Inhaled bacteriophage-loaded polymeric microparticles ameliorate acute lung infections

Rachit Agarwal1,2,7, Christopher T. Johnson2,3, Barry R. Imhoff4,5, Rodney M. Donlan6, Nael A. McCarty4,5 and Andrés J. García1,2*

Lung infections associated with pneumonia, or cystic fibrosis caused by *Pseudomonas aeruginosa* or other bacteria, result in significant morbidity and mortality, in part owing to the development of multidrug resistance, also against last-resort antibiotics. Lytic bacteriophages (that is, viruses that specifically kill bacteria) can reduce lung-associated infections, yet their clinical use is hindered by difficulties in delivering active phages to the deep lung. Here, we show that phage-loaded polymeric microparticles deposit throughout the lung via dry powder inhalation and that they deliver active phages. Phage-loaded microparticles effectively reduced *P. aeruginosa* infections and the associated inflammation in wild-type and cystic fibrosis transmembrane-conductance-regulator knockout mice, and rescued the mice from pneumonia-associated death. These polymeric microparticles might constitute a clinically translatable therapy for eradicating hospital-acquired lung infections and infections associated with cystic fibrosis.

Approximately 5.6 million cases of community-acquired pneumonia occur annually in the USA, and it is the most common cause of death from infectious disease. Another 200,000 cases of healthcare-associated pneumonia result in 36,000 annual deaths in the USA. The lungs of cystic fibrosis patients (~29,000 annually) also become colonized by pathogenic bacteria, resulting in chronic infection and inflammation. *Pseudomonas aeruginosa* is the main causative agent of morbidity and mortality in lung infections. Antibiotic therapy is limited by dosing and multidrug resistance, and even last-resort antimicrobials are ineffective in eradicating infections.

Phages are promising agents for the treatment of bacterial infections due to their ability to infect and lyse bacteria, replicate and degrade biofilm matrix. Because the mechanisms by which phages target bacteria are unrelated to the action of antibiotics, phage therapy is effective against multidrug-resistant bacteria. The use of mixtures of phages reduces the development of resistance against phage therapy. Phages are specific to one, or a few, closely related target bacterial species, and do not infect the commensal microflora of the patient, which can be severely perturbed by antibiotics. In addition, phages have been shown to produce less endotoxin upon bacterial lysis compared with antibiotics such as β-lactams. Phages can be engineered to deliver biofilm-degrading enzymes, increase antibiotic efficacy or provide alternative antibacterial strategies. Recently, compassionate phage treatment of two patients with antibiotic-resistant bacteria in Europe and the USA has shown the translational potential, efficacy and safety of phage therapy. Several studies have shown the potential of phage therapy to reduce lung bacterial infections. A recent study showed that phages and the immune system act synergistically to clear pathogenic lung infections. However, the use of nebulizers or intranasal administration in these studies limits phage stability, clinical translation and patient compliance. To overcome these limitations, dry powder formulations of bacteriophages with or without excipients have also been tested. However, rapid loss of phage activity due to exposure to harsh fabrication conditions, lack of a suitable carrier for deep lung delivery and proof of efficacy in animal models are major challenges for clinical translation of phage therapy when used without suitable delivery vehicles. Here, we describe engineered phage-loaded microparticles (phage-MPs) for pulmonary administration via dry powder inhalation to deliver therapeutic doses of active phages to the site of infection, and demonstrate significant reductions in bacterial counts and enhanced survival of the host. This therapeutic formulation is stable at room temperature and can be translated to conventional inhalers for ease of administration and increased patient compliance.

For pulmonary delivery, particles must have an aerodynamic diameter of ~1–5 μm for efficient deposition to the deep lung. The Δd is related to the physical diameter d and density ρ of the particle \( d_{\text{aer}} = d_{\text{phys}} \sqrt{\text{\(\frac{\rho}{\text{d}}\)}} \). For polymeric particles (d ~ 1–5 μm), the particle diameter should be ~1–5 μm. However, at this size range, alveolar macrophages rapidly clear particles, thereby reducing the therapeutic effect. To avoid clearance, the particle diameter should exceed 5 μm—beyond the uptake capability of alveolar macrophages. This can be accomplished by using larger particles that are porous to reduce their density. Furthermore, large particles have lower aggregation compared with smaller particles and allow for improved aerosolization for dry powder formulations. Such porous particles have been used for the delivery of various therapeutics, but their use for delivery of phages has not been reported.

Results

Phage-MPs effectively kill bacteria. We prepared hollow poly(lactic-co-glycolic acid) (PLGA) MPs via water–oil–water
double emulsion (Fig. 1a,b). Porous particles were generated by including ammonium bicarbonate (ABC) as an effervescent in the inner aqueous phase53. By tuning the processing parameters, we generated highly porous MPs of the appropriate size ($d = 8.0 \pm 4.5 \mu m$) and density ($\sim 0.3 g cm^{-3}$) to yield a $d_{por}$ of 2–5 $\mu m$ (Fig. 1c,d and Supplementary Fig. 1).

Several lytic phages against P. aeruginosa (Supplementary Table 1) were amplified, purified by liquid chromatography and loaded onto MPs by incubation in phage-containing solution. In this system, the phages are not encapsulated within the MPs, but are deposited on the surface of the MPs after the MPs are synthesized. This approach minimizes any loss in phage activity from the solvents used for MP fabrication, and no loss of phage activity was observed upon loading. MPs were loaded with a defined mixture of three to five different phages to increase the infectivity and bactericidal activity and reduce the probability of development of resistance. Porous MPs provide a large surface area for phage adsorption, and phage loading was $2.6 \pm 0.2$ plaque-forming units (p.f.u.) per particle or $\sim 2.6 \times 10^6$ p.f.u. mg$^{-1}$ MPs. Endotoxin levels for the phage-MP formulation were 0.078 $\pm 0.003$ EU (endotoxin units) mg$^{-1}$; that is, two orders of magnitude below the 20 EU per device limit stipulated by the Food and Drug Administration. As expected, these larger porous phage-MPs (8.0 $\mu m$ diameter) exhibited reduced internalization by macrophages compared with nonporous, smaller (1 $\mu m$ diameter) phage particles (Supplementary Fig. 2). To test the bactericidal activity of phage-loaded MPs, phage-MPs or empty MPs were plated onto lawns of P. aeruginosa expressing green fluorescent protein (PAO1-GFP) and incubated for 16 h at 37 °C. Efficient lysis of bacteria was observed around phage-MPs, as shown by zones devoid of fluorescence, whereas control MPs had no effect on PAO1-GFP levels (Fig. 1c,f). Notably, the killing zone for phage-MPs extended beyond the particle area, indicating that phages propagated radially outwards from the MPs, and showing that phages delivered from MPs can infect, replicate, propagate and kill target bacteria beyond those in direct contact with the MPs. Next, we tested whether phage-MPs can infect and kill bacteria in syn}

Inhaled phage-MPs distribute throughout the murine lung. For delivery to the mouse lung, lyophilized phage-loaded MPs suspended in lactose. Dry powder formulations of phage-MPs were tested for 16 d and only showed a slight loss of titre when stored at room temperature (Supplementary Fig. 4). Such dry powder formulations can be easily administered to patients using inhalers, have a long shelf life and increase patient compliance. Next, we performed studies to determine the release kinetics of phages from dry powder MP formulations. An initial burst release of approximately 10–15% of loaded phages was observed followed by very slow release (Supplementary Fig. 5), consistent with stable deposition of phages on the particle surface and slow degradation of the PLGA polymer used56. Attempts to characterize long-term release kinetics were not successful as this requires incubation in aqueous buffer and there is loss of phage activity over time in an aqueous environment at 37°C.
No differences were observed in tissue histology between lungs receiving MP-lactose powder and control lactose powder (no MPs).

Next, we evaluated the distribution of fluorescent MP-lactose dry powder after insufflation via endotracheal intubation. Organs were explanted immediately after delivery and imaged for total fluorescence. All fluorescence was localized to the lungs (Fig. 3a,b), and fluorescent MPs were found throughout the lung tissue (Fig. 3c). These results show targeting of MPs to the lung as well as distribution throughout the lung via dry powder inhalation. To quantify phage delivery, we delivered phage-MPs or phages alone (no MPs) via insufflation. High phage titres (104–105 p.f.u. mg–1 of tissue) were recovered from the lungs treated with phage-MPs, whereas no phages were recovered from the lungs receiving free phages (Supplementary Fig. 8a). These results show that using MPs significantly enhances the delivery of active phages to the lungs. We also examined the deep lung distribution of phage-loaded porous and non-porous MPs. Phages were delivered as previously described and the deep lungs were harvested. Significantly higher deposition of phages in the deep lung was observed for delivery with porous MPs compared with non-porous MPs (Supplementary Fig. 8b).

An attractive attribute of phages compared with other antimicrobials is the ability to persist and replicate at infection sites until the pathogen is cleared. We established an acute model of lung infection using different PAO1-GFP doses, and evaluated the persistence of bacteria at 24 h post-inoculation in healthy mice (Supplementary Fig. 9). A dose of 5×10^6 colony-forming units (c.f.u.) of PAO1-GFP was selected for the subsequent experiments. Phage-MPs were delivered to both control and PAO1-GFP-infected lungs, and 3 orders of magnitude higher phage counts were detected in PAO1-GFP-infected lungs compared with control (uninfected) lungs at 0 h (Supplementary Fig. 8) and 18 h post-delivery (Supplementary Fig. 10). This result shows that phages delivered to the lungs via MPs can infect and amplify in vivo only in the presence of target bacteria.

Phage-MP treatment clears infection from mice lungs. We tested the ability of phage-MPs to reduce acute bacterial lung infections in wild-type mice. Phage-MPs and controls were delivered via insufflation to PAO1-GFP-infected mice, and the lungs were assayed for bacterial and phage counts after 24 h (Fig. 4a,b). Delivery of phage-MPs reduced bacterial counts by an order of magnitude (P = 0.0203) compared with the bacteria + phages group, whereas...
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to-bacteria ratio as the bacteria dose delivered to CFKO mice was present. This modest efficacy could be the result of a higher phage-

the lung of CFKO mice, but significant levels of bacteria were still

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pneumonia-associated death compared with only 13% survival for

of infected mice with phage-MPs rescued 100% of subjects from

pneumonia-associated death.

We also examined the effects of phage-MPs on

P. aeruginosa

infection in the gut-corrected cystic fibrosis transmembrane-

conductance-regulator (CFTR) knockout (CFKO) mouse57. This

model mimics several aspects of the cystic fibrosis human condition,

including increased pathogenesis from

P. aeruginosa

lung infections60. A dosing study with PAO1-GFP identified a suitable
dose to obtain an acute infection (Supplementary Fig. 11). A dose
of 2 × 10^6 c.f.u. PAO1-GFP was used. This dose was lower than that

needed for wild-type mice in the earlier experiment, suggesting that

CFKO mice have reduced capacity to clear bacteria from the lungs.

PAO1-GFP infection and delivery of phage-MPs were performed as
described earlier. The delivery of phage-MPs dramatically reduced
bacterial counts in the lungs by three orders of magnitude, near the

assay detection limit (Fig. 5a,b). In contrast with wild-type mice,
treatment with phages alone also reduced the bacterial burden in
the lung of CFKO mice, but significant levels of bacteria were still
present. This modest efficacy could be the result of a higher phage-
to-bacteria ratio as the bacteria dose delivered to CFKO mice was
lower than that for wild-type mice. Nevertheless, when comparing
free phages with phage-MPs, the use of phage-MPs resulted in
~1.5 orders of magnitude lower bacteria counts compared with free phages. Phages were detected in the lung tissue for both

phage-MPs and free phages. We assessed bacteria levels in the liver
of these mice to determine whether the infection spread to other

organs. Remarkably, no mice treated with phage-MPs showed any
detectable bacteria in the liver (0 out of 7 mice), whereas >70% of
untreated mice (5 out of 6 mice) or mice treated with free phages
(5 out of 7 mice) had measurable levels of bacteria in the liver
(Fig. 5c). Histological analyses revealed massive infiltration of host
immune cells in untreated infected lungs (Fig. 5d). Infected mice
treated with free phages displayed moderate levels of cell infiltra-
tion (Fig. 5e). Importantly, infected mice treated with phage-MPs
had low levels of immune cell infiltration (Fig. 5e), indicating that
this treatment reduces the bacterial load as well as infection-associ-
ated inflammation. Immunostaining analysis on lung sections con-
firmed these observations (Supplementary Fig. 12).

Phage-MPs kill clinical bacterial strains. To evaluate the clinical
potential of the phage-MP formulation, we screened phage-MPs
against clinical strains of bacteria isolated from hospital-associated
lung infections and cystic fibrosis patients. The phage-MP formula-
lation was effective against all acute-infection clinical strains and five
out of six clinical strains from cystic fibrosis patients tested in vitro,
including antibiotic-resistant strains (CFBR 337 and CFBR 505 are
tobramycin-resistant) (Supplementary Table 2). We then evaluated
the in vivo efficacy of phage-MPs against PA103 (a historical strain
derived from the sputum of a patient60) in mice. A dosing study with
PA103 identified a dose (1.6 × 10^5 c.f.u.) suitable to obtain an acute
infection in wild-type mice (Supplementary Fig. 13). To achieve high
phage titres against PA103, our phage library was screened against
PA103, and three different phages were chosen and loaded onto the

MPs (Supplementary Table 3). PA103 infection and delivery of phage-

MPs were performed as described earlier. The delivery of phage-MPs
effectively reduced bacterial counts in the lungs, showing efficacy
against a patient-derived bacterial strain (Fig. 6a,b). To test whether
direct deposition of phages onto MPs (instead of simple co-delivery
of phages and MPs) is required for effective in vivo bacterial clear-
ance, a physical mixture of phages and unloaded MPs with inhalation
grade lactose was evaluated. In contrast with effective bacteria reduc-
tion with phage-MPs, a physical mixture of phages and unloaded

MPs did not reduce the bacteria load in the lung (Fig. 6a), showing
that only phages that are directly deposited on the MPs kill bacteria
in vivo. We also tested 10 bacterial colonies recovered from phage-

MP-treated mice for the development of phage resistance against the
stock phage mixture during the 18 h of incubation in the mouse lung.
All of the recovered colonies were still sensitive to phage-MPs.

Phage-MPs are effective against infection from multiple strains.
Another challenge for antimicrobial therapy against

P. aeruginosa

free phage delivery (same dose as the phage-MPs) had no effect on
bacterial counts (P > 0.12) compared with animals treated with bac-
teria alone. Counts of phages active against

P. aeruginosa

were elev-
ated for both free phages and phage-MPs. Importantly, treatment
of infected mice with phage-MPs rescued 100% of subjects from

pneumonia-associated death compared with only 13% survival for
untreated mice over 6 d (Fig. 4c). These results show that phage-
loaded MPS effectively reduce

P. aeruginosa

lung infections in wild-
type mice and rescue mice from pneumonia-associated death.

We also confirmed these observations (Supplementary Fig. 12).

Fig. 4 | Phage-loaded porous MPs reduce bacteria in the lungs of mice. a, b. The antibacterial efficacy of free phages and phage-MPs was examined in a

mouse model of acute lung infection with

P. aeruginosa

PAO1-GFP. After 24 h of treatment, the bacterial load (a; n = 3 mice for MPs; n = 11 mice for bacteria; n = 6 mice for bacteria + MPs; n = 9 mice for bacteria + phages; n = 11 mice for bacteria + phage-MPs; mean ± s.d.) and phage load (b; n = 3 mice for MPs; n = 6 mice for bacteria; n = 4 mice for bacteria + phages; n = 6 mice for bacteria + phage-MPs; mean ± s.d.) in the lung homogenate were quantified. Data were pooled from two independent experiments. For a, a one-way Kruskal–Wallis non-parametric test was used to detect statistical differences followed by Dunn’s multiple comparison test with adjustment for multiple comparisons. *P = 0.0203. c. Percentage survival of PAO1-GFP-inoculated mice that were untreated or treated with phage-MPs (n = 8 mice per group). The Mantel–Cox test was used to detect differences between survival. Data were collected on male C57BL/6 mice.
infection is the presence of distinct populations and their diversification in the patient lung. To test whether phage-MP treatment can be effective against infection consisting of multiple strains of bacteria, we infected mice with a mixture of PAO1-GFP (2.5 × 10⁴ c.f.u.) and PA103 (8 × 10⁴ c.f.u.) strains. Phages listed in Supplementary Tables 1 and 3 were mixed and loaded on MPs. Treatment with phage-MPs effectively reduced total bacterial counts in the lungs, showing efficacy against infections comprising simultaneous infection by two strains of P. aeruginosa (Fig. 6c). We also tested ten bacterial colonies of each strain recovered from phage-MP-treated mice for the development of phage resistance against the stock phage mixture, and all recovered bacteria were still susceptible to phage-MP treatment.

Finally, to evaluate the efficacy of phage therapy after repeated administration, we delivered empty MPs and phage-MPs in naïve mice. After 21 d, mice in both groups were infected with PA103, and phage-MP treatment was performed. Pre-exposure with phage-MPs did not affect the efficacy of treatment compared with the no pre-exposure control group (Fig. 7). Furthermore, no antibody titres in the blood (0/5 mice) were detected against phages at any time points tested (days 0, 21 and 22). This result indicates that pre-exposure to phage-MPs via dry powder delivery did not reduce its functional performance.

Discussion

We demonstrate that phages can be effectively delivered to lungs using polymeric MPs, and that MP-delivered phages retain their activity to kill host bacteria. Phage-loaded MPs significantly reduced P. aeruginosa infections and associated inflammation in healthy mice and mice with cystic fibrosis, and rescued mice from pneumonia-associated death. Importantly, phage-MPs were effective against clinically derived strains, including antibiotic-resistant strains, as well as lung infections caused by two strains of bacteria. The acute-infection murine model used in our study lacks biofilm formation, mucus plugging, and genetic, phenotypic and spatial diversification of bacterial strains. Nonetheless, we show that phage-MPs were effective against clinically derived strains from acute infections and cystic fibrosis patients, and were able to kill bacteria growing in biofilms. Interestingly, when we delivered free phages to the healthy lung as a dry powder formulation and immediately assayed the lungs, we did not recover any active phages from the lung homogenate. This result is in contrast with previous studies, where free phage administration was shown to be effective against bacterial infections. This discrepancy could be due to differences in the mode of administration. All previous reports have used intranasal administration of phages in liquid, whereas we used a dry powder formulation of free phages. Another reason for this discrepancy could be that the titres delivered for free phages in our experiment (~10⁶ p.f.u. mg–1 MPs) were significantly lower compared with previous reports (10⁹–10¹⁰ p.f.u.). For the acute lung infection model in healthy mice and mice with cystic fibrosis examined in this study, phage-only treatment had modest efficacy in reducing the bacterial burden, even though active phages were recovered. In contrast, phage-MP treatment significantly reduced the bacterial burden, rescued mice from pneumonia-associated death and prevented bacterial colonization of the liver. We attribute this enhanced bactericidal...
The antibacterial efficacy of phage-MPs was examined in a mouse model of lung infection with a clinical strain of \( P. \) aeruginosa (PA103). After 18 h of treatment, the bacterial load (\( a; n = 13 \) mice for bacteria; \( n = 6 \) mice for bacteria + phages + MPs; \( n = 15 \) mice for bacteria + phages + MPs; mean ± s.d.) and phage load (\( b; n = 9 \) mice per group; mean ± s.d.) in the lung homogenate were quantified. Data were collected from two independent experiments. Data were collected on both male and female wild-type CFTR littermates. The antibacterial efficacy of phage-MPs was examined in a mouse model of lung infection with a mixture of \( P. \) aeruginosa strains (PA01-GFP and PA103). After 18 h of treatment, the bacterial load (\( n = 4 \) mice per group; mean ± s.d.) in the lung homogenate was quantified. Data were collected on male wild-type C57BL/6 mice. For a, a one-way ANOVA was used to detect statistical differences, followed by Holm–Sidak’s multiple comparison test with adjustment for multiple comparisons. ** \( P < 0.0001 \) versus the bacteria control and \( P = 0.0001 \) versus the bacteria + phages + MP group. For b and c, a two-tailed Welch’s \( t \)-test was used to detect statistical differences. Measurements were taken from distinct samples.

**Fig. 6 | Phage-MPs significantly reduce infection by a clinically derived strain of bacteria in wild-type mice.** a, b. The antibacterial efficacy of phage-MPs was examined in a mouse model of lung infection with the clinical strain of \( P. \) aeruginosa (PA103). After 18 h of treatment, the bacterial load (\( a; n = 13 \) mice for bacteria; \( n = 6 \) mice for bacteria + phages + MPs; \( n = 15 \) mice for bacteria + phages + MPs; mean ± s.d.) and phage load (\( b; n = 9 \) mice per group; mean ± s.d.) in the lung homogenate were quantified. Data were collected from two independent experiments. Data were collected on both male and female wild-type CFTR littermates. c. The antibacterial efficacy of phage-MPs was examined in a mouse model of lung infection with a mixture of \( P. \) aeruginosa strains (PA01-GFP and PA103). After 18 h of treatment, the bacterial load (\( n = 4 \) mice per group; mean ± s.d.) in the lung homogenate was quantified. Data were collected on male wild-type C57BL/6 mice. For a, a one-way ANOVA was used to detect statistical differences, followed by Holm–Sidak’s multiple comparison test with adjustment for multiple comparisons. ** \( P < 0.0001 \) versus the bacteria control and \( P = 0.0001 \) versus the bacteria + phages + MP group. For b and c, a two-tailed Welch’s \( t \)-test was used to detect statistical differences. Measurements were taken from distinct samples.

In addition, pre-exposure of phage-MPs did not result in a reduction of its functional efficacy to subsequent bacterial challenge. These differences in host responses to phages and phage activity could be due to differences in the route of administration (intraperitoneal versus lung insufflation), phage dose delivered and/or use of MPs as phage delivery carriers. Further studies are needed to establish whether adverse immune responses, antibody production and/or a reduction in phage efficacy arise when phage-MPs are administered several times. Nevertheless, dry formulations of phage-MPs represent a promising alternative to antibiotic regimens, which are severely limited by ineffective dosing and the emergence of antibiotic resistance. The ability to deliver active phages using engineered biomaterials as dry powder formulations provides significant clinical benefits for lung infections, including longer drug stability, ease of administration with fewer side effects and increased patient compliance.

**Methods**

**PLGA MPs.** MPs were prepared by double emulsion of water in oil in water. Some 200 mg PLGA (RG503H; Sigma–Aldrich) was dissolved in 7 ml dichloromethane (DCM). ABC (Sigma–Aldrich) was used as an effervescent to create porous MPs in 1 ml of internal aqueous phase at 4% w/v. The concentration of ABC can be varied to tune the porosity and density of MPs. The solution was homogenized at 3,000 r.p.m. for 2 min and then mixed with 50 ml of 1% poly(vinyl alcohol) (PVA) (Sigma–Aldrich) and homogenized for 2 min. The final emulsion was added to 100 ml 1% PVA and stirred to evaporate all the DCM for 5 h. MPs were washed with deionized water 4 times to remove the PVA and free polymer and lyophilized for 16 h. Fluorescent MPs were prepared by adding 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotritylcyanine iodide (DiR) dye (Thermo Scientific) (5 mg dye per 200 mg PLGA) in DCM.

**Particle size and density.** The size distribution of the MPs was characterized using a Multisizer 3 Coulter counter. To determine the density of the MPs, 1.0 mg lyophilized MPs were suspended and counted in the Coulter counter. The total volume of MPs in 1.0 mg was calculated using the average diameter obtained from the Coulter counter. Density was calculated by theoretical calculation based on mass = density × volume.

**Scanning electron microscopy of MPs.** For scanning electron microscopy (LEO 1550), an MP suspension (5 μl of 1 mg ml⁻¹ solution) was dispensed on a scanning electron microscopy stub, air dried for 2 h at room temperature and sputter coated with 5 nm of gold to make the sample conductive. Imaging was performed with a 5 KeV electron beam.
Fluorescent plaque imaging. PLGA MPs were loaded with phage and washed, and various dilutions were plated with PA01-GFP on trypticase soy agar plates. Plates were incubated for 18 h at 37 °C and imaged on a Nikon C2 laser scanning confocal microscope with a 10x objective. PA01-GFP was excited with a 405 nm laser and emitted light was collected through a 485/35 nm filter cube. Particles were excited with a 561 nm laser and emitted light was collected through a 595/50 nm filter cube.

Phage amplification. Phages (Supplementary Tables 1 and 3; described previously17) were amplified in liquid culture of 25% trypticase soy broth (TSB; Becton Dickinson). Phages were added to an exponentially growing culture of their respective bacterial host at 104–105 c.f.u. ml−1 and incubated at 37 °C until lysis occurred (the culture visibly cleared). The resulting lysate was clarified further by adding 2% v/v chloroform, decanting centrifugation and filtering the supernatant (0.2 μm pore membrane).

Phage purification. For phage purification, an anion-exchange chromatography column, CIM QA-8Fl Tube Column (BIA Separations), was used67. The column was attached to an AKTA fast protein liquid chromatography system (GE Healthcare) with a P900 pump system, and analyzed with UNICORN 5.01 software. Phage lysate was diluted 50% with Tris buffer (40 μM Tris-HCL, pH 7.5) and loaded onto the column. Phages were then washed with Tris buffer (20 mM Tris-HCL, pH 7.5). For elution, a gradient was used by linearly mixing an increasing amount of 1.5 M NaCl, 20 mM Tris-HCL solution with 20 mM Tris-HCL buffer, and collected using an ion collector. Phages were then dialysed with 0.1 M phosphate buffer supplemented with 2 mM MgSO4, passed through an endotoxin removal column and stored at 4 °C.

Active phage counts. Preparations were counted for active phages with plaque assays, using the traditional top agar overlay method68.

Phage loading on particles. MPs (4 mg) were suspended in a solution of phage mixture (10–104 phages ml−1) for 4 h with mild shaking. MPs were then washed three times with phosphate buffered saline (PBS). To determine phage loading levels on MPs, ΦP aeroligus14 was loaded on the particles. Some 500 μl of phage-MP and 500 μl of chloroform were mixed for 20 min at room temperature to dissolve the PLGA. Free phage standards were run in parallel to determine the effects of chloroform on phage stability. Samples were centrifuged at 500 g for 5 min to separate the organic and aqueous phases. Phage titres were performed on the aqueous phase. Particles were enumerated using a haemocytometer, and the number of particles per particle was calculated using the following formula (p.p.f.u. ml−1) = (p.pu ml−1 x adjustment)/(particles ml−1), where the adjustment was determined by dividing the p.f.u. ml−1 of a phage standard by the p.f.u. ml−1 of the same standard exposed to chloroform for 20 min, as described above.

Dry powder preparation. To prepare dry powder formulations, 4.0 mg of washed phage-MPs was suspended in 100 μl solution of lactose (40 mg ml−1), resulting in a 1:1 ratio of particle lactose, and lyophilized for 24 h. For the free phage dry powder formulation, phage titres were adjusted to the amounts of phages loaded on the MPs, suspended in 100 μl solution of lactose (40 mg ml−1), resulting in a 1:1 ratio of particle lactose, and lyophilized for 24 h. For stability testing, the dry powders were stored at room temperature. Phage activity was performed using a LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific) per the manufacturer’s instructions.

Phage release from particles. Lyophilized phage-MPs were suspended in PBS and total p.f.u. were enumerated using the top agar overlay method. For phage release, phage-MPs were centrifuged at 500 g for 5 min and the supernatant was assayed for p.f.u.

In vitro phage-MP phagocytosis. RAW264.7 macrophages were cultured in Dulbecco’s modified Eagle medium supplemented with 4.5 g l−1 glucose and 10% foetal bovine serum in 24-well plates at 37 °C and 5.0% CO2 in a humidified incubator. Fluorescently labeled particles were described as above. Smaller particles (0.94 ± 0.59 μm) were generated by modifying the homogenization speed to 10,000 r.p.m. The PA01 phage mixture was loaded on the MPs. Following loading, MPs of different sizes were added to tissue culture media at a concentration of 0.25 mg ml−1 and incubated with cells for 2 h. Cells were washed 5 times with PBS to remove excess particles, fixed, and stained with 4′,6-diamidino–2-phenylindole (DAPI) and Phallolidin-iFlour 488 (Abcam). Samples were imaged using a Nikon C2 laser scanning confocal microscope through a 10x objective. Three images of each well were acquired. Macrophage phagocytosis was evaluated in ImageJ by performing maximum intensity Z projections and thresholding the green (phallolidin) channel, followed by particle tracking analysis (at a minimum size of 20 pixels) to identify the cell border. Fluorescent blue (DAPI) and red (MP) images were then overlaid, and MPs falling inside cell borders containing a nucleus were counted as having been phagocyted by cells. The total number of cells within an image field was quantified using the ComDet (version 0.3.6) plugin. The percentage of phagocytic cells was computed by dividing the number of phagocytic cells in an image by the total number of cells in the image.

In vitro growth of bacteria in synthetic cystic fibrosis sputum. Synthetic cystic fibrosis sputum was prepared as described previously22. A 50 μl culture of PA103 was grown for 16 h in synthetic cystic fibrosis sputum and used to inoculate 3 ml of the sputum medium in the presence or absence of 0.1 mg of phage-MPs. Cultures were grown at 37 °C and regular optical density measurements were taken to monitor the bacterial growth.

In vitro activity of phage-MPs against bacterial biofilms. To form biofilms, 5 μl culture of PA103 was grown for 16 h in 25% TSB, used to inoculate 500 μl of 25% TSB in each well of a 48-well plate and allowed to grow for 24 h at 37 °C. Biofilms were carefully washed with PBS and incubated with 0.01 mg ml−1 of MPs or phage-MPs. Controls were then washed with PBS containing LIVE/DEAD BacLight (Thermo Scientific) (3μl dye mixture per ml of bacterial medium) and imaged on a Nikon C2 laser scanning confocal microscope with a 10x objective. SYTO 9 (live cell stain) was excited with a 488 nm laser and emitted light was collected through a 525/50 nm filter cube. Propidium iodide (to stain cells with a compromised membrane that are considered dead or dying) was excited with a 561 nm laser, and emitted light was collected through a 595/50 nm filter cube. To prevent bleed-through of the fluorescence signal, each fluorophore was excited, and emitted light was collected in series. The image from each well was analysed for the mean pixel intensity in live and dead channels using ImageJ software (NIH). The ratio of the mean intensity of live and dead cells was plotted.

In vitro activity of phage-MPs against clinical strains of bacteria. To test the activity of phages loaded on the MPs, a 5 μl drop of phage-MPs at 4 mg ml−1 was spotted onto a lawn of respective bacteria (Supplementary Table 2) and allowed to grow for 16 h at 37 °C. The formulation was defined as effective if the spot was visually cleared of any bacterial growth after 16 h of incubation.

Bacterial inoculum preparation. Colonies were resuspended in sterile PBS and the optical density was measured at 600 nm. For each optical density value, serial dilutions were plated on ampicillin-fortified (100 μg ml−1) lysogenic broth agar plates for 24 h at 37 °C with atmospheric CO2. Colonies were counted and plotted against their respective initial optical density values. Bacterial challenges were performed with P. aeruginosa expressing green fluorescence protein (PA01-GFP) or the clinically derived bacteria strain PA103 (America Type Culture Collection 29260). Bacteria were grown on ampicillin-fortified (100 μg ml−1) lysogenic broth agar plates for 24 h at 37 °C with atmospheric CO2. Colonies were removed from the plates, then resuspended in sterile PBS containing LIVE/DEAD BacLight, and resuspended in sterile PBS. Optical density measurements were taken of the solution and adjusted to achieve the required concentration based on a previously determined optical density growth curve for the respective bacteria.

Care and use of mice. All experiments were conducted in accordance with Institutional Animal Care and Use Committee-approved protocols at the Georgia Institute of Technology and Emory University. All animals were housed in specific pathogen-free housing with 12 h light and 12 h dark cycles and received food and water ad libitum. The CFKO mouse Cfr-trim11 mutation (Tg(FABPCTFTR)-1jawl1/J) (C57BL/6 and FVB/N background) was used in some experiments. These Cfr-trim11 null mice lack the body knockout (CFTR) gene and have human CFTR expression regulated by expression of the fatty-acid-binding protein promoter. As such, expression of human CFTR is limited to the small and large intestine with subsequent improved viability and no dietary restrictions or special dietary supplementation compared with the whole-body knockout. C57BL/6 or wild-type littermates of CFKO mice were used as wild-type mice. Both female and male mice were used (randomized) for experiments involving CFTR null mice. Only male mice were used for experiments involving C57BL/6 mice. All animals were used within the age range of 8–14 weeks.

In vivo bacterial challenge. Mice were placed under general anaesthesia with an intraperitoneal injection of ketamine and xylazine (100 mg kg−1 and 10 mg kg−1, respectively). Dry powder phage-MPs (1.0 mg) were delivered to the lungs via a Dry Powder Insufflator (model DP-4M; Penn-Century). Bacteria were inoculated using a MicroSprayer Syringe Assembly (model MSA-250-M; Penn-Century) by injecting 50 μl of the appropriate optical density of bacterial solution through an endotracheal tube. Successful lung delivery was confirmed by a change in the
respiratory status of the animal. Mice were placed under a warming lamp and allowed to recover from general anaesthesia. Some 18–24 h after the intratracheal installation of bacteria, mice were euthanized by intraperitoneal overdose of pentobarbital (150 mg/kg); Euthatal) for organ collection. For assaying phage counts in tissues, organs were explanted, weighed and homogenized in sterile PBS. Homogenates were serially diluted and cultured on lysogeny broth agar plates fortified with ampicillin (100 μg/mL) to select for PAO1-GFP or PA103. Homogenate serial dilutions were also titred for phages on the inoculating bacteria—either PAO1-GFP or PA103. Culture plates were incubated at 37°C under atmospheric CO2, and bacterial colonies or plaques were counted after 16 h incubation. For mixed infections, colony morphology was used to distinguish the different strains of bacteria. The detection limit of colonies was defined as ten colonies in undiluted lung homogenate plates to prevent counting any lung tissue fragments as false positive.

**Immune response against phages.** Mice were given no treatment or dry powder formulations of phage-MPs or empty MPs. After 21 d, all mice were challenged with bacteria and either treated with phage-MPs or left untreated. Mice were euthanized after 18 h and assayed for phages and bacteria as described above. For all time points (days 0, 21 and 22), serum was collected via a checked bleed. To assay for the presence of antibodies, phages were incubated in a 96-well plate for 1 h and washed multiple times with 0.05% w/v Triton X-100 in PBS. Samples were then incubated in 1% w/v casein blocker (Life Technologies) and treated with serum collected at each time point (diluted 1:10 in PBS) for 1 h. Wells were then incubated with alkaline-phosphatase-conjugated anti-mouse antibody (1 μg/mL) (715-05-151; Jackson ImmunoResearch). Following several washes, samples were incubated with 5-μm umbelliferone phosphate substrate (60 μg/mL) and fluorescence read at an excitation of 360 nm and an emission of 465 nm on a HTS 7000 Plus plate reader (Perkin Elmer).

**Phage resistance.** Individual colonies recovered from phage-MP-treated mice were suspended in PBS and plated on trypticase soy agar plates. A 10 μl drop of the original phage mixture was spotted on the soft agar seeded with the appropriate host strain and allowed to grow for 16 h at 37°C. Bacteria were defined as susceptible to phage mixture if the spot was cleared of any bacterial growth after original phage mixture was spotted on the soft agar seeded with the appropriate strain of bacteria. The detection limit of colonies was defined as ten colonies in undiluted lung homogenate plates to prevent counting any lung tissue fragments as false positive. The fluorescence intensity was analysed using the manufacturer’s software.

**Lung histology.** Lungs were harvested at necropsy, fixed in 10% neutral buffered formalin for 16 h, washed with PBS, dehydrated in 70% ethanol and embedded in paraffin. Sections (5 μm) were cut using a Microm 355 H microtome and stained with haematoxylin and eosin. For immunohistochemistry, sections were treated with 0.05% w/v trypsin for 10 min at 37°C, blocked with goat serum and stained with rabbit anti-P. aeruginosa antibody (ab68538; Abcam). Slides were washed, blocked with goat serum and then stained with AlexaFluor 555-conjugated goat anti-rabbit IgG (A21428; Thermo Scientific) and DAPI. For the lung cryosection images, fluorescent MPs were delivered and mice were immediately euthanized. Tissue was cut using a Microm 355 H microtome and stained with rabbit anti-rabbit IgG (A21428; Thermo Scientific) and DAPI. For the lung cryosection, images, fluorescent MPs were delivered and mice were immediately euthanized. Images were acquired at an excitation of 360 nm and an emission of 465 nm on a HTS 7000 Plus plate reader (Perkin Elmer).

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ Confirmed

☐ Exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

☐ Clearly defined error bars

☐ State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

No software was used.

Data analysis

ImageJ was used to analyze images, Graphpad Prism 7.03.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size                          | Sample sizes were selected on the basis of statistical-power calculations and of previous experience with these metrics. |
|--------------------------------------|------------------------------------------------------------------------------------------------------------------------|
| Data exclusions                      | No data were excluded.                                                                                                   |
| Replication                          | Findings were reliably reproduced.                                                                                        |
| Randomization                        | Samples/animals were randomly allocated into experimental groups.                                                         |
| Blinding                             | Blinding was not done. Phage can easily contaminate the samples and hence it is essential for the user to know what groups contain phage. |

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a  Involved in the study

☐ ☑️ Unique biological materials

☐ ☑️ Antibodies

☐ ☑️ Eukaryotic cell lines

☑️ Palaeontology

☐ ☑️ Animals and other organisms

☐ ☑️ Human research participants

Methods

n/a  Involved in the study

☑️ ChIP-seq

☑️ Flow cytometry

☑️ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Phages were isolated and catalogued by the Biofilm Laboratory at CDC or obtained from banks available to scientific community. All are available to the scientific community.

Antibodies

Antibodies used

Primary Antibody: Anti-Pseudomonas antibody from Abcam. Catalog number: ab68538. Clonality: Polyclonal

Abcam website states that it is tested for Immunofluorescence and Immunohistochemistry. It also provides 4 references on the website. No validation was done in our lab.

Secondary Antibody: Alkaline phosphatase (ALP)-conjugated anti-mouse antibody. Jackson Immunoresearch. Catalog Number: 715-055-151. Clonality: Polyclonal

Validation

Primary Antibody: Anti-Pseudomonas antibody from Abcam. Catalog number: ab68538. Clonality: Polyclonal

Abcam website states that it is tested for Immunofluorescence and Immunohistochemistry. It also provides 4 references on the website. No validation was done in our lab.

Secondary Antibody: Alkaline phosphatase (ALP)-conjugated anti-mouse antibody. Jackson Immunoresearch. Catalog Number: 715-055-151. Clonality: Polyclonal
## Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | RAW264.7 (ATCC) |
|---------------------|------------------|
| Authentication      | Cell lines were visually examined for described morphology and growth conditions. No other authentication was used. |
| Mycoplasma contamination | Our lab regularly (once a year) tests all cell lines for Mycoplasma contamination. No mycoplasma contamination was found. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified lines were used. |

## Animals and other organisms

**Policy information about studies involving animals; ARRIVE guidelines** recommended for reporting animal research

| Laboratory animals | Information on the animals used is clearly described in the relevant figure captions. |
|--------------------|----------------------------------------------------------------------------------|
| Wild animals       | The study did not involve wild animals.                                          |
| Field-collected samples | The study did not involve samples collected from the field.                  |