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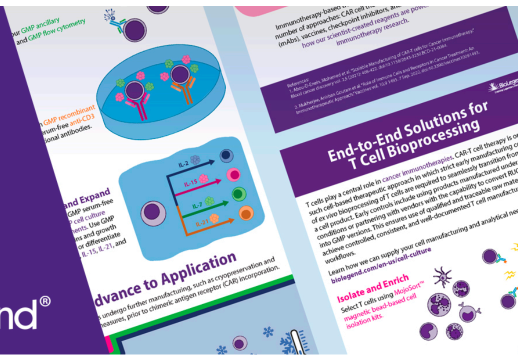

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The Upper and Lower Respiratory Tracts Differ in Their Requirement of IFN-γ and IL-4 in Controlling Respiratory Mycoplasma Infection and Disease[1]

Matthew D. Woolard,[2]* Lisa M. Hodge,[2]* Harlan P. Jones,† Trenton R. Schoeb,‡ and Jerry W. Simecka[3]*

The purpose of this study is to evaluate the significance of IFN-γ and IL-4 production in controlling mycoplasma infection and the pathogenesis of disease in the upper and lower respiratory tract. By using IFN-γ knockout and IL-4 knockout BALB/c mice, we were able to study the contribution of these cytokines in the development of pathogenesis and/or protection in response to mycoplasma respiratory infection, in both the upper and lower respiratory tracts. The loss of either IFN-γ or IL-4 does not affect disease pathogenesis or mycoplasma organism numbers in the upper respiratory tract. However, in the absence of IL-4, the nasal passages developed a compensatory immune response, characterized by higher numbers of macrophages and CD8$^+$ T cells, which may be masking detrimental effects due to IL-4 deficiency. This is in contrast to the lower respiratory tract, where the loss of IFN-γ, but not IL-4, leads to higher mycoplasma numbers and increased disease severity. The loss of IFN-γ impacted the innate immune system's ability to effectively clear mycoplasma, as the number of organisms was higher by day 3 postinfection. This higher organism burden most likely impacted disease pathogenesis; however, the development of Th2 cell-mediated adaptive immune response most likely contributed to lesion severity at later time points during infection. Our studies demonstrate that the upper and lower respiratory tracts are separate and distinct in their cytokine requirements for generating immunity against mycoplasma infection. *The Journal of Immunology,* **2004, 172: 6875–6883.**

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Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States, alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia (1–4). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (5, 6). *Mycoplasma pulmonis* causes a naturally occurring murine respiratory disease with high morbidity and low mortality. *M. pulmonis* is an excellent animal model of *M. pneumoniae*, allowing the characterization of immune responses during the pathogenesis of mycoplasma respiratory disease. Both *M. pulmonis* and *M. pneumoniae* respiratory infections cause rhinitis, otitis media, laryngotrachetis, and bronchopneumonia. In terms of histopathology, both diseases are characterized by the accumulation of mononuclear, macrophage, and lymphocyte cells along the respiratory airway (2, 7–10). This suggests that the activation and recruitment of macrophages and lymphocytes are important in the development of both acute and chronic states of the disease. In support, several studies demonstrate that a component of mycoplasma respiratory disease is immunopathologic (11–15).

It is clear that part of the adaptive immune system contributes to the pathology, while part is protective against *M. pulmonis* infections. SCID mice, which lack T and B cells, do not develop as severe respiratory disease in response to *M. pulmonis* infections as do corresponding immunocompetent mice. However, these SCID mice eventually develop arthritis and a higher mortality rate, demonstrating there is a need for lymphoid immune responses to control mycoplasma respiratory infections. Reconstitution of these SCID mice with splenic lymphocytes results in similar disease compared with wild-type mice (1). Work in T cell-deficient hamsters also demonstrates that these hamsters have less severe disease than hamsters with a full immune arsenal (12, 14). Both of these animal models demonstrate that T and B cell responses can contribute to both pathological as well as protective roles during mycoplasma respiratory disease.

Understanding the T cell environment within the lungs, and how this environment is modulated in the response to mycoplasma infection is important for identifying protective and pathological components of the immune response. The depletion of Th cells results in less severe lung disease, demonstrating that a Th cell response contributes to disease pathology in the lung (16). To the contrary, depletion of CD8$^+$ T cells from mice leads to an exacerbation of mycoplasma pulmonary disease. These results suggest that CD8$^+$ T cells play a unique regulatory role within mycoplasma disease in the lower respiratory tract. Our lab, as well as others, has demonstrated that the lung is a Th2-dominated environment (17, 18). However, in response to mycoplasma infections, there is a mixed mycoplasma-specific Th1/Th2 response in the lung, suggesting that IFN-γ from Th1 cells is playing an important role in mycoplasma respiratory disease (16). Thus, T cell activation and most likely the cytokines they produce are instrumental in the pathogenesis of mycoplasma respiratory disease of the lower respiratory tract.

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The generation of immune response in the upper respiratory tract (nasal passages) can contribute to the progression of mycoplasma respiratory disease. The upper respiratory tract is the initial and major site of Ab production after mycoplasma infection (19); a similar phenomenon has been shown for other infectious agents. Mice immunized for, or infected with, viruses generate a higher titer of Ag-specific Ab responses in the nasal passages vs the lungs (20, 21). These studies suggest the upper and lower respiratory tracts are immunologically separate in their response toward an infectious agent. In support, systemic immunization with *M. pulmonis* Ag can offer some protection to the lungs, but fails to protect nasal passages (1). In fact, recent data demonstrate local immunization of the upper and/or lower respiratory tracts is more effective in protection against mycoplasma disease. These studies suggest the importance as well as the difference in immune responses within the upper and lower respiratory tracts in response to infectious agents.

IL-4 and IFN-γ are pleiotrophic cytokines that have strong immunomodulatory roles on both innate and adaptive immune cells. Early after most infections, NK cells, γδ T cells, and NKT cells release IFN-γ (22–24). IFN-γ then activates macrophages, leading to increased cytokine secretion, and the up-regulation of NO and oxygen radical production and secretion (25). This early source of IFN-γ is also important in directing adaptive immune responses. It causes the phenotypic maturation of Th0 cells to a Th1 phenotype, which aids macrophages in killing intracellular bacteria (26). IL-4 directs eosinophilic responses, IgE generations, and Th2 cell maturation (27). Th2 cells are important in aiding the humoral immune responses. IL-4 also plays a critical role in the maintenance of mucosal immunity (28). Thus, IL-4 and IFN-γ are critical cytokines that are important in the modulation of innate and the generation of adaptive immune responses.

The purpose of this study is to evaluate the significance of IFN-γ and IL-4 in controlling mycoplasma infection and the pathogenesis of disease in the upper and lower respiratory tracts. By using IFN-γ knockout (KO)[4] and IL-4 KO mice, we were able to study the contribution of these cytokines in the development of pathogenesis and/or protection in response to mycoplasma respiratory infection in both the upper and lower respiratory tracts. Studies to characterize differences in cytokine control of the upper and lower respiratory tracts have yet to be done. Information gained from these studies will give insight into the development of effective vaccines that lead to immunity of both the upper and lower respiratory tracts.

Materials and Methods

Mice

Viral- and mycoplasma-free BALB/c, IFN-γ (C.129S7(B6)-IFN-γ^{tm1Ts} on a BALB/c background) KO, and IL-4 (BALB/c-Il4^{tn2Nnt} on a BALB/c background) KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (25, 29), and breeding colonies were established. Mice were housed in sterile microisolator cages supplied with sterile bedding, and sterile food and water were given ad libitum. Mice used in the study were between 8 and 12 wk of age. Female mice were used in all studies, unless where noted in the results. Before experimental manipulation, mice were anesthetized with an i.m. injection of ketamine/xylazine. All lung and nasal passage experiments were conducted on the same mice.

Mycoplasma

The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (30), in mycoplasma medium and frozen in 1-ml aliquots at −80°C. For inoculation, thawed aliquots were diluted to 10^5 CFU/20 μl. Nasal-pulmonary inoculations of 20 μl of diluted mycoplasma were given for experimental infections.

Cell isolation

Mononuclear cells were isolated from lungs, as previously described (19, 31, 32). Lungs were perfused with PBS without magnesium or calcium to minimize contamination of the final lung cell population with those from the blood. The lungs were finely minced. The tissues were suspended in RPMI 1640 medium (HyClone Laboratories, Logan, UT) 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 (Fisher Scientific, Pittsburgh, PA), and antibiotic/antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37°C while mixing on a Nutator (Fisher Scientific) for 90–120 min. During the incubation period, the tissues were vigorously pipetted every 30 min. After incubation, the digestion mixture was passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from cell suspension by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY).

Spleen cells were isolated after centrifugation of all suspensions, followed by red cell removal using ammonium chloride potassium lysis buffer, as previously described (33).

Cells from the nasal passages were isolated, as previously described (19). Briefly, the lower mandibles and skin were removed from the skull. The skull was longitudinally split, and the nasal passages were removed by scraping and transferred to collagenase-DNase digestion medium as used for isolation of lung cells. After 1 h of incubation at 37°C while being mixed on a Nutator, the tissue was passed through a 250-μm nylon mesh, and the red cells were removed using ammonium chloride potassium lysis buffer. This technique isolates cells from both nasal mucosa and nasal-associated lymphoid tissue (NALT).

RNA isolation from nasal passages and lungs

Total RNA was isolated from both whole lungs and nasal passages of mice using the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX). Briefly, nasal passages and lungs were homogenized in the Ultraspec-II RNA reagent using a Pro 200 homogenizer (Pro Scientific, Monroe, CT). The RNA samples were frozen at −80°C until further isolation. Chloroform was added to the homogenate and centrifuged at 12,000 × *g* (4°C) for 30 min. The RNA was precipitated by adding isopropanol to the aqueous phase and centrifuging samples at 12,000 × *g* (4°C) for 10 min. The RNA pellet from each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 × *g*, and then resuspended in diethylpyrocarbonate-treated water. The concentration and quality of RNA in each sample were determined spectrophotometrically (GeneQuant II; Pharmacia Biotech, Piscataway, NJ) and by gel electrophoresis. The RNA samples were stored at −80°C until ready for use.

Cytokine mRNA detection by RT-PCR

RT-PCR was performed using 100 ng of RNA for each sample, as previously described (34). The sequences of the primers and the size of the resulting PCR fragments (in parentheses) for IL-4, IFN-γ, and the housekeeping gene, β_2-microglobulin (β_2m) are given as follows (35): IL-4 (216 bp), 5′-CGGCATTTTGAACGAGGTC and 5′-GAAAAGCCCGAAAGA GTCTC; IFN-γ (227 bp), 5′-GCTCGAGACAATGAACGCT and 5′-AAAGAGATAATCTGGCTCTGC; and β_2m (222 bp), 5′-TGACCGGCT TGTATGCTATC 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 (34, 36). For IFN-γ and β_2m, the samples were amplified for 30 cycles, and for 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, β_2m.

Assessment of gross lesions and histopathology

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 (37).

[4] Abbreviations used in this paper: KO, knockout; β_2m, β_2-microglobulin; NALT, nasal-associated lymphoid tissue.

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Lungs and nasal passages were fixed in alcohol formalin (4% glacial acetic acid (Fisher Scientific), 6% formaldehyde solution (Fisher Scientific), 40% deionized water, and 50% of 95% ethanol); nasal passages were demineralized in acid-decalcifying solution (Richard Allan Scientific, Kalamazoo, MI). Tissues were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with H&E for light microscopy. Each lung lobe was sectioned separately. Histology slides were randomly coded, and subjectively scored at University of Alabama for lesion severity (scale of 0–4) on the basis of the characteristic lesions of murine respiratory mycoplasmosis, as described previously (38). Scores refer to: 1) peribronchial and perivascular lymphoid hyperplasia or infiltration (peribronchial infiltrate), or submucosal infiltrate in nasal passages; 2) mixed neutrophilic and histiocytic exudate in alveoli (alveolar exudate); 3) neutrophilic exudate in airway lumina (airway exudate); and 4) hyperplasia of airway mucosal epithelium (epithelial) (38). A score for each lesion was weighted according to the percentage each lobe contributes to the total lung weight in arriving at a total lesion score for each set of lungs. For each of the four lesions, a lesion index was calculated by dividing the observed lesion score by the maximum lesion score possible. Thus, the maximum lesion index possible for any lesion was 1.0.

Characterization of mycoplasma numbers

The numbers of mycoplasma CFU in lungs and nasal passages were determined, as previously described (39, 40). Briefly, lungs were minced and placed in mycoplasma broth medium. Nasal washes were collected with 1 ml of mycoplasma broth medium that was forced through the nasal passages of mice by inserting a syringe into the soft pallet. In some experiments, we also isolated nasal passage tissue for CFU determination. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell, Newtown, CT) for 2 min at 50 amplitudes without pulsing. After sonication, serial dilutions (1/10) were prepared, and 20 μl of each dilution was plated onto mycoplasma agar medium. After 7 days of incubation at 37°C, the colonies were counted, and the CFU recovered from each tissue was calculated.

M. pulmonis-*specific Ab levels*

To prepare Ag for ELISA, *M. pulmonis* was cultured at 37°C in mycoplasma broth medium for 3 days and harvested. *M. pulmonis* broth was adjusted to 5 mg/ml protein concentration. A total of 20 ml of lysis buffer (4.2 g $NaHCO_3$/L and 5.3 g Na_2CO_3/L, pH 10.0) warmed to 37°C, was added to each 1 ml of *M. pulmonis* stock and incubated at 37°C for 15 min. Then 2.2 g of boric acid was added to 100 ml of lysis buffer and frozen at −70°C. Protein concentration was then determined by Bradford assay.

Falcon Microtest III assay plates (BD Biosciences, Oxnard, CA) were coated with optimal concentrations of *M. pulmonis* Ag (100 μl at 10 μg/ml) in PBS. After overnight incubation at 4°C, the plates were washed three times with PBS-0.05% Tween 20 and blocked with PBS-0.05% Tween 20 supplemented with 10% FBS overnight at 4°C. Serum samples were initially diluted 1/100 and then serially (1/2) diluted with PBS-0.05% Tween 20–10% FBS, and 100 μl was placed in duplicate into wells of the Ag-coated plates. After overnight incubation at 4°C, the plates were washed four times with PBS-0.05% Tween 20. Secondary Ab (biotinylated anti-mouse Ab stock reagents of 0.5 mg/ml; Southern Biotechnology Associates, Birmingham, AL) were diluted 1/2000 (IgA, IgM, or IgG) or 1/500 (IgG1 or IgG2a) in PBS-0.05% Tween 20–10% FBS, and 100 μl was added to the appropriate wells. After overnight incubation at 4°C, the plates were again washed four times with PBS-0.05% Tween 20, and a 1/2000 dilution of HRP-conjugated streptavidin (neutralite avidin; Southern Biotechnology Associates) in PBS-0.05% Tween 20–10% FBS was added to the wells (100 μl). The plates were incubated at room temperature for 2 h, and the plates were washed twice with PBS-0.05% Tween 20 and twice with PBS. The reaction mixtures were developed at room temperature by addition of 100 μl of 3,3′5,5′-tetra methylbenzidine peroxidase substrate (Moss, Pasadena, MD) in each of the wells. Plates were read using MX80 plate reader (Dynatech Laboratories, Chantilly, VA) at an absorbance of 630 nm. Endpoint Ab titers were expressed as the reciprocal dilution of the last dilution that gave an OD at 630 nm of 0.1 U above the OD of negative controls after a 20-min incubation.

To detect Ag-specific IgA Abs in nasal washes, samples were serially diluted 1/2 in PBS containing 10% FBS and added to the appropriate wells of Ag-coated plates. The reactions were developed as for serum samples.

Measurement of total IgA levels in nasal washes

The level of total IgA was measured by luminex suspension array from Upstate Biotechnology (Lake Placid, NY). Ninety-six-well filter bottom plates were used. To each well, 50 μl of anti-IgA beads in assay diluent was added. Fifty microliters of sample or standard were added per well. Plates were incubated at room temperature while shaking in the dark for 15 min. Plates were washed with assay buffer. Then 75 μl of assay buffer and 25 μl of secondary reporter Abs were added to each well. Plates were incubated at room temperature while shaking in the dark for 15 min. Samples were read using Bio-Plex 100 system (Bio-Rad, Hercules, CA). Total IgA levels were determined by comparison with standard curves generated from murine monoclonal IgA and analyzed using Bio-Plex Manager Software (Bio-Rad).

Cell characterization by flow cytometry

Three-color immunofluorescent staining was performed to identify T cell populations using FITC-labeled anti-murine CD4 mAb (L3T4, RM4-5; BD PharMingen, San Diego, CA), PerCB-labeled anti-murine CD8 mAb (Lyt-2, 53-6.7; BD PharMingen), and PE-labeled anti-murine CD3 mAb (KT3; Beckman Coulter, Fullerton, CA). PE-labeled anti-murine F4/80 mAb (F4/80; Caltag Laboratories, Burlingame, CA) was used to identify macrophages, and PE-labeled anti-murine B220 mAb (RA-6B2; Beckman Coulter) was used to identify B cells. Briefly, 10^6 cells per tube were incubated for 30 min at 4°C in 100 μl of optimal concentration of fluorescent Ab. The cells were washed in staining buffer (Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide, 1% FBS) and fixed with 2% paraformaldehyde in PBS for 30 min. After fixation, cells were resuspended in staining buffer for analysis.

The cells were analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter). Cell population gates and detector voltages were set using isotype-stained (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 cell population, their percentages were multiplied by the total number of cells isolated from that tissue.

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 program. When appropriate, data were logarithmically transformed before statistical analysis, and confirmed by a demonstrated increase in power of the test after transformation of the data. A p value ≤ 0.05 was considered statistically significant. If data were analyzed after logarithmic transformation, the antilog of the means and SEs of transformed data were used to present the data and are referred to as the geometric means (×/÷ SE).

Results

Upper and lower respiratory tracts differ in their relative expression of IL-4 and IFN-γ mRNA after mycoplasma infection

From our previous study (16), we found that specific T cell responses in lungs, as characterized by the production of IL-4 and IFN-γ in response to mycoplasma Ag, appear between 10 and 14 days after infection. To determine whether there are qualitative differences between upper and lower respiratory tracts in their cytokine expression after mycoplasma infection, IL-4 and IFN-γ mRNA expression was measured in the nasal passages and lungs 14 days postinfection using RT-PCR. After RT-PCR, the primers generated fragments of the predicted size.

Nasal passages expressed higher relative levels of IL-4 mRNA than IFN-γ mRNA after infection, while the lungs had a mixed expression of both cytokine mRNAs at day 14 postinfection, and uninfected control mice showed no detectable levels of mRNA for IFN-γ and IL-4 in both the nasal passages and the lungs (Fig. 1). At 14 day postinfection, IL-4 and IFN-γ mRNA levels were increased, but mRNA expression for IL-4 was significantly greater relative to IFN-γ in the nasal passages. However, the lungs of mycoplasma-infected animals at day 14 showed little shift in the relative expression of IL-4 and IFN-γ, although the levels were higher than naive mice.

Thus, there were relative differences in IL-4 and IFN-γ mRNA expression levels between nasal passages and lungs in mycoplasma-infected mice. Thus, IL-4 mRNA levels are significantly increased, relative to IFN-γ mRNA levels in the nasal passages, as compared with lungs. This suggests a difference in the contribution

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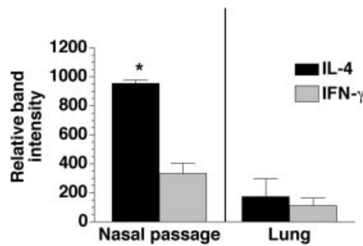

FIGURE 1. Cytokine mRNA expression in the respiratory tract after infection. Fourteen days following mycoplasma infection, lungs and nasal passages were removed, and total RNA was isolated. IL-4 and IFN-γ cytokine mRNA levels were measured in each tissue by using RT-PCR. Relative differences in mRNA expression of cytokines were determined by the relative increase in the ratio of cytokine to the housekeeping gene β_2m. Vertical bars and error bars represent mean ± SE ($n = 6$). *, Denotes statistical difference ($p \leq 0.05$) from all other groups.

of these cytokines in response to *M. pulmonis* disease in upper and lower respiratory tracts.

IFN-γ KO mice have more severe mycoplasma disease in the lungs than control mice

The previous study indicated that there was a difference in the contribution of IL-4 and IFN-γ responses in nasal passages and lungs of mice with mycoplasma respiratory disease. To determine the effect of IFN-γ and IL-4 on mycoplasma disease pathology, age-matched IFN-γ KO and IL-4 KO mice were experimentally infected with *M. pulmonis*, and lungs and nasal passages were collected on days 3, 7, 10, and 14 postinfection. Lungs were first scored for the presence of gross lesions, then lungs and nasal passages were prepared in alcohol formalin for histological staining to determine disease pathology.

IFN-γ KO mice developed more severe mycoplasma disease than IL-4 KO or BALB/c (control) mice. Clinical signs of disease (lethargy and ruffled fur) became apparent in IFN-γ KO mice at a much earlier time point after infection (day 3–7), while IL-4 KO and control mice did not show signs of disease until day 10–14. Consistent with clinical disease, IFN-γ KO mice had significantly higher pulmonary gross lesion scores by day 7 than control mice and continued through day 14 (Fig. 2).

IL-4 KO mice, in contrast, showed comparable lung gross lesion scores to control mice through day 10. However, by day 14, there was a trend for lower gross lesion scores in the lungs of IL-4 KO mice than in control mice.

To determine whether the type of pulmonary lesions and disease severity was affected by cytokine deficiencies, lungs and nasal tissues were collected for histopathology from mycoplasma-infected cytokine KO and normal mice. By day 3 postinfection, there were significantly higher scores in neutrophilic exudate and alveolitis in the lungs of IFN-γ KO mice, and on day 7–14 postinfection, all

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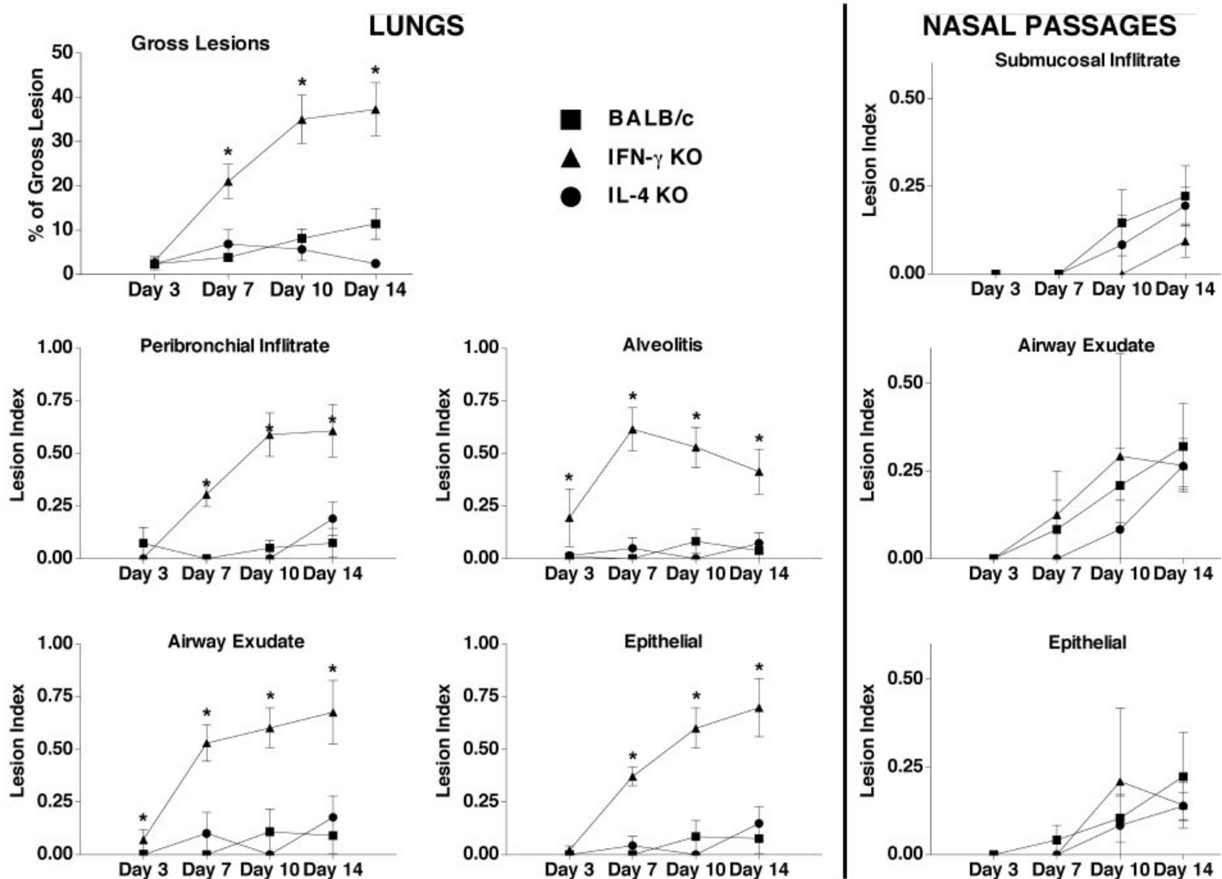

FIGURE 2. Lesion severity of lungs and nasal passages after infection. At days 3, 7, 10, and 14 after mycoplasma infection, lungs and nasal passages were removed. Lungs were scored for percentage of gross lesion, while both lungs and nasal passages were formalin fixed for histology. Lesion index scores refer to: 1) peribronchial and perivascular lymphoid hyperplasia or infiltration (peribronchial infiltrate) in lung or submucosal infiltrate in nasal passages; 2) mixed neutrophilic and histiocytic exudate in alveoli (alveolitis); 3) neutrophilic exudate in airway lumina (airway exudate); and 4) hyperplasia of airway mucosal epithelium (epithelial). Mice in these studies were male and female. Vertical bars and error bars represent mean ± SE ($n = 6$). *, Denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

four histological scores (airway exudates, alveolitis, epithelial, and peribronchial infiltrate) were significantly higher in IFN-γ KO mice than in corresponding controls (Fig. 2). IL-4 KO mice did not show any significant difference at any time point from control animals. Histological lesion scores of the nasal passages show no significant difference in any strain at any time point (Fig. 2). Thus, IFN-γ KO mice have more severe disease in the lungs than control or IL-4 KO mice.

IFN-γ KO mice have higher numbers of mycoplasma in the lungs than control mice

To determine how the loss of IFN-γ and IL-4 affects colonization of mycoplasmas in the lower and upper respiratory tracts, IFN-γ KO, IL-4 KO, and corresponding BALB/c control mice were experimentally infected with *M. pulmonis*.

On days 3, 7, 10, and 14 after infection, nasal washes and lungs were collected, and the number of mycoplasma CFU was determined in upper and lower respiratory tracts.

The IFN-γ KO, but not IL-4 KO, mice had significantly higher numbers of mycoplasmas in their lungs than control mice. By day 3 postinfection, the number of mycoplasma CFU in the lungs of IFN-γ KO mice was almost 2 logs higher than corresponding control mice (Fig. 3.). By day 14, IFN-γ KO mice still had almost 1 log higher mycoplasma CFU in the lung than control mice. IL-4 KO mice, in contrast, tended to have a lower mycoplasma CFU burden within the lungs at days 7 and 10 postinfection; however, the lower CFU numbers in the lungs of IL-4 KO mice were not significantly different from control mice. On day 14 postinfection, mycoplasma CFU burden in the lungs was comparable between IL-4 KO and control mice.

Within the upper respiratory tract, the loss of either IFN-γ or IL-4 did not affect mycoplasma CFU numbers. This is in stark contrast to the lungs, where IFN-γ is critical for controlling mycoplasma CFU numbers. At all time points from day 3 to 14 postinfection, there was no significant difference in CFU numbers obtained from nasal washes between IFN-γ KO, IL-4 KO, and control mice (Fig. 3). To ensure that the lack of differences was not due to the use of nasal washes for sampling, we collected nasal passage tissue in cytokine-deficient and control mice 14 days after infection. As with nasal washes, there were no significant differences in numbers of mycoplasma CFU recovered from the nasal passage tissue between the groups of mice (data not shown).

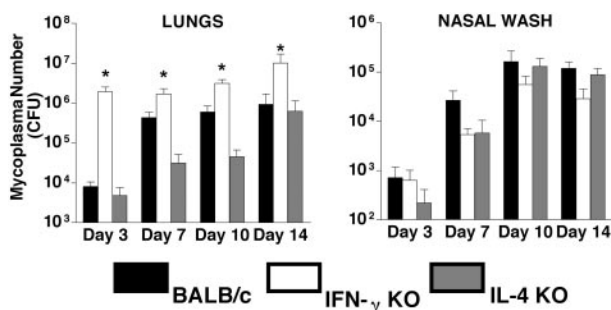

FIGURE 3. Mycoplasma CFU in respiratory tract after intranasal infection. BALB/c, IL-4 KO, and IFN-γ KO mice were infected with *M. pulmonis*. At days 3, 7, 10, and 14 postinfection, the number of mycoplasma CFU in lungs and nasal passages (nasal wash) was determined. Mice used in these studies were male and female. Vertical bars and error bars represent mean ± SE ($n = 9$). *, Denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

IFN-γ KO mice have higher levels of mycoplasma-specific serum IgG than control mice

To determine the generation of Abs in response to *M. pulmonis* in the absence of IFN-γ and IL-4, mycoplasma-specific Ab levels were measured in IFN-γ KO and IL-4 KO mice infected with *M. pulmonis*. Serum was collected from control, IFN-γ KO, and IL-4 KO mice on days 3, 7, 10, and 14 postinfection, and the levels of *M. pulmonis* Ag-specific IgA, IgM, IgG, IgG1, and IgG2a were determined. Nasal washes were also collected at day 14 postinfection, and mycoplasma-specific IgA titers were measured.

There were differences in Ab responses that developed in cytokine-deficient mice in response to mycoplasma infection. At early time points (days 0 and 3), no detectable levels of any *M. pulmonis*-specific Ab classes were found in sera from the three mouse strains (Table I). At day 7, mycoplasma-specific Ab from all classes and subclasses were detectable, but there was no significant difference in the titers of any of the Ab classes between the three strains at this time point. By day 10, there were significantly higher titers of IgM, IgG, IgG1, and IgG2a in the sera of mycoplasma-infected IFN-γ KO mice, as compared with control mice, while IgA was significantly lower. These differences in IgG Ab responses in IFN-γ KO mice at day 10 were also seen at day 14. IL-4 KO mice, in contrast, had serum titer in all Ab classes comparable to that of control mice at days 7 and 10. By day 14, there were significantly higher titers of mycoplasma-specific IgG2a in IL-4 KO than in control mice, although the overall levels of IgG were not significantly different from control mice.

Unlike the sera, IL-4 KO mice had significantly higher titer levels of mycoplasma-specific IgA in the upper nasal passages than control or IFN-γ KO mice (Fig. 4). By day 14 postinfection, IL-4 KO mice had 1 log higher titer of mycoplasma-specific IgA levels in the nasal passages than that of control or IFN-γ KO mice. However, there were no significant differences in total IgA levels in day 14 nasal washes of BALB/c (56 ± 30 ng/ml), IL-4 KO

Table 1. *Comparison of mycoplasma-specific Ab response after Infection*[a]

Isotype[b]	Day[c]	Mouse Strain		
		BALB/c	IFN-γ KO	IL-4 KO
IgA	3	0 (0)[d]	0 (0)	0 (0)
	7	885 (1.3)	800 (1.2)	800 (1.1)
	10	1,233 (1.3)	993 (1.2)[a]	993 (1.2)
	14	21,528 (1.1)	27,825 (1.2)	30,409 (1.1)
IgM	3	0 (0)	0 (0)	0 (0)
	7	2,075 (1.7)	1,466 (1.6)	1,466 (1.5)
	10	2,382 (1.5)	5,309 (1.3)[a]	1,986 (1.5)
	14	46,989 (1.1)	48,989 (1.1)	51,168 (1.0)
IgG	3	0 (0)	0 (0)	0 (0)
	7	475 (1.4)	437 (1.2)	337 (1.2)
	10	497 (1.2)	1,658 (1.5)[a]	325 (1.2)
	14	6,982 (1.3)	28,708 (1.3)[a]	4,932 (1.4)
IgG1	3	0 (0)	0 (0)	0 (0)
	7	337 (1.3)	308 (1.2)	218 (1.2)
	10	497 (1.2)	1,656 (1.5)[a]	325 (1.2)
	14	4,932 (1.3)	19,724 (1.5)[a]	4,932 (1.4)
IgG2a	3	0 (0)	0 (0)	0 (0)
	7	113 (1.3)	104 (1.6)	100 (1.3)
	10	101 (1.5)	3,097 (1.2)[a]	993 (1.2)
	14	2,075 (1.2)	7,603 (1.8)[a]	8,299 (1.2)[a]

[a] BALB/c, IL-4 KO, and IFN-γ KO mice were intranasally *M. pulmonis*. At days 3, 7, 10, and 14 post infection, serum was collected and Ab titers were determined by ELISA.
[b] Mycoplasma-specific Ab responses.
[c] Days post infection when serum was collected.
[d] Geometric mean ×/+ (SE) n = 12.
[e] Denotes significant difference ($p = 0.05$) from BALB/c.

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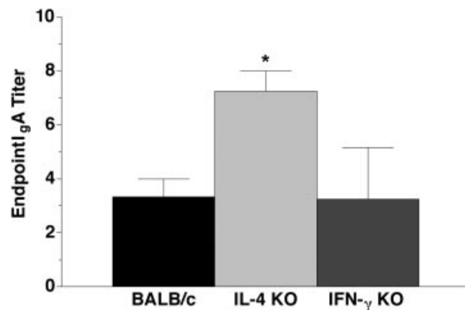

FIGURE 4. Mycoplasma-specific nasal IgA responses after intranasal infection. BALB/c, IL-4 KO, and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. Nasal wash samples were collected 14 days after infection, and endpoint titers of mycoplasma-specific IgA were determined by ELISA. There were no detectable differences in total IgA levels tested in day 14 nasal washes of BALB/c, IL-4 KO, and IFN-γ KO mice. The data were collected from three experiments ($n = 12$), and expressed as the means ± SE. *, Denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

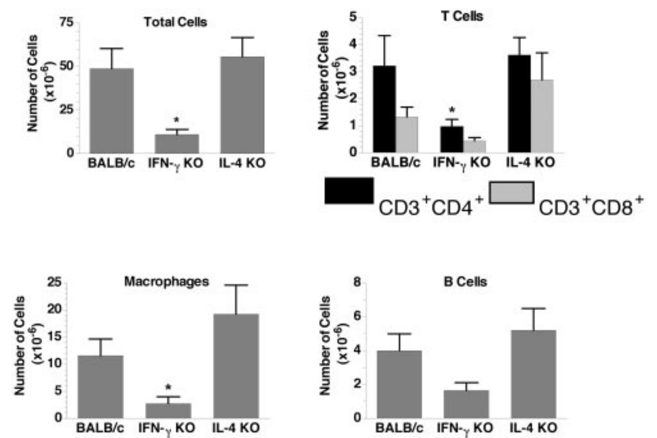

FIGURE 5. Numbers of immune cells in lungs after infection. BALB/c, IL-4 KO, and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. At day 14 postinfection, lung leukocytes were isolated and cell numbers were determined. The number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $B220^+$ B cells, and $F4/80^+$ macrophage populations was determined using flow cytometry. Vertical bars represent mean ± SE ($n = 11$). *, Denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

(88 ± 20 ng/ml), and IFN-γ KO (96 ± 25 ng/ml) mice, and therefore, differences in the titers were not confounded by nasal wash sampling efficiencies. Thus, IFN-γ KO mice had higher titers of mycoplasma-specific serum IgG Ab (and subclasses) responses than the control mice, while IL-4 KO mice had a shift in IgG responses from a mixed IgG1 and IgG2a mycoplasma-specific response, as seen in control, to a predominate IgG2a Ag-specific response.

IFN-γ KO mice have lower numbers of lymphocytes and macrophages in the lungs at day 14 postinfection than control mice

To determine whether lymphocytes or macrophages contribute to the differences in disease severity, BALB/c, IL-4 KO, and IFN-γ KO mice were experimentally infected with *M. pulmonis*, and at 14 days postinfection, cells were isolated from the lungs. Lung cells were stained with fluorescent Ab specific for CD3, CD4, CD8, B220, and F4/80, and analyzed by flow cytometry.

IFN-γ KO mice generated a different cellular response against mycoplasma infection in the lungs than seen in IL-4 KO and control mice. However, after 14 days of infection, there were significantly fewer numbers of cells isolated from the lungs of IFN-γ KO mice than in lungs of control or IL-4 KO mice (Fig. 5). There were also significantly lower numbers of T cell populations ($CD4^+$ Th and $CD8^+$ T cells) and macrophages ($F4/80^+$) present in mycoplasma-infected IFN-γ KO lungs than in control mice lungs. IL-4 KO mice, in contrast, showed no differences from infected control mice in the number of B cells, T cells, or macrophages in the lungs after mycoplasma infection. There were no differences in resident pulmonary lymphocytes and macrophages collected from naive BALB/c, IFN-γ KO, and IL-4 KO mice (data not shown).

Mycoplasma-infected IL-4 KO mice have higher numbers of $CD8^+$ T cells and macrophages in nasal passages at day 14 than control mice

To determine whether lymphocyte or macrophage populations were affected by cytokine deficiencies in the upper respiratory tract, we similarly examined the cell populations of nasal passages from naive and mycoplasma-infected BALB/c, IL-4 KO, and IFN-γ KO mice.

Naive IL-4 KO, IFN-γ KO, and BALB/c mice had similar numbers of $B220^+$ B cells, $CD3^+$ T cells, and $F4/80^+$ macrophages residing in the upper respiratory tract. By day 14 postinfection, there were significantly more cells (~1 million) isolated from IL-4 KO mice than from control or IFN-γ KO mice (Fig. 6). Higher numbers of $CD3^+$ T cells (specifically $CD8^+$ T cells) and macrophages were found in IL-4 KO than in BALB/c or IFN-γ KO mice, while there was no difference in the numbers of B cells isolated from these mice. A trend to an increase in $CD4^+$ T cells was noticed; however, due to high variation of cell counts, numbers of $CD4^+$ T cells between IL-4 KO and BALB/c mice were not significantly different. IFN-γ KO mice did not differ from control mice in numbers of T cells, B cells, or macrophages found in the nasal passages at day 14 postinfection. Thus, IL-4 KO mice have an increased number of macrophages and $CD8^+$ T cells in the nasal passages at day 14 postinfection as compared with control mice, while IFN-γ KO mice have no differences.

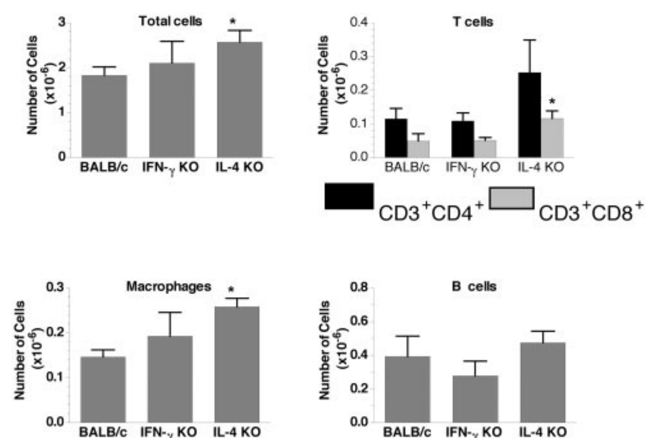

FIGURE 6. Number of immune cells in nasal passages after infection. BALB/c, IL-4 KO, and IFN-γ KO mice were intranasally inoculated with *M. pulmonis*. At day 14 postinfection, nasal passage leukocytes were isolated and cell numbers were determined. The number of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $B220^+$ B cells, and $F4/80^+$ macrophage populations was determined using flow cytometry. Vertical bars represent mean ± SE ($n = 11$). *, Denotes statistical difference ($p \leq 0.05$) from BALB/c mice.

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Discussion

The purpose of this study was to determine the importance of IFN-γ and IL-4 in the upper and lower respiratory tracts after mycoplasma infection. IFN-γ and IL-4 are pleiotrophic cytokines that help direct innate and adaptive immune responses (22, 28, 41–43). These cytokines are also important in the phenotypic development of lymphoid responses, as IFN-γ promotes a cell-mediated response, while IL-4 promotes a humoral response (26). Lymphoid responses are critical in mycoplasma lower respiratory tract disease, as they play both protective and pathological roles; however, immune responses of the upper respiratory tract are unknown (11–15). Studies in the upper respiratory tract do suggest that the nasal passages have a separate and distinct immune response from the lower respiratory tract (1, 19, 21, 40). Although unexplored in the upper respiratory tract, T cells play a multifaceted role within mycoplasma lung disease. The depletion of $CD8^+$ T cells exacerbates lung disease, while depletion of $CD4^+$ T cells decreases lung disease severity (16). Because of the known roles of IFN-γ and IL-4 in Th cell maturation, IFN-γ and IL-4 KO mice have been used in a variety of disease models to begin examining the role of Th cell subsets (44–48), and the use of IFN-γ and IL-4 KO mice in these studies will address similar questions during mycoplasma infection. However, the roles of IFN-γ- and IL-4-mediated cellular responses within mycoplasma respiratory disease have yet to be determined. Given the differences in upper and lower respiratory tract immunity, an in-depth description of these immune mediators within both compartments will shed light on their roles during mycoplasma disease.

The importance of IFN-γ and IL-4 in controlling mycoplasma infections and disease is different between upper and lower respiratory tracts. The loss of either cytokine in the upper respiratory tract did not affect disease pathogenesis or the immune system's ability to control mycoplasma growth. This is in contrast to the lungs, where the loss of IFN-γ results in more severe disease and a 2-log increase in mycoplasma CFU. In support of differences in upper and lower respiratory tract immunity, several studies have demonstrated the upper respiratory tract is in a separate compartment of the immune system from the lung (19–21). The lung is protected from mycoplasma infection by nasal-pulmonary or by systemic immunization with or without an adjuvant, while the nasal passages are protected only with nasal immunizations that contain an adjuvant (1, 40). These studies demonstrated differences in upper and lower respiratory tract immune responses; however, we are unaware of any studies that look at differences in the importance of cytokines in the upper and lower respiratory tracts in an infectious disease model. Our data demonstrate that the loss of either IL-4 or IFN-γ does not affect disease pathology or controlling mycoplasma growth in the upper respiratory tract, while IFN-γ is critical in dampening disease pathology and mycoplasma growth within the lower respiratory tract.

Although the loss of IL-4 does not affect disease pathogenesis or mycoplasma growth in the upper respiratory tract, it does lead to a change in immune response. The fact that the loss of IL-4 does not affect disease pathogenesis was surprising as IL-4 mRNA relative levels were significantly higher than IFN-γ mRNA isolated from the nasal passages of mycoplasma-infected mice. In the lungs, both IL-4 mRNA and IFN-γ mRNA expression were increased after infection, but only the loss of IFN-γ had an effect on pulmonary disease, with increased disease severity and mycoplasma numbers. Although there was no difference in disease, the IL-4 KO mice appear to develop compensatory immune responses that included higher titers of mycoplasma-specific IgA, and higher numbers of $CD8^+$ T cells and macrophages. This increase in mycoplasma-specific IgA may contribute to IL-4 KO mice being able to control mycoplasma upper respiratory tract infection. This would coincide with data that suggest that higher IgA levels play a protective role in *M. pneumoniae* infections (49, 50). Also, the increase in $CD8^+$ T cells and macrophages, which our lab and other labs have demonstrated to be critical cells in controlling mycoplasma disease and numbers (16, 51–53), may be masking any detrimental effects on upper respiratory tract immunity due to the loss of IL-4. It is unclear whether these compensatory immune responses lie in the nasal mucosa or NALT and whether they are related to effector or inductive responses. Similarly, it is possible that IFN-γ KO mice have unrecognized differences in inductor vs effector mechanisms in the upper respiratory tract. Regardless, IL-4 is most likely an important contributor to the development of immunity in the upper respiratory tract toward mycoplasma infection, but compensatory immune mechanisms mask the impact of IL-4 deficiencies on mycoplasma disease and infection. Further studies are needed to evaluate the contribution of IL-4 and related cytokines in NALT and effector immune responses generated in the upper respiratory tract (45), as well as understanding potential compensatory mechanism that can replace or overcome IL-4-mediated immunity.

IFN-γ, but not IL-4, is critical in controlling the level of mycoplasma infection within the lower respiratory tract. IFN-γ KO mice had a 2-log higher number of mycoplasma CFU than control or IL-4 KO mice in the lungs by day 3 postinfection, and this trend of higher CFU continued up to day 14 postinfection. The higher number of mycoplasma at 3 days after infection suggests that innate immune mechanisms that clear mycoplasma from the lung in control mice are impaired in the absence of IFN-γ. This impairment subsequently contributes to the increase in disease severity in IFN-γ KO mice. In support that innate and not adaptive immune mechanisms are contributing to this phenomenon, there were no detectable mycoplasma-specific Ab responses at this time point in any group of mice, demonstrating little to no B cell activity at day 3 postinfection. Work in our lab demonstrated that T cell responses are not seen until day 7 postinfection, further supporting that no adaptive immune response is substantially activated at 3 days after infection (16, 54). Therefore, higher numbers of mycoplasma in the lung by this early time point are most likely due to impairment in innate immunity due to the absence of IFN-γ.

These studies demonstrate the importance of IFN-γ; they do not, however, identify the cell(s) critical in releasing IFN-γ at this early time point. IFN-γ can be released by NK, NKT, and $\gamma\delta$ T cells early after infections with other infectious agents, which activates macrophages and affects adaptive immunity (22–24, 26, 41, 55). In ongoing studies, we demonstrate that NK cells are the major cell population that increased intracellular IFN-γ in response to mycoplasma infection within the first 3 days after infection (M. Woolard, D. Hudig, L. Tabor, and J. Simecka, manuscript in preparation). The loss of NK cell-derived IFN-γ could impact upon macrophage activation, in which macrophages would be unable to kill mycoplasma (56, 57). However, ongoing studies from our laboratory demonstrate that the loss of NK cell-derived IFN-γ is not the cause of increased mycoplasma CFU, but instead, NK cells in an IFN-γ-deficient environment have activities that interfere with normal clearance mechanisms, presumably mediated by macrophages. Thus, IFN-γ plays a complex, but instrumental role in the development of an effective innate immune response toward mycoplasma pulmonary infection.

IFN-γ does not affect innate immune responses solely, but most likely contributes to the development of adaptive immune responses that reduce mycoplasma disease pathology. In the present studies, we found that IFN-γ KO mice had increased lung lesion

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severity compared with other mouse strains, and there was clearly a change in the contribution of lymphocytes and macrophages to the pulmonary inflammatory lesions in IFN-γ KO mice. In contrast to expectations, there were lower numbers of T cell (4-fold) and macrophage (4-fold) populations in the lungs of IFN-γ KO mice at day 14 postinfection. This decrease in the number of macrophages and T cells suggests that there is a change in the recruitment of cells into the site of infection. SCID mice, which are deficient in lymphocytes, develop little to no pulmonary disease at 14 days after infection (11, 14); therefore, lymphoid responses are critical mediators of the characteristic chronic inflammatory lesions in the lungs of mycoplasma-infected mice. This suggests that the lymphocytes present in IFN-γ KO mice, although decreased in numbers, are most likely more pathogenic than the adaptive immune response generated in control mice, as lesion severity continues to increase at later time points. This indicates a shift from a mixed Th1/Th2 cell response (in control mice) in lungs toward a Th2-dominated response (in IFN-γ KO mice) contributing to the lesion severity (17). These results are consistent with pulmonary Th2-type responses generated in the mycoplasma-infected IFN-γ KO mice, contributing to the more severe lung disease seen in the absence of IFN-γ. Thus, it is clear that there are changes in the type of inflammatory lesions in IFN-γ KO mice, and further studies are needed to confirm that the severity of these lesions is mediated by Th2-type cell responses against mycoplasma.

In summary, the present study provides insight into the potential mechanisms of immunity involved in the pathogenesis of mycoplasma respiratory disease. To our knowledge, this is the first study to examine the involvement of cytokines in the pathogenesis of mycoplasma disease in the upper and lower respiratory tracts. We demonstrated that the upper and lower respiratory tracts differ in the contribution of IL-4 and IFN-γ in the pathogenesis of murine mycoplasma respiratory disease. Based on our results, there are most likely multiple (IL-4 and non-IL-4-mediated) immune mechanisms impacting the responses against mycoplasma infection in the upper respiratory tract. In contrast, IFN-γ production in the lung is clearly critical in developing beneficial innate and adaptive immune responses to control infection and inflammatory lesions. Most notably, we believe NK cells, in the absence of IFN-γ, promote activities that are detrimental to the normal clearance of mycoplasma. Although these IFN-γ-deficient NK cell-mediated activities influence disease progression, we also believe that results from the current and previous (16) studies indicate that mycoplasma-specific Th2 cell responses in the lung contribute to the development of immunopathologic reactions. In support, studies using other disease models demonstrate that Th2-mediated pulmonary responses can lead to more severe respiratory disease (58–61). In mycoplasma infections, the ability to generate a Th1 cell-mediated lung immune response may provide more protection against pulmonary infection (40). Additionally, similar mechanisms may be present in other chronic respiratory diseases, and therefore, we believe these studies will also yield insights to a greater understanding of respiratory disease pathogenesis as a whole. By understanding the cytokine requirements in upper and lower respiratory tract immunity and how they differ, they will facilitate the development of nasal-delivered vaccine strategies that can generated protective immunity along the entire respiratory tract.

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