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Contrasting Lifestyles Within the Host Cell

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CHAPTER SUMMARY

Intracellular bacterial pathogens have evolved to exploit the protected niche provided within the boundaries of a eukaryotic host cell. Upon entering a host cell, some bacteria can evade the adaptive immune response of its host, and replicate in a relatively nutrient-rich environment devoid of competition from other host flora. Growth within a host cell is not without its hazards, however. Many pathogens enter their hosts through receptor-mediated endocytosis or phagocytosis, two intracellular trafficking pathways that terminate in a highly degradative organelle, the phagolysosome. This usually deadly compartment is maintained at a low pH, and contains degradative enzymes, and reactive oxygen species resulting in an environment to which few bacterial species are adapted. Some intracellular pathogens, like *Shigella*, *Listeria*, *Francisella*, and *Rickettsia* escape the phagosome to replicate within the cytosol of the host cell. Bacteria that remain within a vacuole either alter the trafficking of their initial phagosomal compartment or adapt to survive within the harsh environment it will soon become. In this chapter, we focus on the mechanisms by which different vacuolar pathogens either evade lysosomal fusion, as in the case of *Mycobacterium* and *Chlamydia*, or allow interaction with lysosomes to varying degrees, such as *Brucella* and *Coxiella*, and their specific adaptations to inhabit a replicative niche.

Keywords

Mycobacterium; *Chlamydia*; *Brucella*; *Coxiella*; endocytosis; phagocytosis; intracellular trafficking

INTRODUCTION

Bacterial pathogens that adopt an intracellular lifestyle often avoid many challenges that are faced by their extracellular counterparts. However, upon entering the host cell, they have a new set of trials with which to contend. Upon phagocytic uptake or entry by receptor-mediated endocytosis, they immediately traffic to a degradative subcellular compartment. Successful intracellular pathogens have adopted different strategies to avoid trafficking of their initial phagosome along the endocytic pathway to fusion with the lysosome, a subcellular compartment that has specifically evolved to degrade them. In the following chapter, we will compare the different molecular mechanisms employed by four intracellular pathogens that have adopted distinct vacuolar niches and lifestyles. Many vacuolar

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pathogens alter their initial phagosomal compartment in order to stall or exit the endocytic pathway, and thereby avoid elimination. Yet, a few species require at least some interaction with lysosomes for completion of their infectious cycle. In this chapter, we will compare the different strategies employed by *Mycobacterium* and *Chlamydia* to avoid lysosomal fusion with those of *Brucella*, whose vacuole interacts with lysosomes transiently and *Coxiella*, a bacterium adapted to growth in a compartment that closely resembles a terminal phagolysosome.

The Benefits of an Intracellular Lifestyle

Pathogens that live within a eukaryotic host cell obtain several benefits of a relatively protected niche. One important advantage to living within a host cell is evasion of acquired immunity. Once inside the confines of a eukaryotic cell, the pathogen is no longer susceptible to complement or neutralizing antibodies. Another benefit of this lifestyle derives from the fact that few bacterial species have evolved to live within a host cell, even fewer within a professional phagocytic cell, like a macrophage. Pathogens that have adapted to this environment experience limited competition from other resident bacteria for nutrients. In fact, the intracellular environment can provide so many vital nutrients to a pathogen that they often have minimal genomes, saving coding capacity and energy for the expression of essential “housekeeping” genes, and virulence factors promoting its infectivity.

The Hazards of an Intracellular Lifestyle

Once a bacterium is taken up into a cell either by phagocytosis or receptor-mediated endocytosis, it begins to traffic along the endocytic pathway towards lysosomal fusion and destruction. There are two main strategies intracellular pathogens have adopted to avoid the mature phagolysosome: modify the vacuole to create a replicative niche, or escape and replicate within the cytosol. Whether a bacterium remains in the phagosome or escapes, it must contend with the host's defenses. Cytosolic pathogens like *Listeria*, *Shigella*, *Rickettsia*, and *Francisella* rapidly escape the initial phagosome and thereby avoid lysosomal fusion (1). However, once in the cytosol, a pathogen must evade xenophagy, a selective form of autophagy that serves as an innate immune defense against cytosolic pathogens (2). Bacteria that remain within the phagosome can avoid xenophagic killing, provided their vacuole remains intact. Taking up residence in a pathogen-tailored vacuole therefore provides a comfortable and protected compartment within a host cell, if phagocytic maturation is quickly curtailed.

The mature phagosome is a dangerous place for most intracellular pathogens

In eukaryotic cells, the specific mechanism of particle internalization depends on the size of the individual item that is consumed. Phagocytosis generally refers to particles $>0.5\ \mu\text{m}$ in size, while smaller objects are taken up by receptor-mediated endocytosis, or pinocytosis. Phagocytosis requires the extrusion of membrane for capture, whereas endocytosed particles submerge into the host cell in response to the binding of a ligand to a specific host receptor. Although the initial signaling events and structural changes at the membrane differ between

these uptake processes, there are many common features of the phagocytic maturation pathway that follows (3).

The phagosomal maturation pathway is characterized by membrane changes in lipid content and the small GTPases that associate with them (**Figure 1**). After a phagosome separates from the membrane, the small GTPase, Rab5, and vacuolar-sorting protein-34 (VPS-34) are recruited to its membrane. VPS-34, a class III PI3K, generates phagosomal phosphatidylinositol-3-phosphate (PtdIns3P) (4), identifying the vacuole as a phagosome/early endosome, distinct from the PtdIns(4,5)P₂- and PtdIns(4)P-enriched plasma membrane (5). Early endosome antigen-1 (EEA1), a Rab5 effector, binds to the PtdIns3P as well as Rab5, and facilitates endosome fusion (6). The early endosome is slightly acidified (7), but a further reduction in pH is achieved later with the recruitment of V-type ATPases. Rab5 is then exchanged for Rab7, in a process that appears to be regulated by SAND-1/Mon1 (8, 9) and requires lysosome-associated membrane proteins (LAMP) 1 and 2 (10).

With the swap of Rab5 for Rab7, the early endosome transitions to a late endosome. The late endosome acquires V-ATPases, which pump protons across the phagosomal membrane, dropping the vacuolar pH to approximately 5.0, making it an increasingly less hospitable place. The vacuoles also acquire RILP (Rab7-interacting lysosomal protein), a Rab7 effector that is required for trafficking of late endosomes to the perinuclear region and subsequent fusion with lysosomes (11). In addition to changes in associated Rabs and their effectors, the lipid profile of late endosomes is distinct from that of early endosomes. Late endosomes and lysosomes are enriched in PtdIns(3,5)P₂, the result of conversion of PtdIns3P by PtdIns(3)P5-kinase.

Upon maturation of the phagosome into a phagolysosome, the pH drops to approximately 4.5, activating proteolytic cathepsins, and contributing to the generation of deadly reactive oxygen species (ROS) (12). In addition to these microbicidal contents, the lysosome also harbors antimicrobial peptides, NO⁻, and natural resistance-associated macrophage protein 1 (NRAMP1), which serves to eject Zn⁺ and Mn⁺ from the lysosome to preserve its low pH and deprive any microbes from essential cofactors (13). Most pathogens that have adapted to life within a host vacuole have to act quickly to halt trafficking or otherwise tailor their environment in order to avoid destruction; maturation can occur within 18 minutes of uptake (14).

To Fuse, or Not to Fuse

As mentioned above, many intracellular pathogens have successfully adopted a vacuolar lifestyle. However, the points at which these pathogen-tailored vacuoles depart from the endocytic pathway are diverse and species-specific. Most vacuolar pathogens have evolved mechanisms to avoid lysosomal fusion by arresting trafficking through the endocytic pathway, as exemplified by *Mycobacterium tuberculosis*. The *Chlamydia trachomatis* inclusion diverges from the pathway early on, modifying its vacuole so that it resembles an exocytic vacuole. Several intracellular bacteria allow acidification. *Brucella* require a transient drop in vacuolar pH to activate the expression and secretion of key virulence factors and complete its infection cycle. In contrast, *Coxiella burnetii* has evolved to require sustained interaction with the lysosome in order to complete its developmental cycle. Over

the course of this chapter, we will discuss the specific adaptations these four different pathogens have evolved to create a hospitable niche inside a host vacuole.

MYCOBACTERIUM TUBERCULOSIS

Tuberculosis (Tb) is a leading cause of death in the world, with 2-3 million fatal cases annually. These deaths really represent global burden on public health, since over one billion people are latently infected with the etiologic agent of Tb, *Mycobacterium tuberculosis* (*Mtb*) (15). While the vast majority of these individuals are asymptomatic for much of their life, they have a small chance (about 10%) of eventually developing active disease. The morbidity and mortality associated with *Mtb* has been amplified by the pandemic of HIV/AIDS patients with MTBC (*Mycobacterium tuberculosis* Complex, of which *Mtb* is a member) infection (16). Additionally, the slow-growing and chronic nature of the infection has lead to widespread hypervirulent multidrug-resistant (MDR and XDR-Tb, extremely drug resistant) cases that are challenging to treat and strain public health systems world-wide (17). The MTBC also includes *M. bovis*, from which the Calmette and Guérin vaccine is derived (BCG). By evolution through a common ancestor, MTBC members can be compared genomically with respect to 14 regions of difference (RD-14) the help to identify specific virulence-associated regions (16). For the majority of comparisons across intracellular bacterial models, we highlight findings from the virulent isolate, H37Rv for lifestyle and intracellular trafficking studies.

Mtb colonize macrophages and persist inside structurally complex granulomas

Macrophages and monocytes are the principally infected cell during *Mtb* infection, and have served as the most common cell type for intracellular trafficking studies. Once inside the macrophage, *Mtb* arrests phagosomal maturation at a very early stage, thereby avoiding degradation and enabling its intracellular replication within this compartment (18). Infected alveolar macrophages initiate cellular responses by nearby epithelial cells that recruit more macrophages to the infection site creating a granuloma within the infected lung. These infection foci function as sites of chronic inflammation, and provide a mechanism for bacterial spread as the newly arriving macrophages provide a growing niche for replication (19). Eventually, acquired immunity can control the bacterial growth, until necrosis develops. *Mtb* released from necrotic cells inside the granuloma can replicate rapidly in the extracellular space, and it is these bacteria that are transmitted to a new host. As a result, the dynamics of the *Mtb* lifestyle is complicated by stage of infection, activation status of the host cell, and the ensuing inflammatory response. For this discussion, we will focus primarily on resident macrophages as replication sites for *Mtb*.

Macrophage infection by *M. tuberculosis* is characterized by an arrest of phagosomal maturation

Mtb avoids digestion by lysosomal enzymes by halting its progression along the phagosomal pathway at the early endosome stage (**Figure 2**) (20, 21). In doing so, it specifically excludes V-ATPase from its vacuole and disables fusogenicity with late endosomes and lysosomes. The result is a parasitophorous vacuole of near-neutral pH that is exclusive of reactive oxygen species and degradative enzymes. While the *Mycobacterium* phagosomal

compartment (MCP) evades fusion with degradative endosomal compartments, it retains the ability to interact with early endosomes, which provide the bacteria with essential nutrients for replication. *Mtb* orchestrates its trafficking arrest through the production of several key virulence factors that interfere with calcium flux, host membrane fusion events, and recruitment of inducible nitrous oxide synthase (iNOS) (18).

(i) Lipoarabinomannan arrests phagosomal maturation and prevents acidification of the MCP

Mtb begin the invasion process by interaction with several distinct receptors, but uptake of complement-opsonized bacteria by complement receptors represents its main route of entry (22, 23). Cholesterol-rich lipid domains may also drive selective entry, and likely affect the signaling response to attachment as well as MPC formation (24). Soon after entry, the early endosome containing *Mtb* acquires Rab5, but Rab7 is altered or absent (25, 26). This block in the canonical trafficking pathway is the result of two converging signaling pathways that are regulated by *Mtb* lipoarabinomannan (LAM) (18). LAM is a glycosylated phosphatidylinositol that is conserved among MTBC members, with the exception of the heterogeneous nature of its sugar cap. The LAM of *Mtb* has a cap of mannose, and is therefore referred to as ManLAM. While ManLAM affects two different signaling pathways, the net effect of its activities is the exclusion of V-ATPase on the MCP.

As mentioned above, the maturation block coordinated by ManLAM has two arms, one that affects calcium signaling, and the other inhibits recruitment of EEA1. ManLAM contributes to the absence of VPS34 on the MCP by inhibiting an increase in cytosolic Ca^{2+} in response to *Mtb* infection (27). In macrophages, an increase cytosolic Ca^{2+} levels, as a response to phagocytosis, activates calmodulin to associate with Ca^{2+} /calmodulin protein kinase CaMKII, an essential factor for phagolysosome maturation (28). This signaling cascade is responsible for the recruitment of VPS34 to the phagosome, which generates the PtdIns3P necessary for EEA1 binding (27). ManLAM may also inhibit association of EEA1 through the activation of p38 MAP kinase (29). Activation of p38 mitogen-activated protein kinase reduces levels of Rab5 on early endosomes and likewise levels of EEA1 (30). EEA1, a membrane tethering molecule, serves as a binding partner for syntaxin 6, a SNARE that delivers V-ATPase and cathepsins from the *trans*-Golgi network (TGN) to endosomes (31). Through the complementary effects of ManLAM on macrophage signal transduction and membrane trafficking, the MCP is protected from acidification and lysosomal hydrolases.

(ii) *Mtb* uses multiple strategies to impede phagosomal maturation

In addition to the expression of ManLAM, *Mtb* employs other virulence factors in an effort to preserve the early endosomal qualities of the MCP. Another lipid produced by *Mtb* called trehalose dimycolate (TDM) can delay phagosomal acidification via an unknown mechanism (32). In addition to lipids, a variety of protein “effectors” have been identified, via transposon library screens, as being involved in arresting phagosomal maturation. Ndk is a nucleoside diphosphate kinase, which is secreted into growth medium and is cytotoxic to macrophages (33). Based on *in vitro* and biochemical data, Ndk appears to dephosphorylate cellular Rab7-GTP and Rab5-GTP, thereby preventing Rab7-dependent heterotypic fusion of the MCP. PtpA is a low-molecular weight tyrosine phosphatase that dephosphorylates

VPS33B, a host protein involved in regulation of membrane fusion in the endocytic pathway. PtpA also binds to the H subunit of V-ATPase, inhibiting its ability to acidify vacuoles (34). The above examples of *Mtb* products that participate in the regulation of intracellular trafficking of the MCP are only a subset of the identified bacterial factors that may play a role in this complex process.

(iii) *Mtb* evades iNOS through interactions with the host actin cytoskeleton

In addition to acidification and lysosomal hydrolases, *Mtb* also avoids the reactive oxygen and nitrogen species that normally accompany phagosomal maturation in macrophages. Inducible nitrous oxide synthase (iNOS) synthesizes NO⁻ gas on the cytosolic face of the phagosomal membrane which then diffuses through the membrane into the phagosomal lumen. The apposition of iNOS to the phagosome is actin-dependent, and *Mtb* interference with the actin cytoskeleton prevented iNOS from contacting the MCP (35). Building on these observations, Davis, *et al.* found that the scaffolding protein EBP50, which normally links iNOS to the actin cytoskeleton and regulates its localization, was rapidly lost from the *Mtb*-containing phagosomes (36). Keeping iNOS at a distance from the MCP excludes NO⁻ from the phagosome, protecting *Mtb* from its harmful affects.

(iv) The *Mtb* lipid PIM counteracts, in part, the effects of ManLAM

ManLAM significantly reduces the fusogenicity of the MCP by the mechanisms detailed above. However, the *Mtb* is capable of bypassing the restrictions that ManLAM sets in place through the production of another bacterial glycosylated phosphatidylinositol called PIM (phosphatidylinositol mannoside). PIM specifically stimulates homotypic fusion of the MCP with early endosomes in a Rab-dependent manner (37). PIM's effect is insensitive to inhibition of PtdIns3P, and the result is a membrane fusion pathway that is independent of that particular host lipid (27). The significance of this bypass pathway is that it solves the problem of how *Mtb* acquires iron, via transferrin, through interaction with early endosomes (38).

***Mycobacterium* expresses several virulence factors that impart resistance to its host's oxidative burst**

While *Mtb* manages to evade interaction with the lysosome of macrophages, it must still contend with the oxidative burst induced by invading its host cell. There are several proteins that either directly or indirectly contribute to the ability of *Mtb* to resist this form of macrophage-mediated killing. Additionally, the integrity of the mycobacterial lipid-rich cell wall may protect *Mtb* from reactive oxygen and nitrogen species (ROS/RNS).

(i) Several *Mtb* proteins directly detoxify ROS and RNS

Mtb employs many classical mediators of ROS/RNS resistance by directly deactivating them during infection. Alkyl hydroperoxidase C (AhpC), an enzyme that reduces organic peroxides, has been shown to exhibit peroxynitrite reductase activity *in vitro* (39). Mutants of this gene show increased susceptibility to peroxynitrite in resting macrophages compared to wildtype *Mtb*, however this difference was nullified upon activation with IFN- γ (40). Another detoxifying enzyme that is important for *Mtb* survival is KatG, which degrades

hydrogen peroxide to water and oxygen. KatG mutants of *Mtb* are attenuated in mice and guinea pigs (41, 42). TpX, a thiol peroxidase, is required for optimal replication in both activated and resting macrophages. A *tpx* mutant was not attenuated in iNOS^{-/-} mice, which exhibit a modest oxidative burst, indicating that TpX is indispensable for resistance to RNS and ROS (43). The outer membrane lipoprotein SodC that bestows *Mtb* with the ability to dismutate extracellular superoxide was also found to promote survival within IFN- γ activated macrophages (44). Having these enzymes in its arsenal are a requirement for *Mtb* virulence.

(ii) Resistance to ROS/RNS is indirectly supplied by other *Mtb* virulence factors

Several *Mtb* proteins or other virulence factors, such as the lipid-rich cell wall, provide resistance to ROS and RNS without directly detoxifying them. Acr2 is one such protein whose expression is up-regulated by the bacterium under various conditions of stress, including oxidative stress (16). While the molecular chaperone Acr2 is not absolutely essential to the pathogenic program of *Mtb*, deletion mutants exhibit a protracted disease progression (45). The role of the hypothetical gene *Rv2136c* in resistance to low pH, heat shock, and ROS, among other stressors, was identified as part of a transposon mutant screen (46). The transposon mutant also displayed attenuation in a mouse infection with regard to its ability to persist in the lung and spleen. The specific function of this protein is unknown, but it has homology to a peptidoglycan synthesis gene in *Escherichia coli*. Other genes of interest identified in the screen include *Rv2224c* and *ponA2*, which had more modest infection defects, but are also predicted to play a role in cell wall synthesis or maintenance. These proteins that impart oxidative stress relief via prevention or repair of damage also play an important role in the survival and therefore pathogenesis of *Mtb*.

The primary survival strategy of *Mycobacterium tuberculosis* is to quickly stall the progression of its internalized phagosome before it becomes too hostile to inhabit. It orchestrates a delicate balance between blocking of phagosomal fusogenicity with the ability to interact with early endosomes as a source of essential nutrients. While the arrested phagosome is free of degradative cathepsins and excludes acidifying V-ATPase, the niche that *Mtb* occupies is not devoid of host defenses. *Mtb* counters the vigorous oxidative burst of the infected macrophage by direct detoxification and indirectly through the expression of protective chaperones. These extremely effective pathogenic attributes have been fine-tuned over the course of millennia, as the relationship *M. tuberculosis* has with its host, is truly ancient.

CHLAMYDIA

There are two main species of *Chlamydiae* that cause the majority of human disease. *Chlamydia trachomatis* is the causative agent of the most commonly reported sexually transmitted bacterial infection in the United States (47). It is also responsible for the blinding disease of trachoma, common in the developing world. *Chlamydophila pneumoniae* frequently causes community-acquired pneumonia, and has been identified as a risk factor for atherosclerosis and asthma (48, 49). Another species, *Chlamydophila psittaci*, is known to cause a zoonotic fever carried by psittacine birds that is transmitted by aerosol and contrasts as an intracellular model. Psittacosis is a severe pulmonary infection that is

difficult to treat, and therefore it has been proposed to have potential for use as a biological weapon (50).

***Chlamydia* has a biphasic developmental cycle**

These Gram-negative obligate intracellular parasites have a characteristic developmental cycle that alternates between two disparate morphologies. The small, extracellular, infectious but metabolically inactive form is termed the elementary body (EB) (**Figure 2**). The metabolically active reticulate body (RB) is larger and non-infectious, and is found exclusively inside host cells. Upon attachment to its host cell, the elementary body induces its uptake by injection of a Type III secreted effector called TARP, which remodels host actin at the site of entry (51). Once inside the host cell, the EB is contained within a specialized parasitophorous vacuole, called the inclusion. EBs rapidly differentiate into RBs, and begin to replicate by binary fission within the inclusion. The bacteria continue to replicate while actively modifying the inclusion by inserting bacterial proteins (Incs) and incorporating host lipids as it traffics to the microtubule organizing center (MTOC) of the cell. The infection cycle completes between 48 and 72 hours later when the RBs asynchronously differentiate back into their infectious form and are released from the host cell by either lysis or a process called extrusion (52).

Over the course of the intracellular phase of chlamydial development, the bacteria are known to subvert a wide variety of host cell functions. Actin is remodeled at the entry site of EBs (51, 53, 54), and the host cytoskeleton is used to build a supporting scaffold for the growing inclusion (55). The lipid composition of the inclusion membrane is changed quickly after the infection is initiated, and the chlamydiae themselves incorporate host lipids into their membranes (56, 57). Rab GTPases that identify recycling endosomes and ER-Golgi trafficking pathways are recruited to the inclusion, and interactions with many host cell organelles have been documented, including the Golgi apparatus, mitochondria, lipid droplets, and multivesicular bodies (MVBs) (58). *Chlamydiae* are also known to interfere with host cell signaling to inhibit apoptosis and promote survival, and block innate immune signaling (59). All of these functions serve to create a parasitophorous vacuole that is separate from the endocytic pathway, thereby evading lysosomal fusion while at the same time, allowing acquisition of essential nutrients and replication within a protected niche.

***Chlamydiae* depart the endocytic pathway early in infection**

In contrast to *Mycobacterium*, which arrests phagocytic trafficking at an early point in the pathway, the chlamydial inclusion diverges from it completely in order to evade destruction and create a hospitable environment for replication. Nascent inclusions do not acquire transferrin receptor, EEA1, mannose 6 phosphate receptor, or LAMP1 following EB uptake. In fact, the vesicles containing endocytosed EBs are nonfusogenic with endocytic vacuoles, including recycling endosomes (60). Inclusions do not acidify, and do not acquire V-ATPases (61, 62), despite the fact that they can be found in close apposition to lysosomes (63). Regulation of these early trafficking events of the chlamydial inclusion requires *de novo* protein synthesis, as chloramphenicol treatment results in an increase in lysosomal fusion. Orchestrating the inclusion's escape involves the co-opting of the host's microtubule network, changing the lipid content of the vacuolar membrane, and recruiting specific Rab

GTPases associated with recycling endosomes and ER-Golgi transport. The end result is a parasitophorous vacuole that operates as a unique pathogen-constructed organelle.

(i) The host cytoskeleton is co-opted by *Chlamydia* for the duration of its infectious cycle

As soon as an EB is irreversibly attached to its host cell, it begins to use the host cytoskeleton to its advantage. EBs translocate the Type III secretion effector TARP (translocated actin-recruiting phosphoprotein) across the host cell membrane to nucleate actin polymerization at the site of entry (51, 53, 54). *C. trachomatis* TARP is also known to activate Rac1, a master regulator of lamellipodium formation (64, 65). This initial actin remodeling induced by TARP is transient, and while the mechanisms governing the subsequent actin depolymerization are not understood, *Chlamydia* expresses two potential regulators for this process: CT166, an inhibitor of Rac1 (66, 67), and CT694, which results in an AHNK-dependent loss of actin stress-fibers (68).

While the actin rearrangements induced by EB attachment are quickly undone, *Chlamydiae* continue to appropriate the host cytoskeleton to support inclusion development. Following entry, the nascent inclusion is trafficked in a dynein-dependent, but dynactin-independent manner to the peri-Golgi region of the host cell (69). Once there, the inclusion maintains an intimate association with the MTOC. Mital, et al. identified cholesterol-rich microdomains on the inclusion membrane that appear to serve as platforms for signaling through Src-family kinases and interaction with centrosomes and dynein (70). Once at the MTOC, the growing inclusion requires a dynamic cytoskeletal scaffold for stabilization (55, 71). The structural integrity maintained by this framework may prevent leakage of inclusion contents into the host cytosol, which might otherwise alert the host cell's innate immune system.

The actin cytoskeleton has a final role to play in the infection cycle as it facilitates extrusion, one of two egress processes described for *Chlamydia* (52). During extrusion, the unbroken inclusion is ejected from the host cell through the plasma membrane, leaving the host cell intact. Pharmacological inhibitors revealed that extrusion requires Wiskott-Aldrich syndrome protein (WASP), myosin II, Rho GTPase, and actin polymerization (52). A recent study also demonstrated that the myosin phosphatase pathway is also involved in extrusion, as siRNA depletion of myosin IIA/IIB, MLC2, and MLCK significantly reduced detectable extrusion events (72). The fate of an extruded inclusion is unknown, but it has been speculated that this release method may facilitate chlamydial persistence or spread to more distant sites of infection (52).

(ii) The inclusion incorporates host lipids that typify exocytic compartments

As discussed previously, the lipid content of a eukaryotic vacuole is highly regulated and helps to define the identity of that specific subcellular compartment. In contrast to *Mycobacterium*, which halts endocytic trafficking by retaining the PtdIns(3)P on its phagosome, *Chlamydia* rapidly incorporates other host-derived lipids into its inclusion membrane, helping to identify it as an organelle separate from the endocytic pathway (73).

The first study to demonstrate host lipid acquisition by the inclusion membrane demonstrated that sphingomyelin (SM) generated from a fluorescent precursor (C6-NBD-

ceramide) in the Golgi accumulated in the parasitophorous vacuole as soon as 2 hours post-infection, and the lipid was also incorporated into the bacteria themselves (57). Since then, it has been shown that *Chlamydia* intercept SM trafficking from the Golgi to the plasma membrane (56, 74, 75), as well as from vesicles trafficking basolaterally (76). Additionally, *Chlamydia* co-opts CERT, a host protein that regulates ceramide traffic from the ER to the Golgi, through binding to chlamydial inclusion membrane protein IncD. CERT interacts with the inclusion membrane to regulate SM incorporation (77, 78).

Additional host lipids have been detected in the inclusion membrane, including cholesterol (79), phosphatidic acid (80), and PtdIns(4)P (81). PtdIns(4)P is an identifying lipid component of the Golgi apparatus, and two of its associated regulatory proteins, OCRL1 and PIK4I α , are also inclusion-localized. Interestingly, depletion of either of these proteins in host cells results in decreased chlamydial infection (81). Other pathways for lipid acquisition include interactions with multivesicular bodies, and lipid droplets (82, 83). By changing the lipid profile of the inclusion from that of a PtdIns(3)P-rich endosome to that resembling a Golgi-derived vesicle, the inclusion can avoid termination within a lysosome.

(iii) The chlamydial inclusion interacts with many host Rab GTPases

In addition to remodeling its lipid profile, *Chlamydia* recruits Rab GTPases to the inclusion that are characteristic of recycling endosomes and vesicles involved in transport to and from the Golgi apparatus. Rab GTPases, like membrane lipid content, also have an important role in organelle identification. As mentioned earlier, Rabs tightly control maturation of phagosomes, with Rab5 and Rab7 being among the most important players in the process. Rab5 regulates fusion of early endosomes and early phagosomes, and is replaced as phagosomes mature by Rab7, which regulates fusion of late endosomes with lysosomes (12). As *Chlamydia* quickly exits the phagocytic pathway, the inclusion membrane does not associate with either Rab5 or Rab7. Rather, the Rabs of recycling endosomes (Rab4 and Rab11), and ER to Golgi transport (Rab1) are recruited the inclusion (84). While the association of Rabs 1, 4, and 11 with the inclusion are universal to *Chlamydiae*, some species-specific interactions have also been described. Golgi-associated Rabs 6 and 14 appear only on the inclusions of *C. trachomatis*. Rab10, which is a regulator of ER dynamics and post-Golgi transport, is found only on the inclusions of *C. pneumoniae* (84, 85). The mechanisms by which Rab GTPases are recruited to the inclusion membrane are not well defined, however, a few chlamydial inclusion membrane proteins (Incs) have been shown to bind Rabs. *C. trachomatis* CT229, a member of the Inc family of chlamydial proteins, interacts directly with Rab4-GTP in a number of assays, including two-hybrid binding and pull-down experiments (86). A *C. pneumoniae* Inc, Cpn0585, is capable of interacting with Rabs 1, 10, and 11 (87).

The specific Rabs that have been observed on the inclusion membrane not only identify the vacuole as a compartment apart from the endocytic pathway, but also enable interaction with host vesicles and organelles that support chlamydial growth and replication. Rabs 6, 11, and 14 coordinate acquisition of Golgi-derived lipids from the host cell (88, 89). *Chlamydiae* depend on host cell transferrin as a source of iron for growth. Concurrent inhibition of Rab4 and Rab11, which coordinate transferrin recycling to the host plasma membrane, resulted in

decreased chlamydial replication and an accumulation of transferrin-containing vesicles adjacent to the inclusion (90). Rab11 is involved in the generation of multivesicular bodies, which have also been observed interacting with inclusions. Inhibition of their trafficking to the inclusion decreases lipid incorporation and bacterial replication (80, 82, 91).

Mitochondria have long been associated with chlamydial inclusions (92, 93), and at least in the case of *C. caviae*, interaction with them is essential for replication (94). This is not surprising, as *Chlamydia* rely on the host cell for ATP (95, 96).

The chlamydial replicative niche is thus characterized by its early departure from the endocytic pathway to escape the terminal lysosome, and rapid association with the Golgi in order to acquire nutrients. While much is known about how *Chlamydia* actively remodels the inclusion to create a replicative niche, there is still much to discover, as many of the predicted Incs encoded within the chlamydial genome lack functional assignments (97, 98). The co-opting of host cytoskeleton by the inclusion provides structural integrity as it quickly takes up the bulk of the host cell's volume, while at the same time provides a network for trafficking away from destruction and reception of host cell cargoes that provide necessary nutrients. Modification of the inclusion membrane further disguises the vacuole as a host organelle that is separate from the endocytic pathway. It is an elegant example of how to successfully hide from the host within one of its cells.

BRUCELLA

While *Brucellae* are known to infect a wide range of host species, there are three that cause the majority of human infection: *Brucella melitensis* (primarily associated with goats and sheep), *Brucella suis* (swine) and *Brucella abortus* (cattle). Humans are incidental hosts for these pathogens, however brucellosis is among the world's most common zoonoses, with over an estimated 500,000 new cases each year (99). The disease is highly infectious, requiring only 10-100 organisms to establish an infection via aerosol (100). While inhalation of the pathogen is certainly an important source of human infection, it is also commonly acquired through ingestion of contaminated animal products, particularly unpasteurized dairy (101). After passing through the mucus membranes to infect phagocytic cells, *Brucella* establishes a protected intracellular niche. As mentioned earlier, an intracellular lifestyle allows a pathogen to evade immune surveillance, and this is particularly important for *Brucella*, which often becomes chronically associated with its host (101, 102). The infection begins with flu-like symptoms, and is characterized by an undulant fever and night sweats. If left untreated, *Brucella* can disseminate via infected phagocytic cells to many different tissues and organs within the body.

***Brucella* transitions between three subcellular compartments to complete its infectious cycle**

Upon entry, *Brucella* begins an intracellular journey in which it will traffic through the endocytic pathway to the ER for replication, and then an autophagosome-like compartment for egress. First, intracellular trafficking of *Brucella* to a replicative compartment is dependent on its LPS status, as only the smooth (virulent) variants with full-length O-antigen can avoid phagocytic maturation and destruction. However, it should be noted that even virulent *Brucella* are often killed within the phagolysosomes of macrophages, as fewer

than 10% of the internalized bacteria survive to establish a replicative *Brucella*-containing vacuole (rBCV) (**Figure 2**) (103). In contrast to *Mycobacterium* and *Chlamydia*, once internalized, the parasitophorous vacuole traffics along the endocytic pathway, acquiring early (104), as well as late endosomal markers (105). There is even some limited interaction with host lysosomes and the BCV acidifies transiently (106). After about 8 hours post-infection, the acidification of the BCV activates expression of the VirB-dependent type 4 secretion system (107) directing the vacuole's escape from the endocytic pathway to set up an ER-associated rBCV (108, 109). It is within this compartment that *Brucella* replicates for up to 48 hours post-infection. Following replication, a subpopulation of *Brucella* traffic to a new subcellular compartment resembling an autophagosome, the aBCV (105). The precise signals that induce aBCV formation are not understood, but these tertiary vacuoles appear to enhance spread of *Brucella* to neighboring cells for a second round of infection. *Brucella* may also exit host cells as rough mutants that sporadically arise within the rBCV causing host cell cytotoxicity and lysis. The released bacteria, an assortment of smooth and rough *Brucella*, then seed a new round of infection (110).

Trafficking to the ER requires the coordination of a number of bacterial and host cell factors

Many different host factors required for efficient *Brucella* replication have been identified, including Rho-family GTPases, SNARES, PtdIns(3) kinases, cytoskeletal proteins, and IRE1 α , a kinase that regulates the unfolded protein response (UPR) and autophagosome formation (111). *Brucella* coordinates its trafficking through the secretion of a number of virulence factors, and expression of proteins to resist the transient acidification of the BCV. Once it reaches the confines of ER and *Brucella* has successfully evaded the terminal phagolysosome, it is safe to begin replication.

(i) Endosomal trafficking of *Brucella* ends with acidification of the BCV

Unlike the other intracellular pathogens discussed in the first part of this chapter, *Brucella* stays within the endocytic trafficking pathway until it reaches a late stage. Smooth, nonopsonized *Brucella* entry into murine macrophages is dependent on lipid rafts (112), PtdIns(3) kinase (113) and TLR4 (114), and fails to activate macrophages. Rough mutants, which lack O-antigen, enter cells independently of lipid rafts and are rapidly killed within activated macrophages (115-117). However, naturally rough *B. ovis* and *B. canis* are capable of lipid raft-dependent entry (118), so there must be additional factors that regulate *Brucella* entry. Similarly to *Chlamydia*, entry is also dependent on the host actin cytoskeleton. The small GTPase, Cdc42, which regulates actin polymerization through interaction with WASP, is recruited to and activated at the entry site (113).

Endosomal trafficking of the BCV proceeds with acquisition of early endosomal markers EEA1 and Rab5 (104, 108). Their association with the BCV is transient, as they are rapidly exchanged for the late endosomal markers Rab7, RILP, and LAMP1 (106). Acquisition of Rab7 and RILP are required for normal intracellular trafficking of *Brucella*, as expression of a dominant-negative Rab7 or overexpression of RILP interfered with conversion of the BCV to an ER-derived vacuole. As they traffic along the endosomal pathway, the BCV allow limited lysosomal fusion. Fusion with lysosomes can be observed up to about 12 hours post-

infection, at which point the BCV loses its late endosomal markers. This study also confirmed that *Brucella* require acidification for completion of their infectious cycle, as pharmacological inhibition of v-ATPases resulted in a dramatic decrease in viable bacteria (106, 108). This is likely because acidification is required for the activation of type IV secretion system gene expression, which is crucial for setting up the rBCV (106-109, 119, 120).

(ii) Adaptations to resist degradation upon lysosomal fusion

Like *Chlamydia*, *Brucella* has evolved to escape destruction by trafficking away from the endocytic pathway before it can be degraded, but not before the BCV fuses with lysosomes. First, it should be noted that lysosomal fusion of the BCV is well controlled by the bacteria, and significantly less fusion occurs with virulent *Brucella* than with heat-killed bacteria (106). Cyclic- β -1,2-glucan, a critical virulence factor, has a role in decreasing lysosomal fusion by depletion of BCV membrane cholesterol (121). Nevertheless, the lumen of the BCV is acidified and does contain degradative enzymes. To mitigate the risk of destruction during this transition, *Brucellae* express several proteins to aid in resistance to the drop in pH, oxidative and nitrosative damage, and antimicrobial peptides (122).

Multiple *Brucella* proteins have been identified as having a role to play in acid resistance. HdeA, a homolog of the *E. coli* acid-resisting periplasmic chaperone, imparts acid resistance *in vitro* (123). CydB was found to contribute to both acid tolerance and resistance to reactive oxygen species (124). Disruption of the *cydB* gene demonstrated that cytochrome *bd* ubiquinol oxidase is required for intracellular survival and virulence. *Brucellae* also express a functional urease that protects the bacteria from extremely low pH and is presumed to allow safe passage of the bacteria through the gastrointestinal tract after ingestion, but it is not required for virulence in cultured cells (125).

Reduced pH is certainly not the only hazard that intracellular *Brucella* encounter, as reactive oxygen (ROS) and nitrogen (RNS) species are key defense mechanisms employed by infected phagocytic cells. In fact, ROS and nitric oxide production have been shown to be important for microbicidal activity against *Brucella* in macrophages (126). SodC, a Cu/Zn superoxide dismutase that counteracts ROS production, is required by *B. abortus* for survival within macrophages and virulence in a mouse model of infection (127). To resist the deleterious effects of RNS, *Brucella* generate nitric oxide reductase to detoxify NO within macrophages (128, 129). *Brucella* also enlist AhpC, a peroxiredoxin that is primarily involved in scavenging endogenous ROS generated by *Brucella* metabolism (130), to impart some resistance to peroxynitrite (122).

Finally, the lysosomes of macrophages contain cationic antimicrobial peptides called defensins that pose a threat to bacteria by creating pores in their outer membranes (12). The BvRS two-component regulatory system controls the expression of genes responsible for the acylation status of lipid A (131). Without this important modification of LPS, BvRS mutants are more susceptible to destruction by cationic antimicrobial peptides due to their altered membrane properties. It is evident that *Brucellae* have evolved many solutions to the problem of requiring lysosomal fusion for replication by expressing proteins that impart resistance to the myriad hazards encountered during endocytic trafficking.

(iii) The carefully coordinated transition to the Endoplasmic Reticulum begins the replicative phase of the *Brucella* infection

In epithelial cells, prior to ER fusion, the BCV has a short-lived association with autophagosomes. The BCVs acquire the autophagosome marker monodansylcadaverine, and have a multilamellar appearance (104, 132). An autophagy-related protein IRE1 α , is required for *Brucella* replication at this stage (111). IRE1 α is a kinase that regulates the host unfolded protein response (UPR) as well as the generation of ER-derived autophagosomes. Its role in autophagy induction was highlighted as part of the ER-fusion process, as other kinases involved in the UPR were not found to be important for *Brucella* replication in that study (111). However, this autophagic transition prior to ER fusion is not observed in macrophages. Interestingly, the UPR is induced in *Brucella*-infected macrophages, and is required for efficient replication (133). The microtubule-associated *Brucella* protein TcpB has been identified as playing an important role in UPR induction in these cells. It is not surprising that the UPR is induced by *Brucella* infection, as a major reorganization of the ER is required for replication, but the idea that *Brucella* may actively induce the process is intriguing.

Brucella-ER fusion, which is required for bacterial replication, is carefully coordinated by secreted virulence factors. BCV-ER fusion requires the small GTPase Rab2, which is part of a multi-protein complex that regulates vesicular trafficking from the Golgi to the ER. Inhibition of Rab2 results in retention of LAMP1 on BCVs (134). A *Brucella* type IV secretion effector RicA has been implicated in initiating Rab2 recruitment through its interactions with the small GTPase (135, 136). Another *Brucella* secreted protein, CstA, which interacts with the ERES-associated protein Sec24A, has recently been discovered as having role in ER fusion of the BCV. Deletion mutants of *cstA* have delayed trafficking to the ER relative to wildtype *B. abortus* (137). Secretion of CstA into cell culture supernatants appears to be dependent on flagellar genes, not type IV secretion, but more work must be done to confirm its secretion in infected cells. Several other type IV substrates have been identified as ER-localized during infection. *Brucella* effectors BspA, BspB, and BspF disrupt the host secretory pathway and inhibit host protein secretion (138). While deletion of these individual effectors does not significantly affect *Brucella* replication, the triple deletion mutant *bspABF* displays reduced intracellular replication and virulence in a mouse model of chronic infection (138).

As BCVs lose fusogenicity with lysosomes, they progressively merge with the ER and acquire markers of this new subcellular compartment that is pH-neutral and represents a safe haven for replication. BCVs initially contact the ER at ER exit sites (ERES) through interactions with the Sar1/COPII complex (139). Inhibition of Sar1 prevents BCV-ER fusion and results in a block in replication. Other ER-specific proteins found on rBCVs include calnexin, calreticulin, and Sec61 (104, 108). The rBCV membrane is also studded with ribosomes. The ER-targeted RNA-interference screen that identified IRE1 α also found 29 other novel host ER proteins to be important for virulence, including important regulators of vesicular trafficking and host protein biosynthesis, as well as cytoskeletal proteins (111). As *Brucella* replicates in this new compartment, the rBCV membrane septates with the bacteria, so they continue to reside singly in tight vacuoles as the host cell fills with bacteria.

Following the replicative phase, egress from the host cells is poorly characterized, but two complementary hypotheses describing an exit route for *Brucella* have been proposed. Starr, et al. described the formation of the late autophagosome-like aBCV, which appears to promote spread of *Brucella* to neighboring uninfected cells (105). aBCV formation was observed at 72 hours post-infection, when rBCVs appear to transition back into a LAMP1-positive subcellular compartment and lose the ER marker calreticulin. Although the cells that harbor aBCVs make up a subset of the infected cell population, those cells had a tendency to form re-infection foci within the monolayer, suggesting that aBCVs offer a mode of egress for *Brucella* (105). Another method of host cell exit may involve a change in the LPS status of replicating *Brucella*. Spontaneous dissociation of smooth *Brucella* to rough mutants with truncated LPS O-antigen has been observed during infection of murine macrophages. Rough *Brucella* are cytotoxic to macrophages, and cause host cell lysis. A recent report demonstrated that the subpopulation of rough mutants that arises during infection with *B. melitensis* enhances cell-to-cell spread of the bacteria by causing host cell lysis and release of smooth *Brucella* along with the sporadic rough mutants (110). Neither exit mechanism excludes the other, nor would it be surprising to find that like *Chlamydia*, *Brucella* uses more than one exit strategy.

Brucella has a unique strategy for setting up its replicative niche. Although trafficking to ER-derived replicative vacuoles is not specific to *Brucella*, the fact that it allows lysosomal fusion prior to arriving in this protected compartment is fairly distinctive. Utilizing the acidic environment of the late endosome as a signal to activate virulence genes and coordinate its escape to the ER is not without risk. Recall that over 90% of the bacteria that attempt to colonize macrophages meet an untimely demise. For the remaining 10%, the tools that *Brucella* employs to resist destruction are effective, and allow it to hide within phagocytic cells for protracted periods of time, making brucellosis a particularly unpleasant disease to contract.

COXIELLA

Coxiella burnetii is a Gram-negative naturally obligate intracellular pathogen that causes Q fever in humans, a flu-like illness that is generally self-limiting and readily resolves without antibiotics. The organism has an extremely wide zoonotic host range that spans mammals, arthropods, fish and avian species. t, a clonal isolate that is avirulent and has a rough LPS phenotype (phase II), which can be studied in a biosafety level 2 environment. *C. burnetii* isolates cause either acute or chronic disease, and phylogenetic studies show that they cluster into three clades corresponding to their clinical presentation. Experimental animal models support the hypothesis that there are three specific pathotypes of *Coxiella* (140). Recent outbreaks have highlighted the continually emerging nature of this pathogen (141), and the significant impact of *Coxiella* on U.S. warfighters in Iraq and Afghanistan demonstrates the endemic nature of the disease (142).

***Coxiella* alternates between two morphologies during its developmental lifecycle**

The highly infectious nature of *C. burnetii* is partially due to its ability to survive in the environment for long periods of time in its metabolically inactive, small cell variant (SCV) form. Replication in a range of developmental stages is similar in design, but not in

mechanism, to that described for *Chlamydia*. In the current model, SCV are predominantly extracellular, and while they are environmentally resistant, they are incapable of replication. After SCV invade host cells, they transition to metabolically active large cell variant (LCV) form following the acidification of the *Coxiella*-containing vacuole (CCV). The percentage of *Coxiella* in the CCV that have transitioned to LCVs approaches 80% after 1 hour of infection, and by 16 hours the CCV contains exclusively LCV (143, 144). LCV replicate in an expanding CCV for 4-7 days, transitioning back to SCV at later stages of the infection cycle, generating stable extracellular forms for future rounds of infection.

Completion of the *Coxiella* infection cycle requires replication in a phagolysosome-like compartment

Unlike the vacuolar pathogens discussed to this point, *Coxiella* allows phagosomal trafficking to proceed all the way to lysosomal fusion. Development of the CCV involves a combination of normal phagosomal trafficking and pathogen-direction manipulation of host processes. The end result is a niche that represents a hostile environment to most other pathogens, and is therefore free of competitors.

(i) Adhesion and invasion

After aerosol transmission, *C. burnetii* targets alveolar macrophages and passively enters these cells by actin-dependent phagocytosis (145, 146). Recent studies using type IV secretion system (T4SS) mutants that have no defect in their ability to infect either phagocytic or non-phagocytic cells (147, 148) support the hypothesis that entry into phagocytic cells proceeds via host-driven actin-dependent phagocytosis. Virulent *C. burnetii* bind to phagocytic cells (monocytes/macrophages) using $\alpha_v\beta_3$ integrin as the main receptor and are taken up into the cell via a Rac1-dependent phagocytosis that depends on membrane ruffling (149, 150).

Histopathologic analysis in both animal models of pneumonia and in humans with atypical pneumonia caused by *C. burnetii* primarily identified monocyte/macrophage infection, but epithelial and endothelial cell infection has also been reported (151). Non-phagocytic cells do not usually engulf large particles, however, intracellular pathogens can invade after direct contact through a zippering of bacterial ligands to host cell receptors. While the identities of the host receptors for *Coxiella* on non-professional phagocytes remain to be determined, the mechanism of entry also appears to be dependent on actin rearrangement (145). Recently, a *Coxiella* protein that is required for invasion of epithelial cells has been identified. Mutants in CBU1260, which encodes the invasin OmpA, exhibit an epithelial cell-specific entry defect (Martinez, 2014). Interestingly, attachment to epithelial cells is unaffected by mutation of CBU1260, indicating that binding and entry of *Coxiella* are independent processes. Despite the invasion defect observed in non-phagocytic cells, actin rearrangement is unaffected by mutation of CBU1260 in THP-1 macrophages, highlighting again the host-driven nature of phagocytic uptake of *Coxiella*.

In a variety of tissue culture models *C. burnetii* phase variants exhibit distinct uptake kinetics whereby avirulent phase II bacteria are more readily internalized than fully virulent phase I bacteria (145, 146). However, both variants replicate with similar kinetics within an

indistinguishable, proteolytically active CCV in a variety of tissue culture cell lines (152), which is important to note, as the majority of studies that examine intracellular trafficking have used the avirulent phase II *C. burnetii* (153).

(ii) Trafficking from the phagosome to the CCV

Phagocytosis results in the formation of the phagosome, which matures into a phagolysosome following a series of highly ordered and regulated fusion and fission events (12). The maturation of the CCV essentially follows the general phagocytic pathway terminating in a compartment that strongly resembles a mature phagolysosome (140). After internalization, the nascent CCV is similar in size to an endosome, and it traffics through the pathway described above as indicated by the sequential recruitment of Rab5 and Rab7 (**Figure 2**) (153). A hallmark of *Coxiella* infection is a marked expansion in CCV size during maturation, prior to when the majority of LCV replication begins, resulting in an extremely spacious vacuole at early stages of infection. Functional inhibition of both Rab5 and Rab7 by overexpression of dominant negative forms of these proteins impairs large vacuole formation (153). Furthermore, formation of a spacious CCV is dependent on *de novo* *C. burnetii* protein synthesis, as well as secretion of T4SS effectors across the CCV membrane into the host cytoplasm (147, 154). Indeed, a growing number of *Coxiella* T4SS substrates are known to be required for CCV expansion and several effectors have recently been shown to localize the CCV membrane during infection (155). However, in the absence of protein synthesis, small CCVs still acquire LAMP proteins and acidify, suggesting that acquisition of LAMPs and acidification are a consequence of the normal progression of phagosomal trafficking (156).

Coxiella is unlike the intracellular pathogens discussed so far, which disrupt the endosomal cascade, or arrest maturation of the phagosome, thereby avoiding lysosomal fusion (157). In the case of *C. burnetii* infection, lysosomal enzymes including cathepsin D and acid phosphatase, accumulate within the CCV by 1 to 2 hours post infection (144), a protracted time course compared to the 15 minutes required for phagosomes harboring inert particles. The observed delay is dependent upon *C. burnetii* protein synthesis, suggesting it is a pathogen-induced process (144, 158, 159). While the delay in lysosomal fusion of the CCV requires bacterial protein synthesis, the T4SS an unlikely mediator of the process, as secretion has not been detected until at least 8 hours post-infection (148). There is some evidence that interaction with the autophagy pathway contributes to this delayed phagosomal maturation (153). Autophagy serves as an innate host defense against many intracellular pathogens (160), and as early as 5 minutes after internalization, the phase II CCV is decorated with the autophagy marker microtubule-associated protein light-chain 3 (LC3) (**Figure 2**) (153, 161). However, *C. burnetii* may benefit from this early interaction with autophagosomes as autophagy induction in infected cells increases CCV size (162).

(iii) Survival inside a degradative vacuole

Coxiella burnetii has adapted to replicate in the very compartment that *Mycobacterium*, *Chlamydia*, and *Brucella* have evolved to avoid. In fact, the metabolic activity of *Coxiella* is activated in response the reduced pH of the nascent CCV (143, 144). As mentioned above, the CCV acquires degradative cathepsin D as well as acid phosphatase soon after infection

(144). Other normally bactericidal contents of a lysosome include ROS and RNS, and *Coxiella* may employ various strategies to resist these deadly host defenses against infection (163). Upon host cell invasion, *Coxiella* upregulates transcription of stress response genes, which may serve to protect its DNA from the damaging effects of ROS (164). To supplement its SOS response, the *Coxiella* genome encodes several genes that enable the bacterium to resist the damaging effects of oxidative stress. An acid phosphatase, encoded by CBU0335, can suppress ROS production in neutrophils by preventing the assembly of host NADPH phagocyte oxidase (165), a major generator of superoxide radicals. *Coxiella* also expresses ROS scavenging systems for the direct detoxification of superoxide. *C. burnetii* encodes a functional cytoplasmic FeSOD (CBU1708) that complemented a double *sodA sodC* mutant strain of *E. coli* under conditions of oxidative stress (166). Other gene products that may take part in ROS scavenging include a periplasmic Cu/ZnSOD (CBU1822), and two alkyl hydroperoxide reductases, AhpC1 (CBU1706) and AhpC2D (CBU1477-1478). Resistance to acid may also be imparted by the expression of a large number of basic proteins, as close to 45% of *C. burnetii* proteins have a predicted pI 9 (167). While *C. burnetii* certainly has a cohort of genetic tools that may impart resistance to damaging phagolysosomal contents, they are not likely to be their first line of defense. A study by Brennan, *et al.* illustrated that *C. burnetii* infection is well-controlled by the host's oxidative burst (168). As a stealth pathogen, it is probably *Coxiella's* ability to infect macrophages without alerting the innate immune response that serves as its primary mechanism to avoid killing due to oxidative stress.

(iv) Expansion of the CCV into a spacious and replication competent compartment

The CCV enlarges dramatically between 8 hours and two days post infection, and can occupy nearly the entire space of the host cell (169). In the case of multiply infected cells, the formation of a large CCV is due to homotypic fusion of several smaller CCV, while the expansion of the compartment proceeds through heterotypic fusion with autophagic, endocytic, and lysosomal vesicles (154, 156). The development of the CCV not only requires bacterial protein synthesis (156), but several host proteins are also necessary. The actin cytoskeleton supports CCV development, and treatment with actin-depolymerizing agents results in the formation of only small CCV (170). Additionally, *C. burnetii* infection activates several host cell kinases, including protein kinase C, protein kinase A, and myosin light chain kinase, all of which are required for the establishment and maintenance of the CCV (171). The parasitophorous vacuole also interacts with the early secretory pathway, as Rab1b accumulates on the CCV membrane, an interaction which is required for the formation of the large CCV (**Figure 2**) (172). Interaction with the ER through the early secretory pathway may provide another source of membrane for the formation of a spacious CCV (172). The large number of cellular processes that are coordinated during *Coxiella* infection suggest that many virulence factors that direct the formation of the CCV remain to be identified.

Coxiella burnetii is a unique bacterium in that it is specifically adapted to infect immune cells that are employed to destroy invading pathogens. Not only does it infect a particularly restrictive cell type, but it replicates within a niche that is especially hostile towards bacteria, in general. It utilizes the low pH of the CCV to activate its metabolism, as well as its

pathogenic program of type 4 secreted substrates. It has adapted to resist the toxic ROS and RNS, as well as the degradative enzymes resident within lysosomes. Life in a CCV affords *Coxiella* some very important benefits in the form of acquired immune evasion and reduced competition from other pathogens. However, it is little wonder why other pathogens have evolved to avoid growth under these same conditions.

CONCLUSION

Intracellular pathogens have evolved many unique and elegant strategies for the survival within their host cells. Entry via phagocytosis does not necessarily sentence a pathogen to death in a phagolysosome. Some bacteria manage to break free of their phagosome in order to replicate in the nutrient-rich cytosol. For those that spend the duration of their intracellular life inside a vacuole, there are myriad ways to customize the replicative niche. The problem of lysosomal fusion can be avoided by stalling trafficking, as in the case of *M. tuberculosis*, or by diverting the vacuole from the endocytic pathway entirely, like *Chlamydia* and *Brucella*. For *C. burnetii*, lysosomal fusion is not avoided, but required for metabolic activation and replication. Whenever a pathogen adapts to a specific intracellular niche, its lifestyle is shaped by the innate immune defenses therein. For bacteria that replicate primarily in phagocytic cells, such as *Mtb* and *Coxiella*, the oxidative burst is a major evolutionary driver. Although they each inhabit and replicate in very different subcellular compartments, *Mtb*, *Chlamydia*, *Brucella*, and *Coxiella* are all very successful pathogens that can serve as valuable tools to enlighten our knowledge of host cell trafficking and immunity.

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Fig 1. Endosomal trafficking is coordinated by exchanges of lipids and Rab-GTPases

The normal endosomal pathway is illustrated here with major regulatory factors highlighted at each step. After uptake of a cargo, the phagosome fuses with early endosomes, acquiring Rab5, its effector EEA1, and VPS34, which coordinate the change in lipid profile of the endosomal membrane. Soon after, the compartment fuses with late endosomes, and Rab5 is exchanged for Rab7 and its effector RILP. LAMP and v-ATPases are also characteristic of the late endosome. Finally, the late endosome fuses with lysosomes, at which point the compartment is fully matured and highly degradative.

Fig 2. Intracellular pathogens have evolved distinct trafficking pathways to arrive within their ideal replicative niche

Intracellular trafficking pathways are shown for *Mycobacterium tuberculosis* (Mtb), *Chlamydia* (Chl), *Brucella* (Br), and *Coxiella burnetii* (Cb). Key regulatory factors, such as Rab-GTPases, lipids, and bacterial factors are shown. Mtb fuses with early endosomes (EE), but inhibits fusion with late endosomes (LE), and replicates in a compartment (MCP) that is stalled at an early point along the endocytic pathway. Chl traffics away from the endosomal pathway, and escapes to replicate in a Golgi-associated Inclusion. Br allows EE and LE fusion as well as limited lysosomal fusion before trafficking to the ER to replicate in rBCVs (replicative *Brucella* containing vacuoles). Cb interacts with EE, autophagosomes, LE, and allows lysosomal fusion to arrive in the CCV (*Coxiella* containing vacuole), which resembles a terminal phagolysosome.