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A Mammalian Cell's Guide on How to Process a Bacteriophage

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Abstract

Bacteriophages are enigmatic entities that defy definition. Classically, they are specialist viruses that exclusively parasitize bacterial hosts. Yet this definition becomes limiting when we consider their ubiquity in the body coupled with their vast capacity to directly interact with the mammalian host. While phages certainly do not infect nor replicate within mammalian cells, they do interact with and gain unfettered access to the eukaryotic cell structure. With the growing appreciation for the human virome, coupled with our increased application of phages to patients within clinical settings, the potential impact of phage-mammalian interactions is progressively recognized. In this review, we provide a detailed mechanistic overview of how phages interact with the mammalian cell surface, the processes through which said phages are internalized by the cell, and the intracellular processing and fate of the phages. We then summarize the current state-of-the-field with respect to phage-mammalian interactions and their associations with health and disease states.

1. INTRODUCTION

Bacteriophages, as the name suggests, are eaters of bacteria. Classically they are defined as specialist viruses that exclusively parasitize bacteria by infecting and replicating inside them (1). Estimated to be at global numbers of 10 to the power of 31, they are the most abundant biological entities on the planet (2, 3). In fact, they outnumber all bacteria and archaea combined by a factor of 10 (2, 4) and all human cells in the world by approximately 43 million (2, 5). Within the human body, phages are found not only across all body surfaces, including the skin (6), oral cavity (7), urinary tract (8), and gastrointestinal tract (9), but also within internal fluids and organs, including blood (10), breast milk (11), lung (12), and even traditionally sterile components, such as cerebrospinal fluid (11). Despite the overwhelming abundance of these phages within us, little is known about their interactions with mammalian cells. It is while considering this abundance of phages within our body that their classical definition of exclusive bacterial parasites becomes limiting. While phages certainly do not infect nor replicate within mammalian cells, they do interact with and gain unfettered access to the eukaryotic cell structure (13, 14). In this review, we focus on emerging research that elucidates the mechanistic interactions between phages and mammalian cells, and how these interactions lead to divergent responses that may have broad implications across health and disease states.

2. A PHAGE WALKS INTO A CELL

Considering the bacterial tropism of phages, it is not surprising that their interactions with mammalian cells remain comparatively underdeveloped (15). Yet with the growing appreciation for the human virome (16, 17), coupled with increased use of phages in clinical settings (18, 19), we are becoming increasingly aware of the potential for phages to impact these tripartite systems. While this has led to an increase in correlative studies linking phages with diverse host-derived responses, including inflammation (20), health and disease states (16), and cognitive function (21), there remains a dearth of mechanistic understanding for these interactions. Further, some phages have been identified as highly immunogenic (22, 23), while others are broadly administered to patients in phage therapy applications with minimal immunological response (19, 24). While preliminary studies have identified the pathways phages can use to access the mammalian host cell, the emergent challenge is to now consider the vast diversity of phage species coupled with their potential interactions across divergent cells, tissues, and organs of the body. Importantly, to understand how bacteriophages influence and affect the mammalian system, we first require a detailed mechanistic understanding of how phages gain access to and subsequently influence the mammalian cell.

2.1. Cell Surface Interactions: First Contact

The first step of any phage-mammalian interaction requires the phage gaining proximity to the mammalian cell surface. Due to the enormous size differential between phage particles and the mammalian cell, there is a large capacity for these cell surfaces to interact with and adsorb phage particles. As phage particles are inanimate, this process is largely driven by random Brownian motion resulting in chance encounters between phages (~100 nm) and the mammalian cell surface. These cells are predominantly organized in structured cellular layers, with compacted cell sizes ranging between 10 and 50 μm (25, 26), reducing most phage-mammalian interactions to boundary conditions along the apical cell surface (**Figure 1**).

The first encounter between phages and mammalian cells typically occurs in the outer glycocalyx where a range of secreted and cell surface–attached glycoproteins and glycolipids reside. It is worth noting that bacteriophages have likely spent billions of years evolving mechanisms to interact with bacterial cell surface glycans (27, 28), with the phage tail representing the most abundant

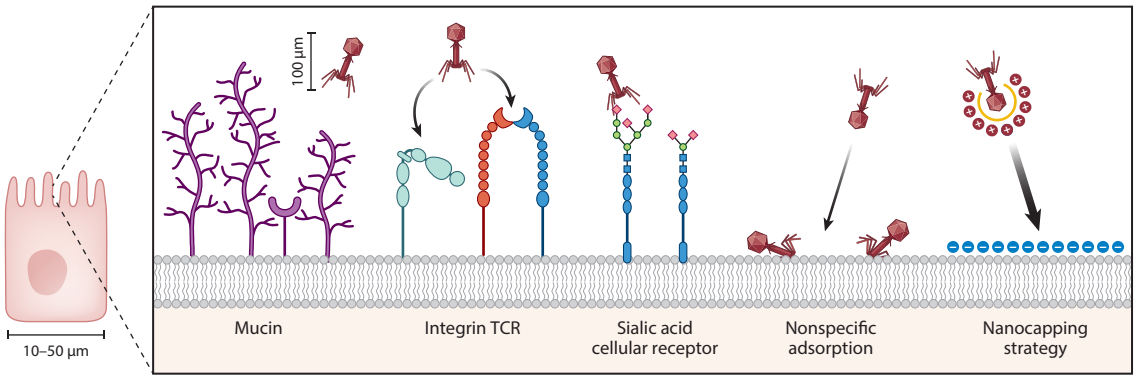

Figure 1

Bacteriophage first contact and surface interactions with the mammalian cell. Phage particles can physically interact with any externally displayed component on the mammalian cell surface. The first interaction is typically with secreted glycosylated mucins, which form a protective layer on many epithelial cell surfaces. Past this layer phages may adsorb directly to integrin and other T cell receptors (TCRs), or alternatively they can directly bind to certain sialic acid residues. Phages can adsorb in a nonspecific manner with the mammalian cell surface, an interaction that can be further enhanced through nanocapping strategies that alter electrostatic interactions.

and diverse glycan-binding structure on the planet. As such, it is perhaps not entirely surprising that phages can also bind to and interact with mammalian-associated glycans. The predominant mammalian-secreted glycosylated compounds are the mucins, which are large, glycosylated proteins that are continually produced by epithelial cell surfaces in locales such as the gut, lung, and oral cavity (29). As these surfaces are in direct contact with the external environments, the mucin glycocalyx acts as a semipermeable barrier that enables efficient nutrient absorption and provides both protection against and niche support for resident microorganisms (30). Certain phage species are known to bind these mucin glycoproteins and while doing so can facilitate a range of symbioses and interactions, including providing an antimicrobial layer of immunity (31, 32), locational targeting of bacterial hosts (33, 34), altered phage infectivity and resistance (35, 36), and the evolution of novel glycan-adherence phenotypes (37).

Past this diffusion-limited glycocalyx, phages can directly interact with the mammalian cell membrane, membrane-bound receptors (including T cell receptors), and other cell-displayed surface structures (38, 39). It is important to emphasize here that phages cannot infect the mammalian cell, but the terminology we use here to describe aspects of this process has parallels with standard phage definitions (e.g., bind, adsorb, internalize). Excluding genetically engineered phage, the potential for a naturally occurring phage to bind a mammalian cell surface receptor that mimics its bacterial host is predictably limited. There are, however, notable examples of this phenomenon occurring. An early study by Dąbrowska et al. (40) found that T4 phage directly bound murine melanoma cells, possibly via interactions between the Lys-Gly-Asp motif on the phage capsid and the β3 integrin receptor. More recently, Lehti et al. (41) demonstrated that the *Escherichia coli* phage PK1A2 bound neuroblastoma cells by adsorbing to the same sialic acid receptor found on its bacterial host. This specific binding to a cellular receptor allowed the PK1A2 phage to be internalized by a receptor-mediated process, although the specific internalization mechanism was not determined in the study by these authors. Similarly, a recent study by Sanmukh et al. (42) demonstrated that both T4 and M13 phage interacted with the human prostate cell line, which led to a subsequent increase in integrin expression. While the exact mechanism here remains to be elucidated, the authors suggested that phage adsorption to cellular integrins resulted in a positive feedback loop leading to increased integrin expression.

While certain phage species are capable of directly binding to mammalian cell surface structures, these specific phage-cell interactions are seemingly rare in the natural world. Despite this, it is expected that most phage particles are capable of nonspecific interactions with the mammalian cell surface. One of the first experimental validations of this was demonstrated by Nguyen et al. (13) whereby naturally occurring phages were shown to nonspecifically interact with tissue culture cell lines. Phages were found to be internalized and trafficked by the cell, facilitating a transcytosis event that was able to transport functional phage particles across the eukaryotic cell architecture. In follow-up work by Bichet et al. (14), it was shown that phages rapidly adsorb to mammalian cell membranes within as short as 30-s exposure, with adsorbed phages being subsequently internalized via nonspecific macropinocytosis events. Macropinocytosis is a process whereby cells create actin-mediated ruffles that elongate from the cytosol and extend the cell membrane toward the external milieu to engulf extracellular fluids, nutrients, and surface-associated microorganisms within large endocytic vesicles (50–1,000 nm) known as macropinosomes. Intriguingly, the work by Bichet et al. (14) also demonstrated that smaller-sized phage particles (<50 nm) were internalized at higher rates. This suggests that smaller-sized phage particles can adsorb to the mammalian surface at a greater density than larger-sized phages. As a result, each subsequent macropinocytosis event internalizes all membrane-bound phage within a given area, leading to increased cellular uptake of smaller-sized phage particles.

As the phage internalization process appears to be limited by the initial phage-cell contact rate, mechanisms to improve this should also enhance phage uptake and internalization. Meng et al. (43) tested this hypothesis using a novel nanocapping strategy whereby phage capsids, which have an inherent net negative charge, were coated by a cationic polymer with minimal loss in bacterial infectivity. This treatment resulted in charge-reversed anti-*Salmonella* N2 phage that were able to adsorb and subsequently enter murine intestinal epithelial cells at greater rates than noncapped virions. Thus, by modulating the electrostatic interactions between phage and mammalian cells, it is possible to increase adsorption and subsequent cellular entry via macropinocytosis processes. This nonspecific macropinocytosis uptake mechanism is likely the predominant interaction that facilitates phage internalization by mammalian cells. It was estimated that the human gut epithelium utilizes these mechanisms to internalize more than 3×10^{10} phages from the gut microbiome every day to provide a circulating intrabody phageome (13, 15). If we further consider the implications of this phage uptake mechanism on phage therapy, it is likely that, following intravenous administration, a significant portion of phages are adsorbed by endothelial cell layers and other organs of the body and subsequently removed from circulation by macropinocytosis events (14). This mechanism may explain the incredibly short half-lives of circulating phages within the blood of both animals and humans (24, 44), which are on the order of minutes to hours. These findings highlight that our mechanistic understanding of phage interactions and spatial affinities with the mammalian cell surface are still limited and that this knowledge has important implications for the later access to the mammalian cell and its downstream effects.

2.2. Phage Internalization: Access All Areas

Following contact with the mammalian cell surface, phages may be internalized via three primary endocytic pathways: clathrin- and caveolae-mediated endocytosis, macropinocytosis, and phagocytosis (**Figure 2**). The routes that phages utilize to gain entry into the mammalian cells establish how the phages are trafficked across different organelles, leading to differing fates. Clathrin- and caveolae-mediated endocytosis constitute the principal endocytic pathway for most mammalian cells. This pathway occurs through an organized multistep process involving the assembly of an ~50- to 100-nm pit where target cargo is concentrated. This pit invaginates and detaches from the plasma membrane, forming a small endocytic vesicle. Importantly, both these endocytic

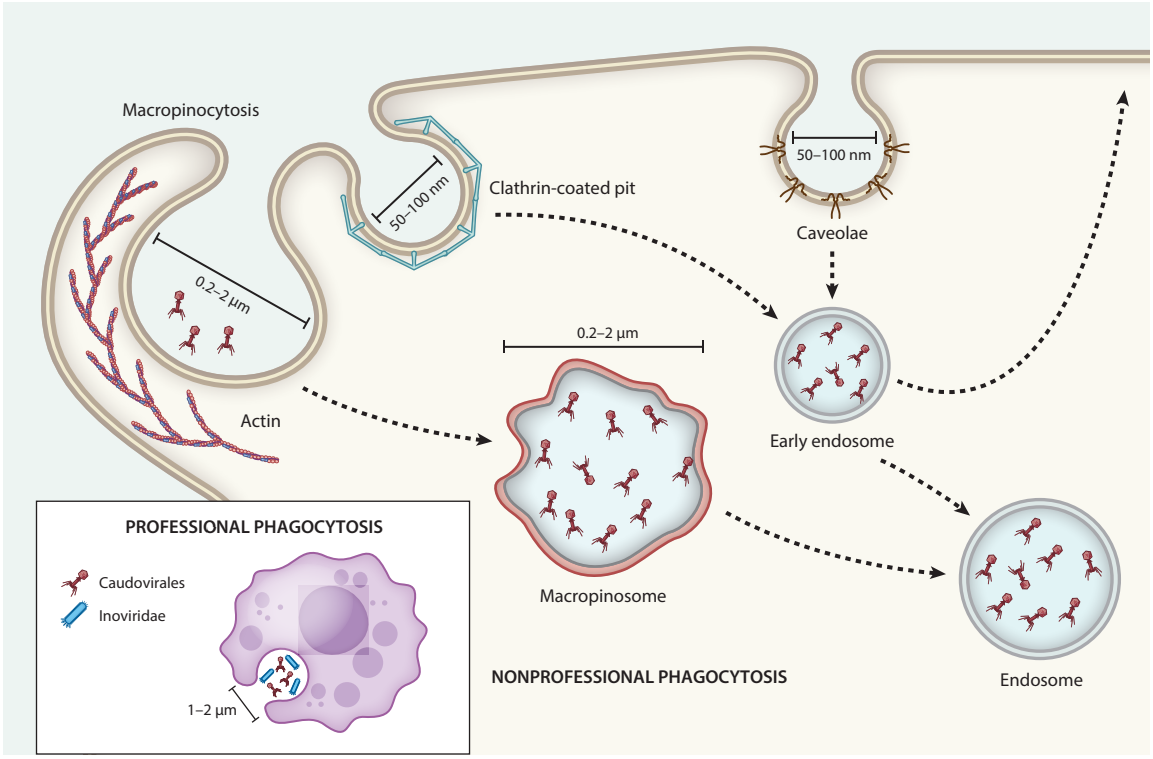

Figure 2

Major endocytic processes involved with bacteriophage uptake and internalization by the mammalian cell. Clathrin- and caveolae-mediated endocytosis is a receptor-mediated process that triggers the formation of a pit (~50–100 nm), leading to internalization; however, there is limited evidence suggesting phages utilize this pathway. Macropinocytosis is a nonspecific uptake pathway that is driven by actin reorganization at the cell membrane leading to the uptake of extracellular milieu (~0.2–2 μm) and is proposed as the primary phage uptake mechanism for nonprofessional cells. Phagocytosis is the cellular uptake of particulates and microorganisms within a plasma membrane envelope (>0.5 μm) and is a known phage uptake mechanism for professional phagocytic cells.

processes require receptor-mediated binding to trigger pit formation and eventual internalization (41, 42, 45). While phages are known to bind specific mammalian surface receptors (41, 42), there is no clear experimental evidence demonstrating that phage uptake occurs via clathrin- or caveolae-mediated endocytosis. Earlier work by Tian et al. (46) demonstrated that the filamentous *E. coli* M13 phage could be internalized by nonprofessional phagocytic cells, including two epithelial (HeLa and MCF-7) and one endothelial cell line (HDMEC), and the use of pharmacological inhibitors suggested the route to be both clathrin- and caveolae-mediated endocytosis. However, due to the size of these filamentous phage particles (>800 nm), it is unlikely they would be internalized via the small ~50- to 100-nm clathrin or caveolae pits, and the authors proposed that broader endocytic mechanisms, including macropinocytosis, were associated with M13 phage uptake.

Macropinocytosis is the nonspecific uptake of fluids and extracellular milieu that is driven by actin reorganization at the cell membrane (47) and leads to the formation of large cytoplasmic vesicles known as macropinosomes (~0.2–2 μm). The role of macropinocytosis has been associated with feeding mechanisms to digest captured proteins or macromolecules. Crucially, this uptake lacks any sorting capacity and forms relatively large endosomes (~1 μm) that are harder to acidify than their smaller endosome counterparts (13, 14, 47). This uptake route appears to

be the predominant mechanism for phage access to the mammalian cell. Using the model phage T4, which is an ~200-nm Myoviridae that infects *E. coli*, Nguyen and colleagues (13) provided a mechanistic breakdown of phage internalization and trafficking across several cell types. Using subcellular fractionation of endocytic vesicles, T4 phages dispersed across all vesicular fractions of the cell and were enriched within the denser endomembrane fractions associated with the Golgi apparatus. Follow-up work by Bichet et al. (14) revealed that cells were capable of accumulating high numbers of phages, with upward of 10^5–10^6 phage per milliliter of cells being observed. There was also large variance in the internalization of phages across cell lines, with certain cell lines having exceedingly high internalization capacities (e.g., A549 lung epithelial) while other cell lines had low capacity for uptake (e.g., HT29 colonic and BJ fibroblast). Once internalized, these phage particles persist within the endomembrane system and its vesicles for hours to days without any loss of phage antimicrobial activity (14). Similar work by Lehti et al. (41) demonstrated that *E. coli* phage PK1A2 internalized by neuroblastoma cells accumulated between 10^4 and 10^5 phages from treated cell layers that persisted within the cells for up to one day without affecting either cell or phage viability.

Finally, phagocytosis is the cellular uptake of particulates (>0.5 μm) within a plasma membrane envelope by professional phagocytic cells, including macrophages, neutrophils, and dendritic cells (DCs), which are responsible for the removal of microorganisms (45). The phagosome then undergoes a series of fusion and fission events with endocytic vesicles that eventually results in the fusion with a lysosome where the gradual decrease in pH leads to the digestion of microorganisms and recovery of antigens for presentation on the surface of the phagocyte and the activation of an adaptive immune response (48). While these uptake pathways have divergent downstream trafficking pathways, they all result in internalized phage particles bounded by membrane-bound vesicles of the cell. Internalization by professional phagocytic cells appears to have the broadest potential to impact mammalian cellular function. Historical work completed by Aronow et al. (49) provided some of the first evidence of phage-mammalian interactions using the *E. coli* T2 phage and rabbit macrophages. Using transmission electron microscopy, the authors presented evidence of T2 phage adsorption to macrophage cell membrane followed by internalization likely through a combination of phagocytosis and macropinocytosis. They specifically highlighted that the interactions between phages and macrophages were a prerequisite for the immunogenic information and subsequent antiphage antibody production, a phenomenon that is now well established (50–52). More recent work by Kaźmierczak et al. (53) studied the interaction between engineered T4 phage displaying a fused green fluorescent protein (GFP) on its capsid along with murine macrophages. The authors showed that the amount of GFP-labeled phage uptake was dependent on incubation time, with fluorescent-positive macrophages detected ~20–40 min post phage incubation. In seminal work by Sweere et al. (23), the filamentous Pf phage that is produce by *Pseudomonas aeruginosa* was demonstrated to be internalized by both human and murine leukocytes and resulted in an antiviral response and antagonized production of cytokines (23, 54). In this work, the authors used a range of professional phagocytic cells, including leukocytes, monocytes, splenocytes, B cells, and DCs, that were all demonstrated to internalize the filamentous Pf phage. These viruses are from the Inoviridae family and have a single-stranded DNA genome packaged within a helical filamentous structure with the virion particles being ~7 nm in diameter but up to ~1–2 μm in length (54, 55). Due to the filamentous Pf virion size, it is likely these particles are preferentially internalized via phagocytosis, which can uptake particles greater than 0.5 μm in size. However, due to their flexibility, it is also possible these virions are internalized via macropinocytosis via wrapping and formation of spaghetti-like structures at the mammalian cell surface (54, 55). Once internalized, Pf phage were trafficked through a range of endosomes and lysosomes, which facilitated interactions with the Toll-like receptors (TLRs) (discussed further below). This was further

confirmed by treatment of the cells with chemical inhibitors for endocytosis, vesicular transport, and microtubule assembly, all of which are consistent with phage trafficking through membrane-bound vesicles. These studies demonstrate that Caudovirale phages and larger filamentous phages can access the mammalian cell through phagocytosis and potentially other endocytic routes.

2.3. Cellular Processing: What Is Thy Fate?

It is evident that several cell types are able to internalize a diverse range of phages through a combination of macropinocytosis and phagocytosis, with preferred uptake route likely being heavily influenced by the mammalian cell type (i.e., professional versus nonprofessional phagocytic cells) (48). Uptake via both of these routes results in phage particles residing within endocytic vesicles within the mammalian cell. Membrane-bound phages can stably persist within tissue culture cells for a number of days and can distribute throughout the endomembrane system (13, 14, 41). In some cases, phages may persist in the cell and trigger a cascade of cellular pathways, leading to a diverse range of functions and fates that await the mammalian-engulfed phages (**Figure 3**).

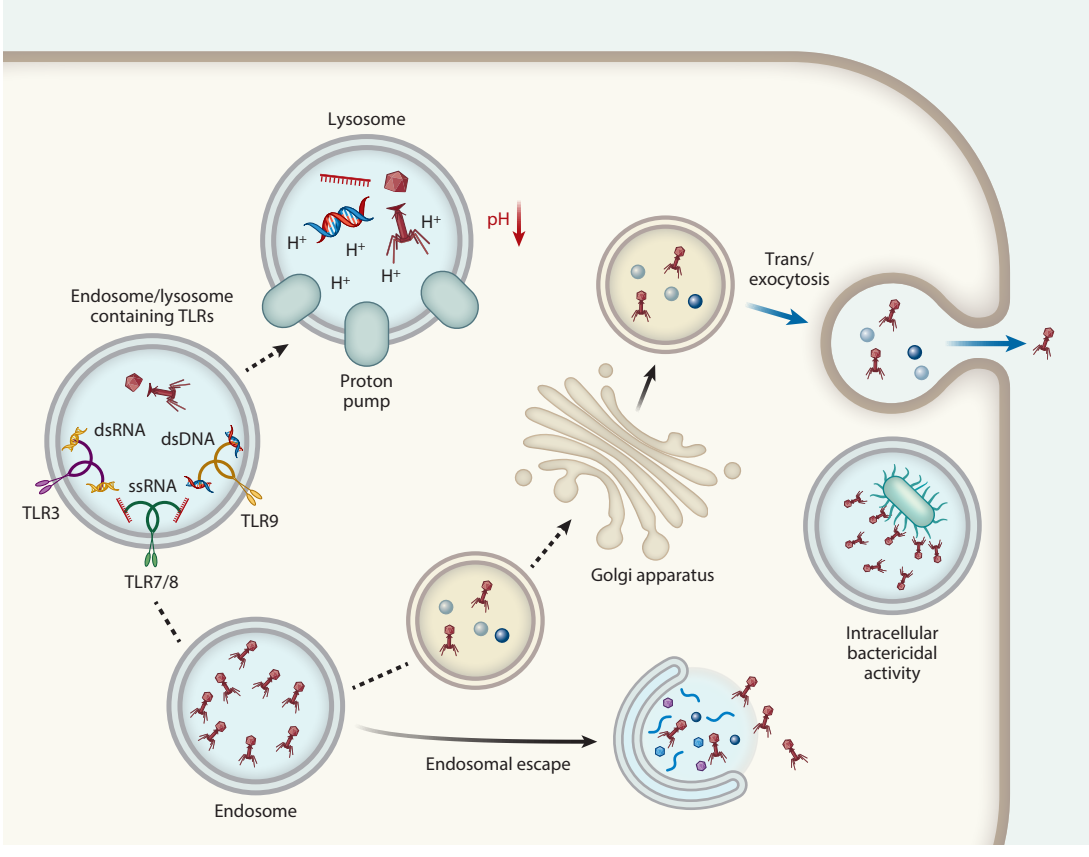

Figure 3

Intracellular processing and fate of internalized bacteriophages. Membrane-bound phage particles and their components may interact with endosomal Toll-like receptors (TLRs) leading to downstream immune response. Alternatively, phage-containing endosomes may fuse with lysosomes leading to phage degradation, may be trafficked across the cell via the trans-Golgi network leading to exocytosis, or could fuse with other vesicles containing membrane-bound intracellular bacteria to facilitate phage infection and lysis of the bacterial host. Finally, phage may escape the endosome leading to release into the cellular cytoplasm.

Following cellular uptake, phages are constrained within the intracellular endocytic vesicles. From here, phages or their components can stimulate TLRs and initiate a cascade of inflammatory reactions. TLRs were the first pattern-recognition receptors (PRRs) to be characterized, and just like any other PRRs, they serve an important role in immune defense by detecting microbial pathogens through the recognition of pathogen-associated molecular patterns (56). TLRs are synthesized in the endoplasmic reticulum, trafficked to the Golgi, and eventually either localized to the cell surface or remain within the endomembrane system (57). Importantly, surface resident TLRs (including TLRs 1, 2, 4, 5, and 6) predominantly recognize microbial ligands, such as lipopolysaccharide, lipoproteins, and flagellin, while endosome TLRs (including TLRs 3, 7/8, and 9) recognize microbial-derived nucleic acids, including double-stranded DNA (dsDNA), single-stranded RNA, and double-stranded RNA (dsRNA). In particular, TLR9 is a sensor of viral DNA, and it has been experimentally demonstrated that phages (and their DNA) from *Lactobacillus*, *Escherichia*, and *Bacteroides* were able to stimulate interferon-γ (IFN-γ)-mediated immune response via TLR9 in vivo (20). Similarly, Pf phages were found to trigger TLR3 response, which recognizes dsRNA in endosomes, leading to inhibition of tumor necrosis factor (TNF) and ultimately the suppression of phagocytosis (23). Other potential phage-sensing TLRs, including TLR2, TLR4, TLR7, and TLR8, may also respond to the intracellular presence of phages due to their ability to recognize viral components, albeit this has not yet been directly observed (56). The stimulation of intracellular receptors can trigger a cascade of inflammatory signaling pathways. In the case of Pf4, stimulation of TLR3 enhanced the production of type I IFN and inhibited TNF production, consequently promoting bacterial infection in the mammalian host (23). M13 phages used in phage display were shown to induce proinflammatory cytokines in murine macrophages in a TLR-dependent manner (58). It remains in contention whether phages can be broadly grouped as proinflammatory or anti-inflammatory entities.

Once internalized, it is likely that a significant proportion of phages are simply degraded by the mammalian cell's machinery. Using the *E. coli* phage K1F, Møller-Olsen et al. (59) demonstrated that phages were unable to escape the phagosome and were degraded via LC3-assisted phagocytosis in a human bladder epithelial cell line. Earlier work by Volcy & Dewhurst (60) using lambda phages in embryonic kidney cells tested a range of pharmacological inhibitors of cell uptake and degradation pathways to determine whether and how these phages were escaping the endomembrane system. Inhibitors of both lysosomal proteases and proteasome increased phage persistence and endocytic escape, while endosome acidification inhibitors had no effect, suggesting that the proteasome complex and lysosomal proteases each impose degradative capacity for internalized phages (60).

Alternatively, phages may evade degradation and undergo intracellular transport. Systematic interrogation of the intracellular pathway revealed that phages likely traffic mammalian cells through the Golgi apparatus via the endomembrane system (13). Eventually these phage-containing membrane-bound vesicles are either recycled back to the plasma membrane, thereby releasing their phage cargo (61), or trafficked further through the endomembrane system. Following trafficking through the Golgi, phages can be exocytosed either in an apical to basolateral direction in polarized cells or recycled and released back across the cell membrane in nonpolarized cells (13). This transcytosis of phage particles was demonstrated to be inhibited by the pharmacological agent Brefeldin A, which inhibits post-Golgi-membrane traffic. As such, the intracellular trafficking of phage particles appears to route via the trans-Golgi network into secretory vesicles (62) that fuse with the cellular plasma membrane to discharge the functional phage particles via exocytosis back into the surrounding milieu. In fact, it was suggested that phage transcytosis is a natural phenomenon that happens to an estimated 31 billion phages every day in a single person, from the gut lumen through the epithelial cell layers into the human body (13). What is even more

fascinating is that phage transcytosis was also observed in a variety of cell layers originating from the lung, liver, kidney, and brain as well, implying this is a universal phenomenon independent of cell types.

This knowledge that phages can enter and traffic throughout the mammalian cell enables approaches to kill intracellular bacterial pathogens. Using the pathogen *Staphylococcus aureus*, which is able to internalize both professional and nonprofessional phagocytic cells, Zhang et al. (63) demonstrated the intracellular killing by the virulent Myoviridae phage vB_SauM_JS25. They found that the longer cells were incubated with phage, the greater the bacterial killing, indicating the phage crossed the cellular membrane and killed intracellular *S. aureus*. Subsequent work by Møller-Olsen et al. (59) fluorescently labeled the *E. coli* phage K1F, demonstrating the phage was internalized by phagocytosis and colocalized with its pathogenic bacterial host within the phagosome causing infection and lysis of the bacteria in the human cell environment (59).

It is well recognized that mammalian cells can transcribe phages' genetic material (64), a characteristic that has been exploited in biomedical research and even gene therapy (65, 66). In fact, the very first scientific evidence that mammalian cells can transcribe phages' DNA came from the gene therapy attempt to introduce a functional version of α-D-galactose-1-phosphate uridyl transferase carried on the lambda phage genome into human fibroblasts from a patient with congenital lack of transferase activity (64). Intriguingly, both lambda-specific RNA and transferase enzyme activity were detected for up to 41 days post phage application with no decrease in levels. This suggests that phage nucleic acid was preserved in these cells by an unknown mechanism. Later research demonstrated that phage DNA can in fact integrate directly into the mammalian cells' genome via site-specific integration mediated by phage integrases (67). In fact, many terminal proteins of phages contain eukaryotic nuclear localization signals, suggesting a ubiquitous biological phenomenon where phages' genetic material can reach the mammalian cell nucleus and facilitate horizontal gene transfer (68). Once integrated, phages may influence the function of eukaryotic cellular genes by increasing DNA methylation (69).

Collectively, these studies suggested that phages can escape the endomembrane system and gain access to the broader mammalian cell cytoplasm. This represents an important mechanistic knowledge gap on how phage particles escape endocytic vesicles and enter the eukaryotic cytoplasm. Once in the cytosol, it has also been suggested that phages' genetic content could stimulate the cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes pathway, which senses cytosolic DNA and produces an inflammatory cellular response (20). The previously discussed nanocapping of phage capsids with a cationic nanopolymer demonstrated enhanced endosomal escape, which was thought to occur via osmotic vesicle swelling and endosomal membrane disruption, thereby releasing phages into the cytoplasm (43). Once there, nanocapped phages were found to inhibit the growth of the intracellular pathogen *Salmonella typhimurium*, showing a significant reduction in intracellular bacterial load over noncapped phages both in vitro and within a mouse intestinal model (43). Finally, an intriguing follow-up study by Zhang et al. (70) demonstrated that treatment of murine macrophage cells with anti-*Staphylococcal* phage vB_SauM_JS25 inhibited the replication of murine Norovirus (70). The authors then applied phages to macrophage cell lines and performed transcriptomics to highlight the increased expression of guanylate-binding proteins (GBPs), which are interferon (IFN)-inducible GTPases that have central roles in cell-autonomous immunity. Importantly, GBPs target and lyse the membranes of pathogen-containing vacuoles, destroying the resident niche and restricting the replication of intracellular protozoal, bacterial, or viral pathogens (71). In this study, Zhang and colleagues (70) suggested that phages may induce GBPs, which subsequently target and lyse phage-containing (and in this instance norovirus-containing) vesicles to disrupt and inhibit norovirus replication. It is intriguing to speculate whether phage-containing vesicles can recruit and activate GBPs, thereby facilitating

a consistent mechanism of cytoplasmic release of membrane-bound virions. In summary, there is emergent evidence that once phage particles are internalized, their cellular distributions and effects are varied but are dependent on both the phage species and the cell type.

3. PHAGE INFLUENCE ON THE MAMMALIAN CELL

In the previous section we examined with a mechanistic lens how a bacteriophage particle interacts with, is internalized by, and is processed throughout the mammalian cell architecture. We now deviate from this perspective and begin to look at the emerging evidence that links phage-mammalian interactions with broader health and diseases states (16, 20, 23). Phages are ubiquitous in the human body, and similarly their capacity to interact with the mammalian host is vast. The array of phage species coupled with the diverse mammalian cell types and route of interaction has resulted in a complex matrix with highly varied responses and outcomes. In certain examples, phages appear to mediate inflammatory responses (20), whereas other scenarios suggest an anti-inflammatory effect (72). It is likely that this phage influence spans the gamut of beneficial through to harmful. Thus, it is of paramount importance to consider that phages (even the same species) may behave differently when in contact with varied mammalian cell types, organs, or systems. Again, we emphasize the dissonance between the more mechanistically focused studies in the section prior and the predominantly correlative studies we discuss below. Importantly, if we are to move beyond associations between potentially disease-causing phage species across mammalian locales, then we must decipher the mechanism of phage-cellular interaction at play.

3.1. Mammalian Virome: Expansive Diversity

It is important to touch on the enormous diversity seen within the mammalian virome and to emphasize how much exploration remains within this ecosystem. Recent work by Shkoporov et al. (17) dissected the viral biogeography of the mammalian virome across two species: the rhesus macaque and the domestic pig. Through careful dissection and shotgun sequencing of the virome, the authors uncovered an abundant phage community, approximately half of which were broadly like previously sequenced viruses, but the remaining half were identifiable as viral only through a de novo assembly approach. Comparing this virome across the gastrointestinal tract revealed extensive mixing of high-abundance gut phages from the caecum through to the colon, highlighting the potential interaction of these viruses across the entirety of the mammalian gastrointestinal tract and its cellular surfaces. Intriguingly, the authors found that the liver, lung, and spleen shared genomic sequences with gastrointestinal phages, suggestive of a translocation mechanism (15, 17). There was a further tendency toward the enrichment of smaller phages in these parenchymal organs, particularly the Microviridae (17), which is in line with mechanistic observations of increased mammalian uptake of smaller viral particles (14). Continuing with the exploration of this intrabody phageome, Haddock et al. (10) performed sequencing of cell-free DNA from bodily fluids and plasma samples collected across three independent human cohorts consisting of infected sepsis patients and healthy asymptomatic controls. The authors demonstrated that all individuals regardless of disease state had a circulating phageome, but that infected patients had an overrepresentation of phages associated with the coinfecting pathogen. While this study was framed toward the detection of circulating phages associated with bacterial sepsis (10), it remains to be determined what the origin of this phageome was, with the potential for a proportion of these intrabody phages to have originated from the gut microbiome through translocation processes (15, 73). Together, these studies emphasize the largely uncharted diversity of the mammalian virome and allude to the extensive interaction, internalization, and trafficking of phages across the body, setting the scene for their correlation with health and disease states.

3.2. The Healthy Virome: Expectant Correlations

The concept of a healthy virome that contributes toward a stable and balanced gut microbiome was recently explored by Manrique et al. (74) whereby they identified clusters of core bacteriophages present in over half of human gut viromes. These phage clusters were also negatively correlated with inflammatory gastrointestinal disease states. While this healthy virome was proposed to modulate its purported health benefits exclusively through the modulation of the gut bacterial community, we are now beginning to appreciate the additional complexities of direct phage-mammalian interactions. It is well established that phages can elicit immune responses and dampen inflammatory pathways (23, 72). Directly testing this hypothesis, Van Belleghem et al. (72) exposed peripheral blood monocytes to 5 different phages and assessed the gene expression profile of immunity-related genes. Through RNA sequencing, a total of 359 differentially expressed genes were identified to be affected by phages. This was followed up by quantitative PCR validation of 12 immunity-related genes. Strong and reproducible immune responses were induced by the phages, and intriguingly, the immune responses elicited by the 5 different phages were largely very similar (72). They concluded that while the 5 diverse phages stimulated a comparable immunological response, there was no definitive pro- or anti-inflammatory signal and it remained unclear whether the response was initiated by whole phage particles or phage-specific components (e.g., phage capsid protein).

In one of the most comprehensive and indicative studies demonstrating the immunomodulatory effect of phages completed to date, Adiliaghdam et al. (16) investigated the potential of healthy gut virome samples to induce a consistent anti-inflammatory phenotype. This study was a comprehensive effort at characterizing viral populations within the upper intestinal tissue, which provide better representations of viruses in the gut than those found in fecal samples. A methodology distinction to note is that the authors prepared viromes from colon resections from noninflamed tissues, followed by 0.22-μm filtration and chloroform treatment to isolate virus-like particles (VLPs), which were found to include both bacteriophages and eukaryotic viruses. Viruses resident to the healthy gut predominantly suppressed the host innate immune response through the broad downregulation of classical antiviral response along with an array of intracellular viral receptors and adaptors. This study also provided one of the first mechanistic and correlative links between the healthy gut virome, whereby resident viruses were readily endocytosed by both primary human macrophages and intestinal epithelial cells, which consistently promoted the production of anti-inflammatory cytokines (16). Crucially, the study also demonstrated that viromes from inflamed or disease patients induced a divergent inflammatory response, which we dissect further in the following section.

3.3. Phages and Disease States: A Signal for Inflammation

Phage virions or their components (DNA, RNA, and protein capsids) can elicit inflammatory immune responses (16, 20, 72). Above we described the approach by Adiliaghdam et al. (16) demonstrating that VLPs from healthy patients induce an anti-inflammatory immune response. The authors further demonstrated that VLPs from inflammatory bowel disease (IBD) and Crohn's disease (CD) patients when exposed to human peripheral blood-derived macrophages revealed prominent induction of inflammatory pathways compared to healthy controls. Similarly, when these VLPs were exposed to intestinal epithelial cells, a proinflammatory response was preferentially induced by the IBD viromes. Most VLPs from the human colon tissue were classified as dsDNA phages, and there was a significant increase in phage reads from CD patients. Differential analysis of the patient viromes did reveal the significantly elevated level of enterovirus B from the eukaryotic picornaviruses in IBD samples compared with non-IBD controls, although there

was no causative link that this specific viral species drove the inflammatory signals. The authors went on to demonstrate that viral nucleic acid sensing by the mammalian host viral receptors, including retinoic acid–inducible gene I, melanoma differentiation–associated protein 5, and cGAS, was absolutely required for the immunomodulation by both healthy and IBD viromes (16). It was proposed that this distinct immunomodulation between health and disease state was the result of fluctuations in certain viruses, which led to deviations in the RNA and DNA moieties that were sensed by the intestinal epithelium PRRs, providing some of the first evidence that the composition of the mammalian phageome has direct functional consequences on the host immune system. Two important questions remain from this study. First, how was the phage DNA or RNA exposed within the intestinal epithelial cells and to which nucleic acid sensor? Second, is there an inflammatory viral community responsible for inflammation, and if so, which species or nucleic acid moieties were the trigger?

Reports of specific phage species triggering inflammatory responses have been described elsewhere. Gogokhia et al. (20) revealed that *Bacteroides*, *Escherichia*, and *Lactobacillus* phages and their genetic materials could stimulate TLR9 and upregulate IFN-γ in germ-free mice. They began by examining the gene expression data of mice administrated with an oral phage cocktail along with the surprising discovery that a substantial number of immune pathways were upregulated in the treatment versus the control group. It was proposed that DCs sampled these orally applied phages while in the intestine, with unmethylated phage DNA being sensed by TLR9 within endosomes and finally presented to $CD4^+$ T cells. This triggered a cascade of inflammatory responses mediated by IFN-γ and led to an exacerbated phenotype of colitis. The involvement of TLR9 and IFN-γ was confirmed by the observation that TLR9 and IFN-γ null mice were unaffected by these phages. This was coupled with a clinical observation that ulcerative colitis (UC) patients responding to fecal microbiota transfer had reduced phages and mucosal IFN-γ compared with nonresponders, reinforcing the idea that phages may have a role in eliciting inflammation in UC (20). Mechanistically, phages from active UC patients also induced higher IFN-γ production than those from healthy donors in naïve $CD4^+$ T cells (20).

In a final example, Sweere et al. (23) described an example of phage-induced inflammation from a pathogenic point of view. The remarkable discovery stemmed from a clinical observation that the filamentous Pf phage were associated with chronic wounds in *P. aeruginosa* infection (23). Pf phages are known to contribute toward biofilm formation of their bacterial host and increase their tolerance to antimicrobial treatment (75). However, it was not known that Pf phages could directly interact with the mammalian immune system to enhance pathogenesis. The authors then experimentally validated that Pf phages contributed to *P. aeruginosa* pathogenesis by inhibiting phagocytosis, thereby reducing bacterial engulfment by professional phagocytic cells in the wound environment. Intriguingly, Pf phage achieved this by first being endocytosed by leukocytes, which led to the subsequent synthesis of Pf phage RNA by the mammalian cells by a yet-to-be-determined mechanism. This further stimulated the antiviral PRR TLR3, leading to Toll/IL-1R domain-containing adaptor-inducing IFN-β–dependent type I IFN production, which subsequently inhibited TNF and ultimately suppressed phagocytosis, allowing *P. aeruginosa* to evade immune clearance (23).

It is worth mentioning that most of the previous studies looking into the interaction between phages and the mammalian immune system focused primarily on disease states. As such, the immunological effects of phages in a healthy state or the interaction between phages and noncanonical immunity-related cell types remain comparatively understudied. It is important to recognize within this context that phages are highly divergent—there are genetic variations across different phyla and strains or even among each viral entity—with the preliminary diseased-focused correlative evidence illuminating certain viral species or moieties that disproportionately

contribute to an inflammatory response. Similarly, which cell or cells of the mammalian immune system first encounter the phage and by which mechanism these particles are internalized and trafficked likely further impact this outcome.

4. CONCLUSIONS AND FUTURE DIRECTIONS FOR THIS EMERGING FIELD

Bacteriophages are enigmatic entities that continually defy definitions. Classically, phages are strict predators of their bacterial hosts. Yet as we have covered within this review, these definitions are restrictive when considering their broader tripartite systems. This emerging field of phage-mammalian interactions is potentially as broad and diverse in their effects as seen within the bacterial-mammalian counterpart. Yet our understanding of this emerging field is very much still in its infancy. While the studies covered in this review provide a preliminary insight into the mechanisms and potential impacts on health and disease, there remain many unanswered questions and implications. Central to this is the dizzying diversity seen within the human virome coupled with the preliminary evidence that the mammalian response to a given phage is driven not only by the phage species itself but also by the cell type it encounters and the mechanism by which it is internalized and processed by the cell. This leaves us with a compounded interaction matrix that seems unattainable to decipher. While several studies have proposed correlations between inflammatory disease states and the virome, many still lack the mechanistic understanding needed to interpret and link these responses. All of this further comes on the precipice of our increased application of phages to human patients in attempts to combat antibiotic-resistant bacterial infections. While currently available data suggest that virulent phages are safe to use, we should continue this application with care, as emerging evidence does suggest that certain phage species are associated with inflammation and disease states. Finally, we should remove our blinders and look beyond diseased and dysbiotic systems to explore the potential symbiotic interactions, positive feedback loops, and broader implications that phage-mammalian interactions may have across diverse ecosystems.

DISCLOSURE STATEMENT

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