ANALYSIS OF *COXIELLA BURNETII*

MEDIATED MODULATION OF HOST CELLS

DURING INFECTION

By

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CHAPTER I

INTRODUCTION: SCOPE OF STUDY AND ABSTRACT

Coxiella burnetii is an obligate intracellular bacterium that replicates within a parasitophorous vacuole (PV) of the eukaryotic host cell [1-3]. C. burnetii infection can manifest as either an acute (Q fever) or chronic disease. Acute Q fever is commonly a self-limiting flu-like illness, with symptoms ranging from sub-clinical to debilitating [4]. The most common sequelae of chronic disease include endocarditis, hepatitis, and/or a chronic fatigue syndrome [5-6]. In addition, C. burnetii infection of heart valves is a leading cause of culture-negative endocarditis [7]. Treatment of Q fever endocarditis is expensive and involves a protracted 18-24 month course of antibiotics [8], and often requires surgical removal of the infected valve.

The molecular mechanisms that C. burnetii uses to parasitize host cells are largely unknown. After inhalation and subsequent contact with alveolar macrophages, C. burnetii are internalized, trafficked through the endocytic pathway, and reside within an acidified PV [3, 9]. Studies suggest that the virulent Nine Mile Phase I (NMI) strain invades a cell by an association with the host cell alpha(v)beta(3) integrin receptor while the avirulent Nine Mile Phase II (NMII) strain enters through an association with alpha(v)beta(3) integrin and Complement Receptor 3 [10-11]. Experiments show that Coxiella protein synthesis is directly involved in a 4-6 hour delay of phagolysosomal maturation with a simultaneous increase in PV fusogenicity with host vesicles that are specifically trafficked to the PV to produce the spacious PV (SPV) [12-13]. Once C. burnetii is established in the PV, they appear to direct an expansion of the PV which becomes spacious in relation to the number of bacteria within the vacuole until late in infection [3]. While this expansion process is not well defined, it appears to be the result of host cell vesicles fusing with the PV through a process which requires C. burnetii

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protein synthesis [12-13].   The PV recruits distinct markers over the course of endocytotic trafficking.   Less than six hours post infection, the PV acquires small GTPases Rab5 (early endosomal marker) and, to a lesser extent, Rab7 (late endosomal marker) while a mature PV maintains the characteristics of a phagolysosome.   Moreover, the PV is also observed to interact with autophagosomes [14-18].   By two days post infection, the mature PV membrane contains vacuolar H+ ATPase, Rab7, LAMP-1, -2, and -3, flotillin 1 and 2, LC3, and Rab24 [3, 18].   If *de novo* bacterial protein synthesis is interrupted, the SPV-specific vesicle trafficking ceases and the SPV collapses into a tight vesicle which eventually destroys the organism.   These studies clearly indicate that PV biogenesis and maintenance is regulated by *C. burnetii* proteins [12-13].

In addition, *C. burnetii* proteins actively inhibit apoptosis. *C. burnetii* infected macrophages treated with inducers of extrinsic and intrinsic apoptotic pathways show a decreased release of cytochrome c from mitochondria, a reduction in caspase activity, and a decline in pro-apoptotic proteolytic cleavage [19-20].   Infection also results in induction of pro-survival transcriptional response, including sustained Akt and Erk 1/2 (host kinases) activation [19, 21].   These anti-apoptotic mechanisms are lost if *C. burnetii* protein synthesis is inhibited.   These studies suggest that *C. burnetii* proteins actively mediate the manipulation of host cell processes; however, little is known about the cell biology mechanisms involved or whether these mechanisms might also be used by the pathogen during *in vivo* infection.   Hence, characterization of the host cell pathways which are specifically regulated by *C. burnetii* derived proteins will contribute to our understanding of host-pathogen interactions, provide valuable targets for intervention

strategies, identify virulence associated cell responses, and establish *in vitro* models of the molecular interactions of this intracellular pathogen.

Intracelluar bacterial pathogens have evolved survival strategies that include bypassing the host's defense systems. One key means of establishing systemic infection is to overcome the host cells innate immune response [22]. The fact that *C. burnetii* invade macrophages and that a subset of infection becomes chronic indicate that *C. burnetii* has the capacity to not only overcome the host cell innate immune response mechanisms, but also modify the immune response to infection over the long term [23]. Macrophages are primary effector immune cells and protect the host by generating an innate immune response against invading pathogens. Upon detecting a pathogen, Pathogen Recognition Receptors (PRRs) such as Toll like receptors (TLR) and Nod like receptors (NLR) trigger a cellular alarm system comprised of intrinsic response pathways that activate surrounding immune cells [24]. This results in an aggressive pro-inflammatory response, phagocytosis and degradation of the pathogen, or migration of infected cells to secondary lymphoid tissue and subsequent antigen presentation. Cell surface TLRs (TLR1-6, TLR 10, and TLR 11) and endosomal membrane TLRs (TLR3 and TLR7–TLR9) signal through MyD88, which activates the NF-κB or IRF3-IRF7 pathway [24-33]. Signaling may also occur through MAP kinase networks by activating the MAPK proteins p38, Jnk, and Erk through phosphorylation [27-29, 32, 34-35]. Whereas MAPKs regulate gene expression through the phosphorylation of other proteins, NF-κB subunits bind directly to DNA to regulate transcription [27]. Hence, PRRs follow specific signaling pathways that facilitate host control of infection via pro-inflammatory responses.

The role of host TLRs and the innate immune response in controlling *C. burnetii* has been investigated. Studies indicate that host TLR-2 is needed for pathogen recognition and growth restriction [36]. Replication of *C. burnetii* is restricted in macrophages in response to TLR2 activation [36] while TLR2 deficient mice permit unrestricted growth of *C. burnetii* [36]. However, alternate findings have suggested that host TLR-4 is associated with initial pathogen uptake and localized actin polymerization within the host [37]. This theory is contrary to findings that demonstrate *C. burnetii* phase II to activate TLR2 but not TLR4 [36]. In addition, the LPS of virulent Phase I organisms, but not Phase II organisms, prevents activation of dendritic cells by masking the TLR-4 ligand [38], further suggesting that TLR4 is primarly affected by *C. burnetii* infection.

Several investigations on Q fever cytokine response indicate that *C. burnetii* triggers an atypical M2 (IL-12^{low}, IL-23^{low}, IL-10^{high}) form of activation in monocyte-derived macrophages (MDM) [39]. Other experiments performed to detect pro-inflammatory cytokines in DCs reveal that phase II *C. burnetii* promotes DC maturation and secretion of IL-12 and TNF [38, 40]. Increased productions of other pro-inflammatory cytokines like RANTES, MCP-1 and transcriptional upregulation of SCYA3, SCYA4, and IL-8 chemokines has also been detected [41-42]. In addition, the studies on apoptosis during *C. burnetii* infection demonstrated an upregulation of genes involved in NFκB signaling as well as an increase in the expression of c-iap2 and A1/bfl-1, antiapoptotic proteins that are positively regulated by NFκB transcription factors [19, 21]. Together these studies suggest that the host innate immune system attempts to restrict *C. burnetii* infections. However, none of these studies indicate how *C. burnetii*

interferes and subverts the signaling pathways of the immune response and establishes a productive infection.

The ability of *C. burnetii* to survive within macrophages while avoiding host immune response and modulating the cellular processes at the molecular level requires the skill to deliver effector molecules out of the PV [3, 18, 23]. The *C. burnetii* type IV secretion system (T4SS) represents a means for the bacteria to deliver effector proteins into the host during the initial infection as well as subsequent PV establishment and bacterial growth, allowing for its survival and cellular pathogenesis. Generally, multiple intracellular pathways might be modulated throughout the infectious cycle, whereby bacteria proteins interact at several points in a pathway to exploit it. Recent studies have identified a list of potential *C. burnetii* T4SS effector proteins whose function are yet unknown [43-45].

Despite recent advances, knowledge of *C. burnetii*'s targets inside a host cell is still limited. In addition, *C. burnetii's* ability to evade detection and grow intracellularly by suppression of the host's immune response is yet to be understood. These properties represent a model for studying virulence determinants which can subsequently unravel specific molecular interactions of *C. burnetii* with its host cell. As such, I sought to address the following questions:

- Does *de novo C. burnetii* protein synthesis regulate host-cell gene expression during infection?

- Does *C. burnetii* actively modulate host cell immune response?

Additionally, no tissue culture model exists to study host cell-pathogen interactions in ticks. In nature, it is possible for domestic animals (cattle, sheep and goat) to acquire *C. burnetii* via tick bites as well as by contact with contaminated tick excreta [4, 46-48]. Ticks likely play a large role in the transmission of this infectious agent between wild and domestic animals [48]. Interestingly, more than 40 species of ticks have been found infected with *C. burnetii* [4]. Historically, crude primary tick cell cultures have been shown to support *C. burnetii* growth, but a modern, established tick cell line has never been employed [49]. In an attempt to develop alternate *in vitro* models of *C. burnetii*-host cell interactions, I sought to determine:

- Is *C. burnetii* capable of infecting a modern continuous tick cell culture?

- Does *C. burnetii* replicate within cultured tick cells, and at what rate?

- Is *C. burnetii* produced within tick cells capable of subsequently infecting mammalian cells?

Abstract

Coxiella burnetii is an obligate intracellular bacteria and the etiologic agent of Q fever. Although discovered over six decades ago, our understanding of the molecular mechanisms involved in disease development remains elementary. Few host cell processes actively modulated by *C. burnetii* have been identified. This study analyses host-cell pathways and processes that are specifically affected by *C. burnetii* proteins. It also defines *C. burnetii* induced temporal modulation of NF-kB activation throughout the infectious cycle. Additionally, it determines *C. burnetii*'s growth cycle in an established tick cell line. First, the global expression of host cell mRNA was characterized following infection with *C. burnetii* Nine Mile Phase II and transient inhibition of bacterial protein synthesis with chloramphenicol. Using comparative microarray analysis, 36 host cell genes were identified to be distinctively modulated by *C. burnetii* proteins. Subsequent gene ontology analysis revealed expression changes in host cell functions such as innate immune response, cell death and proliferation, vesicle trafficking and development, lipid homeostasis, and cytoskeletal organization. A subset of pro-inflammatory cytokine genes was also identified whose expression is classically mediated through the NF-κB signaling pathway. This led to the demonstration that *C. burnetii* infection temporally modulates the activation of the NF-κB signaling pathway. Additionally, I have shown that *C. burnetii* readily infects *Ixodes scapularis*-derived cultured IDE8 cells, followed by a prolonged lag phase, then a doubling time similar to that in eukaryotic cells. Together these studies show that *C. burnetii* replicates and produces infectious progeny in arthropod cells, and temporally modulates mammalian host cell NF-κB signaling

pathway as well as host cell gene expression in a bacterial protein synthesis specific manner.

CHAPTER II

LITERATURE REVIEW

Coxiella burnetii: Introduction

Coxiella burnetii is a Gram-negative, pleomorphic, obligate intracellular bacteria with a worldwide distribution [3-4]. It causes Query fever (Q fever) in humans and Coxiellosis in animals [4, 48, 50-51]. This is thought to be a highly underreported zoonotic disease and accurate estimates of global Q fever cases are unknown. Discovered in the late 1930's, *C. burnetii* is taxonomically placed in the γ-subdivision of the phylum proteobacteria [52]. In nature, domestic animals (cattle, sheep, and goats) are considered to be the primary reservoir, while arthropods contribute in transmission of the bacteria between domestic and wild hosts [53]. Human infection primarily occurs via *C. burnetii* containing aerosol droplets and disease usually manifests as acute Q fever although chronic disease may arise. Acute illnesses are mostly self-limiting while chronic infection is marked by endocarditis, hepatitis, osteomyelitis, or infected aortic aneurysms [4, 8, 54-56]. This organism is extremely infectious, environmentally stable and has been shown to travel as far as 11 miles by wind [57]. Due to such properties, the Center for Disease Control and Prevention (CDC), classifies *C. burnetii* as a category B bio-weapon or bio-terror agent [52, 58-59].

Coxiella burnetii: Brief history of discovery

C. burnetii was independently discovered by Australian and American researchers in the late 1930s [60-68]. In 1935, E H Derrick from the Queensland Health department led an investigation to identify the etiological agent of a mysterious fever outbreak in Brisbane, Australia. The term "Q fever", for querulous fever, was coined by him as he described the illness of infected abattoir (slaughterhouse) workers in a classic paper published in 1937 [64-65, 68]. The clinical symptoms of this illness were initially

considered similar to typhus fever, typhoid and paratyphoid fevers but eventually found to be different [64-66, 68]. Convalescent sera collected from infected patients were titrated against numerous pathogens for antibody detection and none were found positive. The absence of such antibodies raised the suspicion of involvement of a new infectious organism. Derrick failed to identify or isolate the etiological agent and efforts to culture the organism on bacteriological media proved futile. However, he was successful in infecting guinea pigs with blood and urine obtained from infected patients. These experiments led him to believe that the causative agent of this undiagnosed fever was a virus [64-68].

Macfarlane Burnet, a virologist working at Walter and Eliza Hall Institute in Melbourne, was sought-out by Derrick to identify the Queensland agent. Burnet, with the help of his associate Mavis Freeman, began investigations in 1936 [64-67]. They not only infected guinea pigs with tissue material provided by Derrick but also studied the infection in mice and monkey models. Their efforts to grow the pathogen on the chorioallantoic membrane of embryonated eggs were partially successful. Infected mouse spleens stained with haematoxylin and eosin gave the first indications of the rickettsial nature of this unknown organism [64-67]. Later, serological experiments with convalescent sera obtained from typhus and Q-fever patients showed that emulsified spleen tissue agglutinated with Q-fever patient sera but not with Typhus sera. Burnet then cautiously concluded that the causative agent of Brisbane's fever outbreak was a new rickettsial pathogen [64-67].

During the same period, at the Rocky Mountain Laboratory (RML), Montana, US scientists were studying Rocky Mountain spotted fever (RMSF) [60-62, 68-70]. Gordon

Davis, who had joined RML in 1930 as a bacteriologist was investigating the ecology of RMSF. Davis studied rickettsial transmission by feeding ticks onto guinea pigs and noting their rectal temperatures over time. In 1935, Davis received a batch of *Dermacentor andersoni* ticks collected from the Nile Mile Creek area in Western Montana. While conducting his experiments, he observed that one of the guinea pigs developed a febrile illness after feeding by ticks from Nine Mile Creek, but the symptoms differed from those of RMSF. Blood obtained from the infected guinea pig would cause fever in freshly injected guinea pigs, but no bacterial growth was observed in axenic media [62, 68, 70].

Meanwhile, Herald Cox, who obtained his doctoral degree from John Hopkins University, joined RML in 1936. He was assigned to work with Davis on the new infectious agent. After a series of studies, Davis and Cox discovered that the new agent was rickettsia-like and easily passed through Berkefeld filters [62, 68, 70]. In 1938 Cox was successful in cultivating the organism in embryonated eggs [71]. The scientific curiosity about this organism continued until a laboratory worker got infected with the Nine Mile fever and his blood transmitted febrile illness to guinea pigs [63, 68, 70]. The Australian illness was connected to the "Nine Mile" agent when guinea pigs exposed to the Australian organism remained protected when challenged with the laboratory workers blood. These experiments linked the Q fever of the US and Australia [64, 72-75]. At first, the new agent was named *Rickettsia diaporica* by Cox and *Rickettsia burnetii* by Burnet, but in 1948 was renamed *Coxiella burnetii* to honor Cox and Burnet for their extraordinary contributions and because the organism was not similar to true rickettsial organisms [64, 71-72, 76-77].

Coxiella burnetii: Epidemiology

Host Distribution

C. burnetii infects a diverse range of hosts including humans, ruminants (cattle, sheep and goats), pets (cats and dogs), ticks, horses, pigs, camels, buffaloes, birds and occasionally reptiles [4, 78-80]. Among animals, domestic ruminants are considered to be the primary reservoirs and the largest source of infection for humans [4, 48, 53, 81]. Unlike humans, infected animals do not show any symptoms of Q fever [4, 48]. However, *C. burnetii* infection in animals can manifest clinically as late term abortions [4, 48]. Uterus and mammary glands are the most crucial sites of chronic *C. burnetii* infection in ruminants [48]. Infected animals frequently shed *Coxiella* in their urine, feces, milk, and birth products, from which humans are exposed [4, 48]. Maximum shedding occurs during parturition but milk also contains large amounts of *C. burnetii* [4, 82-85]. Epidemiological data clearly show that dairy cows become chronically infected more often than sheep and goats [4, 80, 82, 86-89]. The current prevalence of coxiellosis in domestic ruminants is not known as thorough seroepidemiological studies were last conducted around 30 years ago [4, 48, 52, 81]. Besides domestic livestock, *C. burnetii* is also chronically carried by pets. Dogs are thought to be infected by tick bites, by placenta consumption or by ingestion of milk containing *C. burnetii.* Infection in dogs often leads to the death of pups [4, 48]. Epidemiological studies indicate that humans can acquire Q fever from infected dogs and cats [4, 48, 90-92].

Other crucial hosts of *C. burnetii* include multiple species of arthropods which can act as vectors for disease transmission between animals [4, 48]. Evidence of *C. burnetii* infection has been shown in over 40 tick species, fleas, mites, flies and other

arthropods [4, 46]. Ticks are believed to play a crucial role in the maintenance and transfer of the pathogen between infected wild and domestic mammals [4, 46, 81]. Tick borne transmission of coxiellosis among wild vertebrates like rodents, lagomorphs, and wild birds is prevalent [46, 80, 93]. However, the role of ticks in the passage and cycling of *C. burnetii* between various species needs to be defined precisely. Human Q fever transmission via ticks is rare but possible [74]. *C. burnetii* can also be carried by unusually rare hosts like snakes, tortoises [80], and sea lions [94].

Geographic Distribution

Q fever has a worldwide distribution and has been reported from more than 50 countries in various parts of the world [4, 46]. New evidence shows the presence of infection in New Zealand, the only country previously thought to be free of these bacteria [48, 95-96]. Serological screenings indicate that *C. burnetii* infections are more frequent in tropical than in temperate climates [48]. Most countries in Europe and Asia have diagnosed and reported Q fever cases in humans and animals [4, 97-99]. A major outbreak of Q fever was recently reported from the Netherlands [100]. The outbreak, beginning in 2007, has infected more than 2300 humans and continues, although control measures appear to be lessening the case numbers [52, 100-101]. This epidemic has resulted in severe economic losses as more than 50,000 dairy goats have been slaughtered to prevent disease spread [100, 102]. Countries like France and Australia, where the disease is extensively studied, show higher incidence rates of Q fever than the United States [4, 46, 53, 103]. In the United States, cases of Q fever have increased from 21 cases per year (1978-1999) to 51 cases per year (2000-2004). This increase in the number of Q fever cases is likely linked to the disease becoming reportable in the US in

1999 [81, 104]. Cases of Q fever have also been reported in US military personnel serving in Iraq and Afghanistan [105]. The majority of these patients were found to have been in contact with domestic animals or their products [106].

Transmission

C. burnetii is a highly infectious, stable organism primarily transmitted via aerosols [52, 57, 59, 107-108]. It can survive in the dust of contaminated premises for several months and has a very low infectious dose [4, 59, 109]. *C. burnetii* aerosol contamination often occurs from parturient fluids of infected animals, which may in turn infect humans, new-born animals, and other uninfected animals [4, 48]. Infected animals also shed the bacteria in feces, urine, and milk. When dried, bacteria contained within these products may become aerosolized [4, 48, 81, 109]. Different types of fomites have been identified to serve as vehicles of *C. burnetii* dissemination as well. These include wool, shoes, clothing, and straw and barn yard materials contaminated with infected animal's excreta. Aerosolized bacteria are not only harbored in the contaminated area, but may spread long distances by wind [48, 59, 81]. Wind borne dispersion of *C. burnetii* aerosols plays a major role in infection of humans not in direct contact with infected animals [57].

Transmission via ingestion of contaminated products has also been implicated in *C. burnetii* infections. This has primarily been associated with drinking unpasteurized milk and eating contaminated meat [4, 59, 84, 100, 110]. Direct person to person transmission is very rare, but may be possible in cases of pneumonia [4]. Sexual transmission of *Coxiellae* is yet to be fully established but has been suggested in both humans and mice [111-112]. Bacteria has also been isolated from bull semen [113].

Data on the transmission of *C. burnetii* to humans following a tick bite is lacking [74]. However, it appears that the infected ticks and other arthropods play a significant role in maintaining the pathogen in domestic and wild animals [4, 46, 48, 81, 93]. *C. burnetii* infected ticks commonly pass the bacteria transtadially or transovarially to their offspring. Ticks also transmit *C. burnetii* horizontally (via bite or in feces) to wild vertebrates, wild birds, and domestic animals thereby causing both domestic and wild life coxiellosis [48, 81, 93, 98, 103].

Coxiella burnetii: Disease

C. burnetii causes the zoonotic disease Q fever. Infection can manifest as acute or chronic Q fever [4, 65, 80]. In humans, the primary site of infection is alveolar macrophages [3, 107]. The signs and symptoms of Q fever differs widely. More than 50% of people exposed to *C. burnetii* will not display any symptoms, while in chronically infected cases, the heart and other major organs are typically affected [4, 53].

Acute Q fever

In a typical acute Q fever case, the incubation period ranges from 1 to 3 weeks (depending on inoculation dose). While many cases are inapparent, symptomatic cases present as a non-specific flu like illness [4, 54, 114]. The patient experiences a sudden onset of high fever (104-105° F) and headache. The fever peaks within 2-4 days and returns to normal after 5-14 days. In elderly patients (≥40 years) the fever remains for a longer duration. However, in untreated cases, the fever has been shown to last between 5 to 57 days. A quarter of infected patients experience symptoms of a biphasic fever [4, 54, 114-115]. The first phase has the typical acute Q fever symptoms while the second phase is characterized by an intermittent appearance of low grade fever lasting anywhere

from 1-19 days. Other indicators like malaise, myalgia, sweats, nausea, vomiting, diarrhea, abdominal pain, confusion, sore throat, non-productive cough, chills, and chest pain may also be associated with acute Q fever [4].

Thirty to fifty percent of patients experiencing high fever develop atypical pneumonia, and some may develop hepatitis [4, 54, 114-115]. It is believed that the entry route may also play a role in the disease manifestations. In Nova Scotia, Canada, where Q fever cases often manifest as pnuemonia, infection appears to occur through inhalation of contaminated aerosols [116-118]. Patients diagnosed with atypical pneumonia reveal multiple rounded opacities in both lungs, an increase in reticular markings, atelectasis, and also pleural effusion [4]. Such radiographic results are often confused with viral, *Mycoplasma* or *Chlamydia* infection. In addition, splenomegaly is noted in about 5% of the patients and inspiratory crackles are also reported [4, 116, 119].

In Europe, however, it has been suggested that large numbers of infections occur through the ingestion of raw milk, leading to granulomatus hepatitis. It appears that during the incubation period many patients have a transient *C. burnetii* bacteremia, resulting in the hematogenous spread of the bacteria to multiple organs (liver, spleen, lungs, bone marrow and female genital tract). Such bacterial spreading ultimately causes serious complications such as meningoencephalitis, myocarditis, or pericarditis [4, 8, 56, 114, 120]. Q fever hepatitis is usually detected by elevated enzyme levels of aspartate aminotransferase [AST], alanine aminotransferase [ALT], and alkaline phosphatase. This increase in enzyme levels is often accompanied by abdominal pain (in right hypochondrium), anorexia, nausea, vomiting, and diarrhea [4, 8, 56, 114, 120-124]. In the most severe cases, destruction of hepatic tissue, coma and death might occur. Other

acute Q fever indicators include myocarditis, pericarditis, skin rash and meningoencephalitis [4]. An interesting biological phenomenon observed in over 90% of acute Q fever patients is that the leukocyte count stays normal [4].

Chronic Q fever

It is observed that approximately five percent of *C.burnetii* infections manifest as chronic illness and that the majority of these patients are over 40 years of age. Chronic Q fever may develop a month to many years after an acute infection or exposure. Individuals with no previous history of acute illness have been shown to have chronic Q fever [4, 54, 125-129]. Chronic Q fever is primarily characterized by endocarditis, with 60-70% of diagnosed chronic Q fever cases revealing this complication. If left untreated, the illness usually proves fatal. About 90% of chronic Q fever endocarditis patients are thought to have had previous cardiac valve defects. The bacterium generally affects the aortic and mitral valves, although prosthetic valve Q fever endocarditis has also been reported [4, 54, 126-129]. Clinical symptoms for chronic Q fever endocarditis include cardiac valve dysfunction along with low-grade fever, malaise, weakness, fatigue, weight loss, chills, anorexia, and night sweats. Chest X-rays and electrocardiography show cardiomegaly, arrhythmia and ventricular hypertrophy. Chronic Q fever endocarditis patients often have peripheral manifestations like digital clubbing and purpuric rash (observed in mucosa and extremities) [4, 54, 56, 125-131]. Patients harboring the bacteria for extended periods of time invariably suffer from splenomegaly and hepatomegaly. Mild hematuria has also been noted, which may lead to renal complications [4].

Other prominent clinical presentations of chronic Q fever include vascular infections, osteoarticular infections, chronic hepatitis, chronic pulmonary infections, and chronic fatigue syndrome [4]. Though rare, *C. burnetii* vascular infections can be life-threatening [4]. Q fever has also been associated with fetal morbidity and mortality. Reports show that untreated pregnant women infected with *C. burnetii,* suffered from spontaneous abortion, intrauterine growth retardation, oligoamnios, stillbirth, or premature delivery [4, 131-132]. *C. burnetii* appears to colonize the uterus, placenta, and mammary glands of an infected pregnant woman [4, 101, 131, 133].

Diagnosis, Treatment and Prophylaxis

Clinical detection of acute Q fever is complicated by its resemblance to many infectious diseases [4, 53, 116, 129, 134-137]. Chronic Q fever on the other hand is thought to be an under-diagnosed cause of endocarditis. Q fever cases are primarily diagnosed using serology; immunofluorescent microscopy is used as the primary reference method. Serological testing require detection of both phase II (acute Q fever) and phase I (chronic Q fever) antibodies [138]. Titers of IgM and IgG antibodies directed against *C. burnetii* NM phase II indicate the presence of an acute infection, while titers of IgG and IgA antibodies directed against both virulent (Phase I) and avirulent (Phase II) forms of *C. burnetii* are used to determine the occurrence of chronic infections [4, 52-53, 116, 129, 134-138]. Other diagnostic tools which have been used for *C. burnetii* detection at one time or another include polymerase chain reaction (PCR), immunohistochemistry, complement fixation, microagglutination, ELISA, Western blotting, dot blotting, slide agglutination, indirect hemolysis, radioimmunoassay, and cross-adsorption [4, 52-53, 116, 129, 134-142].

Although *C. burnetii* resides in acidified vacuoles within cells, efficient treatment of acute infections is possible with antibiotics such as teteracycline, cotrimoxazole, ofloxacin, and pefloxacin, using a 14 to 21 day course of treatment [4, 81, 131, 143]. These antibiotics are thought to act bacteriostatically. Combination therapy using doxycycline and chloroquine or OH-chloroquine is a good alternative to antibiotics alone, as chloroquine elevates the pH within the PV, restoring a more bactericidal effect for doxycycline [4, 8, 53, 81, 143]. Chronic Q fever on the other hand requires treatment from18 to 36 months with doxycycline (200 mg daily) and hydroxychloroquine (started at 600 mg daily). In these cases, treated patients require regular eye examination to monitor the development of light sensitivity [4, 8, 53, 81, 143]. Because of possible side effects, pregnant women suffering from Q fever are not treated with doxycycline and chloroquine; instead, cotrimoxazole is substituted although it appears to act bacteriostatically [131].

Preventive measures for Q fever infection control include vaccination of both animals and humans [4, 48, 81]. In animals, vaccination efforts have shown diverse responses; chloroform-methanol residue vaccines are considerably better tolerated by animals than *C. burnetii* whole-cell vaccines [144]. Phase I *C. burnetii* based vaccines have been found to be more protective than phase II bacteria [144]. Vaccination of cattle protected them against *C. burnetii* induced abortion, low fetal weight, and chronic infertility, but failed to eradicate *C. burnetii* in animals naturally infected prior to vaccination [4, 48, 144]. A European vaccine containing both phase II *C. burnetii* and *Chlamydia psittaci* was also reportedly used to protect cattle and goats against fertility problems [4, 144-145]. However, humans in contact with vaccinated goats were found

have developed *C. burnetii* infections. Because of the inconsistent results with animal vaccinations, domestic animals are not currently being vaccinated routinely [4, 48, 144].

In order to prevent Q fever in humans, three different types of vaccine are currently available; a formalin-inactivated Q fever vaccine (Q-Vax), prepared from phase I *C. burnetii* Henzerling strain which is only available in Australia; an Investigational New Drug (IND) experimental phase I formalin-inactivated vaccine in the USA; and the soluble LPS-protein complex chemovaccine extracted from phase I cells by trichloroacetic acid, which has been used in Slovakia [4, 53, 81, 146]. None of these vaccines are commercially available in the United States [147]. The Australian Q-vax vaccine has been found to be highly immunogenic, but requires a skin test prior to administration as it causes adverse effects when administered to previously infected individuals [4, 148]. Chloroform-methanol pretreatment of phase I *C. burnetii* cells significantly reduces these effects, but does not impart the same level of protection [4, 149]. Q fever vaccines are highly recommended for livestock handlers, animal product processors, veterinarians, and laboratory personnel working with phase I *C. burnetii* infected animals [4, 59, 81, 150]. Research on immunoreactive *Coxiella* proteins is ongoing in an effort to manufacture a safe and effective Q fever vaccine.

Coxiella burnetii: Cultivation

C. burnetii was first cultivated in embryonated hen's eggs in 1938 [60]. Culturing the bacteria using this method involves inoculation of 5 to 7 day-old embryonated chicken eggs. Inoculated eggs are incubated at 35°C for 10 to 12 days and then harvested. The yolk sac, specifically the yolk sac membrane, has been found to harbor large numbers of bacteria, with lesser numbers found within the tissues of the embryo. *C.*

burnetii isolation and purification from infected eggs is a difficult and lengthy process involving homogenization of infected yolk sacs, differential centrifugation and passage through density gradients [3, 52, 151-153]. Until the advent of modern tissue culture methods, egg yolk-sac growth was the primary means of culturing *C. burnetii* outside of mammalian hosts.

Historically, *C. burnetii* cultivation was performed in guinea pigs. In modern science, this method has limited use but still remains as an excellent procedure for phase I *C. burnetii* isolation from contaminated environmental samples [4, 52, 151, 154]. Animal models for Q fever include mice, rats, rabbits, guinea pigs and monkeys [4, 155-156]. Guinea pigs are an excellent model for Q fever as it closely mimics the human disease. When infected intranasally or intraperitoneally, guinea pigs develop hyperthermia (≥ 40°C) after 5-12 days of incubation, show signs of pneumonia, excrete bacteria in their urine, and may develop lesions in their spleen, testes and liver [154, 157]. Infected guinea pigs remain latently infected and death during convalescence is frequently due to degenerative myocarditis. Serologically, phase II antibodies appear 15 days post infection, and both phase II and phase I antibodies are seen within the second month of infection [4, 52, 154, 157]. Other than guinea pigs, mice are extensively employed as animal models for *C. burnetii* studies. When infected intranasally or intraperitoneally mice remain asymptomatic and do not acquire fever. However, development of granulomatous lesions in the spleen, liver, kidneys, and adrenals are observed. An increase in bacterial numbers also occurs in the spleen and liver. Chronically infected mice shed *C. burnetii* in their feces and urine [4, 52, 158-161].

C. burnetii has been found to infect a range of cultured cells; this includes primary cells as well as established cell lines. Often monocytic and macrophage cells such as the human acute monocyte leukemia THP-1 cells, J774 and P388D1 macrophage-like tumor cell lines, various fibroblast cell lines, green monkey kidney (Vero) cells, various epithelial cells as well as primary and cultured tick cells [3-4, 151, 162]. While *C. burnetii* purification from cell cultures is far less laborious than isolations from egg culture, it still involves several differential centrifugation and density gradient steps to produce pure *C. burnetii* stocks [3-4, 151].

Recently, a monumental step in the cultivation of *C. burnetii* was accomplished; the growth of *C. burnetii* in host-cell free environment [163]. Omsland *et al*. developed a complex nutrient medium for *C. burnetii* growth under axenic (host cell free) conditions, which will profoundly affect our progress in understanding *C. burnetii's* role in virulence and disease mechanisms [163]. *C. burnetii* acidified citrate cysteine medium (ACCM) was developed by studying *C. burnetii's* metabolic requirements using gene expression arrays, genomic reconstruction and metabolic typing. Using a heavy inoculum and incubation at 2.5% oxygen tension, this medium supports a 3 $\log_{10}$ increase over 6 days time. Additionally, this media allows the conversion of SCVs to LCVs, and the harvested bacteria are highly infectious for Vero cells [163]. This important breakthrough will not only facilitate studies regarding this organism's pathogenesis and genetics, but will ultimately aid in the development of effective Q fever preventatives.

Coxiella burnetii: **Microbiology**

Taxonomically *C. burnetii* is classified under the kingdom: Bacteria, Phylum: Proteobacteria, Class: γ-Proteobacteria, Order: Legionellales, and Family: Coxiellaceae

[4, 164]. It has a bi-phasic life cycle possessing characters for survival both inside and outside of a host cell [9].

Morphology, ultrastructure and developmental biology

C. burnetii is a small Gram-negative pleomorphic coccobacillus with two distinct morphological forms, the large cell variant (LCV) and the small cell variant (SCV) [9, 165]. Both forms differ in size, morphology, peptidoglycan content and resistance to physical disruption. The replicative, metabolically active LCV have an approximate dimension of 0.3 by 1.0 μm while the environmentally stable, metabolically inactive SCVs measure 0.2 to 0.5 μm in length. LCVs possess a typical Gram-negative cell wall structure yet stain in a Gram-variable fashion [9, 165-166]. Therefore, Gimenez staining is often used in observing *C. burnetii* [165]. The LCVs have a thin cell wall with the thickness of the outer and inner membrane being approximately 6.5-8 nm including membranes, peptidoglycan, and periplasmic space. In addition, they contain a filamentous and dispersed nucleiod region [3, 9, 166-167]. In contrast, SCVs are compact, rod shaped with an electron dense nucleoid core surrounded by cytoplasmic and outer membranes. Its cell wall has (i) been measured between 13 and 21 nm in thickness, (ii) no discernable periplasmic space, and (iii) a high protein content within the periplasmic space. These forms can survive for long periods in the environment and are resistant to many physical and chemical treatments. Additionally, tests on *C. burnetii* peptidoglycan content demonstrate that the peptidoglycan protein complex (PG-PC) shifts from being ~2% in the LCVs to ~32% in SCVs [3, 165, 168]. These properties appear to make SCVs insensitive to UV, desiccation, osmotic shock, sonication (in distilled H_2O >30 min.) and temperatures which inactivate many other bacteria [3, 9,

165]. Experiments show that infectious *C. burnetii* were still detectable after 24 h treatments with 0.5% sodium hypochlorite, 2% Roccal, 5% Lysol, or 5% formalin. However, 70% ethanol, 5% chloroform, or 5% Enviro-Chem (diquantinary ammonia product) effectively inactivate them [52, 81, 169].

Differences between the LCV and SCV forms can not only be differentiated by size and membrane structure, but also by proteome expression [3, 9]. For example, the major outer membrane protein, P1, is expressed in large quantities in LCVs yet its expression is reduced in SCVs. Elongation factors EF-Tu and EF-Ts, along with the stationary-phase sigma factor RpoS, also demonstrate increased expressed in *C. burnetii* LCVs. Meanwhile, proteins found uniquely in SCVs include the histone like protein Hq1 (a homolog of eukaryotic H1 histone) and a small basic protein designated ScvA [9, 170-171]. A recent LCV/SCV proteome study has identified several additional proteins with differential protein expression; including 15 in LCVs and 4 in SCVs [170].

Defined studies analyzing the *C. burnetii* growth cycle using a synchronous infection model has revealed that these bacteria follow the typical growth pattern exhibited by a closed bacterial system. There are defined lag, exponential replication, and stationary phases [172]. The lag phase, which extends up to approximately 2 days post infection (PI) primarily involves SCV-to-LCV morphogenesis. The exponential phase spans the next 4 days where LCVs grow and replicate within the PV. The doubling time for *C. burnetii* during this growth phase has been calculated at ~12.4 h. Stationary phase begins ~6 days PI with the re-emergence of SCVs via conversion of LCVs to SCVs through a range of transitional forms [172]. Both LCVs and SCVs are highly infectious in tissue culture settings. Growth cycle kinetics will likely depend on the host cell and

culture conditions. The signals which cause a switch from SCV-to-LCV and LCV-to-SCV inside the PV are still unknown and needs further research. Ultimately, both LCVs and SCVs play crucial roles in *C. burnetii's* life cycle in intracellular and extracellular environments. The conversion of LCVs to SCVs allows *C. burnetii* to stably survive in the extracellular environment while intracellular conversion of SCVs to LCVs allows for growth and replication of the organism [3, 9, 52].

Genetics

C. burnetii carries a single circular genome which had been estimated to be between $1.6 - 2.1 \times 10^6$ base pairs in size using traditional mapping techniques [173-175]. The first *C. burnetii* strain to be completely sequenced was the classic Nine Mile phase I (RSA493 isolate) which was reported by Seshadri *et al.* [176]. Analysis of the sequence revealed a genome of 1,995,275 base pairs with a 42.6% GC content. The genome is predicted to encode 2,094 ORFs. Of these, 1,022 show homology to genes within sequence databases [176-177]. Sequence analysis revealed that a large portion of predicted *C. burnetii* proteins have a pI greater than 9.0, indicating that many of *C. burnetii*'s proteins are very basic in nature. This has lead to the theory that *C. burnetii's* proteins may act as a proton sink; thereby protecting the organism from the high concentration of H^+ ions present within the PV. In contrast to other obligate intracellular bacteria, the genome contains 32 insertion sequence (IS) elements. In the NMI strain, 21 of these IS elements are copies of the IS1111 element. Eighty three pseudogenes have also been detected [176-177]. Analysis of *C. burnetii* genes shows association with various cellular processes like adhesion and invasion (13 ankyrin repeat-containing proteins), intracellular trafficking, detoxification mechanisms (multi-drug efflux pumps), and host-

cell modulation mechanisms (Type I, II, and IVB secretions systems). Genome analysis also indicates that *C. burnetii* may possess antibiotic resistance potential, as it contains a high proportion of multi-drug efflux pumps (MDEP) per mega-base of genome (~11 MDEP/Mbase). It is speculated that the MDEPs are used to remove host defense molecules from the bacteria during growth within the PV Recently, other isolates of *C. burnetii* have also been sequenced – MSU Goat Q177 (length: 2,090,565 nt, GC content: 42%), Dugway 5J108-111 (length: 2,158,758 nt, GC content: 42%), Henzerling strain RSA 331 (Length: 2,016,427 nt, GC content: 42%), and the African clinical isolate RSA 334 (Length: 2,094,010 nt, GC content: 42%). These sequences demonstrate that *C. burnetii* strains possess genomes of strikingly similar lengths, GC content, and ORF content, although gene rearrangements among these strains may provide valuable insight into their differences [177].

Most *C. burnetii* isolates have been shown to maintain one large plasmid. *C. burnetii* NMI carries a 37.4 kb plasmid, designated QpH1, that encodes 40 predicted ORF's [176-177]. In addition to QpH1, three other related plasmid types have been identified and described from different *C. burnetii* isolates. They are designated QpDG, QpDV, and QpRS. Plasmid-less *C. burnetii* strains also exist, yet plasmid-homologous sequences are found in the chromosome of these strains. The four plasmid types contain both plasmid-specific sequences as well as regions of shared homology [177-181]. Recent data shows that QpH1 encodes proteins which can be secreted through the Type 4 Secretion System (T4SS) of *Legionella pneumophila* [43]. QpH1 genes *cpe*C (containing an F-box domain), *cpe*D (possessing kinesin-related and coiled coil regions), and the QpH1-specific gene *cpe*E were all secreted by *L. pneumophila* in a T4SS fashion. Three

other hypothetical proteins (CpeA, CpeB, and CpeF) are shared among the *C. bunetii* plamid types, and are also predicted to be T4SS effectors [43, 176-177].

In order to understand *C. burnetii*'s evolution and pathogenic potential cross-genome comparisons have been conducted. Whole-genome sequences of the K (Q154) and G (Q212) human chronic endocarditis isolates and the naturally attenuated Dugway (5J108-111) rodent isolate were compared to the NMI (RSA493) isolate. These comparisons reveal both novel gene content (numerous IS elements, genomic rearrangements) and distinct collections of pseudogenes which may contribute to the pathogen's virulence potential. Fewer IS elements and pseudogenes suggest that the Dugway strains lineage may be at an earlier stage of patho-adaptation than the NMI, K, and G lineages [182].

The genetic manipulation of bacteria has been a hallmark of modern molecular pathogenesis studies. Until recently, viable systems to manipulate *C. burnetii* did not exist. However, studies on understanding the genetic basis of *C. burnetii* pathogenesis are now within reach. The introduction of *Himar1*-mediated transposition in *C. burnetii* by Beare *et al*., demonstrated the successful cloning and characterization of a *C. burnetii* *ftsZ* mutant generated by *mariner*-based *Himar1* transposon (Tn) mutagenesis [183]. This first report of successful genetic transformation and clonal isolation in *C. burnetii* involved coelectroporation of *C. burnetii* with a plasmid encoding for *Himar1* C9 transposase variant and a plasmid containing *Himar1* transposon encoding chloramphenicol, acetyltransferase, mCherry fluorescent protein, and a ColE1 origin of replication. *Mariner* family transposon *Himar1* randomly mutagenizes *C. burnetii*'s genome [183]. Combined with the ability to now grow *C. burnetii* in ACCM [184], these

developments make possible many molecular techniques previously absent from *C. burnetii* research.

Phase Variation and Lipopolysaccharide

C. burnetii isolated from nature and laboratory animals is virulent and is noted for causing disease in humans and animals [3, 151]. Virulent *C. burnetii* is usually referred to be in "phase I". The bacteria displays a striking phenomenon of phase variation. When phase I organisms are grown for extended periods of time in embryonated eggs or tissue culture, bacteria within the culture may convert into the "phase II", or avirulent form of the organism [3, 151]. In its phase I virulence state the organism produces a full length lipopolysaccharide (LPS) while phase II organisms possess a severely truncated LPS which has lost the O antigen. If these Phase II *C. burnetii* are when injected into guinea pigs, mice, and hamsters without clonal isolation of the phase II strain, the animals become ill and phase I *C. burnetii* can be isolated from these animals, indicating that not all bacteria within the culture had converted to the avirulent form [3, 151]. Animal studies using clonal isolates of phase II bacteria does not produce disease [185].

Phase II *C. burnetii*'s attenuated virulence is due to a chromosomal deletion that eliminates genes associated with *O*-antigen sugar biosynthesis, including the rare sugar virenose (6-deoxy-3-*C*-methlygulose) [3, 186-189]. Three different LPS chemotypes have been reported [40, 187]. Phase I contains full length LPS while Phase II LPS is made up of Lipid A and some core sugars, but lacks *O*-antigen sugars [3, 186-187]. Another cloned LPS variant (designated Nine Mile Crazy (NMC)) promotes intermediate virulence, and produces an LPS of intermediate length [3, 185]. Interestingly, the phase II truncation of LPS has been linked to the *C. burnetii* NM phase II (NMII) strains ability

to infect cultured host cells at a rate ~10-fold greater than that observed in NMI. Even though they infect *in vitro* cells at different rates, both NMI and NMII replicate with the same kinetics once inside the host cell in phenotypically indistinguishable PV [3, 38, 185, 190-191]. In fact, *C. burnetii* phase variants have been shown to grow at similar rates in primary macrophages from guinea pigs, non-human primates and humans. However, it has been suggested that phase II organisms do not grow as well as phase I organisms in primary mouse macrophages [3, 23, 38, 40, 190, 192]. Microscopically the forms of *C. burnetii* are indistinguishable from one another, but the serological responses against the forms is significantly different. Antibody titers against phase II *C. burnetii* antigens is much higher in an acute infection while phase I antigen titers are higher in the sera from chronic infections [4, 23, 155, 190, 193-195].

The LPS chemotype appears to dictate the organism's ability to promote disease [40, 186-187]. Unlike phase I, phase II *C. burnetii* is more susceptible to complement membrane attack complex [192]. Moreover, the full length LPS of phase I bacteria inhibits the binding of antibodies to *Coxiella* surface proteins. Phase I LPS not only protects *C. burnetii* from being detected by host innate immune receptors such as Toll like receptors (TLRs) in dendritic cells, but also prevents interaction with the CR3 receptor of macrophages [23, 38, 40]. As the lipid A moieties of both phase I and phase II *Coxiella* are chemically identical, it might not act as a ligand for TLR4 as suggested by some studies [40, 187].

Coxiella burnetii: Intracellular life style

Once acquired by inhalation, *C. burnetii* initially contacts alveolar macrophages for invasion and subsequently causes systemic infection [3, 196]. To cause disease,

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intracellular bacteria must establish themselves within a host cell, replicate, and exit to invade other uninfected cells [22, 197]. Like other parasites, *C. burnetii* employs multiple uncharacterized attack strategies for establishment, growth, replication, and exiting the a host cell.

Coxiella adherence and internalization via cytoskeleton re-organization

Internalization of *C. burnetii* into host cells is a microfilament-dependent endocytotic process [3, 10, 198-199]. Studies on THP-1 cells reveal that upon cell attachment, phase I *C. burnetii* cause a dramatic reorganization of the actin cytoskeleton. Restructuring of the actin cytoskeleton induces pronounced membrane protrusions at the site of bacterial attachment. F-actin is seen to accumulate in these membrane protrusions, and this accumulation is dependant on host cell tyrosine kinases [200]. When F-actin redistribution is blocked by cytochalasin D, such morphological changes do not occur. Adherence of phase II *C. burnetii* does not generate such cellular modifications [10, 199].

Membrane protrusions such as membrane ruffling have been associated with efficient pathogen uptake, but in the case of *C. burnetii* phase I, uptake is less efficient than phase II uptake. Differential uptake indicates that different host cell receptors may be involved depending on *C. burnetii* phase type [3, 10-11]. In THP-1 cells, uptake of phase II *C. burnetii* involves the participation of both leukocyte response integrin $\alpha_v\beta_3$ receptor and the CR3 receptor, while phase I *C. burnetii* uptake depends only on $\alpha_v\beta_3$ integrin [11]. It is speculated that phase I bacteria restrict the participation of the CR3 co-receptor upon adherence, thereby dampening the efficiency of internalization. It is possible that the full length LPS of phase I *C. burnetii* prevent the CR3 receptor's interaction with the microbe's ligand by sterically masking bacterial surface proteins [10-

11, 201]. Differential uptake of phase variants is also observed in Vero epithelial cells

and L-929 fibroblasts (non phagocytic) which do not contain $\alpha_v\beta_3$ integrin and CR3

receptors [3]. As phase II *C. burnetii* contain a truncated LPS with a much lower

carbohydrate content when compared to phase I organisms, they are extremely

hydrophobic. It is speculated that this increase in phase II surface hydrophobicity

encourages non-specific hydrophobic interactions between host plasma membrane and

cognate receptors, thereby allowing greater eukaryotic interaction and cellular uptake.

The *C. burnetii* ligands mediating such uptake are thought to be proteinaceous since

pretreatment of the bacteria with proteases dramatically inhibits internalization [3].

Type IV Secretion System

Protein export systems or secretion systems in gram negative bacteria specifically

mediate either insertion of proteins into or translocation of proteins across their cell

membranes [202-203]. Secretion of virulence determinants is a crucial process for

bacterial functioning and operation. One secretion system which allows a number of

pathogenic bacteria to deliver proteins with effector functions into the host cytosol is the

Type 4 Secretion System (T4SS) [202-203]. Sequence data indicates that *C. burnetii*

possesses a type IVB secretion system (T4BSS) that has homology to the Dot/Icm

T4BSS of *L. pneumophila* [18, 204-205]. Composed of 26 proteins, the Dot/Icm of *L.*

pneumophila is a type IVB (T4BSS) effector protein delivery apparatus. *C. burnetii* 's

genome contains 23 of the 26 Dot/Icm genes but lacks homologs to the chaperone protein

icmR and inner membrane proteins DotJ and DotV [18, 204-205]. *C. burnetii* T4BSS

genes are located primarily within two loci, designated regions I (RI) and II (RII) [206-

207]. Morgan *et al*. recently demonstrated the polar localization of T4BSS within *C.*

burnetii during infection of host cells [207]. Studies also reveal that *C. burnetii* T4BSS RI genes are expressed early in infection [206].   RI genes were observed to be transcriptionally linked. In addition, it was also observed that *de novo* transcription of *icmX*, *icmV*, and *icmT* begins by 8 hours post infection (hpi).   Further analysis of transcript levels for RI genes - *icmX*, *icmW*, *icmV*, *dotA*, *dotB*, and *icmT* within the first 24 hpi showed an initial increase followed by a late decrease. Protein levels of IcmT increased significantly from 8hpi to 24 hpi. However, IcmT protein levels remained relatively constant from after this marked initial rise [206]. Other gene expression studies using Vero cells infected with avirulent *C. burnetii* NMII and reverse transcriptase-PCR also indicates that T4BSS genes - *icm*S, *icm*W, *icm*Q, and *dot*B were transcribed within 24 hpi [3, 18, 44, 204-205, 208].

Studies have also demonstrated functional similarity of *C. burnetii* T4BSS components with *L. pneumophila* Icm/Dot pathogenesis system [208-209]. Complementation studies using *L. pneumophila* Dot/Icm dependent secretion has shown that *L. pneumophila* mutants of *dotB*, *icmS*, *icmW* and *icmT* can be complemented by the *C. burnetii* homologs while *icmX*, *icmQ*, *dotM*, *dotL*, *dotN* and *dotO* cannot [205, 208-209]. Interestingly, these studies show that four of five *C. burnetii* T4BSS RI genes were able to complement the *L. pneumophila* T4BSS mutants, whereas none of the five RII homologs could compliment, suggesting a functional distinction exists between the *L. pneumophila* and *C. burnetii* T4BSSs [3, 18, 44, 204, 208-210]. An analysis on *C. burnetii icmQ* complementation failure indicates that lack of binding with *L. pneumophila IcmR* may be responsible for this unsuccessful activity. However, research demonstrates that both *C. burnetii* and *L. pneumophila* express non-homologous proteins

which are functionally similar, to *IcmR* [204, 211]. Other protein function studies on *C. burnetii* T4SS chaperones IcmS and IcmW have clearly indicated conservation of Dot/Icm T4BSS substrate recognition [204-205, 208]. Although more than 70 *L. pneumophila* Dot/Icm effector proteins have been recognized, *C. burnetii* does not express homologs of these proteins. This is likely due to the differences between the intracellular environments of these bacteria [18, 212].

As genetic manipulation of *C. burnetii* is still in its infancy, indirect approaches have been used to detect and identify T4BSS effector proteins. Bio-informatic screens have been performed on the pathogens proteome as a means to identify eukaryotic-like motifs/domains that functionally mimic or inhibit the activity of host cell proteins [3, 18, 44-45, 210]. The *C. burnetii* genome encodes multiple proteins with eukaryotic-like features. This includes proteins with ankyrin repeat domains (Anks), tetratricopeptide repeats (TPR), coiled coil domains (CCD), leucine-rich repeats (LRR), GTPase domains, ubiquitination-related motifs, and multiple kinases and phosphatases [18, 44-45, 182, 210]. The predicted function of these identified proteins is divided into two categories; (*i*) proteins containing Ank, TPR, CCD, and LRR domains represent ORFs which might be involved in direct protein-protein interactions with host proteins while (*ii*) proteins containing F-box, GTPase, kinase, and phosphatase homology may participate in host cell signal transduction pathway regulation [18, 44-45, 182, 210].

Multiple screening techniques have been employed in an attempt to identify *C. burnetii* T4BSS substrates. A number have been identified using *L. pneumophila* as a surrogate host in conjunction with a protein fusion-adenylate cyclase (CyaA) enzymatic reporter assay[18, 43-44]. The Identified Dot/Icm T4BSS substrates include four *C.*

burnetii Anks (AnkA, AnkB, AnkF, and AnkG) and several *C. burnetii* plasmid borne proteins (CpeC, CpeD, CpeA, CpeB, and CpeF). Other Ank repeat containing proteins from different *Coxiella* isolates have also been shown to be secreted in a Dot/Icm-dependent fashion [18, 44]. Recent data indicates that the signal for Dot/Icm-mediated translocation resides in the C-terminus of these proteins and that a few of the Anks involve chaperone IcmS for secretion [18, 43-44, 182]. Other *in silico* screens for T4BSS genes identified two candidate effectors with eukaryotic-like features; CBU1206, which encodes a sterol reductase, and CBU1213, which encodes an ankyrin repeat domain-containing protein (AnkI). These substrates are located in the putative *C. burnetii* pathogenicity island [176, 182]. Identifying other *C. burnetii* T4BSS substrates continues in multiple labs. *C. burnetii* Dot/Icm substrates fused to fluorescent proteins and ectopically expressed in mammalian cells suggests that AnkO (CBUD1108) and AnkJ (CBUD1338) traffic to the PV membrane and mitochondria, respectively [18]. A recent study identified 32 new Dot/Icm dependent *C. burnetii* effectors using a fluorescence-based β-lactamase (TEM1) translocation assay and calmodulin-dependent adenylate cyclase (CyaA) assay and *L. pneumophila* as a surrogate host These putative substrates were selected on the basis of their interaction with DotF, a T4BSS component believed to act as a chaperone/substrate binding protein, and bioinformatic approaches [45].

Coxiella phagosome maturation

After inhalation and subsequent infection of alveolar macrophages, *C. burnetii* replicates within a PV that retains many of the features of a mature phagolysosome [3, 190]. At the cellular level, studies suggest that there is a delay in phagolysosomal maturation of *C. burnetii* containing vesicles and that as *C. burnetii* replicates, host

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vesicles are specifically trafficked to the PV to produce a spacious PV (SPV) [3, 213]. Following phagocytosis, the nascent *Coxiella*-containing phagosome proceeds through the endocytic pathway to eventually fuse with the lysosomal compartment. During this trafficking, the *C. burnetii* containing phagosome (< 6 hpi) is observed to recruit the small GTPases Rab5 and Rab7 (in low amounts), which are prototypic markers of early and late endosomes involved in the regulation of membrane trafficking [14, 17, 214]. Rab5 recruitment occurs by 5 minutes pi, peaks at 20 minutes pi and goes down by 60 minutes pi. On the other hand, Rab7 recruitment gradually increases over the infection course and is observed 48-72 hpi. Recent studies show that dominant negative mutants of Rab5 have decreased *C. burnetii* cell entry entry while dominant negative mutants of both Rab5 and Rab7 do not allow *C. burnetii* PV formation [14, 17, 214]. PV formation is also dependent upon F-actin recruitment. Rho GTPases are known to regulate actin dynamics and in Hela cells *C. burnetii* vacuole formation appears to be reliant on two such proteins, RhoA and Cdc42 [215].

The membranes of early *C. burnetii* PVs (5 min pi) have been shown to contain autophagosome markers, microtubule-associated protein light-chain 3 (LC3), and Rab24 [3, 14, 16-17, 214]. The ultimate association of the *C. burnetii* PV with lysosomes is demonstrated by the presence of lysosomal enzymes, acid phosphatase, and cathepsin D [3]. When compared to latex beads, which acquire lysosomal enzymes in approximately 15 min, *C. burnetii* PVs take approximately 2 hours to accumulate these enzymes [3, 213]. *C. burnetii's* interaction with the autophagy pathway is thought to allow an increase in the size of the PV and aid in the initiation of *C. burnetii* SCV to LCV differentiation via delivery of nutrients [3, 214]. Recent data shows that Rab1b is

recruited to the *C. burnetii* PV after 6 hpi. Rab1 is typically involved in secretory pathway transport between the endoplasmic reticulum and the Golgi apparatus and disruption of the secretory pathway has been shown to affect the spaciousness of *C. burnetii* PV [216].

Features of the mature *Coxiella* PV and infected host cells

The first information suggesting that *C. burnetii* grows and replicates inside the phagolysosome of a host cell was based on the cytochemical localization of the lysosomal enzymes acid phosphatase and 5′-nucleotidase [3, 18, 190, 217]. Subsequent experiments demonstrated that the *C. burnetii* PV also acquires thorium dioxide from secondary lysosomes and becomes acidified [213]. When *C. burnetii* enters into the exponential phase at ~2 days pi, the maturing PV often swells to occupy the majority of host cell cytoplasm and interacts extensively with both endolysomal vesicles and autophagosomes [3, 13, 18]. This mature PVs membrane contains vacuolar H+ ATPase, Rab7, lysosome-associated membrane proteins-1, -2, and -3, flotillin 1 and 2, LC3, and Rab24 [2, 12, 16-18, 218]. The lumen of the PV is moderately acidic (~pH 5) as well [3, 18]. Other prominent proteins recruited to the PV membrane include the autophagy pathway protein Beclin 1 and the anti-apoptotic protein Bcl 2 [219].

The detection of lipid raft proteins flotillin 1 and 2 on the PV membrane clearly indicates a cholesterol rich PV [218, 220]. Investigations on the role of host cholesterol in biogenesis and maintenance of the *C. burnetii* PV show that infected Vero cells produce 73% more cholesterol than uninfected cells [3, 218]. An increased transcription of host genes involved in both cholesterol uptake (e.g. LDL receptor) and biosynthesis (e.g. lanosterol synthase) was also observed in *C. burnetii* infected Vero cells.

U18666A, lovastatin, or 25-hydroxycholesterol (cholesterol uptake and/or biosynthesis inhibitors) treatment on infected cells resulted in the alteration of the *C. burnetii* PV and stalling of replication [218, 220]. Together these data suggest that free access to host cholesterol is required for *C. burnetii* growth and replication. Recent studies have suggested that while the bacterium lacks enzymes for *de novo* cholesterol biosynthesis, it might use CBU1206 (a eukaryote-like Δ24 sterol reductase homolog) to modify host cell sterols during its intracellular growth [221].

Host cells often sacrifice themselves to defend against infections from intracellular pathogens. The function of the host cell apoptotic pathway has been shown to be altered during *C. burnetii* infection [19-20]. *C. burnetii* was shown to actively inhibit apoptosis in macrophages exposed to inducers of both extrinsic (treated with TNFα) and intrinsic (treatment with staurosporine) apoptotic pathways in a bacterial protein synthesis dependant manner [19]. Other data indicates that *C. burnetii* mediated the synthesis of host anti-apoptotic proteins A1/Bfl-1 and c-IAP2, which could then directly, or indirectly, prevent the release of cytochrome c from mitochondria, thereby interfering with the intrinsic cell death pathway during infection [20]. In addition, *C. burnetii* was shown to activate the pro-survival host kinases Akt and Erk1/2 during infection, protecting infected host cells from apoptosis [21]. These observations indicate that *C. burnetii* has evolved molecular mechanisms to prolong the life of its replicative niche.

Immune response

Once inhaled by the human host, *C. burnetii* typically infects alveolar macrophages [4]. The fact that *C. burnetii* can survive and grow inside a professional phagocyte reveals the bacterium's capability to overcome host innate immune responses. Cytokine overproduction, a commonly reported feature of primary *C. burnetii* infection, has led to investigations of the role Toll Like Receptors (TLR) and the innate immune response play in controlling *C. burnetii* infections. These investigations show that *C.burnetii* NMI LPS functions as TLR4 antagonists [37]. Studies also show that TLR4 deficient mice clear *C.burnetii* infection successfully, making the role of TLR4 unclear [36]. Contrasting studies have revealed that instead of TLR4, TLR2 plays a major role in macrophage activation during *C. burnetii* NMII infection [36]. Research on TLR2 (not TLR4) deficient murine macrophages show an increased susceptibility to *C. burnetii* infection with a decreased production of tumor necrosis factor-α (TNF-α) and interleukin-12 (IL12). The same study indicated that activation of TLR2 may limit intracellular replication as bacterial numbers in macrophages from TLR2 deficient mice were higher than in wild type or TLR4 deficient macrophages [36].

Interestingly, the LPS of virulent *C. burnetii* Nine Mile Phase I (NMI) organisms, but not *C. burnetii* NMII, interferes with activation of dendritic cells by masking the TLR-4 ligand [38]. However, purified *C. burnetii* NMI Lipid A itself fails to stimulate both TLR2 and TLR4 [36]. Dendritic cells (DC), characterized by their high endocytic activity, function as antigen presenting cells as they scavenge the surrounding environment for pathogens. *C. burnetii* NMI is able to infect and grow within human DCs without initiating an inflammatory burst, whereas *C. burnetii* NMII cause dramatic

DC maturation resulting in surface expression of the CD80, CD83, CD86, CD40, and HLA-DR (maturation markers) and increased IL-12 and TNF secretion [38, 40].

Although the control and clearance of *C. burnetii* infection is T-cell dependent [23], specific data on T-cell activation signals are lacking. The release of cytokines from macrophages is centrally important to many aspects of T cell function and activation [222]. Studies on cytokine expression at a cellular level indicate that an *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) by virulent and avirulent *C. burnetii* causes the production of pro-inflammatory cytokines - RANTES and MCP-1 [223]. Both RANTES and MCP-1 are chemotactic cytokines and play an active role in recruiting T cells and leukocytes into inflammatory sites. However, no data exists on recruitment of T cells or macrophages. A DNA microarray study of host cell transcriptional responses to *C. burnetii* infection also indicates up-regulation of certain chemokines (RANTES, SCYA3, SCYA4, and IL-8) [42]. It appears that the effects of host cell pro-inflammatory cytokine response are being annulled by *C. burnetii* by unidentified mechanisms.

Other cytokine production studies with *C. burnetii* infected cells do not provide a clear picture on how *C. burnetii* is able to prevent activation of professional phagocytes and T cells. Recent data on the programmed activation of monocyte-derived macrophages (MDM) infected with *C. burnetii* suggests that it stimulates an atypical M2 form of activation [39, 224]. Classically, the M1 form of activation is induced with a microbial stimuli (e.g., LPS), cytokines (e.g., TNF and GM-CSF), or by IFNγ alone [39, 224]. Once activated, M1 cells have an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype. They also produce reactive oxygen, nitrogen intermediates, and inflammatory cytokines (IL-1β,

TNF, IL-6) [39, 224]. However, M2 cells typically have an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype with a variable capacity to produce inflammatory cytokines [39, 224]. IL-10 is an anti-inflammatory cytokine, and its role has been extensively studied in chronic Q-fever patients where it has been implicated in the enhanced persistence of *C. burnetii* in infected hosts, possibly due to its anti-inflammatory properties [225]. It is still unclear if this phenomenon is controlled by *C. burnetii* protein synthesis. Many of the innate immune responses seen during *in vitro* and *in vivo* *C. burnetii* studies have been attributed to LPS and intrinsic properties of the bacteria. These approaches have not addressed the possibility that *C. burnetii* actively modulates the innate immune response at the cellular level through bacterial proteins expressed during infection.

CHAPTER III

COXIELLA BURNETII NINE MILE II PROTEINS MODULATE GENE EXPRESSION

OF MONOCYTIC HOST CELLS DURING INFECTION

Abstract

Background. *Coxiella burnetii* is an intracellular bacterial pathogen that causes acute and chronic disease in humans. Bacterial replication occurs within enlarged parasitophorous vacuoles (PV) of eukaryotic cells, the biogenesis and maintenance of which is dependent on *C. burnetii* protein synthesis. These observations suggest that *C. burnetii* actively subverts host cell processes, however little is known about the cellular biology mechanisms manipulated by the pathogen during infection. Here, we examined host cell gene expression changes specifically induced by *C. burnetii* proteins during infection.

Results. We have identified 36 host cell genes that are specifically regulated when *de novo C. burnetii* protein synthesis occurs during infection using comparative microarray analysis. Two parallel sets of infected and uninfected THP-1 cells were grown for 48 h followed by the addition of chloramphenicol (CAM) to 10μg/ml in one set. Total RNA was harvested at 72 hpi from all conditions, and microarrays performed using Phalanx Human OneArray™ slides. A total of 784 (mock treated) and 901 (CAM treated) THP-1 genes were up or down regulated ≥ 2 fold in the *C. burnetii* infected vs. uninfected cell sets, respectively. Comparisons between the complementary data sets (using >0 fold), eliminated the common gene expression changes. A stringent comparison (≥2 fold) between the separate microarrays revealed 36 host cell genes modulated by *C. burnetii* protein synthesis. Ontological analysis of these genes identified the innate immune response, cell death and proliferation, vesicle trafficking and development, lipid homeostasis, and cytoskeletal organization as predominant cellular functions modulated by *C. burnetii* protein synthesis.

Conclusions. Collectively, these data indicate that *C. burnetii* proteins actively regulate the expression of specific host cell genes and pathways. This is in addition to host cell genes that respond to the presence of the pathogen whether or not it is actively synthesizing proteins. These findings indicate that *C. burnetii* modulates the host cell gene expression to avoid the immune response, preserve the host cell from death, and direct the development and maintenance of a replicative PV by controlling vesicle formation and trafficking within the host cell during infection.

Background

Coxiella burnetii is a Gram-negative, pleomorphic, intracellular bacterial pathogen with a worldwide distribution [3-4]. Virulent strains cause human Q-fever, which is usually marked by an acute self-limiting flu-like illness. Persistent infections usually progress into chronic disease [4, 23, 81]. Human infection occurs via inhalation of aerosols contaminated with *C. burneti*. The small cell variant (SCV) form of the bacterium, which are metabolically inactive and environmentally stable, are believed to be responsible for most environmentally acquired infections. SCVs passively ingested by mononuclear phagocytes are trafficked along the endocytic pathway and associate with a variety of endocytic and autophagic markers before ultimately residing within a parasitophorous vacoule (PV) with characteristics of a secondary lysosome [3-4, 81]. Here, they undergo a replicative lag phase of 1-2 days while differentiating into the metabolically active large cell variants (LCVs). Although they are not environmentally stable, LCVs are infectious in laboratory settings and pose a risk of causing disease. After differentiation, LCVs then undergo exponential replication for ~4 days (log phase) before beginning an asynchronous conversion back to SCVs at ~6 days post infection (PI) [9, 172]. LCV replication is accompanied by a remarkable expansion of the PV, which eventually occupies the majority of the host cell [3, 12].

Intracellular bacterial pathogens are known to operate by targeting and subverting vital intracellular pathways of the host [22, 226]. Bacterial proteins are a key factor in this subversion of host cell molecular mechanisms [3, 18, 22, 210]. Biogenesis and maintenance of the PV, interaction with the autophagic pathway, and inhibition of host cell apoptosis are all dependent on *C. burnetii* protein synthesis [3, 19-20, 218, 227].

After ingestion by a host cell, *C. burnetii* PV maturation experiences a delay when compared to vacuoles carrying latex beads or dead *C. burnetii* [12, 213]. This delay in phagolysosomal maturation requires ongoing bacterial protein synthesis [12]. *C. burnetii* protein synthesis is also required for the fusogenicity of *C. burnetii* containing vacuoles, PV fusion with host vesicles, and in the maintenance of a spacious PV (SPV) during logarithmic bacterial growth [12, 213]. Transient interruption of bacterial protein synthesis results in cessation of SPV-specific vesicle trafficking and SPV collapse [12, 213]. The *C. burnetii* PV is thought to interact with the autophagic pathway as a means to provide metabolites to the bacterium. This interaction is also a pathogen driven activity [14]. Additionally, an examination of the PV has revealed increased amounts of cholesterol in the membranes[218]. Interestingly, *C. burnetii* infected cells have been observed to dramatically increase cholesterol production. During log growth, the PV expands and is accompanied by increased transcription of host genes involved in both cholesterol uptake (e.g. LDL receptor) and biosynthesis (e.g. lanosterol synthase) [3, 218].

Recently, the function of the host cell apoptotic pathway has been shown to be altered during *C. burnetii* infection. *C. burnetii* was shown to actively inhibit apoptosis in macrophages exposed to inducers of both the extrinsic and intrinsic apoptotic pathways in a bacterial protein synthesis dependant manner [19]. This antiapoptotic activity causes a marked reduction in activated caspase-3, caspase-9, and poly-ADP (ribose) polymerase (PARP) processing. Other data indicate that *C. burnetii* mediates the synthesis of host anti-apoptotic proteins A1/Bfl-1 and c-IAP2, which might directly or indirectly prevent release of cytochrome C from mitochondria, interfering with the intrinsic cell death

pathway during infection [20]. Moreover, activation of the pro-survival host kinases Akt and Erk1/2 by *C. burnetii* was shown to protect infected host cells from apoptosis [21]. Despite the information on processes that appear to be affected by *C. burnetii* proteins, little is known about the host molecular mechanisms being targeted throughout the course of infection.

A common theme among bacterial pathogens, including *C. burnetii,* is the ability to secrete effector proteins into the host cell as part of their pathogenic strategy [18, 22]. The possession of a type IV secretion system (T4SS) by *C. burnetii* suggests that effector proteins might be delivered to the host cell via this machinery [3, 18, 44, 207]. As the genetic manipulation of *C. burnetii* is in its infancy, indirect approaches such as bioinformatic screens have been useful in predicting putative T4SS substrates. Recent data indicate that *C. burnetii* encodes multiple proteins with eukaryotic-like domains, including ankyrin repeat binding domains (Anks), tetratricopeptide repeats (TPRs), coiled-coil domains (CCDs), leucine-rich repeats (LRRs), GTPase domains, ubiquitination-related motifs, and multiple kinases and phosphatases [3, 176, 182]. Studies have shown that a number of the *C. burnetii* encoded Ank proteins are secreted into the host cell cytoplasm through the *Legionella pneumophila* T4SS [44, 182, 228]. Three of these proteins associate with the PV membrane, microtubules, and mitochondria, respectively, when expressed ectopically within eukaryotic cells [44].

These observations suggest that *C. burnetii* proteins directly interact and exploit mammalian intracellular pathways leading to the establishment and prolongation of the replicative niche. Here, we use the avirulent *C. burnetii* Nine Mile phase II (NMII) strain and the transient inhibition of bacterial protein synthesis as a means to elucidate host

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molecular mechanisms that are being actively targeted by *C. burnetii* during infection. While the *C. burnetii* NMII strain does not cause Q fever, it is a recognized model for the analysis of molecular host cell-pathogen interactions. Recent studies clearly demonstrate that the virulent Nine Mile phase I (NMI) and avirulent NMII strains grow at similar rates and are trafficked to similar intracellular vacuoles during infection of cultured monocytic cells (THP-1) as well as primary monocytes/macrophages [229-230], making NMII an excellent model for molecular studies of this unusual pathogen. In the current study, we have analyzed *C. burnetii* NMII protein induced gene expression changes in infected THP-1 cells. Using microarray technology we have examined the global transcriptional response of THP-1 cells during *C. burnetii* infection by transiently inhibiting (bacteriostatically) bacterial protein synthesis during the logarithmic phase of infection and comparing this to normal (mock treated) infections ran in parallel. Using stringent comparative microarray data analyses, we have discovered 36 previously unidentified host genes whose expression is significantly changed by *C. burnetii* proteins. Gene ontology analysis on these data was performed to define the host cell processes being targeted by this bacterium during infection.

Methods

***C. burnetii* and cell culture growth and infection.** *C. burnetii* Nine Mile phase II was grown in Vero cells (CCL-81; ATCC, Manassas, VA) and purified as previously described [207]. Non-adherent THP-1 human monocytic leukemia cells (TIB-202; ATCC) were propagated in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 1mM sodium pyruvate, and 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. THP-1 cells between passages 6-10 were used in all experiments [19]. Briefly, purified

C. burnetii NMII SCVs at a genome equivalent MOI of 15 were used to establish a synchronous infection. To ensure close host cell-bacteria contact, *C. burnetii* SCVs diluted in RPMI 1640 containing 10% FBS were incubated in $25cm^2$ tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) with $5x10^6$ THP-1 cells in a total volume of 2.5 ml. Incubations were performed at 37°C in an atmosphere of 5% CO_2 for 4 hours. Cells were pelleted by centrifugation at 600g for 5 minutes, washed with fresh media and pelleted again. Cell pellets were then re-suspended in 5ml of fresh media (final concentration = 10^6 cells/ml) and transferred to new $25cm^2$ tissue culture flasks (this represents T=0). Cells were pelleted again at 48 hours post infection (hpi) and re-suspended in fresh media with or without the bacterial protein synthesis inhibitor chloramphenicol (CAM, a final concentration of 10µg/ml), as needed. Cells were then incubated for an additional 24 hours for either total RNA harvest or microscopy analysis (see Figure 3.1). Infected and uninfected cells were handled identically and a total of three experiments (N=3) were carried out for microarray analysis.

Comparative microarray design and analysis. In order to perform the microarray hybridizations, two parallel infection and treatment protocols were employed. A schematic of the comparative microarray experimental design highlighting the separate treatment conditions is shown in Figure 3.1. Using this experimental design, a comparison was made between the THP-1 transcriptional responses of (*i*) uninfected versus *C. burnetii* NMII infected and (*ii*) uninfected versus *C. burnetii* NMII infected THP-1 cells transiently treated with bacteriostatic levels (10µg/ml) of CAM. Briefly, infections were initiated and cultured in parallel with uninfected cells. At 48 hpi media containing CAM (10µg/ml) was added to one set of cells (uninfected and infected THP-1

cells) and culturing was continued. The other set of cells were mock treated with normal media. Total RNA was isolated at 72 hpi from all conditions. Microarrays were performed for both conditions and the results were compared to define the host genes modulated by *de novo* synthesized *C. burnetii* NMII proteins. The 48-72 hpi time frame was used because (*i*) *C. burnetii* would be in logarithmic growth [172] and, (*ii*) previous studies have shown observable changes in PV size within *C. burnetii* infected Vero cells when treated overnight with 10μg/ml of CAM at 48 hpi[12].

RNA extraction, microarray hybridization and data analysis. Following the infection and treatment protocols (Figure 3.1), total RNA was isolated using Tri-Reagent (Ambion, Austin, TX) according to the manufacture's recommendations. All RNA samples were DNase treated using RQ1 DNase (Promega, Madison, WI) and confirmed DNA free by PCR. RNA integrity was assessed by electropherogram using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California). Total RNA (500 ng) from each sample was then amplified using an Epicentre® Biotechnologies (Madison, WI) TargetAmp™ 1-Round AminoallylaRNA Amplification Kit, yielding approximately 6-10μg of aminoallyl-aRNA (AA-aRNA). Alexa Fluor® 555-GREEN (Invitrogen, Carslbad, CA) was used to label the uninfected AA-aRNA, while Alexa Fluor® 647-RED (Invitrogen) was used to label the AA-aRNA from the *C. burnetii* infected cells. Labeled AA-aRNA (2μg) with a dye incorporation efficiency range of 18 -34 picomol/microgram, were mixed pair-wise and hybridized overnight to Human OneArray™ microarrays (Phalanx Biotech Group, Palo Alto, CA). Human OneArrays contain 32,050 oligonucleotides; 30968 human genome probes and 1082 experimental control probes formed as 60-mer sense-strand DNA elements. Arrays were hybridized,

washed, and dried rapidly according to the manufacturer's instructions. Six hybridizations for each condition set (CAM and mock treated) were performed with three biological and two technical replicates. Signal intensity of the hybridized arrays were measured by ScanArray Express (PerkinElmer, Boston, MA, USA) and the images were processed using GenePix Pro version 4.0 (Axon, Union City, CA, USA). The processed GenePix Pro 4.0 output was further analyzed using Loess-Global intensity dependent normalization through the GenePix Auto Processor (http://darwin.biochem.okstate.edu/gpap3/) as previously described [231-233]. Normalized ratio values for each data point were averaged across the three biological replicates and two technical replicates. Significant expression differences were defined as a P-value <0.05 and displayed as a fold change of ≥2 fold [42, 234]. The microarray data were deposited at the NCBI Gene Expression Omnibus (GEO) under the platform accession number GPL6254 and the series number GSE23665. The biological function of the identified genes was determined bioinformatically by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/) [235] as well as by Ingenuity pathway analysis (Ingenuity® Systems, www.ingenuity.com). This software identifies canonical pathways within gene sets using significant associations (P<0.05) calculated by Fisher's exact test and also by a ratio of the number of molecules from the experimental data set that maps to the pathway, divided by the total number of molecules that exists in that canonical pathway.

Immunofluorescence microscopy. Non-adherent THP-1 cells (CAM and mock treated) were analyzed by indirect immunofluorescent antibody (IFA) microscopy. Briefly, 1×10^5 cells were cytocentrifuged onto poly-L-lysine coated slides for 2 minutes

at 1000 rpm using a Shandon Cytospin® 4 Cytocentrifuge (Thermo Scientific) [236]. The cytospun THP-1 cells were air dried and immediately fixed using ice cold acetone for 30 seconds. The fixed preparations were then washed with PBS and stained with a rabbit antibody against whole killed *C. burnetii* NMII (primary antibody) followed by a goat anti-rabbit IgG Alexa Fluor-488 (Molecular Probes, Eugene,OR) secondary antibody. Host and bacterial DNA were also stained using 4',6-diamidino-2-phenylindole (DAPI). Microscopy was conducted using a Nikon Eclipse TE 2000-S microscope with a Nikon DS FI1 camera and NIS-ELEMENTS F 3.00 software. IMAGEJ version 1.42n (Wayne Rasband, NIH) was also used for image processing [207].

RT-qPCR analysis. RT-qPCR was performed using gene-specific primers (shown in Additional file 1-Supplemental Table S1.I), and the SYBR Green Master Mix Kit (Applied Biosystems) on an *Eppendorf Mastercycler*® ep realplex (Eppendorf, Hamberg, Germany) following the manufacturer's recommendations. Briefly, first strand cDNA was synthesized using random hexamers, 1µg of total RNA, and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as suggested by the manufacturer. Oligonucleotide primers were designed using Primer3Plus [237-238]. The primer efficiency of each primer set was determined to be within the efficiency window for the $2^{-\Delta\Delta CT}$ relative fold calculation method [239]. The human β-actin gene was used as the reference gene. Paired T-Test was performed to identify statistical differences between any two conditions. Differences were considered significant at a P<0.05.

Results

SPV morphology within CAM treated *C. burnetii* infected THP-1 cells. As

the transient inhibition of *C. burnetii* protein synthesis within infected THP-1 cells using

CAM is pivotal to testing our hypothesis, we sought to confirm that morphological

changes occur to the PV of infected THP-1 cells after transient CAM treatment in a

manner consistent with that observed in other cell types [12]. Using phase contrast and

IFA microscopy analysis, we assessed the effect of bacteriostatic levels of CAM

(10µg/ml) on infected THP-1 cells during the log growth phase of the *C. burnetii*

infectious cycle in order to coincide with subsequent microarray analysis. Robust

infections (≥90% infected cells) were produced using *C. burnetii* NMII at a genome

equivalent MOI of 15. Infections were either mock or CAM treated at 48 hours post

infection (hpi), and then compared at 72 hpi. Figure 3.2 shows both phase contrast

(Figure 3.2 top panel) and IFA microscopy (Figure 3.2, middle and bottom panels)

images representative of the *C. burnetii* NMII infection of THP-1 cells at 72 hpi.

Multiple, large SPVs can be seen in the mock treated THP-1 infections, while smaller,

dense PVs are observed in the CAM treated infections. These results are in agreement

with published findings where transient CAM treatment resulted in PV collapse in *C.*

burnetii infected Vero cells [12]. Figure 3.2C-H shows a set of similarly treated

infections visualized by IFA microscopy. *C. burnetii* are visualized in green (Figure 3.2,

C and F) and cell nuclei are stained in blue (Figure 3.2, D and G) and the images merged

(Figure 3.2, E and H). Comparing the mock and CAM treated images (Figure 3.2, C and

F), a noticeable decrease in vacuole size and fluorescent intensity is observed, indicating

the collapse of the SPVs within the CAM treated cells when compared to the large, SPVs

observed within the mock treated cells. Comparisons of DNA samples harvested at 48 hpi (prior to CAM treatment) and 72 hpi (after 24 h CAM treatment) using qPCR determined that these samples had similar *C. burnetii* genome equivalents, indicating that the 10µg/ml CAM concentration was acting bacteriastatically (data not shown). In addition, removal of CAM from infected cells after the 24 h transient treatment resulted in the re-establishment of large, SPVs within 48 h as observed by phase contrast microscopy (data not shown). Together, these data indicate that 10µg/ml of CAM is able to transiently arrest *C. burnetii* protein synthesis in the THP-1 cell infection model.

Gene expression in mock and CAM treated infected vs. uninfected THP-1 cells. As outlined in Figure 3.1, two whole genome RNA microarray analyses were performed resulting in the generation of two separate global gene expression profiles. A total of 784 THP-1 genes (Additional file 1-Supplemental table S1.A) were up- or down-regulated ≥2 fold in mock treated infected vs. uninfected cells while a total of 901 THP-1 genes (Additional file 1-Supplemental Table S1.C) were up- or down-regulated ≥2 fold in CAM treated infected vs. uninfected cells. To identify the host cell functions affected by *C. burnetii* infection and proteins, these gene sets were annotated using DAVID. A modified Fisher Exact P-Value test was used to measure gene-enrichment in annotation terms. The top biological function assignments for the mapped genes (P< 0.05) expressed as the percentage of the 784 and 901 significant genes identified in the mock and CAM treated microarrays, respectively, are shown in Additional file 2-Supplemental Figure 3.1. This figure aids in defining the prominent cell functions affected by *C. burnetii* infection and proteins. Identified as affected cell functions under both conditions are immune response, cell migration, regulation of programmed cell death, intracellular

signaling cascades, regulation of cell proliferation, and cytoskeletal organization. Notable differences were observed in the percentage of genes involved with each of these functions under the mock treated and CAM treated conditions, indicating a role for *C. burnetii* proteins in changing gene expression in these pathways. Other important host cell functions influenced under the mock treated condition are protein phosphorylation, lipid storage, gas homeostasis, cell-cell signalling, and cellular ion homeostasis. While major cellular functions seen affected only in CAM treated infected THP-1 cells are cell cycle processes, cell activation, response to DNA damage, lipid (sterol and cholesterol) transport, positive regulation of cytokine biosynthetic processes, and regulation of nitric oxide biosynthetic processes. Additional file 1-Supplemental Tables S1.E and S1.F list the host genes associated with each of these functions. Out of the 784 host genes identified in the mock treated data set, 62 genes were not assigned function by DAVID's biological annotation coverage. In the CAM treated infected vs. uninfected data set, 102 out of the 901 host cell genes remained unassigned.

To further define the prominent host cell pathways affected by *C. burnetii* infection and proteins, an Ingenuity pathway analysis (IPA) was performed on the 784 and 901 significant genes identified in the mock and CAM treated microarrays, respectively. IPA identifies the top canonical pathways represented in a group of genes. Additional file 1-Supplemental Tables S1.G and S1.H list the top canonical pathways associated with the mRNA profiles of the mock treated and CAM treated infected vs. uninfected THP-1 cells, respectively. From the mock treated microarray set, 17 biological functions were influenced by infection while 28 functions were significantly affected by CAM treatment of infections (Additional file 1-Supplemental Tables S1.E

and S1.F). Many of the biological functions identified are the result of the molecular pathways identified by IPA, with several innate immune response and stress pathways implicated when *C. burnetii* protein synthesis is arrested, again indicating a role for *C. burnetii* proteins in managing the host cell response to infection.

Comparative analysis between mRNA profiles of untreated and CAM treated uninfected/infected THP-1 cells. In order to identify the host cell genes differentially expressed (≥2 fold) in response to *de novo C. burnetii* protein synthesis, we compared the two separate mRNA expression profiles. Microarray analysis of mock treated (-CAM), uninfected vs. infected THP-1 cells using a broad cut-off of >0 fold revealed a gene summary list of 2557 genes (P<0.05) (Additional file 1-Supplemental Table S1.B). Within this data set are the 784 genes which changed ≥2 fold (S1.A), and was considered a significant change. Using a >0 fold cut-off for the CAM treated (+CAM) uninfected vs. infected THP-1 cells, a gene summary list of 2584 genes were identified (Additional file 1-Supplemental Table S1.D). The subset of 901 genes that changed significantly (≥2 fold, S1.B) was within this large gene summary list. Figure 3.3 depicts a comparison of these two sets of microarray data using Venn diagrams. To eliminate the insignificantly (<2 fold) expressed genes, (*i*) the subset of significant THP-1–CAM genes (784) was cross-matched to the THP-1+CAM whole gene summary list (>0 fold) of 2584 genes and, (*ii*) the subset of significant THP-1+CAM genes (901) was cross-matched to the THP-1–CAM whole gene summary list (>0 fold) of 2557 genes. This cross comparison identified 28 genes in the THP-1-CAM subset and 35 genes in the THP-1+CAM subset that were significantly changed (≥2 fold) between the two microarray conditions. The overlapping genes from these two data sets were pooled (27

genes) and uniquely expressed genes in the -CAM (1 gene) and +CAM (8 genes) were identified. Comparing the results from these two gene subsets provided us with a list of 36 candidate host cell genes whose expression was ≥2 fold different between the mock treated (–CAM) and CAM treated (+CAM) arrays, indicating genes whose expression is modulated by *de novo* synthesized *C. burnetii* proteins.

Host cell biological functions associated with THP-1 mRNAs modulated by *de novo C. burnetii* **protein synthesis.** To determine the host cell biological pathways being affected by *C. burnetii* protein synthesis, IPA was used. Analysis of the subset of thirty-six differentially expressed host genes modulated by *C. burnetii* protein(s) were classified according to the biological function they are associated with, the protein's cellular location, and its molecular function (Table 3.1). A majority of the proteins in this data set are predicted to reside in the cytoplasm (14 proteins) and cell nucleus (9 proteins). Six proteins are predicted to function in the extracellular space while four proteins are thought to be located on the plasma membrane. Other than cellular location, the host genes were also categorized on the basis of the expressed protein's function – i.e. enzyme, cytokine, transporter, transcriptional regulator, or other. For the thirty-six gene subset, Table 3.1 also lists the fold change found within the separate mock treated and CAM treated microarrays, respectively, as well as the fold difference between the arrays. *C. burnetii* infected host cells had lower RNA levels of twenty-two host genes relative to cells containing *C. burnetii* transiently inhibited with CAM. RNA levels of fourteen genes in this data set are found to be higher due to *C. burnetii* infection when compared to the CAM treated condition. Bioinformatic analysis conducted to determine possible biological functions of these *C. burnetii* modulated host genes indicates that immune

response and cellular movement, cellular signaling, cellular proliferation, cell death, lipid metabolism, molecular transport, as well as vesicle trafficking, and cytoskeletal organization are affected by *C. burnetii* protein synthesis (Table 3.1). These data indicate that the expression of vital genes involved in cellular movement - IL8, CCL2, CXCL1, SPP1 (cytokines) are suppressed via *C. burnetii's* protein synthesis in mock treated conditions when compared to CAM treated conditions. These secretory molecules (IL8, CCL2, CXCL1, SPP1) regulate the infiltration and trafficking of immune cells. Table 3.1 shows other crucial host genes specifically suppressed by *C. burnetii* protein synthesis in THP- 1 infection such as BCL3, CTSB and CTSL1 (apoptosis), MTSS1, SMTN and PLEKHO1 (cytoskeleton organization), APOE, PLIN2 and FABP4 (lipid metabolism), and RAB20, SOD2, PSMA8, MSC, ZFP36L1, and RORA (Miscellaneous). The prominent genes found to be up-regulated (induced) due to *C. burnetii*'s protein synthesis are ITK, DUSP9 & SKP2 (intracellular signaling), SOX11, HELLS & PGR (cell growth and proliferation) SLC22A6, CDH2, PSD4, ZNF573, CHMP5 & MRPL44 (Miscellaneous) and ANLN (cytoskeleton organization).

RT-q PCR analysis of THP-1 gene expression in response to mock and CAM treated *C. burnetii* infection. RT-qPCR was used to validate the expression trends of selected genes identified by microarray analysis. Using the same total RNA samples utilized for the microarray hybridizations, six host genes were selected (IL8, CCL2, ZFP36L1, APOE, RND3, and POU4F2) and analyzed by RT-qPCR using the constitutively expressed β-actin gene as a comparative control. In each case, the RT-qPCR data matched the trends from the microarray analysis with respect to whether expression was increased, decreased, or unchanged. Figure 3.4 shows the fold expression

differences of IL8, CCL2, ZFP36L1, APOE, RND3, and POU4F2 identified by microarray in mock and CAM treated experimental conditions (Figure 3.4A) and the subsequent RT-qPCR analysis (Figure 3.4B).   IL8, CCL2, APOE, and ZFP36L1 represent genes that are increased in mock treated *C. burnetii* infected THP-1 cells but increase further when *C. burnetii's* protein synthesis is transiently inhibited using bacteriostatic levels of CAM. The POU4F2 gene expression is decreased similarly under both conditions and represents a THP-1 gene modulated by C. *burnetii* infection whether or not active protein synthesis is occurring. RND3 expression increases similarly in *C. burnetii* infected THP-1 cells regardless of ongoing bacterial protein synthesis. These results confirm that genes with significant mRNA expression changes by oligonucleotide microarrays analysis are differentially expressed when measured by RT-qPCR.

Discussion

Bacterial effector proteins are crucial to the survival and growth of intracellular pathogens within the eukaryotic cellular environment. These interactions may be at a myriad of pathways or at points within a single pathway. Moreover, the growth of *C. burnetii* within the lumen of the PV would require the mediation of interactions with the host cell using effector proteins, which are predicted to be delivered by the pathogen's type IV secretion system [18, 44, 210]. The goal of this study was to identify host genes that are specifically manipulated by *C. burnetii* proteins. Our hypothesis was that the expression of host cell genes will be changed by infection with *C. burnetii* NMII and that the expression of a subset of these genes will be directly affected by ongoing bacterial protein synthesis. Identification of such genes will aid in the understanding of host molecular mechanisms being targeted by *C. burnetii* during growth. In order to identify

the host genes regulated by *C. burnetii* proteins, we compared CAM and mock treated mRNA profiles of THP-1 cells following a 72 h infection with *C. burnetii*. Microarray data analysis shows that the majority of host genes were up- or down regulated similarly in both the mock and CAM treated array sets, suggesting that most THP-1 genes were not differentially modulated at the RNA level by active *C. burnetii* protein synthesis. We had predicted that the majority of expression changes in the host cell would be in response to the physical presence of bacteria within the cell. Using stringent analysis conditions, the transcriptional response data comparisons identified thirty-six differentially expressed genes, which were uniquely modulated by *C. burnetii* proteins. The targeting of these host genes by the pathogen indicates they may fall within pathways that *C. burnetii* needs to modulate for its own survival.

During infection *C. burnetii* replicates intracellularly, which aids in avoidance of the host immune response. Immune clearance of bacteria is largely dependent on cellular sensors called pattern recognition receptors (PRR) found on phagocytes [27]. Activated macrophages then eliminate bacteria through extrinsic or intrinsic apoptosis and/or inducing pro-inflammatory cytokines [27]. Bacteria employ indirect mechanisms to regulate cytokine production by interfering with the NFkappaB signaling pathway, which is a potent transcriptional activator of cytokines. [240]. Interestingly, of the thirty-six host genes that met our criteria (Table 3.1) for *C. burnetii* protein driven expression changes, four are cytokines (IL8, CCL2, CXCL1 and SPP1). These secretory molecules are noted for chemo-attraction of phagocytic and lymphocytic cells [241-243]. *C. burnetii* protein(s) appear to reduce the RNA levels of each of these four genes in infected THP-1 cells relative to those found in infected cells transiently inhibited with

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CAM. The ability of *C. burnetii* to avoid or suppress host cytokine signalling, even transiently, may well represent an essential part of its ability to survive and cause disease by preventing communication between innate and adaptive immune cells.

Although the control and clearance of *C. burnetii* infection is T-cell dependent, specific data on T-cell activation signals are lacking [23]. One study indicated that an *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) by virulent and avirulent *C. burnetii* strains cause the production of RANTES and CCL2 [41]. Using a 36 h model of *C. burnetii* infection, a DNA microarray study reported an increase in host cell expression of certain chemokines (RANTES, SCYA3, SCYA4, and IL8). The study also observed no induction of TNF-α and IL-1β after 36 h of infection, but the antimicrobial response gene encoding cytochrome b-245 (CYBB) was up-regulated [42]. In the current study, IL8 gene expression was also increased due to *C. burnetii* infection but expression was further increased when *C. burnetii* protein synthesis was inhibited, suggesting that bacterial protein(s) differentially modulate the expression of IL-8 during infection. In addition, the IL8 receptor gene (IL8RB) was found to be down regulated in mock treated, infected THP-1 cells (see Additional file 1-Supplemental Table S1.A). This is the first evidence of host cell cytokine production being modulated by *C. burnetii* protein during an infection.

In addition to the immune response, *C. burnetii* has to overcome another central host defense mechanism, apoptosis. The intracellular pathogens *C. trachomatis*, *Mycobacterium tuberculosis* as well as *C. burnetii* posses mechanisms to subvert cell death pathways [19-20, 244-245]. *C. burnetii* has been shown to inhibit host cell apoptosis by a mechanism that prevents cytochrome C release from the mitochondria

[20]. *C. burnetii* directs the sustained activation of host pro-survival kinases Akt and Erk1/2, which are necessary for anti-apoptotic activity [19] . Table 3.1 shows that seven of the thirty-six *C. burnetii* protein modulated THP-1 genes are associated with apoptosis and cell proliferation within eukaryotic cells. *C. burnetii* protein(s) suppress the expression of three genes (BCL3, CTSB, and CTSL1), when compared to expression levels present in CAM treated THP-1 cells, which can have pro-apoptotic activities.   By modulating these host genes during infection *C. burnetii* appears to promote its own survival by ensuring the survival of the host cell.   The expression of the four cell proliferation/survival genes (C11ORF82, PGR, SOX11 and HELLS) are significantly reduced when *C. burnetii's* protein synthesis is inhibited during infection of THP-1 cells (Table 3.1).   The expression of each of these genes is higher in infected cells than in infected cells where bacterial protein synthesis is inhibited, again indicating that *C. burnetii* protein(s) have an anti-cell death affect.   Interestingly, our microarray analysis also shows a 4-fold expression decrease of TNFRSF10A (Death receptor 4) in mock treated infections of THP-1 cells (Additional file 1-Supplemental Table S1.A). Normally, TNFRSF10A induces apoptosis by binding to TNFSF10/TRAIL ligand in cells [246], suggesting that the expression changes in *C. burnetii* infected cells may represent another means of inhibiting host cell death.

Eukaryotic host cell cytoskeleton (actin filaments, microtubules and intermediate filaments) are a common target of molecular interactions for intracellular microbial pathogens [22]. Virulent *C. burnetii* has been shown to affect F-actin reorganization in THP-1 cells [199-200]. F-actin has also been shown to be associated with PV formation and homotypic fusion of *C. burnetii* containing vacuoles, although PVs are able to

acquire lysosomal markers when F-actin formation is inhibited [215]. Our analysis indicates that MTSS1, ANLN, SMTN and PLEKHO1 are differentially modulated by *C. burnetii* protein synthesis (Table 3.1). Compared to CAM treated THP-1 infections, the relative expression levels of MTSS1, SMTN and PLEKHO1 is lower in THP-1 mock treated infections. The relative expression of ANLN is higher in mock treated *C. burnetii* infections than in CAM treated infections. Interestingly, ANLN interacts with F-actin and is over expressed in dividing cells[247], suggesting that *C. burnetii* infection supports cell growth and division. The structure and integrity of the PV as well as host cell vesicles fusogenicity with the PV is dependent on cytoskeletol structures[215]. Finding that four out of the thirty-six genes are associated with the regulation and function of the cells cytoskeleton supports findings that the cytoskeleton is crucial to *C. burnetii* during infection.

Manipulation of cellular lipids is emerging as an important factor in infectious diseases [248-249]. Host cell cholesterol levels affect the growth of intracellular bacterial pathogens such as *Salmonellae*, *Mycobacteriae*, *Brucellae*, *Anaplasma,* and *Coxiellae* [218, 249]. Little is known about cholesterol levels or imbalance in Q-fever patients, but studies at the cellular level indicate that *C. burnetii* infected Vero cells contain 73% more cholesterol than uninfected cells[218]. Table 3.1 lists three *C. burnetii* protein(s) modulated host genes (APOE, PLIN2, and FABP4) that are associated with lipid metabolism and regulation. These genes have lower relative expression levels in the mock treated THP-1 infections when compared to the CAM treated THP-1 infections. APOE is a multifunctional protein primarily involved in cholesterol homeostasis [250-254]. Endogenously, APOE promotes cholesterol efflux in macrophages to lower

intracellular cholesterol concentrations. Macrophages deficient in APOE are severely compromised in cholesterol homeostasis [250-254]. PLIN2 and fatty acid binding protein 4 (FABP4) are proteins that associate with lipids and fatty acids, respectively, and mediate the stabilization of lipid droplets and fatty acid transport [255-256]. An increase in cholesterol regulating proteins would be expected in response to the profound increases in the cellular concentration of cholesterol seen during *C. burnetii* infection. This makes the increase in APOE expression observed upon inhibition of *C. burnetii* protein synthesis particularly noteworthy. It seems that modulation of these key lipid homeostasis genes allows *C. burnetii* to not only suppress the loss of host cell cholesterol but to also direct lipid trafficking.

Bacterial pathogens often subvert host cell signaling pathways by introducing bacterial effector proteins that interfere with host cell phophorylation cascades [22]. *C. burnetii* dependent regulation of host cell signal transduction pathways are not well understood. Our data identified active modulation of three host cell signal transduction genes (ITK, DUSP9 and SKP2) by *C. burnetii's* protein(s). While ITK and SKP2 play significant roles in inducing host cell proliferation [257-258], DUSP9 is a mitogen-activated protein kinase phosphatase (MKP) that negatively regulates MAPK activity in mammalian cells, thus preserving the cell from apoptosis [259]. The expression of these genes are relatively higher in *C. burnetii* infected THP-1 cells compared to the expression levels found in *C. burnetii* infected THP-1 cells transiently inhibited by CAM. This suggests that *C. burnetii* protein synthesis "encourages" cell proliferation in addition to its anti-apoptotic effects as a means to preserve the host cell environment.

In addition to the outlined host cell processes, we identified a variety of genes involved in diverse functions of a host cell, which were also modulated by *C. burnetii* protein synthesis (Table 3.1). In this miscellaneous cellular functions category, some genes were expressed at relatively higher levels than what was expressed in CAM inhibited infected cells and are of particular interest. The PSD4 gene, which is involved in membrane recycling [260], and CHMP5, which is an essential regulator of late endosome function. CHMP5 null cells show enhanced signal transduction, protein accumulation in enlarged multi vesicular bodies (MVB) and inhibition of MVB trafficking to lysosomes [261]. In addition, we have recently found that markers of multi lamellar/multi vesicular bodies associate with membrane structures within the PV lumen during *C. burnetii* infection of Vero cells (unpublished observations). Given that *C. burnetii's* replication niche possesses markers consistent with those on late endosomes/lysosomes [3], our finding that expression of these genes are markedly lower when *C. burnetii* protein synthesis is inhibited suggests that they play a part in development and maintenance of the PV during infection. This overall manipulation of endocytosis, vesicle trafficking, and late endosome/lysosome maturation is in agreement with studies which found that inhibition of *C. burnetii* protein synthesis at any point during the life cycle changes these processes within *C. burnetii* infected cells [12-13].

Conclusions

Through this study we have discovered thirty-six host cell genes with significant relative expression changes after transient inhibition of *C. burnetii* protein synthesis. The expression changes of these genes in the mock and CAM treatment conditions were confirmed using RT-qPCR analysis. Using bioinformatics, we have also determined the

predominant host cell processes associated with these genes. Collectively, these data support our hypothesis that *C. burnetii* proteins differentially modulate host cell genes during infection. Predominant cellular functions that are modulated by *C. burnetii* proteins include (*i*) innate immune response, (*ii*) cell death and proliferation, (*iii*) vesicle trafficking and development, (*iv*) lipid homeostasis, and (*v*) cytoskeletal function. These findings indicate that *C. burnetii* actively modulates the expression of genes that may play a role in the ability of the pathogen to establish the PV, survive, and replicate within the intracellular environment.

Acknowledgements

Table 3.1. Differentially expressed host genes modulated by *C. burnetii* protein synthesis.

Cellular Function	Gene Symbol	Cellular location	Predicted Function(s)	-CAM[1]	+CAM[2]	FD[3]
Apoptosis	CTSB	Cytoplasm	peptidase	3.102	6.565	↑3.463
	CTSL1	Cytoplasm	peptidase	3.173	6.914	↑3.741
	BCL3	Nucleus	transcription regulator	3.103	5.673	↑2.57
Cell proliferation	C11ORF82	Cytoplasm	other	-1.849	-4.912	↓3.062
	SOX11	Nucleus	transcription regulator	3.127	-2.915	↓6.042
	HELLS	Nucleus	enzyme	-1.551	-4.653	↓3.101
	PGR	Nucleus	ligand-depend. nuclear recept.	-1.539	-6.853	↓5.313
Cell signaling	ITK	Cytoplasm	kinase	2.752	-2.46	↓5.212
	DUSP9	Nucleus	phosphatase	-2.04	-4.388	↓2.348
	SKP2	Nucleus	other	1.581	-2.627	↓4.208
Cytoskeleton	MTSS1	Cytoplasm	other	4.389	6.986	↑2.597
	ANLN	Cytoplasm	other	-1.943	-4.679	↓2.735
	SMTN	Extracell. space	other	-3.319	4.006	↑7.325
	PLEKHO1	Plasma memb.	other	2.162	5.396	↑3.234
Immune response	SPP1	Extracell. space	cytokine	3.351	6.733	↑3.382
	CCL2	Extracell. space	cytokine	5.053	7.451	↑2.398
	CXCL1	Extracell. space	cytokine	5.221	7.275	↑2.054
	IL8	Extracell. space	cytokine	7.839	9.985	↑2.146
Lipid metabolism	FABP4	Cytoplasm	transporter	2.351	4.506	↑2.155
	APOE	Extracell. space	transporter	2.591	4.958	↑2.367
	PLIN2	Plasma memb.	other	3.725	5.772	↑2.047
Miscelleaneous	RAB20	Cytoplasm	enzyme	2.489	4.925	↑2.436
	FAM177B	Unknown	other	5.064	7.125	↑2.061
	SELM	Cytoplasm	other	-2.23	2.531	↑4.761
	PSMA8	Cytoplasm	peptidase	-2.494	3.212	↑5.706
	MSC	Cytoplasm	transcription regulator	3.17	5.49	↑2.32
	MRPL44	Cytoplasm	enzyme	2.775	-1.356	↓4.131
	CHMP5	Cytoplasm	other	1.525	-2.189	↓3.714
	RORA	Nucleus	ligand-depend. nuclear recept.	-6.756	7.147	↑13.903
	ZFP36L1	Nucleus	transcription regulator	3.815	6.842	↑3.027
	ZNF573	Nucleus	other	1.412	-3.322	↓4.734
	SLC22A6	Plasma memb.	transporter	2.097	-2.146	↓4.243
	CDH2	Plasma memb.	other	-1.626	-3.634	↓2.007
	KIAA1279	Unknown	enzyme	7.811	12.888	↑5.077
	SPATA6	Unknown	other	-2.473	19.906	↑22.379
	PSD4	Unknown	other	2.197	-2.149	↓4.346

[1] Fold change of expressed THP-1 genes in response to *C. burnetii* infection under mock treated condition.
[2] Fold change of expressed THP-1 genes in response to *C. burnetii* infection under CAM treated condition.
[3] Fold change difference increase (↑) or decrease (↓) between [1] and [2].
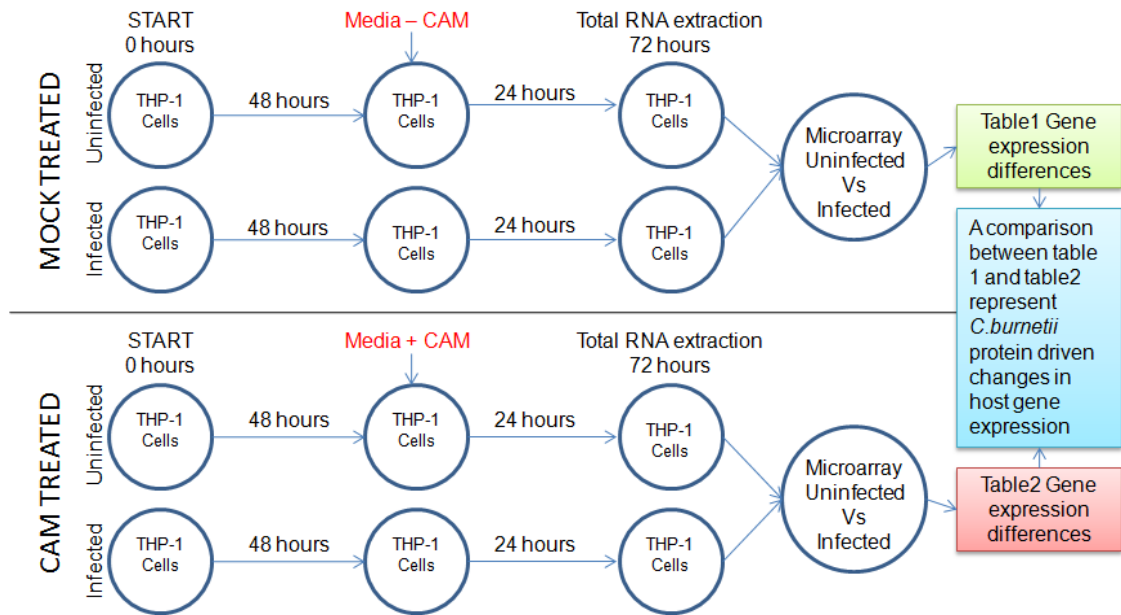
Figure 3.1. Diagram of the experimental design for comparative *C. burnetii* infected host-cell microarrays. The rows of the top panel are untreated and rows of the bottom panel are treated with CAM (10μg/ml) at 48h hpi. Total RNA harvests are performed at 72 hpi for subsequent microarray analysis.
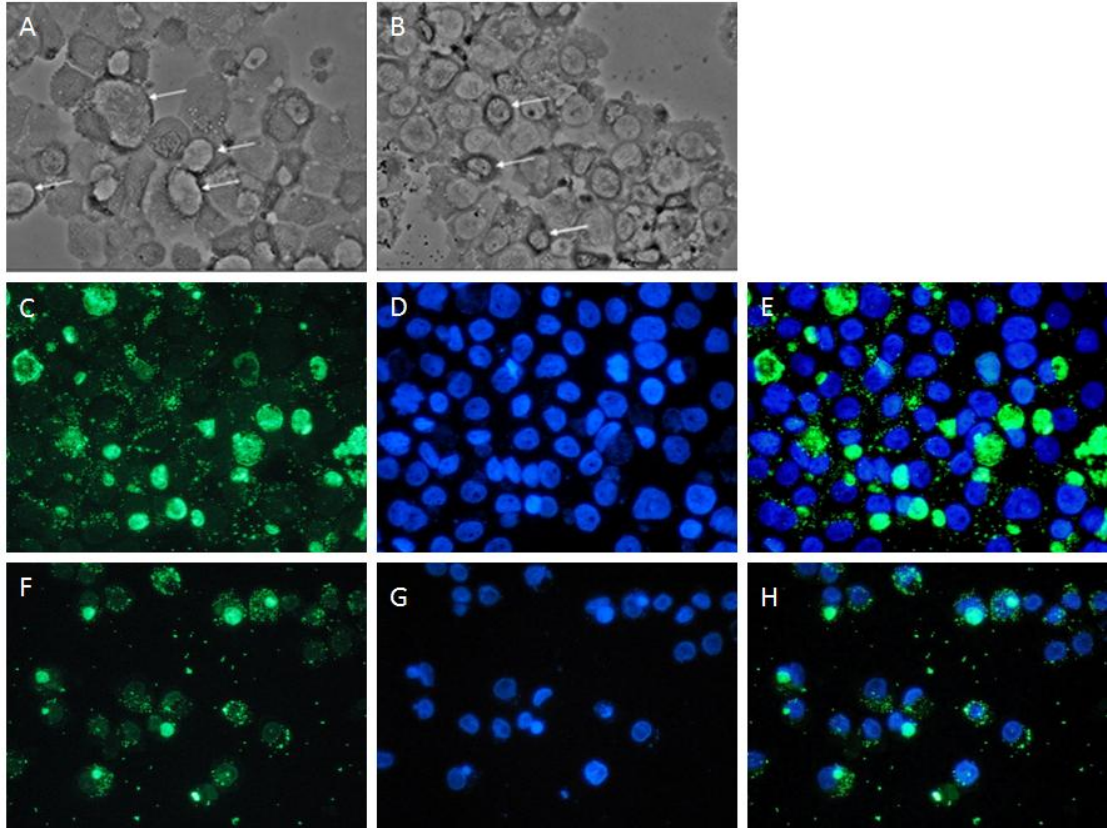
Figure 3.2. Phase contrast and fluorescent microscopy of *C. burnetii* **infected THP-1 cells.** All images are of *C. burnetii* infected THP-1 cells 72 hpi. **Top Panel**, Phase contrast microscopy. **A,** a mock treated infection. **B,** infection treated with 10 μg/ml CAM for the final 24 h. Arrows indicate PVs. **Middle Panel**, IFA microscopy images of a mock treated infection. **C,** Alexa-488 staining of *C. burnetii*. **D,** DAPI staining. **E,** merge of **C** and **D**. **Bottom Panel**, IFA microscopy images of an infection treated with 10 μg/ml CAM for the final 24 h. **F,** Alexa-488 staining of *C. burnetii*. **G,** DAPI staining. **H,** merge of **F** and **G**. 400X magnification was used for all images.
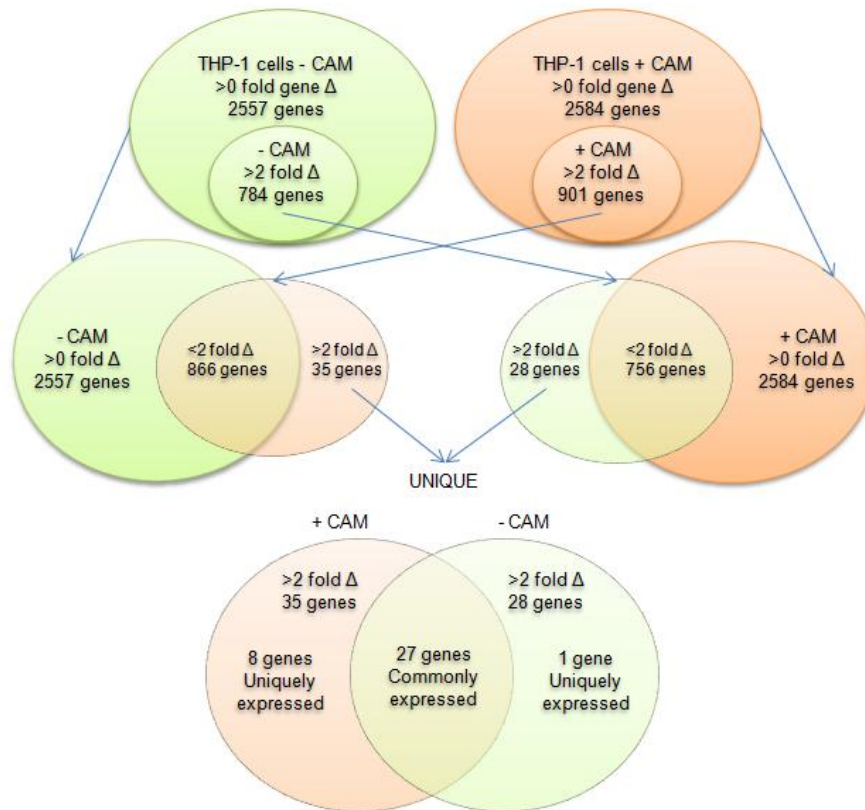
Figure 3.3. Venn diagram of differentially expressed THP-1 genes. A venn diagram visualization showing 784 and 901 differentially expressed host genes in *C. burnetii* infected THP-1 cells under mock (- CAM) and CAM treated (+ CAM) conditions respectively, as determined by oligonucleotide microarray analysis.   Comparisons between differentially expressed genes of –CAM with the gene summary list of + CAM (>0 fold Δ = 2584 genes) and differentially expressed genes of + CAM with the gene summary list of –CAM (>0 fold Δ = 2557 genes) are also shown.   The intersections (areas of overlap) indicate genes regulated in common under both conditions.   Twenty-eight of the differentially expressed genes in - CAM and thirty-five of the differentially expressed genes in + CAM are modulated by *C. burnetii* protein synthesis (>2 fold difference).   Of these, twenty-seven are common between the two conditions, while eight and one genes are uniquely expressed in +CAM and –CAM conditions, respectively.

A

Gene	Fold Change (-CAM)	Fold Change (+CAM)	Difference in Fold Change
IL8	7.84	9.98	2.14
CCL2	5.05	7.44	2.39
ZFP36L1	3.81	6.84	3.02
APOE	2.59	4.95	2.36
POU4F2	-5.27	-4.42	0.85
RND3	5.31	5.35	0.04
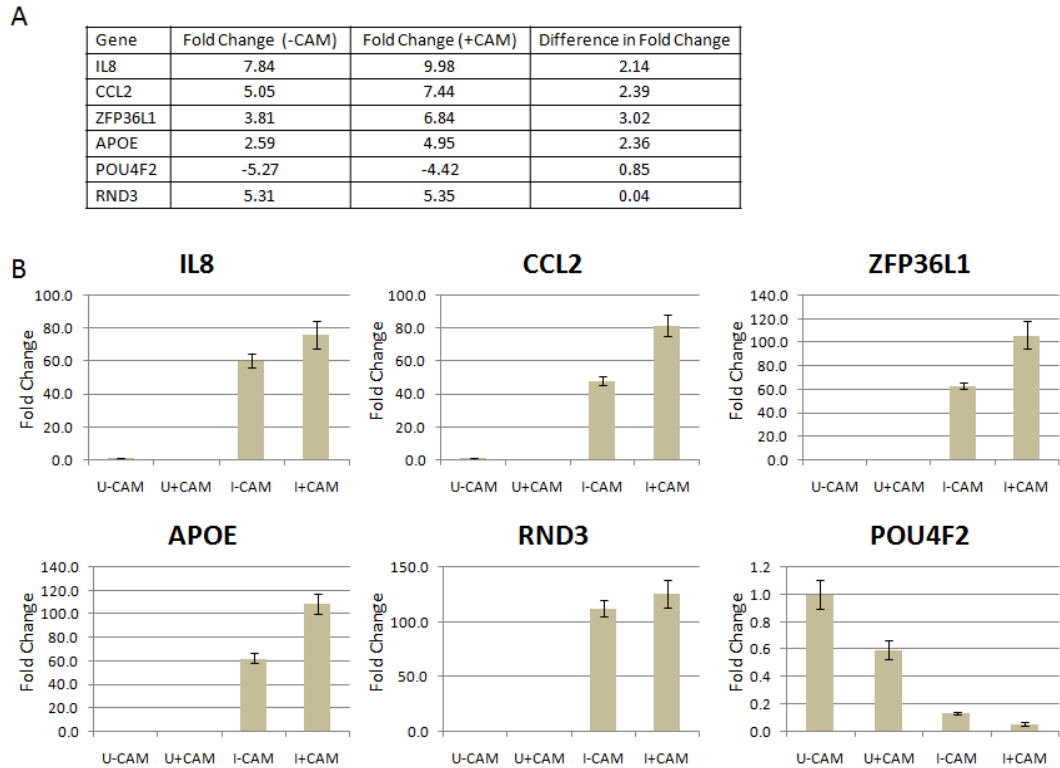
Figure 3.4. RT-qPCR of selected genes confirms microarray expression trends. A, shows the microarray data of the genes used to confirm microarray expression trends. Fold difference (-CAM) is the fold change of differentially expressed THP-1 genes in response to *C. burnetii* infection after mock treatment. Fold difference (+CAM) is the fold change of differentially expressed THP-1 genes in response to *C. burnetii* infection after CAM treatment. **B,** difference in mRNA levels in selected genes relative to β-actin. An equal amount of total RNA from each sample was analyzed by RT-qPCR. The Y-axis represents fold changes in gene expression while X axis shows the conditions under which gene expression was observed (mock and CAM treated, and uninfected and *C. burnetii* infected THP-1 cells). U–CAM, uninfected THP-1 minus CAM. U+CAM, uninfected THP-1 plus CAM. I–CAM, infected THP-1 minus CAM. I+CAM, infected THP-1 plus CAM. The results represent the mean of three biological samples and three technical replicates of each sample. Error bars represent the s.e.m.

Additional file 1

Tables S1.A-I. Excel file containing Tables S1.A through S1.I as individual tab-accessible tables within a single file (Supplemental Table S1.A-I).

OPEN DATA (BMC MICROBIOLOGY)

By open data we mean that it is freely available on the public internet permitting any user to download, copy, analyze, re-process, pass them to software or use them for any other purpose without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself.

Percentage of differentially expressed genes

Prominent biological functions affected in both mock and CAM treated THP-1 infections

- CAM
+ CAM

Prominent biological functions affected only in mock treated THP-1 infections

- CAM

Prominent biological functions affected only in CAM treated THP-1 infections

+ CAM
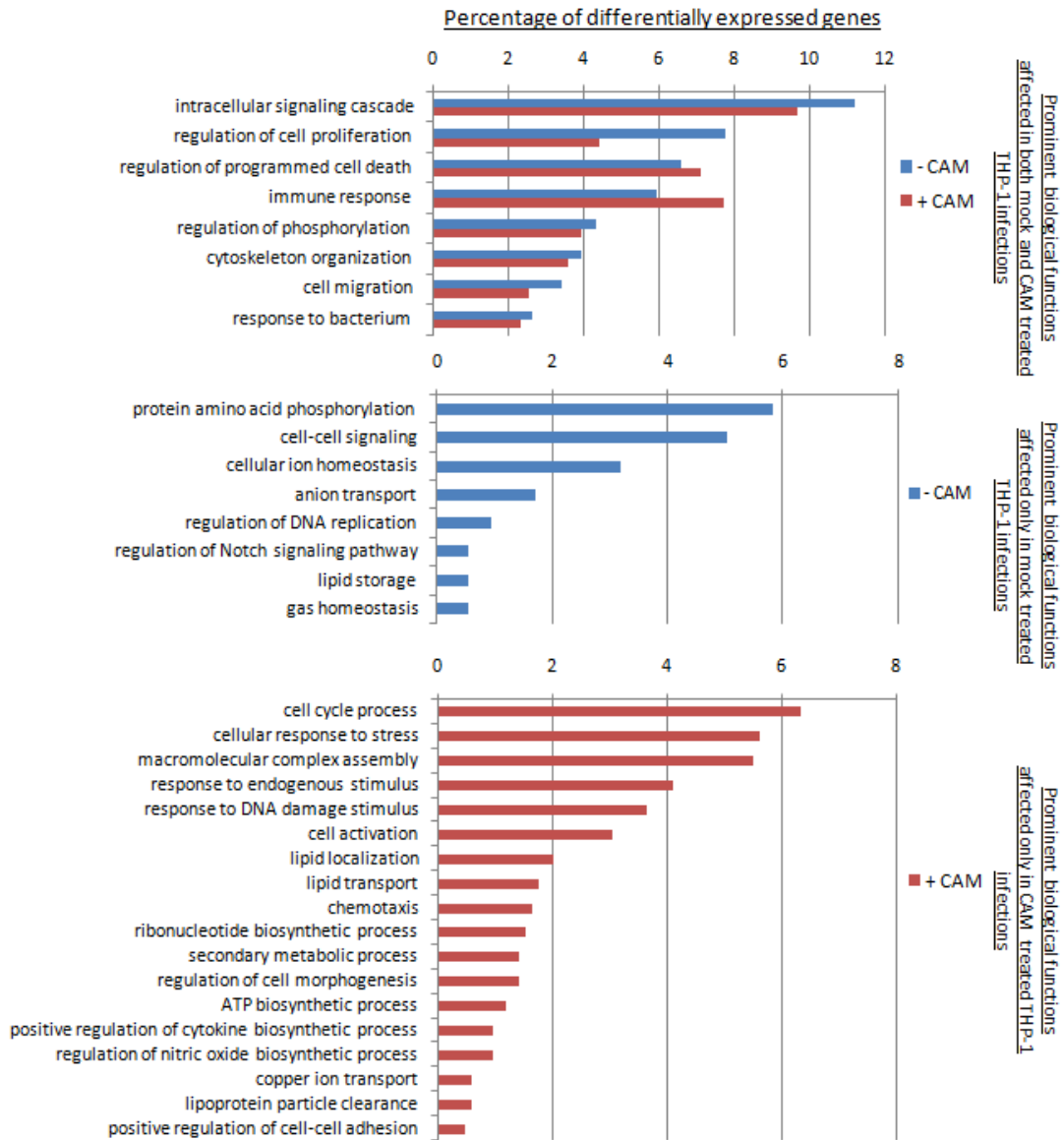
Additional file 2 (Legend on next page)

Figure S1. Biological function assignments of genes differentially expressed in mock and CAM treated THP-1 cells infected with *C. burnetii*. Both sets of microarray data (Additional file 1-Supplemental Tables S1.A and S1.B) containing differentially expressed genes for mock and CAM treated *C. burnetii* infections of THP-1 cells were annotated using DAVID to extract the biological functions of the listed genes. The X axis shows the percentage of differentially expressed genes associated with each annotation term while the Y axis shows the prominent biological functions (annotation terms) obtained through functional annotation of the differentially expressed genes. P-values for each annotation term are calculated using modified Fisher's exact test. A P-value cut off 0.05 or less has been used to identify biological functions. **Top panel**, shows the common host cell functions regulated under both conditions (mock and CAM treatment). **Middle panel** shows the major cellular functions affected only in *C. burnetii* infected THP-1 cells undergoing mock treatment. **Bottom panels** show the crucial host cell functions influenced only in *C. burnetii* infected THP-1 cells undergoing CAM treatment.

CHAPTER IV

COXIELLA BURNETII MODULATES NF-κB ACTIVATION IN HUMAN THP-1

CELLS DURING INFECTION

Introduction

Coxiella burnetii, is an obligate intracellular pathogen and the causative agent of acute Q fever as well as chronic disease in humans[4, 52]. *C. burnetii* infects alveolar macrophages and replicates within parasitophorous vacuoles (PV) resembling phagolysosomes while evading the host immune system [3, 23]. The *C. burnetii* infectious/life cycle is ~6 days long [172] and is highlighted by invasion of the host cell, development of the acidified PV (pH<5), differentiation of *C. burnetii* small cell variant (SCV) forms to large cell variants (LCVs), PV enlargement, log growth of the pathogen, an asynchronous LCV to SCV differentiation, and eventual cell lysis [3, 172]. *C. burnetii* is environmentally stable, acquired through aerosolization, has a low infectious dose (ID_{50}) [185], and classified as a category B select agent [52, 58]. Acute Q fever usually manifests as a self-limiting flu-like illness, with symptoms ranging from sub-clinical to debilitating and can be fatal [4]. Common chronic sequelae include endocarditis, hepatitis, and/or a chronic fatigue syndrome [5-6]. In many countries, *C. burnetii* infection of heart valves is a leading cause of culture-negative endocarditis [7].

Disease often occurs due to the ability of pathogens to subvert the immune system and modulate other cellular processes of the host. Manipulation of host nuclear transcription factor NF-κB signaling pathway(s) is a common strategy used by microbial pathogens to thwart hosts cellular defense responses [22]. NF-κB is a vital regulator of genes involved in pro-inflammatory immune response, cell proliferation, and apoptosis [262]. Normally, NF-κB transcription factors - p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), cRel, and RelB remain in the cytoplasm bound to the IκB inhibitory protein. These factors are activated via the canonical, non-canonical, or IKK independent

(Atypical) signaling pathways [262-263]. In either case, NF-κB activation and nuclear accumulation leads to inflammatory and immunomodulatory responses [262-263]. In general, humans first counter invading microbial pathogens by triggering innate immune inflammatory responses which are typically mediated via rapid activation of NF-κB [264] and subsequent expression of pro-inflammatory cytokine genes [29, 262, 265-266]. However, *C. burnetii* seems to employ unknown mechanisms to successfully avoid host innate immune and other cellular defense mechanisms [19, 23]. It is likely that the type IV secretion system (T4SS) possessed by *C. burnetii* allows for the release of *C.burnetii* effector proteins, which are used to manipulate eukaryotic cellular functions [18]. However, little or no information exists about the regulation of host immune signaling pathways being targeted by *C. burnetii* during the course of infection.

In order to determine if *C. burnetii* triggers host innate immune response, the role of Toll like receptors (TLRs) have been analyzed [36]. Activated TLRs usually signal through the NF-κB signaling pathway [264]. Studies indicate that TLR2 plays a crucial role in *C. burnetii* phase II recognition. It was observed that *C. burnetii* infected TLR2 deficient macrophages fail to produce inflammatory cytokines IL-12 and TNF-α [36]; however, *C. burnetii* surface molecules which activate TLR2 remain undefined. The stimulation of Toll-like receptor 4 (TLR4) in mammalian macrophages induces the release of critical pro-inflammatory cytokines. As TLR4 is typically activated by bacterial LPS [267], the role of TLR4 in *C. burnetii* infection has also been examined. Data shows that upon *C. burnetii* infection macrophages deficient for TLR4 produce elevated amounts of IL-12 and TNF-α. In addition, TLR4 knockout mice effectively arrests *C. burnetii* infection [37]. It seems unlikely that *C. burnetii* signals through TLR4

but one study reports that TLR4 has an association with initial pathogen uptake [37]. Interestingly, experiments on DC maturation using *C. burnetii* NMI and NMII strains show that phase I but not phase II *C. burnetii* prevents macrophage and DC maturation [38]. However, investigations also reveal that LPS chemotypes are not responsible for DC maturation or cytokine production [40]. Hence, it appears that phase I *C. burnetii* uses unknown mechanisms to prevent DC maturation.

Even though the innate immune system is unable to contain primary infections by *C. burnetii,* cytokine production is commonly reported [122]. *C. burnetii* has been shown to stimulate an atypical M2 form of activation [39]. M2 cells typically have an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype with a variable capacity to produce inflammatory chemotactic cytokines [39, 224]. Reports reveal the induction of several other cytokines during *C. burnetii* infection [41, 268]. These include RANTES and MCP-1, SCYA3, SCYA4, and IL8 [42]. The role of IL10 has been extensively studied in chronic Q-fever patients as it has been implicated in the enhanced persistence of *C. burnetii* in infected hosts due to its anti-inflammatory properties [225]. Experiments also show that *C. burnetii* phase II but not *C. burnetii* phase I stimulates increased IL-12 and TNF production [38]. Additionally, *C. burnetii* actively inhibits apoptosis to sustain its host cell [19-20]. However, these studies do not indicate if the cytokine or anti-apoptotic responses during *C. burnetii* infection arise in a NF-κB dependent manner. It is also unknown if NF-κB signaling is being actively modulated by *C. burnetii* proteins during infection.

The fact that *C. burnetii* infects, grows and replicates within alveolar macrophages which are characteristically responsible for phagocytosis and killing of

invading pathogens [269-270] suggests the bacterium is capable of overcoming the mononuclear phagocytes detection and pro-inflammatory response mechanisms. Previously, we used comparative microarray analysis, RT-qPCR and transient inhibition of bacterial protein synthesis to discover a subset of inflammatory cytokine genes (IL8, CCL2, CXCL1, and SPP1), [271] the expression of which are classically mediated through the NF-κB signaling pathway [22, 262, 272]. These secretory molecules normally regulate the infiltration and trafficking of immune cells [273]. *C. burnetii* protein(s) actively reduced the RNA levels of each of these genes relative to those found in cells containing bacteria transiently inhibited with chloramphenicol (CAM) [271]. Here, we hypothesized that the distinct suppression of cytokine genes may be a result of *C. burnetii*'s ability to modulate NF-κB activation in host cells. Modulation of genes regulated by NF-κB may represent a crucial step in *C. burnetii's* virulence. Furthermore, *C. burnetii*'s intracellular survival and growth may also depend on its ability to manipulate molecular components of this signaling pathway. In this study, we have analyzed *C. burnetii* induced modulation of NF-κB signaling and also defined the temporal modulation of NF-κB activation throughout its infectious cycle. In addition, we have also examined whether more than one of the NF-κB signaling pathways participate in NF-κB activation durign *C. burnetii* infection of host cells.

Material and Methods

Growth of *C. burnetii*, tissue culture and infection: *C. burnetii* Nine mile phase II strain was cultivated in African green monkey kidney Vero cells (CCL-81; ATCC, Manassas, VA) and purified as previously described [207]. Human monocytic leukemia derived THP-1 cells (TIB-202; ATCC) were grown in 75-cm^2 tissue culture

flasks using RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS) at 37°C in 5% CO2 [271]. Synchronous infections with *C. burnetii* phase II strains were initiated in 24-well tissue culture plates at a multiplicity of infection (MOI) of 25. Bacteria were added to 2×10^6 THP-1 cells per well in a total volume of 1ml and incubated at 37°C for 4 hours to allow close host cell-bacteria contact. Another 1ml of fresh media was added to the cells after 4 hours to bring the final concentration to 10^6 cells/ml (this time point represents T = 0). To determine whether *C. burnetii* modulate host cell NF-κB activation during infection, experiments were first performed with uninfected or *C. burnetii* infected THP-1 cells at 72 hpi (exponential phase) for total protein extraction. Cells were then incubated in media with (+CAM) or without (-CAM) bacteriastatic levels (10μg/ml) of chloramphenicol (CAM) for the final 24 h of infection [271]. Table 4.1 outlines the experimental design used to assess the temporal modulation of host cell NF-κB during the entire course of infection by *C. burnetii*. Experiments were performed in parallel, with mock treated and chloramphenicol (CAM) treated sets as published previously [271]. At various times post infection (PI) (0, 24, 48, 72, 96, and 120 hpi), one experimental set was transiently treated with 10μg/ml (bacteriostatic) of CAM for 24h while the other set was mock treated. Cell culture media was exchanged daily using centrifugation to harvest the cells and removal of the spent media followed by suspension of the cells in fresh media with or without CAM (10μg/ml). Infected and uninfected cells were handled identically and a minimum of three experiments (n=3) was carried out for each time point and condition. As a control, tumor necrosis factor alpha (TNFα; BD

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Biosciences, San Jose, CA) was added to cell cultures at a final concentration of 50 ng/ml for 8h prior to harvest in order to induce NF-κB activation [19].

Western blot analysis: Collected THP-1 cell pellets were directly lysed in 100μl of 2X laemmli sample buffer (Bio-Rad, Hercules, CA) along with protease and phosphotase inhibitors (Sigma). Samples were then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Pierce, Rockford, IL). The membranes were blocked for an hour at room temperature with 5% nonfat milk in Tris-buffered saline (150 mM NaCl, 100 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBST) [19]. Following blocking, membranes were incubated overnight at 4°C with 5% nonfat milk in TBST having primary antibodies for target proteins. Detection of NF-κB activation was carried out using the rabbit monoclonal anti-human primary antibody specific to the phosphorylated Serine 536 form of NF-κB p65 (Cell Signaling Technology, Danvers, MA), and rabbit anti-human polyclonal antibody against p100 (the precursor), and p52, the active form of NF-kappaB2 (Cell Signaling Technology, Danvers, MA). Mouse monoclonal antibodies directed against human β-actin (Sigma, Saint Louis, MO) were also employed to detect host cell β-actin (used as a loading control). Nitrocellulose membranes were then washed using TBST and incubated with anti-rabbit or anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) for 1h at room temperature. After incubation membranes were washed and target proteins were detected by enhanced chemiluminescence using ECL SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Visualization and digital imaging of the blots was performed on a FluorChem HD2 Imaging System (Alpha Innotech Corporation, Leandro,

CA). The signal density of the detected bands in experimental samples were analyzed by ImageJ [274].

Results

NF-κB activation is modulated by *C. burnetii* proteins during infection. Among all the members of NF-κB complex, p65 is one of the most extensively studied subunits. Additionally, like cRel, and RelB, it contains both a 300-amino acid region with homology to the Rel proto-oncogene (RH domain) and the transactivation domain [262, 275-276]. The RH domain harbors motifs for nuclear localization, and binding to specific DNA sequences while the transactivation domain, which remains bound to the inhibitor IκB in cytoplasm, contains phosphorylation sites [262, 277]. Phosphorylation of the S536 site in the transactivation domain is required for optimal activation [262, 276-277]. In an effort to determine whether host cell NF-κB was modulated by *C. burnetii* during infection, NF-κB activation was assayed via detection of p65 phosphorylation [275]. Total protein from -CAM and +CAM uninfected (U) and *C. burnetii* infected (I) THP-1 cells at 72 hpi (mid-log phase). Figure 4.1A is a representative immunoblot where monoclonal antibody to the activated (Serine 536 phosphorylated) form of NF-κB p65 was used to probe total protein blots. β-actin levels were used to normalize protein sample loading prior to NF-κB p65 analysis. Figure 4.1B clearly shows that host cell NF-κB is activated during infection and that *C. burnetii* protein synthesis modulates the level of this NF-κB activation. Fold changes between samples were calculated by comparing the amount of signal within each band as a percentage of the total signal representing a relative quantitation of the NF-κB activation. When compared to uninfected THP-1 cells, NF-κB p65 phosphorylated protein levels were observed to

increase ~10-fold in *C. burnetii* infected cells. However, NF-κB activation levels are ~20-fold higher in infected cells treated with CAM. These data indicate that while *C. burnetii* infection induces NF-κB activation, bacterial protein synthesis is modulating the levels of this induction.

   ***C. burnetii* modulates NF-κB activation temporally during its infectious cycle.** To measure the dynamics of NF-κB activation throughout the course of *C. burnetii* infection in the presence and absence of CAM, we examined total protein samples as outlined in Table 4.1. Our hypothesis was that the activation of NF-κB would respond directly to *de novo* bacterial protein synthesis depending on the stage of infection (early, mid, or late). Figure 4.2A shows a representative western blot of NF-κB p65 phosphorylated protein at various times PI (24, 48, 72, 96, 120, and 144 hpi) in the presence and absence of transient CAM treatment. Again, β-actin was used as a loading control to normalize the protein samples. Figure 4.2B illustrates the calculated fold changes between the six time points in the +CAM and –CAM experimental sets. *C. burnetii* protein(s) are seen to suppress NF-κB activation at 24hpi. Transient treatment with CAM (0-24h) produces a ~16 fold increase in NF-κB p65 phosphorylation levels during early infection compared to uninfected cells. The data also reveal that *C. burnetii* infection of THP-1 cells induces NFκB activation during mid-infection (48-96h). Compared to 24 hpi levels of p65 phosphorylation, 48 hpi levels are significantly up-regulated ($P < 0.05$) and continue to remain elevated until 96 hpi. Transient application of CAM at 24 and 48hpi results in even higher levels of p65 phosphorylation at 48 and 72hpi respectively. Again suggesting that *C. burnetii* proteins are involved in the regulation of NF-κB activation. Interestingly, application of CAM at 72hpi does not

change the phosphorylation levels of p65 at 96 hpi relative to infected -CAM cells. During late infection (120-144h), NFκB induction is reduced to lower levels and *de novo C. burnetii* protein synthesis does not appear involved in this decrease. These results indicate that infection of THP-1 cells by *C. burnetii* involves modulation of NFκB activation via p65 phosphorylation in a temporal manner. Furthermore, *de novo C. burnetii* protein synthesis causes a significant suppression of NF-κB activation during early and mid stage of the infection.

C. *burnetii* infection does not modulate NF-κB Activation via the Non-Canonical pathway. Results from our previous experiments show the involvement of p65 in NF-κB activation. NF-κB transcription factors are typically activated by either the canonical, non-canonical, or IKK independent (Atypical) signaling pathways. Canonical and atypical pathways signal via NF-κB p65 activation, while the non-canonical pathway signals by NF-κB p52 activation. Formation of active p52 occurs via proteolytic processing of the p100 (precursor) during NF-κB non-canonical pathway signaling. In order to determine the specific NF-κB signaling pathway(s) modulated by *C. burnetii,* we analyzed the role of non-canonical pathway in NF-κB activation over the course of infection. Western blot analysis on total protein samples was carried out to detect NF-κB p100/p52 over the course of *C. burnetii* infectious cycle. Figure 4.3A shows a western blot analysis of NF-κB p100/p52 at various times PI as outlined in Table 4.1. Protein samples were first normalized to human β-actin and subsequently used to detect NF-κB activation. CD 40 induced THP-1 cells were used as positive control. Fold changes in protein expression levels are shown in Figure 4.3B and C respectively. Expression changes were calculated using signal intensities of both NF-κB p100 and p52. Both

Figure 4.3B and C clearly demonstrate that *C. burnetii* does not appear to modulate host cell NF-κB p100 and p52 levels over the course of infection in the presence and absence of bacterial protein synthesis. When compared to *C. burnetii* infected cells at 24hpi, NF-κB p100 expression levels remain relatively constant at various times PI. Addition of CAM does not affect p100 expression. On the other hand, p52 levels are barely detectable and do not change in both –CAM and +CAM experimental sets (Figure 4.3 C). Together, these data reveal that NF-κB activation in infected host cells does not involve the non-canonical NF-κB signaling pathway.

Discussion

The NF-κB signaling pathway plays a crucial role in regulating pro-inflammatory immune response, cell death/apoptosis, and cell proliferation in a human host [262, 277]. Pathogenic microorganisms induce NF-κB activation by triggering PRRs (e.g. TLRs and NLRs), which are expressed on macrophages, DCs and mucosal epithelial cells [22, 278]. NF-κB activation typically induces the expression of a variety of genes involved with the immune response [278-279]. These include pro-inflammatory cytokines, chemokines, and adhesion molecules which regulate recruitment and trafficking of immune cells to the site of infection [22, 278]. NF-κB activation increase the transcription of genes (e.g. defensins) which have direct microbicidal activity [278]. Enzymes which generate reactive intermediates are also induced [278]. NF-κB acts as a major molecular link between the launch of innate and adaptive immune responses by facilitating T cell activation via induction of MHC proteins and CD80/86 in antigen-presenting cells [278]. B cell differentiation is usually stimulated by NF-κB activation as well [278]. Additionally, NF-κB activation plays a critical role in the expression of anti-apoptotic

proteins (e.g. c-IAP-1/2, AI, Bcl-2 and Bcl-XL) [278-279]. Regulation of cell-cycle regulator cyclin D1, which increases cellular survival and proliferation is also dependent on NF-κB activation [278].

Interestingly, pathogenic microorganisms harbor unique strategies to directly interfere with NF-κB activation and its signaling [22]. Bacteria modulate the NF-κB signaling pathway (activation or inhibition) according to the requirement of their life cycle [22, 279]. Studies on *C. burnetii*'s closest phylogenetic neighbor, *Legionella pneumophila,* reveal that the bacteria induce a biphasic pattern of NF-κB activation in human epithelial cells [281]. A short term activation during early infection (< 8 hpi) is followed by a decrease in activation, which is then followed by a long term induction of NF-κB later in infection [281]. However, it is still unclear if the first wave of NF-κB activation is actively suppressed by *L. pneumophila* [281]. Numerous bacterial pathogens appear to produce effector proteins that interfere with host cell NF-κB signaling. Bacterial modulators act both directly or indirectly on the NF-κB signaling pathway to elicit an effect advantageous to the pathogen [279]. Pathogens like *Shigella flexneri* and *Yersinia* spp. use their respective Type III effector proteins OspG and YopP/J to prevent IκB degradation, thereby keeping NF-κB inactive in the host cell cytoplasm [22]. On the other hand, activation of NF-κB protects several intracellular pathogens including *Mycobacterium tuberculosis* [282], *Bartonella henselae* [283], *Chlamydia pneumonia* [284], *Rickettsia rickettsii* [285], and *Legionella pneumophila* [281, 286] from cell death.

Here, we have built on our earlier finding that *C. burnetii* proteins modulate the mRNA abundance of NF-κB mediated cytokine genes (IL8, CCL2, CXCL1, and SPP1)

during infection [271]. The findings of our study clearly demonstrate that infection with *C. burnetii* induces host cell NF-κB activation. In addition, this induction level is effectively modulated by *de novo C. burnetii* protein synthesis. Figure 4.1 shows that host cell NF-κB activation is induced ~10-fold in *C. burnetii* infected THP-1 cells in comparison to uninfected cells. However, this NF-κB activation is further induced to ~20-fold when infected cells are transiently treated with CAM. This increased NF-κB activation in the presence of transient CAM treatment coincides with our findings that the mRNA of some NF-κB mediated pro-inflammatory cytokines (IL8, CCL2, CXCL1, and SPP1) also increases in *C. burnetii* infections of THP-1 cells when bacterial protein synthesis is inhibited. This suggests that while NF-κB is activated by *C. burnetii* infection, bacterial proteins modulate the level of this induction.

NF-κB activation is also associated with anti-apoptosis [262, 277]. Studies analyzing the effect of *C. burnetii* infection on host cell apoptosis show that bacterial protein synthesis inhibits cell death by preventing cytochrome C release from the mitochondria [20] and by activating host cell pro-survival kinases Akt and Erk1/2 [21]. Antiapoptotic genes *c-iap2* and *A1/bfl*-1 are also up-regulated in *C. burnetii* infected cells [19]. Both *c-iap2* and *A1/bfl*-1 are positively regulated via the NF-κB pathway [19]. Together, these studies suggest that two opposing effects of NF-κB activation could be occurring in *C. burnetii* infected cells: Some level of NF-κB activation is required to suppress apoptosis, which is beneficial for the pathogen, while too much NF-κB activation would substantially induce host expression of pro-inflammatory cytokines. However, the host and bacterial factors involved with *C. burnetii* mediated NF-κB modulation remain elusive.

New insights can be obtained from our temporal analysis of NF-κB activation during infection (Figure 4.2. Suppression of NF-κB activation during the first 24hpi may be linked to SCV to LCV morphogenesis or bacterial lag phase [172]. It appears that the bacterium employs unknown mechanisms to protect itself from pro-inflammatory mediators of innate inmmune response during early infection. These unknown mechanisms seems to employ *C. burnetii* proteins which might be directly or indirectly involved with this suppression as transient application of CAM induces a ~16-fold increase in NF-κB activation at 24 hpi. During the exponential phase of *C. burnetii,* i.e between 24-96 hpi [172], NF-κB activation is induced by ~12-fold (48 hpi) in infected cells relative to infected cells at 24 hpi and remains relatively constant till 96 hpi (Figure 4.2). This is a crucial period in *C. burnetii*'s life cycle, where bacterial growth can persist via NF-κB mediated anti-apoptotic effects [19]. It appears that induction of NF-κB activation ensures the integrity of infected cell during bacterial log growth. This pattern of NF-κB activation observed in *C. burnetii* infected cells is similar to *L. pneumophila* mediated NF-κB activation in human epithelial cells and macrophages [281]. Our results support findings from other studies which indicate that both phase I and phase II *C. burnetii* induce antiapoptotic host cell activity during this growth period (48 hpi), leading to reduced caspase processing and PARP cleavage in monocytes and macrophages [19]. However, it appears that *C. burnetii* is carrying out a balancing act by prohibiting NF-κB activation beyond a certain level, indicated by the observation that transient treatment with CAM at 24 and 48 hpi increases NF-κB activation levels by several fold (Figure 4.2). This indicates that *C. burnetii* proteins are playing a crucial role in controlling over production of pro-inflammatory cytokines while allowing the anti-apoptotic activity

necessary for survival. During late infection (between 96-144hpi) there is drop in host cell NF-κB activation and *C. burnetii*'s proteins do not affect this reduction. This coincides with *C. burnetii* LCV to SCV morphogenesis [172]. We are currently pursuing experiments to determine whether induction of host cell NF-κB activation is an absolute requirement for *C. burnetii* survival and growth.

Finally, we have sought to determine whether more than one of the NF-κB signaling pathways (canonical, non-canonical, or atypical) [262] is being targeted by *C. burnetii* over the course of infection. In Figure 4.2 we defined the temporal modulation of host cell NF-κB activation via p65 phosphorylation. Involvement of p65 phosphorylation suggests that *C. burnetii* infection of THP-1 cells induces NF-κB activation by signaling through either canonical or IKK-independent pathways. Figure 4.3 shows that *C. burnetii* infection of THP-1 cells does not induce the non-cannonical NF-κB signaling pathway in the absence or presence of CAM over the course of infection. Therefore, it is likely that *C. burnetii* proteins are interfering with either the canonical or atypical pathways, as evidenced by P65 phosphorylation, and not targeting the non-canonical signaling pathway (P100/p52) to modulate NF-κB activation in infected cells. Identifying the *C. burnetii* infection associated NF-κB pathway(s) modulated during infection is aiding in the design of experiments to define the specific molecular mechanisms modulated by *C. burnetii* during infection.

In summary, we have demonstrated that *C. burnetii* infection induces NF-κB activation in THP-1 cells via p65 phosphorylation. Additionally, *de novo C. burnetii* protein synthesis is able to modulate this NF-κB activation during infection. Induction of NF-κB activation is temporal in *C. burnetii* infected cells, with bacterial proteins

suppressing NF-kB activation early in infection. NF-κB activation levels increase during logarithmic phase but bacterial proteins via yet unknown mechanisms modulate the levels of this activation. Interestingly during late infection, a decline of NF-κB activation is observed and this drop in activation levels is independent of bacterial protein synthesis. In addition, the non-canonical pathway of NF-κB signaling is not induced or modulated by *C. burnetii* infection or protein synthesis.

Table 4.1. Temporal analysis of NF-κB activation in *C. burnetii* infected THP-1 cells.

CAM[a] +/-	SAMPLE[b] COLLECTION
0 hrs	24 hrs
24 hrs	48 hrs
48 hrs	72 hrs
72 hrs	96 hrs
96 hrs	120 hrs
120 hrs	144 hrs

[a] - Times PI when fresh media with 10 µg/ml CAM (I + CAM) or without CAM (I-CAM) was added to *C. burnetii* infected host cells.

[b] - Time points in hrs PI when cells were harvested for total protein extraction and subsequent immunoblot analysis.
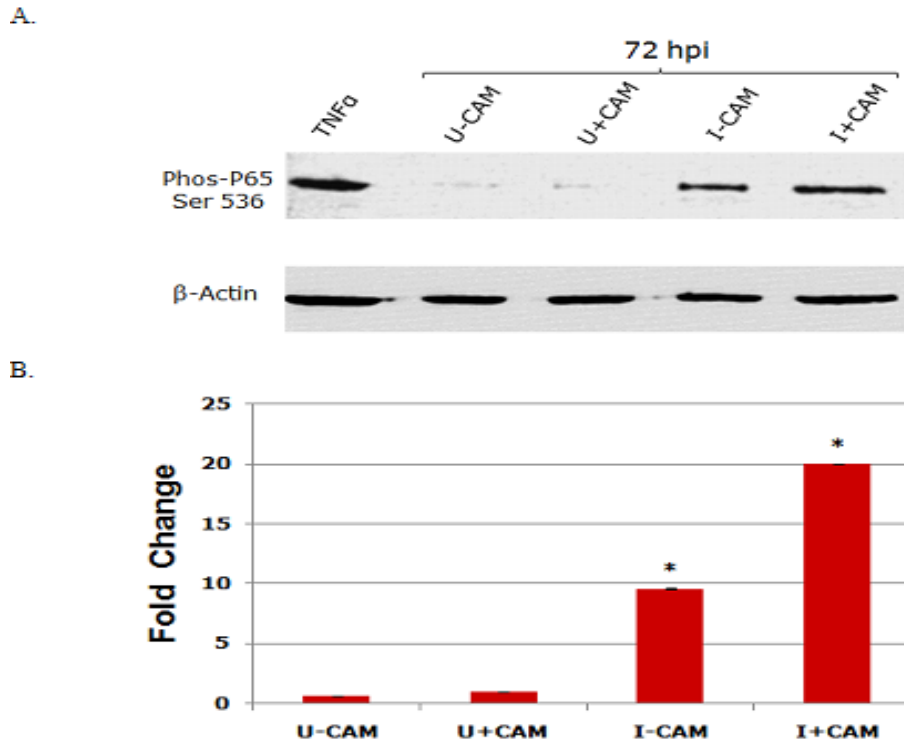
Figure 4.1. **Immunoblot analysis of** *C. burnetii* **modulation of host-cell NFκB activation**. **A**. Top panel was probed with antibody to phosphorylated p65. Lower panel was probed with antibody to β-actin. Uninfected without CAM (U-CAM). Uninfected with CAM (U+CAM). Infected without CAM (I-CAM). Infected with CAM (I+CAM). Time of sample collection is indicated above. **B**. Difference in phosphorylated p65 protein levels relative to normalized β-actin. The Y-axis represents fold changes in phoshorylated protein expression while X axis shows the conditions under which protein expression was observed. The results represent the mean of three independent experiments. Error bars represent +/- SD. Statistically significant differences (* P= <0.001) between the mean values among the samples were measured using One Way Repeated Measures Analysis of Variance and a Pairwise Multiple Comparison Procedure (Holm-Sidak method).
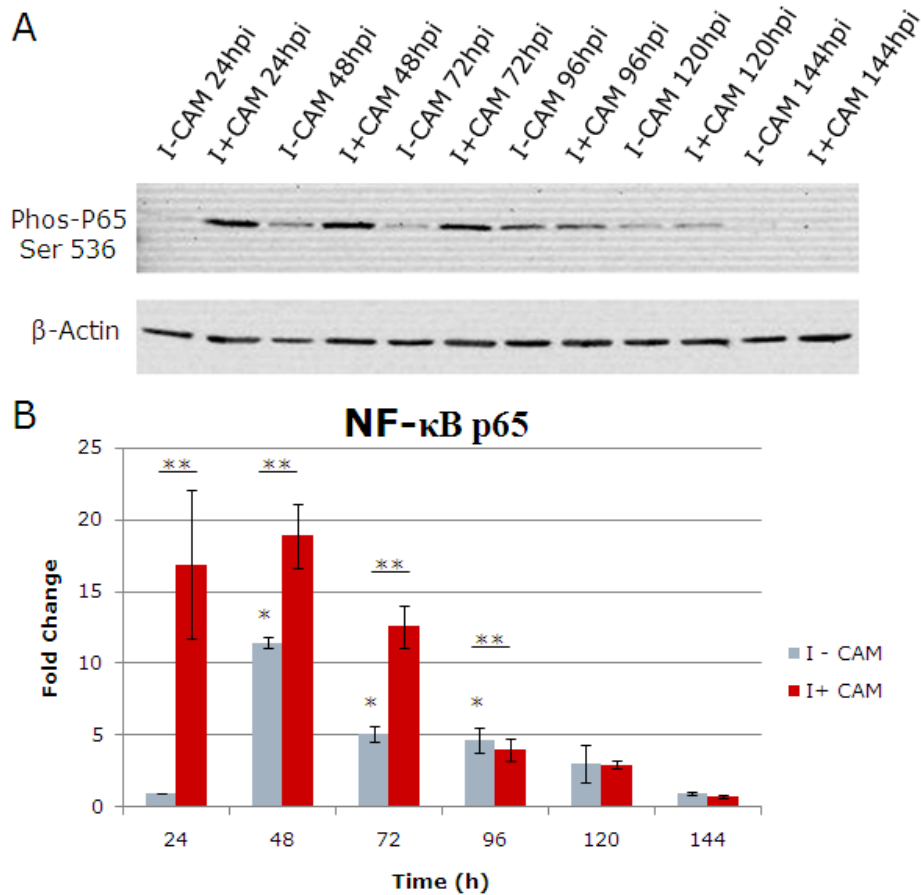
Figure 4.2: Analysis of NFκB activation in *C. burnetii* infected cells throughout the infectious cycle. A. Representative Western blot showing NF-κB activation over the time course of *C burnetii* infection. Top panel shows NFκB p65 phosphorylation levels which was probed with a monoclonal antibody against phosphorylated p65 (Ser 536) of human origin. The bottom panel shows human β-actin expression. Time in hpi at which each untreated (I-CAM) and CAM treated (I+CAM) *C. burnetii* infected cells was harvested is indicated as above. **B.** Fold change of NFκB p65 phosphorylation vs time in the presence and absence of CAM. Results of densitometric analysis (Image J) are means ±S.E.M. of three different experiments. Statistical differences were calculated using T test for paired samples. * signifies *P*<0.05 of I-CAM samples compared to 24 h. ** signifies *P*<0.05 between paired (I-CAM to I+CAM) samples at each time point..
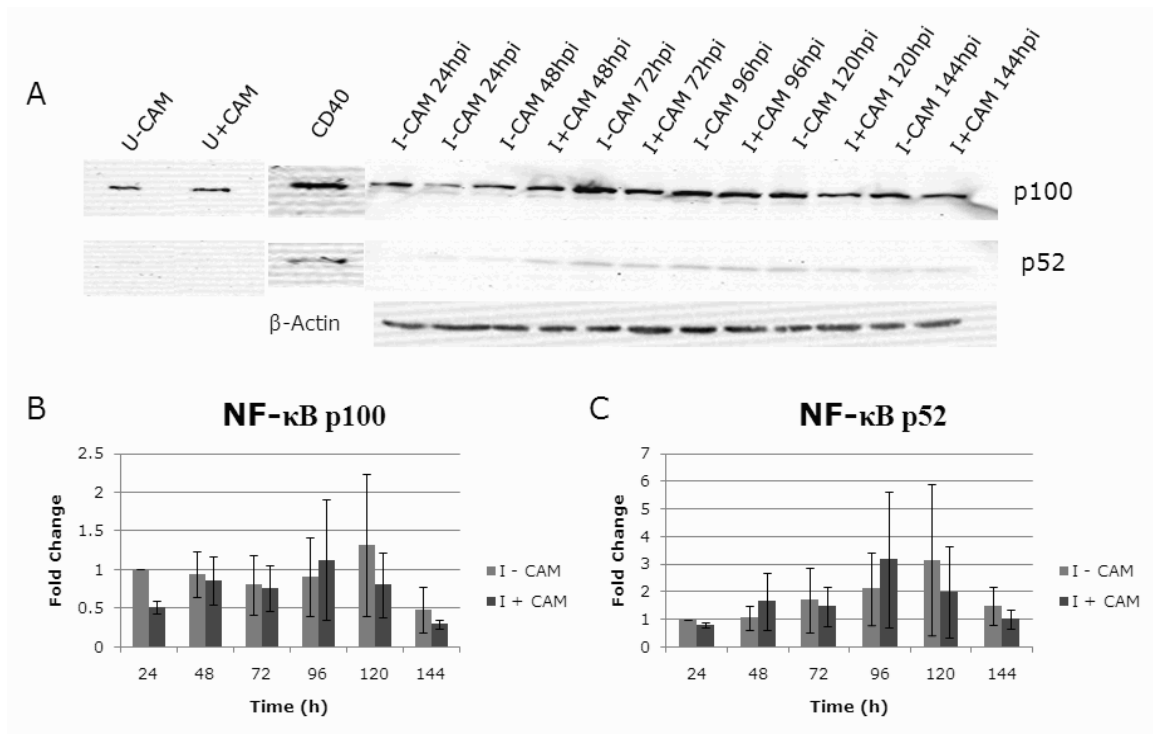
Figure 4.3: Western blot analysis of NFκB p100 and p52 expression over the time course of *C. burnetii* **infection. A.** A reprsentative western blot showing time course expression of p100 and p52 proteins in CAM treated and untreated *C. burnetii* infected cells. Blots were probed with a polyclonal rabbit antibody against NFκB (p100/p52) of human origin (Top and middle panel). The bottom panel shows normalized human β-actin . CAM treated and untreated cells were used as negative controls while CD40 treated THP-1 cells to detect p52 (positive control). Each time point for sample collection is indicated as above. **B.** Results of densitometric analysis showing means ±S.E.M. (fold change) of three biological experiments. **C.** Fold changes of NFκB2 p52 expression over the time course with and without CAM post normalization.

CHAPTER V

GROWTH OF *COXIELLA BURNETII* IN THE *IXODES SCAPULARIS* DERIVED IDE8

TICK CELL LINE

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Abstract

The zoonotic disease, Q fever, is caused by the gram-negative intracellular bacterium, *Coxiella burnetii*. While normally transmitted during exposure to infectious aerosols, *C. burnetii* is also found in arthropod vectors. In the environment, ticks are thought to play a crucial role in bacterial maintenance and transmission by infecting various mammalian species. However, the nature of the pathogen-tick relationship is not well defined. To determine *C. burnetii's* interactions with a cultured tick cell line, we introduced purified *C. burnetii* NMII into *Ixodes scapularis*-derived IDE8 cells and assayed for bacterial presence, replication, gene expression, and subsequent infectivity for mammalian cells. Tick cells were harvested at 24 hrs, 72 hrs, 7 days and 11 days post infection (PI). *C. burnetii* uptake and subsequent replication was demonstrated by indirect immunofluorescence assay (IFA), electron microscopy, and real-time PCR. Using a genome equivalent multiplicity of infection (MOI) of 30, 30-40% of exposed cells were seen to have small, rounded, vacuoles at 72 hrs PI. While at 7 and 11 days PI, 60-70% of cells contained enlarged vacuoles harboring large numbers of bacteria. qPCR analysis of total genomic DNA confirmed that *C. burnetii* genome numbers increased significantly from 24 hrs to 11days PI. The expression of *C. burnetii* type four secretion system homologs at 7 days PI was demonstrated by RT-PCR. Finally, IFA demonstrated that *C. burnetii* propagated within IDE8 cells were infectious for mammalian cells. These studies demonstrate the utility of cultured tick cell lines as a model to investigate *C. burnetii's* molecular interactions with its arthropod vectors.

Introduction

Q fever is a zoonotic disease found throughout the world, with the exception of New Zealand [287-288]. The disease is caused by the Gram-negative intracellular bacterial pathogen *Coxiella burnetii*. Human infection occurs mainly through inhalation of contaminated particulates shed from infected goats, sheep, and cattle [48, 80]. Transmission to animals and humans is facilitated by the ability of *C. burnetii* to survive for extended periods in a spore-like state on objects contaminated with infected tick feces, in water, and in soil [165]. Additionally, wild and domestic mammals, birds, and ticks act as reservoirs for the bacterium[4, 80]. *C. burnetii* infections are usually not clinically apparent in animals, however acute and chronic infection can lead to abortion in sheep and goats, and low birth weights and infertility in cattle[289]. Since ticks are a reservoir, it is thought that they act as vectors in the transmission of *C. burnetii* amongst animals [47, 74, 80] as well as maintaining the pathogen in the environment. Early investigations indicate that *C. burnetii* may replicate in the middle gut or stomach of ticks and subsequently be excreted in the feces [290]. Moreover, studies indicate that transovarial and transstadial transmission of *C. burnetii* may occur in *Hyalomma asiaticum, Hyalomma lusitanicum* and *Dermacentor marginatus* [291-293]. While there is evidence that *C. burnetii* is able to replicate in crude primary tick cell cultures [49], recently established continuous tick cell lines have not been employed to study the host cell-pathogen interactions of *C. burnetii* and these vectors.

Blood feeding Ixodid ticks (subphylum Chelicerata; class Arachnida; subclass Acari; family Ixodidae) are known to transmit a variety of bacterial, rickettsial, viral, and protozoan diseases [294]. Ixodid ticks have recently been shown to harbor *Coxiella* spp.

and *Coxiella*-like pathogens in the wild [295]. Due to the efficiency of *Ixodes spp.* ticks as vectors of pathogens and their worldwide distribution, we have chosen an *Ixodes scapularis*-derived cell line (IDE8) to investigate as an *in vitro* model for studying the tick-pathogen cellular interactions of *C. burnetii*. This cell line has been used to successfully propagate multiple tick-borne pathogens including *Anaplasma phagocytophilum, A. marginale, Ehrlichia canis, E. ruminantium, Borrelia spp. and Rickettsia spp.* [296]. In the current study, we sought to determine *C. burnetii's* infectivity, growth rate, gene expression as well as its ability to reinfect mammalian cells after growth in cultured tick cells. Using the indirect fluorescent antibody (IFA) microscopy assay, electron microscopy (EM), quantitative PCR (qPCR), and reverse transcriptase-PCR (RT-PCR) we determined the ability of *C. burnetii* to invade and replicate within the IDE8 tick cell line, expression levels of genes of the Type Four Secretion System (T4SS) within tick cells and the ability of tick cell derived *C. burnetii* to invade mammalian cells.

Materials and Methods

Bacterial cultivation and purification. *Coxiella burnetii* Nine Mile Phase II Clone 4 (NMII) was propagated in African green monkey kidney (Vero) cells in RPMI 1640 medium, 5% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO_2, and the Small Cell Variant form of the organism was isolated as previously described [172]. The SCVs were resuspended in SPG buffer (0.7 M sucrose, 3.7 mM KH_2PO_4, 6.0 mM K_2HPO_4, 0.15 M KCl, 5.0 mM glutamic acid, pH 7.4) and stored at -80°C. *C. burnetii* genome equivalents were calculated using qPCR [297].

Tissue culture cells: Uninfected Vero cells were propagated as described in medium containing 20 µg/ml gentamicin. The medium was exchanged with fresh RPMI 1640, 5% FBS without antibiotics two hours prior to bacterial infection. The tick cell line IDE8 (ATCC CRL 11973), derived from embryos of *Ixodes scapularis* and maintained in continuous passage for several years, was maintained in a modified Liebovitz's L15 medium at 34°C following the procedures of Munderloh et al [298]. Cultures were washed with antibiotic-free media prior to *C. burnetii* infections.

Infection of IDE8 tick cells: The optimal *C. burnetii* multiplicity of infection (MOI, based on genome equivalents) for IDE8 cells was empirically determine (data not shown). Thereafter, $25cm^2$ flasks containing $1x10^7$ IDE8 cells were infected with *C. burnetii* NMII at a genome equivalent MOI of 30 in 2 mls of L15 medium at 34°C for 4 hours. The flask volume was then brought up to a total of 5 mls with L15 media. Infected cells were incubated at 34°C with culture flask caps closed. Media was replaced every 24-48 hrs as needed.

IDE8 cell sample harvest. The $25cm^2$ flasks containing $1x10^7$ IDE8 cells were divided into five sections. One section of the flask was harvested by scraping just prior to infection (uninfected), and 24, 72, 168 (7 days), and 264 hrs (11 days) post infection (PI). Media was removed prior to each sampling and replaced immediately afterwards, and flasks returned to incubation. Parallel aliquots of infected cells from each time point were (*i*) seeded in 24-well plastic tissue culture plates for 3-4 hours at 34°C to allow for re-attachment, and then fixed with a 4% paraformaldehyde, Tween 20 (0.05%) PBS solution, (*ii*) fixed to glass slides using a cytospin centrifuge followed by fixation for 10 minutes using ice cold methanol, and (*iii*) centrifuged and the total genomic DNA

isolated using the Genomic Isolation Kit (Promega, Madison, WI). A minimum of three biological samples were isolated for each condition and time point.

Indirect Immunofluorescence antibody assay: The 24–well plate seeded and cytospun samples were analyzed by IFA microscopy using rabbit polyclonal antibody against whole-killed *C. burnetii* NMII followed by an Alexa-fluor 488 tagged goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Fluorescent images were captured at 400X magnification using a Nikon eclipse TE-2000 S inverted microscope equipped with a Nikon DS-Fi1 digital camera.

Vero cells seeded on 24-well tissue culture plates were inoculated with *C. burnetii* isolated from 7 day PI IDE8 cell lysates. Lysates were created by scraping *C. burnetii* infected IDE8 cells into PBS, freeze thawing the cells twice at -80°C followed by repeatedly passing the thawed cells through a 26.5-gauge needle. *C. burnetii* were separated from cell debris by differential centrifugation, and resuspended in RPMI 1640 medium, 5% fetal bovine serum (FBS), which was used to inoculate the Vero cells within 24-well culture plates. Cultures were grown at 37°C in an atmosphere of 5% CO_2, for 72 hours, then fixed to the culture plates using methanol. IFA microscopy analysis was performed directly in the culture plates as described above.

qPCR Analysis: Ten-fold serial dilutions of purified *C. burnetii* genomic DNA (10^6, 10^5, 10^4 and 10^3 genomes/sample well) were used to generate a standard quantitative curve in each experiment. Estimation of *C. burnetii* genome equivalents in infected IDE8 cell samples was accomplished using qPCR and the SYBR Green Master Mix kit (Applied Biosystems) in an Applied Biosystems 7500 real-time cycler, with

forward [f] and reverse [r] primers CB594 -5'CGCTTCATGAATTAGCAGCA–3'[f] and

CB595 -5'TGCAGTCAAACGGTTCTTCA-3'[r]. These primers target the *C. burnetii*

*icm*W gene (GenBank accession no. AF318146). Briefly, the reaction mixture contained

0.3 μM of each primer, and 10ng of sample template DNA in a total volume of 15μl.

The resulting fluorescent plots were analyzed and estimated numbers of *C. burnetii*

genomes in the experimental samples were determined based on the standard curve. An

increase in genome equivalents was observed relative to infected IDE8 cells collected 24

hours PI. A minimum of three biological and three technical samples were used in the

analysis of each time point.

RNA isolation and quality control. One half of a 25cm^2 flask containing

infected IDE8 cells was scraped at 7 days PI and cells were pelleted by centrifugation.

Total RNA was then harvested using Tri Reagent (Ambion, San Antonio, TX) following

the manufacturer's recommendations. All RNA samples were DNase treated to remove

contaminating DNA with RQ1 DNase (Promega, Madison, WI) and confirmed DNA-free

by PCR prior to RNA analysis assays.

Reverse Transcriptase-PCR (RT-PCR) analysis. RT-PCR analysis was carried

out using the Access Quick RT-PCR Kit (Promega, Madison, WI) and total RNA isolated

from *C. burnetii* infected IDE8 cells following the manufacturer's directions. Primers

CB40-5'ATGCCAGATCTGTCGC-3'[f] and CB41-5'TAAACCACCTTCCTCAAGAG-

3'[r] (*icmW*), CB70-5'ATGATTCTTTTGGAGTCTTCC-3'[f] and CB71-

5'TTGTTTGGACCCCTTAAAGGTG-3'[r] (*icmV*), and CB703-

5'ATTGGGGCCAGTATCATTCC-3'[f] and CB696-

5'ATGGAGTGTGCGGATTTGAT-3'[r] (*dotH*), were used in RT-PCR analysis.

Electron microscope analysis. One half of a 25cm^2 flask containing infected IDE8 cells was scraped at 7 days PI and cells were pelleted by centrifugation. The *C. burnetii* infected IDE8 cells were fixed with 2.5% paraformaldehyde (v/v)/2.5% glutaraldehyde (v/v) for transmission electron microscope (EM) analysis as previously described (Morgan, Luedtke et al. 2010) . The Imaging Facility in the Department of Molecular Microbiology Center for Infectious Disease Research, Washington University, St. Louis, MO, performed the subsequent sample processing and transmission EM analyses following published techniques [299].

Results

***C. burnetii* infection of IDE8 tick cells:** To determine whether *C. burnetii* infects IDE8 cells, we used an approximate genome equivalent MOI of 30. The infected cells were cytospun to a microscope slide followed by methanol fixation and IFA. These analyses indicate that *C. burnetii* containing vacuoles are present by 72 hrs PI and large, spacious, immunostained *C. burnetii* vacuoles were prominent by 7 days PI (data not shown). Although this indicated that *C. burnetii* were infecting IDE8 cells and replicating within them, the cytospin centrifugation method causes distortion and/or disruption of infected cells resulting in dispersion of many of the bacteria.

In order to observe infected IDE8 cells that are physiologically intact, an alternative method was employed where the tick cells were re-seeded to 24-well tissue culture plates and allowed to adhere prior to fixation and IFA analysis. Figure 5.1A shows that after 72h PI approximately 30-40% of infected cells had small, rounded, vacuoles and at 168 hrs (7 days) PI, swollen enlarged vacuoles containing large numbers

of bacteria were present. By 264 hrs (11 days) PI, the infected IDE8 cells had large fragile vacuoles such that intact infected cells could not be transferred from larger flasks to 24-well culture plates for microscopy analysis without rupturing the cells.

To determine whether *C. burnetii* were growing within membrane bound parasitophorous vacuoles (PVs), EM was performed on infected IDE8 cells fixed at 7 days PI (Figure 5.1B). EM micrographs indicate that *C. burnetii* is replicating in a membrane bound compartment (Figure 5.1B, left panel) and that both replicative large cell variants (LCV) and environmentally stable small cell variant (SCV) forms of the bacteria appear to be present within the vacuole (Figure 5.1B arrows and arrowheads, respectively) at 7 days PI. Combined, these experiments demonstrate that *C. burnetii* can be internalized, survive and grow within IDE8 tick cells *in vitro.* The appearance of spacious vacuoles at the beginning of what might be thought of as the exponential growth phase (72 hrs PI) is similar to that seen in *C. burnetii* infection of cultured mammalian cells [172].

C. burnetii **genome numbers increase after an extended lag phase**: In an effort to quantitate the growth characteristics of *C. burnetii* NMII in IDE8 tick cells, we estimated the number of *C. burnetii* genomes during the course of infection using qPCR. Using primers designed to the *C. burnetii icmW* homolog, and 24 hrs PI as a base line, *C. burnetii* genome equivalents were observed to decrease slightly between 24 and 72 hrs PI, although the decrease was not statistically significant (p<0.05). This was followed by a 3.10 and 17.83 fold increase at 7 , and 11 days PI (Figure 5.2), respectively. After a lag, *C. burnetii* double every ten hours in mammalian cell models [172, 300]. Using our data to calculate the replication rate of *C. burnetii* in the IDE8 cells, a doubling time of

nearly forty hours can be derived over the entire time period. However, if the calculation is made following the approximately 72 hr lag phase, *C. burnetii* genomes double every 10.87 hrs (Figure 5.2) in the IDE8 cells. This rate is very similar to the 10.2 hr (qPCR assay) rate found during the exponential phase of *C. burnetii* growth in mammalian cells following a 48 hr lag phase [172].

Expression of the *C. burnetii* T4SS during infection of IDE8 cells. Secretion systems have been shown to be crucial for the delivery of effector proteins in a number of bacterial pathogens. In particular, the type three secretion system is required for the virulence of bacteria including *E. coli*, *Shigella*, and *Salmonella spp*. [301]. *C. burnetii* possesses T4SS homologs, a system which has been shown to be required for virulence in its closely related neighbor, *Legionella pneumophila* [302-304]. *C. burnetii* T4SS homologs are expressed at the RNA and protein level during infection of mammalian cells in culture [172, 205, 207-208]. To determine whether this virulence determinant is expressed by *C. burnetii* during infection of IDE8 cells, RT-PCR was used to analyze total RNA isolated from infected cells 7 days PI (Figure 5.3). Amplification products following RT-PCR clearly demonstrate that *icmW*, *icmV* and *dotH* are expressed by the bacterium during infection of the IDE8 cell line.

IDE8 derived *C. burnetii* infectivity for mammalian cells. To determine whether the *C. burnetii* surviving within IDE8 cells were infectious for mammalian cells, IFA microscopy analysis was performed on Vero cells that were inoculated with lysates harvested from *C. burnetii* infected IDE8 cells 7 days PI. Figure 5.4 shows an IFA of Vero cells infected with IDE8 derived *C. burnetii*. In this qualitative analysis, it is evident that the *C. burnetii* growing within IDE8 cells are infectious for mammalian

cells. Large PVs containing multiple bacteria are indicated by the large, green fluorescing vacuoles present within the Vero cells (Figure 5.4). This finding indicates that the *C. burnetii* growing within tick cells are readily infectious for mammalian cells, increasing the likelihood that tick-borne exposure to the pathogen could lead to disease.

Discussion

Since the earliest studies of *C. burnetii*, it has been known that this pathogen has an association with arthropod vectors [62, 76]. However, an understanding of whether *C. burnetii* is passively carried in ticks or it is amplified by replication within the tick is not clear. Additionally, it has been demonstrated that *C. burnetii* can grow in a myriad of mammalian cell lines [19, 305-307], yet its ability to invade, replicate, and produce infectious progeny in tick cell lines had not been reported.

Our findings indicate that *C. burnetii* readily infects cultured IDE8 cells. Quantitative PCR of the *icmW* gene indicates *C. burnetii* grown in IDE8 cells undergoes a prolonged lag phase before replication begins relative to growth in mammalian cells [172]. When replication does begin, *C. burnetii's* doubling time in IDE8 cells appears to approach the approximately 10 hrs observed in mammalian cells. These findings suggest a period of adjustment may be required for successful growth. It may be that the organism has to adjust to the lower temperature (34°C) of IDE8 cell culture. It could also be hypothesized that the bacterium has to adjust to a substantially different host cell environment relative to *in vitro* growth in a mammalian cell line. Both factors may influence *C. burnetii's* replication during early infection of these tick cells. Interestingly, while *C. burnetii* genome numbers remained relatively constant between 24 and 72 hrs of

infection (Figure 5.2), vacuoles with appreciable numbers of *C. burnetii* are evident in 72 hrs PI IFA analyses (Figure 5.1). Although difficult to appreciate in the low light fluorescent images (Figure 5.1), a large number of free, or single, bacteria were observed 24 after *C. burnetii* infection of IDE8 cells, suggesting that many of the bacteria used to initiate an infection did not cause a productive infection within a given cell. We speculate that this is the result of overestimation of the MOI due to the inability of the qPCR assay to discriminate between DNA from viable and non-viable bacteria, or that the IDE8 cells were capable of ingesting and killing a portion of the bacteria. In either case, the bacteria that invaded and survived within the IDE8 cells had formed visible vacuoles by 72 hrs PI (Figure 5.1).

The ability of facultative and obligate intracellular pathogens to subvert host cell processes is crucial to their survival. Secretion systems are one of the primary means by which pathogens interact with the host. Our evidence that *C. burnetii* is expressing RNA for the production of a T4SS during infection of IDE8 cells (Figure 5.3) leads us to hypothesize that the pathogen is interacting with the tick cells and are likely manipulating the host cell response, as in mammalian cells [18]. In mammalian cells, the expression of T4SS homologs has been shown at the RNA level throughout infection and that the T4SS machinery is localized to the poles of the bacterial cell [172, 205, 207-208]. Moreover, it is likely that the *C. burnetii* T4SS is crucial to the pathogens ability to subvert cellular pathways for its own benefit.

Ultimately, *C. burnetii's* ability to infect a mammalian host after replication within a tick vector makes ticks a viable and crucial environmental reservoir of this pathogen. Our demonstration that *C. burnetii* isolated from IDE8 cells could readily

infect cultured Vero cells (Figure 5.4) would indicate that the *C. burnetii* propagated within a tick would be capable of causing disease in humans and other mammals. In addition, the production of environmentally stable SCV forms of the pathogen would enable a tick vector to shed infectious particles with the ability to cause disease long after they enter the environment.

The results of these experiments show that *C. burnetii* capable of infecting mammalian cells are produced in IDE8 tick cells after an extended lag phase. Further, we demonstrate that *C. burnetii* expresses homologs of a suggested virulence determinant during infection of the IDE8 cultured cells. These findings demonstrate that cultured tick cells represent a viable *in vitro* model to study the pathogens cellular interactions with tick cells in comparison to those found in mammalian cells while expanding our understanding of *C. burnetii* growth within the tick vector.

Acknowledgments

Fig. 5.1: IFA of *Coxiella burnetii* NMII infection of IDE8 cells. **(A)** Left panel, DAPI stained cells. Middle panel, Alexa-488 labeling of *C. burnetii*. Right panel, merge of left and middle panels. Time of fixation PI, are indicated at the left of each corresponding row. **(B)** EM micrograph of *C. burnetii* infected IDE8 cells fixed at 7 days PI. Arrows indicate *C. burnetii* large cell variants. Arrowheads indicate *C. burnetii* small cell variants. Size bar is 1.0-micron.
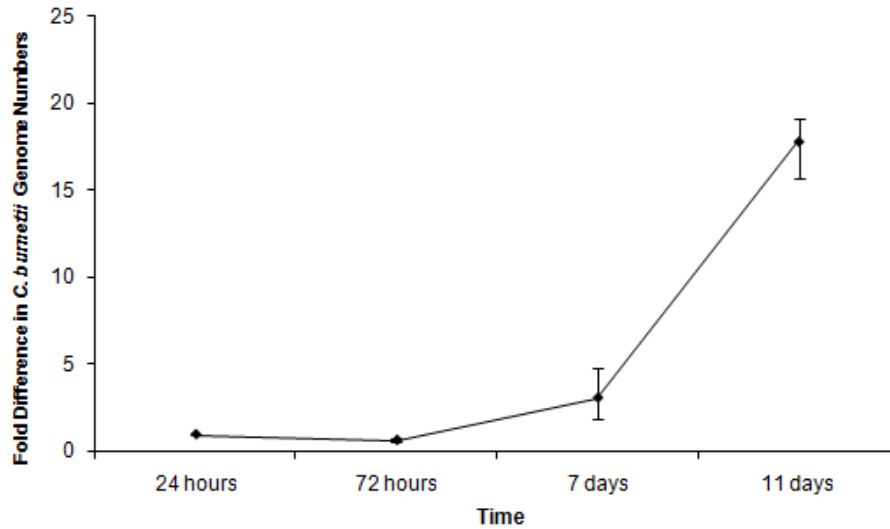
Fig. 5.2: ***C. burnetii*** **genome levels during infection of IDE8 cells.** Fold changes in genome numbers relative to 24 hours PI. An equal amount of total genomic DNA from each sample was analyzed by qPCR. The time (in hours and days) PI when DNA was harvested is indicated below the X-axis. Results represent the mean of three biological samples with no fewer than three technical replicates of each sample. Standard error bars represent the combined standard error of the mean (S.E.M.) per time point.
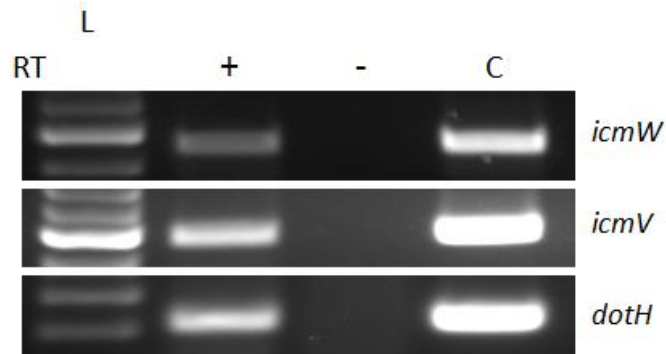
Fig. 5.3: RT-PCR detection of *C. burnetii* T4BSS transcripts, *icmW*, *icmV*, and *dotH* during infection of IDE8 cells. Total RNA template was isolated at 7 days PI from *C. burnetii* infected IDE8 cells. L, 100 bp DNA ladder. +RT, with reverse transcriptase. − RT, without reverse transcriptase. C, genomic DNA positive PCR control.
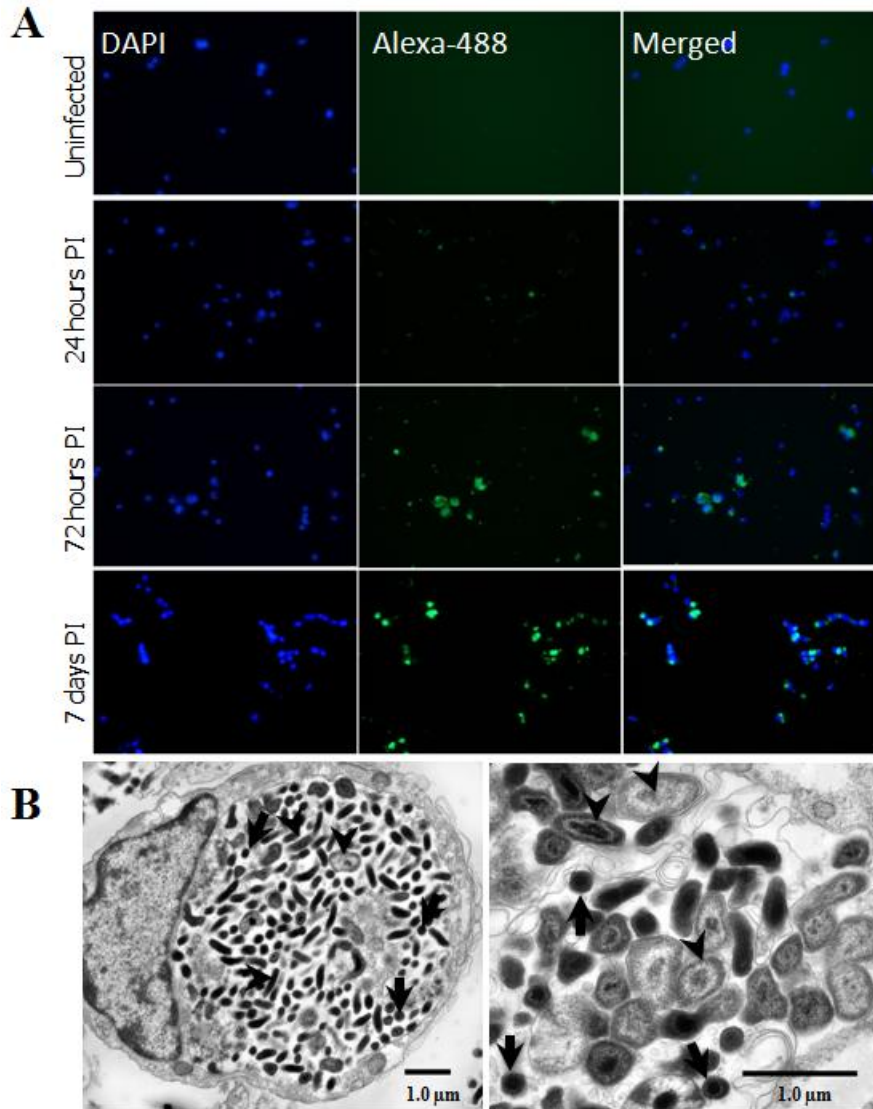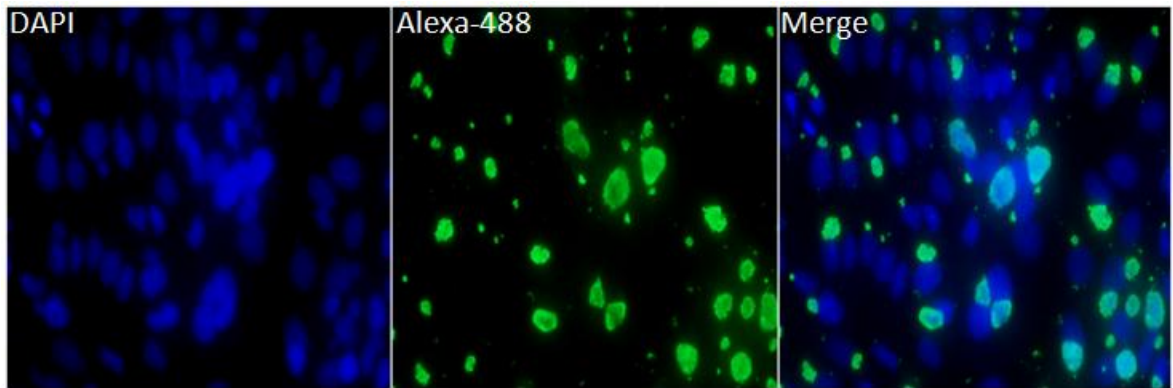
Fig. 5.4: IFA of IDE8 derived *Coxiella burnetii* NMII infecting Vero cells. Left panel, DAPI stained cells. Middle panel, Alexa-488 labeling of *C. burnetii*. Right panel, merge of left and middle panels. Infected cells were fixed at 72 hours PI.

CHAPTER VI

SUMMARY REVIEW OF: ANALYSIS OF *COXIELLA BURNETII*

MEDIATED MODULATION OF HOST CELLS

DURING INFECTION

Coxiella burnetii is a pleomorphic obligate intracellular bacterium that has two distinct stages in its life cycle. The metabolically active/replicative form is called the large cell variant (LCV) whereas the environmentally stable form is termed the small cell variant (SCV). Goats, sheep, and cattle are the main reservoirs of *C. burnetii*'s while ticks, birds, and wild and domestic mammals may also act as reservoirs in the environment. Interestingly, ticks are thought to play a crucial role in the transfer of *C. burnetii* between wild and domestic animals. Transmission to humans and animals primarily occurs due to the ability of SCVs to remain viable for prolonged periods in soil, water and on objects contaminated with tick feces. Chronically infected animals shed bacteria in milk and urine [82, 308-309]. Although overt disease occurs infrequently in these animals, *C. burnetii* has a tropism for birthing (placenta, amniotic fluids) tissues and infection of these tissues can result in abortion [87, 310]. Birthing tissues have been shown to harbor up to 10^9 organisms per gram [80], which can be aerosolized, exposing other animals and/or humans. Infection typically occurs via inhalation of the aerosolized bacteria and usually causes a self-limiting flu-like illness. However, acute Q fever symptoms can range in severity from asymptomatic to debilitating [4]. Chronic Q fever usually manifests as endocarditis or hepatitis.

During the course of an infection, *C. burnetii* invades a host cell, is trafficked to a vacuole resembling a mature phagosome, replicates within this environment avoiding cellular defenses, lyses the cell, encounters a new host and begins the process again. Protein synthesis by *C. burnetii* is required in the process of phagolysomal establishment and maintenance of a spacious parasitophorous vacuole (SPV) during bacterial growth. Interruption of bacterial protein synthesis results in SPV collapse, and eventual death of

the bacterium. The asymptomatic nature of many *C. burnetii* exposures with subsequent

chronic forms of disease highlight the immunomodulatory properties of this organism.

These conditions demonstrate that *C. burnetii* actively manipulates host cell activites and

evades host cell immune responses for its survival and growth. The mechanisms that *C.*

burnetii employs to interact with its host cell are a mystery yet represent crucial systems

for subverting host cell defenses, acquiring specific host-derived molecules, and

maintaining the PV until cell lysis occurs. In an effort to identify host-cell pathways and

processes that are specifically manipulated by *C. burnetii* during the course of infection,

we have characterized the global expression of host cell mRNA following infection with

C. burnetii Nine Mile Phase II strain and discovered host genes specifically modulated by

de novo bacterial protein synthesis.   Additionally, we have also identified a major

eukaryotic immune response signaling pathway regulated by *C. burnetii* during infection.

It was our hypothesis that the expression of host cell genes would be changed by

infection with *C. burnetii* NMII and that a subset of these genes would be in response to

bacterial derived proteins. Using microarray analysis, we compared RNA from THP-1

cells; (*i*) uninfected versus *C. burnetii* NMII infected and (*ii*) uninfected versus *C.*

burnetii NMII infected cells transiently inhibited with 10μg/ml of chloramphenicol.

RNA from mid log (72hpi), infection was used to define expression differences between

the treated and untreated array results. Selected targets were later confirmed by real time

RT-PCR. Through our microarray studies we have generated two separate global mRNA

expression profiles. Analysis of *C. burnetii* infected (-CAM) and uninfected (-CAM)

THP-1 cells revealed a gene summary list of 2557 genes which changed >0 fold. In this

analysis, the mRNA expression of 784 genes changed by at least 2 fold (significant

change). On the other hand, the host transcriptional expression of *C. burnetii* infected (+CAM) and uninfected (+CAM) THP-1 cells revealed a gene summary list of 2584 genes that changed >0 fold. Examination of this data set generated a subset of 901 genes which changed significantly (>2 fold change). Examination of differentially expressed genes in individual microarray data sets to detect the prominent host cell functions affected indicate involvement of the host cell immune response, cell migration, regulation of programmed cell death, intracellular signaling cascades, regulation of cell proliferation, and cytoskeletal organization.

A cross comparison of –CAM and +CAM mRNA data sets (described in Chapter 3) identified 36 host cell genes with significant (≥ 2 fold) gene expression differences across the data sets. Subsequent bio-informatic analysis used to categorize possible biological functions of these 36 genes showed that *de novo C. burnetii* protein synthesis regulates the immune response, cellular movement, cellular signaling, cellular proliferation, cell death, lipid metabolism, molecular transport, as well as vesicle trafficking and cytoskeletal organization of the host cell. Prominent genes that are suppressed during *C. burnetii* infection relative to transiently inhibited infections include IL8, CCL2, CXCL1, SPP1 (cytokines), BCL3, CTSB and CTSL1 (apoptosis), MTSS1, SMTN and PLEKHO1 (cytoskeleton organization), APOE, PLIN2 and FABP4 (lipid metabolism), and RAB20, SOD2, PSMA8, MSC, ZFP36L1, and RORA (Miscellaneous). Notable genes induced during *C. burnetii* infection include ITK, DUSP9 & SKP2 (intracellular signaling), SOX11, HELLS and PGR (cell growth and proliferation) SLC22A6, CDH2, PSD4, ZNF573, CHMP5 and MRPL44 (Miscellaneous) and ANLN (cytoskeleton organization). These findings indicate that *C. burnetii* proteins play a

major role in modulation of host cell functions and pathways during infection. Of particular interest was a subset of immune signaling pro-inflammatory cytokine genes whose expression is lower in *C. burnetii* infections of THP-1 cells relative to parallel infections where bacterial protein synthesis was inhibited [271]. Cytokine genes are typically regulated through the NF-κB signaling pathway. Therefore, we analyzed whether *C. burnetii* NMII infection of THP-1 cells induces NF-κB activation.

Analysis of NF-κB activation was performed on (*i*) uninfected and *C. burnetii* NMII infected and (*ii*) uninfected and *C. burnetii* NMII infected cells transiently treated with 10μg/ml of chloramphenicol. Results of western blots revealed that p65 phosphorylation was induced ~10-fold in *C. burnetii* infected THP-1 cells (72hpi) compared to uninfected cells. However, addition of CAM caused a further increase in phosphorylation levels of p65 to ~20- fold; suggesting that *C. burnetii* infection distinctly induces NF-κB activation yet the levels of this activation is modulated by bacterial protein synthesis. Subsequently, we examined NF-κB activation in *C. burnetii* infected cells over the course of infection. It was our hypothesis that activation of host cell NF-κB will vary with the stage of infection (early, mid, or late) and respond directly to bacterial protein synthesis. Western blot analysis demonstrates that induction of NF-κB activation in human THP-1 cells infected with *C. burnetii* is temporal in nature and levels of induction are modulated by bacterial proteins. Relative NF-κB activation levels in *C. burnetii* infected cells were higher during mid infection (48-96 hpi) compared to early (24 hpi) and late (120-144 hpi) infection. Moreover, *de novo C. burnetii* protein synthesis was observed to modulate the levels of p65 phosphorylation in *C. burnetii* infected THP-1 cells. Analysis of the specific NF-κB pathway(s) targeted by *C. burnetii*

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during infection revealed that the non-canonical pathway remains inactivated during infection both in the absence and presence of CAM. NF-κB p100/p52 expression levels stayed relatively constant throughout the *C. burnetii* life cycle in THP-1 cells indicating that either the canonical or the atypical NF-κB signaling pathway is activated during *C. burnetii* infection.

Together these findings illustrate that *C. burnetii* proteins induce specific gene expression changes in their host cell during infection and that this molecular control extends to modulation of the crucial NF-κB signaling pathway. Figure 6.1 is a proposed model for the changes observed in the transcript levels of host cell genes during *C. burnetii* infection. We propose that *C. burnetii* proteins modulate NF-κB activation, subsequently changing the transcript levels of genes (cytokines, apoptosis regulation, cell proliferation, and cytoskeletal modulation) in order to support bacterial survival and growth in the host. Further studies investigating the specific pathway(s) and the molecular targets within each pathway is necessary to provide insight into the molecular mechanisms involved in *C. burnetii* mediated modulation of NF-κB activation during infection. Such studies will expand our knowledge of the molecular pathogen-host interactions of this unusual bacterium.

Besides analyzing *Coxiellae*-monocyte cellular interactions, we have also investigated *C. burnetii*'s ability to invade and replicate in a cultured tick cell line. This study was performed in an effort to develop a *Coxiellae*-tick host cell model for comparative cellular and molecular interaction studies between tick and mammalian cells. We demonstrated that *C. burnetii* readily infects *Ixodes scapularis*–derived IDE8 cells, followed by a prolonged lag phase prior to the onset of replication. However, after

replication is initiated, the doubling time of *C. burnetii* is ~10h, which is similar to that observed in mammalian cells. In addition, RT-PCR demonstrated that *C. burnetii* T4BSS genes (*icmW, icmV*, and *dotH*) were expressed during infection of IDE8 cells. Finally, indirect immunofluorescence assays demonstrated that the *C. burnetii* propagated within IDE8 cells was infectious for mammalian (Vero) cells. These studies demonstrate the utility of cultured tick cell lines as a model to investigate *C. burnetii's* molecular interactions with its arthropod vectors.
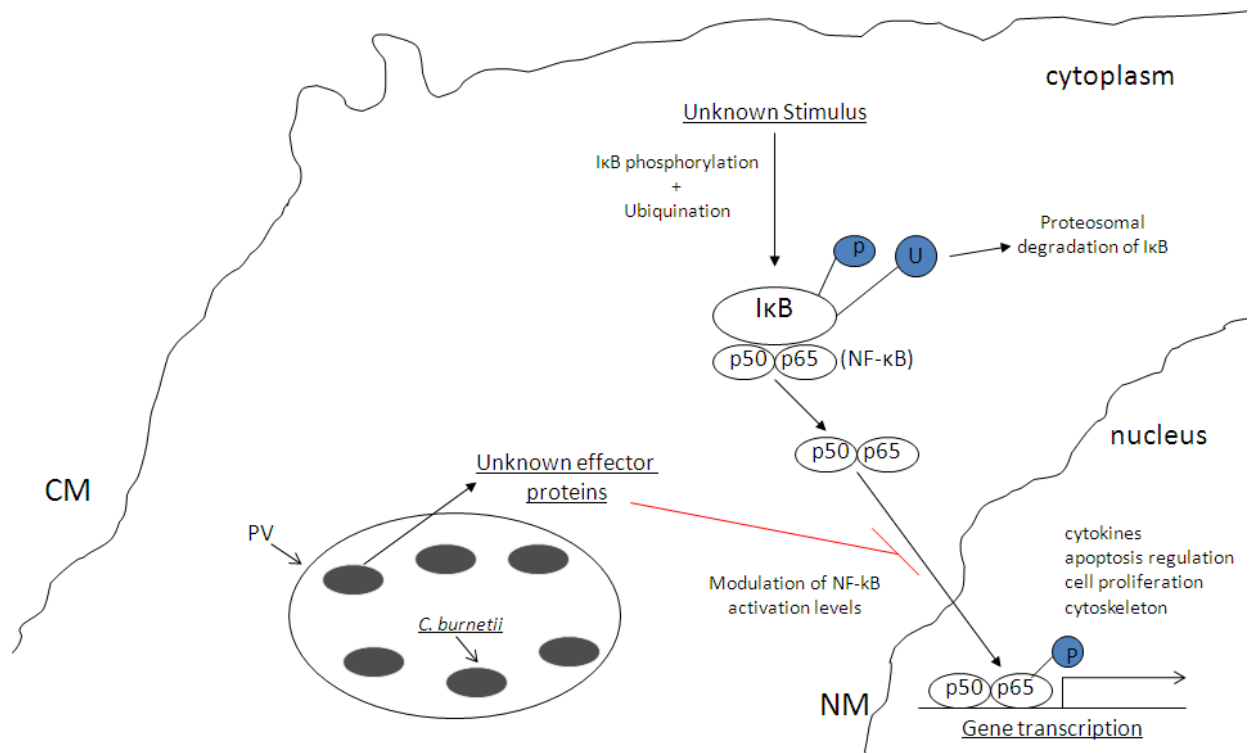
Figure 6.1: Mechanism showing *C. burnetii* mediated modulation of host cell transcripts during infection. Unknown stimulus induces the phosphorylation (P), subsequent ubiquitination (U) and proteosomal degradation of IκBs. Associated p50/p65 NF-κB dimers are released and translocate into the nucleus to bind and express host genes. *C. burnetii* proteins modulate host cell NF-κB activation to regulate host cell gene transcription. CM = Cytoplasmic Membrane, PV = Parasitophorous Vacuole, and NM = Nuclear Membrane.

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: ANALYSIS OF *COXIELLA BURNETII* MEDIATED MODULATION OF HOST
CELLS DURING INFECTION

Major Field: Microbiology / Cell and Molecular Biology

Biographical:

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Completed the requirements for the Doctor of Philosophy in Microbiology and
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Name: Saugata Mahapatra   Date of Degree: July, 2011

Institution: Oklahoma State University   Location: Stillwater, Oklahoma

Title of Study: ANALYSIS OF *COXIELLA BURNETII* MEDIATED MODULATION OF
HOST CELLS DURING INFECTION

Pages in Study: 137   Candidate for the Degree of Doctor of Philosophy

Major Field: Microbiology / Cell and Molecular Biology

Scope and Method of Study: *Scope* - To determine *Coxiella burnetii* mediated
modulation of host cell during infection. *Methods* for microbiological and
molecular analyses include: eukaryotic and prokaryotic cell culture, Ribonucleic
acid (RNA) isolation, Microarray, Real time quantitative polymerase Chain
Reaction (RT-qPCR), reverse transcriptase PCR, indirect immunofluorescent
antibody (IFA), immune-electron microscopy, Western blotting, etc.

Findings and Conclusions: Collectively, these data illustrate that *C. burnetii* proteins are
actively targeting and regulating the expression of specific host cell genes and
pathways. Thirty-six specific host cells genes modulated by *C. burnetii* proteins
were discovered. These findings led to the investigation of host NF-κB activation
in *C. burnetii* infected cells. We determined that over the time course of infection
C. burnetii modulates the host cell NF-κB activation, which might aid the
bacterium to preserve the host cell from death and evade any immune response
surmounted by the host. Additionally we have discovered that *C. burnetii* can
effectively infect and grow in *Ixodes scapularis*-derived IDE8 cultured tick cells.
Bacteria were able to invade and replicate, express T4SS genes associated with
virulence, and retain their ability to re-infect mammalian cells.

ADVISER'S APPROVAL: Edward I. Shaw, Ph.D.