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REVIEW ARTICLE

Phages and their potential to modulate the microbiome and immunity

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Bacteriophages (hence termed phages) are viruses that target bacteria and have long been considered as potential future treatments against antibiotic-resistant bacterial infection. However, the molecular nature of phage interactions with bacteria and the human host has remained elusive for decades, limiting their therapeutic application. While many phages and their functional repertoires remain unknown, the advent of next-generation sequencing has increasingly enabled researchers to decode new lytic and lysogenic mechanisms by which they attack and destroy bacteria. Furthermore, the last decade has witnessed a renewed interest in the utilization of phages as therapeutic vectors and as a means of targeting pathogenic or commensal bacteria or inducing immunomodulation. Importantly, the narrow host range, immense antibacterial repertoire, and ease of manipulating phages may potentially allow for their use as targeted modulators of pathogenic, commensal and pathobiont members of the microbiome, thereby impacting mammalian physiology and immunity along mucosal surfaces in health and in microbiome-associated diseases. In this review, we aim to highlight recent advances in phage biology and how a mechanistic understanding of phage–bacteria–host interactions may facilitate the development of novel phage-based therapeutics. We provide an overview of the challenges of the therapeutic use of phages and how these could be addressed for future use of phages as specific modulators of the human microbiome in a variety of infectious and noncommunicable human diseases.

Keywords: Microbiota; Microbiome; Phages

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INTRODUCTION

Bacteriophages (referred to hereafter as phages) are viruses infecting prokaryotes, which collectively encompass the most genetically variable reservoir on Earth. Phages have been detected wherever a bacterial host exists, including in water sediments, soil, and along mucosal surfaces of the human body.[1–3] Phages are 'adsorbed' on the membrane of the host bacterium, interacting with bacterial receptors through external structures (e.g., tail fibers) to inject their viral DNA through the bacterial cell wall by enzymes present in the tail. During this interaction, the phage capsid remains outside the cell, while the viral genetic material may localize in the cytoplasm, where it can exist in two major states. The first state, characteristic of lytic phages, includes phage exploitation of the replicative machinery of the host to produce more virions, which are then released by lysis of the host cell.[4,5] In the second state, characteristic of temperate phages, the phage genome may be integrated into the bacterial chromosome, where it remains in a dormant state, replicating with the bacterial genome. Importantly, microbial stress signals may induce a transformation between the lysogenic and lytic states in a highly regulated manner (Fig. 1a).

Maintenance of lysogeny requires continuous transcription of repressor proteins to suppress lytic genes (such as CI protein in λ phage). A drop in repressor protein levels, or stress signals such as DNA damage, is interpreted by the integrated phage as a threat to its own genomic stability, inducing excision from the bacterial chromosome and the start of the lytic cycle[4] (Fig. 1a). For example, temperate phages release a pro-lysogenic hexapeptide, named 'arbitrium', that transfers information guiding the next phage generation toward either the lytic or lysogenic cycle. According to this model, aimR, an intracellular phage protein, binds to the phage DNA, thereby increasing transcription of the *aimX* gene, which in turn promotes the lytic cycle through currently unknown mechanisms. Accumulation of arbitrium after several generations of phage production suppresses expression of *aimR*, which leads to prevention of DNA-binding, suppressed activation of *aimX* and lytic induction, thereby promoting lysogeny.[6] As such, at the beginning of a phage infection, few viral particles would increase their ecological success by initiating the lytic cycle, but when infection is spread over an entire bacterial colony, lysogeny could represent a more efficient choice in preserving the phage and its bacterial host. Such a mechanism may help avoid extinction of both bacteria and associated phages[7] or, alternatively, provide phages with information pertaining to the number of available hosts or of lysogens (i.e., bacteria already infected by a prophage), thereby enabling the phage to maximize infection efficiency.[8] Importantly, in this model, the choice between lysis and lysogeny happens independently during infection for each phage, and

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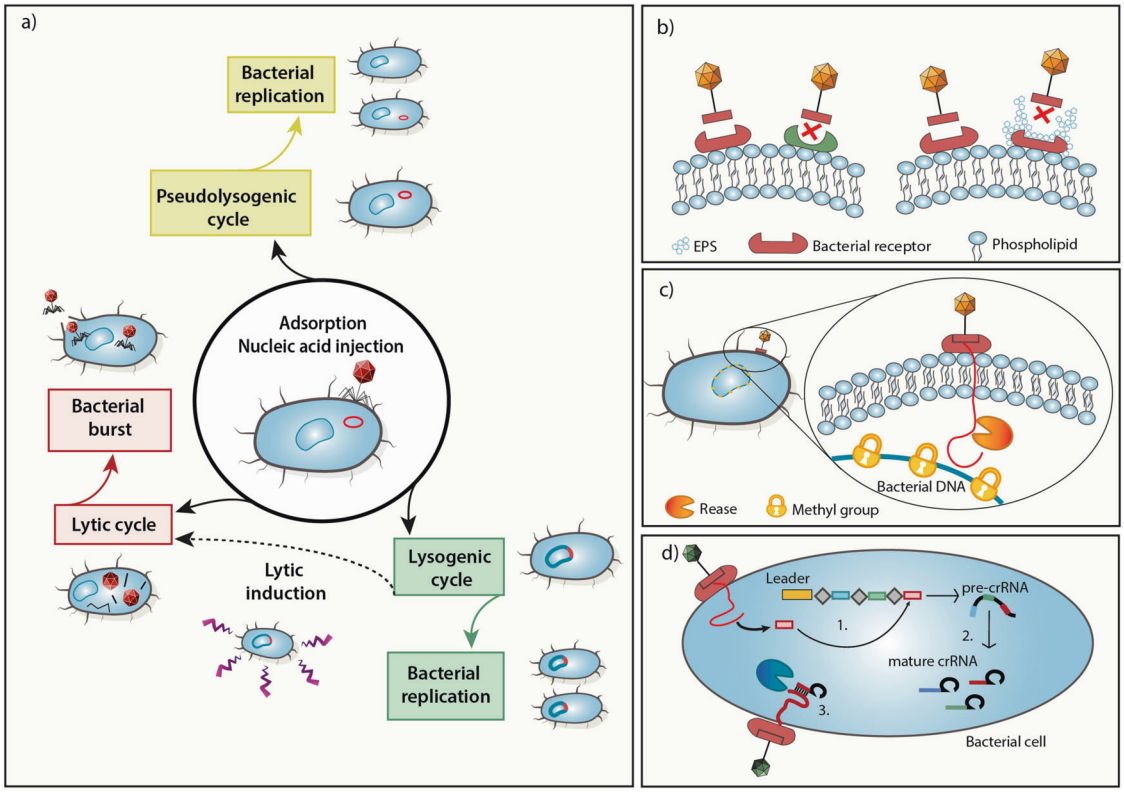

Fig. 1 Mechanisms of phage–bacterial interactions. **a** Following adsorption and injection of a phage genome into a bacterial host, phages can undergo a lytic cycle, leading to viral replication and bacterial cell lysis. Alternatively, in the lysogenic cycle, the phage genome is integrated as a prophage into the bacterial genome, until lytic induction triggered by a stress signal. Pseudolysogeny is the stable persistence of viral DNA in the host when growth conditions become unfavorable, and the phage is not replicating; thus, it will be inherited only by a single progeny cell. Innate bacterial phage evasion mechanisms include (**b**) the generation of mutated phage receptors or receptor masking by extracellular polymeric substances (EPSs), and (**c**) cleavage of the phage genome following its injection into the bacterial cell, while methylated bacterial DNA remains protected. The adaptive bacterial antiphage machinery is exemplified by (**d**) the CRISPR-Cas system, which has several phases: 1. Acquisition: a short viral DNA portion (red rectangle) is inserted between palindromic sequences (gray rhombuses) to form a CRISPR array. 2. Expression: The CRISPR array is transcribed into a pre-crRNA that is then cleaved into mature crRNA ready for recognition. 3. Interference: the endonuclease recognizes and cleaves the complex crRNA-phage nucleic acid

phages coordinate an unanimous decision to enter a lytic versus a lysogenic state only in cases of multiple phages infecting the same host.[9]

Many prophages confer immunity to the lysogen they infect by different mechanisms, collectively termed prophage-mediated superinfection exclusion.[10] For example, phages harboring CRISPR-Cas sequences directed against other phages confer resistance to their lysogenic bacteria upon integration into the chromosome.[11] Interestingly in *Vibrio*'s phage VP882, the lysis-lysogeny decision depends on quorum sensing molecules produced by its bacterial host. The cytoplasmic receptor-transcription factor *Vibrio* quorum modulator A represses virulence- and biofilm-associated bacterial genes. Strikingly, the VP882 phage encodes a homolog of the host's protein that the phage uses as an antirepressor of lytic genes, thereby initiating a lytic cycle, while acquiring direct control over its host's quorum sensing and virulence.[12] Bacteria, in turn, can influence phage fitness by generating metabolic cues, such as resisting phage infection by dampening metabolic functions along the GI tract[13–15] or by inducing prophage production via release of short chain fatty acids.[16,17] Phages may react to these bacterial-induced metabolic signals by altering metabolite composition via targeting of susceptible commensals.[18] Collectively, such an extensive 'arms race' between bacteria and their phages underlines the constant crosstalk between the counterparts at the molecular, cellular, and organ levels.

Given these phage–bacterial interactions, harnessing phageome members for therapeutic purposes was studied in the past century as a promising tool in redirecting bacterial communities toward homeostatic configurations. The first therapeutic use of phages was documented by d'Herelle.[19] Shortly thereafter, phages gained great interest as antimicrobial agents, initially for treating staphylococcal skin infections, and then as therapy against life-threatening diseases such as cholera and bubonic plague, and were marketed by companies such as L'Oréal and by Eli Lilly.[20] However, the efficacy of phage preparations remained controversial,[21] and the concomitant discovery of antibiotics in 1928 led to a decrease in the popularity of phage treatment.[22] Nonetheless, phages were still extensively used as antimicrobial therapy in the former Soviet Union, where antibiotics were not easily accessible. This decades-long experience and the knowledge gained in clinical phage exploitation have remained elusive, as scientific reports from Eastern Europe were mainly written in Russian and were thus inaccessible to the global scientific community. In addition, these reports often lacked adequate randomization and controls,[20] but provided, at least to some extent, proof of the safety of phages as potential therapeutic agents. Currently, the threat of multi-drug-resistant and carbapenem-resistant pathogenic bacteria has led to a renewed interest in antibacterial agents, among them phage therapy.

In this review, we illuminate how phage therapy could potentially reemerge as an effective antimicrobial treatment,

including the potential to modulate the human microbiome. Furthermore, we aim to analyze the biological limitations of phage therapy, including regulatory obstacles that limit its wider application, and the means of addressing these challenges in moving phage therapy toward clinical use.

BACTERIAL ANTIPHAGE DEFENSES

Phages represent a major threat to bacterial species. Reactive to their phage predators, bacteria have developed resistance mechanisms; as a result, phages find strategies to overcome such defenses, resulting in a constantly evolving 'arms race'.[23] The first line of bacterial immunity, defined as innate immunity, akin to more evolved immune systems, includes several nonspecific mechanisms.[24] Following phage infection, bacteria can mutate, downregulate expression or lose receptors exploited by the phage to penetrate the cell.[25] Alternatively, bacteria can mask phage receptors by a layer of extracellular polymers (EPS) or capsular material. Phages can reactively develop the capacity to recognize a novel receptor[26] or to quickly adapt to a mutated receptor.[27] Moreover, tailed phages contain an endosialidase domain in their tail structure, which allows them to digest the capsule or EPS covering masked receptors[28] (Fig. 1b).

After entering the bacterial cell, phages can encounter restriction–modification (R–E) systems that employ a restriction endonuclease (REase) to cleave foreign nucleic acids, while the bacterial DNA is protected by methylation. Phages can resist this defense system in several ways, including naturally integrating modified bases that will alter the restriction site[29] or actively self-methylate their own DNA, as was shown for the *L. lactis* phage φ50[30] (Fig. 1c). Abortive infection systems (Abi) trigger a cascade reaction to induce an 'altruistic cell suicide' of phage-infected bacteria, preventing further infection of the bacterial community.[31] A widespread example of Abi are toxin-antitoxin systems, involving a toxin directed against vital cellular processes, and a protective antitoxin that is constantly expressed, avoiding bacterial extinction.[32] In the presence of stress signals (such as phage infection), the antitoxin, being structurally more labile than the toxin, is degraded, leading to a dormant state or cell death.[31] Phages may overcome this strategic defense by expressing small, repetitive RNA molecules resembling the antitoxin transcript that neutralizes the toxin.[33,34]

The adaptive bacterial antiphage system consists of several more sophisticated systems, such as CRISPR-Cas (clustered regularly interspaced short palindromic repeats; CRISPR-associated), comprising a series of short fragments of foreign DNA (spacers) inserted into repeated palindromic sequences, named CRISPR loci. Small RNAs are then transcribed and associated with Cas proteins, enabling recognition and cleavage of phage genomes matching the RNA sequence[35] (Fig. 1d). This mechanism is characterized by a rapid evolution because of the high intraspecies phage diversity; as a result, point mutations are no longer sufficient to overcome it.[36] However, if directed against a temperate phage inserted in the host genome, CRISPR systems could also cause "autoimmunity" and become deleterious to the host bacterium.[37] In addition to its antiphage properties, CRISPR biology is also recognized as an efficient tool for providing insights into phage-bacteria ecology. Moreover, conservation of ancient spacers in the CRISPR loci allows the identification of unknown phages targeting bacteria residing in the gut.[38] In order to survive, and as part of the bacteria-phage arms race, phages developed anti-CRISPR proteins (Acrs), which can block CRISPR immunity by inhibiting components of its protein complex.[39] Phages can cooperate by producing Acrs to overcome CRISPRs and successfully infect resistant bacteria.[40,41] These counter-regulatory mechanisms were shown to evolve based on the diversity of the surrounding bacterial community.[42]

Besides CRISPR, newly identified antiphage defense systems include, for example, an abortive system inducing cyclic GMP–AMP signaling, leading to membrane damage and bacterial death upon phage infection, even before the phage is able to replicate. Therefore, the death of the single bacterial cell prevents the spreading of the virus and protects the remaining bacterial colony from further infection. Another recently discovered bacterial defense mechanism is termed the defense island system associated with restriction–modification, and is based on a DNA restriction system providing a broad defense against multiple phage types.[43,44]

From these observations, it is clear that phages can drive microbial diversity at a genotypic, phenotypic, and community level, affecting competition between bacteria,[45] and preserving bacterial diversity.[46] Further unraveling the mechanisms by which bacteria develop phage resistance could provide valuable insights into overcoming these mechanisms in effectively targeting bacteria without inducing the onset of phage resistance.

THE GUT PHAGEOME

The last decades have seen a tremendous improvement in understanding the structure of microbial communities inhabiting the human host, collectively termed the microbiome. The microbiome has been increasingly recognized as playing a central modulatory role in the development of noncommunicable or nontransmissible diseases including metabolic syndrome, cardiovascular disease, and cancer. Development and utilization of high-throughput next-generation sequencing technologies allows for characterization of complex, heterogenous, and poorly culturable microbial ecosystems in the gut and elsewhere (see Box 1). Early studies suggested that the viral fraction in the human gut, also defined as the "phageome", encompasses 5–6% of normalized sequencing reads in the human gut.[47] However, the total amount of DNA per gram of stool in the gut derived from phages is still debated and depends on the extraction method.[48–50]

Viruses sequenced from fecal samples of healthy people are mostly temperate and largely composed of the tailed, double-stranded DNA phage *Caudovirales*. It is estimated that 82% of bacteria in the human gut harbor at least one phage sequence, a so-called "prophage".[51,52] This estimation is based on the presence of numerous sequences coding for annotated integrases, utilized by temperate phages to integrate into the bacterial genome.[53,54] The human gut phageome displays a common reservoir of a few highly abundant families shared among unrelated, geographically distant subjects. These include *Siphoviridae*, *Myoviridae*, the ubiquitous crAssphage, and the single-stranded *Microviridae*,[54,55] as well as several low abundance families that are responsible for interpersonal variability.[38,54,56] In contrast to prokaryotic viruses, eukaryotic viruses are less commonly found in human stool samples, although some studies reported their constant presence and fecal shedding.[57,58]

Despite these observations of phage presence as part of the gut microbiome, we are still far from fully elucidating the full repertoire of the phageome composition. Out of 2500 virus-like particles (VLPs) identified in the gut,[53,54] the large majority cannot be linked to a precise bacterial host,[56] and over 90% of obtained sequences cannot be assigned to any of the viral families proposed by the International Committee on Taxonomy of Viruses (ICTV).[59] The major difficulties of characterizing the composition of the gut virome stem from the lack of a common genetic marker that would allow recognition of phage sequences (as in the case of the 16S rRNA gene for bacteria) and the lack of annotated phage genes in confirmed databases. Early studies relied on sequence homology to known viral genes or proteins to identify new prophages. More unbiased and efficient approaches utilizing the percentage of GC compositional skews or the

Box 1

In the last decades, the biological role of phages in modulating complex ecological environments was facilitated by recent advances in high-throughput sequencing (HTS) technologies. However, technical limitations are frequently noted in generating metagenomic libraries from viral DNA, further complicated by variable and often noncomparable protocols utilized in different studies. Establishing validated methods for in-depth study of the human virome would significantly help in understanding the role of the virome in general, specifically the phageome, in health and disease.

After DNA purification, the majority of DNA obtained from a sample is nonviral. DNA is then fragmented to an appropriate size in preparation for next-generation sequencing. Illumina Nextera XT is widely used for creation of virome libraries, although it has limitations in recovery of end sequences. Considering that a low yield of DNA is often obtained from purification, it is necessary to amplify nucleic acids in order to build a library, usually by the use of whole-genome amplification (WGA) or random amplification. Relative abundances of the viral community could be altered by this step, as previously reported for specific enzymes[227] or PCR protocols.[228] Careful selection could help avoid such technical biases.[229] Several efforts in generating an optimized protocol utilized mock viral communities.[230–232] An approach called NETOvir was developed to enrich for viral DNA compared to bacterial DNA.[233] Although it is time consuming and labor intensive, this method allows high-throughput sample preparation with almost total elimination of bacterial reads.[233] Another challenge in preparation of viral metagenomics libraries is the incorporation of contamination from environmental, bacterial, and human sequences, and from commercial kits.[234] To address this problem, multiple negative controls must be included in each run, as a means of differentiating between background noise and true viral sequences of biological relevance. Separation of virus-like particles (VLPs) from contaminants by flow cytometry based on DNA content and size of particles may further address contamination issues. Indeed, such an approach may improve the number of assembled long contigs while reducing contamination from human ribosomal RNA or mitochondrial DNA.[235] Annotation of viral sequences by publicly available databases remains poor, given their high genomic variability, resulting in virome research greatly relying on de novo assembly of short sequencing reads.[236] A study assessing assembling performances of four different datasets reported large database-related variations and low accuracy coverage impacting analyses, with SPADES performing better than the other databases.[237] This result highlights the importance of implementing a multi-assembler approach using several datasets at a time for virome assembly in complex microbiome analyses.

presence of consecutive genes on the same DNA strand may enable recognition of new phages. One example is the cross-assembly phage (later called crAssphage), which is present from early infancy in 50% of healthy adults,[60] but features no encoded proteins matching previously known viral sequences.[61,62] Another major obstacle to accurate phage identification is the possible contamination of prophage sequences inserted in bacterial genomes upon analysis of the "active" part of the virome (lytic phages and activated prophages). These regions are technically impossible to distinguish by sequencing because of their viral origin.[63]

To date, metagenomic studies have identified an increasing number of viral sequences derived from the vast unclassified genetic material, or "dark matter", of the virome in commonly used databases.[60,64–67] Nonetheless, metagenomics approaches lack the high resolution necessary to reconstruct genomes of different, but closely related, microorganisms, which would be important in future research in decoding host–phage relationships, considering the mosaic structure of the genome in viruses and their high degree of interindividual variability.[53,65] Novel technologies such as single-virus genomics,[68] frequently employed in marine ecosystems, and viral tagging (VT) metagenomics[69] could shed new light on phage–bacteria interactions.[70] Likewise, increasing the sequencing depth in the analyses of metagenomic libraries may be more informative in identifying new sequences[50] (see Box 1).

OTHER PHAGEOMES

To date, most of the efforts to characterize the endogenous human virome and phage populations focused on the gut, as the richest ecological niche of the human body. Other viral communities in the human holobiome are only starting to be elucidated. In early studies, phages isolated from human skin were used to distinguish different serotypes of their host.[71,72] Recently, it has been discovered that the skin virome displays a core of double-stranded DNA phages, mainly *Caudovirales*, which are directed against the most prevalent bacteria on the skin, such as *Propionibacterium*, *Streptococcus, and Staphylococcus*. Phages are mostly segregated at a specific site of their bacterial hosts (e.g., sebaceous sites for *Propionibacterium acnes*). Likewise, phage-bacterium dynamics seem to be site-specific. In sebaceous sites, an observed negative host–phage correlation suggests antagonism, while in moist and dry sites, a positive correlation could imply the presence of temperate phages.[73] Phage communities in the skin, and their most abundant genes, display a low variability in composition and diversity over time.[16] For instance, phages targeting *Propionibacterium* spp. in healthy subjects were shown to be dominated by a few strains mostly belonging to the Syphoviridae family,[74] which feature low genetic variability and morphology.[75,76] These skin phages are mostly lytic, but in rare cases, they may undergo a pseudolysogenic cycle,[77] while collectively exhibiting a broad host range toward several *P. acnes* clinical isolates.[78] As such, these phages could be regarded as a promising putative tool for treating *P. acnes*-associated acne.[78] However, they may also induce the generation of phage-resistant clones,[71,72,79] which could limit treatment efficacy.

Comparing phages in the lungs in children affected by multiple acute respiratory tract infections to those in children suffering from a single respiratory infection revealed that phages targeting *Propionibacterium* spp. are more prevalent in the former compared to the latter in a clinical context. Furthermore, the abundance of phages increases with recurrent infections, while the overall diversity of phages is comparable between the two groups. Importantly, *Propionibacterium* spp. colonization of the respiratory tract has been previously associated with a propensity for viral infection[80] and pulmonary inflammation.[81] Indeed, the respiratory phageome has been suggested as a biomarker for the susceptibility of individuals to airway infections, as phages targeting *Propionibacterium* spp. are correlated with higher levels of cytokines, which are associated with respiratory disease.[82] A metagenomic study of the lung phageome in cystic fibrosis (CF) patients revealed a specific composition and metabolic signature associated with the disease and lower species richness in CF than healthy individuals.[83] In addition, the lung phageome differs between smokers and nonsmokers, with the latter featuring higher species richness. Interestingly, the smokers' lung virome configuration was correlated with levels of IL-8, a potential marker of COPD (chronic obstructive pulmonary disease) severity. Strikingly, the differences in the phageome were not reflected by the microbiome composition.[84] Collectively, these observations could provide new insights for the use of the lung phageome as a diagnostic tool in COPD, although further investigation is required to determine its potential causative impacts on human lung physiology.

Little is known about the vaginal phageome, although this ecosystem could play an important role in modulating the onset of microbiome-related diseases, such as bacterial vaginosis (BV). Early in vitro studies suggested that lytic phages could target healthy members of the vaginal microbiome, such as *Lactobacillus jensenii*[85] or other *Lactobacillus* species,[86] thereby contributing to dysbiosis in BV. Conversely, the Human Microbiome Project[87] discovered that the 'healthy' vaginal microbial ecosystem is characterized by low species diversity compared to the gut. In addition, phages were found to correlate with bacterial hosts in a physiological equilibrium, while high microbial diversity was correlated with dysbiosis. Interestingly, BV is more prevalent in women who smoke,[88] while in clinical isolates of *Lactobacillus* spp., prophage activation was induced by cigarette-derived chemicals, such as benzo(a)pyrene diol epoxide, which is also present in the vaginal secretions of smoking women; this might

suggest a possible role of cigarette-derived chemicals in nonphysiological phage activation, potentially disrupting the vaginal microbiome.[89] The vaginal phageome of BV-positive and BV-negative women is mainly composed of dsDNA phages, with a clear correlation noted between phage composition and presence of their specific bacterial hosts in healthy and BV conditions. As such, most of the phages targeting *Lactobacillus spp. (*a hallmark of a healthy vaginal microbiome) are negatively correlated with phages targeting *Gardnerella vaginalis* (a hallmark of the dysbiotic vaginal microbiome in BV). Differences in the composition of the vaginal virome are prominent in women suffering from BV, suggesting that the vaginal phageome could be a marker or even a predictor of this condition.[90] Recently, vaginal microbiome transplantation (VMT) performed on women affected by intractable BV resulted in full long-term remission and reconstitution of a *Lactobacillus*-dominated microbiome configuration.[91] Future studies may assess whether the transferred vaginal phageome could contribute to the observed clinical impacts noted with VMT.

Phages also constitute the majority of viruses in the oral cavity,[92] yet their physiological effects remain unknown.[93,94] Interestingly, the oropharynx is constantly exposed to pathobionts, whose translocation to other microhabitats may be associated with disease states.[95] In accordance with this concept, a plethora of virulence factor homologs was recently found in the salivary phageome,[92] and phages from the mouth were shown to encode for *Streptococcus mitis* virulence factors, which are responsible for platelet-attachment and associated with endocarditis.[96] This link potentially suggests a dynamic network between phages and their pathogenic hosts. Interestingly, the oral cavity is characterized by a high phage diversity compared to the gut, hallmarked by the presence of jumbo phage genomes.[97,98] This diversity could be enhanced by the presence of dense biofilms that were shown to be promoted, among other factors, by phages.[99]

Overall, information is still largely lacking as to the full spectrum and interindividual variability of the phageome in various niches of the human body. Gaining further information in this area could shed light on previously unexplained bacteria-phage networks in unexplored sites of the body and their impacts on host microbiome-related phenotypes, while boosting the potential applications of the phageome as a diagnostic or therapeutic organ- and disease-specific tool.

MODULATION OF GUT PHAGEOME REPERTOIRES

Host intrinsic factors

The phageome is modulated by a complex interplay of endogenous and extrinsic factors, which have remained elusive to date. Longitudinal studies suggested that phageomes generally remain stable in healthy adults featuring a stable lifestyle. However, phageomes can change due to external conditions such as changes in diet,[53,56] differences in mode of delivery[60], and other varying environmental signals. In addition to the role of environmental signals in controlling phageome composition (discussed below), genetic factors were also found to play important roles in phage dynamics modulation. For example, young monozygotic twins shared more similar virotypes than unrelated individuals.[100] Of note, these twins were also more likely to share a similar environment and diet; thus, a key role of the environment in influencing phage dynamics was not ruled out even in this setting. Specific host genotypes influencing phageome composition remain elusive to date. These include genetic factors regulating direct viral nucleic acid recognition or immune-related genes controlling phages directly or through induced alterations in microbiome configuration.

Host extrinsic factors

While host intrinsic factors regulating phageome composition remain largely unknown, key environmental factors determining phageome composition are being increasingly identified. Indeed, in a cohort of adult monozygotic twins, twins exhibiting a similar microbiome also shared more viral taxa in comparison to twins with different microbiomes.[101] Moreover, a positive correlation was noted between the virome and microbiome β-diversity regardless of genetic relatedness, suggesting an association between the virome and microbiome diversity, even in genetically unrelated individuals.[55,102] In a longitudinal study, phage richness was highest at birth and decreased with age.[66] While the mode of acquisition of phages starting from birth has not been fully explained, a significant proportion of phages seem to be transferred to the infant through breast milk.[103] However, phages could also derive from prophages integrated into the genome of gut commensal bacteria,[104] which implies that the mode of delivery may represent an extrinsic factor in shaping the phageome, possibly through its effect on the microbiota.

Similarly, a change in dietary composition also significantly impacts the gut phageome. For example, individuals switching to a similar diet showed reduced interindividual differences in their virome configuration.[53] However, some virome changes exhibit resilience following their modification by diet, suggesting the existence of other intrinsic or extrinsic factors determining phage composition. For example, in mice, the gut virome does not revert to its initial composition following exposure to a diet rich in refined sugars, even if animals are subjected to a period of washout.[105] Different dietary regimens seem to impact the temperate fraction of phages in the gut,[106] possibly contributing to the observed variability in the phageome following a dietary change.[107] Similarly in mice, the fraction of prophages integrated in the genome of different gut bacterial taxa changed in a diet-specific manner.[63] Ingested fructose, possibly acting as a stress signal, could induce the activation of a prophage from the lysogenic gut commensal *Lactobacillus reuteri* in an AckA-dependent manner.[17] Such mechanisms could likely initiate a cascade reaction altering the microbiome composition, both due to further prophage excision following the spread of stress signals and potential phage-induced lysis of microbiome elements closely related to *L. reuteri*.[106] In support of this notion, phages were suggested to modulate microbiome diversity upon increasing consumption of refined foods.[108] Better mechanistic understanding of environmental and indigenous regulators of the human phageome may explain how environmental–phage interactions impact human traits in treatment and disease, while identifying altering phage repertoires as a means of impacting human disease.

PHAGES AND HOST IMMUNITY

Although it remains unclear if different types of phages (i.e., lytic, temperate or pseudolysogenic) directly affect the human host,[109] it has been repeatedly suggested that phages can indirectly modulate the immune system. Such an impact on a pathogenic or commensal bacterial host may alter their host infectivity and virulence. For instance, lysogeny could increase the fitness of bacteria through a mechanism called “lysogenic conversion”,[110] which occurs when integrated DNA from the phage modifies bacteria. Lysogenic conversion often translates into beneficial mutualism, either protecting bacteria from infection by other phages or increasing their virulence through gene transfer (see Table 1). Another interesting example includes “active lysogeny”, in which integration of phage DNA disrupts bacterial genes that increase fitness, thereby enhancing survival chances of both partners.[111] For example, excision of the integrated DNA of phage φ10403S from the *Listeria monocytogenes* genome during infection of mammalian cells leads to restoration of genes encoding for phagosome escape but not to virion production or bacterial lysis.[112] Strikingly, Pasechnek et al. showed that translation of late genes responsible for activation of the lytic cycle in phage

Table 1. Phage-encoded virulence factors transmitted to bacteria

Bacterium	Phage	Acquired gene, phenotype or protein	Bibliography
C. botulinum	Phage C1	*C1;* neurotoxin	204
Corynebacterium diphtheria	β-phage	*tox* gene; diphtheria toxin	205
E. coli K-12	9 cryptic prophages (CP4-6, DLP12, e14, rac, Qin, CP4-44, CPS-53, CPZ-55 and CP4-57)	*kilR and dicB;* resistance to quinolone and β-lactam antibiotics, improved survival to osmotic stress	206
E. coli O157:H7	ΦFC3208	*hly2; Enterohemolysin*	207
P. aeruginosa	ΦCTX	*ctx;* cytotoxin	208
S. aureus	Φ13	*entA, sak; Enterotoxin A, staphylokinase*	209
S. flexneri	Sf6	*oac*; O-antigen acetylase	210
S. pyogenes	T12	*speA, B and C*; pyrogenic exotoxins (SPE) A, B and C	211
S. Typhimurium LT2	Fels-1	*sodCII*; SodCII enzyme	212
Salmonella spp.	Gifsy-2	*SodC1*; periplasmic superoxide dismutase (SodCI)	213
Salmonella spp.	sopEϕ	*sopE;* type III effector	214
Shigella dysenteriae	Phage 7888	*stx;* Shiga toxin	215
V. cholerae O139	CTXΦ	*ctxAB;* cholera toxin (CT)	216

φ10403S are temperature-dependent and downregulated at 37 °C. Thus, when *L. monocytogenes* infects mammalian cells, the phage φ10403S lytic cycle is inhibited at a post-transcriptional level.[113] In addition, persistence of a pseudolysogenic phage may be transmitted unevenly to daughter cells, conferring a metabolic advantage upon genomic integration.[114] Interestingly, maintenance of the pesudolysogenic state in bacteria could lead to generation of phage mutants able to infect the newly generated phage-resistant bacteria, thereby generating larger colonies.[115] Collectively, both lysogenic and pseudolysogenic states could have an impact on host–bacterial virulence and fitness, ultimately bearing an indirect downstream influence on bacterial–mammalian host interactions and the associated immune responses.

In addition to indirect host influences mediated through phage-induced modification of bacterial behaviors, phages may also directly affect the mammalian host immune response.[116] This is important from a therapeutic perspective, as the host immune response has been suggested to limit phage therapeutic efficacy.[117] Some phages, such as phage 536_P1, directly promote an increase in the production of antiviral cytokines like interferon-γ (IFNγ) and interleukin-12, as well as chemokines, even in the absence of host bacteria.[118] This is not surprising, as phages share some features with viruses targeting mammalian cells and are thus recognized by innate host receptors such as members of the Toll-like receptor family. Indeed, evidence in humans suggests that phages of *Lactobacillus*, *Escherichia*, and *Bacteroides* may induce IFNγ via Toll-like receptor 9 (TLR) in the gut in a microbiota-independent manner,[119] which may trigger phage-specific as well as bacteria-specific immunity[119] (Fig. 2). This may be of potential relevance in human disease. For example, ulcerative colitis (UC) patients responding to fecal microbiota transplantation featured reduced levels of phages compared to nonresponders.[119] Phages from UC patients induced more IFNγ than phages from healthy controls, suggesting a potential immunomodulatory role of phages in chronic intestinal autoinflammation.[119] However, such innate immune activation is not observed with all phages. The T4 phage, for example, does not elicit an immune response, including production of reactive oxygen species, at least in the in vitro setting.[120] Another key element in the activation of host immunity by phage preparation is the potential contamination with endotoxins, especially LPS.[117] Endotoxins are potent inducers of immunity and could thus potentially influence the host immune response against the phage. Indeed, phage targeting of gram-negative bacteria leads to massive release of LPS, which in turn will likely increase host immunity toward the phage as well as bacterial clearance.

Conversely, the general inflammatory state of the host was also shown to directly control the number of phages. LPS-induced inflammation in mice reduces phage levels in vivo in several organs, including the spleen, liver, and kidney, through induction of phage-specific antibodies.[121] Interestingly, phages can exhibit some level of commensalism within the host, as high levels of phages were found in the intestinal mucus layer, as well as other mucosal barrier surfaces such as the gums.[122] Phages directly bind to mucin glycoproteins via their capsids. The presence of phages close to the intestinal epithelium likely serves as a defense mechanism of the mammalian host, in preventing bacterial breach of the intestinal barrier, and may constitute an exciting avenue of future research. Conversely, phages can promote loss of intestinal barrier integrity[123] through elusive mechanisms that may involve phage-induced alterations in the composition of the intestinal microbiome.

Collectively, these observations suggest that, similar to bacteria, phages could display commensal versus pathogenic behaviors. Indeed, in sponges, phage-dependent expression of Ankyrin proteins in bacteria can promote host-symbiont coexistence by suppressing eukaryote inflammatory mechanisms including phagocytosis and inflammatory cytokine secretion by macrophages.[124] Conversely, phages may impact bacterial pathogens and their interactions with the host immune system during infection. In respiratory infection with *Pseudomonas aeruginosa*, the presence of specific phages strongly increases their in vivo infectivity, leading to higher morbidity and mortality.[125] This effect is dependent on phage interaction with the host immune system through the activation of TLR3 and subsequent induction of type 1 interferons (Fig. 2). Furthermore, phages may promote inhibition of tumor necrosis factor and phagocytosis.[125] Indeed, *Pseudomonas* phage predation was found to make strains more virulent and less prone to macrophage phagocytosis.[126]

In contrast to the disease-exacerbating roles of some phages mediated through their effects on bacterial pathogens, other phages were found to induce opposite effects. The filamentous phage of *P. aeruginosa* prevents *Pseudomonas*-induced lung injury, mortality, and inflammation, an activity associated with lower cytokine levels and enhanced bacterial adhesion to mucin coupled with reduced invasion of epithelial cells.[127] The phage was also shown to promote M2 macrophage polarization, as well as reduced bacterial phagocytosis[127] (Fig. 2). The underlying molecular mechanisms for these intriguing impacts remain elusive but are

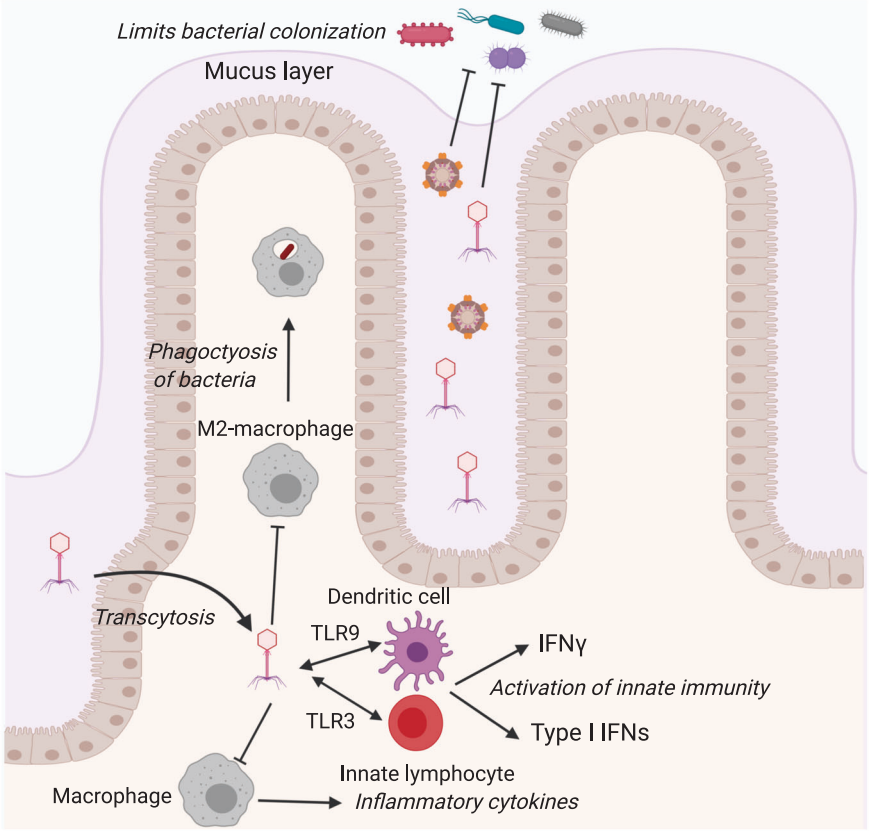

Fig. 2 Phage–immune interactions in the gut. Phages interact with the host immune system in various ways. Depicted are some examples of such interactions occurring in the mammalian gut, including phage-induced stimulation of macrophages to inhibit phagocytosis and production of inflammatory cytokines, activation of dendritic cells and ILCs to produce IFNs, and promoting direct protection against bacterial colonization

speculated to involve immune system modulation. For example, phage therapy against multi-drug-resistant *P. aeruginosa* depends on myeloid differentiation primary response 88 (Myd88), a critical signaling molecule downstream of pattern recognition receptors of the TLR family.[128] Indeed, while neutrophils are also strictly required for efficient phage-mediated therapy of *P. aeruginosa* infection, lymphocytes, including innate lymphoid cells, are immune to their effects[128]. These findings suggest that the adaptive immune system may not play a major role in the antipathogenic effects of phages, at least in some contexts. With increased interest in using phages as a therapeutic for antibiotic-resistant bacterial infection, understanding the molecular basis of phage interactions with the immune system will become crucial for their successful in vivo implementation as an antibacterial treatment.

GUT PHAGEOME IN INTESTINAL STEADY STATE

The healthy gut microbiome features a core community of bacterial phyla that are consistently present in over 90% of healthy individuals.[129] In addition, several studies have attempted to describe the composition of a "healthy gut phageome" (HGP).[53,54,65] Manrique et al. used ultra-deep sequencing to study viral taxa identified in geographically distant individuals.[59] Network analysis of the sequenced viral contigs identified three biological groups based on three fractions: a "core", consisting of 9 phages shared by over 50% of healthy people, a "common" set harbored by 20–50% of individuals, and a rare set of unique or uncommonly shared phages among subjects, containing all remaining phages. The core group, for instance, included the widely spread crAssphage, defined as a benign cosmopolitan element of the gut phageome, which is correlated with different bacterial configurations and dietary habits but not with a particular disease state.[104,130] The authors defined the combination of the core and the common fraction as the HGP, which is commonly present in the intestine of healthy adults, and strikingly, found it to be decreased in individuals with Crohn's disease (CD) and UC.[59]

The bilateral influences of the gut phageome and bacterial microbiome on each other have been challenging to address, as over 80% of temperate phages found in the gut are absent in three commonly used databases.[63] Identifiable phages integrate more frequently in the genome of *Proteobacteria* and *Firmicutes*, rather than *Bacteroidetes* and *Actinobacteria*,[51,52] despite the high abundance of *Bacteroidetes* in the human gut. Whether this stems from an increased abundance of phages targeting these groups, or whether phages specifically targeting *Bacteroidetes* are less well-annotated, remains unclear. Among phage–microbiome interactions, the easiest to identify are the ones that contribute to bacterial virulence.[124] Ankyphages, recently described in sponges but abundant also in humans, promote bacterial protection from macrophage-driven phagocytosis. Recently, contigs encoding similar prophage-derived proteins were also found to be expressed in oral and gut virome metadata.[124] One major challenge in studying the possible effects of the gut phageome on bacterial microbiome configuration stems from the lack of a direct causal relationship between phages and their commensal bacterial hosts. As such, some of the observed dynamics in phage abundances may likely reflect the abundance of their bacterial host, as was recently observed in IBD patients.[65] Moreover, available methods and databases are currently insufficiently equipped to recognize phages targeting some phyla, as is the case for crAssphage.

To address these limitations, researchers modeled the evolution of the lytic and temperate phages in the healthy gut using ecological modeling. For example, in the "kill-the-winner" model, present in several aquatic environments,[131] phages kill the most abundant bacterial hosts, leading to a massive decrease in biomass and replacement by novel bacteria, which will in turn be targeted by specific phages. The resultant reduction in each targeted bacterial population also decreases their respective phage population size, leading to a dynamic cycle.[132] Intriguingly, the "kill-the-winner" model may explain the gut viral kinetics noted in the healthy infant gut, in which a high diversity of phages, or predators, at birth, diminishes because of the low bacterial colonization, followed by an expansion of gut bacteria. This is consequently followed by an enrichment of phage species.[66] Conversely, several studies observed mostly a temperate regime in adult individuals, in which phages are integrated within bacterial chromosomes as prophages, rather than clear predator-prey dynamics.[54,56] Therefore in adults, the phageome seems to fit a stable long-term configuration[54,56] rather than the rapid compositional changes characteristic in the "kill-the-winner" model. However, to date, no longitudinal cohorts have fully assessed the kinetics and 'rules of engagement' by which this diverse phage composition stabilizes into its adult configuration. In summary, changes in the gut microbiome and phageome are tightly linked, and dissecting cause and effect in this relationship is challenging but may lead to a more holistic understanding of the forces shaping the bacterial microbiome.

GUT PHAGEOME IN INFLAMMATORY BOWEL DISEASE

While numerous studies have associated the bacterial microbiome with human disease, including arteriosclerosis,[133] inflammation,[95] amyotrophic lateral sclerosis,[134] nonalcoholic fatty liver disease[135], and other diseases,[136] the drivers of dysbiosis in these conditions remain largely elusive. Phages could represent one such putative dysbiosis-promoting mechanism. Indeed, severe inflammatory states of the gut such as CD and UC have been associated with dysbiosis characterized by a disproportionate outgrowth of *Proteobacteria* and *Bacteroides* phyla over *Firmicutes*.[137] Importantly, abnormal composition of the enteric virome has also been observed in inflammatory bowel disease (IBD). IBD patients from three different geographically distant cohorts showed an increase in *Caudovirales* abundance and species richness and a simultaneous diminishing of *Microviridae* upon comparison to healthy household controls. These changes were inversely correlated with bacterial diversity in these subjects, while a disease-specific phage pattern could be identified.[65] A longitudinal study in a colitis mouse model revealed phage patterns similar to those in humans with IBD, characterized by increased numbers of *Caudovirales* and of phages targeting *Streptococcus* spp. and *Alistipes* spp.[67]

These interesting observations notwithstanding, many alterations in the gut virome in IBD patients do not seem to provide a direct explanation for the changes observed in bacterial communities. For example, the unexpected increase in phages targeting a *Bacteroidetes* member such as *Alistipes* spp. seems contradictory to the decline of *Bacteroidetes* observed during IBD. Other alternative mechanisms may involve release of inflammatory products by the human host, enhancing excision of integrated prophages from bacterial chromosomes, thereby impacting the virome composition. Alternatively, a local lack of nutrients during active inflammation constitutes a known trigger of bacterial stress that could initiate lysogeny excision. As such, an IBD-associated, dysbiotic microbiome consisting of stressed bacteria competing for scarce nutrients would be the ideal environment to trigger the onset of an IBD-associated phage bloom.[67] Further associations between phages and their host could be niche-specific. For example, while *Caudovirales* increased in abundance in the gut lumen of IBD patients,[65,67] species diversity was decreased in the inflamed mucosa compared to biopsies from healthy gut segments of the same individuals or healthy controls.[138] A functional analysis of the mucosa-associated virome in these patients revealed how genes associated with viral fitness (e.g., DNA binding and integrase) were abolished in favor of genes expressing bacterial pathogenicity or antibiotic resistance, highlighting how the mucosal virome could potentially contribute to IBD pathogenesis.[138] After identifying novel prophages in the genome of *Faecalibacterium prausnitzii*, Cornuault et al. hypothesized that these phages could be intestinally induced by IBD-related inflammation, diminishing the presence of the beneficial bacterium and contributing to a pro-inflammatory state. Indeed, they observed a significant intestinal enrichment of *F. prausnitzii*-targeting phages in IBD patients when compared to healthy controls. However, these results were not corroborated in a colitis mouse model.[139]

Overall, a healthy gut phage community is distinct from that of the IBD-associated phage pattern, but the nature of putative phage–bacteria interactions, regulation by the host, and impact on downstream disease pathogenicity merit further study.

MODELING HOST–PHAGE INTERACTIONS IN ANIMAL MODELS

Modeling host–bacterial phage interactions in small animal models may contribute to better understanding of phage impact on the bacterial microbiome and human clinical traits. For example, a striking similarity was recently reported between healthy and IBD-affected human virome composition and that of an established murine model of colitis,[67] suggesting that putative causal commonalities may exist between mammalian species, with respect to phage shifts in inflammatory conditions.

Gnotobiotic mice constitute a key tool in generating mechanistic insights in the microbiome, since transferring microbial communities from disease-affected mice or humans into gnotobiotic mice enables researchers to establish causality between a clinical state and specific bacterial or viral configurations. An elegant study investigated the impact of a pool of human phages on a consortium of 15 bacterial commensals in the gut of adult germ-free (GF) mice.[100] Interestingly, some unknown phages emerged and persisted in the murine gut after 7 days, without a decrease in any related bacterial species, suggesting that they have no apparent bacterial hosts. In contrast, an in vitro model of phage interactions with the same consortium did not show a parallel expansion of any of the unknown phages, highlighting the importance of factors related to the mammalian hosts in impacting these interactions. In addition, some of the phages administered to mice were isolated from stool after only 1 week. This could potentially represent a case of pseudolysogeny, in which a phage is steadily present within a prokaryotic host without undergoing duplication (Fig. 1a).[100] Using a similar approach in GF mice, Hsu et al. observed that phage infection of a known bacterial consortium also impacted other nonbacterial members of the microbiome via indirect effects.[18] Moreover, phages strongly impact the gut metabolite repertoire, either those associated with a single bacterial species (e.g., the neurotransmitters tryptamine and tyramine) or those more broadly associated with microbiome members (such as AAs or bile salts), suggesting that the phageome could be used as an indirect tool to manipulate the host metabolome via microbiome targeting.[18,21] Taken together, these results indicate that utilizing GF mice as a microbiome-free host is a useful tool in decoding the complexity of phage–microbiome interactions and elucidating causal links in vivo. Furthermore, these results highlight the need to study phage–bacteria interactions both in vivo and in vitro, thereby enabling the study of phage-induced indirect effects on commensals and the host.

PHAGE THERAPY

In the 1930s, a full review of all existing literature on phage therapy, requested by the Council on Pharmacy and Chemistry of the American Medical Association, reported insufficient data to recommend phages as a treatment method.[140] During this era, despite the use of phage therapy as treatment for numerous infectious diseases (e.g., typhoid fever, cholera, osteomyelitis, urinary infection, gonorrhea, acne), unambiguous reports demonstrating the clinical efficacy of phage therapy were lacking. The few studies on which these interventions were based lacked preclinical results in animal models and adequate control groups in human studies. In addition, phage preparation methods, administration routes, and dosage varied between groups, while phage formulations, derived from crude bacterial extracts, contained large amounts of endotoxins and other supplements such as mercury, which could have inactivated the phage.[20] Moreover, many of the phage treatment indications at the time focused on self-limited diseases, while phage therapy in life-threatening disease was used as a last-resort compassionate attempt and often did not include adequate placebo controls. Furthermore, limited knowledge was available at the time about serotype identification of phage strains, host range, phage resistance development in bacteria and lysogeny, making it impossible to trace the nature and identity of phage strains utilized by different research groups and clearly establish why some resulted in more effective therapy than others.[141] Consequently, several authors at the time concluded that persuasive therapeutic effects could be observed only in treating localized staphylococcic infections and cystitis.[21] The development of a wide repertoire of antibiotic drugs such as penicillin in the mid-20th century led most clinicians and scientists to dismiss phage therapy as inferior and no longer suitable for use in a clinical setting.

However, major developments in molecular microbiology and genomics now make obtaining highly pure phage preparations possible, including selection of strictly lytic phages or even engineering them by deleting genes for the lysogenic cycle. This broadens their host range or prevents horizontal transfer of pathogenic and antibiotic resistance genes.[20] In the last two decades, renewed interest in clinical phage utilization against antibiotic-resistant infection resulted in numerous clinical applications of phages featuring successful outcomes against pathogenic infections such as *P. aeruginosa*,[142] *Acinetobacter baumanii*,[143] and *Vibrio parahaemolyticus*[144] in mice, *Clostridium difficile* in hamster,[145] and *Staphylococcus aureus* in rabbits[146] (Table 2). Intraperitoneal administration of a single phage strain successfully treated infections caused by B-lactamase producing *Escherichia coli*,[147] vancomycin-resistant *Enterococcus faecium*,[148] and imipenem-resistant *P. aeruginosa*[149] in murine models. However, many challenges still limit the systemic use of phages as pharmaceuticals, including unknown pharmacokinetic and pharmacodynamic properties.[150] Variables affecting phage pharmacokinetics include adsorption (e.g., influx into the blood stream), distribution, (e.g., influx from blood to tissues) and phage clearance by excretion and disintegration. While phage elimination mainly occurs in the liver and spleen and is mediated by serum complement or mononuclear phagocytes, long-circulating phages can be selected by repeated in vivo passages[151] or through decoy peptide expression rendering them less immunogenic.[152] Intravenous injection is the most effective route of administration of phages, and active phages have been observed in the blood stream within minutes of administration.[153]

Many more variables complicate oral phage delivery. An efficient phage encounter with its target bacterium in the gut remains a daunting challenge, potentially limited by factors such as gastric acids (phages are sensitive to low pH), intestinal mucus protection and bacterial biofilm barriers. Some observations suggest that, once phages overcome the gastric barrier, they are mainly absorbed by gut lymph nodes.[154] In a cohort of 120 Bangladeshi children affected by acute diarrhea, no beneficial clinical effects were observed upon oral consumption of T4-like coliphages for 4 days compared to controls. Importantly, fecal phage titers were not higher in patients infected with the bacterial target, suggesting that the phage passively transited along the gastrointestinal tract without ever meeting its target.[155] No detrimental secondary effects were observed in this study, as already previously described, with healthy volunteers receiving phages for 3 weeks in drinking water.[156] Interestingly, several reports noted successful intranasal delivery of phages into the lungs of mice[157,158] and via intraperitoneal injection into the brain.[159,160]

A potential advantage of phage therapy is its self-replicating nature, coupled with a narrow host specificity,[161] thereby limiting off-target effects, preserving microbiome integrity and eubiosis.[20] However, narrow host specificity could also be considered a limitation, as it remains challenging to target all pathogenic strains of a given species by a single phage type. Recently, the use of cocktails of several phages, targeting different host receptors, proved to be an effective therapeutic strategy in increasing host range of phage therapy, while also delaying or preventing the onset of phage resistance.[156,162,163] Alternatively, incorporation of receptor-binding proteins to broaden phage host specificity may also achieve the same task.[164–166] Once they reach the targeted bacterial host, the ability of phages to actively replicate depends on both the ability of the phage to reach a sufficient burst size (i.e., number of new virions produced per infected bacterium) and on the bacterium supporting phage population growth in adequate numbers. Moreover, availability of bacterial surface receptors required for phage infection might vary in in vivo settings in a context-dependent manner and result in the emergence of bacterial phage resistance, as discussed below.

A less specific treatment approach that may involve phages is fecal microbiome transplantation (FMT), mainly reported in *C. difficile* infection (CDI) in humans[167,168] but it has also been increasingly tested in other clinical scenarios with a variable success rate.[169–172] Interestingly, patients affected by CDI display a predominance of *Caudovirales*. After FMT treatment, responders feature a reduced abundance of *Caudovirales*, coupled with an increase in species richness, and a shift in phage composition toward that of the donors.[173–175] In comparison, a pure gut viral transfer (i.e., viruses purified from the bacterial fraction) from mice fed a high-fat diet (HFD) to recipients on HFD or on standard diet was sufficient to alter microbiome composition in recipient mice.[176] Very recently, a high-throughput method called VT was developed to enable the pursuit of novel bacterium–phage interactions and was used to design a rational FMT preparation, in which the donated sample consisted of an in vitro mix of virome and bacterial fractions isolated from stools of different individuals. Of note, a high cross-infectivity, in which phages displayed a very broad range against several bacterial species, was observed, suggesting a potential risk of a nonspecific perturbation of the microbiome following FMT.[177] Another limitation of this interesting methodology relates to its nonspecific nucleic acid staining, which does not allow for discrimination of dsDNA phages from other gut viruses.[178]

The most advanced experimental clinical application of phage therapy involves targeting of multi-drug resistant (MDR) bacteria. With the recent increase in MDR pathogen-mediated infection, the interest and need of the scientific and medical communities to find new ways to manipulate human bacterial ecosystems in this setting are rapidly increasing.[179] Phages and antibiotics share no antimicrobial molecular mechanisms. Thus, cross-resistance is unlikely to occur between these two strategies, while synergistic use of antibiotic-phage therapy, already extensively validated in vitro and in vivo, may constitute an attractive strategy to prevent the development of phage-resistant clones[180–182] (see

Table 2. Overview experimental and clinical studies of phage therapy

Bacterium	Disease	Administration route	Comments	Model	Bibliography
E. coli	Peritonitis	i.p.	Engineered phagemids expressing antimicrobial peptides (AMP) designed to kill bacteria by a nonlytic process to avoid LPS release.	Mouse	[217]
E. coli	Diarrhea	oral gavage	Phage treatment shown to be safe; neither adverse events nor antiphage antibodies were observed in patients.	Humans	[156]
E. coli	Diarrhea	oral gavage	In a phase II randomized and placebo-controlled trial on children, the phage failed to replicate in the gut, with no improvement noted in diarrhea severity.	Humans	[218]
E. coli K1	*E. coli*-induced septicemia	i.v.	The lytic bacteriophage ΦIK1 has successfully rescued mice from lethal *E. coli* K1 infection upon administration within 60 min of infection.	Mouse	[219]
E. faecium (VRE)	Burn wound infection	i.p.[a]	Phage co-injection with the infectious agent rescued 100% of animals from fatal infection. If administered within 45 min of infection, 50% of mice recovered.	Mouse	[148]
Imipimem-resistant *P. aeruginosa*	Bacteremia	i.p.[a]	Phage ØA392 saved 100% of animals if administered within 180 min after infection; after this time, the recovery rate dropped to 50%.	Mouse	[149]
K. pneumoniae	Burn wound infection	i.p.[a]	No therapeutic effect was observed after multiple phage treatments, with multiplicity of infection (MOI) ranging from 0.001 to 900.	Mouse	[220]
K. pneumoniae	Lobar pneumonia	i.p.[a], intranasal	Preventive intranasal phage administration rescued mice from death, but administration after 6 h was not effective against infection.	Mouse	[153]
MDR *A. baumannii*	Wound infection	i.p., topical	A cocktail of four phages lowered the burden of infection by selecting for an unencapsulated, avirulent strain variant of the pathogen.	Mouse	[182]
MRSA *S. aureus*	Lethal lung-derived septicemia	i.p.[a]	Injection of phages 6 h after infection increased survival of infected mice from 10 to 67%.	Mouse	[221]
MRSA *S. aureus*	Chronic osteomyelitis	i.p.	Efficacy of phage therapy was noted when animals were treated either 4 weeks or 6 weeks after infection.	Rabbit	[222]
P. aeruginosa	Burn wound infection	i.m., s.c., i.p.	Mortality was decreased in animals intraperitoneally injected with phages, with an 87% survival rate noted.	Mouse	[223]
P. aeruginosa	Endocarditis	infusion into the superior vena cava	Phages were synergistically administered with ciprofloxacin, achieving a high therapeutic effect. Bacterial mutants resistant to the phage developed but displayed defective virulent phenotype.	Mouse	[181]
P. aeruginosa	Otitis	ear drops[a]	A group of 24 patients suffering of chronic otitis was successfully treated by phage therapy, with reporting no adverse effects.	Humans	[224]
P. aeruginosa	*P. aeruginosa* infection in cystic fibrosis (CF) model	injection into duct of Cuvie	Phage therapy decreased lethality and improved inflammatory status.	Zebrafish	[225]
Shigella sonnei	Diarrhea	oral gavage	A phage cocktail was compared to ampicillin treatment, resulting in equally effective results, with no impact on gut microbiome composition noted.	Mouse	[226]

i.v. intravenous, *i.m.* intramuscular, *i.p.* intraperitoneal
[a]a single dose could completely eradicate the infection

Table 2). Indeed, phage treatment was effective against MDR *Klebsiella pneumoniae* biofilms,[183] and it augmented the effect of antibiotics, which were ineffective when used alone.[184] In 2017, a 68-year old male patient underwent phage therapy against an MDR *A. baumanii*-infected pancreatic pseudocyst due to necrotizing pancreatitis and associated multi-organ failure and featured a significant improvement. Interestingly, following treatment, a phage-resistant clone of *A. baumanii* was isolated, displaying a defective capsule configuration, which was more accessible to penetration by antibiotics.[185] Of note in this case report, no control was included to confirm that the recovery was due to phage administration and not to other confounding factors. Other cases of compassionate intravenous phage therapy against human MDR infections have also been documented,[185,186] including treatment for *Mycobacterium abscessus* infection in a 15-year-old CF patient intravenously administered a cocktail of lytic, genetically engineered phages to remove immunity repressor genes or augment infectivity. The patient showed substantial clinical improvement, with no adverse effects noted.[187]

Despite these promising preliminary reports, phage therapy has to undergo all phases of a clinical trial to ensure efficacy, safety, and absence of adverse effects of the novel modality.[188] In 2016, the European Medicines Agency confirmed that none of the existing regulations suit the peculiar case of phage therapy and that a new regulatory framework toward phage therapy testing should be adapted.[189] The exponential biological diversity of the ecosystem comprising bacteria, phages, and the human host would require a new clinical trial for every new candidate phage of a given phage cocktail, which would lead to an unrealistic and immense number of clinical trials needed for any contemplated phage treatment.[190] Because of the prohibitive costs required to accomplish such standards, phage therapy enterprise has been limited so far only to academia and small biopharmaceutical companies.[188] Given these issues, and the growing need and interest to move phage therapies into the clinic, a dialog is underway between scientists, clinicians, biopharma entities, and regulatory authorities to devise stringent yet viable regulatory pathways ensuring patient safety while not implementing insurmountable obstacles on phage treatment development.

Additional challenges in phage treatment development include the need to develop effective protocols enabling optimization of phage efficacy. Considerations include optimization of phage dosage (due to their self-replicating nature) and mode of application, which should ensure contact with the bacterium to allow replication while not leading to excessive dilution of the phage. Even assessment of the host range mandates standardized protocols using efficacy of plating, which is defined as the number of plaques growing on the target bacterial cultures over the number of plaques growing on the natural bacterial cultures.[191] Standardized criteria should be established to assess phage efficacy, define a minimum bactericidal level, and determine bacteriostatic or bactericidal properties over time against reference pathogens, similar to parallel approaches utilized in the development of novel antimicrobial molecules.[188]

To ensure safety, phages or phage-derived molecules should be produced according to good manufacturing practices (cGMP), obtaining ultrapure preparations that are LPS-free and compatible with physiological solutions to fulfill regulatory requirements.[192] Safety, defined as the lack of adverse effects on healthy volunteers, should be assessed in the initial phase of a clinical trial. Although it is known that the innate and adaptive immune response can be stimulated in a phage-dependent manner as discussed above,[119,193,194] numerous studies report phages to be safe, especially when orally administered.[156,163] Further safety issues could arise from immune reactions to in situ released LPS or other PAMPs, generated from bacterial lysis, which could be addressed by the use of less virulent phages or by co-administration of antibiotics.

Collectively, phage cocktail development as a means of targeting pathogenic or commensal bacteria holds great promise for introducing a new class of patient-tailored precision medicine, but is limited by a number of technical and regulatory challenges. Optimistically, adaptive regulatory paths have been already proposed to address these challenges, allowing the development of in vivo precharacterized phage libraries, enabling mixing and matching of phage cocktails in targeting distinct pathogenic infections[188] (Fig. 3).

PHAGES AS IMMUNOTHERAPY

Another emerging modality of phage therapy involves the exploitation of the immunomodulatory properties of phages to elicit a host immune response against a bacterial pathogen. While binding of antibodies to phages has been shown to limit phage therapy efficacy,[195] immunogenic properties of phages have been exploited to induce potent immune responses. Examples include the use of bacterial strains exposed to phages, leading to their reduced fitness and growth, thereby enabling their exploitation as part of a vaccination strategy.[196–198] Even structural components of phages have been used as vaccines. An example is the use of the phage protein bacterial cell wall lysin of a phage commonly targeting methicillin-resistant *S. aureus* (MRSA), which elicits a potent immune response and subsequently provides protection from MRSA infection.[199] Similarly, a phage-derived peptide has been shown to treat *Mycobacterium tuberculosis* infection by promoting antibacterial immunity.[200] Indeed, phages feature intrinsic immunogenic properties that can be exploited in various contexts, including VLP-mediated vaccination strategies, in which the highly repetitive nature of the page antigen arrangement favors generation of potent antibody responses.[201] Such approaches have even been used to prevent fungal infection, in which recombinant phages expressing fungal antigens provided potent protection.[202] Similarly, a vaccination approach targeting a phage of *P. aeruginosa* was shown to protect against bacterial infection by generating antiphage antibodies which, in turn, promoted opsonization and bacterial uptake.[125] Moreover, specific immunological mechanisms, such as antigen presentation of specific proteins to dendritic cells through phage infection, has been used as a tool to induce potent T-cell immunity.[203] These effects were also dependent on innate pattern recognition receptors such as TLR9, highlighting the role of phages not only as activators of antibody production by B cells but also as key regulators of the mononuclear phagocyte compartment. Overall, these findings highlight the broad potential use of phages or their components for immunomodulation, underlining the importance of phages in contexts outside the classical realm of phage therapy. One may speculate that these unique phage immunomodulatory features may enable future development of the immunotherapies against cancer and other noninfectious diseases.

CONCLUDING REMARKS

The current popularization of research exploring phage–bacterial–host interactions and translating the resulting findings into new forms of human-relevant phage therapy is encouraging but also poses major challenges. One such challenge involves a more comprehensive characterization of phage taxonomy within the 'dark matter' presented in most next-generation sequencing datasets. This will be crucial in providing a holistic description of the phageome, which will, in turn, enable a better understanding of factors, which are orchestrating phage composition under different physiological contexts. With the expected maturation of phage research, an effort should be made to move from descriptive studies to those pursuing deeper mechanistic insights of phage biology and impact on complex ecologic communities such as the human microbiome.

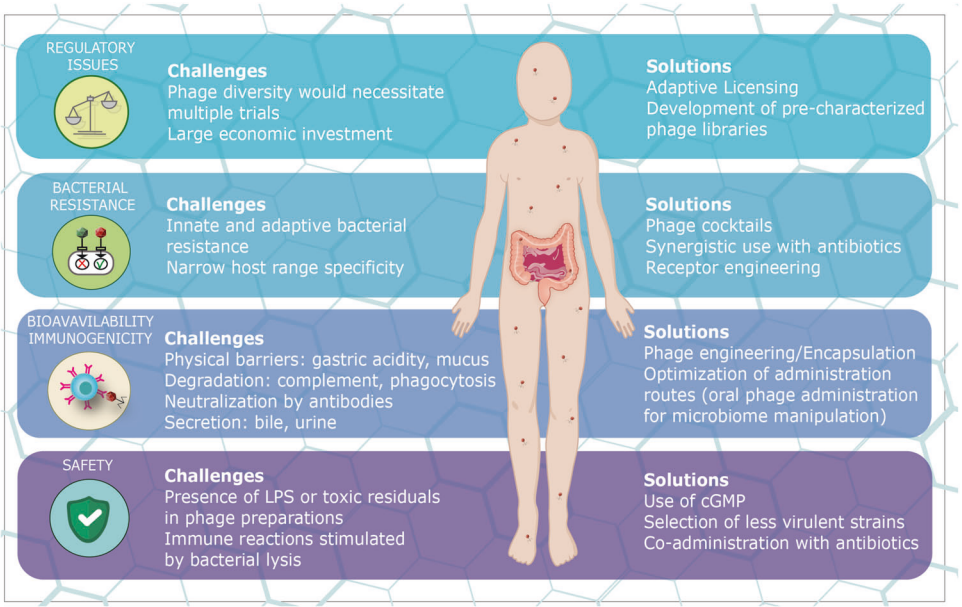

Fig. 3 Challenges and solutions toward clinical application of phage therapy. Depicted are several of the major challenges and proposed solutions for the application of phage therapy. These include regulatory obstacles limiting phage cocktail application that may be addressed by the development of adaptive regulatory protocols; bacterial resistance can be addressed by a rational design of phage cocktails, co-administration with antibiotics, and synthetic alteration of phage structure; challenges related to bioavailability and immunogenicity could be addressed by engineering phage outer structures or phage encapsulation, (e.g., via PEGylation) making them less immunogenic, or through the development of oral routes of administration for gut microbiome modulation; safety challenges may be addressed by following cGMP regulations, pre-selecting phage strains and their co-administration with other antimicrobials

Combinations of modeling, whole-community approaches, and experimental work will be necessary in delineating whether compositional and functional alterations of the microbiome are directly, or indirectly, driven by phageome changes. The driving forces shaping phage modulation of the immune response and, conversely, immune reactivity toward phages merit further mechanistic studies. Understanding this relationship will be crucial for the design of phage therapy, while avoiding immune-mediated inhibition of phage antibacterial activity and harnessing phage-initiated immune responses toward potentiation of their antimicrobial effects. Phage–immune interactions are also likely to affect the microbiome, and how this tripartite relationship is controlled will be key in understanding the complexity of the ecosystem in health and disease.

Conversely, decoding how the phageome is influenced by disease, how it may influence interindividual microbiome differences, and how it may modulate host immune responses will constitute important areas of future research.

With respect to phage therapy, successful application will probably be different from that at the beginning of the twentieth century. It may involve personalization of phage cocktails to the individual and his/her microbiome configurations. It may require genetic engineering of phage structures to ensure safety or the use of phages in combination with other treatments or as an adjuvant for antibiotics, with the goal of maximizing efficacy. Such combinations may better overcome the rising incidence of multi-drug-resistant infections. Importantly, with the advent of data-driven phage therapy against discrete commensals in the microbiome, noncommunicable diseases impacted by dysbiosis, such as metabolic, inflammatory, neoplastic, and neurodegenerative disorders, may also benefit from phage-directed pathobiont elimination therapy. Key limitations include the need to minimize indirect nonspecific immune effects mediated by phages, while specifically targeting disease-associated commensals. This may represent a formidable task, especially when the targeted commensals are rare. In addition, exploiting different routes of phage administration may profoundly affect phage effector function and the resultant activation status of the host immune system. Exploitation of the complex relationship between phages, bacteria, and the host holds promise for future development of immunotherapies against infection and other microbiome-associated diseases.

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AUTHOR CONTRIBUTIONS

All authors performed an extensive literature research, contributed substantially to discussion of the content, and wrote and edited the manuscript.

ADDITIONAL INFORMATION

Competing interests: E.E. is a salaried scientific consultant for DayTwo and BiomX. S.F. and S.P.N. have nothing to declare.

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