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Conventional NK Cells Can Produce IL-22 and Promote Host Defense in *Klebsiella pneumoniae* Pneumonia

Xin Xu,* Ido D. Weiss,* Hongwei H. Zhang,* Satya P. Singh,* Thomas A. Wynn,† Mark S. Wilson,†,1 and Joshua M. Farber*

It was reported that host defense against pulmonary *Klebsiella pneumoniae* infection requires IL-22, which was proposed to be of T cell origin. Supporting a role for IL-22, we found that *Il22*$^{-/-}$ mice had decreased survival compared with wild-type mice after intratracheal infection with *K. pneumoniae.* Surprisingly, however, *Rag2*$^{-/-}$ mice did not differ from wild-type mice in survival or levels of IL-22 in the lungs postinfection with *K. pneumoniae.* In contrast, *K. pneumoniae*–infected *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice failed to produce IL-22. These data suggested a possible role for NK cells or other innate lymphoid cells in host defense and production of IL-22. Unlike NK cell–like innate lymphoid cells that produce IL-22 and display a surface phenotype of NK1.1^{-}NKp46^{+}CCR6^{+}, lung NK cells showed the conventional phenotype, NK1.1^{+}NKp46^{+}CCR6^{-}. Mice depleted of NK cells using anti–asialo GM1 showed decreased survival and higher lung bacterial counts, as well as increased dissemination of *K. pneumoniae* to blood and liver, compared with control-treated mice. NK cell depletion also led to decreased production of IL-22 in the lung. Within 1 d postinfection, although there was no increase in the number of lung NK cells, a subset of lung NK cells became competent to produce IL-22, and such cells were found in both wild-type and *Rag2*$^{-/-}$ mice. Our data suggest that, during pulmonary infection of mice with *K. pneumoniae*, conventional NK cells are required for optimal host defense, which includes the production of IL-22. ***The Journal of Immunology*, 2014, 192: 1778–1786.**

The respiratory tract is an important mucosal site for entry of pathogens; thus, it requires a rapid and effective response to limit and/or clear infection. Th17 cell cytokines, which are critical for protection against various extracellular pathogens, were shown to promote host defense against pulmonary infections (1–5). Roles for the Th17 cell cytokines IL-17 and IL-22 in protection against extracellular bacteria in the lung have been best characterized in the mouse model of pneumonia induced by *Klebsiella pneumoniae* (1, 2, 6). *K. pneumoniae* is an important agent in community-acquired and nosocomial pulmonary infection and is of particular medical importance recently because of the threat from the worldwide spread of multidrug-resistant strains (7–10).

IL-17 and IL-22 were reported to be critical for host defense in the mouse model of *K. pneumoniae* pneumonia (1, 2, 6), and administration of an anti–IL-22 Ab to *K. pneumoniae*–infected mice resulted in 100% mortality within 24 h. This mortality was significantly more rapid than for control or *Il17*$^{-/-}$ mice, suggesting that IL-22 was particularly important in the initial response against *K. pneumoniae* in the lung (1). *K. pneumoniae*–induced IL-22 was proposed to be of T cell origin (1).

IL-22 is a member of the IL-10 family of cytokines (11–13). IL-22R is expressed on several epithelial tissues, including human bronchial epithelial cells and mouse tracheal epithelial cells, but not on cells of hematopoietic origin (1, 14, 15). Studies (1, 15) suggested a critical role for IL-22 in the maintenance of normal mucosal homeostasis and the protection of mucosal sites during infection. The beneficial role of IL-22 in host defense against extracellular bacteria includes maintaining barrier integrity and upregulation of bactericidal proteins (1, 16–19). The primary source of IL-22 is thought to be Th17 cells and/or Th22 cells, with the latter being a subset identified in human skin (16). Nonetheless, certain innate lymphoid cells (ILCs), such as NK cells and other novel non-NK ILC subsets, also can produce IL-22 at mucosal sites in both mice and humans (17–23).

As prototypic ILCs, NK cells are a first line of defense against infection. Although NK cells have been viewed classically as being crucial in providing innate surveillance against viruses and intracellular bacteria through lysis of infected cells and production of IFN-γ (24–27), data also support a role for NK cells in host defense against extracellular bacteria (28). For example, it was shown in mouse models that NK cells play protective roles in host defense against acute respiratory infection by *Staphylococcus aureus* (29) and *Pseudomonas aeruginosa* (30, 31), as well as infection of the gastrointestinal tract with the attaching and effacing bacterium *Citrobacter rodentium* (32, 33). In addition, impairing NK cell responses is one mechanism whereby prior influenza infection increases susceptibility to subsequent *S. aureus* infection (29, 34). The mechanisms whereby NK cells protect against bacterial infections are not extensively characterized, but they may include production of cytokines, such as TNF-α and IFN-γ, production of chemokines to recruit additional leukocytes, interactions with macrophages to regulate bacterial clearance, and direct bacterial killing (32, 33, 35–37).

In our studies of the *K. pneumoniae* pneumonia model in mice, we found that, although IL-22 was indeed important for optimal

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Abbreviations used in this article: ASGM1, asialo ganglio-*N*-tetraosylceramide; ILC, innate lymphoid cell; WT, wild-type.

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host defense, T cells were not required for survival or for the production of IL-22. We found instead that NK cells were essential for protection against *K. pneumoniae*, including preventing extrapulmonary dissemination, contributed directly and/or indirectly to the production of IL-22 in the lungs of *K. pneumoniae*–infected mice, and could acquire the ability to make IL-22 in the absence of T cells. In addition, the lung NK cells exhibited a conventional phenotype rather than the phenotype of NK22/ILC22 cells described at other sites. Together, our data suggest novel and important roles for conventional NK cells in the earliest stages of antibacterial defense and maintenance of mucosal integrity in the respiratory tract.

Materials and Methods

Animals and reagents

Female 8–12-wk-old C57BL/6NTac, $Rag2^{-/-}$ (B6.129S6-$Rag2^{tm1Fwa}$ N12), and $Rag2^{-/-}Il2rg^{-/-}$ (B10;B6-$Rag2^{tm1Fwa}$ $Il2rg^{tm1Wjl}$) mice were obtained from Taconic Farms. Breeding pairs of BALB/c $Il22^{-/-}$ mice were generously provided by Dr. Lynette Fouser (Pfizer, Cambridge, MA), and the production of these mice was as previously described (16). BALB/cAnNTac, BALB/cAnNCr, and BALB/cJ mice were purchased from Taconic Farms, Division of Cancer Treatment and Diagnosis, National Cancer Institute, and The Jackson Laboratory, respectively. All mice were housed under specific pathogen–free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care–approved facility. Animal study protocols were approved by the Animal Care and Use Committee, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Three to five mice/group were used in each experiment, unless otherwise indicated.

Rabbit anti-mouse/rat asialo ganglio-*N*-tetraosylceramide (ASGM1) polyclonal Ab was purchased from Cedarlane Laboratories and reconstituted with 1 ml sterile water. The Ab titer of the lot used in this study was ~1:1000 by immunoflocculation. Mice were injected i.v. with 30 μl reconstituted Ab in 200 μl PBS 1 d before inoculation with *K. pneumoniae* and were injected i.p. every 3 d over the course of the study. Rabbit serum was used as an Ab control.

K. pneumoniae *inoculation model*

Frozen stock aliquots of *K. pneumoniae* strain 43816, serotype 2 (American Type Culture Collection) were grown in tryptic soy broth for 18 h at 37°C. One milliliter of the culture was added to 200 ml fresh tryptic soy broth and grown for another 2 h until the bacteria reached log phase. Bacteria were pelleted by centrifugation at 6000 rpm for 15 min at 4°C, washed twice with normal saline, and suspended in normal saline. Bacterial concentration was determined by measuring the OD at 600 nm and comparing values with a predetermined standard curve, where 0.1 ODU corresponded to 2.8×10^8 bacteria/ml. For inoculation, mice were anesthetized via i.p. injection with ketamine/xylazine, the trachea was exposed, and a 30-μl inoculum of bacterial suspension or normal saline alone was administered via a 30-gauge needle. The inoculum of *K. pneumoniae* was 10^4 CFU for C57BL/6 mice and any mice on the C57BL/6 background, and 10^3 CFU for BALB/c and BALB/c $Il22^{-/-}$ mice. Inoculum sizes were determined based on survival data for wild-type (WT) BALB/c and C57BL/6 mice (data not shown). An aliquot of the inoculated *K. pneumoniae* suspension was serially diluted onto lysogeny (Luria–Bertani) agar plates to confirm the dose of injected bacteria.

K. pneumoniae *CFU in blood and tissues*

At designated times postinfection, mice were anesthetized via i.p. injection with ketamine/xylazine. Heparinized blood was collected from the inferior vena cava. Lungs were perfused through the right ventricle with normal saline. Lungs and livers were removed and homogenized with normal saline. Bacterial burdens were determined in lung, liver, and blood by plating 10-fold serial dilutions of tissue homogenates or blood on lysogeny (Luria–Bertani) agar plates. After 24 h of incubation at 37°C, colonies were counted, and results were calculated as $\log_{10}$ CFU/organ or $\log_{10}$ CFU/1 ml blood.

Cell isolation from lung, spleen, and lymph node

Naive noninfected or infected mice were anesthetized via i.p. injection with ketamine/xylazine. To obtain lung cell suspensions, lungs were perfused with PBS through the right ventricle of the heart and then cut into small pieces and digested with 1 mg/ml Collagenase D (Roche) and 50 U/ml DNase I (Sigma-Aldrich) in PBS for 30 min at 37°C, with vortexing every 10 min. Samples were mashed through 70-μm cell strainers and washed with complete RPMI 1640 media (supplemented with 10% FBS, 1 mM pyruvate, 1 mM nonessential amino acids, and 1 mM L-glutamine). Spleens and mediastinal lymph nodes were mechanically disrupted using a syringe plunger in complete RPMI 1640, and cells were filtered similarly. Remaining erythrocytes in lung and spleen samples were lysed with ACK lysis buffer. Single-cell suspensions were used for subsequent analysis.

Staining of cells and analysis by flow cytometry

Fluorophore-conjugated Abs against mouse CD45, CD3, NK1.1, NKp46, DX5, KLRG1, and CD27 were purchased from BD Biosciences, and anti-mouse IL-22 was purchased from BioLegend. Blue-fluorescent reactive dye for distinguishing live and dead cells was purchased from Invitrogen, and dead cells were excluded from all analyses. NK cells were identified by their scatter profile and as having the surface phenotype $CD45^+CD3^-NK1.1^+$ among cells from C57BL/6 WT and $Rag2^{-/-}$ mice and having the surface phenotype $CD45^+CD3^-DX5^+$ among cells from BALB/c $Il22^{-/-}$ mice. Absolute numbers of cells/sample were calculated by adding a known number of fluorescent counting beads (Spherotech) to each of the samples before analysis on the flow cytometer. Intracellular staining for IL-22 and IFN-γ in isolated lung cells was done after activating cells for 4 h with Leukocyte Activation Cocktail with GolgiPlug (BD Biosciences). Cells were fixed and permeabilized using the Cytofix/Cytoperm Plus kit (BD Biosciences), according to the manufacturer's instructions. All samples were analyzed on an LSR II System flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software (version 8.2; TreeStar).

Cytokine assays

Mouse lungs were homogenized with T-PER tissue protein extraction reagent (Pierce) supplemented with complete mini protease inhibitor mixture tablets (Roche) at a proportion of 1 tablet/10 ml T-PER reagent. Lung homogenates were centrifuged at $10,000 \times g$ for 5 min, and supernatants were collected for cytokine analysis. IL-22 levels in lung homogenates were determined using a mouse IL-22 ELISA Construction Kit (Antigenix), according to the manufacturer's instructions.

RT-PCR

Total RNA was purified using a TRIzol Plus RNA purification kit (Invitrogen), according to the manufacturer's instructions. We used 50 ng RNA with the Platinum Quantitative RT-PCR ThermoScript One Step System (Invitrogen) to perform RT-PCR. Primers and probes for *Gapdh*, *Il17a*, *Ifng*, *Tnfa*, and *Il6* were purchased from Applied Biosystems (catalog numbers Mm99999915_g1, Mm00439619_m1, Mm00801778_m1, Mm00443258_m1, and Mm00446190_m1, respectively), and primers and probes for *Il22* were as previously described (16, 20). The reactions were run on an Applied Biosystems 7900HT system using the standard protocol provided by Invitrogen. All PCR reactions used an annealing temperature of 60°C.

Statistics

All quantitative data are shown as mean ± SD, unless otherwise indicated. All samples were compared using a two-tailed, unpaired Student *t* test. Survival analysis was performed using the Gehan–Breslow–Wilcoxon test. A p value < 0.05 was considered significant.

Results

Reduced survival of IL-22–deficient mice during pulmonary infection with K. pneumoniae

It was reported that treatment with anti–IL-22 Ab leads rapidly to 100% mortality after pulmonary infection of C57BL/6 mice with *K. pneumoniae,* strain 43816, serotype 2 (1). We used the same strain of *K. pneumoniae* in two experiments to infect a total of 17 BALB/c $Il22^{-/-}$ mice and 20 BALB/c WT controls. As shown in Fig. 1, $Il22^{-/-}$ mice started to die on day 2, and WT mice started to die on day 3 postinfection. At days 2, 3, 4, 5, 7, 8, 12, and 17 postinfection, 1, 6, 2, 1, 0, 0, 2, and 0 $Il22^{-/-}$ mice and 0, 1, 1, 4, 1, 2, 0, and 2 WT mice died, respectively. These data demonstrated that $Il22^{-/-}$ mice had significantly reduced survival compared with WT mice ($p < 0.05$), although these results are not as dramatic as those reported using anti–IL-22 Ab (1). To help rule

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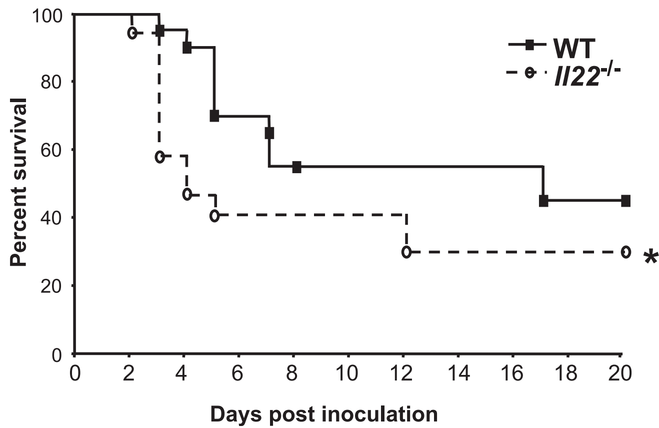

FIGURE 1. IL-22–deficient mice are more susceptible to infection with *K. pneumoniae*. Survival of BALB/c WT (■) and BALB/c *Il22*$^{-/-}$ (○) mice after inoculation in the trachea with 10^3 CFU of *K. pneumoniae*. Data are combined from two independent experiments (total n = 20 in WT group, n = 17 in *Il22*$^{-/-}$ group). *$p < 0.05$, versus WT mice, Gehan–Breslow–Wilcoxcon test.

out an artifactual difference between *Il22*$^{-/-}$ and WT mice that is due to the commercial source of the WT controls, which could differ in their intestinal flora in ways that might affect responses to *K. pneumoniae*, in a separate experiment we compared survival among *Il22*$^{-/-}$, BALB/cAnNTac, BALB/cAnNCr, and BALB/cJ mice. Survival was similar among WT BALB/c mice, irrespective of the commercial colony, and survival was reduced for *Il22*$^{-/-}$ mice versus each of the WT groups ($p < 0.05$, data not shown).

A possible role for NK cells in host defense and IL-22 production

It was proposed that IL-22 production in the first few days after pulmonary infection with *K. pneumoniae* is of T cell origin (1). Given the importance of IL-22 in this model, we were surprised to find that infected *Rag2*$^{-/-}$ mice did not differ from WT mice with regard to survival (Fig. 2A), lung bacterial count (Fig. 2B), and levels of IL-22 in the lungs (Fig. 2C). In contrast, *K. pneumoniae*–infected *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice showed decreased survival (Fig. 2A, $p < 0.05$ for *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ versus *Rag2*$^{-/-}$ and versus WT), had higher bacterial counts compared with WT and *Rag2*$^{-/-}$ mice (Fig. 2B, $p < 0.01$ for *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ versus *Rag2*$^{-/-}$ and versus WT), and failed to produce IL-22 (Fig. 2D). These data suggested a possible role for NK cells or other populations of innate lymphocytes in host defense and the production of IL-22. With regard to non–NK cell innate lymphocytes, a subset of lung-resident ILCs (Lin$^-$CD90$^+$CD25$^+$) was recently identified in both human and mouse that were shown to have a modest ability to

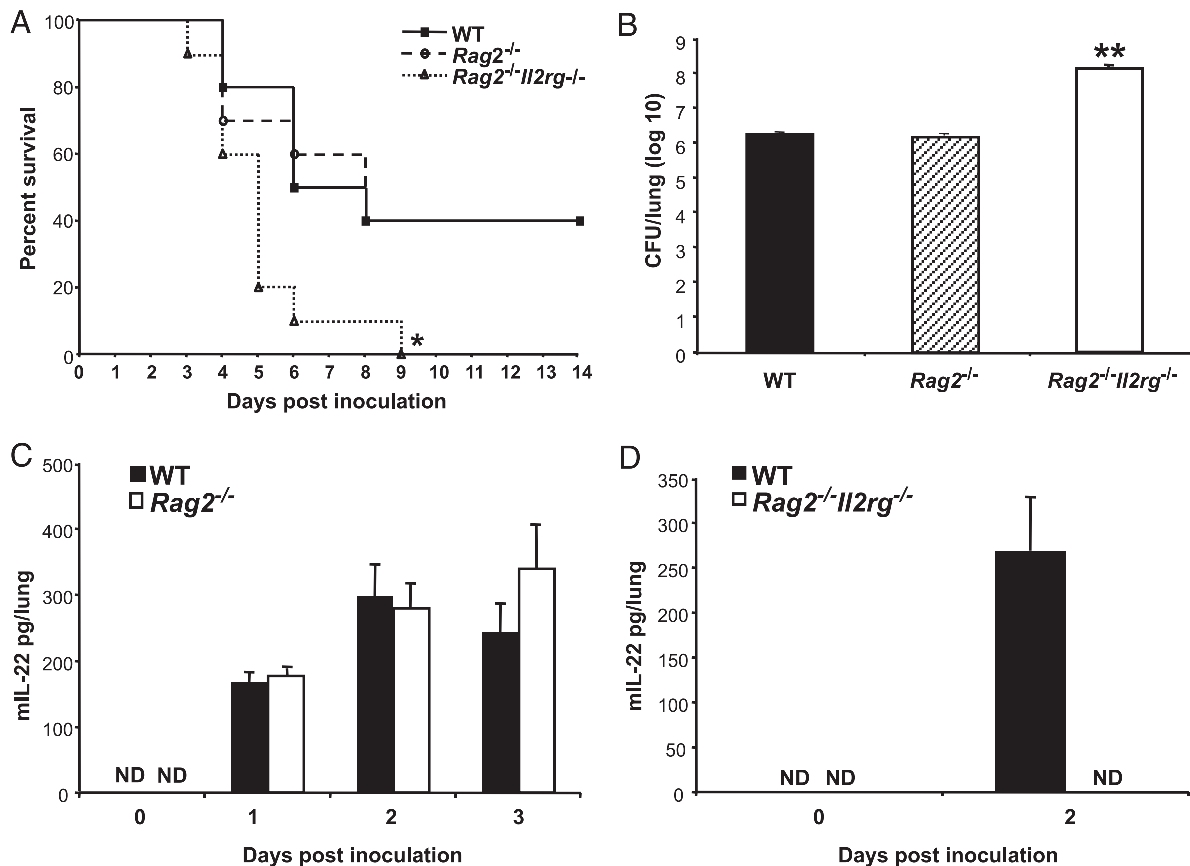

FIGURE 2. *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, but not *Rag2*$^{-/-}$, mice are compromised in their defense against *K. pneumoniae* infection. Mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae* or normal saline alone. (**A**) Survival of C57BL/6 WT, *Rag2*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Data are from 10 mice/group in one representative experiment of two performed. (**B**) *K. pneumoniae* burden in the lung 2 d postinfection in C57BL/6 WT, *Rag2*$^{-/-}$, and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Data are from five mice/group in one representative experiment of two performed. (**C**) ELISA for IL-22 in the lungs of WT and *Rag2*$^{-/-}$ mice. Day 0 data are from mice inoculated with normal saline alone and killed on day 1. Data are from four mice/group in one representative experiment of three performed. (**D**) ELISA for IL-22 in the lungs of WT and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Day 0 data are from mice inoculated with normal saline alone and killed on day 2. Data are from three to five mice/group in one representative experiment of two performed. Error bars represent SD. *$p < 0.05$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice versus other groups, Gehan–Breslow–Wilcoxcon test; **$p < 0.01$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice versus other groups. ND, Not detectable.

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produce IL-22 after stimulation with IL-23 (21). However, in our experiments, we failed to detect a clear population of these cells in the lung either before or postinfection with *K. pneumoniae* (Supplemental Fig. 1).

NK cells contribute to host defense against K. pneumoniae

To examine the involvement of NK cells in this model, a nonactivating Ab, polyclonal rabbit anti-mouse/rat ASGM1 (38), was used successfully to deplete NK cells in vivo (data not shown). NK cell depletion resulted in decreased survival (Fig. 3A, $p < 0.01$) and higher lung bacterial counts (Fig. 3B, $p < 0.01$). One feature of *K. pneumoniae* infection is the dissemination of bacteria to the bloodstream. The effect of NK cells on *K. pneumoniae* dissemination was examined by comparing peripheral blood bacterial counts and liver bacterial counts postinfection between NK cell–depleted and nondepleted mice. Mice treated with anti-ASGM1 displayed significantly increased peripheral blood bacterial counts at days 1 and 2 postinoculation compared with control Ab–treated mice (Fig. 3C, $p < 0.05$ for both days). NK cell depletion also resulted in a significant increase in liver bacterial counts at day 2 postinfection (Fig. 3C, *right panel*, $p < 0.05$). Consistent with these findings, blood bacterial counts were significantly higher in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice versus WT and *Rag2*$^{-/-}$ mice at day 2 postinfection (data not shown).

Decreased production of lung IL-22 postinfection in NK cell–depleted mice

To test our hypothesis that NK cells contribute to IL-22 production in the lung after *K. pneumoniae* infection, we examined the effect of NK cell depletion on lung IL-22, as measured by real-time RT-PCR (Fig. 4A) and ELISA (Fig. 4B). Our results showed that NK cell depletion decreased the production of IL-22 in the lung at 2 d postinfection (Fig. 4A, $p < 0.01$; Fig. 4B, $p < 0.05$), although results for mRNA and protein were discordant, with a dramatic reduction in the former and a more modest reduction in the latter. The effect of NK cell depletion on the induction of mRNAs for other cytokines, including IL-17A, IFN-γ, TNF-α, and IL-6, which were shown to be critical in the protection against infection with *K. pneumoniae* (2, 6, 39–44), was also examined by real-time RT-PCR. NK cell depletion had no effect on the induction of mRNA for IL-17A, but it significantly decreased the induction of mRNAs for IFN-γ, TNF-α, and IL-6 in the lung at 1 d postinfection (Supplemental Fig. 2).

Lung NK cells show conventional, but not NK22/ILC22, surface phenotype

In contrast to conventional NK cells, a population of NK-like cells identified in the mouse intestinal tract, so-called "NK22" or "ILC22" cells, are NKp46$^+$CCR6$^+$ and are limited to NK1.1$^-$ or

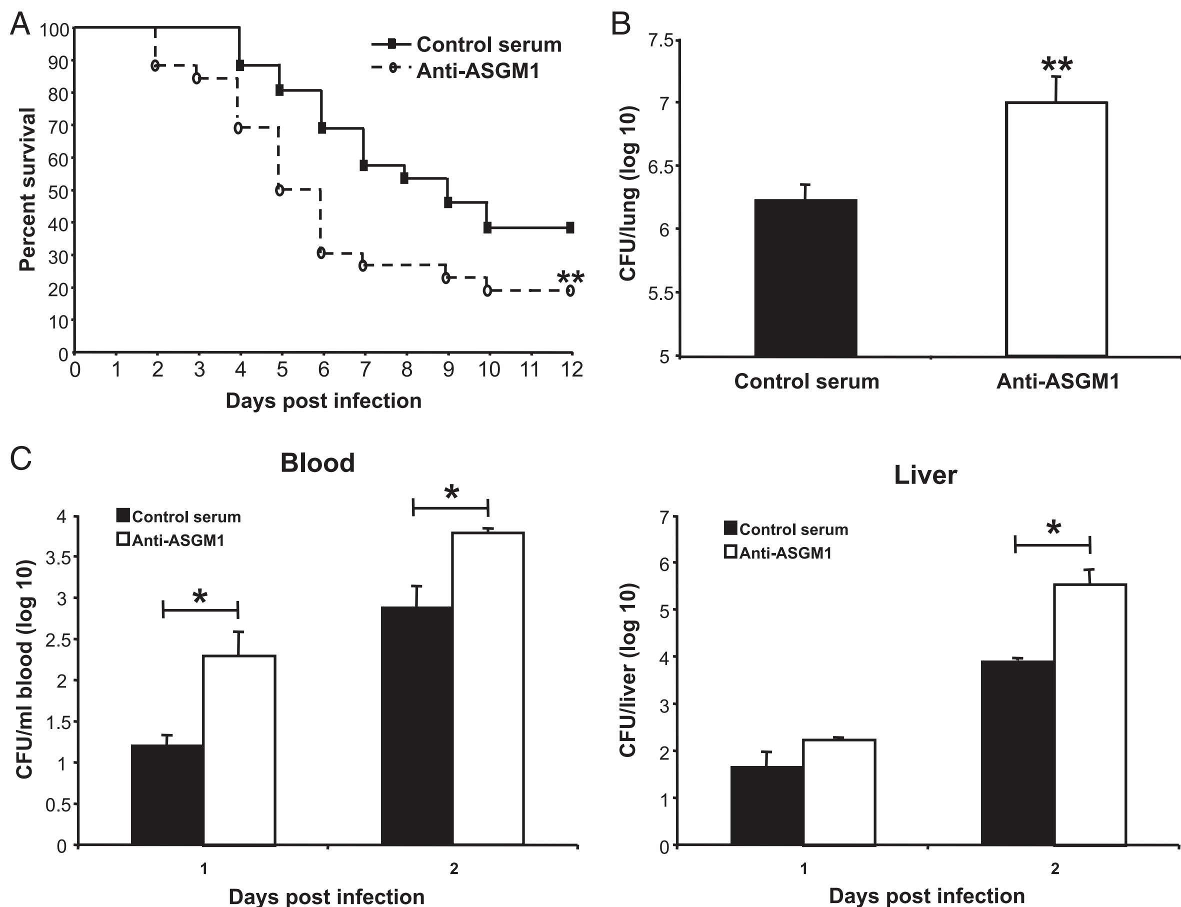

FIGURE 3. NK cell depletion compromises host defense against *K. pneumoniae* infection. C57BL/6 WT mice were injected with anti-ASGM1 or control rabbit serum in PBS; 24 h later, mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae*. (**A**) Survival of anti-ASGM1–treated mice and control rabbit serum–treated mice. Data are combined from two independent experiments (total $n = 26$ in each group). $**p < 0.01$, versus control serum-treated mice, Gehan–Breslow–Wilcoxcon test. (**B**) Lung CFU in anti-ASGM1–treated mice and in control serum-treated mice at day 2 postinfection. Data are from five mice/group in one representative experiment of four performed. $**p < 0.01$, versus control serum-treated mice. (**C**) Blood (*left panel*) and liver (*right panel*) CFU in anti-ASGM1–treated mice and control serum-treated mice at days 1 and 2 postinfection. Data are from four mice/group in one representative experiment of two performed. Error bars represent SD. $*p < 0.05$, anti-ASGM1–treated mice versus control serum-treated mice.

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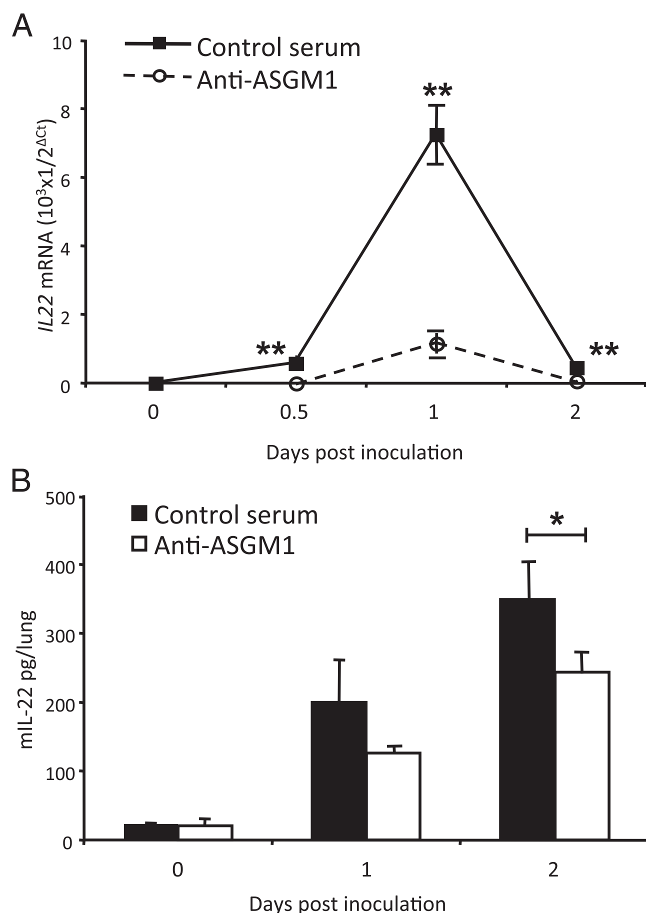

FIGURE 4. NK cell depletion results in decreased production of IL-22 in lungs of infected mice. C57BL/6 WT mice were treated with anti-ASGM1 or control rabbit serum; 24 h later, mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae* or normal saline alone. Lungs were homogenized at various times for quantification of *Il22* mRNA and protein. (**A**) *Il22* mRNA was measured by real time RT-PCR in lungs from mice treated with anti-ASGM1 or control serum. Day 0 data are from mice inoculated with normal saline alone and killed at 0.5 d. Data are from three mice/group in one representative experiment of two performed. (**B**) ELISA for IL-22 in the lungs of anti-ASGM1–treated mice and control serum-treated mice. Day 0 data are from mice inoculated with normal saline alone and killed on day 1. Data are from four mice/group in one representative experiment of three performed. Error bars represent SD. *$p < 0.05$ and **$p < 0.01$, anti-ASGM1–treated versus control serum-treated mice.

NK1.1^{low} subsets. To determine whether these NK-like cells are present in mouse lung, we examined the expression of NK1.1, NKp46, and CCR6 on NK cells from lungs of WT mice. We found no CD3$^-$NK1.1$^-$NKp46$^+$CCR6$^+$ cells. On the contrary, all CD3$^-$NKp46$^+$ cells in the lung were NK1.1$^+$ (Fig. 5A) and lacked CCR6 expression. Because NK cell maturation is associated with KLRG1 upregulation and CD27 downregulation (45, 46), we examined the expression of KLRG1 and CD27 on lung versus splenic NK cells. Overall, lung NK cells showed higher expression of KLRG1 and decreased expression of CD27 compared with splenic NK cells, suggesting that the cells in the lung were more mature (Fig. 5B). The expression of these markers (Fig. 5B) and the numbers of NK cells in the lung (Fig. 5C) were not altered over the first 2 d postinfection with *K. pneumoniae.*

A subset of lung NK cells produces IL-22 early postinfection with K. pneumoniae

Our NK cell–depletion experiments suggested a direct and/or indirect contribution of NK cells to IL-22 production in the *K. pneumoniae*–infected lungs. To examine whether lung NK cells have the ability to produce IL-22 early postinfection, intracellular staining of IL-22 in lung NK cells and T cells was performed following ex vivo stimulation of cells isolated from lungs after 2 d of infection, as well as from lungs of uninfected mice. In WT animals, IL-22 was detected in lung T cells by intracellular staining (Fig. 6A), although only in cells taken from infected mice. These data also show that the infected lungs do not contain a significant population of CD3$^+$NK1.1$^+$ cells (Fig. 6A, *left panel*). Just as for lung T cells, lung NK cells of uninfected WT mice were unable to produce IL-22, whereas a subset of NK cells from *K. pneumoniae*–infected mice was competent to produce IL-22 at day 2 postinfection (Fig. 6B). Similarly, only the NK cells taken from infected lungs were able to produce IFN-γ, and the IL-22–producing NK cells coexpress IFN-γ (Supplemental Fig. 3). In fact, a subset of lung NK cells, but not NK cells in the spleen or in the mediastinal lymph nodes, acquired the ability to produce IL-22 as early as 24 h postinfection (Supplemental Fig. 4).

Although the number of lung NK cells was ∼50–70% of the number of lung T cells in infected animals, the frequency of IL-22–expressing NK cells was ∼3–5-fold that in T cells. Similarly, we presumed that NK cells could be responsible for the production of IL-22 postinfection of *Rag2*$^{-/-}$ mice. As shown in Fig. 6B, infection induced NK cells to become competent to produce IL-22 in *Rag2*$^{-/-}$ mice at a frequency comparable to that in WT mice. In addition, as was described previously (47, 48), *Rag2*$^{-/-}$ mice contained significantly more NK cells in the lung compared with WT mice (Fig. 6C, $p < 0.01$ and $p = 0.01$ at days 0 and 2, respectively), although infection did not alter lung NK cell numbers. Taken together, these data suggest that NK cells could be induced to produce IL-22 early postinfection, that such induction did not require T cells, and that lung NK cells could be a source of IL-22 early postinfection in *Rag2*$^{-/-}$ mice and, more importantly, in WT mice.

Discussion

This study focused on the role of lung NK cells in the production of IL-22 and host defense in a model of bacterial pneumonia using the Gram-negative pathogen *K. pneumoniae*. Previous work showed that pretreatment with anti–IL-22 Ab resulted in 100% mortality after 24 h of infection (1). Consistent with these findings, we demonstrated that *Il22*$^{-/-}$ mice had decreased survival compared with WT mice, although our results showed a less dramatic effect of eliminating IL-22 than did the study using Ab-dependent neutralization. We are not able explain the difference in the magnitudes of the effects. One possibly relevant difference between the Ab-neutralization experiments and our own was that the former were done in C57BL/6 mice, whereas the *Il22*$^{-/-}$ mice we used were in the BALB/c background (and were compared with BALB/c WT mice). Another possible factor, of course, is that the life-long absence of IL-22 produced effects that partially mitigated the lack of IL-22 during *K. pneumoniae* infection. In any case, the data establish a critical role for IL-22 in host defense during *K. pneumoniae* pneumonia.

Published data using models of infection with *K. pneumoniae* or other pathogens suggest several mechanisms whereby IL-22 mediates protection of the host through activities both at epithelial surfaces and systemically. These include stimulating epithelial cell proliferation, enhancing the integrity of the epithelial barrier, activating an acute-phase response, and inducing the production of antimicrobial peptides and immune mediators, such as chemokines and cytokines (1, 15, 18, 49–52). In combination, these activities limit microbial replication and invasion (52–54).

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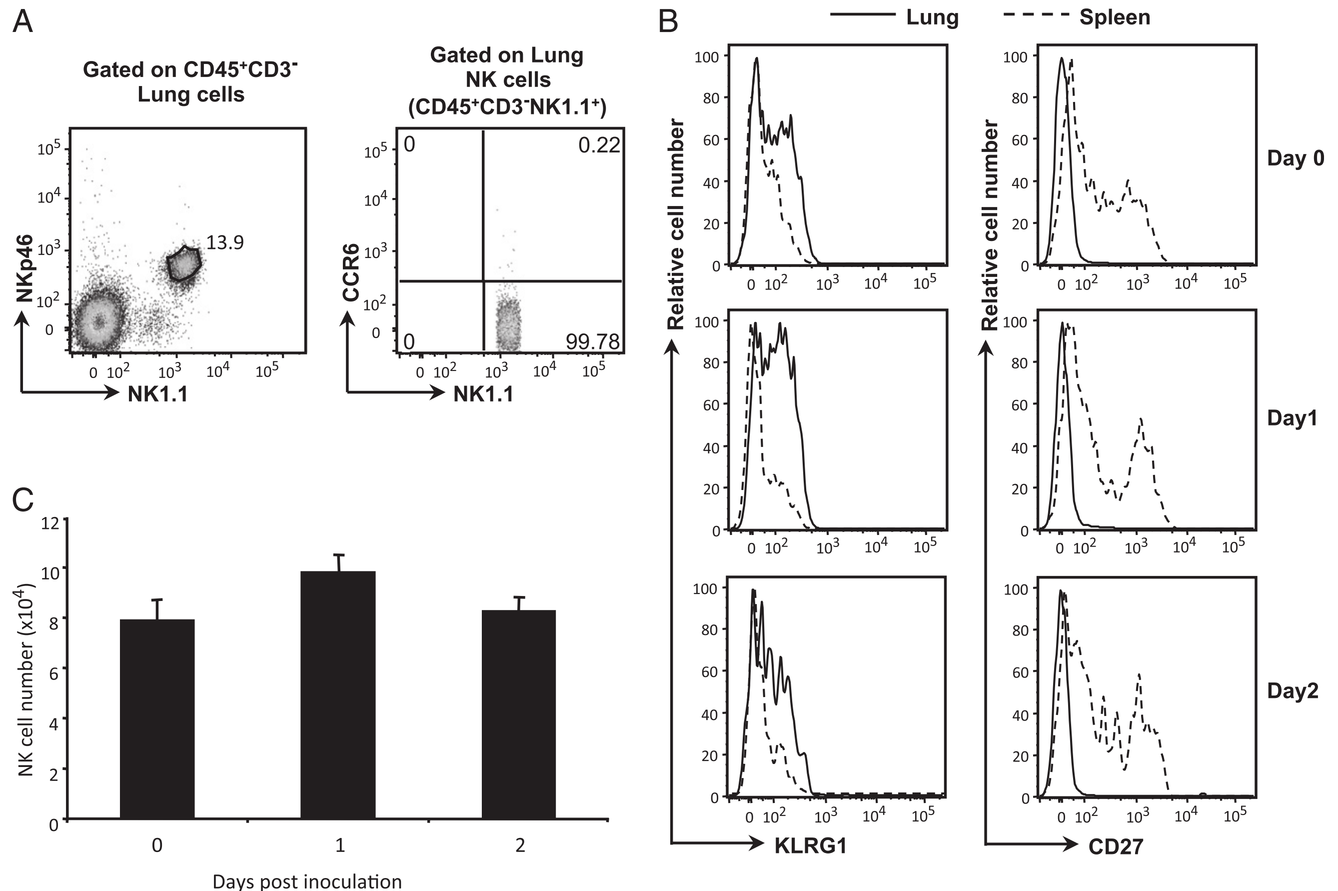

FIGURE 5. Lung NK cells show conventional, mature surface phenotype. Lung cells were isolated from uninfected and *K. pneumoniae*–infected C57BL/6 WT mice. (**A**) Surface expression of NK1.1, NKp46, and CCR6 in $CD45^+CD3^-$ lung cells of uninfected mice. Data are from one mouse, representative of three mice from one experiment, and representative of two experiments performed. (**B**) Expression of KLRG1 (*left panels*) and CD27 (*right panels*) on NK cells ($CD45^+CD3^-NK1.1^+$) from lung and spleen of uninfected mice or mice at days 1 and 2 postinfection by inoculation into the trachea of 10^4 CFU of *K. pneumoniae* or normal saline alone. Day 0 data are from mice inoculated with normal saline alone and killed on day 1. Data are from cells pooled from three mice/group in one representative experiment of two performed. (**C**) Numbers of lung NK cells ($CD45^+CD3^-NK1.1^+$) in uninfected mice or mice at days 1 and 2 postinfection. Day 0 data are from mice inoculated with normal saline alone and killed on day 1. Data are from five mice/group in one representative experiment of two performed. Error bars represent SD.

Although the IL-22 produced during *K. pneumoniae* pneumonia was proposed to be of T cell origin (1), we found no differences in survival or lung bacterial burden or in lung IL-22 in $Rag2^{-/-}$ versus WT mice. In contrast, host defense was significantly compromised in $Rag2^{-/-}Il2rg^{-/-}$ mice, which showed reduced survival and increased bacteria in the lung and produced no detectable IL-22. Together, these results suggested that an ILC contributed to control of pulmonary infection with *K. pneumoniae* through mechanisms that were, at least in part, IL-22 dependent.

We tested this possibility by eliminating NK cells using anti-ASGM1. Treatment with anti-ASGM1 resulted in increased mortality and bacterial burdens in lung, as well as blood and liver, associated with diminished induction of IL-22 and other protective cytokines. Given that IL-22 is of particular importance in maintaining barrier integrity and limiting bacterial invasion (1, 15, 52), a deficiency of IL-22 might have contributed to the extrapulmonary dissemination seen in the NK cell–depleted mice. NK cells also were reported to limit bacterial dissemination through mechanisms that are IL-22 independent (32).

Anti-ASGM1 is used routinely for NK cell depletion, and it has the advantage of being depleting without being activating (38). Nonetheless, there are reports of ASGM1 being expressed on various non-NK cells and/or of anti-ASGM1 depleting non-NK cells in certain experimental models (55–58). For this and other reasons, it was informative to demonstrate that NK cells isolated from infected lungs were able to produce IL-22. Acquiring this ability occurred as soon as 1 d postinfection and was not T cell dependent, because IL-22 could be made by the NK cells from both WT and $Rag2^{-/-}$ mice. Taken together, these data suggested that NK cells could serve as an early and direct source of IL-22 in the lungs of infected animals. NK cells might also contribute to the production of IL-22 through indirect effects mediated by IL-6 and TNF-α, NK cell products (59) that can induce IL-22 in T cells (60). Consistent with this hypothesis, we found that depletion of NK cells also led to decreases in the expression of *Il6* and *Tnfα* in infected lungs. The diminished expression of *Tnfa* might have had broader consequences, because TNF-α was shown to synergize with IL-22 in promoting an inflammatory response (61, 62), and the loss of TNF-α might have compounded the effects of decreased IL-22 in the infected, NK cell–depleted mice.

It is of interest that, even after pharmacological activation ex vivo, NK cells were only able to produce IL-22 if isolated from infected, and not noninfected, lungs. Similarly, only the NK cells taken from infected lungs were able to produce detectable IFN-γ, consistent with reports in other models of infection (63, 64). Because we found no increase in NK cell number in the lungs during infection, these data are consistent with changes in NK cell functionality occurring in situ rather than the recruitment of new,

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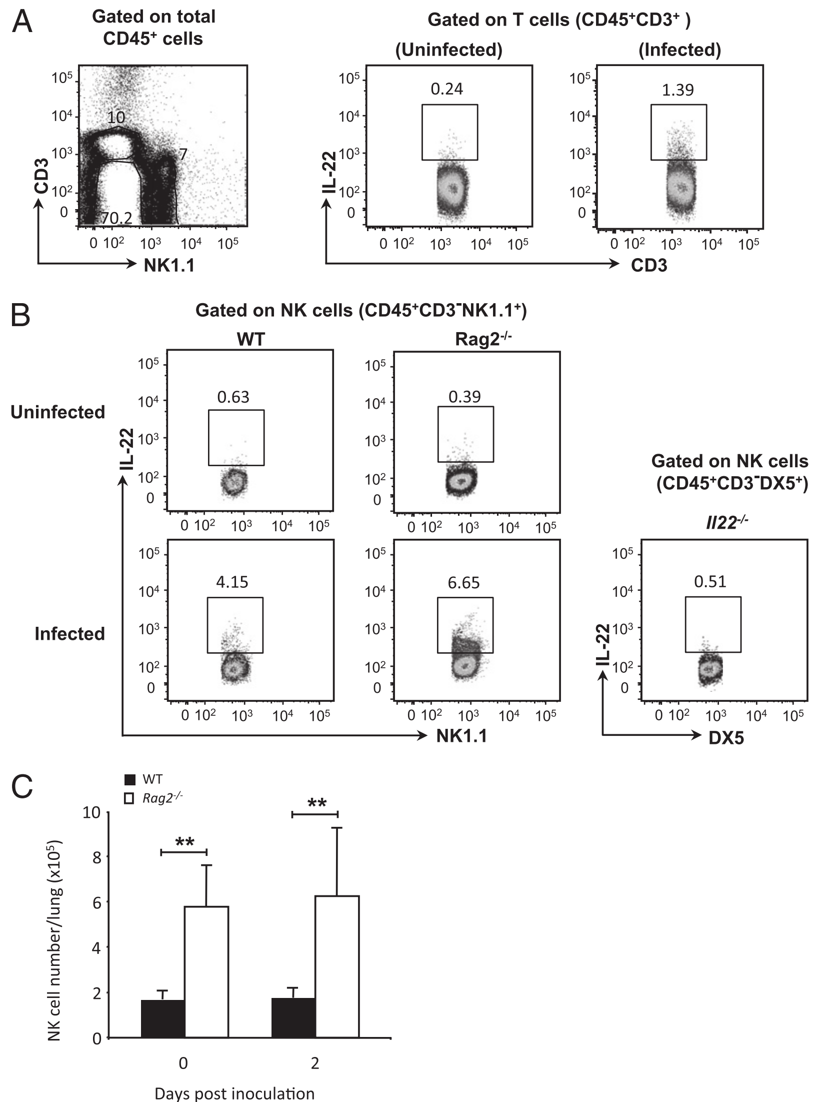

FIGURE 6. A subset of lung NK cells produces IL-22 after *K. pneumoniae* infection. (**A**) WT C57BL/6 mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae* or normal saline alone. Two days postinfection, lung cells were isolated and activated with PMA/ionomycin for 4 h, and intracellular staining was done to detect IL-22 in lung T cells. Dot plot identifying lung T and NK cells (*left panel*). Intracellular staining for IL-22 in lung T cells ($CD45^+CD3^+$) from uninfected and infected WT mice (*right panel*). (**B**) WT C57BL/6 mice and *Rag2*$^{-/-}$ mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae* or normal saline alone. BALB/c *Il22*$^{-/-}$ mice (*Il22*$^{-/-}$), used as a negative control for IL-22 staining, were inoculated in the trachea with 10^3 CFU of *K. pneumonia*e. Two days postinfection, lung cells were isolated and activated with PMA/ionomycin for 4 h, and intracellular staining was done to detect IL-22 in lung NK cells. Staining is shown for $CD45^+CD3^-NK1.1^+$ cells from WT mice and *Rag2*$^{-/-}$mice and for $CD45^+CD3^-DX5^+$ cells from *Il22*$^{-/-}$ mice. Gating to identify IL-22$^+$ cells was set based on staining of the cells from *Il22*$^{-/-}$ mice. Data are for cells pooled from four or five WT or *Rag2*$^{-/-}$ mice and from three *Il22*$^{-/-}$ mice in one representative experiment of two performed. (**C**) C57BL/6 WT mice and *Rag2*$^{-/-}$ mice were inoculated in the trachea with 10^4 CFU of *K. pneumoniae* or normal saline alone. Two days postinfection, lung cells were isolated to quantify the numbers of NK cells ($CD45^+CD3^-NK1.1^+$). Day 0 data are from mice inoculated with normal saline alone. Data are from four or five mice/group in one representative experiment of two performed. Error bars represent SD. $^{**}p < 0.01$, *Rag2*$^{-/-}$ versus WT mice.

IL-22–producing NK cells. In addition, the surface phenotype of the lung NK cells, which suggested that they were more mature than the NK cells in the spleen, did not change during the infection.

Within the T cell population, in addition to Th17 cells (and the Th22 cells in human skin), it was reported that lung invariant NK T cells are able to produce IL-22 (65). Nonetheless, we do not believe that invariant NKT cells were important in our experiments, because we found very few NKT cells in lungs of naive or *K. pneumoniae*–infected mice. Other candidates as sources of IL-22 include non-NK cell populations of ILCs. A subset of lung-resident ILCs ($Lin^-CD90^+CD25^+$) capable of producing IL-22 was recently identified in both human and mouse (21). A second report (47) found very few Lin^-CD90^+ ILCs in lungs of naive mice, and in our experiments we failed to detect a clear population of these cells in the lung either before or postinfection with *K. pneumoniae.*

Other innate lymphocytes of possible relevance are the NK22/ILC22 cells described in the intestinal tract. We found no cells, either in uninfected or infected lungs, with the surface phenotype $NKp46^+CCR6^+$NK1.1-negative or low, which is characteristic of NK22/ILC22 cells. On the contrary, we found that all of the lung NK cells showed a conventional phenotype. Moreover, the IL-22–producing NK cells from infected lungs were also able to make IFN-γ, in contrast to ILC22 cells (36). Our data are consistent with recent reports (15, 66) in an influenza virus model in which IL-22 was produced by conventional lung NK cells that did not express RORγt and were absent from *Il15ra*$^{-/-}$ mice (15). However, these two reports differed somewhat in their findings on the importance of IL-22 in host defense, because neutralizing IL-22 had little effect on clinical outcomes in one study (66), whereas in the second study (15), *Il22*$^{-/-}$ mice showed persistent weight loss after influenza infection. A third report (67) found that NK cells were not a source of IL-22 after influenza infection, and the investigators attributed their apparently discrepant findings to differences in strains of influenza virus.

Although we showed that NK cells are an important component of host defense in the *K. pneumoniae* pneumonia model, the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice died earlier and in greater numbers than did the NK cell–depleted animals. Given the apparent lack of an effect of eliminating T cells, as demonstrated using *Rag2*$^{-/-}$ mice, this finding suggests potential contributions from non-NK, non-T, γ*c*-dependent cells. However, it is also possible that T cells provided functions in the WT, NK cell–depleted mice that were not apparent when comparing *Rag2*$^{-/-}$ versus WT animals. This latter alternative is plausible given the expansion of the NK cell population that we found in the lungs of *Rag2*$^{-/-}$ mice, which may have masked any deficit in IL-22 and other components of host defense resulting from the absence of T cells.

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There are only a small number of studies on the roles for NK cells in bacterial infections of the lung, particularly with regard to extracellular organisms, and both protective and deleterious effects have been described, depending on the model (68). NK cell production of IFN-γ and TNF-α has been implicated in those cases in which NK cells have beneficial activities (68). Our study shows that, during pulmonary infection of mice with *K. pneumoniae*, NK cells are required for optimal host defense, which includes the production of IL-22 in the lung. As far as we are aware, this is the first description of a role for conventional NK cells in producing IL-22 in host defense against extracellular bacteria.

These observations may have clinical relevance. In cases of infection with multidrug-resistant *K. pneumoniae*, not only are there few treatment options among the available antimicrobials, but in colonized and susceptible patients, the adaptive immune system often has been severely compromised, either secondary to underlying disease or iatrogenic factors (10). Based on our data, it is possible that augmenting NK cell function will provide therapeutic benefit in this infection, as was reported in mouse models of viral infection (69) and bacterial sepsis (70). Identifying the factors responsible for enabling NK cells to become producers of IL-22, IFN-γ, and other protective cytokines in response to infection with *K. pneumoniae* may provide avenues worth pursuing in this regard.

Disclosures

The authors have no financial conflicts of interest.

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