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Depletion of CD8⁺ T Cells Exacerbates CD4⁺ Th Cell-Associated Inflammatory Lesions During Murine *Mycoplasma* Respiratory Disease¹

Harlan P. Jones, Leslie Tabor, Xiangle Sun, Matthew D. Woolard, and Jerry W. Simecka²

Mycoplasma infection is a leading cause of pneumonia worldwide and can lead to other respiratory complications. A component of mycoplasma respiratory diseases is immunopathologic, suggesting that lymphocyte activation is a key event in the progression of these chronic inflammatory diseases. The present study delineates the changes in T cell populations and their activation after mycoplasma infection and determines their association with the pathogenesis of murine *Mycoplasma* respiratory disease, due to *Mycoplasma pulmonis* infection. Increases in T cell population numbers in lungs and lower respiratory lymph nodes were associated with the development of mycoplasma respiratory disease. Although both pulmonary Th and CD8⁺ T cells increased after mycoplasma infection, there was a preferential expansion of Th cells. Mycoplasma-specific Th2 responses were dominant in lower respiratory lymph nodes, while Th1 responses predominated in spleen. However, both mycoplasma-specific Th1 and Th2 cytokine (IL-4 and IFN- γ) responses were present in the lungs, with Th1 cell activation as a major component of the pulmonary Th cell response. Although a smaller component of the T cell response, mycoplasma-specific CD8⁺ T cells were also a significant component of pulmonary lymphoid responses. In vivo depletion of CD8⁺ T cells resulted in dramatically more severe pulmonary disease, while depletion of CD4⁺ T cells reduced its severity, but there was no change in mycoplasma numbers in lungs after cell depletion. Thus, mycoplasma-specific Th1 and CD8⁺ T cell activation in the lung plays a critical regulatory role in development of immunopathologic reactions in *Mycoplasma* respiratory disease. *The Journal of Immunology*, 2002, 168: 3493–3501.

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States alone, *Mycoplasma pneumoniae* accounts for up to 30% of all cases of pneumonia and is the second leading cause of tracheobronchitis in children (1–3). Mycoplasma infection is also associated with exacerbation of other respiratory diseases, such as asthma (4, 5). *Mycoplasma pulmonis* respiratory infection in mice is a naturally occurring disease with high morbidity and low mortality and is an excellent paradigm of human mycoplasma disease. Both *M. pneumoniae* and *M. pulmonis* respiratory infections cause rhinitis, otitis media, laryngotracheitis, and bronchopneumonia. Histologically, one of the most consistent characteristics of chronic *M. pulmonis* and *M. pneumoniae* pulmonary disease is the accumulation of large numbers of lymphoid cells into the respiratory tract (3, 6–9). Lymphoid cell infiltration suggests that lymphocyte activation and recruitment are key events that influence progression of chronic inflammatory disease due to mycoplasma infection. In support, several studies demonstrate that a component of mycoplasma respiratory diseases is immunopathologic (10–14). These and other studies (15, 16) demonstrate that recruitment of lymphoid cells and their activation are important in the pathogenesis of mycoplasma respiratory disease.

The mechanisms that determine whether generated immune responses are protective or immunopathologic are unknown for any mycoplasma disease. T cell activation is most likely pivotal in determining the impact of these responses on mycoplasma infection and disease. A role for T cells in immunopathologic responses is supported by studies demonstrating T cell-deficient mice or hamsters have less severe mycoplasma respiratory disease than their immunocompetent counterparts (11, 13). We similarly found that SCID mice, lacking in functional B or T cells, develop significantly less severe *M. pulmonis* lung disease than immunocompetent mice (14). Interestingly, the numbers of mycoplasma in the lungs of SCID mice and immunocompetent mice did not differ, suggesting that lymphoid responses, not an uncontrolled infection, promoted inflammatory lesions due to mycoplasma infection. In fact, reconstitution of SCID mice with splenic lymphocytes resulted in disease as severe as in normal mice (14), and preliminary studies (unpublished data) suggest that components of these lesions were due to T cell activity alone. Although these studies demonstrated that lung disease due to mycoplasma infection was immunopathologic, adaptive immune responses were also shown to be important in preventing extrapulmonary dissemination of infection, leading to arthritis and the eventual death of SCID or nude mice (11, 14). Furthermore, adaptive immunity, also dependent on functional T cells, can promote resistance and recovery from *M. pneumoniae* and *M. pulmonis* disease (8, 17, 18). Thus, adaptive immune responses, mediated by T cell activity, are critical in promoting protective immunity and immunopathologic reactions associated with mycoplasma respiratory disease. However, little is known about these mechanisms, and more work is needed to understand the apparent conflicting roles of immune responses in mycoplasma disease, facilitating the eventual development of vaccines and therapies for this important group of respiratory diseases.

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The role of T cell subsets is unknown for any mycoplasma disease. However, *M. pulmonis* infection in mice provides an opportunity to determine the function of these T cell subpopulations in mycoplasma disease. Based on our previous studies of *M. pulmonis* disease in two strains of rats with differing disease susceptibilities (16, 19–25), we proposed that regulatory T cells play a major role in determining the outcome of mycoplasma infection, although this hypothesis has yet to be directly addressed. The purpose of the present study was to delineate the changes in T cell populations and their activation after mycoplasma infection and determine their impact on the pathogenesis of *M. pulmonis* respiratory disease. We focused on Th cells, as they are known to play a critical role in protection and pathogenesis of many infectious diseases through their ability to mediate humoral and cell-mediated responses. Importantly, Th cells are roughly divided into two major functional populations. Th1 cells promote cell-mediated and inflammatory responses through their production of cytokines, such as IFN- γ (26). In contrast, Th2 cells control Ab responses through their secretion of characteristic cytokines (e.g., IL-4). Studies have shown that Th cell subset activation is important in the control of other infectious diseases (27–35), but their impact on mycoplasma respiratory disease is unknown. In addition, CD8⁺ T cells are also known to impact on the progression of inflammatory diseases, as they can regulate these responses (36, 37) or cause lung injury through CD8⁺ T cell-mediated apoptosis (38–40). Thus, we also examined whether changes in CD8⁺ T cell responses occurred and their contribution to mycoplasma disease.

Materials and Methods

Mice

Female C3H/HeN viral- and mycoplasma free mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed in sterile microisolator cages and supplied with sterile bedding, and food and water were given ad libitum. Mice used in studies were between 8 and 12 wk of age. Before experimental manipulation, mice were anesthetized with an i.m. injection of ketamine/xylazine.

Mycoplasma

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (41), in mycoplasma broth and frozen in 1-ml aliquots at -80°C . For inoculation, thawed aliquots containing 2×10^7 CFU of *M. pulmonis* per milliliter were diluted to 10^5 CFU/20 μl . Inoculations of 20 μl diluted mycoplasma were given nasally.

Cell isolation

Mononuclear cells were isolated from lungs, as previously described (23, 42, 43). Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from blood. The lungs were separated into individual lobes and finely minced. The tissues were suspended in RPMI 1640 (HyClone Laboratories, Logan, UT) medium containing 300 U/ml *Clostridium histolyticum* type I collagenase (Worthington Biochemical, Freehold, NJ), 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO), 10% FBS (HyClone Laboratories), HEPES, and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37°C while mixing on a Nutator (Fisher Scientific, Pittsburgh, PA) for 90–120 min. During the incubation period, the tissue was vigorously pipetted every 20 min. After incubation, the digestion mixture was passed through a 250- μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspensions by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Spleen cells and lower respiratory (mediastinal and hilar) lymph nodes (LRN)³ were isolated after centrifugation of cell suspensions, followed by red cell removal using ACK (ammonium chloride potassium) lysis buffer, as previously described (44).

Immunofluorescent characterization of lymphocyte populations

Two-color and three-color immunofluorescent staining was performed to identify both B and T cell populations using FITC-labeled anti-murine B220 mAb (RA-6B2; Beckman Coulter, Miami, FL), PE-labeled anti-murine CD3 mAb (KT3; Beckman Coulter), PerCP-labeled anti-CD4 mAb (L3T4, RM4-5; BD Pharmingen, San Diego, CA), and FITC-labeled anti-CD8 mAb (Lyt-2, 53-6.7; BD Pharmingen). Briefly, 2×10^6 cells per tube were incubated with purified 2.4G2 mAb (BD Pharmingen) for 5 min at 4°C to reduce nonspecific binding of FcII/III receptors before fluorescent Ab staining. The cells were incubated for 30 min at 4°C with 250 μl fluorescent Ab (2 $\mu\text{g}/\text{ml}$). The cells were washed in staining buffer (Mg^{2+} -free, Ca^{2+} -free PBS with 0.05% sodium azide, 1% FBS (HyClone Laboratories)) and fixed with 4% paraformaldehyde solution for 30 min, and the cells were then resuspended in staining buffer until analysis.

The cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter), with further analysis done using Expo 2 analysis software (Beckman Coulter). Lymphocyte gates and detector voltages were set using unstained (control) lung and splenic cells. The proportion of each cell population was expressed as the percentage of the number of stained cells. To determine the total number of a specific lymphocyte population, their percentage was multiplied by the total number of lymphocytes isolated from a tissue.

RNA isolation from lungs

Total RNA was isolated from the entire lungs of mice using the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX), which is based on a previously described method (45). Briefly, the lungs were homogenized in the Ultraspec-II RNA reagent using a PRO 200 homogenizer (PRO Scientific, Monroe, CT). Chloroform was added to the homogenate and centrifuged at $12,000 \times g$ (4°C) for 30 min. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging the samples at $12,000 \times g$ (4°C) for 10 min. The RNA pellet for each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at $7,500 \times g$ and then resuspended in diethylpyrocarbonate-treated water. The concentration and quality of RNA in each of the samples were determined spectrophotometrically (GeneQuant II; Amersham Pharmacia Biotech, Piscataway, NJ) and by gel electrophoresis. The RNA samples were stored at -80°C until used.

Cytokine mRNA detection by RT-PCR

RT-PCR was performed using 100 ng RNA for each sample, as previously described (46). The sequences of the primers and the size of the resulting PCR fragments (in parentheses) for IL-2, IL-4, IFN- γ , and the housekeeping gene, β_2 -microglobulin ($\beta_2\text{m}$) are given as follows (47): IL-4 (216 bp), 5'-TCGGCATTTTGAACGAGTGC and 5'-GAAAAGCCCCGAAAGAGTCTC; IFN- γ (227 bp), 5'-GCTCGAGACAATGAACGCT and 5'-AAA GAGATAATCTGGTCTGTC; and $\beta_2\text{m}$ (222 bp), 5'-TGACCGGCTTG TATGCTATC and 5'-CAGTGTGAGCCAGGATATAG.

The increase in expression of cytokine mRNA after immunization was determined by the number of cycles of amplification that resulted in little or no PCR product for each cytokine in total lung RNA from sham-inoculated, control mice, as previously described (46, 48). For IFN- γ , IL-5, and $\beta_2\text{m}$, the samples were amplified for 30 cycles, and for IL-2 and IL-4, the samples were run for 35 cycles. The PCR products were separated on 1.8% agarose gels and stained with ethidium bromide. Gels were visualized using Alpha Image 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA). The intensity of each band was determined using densitometry, and the relative cytokine mRNA reactions were compared after normalization to the housekeeping gene, $\beta_2\text{m}$.

Preparation of *M. pulmonis* Ag

A crude preparation of *M. pulmonis* membranes was used for in vitro stimulation and prepared as previously described (22). Briefly, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium and harvested at pH 7. Cells were then centrifuged at 10,000 rpm for 20 min, and pellets were resuspended in 5 ml sterile 0.25 M NaCl. Following a second centrifugation at 9000 rpm for 20 min, pellets were resuspended in a total of 4 ml 2 M glycerol at 37°C . Cells were then sonicated at the highest setting for 15 s using Vibra cell sonicator (Sonics & Materials/Vibrio Cell, Newtown, CT), followed by incubation at 37°C for 10 min. For cell lysis, 0.5 ml of the cell preps was then forced through a 27-gauge needle into 25-ml aliquots of distilled water. To remove unlysed organisms, cells were centrifuged at 10,000 rpm for 20 min. Supernatants were again centrifuged at 20,000 rpm

³ Abbreviations used in this paper: LRN, lower respiratory lymph node; $\beta_2\text{m}$, β_2 -microglobulin.

for 1 h. Membranes were resuspended in 5 ml sterile PBS (HyClone Laboratories) and stored at -80°C . All centrifugations were done at 4°C . Protein concentration was determined by standardization using Bradford protein assay (Bio-Rad, Hercules, CA). A final concentration of $5\text{ }\mu\text{g/ml}$ *M. pulmonis* membrane Ag was used for in vitro studies, as indicated in Results.

In vitro depletion and isolation of T lymphocyte populations

A $100\text{-}\mu\text{l}$ aliquot of mouse anti-CD4 and/or anti-CD8 mAb-labeled magnetic beads (Dynabeads mouse CD4 (L3T4), mouse CD8 (Lyt-2); Dynal Biotech, Lake Success, NY) was added directly to lung and splenic cell suspensions at a final concentration of 1×10^7 cells/ 4×10^7 beads. Lymphoid cells were incubated at 4°C for 20 min on a rotator. After incubation, tubes containing lymphocytes were placed on a magnet (MPC magnet; Dynal Biotech) for 2 min, and both positive and negative fractions were collected for further studies. Confirmation of cell purity was determined using flow cytometry. Cell fractions with a depletion of $>95\%$ were used for subsequent experimentation.

Ag-specific in vitro stimulation of mononuclear cells

Lymphoid cells were cultured in 96-well round-bottom microtiter plates in RPMI 1640 (HyClone Laboratories) supplemented with 5% FBS (HyClone Laboratories), HEPES, 10 U/ml rIL-2 (BD Pharmingen; shown in during earlier studies (49) to amplify, but not shift Th cell responses), antibiotic/antimycotic solution (Life Technologies), and $50\text{ }\mu\text{M}$ 2-ME (Life Technologies). Lymphoid cells were stimulated at 37°C and 5% CO_2 . Cells were stimulated with or without $5\text{ }\mu\text{g/ml}$ prepared *Mycoplasma* Ag in a final volume of $200\text{ }\mu\text{l}$ /well culture media at a cell concentration of 2×10^6 cells/ml. Supernatants were collected 4 days later and stored at -80°C until assayed for cytokine levels.

Cytokine assays

The levels of cytokine were measured by capture ELISA. Murine IL-4 levels were measured using OptEIA IL-4 ELISA set (BD Pharmingen), while murine IFN- γ levels were determined using mouse IFN- γ MiniKit (Endogen, Woburn, MA). Easy-wash 96-well flat-bottom microtiter plates (BD Biosciences, Bedford, MA) were coated overnight at 4°C with $100\text{ }\mu\text{l}$ mAb specific for either murine IL-4 or IFN- γ diluted in 0.1 M Na_2HPO_4 , pH 9. Plates were washed and blocked with $200\text{ }\mu\text{l}$ PBS/Tween 20 supplemented with 10% FBS solution for 30 min. Following PBS/Tween 20 wash, $100\text{ }\mu\text{l}$ sample supernatants were placed into the appropriate wells and incubated overnight at 4°C . Plates were washed three times with PBS/0.05% Tween, and $100\text{ }\mu\text{l}$ biotinylated rat anti-mouse cytokine (IL-4 or IFN- γ) mAb was added to each well and incubated overnight at 4°C . To reveal the reaction, avidin-HRP and 3,3',5,5'-tetramethylbenzidine substrate (Moss, Pasadena, MD) were used. Plates were read using MX80 plate reader (Dynatech, Chantilly, VA) at an absorbance of 630 nm. A total of $100\text{ }\mu\text{l}$ 0.25 M HCl was also added to reaction as needed to increase sensitivity of reaction and read at an absorbance of 450 nm. Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines (IL-4 and IFN- γ ; BD Pharmingen) after log/log quadratic linear regression analysis using Revelation 2.0 software (Dynatech).

In vivo depletion of CD4 and CD8 cells using mAb treatment

Hybridoma cell lines, GK1.5 (L3T4) and 53-672 (LyT3) (American Type Culture Collection (ATCC), Manassas, VA), were grown in MiniPerm Bioreactors (Sartorius, Edgewood, NY) in IMDM culture medium (HyClone Laboratories) containing 15% FBS at 37°C , 10% CO_2 . Ab concentrations in culture supernatants were determined using a rat IgG-specific ELISA. The ability to deplete T cell populations in vivo ($\geq 98\%$ depletion) was confirmed for each batch of Ab by immunofluorescent staining of spleen cells from treated mice.

Three days before infection, mice were given a 1 ml (1 mg) i.p. injection of anti-CD4 (ATCC) and/or anti-CD8 mAb. Ab injections were repeated every 5 days (50, 51). In vivo depletion of lymphocyte populations was confirmed as $\geq 98\%$ depletion by examining splenic cell populations using immunofluorescent staining and flow cytometry.

Mycoplasma numbers

The numbers of CFU in lungs and nasal passages were determined as previously described (52). Briefly, lungs were minced and placed in mycoplasma broth medium. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell) for 2 min at 50 amplitudes without pulsing. After sonication, 1/10 serial dilutions were prepared, and $20\text{ }\mu\text{l}$ of each dilution was plated onto *Mycoplasma* plate media. After 7 days of incu-

bation at 37°C , colonies were counted, and the number of CFU recovered from each tissue was calculated.

Assessment of gross lesions

Lungs were removed, and each lobe was examined by two observers for the presence of gross lesions. The percentage of each lobe with gross lesions was recorded. The gross lesion scores were weighted by the percentage that each lobe contributes to the total lung weight in arriving at the gross lesion index for lungs (53).

Statistical analysis

Data were evaluated by ANOVA, followed by Fisher protected least square differences multigroup comparison. These analyses were performed using the StatView (SAS Institute, Cary, NC) computer programs. A p value ≤ 0.05 was considered statistically significant.

Results

CD4⁺ T cells are the major lymphoid population in lungs at 14 days after M. pulmonis infection

To determine the changes in lymphocyte populations in mice infected with *M. pulmonis*, mononuclear cells were isolated from the lungs, LRN, and spleens at various time intervals (days 0, 7, and 14) after infection. The numbers of B and T cells, including $\text{CD3}^+\text{CD4}^+$ Th cells and $\text{CD3}^+\text{CD8}^+$ T cells, were determined.

T cells were the major lymphocyte population that changed after mycoplasma infection. By 7 days after infection, there was a 3- to 4-fold increase in T cell numbers within the LRN, but no changes were seen in lungs or spleens at this time (Fig. 1). The numbers of B cells, however, did not significantly change in any tissue at this time. Increases in pulmonary T cell numbers were apparent by 14 days after infection. At this time point, there was about a 10-fold increase in T cells in the lungs of infected mice. There was also a 3-fold increase in B cells from lungs of infected mice. In contrast, there was no significant increase in the number of B or T cells isolated from spleens throughout the course of infection. Thus, it is likely that T cells are a major population of lymphocytes contributing to the inflammatory infiltrate.

$\text{CD3}^+\text{CD4}^+$ Th cells were the major T cell population in lungs and LRN of infected mice. In LRN, there was about a 5-fold increase in $\text{CD3}^+\text{CD4}^+$ T cells 7 days after infection (Fig. 1). At this time, there was a small, but not statistically significant, increase in $\text{CD3}^+\text{CD8}^+$ T cells. At 14 days after infection, there was a significant increase in both T cell populations in the lungs of mice infected. There was, however, no significant increase in $\text{CD3}^+\text{CD4}^+$ or $\text{CD3}^+\text{CD8}^+$ T cells in the LRN and spleen between 7 and 14 days after infection. Importantly, there was a higher percentage of $\text{CD3}^+\text{CD4}^+$ Th cells than $\text{CD3}^+\text{CD8}^+$ T cells in all tissues at every time interval after mycoplasma infection (Table I). Furthermore, the percentage of $\text{CD3}^+\text{CD4}^+$ Th cells significantly increased at 7 days after infection in the LRN and at 14 days in the lung. Thus, Th cells were the major T cell population present in lungs and LRN throughout the course of disease, and there was a preferential increase in Th cells in both tissues at selected stages of disease. However, the number of CD8^+ T cells concomitantly increased in the lungs. These changes in T cell populations coincided with the massive cellular infiltrate within the lungs after *M. pulmonis* infection.

IL-4 and IFN- γ cytokine mRNA expression in the lungs of mice infected with M. pulmonis

IL-4 is the characteristic cytokine produced by Th2 cells, while Th1 and CD8^+ T cells produce IFN- γ (26). We examined IL-4 and IFN- γ expression within LRN and lungs during different stages of disease. LRN and lungs from uninfected and *M. pulmonis*-infected mice were collected at different time points (days 0, 7, and 14) after infection. Total RNA was isolated from these tissues, and the

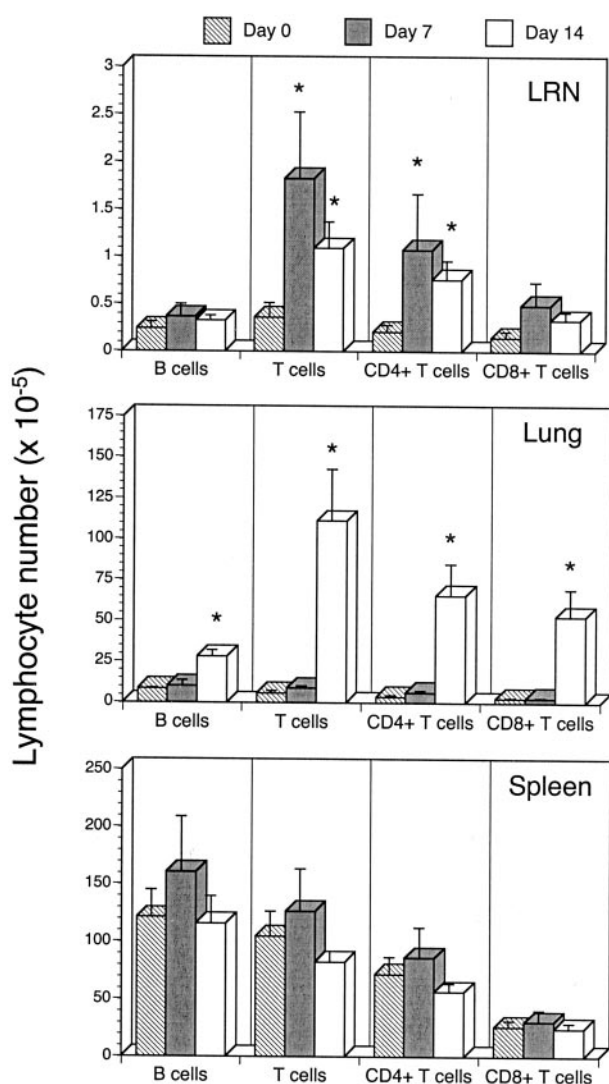


FIGURE 1. CD4⁺ T cells predominate in the lungs 14 days after *M. pulmonis* infection. Mononuclear cells were isolated from the LRN, lung, and spleens at various time intervals (days 0, 7, and 14) after infection. The numbers of B and T cells, including CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells, were determined using flow cytometry. Vertical bars and error bars represent mean \pm SE from two experiments ($n = 6$ per time point). *, A significant ($p \leq 0.05$) difference in number of cells between time points.

expression of IFN- γ and IL-4 mRNA was determined using RT-PCR. There was a significant increase in IL-4 mRNA levels in LRN, but not in lungs, at 7 days after infection (Fig. 2a). In contrast, there was no significant increase in IFN- γ mRNA levels within LRN or lungs at that time point. At 14 days after infection, IL-4 and IFN- γ mRNA levels were significantly higher within LRN and lungs.

As IFN- γ can be produced by CD4⁺ and CD8⁺ T cells and NK cells, CD4⁺ and CD8⁺ T lymphocytes were isolated from uninfected and mice infected with mycoplasma 14 days earlier, and IFN- γ cytokine mRNA expression was determined by RT-PCR. There was an increase in IFN- γ mRNA levels in both CD4⁺ and CD8⁺ T cell populations (Fig. 2b). In addition, there was an increase in IL-4 mRNA expression in CD4⁺ T cells, but not in CD8⁺ T cells (data not shown). Therefore, both CD4⁺ Th and CD8⁺ T cells are sources of IFN- γ within the lung at 14 days after infection, whereas CD4⁺ Th cells also expressed IL-4.

Mycoplasma-specific IL-4 and IFN- γ cytokine production by pulmonary lymphocytes

To monitor mycoplasma-specific T cell responses, IFN- γ and IL-4 production by lymphocytes was measured after stimulation with mycoplasma Ag. Lung, LRN, and spleen cells were isolated from mice on 0, 7, and 14 days after infection and cultured in the presence or absence of mycoplasma Ag in vitro. Four days later, supernatants were collected, and the levels of IFN- γ and IL-4 cytokine were determined.

In response to stimulation with mycoplasma Ag, little, if any, IL-4 or IFN- γ was detected within supernatants from cultures of LRN, lung, and splenic cell collected from mice at day 7 after infection (Fig. 3). However, mycoplasma-specific cytokine responses were readily detected in cells obtained from mice infected for 14 days. IL-4, but not IFN- γ , responses were present in LRN. In contrast, only IFN- γ responses were found in Ag-stimulated spleen cell cultures. Interestingly, lung cells produced both IL-4 and IFN- γ in response to mycoplasma Ag. At this time point (14 days), the levels of IFN- γ cytokine production were significantly higher than IL-4 levels within all lymphocyte cultures. Stimulation of lymphocytes from naive (0 day) mice with mycoplasma Ag did not result in IFN- γ or IL-4 production. Also, there was little or no cytokine production in cell cultures not stimulated with mycoplasma Ag.

To determine the contribution of CD4⁺ and/or CD8⁺ cells to T cell cytokine responses, CD4⁺ and/or CD8⁺ cells were depleted from lung and splenic lymphocytes before in vitro stimulation with mycoplasma Ag. Depletion of CD4⁺ T cells from lung lymphocytes collected from *M. pulmonis*-infected mice significantly reduced the IL-4 response to mycoplasma Ag to $\sim 10\%$ of that produced by whole (undepleted) pulmonary lymphocytes (Table II). Depletion of either lung CD4⁺ Th cells or CD8⁺ T cells significantly reduced IFN- γ levels to $\sim 50\%$ of the levels produced by mycoplasma Ag-stimulated whole lymphocytes. IL-4 and IFN- γ production was diminished to low levels within mycoplasma Ag-stimulated cultures consisting of cells depleted of both CD4⁺ and CD8⁺ T cells from infected mice. In spleen, CD8⁺ T cells were the major source of IFN- γ , although CD4⁺ T cells contributed a smaller, but significant portion of the IFN- γ response.

Impact of T cell populations on disease

To determine the impact of T cell populations, mice were treated with anti-CD4 or anti-CD8 Abs. One group of mice was treated with a mixture of both Abs. Three days later, mice were infected with mycoplasma. Ab treatments were continued throughout the course of disease. At 14 days after infection, the severity of mycoplasma lung disease was determined. In addition, in vivo Ab depletion was confirmed by examining spleen cells from Ab-treated mice for CD8⁺ and CD4⁺ T cells using immunofluorescent staining and flow cytometry. All mice were shown to have $\geq 98\%$ depletion of the appropriate cell population(s). For example, in mice treated with anti-CD4 Ab, $\leq 2\%$ of the CD3⁺ cells were CD4 positive and $\geq 98\%$ were CD8 positive.

No clinical signs of disease were observed between 0 and 10 days after infection in all groups as compared with sham-infected mice. On day 11, mice treated with anti-CD8 Ab showed signs of lethargy and ruffled fur. At this time point, clinical signs were absent in mice treated with anti-CD4 Ab alone or mice treated with a mixture of anti-CD4 and anti-CD8 Abs. By day 14, anti-CD8 Ab-treated mice were very ill. At this time, control-infected mice were showing signs of illness, but these signs were not seen in mice treated with anti-CD4 Ab or the mixture of anti-CD4 and anti-CD8 Abs. In addition, mice treated with anti-CD8 Ab had

Table I. Immunofluorescent characterization of T lymphocyte populations isolated from mice infected with *M. pulmonis*^a

Tissue	Percentage of CD3 ⁺ T Lymphocytes					
	Day 0		Day 7		Day 14	
	CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺	CD8 ⁺
LRN	59.5 (4.3) ^{3b}	40.4 (1.6) ³	69.2 (2.6) ^{5c}	30.6 (6.2) ⁵	60.2 (6.0) ⁴	27.6 (2.3) ⁴
Lung	58.2 (3.6) ³	44.6 (5.8) ³	59.1 (2.2) ⁶	46.1 (2.8) ⁶	68.7 (2.2) ^{5c}	32.5 (3.4) ⁵
Spleen	68.5 (1.7) ⁶	25.1 (1.3) ⁶	66.2 (2.0) ⁶	23.2 (1.0) ⁶	68.3 (1.5) ⁶	28.5 (2.3) ⁶

^a Mice were infected with *M. pulmonis*, and lymphocytes were isolated 0, 7, and 14 days later from the respective tissues.^b Mean \pm SE of the percentage of CD3⁺ cells from respective tissues. The superscripted number denotes the number of experiments that the value was based upon.^c Significant ($p \leq 0.05$) difference increase in CD4⁺ T cell expression between time points.

significantly greater ($p \leq 0.05$) weight loss than any other group of mice (Fig. 4a). There was no significant difference in weight loss between the other groups of mice. Thus, depletion of CD8⁺ T cells resulted in increased disease severity, as indicated by clinical signs.

The severity of pulmonary disease at 14 days after mycoplasma infection was consistent with clinical signs seen in the mice. There was a significant decrease in lesion severity in CD4⁺ T cell-depleted mice as compared with control mice after mycoplasma infection (Fig. 4b). In contrast, mycoplasma-infected mice developed dramatically more severe pulmonary lesions after CD8⁺ T cells were depleted. However, few lesions were found in infected

mice depleted of not only CD8⁺ T cells, but also CD4⁺ T cells. Importantly, Ab treatment had no effect on mice that were not infected with mycoplasma (data not shown). Thus, depletion of CD8⁺ T cells resulted in more severe mycoplasma pulmonary disease, while simultaneous depletion of CD4⁺ T cells reversed this effect.

To determine whether depletion of either population of T lymphocytes had an effect on disease resistance, the above experiments were repeated 14 days after infection, the lungs were removed, and mycoplasma CFU were determined in the lung. There were no significant differences between any of the treatment groups in the numbers of mycoplasma CFU in the lungs (Fig. 4c). Thus, depletion of T cell populations did not affect the level of pulmonary infection by mycoplasma.

Discussion

It is clear that the recruitment and activation of lymphoid cells are important in the pathogenesis of mycoplasma respiratory diseases in humans and animals (10–16), yet the immune mechanisms involved are not fully understood. Most studies have focused on the development of Ab responses after mycoplasma infection (for example, Refs. 20 and 23). Despite past studies demonstrating that T cells are pivotal in the progression of mycoplasma respiratory disease (11), little is known about the T cell responses and their association with pathogenesis of any mycoplasma disease. In particular, the activation of Th1 and Th2 cell subsets has not been examined in any mycoplasma disease, despite their impact in the progression and pathology of other diseases (27–35). Because of the critical role that T cell responses have in modulating immunity, it is important to ascertain which T cell populations are responding to the mycoplasma infection and their contribution to disease pathogenesis. Thus, the purpose of the present study was to delineate the changes in T cell populations and their activation after mycoplasma infection and determine their impact on the pathogenesis of murine mycoplasma respiratory disease.

Increases in T cell population numbers were indeed associated with the development of mycoplasma respiratory disease. Initial changes in lymphocyte numbers were found in LRN. There was a 5-fold increase in Th cells, whereas the numbers of B and CD8⁺ T cell populations did not vary significantly. This indicates that Th responses are the initial lymphocyte population responding after infection, and that in the lower respiratory tract, these responses first appear in the draining lymph nodes. However, by day 14, changes in lymphocyte numbers are readily apparent in the lungs, corresponding with the presence of peribronchial and perivascular mononuclear cell infiltrates. At this time point (data not shown), T cells were the major lymphocyte population recovered from the lungs of infected mice, and the number of T cells was 10 times greater than those recovered from naive mice. Although B cell

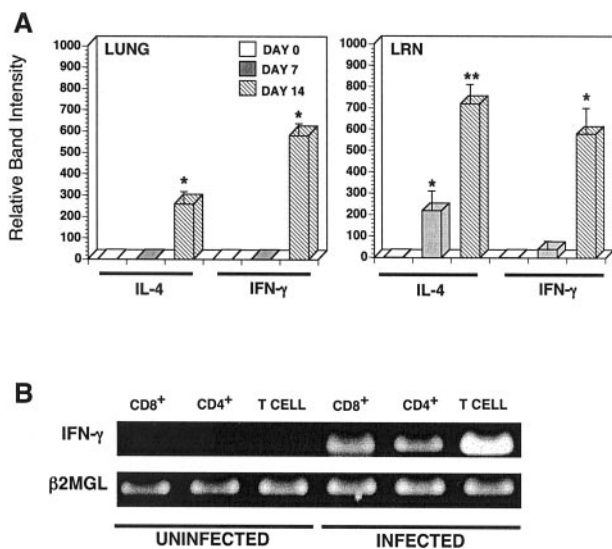


FIGURE 2. Cytokine mRNA expression in pulmonary lymphoid tissues and lung T cells of mice infected with *M. pulmonis*. **A**, IL-4 and IFN- γ mRNA expression in the lungs and LRN of mice infected with *M. pulmonis*. LRN and lungs from uninfected and infected mice were collected at different time intervals (days 0, 7, and 14). Total RNA was isolated from these tissues, and the expression of IL-4 and IFN- γ mRNA was determined using RT-PCR. Differences in the level of mRNA expression were determined by relative increase in band intensity based upon the ratio of cytokine to the housekeeping gene β_2m . *, A significant ($p \leq 0.05$) difference between time points. **, A significant difference in IL-4 mRNA expression between day 0 and day 7 in LRN. Vertical bars and error bars represent mean \pm SE from two experiments ($n = 6$ for each time point). **B**, IFN- γ mRNA expression by purified CD4⁺ and CD8⁺ T cells. Lung cells were isolated from uninfected and *M. pulmonis*-infected mice 14 days after infection. Total RNA was isolated from purified pulmonary lymphocyte populations, and IFN- γ and β_2m mRNA expression was determined by RT-PCR. This experiment was done twice ($n = 6$ for each experimental group), and a representative gel from one of the experiments is shown.

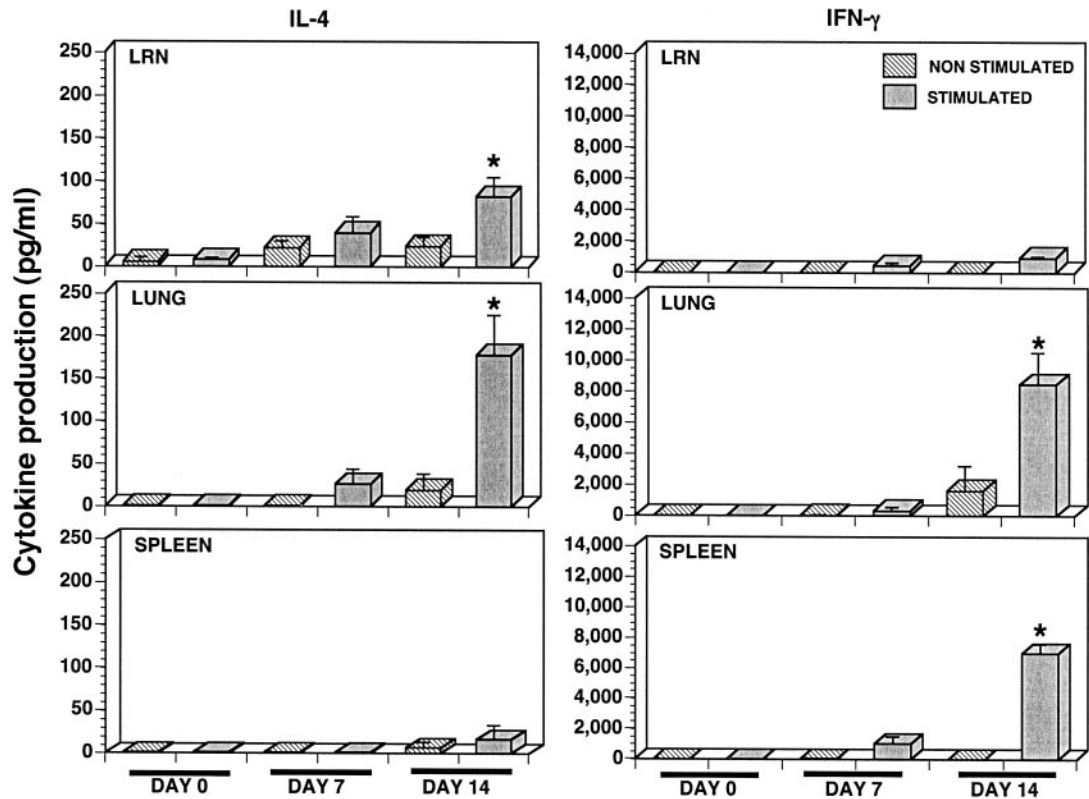


FIGURE 3. Mycoplasma-specific IL-4 and IFN- γ cytokine production by LRN, lung, and splenic lymphocytes. IFN- γ and IL-4 production by lymphocytes was measured after stimulation of lymphocytes with mycoplasma Ag. Lung, LRN, and spleen cells were isolated on 0, 7, and 14 days after infection, and cultured in the presence or absence of mycoplasma Ag. Four days later, supernatants were collected, and the levels of IFN- γ and IL-4 cytokine were determined by ELISA. *, A significant ($p \leq 0.05$) difference from day 0. Vertical bars and error bars represent mean \pm SE from two experiments ($n = 6$ mice for each time point).

numbers also increased in lungs, their increases were significantly smaller. Furthermore, Th cells were the major T cell population in lung, and although both pulmonary Th and CD8⁺ T cells increased after mycoplasma infection, there was a preferential expansion of Th cells. Importantly, the large increases in Th cell numbers are consistent with Th cells being the dominant lymphocyte population in the inflammatory infiltrate associated with mycoplasma respiratory disease, suggesting a major role for Th cell activity in

disease pathogenesis. In support, we demonstrated that in vivo depletion of CD4⁺ T cells results in less severe mycoplasma respiratory disease in both control and most dramatically CD8 cell-depleted mice, without having an effect on mycoplasma numbers in lungs. Thus, Th cells are a major component in the lymphoid responses generated in the lower respiratory tract after mycoplasma infection and are likely to contribute to immune-mediated inflammatory responses in mycoplasma respiratory disease.

Table II. *M. pulmonis*-specific IL-4 and IFN- γ cytokine production by lung and spleen cell cultures depleted of either CD4⁺ and/or CD8⁺ T cells^a

Tissue/Cell Population	Cytokine Production (pg/ml)			
	IL-4		IFN- γ	
	0 μ g/ml	5 μ g/ml	0 μ g/ml	5 μ g/ml
Lung				
Unfractionated	17 (17)	230.9 (40.9)	0 (0)	15,142 (1127)
CD4 negative	0 (0)	9.8 (4.1) ^b	0 (0)	7,438 (3329) ^b
CD8 negative	14.5 (14.5)	312 (63.1)	0 (0)	7,514 (2311) ^b
CD4 and CD8 negative	0 (0)	17.3 (17.3) ^b	0 (0)	3,200 (456) ^b
Spleen				
None	0.5 (0.5)	61 (15.5)	0 (0)	18,520 (78)
CD4 negative	0.5 (0.5)	0.5 (0.5) ^b	0 (0)	11,716 (34) ^b
CD8 negative	3 (2.0)	26.3 (14.3)	0 (0)	5,086 (167) ^b
CD4 and CD8 negative	0.5 (0.2)	6.5 (5.5) ^b	0 (0)	3,108 (233) ^b

^a Lymphocytes isolated from tissues were depleted of either CD4⁺ and/or CD8⁺ T cells using anti-CD4 (CD4-negative) and/or anti-CD8 (CD8-negative) Ab-coated magnetic beads. Lymphocytes were placed in culture and stimulated with (5 μ g/ml)/without (0 μ g/ml) mycoplasma Ag. Four days later, supernatants were collected and IL-4 and IFN- γ levels were determined. Data are expressed as the mean \pm SE of the cytokine levels in culture supernatants for two experiments ($n = 6$ for each experimental group).

^b Significant ($p \leq 0.05$) difference in cytokine levels as compared to unfractionated cells stimulated with mycoplasma Ag.

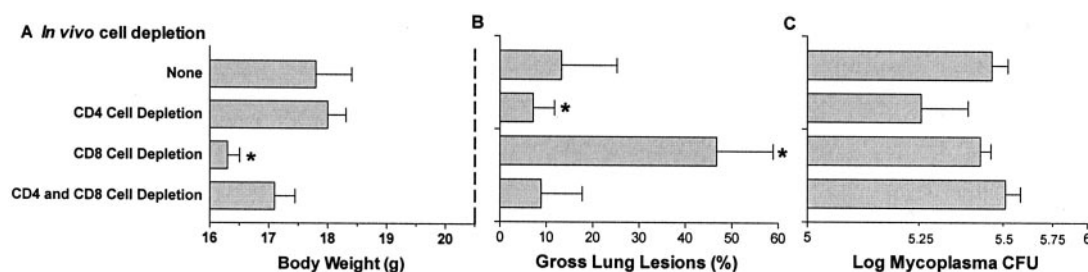


FIGURE 4. Effects of in vivo T cell depletion on mycoplasma lung disease and infection. Mice were treated with anti-CD4 Ab (CD4 Cell Depletion), anti-CD8 Ab (CD8 Cell Depletion), or a mixture of both Abs (CD4 and CD8 Cell Depletion). At 14 days after mycoplasma infection, we measured body weights (in A, the dashed line indicates the mean body weight of uninfected mice). Horizontal bars and error bars represent mean SE from five experiments ($n = 15$ per experimental group). The mice within an experiment had similar body weights, and the variation was due to disease, not variability between starting weights of experimental groups. B, Evaluated gross lung lesions, horizontal bars, and error bars represent mean SE from five experiments ($n = 15$ per experimental group). C, The number of mycoplasma CFU in lungs. Horizontal bars and error bars represent mean \pm SE from two experiments ($n = 6$ per experimental group). *, A significant ($p \leq 0.05$) difference from the untreated mice.

Mycoplasma-specific Th1 and Th2 responses were generated in the lower respiratory tract after infection with *M. pulmonis*, but there were distinct differences in the Th cell responses in each of the tissues examined with the appearance of Th1 responses associated with inflammatory lesions in the lung. At 14 days after infection, mycoplasma-specific IL-4 production by LRN lymphocytes was readily found, while IFN- γ production was low. In contrast, splenic lymphocytes produced high levels of IFN- γ , but not IL-4, after stimulation with mycoplasma Ag. Lung lymphocytes produced both IFN- γ and IL-4 in response to mycoplasma Ag. CD4⁺ Th cells were the major source of IL-4, as their depletion eliminated IL-4 production. We similarly showed that Th cells in spleens and lungs were a source of IFN- γ , although CD8⁺ T cells also contributed to IFN- γ production, particularly in spleens. These results demonstrate that mycoplasma-specific Th2 responses were dominant in LRN, while Th1 responses predominated in spleen. However, both Th1 and Th2 cytokine responses were present in the lungs, but IFN- γ levels were greater, suggesting that Th1 cell activation was a major component of the Th cell response in lungs. The association of IL-4 responses with respiratory tissues, e.g., lungs and LRN, is consistent with our studies demonstrating that resident Th cells in the lung are Th2, which influences immune responses to intranasally given Ag (49). The Th2 dominance of pulmonary immune responses is also consistent with the work of others (54). Thus, the appearance of Th1, as well as Th2, responses in the lung coincides with the development of CD4⁺ cell-mediated chronic inflammatory lesions in mycoplasma respiratory disease.

Mycoplasma-specific Th1 responses in lungs are likely to contribute to the immune-mediated inflammatory reactions in the lungs of mycoplasma-infected mice. Th1 cells promote delayed-type hypersensitivity responses, which are characterized by mononuclear cell infiltration (26), similar to that found in mycoplasma disease (6–8, 24, 25). Although Th2 cell activation can also lead to inflammatory responses, eosinophils are a characteristic cell type within these reactions, but eosinophils are not a major component of mycoplasma respiratory disease. While Th1 cells may be critical in the pathogenesis mycoplasma respiratory disease, Th2 cell activation may also be important. Although Th2 cell responses alone may not be responsible for inflammatory lesions in mycoplasma, interactions between Th1 and Th2 activities have been implicated in increased severity inflammatory reactions associated with asthma (55) and colitis (56). Furthermore, Th2 responses may also contribute to complications associated with mycoplasma respiratory diseases. For example, there are several studies suggesting that mycoplasma respiratory infections in humans can increase the severity of asthma (4, 5). IL-4-mediated Th2 responses pro-

mote IgE production that can lead to allergies and asthma (26, 55, 57–59), and we have shown the generation of mycoplasma-specific IgE responses during the pathogenesis of *M. pulmonis* disease (23). Thus, Th1 cell activity is likely to directly contribute to the inflammatory lesions in mycoplasma respiratory; however, regulatory and effector functions of Th2 cells may lead to increased disease severity and/or complications associated with mycoplasma respiratory diseases. Nevertheless, further studies are needed to uncover the contributions of Th1 and Th2 cell responses to mycoplasma respiratory disease pathogenesis.

Although a smaller component of the T cell response, CD8⁺ T cell numbers also increased in lungs after infection and have a significant impact on the progression of mycoplasma respiratory disease. In addition to cell numbers, we found that mycoplasma-specific CD8⁺ T cell responses were generated in the lungs of *M. pulmonis*-infected mice. This was shown by an increased expression of IFN- γ mRNA in purified CD8⁺ cells, and through our studies demonstrating that depletion of CD8⁺ T cells reduced *Mycoplasma*-specific IFN- γ production in vitro. Importantly, we demonstrated that in vivo depletion of CD8⁺ T cells resulted in dramatically more severe mycoplasma respiratory disease, while depletion of CD4⁺ T cells reduced the severity of these reactions, but there was no change in mycoplasma numbers after depletion. These results suggest that CD8⁺ T cells dampen CD4⁺ T cell-mediated inflammatory reactions in response to mycoplasma infection. There is increasing evidence supporting a similar role for CD8⁺ T cells in pulmonary inflammatory reactions (36, 37, 60). Although not elucidated in the current study, several potential mechanisms could be responsible for the effect CD8⁺ T cells have in regulation of pulmonary inflammatory reactions. There is evidence that CD8⁺ T cells, through IFN- γ cytokine production, have a regulatory role in eosinophilia, neutrophilia, and eotaxin activation observed in a murine model of asthma (37, 61). Additionally, elevated IgE Ab levels occur in mice after in vivo CD8⁺ T cell depletion and immunization with OVA Ag (62, 63). It is likely that the effect of CD8⁺ T cells in regulation of Th2-mediated pulmonary inflammatory responses observed in the above studies is a result of interactions with CD4⁺ T cells. There was, however, no evidence in our in vitro studies that depletion of CD8⁺ cells resulted in an enhancement of IL-4 responses, suggesting that in our in vitro studies, CD8⁺ cells had a major regulatory effect on existing Th2 responses. Additional studies are needed to examine whether CD8⁺ T cell cytokines have an in vivo effect during Th cell differentiation, although preliminary studies do not support a major shift in IL-4 and IFN- γ mRNA expression (data not shown). Alternatively, CD8⁺ T cells have been shown to act as veto cells

in peripheral tolerance against graft rejection through TGF- β 1 production (64, 65), and it is possible that a similar mechanism is involved in the modulation of mycoplasma respiratory disease. An additional mechanism may be through the reduction of APC numbers due to CD8⁺ T cell-mediated cytotoxicity (66), thereby indirectly decreasing Th cell activation. Interestingly, the expansion of CD8⁺ T cells is not found in all inflammatory responses in the lung, as nasal immunization using cholera toxin results in a perivascular and peribronchial mononuclear cell infiltrate that is comprised of CD4⁺ T cells, but not CD8⁺ T cells (49). Thus, CD8⁺ T cells are a significant component of the chronic inflammatory infiltrate, and they play a significant role in mycoplasma respiratory disease pathogenesis through the dampening of CD4⁺ T cell-mediated inflammation. Further studies are needed to determine the mechanisms through which CD8⁺ T cells modulate these responses.

In summary, the present study provides insight to the mechanisms of immunity involved in the pathogenesis of mycoplasma respiratory disease. We delineated the changes in T cell populations and their activation after *Mycoplasma* infection. We also determined their impact on the pathogenesis of murine mycoplasma respiratory disease. Based on our results, mycoplasma-specific Th cell responses in the lung are linked to the development of immunopathologic reactions. Of particular interest is the observation of the shift from a Th2 pulmonary environment to a mixed Th1 and Th2 response and its correspondence with disease. As we have shown in a previous study, a similar change in pulmonary T cell responses occurs after intense immune stimulation (49); future studies need to determine the mechanisms through which this occurs and its impact on resistance and protection against respiratory diseases. Although a smaller component of the T cell response, mycoplasma-specific CD8⁺ T cells are a significant component of the chronic inflammatory infiltrate, and their activation leads to a dampening of the inflammatory reaction in mycoplasma respiratory disease. To our knowledge, this is the first study to examine the development of T cell subset responses and their role in any mycoplasma disease. We have shown that the major T cell subsets, Th and CD8⁺ T cells, have opposing regulatory roles in progression of mycoplasma inflammatory disease of the lung. We anticipate these studies will provide a strong foundation for further studies examining the role of each T cell component in murine mycoplasma respiratory disease. This will undoubtedly benefit our understanding of the detrimental effects of immune-mediated *M. pneumoniae* infection in humans. Additionally, as similar mechanisms may be present in other chronic respiratory diseases, we believe these studies will also yield insights to a greater understanding of respiratory disease pathogenesis as a whole.

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