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Review

A Microbial Strategy to Multiply in Macrophages: The Pregnant Pause

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Humans live in harmony with much of the microbial world, thanks to a sophisticated immune system. As the first line of defense, macrophages engulf, digest, and display foreign material, then recruit specialists to eliminate potential threats. Yet infiltrators exist: certain fungi, viruses, parasites, and bacteria thrive within sentinel macrophages. By scrutinizing the life styles of these shrewd microbes, we can deduce how macrophages routinely mount an effective immune response. The bimorphic life cycles of three pathogens have dramatic consequences for phagosome traffic. In the transmissible state, *Leishmania* spp., *Coxiella burnetii*, and *Legionella pneumophila* block phagosome maturation; after a pregnant pause, replicative forms emerge and thrive in lysosomes.

Key words: autophagy, *Coxiella*, *Legionella*, *Leishmania*, lysosomes, macrophages, morphogenesis, phagosomes

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Pathogens as Tools for Macrophage Biologists

Pathogens employ a variety of strategies to survive in macrophages (1) (Figure 1). *Toxoplasma gondii* completely avoids fusion with the endosomal pathway; during invasion of mammalian cells, parasites direct the assembly of porous vacuoles that exclude host transmembrane proteins, presumably rendering their compartments invisible to the fusion machinery. In contrast, *Mycobacterium tuberculosis* stalls maturation of its phagosome and replicates within a recycling, early endosomal compartment. Even more bold are *Leishmania* spp., *Coxiella burnetii, and Legionella pneumophila*: These pathogens flourish in acidic lysosomal vacuoles, although not one arrives directly, or stays permanently.

To succeed, *Leishmania* spp., *C. burnetii, and L. pneumophila* employ biphasic life cycles. Cellular differentiation likely evolved in response to two selective pressures: the need to multiply and to be transmitted to a new host. To meet this dual challenge, all three pathogens alternate between an intracellular replicative state and a transmissible form that can tolerate harsh environments. In macrophages, the parasites' developmental cycle has dramatic consequences for phagosome biogenesis (Figure 1B). In the transmissible state, *Leishmania* spp., *C. burnetii, and L. pneumophila* block phagosome maturation; after a pregnant pause, replicative forms emerge that thrive in phagolysosomes.

Despite their distinct ecological habitats, *Leishmania* spp., *C. burnetii, and L. pneumophila* apply a similar strategy to surmount the macrophage barrier in humans. Recent conceptual and technical advances by laboratories studying one of the three microbes suggest a comparative approach is opportune. By considering that convergent evolution made these diverse pathogens members of the same exclusive club, we may gain fresh insight into how macrophages govern phagosome maturation and degrade foreign matter. After describing briefly their natural reservoirs and associated diseases, we review the experimental evidence that *L. pneumophila, C. burnetii*, and *Leishmania* spp. alternately avoid and exploit lysosomes to multiply in macrophages, and speculate on virulence mechanisms that may be shared.

Separate Habitats, Diverse Diseases

Leishmania: Prevalent in South America, the *Leishmania* are trypanosomatid protozoans transmitted among mammals by an insect vector (2). When sand flies bite an infected animal, macrophages harboring the pathogen are ingested with the blood meal. Released parasites multiply in the insect midgut, and these can be delivered during subsequent feedings to a new mammalian host. When humans are bitten, some *Leishmania* species establish self-limiting cutaneous lesions, whereas others spread systemically and cause kala azar, a fatal visceral disease.

Coxiella burnetii: *C. burnetii* is a gram-negative bacterium that infects a variety of winged, scaled and hoofed animals and, more rarely, the people in their proximity (3). For example, when infected female sheep give birth, they shed a large burden of micro-organisms. Consequently, humans can inhale the obligate intracellular bacterium in aerosols of con-

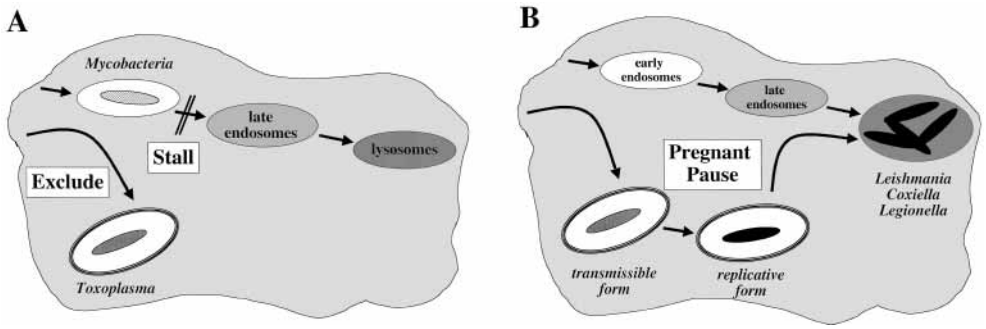

Figure 1: To avoid death in the lysosomes, pathogens short-circuit phagosome maturation. (A) *Mycobacterium tuberculosis* stalls maturation of its phagosome and replicates within an early endosomal compartment, whereas *Toxoplasma gondii* assembles a vacuole that excludes host transmembrane proteins. (B) The transmissible forms of *Leishmania* spp., *Coxiella burnetii, and Legionella pneumophila* establish vacuoles that are separate from the endosomal pathway, but later differentiate to replicative forms that thrive in acidic lysosomes.

taminated soil. When ingested by alveolar macrophages, the microbe can proliferate and disseminate, causing an acute flu–like syndrome known as Q fever. For an unlucky few, the infection progresses to chronic endocarditis or hepatitis.

Legionella pneumophila: Close cousins of *C. burnetii*, the legionellae are common in soil and water, where they thrive within amoebae (4). Humans typically become infected with *L. pneumophila* by inhaling contaminated aerosols generated by man-made devices, such as whirlpools or institutional air conditioners. This facultative intracellular pathogen can colonize alveolar macrophages and, if local or systemic host defenses are impaired, establish a life-threatening pneumonia. However, microbes are not transmitted from one person to another. Since humans are an evolutionary dead-end, it is apparent that pathogenic *L. pneumophila* emerged from communities of fresh-water amoebae (4). In fact, amoebae may be a natural reservoir for a variety of macrophage pathogens, including *C. burnetii*, *M. avium*, *Burkholderia*, *Listeria*, and *Franciscella* (5–7).

Dimorphic Life Cycles

Leishmania: To alternate between insects and mammals, parasites differentiate. Within the midgut of sand flies, *Leishmania* replicate as promastigotes, cells that are long, slender, flagellated, and covered by a dense filamentous glycocalyx. The predominant component of its coat is lipophosphoglycan (LPG), a glycosylphosphatidylinositol-linked polymer of phosphorylated disaccharide repeats. Among other traits, LPG mediates adherence to the midgut wall, protects the parasite from digestive enzymes and, as discussed below, blocks phagosome maturation (8, 9). When ingested by macrophages at the site of an insect bite, the protozoa differentiate to the amastigote form, which lack flagella and LPG, and replicate within acidic phagolysosomes. Upon return to the insect midgut during a subsequent blood meal, amastigotes that are released from macrophages differentiate to the promastigote form, and the cycle continues. In broth cultures, promastigotes become highly infectious in stationary phase (10), but the signal transduction pathway that controls differentiation *in vivo* is not known.

Coxiella burnetii: *C. burnetii* also alternates between two morphologically distinct cell types (11, 12). The spore-like form, known as the small cell variant (SCV), tolerates extreme environments, whereas the large cell variant (LCV) replicates within eukaryotic cells. The architecture of LCVs is typical of gram-negative bacilli; in contrast, SCVs are roughly half their size, and their contents appear condensed. More recent molecular approaches confirmed that SCVs and LCVs are specialized cell types. Abundant in SCVs are a histone H1 homolog and ScvA, a 30-amino-acid basic peptide that localizes to condensed chromatin (13, 14). Conversely, homologs of the sigma factor RpoS and the translation factor EF-Tu are predominantly expressed by LCVs (15, 16). As described below, an acidic pH is required for SCV differentiation to the LCV form, but how the reciprocal event is regulated is not known. To a first approximation, the SCV state differs from the stationary phase of *Escherichia coli*, judging from its low quantity of the stationary phase sigma factor, RpoS (16).

Legionella pneumophila: The bimorphic life cycle of *L. pneumophila* reflects the selective pressures to replicate within and be transmitted among fresh-water amoebae. Growth conditions affect the physiology of this facultative intracellular bacterium (4, 17). Unlike bacteria cultured in broth, *L. pneumophila* released from macrophages or amoebae are short, wide, motile rods with a smooth, thick cell wall and abundant β-hydroxybutyrate. When compared to broth cultures, intracellular bacteria express a different profile of genes, proteins, membrane fatty acids, and lipopolysaccharide. Growth in amoebae also increases *L. pneumophila* resistance to biocides and antibiotics, invasion of mammalian cells, and virulence in mice (18–21). Thus, cellular differentiation enables *L. pneumophila* to alternate between extracellular and intracellular environments.

Recently, a regulatory model for *L. pneumophila* morphogenesis has been generated by genetic and molecular studies of

broth and macrophage cultures. When amino acids become scarce, *L. pneumophila* accumulates the second messenger (p)ppGpp (22). As a result, the two-component regulator LetA/LetS and the sigma factors RpoS and FliA coordinate cellular differentiation to a transmissible form (23, 24). Accordingly, when conditions deteriorate, intracellular bacteria stop replicating and instead express a pore-forming cytotoxin (25, 26) to escape the host, osmotic resistance to tolerate environmental stress, motility to disperse, and factors that block phagosome maturation to establish a protected niche within the next phagocyte (27). When intracellular bacteria sense conditions favorable for growth, they down-regulate traits that promote transmission and convert to the replicative form.

The 'Pregnant Pause' Strategy for Survival

For all three pathogens, morphogenesis from an extracellular infectious form to an intracellular replicative phenotype occurs within a protected vacuole in macrophages. After a period of one to several hours, a new cell type emerges that is equipped not merely to tolerate but to multiply within the harsh lysosomal compartment. Thus, as detailed below, macrophage delivery of these pathogens to lysosomes benefits its intruder, not its host.

Leishmania: In macrophages, the infectious forms of *Leishmania* spp. establish vacuoles that are poorly fusogenic (Table 1). For example, by 2 h after formation, nearly 100% of phagosomes containing latex beads acquire BSA-gold from the endocytic pathway, but only ~50% of those harboring promastigotes do so (28). A series of genetic and biochemical studies have demonstrated that the capacity of certain *Leishmania* spp. to block phagosome maturation is conferred by LPG, its unique surface glycoconjugate (9, 28, 29). Mutants that lack LPG are readily delivered to lysosomes and are killed by macrophages (29–31). In particular, during the first hour of infection of J774 macrophages, almost 100% of the phagosomes formed by lpg2 mutant *Leishmania donovani* promastigotes accumulate Rab7 or LAMP-1, whereas only ~50% of the vacuoles bearing wild-type promastigotes acquire these late endosomal features (30). In a reciprocal experiment, Holm and colleagues found that 30 min after ingestion by J774 macrophages, yeasts colocalize with LAMP-1, but yeasts opsonized with LPG do not (32).

The composition and production of LPG is developmentally regulated by *Leishmania*, and its pattern determines phagosome maturation (9). When cultured in broth, *Leishmania* major promastigotes become highly infectious in the stationary phase, concomitant with an increase in the average number of LPG repeating units and modifications of the side chain termini (10). In macrophages, when promastigotes differentiate to amastigotes, LPG expression is down-regulated. As a result, the parasites traffic to lysosomes, their replication niche (33) (Table 1). For example, 5 days after infection of mouse macrophages, *Leishmania mexicana* vacuoles contain LAMP-1, β-glucuronidase, and cathepsin D, and they are accessible to endocytic probes (34). Similarly, 2 days after

Table 1: Colocalization of young and mature pathogen vacuoles with markers of the endosomal pathway

Assay	*Coxiella burnetii*		*Legionella pneumophila*		*Leishmania* spp Promastigote		Amastigote
	2 h	>6 h	<8 h	>8 h	<3 d	>3 d	
Fluid phase markers							
Lucifer Yellow		+(43)					
Texas Red-ovalbumin			−(49, 51)	+(51)			
FITC-dextran		+(43)		+(51)		+(34)	
BSA conjugates					low (28, 30)	+(34)	
Electron dense colloids	−(38)	+(38, 41)	−(47, 48, 76)			+(33)	
Endosomal proteins							
5′ nucleotidase		+(42)	−(77)				
Transferrin receptor			−(47, 49)				
LAMP-1		+(43)	−(49, 74, 77)	+(51)	low (30)	+(34, 35)	
LAMP-2		+(43)				+(34)	
Proton ATPase		+(43)					
Cathepsin D		+(43)	−(49)	+(51)		+(34, 35)	
Acid phosphatase	−(38)	+(38, 41)	−(48)				
rab7			low (50, 74)		low (30)	+(35)	
pH							
Acridine orange		+(43)					
DAMP						+(36)	
FITC		~5.0 (41, 44)	~7.4 (51, 52)	~5.5 (51)		~5.0 (36)	
Inhibition of growth							
Bafilomycin A		+(43)		+(51)			
Lysosomotropic amines		+(43, 45)		+(51)			

entering macrophages, *L. donovani* amastigotes colocalize with rab7, LAMP-1, and cathepsins B, D, H, and L (35). As expected for lysosomal compartments, vacuoles harboring *Leishmania amazonensis* amastigotes are ~pH 5.0 (36). The ability to culture amastigotes in broth will expedite analysis of the mechanisms that equip parasites to replicate in lysosomes (37).

Coxiella burnetii: Similar to the *Leishmania*, the infectious form of *C. burnetii* perturbs phagosome maturation (38). Exploiting ScvA as a morphogenesis marker (14), Howe and Mallavia monitored in parallel both the fate and the cellular differentiation of bacteria immediately after infection. Fifteen minutes after uptake by J774 macrophages, ~70% of the intracellular bacteria remained in the SCV form, a fraction equal to that of the inoculum. Unlike killed bacteria, the majority remained separate from the lysosomal compartment, as judged by colocalization with acid phosphatase or endocytosed thorium dioxide. By 1 h, ~80% of the *C. burnetii* had differentiated to the metabolically active LCV form. Meanwhile, from 1 to 6 h postinfection, the fraction of lysosomal bacteria increased from ~30% to ~60%. Differentiation to the replicative form likely occurs prior to delivery to lysosomes, as SCV activation *in vitro* is more efficient at pH 5.5 than at pH 7.0 or 4.5 (38). Although correlative, the data are consistent with a model in which SCVs produce factors to block phagosome-lysosome fusion.

Whether the capacity to perturb phagosome maturation in macrophages is unique to the SCV form has not been tested rigorously. In fact, in a chicken egg model, SCVs and LCVs are equally infectious (11), but how accurately growth in eggs predicts a capacity to survive and replicate either in macrophages or an immunocompetent host is not clear. Another indication that LCVs may be infectious is that 2 h after Vero cells are infected with a mixed population of *C. burnetii*, intact LCVs are found in phagosomes that also contain SCVs (39). However, since wild-type *L. pneumophila* can chaperone avirulent trafficking mutants to a replication niche (40), this experiment does not establish that LCVs can alter phagosome biogenesis. More detailed experiments can determine whether *C. burnetii* must delay phagosome maturation to establish a replication niche in macrophages, and whether this attribute is unique to SCVs.

After differentiation to the LCV form, *C. burnetii* thrives in lysosomes (Table 1) (41–43). One detailed immunofluorescence microscopic analysis of a variety of infected eukaryotic cell lines demonstrated that after 40 h of infection, *C. burnetii* is located in vacuoles that contain the vacuolar proton ATPase, the late endosomal and lysosomal proteins LAMP-1 and LAMP-2, and the lysosomal protease cathepsin D (43). In addition, compartments harboring the bacteria are accessible to fluid-phase endocytosis probes, they accumulate the lysosomotropic dye Acridine Orange, and their lumens are ~pH 4.5–5.0 (41, 43, 44). Furthermore, neutralization of the endosomal compartment by treatment with the proton ATPase inhibitor bafilomycin A or the weak bases ammonium chloride, chloroquine, or methylamine inhibits *C. burnetii* growth (43, 45).

Legionella pneumophila: Building on the pioneering work of Horwitz, several investigators have confirmed that, for several hours after formation, phagosomes harboring *L. pneumophila* remain separate from the lysosomal compartment (Table 1). Lysosomes labeled by acid phosphatase cytochemistry or by endocytosis of electron-dense colloids do not fuse with *L. pneumophila* phagosomes aged less than 6 h (46–48), and the late endosomal and lysosomal proteins Rab7, LAMP-1, LAMP-2, and cathepsin D are not typical of bacterial phagosomes younger than 3 h (47, 49, 50). Furthermore, young *L. pneumophila* vacuoles do not accumulate material from the early endosomal compartment, including transferrin receptors, the endocytic tracers Texas Red-ovalbumin and Alexa Fluor-streptavidin, or the lipid dye CM-DiI, probes that are readily detected in phagosomes containing polystyrene beads or avirulent bacteria (47, 49). Therefore, in macrophages, *L. pneumophila* establishes a vacuole that remains functionally separate from the endosomal network for at least 6 h.

In contrast, during their 10–15 h replication period, *L. pneumophila* phagosomes acquire lysosomal characteristics (Table 1) (51). By 18 h postinfection, 70% of the vacuoles contain LAMP-1 and 50% accumulate quantities of cathepsin D and fluorescent endocytic probes that can be detected by fluorescence microscopy. Moreover, whereas nascent *L. pneumophila* phagosomes remain at a neutral pH, by 16–20 h after infection, those vacuoles that are accessible to fluid phase tracers are also acidic (51, 52). Thus, as they mature, *L. pneumophila* replication vacuoles merge with the lysosomal compartment. Four experimental results indicate that the lysosomal compartment promotes, rather than inhibits, *L. pneumophila* growth (51). Vacuoles that contain greater than five bacteria also contain LAMP-1; bacteria within endocytic vacuoles can respond to a metabolic inducer by expressing a reporter Green Fluorescent Protein; neutralization of the endosomal pathway with the proton ATPase inhibitor bafilomycin A1 arrests bacterial replication; and replicating *L. pneumophila* obtained from macrophages are acid tolerant. Together, the data indicate that, like *Leishmania* and *C. burnetii*, *L. pneumophila* differentiates to a replicative form that thrives within macrophage lysosomes.

Autophagy as an Alternate Route to Lysosomes

The immature phagosomes established by some pathogens may be delivered to the lysosomes by autophagy (Figure 2). In this cellular response to stress or developmental cues (53), eukaryotic cells engulf cytoplasmic material and organelles, including mitochondria, within folds of the endoplasmic reticulum, or other as yet unidentified intracellular membranes. Autophagosomes then acquire LAMP-1, the proton ATPase, and lysosomal hydrolases, and the vacuolar contents are de-

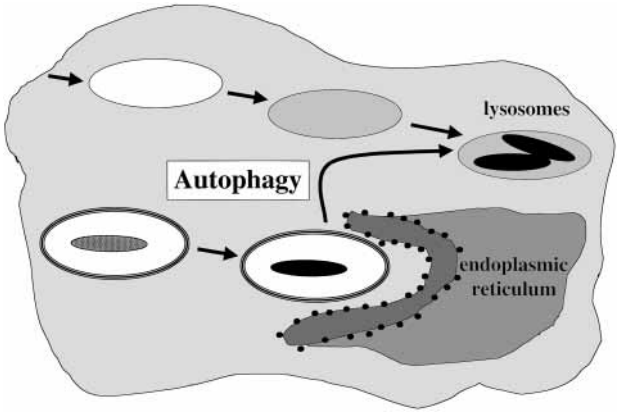

Figure 2: In macrophages, autophagy may act as a quality control mechanism that recognizes and redirects isolated pathogen vacuoles to the lysosomal compartment. See text for details.

graded. Several observations suggest that *L. pneumophila* traffic by autophagy to the lysosomal compartment: Endoplasmic reticulum encloses *L. pneumophila* vacuoles 4–6 h after formation (54, 55); Gsa7p (56), a ubiquitin-E1-like enzyme of the autophagy pathway, colocalizes with young *L. pneumophila* vacuoles (Amer and Swanson, unpublished observations); and conditions that stimulate autophagy promote association of the endoplasmic reticulum with the pathogen vacuole and also modestly stimulate bacterial growth (55). Finally, as expected for autophagosome maturation, the pathogen compartment slowly acquires lysosomal traits (51). Autophagy may also direct *Leishmania* to lysosomes, as vacuoles harboring amastigotes contain detectable calnexin, an endoplasmic reticulum protein, and they slowly accumulate cytosolic markers by a process sensitive to 3-methyladenine, an inhibitor of autophagy (57). Two other gram-negative pathogens, *Brucella abortus* and *Porphyromonas gingivalis,* reside within vacuoles that are enveloped by endoplasmic reticulum and acquire LAMP-1 (58, 59). Perhaps in macrophages, autophagy acts as a quality control mechanism for phagosome maturation. When pathogens assemble nonfusigenic vacuoles, the autophagy pathway may recognize the aborted phagosomes and redirect them to the lysosomal compartment.

Adaptation to Lysosomes

To replicate in the macrophage's most noxious organelle is an astonishing feat. Studies of *C. burnetii* metabolism revealed how pathogens can profit from the lysosomes' acidity. Transport and catabolism of glutamate by freshly explanted extracellular *C. burnetii* is optimal at pH 3–5 and poor at neutral pH (60). These and other data indicate that intracellular *C. burnetii* exploit the proton gradient across its cytoplasmic membrane to drive critical biochemical reactions (45, 61, 62). How lysosomal *Leishmania, C. burnetii*, and *L. pneumophila* cope with the array of degradative enzymes remains a mystery. The majority of cell biological studies demonstrating colocalization of pathogens with lysosomal proteases relied on immunological methods, techniques that do not address biological activity. However, infection by *C. burnetii* of J774 cells does not reduce total activity of lysosomal proteases (41). To investigate whether pathogens exploit the proteases of macrophage lysosomes to generate amino acids needed for replication requires more sophisticated probes of macrophage biology.

Virulence factors that perturb phagosome maturation

In the course of evolution, *Leishmania*, *C. burnetii*, and *L. pneumophila* have learned how to short-circuit phagosome maturation; therefore, they can direct us to critical regulators of this cellular pathway. One clue to how pathogens block phagosome maturation is the limited range of their virulence factors. *Legionella pneumophila* blocks its own progression to lysosomes with no apparent effect on phagosome acidification or maturation elsewhere in the phagocyte (40, 52). Likewise, when J774 macrophages are fed a mixture of latex beads and virulent *Leishmania*, 90% of the beads still traffic to the endosomal compartment (28). Therefore, the virulence factors that block delivery of these pathogenic vacuoles to lysosomes act locally, most likely by altering either the phagosome's membrane or its membrane-associated regulatory factors.

Of the microbial factors that prevent phagosome maturation, the best understood is LPG. The current model for its mechanism of action incorporates the observed effects of purified LPG on target membranes (63). When *L. donovani* LPG partitions into bilayers, the membranes are stabilized and become refractory to fusion with enveloped viruses. Accordingly, LPG may inhibit phagosome maturation by increasing the energy required to alter the bilayer structure to form fusion intermediates. Intercalation of LPG into phagosomal membranes also inhibits protein kinase C activity and increases actin accumulation, two processes antagonistic to phagosome maturation (32, 64).

In light of LPG's potent inhibition of phagosome maturation, it is tempting to speculate that lipopolysaccharide serves an analogous function for the two gram-negative pathogens. By this model, developmental regulation of lipopolysaccharide would account for the pregnant pause of the phagosome maturation. Although lipopolysaccharide (LPS) variants of *C. burnetii* do occur (65, 66), they do not behave as elements of a developmental pathway. Phase 1 forms are isolated from patients or infected animals, have complex oligosaccharide side chains, are serum-resistant, and are infectious in a guinea pig model of disease. After passage in tissue culture or chicken eggs, Phase II organisms emerge; these cells express a less elaborate and more hydrophobic form of LPS (65), are serum-sensitive, adhere more readily to cultured macrophages, but are avirulent in guinea pigs (66). However, since the reciprocal conversion has not been reported, and both phases can establish persistent infections (67), there is no evidence that LPS modifications account for the trafficking pattern of *C. burnetii* in macrophages.

Modifications of *L. pneumophila* lipopolysaccharide have also been correlated to virulence, but a link to growth phase or membrane trafficking has not been described. *Legionella pneumophila* produces an unusually hydrophobic lipopolysaccharide (LPS) that may facilitate its intracellular lifestyle (68). An LPS phase-variant mutant of *L. pneumophila* was isolated after repeat passage (69). Unlike wild-type, the mutant grew poorly within HL60 monocytic cells and in guinea-pig lungs. Furthermore, reversion of the mutant to the wild-type LPS was promoted during animal infections. Therefore, that a developmentally regulated phase variation of LPS contributes to *L. pneumophila* pathogenesis remains an attractive hypothesis.

The best characterized determinant of phagosome biogenesis of *L. pneumophila* is the Dot/Icm type IV secretion system. As discussed by Vogel in this issue (70), this specialized transport apparatus apparently acts during phagocytosis to establish the *L. pneumophila* replication vacuole. It is reasonable to predict that *C. burnetii* uses a similar strategy, as its genome encodes multiple homologs of the *dot/icm* genes. As yet, substrates of the Dot/Icm secretion complex that alter the fate of *L. pneumophila* or *C. burnetii* phagosomes have not been identified. Also contributing to *L. pneumophila* survival in macrophages is a Dot-independent activity (49). A surprising observation is that formalin-killed bacteria and viable *dot* mutant bacteria establish phagosomes that readily acquire LAMP-1, but do not accumulate cathepsin D, nor Texas Red-ovalbumin, CM-DiI, or Alexa Fluor-streptavidin, endocytic probes readily acquired by phagosomes containing *E. coli* or polystyrene beads. Therefore, in the transmissible form, *L. pneumophila* secretes a Dot/Icm-dependent factor and expresses a formalin-resistant surface factor to perturb phagosome maturation.

As observed for *Leishmania,* once *L. pneumophila* establishes a protected vacuole, virulence factors that block phagosome maturation are no longer needed. *L. pneumophila* that express DotA prior to contact with macrophages, but not after, still replicate during the primary infection cycle (50). and when *dotA* mutants share a phagosome with wild-type bacteria, they replicate (40). In addition, recent genetic studies of *L. pneumophila* virulence identified a two-component regulator, LetA/S, that induces transmission traits, including motility, cytotoxicity, and lysosome evasion, yet is dispensable for intracellular replication (24). Consistent with this genetic separation of the transmission and replication phenotypes, *L. pneumophila* that are replicating in macrophages down-regulate flagella production and cytotoxicity, and they traffic to the lysosomal compartment (22, 26, 27, 51). Therefore *L. pneumophila* transmission traits are dispensable for intracellular replication, a phenomenon analogous to the down-regulation of LPG by *Leishmania* spp. during differentiation from the transmissible to the replicative form.

Odd Entrances May Point to Mechanism

Coiling phagocytosis: Macrophages and amoebae engulf *L. pneumophila* within coils of plasma membrane (71,72). Coiling phagocytosis has been observed for a number of other microbes, including *Leishmania donovani* (73). Based on their detailed ultrastructural studies, Rittig and colleagues postulated that coiling phagosomes occur when the membranes of pseudopods that surround a particle fail to fuse (74). Accordingly, plasma membrane whorls may be a product of the same microbial activities that inhibit fusion with lysosomes, although this hypothesis has not been tested directly.

Perturbation of the actin cytoskeleton: Another striking aspect of pathogen entry that may provide insight to virulence mechanisms is aberrant actin dynamics. Isberg and colleagues have found that L. pneumophila can induce diffuse actin ruffles and formation of macropinosomes that contain GPI-linked proteins and also GM1-gangliosides, a hallmark of cholesterol-rich membranes (75). Similarly, *C. burnetii* induce dramatic and transient actin rearrangements in THP-1 monocytes (75), but their consequences for phagosome structure or function have not been determined. Finally, *Leishmania* LPG stimulates a prolonged accumulation of actin filaments around the phagosome, a trait that correlates to isolation from the endosomal pathway (32). How particular actin rearrangements affect pathogen fate is not known.

The formidable microbicidal activities of macrophages also function as strong selective pressures for variants that can replicate intracellularly. *Leishmania* spp., *Coxiella burnetii*, and *Legionella pneumophila* employ a 'pregnant pause' strategy to acquire the time needed to differentiate to a form that can exploit the harsh lysosomal compartment as a replicative niche. Knowledge of the tactics of macrophage pathogens provides a valuable conceptual framework for future research to identify and characterize virulence factors that perturb phagosome maturation. Because of their simplicity relative to mammalian cells, together with their more facile genetics, microbes promise to be powerful experimental tools to investigate macrophage cell biology.

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