MULTIVARIATE ANALYSES OF DISEASE OUTCOMES OF CHLAMYDIAL

INFECTIONS IN CATTLE AND MICE

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MULTIVARIATE ANALYSES OF DISEASE OUTCOMES OF CHLAMYDIAL INFECTIONS IN CATTLE AND MICE

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VITA

Teayoun KIM, son of Sandoo Kim and Soohee Park, was born December 11, 1971, in Pusan, Korea. He graduated from Namil High School in 1989. He graduated from Gyeongsang National University, Jinju, Korea, with a Bachelor of Science degree in the Department of Food Science, College of Agriculture, in 1994. When he was a college student, he was trained as an R.O.T.C. cadet for two years. After two years of military service as a first lieutenant in the Army of the Republic of Korea (ROK) from 1994 to 1996, he went back to Gyeongsang National University to pursue research. He graduated with a degree of Master of Science in 1999, and entered Graduate School at Auburn University, in August 2001 to pursue a Ph.D. degree.

DISSERTATION ABSTRACT

MULTIVARIATE ANALYSES OF DISEASE OUTCOMES OF CHLAMYDIAL INFECTIONS IN CATTLE AND MICE

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In humans, *Chlamydia pneumoniae* infection is implicated in 10 ~ 15% of bronchitis and community-acquired pneumonia, and 50 ~ 70% of adults have antibodies to *Chlamydia pneumoniae*. In livestock animals, ubiquitous *C. abortus* infection is a major, if underestimated, factor influencing subclinical infertility and abortion of farm animals. Numerous factors, including population density of the host and dietary influences on immunity, influence the ultimate manifestation of any infectious disease. Only occasionally chlamydial infections manifest themselves as clinical disease.

The research presented in this dissertation addresses the question of causal involvement of chlamydial infection in a prospective study of fertility in cattle, the natural host of *Chlamydophila abortus*, in association with challenge with this organism.

In a second approach, the effect of dietary components with known influence on the immune response, protein and antioxidants, on chlamydial infection was studied by analyzing lung disease in a murine respiratory challenge model with *C. pneumoniae*.

The first study determined the effects of controlled re-infection on the fertility of cattle naturally pre-exposed to *Chlamydophila abortus*. Virgin heifers were estrus synchronized, artificially inseminated 2 to 3 days later, and challenged immediately by intrauterine administration of *C. abortus* or through indirect exposure by contact with their previously challenged cohorts. Uterine inoculum dose and cohort challenge (or, alternatively, negative pregnancy outcome = infertility) correlated highly significantly with a rise in postchallenge anti-*C. abortus* IgM levels over prechallenge levels. These data proved that local and systemic infection with *C. abortus* suppressed fertility in cattle.

The second study investigated in a balanced multivariate experimental design major factors known to influence *C. pneumoniae* disease: i) host genetic background; ii) pre-challenge immunity against *C. pneumoniae*; iii) duration of the infection; iv) dietary protein; and v) dietary antioxidants. C57BL/6 mice previously immunized against *C. pneumoniae* and fed a low-protein & low-antioxidant diet showed on day 10 post challenge inoculation severe disease due to a pronounced Th1 response as indicated by significantly ($p < 0.05$) upregulated transcripts such as those of IFN-γ, TNF-α, or IL-6. In contrast, immune A/J mice showed lowest disease outcome with Th2-biased immunity and reduced inflammation. This pattern was opposite in the early response on day 3 after inoculation. Immune C57BL/6 mice on LL diet showed a strongly Th2-biased and suppressed immune response, while A/J had a strong, Th1-biased immune response. Of all transcripts of immune C57BL/6 on LL diet on day 3, only the T cell-specific CD3δ

transcript was highly significantly ($p < 0.0001$) reduced by more than four-fold. These results strongly suggest that it is a suppressed early Th1 immune and inflammatory response that primes immune C57BL/6 mice for later severe Th1 inflammatory disease.

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All lab members importantly contributed this work. Senior researcher Dr. Alexander Vaglenov managed experimental mice, established laboratory protocols, instructed in proper laboratory practices. Research associate Dongya Gao assisted with all experiments, especially the management of primer and probe sets for LightCycler FRET-PCR. Chengming Wang provided fundamental concepts in designing of FRET PCR primer and probe sets that he developed in one year-long efforts. Dan Li produced large amounts of highly pure *Chlamydia pneumoniae* bacteria that were used in all experiments.

Finally, the author expresses his deep appreciation to his parents and his younger sister for the love, encouragement, and support during his education abroad.

Style manual or journal used	Infection and Immunity
Computer software used	Office XP (Microsoft® Word 2002) for the text and tables *STATISTICA* version 7.0 for statistical analysis and basic graphs DeltaGraph 5.6 for basic graphs Adobe® Illustrator® CS for editing of graphs

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I. INTRODUCTION

To investigate the relationship between immune response-related genes and disease outcome, many *Chlamydia* research projects use knockout cell lines or animals. This is a useful approach for observation of the function of the target gene since the differential outcome in the absence of the gene can observed. However, knockout approaches create completely new, typically in natural populations non-existent phenotypes. For these reasons, knockout approaches fail to model multifactorial diseases with redundant regulatory pathways. For example, nitric oxide is an inorganic free radical that is implicated in various pathophysiological responses such as vasodilation, nonspecific host defense, ischemia reperfusion injury, chronic inflammation and carcinogenesis (85, 118). Perry et al (126) reported that inducible nitric oxide synthease (iNOS) was not critical for clearance of *C. trachomatis* genital infection, since the kinetics of chlamydial shedding and the time required for complete clearance of infections in iNOS knockout mice were similar to their control group. In their result, the iNOS knockout mice consistently resolved infections at a slightly more rapid rate than their normal counterpart mice. In another study, Ramsey et al (133) reported that although iNOS-knockout C57BL/6 mice could resolve most of a genital *C. trachomatis* infection in a pattern similar to that of normal control mice with a significantly increased disease rate, iNOS was required for the IFN-γ mediated microbicidal effect. They also proposed (134) that iNOS would be required for reducing the disease, because

immune responses during chlamydial infection can be either protective or pathogenic. The disease outcomes of their mouse model of urogenital chlamydial infection such as infertility, histopathological changes, and hydrosalpinx formation showed a reverse relationship with nitric oxide concentration.

Considering otherwise identical experimental conditions, the host genetic background appears to be particularly important in immunity to chlamydial infection and disease. Inbred C57BL/6 mice are resistant against mouse pneumonitis caused by *Chlamydia trachomatis* MoPn, while BALB/c or A/J mice are susceptible strains (182). The C3H/HeN strain is more susceptible to *C. trachomatis* intravaginal infection than BALB/c (121). In humans, the severe inflammatory disease seems to occur in only a minority of infected individuals (18, 32).

Various protective mechanisms and indicators have been proposed for a proper immune response to eliminate the chlamydial-infection. Yang et al (183) reported that B cells are important for the T cell responses in mouse lung-infection, since they found B cell-deficient C57BL/6 mice have a significantly higher mortality rate and *C. trachomatis* growth in their lungs than control mice. On the other hand, Hawkins et al. transferred MoPn-specific CD4Th2 and CD4Th1 clones to *C. trachomatis* infected mice, and found that the $CD4^+$ Th2 clones do not provide protection against genital infection of *C. trachomatis* (55). Could we assume another B cell stimulation pathway than through Th2 cell? Th2 cells play an important role in B cell activation to make them produce antibodies against antigens. In fact, many clinical investigations use immunoglobulin level as an indicator of direct or indirect chlamydial infection (9, 71, 104). Rothfuchs et al (142) reported that macrophage, $CD4^+$, or $CD8^+$ T cell derived IFN-γ is essential for

protection against *C. pneumoniae* lung-infection in IFN-γ deficient C57BL/6 mice while NK cells were not needed for innate resistance against *C. pneumoniae* in their knockout mouse model. This contradicts the general concept about the innate immune response in which an important role of NK cells is assumed. Therefore, although knockout techniques are a useful approach to investigate the direct effect of the absence of a target gene, it might not be easy to extrapolate the results to outbred animals with a normal gene complement, since the immune response involves many factors and complex mechanisms of interaction between them.

On the other hand, Mahony (94) used a cDNA microarray technique to investigate the interaction between *C. pneumoniae* and host cells. Among 268 human genes, IL-1, IL-8, MCP-1, and various cellular growth factors were the most prominently up-regulated genes following the infection of *C. pneumoniae* to HEp2 cells. In addition, inhibition of specific host cell signal-transduction cascades and actin polymerization could abrogate *C. pneumoniae* uptake without blocking attachment to the surface of HEp2 cells. Xia et al (179) also used a cDNA microarray with 15,000 target genes and found 130 genes were differentially transcribed in HeLa cells after infection of *C. trachomatis*. In these two reports, the genes for which the expression level was increased were heparin-binding epidermal-like growth factor, basic fibroblast growth factors, platelet-derived growth factor B chain, apoptosis inhibiting factors, cell differentiating regulatory factors, components of cytoskeleton, transcription factors, and proinflammatory cytokines. These two reports are interesting, but they focused on intracellular molecular signaling mechanisms in a single type of cells. Above all, these cDNA microarray experiments were performed with cell lines that could be different from *in vivo* condition and they did

not observe other major immune cells and the relationship, such as macrophages, neutrophisl, NK cells, B lymphocytes, and various subsets of T lymphocytes.

Although many factors affect the immune response and related disease outcome, many researchers use different approaches with only a limited set of markers or gene expression data for a single cell line. Descriptive observations are needed about immune responses and disease outcome caused by *C. pneumoniae* infection to understand how different factors affect them.

II. LITERATURE REVIEW

Taxonomy of *Chlamydiales*

Based on phylogenetic trees using 16S and 23S rRNA sequence similarities of *Chlamydiales* strains, isolates showing more than 90% sequence identity with prototype strains are categorized as members of the *Chlamydiaceae* family (Figure 1). Isolates showing 80 ~ 90% sequence identity to the *Chlamydiaceae* type strains are classified into three more families termed *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae*. The *Chlamydiaceae* family has two genera; the first genus *Chlamydophila* has six species *Chlamydophila pecorum*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Chlamydophila abortus*, *Chlamydophila caviae*, *Chlamydophila felis*, and the second genus *Chlamydia* has three species *Chlamydia muridarum*, *Chlamydia suis*, and *Chlamydia trachomatis* (43).

Chlamydiaceae spp. have specific host ranges. *Chlamydia muridarum* can be isolated from mice and hamsters. *Chlamydia suis* is found in swine. *Chlamydophila pecorum* has been detected in all mammals; this species is found in ruminants (cows, sheep and goats), swine, and marsupials (koala). *Chlamydophila pneumoniae* and *Chlamydia trachomatis* are human pathogens, but *Chlamydophila pneumoniae* has also been isolated from horses and marsupials. *Chlamydophila felis* is from house cats, and *Chlamydophila psittaci* is found in birds, pigeons, ducks, turkeys, partridges, seagulls,

budgerigars, and even in humans who work in poultry slaughterhouses. *Chlamydophila abortus* has been found in many mammals, including horses, rabbits, guinea pigs, and mice and humans who work with sheep. *Chlamydophila caviae* specifically has been found in guinea pigs (43).

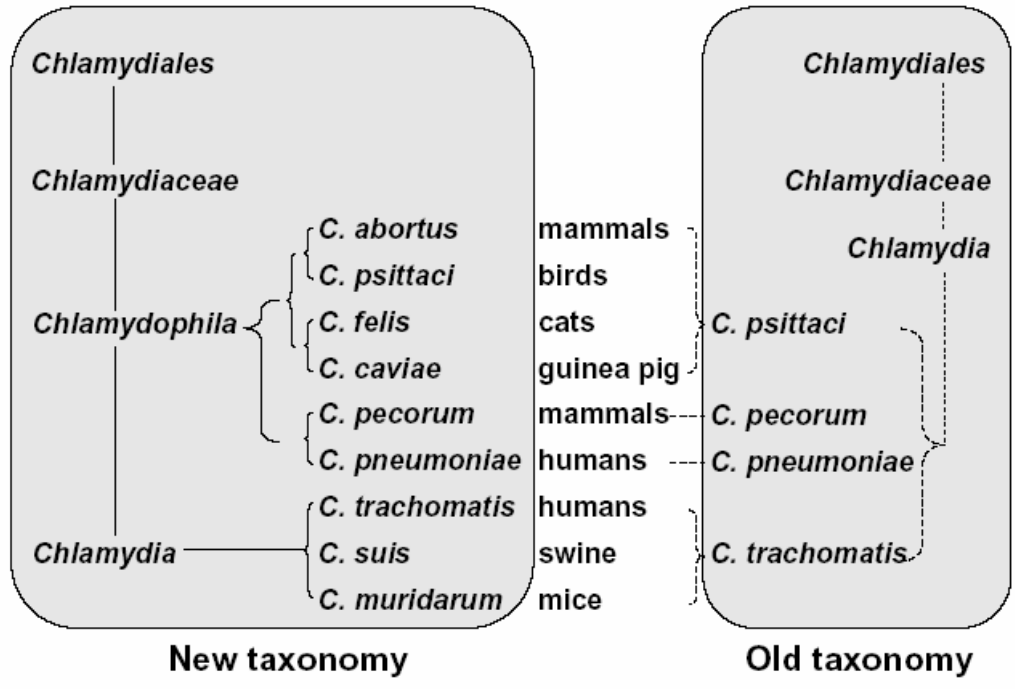

Figure 1. Genetic structure of the order *Chlamydiales*. The tree on the left depicts the recent taxonomic revision of the *Chlamydiales* (Everett et al., 1999), and on the right depicts the old taxonomy of the *Chlamydiales.* Horizontal distances are roughly proportional to genetic distances as measured by 16S rRNA sequence data and DNA-DNA hybridization. A list of typical hosts illustrates the ecological hetero-geneity of *Chlamydiaceae* species. Host ranges of strains outside the *Chlamydiaceae* are not yet well resolved (Bush et al., 2001).

Characteristics of *Chlamydiaceae* spp.

Chlamydiaceae spp. are gram-negative obligate intracellular bacteria that infect eukaryotic cells. They have a unique biphasic developmental cycle among prokaryotes consisting of the elementary body (EB) and reticulate body (RB). The infectious but

metabolically inactive EB is 200 ~ 300 nm in diameter and the membrane structure is similar to that of other gram-negative bacteria. The stable structure and inactive metabolism of EB allows *Chlamydiaceae* spp. to remain viable outside of host cells. The non-infectious but metabolically active RB is 1,000 ~ 1,500 nm in diameter and divides by binary fission, then transforms back to the EB before being released to the exterior. The membrane structure of the RB is a double-layered cytoplasmic membrane without a peptidoglycan layer (24). During transformation of the RB to the EB, the chlamydial DNA is condensed to a nucleoid form, which is unique among bacteria. This condensation confirms previous genomic sequence analysis of *C. trachomatis* in which several genes coding eukaryotic chromatin-associated proteins were identified (158). The *Chlamydiaceae* genome is about 1,100-kbp in size with a well-conserved gene order (137). In Gimenez staining RBs appear basophilic and EBs appear eosinophilic (24). These observations mean the biphasic life cycle of *Chlamydiae* is very different in terms of outer membrane structure and cell size as well as metabolic activity.

Depending on the type of extracellular milieu, *Chlamydiaceae* spp. may also appear in an intermediate phase between EB and RB named the persistent phase. IFN-γ secreted by antigen-specific $CD4^+$ and $CD8^+$ T cells activates the expression of the host enzyme, indoleamine 2, 3-dioxygenase (IDO) which catalyzes the conversion of L-tryptophan to N-formylkynurenine. Since *Chlamydiaceae* spp. are tryptophane auxotrophs, IFN-γ mediated depletion of tryptophane inhibits chlamydial growth, and the bacteria transform into aberrant non-infectious forms that persist within host cells to avoid the stress of nutritional limitation(158). Partial depletion of tryptophane allows maintenance of aberrant chlamydial forms in culture for 42 days, and infectivity could be

maintained in the presence of IFN-γ for up to 38 days. It is not clear how indoleamine 2, 3-dioxygenase activity is related to the differential regulation of chlamydial protein synthesis to maintain the persistent phase (8). An *in vitro* model of IFN-γ mediated persistence showed that chlamydiae in IFN-γ treated HeLa cells continued only DNA replication, but not cell division, thus the mass of chlamydial RB increased dramatically. After removal of IFN-γ, aberrant RBs returned to a normal pattern of cell division within six hours. Highly up-regulated chlamydial genes were those mediating tryptophane synthesis, DNA repair and recombination, phospholipid biosynthesis, translation-related genes, and down-regulated genes were those involved in RB to EB differentiation, proteolysis, peptide transport, cell division, TCA cycle, and genes related to the synthesis of outer membrane proteins (8). Natural persistent infection has also been reported. Among *C. trachomatis* positive urethritis patients, uncured individuals after azithromycin, tetracycline, macrolide, or quinolone treatment showed atypical chlamydial inclusion in urethral or endocervical specimens by electron microscopic observation (17).

Epidemiology of *Chlamydiaceae* spp.

Chlamydial infection is ubiquitous among wild and domestic animals. The prevalence of chlamydial infection in sheep in Switzerland as determined by ELISA was about 20-40% of 775 sheep serum samples (13). In the U.S., the prevalence as determined by fluorescence resonance energy transfer (FRET)-qPCR was about 53% among 51 Holstein heifer vaginal swab samples (35). In Belgium, 96.5% of 258 pig-breeding farms were determined as *Chlamydia*-positive by MOMP (major outer membrane protein)-peptide ELISA (168).

C. pneumoniae appears to be a common human respiratory pathogen, with a wide geographic distribution and affecting all ages (54). In women in the U.K. and U.S., the prevalence of *C. trachomatis* in endocervical swab or urine samples as determined by polymerase chain reaction (PCR) is about 8-12% (23, 116) In Japan, 18.5% among 70 blood donors were *C. pneumoniae* positive as determined by real-time SYBR® Green fluorescence 16S rRNA RT-PCR (181). In Italy, among 613 children who were 2-14 years old and hospitalized between 1998 and 1999 for community-acquired lower respiratory tract infections, 14% were *C. pneumoniae* positive by touchdown-nested MOMP PCR in nasopharyngeal aspirates and ELISA in serum samples (130). The highest report of *C. pneumoniae* seroprevalence in a human population as determined by ELISA using formalin-fixed elementary body antigen was 72% among 433 Taiwanese adults who were older than 20 years (89).

Diseases caused by chlamydial infection

Chlamydiaceae spp. are disseminated by aerosol or by contact, requiring no alternative vector (43). They are one of the major pathogens of sexually transmitted diseases in both animals and humans. In animals, *Chlamydiaceae* spp. cause enteritis, mastitis, encephalomyelitis, and abortion. In humans, infections by *Chlamydiaceae* spp. cause acute and chronic diseases such as pneumonia, trachoma, conjunctivitis, urethritis, and adverse outcomes in pregnancy. Chlamydial infection is also known as one of the potential co-factors enhancing various chronic diseases such as arthritis, Alzheimer's disease, atherosclerosis, and asthma (24).

Subfertility and genital tract disease

Although only 10-20% of human abortion cases worldwide show placentitis with positive culture of *C. trachomatis*, in the U.K. 60% of ectopic pregnancy patients and 70% of tubal factor infertility patients were determined as *C. trachomatis* positive by PCR (6, 32). In the U.S. from 1998 to 1999, 11% of 5,877 high school students were *C. trachomatis*-positive by PCR of urine samples (114). Infection with *C. trachomatis* in pregnant women may cause neonatal infection, premature rupture of the membrane, premature birth, perinatal death, and life-long consequence to their offspring (99). The mechanism for these diseases is acute inflammation and permanent scarring with functional impairment of the infected mucosal membranes in reproductive organs. In the case of women, the inflammation leads to formation of lesions and blockage of the Fallopian tubes. In the case of men, the inflammation progresses throughout the duct system, swells the testicles, or impairs accessory gland function. It has been shown that direct exposure of spermatozoa to *C. trachomatis* reduces the motility and kills the spermatozoa. Six hours of incubation of spermatozoa with live *C. trachomatis* serovar E resulted in 30% decreased motility, and similar exposure to the LGV serovar resulted in 10% decreased motility. The ratio of dead spermatozoa after serovar E exposure was increased by about 15%. Heat-inactivated EB did not show destructive effects, demonstrating that reducing motility and killing spermatozoa is not due to chlamydial antigens such as membrane components (60). Experimental infection with *Chlamydophila abortus* following artificial insemination of cattle showed that pregnancy rates are inversely proportional to the infection dose (36).

Ocular disease

Follicular conjunctivitis is a common ocular disease due to bacterial or viral infection characterized by moderate redness, viscous mucus, follicles in the upper and lower conjunctival tarsus, and ocular pain (53). In young adults, *Chlamydiaceae* spp.-induced conjunctivitis comprises 6-19% of all cases of chronic bacterial conjunctivitis, and mainly *C. trachomatis* is detected in conjunctival scrapings. About 30-80% of all cases of chlamydial conjunctivitis are associated with a genital primary infection; ocular infection is caused by autoinoculation from the urogenital tract or from eye to eye. Once *C. trachomatis* infect the eyelids, mild mucopurulent conjunctivitis is observed which usually is self-limiting and heals without permanent sequelae. However, repeated exposure to this pathogen results in formation of granules of adenoid tissue, and causes chronic keratoconjunctivitis characterized by subepithelial follicles and papillary hypertrophy in the subtarsal conjunctiva. As the scarring progresses, the lid margin is distorted and the lashes turn inwards and rub against the cornea. Constant trauma to the cornea leads to pain, opacity, and finally blindness. This disease caused by *C. trachomatis* was described in ancient Egypt and was given the name trachoma (Greek for "rough") (92).

Joint disease

Evidence of the involvement of *C. trachomatis* in arthritis has been accumulated by use of electron microscopy, immunofluorescence, PCR, ELISA, and rRNA hybridization techniques. In a rat model, the characteristics of arthritis in the early phase were neutrophilic synovitis with accelerated cartilage injury, and viable *Chlamydiaceae*

spp. were observed in the joints, liver and spleen. In the later phase, mononuclear lymphocytic infiltrates appear in the joints along with dysplastic cartilage injuries. Viable *Chlamydiaceae* spp. were not detected, and a distinct humoral immune response developed (67). *C. trachomatis* may disseminate via circulating monocytes/macrophages from the urogenital system to the synovium; however, among individuals who contracted genital infection, only a small portion develop chronic arthritis with a typically asymmetric pattern of joint involvement in the lower limbs. Presumably, *C. trachomatis* breaks immune self-tolerance and causes synovial degradation (173).

Neurological disease

C. pneumoniae is now considered a major co-factor along with type-1 herpes simplex virus (HSV1) in the development of Alzheimer's disease (AD) (68). In the U.K., about 90% of the population was HSV1-positive and about 66% of postmortem elder AD-patients were determined HSV1-positive by PCR (69). *C. pneumoniae* was also found in the brain of a higher proportion (85-90%) of AD-patients by PCR or immunohistochemical analysis. The main routes of chlamydial infection are presumably nasal and respiratory mucus from which *C. pneumonia* infected glial cells, perivascular macrophages, and monocytes migrate to be found around blood vessels in the AD-patients' brain (96). *C. pneumoniae* infection causes intracellular and extracellular accumulation of immunoreactive soluble protofibrillar oligomers of β-amyloid protein in the brain tissue of the AD brain (5). In a mouse model, although intraperitoneally infected *C. pneumoniae* and *C. trachomatis* were not detected in the infected mouse brains by RT-PCR and immunohistochemistry, metabolically active EBs were found in

spleen, lung, or lymph node, from day 3 through day 11 and in spinal cord 18 days after inoculation. Treatment with the antibiotic fluorophenicol reduced the severity of *C. pneumoniae* encephalitis, proving that *C. pneumoniae* could infect the central nervous system via systemic dissemination and could contribute to the pathogenesis of a chronic neurological disease (37).

Cardiovascular disease

More than 25 pathologenesis studies have indicated a correlation between *C. pneumoniae* infection and cardiovascular disease by direct detection at autopsy of *C. pneumoniae* in atherosclerotic lesions of the coronary arteries by electron microscopy, PCR, and immunocytochemistry (113, 136). In addition to the classical risk factors such as high blood pressure, high serum cholesterol, age, gender, and smoking, inflammation is another risk factor. In many cases, these risk factors may cause metabolic changes that result in increased serum C-reactive protein (CRP) concentration after infection (86). Meier et al (103) conducted a population-based case-control analysis in the U.K. from 1992 to 1997. The study included 3,315 patients (on average 75 years old) with first-time acute myocardial infarction (AMI), and 13,139 controls without myocardial infarction. They found that the prescription of tetracyclines or quinolones within 3 years prior to the index date significantly reduced ($p < 0.01$) the risk of AMI diagnosis (Odds Ratio: tetracycline 0.7, quinolones 0.45), while other antibiotics (macrolides, sulfonamides, penicillin) showed same the same odds ratio as the control group. This indirectly indicates that infections with bacteria that are susceptible to tetracyclines or quinolones may play a role in the etiology of heart disease.

However, there were also contradictory case-control studies about the relationship between chlamydial infection and adverse cardiovascular events (2, 27, 41). Luchsinger et al. found 16,139 claims for myocardial infarction among 4,925,347 claims in the pharmacy and health care service database in the U.S. from 1991 to 1997 (90). The patients were above 45 years of age (mean = 65) and received various prescription antibiotics such as macrolides (erythromycin, chlarithromycin, azithromycin), quinolones (ciprofloxacin, ofloxacin), or tetracyclines (doxicycline) for an average of 8 days. The analysis indicated a small detrimental association between antibiotic use and myocardial infarction. This may be reasonable considering the fact that in all animal models, although antibiotic treatments markedly decreased *C. pneumoniae* culture positivity of lung tissue, chlamydial DNA or the antigens could not be totally eradicated with antibiotics such as azithromycin, azithromycin plus rifampicin, doxycycline, amoxicillin, amoxicillin-clavulanate, erythromycin, fluoroquinolones, and telithromycin (164). Successful antibiotic treatment against chlamydial infection requires vigorous, long-term, carefully controlled administration. The EB is not susceptible to antibiotics, while the RB is susceptible but is also capable of entering a persistent phase for an indeterminate time in which it is not susceptible to antibiotics (51).

Pulmonary disease

The clinical signs of *C. pneumoniae* and *C. trachomatis* pulmonary infection are different those precipitated by other pneumonia-causing pathogens. Most cases of pneumonia caused by *C. pneumoniae* infection are asymptomatic. In symptomatic cases (about 30%), patients showed mild symptoms such as decreased pulse rate and white

blood cell counts, and increased diastolic blood pressure and arterial carbon dioxide tension (54, 152). Although occasionally avian *Chlamydophila psittaci* are found in humans with acute respiratory distress symptoms (165), pneumonia is caused mainly by *C. pneumoniae* among *Chlamydiaceae* spp. Of all cases of community-acquired pneumonia (CAP), about 40% are caused by infection with various respiratory pathogens such as influenza virus type A, adenovirus, respiratory syncytial virus, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, or *Moraxella catarrhalis*. The most frequently found microorganisms covering above 30% of CAP cases are *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, and *Haemophilus influenzae*. The proportion of *C. pneumoniae* among CAP, bronchitis, and sinusitis cases is about 3-15% (24, 64, 100, 104). Gender, smoking history, and symptoms such as cough, sputum, sore throat, and chest pain, were not significantly different among *C. pneumoniae*, *S. pneumoniae*, and *M. pneumoniae*-infected patients in Japan (104). However, Marrie reported that a higher frequency of *C. pneumoniae* seropositive cases was observed in males, smokers, and elderly individuals in Canada (100). The pneumonia associated with *C. pneumoniae* was clinically indistinguishable from other pneumonias, and secondary infection with other pathogens was frequent (54).

The mechanism of secondary infection may be explained by an *in vitro* experiment. *C. pneumoniae* completely inhibited the ciliary motion of ciliated human bronchial epithelial cells 48 hours after infection, while *C. trachomatis*-infected and mock-infected cells showed unchanged ciliary activity. Therefore, *C. pneumoniae* may specifically impair the mucociliary clearance function that is the first phase of the

respiratory defense system, and thus and make the host more susceptible to other pathogens (152). Another report showed that the major target cells of *C. pneumoniae* in an acute chlamydial infection model with human vital lung specimens were alveolar macrophages. Within 48 hours after experimental infection, about 30% of alveolar macrophages were infected by *C. pneumoniae* while only about 4% of alveolar epithelial cells were infected and bronchial epithelial cells were not infected. In respiratory specimens from chronic chlamydial infection, the percentage of infected type II alveolar epithelial cells was significantly higher ($p < 0.05$) than of type I alveolar epithelial cells, while bronchial epithelial cells were virtually not infected (143).

Considering the high rate of *C. pneumoniae* seropositivity in epidemiological data, it is plausible to assume that everyone acquires *C. pneumoniae* infection at least once throughout their lifetime, but the disease outcome is variable. There have been several reports about *C. pneumoniae* detection in healthy individuals who do not show symptoms of respiratory tract infection. Birkebaek et al (11) found similar *Chlamydia*-specific IgG titers in patients and healthy individuals: 45% among 201 chronic coughing patients and 40% among 106 healthy blood donors. Miyashita et al (104) found that 14 of 1,018 specimens tested were *C. pneumoniae* positive by PCR or culture, and of the 14 individuals, eight had *Chlamydia*-specific antibodies but four individuals were IgG and IgM antibody titer negative against *C. pneumoniae*. Therefore, immunoglobulin levels or presence of *C. pneumoniae* cannot explain the chronic cough, and complicated underlying mechanisms might precipitate chlamydial lung disease.

Immune responses against chlamydial infection

It has been well known that host immune responses against infection are different depending on the type of pathogen. In 1995, Ferrick et al. reported two different types of $CD4^+$ T-helper cells in C3H/HeJ mice (45). Intraperitoneal infection with intracellularly replicating *Listeria monocytogenes* bacteria made one fraction of peritoneal $CD4^+$ T-helper cells produce IFN-γ. At peak production on day 5, the number of IFN-γ producing cells was 10-fold higher than of $CD4^+$ T-helper cells producing IL-4. Conversely, infection with the extracellular parasite *Nippostrongylus brasiliensis* prompted $CD4^+$ T-helper cells to produce IL-4 at about a 10-fold excess over IFN-γ on day 10. These experiments differentiated $CD4^+$ T-helper cells into two types designated as Th1 or Th2 cells. Th1 cells generate cellular immunity against intracellular pathogens, and Th2 cells promote humoral immunity against extracellular pathogens. Th1 and Th2 cells are not derived from distinct lineages but develop from the same T helper cell precursors in dependent of the cytokine milieu (141). The Th1 response is defined by the production of IL-2 and IFN-γ, while the cytokine profile of the Th2 response is IL-4, IL-5, IL-6, IL-10, and IL-13. Both types of cells produce IL-3, TNF-α and granulocyte-macrophage colony stimulating factor (GM-CSF) (77).

Similar to other intracellular pathogens, the main protective immune response against infection with *Chlamydiaeceae* spp. is cellular Th1 immunity, although there are contradictory reports about the effect of the Th2 response against chlamydial infection. Yang et al (182) reported that B cell-deficient C57BL/6 mice showed a three-fold higher mortality and higher chlamydial lung burden over 30 days after lung infection with *C. muridarum* (*C. trachomatis* mouse pneumonitis biovar MoPn). Interestingly, the

knockout mice also showed two-fold reduced delayed type hypersensitivity (DTH) response, determined by footpad swelling 13 days after infection, as compared to wild type mice. *In vitro* re-stimulated splenic T cells from knockout mice completely or partially failed to produce cytokines such as IFN-γ, IL-6, and IL-10. The results show that B cells are necessary not only for producing antibody against *C. muridarum* but also for the initiation of efficient T cell responses against pulmonary chlamydial infection, and this is related to impaired cytokine production. Hawkins et al (55) produced a *C. trachomatis*-specific $CD4^+$ Th2 cell clone (Th2-MoPn) and a $CD4^+$ Th1 cell clone (Th1-MoPn) for use in a MoPn genital tract infection model. They transferred these cells into *C. muridarum*-susceptible BALB/c mice that had been infected with *C. muridarum* 10 days earlier. Mice that received Th1-MoPn cells cleared the infection to a basal level within 50 days after infection, while mice that received Th2-MoPn cells, non-specific Th1-cells, or control mice showed continuous high levels of MoPn in their genital tracts for up to 80 days after infection, even in the presence of significantly higher ($p < 0.01$) anti-*C. muridarum* IgG and IgA levels than the Th1-MoPn recipient mice. To track cell migration patterns, they labeled T cells with fluorescent dye and transferred them to BALB/c mice on day 7 after vaginal infection. Eighteen hours later, they found significantly fewer ($p < 0.0001$) Th2-MoPn cells than Th1-MoPn cells in the genital tract, but similar numbers of cells were in mesenteric lymph nodes and iliac lymph nodes.

It is difficult to know whether immune responses to chlamydial antigens are linked to the immunopathological mechanisms that operate in chlamydial-chronic diseases. Latvala et al (84) measured IgM levels and *in vitro* proliferative response against *C. pneumoniae* of peripheral blood lymphocytes from 291 patients who had

consulted a doctor because of respiratory tract symptoms and acute fever. Among them, only 16 patients were diagnosed as *C. pneumoniae*-specific IgM positive and nine of the 16 were in the positive range of cell-mediated immune responses. This implies that our understanding of immune mechanisms in natural human chlamydial infection is incomplete.

Experimental infection models offer advantages over epidemiological studies in the analysis of the host immune response against chlamydial infection. Penttila et al (124) reported on the immune response of BALB/c mice against lung infection of *C. pneumoniae*. Although BALB/c mice is a mouse strain susceptible to intracellular infection, primary infection in their model peaked during the first 2 weeks then gradually decreased over 27 days after infection. Mice with secondary infection showed a 100-fold lower bacterial burden in their lungs than mice with primary infection. The expression level of IFN-γ in cells from secondary infected lung tissues increased significantly, while IL-4 and IL-10 levels were low. After secondary infection, the influx of immune cells was significantly increased ($p < 0.001$).

Genetic factors are known to affect the immune response by modulating either resistance or susceptibility against various diseases such as malaria, leprosy, tuberculosis, AIDS, mucocutaneous leishmaniasis, and hepatitis B. Several human case-control studies in Africa indicated that polymorphisms in the promoter region of the TNF-α gene and the IL-10 1082G allele are associated with trachomatous scarring. In addition, various HLA genes, class I allele HLA-A31, HLA class II DQA0102, DQA1010, DQB0501, DQAq0401, and DQB10402 alleles have been linked to differential outcomes of *C. trachomatis* genital disease sequelae (94). Yang et al (182) showed different

immune responses of the C57BL/6 and BALB/c mouse strains against *C. muridarum*. C57BL/6 mice showed lower weight loss with faster weight recovery, lower mortality, and a faster clearance rate of *C. muridarum* than BALB/c mice. Twenty-five days after intranasal infection with 10^5 IFU of MoPn, C57BL/6 mice completely cleared the organism, while BALB/c mice still had a titer of about 10^4 IFU/lung. C57BL/6 mice showed stronger DTH to footpad injection of *C. muridarum* antigens than BALB/c mice, but the IgG1antibody titer was higher in BALB/c mice. The IFN-γ and IL-10 ratio of the two strains showed exactly opposite patterns, with a Th1 response in C57BL/6 mice and Th2 response in BALB/c mice.

In summary, complex mechanisms underlie the disease outcome of chlamydial infection, and we need to consider host genetic factors as well as the bacteria to understand the mechanisms of ubiquitously present chlamydial infections in animals as well as the human population.

III. RESEARCH OBJECTIVES

Understanding the mechanism of ubiquitous chlamydial infection requires animal models with proper indicators or predictors. To increase the understanding of natural *C. abortus* infection as well as of murine experimental lung infection with *C. pneumoniae*, investigations with the following specific research objectives were performed:

1) Investigate the immune response of cattle against experimental *Chlamydophila abortus* infection by use of ELISA detection of anti-*C. abortus* immunoglobulins, and determine the relationship between the immune response and fertility in cattle experimentally infected with different challenge doses. The results of this research have been published in *Infection and Immunity*. 2004. **72**:2538-2545.

2) Investigate the major parameters that influence disease outcome and bacterial burden in the lungs of mice infected with *Chlamydia pneumoniae*. This will be accomplished primarily by use of one-step duplex FRET real-time RT-PCR for the quantification of target transcripts.

IV. RE-INFECTION WITH *CHLAMYDIA ABORTUS* BY UTERINE AND INDIRECT COHORT ROUTES REDUCES FERTILITY IN CATTLE PRE-EXPOSED TO *CHLAMYDOPHILA*

INTRODUCTION

The intracellular bacterium *Chlamydia* (*C.*) *abortus* is well recognized as the infectious agent of epizootic bovine abortion, and its role in this disease has been reviewed previously (157). Abortion probably follows systemic infection subsequent to inhalation or ingestion of elementary bodies (EB), the infectious form of *Chlamydia* spp., but may also result from direct inoculation of the reproductive tract with EB during sexual contact. Although cattle may experience clinical disease subsequent to inoculation with *C. abortus*, the more typical response is a balance between host and parasite that results in chronic inapparent infection (157). Although economic losses caused by epizootic bovine abortion are readily apparent, infection by the routes described above also occurs during breeding and may result in unrecognized economic losses as the result of subclinical infertility (27, 125).

The ruminant abortion strain of *C. abortus* (previously termed *Chlamydia psittaci* serovar 1, biovar1, *omp1* strain B577) (57) is not host species- or tissue-specific, and is also a leading cause of abortion in sheep (123). Human beings are also susceptible to infection with this strain of *C. abortus*, contracted predominantly during unprotected aid

in delivery of aborted lambs (62, 93, 157). Infected human beings may exhibit flulike symptoms, and pregnant women have a high probability of abortion if clinical signs of disease are apparent (63, 93). Cattle raised under normal animal husbandry conditions invariably have high antibody titers against *Chlamydia* spp., presumably due to the ubiquitous presence of infections such as genital infection with the ruminant abortion strain of *C. abortus* (34, 43). Bovine infertility associated with *C. abortus* infection has been addressed, but not extensively characterized (34, 43, 74, 123, 148, 150). The role of *C. abortus* challenge dose on fertility and relative importance of route of inoculation, is unknown, even though it is recognized that bulls can shed *C. abortus* in semen, that breeding bulls that shed *C. abortus* in semen have decreased fertility, that semen spiked with *C. abortus* is associated with reduced fertility, and that uterine challenge with *C. abortus* can cause metritis (31, 75, 175).

Cattle typically experience a first infection with *C. abortus* and other chlamydial strains at an early age and respond by establishing immune responses (33, 43). This presents the interesting question that if cattle with established immunity have the ability to effectively control infection, what are the consequences when these animals eventually and almost invariably become reinfected?

In this study we analyzed the consequences of re-infection with *C. abortus*, by uterine and cohort challenge, on the fertility of heifers pre-exposed to this agent. We report that pre-exposed heifers are fully susceptible to depression of fertility, with fertility being dependent on uterine and cohort challenge dose and anti-*Chlamydia* immune status. The results indicate that subclinical infection with *C. abortus* may profoundly affect bovine herd health and production.

MATERIALS AND METHODS

Cultivation of *Chlamydia*. *C. abortus* strain B577 (VR-656; American Type Culture Club, Manassas, VA, USA), isolated from ovine abortion (160) was cultured in buffalo green monkey kidney cells (BGMK) and NCI-H292 cells (BioWhittaker, Walkersville, MD), as described previously (73). Briefly, cells were maintained in MEM (Life Technologies, Gaithersburg, MD, USA) containing 5% fetal bovine serum (FBS), 25 μg/ml gentamicin, and 2 μg/ml amphotericin B. Confluent monolayers were inoculated with *C. abortus* by centrifugation, and the medium changed to Iscove's modified Dulbecco's medium (Life Technologies) supplemented with gentamicin, amphotericin B, and 10% FBS. One week later, 0.25% glucose was added and released chlamydial EB were harvested in culture medium 3-4 days later. Crudely purified EB were prepared by sonication and centrifugation, as described previously (73). Briefly, cell culture medium containing released EB was sonicated on ice and cell nuclei removed by centrifugation. The EB in supernatant were sedimented by high-speed centrifugation on a step gradient composed of 50% sucrose and 30% renografin-76 in 30 mM Tris-HCl (pH 7.3, Bracco Diagnostics Inc., Princeton, NJ, USA). Sedimented EB were washed twice in sucrose-phosphate-glutamate buffer (SPG), were frozen at -85°C, and served as concentrated *C. abortus* stock preparation. Inclusion forming units (IFU) of the *C. abortus* stock were determined in shell vial cultures by staining of 30-hour infected BGMK cells with monoclonal antibody against chlamydial LPS (BioRad, Woodinville, WA, USA). Five different doses of *C. abortus* B577 (0, 10^4, 10^5, 10^6, and 10^8 IFU) were produced, by diluting stock *C. abortus* preparation in SPG buffer to a final volume of

400 µl, and placed in ½ ml wick and powder polyvinyl chloride embryo transfer straws (Professional Embryo Transfer Supply, Inc, Canton, TX, USA). Straws were sealed with polyvinyl powder (Professional Embryo Transfer Supply, Inc., Canton, TX, USA), cooled slowly to -85°C, and stored in liquid nitrogen.

***C. abortus* serology.** Sera were harvested from blood collected at the start of the experiments before heifers were challenged with *C. abortus* and again at the conclusion of the experiments. Sera were tested by enzyme-linked immuno sorbent assay (ELISA) for antibodies against *C. abortus* strain B577 elementary body lysate, using a previously reported method that has been modified as a chemiluminescent assay (123, 160).

Holstein heifer model. Virgin Holstein heifers (n=32) were fed Bermudagrass hay and supplemented twice a day with a grain-based concentrate, and housed on Bermudagrass pasture. Mean ±SD age of heifers was 15.2 ±1.16 months old. All animal procedures were approved by Auburn University's Institutional Animal Care and Use Committee.

Heifers were observed for standing estrus twice daily over 30 minute observation periods. Standing estrus was defined as a heifer that would stand to permit another heifer to mount. In addition, heifers were fitted with pressure-sensitive radio transmitters applied immediately cranial to the tailhead (HeatWatch®, DDx Inc., Denver, CO, USA). Mounting data were continually collected by a radio receiver that down loaded to a desktop computer. Commercial software was used to analyze mounting data, to determine onset of estrus (HeatWatch®, DDx Inc., Denver, CO, USA). Estrus was synchronized in all heifers with a single injection of dinoprost tromethamine (25 mg, intramuscular injection, prostaglandin F2, Lutalyse®, Pharmacia & Upjohn Company,

Kalamazoo, MI, USA; n=32). Two weeks later, heifers that were not bred after the first injection (Group 2, n=10), received an additional injection of dinoprost tromethamine.

Heifers in standing estrus were bred by artificial insemination using semen from a bull of proven fertility (Rubytom, Southeast Select Sires, Franklin, TN, USA). Semen was stored in liquid nitrogen and thawed in a water bath at 35°C for 45 seconds and placed immediately into the uterine body, approximately 1 cm from the cervix.

Heifers were challenged with uterine *C. abortus* five minutes after breeding. The inoculum was thawed in a water bath at 35°C for 45 seconds and placed approximately 1 cm into the uterine body. Heifers were randomly assigned to five groups and each group was challenged with a different dose of *C. abortus* (0, 10^4, 10^5, 10^6, and 10^8 IFU). There were six heifers per treatment group for a total of 30 heifers. Two additional heifers, reserved as "spares", were not used in the experiment.

Heifers that received intra-uterine challenge with 0 to 10^8 IFU *C. abortus* were randomly assigned to one of two groups. Heifers assigned to Group 1 were bred and challenged at the onset of the experiment and served as a group with no cohort challenge to *C. abortus*. Breeding and challenge of Group 2 heifers were delayed for 2 weeks, so that Group 2 heifers would be exposed to maximum genital tract shedding of *C. abortus* from the heifers in Group 1, and served as a group with cohort challenge to *C. abortus* (57). The goal of this approach was to coincide maximum vaginal shedding of Group 1 heifers with the implantation of embryos into the uterine wall of Group 2 heifers, at which time the Group 2 heifers were at the highest level of susceptibility to infection with *Chlamydia*. Pregnancy status was determined by manual rectal palpation at 42 days post breeding. Two months after the completion of this experiment, all heifers that were not

diagnosed pregnant were synchronized again with dinoprost tromethamine and bred a second time.

Validation of the cohort challenge model. Two additional experiments were conducted to validate the cohort challenge model. The first validation experiment was designed to provide a cohort challenge that was similar to the original experiment. The estimated relative cohort challenge of the initial experiment was duplicated with a uniform first-round uterine inoculum of 3×10^7 IFU *C. abortus* strain B577 in 15 out of 32 heifers (47%). Relative cohort challenge was estimated as the sum of the log inoculum IFUs of first-round uterine-inoculated heifers multiplied by the number of first-round uterine-inoculated heifers divided by the total number of heifers: relative cohort challenge = (15×7.5)(15/32) = 52.7; relative cohort challenge of the initial experiment = (5×8+5×6+3×5+3×4)(16/32) = 48.5. The experiment was also modified to reduce the effect of direct uterine challenge on fertility, by using a uterine challenge dose of 3×10^7 IFU *Chlamydia* that was 33% of the high dose used in the initial experiment (10^8 IFU). The goal of this approach was to retain the overall amount of vaginal shedding, compared to the initial experiment, while allowing some of the uterine challenged heifers to become pregnant.

The second validation experiment was designed to reduce the effect of both the overall cohort challenge and direct uterine challenge with *Chlamydia* on fertility. This was achieved by 1) reducing the number of heifers that received uterine *Chlamydia* in the first period to 7 heifers, as compared to 15 heifers in the first validation experiment; 2) reducing the challenge dose to 6.67×10^6 IFU, compared to the 3×10^7 IFU dose that was used in the first validation experiment. This resulted in a relative cohort challenge of

(7×6.82)(7/32) = 10.4. Two months after the completion of the first and second validation experiments, heifers that did not become pregnant were again synchronized with dinoprost tromethamine and bred a second time.

Statistical analysis. Paired sera from heifers, collected before and after challenge with *C. abortus* and tested by ELISA, were evaluated for changes in immunoglobulin isotype concentration with the Wilcoxon Signed Rank Test (159). The influence of uterine and cohort challenge, and pre-challenge antibodies on post-challenge antibody levels was analyzed by multivariate analysis of covariance (MANCOVA). In all analyses in this study, uterine chlamydial challenge was evaluated as a continuous variable using log10 transformed IFU values of the intrauterine inocula. Changes in concentrations of anti-*Chlamydia* antibodies over time were evaluated by using the ratio of post-challenge to pre-challenge antibody concentration, with values above and below 1 indicating increases and decreases of antibodies over time, respectively. This ratio was termed "antibody trend".

Antibody trends, by immunoglobulin isotype, for fertile versus infertile, cohort versus non-cohort challenged heifers, and low versus high pre-challenge antibody levels, were analyzed by Student's t-test (177). The effects of uterine *C. abortus* dose and cohort challenge, and alternatively of pregnancy outcome, on antibody trend, by anti-*C. abortus* immunoglobulin isotype, were evaluated using the general linear model for analysis of variance (27).

Pregnancy outcome versus logarithm uterine *C. abortus* IFU dose, cohort challenge with *C. abortus*, and antibody concentration against *C. abortus* was statistically modeled with logistic regression, using iteratively reweighted least squares to estimate

model parameters and forward selection (33, 150). IFU dose data were logarithmically transformed before modeling. Antibody data were transformed to dichotomous data as either above or below median values before modeling. Log (odds of infertility) was modeled as Log (odds) = $b_0 + b_1x_1 + b_2x_2 + b_3x_3$, where b_0 is the intercept, b_1, b_2, and b_3 are regression coefficients, and x_1, x_2, and x_3 are the independent variables log IFU challenge dose (0, 4, 5, 6, 8), cohort challenge (0, 1), and antibody level (0, 1), respectively. Probability of infertility was calculated as probability = odds/(1 + odds) (2, 6, 7, 37). Values of $p < 0.05$ were considered to be significant.

RESULTS

Holstein heifers have high pre-challenge anti-*C. abortus* antibody levels.

The levels of pre-challenge immunoglobulin isotypes were high in all heifers, with median (minimum-maximum) values of 17298 (5248-40376), 15761 (3960-28321), 7059 (3168-14838), and 837 (36-9605) for IgM, IgG, IgG_1, and IgG_2, respectively, for the entire group of heifers (Figure 2). These data indicate that all of these virgin heifers had been exposed to *C. abortus* prior to challenge infection, and that the infection occurred by non-sexual transmission (34). Antibody levels of this magnitude are consistent with recent or ongoing infection with *Chlamydophila*. For each uterin *C. abortus* inoculation dose, levels of the antibody isotypes in the single pre- and post-challenge paired serum samples were compared using the Wilcoxon Signed Rank Test, for each uterine *C. abortus* inoculation dose.

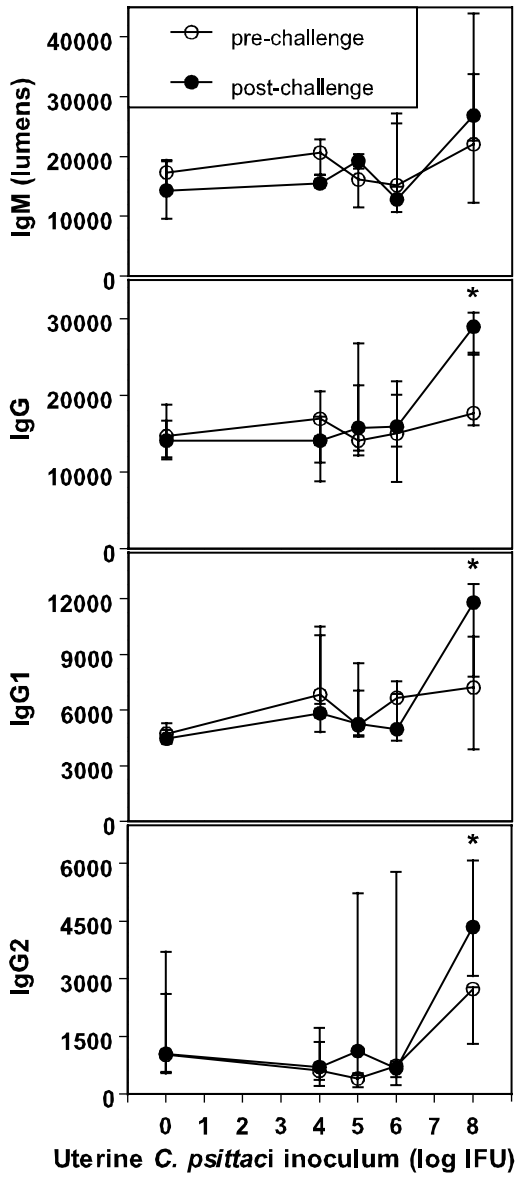

Figure 2. **Uterine inoculation of heifers with *C. abortus* increases pre-existing anti-*C. abortus* serum antibody levels in a dose-dependent manner.** Estrus was induced in a total of 32 Holstein heifers by injection with dinoprost tromethamine. Thirty heifers responded with estrus and received a uterine challenge of *C. abortus* strain B577 via the cervix, 6 heifers each per challenge dose of 0, 10^4, 10^5, 10^6, or 10^8 IFU. Serum samples were collected 3-5 weeks before and 7-9 weeks after inoculation. Median and quartile lumen values are shown for bovine immunoglobulin isotypes IgM, IgG, IgG1, and IgG2 in a chemiluminescent ELISA using *C. abortus* strain B577 elementary body lysate antigen. Asterisks indicate significantly different ($p < 0.05$) pre- and post-challenge serum antibody levels. The increase in antibody levels at high challenge inocula suggests that these inocula sufficiently breached anti-chlamydial immunity to establish infections that resulted in substantial stimulation of the immune response to *C. abortus*.

Values for IgG, IgG_1, and IgG_2 of heifers that received the highest uterine challenge dose of 10^8 IFU of *C. abortus* increased significantly after challenge with *C. abortus* ($p = 0.028$). IgM antibody levels also increased in heifers challenged with 10^8 IFU, but this increase was not significant. Anti-*C. abortus* IgM, IgG, IgG_1, and IgG_2 did not significantly increase in heifers challenged with 0, 10^4, 10^5, or 10^6 IFU of *C. abortus* (Fig. 2). The pattern of significant changes suggests that the three significant increases in post-challenge antibody concentrations are not related to type I error in the 20 tests performed (4 antibody isotypes × 5 *C. abortus* inocula).

The increased post-challenge serum anti-*C. abortus* antibody concentrations after the highest uterine challenge suggest that this inoculum sufficiently breached anti- *C. abortus* immunity to establish an infection that substantially stimulated the immune response to *C. abortus*. Despite this clear antibody reaction to *C. abortus* infection, none of these heifers, nor any heifer challenged with lower inocula, showed any signs of overt systemic or genital disease (e.g., anorexia, clear signs of vaginal inflammation) throughout the course of the experiment. The only clinical deviation observed after uterine inoculation with *C. abortus* was that none of the heifers that failed to conceive had a normal estrus cycle of 21 days. These non-pregnant heifers returned to standing estrus after 30 (±10) days.

Cohort and high uterine challenge with *C. abortus*, or infertility, predict rises of anti-*C. abortus* IgM.

To gain further insight into the factors affecting the antibody responses, we used the ratios of post-challenge to pre-challenge serum anti-*C. abortus* antibody concentrations as indicators of the change in antibody levels over the course of the

experiment, i.e. as marker for the stimulation of the antibody response. These ratios, subsequently termed antibody trends, indicate a rise in antibodies if they are larger than 1 while they indicate a decline if they are lower than 1 (Fig. 3). Cohort challenge correlated with an increased IgM trend versus no cohort challenge (mean of all intra-uterine challenge groups: 1.30±0.46 vs. 0.95±0.38, $p = 0.04$), but IgG isotype trends did not differ significantly between cohort and non-cohort challenged animals. The antibody trends for all immunoglobulin isotypes were higher in infertile (non-pregnant) animals than in fertile ones (mean of all intra-uterine challenge groups: IgM: 1.39±0.46 vs. 0.85±0.24, $p = 0.00$; IgG: 1.58±0.74 vs. 0.99±0.24, $p = 0.01$; IgG1: 1.53±0.75 vs. 0.98±0.34, $p = 0.01$; IgG2: 3.69±5.04 vs. 1.88±2.41, $p = 0.20$). Antibody trend data for IgM, the isotype with the strongest association to experimental parameters and outcomes, are shown in dependence on uterine inoculum in Fig. 3. Overall, the antibody trend data suggest that IgM anti-*C. abortus* antibodies, more than IgG antibody isotypes, might be closely associated with parameters affecting the stimulation of the immune response by the *C. abortus* infection, and potentially the functional outcome of the infection.

The high levels of post-challenge IgM of heifers that were infertile in the challenge experiment predicted increased resistance against *Chlamydophila*-mediated infertility and likely high fertility in a second breeding, provided the challenge inoculation had not caused irreparable uterine injury. Sixty days after initial challenge with *C. abortus*, 10 non-pregnant heifers were bred a second time. Fertility was high for all previous uterine challenge groups, with 80% of the heifers becoming pregnant after the second breeding.

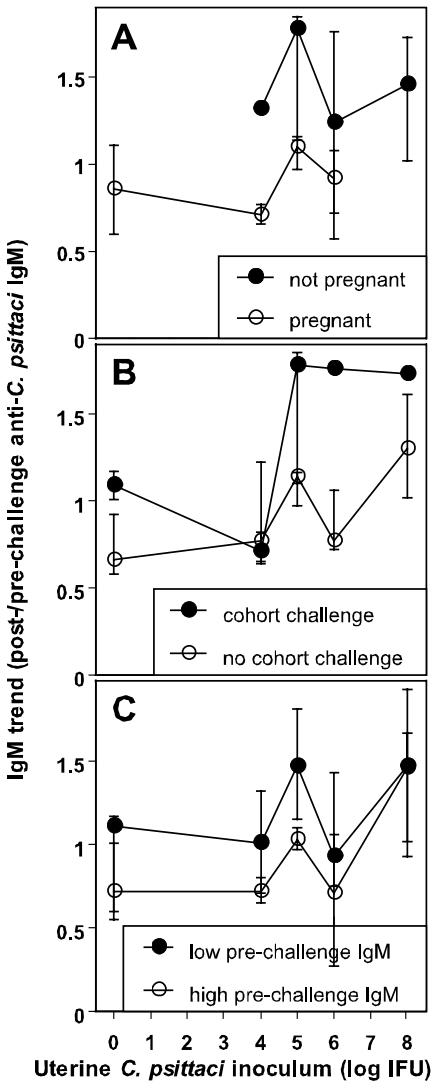

Figure 3. **Infertility, cohort challenge, and low pre-challenge anti-*C. abortus* IgM are associated with an increased antibody trend for IgM.** Median and quartile antibody trends for IgM for all uterine *C. abortus* inoculum doses are shown. A) Antibody trend for IgM of infertile heifers (non-pregnant, n=12) are consistently higher than those of fertile heifers (pregnant, n=18). B) IgM trends of cohort-challenged heifers (n=10) are higher than of herd mates that were not cohort-challenged (n=20). Cohort challenge indicates the exposure of inseminated heifers to *C. abortus* infection by shedding from heifers that had received uterine *C. abortus* inocula 2 weeks earlier. Animals that were inseminated during the first round of estrus were scored as not cohort challenged by herd mates that received uterine *C. abortus* inocula during the same time span. Animals inseminated 2 weeks later, during the second round of induced estrus, were scored as having been cohort challenged by their herd mates that were inoculated in the first round. Increased antibody trends for IgM were significantly associated with infertility ($p = 0.001$) and cohort challenge ($p = 0.04$) with *C. abortus* (Student's *t*-test; mean of all intra-uterine challenge groups).

High uterine *C. abortus* challenge, cohort challenge with *C. abortus*, and low pre-challenge anti-*C. abortus* IgM levels reduce fertility.

Uterine challenge and cohort challenge with *C. abortus*, and low (below-median) pre-challenge anti-*C. abortus* IgM levels were associated with decreased fertility in heifers as shown in Fig. 4. When modeled with logistic regression, uterine *C. abortus* inoculum, cohort challenge with *C. abortus*, and IgM levels against *C. abortus* predicted fertility as shown in Fig. 5. Uterine *C. abortus* inoculum, cohort challenge with *C. abortus*, and IgM antibody levels against *C. abortus* significantly affected the models at *p* values < 0.05. The Odds Ratios (Wald 95% Confidence Intervals) per log *C. abortus* IFU for infertility were 5.4 (1.4-21; model A, Fig. 5) and 3.3 (1.2-9.5; model B, Fig. 5), i.e. the models predicted infertility to increase by a factor of 5.4 or 3.3 for each log increase in IFU of uterine *C. abortus* inoculum. For cohort challenge with *C. abortus* the Odds Ratio for infertility was 4.6 (1.01-21.15), so that the model predicted infertility to increase by a factor of 4.6 with cohort challenge with *C. abortus*. For low pre-challenge IgM levels against *C. abortus* EB the Odds Ratio for infertility was 4.4 (1.05-18.50), so that the model predicted infertility to increase by a factor of 4.4 for heifers with lower than median values of IgM. Overall, the logistic regression models demonstrate that the uterine *C. abortus* inoculum required to cause infertility is 8.5-fold higher for heifers without *Chlamydophila* cohort challenge, and 17-fold higher for heifers with high IgM antibody levels against *Chlamydophila*, compared to heifers with cohort challenge or with low IgM antibody levels against *Chlamydophila*.

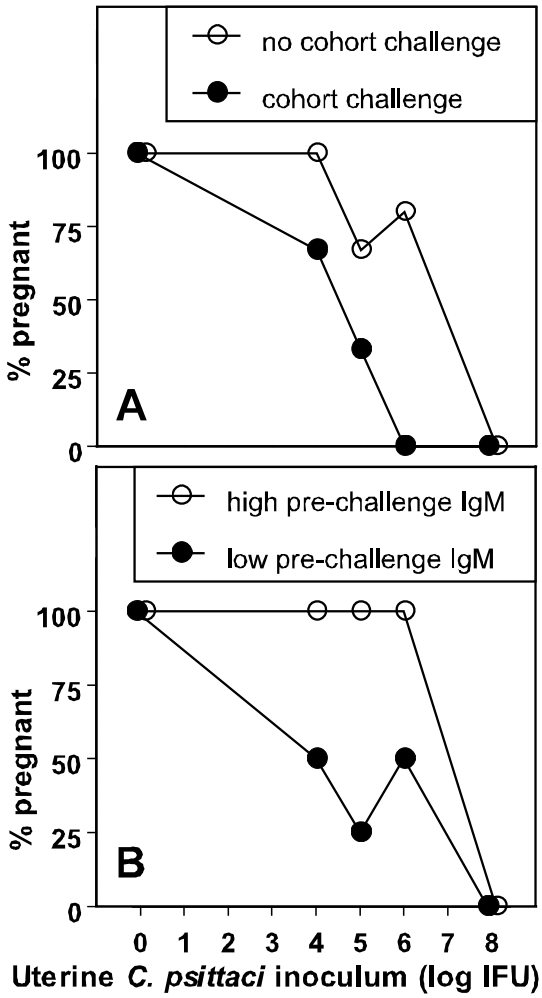

Figure 4. **High inoculum and cohort challenge with *C. abortus* and low pre-challenge anti-*C. abortus* serum IgM levels are associated with reduced fertility in heifers previously exposed to *C. abortus*.** Pregnancy as an indicator of fertility was evaluated 6 weeks after uterine challenge with *C. abortus* strain B577. A) Overall pregnancy data for Holstein heifers (n=30) inoculated in the uterus with 0, 10^4, 10^5, 10^6, or 10^8 IFU, with or without cohort challenge. B) Pregnancy data for the same group of 30 heifers, with high or low pre-challenge anti-*C. abortus* IgM levels. Taken together, these data suggest that reduction of bovine fertility by re-infection with *C. abortus* depends on the epidemiological parameters of herd infection with *C. abortus*, specifically total chlamydial challenge, via direct uterine inoculation and via cohort exposure, and the immune response to previous infection.

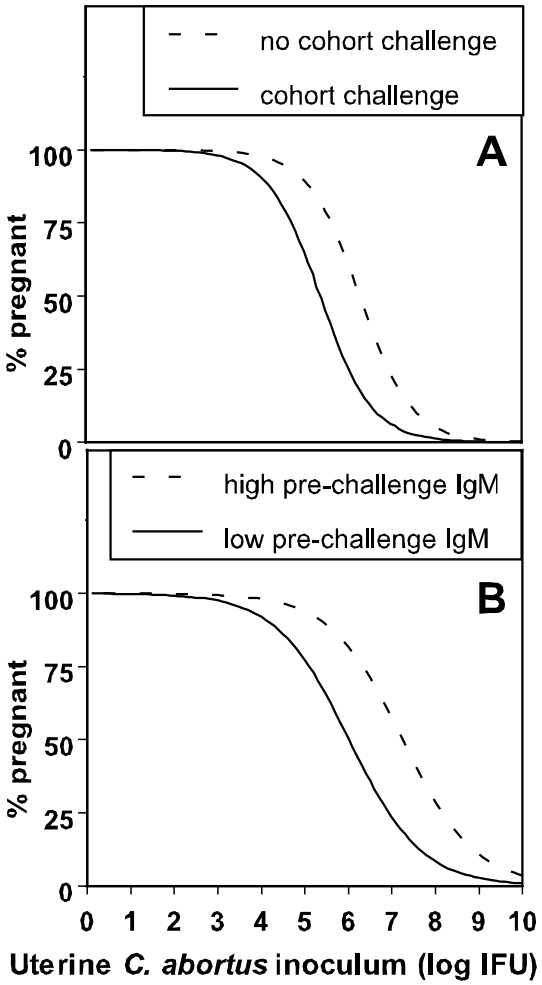

Figure 5. **Cohort challenge and pre-challenge anti-*C. abortus* serum IgM levels strongly modulate fertility after uterine challenge with *C. abortus*.** Fertility of challenged heifers (i.e. percentage of pregnant animals) is significantly predicted in logistic regression models by the uterine *C. abortus* inoculum dose and cohort challenge by *C. abortus* (A) or by the uterine inoculum dose and concentration of preformed IgM against *C. abortus* (B). The solid line represents fertility dependence on the uterine inoculum under conditions of cohort challenge to *C. abortus* (A) or under conditions of below-median (low) levels of anti-*C. abortus* IgM (B). The dashed line represents fertility without cohort challenge (A) or with above-median (high) levels of anti-C. abortus IgM (B). The WALD *p* values are as follows: for inoculum dose, 0.016 (A) and 0.024 (B); for cohort challenge, 0.048; and for IgM levels, 0.043. These logistic regression models of fertility of heifers with established immunity against C. abortus indicate that (i) with cohort challenge a uterine infection of $10^{5.38}$ IFU of C. abortus is necessary to reduce fertility of heifers from 100 to 50% compared to the 8.5-fold-higher dose of $10^{6.31}$ IFU required for the same reduction without cohort challenge (A) and (ii) at low prechallenge anti-*C. abortus* IgM levels $10^{6.01}$ intrauterine IFU of *C. abortus* reduce fertility of heifers from 100 to 50% compared to the 17-fold-higher dose of $10^{7.24}$ IFU required for the same reduction at high prechallenge anti-*C. abortus* IgM levels (B).

Validation experiments corroborate reduction of fertility by cohort challenge.

Results for the validation experiments are shown in Fig. 6. The first validation experiment approximated the total cohort challenge of the initial experiment using a uniform challenge dose. Three of 14 (21%) and 4 of 5 (80%) heifers without cohort challenge became pregnant when challenged or not challenged by uterine inoculation with *Chlamydophila*, respectively. In contrast, 0 of 7 (0%) and 0 of 3 (0%) heifers with cohort challenge became pregnant, with or without uterine challenge with *Chlamydophila*, respectively. These results suggested that cohort challenge with *Chlamydophila abortus* induces infertility in cattle.

In the second validation experiment the relative cohort challenge with *Chlamydophila* was reduced by 80% as compared to the initial experiment. One of 7 (14%) and 5 of 8 (63%) heifers without cohort challenge became pregnant, with or without uterine *Chlamydophila* challenge, respectively. In contrast, 0 of 8 (0%) and 8 of 8 (100%) heifers with cohort challenge became pregnant when challenged or not challenged with uterine *Chlamydophila*, respectively. These results suggest that cattle that are cohort-challenged with *Chlamydophila*, at a level below a discrete threshold value, will resist re- infection. However, when cattle are simultaneously inoculated with uterine *Chlamydophila*, this cohort-challenge threshold value is breeched and fertility of these cattle, which is already reduced by the uterine challenge, will be further reduced by the cohort challenge. These results further suggest that the herd dynamics of infection, such as total herd density, ratio of *Chlamydophila* shedding vs. non-shedding animals, and level of *Chlamydophila* shedding, strongly influence *Chlamydophila*-mediated depression of bovine fertility.

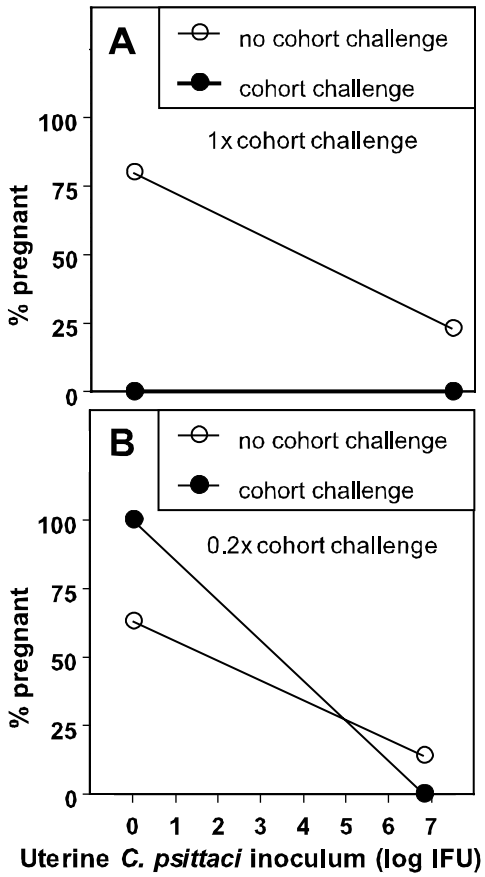

Figure 6. **Uterine *C. abortus* inoculation experiments at high and low *C. abortus* cohort challenge confirm the influence of cohort challenge on bovine fertility.** Validation experiments for the effect of cohort challenge on fertility were performed with 2 groups of heifers. Group A received a cohort challenge estimated to be similar to the first experiment, group B received 20% of the estimated original cohort challenge. A) Pregnancy data for Holstein heifers (n=29) with or without cohort challenge to *C. abortus*. Uterine inoculum dose was either 3×10^7 IFU *C. abortus* strain B577 or placebo challenge. In this experiment, the estimated relative cohort challenge of the initial experiment was duplicated with a first-round uterine inoculum of 3×10^7 IFU *C. abortus* strain B577 in 15 heifers. Relative cohort challenge was estimated as 52.7, compared to that of the initial experiment as 48.5. B) Pregancy data for Holstein heifers (n=32) with or without an estimated 20% of the relative cohort exposure of group A. Relative cohort exposure to *C. abortus* was reduced to 10.4, approximately 20% of the cohort challenge in the initial and the first validation experiment A, by reducing the number of heifers (n=7) uterine-inoculated with *C. abortus* and by reducing the inoculation dose per heifer (6.67×10^6 IFU). Uterine inoculum dose was either 6.67×10^6 IFU *C. abortus* strain B577 or placebo challenge. The results of these experiments confirm that there is a reduction of bovine fertility subsequent to cohort challenge. Reduction of the estimated relative cohort challenge by 80% abolished the cohort-induced depression of fertility in heifers that did not receive uterine challenge, but this cohort challenge still negatively influenced fertility in heifers that also received a uterine challenge.

DISCUSSION

Investigations conducted over the last 30 years have suggested that *C. abortus* causes ubiquitous genital infection in cattle (33, 34, 93, 148, 150, 177). Recently, we detected with high frequency the presence of low levels of *C. abortus* strain B577 DNA in vaginal mucosal samples collected from virgin heifers (33, 34). These data also suggest that nonsexual transmission of the organism, most likely by ingestion from a contaminated environment or by direct contact with shedding herd mates, is an important mode of transmission for this organism in cattle (33, 34). The study reported here provides additional evidence that *C. abortus* is nonsexually transmitted to the reproductive tract and that this transmission has the potential to cause infertility. The present study confirms that environmental and direct cohort exposure may both serve as important sources of infection and subsequent infertility, caused by *C. abortus* in cattle.

C. abortus may cause infertility in a number of ways. Bulls with natural or experimentally induced *C. abortus* genital infection often have pyospermia with a high occurrence of morphological abnormalities (27, 160). Cows and heifers with *C. abortus* genital infection may also have reduced fertility caused either by direct infection of the embryo after loss of the zona pellucida and/or as a result of local infection and related inflammation creating an uterine environment that is not conducive to embryo implantation. Experimental evidence strongly suggests that uterine inflammation, but not direct infection of the fertilized egg, is the dominant factor in bovine female infertility caused by *C. abortus* (27). Our observation of extended post-insemination estrus cycles of infertile heifers in this study supports this concept.

The highest possible fertility for all heifers was essential for meaningful results in this investigation. The combination of estrus synchronization with prostaglandin F2 and electronic estrus detection provided optimum information for the best time of insemination. Precise identification of the onset of estrus and a high plane of nutrition were important factors that contributed to the high conception rate of heifers that were not challenged with uterine *C. abortus* in this study.

Logistic regression models for the data from our study indicate that a uterine challenge with 2.4×10^5 *C. abortus* IFU would reduce the first service conception rate of highly fertile cattle from 100% to 50%, if the cattle are exposed to *C. abortus* shed by the herd mates. In contrast, when cattle experience low environmental and cohort exposure to *C. abortus*, the increased uterine dose of *C. abortus* required for a 50% reduction in the first service conception rate would be 2×10^6 IFU, an 8.5-fold difference. Similarly, the dose required to reduce fertility by 50% increases 17-fold from 10^6 to 1.7×10^7 IFU for cattle with high anti-*C. abortus* IgM antibody levels as compared to cattle with low antibody levels. Similar ranges of *C. abortus* challenge doses are typically used in mouse studies, irrespective of a greater than 10,000-fold difference in body weight between cattle and mice, suggesting a high natural susceptibility of cattle to *C. abortus* infection and disease. Doses of *C. abortus* that caused infertility in this study correspond well to natural challenges that are likely to occur in commercial cattle operations (148). Vaginal infection and fertility rates commonly observed in commercial cattle operations are consistent with low dose exposure to *Chlamydophila*. In the second confirmation experiment of this study using a reduced cohort challenge, a cohort-induced depression of fertility was not observed in heifers that did not receive a uterine challenge. However,

the cohort-induced reduction in fertility was still evident in heifers that received uterine challenge. This result suggests that a threshold of infection needs to be overcome, before fertility is depressed in heifers re-infected with *Chlamydophila.* We assume that this threshold might be lowered in cattle that experience *C. abortus* infection concurrent with reduced immune function due to dietary deficiencies, stressors, and/or concurrent disease, all of which occur frequently in commercial cattle operations.

The high concentrations of serum antibodies against *C. abortus* suggest that all heifers in this study experienced active infection with *C. abortus*, or had recently recovered from infection. Thus, the heifers most likely had a competent naturally acquired immune response to *C. abortus*. In this study, above-median IgM concentration was identified as a predictor of subsequent fertility in cattle following challenge with *C. abortus*. The association of improved fertility with above-median pre-challenge IgM concentrations is an important finding with several possible explanations. One possible explanation is that IgM protect directly from infection by neutralizing circulating *C. abortus* organisms or mediating lysis of infected cells, resulting in decreased disease such as infertility. Epidemiological data suggesting functional effects of antibodies against *C. abortus* are limited (7, 62). Data from *Chlamydiaceae* disease models and epidemiological investigations of human disease suggest that humoral antibodies against *C. abortus* provide only limited protection from disease (7, 16, 62, 73, 74, 123). In this study, infertile heifers with below-median pre-challenge serum IgM levels still had considerable concentrations of IgM. This fact, and the lack of significant differences for other antibody isotypes, argues against causality of antibody, including IgM, in protection from infertility, and suggests an associative relationship of pre-challenge IgM levels with

protection. The primary mechanism of this protection is most likely based on Th1 cellular immunity, as suggested by a large body of evidence from human epidemiological and animal model studies (7, 16, 62, 72, 73, 131-134, 175). Above-median anti- *C. abortus* IgM levels may indicate a recent episode of *C. abortus* infection that elicits transient protection from disease. Thus, elevated IgM may provide a surrogate marker of recently stimulated anti- *C. abortus* cellular immunity, indicating circulating specific effector T cells or high levels of memory T cells. This explanation is consistent with our observation that only elevated IgM levels predicted protection, but not elevated total IgG, or IgG1 or IgG2 antibodies, which are less indicative of a recent infection.

The strong association of an increased antibody trend for IgM with infertility (Fig. 3) suggests a common mechanism that results in both infertility and elevated anti- *C. abortus* serum IgM levels. We assume that this mechanism is associated with reduced elimination kinetics of *C. abortus* from tissues, including the uterus. This would result in a prolonged uterine inflammatory response in animals with below-median IgM that prevents implantation of the embryo and represents a stronger immune stimulus than the stimulus of an infection that is rapidly eliminated by an effective immune response. This concept is consistent with the high fertility of heifers in this study that were bred a second time, after failing to conceive 60 days earlier following the initial breeding and challenge. These heifers most likely mounted effective cellular immune responses subsequent to the earlier *C. abortus* challenge, so that infection-associated inflammation was reduced, resulting in a high pregnancy rate.

The present investigation clearly demonstrates that previous exposure to *C. abortus* does not provide complete protection from disease in a subsequent challenge,

even in the absence of clinical signs of disease. However, relative immune protection can be accomplished, as indicated by the association of elevated anti- *C. abortus* serum IgM levels with reduced disease. Simple models with a total of three predictor variables accounted for the variation in fertility observed in a herd of dairy cattle that was pre-exposed and then re-challenged with *C. abortus*. These models addressed the main factors that impact the fertility of cattle infected with *C. abortus*, and provided valuable insight into the population dynamics of infection with *Chlamydophila*. One of the logistic regression models clearly specifies profound effects on fertility of both sexual and non-sexual transmission of *C. abortus* from herd mates. Overall size and density of breeding and *Chlamydophila*-shedding cohort populations influence the frequency of sexual and cohort transmission, and are therefore likely critical determinants of *Chlamydophila*-mediated fertility disorders. Another major determinant of *C. abortus* -mediated fertility disorders is the time-dependent response to boost cycles of *Chlamydophila*-specific host immunity by natural re-infection. Serum anti- *C. abortus* IgM levels relative to the herd average provide a surrogate marker for immunity to *Chlamydophila* infection, as specified in the second logistic regression model. Aside from type and dose of re-infection, the immune response will be influenced by a wide range of herd-specific immunosuppressive factors including quantitative and qualitative malnutrition, numerous well-characterized herd stressors, and/or concurrent disease, all frequently observed in commercial cattle operations. Thus, the quality of herd management will likely affect *Chlamydophila*-mediated bovine fertility disorders. Another profound influence on any immune response will be genetic variation of the host that is characteristic of an outbred population. This resistance would most likely be

expressed through immune function, but could also be expressed as behavioral change, such as altered social interaction with herd mates shedding *Chlamydophila*.

Chronic infections with *C. abortus* are ubiquitous in cattle, and subtle manifestations of these infections are essentially not recognized and subject to prophylactic or therapeutic measures. This study demonstrates that these subclinical infections may potentially have a profound negative effect on bovine fertility, and that these losses may be reduced with appropriate herd management practices. Data from this study and *C. abortus* prevalence data (33, 34) suggest a steady-state equilibrium of *C. abortus* infection at the herd level, which is driven and maintained by asynchronous cycles of relative resistance and susceptibility of individual animals and by the kinetics of sexual and cohort transmission as determined by breeding regimen, herd size and population density. This equilibrium may be induced to shift by a variety of mechanisms, such as a nutrition-related decrease in overall herd immunity, which may lead to increased susceptibility to infection and enhanced transmission, resulting in a proportional reduction in fertility.

The risk factors identified in this study may provide insight for the development of viable prophylactic and therapeutic measures. Numerous herd management practices, most importantly high quality nutrition, need to ensure high immunocompetence of herds. Routine monitoring of sires for the presence of *C. abortus* in semen and/or the prepuce would provide useful information for reducing sexual transmission. Cohort transmission may be decreased by reducing group size and density, and by the elimination or isolation of debilitated animals that chronically shed *Chlamydophila*. Vaccination may also serve as a component of a prophylactic or therapeutic program, by

protecting individual animals from disease (57) and by reducing *Chlamydophila* transmission within the herd. The results of this investigation provide encouraging evidence that effective vaccines against *Chlamydophila* can be constructed. It is likely that this protection will require circulating *Chlamydophila*-specific Th1 cells and consequently this protection is likely to be transient, with frequent vaccination required to maintain continuous protection.

V. MULTIVARIATE ANALYSIS OF DIETARY PROTEIN AND ANTIOXIDANT EFFECTS ON MURINE *CHLAMYDIA PNEUMONIAE* INFECTION

INTRODUCTION

Chlamydia pneumoniae is an obligate intracellular bacterial pathogen. It is highly prevalent in the human population, and has been associated with community-acquired upper respiratory tract disease (118, 174). Chronic infections with *C. pneumoniae* have been associated with chronic inflammatory diseases such as coronary atherosclerosis and Alzheimer's disease (68, 71, 86). These epidemiological data provide a strong incentive to explore factors that potentially prolong or exacerbate *C. pneumoniae* infections since such factors may also enhance natural human infection and disease.

Many factors that may affect the outcome of chlamydial disease in animal models have been reported including specific and non-specific immune defenses and host genetic background (31, 38, 59, 140). The effect of diet on the immune response to chlamydal infection, and its influence on disease outcome, has been mainly studied for dietary additives (41), but not for main diet components such as protein concentration. However, numerous studies document the influence of diet composition on other respiratory diseases such as tuberculosis (26, 44). The shortcoming in many of these investigations is that they contrast extreme diet compositions, e.g., a virtually complete absence of

proteins with diets that contain normal amounts of protein (26, 139). While these studies typically show profound effects of diet composition on disease outcome, they do not reflect real-life fluctuations of diet composition that may have subtle, but more important consequences. Because most nutrients such as fatty acids, vitamins, minerals, and amino acids in diet have specific roles in maintaining homeostasis, it is difficult to define the optimal levels of each component to prevent various infectious diseases (28, 146).

Protein malnutrition causes immunodeficiency since rapid proliferating immune cells require amino acids and high-energy for cell division and protein synthesis (44, 50). In a study of the effect of protein malnutrition on *Mycobacterium tuberculosis* lung infection, protein malnourished C57BL/6 mice had a severely compromised immune response (26). However, in another investigation studying peritoneal infection with *Staphylococcus aureus* and *E. coli*, a low-protein diet improved the survival rate of guinea pigs during the ensuing bacterial sepsis (110). These conflicting results may be reconciled by the fact that different mechanisms are at work during immune protection form these infections (26, 110). The amino acid L-arginine is a crucial component of the immune response as substrate for both inducible nitric oxide synthase (iNOS) and arginase in the cytotoxic pathways of macrophages. These enzymes are highly increased after challenge with various immunogenic antigens (82, 106).

Reactive oxygen intermediates (ROI) produced by phagocytic cells may damage cellular components and lead to death due to the molecular damage resulting from oxidative stress (47). Immune cells are particularly susceptible to oxidative damage because of the high percentage of polyunsaturated fatty acids in their plasma membranes and their ability to produce large amounts of free radicals during inflammation (22).

Although the ROI (and NO) also act as signaling molecules, oxidative stress by exogenously derived ROI decreases the activity of transcription factors and the proliferative response of T lymphocytes (98, 131). Furthermore, Th2 cytokine production is more rapidly reduced by oxidative stress than Th1 cytokine production (83, 98). Antioxidants such as vitamins C and E stabilize the immune system by protecting against ROI-induced cellular damage, since high levels of vitamin C accumulate in immune cells and are oxidized rapidly during infection. This prevents oxidative damage to DNA by reducing ROI and by regenerating vitamin E that prevens lipid oxidation and damage to cell membranes (151).

The effects of protein and antioxidants on *C. pneumoniae* infection are not understood. We designed this investigation to study the effects at nutrient levels that are well within those of commercially available rodent diet formulations. We designed diets that provide protein in the range of low or high concentrations found in commercial diets, and which provide antioxidants at the customary or twice the customary level. The purpose of this study was to determine if relevant differences in protein and antioxidant levels in diets affected lung disease outcome caused by *C. pneumoniae* infection in two mouse strains that were naïve or immune to *C. pneumoniae* through previous low-level exposure.

MATERIALS AND METHODS

Experimental Design. This balanced multivariate experiment was designed to test the contribution of dietary protein and antioxidant content to the outcome of *C. pneumoniae*

infection (Table 1). The contribution of these dietary parameters was evaluated on the genetic background of two mouse strains (A/J or C57BL/6), in the presence or absence of an established immune response against *C. pneumoniae* (immune or naïve), and at two different time points after infection (day 3 or 10 post inoculation). To exclude a confounding sex influence, only female mice were used. Four weeks prior to challenge groups of 10 mice received a low-dose immunizing *C. pneumoniae* inoculum (immune) or a mock inoculum (naïve). From two weeks before *C. pneumoniae* challenge until sacrificing, all mice (Harlan Sprague & Dawley) received one of the following four diets ad libitum: high-protein & high-antioxidants diet (HH), high-protein & low-antioxidants diet (HL), low-protein & high-antioxidants diet (LH), or low-protein & low-antioxidants diet (LL) (Table 2). Mice were sacrificed 3 or 10 days after *C. pneumoniae* challenge. The whole experiment consisted of 32 different groups of two mouse strains, four diets, two immune conditions, and two time points, comprising a total of 320 mice (10 mice per group).

Chlamydia pneumoniae. *C. pneumoniae* strain CDC/ CWL-029 (ATCC VR-1310) was grown, purified and quantified as described (87). Briefly, Buffalo Green Monkey Kidney cells (Diagnostic Hybrids Inc., Athens, OH) were used as host cells for propagation of chlamydiae. For purification, EBs in supernatant culture medium were concentrated by sedimentation followed by low-speed centrifugation for removal of host cell nuclei and by step-gradient centrifugation of the supernatant in a 30% RenoCal-76 - 50% sucrose step-gradient. Sediments of purified infectious EBs were suspended in sucrose-phosphate-glutamate (SPG) buffer and stored in aliquots at -80°C.

Table 1. Balanced multivariate design of the *C. pneumoniae* challenge experiments.

Mouse Strain	Immune Status	Sacrificing Day[a]	Diet	
			Protein	Antioxidants
A/J	immune	3	high	low[b]
				high
			low	low
				high
C57BL/6			high	low
				high
			low	low
				high
A/J		10	high	low
				high
			low	low
				high
C57BL/6			high	low
				high
			low	low
				high
A/J	naive	3	high	low
				high
			low	low
				high
C57BL/6			high	low
				high
			low	low
				high
A/J		10	high	low
				high
			low	low
				high
C57BL/6			high	low
				high
			low	low
				high

[a] Day 10 or 3 is the time after lung infection at which the mice were sacrificed.

[b] Ten mice (2 cages of 5 mice) were used for each combination of experimental categorical variables.

Animals and diets. Inbred A/J and C57BL/6 female mice were obtained from Harlan, Sprague and Dawley (Indianapolis, IN) at 5 weeks of age. Udel "shoebox" type cages with spun fiber filter top were maintained in static air or ventilated cage racks. Five animals were housed per cage in a temperature-controlled room with a 12-hour light/dark cycle. They were allowed ad libitum access to water and one of four diets. Mice were fed a 19% protein/1.33% L-arginine standard rodent maintenance diet. All animal protocols followed NIH guidelines and were approved by the Auburn University Institutional Animal Care and Use Committee (IACUC).

Two weeks before challenge infection and during challenge infection, mice were fed one of 4 custom diets (Table 2) containing either 24% protein/1.8% L-arginine or 14% protein/0.7% L-arginine and either high or low antioxidants (low: 3.56 g vitamin C + 0.85 g vitamin E acetate/kg diet; high: 6.42 g vitamin C + 1.57 g vitamin E acetate/kg diet). Diets were manufactured by Harlan Teklad (Madison, WI, USA). The composition of the four custom diets is listed in Table 2.

***C. pneumoniae* lung challenge infection**. Mouse intranasal inoculation was performed as previously described (63). For intranasal inoculation, mice received a light isoflurane inhalation anesthesia. Six-week-old mice received a low dose challenge of 5×10^6 *C. pneumoniae* viable elementary bodies in 30 µl SPG buffer (immune mice) or a mock inoculum of 30 µl SPG buffer (naïve mice). Beginning at 8 weeks of age, all mice were fed a standardized custom diet containing either 24% protein/1.8% arginine or 14% protein/0.7% arginine and either high or low antioxidants (Table 2). Four weeks after the priming inoculation, at 10 weeks of age, the mice were challenged by intranasal inoculation of 1×10^8 *C. pneumoniae* bacteria and sacrificed by CO_2 inhalation 3 or 10

Table 2. Composition of four custom rodent diets.

Components (g/kg)	HH[a]	HL	LH	LL
Casein	275.90	275.90	160.92	160.92
L-Cystein	4.41	4.41	2.41	2.41
L-Arginine HCl	9.37	9.37	2.46	2.46
Corn Starch	302.82	306.30	426.88	430.36
Maltodextrin	132.00	132.00	132.00	132.00
Sucrose	100.00	100.00	100.00	100.00
Soybean Oil	70.00	70.00	70.00	70.00
Cellulose (fiber)	50.00	50.00	50.00	50.00
Mineral Mix, AIN-93G-MX	35.00	35.00	35.00	35.00
Calcium Phosphate Monobasic	--	--	2.02	2.02
Calcium Carbonate	2.31	2.31	0.12	0.12
Vitamin Mix, AIN-93-VX	12.00	12.00	12.00	12.00
Choline Bitartrate	2.50	2.50	2.50	2.50
TBHQ (antioxidant)	0.01	0.01	0.01	0.01
Vitamin E Acetate (500 U/g)	0.72	0.10	0.72	0.10
Stay-C 35	2.86	--	2.86	--
Food Color (soluble)	0.10	0.10	0.10	0.10
Protein concentration	24%	24%	14%	14%
Antioxidant (Vit C & E) concentration	0.8%	0.44%	0.8%	0.44%

[a] HH, high-protein & high-antioxidant: 24% protein, 1.8% L-arginine, Vit. E + Vit. C
HL, high-protein & low-antioxidant: 24% protein, 1.8% L-arginine
LH, low-protein & high-antioxidant: 14% protein, 0.8% L-arginine, Vit. E + Vit. C
LL, low-protein & low-antioxidant: 14% protein, 0.8% L-arginine.

days later. Lungs were weighed, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Percent lung weight increase was based on naïve lung weights of 138.4 mg for adult A/J mice and 133 mg for adult C57BL/6 mice.

Mouse lung nucleic acid extraction. Mouse lungs were homogenized in guanidinium isothiocyanate Triton X-100-based RNA/DNA stabilization reagent in disposable tissue grinders (Fisher Scientific, Atlanta, GA) to create a 10% (wt/vol) tissue suspension. This suspension was used for total nucleic acid extraction by the High Pure® PCR template preparation kit (Roche Applied Science, Indianapolis, IN) and for mRNA extraction using oligo $(dT)_{20}$ silica beads (35, 172).

For total nucleic acid extraction, 100 µl of 10% lung suspension was mixed with 240 µl of DNA/RNA stabilization reagent and 40 µl of 10% proteinase K solution and incubated for 1 hr at 72°C at 600 rpm in a shaking heater. This suspension was mixed with 100 µl isopropanol and centrifuged through glass fiber filter tubes for 1 min at 5,000 × g to bind total nucleic acids. After two washing steps with 500 µl wash buffer for 1 min at 13,000 × g, total nucleic acids bound to the glass fiber filter were incubated with 100 µl of 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA (35) for 5 min at 72°C and eluted by centrifugation for 1 min at 13,000 × g.

For mRNA extraction, a suspension of oligo $(dT)_{20}$-coated silica beads (25 mg/ml in dH_2O; 1 µm particle size, Kisker GbR, Steinfurt, Germany) was used. First, 100 µl of 10% lung suspension was mixed with 10 µl oligo $(dT)_{20}$ silica bead suspension diluted in 230 µl dilution buffer (0.1 M Tris-HCl, pH 7.5, 0.2 M LiCl, 20 mM EDTA). For mRNA binding, samples were incubated at 72°C for 3 minutes followed by room temperature for 10 minutes. The silica beads were sedimented by centrifugation at 13,000 × g for 2

Table 3a. Oligonucleotide primers and probes used in real-time PCRs.

gene name length exon	primers probes[a]	Sequence (5'→3')[a]
Chlamydia 23S rRNA 168 bp	UP	GGGGTTGTAGGGTYGAGRAIAWRRGATC
	DN	GAGAGTGGTCTCCCCAGATTCARACTA
	BOD	BOD-CCTGAGTAGRRCTAGACACGTGAAAC-P
	FLU	ACGAAARAACAARAGACKCTAWTCGAT-6-FAM
PBGD intron 176 bp	UP	CCTAGTAGAGACACACCTGAATTGCTATTGTGA
	DN	TTCATAGCCAAGACTACTGCTTACTGCCTG
	BOD	BOD-TGAGCTGGGAAGCTTGCTTATCTCCT-P
	FLU	TGGGCAGGCTGCCTGAGATAAGG-FAM
Lactoferrin 189 bp exon 4-6	UP	CCTGCATCCCTTGAGGAAGCGGTA
	DN	TCCAGCATTGTCTCTCAGACACCTCAAG
	BOD	BOD-GAGGAGCCATACTCAGGTTATGCTGGA-P
	FLU	AGCCAACAAATGTGCCTCTTCCCC-6-FAM
F4/80 229 bp exon 7-9	UP	CAAAGACCTAGAGGTGACATGTGAAGATATTGA
	DN	TTGCTGTATCTGCTCACTTTGGAGTATCAAGT
	BOD	BOD-CTGCAAAAGGATCCTCTTCAAGTGTAAGGA-P
	FLU	GAAGGCTCCCAAGGATATGGAAACTTCA-6-FAM
NKp46 180 bp exon 3-6	UP	TGGCTCTTACAACGACTATGCATGGTCT
	DN	GATCCCAGAAGGCGGAGTCCTTTTG
	BOD	BOD-GAGTTTGACCTTTCAACCAATGAATCAGGA-P
	FLU	CCTACCGACCCTACTTCTTCTCTTGATTATTG-6-FAM
CD3δ 215 bp exon 2-4	UP	TGCAAGTCCATTACCGAATGTGCCA
	DN	ATCTTCACGATCTCGAAGAGGCTGATACAG
	BOD	BOD-CTTCTGGGGCTGCTGAGGTTCAAG-P
	FLU	TTTGCAGGACATGAGACCGGAAGG-6-FAM
Tim3 200 bp exon 2-5	UP	CTGAAATTAGACATCAAAGCAGCCAAGGT
	DN	GTTCTGATCGTTTCTCCAGAGTCCTTAATTTC
	BOD	BOD-GGAACAAAAATTTCCACATGGGCTGA-P
	FLU	ACAGACACTGGTGACCCTCCATAATAACAA-6-FAM
GATA3 171 bp exon 3-5	UP	AAGCCCAAGCGAAGGCTGTCG
	DN	TTTCTTCATAGTCAGGGGTCTGTTAATATTATGAAG
	BOD	BOD-GCAATGCCTGTGGGCTGTACTACAAG-P
	FLU	GAACGCTAATGGGGACCCGGTC-6-FAM

[a] BOD, amine-reactive Bodipy fluorophore attached to 5' terminus; 6-FAM, 6-carboxyfluorescein attached to 3'-O-ribose; P, Phosphate group attached to the 3' terminus; I=deoxy inosine; K=G/T; R=A/G; W=A/T; Y=C/T.

Table 3b. Oligonucleotide primers and probes used in real-time PCRs.

gene name length exon	primers probes[a]	Sequence (5'→3')[a]
CD45RB 209 bp exon 2-7	UP	CACAGAAGTCTTTGTCACAGGGCAAACA
	DN	GAGTTGTGAGGCTGGCACCTGGTG
	BOD	BOD-CGTGGATAACACACCATCACTGGGTGTA-P
	FLU	TGTGGGGACAGGTGAGGCAGCA-6-FAM
CD45RO 157 bp exon 2-9	UP	CACAGAAGTCTTTGTCACAGGGCAAACA
	DN	GTTCCCAAACATGGCAGCACATGTT
	BOD	BOD-GGCTGGCACCATCACTGGGTGTA-P
	FLU	GCCCAGAGTGGATGGTGTAAGAGTTGTG-6-FAM
Perforin 1 194 bp exon 1-3	UP	ATGTGAACCCTAGGCCAGAGGCAAAC
	DN	TCTGAGCGCCTTTTTGAAGTCAAGGT
	BOD	BOD-CGCATGTACAGTTTTCGCCTGGTACAA-P
	FLU	GACCAGTACAACTTTAATAGCGACACAGTAGAGTG-6-FAM
CD19 198 bp exon 4-7	UP	GGTCATTGCAAGGTCAGCAGTGTGG
	DN	GGAGGCGTCACTTTGAAGAATCTCCTG
	BOD	BOD-CCTGAGAAGGAAAAGGAAGCGAATGACT-P
	FLU	GTGGCTTTTCTCTATTGTCAAAGAGCCTTTA-6-FAM
Arg1 212 bp exon 3-5	UP	GTGGAGACCACAGTCTGGCAGTTGGA
	DN	GCAGGGAGTCACCCAGGAGAATCCT
	BOD	BOD-GAAGGAACTGAAAGGAAAGTTCCCAGATGT-P
	FLU	CATGGGCAACCTGTGTCCTTTCTCC-6-FAM
Arg2 160 bp exon 1-3	UP	TTTCTCTCGGGGACAGAAGAAGCTAGGA
	DN	CAGATTATTGTAGGGATCATCTTGTGGGACA
	BOD	BOD-TCTTCAGCAAGCCAGCTTCTCGAATGG-P
	FLU	GGTGGCATCCCAACCTGGAGAGC-6-FAM
Nos2 189 bp exon 12-15	UP	GCTACGCCTTCAACACCAAGGTTGTCT
	DN	CAAACACAGCATACCTGAAGGTGTGGTT
	BOD	BOD-GCAATGGGCAGACTCTGAAGAAATCTCTG-P
	FLU	GCACATTTGGGAATGGAGACTGTCCC-6-FAM
Cybb 176 bp exon 5-7	UP	CTATTCAATGCTTGTGGCTGTGATAAGCA
	DN	CCTGCACAGCCAGTAGAAGTAGATCTTTTTG
	BOD	BOD-CAACTGCTATCTTAGGTAGTTTCCAGGCATC-P
	FLU	TCTTCACTGGCTGTACCAAAGGGTCCA-6-FAM
IL-4 226 bp exon 2-5	UP	TCACAGGAGAAGGGACGCCATGCA
	DN	GTGCAGCTTATCGATGAATCCAGGCA
	BOD	BOD-GTGAGCTCGTCTGTAGGGCTTCCAA-P
	FLU	CTCACAGCAACGAAGAACACCACAGAG-6-FAM

[a] BOD, amine-reactive Bodipy fluorophore attached to 5' terminus; 6-FAM, 6-carboxyfluorescein attached to 3'-O-ribose; P, Phosphate group attached to the 3' terminus.

Table 3c. Oligonucleotide primers and probes used in real-time PCRs.

gene name length exon	primers probes[a]	Sequence (5'→3')[a]
Ptgs2 176 bp exon 5-7	UP	AGGACTGGGCCATGGAGTGGACTTA
	DN	CAGGGATGTGAGGAGGGTAGATCATCTC
	BOD	BOD-GTATCCCCCCACAGTCAAAGACACTCA-P
	FLU	AATTGAAATATCAGGTCATTGGTGGAGAGG-6-FAM
IL-6 210 bp exon 1-3	UP	GTTCCTCTCTGCAAGAGACTTCCATCCA
	DN	CCATTGCACAACTCTTTTCTCATTTCCAC
	BOD	BOD-ACCAGCATCAGTCCCAAGAAGGCAA-P
	FLU	TTGTGAAGTAGGGAAGGCCGTGGTTGT-6-FAM
TNF 177 bp exon 2-5	UP	GCCACCACGCTCTTCTGTCTACTGAACT
	DN	CTCCACTTGGTGGTTTGCTACGACGT
	BOD	BOD-TTGGGAACTTCTCATCCCTTTGGGGA-P
	FLU	CCATAGAACTGATGAGAGGGAGGCCA-6-FAM
Ifng 166 bp exon 3-4	UP	CCTTCTTCAGCAACAGCAAGGCGAA
	DN	CAGCAGCGACTCCTTTTCCGCTTC
	BOD	BOD-GGGTTGTTGACCTCAAACTTGGCAATAC-P
	FLU	TGAATGCTTGGCGCTGGACCTG-6-FAM
Cxcl2 241 bp exon 2-5	UP	ACGTGTTGGCTCAGCCAGATGCAGTTAA
	DN	TGGACCCATTCCTTCTTGGGGTCA
	BOD	BOD-TGTCCCAAAGAAGCTGTAGTTTTTGTCACC-P
	FLU	AGAGCTACAAGAGGATCACCAGCAGCAG-6-FAM
IL-10 227 bp exon 2-4	UP	GCACTGCTATGCTGCCTGCTCTTACTG
	DN	CAACCCAAGTAACCCTTAAAGTCCTGCAT
	BOD	BOD-CCAGCTGGACAACATACTGCTAACCGACT-P
	FLU	CCAGGTGAAGACTTTCTTTCAAACAAAGG-6-FAM
Ccl2 193 bp exon 2-4	UP	ACGTGTTGGCTCAGCCAGATGCAGTTAA
	DN	TGGACCCATTCCTTCTTGGGGTCA
	BOD	BOD-TGTCCCAAAGAAGCTGTAGTTTTTGTCACC-P
	FLU	AGAGCTACAAGAGGATCACCAGCAGCAG-6-FAM
Serp1 222 bp exon 5-8	UP	CGCCTCCTCATCCTGCCTAAGTTCTC
	DN	TGAGATGACAAAGGCTGTGGAGGAAGAC
	BOD	BOD-TAGCACAGGCACTGCAAAAGGTCAGG-P
	FLU	AGTCTTTCCGACCAAGAGCAGCTCTCT-6-FAM
CRP 189 bp exon 1-3	UP	GGACTCCTTGTCCTTGATCTTTCAGACAA
	DN	ACACATAGGAAGTATCTGACTCCTTGGGAAA
	BOD	BOD-TCATGAAGACATGTTTAAAAAGGCCTTTGT
	FLU	ATCATGATCAGCTTCTCTCGGACTTTTG-6-FAM

[a] BOD, amine-reactive Bodipy fluorophore attached to 5' terminus; 6-FAM, 6-carboxyfluorescein attached to 3'-O-ribose; P, Phosphate group attached to the 3' terminus.

minutes, supernatants removed by decanting, the beads resuspended in 100 µl DNase buffer (20 mM Tris-HCl, pH 7.0, 1 M NaCl, 10 mM $MnCl_2$) containing 100 U of RNase-free bovine pancreatic DNase I (Roche Applied Science, Indianapolis, IN) and incubated for 15 minutes at room temperature. Subsequently, beads were washed three times with wash buffer (10 mM Tris-HCl, pH 7.5, 0.2 M LiCl, 1 mM EDTA) by vigorous vortexing for 2 minutes followed by sedimentation at 13,000 × g, and mRNA was eluted by resuspension of the beads in 200 µl DEPC-treated ddH_2O followed by incubation at 72°C for 7 minutes, centrifugal sedimentation, and removal of the supernatant mRNA. The purified total nucleic acid and mRNA samples were stored at -80°C until used later.

Analysis of lung nucleic acids by real-time quantitative PCR. The primers and probes used in all PCR assays were obtained from Operon, Alameda, CA, and the sequences are listed in Table 3a, b, c.

C. pneumoniae and host cell DNA: The copy number of *C. pneumoniae* genomes per lung were determined by *Chlamydia* genus-specific 23S rRNA FRET (fluorescence resonance energy transfer) qPCR (35). Copy numbers of murine genomes per lung, as markers of total number of cells, were determined by real-time amplification and quantification of an intron sequence of the porphobilinogen deaminase gene (PBGD). The qPCRs were performed in 20 µl volumes consisting of 15 µl reaction master mixture and 5 µl sample aliquot in glass capillaries in a LightCycler® real-time thermal cycler. The PCR buffer was 4.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, supplemented with 0.05% each Tween-20 and Nonidet P-40, and 0.03% acetylated bovine serum albumin (Roche Applied Science, Indianapolis, IN). Nucleotides were used at 0.2 mM (dATP, dCTP, dGTP) and 0.6 mM (dUTP).

For each 20 µl total reaction volume, 1.5 U hot-start Platinum *Taq* DNA polymerase (Life Technologies, Grand Island, New York) and 0.2 U heat-labile uracil-DNA glycosylase (UNG) (Roche Molecular Biochemicals, Indianapolis, IN) was used. Heat-labile UNG is active at room temperature and is inactivated at 50°C. This allows the use of UNG carry-over prevention in one-step RT-qPCR. Thermal cycling consisted of a 2 min denaturation step at 95°C followed by 18 high-stringency step-down thermal cycles, 40 low-stringency fluorescence acquisition cycles, and melting curve determination between 50°C and 80°C. The parameters for the 23S rRNA qPCR were 6× 12 sec at 64°C, 8 sec at 72°C, 0 sec at 95°C; 9× 12 sec at 62°C, 8 sec at 72°C, 0 sec at 95°C; 3× 12 sec at 60°C, 8 sec at 72°C, 0 sec at 95°C; 40× 8 sec at 54°C and fluorescence acquisition, 8 sec at 72°C, 0 sec at 95°C (35, 61). The parameters for the PBGD intron qPCR were 40× 8 sec at 58°C and fluorescence acquisition, 8 sec at 72°C, 0 sec at 95°C.

Transcripts: One-step duplex RT-qPCR was performed in a LightCycler as described (172). In one-step RT-qPCR, RT reaction and PCR amplification for one of the 23 analyte transcripts and an internal reference gene transcript (porphobilinogen deaminase, PBGD, for 22 analyte transcripts; or hypoxanthine guanine phosphoribosyl transferase, HPRT, for Arginase 1 analyte transcript) were performed in the same tube. All analyte transcript numbers are expressed as copies per 1000 reference transcripts. Twenty-three murine analyte transcripts for which one-step duplex PCRs have been developed include cellular markers, chemokines and cytokines, and inflammatory effectors and regulators (Tables 3a-c, 4a, b).

Table 4a. Functional significance, key references, and GenBank accession of transcripts of cellular marker genes.

Marker for:	Transcript	Function	GenBank accession number & references
Total cells	PBGD intron	The 3rd enzyme of the heme biosynthetic pathway	J04981 (9)
Neutrophils	Lactoferrin	Neutrophil secreted antimicrobial-globular protein	NM_008522 (140, 154)
Macrophages	F4/80	Macrophage surface antigen with unknown function	NM_010130 (88, 149)
Natural Killer cells	NKp46	NK cell natural cytotoxicity triggering receptor 1	NM_010746 (131, 175)
T cells	CD3δ	T cell-specific, expressed T cell receptor component	NM_013487 (30, 39, 76)
T helper 1 cells	Tim 3	CD4 Th1 cell surface protein	AF450241 (105, 171, 184)
T helper 2 cells	GATA-3	CD4 Th2 cell-specific GATA-3 transcription factor	X55123 (82, 109, 120, 184)
Naïve T cells	CD45RB	Naïve T cell surface protein	NM_011210 (56, 145)
Memory T cells	CD45RO	Memory T cell surface protein	NM_011210 (55, 145)
Cytotoxic T cells	Perforin 1	CD8 CTL and NK cell secreted cytolytic granule protein	J04148 (129, 166,174)
B cells	CD19	B cell surface protein	NM_009844 (48, 49, 147, 185)

Table 4b. Functional significance, key references, and GenBank accession of transcripts of inflammatory regulator genes.

Marker for:	Transcript	Function	GenBank accession number & references
Arginase 1	Arginase1	Liver isoform, cytosolic enzyme	NM_007482 (106, 161)
Arginase 2	Arginase2	Extra-hepatic (macrophage) isoform, mitochondrial enzyme	NM_009705 (106, 153)
NOS2	NOS2	Macrophage produced Nitric oxide synthase 2	NM_010927 (92, 118)
Oxidative burst oxidase	Cybb	Cytochrome b-245, beta polypeptide (formerly $gp91^{phox}$ subunit of the phagocyte oxidative burst NADPH oxidase)	NM_007807 (12, 25)
Cyclooxygenase 2	Ptgs2	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2, COX-2)	NM_01198 (38, 115)
IL-6	IL-6	Interleukin-6	NM_031168 (102, 168)
TNF-α	TNF-α	Tumor necrosis factor	NM_013693 (15, 46)
IFN-γ	IFN-γ	Interferon gamma	NM_008337 (15)
MIP-2	Cxcl2	C-X-C motif ligand 2 (formerly macrophage inflammatory protein-2, MIP-2, homolog to human IL-8)	NM_009140 (58, 162)
IL-10	IL-10	Interleukin-10	NM_010548 (80, 108)
MCP-1	Ccl2	C-C motif ligand 2 (monocyte chemotactic protein-1, MCP-1)	NM_011333 (14, 144)

Statistical Analysis. All statisticial analyses were performed with the Statistica 7.0 software package (StatSoft, Inc., Tulsa, OK). Data of *C. pneumoniae* genome and transcript numbers were Log_{10} transformed. Normal distribution of data was confirmed by Shapiro-Wilk's W test, and homogeneity of variances by Levene's test. Data were analyzed by mean plots ± 95% confidence intervals in factorial analysis of variance (ANOVA). Comparisons of means under the assumption of no *a priori* hypothesis were performed by the Tukey honest significant difference (HSD) test. The main effects dietary protein and antioxidants on the 25 outcome parameters were visualized in plots of the Log_2 transformed ratios of the mean value at low main effect level over the mean value at high main effect level. Differences at $p \leq 0.05$ in Tukey HSD were significant.

RESULTS

Dietary protein and antioxidants are associated with a significant modification of B and $CD4^+$ T lymphocyte marker transcripts and their ratio. After inoculation with *C. pneumoniae*, mice were sacrificed on day 3 or 10, lungs were processed, and a total of 28 outcome parameters were determined. Transcripts of IL-4 and C-reactive protein were not detected in any specimen, and are therefore not included in analyses. The main effect of the factors protein and antioxidant on the remaining 26 parameters was determined by analysis of variance using the complete data of the balanced experiment with 320 mice. The results are shown in Figure 7 as the $\log_2$ of the ratio of the mean of the low factor level (protein or antioxidant) divided by the mean of the high factor level ($\log_2$ ratio = $\log_2(10^{\log 10 \text{ low level}}/10^{\log 10 \text{ high level}})$. Thus, a value above zero for a parameter indicates an

increase at low factor level over the value at high factor level, and a value below zero indicates a decrease. The significance of the changes in Figure 7 was ascertained by the Tukey HSD test.

Differences in most outcome parameters between high and low diet protein or antioxidants oscillated marginally around the zero baseline and were not significant. Interestingly however, transcripts of markers of major lymphocyte populations showed a significant and opposite pattern. CD19 transcripts, a marker of B lymphocytes, were significantly higher in lung tissue of mice fed a low protein diet as compared to a high protein diet ($p = 0.002$; mean low protein = $10^{3.56}$ CD19 transcripts per 1000 PBGD transcripts, n = 160; mean high protein = $10^{3.47}$ CD19 transcripts, n = 160). Dietary antioxidant levels had the opposite effect, and CD19 transcripts were significantly lower in mice fed a low antioxidant diet ($p < 0.001$; mean low antioxidant = $10^{3.46}$; mean high antioxidant = $10^{3.57}$). An opposite effect was evident for CD45RB transcripts, a surface protein predominantly expressed in naïve $CD4^+$ T_{helper} cells, with a significant reduction at low protein ($p = 0.03$), but higher at low diet antioxidant content ($p = 0.122$). Thus, dietary protein and antioxidants exerted a significant main effect on B and $CD4^+$ lymphocyte transcripts and their ratio: low dietary protein was associated with a high B/$CD4^+$ T cell ratio (Log_2 CD19/CD45RB = 0.872, $p = 0.035$), and low antioxidants with a low ratio (Log_2 CD19/CD45RB = -0.845, $p = 0.041$).

Immune C57BL/6 mice on low-protein & low-antioxidant diet have a profoundly severe day-10 disease. As the next step, we analyzed the full factorial interaction effect on the two principal outcome measures of *C. pneumoniae* inoculation - disease intensity expressed as percent lung weight increase and total *C. pneumoniae* lung burden.

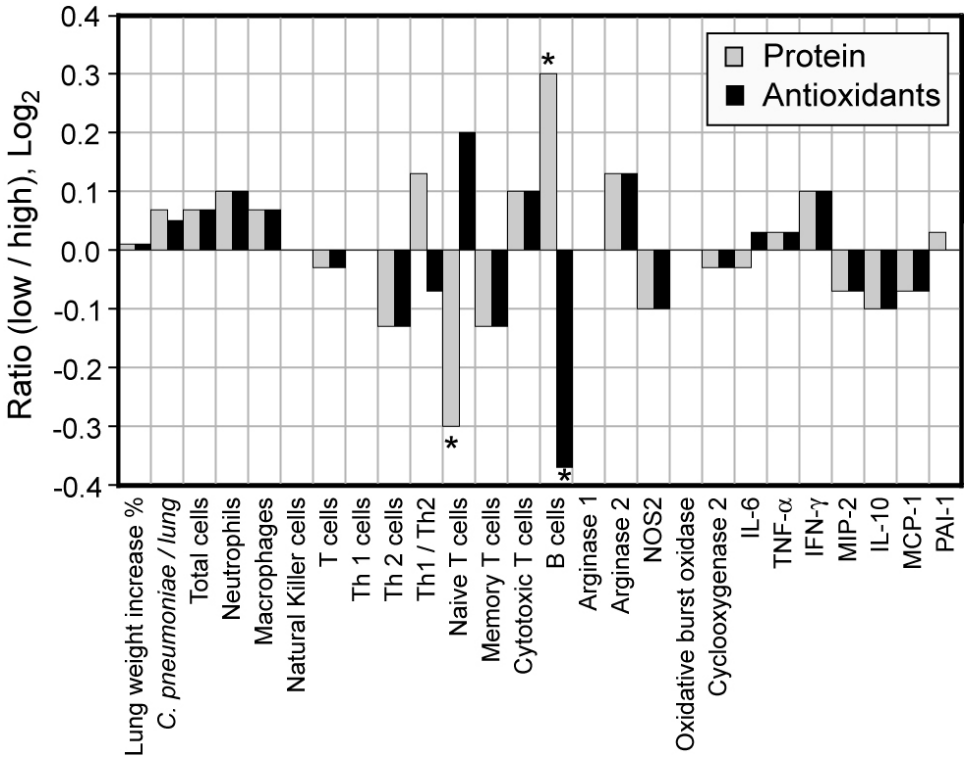

Figure 7. **Influence of dietary protein and antioxidants on 25 outcome parameters of *C. pneumoniae* lung infection.** Female A/J or C57BL/6 mice received a pre-challenge mock inoculum (naïve) or a low-dose *C. pneumoniae* inoculum (5×10^6 EB; immune), were fed a diet containing low (14%) or high (24%) protein and low antioxidant (3.56 g vitamin C + 0.85 g vitamin E acetate per kg diet) or high antioxidant (6.42 g vitamin C + 1.57 g vitamin E acetate per kg diet), intranasally challenged 4 weeks later with 1×10^8 *C. pneumoniae*, and sacrificed during early disease (3 days post inoculation, pi) or peak disease (10 days pi). Results are shown as $\log_2$-transformed values of the mean of all 16 groups of 10 mice with the low level over the mean of all 16 groups with the high level of a dietary main effect (protein or antioxidant). Open circles denote the ratio of parameters for the factor protein content and closed circles for antioxidant content. Asterisks indicate significant differences in parameters between high and low factor levels ($p < 0.05$, Tukey HSD test). The data indicate that low dietary protein results in decreased CD45RB ($p = 0.03$), and in increased CD19 ($p = 0.002$) lung transcripts. Low dietary antioxidants resulted in decreased CD19 transcripts ($p < 0.001$).

The factors and their levels are: mouse strain (A/J - C57BL/6), immune status (naïve - immune), sacrificing time after inoculation (day 3 - day 10), dietary protein (high - low), and dietary antioxidants (high - low). Data shown in Figure 8 indicate that dietary protein and antioxidants did not influence lung weight increases of naïve animals of either strains or time points. However, for immune mice a highly significant 4-way interaction between mouse strain, time after inoculation, diet protein, and diet antioxidants was observed. Immune C57BL/6 mice on LL diet (low-protein & low-antioxidant) had more severe disease (89% lung weight increase) on day 10 as illustrated in Figure 9 than other groups ($p \leq 0.003$; n=10). In Fig. 8C, the corresponding lung weight increase of 26% on day 3 for these mice is lower than for the three other diets (HH, LH, HL), but only the difference to C57BL/6 mice on HL (high-protein & low-antioxidant) diet is significant ($p = 0.008$).

Similar to the effect on lung weight increases, dietary protein and antioxidants did not significantly influence *C. pneumoniae* lung burdens of naïve mice of either strain or time point (Figure 10) ($p > 0.05$). The chlamydial lung burden of immune A/J mice was almost always significantly different from naïve mice, higher on day 3 and lower on day 10. In immune C57BL/6 mice on day 3, the chlamydial lung burden was significantly higher than in naïve mice for all diets except the LL diet. On day 10, lung *C. pneumoniae* loads of naïve and immune C57BL/6 mice were not statistically different for all diets ($p > 0.05$). Naïve mice of both strains did not achieve a net elimination of *C. pneumoniae* between days 3 and 10 after inoculation. In contrast, immune mice reduced lung *C. pneumoniae* significantly ($p \leq 0.047$) between days 3 and 10, except for C57BL/6 mice on LL diet, which had essentially identical chlamydial loads on day 3 and 10 post

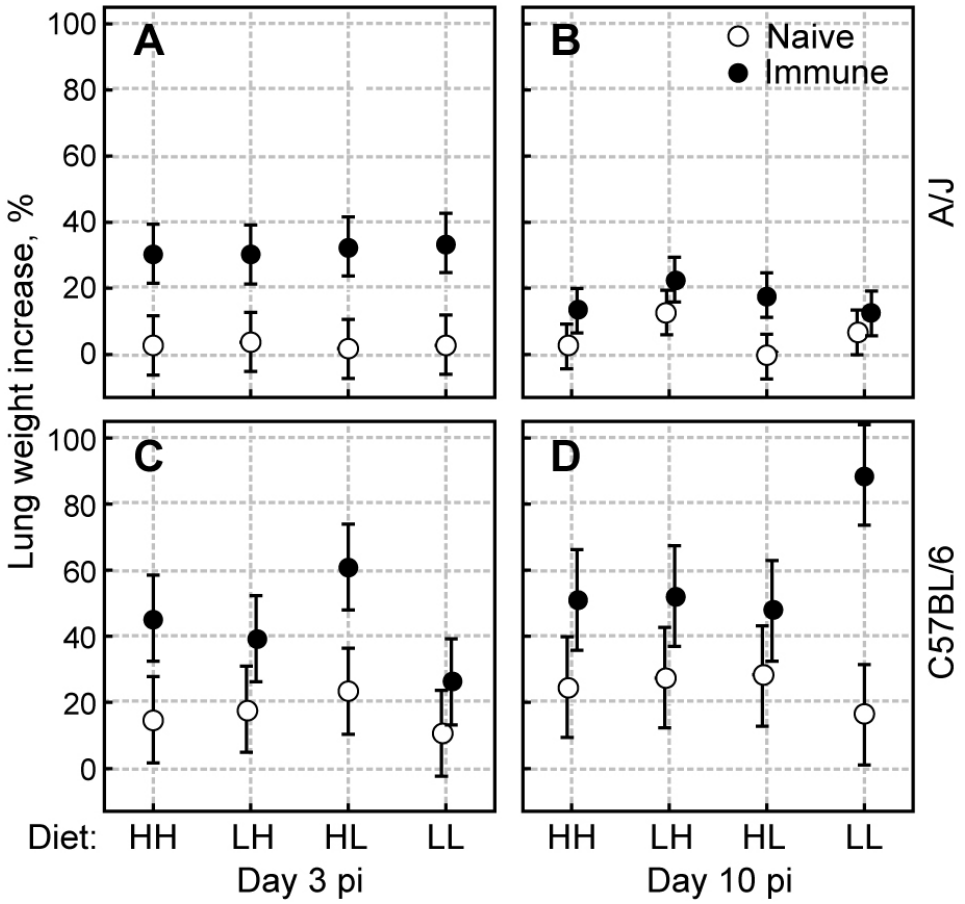

Figure 8. **Influence on the lung weight increase outcome of the interaction between mouse strain, immune status, time after inoculation, and dietary protein and antioxidants.** Naïve mice received a pre-challenge mock inoculum, immune mice received a low-dose *C. pneumoniae* inoculum (5×10^6 EB). All mice were challenged 4 weeks later intranasally with 1×10^8 *C. pneumoniae* EB. HH, high-protein & high-antioxidant; LH, low-protein & high-antioxidant; HL, high-protein & low-antioxidant; LL low-protein & low-antioxidant. Data represent means per treatment group (n=10) ± 95% confidence interval. **A**. Immune A/J mice had significantly higher lung weight increases than naïve mice on day 3 pi ($p < 0.05$, Tukey HSD test). **B**. On day 10 pi lung weight increases of immune A/J mice had declined and were not significantly higher than those of naïve mice. **C**. C57BL/6 mice had generally higher lung weight increases than A/J mice, and immune C57BL/6 mice had significantly higher increases than naïve C57BL/6 mice ($p < 0.05$). The exception are immune C57BL/6 mice on low-protein & low-antioxidant diet on day 3, which have a low lung weight increase (26%) that does not differ from that of naïve C57BL/6 mice. **D**. In contrast, on day 10 pi, immune C57BL/6 mice on low-protein & low-antioxidant diet show the most severe disease of all mice in this experiment. The lung weight increase of 89% is highly significantly higher than that of any other treatment group at peak disease on day 10 pi ($p \leq 0.003$).

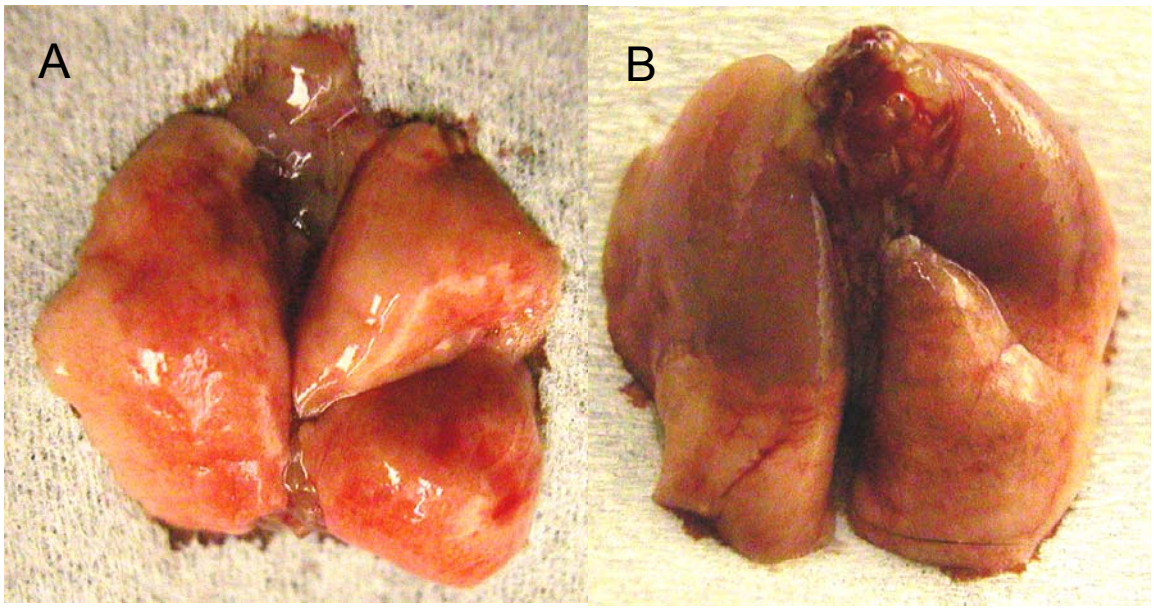

Figure 9. Comparison of mock-inoculated and *C. pneumoniae* inoculated mouse lungs. A. C57BL/6 mouse lung 10 days after mock inoculation. **B**. Lung of immune C57BL/6 mouse on LL diet on day 10 after inoculation with *C. pneumoniae*. Mice were inoculated intranasally with purified *C. pneumoniae* EBs suspended in SPG buffer or with SPG buffer only. Under light isoflurane anesthesia, 30 µl of SPG containing chlamydial EBs or control inoculum was applied onto the nostrils with a pipette. Mice were sacrificed by CO_2 inhalation 10 days after inoculation. The lung of the mock-inoculated mouse is collapsed, while the *C. pneumoniae*-inoculated lung tissue is of the anterierior lobe is consolidated due to interstitial cellular infiltration.

inoculation. In the combined analysis of both principal infection outcomes, it is clear that only the low-protein & low-antioxidant diet in previously immunized C57BL/6 mice resulted in profound peak disease on day 10. This peak disease was coupled to reduced disease on day 3 and a lack of elimination of *C. pneumoniae* between days 3 to 10 after inoculation.

The severe day-10 disease in immune C57BL/6 mice on low-protein & low-antioxidant diet is driven by extensive Th1 inflammation. The increased lung weight on day 10 was not due to increased lung cellularity as indicated by the PBGD intron levels, since day 3 and day 10 C57BL/6 lungs had virtually identical copy numbers of mouse genomes. To further characterize the profound disease in C57BL/6 mice, we analyzed differences in day-10 outcome parameters of immune C57BL/6 mice between high and low dietary protein levels at the low antioxidant level, and between high and low dietary antioxidant levels at the low protein level. The differences were computed as means at the low level of the variable dietary component divided by the respective means at the high level (Figure 11). Day-10 PBGD intron levels in C57BL/6 mice on the disease-precipitating LL diet did not change significantly as compared to the HL or LH diets, confirming that a quantitatively increased cellular infiltrate was not the cause of the severe disease. Transcripts of inflammatory cytokines, regulators, and effectors such as IFN- γ, TNF- α, IL-6, IL-10, Arg1, Arg2, and Ptgs2 were significantly higher at low protein than high protein at the low antioxidant level (Figure 11A) ($p < 0.05$, Tukey HSD). Interestingly, NKp46 and Serpine1 transcripts were lower ($p \leq 0.001$). The Arg1 transcript was higher at low antioxidant low protein and the Th1/Th2 balance shifted

towards Th1 as indicated by the higher Tim3/GATA3 ratio in the same condition (Figure 11B) ($p \leq 0.035$).

In contrast to C57BL/6 mice, immune A/J mice had the lowest disease on LL diet (Figure 8). Numerous transcript levels were significantly different between either high protein or high antioxidants and the respective low factor level (Figure 11). The most significant transcript changes are increases at low antioxidants and low protein in CD3δ, GATA3, Perforin1, and CD19 by more than 2-fold (Figure 11B) than at high antioxidant and low protein, and increases in Tim3, Arg2, and Tim3/GATA3 ratio by up to 50% at high antioxidants and low protein over levels at low antioxidants and low protein ($p \leq 0.019$). Differences between high and low dietary protein were observed for fewer parameters, most notably significantly higher GATA3 transcripts ($p = 0.005$) and lower CD45RO memory T cell transcripts ($p = 0.033$).

Overall, the data indicate that A/J mice on LL diet have the lowest day-10 disease and show a change in transcripts consistent with an enhanced T cell response, a Th2 shift, and reduced inflammation. In contrast, in highly diseased immune C57BL/6 mice, the low antioxidant content of the LL diet was associated with transcript changes consistent with a Th1 shift of the T cell response, and the low protein content was associated with with higher effectors and regulators of Th1 inflammation. This response in C57BL/6 mice likely was driven by $CD4^+$ T cells since transcript levels of the natural killer cell marker NKp46 were reduced.

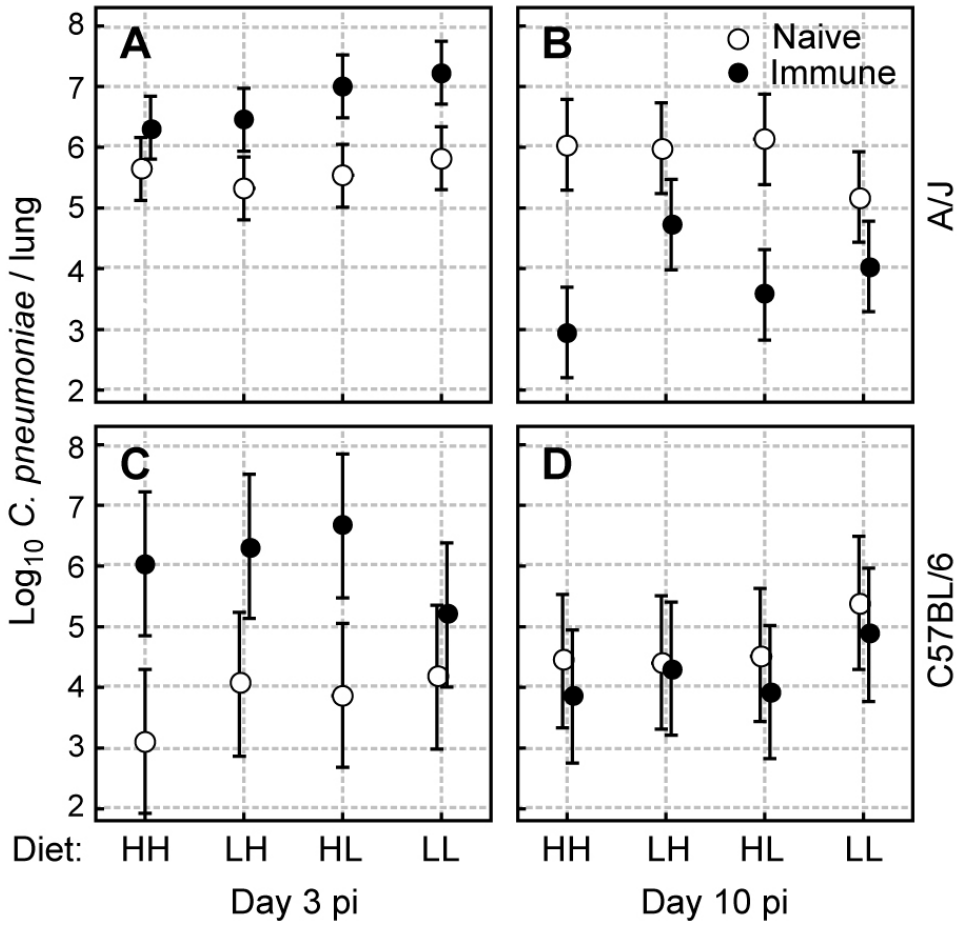

Figure 10. **Influence on the *C. pneumoniae* lung load outcome of the interaction between mouse strain, immune status, time after inoculation, and dietary protein and antioxidants.** Data are shown as means (n=10) ± 95 % confidence interval. **A**, **C**. On day 3 pi, immune A/J and C57BL/6 mice have generally higher *C. pneumoniae* lung burden than naïve mice, except for A/J mice on HH diet, and C57BL/6 on LL diet. **B**, **D**. Both naïve A/J and C57BL/6 mice did not achieve a net elimination of *C. pneumoniae* between days 3 and 10 days after infection. In contrast, immune A/J mice, but not C57BL/6 mice, significantly reduced *C. pneumoniae* lung burdens between day 3 and day 10 ($p < 0.05$, Tukey HSD test). Between day 3 and day 10 pi, the lung load of C57BL/6 mice on LL diet remained essentially unchanged at approximately 10^5 *C. pneumoniae* genomes per lung.

Reduced early T cell responses and Th1 inflammation associated with severe day-10 disease in immune C57BL/6 mice on low-protein & low-antioxidant diet. To associate early transcriptional events with the late severe Th1-driven inflammatory disease of immune C57BL/6 mice on LL diet, and to identify possible disease-regulatory mechanisms, we analyzed diet-associated differences in lung transcripts on day 3. The differences were visualized for both A/J and C57BL/6 mice separately for the comparison between high and low dietary protein at the high (Figure 12A) or low dietary antioxidant level (Figure 12B), and for the difference between high and low dietary antioxidants at the high (Figure 13A) or low dietary protein level (Figure 13B).

The differences of global day-3 transcript profiles between high and low dietary protein (Figure 12) were more pronounced than between high and low dietary antioxidants (Figure 13). Transcript profiles, particularly for changes in dietary protein, differed profoundly between A/J and C57BL/6 mice. The pattern of changes, discernible through lines between transcripts, is remarkably similar at both high and low levels of the respective unchanged dietary factor. Low dietary protein increased in A/J mice at high dietary antioxidant levels (Figure 12A) CD3δ, Tim3/GATA3, Cybb, and Ptgs2 ($p \leq 0.007$), and decreased F4/80, GATA3, and CD45RB transcripts ($p \leq 0.003$). C57BL/6 mice showed increased CD19 ($p < 0.001$), and decreased PBGD intron, CD45RB, CD45RO, Arg1, NOS2, Ptgs2, and IL-6 transcripts ($p \leq 0.05$). In A/J mice on low dietary protein at low dietary antioxidant content (Figure 12B), the level of PBGD intron, lactoferrin, CD3δ, Tim3/GATA3 ratio, perforin, and Ptgs2 increased ($p \leq 0.048$), but only GATA3 transcripts were significantly decreased ($p = 0.011$), and all other transcripts remained essentially unchanged.

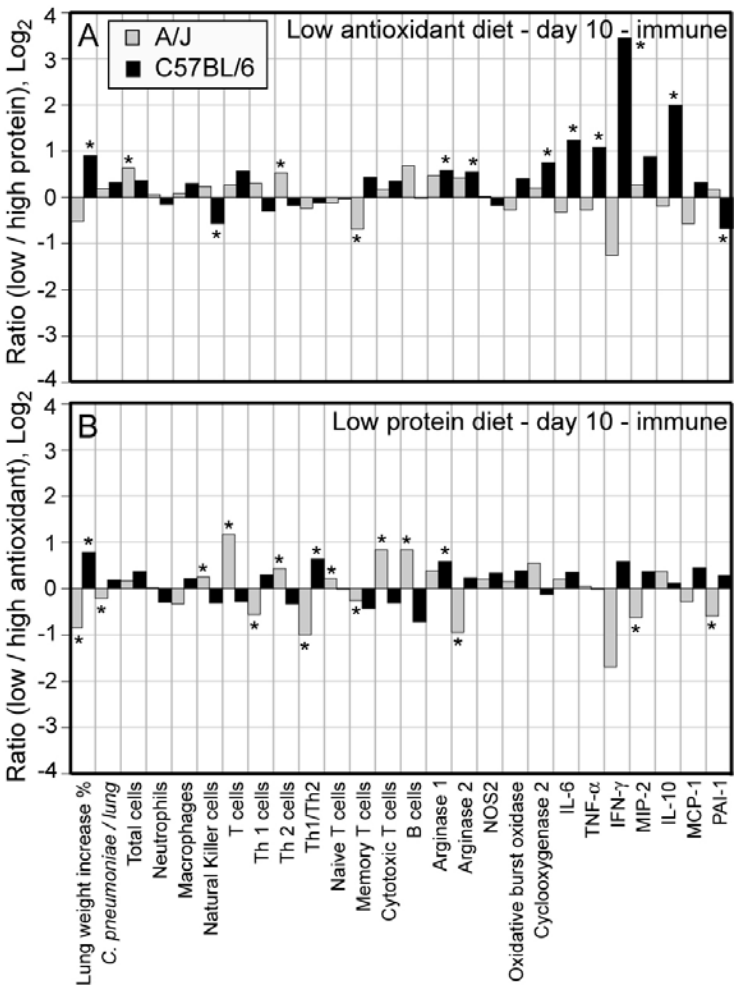

Figure 11. **Comparison of transcript levels at high and low dietary protein or antioxidants in day-10 lungs of mice.** Data describe **A**, the difference of parameters between high and low dietary protein at low dietary antioxidant content by comparing the HL to the LL diet (protein), or **B**, the difference of parameters between high and low dietary antioxidants at low dietary protein content by comparing the LH to the LL diet (antioxidants). In C57BL/6 mice the level of Th1-associated transcripts such as IFN-γ, TNF-α, IL-6, IL-10, Arg1, Arg2, and Ptgs2 was higher for low protein at low antioxidant level than for high protein at low antioxidant level (panel A; $p < 0.05$, Tukey HSD). NKp46 and Serpine1 transcripts were reduced ($p \leq 0.001$). A/J mice had higher PBGD intron and GATA3 levels at low protein and low antioxidants than at high protein and low antioxidants, but lower CD45RO transcript levels under identical conditions ($p \leq 0.033$). C57BL/6 mice had higher Arg1 transcripts and the Tim3/GATA3 ratio at low antioxidants and low protein than at high antioxidants and low protein (panel B; $p \leq 0.035$). A/J mice under identical conditions had numerous differences in transcripts, most prominently higher CD3δ, GATA3, Perforin1, and CD19 transcripts, and lower Tim3, Tim3/GATA3, and Arg2 levels ($p \leq 0.019$). The data show that the severe disease on day 10 pi of immune C57BL/6 mice fed the LL diet is associated with a Th1-driven inflammatory response, and that conversely the low disease in A/J mice is associated with an enhanced T cell response, a Th2 shift, and reduced inflammation.

C57BL/6 mice showed pronounced reduction of numerous parameters related to the lung cellular infiltrate (PBGD intron, CD3δ, Tim3, CD45RB, CD45RO, $p \leq 0.004$) and the inflammatory response (Arg1, NOS2, Cybb, Ptgs2, IL-6, TNF-α, IFN-γ,CxCl2, and IL-10, $p \leq 0.028$). F4/80 transcripts were increased ($p = 0.023$). The pattern of C57BL/6 mice on low dietary protein and low antioxidants resembled that at the low protein and high antioxidant level, but was much more pronounced (Figure 12).

Low dietary antioxidants at high dietary protein levels (Figure 13A) increased Tim3, CD45RB, CD45RO, and Ptgs2 transcripts in C57BL/6 mice, and decreased F4/80 transcripts ($p \leq 0.049$), but were not associated with any significant change in A/J mice. At low dietary protein levels (Figure 13B), low dietary antioxidants increased F4/80, Tim3, GATA3, CD45RB, and IL-10 transcripts in A/J mice ($p \leq 0.047$), and decreased CD3δ and CD19 transcripts in both A/J and C57BL/6 mice ($p \leq 0.037$). These data confirm that a fundamentally different early transcriptional response to changes in dietary protein and antioxidants is associated with the diametrically opposed late *C. pneumoniae* disease outcomes in immune A/J and C57BL/6 mice.

A/J mice showed fewer overall transcriptional changes, with the exception of an increase in CD3δ T cell marker transcripts and a profound shift of the Th1/Th2 balance towards Th1 at low protein levels. In contrast, immune C57BL/6 mice on day 3 on low dietary protein and low dietary antioxidant content showed a global pattern of reduced transcription of markers of the lung T cell population, of T cell-driven regulators and effectors of the inflammatory response, and of cytokines associated with Th1-driven inflammation. This transcriptional profile is consistent with suppression of the Th1 cell immune response.

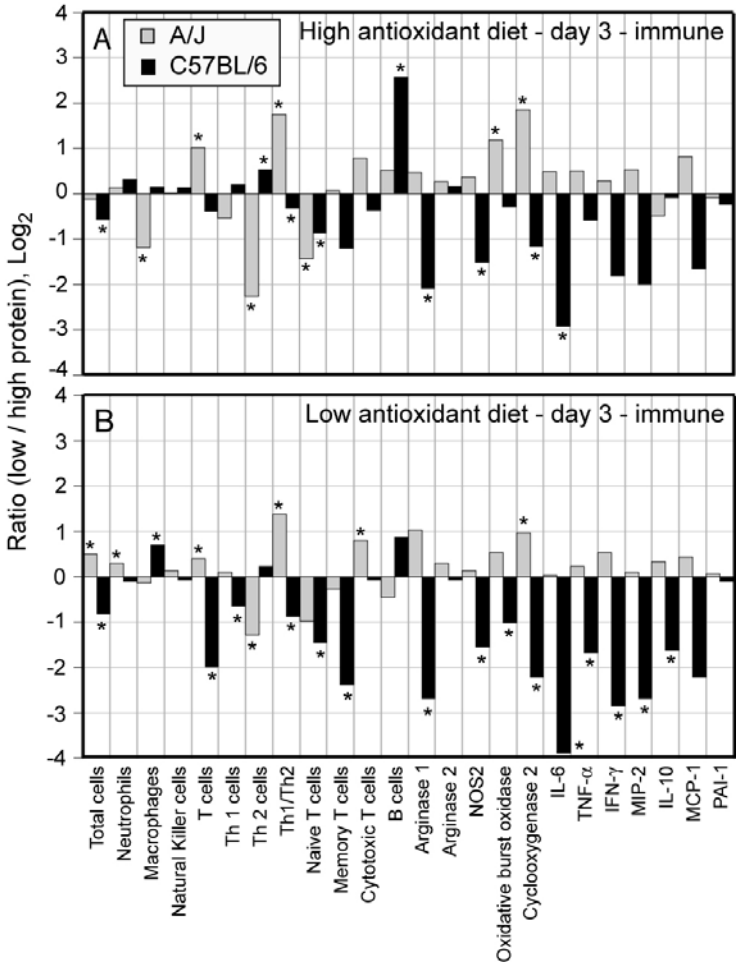

Figure 12. **Influence of diet protein on transcript levels of immune mice on day 3 pi.** Data (n=10) are shown as the $\log_2$ of the ratio of transcript levels at low-protein diet over transcript levels at high-protein diet. **A**. At high diet antioxidant levels, low diet protein as compared to high diet protein (LH versus HH diet) increased in A/J mice CD3δ, Tim3/GATA3, Cybb, and Ptgs2 ($p \leq 0.007$), and decreased F4/80, GATA3, and CD45RB transcripts ($p \leq 0.001$). C57BL/6 mice showed increased CD19 ($p < 0.003$), and decreased PBGD intron, CD45RB, CD45RO, Arg1, NOS2, Ptgs2, and IL-6 levels ($p \leq 0.05$). **B**. At low diet antioxidant levels, low diet protein (LL versus HL diet) increased in A/J mice the PBGD intron and CD3δ level, but most prominently Tim3/GATA3 ratio, Perforin1, and Ptgs ($p \leq 0.044$), GATA3 transcripts were significantly decreased ($p = 0.011$), and all other transcripts remained essentially unchanged. C57BL/6 mice showed reduction of numerous parameters related to the lung cellular infiltrate (PBGD intron, CD3δ, Tim3, CD45RB, CD45RO, $p \leq 0.004$) and the inflammatory response (Tim3/GATA3, Arg1, NOS2, Cybb, Ptgs2, IL-6, TNF-α, IFN-γ, CxCl2, and IL-10, $p \leq 0.028$). The macrophage marker F4/80 was increased ($p = 0.023$).

Suppression of the day-3 T cell response is significantly associated with late severe disease in immune C57BL/6 mice fed a low-protein & low-antioxidant diet. In a final analysis, we sought to identify principal transcript parameters of the early disease response that potentially precipitated the late severe disease of immune C57BL/6 mice. Using ANOVA, the full factorial interaction effects of dietary protein and antioxidant levels on day-3 transcripts of immune mice were evaluated. The objective was to identify significant differences in parameters that were uniquely present only during feeding of the low-protein & low-antioxidant diet.

Of all transcripts, only the T cell-specific CD3δ transcript was highly significantly ($p = 0.0001$) reduced by more than four-fold specifically in C57BL/6 mice on the low-protein & low-antioxidant diet (Figure 14A). Other transcripts were changed as evident in Figures 12 and 13, but the changes did not appear specifically only in the LL diet. Figure 14 illustrates the changes in the Th1/Th2 ratio (Tim3/GATA3, Figure 14B), in memory (CD45RO, Figure 14C) and naïve (CD45RB, Figure 14D) T cell transcripts, and in B cell transcripts (CD19, Figure 14F). Figure 7E shows changes in Arginase 1 transcripts, to illustrate the pattern of inflammatory marker molecules, which is repeated in virtually all significantly different inflammation-associated parameters (Figures 12, 13).

These results strongly suggest that suppression of the T cell response is the unique feature of the early disease response that primes immune C57BL/6 mice to severe disease later. While C57BL/6 mice have in general a higher disease response than A/J mice, the pattern of four-fold lower CD3δ transcription is specific for the low-protein & low-antioxidant diet, and does not appear in any other combination of these nutrients.

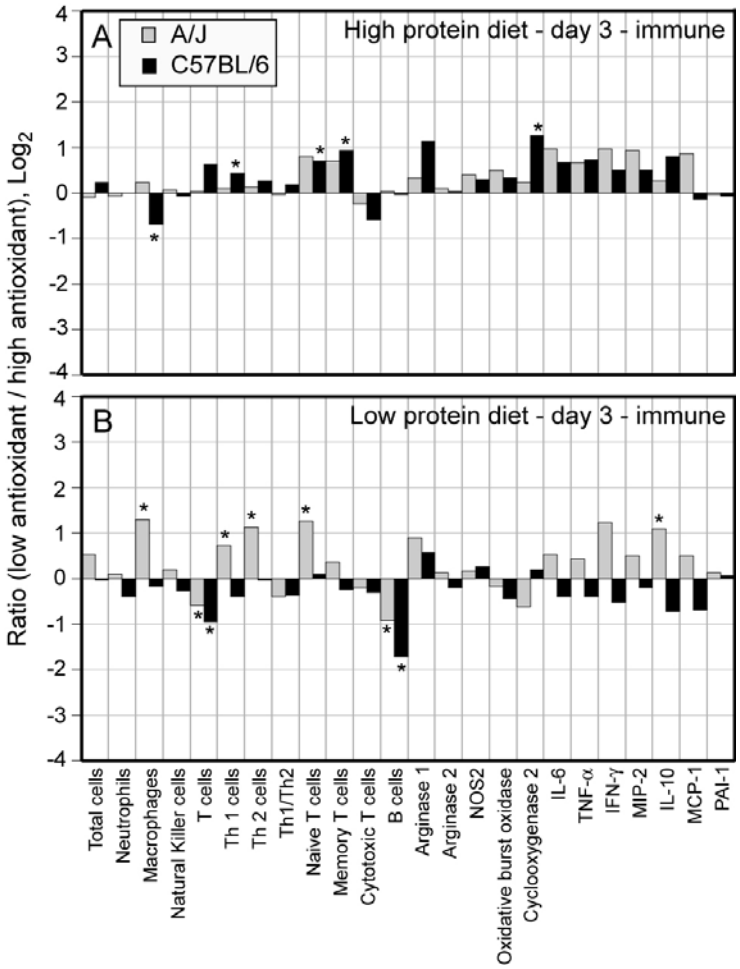

Figure 13. **Influence of diet antioxidants on transcript levels of immune mice on day 3 pi.** Data (n=10) are shown as the $\log_2$ of the ratio of transcript levels at low-antioxidant diet over transcript levels at high-antioxidant diet. **A**. At high diet protein levels, low diet antioxidants as compared to high diet antioxidants (HL versus HH diet) increased Tim3, CD45RB, CD45RO, and Ptgs2 transcripts, decreased F4/80 in C57BL/6 mice ($p \leq 0.05$), but did not change any transcript significantly in A/J mice. **B**. At low diet protein levels, low diet antioxidants (LL versus LH diet) increased F4/80, Tim3, GATA3, CD45RB, and IL-10 transcripts in A/J mice ($p \leq 0.047$), while they decreased CD3δ and CD19 transcripts in both A/J and C57BL/6 mice ($p \leq 0.037$).

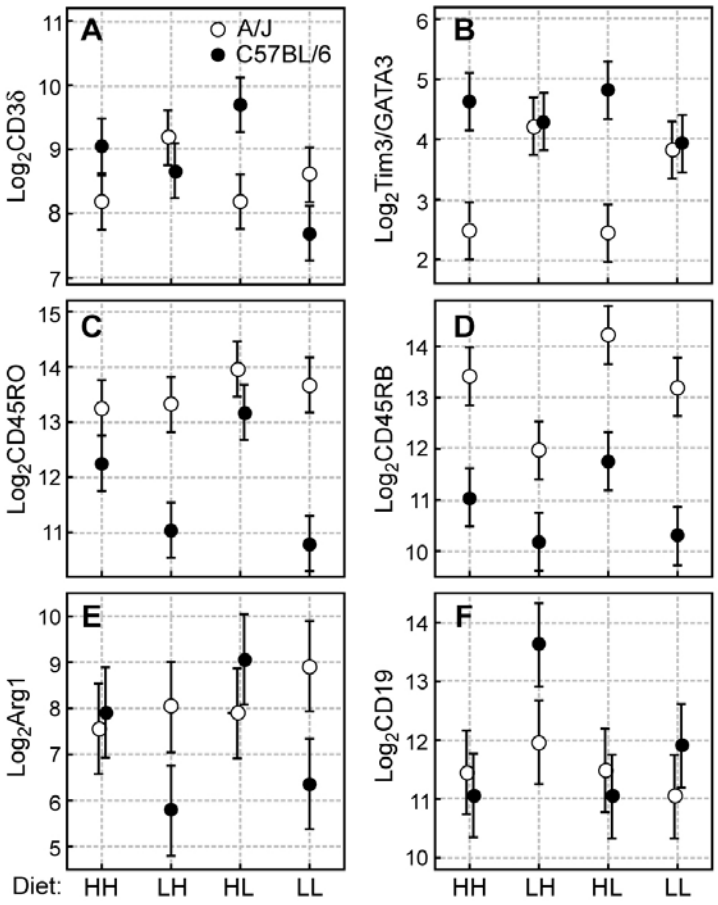

Figure 14. Influence of the interaction between mouse strain and dietary protein and antioxidants on day 3 in immune mice on highly significant outcome parameters. Data represent means per treatment group (n=10) ± 95% confidence interval. **A**. CD3δ expression level was significantly lower in C57BL/6 mice fed the low-protein & low-antioxidant (LL) diet as compared to the high-protein & low-antioxidant (HL) diet ($p < 0.001$). **B**. The Tim3/GATA3 ratio of A/J mice increased highly significantly in response to low diet protein ($p \leq 0.003$), but remained unchanged in C57BL/6 mice. **C**. CD45RO expression in C57BL/6 mice decreased in response to reduction of diet protein ($p \leq 0.022$), while it remained unchanged in A/J mice. **D**. CD45RB expression was always significantly higher in A/J mice than in C57BL/6 mice ($p \leq 0.001$). Both mouse strains responded to low diet protein with a decrease in CD45RB transcripts, but this difference was not consistently significant. **E**. Arginase1 expression decreased in response to reduced diet protein in C57BL/6 mice ($p \leq 0.05$), but not A/J mice. **F**. CD19 expression increased significantly in C57BL/6 mice on low-protein & high-antioxidant (LH) diet ($p \leq 0.023$), but did not change in A/J mice in response to diet protein or antioxidants.

In contrast, A/J mice are not negatively affected by this combination of dietary components, and in fact appear even more resistant to *C. pneumoniae* when fed the LL diet.

DISCUSSION

In this report, we examined the influence of dietary protein and antioxidants on disease, *C. pneumoniae* burden, and gene transcription outcomes at early and peak disease following intranasal challenge with *C. pneumoniae*. To maximize the analytical accuracy, we examined these parameters directly in the affected organ, the lung. In contrast to microarray analyses, this approach targets a smaller number of transcripts, but those with higher accuracy in one-step duplex real-time PCR in which the analyte and reference transcript are simultaneously quantified. This approach allows absolute comparison of multiple factor levels (32 overall) with appropriate statistical resolution (n=10/factor level). We further evaluated if the host genetic background of susceptible C57BL/6 mice or resistant A/J mice, and the presence or absence of specific immunity against *C. pneumoniae*, modified the outcomes. These categorical variables were assessed in a balanced experiment by feeding four controlled custom diets containing all combinations of two concentrations of protein and antioxidants. The concentrations of these main dietary components were close to, but within, the upper and lower boundaries of recommended concentrations in rodent diets.

We report profoundly different outcomes of disease and gene transcription in dependence of the dietary variables, host strain, and pre-existing immunity against *C. pneumoniae*. Differential gene transcription early after inoculation is associated with a

minimal or a severe disease response one week later. These late responses are again characterized by fundamentally different gene transcription profiles.

Our analytical approach was to initially identify main effects of the dietary components, followed by full factorial interaction analysis of the effect of all categorical variables on the principal outcomes of the infection with *C. pneumoniae* - lung disease quantified as percent lung weight increase over the lung weight of matched healthy mock-infected mice, and *C. pneumoniae* lung burden quantified by the number of *C. pneumoniae* genomes per lung. A unique dichotomy of the day-10 disease outcome in immune mice was apparent at the lower levels of both dietary protein and antioxidants. A/J mice fed the low-protein & low-antioxidant diet showed the lowest disease while C57BL/6 mice showed the most severe disease of all groups in the experiment. We identified the transcriptional profiles uniquely associated with the low levels of both dietary components by a series of analyses contrasting in each immune mouse strain the transcription between high and low level of one dietary factor while the other factor was kept constant at high or low dietary content. In a final approach, we identified, by quantitative comparison of the full interaction of dietary factor levels, early transcripts that uniquely differentiated the severe disease outcome of C57BL/6 mice from the full protection outcome of A/J mice.

Using this analytical strategy, we first found that dietary protein and antioxidant significantly ($p \leq 0.029$) and reciprocally modified marker transcript concentrations of B and $CD4^+$ lymphocytes. Specifically, low dietary protein increased the B/$CD4^+$ lymphocyte transcript ratio, and low antioxidants decreased this ratio. Thus, the combined effect of low protein and antioxidants resulted in an unchanged ratio, and we

did not find an association of the B/CD4$^+$ lymphocyte transcript ratio with disease severity.

Analysis of day-10 relative transcript profiles indicated that the severe day-10 disease in immune C57BL/6 mice on low-protein & low-antioxidant diet had an extensive Th1 inflammation transcript profile. In contrast, disease-free A/J mice had transcript changes indicative of an enhanced T cell response, a Th2 shift, and reduced inflammation. In C57BL/6, low levels of dietary protein and antioxidants had a differential effect: low protein associated with an upregulation of effectors and regulators of Th1 inflammation, and low antioxidants associated with a Th1 shift of the T cell response. This response in C57BL/6 mice likely was driven by CD4$^+$ T cells since transcript levels of the natural killer cell marker NKp46 were reduced.

In efforts to uncover potential factors causing the late disease dichotomy between immune A/J and C57BL/6 mice, the day-3 transcript profiles were analyzed. C57BL/6 mice on low-protein & low-antioxidant diet had highly reduced transcripts associated with early T cell responses and Th1 inflammation while A/J mice had increased T cell marker transcripts and a profound shift of the Th1/Th2 balance towards Th1. These data confirmed that a fundamentally different early transcriptional response to changes in dietary protein and antioxidants was associated with the diametrically opposite late *C. pneumoniae* disease outcomes in immune A/J and C57BL/6 mice. C57BL/6 mice on the low-protein & low-antioxidant diet that precipitated severe day-10 disease had a day-3 transcription profile of suppression of the Th1 cell immune response while A/J mice with no day-10 disease had an enhanced day-3 Th1 immune response.

The final quantitative comparison of the full interaction effects of dietary factor levels on early transcripts indicated that transcription of the pan-T cell marker CD3δ uniquely differentiated C57BL/6 on LL diet from C57BL/6 mice on any other diet, and from all A/J mice. Thus, suppression of the day-3 T cell response was uniquely and highly significantly ($p < 0.0002$) associated with late severe disease in immune C57BL/6 mice fed a low-protein & low-antioxidant diet. These results strongly indicate that suppression of the T cell response is the unique feature of the early disease response that primes immune C57BL/6 mice to severe disease later. While C57BL/6 mice have, in general, a higher disease response than A/J mice, the pattern of four-fold lower CD3δ transcription is specific for the low-protein & low-antioxidant diet, and does not appear in any other combination of these nutrients. In contrast, A/J mice are not negatively affected by this combination of dietary components, and in fact appear even more resistant to *C. pneumoniae* when fed the LL diet.

The immunosuppressive effect of protein malnutrition and the associated reduction of the resistance to infectious diseases have been documented before (26, 28, 138). Other reports have demonstrated a deregulation of the macrophage proinflammatory cytokine network (1), altered distribution of T-cell subsets under protein deficiency (97, 178), and the modulation of such immune responses by oxidative stress and antioxidant exposure (83, 98, 151, 163). However, reports about the effect of protein calorie malnutrition on immune response have not agreed (110, 128, 156).

Our data conclusively indicate that the profound effect of protein malnutrition that results in severe disease in *C. pneumoniae* infection depends on co-factors that modulate this effect. First, enhanced disease at low dietary protein is restricted to one mouse strain

and to animals with a preformed *C. pneumoniae*-specific immune response. Second, even in immune C57BL/6, severe disease and the full early transcriptional profile associated with it, develop only at simultaneous relative protein and antioxidant deficiency. Thus, modeling of nutritional influence on disease and the associated immune response requires a multivariate approach.

In our model, combined protein-antioxidant nutritional deficiency resulted in the early suppression of specific Th1 immunity. The suppressed and Th2-biased T-cell response against *C. pneumoniae* resulted in low early inflammation and disease, but failed to reduce the chlamydial burden. The absolute requirement for a Th1 immune response for chlamydial elimination has been well established (63, 107). Later, on day 10, severely diseased C57BL/6 mice had developed a strong Th1 immune and inflammatory response that was associated with the disease. In contrast, disease-protected A/J mice had mounted a strong Th1 immune response on day 3, by day 10 had substantially eliminated *C. pneumoniae*, and had modified their response towards reduced and Th2-shifted T-cell immunity.

Collectively, by multivariate modeling of nutritional influence on chlamydial disease, we have been able to identify a set of restrictive factors that maximize the amplitude between disease and protective outcome of chlamydial infection. We have not only identified the nutritional conditions for disease, but also general mechanisms of chlamydial pathogenesis. Our data clearly support the concept that an early, vigorous, specific Th1 immune response against chlamydial infection is crucial for avoidance of later disease. While early immune suppression and/or Th2 bias somewhat reduce early disease, the price is an exacerbated late Th1 immune and inflammatory response that

represents the full manifestation of chlamydial disease. While nutrition and immune status are indispensable co-factors, the ultimate regulator of chlamydial disease is the host, and its genetic background that determines the principal susceptibility to disease such as that of the C57BL/6 mouse strain, and the principal resistance of the A/J strain.

VI. OVERALL CONCLUSIONS

In this research two fundamentally different animal models were used to examine the disease potential of clinically inapparent chlamydial infection and to investigate mechanisms and factors that promote protection from, or manifestation of disease. In the heifer uterine *C. abortus* infection model, an immune response activated by recent infection, as indicated by increased IgM levels, provided protection against *Chlamydia*-mediated suppression of fertility. Protection presumably was mediated by reduction of uterine inflammation in response to the influx of *Chlamydia*-infected macrophages. The short duration of protection, 3-4 months, suggests that circulating effector T cells rather than antibodies were protective. This concept is consistent with our understanding from rodent animal models of the immune mechanisms that lead to clearance of chlamydiae.

Knowledge accumulated over decades of research indicates that chlamydial disease is caused not by direct action of chlamydial toxins, but rather by the host immune and inflammatory response to the organisms. In the mouse lung infection model we therefore analyzed which mechanisms and dietary factors precipitated a disease response to chlamydial infection versus elimination of chlamydiae without apparent disease. These studies unambiguously revealed that a vigorous T cell response in the early phase of chlamydial infection is required for protection from disease. In addition to high intensity, a functional profile of T helper cell 1 immunity is indispensable for this specific T cell responsere to chlamydial infection. While the vigorous Th1 response resulted in

inflammation and somewhat enhanced disease in the early phase of chlamydial infection, it ensured reduction of chlamydial burden and avoidance of much more sever disease later. In contrast, early immune suppression and/or Th2 bias somewhat reduce early disease, but at the price of an exacerbated late Th1 immune and inflammatory response that represents the full manifestation of chlamydial disease. While nutrition and immune status are indispensable co-factors, the ultimate regulator of chlamydial disease was the host, and it is the genetic background of the host that determines the principal susceptibility to disease.

In conclusion, both models employed in this research point to the importance of the time sequence of the immune response: if vigorous Th1 immunity occurs early after infection with chlamydiae it protects from disease. If it appears only later in reponse to prolonged chlamydial antigenic stimulus, the same Th1 immune response results in profound disease. Chlamydial infections are ubiquitous, and multiple infections are common in animal and humans. While we cannot control the host genetic background that is responsible for principal susceptibility or resistance, we are able to favorably influence epidemiological and dietary factors that can cooperate to result in severe disease, but in optimum combination can also prevent manifestation of disease in susceptible individuals.

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