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A bacteriophages journey through the human body

Jeremy J. Barr

School of Biological Sciences, Monash University, Melbourne, VIC, Australia

Correspondence
Jeremy J. Barr, School of Biological Sciences, Monash University, Melbourne, VIC, Australia.
Email: jeremybarr85@gmail.com

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Summary

The human body is colonized by a diverse collective of microorganisms, including bacteria, fungi, protozoa and viruses. The smallest entity of this microbial conglomerate are the bacterial viruses. Bacteriophages, or phages for short, exert significant selective pressure on their bacterial hosts, undoubtedly influencing the human microbiome and its impact on our health and well-being. Phages colonize all niches of the body, including the skin, oral cavity, lungs, gut, and urinary tract. As such our bodies are frequently and continuously exposed to diverse collections of phages. Despite the prevalence of phages throughout our bodies, the extent of their interactions with human cells, organs, and immune system is still largely unknown. Phages physically interact with our mucosal surfaces, are capable of bypassing epithelial cell layers, disseminate throughout the body and may manipulate our immune system. Here, I establish the novel concept of an "intra-body phageome," which encompasses the collection of phages residing within the classically "sterile" regions of the body. This review will take a phage-centric view of the microbiota, human body, and immune system with the ultimate goal of inspiring a greater appreciation for both the indirect and direct interactions between bacteriophages and their mammalian hosts.

KEYWORDS
immune system, microbiome, mucus barrier, tolerance, virus

1 | INTRODUCTION

Viruses are the most abundant and diverse entities on the planet.[1] They are capable of infecting organisms across the tree of life and are found within all biospheres.[2] Bacterial viruses, also known as bacteriophage, are by far the most numerous virus type, encoding the majority of global genetic diversity and biological "dark matter".[3,4] Bacteriophages are intrinsic components of our microbiomes, which consist of diverse communities of microorganisms, including bacteria, fungi, and viruses, that occupy habitats such as the gut, skin, lung, and urinary tract.[5]

The human body contains an estimated thirty trillion microbial cells,[6] with the large intestine harboring the most densely populated microbial ecosystem with 10^{13}-10^{14} estimated microbial cells per gram of fecal matter.[7] It is well established that our gut microbial flora is largely responsible for our overall health. Gut microbes have coevolved symbiotic relationships with our bodies, imparting specific functions associated with; nutrient metabolism, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and protection against pathogens.[7–9] In conditions of dysbiosis, the composition and function of the microbiota is altered, which can result in pathophysiological processes such as inflammation and immune activation. These dysbiotic gut conditions have been associated with chronic inflammatory bowel disorders (IBDs)[10] and there is a growing interest in the characterization of these microbial signatures with the hopes of using dietary and microbial strategies to mitigate their effects.[11–13] Yet, the microbial pathogenesis of the gut is a complex affair oft associated with multiple pathogens and treatment strategies, which are further confounded by subsets non-responding individuals.[11,14,15] In many cases, the etiology of gut diseases and disorders remain unclear.

Bacteriophages, or phages for short, are ubiquitous in the gut.[16–18] Phages exert significant selective pressure on their bacterial hosts and

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undoubtedly influence the human microbiome and its impact on our health. Our bodies are frequently and continuously exposed to diverse communities of phages.[19–22] Despite the prevalence of phages throughout our bodies, the extent of their interactions with human cells and organs is still largely unknown. The focus of this review are the bacteriophage populations that reside on, within, and throughout the human body and their interactions with both the microbiota and the cells, tissues and immune systems of the body. Here, we will take a phage-centric view of these interactions. First, we review the bacteriophage communities that populate our body, tracking their movements through the gastrointestinal tract and detailing phage adherence mechanisms to the mucosal linings of the gut. Next, we describe the processes by which gut phages might access epithelial cells and eventually the body. Finally, we discuss the novel concept of the "intra-body phageome" and its potential involvement with the immune systems of the body. In many cases, the number of studies investigating and discussing these phage-eukaryotic interactions is limited and as such I extrapolate findings and associations from previous microbiome studies where phages were not the primary focus. Nevertheless, the enormous potential for phages to directly affect the microbiome, body, health, and immunity represents a relatively untouched research area that urges to be explored.

2 | BACTERIOPHAGE POPULATIONS WITHIN THE BODY

From birth, the human body is constantly exposed to and colonized by diverse bacteriophage populations. Upon delivery, the neonate is immediately exposed to a diverse spectrum of phages, bacteria and fungi from a variety of sources, including the vaginal flora of the mother, as well as other body fluids and environmental communities.[23–25] From here, phages colonize all body niches, including the skin,[26,27] oral cavity,[28,29] lungs,[30,31] gut [17,18], and urinary tract.[32] Of all the microbial communities within the body, the intestinal community is by far the most complex and dense, harboring an estimated ten trillion bacteria[6,33] and an estimated two trillion phages.[6,34–36]

Phage replication within these communities proceeds through numerous lifecycles involving their bacterial hosts.[37,38] The lytic lifecycle begins with adsorption of a phage to a susceptible bacterial host, followed by; injection of phage genetic material into the host cell, repurposing of the bacterial host as a virocell for the production of phages particles, and finally cellular lysis and release of newly infective phages into the surrounding environment. Alternatively, some phages can postpone virion production by integrating within a host bacterium's genome as a prophage, in a lifecycle referred to as a temperate or lysogenic. Although not actively replicating, the prophage is propagated and spread to daughter cells every time its bacterial host replicates. Prophages can favor the survival and growth of their host bacterial cells through diverse mechanisms, including; protection against infection by closely related lytic phages via superinfection immunity, increased host fitness through lysogenic conversion or transduction of genes, and removal of competing bacteria through self-induction and the "weaponization" of temperate phages.[39–42] The proportion of phages existing within the lytic vs temperate lifecycles has important ecological, biochemical, and pathological implications within the body.

Within the human gut, temperate appears to be the preferred phage lifecycle.[17,43] This is in contrast to aquatic ecosystems where the lytic phage lifecycles dominate, with phages following classical Kill-the-winner dynamics.[44,45] The established model in viral ecology predicts that these lytic dynamics dominate at conditions of high host density, whereas lysogeny is favored at low host densities.[46] In this light, the predominance for lysogeny within the gut is surprising, when considering that high bacterial density should favor lytic viral dynamics. Recently, Knowles et al.[47] proposed an alternative explanation whereby lysogeny became increasingly important in ecosystems with high microbial densities, as viruses exploit their hosts through lysogeny rather than killing them. This same mechanism is thought to also extend to the viral lytic-lysogeny dynamics within the gut.[48] Gut phages encode a surprisingly rich repertoire of functional genes that confer beneficial traits to their bacterial hosts, which in turn bestow beneficial functions to their metazoan hosts in an intriguing tripartite symbiosis.[34,41,49–52]

The potential for bacteriophages to influence the structure and function of the gut microbiome is being increasingly recognized and appreciated. Reyes et al.[17] provided one of the first detailed metagenomics analyses of human gut viromes, demonstrating remarkable interpersonal diversity within fecal viromes while the intrapersonal viral diversity was very low; with >95% of virotypes persisting within the gut of an individual over a 1 year period. An important note from this study was that >80% of the virome reads did not match any known sequences in public databases, highlighting both the inherent difficulties associated with the molecular identification of bacteriophage communities and the limitations of these high interpersonal diversity estimates. To address these issues, Dutilh et al.[53] applied a cross-assembly approach on the unidentified sequences from this same viromic data set, identifying the a ~97 kbp circular genome sequence of a novel bacteriophage termed "crAssphage". CrAssphage was found to be highly abundant and ubiquitous within all human fecal viromes sequenced to date, suggesting that interpersonal viral diversity may not be as large as previously thought. The function and bacterial host of crAssphage remains unknown, although the authors did speculate a *Bacteriodes* host based on co-occurrence profiles. In further studies, Minot et al.[43] used a deep metagenomics sequencing approach to show that 80% of viral types persisted within the gut of a single individual over a two and a half year period, highlighting the long-term viral stability within the human gut. The current collective of evidence suggests that the human gut virome is highly personalized; consisting of a stable collection of long-term phages that exhibit temperate lifestyles.[17,43,49,53] Gut phages fix and adapt with our microbiome over the course of our life span, encoding a rich repertoire of genes that provide functional attributes affecting their bacterial hosts, diseases and immune responses.[41,50,51,54]

The impact of the bacterial component of the human gut microbiome on health and well-being is well established.[55–58] Research into the genetic potential of gut microbes has led to the concept of a "healthy gut microbiome," where similar but not identical microbes

provide common functions that promote human health.[55] Manrique et al.[50] extended this concept to include a "healthy gut phageome," which is composed of a core and common set of phages shared among healthy individuals that are likely globally distributed. These core phages were found to be significantly decreased in individuals with gastrointestinal disease [ulcerative colitis (UC) and Crohn's disease]. Similar studies have shown discordant gut viromes being increasingly associated with malnutrition, diet, obesity and IBD.[54,59,60] The pertinent question is whether discordant phage communities are implicated with these disease states, or are they merely a consequence of changes in the microbiota.

Gnotobiotic mouse studies represent the gold standard for in vivo experimental elucidation of these effects. Pioneering studies using these germ-free animal models have described the impact of commensal bacteria on host gene responses,[61] activation of the immune system,[62] the regulation of fat storage,[63] and have established interactions between the microbiota, diet, and energy utilization within the gut.[64] Comparatively, gnotobiotic studies investigating the impact phages have on the microbial ecosystem of the gut are less well studied. Weiss et al.[65] investigated the in vivo replication of T4 and T7 phages within the gut of mice mono-colonized with a non-pathogenic *Escherichia coli* strain, showing that T7 phage was capable of significantly higher replication within the gut. Duerkop et al.[40] investigated *Enterococcus faecalis* prophages within germ-free and antibiotic-treated mice, showing that prophage production was ~200 times higher in the mouse intestine than observed in vitro assays. Finally, Reyes et al.[36] colonized gnotobiotic mice with a simplified 15-member bacterial community and staged a phage attack on this community using a pool of phages extracted from human faeces. This resulted in a reproducible phage attack over a 25-day period, with increases in specific phages correlating with a reduction in particular bacterial taxa. Phage resistance developed through ecological or epigenetic mechanisms, rather than by adaptive immune responses in CRISPR elements or cell-surface markers. Surprisingly, even this simplified intestinal microbiome was remarkably resilient to the bacteriophage invasion, with changes in specific bacterial taxa occurring for only brief periods of time.[36]

These gnotobiotic studies provide unparalleled information on the in vivo interactions and dynamics of phages within the mammalian gut. Yet, the cost and technical expertise required to operate and maintain a gnotobiotic facility make these experiments prohibitive to many researchers. Furthermore, a growing body of evidence is highlighting the limitations of animal models to recapitulate human diseases, drug pharmacokinetics and the human microbiome.[66–68] Surrogate models that can reproduce the complex structure and functionality of living human cells, organs and systems are needed. The construction of biomimetic microsystems, called "lab-on-chip" that are capable of reconstituting organ level functions may provide an alternative experimental system to elucidate these effects.[69] "Lab-on-chip" approaches have successfully microfabricated models of airways, gut, and immune cells among others.[70,71] The challenges for phage researchers looking to utilize these systems lies in fabrication, optimization of experimental design, and extraction of a suitable and continuous data set that addresses the fundamental and functional impacts of phages within these devices.[72]

3 | THE MUCOSAL BARRIER

Bacteriophages are integral components of the gastrointestinal tract and are associated with health, nutrition, and disease. During their residence within the gut, these phage communities eventually encounter the mucosal barrier, which forms an active lining covering the gastrointestinal epithelial cells.[73–76] Mucosal surfaces are among the most microbe-rich sites within the body, being heavily colonized by bacterial symbionts that contribute additional genetic and metabolic potential.[61,77–79] Mucus layers are also breeding grounds for large and diverse communities of bacteriophages.[51,72]

Our research found that bacteriophages were significantly enriched within the mucus surfaces of diverse metazoans compared to the adjacent non-mucosal environment.[51] On average phage concentrations were 4.4-fold higher within mucus layers, with similar phage-mucosal enrichments having been reported by other research groups.[80,81] Phage enrichment was found to be dependent on the presence of mucus, rather than general properties of the cell surface or other macromolecular components.[51] On the basis of these observations, we proposed the bacteriophage adherence to mucus, or BAM model, whereby phages adhere to the mucosal surfaces of diverse animals, reducing the microbial colonization and pathology of the surfaces, and providing a non-host-derived layer of immunity.[42,51] The BAM model is a result of a complex interplay of biophysical interactions, environmental fluxes, and population dynamics that has implications for symbioses, infection, and immunity of the mucosal layer.

To describe these interactions, we must first understand the structure and complexity of the mucosal environment. Mucus is primarily composed of water (normally >98%) with mucin glycoproteins representing the major structural components. Mucins form some of the largest macromolecules in biology (up to 10^6-10^9 Da) and contain an amino acid backbone that is heavily glycosylated.[73] The extensive glycosylation results in the display of hundreds of variable, branched, negatively charged glycan chains that extend 0.5-5 nm outward from the peptide core into the surrounding environment. This gives the mucins an extended, stiff and voluminous confirmation that resembles a "bottle brush".[82] This structure gives mucins an extended confirmation with a high capacity to bind water, leading to distinctive gel-forming properties.[76] There are two major classes of mucins; the cell-tethered or transmembrane mucins, which are anchored to the cell wall and can contain an intracellular domain; and the gel-forming or secreted mucins, which are secreted by specialized epithelial cells and make up the scaffolding of the mucus layer.[83] Gel-forming mucin monomers aggregate together through disulphide bond linkages and form large, polymer-stabilized, net-like structures that spread outward as organized and distinct sheets. Conceptually, the mucus layer exists as a three dimensional network of mucin cables, each covered with thousands of variable glycan chains.

The underlying mechanism of action for the BAM model is a biophysical binding between proteins displayed on phage capsids and the mucin network (Figure 1). The first hint of these binding interactions came from a report by Minot et al.[84], revealing that human gut phage communities encoded hypervariable loci associated with genes

encoding immunoglobulin (Ig) superfamily proteins. The Ig-like protein fold is one of the most common and widely dispersed in nature,[85] and is commonly associated with binding interactions. The Ig-like fold is comprised of at least seven β-strands arranged into two distinct and parallel sheets, allowing for a high degree of variation (supporting >10^{13} potential alternatives) while still maintaining the structural stability of the protein fold. Bacteria utilize Ig-folds for cell-to-cell adhesion, the same fold plays a varied but essential role in the vertebrate immune response, and ~25% of sequenced *Caudovirales* phages encode structural proteins containing predicted Ig-like domains.[86–88] While phage tail fibers encoding Ig-like domains are known to be involved in bacterial host binding and recognition processes,[89] a surprisingly large number of Ig-like domains are associated with phage head accessory proteins with undescribed functions.[88,90] A case example is seen in phage T4, which encodes four Ig-like domains within an outer capsid protein termed "Hoc." The Hoc capsid protein is completely dispensable for phage growth under laboratory conditions and while its function remained largely unknown,[90–92] it was hypothesized to mediate interactions with mammalian organisms.[93,94] The high prevalence of phages in mucus, along with the observations of hypervariation within the Ig-like domains of gut phages, led us to hypothesize that these capsid-displayed Ig-like domains might mediate phage adherence to mucus.[51]

Using the wildtype T4 phage and a mutant T4Δ*hoc* phage—a deletion mutant that does not encode the Hoc protein that displays four Ig-like protein folds—we demonstrated that the presence of these Ig-like folds on the phage capsid was required for mucus adherence.[51,72] A search of publically available viral metagenomes for homologs of the T4 phage Ig-like domain revealed that mucosal associated environments contained higher proportions of these domains.[51] Furthermore, all identified domains displayed high structural homology to a plant-sugar binding domain known for its promiscuous carbohydrate binding; providing our first clue that phages bound the glycan component of mucin. To confirm this, we exposed fluorescently labelled T4 and T4Δ*hoc* phage to a glycan microarray printed with 610 diverse mammalian glycans. Phages were washed across the array and the relative strength of binding to a specific glycan recorded as a normalized relative fluorescence unit (RFU). T4 phage bound weakly (5000-2000 RFU) to >200 mammalian glycans, while T4Δ*hoc* phage did not strongly associated with any glycans.[51] When compared to other biological examples, including human parainfluenza virus (>30 000 RFU) and the gut symbiont *Bifidobacterium longum* subsp. *infantis* (>20 000 RFU), both of which strongly associate with mammalian glycans for infection and metabolism respectively,[95,96] T4 phage-glycan binding interaction was both weaker and more promiscuous. T4 phage compensates for this weak binding by evenly covering its capsid in hundreds of these Ig-like folds; with each phage displaying 155 copies of the Hoc structural protein, each of which encode four Ig-like folds for a total of 620 structurally exposed domains (Figure 1). This multimeric display increases the avidity of T4 phages adherence to mucus glycans, even though each individual Ig-like fold has a relatively weak affinity. Increased avidity via multimeric display appears to be a common strategy for phage adherence to mucus. The recently described crAssphage

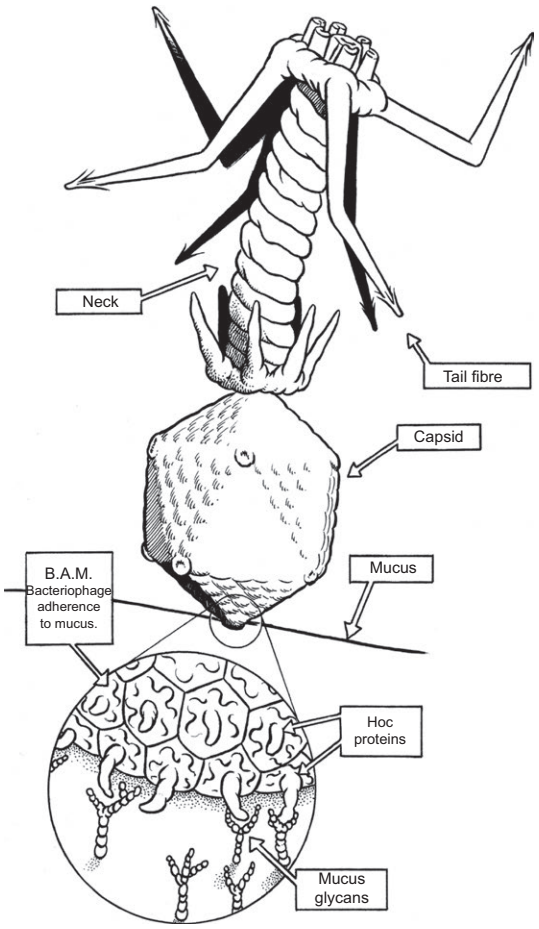

FIGURE 1 The bacteriophage adherence to mucus (BAM) model. Phages adhere to mucus glycans through weak binding interactions with the Hoc proteins displayed on their capsid. This binding mechanism enables subdiffusive motion of phages within mucosal surfaces, providing significantly enhanced encounter rates with bacterial hosts. These benefits allow mucus-adherent phage to propagate throughout the mucus layer, forming a non-host-derived layer of immunity. Figure reproduced with permission from The Invisible War a Tale on Two Scales[247]

is hypothesized to mediate adherence to mucus by covering its capsid with structural proteins, each of which display eight *Bacteriodetes*-associated carbohydrate-binding (BACON) domains that are predicted to bind glycans.[53,97] Many other phages including Vibriophage VP2 and Enterobacteria phage RB69 encode multimeric Ig-like folds that may also mediate mucus adherence.[88] Thus, phage adherence to mucus is the result of hundreds of weak binding interactions between multimeric capsid proteins (such as the T4 phage Hoc protein that encodes four Ig-like folds) and the highly diverse glycans covering the mucin glycoproteins.

Mucus is an optimal environment for microbial growth. Mucin glycoproteins provide both structure and nutrients for bacterial residents. It is important to note that the structured mucin network traps particles, including bacteria and phage, based on their size.[98–100] We demonstrated this by showing the equal accumulation and persistence of both mucus adherent and non-adherent T4 phage within a "gut-like"

mucosal surface that was exposed to fluid flow and shear forces,[72] indicating that mucus secretion dynamics govern phage abundance in mucus rather than the ability of any phage to adhere to mucus. How then do mucus-adherent phages mediate antimicrobial and immune-protective effects in mucus? To answer this question, we conceptualized the way phages moved in mucus as a way to "hunt" for a bacterial host; similar to the way larger predators hunt for prey within their respective environments.[72] This search for food is a ubiquitous process throughout biology. Many predators, such as albatross, blue fin tuna and humans, utilize search strategies to statistically increase their chances of encountering prey.[101–104] Phages proved to utilize a similar albeit unique strategy to improve their chances of encountering bacteria in mucus layers.

Phages are inert particles that rely on diffusion processes to bring about chance encounters with bacterial hosts. Within the mucus layer the diffusion of any particle: phage, bacterium or otherwise, is slowed by the mucin network.[99] Within this layer, mucus adherent phages weakly bind to the glycans covering the mucins (Figure 1). This binding slows the diffusive motion of the phages and keeps them nearer to the mucin strands. Bacteria residing within the mucus layers are also attracted to, and caught by these same mucins; enabling the mucus adherent phages a higher statistical chance of infecting a bacterial host—akin to a search strategy. The exponential growth of the mucus adherent phages results in their propagation throughout the surrounding mucus, which leads to more productive infections and further reductions in bacterial load.[72] Thus, while the replicative benefit for any singular mucus-adherent phage particle is quite small, the rapid propagation of these phages provides a dynamic antimicrobial and immunological barrier whose collective effects are propagated throughout the mucosal surface.

Applying these concepts for the manipulation of bacterial hosts within the gut remains a complicated endeavor.[105,106] Although the human gut is anatomically external to the body, it is not easily amenable to direct sampling, with many studies using fecal samples as a proxy. Fecal samples are inherently biased toward lumenal-derived microbes and may under represent the more active bacterial and phages communities residing within the mucosa. These mucosal communities are inherently difficult to sample and manipulate, with mucosal microbial communities exhibiting surprising stability and resilience through selective epithelial secretions, ecological niche competition, immense diversity, and functional adaptability.[107–109] Phages undoubtedly play a role in the selection and maintenance of the mucosal communities, yet their complexity and diversity increases ad infinitum.

How then can we best apply phages at the mucosal surface of the gut? First, we must understand not only the bacteriophages within this environment, but also the physiological status and spatial location of their bacterial host. Many species in the gut are considered to be nutritionally deprived and non-replicating, making them poor targets for replicating bacteriophages.[39,110–113] Identifying keystone bacterial species that are physiologically and metabolically active within the mucosal surfaces of the gut represents an important advance. Second, we must assess the strain-level diversity of these species and their bacteriophages, likely on a personalized basis. Therapeutic phages typically have narrow host ranges and require minimum host densities of $>10^3$ cfu/mL to support exponential growth[114]; conditions which are effectively countered by increased bacterial strain diversity. These constraints were evident in a recent phage therapy trial of acute bacterial diarrhea performed in Bangladesh.[14] The trial confirmed the safe application of an oral coliphage cocktail in children infected with *Escherichia coli*, but failed to demonstrate a quantitative impact on diarrheal parameters over the placebo. Results revealed that although the oral phage cocktail reached the gut, it did not achieve substantial in vivo replication, likely due to the low carriage of pathogenic *E. coli* (typically less than 5% of total fecal bacteria) and the polymicrobial nature of diarrheal infections (which can include *Vibrio cholera* and *Campylobacter jejunni*).[115,116]

The BAM model offers the premise of increased phage replication within the mucosal surfaces of the gut. Mucus-adherent phages exhibit increased bacterial encounter rates when their bacterial host concentrations are low and mucus layer thickness is increased—conditions that are prevalent within the gut.[42,51,72] Our theoretical model predicts that mucus-adherent phages are 19 times more likely to encounter a bacterial host within mucus when hosts densities are low ($\sim 10^3$ cfu/mL), compared to a non-adherent phage in the same environment. Thus by utilizing phages with mucus-adherent properties, it may be feasible to increase phage infection and replication with low carriage gut bacteria. The Ig-like domains that mediate this adherence also possess the capacity for adaptive evolution to divergent glycosylation profiles seen across mucosal surfaces.[51,117] The co-evolution between phage and the eukaryotic mucosal surface may provide a further mechanism to enhance the control of bacterial species at specific mucosal locales across the body.

4 | ACROSS THE CELL LAYER

Following interactions with the microbiome and mucosal layers of the gut, most phages are inevitably degraded or discharged from the intestinal tract. Yet, a select few phages may continue through the body via interactions with the intestinal epithelial cell layer. Past the secreted mucus layer, the intestinal epithelium is covered in a final layer of transmembrane mucins that cover the apical cellular surface.[83] The transmembrane mucins consist of; an extracellular region that is apically expressed and highly glycosylated; a single, hydrophobic transmembrane domain; and a cytoplasmic tail that is exposed within the cellular cytoplasm.[74,82,118] As these transmembrane mucins contain both an intracellular domain and an external glycocalyx, which is extended into the intestinal lumen, they are capable of acting as both a physiological barrier and as a cellular receptor.[119]

Within the human body, there are approximately 10 different membrane-tethered mucins. These mucins have been observed to act as cellular receptors for the external environment; mediating various functions, including signal transduction, regulation of ion channels and inflammation.[120–124] As an example, the MUC1 transmembrane mucin contains a long cytoplasmic tail with several tyrosine phosphorylation sites, which are known to be involved in signal transduction and

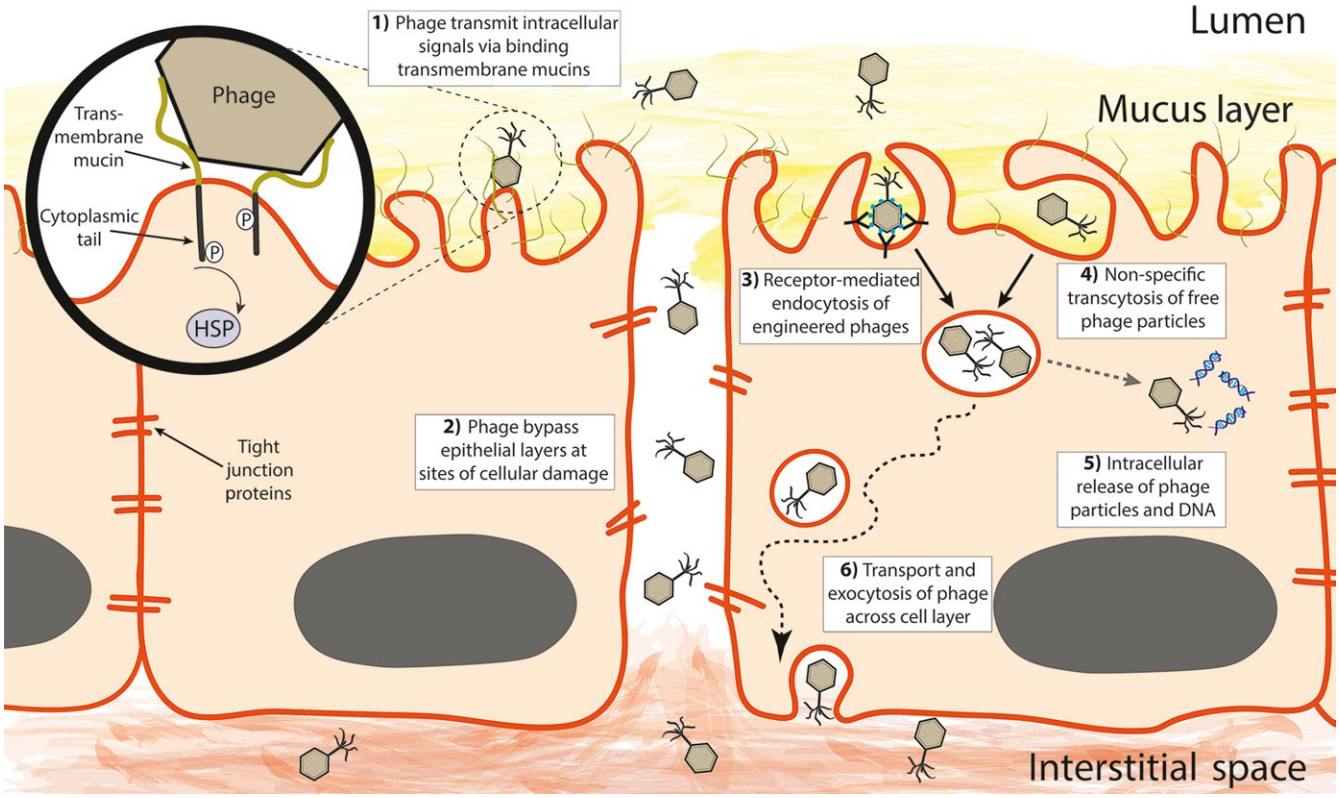

FIGURE 2 Bacteriophage interactions with the mammalian epithelial cell layer. (1) Binding interactions between phages and transmembrane mucins may allow signal transduction in the epithelial cell. Membrane associated mucins serve as ligands for diverse molecules, such as bacteriophages. Following engagement transmembrane mucins can undergo primary changes in the conformation leading to the phosphorylation of the cytoplasmic tail, which can trigger downstream signal transduction and the activation of Heat Shock Proteins (HSP) that may down regulate apoptosis and affect transcription factors.[120,132] (2) Phage may gain access to the body via a "leaky gut", where they bypass the epithelial cell barrier at sites of cellular damage and punctured vasculature.[142,143] (3) Viral particles can be transported into the cell by receptor-mediated endocytosis whereby a specific receptor on the cell-surface binds tightly to an extracellular ligand that is displayed on the phage capsid. Phage particles can be engineered to display diverse ligands on their capsids, triggering receptor-mediated endocytosis and uptake by a specific cell type.[148–153] (4) Phages may also enter eukaryotic cells by non-specific uptake of free phage particles.[147,152,154,155] (5) Internalized phage particles may be degraded leading to the intracellular release of phage particles and genetic material, which is capable of being transcribed and translated by eukaryotic cellular machinery.[153,167] (6) Internalized phage particles are hypothesized to cross the eukaryotic cell enabling phage dissemination to the body

thought to coordinate cellular responses involved with proliferation, differentiation, apoptosis, and secretion of cellular products.[125,126] This was demonstrated using *Pseudomonas aeruginosa* flagellin proteins that were shown to bind the external MUC1 glycocalyx region, resulting in phosphorylation of the cytoplasmic tail and activation of downstream extracellular signal-regulated kinase (ERK) signalling pathway, which subsequently initiated an inflammatory response.[127,128]

Could mucus-adherent phages also bind transmembrane mucins and coordinate a cellular-level response? Although it was traditionally thought that intrinsic interactions between phages and eukaryotic cells did not occur, a growing body of research is now questioning this assumption. Bloch demonstrated potential interactions between phages and malignant tumour cells isolated from different animals, showing that phages inhibited the growth of these tumours.[129] Decades later, Dabrowska et al.[94,130,131] tested this observation showing that T4 phages bound the membranes of cancer cells, attenuating tumour growth and inhibiting lung cancer metastasis of murine B16 melanomas. Recently, Talago presented preliminary results on the binding of T4 phage to the MUC1 transmembrane mucin within A549 lung cells using two-dimensional gel electrophoresis (2DGE).[132] Total protein extracts from untreated and phage exposed cells were analyzed by 2DGE, and protein spots that changed in either intensity or isoelectric point shift were extracted and analyzed using liquid chromatography mass spectrometry (LC-MS). Phage-response proteins identified included heat shock protein (HSP) 70 and 90, both of which are known to interact with the MUC1 cytoplasmic tail leading to the downregulation of apoptosis and translocation of transcription factors to the nucleus.[120,132] Taken together these result support interactions between phages and transmembrane mucin domains, although further research is required to elucidate the subsequent effects on the eukaryotic cell (Figure 2).

Phage cannot infect eukaryotic cells in the same way they infect their bacterial hosts. This is primarily due to fundamental differences in cell-surface receptors and intracellular machinery between the prokaryotic and eukaryotic cell. Despite these differences, phages have long been known to completely and profusely permeate the bodies

of humans and other vertebrate organisms.[19–21,133–137] In 1921, Felix d'Hérelle first observed the transitory appearance of phages targeting *Salmonella typhimurium* in the blood of infected rats.[22] Subsequent studies involving intraperitoneal injections of phages in mice resulted in their accumulation within the kidney, spleen, liver, and brain.[133,134] Oral, intranasal, and gastric application of phages to rats all resulted in the detection of phages within the blood as little as 10 min post-application.[135–137] The oral administration of phages to humans also resulted in their detection within the blood stream and urinary tract.[138,139] Diverse phages have also been found within commercial animal serum, with their presence thought to have arisen through either absorption from the gut or synthesis somewhere else in the animal.[140,141]

The presence of phages within these classically "sterile" regions of the body and their apparent permeation throughout diverse animal hosts raises the question as to how phages enter the body? There are several hypothetical possible routes by which phages could penetrate the body (Figure 2). Perhaps the most rudimentarily proposed route of access is via a "leaky gut"—characterized by cellular damage and punctured vasculature at sites of inflammation, thereby allowing phages to bypass confluent epithelial layers.[142,143] Phages may also gain entry into the body via eukaryotic cell uptake through a number of proposed routes. Phages have been documented to enter eukaryotic cells through a Trojan Horse mechanism, whereby phages infect or integrate into a bacterial host, which is in turn engulf by, or enters a eukaryotic cell and subsequently releases the phage particles.[144–147] Numerous studies have also reported targeted gene delivery to eukaryotic cells utilizing phage-display mechanisms, whereby phages are engineered to display peptides complementary to cell-surface integrins that trigger receptor-mediated endocytosis.[148–153] The majority of these studies utilize the filamentous M13 phage by fusing the phage coat protein with a cell targeting ligand, which subsequently triggers receptor-mediate endocytosis of recombinant phages on contact with the eukaryotic cell of interest. Phage may also access the eukaryotic cell through the uptake of free phage particles via endocytosis, although the mechanisms for this uptake remain unclear.[147,152,154,155] There is supporting and contrasting evidence for all of these mechanisms, suggesting that phages may access the body via diverse routes. Aronow et al.[154] presented one of the first images of free T2 phage particles within phagocytic cells, showing phages within endocytic vacuoles that fused with denser bodies of the cytoplasm as they migrated towards the cellular interior. Similar studies have shown evidence of filamentous phage and *Myoviridae* within mammalian cells, although these phages were either engineered to display exogenous protein markers or the mechanism of cellular entry was not clearly delineated.[152,155,156] Several review articles have broached the topic of phage-epithelial transcytosis, but few attempts have been made to experimentally validate whether phage transcytosis occurs naturally and via which route.[19,146,147,157] Thus, the primary mechanism that native phages use to access the eukaryotic cell remains unknown.

The dissemination of phages throughout the cell could enable direct interactions with eukaryotic organelles, cellular machinery and may stimulate cellular autonomous immunity through interactions with pattern recognition receptors (PRRs).[158–160] Whether intracellular PRRs recognize phage capsids or genetic material as pathogen-associated molecular patterns remains to be seen.[161,162] There is evidence suggesting that key components of the mitochondrial transcription and replication apparatus were derived from the T-odd phage lineage,[163] yet how and from where these genes originated is unknown. One current theory is that a phage-derived gene replaced that of the proto-mitochondrion by non-orthologous gene displacement.[164] Recently, these same mitochondrial genes and other Eukaryotic genes have been discovered in the genomes of cyanophages, suggesting potential horizontal gene transfer (HGT) events occurred between phages and the proto-mitochondrion.[165,166] The uptake of phages into eukaryotic cells could provide a missing link for the viral origin of these mitochondrial genes.

Perhaps the greatest potential function of these intracellular phages is the utilization of their genetic material by the eukaryotic cell directly. Merril et al.[167] provided evidence that lambda bacteriophages were capable of transducing mammalian tissue culture cells, with phage encoded genetic material being transcribed and translated by the eukaryotic cellular machinery. The study used galactosemic fibroblast cells, which lack α-D-galactose-1-phosphate uridyl (GPU) transferase activity, which were infected with GPU-containing lambda phages. To obtain a functional enzyme from the phage, the mammalian cell would have to transcribe at least part of the phage carried DNA into mRNA and then translate this into protein. Following phage transduction, the fibroblast cells maintained GPU enzyme activity for up to 41 days after phage infection with no decrease in expression, suggesting that the phage transduced genes were preserved within the cell by an unknown mechanism.[20,167] Further experiments by Geier and Merril[168] highlighted that human cells are capable of effecting transcription of phage carried genes. Numerous other studies have shown the capacity for phage particles to deliver and express genes in eukaryotic cells.[153,169,170] Indeed phages are commonly used as nanocarriers and viral gene delivery vectors, primarily due to their ease of use, capacity for nucleic acid packaging and relative safety in humans.[143,153] In these approaches, therapeutic agents were translocated and packaged into emptied phage heads that were engineered to display specific cellular recognition molecules or peptides covering their surface. This allowed for the conjugated delivery of therapeutic agents into a specific cell type of interest through receptor-mediated endocytosis. Using this approach, Poul and Marks[171] engineered filamentous phage F5 to display the growth factor receptor ErbB2, which enabled receptor-mediated endocytosis into breast tumour cells and the delivery and subsequent expression of GFP reporter genes. Tao et al.[153] reconfigured the T4 phage packaging machinery to deliver reporter genes, vaccine genes and functional enzymes into mammalian cells using targeting ligands incorporated into the T4 phage head that induce receptor-mediated endocytosis, with the delivered genes being abundantly expressed both in vitro and in vivo. These approaches utilized recombinant phages to successfully deliver and express genes, proteins and drugs into a range of mammalian cells both in vitro and in vivo,[143,151,153,171,172] demonstrating the capacity for engineered phages to effect eukaryotic cells directly.

Gut phages are a known repository of accessory genes within the gut microbiome, harboring genes associated with carbohydrate and amino acid metabolism, and antibiotic resistance genes among others.[17,18,173] The gut virome encodes an astounding amount of uncharacterized genetic diversity that is commonly termed biological "dark matter".[3,4] Might this phage "dark matter" also contain accessory genes capable of being directly expressed by eukaryotic cells? Extensive differences between viral and mammalian transcriptional sites, phosphorylation, restriction endonucleases, codon usage, and tRNA distribution would limit potential transcription and translation of native phage genes within the eukaryotic cell.[20,168] Even in consideration of these limitations, the potential for phage genetic material to directly affect our cells, metabolism, immunity, and health and disease states is too great to dismiss. If true, this would allow our cells and body access to a huge external reservoir of genetic material encoded by the resident phage populations in our gut.

5 | BODY-LEVEL FUNCTIONS AND IMMUNOMODULATION

Whether native phage populations residing within our gut are capable of entering and crossing the epithelial cell layer of the gut remains to be thoroughly characterized, although current evidence suggests this is conceivable. This is supported by long-standing observations of phages within regions of the body that are classically considered sterile, including; the blood, lymph, liver, kidney, and brain.[133,134,138,174–176] Based on currently available experimental evidence, I hypothesize that gut phages are capable of crossing the epithelial cell layers of the gut, gaining access to the body and resulting in the accumulation and assembly of an "intra-body phageome."

Few studies have investigated the presence and diversity of "intra-body phages" using culture-independent methods within humans. Breitbart and Rohwer[175] provided one of the first culture-independent characterizations of the intra-body phageome by obtaining shotgun library sequences from healthy human plasma, detecting four contigs with significant tBLASTx similarities to known bacteriophages. Fancello et al.[174] investigated the viral DNA communities present within human pericardial fluids, identifying sequences related to phages infecting *Staphylococcus*, *Enterobacteria*, *Streptococcus*, *Burkholderia*, and *Pseudomonas*. Dinakaran et al.[177] studied the circulating virome of healthy subjects and cardiovascular disease (CVD) patients, finding that the viromes of CVD patients were predominantly populated by phages, which contributed 63% of total viral sequences compared to 18% within the healthy subjects. Recently, Thannesberger et al.[178] investigated the virome of mammalian cell cultures and human clinical samples where they found an extensive and diverse population of phages and novel viruses in both samples. The complex virome discovered in mammalian cell cultures was particularly astonishing, with the detection of a high number of virus-like particles with >90% of the reads being taxonomically classified as phages. The authors did not identify where these phages particles originate from, but hinted that the most likely source was commercial animal serum. To address this unknown viral source, Kowarsky et al.[179] performed a massive shotgun sequencing of circulating cell-free DNA from 1351 blood samples collected from 188 patients. They revealed previously unknown and highly prevalent microbial and viral diversity within the blood of humans, with many of the sequences being placed in distant sectors of the tree of life. Numerous novel phage sequences were identified, although it should be noted that the cell-free DNA extraction procedure utilized would be unlikely to efficiently extract phage DNA from intact viral capsids.[179] Outside of the above mentioned studies, very little information is available regarding the diversity, abundance and persistence of this intra-body phageome. This is partly due to the inherent difficulties associated with the sufficient sampling and subsequent molecular identification of low abundance phages within the body.

What would this intra-body phageome look like and what is its function? To address the first question I assume that intra-body phages primarily originate from the gut, which houses the largest aggregation of phages within the body.[17,180] Within the gut, there are an estimated 5.09×10^{9} phage per gram of faeces,[34–36] yielding 2.09×10^{12} total phage within the colon of an average adult human.[6] These gut phages are highly diverse, engaged in predatory-prey relationships with their bacterial hosts, and have co-evolved with our microbiome over our life span.[18,43] Next, we assume these gut phages are capable of by passing the gut epithelial cell layer and accessing the body through an as yet unidentified mechanism.[19,146,147,157] Phages that successfully cross the epithelial cell layer likely enter into the interstitial matrix. From here, the intra-body phages drain into the lymphatic system [181,182] and are subsequently able to access the regional lymph nodes, circulatory system, and disseminate to organs throughout the body. Finally, the body is adept at the removal of circulating phages, with half-lifes on the order of hours rather than days.[183–185] Thus, the intra-body phageome likely originates from the highly diverse phage communities of the gut and provides a representative snapshot of our gut microbial status quo that is disseminated throughout the blood, lymph, and organs of the body. These assumptions are important for subsequent inferred interactions between phages, eukaryotic cells, and the immune system.

The first potential role of the intra-body phageome is its action as a non-host-derived, circulating antimicrobial agent protecting against the invasion of opportunistic gut bacterial symbionts. If given the opportunity to bypass the mucosal epithelium, many gut symbionts quickly turn from commensal to opportunistic pathogens.[186,187] Due to the predatory-prey relations between phages and their bacterial hosts, the circulating intra-body phageome likely parallels the bacterial diversity found within the gut. Accordingly, a low-level, circulating supply of phages from the gut may provide the body with a first step antimicrobial against these opportunistic invasions. However as discussed previously, temperate is the preferred phage life lifecycle within the gut and many bacterial symbionts are resistant to their own phages through superinfection immunity mechanisms, raising questions regarding the efficacy of this mechanism. Identifying the intra-body phageome remains an important first step in elucidating its subsequent effects within the body.

The intra-body phageome may interact with the mammalian immune system, mediating humoral immunity, and immunomodulatory effects. The mammalian immune system functions to protect the host against a broad range of pathogenic microorganisms while minimizing erroneous or excessive inflammatory responses that may be deleterious. Phages are exogenous and immunogenic protein particles that are capable of stimulating humoral immunity and inducing anti-phage antibodies.[138,185,188,189] The continual exposure of the human body to intra-body phages raises the question as to how the mammalian immune system can sustain a continuous influx of these foreign, proteinaceous, and immunogenic particles without eliciting inflammatory immune responses? It should be noted that the mammalian immune response to phages is incredibly mild, with no incidences of phage initiated anaphylaxis having been reported.[14,190,191] This apparent lack of a phage-mediated inflammatory immune response likely originates from metazoans early exposure to phages across their evolutionary history, mediating greater levels of tolerance to phages than to other immunostimulatory particles.[190,192]

The activation of humoral immunity typically requires large inoculations of a singular cell-surface associated component originating from a bacterium, virus or other foreign entity. Majewska et al.[185] recently demonstrated the activation of humoral immunity in response to phages through the long-term application of high-titre T4 phages to mice. In this study, mice were given T4 phage either orally in their drinking water at a concentration of 4×10^9 phage/mL for 100 days, or via subcutaneous injection at a concentration of ~5×10^9 phage/mL on three separate occasions over 48 days. Researchers observed that both treatments routes stimulated humoral immunity and the production of anti-phage antibodies, however, the immunostimulatory responses were relatively weak, requiring persistent treatment with high-titre phages to achieve a marked immunological response.[185] Such immunostimulatory responses are likely of limited relevance outside of therapeutic interventions using high-titre, phage monocultures, and do not directly address how the highly diverse, intra-body phageome may affect the mammalian immune system.

Rather than stimulating humoral immunity, might the intra-body phageome have an immunomodulatory or tolerance effect? Research by Górski et al.[93,157,193,194] has provided evidence of this, showing phage-mediated inhibition of T-cell proliferation, downregulation of antibody production, and even demonstrated the ability of phages to extended allograft survival in mice. Outside of these studies, very little is known about the specific mechanisms and direct interactions between phages and immune cells. Over the past decade, it has emerged that adaptive immune responses in animals involve the recruitment of not only effector T and B cells, but also T cells that disrupt or suppress immune system functioning.[195,196] These specialized immunosuppressive T cells, called regulator T cells (Tregs), strike a balance with effector cells to control the quality and magnitude of the adaptive immune response. Treg cell populations and function are critical for either establishing or breaching tolerance to self- and non-self-antigens.[197]

Could interactions between intra-body phages and Treg cells be mediating immunosuppressive effects, and if so how might these interactions occur? We assume that gut bacteriophages that cross the gastrointestinal epithelial layer are likely secreted into the interstitial matrix. From here, the interstitial matrix is drained into the lymphatic system [181,182] and the intra-body phages are disseminated to regional lymph nodes, circulatory system and organs throughout the body. Research suggests that Treg cells migrate to and are enriched within regional lymph nodes and sites of infection, where they may become activated by either tissue-specific self-antigens or microbial antigens that are presented to them.[198,199] It is still contentious whether Treg cells can recognize foreign antigens directly, although there is growing experimental evidence in support of these direct interactions.[200,201] Following antigenic stimulation, activated Tregs can clonally expand; with subpopulations retaining their immunosuppressive functions, allowing for long-term persistence of activated Tregs and their immune-tolerance.[202,203] It is plausible that it is within the regional lymph nodes, where intra-body phages interact directly or in-directly with Treg cells to mediate immunosuppressive functions.

The capacity of the mammalian body to mount an effective immune response depends on a complex equilibrium between Tregs and effecter cell populations. Effector cells that boost immune responses, favoring pathogen control and removal, also abrogate natural Treg cell functions. Conversely excessive Treg cell numbers or function can depress or prevent effector mediated immune responses.[204,205] The immunomodulatory role of phages is likely heavily influenced by this equilibrium. Adequate numbers and function of Treg cells may prove critical for the persistence of intra-body phages and the suppression of phage-induced inflammatory immune responses. Conversely, reduction in Treg numbers or function may lead to enhance activation of humoral immunity and the increased clearance of intra-body phages, both of which may exacerbate inflammatory responses following subsequent re-exposures to intra-body phages.

Aberrant phage uptake by gut epithelial cells or drastic shifts in the gut microbiome likely affect the intra body phageome and its immunomodulatory functions. Phages may bypass epithelial cell layers at sites of cellular damage or be internalized and cross the epithelium directly by as yet unidentified mechanisms. It is likely that many of the internalized phages do not successfully make this cross-cell transit, instead remaining within the eukaryotic cell and being degraded through autophagy processes. Excessive phage uptake may result in ER stress and the consequent unfolded protein response, which has been linked with the onset of intestinal inflammatory disorders.[206–208] Conversely, cellular processes that reduce or block phage epithelial uptake or bypass may impede phage-mediated immunosuppressive mechanisms, leading to increased activation of humoral and inflammatory immune responses when the body next re-encounters these phages. Similarly, drastic shifts in the gut microbial community are likely accompanied by the appearance of new intra-body phage species, which may not have been previously been encountered by the body. The immune system may lack immunological tolerance for these new phage species, leading to activation of humoral immunity and inflammatory responses. This hypothesis is supported by recent work from Norman et al.[54] showing the significant expansion in the taxonomic richness of *Caudovirales* bacteriophages in IBD patients. This increase in phage richness was

inversely associated with bacterial richness and diversity, suggesting that phage expansion was not simply the result of increased richness of the bacterial hosts. These drastic shifts and expansion of the gut virome seen in IBD patients are likely reciprocated by the intra-body phageome, potentially resulting in exposure of inapposite phages that lack activated Tregs with immunosuppressive functions and lead to the stimulation of humoral immunity and inflammatory responses. These phage-immunomodulatory interactions may provide a missing link in the study of inflammatory bowel diseases and disorders, including IBD, UC, and Crohn's disease, which despite dozens of years of study their etiology still remains elusive.[15,209] Determining how the apparent unresponsiveness of the adaptive immune system to intra-body phages is established and maintained, and whether disruptions in phages access to body is associated with inflammatory diseases needs to be a research priority.

6 | DISSEMINATION, TRANSMISSION, AND MODULATION

Once the intra-body bacteriophages enter the lymphatic and circulatory system they would be quickly disseminated throughout the body. From here they may; directly interact with immune cells, tissues or organs; be further bound and endocytosed by specific mammalian cells; bind co-circulating bacterial hosts; or be removed by the mononuclear phagocytic system. Their dissemination throughout the body may also enable the vertical transmission of the intra-body phageome to a new human host.

Maternal transmission of microbes to developing offspring is pervasive throughout the Animalia kingdom.[210,211] The surprising diversity and plasticity by which microbes gain access to germ cells, embryos or developing offspring signifies that maternal transmission of microbial symbionts is an ancient and evolutionarily advantageous mechanism inherent in animals.[210] Examples of this maternal transmission of microbial symbionts include; sessile marine sponges, which are among the most ancient metazoans, that vertically transmit diverse bacterial symbionts to their embryos[212]; the Beewolf wasp, which cultivates symbiotic *Streptomyces* bacteria in specialized glands in their antennae that are subsequently acquired by their young through an external coating[213]; and in vertebrates as documented during the egg formation in turtles [214] and during child birth in humans.[23]

Are our intra-body phages vertically transmitted from mother to infant? The "sterile womb paradigm" suggests that the placental barrier keeps human infants sterile throughout pregnancy. However, recent studies suggest that infants incorporate an initial microbiome before birth.[215–217] In a seminal study, Jiménez et al.[218] fed a group of pregnant mice genetically labelled *Enterococcus faecium* that was previously isolated from the breast milk of a healthy woman. The labelled strain was subsequently detected from the meconium of cesarean section mice, but not within control mice that did not receive the labelled strain, providing some of the first evidence for mammalian maternal microbe transmission to the fetus in utero. Whether circulating intra-body phages are also able to access the placental barrier and subsequently colonize the human fetus prior to birth remains to be addressed.

Infants receive copious maternal microbes through birthing and breastfeeding.[23,215,216,219,220] In early infancy, mammalian young almost exclusively consume breast milk as a food source, which is in stark contrast to the varied diets consumed post-weaning.[220] At its core, breast milk is a nutrient delivery system; additionally, it educates the infant immune system, confers a certain degree of protection against intestinal pathogens, is a prebiotic and introduces a source of commensal bacteria to the infant gut.[219,221,222] Human breast milk is known to contain diverse populations of bacteria, which likely influence the colonization of the infant gut and development of the immune system.[223–225] Ward et al.[223] investigated the microbiome of pooled human breast milk, identifying a community of over 360 prokaryotic genera that was dominated by the phyla of Proteobacteria (65%) and Firmicutes (34%). However, these samples were collected from non-sterilized breasts, and the presence of contaminating skin or infant oral microbiota cannot be ruled out as an inoculum source. In an attempt to address this issue, Urbaniak et al.[226] characterized the bacterial microbiota of sterilely collected human breast tissue from women with and without cancer. A diverse community of bacteria were detected within tissues collected from sites throughout the breast in women ages 18-90, not all of whom had a history of breast feeding, with the principal phylum identified being again Proteobacteria and Firmicutes. These studies demonstrate that human breast tissue and milk is not sterile and harbors a diverse community of bacteria. However, the exact mechanisms by which bacteria reach the mammary gland is still contentious, with potential mechanisms including; contamination from the mother's skin or infant's oral cavity, active migration of maternal gut microbes to the mammary gland through endogenous routes, and increased gut permeability during pregnancy.[219,224,227,228]

Could the intra-body phageome be vertically transmitted from mother to infant through breast milk, and would these phages provide the initial microbial inoculum for the infant's gut? Breitbart et al.[229] described the first gut virome from a 1-week old infant, revealing a viral community with extremely low diversity. The most abundant gut viral sequences detected were not found in either breast milk or infant formula, leading the authors to speculate a non-dietary source of these phages. However, it should be noted that the sequencing depth and bioinformatic analyses used at the time of this study may have precluded the identification of low abundance bacteriophage communities.[229] Recent research by Lim et al.[230] characterized the viral and bacterial microbiome from the gut of healthy infant twins from birth through to 2 years of ages. They found that the eukaryotic virome richness was lowest early in life, suggesting eukaryotic viruses establish after birth from environmental sources. In contrast, the bacteriophage richness was greatest earliest in life and decreased with age. This contraction of infant bacteriophage community was inversely related with the bacterial community, which increased in richness and diversity with age, eventually maturing into a more "adult-like" microbiome.[231] Thus the infant microbiome shifted from a high bacteriophage-low bacterial diversity community from birth toward a

low bacteriophage-high bacterial diversity community by 2 years of age.[230] This study was again unable to address the source of the early bacteriophage diversity, but suggested a shared environmental exposure early in life was the most likely source.

The presence of an intra-body phageome suggests that these phages might be able access the mammary gland and subsequently the breast milk, although the cellular mechanisms for this transport remain unclear. If true, this would enable the vertical transmission of maternal gut phage communities from mother to infant. Current knowledge suggests that the adult human gut virome consists of a core and common set of phage communities that fix and adapt with our microbiome over the course of our life span.[17,43,50] The vertical transmission of these maternally adapted gut phage communities to an infant may represent a critically important step in the transmission and establishment of a healthy infant microbiome. Further research is needed to identify whether bacteriophage populations persists within mammalian breast milk, and the identification of viral species will provide further evidence for the origin of these communities, which likely include a mixture of skin, oral and possibly gut microbes.

It is well established that our microbiome regulates our nutrition, metabolism and is critical for the development and function of our immune system, which can have lasting implications for our health. More recently, it has been suggested that the microbiota are capable of regulating the nervous system, influencing behavioral and neurological functioning.[232,233] Gut microbes use diverse mechanisms to mediate psychoactive effects, including; fermentation products, such as short chain fatty acids, that can inhibit neuronal function; the direct production of neurotransmitters; shedding of microvesicles that can recapitulate effects of the parent bacteria on nervous system; and the activation of the vagus nerve, which provides a direct neurochemical pathway linking the gut and brain.[233–235] Bravo et al.[235] showed that the lactic acid bacterium *Lactobacillus rhamnosus* had direct effects on neurotransmitter receptors in the central nervous system (CNS) in mice. These *L. rhamnosus*-induced neurochemical and behavioral effects were not seen in vagotomized mice, indicating the vagus nerve as the modulatory pathway between the bacteria, gut and brain. Hsiao et al.[236] provided evidence that oral treatment with the commensal *Bacteriodes fragilis* altered the microbial gut composition, corrected gut permeability and subsequently ameliorated behavioral defects in mice. These effects are thought to occur through *B. fragilis* mediated serum metabolites that impact host behavior. This concept of "microbial mind control" is not just limited to bacteria; the protozoan parasite *Toxoplasma gondii* induces loss of defence behavior and attraction to feline odors in rodents[237]; and the fungal pathogen *Ophiocordyceps unilateralis* is known to manipulate the behaviors of foraging ants, making them climb foliage and latch onto vegetation to increase the spread of fungal spores.[238] The link between our microbiota and behavior is steadily becoming more apparent.

Undoubtedly phages affect the microbiome and can therefore impact the behavior and neurological functioning of mammals through indirect mechanisms with their bacterial hosts. Yet there is a growing body of evidence suggesting that direct neurological interactions may also occur. Phages have long been known to be able to cross the blood-brain barrier (BBB), accumulating within the brain and mediating protective antimicrobial affects there.[21,133,176,239] A defining characteristic of the BBB are the tight junction proteins that rigidly link together the endothelial cells of the CNS. This linkage creates a physical barrier that prevents paracellular transport and regulates both passive and active transcytosis. This process is so selective that passive transcytosis is typically limited to small, lipophilic molecules of less than 500 Daltons, and has been shown to exclude ~98% of all small molecule drugs and ~100% of large molecule neurotherapeutics, including monoclonal antibodies and recombinant proteins.[240,241] In 1943 Dubos et al.[21] demonstrated the accumulation and replication of anti-*Shiga* bacteriophage within the brains of mice following intravenous injection, subsequently preventing bacterial meningitis. In 1958, Keller & Engley showed that anti-*Bacillus* phages accumulated and persisted within the brains of mice for upwards of 6 hours following intraperitoneal injections.[133] More recently, Ksendzovsky et al.[242] perfused primate brains with native filamentous M13 phages (~900 nm particle size) using convection enhanced delivery. During treatment, all animals remained neurologically active with no evidence of toxicity, while the phages were seen to disseminate throughout the primate brain via axonal transport. One of the more surprising studies was completed by Carrera et al.,[172] where filamentous *fd* phages displaying cocaine-binding proteins on its surface were delivered into the brains of mice to sequester and block the psychoactive effects of cocaine. All of these studies highlight capacity of diverse phages to penetrate the CNS and the BBB to gain direct access to the brain in mammalian hosts.

The fact that these large, proteinaceous and foreign phage particles can readily access the CNS and cross the BBB is nothing short of remarkable.[240,241] This raises the question as to whether intra-body phages can accumulate within the CNS or brain, and mediate direct behavioral and neurological affects in mammals? Practically no research has been done investigating the role and function of native intra-body phages on the CNS and brain. The most obvious (and least distressing) potential function of phages in the CNS and brain is to protect against bacterial meningitis caused by commensal bacteria.[243] In the 1970s and 1980s, over 80% of bacterial meningitis cases were found to be caused by five pathogens; *Haemophilus influenza*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, group B *Streptococcus* (GBS), and *Listeria monocytogenes*, four of which can be considered commensal flora. The presence of these commensal bacteria within the body also suggests the presence of phages targeting them, which may be disseminated throughout the body, CNS and brain for protective anti-microbial effects. Phages have also been shown to bind amyloid beta plaques, cancers and tumour cells, suggesting a potential therapeutic role within the brain.[94,130,176] Frenkel et al.[176] intranasally administered engineered filamentous M13 phages displaying anti-β amyloid antibodies, resulting in phage co-localization with β amyloid plaques within the brains of mice. Phages binding to β-amyloid and α-synuclein plaques, which are associated with Alzheimer and Parkinson disease respectively, facilitated the disintegration and removal of these misfolded proteins from the

brain.[242,244,245] Supporting these interactions, Dabrowska et al.[94,130] provided evidence of T4 phage binding to both cancer and tumour cells, reducing metastasis, and tumour invasiveness. Thus, it is possible that phages act as cleaners of the brain, and other immune segregated regions of the body by directly binding with plaques, cancer, and tumour cells, facilitating their disintegration and removal by the immune system. Finally, we must consider the possibility of "bacteriophage mind control." To date no experimental evidence exists showing the capacity of native phages to mediate direct behavioral or neurological affects within mammals. But the breadth of microorganisms capable of either direct or indirect behavioral and neurological changes, which include protozoa, fungi, bacteria, and eukaryotic viruses,[235,237,238,246] along with the prevalence of phages throughout the body, CNS and brain of higher organisms calls for additional investigative research within this area.

7 | CONCLUSIONS AND PERSPECTIVE

A growing body of literature is beginning to unveil the multitudes by which bacteriophages can interact with our microbiota, cells, organs, immune systems, and body. The phage communities found on, within and throughout our bodies and the bodies of other higher vertebrates are diverse, dynamic and pervasive, both adapting with us over the course of our life span and being associated with our health.[17,43,50] Phages residing within our mucosal linings form an adaptive, non-host-derived immunity that may enable additional mechanisms to manipulate bacterial abundances and populations within the gut.[51,72] Once across the mucosal layer phages can interact with and cross the epithelial cell layers, entering both the lymphatic and circulatory systems of the body,[133,134,138,174–176] leading to the establishment a of hypothesised "intra-body phageome." What functions these intra-body phages have on the body and immune system remains largely unknown, but supporting evidence suggests associations with immune tolerance, inflammatory disorders, vertical transmission, and neuronal interactions.[54,172,185,230] The utilization of these phage populations and their biological processes offer unforetold scientific and commercial opportunities for the control and manipulation of microbial, immunological, and cellular processes within our bodies. A combined effort of researchers in fields of bacteriophage biology, microbiology, immunology, cell biology, nutrition, and bioinformatics will be required to obtain a fundamental understanding of these interactions and achieve practical results.

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CONFLICTS OF INTEREST

The author has no conflicts of interest to disclose.

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