Coxiella burnetii Type IV Secretion-Dependent Recruitment of Macrophage Autophagosomes

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Coxiella burnetii is an intracellular Gram-negative bacterium that causes human Q fever, a flu-like disease that can progress to chronic, life-threatening endocarditis. In humans, C. burnetii infects alveolar macrophages and promotes phagosomal fusion with autophagosomes and lysosomes, establishing a unique parasitophorous vacuole (PV) in which to replicate. The pathogen uses a Dot/Icm type IV secretion system (T4SS) to deliver effector proteins to the host cytoplasm, where they alter cellular processes to benefit the pathogen. The T4SS is required for PV expansion and prevention of apoptosis, but little else is known about the role of the system during intracellular growth. Recent reports suggest that C. burnetii actively recruits autophagosomes to the PV to deliver nutrients to the pathogen and provide membrane for the expanding vacuole. In this study, we examined the role of the T4SS in mediating PV interactions with autophagosomes. We found that the autophagy-related proteins LC3 and p62 localized to wild-type PV but not to T4SS mutant organism-containing phagosomes in human macrophage-like cells, primary human alveolar macrophages, and Chinese hamster ovary cells. However, while lipidated LC3 levels were elevated regardless of T4SS activity, no p62 turnover was observed during C. burnetii growth in macrophages, suggesting that the pathogen recruits preformed autophagosomes. When the T4SS was activated 24 h after infection, autophagosome recruitment ensued, indicating that autophagosome interactions are dispensable for initial PV maturation to a phagolysosome-like compartment but are involved in vacuole expansion. Together, these results demonstrate that C. burnetii actively directs PV-autophagosome interactions by using the Dot/Icm T4SS.

Coxiella burnetii is the highly infectious intracellular bacterial agent of human Q fever. Humans typically inhale C. burnetii while working with infected livestock and present with influenza-like symptoms of acute Q fever, including high fever and pneumonia (1). Chronic Q fever, which is less common but severe, often presents as endocarditis, a condition associated with a high fatality rate that is notoriously difficult to treat with current antibiotics (2). C. burnetii is considered an emerging pathogen, and a recent outbreak of Q fever in the Netherlands underscores our lack of understanding of this underdiagnosed disease and understudied pathogen (3, 4). However, at the root of Q fever is the inability of large numbers of LCVs, containing millions of bacteria, to replicate within a lysosome-like parasitophorous vacuole (PV) in eukaryotic cells (5).

Following inhalation by a mammalian host, C. burnetii targets alveolar macrophages, which engulf the organism by canonical phagocytosis. The initial C. burnetii-containing, tightly fitting phagosome is decorated with markers of early phagosomes, such as Rab5, and interacts with autophagosomes, as evidenced by the presence of LC3 on the phagosome membrane (6). Following an initial 4- to 6-h stall at the early phagosomal stage (7), the C. burnetii-containing compartment fuses with lysosomes and expands to occupy most of the host cell cytoplasm (8). During phagolysosomal maturation, the organism converts into an environmentally stable small-cell variant (SCV) into a replicatively active large-cell variant (LCV) (9). LCVs replicate for many days within an expanding PV that contains elements of secondary lysosomes, including an acidic luminal pH (~5.0) and degradative proteases, such as cathepsin D. In fact, other bacteria, such as Escherichia coli, are destroyed in the C. burnetii PV, demonstrating the degradative, lysosomal nature of the vacuole (10). Following accumulation of large numbers of LCVs, C. burnetii converts back into SCVs, which are released from heavily infected cells by an undefined mechanism. Many infection events, including PV formation and prevention of host cell death, are controlled by C. burnetii proteins secreted by a Dot/Icm type IV secretion system (T4SS) (11, 12).

Autophagy is a eukaryotic remodeling system that processes and removes damaged organelles, misfolded proteins, and intracellular pathogens via delivery to lysosomes for degradation (13). Autophagy initiates with formation of a phagophore that surrounds cargo, elongates, and eventually closes to form a double-membrane autophagosome. Autophagosome formation requires the activity of phosphatidylinositol 3-kinase and numerous Atg proteins originally identified in Saccharomyces cerevisiae, with Atg5 and Atg12 controlling elongation of the phagophore. As the phagophore forms a double-membrane autophagosome, Atg12 is covalently conjugated to a cysteine residue in Atg5, with C-terminal membranes, followed by LC3 allowing for LC3 to undergo lipidation, allowing for LC3 to coat the autophagosome. LC3 interacts with Atg3 and Atg7 to seal the autophagosome, which then matures into an autophagolysosome upon fusion with lysosomes. Prior to sealing, LC3 is lipidated, converting from LC3-I to LC3-II, and this conversion is often monitored as a read-out of autophagic activity (14). Macroautophagy is typically used to engulf large amounts of cytoplasm, organelles, and proteins (15), and selective forms of macroautophagy, such as mitophagy, have been described (16).
A form of macroautophagy termed xenophagy is used to dispose of infectious bacteria in an autophagolysosome (17). However, autophagosome fusion with the C. burnetii PV is predicted to provide a membrane source for the expanding vacuole and nutrients for harbored bacteria. During infection of Chinese hamster ovary (CHO) cells, the PV membrane is labeled with LC3, and treated of infected cells with the autophagy antagonist 3-methyladenine (3-MA) or wortmannin prevents typical vacuole formation (6). In contrast, induction of autophagy stimulates PV formation in CHO cells, enhancing C. burnetii replication (18). PV-autophagosome interactions are abolished in the presence of chloramphenicol, an antibiotic that inhibits bacterial protein synthesis, indicating that C. burnetii actively directs recruitment of autophagosomes (19). A recent study also showed that the autophagic protein Beclin-1 decorates the PV membrane (20), further supporting a role for autophagosome interactions in C. burnetii parasitism of host cells.

Although previous studies demonstrated PV fusion with autophagosomes, this event was not assessed in the pathogen's in vivo target cell, the human macrophage, nor was the role of the T4SS directly analyzed. In the current study, we assessed the subcellular localization of autophagy-related proteins in human macrophage-like cells and primary human alveolar macrophages (hAMs). We found that C. burnetii effector proteins control interactions with autophagosomes in macrophages and that fusion with autophagosomes at early stages of infection is dispensable for PV fusion with lysosomes. Finally, we show, for the first time, the presence of host p62 (sequestosome-1) on wild-type PV membranes, suggesting that presence of host p62 (sequestosome-1) on wild-type PV membranes, enhancing C. burnetii replication (18). PV-autophagosome interactions are abolished in the presence of chloramphenicol, an antibiotic that inhibits bacterial protein synthesis, indicating that C. burnetii actively directs recruitment of autophagosomes (19). A recent study also showed that the autophagic protein Beclin-1 decorates the PV membrane (20), further supporting a role for autophagosome interactions in C. burnetii parasitism of host cells.

Immunoblot analysis. Cells were infected with C. burnetii in 6-well plates for the indicated times. Cells were harvested in lysis buffer (50 mM Tris, 5 mM EDTA, 1% sodium dodecyl sulfate [SDS]) containing protease and phosphatase inhibitor cocktails (Sigma) by 10 passages through a 26-gauge syringe. Total protein concentrations were determined using a DC protein assay (Bio-Rad). Ten micrograms of total protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and then transferred to a 0.2-μm-pore-size polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked at room temperature for 1 h in TBS (150 mM NaCl, 100 mM Tris-HCl [pH 7.6]) containing 0.1% Tween 20 and 2.5% nonfat milk. Equal loading was confirmed by probing membranes with an anti-B-tubulin mouse monoclonal antibody (clone SAP.4G5; Sigma) overnight at 4°C. An anti-LC3 rabbit monoclonal antibody (clone D11; Cell Signaling) was used to detect LC3-I (16 kDa) and LC3-II (14 kDa). p62 (sequestosome-1) was detected using a mouse monoclonal (clone 2C11; Sigma) or rabbit monoclonal (clone D101E10; Cell Signaling) antibody. Reacting proteins were detected by enhanced chemiluminescence, using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) and SuperSignal Pico and Femto substrates (Thermo).

Fluorescence microscopy. Cells cultured on 12-mm glass cover slips were washed with cold PBS and then fixed and permeabilized with 100% cold methanol for 3 min. After washing, slides were blocked with 0.5% bovine serum albumin (BSA; Cell Signaling) in PBS overnight at 4°C. C. burnetii was detected using the rabbit monoclonal antibody listed above in 0.5% BSA. A mouse monoclonal CD63 antibody (clone HcA5; BD Biosciences) was used to detect PV membranes. LAMP-1 was detected using a monoclonal antibody (clone HA43) obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology (Iowa City, IA). Samples were incubated for 1 h at room temperature and then washed with cold PBS. Next, samples were incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen) in PBS overnight at 4°C. After washing, cells were incubated with 4′,6-di- amidino-2-phenylindole (DAPI; Invitrogen) at room temperature for 5 min to detect host and bacterial DNAs. Cover slips were mounted on glass slides with Mowiol and visualized under oil immersion, using a 60 × objective and a Nikon Ti-U microscope (Nikon). Images were obtained using a D5-QIMR digital camera and analyzed with NIS-Elements software (Nikon). For quantification, at least 100 cells per sample were scored for the presence of LC3 on the PV membrane. A Nikon Ti-Eclipse confocal microscope was used to acquire primary hAM images.

RESULTS

C. burnetii engages autophagosomes in a T4SS-dependent manner. To probe the requirement of the Dot/Icm T4SS for PV-au-

MATERIALS AND METHODS

C. burnetii. Coxella burnetii Nine Mile phase II (NMI; also known as RSA439) organisms and IcmD mutant bacteria (icmD::Tn) (11) were propagated in acidified citrate cyanide medium (ACCM) at 37°C in 5% CO2 and 2.5% O2. After 7 days of growth, organisms were collected by centrifugation, washed three times in sucrose phosphate buffer, and stored at −80°C. IcmD mutant organisms were cultured in ACCM or with eukaryotic cells in the presence of kanamycin, and the inducible IcmD strain was incubated with kanamycin and chloramphenicol. IcmD expression was triggered by addition of 200 ng/ml or 400 ng/ml anhydrotetracycline (ATc; Sigma) to ACCM or eukaryotic cultures, respectively, as previously described (11). Autophagy was stimulated in control, uninfected cells by incubation in Earle’s balanced salt solution (EBSS) or serum-free medium for 2 to 48 h.

Mammalian cell culture. THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS; Invitrogen) at 37°C and 5% CO2. For infections, THP-1 cells were cultured in 6-well or 24-well tissue culture plates with 200 nM phorbol 12-myristate 13-acetate (PMA; EMD Biosciences) for 18 h to differentiate cells into adherent, macrophage-like cells. PMA-containing medium was then removed and replaced with PMA-free medium. Infections were performed by adding organisms to the culture medium at a multiplicity of infection (MOI) of ~10, and this point was considered to be 0 h postinfection (hpi). CHO cells stably expressing either LC3-GFP or green fluorescent protein (GFP) alone were grown in modified Eagle’s alpha medium (Invitrogen) supplemented with 10% FBS at 37°C in 5% CO2. Geneticin (0.2 mg/ml) was added to cells for plasmid selection and removed for infection experiments. Infection of CHO cells with C. burnetii was performed by addition of organisms to cells at an MOI of ~50 (time = 0 hpi). Rapamycin (Enzo Life Sciences) was used as a positive control for activation of autophagy. CHO cells were treated with 50 μg/ml rapamycin for 2 h, while THP-1 cells were treated with 25 μg/ml rapamycin for 2 h, prior to assessment by immunoblotting or fluorescence microscopy.

Primary hAMs were isolated by bronchoalveolar lavage from lung tissue postmortem (National Disease Research Interchange) as previously described (21). No donors were excluded unless obvious contamination, indicative of infection, was observed. Cells were cultured in Dulbecco’s modified Eagle medium-F-12 medium containing 10% FBS, penicillin (50 U/ml), streptomycin sulfate (50 μg/ml), gentamicin sulfate (50 μg/ml), and amphotericin B (0.25 μg/ml) (Invitrogen) prior to infection. For electron microscopy, hAMs were infected for 72 h, washed with phosphate-buffered saline (PBS), and then fixed on ice for 15 min in PBS containing 4% paraformaldehyde and 2.5% glutaraldehyde (Invitrogen). Cells were then gently scraped from plates and incubated in fixative for an additional 45 min at room temperature. Cells were then resuspended in PBS and analyzed by Wandy Beatty at the Washington University School of Medicine Molecular Imaging Facility.

Fluorescence microscopy. Cells cultured on 12-mm glass coverslips were washed with cold PBS and then fixed and permeabilized with 100% cold methanol for 3 min. After washing, slides were blocked with 0.5% bovine serum albumin (BSA; Cell Signaling) in PBS overnight at 4°C. C. burnetii was detected using the rabbit monoclonal antibody listed above in 0.5% BSA. A mouse monoclonal CD63 antibody (clone HcA5; BD Biosciences) was used to detect PV membranes. LAMP-1 was detected using a monoclonal antibody (clone HA43) obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology (Iowa City, IA). Samples were incubated for 1 h at room temperature and then washed with cold PBS. Next, samples were incubated with Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen) in 0.5% BSA for 1 h at room temperature. After washing, cells were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) at room temperature for 5 min to detect host and bacterial DNAs. Cover slips were mounted on glass slides with Mowiol and visualized under oil immersion, using a 60 × objective and a Nikon Ti-U microscope (Nikon). Images were obtained using a D5-QIMR digital camera and analyzed with NIS-Elements software (Nikon). For quantification, at least 100 cells per sample were scored for the presence of LC3 on the PV membrane. A Nikon Ti-Eclipse confocal microscope was used to acquire primary hAM images.

RESULTS

C. burnetii engages autophagosomes in a T4SS-dependent manner. To probe the requirement of the Dot/Icm T4SS for PV-au-
tophagosome interactions, we initially used an established CHO cell model that overexpresses GFP-tagged LC3 as an autophagosome marker (6). CHO cells expressing LC3-GFP or GFP alone were infected with wild-type C. burnetii or T4SS-defective bacteria (IcmD mutant) for 4, 24, or 48 h and then processed for fluorescence microscopy. As shown in Fig. 1, PV containing wild-type C. burnetii were decorated with LC3 from 24 to 48 hpi but not at 4 hpi. In contrast, T4SS-deficient organism-containing phagosomes were not significantly decorated with LC3 at any time point examined. When quantified, approximately 95% of wild-type C. burnetii PV were LC3 positive from 24 to 48 hpi, while only approximately 10% of IcmD mutant-containing phagosomes were labeled with LC3-GFP at the same times postinfection. These results indicate that C. burnetii actively promotes interactions with autophagic vesicles by using the Dot/Icm T4SS.

PV-autophagosome interactions occur in human macrophages. Because previous studies defined PV-autophagosome interactions within the context of nonhuman cells, we next assessed whether C. burnetii directs PV-autophagosome interactions in the THP-1 in vitro model of human macrophage infection. Following differentiation into macrophage-like cells, THP-1 cells were infected with wild-type or IcmD mutant C. burnetii for 4, 24, 48, 72, or 96 h. A longer time course was assessed in THP-1 cells to determine if the pathogen promotes interactions with autophagosomes throughout growth in macrophages. Approximately 80% of wild-type C. burnetii PV were decorated with native LC3 (present as punctate spots) in THP-1 cells from 24 to 96 hpi, while only 15 to 20% of IcmD mutant-containing compartments colocalized with LC3 at any time postinfection (Fig. 2). Together, the CHO and THP-1 cell results indicate that C. burnetii actively directs autophagosome recruitment via T4SS effector protein activity and that these interactions are involved in parasitism of macrophages.

We recently developed a primary hAM model of C. burnetii infection to provide human disease relevance for in vitro studies (21). Here we used the hAM model to probe whether autophagosomes are recruited to the PV in the pathogen’s in vivo target cell. When assessed by electron microscopy, infected hAMs contained numerous double-membrane compartments adjacent to and fusing with the PV, delivering enclosed material to the vacuole lumen (Fig. 3). Double-membrane compartments in the eukaryotic cell cytosol are reliable indicators of autophagic vesicles (22), suggesting that autophagosomes are recruited to and fuse with the PV in primary hAMs. To confirm that these compartments were autophagosomes, infected hAMs were subjected to confocal microscopy analysis using an LC3-specific antibody. As shown in Fig. 3, LC3 trafficked to approximately 70% of wild-type PV in hAMs, while only 15 to 20% of IcmD mutant-containing phagosomes were decorated with LC3. These results demonstrate that T4SS-dependent PV-autophagosome interactions occur during C. burnetii growth in its target human cell.

Early autophagosome interactions are dispensable for PV fusion with lysosomes. Previous studies demonstrated PV-autophagosome interactions in CHO cells as early as 5 min postinfection (19), a time when C. burnetii is still in the SCV form, which does not replicate or display metabolic activity. However, protein synthesis inhibitors prevented this early association (19), suggesting that C. burnetii manipulates the autophagic response from

![FIG 1 LC3 localization in C. burnetii-infected CHO cells. LC3-GFP- or GFP-expressing CHO cells were infected with wild-type or T4SS-deficient (IcmD mutant) C. burnetii and processed for fluorescence microscopy at 4, 24, and 48 hpi. DAPI was used to stain DNA (blue). Meanings for other colors are indicated in the figure. LC3 localization around the PV indicates autophagosome fusion with the PV. Numbers of LC3-positive PV were determined for at least 100 cells in triplicate; error bars indicate standard errors of the means. *, P < 0.0001 according to the Student t test. Bar, 10 μm. Wild-type C. burnetii PV were positive for LC3 at 24 and 48 hpi, while significantly fewer IcmD mutant PV were positive for LC3, indicating that the T4SS is required for autophagosome recruitment. No colocalization was seen between GFP and PV (panels below the graph).]
within the nascent phagosome. To determine if early association with LC3 is essential for cellular infection, we used a T4SS-defective, ATc-inducible IcmD strain (11) in combination with the CHO cell model of autophagy and THP-1 cells. Cells were infected with noninduced C. burnetii for 24 h, and then ATc was added to stimulate IcmD expression. As shown in Fig. 4, noninduced organisms were enclosed in LC3-negative, CD63-positive THP-1 cell phagosomes, demonstrating that early phagosomes progressed to the lysosomal stage of maturation but failed to expand. However, when IcmD expression was induced, C. burnetii repli-

![Image of T4SS-dependent recruitment of LC3 to PV in THP-1 cells.](http://iai.asm.org/)
FIG 3 PV interact with autophagosomes in primary human alveolar macrophages. hAMs were infected for 72 h with wild-type C. burnetii and then processed for electron (A) or confocal (B) microscopy at the indicated times, using a polyclonal LC3 antibody. Antibodies were used to detect LC3, CD63, and C. burnetii, and nuclei were stained with DAPI (blue). Meanings for other colors are indicated in the figure. Double-membrane compartments indicative of autophagosomes fused with the PV (arrows) in hAMs, and native LC3 localized to the PV throughout infection. LC3-positive vacuoles were quantified for at least 100 cells under each condition from two different donors, and arrowheads indicate representative LC3 puncta. Error bars indicate standard deviations from the means. *, *P < 0.0001 according to the Student t test. Bar in panel B, 10 μm. A total of 65 to 80% of wild-type PV in hAMs colocalized with LC3-positive compartments throughout intracellular growth.
cation ensued, and the expanding PV were decorated with LC3 for the remainder of the infection of CHO cells and THP-1 cells (Fig. 4). These results further indicate that *C. burnetii* uses T4SS effector activity to regulate interactions with autophagosomes that are not absolutely required for initial maturation to a lysosomal compartment but, rather, are involved in PV expansion.

**LC3-II levels are elevated and p62 levels remain constant during *C. burnetii* intramacrophage growth.** During autophagosome formation, LC3 activity is altered following lipidation by phosphatidylethanolamine (PE), which promotes sealing of the phagophore prior to lysosomal fusion (23). Lipidation converts the protein from LC3-I to LC3-II, and this change is commonly monitored by immunoblot analysis in combination with fluorescence microscopy to confirm autophagy activation. Indeed, a previous study demonstrated an accumulation of LC3-II in *C. burnetii*-infected CHO cells overexpressing LC3-GFP from 72 to 96 hpi (19). In addition to LC3 levels, p62 (sequestosome-1) levels are also commonly analyzed, as this protein should be degraded upon LC3-mediated delivery to a lysosome (24). Therefore, autophagy activation is characterized by increasing levels of LC3-II.

**FIG 4** Early autophagosome recruitment is dispensable for PV fusion with lysosomes. CHO cells expressing LC3-GFP (A) or THP-1 cells (B) were infected with IcmD-inducible *C. burnetii* without ATc for 24 h, and then ATc was added and cells processed for immunofluorescence microscopy at 120 hpi. Antibodies were used to detect LC3, CD63, and *C. burnetii*, and nuclei were stained with DAPI (blue). Meanings for other colors are indicated in the figure. Bars, 10 μm. In the absence of IcmD production (−ATc), LC3 was not recruited to CD63-positive, pathogen-containing THP-1 cell phagosomes. In contrast, LC3 recruitment to the PV resumed in both cell types following induction of IcmD expression (+ ATc), further demonstrating that the pathogen uses T4SS effectors to control PV-autophagosome interactions.
In C. burnetii-infected THP-1 cells and hAMs, native p62 levels did not deviate from uninfected control cell levels during infection with wild-type or IcmD-deficient bacteria assessed from 4 to 96 hpi, while LC3-II levels were elevated above uninfected cell levels during wild-type and mutant infections of THP-1 cells, while p62 levels remained constant, suggesting that C. burnetii does not induce excess formation of autophagosomes by using the T4SS during intracellular growth. In contrast, induction of autophagy by starvation triggered elevated LC3-II levels and corresponding decreases in the level of p62 (Fig. 5B). These results indicate that while LC3-II levels are dynamic in macrophages, C. burnetii does not promote the turnover of autophagic p62 indicative of increased autophagic flux.

Ubiquitin-related p62 associates with LC3 at the PV membrane. Recent studies showed that eukaryotic cells use p62 to target intracellular pathogens for degradation in autophagolysosomes (25, 26). p62 also tethers ubiquitinated protein aggregates to autophagosomes via interactions with LC3, resulting in protein degradation (24). This process ultimately results in delivery of cytosolic proteins, including antibacterial molecules, or bacteria to the autophagolysosome. Because LC3 was recruited to wild-type C. burnetii PV, we also assessed p62 localization to determine if the protein associates with the vacuole. In THP-1 cells, p62 colocalized with LC3 (Fig. 6A) and lysosomal CD63 (Fig. 6B) on wild-type PV but not on IcmD mutant-containing phagosomes, suggesting that C. burnetii actively recruits host proteins that function at the autophagy-ubiquitination interface during infection. In contrast to the case with other intracellular pathogens (25, 26), p62 recruitment did not have an antibacterial effect, as C. burnetii replicated to high levels in p62-positive PV.

**DISCUSSION**

In the current study, we show that C. burnetii uses a Dot/Icm T4SS to regulate interactions with autophagy components in macrophages. Previous studies demonstrated that the PV fuses with LC3-positive compartments at early times postinfection and that this interaction requires bacterial protein synthesis (18, 19). PV fusion with autophagosomes is predicted to provide membrane for the growing vacuole and a nutrient pulse during the SCV-to-LCV transition prior to replication. However, previous studies did not assess the role of the Dot/Icm T4SS in PV-autophagosome interactions during infection of macrophages, the pathogen’s in vivo target cells. Actively promoting fusion with autophagosomes is an unusual activity for an intracellular pathogen. Indeed, autophagy is commonly used to envelop bacterial pathogens, followed by fusion with lysosomes to form an autophagolysosome and efficiently degrade harbored organisms, in a process termed xenophagy (17). For example, Legionella pneumophila, Mycobacterium tuberculosis, and Salmonella spp. actively prevent delivery to autophagolysosomes, allowing intracellular replication to high levels.
levels (27–29). In contrast, C. burnetii replicates throughout its infectious cycle within a PV highly similar to host cell lysosomes (5). Therefore, fusing with autophagosomes promotes development of this typically hostile replication niche.

To initiate this study, we used the established CHO cell model of autophagy to probe PV-autophagosome interactions, similar to the case in previous reports (18,19). This model allows simple visualization of LC3 trafficking and shows that LC3-GFP is present at most PV from 24 to 48 hpi. Romano et al. reported that LC3-GFP labels the C. burnetii PV as early as 5 min postinfection (19). In contrast, in our hands, no significant LC3-GFP labeling was detected at 4 hpi. One potential reason for this difference is that LC3 associated with individual bacteria may have been below the limits of detection in our analysis. However, CHO cell overexpression data suggest that this was not the case, as LC3 was easily detected in these cells. One difference between our experiments and previous studies is the use of in vitro-cultured C. burnetii at a low MOI (~10). When a higher MOI is used, large clumps of five or more bacteria are labeled with LC3 at 4 hpi (data not shown), while individual organisms are not. Because C. burnetii NMII lipopolysaccharide (LPS) is a Toll-like receptor 2 (TLR2) agonist (30), a higher MOI may stimulate excess TLR2-driven autophagosome formation. Additionally, C. burnetii does not translocate known effector proteins prior to 8 hpi (31), supporting results showing that neither individual wild-type nor T4SS-defective organisms recruit LC3 at 4 hpi. These results suggest that early (<4 hpi) interactions with autophagosomes are dispensable for the C. burnetii-containing phagosome to mature into an early lysosome-like PV but are likely required for optimal expansion of the vacuole.

In further support of these results, T4SS-defective organisms are enclosed in a phagosome that transits to a CD63-positive compartment (11) in the absence of LC3 labeling, indicating that the early phagosome matures to a tight-fitting phagolysosome in the absence of autophagosome interactions. We further assessed the importance of early PV-autophagosome interactions by using a recently constructed strain of C. burnetii containing icmD under the control of an ATc-inducible promoter (11). When icmD was not expressed, organisms behaved similarly to an icmD mutant and trafficked to a tight-fitting, CD63-positive lysosome devoid of LC3. However, when icmD expression was triggered at 24 hpi, the

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**FIG 6** p62 colocalizes with LC3 on wild-type C. burnetii PV membranes containing ubiquitinated proteins. THP-1 cells were infected with wild-type or IcmD mutant C. burnetii for 72 h, and a monoclonal p62 (A and B) antibody was used to assess protein recruitment to the PV. Bacteria were detected with a C. burnetii-specific antibody, and nuclei were detected using DAPI (blue). Meanings for other colors are indicated in the figure, and the arrowhead indicates representative p62 colocalization with LC3 at the PV membrane. Intensity profiles in panel B represent the region indicated by an arrow in each micrograph. Arrowheads indicate the wild-type PV limiting membrane or individual IcmD mutant organisms. Bars, 10 μm. p62 colocalized with LC3 on wild-type C. burnetii PV at 72 hpi but did not associate with IcmD mutant-containing phagosomes in THP-1 cells.
phagosome expanded, bacterial replication ensued, and the PV membrane was decorated with LC3. These results confirm the dispensability of PV-autophagosome interactions at early times postinfection for phagolysosome formation.

Although CHO cells are a reliable model of LC3 trafficking, they do not represent *C. burnetii*’s target cell during natural infection. To better understand the importance of autophagy in vivo, we used two models of *C. burnetii*-macrophage interactions. First, we used macrophage-like THP-1 cells, an established in vitro model of *C. burnetii*-host cell interactions (32–34). PV in THP-1 cells interacted with autophagosomes from 24 to 96 hpi, suggesting that these interactions occur throughout intracellular growth in macrophages. Additionally, IcmD mutant organisms trafficked to a lysosomal compartment in THP-1 cells but did not recruit LC3, further supporting a critical role for the T4SS during intramacrophage growth. Second, we used our newly developed primary hAM model to probe PV-autophagosome interactions in the cell type most relevant to human disease. Similar to the case in THP-1 cells, *C. burnetii* actively directed recruitment of hAM autophagosomes to the PV, in a T4SS-dependent manner. Together, these results confirm those derived from other cell models and lend disease relevance to the interaction between *C. burnetii* and autophagosomes.

The absence of p62 turnover during *C. burnetii* infection of macrophages suggests that the pathogen recruits preformed autophagosomes as opposed to purposely triggering autophagy during natural infection. This aspect of cellular infection represents a potential benefit for *C. burnetii*, as less energy would be devoted to continually stimulating autophagy. These results support a recent small interfering RNA (siRNA)-based study that found no substantial association of decreased autophagic protein expression with impaired PV formation (35). However, fusion with autophagosomes likely supports optimal PV expansion, and previous inhibitor-based studies indicate that autophagy is involved in optimal PV formation (6, 18). T4SS-defective organisms are unable to recruit autophagosomes, despite elevated LC3-II levels, further suggesting that *C. burnetii* actively targets preformed organelles. Although not absolutely required (6, 18), autophagosomes likely serve as a consistent nutrient and membrane source during *C. burnetii* growth in macrophages. However, in the absence of an autophagic response, *C. burnetii* may use other membrane sources present in macrophages actively engulfing extracellular material. Indeed, the PV is highly fusogenic and interacts with fluid-phase endosomes and early and late phagosomes, all of which could provide membrane for the expanding PV in the absence of autophagosome fusion.

Individual T4SS effectors directing *C. burnetii* interaction with autophagosomes are unknown. Over 100 effectors have been identified to date, but only 3, AnkG, CaaE, and CaeB, have known activities, with each protein preventing apoptotic cell death (36, 37). In two recent studies, we identified novel effectors encoded by *C. burnetii* plasmid genes that colocalize with LC3-positive compartments when ectopically expressed in human cells (38, 39). These effectors are candidate proteins for controlling PV-autophagosome interactions. However, numerous bacterial effectors often regulate replication vacuole fusion events. For example, *L. pneumophila* produces many Dot/Icm substrates that recruit host proteins, such as Rab GTPases, to the pathogen’s replication vacuole membrane, where they influence phagosome maturation (40–43). Thus, T4SS effector control of the *C. burnetii* PV-autophagosome interface and other vacuole biogenesis events is an area of research ripe for exploration. Finally, the T4SS-dependent presence of p62 at the PV membrane suggests that *C. burnetii* interacts with the host ubiquitination machinery. In support of these findings, T4SS-secreted CpeC colocalizes with p62-positive proteins in the cytoplasm of host cells (C. G. Winchell and D. E. Voth, unpublished results) and may be involved in ubiquitin-related PV formation and maintenance processes. Combined with results in the current study, these data indicate that *C. burnetii* purposefully interacts with two host processes, autophagy and ubiquitination, that are critical for host cell control of intracellular pathogen infection.

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