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NK Cells in Gamma-Interferon-Deficient Mice Suppress Lung Innate Immunity against *Mycoplasma* spp.

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The purpose of this study was to examine the 100-fold difference in mycoplasma levels in lungs of gamma interferon knockout (IFN-$\gamma^{-/-}$) mice compared to those seen with wild-type BALB/c mice at 3 days postinfection. NK cells secreted IFN-γ; however, their cytotoxic granule extracts failed to kill mycoplasma. We found a conundrum: the clearance of organisms was as effective in NK-depleted IFN-$\gamma^{-/-}$ animals as in wild-type mice (with both IFN-γ and NK cells). NK^+ IFN-$\gamma^{-/-}$ animals had high mycoplasma burdens, but, after NK-like cell depletion, mycoplasma numbers were controlled. Essentially, IFN-γ was important in animals with NK-like cells and unimportant in animals without NK cells, suggesting that IFN-γ counters deleterious effects of NK-like cells. Impairment of innate immunity in IFN-$\gamma^{-/-}$ mice was not due to NK-like cell killing of macrophages. The increased levels of inflammatory cytokines and neutrophils in lung fluids of NK^+ IFN-$\gamma^{-/-}$ mice were reduced after NK cell depletion. In summary, in the murine model that resembles chronic human disease, innate immunity to mycoplasma requires IFN-γ when there are NK-like cells and the positive effects of IFN-γ counteract negative effects of NK-like cells. When imbalanced, NK-like cells promote disease. Thus, it was not the lack of IFN-γ but the presence of a previously unrecognized NK-like cell-suppressive activity that contributed to the higher mycoplasma numbers. It appears that pulmonary NK cells may contribute to the immunosuppressive environment of the lung, but when needed, these dampening effects can be counterbalanced by IFN-γ. Furthermore, there may be instances where perturbation of this regulatory balance contributes to the susceptibility to and severity of disease.

Mycoplasma infection is a leading cause of pneumonia worldwide. In the United States alone, *Mycoplasma pneumoniae* accounts for 30% of all cases of pneumonia (18, 19, 40). Mycoplasma disease is also associated with the exacerbation of other respiratory diseases, such as asthma (20, 46). *Mycoplasma pulmonis* causes a naturally occurring murine respiratory disease with high morbidity and low mortality. *M. pulmonis* infection is an excellent animal model for human infection with *M. pneumoniae*, allowing the characterization of immune responses against mycoplasma during the pathogenesis of mycoplasma respiratory disease in its natural host. Both *M. pulmonis* and *M. pneumoniae* respiratory infections cause rhinitis, otitis media, laryngotracheitis, and bronchopneumonia. In terms of histopathology, both diseases are characterized by the accumulation of mononuclear cells along the respiratory airways (6, 7, 9, 18, 76). This infiltrate suggests that activation and recruitment of inflammatory cells are key in the development of both acute and chronic disease. Furthermore, several studies demonstrate that a component of mycoplasma respiratory disease is immunopathologic (5, 16, 38, 47, 70). Mycoplasma infections persist despite host immune responses. Both innate and adaptive immune mechanisms critically affect the level of infection and the progression of disease (65).

Innate immunity is critical in the early stages of mycoplasma infection, reducing the growth of mycoplasma in the lungs and improving resistance to mycoplasma lung disease. Strains of mice that differ in susceptibility to pulmonary disease differ in their ability to clear mycoplasma infection (11, 27, 56, 57), and these differences in mycoplasma clearance occur in the absence of adaptive immunity (5). Alveolar macrophages can effectively control mycoplasma growth in vitro (12, 26, 33–36), and when pulmonary macrophage function is impaired in vivo, this impairment increases disease severity and leads to chronic inflammatory disease (25, 27). Macrophages can kill mycoplasma in vitro in the presence of opsonins (e.g., surfactant protein A or immunoglobulin G antibody) (12, 25, 33). Alveolar macrophages may also require in vitro activation by interferon gamma (IFN-γ) to kill opsonized mycoplasma (25). In support of an in vivo role for IFN-γ, we found that *M. pulmonis* infection of IFN-γ knockout (KO) (IFN-$\gamma^{-/-}$) mice results a 2-log-higher CFU count at day 3 postinfection than that seen with wild-type mice (75). This IFN-γ-dependent effect occurs before significant development of adaptive immune responses (32, 75), supporting the in vivo importance of IFN-γ in anti-mycoplasma innate immune responses.

IFN-γ is, however, a pleiotropic cytokine that has a very intricate and complex role in the development of both innate and adaptive immune responses (61, 62). IFN-γ activity in innate immune responses promotes robust inflammation that is capable of clearing many bacterial infections. Early release of IFN-γ plays an important role in the early development of

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this inflammatory response. Several studies demonstrate that the loss of IFN-γ leads to more-severe disease (50, 53) in response to numerous pathogens. IFN-γ activates macrophages and directs Th1 development and antibody class switching (1, 8, 49). Macrophages and IFN-γ are clearly key components in innate immunity against mycoplasma infection, but the role of IFN-γ in modulating mycoplasma respiratory disease needs to be further investigated.

The purpose of this study was to determine the source of IFN-γ and its function within innate immune responses to pulmonary mycoplasma infection. Previous studies (43) suggested that NK cells are the important early source of IFN-γ during mycoplasma disease and are beneficial in clearance of mycoplasma organisms from the lung, but these studies left many unanswered questions. On the basis of previous literature (3, 15, 43, 45, 50, 53), we initially hypothesized that NK cells produced IFN-γ and that this IFN-γ was critical for clearance of mycoplasma from the lung. In contrast to our expectations, after depletion of NK cells, wild-type mice still cleared mycoplasma. An even more unexpected finding was that, upon depletion of NK-like cells from IFN-$\gamma^{-/-}$ mice, the mice cleared mycoplasma as efficiently as fully constituted wild-type mice. Essentially, IFN-γ was important in animals with NK-like cells and unimportant in animals without NK-like cells during initial containment of mycoplasma growth. Our results also suggest a novel disease-promoting role for NK cells of the lung during mycoplasma disease and that IFN-γ is critical for counterbalancing this activity. Once the IFN-γ counteracts the deleterious NK cell effects, other innate immune responses are able to control mycoplasma infection in the first days after infection.

MATERIALS AND METHODS

Mice. BALB/c wild-type and IFN-γ knockout (IFN-$\gamma^{-/-}$) [C.129S7(B6)-ifng^{tm1Ts} on a BALB/c background] mice, tested to be virus and mycoplasma free, were obtained from The Jackson Laboratories (Bar Harbor, ME) (10). Mice were housed in sterile microisolator cages supplied with sterile bedding, food, and water given ad libitum. Mice used in the study were between 8 and 12 weeks of age. Female mice were used in all studies. Before experimental infection, mice were anesthetized with an intramuscular injection of ketamine-xylazine.

Mycoplasma. The UAB CT strain of *M. pulmonis* was used in all experiments. Stock cultures were grown, as previously described (61), 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.

BAL. At time of harvest, mice were euthanized with ketamine-xyalzine. The tracheas were exposed, and intravenous catheters (Becton Dickinson, Sandy, Utah) were inserted. For bronchoalveolar lavage (BAL), 1 ml of phosphate-buffered saline (PBS) without magnesium or calcium was infused into the lungs of each mouse and then removed for analyses. BAL suspensions were spun at 200 × *g* for 10 min to pellet BAL cells. Supernatants (BAL fluids) were removed and placed into new tubes and frozen at −80°C for later analyses. Cells were placed into Ultraspec-II RNA isolation buffer (Biotecx Laboratories, Inc., Houston, TX) to extract total RNA, labeled with fluorescent antibodies, or stained with modified Wright-Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL) for cell characterization. Corresponding lungs, devoid of the BAL cells, were either placed into Ultraspec-II RNA isolation buffer or made into cell suspensions so that the lung lymphocytes could be characterized by flow cytometry. Differential counts on 300 cells were performed by light microscopy, using a single-blind method after application of a modified Wright-Giemsa stain.

Lung cell isolation. Mononuclear cells were isolated from lungs, as previously described (22, 54, 66). Perfusion of the lungs with PBS without magnesium or calcium minimized contamination with blood cells. 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% fetal bovine serum (HyClone Laboratories), HEPES, and antibiotic-antimycotic solution (Life Technologies, Grand Island, NY). The tissues were incubated at 37°C while being mixed on a Nutator apparatus (Fisher Scientific, Pittsburgh, PA) for 90 to 120 min. During the incubation period, the tissues were vigorously pipetted every 30 min. After incubation, the digestion mixtures were passed through a 250-μm nylon mesh to remove undigested tissue. Mononuclear cells were purified from the cell suspensions by density gradient centrifugation using Lympholyte M (Accurate Chemicals, Westbury, NY) (42).

Cell characterization by flow cytometry. Three-color immunofluorescent staining was performed to identify T cells, NK T cells, NK cells, and intracellular IFN-γ by use of phycoerythrin (PE)-CY7-labeled anti-murine CD3 monoclonal antibody (Caltag, Burlingame, CA), biotin-labeled anti-murine DX5 monoclonal antibody (Caltag), PE-TC (TriColor)-conjugated streptavidin (Caltag), and PE-labeled anti-murine IFN-γ monoclonal antibody (Caltag). Briefly, 10^6 cells per tube were incubated with Fc block (Caltag) for 30 min at 4°C. The cells were washed in staining buffer (Mg^{2+}-free, Ca^{2+}-free PBS with 0.05% sodium azide–1% fetal bovine serum [HyClone Laboratories]). Cells were then stained with a 100 μl cocktail of fluorescent antibody for NK and T cells (2 μg/ml) for 30 min at 4°C. The cells were washed in staining buffer. Cells were then incubated with streptavidin PE-TC (1 μg/ml) for 30 min at 4°C. The cells were washed again in staining buffer and then fixed and permeabilized using a Caltag Fix and Perm kit and stained for intracellular IFN-γ per the Caltag instructions. After fixation, cells were resuspended in staining buffer for analysis.

Two-color immunofluorescent staining was performed to identify apoptotic macrophage populations by use of PE-labeled anti-murine F4/80 monoclonal antibody (Caltag) and fluorescein isothiocyanate-VAD-FMK (Promega, Madison, WI). Briefly, 10^6 cells per tube were incubated for 30 min at 4°C in 100 μl of fluorescent antibody (2 μg/ml). The cells were washed in staining buffer and fixed with 2% paraformaldehyde solution 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, Fullerton, CA). 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 each cell population within a tissue. The total number of cells isolated from the tissue was multiplied their percentage.

RNA isolation from BAL cells and lungs. Total RNA was isolated from lungs and BAL cells of mice by use of the Ultraspec-II RNA isolation system. Briefly, BAL cells were isolated; corresponding lungs were homogenized using a Pro 200 homogenizer (Pro Scientific, Monroe, CT); and both samples were placed in the Ultraspec-II RNA reagent. 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 for each sample was washed twice with 75% ethanol by vortexing and subsequent centrifugation for 30 min at 12,000 × *g* and then resuspended in diethylpyrocarbonate-treated water. The concentration and quality of RNA in each sample was 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 quantitative real-time reverse transcription-PCR (RT-PCR). RNA from either BAL cells or corresponding whole lungs was transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Branchburg, NJ) to generate cDNA. cDNA was subjected to real-time PCR using a Cepheid Real Time Smart Cycler I system (Cepheid, Kingwood, TX) with a TaqMan Universal PCR Master Mix kit (Applied Biosystems). Amplification conditions were a single cycle at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Probes and primers for interleukin-4 (IL-4), IL-10, IL-12, IFN-γ, tumor necrosis factor alpha (TNF-α), and GAPDH were purchased from Biosource (Camarillo, CA). Relative quantification of cytokine mRNA expression was determined by comparing cycle threshold values after normalization with the data obtained for GAPDH housekeeping gene expression. The data are expressed as severalfold differences with average baseline values of samples from uninfected (control) mice.

NK cell granule preparation. Granules from NK cells were isolated as previously described (73). The granules (74) were obtained from RNK-16 NK leukemia cells (23) grown as an ascites line in F344 rats obtained from the U.S. National Cancer Institute. The ascites cells were washed with Borregard's relaxation buffer (4) and then ruptured using a nitrogen cavitation bomb (Parr Instrument Co., Moline, IL) pressurized to 450 lb/in^2. The lysate was layered over 54% Percoll (Sigma Chemical Co.), which formed a gradient during a 20 min of

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centrifugation at 45,000 × *g* in a Beckman Ti50.2 rotor at 4°C. The dense fractions (up to 1.068 g·cm^{-3}) were pooled. Nuclei were removed by filtration through a 3-μm Nuclepore filter (Millipore, Bedford, MA) (23). Percoll was then removed by a high-speed spin for 4 h at 145,000 × *g*. Granules were collected from above the Percoll pellet, disrupted by three freeze-thaw cycles after NaCl was added to bring the salt concentration up to 1 M (4), and stored at −20°C. Protein concentrations were determined by a bicinchoninic assay (Pierce, Rockford, IL) using bovine serum albumin for calibration.

NK cell granule cytotoxicity and mycoplasma bactericidal assays. Cytolytic assays were done as previously described (73). Cytolytic activity was determined by the hemoglobin released from lysed red blood cells (RBC) (23, 29). Highly hemolytic concentrations of perforin-containing granule extracts were incubated with mycoplasma for 20 min at 37°C, and then the surviving numbers of mycoplasma were determined by the CFU assay. In addition, we determined whether mycoplasma could inactivate the perforin hemolytic activity. NK cell granules and various concentrations of mycoplasma were incubated with 0.5% (vol/vol) RBC at room temperature for 2 min in 0.2 ml round-bottom microtiter plates (Falcon 3910; Becton Dickinson Labware, Lincoln Park, NJ). The assay buffered contained 10 mM HEPES, 0.15 M NaCl, and 10 μg/ml bovine serum albumin (Sigma A4503), pH 7.5, with the addition of calcium to 1 mM during incubation to start lysis (23). The reaction was halted by acidification with pH 6.0 2-[*N*-morpholino]ethane-sulfonic acid (MES; Sigma M-8250) (29). The microtiter plates were spun at 1,500 × *g* for 10 min. The cell-free supernatants transferred to a second microtiter plate, and the released hemoglobin was detected with an MX80 microplate (Dynatech, Chantilly, VA) reader at 412 nm. Percent lysis was calculated as follows:

$$\%\ \text{lysis} = \frac{\%\ \text{experimental hemolysis} - \%\ \text{spontaneous hemolysis}}{\%\ \text{maximal hemolysis} - \%\ \text{spontaneous hemolysis}} \times 100$$

Addition of 0.01% saponin (Sigma Chemical Co.) produced maximal hemolysis. In addition, the number of mycoplasma was determined by CFU determination.

NK cell depletion. To deplete mice of NK cells, mice were given an intraperitoneal injection of anti-asialo GM-1 antibody (Wako, Osaka, Japan) (100 μg/100 μl) on day −1 of infection. Depletion was confirmed by staining of splenic and pulmonary lymphocytes with biotinylated anti-DX5 (NK marker) antibody (Caltag), followed by incubation with PE-TR-conjugated streptavidin (Caltag), and analyzed using an EPICS XL-MCL flow cytometer (Beckman Coulter). More than 95% of the NK cells were still depleted 3 days after mycoplasma infection. We also confirmed that these antibodies did not react with *M. pulmonis*. Lymphocytes were collected from the lungs of anti-asialo GM1 antibody-treated mice, and cells were labeled with fluorescently tagged antibodies for T cells and NK cells. Only the NK ($DX5^+$ $CD3^-$) cell fraction was consistently depleted, while the numbers of T cells were unchanged (data not shown). NK T cells were reduced in some mice to up to 50% of the numbers seen with untreated mice; however, depletion of NK T cells was not consistent, whereas NK cell depletion was reliable. Therefore, the effects due to anti-asialo GM-1 antibody treatment were most likely due to the loss of NK cells and not due to the presence of NK T cells.

Mycoplasma numbers. The numbers of mycoplasma CFU in lungs and nasal passages were determined as previously described (27a, 75). Briefly, lungs were minced and placed in mycoplasma broth medium. The samples were sonicated (Vibra cell sonicator; Sonics & Materials/Vibro Cell, Newtown, CT) 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 CFU recovered from each tissue was calculated.

Cytokine assays. Levels of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40, IL-12 p70, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, granulocyte colony-stimulating factor(G-CSF), TNF-α, KC, macrophage inflammatory protein 1α (MIP-1α), and RANTES were measured with a Bio-Plex 18-plex cytokine panel (Bio-Rad, Hercules, CA). Microtiter 96-well filter bottom plates were used. To each well, 50 μl of anti-cytokine beads in assay diluent was added. After each step, plates were washed with Bio-Plex washing buffer. A 50-μl volume of sample or standard was added per well. Plates were incubated at room temperature while being shaken in the dark for 30 min. A total of 25 μl of biotinylated secondary antibodies was added to each well. Plates were incubated at room temperature while being shaken in the dark for 30 min. A 50-μl volume of streptavidin-PE was added to each well. Plates were incubated at room temperature while being shaken in the dark for 30 min. 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 and analyzed using Bio-Plex Manager software (Bio-Rad).

Statistical analysis. Data were evaluated by analysis of variance, followed by Fisher protected least-square differences multigroup comparison. A simple regression was used to correlate KC and neutrophil numbers in BAL at 3 days postinfection. These analyses were performed using the StatView (SAS Institute, CARY, NC) computer program. When appropriate, data were logarithmically transformed prior to statistical analysis, and results were confirmed by a demonstrated increase in the 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 antilogs of the means and standard errors (SE) of transformed data were used to present the data and are referred to as the geometric means (×/÷ standard error). All experiments were repeated at least twice.

RESULTS

IFN-γ mRNA levels increase along the airway after infection. In a previous study (75), we demonstrated that innate immune mechanisms against pulmonary mycoplasma were impaired in the absence of IFN-γ. To determine whether mycoplasma infection caused a localized change in IFN-γ transcript levels, total RNA was isolated from cells collected by BAL and the corresponding lungs. Real-time RT-PCR was used to quantify the IFN-γ mRNA transcript levels within cells isolated by BAL of naïve mice and BALB/c (wild-type) mice infected for 3 days.

After infection, the IFN-γ mRNA transcript levels increased in BAL cells but not in the corresponding lungs after the lavage (Fig. 1a and 1b). Transcripts of IFN-γ were approximately threefold higher in cells isolated from the BAL of 3-day-infected mice than transcripts from the BAL of uninfected mice. The corresponding lungs, on the other hand, showed no detectable difference in IFN-γ transcript levels after infection. Thus, IFN-γ mRNA was increased in cells within the respiratory airways and alveoli but not in the cells within the parenchymal lung tissue.

Intracellular IFN-γ increases in pulmonary NK cells during early infection. To determine which IFN-γ-producing cells were responding to mycoplasma infection, lymphocytes were isolated from cell suspensions from the total lungs of naïve and 3-day-infected wild-type mice. Lymphocytes were characterized by flow cytometry after staining for cell surface expression CD3 (to distinguish T cells) and DX5 (to distinguish NK cells) as well as for intracellular IFN-γ. The fraction of NK cells with intracellular levels of IFN-γ within the total lung cell population significantly increased after infection (Fig. 1c). The frequency of IFN-γ-positive NK cells was increased by about 100% after infection in wild-type mice. Neither $CD3^+$ $DX5^-$ T cells nor $CD3^+$ $DX5^+$ NK T cells significantly increased in their intracellular IFN-γ levels after infection (data not shown). Therefore, within the lungs early postinfection, only NK cells increased their production of IFN-γ in response to mycoplasma infection.

***M. pulmonis* organisms survive treatment with highly cytotoxic granules from NK cells.** To determine whether NK cell mechanisms could directly kill mycoplasma organisms, perforin-containing granule extracts isolated from NK cells were incubated with *M. pulmonis*, and the surviving number of mycoplasma CFU was determined after incubation with granules. The mycoplasma survived highly toxic levels of NK cell granule extracts (Fig. 2a) that were sufficient to lyse nucleated cells. To determine whether mycoplasma can interact with perforin and block the lytic activity of the granule extracts, various concen-

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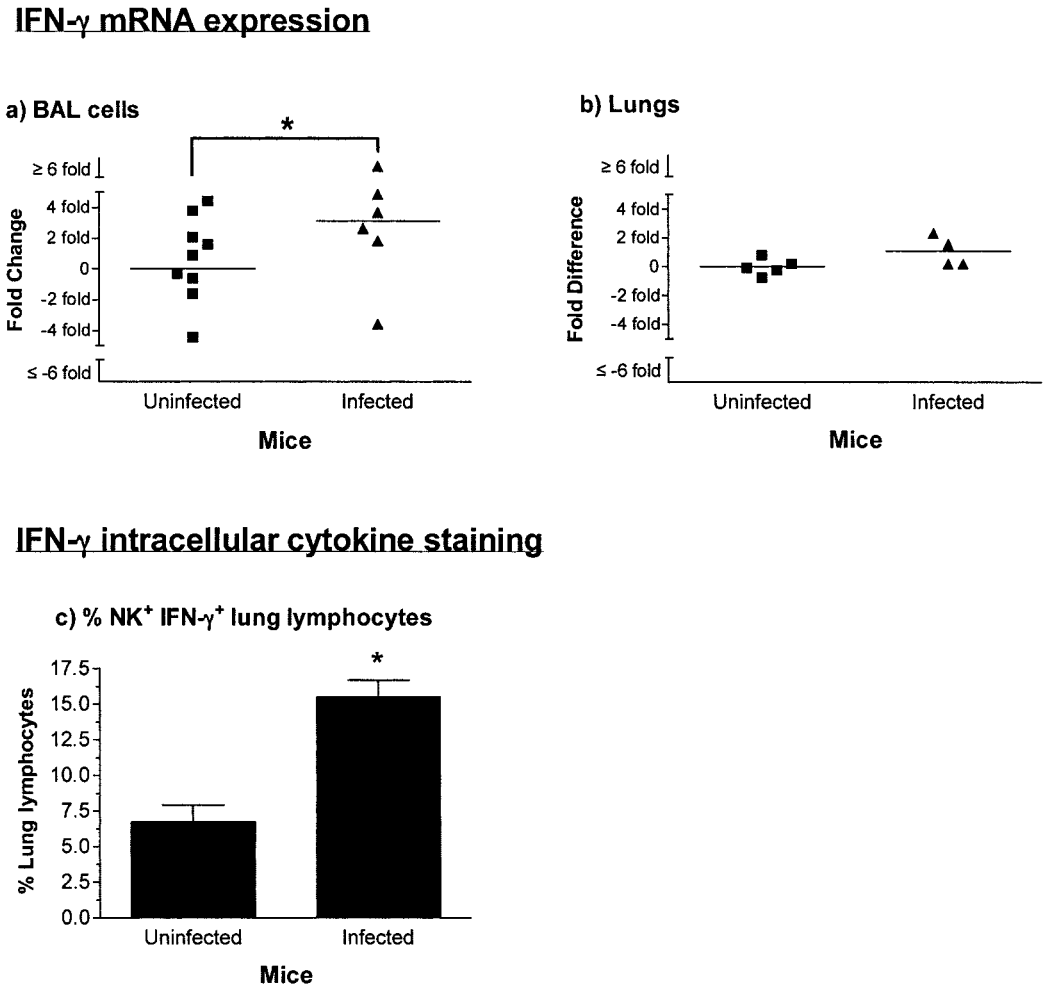

FIG. 1. mRNA and intracellular level of IFN-γ in wild-type mice 3 days after infection. Three days after infection, cells from BAL (a) and the corresponding lungs (b) were isolated, and total RNA was collected. IFN-γ transcript levels were measured by real-time RT-PCR. Levels of mRNA were standardized to GAPDH housekeeping gene. The lines represent the geometric means of the severalfold differences in IFN-γ mRNA levels relative to the average baseline values of samples from uninfected (control) mice. "*" denotes a statistical difference ($P \leq 0.05$) from uninfected mouse results. (c) Three days after infection, lung cells were isolated and stained for CD3 (T cell), DX5 (NK), and intracellular IFN-γ and analyzed by flow cytometry. Shown are the populations of IFN-γ$^+$ NK$^+$ cells before and after infection. Vertical bars represent means ± SE ($n = 8$). "*" denotes a statistical difference ($P \leq 0.05$) from uninfected mouse results.

trations of mycoplasma were incubated with granule extracts during red blood cell lysis. After 20 min, regardless of the concentration of mycoplasma used, 50% of target cells were still lysed (Fig. 2b). These results demonstrate that mycoplasma are refractory to NK cell exocytosis of cytotoxic granules and that, in concentrations comparable to the lung infections, they are unlikely to interfere with NK-cell-mediated cytotoxicity.

Depletion of NK-like cells from IFN-γ$^{-/-}$ mice allows the mice to clear mycoplasma organisms from the lungs. To determine whether the loss of NK-derived IFN-γ was responsible for the inability of IFN-γ$^{-/-}$ mice to control mycoplasma growth within the lungs, NK-like cells were depleted from IFN-γ$^{-/-}$ and wild-type mice. In these and subsequent experiments, NK-like cells were depleted by anti-asialo GM1 antibody treatment 1 day prior to infection. Persistent depletion was verified at day 3 after mycoplasma inoculation. Furthermore, we found that only the NK (DX5$^+$ CD3$^-$) cells were consistently depleted, while the numbers of T cells were unchanged (data not shown). NK T cells were reduced in some mice up to 50% of numbers in untreated mice; however, depletion of NK T cells was not consistent, whereas NK cell depletion was reliable. Therefore, the effects due to anti-asialo GM-1 antibody treatment were most likely due to the loss of NK cells and not due to the presence of NK T cells.

Contrary to our expectations, the mycoplasma infection was still cleared when NK cells were depleted from the wild-type mice (Fig. 3). Surprisingly, the depletion of NK-like cells from IFN-γ$^{-/-}$ mice significantly reduced the numbers of mycoplasma in lungs to levels comparable to infected wild-type mouse levels. Thus, the NK$^-$ IFN-γ$^{-/-}$ mice had a potent form of innate immunity that was independent of NK cells and of IFN-γ. It appeared that the IFN-γ was necessary only when there were NK cells. These results demonstrate that NK cells have deleterious effects on innate immune responses during mycoplasma disease when there is no IFN-γ in the environment.

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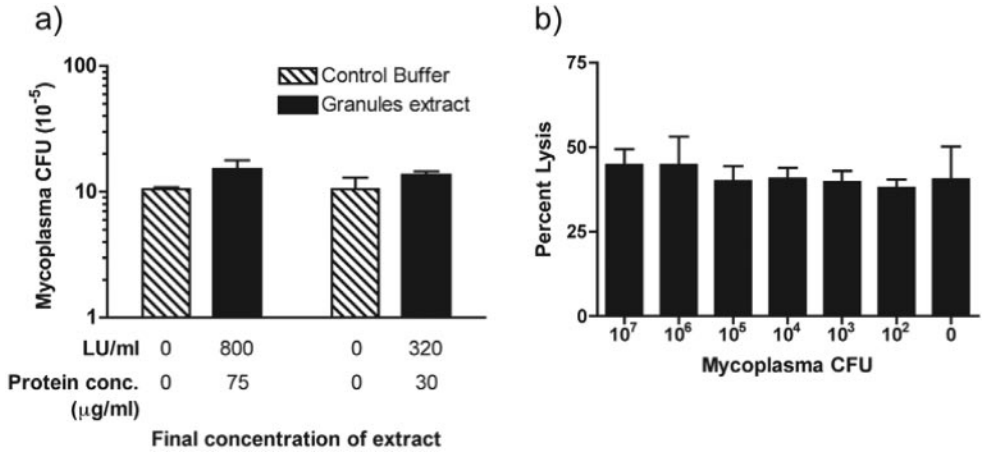

FIG. 2. Interaction of mycoplasma and NK cell derived granules. (a) 10^5 CFU of mycoplasma were incubated for 20 min with either a 1/10 or 1/25 dilution of control buffer or NK cell-derived granules [800 or 320 lytic units (LU)/ml] for 20 min, and then the numbers of mycoplasma CFU were determined. (b) Various numbers of mycoplasma CFU were incubated with a constant concentration of NK cell-derived granules and SRBC for 20 min, and afterwards, the percentage of RBC lysis was determined.

Macrophage death in infected lungs is independent of NK cells. We examined the possibility that NK cells could damage macrophages during mycoplasma infection, particularly in the IFN-$\gamma^{-/-}$ animals. The viable and apoptotic macrophage numbers were significantly elevated in the BAL of IFN-$\gamma^{-/-}$ mice and reduced after NK-like cell depletion (data not shown). However, the fractions of macrophages that were apoptotic (percent viability), as indicated by activated caspases, were similar, regardless of the IFN-$\gamma^{-/-}$ or NK cell status of the animals. There were no significant changes in macrophages isolated from the corresponding, lavaged lungs. Thus, we were unable to observe deleterious effects of NK cells on macrophage viability.

The presence of NK-like cells is associated with an increase in cellular inflammatory infiltrate into the airways of infected mice. As NK-like cells affected mycoplasma numbers in lungs of IFN-$\gamma^{-/-}$ mice, wild-type and IFN-$\gamma^{-/-}$ mice were either treated with anti-asialo GM1 antibody to deplete NK-like cells or sham treated. At day 3 postinfection, modified Wright-Giemsa staining was used to characterize BAL and lung cells.

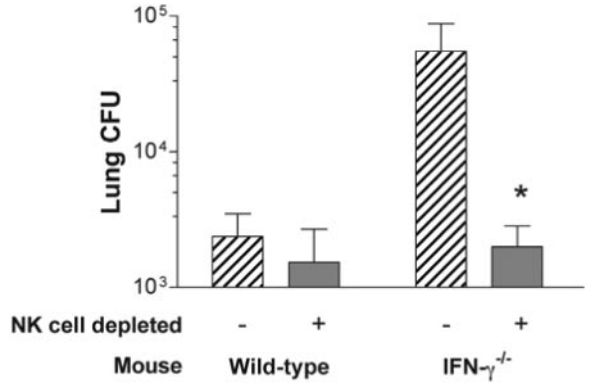

FIG. 3. Mycoplasma CFU found in lungs of anti-asialo GM-1 antibody-treated and untreated wild-type and IFN-$\gamma^{-/-}$ mice. Wild-type and IFN-$\gamma^{-/-}$ mice were depleted of NK-like cells on day −1 by use of treatment with anti-asialo GM1 antibody. Three days postinfection, the numbers of mycoplasma CFU in lungs were determined. Vertical bars and error bars represent means ± SE (n = 8). "*" denotes a statistical difference ($P \leq 0.05$) from strain control results.

The depletion of NK-like cells from IFN-$\gamma^{-/-}$ mice led to a significant decrease in the number of neutrophils and macrophages in the bronchoalveolar spaces and lungs (Fig. 4). IFN-$\gamma^{-/-}$ mice had more total cells recruited to the bronchoalveolar regions after infections than seen in wild-type mice, though no one cell type was significantly increased (data not shown). The depletion of NK-like cells led to a significant decrease in total cells in IFN-$\gamma^{-/-}$ mice but specifically decreased the number of neutrophils and macrophages seen in the BAL. Anti-asialo GM1 antibody treatment also affected wild-type mice, whereas a decrease in macrophages and neutrophils was also seen in the BAL.

Cytokines were elevated in the BAL cells and fluids of IFN-$\gamma^{-/-}$ mice, and the depletion of NK-like cells reduced the levels of these cytokines to wild-type levels. To determine whether there was a change in the types of cytokine responses generated, cytokine mRNA transcript levels were determined before and after infection in both wild-type and IFN-$\gamma^{-/-}$ mice. Mice were either infected with mycoplasma or sham inoculated. At 3 days postinfection, total RNA from BAL cells and corresponding lungs after BAL was isolated and the levels of IL-4, IL-10, IL-12, and TNF-α were determined. The mRNA isolated from the BAL cells or the corresponding lungs of wild-type mice showed no significant change in the levels of any of these cytokines after infection (data not shown). IFN-$\gamma^{-/-}$ mice, on the other hand, showed an increase in mRNA transcript levels of TNF-α, IL-12, and IL-10 in cells isolated from the BAL after infection, whereas only TNF-α mRNA transcript levels increased in the corresponding lungs of infected IFN-$\gamma^{-/-}$ mice.

To further understand the impact of NK-like cells on the development of innate immune responses to mycoplasma infections, cytokine concentrations were determined in the BAL fluids from wild-type and IFN-$\gamma^{-/-}$ mice 3 days after mycoplasma infection. Wild-type and IFN-$\gamma^{-/-}$ mice were depleted

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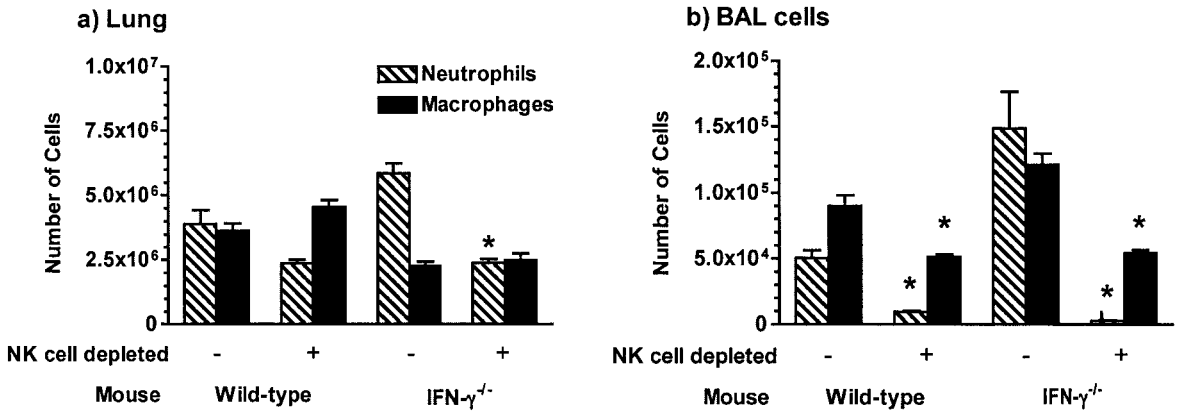

FIG. 4. The influence of NK cell depletion on numbers of macrophage and neutrophils in lungs and BAL in wild-type and IFN-γ KO mice. On day −1, wild-type and IFN-γ$^{-/-}$ (IFN-γ KO) mice were either intraperitoneally injected with anti-asialo GM-1 antibody or sham treated. Three days after infection, BAL cells and the remaining lung cells were collected and stained using a modified Wright-Giemsa stain. Vertical bars and error bars represent means ± SE (n = 8). "*" signifies a significant difference ($P \leq 0.05$) from corresponding sham-treated strain mouse results.

of NK-like cells by use of treatment with anti-asialo GM1 antibody. At 3 days postinfection, BAL fluids were collected, and the levels of 18 cytokines were determined. Cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40, IL-12 p70, IL-17, GM-CSF, IFN-γ, G-CSF, TNF-α, KC, MIP-1α, and RANTES were measured.

IFN-γ$^{-/-}$ mice had significant increases in the levels of several cytokines (Fig. 5). Three cytokines (IL-3, GM-CSF, and IFN-γ) were below the level of detection, while there were no detectable changes in IL-2, IL-4, IL-5, and IL-12p40 levels. Protein levels of IL-1α (1.4 logs higher), IL-6 (3.2 logs higher), IL-10 (1.6 logs higher), IL-17 (1.5 logs higher), G-CSF (3.4 logs higher), and TNF-α (at least 2.5 logs higher) were all significantly higher 3 days after infection than wild-type mouse levels. In addition, IL-1β, IL-12p70, and KC all tended to be higher in IFN-γ$^{-/-}$ mice, though this change was not statistically significant. Importantly, the depletion of NK-like cells from wild-type mice did not change the levels of any cytokine tested. The depletion of NK-like cells from IFN-γ$^{-/-}$ mice, on the other hand, significantly reduced the levels of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, G-CSF, TNF-α, KC, and MIP1-α to wild-type levels. Thus, NK-like cells impact the type of immune response generated in IFN-γ$^{-/-}$ mice.

DISCUSSION

Innate immunity is a critical determinant in the course of mycoplasma disease (5, 17, 24, 25, 27, 28, 43, 44, 69). It influences the initial establishment of infection and progression of disease. In other infectious diseases, both cells and cytokines play intricate roles in the development of innate immune responses. Early after infection, macrophages release a variety of cytokines and chemokines that attract NK and other inflammatory cells into the site of infection (21, 49). This early recruitment of NK cells is critical, as they can release a large amount of IFN-γ into the area, leading to activation of macrophages (50). NK cell-derived IFN-γ has been suggested to be important during mycoplasma disease (43, 44); however, the role of NK cells during mycoplasma disease is still unclear. IFN-γ is a pleiotropic cytokine that has a very intricate role in the development of innate immune responses (61, 62). In fact, previous work in our lab demonstrated that IFN-γ is critical in clearing mycoplasma early after infection, as IFN-γ$^{-/-}$ mice have a 2-log-higher CFU burden than wild-type BALB/c mice by day 3 postinfection (75). Furthermore, we showed there is no significant contribution of adaptive immune responses at this time (31, 32). The purpose of this study was to determine the source of IFN-γ and its function within innate immune responses to pulmonary mycoplasma infection. On the basis of current literature, we hypothesized that NK cell-derived IFN-γ would be important in the clearance of mycoplasma by day 3 postinfection in the lung.

Consistent with our original hypothesis, NK cells respond to pulmonary mycoplasma infection with an increase in IFN-γ production. The BAL cells show an increase in IFN-γ mRNA transcript levels 3 days after infection. The number of intracellular IFN-γ-expressing NK (CD3$^-$DX5$^+$) cells increased in the lung, whereas there was no increase in IFN-γ$^+$ cells numbers in other cell populations. As IFN-γ is an important mediator in innate immunity in mycoplasma disease, these data suggest that NK cells could influence mycoplasma disease through the secretion of IFN-γ, in similarity to the results of studies with leishmania (53) and studies on mycoplasma immunity by Lai et al. (43). Alternatively, our results demonstrate that NK cells were becoming activated in vivo in response to mycoplasma, which has been demonstrated in an in vitro model (14), and upon activation, NK cells could release their cytoplasmic granules, which contain granulysin (41) and other mediators, to directly kill host cells and/or bacteria. However, NK cell-derived granules were not able to directly kill mycoplasma, and mycoplasma do not interfere with granule activity. In addition, NK cell granules could cause bystander damage and kill macrophages, especially in an IFN-γ-deficient environment, which could lead to impaired innate immunity (68). However, there was no significant increase in apoptotic macrophages in IFN-γ$^{-/-}$ mice after mycoplasma infection. Thus, NK cells are the major source of IFN-γ early after mycoplasma infection. Furthermore, the involvement of NK cells during mycoplasma infection is most likely through cytokine cascades

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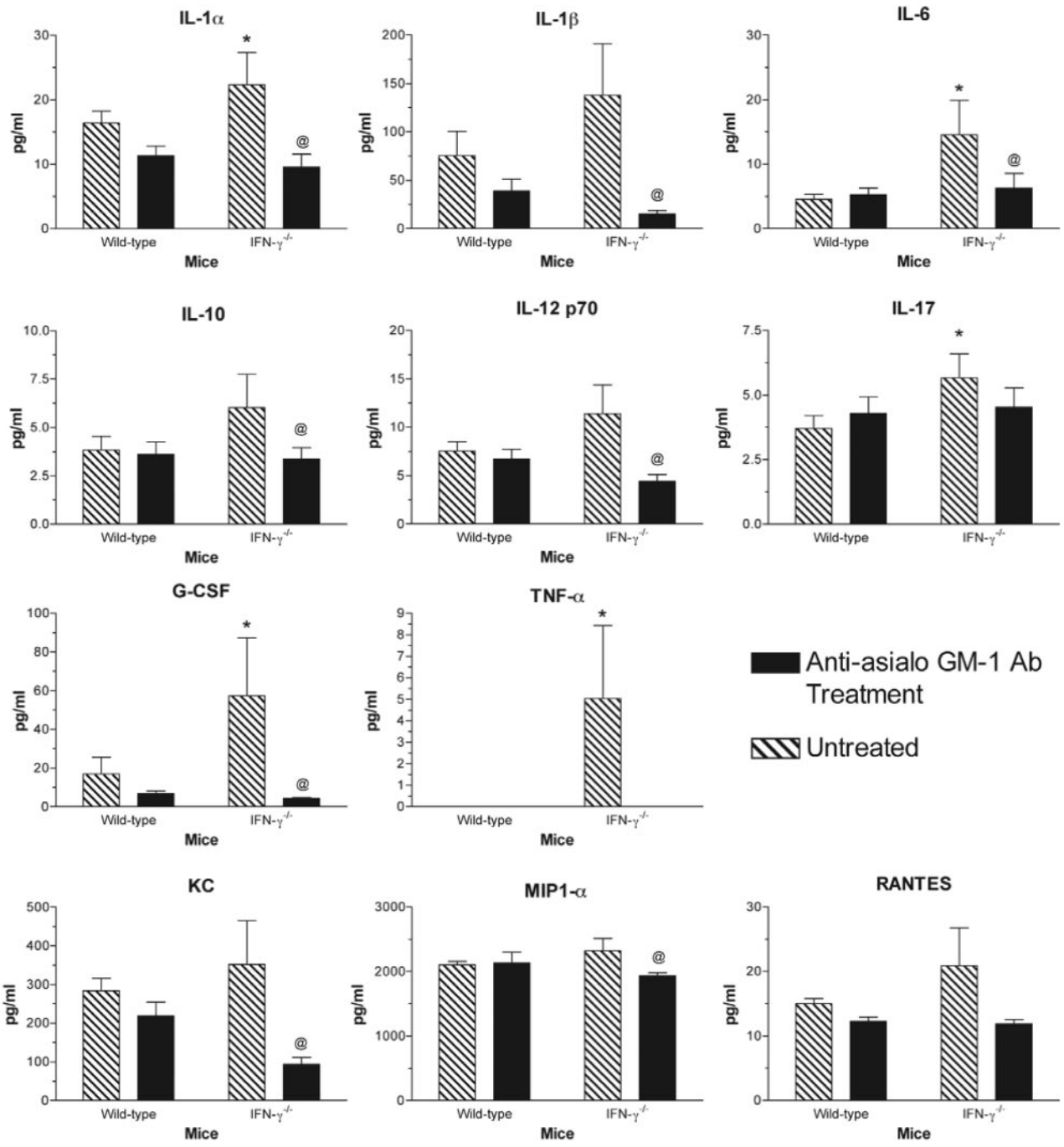

FIG. 5. Cytokine and chemokine levels in BAL fluids from anti-asialo GM1 antibody-treated or untreated wild-type and IFN-γ KO mice after infection. Mice were depleted of NK-like cells by use of anti-asialo GM1 antibody treatment on day −1. After 3 days of infection, BAL fluids were collected, and cytokine and chemokine levels were determined using a Bio-Plex suspension array. Vertical bars and error bars represent means ± SE ($n = 8$). "*" signifies a significant difference ($P \leq 0.05$) from untreated wild-type mouse results. "@" signifies a significant difference ($P \leq 0.05$) from corresponding strain control results.

that influence the effectiveness of innate immune responses rather than cytotoxicity.

Contrary to our original hypothesis, NK cells were not necessary to control mycoplasma numbers in wild-type mice, and in contrast to our expectations, the loss of NK cells allowed IFN-$\gamma^{-/-}$ mice to readily clear mycoplasma from the lung. Our initial hypothesis was that NK-derived IFN-γ was critical for activation of key immune cells that would lead to the control of pulmonary mycoplasma infection. We showed that NK cells were the major source of IFN-γ early after mycoplasma infection. Therefore, by removing NK-like cells from wild-type mice, we expected to eliminate the critical early source of IFN-γ and mimic an environment in the wild-type mice similar to that which is seen in IFN-$\gamma^{-/-}$ mice. In contrast to our

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expectation, wild-type mice and wild-type NK-like cell-depleted mice both effectively cleared mycoplasma from the lung, as immunocompetent and NK-like cell-depleted wild-type mice had similar numbers of CFU in their lungs 3 days after infection. However, IFN-$\gamma^{-/-}$ mice without NK cells cleared mycoplasma as effectively as wild-type mice. Although other cell populations such as NK T cells could be involved, we found that the NK ($DX5^+$ $CD3^-$) cells were consistently depleted using anti-asialo GM1 antibody treatment. In contrast, depletion of NK T cells was not consistent, and there was no effect on other T-cell populations. Most likely, the effects of anti-asialo GM1 antibody treatment were due to the loss of NK cells and not due to the presence of NK T cells. After depletion of NK cells in IFN-$\gamma^{-/-}$ mice, there was also a decrease in proinflammatory (IL-1α, IL-6, and TNF-α) and anti-inflammatory (IL-10, IL-17, and G-CSF) cytokine levels in BAL fluids and fewer phagocytic cells in the BAL cells in response to the mycoplasma infection. This complex scenario demonstrates that NK cells in the absence of IFN-γ have a detrimental role and indicates that removal of these cells allows IFN-γ-independent innate immune responses to function more effectively. This is contrast to the work of Lai et al. (43, 44) suggesting that NK cells and their IFN-γ are critical for the clearance of mycoplasma from the lung. However, his studies were done using C57Bl/6 mice which are resistant to mycoplasma lung disease (44) and which have more-effective macrophage-mediated clearance mechanisms of mycoplasma than susceptible mouse strains (13, 55). It is possible that the NK cell activities in BALB/c (wild-type) but not C57Bl/6 mice may contribute to the increased susceptibility to mycoplasma disease. Regardless, we demonstrated that NK-like cells (most likely NK cells) of the lung have novel activities that modulate innate immunity and can, in some instances, play a detrimental role against pulmonary mycoplasma infection. Furthermore, the ability to produce IFN-γ apparently counters this activity.

As indicated above, the mechanisms through which NK cells can interfere with the effectiveness of innate immunity against mycoplasma infection are most likely cytokine mediated. This viewpoint is based on our observations that NK cell granules were unable to kill mycoplasma and a lack of any evidence of significant innocent bystander damage to macrophages in vivo. NK cell function includes not only cytotoxic activity but also cytokine production. In addition to IFN-γ, NK cells can secrete TNF-α, IL-10, and G-CSF (48, 58), and thus, it is possible that NK cells release anti-inflammatory cytokines (IL-10 and G-CSF) that, in the absence of IFN-γ, dampen key innate immune mechanisms (8, 63, 64, 71, 72). In support of this potential mechanism, there were increased IL-10 and G-CSF levels found in BAL fluids recovered from IFN-$\gamma^{-/-}$ mice, which were reduced to the levels found in infected wild-type BALB/c mice after depletion of NK-like cells. IL-10 is of particular interest, as this cytokine is associated with impaired innate immunity and facilitating the persistence of other infectious agents (2, 30, 37, 51, 52, 55, 59). Presently, it is not clear whether NK cells are the source of IL-10 or other anti-inflammatory cytokines or whether the same or independent NK cell populations in the lung produce the IL-10 and IFN-γ in response to mycoplasma infection. Alternatively, NK cells could indirectly impair innate immunity against mycoplasma through the recruitment of neutrophils. There were more neutrophils within the BAL fluids and lungs from IFN-$\gamma^{-/-}$ mice than in those from wild-type mice, and the depletion of NK-like cells caused a decrease in both neutrophil and macrophage numbers in airways of both wild-type and IFN-$\gamma^{-/-}$ mice. Furthermore, the levels of KC, a chemokine that can be released by NK cells (39), correlate with the number of neutrophils isolated from the airways. Neutrophils could have several devastating effects on mycoplasma disease. Neutrophils could release anti-inflammatory cytokines, dampening activation of immune response (60), or neutrophils, which are unable to effectively kill mycoplasma, could impair of normal clearance (28, 69). Therefore, NK cells could influence the effectiveness directly through the production of anti-inflammatory cytokines and/or indirectly by the recruitment of neutrophils, or other cells, that interfere with macrophage killing of mycoplasma.

In summary, our results demonstrate that NK-like cells, most likely NK cells, can have a novel activity in the lung which is balanced by their ability to produce IFN-γ. In a previous study, we found that IFN-$\gamma^{-/-}$ mice were impaired in their ability to control mycoplasma infections in the lung, and this was consistent with a defect in innate immune mechanisms (75). As previous literature showed for other diseases (3, 15, 43, 45, 51, 53), we hypothesized that NK cell-derived IFN-γ would be critical for clearance of mycoplasma from the lung. This was consistent with earlier in vitro and in vivo studies with mycoplasma (26, 45, 75). In our studies, NK cells were indeed found to be the early source of IFN-γ. In contrast to our expectations, NK-like cells, in the absence of IFN-γ, interfered with the innate immune mechanisms involved in controlling mycoplasma numbers in the lung, resulting in the higher numbers of mycoplasma recovered from lungs in IFN-$\gamma^{-/-}$ mice. Thus, it was not the lack of IFN-γ but the presence of a previously unrecognized NK-like cell-suppressive activity that contributed to the higher mycoplasma numbers. There are several other immunosuppressive mechanisms found in the lungs, such as alveolar macrophages and surfactant, thought to minimize unnecessary immune and inflammatory activity to maintain the integrity of gas exchange. It appears that NK cells in the lungs can contribute to this immunosuppressive environment, but when needed, these dampening effects can be counterbalanced by IFN-γ. These data suggest that there may be instances where perturbation of this regulatory balance may contribute to the susceptibility to and severity of disease. However, further studies must be done to characterize these NK-like cells and the mechanisms that influence their interactions with innate immune mechanisms in the lung. Regardless, these results open new avenues and theories into immune modulation in lungs and by NK cells and impact the design of NK cell-based therapies for disease treatment.

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