Specificity of *Legionella pneumonia* and *Coxiella burnetii* Vacuoles and Versatility of *Legionella pneumonia* Revealed by Coinfection

John-Demian Sauer, Jeffrey G. Shannon, Dale Howe, Stanley F. Hayes, Michele S. Swanson, and Robert A. Heinzen*

Coxiella Pathogenesis Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, 903 S. 4th St., Hamilton, Montana 59840, and Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109

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*Legionella pneumonia* and *Coxiella burnetii* are phylogenetically related intracellular bacteria that cause aerosol-transmitted lung infections. In host cells both pathogens proliferate in vacuoles whose biogenesis displays some common features. To test the functional similarity of their respective intracellular niches, African green monkey kidney epithelial (Vero) cells, A/J mouse bone marrow-derived macrophages, human macrophages, and human dendritic cells (DC) containing mature *C. burnetii* replication vacuoles were superinfected with *L. pneumonia*, and then the acidity, lysosome-associated membrane protein (LAMP) content, and cohabitation of mature replication vacuoles was assessed. In all cell types, wild-type *L. pneumonia* occupied distinct vacuoles in close association with acidic, LAMP-positive *C. burnetii* replication vacuoles. In murine macrophages, but not primate macrophages, DC, or epithelial cells, *L. pneumonia* replication vacuoles were acidic and LAMP positive. Unlike wild-type *L. pneumonia*, type IV secretion-deficient *dotA* mutants trafficked to lysosome-like *C. burnetii* vacuoles in Vero cells where they survived but failed to replicate. In primate macrophages, DC, or epithelial cells, growth of *L. pneumonia* was as robust in superinfected cells as in those singly infected. Thus, despite their noted similarities, *L. pneumonia* and *C. burnetii* are exquisitely adapted for replication in unique replication vacuoles, and factors that maintain the *C. burnetii* replication vacuole do not alter biogenesis of an adjacent *L. pneumonia* replication vacuole. Moreover, *L. pneumonia* can replicate efficiently in either lysosomal vacuoles of A/J mouse cells or in nonlysosomal vacuoles of primate cells.

Most intracellular bacterial pathogens inhabit replication vacuoles that exhibit a wide range of interactions with the endocytic pathway. Elucidation of bacterial and cellular signaling pathways that promote development of a vacuole permissive for growth is critical to gaining a better understanding of pathogenic mechanisms of intracellular bacteria. Moreover, the biochemical composition of the luminal milieu of vacuoles that promotes pathogen replication is largely undefined. As one approach to improve our understanding of replication vacuole biogenesis and composition, host cells have been doubly infected with pathogens that reside in distinct vacuolar compartments. Dual infections can be used to probe the dominance of pathogen signals that direct replication vacuole maturation and the permissiveness of vacuole growth environments. The dual infection model used in the present study used *Coxiella burnetii* and *Legionella pneumophila*, phylogenetically related macrophage pathogens that have both common and unique associations with the host cell. *C. burnetii* is an obligate intracellular gram-negative bacterium and the causative agent of human Q fever. *C. burnetii* is resistant to physical and chemical disruption in the small cell variant form (SCV) of its biphasic life cycle. The SCV is presumably the environmentally stable form of *C. burnetii* that, when inhaled, infects alveolar macrophages and differentiates into the replicative large cell variant (13). *L. pneumophila* is a facultative intracellular gram-negative bacterium that parasitizes freshwater amoebas and protozoa that serve as an environmental reservoir. *L. pneumophila* also undergoes a biphasic life cycle where the transmissive form (TF) (or exponential phase) is resilient and efficient at infection and the replicative form (RF) (or exponential phase) carries out intracellular growth. *L. pneumophila* can colonize human alveolar macrophages via inhalation of contaminated aerosols and cause a severe pneumonia known as Legionnaires’ disease (7).

The establishment of a replication vacuole is absolutely required for infection by *L. pneumophila* and *C. burnetii*. Replication vacuoles formed by most intracellular pathogens are permissive for growth because they do not fully mature through the endocytic pathway to fuse with the lysosomal compartment. Maturation of *L. pneumophila* and *C. burnetii* vacuoles through the endocytic pathway is stalled relative to phagosomes containing inert particles or killed organisms. The delay in *L. pneumophila* replication vacuole maturation is thought to allow differentiation of the environmentally stable TF to the replicating but environmentally sensitive RF. The functional significance of the delay in *C. burnetii* replication vacuole maturation is unclear since both SCV and LCV are inherently resistant to long-term exposure to the lysosomes. Indeed, the replication vacuole of *C. burnetii* is unusual in that it ultimately

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* Corresponding author. Mailing address: Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, 903 S. 4th St., Hamilton, MT 59840. Phone: (406) 375-9695. Fax: (307) 363-9380. E-mail: rheinzen@niaid.nih.gov.
becomes acidified and acquires characteristics of a phagolysosome (21). The maturation of vacuoles harboring *L. pneumophila* appears to be cell type specific with vacuoles acquiring lysosomal characteristics in primary murine macrophages (47) but not in primary human monocytes (53). *L. pneumophila* replication vacuoles form by interacting with the endoplasmic reticulum (ER) and the autophagic pathway (1, 49, 50). The *C. burnetii* replication vacuole also appears to interact with autophagic vesicles during maturation, but specific interactions with the biosynthetic pathway have not been demonstrated (5).

The biogenesis of bacterial replication vacuoles is regulated by the complex interplay of both host and bacterial proteins that modulate vesicular trafficking (28). Bacterial effectors of replication vacuole maturation can be translocated by type III or type IV secretory systems (28). The *L. pneumophila* type IV secretory apparatus is encoded by 26 dot/icm genes (42, 52). Although an intact Dot/Icm transporter is essential for establishment of the replication vacuole (42, 52), the absence of individual secreted substrates identified thus far does not result in strong defects in intracellular growth, suggesting that Dot/Icm translocated proteins are functionally redundant (14, 29, 35). The *C. burnetii* genome contains a nearly complete set of the *L. pneumophila* dot/icm genes with the exception of icmR (44), and *C. burnetii* homologs of *L. pneumophila* dotB, icmS, icmW, and icmT complement corresponding mutants in *L. pneumophila* (56, 57). A role for Dot/Icm in *C. burnetii* replication vacuole formation has not been established, owing in large part to the lack of workable genetic systems for this bacterium. However, protein synthesis is necessary for maturation and fusogenicity of the *C. burnetii* replication vacuole, and it is logical to suspect that Dot/Icm translocation substrates govern these events (24). Based on their genetic and cell biological similarities, it is possible that *L. pneumophila* and *C. burnetii* share common type IV secretion system-dependent effector molecules that mediate formation of their respective replication vacuoles.

To gain insight into mechanisms mediating the formation of replication vacuoles, *C. burnetii*-infected cells have been superfected and coinfected with other pathogens. Mature *C. burnetii* replication vacuoles readily fuse with vacuoles harboring the intracellular pathogens *Mycobacterium avium* (15, 18), *Mycobacterium tuberculosis* (18), *Leishmania amazonensis* (51), and *Trypanosoma cruzi* (2). Of these organisms, *M. avium* and *L. amazonensis* efficiently replicate in this environment (18, 51). *C. burnetii* vacuoles fuse at a very low frequency with replication vacuoles of *Toxoplasma gondii* (45) and not at all with vacuoles harboring *Chlamydia trachomatis* (21), two pathogens whose vacuoles appear completely disconnected from the endocytic pathway (21, 25, 45).

*C. burnetii* and *L. pneumophila* are related bacterial pathogens that have a superficially similar infectious cycle. To compare and contrast the development of their respective replication vacuoles, we superinfected with *L. pneumophila* a variety of cell types previously infected with *C. burnetii*. By this dual-infection approach, we sought to determine whether virulence factors/type IV effectors are restricted to pathogen vacuoles or instead act globally on host cell biology. In particular, we wanted to determine whether maintenance of an existing replication vacuole inhibits subsequent development of a different replication vacuole, whether respective pathogen vacuoles are capable of heterotypic fusion, and whether a coinfected vacuole is permissive for growth of both pathogens. Our results indicate that preexisting *C. burnetii* vacuoles do not compete with *L. pneumophila* for either establishment of its distinct vacuole or its subsequent replication.

**MATERIALS AND METHODS**

**Bacterial strains.** *C. burnetii* (Nine Mile strain in phase I or phase II) were propagated in African green monkey kidney epithelial (Vero) cells (CCL-81; American Type Culture Collection) grown in RPMI medium (Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Organisms were purified by renografin density gradient centrifugation as previously described (19) and stored at −80°C. *L. pneumophila* strain Lp02 (thymine auxotroph), and an isogenic dotA strain defective in type IV secretion, were cultured at 37°C on charcoal-yeast extract (CYE) plates or in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (AYE) broth supplemented with 100 μg of thymidine/ml (4, 16). The TF of *L. pneumophila* was used for all infections. Briefly, AYE broth was inoculated with *L. pneumophila* colonies from CYE plates less than 2 weeks old and incubated overnight (16 to 20 h) at 37°C. *L. pneumophila* organisms were generated from these cultures by subculturing to fresh AYE broth and incubation until they entered post-exponential phase (optical density at 600 nm of 3.5 to 4.0).

**Infection of Vero cells, primary BMDC, and primary human macrophages, and DC.** Vero cells were propagated as described above. Murine bone marrow-derived macrophages (BMDM) were isolated from the bone marrow exudates of female A/J mouse femurs and cultured as previously described (49). Human primary macrophages and dendritic cells (DC) were derived from human peripheral blood mononuclear cells. Briefly, peripheral blood mononuclear cells were isolated from buffy coats that were generated by centrifugation through Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were enriched for monocytes (CD14+) by using a RosetteSep monocyte enrichment kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). DC were generated by culturing purified monocytes (10⁵ cells per ml) in DC medium (RPMI plus Glutamax, 1 mM sodium pyruvate, and 100 μg of penicillin-streptomycin/ml) containing interleukin 4 (10 ng/ml) and granulocyte-macrophage colony-stimulating factor (10 ng/ml) (Peprotech, Rocky Hill, N.J.). Cells were cultured for 6 days with fresh cytokines added every 48 h. Nonadherent cells were harvested and determined to be >95% DC by positive staining for CD209^+CD11c^+CD1a^− as measured by flow cytometry. Macrophages were generated by culturing purified monocytes (10⁵ cells per ml) in macrophage medium (RPMI plus 10% FBS) containing macrophage colony-stimulating factor (10 ng/ml). After culture for at least 7 days, adherent macrophages were harvested by scraping and replated at the desired concentration.

For infections, all host cells were cultivated in 35-mm coverslip-bottom petri dishes (MatTek, Ashland, Mass.) or 24-well tissue culture plates (Corning, Inc., Corning, N.Y.) with or without 12-mm glass coverslips (Menzel, Germany). Cells and human macrophages were infected with the *C. burnetii* Nine Mile phase II strain at a multiplicity of infection (MOI) of 10. Murine BMDM were infected with the Nine Mile phase II strain or the Nine Mile phase I strain at MOIs of 10 and 20, respectively. Organisms in tissue culture media were added directly to the host cells and incubated for 2 h. Cell cultures were then washed three times with fresh tissue culture media and replenished with fresh medium. The *C. burnetii* inoculum was not washed from nonadherent DC cultures. *L. pneumophila* were used to infect BMDM, human macrophages and DC at an MOI of 2, and Vero cells at an MOI of 100. (The lower MOI used with BMDM, human macrophages, and DC was necessary to minimize *L. pneumophila* contact-dependent cytotoxicity [27], whereas the higher MOI used with Vero cells was necessary to maximize entry into these nonphagocytic cells, which are not susceptible to *L. pneumophila* cytotoxicity.) Infections were conducted as described above for *C. burnetii*. Thymidine was added to all tissue culture media at a final concentration of 100 μg/ml to support *L. pneumophila* growth. All coinfecions were carried out as superinfections; *C. burnetii* infection preceding *L. pneumophila* because of its slower growth rate.

**Light and fluorescence microscopy.** Fixation and staining procedures for indirect immunofluorescence localization of internalized bacteria and lysosomal characteristics were conducted as previously described (24). Mouse anti-human lysosome-associated membrane protein 1 (LAMP-1; clone HA43) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa), rat anti-mouse LAMP-2 antibody was acquired from BD Pharmingen (San Diego, Calif.), rabbit anti-*L. pneumophila* antibody was a generous gift...
from R. Isberg (Tufts University School of Medicine, Boston, Mass.), and con
cvalent C. burnetii antisera was derived from infected guinea pigs. Anti-
mouse, anti-rabbit, anti-rat, and anti-guinea pig Alexa Fluor 488, 594, or 647
immunogoldoglutin G antibodies were purchased from Molecular Probes (Eugene,
Oreg.). Images were acquired by using a Perkin-Elmer UltraView spinning disk
confocal laser illuminator system connected to a Nikon TE-2000S microscope, a
×/60 objective lens, and Metamorph software (Universal Imaging Corp., Down-
ingtown, Pa.).

The acidity of bacterium-containing vacuoles was qualitatively evaluated by
acidine orange staining (21). Acidic vesicles were stained with acidine orange
at a final concentration of 5 µg/ml in culture medium. After 1 h at 37°C, the stain
was removed and the monolayer washed three times with phosphate-buffered
saline (150 mM NaCl, 10 mM NaPO4, pH 7.2). Phase-contrast and fluorescent
images of live acidine orange-stained cells were obtained by using a Nikon
TE-2000-E inverted microscope, a MicroPublisher 3.3 RTV digital color camera
(QImaging, Burnaby, British Columbia, Canada), and Metamorph software. All
images were processed by using Image J (written by Wayne Rasband at the U.S.
National Institutes of Health and available by anonymous FTP from zippy.nih.gov)
and Adobe Photoshop software (Adobe Systems, Mountain View, Calif.).

Electron microscopy. Infected cells in six-well cell culture plates were fixed for
5 min with 2.5% glutaraldehyde–4.0% paraformaldehyde (vol/vol) in 0.1 M
sodium phosphate buffer at pH 7.0 to 7.2. Adherent cells were detached by gently
scraping cells with a cell scraper and transferred with fixative to a 1.5-ml mi-
crofuge tube. Samples were pelleted and stored in fixative for 24 to 48 h at 4°C.
Fixed pellets were washed with sodium phosphate buffer and then water and then
postfixed with 0.5% OsO4–0.8% K4Fe(CN)6 ·3 H2O in distilled water for 30 min
at room temperature. Pellets were then washed three times with water and
then treated with 0.1% tannic acid for 5 min. After a three washes with water, pellets
were stained en bloc with 1% aqueous uranyl acetate for 30 min at room
temperature. Pellets were then dehydrated through a graded ethanol series and
embedded in Spurr’s low-viscosity resin (Ted Pella, Inc., Redding, Calif.). Thin
sections were viewed at 80 kV on a Hitachi H7500 transmission electron micro-
scope. Images were captured by using a Hamamatsu C4742-57-12NR side-
mounted charge-coupled device camera and Advantage HR/HR-B digital imag-
ing software (AMT, Danvers, Mass.).

Quantification of L. pneumophila replication. To assess the effect of C. burnetii
replication on L. pneumophila replication, CFU growth assays were performed on
L. pneumophila as previously described (3). L. pneumophila was used to infect
Vero cells, human macrophages, and human DC, previously infected with the C.
burnetii Nine Mile phase II strain, which produces a truncated lipopolysaccharide
and is more efficiently internalized than the Nine Mile phase I strain (34). Using
this approach, >90% of L. pneumophila-infected cells also contained replicating
C. burnetii. Growth of L. pneumophila in superinfected murine BMDM was not
evaluated since only the poorly internalized C. burnetii L. pneumophila on
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was used to infect Vero cells. The
phila phase I variant efficiently
and is more efficiently internalized than the Nine Mile phase I strain (34). Using
A/J mice infected with both pathogens. Thus,
result corroborates and extends a previous study
was demonstrated (21). Infection of Vero cells with wild-type L. pneumophila
for 48 h (Fig. 2). If each pathogen uses a similar
mutant defective for type IV secretion did
traffic to the C. burnetii replication vacuole. Vacuoles harboring
both L. pneumophila dotA and C. burnetii were observed in
60.7% ± 5.0% of cells infected with both pathogens. Thus,
avoidance of the lysosome-like compartment of C. burnetii by
wild-type L. pneumophila is contingent upon expression of an
intact type IV secretion system. Only one dotA mutant organ-
ism was typically observed in C. burnetii vacuoles, suggesting
the environment is not amenable for growth of L. pneumo-

L. pneumophila replication is enhanced in C. burnetii-infected Vero cells. We next investigated whether the growth rate of L. pneumophila was altered in Vero cells infected with C. burnetii for 48 h (Fig. 2). If each pathogen uses a similar
mechanism(s) for acquisition of nutrients and vacuolar mem-
brane, then exponentially replicating C. burnetii might inter-
fere with subsequent growth of L. pneumophila. Replication of
wild-type L. pneumophila was first quantified in singly infected
cells. A 53-fold increase in CFU was observed between 2 and
48 h postinfection (p.i.) with growth slowing between 24 and
48 h p.i. Large, distended replication vacuoles were apparent
by phase microscopy at 48 h with no obvious cytolyis. Surpris-
ingly, an 86-fold increase in L. pneumophila CFU over the
same time course was observed in superinfected cells, which
was significantly greater (P < 0.014) than the increase
observed in singly infected cells. There was no replication of the
dotA mutant in singly infected or superinfected cells as indi-
cated by similar CFU counts at 2 and 48 h p.i. As described
above, a high percentage of C. burnetii replication vacuoles
harbor dotA mutants in superinfected cells. Consistent with
microscopic observations (Fig. 1), growth curve data indicate
that these organisms survive but do not replicate. Taken
together, these results indicate that replicative vacuoles occupied
by dividing C. burnetii do not impede biogenesis of vacuoles
harboring L. pneumophila and subsequent replication of the
organism.

RESULTS

L. pneumophila and C. burnetii replicate within distinct rep-
ication vacuoles in superinfected Vero cells. To investigate the
degree of similarity of replication vacuoles of L. pneumophila
and C. burnetii in an epithelial host cell, we superinfected C.
burnetii (phase II)-infected Vero cells with L. pneumophila
(Fig. 1). Vero cells are a standard experimental host cell of C.
burnetii (21) and have occasionally been used to study this
pathogen (54). Of a number of mouse strains
tested, A/J mice also rank as one of the more sensitive to
C. burnetii infection (41). Therefore, to compare replication vac-
ule formation in a professionally phagocytic cell type, A/J
mouse BMDM infected with C. burnetii for 48 h were super-


FIG. 1. Replication vacuole formation in Vero cells infected with *C. burnetii* and/or *L. pneumophila*. Vero cells infected with *C. burnetii* phase II (Cb; arrows) for 48 h were superinfected with wild-type *L. pneumophila* (Lp; arrowheads) or the isogenic dotA strain (Lp dotA; arrowheads) for 24 h. Vero cells singly infected with *C. burnetii* for 72 h or *L. pneumophila* for 24 h were used as controls. Live cells were stained with the acidotropic base acridine orange, and the same field was viewed by phase-contrast and fluorescence microscopy. Fixed cells were examined for localization of the lysosomal glycoprotein LAMP-1 by indirect immunofluorescence. Representative electron micrographs of infected cells are also depicted. In both singly and superinfected cells, acridine orange was sequestered by replication vacuoles harboring *C. burnetii* but not *L. pneumophila*. In singly infected cells, LAMP-1 (green) localized to the membrane of distinct replication vacuoles harboring *C. burnetii* (red) but not *L. pneumophila* (red). In superinfected cells, LAMP-1 (green) also localized to the *C. burnetii* (blue) replication vacuole membrane but not to the *L. pneumophila* (red) replication vacuole membrane. *C. burnetii* and *L. pneumophila* were never observed in the same replication vacuole. *C. burnetii*-infected cells superinfected with *L. pneumophila* dotA type IV secretion mutants, nonreplicating *L. pneumophila* (red) localized to the LAMP-1-positive (green) vacuole containing replicating *C. burnetii* (blue). Vacuoles harboring both *L. pneumophila* dotA and *C. burnetii* were observed in 60.7% ± 5.0% of cells infected with both pathogens. In electron micrographs, *L. pneumophila* are distinguished from *C. burnetii* by their larger size (>1 μm in length), uniform rod morphology, electron-translucent hydroxybutyrate granules, and lack of filamentous chromatin. Percent colocalization of dotA mutants is expressed as the mean ± the standard deviation of three independent experiments in which at least 50 superinfected cells were evaluated. Images are representative of three independent experiments in which at least 100 singly infected or superinfected cells were examined.
shown). Therefore, to compare and contrast replicates in human macrophages and DC with an /H11011 components of this response (31). We have found that, unlike presentation and pathogen killing, respectively, are critical immune response (31). DC and macrophages, adept at antigen intracellular pathogens generally requires a vigorous Th-1 type vacuoles occurs in human DC.

2-positive vacuoles in super-infected cells. However, despite singly infected cells, that harbored numerous replicating organisms. At 18 h p.i. in singly infected cells, L. pneumophila also trafficked to an acidic LAMP-2-positive vacuole, where it replicated as previously described (47). As in singly infected cells, L. pneumophila and C. burnetii (phase I) both trafficked to distinct acidic, LAMP-2-positive vacuoles in super-infected cells. However, despite the similar vacuole phenotype, cohabitation of both pathogens within a single vacuole was never observed.

Rare trafficking of L. pneumophila to C. burnetii replication vacuoles occurs in human DC. Control of human infection by intracellular pathogens generally requires a vigorous Th-1 type immune response (31). DC and macrophages, adept at antigen presentation and pathogen killing, respectively, are critical components of this response (31). We have found that, unlike the situation in murine A/J mouse BMDM, phase II C. burnetii replicates in human macrophages and DC with an ~10-fold increase in genome equivalents after 6 days of growth (data not shown). Therefore, to compare and contrast L. pneumophila and C. burnetii replication vacuole development in macrophages and DC, human cells infected with phase II C. burnetii for 36 h were superinfected with L. pneumophila for 12 h (Fig. 4 and 5). The shorter incubation time after L. pneumophila infection used here relative to other cell types was necessary to prevent the pronounced cell detachment that was observed for an incubation time of 18 h. In singly infected macrophages at 48 h p.i., replicating C. burnetii localized to multiple large and spacious, acidic (data not shown) LAMP-1-positive vacuoles (Fig. 4). Conversely, at 12 h p.i. L. pneumophila localized to a nonacidic (data not shown) LAMP-1-negative vacuole where robust replication was evident (Fig. 4). The vacuole phenotypes of each pathogen in singly infected macrophages were conserved in superinfected macrophages (Fig. 4). The pathogen vacuoles of superinfected cells were distinct and clearly separate with no cohabitation observed. In singly infected DC, multiple large and spacious, acidic (data not shown) LAMP-1-positive vacuoles containing replicating C. burnetii were observed at 48 h p.i. (Fig. 5). Like the situation in human macrophages, at 12 h p.i. L. pneumophila were harbored in nonacidic (data not shown), LAMP-1-negative vacuoles (Fig. 5). The vacuole phenotypes of each pathogen in singly infected DC were conserved in superinfected DC, with the most common occurrence being the formation of distinct and clearly separate replication vacuoles (Fig. 5). However, unlike the other cell types examined, trafficking of wild-type L. pneumophila to the C. burnetii vacuole was observed, albeit at a very low frequency (<1% of cointfected cells). In cointibated vacuoles, only C. burnetii appeared to be actively replicating, since only one or two L. pneumophila organisms were present. Thus, although an acidic, LAMP-1-positive vacuole is permissive for growth of wild-type L. pneumophila in murine BMDM, in DC the organism apparently does not replicate in C. burnetii replication vacuoles with the same traits.

Overall, the L. pneumophila replication is unaltered in human macrophages and DC infected with C. burnetii. We next investigated whether the growth rate of L. pneumophila was altered in human macrophages and DC infected with C. burnetii (phase II) for 36 h (Fig. 6). Between 2 and 48 h p.i., L. pneumophila replicated at a statistically similar rate in singly infected and superinfected human macrophages that approximated the rate previously described for L. pneumophila in murine BMDM (49). Host cell lysis occurred by 24 h p.i., allowing a secondary round of infection that resulted in 188- and 165-fold increases in CFU in singly infected and superinfected cells, respectively. Between 2 and 72 h p.i., L. pneumophila also replicated in singly infected and superinfected human DC at statistically similar rates. Host cell lysis also occurred by 24 h p.i., allowing a secondary round of infection. In DC, the fold increase in CFU was significantly lower than the yield from macrophages over a shorter time course with 27- and 16-fold increases in CFU in singly infected and superinfected cells, respectively. The lower absolute increase in CFU from DC relative to macrophages likely reflects the fact that the starting inoculum was not removed from infected cells.

**DISCUSSION**

Signals involved in maturation and maintenance of lysosome-like C. burnetii replication vacuoles do not appear to act

**FIG. 2. Growth of L. pneumophila in C. burnetii-infected Vero cells.** Vero cells infected with C. burnetii (phase II) for 48 h were superinfected with wild-type L. pneumophila or the isogenic dotA mutant. Vero cells were singly infected with L. pneumophila as a control. Intracellular L. pneumophila were released from host cells at 2, 24, and 48 h p.i., and CFU assays performed. The 0 h time point represents the titer of the starting inoculum prior to washing extracellular organisms from the monolayer at 2 h p.i. Wild-type L. pneumophila CFU increased 53- and 86-fold between 2 and 48 h p.i. in singly infected (solid line with squares) and superinfected cells (dotted line with squares), respectively. No replication of the dotA mutant was observed in singly infected (dotted line with circles) or superinfected (solid line with circles) cells. The results are expressed as the mean from three independent experiments with error bars representing the standard deviation.
in trans to circumvent Dot/Icm-dependent signals that promote formation of *L. pneumophila* replication vacuoles. Likewise, factors that promote development of *L. pneumophila* replication vacuoles are apparently specific to this compartment and do not disrupt acidification or the structural integrity of existing *C. burnetii* vacuoles. Consistent with this model are results showing that *L. pneumophila* and *C. burnetii* replicate in distinct vacuoles within superinfected BMDM, human macro-

FIG. 3. Replication vacuole formation in murine BMDM infected with *C. burnetii* and/or *L. pneumophila*. BMDM from A/J mice were infected with phase I *C. burnetii* (Cb I; arrows) for 48 h and then superinfected with *L. pneumophila* (Lp; arrowheads) for 18 h. BMDM singly infected with phase I or phase II *C. burnetii* (Cb II; arrows) for 66 h or *L. pneumophila* for 18 h were also evaluated. Live cells were stained with the acidotropic base acridine orange, and the same field was viewed by phase-contrast and fluorescence microscopy. Fixed cells were examined for localization of the lysosomal glycoprotein LAMP-2 by indirect immunofluorescence. In singly infected cells, acridine orange was sequestered by replication vacuoles harboring both phase I *C. burnetii* and *L. pneumophila*. In singly infected cells, LAMP-2 (green) localized to the vacuole membrane surrounding both phase II (red) and phase I *C. burnetii* (red), as well as *L. pneumophila* (red). In phase II *C. burnetii*-infected cells, single organisms were observed scattered throughout the cytoplasm tightly bounded by a LAMP-2-positive membrane. In *C. burnetii* phase I- or *L. pneumophila*-infected cells, replicating organisms were observed in multiple large replication vacuoles. In superinfected cells, LAMP-2 (green) also localized to vacuoles harboring phase I *C. burnetii* (blue) and *L. pneumophila* (red). In no instance were phase I *C. burnetii* and *L. pneumophila* observed in the same vacuole. Images are representative of three independent experiments where at least 100 singly infected or superinfected cells were examined.
phages, and DC, professionally phagocytic cell types, and within Vero epithelial cells. With the exception of murine BMDM, *L. pneumophila* resides in a nonacidic, LAMP-negative compartment. The occurrence of pathogen-specific replication vacuoles in murine BMDM is somewhat surprising since in this cell type *L. pneumophila* replicates in acidic, LAMP-positive vacuoles, and the *C. burnetii* vacuole is promiscuously fusogenic with vacuole compartments having lysosomal characteristics (24). Effectors of *C. burnetii* vacuole formation also do not disrupt development of *T. gondii* and *C. trachomatis* vacuoles in doubly infected cells (21, 45) However, unlike the *L. pneumophila* vacuole, replication vacuoles of these organisms have negligible interactions with the endocytic pathway (21, 25, 45). Conversely, effectors of *C. burnetii* replication vacuole formation appear to override regulatory mechanisms that normally stall maturation of vacuoles harboring *M. avium* or *M. tuberculosis* at an early endosome stage (11, 15, 18, 46).

The occurrence of distinct replication vacuoles in superinfected cells indicates that *L. pneumophila* and *C. burnetii* effectors of vacuole maturation specifically act in cis to mediate their formation. This specificity may reflect the fact that, although the genomes of *L. pneumophila* and *C. burnetii* encode similar Dot/Icm secretion systems (42, 44, 52), to date, nearly all defined and putative *L. pneumophila* type IV effectors lack homologs in *C. burnetii* (9, 10, 43). Type IV secretion may also be differentially regulated by the two pathogens. In *L. pneumophila*, type IV secretion is required prior to and immediately after infection for establishment of a replication vacuole in BMDM (40). Once the replication vacuole is formed, continued expression of dot/icm is not required for intracellular growth (12, 40). Conversely, continuous synthesis of *C. burnetii* protein (possibly translocated by a type IV apparatus) is required for maintenance of the spacious character and fusogenicity of its replication vacuole (24).

Further evidence supporting noncompetitiveness of *L. pneumophila* and *C. burnetii* replicative vacuoles are data showing that *L. pneumophila* replicate at approximately the same rate in singly infected and *C. burnetii*-infected human macrophages and DC. Indeed, in Vero cells, *L. pneumophila* grows more robustly in *C. burnetii*-infected cells. This behavior may be due to nonlytic growth of *L. pneumophila* in this cell type, combined with the possibility that *C. burnetii* infection upregulates nutrient trafficking pathways that augment *L. pneumophila* replication. Our results suggest that host cells can metabolically adapt to support vigorous growth of two intracellular pathogens that possibly exploit a common pathway for nutrient acquisition. Alternatively, pathogens may adapt to exploit non-competitive nutrient sources.

The idea that the *C. burnetii* vacuole is toxic to, and/or nutritionally deficient for, *L. pneumophila* is supported by data showing dotA mutants and wild-type organisms do not replicate in this compartment in Vero cells and human DC, respec-

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FIG. 4. Replication vacuole formation in human primary macrophages infected with *C. burnetii* and/or *L. pneumophila*. Cells were infected with phase II *C. burnetii* (Cb; arrows) for 36 h and then superinfected with *L. pneumophila* (Lp; arrowheads) for 12 h. Cells singly infected with *C. burnetii* for 48 h or *L. pneumophila* for 12 h were used as controls. Fixed cells were examined for localization of the lysosomal glycoprotein LAMP-1 by indirect immunofluorescence. In singly infected cells, LAMP-1 (green) localized to the membrane of distinct replication vacuoles harboring *C. burnetii* (red) but not *L. pneumophila* (red). In superinfected cells, LAMP-1 (green) also localized to the *C. burnetii* (blue) replication vacuole membrane but not to the *L. pneumophila* (red) replication vacuole membrane. In no instance were *C. burnetii* and *L. pneumophila* observed in the same replication vacuole. Images are representative of three independent experiments in which at least 100 singly infected or superinfected cells were examined.

FIG. 5. Replication vacuole formation in human primary DC infected with *C. burnetii* and/or *L. pneumophila*. Cells were infected with phase II *C. burnetii* (Cb; arrows) for 36 h and then superinfected with *L. pneumophila* (Lp; arrowheads) for 12 h. Cells singly infected with *C. burnetii* for 48 h or *L. pneumophila* for 12 h were used as controls. Fixed cells were examined for localization of the lysosomal glycoprotein LAMP-1 by indirect immunofluorescence. Representative electron micrographs of infected cells are also depicted. In singly infected cells, LAMP-1 (green) localized to the membrane of distinct replication vacuoles harboring *C. burnetii* (red) but not *L. pneumophila* (red). In superinfected cells, LAMP-1 (green) also localized to distinct vacuoles harboring replicating *C. burnetii* (blue) but not replicating *L. pneumophila*. On rare occasions one or two *L. pneumophila* organisms were observed in *C. burnetii* replication vacuoles in superinfected cells (lower dual-infection panels). *L. pneumophila* are distinguished from *C. burnetii* in electron micrographs by their larger size (>1 μm in length), uniform rod morphology, electron-translucent poly-3-hydroxybutyrate granules, and lack of filamentous chromatin. Images are representative of three independent experiments where at least 100 singly infected or superinfected cells were examined.
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tively. Lack of replication is likely not due to acidity per se because, as demonstrated here and elsewhere (47), *L. pneumophila* replicate in acidic vacuoles in BMDM. Moreover, *dotA* mutants can replicate when presented with an appropriate intracellular environment, since synchronous infection of this strain with wild-type *L. pneumophila* results in coinhabited vacuoles where both strains replicate (12). Interestingly, the *C. burnetii* vacuole is permissive for growth of the more distantly related organisms *M. avium* and *L. amazonensis* (18, 51). Human DC were the only cell type where trafficking of wild-type *L. pneumophila* to the *C. burnetii* replication vacuole was observed, albeit at a very low frequency. This specific trafficking behavior may reflect the dominant antigen presentation pathway of DC (31).

As demonstrated here and elsewhere (22, 53), trafficking of *L. pneumophila* to an acidic, LAMP-positive vacuole does not occur in primate macrophages, DC, and epithelial cells. Thus, the lysosomal trafficking observed in murine BMDM is likely a host cell-directed process that is not required for pathogen replication. After internalization by BMDM from A/J mice, *L. pneumophila* initially avoid the endosomal network and delay phagosome maturation by a mechanism that requires *dot/icm* type IV secretion (4, 6, 22). The *L. pneumophila* vacuole first acquires ER markers (22, 26, 49) and, after 8 to 12 h, during which time the bacterium differentiates from the TF to RF, the replication vacuole acquires markers of terminal lysosomes, including low pH, LAMP-1, fluid-phase markers, and cathepsin D (47). Replication of *L. pneumophila* in lysosomal and nonlysosomal compartments also illustrates the metabolic flexibility of the organism. Conversely, *C. burnetii* absolutely requires an acidic environment for replication (20).

Both replicating virulent phase I *C. burnetii* and nonreplicating avirulent phase II organisms traffic to LAMP-positive vacuoles in A/J mouse BMDM. The lack of replication of phase II bacteria is consistent with a previous study showing little replication of this variant in BMDM derived from a number of mouse strains (55). In contrast to murine BMDM, we observed vigorous replication of phase II *C. burnetii* in acidic, LAMP-positive vacuoles of human macrophages and DC. This result contrasts with previous reports showing no replication and subsequent killing of phase II organisms in primary human monocytes and human monocyte-like lines (8, 17). These studies reported that phase II, but not phase I *C. burnetii*, engage complement receptor 3 to result in delivery of the organism to a microbial lysosomal compartment (8, 17). Maturation of vacuoles containing phase I is proposed to stall at a late endosome stage (17). Permissiveness for *L. pneumophila* growth in DC also appears to be host species specific. Here we show proficient replication of *L. pneumophila* in human DC, whereas a previous study demonstrated severe growth restriction in murine DC (36). These disparate results may be due to innate differences between human and mouse DC.

In conclusion, both *L. pneumophila* and *C. burnetii* appear exquisitely adapted for replication in unique vacuolar environments that noncompetitively coexist within the same cell. *L. pneumophila* is the more adaptable intracellular pathogen since it can prosper in either a lysosomal or nonlysosomal replication vacuole during its infectious cycle.

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REFERENCES


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