



Adaptive immunity to *Chlamydia pneumoniae* respiratory infection
by Thomas Gordon Day, III

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Microbiology

Montana State University

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Abstract:

Chlamydia pneumoniae is a significant cause of respiratory infection in humans and has been associated with cardiovascular disease. Many studies have investigated the relationship between *C. pneumoniae* infection and cardiovascular disease, but few have examined host immunity to respiratory infection. Using a murine model of respiratory infection, we sought to characterize the course of primary and secondary *C. pneumoniae* infection, and to determine the role of humoral and cell mediated immune responses in adaptive immunity to infection. Intranasal inoculation with *C. pneumoniae* resulted a self-limiting respiratory infection that resolved within 21 days. Histological evaluation of lung sections revealed an interstitial pneumonia composed of polymorphonuclear neutrophils and mononuclear cells, and the association of *C. pneumoniae* inclusions with bronchial epithelial cells. Inflammation peaked at 10-14 days post infection and had completely resolved by five weeks following primary infection. The inflammatory response consisted primarily of CD4⁺ T cells and B cells when analyzed by immunohistochemistry. Anti-*C. pneumoniae* serum and bronchial antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), and were characterized by a predominance of IgG2a and IgG2b and the lack of IgA. Cytokine production by immune splenocytes was analyzed as a measure of cell mediated immunity, and was characterized by the production of IFN- γ and IL-10. Mice were susceptible to reinfection, but showed a level of protective immunity as evidenced by a shortened course of infection. Reinfection was also characterized by anti-*C. pneumoniae* IgA in bronchial washes, and a marked CD4⁺ T cell and B cell response in lung tissues. To assess the role of B cells in protective immunity to *C. pneumoniae* respiratory infection, primary and secondary infections were evaluated in B cell gene knockout mice. Infection of B cell deficient mice was indistinguishable from that of wild-type C57BL/6 mice.

Thus, a level of adaptive immunity developed following resolution of primary infection, and B cells appeared unnecessary for the resolution of primary or secondary infection. If *C. pneumoniae* is proven to cause cardiovascular disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal.

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Thomas Gordon Day III

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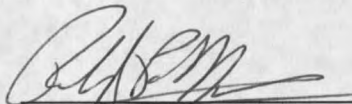
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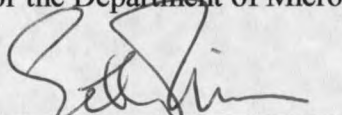
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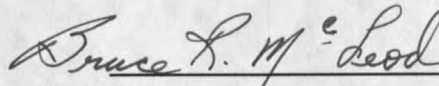
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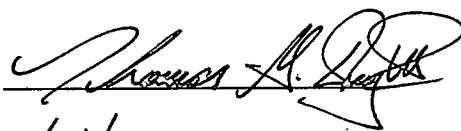

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I dedicate the completion of this thesis and this degree to the memory of my father, Dr. Thomas Gordon Day Jr. M.D., whose strength and courage serve as a constant source of inspiration and motivation. He taught me so much without ever knowing it.

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ABSTRACT

Chlamydia pneumoniae is a significant cause of respiratory infection in humans and has been associated with cardiovascular disease. Many studies have investigated the relationship between *C. pneumoniae* infection and cardiovascular disease, but few have examined host immunity to respiratory infection. Using a murine model of respiratory infection, we sought to characterize the course of primary and secondary *C. pneumoniae* infection, and to determine the role of humoral and cell mediated immune responses in adaptive immunity to infection. Intranasal inoculation with *C. pneumoniae* resulted a self-limiting respiratory infection that resolved within 21 days. Histological evaluation of lung sections revealed an interstitial pneumonia composed of polymorphonuclear neutrophils and mononuclear cells, and the association of *C. pneumoniae* inclusions with bronchial epithelial cells. Inflammation peaked at 10-14 days post infection and had completely resolved by five weeks following primary infection. The inflammatory response consisted primarily of CD4⁺ T cells and B cells when analyzed by immunohistochemistry. Anti-*C. pneumoniae* serum and bronchial antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), and were characterized by a predominance of IgG2a and IgG2b and the lack of IgA. Cytokine production by immune splenocytes was analyzed as a measure of cell mediated immunity, and was characterized by the production of IFN- γ and IL-10. Mice were susceptible to reinfection, but showed a level of protective immunity as evidenced by a shortened course of infection. Reinfection was also characterized by anti-*C. pneumoniae* IgA in bronchial washes, and a marked CD4⁺ T cell and B cell response in lung tissues. To assess the role of B cells in protective immunity to *C. pneumoniae* respiratory infection, primary and secondary infections were evaluated in B cell gene knockout mice. Infection of B cell deficient mice was indistinguishable from that of wild-type C57BL/6 mice. Thus, a level of adaptive immunity developed following resolution of primary infection, and B cells appeared unnecessary for the resolution of primary or secondary infection. If *C. pneumoniae* is proven to cause cardiovascular disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal.

INTRODUCTION

The genus *Chlamydia* consists of three species of obligate intracellular bacterial pathogens known to be infectious to humans: *C. pneumoniae*, *C. trachomatis*, and *C. psittaci*. Members of the genus *Chlamydia* are considered Gram-negative and have a unique biphasic developmental cycle that consists of two distinct forms: the elementary body (EB), the infectious, non-replicative form; and the reticulate body (RB), the non-infectious, replicative form. Following attachment to host cells EBs are internalized into a vacuole that avoids fusion with the phagolysosomal pathway of the host cell. Within hours EBs differentiate into RBs. RBs undergo binary fission, and by 48-72 hours the RBs reorganize back to EBs, which are released from the cell to begin a new infectious cycle. The manner in which the intracellular inclusion interacts with the host cell is still somewhat of a mystery, but the ability of the vacuole to avoid fusion with the lysosomal pathway may contribute to *Chlamydia* being such elusive pathogens.

C. trachomatis has been studied as a human pathogen for years. Although it is most commonly associated with sexually transmitted diseases, *C. trachomatis* is also associated with trachoma, the leading cause of preventable blindness in the world. *C. psittaci* infection is limited primarily to lower animals, and is not a major cause of human disease. *C. pneumoniae* causes a mild form of pneumonia that can be asymptomatic or present as acute respiratory disease in the form of bronchitis and/or pharyngitis. *C. pneumoniae* has also been associated with a number of other diseases including, but not

limited to, reactive arthritis and atherosclerosis. Although there is far less known about *C. pneumoniae*, it has emerged as a very relevant human pathogen.

Classification of *C. pneumoniae*

C. pneumoniae was recognized formally by Grayston and colleagues in 1989 as a distinct species of *Chlamydia* (15). The first step in differentiating *C. pneumoniae* as a new species was taken in 1986 when, in an effort to determine if *C. psittaci* was an important respiratory pathogen, a unique isolate of *Chlamydia* was isolated from the oropharynx of several university students that presented with acute respiratory infection (16). Symptoms of the students included pneumonia, bronchitis, and pharyngitis. The unique chlamydial isolate was termed TWAR, and through the use of genus and species specific monoclonal antibodies the unique antigenic identity of the TWAR isolates was demonstrated (16). *Chlamydia* specific antibodies react with *C. trachomatis*, *C. psittaci*, and TWAR, whereas TWAR specific antibodies react only with TWAR isolates (16). Previous studies had postulated a role for certain strains of *C. psittaci* in epidemics of pneumonia (16), but this was the first study in which the organism was isolated from multiple patients that presented with pneumonia or other respiratory illnesses. Due to its morphological similarity to *C. psittaci* and *C. psittaci*'s potential to cause human disease, Grayston et al hypothesized that the respiratory infections had arisen from bird to human contact, but they were unable to verify that mode of transmission. Despite the inability to establish a mode of transmission for TWAR outside of human-to-human contact, this

study laid the groundwork for a causative relationship between this unique isolate and human respiratory infection.

Inclusion morphology has been used to distinguish TWAR from *C. trachomatis* (30). The inclusion bodies formed by TWAR, when cultured in HeLa cells, are more characteristic of *C. psittaci* than *C. trachomatis*. *C. pneumoniae* inclusions are oval in shape and dense, and do not contain glycogen, which is similar to the inclusion morphology of *C. psittaci* (30). *C. trachomatis* inclusions are more vacuolar and contain glycogen (30). *C. pneumoniae* has also been differentiated from the other two species by its unique pear-shaped EB. Electron microscopy demonstrates pear-shaped EBs that are typically .38 micrometers with a large periplasmic space. *C. trachomatis* and *C. psittaci* generally exhibit round EBs with narrow periplasmic spaces (15).

C. trachomatis and *C. psittaci* share <10% DNA homology, and as such are easily differentiated by restriction endonuclease analysis and DNA-DNA hybridization (4). TWAR isolates are readily distinguishable from *C. trachomatis* and *C. psittaci* by restriction endonuclease analysis (4), and different TWAR isolates have identical restriction endonuclease banding patterns. Thus, TWAR isolates appear to be of a single strain (4). Finally, when TWAR DNA is used to probe *C. trachomatis*, *C. psittaci*, and TWAR DNA, only DNA from TWAR isolates hybridizes. These data suggest that there is a single strain of TWAR, and that it is distinct from both *C. trachomatis* and *C. psittaci*. In addition, TWAR does not contain a plasmid, a trait shared by the other two *Chlamydia* species (4). In 1988 Cox et al confirmed that TWAR strains possess between 94-100% DNA homology with each other and less than 10% homology with either *C.*

trachomatis or *C. psittaci* (various strains) (7). Collectively the molecular data suggest that TWAR is a unique species within the genus *Chlamydia*, and in 1989 *Chlamydia pneumoniae* was proposed and accepted as a new species of *Chlamydia* (15).

In addition to the lack of DNA relatedness between *C. pneumoniae* and the other chlamydial species, *C. pneumoniae* is also differentiated serologically based on the observation that TWAR specific monoclonal antibodies react with TWAR antigen, but not with *C. trachomatis* or *C. psittaci*, and vice versa (15, 16, 30). The recent sequencing of the *C. trachomatis* and *C. pneumoniae* genomes (22, 60) provides a great deal of insight into the genotypic commonalities and differences of the two human pathogens. Genomic sequences confirm the lack of DNA relatedness between the two species. Based on additional nucleic acid analysis, it has recently been proposed that the organisms presently contained in the genus *Chlamydia* be reclassified. The new classification would place *C. pneumoniae* into a new genus *Chlamydophila* (i.e. *Chlamydophila pneumoniae*)(10). Since the official reclassification has yet to occur, *Chlamydia pneumoniae* will continue to be used throughout the current thesis.

C. pneumoniae and Human Disease

First isolated as a human respiratory pathogen in 1983 (16), *C. pneumoniae* has since been associated with a number of other human diseases including reactive arthritis, atherosclerosis, erythema nodosum, Reiter syndrome, aortic aneurysm, stroke (5), malignant lymphoma (2), and cerebellar dysfunction (28). Of these associations, the connection between *C. pneumoniae* and cardiovascular disease has received the most

attention. Initial evidence linking *C. pneumoniae* and cardiovascular disease was based upon seroepidemiological studies (57). Saikku et al were the first to make the association between elevated *C. pneumoniae* antibody levels and acute myocardial infarction, and between elevated anti-*C. pneumoniae* antibody titers and the increased likelihood of cardiovascular disease (56, 57). Subsequently, numerous reports have described the presence of *C. pneumoniae* specific antibodies in patients with atherosclerosis (1, 11, 33, 42, 56, 65). However, these associations are confounded by the finding that in some studies matched controls demonstrated titers similar to those seen in patients suffering from cardiovascular disease (57). Approximately 54% of the adult population exhibits a positive antibody titer for *C. pneumoniae* serology. Since so many adults have a positive *C. pneumoniae* antibody titer, it is difficult to determine serologically if the heart disease is an unrelated event.

Microbiological and molecular techniques have been used to provide additional evidence linking *C. pneumoniae* with cardiovascular disease. *C. pneumoniae* DNA has been detected in atherosclerotic plaques by PCR (6, 18, 31, 32, 42, 64), and *C. pneumoniae* mRNA has been detected in atheromas by RT-PCR (9). However, some studies attempting to detect *C. pneumoniae* in association with atherosclerotic plaques have yielded disparate results, ranging from 1.7% to 100% of diseased tissues being culture positive or nucleic acid positive for *C. pneumoniae* (51). Specimens examined include coronary arteries, aorta, carotid arteries, iliac arteries, and smooth muscle (6, 32, 51). In most cases *C. pneumoniae* nucleic acid is not detected in explanted heart tissues without overt heart disease (49). However, in one study 9% of control tissues were

positive for *C. pneumoniae* (58). The accuracy and precision of molecular detection methods is still somewhat questionable, and as a result most studies still depend on serology as a correlative aspect of evaluation. In one such study (6) of 38 patients presenting with overt atherotic disease, 72% were positive for *C. pneumoniae* specific antibodies. This percentage is similar to that seen in population studies of older adults without heart disease, which reiterates the difficulty in interpreting seroepidemiological studies. However, despite this ambiguity 20/38 of the atherectomy specimens were positive for *C. pneumoniae* by immunocytochemical (ICC) staining and/or PCR. A large percentage of specimens (9/12), were positive by PCR and by ICC. There was no correlation between a positive serologic titer and detection of *C. pneumoniae* antigen (by ICC) or DNA (by PCR). In fact, the patient with the highest titer of *C. pneumoniae* specific IgG, was not positive for *C. pneumoniae* by either of the other methods. This study highlights some of the inconsistencies linking *C. pneumoniae* infection and cardiovascular disease.

Data from other studies (18, 33, 41) suggest that the relationship between *C. pneumoniae* infection and heart disease is arguable. In those studies the investigators were unable to establish a positive correlation between *C. pneumoniae* antibody titer and atherogenesis (18, 33, 41, 49). In addition, only 8% of coronary atherectomy specimens tested by RT-PCR were positive for *C. pneumoniae* (18), and *C. pneumoniae* DNA was not detected in any of the samples by in situ hybridization (18). In a separate study using PCR (8), *C. pneumoniae* DNA was not detected in the atherosclerotic plaques of the 29

patients tested. Collectively, these studies highlight the inconsistent nature of associating *C. pneumoniae* and coronary artery disease.

To further examine the inconsistencies associating *C. pneumoniae* with cardiovascular disease, specimens from 12 patients undergoing heart transplant were sent to different laboratories for analysis (49). Ten of the 12 patients presented overt atheromas. Specimens were sent to seven different labs where a series of tests were performed to isolate and identify *C. pneumoniae*. One patient provided a convincing argument that *C. pneumoniae* was associated with heart disease. This male patient was culture positive for *C. pneumoniae* by all three labs that performed the test, PCR positive for *C. pneumoniae* by two of three labs tested, positive by ICC in one of two labs tested, and positive by transmission electron microscopy (TEM) and in situ hybridization by 1 lab. Unfortunately, the remaining patients involved in the study demonstrated inconsistent detection of *C. pneumoniae* by different methods in different labs. In fact, none of the other labs involved in the study were able to culture *C. pneumoniae* from the specimens they studied. These inconsistencies may be attributed to the fact that experimental protocols varied between some of the labs.

Examining the nature of atherosclerosis may provide some insight into the role of *C. pneumoniae* in the development of atherosclerotic plaques. The current paradigm regarding the development of atherosclerosis is the "response to injury" hypothesis (53), in which the protective inflammatory response associated with injury to the endothelium persists, and eventually becomes a chronic inflammatory process. The initiating step in atherosclerosis is endothelial cell dysfunction, which is often due to cell injury.

Endothelial cell injury may include the infection of resident endothelial cells by *C. pneumoniae*. Infection of human aorta derived endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) with *C. pneumoniae* promotes endothelial cell activation characterized by enhanced rolling, adhesion, and transmigration of monocytes and PMNs (29). Endothelial cell activation is also evidenced by the up regulation of cell surface adhesion molecules on leukocytes associated with the activated endothelial cells. Endothelial cell injury leads to increased adherence and diapedesis of leukocytes, increased transcytosis of low-density lipoprotein (LDL), and subsequent oxidation of LDL by macrophages and smooth muscle cells. While native LDL is not considered atherogenic, oxidized LDL is highly atherogenic, and accumulation of ox-LDL, smooth muscle cells, and macrophages in the arterial intima leads to the progression of atherosclerotic plaques.

Macrophages are very important cells in the progression of atherogenesis and are present throughout plaque development in the atherosclerotic microenvironment. Studies of the interaction of *C. pneumoniae* and macrophages have provided further insight into possible mechanisms of *C. pneumoniae* induced atheroma formation. Macrophages extravasate to the intima where they phagocytose excessive lipids (LDL). Phagocytosed LDLs reside in cytoplasmic vacuoles, and due to their histological appearance, macrophages containing an abundance of LDLs are termed foam cells. Foam cells make up the initial fatty streak in atherogenesis (53). It has been shown that *C. pneumoniae* infected macrophages more readily phagocytose LDL, and take on the appearance of foam cells (19, 20). Data suggest that foam cell formation is increased an average of

30%-50% in *C. pneumoniae* infected macrophages, and that lipid accumulation occurs through dysregulation of the native LDL receptor (20). In vitro inhibition of chlamydial lipopolysaccharide with lipid X results in reduced foam cell formation, indicating that chlamydial LPS induces macrophage foam cell formation (19). LDLs activate macrophages, and mediators released by these activated cells attach additional macrophages, smooth muscle cells, and lymphocytes to the intima causing the fatty streak to progressively form a fibrous plaque (53).

Chlamydial and human heat shock protein 60s have both been shown to localize in human atheromas and up-regulate vascular cell functions including the expression of adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (24), and macrophage/monocyte production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and IL-6 (25, 26). Furthermore, *C. pneumoniae* infected monocytes and purified chlamydial HSP-60 (in the presence of monocytes) have been shown to induce oxidation of LDL (21). Thus of the cell types involved in the development of atheromatous plaques *C. pneumoniae* has been shown to infect endothelial cells, smooth muscle cells, and macrophages. While *C. pneumoniae* growth in these cell types is not as robust or as consistent as that of *C. pneumoniae* growth in epithelial cells, infection and replication do occur (13). Together those findings suggest that *C. pneumoniae* infected macrophages may participate in foam cell formation and oxidation of LDL, both of which contribute to the pathogenesis of atherosclerosis.

It has been postulated that macrophages have the ability to relocate from the respiratory tract to coronary arteries by way of arterial and venous transmission, and that *C. pneumoniae* could infect alveolar macrophages that in turn travel to coronary arteries and undergo foam cell formation (36). Subsequent oxidation of LDL by *C. pneumoniae* infected macrophages generates an atherogenic environment in which respiratory pathogen and host immune response combine to create a persistent state of inflammation. Thus dissemination of *C. pneumoniae* infected macrophages provides a link between respiratory infection and cardiovascular disease, and corroborates the role of *C. pneumoniae* infected macrophages in atherogenesis.

Thus far *C. pneumoniae* has been linked to atherosclerosis by a variety of means. In vivo, the two have been associated by serology and the detection of *C. pneumoniae* in atherosclerotic plaques by RT-PCR, in situ hybridization, and immunocytochemistry, and by culturing and propagating *C. pneumoniae* from atherosclerotic plaques. In vitro, *C. pneumoniae* has been associated with atherosclerosis by its ability to infect the cell types associated with atherogenesis, induce foam cell formation, oxidize LDL, and stimulate a local inflammatory response via chlamydial HSP60. Although there remains a large degree of uncertainty regarding the role of *C. pneumoniae* in atherogenesis, if a causal relationship is proven, a more complete understanding of the host immune response to respiratory infection will be required if vaccination is deemed a reasonable approach to controlling this infectious disease.

Animal Models of *C. pneumoniae* Cardiovascular Infection

The rabbit and mouse have been evaluated as animal models for studying the influence of *C. pneumoniae* on the development of experimental atherosclerosis. Rabbits fed a moderately enhanced cholesterol diet and challenged intranasally with *C. pneumoniae* develop intimal thickening of coronary arteries similar to that associated with atherosclerotic plaques of humans (40). Because cholesterol is a well-established risk factor in the development of atherosclerosis, it is not known whether chlamydiae home to sites of lipid accumulation and exacerbate atherosclerotic plaque formation, or if chlamydiae initiate the pathologic process. However, in a separate study *C. pneumoniae* infected rabbits on a noncholesterol diet developed atherosclerotic pathology (12). Furthermore, *C. pneumoniae* is detected in aortic sections of infected rabbits by PCR and immunocytochemistry (40). Lastly, azithromycin reduces intimal thickening in *C. pneumoniae* infected rabbits (40). That finding is particularly relevant because of the ongoing studies in humans to reduce the number of cardiac events associated with atherosclerosis through antibiotic therapy (14). Thus, studies using the rabbit model of *C. pneumoniae* infection suggest that *C. pneumoniae* may not only have a predilection for fatty streaks, but also induce atherosclerotic pathology.

Although studies from the rabbit model have provided some encouraging results concerning *C. pneumoniae* infection and atherosclerosis, the usefulness of the model for detailed microbiological, immunological and pathological studies is limited. Alternatively, mice provide an experimental system that is more amenable to

experimental manipulation. Apolipoprotein (apo)E-deficient mice develop atherosclerotic lesions spontaneously in the absence of an atherogenic diet, and thus provide a model to test the ability of *C. pneumoniae* to enhance atherosclerotic development. In apoE-deficient gene knockout mice challenged intranasally at eight weeks of age, *C. pneumoniae* DNA persists for 20 weeks in atheromatous lesions, compared to two weeks in wild type C57BL/6 mice. However, *C. pneumoniae* DNA was only detected in 35% of the apoE aorta samples by PCR. Interestingly though, atheromatous lesions from apoE-deficient mice challenged at an older age (16weeks) were consistently positive for *C. pneumoniae* DNA at eight weeks following infection, implying perhaps, that older mice develop more severe atherosclerotic plaques (37). ApoE-deficient mice challenged intranasally with *C. pneumoniae* also develop atherosclerotic lesions at an accelerated rate compared to sham inoculated apoE-deficient mice (35). Collectively, these data suggest that *C. pneumoniae* is associated with fatty streaks, and has the ability to enhance atherosclerotic pathology in a murine model.

Animal Models of *C. pneumoniae* Respiratory Infection

The majority of research involving *C. pneumoniae* to date has focused primarily on the possible role of *C. pneumoniae* in atherogenesis. For example, positive serology for *C. pneumoniae* correlates with an increased risk of heart disease; *C. pneumoniae* infects vascular endothelial cells, macrophages, and smooth muscle cells; *C. pneumoniae* antigen and/or DNA is present in atherosclerotic plaques; and *C. pneumoniae* has been isolated and propagated from atherosclerotic plaques. These observations have generated

a considerable body of information regarding the association of *C. pneumoniae* and atherosclerosis. What is less clear is how respiratory infection relates to atherosclerosis.

It is generally believed that *C. pneumoniae* attains residency in the heart following respiratory infection. In a study by Moazed et al. (36) *C. pneumoniae* infected alveolar and peritoneal macrophages were collected from mice that had been challenged intranasally or intraperitoneally, with *C. pneumoniae*, respectively. Naïve mice were then injected intraperitoneally with infected macrophages, and tissues were analyzed three days later for the presence of *C. pneumoniae*. Using this model the lung, spleen and thymus of mice inoculated with *C. pneumoniae* infected alveolar or peritoneal macrophages contain *C. pneumoniae* DNA. *C. pneumoniae* was detected in peripheral blood mononuclear cells (PBMC) by culture and by PCR indicating dissemination of *C. pneumoniae* in the peripheral circulation. In addition, viable organisms were re-isolated from macrophages of infected mice. This study demonstrates the ability of *C. pneumoniae* to remain infectious in macrophages and to disseminate systemically. The route of initial inoculation with the *C. pneumoniae* EBs appeared to have a dramatic effect on the subsequent recovery of *C. pneumoniae*. *C. pneumoniae* was found in alveolar macrophages of mice inoculated intranasally, but not intraperitoneally. Similarly, *C. pneumoniae* was recovered from peritoneal macrophages of mice inoculated intraperitoneally but not intranasally. This study begins to address the route of *C. pneumoniae* infection as it relates to atherosclerosis. Thus, examining the basic immunology surrounding the respiratory infection may prove to be critical in developing intervention strategies for preventing the subsequent pathology in the heart.

Initial studies involving *C. pneumoniae* respiratory infection established the basic parameters of the murine model (66, 67, 70). Intranasal inoculation results in a primary course of infection that is self limiting, generally resolving by three weeks (67, 70). However, *C. pneumoniae* organisms have been recovered from lung samples of some strains of mice until day 42 post challenge (70). Mice develop a *C. pneumoniae* specific humoral immune response characterized by IgM and IgG antibodies. Anti-*C. pneumoniae* IgG antibodies become apparent between days 9 and 11 post infection and the titer continues to rise until day 40 (67). IgM has been detected by some investigators until day 17 post challenge (67), but not detected by others (70). The role of *C. pneumoniae* specific antibodies in protective immunity to respiratory infection has not been clearly established, but in one report immune sera conferred a level of protection to naïve recipient mice as demonstrated by fewer recoverable infectious chlamydiae following intranasal challenge (66). Histologically, respiratory infection causes interstitial pneumonia, characterized by alveolar septae thickening and the absence of exudate in the alveolar spaces. Some studies report the presence of PMNs two days post challenge, followed by mononuclear cells during the resolution of infection (69, 70), while others detect only mononuclear cells (67). All histopathological studies however, note perivascular and peribronchial aggregation of inflammatory cells. Mice that resolve primary infection are partially resistant to reinfection as evidenced by fewer recoverable infectious chlamydiae following secondary challenge (66).

More recent studies have examined the local immune response to *C. pneumoniae* respiratory infection. The number of mononuclear cells in lungs of infected mice is

approximately three times that of uninfected mice (44). In addition, a novel cell population ($CD3^{-}$, $CD4^{+}$, $CD8^{+}$) increases by as much as 70% during infection (46).

While those data begin to examine the role of cell mediated immunity in *C. pneumoniae* infection, the role of the $CD3^{-}$, $CD4^{+}$, $CD8^{+}$ cell population remains unclear. The nature of the histological and cytokine response during secondary challenge has also been examined (44). Fewer cultivatable bacteria characterize reinfection, and there is a significant increase in the magnitude of the cellular response characterized by a moderate perivascular and peribronchial lymphocytic infiltration. T cells are the predominant lymphocyte population during the resolution of secondary infection (44), yet the importance of this increase in T cell number in imparting immunity is unclear. Consistent with the in vivo data, in vitro proliferation assays reveal a marked increase in mononuclear cell proliferation and cytokine (IFN- γ) production following rechallenge compared to primary infection (44).

$CD4^{+}$ T cells are critical for acquired immunity to *C. trachomatis* infection, whereas $CD8^{+}$ T cells play more subordinate roles (38). Cellular immunity to *C. pneumoniae* infection is less well understood. In recent studies, investigators have concluded that unlike *C. trachomatis* infection, $CD8^{+}$ T cells contribute to protective immunity in *C. pneumoniae* infection (45, 54). A variety of gene knockout mice have also been used to analyze the cellular response to *C. pneumoniae* respiratory infection. Mice deficient for the following molecules, among others, have been studied: $CD4^{-/-}$, $CD8^{-/-}$, IFN- $\gamma R^{-/-}$, perforin $^{-/-}$, TAP-1 $^{-/-}$, $\beta 2$ microglobulin(μ) $^{-/-}$, MHC-II $^{-/-}$, $CD4^{-/-}$ // $CD8^{-/-}$, and TAP-1 $^{-/-}$ // $\beta 2$ microglobulin(μ) $^{-/-}$. Mice genetically depleted for $CD8^{+}$ T cells

experience a 10-fold increase in IFU recovery compared to normal mice, 14 and 24 days following primary infection with *C. pneumoniae*, while mice depleted for CD4⁺ T cells resolve in a manner similar to normal mice (54). Mice genetically depleted for MHC-II experienced a course of infection similar to that seen in CD4^{-/-} and wild type mice, while mice genetically depleted for TAP-1/β2μ experienced an enhanced recovery of *C. pneumoniae*, corroborating the relative importance of CD4⁺ T cells and CD8⁺ T cells respectively. Early during the course of infection, CD4^{-/-}/CD8^{-/-} and severe combined immunodeficient (SCID) mice challenged intranasally develop an infection similar to that seen in normal mice, but fail to resolve infection and remain culture positive to day 60 post primary challenge. In addition, SCID mice reconstituted with CD4⁺ T cells and challenged intranasally, shed higher numbers of IFUs following primary challenge than non-reconstituted mice. Collectively, those data suggest that CD8⁺ T cells are critical in the resolution of primary infection, and that CD4⁺ T cells have a detrimental effect early in infection. However, reconstituted SCID mice were only tested at one time point (14 days) following primary challenge, and thus it is difficult to assess the overall effect on the course of infection. If CD8⁺ T cells play a role in the resolution of infection, it appears they do so without using perforin, as perforin deficient mice resolve infection without incident (54). The role of T cells is further implicated by the fact that mice deficient in IFN-γ experience a 100-fold increase in bacterial burden throughout the course of primary infection. Helper T cells are generally considered requisite for the development of an adaptive immune response, and as such these data are counterintuitive. In addition, the data from several of the experiments was collected at a single time point,

and thus the significance of those data is arguable. Others have examined the role of CD8⁺ T cells and CD4⁺ T cells in *C. pneumoniae* respiratory infection, and shown that nude mice experience a prolonged period of primary infection that does not resolve, while normal mice resolve infection within 3 weeks (45). These data once again suggest a role for T cells in the resolution of primary *C. pneumoniae* respiratory infection. In vivo depletion of CD4⁺ T cells and CD8⁺ T cells has also been used to study the contribution of these cell populations to immunity. Mice depleted of CD4⁺ T cells resolve infection normally, and show enhanced clearance of bacteria at one time point, while mice lacking CD8⁺ T cells have an increased bacterial load. Both groups of mice however, resolve primary infection in a time span similar to that seen in normal mice. The course of secondary infection is exacerbated in CD8 depleted mice (compared to wild type mice), yet once again infection resolves within 3 weeks. Past literature supports the need for MHC-II restricted T cell responses in the development of adaptive immunity. These data from the *C. pneumoniae* infection model seem to contradict that paradigm, and clearly additional work is required to clarify the existing data and to elucidate the mechanisms involved in the host immune response to *C. pneumoniae* respiratory infection.

A growing body of evidence supports the role of *C. pneumoniae* infection in the development of atherosclerosis, although a causal relationship has not been precisely defined. If *C. pneumoniae* is shown to cause coronary artery disease, then vaccination against respiratory infection is a logical goal, and an understanding of host immunity to respiratory infection will prove paramount in achieving this goal. Our current

understanding of the elements of the immune response that contribute to the resolution of *C. pneumoniae* respiratory infection is quite meager, and the relative importance of the various cell populations is arguable. The current study seeks to characterize the course of primary and secondary *C. pneumoniae* infection of C57BL/6 mice, and to determine the role of adaptive immunity in the resolution of infection. We have confirmed and expanded upon previous studies by analyzing the evolution of the humoral and cell mediated immune responses during the course of primary and secondary *C. pneumoniae* respiratory infection, and demonstrate that specific antibody is not necessary for the resolution of respiratory infection.

MATERIALS AND METHODS

Experimental Animals

Female C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, Md.), and used at 8-10 weeks of age. B cell deficient C57BL/6-Igh-6 mice (23) were purchased from Jackson Laboratories (Bar Harbor, ME.), and were bred and maintained at the Montana State University Animal Resource Center. B cell maturation is arrested at the pre-B cell stage in B cell deficient mice, and they fail to produce detectable levels of immunoglobulin. B cell deficient mice were used at 8-14 weeks of age.

Growth and Purification of *Chlamydia pneumoniae*

HL cell (Washington Research Corporation, Seattle, Wa.) monolayers were grown in Dulbecco's Modification of Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, Ut.), L-glutamine (2mM) (Gibco Life Technologies, Grand Island, NY), HEPES (1mM)(Gibco Life Technologies, Grand Island, NY), sodium pyruvate (10μM) (Gibco Life Technologies, Grand Island, NY), beta mercaptoethanol (.6μM) (Gibco Life Technologies, Grand Island, NY), and gentamycin (10μg/ml) (Gibco Life Technologies, Grand Island, NY), (DMEM-10) at 35°C, in a humidified atmosphere containing 5% CO₂. *C. pneumoniae*, strain AR-39, was obtained from ATCC and stored at -80°C until used. *C. pneumoniae* was

propagated and EBs were purified following previously described methods for *C. trachomatis* (3). Briefly, 2.5ml of *C. pneumoniae* diluted in 250mM sucrose, 10mM sodium phosphate, 5mM L-glutamic acid (SPG) was inoculated onto HL cell monolayers in 150cm² flasks at a multiplicity of infection (MOI) of 2.5. Flasks were rocked for two hours at 35°C, and then 50ml of DMEM-10 containing 0.8µg/ml cyclohexamide was added. Infected monolayers were incubated for an additional 72 hours at 35°C. Media was removed and replaced with 10ml of Hank's Balanced Salt Solution (HBSS). Monolayers were disrupted with glass beads, and briefly sonicated (2 X 20 sec., 90 watts). EBs were pelleted by centrifugation at 30,000 X g for 60 minutes at 4°C. The pellets were resuspended in SPG, and sonicated as before. Sonicates were centrifuged over 30% renografin (Squibb Diagnostics, Princeton, NJ) at 80,000 X g for 30 minutes at 4°C. The pellets were resuspended in SPG, and briefly sonicated (4 X 5 sec., 56 watts). Sonicates were placed on a discontinuous renografin (Squibb Diagnostics, Princeton, NJ) gradient (40%, 44%, 54%) and centrifuged at 72,128 X g for 60 minutes at 4°C. EBs, which band at the interface of the 44% and 54% renografin layers, were collected, washed in SPG, and pelleted at 43,667 X g for 20 minutes at 4°C. Pellets were resuspended in SPG, aliquotted and frozen at -80°C until used. EBs purified as described above are referred to as density gradient purified EBs, and were used for antigen in ELISA and for in vitro stimulation of lymphocytes. For infection of mice, *C. pneumoniae* was propagated as described above, but EB preparations were purified by pelleting EBs through 30% renografin, resuspending in SPG, and freezing at -80°C until used.

Enumeration of Infectious Chlamydiae

C. pneumoniae were enumerated as described previously (38), with the following exceptions. Three hundred microliters of diluted, purified stocks or lung homogenates were added to triplicate wells of HL cell monolayers grown in 48 well plates. Plates were centrifuged at 540 X g for 60 minutes at 35°C, and then incubated at 35°C, 5% CO₂ for 30 minutes. The inoculum was removed and replaced with DMEM-10 containing 0.8µg/ml cycloheximide. Following 72 hours of incubation at 35°C, 5% CO₂, media was aspirated and monolayers were washed once with phosphate buffered saline (PBS) (10mM phosphate, 0.13M NaCl, pH 7.4), and fixed with 100% methanol at room temperature for ten minutes. Methanol was aspirated and monolayers were washed once with PBS. *C. pneumoniae* inclusions were visualized using indirect immunofluorescent staining. Anti-chlamydial LPS monoclonal antibody (EVI-H1) (a kind gift from Dr. Harlan Caldwell, NIH) diluted in PBS containing 2% BSA was added to fixed monolayers and incubated for 45 minutes at room temperature on a rocking platform. Monolayers were washed twice with 1.0ml of PBS. Fluoresceinated goat anti-mouse IgG (Sigma, St. Louis, Mo.), diluted 1:400 in PBS containing 2% BSA was added to each monolayer, and plates were rocked at room temperature for 45 minutes. Wells were washed twice with PBS and inclusion forming units (IFUs) were visualized using an inverted fluorescent microscope. IFUs were enumerated and expressed as log₁₀ IFU.

Primary and Secondary Infectious Challenge

Initial challenge experiments were based on previous experiments by Yang et al. (70) in which Swiss Webster mice were challenged intranasally with 50 μ l of *C. pneumoniae* containing 3×10^7 IFUs. We found that intranasal inoculation of C57BL/6 with $>1 \times 10^7$ IFUs resulted in death of some mice. Therefore, an initial experiment was performed to determine a dose of *C. pneumoniae* that would result in a self-limiting respiratory infection. Mice were anesthetized with either Metofane or Isoflurane and inoculated intranasally with 40 μ l of diluted, partially purified (30%), *C. pneumoniae* EBs ($4 \times 10^7 - 1 \times 10^4$ IFUs). Mice were sacrificed seven days following infection, their lungs were removed, placed in 10ml of SPG and ground using glass homogenization tubes. Homogenates were centrifuged briefly (~1minute) at 200 X g to remove tissue debris. Supernatants were transferred to sterile tubes and stored at -80°C until IFUs were enumerated as described above. From our initial experiments, it was determined that intranasal infection with 5×10^6 IFUs, produced an infection that did not result in mortality. Thus, for primary infections, anesthetized mice were inoculated intranasally with 5×10^6 IFUs. Groups of five mice were sacrificed at various times following infection, lungs were removed, and IFUs were enumerated as described above. For secondary challenge experiments mice were allowed to resolve primary infection and then re-challenged with 5×10^6 IFUs on day 37 following primary infection. Lungs were removed at various times following secondary challenge and IFUs enumerated.

Histopathology

Lungs were removed from infected and non-infected mice, and fixed in 10% buffered formalin. Fixed tissues were forwarded to a veterinary pathologist for embedding, sectioning, staining, and histological evaluation (William Quinn, DVM, Bozeman, MT.)

In situ Detection of Chlamydial Inclusions

Formalin-fixed, paraffin embedded lung sections were deparaffinized, and stained with an anti-chlamydial 60 kilodalton heat shock protein (hsp60) monoclonal antibody (71) to visualize chlamydial inclusions as previously described (38). Briefly, paraffin embedded lung sections were deparaffinized by heating at 60°C for 40 minutes, followed by three successive xylene washes of 5 minutes each. Slides were placed in 100% ethanol for 5 minutes, and endogenous peroxidase activity was blocked by a 30 minute incubation in methanol containing 0.5% hydrogen peroxide. Slides were rehydrated by sequential 3 minute washes in 100% ethanol, 70% ethanol, 50% ethanol; then washed 3 times in PBS for 3 minutes each, and washed a final time in PBS for 5 minutes. Tissues were blocked with avidin, biotin and 5% normal mouse serum following the manufacturer's recommendations (Vector Labs, Burlingame, CA.). Following blocking, sections were rinsed with PBS, and incubated with biotinylated, anti-chlamydial hsp-60 antibody A57-B9 (71) diluted in PBS containing 5% normal mouse serum. Slides were rocked in a humidified chamber for 2 hours at room temperature, and washed with PBS for 5 minutes. Vectastain ABC complex (Vector Labs, Burlingame, CA.) was added and

tissue sections were incubated in a humidified chamber at room temperature for 30 minutes. Slides were rinsed in PBS for 2 minutes and developed with diaminobenzidine (DAB) substrate for 10 minutes. Slides were then rinsed in water for 2 minutes and counterstained with hematoxylin for 1-2 minutes. Slides were rinsed quickly with distilled water, followed by PBS for 1-2 minutes, and distilled water for 1-2 minutes. Finally, slides were de-hydrated with two successive washes of 100% ethanol, and xylene, and cover slides were added using Permount (Fisher Scientific, Pittsburgh, PA.).

Immunohistochemical Analysis of Cell Surface Phenotypes

Lungs were harvested, placed into OCT compound (Tissue-Tek ,Sakura, Torrance, CA.), and frozen in dry ice-cooled, 2-methyl butane. Thin sections (~5µm) were placed onto Superfrost slides (Fisherbrand, Pittsburgh, PA.), fixed with acetone for 5 minutes, and allowed to air dry. Tissue sections were re-hydrated in PBS for 15 minutes. Endogenous peroxidase activity was blocked by covering tissue sections with Peroxobloc (Zymed, San Francisco, CA.) for 40 seconds. Tissues were blocked with avidin, biotin and 5% normal mouse serum following the manufacturer's (Vector Labs, Burlingame, CA.) recommendations. Sections were rinsed with PBS for 2 minutes, and then incubated with primary antibody [rat anti-mouse CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-CD45R (clone RA3-6B2), anti-Ly6G clone (RB6-8C5), purchased from Pharmingen, San Diego, CA.] for 30 minutes in a humidified chamber at room temperature. Tissues were rinsed with PBS for 2 minutes, and incubated with biotin-labeled goat anti-rat Ig (Sigma, St. Louis, MO.) for 30 minutes at room temperature in a

humidified chamber. Sections were rinsed in PBS for 2 minutes, Vectastain ABC complex was added (following manufacturer's recommended procedure), and slides were incubated in a humidified chamber at room temperature for 30 minutes. Slides were developed, counterstained and de-hydrated as described above.

Analysis of Serum and Bronchiolar Alveolar Lavage (BAL) Fluid
for Anti- *C. pneumoniae* Antibody

Sera and BAL were analyzed for anti-chlamydial class and sub-class specific antibody responses by enzyme linked immunosorbent assay (ELISA) as previously described (38). Briefly, 100µl of formalin-fixed, density gradient purified *C. pneumoniae* EBs (10µg/ml) diluted in buffer (50mM Tris, 150mM NaCl, pH 7.5) was aliquotted into wells of 96 well Immulon 2, U bottom plates (Dynatech Laboratories Inc., Chantilly, VA.) and incubated overnight at 4°C. Plates were washed three times with Tris HCl (12mM, pH 7.5) containing 0.5% Tween-20 (wash buffer), and blocked with Tris (12mM, pH 7.5) containing 2% bovine serum albumin (BSA) (blocking buffer) for 2 hours at 35°C. Wells were washed once with wash buffer, and fourfold serial dilutions of sera diluted in blocking buffer were added to the wells. Plates were covered and incubated at 35°C for 90 minutes. Wells were washed twice with wash buffer and alkaline-phosphatase labeled class and sub-class specific secondary antibodies for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnologies, Birmingham, AL.) were diluted 1:250 in blocking buffer and added to the appropriate samples. Plates were covered and incubated at 35°C for 75 minutes. Wells were washed twice with wash buffer, phosphatase substrate (Sigma, St. Louis MO.) was added, and the reaction was

terminated after 30 minutes with 5N NaOH. Absorbance was read at 405nm. Purified EVI-H1 anti-chlamydial LPS was used as a positive control, and wash buffer alone was used as a negative control in all experiments. Titer was defined as the inverse of the greatest dilution that generated an optical density (OD_{405}) >0.20 . Sera from non-infected mice were consistently negative ($OD_{405} <0.20$) for anti-*C. pneumoniae* antibodies when diluted 1/16. Data were reported as mean serum titers $\log_2$ of five mice at each time point $\pm$ standard error of the mean (SEM). For some experiments, BAL was collected by passing a nylon tube attached to a 21-gauge needle into resected lungs, and washing the lungs twice with 0.5ml of SPG. The BAL washes were centrifuged at 14,926 X g for 15 minutes to remove cellular debris, and the supernatant frozen at -20°C , until analyzed by ELISA as described.

Heat Inactivation of *C. pneumoniae* EBs

Density gradient purified *C. pneumoniae* EBs (3×10^9 IFUs/ml) were thawed in a 37°C water bath and diluted to a concentration of 1×10^9 IFUs/ml in DMEM-10 containing 10mM, 3-N-morpholino-propane sulfonic acid (MOPS). Diluted EBs were heat inactivated at 56°C for 30 minutes. Heat inactivated EBs were nonviable as determined by in vitro culture.

Ultraviolet (UV) Inactivation of *C. pneumoniae* EBs

Partially purified *C. pneumoniae* EBs were thawed in a 37°C water bath and diluted to a concentration of 5×10^6 IFUs/ml in 0.5ml SPG. Diluted EBs were placed in

one well of a 24 well plate and exposed to an ultraviolet lamp in a biosafety cabinet for 30 minutes. UV inactivated EBs were nonviable as determined by in vitro culture.

Cytokine ELISA

Splenocytes (1×10^7 /ml) from normal or infected mice were mixed with or without heat inactivated EBs at a splenocyte:EB ratio of 1:4. The splenocyte/EB suspensions were incubated at 35°C for 60 minutes with constant rotation, and then plated into wells of a 24 well plate at 1×10^7 splenocytes/well. Splenocyte cultures were incubated at 35°C, 5% CO₂ for 72 hours. Supernatants were collected, centrifuged at 350 X g for 15 minutes to remove cellular debris, and frozen at -80°C until analyzed. Frozen supernatants were thawed in a 37°C water bath and analyzed for IL-4, IL-10, IL-12, and IFN- γ using Opt-EIA cytokine kits (Pharmingen, San Diego, CA.), and following the manufacturer's instructions. Briefly, Maxisorp 96 well round bottom plates (NUNC) were coated with either anti-mouse IL-4, IL-10, IL-12, or IFN- γ overnight at 4°C. Plates were washed three times (five for IFN- γ) with wash buffer (PBS with 0.05% Tween-20) and blocked with assay diluent (Pharmingen, San Diego, CA.) for one hour at room temperature. Plates were washed three times with wash buffer (five for IFN- γ), and cytokine standards or sample was added and incubated for two hours at room temperature. All plates were washed five times with wash buffer. Biotin labeled anti-mouse cytokine antibody, and avidin labeled horseradish peroxidase conjugate were added to wells and incubated for one hour at room temperature. Plates were washed seven times (10 times for IFN- γ) and developed with substrate [H₂O₂, tetra-

methylenediamine(TMB)] for 30 minutes in the dark. Reactions were terminated with 2N H_2SO_4 and read at 450nm.

Purification of T lymphocytes

Immunoaffinity chromatography columns were used to isolate normal and immune T lymphocytes from splenocyte preparations. Immune splenocytes were obtained from mice that had resolved primary *C. pneumoniae* infection and received a secondary challenge 20 days later. Ten days following secondary challenge splenocytes were harvested. T lymphocytes were purified according to the manufacturer's procedure (Cytovax Biotechnologies Inc., Edmonton, Alberta, Canada). Briefly, columns were washed with 15ml of HBSS containing 2%FBS (wash buffer), and then incubated with 1.5ml Column Reagent [polyclonal goat anti-mouse IgG (H+L)] for 1-4 hours at room temperature. Freshly isolated immune and normal splenocytes were adjusted to a concentration of 5×10^7 cells/ml in column wash buffer. Columns were washed with 20ml wash buffer, and the flow rate was adjusted to 6-8 drops per minute. Samples were added to the columns and non-adherent cells (T cells) were washed through with 10-15ml of wash buffer. T cell suspensions were centrifuged at $200 \times g$ for 10 minutes. Supernatant was discarded and pellets were resuspended in HBSS. Cells were counted and adjusted to 2×10^7 cells/ml. Viability was >92% as determined by Trypan blue. Homogeneity of the T lymphocytes was >90% as determined by immunohistochemical staining for cell surface CD3e.

Adoptive Transfer of Immune T Lymphocytes

Purified immune or non-immue T lymphocytes were adjusted to 2×10^7 cell/ml in HBSS, and 0.5ml (1×10^7 cells) was injected intravenously into naïve mice. Recipient mice were subsequently challenged intranasally with 5×10^6 IFUs of *C. pneumoniae* 24 hours following the administration of T cells, and the course of infection was followed as described previously.

RESULTS

Determination of Infectious Dose

To establish a challenge inoculum that would result in a self-limiting respiratory infection, with manifestations similar to human infection, groups of mice were challenged intranasally with doses of *C. pneumoniae* ranging from 1×10^4 to 4×10^7 IFUs. Mice were sacrificed seven days following infection, lungs were removed and homogenized, and infectious chlamydiae were enumerated (Table 1). Mice challenged with 4×10^7 IFUs experienced greater mortality (7/16), but had a bacterial burden similar to that seen in mice challenged with 1×10^7 . A challenge dose of 5×10^6 IFUs produced a non-lethal infection, and a bacterial burden that was ~10 fold less than that produced by higher challenge inoculums. Because the 5×10^6 IFU challenge inoculum did not result in death of experimental animals, and produced a respiratory infection having a bacterial burden only somewhat less than that found in animals challenged with a lethal dose, we chose the 5×10^6 inoculum to use in our studies of adaptive immunity.

Table 1. Determination of an Infectious Dose^a

Challenge Inoculum ^b	Number of Deaths/ Number Tested ^c	Mean IFU Recovery (log ₁₀)	SEM ^d (log ₁₀)
1 x 10 ⁴	0/5	2.69	0.11
1 x 10 ⁵	0/5	3.54	0.12
1 x 10 ⁶	0/5	4.45	0.24
5 x 10 ⁶	0/5	5.50	0.48
1 x 10 ⁷	1/5	5.80	0.69
4 x 10 ⁷	7/16 ^e	6.77	0.09

^a Groups of mice were challenged intranasally with various doses of *C. pneumoniae*, and IFUs were enumerated 7 days following primary infection

^b Mice were inoculated intranasally with the specified challenge inocula

^c Number of mice that died from the challenge dose prior to day 7 post infection (# deaths/ # tested)

^d Standard error of the mean of 5 mice per experimental group

^e Data are pooled from two separate experiments

Time Course of *C. pneumoniae* Primary and Secondary Respiratory Infection

Mice were challenged intranasally with 5 x 10⁶ IFUs, and on days 3, 7, 10, 14, 21, and 28 following primary challenge animals were sacrificed and infectious chlamydiae were enumerated. Mice developed a self-limiting respiratory infection that resolved within 21 days (Figure 1). Peak IFU recovery (10^{6.7}) occurred early during the course of infection, and IFUs were no longer detectable by three weeks. To determine if adaptive immunity developed following the resolution of primary infection, mice were infected, allowed to resolve primary infection, and then re-challenged with 5 x 10⁶ IFUs on day 37 post primary infection. Lungs were harvested and infectious chlamydiae were enumerated on days 3, 7, 10, 14, and 21 following secondary challenge (Figure 2). Mice were not protected from reinfection. However, compared to primary infection, the course of secondary infection was characterized by a reduced bacterial burden at all sampling points (P < 0.05 at days 7 and 10), and a more rapid resolution of infection (Figure 2).

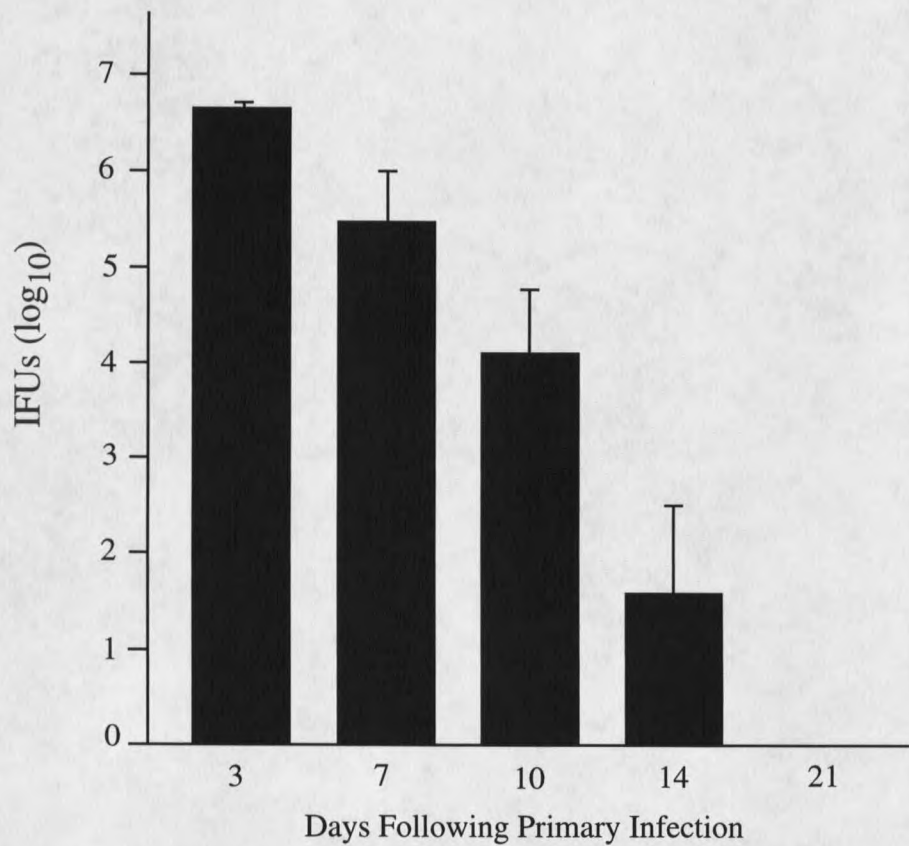


Figure 1. Course of primary *C. pneumoniae* respiratory infection. Mice were challenged intranasally with 5×10^6 IFUs, and infectious chlamydiae were enumerated on the specified days following infection. Bars represent the mean $\pm$ the standard error mean (SEM) of IFUs recovered from the lungs of 4 to 5 mice at each time point. Each animal was assayed in triplicate.

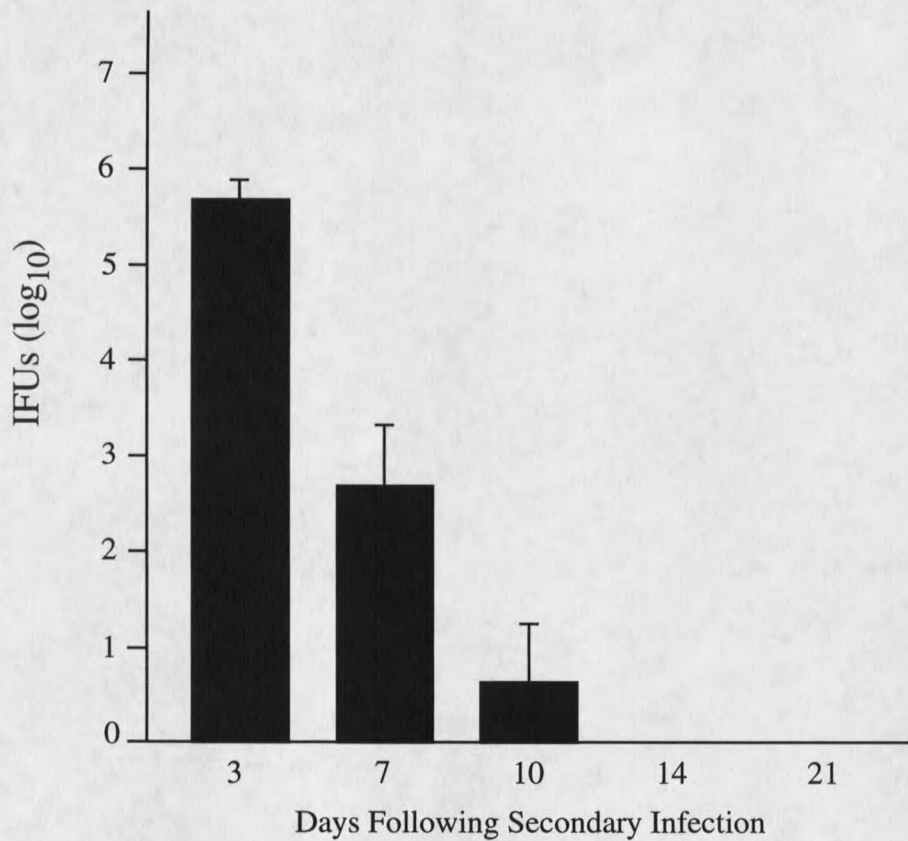


Figure 2. Course of secondary *C. pneumoniae* respiratory infection. Mice were challenged intranasally with 5×10^6 IFUs, allowed to resolve infection, and then re-challenged 37 days following primary infection. Infectious chlamydiae were enumerated on the specified days following secondary challenge. Bars represent the mean $\pm$ the SEM of IFUs recovered from the lungs of 5-10 mice at each time point. Each animal was assayed in triplicate.

Thus a significant level of adaptive immunity developed following the resolution of primary infection.

Characterization of the Local Inflammatory Response Following *C. pneumoniae* Respiratory Infection

To determine the nature of the inflammatory response, lungs of mice were removed on specified days following primary infection and processed for histological evaluation. Hematoxylin and eosin stained lung tissue sections from mice at 7 days post primary infection revealed an acute inflammatory response characteristic of interstitial pneumonia. The inflammatory response was characterized by mononuclear and polymorphonuclear (PMN) cellular infiltration in the perivascular and peribronchial regions, and by thickening of the alveolar septa (Figure 3B). The intensity of the inflammatory response peaked on days 7-10 post infection and declined thereafter. By 37 days post infection inflammation had subsided (Figure 3C), and histologically the tissue was indistinguishable from normal lung tissue (Figure 3A). To confirm that the pathological changes observed in the lungs of infected mice were a consequence of infection and not merely an inflammatory response to the challenge inoculum alone, a group of five mice was challenged intranasally with 5×10^6 UV inactivated IFUs. Seven days following challenge with UV-inactivated chlamydiae, lungs were removed and processed for histological evaluation. Hematoxylin and eosin stained lung tissue sections revealed no pathological changes (Figure 3D). UV-inactivated chlamydiae did not produce inclusions when titrated on HL cell monolayers. Lung tissues from mice infected with viable *C. pneumoniae* were also analyzed by immunoperoxidase staining

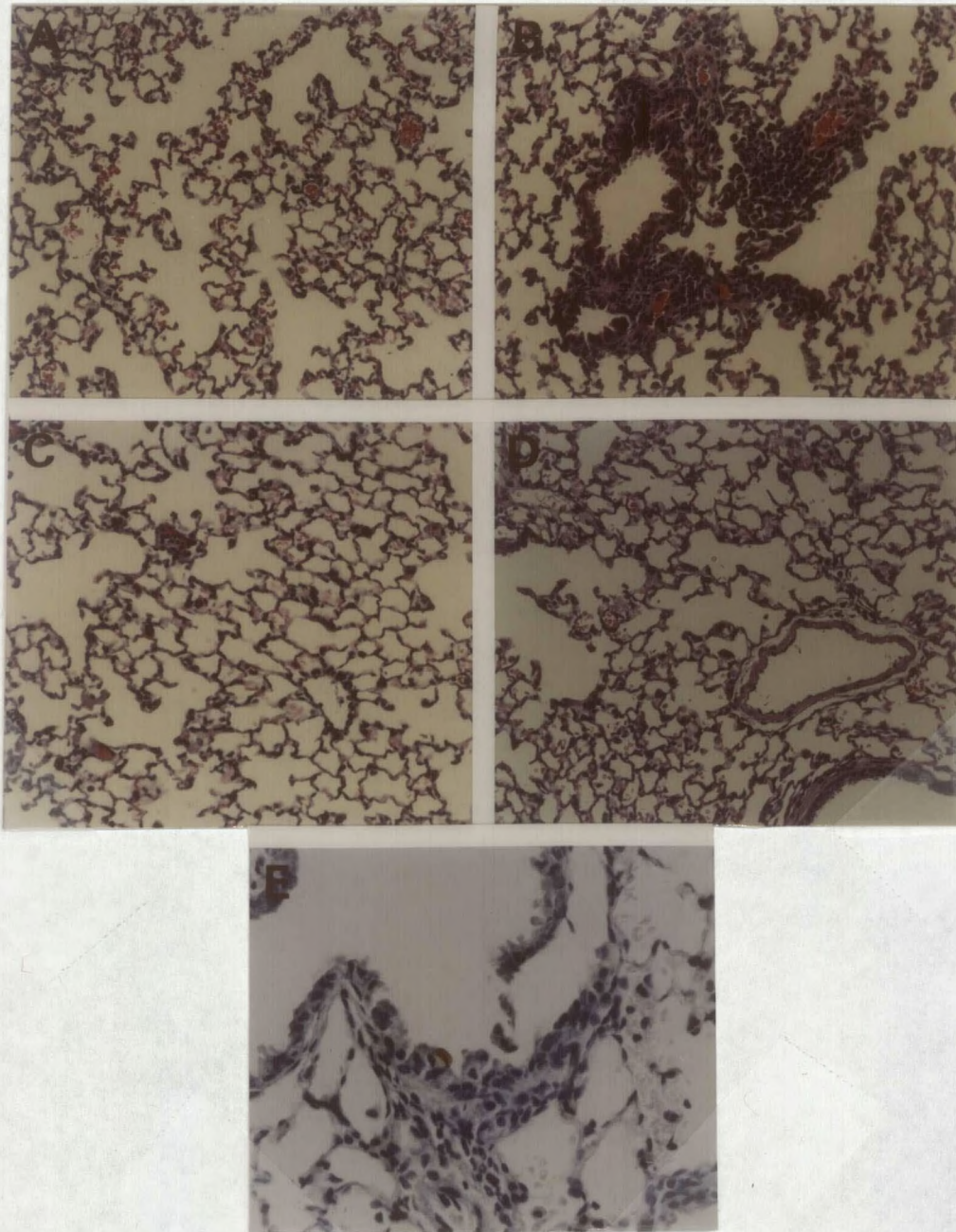


Figure 3. Histological response to *C. pneumoniae* respiratory infection. Representative hematoxylin and eosin stained lung tissues from non-infected mice (A), and from mice at 7 days post primary infection (B), 37 days post primary infection (C), and 7 days following challenge with UV-inactivated *C. pneumoniae* (D). *C. pneumoniae* inclusions were detected in lung tissue of infected mice by immunoperoxidase staining. Chlamydial inclusion in bronchial epithelial cell 7 days post infection (E). Final magnification (320X).

for the presence of *C. pneumoniae* inclusions. Inclusions were observed in association with the bronchial epithelium on days 3 (data not shown), 7 (Figure 3E), and 10 (data not shown) following primary infection. Thus, primary respiratory infection was characterized by an infection of bronchial epithelial cells with *C. pneumoniae* and a histopathological response consistent with interstitial pneumonia.

Characterization of Cell Surface Phenotypes of Lung Inflammatory Infiltrates

To further characterize the evolution of the inflammatory response following primary infection, sections of lung tissues from normal and infected mice were analyzed by immunohistochemistry for granulocytes, B cells, and T cells. Granulocytes (Figure 4A), B cells (Figure 4E), CD4⁺ T cells (Figure 4I), and CD8⁺ T cells (Figure 4M), were scattered throughout the lung tissue of non-infected mice, which is consistent with other published reports (27). Two weeks following *C. pneumoniae* infection the inflammatory response was dominated by CD4⁺ T cells (Figure 4J) and B cells that tended to aggregate around the vessels and airways (bronchi and/or bronchioles) (Figure 4F). CD4⁺ T cells were diffusely localized around vessels and airways (Figure 4J) while B cells aggregated in larger clusters (Figure 4F). The presence of PMNs was primarily diffuse with very few focal areas, and CD8⁺ T cells appeared throughout the tissue with occasional areas of perivascular and peribronchiolar aggregation (Figure 4B, N). By five weeks post primary infection the cellular infiltrate was indistinguishable from non-infected tissue (Figure 4 C, G, K, O). Control rat IgG was negative in all tissues analyzed.

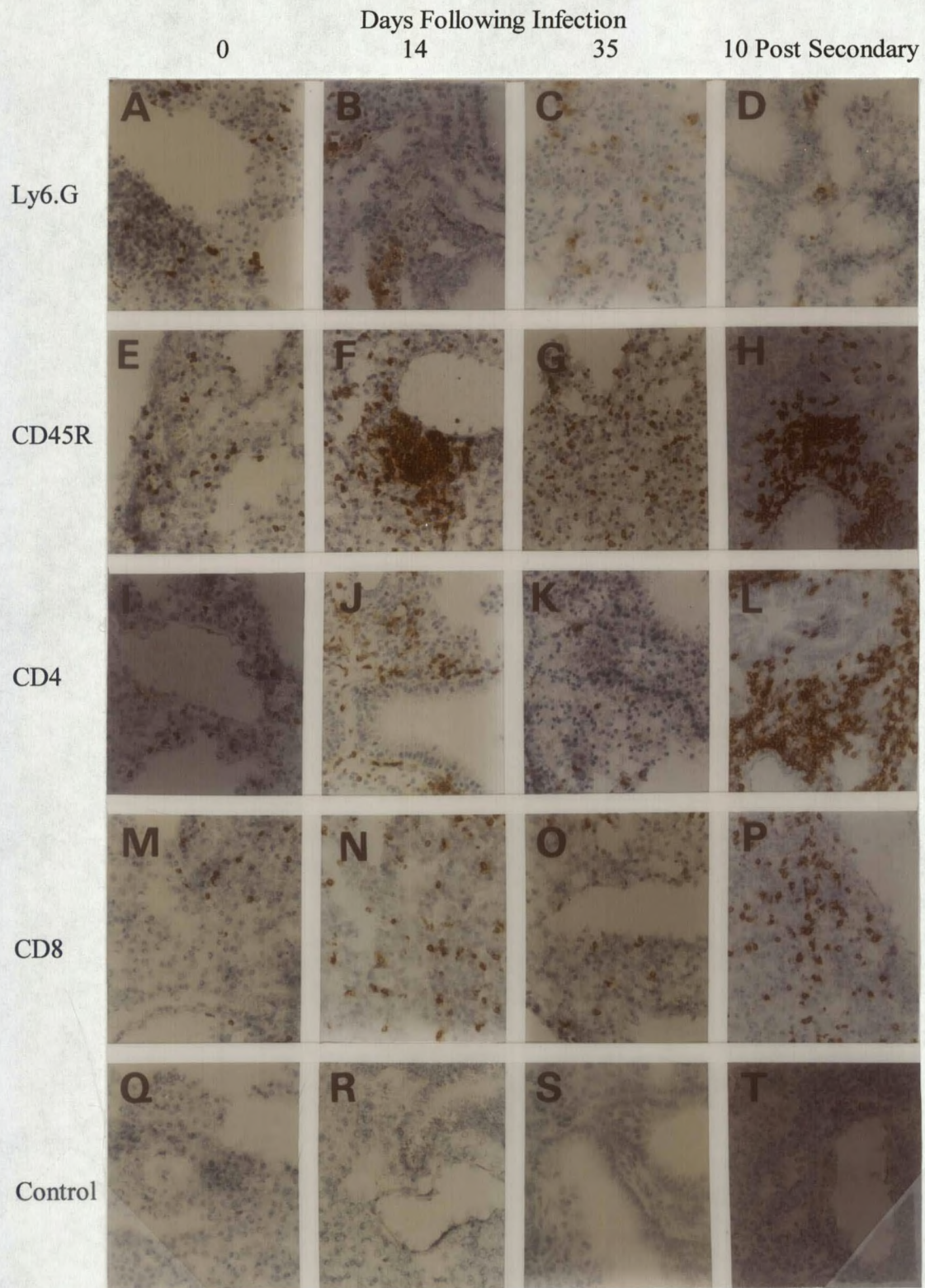


Figure 4. See figure legend next page.

Figure 4. Immunohistological detection of cell populations in lung tissue sections from non-infected and infected animals. Immunoperoxidase staining was used to characterize the local inflammatory response during the course of *C. pneumoniae* infection. Tissue sections were stained with anti-Ly6.G (PMNs) (A-D), anti-CD45R (B cells) (E-H), anti-CD4 (CD4+ T cells) (I-L), or anti-CD8 (CD8+ T cells) (M-P). Sections were also stained with either IgG2a (Q) or IgG2b (R-T) control antibody. Tissues from non-infected mice (A, E, I, M, Q), mice at 14 days following primary infection (B, F, J, N, R), mice at 35 days following primary infection (C, G, K, O, S), and mice 10 days following secondary challenge (D, H, L, P, T) were evaluated. Figures are representative of 3 mice at each time point. Final magnification (320X).

Protective immunity develops following the resolution of primary infection (Figure 2). Therefore, to determine if changes in the inflammatory response were evident at that time, we analyzed lung tissue by immunohistochemistry, from mice given a secondary infection. Ten days following secondary challenge the inflammatory response was very remarkable (Figure 4 H, L, P). CD4+ T cells were extremely abundant around the vessels and airways (Figure 4 L). B cells were more numerous than that observed following primary infection (Figure 4 H), but less so than CD4+ T cells. CD8+ T cells (Figure 4 P) and PMNs (Figure 4 D) were present, but with an intensity similar to that seen following primary infection.

Kinetics of Humoral Immune Response

Serum samples were collected from groups of mice on specified days following primary and secondary infection, and analyzed for the presence of anti-*C. pneumoniae* class and sub-class specific antibodies (Figure 5). *C. pneumoniae* specific antibodies were undetectable until two weeks following infection. As the infection resolved, IgG2a and IgG2b anti-*C. pneumoniae* antibodies predominated. Serum anti-*C. pneumoniae* IgA was not detected at any of the time points tested. The anti-*C. pneumoniae* antibody response did not change significantly following secondary challenge, and continued to be dominated by IgG2a and IgG2b (Figure 5). Animals challenged with UV-inactivated chlamydia never produced a detectable anti-*C. pneumoniae* antibody response (Data not shown). The absence of serum anti-*C. pneumoniae* IgA was somewhat surprising because in other murine models of chlamydial infection (62), and following human

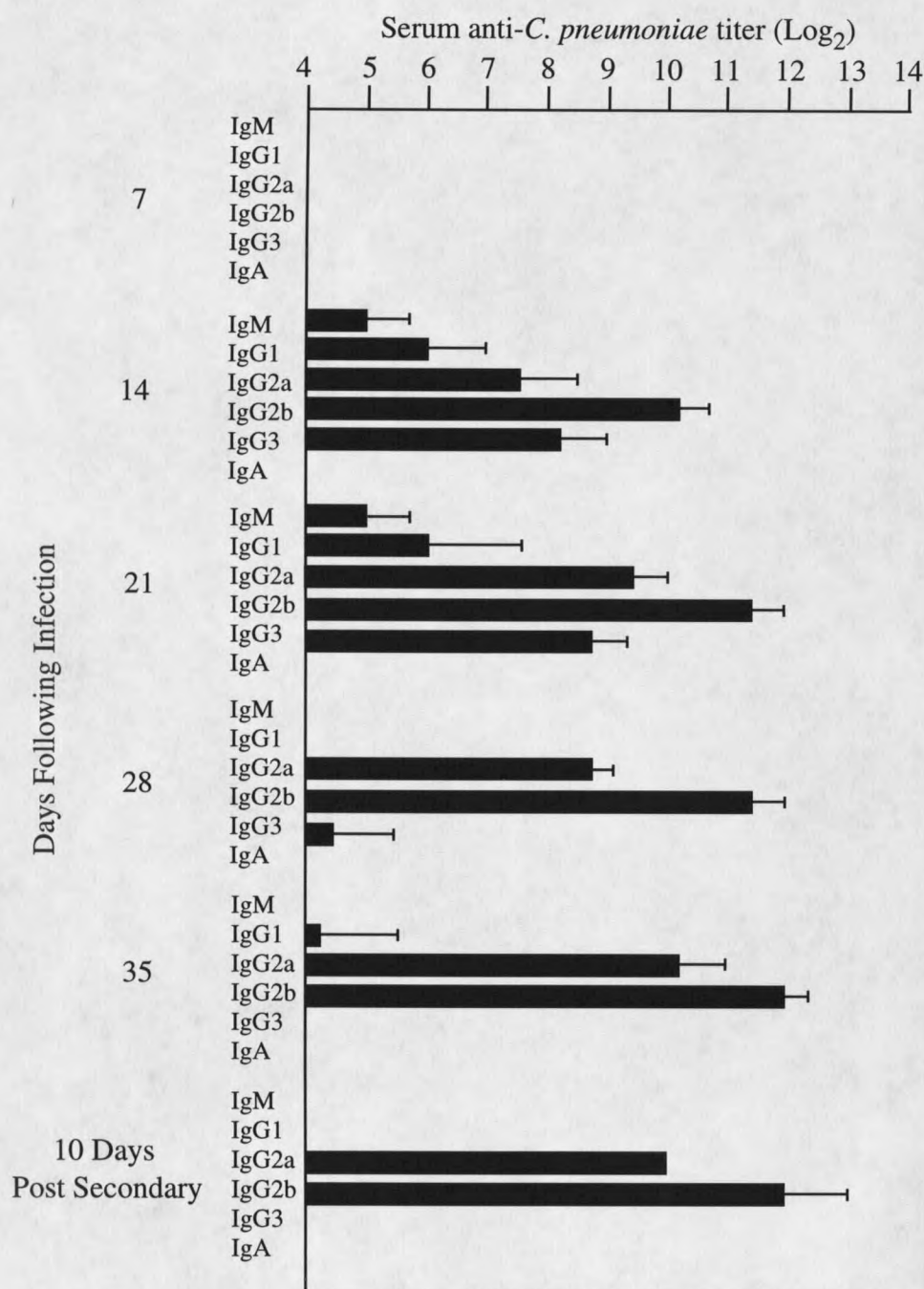


Figure 5. Time course of serum immunoglobulin class and subclass specific anti-*C. pneumoniae* antibody response following primary and secondary *C. pneumoniae* respiratory infection. ELISA was used to analyze serum samples for anti-*C. pneumoniae* specific antibodies on the specified days following primary and secondary infection. Data are presented as the mean titer (Log₂) ± SEM of 2-5 mice/group. See Material and Methods for description of a positive response. Non-infected mice were consistently negative for anti-*C. pneumoniae* antibodies.

infection (55, 57), anti-chlamydial IgA is a predominant response. Therefore, we investigated that finding further by analyzing bronchiole alveolar lavage (BAL) fluid collected from mice following primary or secondary infection (Table 2). BAL washes were negative for *C. pneumoniae* specific IgA during primary infection, but 75% of samples were positive following secondary challenge. Hence, while *C. pneumoniae* specific serum antibodies remained unaltered between primary and secondary infection, BAL fluids demonstrated a significant increase in IgA following secondary challenge.

Table 2. Anti-*C. pneumoniae* Antibody Response in Bronchiole Alveolar Lavage^a

Sample Period	Number Positive/Number Tested ^b					
	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
Primary Infection	0/6	1/6	2/6	3/6	0/6	0/6
Secondary Infection ^c	0/4	0/4	4/4	4/4	1/4	3/4
Non-infected	0/3	0/3	0/3	0/3	0/3	0/3

^a BAL fluids were collected from the lungs of mice either before infection, following primary infection (3-5 weeks), or 10 days post secondary infection

^b Data are presented as the number of positive samples per number of mice tested; positive titer was set at an OD > .20 in samples diluted 1:4

^c Data points are pooled from two different experiments

Kinetics of the Cell Mediated Immune Response

Immune and normal splenocytes were harvested from mice and incubated with or without heat inactivated *C. pneumoniae* EBs. Supernatants were harvested and analyzed for the presence of IL-4, IL-10, IL-12, and IFN- γ by ELISA. Splenocytes from *C. pneumoniae* infected mice consistently produced IL-10 and IFN- γ throughout the course of primary infection and following secondary infection while IL-4 and IL-12 were never detected (Table 3). Thus it appeared that the cells associated with the host immune

response to *C. pneumoniae* respiratory infection produced cytokines that were characteristic of both a T helper 1 response (IFN- γ), and a T helper 2 response (IL-10).

Table 3. *C. pneumoniae* Induced Cytokine Production by Splenocytes

<u>Days Following Infection</u>	<u>pg/ml Cytokine Protein^a</u>	
	<u>IL-10</u>	<u>IFN-γ</u>
0	273 ^b (32)	<10
7 ^c	1868 (478)	1634 (494)
14 ^c	2022 (339)	1066 (495)
21 ^c	1332 (239)	1166 (305)
10 Days Post Secondary ^c	1470 (202)	1100 (192)

^a Mean $\pm$ Standard error of the mean (SEM)

^b Detectable levels of IL-10 at day 0 represent nonspecific background activity

^c Values for IL-10 and IFN- γ from days 7, 14, 21 post primary infection and 10 days post secondary infection were different from non-infected mice ($P < 0.0003$)

Adoptive Transfer of Immune T lymphocytes

Because mice show a level of protective immunity to secondary infection (Figure 2), a vigorous CD4⁺ T cell inflammatory response (Figure 4), and production of T cell cytokines (Table 3), we investigated whether immune T cells could transfer immunity to naïve animals. Naïve mice were given immune T cells (see Material and Methods), and then challenged with 5×10^6 IFUs. Infectious chlamydiae were enumerated at 7 and 10 days following challenge. IFU recovery in immune T cell recipient mice was not significantly different from that of normal mice (Data not shown). Thus, we were unable to transfer immunity to naïve mice by immune T cells.

Evaluation of Immune Response in B cell Deficient Gene Knockout Mice

The enhanced resistance of mice to secondary *C. pneumoniae* infection supports the notion of adaptive protective immunity. It was somewhat surprising therefore, that the passive transfer of immune T cells to naïve mice did not confer any level of immune resistance. To further address the role of CMI and the humoral immune response in immune protection, we evaluated respiratory infection in mice that were deficient in mature B cells and antibody. Groups of B cell knockout mice were challenged intranasally with 5×10^6 IFUs and infectious chlamydiae were enumerated on specified days following primary and secondary challenge. The course of primary infection was similar to that seen in immunocompetant mice, with infection resolving within three weeks (Figure 6); however, B cell deficient mice shed significantly greater numbers of chlamydiae 13-14 days following infection ($P < 0.05$). Interestingly, B cell deficient mice developed a level of adaptive immunity following primary infection as demonstrated by a shortened course of infection upon secondary infectious challenge (Figure 7). B cell deficient mice tended to shed higher numbers of chlamydiae at 7 and 10 days following secondary infection compared to wild type mice ($P < 0.05$). The duration of secondary infection was shortened and comparable to secondary infection of immunocompetant wild type mice. The ability of B cell deficient mice to resolve infection in a manner similar to wild type mice suggested that B cells and/or antibody were not necessary for the resolution of primary infection or for the development of adaptive immunity to re-infection. However, B cell deficient mice tended to experience a greater bacterial burden

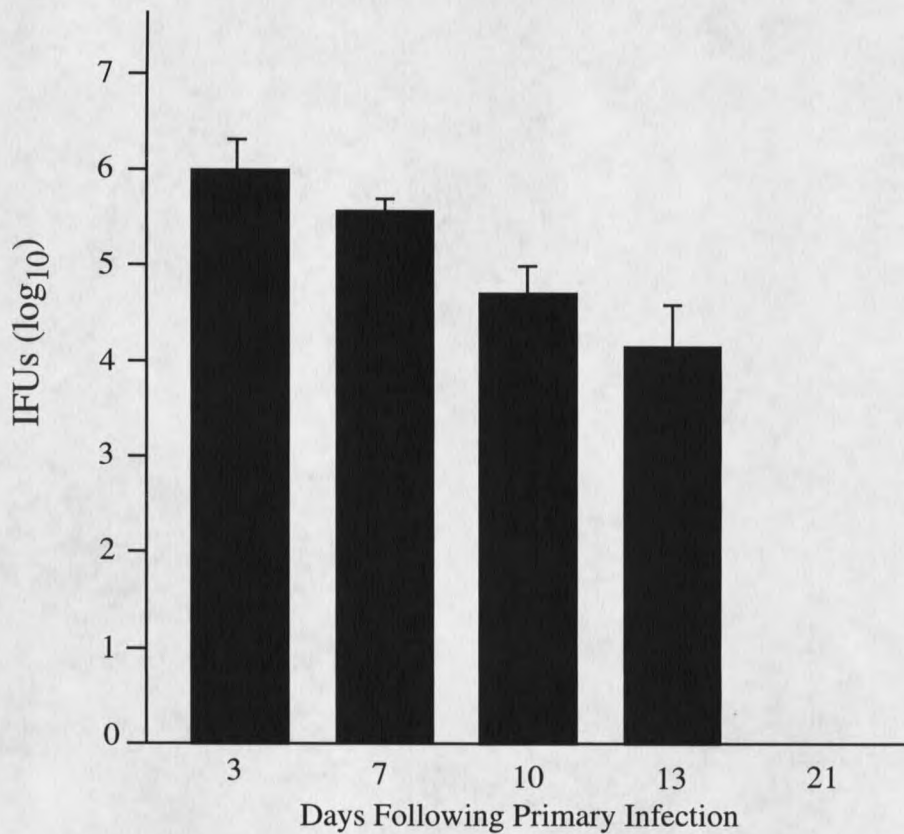


Figure 6. Time course of primary *C. pneumoniae* respiratory infection in B cell gene knockout mice. B cell deficient mice were challenged intranasally with 5×10^6 IFUs, and infectious chlamydiae were enumerated on the indicated days following infection. Data are presented as the mean IFUs $\pm$ SEM of triplicate determinations of 4-6 mice at the indicated days following primary infection.

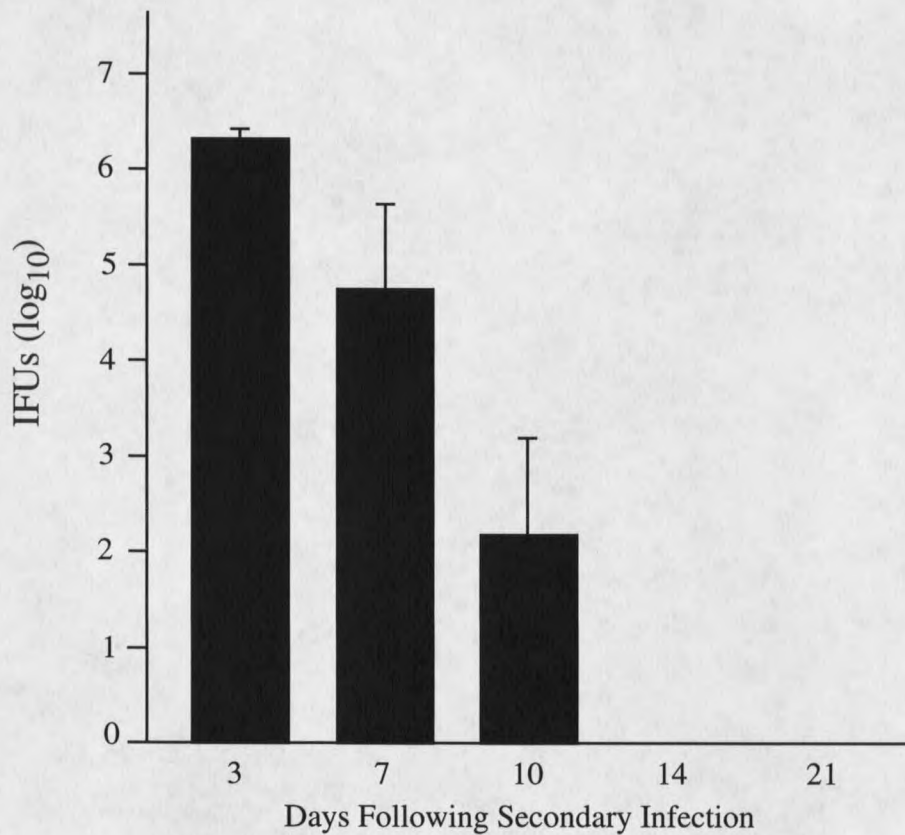


Figure 7. Time course of secondary *C. pneumoniae* respiratory infection in B cell gene knockout mice. B cell deficient mice were challenged intranasally with 5×10^6 IFUs, allowed to resolve infection, and then re-challenged with 5×10^6 IFUs 40 days following primary infection. Data are presented as the mean IFUs $\pm$ SEM of triplicate determinations of 4-5 mice at the indicated days following secondary infection.

following primary and secondary infectious challenge and thus antibody may play a subordinate role in protective immunity.

DISCUSSION

The recent association between infectious organisms and cardiovascular disease has stimulated a great deal of interest, and *Chlamydia pneumoniae* is one of the microorganisms that has been implicated as a potential causative agent in coronary heart disease (17) (31). Should there prove to be a causative relationship between *C. pneumoniae* and atherosclerosis, preventing infection through vaccination might be an effective means of preventing heart disease. Preparation of an effective vaccine will require detailed knowledge of the host's immune response to infection. In the current study we used a murine model of *C. pneumoniae* respiratory infection to characterize the local infection and inflammatory response, and to define some of the cell mediated and humoral immune responses that develop following infection.

The peak recovery of chlamydiae from infected lungs (day 3 post infection) was equivalent to the challenge inoculum, and the number of recoverable IFUs decreased throughout the course of primary infection. One could argue that the decrease in recoverable IFUs did not necessarily represent resolution of infection per se, but instead represented the elimination of the inoculum. However, several lines of evidence support an infection process. Histopathological evidence of *C. pneumoniae* inclusions within bronchial epithelial cells is indicative of an active infection process since inclusions are the product of active replication of RBs. Moreover, the replicative cycle of *C. pneumoniae* is characterized by the conversion of RBs back to EBs between 48-72 hours,

which coincides with peak IFU recovery at day 3. The relatively infrequent number of inclusions observed in any single tissue section was concerning since we recovered $10^{5.5}$ IFUs 7 days following infection. To control for this observation, mice were challenged with 16 times the standard inoculum (8×10^7 IFUs), and evaluated by immunohistology for the presence of inclusions. Despite infecting mice with a dose that results in mortality, only a slight increase in the number of inclusions was observed in lung tissues (data not shown). Furthermore, active infection is indicated because mice challenged with viable chlamydiae demonstrated lung pathology, cytokine production by immune splenocytes, and the development of specific immune responses, while mice challenged with UV-inactivated chlamydiae did not. Our data are consistent with a previous study in which chlamydial DNA is not detected in the lungs of mice within minutes of intranasal challenge with non-infectious chlamydiae, but is detected when mice are challenged with infectious chlamydiae (36).

Histopathological evaluation of lungs of infected mice revealed an inflammatory response that was characteristic of interstitial pneumonia, and remarkably similar to that seen in human interstitial pneumonia (52). Our results are consistent with those of others in which perivascular and peribronchial inflammation is observed (67, 70). Exudate was not observed in the alveolar sacs or bronchial lumens, but marked septal thickening was observed. Some investigators however, report exudate in the alveolar spaces and bronchial lumens (70), while others report no inflammation of the alveolar septa (67). The differences in histopathology observed among laboratories is concerning, but may be attributed to differences in mouse strains or in the strains of *C. pneumoniae* used.

The evolution of the inflammatory response was analyzed by identifying the cell populations present throughout the course of infection. The inflammatory infiltrate was characterized initially (day 3 post infection) by the presence of polymorphonuclear cells (PMNs). A significant decrease in the bacterial burden of infected lungs is observed within the first week of infection. Because specific immune responses develop over time, PMNs and other innate immune mechanisms may contribute to this early decrease. By day 7, lymphocytes, especially B cells, began to accumulate around vessels and airways, and granulocytes became less evident. Two weeks following infection, the aggregation of B cells, and to a lesser extent CD4⁺ T cells, around vessels and bronchi was notable. The accumulation of lymphocytes has been reported in other chlamydial models of infection (39, 43), and has been implicated as a mediator of pathogenesis rather than protection. In addition, previous work (38, 61) has demonstrated that CD4⁺ T cells are important in the resolution of infection in *C. trachomatis* mouse models, and in vitro work done with *C. pneumoniae* has shown that human mononuclear cells responding to *C. pneumoniae* are predominantly of the CD4⁺ subtype (63). CD8⁺ T cells also accumulate during the resolution of infection, but are generally less numerous than CD4⁺ T cells and B cells. Inflammation subsides, and as infection resolves only a few inflammatory cells remain. The remaining inflammatory cells may contribute to the vigorous cellular response observed during reinfection.

The importance of serology in chlamydial infections is controversial. While antisera to *C. trachomatis* and *C. pneumoniae* have been shown to neutralize infectivity in vitro (48), it is unclear whether immune sera confers the same level of protection in

vivo. We used serum antibody response as one means of characterizing the humoral immune response to *C. pneumoniae* respiratory infection. In the current study, the *C. pneumoniae* specific serum antibody response was dominated by IgG2a and IgG2b, which is similar to the response seen in other models of chlamydial infection (38). An interesting aspect of the humoral response to primary and secondary *C. pneumoniae* respiratory infection was the lack of a serum IgA response. Chlamydial infections are generally considered mucosal in nature, and IgA is an antibody that is generally associated with mucosal surfaces. As such it was surprising that serum *C. pneumoniae* specific IgA levels were undetectable. One possible explanation is that when measuring antibodies by ELISA, IgG isotypes compete with IgA for antigenic epitopes and simply do not allow IgA to bind, thus giving a false negative result. Because serum was negative for IgA at all times analyzed, we examined the possibility that IgA was present at the mucosal surface. We analyzed bronchiolar washes for the presence of *C. pneumoniae* specific immunoglobulins and IgA antibodies were not detected in BALs during primary respiratory infection.

In an effort to further characterize the role of antibodies in the host immune response to primary *C. pneumoniae* respiratory infection, we evaluated infection in mice incapable of producing mature B cells or antibodies. Resolution of primary infection in B cell deficient mice occurred within three weeks. While this course of infection was similar to that seen in immunocompetent wild type mice, B cell deficient mice shed significantly ($P < 0.05$) greater numbers of IFUs two weeks following infectious challenge. This is in accordance with other models which report that B cell deficient

mice challenged intranasally with *C. trachomatis* strain MoPn resolve primary infection in a fashion similar to normal mice (68). The ability of B cell deficient mice to resolve infection suggests that B cells are not necessary for the resolution of primary infection and implies that cell mediated immunity may be more critical.

We attempted to identify the cell populations involved in the protective immune response by adoptively transferring immune T cells to naïve mice. Following adoptive transfer and infectious challenge, mice receiving immune T cells experienced a course of infection indistinguishable from that seen in wild type mice. The absence of protection was surprising given the vigorous CD4⁺ T cell response to secondary infection, and the ability of B cell deficient mice to resolve infection. T cells comprised greater than 90% of the purified cell preparation, and greater than 90% of the purified cells were viable. Together these facts argue against a contaminated transfer preparation. Possibly the number of immune T cells that was transferred was too small to impart immunity. It has been shown that *C. pneumoniae* is found in alveolar macrophages of mice inoculated intranasally, but not intraperitoneally and vice versa (36). Perhaps there is a tissue specific response associated with intranasal infection. Immune splenic T cells transferred intravenously may not home to the site of infection quickly enough to impart a protective response. We isolated immune T cells from the spleen when it may have been more effective to isolate them from lymph nodes near the source of the infection. Also, because of the marked CD4⁺ T cell response in the lungs of mice given a secondary challenge, it is possible that several infections may be required to prime T cells so they are capable of conferring protection to naïve mice.

Cytokine analysis is another way in which investigators have studied adaptive immunity to chlamydial infections. The importance of IL-12 and IFN- γ in protective immunity to *C. trachomatis* infection is well characterized (47), but the importance of cytokines in immunity to *C. pneumoniae* infection is less clear. In the present study we measured production of IL-4 and IL-10 (characteristic of a T helper cell-type 2 response), and IL-12 and IFN- γ (characteristic of a T helper cell-type 1 response) by immune lymphocytes as another measure of cell mediated immunity. Cytokines of both the Th-1 type (IFN- γ), and Th-2 type (IL-10) were detected in supernatants from antigen pulsed splenocytes collected at various times following infection. The production of seemingly counteractive cytokines (Th-1 & Th-2) has been reported in other chlamydial infections (47). In contrast to other studies (44), we detected significant production of IL-10 and IFN- γ during primary infection. There is evidence that IFN- γ mediates the catabolism of tryptophan, an amino acid critical to chlamydial growth (34). This phenomenon is characterized by chlamydia entering an atypical developmental stage from which they emerge once the IFN- γ pressure has subsided. This paradigm may help to explain the ability of *Chlamydia* to establish persistent infections. Furthermore, IFN- $\gamma^{-/-}$ mice have been shown to be more susceptible to chlamydial infection (54). Collectively, those data suggest that IFN- γ could play a critical role in the resolution of *C. pneumoniae* infection.

In the current study reinfection of mice that had resolved primary challenge resulted in a protective immune response that was characterized by a reduced bacterial burden and an abbreviated course of infection. The number of recoverable IFUs during reinfection was reduced by as much as 3.5 logs on days 7 and 10 following secondary

challenge. Although secondary infection resolved more quickly, the protective response did not protect mice from reinfection. This finding is analogous to *C. pneumoniae* and other chlamydial infections in humans, which are characterized by recurrent infection (50).

Of interest in our model was the lack of mucosal anti-chlamydial IgA following primary infection. Interestingly though, mucosal IgA was present in BALs following secondary respiratory challenge (Table 2). The genital tract model of *C. trachomatis* infection suggests that dimeric secretory IgA may prevent infection by neutralizing EBs in the lumen (59). In addition, it has been shown that mice genetically depleted for CD4 demonstrate a delayed production of mucosal IgA, the subsequent appearance of which coincides with resolution of infection (38). Such evidence might help explain the protective response observed in our study following secondary respiratory infection. For example, because local (lung) anti-chlamydial IgA antibodies were not found following primary infection, animals were not protected from reinfection. However, secondary infection recalls and enhances anti-chlamydial responses, which lead to a more rapid resolution of infection. This notion was corroborated by the fact that B cell deficient mice experienced a greater bacterial recovery following secondary challenge than did wild type mice ($P < 0.05$), but were protected as evidenced by a shortened course of infection. Those data demonstrate that B cells were not required for resolution of primary or secondary infection, and suggest indirectly that cell mediated immunity was critical to protective immunity. The remarkable appearance of both secretory IgA and CD4⁺ T cells following secondary infection suggests that multiple infections may be

necessary to generate a sufficient protective immune response. Perhaps if mice were allowed to resolve secondary infection and challenged a third time, a more complete, if not sterile, immunity would develop. It is also possible however, that the marked immune response that develops locally following secondary infectious challenge contributes to an enhanced pathology that would only be exacerbated upon reinfection. It has been reported that upon chlamydial reinfection mice experience a reduced bacterial burden, but an enhanced inflammatory response follows (66). The relative importance of the immune mediators that we examined during secondary infection is unclear at this point, but studies attempting to prime specific mediators of the protective response through multiple infections, may prove useful.

The data presented in the current study established an animal model of *Chlamydia pneumoniae* respiratory infection in which the basic immune parameters of infection were studied. We demonstrated that mice develop a protective immune response following primary intranasal challenge with *C. pneumoniae*, and that B cells are not required for the resolution of infection. In addition, mice generated a cell mediated immune response characterized by the production of T cell cytokines, and a humoral immune response characterized by serum anti-chlamydial IgG2a and IgG2b. Remarkably, anti-chlamydial IgA was not detected in serum or BAL following primary infection. Reinfection was characterized by a strong T cell response around airways and vessels, and the production of local (BAL), but not serum, anti-chlamydial IgA antibodies. This study provides a foundation on which more elaborate immunological studies can begin to target the aspects of protective immunity that might be conducive to vaccine design.

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