

REVIEW

Defying Death – How *Coxiella burnetii* Copes with Intentional Host Cell Suicide

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The obligate intracellular pathogen *Coxiella burnetii* is the causative agent of the worldwide zoonotic disease Q fever. This Gram-negative bacterium infects macrophages where it establishes a replicative niche in an acidic and phagolysosome-like vacuole. Establishing and maintaining the niche requires a functional type IV secretion system (T4SS[†]) which translocates multiple effector proteins into the host cell. These effector proteins act by manipulating diverse cellular processes allowing the bacterium to establish an infection and complete its complex biphasic developmental cycle. The lengthy nature of this life cycle suggests that *C. burnetii* has to successfully deal with cellular defense processes. Cell death is one mechanism infected cells frequently utilize to control or to at least minimize the impact of an infection. To date, four effector proteins have been identified in *C. burnetii*, which interfere with the induction of cell death. Three, AnkG, CaeA, and CaeB, affect intrinsic apoptosis, CaeA additionally extrinsic apoptosis. The proteins target different steps of the apoptotic pathway and are not conserved among isolates suggesting redundancy as an important feature of cell death inhibition. The fourth effector protein, IcaA, interferes with the non-canonical pathway of pyroptosis, an important inflammatory cell death pathway for controlling infectious disease. Autophagy is relevant for the *C. burnetii* life-cycle, but to which extent autophagic cell death is a factor in bacterial survival and proliferation is still not clear. To convincingly understand how bacterial manipulation of autophagy affects cell death either directly or indirectly will require further experiments. Collectively, *C. burnetii* modulates the extrinsic and intrinsic apoptotic pathways and non-canonical pyroptosis to inhibit host cell death, thereby providing a stable, intracellular niche for the course of the pathogen's infectious cycle.

INTRODUCTION

In multicellular organisms, the death of individual cells is frequently a fundamental and essential biological activity and not just an accidental event. Without

purposeful, active cell death, physiological processes in animal development, stress response, tissue homeostasis, and defense against infection are impaired in their course of action [1]. Since the initial descriptions of necrosis in the 19th century and of apoptosis in 1972 [2], new types of

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†Abbreviations: T4SS, type IV secretion system; NET, Neutrophil extracellular trap; CCV, *Coxiella*-containing vacuole; SCV, small cell variant; LCV, large cell variant; ER, endoplasmic reticulum; MOMP, mitochondrial outer membrane permeabilization; FADD, Fas-associated protein with death domain; NM, Nine Mile; LPS, lipopolysaccharide; GFP, green fluorescent protein; HA, human influenza hemagglutinin; IL, interleukin; ASC, apoptosis-associated speck protein containing a CARD or Caspase activation and recruitment domain; NMI, Nine Mile phase I; NMII, Nine Mile phase II; ORF, open reading frame.

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active, regulated cell death, such as pyroptosis, NETosis, entosis, ferroptosis, and others, have been described (for a review, see [3]). Several of these cell death pathways have important and sometimes diverse roles during microbial infection. In some cases, cell death is detrimental for the host. *Mycobacterium tuberculosis*, for example, triggers macrophage ferroptosis in a mouse infection model to further bacterial growth and dissemination [4]. Enteropathogenic yersiniae induce apoptosis in macrophages [5] and in dendritic cells *in vitro* [6]. Apoptosis induction not only counteracts innate immunity, but also contributes *in vivo* in a murine infection model to the inhibition of the adaptive immune system by suppressing the development of an effective CD8 T cell response [6]. In other cases, death of the infected cells benefits the host. Apoptosis is viewed as an important means of innate immunity to clear cells damaged by an infectious agent from the host [7]. Apoptosis of alveolar macrophages, for example, contributes to clearance of *Streptococcus pneumoniae* in a resolving mouse model of pulmonary infection [8]. Pyroptosis, on the other hand, protects mice from lethal infection with cytosol-invasive bacteria, such as *Burkholderia thailandensis* [9].

Not surprisingly, pathogens have developed many different mechanisms which actively engage these cellular defenses to allow microbial dissemination within the host organism or which, alternatively, inhibit pathway execution to ensure pathogen survival within the host cell. The latter is especially important for obligate intracellular bacterial pathogens, such as *Coxiella burnetii*, because they rely entirely on the host cell to provide them with essential substrates to maintain bacterial physiology and replication. This requires sophisticated modulation of host cell death pathways by bacterial virulence factors (for a review, see [10]).

THE LIFE AND TIMES OF COXIELLA BURNETII AS AN INTRACELLULAR PATHOGEN

Coxiella burnetii is the highly-infectious causative agent of the zoonosis Q fever. It is an obligate intracellular, Gram-negative bacterium with worldwide distribution, except in New Zealand [11,12]. Infection typically occurs by inhalation of an environmentally stable, highly infectious form with a minimal infectious dose of less than ten bacteria. *C. burnetii* infects many different vertebrate and invertebrate host species including, for example, humans, ruminants, birds, reptiles, and ticks [13], but domestic ruminant livestock are considered to represent the main natural reservoir. Their infection is often symptom-free. However, infected sheep and goats may experience abortion, stillbirth, and delivery of enfeebled lambs [14,15]. Infected animals excrete the bacteria through

vaginal secretions, milk, and feces [16], but primarily and in large numbers in birth products. Bacteria shed in such a manner are responsible for the majority of human Q fever cases and can even lead to large outbreaks, as reported in the Netherlands, with roughly 4000 cases from 2007 to 2010 [17], Germany [18], Israel [19], and other countries throughout the world. The clinical presentation of a *C. burnetii* infection can vary widely, depending on both bacterial and host factors (reviewed in [11]). In humans, the acute course of Q fever can be asymptomatic or flu-like, accompanied with fever and headache. It is usually self-limiting. In severe cases, an atypical pneumonia and hepatitis may occur. Pregnant women also show abortion, stillbirth, premature birth, and birth of weak infants. In rare cases, the infection can become chronic years after infection, with endocarditis as the most common manifestation. It has also been put forward that a chronic fatigue syndrome can develop as long-term complication of an infection (reviewed in [11]).

An infection begins after passive uptake of the bacterium by the host cell, which requires actin rearrangement. The *Coxiella*-containing vacuole (CCV) then traverses the default endocytic pathway maturing from an early endosome to the phagolysosome stage (reviewed in [20,21]). *C. burnetii* is one-of-a-kind among intracellular bacteria by not only replicating in such an acidic phagolysosome-like compartment, but to actually require these harsh conditions for metabolic activation and translocation of the effector proteins used to manipulate cellular processes [22,23]. *C. burnetii* uses a type IV secretion system (T4SS) [24] to transport roughly 150 effector proteins into the host cell where they interact with diverse host cell processes, such as apoptosis, autophagy, and endolysosomal trafficking (see [25] for a review). This interaction network establishes the replicative niche for the biphasic developmental cycle of the pathogen, which alternates between a metabolically dormant, environmentally more stable, spore-like small cell variant (SCV) and a metabolically-active, replicating large cell variant (LCV) [26]. Since the infectious cycle is lengthy and occurs without gross adverse effects to the host cell, the bacteria must have found ways to maintain cell viability possibly by disrupting cell death processes typically used to limit infections. This interference might even be relevant *in vivo*, as fewer apoptotic cells were detected in placentas from northern fur seals infected with *C. burnetii* than in placentas from non-infected seals [27]. How the bacterial pathogen interferes with the different types of host cell death is the topic of this review. Apoptosis, pyroptosis, and autophagy will preferentially be in the spotlight, since necrosis/necroptosis and other cell death pathways have not been linked to *C. burnetii* infections to date.

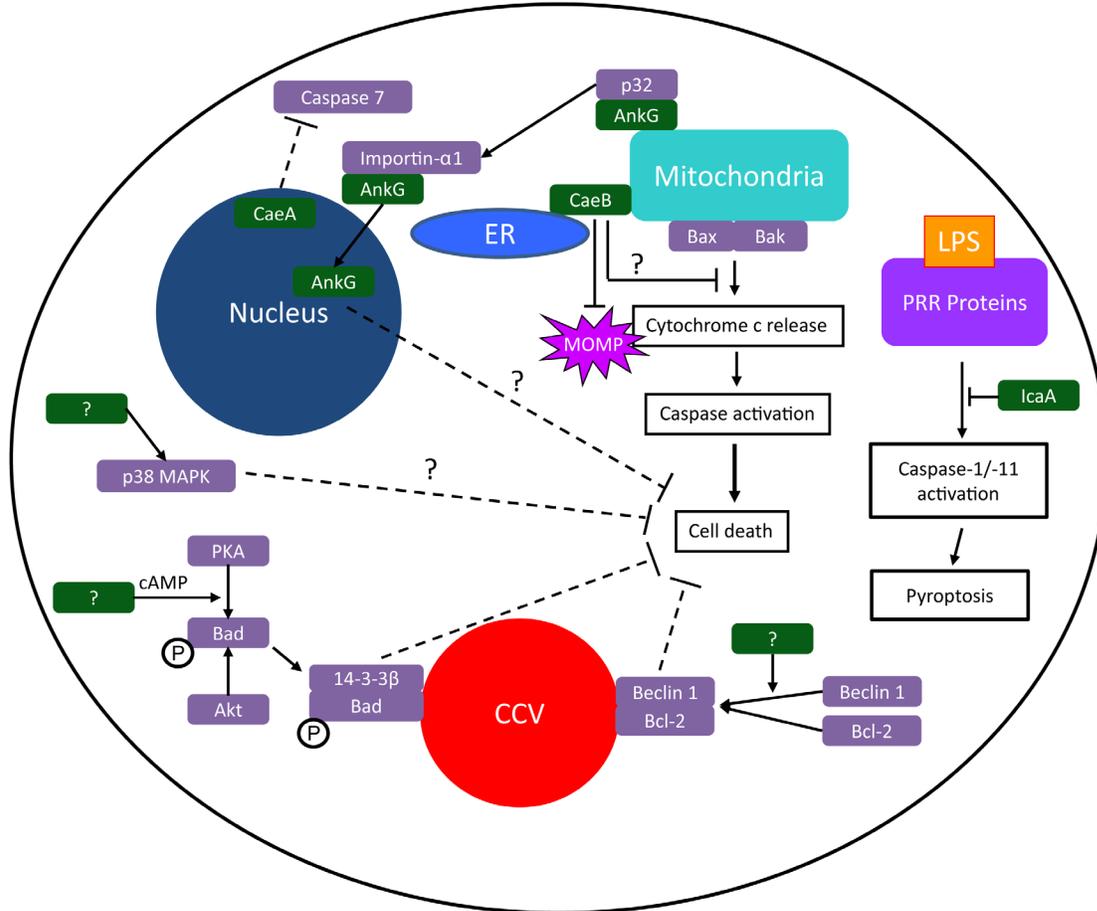


Figure 1. Schematic overview of cell death pathways affected by *Coxiella burnetii* effector proteins. Relevant sections of different cell death pathways and the interference of *C. burnetii* T4SS effector proteins (green boxes) with these pathways are shown. The anti-apoptotic effector protein AnkG migrates to the nucleus (blue circle), is imported, and fulfills its activity by a hitherto unknown mechanism. CaeB is localized to the ER (light blue oval) and possibly also to the mitochondrion (petrol box). CaeB inhibits apoptosis by interference with the mitochondrial pathway after Bax/Bak activation and inhibition of MOMP (light purple star). CaeA is localized in the nucleus, but acts far downstream in the apoptotic pathway by inhibiting the activation of Caspase-7 (interrupted line). Furthermore, an infection with *C. burnetii* facilitates the dimerization of Beclin-1 and Bcl-2 by an unknown mechanism to inhibit cell death. Additionally, there is an influence on cAMP signaling leading to phosphorylation of Bad, which is recruited to the *Coxiella*-containing vacuole (CCV; red circle) thereby inhibiting its pro-apoptotic features. The p38 signaling pathway is also influenced, leading to anti-apoptotic transcription/translation activity. *C. burnetii* also inhibits Caspase-11 activation via the effector protein IcaA. Hence, there is no inflammasome formation and no Caspase-1 activation, which in concert prevents pyroptosis. Bacterial lipopolysaccharide (LPS) is depicted as an orange box, which interacts with cellular pattern recognition receptors (purple box). Other cellular proteins are also represented by purple boxes.

APOPTOSIS AND ITS MANIPULATION BY *C. BURNETII*

Apoptosis, reviewed by [1,3,28], is morphologically characterized by blebbing of the plasma membrane, shrinking of the cell body, nuclear condensation and fragmentation, as well as formation of membrane-bound apoptotic bodies. If the latter are rapidly phagocytosed, apoptosis does not induce inflammation, since cytoplasmic content is not released to the extracellular milieu.

There are two major subtypes of apoptosis – intrinsic and extrinsic apoptosis. Intrinsic apoptosis is often initiated by cellular stress and involves mitochondrial outer membrane permeabilization which is regulated by members of the Bcl-2 family of proteins. Permeabilized mitochondria release cytochrome c resulting in the formation of a large macromolecular complex, termed the apoptosome. Subsequently, initiator and executioner cysteine proteases, known as caspases, are activated. Extrinsic apoptosis, which pathogenic bacteria can also trigger, involves

ligand binding at death receptors, followed by their dimerization, recruitment of the adaptor protein FADD and activation of initiator and executioner caspases. Processed executioner caspases then cleave many different cellular substrates, ultimately resulting in cell death.

Coxiella burnetii inhibits both intrinsic and extrinsic apoptosis in different cell types (Figure 1), such as immune and epithelial cells, and in its virulent phase I and avirulent phase II forms [29,30]. The bacterium also promotes survival by activating Akt, Erk1/2, and p38 signaling [31,32] and by subverting cAMP-dependent protein kinase activity [33]. A functional T4SS is essential for the anti-apoptotic activity [34] and several secreted T4SS effector proteins have been identified which interfere with apoptosis [35,36]. So far, AnkG (CBU0781) is the best-characterized of these. AnkG exhibits its anti-apoptotic activity in the cell nucleus where it is transported by two direct interactions with cellular proteins, which bind to different sites in its N-terminal 69 residues [37,38]. The first involves binding to p32 via an arginine rich region encompassing arginine residues 22 and 23 of AnkG [37]. This interaction with p32 is ingenious, because it couples transport of AnkG with stress-induced p32 trafficking to the cell nucleus. After migration to the nucleus, AnkG uptake is mediated by binding of importin- α 1 to a serine/threonine-rich region at residues 8-14, which acts as an alternative nuclear localization signal [38]. This interaction was discovered, because the *ankG* allele from the Dugway strain, with a leucine codon at residue eleven instead of an isoleucine codon like the Nine Mile (NM) strain, displayed better protection from staurosporine-induced apoptosis than the NM variant, when ectopically expressed in CHO cells [38]. The anti-apoptotic activity of AnkG also resides within these first 69 amino acids. However, neither its mechanism of action nor any host proteins involved have been identified or characterized to date. Another open question is if the remaining residues 70-338 of AnkG have a biological function apart from T4SS-mediated translocation and possibly, a role in intracellular trafficking, since ectopically expressed deletion mutants containing either residues 1-69 or 70-388 of AnkG are localized mainly in the nucleus or in the cytoplasm, respectively, under both healthy and apoptotic conditions [37]. A caveat that should also be kept in mind, is that secreted, 3xFlag-tagged AnkG is located in the cell nucleus during an infection [38], indicating cell stress. But AnkG, when ectopically expressed as a GFP fusion protein in transfected cell lines, is mainly mitochondria-associated and only transported to the nucleus after the cell has received an apoptotic stimulus [35,37]. Thus, even though heterologous expression experiments are extremely helpful in dissecting the molecular mechanism(s) of a process, they do not necessarily reflect the physiological status of an interaction.

Another anti-apoptotic protein [39] secreted by *C. burnetii* is CaeB (CBU1532), a potent inhibitor of intrinsic apoptosis [36,40]. Narrowing down the point of action of CaeB using an inducible expression system, immunoblotting and staining of the mitochondrial potential revealed that CaeB acts after Bax activation and mitochondrial targeting, but before mitochondrial outer membrane polarization, indicating an intra-mitochondrial block in apoptotic signaling (Figure 1) [36]. Formation of the apoptotic pore in the mitochondrial outer membrane, which is a complex process involving protein constitution, lipid composition and mitochondrial architecture [41], would be such an attractive potential candidate. Where and how CaeB blocks mitochondrial outer membrane permeabilization remains an open question, as also the subcellular localization of CaeB is unclear. Thus, one report suggests mitochondrial localization of a 3xFlag-tagged protein [39] and another demonstrated colocalization of GFP- or HA-tagged CaeB with the ER [42]. Further research is required to determine the molecular mode of action of CaeB.

The *C. burnetii* effector protein CaeA (CBU1524) inhibits both extrinsic and intrinsic host cell apoptosis by a yet unknown molecular mechanism [43]. CaeA, when ectopically expressed in HeLa cells, is found in the cell nucleus [39], but acts far downstream in the cell death cascade by preventing cleavage of the executioner caspase-7, but not of the initiator caspase-9 (Figure 1) [43]. This might explain the observed inhibition of both apoptosis subtypes. It also suggests that CaeA acts indirectly, possibly by affecting expression of anti-apoptotic proteins. The presence of CaeA does lead to higher levels of survivin in host cells, but this is not responsible for apoptosis inhibition by CaeA. Sequence comparison of *caeA* alleles from different *C. burnetii* isolates revealed a crucial EK (glutamic acid/lysine) short tandem repeat motif, with CaeA variants containing three, four, or six copies having strong anti-apoptotic activity [43].

PYROPTOSIS IS INHIBITED BY *C. BURNETII*

Pyroptosis, reviewed in [44-46], is a proinflammatory form of regulated cell death, characterized by the activation of inflammatory caspase proteases. Pyroptotic cell death is inflammatory, but shares several morphological features with apoptosis, such as DNA fragmentation, caspase-3 activation, and annexin V staining. In murine cells, caspases-1 and -11 cleave the pro-pyroptotic factor gasdermin D to induce pore formation in the plasma membrane, while caspase-1 additionally cleaves pro-interleukin (IL)-1 β and pro-IL-18 yielding the mature inflammatory cytokines. These cytokines, together with other intracellular contents, are released into the extracel-

lular space through the pores formed by cleaved gasdermin D, and constitute the inflammatory nature of the process. Pyroptosis can be divided into two major subtypes: a caspase-1 dependent canonical form and a caspase-1 independent non-canonical form. The caspase-1 dependent pathway is activated by the recognition of pathogen-associated molecular patterns or danger-associated molecular patterns by sensor proteins, followed by their oligomerization and the recruitment of the adaptor protein ASC. Oligomerized ASC binds and activates caspase-1 in the so-called inflammasome complex. Activated caspase-1 then cleaves its target molecules. The caspase-1 independent pathway (Figure 1) is specific for Gram-negative bacteria and involves caspases-4 and -5 in humans and caspase-11 in mice. These proteases are directly activated by the recognition of cytoplasmic lipopolysaccharide (LPS) and then cleave gasdermin D. Pore formation in the plasma membrane acts as a feedback loop for canonical inflammasome formation, amplifying the pyroptotic inflammatory response. Pyroptosis initiates a strong inflammatory response to pathogens in immune cells, such as macrophages and monocytes, but also in other cell types, including intestinal epithelial cells [45]. This cell death pathway is, thus, an important defense mechanism of the innate immune system of humans as well as of animals.

Depending on the cell type studied, the avirulent *Coxiella* Nine Mile phase II (NMII) variant can either induce or inhibit pyroptosis. In murine peritoneal B1a cells, which are predominantly found in the periphery and are considered carriers of innate immunity [47], NMII activates, in a T4SS-dependent manner [48], pyroptosis via Toll-like receptor 2 signaling and canonical inflammasome formation. In contrast, the virulent Nine Mile phase I (NMI) strain does not trigger a pyroptotic response [48]. A similar pattern was observed in primary human alveolar macrophages [49]. Although the authors did not check for pyroptosis, infection with the NMII strain led to strong and sustained production of pro- and mature IL-1 β , a hallmark of pyroptotic cell death, through 72 hours post infection. In contrast, levels of pro- and mature IL-1 β in cells infected with the phase I strains NMI, G and Dugway were only transiently and negligibly detectable 24 hours post infection [49]. The observation that phase I strains did not induce IL-1 β production and that the NMI strain did not induce pyroptosis suggested the presence of a mechanism to inhibit this kind of cell death thereby ensuring bacterial survival. Studies with bone marrow-derived and alveolar macrophages from different mouse strains revealed *C. burnetii* NMII inhibition of non-canonical inflammasome formation by preventing caspase-11 activation (Figure 1) [50]. The authors used a genetic screen in *Legionella pneumophila* to identify a responsible T4SS-secreted effector protein. The respective protein, previously known as CBU1823,

was named IcaA (inhibition of caspase activation). Sequence analysis showed no known protein domains and no homology to other eukaryotic or prokaryotic proteins. Hence, its mechanism of action remains elusive. Inhibition of non-canonical, caspase-11 mediated pyroptosis by *C. burnetii* indicates that bacterial LPS does become exposed in the host cell cytoplasm where it would trigger caspase-11 activation.

COXIELLA AND AUTOPHAGY INVOLVEMENT IN CELL DEATH

Macroautophagy (hereafter called “autophagy”) is a ubiquitous, evolutionary conserved, stress-responsive, degradative pathway. It disposes of superfluous or potentially dangerous cytosolic material, such as damaged organelles, misfolded protein aggregates, and invading pathogens, by sequestration within so-called autophagosomes, which are double-membraned vesicles. The autophagosomes are delivered to lysosomes for degradation so that the material can be recycled. Physiological levels of autophagy promote cell survival (reviewed in [51-53]). Autophagy occupies a central position in the biology of most eukaryotes and many components of the autophagy apparatus partake in non-autophagic cell functions, with cell death being one of them [54]. It is still controversial whether cells truly die via autophagy or whether autophagy is merely a bystander in dying cells, although increasing evidence suggests that autophagic components may be essential for cell death in certain settings (reviewed in [54,55]). Likewise, excessive or uncontrolled levels of autophagy can also induce autophagy-dependent cell death [56]. With respect to bacterial infection, autophagy can have both pro- and anti-microbial effects [57].

The interaction of *C. burnetii* with the autophagy pathway has been intensively studied (nicely summarized by [25]). The phagolysosome-like character of the *Coxiella*-containing vacuole makes it an attractive target for fusion with autophagosomes and several autophagy markers are found in the CCV membrane. CCV fusion with autophagosomes is assumed to contribute to *C. burnetii* proliferation by providing membrane material for vacuole expansion and nutrients for macromolecular synthesis [58-60]. Autophagy is functionally connected to apoptosis by a regulatory interaction between Beclin-1 and Bcl-2 [56], but this does not necessarily lead to cell death. Overexpression of wild-type Beclin-1 does not affect the anti-apoptotic activity of *C. burnetii*, but it can titrate overexpressed Bcl-2, reducing its non-specific survival activity [61]. This indicates that autophagy can influence, via the level of free Beclin-1, the threshold for intrinsic apoptosis. Any control exerted here would be indirect. Overexpression of a Beclin-1 mutant, that does not interact with Bcl-2 and that can induce cell death [56],

led to increased apoptotic cell death in transfected cells, even in cells infected with *C. burnetii* [61]. Unfortunately, autophagic flux was not measured simultaneously, so that it is not clear if the increase in cell death is due to excessive autophagy. While the effector protein Cig2/CvpB has been shown to be essential for converting the CCV to an organelle that is similar to the autolysosome [62,63], it does not seem that effector proteins are present to protect against the induction of excessive autophagy. If and how the bacterium controls autophagic flux to maintain levels beneficial for its survival and growth, is an interesting and open question that warrants further investigation.

CONCLUSION

The data presented above paint a picture of strong bacterial interference with pathways leading to host cell death. *C. burnetii* actively deploys multiple effector proteins to impede process progression. The redundancy observed here is one standout feature. In the case of apoptosis, three different effector proteins (AnkG, CaeA, and CaeB) have been identified to date. It can be expected that more anti-apoptotic proteins exist, as Martinez *et al.* [64] identified six *C. burnetii* transposon mutants which were unable to protect the cell from apoptosis-induction. Thus, the genes disrupted by the transposon might either encode for anti-apoptotic effector proteins or for proteins that influence the expression or translocation of anti-apoptotic effector proteins or of proteins that trigger pro-apoptotic signaling within the host cell. In addition, *C. burnetii* might harbor pro-apoptotic effector proteins [65]. The results obtained with the *icaA* deletion strain also clearly indicate that additional effector proteins must be present, which interfere with pyroptosis as well. The reasons for this redundancy are not clear and several explanations, not necessarily mutually exclusive, are possible. These effector proteins manipulate the cell death pathways at different steps, so that one could expect additional, novel anti-cell death effector proteins also to interfere with different steps in the various pathways. Thus, the anti-cell death effector proteins might be required *in toto* to address the many different extra- and intracellular signals and stresses that can lead to cell death, to ensure that all loopholes are covered. Alternatively, individual sets of anti-cell death effector proteins might be needed in each of the different cell types (macrophages, trophoblasts, epithelial cells) that can be infected by *C. burnetii* to address their specific requirements for maintaining cell viability.

Although it is possible that these proteins work in concert to subvert apoptotic cell death, there is no evidence for this to date. So far, each anti-cell death effector protein is able to inhibit apoptosis independently. But, since they are all actively secreted during infection, si-

multaneous, accumulated effects could be expected and it will be exciting to see if and how mutant strains containing single and multiple gene knock-outs are affected in their survival, pathogenicity, and virulence in cell culture and *in vivo* infection models.

None of the four effector proteins, which have been identified to date to interfere with cell death, are intact in all sequences of five different strains [25]. In the sequence of the CbuK-Q154 strain, the open reading frames (ORF) of all four genes are disrupted and only the sequence of the Dugway 5J108-111 strain features four intact ORFs [66]. In addition, their sequences display notable heterogeneity. This can be of functional relevance, with the AnkG variant from the Dugway strain displaying better protection from staurosporine-induced apoptosis than AnkG from the NM isolate [38]. Likewise, the number of EK repeats in CaeA correlates with protein integrity and anti-apoptotic activity [43]. Again, in both these and possibly in other cases as well, this could be due to one or more different reasons. For example, the mutational variation, at least in the intact mutants, reflects the threshold of activity required to fulfill the respective protein's biological function. Or, the redundancy present in the total effector pool is still sufficient to ensure that the biological function is met in the absence of one or more effector proteins. It is also possible, that the characteristics of the cell death pathways encountered in the host cell types preferentially infected do not require the presence of the mutated effector protein(s). The resulting mosaic pattern of active effector proteins might then contribute to pathotype development or to a speciation process, if certain genes become dispensable in specific hosts.

C. burnetii also manipulates the autophagy pathway, most likely for membrane and nutrient acquisition. Since the Bcl-2/Beclin-1 interaction indicates a strong connection between autophagy and apoptosis pathways and both proteins are recruited to the CCV membrane, it appears highly likely that effector proteins targeting one pathway might also affect the other. Controlling this interaction can also serve as an elegant means to optimize the conditions for intracellular bacterial survival and replication, by inhibiting one (apoptosis) and, at the same time, facilitating the other (autophagy). The bacteria would additionally have to safeguard against excessive autophagy, which, by itself, can lead to cell death. These assumptions have to be validated and any potential effector proteins involved in the process identified.

Of the four *Coxiella* effector proteins identified so far to interfere with host cell death pathways, one, namely AnkG, displays at least transient mitochondrial localization. Mitochondria are not only important for apoptotic cell death. They are also the power stations of the eukaryotic cell and, furthermore, involved in maintaining ion homeostasis, the production of macromolecular pre-

cursors or the sequestration of potentially damaging metabolic byproducts [67]. Additionally, mitochondria take part in cellular communication, including a prominent role in cell death activation, via signaling pathways and contact with other cellular organelles [68]. It is therefore not astonishing that mitochondria are frequently targeted by pathogenic microorganisms [69,70]. Several other putative *C. burnetii* effector proteins also display mitochondrial localization – CBU1825 [39,71], CBU1425 and the pQ1H1 plasmid-encoded CBUA0020 [71], and also MceA (CBU0077; [72]). These effector proteins were identified by a multi-stage screening process. The first step involved either a bioinformatics-guided approach [71] or random shotgun cloning [39]. Candidates were tagged and tested in a bacterial translocation assay for delivery into the infected host cell. The localization of the translocated effector protein was then visualized by fluorescence microscopy of cells transiently transfected with plasmids ectopically expressing fusion proteins. Further experiments are required to determine if these mitochondrially localized effector proteins interfere with host cell viability or with other mitochondrial functions.

OUTLOOK

As many of the mechanisms *C. burnetii* employs to manipulate host cell pathways are not understood in detail yet, especially at the molecular level, further interesting results can be expected. The recent description that T4SS-dependent recruitment of p62 to the CCV [60] leads to protein stabilization, which coincides with activation of the cytoprotective Nrf2-Keap1 pathway in infected cells [73], offers an exciting glimpse into novel fields for future research on cell survival and niche formation. With the bacterial T4SS secreting approximately 150 different known effector proteins and possibly even more, hitherto unknown proteins, it is also highly likely that progress in identifying additional actors involved and in characterizing the processes that they affect and the partners they interact with will be made. This has already become easier due to the concurrent development of defined axenic media that support host cell-free growth [74,75] and of genetic tools for generating chromosomal mutants [75-77]. Further improvement of and additions to the genetic toolbox will contribute to the functional characterization of effector proteins.

Cross-talk between the different cell-death pathways and functional redundancy of the *C. burnetii* effector proteins, as well as their importance in manipulating host cell pathways, suggest the presence of a kind of effector protein network established by *C. burnetii* to reprogram cellular physiology by affecting pivotal reactions and the corresponding proteins involved. Unraveling this network at the level of the exact molecular mechanisms

and its temporal progression will be major challenges for future research, together with elucidating the external and internal stimuli leading to network setup and the concomitant regulation involved in component expression and delivery. The insight gained will not only advance our knowledge of bacterial pathogenicity and virulence, but also pinpoint and highlight key steps in the affected intracellular pathways, providing us with the added benefit of better understanding host cell physiology, too.

Many experiments have so far been performed with the NMII strain, due to its easier handling under BSL-2 conditions, and with murine and human cells and cell lines or mouse infection models, which is self-explanatory, due to the excellent characterization of both model systems and the depth of the method and reagent toolboxes, especially for the murine system. Nevertheless, to fully understand *C. burnetii* pathogenicity and virulence, we will have to improve our knowledge of the infection in reservoir hosts and host cells [78,79], also using highly virulent wild-type strains from different hosts and with different pathotypes, even if this means adapting and developing new protocols and reagents. Analysis of field isolates will be quite important, considering the differential conservation of effector proteins between individual isolates. But only then will we be able to better understand and explain strain differences in pathogenicity and/or virulence [80].

Ongoing effector protein structure-function analysis, improved host cell pathway and network characterization, expansion of *ex vivo* and *in vivo* model systems to reservoir hosts will, when combined, offer deeper insight into the mechanisms of intentional death of eukaryotic cells with a focus on the general interplay between host cell and pathogen. This should ultimately result in new approaches to better fight *C. burnetii* infections.

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