

Chapter 7

Coxiella Subversion of Intracellular Host Signaling

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Abstract *Coxiella burnetii* is a highly infectious bacterial pathogen that replicates in a specialized vacuole inside eukaryotic cells. Due to a prolonged growth cycle, *Coxiella* continuously manipulates cellular processes to generate this parasitophorous vacuole (PV) and promote host cell viability. Here, we discuss recent findings that indicate *Coxiella* modulates several host signaling pathways to influence survival and ensure intracellular replication. The pathogen actively inhibits apoptotic cell death and activates the pro-survival kinases Akt and Erk1/2 to promote host viability. *Coxiella's* anti-apoptotic activity also involves the interface between autophagy and apoptosis, which is regulated by the interaction of autophagy-related Beclin-1 and anti-apoptotic Bcl-2. Additionally, *Coxiella* requires host kinase activity for PV biogenesis and maintenance. Thus, signaling modulation by *Coxiella* is critical for multiple aspects of host cell parasitism. Collectively, recent signaling studies

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have enhanced our understanding of the unique *Coxiella*-host cell interaction. Identification of bacterial factors that regulate signaling events will further our ability to model this intriguing infectious process.

Keywords *Coxiella* • Vacuole • Apoptosis • Signaling • Autophagy

7.1 Introduction

Bacterial pathogens have evolved a battery of mechanisms to modulate the hostile host environment encountered during infection and cause disease. Intracellular bacterial parasites comprise a group of intriguing organisms that rely on the internal eukaryotic cell environment for nutrient acquisition and formation of a protected replication niche. Intracellular pathogens have adapted to their unique lifestyle by counteracting antibacterial responses such as cytokine production, immune detection, lysosomal degradation, and cell death. Manipulation of these processes intricately controls the infectious process and ultimately ensures an efficient infection. Conversely, combating these activities often results in clearance of the pathogen and resolution of disease. Thus, regulation of intracellular host processes has become a major focus of this growing field of infectious disease.

Coxiella burnetii is the intracellular bacterial agent of the zoonosis human Q fever, which can present as acute or chronic disease. Humans are typically exposed to *Coxiella* via inhalation of contaminated aerosols, resulting in deposition of infectious organisms in the alveolar spaces (Raoult et al. 2005). Hence, alveolar phagocytic cells are considered the initial reservoir wherein *Coxiella* promotes formation of a parasitophorous vacuole (PV) in which to replicate (Voth and Heinzen 2007). The early *Coxiella*-containing phagosome matures through interactions with host autophagosomes as a potential source of nutrients and membrane for the expanding vacuole (Romano et al. 2006). As the vacuole enlarges through continual fusion with lysosomes, autophagosomes, and fluid phase endosomes, *Coxiella* converts from a small cell variant morphological form into a replication-proficient large cell variant form that divides by binary fission throughout a prolonged growth cycle (Coleman et al. 2004). The mature PV is unique among intracellular pathogen compartments in possessing an acidic (pH ~ 5.0), phagolysosome-like nature (Akpouriaye et al. 1983). Indeed, the PV contains active acid hydrolases and retains degradative activity against other bacterial cells (Howe et al. 2010). Strikingly, *Coxiella* replicates to high numbers and thrives in this hostile environment designed to dispose of invading pathogens. Therefore, PV maintenance is of utmost importance to *Coxiella* and the bacterium actively promotes vacuole biogenesis and maintenance to ensure a successful infection.

Coxiella protein synthesis is required for PV biogenesis and antibiotic treatment results in accumulation of tight-fitting phagolysosomes containing individual organisms (Howe et al. 2003). These small vacuoles are unable to fuse with other PV or host vesicles and expansion and replication is stalled. To direct PV biogenesis,

Coxiella likely employs its Dot/Icm type IV secretion system (T4SS), which delivers bacterial proteins, termed substrates or effectors, to the host cytosol where they interact with eukaryotic proteins to regulate infection events. Recent evidence indicates that *Coxiella* encodes numerous Dot/Icm substrates predicted to control a vast array of host processes. For example, *Coxiella* isolates collectively encode 11 Dot/Icm substrates with ankyrin repeat domains (Voth et al. 2009; Pan et al. 2008), which are eukaryotic motifs that mediate protein-protein interactions. These and other Dot/Icm-translocated proteins are predicted to be major virulence factors used by *Coxiella* to control infection events, including subversion of signaling pathways. In this chapter, recent discoveries will be discussed regarding intracellular host signaling pathways manipulated by *Coxiella* to control host viability and PV formation.

7.2 *Coxiella* Exhibits Potent Anti-apoptotic Activity

A costly detriment to an intracellular parasite is the demise of its preferred host cell. Many intracellular pathogens, including *Coxiella* (Coleman et al. 2004), display slow replication rates, necessitating a viable host cell throughout a prolonged growth cycle. Thus, these organisms have developed methods to promote host cell survival until replication is complete and cellular release can ensue. A common target of these organisms is apoptosis (Labbe and Saleh 2008), a form of ordered, non-inflammatory eukaryotic cell death broadly consisting of two pathways: extrinsic and intrinsic (Fig. 7.1). Extrinsic apoptosis is mediated by ligation of cell surface death receptors that activate intracellular proteolytic caspase cascades (Jin and El-Deiry 2005). Subsequent activation of downstream, or effector, caspases such as caspase-3 ultimately results in DNA fragmentation and cell death. Intrinsic apoptosis is regulated by intracellular events that trigger mitochondrial release of cytochrome *c* (Jin and El-Deiry 2005). Cytochrome *c* release is regulated by a panel of interacting anti- and pro-apoptotic mitochondrial surface proteins containing BH3 homology domains. In the cytosol, cytochrome *c* forms a complex with Apaf-1 and caspase-9 termed the apoptosome, which activates effector caspases, triggering DNA damage and death. It is increasingly clear that intracellular pathogens can completely halt cell death.

Anti-apoptotic activities have been described for multiple intracellular pathogens including *Rickettsia*, *Salmonella*, *Mycobacterium*, *Legionella*, and *Chlamydia*. *Salmonella typhimurium* secretes SopB, a type III effector protein that activates the host pro-survival kinase Akt during early stages of epithelial cell infection, resulting in decreased caspase activation (Knodler et al. 2005). *Mycobacterium tuberculosis* inhibits mitochondrial-dependent death by directly altering activity of the BH3 domain protein Bad (Maiti et al. 2001). *Legionella pneumophila* also directly engages mitochondrial proteins by secreting effector proteins that target pro-apoptotic BNIP3 and Bcl-rambo (Banga et al. 2007). *Chlamydia spp.* are perhaps the most prolific anti-apoptotic organisms described to date. *Chlamydia spp.* use a multi-faceted approach to inhibit apoptosis including activation of Akt

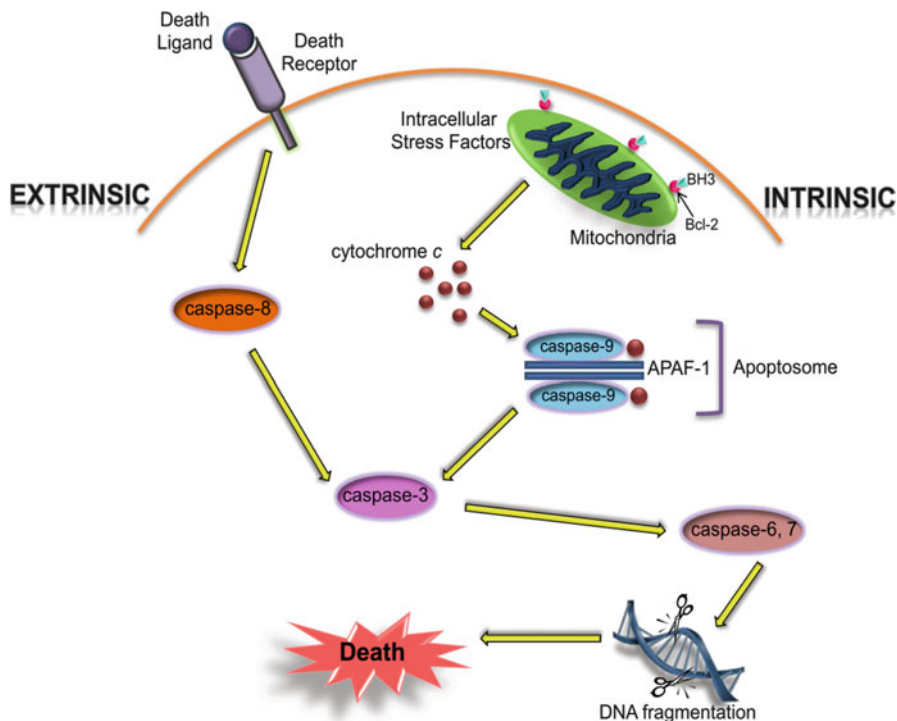


Fig. 7.1 Overview of extrinsic and intrinsic apoptosis. Extrinsic apoptosis ensues following ligation of cell surface death receptors that trigger activation of proteolytic initiator caspase proteins, such as caspase-8. Intrinsic apoptosis is initiated by stress factors that allow mitochondrial release of cytochrome *c* through the interaction of pro-apoptotic BH3 domain proteins and anti-apoptotic proteins such as Bcl-2. Cytochrome *c* release results in formation of a multi-protein complex, termed the apoptosome, that mediates caspase-9 activation with the assistance of apoptotic protease activating factor-1 (Apaf-1). Following activation of initiator caspases, both apoptotic pathways converge on effector caspases, such as caspase-3 and -7, which trigger downstream DNA fragmentation and cell death

(Verbeke et al. 2006), inhibition of cytochrome *c* release (Fan et al. 1998), and degradation of pro-apoptotic BH3 domain mitochondrial proteins (Fischer et al. 2004; Dong et al. 2005; Ying et al. 2005), an activity mediated by the chlamydial protease CPAF (Pirbhai et al. 2006). Finally, many intracellular pathogens promote a pro-survival host gene expression program that relies on the transcription factor NF- κ B (Clifton et al. 1998; Losick and Isberg 2006; Abu-Zant et al. 2006; Wahl et al. 2003; Dhiman et al. 2007).

Coxiella has taken a page from the pathogens above and actively promotes host cell survival by altering multiple steps of the apoptotic pathway. The organism antagonizes caspase-3, caspase-9, and poly (ADP-ribose) polymerase (PARP) processing following either staurosporine (intrinsic apoptosis) or TNF- α (extrinsic apoptosis) treatment of THP-1 macrophage-like cells (Voth et al. 2007b). *Coxiella* also inhibits

caspase-3 activation in primary primate alveolar macrophages, which represent a *Coxiella* target cell, implicating the *in vivo* importance of promoting cell survival. *Coxiella* also exhibits anti-apoptotic activity in HeLa (human epithelial) and CHO (Chinese hamster ovary) cells via inhibition of mitochondrial cytochrome *c* release (Luhmann and Roy 2007). However, unlike *Chlamydia spp.*, mitochondrial BH3 domain proteins are not degraded during *Coxiella* infection (Luhmann and Roy 2007; Voth et al. 2007b). Importantly, virulent *Coxiella* also inhibits caspase-3 and PARP processing (Voth et al. 2007b), indicating anti-apoptotic activity is important during natural infection by disease-causing organisms. Furthermore, bacterial protein synthesis is required for the organism's anti-apoptotic effects, indicating active regulation of host cell survival. It is reasonable to predict that one or more Dot/Icm substrates are responsible for this potent anti-apoptotic activity. Indeed, closely related *Legionella pneumophila* secretes the Dot/Icm substrates SidF (Banga et al. 2007), SdhA (Laguna et al. 2006), and LegK1 (Ge et al. 2009) to antagonize mitochondrial-mediated apoptosis and activate NF- κ B. However, *Coxiella* does not encode homologs of these proteins, suggesting a pathogen-specific repertoire of anti-apoptotic effectors.

Coxiella also regulates host apoptosis at the transcriptional level by altering expression of 30 survival-related genes (Voth et al. 2007b). For example, infected THP-1 cells show increased expression of *cIAP2*, *a1/bfl-1*, and *bag1*, which promote survival, and decreased expression of pro-apoptotic *bax*, *bim*, *bik*, *casp2*, and *casp6*, contributing to an overall anti-apoptotic state. cIAP2 and A1/Bfl-1 protein production also dramatically increases during infection. Previous studies demonstrated that *cIAP2* and *a1/bfl-1* are regulated by NF- κ B transcriptional activity (Zong et al. 1999; Chu et al. 1997), which is important for the anti-apoptotic potential of other intracellular pathogens (see above). Interestingly, NF- κ B translocates to the host nucleus during early stages of *Coxiella* infection and substantial nuclear levels persist throughout intracellular growth (Voth et al. 2007a), suggesting *Coxiella* promotes sustained activation of this versatile transcription factor.

7.3 A Link Between Autophagy and *Coxiella* Anti-apoptotic Activity

Coxiella engages host autophagosomes throughout infection as a potential source of nutrients and membrane for the maturing PV. In fact, the PV decorates with the autophagy marker LC3 as early as 5 min post-infection (Gutierrez et al. 2005; Romano et al. 2006). Additionally, activation of autophagy by amino acid deprivation or exogenous treatment with rapamycin stimulates infection and enhances PV formation. Recent evidence indicates a potential link between *Coxiella* interactions with autophagosomes and the pathogen's ability to antagonize apoptosis. During intracellular growth, the PV decorates with the autophagy-related protein Beclin-1 and the anti-apoptotic mitochondrial protein Bcl-2 (Vazquez and Colombo 2010). The interaction between these two proteins is critical for both PV biogenesis and

inhibition of apoptosis as evidenced by increased death of cells expressing low levels of Beclin-1 or a Beclin-1 mutant deficient for Bcl-2 binding. These intriguing new studies underscore the importance of crosstalk between multiple pathways for proper PV formation and sustenance of host viability.

7.4 *Coxiella* Activates Host Pro-survival Signaling Proteins

It is clear that intracellular pathogens control apoptosis at both transcriptional and post-translational levels to ensure a viable niche throughout their growth cycle. As discussed above, *Coxiella* inhibits mitochondrial cytochrome *c* release and caspase activation and induces a pro-survival transcriptional program (Luhmann and Roy 2007; Voth et al. 2007b). A recent study also demonstrates the role of host kinase-directed signaling in the *Coxiella* anti-apoptotic response (Voth and Heinzen 2009). During infection of THP-1 cells, two kinase activation events are observed. First, c-Jun, Hsp27, JNK, and p38 MAPK are phosphorylated at 2 hours post-infection (hpi), then de-phosphorylated at 12–24 hpi, suggesting these proteins are activated as an initial host response to phagocytosis of *Coxiella*. Conversely, the pro-survival kinases Akt and Erk1/2 are phosphorylated at 6 hpi and remain phosphorylated throughout infection, indicating prolonged activation of both proteins and regulation of downstream targets by *Coxiella*. The pathogen actively promotes increased Akt and Erk1/2 activation as elevated phosphorylation levels are not observed in cells infected in the presence of bacterial protein synthesis inhibitors. Akt and Erk1/2 are heavily involved in maintaining a viable eukaryotic cell via phosphorylation. Akt regulates numerous pathways that promote survival including activation of the FOXO family of transcription factors and direct regulation of caspase-9 activity (Manning and Cantley 2007), while Erk1/2 activates survival-related transcription factors such as Elk-1 and cAMP response binding element, or CREB (McCubrey et al. 2006). Thus, Akt and Erk1/2 are prime targets for apoptosis intervention by *Coxiella*. Furthermore, treatment of infected cells with Akt and Erk1/2 pathway inhibitors negates *Coxiella*'s anti-apoptotic effect, suggesting these pathways are critical for the pathogen's ability to promote survival. However, the upstream and downstream components of these cascades that are modulated during infection, and the bacterial proteins responsible, are currently unknown.

7.5 A Role for Host Signaling Cascades in PV Development

Mammalian cells also use phosphorylation-based signaling to control a diverse array of responses not directly related to apoptosis. Kinase-based cascades, such as Akt and Erk1/2, are efficient scaffolds that control sequential phosphorylation events to regulate downstream substrate activity (McCubrey et al. 2006). Eukaryotic

kinases are also controlled by their own phosphorylation, providing for tightly regulated responses. Not surprisingly, bacterial pathogens have evolved mechanisms to subvert distinct signaling pathways and influence infection events for the benefit of the pathogen (Bhavsar et al. 2007). Aside from apoptosis, intracellular pathogens utilize eukaryotic signaling for cellular entry, modulation of cytokine production, and alteration of the host cell cycle (Knodler et al. 2001; Bhavsar et al. 2007). However, the role of mammalian kinase signaling in formation of bacterial replication vacuoles has not been elucidated.

Recent studies in our laboratory have uncovered a role for host phosphorylation signaling in PV biogenesis and maintenance (Hussain et al. 2010). Thirteen signaling proteins including protein kinase C (PKC), cAMP-dependent protein kinase, and calmodulin kinase II are involved in PV formation, and inhibition of these molecules adversely affects *Coxiella* growth. These results suggest the organism must engage host signaling that is not directly involved in survival to promote PV formation and maintenance. Additionally, several kinases are differentially phosphorylated throughout intracellular growth. Furthermore, virulent *Coxiella* isolates activate PKC during infection, suggesting PKC signaling is regulated during natural infection. However, the ultimate effects of pathogen-modulated kinase activity are unknown.

7.6 Conclusions and Future Perspectives

Intracellular pathogens are adept at directing host pathways during intracellular growth to ensure a viable, sustainable niche in which to replicate. As discussed in this chapter, *Coxiella* actively regulates host apoptosis, autophagy, and phosphorylation cascades to provide a proper replication environment (Fig. 7.2). However, the bacterial proteins that regulate signaling events have not been identified and represent a substantial void in our understanding of *Coxiella*-host cell interactions. As mentioned above, *Coxiella* produces a specialized Dot/Icm T4SS during infection that delivers bacterial proteins directly to the host cytoplasm. It is intriguing to predict that a subset of effector proteins controls the pathogen's anti-apoptotic activity through direct interactions with host proteins. Indeed, exciting new evidence demonstrates *Coxiella* Dot/Icm-translocated AnkG binds to host p32, inhibiting the pro-apoptotic properties of this protein and promoting cell survival (Luhrmann et al. 2010). In addition to identifying novel Dot/Icm substrates using heterologous models such as closely-related *L. pneumophila*, recent advances in *Coxiella* host cell-free growth (Omsland et al. 2009) and genetic manipulation (Beare et al. 2009) will enhance our ability to identify and functionally characterize pathogen virulence factors. Furthermore, recent success using transposon mutagenesis (Beare et al. 2009) will allow screening of *Coxiella* mutant libraries to identify strains defective for anti-apoptotic activity and kinase activation. These anticipated studies will shed important insight into *Coxiella* virulence factors and pathogenic mechanisms.

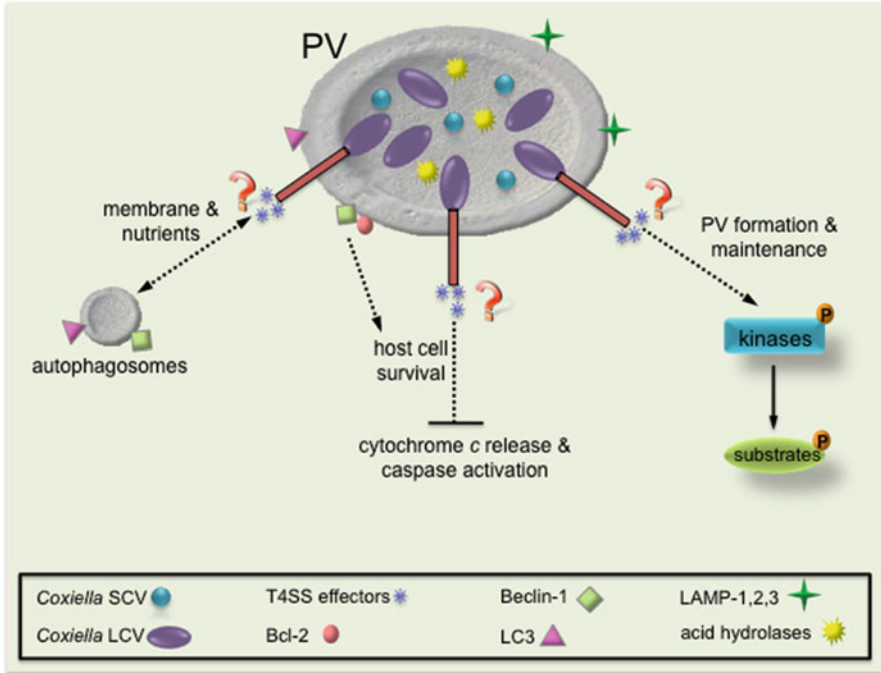


Fig. 7.2 Manipulation of intracellular host signaling by *Coxiella*. In susceptible host cells, *Coxiella* generates a membrane bound vacuolar compartment with features of phagolysosomes, including acidic pH and the presence of acid hydrolases and lysosomal membrane markers (e.g., LAMP-1, -2, -3). PV formation is aided by interactions with autophagosomes (denoted by labeling with LC3) that provide membrane for the expanding vacuole and may provide nutrients to replicating organisms. PV biogenesis relies on functional host kinase activity as evidenced by phosphorylation (p) of downstream substrates. During a lengthy infectious cycle, *Coxiella* actively antagonizes apoptotic cell death through inhibition of cytochrome *c* release and caspase processing, up-regulation of an anti-apoptotic transcriptional program, and activation of pro-survival kinases. Additionally, the autophagy-related protein Beclin-1 interacts with anti-apoptotic Bcl-2 to promote cell survival. These processes are likely controlled by the activity of numerous *Coxiella* proteins, such as anti-apoptotic AnkG, that are delivered to the host cytosol by the Dot/Icm T4SS

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