Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*

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A 15-year-old patient with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection was treated with a three-phage cocktail following bilateral lung transplantation. Effective lytic phage derivatives that efficiently kill the infectious *M. abscessus* strain were developed by genome engineering and forward genetics. Intravenous phage treatment was well tolerated and associated with objective clinical improvement, including sternal wound closure, improved liver function, and substantial resolution of infected skin nodules.

Mycobacterial infections impose a substantial global health burden, and drug resistance in *Mycobacterium tuberculosis* and non-tuberculous mycobacteria (NTM) is widespread1,2. Patients with cystic fibrosis (CF) frequently have NTM infections (including *Mycobacterium abscessus*3,4), which are antibiotic resistant and difficult to manage clinically3,5. CF is the third most common indication for lung transplantation, but persistent infection — particularly when NTM infections are present prior to surgery — can result in substantial post-transplant morbidity and mortality6,7. Therapeutic bacteriophages are a plausible alternative treatment to antibiotics, but have not been used for mycobacterial infections in humans2,8; however, personalized intravenous phage treatments for other bacterial infections have been described (Supplementary Information)9,10.

A 15-year-old patient with CF (homozygous for AF508) with comorbidities of pancreatic insufficiency, insulin-dependent diabetes, CF-related liver disease, Nissen fundoplication and gastrostomy, CF-related osteoporosis, and failure to thrive was referred for lung transplant. The patient was chronically infected with *Pseudomonas aeruginosa* and *M. abscessus* subspecies *massiliense* and had been on anti-NTM treatment for 8 years prior to lung transplantation (Extended Data Fig. 1). Despite compassionate lumacaftor/ivacaftor treatment for 6 months prior to placement on the transplant waiting list, the forced expiratory volume in 1 s (FEV1) declined to 0.63 l (29% of predicted normal function for height; Fig. 1a), with hypercapnia and main pulmonary artery enlargement on CT scan. After an uncomplicated bilateral lung transplant, immunosuppressive drugs and multiple intravenous (i.v.) antibiotics were administered (Extended Data Fig. 1). Severe side effects including nausea, anorexia, diarrhea, and electrolyte derangement necessitated total parenteral nutrition and discontinuation of i.v. antibiotics. Within 1 week of stopping intravenous therapy, redness was noted at the surgical incision. The chest X-ray showed consolidation, and *M. abscessus* grew from sputum (Supplementary Table 1). Clofazimine and bedaquiline were added and previous i.v. antibiotics were recommended (Extended Data Fig. 1 and Supplementary Information). A positron-emission tomography (PET)-CT scan to evaluate ongoing abdominal pain, hepatomegaly, deranged liver function tests, and Epstein–Barr virus (EBV) viremia (11 million copies per ml) revealed fluorodeoxyglucose (FDG) activity in a 3.5 cm × 4 cm porta hepatitis lesion and a destructive process at the sternum with abnormal adjacent soft tissue (Fig. 1b,c). Mycophenolate motefol was stopped and four doses of i.v. rituximab were given for presumed post-transplant lymphoproliferative disease, but the node enlarged to 6.8 cm × 4 cm (Fig. 1c). Two skin lesions developed on the forearm, and sternal wound and skin biopsies revealed granulomatous inflammation. The patient was discharged 7 months after transplant with a diagnosis of disseminated mycobacterial infection. Antimicrobial therapy was continued with a palliative care plan in place. Over 8 weeks, 20 additional skin nodules appeared on arms, legs, and buttocks, and the surgical wound showed areas of breakdown (Fig. 1d).

*M. abscessus* subsp. *massiliense* with a rough colony morphology (designated strain GD01; Supplementary Information) isolated 1 month post-transplantation was used to identify potentially therapeutic phages. We exploited a collection of >10,000 phages isolated using *Mycobacterium smegmatis* by students in the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program, 1,800 of which are genomically defined14. Some of these infect *M. tuberculosis*15, but other NTM specificities are unknown16. Screening of a representative subset identified one (Muddy) that kills GD01 efficiently (Fig. 2a and Supplementary Table 2). A second phage, ZoeJ, infects GD01 with reduced efficiency of plating (EOP), although the plaques are extremely turbid and difficult to visualize (Fig. 2a). A lytic derivative of ZoeJ was engineered using Bacteriophage Recombineering of Electroporated DNA (BRED)17 to precisely remove its repressor gene (45);18 this efficiently infects and kills GD01 (Fig. 2a). A third phage (BPs) and its lytic derivative (BPsD3HTTH19) infect GD01 poorly, but we isolated host range mutants (HRM1 and HRM10) that infect GD01 efficiently and retain *M. smegmatis* infection (Fig. 2a and Supplementary Information). HRM1 and HRM10 have single base changes in the portal gene 3 (C2083T and A2695G) conferring R66W and N270D amino acid substitutions, respectively.

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Phages Muddy, ZoeJ, and BPs are morphologically siphoviral (Fig. 2b) and are genomically distinct — grouped in clusters AB, K, and G, respectively (Fig. 2c) — with limited segments of shared sequence similarity (Extended Data Fig. 2). A subsequent search for additional phages included screening of 100 environmental samples, and, as a result, phage Isca was isolated. Isca does not multiply on M. smegmatis, but several close relatives (BabyRay, Heldan, Fred313, Puppy, and TNguyen7) do (Supplementary Table 2). GD01 was also used to enrich large pools of unsequenced phages and we thereby isolated Gabriela and Itos (Supplementary Table 2).

Fig. 1 | Patient status before and after phage treatment. a, Lung function as percent predicted FEV1 (blue) and forced vital capacity (FVC; red). b,c. Whole-body (b) and cross-section (c) PET-CT scans 12 weeks before and 6 weeks post phage treatment. Arrows show (1) the sternal area and surrounding soft tissue, (2) abdominal lymph nodes at the porta hepatis, and (3) skin nodules. Arrow 4 indicates normal kidney excretion. d, Upper and lower panels show the patient’s left arm and sternal wound, respectively, immediately prior to and 6 months after phage treatment. e,f, Phage titers by plaque assay (e) or dPCR (f) following phage administration (vertical arrow). Serum (black bars), sputum (purple bars), feces (green bars), and wound swab (red bars) were tested on the dates indicated (asterisks).
Thus, phages for GD01 are uncommon, but can be identified with extensive searches. A search for phages of a second strain (M. abscessus GD02) from a clinically similar patient identified few useful phages (Supplementary Table 3); this patient subsequently died. Muddy, BPs, and ZoeJ do not kill other M. abscessus clinical isolates efficiently, and the three-phage cocktail is not a generalizable treatment (Fig. 2d).

Each of the three phages — Muddy, BPsΔ33HTH-HRM10, and ZoeJΔ45 — infect and kill GD01 over a wide range of cell and phage concentrations, although ZoeJΔ45 and BPsΔ33HTH-HRM10 kill inefficiently at low phage concentrations and higher bacterial numbers (Fig. 2c); however, no bacterial survival in vitro was observed using the three-phage cocktail (Fig. 2c). After challenging a larger GD01 culture with the cocktail, some survivors were recovered and shown to be resistant to BPs33ΔHTH-HRM10 and ZoeJΔ45 but at least partially sensitive to Muddy (Extended Data Fig. 3).

Twenty-four hours following a single topical test dose in the sternal wound, i.v. therapy was initiated with the three-phage cocktail (10⁹ plaque forming units (PFU) per dose of each phage) every 12 h for at least 32 weeks (Supplementary Information). During the first 2 d of treatment, the patient felt sweaty and flushed but had no fever or changes on physical examination. Otherwise phage treatment was well tolerated throughout, without significant side effects. After 9 d, the patient was discharged and 12 h i.v. administration of the cocktail was continued. After 1 month of treatment, the sternal wound, which had received a topical test dose, had improved more than the other skin lesions and topical daily phage therapy was commenced for both. Over the next 6 months (to the time of writing) the patient...
continued to improve clinically with gradual healing of surgical wound and skin lesions (Fig. 1d) and improvement of lung function (Fig. 1a) and liver function. Weight increase was also observed, despite cessation of overnight supplemental feedings. A repeat CT-PET scan 6 weeks into phage treatment showed resolution of FDG activity of the previously enlarged node at the porta hepatitis, although sternal and skin lesion activity remained (Fig. 1b–d).

M. abscessus was not isolated from serum or sputum at any point after initiation of phage treatment, although M. abscessus was cultured from swabs of slowly resolving skin nodules at 1, 3, 4, and 5 months. Sera showed no evidence of phage neutralization, although weak antibody responses to phage proteins were seen (Extended Data Fig. 4). Among the weak cytokine responses were interferon-γ (IFNγ), interleukin-6 (IL-6) and IL-10 after 16 d of treatment, tumour necrosis factor-α (TNFα), IL-6 and IL-10 after 1 month of treatment, and IL-6 after 3 and 4 months of treatment (Supplementary Table 4).

Phages were detected in serum 1 d after starting treatment, and reached titers in excess of 10^10 PFU ml^−1 (Fig. 1e); digital polymerase chain reaction (dPCR) performed on EDTA blood samples had a similar temporal profile, although the maximum phage load detected was ~10^8 copies per ml (Fig. 1f); these observations are consistent with phage replication. Serum phage concentrations fell below detection limits 1 week after starting treatment, although two later samples had mycobacteriolytic activity (Fig. 1e,f). Sputum samples were predominantly salivary and did not contain detectable lytic activity, although a purulent sputum sample collected 9 d after initiation of treatment had a high phage titer (10^10 PFU ml^−1). Lower phage concentrations were detected in feces 4 and 6 d post treatment, and in wound swabs at 3 and 5 d post treatment (Fig. 1e,f).

M. abscessus isolates were recovered from skin nodule swabs at 20, 72, 107, and 121 d after treatment initiation but remained sensitive to each phage in the cocktail (Extended Data Fig. 5). It is plausible that phage resistance is associated with reduced virulence.

To our knowledge, this is the first therapeutic use of phages for a human mycobacterial infection, and the first use of engineered phages. Phage treatment was associated with clinical improvement, although we cannot exclude the possibility that patient gains would have occurred without phage treatment. However, we note that patients with similar clinical conditions typically have high morbidity and mortality, that improvement was not temporally associated with cessation or initiation of other drug administrations, and we show evidence to support in vivo phage replication. There were no adverse reactions to phage administration. We note that there is substantial variation in M. abscessus phage susceptibilities, and phage treatment of similar patients will require expansion of our understanding of phage infection of these strains.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41591-019-0437-z.

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Author contributions
R.M.D., C.A.G.-B., R.A.G., D.A.R., D.J.S., K.H., and K.C.G. contributed to data collection, analysis, interpretation, and writing. K.F. contributed data collection, analysis, interpretation, writing, and regulatory approvals. J.S. contributed to literature search, data collection, analysis, interpretation, and writing. R.T.S., G.E.H., and H.S. contributed to the study design, data interpretation, and writing.

Competing interests
R.T.S. serves as an uncompensated member of the AmpPhili Scientific Advisory Board. Other authors declare no competing interests.

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Methods

Bacterial strains. *M. smegmatis mc²155* is a laboratory stock strain and was grown as described previously14. *M. abscessus* ATCC19977 was obtained from the American Type Culture Collection. *M. abscessus* GD01, GD02, GD03, GD04, and GD05 are patient isolates from London, UK (GD01 and GD02), from Seattle, WA (GD03), Los Angeles, CA (GD04), and Winston-Salem, NC (GD05), USA.

*M. abscessus* strains were grown in Middlebrook 7H9 medium with OADC and 1 mM CaCl₂ for 4–5 d at 37°C, with shaking. These *M. abscessus* strains grow with a doubling time of approximately 6 h, with isolated colonies visible on solid medium in 5–7 d. For plaque assays, *M. abscessus* cultures were sonicated briefly in a cup-horn sonicator (Qsonica) at 30% amplitude with 15 s on and 10 s off until visibly dispersed.

Genome sequencing. DNA was isolated from a liquid culture of *M. abscessus* GD01 and sequenced using illumina (421-fold coverage, 250-bp paired-end reads) and Oxford Nanopore (12-fold coverage, average length 2.6 kb) technologies, assembled with SPAdes16, and evaluated and finished using Cnsed. The GD01, A and GD01_B sequences are deposited in GenBank with accession numbers CP035923 and CP035932, respectively. Phage DNAs were isolated and sequenced as described previously14. Sequence features of phages are shown in Fig. 2c; other phage information including genome sequences are available at https://phagesdb.org.

Phage susceptibility screening. Phage susceptibility profiles were screened using a standard plaque assay15, and efficiencies of plating (EOP) were determined by a standard plaque assay15, and efficiencies of plating (EOP) were determined by a standard plaque assay15, and efficiencies of plating (EOP) were determined by a standard plaque assay15.

Phage killing of *M. abscessus* GD01. Bacterial survival assays were conducted in 96-well trays (Falcon), and were composed of a grid containing 200 µl volumes of tenfold serial dilutions of bacterial culture (x axis) and tenfold serial dilutions of phage lyase (y axis). Undiluted bacterial cultures contained ~5 × 10⁹ CFU ml⁻¹, such that each well contained totals of 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹ CFU. Undiluted phage lysates had titers of 10⁴ PFU ml⁻¹, such that each well contained 2 × 10⁵, 2 × 10⁶, 2 × 10⁷, 2 × 10⁸, 2 × 10⁹, 2 × 10⁴, 2 × 10³, and 2 PFU. Cultures were incubated at 37°C for 24 h, and 3 µl of each sample spotted on Middlebrook 7H10 solid media, incubated for 7 d, and photographed.

Phage quantification in patient samples. Samples were collected for measuring phage counts as follows: for wounds, shortened dry cotton swabs were placed under sterile conditions in 20 ml universal bottles, closed, and weighed. After application, the swab was returned to the bottle, which was re-weighed. After addition of a measured volume of phage buffer and vortexing, the sample was passed through a 0.2 µm filter, serially diluted, and 0.05 ml volumes added to *M. smegmatis* agar overlays. For sputum, a small sample of sputum was transferred to a pre-weighed bijou bottle, which was then re-weighed. A measured volume of phage buffer (usually 0.5 ml) was added, vortexed, filtered, and titered as above. For serum, clotted blood samples were centrifuged at 3,000 rpm and the serum serially diluted and counted as above. Phage DNA was quantified by dPCR on a QX100 Droplet Digital PCR system (BioRad); quantitative PCR (qPCR) was performed using the 7500 Fast real-time PCR system (Thermofisher). The primer and probe sequences used for dPCR and qPCR assays are shown in Supplementary Table 5.

Patient antibody and cytokine responses. Patient serum was diluted 1:100 in PBS, incubated with 10⁵ PFU of each phage separately, and incubated at room temperature for 30 min. Tenfold serial dilutions were spotted onto top agar overlays of *M. smegmatis* mc²155 and incubated at 37°C for 1–2 d to determine titer. Cytokines were measured using a Th1/Th2 Cytokine Bead Array (BD Biosciences) according to the manufacturer's instructions.

Western blotting. Phage samples were prepared by adding 75 µl of 20 mM Dithiothreitol (DTT) to 20 µl of phage lyase (10⁴ PFU ml⁻¹) and mixed, following by addition of 2 µl of 0.5 M EDTA and incubation at 75°C for 5 min. Sample viscosity was reduced by adding 4 µl of 1 M MgSO₄ and 2 µl of 1 mg ml⁻¹ DNase I (Invitrogen) and incubated further for 30 min at 37°C. Loading buffer was added and samples were run on an SDS polyacrylamide gel and transferred to a PVDF membrane (BioRad) using BioRad wet transfer apparatus according to the manufacturer's instructions. The membrane was blocked with Tris buffered saline (TBS) and 5% milk powder for 1 h at room temperature and then rinsed with TBS and 0.1% Tween (TBST) for 5 min. The primary antibody (patient serum from 29 August 2018) was diluted 1:100 into TBST and incubated with the membrane for 1 h at room temperature. The membrane was washed with TBST three times for 5 min each. The secondary antibody, Goat anti-human HRP (Novex), was diluted 1:2,000 in TBST and added to the membrane and incubated at room temperature for 45 min. The membrane was then washed with TBST three times for 5 min each and then once with TBS for 5 min. The SuperSignal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific) was used to develop the blot according to the manufacturer's instructions.

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Extended Data Fig. 1 | Timeline of drug administration. Timeline showing administration of antibiotics, immunosuppressive drugs, and the phage cocktail. Levels of the immunosuppressive drug Tacrolimus are shown at the top, and the administration of drugs is as indicated.
Extended Data Fig. 2 | Genome maps of phages Muddy, BPs, and ZoeJ. Genes are shown as colored boxes above or below a genome track, reflecting rightwards and leftwards transcription, respectively. Pairwise nucleotide sequence similarity is indicated by spectrum-colored shading between genomes, with violet representing closest similarity.
Extended Data Fig. 3 | In vitro selection of phage resistance. a, Approximately $5 \times 10^8$ cells of *M. abscessus* GD01 in one ml were incubated with a cocktail of $10^9$ pfu each of three phages for one week in liquid culture. Aliquots (100 µl) were plated onto solid media and incubated at 37 °C. In the absence of phage, a confluent lawn grew (left), and in the presence of phage (right), approximately 150 small colonies were observed. b, Six individual colonies were picked, grown and retested for phage susceptibilities. Top agar overlays with each strain were plated on solid media and 10-fold serial dilutions of phages (as indicated) were spotted onto each plate.
Extended Data Fig. 4 | Detection of antisera recognizing phage proteins. Phage preparations of Muddy, ZoeΔ45, and BPs33ΔHTH-HRM10 (as shown) each containing approximately $2 \times 10^{10}$ phage particles were separated by SDS-PAGE, together with protein markers (M) and a control sample of 10 µl of a 1:100 dilution of patient serum (serum) collected 72 days after initiation of phage treatment. The gel was stained with Coomassie Blue (left), transferred to a membrane for a Western blot which was probed with the same patient serum and an anti-human Horse Radish Peroxidase conjugated secondary antibody. These assays were repeated three times with similar results; a representative experiment is shown.
Extended Data Fig. 5 | Phage susceptibilities of GD01 clinical isolates. *M. abscessus* were recovered at 20-, 72-, 107, and 121-days after initiation of phage treatment, propagated, and tested for susceptibilities to each of the phages in the cocktail. Each phage was diluted serially 10-fold and spotted onto bacterial lawns. These assays were repeated at least twice with similar results; a representative experiment is shown.
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Mycobacterium abscessus subsp. massiliense FLAC047 (GenBank accession # CP021122); phage Muddy (GenBank accession #KF024728); phage BPs (GenBank accession #EU568876); phage ZoeJ (GenBank accession #KJ510412). Information on all phages tested here including genome sequence data and GenBank accession numbers are all available at https://phagesdb.org. Genome sequence and raw reads of M. abscessus GD01_A and GD01_B sequences are available in GenBank with Accession #'s CP035923 and CP03592, respectively.
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### Antibodies

- **Antibodies used**: Primary antibody source was patient serum; Secondary antibody used for Western blotting was Goat anti-human HRP (Novex).
- **Validation**: No validation is available.

### Human research participants

- **Population characteristics**: A 15-year-old female patient with Cystic Fibrosis (homozygous DF508) with a chronic, multi-drug resistant, Mycobacterium abscessus subspecies massiliense infection.
- **Recruitment**: The patient had no other viable options to treat a disseminated Mycobacterium abscessus infection.
- **Ethics oversight**: The Great Ormond Street Hospital (London) Institutional Ethics, Drugs and Therapeutics committee approvals and patient and parental consent for experimental treatment were obtained. Details are provided.

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