MAJOR ARTICLE

Efficacy of Bacteriophage Therapy in a Model of *Burkholderia cenocepacia* Pulmonary Infection

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The therapeutic potential of bacteriophages (phages) in a mouse model of acute *Burkholderia cenocepacia* pulmonary infection was assessed. Phage treatment was administered by either intranasal inhalation or intraperitoneal injection. Bacterial density, macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor α (TNF-α) levels were significantly reduced in lungs of mice treated with intraperitoneal phages ($P < .05$). No significant differences in lung bacterial density or MIP-2 levels were found between untreated mice and mice treated with intranasal phages, intraperitoneal ultraviolet-inactivated phages, or intraperitoneal λ phage control mice. Mock-infected mice treated with phage showed no significant increase in lung MIP-2 or TNF-α levels compared with mock-infected/mock-treated mice. We have demonstrated the efficacy of phage therapy in an acute *B. cenocepacia* lung infection model. Systemic phage administration was more effective than inhalational administration, suggesting that circulating phages have better access to bacteria in lungs than do topical phages.

Species within the *Burkholderia cepacia* complex (Bcc) are life-threatening, opportunistic bacterial pathogens for persons with cystic fibrosis (CF) or chronic granulomatous disease (CGD) [1, 2]. In CF, respiratory tract infection with these species can result in acute pulmonary disease and sepsis ("cepacia syndrome") or chronic infection characterized by an accelerated decline in lung function [3, 4]. Although most of the 17 species currently included in the Bcc have been recovered from patients with CF, *B. cenocepacia* is particularly problematic, accounting for ~45% of Bcc infections in patients with CF in the United States [5]. *B. cenocepacia* is also generally considered a contraindication to lung transplantation in patients with CF with end-stage pulmonary disease [6]. Effective antimicrobial therapy of Bcc lung infection in CF and CGD is severely limited by the constitutive and inducible multidrug-resistant phenotypes exhibited by most strains [7–9]. Consequently, there is a critical need for alternative strategies to treat Bcc infection in these vulnerable patient populations.

Bacteriophages (phages) have long been envisioned as a potential therapy for bacterial infections. Indeed, after the discovery of bacteriophages in 1917, phage therapy was used to treat a variety of infectious diseases. Although this practice has continued in Eastern Europe [10, 11], a mixed record of success and the discovery of antibiotics resulted in the decline of phage therapy in Western medicine [12]. The emergence of multidrug-resistant bacteria, however, has renewed interest in phage therapy as an alternative or complement to conventional antibiotic therapy. Several recent, well-controlled animal studies have demonstrated the potential of phages as antibacterial therapy in vivo [13–22].

The development of a successful phage therapy pro-

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gram involves the careful selection and characterization of candidate phages [23]. Lysogenic phages with potential for horizontal transfer of toxin or antibiotic resistance genes between bacteria should be avoided in favor of virulent phages, incapable of a lysogenic lifestyle [24]. Several virulent phages for Bcc species have been well characterized [25]. The broad-spectrum antimicrobial resistance of *B. cenocepacia*, an abundance of novel phages that target this clinically important species, and the availability of tools to characterize these phages make *B. cenocepacia* infection a reasonable choice for a trial of phage therapy.

The objective of this study was to demonstrate the in vivo efficacy of phage therapy for *B. cenocepacia* respiratory tract infection. Using a mouse model of acute lung infection, we examined the effect of treatment with a single phage strain on bacterial load and lung inflammation.

METHODS

Bacterial strains and growth conditions. *B. cenocepacia* strains AU0728 and K56-2 were isolated from the sputum of patients with CF and represent the Midwest and ET12 epidemic lineages, respectively. For infection of mice, bacteria were grown on brain-heart infusion agar (Fisher Scientific) and then subcultured in tryptic soy broth (Fisher). Bacteria were washed in phosphate-buffered saline (PBS; Invitrogen) and resuspended in PBS to the desired concentration.

Bacteriophages. Phage BcepIL02 was isolated from soil collected from a corn field in Champaign County, Illinois, in 2006, by enriching 20 g of soil with *B. cenocepacia* PC184 (another representative of the Midwest lineage) and incubating at 37°C overnight. The culture was centrifuged, and the supernatant was filter-sterilized, diluted, and plated to lawns of PC184. An isolated plaque was recovered and suspended in 1 mL of super optimal broth medium (SM) buffer (50 mmol/L Tris-HCl pH 7.5, 100 mmol/L NaCl, 8 mmol/L $MgSO_4$, 0.01% [wt/vol] gelatin). The phage isolate, designated BcepIL02, was purified by 3 passages through this single-plaque isolation procedure and was routinely propagated in PC184 using standard techniques [26]. BcepIL02 is a virulent phage active against a range of *B. cenocepacia* strains, including the Midwest and PHDC epidemic lineages; however, it shows no activity against *B. cenocepacia* in the ET12 clonal lineage.

For administration to mice, BcepIL02 was propagated in tryptone nutrient broth (TNB; 150 mmol/L NaCl, 5 mmol/L glucose, 5 g/L tryptone, 2.5 g/L yeast extract) using PC184 as a host. The phage liquid lysate was purified using methods modified from Sambrook et al [27]. Endotoxin levels in the finished phage concentrates were <0.125 EU/mL, as determined by Limulus amoebocyte gel-clotting assay (Pyrogent Plus; Lonza Biosciences). Phage λ was prepared by thermal induction of *Escherichia coli* MC4100, containing a λ cI857 Sam7 lysogen [28]. Endotoxin content of the λ concentrate was no greater than 6 EU/mL. When necessary, phages were inactivated by exposure to an ultraviolet (UV) transilluminator for 45 min. An absence of plaques in a top agar plaque assay confirmed 100% inactivation.

Mouse model. The 9- to 12-week-old C57BL/6 mice were infected via tracheotomy with 1×10^7 or 1×10^8 CFUs *B. cenocepacia*, suspended in 50 μL sterile PBS. Control mice were mock-infected with 50 μL PBS without bacteria. Twenty-four hours after infection, mice were treated with either intranasal inhalation or intraperitoneal injection of phage suspended in 50 or 100 μL of SM buffer, respectively, at a multiplicity of infection of 100. Preliminary experiments with dye indicated that 50 μL was a sufficient volume to deliver the suspension uniformly to lower airways via inhalation. Control mice were mock-treated with 50 μL intranasally or 100 μL intraperitoneally of SM buffer or intraperitoneal injection of UV-inactivated BcepIL02 or λ phages. Forty-eight hours after phage treatment, mice were weighed and euthanized, and lungs were collected, weighed, and homogenized in sterile PBS. Lung homogenates were serially diluted and plated for viable bacteria counts on *B. cepacia* selective agar [29]. An aliquot of each homogenate was centrifuged, and supernatants were passed through a 0.45-μm filter and assayed for phage titer via top agar plaque assay. Animal experiments were approved by the University Committee on Use and Care of Animals of the University of Michigan.

MIP-2 and TNF-α. MIP-2 and TNF-α levels in lung were measured using Quantikine enzyme-linked immunosorbent assay kits (R & D Systems) following the manufacturer's instructions. To prepare samples, Complete Protease Inhibitor (Roche) was added to lung homogenates. Samples were assayed in duplicate.

Antibodies. Polyclonal rabbit antibody (R418) to whole lysed *B. cenocepacia* has been described elsewhere [30]. Antibody to BcepIL02 was generated in chickens using 3×10^9 plaque-forming units of UV-inactivated phage as antigen (Virusys). Total immunoglobulin Y was purified from egg yolk and adsorbed against heat-killed AU0728. Antigen affinity and specificity were confirmed by Western blot with AU0728 as a negative control (data not shown). Secondary antibodies were conjugated with AlexaFluor-488 or AlexaFluor-594 (Invitrogen).

Immunofluorescence. Lungs of 2–3 mice from each experimental group were collected 24 or 72 h after infection, fixed in 10% neutral buffered formalin for 24 h, and embedded in paraffin. Lungs were cut into 5 μm sections, air-dried, deparaffinized in xylene, and rehydrated in graded alcohols. Sections were blocked with 5% normal goat serum in tris-buffered saline for 1 h and then incubated with the appropriate primary antibody overnight at 4°C. Bound antibodies were detected by anti-chicken or anti-rabbit antibody conjugated to AlexaFluor-

488 or AlexaFluor-594 as indicated. Sections were counterstained with 4′,6-diamidino-2-phenylindole, mounted, and visualized using a Zeiss LSM 510 confocal laser microscope.

Statistical analysis. Statistical analyses were performed using SPSS software. Parametric 1-way analysis of variance with Games-Howell post-test was used to compare 3 or more groups with data expressed as mean and standard error of mean (SEM). Differences were considered statistically significant if $P < .05$. When sample distributions were not normal, a nonparametric Kruskal-Wallis test was performed. Multiple pairwise Mann-Whitney U tests with Bonferroni correction were performed as post-tests with data expressed as median values.

RESULTS

Phage treatment of pulmonary* B. cenocepacia *infection. We used a model of mouse lung infection to assess the ability of phage BcepIL02 to kill *B. cenocepacia* in vivo. Twenty-four hours after bacterial infection, mice were treated with phage at a multiplicity of infection of 100, administered by either intranasal inhalation or intraperitoneal injection. Infected control mice were mock-treated with either intranasal or intraperitoneal SM buffer without phage. Bacterial densities in lungs were determined 48 h later (or 72 h after infection). The treatment of AU0728-infected mice with BcepIL02 via intraperitoneal injection resulted in a significant reduction in lung bacterial density 48 h after administration relative to mock-treated mice ($P = .002$; Figure 1). AU0728-infected mice treated with intraperitoneal BcepIL02 also had significantly lower lung weights and lung weight to body weight ratios than mock-treated mice (data not shown). A reduced density of bacteria in lungs 48 h after treatment was also observed in AU0728-infected mice treated with intranasal inhalation of BcepIL02; however, the difference between treated mice and mock-treated control mice was not statistically significant.

To rule out the possibility that a nonspecific host immune response to the presence of phage was responsible for the decrease in bacterial density in lungs, we treated AU0728-infected mice with intraperitoneal injection of either UV-inactivated BcepIL02 or λ phage, both of which have no activity against *B. cenocepacia* in vitro. We observed no significant difference in lung bacterial densities 48 h after treatment between mock-treated control mice and mice treated with either UV-inactivated BcepIL02 or λ phage (Figure 1). In mice infected with *B. cenocepacia* strain K56-2 (against which BcepIL02 has no activity in vitro), we found no significant differences in lung bacterial densities between mice treated with intraperitoneal BcepIL02 and mock-treated control mice (Figure 2).

A significant advantage of therapeutic phages over conventional antibiotics is the ability of phage to replicate in the presence of a susceptible bacterial target. We measured phage titers in lungs to assess the degree of phage replication in vivo in the presence of bacteria. Phage titers in AU0728-infected mice 48 h after treatment with BcepIL02 via intranasal inhalation were significantly higher than those in mock-infected mice similarly treated with BcepIL02 ($P = .018$; Figure 3). Phage titers in AU0728-infected mice 48 h after treatment with BcepIL02 via

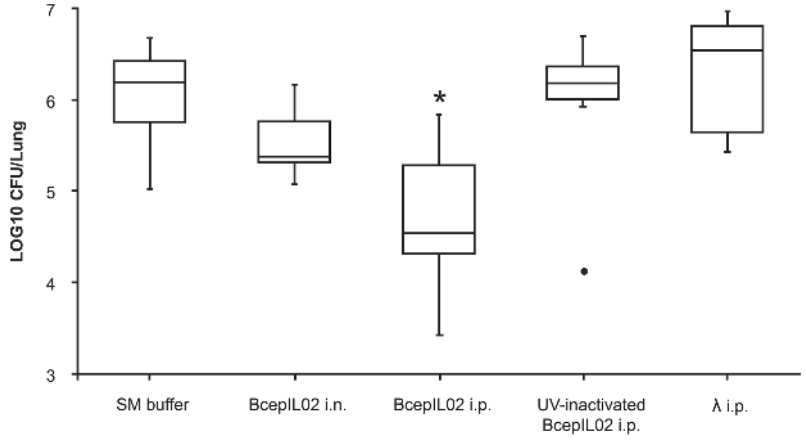

Figure 1. Effect of phage treatment on bacterial density. Density of bacteria in lungs was determined in mice that were infected with *Burkholderia cenocepacia* AU0728 and treated 24 h later with intranasal inhalation (6 mice per group) or intraperitoneal injection (6 mice per group) of BcepIL02. Infected control mice were mock-treated with intranasal (5 mice per group) or intraperitoneal (11 mice per group) super optimal broth medium (SM) buffer without phage (because there was no difference between these 2 groups, results were combined). Other control mice were treated with intraperitoneal injection of ultraviolet (UV)-inactivated BcepIL02 (6 mice per group) or intraperitoneal injection of λ phage (8 mice per group). The horizontal line in each box is the median colony-forming units (CFUs) per lung for the group, calculated from 2 or more independent experiments, each performed in triplicate or quadruplicate; the boxes indicate the interquartile range of CFUs per lung, and the whiskers indicate the range of CFUs per lung for the group. •Extreme outlier; *$P < .05$ (by Mann-Whitney U test) for difference between BcepIL02 intraperitoneally treated mice and SM buffer mock-treated control mice; i.n., intranasal inhalation; i.p., intraperitoneal injection.

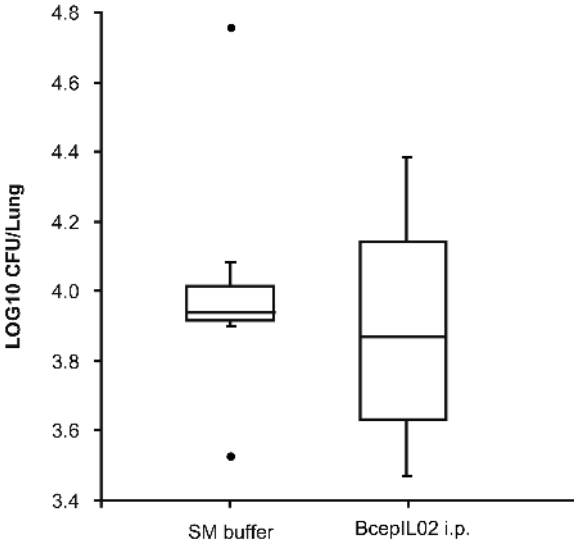

Figure 2. Recovery of *Burkholderia cenocepacia* K56-2 from lungs 48 h after treatment. Density of bacteria was determined in mice that were infected with *B. cenocepacia* K56-2 and were mock-treated 24 h later with super optimal broth (SM) buffer (7 mice per group) or treated with intraperitoneal injection of BcepIL02 (6 mice per group). The horizontal line in each box is the median colony-forming units (CFUs) per lung for the group, calculated from 2 independent experiments, each performed in triplicate or quadruplicate. The boxes indicate the interquartile range of CFUs per lung, and the whiskers indicate the range of CFUs per lung for the group. •Extreme outlier; i.p., intraperitoneal injection.

intraperitoneal injection were also higher than those in mock-infected mice similarly treated with BcepIL02; however, this difference was not significant. In both *B. cenocepacia*–infected and mock-infected mice, ~2 logs more phage were recovered from the lungs of mice treated with BcepIL02 via intranasal inhalation than from those treated via intraperitoneal injection (Figure 3).

Inflammatory markers in lungs after phage treatment. To determine if phage treatment could attenuate infection-associated lung inflammation, we measured levels of proinflammatory cytokine TNF-α and neutrophil chemokine MIP-2 in lungs 48 h after treatment. In AU0728-infected mice, we observed that BcepIL02 treatment resulted in significantly reduced TNF-α levels relative to mock-treated controls, whether phage were administered via intranasal inhalation ($P = .017$) or intraperitoneal injection ($P < .001$) (Figure 4*A*). We also found that AU0728-infected mice treated with BcepIL02 via intraperitoneal injection had significantly reduced levels of MIP-2 in lungs, compared with levels in mock-treated control mice ($P = .026$; Figure 4*B*). Treatment of AU0728-infected mice with BcepIL02 via intranasal inhalation also resulted in reduced MIP-2 levels; however, this reduction was not statistically significant (Figure 4*B*).

AU0728-infected mice treated with UV-inactivated BcepIL02 or λ phage showed no significant differences in TNF-α (Figure 5*A*) or MIP-2 (Figure 4*B*) levels in lungs, compared with those in mock-treated control mice. There were no significant differences in TNF-α or MIP-2 levels in lungs of mice infected with K56-2 and treated with BcepIL02 via intraperitoneal injection, compared with levels in mock-treated control mice (data not shown). To assess the proinflammatory potential of phage alone, we measured lung cytokine levels in mock-infected mice treated with phage. Mice that were mock-infected with PBS and then treated 24 h later with BcepIL02 via either intranasal or intraperitoneal administration had no appreciable levels of either TNF-α or MIP-2 in lungs 48 h after treatment (data not shown).

Localization of bacteria and phage in lungs. To evaluate how phage and bacteria interact spatially in lungs, we used immunofluorescence microscopy to localize phage and bacteria in infected and phage-treated lungs. Twenty-four h after infection with AU0728 (but before treatment), bacteria were found primarily in lung parenchyma in peribronchiolar and perivascular areas, occasionally colocalized with alveolar macrophages. Relatively few bacteria were located within the airway lumen (Figure 5*A*). Seventy-two h after infection, without treatment, bacteria were found predominantly in consolidated lung parenchyma, often forming microcolonies (Figure 5*B*).

When AU0728-infected mice were treated 24 h after infection with BcepIL02 via intranasal inhalation, most phage were colocalized with alveolar macrophages 48 h later (Figure 6*A*).

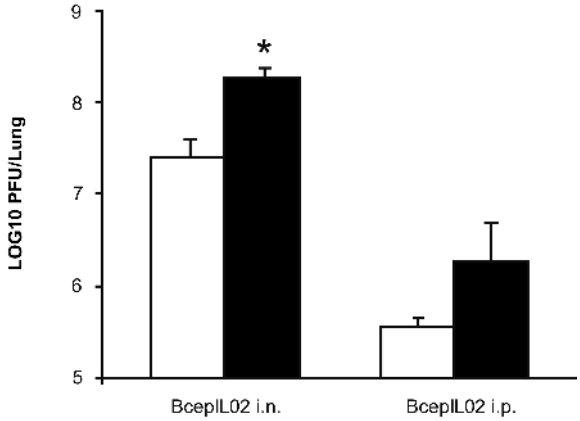

Figure 3. Recovery of phage from treated mice. Titer of BcepIL02 was determined in the lungs of mice that were infected with *Burkholderia cenocepacia* AU0728 (*black bars*; 6 lungs per group) or mock-infected with super optimal broth medium (SM) buffer (*white bars*; 6 lungs per group) and treated 24 h later with either intranasal inhalation or intraperitoneal injection of BcepIL02. Data are mean plaque-forming units (PFUs) per lung 48 h after phage treatment (± standard error of the mean), calculated from 2 independent experiments, each performed in triplicate. *$P < .05$ (Games-Howell) for difference between AU0728-infected mice and mock-infected mice treated with intranasal BcepIL02; i.n., intranasal inhalation; i.p., intraperitoneal injection.

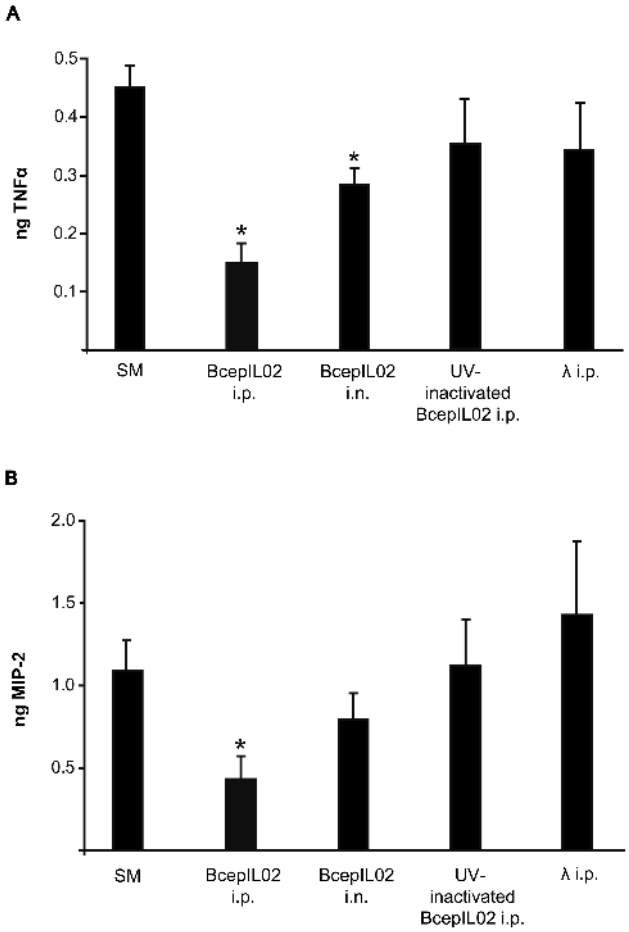

Figure 4. Tumor necrosis factor α (TNF-α) and macrophage inflammatory protein 2 (MIP-2) levels in lungs after phage treatment. Levels of TNF-α (*A*) and MIP-2 (*B*) were measured in mice lungs that were infected with AU0728. Twenty-four hours after infection, mice were mock-treated with super optimal broth medium (SM) buffer (16 mice per group) or treated with BcepIL02 by intraperitoneal (i.p.) injection (6 mice per group) or intranasal (i.n.) inhalation (6 mice per group). Other control mice were treated via i.p. injection with ultraviolet-inactivated phage BcepIL02 (6 mice per group) or λ phage (8 mice per group). Data are mean ng of TNF-α or MIP-2 per lung ($\pm$ standard error of the mean), calculated from 2 or more independent experiments, each performed in triplicate or quadruplicate. *$P < .05$ (Games-Howell) for differences between mice that received the treatments indicated and the control mice that received mock treatment with SM buffer.

Phages and macrophages occasionally colocalized with degraded bacteria in alveolar septa (Figure 6*B*). Few intact bacteria were observed in alveolar septa or within macrophages and no bacteria were observed in the airway lumen at this time. Mice that were mock-infected with PBS alone and then treated 24 h later with BcepIL02 via intranasal inhalation showed phage colocalized with alveolar macrophages 48 h later (data not shown). Forty-eight hours after treatment of infected mice with BcepIL02, far fewer phages were observed in lungs from mice treated via intraperitoneal injection than in lungs from intranasal-treated mice. The greatest concentrations of phage in the intraperitoneally treated mice were observed in perivascular areas (Figure 6*C*) and alveolar septa, where phage could be found colocalized with degraded bacteria (Figure 6*D*). Few intact bacteria were observed in sections from mice treated with phage via intraperitoneal injection.

DISCUSSION

Because *Burkholderia* species typically are nonpathogenic in animals, studies investigating the virulence of these species often employ the agar bead model of respiratory infection, where bacteria are embedded in agar beads prior to instillation into murine lungs [31]. Animals develop nonlethal pulmonary disease, and viable bacteria may be recovered from lungs for 2–3 weeks. However, because phage killing depends on unobstructed access of phages to their bacterial target, we chose to employ a model of acute infection that avoids encasing bacteria in agar. In a series of preliminary experiments, we determined that when inoculated into the lungs of mice via intratracheal instillation, *B. cenocepacia* causes nonlethal pulmonary disease and can be recovered for up to 7 days, thus providing a tractable model to assess the relative efficacy of anti-infective treatments. We also chose to use *B. cenocepacia* strain AU0728, which was recovered from respiratory culture of a patient with cystic fibrosis, and is a representative of the *B. cenocepacia* Midwest clone, a lineage that accounts for a great deal of infection among patients with cystic fibrosis in the United States [32]. Furthermore, our previous work with AU0728 indicates that it is multidrug-resistant and capable of robust biofilm formation in vitro (unpublished data) and thus ought to provide for a rigorous test of novel anti-infective therapies.

With this acute respiratory infection model, we demonstrated in vivo efficacy of phage BcepIL02 in decreasing bacterial den-

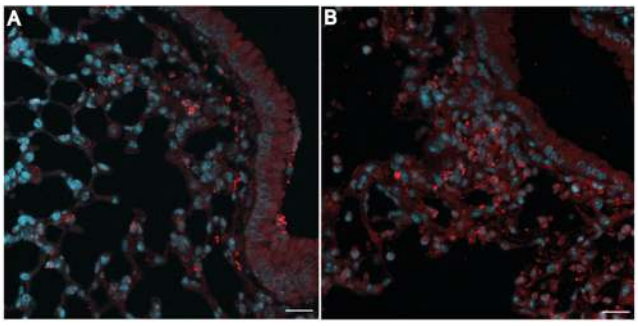

Figure 5. Immunofluorescent localization of *Burkholderia cenocepacia* AU0728. C57/BL6 mice were infected with *B. cenocepacia* AU0728. Lungs were removed 24 h (*A*) or 72 h (*B*) later, fixed, sectioned, stained for bacteria (*red*), and counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *A*, Bacteria localized primarily in lung parenchyma, with few bacteria observed in airway lumen. *B*, By 72 h after infection, bacteria formed microcolonies in areas of consolidated lung parenchyma (*bar*, 20 μm).

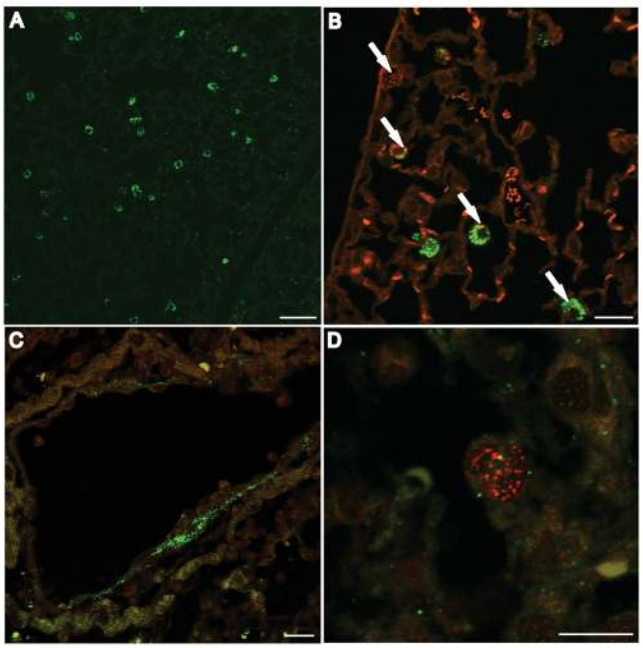

Figure 6. Immunofluorescent localization of *Burkholderia cenocepacia* AU0728 and phage BcepIL02 in mouse lungs. C57BL/6 mice were infected with *B. cenocepacia* AU0728 and treated 24 h later by intranasal inhalation (*A, B*) or intraperitoneal injection (*C, D*) of BcepIL02. Lungs were removed 48 h later, fixed, sectioned, and stained for phage (*green*) and bacteria (*B, C, D*; *red*). Phage administered via intranasal inhalation primarily localized in alveolar macrophages (*A, B*). Arrows (*B*) indicate localization of degraded bacteria inside macrophages. Phage administered via intraperitoneal injection were found primarily in perivascular areas (*C*) and alveolar septa, where they could be observed colocalized with degraded bacteria (*D*). Bar indicates 50 μm (*A*), 20 μm (*B, C*), or 10 μm (*D*).

sity and lung inflammation during infection. These positive treatment effects were not observed with either UV-inactivated phage or λ phage, and BcepIL02 was ineffective against infection with *B. cenocepacia* K56-2, a strain resistant to BcepIL02. These findings indicate that active and specific phages are required for effective treatment.

We found that phages were more effective when administered via intraperitoneal injection than when administered by intranasal inhalation, despite the significantly greater titers of phages observed in lungs after treatment via inhalation. The reasons for this observation are not clear, although previous studies have similarly shown the superiority of systemically administered phage, compared with topical phage, in treating infection [16, 18, 22]. In preliminary experiments, we established that the volume used to administer phage by inhalation was sufficient to deliver the suspension to lower airways. Nevertheless, it appears that phages delivered via inhalation have less access to (and/or are less active against) bacteria than are phages delivered via systemic circulation. This suggests that the site of greatest bacterial killing may be within lung parenchyma rather than within the airway lumen. Phages delivered directly to the airway lumen may not be able to effectively penetrate the respiratory epithelium to encounter bacteria that have translocated from the lumen to the lung interstitium. Indeed, it appears that bacteriophage are not able to readily penetrate eukaryotic cells [33]. In a set of preliminary in vitro experiments using polarized 16HBE14o human bronchial epithelial cells, which form intercellular tight junctions, we observed that BcepIL02 delivered to the apical cell surface did not penetrate the intact epithelium (data not shown). It is also possible that the lower phage titers we observed in infected lungs after intraperitoneal injection (relative to phage titers after inhalation) reflect a more rapid decrease in bacterial density and the subsequent clearance of phage in the absence of sufficient bacterial hosts [34]. Finally, we found no significant differences between any of the treatment or control groups in the density of bacteria recovered from spleens of infected mice (data not shown). Although it is possible that systemic phage may have greater access to such extrapulmonary sites than do intranasal phage, this finding suggests that bacterial reinfection of lung from infected spleen does not account for the differences in lung bacterial density observed between groups.

Several previous studies of phage therapy in animal models of infection found that delaying intervention for more than a few hours after infection mitigated or eliminated entirely any positive treatment effects [15, 16, 21, 22, 35]. In these models, which generally employ a lethality end point, phage intervention must be timed to interrupt the exponential expansion of the infecting bacterial population before the host succumbs. In contrast, in our nonlethal infection model, we found that phages administered well after infection was established were effective in producing positive treatment effects.

To better assess the dynamics of phage-bacteria interaction in infected lungs, we employed confocal immunofluorescent microscopy to localize phage, bacteria, and host cells. We found that within 24 h of intratracheal instillation, relatively few bacteria were located in the airways; rather, bacteria were found primarily in lung parenchyma occasionally colocalized with alveolar macrophages. By 72 h after infection, bacteria were found predominantly in areas of consolidated lung, often forming large microcolonies. After phage treatment via inhalation, phages were found primarily inside alveolar macrophages, suggesting that a large proportion of inhaled phage may be sequestered in macrophages and therefore unavailable to infect and kill bacteria within the lung parenchyma. In striking contrast, phages administered via intraperitoneal injection were not found within alveolar macrophages. Rather, phages were observed primarily in vascular and perivascular areas, as well as in alveolar septa, where they could be observed colocalized with degraded bacteria. Very few intact bacteria were observed in lungs from mice treated with intraperitoneal phage.

After inhalation, phage titers in lungs were significantly greater in *B. cenocepacia*–infected mice than in mock-infected control mice ($P < .05$), suggesting that inhaled phages were able to reach some target bacteria and replicate. Whether phages are taken up with bacteria by macrophages after primary infection, preventing efficient secondary infection, or whether phages replicate within bacteria inside macrophages is unclear and is the subject of ongoing research. Broxmeyer et al [36] observed that a *Mycobacterium* phage was able to kill *Mycobacterium avium* and *Mycobacterium tuberculosis* residing within macrophages; however, phages were carried into the intracellular compartment by phage-infected *Mycobacterium smegmatis*. In our study, phages were found localized in alveolar macrophages in mock-infected mice administered phage by inhalation, suggesting that a significant proportion of inhaled phage are taken up by macrophages even in the absence of bacteria. In any event, the high phage titers observed in lungs 48 h after inhaled phage treatment suggests that a significant proportion of phage remains viable after uptake by macrophages.

A number of concerns have been raised regarding the therapeutic use of phages. Chief among these is that the mammalian host immune response will render the phage inactive and repeat dosing ineffective. Although it has been suggested that innate immunity may be sufficient for rapid elimination of phage from circulation [19], studies have shown the persistence of high phage titers in blood as long as a suitable bacterial host was present [14, 17]. Selection of long-circulating phages is a strategy that has been suggested to avoid rapid immune clearance [19]. The development of neutralizing antibodies could also potentially inactivate phages. The presence of antiphage antibodies after phage therapy has been reported [13, 20], although the impact that these antibodies have on efficacy of repeated phage dosing is unclear. Although the short course of our infection model makes it unlikely that we would have observed such an induction of humoral immunity, the effect of long-term exposure to circulating BcepIL02 is unknown. As has been suggested by others, and as we observed here, the effect of phage therapy is rapid, likely faster than specific immunity can develop [37]. Repeated dosing using phages with different antigenicities to avoid immunologic cross-reactivity is another means to evade immune inactivation. Moreover, at least with the model used here, treatment with only a single phage had a positive treatment effect, indicating that the emergence of resistant bacteria, often cited as a major weakness for phage-based treatment, might not always limit efficacy. In any case, most scenarios in which phages would be deployed in clinical settings would involve cocktails of multiple phages with different receptor specificities. Such cocktails may consist of several distinct phages or a single phage modified to extend its host range.

In summary, we have shown that phage therapy may be a viable option for treating life-threatening *B. cenocepacia* respiratory infections. Future studies that assess the efficacy of phage therapy in chronic infection models of Bcc infection are worthwhile, as are studies to determine the optimal dosing and timing to achieve maximal therapeutic effect. The route of phage delivery appears to be an important determinant of successful therapy. A better understanding of the pharmacokinetics of phage treatment and the specific interactions of phages with the mammalian host immune system will be critical in optimizing a phage therapy program.

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