

Flagellin is critical for *Legionella* motility and macrophage recognition

by

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*For
Aziah and Karen (Mom)*

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ABSTRACT

The causative agent of Legionnaires' disease is *L. pneumophila*, an intracellular pathogen that infects aquatic amoebae and alveolar macrophages. *L. pneumophila* expresses virulence factors that are important for growth in mammalian macrophages and transmission from one host cell to the next, specifically motility, stress resistance and cytotoxicity to macrophages. A correlative study of nosocomial Legionnaires' disease and colonization of the corresponding hospital water systems provided the opportunity to determine how well widely used laboratory assays correlate with the virulence potential of *Legionella* isolates. I found that disease incidence of the *L. pneumophila* isolates correlated with one laboratory test of virulence, the ability to survive in the stringent environment of primary mouse macrophages; nevertheless, motility and cytotoxicity were conserved across all strains.

The flagellum is essential for motility and dispersal of *Legionella* in aquatic environments. Furthermore, mouse resistance to *L. pneumophila* is accomplished through macrophage recognition of the major flagellar protein, flagellin. Macrophage innate defenses are triggered by cytosolic flagellin, independently of TLR5, by a pathway that includes the NOD-like cytosolic protein Naip5, requires caspase-1, and that effectively restricts replication of *L. pneumophila* within cultured macrophages and mouse lungs. To elucidate the factors that contribute to restriction in C57Bl/6 macrophages, I analyzed the ability of flagellate *Legionella* species that replicate to

trigger a pro-inflammatory innate response. In summary, I provide evidence that *L. pneumophila* is a potent trigger of the innate immune system of macrophages as a result of cytosolic contamination that requires two key bacterial factors: pore formation and flagellin. Studying non-*pneumophila* species of *Legionella* has extended the evidence that translocation by the type IV secretion system is critical to recognition of flagellin.

CHAPTER ONE

Introduction

AN UNEXPLAINED PNEUMONIA

Although originally identified earlier, the bacterium *Legionella pneumophila* was first recognized as a pathogen during a 1976 American Legion convention in Philadelphia (Fraser *et al.*, 1977). Identified as the causative agent of the mysterious pneumonia-like illness that afflicted the attending members, it was subsequently established that *L. pneumophila* causes two distinct illnesses—Legionnaires’ disease, a severe pneumonia, and Pontiac fever, a flu-like illness. *Legionella* is ubiquitous in aquatic environments and is a facultative intracellular pathogen of eukaryotic cells, both amoebae and macrophages.

Person-to-person transmission of *Legionella* has never been demonstrated. Instead, *Legionella* are transmitted through aerosols from colonized devices such as showers and faucets, cooling towers, or any aerosolized contaminated water. When the infected aerosols are inhaled, *Legionella* reach the human lung. Subsequently, *Legionella* that are ingested by alveolar macrophages, protectors of the lung environment, turn their temporary intracellular lodging into the prime location for replication by evading the destructive equipment of the macrophage—the degradative lysosomes. After undergoing a

regulated phenotypic switch, the bacteria then replicate profusely in the macrophages, ultimately resulting in an acute, severe pneumonia.

Legionella infection of the human lung is a dead end, in stark contrast to other respiratory pathogens, such as *Bordetella pertussis* or *Mycobacterium tuberculosis*, which are transmitted from person-to person. Absence of human-to-human transmission indicates that *L. pneumophila* has not adapted to the human host; therefore, evolution of *Legionella* has likely occurred in an alternative environment (Swanson *et al.*, 2002). For example, the similarities between *Legionella*'s life cycle within lung macrophages and protozoa in an aquatic environment indicate that selective pressure on the microbe has likely been exerted by protozoa, rather than humans. Indeed, it is conceivable that the natural reservoir for several macrophage pathogens such as *Franciscella*, *Coxiella*, *Burkholderia*, *Listeria* and *Mycobacterium avium* may be environmental amoebae (Cirillo, 1997; La Scola and Raoult, 2001; Winiacka-Krusnell and Linder, 1999).

CLINICAL RELEVANCE AND EPIDEMIOLOGY OF LEGIONNAIRES' DISEASE

An estimated 8,000 to 18,000 cases of Legionnaires' disease occur every year in the United States, however each year a large number remain unreported, perhaps 90-98% of total cases. This discrepancy is due in part to difficulties in culturing the bacteria as well as inconsistency in diagnosis from secretions (Abu Kwaik *et al.*, 1998; Jaulhac *et al.*, 1998; Koide and Saito, 1995). Also, many physicians do not order the specific test, but simply treat with broad-spectrum antibiotics, due to cost and prevention measures. The majority of arising cases are sporadic, community acquired cases, whereas only a small

number of cases are attributed to outbreaks (10-20%), according to data from the Centers of Disease Control and Prevention (Fields *et al.*, 2002).

Attendees of the convention that gave the bacterium its name fit well into the “at risk” demographic—older men, drinkers, and smokers. The demographic most vulnerable for a *Legionella* infection are the elderly, the immunocompromised, smokers and those with underlying respiratory conditions. These risk factors also make the hospital a primary setting for the morbidity and mortality associated with Legionnaires’ disease. Nosocomial (hospital-acquired) Legionnaires’ disease has mortality rates that range from 25-70% (Marra and Shuman, 1992).

The water sources predominately responsible for Legionnaires’ disease in hospitals are contaminated plumbing systems or cooling towers. Eradication of *Legionella* from these water sources has resulted in a substantial decrease in the number of cases in the hospital (Guiguet *et al.*, 1987; Stout *et al.*, 1982). As a result, the Centers for Disease Control and Prevention has issued guidelines for the best methods to remove *Legionella* from hospital water sources.

In addition, changes in the diagnosis of Legionnaires’ disease has led to a decrease in the mortality rates of both community acquired and nosocomial cases of disease. Previously the gold standard for detection of a *Legionella* infection was growth on buffered charcoal yeast extract (Edelstein, 1987). Although respiratory secretions (sputum, bronchial lavage and aspirates) are the specimens of choice, in some cases *Legionella* can be isolated from blood, lung tissue, lung biopsies, and stool. Expertise in culturing *Legionella* from respiratory secretions and efficient handling of specimens highly affects culturability. The reported sensitivity of using respiratory secretions as a

source ranges from 0-80%, indicating that several factors attribute to this wide range of sensitivity. Other widely used tests include direct immunofluorescence of *Legionellae* in lung tissue and respiratory secretions, serology and detection of antigen in urine specimens. Direct immunofluorescence is a rapid, highly sensitive (99-100%) method to detect *Legionella* (33-66% of cases), however it can be technically demanding (Fields *et al.*, 2002). An increase in the number of urine antigen tests from 1990 to 1998 has caused a decrease in the mortality rate from 26 to 10% in community acquired cases and 46 to 14% in nosocomial cases (Benin *et al.*, 2002).

The majority of isolates associated with Legionnaires' disease are *L. pneumophila*, despite the existence of 48 described species and several *Legionella*-like amoebal pathogens (Benson and Fields, 1998; Fields *et al.*, 2002; Muder, 1989). In addition, the most prevalent serogroup that is attributed to these infections is serogroup 1, accounting for 79% of culture-confirmed or urine antigen confirmed cases (Fields *et al.*, 2002). Infections have also been attributed to the other species of *Legionella*, however since they occur rarely, and diagnostic techniques are lacking, these go unreported for the most part (Fields *et al.*, 2002). A multi-genome analysis of 217 *L. pneumophila* strains and 32 non-*pneumophila* strains of *Legionella* determined that no specific hybridization profile distinguished clinical from environmental strains or different serogroups; however, the majority of genes found on a 33 kb element that encodes proteins for lipopolysaccharide biosynthesis were only found in strains of the most prevalent serogroup—serogroup 1 (Cazalet *et al.*, 2008). For a case or outbreak to occur, three events must coincide: (1) *Legionella* must be present in an aquatic environment, (2) the

bacteria must replicate to an unknown infectious dose, and (3) aerosols laden with bacteria must be transmitted to a susceptible human host (Fields *et al.*, 2002).

Legionnaires' disease is characterized by an acute alveolitis and bronchiolitis, where patient exudates are littered with macrophages, polymorphonuclear cells, fibrin, red blood cells, proteinaceous material, and cellular debris indicative of the inflammation, cell death, and cell lysis typical of a *Legionella* infection (Glavin *et al.*, 1979; Winn, 1981). Although, *Legionella* are found intracellularly, the bacteria can also cause extensive lysis of infected white blood cells in the alveoli. *L. pneumophila* can insert pores into membranes of white blood cells using its type IV secretion system and cause lysis (Kirby *et al.*, 1998), yet cell death requires type IV secretion and flagellin (Molofsky *et al.*, 2005; Vinzing *et al.*, 2008a). Flagellate *L. pneumophila* activate inflammasomes and stimulate the release of pro-inflammatory cytokines from cultured macrophages to combat infections (Molofsky *et al.*, 2006). Also, when injected into mice, *L. pneumophila* flagellin is immunogenic (Ricci *et al.*, 2005). Taken together, these data indicate that *L. pneumophila* flagellin is an important feature of the inflammatory response mounted against the infection.

THE INTRACELLULAR LIFE OF *LEGIONELLA*

From transmission to replication

Legionella lead a multi-faceted lifecycle whereby they switch between replicative, transmissive, and dormant phases (Figure 1.1). After ingestion by a eukaryotic host, *Legionella* avoid the endocytic network and degradation within acidic lysosomes. Once

the bacteria establish an environment fit for replication, the first intracellular phenotypic switch occurs, as the bacteria switch from the transmissive phase to the replicative phase. Once the replicating bacteria (replicative phase) have exhausted the available nutrients within their replication vacuole, they switch to the form that is fit for transmission to a new host (transmissive phase).

Transmissive *Legionella* express several traits important for its dispersal, including but not limited to: a flagellum and motility, resistance to osmotic and environmental stresses, sensitivity to sodium, ability to evade phagosome-lysosome fusion, and cytotoxicity (Bachman and Swanson, 2001, 2004a, b; Hammer *et al.*, 2002b; Jacobi and Heuner, 2003; Lynch *et al.*, 2003). Ultimately, the transmissive bacteria lyse the infected cell and are equipped to infect a new host cell; however, if a new host cell is not present *Legionella* likely persevere by establishing residence within a biofilm. Under particular starvation conditions, they instead persist as a mature intracellular form (MIF), a cell type that is extremely infectious and durable, capable of surviving several months (Garduno *et al.*, 2002; James *et al.*, 1999; Lee and West, 1991; Schofield, 1985; Skaliy and McEachern, 1979).

Experimentally, differentiation can be observed in a broth culture model, allowing study of the contributions of phenotypes characteristic of each phase. In particular, exponential (E) phase bacteria mimic the replicative stage and post-exponential (PE) express transmissive phenotypes (Fig. 1.1). Originally, the shared similarities of broth growth with the intracellular lifecycle were recognized by a comparative analysis of several transmissive phenotypes within primary murine macrophages and broth (Byrne and Swanson, 1998). More recently, the model has been extended by transcriptional

profiling, which documented that a large proportion of genes upregulated during each phase of broth growth correspond to those genes upregulated during the life cycle within the amoebae *Acanthamoeba castellanii* (Bruggemann *et al.*, 2006).

A critical aspect of the ability of *Legionella* to establish an infection is the mechanism by which it avoids immediate delivery to the lysosomes (Horwitz, 1983a, b). Although studied extensively, a single factor sufficient to block phagosome-lysosome fusion has not been determined. It is known that, upon infection *Legionella* in the transmissive phase are within vacuoles that are separate from the endosomal network, since they do not colocalize with the endosomal markers, LAMP-1, transferrin receptor and cathepsin D (Joshi *et al.*, 2001) (Fig. 1.2). Instead, within 30 minutes, the sequestered vacuole intercepts secretory vesicles as they exit the endoplasmic reticulum (ER), which surround the vacuole (Kagan *et al.*, 2004; Shin and Roy, 2008; Swanson and Isberg, 1995). Several hours later, the bacteria replicate in a vacuole that acquires lysosomal characteristics (Sturgill-Koszycki and Swanson, 2000); after several rounds of replication, the cell is lysed (Swanson and Hammer, 2000). Moreover, when acidification or another early event in biogenesis of the *L. pneumophila* replicative organelle is inhibited by bafilomycin treatment, replication ceases (Kagan and Roy, 2002; Sturgill-Koszycki and Swanson, 2000). Its replication in an acidic environment is analogous to the pathogens *Coxiella burnetii* and *Leishmania*, which likewise multiply within acidic phagolysosomes of macrophages (Akporiaye *et al.*, 1983; Heinzen *et al.*, 1996; Maurin *et al.*, 1992; McConville *et al.*, 1992; Schaible *et al.*, 1999; Turco and Sacks, 1991).

In contrast to phagosomes of transmissible (PE) bacteria, vacuoles containing replicative (E) *Legionella* are immediately delivered to the lysosomes where the bacteria are subsequently degraded (Fig. 1.2) (Joshi *et al.*, 2001). Traits specific to transmissible *Legionella* contribute to evasion of phagolysosomal fusion, including the factors dependent on the Dot/Icm type IV secretion system and a formalin-resistant surface factor. In A/J mouse macrophages, both *dot* mutant bacteria and formalin-killed bacteria reside in a phagosome that is LAMP-1 positive but Texas-red ovalbumin and cathepsin D negative, two markers that decorate phagolysosomes containing *E. coli* and polystyrene beads (Joshi *et al.*, 2001). In contrast, heat killed transmissible *L. pneumophila* are found in canonical phagolysosomes, suggesting that a heat-labile component contributes to lysosome evasion. Taken together these data suggest that more than one surface-associated property of transmissible phase *L. pneumophila* contributes to inhibition of phagosome maturation.

SURFACE PROPERTIES THAT CONTRIBUTE TO INFECTION

Legionella has evolved mechanisms sufficient to exploit both mammalian cells and aquatic protozoa as a replication niche. The initial encounter between *Legionella* and eukaryotic cells requires direct contact, pointing to a critical interface between the phagocyte's plasma membrane and the surface of the bacterial cell. Contact is promoted by a surface appendage—the flagellum—that allows the bacteria to move freely in an aqueous environment and also toward phagocytic cells. Contact during mammalian infections may be achieved not only by expression of motility but also by the unusual hydrophobic nature of *Legionella*'s lipopolysaccharide by facilitating its contact with

target cell membranes of the lung airway (Thomas and Brooks, 2004). Once alveolar macrophages internalize the bacteria, the Dot/Icm type IV secretion system of *Legionella* is thought to modify the resulting phagosome.

Dot/Icm Type IV Secretion

The Dot/Icm type IV secretion system of *Legionella*, encoded by the *defective in organelle transport (dot)/intracellular multiplication (icm)* loci, has been its most widely studied virulence factor. Specialized secretion systems are one mechanism by which several pathogens deliver their proteins to host cells. The *Legionella* type IV secretion systems, functionally analogous to type III secretion systems, are multicomponent complexes that transport proteins across host cell membranes and alter host cell processes. Playing a similar role in virulence in *Brucella*, *Bordetella*, and *Helicobacter pylori* (Censini *et al.*, 1996; Sexton and Vogel, 2002; Weiss *et al.*, 1993), type IV secretion systems were originally recognized as classical conjugation systems (Christie and Vogel, 2000; Sexton and Vogel, 2002).

To prepare its replication niche, *Legionella* is thought to evade lysosomes by a Dot/Icm dependent event that prevents fusion with the endosomal pathway (Roy *et al.*, 1998). Essentially every mutant of the *dot/icm* apparatus is defective for intracellular growth (Andrews, 1998; Berger *et al.*, 1994; Horwitz, 1987; Marra *et al.*, 1992; Swanson and Isberg, 1996a). Unlike wild-type *L. pneumophila*, *dot/icm* structural mutants are targeted quickly to endocytic vacuoles, where they acquire the immunofluorescence markers Rab5, lysosomal-associated membrane protein (LAMP-1), and the vacuolar

ATPase (V-ATPase)(Coers, 1999; Lu and Clarke, 2005; Swanson and Hammer, 2000). Once this vacuole is distinct from the endosomal pathway, the type IV machinery is no longer necessary, indicating the critical modifications occur very early during the internalization process (Roy *et al.*, 1998; Wiater *et al.*, 1998). Moreover, when *dotA* mutants, which lack an integral cytoplasmic membrane protein, reside within the same phagosome as a wild-type bacterium, the mutants do replicate (Coers, 1999). Also, expression of DotA by *Legionella* before macrophage contact, but not afterward, relieves the mutants' replication defect (Roy *et al.*, 1998).

Not only is *dot/icm* type IV secretion critical for establishing the replication vacuole, several of the secretion components are also required for other transmissible traits associated with virulence (Byrne and Swanson, 1998; Kirby *et al.*, 1998). For example, even at high multiplicities of infection, *dot/icm* mutants cause less than 10% cytotoxicity; in contrast, wild-type *L. pneumophila* kill more than 90% of macrophages at the low MOI of 10. Similarly, *dot/icm* mutants fail to lyse red blood cells or perforate eukaryotic cell membranes (Kirby *et al.*, 1998). Thus, *dot/icm* function is required for cytotoxicity, red blood cell hemolysis, sodium sensitivity, and intracellular growth. Interestingly, our data suggest that intracellular growth may not be solely dependent on *Dot*-specific factors and this phenotype may be specific to *L. pneumophila* (Chapter 4).

Lipopolysaccharide

Biological studies of the lipophosphoglycan (LPG) of *Leishmania* and the lipoarabinomannan (LAM) of *Mycobacterium tuberculosis* have given insight into how

surface properties can determine the intracellular fate of a pathogen. Descoteaux and colleagues have shown that, as *Leishmania* promastigotes enter macrophages, they transfer LPG, a polymer of disaccharide-phosphate units anchored into the membrane, to the phagosomal membrane; consequently, the parasites reside in phagosomes sequestered from the endosomal pathway (Desjardins and Descoteaux, 1997; Scianimanico *et al.*, 1999). However, mutants that lack LPG colocalize with the endosomal markers Rab7 and LAMP-1 (Scianimanico *et al.*, 1999), indicating that the LPG mutants cannot evade lysosomes. Subsequently, the LPG is downregulated during differentiation into the amastigote form, and the phagosome matures into lysosomes, where, similar to *Legionella*, the parasites replicate (Beverley and Turco, 1998; Turco, 1992). A similar strategy of altering their surface properties is used by other pathogenic species as well, including *Salmonella* spp., *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Campylobacter jejuni* (Guerry *et al.*, 2002; Guo *et al.*, 1997; Luneberg *et al.*, 2000; van Putten, 1993; Weiser and Pan, 1998).

The formidable barrier that protects *Legionella* from its outside environment is its outer layer, the lipopolysaccharide. The lipopolysaccharide (LPS) produced by *L. pneumophila* is extremely hydrophobic and is thought to promote its transmission in aerosols and during its intracellular life cycle (Knirel *et al.*, 1994; Luneberg *et al.*, 2000). LPS is a major component of the outer membrane of gram-negative bacteria and is composed of three domains: lipid A, core polysaccharide and O-antigen. The O-antigen of *L. pneumophila* LPS is a homopolymer of legionaminic acid (Fig. 1.3). Legionaminic acid is an unusual hydrophobic sugar that lacks free hydroxyl groups and may be synthesized by a biosynthetic pathway similar to that of sialic acid (Zahringer *et al.*,

1995). The only characterized avirulent strain of *L. pneumophila* serogroup 1 that is known to express an LPS structural variant is *Mutant 811*, yet the molecular mechanism of its avirulence is unknown (Luneberg *et al.*, 1998; Luneberg *et al.*, 2001). Other direct tests of the contribution of LPS in virulence have yielded negative results (Kooistra *et al.*, 2001; Luneberg *et al.*, 2000).

Previous studies from our lab indicate that *L. pneumophila* alters its surface during its lifecycle. By analyzing the LPS profile and the ability to bind either sialic acid lectins or the hydrocarbon hexadecane, it was found that developmental changes of its surface during the transition from E to PE phase correlated with evasion of phagosome-lysosome fusion by *L. pneumophila* (Fernandez-Moreira *et al.*, 2006). Developmental regulation of LPS is not limited to the bacterial surface but is also evident in membrane vesicles shed by *L. pneumophila*, organelles sufficient to inhibit fusion of phagosomes with lysosomes. Thus, the pathogen has a dynamic surface that *L. pneumophila* modifies according to its changing environment.

Flagella

L. pneumophila induces expression of its flagellum when nutrients become scarce and the bacteria undergo differentiation into the transmissive phase (Byrne and Swanson, 1998). The flagellum is a large protein complex that is expressed by a highly coordinated hierarchy of flagellar regulon genes (Aldridge and Hughes, 2002). The FliA sigma factor not only regulates expression of flagellin (encoded by *flaA*) but also motility, lysosome evasion, cytotoxicity, and, in the amoebae *Dictyostelium discoideum*, replication of *L.*

pneumophila (Hammer *et al.*, 2002a; Heuner *et al.*, 2002). The flagellar secretion apparatus is very similar to the type III secretion system and is responsible for the export of many of the flagellar structural components. In addition to motility, flagellin contributes most significantly to inflammation via recognition by the innate immune system of macrophages (Molofsky *et al.*, 2005; Molofsky *et al.*, 2006).

Although, flagellar expression is not required for intracellular survival or replication, flagellin itself is required for cytotoxicity (Molofsky *et al.*, 2005). When *flaA* mutants are incubated with macrophages, and then mildly centrifuged to promote contact, bacteria are not cytotoxic, regardless of whether multitudes of bacteria are co-incubated with macrophages (~100/macrophage) (Molofsky *et al.*, 2005). In contrast, <10% of macrophages survive when incubated with wild-type *L. pneumophila* at an MOI of 10. The presence of flagellin is also critically important for a macrophage's ability to detect *L. pneumophila*: restrictive macrophages cannot sense *flaA* mutants, which replicate profusely (Molofsky *et al.*, 2006). Previously, the mechanism by which flagellin promotes macrophage death was unknown; however, our research has provided insight to the interaction between the macrophage and flagellin that leads to a specific type of pro-inflammatory cell death, termed "pyroptosis" (Chapter 4).

PYROPTOSIS—“THE FIERY DEATH”

The type of death triggered in macrophages by *L. pneumophila* had been characterized as necrotic cell death, similar to the necrosis of the alveolar epithelium in mice, that leads to extensive inflammation (Blackmon *et al.*, 1978; Brieland *et al.*, 1994a; Katz and Hahemi, 1982). As part of the recent wave of research on cell death, the pro-

inflammatory death induced by *L. pneumophila* has been attributed to pyroptosis, which depends upon caspase-1 and is distinct from apoptosis, a non-inflammatory cell death (Brennan and Cookson, 2000; Hersh *et al.*, 1999; Molofsky *et al.*, 2006). Pyroptotic cell death has been most extensively studied with the intracellular pathogen *Salmonella* (Fink and Cookson, 2007). Pyroptosis is characterized by loss of membrane integrity and ultimately the release of pro-inflammatory intracellular contents. Accordingly, the term is derived from the Greek *pyro*, to invoke fire or fever, and *ptosis*, or falling (Fink and Cookson, 2007).

Caspase-1 is activated by multi-protein, cytosolic complexes called inflammasomes (Mariathasan and Monack, 2007; Martinon *et al.*, 2002), which process the inactive cytokines pro-IL-1 β and pro-IL-18 into their active forms, IL-1 β and IL-18 respectively. Once processed, these cytokines are secreted from the cell, initiating the recruitment and activation of immune cells as a response to microbe-associated molecular patterns (MAMPS) that have contaminated the cytosol. Recently, multiple inflammasome components have been identified and characterized (Mariathasan and Monack, 2007). Of these the cytosolic proteins Naip5 and Ipaf (Nlrc4) have been specifically implicated in defense against *L. pneumophila* infections (Amer *et al.*, 2006; Lamkanfi *et al.*, 2007; Mariathasan, 2007; Ren *et al.*, 2006; Vinzing *et al.*, 2008b).

Naip5 and Ipaf are nucleotide oligomerization domain-like receptors (NLRs) that are similar to the extracellular toll-like receptors (TLRs) but are located in the cytosol. Murine Naip5 (Birc1e) restricts replication of *L. pneumophila*, since unlike mouse strains that are resistant to *L. pneumophila*, the A/J strain encodes a *naip5* mutant allele that confers susceptibility to *L. pneumophila* (Diez *et al.*, 2003; Fortier *et al.*, 2005; Wright *et*

al., 2003). Restriction of infection depends on the presence of a wild-type Naip5 allele, the ability of the macrophages to sense flagellin that has contaminated the cytosol, and the type IV secretion system (Molofsky *et al.*, 2006; Zamboni *et al.*, 2006) (Chapter 3). The model generated by these studies holds that, upon uptake of *L. pneumophila*, perforations in the phagosomal membrane by the type IV secretion system lead to contamination of the cytosol with flagellin monomers. Flagellin detected by the inflammasome, in turn, activates caspase-1 and causes the processing and secretion of pro-inflammatory cytokines to combat the infection (Fig. 1.4).

OUTLINE OF THESIS

L. pneumophila is a ubiquitous resident of the aquatic environment, yet it has evolved mechanisms to reside and multiply within the human host. Several virulence factors contribute to *L. pneumophila*'s ability to establish an infection in humans; conversely, the mammalian immune system has acquired several means to recognize a bacterial invader and initiate an immediate response. Chapter Two investigates the association between water colonization of *Legionella* and the ability to establish an intracellular infection. I show that an environmental isolate of *L. pneumophila* that fails to cause human cases of disease is competent to replicate within eukaryotic cells but cannot replicate within primary macrophages derived from the A/J mouse, a widely used murine model for *L. pneumophila* infection. The environmental strains that do not cause human disease have a modified lipopolysaccharide (LPS) and lack the epitope recognized by monoclonal antibodies and characterized as a "virulence-associated" epitope. In

addition, the traits of motility and cytotoxicity are conserved despite whether the isolates were obtained from patients or water sources.

It is widely known that motility is an important virulence factor of *L. pneumophila* that is required for its transmission and is highly expressed during the transmissive phase. Chapter Three demonstrates that the protein subunit flagellin of the flagellum is also a smoke signal that alerts the innate immune system of mice to the presence of *L. pneumophila*. Macrophage death in response to a *L. pneumophila* infection is not specific to *L. pneumophila* but is also initiated in response to other bacteria whose flagellin is highly similar. Chapter Five exploits a panel of non-*pneumophila* species of *Legionella* that can replicate in restrictive macrophages to analyze the surface factors that contribute to innate immune system recognition. Finally, Appendix A describes lipopolysaccharide as a virulence factor that is modified by *L. pneumophila* during its multi-phasic lifecycle as an additional strategy to evade the macrophage degradation machinery. I show that acetylations of the *O*-antigen of LPS by Lag-1 are implicated in this evasion.

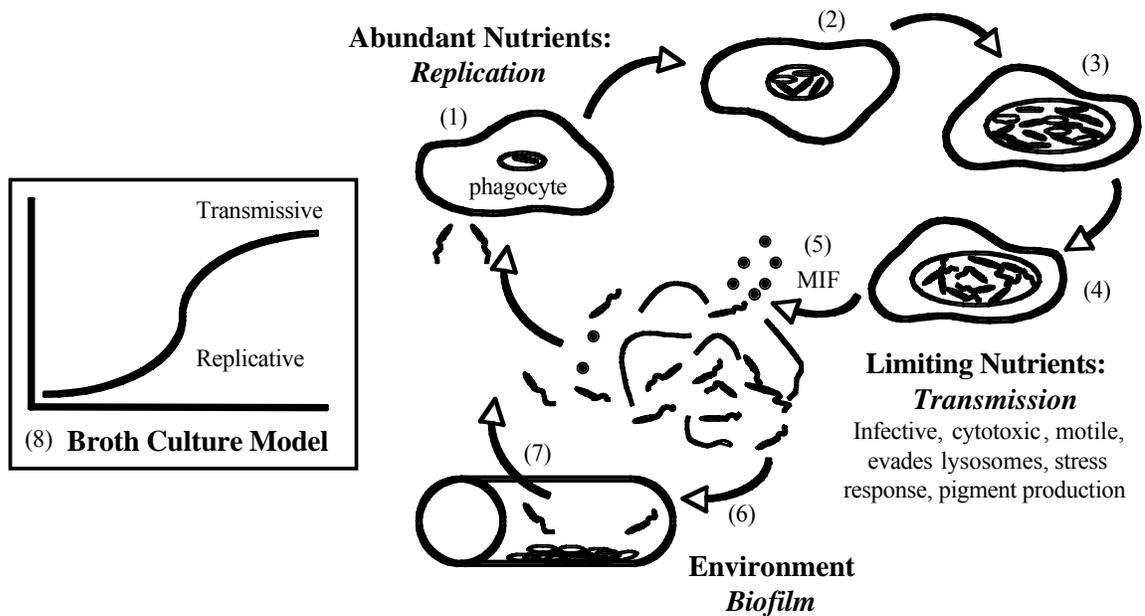


Figure 1.1. Life cycle of *L. pneumophila*.

(1) Transmissive *L. pneumophila* engulfed by phagocytic cells reside in a protective vacuole from lysosomal degradation. (2) Under favorable conditions transmissive bacteria begin to replicate. (3) When nutrient levels decrease, replicating bacteria stop dividing and begin to express transmissive traits. (4) Transmissive phase bacteria become more resilient to environmental conditions and more infectious. (5) The host cell is lysed and transmissive *L. pneumophila* are released into the environment and can infect a new host. (6) *L. pneumophila* can establish biofilms in water systems and ponds, where they become highly resistant. (7) *L. pneumophila* can reinstate the cycle by infecting a new host cell. (8) *L. pneumophila* cultured in broth exhibit similar traits to those observed in phagocyte cultures.

Adapted from Molofsky and Swanson, *Molecular Microbiology* 53(1), 29-40, 2004.

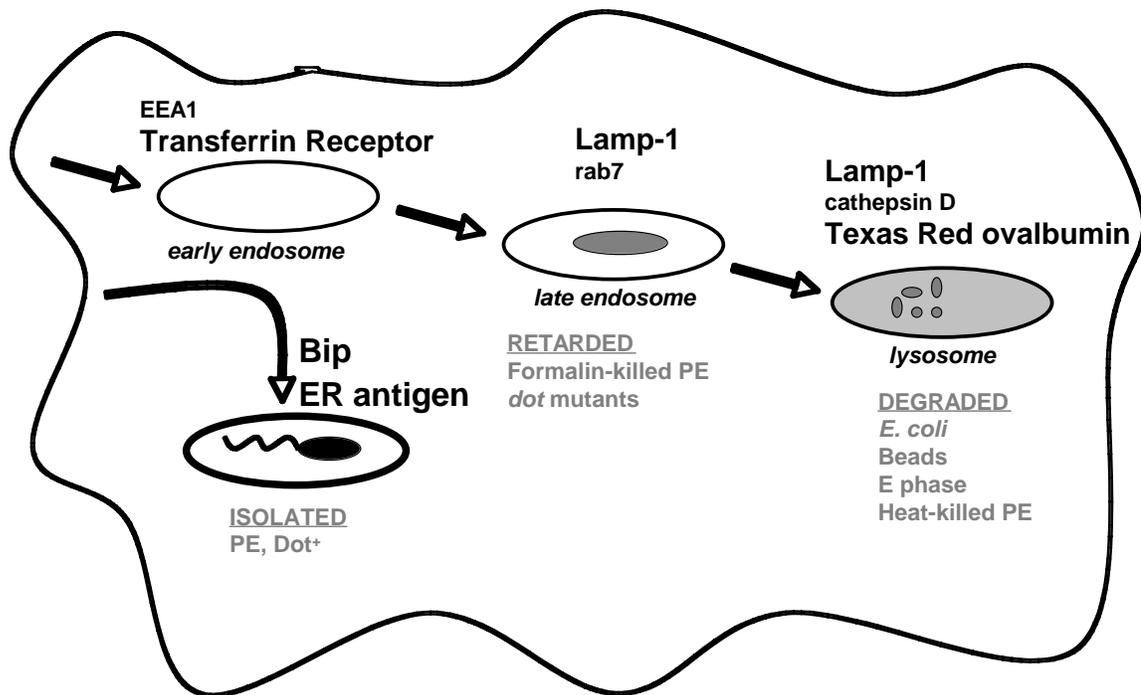


Figure 1.2. Immunofluorescence trafficking markers.

Depicted in bold are the immunofluorescence markers that label compartments of the endosomal network, evaded by transmissive *L. pneumophila*. (Fernandez-Moreira *et al.*, 2006; Joshi *et al.*, 2001)

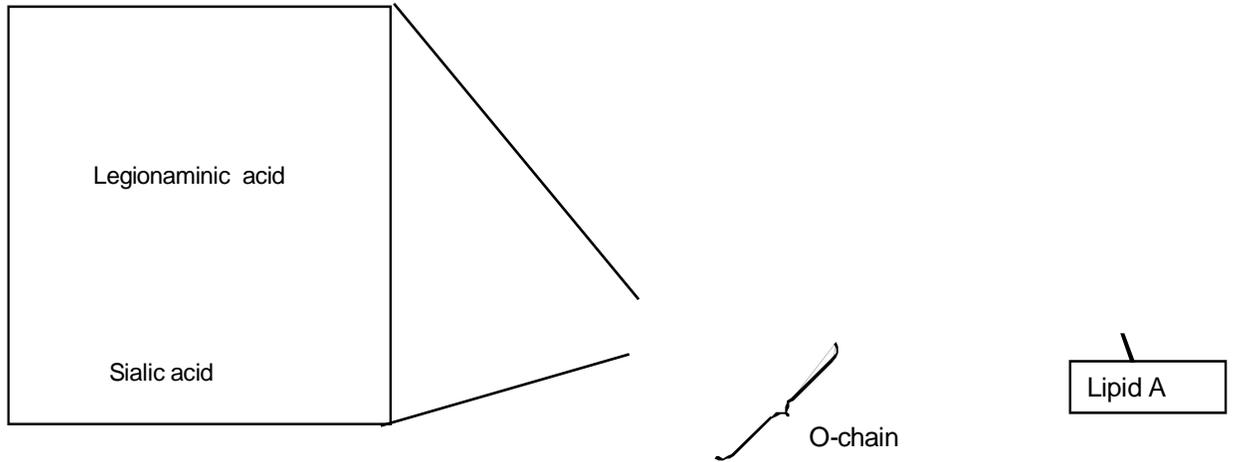


Figure 1.3. Lipopolysaccharide structure of *L. pneumophila*.
Adapted from Kooistra *et al.*, 2001.

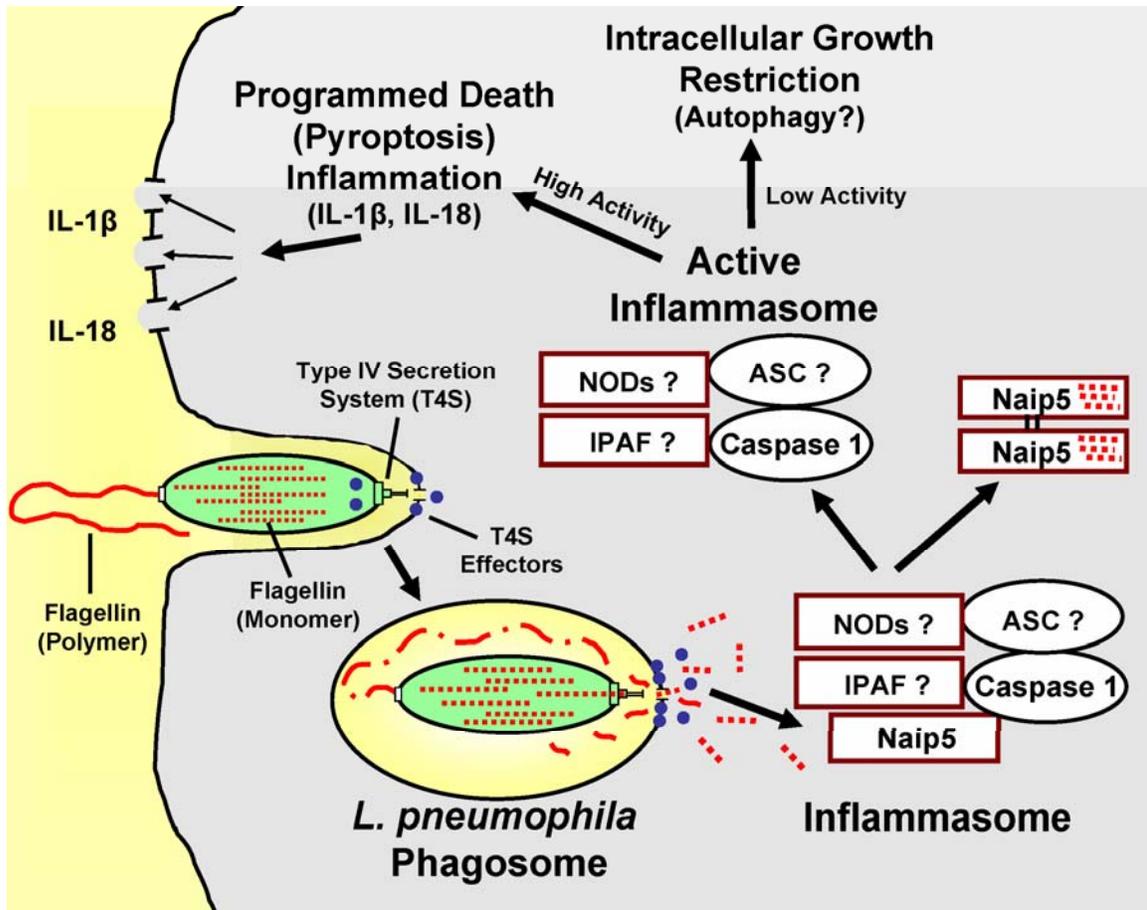


Figure 1.4. Model of *L. pneumophila* flagellin triggering pyroptosis.

During phagocytosis, the type IV secretion system (T4S) inserts pores into the membrane to deliver virulence factors. Flagellin protein leaks through the pores and is detected by Naip5 and IPAF activating the inflammasome, leading to secretion of pro-inflammatory cytokines to combat the infection. Modified from Dubuisson and Swanson, 2006.

CHAPTER TWO

***Legionella* survival in mouse bone marrow derived macrophages as a useful marker of clinical risk**

SUMMARY

A correlative study of nosocomial Legionnaires' disease and colonization of corresponding hospital water systems revealed that the majority of institutions reported identical isolates in the water supply and patients. In contrast, no disease cases were detected in one hospital despite its colonization with *L. pneumophila* serogroup 1 isolates (*NE-2733*, *NE-2735*). In this chapter we sought to determine how well widely used laboratory assays correlate with the virulence potential of *Legionella* isolates. Using quantitative assays for cellular cytotoxicity, intracellular replication, and lysosomal degradation, we show that all isolates (*Pitt-1515*, *NY-2425*, *NE-2733*, *NE-2735*, and *PA-2591*) were cytotoxic and avoided lysosomes. Likewise, all five isolates replicated proficiently in the U937 human monocytic cell line. In primary mouse macrophages, four of five isolates survived, although they replicated poorly and caused little destruction of the macrophages. In contrast, isolate *NE-2733*, which colonized water supplies but was not associated with disease, successfully entered macrophages, but was defective for subsequent survival and replication. Notably, *NE-2733* lacks an efflux pump locus and the 65 kb pathogenicity island of *L. pneumophila* strain Philadelphia 1. In summary,

disease incidence of the isolates correlated with one laboratory test of virulence, the ability to survive in the stringent environment of primary mouse macrophages, as well as the presence of the 65 kb pathogenicity island and an efflux pump locus; in contrast, the 65 kb pathogenicity island and efflux locus seem to be dispensable for water supply colonization and growth in U937 cells.

INTRODUCTION

Legionella pneumophila, an intracellular pathogen of alveolar macrophages, is the causative agent of both Legionnaires' disease and a milder illness, Pontiac Fever. *L. pneumophila* surfaced during a 1976 American Legion Convention in Philadelphia, when an outbreak of pneumonia afflicted the attending Legionnaires', thus giving the bacterium its name (Fraser *et al.*, 1977; McDade *et al.*, 1977). The natural hosts for *Legionella* are freshwater protozoa, which exert selective pressure for the bacterium to acquire mechanisms not only to evade killing but also to replicate within a variety of professional phagocytes (Fields, 1996; Rowbotham, 1980). *Legionella* can opportunistically infect humans, especially the immunocompromised; therefore, hospital populations are especially vulnerable to infection (Carratala, 1994; Strampfer, 1988).

L. pneumophila is well adapted to its environmental niche, where the bacteria can alternate between at least two phases: an intracellular "replicative" and an extracellular "transmissive" phase (Byrne and Swanson, 1998; Molofsky and Swanson, 2004; Rowbotham, 1986). *Legionella* can survive within normally bactericidal amoebae and macrophages by subverting the phagosome maturation pathway. When conditions are optimal, transmissive *Legionella* differentiate to the replicative form and replicate within

a host vacuole. When intracellular resources become scarce, the bacteria differentiate back to the transmissive form, becoming cytotoxic, motile, sodium sensitive, osmotically sensitive, and competent to evade phagosome-lysosome fusion (Byrne and Swanson, 1998). The coordinated expression of these traits permits *Legionella* to survive and disperse in the environment until another host cell is encountered, wherein a new intracellular replication niche can be established. Thus, *Legionella*'s cellular differentiation is an integral component of its pathogenesis (Byrne and Swanson, 1998; Molofsky and Swanson, 2004).

The primary route of human infection is inhalation of aerosols from contaminated water sources; person-to-person transmission of the bacteria does not occur. The majority of the cases of Legionnaires' disease reported to the Centers for Disease Control and Prevention (CDC) are hospital-acquired infections, accounting for 25 – 45% of cases (Benin *et al.*, 2002). At least 48 species and 70 serogroups of *Legionella* exist, yet >85% of disease cases are attributed to serogroup 1 (Fields *et al.*, 2002). Healthy individuals who have been exposed to *Legionella*, as judged by seroconversion, usually remain asymptomatic. The mortality rate for hospital-acquired Legionnaires' disease is roughly double that for community-acquired cases, 28 vs. 14%, respectively (Benin *et al.*, 2002). Diagnosis of *Legionella* infections relies extensively on specialized laboratory testing, a capability that most hospitals lack (Stout *et al.*, 2007). As a result, many cases of hospital-acquired Legionnaires' disease remain undetected (Mulazimoglu and Yu, 2001).

Routine culturing of the hospital water system has been recommended as part of a proactive plan for the prevention of hospital-acquired Legionnaires' disease (Squier *et al.*). Epidemiological investigations involve determination of the presence and serotype

distribution of *Legionella* in the hospital water system, where a high level of colonization (>30% of water outlets positive for *Legionella*) has been associated with increased risk of disease (Best *et al.*, 1983; Sabria *et al.*, 2004). Different serogroups and subtypes have been associated with increased risk of disease. For example, *L. pneumophila* serogroup 1 isolates have been associated with more disease than other serogroups (Yu, 2002) and certain monoclonal antibody subtypes of *L. pneumophila* serogroup 1 (mAb-2) have been shown to be more likely to cause disease (Dournon *et al.*, 1988). Genotypic analysis using amplified fragment length polymorphism (AFLP) has also shown that a particular AF type was recovered from patients (Huang *et al.*, 2004). However, recent genomic analyses indicate that clinical and environmental *Legionella* strains or strains of different serogroups could not be differentiated based on a specific DNA hybridization profile (Cazalet *et al.*, 2008). Since knowledge of whether isolates inhabiting a hospital's water system pose a threat would decrease the time and resources exhausted on laboratory diagnosis, environmental surveillance for virulent strains is an attractive management strategy.

The *Legionella* Study Group conducted a multi-center, correlative study consisting of twenty hospitals in 14 different states (Stout *et al.*, 2007). Each hospital performed environmental and clinical surveillance for *Legionella* from 2000 to 2002 and provided specimens to the VA Pittsburgh Special Pathogens Laboratory. Urine and sputum specimens were tested for *Legionella*, and isolates of *L. pneumophila* were subtyped by serogrouping and pulsed-field gel electrophoresis (PFGE). A case of nosocomial Legionnaires' disease was defined as a patient with *Legionella*-induced pneumonia whose infecting strain matched the strain recovered from the hospital's water

system (Stout *et al.*, 2007). High-level colonization of the water systems of four hospitals highly correlated with disease incidence. However, one site that was 83% positive for *L. pneumophila* colonization was completely void of clinical cases of Legionnaires' Disease over a 2 year period (Stout *et al.*, 2007), generating the opportunity to investigate whether the virulence assays commonly used by laboratories in the pathogenesis field (Catrenich and Johnson, 1989; Horwitz and Silverstein, 1980; Horwitz, 1983b; Husmann and Johnson, 1994; Pruckler *et al.*, 1995) can distinguish those isolates that inhabit the water systems and also cause disease from those environmental isolates that highly colonize the water system but cause no disease. By comparing the virulence trait profile of this set of environmental and clinical isolates, we asked which, if any, of the assays correlated with the potential of *Legionella* isolates to cause disease.

EXPERIMENTAL PROCEDURES

Bacterial strains and media

The *L. pneumophila* serogroup 1 isolates *Pitt-1515*, *NY-2425*, *NE-2733*, *NE-2735* and *PA-2591* used in this study were obtained from the *Legionella* Study Group at the University of Pittsburgh (Table 2.1) (Stout *et al.*, 2007). Isolates *Pitt-1515*, *NY-2425*, and *PA-2591* were isolated from patients diagnosed with human disease and matched by PFGE to those isolated from the water outlets. The prototype, *L. pneumophila* *Lp02*, is a virulent thymine auxotroph derived from the *L. pneumophila* serogroup 1 Philadelphia-1 strain that has been extensively studied by the Isberg, Swanson, Roy, and Vogel laboratories. Strains from glycerol stocks maintained at -80° C were colony-purified onto

N-(2-acetomido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered charcoal-yeast extract agar (CYE) or CYE supplemented with 100 µg thymidine ml⁻¹ (CYET). Bacterial strains were cultured in ACES-buffered yeast extract broth (AYE) or in AYE supplemented with thymidine (100 µg ml⁻¹; AYET) at 37° C with aeration.

Previous studies have shown that broth cultures of *L. pneumophila* express several virulence traits upon entry to the post-exponential (PE-transmissive) phase of growth: flagellar-based motility, contact-dependent cytotoxicity, and evasion of macrophage lysosomes (Byrne and Swanson, 1998). Therefore, to examine expression of these virulence traits by the isolates, growth kinetics of each strain was analyzed using optical density at 600 nm (OD₆₀₀). An exponential (E-replicative) phase culture was diluted 1:500, 1:100, 1:50 or 1:10 and incubated at 37° C for 20 hours, and then the OD₆₀₀ of the subcultures were obtained at 3 h intervals.

Macrophage culture

Bone marrow-derived macrophages were obtained from femurs of female A/J mice (Jackson Laboratory) as described previously (Swanson and Isberg, 1995). Macrophages were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (RPMI-FBS; GIBCO/BRL) and plated as described below for each assay. The human cell line, U937, was obtained from the ATCC (Rockville, MD) and cultured as described previously (Berger and Isberg, 1993). After thawing, cells were cultured as non-adherent cells in RPMI-FBS at 37° C in 5% CO₂ and were passaged no more than five times. Cells were then transformed into an adherent macrophage-like cell by treating with phorbol 12-myristate 13-acetate (PMA, Sigma) for a minimum of 36 hr. Cells were

removed from tissue culture flasks with trypsin in RPMI-FBS, pelleted by centrifugation, then resuspended in fresh media and plated as indicated for intracellular assays.

Motility

The motility of broth cultures was scored by examining wet mounts by phase microscopy at a magnification of 320X. Cultures were defined as motile when >75% of bacteria in a field of ≥ 100 cells showed rapid, directed movement.

Infectivity

The efficiency of binding, entering, and surviving within macrophages by *L. pneumophila* or “infectivity”, was assessed as described previously (Byrne and Swanson, 1998). Macrophages were plated in 24-well tissue culture plates at a density of 2.5×10^5 macrophages per well and infected at a 1:1 ratio with transmissive bacteria for 2 h at 37° C. Extracellular bacteria were removed by rinsing the macrophage monolayer three times with 0.5 ml of RPMI-FBS at 37° C, a medium that is not permissive to *Legionella* replication. Intracellular bacteria were quantified by lysing monolayers by scraping and forceful pipetting with ice-cold (PBS) and plating duplicate aliquots on CYE and CYET. Colony forming units added at 0 h was determined by diluting the infection inocula with PBS and plating on CYE/CYET. PBS did not affect the viability of the *Legionella* isolates (data not shown). The initiation of infection was calculated from triplicate samples by the following equation: $(\text{CFU from lysates at 2 h})/(\text{CFU added at 0 h}) \times 100$.

Lysosomal degradation

The ability of the bacteria to evade lysosomal degradation after a 2 h infection was analyzed by fluorescence microscopy as described previously using rabbit anti-*L. pneumophila* (a gift from Dr. Ralph Isberg, Howard Hughes Medical Institute and Tufts University School of Medicine, Boston, MA) (Swanson and Isberg, 1996b). Macrophages were cultured on 12-mm glass coverslips at a density of 2×10^5 and infected at a MOI of $\sim 1-2$. Isolates *NE-2733* and *NE-2735*, which do not react with mAb2 (Joly *et al.*, 1986) also stained poorly with the *L. pneumophila*-specific monoclonal and polyclonal antiserums used in this study. Thus, to detect intact and degraded bacteria, the bacteria were labeled fluorescently prior to infection using a previously described method (Sturgill-Koszycki and Swanson, 2000). Bacteria were incubated with 5(6)-carboxyfluorescein-*N*-hydroxysuccinamide ester (FLUOS; Boehringer Mannheim Biochemica) for 30 min on ice. The cells were washed twice with PBS and once with RPMI-FBS by centrifugation, then resuspended in RPMI-FBS prior to use. FLUOS had no detrimental effects on the macrophages (data not shown). Macrophage nuclei were labeled by incubating fixed cells with $0.1 \mu\text{g}$ 4',6-diamidino-2-phenylindole (DAPI) ml^{-1} of PBS.

Intracellular bacterial growth

To quantify replication of bacteria in macrophages at 24 h intervals, cells were infected at an MOI of 1 as described for infectivity. To enumerate CFU, lysates were prepared from triplicate samples and plated on CYE/CYET for CFU. U937 cells were

plated in 24 well plates at a density of 1×10^6 per well. Cells were allowed to adhere overnight before incubation with bacteria. Cells were lysed, by treating monolayers with 2% saponin (Sigma) in PBS, at the indicated time intervals. Lysates were prepared from triplicate samples and plated on CYE/CYET for CFU enumeration.

Cytotoxicity

Contact-dependent cytotoxicity was quantified as the percent of macrophages killed during a 1 h incubation with *L. pneumophila*. Macrophages were cultured at a density of 5×10^4 per well in 96-well tissue culture plates. Transmissible bacteria suspended in RPMI-FBS at varying ratios were co-incubated with the macrophages for 1 h at 37° C. After bacteria were washed away, the monolayers were subsequently incubated with 0.5 ml of 10% (vol/vol) Alamar Blue (TREK Diagnostics) in RPMI-FBS for from 4 h to overnight. The redox-specific absorbance resulting from the reduction of Alamar Blue to its reduced form by viable macrophages was measured with a SpectraMax 250 spectrophotometer (Molecular Devices) at OD₅₇₀ and OD₆₀₀. The percent of viable macrophages was calculated in triplicate from the standard curve, the slope of a plot of A_{570}/A_{600} determined for triplicate samples of six known densities of uninfected macrophages in the range of 10^3 to 5×10^4 macrophages per well. The actual MOI was determined by plating duplicate samples of the infection inocula onto CYE/CYET.

Statistical analysis

P-values for infectivity and lysosomal evasion were calculated using a one-way analysis of variance (ANOVA) for three independent experiments.

RESULTS

Previous studies have shown that the profile of virulence traits expressed by *L. pneumophila* is coordinated with the post-exponential (PE; transmissive) broth growth phase (Byrne and Swanson, 1998; Molofsky and Swanson, 2004). Therefore, the clinical and environmental isolates *Pitt-1515*, *NY-2425*, *NE-2733*, *NE-2735*, and *PA-2591* were cultured in broth to the PE phase, and then their phenotypic characteristics and virulence traits were examined. Each of the isolates was fully capable of growing in AYET broth (data not shown) and becoming motile in the PE phase, a trait correlated genetically with virulence (Merriam *et al.*, 1997; Molofsky *et al.*, 2005; Pruckler *et al.*, 1995; Steinert and Heuner, 2005). Expression of motility was used as an indicator of transmissive phase *L. pneumophila* for all subsequent analysis.

Cytotoxicity

To determine whether each of the hospital isolates intoxicate macrophages during a 1 h incubation, we quantified macrophage viability by the capacity of cells to reduce the colorimetric dye Alamar Blue (Byrne and Swanson, 1998). Replicative bacteria of the

clinical and environmental strains behaved similar to replicative *Lp02*: 95% of macrophages incubated with replicative phase *Lp02* were viable (Fig. 2.1; data not shown). In contrast, less than 20% of macrophages incubated with transmissive *Lp02*, *Pitt-1515*, *NY-2425*, *NE-2733*, *NE-2735*, or *PA-2591* survived.

Infectivity

The capacity of isolates *Pitt-1515*, *NY-2425*, *NE-2733*, *NE-2735*, *PA-2591* to bind and enter macrophages was compared with the virulent laboratory strain *Lp02* (Berger and Isberg, 1993). Isolates were cultured to the transmissive phase, then incubated with macrophages at an MOI of ~1 bacterium per macrophage. Two hours after their addition to macrophage cultures, approximately 20% of the transmissive *Lp02* inoculum initiated an infection, whereas only 2% of the replicating *Lp02* inoculum was viable and cell associated (Byrne and Swanson, 1998), as expected. For each of the hospital isolates ~6 to 20% of the inoculum was intracellular and viable after the 2 h incubation. Compared with the other isolates, *PA-2591* had an intermediate phenotype. Nevertheless, each of the isolates appeared competent to bind, enter and survive in macrophages (Fig. 2.2).

Lysosomal degradation

To test whether the relative amount of cell-association of the isolates reflected their ability to evade lysosomes, macrophages were infected for 2 h with each strain, and then bacterial degradation was quantified by immunofluorescence microscopy. As

expected (Byrne and Swanson, 1998), nearly 80% of transmissible *Lp02* were intact after a 2 h infection, whereas < 20% of replicative *Lp02* were intact (Fig. 2.3 A,B). All five hospital isolates effectively avoided lysosomal degradation, although *PA-2591* did so somewhat less efficiently ($\leq 60\%$; Fig. 2.3 A,B). Thus, each of the isolates was capable of infecting macrophages by evading lysosomal degradation.

Intracellular bacterial replication

We next analyzed whether each of these isolates could multiply in cultured macrophages. First we infected the U937 human monocytic cell line, which supports robust replication of laboratory strains (Pearlman *et al.*, 1988). All five strains were proficient for replication within U937 macrophages; their yield increased at least 500-fold during a 48 h period (Fig. 2.4). Next we examined the intracellular growth profile of each strain in primary macrophages derived from the bone marrow of A/J mice, a host cell model that is more stringent than the human cell line (Yamamoto *et al.*, 1988). In mouse macrophages, transmissible and replicative *Lp02* replicated robustly; by 72 h their yield increased 100-1000-fold (Table 2.2), and the macrophage monolayer was visibly destroyed (data not shown). In contrast, by 72 h after infection, isolates *Pitt-1515*, *NY-2425*, *NE-2735*, and *PA-2591* had replicated poorly in macrophages (<1 log), and there was no detectable damage to the macrophage monolayer. Nevertheless, the intracellular bacteria of each of these four strains survived fairly well (Table 2.2). In contrast, isolate *NE-2733* was unique in that it failed either to replicate or survive within primary mouse macrophages during the 72 h incubation (Table 2.2, Fig. 2.5) or when incubated up to

120 h (data not shown). Thus, although each of the hospital isolates survived their initial encounter with primary mouse macrophages (Fig. 2.2,5), none of the strains replicated significantly, and one strain was eventually cleared by macrophages (Fig. 2.5).

DISCUSSION

To investigate whether particular *L. pneumophila* laboratory assays commonly analyzed in the pathogenesis field correlate with the ability to cause disease and to colonize water supplies, we took advantage of strains and data collected from a recent multi-center environmental and clinical surveillance study (Stout *et al.*, 2007). By considering the results of our laboratory assays in light of the epidemiological data and recent genomic analyses (Cazalet *et al.*, 2008), two phenotypic profiles are noteworthy. Each of the strains known to colonize water supplies expressed motility, contact-dependent cytotoxicity, and immediate evasion of macrophage lysosomes, suggesting these traits confer fitness in the environment. Strains that also caused nosocomial disease expressed one additional phenotype: the ability to persist in primary macrophages derived from A/J mice.

Contact-dependent cytotoxicity is characteristic of virulent *L. pneumophila*, occurring independently of intracellular replication (Husmann and Johnson, 1994). The three clinical isolates and both environmental isolates were cytotoxic to macrophage monolayers, similar to transmissible *Lp02*. Therefore, each of these motile isolates likely express the pore-forming cytotoxin, since at high MOI they are toxic to macrophages. A toxin is presumably secreted through pores produced in the nascent phagosomal membrane of the host cell by the Dot/Icm type IV secretion apparatus of *L. pneumophila*

(Dumenil *et al.*, 2004). The cytotoxin is thought to be necessary for the bacteria to lyse the host cell when nutrients have been expended during replication, thus freeing the bacteria to infect new host cells (Alli *et al.*, 2000; Byrne and Swanson, 1998). Expression of contact-dependent pore-forming toxin by *L. pneumophila* is dependent upon *dotA* and other virulence loci (Kirby *et al.*, 1998). However, in both human and mouse macrophages, cytotoxicity also requires flagellin (Vinzing *et al.*, 2008b). Moreover, cytosolic flagellin triggers intoxicated cells to release the pro-inflammatory cytokine IL-1 β (Molofsky *et al.*, 2006), which could contribute to the extensive inflammation typical of disease (Alli *et al.*, 2000; Blackmon *et al.*, 1978; Brieland *et al.*, 1994a). In the environment, the flagellar regulon likely promotes dispersal, contact with host cells, and also lysosome evasion (Molofsky *et al.*, 2005). Accordingly, the ability of all of the isolates to escape from an amoebal host once resources are depleted, to disperse in the environment, and to evade lysosomes efficiently likely provides the selective pressure to maintain cytotoxicity to human macrophages, a trait shared by all of the hospital isolates examined here.

To establish infection, virulent *L. pneumophila* must enter host cells and avoid killing within lysosomes (Horwitz, 1987). When the isolates were incubated with murine macrophages, they successfully avoided immediate demise within the degradative lysosomes (Fig. 2.3). Although all five of the hospital isolates survived their initial encounter with primary mouse macrophages, only four were able to persist there (Table 2.2). Therefore, to persist in the water supplies and to cause infections in humans, *L. pneumophila* evidently must express factors that also inhibit phagosome maturation in mouse macrophages.

After successful entry and lysosome evasion, *L. pneumophila* initiates replication within macrophages, a critical feature of its pathogenesis (Horwitz and Silverstein, 1980). With the exception of *NE-2733*, the CFU of the other strains remained constant, and viable cells could be recovered even 120 h post-infection. However, unlike the laboratory strain *Lp02*, none of the isolates were able to replicate within primary murine macrophage cultures, despite their prolific replication in U937 cells (Fig. 2.4). Whether the hospital isolates persist in a transmissible form throughout the 72 h intracellular infection can be determined by additional molecular analysis (Sauer *et al.*, 2005). In any case, the ability of the hospital isolates to persist in cultured mouse macrophages correlated with the incidence of clinical cases of Legionnaires' disease (Tables 2.1, 2.2), whereas growth in U937 cells does not appear to be valuable as a predictor of virulence potential.

Genomic analysis of *NE-2733* and *NE-2735* has presented some insight into the phenotypic analysis presented here. The environmental isolates *NE-2733* and *NE-2735* are highly similar and phylogenetically cluster into the same lineage (Cazalet *et al.*, 2008). The gene *lag-1*, encoding an acetyltransferase that acetylates the *O*-antigen to generate a "virulence-associated" lipopolysaccharide epitope (Luck *et al.*, 2001) that is required for recognition by some *Legionella* specific antibodies (Cazalet *et al.*, 2008), was not detected in *NE-2733* and *NE-2735* by genomic micro array hybridization. Lack of reactivity to the monoclonal *Legionella* antibody used in this study may be due to this missing epitope. On the other hand, among this panel of strains, isolate *NE-2733* was unique in that it lacked two other loci. First, *NE-2733* lacked the genes encoding for proteins of a cation/multidrug efflux pump cluster found in *L. pneumophila* Paris strain

(Cazalet *et al.*, 2008); however, this genome does hybridize to a probe for *cadAI*, a gene upstream of the efflux cluster that is similar to a cadmium transporting ATPase. Second, missing is the 65 kb pathogenicity island that is unique to *L. pneumophila* Philadelphia 1 and that contains type IV secretion homologues, virulence genes and other pathogenicity island hallmark elements (Brassinga *et al.*, 2003). In addition, *NE-2733* contains a small cluster of unknown genes, which are not present in *NE-2735*. Therefore, the 65 kb pathogenicity island and the efflux pumps appear to be dispensable for growth in U937 cells, but these two loci correlate with growth in restrictive mouse macrophages.

In general, cases of Legionnaires' disease are detected in hospital patients when > 30% of a hospital's water outlets are positive for *Legionella* serogroup 1 (Best *et al.*, 1983). Yet, the hospital colonized by *NE-2733* and *NE-2735* had 83% of its sites positive for *L. pneumophila* (Table 2.1), but no cases of disease. Although isolate *NE-2735* established an infection poorly (Fig. 2.2), the intracellular bacteria persisted in cultured macrophages (Fig. 2.4). In contrast, *NE-2733* failed to survive in permissive mouse macrophages, consistent with the observation that this strain did not cause hospital-acquired Legionnaires' disease (Table 2.2). To account for the environmental, clinical, and laboratory data obtained, we speculate that the avirulent strain *NE-2733* was more fit to colonize hospital water outlets, therefore out-numbering the more virulent strain *NE-2735*. By this reasoning, less than 30% of the sites would be positive for the virulent strain *NE-2735*, so no disease cases would occur. Since the relevant hospital sites were promptly decontaminated after the initial clinical surveillance was conducted, the relative prevalence of *NE-2733* and *NE-2735* in the water supply could not be assessed.

Although differences in plumbing systems and patient populations likely also contribute to the incidence of nosocomial Legionnaires' disease, our strain survey data suggest that multiple bacterial factors also contribute to the ability of *Legionella* to establish an infection. The plasticity of the *L. pneumophila* genome is extraordinarily high: ~13% of the genes encoded by strains Paris, Lens and Philadelphia 1, three serogroup 1 *L. pneumophila* clinical isolates, are not common to the other strains (Cazalet *et al.*, 2004). Indeed, phenotypic heterogeneity is well documented (Izu *et al.*, 1999; Joshi and Swanson, 1999) and has been the basis for a classification scheme of *Legionella* strains (Alli *et al.*, 2003). Accordingly, phenotypic markers of disease potential, such as growth in primary A/J mouse macrophages, could be a valuable complement to molecular markers of virulence.

Table 2.1. Epidemiological Data of Hospital Isolates*

Strain	<i>Environmental Surveillance</i>		<i>Clinical Surveillance</i>
	Hospital Location	Mean Percent Positive Sites With <i>L. pneumophila</i> (No. pos./No. tested)	Clinical Cases of Legionnaires' Disease
1515 [†]	Pitt	N/A	Yes
2425	NY	36% (8/22)	Yes
2733/2735	NE	83% (58/70)	No
2591	PA	43% (17/40)	Yes

*Data summarized from (Stout et al., 2007).

[†]Positive control strain for serogrouping and subtyping of hospital isolates.

Table 2.2. Intracellular Replication

Strain	% Uptake *	Fold Change CFU †
<i>Lp02</i> PE	23±4.2	150
<i>Lp02</i> E	2.2±0.9	1400
<i>Pitt-1515</i>	18.9±6.1	0.34
<i>NY-2425</i>	18±5.7	0.29
<i>NE-2733</i>	13.2±4.9	0.0
<i>NE-2735</i>	12.9±4.1	4.1
<i>PA-2591</i>	6.3±1.1	1.1

*Mean percent of CFU. associated with primary mouse macrophages 2 h after infection (see Materials and Methods).

†Increase in CFU yield, expressed as (mean cell-associated CFU @ 72 h / mean cell-associated CFU @ 2 h).

Representative of two or more experiments in triplicate.

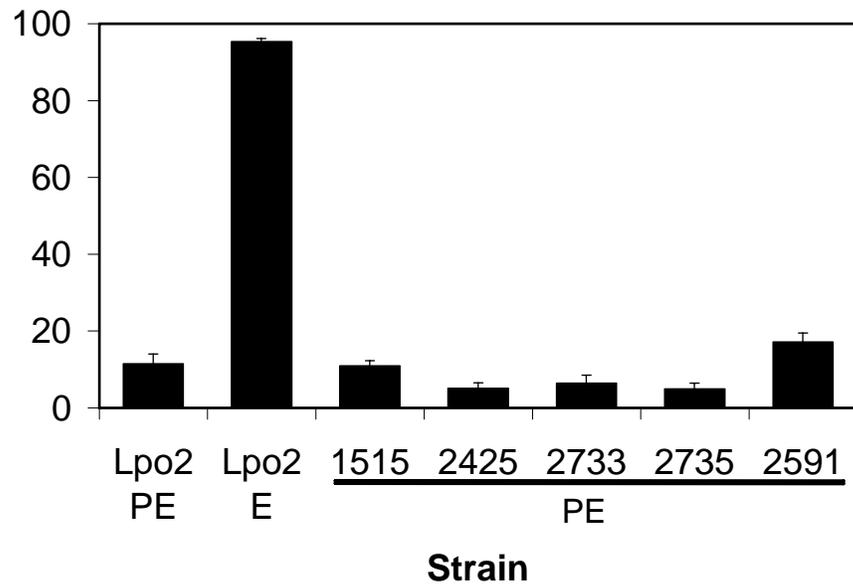


Figure 2.1. *L. pneumophila* cytotoxicity for macrophages.

Macrophage viability was quantified by determining the capacity of macrophages to reduce the colorimetric dye Alamar Blue after a 1 hr incubation with replicative (E) or transmissive (PE) bacteria (MOI~15). The means calculated for triplicate samples from three different experiments are shown with standard error. The MOI was calculated by plating the respective broth culture on CYET.

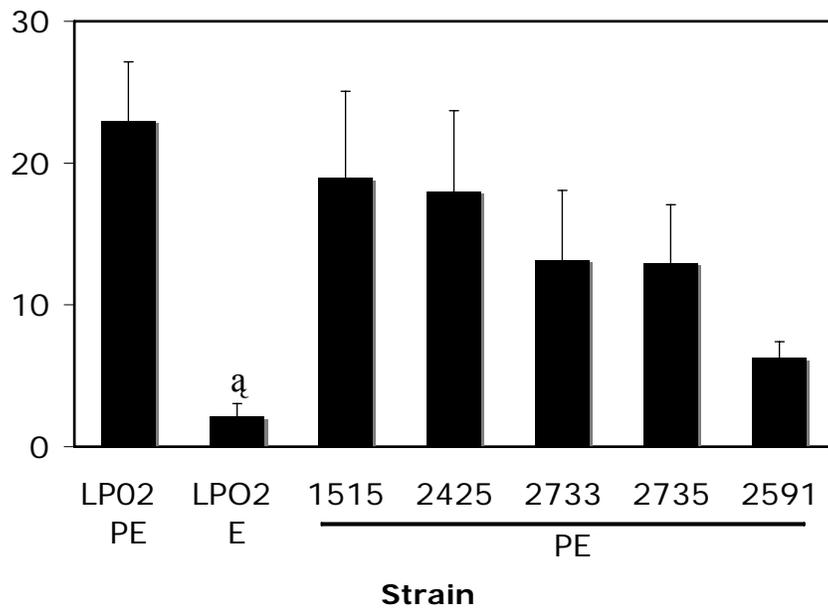


Figure 2.2. Binding, entry, and survival.

The ability of *L. pneumophila* to enter and survive in macrophages was assessed by incubating replicative (E) or transmissive (PE) bacteria with macrophages at an MOI ~1 for 2 h, then determining the percent of viable and cell associated bacteria. The mean percent of infectious *L. pneumophila* was determined for duplicate or triplicate samples in four experiments. Bars indicate standard errors. A statistically significant difference when compared to PE *Lp02* is indicated by ‡, where $P < 0.01$.

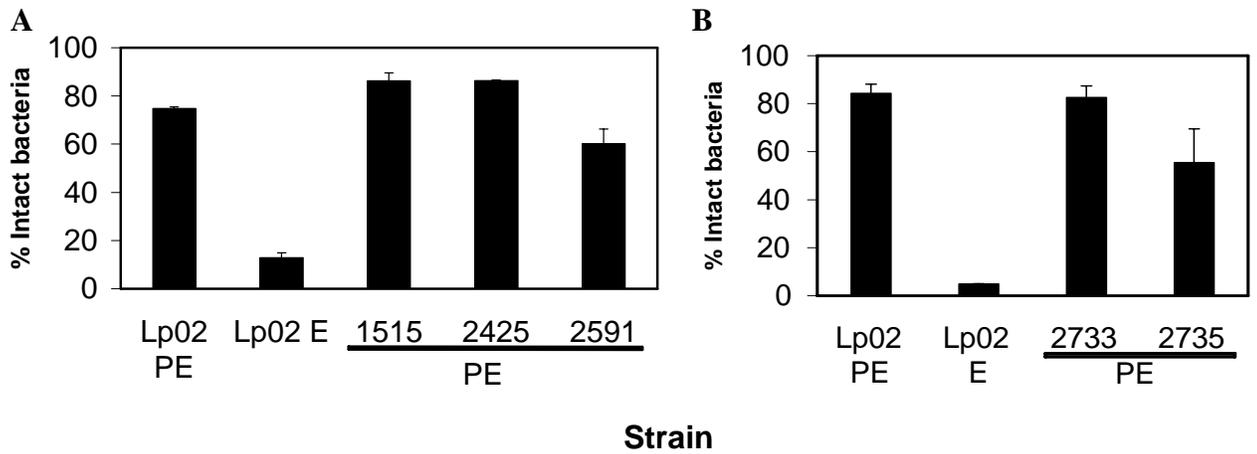


Figure 2.3. Lysosomal degradation.

The ability of replicative (E) or transmissive (PE) *L. pneumophila* to evade macrophage lysosomes was quantified by fluorescence microscopy using anti-*Legionella* antibody (A) or FLUOS (B) to label bacteria. The mean percent intake bacteria for three experiments is shown. A statistically significant difference when compared to PE *Lp02* is indicated by ‡, where $P < 0.01$ and *, where $P < 0.05$.

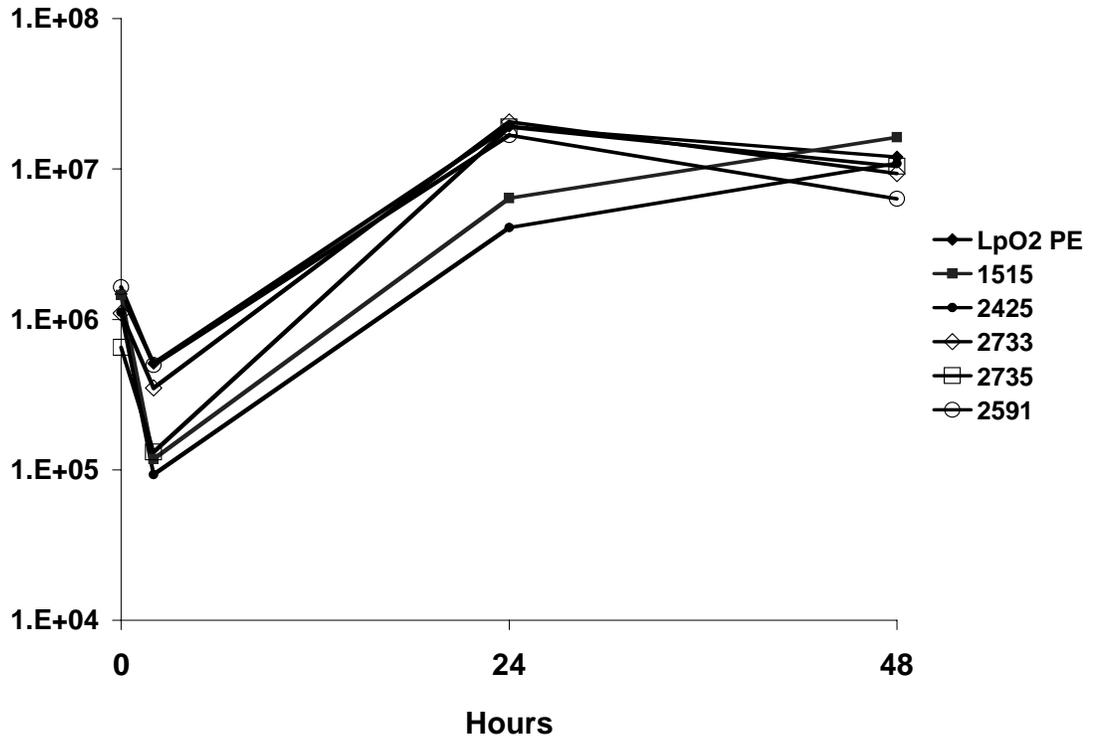


Figure 2.4. U937 intracellular replication.

The relative growth of PE *L. pneumophila* of each strain incubated with the U937 human macrophage cell line for 24 h intervals was determined by quantifying viable and cell associated bacteria. Representative of three experiments where each isolate was analyzed in triplicate. Standard deviations for each point were too small to be detected.

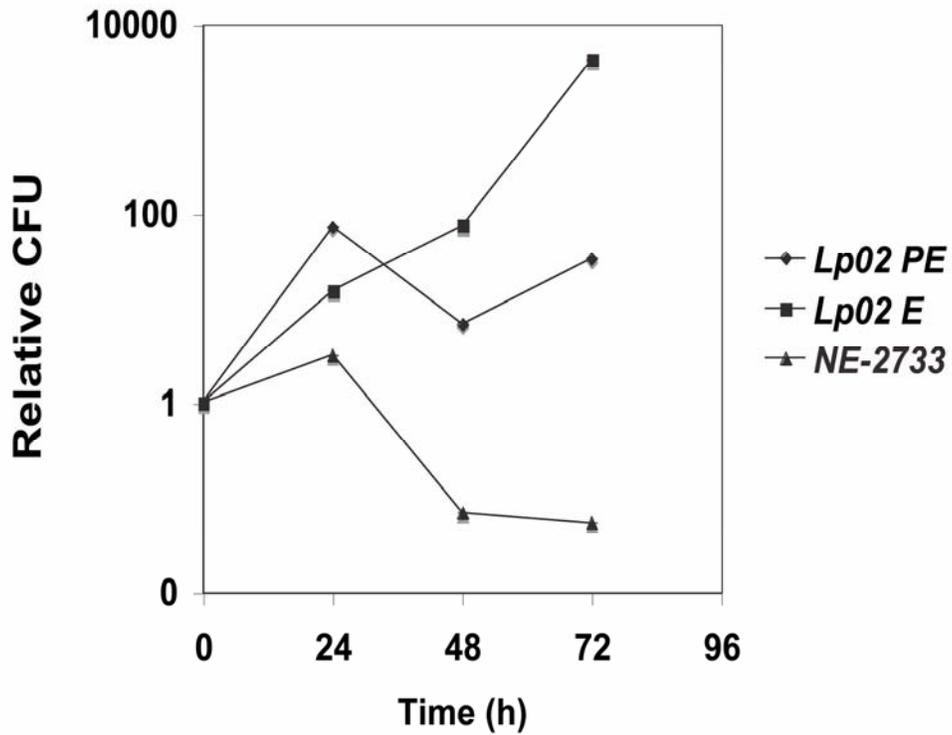


Figure 2.5. Mouse macrophage intracellular replication.

The relative growth of *L. pneumophila* incubated with primary mouse macrophages for 24 h intervals was determined by quantifying viable and cell associated bacteria. Representative of three experiments where each isolate was analyzed in triplicate. (PE-transmissible, E-replicative). Standard deviations for each point were too small to be detected.

CHAPTER THREE

Cytosolic recognition of flagellin by murine macrophages restricts *Legionella pneumophila* infection

SUMMARY

To restrict infection by *Legionella pneumophila*, murine macrophages require Naip5, a member of the NOD-LRR family of pattern recognition receptors, which detect cytoplasmic microbial products. We report that murine macrophages restricted *L. pneumophila* replication and initiated a pro-inflammatory program of cell death when flagellin contaminated their cytosol. Nuclear condensation, membrane permeability, and interleukin-1 β secretion were triggered by type IV secretion-competent bacteria that encode flagellin. The macrophage response to *L. pneumophila* was independent of Toll-like receptor signaling but correlated with Naip5 function and required caspase 1 activity. The *L. pneumophila* type IV secretion system provided only pore-forming activity, since listeriolysin O of *Listeria monocytogenes* could substitute for its contribution. Flagellin monomers appeared to trigger the macrophage response from perforated phagosomes: Once heated to disassemble filaments, flagellin triggered cell death, but native flagellar preparations did not. Flagellin made *L. pneumophila* vulnerable to innate immune mechanisms, since Naip5⁺ macrophages restricted growth of virulent microbes, but

flagellin mutants replicated freely. Likewise, after intra-tracheal inoculation of Naip5⁺ mice, the yield of *L. pneumophila* in the lungs declined, whereas the burden of flagellin mutants increased. Accordingly, macrophages respond to cytosolic flagellin by a mechanism that requires Naip5 and caspase 1 to restrict bacterial replication and release pro-inflammatory cytokines that control *L. pneumophila* infection. This work has been published in J Exp Med. 2006 April 17; 203(4): 1093–1104. My contributions to this work were Figures 3.3 and 3.9, molecular cloning of MB589 and MB593, several control experiments, and participation in the discussions and writing relevant to this chapter.

INTRODUCTION

Macrophages are the guardians of the innate immune system, recognizing a broad array of pathogen-associated molecular patterns (PAMPs) to initiate immediate defenses and to recruit the adaptive branch of the immune system. Toll-like receptors (TLR) detect extracellular microbial products, such as lipopolysaccharide, peptidoglycan, lipoteichoic acid and flagellin (Iwasaki and Medzhitov, 2004), whereas surveillance of the cytosol is the task of nucleotide-binding oligomerization domain-leucine rich repeat (NOD-LRR) proteins. The best characterized members of the NOD-LRR family are NOD1 and NOD2, which recognize distinct elements of bacterial cell wall peptidoglycan in the cytosol to mount or modulate a pro-inflammatory immune response or to promote apoptosis (Inohara, 2004).

In murine macrophages, the NOD-LRR protein Naip5 (Birc1e) restricts intracellular replication of the opportunistic human pathogen *Legionella pneumophila* (Diez *et al.*, 2003; Fortier *et al.*, 2005; Wright *et al.*, 2003). Naip5 is comprised of three

modules: N-terminal baculoviral inhibitor-of-apoptosis repeats (BIR), a central nucleotide-binding oligomerization (NBS or NOD) domain, and C-terminal leucine-rich repeats (Inohara, 2004). By analogy to other NOD-LRR proteins, the LRR region is thought to recognize microbial products, triggering oligomerization via the NOD domain, then activation of a cellular response that is governed by various N-terminal effector-binding domains (Inohara, 2004). Whereas virtually all mice are resistant to *L. pneumophila*, the A/J strain encodes a *naip5* allele that confers susceptibility to infection (Fortier *et al.*, 2005). Whether the reduction in Naip5 protein (Wright *et al.*, 2003) or a change in its function accounts for the failure of A/J macrophages to restrict *L. pneumophila* replication has not been unequivocally established.

The biochemical activity of murine Naip5 is not yet known, but testable models can be drawn by analogy to related proteins. Human Naip/Birc1 inhibits apoptosis by binding effector caspases through its BIR domain (Davoodi *et al.*, 2004; Maier *et al.*, 2002). Other NOD family members, including Ipaf and the NALPs, interact with the inflammasome, a caspase 1-containing complex that can be triggered by microbial products (Martinon *et al.*, 2004). In response to the intracellular pathogens *Salmonella enterica*, *Shigella flexneri*, or *L. pneumophila*, the inflammasome can initiate a caspase 1-dependent pro-inflammatory cell death (Brennan and Cookson, 2000; Chen *et al.*, 1996; Fink and Cookson, 2005; Mariathasan *et al.*, 2004; Suzuki *et al.*, 2005; Zamboni *et al.*, 2006).

Extensive inflammation, destruction of lung epithelium, and lysis of macrophages are clinical hallmarks of Legionnaires' disease pneumonia thought to be a result of cytotoxin(s) (Alli *et al.*, 2000; Blackmon *et al.*, 1978; Brieland *et al.*, 1994b). *L.*

pneumophila can utilize its Dot/Icm type IV secretion system to insert pores into the membranes of either red or white blood cells (Kirby *et al.*, 1998; Vogel *et al.*, 1998). However, to kill mouse macrophages, *L. pneumophila* require not only type IV secretion, but also flagellin (Molofsky *et al.*, 2005), the major subunit of the flagellum. Flagellar genes are conserved in the species, as they equip *L. pneumophila* to build a flagellum and become motile, infect host cells efficiently, and avoid degradation (Heuner *et al.*, 2002; Molofsky *et al.*, 2005).

Both humans and mice detect *L. pneumophila* flagellin to mount an immune response. In humans, its recognition by Toll-like Receptor 5 correlates with resistance to Legionnaires' Disease (Hawn *et al.*, 2003). When injected into mice, *L. pneumophila* flagellin triggers a robust inflammatory response (Ricci *et al.*, 2005), a trait of other flagellins (Ramos *et al.*, 2004). Therefore, we tested the hypothesis that a Naip5- and caspase 1-dependent pathway equips mouse macrophages to detect cytosolic flagellin, induce a pro-inflammatory programmed death, and restrict growth of intracellular *L. pneumophila*.

EXPERIMENTAL PROCEDURES

Bacteria

L. pneumophila (Table 1) was cultured on CYET agar or in AYET broth to exponential (E) or post-exponential phase (PE). The flagellar regulon is expressed exclusively in the PE phase (48). In synchronous PE broth cultures, >95% of strain Lp02 cells are motile, but only ~ 10 % of Lp01 bacteria are (Molofsky *et al.*, 2005).

Intracellular growth was calculated from duplicate wells as [total CFU] / [cell-associated CFU at 0 h] X 100. PE

Macrophages

Macrophages prepared from bone marrow of permissive A/J mice or restrictive BALb/C, C57Bl/6J (Jackson Laboratory, Bar Harbor, ME), or C57Bl/6 MyD88^{-/-} mice (gift of Dr. C. Hogaboam, Ann Arbor, MI) were cultured in RPMI + 10% heat inactivated FBS (Molofsky *et al.*, 2005). Caspase activity was inhibited by 100 μ M Ac-YVAD-cmk (caspase 1), Z-VAD-fmk (pan-caspase), or Ac-DEVD-cho (caspase 3; Fisher).

Macrophage viability after a 1 h infection (Fig. 3.2 A) was quantified by reduction of Alamar Blue (AccuMed) after 6-12 h (Molofsky and Swanson, 2003; Molofsky *et al.*, 2005). Macrophage permeability was indicated by lactate dehydrogenase (LDH) in supernatants using the Cytotox96 Non-Radioactive Cytotoxicity Kit (Kirby *et al.*, 1998). Where noted, results were pooled by averaging % viability in serial two-fold Multiplicity of Infection (MOI) bins; mean % viability \pm SE are shown. For co-infections, WT or *listeriolysin O* *Listeria monocytogenes* (*Lm*; Table 1; gift of Dr. M. O’Riordan, Ann Arbor, MI) at a final MOI of 25 was mixed with *L. pneumophila* at each MOI indicated (up to 100), centrifuged at 5000g for 5 min, gently resuspended in medium, then added to macrophages. Duplicate or triplicate wells were analyzed for LDH release; one experiment representative of > 3 is shown.

Hemolysis

A 2-fold dilution series of microbes in 100 μ L of RPMI/FBS was distributed to 10 μ L of 10^7 fresh washed sheep red blood cells (Becton Dickinson) in a 96 well plate, samples were incubated at 37° for 1 h, and lysis assayed as described (Kirby *et al.*, 1998). Results were calculated for triplicate samples as % hemoglobin released by detergent lysis prepared for a standard curve. Data were pooled in bins from 3 or more experiments.

Toxicity of cytosolic flagellin

To eliminate motility but retain viability (Fig. 3.1 A), flagella were dissociated by treating PE bacteria with PBS pH 2.0 for 5 min at 37° (Molofsky *et al.*, 2005). Crude flagellar preparations were obtained from WT, *dotA*, and *flaA* mutant *L. pneumophila* as described (Molofsky *et al.*, 2005); protein concentration was determined by the Bradford assay. The flagellin preparations were analyzed by SDS-PAGE using 12% acrylamide and Coomassie Blue staining. The protein concentration of the *flaA* mock flagellin preparation was adjusted to that of WT by addition of BSA. To promote disassembly of flagellar filaments into monomers, CFP were incubated at 78° for 15 min (Smith *et al.*, 2003). To affinity purify flagellin (Fig. 3.3 C), the rabbit monoclonal antibody (mAb) 2A5 specific to *L. pneumophila* flagellin (Byrne and Swanson, 1998) was incubated overnight at 4° with Protein G carboxylate beads (Polysciences, Inc.) in PBS-BSA. After four washes with PBS, mAb-beads were incubated overnight at 4° with or without heat-

treated CFP. Bead preparations were washed 4x, then diluted into RPMI-FBS for macrophage infections. *Salmonella typhimurium* and *Bacillus subtilis* flagellin was purchased from InvivoGen.

To perforate macrophages, a recombinant His-tagged listeriolysin O protein (LLO) was purified essentially as described (Mandal and Lee, 2002) except that *E. coli* were lysed by the French press technique. The purity and concentration of the LLO toxin was determined by SDS-PAGE and Coomassie Blue staining, and its activity was verified by hemolysis assay. To analyze toxicity of cytosolic flagellin, LLO (1 µg/ml in RPMI/FBS) was incubated with either native or heat-treated CFP or affinity-purified flagellin bound to beads in RPMI/FBS at the concentration indicated, centrifuged onto 8×10^4 macrophages per well in 96 well plate and incubated at 37° for 2 h, then supernatants were assayed for the cytosolic enzyme LDH.

Microscopy

Duplicate coverslip cultures were stained with rabbit anti-*Legionella* and rat anti-LAMP1 antibody and DAPI as described (Molofsky and Swanson, 2003; Molofsky *et al.*, 2005). Macrophage permeability was quantified using the Live/Dead Reduced Biohazard Cell Viability Kit (Molecular Probes). After 1 h with a high (50-100) or low (<1) MOI, cells were incubated with dyes for 15 min and examined immediately. The % of total cells (high MOI) or % singly infected cells (low MOI, phase contrast or SYTO-10 green stain) that were permeable (ethidium homodimer-2 red stain) was scored for > 100 macrophages on duplicate coverslips; the means \pm SD from 3 or more independent

experiments are shown. To calculate the % “condensed nuclei” \pm SD in 3 or more experiments, duplicate samples were infected at the MOI indicated by centrifugation at 400 g for 10 min, then incubated 1 or 2 h. After fixation and staining with DAPI, 100 macrophages from several fields were scored for phase dense, rounded, shrunken nuclei.

Interleukin-1 β

4-5 x 10⁵ macrophages were infected by centrifugation at 400g for 10 min, then incubated for 1 or 6 h. After centrifugation at 400g for 5 min, supernatants were stored at -80 °C until murine IL-1 β levels were determined in duplicate by the Quantikine ELISA (R&D Systems, MLB00B). Negligible IL-1 β was detected when uninfected cells were lysed with 0.1% SDS, verifying that the mature form of IL-1 β is not readily detected by this assay (Dinarello, 1992; Herzyk *et al.*, 1992). Mean \pm SD are shown for one experiment that is representative of 2-3 others.

Lung infections

Female A/J and C57BL/6 mice, 6-8 weeks old (Sankyo laboratory, Tokyo, Japan), were cared for in the Toho University School of Medicine animal facility. Mouse infections were performed according to a protocol approved by the animal facility of Toho University School of Medicine. After inducing anesthesia with 6 mg and 100 mg of xylazine and ketamine per kg, respectively, i.p., mice were infected with PE *L. pneumophila* strain Lp01 as described (Tateda *et al.*, 2001). To quantify CFU, whole

lungs were harvested and homogenized in 1.0 ml of PBS using a tissue homogenizer (Biospec Products, Inc.), and then 10 ml aliquots of a 1:10 dilution series in PBS were spread on CYE.

Apoptosis

Apoptosis was induced by treating macrophages with staurosporine (1 μ M; Sigma). Caspase 3 activation was evaluated by immunofluorescence microscopy using a rabbit antibody specific for the activated form of caspase 3 (1:1000 dilution; Molecular Probes) and DAPI as described (Molofsky *et al.*, 2005).

RESULTS

L. pneumophila requires flagellin to kill macrophages but not to perforate membranes

To test whether macrophages respond to *L. pneumophila* flagellin, a panel of previously characterized mutants (Molofsky *et al.*, 2005) was analyzed for cytotoxicity to macrophages (Fig. 3.1 A). Compared to motile wild-type post-exponential phase (WT PE) *L. pneumophila*, little macrophage toxicity was induced even by large numbers of the non-motile strains that either lack flagellin or contain scant amounts (flagellin mutant *flaA*, MB534; regulatory mutant *letA*, MB413; and flagellar sigma factor *fliA*, MB410). Flagellar mutants with intermediate amounts of flagellin induced corresponding levels of death (Molofsky *et al.*, 2005). Flagellin on the surface of bacteria was implicated in cell death, since a brief acid wash substantially reduced toxicity but not viability or intracellular multiplication of WT, *motAB* and *flhB* microbes (Fig. 3.1 A, unpublished

data). Poor contact by non-motile microbes did not account for the flagellin-dependence of death, as *flaA* (flagellin mutant) and *fliD* (flagellin polymerization mutant, MB552) bind macrophages to a similar extent (Molofsky *et al.*, 2005), yet *fliD* mutants were substantially more toxic (Fig. 3.1). Flagellin also appeared more potent when non-polymerized: Compared to *motAB* motility motor mutants, *fliD* polymerization mutants have less total flagellin (Molofsky *et al.*, 2005) but they more readily killed cells (40% versus 65% viable BMM; $p < 0.05$, MOI bin 30-60). As expected, toxicity of *fliD* polymerization mutants required flagellin and type IV secretion (Fig. 3.1 B; *flaA fliD*, MB567; *dotA fliD*, MB569). Thus, by a process that requires type IV secretion, flagellin exported by *L. pneumophila* promoted macrophage death.

In stark contrast to its contribution to macrophage death, flagellin was dispensable for *L. pneumophila* to insert pores into red blood cells (Alli *et al.*, 2000; Kirby *et al.*, 1998). When equipped for Dot/Icm type IV secretion, non-motile mutants were as hemolytic as motile WT, whether or not they expressed flagellin (Fig. 1 C, unpublished data; *flaA*, *fliD*, *fliA*, *fliI*, *flhB* and *motAB*). Therefore, the ability to perforate eukaryotic membranes was not sufficient for *L. pneumophila* to kill macrophages rapidly; flagellin was also required.

Flagellin⁺ L. pneumophila trigger macrophage death independently of MyD88 Toll-like Receptor signaling but not Naip5

Extracellular flagellin is recognized by the human innate immune system through TLR5 (Hayashi *et al.*, 2001). However, four observations discounted a role for TLR

proteins in murine macrophage intoxication by flagellin. Even in high concentrations, a crude flagellin preparation was not toxic to macrophages (Fig. 3.3 B, unpublished data). TLR5 is not detectable on mouse peritoneal or bone marrow-derived macrophages (Manes *et al.*, 2003). Macrophages that lack MyD88, the adaptor protein that mediates TLR5 and most other TLR signaling (Hayashi *et al.*, 2001; Iwasaki and Medzhitov, 2004), were as sensitive to flagellin-dependent death as the isogenic control cells (Fig. 3.2 A), and they also efficiently restricted *L. pneumophila* growth (Fig. 3.2 B).

The cytosolic NOD-LRR protein Naip5 confers not only resistance of mice to *L. pneumophila* (Diez *et al.*, 2003; Fortier *et al.*, 2005; Wright *et al.*, 2003), but also susceptibility of infected macrophages to a caspase 1-dependent, pro-inflammatory death (Zamboni *et al.*, 2006)

. Therefore, we investigated whether Naip5 contributes to detection of Flagellin⁺ microbes. Restrictive Naip5⁺ C57Bl/6 macrophages were more sensitive than permissive *naip5* mutant A/J cells to Flagellin⁺ *L. pneumophila* equipped for type IV secretion, as judged by LDH released at 1 or 6 h (Fig. 3.2 C, D; unpublished data). Compared to resistant cells, A/J macrophages express reduced levels of a Naip5 mutant protein that harbors 14 amino acid substitutions (Diez *et al.*, 2003; Fortier *et al.*, 2005; Wright *et al.*, 2003). Thus, the macrophage response to Flagellin⁺ pore-forming *L. pneumophila* correlated with the amount of Naip5 protein.

When present with a pore-forming activity, flagellin triggers macrophage death

To learn if *bona fide* substrates of the type IV secretion are required for macrophage cytotoxicity, we exposed Flagellin⁺ *L. pneumophila* to the cytosol by another means. To perforate phagosomes, we exploited *Listeria monocytogenes*, a pathogen that escapes into the cytosol when listeriolysin O (LLO) forms pores in macrophage vacuoles. Like *L. pneumophila* that lack either type IV secretion (*dotA*) or flagellin (*flaA*), WT *L. monocytogenes* (*Lm*; 1040S) did not rapidly cause significant permeability of C57Bl/6 macrophages, as measured by release of cytosolic lactate dehydrogenase (LDH; Fig. 3.3 A). However, macrophages released >30% of their LDH when co-infected with *L. monocytogenes* and either motile Flagellin⁺ *dotA* or non-motile Flagellin⁺ *dotA fliD* *L. pneumophila*. The combination of pore-formation and flagellin was required for the macrophage response, since cells released little LDH when incubated either with a mixed suspension of *L. monocytogenes* and *L. pneumophila* flagellin null mutants (Fig. 3.3 A, unpublished data; *flaA* or *dotA flaA* MB600) or when co-infected with non-hemolytic *llo*⁻ *L. monocytogenes* (DP-L2161) and Flagellin⁺ *dotA L. pneumophila* (unpublished data). Thus, substrates of the *L. pneumophila* type IV secretion system were dispensable for macrophage cytotoxicity, whereas pore-formation and flagellin were both required.

We investigated in more detail whether flagellin protein that has access to the cytosol triggers macrophage death. In either the presence or absence of the LLO toxin, crude flagellar preparations (CFP) of either WT, *dotA* or *flaA* mutant *L. pneumophila* failed to trigger significant LDH release from macrophages (Fig. 3.3 B). However, it is known that the flagellin epitopes that are recognized by TLR5 are buried within

polymerized filaments (Smith *et al.*, 2003), and we observed that *L. pneumophila* that secrete but cannot assemble flagellin protein are the most cytotoxic of the non-motile mutants analyzed (Fig. 3.1, *fliD*). Therefore, to test whether, when disassembled, cytoplasmic flagellin is toxic to macrophages, the CFP was incubated at 78 °C for 15 min, a treatment that promotes filament depolymerization (Smith *et al.*, 2003). When exposed to heat-treated CFP and LLO, macrophages rapidly released LDH (Fig. 3.3 B).

Flagellin and not other microbial products appeared to trigger the macrophage response, based on results of several control experiments. First, ~ 3 ng of affinity-purified flagellin induced nearly the same amount of LDH release as either ~ 30 or 300 ng of flagellin in CFP (Fig. 3.3 C; unpublished data). Second, macrophages did not release significant amounts of LDH when incubated with LLO protein (1 µg/ml), which had been obtained from *E. coli* lysates (Fig. 3.3 B, C). Third, when mixed with LLO, a mock flagellin preparation isolated from *flaA* mutant *L. pneumophila* was not toxic (Fig. 3.3 B, C). Fourth, either in the presence or absence of LLO, even 1 µg of native CFP did not induce significant macrophage permeability (Fig. 3.3 B, unpublished data). Fifth, proteinase K treatment substantially reduced the toxicity of heat-treated CFP (unpublished data). Therefore, we postulate that macrophages are equipped with a cytosolic surveillance system that detects flagellin, specifically recognizing epitopes that are masked within *L. pneumophila* flagellar filaments.

The mouse macrophage response did not appear to be specific to cytosolic flagellin of *L. pneumophila*. Flagellin purified from the intracellular gram-negative pathogen *S. typhimurium* or the soil gram-positive bacterium *Bacillus subtilis* also elicited LDH release from macrophages, but only when LLO was present (Fig. 3.3 D).

Heating had no effect on the potency of these commercial flagellins (unpublished data), which had already been treated with acid and heat. Thus, macrophages appear to recognize when their cytosol is contaminated with the flagellin from either *L. pneumophila* or at least two other microbes.

Flagellin⁺ L. pneumophila trigger a rapid pro-inflammatory programmed cell death

Since macrophages release cytosolic components soon after exposure to flagellin (Fig. 3.2, 3), we postulated that this PAMP triggers “pyroptosis”, a rapid pro-inflammatory death that is accompanied by membrane permeability and nuclear condensation and requires caspase 1 (Fink and Cookson, 2005; Suzuki *et al.*, 2005). After exposure for 1 h to Flagellin⁺ *L. pneumophila*, macrophages were permeable and had condensed nuclei (Fig. 3.4 A, B; WT or *fliD*). Both morphological changes were rare for macrophages infected by *L. pneumophila* that lack type IV secretion (*dotA*) or harbor little or no external flagellin (WT E, *flaA*, WT PE acid wash), even after prolonged incubations at a high MOI (Fig. 3.4 B, unpublished data). A caspase 3 apoptosis inhibitor had little effect on LDH release (Fig. 3.4 D) or macrophage viability, as measured by reduction of Alamar Blue (unpublished data). In contrast, macrophages were protected from flagellin-dependent toxicity by Ac-YVAD-CHO (Fig. 3.4 A, C, D), a peptide inhibitor that exhibits a $K_i > 200$ -fold lower for caspase 1 than caspases 2 – 10 (Fink and Cookson, 2005; Garcia-Calvo *et al.*, 1998; Suzuki *et al.*, 2005). Indeed, caspase 1 mutations render mice susceptible to infection by *L. pneumophila*, whereas mice that lack caspase 3 remain resistant (Zamboni *et al.*, 2006). Even when infected for 1 h with one

Flagellin⁺ bacterium, >35 % of A/J macrophages exhibited caspase 1-dependent membrane permeability and nuclear condensation (Fig. 3.4 A, C).

During pyroptosis, caspase 1 cleaves pro-interleukin 1 β (IL-1 β) and pro-interleukin 18 (IL-18), which are discharged as active cytokines (Brennan and Cookson, 2000). After a 1 h exposure to Flagellin⁺ *L. pneumophila*, macrophages released IL-1 β (Fig. 3.4 E; WT, *fliD*, or *flaA* pFlaA); by 6 h, IL-1 β levels were an additional ~5 fold higher (unpublished data). Liberation of active IL-1 β required not only *L. pneumophila* type IV secretion and flagellin (Fig. 3.4 E, unpublished data; *flaA*, *dotA*), but also macrophage caspase 1 activity (Fig. 3.4 F). Macrophages from mice that lack caspase 1 also fail to secrete IL-1 β in response to *L. pneumophila* (Zamboni *et al.*, 2006). Furthermore, Naip5⁺ C57Bl/6 macrophages released more IL-1 β than did *naip5* mutant A/J macrophages (Fig. 3.4 F), consistent with their degree of permeability after exposure to Flagellin⁺ *L. pneumophila* (Fig. 3.2 B, C). Thus, when exposed to flagellin of type IV secretion-competent *L. pneumophila*, murine macrophages initiated a rapid Naip5- and caspase 1-dependent pro-inflammatory death program.

Since *L. pneumophila* can induce apoptosis in numerous cell types (Abu-Zant *et al.*, 2005; Gao and Abu Kwaik, 1999; Hagele *et al.*, 1998; Molmeret *et al.*, 2004; Muller *et al.*, 1996; Neumeister *et al.*, 2002; Zink *et al.*, 2002), we tested whether flagellin triggers classic apoptosis in mouse macrophages. After a 5 h treatment with staurosporine, an inducer of apoptosis (Molmeret *et al.*, 2004), 30% of the cells contained activated caspase 3, as judged by immunofluorescence microscopy (Fig. 3.8 A, B), and a majority showed hallmarks of an apoptotic response, including chromatin condensation and nuclear blebbing; intact plasma membranes, as measured by LDH release and

Live/Dead staining; and viability, as measured by Alamar Blue reduction (unpublished data). A different pattern was observed for macrophages incubated with a high MOI of *L. pneumophila*: < 5% contained appreciable activated caspase 3 even 5 h after infection (Fig. 3.8) and, by 1 h, ~75% of the cells were permeable and had condensed nuclei (Fig. 3.4 B, Fig. 3.8 C). Thus, in mouse macrophages, *L. pneumophila* induces a pro-inflammatory death that is distinct from classic apoptosis, as judged by caspase 3 activation, nuclear morphology, plasma membrane permeability, sensitivity to caspase inhibitors and mutations (Zamboni *et al.*, 2006), and the speed of the response.

Flagellin makes L. pneumophila vulnerable to innate immune defenses of mouse macrophages

When infected by Flagellin⁺ *L. pneumophila*, Naip5⁺ murine macrophages not only exhibit pyroptosis (Figs. 3.2-4), but also restrict bacterial replication (Diez *et al.*, 2003; Wright *et al.*, 2003). Accordingly, we postulated that the Naip5 cytosolic surveillance pathway would be futile if the intracellular pathogens lack flagellin. During a 72 h incubation with permissive *naip5* mutant A/J macrophages, all *L. pneumophila* strains equipped for type IV secretion replicated >100 fold, regardless of motility, flagellin production, or assembly (Fig. 3.5 A and unpublished data; WT, *flaA*, *fliD*, *flhB*, *fliI*, and *motAB*). As expected, Naip5⁺ C57Bl/6 macrophages restricted replication of WT *L. pneumophila* and other strains that encode flagellin (WT, *fliD*, *flaA pFlaA*, *flhB*, *fliI*, *motAB*) to the level observed for type IV secretion mutants (*dotA*; Fig. 3.5 B and unpublished data). In stark contrast, two independent flagellin null mutant strains

replicated freely in restrictive Naip5⁺ C57Bl/6 or BALB/c macrophages (Fig. 3.5 B, MB534 *flaA:kan* and MB532 *flaA:gent*; unpublished data). A flagellin null mutant of *L. pneumophila* strain Lp01 also escaped restriction by C57Bl/6 macrophages (unpublished data).

Restrictive C57Bl/6 cells exerted strong selective pressure against flagellin expression by intracellular *L. pneumophila*. In several experiments, 72-96 h after infecting macrophages with *flaA* mutants that carried the complementing plasmid pFlaA (MB557), a population of pFlaA-free microbes emerged, as quantified by loss of the plasmid's selectable marker (unpublished data). Enrichment for plasmid-cured bacteria was attributable to the *flaA* locus, since *flaA* mutants maintained the pMMB vector (MB558) during replication in C57Bl/6 macrophages, and they retained pFlaA (MB557) during growth in *naip5* mutant A/J cells (unpublished data).

Microscopy provided additional insight to how Naip5⁺ murine macrophages restrict *L. pneumophila* replication (Fig. 3.5 C). After a 2 h infection of either A/J or C57Bl/6 macrophages, the majority of WT, *flaA*, and *fliD* microbes were intact, and <30% resided in LAMP-1-positive endosomal vacuoles, whereas >70% of *dotA* type IV secretion mutants were delivered to the endosomal pathway (Derre and Isberg, 2004a; Molofsky *et al.*, 2005). After a 24 h infection of permissive A/J macrophages, both WT and *flaA* microbes had replicated profusely, mainly clustered in large LAMP-1-positive vacuoles, as previously noted (Sturgill-Koszycki and Swanson, 2000). A similar pattern was observed 24 h after Naip5⁺ C57Bl/6 cells had ingested *flaA* mutants. In contrast, after 24 h in restrictive C57Bl/6 cells, WT *L. pneumophila* had begun to replicate, but the progeny were less numerous, and they were dispersed throughout the cytoplasm in small

LAMP-1-positive vacuoles (Derre and Isberg, 2004a). By 48 h, no further replication of WT microbes was evident; instead, a sub-population of apparently healthy macrophages contained dispersed bacteria and bacterial debris. Thus, it was apparent that Naip5⁺ macrophages can restrict infection by Flagellin⁺ *L. pneumophila* either by committing pyroptosis or by slowly delivering microbes to degradative vacuoles.

Flagellin makes L. pneumophila vulnerable to innate immune defenses of mouse lungs

To evaluate whether the Naip5 surveillance pathway for cytosolic flagellin contributes to control of *L. pneumophila* infections in lungs, the fates of WT and *flaA* mutant bacteria were compared after intra-tracheal inoculation of *naip5* mutant A/J and Naip5⁺ C57Bl/6 mice. As expected, within the lungs of restrictive Naip5⁺ mice, WT *L. pneumophila* failed to replicate; by the third day, the yield of CFU had decreased ~ 50 fold (Fig. 3.6). In striking contrast, *L. pneumophila* that lack flagellin replicated in Naip5⁺ C57Bl/6 mice: Their yield gradually increased for two days, then rapidly declined, a pattern similar to that of both WT and *flaA* mutant *L. pneumophila* within the lungs of *naip5* mutant mice (Fig. 3.6). Thus, detection of flagellin is critical to the robust murine innate immune response that controls *L. pneumophila* infection.

DISCUSSION

Cytosolic flagellin not only induced a rapid caspase 1-dependent pro-inflammatory macrophage death, but also made *L. pneumophila* vulnerable to the innate immune system of mice that encode the NOD-LRR protein Naip5. Recent molecular

genetic analysis of the signal transduction pathway that mediates the mouse response to *L. pneumophila* identified as critical components not only Naip5 but also the NOD-LRR proteins Ipaf and ASC (Zamboni *et al.*, 2006). Accordingly, we propose a model in which the macrophage response to Flagellin⁺ *L. pneumophila* is governed by Naip5 regulation of the inflammasome, a protein complex that contains the pro-inflammatory enzyme caspase 1 (Martinon *et al.*, 2002). During phagocytosis, the *L. pneumophila* type IV secretion system inserts pores into the macrophage membrane to deliver virulence effectors that perturb phagosome maturation (Sexton and Vogel, 2002). Flagellin protein that diffuses through these pores is detected by Naip5, either directly via its LRR region or indirectly by hetero-oligomerization with another NOD-LRR protein that binds flagellin (Damiano *et al.*, 2004). Consequently, Naip5 activates the inflammasome, either directly or by interacting with the caspases 1-adaptor proteins Ipaf and ASC (Zamboni *et al.*, 2006) and perhaps other NOD-LRRs. The activated inflammasome then coordinates secretion of mature pro-inflammatory cytokines and degradation of intracellular microbes to combat the infection (Figs. 3.4-6).

The inflammasome is a versatile sensor of infection whose specificity is conferred by adaptor proteins. Using mutant mice that lack particular components of the inflammasome, Mariathasan and colleagues demonstrated that detection of *S. typhimurium* requires the adaptor Ipaf; the response to *Listeria monocytogenes*, *Staphylococcus aureus*, or LPS, in the presence of pore-forming agents, requires cryopyrin, whereas the mouse response to cytosolic *Francisella tularensis* occurs independently of both (Mariathasan *et al.*, 2004; Mariathasan *et al.*, 2005; Mariathasan *et al.*, 2006). When macrophages are infected with *L. pneumophila*, Ipaf and Naip5 are

required to restrict bacterial growth, whereas Naip5, Ipaf and ASC coordinate maximal secretion of IL-1 β (Zamboni *et al.*, 2006). In a transfected 293T cell model, the inflammasome can be activated by the muramyl dipeptide of peptidoglycan via cyropyrin (Nalp3), or Nalp 1 or 2 (Martinon *et al.*, 2004). When incubated with bacterial RNA, macrophages release IL-1 β by some mechanism that utilizes cryopyrin and caspase 1 (Kanneganti *et al.*, 2006a). Here, we implicate Naip5 as an adaptor that senses cytosolic flagellin of *L. pneumophila*. Once activated, the inflammasome equips macrophages to combat intracellular pathogens by one or more methods: releasing inflammatory cytokines, degrading intracellular bacteria, and committing suicide (Kanneganti *et al.*, 2006b; Mariathasan *et al.*, 2004; Mariathasan *et al.*, 2005; Mariathasan *et al.*, 2006; Zamboni *et al.*, 2006).

In our model's simplest form, flagellin itself is detected by the macrophage cytosolic surveillance system. Formally, flagellin could instead mediate the release or the translocation of another PAMP, such as peptidoglycan or LPS, which then activates the inflammasome (Damiano *et al.*, 2004; Mariathasan *et al.*, 2004). For example, recent data indicate that *Shigella flexneri* delivers the LPS component lipid A to the cytosol, which induces a lytic death with features of pyroptosis, including nuclear condensation (Suzuki *et al.*, 2005). However, we favor the model that flagellin protein is detected by the cytosolic surveillance system, for several reasons. We have ruled out indirect contributions of flagellin to macrophages adherence (Molofsky *et al.*, 2005) and type IV secretion. Macrophages are not intoxicated when exposed to native, crude preparations of flagellin in the presence of the pore-forming LLO toxin, but do respond to flagellin preparations that have been heated to disassembly filaments and affinity-purified (Fig.

3.3). Most strikingly, loss of *flaA* was sufficient to permit type IV secretion-competent *L. pneumophila* to replicate freely in restrictive mouse macrophages (Fig. 3.5) and also within lungs (Fig. 3.6). Whatever the exact mechanism, Naip5⁺ macrophages can efficiently restrict replication of any microbe that harbors even minute quantities of flagellin (Molofsky *et al.*, 2005).

How flagellin of type IV secretion-competent *L. pneumophila* is exposed to cytosolic NOD-LRR proteins remains to be determined. Since both the flagellar and type IV secretion systems are positioned at the bacterial pole (Bardill *et al.*, 2005) and become active exclusively in the PE phase of growth (Molofsky *et al.*, 2005), sufficient flagellin may diffuse through the secretion channel into the cytoplasm to trigger the host response. Although flagellin does encode the two C-terminal leucine residue motif common to known substrates of type IV secretion (Nagai *et al.*, 2005), *L. pneumophila* does not translocate flagellin as efficiently as *bona fide* effectors (Bardill *et al.*, 2005; Nagai *et al.*, 2005), as judged by quantifying cAMP in macrophages infected with *L. pneumophila* expressing a CyaA-FlaA or a CyaA-RalF fusion protein (unpublished results). The type IV secretion system of *Helicobacter pylori* provides a conduit to the cytoplasm for peptidoglycan (Viala *et al.*, 2004). Therefore, we favor the model that the *L. pneumophila* type IV secretion system inadvertently contaminates the macrophage cytosol with trace amounts of flagellin.

By analogy to TLR proteins, NOD-LRR proteins likely detect a variety of cytosolic PAMPs. Mice carry numerous tandem copies of closely related *naip* sequences and putative pseudogenes (Fortier *et al.*, 2005); presumably these loci have diverged to detect distinct microbial components or fulfill distinct roles. Degradation products of

bacterial peptidoglycan are detected by specific NOD-LRR family members (Inohara, 2004), and our data indicate that flagellin is detected by a Naip5-dependent pathway. Compared to C57Bl/6 cells, A/J macrophages contain reduced amounts of Naip5 protein (Wright *et al.*, 2003), and they are less responsive to flagellin-mediated pyroptosis (Figs. 3.1, 2), release less IL-1 β (Fig. 3.4 F), and fail to restrict *L. pneumophila* replication in macrophages either in culture (Fig. 3.5) or in lungs (Fig. 3.6). Whether a component of the A/J mouse Naip5 pathway binds the flagellin-dependent PAMP less avidly or is mis-regulated requires further study. Although detection of flagellin is one critical component of the murine innate immune response to *L. pneumophila* infection, it is clear that other mechanisms also contribute: The burden of flagellin mutants does begin to decline after three days (Fig. 3.6 A). Unlike mice, humans encode a single Naip protein, so they may or may not use a similar mechanism to combat *L. pneumophila* infection.

Together with the TLR5 pathway, the Naip5 cytosolic surveillance system likely exerts selective pressure for intracellular pathogens equipped for flagellar motility to acquire sophisticated mechanisms to evade detection (Ramos *et al.*, 2004). *S. enterica* and *L. monocytogenes* repress flagellar expression in mammalian hosts (Bergman *et al.*, 2005), and not all species of flagellate microbes encode the epitope that is recognized by TLR5 (Andersen-Nissen *et al.*, 2005). Since *L. pneumophila* co-evolved with freshwater amoebae, perhaps this opportunistic human pathogen has not been subjected to selective pressures exerted by mammalian immune systems (Molofsky and Swanson, 2004).

In addition to pyroptosis (Fig. 3.4), Naip5⁺ mouse macrophages also restrict infection by digesting intracellular *L. pneumophila* (Derre and Isberg, 2004b). During infections of *S. enterica*, an altered level of the NOD-LRR protein Ipaf affects whether

host cells commit pyroptosis or restrict bacterial replication (Damiano *et al.*, 2004).

Autophagy is a degradative pathway of macrophages whose activity correlates with *naip5* status (Amer and Swanson, 2005). Many microbes interact with the autophagy machinery (Kirkegaard *et al.*, 2004), there is regulatory cross-talk between autophagy and programmed cell death (Debnath *et al.*, 2005). Accordingly, we have postulated that the degree of the microbial contamination determines whether macrophages initiate autophagy as a cyto-protective measure or pyroptosis as a failsafe response to infection (Molofsky *et al.*, 2005). By applying bacterial and mouse genetics, *L. pneumophila* infection of macrophages and lungs can be exploited to reveal how this cytosolic surveillance system detects and restricts infection by intracellular pathogens.

Figure 3.1. *L. pneumophila* flagellin contributed to macrophage death, but not pore formation.

A, B) After centrifugation with 2-fold dilutions of the strains indicated, A/J mouse macrophages and microbes were incubated for 1 h, then viability was determined by Alamar Blue reduction. Shown are mean % viable macrophages \pm SE pooled from 3 or more experiments in MOI bins of 2-fold dilutions; the middle value for each bin is indicated. To facilitate comparisons between strains, the WT and *dotA* values are displayed in both A and B. **C)** To quantify red blood cell lysis after incubation for 1 h with the microbes at each MOI indicated, soluble hemoglobin was measured spectrophotometrically. E, exponential phase (non-motile); PE, post-exponential phase (motile); acid, bacteria washed with acid to remove flagella; pGene, complementation plasmids carried by strains and described previously (Molofsky *et al.*, 2005).

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are needed to see this picture.

Figure 3.2. Flagellin⁺ *L. pneumophila* induced death by a mechanism independent of MyD88 but sensitive to Naip5.

A) To test if TLR signaling induces death, viability of C57Bl/6 (solid lines, B6) or C57Bl/6 *myD88*^{-/-} macrophages (dashed lines) was determined after infection for 1 h as shown and described in Figure 3.1. **B)** To test if TLR signaling is required to restrict *L. pneumophila* growth, A/J, C57Bl/6 (B16) or C57Bl/6 *myD88*^{-/-} macrophages (*myD88*) were infected for the periods shown, and bacterial yield was determined by enumerating colony forming units (CFU). **C, D)** To test if Naip5 contributes to the host response, mean % (± SE) LDH released from *naip5* A/J (**B**) or Naip5⁺ C57Bl/6 (**C**) macrophages was quantified 1 h after the infections indicated in 3 or more experiments performed in triplicate, pooling results into MOI bins of two-fold dilutions. E, exponential phase (non-motile); PE, post-exponential phase (motile); acid, bacteria washed with acid to remove flagella.

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Figure 3.3 When present with a pore-forming activity, flagellin triggered macrophage death.

A) To test if pore-formation or substrates of the type IV secretion system are required for flagellin to stimulate death, LDH released by C57Bl/6 cells incubated for 1 h with WT *Listeria monocytogenes* (*Lm*, constant MOI 25), or *dotA* or *flaA* mutant *L. pneumophila* either alone or mixed (+) was quantified. To test if cytosolic flagellin is toxic to macrophages, C57Bl/6 macrophages were incubated with or without the pore-forming toxin LLO (1ug/ml) for 2 h after an initial centrifugation with heat-treated or native crude flagellar preps (~300 ng flagellin) (**B**) or heat-treated flagellin (~3 ng) that had been affinity-purified and affixed to beads via a flagellin-specific monoclonal antibody (**C**). **D)** To test if macrophages responded to cytosolic flagellin from other microbes, C57Bl/6 macrophages were incubated for 2 h without (black bars) or with LLO (1 ug/ml; gray bars) and 1.25 µg of either heated crude flagellin from *L. pneumophila* (*Lp*) or commercial preparations of *S. typhimurium* (*St*) or *B. subtilis* (*Bs*) flagellin. Shown are the mean (± SD) % total LDH released from permeable macrophages calculated from one experiment that is representative of at least two performed.

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Figure 3.4. Pyroptosis was induced by pore-forming Flagellin⁺ *L. pneumophila*.

A) After infection at MOI < 1.0 for 1 h with WT *L. pneumophila* expressing GFP, A/J macrophage permeability was analyzed by phase (left) and fluorescence microscopy (right). Arrows indicate single *L. pneumophila*; arrowheads indicate infected cells that have permeable membranes and phase dark condensed nuclei. **(B)** After infection with MOI 50-100 for 1 h as shown, mean % ± SD of A/J macrophages that were permeable (dark bars) or contained phase dark condensed nuclei (grey bars) was calculated from 3 or more independent experiments. **(C)** After infection at MOI of <1.0 for 2 h as shown, mean % ± SD of A/J macrophages containing one bacterium that had phase dark nuclei was determined. **(D)** After infecting C57Bl/6 macrophages for 1 h at MOI of 30-60 as shown, mean % ± SD of LDH release was calculated from 2-3 experiments. **E, F)** After infecting the macrophages shown for 1 h as indicated, secreted interleukin-1β was quantified. Results from one experiment representative of 2-3 others are shown. Where indicated, macrophages were treated for 1 h before and during the infection with 100 μM of inhibitors of caspase 1 (Ac-YVAD-cmk), pan-caspases (Z-VAD-fmk), or caspase 3 (Ac-DEVD-cho). Student's t-test (p<0.05*) indicates significant differences ± caspase inhibitors. ND, not determined.

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Figure 3.5. Naip5+ C57Bl/6 macrophages restricted growth of *L. pneumophila* that encode flagellin, in part by degrading the intracellular progeny.

Growth of the *L. pneumophila* strain shown in macrophages of (A) permissive A/J mice or (B) restrictive C57Bl/6 mice was quantified in 3 or more experiments; representative data are shown. C) The macrophages indicated were infected for 2 h with an MOI <1 of WT or *flaA* mutant *L. pneumophila*, then at 24 or 48 h the integrity of *L. pneumophila* and macrophages was analyzed by immunofluorescence (left) and phase contrast (right) microscopy, respectively.

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are needed to see this picture.

Figure 3.6. Naip5+ C57Bl/6 mice restricted growth of *L. pneumophila* that encode flagellin.

C57BL/6 and A/J mice were infected via the trachea with *L. pneumophila* Lp01 or its *flaA*-deficient mutant, and the lung bacterial burden was quantified 1, 2 and 3 days later. Shown are mean CFU +/- SD, each calculated from 5 animals.

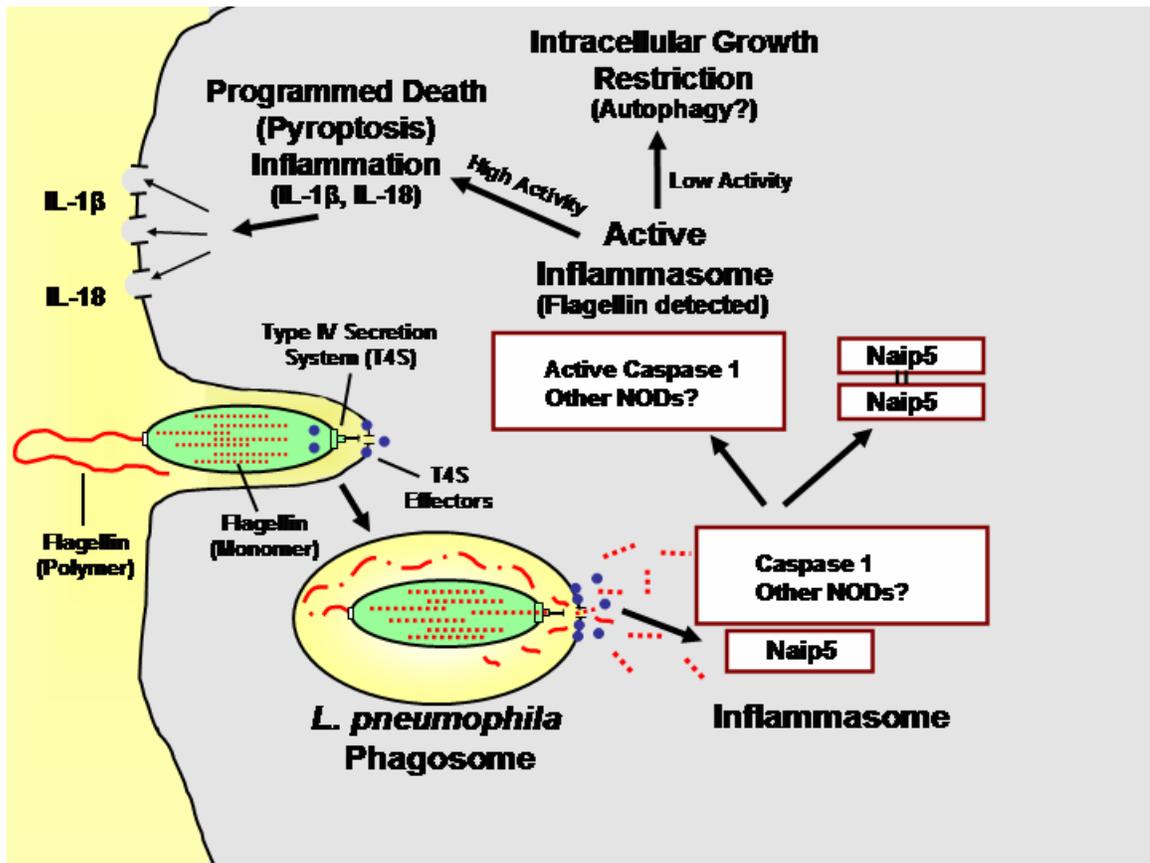


Figure 3.7. Model for induction of a caspase 1- and Naip5-dependent murine macrophage innate immune response to cytosolic *L. pneumophila* flagellin.

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are needed to see this picture.

Figure 3.8. *L. pneumophila* induced a macrophage death with features distinct from classical apoptosis.

A/J macrophages were incubated for 1 or 5 h with the *L. pneumophila* strain indicated or the apoptosis inducer staurosporine, then caspase 3 activation was analyzed by immunofluorescence microscopy (**A**), and the fraction of positive cells enumerated (**B**). In parallel, the % of cells with phase dark, condensed nuclei was quantified (**C**). Shown are results from one experiment that is representative of >2 others.

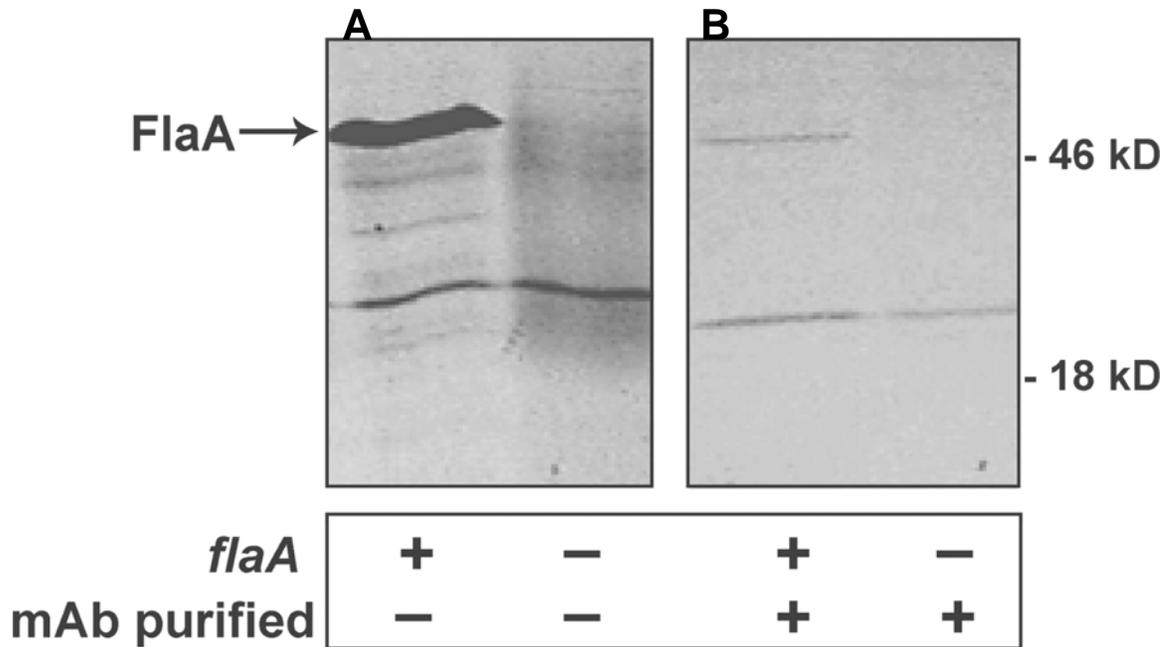


Figure 3.9. Analysis of *L. pneumophila* flagellin preparations.

A 20 μ l sample of each crude (A) or affinity-purified (B) flagellin preparation that had been prepared in parallel from cultures of PE phase *L. pneumophila* that either encode or lack *flaA*, the structural gene for flagellin, was separated by SDS-PAGE on a 12% acrylamide gel and stained with Coomassie Brilliant Blue. The positions of molecular weight standards are indicated. The crude flagellin preparation lane contains 5 μ g and the affinity-purified sample contains 0.1 μ g of flagellin protein, as calculated using densitometry and a standard curve.

Table 3.1 Bacterial strains

Strain	Relevant genotype/phenotype	Reference
<i>E. coli</i>		
DH5 α	F-endA1 <i>hsdR17</i> (r- m+) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacZYA-argF</i>) _{U169} Φ 80dLacZ Δ M15 λ pirRK6	Laboratory collection
<i>L. monocytogenes</i>		
10403S	wild type	66
DP-L2161	<i>listeriolysin O</i> mutant	67
<i>L. pneumophila</i>		
MB110	Lp02 wild type, <i>thyA hsdR rpsL</i>	
MB413	Lp02 <i>letA 22-3::kan</i> mutant (lpg2646)	(Hammer <i>et al.</i> , 2002a)
MB416	Lp02 <i>letS 36::kan</i> mutant (lpg1912)	(Hammer <i>et al.</i> , 2002a)
MB410	Lp02 <i>fliA 35::kan</i> mutant (lpg1782)	(Hammer <i>et al.</i> , 2002a)
MB460	Lp02 <i>dotA::gent</i> mutant (lpg2646)	(Molofsky and Swanson, 2003)
MB473	Lp02 pMMBGent Δ mob, vector control	(Hammer, 1999)
MB552	Lp02 <i>fliD::kan</i> mutant (lpg1338)	(Molofsky <i>et al.</i> , 2005)
MB553	Lp02 <i>fliD::gent</i> mutant	(Molofsky <i>et al.</i> , 2005)
MB554	Lp02 <i>flhB::gent</i> mutant A (lpg1786)	(Molofsky <i>et al.</i> , 2005)
MB556	Lp02 <i>flhB::kan</i> mutant	(Molofsky <i>et al.</i> , 2005)
MB534	Lp02 <i>flaA::kan</i> mutant (lpg1340)	(Molofsky <i>et al.</i> , 2005)
MB557	Lp02 <i>flaA::kan</i> mutant pMMBGent- <i>flaA</i>	(Molofsky <i>et al.</i> , 2005)
MB558	Lp02 <i>flaA::kan</i> mutant pMMBGent Δ mob vector	(Molofsky <i>et al.</i> , 2005)
MB532	Lp02 <i>flaA::gent</i> mutant	(Molofsky <i>et al.</i> , 2005)
MB559	Lp02 pMMBGent- <i>flaA</i>	(Molofsky <i>et al.</i> , 2005)
MB560	Lp02 <i>motAB::gent</i> mutant A (lpg1780-81)	(Molofsky <i>et al.</i> , 2005)
MB562	Lp02 <i>motAB::gent</i> mutant A pMMB206- <i>motAB</i>	(Molofsky <i>et al.</i> , 2005)
MB563	Lp02 <i>motAB::gent</i> mutant A pMMB206, vector control	(Molofsky <i>et al.</i> , 2005)
MB564	Lp02 <i>fliI::cam</i> mutant (lpg1757)	(Merriam <i>et al.</i> , 1997)
MB567	Lp02 <i>flaA::gent fliD::kan</i> double mutant A	(Molofsky <i>et al.</i> , 2005)
MB569	Lp02 <i>dotA::gent fliD::kan</i> double mutant A	(Molofsky <i>et al.</i> , 2005)
MB593	Lp02 <i>dotA::gent flaA::kan</i> double mutant A	This work
MB571	Lp02 <i>motAB::gent fliD::kan</i> double mutant A	(Molofsky <i>et al.</i> , 2005)
MB314	Lp01 wild type prototroph, <i>hsdR rpsL</i>	(Berger and Isberg, 1993)
MB589	Lp01 <i>flaA::kan</i> mutant (lpg1340)	This work

CHAPTER FOUR

Mouse macrophages are permissive to motile *Legionella* species that fail to trigger pyroptosis

SUMMARY

The motile intracellular pathogen *Legionella pneumophila* is restricted from replicating in macrophages obtained from C57Bl/6 mice. Resistance to *L. pneumophila* depends on macrophage recognition of the major flagellar protein, flagellin. Murine macrophages detect flagellin that contaminates the cytoplasm, triggering a pro-inflammatory cell death that results in secretion of IL-1 β . In contrast, when C57Bl/6 macrophages are infected with motile *L. parisiensis* and *L. tucsonensis*, the bacteria replicate, a pattern similar to a flagellin deficient mutant of *L. pneumophila*. To understand how these species escape innate defense mechanisms that restrict *L. pneumophila*, we compared their impact on macrophages. Despite conservation of motility, *L. parisiensis* and *L. tucsonensis* do not induce pro-inflammatory cell death, as measured by LDH release and IL-1 β secretion. In addition, neither species displays characteristics typical of a canonical type IV secretion system, which can perforate phagosomal membranes. However, when transfected into the cytosol of macrophages, flagellin isolated from broth-grown *L. parisiensis* and *L. tucsonensis* does trigger cell death and IL-1 β secretion. Therefore, when *L. parisiensis* and *L. tucsonensis* invade a

macrophage, flagellin must be confined to the phagosome, protecting the bacteria from recognition by the innate immune response and allowing the bacteria to replicate within macrophages.

INTRODUCTION

Legionella is a gram-negative bacterium that opportunistically infects the alveolar macrophages of the mammalian lung. Although 48 species of *Legionella* that comprise 70 serogroups have been identified, only a subset has been found to cause disease (Benson and Fields, 1998). The most prevalent species to cause disease is *Legionella pneumophila*, which accounts for the majority of cases around the world (Benin *et al.*, 2002). Of the non-*pneumophila* species implicated in disease, infections primarily occur in immunocompromised hosts. In particular, the non-*pneumophila* species *L. birminghamsensis*, *L. parisiensis* and *L. tucsonensis* have been isolated from transplant patients (Lo Presti *et al.*, 1997; Thacker *et al.*, 1989; Wilkinson *et al.*, 1987). Whether these species use similar strategies as *L. pneumophila* to establish a replication niche and to evade the innate immune system is not known since these rare species have not been extensively studied and little more than whether they can replicate in various cell types in culture has been analyzed.

Macrophages are key phagocytic defenders of the innate immune system that scout various tissues for the presence of foreign materials, including pathogens. This recognition is accomplished by several types of receptors that are found on the surface of the cell and within the intracellular milieu, called pattern recognition receptors (PRRs) (Kumagai *et al.*, 2008). The toll-like receptors (TLRs), present on the surface of many

cell types, recognize various microbe-associated molecular patterns (MAMPs) such as lipopolysaccharide, peptidoglycan, lipoproteins, microbial nucleic acids and flagellin. Much like the TLRs in function, the nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs) monitor the cytoplasm (Akira and Takeda, 2004; Mitchell *et al.*, 2007). Detection of microbial products by these receptors initiates a signaling cascade that culminates with the secretion of pro-inflammatory mediators that recruit other lymphocytes to respond to an infection (Delbridge and O'Riordan, 2007; Drenth and van der Meer, 2006; Martinon *et al.*, 2002; Petrilli *et al.*, 2005).

NLRs participate as components of a protein complex known as the inflammasome. Several different inflammasomes have been characterized based on the MAMPs that initiate their formation and activation. The NLR proteins Nalp1 and Nalp3 bind muramyl dipeptide of peptidoglycan, which also binds to the more extensively studied NOD proteins (Akira and Takeda, 2004; Mitchell *et al.*, 2007). Another NLR, neuronal apoptosis inhibitory protein 5 (Naip5, Birc1e), which restricts replication of *Legionella pneumophila* in resistant mouse macrophages, is composed of three domains: (1) an amino-terminal baculoviral inhibitor of apoptosis repeats, (2) a central NOD domain, and (3) carboxy-terminal leucine rich repeats (LRRs) (Diez *et al.*, 2003; Fortier *et al.*, 2005; Inohara *et al.*, 2005; Wright *et al.*, 2003). Studies of other NLR proteins indicate that the LRR region is critical for recognition of microbial products, which then triggers oligomerization of the NLR with other inflammasome components through the NOD domain. Subsequently, activation of downstream signaling is carried out by the amino-terminal domains, such as the caspase recruitment domains (CARDs) (Inohara *et al.*, 2005). Unlike the NODs, the Nalps and Naip5 control posttranslational

processing and secretion of the pro-inflammatory cytokines IL-1 β and IL-18 (Martinon and Tschopp, 2005). Once formed, the inflammasome recruits and activates caspase-1, which in turn processes pro-IL-1 β and pro-IL-18 into their mature form before their release into the extracellular space. Recruitment and activation of caspase-1 is mediated directly or through adapter proteins, like ASC and Ipaf (Agostini *et al.*, 2004; Yu *et al.*, 2006). This pro-inflammatory reaction is a key element of *pyroptosis*, the “fiery” cell death seen in response to infection by the pathogens *Salmonella enterica*, *Shigella flexneri*, and *L. pneumophila* (Fink and Cookson, 2006, 2007; Fink *et al.*, 2008)

L. pneumophila has become a model intracellular organism to study both bacterial replication and host detection of pathogens (Fortier *et al.*, 2005). The pathogen requires a type IV secretion system to establish a replication vacuole, a key determinant of infection. However, mice can restrict *L. pneumophila* infection when the NLR protein Naip5 detects flagellin. For example, C57BL/6 mice do not support *L. pneumophila* replication beyond the first 24 hours (Derre and Isberg, 2004b; Izu *et al.*, 1999; Yamamoto *et al.*, 1991; Yoshida *et al.*, 1991). In contrast, *L. pneumophila* replicate profusely in macrophages derived from the A/J mouse strain (Yamamoto *et al.*, 1988), a widely used mammalian host model. The A/J Naip5 allele is hypofunctional, based on experiments using transgenic complementation, RNA knockdown, and Naip5^{-/-} mutant mice, which each indicate that A/J mice produce significantly less of the protein than resistant mice (Diez *et al.*, 2003; Lightfield *et al.*, 2008; Wright *et al.*, 2003). Naip5^{-/-} mice fail to activate the inflammasome during a *L. pneumophila* infection (Lightfield *et al.*, 2008; Molofsky *et al.*, 2006; Ren *et al.*, 2006). Furthermore, mutants of *Legionella* deficient in flagellin, the major protein subunit of the flagellum, subvert the Naip5-

mediated defenses and replicate in restrictive C57BL/6 macrophages (Molofsky *et al.*, 2006; Ren *et al.*, 2006). Flagellin (*flaA*) deficient mutants are also not cytotoxic to macrophages, a trait that also requires type IV secretion.

The *Legionella* factors contributing to its detection by the inflammasome pathway of macrophages is the focus of this chapter. Whether components of the inflammasome directly bind flagellin is not yet known, but this study will shed light on the importance of bacterial factors that affect macrophage processes. In this chapter, I show that highly motile non-*pneumophila* species of *Legionella* can evade replication restriction in C57BL/6 murine macrophages despite their ability to detect the divergent bacterial flagellin when it contaminates the cytosol. Macrophage restriction of replication correlates with a canonical feature of the *L. pneumophila* type IV secretion system, specifically phagosome perforation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture

Legionella pneumophila (Lp02, thyA hsdR rpsL) derived from Philadelphia 1, the *dotA* mutant (Lp03) and the *flaA* mutant have been previously described (Berger and Isberg, 1994; Molofsky *et al.*, 2005). *Legionella parisiensis* and *Legionella tucsonensis* were gifts from Dr. Cary Engleberg (University of Michigan). Strains, maintained at -80° C in glycerol stocks, were colony-purified onto *N*-(2-acetomido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered charcoal-yeast extract agar (CYE) supplemented with 100 ug/ml of thymidine (CYET). Bacterial strains were cultured in

ACES-buffered yeast extract broth (AYE) supplemented with thymidine (100 ug/ml; AYET) at 37° C with aeration. *Legionella* were subcultured in AYET from an overnight primary culture and grown to the exponential and post-exponential phases for experimentation. Exponential phase cultures (E-replicative) were defined as OD₆₀₀ 0.5-2.0, while post-exponential phase (PE-transmissive) cultures were defined as OD₆₀₀ 3.0-4.0 with high motility. Cultures were defined as motile when >75% of bacteria in a field of ≥100 cells showed rapid, directed movement.

Macrophage Culture

Bone-marrow derived macrophages were isolated from the femurs of female A/J and C57BL/6 mice (Jackson Laboratories) as previously described (Swanson and Isberg, 1995). Macrophages were maintained in RPMI supplemented with 10% fetal bovine serum (RPMI-FBS, Gibco) and were plated at the density indicated for each assay.

Intracellular Bacterial Growth

The efficiency of binding, entering, and surviving within macrophages by *L. pneumophila* was assessed as described previously (Byrne and Swanson, 1998). Macrophages were plated in 24-well tissue culture plates at a density of 2.5 X 10⁵ macrophages per well. Cells were allowed to adhere overnight before incubation with bacteria and infected at a 1:1 ratio with transmissive bacteria for 2 h at 37° C. Extracellular bacteria were removed by rinsing the macrophage monolayer three times with 0.5 ml of RPMI-FBS at 37°C, a medium that is not permissive for *Legionella*

replication. Intracellular bacteria were quantified by lysing monolayers with 2% saponin (Sigma) in PBS and plating triplicate aliquots on CYET. Colony forming units (CFU) added at 0 h was determined by diluting the infection inocula with PBS and plating on CYET. PBS did not affect the viability of the *Legionella* isolates (data not shown). The initiation of infection was calculated from triplicate samples by the following equation: (CFU from lysates at 2 h)/(CFU added at 0 h) x 100.

To quantify replication of bacteria in macrophages at 24 h intervals, cells were infected at an MOI of 1 as described for infectivity. At the indicated time intervals, cells were lysed by treating monolayers with 2% saponin (Sigma) in PBS. Lysates were prepared from triplicate samples and plated on CYET for CFU enumeration.

Cytotoxicity

Contact-dependent cytotoxicity was quantified as the percent of macrophages killed during a 1 h incubation with *L. pneumophila*. Macrophages were cultured at a density of 8×10^4 per well in 96-well tissue culture plates. Transmissible bacteria suspended in RPMI-FBS, at varying ratios, were co-incubated with the macrophages for 1 h at 37° C. After bacteria were washed away, the monolayers were subsequently incubated with 10% (vol/vol) Alamar Blue (TREK Diagnostics) in RPMI-FBS for from 4 h to overnight. The redox-specific absorbance resulting from the reduction of Alamar Blue to its reduced form by viable macrophages was measured with a SpectraMax 250 spectrophotometer (Molecular Devices) at OD₅₇₀ and OD₆₀₀. The percent of viable macrophages was calculated in triplicate from the standard curve, the slope of a plot of

A_{570}/A_{600} determined for triplicate samples of six known densities of uninfected macrophages in the range of 10^3 to 8×10^4 macrophages per well. The actual MOI was determined by plating duplicate samples of the infection inocula onto CYET.

Red Blood Cell Hemolysis

Hemolysis of sheep red blood cells by *Legionella* was quantified by a method modified from Kirby and colleagues (Kirby *et al.*, 1998). Defibrinated sheep red blood cells (RBCs) were diluted in PBS and washed three times by centrifugation at $750 \times g$ for 10 min at 4 C, until the supernatant cleared. The RBCs were then counted on a hemocytometer and diluted to a working concentration. The diluted RBCs were then added to bacteria grown to the PE phase diluted 1:2 in a 96-well plate. For a negative control, these wells were filled with media alone. To obtain a standard curve of hemolysis, RBCs were added to a serial dilution of RPMI media plus 0.1% sodium dodecyl sulfate (SDS), and these wells with RBCs but not SDS served as the background control. To pellet RBCs with the *Legionella*, the 96-well plate containing bacteria and RBCs was centrifuged for 10 min at $400 \times g$. The plate was incubated at 37°C for 1 h; subsequently the pellet was resuspended gently and re-pelleted by centrifugation at $400 \times g$. Supernatants were transferred to a fresh 96-well plate without disturbing the pellet, and the optical density (A_{415}) was determined with a spectrophotometer.

NaCl Resistance

Bacterial cultures were grown to the phase indicated and plated in serial dilutions on CYET alone or CYET containing 100 mM NaCl. After incubation at 37°C for 4 days, CFU were counted and expressed as (CFU CYET+NaCl / CFU CYET) X 100.

Ethidium Bromide Permeability

Bacterial were cultured to the PE phase until highly motile. Macrophages plated at 2.5×10^5 per glass coverslip were infected at an MOI of 50 for 1 h. Coverslips were inverted onto a 5 μ l drop of PBS containing 25 mg ml⁻¹ ethidium bromide and 5 mg ml⁻¹ acridine orange placed on the surface of a glass slide. Samples were scored immediately, by fluorescence microscopy (Kirby *et al.*, 1998).

DNA Hybridization

Genomic DNA from each *Legionella* strain was transferred to positively charged nylon membranes using the Bio-Dot SF (BioRad) apparatus. Flagellin-specific probes were labeled with DIG-dUTP (Roche) by PCR amplification and detected by nonradioactive CSPD (Roche) chemiluminescence detection. Hybridization was performed under low stringency conditions at 42°C. Films were analyzed using Image J software (NIH).

Crude Flagellin Preparations

Crude flagellin preparations (CFP) were obtained from WT and *flaA* *L. pneumophila*, *L. parisiensis*, and *L. tucsonensis* essentially as described (Molofsky *et al.*, 2005). Broth cultures were centrifuged at 8000 X g for 20 min at 4°C to collect the bacteria. Supernatants were discarded, and the bacterial pellets were resuspended in 50 ml of sterile PBS. To shear flagella from the bacteria, suspensions were vortexed at high speed for 5-10 min and then centrifuged as before to remove bacteria from the suspension. To remove any remaining bacteria, supernatants were collected and filtered through a 0.45 µm filter. Filtered supernatants, containing flagellin, were ultracentrifuged at 100,000 X g for 3 h at 4° C and supernatants were discarded. The pellet was resuspended in 1 ml sterile PBS and analyzed by SDS-PAGE and Coomassie blue staining. Protein concentration was determined by the Bradford assay (Pierce).

Immunoblot

CFP were boiled for 5 min in Laemmli buffer, resolved by SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked in BLOTTO (TBST containing 5% non-fat milk) and incubated at 4°C overnight with MAb 2A5 anti-*Legionella* flagellin diluted in BLOTTO. Membranes were then washed 5 times in TBST and incubated in secondary goat anti-mouse conjugated to horseradish peroxidase diluted

in BLOTTO for 1 h at RT with shaking. Membranes were washed as before and developed using the ECL system (Pierce, West Pico).

Toxicity of Cytosolic Flagellin

A/J and C57BL/6 macrophages were plated in 96-well plates at the densities 5×10^4 and 8×10^4 , respectively. Toxicity of cytosolic flagellin was analyzed by incubating the protein transfection reagent Profect P1 (Targeting Systems) with either native, heat-treated or proteinase K-treated CFP at the concentration indicated. Suspensions were centrifuged onto macrophages and incubated at 37°C for 2 h. After incubation, the supernatants were assayed for the cytosolic enzyme LDH using the CytoTox96 NonRadioactive Cytotoxicity Assay (Promega; reference (Kirby *et al.*, 1998)). Profect P1 without flagellin preparations or with proteinase K served as a background controls.

IL-1 β Secretion

Macrophages were seeded in 24-well plates at a density of 1×10^6 and were left either untreated or pretreated with 50 ng ml^{-1} of LPS overnight. Prior to transfection, cells were washed with serum-free media, and then infected with bacteria at an MOI of 5 or transfected with Profect-P1 and CFP complexes. Contact of the bacteria was promoted by centrifuging the plates at $250 \times g$ for 5 min. After a 2-hour incubation, the concentration of IL-1 β in the supernatants was determined by ELISA (eBioscience).

RESULTS

The flagellated species L. parisiensis and L. tucsonensis replicate in C57Bl/6 macrophages

The innate immune system of C57Bl/6 mouse macrophages restricts *L. pneumophila* by detecting cytosolic flagellin (Lightfield *et al.*, 2008; Molofsky *et al.*, 2006; Ren *et al.*, 2006). Like *L. pneumophila*, when cultured to the stationary phase *L. parisiensis* and *L. tucsonensis* display rapid, directed movement that is apparent when analyzed by light microscopy. To investigate whether other flagellated species of *Legionella* are restricted by mouse macrophages, we compared the intracellular growth of *L. parisiensis* and *L. tucsonensis* to *L. pneumophila*. Macrophages were infected with stationary bacteria, and then replication was assessed at 24 h intervals by quantifying CFU of *L. pneumophila*, *L. pneumophila flaA*, *L. parisiensis*, and *L. tucsonensis*. As expected, the number of *L. pneumophila* increased 10-fold for the first 24 h, but subsequently the infection was suppressed (Fig. 5.1). In contrast, the yield of *L. parisiensis* increased 100-fold over a 2-day period, consistent with previous reports (Alli *et al.*, 2003) and similar to non-flagellated *L. pneumophila flaA* mutants. In addition, *L. tucsonensis* effectively infected C57Bl/6 macrophages, replicating to CFU yields similar to *L. parisiensis*. Therefore, evasion of the innate defenses of C57Bl/6 macrophages is not unique to non-flagellated bacteria, since two motile *Legionella* species established robust infections.

L. parisiensis and *L. tucsonensis* do not trigger pyroptosis.

L. pneumophila elicits pyroptosis by a mechanism that requires flagellin since *flaA* mutants are not cytotoxic and do not induce IL-1 β secretion from macrophages (Molofsky *et al.*, 2006; Ren *et al.*, 2006). To assess whether flagellated *L. parisiensis* and *L. tucsonensis* fail to trigger an innate immune response, we analyzed their cytotoxicity to macrophages and secretion of the pro-inflammatory cytokine IL-1 β . When infected for 1 h at high MOI, PE phase *L. pneumophila* was cytotoxic; less than 35% of macrophages were viable (Fig. 5.2 A). In contrast, nearly 100% of macrophages cultured with *L. parisiensis* and *L. tucsonensis* were viable, a pattern similar to macrophages incubated with flagellin-deficient *flaA* mutants of *L. pneumophila*. Furthermore, after a 2 h incubation, *L. parisiensis* and *L. tucsonensis* triggered secretion of only negligible amounts of IL-1 β from macrophages, whereas *L. pneumophila* induced release of 1500 pg/ml, (Fig. 5.2 B). Increasing the incubation period or the MOI had no effect on the amount of IL-1 β secreted (data not shown). Therefore, *L. parisiensis* and *L. tucsonensis* fail to induce pyroptosis, despite their motility.

Flagellins from L. parisiensis and L. tucsonensis are divergent

To verify that *L. parisiensis* and *L. tucsonensis* encode flagellin, we used conventional methods to isolate flagellin from *L. pneumophila* and then analyzed the

crude preparations (CFP) by SDS-PAGE and western blot analysis. *L. parisiensis* and *L. tucsonensis* produce protein consistent with the molecular size of *L. pneumophila* flagellin (Fig. 5.3 A). However, CFP from *L. parisiensis* and *L. tucsonensis* did not react with anti-*L. pneumophila* flagellin antibody (Fig. 5.3 B), indicating divergence at the protein level. A recent DNAarray study of several *Legionella* species also reported divergence of *flaA* in *L. parisiensis* and *L. tucsonensis*, since probes representing the *flaA* locus of three *L. pneumophila* strains did not hybridize to *L. parisiensis* or *L. tucsonensis* genomic DNA (Cazalet *et al.*, 2008). Furthermore, several attempts using conventional and degenerate PCR to amplify the complete flagellin gene from these species were unsuccessful. To estimate the extent of this divergence, we generated labeled probes for each of three regions of *L. pneumophila flaA* and analyzed their homology to genomic DNA by dot-blot hybridization. *L. parisiensis* DNA hybridized weakly at the N-terminus and the core region, whereas *L. tucsonensis* was most divergent in the core region (Fig. 3 C). Significant homology was observed for *L. parisiensis* and *L. tucsonensis* in the C-terminus, where >90% hybridization was evident in the region that also harbors the *L. pneumophila* flagellin carboxy-terminal “death” domain (Lightfield *et al.*, 2008). Therefore, it was formally possible that the divergence observed for the flagellin genes and proteins of *L. parisiensis* and *L. tucsonensis* is sufficient to circumvent detection by the innate immune machinery that induces pyroptosis and restricts replication.

Cytosolic L. parisiensis or L. tucsonensis flagellin induces pro-inflammatory cell death

Pyroptosis in murine bone marrow-derived macrophages is induced when flagellin that has leaked to the cytosol is detected by Naip5 and Ipaf (Lightfield *et al.*, 2008; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Vinzing *et al.*, 2008b). To determine if the divergent *L. parisiensis* and *L. tucsonensis* flagellins elude detection, we introduced crude flagellin from broth grown cultures into the cytosol and then tested if it could trigger pyroptosis. C57Bl/6 mouse macrophages were incubated for 2 h with the protein transfection reagent Profect P1 complexed with CFP obtained from *L. pneumophila*, *L. pneumophila flaA*, *L. parisiensis*, and *L. tucsonensis*, and then the LDH released by intoxicated cells was quantified. Macrophages whose cytosols were contaminated with CFP from *L. pneumophila*, *L. parisiensis*, and *L. tucsonensis* released >80% of LDH, significantly more than the quantities measured for transfection reagent alone or the *flaA* mock prep (Fig. 5.4 A). The toxicity was due to protein and not some other bacterial product in the CFP, since treatment of the CFP with proteinase K prior to transfection drastically reduced their toxicity (Fig. 5.4 A, gray bars).

A hallmark of pyroptosis is caspase-1 activation, which results in the secretion of IL-1 β from macrophage supernatants. Not only did cytosolic CFP trigger cell death, but they also induced secretion of IL-1 β from macrophages. When transfected with CFP, ~70% more IL-1 β was secreted from macrophages than those CFP pretreated with proteinase K (Fig. 5.4 B, gray bars) and >95% more from macrophages that were treated with *L. pneumophila flaA* mock CFP or the negative control samples (Fig. 5.4 B). Thus, when presented to the macrophage cytosol, flagellin from *L. parisiensis* and *L. tucsonensis* are potent triggers of pyroptosis.

L. parisiensis and *L. tucsonensis* do not perforate macrophage membranes

To activate the inflammasome complex and subsequently induce secretion of IL-1 β , *L. pneumophila* require not only flagellin but also type IV secretion, which is thought to provide a conduit to the cytoplasm (Miao *et al.*, 2006; Molofsky *et al.*, 2006; Ren *et al.*, 2006). Therefore, we next tested whether the cytotoxicity defect of *L. parisiensis* or *L. tucsonensis* is attributed to their type IV secretion systems. Although a mechanism has not been established, *L. pneumophila* that are defective for type IV secretion are resistant to sodium; indeed, several of the *dot/icm* type IV secretion mutants were originally isolated based on their ability to grow on high concentrations of NaCl (Vogel *et al.*, 1996). Accordingly, as one probe of their type IV secretion systems, we tested the ability of *L. parisiensis* and *L. tucsonensis* to form colonies on media containing 100 mM NaCl. *L. pneumophila* is sensitive to NaCl in the PE phase of growth, yet exponential phase bacteria and PE *dotA* mutants are NaCl resistant (Fig. 5.5 A). In contrast to *L. pneumophila*, PE phase *L. parisiensis* and *L. tucsonensis* were resistant: compared to the *dotA* mutant salt-resistant control, these *Legionella* species were 10-fold more tolerant to NaCl (Fig. 5.5 A).

A second hallmark of type IV secretion is the ability to perforate macrophage membranes, an activity evident by staining the cells after a 1 h infection with the fluorescent dyes ethidium bromide (EtdBr) and acridine orange (Kirby *et al.*, 1998). Cells with intact membranes exclude ethidium bromide; as such, cells with red nuclei are scored as EtdBr-permeable and those with green nuclei are scored as alive or EtdBr-impermeable. As shown previously, WT *L. pneumophila* permeabilize 70% of

macrophages, whereas *L. pneumophila dotA* mutants do not (<10% of macrophages nuclei were red; Fig. 5.5 B). Similar to *dotA* mutants, the majority of nuclei from macrophages infected with *L. parisiensis* and *L. tucsonensis* remained green. Thus, *L. parisiensis* and *L. tucsonensis* fail to induce macrophage permeability. Taken together, the phenotypes of *L. parisiensis* and *L. tucsonensis* indicate that their type IV secretion systems differ from the canonical *L. pneumophila* apparatus, which forms pores in macrophage membranes and likely provides a conduit for flagellin to the macrophage cytoplasm.

DISCUSSION

The response to cytosolic flagellin by the innate immune machinery of C57Bl/6 macrophages results in resistance to *L. pneumophila* infection. Yet, other flagellated species of *Legionella* establish a productive replication niche in these macrophages (Fig 4.1; (Alli *et al.*, 2003; Derre and Isberg, 2004b; Yamamoto *et al.*, 1991, 1992). We show here that the flagellated species *L. parisiensis* and *L. tucsonensis* replicate in C57Bl/6 macrophages, at least in part because they fail to stimulate pro-inflammatory cell death. Pyroptosis is likely one rapid response by C57Bl/6 macrophages that leads to the elimination of infected cells and recruitment of leukocytes to the site (Derre and Isberg, 2004b; Fink and Cookson, 2007). Since stationary phase *L. parisiensis* and *L. tucsonensis* fail to perforate macrophage membranes and do not become sodium sensitive, their type IV secretion systems differ from that of *L. pneumophila*, which provides a conduit for toxic flagellin to the macrophage cytosol. We postulate that,

without release of flagellin into the cytoplasm, macrophages remain blind to these intracellular *Legionella* species and fail to trigger pyroptosis to combat the infection.

Flagellin is a potent stimulator of innate immune signaling pathways. By analogy to the TLR5 epitope of flagellin that is conserved across bacterial species, it is likely that the epitope recognized by the NLR cytoplasmic receptors is highly conserved (Andersen-Nissen *et al.*, 2005; Miao *et al.*, 2006; Miao *et al.*, 2007; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Takeda and Akira, 2004; Zamboni *et al.*, 2006). For example, mouse macrophages detect cytosolic flagellin from *Legionella*, *Salmonella*, *Bacillus* and *Pseudomonas* through the NLRs Ipaf and Naip5 (Franchi *et al.*, 2007a; Franchi *et al.*, 2007b; Lightfield *et al.*, 2008; Miao *et al.*, 2006; Miao *et al.*, 2008; Molofsky *et al.*, 2006; Ozoren *et al.*, 2006; Warren *et al.*, 2008). By genomic hybridization, the *flaA* sequences from *L. parisiensis* and *L. tucsonensis* are most similar to the *L. pneumophila* gene in the region that harbors the toxic epitope recognized by Naip5 and required for activation of the inflammasome (Lightfield *et al.*, 2008). Furthermore, *L. parisiensis* and *L. tucsonensis* flagellin can trigger pyroptosis when delivered directly to cytosol (Fig. 5.4). Therefore, divergence of the *L. parisiensis* and *L. tucsonensis* flagellin species that is evident at the DNA and protein level is not sufficient to account for the lack of cytotoxicity of these bacteria (Fig. 5.2). Instead, we favor the model that during a *L. parisiensis* or *L. tucsonensis* infection, flagellin does not escape from the vacuole to contaminate the cytoplasm.

Bacterial secretion systems are one route to the cytosol for bacterial products (Aroian and van der Goot, 2007; Hueck, 1998; Viala *et al.*, 2004). For example, *Salmonella* activates the innate immune system through not only TLRs but also NLRs,

since mutants that lack a type III secretion system do not activate NLR signaling (Mariathasan *et al.*, 2004; Mariathasan *et al.*, 2006). Therefore, the cytosolic surveillance system provides macrophages a mechanism to respond specifically to invading pathogens that express virulence factors that breach the phagosome, specifically toxins and specialized secretion systems (Delbridge and O'Riordan, 2007; Fink *et al.*, 2008). The cytosolic surveillance system detects *L. pneumophila* that have a functioning type IV secretion apparatus (Molofsky *et al.*, 2006), a machinery the bacteria require to establish replication vacuoles (Roy *et al.*, 1998; Segal *et al.*, 1999). Although translocation of flagellin by *L. pneumophila* through its type IV secretion system has not been unequivocally demonstrated, it is hypothesized that, as for *Salmonella*, when virulence effectors are secreted, minute amounts of flagellin are also translocated from the bacterium to the macrophage cytosol (Lightfield *et al.*, 2008; Miao *et al.*, 2007; Sun *et al.*, 2007). Compared to *L. pneumophila*, *L. parisiensis* and *L. tucsonensis* lack phenotypes characteristic of the canonical type IV secretion system.

It remains to be determined whether *L. parisiensis* and *L. tucsonensis* evade detection by the inflammasome due to incompatibility of their divergent flagellin species with the secretion system, a pore size that is not permissive for flagellin to escape, or a defective secretion system. Genomic DNA of *L. parisiensis* and *L. tucsonensis* fails to hybridize with many of the type IV secretion system structural and secreted effector genes of three different *L. pneumophila* strains, indicating that the genes are either missing or highly divergent (Cazalet *et al.*, 2008; C. Buchrieser, personal communication). In some conditions, the canonical type IV secretion system is dispensable. For example, when amoebae are infected with water- or Ers-treated *dot/icm*

mutants, the bacteria replicate intracellularly and avoid phagosomal acidification as efficiently as the wild-type parental strain (Bandyopadhyay *et al.*, 2004; Bandyopadhyay *et al.*, 2007). The canonical type IV secretion system may be functionally redundant with the Lvh type IV secretion system, since the homologous Lvh system was required for entry and intracellular multiplication in *dot/icm* mutants following incubation with water (Bandyopadhyay *et al.*, 2007). Together these studies illustrate that the *Legionellae* can utilize a variety of specialized secretion systems to parasitize macrophages.

In humans, *L. pneumophila* infection causes an acute broncheolitis and severe inflammation where patient exudates are filled with macrophages, polymorphonuclear cells, fibrin, red blood cells, proteinaceous material, and cellular debris indicative of cell death, and cell lysis (Glavin *et al.*, 1979; Winn, 1981). The type IV secretion system can lyse white blood cells, flagellin is pro-inflammatory, and both factors are required for activation of the inflammasome. Therefore, both flagellin and type IV secretion are key inducers of the inflammatory response to *L. pneumophila* (Kirby *et al.*, 1998; Molofsky *et al.*, 2005; Molofsky *et al.*, 2006; Ricci *et al.*, 2005; Rota *et al.*, 2005; Scaturro *et al.*, 2005; Scaturro *et al.*, 2007; Sposato *et al.*, 2003; Vinzing *et al.*, 2008a). It is plausible that *L. parisiensis* and *L. tucsonensis* are less frequent causes of Legionnaires' disease because their flagellin is not translocated to the macrophage cytoplasm. By this model, the inflammation and cellular damage characteristic of the disease is a manifestation of robust activation of the inflammasome and extensive pyroptosis triggered by the *L. pneumophila* flagellin that contaminates the cytosol during type IV secretion system.

Although the innate immune system is critical for early detection of *L. pneumophila*, the overall host defense is a culmination of many factors. Indeed, wild-

type *Legionella* and *flaA* mutants are eventually cleared from A/J *naip5* and C57Bl/6 mice, respectively, indicating that other mechanisms and host immune response pathways contribute to restrict *Legionella* replication (Molofsky *et al.*, 2006). Many studies of infections in animals and humans have shown an important role for several mediators of the immune system, including neutrophils, B cells, T cells, cytokines, and chemokines (Vance and Hawn, 2008). Once *Legionella* is recognized by the innate immune system, macrophages secrete a number of cytokines, chemokines, and other molecules that coordinate the adaptive immune system (Neild and Roy, 2004; Park and Skerrett, 1996). Notably, increased permissiveness to *Legionella* replication in macrophages and human cells has been shown as a result of defective IFN- α/β and IFN γ signaling (Coers *et al.*, 2007; Opitz *et al.*, 2006; Vance and Hawn, 2008).

In conclusion, we propose a model where *L. parisiensis* and *L. tucsonensis* replicate in macrophages by eluding early detection by the innate immune system to combat the infection. As opposed to *L. pneumophila*, without the formation of a canonical pore in the *Legionella*-containing phagosome, the macrophage cytosol does not become contaminated with flagellin and the inflammasome is not activated allowing *Legionella* to establish a its replication niche. In our analysis of non-*pneumophila* *Legionella* species we have independently extended the evidence that translocation by the type IV secretion system is required for early detection of flagellin by the innate immune system and subsequent restriction of replication.

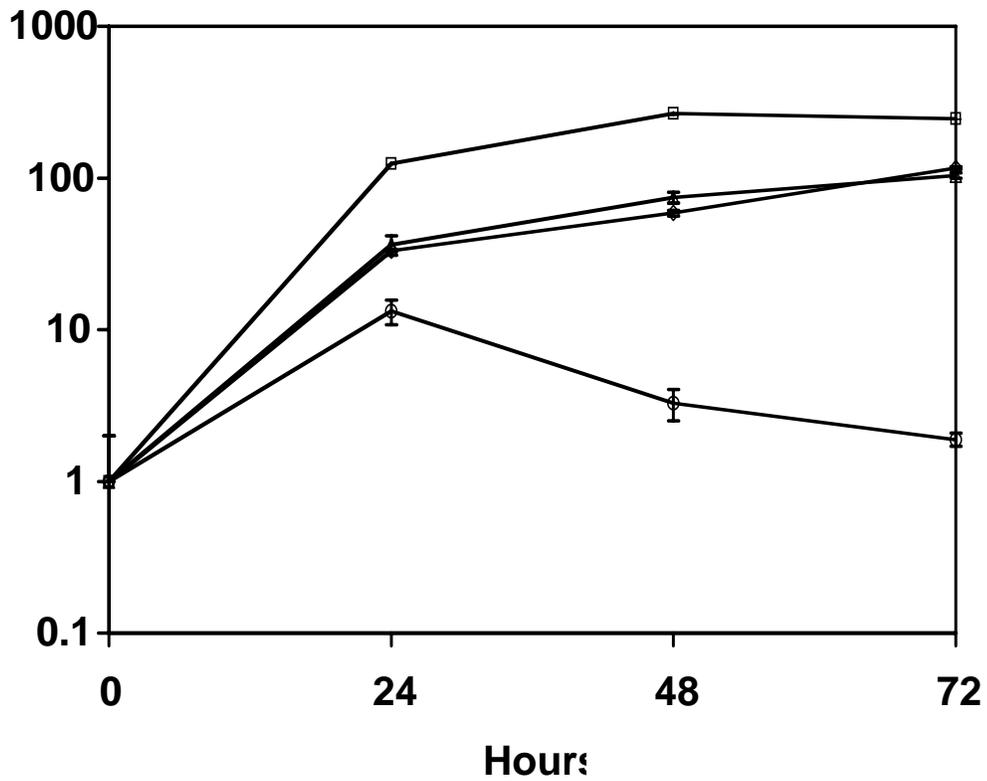


Figure 4.1. Flagellated *L. parisiensis* and *L. tucsonensis* evade C57BL/6 macrophage restriction of replication.

Intracellular growth of *Legionella* in C57BL/6 macrophages shown is representative of three or more experiments for *L. pneumophila* (○), *L. pneumophila* *flaA* mutant (□), *L. parisiensis* (◇), and *L. tucsonensis* (△).

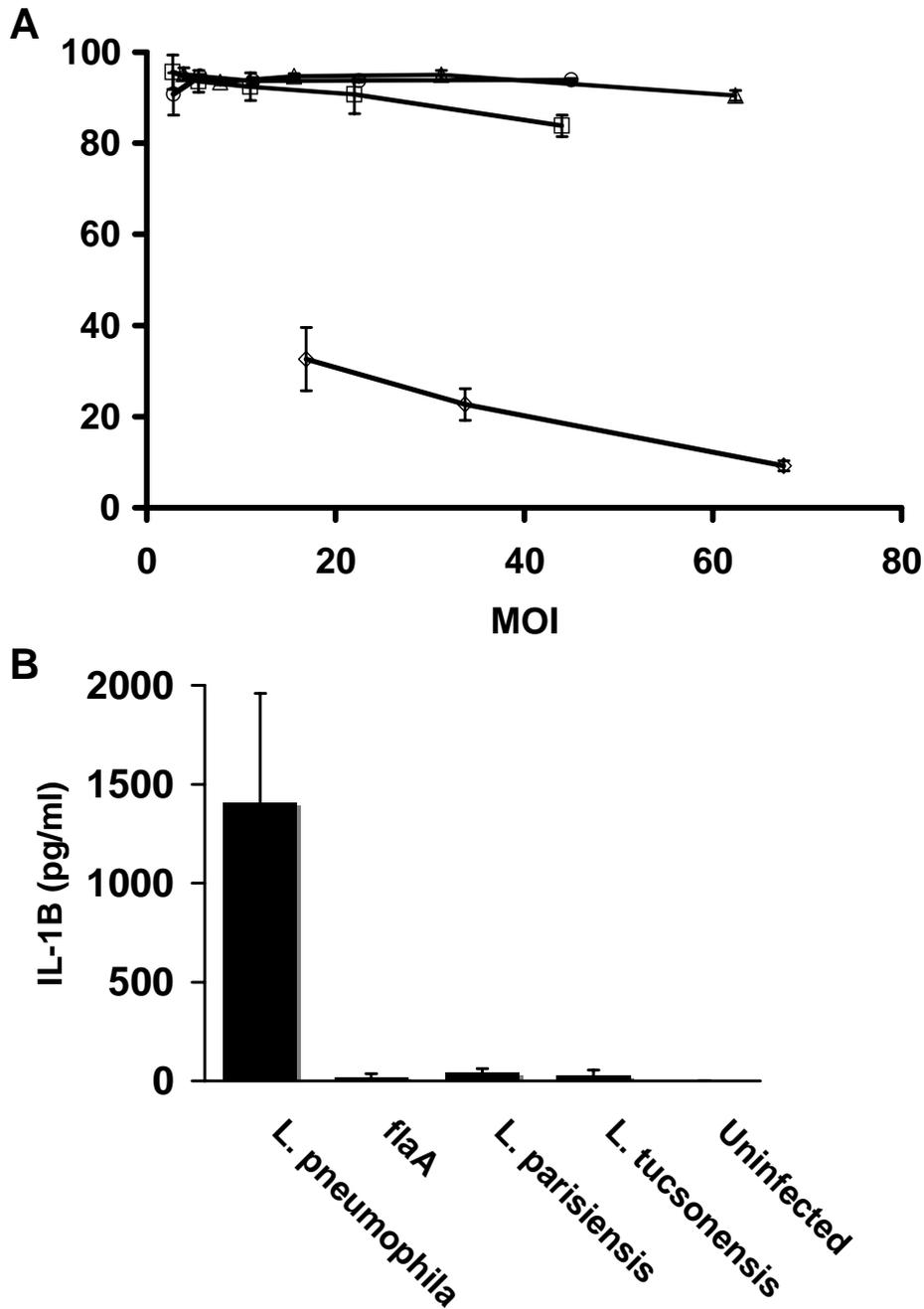


Figure 4.2. *L. parisiensis* and *L. tucsonensis* do not trigger cell death or IL-1B secretion.

(A) C57Bl/6 macrophages were incubated for 1 h with 2-fold dilutions of the strain indicated, and cell viability was quantified by Alamar blue reduction. *L. pneumophila* (◇), *L. pneumophila flaA* mutant (□), *L. parisiensis* (△) and *L. tucsonensis* (○). Results from one experiment representative of three is shown. (B) Macrophages, pre-treated with LPS, were infected with the strain indicated for 2 h and secreted IL-1 β was quantified in macrophages. Results shown are means \pm SE for three experiments assaying duplicate wells.

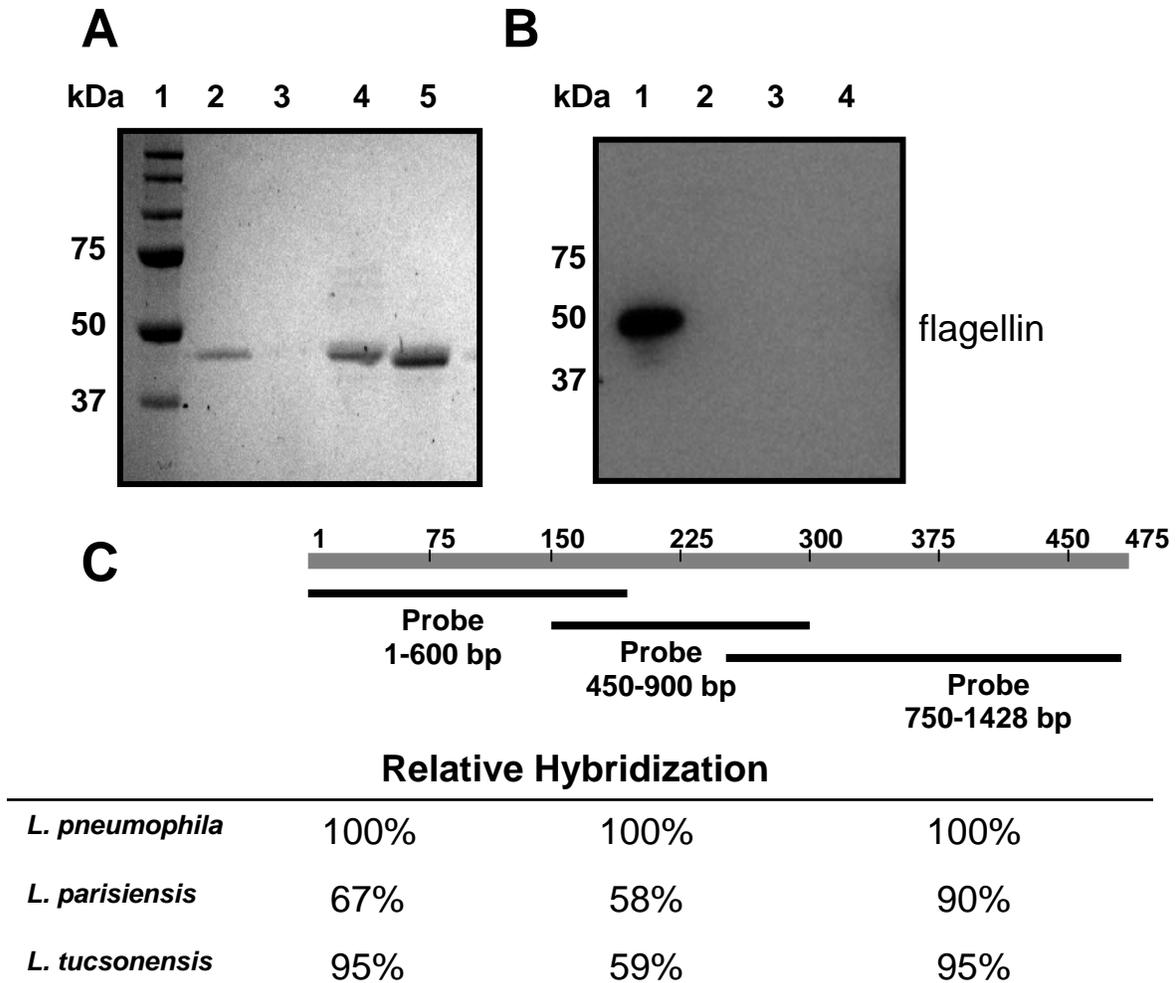


Figure 4.3. Analysis of crude flagellin preparations.

Flagellin samples prepared in parallel from broth cultures were separated by SDS-PAGE and analyzed by (A) Coomassie blue staining of *L. pneumophila* (Lane 2), *L. pneumophila flaA* (Lane 3) *L. parisiensis* (Lane 4), and *L. tucsonensis* (Lane 5) or (B) western analysis using anti-*L. pneumophila* flagellin antibody of *L. pneumophila* (Lane 1), *L. pneumophila flaA* (Lane 2) *L. parisiensis* (Lane 3), and *L. tucsonensis* (Lane 4). Positions of molecular mass standards are shown (Lane 1). (C) Relative hybridization under low stringency with DNA probes to the indicated regions of *flaA*.

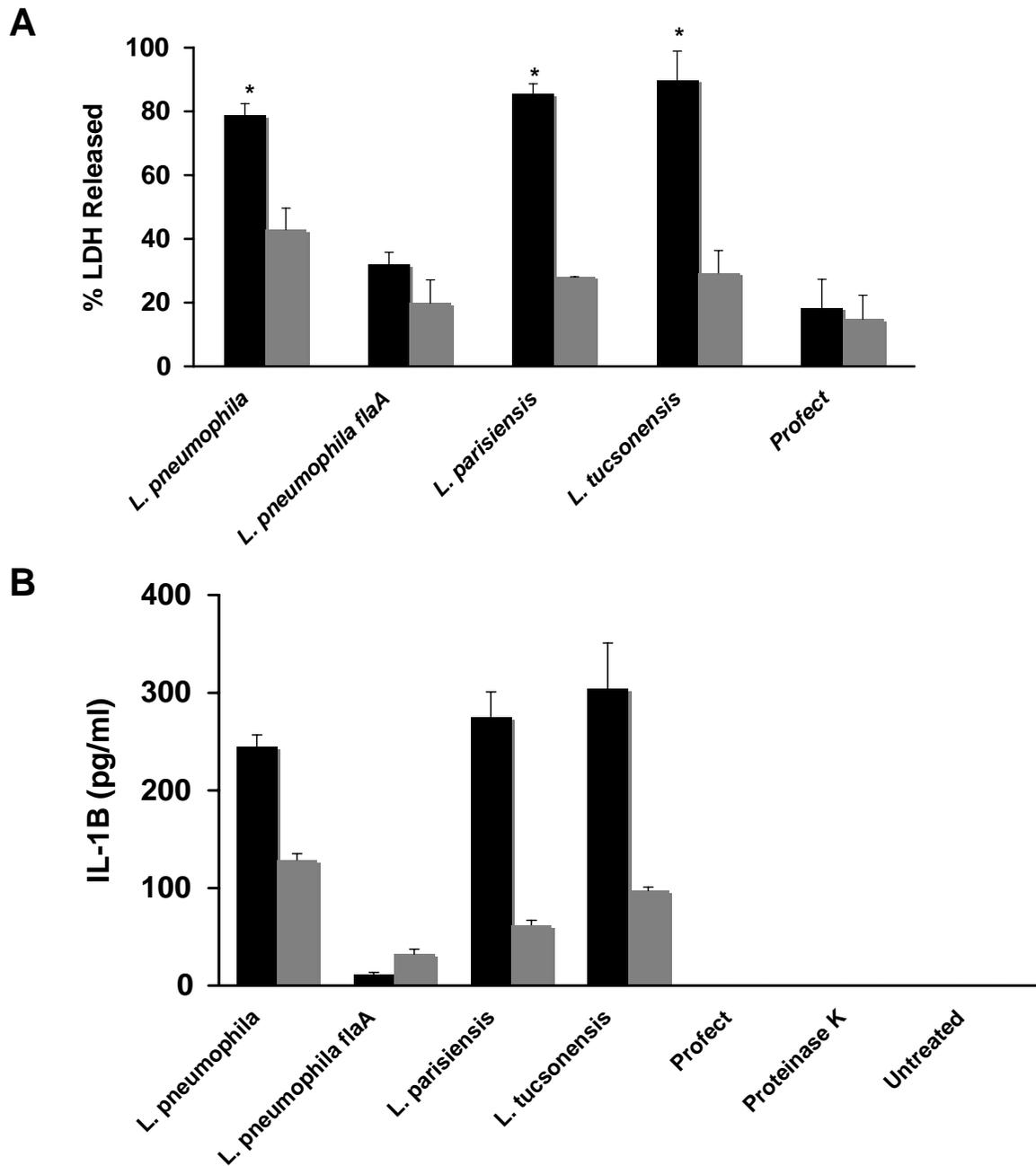


Figure 4.4. Like *L. pneumophila* flagellin, flagellin from *L. parisiensis* and *L. tucsonensis* triggers macrophage cell death.

To determine whether flagellin from *L. parisiensis* and *L. tucsonensis* triggers pyroptosis, (A) LDH and (B) IL-1 β released from C57Bl/6 macrophages transfected with heat-treated crude flagellin (1.25 μ g) or proteinase K treated (gray bars) and incubated for 2h was quantified. Shown are means \pm SE for three experiments, where * indicates statistical difference from *L. pneumophila flaA* of $p < .05$ using Student's *t* test.

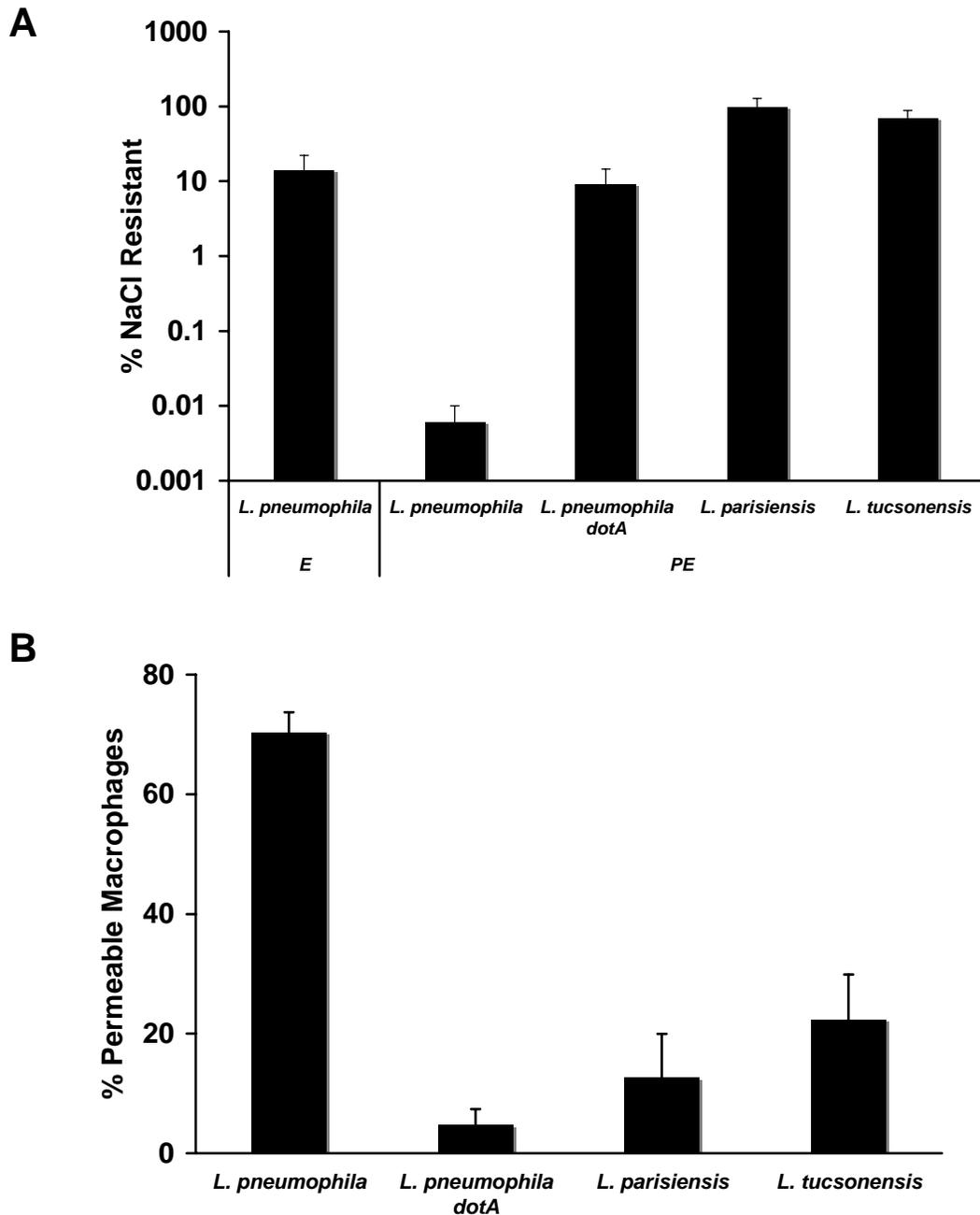


Figure 4.5. *L. parisiensis* and *L. tucsonensis* do not display features of the classic type IV secretion system.

(A) Sodium resistance was quantified by plating broth grown CFU on media with or without 100 mM NaCl and calculating $[(\text{CFU on CYET+NaCl})/(\text{CFU on CYET})] \times 100$. (B) PE phase *Legionella* were incubated for 1 h with primary macrophages, and then permeability was determined by uptake of ethidium bromide. Shown are means with SE for three experiments.

CHAPTER FIVE

Conclusion

L. pneumophila has evolved an array of virulence factors that allow the bacteria to reside within the accidental mammalian host, where cases of person-to-person transmission have yet to be observed. Yet, the interplay between *L. pneumophila* and mammalian macrophages has been widely studied making *L. pneumophila* a model organism to study intracellular pathogenesis. My thesis work has focused on *Legionella*'s interaction with the mammalian host at the cellular level, specifically the features of the bacterium that contribute to disease in the host. *L. pneumophila* has a phasic lifestyle, whereby it expresses many transmission traits that are important for infecting host cells and establishing an intracellular replication niche (Chapter 1; as reviewed in Molofsky and Swanson, 2004). Subsequently, the bacteria differentiate into the replicative phase and multiply until necessary nutrients are exhausted. Then the bacteria lyse the occupied cell and start the cycle anew. *Legionella* can also persist in a dormant form, a trait that may enhance its resilience until a new host is encountered. *Legionella*'s differentiation process is a response to metabolic cues that control its transcriptional profile to allow expression of phase-specific traits to cope with environmental fluctuations.

To explore whether virulence assays used regularly in basic science laboratories distinguish virulent strains from environmental isolates, I began by characterizing several *L. pneumophila* isolates obtained from hospital water sources and infected patients. We found that several transmission traits required for contacting and infecting a new host cell were conserved by all the strains analyzed, despite *Legionella*'s genetic and phenotypic heterogeneity (Chapter 1; Amemura-Maekawa *et al.*, 2005; Brassinga *et al.*, 2003; Cazalet *et al.*, 2004; Cazalet *et al.*, 2008; Izu *et al.*, 1999; Samrakandi *et al.*, 2002; Sexton and Vogel, 2004). Each of these traits, namely dispersal, contact with host cells, and lysosome evasion, are promoted by the flagellar regulon (Molofsky *et al.*, 2005). In particular, the protein subunit, flagellin, is also required for *L. pneumophila* to kill mouse macrophages (Molofsky *et al.*, 2005). In the environment, once intracellular resources are depleted, the demand to escape from an amoeba host, to disperse in the fresh water, and to evade degradation upon infecting a new host may provide the selective pressure to maintain the transmission traits, motility and cytotoxicity.

A second consistent phenotype observed in this study was the ability to persist and replicate in macrophages, a critical feature of *Legionella* pathogenesis (Horwitz and Silverstein, 1980). Even though all environmental and clinical isolates replicated robustly in the macrophage monocytic cell line U937, only four of the isolates analyzed persisted in murine macrophages. In contrast, the environmental isolate *NE-2733* was unable to persist or survive a mouse macrophage infection. Thus, murine macrophages provide a less tolerant environment for *Legionella*, and replication in U937 cells is not a valuable predictor of virulence. Recent genomic analysis of the *L. pneumophila* isolates studied indicates that the avirulent strain is missing several genetic elements that may

contribute to its ability to persist in mammalian macrophages and to cause disease (Chapter 1; Cazalet *et al.*, 2008). However, a more detailed replication study in several macrophage types and amoebae is required to further analyze these correlations. Since genetic manipulation in *L. pneumophila* is relatively easy, it would be interesting to determine if the absence of the gene *lag-1* encoding for the “virulence-associated” epitope of LPS accounts for the lack of disease cases attributed to *NE-2733* and *NE-2735*, by providing the gene in *trans* (Helbig *et al.*, 1995). These epidemiological and laboratory data support the notion that selective pressures in its natural environment contribute to the virulence of *L. pneumophila* in a clinical setting.

Moreover, conservation of motility and cytotoxicity has rendered *Legionella* vulnerable to the defenses of the macrophage. As pathogens evolve mechanisms to subvert the defenses of macrophages, hosts have evolved the means to detect invasion by a pathogen and combat infection (Delbridge and O’Riordan, 2007; Kumagai *et al.*, 2008; Sutterwala *et al.*, 2007). One way that cells first detect pathogens is through the Toll like receptors (TLRs) found on the surface of cells that recognize several microbial products known as microbe associated molecular patterns or MAMPS (Akamine *et al.*, 2005; Andersen-Nissen *et al.*, 2005; Kaisho and Akira, 2004; Smith *et al.*, 2003; Takeda and Akira, 2004; Weiss *et al.*, 2004). Second, a class of factors located within the cell, NOD-like receptors (NLRs), can detect MAMPs that have contaminated the cytosol (Delbridge and O’Riordan, 2007; Fortier *et al.*, 2005; Fritz and Girardin, 2005; Martinon and Tschopp, 2005). In the case of *L. pneumophila*, the latter is a major pathway macrophages use to combat infection (Lightfield *et al.*, 2008; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Vinzing *et al.*, 2008a).

In the simplest model, after phagocytosis and modification of the phagosome by type IV secretion, flagellin is detected in the cytoplasm by NLRs, and its detection leads to a pro-inflammatory cell death that is characterized by activation of caspase-1 and secretion of IL-1 β (Chapter 3). Detection of cytosolic flagellin is a general defense mechanism, since flagellin from *Salmonella typhimurium* and *Bacillus subtilis* initiate a similar response. The host pathway is triggered by very low amounts of flagellin, suggesting that macrophages can detect small numbers of bacteria. Although flagellin is detected by TLRs and NLRs, the epitope that each recognizes is quite different (Lightfield *et al.*, 2008). This is indicative of a system where macrophages use distinct types of receptors to differentiate between extracellular pathogens and those intracellular pathogens that express virulence factors to evade the normal degradative machinery (as reviewed in Miao *et al.*, 2007).

One such virulence factor, the type IV secretion system of *L. pneumophila*, modulates the replication vacuole by secreting effector proteins through the phagosome membrane (Christie and Vogel, 2000). Translocation of *Salmonella* flagellin into the macrophage cytosol requires the *Salmonella* Pathogenicity Island 1 type III secretion system but not the flagellar type III secretion system, providing a precedent for the idea that the type IV secretion system may be a conduit for *L. pneumophila* flagellin into the macrophage cytosol (Sun *et al.*, 2007). Flagellin translocation by the type IV secretion was not as efficient as delivery of *bona fide* effectors when analyzed using a Cya-fusion to FlaA and RalF (Chapter 3) (Molofsky *et al.*, 2006). In any case, the type IV secretion system seems to be a highly promiscuous system that secretes a wide variety of proteins, including several families of proteins with a high level of redundancy. Therefore, it is

feasible that small amounts of flagellin are secreted non-specifically (reviewed in Ninio and Roy, 2007; Isberg *et al.*, 2009; Luo and Isberg, 2004).

Although current data are consistent with the simple model that flagellin protein that has diffused through phagosomal pores is detected by Naip5 (Chapter 3), several observations indicate that other host and bacterial components also contribute. For example, the inflammasome component Nlrc4 (Ipaf) also detects cytosolic flagellin of *Legionella*, *Listeria*, *Salmonella*, *Bacillus* and *Pseudomonas*, yet how it does so either alone or in conjunction with Naip5 or the adapter protein ASC remains to be established (Franchi *et al.*, 2007a; Franchi *et al.*, 2007b; Miao *et al.*, 2006; Miao *et al.*, 2008; Ozoren *et al.*, 2006; Warren *et al.*, 2008). Recent studies with Naip5^{-/-} mice suggest that, upon stimulation, Nlrc4 and Naip5 cooperate to activate caspase-1 (Lightfield *et al.*, 2008). Nlrc4 induced caspase-1 activation also occurs in a flagellin-independent manner during infection by *Shigella* and *Pseudomonas* (Franchi *et al.*, 2007b; Suzuki *et al.*, 2007). However, in each case a functional secretion system is required to activate the inflammasome, including the type IV secretion system of *Legionella* (Franchi *et al.*, 2007b; Molofsky *et al.*, 2006; Ren *et al.*, 2006; Suzuki *et al.*, 2007). These data suggest that a functional bacterial secretion system is critical for macrophages to detect bacterial invaders, including *Legionella*.

Although detection of microbial products and downstream signaling events has provided a foundation for the field of innate immune recognition, several questions remain. Does direct binding between the NLRs and flagellin occur? Is it leakage or diffusion through pores in the phagosomal membrane that delivers flagellin to the cytosol? Does *Legionella* actively secrete flagellin by type IV secretion or another

system? Is flagellin translocated from the inside or outside of the bacterial cell to the macrophage cytosol? Is flagellin disassembled in the phagosome prior to crossing the membrane?

The NLR Naip5 was originally identified as Bircl1e or Lgn1 when mutations in the locus were mapped to the chromosomal region that confers susceptibility of the A/J mouse strain to a *Legionella* infection (Derre and Isberg, 2004b; Diez *et al.*, 2003; Fortier *et al.*, 2005; Growney and Dietrich, 2000; Wright *et al.*, 2003). Permissiveness in A/J mice is due to mutations in the Naip5 allele, which produces a hypofunctional protein, whereas wild-type mice such as C57Bl/6 macrophages restrict infection (Chapter 3 and 4). However, when infected with *L. pneumophila* that lack flagellin, C57Bl/6 macrophages do not restrict the bacteria from replicating, and the mutants establish a robust infection. Thus when macrophages are unable to sense flagellin, the innate immune system is blind to the presence of the bacteria for a period sufficient to allow the infection to progress. One study concluded that Naip5 is dispensable for inflammasome activation and restriction of bacterial growth, based on the measurable caspase-1 activation in A/J macrophages in response to *L. pneumophila*. However these studies analyzed caspase-1 activation only using qualitative assays of macrophages that still make a hypofunctional Naip5 protein (Lamkanfi *et al.*, 2007). Subsequent work using single-celled quantitative assays in Naip5 knockout macrophages determined that Naip5 functions upstream of caspase-1 activation and is required for detection of flagellin. Therefore, inflammasome activation plays a critical role in restricting *L. pneumophila* infection (Lightfield *et al.*, 2008).

Later in the infection, clearance of wild-type *L. pneumophila* by A/J *naip5* mutant mice and *flaA* *L. pneumophila* mutants by C57Bl/6 mice demonstrates that macrophages have alternative recognition mechanisms, albeit sluggish, that are sufficient to control the infection (Molofsky *et al.*, 2006). Once *Legionella* is detected by the innate immune system, cytokines, chemokines and other proteins are secreted by alveolar macrophages and other immune cells. For example, growth of *Legionella* is also restricted when macrophages are activated by IFN- γ , yet defective IFN- α/β signaling increases permissiveness of *Legionella* replication (as reviewed in Vance and Hawn, 2008). Accordingly, the immune host response to *Legionella* is a cooperation of diverse interactions between several immune cell types and secreted factors.

To learn more about restriction of *Legionella* infections by C57Bl/6 mice and additional factors that may contribute, in Chapter 4 I exploited flagellated *Legionella* species that replicate in these macrophages. I determined that the flagellated species *L. parisiensis* and *L. tucsonensis* replicate in restrictive macrophages despite production and expression of the toxic epitope of flagellin. The bacteria failed to induce pyroptosis and Il-1 β secretion, unless flagellin protein was transfected directly into the cytosol, indicating that during a normal infection some *Legionella* species fail to contaminate the cytosol with flagellin and activate caspase-1. Their ability to evade the innate immune system involves the requirement of a canonical bacterial secretion system, since they are resistant to NaCl and do not cause ethidium bromide uptake in macrophages, two phenotypes that are dependent on the type IV secretion system of *L. pneumophila* (Byrne and Swanson, 1998; Kirby and Isberg, 1998; Kirby *et al.*, 1998).

Our initial study (Chapter 3) focused primarily on flagellin as the major factor contributing to the ability of C57Bl/6 macrophages to suppress an *L. pneumophila* infection. My subsequent data reinforce that, in addition to flagellin, pore formation or production of a conduit for flagellin to the cytosol is also required for macrophages to restrict replication, an end result of inflammasome activation. Since there is divergence of both the gene and the protein, flagellin produced by *L. parisiensis* and *L. tucsonensis* may be incompatible with their secretion systems such that appreciable translocation into the cytosol does not occur. To increase our understanding of whether the activation of caspase-1 influences replication by *Legionella*, we could genetically provide a mechanism for pore formation to these flagellated species, and then analyze pyroptosis and intracellular replication.

A number of observations indicate that processing of flagellin occurs before its translocation by the type IV secretion system (Lightfield *et al.*, 2008; Smith *et al.*, 2003). Disassembly of flagellin into monomeric form is required for recognition by TLR5 (Smith *et al.*, 2003) and pyroptosis (Fink and Cookson, 2007). It is also likely that the flagellin expressed using a retroviral transduction system in macrophages was also in monomeric form (Lightfield *et al.*, 2008). De Jong and colleagues successfully used a *Salmonella* beta-lactamase reporter assay in *Legionella* to measure translocation of *dot/icm* effectors, so this system could potentially be used to analyze flagellin translocation (de Jong *et al.*, 2008). Future studies to closely analyze how translocation occurs and if it results in modification of flagellin would greatly contribute to understanding the interaction of flagellin with NLRs.

In conclusion, my thesis has emphasized how the conserved traits of motility and cytotoxicity both make *Legionella* a versatile pathogen, but have also allowed the host a means to fight the infection. Evolution of *L. pneumophila* to survive within its natural host, amoebae, has poorly prepared the pathogen for the innate defense mechanisms it encounters in a mammalian host. The pro-inflammatory response, triggered by *L. pneumophila* flagellin, likely results in the infiltration of neutrophils and macrophages to sites of infection causing diffuse alveolar damage and extensive inflammation. Indeed, the pro-inflammatory response to *L. pneumophila* likely accounts for much of the pathology of Legionnaires' disease, which ultimately represents a dead end for *L. pneumophila*. Moreover, the potential of the *Legionellae* to cause human disease appears to be inversely correlated with the capacity to replicate in macrophages of C57Bl/6 mice. Ultimately, this thesis also points to the complexity of human disease, a product of both microbial virulence traits, defined by laboratory assays and the capacity of the host to mount a measured immune response.

APPENDIX

APPENDIX A

Acetylations of the lipopolysaccharide of *Legionella pneumophila* contribute to lysosomal evasion

SUMMARY

Legionella pneumophila inhibition of phagosome maturation is correlated with modifications of its surface properties during the post-exponential (PE) phase of growth. Our objective was to identify the modification of the surface glycoconjugates during the transition to *L. pneumophila's* virulent phase that affects phagosome maturation. Lag-1 is an O-acetyl transferase that acetylates the hydrophobic legionaminic acid monomers of the O-antigen; this acetylation can alter *Legionella's* serum sensitivity and is a known virulence determinant. A mutant that lacks the gene encoding a particular LPS acetyltransferase was constructed to determine if the corresponding locus is required to block phagosome maturation. A *lag-1* mutant lacks an acetylated O-antigen, allowing us to test whether the charge and hydrophobicity of the O-antigen affects the fate of *Legionella* in macrophages. Our data suggests that the LPS of *L. pneumophila* is more hydrophobic in the replicative phase; as the bacteria differentiate, they de-acetylate their LPS. Accordingly, a hydrophilic LPS may increase fitness when the bacteria egress from phagocytic cells and are released into an aqueous environment.

INTRODUCTION

Legionella pneumophila is a ubiquitous environmental microorganism that parasitizes aquatic amoebae. However, in the event that aerosolized *Legionella* are ingested by humans, *Legionella* have the opportunity to parasitize the alveolar macrophages of the lung and establish a severe pneumonia. First implicated as a pathogen in 1976 when American Legion members attending a convention in Philadelphia were afflicted with a serious illness, *Legionella pneumophila* serogroup 1 has been responsible for the majority of Legionnaires' disease cases.

A major virulence trait of *Legionella pneumophila* is the ability to avoid lysosomal degradation by both amoebae and macrophages. When engulfed by phagocytic cells, transmissible *L. pneumophila* cells establish a vacuole that is separate from the endosomal network and instead interacts with the secretory pathway (Horwitz and Silverstein, 1983; Horwitz and Maxfield, 1984; Molofsky and Swanson, 2004; Roy *et al.*, 1998; Swanson and Isberg, 1996a). Within thirty minutes, the vacuole recruits Rab1 and is subsequently decorated with secretory vesicles intercepted from the endoplasmic reticulum (ER) exit sites (Kagan and Roy, 2002; Kagan *et al.*, 2004). Rab7 and lysosomal associated membrane protein (LAMP-1), both late endosomal markers, do not colocalize with *Legionella*-containing phagosomes at these early times (Roy *et al.*, 1998; Swanson and Isberg, 1996a). In A/J mouse macrophages, the pathogen persists for at least six hours in this sequestered autophagosome-like compartment characterized by the recruitment of ER (Amer and Swanson, 2005; Swanson and Isberg, 1995). Subsequently, replicative *L. pneumophila* multiply within this vacuole, acquiring the

lysosomal characteristics, LAMP-1, cathepsin-D and an acidic pH (Sturgill-Koszycki and Swanson, 2000). As nutrient resources within the vacuole diminish, the replicating bacteria differentiate and express several known virulence traits thought to promote transmission to a new host cell (Byrne and Swanson, 1998).

The Dot/Icm type IV secretion system of *L. pneumophila* is essential for establishment of the replication vacuole, since *dot* mutants fail to alter endocytic trafficking, reside within a compartment with late endosomal markers, and never acquire characteristics of the wild-type *L. pneumophila* vacuole (Berger, 1994). Although Dot/Icm mutants fail to evade the endolysosomal pathway, *dotA* mutants reside in Rab7/LAMP-1 positive vacuoles that do not acquire lysosomal characteristics (Roy *et al.*, 1998). In addition to Dot/Icm function, an independent formalin resistant component is required to inhibit phagosome-lysosome fusion, since formalin-killed *L. pneumophila* fail to accumulate lysosomal markers (Joshi *et al.*, 2001). Consequently, *L. pneumophila* must possess other factors that contribute to its ability to inhibit fusion and degradation in lysosomes.

The capacity to inhibit phagosome-lysosome fusion is correlated with changes in the lipopolysaccharide (LPS) surface and the shedding of outer membrane vesicles as *Legionella* differentiates (Fernandez-Moreira *et al.*, 2006). Several intracellular pathogens use modifications of their surface properties as a virulence strategy to redirect phagosomal trafficking and inhibit their demise (Guerry *et al.*, 2002; Guo *et al.*, 1997; van Putten, 1993; Weiser and Pan, 1998). Similar to *Legionella*, *Mycobacterium tuberculosis* can alter normal phagosomal trafficking, a process that is partly dependent on the phospholipid lipoarabinomannan (Fratti *et al.*, 2001), since latex beads coated with lipoarabinomannan failed to recruit endosomal antigen and mature into phagolysosomes.

Similarly, interaction of vacuoles containing *Leishmania donovani* promastigotes rarely colocalize with endocytic compartments; however, vacuoles containing *Leishmania* mutants, lacking the cell surface lipophosphoglycan (LPG), colocalize with endosomes and lysosomes (Desjardins, 1997; Sacks *et al.*, 2000). These data indicate that particular surface glycoconjugates can provide some protection from delivery to lysosomes.

Developmental regulation of surface modifications by *L. pneumophila* contributes to its ability to block phagosome-lysosome fusion (Fernandez-Moreira *et al.*, 2006). Modifications of the lipopolysaccharide of *L. pneumophila* serogroup 1 strains have been correlated with virulence and serum sensitivity (Luneberg *et al.*, 1998). The “virulence-associated” LPS epitope recognized by MAb 3/1 is prevalent among clinical isolates of *L. pneumophila* serogroup 1 (Luck *et al.*, 2001). The epitope of MAb3/1 is dependent upon a functional *lag-1* (lipopolysaccharide-associated gene) gene, which encodes the *O*-acetyl transferase that modifies the eighth carbon position on the legionaminic acid monomers of the *L. pneumophila* *O*-antigen. Spontaneous mutants lacking the *lag-1* gene lose reactivity with MAb3/1, and expression of the gene *in trans* restores MAb3/1 reactivity as well as acetyltransferase activity (Kooistra *et al.*, 2001; Luck *et al.*, 2001; Zou *et al.*, 1999). In addition to their role in specific antibody recognition, these *O*-acetyl groups are a major contributor to the hydrophobicity of the LPS (Kooistra *et al.*, 2001). These alterations in hydrophobicity did not affect uptake and intracellular replication in macrophages and *Acanthamoeba castellanii* (Luck *et al.*, 2001; Zou *et al.*, 1999), however any contribution to intracellular trafficking was not analyzed.

In addition to modifications to its surface, *Legionella* shed outer membrane vesicles (OMV), also developmentally regulated, that are sufficient to inhibit phagosome-lysosome fusion (Fernandez-Moreira *et al.*, 2006; Galka *et al.*, 2008). The lysosomal

avoidance associated with OMV does not require type IV secretion and persists for several hours (Fernandez-Moreira *et al.*, 2006). Galka et al. recently (Galka *et al.*, 2008) confirmed that OMV are shed within the phagosomes of an infected host cells, can intercalate with host cell membranes. Further, they also demonstrated that non-protein components of the OMV stimulate a cytokine response. Taken together these data suggest that the LPS is a dynamic molecule and that modifications, either to membrane-associated or shed LPS, can play a critical role in the host cell response and the trafficking of *Legionella* within the cell.

Analogous to the developmentally regulated lipophosphoglycan of *Leishmania* spp. that blocks phagosome maturation, we hypothesize that alterations in the lipopolysaccharide affects the ability of *L. pneumophila* to modify phagosome maturation. The main modification of *L. pneumophila* LPS is the acetylations of its O-antigen (Zahringer *et al.*, 1995). Therefore, to test whether acetylation of the O-antigen affects phagosome maturation, we constructed a *lag-1* mutant and compared its fate in murine macrophages to a wild-type serogroup 1 *L. pneumophila* strain (Lp02).

EXPERIMENTAL PROCEDURES

Bacterial strains and culture.

Legionella pneumophila, Lp02 (*thyA hsdR rpsL*), derived from Philadelphia 1, was the source of the *lag-1* region clone. The *dotA* mutant, Lp03, was also used in this analysis and has been previously described (Berger and Isberg, 1994) Strains, maintained at -80°C in glycerol stocks, were colony-purified onto

N-(2-acetomido)-2-aminoethanesulfonic acid (ACES; Sigma)-buffered charcoal-yeast extract agar (CYE) supplemented with 100 ug/ml of thymidine (CYET). Bacterial strains were cultured in ACES-buffered yeast extract broth (AYE) supplemented with thymidine (100 ug/ml; AYET) at 37°C with aeration. *Legionella* was subcultured in AYET from an overnight primary culture and grown to the exponential and post-exponential phases for experimentation. Exponential phase cultures (E-replicative) were defined as OD₆₀₀ 0.5-2.0, while post-exponential phase (PE-transmissive) cultures were defined as OD₆₀₀ 3.0-4.0 with high motility. Cultures were defined as motile when >75% of bacteria in a field of ≥100 cells showed rapid, directed movement.

Mutant construction

A *lag-1* mutant was constructed by amplifying the ~2000-bp genomic locus from Lp02 genomic DNA using the primers *lag-1*UPPER (ATCGGATGAACTGAAAATAAAAA) and *lag-1*LOWER (CTCCCGACAAAACCAACTGA) and ligating with the plasmid vector pGEM-T (Promega) to create pGEM-*lag-1*. Digestion with BsrGI and BglII removed ~2-kb of the open reading frame of *lag-1*, and the DNA remaining was blunted with Klenow fragment and dephosphorylated with calf intestine phosphatase (New England Biolabs). A 1.3 kb kanamycin cassette was removed from puc4K by digestion with EcoRI, blunted with Klenow fragment and ligated into the digested pGEM-*lag-1*, creating pGEMΔ*lag-1*::kan. The Δ*lag-1*::kan locus was transferred to Lp02 by natural competence with PCR amplified product as previously described (Bachman and Swanson, 2001). Putative *lag-*

I::kan mutants were selected on CYET containing kanamycin and screened for mutant loci by PCR. Two independent isolates (NW029 and NW031) were tested for similar phenotypes; results shown are NW029. The $\Delta lag-I::kan$ locus was also transferred to Lp03 by natural competence with PCR amplified product to create $\Delta lag-I::kan dotA::gent$, subsequently annotated as *lag-1dotA*.

Bacterial adherence to hexadecane

Adherence to the hydrocarbon *N*-hexadecane was performed as previously described (Fernandez-Moreira *et al.*, 2006; Rosenberg, 1984). Cells were pelleted by centrifugation at 5000 X g for 5 min and resuspended in sterile phosphate buffered saline (PBS; pH 7.4; Gibco). Culture densities were normalized to OD₆₀₀ 0.2 in a final volume of 6 mls PBS in glass test tubes. Each sample was then agitated, in the presence of 1 ml of *N*-hexadecane (Sigma) at 37 C for 10 min. Following incubation, samples were vortexed for 60 sec. Samples were then allowed to partition the aqueous phase from the hydrocarbon phase at room temperature for 30 minutes. The hydrocarbon phase was gently removed, and the optical density of aqueous phase was assessed. The percentage of bacteria that remains in the aqueous phase after agitation with hexadecane was calculated as $(1 - \frac{\text{the OD}_{600} \text{ of the cell suspension after agitation}}{\text{OD}_{600} \text{ of the cell suspension before agitation}}) \times 100$.

Western analysis

Lipopolysaccharide (LPS) was isolated from *Legionella* by resuspending cells grown on plates for 48 h in sterile double distilled H₂O (ddH₂O) to an OD₅₅₀ of 1.00. The cell suspension was then pelleted at 12000 X g for 2 min and resuspended in 200 ul of Proteinase K (Sigma) Buffer (0.5% SDS, 10mM Tris-HCl, 5mM EDTA in ddH₂O). Cells were then boiled for 5 min, and 0.5 mg/ml of Proteinase K was added. The suspension was incubated for 1 h at 37° C and then centrifuged at 12,000 X g for 2 min. Supernatants containing LPS were stored at -20° C. Samples were separated by SDS-PAGE and immunoblotted with anti-*Legionella* (a gift from Dr. Ralph Isberg, Howard Hughes Medical Institute and Tufts University School of Medicine, Boston, MA) (Swanson and Isberg, 1996b) or monoclonal antibody 3/1 (MAb 3/1)(Helbig *et al.*, 1995) and detected by ECL (Pierce).

Macrophage culture

Bone-marrow derived macrophages were isolated from the femurs of female A/J mice (Jackson Laboratories) as previously described (Swanson and Isberg, 1995). Macrophages were maintained in RPMI supplemented with 10% fetal bovine serum (RPMI-FBS, Gibco) and were plated at the density indicated for each assay.

Infectivity

The ability of *L. pneumophila* to enter and survive within macrophages was assessed as described previously (Byrne and Swanson, 1998). Macrophages were plated at a density of 2.5×10^5 per well in 24-well tissue culture plates and infected at a 1:1 ratio with post-exponential bacteria for 2 h at 37°C with 5% CO₂. Extracellular bacteria were removed by rinsing the macrophage monolayer three times with RPMI-FBS at 37°C, a medium that is not permissive to *Legionella* replication. Intracellular bacteria were quantified by lysing monolayers by trituration with ice-cold phosphate buffered saline (PBS) and plating duplicate aliquots on CYET. Colony forming units (CFU) added at 0 h were determined by plating an aliquot of the infection media on CYET. The initiation of infection was calculated from triplicate samples by the following equation: (CFU from lysates at 2 h)/(CFU added at 0 h) x 100.

Lysosomal degradation

The ability of the bacteria to evade lysosomal degradation during a 2 h infection was analyzed by fluorescence microscopy as described previously using rabbit anti-*L. pneumophila* antibody (a gift from Dr. Ralph Isberg, Howard Hughes Medical Institute and Tufts University School of Medicine, Boston, MA) (Swanson and Isberg, 1996b). Macrophages were cultured on 12-mm glass coverslips at a density of 2×10^5 and infected at a MOI of ~1-2 for 2 h at 37 C with 5% CO₂. Macrophages were then washed and fixed with periodate-lysine-paraformaldehyde(PLP)-sucrose for 30 min at room

temperature. Macrophage membranes were permeabilized with ice-cold methanol for 10 sec and subsequently rinsed with sterile PBS. Intracellular bacteria were then labeled by incubating fixed and methanol extracted cells with polyclonal anti-*Legionella* antibody and Texas Red conjugated anti-rabbit secondary antibody (Molecular Probes).

Macrophage nuclei and all bacteria were labeled by incubating fixed cells for 5 min with 0.1 ug of 4', 6-diamidino-2-phenylindole (DAPI) per ml of PBS. Stained coverslips were mounted using Profade Mounting medium (Molecular Probes) and viewed on a Zeiss Axioplan 2 fluorescence microscope. Bacteria were scored as intact if a distinct Texas Red positive rod shape was visible. Non-intact bacteria were defined as dispersed particles of Texas Red-positive fluorescence.

Lamp-1 colocalization

Interaction between *Legionella* and late endosomal or lysosomal compartments was determined by quantifying colocalization of bacteria with the marker LAMP-1. Association was determined as described for lysosomal degradation, however lysosomal compartments were labeled with anti-LAMP-1 (Santa Cruz) and Oregon Green anti-rat secondary (Molecular Probes) antibodies.

Intracellular bacterial growth

Macrophages were infected at a multiplicity of infection (MOI) of 1 as described for infectivity, then replication of bacteria within macrophages was measured at 24 h intervals. Cells were allowed to adhere overnight before incubation with bacteria. Cells were lysed by treating the monolayers with 2% saponin (Sigma) in sterile PBS at the selected time intervals. Lysates were prepared from triplicate samples and aliquots of each were plated on CYET for CFU enumeration.

Cytotoxicity

Contact-dependent cytotoxicity was quantified as the percent of macrophages killed during a 1 h incubation with *L. pneumophila*. Macrophages were cultured at a density of 5×10^4 per well in 96-well tissue culture plates. Transmissible bacteria suspended in RPMI-FBS at varying ratios were co-incubated with the macrophages for 1 h at 37°C. After extracellular bacteria were washed away, the monolayers were subsequently incubated with 0.5 ml of 10% (vol/vol) Alamar Blue (TREK Diagnostics) in RPMI-FBS for from 4 h to overnight. The redox-specific absorbance resulting from the reduction of Alamar Blue to its reduced form by viable macrophages was measured with a SpectraMax 250 spectrophotometer (Molecular Devices) at OD₅₇₀ and OD₆₀₀. The percent of viable macrophages was calculated in triplicate from the standard curve, the slope of a plot of A_{570}/A_{600} determined for triplicate samples of six known densities of

uninfected macrophages in the range of 10^3 to 5×10^4 macrophages per well. The actual MOI was determined by plating duplicate samples of the infection inocula onto CYET

RESULTS

To analyze whether the LPS of the *lag-1* mutant differed from wild-type and lost reactivity with MAb3/1, indicative of loss of 8-*O*-acetyl groups in legionaminic acid (Luck *et al.*, 2001), we used western, immunofluorescence, and bacterial adherence to hydrocarbon (BATH) assays. As expected, *lag-1* mutants lack the acetylations required for recognition by MAb3/1, as determined by both immunofluorescence and western analysis, since *lag-1* mutants failed to react with the MAB3/1 antibody but reacted with polyclonal anti-*Legionella* (Fig. A.1 and 2).

Bacterial adherence to the hydrocarbon *n*-hexadecane, which reflects surface hydrophobicity or charge, is developmentally-regulated, dependent on the growth phase of the broth cultures from which the cells are isolated, and correlates with lysosomal avoidance (Fernandez-Moreira *et al.*, 2006 ; Pembrey *et al.*, 1999). Unlike wild-type, replicative phase *L. pneumophila lag-1* mutants did not bind to *n*-hexadecane (Fig. A.3). Thus, *L. pneumophila* binding to hexadecane correlated with reactivity to MAb3/1; both activities required the Lag-1 acetyltransferase and were maximal in the replicative phase. Confident that the *lag-1* mutants lacked LPS-specific acetylations, we proceeded to examine their virulence phenotypes using several well-characterized assays (Byrne and Swanson, 1998).

Hydrophobicity of bacterial cells has been shown to affect association with mammalian cells (Absolom, 1988; Magnusson *et al.*, 1980), and alterations in the O-antigen may increase infectivity of *Salmonella* and protect the bacteria from replication. (Bjur *et al.*, 2006). Therefore to assess the ability of *lag-1* mutants to enter and replicate in macrophages, bacteria was incubated with primary murine bone marrow-derived macrophages for 2-72 hours, and then the fraction of the inoculum that was cell-associated and viable at 2 hours was determined. Additionally, the total yield at subsequent times was enumerated. Altering the hydrophobicity of the *Legionella* surface did not play a direct role in either the infectiousness of the bacteria or in their ability to replicate, since *lag-1* mutants entered and replicated within macrophages as efficiently as wild-type (Fig. A.4 A-B).

Pseudomonas mutants lacking an O-antigen have increased cytotoxicity to epithelial cells that is mediated by type III secretion (Augustin *et al.*, 2007). *Legionella* cytotoxicity to macrophages is contact dependent and requires both flagellin and type IV secretion (Kirby *et al.*, 1998; Molofsky *et al.*, 2005). Thus, it is plausible that variations in *Legionella* LPS architecture could modify exposure and secretion dynamics of the type IV secretion system. To determine whether surface properties contribute to contact-dependent cytotoxicity (Kirby *et al.*, 1998; Molofsky *et al.*, 2005) of *L. pneumophila*, we analyzed macrophage viability after a one-hour incubation with bacteria at a high multiplicity of infection. An acetylated LPS was not required for *L. pneumophila* to be cytotoxic to macrophages since an MOI ~10 of wild-type or *lag-1* mutant bacteria killed ~95% of the macrophages, while the type IV secretion mutants, *dotA* and *lag-1dotA*, had no effect on the macrophages (Fig. A.5).

To assess whether the deacetylation pattern of *L. pneumophila* is critical to the fate of the bacteria within the macrophage, macrophages were infected with wild-type and mutant bacteria. The bacterial status, intact vs. degraded, was determined by immunofluorescence, as previously described (Byrne and Swanson, 1998; Molofsky *et al.*, 2005). After a 2 hr incubation with macrophages, wild-type PE cells avoid degradation, since ~80% of bacteria remain intact (Fig. A.1 and 6). Only 10% of wild-type replicative cells are equipped to evade the lysosomes and subsequent degradation; however, when macrophages are infected with *L. pneumophila* lacking an acetylated LPS, ~60% of replicative phase *lag-1* mutant bacteria avoided lysosomal degradation in macrophages (Fig. A.6). The ability of E phase *lag-1* bacteria to evade degradation is consistent with the ability of hydrophilic wild-type PE bacteria to arrest phagosome maturation. A large percentage of those replicative *lag-1* mutant bacteria that avoided degradation however, co-localized with the late endosomal and lysosomal protein LAMP-1, suggesting they are stalled before reaching the degradative lysosomes.

The aberrant intracellular trafficking pattern of E phase *lag-1* mutants is also typical of post-exponential phase *dotA* mutants and formalin-killed post-exponential phase wild-type *L. pneumophila* (Joshi *et al.*, 2001). Dot/Icm is thought to primarily act in the PE phase, indicating that E phase *lag-1* mutants evade the endosomal pathway by a type IV secretion independent mechanism. To test the contribution of Lag-1, independently of type IV secretion, we constructed a *lag-1 dotA* double mutant. Intact rods that co-localized with LAMP-1 were also evident within macrophages infected with replicative *lag-1dotA* mutant bacteria (Fig. A.6), indicating that a significant number of E phase *lag-1* bacteria arrest maturation of their vacuole by a Dot/Icm-independent

mechanism. Thus, lack of acetylation of LPS by *L. pneumophila* correlated with residence and survival in a vacuole whose progression to degradative lysosomes was arrested.

DISCUSSION

O-acetylations have been implicated as an important virulence factor of bacterial pathogens, by generating antigenic variation that may enhance survival and prevent an immune response. The ability of *Staphylococcus aureus* to colonize mouse kidneys and resist opsonophagocytic killing was drastically reduced when *o*-acetylations of the capsular polysaccharide were lost (Bhasin *et al.*, 1998). Recognition of LPS by seven different antibodies against *Salmonella typhimurium* were all affected by acetylation, both as a required epitope for recognition and by interfering with recognition (Slauch *et al.*, 1995). Past studies have indicated that modifications of *L. pneumophila* lipopolysaccharide by the Lag-1 *O*-acetyltransferase do not influence virulence in host cells (Luck *et al.*, 2001; Zou *et al.*, 1999). Yet in this study, we have expanded those observations and now implicate LPS acetylations as one determinant of the intracellular fate of *L. pneumophila* in mouse macrophages. We have shown that in the replicative phase, the *lag-1* mutant lacks acetylations that contribute to hydrophobicity, and do not attach to *n*-hexadecane or react with MAb3/1, traits that correlate with the ability of wild-type *L. pneumophila* to evade degradation in lysosomes (Fernandez-Moreira *et al.*, 2006). We have shown that like other strains of *Legionella*, Lag-1 is dispensable for many of the well-characterized virulence traits of strain Lp02 (Fig. A.4 and 5). However, this is the

first study to show that replicative phase *lag-1* mutants avoid degradation (Fig. A.6). This is indicative of *Legionella* whose LPS acetylation-status resembles that of transmissible wild-type *Legionella* that are able to avoid immediate degradation and encounter an aqueous extracellular environment, where a hydrophilic LPS would be suitable.

The MAb3/1 LPS epitope is associated with the ability of *Legionella* to cause disease, and *o*-acetylations of the LPS increase the hydrophobicity of the bacteria (Luck *et al.*, 2001; Zahringer *et al.*, 1995). We postulate that by acetylating its LPS during intracellular replication, *Legionella* release the inhibition to fuse with endosomal vacuoles while increasing their resistance to lysosomal enzymes (Sturgill-Koszycki and Swanson, 2000). As a consequence, the pathogen can exploit the host endosomal pathway as a source of not only nutrients but also the membrane needed to expand its replication niche. Once replication ceases and the bacteria differentiate into the transmissible form, deacetylation of their LPS provides a more hydrophilic surface that is more conducive to the extracellular aqueous environment. It is thought that modifications of the surface may be important for establishing stable aerosols (Dennis and Lee, 1988) or in the establishment of an infection in a mammalian host where *Legionella* come into close contact with mucus membranes and must adhere to the upper respiratory tract.

There is evidence that LPS participates in the attachment of pathogens to extracellular matrices and host cells (Jacques, 1996). Since all studies with *lag-1* mutants have only analyzed persistence *in vitro* (Luck *et al.*, 2001; Zou *et al.*, 1999) and other *Legionella* mutants are attenuated in mouse infections but not *in vitro* (DebRoy *et al.*, 2006), it would be worthwhile to determine the effects of the LPS modifications rendered by Lag-1 with an *in vivo* model system. The LPS of *Legionella* is versatile and is in

constant flux to adapt the bacteria to its ever-changing environment, survival outside of a host cell and within. By modifying the pathogen's surface according to growth phase, the LPS armor can alternately contribute both to transmission and to replication within key defenders of the human immune system.

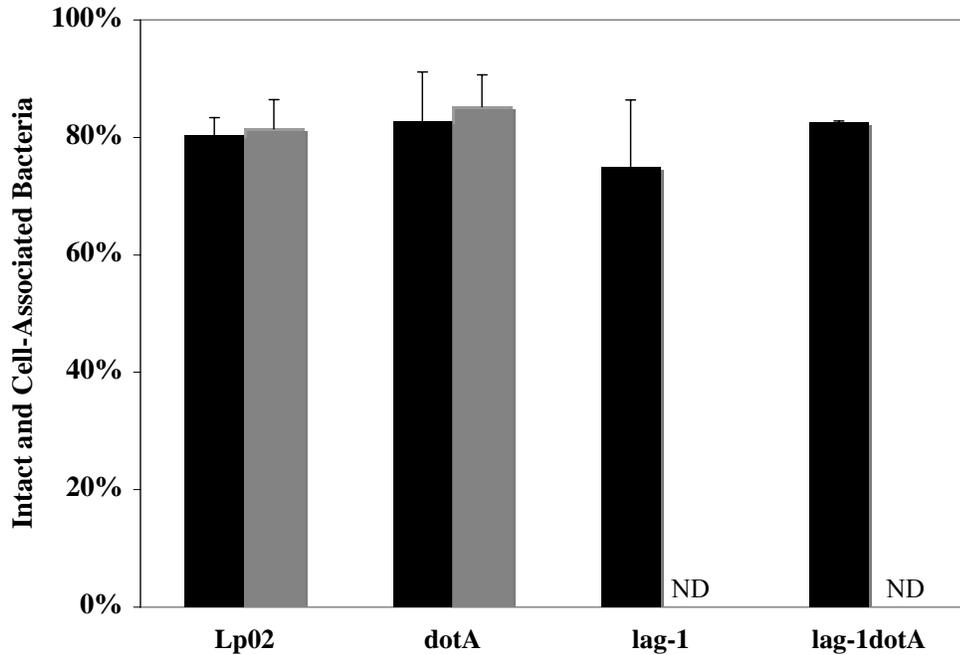


Figure A.1. Acetylations required for recognition by the virulence-associated monoclonal antibody 3/1 are not present *lag-1* mutants.

The ability of PE *L. pneumophila* to evade macrophage lysosomes was quantified by fluorescence microscopy, using anti-*Legionella* antibody. Macrophages were incubated with bacteria at an MOI of 1 for 2 h, rinsed of extracellular bacteria and fixed. Macrophages were then stained and mounted for viewing. Black bars correspond to polyclonal anti-*Legionella* antibody and gray bars correspond to monoclonal 3/1 antibody. ND: not detected.

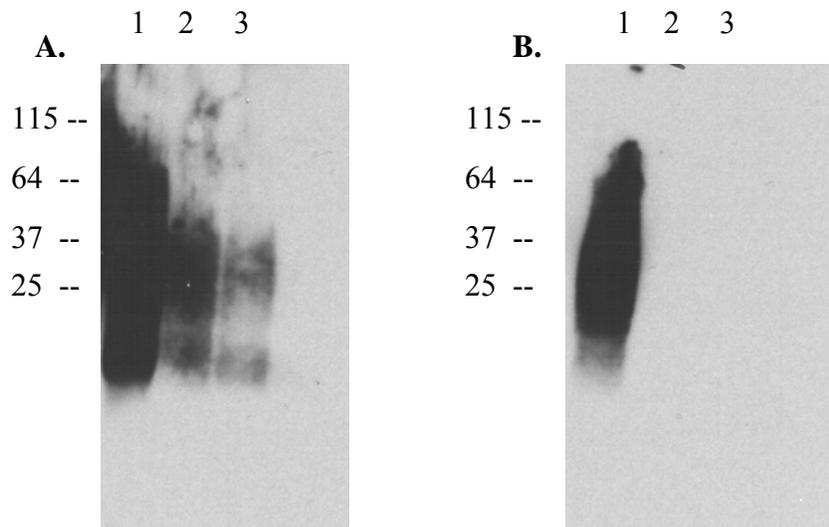


Figure A.2. LPS from *lag-I* mutants lack the MAb3/1 epitope.

LPS preparations from cultures grown on CYET were resolved by SDS-PAGE and immunoblotted with (A) polyclonal anti-*Legionella* and (B) MAb3/1 antibodies. Lanes are as follow: wild-type (1) Lp02 and two independent *lag-I* mutant isolates (2) NW029 (3) NW031.

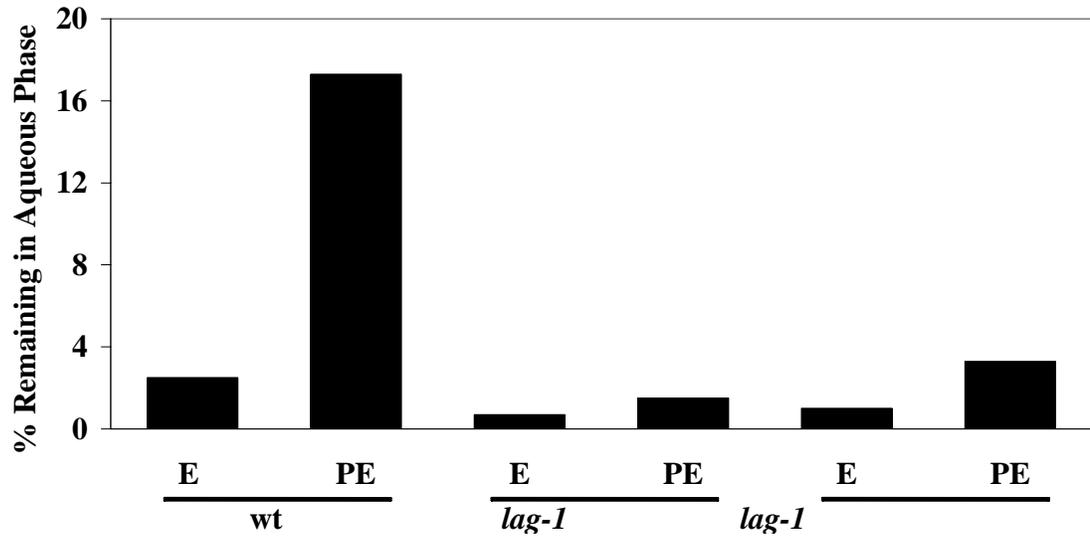


Figure A.3. *Legionella* lacking an acetylated LPS are unable to differentially bind to hexadecane.

Strains were grown to the phase indicated, then normalized to an OD of 0.85 in a buffered solution. The samples were then vortexed for 1 min with *n*-hexadecane, then incubated at room temperature for 30 min to allow the phases to separate. The *n*-hexadecane layer was carefully removed and the OD of the aqueous layer was read to determine the percentage of bacteria that attached to the *n*-hexadecane layer.

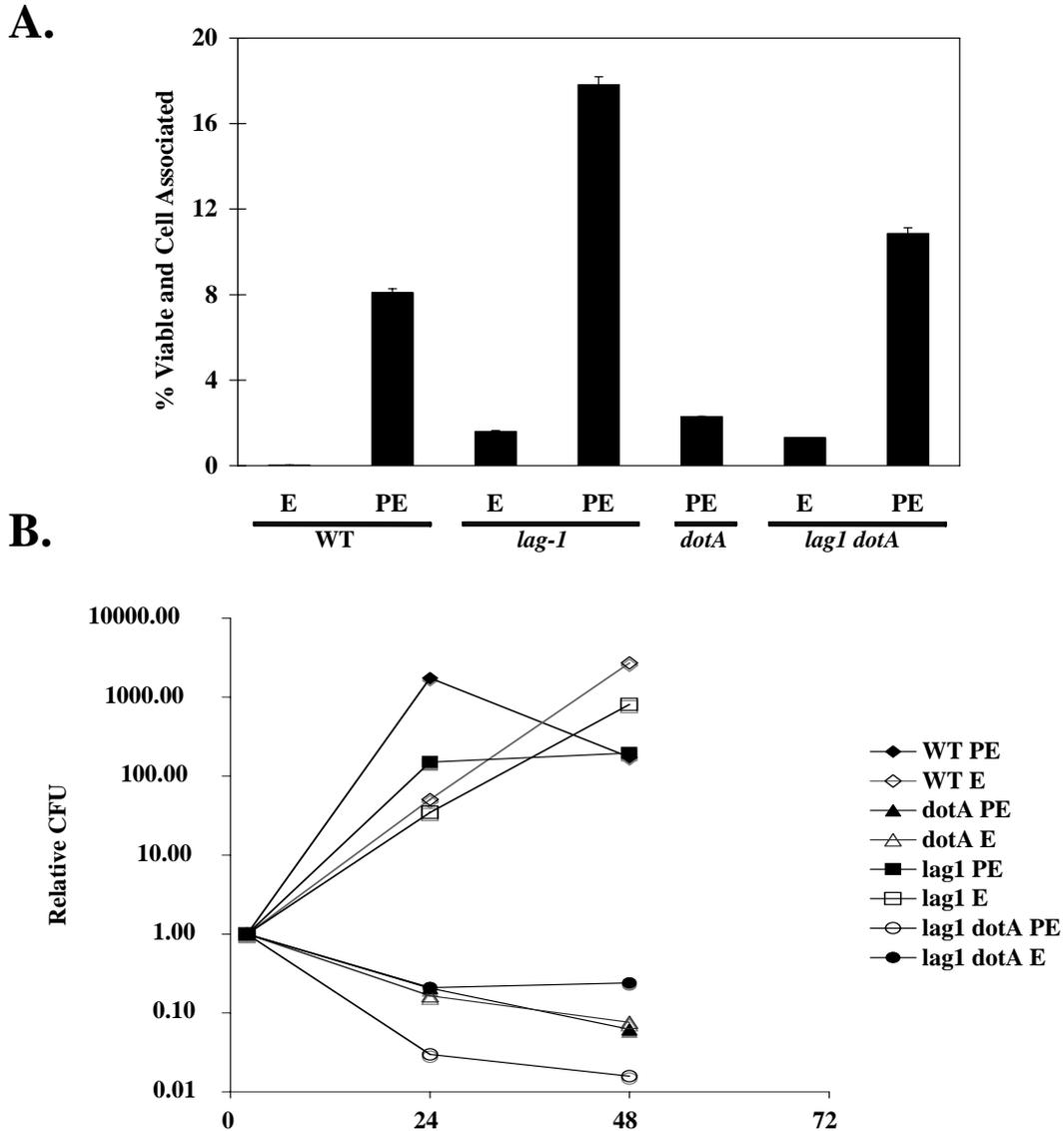


Figure A.4. An acetylated LPS is not required for *Legionella* to efficiently infect and replicate in macrophages.

(A) The ability of *L. pneumophila* to enter macrophages was assessed by incubating PE bacteria with macrophages at an MOI ~1 for 2 h, then determining the percent of viable and cell associated bacteria. Shown is a representative figure of triplicate samples in four experiments. Bars indicate standard deviations of samples. (B) To quantify the ability of each *L. pneumophila* isolate to replicate and survive within macrophages, cells were infected with PE bacteria at an MOI ~1, and then 2 h post-infection extracellular bacteria were washed from the macrophage monolayer. Viable bacteria at 72 hr were quantified by determining total colony-forming units in each sample. The mean yield of *L. pneumophila* was determined for duplicate or triplicate samples in three experiments.

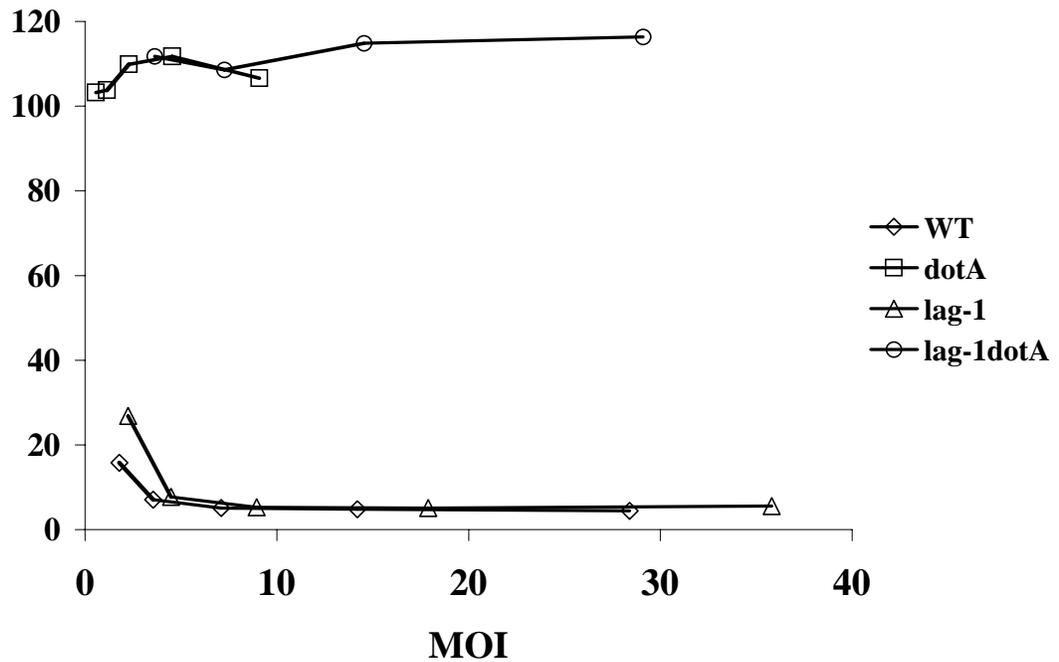


Figure A.5. LPS acetylations by Lag-1 do not contribute to cytotoxicity of macrophages.

Macrophage viability was analyzed by determining the capacity of viable macrophages to reduce the colorimetric dye Alamar Blue after a 1 h incubation with PE bacteria. The multiplicity of infection (MOI) was calculated by plating the respective broth culture on CYET. Shown is a representative figure of triplicate samples of three or more experiments.

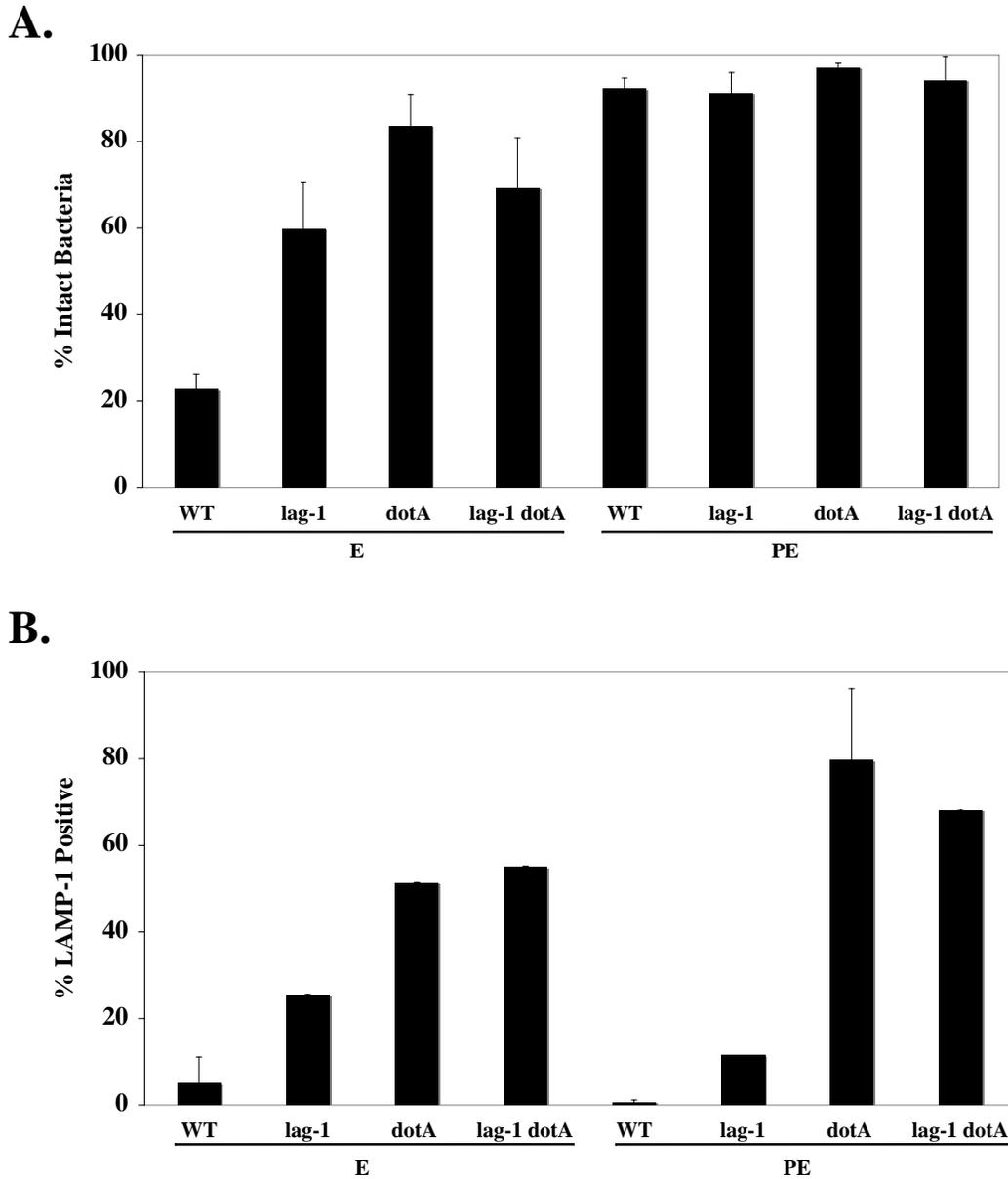


Figure 6. Lack of an acetylated LPS protects replicative (E) *lag-1* mutants from degradation.

The ability of PE *L. pneumophila* to evade macrophage lysosomes was quantified by fluorescence microscopy. Macrophages were co-incubated with the strains indicated for 2 h and then fixed and stained with anti-*Legionella* and with a monoclonal specific to the lysosomal protein Lamp-1 to identify the macrophage lysosomal compartment.

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