UC San Diego
UC San Diego Previously Published Works

Title

Development of Host Immune Response to Bacteriophage in a Lung Transplant Recipient on Adjunctive Phage Therapy for a Multidrug-Resistant Pneumonia

Permalink

https://escholarship.org/uc/item/2gq4m27w

Journal

The Journal of Infectious Diseases, 227(3)

ISSN

0022-1899

Authors

Dan, Jennifer M
Lehman, Susan M
Al-kolla, Rita
et al.

Publication Date

2023-02-01

DOI

10.1093/infdis/jiac368

Peer reviewed

Development of Host Immune Response to Bacteriophage in a Lung Transplant Recipient on Adjunctive Phage Therapy for a Multidrug-Resistant Pneumonia

Jennifer M. Dan,[1,2] Susan M. Lehman,[3,a] Rita Al-kolla,[1] Samuel Penziner,[2] Kamyar Afshar,[4] Gordon Yung,[4] Eugene Golts,[5] Nancy Law,[2] Cathy Logan,[2] Zsuzsanna Kovach,[6] Gill Mearns,[6] Robert T. Schooley,[2] Saima Aslam,[2] and Shane Crotty[1,2]

[1]Center for Infectious Diseases and Vaccine Research, La Jolla Institute for Immunology, La Jolla, California, USA; [2]Department of Medicine, Division of Infectious Diseases and Global Public Health, University of California, San Diego, La Jolla, California, USA; [3]AmpliPhi Biosciences, San Diego, California, USA; [4]Department of Medicine, Division of Pulmonary and Critical Care Medicine, University of California, San Diego, La Jolla, California, USA; [5]Department of Surgery. Division of Cardiovascular and Thoracic Surgery, University of California, San Diego, La Jolla, California, USA; and [6]AmpliPhi Australia, Sydney, Australia

Bacteriophage therapy is the use of viruses to kill bacteria for the treatment of antibiotic-resistant infections. Little is known about the human immune response following phage therapy. We report the development of phage-specific CD4^{+} T cells alongside rising phage-specific immunoglobulin G and neutralizing antibodies in response to adjunctive bacteriophage therapy used to treat a multidrug-resistant *Pseudomonas aeruginosa* pneumonia in a lung transplant recipient. Clinically, treatment was considered a success despite the development phage-specific immune responses.

Keywords. AIM; PBMC; anti-phage antibody; bacteriophage; immune response; phage therapy; T follicular helper cell.

Owing to the global prevalence of antibiotic resistance, bacteriophages ("phages") represent a promising therapeutic. Although phage therapy is not routinely used in medical practice, many patients have received it when antibiotics have failed [1–3]. Despite case reports describing the benefit of phage therapy, the full interplay of phages with the human immune system remains unclear.

Observations suggest that immunity against phage may not affect that phage's ability to successfully treat a bacterial infection [3, 4]. Robust immune responses do develop against phages, as these are new antigens. Extrapolating from animal models, phages are subject to innate immune clearance. Because phages do not replicate within human cells, CD8^{+} T-cell responses might not be significant. However, phagocytic cells are capable of processing new phage antigens to induce a CD4^{+} T-cell response, leading to an antibody response against phages.

Interestingly, there are reports of preexisting phage-specific humoral immunity in healthy individuals never exposed to phage therapy [5, 6]. Presumably, preexisting humoral immunity results from natural exposure, as phages are part of the human microbiota [7]. Therefore, treatment-naive patients are likely not affected by preexisting phage-specific immunoglobulin, as it takes approximately 7–14 days to develop a CD4^{+} T-cell–dependent humoral response to a new antigen [8]. However, patients who have previously received structurally similar phages may have phage-specific immunoglobulin (Ig) G, which could minimize the impact of further phage-specific therapies [9]. We therefore analyzed the development of phage-specific immune responses in a bilateral lung transplant recipient who received adjunctive intravenous and inhaled phage therapy for a multidrug-resistant *Pseudomonas aeruginosa* pneumonia [2]. The patient's maintenance immunosuppression consisted of sirolimus and prednisone, monthly intravenous immunoglobulin (IVIG) for acquired posttransplant hypogammaglobulinemia, and monthly photopheresis for chronic lung allograft dysfunction.

METHODS

Human Subjects

The patient was treated under an emergency investigational new drug application 171077, according to the Food and Drug Administration's expanded access program. Informed consent was obtained for investigational new drug 171077 and institutional review board 200163. AB-PA01 was registered for expanded access use (NCT03395743). Blood samples were collected weekly.

Activation-Induced Marker Assay

Peripheral blood mononuclear cells were cultured at 1×10^6 cells per well in 5% human serum medium for 24 hours, as described elsewhere [10, 11], using no stimuli, 5 µg/mL phage antigen, or 100 ng/mL staphylococcal enterotoxin B. Cells were stained, acquired on a BD Celesta, and analyzed using FlowJo Software, version 9.9.6.

Total Anti-Phage IgG

Each phage cocktail was coated at 1 μg/mL overnight at 4°C on 96-well MaxiSorp enzyme-linked immunosorbent assay plates. Plates were blocked with phosphate-buffered saline containing 0.2% Tween and 1% bovine serum albumin at room temperature for 90 minutes. Plates were washed 5 times with phosphate-buffered saline containing 0.5% Tween. Anti-human IgG was added at 1:5000 for 1 hour at room temperature (Hybridoma Reagents Laboratory HP6043). Plates were developed with Pierce TMB Substrate Kit (Thermo Fisher Scientific) and stopped with 1 mol/L sulfuric acid. Plates were read on a SpectraMax plate reader at an optical density of 450 nm, using SoftMax Pro software (Version 7.1).

Pooled plasma from 6 normal healthy donors (NHDs) was used as a standard to establish the relative units (RUs) of anti-phage IgG. Pooled plasma was diluted 1:10. Serial dilutions by 2 were performed to generate a standard curve. Serial dilutions were performed on the patient's plasma samples, intravenous immunoglobulin (IVIG) from the same manufacturer as the IVIG the patient received, and 5 other NHDs to determine an end-point titer. Comparison of the end-point titer with the standard curve allowed for quantification of anti-phage IgG present. RUs were plotted for each time point and compared with the RUs present in IVIG and NHDs.

Neutralizing Antibody Titer

Serum samples were incubated for 30 minutes at 56°C to inactivate complement. Heat-inactivated serum was diluted to between 1/10 and 1/4000 in SMB (sodium, magnesium, Tris Base) (sodium chloride, 5.2 g/L; magnesium sulfate, 2 g/L; Tris-HCl, 6.35 g/L; tris Base, 1.18 g/L; 2% gelatin, 5 mL; reverse osmosis water, 995 mL; pH 7.4), spiked with phage to a final concentration of 4×10^5 plaque-forming units (PFUs)/mL (AB-PA01), 5×10^5 PFUs/mL (AB-PA01-m1), or 1×10^5 PFUs/mL (unrelated *Staphylococcus aureus* phage used as negative control) and incubated for 1 hour at 37°C. Each individual phage was present at a concentration of 1×10^5 PFUs/mL. For positive controls, SMB was spiked and incubated in parallel. The postincubation concentration of active phages in each serum dilution was compared with the concentration in SMB using a plaque assay. Briefly, using nutrient broth–based medium (Oxoid CM0001), 100 μL of 16–18-hour *P. aeruginosa* culture was added to 6 mL of molten 0.4% agar and poured over a square 1.5% agar plate. Serial dilutions of serum-incubated samples were spotted (5 μL per spot) onto the overlays and incubated overnight at 37°C.

Plaque counts are expressed as concentrations using (200 × PFUs in spot × dilution factor of spot). Antibody titer was calculated as the serum dilution that resulted in a 1.0 $\log_{10}$ reduction in phage concentration (measured in PFUs per milliliter) relative to serum-free SMB. This threshold was chosen because it represented a clear titer reduction beyond the assay's established variability of ± 0.5 $\log_{10}$ (for values measured in PFUs per milliliter). To quantify cytokines and chemokines in serum, a serum sample from the patient was sent to Eve Technologies in Calgary, Alberta, Canada.

Statistical Analysis

For Figure 1C and 1D and Supplementary Figure 1C and 1D, the mean frequency with standard error of the mean of phage-specific CD4+ T cells for NHDs was plotted. For Figure 2A and 2B, the mean frequency with standard error of the mean of phage-specific IgG for NHDs was plotted.

RESULTS

The patient received AB-PA01 intravenously on days 1–14 and inhaled AB-PA01 on days 7–28 (Supplementary Table 1). Blood samples were collected weekly to track the development of phage-specific immunity. We first focused on activated circulating T follicular helper (cTfh) cells. A T follicular helper cell is a specialized CD4+ T-cell whose function is to facilitate B-cell differentiation, thereby leading to the generation of high-affinity antibodies [12]. Over the first course of intravenous therapy, activated cTfh cells (PD-1+ICOS+ of CXCR5+CD45 RA−CD4+ T cells) increased, peaking at day 14 (Figure 1A and Supplementary Figure 1A). After completion of intravenous therapy, the frequency of activated cTfh cells decreased but remained higher than pretreatment frequencies. After 4 weeks, the patient became ambulatory and was weaned off a ventilator.

Unfortunately, the patient experienced worsening pneumonia and resumed a second course of phage therapy on day 53, with a modified version of AB-PA01 (AB-PA01-m1). The patient received AB-PA01-m1 on days 53–63, an additional antipseudomonal phage cocktail from the United States Navy on days 64–78, and AB-PA01-m1 on days 78–92. Levels of activated cTfh cells peaked at day 59, 7 days after starting intravenous and nebulized phage therapy (Figure 1A) and remained high throughout the second course of therapy. We also analyzed the frequency of circulating plasmablasts (the CD38hiCD27hi proportion of CD19+ cells). Although the patient was hypogammaglobulinemic, there was a subtle trend toward increasing plasmablast frequencies after the second round of phage therapy (Figure 1A and Supplementary Figure 1B).

To determine whether these activated cTfh cells were phage specific, we used the activation-induced marker assay to quantify antigen-specific CD4+ T cells [10]. Using coexpression of activation markers OX40 and PD-L1, we observed AB-PA01–specific and AB-PA01-m1–specific CD4+ T cells (Figure 1B). The patient had AB-PA01–specific cTfh cells at day 21 (Figure 1C), which peaked at day 73. The patient had preexisting AB-PA01-m1–specific cTfh cells, as AB-PA01-m1 consisted of AB-PA01 plus an additional phage Pa176 (Figure 1D and Supplementary Table 2). Similarly, AB-PA01-m1–specific cTfh
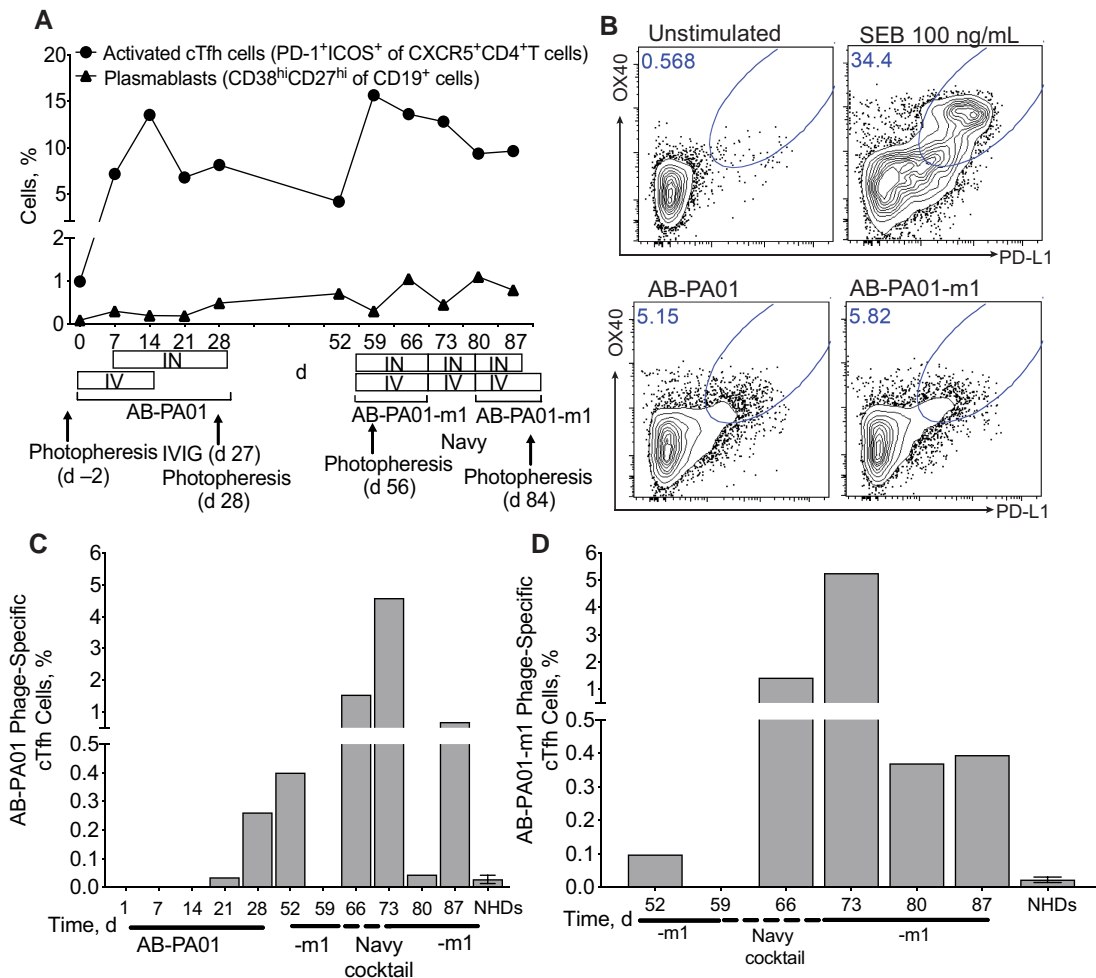
Figure 1. Phage-specific CD4$^+$ cells. *A,* Timeline of adjunctive phage therapy showing frequency of activated circulating T follicular helper (cTfh) cells (PD-1$^+$ICOS$^+$ proportion of CXCR5$^+$CD45RA$^-$CD4$^+$ T cells) and plasmablasts (CD38$^+$CD27^{hi} proportion of CD19$^+$ B cells). Abbreviation: IN, intranasal; IV, intravenous; IVIG, intravenous immunoglobulin; -m1 stands for modified version of AB-PA01 (AB-PA01-m1). *B,* Flow cytometry gating of phage-specific cTfh cells. Peripheral blood mononuclear cells were cultured for 24 hours and stained for coexpression of OX40 and PD-L1. Unstimulated cells (medium alone) served as the negative control, and staphylococcal enterotoxin (SEB; 100 ng/mL) as the positive control. *C, D,* Frequency of AB-PA01–specific (*C*) and AB-PA01-m1–specific (*D*) cTfh cells over the course of adjunctive phage therapy. As a negative control, the frequency of phage-specific total memory CD4$^+$ cells was also quantified in 3 normal healthy donors (NHDs).

cells peaked at day 73. We observed minimal phage-specific cTfh cells in NHDs who had not received phage therapy (Figure 1C and 1D). With continuous phage exposure, AB-PA01 and AB-PA01-m1 memory CD4$^+$ T-cell responses occurred (Supplementary Figure 1C and 1D), following the trend of phage-specific cTfh cells.

Serum cytokines and chemokines were also monitored over the course of phage therapy. Low levels of interferon γ and interleukin 2, were observed with a peak in interleukin 2 at day 76 (Supplementary Figure 2A). The level of interleukin 6 peaked at day 6 (1367.86 pg/mL; Supplementary Figure 2B). Interleukin 8 and tumor necrosis factor α remained at steady levels (Supplementary Figure 2C). The level of granulocyte-macrophage colony-stimulating factor peaked at day 76 (24.81 pg/mL), and that of interleukin 12p70 at day 82

(11.71 pg/mL; Supplementary Figure 2D). Interleukin 10 also peaked at day 76 (12.69 pg/mL; Supplementary Figure 2E). Interestingly, peak cytokine responses corresponded with peak phage-specific CD4$^+$ T-cell responses (Figure 1C and 1D and Supplementary Figure 1C and 1D).

We next tested whether phage-specific cTfh cells instructed the development of phage-specific IgG. Over the course of treatment, the patient had rising levels of anti–AB-PA01 IgG (Figure 2A) and anti–AB-PA01-m1 IgG (Figure 2B). At the end of treatment, the patient's anti–AB-PA01 IgG level was 100-fold higher than that of IVIG and 1000-fold higher than that of NHDs (Figure 2A).

We finally tested whether these phage-specific IgG were neutralizing by quantifying the ability of heat-inactivated serum to reduce phage activity. Two strains of *P. aeruginosa* (control host 1 and 2 [CH1 and CH2]) were used because the individual
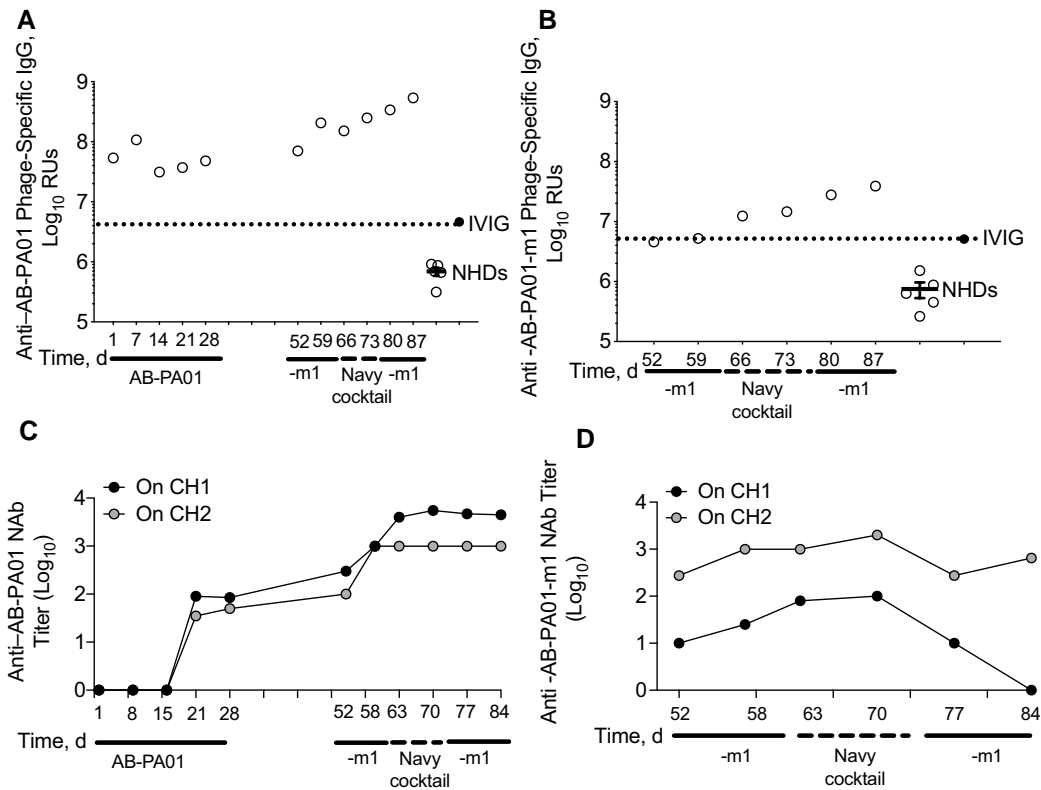
Figure 2. Phage-specific antibodies. *A, B,* AB-PA01-specific (*A*) and AB-PA01-m1–specific (*B*) immunoglobulin (Ig) G determined by enzyme-linked immunosorbent assay. As a negative control, anti–phage-specific IgG titers were quantified in 3 normal healthy donors (NHDs). Anti–phage-specific IgG was quantified in intravenous immunoglobulin (IVIG). Abbreviation: RUs, relative units; -m1 stands for modified version of AB-PA01 (AB-PA01-m1). *C, D,* Titers of neutralizing antibodies (NAbs) to AB-PA01 (*C*) and AB-PA01-m1 (*D*) were quantified based on the serum dilution that caused a 1-log drop in phage titer on either control host 1 (CH1) or control host 2 (CH2). Note that CH2 preferentially supports plaque formation by Pa222 and Pa223, which are closely related with very similar structural proteins and are therefore likely to be affected by the same neutralizing antibodies. Therefore, neutralizing activity visualized using CH2 reflects the total neutralizing activity of antibodies that are specific to Pa222 and Pa223 and of antibodies that cross-react with multiple phages. Similarly, neutralizing activity visualized on CH1 should be specific to the 2 closely related PB1-like phages, Pa193 and Pa204; after the transition to AB-PA01-m1, neutralizing activity on CH1 also reflects total neutralizing activity against Pa176. The effect of adding Pa176 is seen when comparing panels *C* and *D*. Specifically, antibody titers quantified using CH2 are similar in *C* and *D* because AB-PA01 and AB-PA01-m1 do not differ in terms of phages that plaque on CH2 (Pa222 and Pa223). In contrast, AB-PA01-m1 contains 2 types of phages that both plaque on CH1 but that are distantly related and may not be inactivated by the same antibodies. Therefore, the apparent drop in total neutralizing activity assayed on CH1 in *D* suggests that the antibodies initially present when AB-PA01-m1 treatment was initiated are antibodies that neutralize the 2 closely related phages Pa193 and Pa204 (based on *C*) and that these antibodies are not as good at inactivating Pa176 (the distantly related phage present only in AB-PA01-m1).

phages plaque differently. The 2 PB1-like phages (Pa193 and Pa204) and the Lit1-like phage (Pa176) plaque well on control host 1 but very faintly on CH2, while the 2 LUZ24-like phages (Pa222 and Pa223) plaque well on CH2 but faintly on CH1. This permits some phage-specific inferences about neutralizing antibodies even though the serum was tested on phage cocktails rather than single phages. The patient had no preexisting neutralizing antibodies (Figure 2C and 2D; day 1). Neutralizing antibodies for AB-PA01 were first observed on day 21 (Figure 2C), coinciding with the development of AB-PA01-specific cTfh (Figure 1C). Neutralizing titers for AB-PA01 peaked at day 63 and then plateaued during the transition between AB-PA01-m1 and the Navy phage cocktail (Figure 2C). Neutralizing titers for AB-PA01-m1 peaked at day 70 (Figure 2D), with titers against CH1 decreasing by day 87. This suggests that at day 87 there was no neutralizing IgG

against the new phage Pa176 (Figure 2D) when compared with the neutralizing activity of anti–AB-PA01 against CH1 (Figure 2C).

DISCUSSION

To our knowledge, this is the first study to demonstrate the development of phage-specific CD4+ T cells, specifically cTfh cells, and correlate this with phage-specific IgG and neutralizing antibodies. The patient had a high frequency of activated cTfh cells on day 14, followed by phage-specific cTfh cells and neutralizing antibodies on day 21. On reexposure to AB-PA01-m1, the patient had a quick recall response, with activated cTfh cells increasing on day 59 (7 days after initiation of second treatment), followed by phage-specific cTfh cells and neutralizing AB-PA01 antibodies on day 66. The timeline of

his phage-specific immunity is consistent with the immunologic response after vaccination with a new antigen, in which day 21 marks the peak of germinal center activity in lymph nodes [13]. Similar to a finding in a nonhuman primate vaccination model using an osmotic pump to continually deliver antigen [14], our patient had phage-specific cTfh cells and neutralizing antibodies on day 21.

Two case reports assessed the development of phage-specific neutralizing antibodies following intravenous phage therapy to treat *Mycobacterium abscessus* pneumonia. One reported no neutralizing antibodies in a 15-year-old lung transplant recipient, though the patient had received rituximab for possible posttransplant lymphoproliferative disorder [3]. The second report noted strong phage-neutralizing antibody responses after 2 months in an immunocompetent 81-year-old patient [9].

Our patient received photopheresis, monthly IVIG, and maintenance sirolimus and prednisone. Photopheresis is associated with an increase in circulating regulatory T cells, inducing immune tolerance [15]. This may explain the lack of phage-specific $CD4^+$ T cells at day 59, which was 3 days after photopheresis. Despite sirolimus, prednisone, and monthly IVIG, our patient experienced a robust phage-specific immune response. Regardless, our study suggests that the robust phage-specific immune response did not prevent treatment success. We defined success in the first treatment episode as progress from sedation and ventilation to an ambulatory condition (albeit without microbiological cure), and success in the subsequent treatment episode as the absence of pneumonia and microbiological cure (Supplementary Table 2). This may partly be due to the vast number of phages administered to surmount neutralizing antibodies. Further studies in immunocompetent individuals are needed to determine whether the development of an immune response to one type of phage precludes the future use of that phage type or other types. Use of the activation-induced marker and plaque neutralization assays can further elucidate the interplay of the human immune system with phage therapy.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

References

1. Schooley RT, Biswas B, Gill JJ, et al. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. Antimicrob Agents Ch **2017**; 61:e00954–17.
2. Aslam S, Courtwright AM, Koval C, et al. Early clinical experience of bacteriophage therapy in 3 lung transplant recipients. Am J Transplant **2019**; 19:2631–9.
3. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. Nat Med **2019**; 25:730–3.
4. Ochs HD, Davis SD, Wedgwood RJ. Immunologic responses to bacteriophage ϕX 174 in immunodeficiency diseases. J Clin Invest **1971**; 50:2559–68.
5. Żaczek M, Łusiak-Szelachowska M, Jończyk-Matysiak E, et al. Antibody production in response to staphylococcal MS-1 phage cocktail in patients undergoing phage therapy. Front Microbiol **2016**; 7:1681.
6. Lusiak-Szelachowska M, Zaczek M, Weber-Dbrowska B, et al. Antiphage activity of sera during phage therapy in relation to its outcome. Future Microbiol **2017**; 12:109–17.
7. Federici S, Nobs SP, Elinav E. Phages and their potential to modulate the microbiome and immunity. Cell Mol Immunol **2021**; 18:889–904.
8. Bentebibel SE, Lopez S, Obermoser G, et al. Induction of $ICOS^+CXCR3^+CXCR5^+$ T_H cells correlates with antibody

responses to influenza vaccination. Sci Transl Med **2013**; 5: 176ra32–176ra32.

9. Dedrick RM, Freeman KG, Nguyen JA, et al. Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary *Mycobacterium abscessus* infection. Nat Med **2021**; 27:1357–61.

10. Dan JM, Arlehamn CSL, Weiskopf D, et al. A cytokine-independent approach to identify antigen-specific human germinal center T follicular helper cells and rare antigen-specific CD4^{+} T cells in blood. J Immunol Baltim Md **2016**; 197:983–93.

11. Reiss S, Baxter AE, Cirelli KM, et al. Comparative analysis of activation induced marker (AIM) assays for sensitive identification of antigen-specific CD4 T cells. PLoS One **2017**; 12:e0186998.

12. Crotty S. Follicular helper CD4 T cells (T$_{FH}$). Annu Rev Immunol **2011**; 29:621–63.

13. Havenar-Daughton C, Carnathan DG, Boopathy AV, et al. Rapid germinal center and antibody responses in non-human primates after a single nanoparticle vaccine immunization. Cell Rep **2019**; 29:1756–1766.e8.

14. Cirelli KM, Carnathan DG, Nogal B, et al. Slow delivery immunization enhances HIV neutralizing antibody and germinal center responses via modulation of immunodominance. Cell **2020**; 180:206.

15. George JF, Gooden CW, Guo L, Guo WH, Kirklin JK. Role for CD4^{+}CD25^{+} T cells in inhibition of graft rejection by extracorporeal photopheresis. J Hear Lung Transplant **2008**; 27:616–22.