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# Phage therapy of pneumonia is not associated with an over stimulation of the inflammatory response compared to antibiotic treatment in mice

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## **Abstract (word count: 234)**

**Background.** Supported by years of clinical use in some countries and more recently by literature on experimental models as well as compassionate use in Europe and in USA, bacteriophage (phage) therapy is providing a solution for difficult to treat bacterial infections. However, studies of the impact of such treatments on the host remain scarce.

**Methods.** Murine acute pneumonia initiated by intranasal instillation of two pathogenic strains of *Escherichia coli* (536 and LM33) were treated by two specific bacteriophages (536\_P1 and LM33\_P1; intranasal) or antibiotics (Ceftriaxone, Cefoxitin, Imipenem-Cilastatin; intraperitoneal). Healthy mice also received phages alone. Severity of pulmonary edema, acute inflammatory cytokines (blood and lung homogenates), complete blood count, bacteria and bacteriophages counts were obtained at early ( $\leq 12$ h) and late ( $\geq 20$ h) time points.

**Results.** Bacteriophage's efficacy to decrease bacterial load was faster than antibiotics, but both displayed similar endpoints. Bacteriophage treatment was not associated with an over-inflammation but in contrast tended to lower inflammation and provided a faster correction of blood cell count abnormalities compared to antibiotics. In absence of bacterial infection, bacteriophage 536\_P1 promoted a weak increase in the production of anti-viral cytokines (INF- $\gamma$  and IL-12) and chemokines in the lungs, but not in the blood. However, such variations were no longer observed when bacteriophage 536\_P1 was administered to treat infected animals.

**Conclusions.** The rapid lysis of bacteria by bacteriophages *in vivo* does not increase the innate inflammatory response compared to an antibiotic treatment.

## INTRODUCTION

Among the life threatening infections caused by multi-drug resistant bacteria, ventilator-associated pneumonia is the most severe and frequent healthcare-related infection encountered by mechanically ventilated patients in intensive care unit (1). In this particular context, unconventional antibacterial treatments such as bacteriophages (phages) must be particularly discreet in terms of immune-stimulation to avoid any additional inflammation in the lungs. Indeed, the alveolar epithelium is already highly stressed by mechanical ventilation (2, 3), bacteria and patient's critical condition, which provides independent and underestimated sources of inflammation (4).

Supported both by years of clinical use in some countries and by an increasing amount of experimental data from independent research teams worldwide, phage therapy is now considered *per se* as a realistic therapeutic strategy to treat multidrug resistant and severe infections, as recently illustrated by several compassionate treatments (5-8).

Paradoxically, phage therapy safety is under-investigated despite some clinical feedback (9-12) and *in vivo* as well as *in vitro* data on many phages (13-16). We recently showed *in vitro* that the endotoxin release from two pathogenic strains of *Escherichia coli* cells lysed by two unrelated virulent phages was lower than the one provoked by beta-lactams (13). However, knowing if the phage-induced bacterial lysis and/or the phage particle itself could be a pro-inflammatory stimulus *in vivo* is still a bone of contention.

To address this question, we monitored the effect of two independent phage treatments on the inflammatory response of healthy and infected animals in a murine acute pneumonia model. A reference antibiotic treatment was used as a comparator. We measured the early (10-12 hours post-infection) and late (20-22 hours) host responses based on complete blood counts (CBC), lung edema, cytokine assays in both lung homogenates and blood.

## MATERIAL AND METHODS

**Bacteria and phages.** Strains 536 (17) and LM33 (18) were grown in lysogeny broth at 37°C. Stock solution of phages 536\_P1 and LM33\_P1 were obtained from a high titer lysate on their respective host (strains 536 and LM33) and purified as described in (13). The residual endotoxin content was assessed

using an endpoint fluorescent assay based on the *Limulus ameobocyte* lysate method (EndoZyme II recombinant factor C, Hyglos, Germany): the endotoxin concentration, measured in the solutions of 536\_P1 and LM33\_P1 administered to mice, was 0.072 and 0.003 EU/mL, respectively.

**Antibiotics preparation and doses.** Clinical grade powdered antibiotics (Ceftriaxone, Cefoxitin, Imipenem-Cilastatin) were suspended in sterile LPS free normal saline (B. Braun, Germany) to reach the adequate concentration and administered subcutaneously. Doses and dosing frequency were decided according to the pharmacodynamics/pharmacokinetic data available in mice model (19, 20) and previous experiments (17), to maintain a plasma concentration above the minimal inhibitory concentration (time-dependent antibiotics: : Ceftriaxone (Mylan, USA) was administered every 6 hours (75 mg/kg per dose, 3 doses), Cefoxitin (Hospira, USA) every 2 hours (150 mg/kg per dose, 8 doses) and Imipenem-Cilastatin (Panpharma, France) every 2 hours (100 mg/kg of Imipenem per dose, 8 doses).

**Ethic statement.** Eight-weeks-old BALB/cJRj male specific pathogen-free mice (Janvier, France) were housed in animal facility at Institut Pasteur (Paris, France) in accordance with the French and European regulations on the care and protection of the laboratory animals. Animal experiments were approved by the committee on animal experimentation of the Institut Pasteur (approval 10.565) and by the French Ministry of Research (approval 2015-0041). A total of 121 mice was used in the experimental setting. Food and drink were provided *ad libitum*.

**Murine model of pneumonia.** Mice were infected with  $1 \times 10^7$  colony-forming unit (CFU) of strain 536 or  $5 \times 10^7$  CFU of strain LM33. Bacterial suspensions were prepared as needed from exponentially growing cells. Cells were collected by centrifugation, gently washed 3 times with PBS (phosphate buffered saline) to remove culture medium and resuspended in PBS. Final cells concentration was determined spectrophotometrically (OD 600 nm) and adjusted with PBS, based on prior experiments where CFU/OD relationship has been determined for each strain.

The time of infection was defined as H0. Regarding uninfected mice (receiving only the phage preparation), H0 refers to phage administration. Four hours later (H4), the infected mice received an intranasal administration of 20  $\mu$ L of PBS (control and antibiotic groups) or the therapeutic bacteriophage suspensions (20 $\mu$ L) with a bacterium:phage ratio of 1:100 (calculated from the CFU for each strain inoculated at H0). At H4, the antibiotic treatment was also started: mice received a

subcutaneous injection of antibiotic or isotonic saline (control and phage groups), continued by subsequent injections according to the antibiotic dose regimen.

The following time points were chosen for analysis. H0; H4; H10 (strain 536) or H12 (strain LM33) for early assessment post-treatment; H22 (strain 536) or H20 (strain LM33) for late assessment. The healthy mice that received phages only were analyzed at H7 (early) and H17 (late). These time points were chosen to match those used during experiments with infected mice and to tally with the antibiotic injection schedule to limit mice handling.

Following sacrifice, by a lethal intraperitoneal injection of pentobarbital (10 mg/200  $\mu$ L), blood was collected by a retro-orbital sampling and instantaneously mixed with 5  $\mu$ L of sodium heparinate (5000 UI/mL, Panpharma, France), lungs were removed, weighed and mechanically homogenized (gentleMACS Octo Dissociator, Miltenyi Biotec, Germany) in PBS supplemented with protease inhibitors (Complete Ultra EDTA-free, Roche, Germany). Lungs and blood were kept on ice and serially diluted within one hour to count CFU on selective agar plates (Drigalski medium) and plaque-forming units (PFU) of phage 536\_P1 or LM33\_P1 using routine method in triplicate (17). At least 2 independent experiments were performed.

**Cytokine assays (see Supplemental Material).** Once collected, lung homogenates and blood were centrifuged at 4000 g and 1500 g, respectively, for 10 minutes at 4 °C to pellet either debris or blood cells. Supernatant and plasma were subsequently frozen at -20 °C. Cytokines were quantified using a multiplex magnetic bead-based kit (Mouse Cytokine Magnetic 20-Plex Panel, Life Technologies, USA) relying on the Luminex® Xmap™ technology. Plates were read on a Bio-plex 200 system (Bio-Rad, USA).

**Complete blood count (CBC).** Freshly obtained heparinized blood was analyzed by an automated veterinary hematology cell counter within 30 minutes following collection (Vet ABC+, Scil, Germany) with mice-dedicated gating parameters.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software, California, USA). The normal distribution of all variables was checked using the Kolmogorov-Smirnov test. Statistical tests (Student's t-test or Mann-Whitney test) were chosen accordingly. Comparisons of proportion were performed using the Fisher's exact test. A two-way ANOVA was used to determine how a response (the cytokine variation) is affected by two factors (the time and the treatment). A p value less than 0.05 was considered as statistically significant.

## RESULTS

### **Phages 536\_P1 and LM33\_P1 do not induce a pro-inflammatory response after their administration in the lungs of healthy uninfected mice**

We first examined separately the capacity of phages 536\_P1 and LM33\_P1 (main characteristics detailed in **Table 1**) to induce the synthesis of acute-phase inflammatory cytokines (APCyt) in the lungs and blood of non-infected mice.

CBC and cytokine assays in lung homogenates and plasma were performed 7 hours (n=5) and 17 hours (n=5) following phage administration. We compared these results to those obtained in control mice (n=5) that received PBS.

**Phages 536\_P1.** At H7, no significant change in blood cells parameters was observed between phage and PBS groups (**Figure 1**). At H17, phage 536\_P1 was associated with mild changes in the CBC, namely a slight decrease in platelet count (not statistically significant but below the normal lower limit) and a significant increase in neutrophil granulocytes ( $p=0.03$ ) that nonetheless remained within the normal values (**Figure 1**). No significant increase in concentration of the APCyt was observed in the blood compartment (**Figure S1**), whereas in the lungs, concentration of antiviral cytokines (IFN- $\gamma$  and IL-12) as well as chemokines (such as MIG, MCP-1 and KC) displayed increased levels at H17 ( $p\leq 0.01$ ; for all listed cytokines compared to PBS, see **Figure 2 and S2**).

**Phage LM33\_P1.** The pulmonary administration of LM33\_P1 by the intranasal route was not associated with significant changes in blood cell counts except for lymphocyte count, which was reduced at H7 but returned to the normal level at H17, in comparison with the PBS control group (**Figure S3**). No variations in the concentration of the APCyt could be detected, whether it was in the lung or in the blood. Contrary to phage 536\_P1, we observed no significant increase in the antiviral cytokines or chemokines concentrations in the lungs or in the blood, regardless of the time points considered (**Figures 3, S5, S4**, respectively).

Additionally, two groups of four healthy mice, having each received a single dose of either 536\_P1 or LM33\_P1, were closely monitored for 7 days following the intranasal phage administration. Compared to control animals (PBS, n=4), no difference in their behavior and weight evolution was noticed.

### **Phage 536\_P1 clears strain 536 from lungs of infected mice as efficiently as ceftriaxone, prevents bacteremia and limits pulmonary inflammatory edema**

Following the intranasal instillation of strain 536 (n=43), the bacterial load in the lungs of untreated mice (n=19) continuously increased over time (**Figure 4A**). By contrast, phage treatment (PT) (536\_P1, n=12) resulted in a decrease in bacterial load, significantly more pronounced than the decrease observed upon the ceftriaxone treatment (n=12; p=0.05 at H10, p=0.01 at H22).

Strain 536 has many virulence factors which can favor its dissemination into the blood. Indeed, over the course of the infection, the number of animals with live *E. coli* cells in their blood (limit of detection: 40 CFU/mL) progressively increased over time from 0% (H4) to 40% (H10) and to 83% (H22) with an increasing bacterial load. By contrast, *E. coli* cells could not be detected in the bloodstream of animals treated by phage 536\_P1 or Ceftriaxone (**Figure 4B**).

Concomitant with the severity of the lung infection, was the development of a pulmonary inflammatory edema that we monitored by weighting the lungs over time. Lung weight from the infected untreated mice was about two-fold higher than from non-infected animals at H22 (mean weight 360 vs 171 mg respectively). Compared to infected untreated animals, both phage and ceftriaxone treatments significantly limited the weight increase at late time point (H22; p<0.01; 266 and 271 mg respectively, **Figure 4C**).

### **Phage 536\_P1 and ceftriaxone treatments have a similar beneficial impact in reducing host inflammation over the course of pneumonia**

Having shown that phage and antibiotic treatments were both effective, we then investigated the host acute inflammatory response during these treatments.

The infected untreated animals (n=19) displayed a profound disturbance in their CBC characterized by an early, progressive and marked decrease in leukocytes, from H4 post-infection onwards (**Figure 1A**). This leukopenia was related to a depletion in neutrophil granulocytes and to a greater extent in lymphocytes (**Figures 1B, 1C**). The same kinetic of decrease was observed with platelets (**Figure 1D**). Compared to Ceftriaxone treatment (n=12), PT (n=12) was associated with a higher leukocyte count at H10 (p=0.04, **Figure 1A**). Similarly, the platelet count decrease in phage-treated mice was less pronounced than in those treated with ceftriaxone (**Figure 1D**).

The infected untreated animals displayed an expected increase in the APCyt (IL-6, IL-1 $\beta$ , TNF $\alpha$ , KC, MCP-1), especially as infection progressed over time. The inflammation was essentially present in the lungs while observed in a lower extent in the blood compartment (**Figures 2, S1, S2**). The main findings

regarding the acute-phase host response in treated animals can be summarized as follows: (1) ceftriaxone and 536\_P1 were both responsible for a marked decrease in the levels of APCyt in the lungs, at the late time point, (2) the extent of this decrease did not differ between ceftriaxone and 536\_P1 treatment, (3) the level of the inflammatory response in the blood was weaker than in the lungs, with a quicker resolution as attested by very low levels of inflammation at H22, comparable to those observed in the control animals (untreated, uninfected, n=5), (4) interestingly, the anti-viral cytokine levels at the late time point in 536\_P1-treated mice were not higher (significantly lower) than those recorded in uninfected mice receiving the same amount of phage (IFN- $\gamma$ : 40 vs 69 pg/mL, p=0.03; IL-12: 23 vs 115 pg/mL, p<0.001, respectively).

**A second couple of *E. coli* strain/phage give rise to similar results regarding inflammation and bacterial control.**

To test whether our observations could be extended, we performed a second study using another *E. coli* isolate (strain LM33) and a different virus (phage LM33\_P1). LM33 (18) is an *E. coli* strain producing extended-spectrum beta-lactamase, belonging to the O25b-ST131 clonal complex (21) and isolated from a mechanically-ventilated patient who developed a pneumonia. LM33\_P1 is one of the most rapid and productive coliphage known to date (18), which could potentially cause a massive endotoxin release associated with the bacterial lysis and therefore lead to an excessive pro-inflammatory stimulation. However, preliminary *in vitro* data do not support such a scenario as we previously showed that the endotoxin release upon LM33\_P1 treatment is actually less important than upon a beta-lactam treatment (13).

Because of its lower virulence, the course of the infection and the host response to strain LM33 were anticipated to be different from strain 536. Indeed, mice infected with  $5 \times 10^7$  CFU of strain LM33 but untreated (n=12) controlled the bacterial growth (**Figure S6A**). Consequently, the lung inflammatory edema was weaker than in the animals infected by strain 536 (compare **Figure 4C and S6B**), which overall confirmed the lower virulence of strain LM33 compared to strain 536.

We then compared the efficacy of phage LM33\_P1 (n=8) to two different antibiotic treatments using Cefoxitin (n=8) and Imipenem-Cilastatin (n=8). We observed, as previously (18), that the bacterial load was significantly reduced by PT (p=0.02 at H12 and H20, compared to PBS), in a greater extent than what was observed with antibiotic treatments which turned out to be weakly and slowly active, as they did not lead to a significant decrease in bacterial load (p $\geq$ 0.1 compared to PBS, for both antibiotic at H12 and H20, **Figure S6A**). We also observed that the lung edema was significantly reduced by PT (compared to

PBS), at both early and late time points ( $p=0.02$ , see **Figure S6B**). This was not observed with the antibiotic treatments, except with Cefoxitin at H20 ( $p=0.02$ ).

Infection with strain LM33, in contrast to 536, was associated with a progressive increase in leukocyte and platelet counts, before a return to the initial values at H20 (**Figure S3**). Treatment with LM33\_P1 was associated with a stable count of leukocytes and platelets at H12 and H20. Similar to antibiotic treatments, LM33\_P1 seemed to prevent pathological changes (**Figure S3**).

In animals infected by strain LM33, cytokine concentrations were measured only in the lungs due to invalid blood sampling. Compared to strain 536, the infection caused by strain LM33 was associated with an earlier and more pronounced pulmonary inflammation in regards to the APCyt and chemokines while it was weaker at late time points (**Figures 2, 3, S2, S5, S7**). The level of inflammation recorded in the lungs from LM33\_P1-treated mice was not significantly different when compared to untreated animals (**Figures 3 and S5**). When comparing PT to antibiotic treatments, a two-way ANOVA showed that the time was the only independent factor explaining the variability in the concentration of APCyt (IL-1 $\beta$ , TNF $\alpha$ , IL-6, MCP-1, KC), meaning that a given treatment regimen had no significant impact over another one.

## DISCUSSION

The vital function of the lung (blood-air interface) can rapidly be jeopardized during a bacterial pneumonia through the direct interaction between bacteria and the immunologically highly reactive epithelial surface. In particular, an excessive and uncontrolled inflammation appears to be a key factor in the pathophysiology of the acute respiratory distress syndrome, a life-threatening disease that may complicate the course of severe pneumonia (22, 23). Therefore, the thorough evaluation of the host immune response during an anti-bacterial treatment is of prime interest.

The main findings of our work could be summarized as follows:

- PT efficiently decreases bacterial loads regardless of the virulence and antibiotic resistance profiles of the targeted *E. coli* strains;
- the lung inflammation is differentially affected by each phage and each strain;

- the acute cytokine response to phages is localized, phage-dependent, has no clinically observable consequence and is no longer detectable in infected animals nor responsible for an immune over-stimulation.

We could not find major differences in treatment efficacy between phages and antibiotics. Both decreased the bacterial load in the lungs regardless of the causative pathogen, as well as in the blood of mice infected by strain 536. In each case, both phages acted faster than antibiotics (**Figures 4 and S5**). During treatments of strain 536-infected mice, the inflammatory parameters were strongly reduced by phage 536\_P1 (as ceftriaxone), while in strain LM33-infected mice, phage LM33\_P1 provided a limited decrease despite a faster control of the bacterial load. This limited decrease of inflammatory parameters observed in phage- or antibiotic-treated animals was not different than the one spontaneously observed in untreated animals. This could be interpreted in two ways: either the animal's immune response is sufficient to clear this low-virulence strain, or the treatment (phage or antibiotic) is responsible for a pro-inflammatory stimulation compensating the inflammation reduction usually associated to the treatment. Even though we cannot strictly rule out this latter hypothesis, indirect data (lung weight, decrease in bacterial load) and levels of inflammatory markers from healthy mice exposed to phage LM33\_P1 do not support it.

We also observed an unexpected decrease in leucocyte counts during the course of the infection caused by strain 536. Nonetheless, it is known that severe infections can lead to a paradoxical decrease in leukocyte counts which represents, by itself, a severity criterion in human infectious diseases (24). Several mechanisms (decrease in production, increase in consumption and migration) are intertwined but seem to be the consequence of an immune overstimulation (25). The observed lower inflammation and higher/normal leucocyte counts in treated animals (phages or antibiotics) are in accordance with such interpretation and provide additional data supporting the safety of phages.

The presence of an anti-viral signature in the lungs of healthy uninfected mice, following an intranasal instillation of a purified solution of phage 536\_P1, but not phage LM33\_P1, raises two comments. First, such triggering of anti-viral immunity is phage specific, in agreement with data regarding phage-induced adaptive immunity (26-28). Second, the increase in anti-viral cytokine levels was no longer observed when animals were infected and treated by this phage, despite a major increase in phage particle concentration in the lungs resulting from its amplification when killing bacteria (see **Figure S8**). To our

knowledge, this anti-viral response towards a phage particle is reported here for the first time and its biological (and clinical) significance remain unknown.

Another intriguing result concerns experiments with LM33 where mice treated with phage LM33\_P1 displayed similar concentration of APCyt in the lung homogenates (compared to antibiotic treatments), except for MIG (Monocyte Induced by Interferon- $\gamma$ ) at H20 post-infection (**Figure 3H and S5**). MIG (Monokine Induced by Gamma interferon) is a member of the CXC chemokine family, known to be a T-cell chemoattractant. It is involved in various immunological pathways, including anti-viral (eukaryotic viruses) (29-31) and anti-bacterial immunity, especially when epithelial interface are involved (32-36). Its expression is mediated by IFN- $\gamma$  but not exclusively. The fact that we observed an increased level of MIG in lung homogenates of mice treated by LM33\_P1 and not those receiving LM33\_P1 solely, without a significant increase in IL-12 and IFN- $\gamma$  tends to favor the hypothesis of a bacteria-dependent stimulation of MIG. However, our model was not designed to fully answer this aspect of the question. Part of the explanation lies on the different mechanisms of bacterial lysis performed by a beta-lactam drug or a phage, with important consequences in terms of LPS (and other antigens) release (kinetic and quantity), cell morphology changes and death timing as we previously reported for these two phages (13).

These findings underline the complex modulation of the immune response and its different behavior according to the immune situation (dormant or primed) when a viral antigen presentation takes place. Such multifaceted interactions are progressively being highlighted between eukaryotic viruses and the human immune system, as well as the role of a virus-associated modulation of the immune response towards non-viral antigens. With strong clinical implications, these interactions are observed for example during allograft rejection in solid organ transplantation (37) or during influenza virus-associated bacterial pneumonia (38). Data related to similar interactions regarding prokaryotic viruses (mainly phages) are scarce but some authors pointed out a potential immuno-modulatory role of phages by themselves (39-41). As recently stressed by Krut *et al.*, our ignorance is substantial regarding the immunological aspects of phage therapy despite evidences that phages interact with the immune system on top of their synergistic activity with it (28). All these data point out that phage particles are first able to physically kill bacterial cells and second may also be capable of modulating the immune response, in a phage specific manner, with to date unknown biological significances but without any adverse observable clinical ones.

Our work has two important limits: first, only two phages were investigated here and our results could not be generalized to a greater extent, even if they agree with the general assumption that phages are

safe (42). Second, we only investigated the “acute sensing” through a large but limited number of cytokines and we cannot predict the answer of the adaptive immune response (selection of phage-specific lymphocytes and antibodies synthesis) after few days/weeks.

We conclude that PT does not elicit a pro-inflammatory response whereas it is associated with a comparable (if not greater) microbiological control of the infection, overall strengthening arguments in favor of phage therapy. Together with the increasing compassionate use of phages (5-8) and the recent creation of a new center for *Innovative Phage Applications and Therapeutics* (IPATH) in San Diego, USA (43), our safety data will support further the re-deployment of phage therapy to patients.

## References

1. Nair GB, Niederman MS. 2015. Ventilator-associated pneumonia: present understanding and ongoing debates. *Intensive Care Med* 41:34-48.
2. Chu EK, Whitehead T, Slutsky AS. 2004. Effects of cyclic opening and closing at low- and high-volume ventilation on bronchoalveolar lavage cytokines. *Crit Care Med* 32:168-74.
3. Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS. 1997. Injurious ventilatory strategies increase cytokines and c-fos m-RNA expression in an isolated rat lung model. *J Clin Invest* 99:944-52.
4. Alkharfy KM, Kellum JA, Matzke GR. 2000. Unintended immunomodulation: part I. Effects of common clinical conditions on cytokine biosynthesis. *Shock* 13:333-45.
5. Jennes S, Merabishvili M, Soentjens P, Pang KW, Rose T, Keersebilck E, Soete O, Francois PM, Teodorescu S, Verween G, Verbeken G, De Vos D, Pirnay JP. 2017. Use of bacteriophages in the treatment of colistin-only-sensitive *Pseudomonas aeruginosa* septicemia in a patient with acute kidney injury-a case report. *Crit Care* 21:129.
6. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, Barr JJ, Reed SL, Rohwer F, Benler S, Segall AM, Taplitz R, Smith DM, Kerr K, Kumaraswamy M, Nizet V, Lin L, McCauley MD, Strathdee SA, Benson CA, Pope RK, Leroux BM, Picel AC, Mateczun AJ, Cilwa KE, Regeimbal JM, Estrella LA, Wolfe DM, Henry MS, Quinones J, Salka S, Bishop-Lilly KA, Young R, Hamilton T. 2017. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant *Acinetobacter baumannii* Infection. *Antimicrob Agents Chemother* 61.
7. Pirnay JP, Verbeken G, Ceyssens PJ, Huys I, De Vos D, Ameloot C, Fauconnier A. 2018. The Magistral Phage. *Viruses* 10.
8. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. 2018. Compassionate Use of Bacteriophage Therapy for Foot Ulcer Treatment as an Effective Step for Moving Toward Clinical Trials. *Methods Mol Biol* 1693:159-170.
9. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. 2011. Phage treatment of human infections. *Bacteriophage* 1:66-85.
10. Bruttin A, Brussow H. 2005. Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrob Agents Chemother* 49:2874-8.
11. McCallin S, Alam Sarker S, Barretto C, Sultana S, Berger B, Huq S, Krause L, Bibiloni R, Schmitt B, Reuteler G, Brussow H. 2013. Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. *Virology* 443:187-96.
12. Miedzybrodzki R, Borysowski J, Weber-Dabrowska B, Fortuna W, Letkiewicz S, Szufnarowski K, Pawelczyk Z, Rogoz P, Klak M, Wojtasik E, Gorski A. 2012. Clinical aspects of phage therapy. *Adv Virus Res* 83:73-121.
13. Dufour N, Delattre R, Ricard JD, Debarbieux L. 2017. The lysis of pathogenic *Escherichia coli* by bacteriophages releases less endotoxin than betalactams. *Clin Infect Dis* doi:10.1093/cid/cix184.
14. Miernikiewicz P, Dabrowska K, Piotrowicz A, Owczarek B, Wojas-Turek J, Kicińska J, Rossowska J, Pajtasz-Piasecka E, Hodyra K, Macegoniuk K, Rzewucka K, Kopciuch A, Majka T, Letarov A, Kulikov E, Maciejewski H, Gorski A. 2013. T4 phage and its head surface proteins do not stimulate inflammatory mediator production. *PLoS One* 8:e71036. doi: 10.1371/journal.pone.0071036. eCollection 2013.
15. Roach DR, Leung CY, Henry M, Morello E, Singh D, Di Santo JP, Weitz JS, Debarbieux L. 2017. Synergy between the Host Immune System and Bacteriophage Is Essential for Successful Phage Therapy against an Acute Respiratory Pathogen. *Cell Host Microbe* 22:38-47 e4.
16. Barr JJ. 2017. A bacteriophages journey through the human body. *Immunol Rev* 279:106-122.

17. Dufour N, Debarbieux L, Fromentin M, Ricard JD. 2015. Treatment of Highly Virulent Extraintestinal Pathogenic *Escherichia coli* Pneumonia With Bacteriophages. *Crit Care Med* 43:e190-8.
18. Dufour N, Clermont O, La Combe B, Messika J, Dion S, Khanna V, Denamur E, Ricard JD, Debarbieux L, ColoColi g. 2016. Bacteriophage LM33\_P1, a fast-acting weapon against the pandemic ST131-O25b:H4 *Escherichia coli* clonal complex. *J Antimicrob Chemother* doi:10.1093/jac/dkw253.
19. Lepeule R, Ruppe E, Le P, Massias L, Chau F, Nucci A, Lefort A, Fantin B. 2012. Cefoxitin as an alternative to carbapenems in a murine model of urinary tract infection due to *Escherichia coli* harboring CTX-M-15-type extended-spectrum beta-lactamase. *Antimicrob Agents Chemother* 56:1376-81.
20. Soubirou JF, Rossi B, Couffignal C, Ruppe E, Chau F, Massias L, Lepeule R, Mentre F, Fantin B. 2015. Activity of temocillin in a murine model of urinary tract infection due to *Escherichia coli* producing or not producing the ESBL CTX-M-15. *J Antimicrob Chemother* 70:1466-72.
21. Nicolas-Chanoine MH, Bertrand X, Madec JY. 2014. *Escherichia coli* ST131, an intriguing clonal group. *Clin Microbiol Rev* 27:543-74.
22. Han S, Mallampalli RK. 2015. The acute respiratory distress syndrome: from mechanism to translation. *J Immunol* 194:855-60.
23. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. 1995. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. *Chest* 108:1303-14.
24. Reilly JP, Anderson BJ, Hudock KM, Dunn TG, Kazi A, Tommasini A, Charles D, Shashaty MG, Mikkelsen ME, Christie JD, Meyer NJ. 2016. Neutropenic sepsis is associated with distinct clinical and biological characteristics: a cohort study of severe sepsis. *Crit Care* 20:222.
25. Goyette RE, Key NS, Ely EW. 2004. Hematologic changes in sepsis and their therapeutic implications. *Semin Respir Crit Care Med* 25:645-59.
26. Dabrowska K, Miernikiewicz P, Piotrowicz A, Hodyra K, Owczarek B, Lecion D, Kazmierczak Z, Letarov A, Gorski A. 2014. Immunogenicity studies of proteins forming the T4 phage head surface. *J Virol* 88:12551-7.
27. Shiley JR, Comfort KK, Robinson JB. 2017. Immunogenicity and antimicrobial effectiveness of *Pseudomonas aeruginosa* specific bacteriophage in a human lung in vitro model. *Appl Microbiol Biotechnol* 101:7977-7985.
28. Krut O, Bekeredjian-Ding I. 2018. Contribution of the Immune Response to Phage Therapy. *J Immunol* 200:3037-3044.
29. Salazar-Mather TP, Hamilton TA, Biron CA. 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest* 105:985-93.
30. Hokeness KL, Deweerd ES, Munks MW, Lewis CA, Gladue RP, Salazar-Mather TP. 2007. CXCR3-dependent recruitment of antigen-specific T lymphocytes to the liver during murine cytomegalovirus infection. *J Virol* 81:1241-50.
31. Salazar-Mather TP, Hokeness KL. 2006. Cytokine and chemokine networks: pathways to antiviral defense. *Curr Top Microbiol Immunol* 303:29-46.
32. Yung SC, Murphy PM. 2012. Antimicrobial chemokines. *Front Immunol* 3:276.
33. Reid-Yu SA, Tuinema BR, Small CN, Xing L, Coombes BK. 2015. CXCL9 contributes to antimicrobial protection of the gut during *Citrobacter rodentium* infection independent of chemokine-receptor signaling. *PLoS Pathog* 11:e1004648.
34. Park MK, Amichay D, Love P, Wick E, Liao F, Grinberg A, Rabin RL, Zhang HH, Gebeyehu S, Wright TM, Iwasaki A, Weng Y, DeMartino JA, Elkins KL, Farber JM. 2002. The CXC chemokine murine monokine induced by IFN-gamma (CXC chemokine ligand 9) is made by APCs, targets

- lymphocytes including activated B cells, and supports antibody responses to a bacterial pathogen in vivo. *J Immunol* 169:1433-43.
35. Eliasson M, Frick IM, Collin M, Sorensen OE, Bjorck L, Egesten A. 2007. M1 protein of *Streptococcus pyogenes* increases production of the antibacterial CXC chemokine MIG/CXCL9 in pharyngeal epithelial cells. *Microb Pathog* 43:224-33.
  36. Egesten A, Eliasson M, Johansson HM, Olin AI, Morgelin M, Mueller A, Pease JE, Frick IM, Bjorck L. 2007. The CXC chemokine MIG/CXCL9 is important in innate immunity against *Streptococcus pyogenes*. *J Infect Dis* 195:684-93.
  37. Martin-Gandul C, Mueller NJ, Pascual M, Manuel O. 2015. The Impact of Infection on Chronic Allograft Dysfunction and Allograft Survival After Solid Organ Transplantation. *Am J Transplant* 15:3024-40.
  38. Robinson KM, Kolls JK, Alcorn JF. 2015. The immunology of influenza virus-associated bacterial pneumonia. *Curr Opin Immunol* 34:59-67.
  39. Gorski A, Jonczyk-Matysiak E, Lusiak-Szelachowska M, Weber-Dabrowska B, Miedzybrodzki R, Borysowski J. 2018. Therapeutic potential of phages in autoimmune liver diseases. *Clin Exp Immunol* 192:1-6.
  40. Gorski A, Miedzybrodzki R, Borysowski J, Dabrowska K, Wierzbicki P, Ohams M, Korczak-Kowalska G, Olszowska-Zaremba N, Lusiak-Szelachowska M, Klak M, Jonczyk E, Kaniuga E, Golas A, Purchla S, Weber-Dabrowska B, Letkiewicz S, Fortuna W, Szufnarowski K, Pawelczyk Z, Rogoz P, Klosowska D. 2012. Phage as a modulator of immune responses: practical implications for phage therapy. *Adv Virus Res* 83:41-71.
  41. Van Belleghem JD, Clement F, Merabishvili M, Lavigne R, Vaneechoutte M. 2017. Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by *Staphylococcus aureus* and *Pseudomonas aeruginosa* phages. *Sci Rep* 7:8004.
  42. Loc-Carrillo C, Abedon ST. 2011. Pros and cons of phage therapy. *Bacteriophage* 1:111-114.
  43. Servick K. 2018. U.S. center will fight infections with viruses. *Science* 360:1280-1281.
  44. Russell ES, Neufeld EF, Higgins CT. 1951. Comparison of normal blood picture of young adults from 18 inbred strains of mice. *Proc Soc Exp Biol Med* 78:761-6.

**Supplementary Data:** Supplementary materials (Figures S1 to S7)

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## Table and Figures

**Table 1.** Main characteristics of phages LM33\_P1 and 536\_P1.

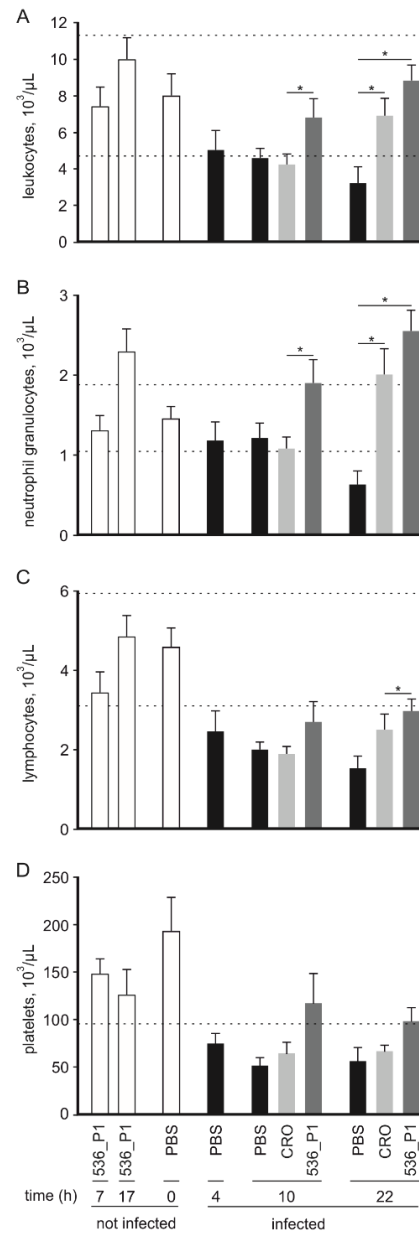
	LM33_P1 (18)	536_P1 (17)
Family	<i>Podoviridae</i>	<i>Myoviridae</i>
Genome size	38.9 kbp	149.4 kbp
Primary host	<i>E. coli</i> LM33 (ESBL, MDR)	<i>E. coli</i> 536 (wild type)
Adsorption constant <sup>a</sup> (mL/min, [95% CI])	$K_1 = 1.2 \times 10^{-8}$ [1.1 to $1.3 \times 10^{-8}$ ]	$K_1 = 6.7 \times 10^{-8}$ [5.0 to $8.4 \times 10^{-8}$ ] $K_2 = 5.1 \times 10^{-9}$ [4.4 to $5.8 \times 10^{-9}$ ]
Latent period <sup>b</sup> (min)	9	19
Eclipse period <sup>c</sup> (min)	7	17
Burst size (PFU, [95% CI])	317 [289-345]	176 [161-193]

<sup>a</sup> Phage LM33\_P1 has a one-step adsorption kinetic while phage 536\_P1 has a two-step kinetic (a fast one followed by a slower one), hence the two different adsorption constants.

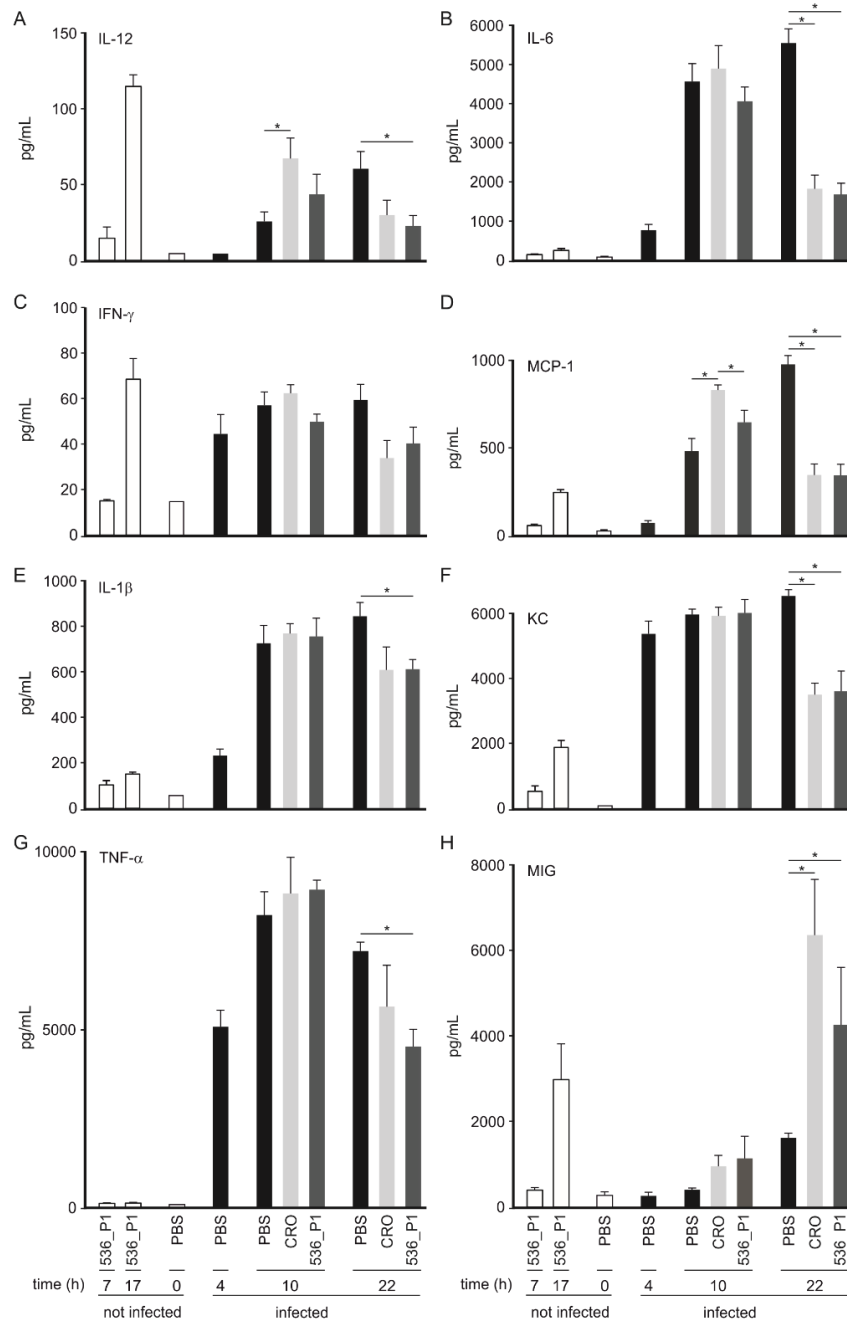
<sup>b</sup> The latent period corresponds to the time elapsed between cell infection and cell lysis.

<sup>c</sup> The eclipse period corresponds to time elapsed between cell infection and the detection of the first functional intracellular virions, before cell lysis.

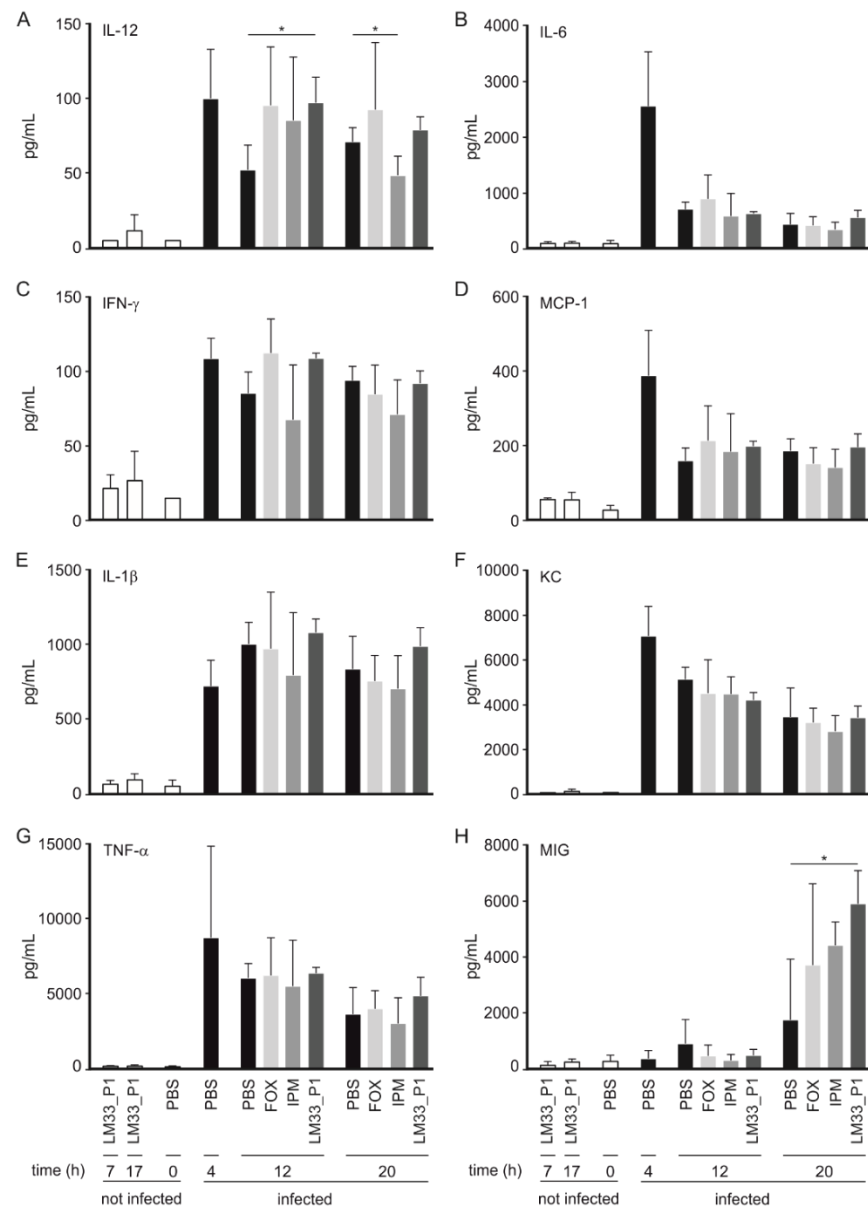
ESBL: extended-spectrum beta-lactamase, MDR: multi-drug resistant, PFU: plaque-forming unit, CI: confidence interval.



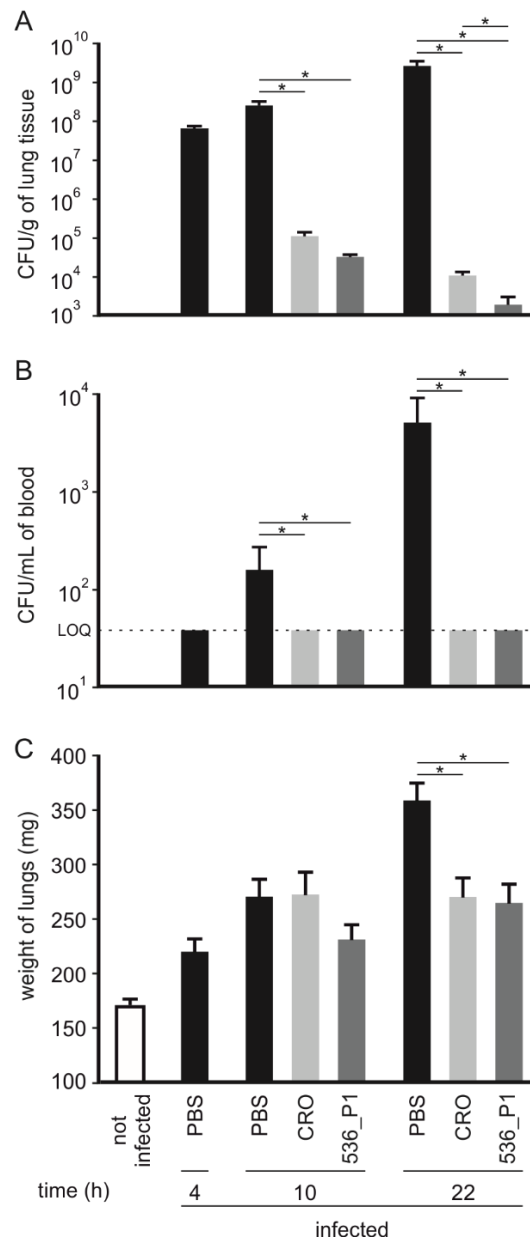
**Figure 1. Complete blood counts observed in the experiments with phage 536\_P1, Ceftriaxone and strain 536.** Total leukocytes (A), neutrophil granulocytes (B), lymphocytes (C) and platelets (D) were measured at indicated time points. Mice infected with strain 536 (n=43), received no treatment (PBS, black bars, n=19) or were treated 4 hours post infection either by Ceftriaxone (CRO, light grey, n=12) or phage 536\_P1 (dark grey, n=12). Uninfected healthy mice (white bars, n=15) either received phage 536\_P1 (assessed 7 and 17 hours after administration, n=10) or PBS (n=5). Dotted horizontal lines represent upper and lower limits of normality for the considered parameter, defined as the 95% confidence interval deduced from the control mice. Data are means with standard errors. (\*) refers to comparisons performed exclusively within the same time point with a p value <0.05.



**Figure 2. Cytokine concentration in lung homogenates observed in the experiments with phage 536\_P1, Ceftriaxone and strain 536.** Mice infected with strain 536 (n=35) received no treatment (PBS, black bars, n=15) or were treated 4 hours post infection either by Ceftriaxone (CRO, light grey, n=10) or phage 536\_P1 (dark grey, n=10). Uninfected healthy mice (white bars, n=15) either received phage 536\_P1 (assessed 7 and 17 hours after administration, n=10) or PBS (n=5). Data are means with standard errors. (\*) refers to comparisons performed exclusively within the same time point with a p value <0.05.



**Figure 3. Cytokine concentration in lung homogenates observed in the experiments with phage LM33\_P1, Cefoxitin, Imipenem-Cilastatin and strain LM33.** Mice infected with strain LM33 (n=36) received no treatment (PBS, black bars, n=12) or were treated 4 hours post infection either by Cefoxitin (FOX, light grey, n=8), Imipenem-Cilastatin (IPM, medium grey, n=8) or phage LM33\_P1 (dark grey, n=8). Uninfected healthy mice (white bars, n=15) either received phage LM33\_P1 (assessed 7 and 17 hours after administration, n=10) or PBS (n=5). Data are means with standard errors. (\*) refers to comparisons performed exclusively within the same time point with a p value < 0.05.



**Figure 4. Bacterial loads and lung weights observed in the experiments with phage 536\_P1, Ceftriaxone and strain 536.** Bacterial loads in lung tissue (A), in blood (B) and weight of lungs (C) of mice infected with strain 536 (n=43) were measured at indicated time points. Mice received no treatment (PBS, n=19, black bars) or were treated 4 hours post infection either by Ceftriaxone (CRO, light grey, n=12) or phage 536\_P1 (dark grey, n=12). Uninfected healthy mice (n=7) are represented by a white bar. LOQ: limit of quantification. Data are means with standard errors. (\*) refers to comparisons performed exclusively within the same time point with a p value <0.05.