Molecular Pathogenesis of Infections Caused by
Legionella pneumophila

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INTRODUCTION

The genus *Legionella* was first described following a large outbreak of severe pneumonia among attendees at an American Legion convention in Philadelphia, PA, in 1976. Overall, there were 182 cases that resulted in 29 deaths and the hospitalization of 147 people (131). Following months of intense investigation, the causative agent was identified as a Gram-negative bacillus that was subsequently termed *Legionella pneumophila*, its name reflecting both its victims and the newly described Legionnaires' disease (53). The discovery of *Legionella* in 1977 as well as the development of successive growth media provided a means for the retrospective study of previously unsolved outbreaks of respiratory disease as well as the environmental isolation of *Legionella*. The ability to isolate and grow the bacteria led to the rapid classification of other *Legionella* species associated with human pneumonia. Human bacterial isolates originally identified as rickettsia in the 1940s through the 1960s were also definitively identified as *Legionella* spp. by using these new techniques (121, 161).

In the relatively short period since *L. pneumophila* was first identified as a human pathogen, more than 50 species of *Legionella* have been recognized, and at least 24 of these have been associated with human disease. It is possible that under the appropriate conditions, immunocompromised people can

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be infected with any species of *Legionella*. The great majority of Legionnaires' disease, approximately 90%, is caused by *L. pneumophila*, and despite the description of at least 15 serogroups, *L. pneumophila* serogroup 1 is responsible for over 84% of cases worldwide (218, 237, 362). The bias toward *L. pneumophila* as the most prevalent *Legionella* species to infect humans does not reflect the environmental distribution of the genus. A large study comparing clinical and environmental *Legionella* isolates in France showed that *L. pneumophila* serogroup 1 accounted for 28% of environmental *Legionella* isolates compared to 95% of clinical isolates (106). That study and similar studies provided strong evidence that *L. pneumophila* is more pathogenic to humans than other *Legionella* species. The public health impact of *L. pneumophila* is therefore reflected in the greater research focus on this organism. *Legionella bozemanae*, *L. micdadei*, and *L. longbeachae* are the next most common etiological agents of Legionnaires' disease and together account for approximately 2 to 7% of *Legionella* infections worldwide (237). Interestingly, this trend does not hold true for Australia and New Zealand, where approximately 30% of Legionnaires' disease is attributed to *L. longbeachae* (362). The remaining pathogenic species of *Legionella* are rarely isolated from humans, and some species are classified as pathogenic based on a single reported human infection.

An Environmental Organism and Accidental Pathogen

Legionella bacteria are ubiquitous organisms within freshwater environments (128). *L. pneumophila* has been recovered from a wide range of both human-made and natural aquatic habitats, from lakes and streams to air-conditioning cooling towers, fountains, and spa baths (130, 198, 235, 300, 314). The exception to this ecological niche is *L. longbeachae*, which resides primarily in soil, and infection is often associated with exposure to commercial potting soil (317, 318). *Legionella* bacteria are not free-living aquatic bacteria; rather, they parasitize or form a commensal relationship with free-living, freshwater, and soil amoebae (288, 307, 319). *Legionella* species multiply intracellularly in many types of protozoa, and this relationship is central to the ecology of the organism in both aquatic and soil environments. While providing a niche for *Legionella* replication, amoebae also protect *Legionella* from harsh environmental conditions. This relationship increases the resistance of *L. pneumophila* to biocides, antibiotics, acid, and osmotic and thermal stress (15, 31, 84, 85, 191). Furthermore, some amoebal species expel biocide-resistant vesicles containing large numbers of *L. pneumophila* bacteria, which may act as airborne agents for the transmission of the bacteria (40).

Within human-made water systems, *Legionella* bacteria are found almost exclusively within complex biofilms (282). The characterization of *Legionella* within these ecosystems is difficult, but model biofilm systems have demonstrated that the replication of *L. pneumophila* within this niche depends on the presence of a protozoan host (239). Legionnaires' disease is most strongly associated with human-made aquatic environments that contain water at elevated temperatures. In particular, many disease outbreaks are linked to air-conditioning cooling towers and evaporative condensers, which can produce contaminated water droplets that are inhaled by passers by. The increased presence of these large-scale, human-made aquatic reservoirs has likely led to the increased human exposure to *Legionella* and subsequently an increased incidence of *Legionella* infection in the latter half of the 20th century. It should be emphasized that accidental human infection is a dead end for *Legionella* replication, and person-to-person transmission has never been reported. Therefore, the evolution of virulence traits in *L. pneumophila* has resulted largely from the organism's need to replicate in an intracellular niche and also avoid predation by environmental protozoa.

REPLICATION OF *LEGIONELLA PNEUMOPHILA* IN EUKARYOTIC CELLS: A KEY VIRULENCE MECHANISM

The innate ability of *Legionella* to replicate within different protozoa has equipped the bacteria with the capacity to replicate in human alveolar macrophages. The interaction of *L. pneumophila* with eukaryotic cells is therefore key to understanding the ability of the pathogen to cause disease. This relationship has been studied for a wide variety of protozoan and mammalian host cells. In particular, *Acanthamoeba castellanii*, *Hartmannella vermiformis*, *Naegleria* spp., and *Dictyostelium discoideum* have been used to examine the association of *L. pneumophila* with an environmental host. *D. discoideum* provides the unique advantage of being genetically tractable so that host factors involved in *L. pneumophila* pathogenesis can be studied by mutagenesis (320). For mammalian cells, much work has been performed by using macrophage-like tissue culture cells and mouse bone marrow-derived macrophages to characterize the relationship between *L. pneumophila* and the cell types implicated in human infection. Other tissue culture models such as HeLa, A549, and CHO-K1 epithelial cell derivatives have been used to characterize the intracellular niche of *L. pneumophila*. While some features are unique to the interactions between *L. pneumophila* and specific host cells, the primary mechanisms of infection appear to be the same. In all models, following internalization by the host cell, the bacterial vacuole associates transiently with mitochondria and then acquires characteristics of the endoplasmic reticulum (ER) (Fig. 1).

The Unusual Intracellular *L. pneumophila*-Containing Vacuole

Phagosomes traditionally mature into digestive vacuoles via the endocytic pathway. This involves a progressive interaction with the endosomal network, leading to the acidification of the vacuole and the degradation of most microbes (141). The endocytic pathway is characterized by the acquisition of early endosome markers, including Rab5, a small GTPase predicted to confer specificity to membrane fusions, and early endosome antigen 1 (EEA1) (306, 365). Following the recruitment of Rab5 and EEA1, endosomes acquire Rab7 and other late endosomal proteins, including lysosome-associated membrane glycoproteins (LAMPs). The accumulation of LAMPs, cathepsin D, and other acid hydrolases ultimately leads to phagolysosome formation (210). The unusual biogenesis of the *Legionella* vacuole arises from the simultaneous delay of endosome fusion and the recruitment of vesicles and membrane from the host cell secretory pathway (186, 187, 241). An initial examination of *L. pneumophila*-containing vacuoles (LCVs) re-
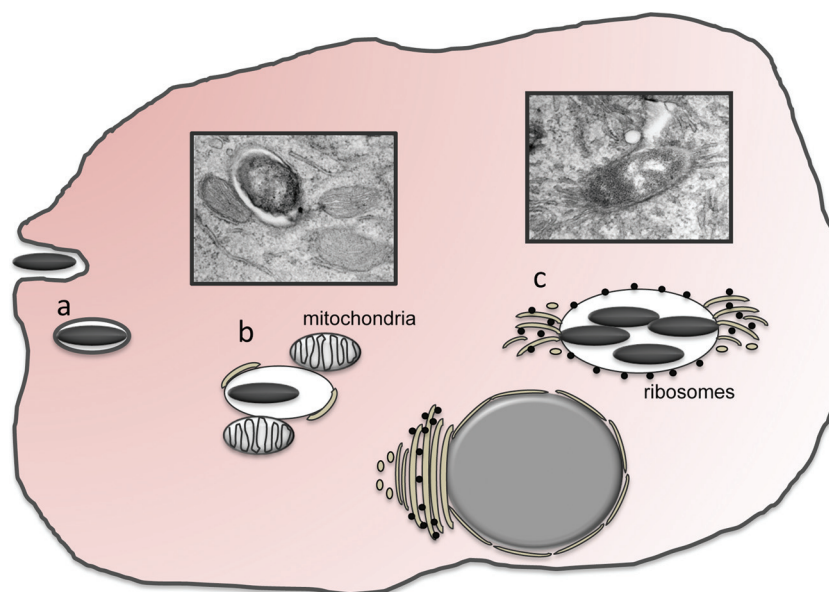
FIG. 1. Biogenesis of the *Legionella*-containing vacuole (LCV). Following phagocytosis through PI-3-kinase-dependent and/or -independent mechanisms (a), within minutes, the nascent LCV avoids interactions with endosomes, fuses transiently with mitochondria, and intercepts ER exit vesicles bearing COP II markers (b). For the next several hours, the LCV maintains interactions with ER-derived vesicles, and the bacteria replicate in a vacuole surrounded by a membrane that resembles rough ER (c).

ported the formation of a novel ribosome-studded vacuole that did not undergo acidification (173, 174). Indeed, within 5 min of phagocytosis, LCVs begin to recruit ER vesicles that can be detected by the presence of the resident ER protein BiP and analysis of membrane width by electron microscopy (337). After 15 min, the phagosomal membrane becomes thinner, resembling that of the intimately attached ER vesicles rather than the plasma membrane (328, 337). Most endocytic markers, including LAMP-1, cathepsin D, and Rab5, are absent until 18 to 24 h after LCV formation (88, 171, 325, 328). Interestingly, Rab7, a small GTPase that regulates vesicular traffic from early to late endosomal stages of the endocytic pathway, is also found abundantly on LCVs (89, 339). Therefore, although the LCV does not mature along the classical endocytic pathway, there are nevertheless interactions with vesicles trafficking in both the secretory and endocytic pathways (338).

Soon after phagocytosis, the LCV rapidly acquires proteins from secretory vesicles that cycle between the ER and Golgi apparatus. The LCV specifically recruits Rab1, but not Rab2 or Rab6, to the LCV within minutes of uptake, preceding any remodeling of the vacuole (187). Rab1, Rab2, and Rab6 are small GTPases that recruit factors necessary for the fusion of ER-derived vesicles with the Golgi apparatus (7, 236). The inhibition of Rab1 activity impedes the intracellular replication of *L. pneumophila*, providing further evidence that its recruitment to the LCV is important for the biogenesis of the *L. pneumophila* replicative organelle (187). Aside from GTPase activity, vesicle tethering and fusion also involve interactions between soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) on both the vesicle and target membranes (312). In mammalian cells, the SNARE Sec22b is found on ER-derived vesicles and normally binds with a SNARE complex formed by membrin, syntaxin-5, and Bet1 on the pre-Golgi intermediate compartments (160, 360). Sec22b is delivered to the LCV membrane, providing clear evidence of fusion between LCVs and ER-derived vesicles (187). While most research examining the biogenesis of the LCV has utilized mammalian tissue culture models of infection, the formation of the replicative niche within protozoan hosts also involves a substantial diversion from the default endosomal pathway and the recruitment of rough ER (1, 49). This finding suggests that the ability of *L. pneumophila* to replicate in protozoa is closely linked to its ability to replicate in alveolar macrophages and that the fundamental processes are conserved between the two hosts.

Apart from Rab1 and Sec22b, double-stranded RNA interference techniques using *Drosophila melanogaster* Kc167 tissue culture cells have shown that the knockdown of the small GTPases Sar1 and ADP-ribosylating factor 1 (Arf1) decreases the intracellular replication of *L. pneumophila* (108). Reduced levels of expression of many other transport proteins had no effect on replication despite resulting in a severe disruption of normal host organelle biogenesis. The disruption of multiple pathways in this system, particularly Sec22 and the Golgi transport protein particle (TRAPP), did hinder *L. pneumophila* replication, suggesting that multiple sources of vesicles supply the LCV (108). Interestingly, the depletion of Cdc48/p97, which functions as an ATPase in a complex that recognizes ubiquitinated proteins targeted for ER-associated degradation, resulted in diminished *L. pneumophila* replication in both *Drosophila* and mammalian tissue culture cells (108). This complex was subsequently shown to associate with the LCV. Although the role of Cdc48/p97 in *L. pneumophila* infection remains unclear, it may assist in the translocation of bacterial effector proteins into the host cell. Overall, the ability of *L. pneumophila* to establish and maintain this intracellular niche

indicates that the bacteria actively and continually manipulate host cell trafficking from within the replicative vacuole.

Within 1 to 2 h of the recruitment of ER-derived vesicles to the LCV, resident ER proteins such as calnexin also become associated with the LCV, and within 4 to 6 h, the LCV membrane appears to be completely comprised of rough ER (186, 328). Replication of *L. pneumophila* bacteria then commences between 4 and 10 h after phagocytosis, and the bacteria continue to replicate within a vacuole that maintains a neutral pH (356). This finding concurs with the fact that the optimal growth of *L. pneumophila* bacteria *in vitro* occurs between pH 6.3 and 7.2 (2, 278). The nutritional environment of the LCV is poorly defined but presumably supplies the bacteria with amino acids for replication. Indeed, amino acid transporters are important for bacterial viability during intracellular replication (294). The oxidation of amino acids may also contribute to pH balance in the LCV.

Other evidence suggests that the manipulation of autophagy by *L. pneumophila* is important for bacterial replication. Autophagy is an important process for cellular homeostasis in which double-membrane vesicles (autophagosomes) derived from the engulfment of cytoplasmic components and organelles traffic to lysosomes for degradation (114). This commonly occurs in response to nutrient starvation, as the breakdown of macromolecules provides substrates for essential biosynthetic pathways. Within permissive bone marrow-derived macrophages, *L. pneumophila* appears to activate the autophagy process upon infection (14). The autophagy enzymes Atg7 and Atg8 show a transient association with the LCV that eventually acquires lysosomal markers in a manner similar to that of traditional autophagosomes (14). It is possible that interactions with the autophagic pathway provide a mechanism for supplying replicating *L. pneumophila* bacteria with a ready source of nutrients (109). However, investigations of *L. pneumophila* replication within *D. discoideum* have shown that autophagy is not linked to *L. pneumophila* replication. Mutations within five different genes involved in autophagy produced defects in the autophagy process but had no impact on LCV development or the replication of *L. pneumophila* (257). Therefore, the contribution of autophagy to *L. pneumophila* replication may be a peculiarity of cell type rather than a core element of LCV formation.

LCV Formation by Other *Legionella* Species

Other species of *Legionella* replicate within various protozoan species and human-derived cell lines but with differing levels of efficiency, and very little is known about the intracellular vacuoles of these opportunistic pathogens. The properties of the replicative vacuole have been examined only for *L. micdadei* and *L. longbeachae*. Within mammalian macrophages, alveolar epithelial cells, and *H. vermiformis*, *L. micdadei* multiplies within a spacious vacuole that shows no association with rough ER (138, 353). However, in MM6 human monocytic cells, the *L. micdadei*-containing vacuole colocalizes with calnexin, a resident protein of the ER (145). These differences suggest that different strains of *L. micdadei* may evoke different mechanisms to establish an intracellular niche. Virulent strains of *L. longbeachae* show the same intracellular phenotype as *L. pneumophila*, with the replicative vacuole asso-

ciated with rough ER and studded with ribosomes (145). The *L. longbeachae*-containing vacuole does, however, acquire LAMP-2, a marker of late endosomes (22). Interestingly, cytoplasmic replication has also been reported for some *Legionella* species, including *L. dumoffii* (181, 219), suggesting that there is great diversity in the mechanisms used by *Legionella* species to multiply in eukaryotic cells.

Bacterial Entry and Exit

Phagocytosis or invasion? In contrast to our understanding of LCV biogenesis, there is a surprising lack of knowledge surrounding how the bacteria are internalized by eukaryotic cells and ultimately released to infect new cells. Several competing theories regarding *Legionella* entry and exit differ on whether it is a pathogen-driven or host-directed response. In general, the bacteria appear to be taken up into phagocytes by conventional phagocytosis. A less common, atypical mechanism of coiling phagocytosis has been observed during the uptake of *L. pneumophila* bacteria in mammalian cells and amoebae (1, 49, 172); however, due to its infrequency, the significance of coiling phagocytosis to *Legionella* pathogenesis is questionable. Likewise, a form of macropinocytosis observed for bone marrow-derived macrophages (349) appears not to be a universal trait of *Legionella* uptake, as *L. pneumophila* Knoxville 1 and *L. micdadei* are phagocytosed via a classical zipper-like mechanism (271, 353). The relevance of opsonin-dependent phagocytosis to *L. pneumophila* infection is also dubious, given that complement levels in the lungs are generally very low (274). In addition, the phagocytosis of *L. pneumophila* is clearly observed in the absence of serum and occurs in cells that express complement receptors at only low levels (146, 281, 354). Furthermore, the ability of *L. pneumophila* to invade nonprofessional phagocytes such as A549, CHO-K1, and HeLa epithelial cells supports the hypothesis that the uptake of *L. pneumophila* is a virulence-directed property of the pathogen (112, 139, 222, 250).

The involvement of phosphatidylinositol 3 (PI-3)-kinase in conventional phagocytosis is well established; however, its involvement in the uptake of *L. pneumophila* is controversial. One research group found that the PI-3-kinase inhibitor wortmannin had no significant impact on the uptake of wild-type *L. pneumophila* bacteria by macrophages (189). This finding was supported by a recent study utilizing *Dictyostelium* mutants that lack two PI-3-kinase isoforms (351). However, other recent work has contradicted this finding, reporting that the nonopsonic phagocytosis of *L. pneumophila* does involve PI-3-kinase (330, 351). Regardless of whether PI-3-kinase assists *L. pneumophila* uptake, the mode of entry does not appear to influence LCV biogenesis, although PI-3-kinase-mediated uptake may influence the efficiency of LCV formation (71).

Bacterial factors implicated in *L. pneumophila* host cell invasion include at least five proteins, EnhC, LpnE, RtxA, LvhB2, and HtpB (86, 87, 140, 249, 277), although few of these have been shown definitively to play a direct role in bacterial uptake. For example, EnhC is a periplasmic protein that is required for the maintenance of cell wall integrity, and therefore, its contribution to bacterial invasion is likely to be indirect (205). The best-characterized of the entry proteins is the surface-located chaperonin HtpB or Hsp60. HtpB is upregulated

in the presence of eukaryotic cells and accumulates in the LCV after bacterial uptake. Recent work suggested that HtpB contributes to the recruitment of mitochondria to the nascent LCV, as inert beads coated with HtpB are associated with mitochondria following invasion (77). Similar to LpnE, which contributes to endosome evasion (251), HtpB may play a dual role in bacterial entry and the early development of the LCV, suggesting that these two events are closely linked.

Pore formation, cytotoxicity, and bacterial egress. An essential step in the development of infection and disease progression is the ability of intracellular pathogens to exit the host cell once replication has ceased, thereby allowing infection of new host cells. Tissue samples from patients and experimental animals show that *L. pneumophila* is free in the cytoplasm during late stages of replication, and the ability of *L. pneumophila* to escape from the replicative vacuole appears to be mediated by pore formation and membrane lysis (10, 54, 188, 192). Release into the host cell cytoplasm was hypothesized to cause the functional and structural disruption of cytoplasmic organelles and the disintegration of the plasma membrane, probably via further pore formation, leading to host cell osmotic lysis and bacterial egress (227).

Two main observations support a regulated mechanism of egress. First, *L. pneumophila*, in the postexponential phase, undergoes a number of phenotypic changes, converting to a more virulent or transmissive phenotype (10, 63). These mature, transmissive bacteria show increased levels of expression of virulence-related genes and are highly motile (59). The differentiation of *L. pneumophila* from a replicative form to a highly infectious mature intracellular form (MIF) occurs during intracellular growth but not *in vitro*. This development leads to ultrastructural changes in the bacteria, including an altered cell envelope and the presence of large inclusions within an electron-dense cytoplasm (120, 139). MIFs do not undergo cell division and are more resistant to environmental stress than replicative bacteria. MIF formation is enhanced by transient passage through the freshwater ciliate *Tetrahymena*, which expels live *L. pneumophila* bacteria as MIFs in a membrane-bound food vacuole (39). Mutants with regulatory defects that are unable to differentiate from the replicative form to the transmissive form are unable to survive within the *Tetrahymena* food vacuole and show an impaired ability to form MIFs (119). Hence, this regulated developmental biology of *L. pneumophila* likely contributes to the environmental persistence of the organism by aiding its transit through a variety of eukaryotic hosts.

The second observation is that at high multiplicities of infection with *L. pneumophila* in the transmissive phase, the bacteria induce contact-dependent cell cytotoxicity mediated by the development of pores less than 3 nm in diameter in the host cell membrane (175, 192). Originally, pore formation was thought to result from the insertion of Dot/Icm channels in the host membrane upon bacterial contact (192). Recent evidence suggests, however, that in mammalian cells, the pores are derived from the activation of the host cell inflammasome in response to bacterial flagellin (305). This conclusion is based on the fact that *L. pneumophila* mutants lacking flagellin do not induce pore formation and that caspase-1-deficient murine macrophages are resistant to pore formation regardless of Dot/Icm activity (305). Thus, contact-dependent cytotoxicity in mammalian cells appears to result from pores formed by the host cell rather than Dot/Icm pores inserted by the bacteria. Whether this mechanism aids bacterial egress in mammalian cells remains to be determined. For both protozoa and macrophages, a curious egress phenotype can be observed for *icmT* (*rib*) mutants that exhibit no contact-dependent lysis of cells (10). *L. pneumophila* bacteria carrying *icmT* mutant alleles become trapped within the host cell, growing to very high numbers, and are only gradually released from macrophages in the late stages of infection (10, 229). Based on mutational analysis of the C terminus, IcmT was described to be a pore-forming protein (228); however, no direct biochemical evidence exists yet to support functional pore formation by the IcmT protein, and the mechanism behind the *icmT* mutant phenotype may be indirect.

LESSONS FROM *LEGIONELLA PNEUMOPHILA* GENOMICS

In the last 5 years, four *L. pneumophila* serogroup 1 genome sequences have been completed. The genomes not only are an invaluable reference for molecular epidemiology and the analysis of *L. pneumophila* phylogeny but also have allowed researchers to gain insights into fundamental mechanisms of pathogenesis and pathogen evolution. Most striking is the genetic evidence for the interkingdom transfer of genes from protozoa to the *L. pneumophila* genome (68, 75).

Genome Structure and Diversity

The four *L. pneumophila* genome sequences available are all human clinical isolates with worldwide distributions and include Philadelphia-1, derived from the original Philadelphia outbreak (223); the Paris strain, an endemic strain responsible for around 12% of cases of Legionnaires' disease in France (23); the Lens strain, the causative agent of a large outbreak in France (252); and the Corby strain (184). The general features of the genomes are conserved in size (3.3 to 3.5 Mb), G+C content (~38%), number of predicted genes (3,001 to 3,259), percentage of coding regions (88 to 90.2%), and average length of the coding regions (68, 75, 147, 150). In general, the genetic order of the chromosome is conserved; however, synteny between the Lens strain and Paris, Philadelphia-1, and Corby is disrupted by a 260-kb inversion. Overall, the strains share around 80% of genes that constitute the core genome, while around 10% of the genome is strain specific (150). Much of the strain-specific genetic content is present in genomic regions associated with different G+C contents that may represent pathogenicity islands (321). For example, a 65-kb Philadelphia-1-specific genomic island harbors a plasmid-like element comprising a new *trb/tra* region and several efflux pumps and putative membrane transporters (51). Both Paris and Lens harbor plasmids that in the Paris strain include virulence determinants also found on a large *L. longbeachae* plasmid (111). The very high level of conservation between the sequenced section of the *L. longbeachae* plasmid and the 132-kb *L. pneumophila* Paris plasmid suggests recent horizontal transfer between these species (68). Another 60-kb plasmid present in the Lens strain encodes several proteins with homology to the transfer region of the F plasmid of *Escherichia coli*. Interestingly, the region encoding the Lvh type IVa secretion system of

L. pneumophila Paris may be present as a multicopy plasmid or integrated in the chromosome (68, 107). This kind of genetic mobility has undoubtedly contributed to the high overall level of plasticity in the *L. pneumophila* genome and diversity among different strains (67, 68, 75, 147).

The core *L. pneumophila* genome contains many of the factors associated with the ability of the bacteria to infect eukaryotic cells and replicate (67). In fact, a DNA array screen of 217 *L. pneumophila* strains showed that there is a high degree of conservation among virulence-associated genes (67). Although no specific hybridization pattern was associated with environmental or clinical isolates in that study, those authors identified *L. pneumophila* Paris as a globally distributed epidemic strain, suggesting that genomic background does contribute to environmental persistence and clinical disease. That work also identified four phylogenetic lineages (lineages I, II, III, and IV) of *L. pneumophila*, but these lineages were not associated with geographic region or epidemiology (67, 150). In short, it appears that *L. pneumophila* is a highly diverse species, and the capacity of any one strain to cause human infection cannot be predicted from its gene content.

Preponderance of Eukaryotic Protein Motifs and Domains

Perhaps the most intriguing aspect of the *L. pneumophila* genome is the unusual number of genes predicted to encode products with amino acid sequence similarity to eukaryotic proteins and/or containing eukaryotic domains (68, 150, 207). Although genes of apparent eukaryotic origin have also been identified for other intracellular pathogens, including *Coxiella*, *Wolbachia*, *Mycobacterium*, and *Rickettsia* species, *L. pneumophila* possesses the widest variety. Upon subsequent investigation, many of the gene products were found to play a role in host-pathogen interactions, and many of them are translocated into the eukaryotic host cell, where they presumably interfere with pathways through functional mimicry (207).

One interesting set of eukaryote-type enzymes are the three serine/threonine protein kinases (STPKs) identified in all the *L. pneumophila* genomes. *Mycobacterium tuberculosis* produces 11 eukaryote-like STPKs, with 1 clearly involved in the inhibition of phagosome-lysosome fusion and the promotion of intracellular survival (91). *L. pneumophila* may utilize eukaryotic STPKs for a similar purpose. *L. pneumophila* also secretes two proteins exhibiting a significant level of similarity to the mammalian CD39 family of ectonucleoside triphosphate diphosphohydrolases (NTPDases) (134, 291). The presence of two NTPDase genes in *L. pneumophila* is surprising since these enzymes are associated almost exclusively with eukaryotes. In fact, the recently determined crystal structure of Lpg1905 shows that the bacterial protein is a conserved structural mimic of mammalian NTPDases (344). Lpg1905 is a functional NTPDase that hydrolyzes both ATP and GTP with similar efficiencies (291, 292). The inactivation of *lpg1905* results in the defective replication of *L. pneumophila* bacteria in amoebae, epithelial cells, and macrophages (291). Substantial levels of enzyme activity are also required for the full virulence of *L. pneumophila* in a mouse model of infection, although it is not yet clear if the ATPase or GTPase activity or both activities contribute to *L. pneumophila* infection (292).

Another eukaryote-type enzyme and a further addition to the family of secreted metalloproteases predicted for the *L. pneumophila* genome is an M12 zinc metalloprotease (68, 95). The eukaryotic M12 family of metalloproteases has diverse biological roles and includes members such as adamalysin, which is a component of rattlesnake venom (129), as well as ADAM17, a tumor necrosis factor alpha (TNF-α)-converting enzyme, and ADAMTS13, which cleaves plasma von Willebrand factor (259). Other members of this family target components of the extracellular matrix such as collagen (259). The M12 metalloprotease of *L. pneumophila* is secreted *in vitro* and translocated into the host cell, but to date, its substrate specificity and function are unknown (95, 99). Other gene products with eukaryotic similarity include enzymes involved in sphingomyelin degradation, a pathway that is not present in prokaryotic cells. *L. pneumophila* possesses a sphingomyelinase, sphingosine kinase, and sphingosine-1-phosphate lyase, strongly suggesting that the bacteria interfere with sphingomyelin degradation, although the consequence of this for bacterial replication and LCV formation is not yet known (150). The sphingosine-1-phosphate lyase is at least translocated into eukaryotic cells, where it is targeted to mitochondria (100).

Eukaryotic domains found within predicted *L. pneumophila* proteins include at least 5 proteins with multiple Sel1 repeats and 20 proteins with ankyrin repeat domains (Ank). Sel1 repeat domains are linked to a range of protein-protein interactions in both prokaryotic and eukaryotic cells (149). Three of the Sel1 repeat proteins, EnhC, LidL, and LpnE, are linked to *L. pneumophila* entry and/or endosome evasion, although it is not yet known if they play a direct role in host-pathogen interactions (87, 92, 205, 249, 251). For example, EnhC function has been linked to the maintenance of cell wall integrity (205). The Ank proteins are the most abundant group of eukaryote-type proteins encoded by the *L. pneumophila* genome and exhibit significant diversity among different *L. pneumophila* strains (67). In eukaryotes, ankyrin repeats mediate protein-protein interactions and are commonly found together with other domains that determine protein function. Therefore, the Ank proteins of *L. pneumophila* are predicted to have varied activities. The carriage of a large number of Ank proteins is a property shared by the closely related bacterial pathogen *Coxiella burnetii* (346). In the case of both pathogens, the Ank proteins are translocated into the host cell, where they may interfere with various cell functions, including vesicular transport (258, 346).

Other *L. pneumophila* proteins carry domains found in eukaryotic guanine exchange factors (GEFs). The Sec7 domain protein RalF is translocated into the host cell and functions as a GEF for the host cell GTPase Arf1, which regulates COPI vesicle trafficking (241). Another protein with a predicted Ras GEF domain (Lpg0276/Lpp0350/Lpl0328/LPC_0353) has not been characterized. Some *L. pneumophila* proteins appear to target host ubiquitination pathways though U-box and F-box domains, and these too are translocated into the host cell (6, 195; M. Lomma, D. Dervins-Ravault, H. J. Newton, F. M. Sansom, M. Jules, T. Sahr, T. Nora, M. Bonazzi, E. L. Hartland, and C. Buchrieser, submitted for publication). The carriage of such diverse proteins with eukaryotic similarity and/or eukaryotic domains suggests that a major survival strategy of *L. pneumophila* is the functional mimicry of multiple eukaryotic pathways.
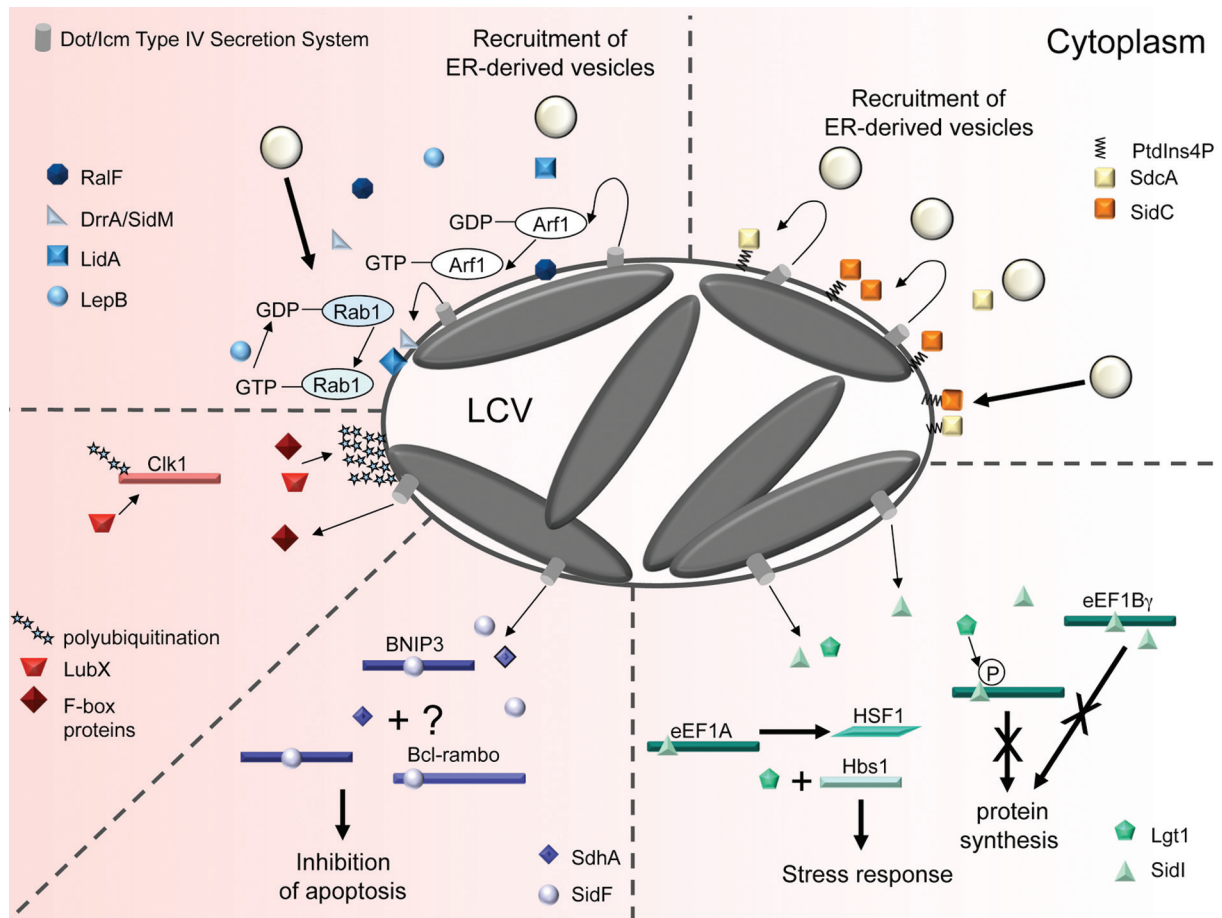
FIG. 2. Selected host cell processes targeted by Dot/Icm effectors demonstrating the complexity of LCV biogenesis and maintenance as well as the broad range of effectors and pathways involved. *L. pneumophila* effectors attack numerous cellular structures and host proteins; major targets include (clockwise from top left) host GTPases regulating vesicle trafficking, membrane phosphoinositides, host protein synthesis and stress response machinery, cell apoptosis, and host ubiquitination pathways. See the text for details. Larger circles represent ER-derived vesicles.

The origin of the eukaryotic proteins of *L. pneumophila* is an intriguing issue. The proteins seem unlikely to have emerged through convergent evolution, and hence, the current hypothesis is that genes were acquired by horizontal gene transfer from a range of eukaryotic hosts (150). *L. pneumophila* is capable of exogenous DNA uptake through natural transformation and may have acquired new genetic information through its long evolutionary association with protozoa. In support of this hypothesis, there is increasing phylogenetic evidence of interkingdom DNA transfer between the intra-amoebal bacterium *Legionella drancourtii* and its amoebal host (225, 226). However, the integration of unrelated DNA would present a significant challenge for the bacterium, and until more protozoan genome sequences become available, the evolution and origin of the eukaryotic genes in *L. pneumophila* will remain unexplained (150).

VIRULENCE FACTORS OF *LEGIONELLA PNEUMOPHILA*

L. pneumophila possesses many of the traditional bacterial determinants that are important for pathogenicity in other bacteria, including lipopolysaccharide (LPS), flagella, pili, a type II secretion system (T2SS), and outer membrane proteins.

However, the ability to manipulate host cell processes from within an intracellular vacuole requires a unique arsenal. In the case of *L. pneumophila*, this includes a type IV secretion system (T4SS) that translocates around 200 effector proteins, including many proteins with eukaryotic similarity, into the host cell, where they act on diverse host cell pathways (Fig. 2).

The Dot/Icm Type IV Secretion System

The Dot/Icm secretion apparatus. Components of the *L. pneumophila* Dot/Icm system appear to be involved in all aspects of the intracellular biology of *L. pneumophila*. Not only is the Dot/Icm system required for intracellular replication and the establishment of the LCV, it is also involved in bacterial entry, the inhibition of host cell apoptosis, and the egress of *L. pneumophila* from host cells (166, 192, 208). The dual name of the type IV secretion system in *Legionella* comes from its codiscovery by two research groups. In one instance, a single genetic locus able to complement an avirulent mutant was termed *icm*, for intracellular multiplication (170, 217). This locus comprised four putative genes, *icmWXYZ* (50). In parallel, another mutant with defective intracellular replication and an inability to establish the ER-derived LCV was comple-

mented with a single genetic locus, designated *dot*, for defect in organelle trafficking (37). This complementing locus contained one open reading frame encoding a 1,048-amino-acid protein that was designated *dotA*, and the split nomenclature for this secretion system has remained (38, 242). These initial discoveries were followed rapidly by the identification of more *dot/icm* genes required for *L. pneumophila* virulence, including *dotH*, *dotI*, and *dotO*, which are essential for intracellular growth and the evasion of the endocytic pathway, and *icmGCDJBF* and *icmTSRQPO*, which were implicated in macrophage cell death (16, 266, 289, 298, 299, 345). The genes are found on two distinct regions of the chromosome, each approximately 20 kb in length. Region I comprises *dotDCB* and *dotA-icmVWX* (221, 345). Region II contains 18 genes, most of which have both *dot* and *icm* designations (298, 345).

While some of the *dot/icm* genes show no sequence homology to other known open reading frames, at least 18 of the genes show similarity to components of bacterial conjugative DNA transfer systems, particularly of the IncI plasmids ColIB-P9, from *Shigella flexneri*, and R64, from *Salmonella enterica* (194, 345). Indeed, the Dot/Icm system is ancestrally related to DNA conjugation systems and has retained the ability to mobilize certain plasmids (298, 345). These T4SSs are important for the virulence of several pathogens and may transfer nucleic acids, proteins, or complexes of both to recipient cells. A range of pathogens utilize T4SSs to secrete virulence determinants, including *Bordetella pertussis*, *Helicobacter pylori*, *Brucella* sp., *Rickettsia prowazekii*, and *C. burnetii* (78). There are two distinct categories of T4SSs: T4SSa includes those systems that resemble the prototypic *Agrobacterium tumefaciens* Vir system, and T4SSb comprises systems with homology to IncI plasmids. The Dot/Icm system and the T4SS of *C. burnetii* are both members of the T4SSb category (78), and recent findings suggest that the T4SS of *C. burnetii* is functionally related to the Dot/Icm system (364, 368).

Recent evidence suggests that the Dot/Icm components form a multiprotein apparatus that spans the inner and outer membranes of the bacterial cell wall (341). Although there is no experimental evidence yet, a model for protein secretion and translocation exists whereby one or more of the Dot/Icm components form a pilus-like organelle on the bacterial surface that inserts a translocation pore into the host cell plasma membrane and vacuolar membrane of the LCV (179). Thus, the Dot/Icm apparatus forms a translocon that delivers multiple bacterial effector proteins into the host cell in a manner that is functionally analogous to that of a type III secretion system (T3SS). The identity of the translocation pore has not been resolved, but a candidate pore-forming protein, IcmQ, may be involved, along with its cytoplasmic chaperone, IcmR (113, 270). Indeed, similar to a T3SS, the Dot/Icm system is believed to utilize a network of cytoplasmic chaperones, the best-characterized of which are IcmS and IcmW, that bind to effector proteins and facilitate their translocation (254). Unlike T3SS effectors, the secretion signal of Dot/Icm effectors appears to be located in the C terminus of the proteins (64, 240).

Substrates of the Dot/Icm system. A major advance in our understanding of the complexity of *Legionella*-host cell interactions has been the discovery of around 200 proteins that are translocated into the cell by the Dot/Icm secretion system. Dot/Icm effectors therefore constitute around 10% of the *L.*

pneumophila proteome, which suggests that the effectors are a major determinant of *L. pneumophila* survival and that the selection for their retention is strong. Nevertheless, there are differences in effector repertoires between strains, suggesting that some are not essential and/or are niche specific (207). In contrast to substrates of the Lsp system described below, few of the Dot/Icm effectors can be detected in culture supernatants of *L. pneumophila* bacteria grown *in vitro*. This may be explained by the observation that effector translocation and phagocytosis are supported by intimate contact between the bacteria and the host cell as well as certain host cell factors, including the cytoskeletal components actin, tubulin, and N-WASP and host tyrosine kinases (71).

Substrates of the Dot/Icm secretion system have been identified through a range of approaches, including the presence of eukaryotic motifs such as the Sec7 domain of RalF that is characteristic of ADP-ribosylation factor (Arf) GEF proteins (74, 241). Dot/Icm substrates have also been identified through screening for Dot/Icm misregulation, interbacterial protein transfer, genes that cause lethality or mistrafficking of vacuolar proteins in *Saccharomyces cerevisiae*, and proteins that can interact with Dot/Icm chaperones (65, 92, 209, 254, 304). A recent bioinformatic machine learning approach also identified multiple new effectors (61). Many of the effector proteins comprise families of paralogues, and the function for the vast majority of these proteins is unknown. In general, the inactivation of genes encoding Dot/Icm effectors leads to, at most, a modest defect in intracellular replication, certainly none as severe as mutations in the Dot/Icm apparatus itself. This has led researchers to conclude that much functional redundancy exists among the Dot/Icm effectors, arising from significant functional redundancy among multiple Dot/Icm effector paralogues and from unrelated Dot/Icm effectors targeting the same host cell pathway (108). Emerging work also supports the idea that *L. pneumophila* passes through a variety of eukaryotic hosts. Therefore, some effectors may assist in the evasion of killing by different protozoan predators rather than contribute to intracellular replication *per se*.

Host Cell Processes Targeted by Dot/Icm Effector Proteins

Vesicular trafficking pathways. Many studies have sought to identify Dot/Icm effectors that interfere with host cell organelle trafficking (92, 99, 108, 163, 304). Frequently, the involvement of the effectors in subverting vesicle trafficking has been based on their ability to interfere with protein secretion in yeast or to induce yeast lethality (65, 304). The subcellular localization of ectopically expressed Dot/Icm effectors has also been key to understanding their function, and several effectors show distinct Golgi apparatus and ER localizations (99, 253, 258). In one study, a cohort of genes was identified that, when disrupted, led to reduced LAMP-1 avoidance; however, with the exception of LidA (see below), the mechanisms are unknown (58, 92, 103, 205). Similarly, a large number of Dot/Icm effector proteins that interfere with the trafficking of secretory proteins in yeast were identified recently, and while the mechanisms behind this interference are unclear, at least one protein, SetA, mediates its effect through glycosyltransferase activity (163). Another Dot/Icm effector, AnkX, interferes with microtubule-dependent vesicle trafficking, although the mech-

anism is unknown (258). The precise elucidation of the function of effectors that induce aberrant host vesicle trafficking will be critical to an understanding of the steps involved in LCV biogenesis.

Regulation of host GTPases. A key aspect of manipulating trafficking events within eukaryotic cells is the ability of *L. pneumophila* to recruit GTPases and control GTP cycling. Many intracellular bacterial pathogens employ similar approaches, particularly targeting Rab GTPases (60). Rab GTPases are found on membranes of organelles and act as molecular switches depending on whether they are bound to GTP or GDP. There are many additional proteins that regulate this process, including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs) that bind to cytosolic inactive GTPases, and GDI displacement factors (GDFs).

Specific Dot/Icm effector proteins of *L. pneumophila* influence GTP cycles through competing with endogenous GEFs to enable the rapid recruitment, redirection, and activation of Arf1 and Rab1 on the LCV (102, 187, 241). As mentioned previously, the Dot/Icm effector protein RalF acts as a specific GEF for Arf1, which normally regulates COPI-coated vesicle formation (241). While Arf1 function is required for ER fusion with the LCV, the mutation of *ralF* does not have an impact on the ability of *L. pneumophila* to replicate intracellularly (241, 280). The COPII GTPase SarI also appears to be involved in the establishment of the LCV, as the expression of a dominant inactive form of Sar1 demonstrated that Sar1 function is required for the recruitment and tethering of ER vesicles with LCVs (280).

Rab1, the GTPase that promotes the fusion of ER-derived vesicles with Golgi compartments, is recruited to the LCV within minutes of bacterial uptake (102, 187). Several *L. pneumophila* Dot/Icm effector proteins play distinct roles in enabling the exploitation of Rab1. DrrA (defect in Rab1 recruitment)/SidM (substrate of Icm/Dot) was shown by two laboratories to be the Dot/Icm effector protein essential for Rab1 manipulation (213, 238). This protein is bifunctional, with the ability to act both as a GDF, allowing more Rab1 to become available, and as a GEF (177, 212). In addition, the Dot/Icm effector protein LidA binds Rab1 and acts as an accessory protein for DrrA/SidM, enhancing Rab1 recruitment (213).

The screening of an *L. pneumophila* mutant library for mutants defective in Rab1 recruitment identified several other proteins linked to this process, including the Dot/Icm effector protein LepB (238). Most recently, it was demonstrated that LepB acts as a Rab1 GAP, inactivating Rab1 by stimulating GTP hydrolysis, leading to the removal of Rab1 from the membrane (177). This finding demonstrates that *L. pneumophila* has the capacity to control all aspects of Rab1 cycling on the LCV membrane.

Phosphoinositide binding and the LCV. Several Dot/Icm effector proteins localize to the LCV through interactions with phosphatidylinositol (PI) derivatives. PIs are negatively charged glycerolipids that can be phosphorylated via the inositol head group. These molecules play crucial roles in a variety of cellular functions, including cell signaling and membrane trafficking. The surface of the LCV is rich in phosphatidylinositol 4-phosphate [PtdIns(4)P] which is preferentially found on

the *trans*-Golgi network and acts as a second messenger to mediate the export of early secretory vesicles from ER exit sites (267).

The Dot/Icm effector protein SidC and its homologue, SdcA, anchor to the membrane of the LCV by binding specifically to PtdIns(4)P (209, 351). The PtdIns(4)P binding portion of SidC has been mapped to the C terminus, while the N-terminal portion of the protein promotes interactions with the ER (267). A mutant strain lacking both *sidC* and *sdcA* is still able to replicate intracellularly; however, the LCV acquires ER markers less efficiently (267). Further screening for other *L. pneumophila* phosphoinositide binding proteins identified the Dot/Icm effector SidM/DrrA (described above) as a PtdIns(4)P binding protein (58). LidA, the auxiliary protein for SidM/DrrA function, also binds PtdIns(4)P but binds the less abundant PtdIns(3)P with a higher affinity (58). A further PtdIns binding protein, LpnE, is not a classical Dot/Icm effector (our unpublished data), although the exported protein has the ability to bind both PtdIns(4)P and PtdIns(3)P as well as the 4′ phosphatase OCRL1. The LpnE-PtdIns interactions may be significant for LCV biogenesis, as *lpnE* mutant vacuoles exhibit an increased association with LAMP-1, and OCRL-1 restricts intracellular *Legionella* growth (251, 350). These examples clearly demonstrate that *L. pneumophila* can manipulate host cell PtdIns to assist in the formation of the LCV, perhaps by providing a means for multiple effector proteins and/or host proteins to anchor to the LCV membrane.

Host protein translation and induction of stress responses. The large GTPase eEF1A acts as a component of the protein synthesis elongation complex and is a target for multiple effector proteins of *L. pneumophila*. Most recently, the Dot/Icm substrate SidI was shown to bind both eEF1A and eEF1Bγ, which led to the arrest of host protein synthesis (301). The interaction with eEF1A simultaneously induced a stress response in host cells, leading to the activation of the heat shock regulatory protein HSF1 (301). This may make the host cell environment more conducive to bacterial replication. A second effector protein, the *L. pneumophila* glycosyltransferase Lgt1, glycosylates eEF1A at serine 53, which also results in the inhibition of protein synthesis (35). Furthermore, Lgt1 has the capacity to modify the heat shock protein 70 subfamily B suppressor Hbs1, which may have an added influence on the induction of a stress response in infected cells (36). Interestingly, the deletion of both *lgt1* and *sidI* does not alter the intracellular replication of *L. pneumophila* bacteria, which suggests either that this property is not important for bacterial replication or that further effectors influence the pathways controlled by eEF1a (301). Nevertheless, this is a clear demonstration of *L. pneumophila* providing multiple independent mechanisms to influence a common host cell process.

Inhibition of apoptosis. While several early reports suggested that *L. pneumophila* induces the apoptosis of epithelial cells and macrophages through the activation of caspase-3 (137, 247), it is now evident that despite activating caspase-3, *L. pneumophila* inhibits apoptosis in macrophages until the later stages of infection (3, 230). The finding that caspase-3 is important for the intracellular survival of *L. pneumophila* led to the suggestion that caspase-3 activation may be connected to Naip5-mediated protection against *L. pneumophila* infection of mice (see below) (151, 359). A close homologue of Naip5,

XIAP, is an apoptosis inhibitor protein that can bind to caspase-3 and render it inactive (214, 327). Recent data have shown that *L. pneumophila* also activates caspase-7 downstream of caspase-1 and the Ipaf/Nlrc4 inflammasome (4). The authors of that report suggested that caspase-3 and caspase-7 act differentially to modulate LCV trafficking where caspase-3 inhibits and caspase-7 promotes phagolysosome fusion (13). Clearly, the *L. pneumophila*-induced activation of the inflammasome and the pathogen-mediated modulation of apoptosis are likely to be closely connected, and the dissection of these pathways and their relative contributions to *L. pneumophila* replication in macrophages will be an intriguing puzzle to solve. The inhibition of apoptosis may, in part, be due to the activation of NF-κB upon *L. pneumophila* infection of macrophages (208). *L. pneumophila* induces an increase in the level of expression of antiapoptotic genes, which is regulated through NF-κB (208). In fact, the activation of NF-κB by *L. pneumophila* is biphasic, where, following an initial stimulation by flagellin through Toll-like receptor 5 (TLR5), NF-κB activation is sustained for several hours in a flagellin-independent and Dot/Icm-dependent manner (32). The inhibition of apoptosis varies with cell type, and recently, it was established that dendritic cells (DCs) limit the replication of *L. pneumophila* by caspase-3-mediated apoptosis. The rapid induction of apoptosis upon infection in dendritic cells may provide the host with a means to restrict infection while also inducing important innate immune responses (255).

Given that *L. pneumophila* infection inhibits apoptosis in macrophages, it is not surprising that the Dot/Icm secretion system delivers effectors with specific antiapoptotic properties. The effectors SdhA and SidF play a direct role in preventing host cell apoptosis during infection, although the mechanism of SdhA function is unknown (30, 196). SidF inhibits apoptosis through direct interactions with two proapoptotic members of the Bcl2 protein family, BNIP3 and Bcl-rambo (30). The evolution of these proteins is intriguing, as amoebae do not have the genes required for apoptosis, so their presence suggests either a substantial interaction of *L. pneumophila* with a higher eukaryotic organism or a fortuitous dual function of the effectors. In support of the former suggestion, *L. pneumophila* was recently shown to colonize the intestinal tract of *Caenorhabditis* nematodes and induce worm death (52). This raised the possibility that *L. pneumophila* evolved with both protozoan and metazoan hosts. In addition, several Dot/Icm effectors have been implicated in the sustained activation of NF-κB, including LegK1, which has been reported to mimic host IKK and directly phosphorylate and induce the degradation of the NF-κB inhibitor IκB in macrophages (142). SdbA and LubX also contribute to sustained NF-κB activation in A549 epithelial cells, and both are essential for *L. pneumophila* replication, although their mechanism of action is unknown (32).

Mimicking host ubiquitination pathways. During infection, the LCV is decorated with polyubiquitinated proteins in a Dot/Icm-dependent manner (108, 180). The *L. pneumophila* genome encodes several putative Dot/Icm effector proteins with eukaryotic motifs that function in the eukaryotic ubiquitination pathway (68). These include three proteins with F-box domains that in eukaryotes recruit specific target proteins to the SCF (Skp1/cullin 1/F-box) ubiquitin ligase complex. Another protein, LubX, has two U-box domains that are impor-

tant for E3 ubiquitin ligase target recognition (167). LubX has been confirmed to be a Dot/Icm substrate and acts as a ubiquitin ligase in the polyubiquitination of Clk1 (Cdc2-like kinase 1) (195). It is not clear how the function of LubX aids *L. pneumophila* infection, but the inhibition of Clk kinase significantly reduces *L. pneumophila* intracellular replication (195). Similarly, all three F-box proteins are translocated into host cells via the Dot/Icm system (6; Lomma et al., submitted). One of these, AnkB, or Lpp2082, recruits ubiquitinated proteins to the LCV and is essential for intracellular replication and virulence in mice (265; Lomma et al., submitted). AnkB/Lpp2082 binds Skp1 in the SCF (265; Lomma et al., submitted) and also interferes with the ubiquitination and degradation of the focal adhesion protein ParvB. In cells depleted of ParvB or Skp1, *L. pneumophila* intracellular replication is diminished, suggesting that host cell-mediated ubiquitination and possibly ParvB itself support LCV biogenesis (265; Lomma et al., submitted).

The Lsp Type II Secretion System

In addition to the Dot/Icm T4SS, *L. pneumophila* possesses a type II protein secretion system (T2SS) termed Lsp, for *Legionella* secretion pathway, which is required for full virulence and environmental persistence (153, 310). T2SSs are highly conserved protein secretion machines of Gram-negative bacteria that play an important role in the disease progression of many bacterial pathogens through the export and directed release of toxins, proteases, and other enzymes (80). Substrates of T2SSs possess an N-terminal signal sequence that mediates their translocation across the inner membrane via Sec or Tat machinery (80). Once in the periplasm, the signal peptide is removed and the folded protein is transported across the outer membrane by a multiprotein complex comprising the T2SS secretin. The *L. pneumophila* T2SS has 12 components located in five loci throughout the chromosome. These include the prepilin peptidase *pilD*, which processes the pilin of type IV pili and pseudopilins of the T2SS (201); the outer membrane secretin and ATPase *lspDE*; the pseudopilins *lspFGHIJK*; and *lspC* and *lspLM*, which are predicted to promote secretion (287). Mutant strains of *L. pneumophila* lacking *lspDE*, *lspG*, or *lspK* demonstrate drastically impaired replication within *A. castellanii* and *H. vermiformis* cells and a more moderate replication defect in macrophages (263, 284). In addition, *lsp* mutants show a reduced capacity to replicate in the lungs of mice compared to wild-type *L. pneumophila* (287).

The ability of *L. pneumophila* to remain viable under a wide range of conditions is fundamental to its environmental persistence. A disruption of the Lsp secretion system results in an inability of *L. pneumophila* to survive at low temperatures, both in tap water at temperatures ranging from 4°C to 17°C and in the presence of aquatic amoebae at temperatures of 22°C to 25°C (310, 311). The Lsp system presumably secretes factors that aid *L. pneumophila* persistence at low temperatures, as the growth of *lsp* mutants was stimulated by the addition of culture supernatant from the wild-type strain (310). One of these factors is a secreted peptidyl-prolyl *cis-trans* isomerase present in the culture supernatant (308). Additional factors were recently identified from a transposon mutant screen for growth defects at low temperatures. The screen identified 11 mutants with a compromised ability to replicate at

17°C, and these included mutants with insertions into components of the T2SS. Other major factors included a homologue of the LpxP lipid A acyltransferase; an RNA helicase, CsdA, which was particularly important for bacterial survival; and RNase R, which was implicated previously in replication at low temperatures (70, 309).

Recently, *L. pneumophila* was observed to undergo sliding motility on agar plates, and this phenotype was linked to the function of the Lsp secretion system (323). Sliding motility was independent of flagella and pili but required a functional Lsp secretion system. Sliding motility appears to be linked to the production of an undefined amphipathic, diffusible surfactant, which may be directly secreted by the T2SS or processed by an Lsp effector protein (323). Surfactant production still occurs in mutants lacking characterized Lsp substrates and in a *tatB* mutant; therefore, the molecular basis of surfactant production is yet to be identified. Other species of *Legionella* also demonstrate sliding motility, albeit with various efficiencies, except *L. micdadei*. This finding is consistent with data from previous reports showing that while *L. micdadei* harbors the *lsp* genes, this *Legionella* species does not express Lsp-dependent phenotypes, including phosphatase, hemolytic and lipolytic activity, and growth at low temperatures (26, 125, 287, 310). It is unclear whether these phenotypic differences are due to a lack of expression of the components of the Lsp secretion system or a specific lack of the Lsp substrates that mediate these phenotypes.

Substrates of the Lsp Secretion System

The Lsp secretome has been characterized by proteomics and comprises a cohort of at least 27 proteins (95). Curiously, the Dot/Icm component IcmX is among the secreted proteins (95). A chitinase enzyme, ChiA, is also secreted by the Lsp T2SS, and further investigation demonstrated that while this protein is not required for *in vitro* intracellular growth, it does promote persistence within the mouse model of infection. Infected mice also produce antibodies to ChiA (95). A range of proteins with enzymatic activities are secreted by the Lsp system, including the most abundant secreted protein, the zinc metalloprotease ProA or Msp, as well as acid phosphatases, multiple lipases, phospholipases C and A, lysophospholipase A (LPLA), an RNase, both tartrate-resistant and tartrate-sensitive acid phosphatases, and an endoglucanase (17–19, 153, 201, 260, 284, 287). An analysis of mutants lacking the tartrate-sensitive acid phosphatase, LipA and LipB lipase, LPLA, phospholipase C, or two aminopeptidases, LapA and LapB, found no impairment in intracellular replication (17, 19, 126, 224, 286, 329). Additionally, these single mutants showed no growth impairment at low temperatures (311). Recently, ProA/Msp and the RNase SrnA were shown to be required for the optimal replication of *L. pneumophila* bacteria in *H. vermiformis* (285, 286). This is an interesting finding, as these enzymes are dispensable for growth in all other host cell models tested, which may implicate substrates of the Lsp secretion system in host-specific responses. Indeed, not all *Legionella* strains have the same Lsp substrate repertoire. For example, the T2SS-dependent secreted endoglucanase is not found in all *L. pneumophila* strains (260).

As with the substrates of the Dot/Icm secretion system, the vast majority of Lsp substrates confer no phenotype that can be measured by comparing the replications of wild-type and mutant strains. With examples such as ProA and SrnA, this can perhaps be attributed to the substrate playing a specific role in a subset of environmental hosts. However, functional redundancy is also prevalent among Lsp substrates. *L. pneumophila* encodes at least 15 proteins with phospholipase A (PLA) and LPLA activities, and at least 2 of these proteins, PlaA, the major secreted phospholipase, and PlaC, are secreted via the Lsp system (29, 126; for a review, see reference 28). Phospholipases cleave fatty acids from lipids having a direct impact on host cells through membrane destruction. In the case of a lung pathogen, phospholipases may also act to break down lung surfactant. In addition, by-products of phospholipase activity can act as signaling molecules, influencing important host functions such as inflammation and apoptosis (28). PlaA and PlaC are therefore likely to be important for *L. pneumophila* virulence, yet single mutants demonstrate no discernible phenotype (29, 126). Further functional redundancy may be expected, as *L. pneumophila* also has phospholipase activity from a third GDSL-like phospholipase, PlaD (28), and a cell-associated phospholipase, PlaB (127). A cohort of patatin-like phospholipases are also encoded by the *L. pneumophila* genome, at least two of which are translocated into host cells by the Dot/Icm T4SS (304, 340).

Future studies on the Lsp system will continue to unravel the contribution of Lsp substrates to *Legionella* biology and may also uncover other unexpected roles of the T2SS, such as its possible function in Rab1 recruitment to the LCV and inhibition of interleukin-8 (IL-8) secretion (N. Cianciotto, personal communication). The putative Lsp/Rab1 connection was made when an *lspE* mutant was identified among a small group of transposon mutants that recruited Rab1 poorly to the LCV (238). In addition, there is likely to be cross talk between the Lsp and Dot/Icm secretion systems. For example, the M12 zinc metalloprotease is secreted by the Lsp system and is also translocated into host cells by the Dot/Icm system (95, 99). How these two secretion systems may function together at a molecular level remains to be determined.

The Tat, Lss, and Lvh Secretion Systems of *L. pneumophila*

In addition to the Dot/Icm and Lsp protein secretion systems, *L. pneumophila* harbors a type I secretion system (Lss), a twin-arginine translocation system (Tat), and a second type IV secretion system (Lvh). The *lssXYZABD* locus includes the typical components of a type I secretion system, including an ABC transporter (LssB) and a membrane fusion protein (LssD) (182). Lss may play a role in the biology of *L. pneumophila*, although the secretion system appears to be largely dispensable for host-pathogen interactions (182). In contrast, the Lvh system may play a role in certain aspects of *L. pneumophila* intracellular replication by complementing Dot/Icm function (27). Following the incubation of *L. pneumophila* bacteria in water or the encystment of *L. pneumophila* bacteria in amoebae, the Lvh region was required to rescue the host cell uptake of Dot/Icm mutants (27). This finding suggests that both the Dot/Icm and Lvh systems are conditionally required for certain virulence attributes such as host cell invasion. Another curious feature of the Lvh system is that the locus may

exist as an extrachromosomal plasmid or as an integrated genomic island (68). The excision and integration of the *lvh* region into a chromosomal tRNA gene depend on the growth phase-dependent expression of genes carried by the plasmid-like element (107). For both Lss and Lvh, the proteins or substrates exported by these secretion systems have not yet been identified.

The Tat system complements the general secretory pathway (Sec) by transporting folded proteins across the inner membrane. In the case of *L. pneumophila*, the Tat pathway contributes to biofilm formation and intracellular replication in macrophages and amoebae and aids growth under low-iron conditions (98, 283). Several substrates of the Tat pathway have been confirmed, including phospholipase C, LvrE, and cytochrome *c* oxidase, although the list of likely Tat substrates is much longer and also includes components of the Lss and flagellar biosynthesis systems (96–98, 283). There appears to be little crossover between the Lsp and Tat secretion systems. Very few of the Lsp substrates seem to utilize the Tat pathway to reach the periplasm, and the supernatants of *tatB* mutants exhibit normal levels of Lsp-dependent enzymatic activity (283). In addition, a mutant lacking both Tat and Lsp systems demonstrates a defect in replication greater than that of either of the single mutants (283), which supports the idea that the Tat and Lsp systems work independently.

The Peptidylprolyl *cis-trans* Isomerase Mip

The gene encoding the peptidylprolyl *cis-trans* isomerase (PPIase) Mip was one of the first genes associated with the ability of *L. pneumophila* to replicate in eukaryotic cells, and the *mip* nucleotide sequence has been used extensively as a target for *Legionella* molecular diagnostics and typing for 2 decades (69, 115, 118, 185, 203, 269). Mip stands for macrophage infectivity potentiator, and *mip* mutants of *L. pneumophila* are defective for replication in macrophages, epithelial cells, and amoebae and are attenuated in both guinea pig and mouse models of infection (81, 83, 358).

The *mip* product is a 24-kDa protein with PPIase activity that shares amino acid sequence similarity with the mainly eukaryotic family of FK506 binding proteins, a class of immunophilins (123, 152). Mip is exported to the cell surface, where the protein exists as a stable homodimer (82, 275, 297). The catalytic PPIase domain of the enzyme is located in the C terminus of the protein, which is connected to the N-terminal dimerization domain by a long alpha-helix that acts as a hinge between the two domains (168, 275). The crystal structure of Mip revealed that the fold of the catalytic domain resembles the family of human protein FK506 binding proteins; therefore, Mip is another structural and functional mimic of a eukaryotic protein (275). Recently, the PPIase activity of Mip was associated with the ability of *L. pneumophila* to penetrate an epithelial barrier and to disseminate from the lung in a guinea pig model of infection (347). Mip binds to collagen and may sensitize the extracellular matrix to proteolysis by converting protease-insensitive *cis* prolyl bonds to protease-sensitive *trans* prolyl bonds (347). Mip then acts in concert with a serine protease of either bacterial or host origin to degrade the extracellular matrix. Mip may also act synergistically with other

secreted proteins of *L. pneumophila*, such as a *p*-nitrophenol phosphorylcholine hydrolase (94).

Iron Acquisition Mechanisms and Vacuolar Nutrition

Many studies have highlighted the importance of iron for *L. pneumophila* virulence and replication by exposing *L. pneumophila* bacteria to iron-limiting conditions *in vitro* (183, 272). Iron is also important during intracellular replication, as monocytes and macrophages that have been treated with an iron chelator do not support *L. pneumophila* replication (143, 342). In addition, a low level of expression of the transferrin receptor in monocytes and macrophages correlates with a nonpermissive state (62).

Given that a source of iron is essential for *L. pneumophila* replication, it is not surprising that the pathogen has developed mechanisms to regulate iron utilization and obtain iron from the environment and from host cells (79). A key development in our understanding of the acquisition of iron by *L. pneumophila* was the discovery that the bacteria secrete a siderophore although only at particular phases of growth under iron-limiting conditions (202). Representative strains from all *L. pneumophila* serogroups and other *Legionella* species also have siderophore activity under defined conditions (316). The production and export of the *L. pneumophila* siderophore legiobactin have been linked to the genetic locus *lbtAB*. LbtA is the predicted siderophore synthetase, and LbtB exhibits homology to multidrug efflux pumps and is likely responsible for the export of legiobactin (9). Most recently, *lbtA* or *lbtB* was confirmed as being essential for the production of legiobactin, and *lbtA* was identified as being important for infection of mice. Coinfection studies were able to show that legiobactin secreted from wild-type *L. pneumophila* bacteria can promote the replication of the *lbtA* mutant in *trans* (8).

Once the iron-siderophore complex is internalized across the outer membrane, it is likely acted upon by a periplasmic ferric reductase to yield ferrous iron that is transported across the inner membrane by FeoB (79). *L. pneumophila* also secretes pyomelanin, which has ferric reductase activity and may supply ferrous iron substrate to FeoB (72). Mutational analysis has shown that *feoB* is important for extracellular growth and promotes replication in *H. vermiformis* and macrophage cell lines as well as infection of mice (279). *L. pneumophila* also produces a multicopper oxidase, McoL, that is essential for aerobic extracellular growth under iron-limiting conditions or where ferrous iron is the only iron source (176). Rather than aiding iron acquisition, McoL may help prevent the toxic effects of utilizing ferrous iron during aerobic growth. In addition to these fundamental mechanisms, several other factors appear to be important for iron acquisition and assimilation, most notably the cytochrome *c* maturation (*ccm*) locus, which is essential for intracellular multiplication, and *iraAB* (for iron acquisition/assimilation), where *iraA* is important for intracellular replication and *iraB* contributes to extracellular growth under iron-limiting conditions (244, 264, 342, 343).

While iron is a critical limiting nutrient for many pathogens, other nutrient acquisitions by *L. pneumophila* must also occur intracellularly during the replicative phase of *L. pneumophila* growth. In fact, the nutritional environment of the LCV is quite poorly defined. It is clear that *in vitro*, amino acids are

preferentially utilized by *L. pneumophila* as a source of carbon, nitrogen, and energy in broth culture. Early reports demonstrated that *L. pneumophila* requires the presence of arginine, isoleucine, leucine, methionine, serine, threonine, valine, cysteine, and glutamic acid to replicate (144, 332, 333). Interestingly, genome sequencing revealed that *L. pneumophila* does possess genes for the synthesis of cysteine and methionine, suggesting that under certain conditions, the bacterium may not be auxotrophic for these amino acids (68, 75).

Clear evidence for the importance of amino acids for *L. pneumophila* replication is demonstrated by the discovery of a family of amino acid transporters that are crucial for the ability of *L. pneumophila* bacteria to differentiate and replicate within host cells (73, 294). These transporters have been termed phagosomal transporters (Pht) and belong to a subfamily of the major facilitator superfamily transporters. The first member of the family to be characterized was PhtA, which was shown to be a threonine transporter (294). *L. pneumophila phtA* mutants have an inability to replicate in macrophages, as the transmissive bacteria that invade and establish the LCV do not differentiate into a replicative phase (294). This demonstrates that the phagosomal transporters not only act to provide essential nutrients but in doing so can also assess the nutrient supply in a given environment, thereby providing a cue for replication. In addition, host amino acid transporters likely contribute to the vacuolar conditions that support *L. pneumophila* intracellular replication (357).

Including PhtA, all sequenced *L. pneumophila* strains possess 11 Pht paralogues, with strain Lens also having a strain-specific Pht (73). PhtJ, which transports valine, is at this stage the only additional Pht with a defined function (73); however, recent evidence suggests that other Pht family members, namely, PhtC and PhtD, may act as thymidine nucleoside transporters (M. Swanson, personal communication). Interestingly, several other intracellular human pathogens, including *Chlamydia* and *Coxiella*, possess Pht genes, indicating that this may be a common mechanism for nutrient sensing and acquisition by vacuolar pathogens (73).

The Biphasic Replicative Cycle of *L. pneumophila*

The environmental and intracellular niches of *L. pneumophila* expose the organism to a range of nutrient and temperature conditions that require the bacteria to be highly adaptable. Similar to other intracellular pathogens such as *Chlamydia* and *Coxiella*, *L. pneumophila* has a biphasic growth cycle where, under nutrient-rich conditions, the bacteria are in a replicative phase and express few virulence traits, but under nutrient-limited conditions, the bacteria enter a transmissive phase and become highly motile and resistant to various stresses (63). The transmissive phase allows bacteria to egress from spent host cells and commence the next round of infection, and this coincides with the expression of many virulence determinants.

A comprehensive microarray analysis examining and comparing the transcription profiles of *L. pneumophila* bacteria in replicative and transmissive phases both during infection of *A. castellanii* and *in vitro* highlighted the dramatic changes that differentiate these phases (59). That study found an increased transcription of metabolic genes involved in carbohydrate utilization and amino acid metabolism during the replicative phase (59). In contrast, during the transmissive phase, *L. pneumophila* upregulates the expression of many Dot/Icm substrates, genes involved in flagellar biosynthesis, and many other known virulence determinants. A large class of GGDEF and EAL genes, known to modulate the concentration of the second messenger cyclic di-GMP, are also upregulated during the transmissive phase, suggesting that cyclic di-GMP signaling is important for the regulation of this biphasic cycle (59).

A range of regulatory genes interact in a complex manner to control the transition of *L. pneumophila* between replicative and transmissive forms. Initial studies examining regulation during broth culture growth of *L. pneumophila* bacteria found that amino acid depletion, occurring following exponential growth, triggered the bacteria to release the alarmone guanosine 3′,5′-bispyrophosphate (ppGpp) (154). This response has been reported for a variety of microbes and involves uncharged tRNAs binding to ribosomes and activating ppGpp synthetases such as RelA and SpoT in *L. pneumophila* (93, 154). The accumulation of ppGpp stimulates the transition of *L. pneumophila* from its replicative form to its transmissive form via the sigma factors RpoS and FliA and the two-component response regulator LetA/LetS (for *Legionella* transmission activator and sensor) as well as the positive regulator LetE (25, 155, 367).

L. pneumophila RpoS regulates motility, sodium sensitivity, and evasion of the endocytic pathway (24, 153a). As such, it is not surprising that an isogenic mutant of *rpoS* is unable to replicate within *A. castellanii* (153a). Among other factors, RpoS regulates the expression of *fliA*, which itself is important for regulating contact-dependent cytotoxicity, infectivity, lysosomal avoidance, and biofilm formation (155, 215, 232). In concert or in parallel with RpoS and FliA, other two-component response regulators contribute to the regulation of transmissive traits. Both PmrAB and CpxRA directly regulate the transmissive-phase expression of Dot/Icm components and substrates (5, 11, 135, 366).

Perhaps the most important regulatory switch controlling the replicative- to transmissive-phase cycle is the two-component system LetAS (136, 155, 211). LetAS initiates a global regulatory mechanism that acts through the derepression of CsrA (233). Only recently have the small noncoding RNAs that mediate this process been identified (268, 290). A signal is relayed from LetAS to increase the expression levels of the small RNAs RsmY and RsmZ. These noncoding RNAs possess multiple CsrA binding motifs that act to sequester the transcriptional repressor CsrA and allow the transcription of genes that mediate the transmissive phenotype (268, 290).

An additional layer of complexity is incorporated into this regulatory network by quorum sensing. The response regulator LqsR contributes to virulence processes such as phagocytosis and the formation of the LCV by relaying signals from the adjacent sensor kinase LqsS (334, 335). Neighboring this system is an autoinducer synthase, LqsA, which was recently shown to synthesize 3-hydroxypentadecan-4-one, the small *Legionella* autoinducer 1 (LAI1) (313). Although an *lqsA* mutant of *L. pneumophila* showed only a modest decrease in phagocytosis compared to the wild-type strain, the overexpression of LqsA in an *lqsR* or *lqsS* mutant background restored the more severe uptake defects of these mutants, suggesting that LqsA does have a role in modulating virulence, possibly

through regulating the expression of a novel genomic island (336).

IMMUNE RESPONSES TO LEGIONELLA INFECTION

L. pneumophila is an environmental organism and, therefore, an opportunistic and accidental pathogen of humans. As a result, there has been no selective pressure on the evolution of *L. pneumophila* from the mammalian immune system. In general, a robust early inflammatory response is believed to control bacterial replication while cell-mediated immunity contributes to the resolution of the infection and bacterial clearance. Different animal models of *L. pneumophila* infection have been established to examine the host-pathogen relationship. Indeed, the isolation of *L. pneumophila* from the 1976 outbreak was facilitated by the intraperitoneal inoculation of guinea pigs with lung tissue from infected humans (223). Guinea pigs are highly susceptible to *L. pneumophila* infection, die within 3 days of aerosol exposure to the bacteria, and exhibit many clinical features associated with Legionnaires' disease of humans (33). In contrast, inbred strains of mice are largely resistant to *L. pneumophila* infection, with the notable exception of the A mouse strain (often called A/J, although this terminology refers only to mice derived directly from the Jackson Laboratories), which, when inoculated intratracheally with *Legionella* bacteria, develop acute lung inflammation (54, 124). However, even in A mice, the infection is self-limiting. The exception to the general rule of murine resistance to *Legionella* infection is *L. longbeachae*, which is highly virulent for a variety of inbred mouse strains (148). Several other *Legionella* species also replicate in murine macrophages (181, 355) and, if given a high-enough inoculum, may also replicate in whole animals. Nevertheless, while the guinea pig infection model is superior in replicating much of the clinical course of Legionnaires' disease, the mouse model has been favored by research groups around the world due to the availability of reagents and transgenic animals to study immune responses and pathogenesis.

Importance of Innate Immunity to *Legionella* Responses in Mice

Despite the significant shortcomings of the mouse infection model for Legionnaires' disease, the fact that mice are by and large resistant to *L. pneumophila* infection has allowed investigators to identify elements of the immune response that are critical to the control of *L. pneumophila* replication in macrophages and in the lung. Crosses between the permissive A strain and highly resistant C57BL/6 mice allowed the unique susceptibility of A mice to be mapped to the lgn1 locus on chromosome 13 (34, 104). Within this region are a series of highly polymorphic repeats of neuronal apoptosis-inhibitory protein (Naip), also known as baculoviral IAP (inhibitor of apoptosis) repeat-containing 1 (Birc1) (151, 361). Variations, either by missense polymorphisms or altered expression levels, in Naip5/Birc1e alleles have been defined as the basis for the susceptibility of the A mouse strain to *L. pneumophila* (105, 359). Naip5 is an intracellular flagellin recognition molecule that shows homology to plant resistance proteins involved in innate immune responses to secreted bacterial virulence pro-

teins (178). In macrophages, Naip5 activates caspase-1 upon the phagocytosis of *L. pneumophila*, leading to IL-1β production and an increased fusion of the LCV to endosomes, followed by bacterial degradation (363). Although a Naip5 equivalent is not found in humans, a study of Ipaf, another intracellular flagellin recognition molecule, produced similar findings, where Ipaf was shown to restrict *L. pneumophila* replication (12). Both Naip5 and Ipaf detect cytosolic flagellin, and either the deletion of flagellin from *L. pneumophila* or the deletion of Naip5 or Ipaf from nonpermissive macrophages reverses the restriction of *L. pneumophila* intracellular replication (12, 231, 273).

Since the publication of the first *in vivo* mouse model of *L. pneumophila* infection (162), various studies have identified key components of the innate immune response as being important for limiting and eliminating infection. These components include cytokines such as gamma interferon (IFN-γ) (54, 303), tumor necrosis factor alpha (TNF-α) (56, 133), IL-12 (57), and IL-18 (55) as well as the cell types that produce these cytokines, namely, neutrophils (101) and natural killer (NK) cells (20, 101).

More recently, investigations of the innate immune response to *L. pneumophila* have focused on Toll-like receptors (TLRs), a group of receptors that recognize various microbial products that trigger an innate immune response upon binding a specific ligand. Considerable study has also been directed toward myeloid differentiation primary response gene 88 (MyD88), an important adaptor molecule for most TLR signaling. MyD88-deficient mice infected with *L. pneumophila* have an increased bacterial burden in the lung and decreased survival rates, develop more severe lung pathology, and suffer disseminated bacterial infection in the spleen compared to wild-type mice (20, 157). However, investigations of individual TLRs such as TLR2, TLR4, TLR5, or TLR9 have revealed that their contribution to *L. pneumophila* resistance is negligible or, at best, very modest compared to that of MyD88 (21, 41, 156, 157, 197, 248, 315). For example, in an attempt to determine if multiple TLRs were responsible for the MyD88-dependent immune response to *L. pneumophila*, TLR2/9-deficient mice were infected with a flagellin-null (ΔflaA) mutant strain of *L. pneumophila*. By using this strategy, the combined contribution of TLR2, TLR5, and TLR9 to the immune response to *L. pneumophila* could be assessed. The ΔflaA bacterial burden in TLR2/9-deficient mice was similar to that of control mice, suggesting that the MyD88-dependent immune response was not restricted to the actions of TLR signaling (20). MyD88 also functions as an adaptor molecule for IL-1 and IL-18 receptor signaling, both of which are induced upon *L. pneumophila* infection (12, 216, 293, 363). The fact that *L. pneumophila* infection of caspase-1-deficient mice results in significantly higher bacterial loads in the lung and significantly less IL-18 production than that in control mice indicates that caspase-1- and Ipaf-deficient mice are more susceptible to *L. pneumophila* infection due to reduced IL-18 (or IL-1β) signaling that is MyD88 dependent (66).

Apart from the activation of the inflammasome through Ipaf and Naip5, *L. pneumophila* has diverse effects on cytokine signaling pathways. In macrophages, *L. pneumophila* stimulates cytokine activity in a TLR- and RIP2-independent and Dot/Icm-dependent manner (302). In fact, robust mitogen-

activated protein kinase (MAPK) signaling (p38 and Jun N-terminal protein kinase [JNK]) is induced in response to the Dot/Icm system in infected macrophages, and the fact that *icmS* and *icmW* mutants do not induce MAPK activation suggests that one or more Dot/Icm effectors are either directly or indirectly responsible (302). The consequences of MAPK activation for *L. pneumophila* replication are unknown, but this activity may constitute an important host response to infection *in vivo*. Certainly, in amoebae, *L. pneumophila* inhibits the equivalent of the MAPK pathway by inducing the expression of DupA, a tyrosine kinase/dual-specificity phosphatase, which is likely to be a negative regulator of MAPK signaling (200). *L. pneumophila* upregulates MAPK phosphatases in macrophages, which supports the idea that this pathway is manipulated during infection (208).

The induction of a robust type I interferon response was also observed during infection of macrophages and epithelial cells with *L. pneumophila* (204, 256, 295, 302). Exogenous type I interferon restricts *L. pneumophila* replication in permissive macrophages, while endogenous type I interferon restricts *L. pneumophila* replication in nonpermissive macrophages (234, 262, 295). As evidence for the latter, macrophages from type I interferon receptor-deficient mice support *L. pneumophila* replication (90, 234, 262, 295). The type I interferon response is independent of flagellin and results from the transfer of nucleic acid to the cell cytosol through a Dot/Icm-dependent process (90, 234, 322). Until recently, it was thought that bacterial DNA was recognized by a cytosolic DNA sensor (322). However, it has now been shown that RNA stimulates the type I interferon response through the host helicases RIG-I and MDA5 and the adaptor Ips-I, a pathway previously known to respond only to viral infections (76, 234). The source of RNA may originate from the pathogen or from RNA generated by host RNA polymerase III acting on pathogen-derived DNA (76, 234). The latter possibility seems unlikely given that cytosolic *L. pneumophila* DNA alone does not stimulate an Ips-I-dependent type I interferon response in macrophages, even in the presence of RNA polymerase III (234). Therefore, *L. pneumophila* may translocate RNA into the host cytosol, or alternatively, as-yet-unknown Dot/Icm effectors may stimulate the production of RNA intermediates in the host cell that then stimulate RIG-I/MDA5 signaling (234). Given the currently accepted view that *L. pneumophila* has not evolved to avoid the mammalian immune system, it is surprising that the Dot/Icm effector SdhA is a potent inhibitor of the type I interferon response (234). Ectopically expressed SdhA inhibits the RIG-I/MDA5 pathway, although it is not yet clear if this occurs through a direct interaction or whether the inhibition results from an indirect effect of SdhA on mitochondria. Since SdhA is also implicated in inhibiting host cell death (196), the two effects of SdhA on macrophages could be linked. Despite the effort that has gone into characterizing the mechanism of type I interferon induction, Ips-I- and type I interferon receptor-deficient mice are no more susceptible to *L. pneumophila* infection, indicating that the type I interferon response is dispensable for limiting *L. pneumophila* infection *in vivo*, at least in mice (234; D. K. Y. Ang, E. L. Hartland, and I. R. van Driel, unpublished data).

Few studies have examined other *Legionella* species in animal infection models; however, *L. micdadei* does not infect the lungs of A mice as readily as *L. pneumophila*, highlighting differences in pathogenicity between *L. pneumophila* and *L. micdadei* in mice (138). A study of the ability of *L. longbeachae* isolates to infect guinea pigs also demonstrated that there was a substantial variation in the virulences of different *L. longbeachae* strains in this infection model (110). Strains of *L. longbeachae* serogroup 1 are highly virulent in mice, with the intratracheal inoculation of 10^3 bacteria capable of causing disease (148). Unlike *L. pneumophila*, mouse infection with *L. longbeachae* is not restricted to A mice, and both BALB/c and C57BL/6 mice are also susceptible to infection with serogroup 1 strains (148). This difference in virulence may in part be related to the fact that *L. longbeachae* serogroup 1 strains are nonmotile, as the deletion of flagellin from *L. pneumophila* also increases the virulence and host range of the pathogen for mice (148, 273).

Innate Immune Responses to *Legionella* during Human Infection

Although the characterization of innate immune responses to *L. pneumophila* infection of mice is well investigated, far less is known about the innate immune response during legionellosis in humans. Certainly, the great majority of people exposed to *L. pneumophila* remain asymptomatic or suffer only a mild self-limiting infection. Risk factors associated with the development of severe disease include age, smoking status, male gender, chronic obstructive pulmonary disease, alcohol intake, and immune suppression (324). A retrospective analysis of Legionnaires' disease patients showed that these individuals released less IFN-γ in response to bacterial LPS than non-Legionnaires' disease patients, suggesting that an impairment in the IFN-γ response may also increase susceptibility to the disease (199). In general, patients with acute Legionnaires' disease have elevated serum levels of IFN-γ and IL-12, which are typical of a Th1-type response (331). This finding is supported by *in vitro* studies showing that primary human macrophages or cell lines derived from human macrophages or epithelial cells produce a Th1-type cytokine response following *L. pneumophila* infection that ultimately restricts bacterial replication (42, 220, 243, 348). Thus, an early and robust inflammatory response appears to be critical to limit infection.

Attempts to examine possible genetic susceptibilities to Legionnaires' disease are frustrated by the small numbers of patients in disease cohorts. Nevertheless, the examination of patient genotypes in a large outbreak of Legionnaires' disease at a flower show in the Netherlands correlated some human TLR polymorphisms with the development of disease independently of other risk factors. A common TLR5 polymorphism that introduces a premature stop codon (TLR5[392STOP]) occurs in around 10% of the population and is associated with a small but significantly increased risk of Legionnaires' disease (159). In heterozygotic individuals, the TLR5[392STOP] polymorphism is dominant and impairs the production of proinflammatory cytokines. As the recognition of bacterial flagellin by TLR5 on alveolar epithelial cells is a major driver of an IL-8 and IL-6 response, the TLR5[392STOP] mutation likely increases an individual's risk of Legionnaires' disease by weakening the cytokine response (159). These results support findings from the mouse model of *L. pneumophila* infection where deficiencies in

the recognition of flagellin by the innate immune system also increase susceptibility to infection. In contrast, a polymorphism in the TLR4 receptor TLR4^{A896G} was associated with an increased resistance to Legionnaires' disease despite the fact that *L. pneumophila* LPS does not strongly activate inflammatory signaling through TLR4 (158). Another innate immune factor that has been associated with resistance to *L. pneumophila* infection is mannose binding lectin (MBL). Patients with Legionnaires' disease show impaired MBL-mediated complement activation (116, 164). However, this deficiency does not reflect an underlying genetic predisposition to Legionnaires' disease but rather appears to be a physiological consequence of severe *L. pneumophila* infection (117, 164).

Interactions with the Adaptive Immune System

Although a vigorous inflammatory response is critical for limiting bacterial infection of the lung during Legionnaires' disease, T and B cells are ultimately required for the clearance of the infection. Evidence for the role of T cells comes from the depletion of CD4 or CD8 T cells using monoclonal antibodies, which resulted in decreased survival rates for mice infected with high doses of *L. pneumophila* compared to nontreated animals (326). For mice infected with lower doses of *L. pneumophila*, CD4 or CD8 T-cell-depleted animals were unable to clear the bacteria from their lungs for up to 11 days after inoculation (326). In all cases, animals depleted of both CD4 and CD8 T cells were more severely affected than mice depleted of only one subset (326). Another study showed that mice immunized with *L. pneumophila*-pulsed, fractalkine-expressing DCs were protected from a lethal dose of *L. pneumophila*. This protection was completely abolished in CD4 T-cell-, CD8 T-cell-, and B-cell-deficient animals (190). These results demonstrate that T and B cells are essential for protecting mice against *L. pneumophila* infection. However, the exact mechanisms by which these cells become activated and confer protection are still largely unexplored.

T cells become activated after recognizing their antigen via the presentation of peptide-major histocompatibility complexes (MHCs) by antigen-presenting cells (APCs). The primary APC is the dendritic cell, and several studies performed with mice have implicated this cell type as the APC responsible for initiating the adaptive immune response to *L. pneumophila*. For example, murine bone marrow-derived dendritic cells (BMDCs) infected with *L. pneumophila* are able to stimulate IFN-γ production by CD4 T cells *in vitro* (245). This activation of CD4 T cells was dependent on the successful formation of the LCV and the prevention of lysosome fusion by the bacteria (245). Interestingly, this scenario also occurred with murine bone marrow-derived macrophages, although BMDCs stimulated more IFN-γ production in CD4 T cells than did macrophages (245, 246). These data indicate that although *L. pneumophila* is sequestered in the LCV, this is insufficient to prevent the presentation of bacterial antigens to CD4 T cells. The exact mechanisms by which the bacterial antigens are processed and presented by MHC class II molecules are still unknown. BMDCs that come into contact with *L. pneumophila* also upregulate fractalkine (CX3CL1), a strong chemoattractant for T cells (190). In contrast to macrophages, murine BMDCs restrict the intracellular growth of *L. pneumophila*

(245). This and the fact that BMDCs undergo rapid apoptosis upon *L. pneumophila* infection may be a mechanism that not only prevents bacterial dissemination but also delays antigen presentation and the activation of *L. pneumophila*-specific T cells (255).

Early studies showed that a cell-mediated immune response to *L. pneumophila* also occurs in humans (43, 169). Although there is a paucity of information on the nature of this response, patients with immune deficiencies resulting from corticosteroid therapy and some types of leukemia are at an increased risk of severe and persistent Legionnaires' disease (165, 296). *L. pneumophila* infections are uncommon in patients with AIDS (122), although some reports suggested that the disease is more severe (261). The nature of the effector cells controlling *L. pneumophila* infection in humans is not clear, but *in vitro* studies show that Th1-type CD4 T cells respond to *L. pneumophila* by secreting IFN-γ (193).

Immune regulation has emerged in recent times as an important concept describing the delicate balance between protective immune responses to pathogens and damaging immune-mediated pathology. During *L. pneumophila* infection of mice, it appears that this balance may be achieved via the production of prostaglandins by infected macrophages, which in turn inhibit IFN-γ production in T cells (246). It will be interesting to see if other aspects of immune regulation such as CD4$^+$ Foxp3$^+$ T-regulatory cells also play a role in regulating the cell-mediated immune response to *L. pneumophila*.

Both patients and laboratory animals produce antibodies in response to *L. pneumophila* infection. Major characterized antigens include lipopolysaccharide, the Msp/ProA protease, flagellin, Hsp60, and OmpS (45, 48, 132, 276, 352). Early studies investigated the potentials of several of these antigens in the development of vaccines to *L. pneumophila*, and although some antigens showed significant promise in animal infection models, there has been little activity in this area in recent years (44–48, 276). Nevertheless, vaccine studies have informed our understanding of the immune control of *L. pneumophila* infection and support a role for both antibody- and cell-mediated immunity in the clearance of the bacteria from the lung (46, 48, 276).

CONCLUDING REMARKS

Although *L. pneumophila* is an environmental organism, its innate ability to replicate inside eukaryotic cells and its capacity to inadvertently avoid regular pathogen control mechanisms in the host have led to its emergence as an important yet accidental cause of community- and hospital-acquired pneumonia. The rapid advances made in the understanding of *L. pneumophila* molecular pathogenesis have resulted largely from the availability of genome sequences that have uncovered unusual features of the pathogen, such as the presence of virulence determinants with similarity to eukaryotic proteins. This information has been used to gain insights into the most important aspects of *Legionella* virulence and its potential to manipulate various aspects of eukaryotic cell biology and innate immunity. The ongoing use of genomics to provide reference sequences of more strains and serogroups of *L. pneumophila* and other *Legionella* species will no doubt continue to uncover novel virulence effectors of *Legionella* species and help

us to understand the evolution and emergence of *L. pneumophila* as a major respiratory pathogen. In addition, through investigations of host-pathogen interactions, *L. pneumophila* has emerged as an important model for the study of innate immune responses to intracellular pathogens as well as a useful tool for investigations into eukaryotic cellular trafficking events. The ongoing study of *Legionella* virulence proteins and their effects on host cell biology and immunity will continue to deepen our understanding of the roles that various host cell signaling and trafficking pathways play in resistance to infection.

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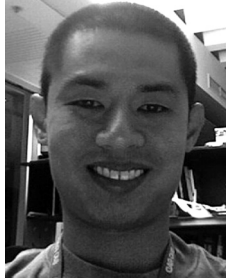
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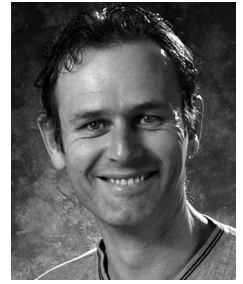
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