CellPress

Trends in Microbiology

Review

The Enemy of My Enemy: New Insights Regarding Bacteriophage–Mammalian Cell Interactions

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Bacteriophages (phages) are the most abundant biological entity in the human body, but until recently the role that phages play in human health was not well characterized. Although phages do not cause infections in human cells, phages can alter the severity of bacterial infections by the dissemination of virulence factors amongst bacterial hosts. Recent studies, made possible with advances in genome engineering and microscopy, have uncovered a novel role for phages in the human body – the ability to modulate the physiology of the mammalian cells that can harbor intracellular bacteria. In this review, we synthesize key results on how phages traverse through mammalian cells – including uptake, distribution, and interaction with intracellular receptors – highlighting how these steps in turn influence host cell killing of bacteria. We discuss the implications of the growing field of phage–mammalian cell interactions for phage therapy.

Highlights

Phages can enter mammalian cells through similar pathways to mammalian viruses.

With high-resolution microscopy and fluorescent reporters, phages have been found inside endosomes, lysosomes, Golgi, cytoplasm, and the nucleus of mammalian cells.

Inside endosomal compartments, phages can activate Toll-like receptors, stimulate cytokine expression, and alter immune cell polarization.

In both the phagosome and the cytosol, phages can interact with and kill host bacteria.

Phage DNA can reach the nucleus of mammalian cells and express native genes.

How Phages Influence Human Health

The most abundant biological entity on Earth is the phage – a virus that only infects bacteria. Close to 10^{31} phages populate diverse ecosystems such as the ocean, soil, and the human gut microbiome [1]. Recent work has uncovered a healthy phage-ome within the human body that influences the stability of the gut bacterial community [2–4]. Phage-induced changes to susceptible hosts in the bacterial microbiome can ultimately change the composition of nonhost bacteria through network effects, which influence the composition of the bacterial-derived metabolome [5]. Beyond manipulating bacterial populations, phages can directly alter the pathogenicity of bacteria through horizontal gene transfer, a process that disseminates virulence factors and antibiotic-resistance genes [6]. Furthermore, transcription of phage genes leads to higher expression of phage-encoded toxins such as the Shiga toxin in pathogenic *Escherichia coli* [7]. Thus, phages affect human health and disease both directly and indirectly.

In the body, phages directly encounter human cells, as has previously been reviewed [8–11], and niches exist in almost every organ – the skin, mouth, lungs, urinary tract, and even the brain, with the gut having the highest estimated abundance of phage, more than two trillion [9]. Innovations in genome engineering, such as the placement of fluorescent proteins into phage genomes, and microscopy, are now allowing for the interactions between phages and human cells to be delineated. It is now possible to observe phage **lysogenic** (see Glossary) or **lytic** life cycle dynamics in individual cells in a high-throughput manner [12,13]. Phage genomes, which range in size from 2.3 kb to greater than 540 kb [14], are difficult to engineer because capsids have limited tolerance for change in genomic DNA size, and many phage genes have unknown function. Additionally, making many modifications to phage genomes in parallel is difficult with traditional cloning techniques but is more efficient with recently developed yeast-based recombineering methods [15,16]. In addition to advances in engineering, high-resolution microscopy techniques

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have allowed the visualization of phages in precise intracellular compartments. Super-resolution microscopy of green fluorescent protein (GFP)-tagged *Pseudomonas* phage P8 revealed that splenic macrophages directly uptake and degrade phage particles [17]. Similarly, dendritic cells can phagocytose *Pseudomonas* phage Pf and activate Toll-like receptor (TLR) 3 as observed by confocal microscopy [18], suggesting that intracellular phages manipulate mammalian cell behavior.

Rekindled interest in the field of **phage therapy** has brought about key developments in the understanding of phage–mammalian cell interactions. Phage therapy is not yet widely accepted in Western medicine but offers a potentially game-changing solution to the rise of, and threat posed by, antibiotic-resistant bacterial infections (also reviewed by Altamirano and Barr [19]). Characterization of phage–mammalian interactions will inform the pharmacological properties of phages, including distribution, half-lives, and toxicity in the body.

In this review we discuss how phages gain entrance to mammalian cells and which organelles phage particles can access. Next, we cover how intracellular location influences the host immune response to phage and how functional phage particles remain active against bacteria in different organelles. Finally, we examine how phages act as a source of foreign DNA in the nucleus with the potential for transcription and genomic integration. As shown in Figure 1, Key Figure, our review focuses on each of these areas – with special attention to the immune response to phage, the functionality of phage proteins and nucleic acids in various locations, and the beneficial and/or harmful cues phages may provide to mammalian cells.

Mechanisms of Phage Entry into Mammalian Cells

Phages have the potential to enter mammalian cells by taking advantage of nonspecific uptake mechanisms such as phagocytosis, as shown in Figure 1A and Figure 2 pathway 1 (see also Table 1 for a comparison to eukaryotic viruses) [17,18,20–22]. For example, the **filamentous** *E. coli* phage M13 can enter epithelial and endothelial cells through either clathrin-mediated endocytosis, macropinocytosis, or caveolae-mediated endocytosis [20], and filamentous *Pseudomonas* phage Pf4 enters monocytes by clathrin-mediated endocytosis involving vesicular transport and microtubule assembly [18]. Finally, epithelial cells from colon, lung, liver, and brain can transcytose T-odd phages of *E. coli*, T4-like phages of *Bacillus*, and P22 of *Salmonella* [22]. Tissue type of origin (for epithelial cells, lung is best, followed by cervix and then skin), and the size of phage particles, play a role in propensity of uptake for three *E. coli* phages [23]. Whether phages enter mammalian cells through transcytosis [22] and macropinocytosis [20] predominantly *in vivo*, and whether prevalent gut phages such as crAssphage [24] utilize the same pathways, remain to be characterized.

Phages also enter mammalian cells through pathways involving cell-surface receptors, (Figure 2, pathways 2 and 3), similar to eukaryotic viruses (Table 1). Eukaryotic viruses coated with antibodies can enter monocytes by binding to antibody receptors – leading to antibody-dependent enhancement, whereby the antibody fails to neutralize the virus and instead leads to uptake (Table 1). Phage λ coated with poly clonal antiserum against a capsid protein can enter kidney fibroblasts *in vitro* expressing the receptor FcγRI, through receptor-based and clathrin-mediated endocytosis [25], and this may occur during phage therapy if phages are delivered in high titer for a long duration, inducing an adaptive immune response, as observed in mice dosed for at least 15 days with 10^9 plaque-forming units/ml of *Staphylococcus aureus* phages [26]. Phages can also use receptor molecular mimicry, as *E. coli* phage PK1A2 enters neuroblastoma cells through an endosomal route by binding to a surface protein, NCAM, that contains polysialic acid like the bacterial receptor [27].

Glossary

Attachment sites: for lysogenic phages, the locations on the phage genome and the bacterial genome where there is recombination that leads to phage integration.
Filamentous: refers to a type of phage with a single-stranded DNA genome that has a morphology that includes long filaments. These are typically lysogenic phages but they cannot lyse their host and, instead, are secreted during the lytic cycle.
Integrase: a phage recombination enzyme that directs site-specific integration of a lysogenic phage genome into its host.
Lyse: phage-mediated killing of a bacterial host; lysis is typically due to secreted phage proteins that destabilize the bacterial cell wall and burst out. The noun form is lysis.
Lysogen: a bacterium that stably carries a phage (as a plasmid or genomic integration).
Lysogenic: refers to a type of phage that can either undergo a lytic cycle or integrate its genome into its host.
Lytic: refers to a type of phage that has one life-cycle that involves DNA injection, genome replication, and killing of its bacterial host.
Pathogen-associated molecular patterns (PAMPs): bacterial-derived products, such as lipopolysaccharide, proteins, and nucleic acids, that stimulate surface and interior receptors on immune cells.
Pattern-recognition receptors (PRRs): innate immune receptors that recognize PAMPs.
Phage therapy: dosing patients with phage particles against a specific species of bacteria that is causing an infection.
Phagosome: a vesicle, formed in cells such as macrophages, neutrophils, and dendritic cells, where the plasma membrane engulfs a bacterium. Phagosomes can mature to phagolysosomes by fusion with lysosomes, leading to harsh environmental conditions such as acidic pH, proteases, and reactive oxygen species.
Prophage: a phage genome carried by a lysogen that is dormant (not undergoing genome replication). Prophages can be integrated into the host bacterium's genome or on mobile elements such as plasmids.

Key Figure

An Overview of How Phages Enter and May Interact with Mammalian Cells and the Implications for Phage Therapy

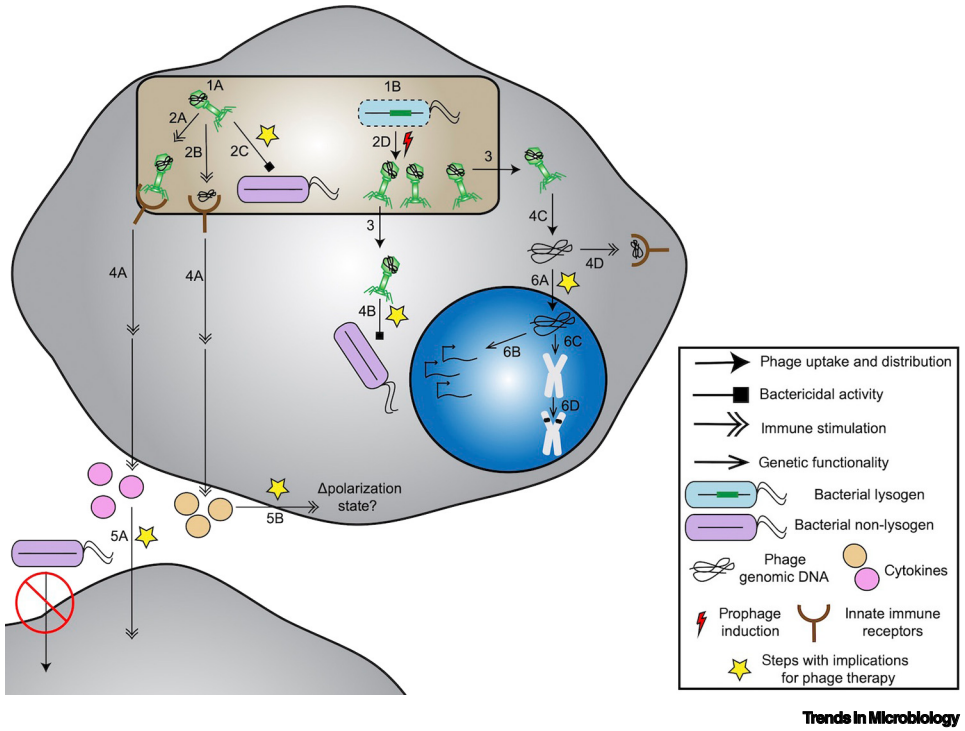

Figure 1. (1) Phages can enter mammalian cells either (A) directly through nonspecific uptake mechanisms [17,18,20–22] or (B) indirectly through a bacterial carrier [9,29,30]. Phage uptake can reduce half-life for therapy. (2) Once inside endosomal compartments, phages have the potential to (A) stimulate endosomal receptors, (B) uncoat and release nucleic acids that trigger the activation of immune receptors such as the Toll-like receptors (TLRs) [18,43], (C) kill other nonlysogenic bacteria present [29,30], or (D) undergo prophage induction and release from internalized lysogenic bacteria undergoing prophage induction [13]. Characterization of intracellular killing by phages will inform pharmacodynamics (PD) of phage therapy for intracellular pathogens. (3) Phage particles may escape endosomes and reach the cytoplasm. (4) In the cytoplasm, (A) a signaling cascade from TLRs activated in the endosome could lead to the upregulation of cytokine expression [18,41], (B) phage particles could infect and kill cytoplasmic bacteria, implicating PD, or (C) phage particles could be degraded by the proteasome, leading to the release of phage nucleic acids [54]; (D) cytoplasmic nucleic acids could trigger receptors such as cGAS-STING or RIG-I. (5) Phage-derived products may influence mammalian cell physiology, in turn, influencing the bacterial-killing ability of immune cells, a potential for either efficacy boost or toxicity of phage therapy. Cytokines released from phage-infected mammalian cells may (A) be involved with paracrine signaling to other cells, and alter the propensity for bacterial uptake [18], or (B) change macrophage polarization to an M1 inflammatory state through autocrine signaling [49]. (6) Nucleic acids released from phages may interact with host DNA in the nucleus, a potential toxicity of phage therapy. If (A) phage genomic DNA reaches the nucleus, then (B) native phage genes may be transcribed [71] or (C,D) may be integrated and persist long term [69,70].

Prophage induction: a bacterial stress response whereby a prophage excises from the genome of its host, replicates, and undergoes the lytic cycle.
SOS response: the bacterial stress response pathway to DNA damage mediated, at least in *Escherichia coli*, by the gene *recA*. The SOS response fixes double-stranded DNA breaks.

One mechanism by which a large amount of phage particles could enter phagocytic cells, the most likely cell type that phages may encounter [28], is through a Trojan-horse, bacterial carrier (Figure 2, pathways 4 and 5). Lytic phage applied to *Chlamydia* infected-HeLa cells attached to extracellular *Chlamydia* and replicated, and **lysed** the bacterium once phagocytosed [29]. Similarly, lytic phage TM4 applied to *Mycobacterium smegmatis* – prior to infection into *Mycobacterium tuberculosis* (*M. tb*)-infected macrophages – was able to help clear *M. tb.* [30]. Bacterial **lysogens** could also serve as a Trojan-horse carrier of phage into mammalian cells (Figure 2,

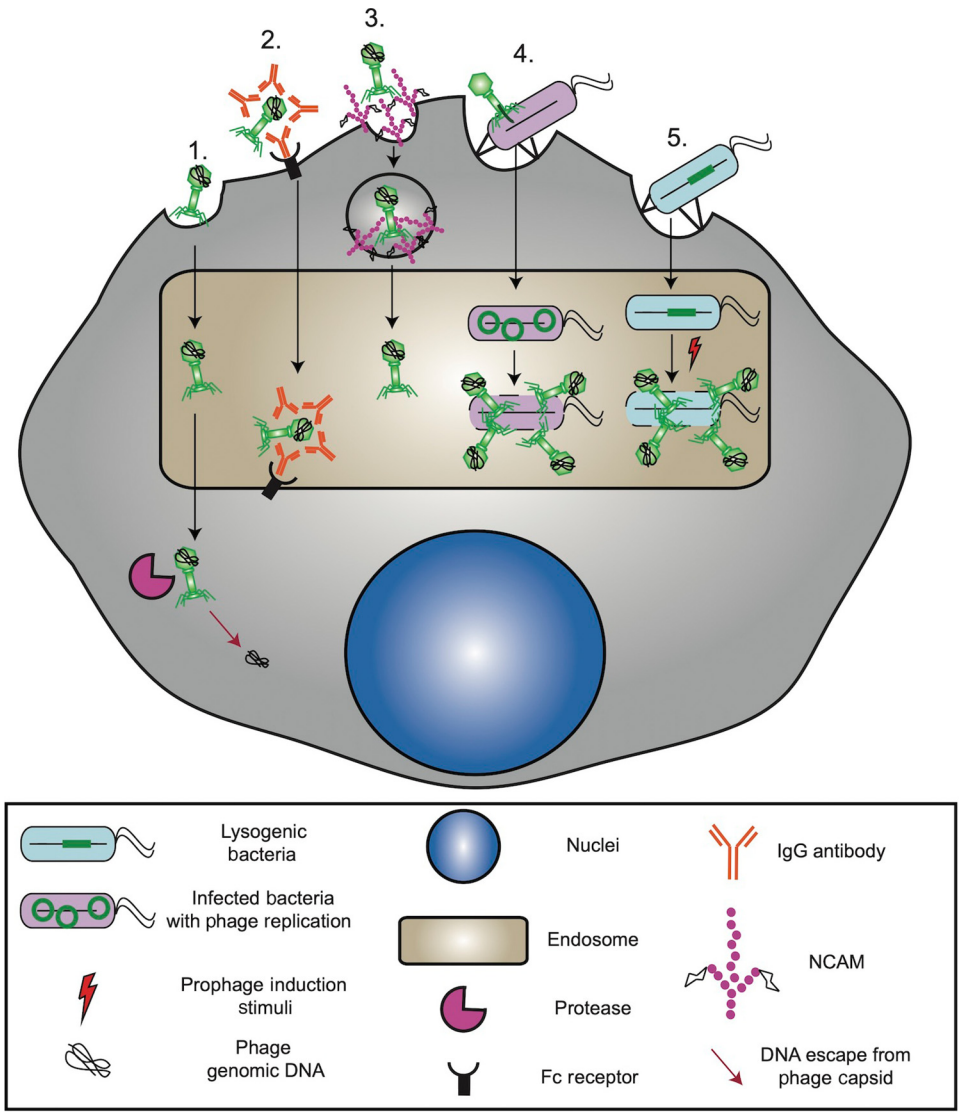

Figure 2. Mechanisms of Entry of Phages into Mammalian Cells. (1) Phage entry through nonspecific endocytic pathways such as macropinocytosis [20], phagocytosis [17], or transcytosis [22]. If phage particles escape the endosome there is a chance that the proteasome may degrade the capsid, leading to release of genomic DNA. (2) Antibody-dependent uptake. Phages coated with serum IgG may be taken up through antibody-dependent receptor endocytosis [25]. This process may stabilize the phage capsid. (3) Phage uptake through molecular mimicry. Certain surface molecules, such as neural cell adhesion molecule (NCAM), on neuroblastoma cells contain polysialic acid, similar to the receptor present on the surface of bacterial species that are recognized by certain phages [27]. Phage binding can trigger endocytosis. Each of these pathways leads to the transport of phage particles into an endosomal compartment, from which phage DNA or RNA may have the chance to escape into the host cell cytoplasm as in pathway 1. (4) Phage entry through an active bacterial infection. Bacteria that are infected with lytic phage prior to entry into mammalian cells (either by phagocytosis, as indicated, by binding to surface receptors or by active entry by pathogenic bacteria) may continue on the phage life cycle [29,30]. Replicated phages may package and form particles that release within the bacterial compartment of the cell. (5) Phage entry through activation of a bacterial carrier. Lysogens containing integrated prophages may enter mammalian cells through bacterial entry. Once inside the phagolysosomal environment, harsh stress may activate prophage induction, leading to bacterial lysis and release of phage particles [13].

Table 1. Pathways that Eukaryotic Viruses Use to Traverse Host Cells Hijacked by Phages.

Step	Process	Examples of eukaryotic viruses	Refs	Examples of prokaryotic viruses	Refs
Entrance	Macropinocytosis	Enveloped and non-enveloped viruses: Vaccinia, Adenovirus, Coxsackievirus HSV1, HIV	Reviewed in [91]	M13 phage of *E. coli* in epithelial and endothelial cells	[20]
Entrance	Antibody-dependent enhancement	Coronaviruses, dengue virus, Zika virus, yellow fever virus, HIV	[92]	λ phage of *E. coli* in fibroblasts	[25]
Replication	Protected viral factories	Alphavirus, Poxvirus	[93]	Phages replicate inside mammalian cells in 'protected' bacterial hosts. Phage can set up protected replication factories in bacterial hosts	[13,68]
Nuclear entry	Nuclear envelope breakdown	HPV, murine leukemia virus	[64]	Phage particles have been observed in the nucleus; the mechanism is unknown	[65]
Nuclear entry	Nuclear localization sequence (NLS) on capsid	Hepatitis B virus	[64]	Phi29, Nf, PRD1, Bam35, and Cp-1 phages have terminases with NLSs that can enter the nucleus	[66]
DNA integration	Homologous recombination of microhomology regions	Epstein–Barr, HPV	[74]	Theoretically possible, but has not directly been proven	Not applicable

pathway 5) [31]. Lysogens may induce a **prophage** while inside endosomes or **phagosomes** of mammalian cells, leading to phage particle production or defective induction and alteration in bacterial gene expression (Figure 1, 1B). A *Listeria monocytogenes* prophage inside bone-marrow-derived macrophages (BMDM) is able to excise, as detected by PCR, without intracellular phage particle production or expression of late lysis genes [32]. In contrast, we have demonstrated that macrophages trigger **prophage induction** of λ phage from phagocytosed *E. coli*, leading to production of functional particles that lyse host *E. coli* and propagate infection to other bacteria in the same phagosome.

The field first postulated that DNA-damaging agents found inside macrophage phagosomes could induce prophages from intracellular bacteria such as *Salmonella* [33]. From mouse fecal samples, it was determined that 1–2% of *E. coli* carrying λ prophage induce phage, mediated by the RecA-dependent **SOS response** to DNA damage – although the exact location inside the mouse where prophage induction occurs has not been determined [34]. Similarly, commensal *Lactobacillus reuteri* phages induce in the guts of mice, in a RecA-mediated manner [35]. In contrast, we have shown a specific role for outer-membrane damaging agents found in the phagosome of macrophages in triggering of prophage induction in nonpathogenic *E. coli*, including, most surprisingly, antimicrobial peptides (AMPs) such as mCramp1 [13]. In contrast, a *Listeria* prophage excises but does not propagate inside macrophages (in a process termed active lysogeny) [32] due to a cryptic prophage that expresses an antirepressor, specifically in intracellular conditions [36]. Intriguingly, the activator of late-lysis genes is expressed in this prophage, but late-lysis genes are not, suggesting that the activator may be inhibited by destabilization [37]. Reactive oxygen species (ROS), generated by macrophage enzymes that induce bacterial DNA damage, were shown to be indispensable for phage transfer across *Salmonella* strains in mice, suggesting that other mechanisms cause the induction [38]. Future work should identify the generalizability of these induction mechanisms, such as PhoP [39], across pathogenic and commensal strains of bacteria.

Phage Access to Organelles and Influence on Signaling

Phages have been isolated from many mammalian cellular compartments involved in internal transport of cargo, including endosomes [40], lysosomes [21,41], and the Golgi apparatus [22]. Most studies to date have characterized the uptake of phages of the common laboratory bacterial

strain, *E. coli*; however, even within a particular species of phage, a comprehensive pathway from uptake to distribution has not been fully delineated. The most comprehensive characterization of the distribution of phages within organelles found that T4 phage applied to MDCK and A549 epithelial cells localizes to all subcellular fractions and endomembrane components, with specific enrichment in the Golgi apparatus [22]. Whether these are the predominant intracellular locations for other phage species remains to be characterized.

Phages present in phagosomes or endosomes may stimulate **pattern-recognition receptors** (**PRRs**) that sense **pathogen-associated molecular patterns** (**PAMPs**) (Figure 3). TLR9 is a detector of endosomal DNA, specifically unmethylated CpG dinucleotides [42]. A combination of *Lactobacillus, Escherichia* and *Bacteroides* phages applied to dendritic cells stimulated the production of interferon-gamma (IFN-γ) due to stimulation of TLR9 and its adapter, Myd88 [43]. Another endosomal receptor, TLR3, is a sensor of double-stranded viral RNA, which may be present in phages with dsRNA genomes [42]. BMDMs that phagocytose single-stranded DNA Pf phages have been shown to produce type 1 interferons, mediated by TLR3 and its adapter Trif, suggesting that phage RNA transcription occurs after phagocytosis [18]. Phages may also stimulate endosomal receptors that sense single-stranded RNA, such as TLR7/8 [42], as posited by Duerkop and Hooper [44]; however, this has not been directly observed.

Phage proteins, nucleic acids, or whole phage particles may also escape the endosome and access the cytosol. The phagosome may have an intrinsic level of 'leakiness', allowing phage protein or nucleic acid to escape, as exemplified by the findings that bacterial peptidoglycans from an escape-deficient mutant of *Listeria* can trigger cytosolic NOD2 receptors [45] and that *E. coli* auxotrophs inside bone-marrow-derived dendritic cells (BMDCs) shed RNA that activates cytosolic receptors such as NLRP3 [46]. Perhaps, phages could also access the cytoplasm by prophage induction inside pathogens with known phagosomal escape mechanisms, as most of these bacteria carry prophages [47]. For example, *Chlamydia* carrying lytic phage is able to lyse and release phage particles into the cytosol of HeLa cells through an unknown mechanism [29]. Phage proteins involved in lysis, such as holin from phage λ, can also localize to the mitochondria and endoplasmic reticulum (ER) when expressed in epithelial cells, leading to loss of mitochondrial membrane potential [48].

Once in the cytosol, there are several other receptors for foreign nucleic acids that may be stimulated by phages. The cGas/Stimulator of Interferon Genes (STING) pathway senses cytosolic single-stranded DNA and is hypothesized to sense phage DNA in the cytosol – but in phagocytes it does not respond to phage Pf [18,43]. Receptors in the cytoplasm that recognize RNA, such as the RIG-I-like receptors [42], have also been proposed to recognize phage DNA if it was converted to 5′-phosphorylated RNA by RNA polymerase III [44]. Future work should characterize immune stimulation following infection with bacteria that are known to access the cytoplasm of host cells and can also carry phages.

Phage particles that stimulate intracellular receptors trigger a downstream inflammatory cytokine response [18], and in the case of macrophages, alter polarization [49]. There is a growing body of literature concerning cytokine expression profiles, predominantly anti-inflammatory, of phage-stimulated cell types such as peripheral blood mononuclear cells (PBMCs) [50]. However, these studies typically do not consider whether the phage particles triggered surface or internal receptors, and here we are concerned primarily with internalized phage. In that context, BMDMs upregulate type 1 interferon expression and downregulate tumor necrosis factor-alpha (TNF-α) in response to Pf phage but not to a similar filamentous phage of *E. coli*, Fd, suggesting a species-dependent cytokine response [18]. Moreover, *E. coli* phages upregulate interleukin-12

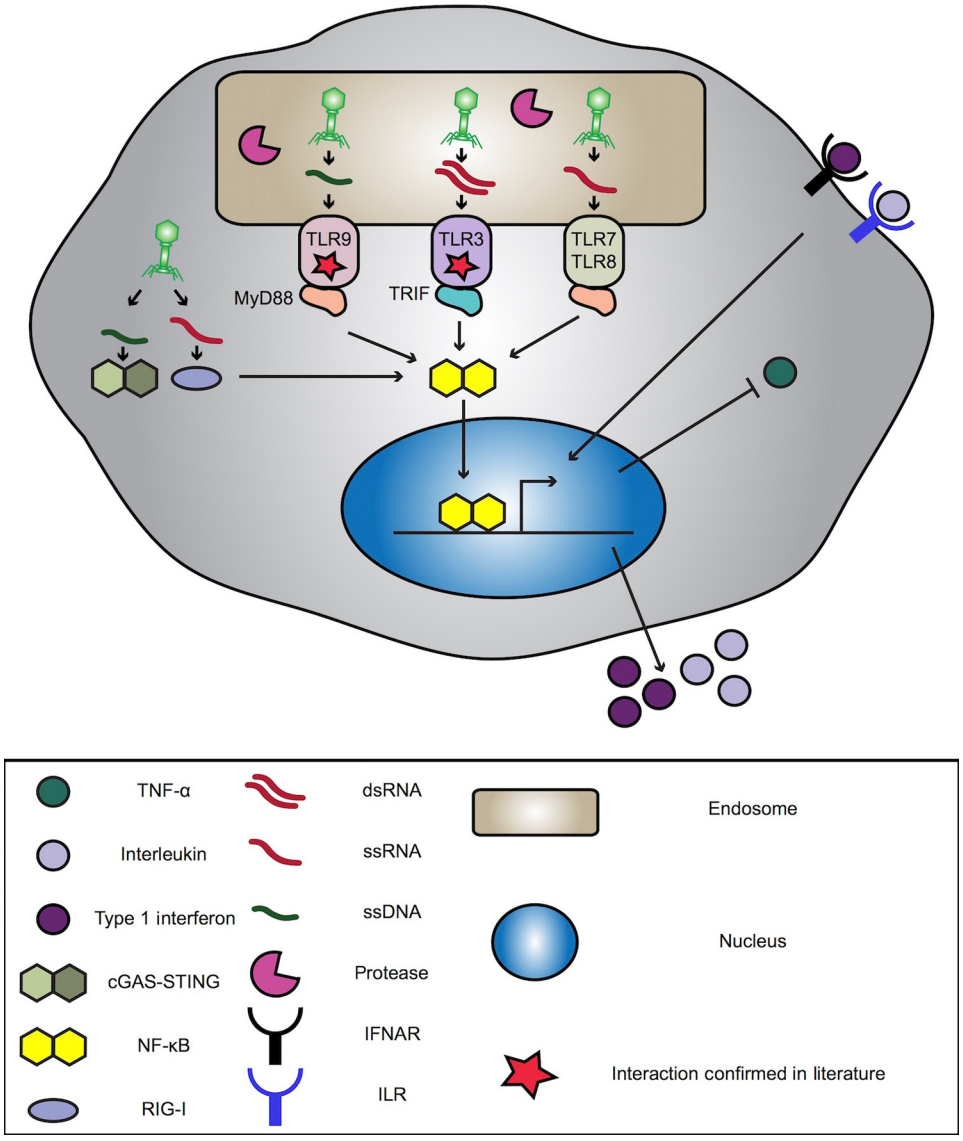

Figure 3. Innate Immune Signaling Responses to Phage. Nucleic acids from degraded phage in the endosome have been demonstrated to activate TLR9 [43] and TLR3 [18] which signal to activate the transcription factor NF-κB. TLR7/8 activation is hypothesized to occur but has not been proven [44]. Phage in the cytosol is also hypothesized to activate the RIG-I and STING pathways [44]. Active NF-κB can lead to a proinflammatory cytokine upregulation of various interleukins (ILs) and interferon-gamma (IFN-γ) [43]. These cytokines can signal in an autocrine fashion, for example by signaling through the interferon-α/β receptor (IFNAR), or they can alter the expression of other cytokines, such as tumor necrosis factor-alpha (TNF-α) [18].

(IL-12), IL-6, and IL-10, but not IL-1α or TNF-α in dendritic cells cocultured with $CD4^+$ T cells which, in turn, produce IFN-γ [43]. Cytokine expression shifts could ultimately shape host physiology beyond control of bacterial infection, as M13 phage causes tumor-associated macrophages to shift from an M2 to an M1 phenotype [49], and phage T4 reduces T cell activation *in vitro* and induces tolerance of an allogeneic skin graft in mice [51]. Immunosuppressive responses have also been observed with *Staphylococcus* phage vB_SauM_JS25 in MAC-T epithelial cells [52], and a lack of inflammatory cytokine response has been observed in *E. coli*

phage K1F internalized by endothelial cells [53]. Thus, both proinflammatory cytokines (type 1 interferons, IFN-γ, IL-12, IL-6) and anti-inflammatory cytokines (IL-6, IL-10) are induced by internalized phage in a cell-type-dependent manner.

Intracellular Degradation of Phage Particles

In various intracellular compartments phages will encounter harsh environmental factors, potentially limiting their capacity to kill their host bacteria. Similar to the factors that eukaryotic viruses may encounter after endocytosis, phages may be degraded by mammalian proteases and proteasomal machinery. Phage K1F is degraded by LC3-assisted phagocytosis, an autophagy mechanism in human bladder epithelial cells [21]. Phage λ introduced into HEK293 cells exhibits higher expression of a mammalian promoter-driven luciferase reporter, if the cells are pretreated with inhibitors of the proteasome, lysosomal proteases, and lysosomal acidification [54]. Since phages are stable *in vitro* at an acidic pH [55] characteristic of phagolysosomes [56], phagosomal pH alone is unlikely to degrade phage particles. Rather, the predominant mechanism of degradation inside organelles is likely due to the activity of lysosomal proteases at acidic pH.

Although environmental conditions alter phage particle stability, phage particles reisolated from mammalian cells remain functional for bacterial lysis. Pretreatment of a human endothelial cell line with chloroquine improves the recovered functional titer of internalized M13 phage [57]. Additionally, we have shown that phage λ particles produced from *E. coli* lysogens in the phagosome lyse host bacteria and remain functional to lyse coinfecting *E. coli* within the same macrophage [13]. *S. aureus* phage MSa can also destroy bacteria inside murine macrophages, but only after first infecting extracellular *S. aureus* [58]. However, this appears to be phage species-specific as phage φA1122 cannot kill *Yersinia pestis* inside mouse macrophages [59]. More *Y. pestis* phages should be tested intracellularly to determine if this class of phages is more sensitive to pH as the similarly structured phage T7 is stable only at a pH that is higher than that of the phagolysosome [56,60].

Phage function inside mammalian cells serves to benefit both the population of phages and the innate immune system. Computational modeling demonstrated that phage–neutrophil synergy is necessary for the efficacy of phage therapy in order to allow the immune system to clear phage-resistant bacteria [61]. However, phages may cause toxicity due to the release of highly immunogenic bacterial endotoxins or exotoxins. At least *in vitro*, *Clostridium difficile* exposed to its phage phiCDHS1 inside human epithelial cells did not release *C. difficile* toxin after phage lysis, suggesting that this may not be a large concern [62]. The released phage particles that survive lysosomal degradation could also recycle back into the extracellular medium, along with bacterial debris, by the process of exocytosis [63] and encounter other bacterial hosts to infect and propagate. For example, up to 0.1% of phage T7 applied to epithelial cells that transcytose from the apical membrane can be reisolated intact from the basal membrane [22]. Identifying the factors that determine the stability of phages inside mammalian cells will allow for engineering of phages with enhanced pharmacological properties.

Delivery and Function of Phage Components in the Nucleus

Phage nucleic acids may also function inside cells if their capsids are destabilized inside endosomes. Like eukaryotic viruses, phage particles or nucleic acids may reach the nucleus during breakdown of the nuclear envelope during cellular division (Figure 4, pathway 1). For example, several eukaryotic viruses, such as the human papilloma virus (HPV) and the murine leukemia virus, enter the nucleus during mitosis after breakdown of the nuclear envelope (Table 1) [64]. Although the mechanism was not determined, proteins of the lytic *S. aureus* phage vB_SauM_JS25, delivered in high doses to bovine mammary epithelial cells, were detected in

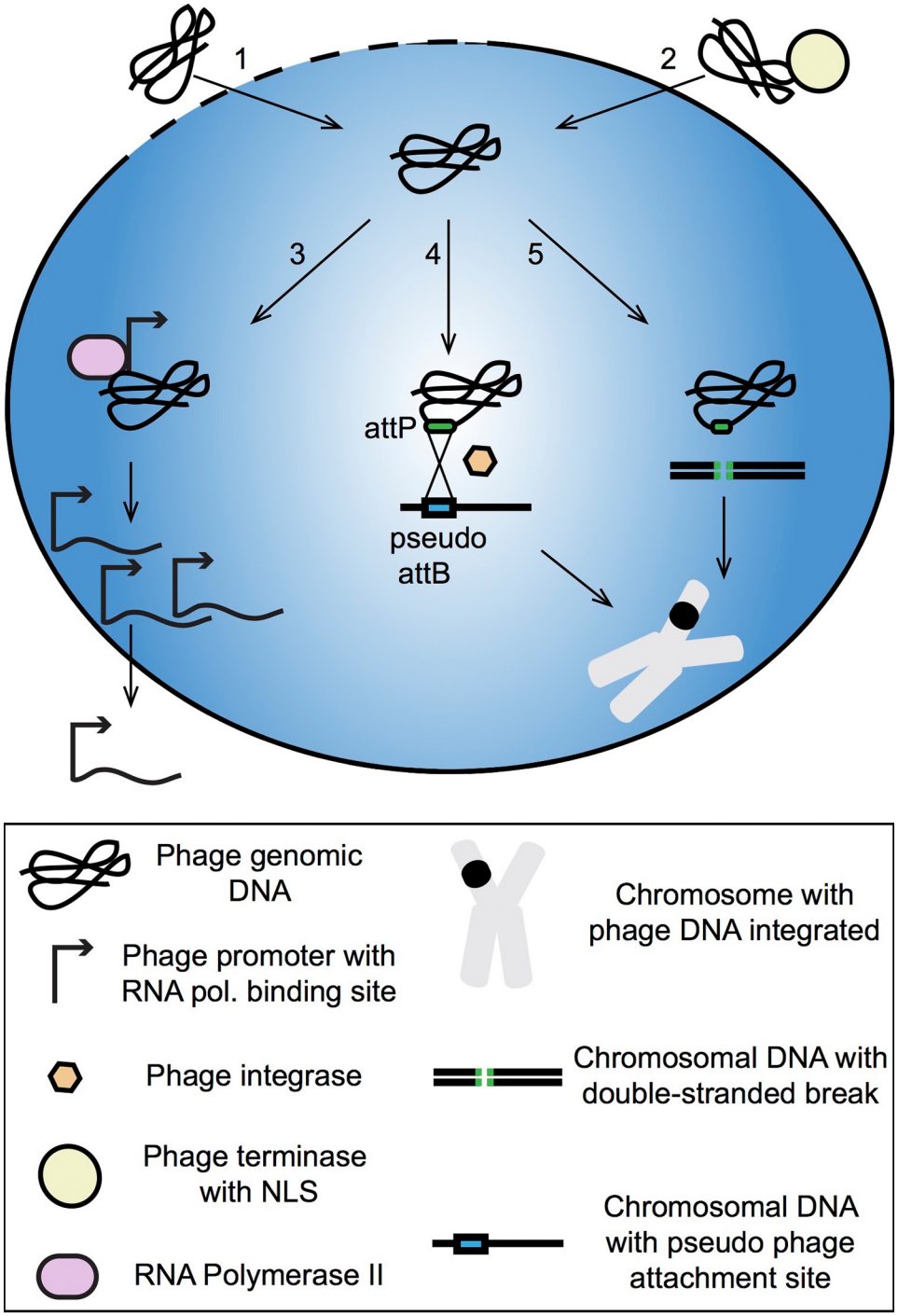

Figure 4. Mechanisms of Phage DNA Entry and Function Inside Mammalian Cell Nuclei. (1) Phage genomic DNA may enter during nuclear envelope breakdown during mitosis. (2) Phage DNA, if bound to a DNA-packaging enzyme such as terminase, may transport into the nucleus due to nuclear localization sequences (NLSs) in the terminase [66]. (3) If phage genomic DNA has a TATA box and sequence homology to transcription factor binding sites, then RNA polymerase II may

(Figure legend continued at the bottom of the next page.)

the nucleus with a capsid-specific dye [65]. Eukaryotic viruses also use nuclear localization sequences (NLSs) on capsid proteins to facilitate binding to nuclear pore complexes and deliver genomic DNA to the nucleus (Table 1) [64]. Some phage terminases, enzymes responsible for packaging phage DNA into capsids and priming for replication, contain an NLS and function in mammalian cells, in particular for phages phi29, Nf, PRD1, Bam35, and Cp-1 (Figure 4, pathway 2) [66]. Finally, phages can form nucleus-like structures, in *Xenopus* eggs [67], and intriguingly, in the context of bacterial hosts, certain *Pseudomonas* phages replicate inside proteinaceous shells that resemble nucleus-like structures, possibly to protect from defenses such as clustered regularly interspaced short palindromic repeats (CRISPR) [68]. These structures could function like the replication factories of eukaryotic viruses (Table 1). To avoid delivery of phage into the nucleus during phage therapy, phage proteins should be screened for NLS domains.

Mammalian cells may transcribe genes from phage genomes, a fact that has been exploited for gene therapy research (Figure 4, pathway 3). Applied to human fibroblast cells, λ phage containing the *E. coli* galactosyltransferase gene have been shown to transcribe RNA [69]. RNA transcripts from wild-type λ could also be isolated from fibroblasts, suggesting transcription from native phage promoters [70]. In primary human monocytes, Pf4 phage RNA transcripts were detected by quantitative PCR a day after phagocytosis [18]. The mechanism of transcription in this case is intriguing because phage particles or DNA were not observed in the nucleus [18]. Expression of a GFP reporter under the control of the Shiga toxin (Stx) 2 promoter from enterohemorrhagic Stx-producing *E. coli*, located inside a lambdoid prophage, was observed in kidney epithelial and fibroblast cells, as the native promoter was predicted to have a TATA box, eukaryotic transcription factor sites, and polyadenylation recruitment sequences within the transcript [71]. Together, these studies make the case that phage DNA can be transcribed by mammalian transcription machinery and suggest that phages utilized for phage therapy should be screened for toxin-encoding genes.

Once in the nucleus, phage genomes, as with any foreign DNA, have the potential to integrate into chromosomes either through site-directed (Figure 4, pathway 4) or random integration (Figure 4, pathway 5) processes. Phage integration enzymes (**integrases**), such as phiC31, can function in mammalian cells and integrate foreign DNA into pseudo-**attachment sites** (attP) that natively exist in the human genome [72]. With only three amino acid mutations, and no need for further cofactors, the phage λ integrase can perform site-specific integration of DNA into endogenous LINE-1 elements in the human genome in embryonic stem cells [73]. While useful for gene therapy, integrases should either be removed from phages prior to use in phage therapy, or lytic phages should be utilized.

Random integration of foreign DNA may also occur through the nonhomologous end-joining machinery, which repairs double-stranded DNA breaks, or through homologous recombination of microhomology regions. Such an approach is used by HPV, inducing integration-driven carcinogenesis (Table 1) [74]. Multiple studies support that random integration of phage DNA into mammalian chromosomes occurs, at least in culture, as phage λ RNA expression persists in fibroblasts for more than 40 days [69,70]. Similarly, radiolabeled phages phiX174 and T2 introduced into lymphocytes incorporated segments of DNA into chromosomes at random and

transcribe RNA, leading to transcripts that have the potential for protein expression [71]. (4) If cognate attachment sites exist in the mammalian chromosome to phage attachment sites, then DNA integration may occur in a site-directed fashion [72]. This recombination is driven by the phage integration enzyme, integrase, if the phage is lysogenic. (5) If the chromosome DNA undergoes a double-stranded break, then, during repair, phage DNA in the nucleus may be incorporated in the process of microhomology-mediated end-joining (MMEJ).

led to a suppression of DNA replication [75]. These results warrant further investigation to determine whether phages have carcinogenic potential, possibly by integration into tumor suppressor genes, if in proximity to the pseudo phage attachment sites [72]. Phage λ has also been identified within human tumor tissue genomic sequences in The Cancer Genome Atlas – although there was not conclusive evidence precluding the reads from being contaminants [76]. Regardless of the integration method, barriers such as methylation exist to prevent phage gene expression [77].

Phage Influence on the Progression of Intracellular Bacterial Infection

Perhaps, in the context of mammalian cells, prophages may function because the phage–bacteria evolutionary arms race has allowed for prophage induction to occur without depleting the whole bacterial population. For example, similar to what has been observed in the *E. coli* response to the AMP LL-37, which is primarily absorbed by a small fraction of individual bacteria to save the rest of the population, one bacterium may induce a phage that expresses beneficial genes, even though that bacterium is killed [78]. When a prophage induces, excises, and replicates, the virulence gene will likely be expressed at high copy number without endangering the entire population [33]. One example of a phage-encoded, secreted virulence factor is GtgE, a protease in the Gifsy-2 phage of *Salmonella typhimurium*, that cleaves macrophage Rab32 to block phagolysosome fusion and is known to be expressed in environmental contexts similar to macrophage phagosomes [79,80]. Future work should examine whether pathogenic prophages induce and produce particles inside phagosomes.

The Importance of Phage–Mammalian Interactions for Phage Therapy

Further characterization of the pathways described in this review will inform the design of phage therapies with improved pharmacological properties. Phages have been extensively developed as nanomaterials for biomedical applications, for example as MRI reagents (reviewed in [81]) and as drug-delivery vectors to package adeno-associated virus genomes [82]. Phages have also been conjugated to chemotherapeutics to treat colorectal cancer associated with a pathogenic bacterial infection [83] and delivered directly via intravenous injection to treat resistant bacterial infections such as *Acinetobacter baumannii* (reviewed in [19]). However, to date, phage therapy is only approved by the FDA for emergency, investigational, and compassionate use [84]. The factors that limit the uptake, distribution, and degradation of phages in the body, and the toxicity, must be better characterized. For example, splenocytes rapidly uptake and degrade phage particles, limiting their half-life in the body [85], and pharmacokinetic modeling suggests that the endothelial and epithelial cell layers are a major sink for phages [23]. Additional limitations of phage therapy include the lack of a delivery modality to bacteria that survive within intracellular niches [40] and the ability for the immune system to develop neutralizing antibodies, limiting redosing [86]. We anticipate that more mechanistic characterization of phage–bacteria–mammalian cell interactions will enable scientists to engineer phage pharmacological properties, allowing phage therapy to work in the proper niches.

Concluding Remarks and Future Perspectives

Phage–mammalian cell interactions have broad implications, including design principles for phages for phage therapy, the role of phages in human health (e.g., enhancing immune responses), and disease promotion (such as disrupting genomic DNA or expressing toxic phage genes). As discussed, phages enter cells by means similar to those of mammalian viruses, can access many intracellular compartments, including endosomes and lysosomes, and stimulate endosomal receptors such as TLR3/9. Phages can function and kill bacteria in intracellular compartments and also have the potential to escape into the cytoplasm and even function in the nucleus of mammalian cells.

Outstanding Questions

What are the dominant mechanisms of phage uptake and immune stimulation *in vivo*?

Are there other examples of natural phage uptake by mammalian cells with receptors mimicking bacterial receptors?

How do the uptake and distribution of nonfilamentous phages look in different mammalian cell types (phagocytic, epithelial, endothelial, etc.) and especially phage species that are more relevant to the gut microbiome composition, such as crAssphage?

What mechanism drives prophage induction *in vivo*, and how does this mechanism differ between pathogenic and nonpathogenic bacteria?

Can host/bacteria transcriptomics datasets be mined for evidence of phage induction in various intracellular conditions?

How can phages naturally escape from the endosome, and does this follow after bacterial pathogen escape?

What is the fate of various phage/bacteria-containing vacuoles? Do phages get recycled back out to the extracellular medium?

How can phages be used for therapy of intracellular pathogens? What information from this field can better inform the pharmacodynamics and pharmacokinetics of phage therapy?

Is there evidence for phage integration into mammalian cell genomes, either experimentally or through sequencing datasets?

What role may phages play in disease pathologies (e.g., by changing cytokine expression patterns and by potential genomic disruption or gene expression)?

Further technological development will also allow for improved tracking of phages taken up by mammalian cells. If the phage particles are produced by bacteria *de novo* once inside the cell, then tracking induced phage particles will require the attachment of fluorescent proteins to capsid proteins and high-resolution imaging techniques such as confocal microscopy. A split-GFP system, such as that used for tracking of *Salmonella* effector proteins injected into the cytoplasm of macrophages [87], can be engineered [15,16] into phage capsids.

Finally, the reason why phage DNA can function inside eukaryotic cells may come from ancient endosymbiosis of bacteria that carry phages. A cytoplasmic DNA element with homology to *Acinetobacter* phages can propagate inside neuronal cells and is speculated to come from intestinal bacteria [88]. Our own human endosymbionts, the mitochondria, are also derived from bacterial origins as the RNA polymerase has significant homology to that of T-odd phages from eubacteria [89]. In contrast, eukaryotic DNA has been identified in the phage WO genome that inhabits the obligate intracellular bacterium, *Wolbachia*, an endosymbiont of wasps [90]. Whether the genetic transfer goes from phage to eukaryote or eukaryote to phage, lysogenic bacterial endosymbionts are likely to provide a rich space to study interactions between phage and eukaryotic DNA (see Outstanding Questions).

Acknowledgments

We gratefully acknowledge funding from the Allen Discovery Center grant to M.W.C. from the Paul G. Allen Frontiers Group. We further acknowledge the following support: Gabilan Stanford Graduate Fellowship (K.B.), Fannie and John Hertz Foundation Fellowship – Hertz-Draper Fellow (K.B.) and Stanford EDGE-STEM Doctoral Fellowship (K.B.). We would also like to thank members of the Covert laboratory, including S. Jeknić, for helpful discussions.

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