Phage Therapy of Pneumonia Is Not Associated with an Overstimulation of the Inflammatory Response Compared to Antibiotic Treatment in Mice

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ABSTRACT Supported by years of clinical use in some countries and more recently by literature on experimental models, as well as its compassionate use in Europe and in the United States, 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. Murine acute pneumonia initiated by intranasal instillation of two pathogenic strains of *Escherichia coli* (536 and LM33) was treated by two specific bacteriophages (536_P1 and LM33_P1; intranasal) or antibiotics (ceftriaxone, cefoxitin, or imipenem-cilastatin; intraperitoneal). Healthy mice also received phages alone. The severity of pulmonary edema, acute inflammatory cytokine concentration (blood and lung homogenates), complete blood counts, and bacterial and bacteriophage counts were determined at early (≤ 12 h) and late (≥ 20 h) time points. The efficacy of bacteriophage to decrease bacterial load was faster than with antibiotics, but the two displayed similar endpoints. Bacteriophage treatment was not associated with overinflammation but in contrast tended to lower inflammation and provided a faster correction of blood cell count abnormalities than did antibiotics. In the absence of bacterial infection, bacteriophage 536_P1 promoted a weak increase in the production of antiviral cytokines (gamma interferon [IFN- γ] and interleukin-12 [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. The rapid lysis of bacteria by bacteriophages *in vivo* does not increase the innate inflammatory response compared to that with antibiotic treatment.

KEYWORDS antibiotic resistance, bacteriophage therapy, cytokines, host response, inflammation, innate immunity, lung infection, pneumonia

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

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TABLE 1 Main characteristics of phages LM33_P1 and 536_P1

Characteristic	LM33_P1 (17)	536_P1 (41)
Family	<i>Podoviridae</i>	<i>Myoviridae</i>
Genome size (kbp)	38.9	149.4
Primary host	<i>E. coli</i> LM33 (ESBL, MDR ^a)	<i>E. coli</i> 536 (wild type)
Adsorption constant ^b (ml/min [95% CI])		
K_{a1}	1.2×10^{-8} (1.1 to 1.3×10^{-8})	6.7×10^{-8} (5.0 to 8.4×10^{-8})
K_{a2}		5.1×10^{-9} (4.4 to 5.8×10^{-9})
Latent period ^c (min)	9	19
Eclipse period ^d (min)	7	17
Burst size (PFU [95% CI])	317 (289–345)	176 (161–193)

^aESBL, extended-spectrum β -lactamase; MDR, multidrug resistant.

^bPhage 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. 95% CI, 95% confidence interval.

^cTime elapsed between cell infection and cell lysis.

^dTime elapsed between cell infection and the detection of the first functional intracellular virions, before cell lysis.

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 to be 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 underinvestigated despite some clinical feedback (9–12) and *in vivo* and *in vitro* data on many phages (13–16). We recently showed *in vitro* that endotoxin release from two pathogenic strains of *Escherichia coli* lysed by two unrelated virulent phages was lower than that provoked by β -lactams (13). However, knowing if the phage-induced bacterial lysis and/or the phage particle itself could be a proinflammatory stimulus *in vivo* is still a bone of contention.

To address this question, we monitored the effects 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 to 12 h postinfection) and late (20 to 22 h) host responses based on complete blood counts (CBC), lung edema, and results of cytokine assays in both lung homogenates and blood.

RESULTS

Phages 536_P1 and LM33_P1 do not induce a proinflammatory 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 noninfected mice.

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

(i) Phage 536_P1. At hour 7 (H7), no significant change in blood cell parameters was observed between the phage and PBS groups (Fig. 1). At hour 17 (H17), phage 536_P1 was associated with mild changes in the CBC, namely, a slight decrease in platelet count (not statistically significant) and a significant increase in neutrophil granulocytes ($P = 0.03$, Fig. 1). No significant increase in concentration of the APCyt was observed in the blood compartment (see Fig. S1 in the supplemental material), whereas in the lungs, the concentrations of antiviral cytokines (gamma interferon [IFN- γ] and IL-12) and chemokines (such as monokine induced by IFN- γ [MIG], monocyte chemoattractant protein-1 [MCP-1], and keratinocyte chemoattractant [KC]) displayed increased levels at H17 ($P \leq 0.01$; for all listed cytokines compared to PBS, see Fig. 2 and S2).

(ii) Phage LM33_P1. Pulmonary administration of LM33_P1 by the intranasal route was not associated with significant changes in blood cell counts, except for the lymphocyte count, which was reduced at H7 but returned to the normal level at H17,

FIG 1 Complete blood counts observed in the experiments with phage 536_P1, ceftriaxone, and strain 536. (A to D) Total leukocytes (A), neutrophil granulocytes (B), lymphocytes (C), and platelets (D) were measured at indicated time points. Mice were infected with strain 536 ($n = 43$), received no treatment (PBS, black bars, $n = 19$), or were treated 4 h postinfection either by ceftriaxone (CRO, light gray, $n = 12$) or phage 536_P1 (dark gray, $n = 12$). Uninfected healthy mice (white bars, $n = 15$) either received phage 536_P1 (assessed 7 and 17 h after administration, $n = 10$) or PBS ($n = 5$). Dotted horizontal lines represent the 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. *, comparisons performed exclusively within the same time point with a P value of <0.05 .

in comparison with the PBS control group (Fig. 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 cytokine or chemokine concentrations in the lungs or in the blood, regardless of the time points considered (Fig. 3, S5, and S4, respectively).

FIG 2 Cytokine concentrations 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 h postinfection either by ceftriaxone (CRO, light gray, $n = 10$) or phage 536_P1 (dark gray, $n = 10$). Uninfected healthy mice (white bars, $n = 15$) either received phage 536_P1 (assessed 7 and 17 h after administration, $n = 10$) or PBS ($n = 5$). Data are means with standard errors. *, comparisons performed exclusively within the same time point with a P value of <0.05 .

FIG 3 Cytokine concentrations in lung homogenates observed in the experiments with phage LM33_P1, ceftiofur, imipenem-cilastatin, and strain LM33. Mice infected with strain LM33 ($n = 36$) received no treatment (PBS, black bars, $n = 12$) or were treated 4 h postinfection either by ceftiofur (FOX, light gray, $n = 8$), imipenem-cilastatin (IPM, medium gray, $n = 8$), or phage LM33_P1 (dark gray, $n = 8$). Uninfected healthy mice (white bars, $n = 15$) either received phage LM33_P1 (assessed 7 and 17 h after administration, $n = 10$) or PBS ($n = 5$). Data are means with standard errors. *, comparisons performed exclusively within the same time point with a P value of <0.05 .

Additionally, two groups of four healthy mice, each having 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 differences in their behavior and weight evolution were noticed.

Phage 536_P1 clears strain 536 from lungs of infected mice as efficiently as ceftriaxone, and it prevents bacteremia and limits pulmonary inflammatory edema. Following the intranasal instillation of strain 536 ($n = 43$), the bacterial load in

FIG 4 Bacterial loads and lung weights observed in the experiments with phage 536_P1, ceftriaxone, and strain 536. (A to C) Bacterial loads in lung tissue (A) and 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 h postinfection either by ceftriaxone (CRO, light gray, $n = 12$) or phage 536_P1 (dark gray, $n = 12$). Uninfected healthy mice ($n = 7$) are represented by a white bar. LOQ, limit of quantification. Data are means with standard errors. *, comparisons performed exclusively within the same time point with a P value of <0.05 .

the lungs of untreated mice ($n = 19$) continuously increased over time (Fig. 4A). In contrast, phage treatment (PT) (536_P1, $n = 12$) resulted in a decrease in bacterial load that was significantly more pronounced than the decrease observed upon the ceftriaxone treatment ($n = 12$; $P = 0.05$ at H10, $P = 0.01$ at hour 22 [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. In contrast, *E. coli* cells could not be detected in the bloodstream of animals treated by phage 536_P1 or ceftriaxone (Fig. 4B).

Concomitant with the severity of lung infection was the development of a pulmonary inflammatory edema that we monitored by weighing the lungs over time. The lung weight from the infected untreated mice was about 2-fold higher than that from noninfected animals at H22 (mean weight, 360 ng versus 171 mg, respectively). Compared to infected untreated animals, both phage and ceftriaxone treatments signifi-

cantly limited the weight increase at late time point (H22; $P < 0.01$; 266 and 271 mg, respectively; Fig. 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 postinfection onwards (Fig. 1A). This leukopenia was related to a depletion in neutrophil granulocytes and to a greater extent in lymphocytes (Fig. 1B and C). The same kinetic of decrease was observed with platelets (Fig. 1D). Compared to ceftriaxone treatment ($n = 12$), PT ($n = 12$) was associated with a higher leukocyte count at H10 ($P = 0.04$, Fig. 1A). Similarly, the platelet count decrease in phage-treated mice was less pronounced than in mice treated with ceftriaxone (Fig. 1D).

The infected untreated animals displayed an expected increase in the APCyt (IL-6, IL-1 β , tumor necrosis factor alpha [TNF- α], KC, and MCP-1), especially as the infection progressed over time. The inflammation was essentially present in the lungs while observed to a lower extent in the blood compartment (Fig. 2, S1, and S2). The main findings regarding the acute-phase host response in treated animals can be summarized as follows: (i) ceftriaxone and 536_P1 were both responsible for a marked decrease in the levels of APCyt in the lungs at the late time point, (ii) the extent of this decrease did not differ between ceftriaxone and 536_P1 treatments, (iii) 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, $n = 5$), and (iv) interestingly, the antiviral 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- γ , 40 versus 69 pg/ml, respectively, $P = 0.03$; IL-12, 23 versus 115 pg/ml, respectively, $P < 0.001$).

A second couple of *E. coli* strain/phage gives 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 (17) is an *E. coli* strain producing extended-spectrum β -lactamase, belonging to the O25b-ST131 clonal complex (18), and isolated from a mechanically ventilated patient who developed a pneumonia. LM33_P1 is one of the most rapid and productive coliphages known to date (17), which could potentially cause a massive endotoxin release associated with the bacterial lysis and therefore lead to excessive proinflammatory 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 that upon β -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 those with strain 536. Indeed, bacterial growth was controlled in mice infected with 5×10^7 CFU of strain LM33 but untreated ($n = 12$) (Fig. S6A). Consequently, the lung inflammatory edema was weaker than that in the animals infected by strain 536 (compare Fig. 4C and S6B), which overall confirmed the lower virulence of strain LM33 compared to that of 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 noted previously (17), that the bacterial load was significantly reduced by PT ($P = 0.02$ at H12 and H20, compared to PBS) to 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; Fig. 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 Fig. 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 (Fig. 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 (Fig. 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 earlier and more pronounced pulmonary inflammation with regard to APCyt and chemokines, while it was weaker at late time points (Fig. 2, 3, S2, S5, and S7). The level of inflammation recorded in the lungs from LM33_P1-treated mice was not significantly different from that in untreated animals (Fig. 3 and S5). In a comparison of PT to antibiotic treatments, a two-way analysis of variance (ANOVA) showed that the time was the only independent factor explaining the variability in the concentration of APCyt (IL-1 β , TNF- α , IL-6, MCP-1, and 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, 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 (19, 20). Therefore, a thorough evaluation of the host immune response during an antibacterial 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; lung inflammation is differentially affected by each phage and each strain; and the acute cytokine response to phages is localized and phage dependent, has no clinically observable consequence, and is no longer detectable in infected animals nor responsible for immune overstimulation.

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 (Fig. 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 in 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, as follows: either the animal's immune response is sufficient to clear this low-virulence strain, or the treatment (phage or antibiotic) is responsible for a proinflammatory stimulation compensating for the inflammation reduction usually associated with the treatment. Even though we cannot strictly rule out the latter hypothesis, indirect data (lung weight and 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 leukocyte 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 (21). Several mechanisms (decrease in production and increase in consumption and migration) are intertwined but seem to be the consequence of immune overstimulation (22). The observed lower inflammation and higher/normal leukocyte counts in treated animals (phages or antibiotics) are in accordance with such an interpretation and provide additional data supporting the safety of phages.

The presence of an antiviral 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 antiviral immunity is phage specific, in agreement with data regarding phage-induced adaptive immunity (23–25). Second, the increase in antiviral 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 Fig. S8). To our knowledge, this antiviral response toward a phage particle is reported here for the first time, and its biological (and clinical) significance remains unknown.

Another intriguing result concerns experiments with LM33, where mice treated with phage LM33_P1 displayed a concentration of APCyt in the lung homogenates similar to that with antibiotic treatments, except for monokine induced by interferon- γ (MIG) at H20 postinfection (Fig. 3H and S5). MIG is a member of the CXC chemokine family known to be a T-cell chemoattractant. It is involved in various immunological pathways, including antiviral (eukaryotic viruses) (26–28) and antibacterial immunity, especially when the epithelial interface is involved (29–33). Its expression is mediated by IFN- γ , 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- γ tends to favor the hypothesis of a bacterium-dependent stimulation of MIG. However, our model was not designed to fully answer this aspect of the question. Part of the explanation relies on the different mechanisms of bacterial lysis brought into play by a β -lactam antibiotic or a phage, with important consequences in terms of lipopolysaccharide (LPS) (and other antigens) release (kinetic and quantity), cell morphology changes, and death timing, as 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 virus-associated modulation of the immune response toward nonviral antigens. With strong clinical implications, these interactions are observed, for example, during allograft rejection in solid organ transplantation (34) or during influenza virus-associated bacterial pneumonia (35). Data related to similar interactions regarding prokaryotic viruses (mainly phages) are scarce, but some authors have pointed out a potential immunomodulatory role of phages by themselves (36–38). As recently stressed by Krut et al., our ignorance is substantial regarding the immunological aspects of phage therapy, despite evidence that phages interact with the immune system on top of their synergistic activity with it (25). All these data indicate that phage particles are first able to physically kill bacterial cells; second, they 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, as follows: 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 (39). 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 antibody synthesis) after a few days/weeks.

We conclude that PT does not elicit a proinflammatory 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, CA (40), our safety data will support further the redeployment of phage therapy to patients.

MATERIALS AND METHODS

Bacteria and phages. Strains 536 (41) and LM33 (17) were grown in lysogeny broth at 37°C. Stock solutions 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 reference 13. The residual endotoxin content was assessed using an endpoint fluorescent assay based on the *Limulus amoebocyte* lysate

method (EndoZyme II recombinant factor C; Hyglos, Germany); the endotoxin concentrations, measured in the solutions of 536_P1 and LM33_P1 administered to mice, were 0.072 and 0.003 endotoxin units (EU)/ml, respectively.

Antibiotics preparation and doses. Clinical-grade powdered antibiotics (ceftriaxone, ceftiofur, and 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 a mouse model (41, 42) and previous experiments (43) to maintain a plasma concentration above the MIC (time-dependent antibiotics), ceftriaxone (Mylan, USA) was administered every 6 h (75 mg/kg per dose, 3 doses), ceftiofur (Hospira, USA) every 2 h (150 mg/kg per dose, 8 doses), and imipenem-cilastatin (Panpharma, France) every 2 h (100 mg/kg imipenem per dose, 8 doses).

Ethics statement. Eight-week-old BALB/cJrj male specific-pathogen-free mice (Janvier, France) were housed in an animal facility at the 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 1×10^7 CFU of strain 536 or 5×10^7 CFU of strain LM33. Bacterial suspensions were prepared as needed from exponentially growing cells. Cells were collected by centrifugation, gently washed 3 times with phosphate-buffered saline (PBS) to remove culture medium, and resuspended in PBS. Final cell concentrations were determined spectrophotometrically (optical density at 600 nm [OD₆₀₀]) and adjusted with PBS, based on prior experiments where the CFU/OD relationship has been determined for each strain.

The time of infection was defined as H0. Regarding uninfected mice (those receiving only the phage preparation), H0 refers to the time of phage administration. Four hours later (H4), the infected mice received an intranasal administration of 20 μ l of PBS (control and antibiotic groups) or the therapeutic bacteriophage suspensions (20 μ l) with a bacterium-to-phage ratio of 1:100 (calculated for each strain from the CFU 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), followed by subsequent injections according to the antibiotic dose regimen.

The following time points were chosen for analysis: H0, H4, and H10 (strain 536) or H12 (strain LM33) for early assessment posttreatment, and 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 coincide with the antibiotic injection schedule to limit mouse handling.

Following sacrifice by a lethal intraperitoneal injection of pentobarbital (10 mg/200 μ l), blood was collected by a retro-orbital sampling and instantaneously mixed with 5 μ l of sodium heparinate (5,000 IU/ml; Panpharma, France); the 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). The lungs and blood were kept on ice and serially diluted within 1 h to count the CFU on selective agar plates (Drigalski medium) and PFU of phage 536_P1 or LM33_P1 using a routine method in triplicate (41). At least 2 independent experiments were performed.

Cytokine assays. Once collected, lung homogenates and blood were centrifuged at $4,000 \times g$ and $1,500 \times g$, respectively, for 10 min at 4°C to pellet either debris or blood cells. The 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) (see supplemental material).

Complete blood count. Freshly obtained heparinized blood was analyzed by an automated veterinary hematology cell counter within 30 min following collection (Vet ABC Plus+; scil, Germany) with mouse-dedicated gating parameters.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software, CA, 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 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 statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00379-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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We declare no conflicts of interest.

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