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NK Cells Respond to Pulmonary Infection with Mycobacterium tuberculosis, but Play a Minimal Role in Protection

Ana Paula Junqueira-Kipnis,²* Andre Kipnis,∗ Amanda Jamieson, † Mercedes Gonzalez Juarrero, * Andreas Diefenbach, † David H. Raulet, † Joanne Turner,* and Ian M. Orme*

Both innate and adaptive immune systems contribute to host defense against infection with Mycobacterium tuberculosis. NK cells have been associated with early resistance against intracellular pathogens and are known to be potent producers of the cytokine IFN-γ. In C57BL/6 mice infected by aerosol exposure with M. tuberculosis, NK cells increased in the lungs over the first 21 days of infection. Expansion of the NK cell subset was associated with increased expression of activation and maturation markers. In addition, NK cells isolated from the infected lungs were capable of producing IFN-γ and became positive for perforin. In vivo depletion of NK cells using a lytic Ab had no influence on bacterial load within the lungs. These findings indicate that NK cells can become activated during the early response to pulmonary tuberculosis in the mouse model and are a source of IFN-γ, but their removal does not substantially alter the expression of host resistance. The Journal of Immunology, 2003, 171: 6039–6045.

Mycobacterium tuberculosis is one of the leading causes of death among infectious diseases (1–4). Although the roles for acquired immune mechanisms that involve T lymphocytes and the Th1 cytokine pathway are now thoroughly established (5–13), work in the last decade has shown a substantial underlying network of more innate mechanisms that also contribute to host resistance. These mechanisms include mycobacterial cell wall molecules such as lipopolysaccharides and lipoproteins that can directly bind to Toll-like receptor-2 on macrophages, stimulating IL-12 secretion (14). In addition, similar lipid molecules can be sampled from within the endosomal pathway, leading to their presentation by CD1 molecules and subsequent recognition by CD1-restricted leukocyte subsets (15, 16).

Studies by Flynn (11, 17) as well as our laboratory (18) have demonstrated that mice deficient in the β₂-microglobulin gene are more susceptible to infection with M. tuberculosis. NK1.1⁺ T cells, a specialized subset of T cells that recognize CD1, are reportedly absent in young β₂-microglobulin-deficient (β₂-m-KO) mice (19, 20); supporting the hypothesis that NK T cells may contribute to early protective innate immunity against the challenge infection. In fact, there is substantial evidence that NK and NKT cells may be a potent source of IFN-γ during the initial phase of resistance to infections such as Cryptococcus neoformans (21) and may induce a granulomatous reaction to purified mycobacterial cell wall (22). More recently, it was shown that increasing the activity of NK T cells by administration of the known CD1 ligand α-galactosylceramide can increase the resistance of mice to a subsequent infection with M. tuberculosis (23).

To determine whether NK or NKT cells could participate in the early innate resistance to infection with M. tuberculosis, we examined the kinetics and function of these cell subsets following aerosol infection with M. tuberculosis. A steady increase in NK, but not NKT, subsets was observed within the lung over the first 3 wk of infection. NK cells increased the expression of cell surface activation markers, were IFN-γ positive and labeled positive for intracellular perforin after in vitro stimulation. Despite this, the depletion of NK cells using lytic Abs had no influence on the pulmonary bacterial load. These data suggest that in response to an infection with M. tuberculosis, lung NK cells can become activated, but are not an essential protective mechanism.

Materials and Methods

Animals

Specific pathogen-free female, 8- to 10-wk-old, C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under barrier conditions with sterile mouse chow and water ad libitum. The specific pathogen-free nature of the mouse colonies was demonstrated by testing sentinel animals, which were shown to be negative for 12 known mouse pathogens. All experimental procedures were approved by the Colorado State University animal care and use committee.

Aerosol infection with M. tuberculosis

M. tuberculosis strain Erdman, originally obtained from Trudeau Institute (Saranac Lake, NY), was grown in Proskauer-Beck liquid medium containing 0.05% Tween 80 to mid-log phase and then frozen in aliquots at −70°C until needed. Mice were infected using procedures described previously (8). Briefly, bacterial stocks were diluted in 5 ml of sterile distilled water to 2 × 10⁶ CFU/ml and placed in a nebulizer attached to an airborne infection system (Glass-Col, Terre Haute, IN). Mice were exposed to 40 min of aerosol, during which ~100–200 bacteria were deposited in the lungs of each animal. Bacterial load was determined by plating whole organ homogenates onto nutrient 7H11 agar supplemented with OADC. Colonies were enumerated after 21-day incubation at 37°C.

Flow cytometric analysis of cell surface markers

Cells were labeled with the following specific Abs: FITC-anti-2B4 (clone 2B4), -anti-NKG2a-c (clone 20d5), -anti-CD44 (clone 1M7), -anti-CD117 (clone 2B8), -anti-Ly-6C (Gr-1, clone RB6-8C5), or -anti-MAC-3 (clone M3/84); PE-anti-CD3 (clone 145-2C11), -anti-CD8 (clone 53-6.7), -anti-CD69 (clone H1.2F3), -anti-CD16/32 (clone 2.4G2), -anti-CD122 (clone 5H4), -anti-CD43 (clone S7), -anti-CD62L (clone MEL-14), or -anti-
CD51 (clone RMV-7); PerCP-anti-CD4 (clone H129.19) or -anti-CD11b (clone M1/70); and allophycocyanin-anti-NK1.1 (clone PK136), -anti-CD11c (clone HL3), or -anti-CD8 (clone 53-6.7). All Abs were purchased from BD PharMingen (San Diego, CA) and were used at 0.2 μg/10^7 cells in PBS containing 10% mouse serum and 0.1% sodium azide for 30 min at 4°C. Biotin-labeled anti-NKG2D was supplied by Dr. Rautel (University of California, Berkeley, CA) and was used at a dilution of 1:50, followed by incubation with streptavidin-PE. After washing with PBS containing 0.1% sodium azide, the cells were analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA), and the data were analyzed using CellQuest software (BD Biosciences, San Jose, CA). Cells were gated on lymphocytes based on characteristic forward and side scatter profiles. Individual cell populations were identified according to the presence of specific fluorescent-labeled Ab. All analyses were performed with an acquisition of at least 50,000 events.

### In vivo NK depletion

C57BL/6 mice were treated every 3 days i.p. with 100 μg of rabbit anti-asialo-GM1 (AAGM1; WAKO, Richmond, VA) or rabbit IgG isotype control. Two weeks after the initiation of AAGM1 treatment, the animals were infected by aerosol with *M. tuberculosis*. Ab treatment was continued for the duration of the experiment. Noninfected mice that received AAGM1 or saline were included for each experimental time point. Flow cytometric analysis of lung, spleen, and liver cells was used to monitor the efficacy of the depletion of NK1.1+ cells. Following depletion, <0.5% of the lymphocytes in all three organs were NK cells. Alternatively, mice were treated with anti-NK1.1 (clone PK136; BD PharMingen) with similar results.

### Culture and infection of macrophages

Bone marrow macrophages (2 × 10^5 cells/ml) were cultured for 7 days in DMEM (Life Technologies, Gaithersburg, MD) containing 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO), 2 mM nonessential amino acids (Sigma-Aldrich), 2 mM sodium pyruvate (Sigma-Aldrich), and 10 mM HEPES (Sigma-Aldrich; cDMEM) and supplemented with L929 cell line supernatant. The adherent cells were washed twice with cDMEM and infected with 2.5 × 10^6 CFU/ml of *M. tuberculosis* Erdman in a final volume of 1 ml of cDMEM. Cells were infected for 10–12 h. Extracellular bacteria were removed from the cells by washing with cDMEM. The plates were incubated for 18 h at 37°C in 5% CO₂, washed, and cultured with cDMEM for an additional 24 h.

### Lung cell digestion

Mice were euthanized by CO₂ asphyxiation, and the pulmonary cavity was opened. The lung was cleared of blood by perfusion through the pulmonary artery with 10 ml of ice-cold PBS containing 50 U/ml heparin (Sigma-Aldrich). Lungs were aseptically removed, teased apart, and treated with a solution of DNase IV (Sigma-Aldrich; 30 μg/ml) and collagenase XI (Sigma-Aldrich; 0.7 mg/ml) for 45 min at 37°C. To obtain a single-cell suspension, the organs were gently passed through cell strainers (BD Biosciences, Lincoln Park, NJ). Remaining erythrocytes were lysed with Gey’s solution (0.15 M NH₄Cl and 10 mM KHCO₃), and the cells were washed with cDMEM. Total cell numbers were determined using a Neubauer chamber (IMV International, Minneapolis, MN).

### Macrophage overlays

Single-cell suspensions from the lungs were cocultured with noninfected or *M. tuberculosis*-infected macrophages for 18 h. Then 3 μM monensin (Golgi Stop solution; BD PharMingen) was added to the cultures for the final 6 h of the incubation period. The lung cells were then stained with allophycocyanin-anti-NK1.1, anti-perforin (rat IgG2a, clone KM585; Kamiya Biomedical, Seattle, WA), and a secondary Ab, anti-rat IgG2a-FITC (Serotec, Raleigh, NC).

### Intracellular cytokine staining

Measurement of intracellular IFN-γ was conducted by preincubating lung cells with monensin (3 μM) for 4 h at 37°C in 5% CO₂. The cells were then stained with allophycocyanin-anti-NK1.1, PerCP-anti-CD4, and PE-anti-CD8 for 30 min; washed with PBS containing 0.1% sodium azide; fixed and permeabilized with Perm/Fix/Perm Wash (BD PharMingen); and stained for intracellular IFN-γ (FITC, clone XMG1.2) or FITC-rat IgG1 isotype control (BD PharMingen) for an additional 30 min. The Abs were diluted in PBS containing 0.1% sodium azide and 10% normal mouse serum (Serotec, Raleigh, NC).

### Statistics

Statistical significance was determined using Student’s *t* test.

### Results

**NK cells accumulate early in the lungs after aerosol infection with** *M. tuberculosis*

Mice were infected by aerosol with *M. tuberculosis*, and at various times after exposure the lung cells were isolated and analyzed by flow cytometry for the presence of NK or NKT cells. NK cells were defined as NK1.1+CD3− cells, and NKT cells as NK1.1+CD3+ cells (Fig. 1A). A steady and significant increase in NK cells was observed in the lungs of mice infected with *M. tuberculosis*, which was most apparent after 7 days of infection.
The number of NK cells within the lungs remained elevated for 14 days, representing ~10% of the total lung cells at this time, after which there was a slight decrease in the infected mice. No such increase was observed in the NKT cell subset (NK1.1⁺CD3⁻), which consistently represented <1% of the total lung cells (Fig. 1C).

**NK cells express distinct phenotypic cell surface markers**

To further characterize the NK cells that accumulated in the lung during infection, NK cells were analyzed for the expression of several cell surface markers. Most NK cells within the lungs stained brightly for NKG2D (Fig. 2A) and CD11b (Fig. 2B). The expression of NKG2D and CD11b did not change during infection with *M. tuberculosis*. Analysis of NKG2a-c expression identified two distinct NK cell subsets within the lungs: NKG2a-c⁺ and NKG2a-c⁻ (Fig. 2C). Upon infection with *M. tuberculosis*, however, NK cells expanded equally within the two NKG2a-c subsets. Collectively, these data show that the number of NK cells within the lungs increased in response to an infection with *M. tuberculosis*, but we found no evidence of a selective increase in any particular NK subset.

**FIGURE 2.** Phenotype of NK cells within the lung. NK1.1⁻CD3⁻ cells were analyzed for the expression of NKG2D, CD11b, and NKG2a-c by flow cytometry. Single-cell suspensions from control C57BL/6 mice were incubated with fluorescently labeled Abs for NK1.1 and were gated and analyzed for the expression of NKG2D (A), CD11b (B), and NKG2a-c (C). Numbers indicate the percentage of positive cells. Figures are representative of four mice per group from three independent experiments. The same results were observed for the infected mice at all time points analyzed.

**NK cells in the lungs of mice infected with M. tuberculosis express markers of activation**

To determine whether NK cells within the lungs were activated in response to infection with *M. tuberculosis*, we examined the expression of two activation markers, 2B4 and CD69. The majority of NK cells present in the lungs of noninfected mice expressed 2B4 (Fig. 3A). Following infection, there was a significant increase in the expression of the early activation marker CD69 (Fig. 3, A and B). Increased expression of CD69 by NK cells occurred in both 2B4⁺ and 2B4⁻ subsets. The kinetics of CD69 expression are shown in Fig. 3C, showing a steady increase in the proportion of NK cells that expressed CD69 over the early course of the infection.

**FIGURE 3.** NK cell activation in response to *M. tuberculosis* infection. Mice were aerosol-infected with *M. tuberculosis*, and at specific time points postinfection lung cells were isolated and incubated with fluorescently labeled Abs for NK1.1 and CD3. Cells NK1.1⁻CD3⁻ were analyzed for the expression of CD69 and 2B4 by flow cytometry. A, Control, age-matched mice (inset, isotype control (~0.6% positive cells in all three quadrants)); B, infected mice at 21 days postinfection. Numbers in quadrants indicate the percentage of positive cells. C, Kinetics of NK1.1CD69⁺ cells during the early phase of the infection. C, Control mice; ○, infected mice. *, Statistical significance between control and infected mice, p < 0.01.
Lung NK cells were also analyzed for the expression of CD122 and CD16/32; however, the expression of these two cell surface markers was not altered in response to infection (data not shown). In addition, we found no expression of either CD95 or CD95L on NK cells (data not shown).

Examination of the maturation status of NK cells in the lungs, defined by the expression of c-Kit, CD51, CD11b, and CD43, showed that the majority of NK cells expressed CD11b and CD43 (Table I) and were therefore either expanding or fully mature. No changes in these markers were seen over the first 21 days of the infection.

**NK1.1+ cells in the lungs of M. tuberculosis-infected mice can produce IFN-γ and perforin**

The early secretion of cytokines by NK cells is thought to dictate the subsequent developing acquired immune response (24, 25). We therefore analyzed NK cells for their capacity to produce either IFN-γ or IL-4 in response to *M. tuberculosis*. Significantly more NK cells from the lungs of infected mice produced IFN-γ than similar cells from the lungs of noninfected mice (Fig. 4). This was most apparent between days 14–21 of infection. Finally, given the knowledge that NK cells can also produce IL-4, this was also examined, but only background levels (<0.5%) were observed.

A primary function of NK cells is their capacity to act as cytolytic cells; therefore, NK cells were analyzed for their capacity to use the lytic molecule perforin (26). NK cells obtained directly from the lungs of both noninfected and infected mice contained small amounts of perforin (<5%), but when restimulated in vitro with *M. tuberculosis*, cells from infected mice showed high levels of perforin staining (Fig. 5). We previously observed that NK cells from the lungs of *M. tuberculosis*-infected mice were negative for Fas or Fas ligand expression (data not shown).

**Depletion of NK cells does not influence the bacterial load or the immune response elicited within the lungs of mice infected with M. tuberculosis**

It was reasonable to hypothesize that if the influx of NK cells into the lungs and their potential cytolytic activity contributed to early resistance to *M. tuberculosis*, then depletion of the NK population using AAGM-1 or anti-NK1.1 Abs should increase the susceptibility of such mice to infection. No overt differences were observed in the bacterial load of Ab and isotype control-treated mice, indicating a similar course of infection in the presence and in the absence of NK cells (Fig. 6). Furthermore, the depletion of NK cells did not influence the granulomatous response, as the quality of this cellular response was similar in the two groups (data not shown). Analysis of the individual T cell subsets within the lungs of infected mice demonstrated no apparent difference in the activation status or the capacity to produce IFN-γ between mice that had received AAGM-1 or isotype control Ab (Table II).

**Discussion**

The results of this study show that the NK cell population expands within the lung in response to an aerosol infection with *M. tuberculosis*. Furthermore, lung NK cells acquired cell surface markers associated with activation, produced IFN-γ, and stained positively for intracellular perforin. In contrast to our initial hypothesis that NK cells might play an essential contribution to early resistance to an infection with *M. tuberculosis*, we observed no alteration in the bacterial load within the lungs of mice that had been depleted of NK cells.

![Figure 4](http://www.jimmunol.org/)

**Figure 4.** IFN-γ expression by NK1.1+ cells after *M. tuberculosis* infection. Mice were infected with *M. tuberculosis*, and at specific time points postinfection lung cells were incubated for 4 h with monensin, followed by incubation with fluorescently labeled Abs for NK1.1 and IFN-γ. Representative dot plots are shown in A (control mice) and B (infected mice at 21 days postinfection), where NK1.1-gated cells were analyzed for IFN-γ expression. Numbers in upper left quadrant indicate the percentage of positive cells for both markers analyzed. C, Kinetics of NK1.1 IFN-γ-positive cells during the early phase of the infection. ○, Control mice; ●, infected mice. Data shown are the mean (n = 4) ± SEM and are representative of three independent experiments. *p < 0.01.
NK cells. These data suggest that NK cells could certainly contribute to early resistance, but their presence is not essential, probably due to redundancy in the evolving cellular response in the lungs.

The NK cell kinetics in the lungs in response to infection were quite intriguing. Infection was followed by an increase in the number of NK cells producing IFN-γ at 3 days postinfection, followed by a period of apparent inactivity after which we detected a significant increase in IFN-γ-positive cells within the lungs. This suggests a gradual increase in NK cell activation and IFN-γ production over the first 2 wk as the infection begins to be detected in the lungs. These data may indeed fit the current hypothesis that NK cells are a first line of defense to infection, as we observed a moderate increase in NK cell activity as early as 3 days after aerosol exposure. However, we also showed that NK cell activation and responsiveness were most apparent after 14 days of infection, perhaps suggesting two early waves of NK cell responsiveness during an infection with *M. tuberculosis*.

The method by which NK cells responded to infection with *M. tuberculosis* was not fully determined in this study. One method would be the capacity of NK cells to kill target cells by lytic or FAS-mediated mechanisms. While, we found minimal expression of FAS (CD95) on the surface of NK cells from the infected lungs, considerable increases in perforin expression by NK cells were observed after in vitro stimulation. Whether perforin-mediated killing is used by NK cells in the lungs of *M. tuberculosis*-infected mice is unclear. Other studies (27, 28) have shown no differences in the course of *M. tuberculosis* infection in mice in which the perforin gene has been disrupted, suggesting that this mechanism is not important. NK cells are also capable of producing IFN-γ, and while this response could well provide a source of this cytokine to activate infected macrophages within the lungs, depletion of NK cells did not have any influence on the bacterial load.

We found no significant expansion of the NKT cell population within the lungs during infection with *M. tuberculosis*, suggesting that NKT cells do not appear to respond throughout the course of a pulmonary infection with this pathogen. A similar conclusion was recently made in a study (29) of mice lacking NKT cells through gene deletion, in which the course of an aerogenic infection with *M. tuberculosis* did not change compared with that in normal mice. These results are in contrast to an earlier study (22) that implicated NKT cells in the granulomatous process. However,

![Figure 5](image-url)  
**FIGURE 5.** Lung NK1.1⁺ cells become perforin positive during *M. tuberculosis* infection. Mice were aerosol-infected with *M. tuberculosis*, and 21 days later lung cells were overlaid onto macrophage cultures infected 24 h previously with *M. tuberculosis*. Cells were then incubated with fluorescently labeled Abs for NK1.1 and perforin, and the samples were analyzed by flow cytometry. Numbers in histograms indicate the percentage of positive cells for both markers analyzed. The data represent one of three independent experiments.

![Figure 6](image-url)  
**FIGURE 6.** Depletion of NK cells has no influence on the course of *M. tuberculosis* infection. Mice were treated weekly with AAGM1, rabbit IgG, or saline before and after infection with *M. tuberculosis*. Lung cells were incubated with fluorescently labeled Abs for NK1.1, and the depletion efficiency was analyzed by flow cytometry. **A**−**C**, Representative dot plots of lung cells from 14-day-infected mice. **A**, Lung NK cells from infected mice treated with saline; **B**, lung cells from infected mice treated with rabbit IgG (isotype control); **C**, lung cells from infected mice treated with AAGM1. The dot plots are representative of three independent experiments. Bacteria in the lung were enumerated at 3, 7, 14, and 21 days postinfection by culturing whole organ homogenates on 7H11 agar and counting colonies after 21 days of incubation at 37°C. Data are expressed as the log_{10} CFU per lung from four mice per group (mean ± SD). **D**, Infected mice; •, NK-depleted and infected mice. Data are representative of three experiments.
Table II. Cell dynamics during M. tuberculosis acute infection in NK-depleted mice

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<tr>
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<th>NK-Depleted, Infected 14 Days&lt;sup&gt;d&lt;/sup&gt;</th>
<th>NK-Depleted, Infected 21 Days&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NK-Depleted, Infected 21 Days&lt;sup&gt;d&lt;/sup&gt;</th>
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<sup>a</sup>These results represent two independent experiments.
<sup>b</sup>C57BL/6 mice were aerosol-infected with M. tuberculosis.
<sup>c</sup>Data represent the mean and SD of six to eight mice per group.
<sup>d</sup>C57BL/6 mice were treated with AAGM1 Abs for 2 wk and then aerosol-infected with M. tuberculosis.
<sup>e</sup>These data are the percentages within the CD4 and CD8 cell populations.
<sup>f</sup>The cell population CD11b⁻/CD11c⁻/MAC3<sup>+</sup> was defined as macrophages.

in that study mice were not infected with live bacilli, but were injected with chemically modified mycobacterial cell wall.

The lack of participation of NKT cells in immunity to tuberculosis in the mouse may reflect the fact that mice only possess CD1d and lack the related CD1 family of molecules that are needed to present mycobacterial lipids (30, 31). For this reason, mice in which the CD1d gene has been disrupted are no less resistant to M. tuberculosis infection (18, 32). In fact, a recent study (23) clearly demonstrated that if NKT cells were stimulated by α-galactosylceramide, the ligand for CD1d, these mice became more resistant to an i.v. challenge infection (23, 32). These data suggest that targeting the NKT cell response may have therapeutic potential.

Our findings do not help to explain the observation that β2-m-KO mice are susceptible to M. tuberculosis delivered by the i.v. (11, 17) or aerogenic (18) route. In fact, this remains even more enigmatic with the recent observations by Urdahl and colleagues (33) that mice lacking class Ia MHC molecules, but retaining class Ib molecules, were more susceptible to infection with M. tuberculosis, whereas these mice were less susceptible than mice lacking all class I molecules. The class Ia-deficient mice still retained a class Ib-restricted CD8 T cell population, but this did not appear to contribute to protection. Instead, such mice developed lesions in which lymphocytes aggregated on the periphery of poorly formed granulomas, a histological profile very similar to our own observations in β2-m-KO mice (18). Collectively, these data suggest that neither NKT cells nor CD8 cells, both of which can recognize class I molecules, contribute to the very early expression of host resistance to M. tuberculosis. Other class Ib-related molecules may be involved, but at the current time this molecule has not been identified, nor has the cell type responsible for recognition and IFN-γ production been characterized. In fact, virtually all αβ and γδ TCR T cells are now known to be capable of making IFN-γ, and even macrophages and neutrophils may make small amounts (34).

Little is known about the developmental status and phenotype of NK cells within the lungs. We demonstrate here that the majority of resident NK cells were mature, and no expansion of any immature subset in response to infection with M. tuberculosis was observed. These observations confirm the results of Kim and colleagues (35), who have demonstrated that mature NK cells do not undergo readily detectable proliferation unless stimulated by an infection. Our results demonstrate that mature NK cells reside within the lung, and that those cells could be further activated by the presence of M. tuberculosis, as demonstrated by the increased expression of CD69.

We further characterized the NK cell population within the lungs by the expression of NK receptors. A balance between the expression of activating or inhibitory receptors governs NK cell activity (36–38), and therefore the characterization of two distinct subsets of NK cells based on their expression of the NKG2a-c receptor was an important finding. Interestingly, we found no alteration in the expression of these receptors in response to infection with M. tuberculosis, suggesting that although NKG2D receptors are known to recognize stressed and viral infected cells (26, 38–40), the expression of these specific receptors may not be required for the overall defect to M. tuberculosis. This cannot be firmly concluded, however, since cells other than NK cells can express these receptors.

In summary, we have demonstrated that in response to an aerosol infection with M. tuberculosis, the NK cell population within the lung expands, expresses cell surface markers associated with activation, and can produce IFN-γ and perforin. Despite the observed increases in activity within the NK cell population in response to infection, depletion of NK cells using lytic Abs did not alter the bacterial load within the lungs. This depletion is clearly removing a cellular source of IFN-γ, which therefore suggests that some element of redundancy may be involved.

Acknowledgments

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References


