AN INHALATION MODEL OF ACUTE Q FEVER IN GUINEA PIGS

A Dissertation

by

KASI ELIZABETH RUSSELL-LODRIGUE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Veterinary Microbiology

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Approved by:

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ABSTRACT

An Inhalation Model of Acute Q Fever in Guinea Pigs. (December 2006)

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Co-Chairs of Advisory Committee: Dr. James E. Samuel
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Coxiella burnetii is an intracellular pathogen that can cause both acute and chronic disease (Q fever) in humans and infects many animals with varying clinical illness and persistence. A guinea pig aerosol-challenge model of acute Q fever was developed using infection with *C. burnetii* across a 5-log range of challenge doses. Clinical signs included fever, weight loss, respiratory difficulty, and death, with degree and duration of response corresponding to dose of organism delivered. Histopathologic evaluation revealed coalescing panleukocytic bronchointerstitial pneumonia 7 days after a high-dose challenge, resolving to multifocal lymphohistiocytic interstitial pneumonia by 28 days. Clinical and pathologic changes noted in these guinea pigs were comparable to those seen in human acute Q fever, making this an accurate and valuable animal model. This model was used to compare the relative virulence of eight isolates from four different genotypic groups: I (RSA493, RSA334, and RSA270), IV (Q177 and Q173), V (Q212 and Q217), and VI (5J108-111). Guinea pigs infected with group I acute-disease-associated isolates had severe respiratory disease, while no to moderate clinical illness was observed in animals given group IV or V chronic-disease-associated isolates. 5J108-111 appeared avirulent. These data suggest that *C. burnetii* isolates have a range of disease potentials and support a distinction in strain virulence between established

genotypic groups, though isolates within the same genomic group cause similar pathologic responses. Heterologous protection was confirmed by cross vaccination and challenge with RSA493 and Q217. A marked non-specific suppression of lymphoproliferation was noted at 14 and 28 days post infection with RSA493; similar suppression was seen after infection with Q173 and Q212 but not 5J108-111. Pro-inflammatory cytokines IFN-γ and TNF-α were produced during early *C. burnetii* infection, at which time anti-inflammatory cytokines TGF-β and IL-10 were repressed. A vaccine made from phase I *C. burnetii* was found to be completely protective against lethal infection in the guinea pig model, while vaccination with killed phase II organisms conferred only partial protection, preventing death and reducing but not precluding fever and respiratory illness. Protective vaccination significantly stimulated cell-mediated immunity and elicited increases in IFN-γ, TNF-α, and IL-12p40 mRNA levels.

DEDICATION

To Mom and Granny, for everything

and

To KJ, for everything else

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I. INTRODUCTION

Q ("Query") fever is a zoonotic disease of worldwide significance caused by the obligate intracellular bacterium *Coxiella burnetii*. The first published account of the disease was in Australia in 1937, and it was originally suspected to be caused by a virus (34). The following year, researchers in the United States reported the isolation of a rickettsial agent from ticks that they called "Nine Mile agent," now identified as *C. burnetii* (30, 35). The organism commonly infects sheep, goats, and cattle, and human infection arises primarily from inhalation of contaminated materials from these animals (6). *C. burnetii* can withstand desiccation and remain infectious in contaminated soils for years (163) and has a high degree of resistance to physical and chemical agents (8, 119, 143). Due to the highly infectious nature of *C. burnetii* and its hardiness in adverse environmental conditions, the organism has been included in the list of weapons of mass destruction likely to be used in bioterrorism and biological warfare and is considered a Category B agent by the Centers for Disease Control and Prevention (39, 85). The weaponization and mass-production of *C. burnetii* has already been achieved (133, 151), accentuating the need for a safe, effective vaccine to protect populations at risk subsequent to a deliberate or natural outbreak.

This dissertation follows the style of Infection and Immunity.

THE ORGANISM

C. burnetii is the only member of the genus *Coxiella*. Phylogenetic investigations indicate that *C. burnetii* is most closely related to *Wolbachia, Francisella,* and *Legionella*, as evidenced by 16S RNA sequencing (173) and multiple individual gene sequences (114). The genome of *C. burnetii*, Nine Mile isolate, has recently been sequenced by the Institute for Genomic Research. The chromosome is 1.99 Mb and circular in nature with ~2100 open reading frames (ORF). Additional genetic information is present in the form of circular plasmid DNA that ranges in size among isolates from 32 to 56 kb; plasmidless isolates contain plasmid-related sequences integrated into their chromosomes (145).

C. burnetii isolates have been obtained worldwide from natural acute and chronic Q fever infections in humans and other animals. Studies comparing the plasmid profiles (140), chromosomal RFLPs (55), and LPS banding patterns (47) established a distinction between acute isolates and chronic isolates, and six major strains were described based on these biochemical markers (91). Genomic groups I-III have a QpH1 plasmid and were historically associated with isolates found in animals and acute human Q fever; groups IV and V, containing a QpRS plasmid or no plasmid, respectively, were associated with Q fever endocarditis or disease in livestock; and group VI isolates with the QpDG plasmid from feral rodents in Utah had undefined pathogenicity (107). A recent study using a comprehensive microarray- based whole genome comparison confirmed the relatedness of RFLP-grouped isolates and added two more genomic groups, VII and VIII (12). Unique sequence differences between genomic groups could

influence the clinical expression of Q fever (140). However, predisposing host factors may be as important as strain variety in the development of acute or chronic illness (82, 126, 188).

C. burnetii has evolved the ability to survive in harsh environments that are lethal to most bacterial species. It has developed specialized forms for propagation, enduring intracellular stresses, and surviving and persisting in an extracellular environment. These forms have been studied and described since the organism's discovery in the 1930's (30). Numerous models of the developmental cycle of *C. burnetii* have been proposed over the years, many describing it as similar to the life cycle model of *Chlamydia*. The most recent new developmental life cycle model (52, 139) was based on evidence of differential protein expression between separable forms designated large cell variants (LCV) and small cell variants (SCV). LCV and SCV may represent a novel adaptation for survival in both the phagolysosome and the extracellular environment (146). Both SCVs and LCVs have a Gram-negative cell wall typical of eubacteria with two layers separated by a periplasmic space. In SCVs, the periplasmic space is densely filled with proteins and peptidoglycan, which may enhance the resistance of this variant to environmental conditions. LCVs and SCVs, separated by a density gradient system, have both been demonstrated to be infectious (175).

Antigenic variations seen in *C. burnetii* are similar to those recognized in enterobacteria like *Escherichia coli*. Phase variation is the only well characterized phenotypic difference that relates to virulence in *C. burnetii*(160); phase variation and virulence are primarily related to mutational variation in the lipopolysaccharide (LPS)

(47-49). Virulent phase I *C. burnetii* possesses a smooth LPS layer; deletions of the O-side chain and certain sugars result in the rough LPS of avirulent phase II(142). Recent microarray-based whole genome comparison of Nine Mile phase I to variants with attenuated virulence showed that the only difference between the virulent and avirulent forms lie in the deletion of LPS biosynthesis genes(12), as has been previously suggested(59). Virulence is clearly defined in phase I, the highly infectious phase isolated in natural infections. Strains convert to phase II after multiple serial passages in cell or embryonated egg cultures and require a large number of organisms for infection to occur (40, 125). One study suggests that this relative avirulence of phase II organisms may be due to preferential complement receptor uptake targeting phase II for killing by macrophages/monocytes (111). The smooth LPS expressed by phase I cells may play an important role in protecting the microorganism from the host's microbicidal activities (178), thus allowing the infection to progress. Genetically isolated groups may also differ in phase I LPS (47). LPS differences are known to be responsible for many of the virulence effects of other Gram-negative bacteria; whether a variation in the phase I LPS of *C. burnetii* isolates is linked to virulence is not yet known. *C. burnetii* LPS is less pyrogenic than that of most Gram-negative bacteria (72) and may therefore only have a minor role in the fever response to infection. The combination of a variety of factors expressed by phase I bacteria likely govern the ability of *C. burnetii* to infect cells and maintain continuous growth within the phagolysosome. The recent study by Beare, et al, comparing all open reading frames (ORF) of Nine Mile phase I to several other isolates showed that a majority of the ORFs deleted from Nine Mile in the other isolates were

either hypothetical or nonfunctional, but some were associated with assorted cellular functions(12).

C. burnetii invades the host cell by a microfilament-dependent, parasite directed, endocytic process (9). Once inside the cell, the organism takes up residence in a parasitophorous vacuole with characteristics of a secondary lysosome and may be the only intracellular bacteria to replicate in this acidified space. *C. burnetii* enter and replicate in a variety of epithelial, fibroblast, and macrophage-like cell lines (10). *In vivo*, the initial target in aerosol infection is the alveolar macrophage, after which the organism is disseminated to replicate in a multitude of tissues. Despite a relatively slow replication rate (12-48 hour doubling time), the organism reaches high numbers within the parasitophorous vacuole and survives many toxic host factors normally considered to be bactericidal, such as acid hydrolase, oxygen and nitrogen radicals, and defensins (10).

The nature of the intracellular killing mechanisms that *C. burnetii* is exposed to and mechanisms to evade or overcome these host factors are not well understood. Two early studies demonstrated that interferon gamma (IFN-γ) could induce both guinea pig monocytes and L929 fibroblasts to inhibit the replication of *C. burnetii*, though growth was not hindered by type I interferons (56, 167). Another study evaluated the potential killing mechanisms of IFN-γ-stimulated monocytes to control *C. burnetii* replication using the THP-1 monocyte cell line(32) where THP-1 cells infected with *C. burnetii* for 24 hours and treated with IFN-γ expressed a high level of TNF-α; this cytokine appeared to drive infected cells into an apoptotic death. Studies by Howe, et al. (62), and Brennan, et al. (16), demonstrate that nitric oxide in L929 fibroblasts and nitric oxide plus oxygen

radicals, respectively, participate in control of *C. burnetii* replication by IFN-γ-stimulated cells. Zamboni (190) and Brennan (16) obtained similar results in mouse peritoneal macrophages and also showed that the effects of IFN-γ are not exclusively reliant on nitric oxide production since IFN-γ also controlled the infection in macrophages obtained from inducible nitric oxide synthase (iNOS) knockout mice. This may be due, in part, to the action of indoleamine 2,3-dioxygenase(136).

THE DISEASE

Q fever is considered primarily an occupational hazard in people who frequently have contact with domestic livestock such as sheep, goats, and cattle (107), though the disease may be contracted from pets including dogs and cats (20, 101, 124). *C. burnetii* has also been documented in several other mammals, birds(156, 165), reptiles(84, 157, 186), and arthropods(14, 30, 153). *C. burnetii* has a high affinity for the placenta and is routinely shed in birthing fluids and tissues, milk, urine, and feces. Humans are most often infected through inhalation of the organism in fine-particle aerosols, with as few as 10 organisms leading to disease (13). Transmission may also occur through ingestion of the organism from contaminated, unpasteurized, dairy products (63, 86). Due to the bacteria's ability to persist in the environment, even inhalation of contaminated dust particles can lead to infection(31, 187). Individuals at the highest risk of contracting Q fever include farmers, abattoir workers, veterinarians, and laboratory personnel performing *C. burnetii* cultures or working with infected animals. In the past two

decades, several outbreaks of infection in medical research facilities working with pregnant ewes have been described(113, 147, 152).

Q fever can manifest as either an acute or chronic illness. Humans developing acute disease often present with a flu-like illness with hallmark cyclic fever (104-105°F) and severe periorbital headache(129); other symptoms may include malaise, myalgia, fatigue, non-productive cough, nausea/vomiting, diarrhea, and weight loss. Most cases of Q fever are not recognized or diagnosed since acute illness often falls within the group of "fever of unknown origin" syndromes (37), and as many as 50% of exposed persons may develop an antibody response without showing signs of clinical illness (110). Common complications include pneumonia and hepatitis, but acute disease is almost always self-limiting. Q fever pneumonia is characterized by gross consolidation of the lungs and an interstitial pneumonia with bronchial and alveolar exudates and inflammatory infiltrates mainly consisting of lymphocytes and macrophages (98, 100, 118). Liver biopsy samples from human acute Q fever cases primarily exhibit granulomatous hepatitis with characteristic "doughnut" granulomas, a central vacuole surrounded by a fibrin ring. Though this type of granuloma is often found in Q fever cases, it is not specific to this disease, also occurring in cases of infectious mononucleosis and Hodgkin's disease, among others (99). Portal triaditis, Kupffer cell hyperplasia, and moderate fatty change have also been described in association with acute Q fever hepatitis (107). Acute illness is most successfully treated with tetracyclines, though various other antibiotics have also shown some efficacy (127).

Chronic infection is contracted less frequently than acute disease but bears a poorer prognosis. Chronic Q fever most often presents as endocarditis and/or hepatitis but has also has been diagnosed in cases of osteomyelitis (117, 130) Recognition of these infections is increasing worldwide, though Q fever is still considered a rare cause of endocarditis overall. Endocarditis caused by *C. burnetii* has an atypical presentation: bacterial vegetation with bacteria embedded in necrotic inflammatory debris and fibrin does not occur (as would be expected with the typical valvular endocarditis), and blood cultures are negative (82). It has been suggested that acute infection with *C. burnetii* is a predisposing factor for long-term vascular inflammation, and latent vascular infection may occur similar to that noted for *Chlamydia pneumoniae* and other infectious agents (80, 138).

The doughnut granulomas typical of acute Q fever are not found in chronic Q fever hepatitis, which primarily presents as lymphocytic infiltration along portal tracts, nonspecific granulomas, and focal necrosis (37, 174). A relationship has also been discovered between Q fever and a chronic fatigue-like syndrome (93, 96, 122, 177). Chronic infections appear to be associated with a suppression of the cell-mediated immune system (126), and patients with HIV are known to be at particular risk (131). In these immunocompromised individuals, antigen-specific lymphoproliferation and IFN-γ synthesis are down regulated (65, 77, 78), and tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are up regulated in patients with endocarditis (26). The antigen-specific lymphocyte unresponsiveness is mediated in part by adherent suppressor cells via prostaglandin E_2 (PGE_2) production (77). Chronic Q fever has been linked to

overproduction of interleukin-10 (IL-10) and deficient killing of *C. burnetii* by monocytes(42). Elevated IL-10 expression is associated with a poor disease outcome and has been specifically linked with the incidence of relapses (25). Unlike acute infections, chronic infections do not respond well to antibiotic therapy (30, 35). Combination treatment regimens such as doxycycline and chloroquine taken for extended periods are most successful (105, 106).

ANIMAL MODELS

The majority of *C. burnetii* studies utilize mice or guinea pigs as experimental models, though sheep (18) and non-human primates (21, 46) have also been used. Mice are most commonly used due to the many genetic and immunologic tools available for this species. Acute infection in various mouse strains is defined as lethality (144) or splenomegaly (38), whereas the development of fever is used as the indicator of disease in guinea pigs (51). Mice are essentially a clearance model, relying on a mouse's ability to control infection after uptake of the organism. Guinea pigs can be used as a model of protection against clinical disease and are thus more relevant for testing vaccines for human use. Mice have generally been inoculated intraperitoneally (IP), and as with many diseases, the strain of mouse is significant and determines the course of infection with *C. burnetii*, with responses ranging from very low morbidity in less susceptible mice (e.g., C57BL/6J mice) to severe illness and death in the most susceptible strains (e.g., A/J mice) (144). Even susceptible mice must be inoculated with large quantities of *C. burnetii* ($>10^8$ organisms) to develop overt clinical signs of disease. On the other

hand, guinea pigs inoculated IP with as few as ten virulent organisms develop fever within five days (116), and bacteria can be isolated from a variety of tissues, including the spleen, for several months post-infection. A majority of the research done with guinea pigs and *C. burnetii* has involved infection via IP inoculation; however, the clinical signs developed in this model do not adequately mirror the respiratory component seen in natural human infections. Both infection route and inoculum size have been shown to determine the clinical manifestations of disease (83), with a higher incidence of pneumonia, a common complication of Q fever in humans (68), seen in guinea pigs infected via aerosol and more severe signs of hepatitis, which is associated with ingestion of *C. burnetii* contaminated dairy products in humans(164), in IP-infected animals. A comparison of the relative virulence of isolates from acute genomic group I Nine Mile phase I and chronic genomic group IV Priscilla phase I infections showed that $>10^4$ inclusion forming units (IFU) of chronic isolate were required for demonstrable fever in guinea pigs versus the 10 IFU needed with acute isolates (116). Guinea pigs have also been used to study the endocarditis aspect of *C. burnetii* infection by inoculation with *C. burnetii* Nine Mile phase I after electrocoagulation of native aortic valves (82).

Previous studies exposing guinea pigs to *C. burnetii* via aerosol have used only single, moderate to high doses of the organism for disease induction. The methods used to determine the amount of organism delivered in a majority of these studies did not quantify *C. burnetii*, instead relying on indirect assessments of bacterial burden, such as egg and mouse median infectious doses (71, 73). Other common assays used to

determine the amount of organism delivered by IP and aerosol infection include direct particle count, inclusion forming units (IFU), and plaque forming units (PFU). Guinea pigs infected with *C. burnetii* intranasally develop a higher incidence of pneumonia; those infected IP develop more severe signs of hepatitis (83), a significant finding since the route of infection is also known to determine the clinical manifestations of acute Q fever in humans (102). It is unknown at this time what effect low doses of organism delivered by aerosol would have on the development of respiratory and/or hepatic changes in the guinea pig. This information is of vital importance in the development of an adequate inhalation model of Q fever for multiple reasons, including inference of human response to similar doses and establishment of baseline data for vaccine and other studies.

VACCINE STUDIES

A lot of the work with animal models of *C. burnetii* infection has been to further the development of a safe, effective vaccine for use in humans. Formalin-killed bacteria have been used historically to produce a protective immune response and are known to be highly immunogenic and protective in both humans and animals (1, 179). Unfortunately, this vaccine can lead to the development of severe necrotic lesions at the site of injection in a small fraction of patients, usually due to prior exposure to *C. burnetii*. Organic solvent extraction partially eliminated the primary antigenic components that caused the DTH-type necrotic reactions (180), and this material has been used in a range of vaccine studies showing good efficacy (183). A killed, whole-

cell vaccine has been developed and is used in Australia (Qvax; CSL Ltd., Melbourne, Australia) in conjunction with a skin test prior to vaccination to avoid potential adverse reactions (95). These vaccines are currently not available for use in most countries, including the United States. The immune response seen post-vaccination in humans has been evaluated through cell mediated immunity, known to be a factor in resistance to *C. burnetii* infection(107), and antibody response(7, 185). The killed whole cell vaccine (WCV) has been shown to elicit humoral antibodies and protect against disease in man and guinea pigs (112, 150). IgG1, IgG2 and IgA1 subclasses were found to predominate both in natural infection and post vaccination with a soluble phase I *C. burnetii* extract (22). Lymphoproliferation is noted in humans after vaccination with WCV (65, 66), and T cell immunity is known to be effective in the control of natural Q fever infection (107). Studies in the early 60's showed that a killed whole cell vaccine made from phase I and phase II organisms have differential protectivity against infection with phase I bacteria, with phase I vaccines being 100-300 times more effective (120). *In vitro* studies with peritoneal macrophages from phase I and phase II immunized guinea pigs subsequently infected with phase I or II organisms showed that only phase I organisms were destroyed by macrophages from phase I immunized animals (76). Other mouse protective immunity studies may indicate a Th1 requirement for protection, based on the cytokines detected (166, 170).

Subunit vaccine development efforts for *C. burnetii* have been moderate to date (181, 182), but are supported by limited prior studies. BALB/c/Han mice immunized twice with various amounts of a partially purified 29 kDa membrane antigen in the

context of complete Freund's adjuvant and then challenged with 10^5 EID_{50} (50% egg infectious dose) had splenic bacterial burdens decreased by ~3 logs at the highest immunization doses (88). A partially purified 67 kDa antigen in another study used in both guinea pigs and BALB/c mice was determined to be fully protective at two doses of 100μg in incomplete Freund's adjuvant (191).

The guinea pig model of aerosol infection with *C. burnetii* provides an excellent resource for a second level of evaluation of potential vaccine candidates after initial testing in the mouse model and prior to proposal for human studies. Once the guinea pig aerosol challenge model is available and adequately characterized, it can be used to evaluate multiple alternate vaccine strategies including recombinant antigens, T-cell epitopes, and DNA vaccination. This model can also be integrated into diverse projects and will be supportive for testing pathogenesis of disease, diagnostic techniques, and treatment/prevention strategies.

II. CLINICAL AND PATHOLOGIC CHANGES IN A GUINEA PIG AEROSOL-CHALLENGE MODEL OF ACUTE Q FEVER*

INTRODUCTION

Q fever is a disease of worldwide importance to both humans and other animals caused by the obligate intracellular bacterium *Coxiella burnetii*. Sheep, goats, and cattle are the primary reservoirs of the organism and can shed it in milk, urine, feces, and birth products (6). Thus, occupational exposure of persons in contact with these animals, such as abattoir workers, farmers, and veterinarians, is associated with a higher risk of contracting Q fever. *C. burnetii* has also been found in numerous other mammals, birds (156, 165), reptiles (84, 157, 186), and arthropods (14, 30, 153). Humans are primarily infected through inhalation, and as few as 10 organisms are known to cause disease (13). Ingestion of contaminated dairy products (63, 86, 164) or bites from infected ticks (30, 35, 134)may also lead to infection.

C. burnetii has a high degree of resistance to physical and chemical agents (119, 143) and can withstand desiccation and remain infectious in contaminated soils for years (163). Due to the highly infectious nature of *C. burnetii* and its hardiness in adverse environmental conditions, the organism is considered a Category B agent by the Centers for Disease Control and Prevention and has been included in the list of weapons of mass destruction likely to be used in bioterrorism and biological warfare (39, 85).

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Weaponization and mass-production of this organism has already been accomplished (133, 151), reinforcing the need for a safe, efficacious vaccine that could be used to protect populations at risk following a deliberate or natural outbreak.

Human Q fever can present either as acute or chronic infection. Acute Q fever generally presents as a flu-like illness with severe periorbital headache, high fever, malaise, myalgia, rare non-productive cough, and weight loss (98, 107). This illness can progress to Q fever pneumonia, characterized by gross lung consolidation and an interstitial pneumonia with bronchial and alveolar exudates and inflammatory infiltrates primarily consisting of lymphocytes and macrophages (98, 100, 118). Acute Q fever patients may also develop hepatitis (27), alone or in combination with the respiratory illness (99). Chronic Q fever often presents as endocarditis (41) and/or hepatitis and has been occasionally diagnosed in osteomyelitis cases (117, 130). An association has also been made between a chronic fatigue-like syndrome and Q fever (96, 122, 172, 177).

Animal models commonly used in the study of Q fever include mice, guinea pigs, non-human primates, and livestock. Mice are most often utilized due to the many genetic and immunologic tools available, and differences in strain susceptibility have been noted (144). Immunocompetent mice require a large number of organisms to develop clinical signs of illness, and splenomegaly is recognized as the primary indicator of disease (38), compared to fever as the primary indicator in guinea pigs (51), which can develop clinical illness after intraperitoneal (IP) infection with as few as 10 organisms (116). It has been shown that guinea pigs inoculated IP exhibit dose dependent fever and have more pathologic changes associated with the liver, whereas

those infected intranasally have greater involvement of the lungs (83). Mice are essentially a clearance model, relying on a mouse's ability to control infection after uptake of the organism. Guinea pigs can be used as a model of clinical disease and, therefore, would be more relevant for testing vaccines or antibiotic regimens for human use.

Guinea pigs in this study were infected with *C. burnetii* across a 5-log range of challenge doses through inhalation of small particle aerosols. These exposures resulted in a dose responsive relationship to clinical and pathologic changes. The disease produced in the guinea pig aerosol-challenge model closely mimics human acute Q fever and Q fever pneumonia. Experiments presented here fill gaps in the current knowledge concerning consequences of aerosol infection with *C. burnetii* using a physiologically relevant model, including dose response to infection, kinetics of extrapulmonary dissemination, and pathologic and histopathologic changes resulting from aerosol exposure.

MATERIALS AND METHODS

Animals. Six- to eight-week old female, outbred, Hartley guinea pigs obtained from Charles River Laboratories (Wilmington, MA) were housed in microisolator caging in a BSL-3 facility with a 12:12 light:dark cycle and were given Harlan Teklad (Madison, WI) guinea pig diet and water *ad libitum*. Guinea pigs were acclimated to the facility and assessment procedures for one week prior to infection to reduce stress-related abnormalities. A modified Karnofsky performance status scoring system was

used to determine if humane euthanasia was necessary after infection. All animal experimentation was reviewed and approved by the Texas A&M University Laboratory Animal Care Committee and was performed in an AAALAC approved facility in accordance with university and federal regulations.

Purification of *C. burnetii*. *C. burnetii* Nine Mile (RSA493), an isolate originating from a tick pool, was harvested from infected L929 mouse fibroblast cells by pooling infected cells and centrifuging at 1000 x g for 5 min. The supernatant was then centrifuged at 15000 x g for 30 min to collect the naturally released bacteria, and the resulting pellet was re-suspended in 0.25M sucrose phosphate (SP) buffer (53.9 mM Na_2HPO_4, 12.8 mM KH_2PO_4, 72.6 mM NaCL, 250 mM sucrose). Double distilled H_2O was added to the original pellet. This suspension was passaged through an 18 gauge canula, lysis was monitored by visual inspection, and equal volumes of .25M SP were added when a majority of the cells were lysed. Benzonase, a non-specific nuclease (Novagen; Madison, WI), was added to the lysed cell suspension for 5 min at room temperature. Cells were re-suspended several times with a pipet before pelleting host cells by centrifugation at 1,000 x g for 5 min. The supernatant was combined with the naturally released bacteria, and the combination was pelleted by centrifugation at 14,000 x g for 30 min. The supernatant was discarded, and the pellet was re-suspended in SP. Host cell debris was pelleted by centrifugation at 1,000 x g for 5 min, and the pellet was discarded. Bacteria in the supernatant were pelleted by centrifugation at 14,000 x g for 30 min, the pellet was re-suspended in SP, and the purity of the bacterial pellet was ascertained by examination of heat fixed cells with Gimenez stain.

Quantification of *C. burnetii* inoculum. *C. burnetii* Nine Mile (RSA493) was quantified using three methods. Optical density (OD) was used to determine the number of particles in the stock solution as previously described (121). Particle count was performed using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes; Eugene, OR) according to the manufacturer's instructions. Primers amplifying the *com-1* gene were used to enumerate *C. burnetii* genome copies by real-time PCR as previously described (17). A dose of $2x10^6$ organisms as determined by OD corresponded to $1.7x10^6$ by particle count with 95% viability and $1.1x10^6$ genome equivalents by real-time PCR. Infectious doses described in this paper are as enumerated by OD.

Infection. Guinea pigs were exposed to phase I *C. burnetii* Nine Mile (RSA493) in PBS or PBS alone (negative control) using a chamber specially designed to deliver droplet nuclei directly to the alveolar spaces (College of Engineering Shops, University of Wisconsin, Madison, WI) (108, 176). This chamber allows the infection of multiple guinea pigs simultaneously, ensuring uniform infection within each group.

Experimental design. 1) Fourteen guinea pigs were exposed to ~$2x10^6$ *C. burnetii*; two negative control animals were sham-infected with sterile phosphate buffered saline (PBS). Clinical assessment of disease progression was performed daily for 28 days including behavior, weight, rectal temperature, thoracic auscultation, and abdominal palpation. Temperatures ≥39.5°C were defined as fever. Euthanasia using a ketamine/xylazine overdose followed by exsanguination was performed at 7, 14, and 28 days post-infection or as indicated by the Karnofsky score. Spleen and liver were

weighed at necropsy, and heart, lung (perfused), liver, spleen, and kidney were collected and fixed in formalin for evaluation by histopathology and immunohistochemistry.

2) Three guinea pigs per dose were exposed to ~$2x10^6$, $2x10^5$, $2x10^4$, $2x10^3$, $2x10^2$, or $2x10^1$ *C. burnetii* based on optical density determined bacterial number correlation; three negative control animals were sham infected with PBS. Three vaccinated guinea pigs (see below) were infected with ~$2x10^6$ *C. burnetii*. Clinical and pathologic assessments were performed as described for experiment 1.

Guinea pigs infected with $2x10^6$ *C. burnetii* from experiment 2 were combined with those from experiment 1 to develop a survival curve.

Vaccination. Guinea pigs were given subcutaneous injections of 100μg formalin-killed phase I Nine Mile *C. burnetii* in incomplete Freund's adjuvant twice, with 2-week intervals between vaccinations and between the final vaccination and infection. Blood was collected from all animals prior to infection for serological confirmation of vaccination efficacy.

Histopathology and immunohistochemistry. Tissues collected at necropsy were fixed in 10% buffered formalin for a minimum of 48 hours. Tissues were sectioned and embedded in paraffin, cut to a thickness of 5μm, adhered to slides, and stained routinely with hematoxylin and eosin. Unstained slides were prepared for immunohistochemical staining. Vectastain® ABC kit and Vector NovaRED substrate kit (Vector Laboratories; Burlingame, CA) were used with rabbit anti-Nine Mile *C. burnetii* serum generated in our laboratory for immunostaining of *C. burnetii* in tissue sections

(11); slides were counterstained with hematoxylin. All slides were evaluated in a blinded fashion.

Serology. Serum samples collected at necropsy were tested by ELISA in 96-well U-shaped polystyrene plates (Fisher Scientific; Houston, TX) coated with 50µl of 0.5µg/ml phase I Nine Mile *C. burnetii* antigen (formalin-killed whole cells) diluted in 0.1M sodium carbonate buffer (pH 9.6) per well. After 24 h of incubation at 4ºC, plates were emptied and blocked for 1 h at 37ºC in 1% BSA-PBST. Serum was heat inactivated at 56ºC for 30 min, dilutions of 1:400 to 1:102,400 were prepared in 1% BSA-PBST, and 50µl of sample and 50µl of 1% BSA-PBST were added to each well and incubated at room temperature for 2 h. Plates were washed four times with dH_2O then incubated with 100 µl of goat anti-guinea pig IgG (Bethyl Laboratories; Montgomery, TX) diluted 1:1000 for 2 h at room temperature before washing again 4 times with dH_2O. 100µl substrate (Sigma Fast ™ o-phenylenediamine dihydrochloride; Sigma-Aldrich; St. Louis, MO) was incubated in each well for 5-10 min in the dark at room temperature, and the reaction was stopped with 100µl 1M H_2SO_4 per well. Plates were read at 490nm in a Dynatech MR5000 microplate reader (Dynatech Laboratories; Chantilly, VA). Uninfected guinea pig serum was used as a negative control.

Statistical analysis. Results were expressed as the means +/- the standard error and were compared using a student's t-test. Differences were considered significant at $p<0.05$.

RESULTS

Clinical signs of infection with *C. burnetii* delivered via small particle aerosol. Guinea pigs infected with $2x10^6$ *C. burnetii* developed fever (≥39.5°C) by day 5 post-infection (p.i.). The temperature peaked at 40.8°C on day 7 p.i. and returned to normal by day 13 p.i. in surviving animals. Temperature thereafter remained within normal parameters out to 28 days. Animals infected at all lower doses showed initial fever responses on later days. A dose-dependent relationship was noted for both onset and degree of fever (Table 2.1).

All guinea pigs infected with $2x10^4$-$2x10^6$ *C. burnetii* experienced inappetance and lethargy corresponding with the onset of fever. Weight loss began as early as day 4 p.i. in $2x10^6$ infected animals, was statistically significant ($p<.01$) by day 5, and continued to death or to day 11-13 in surviving guinea pigs (Fig. 2.1). Mild to moderate clinical dehydration was apparent by day 6-7 p.i. in these animals and only resolved with the alleviation of fever. Guinea pigs given $2x10^1$-$2x10^3$ organisms had no significant weight loss or dehydration associated with disease.

Increased respiratory rate, sounds, and effort were detected starting at days 5 and 6 p.i. in the $2x10^6$ infection group. Consolidation and crackles (rales) were noted on auscultation with uniform distribution between right and left sides. Upper respiratory obstruction was minimal. Paling and faint cyanosis of the muzzle was evident. Nasal discharge, though present, was rapidly cleaned away by the guinea pigs and therefore only noted sporadically throughout the main course of infection.

A 70.6% total mortality rate (12/17 animals from experiments 1 and 2) was noted in non-vaccinated guinea pigs infected with $2x10^6$ *C. burnetii*, with spontaneous death (75%) or humane euthanasia (25%) occurring from days 7-10 p.i (Fig. 2.2); no animals died after 10 days post infection. Groups infected at all lower doses had no fatalities, and the LD_{50} was calculated to be $2x10^{5.7}$ by the Reed and Muench method (132).

Vaccination prior to infection conferred complete protection against the development of fever, respiratory abnormalities, and death. The lack of clinical signs of illness was comparable between the vaccinated, infected animals and those sham-infected with PBS (Table 2.1).

Pathologic changes in guinea pigs infected with *C. burnetii.* At 7 days p.i. guinea pigs exposed to $2x10^6$ *C. burnetii* displayed complete gross consolidation of cranial lung lobes and partial consolidation of caudal lobes at necropsy. Several multifocal, pinpoint, white foci were noted throughout the lungs in both cranial and caudal lobes. The liver was mottled and pale with a yellowish discoloration. These animals also had a noticeable lack of abdominal fat. By 14 days p.i. lung consolidation had largely resolved in surviving guinea pigs. The liver appeared as described for 7 days p.i, and there was still a lack of abdominal fat. Moderate, transient splenomegaly ($p < 0.01$) was also noted (Fig. 2.3). A calculation of relative spleen to body weight ratio also shows statistical significance ($p<.05$) at 7 days p.i., though this is likely artifact due to the rapid, severe weight loss of the animals at that time. Multifocal pinpoint to 4mm white foci were present in the lungs 28 days p.i. The liver continued to appear somewhat

mottled, but coloration returned to normal, and pinpoint to 2mm white to tan foci were present in some animals.

Histopathologic evaluation (Table 2.2, Fig.2. 4) 7 days p.i. revealed a coalescing, panleukocytic, bronchointerstitial pneumonia (Fig. 2.4, B1-B3). Lungs were hyperemic, and alveolar walls were thickened due to cellular infiltration primarily consisting of neutrophils and lymphocytes, though all inflammatory cell types were present to some extent. The amount of bronchial associated lymphoid tissue (BALT) was increased (Fig. 2.4, B1, arrows). Extensive bronchial and alveolar exudates were noted throughout the sections. Purulent bronchial exudates (Fig. 2.4, B2) were prominent, with degeneration and sloughing of the bronchial epithelial lining. Alveolar exudates (Fig. 2.4, B3) were a deep pink, indicating high protein content. By 28 days p.i. lung changes resolved to multifocal, lymphohistiocytic, interstitial pneumonia with granuloma formation (Fig. 2.4, C1-C3). Granulomas (Fig. 2.4, C2 arrows, C3) varied in size and distribution and consisted primarily of aggregates of macrophages surrounded by lymphocytes.

Severe, diffuse hepatic lipid accumulation (Fig. 2.5A) was present at 7 days p.i. Centrilobular hepatocellular degeneration and vacuolization were noted in the liver along with periportal lymphocyte infiltration and multiple small granulomas at 14 and 28 days p.i. (Fig. 2.5B). One guinea pig evaluated at 14 days p.i. was noted to have extensive focal necrosis and mineralization in a section of the liver. Disruption of normal splenic architecture (Fig. 2.5C) was most apparent at 14 days p.i., corresponding to the gross splenomegaly observed at necropsy, and granulomas were present in the spleen at both 14 and 28 days p.i. (Fig. 2.5D). Vascular thrombi were noted in several tissues 28

days p.i. Mineralization of kidney tubules was occasionally noted. Though some guinea pigs exhibited a mild lymphocytic myocarditis or pericarditis, there was no evidence of valvular endocarditis at any time post infection.

Animals infected at lower doses necropsied at 28 days p.i. displayed progressively less severe signs of pneumonia and hepatic degeneration (Table 2.3). No significant pathologic changes were noted in negative control and vaccinated guinea pigs (Fig. 2.4, A1-A3 and D1-D3).

Immunohistochemistry confirmed the presence of *C. burnetii* organisms in infected animals in the lungs, liver, and spleen (Table 2.3). Bacteria were most prominent in macrophages but were also noted in other cell types, primarily pneumocytes in the lungs.

Serologic response to infection. Infected guinea pigs in all dose groups seroconverted by the time of euthanasia, with the exception of animals necropsied at 7 days p.i. The extent of seroconversion was dose dependent (Table 2.1). No PBS control animals seroconverted.

DISCUSSION

A need exists in *Coxiella* research for an animal model that simulates both the natural route of infection and common clinical presentations associated with human acute Q fever infection. Such a physiologically relevant model would be valuable not only for testing vaccines and therapeutic agents but also in evaluating the comparative virulence of different *C. burnetii* isolates. Moos and Hackstadt showed a difference in

virulence between two isolates, Nine Mile and Priscilla, in an IP challenge guinea pig model (116), and Kazar, et al., also demonstrated a lower virulence of Priscilla compared to the Nine Mile and S isolates (71). No histologic changes were described by Stein, et al., for Q212 infected mice as compared to multiple pathologic changes in Nine Mile infected animals (154). The guinea pig aerosol-challenge model described here is able to discern differences in disease manifestation within and between the six *C. burnetii* genomic groups (manuscript in preparation). This model would also be useful for examining the kinetics of extrapulmonary distribution of *C. burnetii,* especially in whole blood and serum, in very early stages of infection for the purposes of improving diagnostic tests for use in humans following a suspected exposure or outbreak.

The dose responsive nature of infection in the guinea pig aerosol-challenge model was expected and consistent within each group. This clinical dose-response effect has been previously noted in humans (13, 101) and cynomolgus macaques (46). Guinea pigs receiving lower numbers of organisms took longer to develop a lower grade fever for a brief period of time, in some cases as little as one day. Conversely, guinea pigs receiving larger numbers of organisms developed a high fever which persisted longer. Fatalities noted in animals exposed to $2x10^6$ *C. burnetii* were likely due to severe respiratory insufficiency. Guinea pigs receiving $2x10^3$-$2x10^5$ organisms, though also experiencing respiratory difficulty and showing histologic evidence of pneumonia, were able to overcome the illness. An LD50 of $2x10^{5.7}$ *C. burnetii* was determined in this model. The aerosol-challenge mouse model described by Stein, et al., required 10^8 *C. burnetii* before any pathologic changes were noted at early time points post infection and

asserts that a large amount of the organism must be present in aerosols for the development of Q fever (154), whereas the guinea pig model displays clinical and pathologic evidence of Q fever over a wider and lower range of doses at more time-points post infection, and the consistent development of fever, easily measurable antemortem, makes the guinea pig a much more sensitive model. As opposed to reports using high doses of *C. burnetii* for infection via inhalation in guinea pigs (83), this study showed that guinea pigs exposed to as few as $2x10^1$ organisms are able to develop acute Q fever, as is the case in humans.

The histologic characteristics of the pneumonia which developed in the guinea pigs in this study corresponded to those seen in human Q fever pneumonia (98, 100, 118). The change from the panleukocytic bronchointerstitial pneumonia seen 7 days p.i. in $2x10^6$ infected animals to a lymphohistiocytic or granulomatous pneumonia by 28 days p.i. showed resolution of the pneumonia and recovery, but it is unknown at this time how long the pulmonary (and hepatic) granulomas may persist post-infection. Should these granulomas remain with viable organisms, it is possible that they could be sites of persistent or latent infection, like the placenta (130), bone marrow (97), and liver (50) in humans.

In many animals, hepatic lipidosis has been associated with periods of anorexia, similar to that seen in guinea pigs infected at higher doses in this study, leading to an excess of triglycerides in the liver, intrahepatic cholestasis, and liver failure. Guinea pigs are known to develop fatty liver as a result of reduced carbohydrate intake and mobilization of fat as an energy source, most often associated with fasting metabolic

pregnancy toxemia (141). The hepatic steatosis seen at 7 days p.i. in our guinea pigs infected via aerosol with $2x10^6$ *C. burnetii* has been previously reported in IP inoculated guinea pigs 3-4 days p.i. An increase in triglycerides, unesterified fatty acids, and cholesterol have been noted during early Q fever infection in these animals, and it is thought that the development of fatty liver is due to failure of the liver to export these lipids after their mobilization to the liver (15). An association has also been suggested between fatty liver development and changes in the plasma membrane peptide composition, which alters lipid transport (92). This steatohepatitis resolved after the main course of infection when the animals resumed eating and was no longer apparent at 14 and 28 days p.i. when centrilobular hepatocellular degeneration and vacuolization were noted. Centrilobular hepatic degeneration is a zonal change centered about the central vein (hepatic venule) and is generally caused by low oxygen tension or high concentration of enzymes associated with bioactivation of toxic compounds. Low oxygen tension, in this case, could be caused by the severe respiratory insufficiency and resulting hypoxia displayed by high-dose infected guinea pigs.

There is currently no licensed vaccine against Q fever in the United States, although a killed whole cell vaccine (QVax, CLS Ltd.) available in Australia has been shown to be highly successful in the prevention of clinical disease in humans (66) as well as in rodent (168) and non-human primate (169) models. However, this vaccine can cause severe necrotic lesions or granuloma development at the injection site in humans previously exposed to *C. burnetii* (36, 94), which requires skin testing prior to vaccination. A new, efficacious vaccine without such deleterious side effects is needed,

and appropriate animal models of human Q fever will be required to evaluate the safety and efficacy of new vaccine candidates. Guinea pigs in this study that were vaccinated with whole killed cells were completely protected from fever development and death when given a high dose challenge, again correlating with human response to vaccination and challenge (1). The guinea pig aerosol-challenge model presented here mimics both the clinical and pathologic changes seen in human acute Q fever and Q fever pneumonia cases and will provide an accurate and valuable tool for the study of the general pathogenesis of *C. burnetii* infection, vaccine assessment, and evaluation of host immune responses.

III. GENOGROUP-SPECIFIC VARIATIONS IN DISEASE POTENTIAL OF *COXIELLA BURNETII* ISOLATES IN A GUINEA PIG AEROSOL-CHALLENGE MODEL OF ACUTE Q FEVER

INTRODUCTION

Coxiella burnetii, the etiologic agent of acute and chronic Q fever, is an obligate intracellular bacterium of worldwide distribution with a diverse host range. Livestock infected with *C. burnetii* may be asymptomatic carriers or exhibit reproductive disorders and serve as the organism's primary reservoir. Humans are most often infected through inhalation of the bacterium in fine-particle aerosols, though transmission may also occur through ingestion of the organism from contaminated, unpasteurized, dairy products (63, 86). Humans can become ill from exposure to as few as 10 organisms (13) and may display signs of: 1) an acute flu-like illness with or without pneumonia and/or hepatitis (98, 99); or 2) a chronic endocarditis and/or hepatitis (129, 130).

C. burnetii isolates have been obtained from natural Q fever infections in humans and other animals. Several theories have been proposed to explain the dichotomy in development of acute or chronic Q fever. One hypothesis is that unique genome sequence differences between genomic groups correlate with the clinical expression of Q fever (140). Biochemical markers have grouped *C. burnetii* isolates from chronic disease patients separately from acute disease/arthropod/domestic animal isolates, but whether these groupings predict virulence potential and acute/chronic disease outcome has not

yet been fully resolved. Samuel, et al., were the first to separate these isolates and their resulting diseases based on plasmid patterns (140). Hackstadt used variations in LPS banding patterns to divide isolates of *C. burnetii* into 3 groups, and group distinction was noted in correlation with acute or chronic disease (47). Hendrix, Samuel, and Mallavia separated *C. burnetii* isolates into six genomic groups (55). Group I-III isolates have a QpH1 plasmid and have been isolated from ticks, acute human Q fever cases, cow's milk, and livestock abortions. Groups IV and V have a QpRS plasmid or no plasmid, respectively, and have been associated with livestock abortions and human chronic endocarditis or hepatitis. Group VI isolates were collected from wild rodents in Dugway, Utah, and are considered infectious but avirulent (158, 159). Jager, et al., used restriction fragment length polymorphism (RFLP) to differentiate 80 *C. burnetii* isolates and reproduced distinguished patterns for reference isolates in groups I, IV, V, and VI (67). More recently, multiple locus variable nucleotide tandem repeat (VNTR) analyses (MLVA) (161) have validated these groupings. A comprehensive microarray- based whole genome comparison by Beare, et al., confirmed the relatedness of RFLP-grouped isolates and added two more genomic groups, VII and VIII (12). Infrequent restriction site-PCR (IRS-PCR) of 14 livestock and tick isolates resulted in six groups; subsequent MLVA typing of 42 isolates revealed 36 genotypes (5). Glazunova, et al., used multispacer sequence typing (MST) to analyze 173 isolates, a majority of which were acquired from chronic disease patients, and identified thirty genotypes in three monophyletic groups; an association between plasmid type, some genotypes, and disease type was observed (45). These monophyletic groups supported the early RFLP groups

and placed group I, II, and III as one monophyletic group, group IV representative of the second monophyletic group, and group V representative of the third monophyletic group. It has been shown in an intraperitoneal (IP) challenge guinea pig model that while only 10^1 organisms of the acute-disease-associated group I isolate RSA493 caused fever, 10^6 chronic-disease-associated group IV isolate Q177 organisms were required for fever development (116). In opposition to the theory of genotype/pathotype correlation, Stein and Raoult evaluated 28 human isolates and found that isolates bearing the QpH1 plasmid were present in both acute and chronic Q fever patients in France, and isolates without the QpH1 plasmid were able to cause acute disease (155). QpH1 plasmid-containing isolates have also been isolated from chronic endocarditis patients (162).

Several groups have speculated that host factors are primarily responsible for the outcome of infection with *C. burnetii*. Individual differences in immune function and competence lead to varying sensitivity to infection and disease development. In this case, acute and chronic disease could be caused by the same isolate, and chronic disease would develop because of compromised resistance of the host rather than any specific property of the pathogen. It is known that HIV infection is a risk factor for the development of chronic Q fever endocarditis (19, 90). Deficiencies in the host specific cell-mediated immune response have been associated with the suppression of monocyte and macrophage activity (77), and monocytes from chronic Q fever patients are known to have defective phagosome maturation and impaired *C. burnetii* killing, regulated by IL-10 (44). There is strong evidence to support the role of increased host production of

interleukin-10 in the development of chronic Q fever endocarditis and fatigue syndrome (25, 42, 58, 122).

Route of infection may also be a determining factor in the manifestation of acute or chronic Q fever. LaScola and Marrie demonstrated that the route of infection and size of inoculum affect clinical illness and pathology associated with infection in mouse and guinea pig models (83, 102). Differences in the geographic distribution of disease have also been noted (100); in Nova Scotia the primary manifestation of acute Q fever is pneumonia (107), but in France it is hepatitis, possibly due to ingestion of raw milk (164).

The establishment of the aerosol model of *C. burnetii* infection in guinea pigs (137) provides a relevant model in which to test isolate virulence. Eight isolates from four genomic groups (Table 3.1) were evaluated for their ability to cause acute disease. We hypothesized that isolates within the same genotypic group would cause similar disease and that there would be a distinct difference in disease manifestation between isolate groups. Lesny, et al., compared the cross-immunity of whole-cell and soluble Q fever vaccines made from phase I Nine Mile, S, Priscilla, and Luga strains. They found that vaccines from Nine Mile and Priscilla afforded a higher degree of protection than S and Luga vaccines and that whole-cell vaccines were more effective than soluble vaccines (87).We also evaluated the potential protection of a vaccine made against one *C. burnetii* isolate in protecting against infection with an isolate from another group. We speculated that such a vaccine would confer heterologous protection against virulent high-dose challenge.

MATERIALS AND METHODS

Animals. Female Hartley guinea pigs (Charles River Laboratories; Wilmington, MA) weighing approximately 350-450g were housed in an approved BSL-3 facility under microisolator caging and a 12:12 light:dark cycle and were provided with Harlan Teklad guinea pig diet (Madison, WI) and water *ad libitum*. All guinea pigs used in this study were acclimated to the facility and assessment procedures during the week prior to infection to decrease stress-related abnormalities. Texas A&M University Laboratory Animal Care Committee reviewed and approved all animal experimentation, which was performed in AAALAC approved facilities in accordance with university and federal regulations.

Purification and quantification of *C. burnetii*. *C. burnetii* Nine Mile (RSA493) and S (Q217) isolates were harvested from infected L929 mouse fibroblast cells as previously described (137). *C. burnetii* African (RSA334), Ohio 314 (RSA270), MSU Goat (Priscilla) (Q177), P (Q173), G (Q212), and Dugway (5J108-111) isolates were purified from infected yolk sacs as formerly reported (54, 140).

C. burnetii was quantified by optical density (121), direct viable particle count using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes; Eugene, OR), and quantitative real-time PCR (qPCR) using primers amplifying the *com-1* gene (17) (Table 3.2).

Experimental design. A chamber specially designed to deliver droplet nuclei directly to the alveolar spaces (College of Engineering Shops, University of Wisconsin,

Madison, WI), allowing the infection of multiple guinea pigs simultaneously and ensuring uniform infection within each challenge group (108, 176), (137), was used for all infection studies.

Three guinea pigs per group were infected with low, mid, or high doses of one of the following phase I *C. burnetii* isolates (Table 3.1): Nine Mile RSA 493, African RSA334, Ohio 314 RSA270, MSU Goat (Priscilla) Q177, P Q173, G Q212, S Q217, or Dugway 5J108-111. Four negative control animals were sham-infected with sterile phosphate buffered saline (PBS). Guinea pig health was assessed by a veterinarian daily. Weight, rectal temperature, and behavioral attitude were recorded along with any abnormalities noted on thoracic auscultation and abdominal palpation. A rectal temperature ≥39.5°C was defined as fever. Guinea pigs were euthanized with a ketamine/xylazine overdose followed by exsanguination 28 days post infection (p.i.). Spleen and liver were weighed at necropsy. These and other tissues were collected and fixed in 10% buffered formalin for histopathologic evaluation. Serum was obtained from each animal for serologic testing.

In a separate experiment, three guinea pigs per group were exposed to PBS or high-dose RSA493, Q173, Q212, or 5J108-111. Daily assessment of these animals was performed as described above, and organs were weighed at necropsy 14 days p.i. to detect splenomegaly and/or hepatomegaly.

In the heterologous protection study, guinea pigs were vaccinated with 40μg of formalin-inactivated phase I *C. burnetii* Nine Mile RSA493 or S Q217 in Freund's incomplete adjuvant or adjuvant alone twice, with two week intervals between

vaccinations and infection. Animals were then infected with high doses of either Nine Mile RSA493 or S Q217. Three animals per group were separated into the following six groups: 1) non-vaccinated, RSA493 infected; 2) non-vaccinated, Q217 infected; 3) RSA493 vaccinated, RSA493 infected; 4) Q217 vaccinated, Q217 infected; 5) RSA493 vaccinated, Q217 infected; and 6) Q217 vaccinated, RSA493 infected. Guinea pigs were monitored for 14 days p.i. for development of fever and other clinical signs of illness.

Histopathology and immunohistochemistry. Formalin fixed tissue samples from the high-dose infection group of each isolate were sectioned and embedded in paraffin, cut to a thickness of 5µm, adhered to slides, and routinely stained with hematoxylin and eosin. Unstained slides were prepared for immunohistochemistry on which Vectastain® ABC kit and Vector NovaRED substrate kit (Vector Laboratories; Burlingame, CA) were used with in-house generated rabbit anti-Nine Mile *C. burnetii* for immunostaining of *C. burnetii* in tissue sections (11). Slides were counterstained with hematoxylin. All slides were evaluated in a blinded fashion.

Serology. Serum samples collected at necropsy were tested by ELISA in 96-well U-shaped polystyrene plates (Fisher Scientific; Houston, TX) coated with 50µl of 1.5µg/ml phase I Nine Mile *C. burnetii* antigen diluted in 0.1M sodium carbonate buffer (pH 9.6) per well. After 24 h of incubation at 4ºC, plates were emptied and blocked for 1 h at 37ºC in 1% BSA-PBST. Serum was heat inactivated at 56ºC for 30 min, dilutions of 1:400 to 1:102,400 were prepared in 1% BSA-PBST, and 50µl of sample and 50µl of 1% BSA-PBST were added to each well and incubated at room temperature for 2 h. Plates were washed four times with dH_2O then incubated with 100 µl of goat anti-guinea pig

IgG (Bethyl Laboratories; Montgomery, TX) diluted 1:1000 for 2 h at room temperature before washing again 4 times with dH_2O. 100µl substrate (Sigma Fast ™ o-phenylenediamine dihydrochloride; Sigma-Aldrich; St. Louis, MO) was incubated in each well for 5-10 min in the dark at room temperature, and the reaction was stopped with 100µl 1M H_2SO_4 per well. Plates were read at 490nm in a Dynatech MR5000 microplate reader (Dynatech Laboratories; Chantilly, VA). Serum from uninfected guinea pigs was used as a negative control.

Statistical analysis. Results were expressed as the means +/- the standard error and were compared using a student's t-test. Differences were considered significant at $p<0.05$.

RESULTS

Group I and V *C. burnetii* isolates cause acute Q fever and Q fever pneumonia in the guinea pig model. Guinea pigs challenged with group I and V isolates developed a significant fever in response to infection ($p<0.01$), whereas those given isolates from groups IV and VI were afebrile even at the highest challenge dose (Table 3.3, Fig. 3.1).

Fever response, weight loss, and other clinical signs displayed a dose dependent relationship in guinea pigs infected with group I *C. burnetii* isolates RSA334 and RSA270 as has been described for the reference isolate in this group, RSA493 (137). All animals receiving high-dose RSA334 or RSA270 organisms died within 7-9 days post infection, as did 2/3 receiving RSA493; lower infectious doses were not lethal. Gross

lung consolidation and overall lack of normal body fat were noted on necropsy at 7-9 days p.i. in guinea pigs infected with the highest dose of organisms. Histologically, these animals had severe panleukocytic bronchointerstitial pneumonia with bronchial and alveolar exudates. Lung tissues from the surviving RSA493-infected guinea pig and those given the mid-dose group I organisms were evaluated at 28 days p.i. for comparison to animals infected with other isolates evaluated at this time-point. Grossly, multiple small white foci were noted in right and left lung lobes; moderate multifocal lymphohistiocytic pneumonia with granuloma formation was present in lung sections.

No significant fever or other overt clinical signs were noted in guinea pigs infected with group IV isolates Q177 and Q173. Mild lymphohistiocytic pneumonia was seen histologically in animals delivered the highest dose of organism.

Group V isolate Q212- and Q217-infected guinea pigs all developed fever when delivered the highest challenge dose, and dose-dependent temperature increase and other clinical signs were again noted, with no fever development in those animals receiving the lowest dose of organism. Though auscultation confirmed respiratory compromise, none of the infections were lethal. At 28 days p.i., the lungs had a mild to moderate lymphohistiocytic interstitial pneumonia, and small granulomas were occasionally noted.

No major clinical or pathologic changes were noted in negative control animals or in guinea pigs infected with the group VI isolate, 5J108-111. Table 4 compares the severity of histopathologic changes in guinea pigs infected with high dose *C. burnetii* isolates from each group at 28 days p.i. Immunohistochemistry confirmed the presence

of *C. burnetii* organisms, primarily in macrophages, in the lungs, liver, and spleen of high-dose infected animals.

Splenomegaly in response to *C. burnetii* infection. There were no significant differences in spleen weight at 28 days p.i. within or between genomic or dose groups. Animals infected with all isolates examined at 14 days p.i. (RSA493, Q173, Q212, and 5J108-111) had significantly larger spleens than PBS control animals, and spleens from RSA493 and Q212 guinea pigs were significantly larger ($p<0.01$ and $p<0.05$, respectively) than those of Q173 and 5J108-111 animals (Fig. 3.2). Pathologic findings included multiple small granulomas in the spleens of group I-infected guinea pigs; fewer small granulomas were occasionally noted in animals infected with group IV and V isolates.

Increased incidence and severity of hepatitis in group IV infected guinea pigs. The characteristic doughnut granulomas of human acute Q fever hepatitis (99) have not been previously described in animal infection experiments and were likewise not seen in guinea pigs infected in this study. RSA 493, RSA334, and RSA270 (group I) liver sections showed a mild hepatitis and severe hepatic lipidosis at death 7 days p.i. Tissue sections from the remaining RSA493 guinea pig and those infected with the mid-dose group I organisms were evaluated for comparison with animals infected with other isolates at 28 days p.i. Few pinpoint white to tan foci were present on the surface of the liver and histologically exhibited centrilobular hepatocellular degeneration and vacuolization, periportal lymphocyte infiltration, and multiple small granulomas consisting primarily of lymphocytes with few macrophages.

Few pinpoint to 4mm tan to white foci, singly and coalescing, were observed on the liver surface of guinea pigs infected with group IV isolates Q177 and Q173 (Fig. 3.3A), and periportal lymphocytic infiltration and multiple granulomas of varying sizes were noted histologically. These granulomas were more defined and had a greater amount of histiocytic involvement than those seen in guinea pigs infected with group I isolates. Subjectively, of all animals necropsied from each isolate group, hepatic granulomas from those infected with Q173 were the greatest in size and number (Fig. 3.3B).

Few small granulomas and infiltration of lymphocytes along portal tracts were noted in the livers of group V isolate Q212- and Q217-infected guinea pigs, and the degree of hepatic change indicated that isolates from this group are more hepatovirulent than group I isolates but less than group IV isolates.

Necrotic lesions were associated especially with peripheral granulomas in guinea pigs infected with isolates from groups I, IV, and V, and were again most common in animals given group IV isolates. No hepatic granulomas were noted in guinea pigs infected with the group VI isolate 5J108-111. Liver weights did not vary significantly within or between genomic groups.

Gallstone formation associated with *C. burnetii* infection. Gallstones were present in five guinea pigs from the following groups: mid-dose RSA270, mid- and high-dose Q177, low-dose Q173, and low-dose 5J108-111. The biliary calculi were the size of sand and were black (pigment) in RSA270, Q177, and Q173; white (cholesterol) in 5J108-111; and mixed black and white in Q177.

Serologic response to infection. Infected guinea pigs in all dose groups for each isolate had seroconverted by the time of euthanasia, with the exception of high-dose infected RSA493, RSA334, and RSA270 animals necropsied at one week p.i. and low-dose 5J108-111 infected guinea pigs (Table 3.3). The degree of seroconversion was dose dependent and varied among isolates (data not shown). No PBS control animals seroconverted.

Heterologous protection of cross vaccination and challenge. Guinea pigs were given RSA493 or Q217 vaccines and cross-challenged to evaluate the potential heterologous protection against high-dose infection. Non-vaccinated guinea pigs developed a noticeable fever response by day 5 p.i., and infection was lethal in 3/3 RSA493 and 1/3 Q217 challenged animals. Guinea pigs vaccinated with either formalin-killed RSA493 or Q217 were completely protected against fever development and death when challenged with either RSA493 or Q217 (Fig. 3.4).

DISCUSSION

The ability to cause fever and respiratory illness was isolate and dose dependent in the guinea pig aerosol-challenge model, with isolates from groups I and V exhibiting disease consistent with human acute Q fever. Isolates within the same genomic group produced similar clinical illness, strongly supporting the hypothesis that genogroup-specific genetic differences in the bacterial isolates do play a role in virulence. It was shown here that isolates associated with chronic disease, Q212 and Q 217, have the ability to cause acute disease in the guinea pig model. These group V isolates have no

plasmid, though a portion of the QpRS plasmid of genogroup IV has been integrated into their genome (140). This is interesting in that the clinical illness produced by these isolates lay somewhere between what was seen with group I and group IV isolates, suggesting the genetic differences in virulence are not plasmid related, but plasmid groupings can be used to anticipate the expected disease occurrence in a given outbreak. Although *C. burnetii* isolates are considered to belong to a single species according to serologic methods and sequence analysis of 16S rRNA (155), data presented in this study emphasize that the genetic diversity among isolates correlates with disease manifestation. Our study confirmed and expanded the observations by Kazar, et. al., as to the greater virulence of Nine Mile and S isolates compared to that of Priscilla (71).

Phase variation is the only well characterized phenotypic difference that relates to virulence in *C. burnetii* (160). Although LPS may be a major virulence determinant, other components alone or in association with LPS may be responsible for differences in fever development in aerosol-challenged guinea pigs. The combination of a variety of factors expressed by phase I bacteria likely govern the ability of *C. burnetii* to infect cells and maintain continuous growth within the phagolysosome. A recent report compared all open reading frames (ORF) of Nine Mile phase I to African RSA334, Ohio 314 RSA270, P Q173, G Q212, S Q217, and Dugway, among others (12), and a majority of the ORFs deleted from Nine Mile in the other isolates were either hypothetical or nonfunctional; however, a few were associated with assorted cellular functions.

Based on the persistent presence of the antigen in humans during chronic Q fever, host factors could be an important determinant in the development of chronic

disease. Findings of this study do not preclude the importance of host factors in causing disease as only immunocompetent animals were used for challenge. Immunodeficient animals may develop acute disease from infection with group IV and VI isolates that were unable to produce clinical signs of illness in the model presented. It has been shown that humans who are immunocompromised are more prone to develop chronic disease (128), but whether host factors or the genetic properties of the pathogen play a more important role is not yet clear. It is likely that chronic Q fever results from a complex combination of patient predisposition and infection with specific *C. burnetii* isolates. In a previously reported study, severe combined immunodeficient (SCID) mice infected with *C. burnetii* Nine Mile RSA493 (genomic group I) showed severe lesions consistent with those seen in chronic Q fever (3). These findings indicate that this isolate, which is typically associated with acute disease, can induce chronic disease lesions in the absence of a normal immune response. Humans that have recovered from acute Q fever can remain latently infected, and it is possible that patients develop chronic disease because the organism continues to multiply in various organs (184). As the experimental infection of guinea pigs with various isolates in our study was only conducted out to 28 days p.i., it was a measure of the potential to cause acute disease only. Further studies must be conducted on the ability of each isolate to induce chronic disease and on disease manifestation in immunocompetent vs. immunodeficient animals.

Killed whole cell vaccines made from isolates differing in LPS banding pattern (47), plasmid type (140), and genomic group (55), specifically isolates from groups I and V, confer heterologous protection against virulent high dose challenge in accordance

with previous studies (87). This suggests that although the manifestation of disease may differ among various isolate groups, the antigenic properties of whole cell vaccines are similar enough that cross protection is possible. This could be of utmost importance in offering reliable protection in the event of an outbreak.

The differences in infectious dose noted when OD, particle count, and genome copy enumeration were compared underline the importance of using multiple quantitation methods. Some of the differences in disease manifestation seen in this study could be due to slight differences in infectious dose delivered. For instance, Q177 and Q173 both induced hepatic changes, though guinea pigs infected with Q173 appeared to induce more severe lesions than Q177, which had a lower infectious dose by OD and qPCR. The difference in infectious dose as determined by genome copy number could account for this variation. However, Q212 and Q217 both caused fever, and although guinea pigs infected with Q212 did not attain the same degree of febrile response as Q217 infected animals, quantitation by particle count and real-time PCR showed infectious doses of Q217 to be over a log lower than Q212. It could be argued that Q177 infected guinea pigs did not develop fever because fewer bacteria were present in the aerosol challenge; however the group IV isolates did not induce fever at any of the challenge doses while group I isolates induced fever even at the lowest dose. We believe that despite the occasional variation in infectious dose depending on enumeration technique, the significant differences noted among genotypic groups are valid.

Splenomegaly has long been associated with virulent *C. burnetii* infection in animal models (38), possibly due to excessive antigenic stimulation. The lack of a

statistically significant difference in spleen and liver weights among animals infected with different doses and isolates at 28 days p.i. is likely due to the time at which animals were necropsied post infection. We have previously found that splenomegaly in the aerosol-challenge guinea pig model is transient and peaks around 14 days post infection in animals given 10^6 Nine Mile *C. burnetii* (137), resolving by the 28-day time point used as the termination point for this study. An endpoint of 28 days was initially chosen since fever was used as the primary indicator of disease, and it has been determined in previous studies that lower doses of virulent organisms take longer to induce fever; it was possible that less virulent isolates could take even longer. By examining animals infected with select isolates at 14 days p.i., differences in spleen size were discovered. Splenomegaly was noted to some degree in all infected guinea pigs and was significantly greater in those infected with isolates that also induced a fever response, representing yet another difference in virulence among genogroups. It was the desire of the authors to have quantitative PCR counts of *C. burnetii* from splenic tissue to correlate to the observed splenomegaly, as has been done in the mouse model (unpublished data); however, due to as yet unresolved technical issues in the extraction process, this was not possible at this time.

The presence of gallstones may be an incidental finding due to the occasional occurrence of this condition in guinea pigs, though it is generally only noted in aged animals (103). However, as gall bladder disease in the form of acute acalculous cholecystitis has been shown to occur in humans in connection with *C. burnetii* infection (115, 135), it is possible that infection with *C. burnetii* contributed to the development of

cholelithiasis in this study. Elevated cholesterol enhances stone formation in the guinea pig gallbladder (69), and recently *C. burnetii* infection has been reported to lead to an increase in cellular cholesterol and transcriptional up-regulation of host cholesterol trafficking and biosynthesis genes (61), suggesting another possible explanation for gallstone formation in association with *C. burnetii* infection. It is interesting to note that in the author's experience, though cholelithiasis was found in 5/63 guinea pigs infected with various other *C. burnetii* isolates in this study, it has not yet been found in over 100 guinea pigs infected with Nine Mile isolate alone. Similar findings have occurred in other rodents where certain species of *Helicobacter* have been identified as cholelithogenic, whereas others have no effect on gallstone development. It has been proposed that the formation of cholesterol gallstones may be due to species-specific bacterial products or an immunologic response of the host to infection (104).

Isolates of phase I *C. burnetii* have the potential to cause a range of clinical signs, including fever, pneumonia, hepatitis, and splenomegaly. Isolates from one human chronic disease group induced mild to moderate acute disease in the guinea pig aerosol-challenge model while a separate isolate group representing several chronic disease isolates caused no acute disease. In this study, isolates within the same genomic group caused similar pathologic responses, with a distinction in strain virulence between established genomic groups, sustaining the theory that genetic differences in the bacterial isolates do affect their virulence.

IV. IMMUNOLOGY OF *COXIELLA BURNETII* INFECTION AND PROTECTIVE VACCINATION

INTRODUCTION

Q fever is a zoonotic disease with worldwide distribution caused by the obligate intracellular bacterium *Coxiella burnetii*. Infection may be subclinical, acute, or chronic. Acute illness can develop from exposure to as few as 10 organisms (13) and is most commonly characterized by a self-limiting, flu-like illness with high fever and periorbital headache that may develop into a pneumonia. Chronic disease may present as endocarditis, hepatitis, or a chronic-fatigue-like syndrome. The disparity in the development of acute or chronic Q fever may be due to unique virulence differences between genomic groups, host factors involving individual differences in immune function, or a combination of the two. Vaccination with a killed whole cell vaccine (Q-Vax, CLS Ltd.; Australia) made from virulent phase I *C. burnetii* is successful in preventing clinical disease in humans (66) as well as in rodent (168) and non-human primate (169) models; vaccines made from avirulent phase II organisms are less effective (120).

The cell-mediated immune response (CMI) is considered essential for the control of Q fever infection, although humoral immunity may accelerate the process (64). Deficiencies in the host-specific CMI have been associated with the suppression of monocyte and macrophage activity (77). Comparatively little has been reported on the host immune response to acute infection with *C. burnetii* compared to that of chronic

infection. In one study, patients with uncomplicated acute Q fever had an increased release of tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-12, and IL-10 compared to chronic endocarditis patients, with a greater increase in acute Q fever hepatitis patients than in those presenting with fever or pneumonia (58). Another report indicated an increase in TNF and IL-6 but not IL-1β in acute Q fever and Q fever endocarditis, with an increase in IL-1 receptor agonist (IL-1Ra) and TNF receptor type II in patients with the acute disease (23). It was speculated that this decrease in IL-1β and increase in IL-1Ra may contribute to decreased resistance to *C. burnetii* infection. In blood cell cultures from healthy donors stimulated with antigens of either Nine Mile or Priscilla strain *C. burnetii*, it was shown that Nine Mile exhibited less cytokine-inducing activity with respect to TNF-α, IL-6, and IL-8 than Priscilla, but had a greater inhibitory effect on IL-1β and IFN-γ secretion (81). Several reports have shown a strong correlation between increased host production of interleukin-10 in the development of chronic Q fever endocarditis and fatigue syndrome (25, 42, 58, 122) Increases in transforming growth factor-beta (TGF-β) have also been associated with Q fever endocarditis and chronic fatigue syndrome (25, 122).

Suppression of lymphoproliferation has been reported following both acute and chronic infection (75, 171) due to the production of prostaglandin E_2 (PGE_2) by stimulated monocytes (77). Vaccination with phase I. *C. burnetii* results in an increase in the blastogenic response (65, 74, 179).

The purpose of this study was to characterize the host immune responses to acute Q fever infection by systematic evaluation of cytokine and chemokine profiles in a

clinically relevant model. We evaluated the immune response of guinea pigs infected via aerosol with a sub-lethal challenge of *C. burnetii* at 7, 14, and 28 days post infection, corresponding to early, late, and convalescent acute disease. Lymphoproliferative response was assessed, and message RNA was measured for IFN-γ, TNF-α, TGF-β, RANTES, IL-1β, IL-8, IL-10, and IL-12p40 in response to infection. An effort was then made to discern differences between protective phase I and non-protective phase II vaccination strategies that were established in a mouse model (GQ Zhang, manuscript in preparation). Once the baseline responses were determined for the reference isolate, Nine Mile RSA493, we sought to discriminate between the lymphoproliferative responses of a selection of isolates previously demonstrating differing virulence and disease outcomes after aerosol challenge.

MATERIALS AND METHODS

Bacteria. *C. burnetii* Nine Mile RSA493 was harvested and purified from infected L929 mouse fibroblast cells as described (137); P Q173, G Q212, and Dugway 5J108-111 isolates were purified from infected yolk sacs as formerly reported (54, 140). *C. burnetii* was quantified by optical density (121), particle count using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes; Eugene, OR), and genome copy number using quantitative real-time PCR (qPCR) *com-1* gene primers(17).

Animals. 350-450g female Hartley guinea pigs (Charles River Laboratories; Wilmington, MA) were housed in microisolator caging with a 12:12 light:dark cycle and were provided with Harlan Teklad guinea pig diet (Madison, WI) and water *ad libitum*. All guinea pigs used in this study were acclimated to the facility and assessment

procedures during the week prior to infection to decrease stress-related abnormalities. Infected animals were housed in an approved BSL-3 facility. The University Laboratory Animal Care Committee of Texas A&M University reviewed and approved all animal experimentation, which was performed in AAALAC approved facilities in accordance with university and federal regulations.

***C. burnetii* antigen preparations.** Formalin-killed phase I (WCV-I) and phase II (WCV-II) Nine Mile *C. burnetii* were prepared for use in vaccination and *in vitro* antigenic stimulation by inactivating live organisms in a 1% formalin solution for 48 h. The product was then dialyzed against phosphate buffered saline (PBS) for 48 hours with 4 changes of PBS. Additional *C. burnetii* antigens were developed to determine the optimum antigen for use in lymphoproliferation assays. Samples were frozen in liquid nitrogen for 1 min, thawed in a 37°C water bath for 1 min, then disrupted using FastProtein Blue Matrix in a FastPrep instrument (Q-biogene, Irvine, CA) according to manufacturer's instructions, and the resulting supernatant was collected. This cycle was repeated 3 times, and the supernatants were pooled. The final pellet from this procedure was then extracted by detergent treatment. The pellet was re-suspended with 2% weight/volume N-lauroylsarcosine (Sigma, St. Louis, MO) in dH_2O and mixed for 1 h. The supernatant was collected after 5 min of centrifugation at 13,000 x g and dialyzed against dH_20 for 12 h and 3 water changes. The final product was then combined 1:1 with the soluble antigen obtained from the FastPrep homogenization.

The original phase I *C. burnetii*, FastProtein Blue extract (bbAg), detergent extract (detAg), final pellet, and formalin-killed *C. burnetii* (WCV-I) were compared by

western-blot and silver stain; 8µg per well were loaded for each protein. The gel transfer was blocked for 1h in 10% milk then incubated for 1h with 1:400 mouse infection-derived serum in 5% milk PBS-T and washed for 15 min with PBS-T. A 1 h incubation with 1:10,000 goat anti-mouse IgG HRP in 5% milk PBS-T was followed by a 45 min wash.

Experimental design. 1) Guinea pigs were infected via aerosol with 10^5 phase I *C. burnetii* Nine Mile (RSA493) in PBS using a chamber expressly designed to deliver droplet nuclei to the alveolar spaces (College of Engineering Shops, University of Wisconsin, Madison, WI) (108, 176),(137). Negative control animals were given PBS alone. Four animals per time point were euthanized at 7, 14, and 28 days post-infection. Blood, inguinal and axillary lymph nodes and spleen were collected aseptically for use in lymphoproliferation and cytokine assays. A second group of animals was challenged with a potentially lethal dose of 10^6 organisms and euthanized at 28 days post infection for evaluation of lymphoproliferative response.

2) Three guinea pigs per group were given 10^6 *C. burnetii* of Nine Mile RSA 493, P Q173, G Q212, or Dugway 5J108-111 isolates and euthanized at 14 days post infection for assessment of differential lymphoproliferative responses.

2) Guinea pigs were given subcutaneous injections of 0.2µg, 2µg, or 20µg formalin-killed phase I (WCV-I) or phase II (WCV-II) Nine Mile *C. burnetii* in incomplete Freund's adjuvant twice, with a two week interval between vaccinations and between the final vaccination and infection with $2x10^6$ phase I *C. burnetii*. Temperature

and weight measurements were taken daily; temperatures ≥39.5°C were considered fever. Animals were necropsied 14 days post infection, and the spleen was weighed.

3) Guinea pigs were given subcutaneous injections of 20μg WCV-I or WCV-II in incomplete Freund's adjuvant twice, with a two week interval between vaccinations. Four weeks after the final vaccination, blood, lymph nodes, and spleen were aseptically collected at necropsy for use in lymphoproliferation and cytokine assays.

Isolation and preparation of lymphocytes. Single-cell suspensions were prepared from the aseptically collected spleen and lymph nodes by gently homogenizing the tissues in sterile glass homogenizers with tissue culture medium [RPMI 1640 supplemented with HEPES buffer, 10% fetal bovine serum, 2-mercaptoentanol (10 μm), L-glutamine (2 μm), penicillin (100 U/ml), and streptomycin (100 μg/ml)] and passing them through wire filters (109). Cells were spun down and washed in media twice before final suspension. Blood collected at necropsy was diluted in Hank's PBS + 5mM EDTA and incubated at room temperature for 1 h. The suspension was then layered over Histopaque 1.107 (Sigma Chemicals, St. Louis, MO) and centrifuged at 250 x g for 40 min at room temperature as previously described (109, 123). The buffy coat was removed and peripheral blood mononuclear cells (PBMC) were washed 3x with Hank's PBS. After the final wash, cells were resuspended in tissue culture media. The viability of the lymphocyte suspensions was determined by trypan blue exclusion.

Lymphoproliferation assay. Lymphocytes harvested from blood, lymph nodes, and spleens of control, infected, and vaccinated guinea pigs were analyzed for their ability to proliferate *in vitro* according to an established procedure (28, 109, 123) in

response to concanavalin A (ConA), phytohemagglutinin-P (PHA), pokeweed mitogen (PWM) (Sigma Chemicals, St. Louis, MO), or WCV-I. Lymphocytes were seeded into 96-well plates at a concentration of $2x10^5$ per well for splenocytes and PBMC and at $4x10^5$ per well for lymph node-derived cells. Triplicate cultures were stimulated with the mitogens or antigen at a concentration of 10 μg/ml and incubated for 4 days at 37°C and 5% CO2. [^{3}H]-thymidine was added for the last 6 hours of culture, and the contents of each well were then harvested onto glass fiber filters and quantitated using a liquid scintillation counter (LS 8000; Beckman, Irvine, CA). The stimulation index was determined by dividing the counts per minute of [^{3}H]-thymidine taken up by stimulated cells by the counts per minute of unstimulated cells from the same source.

Cell stimulation. Splenocytes were cultured in 24-well tissue culture plates at $5x10^5$ cells per well and incubated in either medium alone or medium plus 10 μg/ml WCV-I. Cells were collected at 0, 3, 6, 12, and 18 h for RNA isolation and analysis by quantitative PCR.

Total RNA isolation and quantitative PCR. Real-time PCR was performed on splenocyte cultures to determine mRNA levels for IFN-γ, TNF-α, TGF-β, RANTES, IL-1β, IL-8, IL-10, IL-12p40, and HPRT (hypoxanthine phosphoribosyl-transferase). Total RNA was isolated from splenocyte cultures at 1, 3, 6, 12, and 18 h using RNeasy columns (Qiagen, Valencia, CA) treated with RNase-free DNase to remove contaminating DNA. Reverse transcription with Taqman Reverse Transcription Reagents and real-time PCR using SYBR Green I double-stranded DNA binding dye (Applied Biosystems, Foster City, CA) were used according to established procedures

(89). The real-time primer sequences used have been previously reported (2, 89, 148, 149)and are given in Table 4.1. Fold induction of mRNA was determined from the threshold cycle values normalized for HPRT expression and then normalized to the value derived from unstimulated cells from the same source. *Ex vivo* samples were normalized to HPRT and the mean value derived from normal, healthy control animals.

Statistical analysis. Results were expressed as the means +/- the standard error and were compared using one- and two-way ANOVAs, Tukey's multiple comparison, and student's t-tests. Differences were considered significant at $p<0.05$.

RESULTS

The guinea pig immune response to infection and vaccination was evaluated by determining lymphoproliferative responses to mitogenic and antigenic stimulation and examining the production of mRNA for eight cytokines depicting both pro- and anti-inflammatory responses.

Stimulation with *C. burnetii* antigen enhanced cytokine mRNA production. To establish times of peak inducible cytokine mRNA expression in response to *C. burnetii* antigens, splenocytes from normal, control guinea pigs were stimulated with WCV-I. WCV-I did induce expression of cytokine message RNA in normal cells compared to unstimulated controls, although at 10μg/ml WCV-I was found to be only a weak stimulating antigen. Hours of peak cytokine mRNA induction in response to antigenic stimulation were as follows: IFN-γ, 12-18 h; TNF-α, 6 h ($p<0.05$); IL-1β, 6 h; IL-8, 6 h; IL-10, 12 h; and IL-12p40, 6-18 h. *C. burnetii* antigenic stimulation did not

increase mRNA production in the time range evaluated for TGF-β or RANTES (Fig. 4.1).

Increased pro-inflammatory cytokine mRNA with *C. burnetii* infection *ex vivo* and post-stimulation. Once it was determined that WCV-I stimulation of cells from healthy donors induced cytokine expression, induction of mRNA from *ex vivo* splenocytes following 7, 14, or 28 days of infection were evaluated and normalized to values of non-infected control animals with differences noted for several cytokines through the course of infection. A statistically significant increase in IFN-γ at 28 days p.i. was identified when compared to samples from non-infected controls and infected animals at 7 and 14 days p.i. ($p<0.01$). A significant decrease was noted at 14 days p.i. in TNF-α compared to non-infected controls and infected animals at 7 and 28 days p.i. ($p<0.01$). IL-10 mRNA expression was reduced at 7 days p.i. ($p<0.05$). Minor, though statistically significant, decreases in expression of TGF-β were present at 7 and 28 days p.i ($p<0.05$). Changes in mRNA expression of IL-12p40 were also dependent upon infection duration ($p<0.05$), with significantly reduced levels at 7 days p.i. rising to levels above controls by 28 days p.i. No significant change in *ex vivo* mRNA expression was recognized for RANTES, IL-1β, or IL-8 (Fig. 4.2).

Response to recall antigen provided another means to evaluate the immune response to *C. burnetii* infection. IFN-γ mRNA fold induction was increased at 7, 14, and 28 days p.i, at 12 and 18 h after stimulation with *C. burnetii* antigen, with peak induction occurring at 14 days p.i. IL-12p40 was significantly induced at 28 days post infection and 6 h post stimulation ($p<0.05$). IL-10 induction was also increased at 28

days p.i. at 6 ($p<0.05$), 12, and 18 h post stimulation. Splenocytes from 28 day p.i. animals displayed increased TNF-α induction at 3 h post stimulation, whereas those from 7 and 14 days p.i. had a slight reduction. An approximately 2-fold reduction in IL-1β was noted at 12 and 18 h post antigenic stimulation in all infected animals when compared to controls (Fig. 4.3). There was no detectable induction of TGF-β, RANTES, or IL-8 at any point post infection, as with *ex vivo* samples. It should be noted that significances in fold change were as compared to stimulated, non-infected controls.

Suppression of lymphoproliferation following infection with *C. burnetii*. The lack of general cytokine induction after infection with *C. burnetii*, especially at 14 days p.i., was unexpected given the clinical development of fever and pulmonary pathology. An early report of *C. burnetii* infection in monkeys and guinea pigs (75) suggested that infection induces immunosuppression. Therefore, the general function of lymphocyte activity was evaluated following infection.

Non-specific suppression of mitogen-stimulated lymphoproliferation was noted in infected guinea pig lymph node cells (Fig. 4.4), splenocytes, and PBMC at 14 and 28 days p.i. when compared to non-infected controls ($p<0.05$). No such suppression was seen at 7 days p.i.

Differential protectivity of phase I and phase II vaccination. Animals, including guinea pigs, as well as humans, develop long-lived protective immunity after vaccination with whole killed phase I *C. burnetii*. In contrast, whole killed phase II *C. burnetii* vaccination results in a strong immune response with limited or inapparent protection from challenge by virulent organisms. This distinction in the ability to

engender protection was noted early in *C. burnetii* research (120), but the molecular correlates that distinguish these two vaccination responses have not been characterized.

Before analysis of immunologic responses to vaccination, protective efficacies of vaccine preparations from phase I and phase II *C. burnetii* organisms were determined. Guinea pigs vaccinated with 0.2μg WCV-I and WCV-II developed fever similar to non-vaccinated controls in response to challenge with 10^6 virulent *C. burnetii*, but those receiving WCV-I were protected against death while 3/3 non-vaccinated and 2/3 WCV-II-vaccinated animals succumbed to infection. Guinea pigs vaccinated with 2 and 20μg WCV-II had fever of a lesser degree and shorter duration than non-vaccinated controls with no fatalities. Administration of 2 or 20μg WCV-I provided complete protection; no guinea pigs vaccinated with 2 or 20μg WCV-I exhibited clinical signs of illness (Fig. 4.5). Peak splenomegaly in response to high-dose *C. burnetii* infection occurs at 14 days p.i. (137). Because non-vaccinated control animals died before this point, an adequate comparison of spleen weights between vaccinated and control animals could not be performed. However, spleen weight and spleen/body weight ratios decreased with increasing doses of vaccine, both WCV-I and WCV-II, and animals receiving 20μg WCV-I had no discernible splenomegaly (data not shown).

Increased expression of pro-inflammatory cytokine IFN-γ with protective vaccination. *C. burnetii* infection appeared to drive a Th1-dominant immune response, as described above, but it was unknown what similarities infection-derived immunity would share with vaccination-induced immunity. Splenocytes from vaccinated, non-infected guinea pigs were evaluated for their production of cytokine mRNA *ex vivo* and

upon stimulation with WCV-I to assess differences resulting from fully-protective WCV-I and partially-protective WCV-II vaccination. A minor but significant reduction of TNF-α ($p<0.05$) in *ex vivo* splenocytes from WCV-II-vaccinated animals was noted when compared to controls. There was also a non-significant ~2-fold reduction in IL-1β. No significant induction or reduction of IFN-γ, TGF-β, RANTES, IL-8, or IL-10 were evident post vaccination in *ex vivo* samples.

Increased IFN-γ induction was noted at 12 and 18 h post antigenic stimulation in splenocytes from WCV-I-vaccinated guinea pigs when compared to WCV-II-vaccinated animals and controls. TNF-α was increased in samples from WCV-I animals at 3 ($p<0.05$) and 6 h post stimulation. WCV-I vaccination induced IL-12p40 at 6 and 12 h post stimulation. Vaccination with WCV-I and WCV-II caused slight increases in IL-8 induction at 3 and 6 h post stimulation and in IL-10 at 6 ($p<0.05$) and 12 h, with IL-10 mRNA production greater in samples from WCV-II vaccinated animals (Fig. 4.6). Vaccination had no effect on TGF-β, RANTES, IL-1β.

Protective vaccination stimulates lymphoproliferation. Increased blastogenesis in response to mitogenic and *C. burnetii* antigenic stimulation was noted in splenocytes, lymph node-derived lymphocytes (Fig.4.7), and PBMC from vaccinated guinea pigs, with WCV-I eliciting stronger mean responses than WCV-II. The antigen-specific enhancement of the lymphoproliferative response was significantly greater in WCV-I vaccinated animals ($p<0.05$). In order to illustrate that differences in lymphoproliferative responses were not due to potential differences in the ability to respond to fixed antigen, additional soluble antigen preparations were also evaluated. As

seen in both western-blot and silver stain (Fig. 4.8), the majority of the proteins in the whole bacteria (Lane 1) were also present in the FastProtein Blue extract (Lane 2) and the detergent extract mixture (Lane 3). Only a few strongly antigenic proteins remained in the unsoluble fraction (Lane 4). No significant differences were noted in fixed whole cell vs. soluble antigen stimulation of lymphoproliferation.

***C. burnetii* virulence is associated with suppression of lymphoproliferation.** To test whether suppression of lymphocyte proliferation was unique to infection with Nine Mile isolate, we compared lymphocytes from animals infected with isolates with distinct virulence phenotypes, as shown in our previous study (manuscript in preparation). Nine Mile RSA493 caused fever and severe atypical pneumonia while lesser disease was seen with P Q173 and G Q212, and no evidence of clinical illness was noted following Dugway 5J108-111 infection. Lymphoproliferation of PHA-stimulated lymph node cells was significantly suppressed in cultures from guinea pigs infected with RSA493 ($p<0.01$), Q173 ($p<0.01$), and Q212 ($p<0.05$) when compared to non-infected controls and those challenged with 5J108-111 (Fig. 4.9), adding differences in immunosuppressive response to infection to differences in clinical disease presentation between isolates.

DISCUSSION

Infection with *C. burnetii* leads to a range of immune responses in the host dependent on the stage of infection. In the guinea pig model, infection with 10^5 *C. burnetii* organisms via aerosol results in fever starting around day 6 p.i and lasting

approximately 7 days (137). The time-points evaluated here, 7, 14, and 28days p.i., were intended to allow evaluation of the host immune response in early, late, and convalescent stages of acute infection.

IFN-γ activates macrophages and enhances their ability to destroy ingested microorganisms. IFN-γ is produced by Th1 cells in response to stimulation by IL-12, which is produced by activated macrophages, and IL-2, which is also produced by Th1 cells, creating a strong stimulatory loop in response to infection. IFN-γ also enhances NK cell activity and expression of TNF-α receptors. Reactive oxygen and nitrogen intermediates produced in response to IFN-γ stimulation have been shown to inhibit *C. burnetii* replication and are likely to play a role in the host control of infection (16, 60), though the effects of IFN-γ may not be exclusively due to nitric oxide production since IFN-γ also controlled the infection in macrophages derived from inducible nitric oxide synthase knockout mice (190). It has also been proposed that IFN-γ stimulates the killing of *C. burnetii* by causing phagosome maturation and alkalinization (43) and promotes apoptosis of infected monocytes by activation of caspase-3, which is dependent on the expression of membrane TNF in cell culture systems (33), although these reports have not been substantiated by other studies. Here we show that IFN-γ mRNA was primarily induced at 12 and 18 h post stimulation with WCV-I in splenocytes from both infected and non-infected guinea pigs. Though it was expected that an increase in IFN-γ would be seen in response to active infection due to its established role in the inflammatory response, ex vivo expression was only noted during the convalescent stage at 28 days p.i. Induction following antigenic stimulation, though evident at both 14 and 28 days p.i,

was slightly higher at the 14 day time-point. These increases in IFN-γ during late infection and early convalescence suggest a role for IFN-γ in the control of *C. burnetii* infection in the guinea pig model. Splenocytes from animals receiving phase I vaccination were more capable of IFN-γ mRNA induction after antigenic stimulation than those of phase II vaccinees and normal guinea pigs. It has been demonstrated that stimulation of human PBMC with WCV-II causes a greater IFN-γ response than WCV-I, and the presence of phase I LPS actually has a down-regulatory action on IFN-γ production, which is offset by the addition of IL-2 to the cultures (65). It has also been shown in mice that the non-specific anti-viral immunity induced by vaccination with *C. burnetii* is dependent on IFN-γ, since signaling through the IFN-γ receptor is essential for the production of nitric oxide, indoleamine 2,3-dioxygenase, 2'-5'-oligoadenylate synthetase, and protein kinase (136). An increase in IFN-γ has been noted in patients with post-Q fever fatigue syndrome (122) but is not commonly reported in Q fever endocarditis patients. Q fever patients deficient in specific T cell activity may be unable to contain the organism in phagocytes and granulomas (78), as in $TLR4^{-/-}$ mice where impaired formation of granulomas has been associated with decreased IFN-γ and TNF-α production (57). Other studies have shown that TLR2, not TLR4, is important for recognizing *C. burnetii* and limiting its growth, where macrophages lacking TLR2 but not TLR4 produce less TNF-α and IL-12 when infected with the organism (189)..

TNF-α is a major immune response-modifying cytokine produced principally by activated macrophages and is involved in inflammation (including fever) and cell death

and may induce IL-1 release. *C. burnetii* antigen caused increased induction of TNF-α mRNA in splenocytes from normal, healthy animals. Interestingly, TNF-α mRNA levels were decreased in *ex vivo* cells from infected guinea pigs when clinical illness was resolving, and no significant change was noted upon stimulation of splenocytes with recall antigen. It is possible that the window of TNF-α induction was missed due to experimental design; daily evaluation during the infectious process might reveal the expected rise in TNF-α. Increases in TNF-α secretion have been shown from *ex vivo* PBMC of patients with acute Q fever, though levels were reportedly higher in samples from patients with acute hepatitis than those with pneumonia, but *C. burnetii*-stimulated cytokine production was no different than controls (58). Another study showed that both TNF-α and TNF receptor II, a cytokine antagonist, were increased in acute Q fever and Q fever endocarditis (23). Overproduction of TNF-α has commonly been reported in association with chronic Q fever endocarditis (26, 58). It has been proposed that differences in TNF-α induction might play a role in the pathobiology of Q fever, supported by the findings that the most effective inducers in a TNF-bioassay of peritoneal macrophages from naïve mice were the LPS and whole cell antigens of Priscilla (Q177), Scurry (Q217), Nine Mile (RSA493), Luga (Q216), and Henzerling (RSA331), in that order, whereas by ELISA the best inducers were the LPS of Luga, Henzerling, Nine Mile, Priscilla, then Scurry (79). Neither infection with *C. burnetii* Luga strain nor vaccination with whole killed cells lead to an increase in TNF in the serum of mice, though stimulation by intravenous injection of WCV-I post-infection caused marked TNF production (166). In our study, vaccination of guinea pigs with

WCV-I provoked an increase in TNF-α mRNA induction compared to WCV-II and non-vaccinated controls. Blood cell cultures from healthy humans incubated with WCV-I from Nine Mile and Priscilla strains of *C. burnetii* induced significantly higher levels of TNF-α secretion than WCV-II of the same isolates. Those experiments demonstrated that the higher the LPS content, the more pronounced the induction of TNF-α, as well as IL-6 and IL-8, secretion (81).

IL-1 is a major pro-inflammatory cytokine secreted by macrophages in response to phagocytosis of foreign particles or T cell contact with macrophages that activates T cells in response to antigen and induces T-cell expression of IFN-γ. It was anticipated that an increase in IL-β mRNA would be seen during early infection since IL-1 is known to cause fever by stimulating the release of prostaglandins and stimulates the release of and is chemotactic for neutrophils, which have been found in abundance in *C. burnetii* infected tissues at 7 days p.i. (137). Interestingly, IL-1β does not appear to be increased during the febrile stage of infection in the guinea pig aerosol-challenge model, and mRNA levels were in fact lower in infected animals compared to non-infected controls in *ex vivo* and stimulated splenocytes. It would be desirable to test for IL-1 receptor agonist (IL-1RA) in the guinea pig model since it has previously been found that IL-1RA was significantly increased in plasma from patients with acute Q fever (23). On the contrary, IL-1β production by PBMC from patients with chronic Q fever endocarditis is greatly increased (26). Normal human blood cell cultures incubated with antigens from both the acute disease-associated strain Nine Mile and the chronic disease-associated

strain Priscilla showed no increase in IL-1β secretion (81), and similar results were found in cultured splenocytes in the guinea pig model.

IL-12 is a pro-inflammatory cytokine produced by activated macrophages that acts on Th1 cells to enhance the secretion of IFN-γ. IL-12p40 mRNA induction in guinea pig splenocytes in response to *C. burnetii* antigenic stimulation occurred between 6 and 18 h post-stimulation. *Ex vivo* samples exhibited a significant reduction at 7 days p.i., gradually returning to control levels by 28 days p.i., at which time splenocytes exposed to antigen had a significant increase in IL-12p40. Vaccination with WCV-I also lead to an increase in IL-12p40 mRNA induction compared to WCV-II administration or non-vaccination. Patients with uncomplicated acute Q fever and Q fever valvulopathy have exhibited an increased release of IL-12 from unstimulated PBMC, but the level of cytokine production was not affected by the amount of time between disease onset and cytokine analysis (58).

IL-10 is produced by activated macrophages and Th2 cells (and Th1 cells in humans) and directly inhibits cytokine synthesis by macrophages, reducing the production of IL-12 and TNF-α. The reduction in IL-12 production in turn results in a decrease in Th1 derived IFN-γ. Overproduction of TNF can stimulate monocyte production of IL-10, causing a negative feedback loop. IL-10 can also stimulate the production of IL-1RA, leading to a decrease in IL-1. IL-10 mRNA was reduced in *ex vivo* guinea pig splenocytes after infection with *C. burnetii*, with the greatest degree of reduction corresponding to the early stage of febrile illness. However, IL-10 was significantly induced in 28 day p.i. samples after antigenic stimulation, as would be

expected to control the host inflammatory response caused by active infection. A slightly greater induction of IL-10 was noted in guinea pigs after vaccination with WCV-II at 12 hours post-stimulation when compared with WCV-I, indicating a stronger Th1 response to protective vaccination and associating a possible Th2 skew with phase II vaccination. The overproduction of IL-10 has been associated with chronic Q fever endocarditis and fatigue syndrome (42, 122) due to defective phagosome maturation and impaired *C. burnetii* killing (44) and is specifically associated with the occurrence of relapses (25). Increased IL-10 release has also been noted in acute Q fever hepatitis patients (58).

Very few changes in TGF-β, RANTES, and IL-8 mRNA were observed in infected or vaccinated guinea pigs. TGF-β is produced by activated macrophages and T cells and inhibits IL-1 and IFN-γ production. TGF-β is also known to inhibit T cell proliferation, so it is somewhat interesting that TGF-β mRNA levels were not increased at times when suppression of lymphoproliferation was noted with the [^{3}H]-thymidine assay. Monocytes from patients with Q fever endocarditis have been shown to release TGF-β (25), and levels are increased in the serum of post-Q fever fatigue syndrome patients (122). RANTES is a β-chemokine produced by circulating T cells and, as its name implies (Regulated upon Activation, Normal, T Expressed and Secreted), is reduced when T cells are activated. It has been suggested that RANTES plays a role in regulating the inflammatory response to LPS in the guinea pig by altering macrophage production of pro-inflammatory cytokines (148), but no significant changes were noted after *C. burnetii* infection or vaccination. RANTES has been shown to increase phagocytosis of virulent *C. burnetii* by THP-1 cells, preventing *C. burnetii* replication

but not elimination (24). IL-8 is produced by macrophages and is chemotactic for neutrophils and certain T cells. Slight increases in IL-8 were seen at 6 h post-stimulation in normal cells and in those acquired from animals vaccinated with WCV-I and WCV-II. Secretion of IL-8 has been noted in normal blood cell cultures stimulated with Nine Mile and Priscilla antigens and was independent of the phase of antigen used (81).

The formalin-killed, whole-cell *C. burnetii* vaccine is known to be highly immunogenic and protective in both humans and animals (1, 179). The immune response seen post-vaccination in humans has been evaluated through cell mediated immunity, known to be a factor in resistance to *C. burnetii* infection (107), and antibody response (7, 185). Formalin-killed phase I *Coxiella* have been shown to be 100-300 times more protective than phase II organisms (120). Comparison of these two immunizing materials provides an important approach to examining protective immunity. In this study, we showed that as little as 2μg of WCV-I conferred complete protection against fever and death in the guinea pig aerosol-challenge model in response to infection with a potentially lethal dose of *C. burnetii*. WCV-II vaccination conferred only partial protection at higher doses, preventing death and reducing but not eliminating the febrile response. As discussed above, increased induction of certain cytokines such as IFN-γ, TNF-α, and IL-12p40 after vaccination with WCV-I, in conjunction with the increase in IL-10 seen with WCV-II vaccination, support the necessity of a Th-1 dominant immune response in protective vaccination.

In our study, guinea pig splenocytes, PBMC, and lymph node cells exhibited marked lymphocyte hyporesponsiveness at 14 and 28 days p.i. but not during clinically

apparent infection at 7 days p.i. This suppression of lymphoproliferation may be due to corresponding increases in IFN-γ, which can have immunosuppressive as well as immunostimulatory effects. Suppression of blastogenesis of PBMC from cynomolgus macaques in the presence of PHA has been shown to occur between 14 and 28 days post infection with *C. burnetii* Henzerling at which times lymphocyte counts were within normal limits and clinical signs of illness had abated; similar results were seen in guinea pigs during early convalescence. Cynomolgus lymphocytes were again responsive on day 35 p.i. when cultured with WCV-I or WCV-II (75). PBMC from patients that have recovered from acute Q fever and those from patients with active Q fever hepatitis exhibit a strong proliferative response when cultured with *C. burnetii* antigens (78). Q fever endocarditis patients have been shown to have an antigen-specific lymphocyte unresponsiveness, mediated in part by adherent suppressor cells that work through the production of prostaglandin E_2 (PGE_2) (77, 78). It has been demonstrated that *C. burnetii* contains an immune suppressive complex (ISC) attached to the cell matrix by disulfide bonds (171). This ISC was expressed by phase I but not phase II organisms and lead to mitogen hyporesponsiveness and antigen-specific down-regulation of splenic and nodal lymphocytes from C57BL/10 mice infected with phase I *C. burnetii* (29). In that model, vaccination also induced dose-dependent lymphocyte hyporesponsiveness to phytomitogens. On the other hand, lymphocytes from humans vaccinated with Q-Vax or other phase I vaccines exhibit a mitogenic response to *C. burnetii* antigens (65, 70), and PBMC from guinea pigs vaccinated with a killed phase I *C. burnetii* vaccine had increased stimulation indices when stimulated with PHA (74). An increase in

lymphoproliferation in response to mitogenic and *C. burnetii* antigenic stimulation in vaccinated guinea pigs, particularly after vaccination with WCV-I, was also demonstrated in our study. Previously, no immunosuppression was seen in mice injected with phase I strains isolated from heart valves of individuals with chronic Q fever endocarditis, KAV and PAV (171). However, we have shown here that lymphoproliferation of PHA-stimulated lymph node cells was significantly suppressed in cultures from guinea pigs infected with two endocarditis-derived isolates, P Q173 and G Q212, and the acute disease-associated Nine Mile RSA493, but not from those challenged with Dugway 5J108-111, elucidating yet another facet of the complex interactions between *C. burnetii* isolates of differing genetic compositions and the host immune response and showing similar suppression of lymphoproliferation in isolates inducing different degrees and types of clinical illness in the guinea pig model.

We evaluated the guinea pig immune response to infection via aerosol and vaccination with protective and partially protective vaccine preparations. Cytokine analyses and lymphoproliferative responses indicate a role for Th1 dominant cell-mediated immunity in infection and protective vaccination. Few immunologic tools have been developed for analysis of the guinea pig immune response, but with the recent completion of the guinea pig genome sequence analysis, it is anticipated that primers for more key cytokines will soon be available. This will allow further much-needed analysis of the immune response associated with infection and required for effective vaccination.

V. SUMMARY AND CONCLUSIONS

There was a great need in the *Coxiella burnetii* research field for a fully-characterized animal model system that could adequately mimic both the common route of infection and major manifestations of clinical illness of human acute Q fever. The aerosol-challenged guinea pig model presented here meets these criteria and evaluation of this model has lead to further appreciation of the host response to *C. burnetii* infection. The objectives of this dissertation were to develop and characterize a guinea pig model infected via aerosol with *C. burnetii*, assessing clinical, pathologic, and immunologic responses to infection. This model was then used to determine the consequences of infection with multiple *C. burnetii* isolates representing four separate genomic groups and to compare the protective efficacies of vaccination with killed phase I and phase II organisms.

The dose-responsive nature of infection with *C. burnetii* in the guinea pig aerosol-challenge model corresponded with what had been previously, albeit to a lesser extent, characterized in other models, including humans (13, 83, 116), and spanned disease ranging from largely subclinical illness with minor febrile response to lethal pneumonia. The development of exceptionally high fever (40-40.5°C;104-105ºF) in the guinea pig model was reliably reproducible and one of the hallmarks of human infection with *C. burnetii*. Histopathologic evaluation during active infection in guinea pigs receiving a large number of *C. burnetii* Nine Mile RSA493 organisms revealed coalescing panleukocytic, bronchointerstitial pneumonia with extensive bronchial and alveolar exudates that resolved to multifocal, lymphohistiocytic, interstitial pneumonia

with granuloma formation during convalescence. These findings are compatible with those seen when human cases of acute Q fever develop pneumonic complications (98, 100, 118). The development of pneumonia proved to be one of the outcomes of infection with not only the genogroup I isolates Nine Mile RSA493, African RSA334 and Ohio 314 RSA270, but also to lesser degrees with other isolates more commonly associated with chronic disease symptomology, including, in descending order of severity of pneumonic disease, group V isolates S Q217 and G Q212 and group IV isolates P Q173 and MSU Goat (Priscilla) Q177. No clinically significant abnormalities were noted in guinea pigs infected with any dose of the group VI isolate 5J108-111 as was expected since isolates in this group have generally been considered avirulent, though infectious (158, 159). A large amount of lipid accumulation was noted in the livers of severely ill animals necropsied during early infection, which, though not a common manifestation of human Q fever, has been previously reported in guinea pigs infected IP (15). Infection with group IV isolates, particularly P Q173, was associated with a notably increased incidence and severity of granulomatous hepatitis, similar to that seen in human acute Q fever hepatitis cases exhibiting primarily granulomatous inflammation, though the "doughnut" granulomas associated with that condition were not noted in any infected guinea pigs. In general, where pneumonia was more advanced with I, V, then IV infection, hepatitis was more severe in IV, V, then I-infected animals, indicating that although route of infection is likely to play some role in the manifestation of disease as has been previously demonstrated (83, 102), the genetic composition of the infecting bacteria is also a major, and possibly more important, contributing factor.

In addition to unique sequence differences between genomic groups that may contribute to differences in disease manifestation, host factors such as immunocompetence/suppression are also likely to play a role, and, indeed, host immunogenetic differences have been noted in patients with acute Q fever, Q fever endocarditis, and post-Q fever fatigue syndrome (53). Severe combined immunodeficient mice, lacking both B and T cell responses, are highly susceptible to infection with *C. burnetii* and studies have suggested that innate immunity cannot control replication of the bacteria and acquired immunity is essential for mice to survive infection (3, 4). The cell-mediated immune response is considered an essential component for controlling *C. burnetii* infection (10), and experiments in knock-out mice have shown that T cells are crucial for the clearance of both phase I and phase II *C. burnetii* (M Andoh, manuscript in preparation). These findings concur with evidence that infection with human immunodeficiency virus is a risk factor for the development of chronic Q fever endocarditis (19, 90). It was interesting to note that similar suppression of lymphoproliferation was seen after infection with isolates producing disparate diseases (RSA493, Q173, and Q212) while no suppression was noted in an avirulent isolate (5J108-111). The suppression of lymphoproliferative responses seen after active infection with isolates from three separate genomic groups (I, IV, and V) in the guinea pig model has also been reported following both acute and chronic human infections (75, 171) and was found to be due to prostaglandin E_2 production by stimulated monocytes (77).This suppression following acute infection may be a contributing factor in latent infection, which could in turn develop into chronic disease, particularly in individuals

with prior valvulopathies or immunosuppressive events such as chemotherapy. Infection of guinea pigs with *C. burnetii* via aerosol lead to an assortment of modifications of the host immune response dependent on the stage of infection. Increased induction of mRNA was noted in Th1 cytokines such as IFN-γ and IL-12p40 in response to infection and re-stimulation with *C. burnetii*, primarily in animals sampled at 28 days p.i., though IL-12p40 was significantly reduced in *ex vivo* samples during early infection. The mRNA production was also increased in the Th2 cytokine IL-10 at 28 days p.i. The other cytokines monitored were either slightly decreased (IL-1β) or showed little to no change (TGF-β, RANTES, IL-8). A major limitation of the assessment of cytokine response to infection was the amount of time between sample collections, potentially leading to missing the window of expression of TNF-α and other cytokines.

Vaccination with phase I antigens derived from two separate genomic groups showed complete heterologous protection against lethal challenge. Comparison of phase I (WCV-I) and phase II (WCV-II) antigenic preparations of RSA493, however, revealed only partial protection in response to infection (protection against death but not fever) after WCV-II administration as opposed to the complete protection offered by WCV-I, confirming the results of a similar early study (120). WCV-I , and to a lesser extent WCV-II, administration stimulated an increase in lymphoproliferation in a [^{3}H]-thymidine uptake assay upon stimulation with the mitogens ConA, PHA, and PWM as well as three different preparations of phase I *C. burnetii* antigen. This increased blastogenic response after vaccination with phase I. *C. burnetii* has also been noted in humans (65, 74, 179). Th1 cytokine response to vaccination was clearer than that of

infection with increased production of IFN-γ, TNF-α, IL-1β, and IL-12p40 mRNA after antigenic stimulation associated with WCV-I vaccination when compared to WCV-II vaccination, and an increase in IL-10 in samples from WCV-II vaccinated animals. Overall, cells from guinea pigs vaccinated with WCV-II did not respond to recall antigen with the same efficiency as cells from animals vaccinated with WCV-I. It would be attractive to infer that a Th1 immune response is required for protective vaccination, while the WCV-II vaccine is less protective due to a skew towards a Th2 response. However, it is more likely that the difference is simply a stronger vs. less strong Th1 response to WCV-I vs. WCV-II vaccination.

Clinical signs, pathologic changes, and immunologic responses noted in guinea pigs infected via aerosol are comparable to those seen in human acute Q fever, making this an accurate and valuable animal model of human disease. This physiologically relevant model will be important for testing prospective preventative and therapeutic agents, evaluating potential improved diagnostic tests, and further exploration of the general pathogenesis of *C. burnetii* infection, including a more detailed analysis of host immune response to infection and protective vaccination. Key findings in the series of experiments presented here were: 1) the dose-responsive nature of *C. burnetii* infection, including in depth examination of the associated pathologic changes; 2) genetic differences among *C. burnetii* isolates are responsible for variations in clinical disease presentation, and phylogenetic groupings may be used to anticipate the course of infection; 3) vaccination with whole killed cells from an isolate of one genetic group are protective against lethal infection with an isolate from another group; 4) vaccination

with whole killed cells from phase I, but not phase II, organisms is completely protective against lethal challenge; and 5) a stronger Th1 immune response is present in association with phase I vaccination when compared to phase II vaccination.

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APPENDIX A

FIGURES

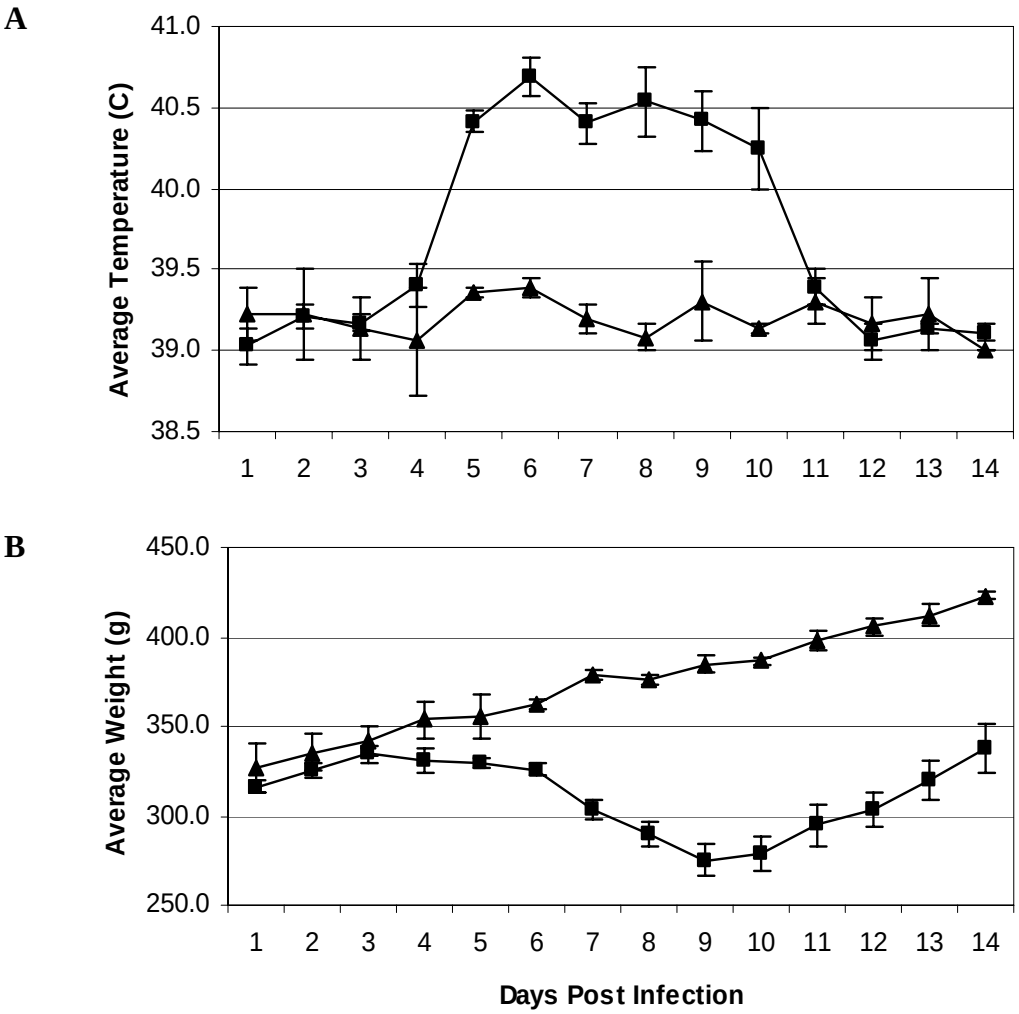

FIG. 2.1. Fever response and weight loss. Average daily temperatures (A) and weights (B) of control guinea pigs (▲) and those infected with $2x10^6$ *C. burnetii* (■). Temperatures in excess of 39.5°C are considered fever. Weight loss and gain corresponded with the onset and resolution of fever.

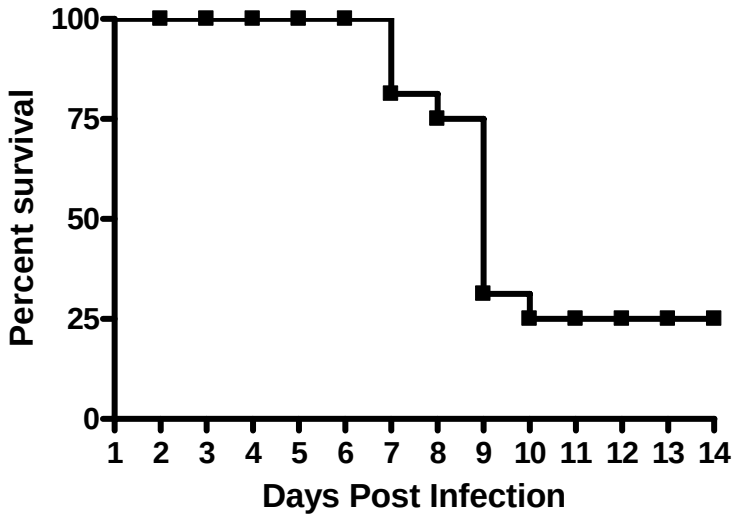

FIG. 2.2. Percent survival after infection with $2x10^6$ *C. burnetii*. Inoculation with $2x10^6$ *C. burnetii* resulted in mortality in 12/17 guinea pigs within the first 10 days of infection, with no deaths occurring thereafter.

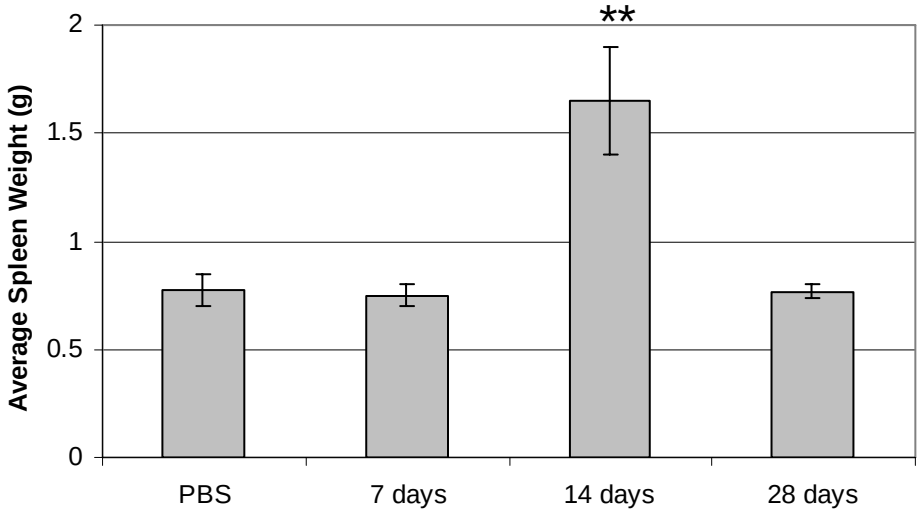

FIG. 2.3. Transient splenomegaly in animals surviving $2x10^6$ *C. burnetii* challenge. Spleen weights are the average of 3 guinea pigs infected with $2x10^6$ *C. burnetii* euthanized at 7, 14, and 28 days post infection. Statistically significant splenomegaly was present at 14 days post infection ($p<0.01$).

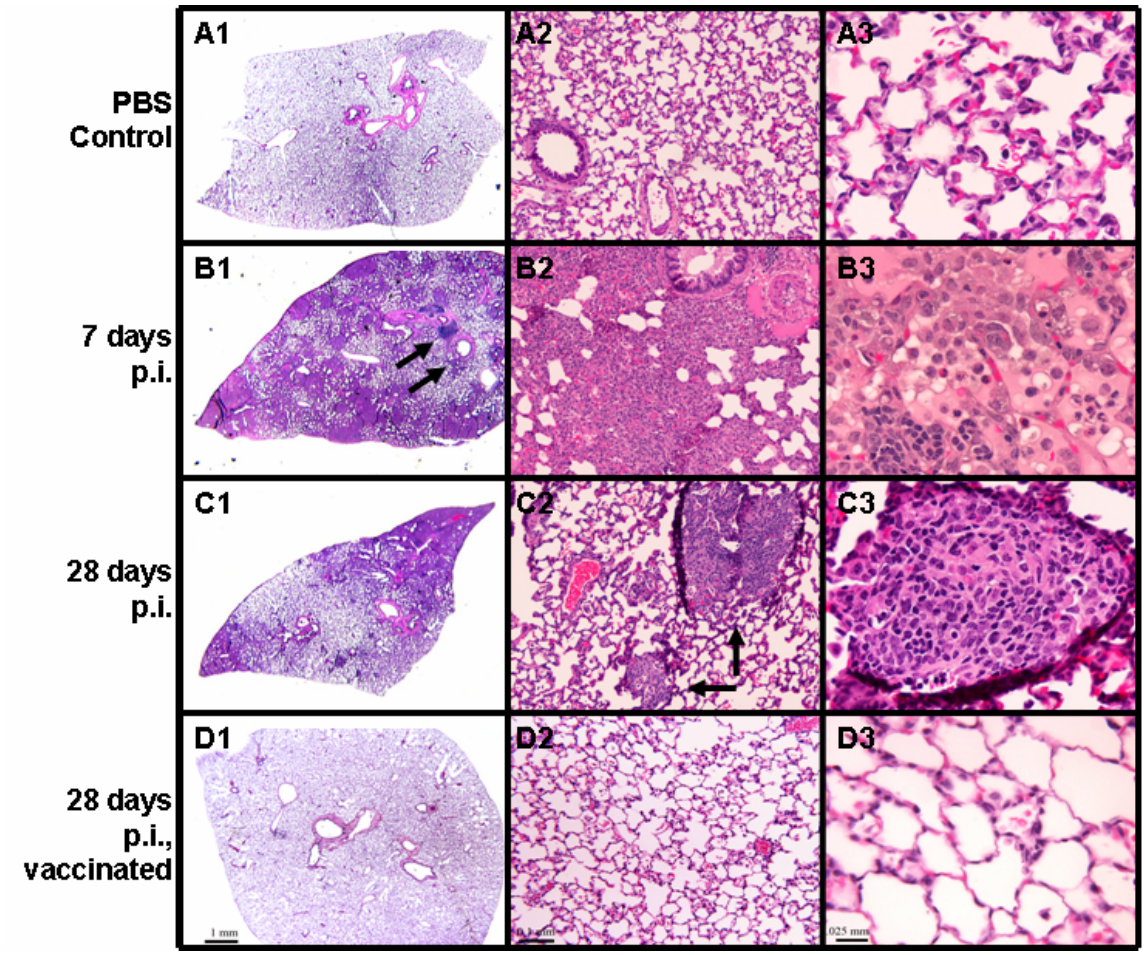

FIG. 2.4. Q fever pneumonia. H&E stained lung tissues from guinea pigs infected with $2x10^6$ *C. burnetii* 7 and 28 days p.i. compared to PBS controls (A1-A3). Panleukocytic bronchointerstitial pneumonia with bronchial and alveolar exudates can be seen at 7 days p.i. (B1-B3); arrows indicate BALT. Granulomatous pneumonia was present 28 days p.i. (C1-C3); arrows indicate pulmonary granulomas. No significant pathologic change was noted in guinea pigs vaccinated prior to infection (D1-D3).

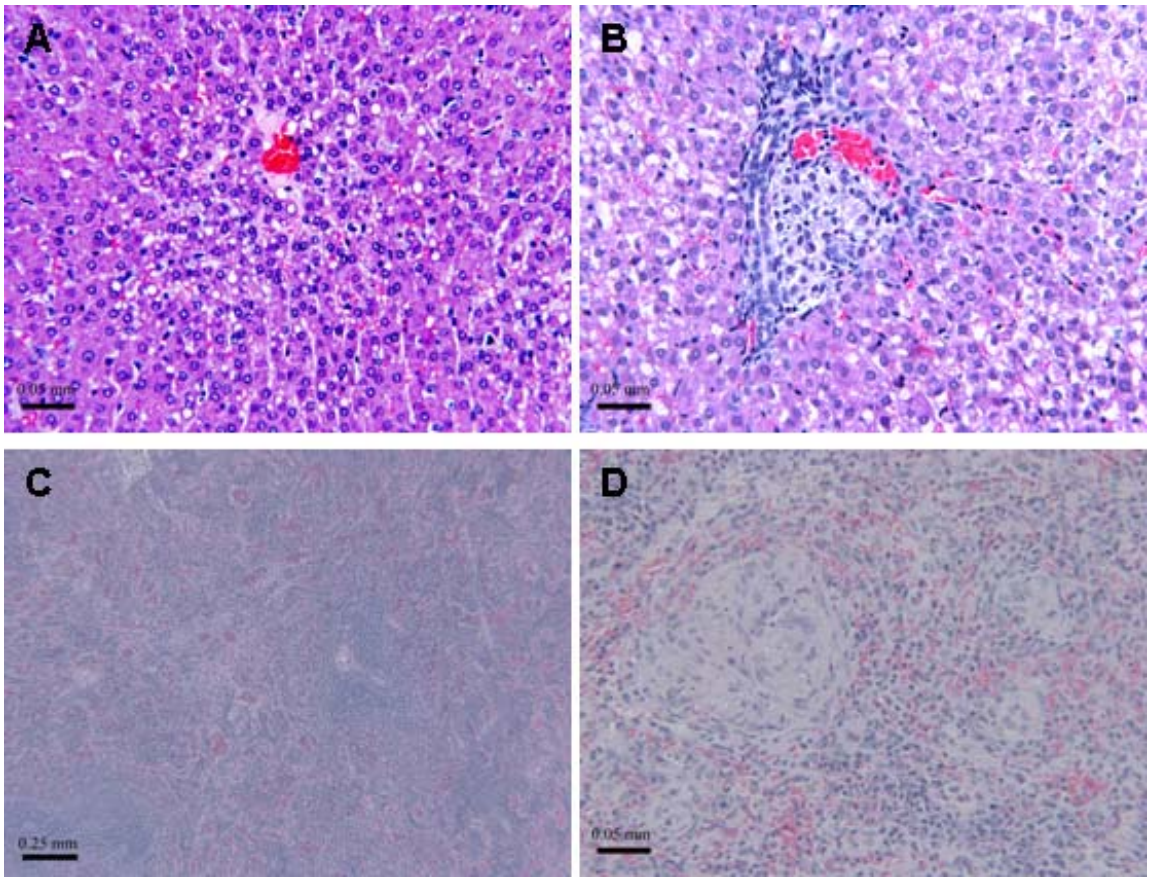

FIG. 2.5. Q fever hepatopathies and splenic changes. H&E stained liver and spleen sections showing (A) hepatic lipidosis, 7 days p.i.; (B) hepatic granuloma, 14 days p.i.; (C) disruption of normal splenic architecture, 14 days p.i.; and (D) splenic granulomas, 14 days p.i., in guinea pigs infected with $2x10^6$ *C. burnetii*.

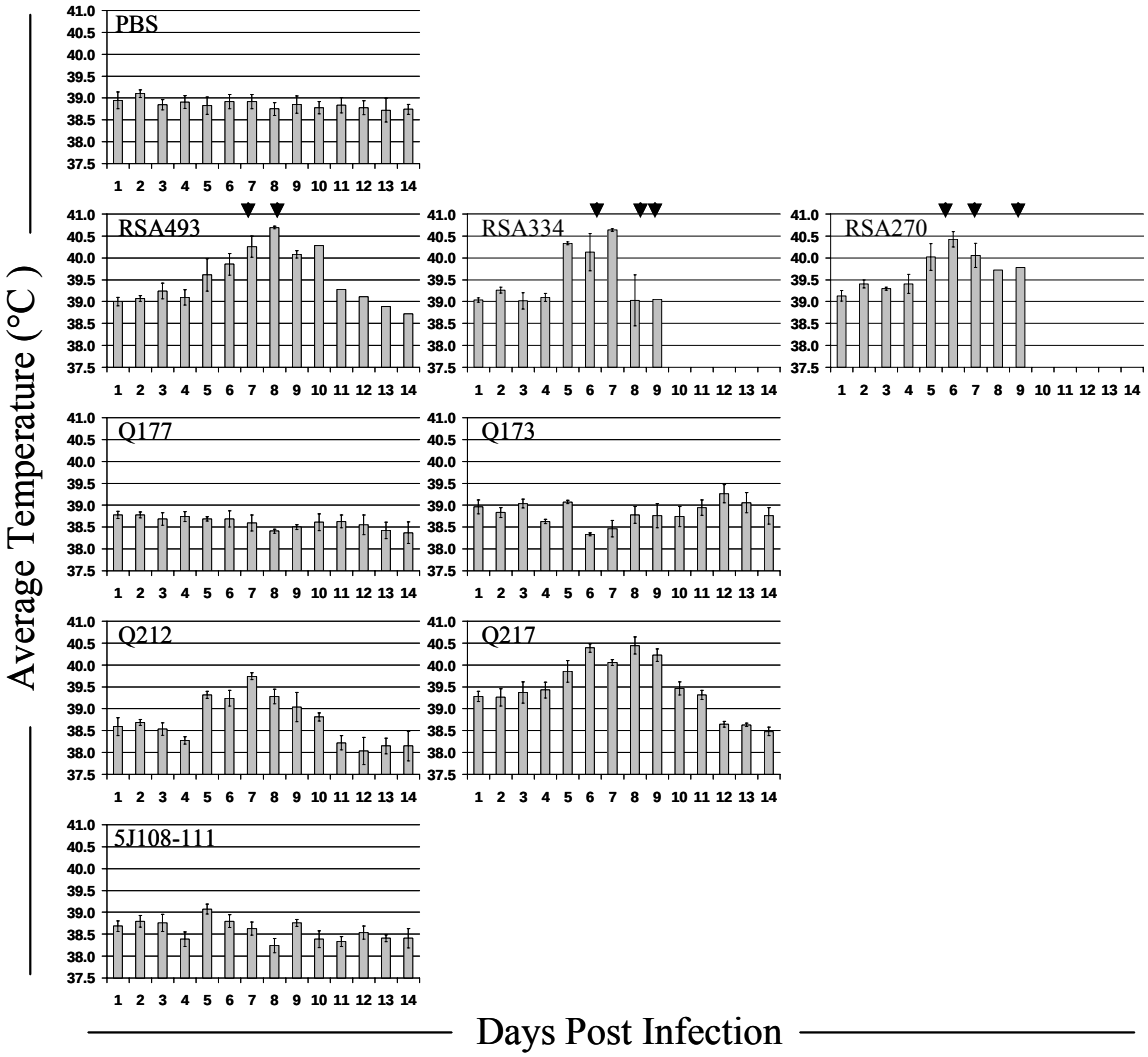

FIG. 3.1. Fever response to infection with high dose *C. burnetii* isolates. Mean daily temperatures +/- SEM (n=3) of guinea pigs infected with $2x10^6$ of each *C. burnetii* isolate. Temperatures ≥39.5°C are considered fever. Arrows indicate days on which death occurred in RSA493, RSA334, and RSA270 groups.

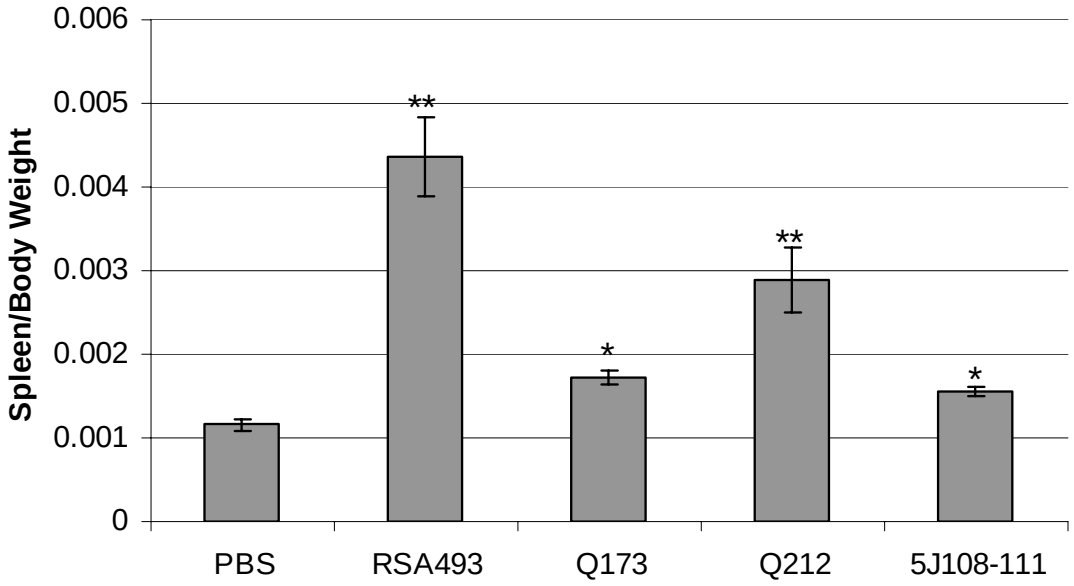

FIG. 3.2. Splenomegaly 14 days p.i. Spleen weights are the average of 3 guinea pigs per group infected with $2x10^6$ *C. burnetii* euthanized at 14 days post infection. Statistically significant ($p<0.05$) splenomegaly was present in all guinea pigs receiving each *C. burnetii* isolate. Q173 and 5J108-111 were also statistically different from RSA493 ($p<0.01$) and Q212 ($p<0.05$). *=$p<0.05$, **=$p<0.01$ compared to PBS controls.

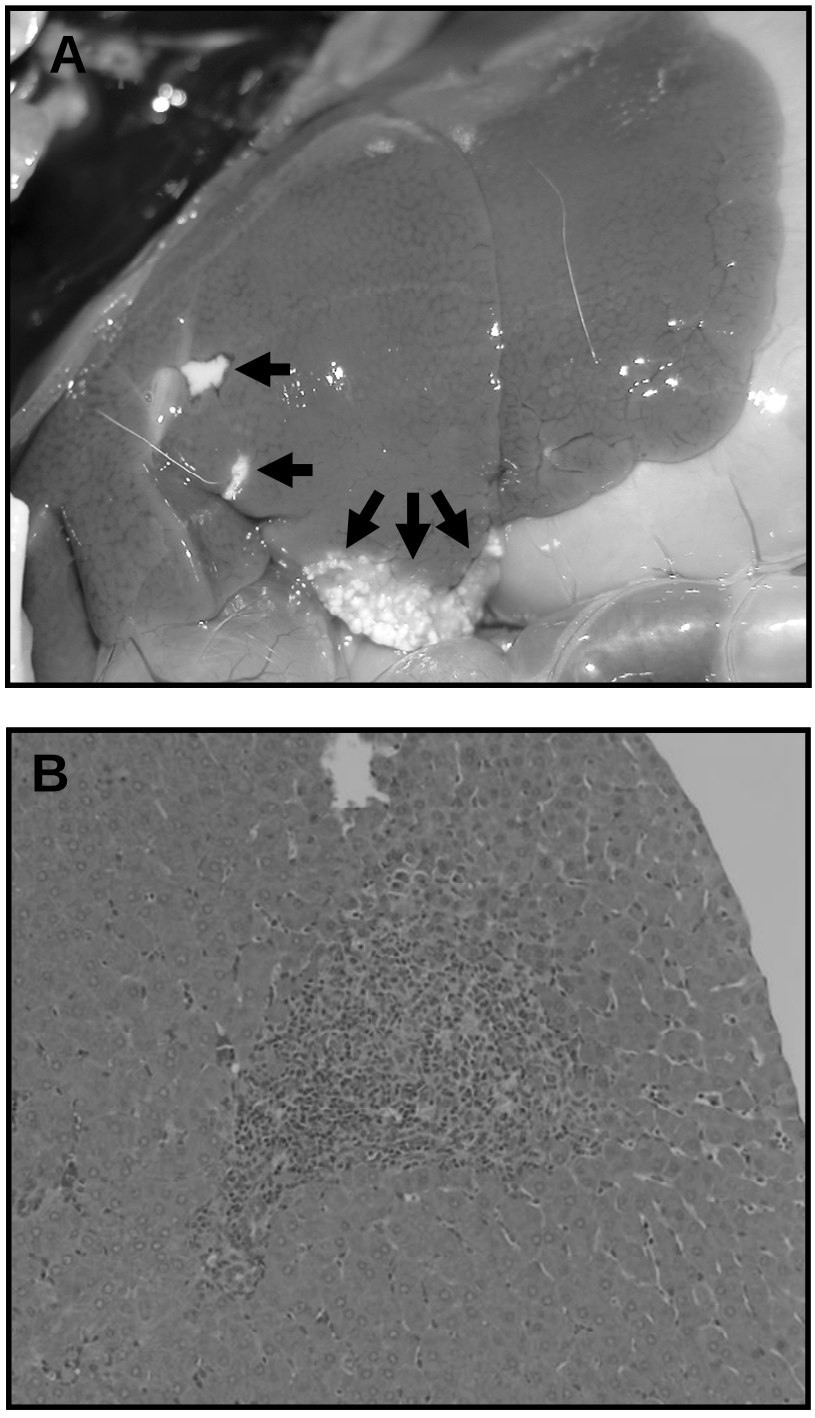

FIG. 3.3. Q173 infection-associated hepatic granulomas. A) Pinpoint to 4mm, individual to coalescing, white foci in the liver of a guinea pig 28 days post infection with high-dose phase I Q173 *C. burnetii*. B) 100x magnification of a single hepatic granuloma from the same guinea pig.

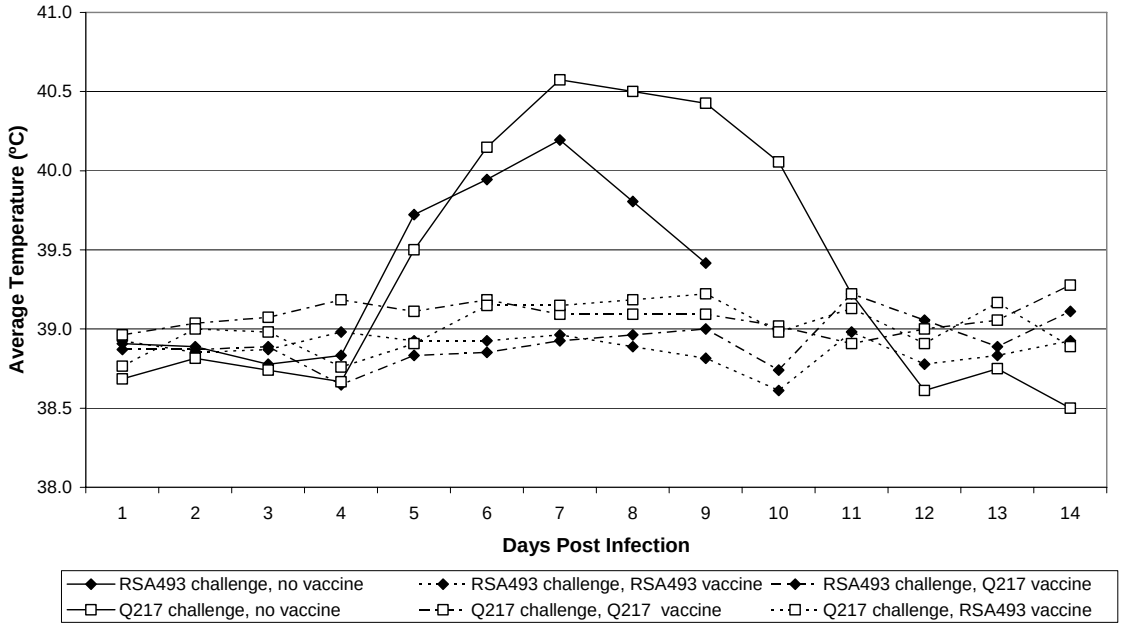

FIG. 3.4. Heterologous vaccination and challenge. Average daily temperatures of guinea pigs vaccinated with RSA 493 (dotted line), Q217 (dashed line), or adjuvant alone (solid line) and challenged with high dose RSA493 (♦) or Q217 (□). Temperatures ≥ 39.5°C were considered fever.

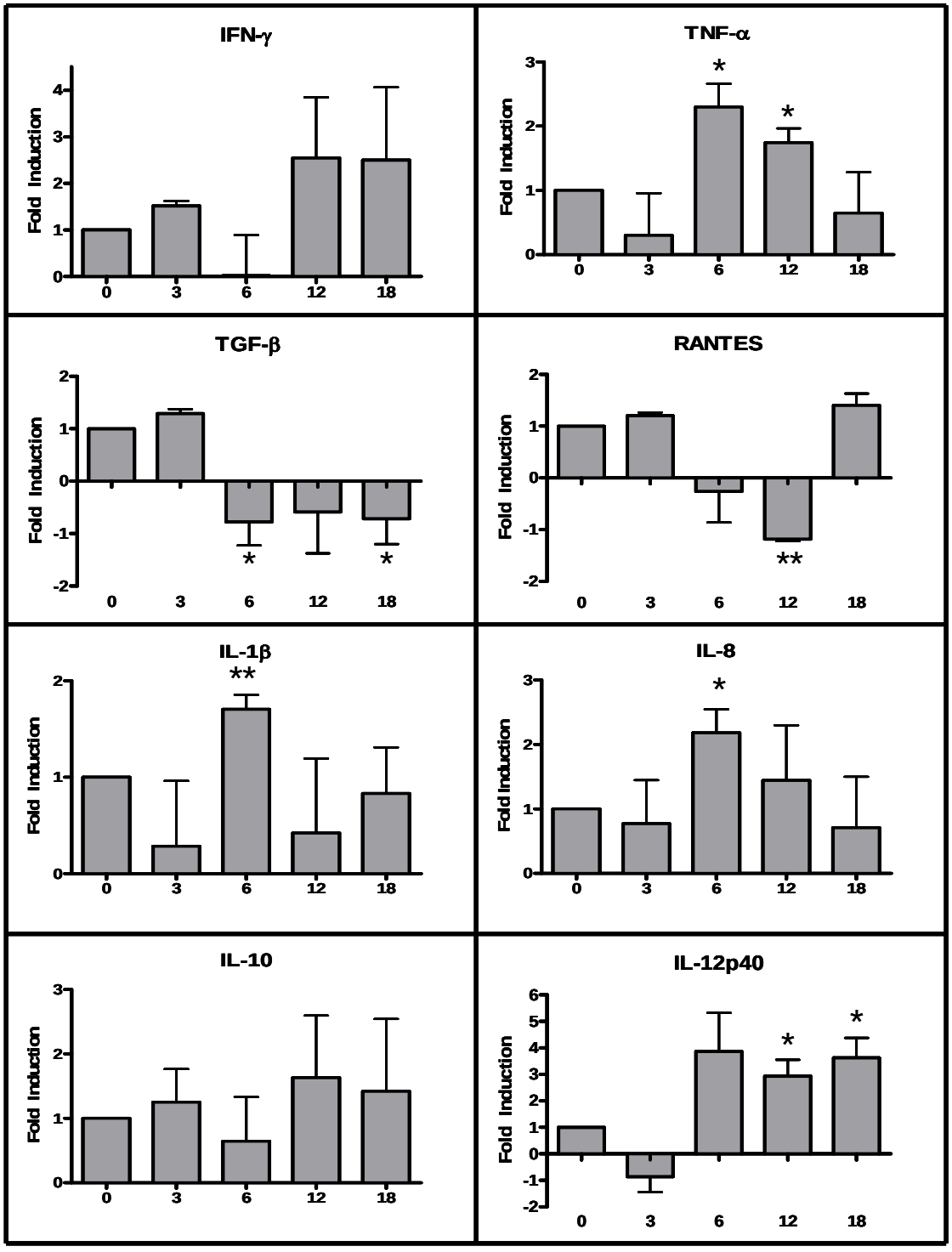

Fig. 4.1. Splenocyte cytokine response to *C. burnetii* antigen stimulation. Fold induction of mRNA derived from healthy control guinea pig splenocytes after stimulation with phase I *C. burnetii* antigen normalized to time zero. Y axis depicts hours post stimulation. *p<0.05; **p<0.01.

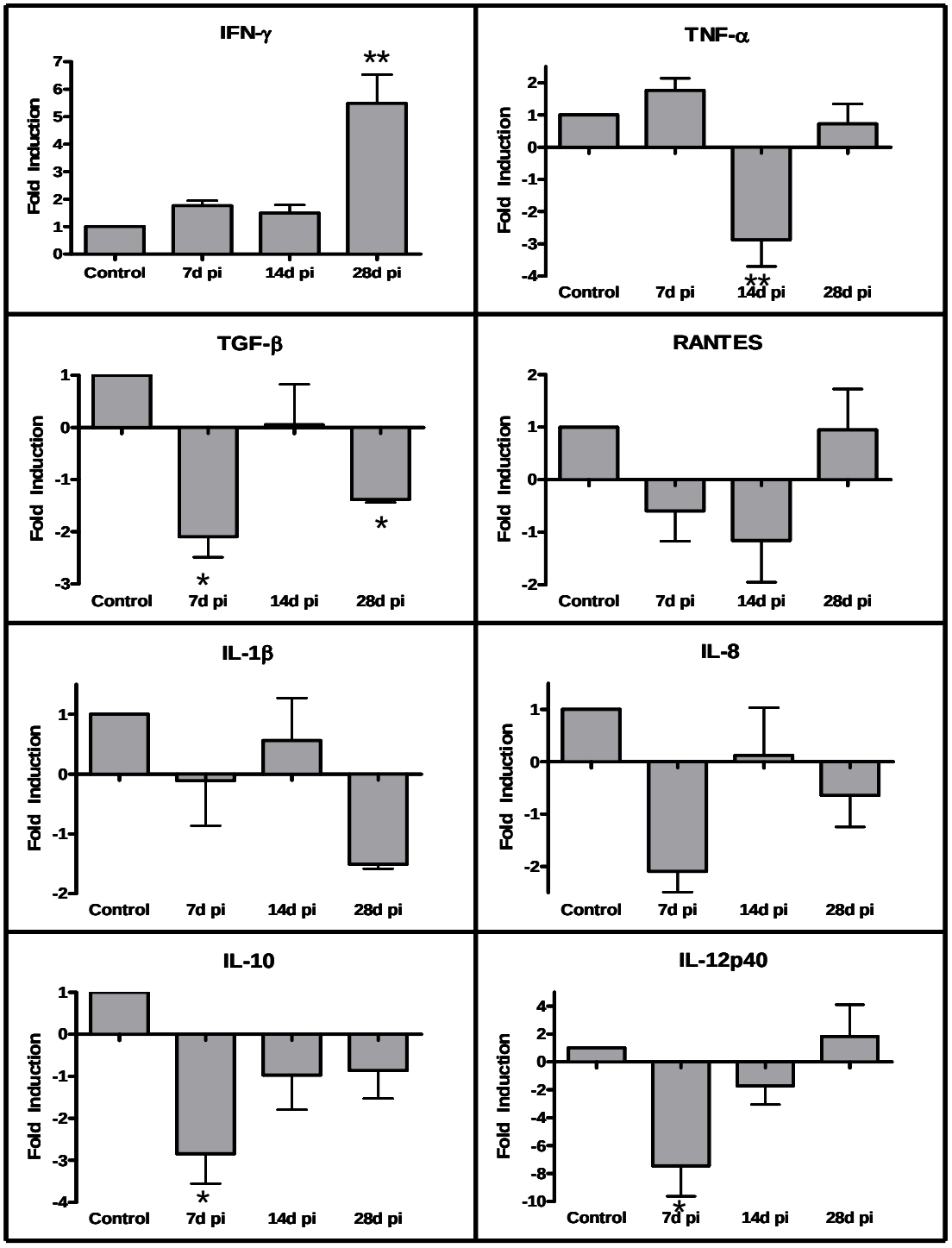

Fig. 4.2. *Ex vivo* splenocyte cytokine levels following *C. burnetii* infection. *Ex vivo* fold induction of mRNA from splenocytes derived from guinea pigs aerosol-challenged with 10^5 *C. burnetii* collected at 7, 14, and 28 days post infection compared to non-infected control animals. *p<0.05; **p<0.01.

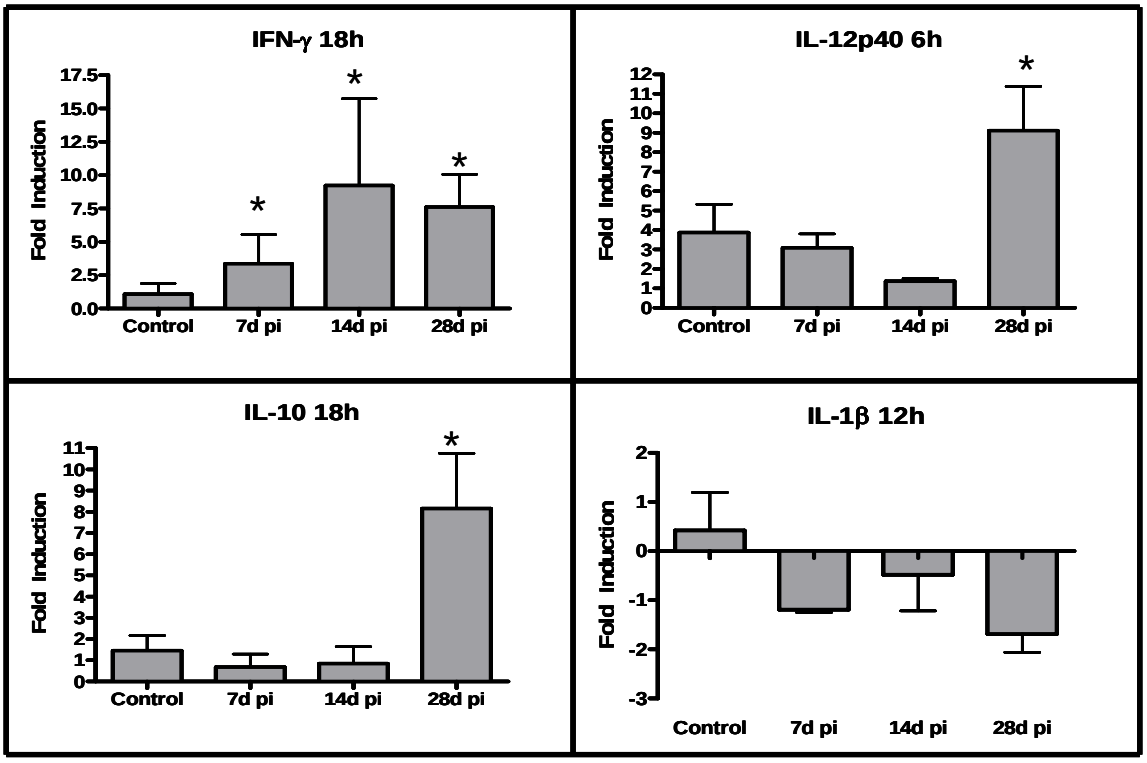

Fig. 4.3. Peak cytokine response to *C. burnetii* antigen stimulation in splenocytes from infected guinea pigs. Cytokine mRNA fold induction in response to WCV-I stimulation of splenocytes from non-infected guinea pigs and those infected for 7, 14 and 28 days. Hours reported represent peak stimulation. *$p<0.05$; *$p<0.01$.

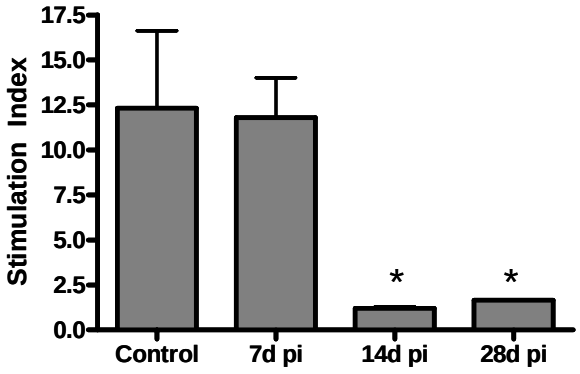

Fig. 4.4. Non-specific suppression of lymphoproliferation post infection. Non-specific suppression of lymphoproliferation of lymph node-derived cells with ConA mitogenic stimulation in response to 10^6 *C. burnetii* infection at 7, 14, and 28 days p.i. Stimulation index = counts per minute of [^{3}H]-thymidine taken up by stimulated cells ÷ the counts per minute of unstimulated cells from the same source. *$p<0.05$; **$p<0.01$.

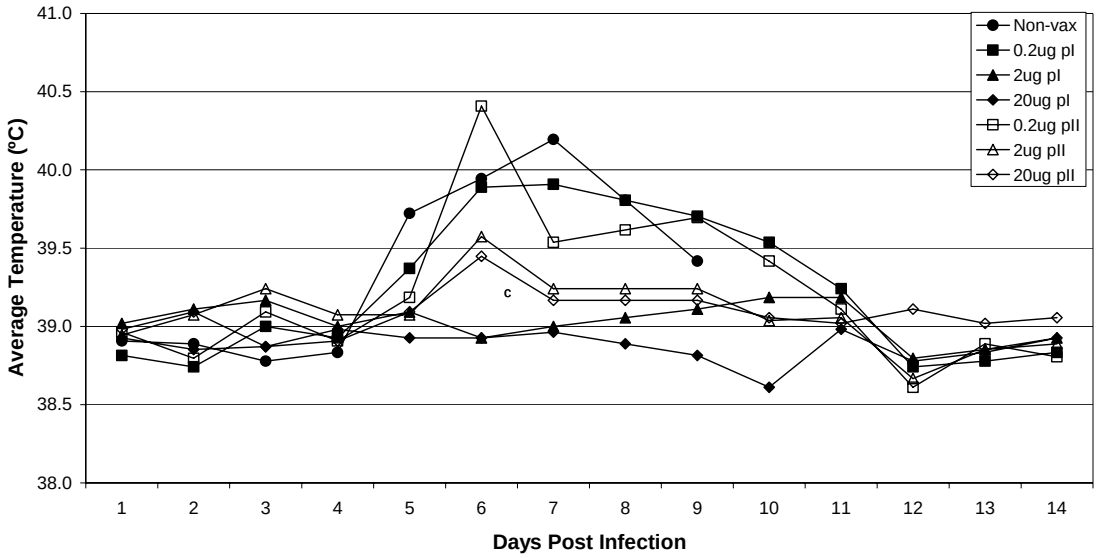

Fig. 4.5. WCV-I vs. WCV-II vaccination. Fever development post WCV-I and WCV-II vaccination. Guinea pigs receiving 2 or 20μg of WCV-I did not develop fever while those receiving WCV-II did. Fever was defined as temperatures ≥39.5°C. The non-vaccinated animals' data ends on day 9 due to death of all subjects in that group.

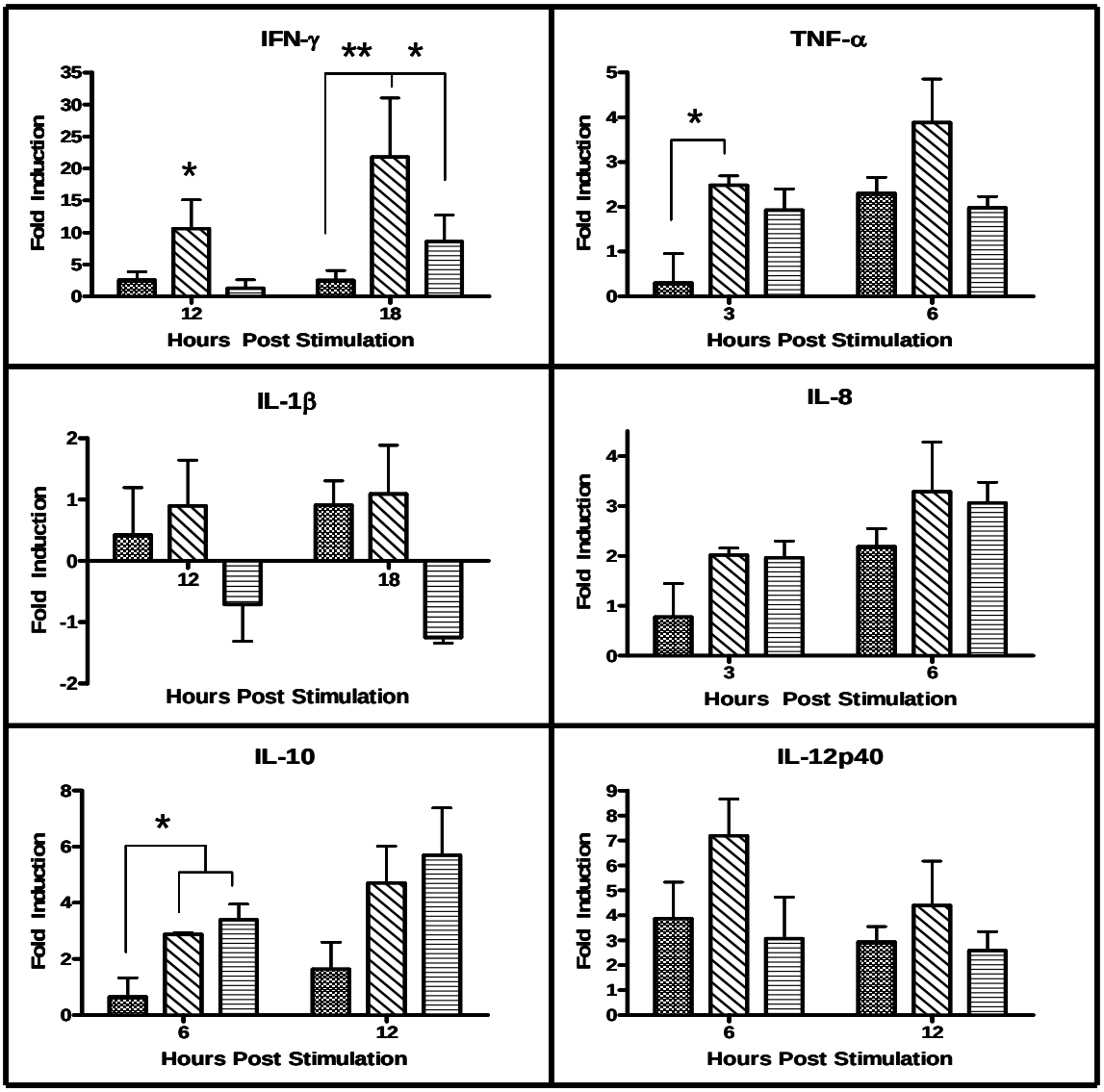

Fig. 4.6. Peak cytokine response to *C. burnetii* antigen stimulation in splenocytes from vaccinated guinea pigs. Cytokine mRNA induction in response to WCV-I stimulation of spleen cells from WCV-I (diagonally lined) and WCV-II (horizontally lined) vaccinated and non-vaccinated (checkered) guinea pigs. *$p<0.05$; **$p<0.01$.

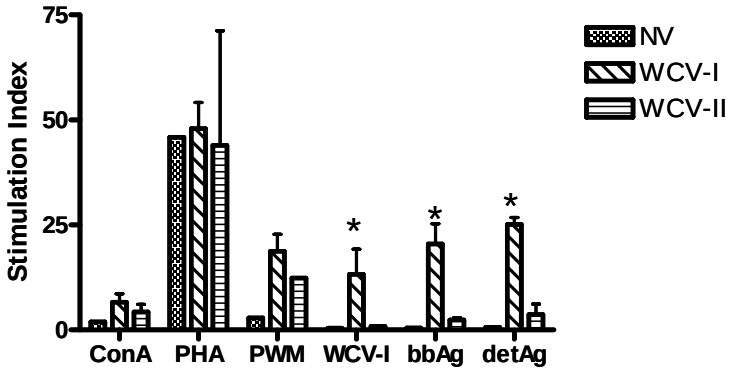

Fig 4.7. WCV-I stimulation of lymphoproliferation. Enhanced lymphoproliferation of lymph node-derived cells with WCV-I vaccination in response to mitogenic (ConA, PHA, PWM) and antigenic (WCV-I, bbAg, detAg) stimulation. Stimulation index = counts per minute of [^{3}H]-thymidine taken up by stimulated cells ÷ the counts per minute of unstimulated cells from the same source. *$p<0.05$.

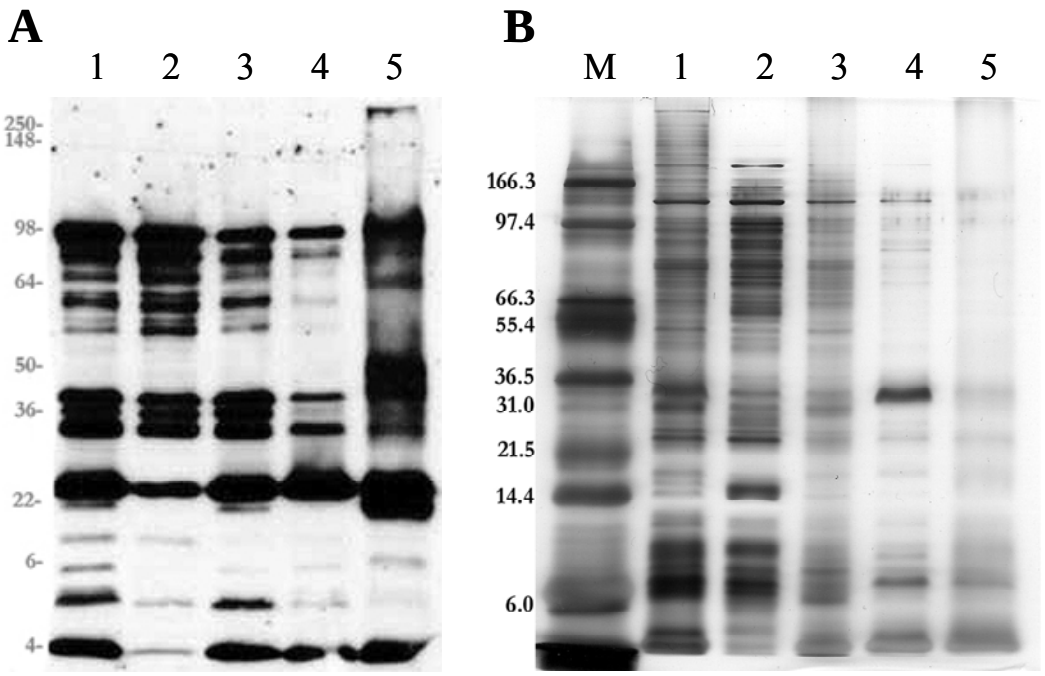

Fig. 4.8. Western blot (A) and silver stain (B) analyses of *C. burnetii* proteins. M: Mark 12 molecular weight marker; Lane 1: Phase I *C. burnetii*; Lane 2: FastProtein Blue extract; Lane 3: FastProtein Blue extract/detergent extract mixture; Lane 4: final pellet; Lane 5: WCV-I. 8µg of protein were loaded per well.

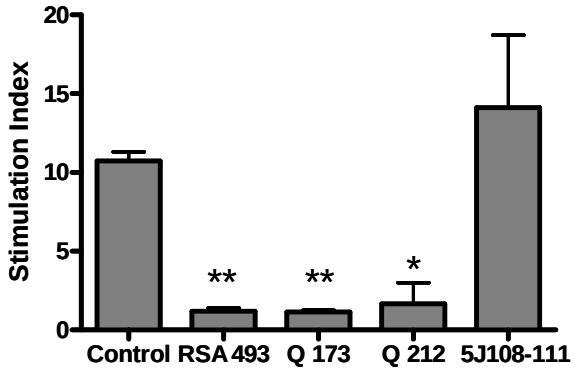

Fig 4.9. Non-specific suppression of lymphoproliferation following infection with virulent *C. burnetii* isolates. Lymphoproliferation of PHA-stimulated lymph node-derived cells from guinea pigs infected for 14 days with various *C. burnetii* isolates. Stimulation index = counts per minute of [^{3}H]-thymidine taken up by stimulated cells ÷ the counts per minute of unstimulated cells from the same source. *$p<0.05$; **$p<0.01$.

APPENDIX B

TABLES

TABLE 2.1. Response to infection with *C. burnetii*

Infectious Dose	Fever/ Total	Earliest Onset of Fever	Average Fever Duration (days)	Highest Temperature (°C)	Death/ Total	Seroconversion/ Total	ELISA Titer (range) 28days p.i.
PBS control	0/3	N/A	N/A	39.3	0/3	0/3	0
$2x10^1$	2/3	Day 14	1	39.6	0/3	3/3	100
$2x10^2$	3/3	Day 14	1.67	39.9	0/3	3/3	400-800
$2x10^3$	3/3	Day 10	2.67	39.9	0/3	3/3	1600-3200
$2x10^4$	3/3	Day 6	4	40.6	0/3	3/3	3200-51200
$2x10^5$	3/3	Day 6	6	40.9	0/3	3/3	51200-102400
$2x10^6$	3/3	Day 5	5*	40.8	2/3	1/3*	25600
$2x10^6$, vaccinated	0/3	N/A	N/A	39.4	0/3	3/3	25600-102400

*Number may be skewed due to death

TABLE 2.2. Severity* of histopathologic changes in selected tissues of guinea pigs infected with $2x10^6$ *C. burnetii*

	7 days p.i.	**14 days p.i.**	**28 days p.i**
Lung	5	4	3
Liver	4	3	3
Spleen	2	3	2

*Severity is based on the amount of architectural change, cellular infiltration, and the presence or absence of necrosis: 0, none; 1, mild; 2, mild to moderate; 3, moderate; 4, moderate to severe; 5, severe. Scores are the average of sections evaluated for 2 guinea pigs per time point.

TABLE 2.3. Histopathology* and immunohistochemistry[#] in selected tissues of guinea pigs infected with *C. burnetii*

	Lung		Liver		Spleen	
	H*	**I#**	**H***	**I#**	**H***	**I#**
PBS Control	0	-	0	-	0	-
$2x10^1$	1	-	0	-	0	+
$2x10^2$	1	+	1	-	0	+
$2x10^3$	2	+	1	+	1	++
$2x10^4$	3	+	2	++	1	++
$2x10^5$	3	++	2	++	1	+++
$2x10^6$	3	++	3	++	2	+++
$2x10^6$, vaccinated	0	-	0	-	0	+

*Histopathology: severity is based on the amount of architectural change, cellular infiltration, and the presence or absence of necrosis: 0, none; 1, mild; 2, mild to moderate; 3, moderate; 4, moderate to severe; 5, severe.

[#]Immunohistochemistry: -, no organisms present in the tissue section; +, the presence of the organism is rare and focal; ++, few organisms are multifocally present; +++, many organisms are diffusely present; ++++, abundance of organisms are diffusely present. Scores are the average of sections evaluated for 3 guinea pigs per group at 28 days p.i. The evaluation of tissue sections was performed in a blinded fashion.

TABLE 3.1. Isolates tested for virulence in aerosol-challenged guinea pigs

Genomic Group	Isolate	Original Source			
		Sample	**Year**	**Location**	**Disease**
I	Nine Mile RSA493	Tick	1935	Montana, US	n/a (acute, flu-like in humans)
	African RSA334	Human Blood	1949	Central Africa	Acute, Congolese Red Fever
	Ohio 314 RSA270	Cow's Milk	1956	Ohio, US	Persistent
IV	MSU Goat Q177	Goat Cotyledon	1980	Montana, US	Abortion
	P Q173	Human Heart Valve	1979	California, US	Endocarditis
V	G Q212	Human Heart Valve	1981	Nova Scotia, Canada	Endocarditis
	S Q217	Human Liver Biopsy	1981	Montana, US	Hepatitis
VI	Dugway 5J108-111	Rodents	1958	Utah, US	n/a

TABLE 3.2. Comparison of OD, particle count, and real-time PCR quantification of *C. burnetii* inoculum

Group	Isolate	OD	Particle Count	Real-time PCR
I	RSA493	$2x10^6$	$1.7x10^6$	$1.1x10^6$
I	RSA334	$2x10^6$	$2.6x10^6$	$3.6x10^6$
I	RSA270	$2x10^6$	$2.8x10^6$	$4.2x10^6$
IV	Q177	$2x10^6$	$8.9x10^4$	$5.5x10^4$
IV	Q173	$2x10^6$	$1.0x10^6$	$9.8x10^5$
V	Q212	$2x10^6$	$4.1x10^5$	$6.3x10^5$
V	Q217	$2x10^6$	$3.4x10^4$	$2.6x10^4$
VI	5J108-111	$2x10^6$	$6.5x10^5$	$1.3x10^5$

High dose delivered to infected guinea pigs as determined by optical density, viable particle count, and real-time PCR amplification of *com-1*.

TABLE 3.3. Response to infection with different *C. burnetii* isolates

Strain	Dose	Fever/ Total	Death/ Total	Seroconversion/ Total
RSA493	$2x10^2$	3/3	0/3	3/3
	$2x10^4$	3/3	0/3	3/3
	$2x10^6$	3/3	2/3	1/3*
RSA334	$2x10^2$	3/3	0/3	3/3
	$2x10^4$	3/3	0/3	3/3
	$2x10^6$	3/3	3/3	0/3*
RSA270	$2x10^2$	3/3	0/3	3/3
	$2x10^4$	3/3	0/3	3/3
	$2x10^6$	3/3	3/3	0/3*
Q177	$2x10^2$	0/3	0/3	3/3
	$2x10^4$	0/3	0/3	3/3
	$2x10^6$	0/3	0/3	3/3
Q173	$2x10^2$	0/3	0/3	3/3
	$2x10^4$	0/3	0/3	3/3
	$2x10^6$	1/3	0/3	3/3
Q212	$2x10^2$	0/3	0/3	3/3
	$2x10^4$	2/3	0/3	3/3
	$2x10^6$	3/3	0/3	3/3
Q217	$2x10^2$	3/3	0/3	3/3
	$2x10^4$	3/3	0/3	3/3
	$2x10^6$	3/3	0/3	3/3
5J108-111	$2x10^2$	0/3	0/3	0/3
	$2x10^4$	0/3	0/3	3/3
	$2x10^6$	0/3	0/3	3/3
PBS Control	N/A	0/4	0/4	0/4

*Numbers may be skewed due to death.

TABLE 3.4. Severity* of histopathologic changes in guinea pigs infected with high dose *C. burnetii* 28 days post infection

	Lung	**Liver**	**Spleen**
RSA493	3	2	2
Q177	1	2	1.33
Q173	1	3	2
Q212	2	1.33	1.33
Q217	2.67	2	1
5J108-111	0	0	0

*Severity is based on the amount of architectural change, cellular infiltration, and the presence or absence of necrosis: 0, none; 1, mild; 2, mild to moderate; 3, moderate; 4, moderate to severe; 5, severe. Scores are the average of sections evaluated in a blinded fashion for 3 guinea pigs per group, with the exception of RSA493.

Table 4.1. Real-time PCR primers for guinea pig genes

mRNA		Primer
IFN-γ	Forward	5’ ATTTCGGTCAATGACGAGCAT 3’
	Reverse	5’ GTTTCCTCTGGTTCGGTGACA 3’
TNF-α	Forward	5’ CCTACCTGCTTCTCACCCATACC 3’
	Reverse	5’ TTGATGGCAGAGAGAAGGTTGA 3’
TGF-β	Forward	5’ CATCGATATGGAGCTGGTGAAG 3’
	Reverse	5’ GCCGTAATTTGGACAGGATCTG 3’
RANTES	Forward	5’ CTGGCCCACTGCTTAGCAAT 3’
	Reverse	5’ CCTTGCTTCTTTGCCTTGAAA 3’
IL-1β	Forward	5’ GCCCAGGCAACAGCTCTC 3’
	Reverse	5’ GGAGTCTCTACCAGCTCAACTTGG 3’
IL-8	Forward	5’ GGCAGCCTTCCTGCTCTCT 3’
	Reverse	5’ CAGCTCCGAGACCAACTTTGT 3’
IL-10	Forward	5’ CCTTACTGGCCGGGGTCA 3’
	Reverse	5’ GCTGATCCTGTGTTTGGAAGAAAG 3’
IL-12p40	Forward	5’ CCACAGTTTCATGCCACAAGA 3’
	Reverse	5’ CCATTCGCTCCACGATGAG 3’
HPRT	Forward	5’ AGGTGTTTATCCCTCATGGACTAATT 3’
	Reverse	5’ CCTCCCATCTCCTTCATCACAT 3’

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EDUCATIONAL BACKGROUND

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