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NK Cells Interfere with the Generation of Resistance against Mycoplasma Respiratory Infection following Nasal-Pulmonary Immunization

Sheetal Bodhankar,* Mathew D. Woolard,† Xiangle Sun,* and Jerry W. Simecka2*

The purpose of the present study was to determine the impact of NK cells on the development of protective adaptive immunity in response to nasal-pulmonary immunization against mycoplasma. Depletion of NK cells before nasal-pulmonary immunization enhanced resistance to mycoplasma respiratory infection. The effect of NK cells on the generation of protective immunity in lungs was dependent on lymphoid cells, as immunization of either SCID mice or immunocompetent mice depleted of CD4+ T cells did not demonstrate any increased resistance in the presence or absence of NK cells. The presence of NK cells at the time of nasal-pulmonary immunization modulated mycoplasma-specific cytokine responses in lungs and lower respiratory nodes. In particular, NK cells skewed the mycoplasma-specific T cell cytokine responses in the draining lymph nodes to higher IL-4, IL-13, and IL-17 while lowering IFN-γ responses. Adoptive transfer of total lung lymphocytes isolated from immunized mice into naive mice led to a significant reduction in the mycoplasma numbers in lungs, and the resistance was greater if cells were obtained from immunized mice that were depleted of NK cells. Similar results were obtained if purified B cells, T cells, or CD4+ T cells were used. Interestingly, this is the first time that a favorable role of functional CD4+ T cells in mediating protection in mycoplasma respiratory disease was demonstrated. Thus, NK cells can influence the responses of multiple lymphocyte populations capable of mediating resistance to mycoplasma infection. The Journal of Immunology, 2009, 183: 2622–2631.

Mycoplasma infections are a leading cause of respiratory diseases in humans and animals worldwide. Mycoplasma respiratory diseases have a major economic impact in livestock, such as cattle, pigs, and goats (1). In humans, Mycoplasma pneumoniae is a major cause of respiratory disease, accounting for 8–15 million cases of pneumonia every year in the United States alone (2). It is the second leading cause of tracheobronchitis in children and is also associated with the exacerbation of asthma in humans (3, 4). Mycoplasma pulmonis causes a naturally occurring respiratory disease in mice and rats with high morbidity and low mortality (5–7). Mycoplasma pneumoniae causes rhinitis, otitis media, laryngotracheitis, and bronchopneumonia in mice, similar to that caused by M. pneumoniae in humans and mycoplasma infections in other animals. M. pulmonis serves as an excellent animal model for mycoplasma respiratory diseases, including M. pneumoniae infection in humans, allowing the study of host immune responses to these infections. In terms of histopathology, these diseases are characterized by the accumulation of mononuclear cells, macrophages, and lymphocytes along the respiratory airways (8–11). This infiltration suggests that the activation and recruitment of macrophages and lymphocytes are critical in the development of both acute and chronic states of the disease. In fact, several studies have demonstrated that immune responses not only protect from disease, but they can also contribute to the pathology (12–14). This has complicated the development of mycoplasma vaccines. Thus, it is critical to understand the regulatory mechanisms affecting immune responses that contribute to resistance against mycoplasma respiratory diseases to develop optimal approaches for vaccination.

Both adaptive and innate immune mechanisms are involved in determining the outcome of mycoplasma respiratory infection. The initial interactions between mycoplasma and the mediators of innate immunity have a major influence on determining the establishment of infection in the lungs (11, 13, 15). Adaptive immunity clearly contributes to both the pathology in mycoplasma respiratory disease and resistance to infection. The contribution of lymphoid cells, particularly T cell responses, to the severity of inflammatory lesions is supported by studies using SCID mice, T cell-deficient mice, and hamsters (12–14). Furthermore, depletion of Th cells results in less severe lung diseases, demonstrating that Th cell responses can contribute to disease pathology in the lung (16). To the contrary, CD8+ T cells dampen the severity of mycoplasma pulmonary disease. Although lymphoid cell responses can contribute to lesion severity, immune responses along the respiratory tract can also confer resistance to mycoplasma infection (17, 18). Local (nasal-pulmonary) immunization is more effective than systemic routes of immunization against mycoplasma infection (18). Thus, there appears to be a complex balance between beneficial and detrimental adaptive host responses, and an understanding of the mechanisms involved in modulating these responses throughout the respiratory tract is critical for vaccine development and evaluation. Importantly, little is known about how innate immune mechanisms may influence the type and effectiveness of adaptive immunity against mycoplasma infection.
NK cells play a role in the initial phases of mycoplasma infection (19). NK cells are lymphocytes of the innate immune system that are involved in the early defense against foreign cells and autologous cells undergoing various forms of stress, such as microbial infection or tumor transformation (20). NK cells also produce cytokines, such as IFN-γ, TNF-α, and GM-CSF, and chemokines such as CCL3 (MIP1-α), CCL4 (MIP1-β), and CCL5 (RANTES), when stimulated with susceptible target cells. Recent studies in our laboratory demonstrate that IFN-γ is critical in controlling mycoplasma disease early in infection (21). Three days after mycoplasma infection of BALB/c mice, NK cells are the major population in the lungs producing IFN-γ (19). Surprisingly, depletion of NK cells from IFN-γ−/− mice before infection leads to a more effective clearance of mycoplasma from the lung than in NK cell–competent IFN-γ−/− mice; this effect was not seen in NK-depleted wild-type mice. There were also lower levels of inflammatory cytokines and less infiltration of neutrophils in the lungs of the NK-depleted IFN-γ−/− mice, indicating a corresponding decrease in inflammatory disease. These results demonstrate that in the absence of IFN-γ, NK cells can dampen innate immune mechanisms that help control mycoplasma numbers in the lung.

The purpose of the present study was to extend our previous work (19) and determine the impact of NK cells on the development of protective adaptive immunity in response to nasal-pulmonary immunization against mycoplasma. NK cells can regulate adaptive immune responses through the production of Th1-type cytokines early after viral infection (22) or through the activation of dendritic cells (23). The depletion of NK1.1+ cells before immunization with OVA inhibits pulmonary eosinophilic and T cell infiltration, with decreased levels of IL-4, IL-5, and IL-12 in the bronchoalveolar lavage fluid in a murine model of allergic asthma (24). However, the role of NK cells in the generation of adaptive immunity against mycoplasma infection is unknown. Based on our previous studies (19), we hypothesized that NK cells would negatively regulate the generation of protective immunity against mycoplasma infection. Our results demonstrate that depletion of NK cells before nasal-pulmonary immunization led to improved resistance against mycoplasma infection along the respiratory tract. Furthermore, protection rendered in mice depleted of NK cells was due to lymphoid cells. Interestingly, this is the first time that a favorable role of functional CD4+ T cells in mediating protection in mycoplasma respiratory disease was demonstrated. These results indicate that NK cells influence the generation of protective adaptive immunity, which is mediated by CD4+ T cells, and that NK cells may interfere with the development of optimal immunization strategies against mycoplasma infections.

Materials and Methods

Mice
Female BALB/cJ wild-type and SCID (CB6Smm.CB17-Prkd-wd+/ld on a BALB/cBySmm background) mice, tested to be virus- and mycoplasma-free, were obtained from The Jackson Laboratory. Mice were housed in sterile microisolator cages supplied with sterile bedding, with food and water provided ad libitum. Mice used in the study were between 6 and 8 wk of age. Female mice were used in all studies. Before experimental immunization and infection, mice were anesthetized with an i.p. injection of diluted ketamine-xylazine. The animal studies were reviewed and approved by the University of North Texas Health Science Center Institutional Animal Use and Care Committee.

Mycoplasma
The UAB CT strain of M. pulmonis was used in all experiments. Stock cultures were grown, as previously described (25), in mycoplasma pleuropneumonia-like organism medium (Acumedia) and frozen in 1-ml aliquots at −80°C. For inoculation, thawed aliquots were diluted to 2 × 10⁸ CFU/20 μl. Nasal-pulmonary inoculations of 20 μl of diluted mycoplasma were given for experimental infections.

Preparation of M. pulmonis Ag
Crude preparations of M. pulmonis membrane were used for immunization and in vitro stimulation and prepared as previously described (26). 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 of sterile 0.25 M NaCl. Following a second centrifugation at 9000 rpm for 20 min, pellets were resuspended in a total of 4 ml of 2 M glycerol at 37°C. Cells were then sonicated at the highest setting for 15 s using a Vibra cell sonicator (Sonic & Materials/Vibrio Cell), followed by incubation at 37°C for 10 min. For cell lysis, 0.5 ml of the cell preparations were 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 for 1 h. Membranes were resuspended in 5 ml of sterile PBS (HyClone Laboratories) and stored at −80°C. All centrifugations were done at 4°C. Protein concentration was determined using a Bradford protein assay (Bio-Rad).

Immunization of mice
Immunizations were done on days 1 and 7 with M. pulmonis Ag (5 μg) in a 24-μl volume, resulting in nasal-pulmonary deposition of the inoculum (18). Control mice were inoculated with 24 μl of sterile PBS.

NK cell depletion
To deplete mice of NK cells, mice were injected i.p. with 50 μl of anti-asialo ganglio-N-tetraosylceramide (anti-asialo GM1) Ab (Wako). NK cell depletions were done 1 day before each of the nasal-pulmonary immunizations. Staining of splenic and pulmonary lymphocytes with FITC-labeled DX5 mAb (Caltag Laboratories) followed by flow cytometry was used to monitor the depletion of NK cells. Control mice were injected with sterile PBS.

Determination of mycoplasma numbers
The numbers of CFU in the lungs and nasal passages were determined as previously described (25). Briefly, lungs were placed in 1 ml of mycoplasma broth medium and minced. The samples were sonicated (Vibra cell sonicator; Sonic & Materials/Vibrio Cell) for 1 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, colonies were counted and the numbers of CFU recovered from each tissue were calculated.

Lymphocyte isolation
Mononuclear cells were isolated from lungs, as previously described (27). Briefly, 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) containing 300 U/ml Clostridium histolyticum type I collagenase (Worthington Biochemical), 50 U/ml DNase (Sigma-Aldrich), 10% FBS (HyClone Laboratories), and antibiotic/antimycotic solution (Invitrogen). The tissues were incubated at 37°C while mixing on a Nutator (Fisher Scientific), and antibiotic/antimycotic solution (Invitrogen). 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).

Lower respiratory lymph node (LRN) cells were isolated after centrifugation of all suspensions, followed by red cell removal using ammonium chloride potassium lysing buffer, as previously described (28).

Immunofluorescent characterization of lymphocyte populations
Two-color and three-color immunofluorescent staining was performed to identify NK, T, B, monocyte, macrophage, and dendritic cell populations using FITC-labeled anti-mouse NK mAb (DX5; Caltag Laboratories), PE-labeled anti-mouse CD3e mAb (145-2C11; Caltag Laboratories), FITC-labeled anti-mouse CD4 mAb (L3T4, RM4-5; BD Pharmingen), FITC-labeled anti-mouse CD8 mAb (Ly-2, 53-6.7; BD Pharmingen).

3 Abbreviations used in this paper: LRN, lower respiratory lymph node; anti-asialo GM1, anti-asialo ganglio-N-tetraosylceramide.
FITC-labeled anti-mouse y8 T cell receptor (GL3; Caltag Laboratories); PE-Cy7-labeled anti-mouse CD45R mAb (RA3-6B2; BD Pharmingen); PE-Cy7-labeled anti-mouse CD11b mAb (integrin α6 chain, Mac-1e-chain, M1/70; Caltag Laboratories); FITC-labeled anti-mouse F4/80 mAb (BM8; Caltag Laboratories); and PE-labeled anti-CD11c (integrin α2 chain, HL3; BD Pharmingen), respectively. Asialo GM1-expressing cells were identified using rabbit-anti-asialo GM1 IgG (Wako) and allophycocyanin-labeled goat anti-rabbit IgG (H+L) Ab (Caltag Laboratories). Briefly, 5 × 10^6 cells per tube were incubated with purified anti-mouse CD16/CD32 Ab (2.4G2, Fcγ III/II receptor; BD Pharmingen) for 5 min at 4°C to reduce nonspecific binding of FcII/IIIr before fluorescent Ab staining. The cells were incubated for 30 min at 4°C with 200 µl of fluorescent Ab staining. The cells were washed in staining buffer (Mg^2+ -free, Ca^2+ -free PBS with 0.05% sodium azide, 1% FBS) and fixed with 4% paraformaldehyde solution for 30 min; cells were then resuspended in staining buffer and data were acquired using a Beckman Coulter Cytomics FC 500. Data analysis was performed using the CXP analysis software provided by 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, the total number of lymphocytes isolated from each tissue was multiplied by their percentage.

Adoptive transfers of isolated T lymphocyte populations
T cells were purified using paramagnetic bead-conjugated Abs and autoMACS (Miltenyi Biotec) following the manufacturer’s instructions. Pulmonary T cells (negative fraction) were isolated by a Pan T cell isolation kit (Miltenyi Biotec) by depleting non-T cells (positive fraction) using a cocktail biotin-conjugated Ab followed by anti-biotin microbeads. Both positive and negative fractions were collected and used for further adoptive transfer studies. Confirmation of cell purity was determined using flow cytometry. Cell fractions with a depletion of >95% were used for subsequent experimentation.

Fluorescent-activated cell sorting
The purification of pulmonary CD4^+ T cell lymphocytes was performed by cell-sorting techniques. Briefly, total pulmonary lymphocytes were labeled with FITC anti-CD4 (L3T4, RM-45; BD Pharmingen) after blocking the Fc receptors. After labeling, total lung lymphocytes were sorted using an InFlux cell sorter (Cytopeia). CD4^+ T cell populations were collected. Selected cell populations were determined to have a purity of at least 99%, as analyzed using Sigmoid software (Cytopeia).

Whole lung lymphocytes or purified pulmonary T cells and non-T cells were obtained from immunized and NK cell-depleted, immunized mice on day 14 after immunization and were adoptively transferred into naive mice via tail vein injections. Total lung lymphocytes (10^6) or 5 × 10^5 purified T cells or CD4^+ T cells in 100 µl were given to each naive mouse. The naive mice were infected with mycoplasma 1 day after the adoptive transfers.

In vivo depletion of CD4^+ and CD8^+ cells using mAb treatment
Clones, that is, CD4-specific (GK1.5 (L3T4)) and CD8-specific (53-6-72 (LyT3)) mAbs, were obtained (Bio X Cell) and were used to deplete CD4^+ and CD8^+ T cells. To deplete CD4^+ and CD8^+ T cells, 300 µg per 500 µl of Ab was injected i.p. 1 day before each of the nasal-pulmonary immunization or mycoplasma infection (16). In vivo depletion of lymphocyte population was confirmed as ≥98% depletion by examining splenic and pulmonary lymphocyte populations using immunofluorescent staining, followed by flow cytometry. More than 95% of CD4^+ and CD8^+ T cells were depleted 7 days after injection of the respective depleting Abs.

Ag-specific in vitro stimulation of mononuclear cells
LRN and pulmonary lymphoid cells, isolated from control, immunized, and NK cell-depleted immunized mice at day 14 postimmunization, were cultured in 96-well round-bottom microtiter plates in RPMI 1640 (HyClone Laboratories) supplemented with 5% FBS (HyClone Laboratories), HEPES, antibiotic/antimycotic solution (Invitrogen), and 50 µM 2-ME (Invitrogen). Lymphoid cells were cultured at 37°C and 5% CO2. Cells were stimulated with or without 5 µg/ml mycoplasma membrane Ag in a final volume of 200 µl/well of culture medium 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 cytokines were measured by capture ELISA. Murine IFN-γ and IL-4 levels were measured using OptEIA IL-4 and IFN-γ ELISA sets (BD Pharmingen). Easy-wash 96-well flat-bottom microtiter plates (BD Biosciences) were coated overnight at 4°C with 100 µl of mAb specific for either murine IL-4 or IFN-γ diluted in 0.1 M NaOH, pH 9. Plates were washed and blocked with 200 µl PBS/Tween 20 supplemented with 10% FBS for 30 min. Following a PBS/Tween wash, 100 µl of sample supernatant was placed into the appropriate wells and incubated overnight at 4°C. Plates were washed three times with PBS/0.05% Tween 20, and 100 µl of 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 tetramethylbenzidine substrate (Moss) were used. Plates were read using an MX80 plate reader (Dynatech) at an absorbance of 630 nm. One hundred microliters of 0.25 M HCl was also used to read the reaction if needed to increase its sensitivity, 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). Additionally, levels of IL-10, IL-13, and IL-17 were measured using a 5-plex LINCOplex mouse cytokine kit (LINCO Research). IL-10, IL-13, and IL-17 concentrations were assessed in a total of 50 µl of volume of the supernatant sample or standards using Abs for each cytokine covalently immobilized to a set of microspheres, according to the protocol provided in the LINCOplex kit. The cytokines bound to the surface of the microspheres were detected using biotinylated Abs directed against IL-10, IL-13, and IL-17, followed by a streptavidin-PE conjugate. Samples were read using a Bio-Plex 100 system (Bio-Rad). Cytokine levels were determined by comparison with standard curves generated from murine recombinant cytokines provided in the LINCOplex kit and analyzed using Bio-Plex Manager software (Bio-Rad).

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) 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 of ≤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 referred to as the geometric means (x̄/± SE).

Results
Depletion of asialo GM1^+ cells, before immunization, enhances protection from mycoplasma respiratory infection
A previous study from our laboratory (19) demonstrated that NK cells could have deleterious effects on innate immune responses during mycoplasma infection. To determine whether the presence of NK cells affects the generation of mycoplasma-specific protective immunity along the respiratory tract, NK cells were depleted from wild-type BALB/cJ mice before nasal-pulmonary immunization (Fig. 1). Mice were immunized with mycoplasma Ag twice, on day 1 and on day 7; however, NK cells (asialo GM1^+) were depleted in a group of mice by anti-asialo GM1 Ab treatment 1 day before each of the immunizations. Another group of mice was injected with sterile PBS instead of anti-asialo GM1 Ab. All of the mice in each group were infected 7 days after the second immunization with mycoplasma Ag or inoculation with PBS. On days 3, 7, and 14 after mycoplasma infection, lungs and nasal washes were collected from each group of mice, and the numbers of mycoplasma in the upper and lower respiratory tracts were enumerated.

The presence of NK cells had a detrimental effect on the development of mycoplasma protective immunity along the respiratory tract. The depletion of asialo GM1^+ cells, before immunizations, significantly reduced (p ≤ 0.05) the numbers of mycoplasmas recovered from the lungs as compared with immunized mice with intact NK cells (Fig. 2). The numbers of mycoplasma in lungs of the asialo GM1^+ cell-depleted immunized mice were at least 1 log lower than in the immunized mice at all time points. On day 14,
Passages to mycoplasma infection. Fers better protection in lungs and nasal passages of the asialo GM1⁺ cell-depleted immunized group of mice as compared with the control and immunized mice. Although immunization in the absence of NK cells conferred resistance early after infection, the enhanced resistance due to NK cell depletion in nasal passages disappeared at later time points. These results demonstrate that the presence of asialo GM1⁺ cells during the development of mycoplasma-specific protective adaptive immune responses has detrimental effects on the clearance of mycoplasma infection, and this was most consistently found in the lower respiratory tract.

To determine the efficiency of anti-asialo GM1 Ab treatment in depleting NK cells, mice were injected with the Ab, and a day later, pulmonary lymphocytes were isolated to enumerate the NK cells by immunofluorescent staining and flow cytometry analysis. The mice injected with anti-asialo GM1 Ab showed a preferential depletion of NK (DX5⁺CD3⁻) cells. The anti-asialo GM1 Ab treatment did not significantly reduce other cell populations that potentially could be affected, that is, CD4⁺ T cells, CD8⁺ T cells, NKT (DX5⁺CD3⁻) cells, γδ T cells, or dendritic cells (CD11c⁺) (Table I). Also, to ensure the specificity of the anti-asialo GM1 Ab, asialo GM1⁺ cell populations were determined in the lungs and spleens. Most anti-asialo GM1⁺ cells in the lungs and spleens were DX5⁺. However, a small percentage (5%) of asialo GM1⁺ cells were also CD11b⁺. Since NK cells can also express CD11b (29), a three-color stain to enumerate asialo GM1, DX5, and CD11b expression was done. Only 1% of the CD11b⁺ cell population was found to be asialo GM1⁺DX5⁻. Asialo GM1 did not co-stain with F4/80⁺ cells or with GR1⁺ cells, being markers for mature macrophages (30) and neutrophils (31), respectively. Therefore, the enhanced mycoplasma-specific protective immune responses due to asialo GM1 Ab administration most likely were due to depletion of NK cells.

Furthermore, we found that the numbers of splenic and pulmonary NK (DX5⁺) cells in the anti-asialo GM1 Ab-injected mice were restored to levels comparable to the NK cells in mice that were infected and unimmunized mice. However, no significant difference in lesion formation was observed between the asialo GM1⁺ cell-depleted immunized and the immunized mice (data not shown). The loss of asialo GM1⁺ cells, before immunization, also led to the reduction in mycoplasma numbers within the upper respiratory tract. On day 3 after infection, a significant reduction of mycoplasma CFU was found in the nasal passages. The numbers of splenic and pulmonary mycoplasma CFU were determined in the lungs and nasal passages of mycoplasma infection. Mice were immunized with mycoplasma membrane Ag once (day 1) and again 7 days later. Control mice received PBS instead of immunizations. The mice with NK cell depletion without immunizations served as control for the NK cell-depleted immunized mice group. To monitor resistance to infection, mice were infected 7 days after the second immunization with mycoplasma Ag (day 14 after immunization/day 0 after infection). On days 3, 7, and 14 after mycoplasma infection, lungs and nasal washes were collected from each group of mice, and the numbers of mycoplasma CFU in the upper and lower respiratory tracts were enumerated. To evaluate mycoplasma-specific immune responses, including lung lymphocyte purification for adoptive transfers, mice were on day 14 after immunization (uninfected) were used.

**FIGURE 1.** Study design. NK cells were depleted from wild-type BALB/cJ mice, by i.p. injection of rabbit polyclonal anti-asialo GM1 Ab on days 0 and 6, 1 day before each of the nasal-pulmonary immunization. Mice were immunized with mycoplasma membrane Ag once (day 1) and again 7 days later. Control mice received PBS instead of immunizations. The mice with NK cell depletion without immunizations served as control for the NK cell-depleted immunized mice group. To monitor resistance to infection, mice were infected 7 days after the second immunization with mycoplasma Ag (day 14 after immunization/day 0 after infection). On days 3, 7, and 14 after mycoplasma infection, lungs and nasal washes were collected from each group of mice, and the numbers of mycoplasma CFU in the upper and lower respiratory tracts were enumerated. To evaluate mycoplasma-specific immune responses, including lung lymphocyte purification for adoptive transfers, mice were on day 14 after immunization (uninfected) were used.

**FIGURE 2.** Depletion of asialo GM1⁺ cells before immunization confers better protection in lungs and nasal passages to mycoplasma infection. α, NK (DX5⁺) cells in the lungs were confirmed to be depleted 1 day after treatment of mice with anti-asialo GM1 Ab (flow data shown are from one representative experiment). Immunized and control mice were infected with mycoplasma, and 3, 7, and 14 days later, the numbers of mycoplasma CFU were determined in (b) lungs and (c) nasal passages. Control (hatched bar) and NK cell-depleted (gray bar) mice were not immunized. Immunized mice (open bar) were nasal-pulmonary immunized with mycoplasma Ag alone, while NK cell-depleted immunized mice (black bar) were also given anti-asialo GM1 Ab 1 day before each of the immunizations. NK cell numbers recovered in NK cell-depleted animals by the time of infection (day 14). Vertical bars and error bars represent means ± SE (n = 8) from two separate experiments. *, Significant difference (p ≤ 0.05) from PBS-inoculated mice; †, significant difference (p ≤ 0.05) from immunized mice.
were not treated with Ab before mycoplasma infection (data not shown). Thus, the presence of NK cells during the generation of protective immunity is most critical, and their presence during the course of mycoplasma infection did not affect the outcome of the disease.

The effect of NK cells on the generation of protective immunity in lungs is dependent on lymphoid cells

The above studies demonstrate that NK cells dampen the generation of protective immunity. There is a possibility that the protection being rendered was due to an enhanced generation of non-specific immune responses upon immunization. To ascertain whether NK cells modulate protection through lymphocytes of the adaptive immune system, SCID mice, lacking functional T and B lymphocytes, were NK cell depleted and immunized, similar to the experimental protocol in the previous studies, and the mice were infected with mycoplasma 7 days after the second immunization. Three days after mycoplasma infection, the numbers of mycoplasma in the lungs were determined. There were no significant differences in mycoplasma numbers in the lungs of any of the groups of SCID mice (Fig. 3). Thus, in the absence of lymphocytes, NK cell depletion and/or nasal-pulmonary inoculation of mycoplasma Ag did not result in any indication of the generation of resistance to infection. These results demonstrate that the presence of NK cells have a detrimental impact on the cells of the adaptive immunity, which results in an impaired development of mycoplasma-specific resistance.

Cells from lung-draining lymph nodes of NK cell-depleted immunized mice have higher IFN-γ and lower IL-4 levels than do immunized mice

Preliminary studies indicate that NK cells do not significantly affect either the generation of serum Ab responses or the numbers of lymphoid cell populations (NK, T cell populations, and B cells) in the lungs in response to nasal immunization. To determine whether the NK cells influenced pulmonary T cell cytokine responses against mycoplasma Ag, cells from lungs and the lung-draining lymph node (LRN) were isolated from mice 7 days after the second immunization and cultured in the presence or absence of mycoplasma Ag in vitro. Four days later, supernatants were collected, and the levels of IFN-γ, IL-4, IL-10, IL-13, and IL-17 were measured. Lung and lymph node lymphocytes from immunized or NK cell-depleted immunized mice produced significant levels of IFN-γ IL-4, IL-10, IL-13, and IL-17 in response to mycoplasma Ag (Fig. 4), whereas lymphocytes from unimmunized mice did not demonstrate mycoplasma-specific cytokine responses. Lung lymphocytes from NK cell-depleted immunized mice produced lower levels of IFN-γ, IL-4, and IL-17 (p ≤ 0.05). Notably, LRN cells from NK cell-depleted immunized mice produced significantly (p ≤ 0.05) higher levels of IFN-γ in response to mycoplasma Ag stimulation than did cells from immunized mice that were not given anti-asialo GM1 Ab. Conversely, the cells from NK cell-depleted immunized mice produced significantly lower levels of IL-4, IL-13, and IL-17 (p ≤ 0.05) than did their immunized counterparts. There was no effect on the levels of IL-10 produced in response to mycoplasma Ag stimulation (data not shown). These results indicate that the presence of NK cells during the generation of protective immunity, due to nasal-pulmonary immunization, modulates immune responses in lungs and LRNs. In particular, NK cells skewed the mycoplasma-specific T cell cytokine responses in the draining lymph nodes to higher IFN-γ and lower IL-4, IL-13, and IL-17 levels.

CD4+ T cells are critical both in the generation of protective immunity and during the effector phase of mycoplasma infection in mice depleted of NK cells

As demonstrated above, the effect of NK cells on the generation of protective immunity in lungs is dependent on lymphoid cells. Previous studies (16) have demonstrated that T cells play a significant role in regulating the severity of disease without affecting mycoplasma numbers. These studies demonstrated that CD4+ T cells promote inflammatory disease due to mycoplasma infection, whereas CD8+ T cells dampen disease severity. To determine the relative contributions of CD4+ and CD8+ T lymphocyte populations in generating protective immunity and to characterize which cell population was affected by NK cells during the development resistance to infection, mice were depleted of CD4+ and/or CD8+ lymphocytes a day before immunization (i.e., days 0 and 6). Mice were infected with M. pulmonis 7 days after the second immunization. Three days after infection the numbers of mycoplasma in the lungs were determined.

The depletion of CD4+ T cells in both groups of immunized mice, irrespective of NK cell status, resulted in the recovery of more mycoplasma from lungs (Fig. 5, a and b). Although CD8+ T cells contributed to the response with about a 1 log increase in mycoplasma, depletion of CD4+ T cells had the greatest effect on

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<th>Table 1. Immunofluorescent characterization of pulmonary cell populations isolated from mice after anti-asialo GM1 Ab treatmenta</th>
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<tr>
<td><strong>Cell Type</strong></td>
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<td>DX5+CD3+ (NK cells)</td>
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a Mice were given PBS or anti-asialo GM1 Ab i.p., and lung cells from five mice were isolated 1 day later and pooled. Cells were immunofluorescently stained and analyzed by flow cytometry. The data shown are from one experiment, where all staining combinations were done; however, the presence or lack of depletion was shown reproducible for each of individual cell populations in at least one additional experiment.
The resistance found in NK cell-depleted immunized mice, as there was about a 3 log increase in mycoplasma numbers. In contrast, there was less than a log increase in the number of mycoplasma recovered from immunized mice depleted of T cell populations. These results clearly indicate that CD4+ T cells are most critical in the development of protective immunity in mice depleted of NK cells.

In additional experiments, we examined the contribution of the CD4+ T cell population during the effector phase of adaptive resistance after mycoplasma infection. One day before mycoplasma infection, immunized and NK cell-depleted immunized mice were treated with Ab to in vivo depleted CD4+ T cells, and 3 days later, mycoplasma CFU in lungs were determined. As shown, the absence of primed CD4+ T cells during the period of mycoplasma infection resulted in both the immunized and the NK-depleted and immunized groups to lose the protective immunity generated (Fig. 5c). Although depletion of CD4+ T cells from the immunized group led to an almost 1 log increase in the CFU numbers recovered from the lungs, this effect was more pronounced in the NK cell-depleted and immunized group depleted of CD4+ T cells (almost 2 log higher CFU in the lungs). These results clearly indicate that CD4+ T cells are essential, not only during the development of protective immunity, but also in mediating resistance to mycoplasma infection in immunized mice depleted of NK cells.

Both T and non-T lymphocytes contribute to the increased resistance to mycoplasma infection in NK cell-depleted and immunized mice

To further identify the lymphoid cells involved in the improved protection against mycoplasma infection in the NK cell-depleted immunized mice, pulmonary lymphocytes were isolated from untreated and anti-asialo GM1 Ab-treated mice 7 days after the second immunization, and these cells were adoptively transferred into naive mice via tail vein injections. The mice were infected with mycoplasma a day after the adoptive transfer, and the mycoplasma numbers in the lungs and nasal washes were enumerated 14 days postinfection.

Adoptive transfer of total lung lymphocytes isolated from NK cell-depleted immunized mice into naive mice led to a significant reduction (p ≤ 0.05) in the mycoplasma CFU numbers in the lungs as compared with mice that received lymphocytes from the control mice, as well as in those from immunized mice. The mice that received pulmonary lymphocytes from NK cell-depleted immunized mice had almost a 3 log reduction in mycoplasma CFU numbers in the lungs as compared with the controls, whereas the adoptive transfer of lymphocytes from immunized, but Ab untreated, mice resulted in only about a 1 log reduction in mycoplasma numbers in lungs (Fig. 6). Although immunization conferred resistance, there was no significant effect of NK cell depletion at later time points in nasal passages. Thus, these results demonstrate that the increased protection in lungs rendered in the NK cell-depleted immunized mice can be adoptively transferred using pulmonary lymphocytes.

To determine whether T cells, non-T cells, or both contribute to the increased protection resulting from immunization in the absence of NK cells, pulmonary T and non-T cells were purified by paramagnetic bead selection from immunized and NK cell-depleted immunized mice and were adoptively transferred to naive mice, which were subsequently infected, as described above. The results show that adoptive transfer of purified pulmonary T lymphocytes from NK cell-depleted immunized mice led to significantly (p ≤ 0.05) lower numbers of mycoplasma (1 log) recovered from the lungs of recipient mice as compared with those that received T lymphocytes from immunized mice (Fig. 7). Non-T lymphocytes from the lungs of NK cell-depleted and immunized mice also showed improved protection in the recipient mice (0.6 log lower CFU). Thus, both T and non-T lymphocyte (>90% B cells) populations contributed to the increased protection against mycoplasma infection in NK cell-depleted immunized mice.

CD4+ T lymphocytes from the NK cell-depleted immunized mice contribute to protection against mycoplasma respiratory infection

To determine whether CD4+ T cells alone can mediate resistance to infection, pulmonary CD4+ T cells were fluorescent cell-sorted from immunized mice and NK cell-depleted immunized mice 7 days after the second immunization. Purified CD4+ T cells from each of the groups of mice were adoptively transferred, via tail vein injections, into naive mice. A group of naive mice receiving PBS via tail vein injection served as control. All of the recipient mice were infected with M. pulmonis 1 day after the adoptive transfers. Fourteen days after the infection, the numbers of mycoplasma in the lungs and nasal passages were determined.
CD4+ T cells from either group of immunized mice promote resistance to mycoplasma infection from both lungs and nasal passages, as compared with control mice (Fig. 8). Adoptive transfer of CD4+ T cells from NK cell-depleted immunized mice resulted in about a 1 log decrease in the numbers of mycoplasma recovered from lungs of infected mice ($p \leq 0.05$). In the nasal passages, there was a lower, but not statistically, significant reduction in the numbers of mycoplasmas found. Thus, pulmonary CD4+ T cells directly contributed to protection against mycoplasma infection, and the results indicate that CD4+ T cells, primed in an NK cell-deficient environment, were most effective.

**Discussion**

The lack of efficient vaccines against mycoplasma infection necessitates understanding of the regulatory mechanisms affecting the adaptive immune responses, thereby aiding in the development of optimal approaches for vaccination. Adverse immunopathologic reactions, characterized by pulmonary airway inflammation (18, 32, 33), after intranasal immunization are a major concern in the development of effective vaccines against mycoplasma respiratory diseases. However, the immune mechanisms involved are not fully understood. The purpose of the present study was to determine the contribution of NK cells in the generation of protective adaptive immunity against mycoplasma infection upon nasal-pulmonary immunization. Although NK cells are the primary effector cells of the innate immune system (34), they are also able to influence the adaptive immune responses (35–41). Previous studies in our laboratory (19) demonstrated that the pulmonary NK cells can suppress innate immune responses generated against mycoplasma respiratory infection; however, the contribution of NK cells in the development of adaptive immune responses against any mycoplasma had not been examined. Similar to what we found in innate

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** CD4+ T cells are critical both in generation of protective immunity and during the effector phase of mycoplasma infection in mice depleted of NK cells. NK cell depletions were done using rabbit anti-asialo GM1 Ab 1 day before each of the immunizations. Mycoplasma membrane Ag was used for immunizations on days 1 and 7. To examine the role of T cells in the generation of protective immunity, CD4+ and/or CD8+ T cells were depleted 1 day before the immunizations on days 1 and 7 from (a) immunized and (b) NK cell-depleted and immunized mice. Control mice received PBS instead of immunizations. On day 14, following the in vivo cell depletions and immunizations, all mice were challenged with *M. pulmonis*. The numbers of mycoplasma CFU in lungs were determined 3 days later. Vertical bars and error bars represent means $\times \sqrt{N}$ SE ($n = 8$) from two separate experiments. * Significant difference ($p \leq 0.05$) from PBS inoculated mice; **, significant difference ($p \leq 0.05$) from immunized mice with no CD4+ and CD8+ T cell depletions; and ■, significant difference from CD8+ T cell-depleted mice. To examine the role of primed Th cells to mediate protective immunity, CD4+ T cells were depleted 1 day prior mycoplasma infection of (c) immunized and NK cell-depleted immunized mice. The numbers of mycoplasma CFU in lungs were similarly determined 3 days later. * Significant difference ($p \leq 0.05$) from immunized mice; **, significant difference ($p \leq 0.05$) from NK-depleted and immunized mice with no CD4+ T cell depletions. Vertical bars and error bars represent means $\times \sqrt{N}$ SE ($n = 8$) from two separate experiments.

![FIGURE 6](http://www.jimmunol.org/)

**FIGURE 6.** Total lung lymphocytes from NK cell-depleted immunized mice are capable of rendering increased protection upon being adoptively transferred in recipient mice. On day 14, following the NK cell depletions and immunizations, total lung lymphocytes were isolated from all groups of mice and adoptively transferred into wild-type naive mice via tail vein injections. The recipient mice were challenged with *M. pulmonis* 1 day later. Lungs and nasal washes were obtained 14 days after infection. Vertical bars and error bars represent means $\times \sqrt{N}$ SE ($n = 8$) from two separate experiments. * Significant difference ($p \leq 0.05$) from PBS inoculated mice; **, significant difference ($p \leq 0.05$) from immunized mice.

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** Both T and non-T pulmonary lymphocytes contribute to the increased resistance to mycoplasma infection in the NK cell-depleted immunized mice. On day 14, following the NK cell depletions and immunizations, T and non-T cells from lungs were isolated from immunized and NK cell-depleted immunized mice and adoptively transferred into wild-type naive mice. The recipient mice were challenged with *M. pulmonis* 1 day later. Fourteen days later, mycoplasma numbers in lungs and nasal washes were determined. Vertical bars and error bars represent means $\times \sqrt{N}$ SE ($n = 7$) from two separate experiments. * Significant difference ($p \leq 0.05$) from purified lung lymphocytes transferred from immunized mice; **, significant difference ($p \leq 0.05$) between immunized mice; and ■, significant difference ($p \leq 0.05$) between NK cell-depleted immunized mice.
tracts.
sistance to the organism in both the upper and lower respiratory
coplasma is most likely responsible for the less than optimal re-
during the development of adaptive immune responses against my-
at the time respiratory infection. Thus, the presence of NK cells
administered anti-asialo GM1 Ab, were restored to normal levels
tantly, NK cell numbers, both in the lungs and spleens of the mice
ated immunized mice had almost 2 log lower numbers of myco-
immunity (19), we hypothesized that NK cells would modulate,
and perhaps play a detrimental role, in generating mycoplasma-
specific adaptive immune responses that resist infection.
The depletion of NK cells before mycoplasma-specific immu-
nizations indeed led to a significantly better clearance of myco-
plasma both from the upper and lower respiratory tracts as early as
3 days postmycoplasma infection. In the lungs, the NK cell-de-
pleted immunized mice had almost 2 log lower numbers of my-
coplasma as compared with the immunized mice at day 14 after
fection. In the nasal passages, however, the effect of NK cells
was not as pronounced, particularly on days 7 and 14 when there
were no significant differences between the two immunized
groups. This difference in immune responses between the upper
and lower respiratory tract is consistent with previous studies (18,
33). We were unable to detect an effect on any other innate cell
population due to administration of anti-asialo GM1 Ab, which is
widely used to deplete the functional and phenotypic NK cell ac-
activity in vivo (42, 43). In support, almost all of the asialo GM1−
cells were found to be DX5− cells (a NK cell marker). Impor-
tantly, NK cell numbers, both in the lungs and spleens of the mice
administered anti-asialo GM1 Ab, were restored to normal levels
at the time of infection with viable mycoplasma. This indicates that
the critical role of NK cells in modulating the adaptive responses
to mycoplasma is limited only to the immunization phase and not
at the time respiratory infection. Thus, the presence of NK cells
during the development of adaptive immune responses against my-
coplasma is most likely responsible for the less than optimal resis-
tance to the organism in both the upper and lower respiratory
tracts.
The effect of NK cells on the generation of resistance to infec-
tion was dependent on lymphoid cells. It is possible that inocula-
tion of mycoplasma Ag nonspecifically activated innate immune
responses, resulting in resistance to infection independent of lymph-
ocyte responses. However, nasal pulmonary inoculation of SCID mice with mycoplasma Ag, irrespective of NK cell deple-
tion, did not affect resistance to mycoplasma, indicating that NK
cells indeed impaired the generation of mycoplasma-specific re-
sistance through their effects on adaptive immune responses. This
was further supported by the elimination of resistance when CD4+T
cells were depleted in immunocompetent mice during immuni-
ization, regardless of whether NK cells were present. Interestingly,
depletion of CD8+ T cells did not have a major effect on gener-
ation of resistance. The literature indicates (44) that the early ap-
ppearance of NK cells at the site of immunization implicates their
critical role in the development of adaptive immunity. Also, acti-

CD4+ T cells from the NK cell-depleted immunized mice
mediate protection against mycoplasma respiratory infection. On
day 14, following the NK cell depletions and immunizations, CD4+
lymphocytes from lungs of immunized (open bars) and NK cell-depleted immunized
mice (filled bar) were obtained and adoptively transferred into wild-type
naive mice. The recipient mice were challenged with M. pulmonis 1 day later.
Control mice (hatched bars) were included that were not given cells but were
infected. Fourteen days later, mycoplasma numbers in lungs and nasal washes
were determined. Vertical bars and error bars represent means ± SE (n =
9) from two separate experiments. *, Significant difference (p ≤ 0.05) from
PBS-inoculated mice (mice not given adoptive transfers); ♦, significant dif-
ference (p ≤ 0.05) from immunized mice.

FIGURE 8. CD4+ T cells from the NK cell-depleted immunized mice
mediate protection against mycoplasma respiratory infection. On day 14,
following the NK cell depletions and immunizations, CD4+ T lymphocytes
from lungs of immunized (open bars) and NK cell-depleted immunized
mice (filled bar) were obtained and adoptively transferred into wild-type
naive mice. The recipient mice were challenged with M. pulmonis 1 day later.
Control mice (hatched bars) were included that were not given cells but were
infected. Fourteen days later, mycoplasma numbers in lungs and nasal washes
were determined. Vertical bars and error bars represent means ± SE (n =
9) from two separate experiments. *, Significant difference (p ≤ 0.05) from
PBS-inoculated mice (mice not given adoptive transfers); ♦, significant dif-
ference (p ≤ 0.05) from immunized mice.
that resistance to infection can be mediated through multiple cell types, with either immune B or T cells having the capacity to provide protection against infection. Furthermore, NK cells dampened the capabilities of each of these cell types to respond optimally against mycoplasma infection. Mycoplasma-specific serum Ab titers and experiments using passive immunization with serum (data not shown) from immunized mice did not demonstrate an effect of NK cells on systemic B cell responses. This may indicate that local Ab responses may be more affected by NK cell activity, and, in support, preliminary studies do indicate that mycoplasma-specific IgA responses in lungs and LRNs, as measured by ELISPOT assays, are ~50% higher in immunized mice that were NK cell depleted. Consistent with our results using adoptive transfer of T cells, NK cells modulated the mycoplasma-specific T cell cytokine responses in both lungs and LRNs, as discussed above. The depletion of immune Th cells, before infection, severely curtailed protection due to immunization. Furthermore, the adoptive transfer of pulmonary CD4+ T cells alone from immunized mice also promoted resistance to infection, and immune CD4+ T cells derived from lungs of NK cell-depleted mice were significantly better in doing this. Thus, these data suggest that NK cells can shape the responses of multiple lymphocyte populations capable of mediating resistance to mycoplasma infection. Further studies, however, are needed to determine whether the effects of NK cells are directly or indirectly on these cells, but previous studies (39, 49) demonstrate that NK cells, in some cases, can directly influence maturation of dendritic cells or Th cell responses, which could affect both B and T cell responses.

In summary, NK cells can impair the success of nasal-pulmonary immunization against mycoplasma infection. Mycoplasmas cause persistent infections characterized by chronic inflammatory lesions. Development of mycoplasma vaccines are problematic, often exhibiting only variable success and, in some cases, more severe immunoreactivity (50). To our knowledge, this is the first study to examine the critical role played by the NK cells during the development of protective adaptive immunity in a mycoplasma infection. Significantly, local immunization of the respiratory tract is most effective in protecting against mycoplasma infection (18), and the recognition that an innate cell population, the NK cells, can modulate resistance mediated by T and B cells provides insight to mechanisms through which vaccine approaches can be optimized. Furthermore, previous work from our laboratory (16) demonstrates that CD4+ T cells contribute to disease pathology in the lungs of mycoplasma-infected mice. However, in the present study, for the first time, we show that purified pulmonary CD4+ T cells alone from immunized mice can confer resistance to mycoplasma infection. These results support our earlier hypothesis that different Th cell populations are involved in determining the balance between beneficial (protective) and detrimental (immunopathologic) host responses resulting from mycoplasma infection (11, 51). The present studies suggest that NK cells are one of the factors that influence this balance, and that optimal approaches for vaccination may require minimizing the impact of NK cells on Th cell responses during the generation of protective adaptive immunity.

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Disclosures

The authors have no financial conflicts of interest.

References


