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ALTERATION OF HOST CELL UBIQUITINATION BY THE INTRACELLULAR
BACTERIAL PATHOGEN *COXIELLA BURNETII*

An Undergraduate Honors Thesis

in the

Department of Poultry Science

Submitted in partial fulfillment of the requirements for the
University of Arkansas
Dale Bumpers College of Agricultural, Food and Life Sciences
Honors Program

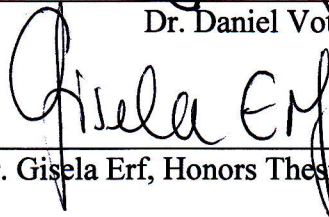
by

Lindsey Elizabeth Pritchett

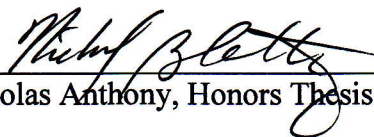
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ABSTRACT

The intracellular bacterial agent of Q fever, Coxiella burnetii, replicates within a phagolysosome-like parasitophorous vacuole (PV) in human macrophages and delivers effector proteins to the host cytosol via a Dot/Icm type IV secretion system (T4SS). T4SS effectors are critical for PV formation and prevention of host cell death that allows sufficient time for bacterial replication. Recruitment of ubiquitin-related components to the C. burnetii PV is also predicted to be involved in PV formation and bacterial replication and is likely controlled by effector proteins. In this study, we assessed the role of the Dot/Icm T4SS in regulating ubiquitination by comparing subcellular localization of ubiquitinated proteins between cells infected with wild type C. burnetii and a mutant that lacks a functional T4SS. Fluorescence microscopy showed ubiquitinated proteins surrounding wild type C. burnetii PV but not phagosomes harboring T4SS-defective organisms. Immunoblot analysis showed altered ubiquitinated protein profiles throughout infection, suggesting C. burnetii impacts post-translational modification of host cell and/or bacterial proteins. Future studies will determine how T4SS-mediated recruitment of ubiquitinated proteins impacts C. burnetii-host cell interactions and eventual development of disease.

INTRODUCTION

Ubiquitination is a post-translational protein modification used by eukaryotic cells to control protein fate and location. This three-step process begins with free ubiquitin binding to the E1 ubiquitin-activating enzyme followed by transfer of activated ubiquitin to the E2 ubiquitin-conjugating enzyme. Finally, the ubiquitin molecule is either tethered to its target substrate directly by the E3 ubiquitin-protein ligase or using an E3 adaptor that binds to E2 and the substrate. Eukaryotes encode two E1 proteins, 37 E2 proteins, and over 600 E3 ligases, providing specificity (Komander, 2009). Two general types of ubiquitination can occur and have distinct effects on protein activity. Mono-ubiquitination acts as a protein-targeting signal and often guides plasma membrane-associated proteins to endosomes (Hicke and Dunn, 2003; Patel *et al.*, 2009; Collins and Brown, 2010). Poly-ubiquitination targets proteins to the 26S proteasome or lysosomes for degradation (Thrower *et al.*, 2000; Chen and Sun, 2009). Poly-ubiquitination is typically controlled by the Skp1-Cul1-F-box (SCF) complex that functions as an E3 ligase (Jackson and Eldridge, 2002).

Many intracellular pathogens exploit host ubiquitination to alter host or bacterial protein stability and function. For example, *Listeria monocytogenes* secretes a pore-forming toxin known as listeriolysin O (LLO) that lyses endosomal membranes, allowing bacterial escape to the host cell cytoplasm and subsequent replication. LLO is then ubiquitinated for degradation to avoid destroying the host cell (Collins and Brown, 2010). *Salmonella typhimurium* uses a needle-like type III secretion system (TTSS) to secrete effector proteins, such as SopB, into the host cytosol. In the cytosol, SopB promotes bacterial replication following mono-ubiquitination (Collins and Brown, 2010). *Legionella pneumophila* also secretes effector proteins, via a Dot/Icm type IV secretion system (T4SS), which interact with ubiquitination machinery. Poly-

ubiquitinated proteins also accumulate on wild type *L. pneumophila* vacuoles (Ivanov and Roy, 2009).

Coxiella burnetii is an intracellular, Gram-negative bacterium that causes the zoonosis human Q fever. Humans are typically infected with *C. burnetii* following inhalation of contaminated aerosols while working with infected livestock (Marrie, 1990). Q fever presents acutely as a flu-like illness, but can also persist chronically, causing life-threatening endocarditis. Due to the flu-like nature of acute Q fever symptoms, many cases are misdiagnosed. However, *C. burnetii* has the potential for use as a biological weapon due to a low infectious dose (<10 organisms), aerosol-mediated transmission, and pronounced environmental stability (Williams, 1991; Gilk *et al.*, 2009). As a United States Center for Disease Control and Prevention category B select agent, the organism warrants extensive research. Additionally, the number of Q fever cases is on the rise worldwide and a better understanding of pathogenic mechanisms is needed to identify new therapeutics.

In vivo, *C. burnetii* displays tropism for alveolar macrophages and thrives within a lysosome-like compartment termed the parasitophorous vacuole (PV). PV formation and maturation occurs through the normal phagolysosomal pathway. The phagosome fuses with host autophagosomes, recruits GTPases involved in vesicle fusion (Rab5 and Rab7), and eventually acquires lysosomal hydrolases following lysosome fusion with the expanding vacuole. After maturation to a phagolysosome, the PV expands and is maintained by proteins that interact with the host cell following T4SS-mediated translocation to the cytosol (Gilk *et al.*, 2009).

Two recently identified T4SS effectors co-localize with ubiquitinated proteins when expressed in HeLa cells, suggesting the pathogen interacts with host ubiquitin machinery. CpeC is an effector protein that contains an F-box domain, which is part of the three-component SCF

ubiquitination complex in eukaryotic cells (Voth *et al.*, 2011). CpeL was also recently identified as an effector protein that co-localizes with ubiquitinated proteins (Maturana *et al.*, 2013). To test the hypothesis that *C. burnetii* uses T4SS effectors, such as CpeC and CpeL, to exploit host cell ubiquitin machinery, we compared subcellular localization of ubiquitinated proteins between cells infected with wild type *C. burnetii* and a mutant that lacks a functional T4SS. Ubiquitinated proteins were only recruited to wild type PV, suggesting secreted effector proteins direct ubiquitin-related events throughout intracellular growth.

MATERIALS AND METHODS

Bacteria and mammalian cell culture. Avirulent *C. burnetii* (Nine Mile phase II, clone RSA439) and a mutant strain with a disrupted *icmD* gene (Beare *et al.*, 2011) were used for infections in this study. Cells infected with the IcmD mutant were grown in the presence of kanamycin (375 µg/ml) for selection. THP-1 human monocytic cells and HeLa (human epithelioid carcinoma) cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C and 5% CO₂. Prior to infection, THP-1 cells were differentiated into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (200 nM) for 24 h as previously described (Voth *et al.*, 2007). In the final portion of this study, HeLa cells were grown in the presence of lactacystin (5 mM), a known proteasome inhibitor.

Immunofluorescence microscopy. After infection of THP-1 or HeLa cells with *C. burnetii* at a multiplicity of infection ~ 10 for 4, 24, 48, 72, or 96 hours, cells were fixed and permeabilized with 4% paraformaldehyde for 15 minutes, then blocked for 1 hour in PBS containing 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100 at room temperature. Cells were then incubated at room temperature with either mouse anti-FK1 antibody (1:100) or

mouse anti-FK2 antibody (1:250) and rabbit anti-*C. burnetii* antibody (1:1000) for 1 hour. Cells were then washed three times with ice-cold PBS and incubated for 1 hour in 0.3% Triton X-100-0.5% BSA-PBS containing AlexaFluor-488 or -594-conjugated secondary antibodies at room temperature. Cells were then washed twice with ice-cold PBS and incubated with DAPI for 5 minutes at room temperature to detect host and bacterial DNA. Fluorescence microscopy was performed using a Nikon Ti-U microscope with a 60X oil immersion objective. Images were obtained with a D5-QiIMc digital camera and analyzed using NIS-Elements software. PV were quantified from 5-10 fields, containing at least 10 cells/field. Wild type PV were compared to mutant-containing phagosomes at each time point. Graphpad software was used for statistical analyses, and significance of differences was assessed using a Student's t-test where $P < 0.01$.

Immunoblot analysis. After infection of THP-1 cells for 4, 24, 48, or 72 hours, cells were harvested in lysis buffer containing 1% sodium dodecyl sulfate (SDS) and total protein concentration was determined using a DC protein assay. Nine micrograms of total protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and immunoblotting was performed using mouse antibodies directed against ubiquitin-related components (1:1000; Table 1). Samples were also probed for equal protein loading using a mouse antibody directed against β -tubulin (1:1000). Reacting proteins were detected using either an anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:2000) and enhanced chemiluminescence following exposure to film.

RESULTS

Recruitment of ubiquitinated proteins to the *C. burnetii* PV. To determine if *C. burnetii* infection influences host ubiquitin processes, we first assessed whether ubiquitinated

proteins are recruited to the PV membrane similar to reports for other intracellular pathogen replication vacuoles (Ivanov and Roy, 2009; Huang *et al.*, 2012). Cells were assessed for ubiquitinated protein localization over the course of a 96 hour infection using fluorescence microscopy (Figure 1). Samples were incubated with FK1 antibody to detect poly-ubiquitinated proteins or FK2 antibody to detect mono- and poly-ubiquitinated proteins. FK1- and FK2-labeled proteins were present on wild type PV at all time points of infection with 40-60% of wild type PV decorating with FK1- and FK2-labeled proteins by 96 hours post infection (hpi). In contrast, only 10-20% of IcmD mutant-containing phagosomes associated with these proteins. These results indicate that mono- and poly-ubiquitinated proteins are recruited to the PV membrane in a T4SS-dependent fashion.

Of note, among wild type *C. burnetii*-infected cells, there was a high prevalence of large aggregations of ubiquitinated proteins. Similar structures, termed dendritic cell aggresome-like structures (DALIS), have been previously observed around *L. pneumophila*-containing vacuoles (Ivanov and Roy, 2009). Thus, future studies will examine the formation and potential importance of DALIS in *C. burnetii*-infected cells.

Alteration of ubiquitinated protein levels during *C. burnetii* infection. Because ubiquitinated proteins were recruited to the wild type PV membrane, we next assessed whether *C. burnetii* alters ubiquitinated protein profiles using immunoblot analysis (Figure 2). Levels of poly-ubiquitinated and mono-ubiquitinated proteins were analyzed using FK1 and FK2 antibodies, respectively. Over the course of infection (4-72 hpi), overall levels of FK1-labeled proteins decreased while levels of FK2-labeled proteins increased in wild type *C. burnetii*-infected cells. In contrast, levels of FK1- and FK2-labeled proteins remained constant in IcmD

mutant-infected cells. These results suggest *C. burnetii* uses T4SS effectors to promote accumulation of mono-ubiquitinated proteins at 48 hpi and beyond.

We next evaluated levels of Skp1, a critical component of the SCF complex, and UBC3 throughout intracellular growth (Figure 3). Over the course of infection, levels of Skp1 decreased, while levels of UBC3 increased. These results correlate with accumulation of mono-ubiquitinated proteins at later times post-infection. We also probed the presence of K48- and K63-linked proteins during infection (Figure 4). Differentiating these linkages is important because they exert differing effects on target protein function. K48-linked poly-ubiquitin chains serve proteolytic functions, while K63-linked chains are involved in non-proteolytic functions similar to mono-ubiquitinated proteins. As infection progressed, K48-linked protein levels decreased while K63-linked protein levels increased. These results further suggest that *C. burnetii* promotes accumulation of proteins involved in cellular localization processes (mono-ubiquitination), rather than proteasome-mediated degradation.

Proteasome inhibition does not affect infection progression. Previous results suggested that *C. burnetii* interacts with mono-ubiquitinated proteins. To assess the potential importance of poly-ubiquitination during intracellular growth, we treated *C. burnetii*-infected cells with lactacystin, a known proteasome inhibitor, and monitored PV formation. When comparing treated cells to untreated cells, there was no substantial change in PV formation or the progression of infection (Figure 5), further suggesting mono-ubiquitination events are more important for *C. burnetii* manipulation of host cells.

DISCUSSION

In the current study, we show that a functional T4SS is necessary for recruitment of ubiquitinated proteins to the *C. burnetii* PV and alteration of ubiquitinated protein levels. Ubiquitinated proteins label the PV only in wild type *C. burnetii*-infected cells, suggesting ubiquitination is involved in pathogenesis. Additionally, mono-ubiquitinated proteins and K63-linked poly-ubiquitin chains accumulate during infection, suggesting *C. burnetii* manipulates host cell and/or bacterial protein trafficking. Future studies will define a mechanism for T4SS-mediated recruitment of ubiquitin machinery and the impact of this event on *C. burnetii*-host interactions.

The decrease observed in poly-ubiquitinated proteins from 24-72 hpi could be attributed to infection-specific degradation. However, treatment of infected cells with the proteasome inhibitor lactacystin does not alter PV formation or bacterial replication. These results correspond to the presence of DALIS in wild type *C. burnetii*-infected cells. DALIS can extend the half-life of poly-ubiquitinated proteins by preventing proteasome-directed degradation (Lelouard *et al.*, 2004), and this may benefit *C. burnetii*. DALIS contain masses of ubiquitinated proteins (Ivanov and Roy, 2009) and sequester defective ribosomal products (DRiPs) targeted for degradation (Lelouard *et al.*, 2004). Previous studies also found the ubiquitin-related proteins E1 and E2 and the C-terminus of an E3 ligase in DALIS. Cells infected with closely-related T4SS-deficient *L. pneumophila* produce DALIS, while organisms with a functional T4SS fail to trigger DALIS formation. T4SS-deficient *L. pneumophila*, however, fails to avoid lysosome fusion and is degraded (Ivanov and Roy, 2009). In contrast to *L. pneumophila*, DALIS-like structures are present in wild type *C. burnetii*-infected cells, suggesting they are not detrimental to pathogen

viability. Future studies will define the role of DALIS formation in PV formation and *C. burnetii* parasitism of host cells.

Overall, the data obtained from this study contribute to increased understanding of the *C. burnetii*-host cell dynamic and provide new ubiquitin-related hypotheses for future testing. Specifically, the presence of DALIS in infected cells represents a novel area of research in *C. burnetii* pathogenesis. Additionally, linking ubiquitin modulation to a specific T4SS effector(s) will establish novel bacterial protein activity that could be targeted in design of new therapeutic strategies.

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Table 1*Antibody concentrations for Western blot analysis*

Antibody	Host	Specificity
anti-FK1	mouse	poly-ubiquitinated conjugates
anti-FK2	mouse	mono- and poly-ubiquitinated conjugates
anti-Skp1	rabbit	endogenous levels of total Skp1
anti-UBC3	rabbit	endogenous levels of total UBC3 and UBC3B
anti-K48	rabbit	poly-ubiquitin chains formed by Lys48 linkage
anti-K63	rabbit	poly-ubiquitin chains formed by Lys63 linkage

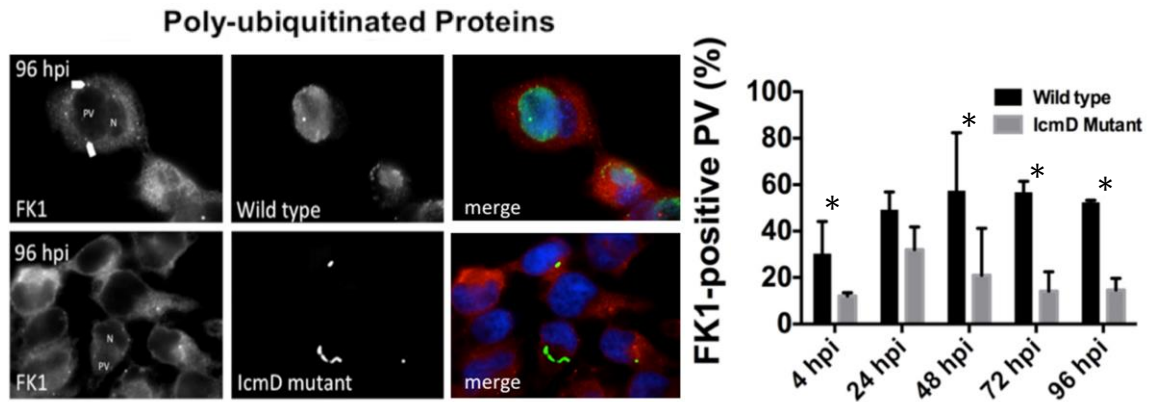
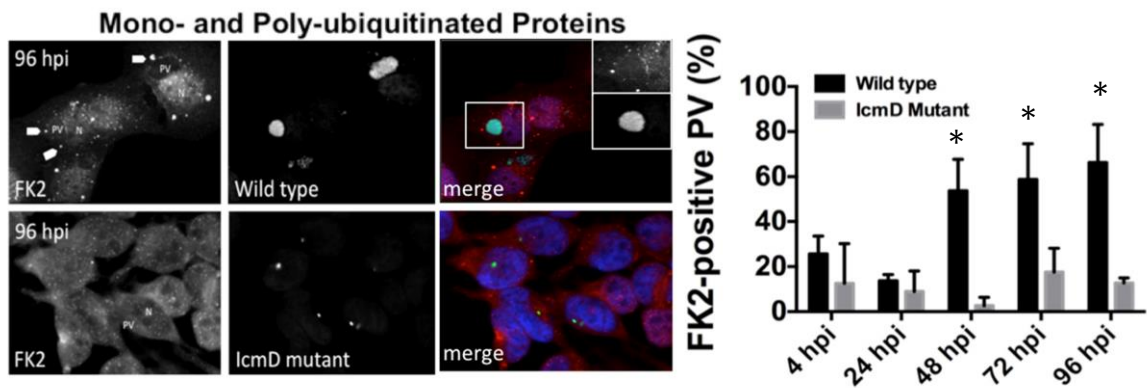
A**B**

Fig. 1. Recruitment of ubiquitinated proteins to the PV. HeLa cells were infected with wild type or T4SS-deficient (lcmD mutant) *C. burnetii* and prepared for fluorescence microscopy at 4, 24, 48, 72, and 96 hours post infection (hpi). DAPI was used to stain DNA. FK1- (A) and FK2- (B) labeled proteins were present on wild type PV (markers) from 24-96 hpi and fewer mutant-containing phagosomes. These results suggest that ubiquitinated proteins are recruited to wild type PV membranes during infection. Quantification showed that significantly more FK1+ and FK2+ vacuoles are present in wild type infections compared to lcmD-mutant infected cells. These results indicate that recruitment of ubiquitinated proteins to the PV is T4SS-dependent. Statistical analysis was performed using a Student's t-test. Asterisks indicate significant differences where $P < 0.01$

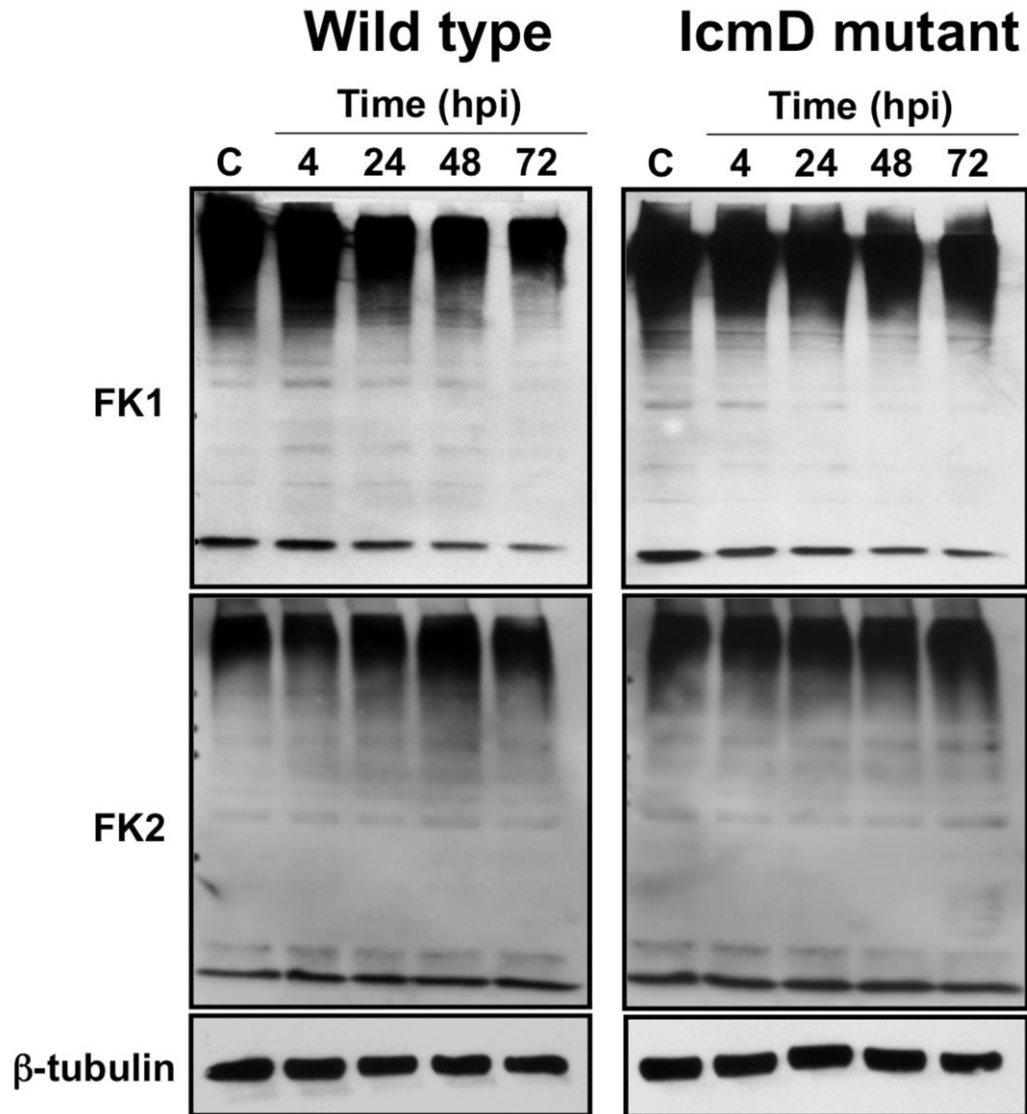


Fig. 2. Alteration of ubiquitinated protein levels in *C. burnetii*-infected cells. THP-1 cells were infected with wild type or IcmD mutant *C. burnetii* and cellular lysates harvested at 4, 24, 48, and 72 hpi. Lysates were assessed for ubiquitinated protein levels by immunoblot, and β -tubulin was probed to ensure equal protein loading. Immunoblot analysis showed decreasing levels of FK1-labeled proteins and increasing levels of FK2-labeled proteins in wild type-infected cells, suggesting *C. burnetii* promotes accumulation of mono-ubiquitinated proteins during intracellular growth.

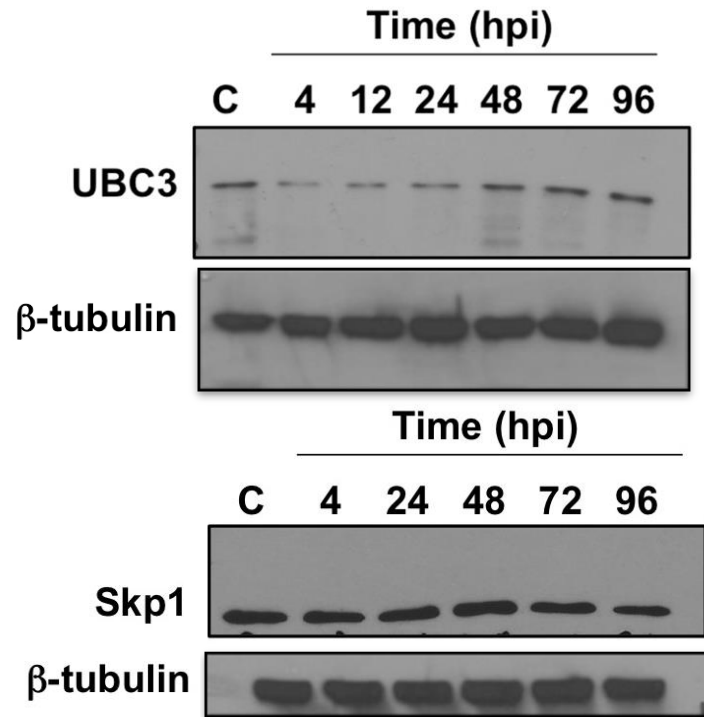


Fig. 3. Alteration of ubiquitin-related components in *C. burnetii*-infected cells. THP-1 cells were infected with wild type *C. burnetii* and cellular lysates harvested at various time points post-infection. Lysates were assessed for UBC3 and Skp1 levels by immunoblot. Analysis showed increasing levels of UBC3 and decreasing levels of Skp1 in wild type-infected cells, suggesting *C. burnetii* promotes accumulation of mono-ubiquitinated proteins.

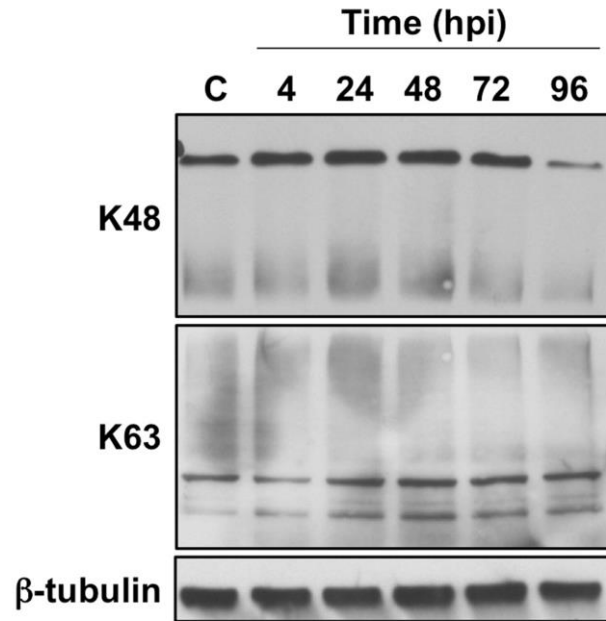


Fig. 4. K48- and K63-linked protein levels in *C. burnetii*-infected cells. THP-1 cells were infected with wild type *C. burnetii* and whole cell lysates were taken at 4, 24, 48, 72, and 96 hpi. Lysates were assessed for levels of K-48 and K-63 linkages using immunoblot, and β -tubulin was probed to ensure equal protein loading. Immunoblot analysis showed decreasing levels of K-48 linked poly-ubiquitin chains and increasing levels of K-63 linked chains. These results further suggest *C. burnetii* promotes accumulation of mono-ubiquitinated proteins involved in cellular localization processes.

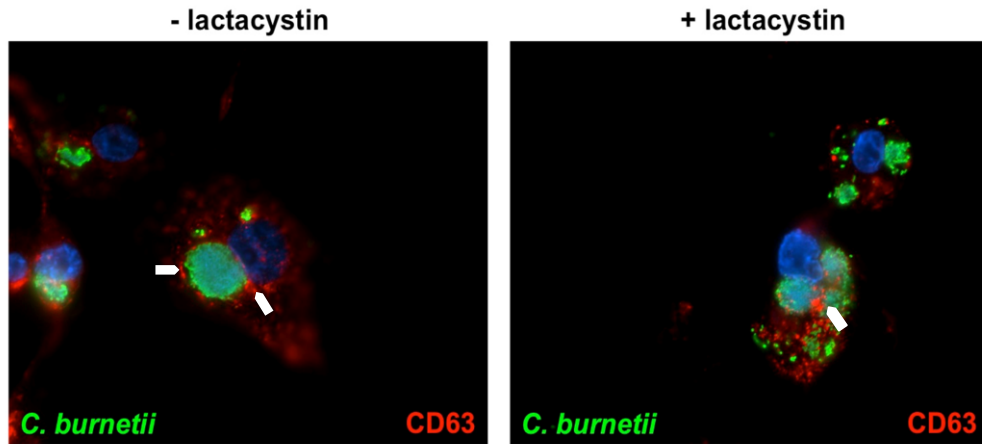


Fig. 5. Lactacystin treatment of wild type *C. burnetii*-infected cells. HeLa cells were infected with wild type *C. burnetii* and prepared for fluorescence microscopy at 4, 24, 48, 72, and 96 hours post infection (hpi). DAPI was used to stain DNA and CD63 was used to label the PV. There was no difference in the progression of infection between control (left) and lactacystin-treated cells (right). These results suggest mono-ubiquitinated proteins are more important for proper *C. burnetii* infection.