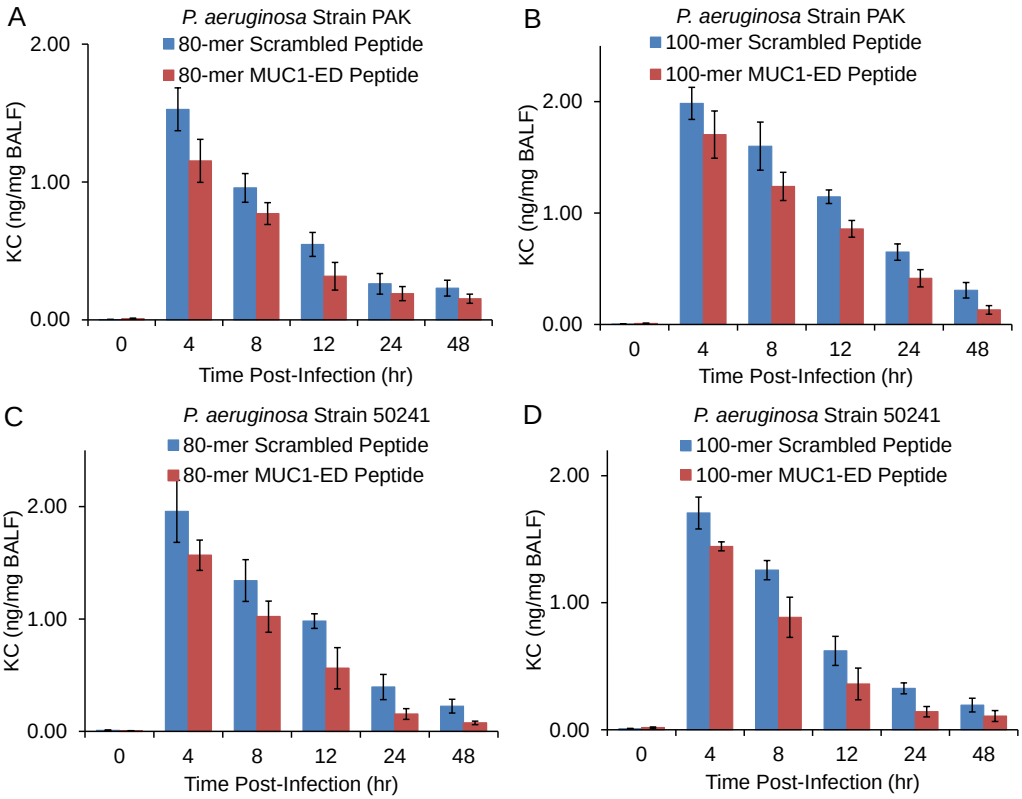
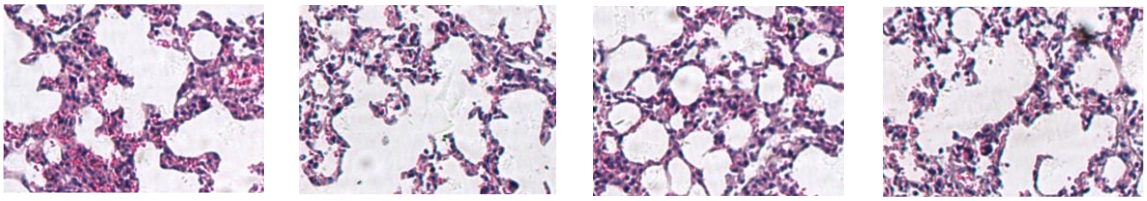


Figure 29. KC cytokine levels in BALF of mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.



80-mer Scrambled Peptide | 80-mer MUC1-ED Peptide | 100-mer Scrambled Peptide | 100-mer MUC1-ED Peptide

Figure 30. Lung inflammation by H&E staining in mice infected with a sublethal dose of *P. aeruginosa* laboratory strain PAK 4 hr prior to administration of the 80-mer or 100-mer MUC1-ED or scrambled control peptides.

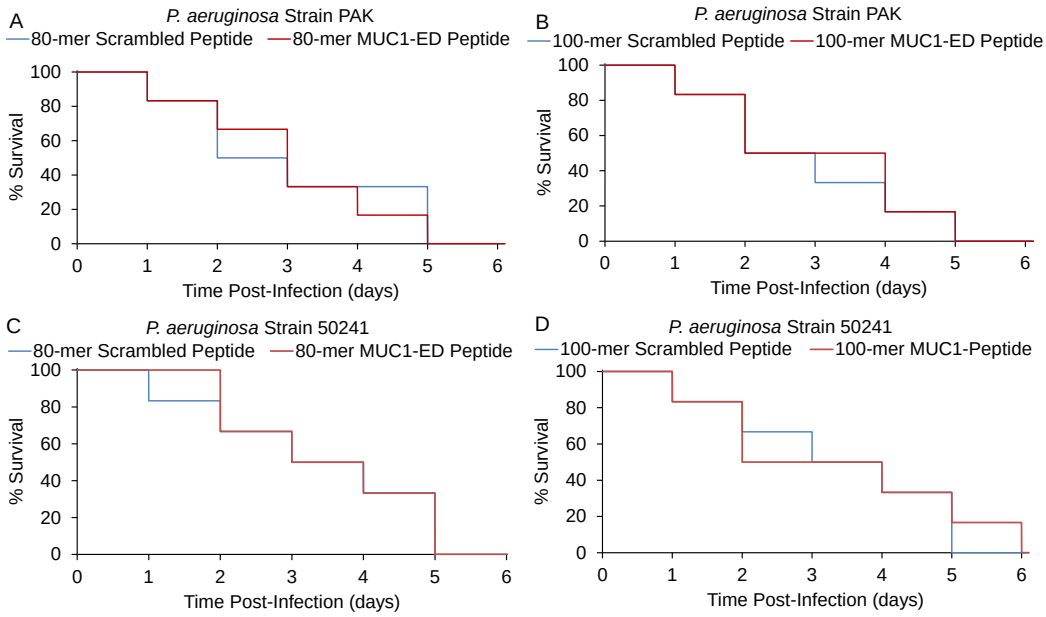


Figure 31. Survival of mice infected with a lethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 4 hr prior to administration of 10 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

Because neither the 80-mer nor 100-mer peptides administered at 10 µM at 4 hr following intranasal infection with a lethal dose of *P. aeruginosa* strains PAK or 50241 increased mouse survival compared with the scrambled control peptides (Figure 31), additional survival experiments were conducted using higher peptide doses and a shorter time interval following bacterial challenge. More specifically, the 80-mer and 100-mer peptides were administered at 25 or 100 µM to mice at 1 hr following lethal challenge infection with $2x10^7$ CFU/mouse of *P. aeruginosa* laboratory strain PAK or clinical strain 50241, equal to 2-times the half-maximal lethal bacterial dose ($2xLD_{50}$). At 0, 1, 2, 3, 4, 5, and 6 days post-infection, mouse survival was assessed by Kaplan-Meier analysis (Figures 32, 33). Statistical analysis by the Mantel-Cox log-rank test revealed that administration of 25 µM of the 80-mer or 100-mer MUC1-ED peptides at the 1 hr time point following lethal infection with *P. aeruginosa* strains PAK or 50241 (Figure 32), or at 100 µM following lethal infection with strain PAK (Figure 33, panels A, B), had no effect on mouse survival compared with the respective scrambled control peptides (Table 4). However, significantly increased survival was seen following administration of 100 µM of the 80-mer (p=0.035) or 100-mer (p=0.033) MUC1-ED peptides at 1 hr following infection with *P. aeruginosa* strain 50241 compared with the 80-mer or 100-mer scrambled control peptides (Figure 33, panels C, D; Table 4).

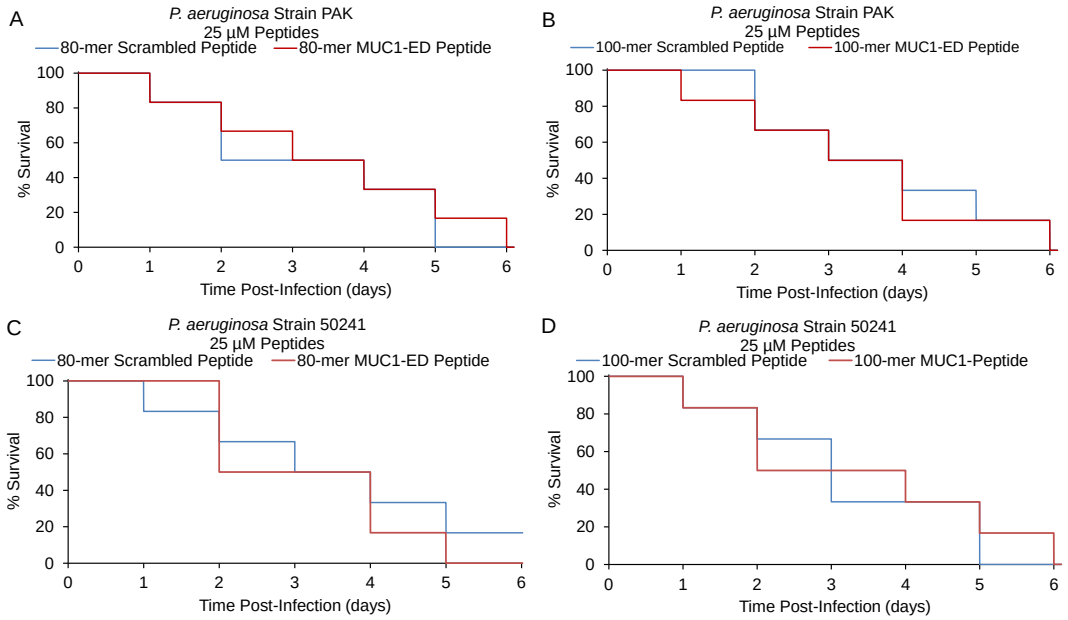


Figure 32. Survival of mice infected with a lethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 1 hr prior to administration of 25 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

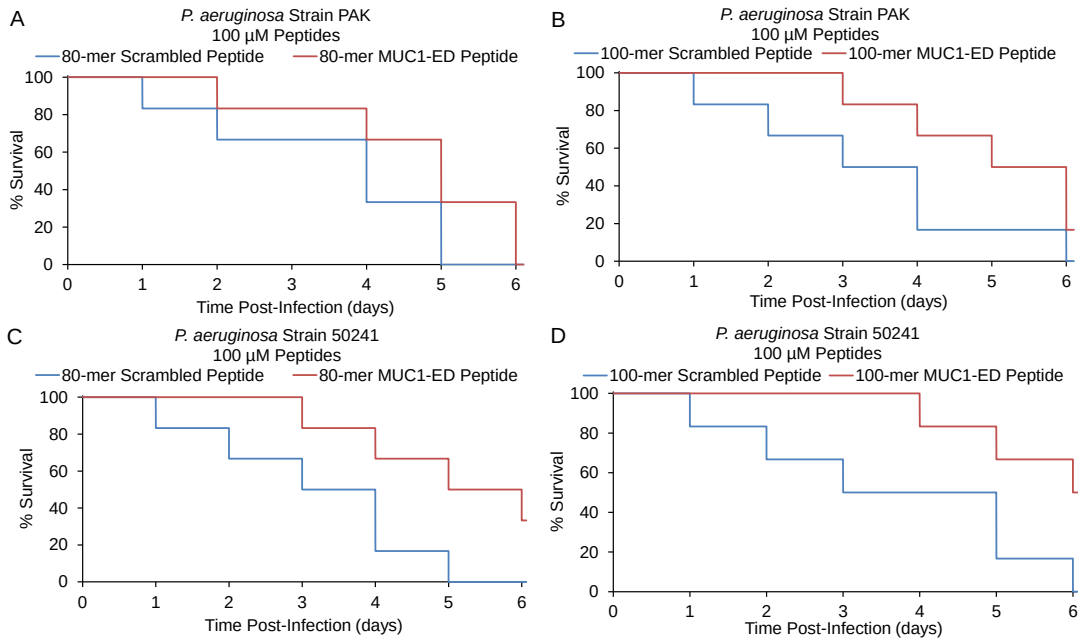


Figure 33. Survival of mice infected with a lethal dose of *P. aeruginosa* laboratory strain PAK (A, B) or clinical strain 50241 (C, D) 1 hr prior to administration of 100 µM of the 80-mer (A, C) or 100-mer (B, D) MUC1-ED or scrambled control peptides.

Peptide	Concentration	*P. aeruginosa* Strain	Time Post-Infection	p-Value	Figure Correspondence
80-mer	10 µM	PAK	4 hr	0.83	Figure 31A
100-mer				0.82	Figure 31B
80-mer	10 µM	50241	4 hr	0.92	Figure 31C
100-mer				0.76	Figure 31D
80-mer	25 µM	PAK	1 hr	0.68	Figure 32A
100-mer				0.83	Figure 32B
80-mer	25 µM	50241	1 hr	0.32	Figure 32C
100-mer				0.74	Figure 32D
80-mer	100 µM	PAK	1 hr	0.13	Figure 33A
100-mer				0.10	Figure 33B
80-mer	100 µM	50241	1 hr	0.035	Figure 33C
100-mer				0.033	Figure 33D

Table 4. Statistical analyses by the Mantel-Cox log-rank test comparing survival of mice administered with 10, 25, or 100 µM of the 80-mer or 100-mer MUC1-ED peptides at 1 or 4 hr following lethal infection with *P. aeruginosa* strains PAK or 50241, compared with the respective 80-mer or 100-mer scrambled control peptides.

3C. Opportunities for training and professional development: Nothing to Report
3D. Dissemination of results to communities of interest: Nothing to Report
3E. Plans for the next reporting period to accomplish the goals: Nothing to Report

4. IMPACT
4A. Impact on development of the principal discipline(s) of the project: Nothing to Report
4B. Impact on other disciplines: Nothing to Report
4C. Impact on technology transfer: Nothing to Report
4D. Impact on society beyond science and technology: Nothing to Report

5. CHANGES/PROBLEMS: There were no changes in the approach, objectives, scope, or direction of this project as described in the original SOW. However, as indicated above in section 3A, due to University of Maryland, Baltimore research restrictions as a result of the COVID-19 pandemic, two sequential 12 month no-cost extensions were requested and approved, the first covering the time period 8/15/20-8/14/21 and the second covering the time period 8/15/2021-8/14/2022. The first extension approval was communicated in an email dated 7/7/20 10:07 AM from Ms. Sherry M. Apperson and cc'ed to Scientific Officer Dr. Annmarie Gersch, Ph.D., and Grants Management Specialist Ms. Catherine C. Henry. The second extension approval was communicated in an email dated 7/7/21 8:23 AM from Ms. Apperson and cc'ed to Dr. Gersch and Ms. Henry. As a result, Major Task 6 which was originally scheduled for months 12-14, was extended to months 12-20, Major Task 7, which was originally scheduled for months 15-16, was extended to months 21-30, and Major Task 8, which was originally scheduled for months 17-18, was extended to months 31-42.

6. PRODUCTS
6A. Publications, conference papers, and presentations: Nothing to Report
6B. Website(s) or other Internet site(s): Nothing to Report
6C. Technologies or techniques: Nothing to Report
6D. Inventions, patent applications, and/or licenses: Nothing to Report
6E. Other products: Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7A. Individuals who have worked on the project:

Name	Erik P. Lillehoj, PhD
Project Role	PI
Research Identifier	ORCID: 0000-0003-0910-6074
Nearest person month worked	6
Contribution to Project	Dr. Lillehoj performed the studies, analyzed the results and prepared data graphs and tables.
Funding Support	Nothing to Report

7B. Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period: Nothing to Report

7C. Other organizations were involved as partners: Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: Nothing to Report

9. APPENDICES: Nothing to Report