

1 **Pharmacokinetics and pharmacodynamics of inhaled** 2 **antipseudomonal bacteriophage therapy in mice**

3 Running title: PK and PD of inhaled phage therapy in mice

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29 **Abstract**

30 Inhaled bacteriophage (phage) therapy is a potential alternative to conventional antibiotic therapy
31 to combat multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections. However,
32 pharmacokinetics (PK) and pharmacodynamics (PD) of phages are fundamentally different to
33 antibiotics and the lack of understanding potentially limits optimal dosing. The aim of this study
34 was to investigate the *in vivo* PK and PD profiles of antipseudomonal phage PEV31 delivered by
35 pulmonary route in mice. BALB/c mice were administered phage PEV31 at doses of 10^7 and 10^9
36 PFU by the intratracheal route. Mice ($n = 4$) were sacrificed at 0, 1, 2, 4, 8 and 24 h post-
37 treatment and various tissues (lungs, kidney, spleen and liver), bronchoalveolar lavage and blood
38 were collected for phage quantification. In a separate study, mice ($n = 4$) were treated with
39 PEV31 (10^9 PFU) or PBS at 2 h post-inoculation with MDR *P. aeruginosa*. Infective PEV31 and
40 bacteria were enumerated from the lungs. In the phage only study, PEV31 titer gradually
41 decreased in the lungs over 24 hours with a half-life of approximately 8 h for both doses. In the
42 presence of bacteria, PEV31 titer increased by almost 2- \log_{10} in the lungs at 16 h. Furthermore,
43 bacterial growth was suppressed in the PEV31-treated group, while the PBS-treated group
44 showed exponential growth. Some phage-resistant colonies were observed from the lung
45 homogenates sampled at 24 h post-phage treatment. These colonies had a different antibiogram
46 to the parent bacteria. This study provides evidence that pulmonary delivery of phage PEV31 in
47 mice can reduce the MDR bacterial burden.

48 **Keywords:** bacteriophage (phage), pseudomonas, pulmonary delivery, pulmonary infection;
49 pharmacokinetics (PK), pharmacodynamics (PD), multidrug-resistant (MDR) infection

50

51 **Introduction**

52 Since the discovery of penicillin, much effort has been targeted towards understanding the
53 pharmacokinetics (PK) and pharmacodynamics (PD) of antibiotics to guide safe and effective
54 treatment regimens. While bacteria can be intrinsically resistant to antibiotics, the inappropriate
55 use of antibiotics has subjected bacteria to high selective pressure, leading to the advent of
56 resistant strains at an alarming rate and now poses a serious global threat to human health. The
57 severe threat of antimicrobial resistance remains imminent (1) and the World Health
58 Organization has called for global action to tackle this crisis (2). Unfortunately, the antibiotic
59 discovery pipeline is drying with a lack of novel antimicrobial agents against Gram-negative
60 bacteria (3). In particular, the emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa*
61 strains presents a major public health risk due to their prevalent intrinsic and acquired resistance
62 to most antibiotics (4). MDR *P. aeruginosa* causes complications of respiratory infections
63 associated with high morbidity and mortality rates in many diseases, including bronchiectasis,
64 cystic fibrosis, chronic obstructive pulmonary disease and pneumonia (4).

65 Bacteriophages (phages) are naturally occurring bactericidal virus that infect targeted host
66 bacteria. They are recently rediscovered and reintroduced as potential antimicrobial treatment
67 and are considered an attractive solution to the increasing failure of antibiotics (5). Phage therapy
68 predominantly relies on the lytic life cycle of phages. Virulent (lytic) phages recognize and
69 attach to surface receptors of host bacterium, inject their genetic material and then utilize the
70 metabolic machineries of the host for self-replication (5). Up to hundreds of progenies can be
71 produced and then released into the surrounding during bacteriolysis. Phage therapy has distinct
72 advantages over conventional antibiotic treatment in that phages are (i) a naturally occurring
73 antibacterial, (ii) self-replicating, (iii) self-limiting upon resolution of infection, (iv) effective
74 against both MDR or antibiotic sensitive bacteria, (v) highly specific with low inherent toxicity,
75 (vi) able to co-evolve with bacteria, and (vii) able to penetrate biofilms (5). The potential use of
76 phages as antibacterial agents has been demonstrated in *in vitro* (6, 7), preclinical (8-11) and in
77 compassionate single case studies (12-14).

78 Despite these advantages and potential, development and application of phage therapy has been
79 relatively slow. A possible reason is that the current understanding and paradigm associated with

80 antibiotic treatment cannot be transferred directly to phages (15). The PK and PD of phages are
81 fundamentally different from those of conventional antibiotics. While many antibiotics are small
82 molecules, phages are nano-sized virus composed of proteins, nucleic acids (DNA or RNA) and
83 sometimes lipids. In addition, phages have a unique dynamic with their bacterial host as self-
84 replicating biopharmaceuticals (15). The PK/PD of phages are determined by their antibacterial
85 activities featuring self-replication, phages and bacteria coevolution, as well as the human
86 immune system in response to the two concurrent events (16).

87 Inhaled phage therapy holds remarkable potential to treat respiratory infections caused by
88 bacteria, including MDR isolates (5). With oral inhalation route for delivery to the lung, high
89 concentration of phages can be delivered to the site of infection in the respiratory tract, achieving
90 high pulmonary bioavailability. Inhaled phage therapy has been used in Eastern European
91 countries to treat bacterial respiratory infections that was otherwise untreatable with antibiotics.
92 A 7-year old cystic fibrosis patient received inhaled phage therapy in 2011, which dramatically
93 reduced the MDR *P. aeruginosa* and *Staphylococcus aureus* numbers in the lungs (12). Although
94 inhaled phage therapy has been practiced in Eastern Europe for many decades, the phage
95 viability in nebulized aerosol droplets has only recently been investigated. Our group and others
96 have demonstrated that inhalable aerosolized *Pseudomonas* phages remain biologically active
97 when a suitable nebulizer system is used (17-20). Although the feasibility of producing infective
98 phage aerosols have been well-established, there is a lack of understanding of *in vivo* PK and PD
99 profiles of phages and bacteria in the lungs. The aim of this study is to investigate the PK and PD
100 profiles of *Pseudomonas* phage PEV31 administered by pulmonary delivery in a neutropenic
101 murine model of respiratory infection.

102 **Materials and methods**

103 *Bacteriophage*

104 Anti-Pseudomonas phage PEV31 was isolated from the sewage plant in Olympia (WA, USA) by
105 the Evergreen Phage Lab (Kutter Lab). PEV31 belongs to the *Podoviridae* family. Stocks of the
106 phage were amplified using the Phage-on-Tap protocol (21) with minor modifications. Briefly,
107 200 mL of Nutrient Broth (NB, Amyl Media, Australia) supplemented with 1 mM of CaCl₂ and
108 MgCl₂ were mixed with 0.1 volumes of overnight bacterial host (*P. aeruginosa* dog-ear strain
109 PAV237). The mixture was incubated for 1 h with continuous shaking (220 rpm) at 37°C. A
110 volume (200 µL) of PEV31 lysate at 10⁹ plaque forming units (PFU)/mL was added, followed by
111 further incubation for 8 h. The mixture was centrifuged at 4000 × g for 20 min and the
112 supernatant was filter-sterilized using 0.22 µm polyethersulfone membrane filter. The phage
113 lysate was further purified and concentrated using ultrafiltration (100 kDa Amicon® Ultra-15
114 centrifugal filter, Sigma, Australia), and the media was replaced with phosphate-buffered saline
115 (PBS) supplemented with 1 mM CaCl₂. Bacterial endotoxins were removed by adding 0.4
116 volumes of 1-octanol, followed by vigorous shaking at room temperature for 1 h. The mixture
117 was centrifuged at 4000 g for 10 min and then the aqueous phase was collected. Residual organic
118 solvent was removed by centrifuging down the phages at 20,000 g for 1.5 h and then replacing
119 the buffer with fresh PBS supplemented with 1 mM CaCl₂.

120 *Endotoxin level quantification*

121 The Endosafe® Portable Test System (Charles River Laboratories, Boston, USA) was used as per
122 the manufacturer's instructions to quantify endotoxin level in the resulting phage lysates. The
123 single use LAL assay cartridges contain four channels to which the LAL reagent and a
124 chromogenic substrate have been pre-applied. A single cartridge enables duplicate measurements
125 of the sample and positive control with a known endotoxin concentration. The sensitivity of the
126 Readouts between 50% and 200% spike recovery are deemed acceptable. The sensitivity of the
127 assay was 1 – 10 EU/mL. Endotoxin-free water, tips and tubes were used at all times.

128 *Bacterial strain and phage-susceptibility testing*

129 *P. aeruginosa* FADDI-PA001 was used to induce bacterial infection in the mice used in this
130 study. The strain is an MDR clinical isolate provided by Li lab, Monash University, Australia (8).
131 Phage-susceptibility of this isolate was assessed using a spot test (6). Briefly, 5 mL of 0.4%
132 Nutrient Broth top agar was mixed with overnight culture of FADDI-PA001 (approximately $2 \times$
133 10^8 colony forming units, CFU) and then overlaid onto a 1.5% Nutrient Agar plate. Then, 10
134 μ L of a phage stock solution was spotted on the top agar plate, air-dried and incubate at 37°C for
135 24 h. After incubation, the appearance of the lysis zone was assessed for phage-susceptibility.

136 *Animals*

137 Female BALB/c mice of 6 – 8 weeks were obtained from Australian BioResources Ltd (Moss
138 Vale, New South Wales, Australia). The mice were housed under a 12-hour dark-light cycle with
139 *ad libitum* supply to standard chow diet and water. All animal experiments conducted were
140 approved by the University of Sydney Animal Ethics Committee.

141 *Pharmacokinetics of PEV31 after intratracheal administration*

142 Healthy (non-infected) mice were anaesthetized by intraperitoneal injection of ketamine /
143 xylazine mixture (80 mg/kg and 10 mg/kg, respectively) in 150 μ l PBS. Upon deep anesthesia as
144 confirmed by the absence of pedal reflex, the anaesthetized mouse was suspended on a nylon
145 floss by its incisor teeth and placed on an inclined intubation board. The trachea was then gently
146 intubated with a soft plastic guiding cannula. PEV31 at two different doses (10^7 and 10^9 PFU)
147 suspended in 25 μ L PBS was administered into the trachea through the guiding cannula with a
148 micropipette and a 200 μ L gel-loading pipette tip. At 0 (immediately after administration), 1, 2, 4,
149 8 and 24 h post-phage administration, mice ($n \geq 4$) were terminally anaesthetized by
150 intraperitoneal injection of an overdose of ketamine / xylazine mixture (300 mg/kg and 30 mg/kg,
151 respectively). Broncho-alveolar lavage (BAL), lung, kidney, spleen, liver and blood samples
152 were collected (**Figure 1A**). BAL was performed by instilling 1.5 mL PBS (as three aliquots of
153 0.5 mL) through the trachea to the lung and collecting the lavage suspension. Approximately 1.2
154 to 1.3mL of lavage suspension was recovered. Harvested tissues were homogenized by
155 TissueRuptor II with plastic probes (QIAGEN, Hilden, Germany) in cold PBS under ice-water

156 bath for 30 seconds. Tissue homogenates were kept at 2 – 8 °C until phage quantification by
157 plaque assay (described below). Plaque assay was performed within 3 hours of sample collection.

158 *Pseudomonas pulmonary infection*

159 A neutropenic murine model (8) was used to establish pulmonary *P. aeruginosa* infection. Two
160 doses of cyclophosphamide were intraperitoneally administered 4 days (150 mg/kg) and 1 day
161 (100 mg/kg) prior to infection. On the day of infection, the FADDI-001 bacterial suspension at
162 its early logarithmic growth phase was inoculated intratracheally at a concentration of 10⁶ CFU
163 in 25 µL, as described above. At 2 h post-infection, PEV31 suspension (10⁹ PFU in 25 µL PBS)
164 or sterile PBS of equal volume (as untreated control) was intratracheally administered to the
165 infected mice. Following terminal anesthesia as described above, BAL and other tissues were
166 collected at 0 (immediately after bacteria inoculation), 2 (immediately after phage
167 administration), 4, 8, 16 and 26 h post-infection ($n = 4$) (**Figure 1B**). Collected tissues were
168 homogenized in cold PBS. Bacterial load and phage concentrations in the tissue homogenates
169 and BAL were stored on ice at all times and analyzed within two hours using plaque assay and
170 colony counting, respectively, as described below. Bacteria enumeration was performed as soon
171 as practically possible and was not later than 2 hours after sample collections. Plaque assay was
172 performed within 3 hours of sample collection.

173 *Plaque assay*

174 BAL and tissue homogenates were serially diluted in PBS for phage quantification. For samples
175 from infected animals, bacteria were first removed from the homogenates and BAL samples by
176 filtering through 0.22 µm polyethersulfone membrane filter before dilution. A volume of
177 reference bacterial host (PAV237) containing 2×10^8 CFU at stationary phase was mixed with 5
178 mL of Nutrient Broth top agar. The mixture was overlaid on top of a Nutrient Agar plate and
179 dried for 15 min. Then, 20 µL of serially diluted phage suspension were dropped on top of the
180 top agar plate, left to air dry, and then incubated for 24 h at 37°C. The diluted samples were
181 analyzed in triplicate.

182 *Bacteria enumeration*

183 Phage-inactivation was performed prior to bacteria enumeration to prevent bactericidal activities
184 of phages and impede reduction of CFU counts *ex vivo*. Furthermore, all samples were kept on
185 ice at all times to minimize the risk of phage-bacteria interactions *in vitro*. The samples were
186 treated with tannic acid (20 mg/L) and ferrous sulfate (2.5 mM) to inactivate the phage PEV31
187 and then treated with 2% Tween 80 in PBS to stop the interaction. The phage-inactivated lung
188 homogenate was filtered through a sterile filter bag (bag stomacher filter with a pore size of 280
189 μm , Labtek Pty Ltd., Australia). Filtrate samples and BAL were serially diluted in PBS and then
190 spiral plated on Nutrient Agar plates using an automatic spiral plater (WASP, Don Whitley
191 Scientific, United Kingdom). The plates were air-dried and then incubated for 24 h at 37°C.
192 Colonies were counted using a ProtoCOL automated colony counter (Synbiosis, United
193 Kingdom).

194 *Minimum inhibitory concentration*

195 Bacterial colonies from the spiral plates ($t = 0$ and 26 h) were taken and inoculated in Nutrient
196 Broth. The antibiogram of these colonies was assessed by determining the minimum inhibitory
197 concentrations (22) of selected antibiotics, including ciprofloxacin, tobramycin, colistin and
198 aztreonam. A volume (190 μL) of early-log phase bacterial culture (1×10^6 CFU/mL) was mixed
199 with 10 μL of antibiotics (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 $\mu\text{g}/\text{mL}$). The treated bacterial culture
200 was incubated for 24 h at 37°C with continuous shaking at 220 rpm. Optical density at 600 nm
201 (OD_{600}) was measured using a microplate reader (Victor multilabel Plate Reader, Perkin Elmer,
202 United States).

203 *Cytokine quantification*

204 The BAL collected was centrifuged at $400 \times g$ for 10 min, and the supernatant was collected as
205 the broncho-alveolar lavage fluid (BALF). The levels of interleukin (IL)-6, TNF-alpha and IL-1 β
206 (in infected mice) in BALF were quantified by enzyme-linked immunosorbent assay (ELISA)
207 according to the manufacturer's protocol (DY406, DY410 and DY401 from R&D systems;
208 Minnesota, USA). The UV absorbance at 450 nm and 570 nm were measured for the primary
209 signal and for plate correction, respectively (Victor multilabel Plate Reader). The standard curves
210 were constructed by 4-parameter logistic non-linear regression.

211 *Data analysis*

212 For the pharmacokinetics study, regression analysis on the phage titer (PFU) in lungs (lung
213 tissues and BALF combined) over time was performed using simple exponential decay model.
214 The exponential decay takes the form of $P_t = P_0 \times e^{-kt}$ where P_t and k are the relative phage
215 titer at time t and the rate constant (in h^{-1}) respectively (P_0 denoted the phage titer at time = 0). It
216 follows that the half-life of the decay was given by $\ln 2/k$. The regression was performed using
217 Prism software version 8.3 for Windows (GraphPad Software Inc., California, USA). The
218 regressions were done with the sums of the squares weighted by the reciprocal of the dependent
219 variables squared (*i.e.* $1/y^2$).

220

221 **Results**

222 *Phage preparation and in vitro phage susceptibility*

223 The titer of purified PEV31 enumerated against the reference strain (PAV237) used for phage
224 amplification was 4×10^{10} PFU/mL. Phage PEV31 formed a clear zone of lysis on top of overlay
225 plate containing MDR *P. aeruginosa* isolate FADDI-001. PEV31 was highly efficacious *in vitro*
226 against FADDI-001 with an efficiency of plating of 1. Endotoxin level in the purified phage
227 lysate was 3.8 EU/mL (i.e. 0.095 EU in 25 μ L). The spike recovery was 130% and the
228 coefficient of variation of the assay was 4%, which were all considered acceptable as per the
229 manufacturer's recommendations.

230 *Pharmacokinetics of intratracheally administered PEV31*

231 Infectious PEV31 gradually decreased in the lungs (lung homogenate and BALF combined) over
232 time regardless of the administered dose (**Figure 2**). At 24 h after IT administration, the phage
233 titer dropped to 12.3% and 15.2% of the administered dose for the low (10^7 PFU) and high (10^9
234 PFU) doses, respectively. The elimination of active phage could be adequately described using
235 simple exponential decay model, with the adjusted weighted R^2 being 0.864 and 0.956 for the
236 low and high dose, respectively. The rate constant k was estimated to be 0.0875 h^{-1} (95% CI
237 0.0527 to 0.0924) for the low dose and 0.0797 h^{-1} (95% CI 0.0700 to 0.0871) for the high dose,
238 which are equivalent to a half-life of 7.9 (95% CI 7.5 to 13.2) and 8.7 (95% CI 8.0 to 9.9) hours,
239 respectively. Infective PEV31 titer in other organs including kidney, liver, blood and spleen was
240 extremely low and only accounted for less than 0.01% of the administered doses (**Figure 3**). The
241 phage titer gradually increased over 24 hours in the liver of mice that received 10^9 PFU of
242 PEV31. PEV31 suspension was well tolerated at a low dose without changes in inflammatory
243 cytokine level (**Figure 4**). On the contrary, a transient upregulation of TNF- α and IL-6 activity
244 was observed at 4 and 8 h post-administration, respectively when the mice were given a high
245 dose of PEV31. Both cytokines returned to baseline at 24 h after the single IT dose.

246 *Pharmacodynamics of intratracheally administered PEV31*

247 In the infected only group, the bacteria continued to replicate without any significant stationary
248 period (**Figure 5**). Initially, the bacteria grew exponentially for up to about 8 h post-infection
249 (hpi), after which the growing rate decreased. In the infected mice treated with 10^9 PFU of
250 PEV31 at 2 hpi, the bacterial load in lung remained mostly unchanged except for the initial drop
251 at 4 hpi (2 h post-phage IT administration), conferring to more than 4-log reduction in bacterial
252 load at 26 hpi. Some of the survived bacterial colonies at 26 hpi in the phage-treated group
253 showed a different antibiogram profile in comparison with the parent bacteria used to inoculate
254 each mouse (**Table 1**). The MIC value of ciprofloxacin decreased from 8 to 2 $\mu\text{g/mL}$, while
255 tobramycin and colistin increased from 8 to 64 $\mu\text{g/mL}$ and 4 to 32 $\mu\text{g/mL}$, respectively. There
256 were no apparent sensitivity changes to other antibiotics tested and all the tested bacterial
257 colonies from PBS-treated group had the same MIC as the parent stock.

258 The phage-mediated bacterial killing was evident by increase of infectious phage particles over
259 time (around 2-log_{10}) at 24 h post-phage administration (**Figure 6**). Inflammatory cytokines
260 activity (TNF- α , IL-1 β and IL-6) in BALF were also measured as an evaluation of lung
261 inflammation. In bacteria-infected only group, a substantial upregulation of all three cytokines
262 was observed. TNF- α peaked at 4 hpi while the other two cytokines displayed peak activity at
263 later time points. The upregulation of IL-1 β activity considerably diminished at 26 hpi and to a
264 lesser extent for TNF- α , but not for IL-6. Interestingly, the upregulation in cytokines was only
265 partially suppressed by the phage treatment for IL-1- β (23), but not for TNF- α and IL-6. In the
266 phage-treated group, the peak of TNF- α appeared delayed to 8 hpi.

267 **Discussion**

268 This is the first study investigating PK and PD of intratracheally administered *Pseudomonas*
269 phages *in vivo*. In previous studies with mice, the intranasal route has been widely used for
270 initiation of lung infection and then phage treatment (24-26) likely due to ease of administration.
271 These studies provide strong support for inhaled phage therapy with reduction in bacterial load
272 and inflammation in the mouse lung infection model. Compared with intranasal route,
273 intratracheal administration enables direct application of bacteria and phages to the mouse lungs
274 with minimal loss in other parts of the respiratory route, including nose, throat and upper airways
275 (8, 27). Hence, the exact phage doses of interest were given in the PK study, and in the PD study.

276 Despite these advantages, studies on intratracheal administration of phages for lung infections
277 have been scarce (8, 28). In this study, intratracheal instillation was used to administer and assess
278 the PK of phage PEV31 at two different doses.

279 The infectious phage PEV31 as quantified using plaque assay exhibited an exponential decay in
280 the lung at both low and high doses with similar half-life (rate constant, **Figure 2**). After oral
281 inhalation of phages to the lung, the phage titer dropped by 1-log_{10} over 24 hours at both doses.
282 Liu *et al.* studied the PK profile of *Siphoviridae* lytic mycobacteriophage D29 after doing 5×10^8
283 PFU via intra-tracheal route (28). The titer of D29 dropped to 1.2-log_{10} by 24 hours post-
284 administration. Using the same regression methodology on the titers reported by Liu *et al.*, we
285 determined the half-life of D29 to be 5.8 hours, which is lower than our values of 7.9 – 8.7 hours
286 for PEV31. Phage D29 belongs to the *Siphoviridae* family and has a longer phage tail as
287 compared with PEV31 (*Podoviridae*). Whether there is a correlation between the family and/or
288 the geometry of the phage particle and the rate of elimination warrants further investigations.
289 Compared with phage delivered via intravenous injection (10), intra-tracheal route resulted in
290 reduced systemic exposure (**Figure 3**).

291 Our current work has shown that the total titer of administered PEV31 phages in various organs
292 do not add up to 100% of the delivered dose. No phage titer reduction was observed during the
293 sample processing, including homogenization, filtration (0.22 μm PES membrane and BagPage
294 filter) and sample dilution. This implies that phage inactivation or degradation in the lungs
295 and/or other organs are likely. Hence, both biodistribution of phages as well as
296 degradation/inactivation may contribute to the titer reduction observed in the lungs over time.
297 Plaque assay is the method of choice for quantifying infectious phages (29, 30). In a plaque assay,
298 a zone of clearance (plaques) are formed on top of a bacterial lawn as a result of cycles of
299 infection of the bacterial cells with phage progeny radiating from the original source of infection
300 (31). It follows that only infectious phages can be enumerated. To evaluate the total number of
301 viral particles – infectious, non-infectious and defective, genome quantification using qPCR can
302 potentially be utilized (32, 33). The combination of qPCR and plaque assay could potentially
303 help understand the biodistribution of infective phages as well as those that have been broken
304 down or inactivated in different organs. This information may be particularly useful for
305 estimating the total phage burden over time and correlating any long-term side effects associated

306 with accumulation of nano-sized virus particles in the various cavities during a prophylactic
307 treatment. Nano-sized particles (<10 nm) can easily enter human tissues and disrupt the
308 biochemical environment of normal cells (34-38). Nanoparticles mostly accumulate in the liver
309 tissues and adverse unpredictable health outcomes have recently surfaced (39, 40). Our current
310 study has shown that infective phage titer gradually increases in the liver over time, and there
311 may be inactive or degraded phage particles further accumulated in the organ. The most
312 significant phage phagocytosis function is thought to be played by the liver. Phages mostly
313 accumulate in the liver (99%) after intravenous administration and the rate of phagocytosis by
314 Kupffer cells are four times faster than splenic macrophages (41). The current study has shown
315 that by delivering phages directly to the lungs, systemic exposure and liver-induced phagocytosis
316 is substantially minimized.

317 In the current study, phage PEV31 at a high dose [0.095 EU; 4.75 EU/kg] resulted in an
318 upregulation of the inflammatory cytokine TNF- α at 2 h post-administration, which then
319 substantially increased at 4 h (**Figure 4**). The upregulation of IL-6 followed and then peaked at a
320 later timepoint of 8 h. Upon phage administration, TNF- α and other inflammatory cytokines (e.g.
321 IL-1) secreted from resident macrophages stimulated the release of other chemoattractant factors
322 such as MIP-2, MCP-1 and IL-6, promoting the adherence of circulating inflammatory cells to
323 the endothelium (28). The upregulation of both cytokines subsided at 24 h. Overexpression of
324 these cytokines were absent when the mice were administered a lower dose. It has been reported
325 that phages could trigger both inflammatory and anti-inflammatory responses (42), and
326 endotoxin alone cannot explain all the observed upregulation of cytokines in the current study.
327 Liu *et al.* reported no significant differences in leukocyte, neutrophils, lymphocytes and TNF- α
328 levels in the BALF at 24 h post-administration of D29 in healthy mice (28). However, the levels
329 of these cytokines between 0 and 24 h post-administration is unknown and unfortunately, the
330 endotoxin level in the phage preparations was unreported. In another study, intra-nasal
331 administration of phages with endotoxin level of 0.0063 EU/mice (approx. 0.3 EU/kg) did not
332 exhibit appreciable levels of TNF- α at 48 h post-treatment (9). Extremely low TNF- α and IL-6
333 levels were similarly observed in the lungs of mice that received *Pseudomonas* phages via intra-
334 nasal route at 24 h post-administration, although the endotoxin level of the phage lysate was
335 unreported (24). These findings aligned with our observation, where the upregulation of

336 inflammatory cytokines subsided at 24 h post-administration. Hence, phage preparations with
337 endotoxin levels even lower than that for parenteral and free of bacterial impurities should be
338 considered for respiratory delivery (42) to ensure minimum toxicity (43), particularly in the case
339 of prophylactic use. The current consensus is that phage therapy is safe (and has been so for
340 decades) provided the phage preparation is sufficiently purified with low endotoxin level and
341 other bacterial impurities (5, 44, 45). However, phage lysates originated from Gram-negative
342 pathogens can be contaminated with endotoxins (lipopolysaccharides) and other proteins that are
343 toxic to humans. Endotoxins are highly immunogenic and may cause septic shock by triggering
344 cytokine signaling (46-48). The highest permitted endotoxin concentration for injection is 5
345 EU/kg/h. Even purified phage preparations with extremely low endotoxin level (<0.1 EU) may
346 induce some pro-inflammatory responses, which are caused by other bacterial proteins and
347 nucleic acids present in the phage lysate (42). Hence, despite low endotoxin level in our PEV31
348 preparation, bacterial proteins and other contaminants may have caused pro-inflammatory
349 response in the murine lungs. Lung infection with *P. aeruginosa* caused significant increase in
350 inflammatory cytokines (TNF- α , IL-1 β and IL-6), with levels of IL-1 β partially suppressed by
351 phage treatment (**Figure 7**). Immune responses of phages are phage specific and some phages
352 can even be anti-inflammatory such that bacteria clearance is reduced to promote phage
353 propagation, as well as minimize the clearance of phages from the site of infection (42). Anti-
354 inflammatory cytokines (e.g. IL-10) can be investigated in future studies to assess the potential
355 role of phages as an anti-inflammatory agent.

356 MDR *P. aeruginosa* isolate FADDI-001 used in this study is a clinical strain isolated from an
357 ICU patient and is resistant to multiple antibiotics including rifampicin, doxycycline,
358 ciprofloxacin, amikacin, aztreonam and tobramycin. When PEV31 was administered two hours
359 post infection, the growth of FADDI-001 was suppressed, suggesting the rate of bacteria
360 replication was similar to that of bacteria removal through phage-killing or clearance by host
361 immune response, with the former evident by the increase in phage titer (**Figure 6**). FADDI-001
362 was highly susceptible to PEV31 *in vitro* but developed phage-resistance over time (upon
363 overnight incubation) as reported for many naturally occurring phages under *in vitro* conditions.
364 Bacteria can resist phage infection through different mechanisms, including (i) spontaneous
365 mutations to prevent phage adsorption or phage DNA entry, (ii) restriction modification systems

366 to cut phage nucleic acids, and (iii) CRISPR-Cas system mediated adaptive immunity (49-53).
367 When bacteria are pressured with a high number of infective phages, resistance can develop
368 rapidly (54) by changing the bacterial surface components that act as phage-binding receptors.
369 These receptors can be blocked by producing extracellular matrix or competitive inhibitors, or
370 even be removed to prevent phage adsorption (49). Contrary to *in vitro* results (data not shown),
371 some but not all bacteria at 24 hpi remained susceptible to the phage despite high initial MOI.
372 This may be due to fundamental differences between *in vitro* and *in vivo* systems, such as the
373 involvement of mammalian immune responses and heterogeneous mixing. For the latter, it is
374 possible that bacteria and phages were not evenly mixed within the mouse lungs during
375 administration (*i.e.* spatial constrain). Hence, not all the bacteria may have been exposed to the
376 same stress and selective pressure despite high phage titer used in this study. Those colonies that
377 became resistant to phage PEV31 showed a different antibiogram to phage-susceptible bacteria.
378 In the fight to become phage-resistance, FADDI-001 developed increased sensitivity to
379 ciprofloxacin (quinolone), but also developed increased resistance to tobramycin
380 (aminoglycoside) and colistin (polymyxin) (Table 1). The changes to antibiogram suggests
381 possible modifications in the bacterial cell envelope as a result of acquiring phage resistance.
382 The susceptibility of amikacin (another aminoglycoside) remained the same, suggesting the
383 phage-mediated mechanisms for the PEV31-FADDI-001 system to antibiotic susceptibility is
384 antibiotic-specific. Reversal of antibiotic resistance of *P. aeruginosa* under selective pressure of
385 phage has been reported (55). Chan *et al.* isolated a lytic *Pseudomonas* phage OMKO1 that binds
386 to outer membrane porin M of the multidrug efflux systems. MDR *P. aeruginosa* developed
387 resistance to OMKO1 within 24 h of incubation *in vitro*, while these phage-resistant bacteria
388 regained sensitivity to ciprofloxacin, erythromycin, ceftazidime and tetracycline. Understanding
389 the phage-mediated mechanisms to antibiotic susceptibility is outside the scope of this work.
390 However, the data suggested the need to assess the impact of phage treatment on antibiotic
391 susceptibilities for each phage-bacteria system, particularly if combined phage-antibiotic
392 treatment is being considered in a clinical setting.

393 The current study used an acute lung infection mouse model and does not necessarily inform the
394 PK and PD data in chronic infections, such as cystic fibrosis. One of the major challenges in
395 conducting simultaneous PK/PD study of phage therapy in bacterial infection lies in the

396 continued interactions between bacterial host and phage even after sample collection. Procedures
397 have been taken to minimize lysis of bacteria and phage propagation once the lung tissues have
398 been harvested through physical separation by filtration and chemical inactivation by viricides.
399 However, complete elimination of phage from bacteria in the tissue homogenates and removing
400 phages that have already infected the bacteria could be difficult. Any remaining phages that have
401 not been removed or inactivated could reduce the bacteria count and thus overestimate phage
402 killing efficacy.

403 **Conclusion**

404 This is the first study investigating the PK and PD profiles of antipseudomonal phage in the
405 lungs of healthy and *P. aeruginosa*-infected mice, respectively. The safety and biodistribution of
406 phage PEV31 over time were assessed in the lungs of healthy mice. Importantly, inhaled phages
407 not only reduced the lung bacterial load, but also suppressed pro-inflammatory cytokines in the
408 lungs. Bacterial antibiogram was altered upon phage treatment, where bacteria became
409 susceptible to some, and more resistant to other antibiotics. Nonetheless, more work is required
410 to examine the influence of phage exposure on antibiotic susceptibility of bacteria. Further *in*
411 *vivo* toxicity and PK/PD studies evaluating various dose regimes in both acute and chronic
412 models are urgently needed to better understand the phage and bacteria kinetics in the lungs.

413 **Acknowledgement**

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415 Grant APP1140617).

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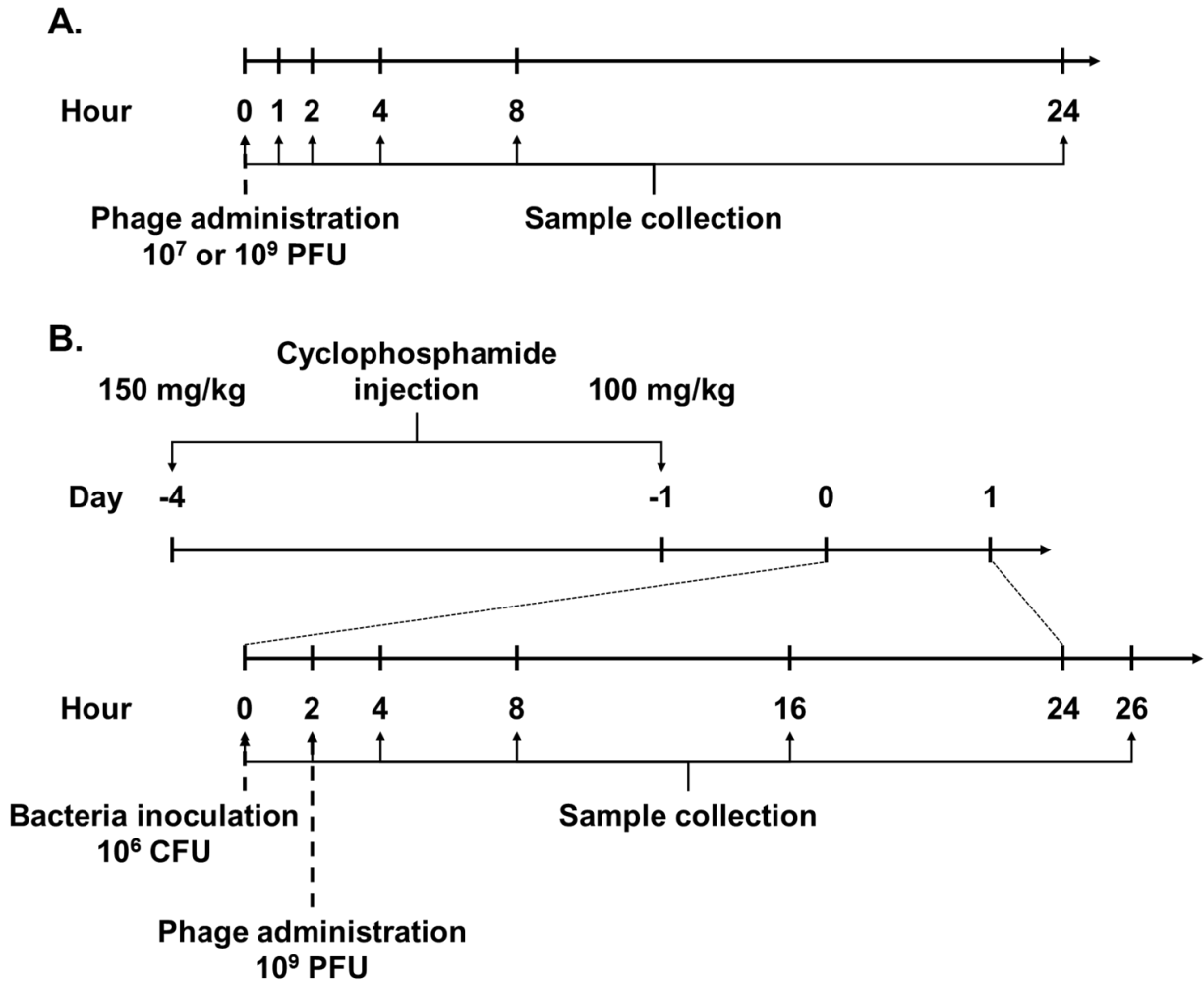
572 **Table 1.** Antibiotic and phage susceptibility of bacterial colonies isolated from the lung
573 homogenate before and after treatment with phage PEV31 or PBS.

574

Time post-infection (h)	Treatment	Number of colonies observed (tested)	MIC ($\mu\text{g/mL}$)					PEV31 susceptibility
			Amikacin	Ciprofloxacin	Tobramycin	Aztreonam	Colistin	
0	n/a	4 (4)	32	8	8	>64	4	S
26	PBS	4 (4)	32	8	8	>64	4	S
26	Phage	3 (8)	32	2	64	>64	32	R
26	Phage	5 (8)	32	8	8	>64	4	S

575 NOTE: MIC, minimum inhibitory concentration; S, susceptible; R, resistant.

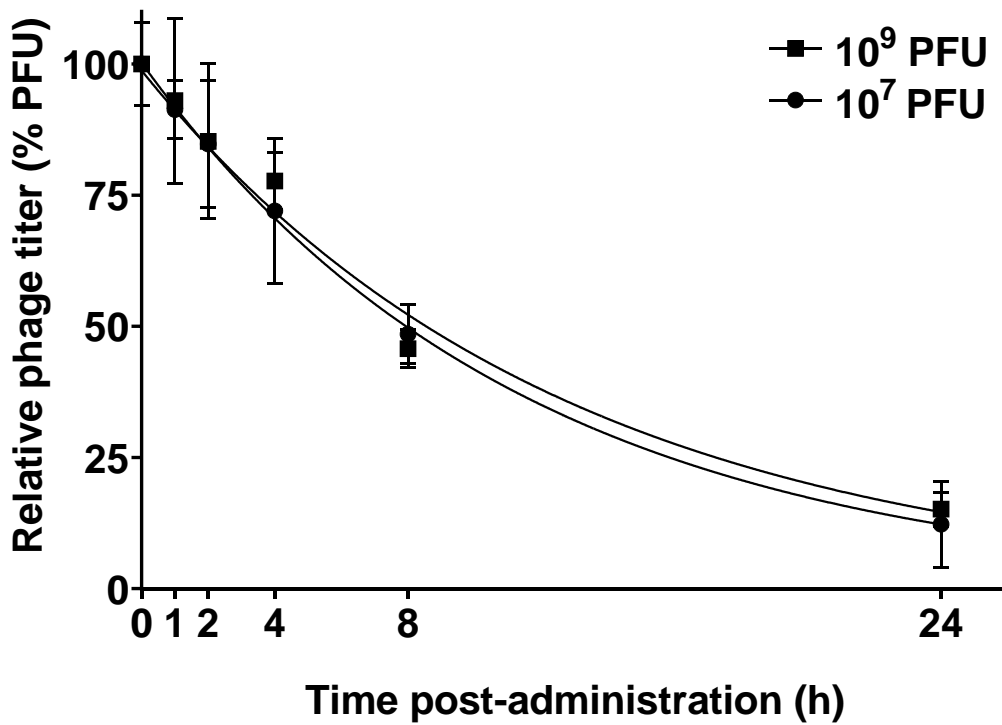
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578 **Figure 1.** Timeline of experimental procedures to investigate the pharmacokinetics (A) and
579 pharmacodynamics (B) of intratracheally administered PEV31 in mice.

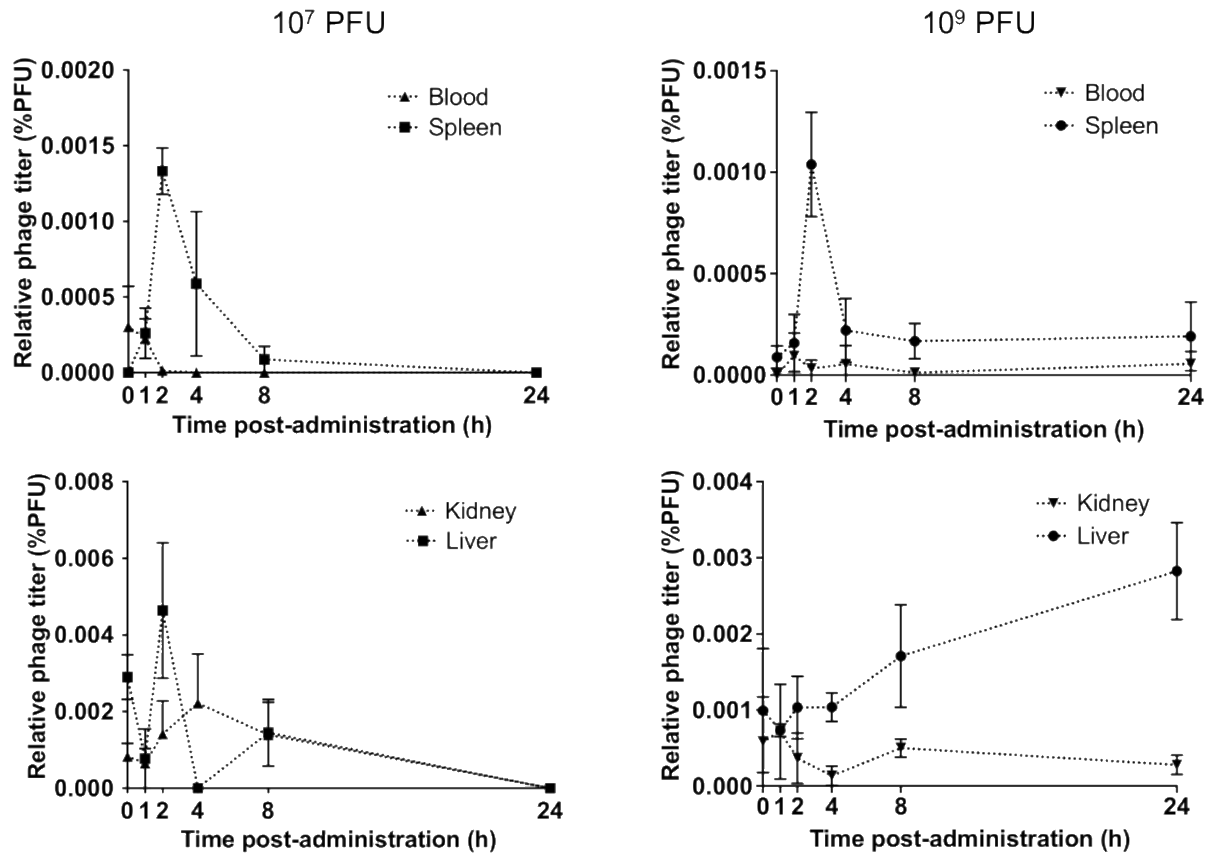
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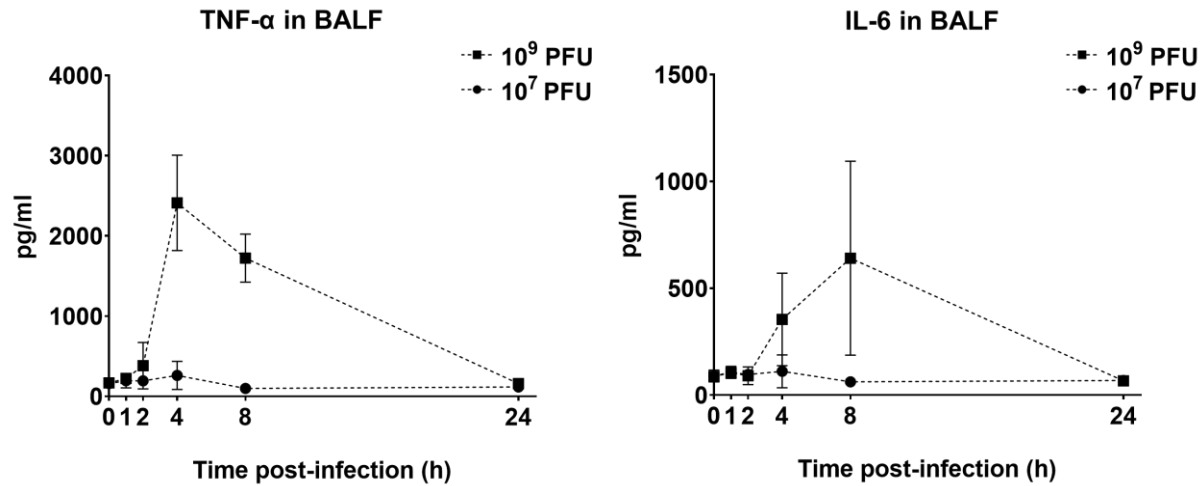
583 **Figure 2.** Relative phage titer in the lungs (lung tissues and BALF combined) of healthy mice
584 after intratracheal administration of phage PEV31 at doses of 10⁷ and 10⁹ PFU. Phage titer is
585 expressed as number of PFU relative to the administered dose. Error bar denotes standard
586 deviation ($n \geq 4$ except for $t = 2$ h of the 10⁷ PFU group, and $t = 1$ h and 4 h of the 10⁹ PFU
587 group where $n = 3$).



588

589 **Figure 3.** Relative phage titer in kidney, liver, blood and spleen at two phage doses. Phage titer
590 is expressed as number of PFU relative to the administered dose. Error bar denotes standard error
591 (n \geq 4).

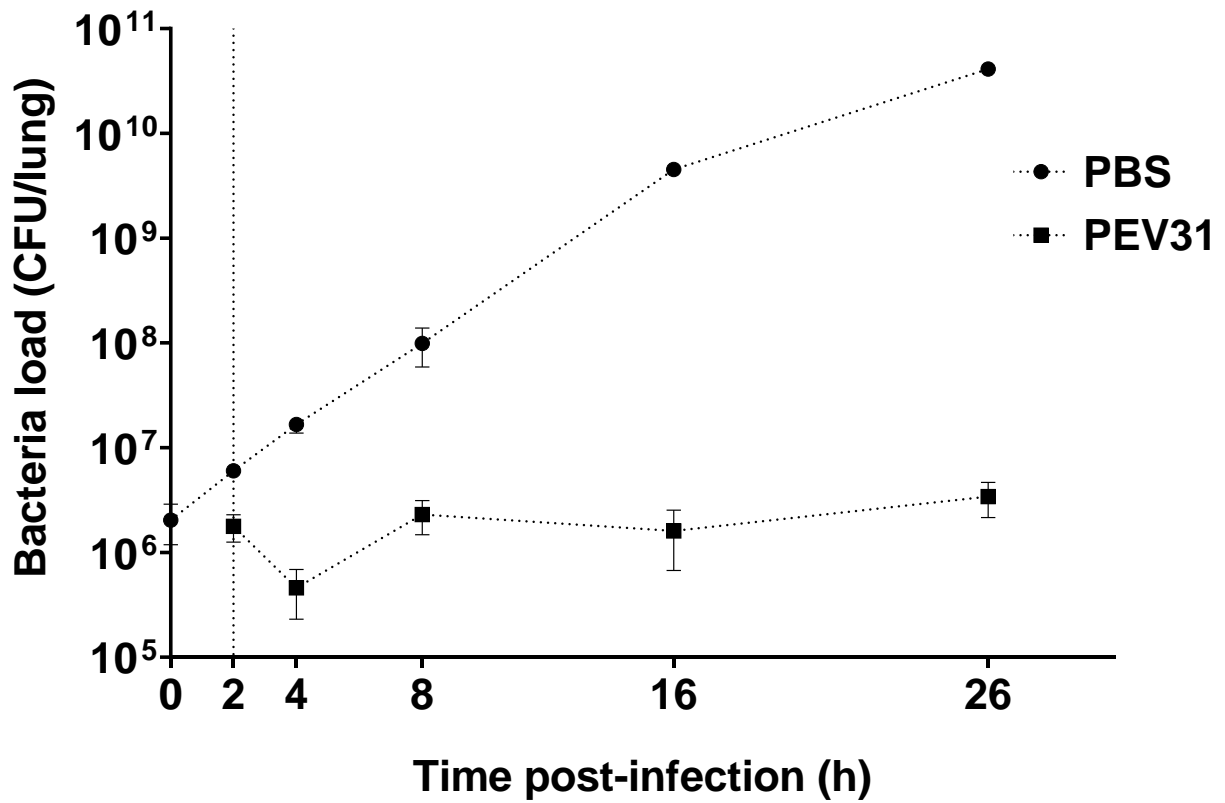
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594 **Figure 4.** Levels of TNF- α and IL-6 in BALF in healthy mice after phage administration at doses
595 of 10^7 and 10^9 PFU. Error bar denotes standard deviation ($n \geq 4$ except for $t = 2$ h of the 10^7 PFU
596 group where $n = 3$).

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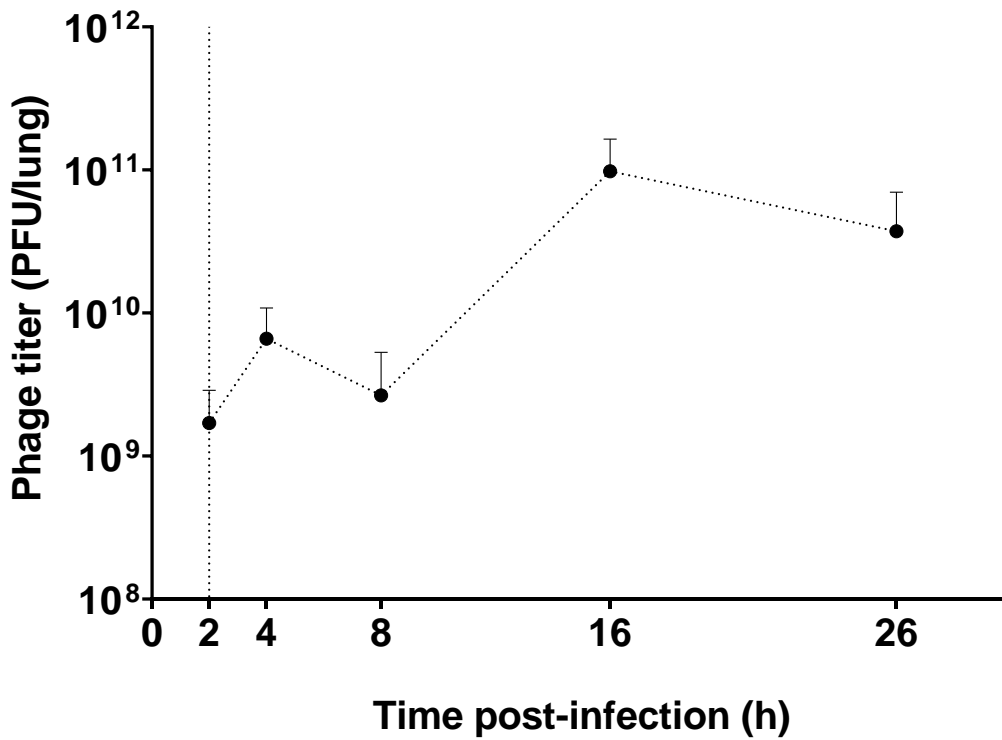


598

599 **Figure 5.** Bacterial load in the lungs of mice treated with PBS and phage over 26 hours. Dotted
600 vertical line represents time of phage administration ($t = 2$ h). Error bar denotes standard
601 deviation ($n = 2 - 4$).

602

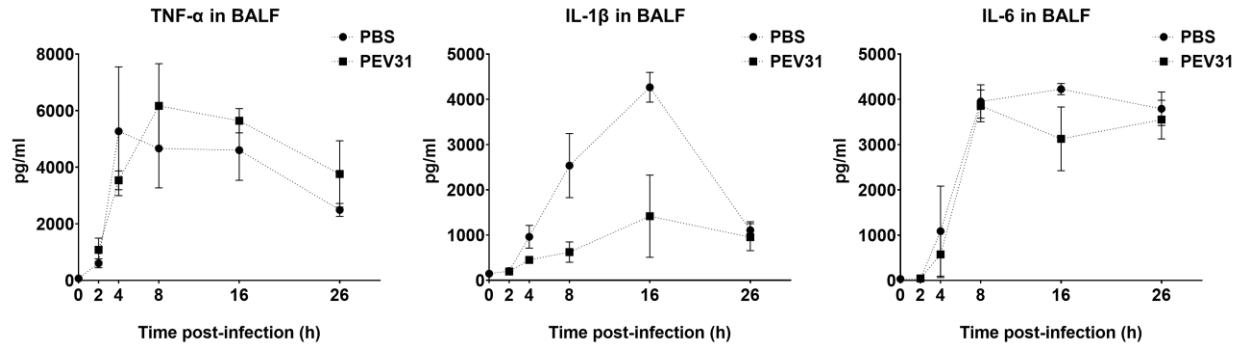
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605 **Figure 6.** Phage titer in the lungs of mice infected with *P. aeruginosa*. Dotted vertical line
606 represents the time ($t = 2$ h) of phage administration (10^9 PFU). Error bar denotes standard
607 deviation ($n = 3 - 4$).

608



609

610 **Figure 7.** Levels of IL-6, TNF- α and IL-1 β in BALF of *P. aeruginosa*-infected mice treated with

611 PBS or phage PEV31 (10^9 PFU) over time. Error bar denotes standard deviation ($n = 2 - 4$).