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NKG2D Is Critical for NK Cell Activation in Host Defense against Pseudomonas aeruginosa Respiratory Infection

Scott C. Wesselkamper,* Bryan L. Eppert,* Gregory T. Motz,* Gee W. Lau,† Daniel J. Hassett,‡ and Michael T. Borchers²§

Pseudomonas aeruginosa is a major cause of nosocomial respiratory infections. The eradication of P. aeruginosa from the lung involves the orchestrated actions of the pulmonary epithelium and both resident and recruited immune cells. The NKG2D receptor is constitutively expressed on the surface of circulating and tissue-resident NK cells (and other cytotoxic lymphocytes), and is capable of controlling NK cell activation and production of cytokines, such as IFN-γ via interactions with ligands expressed on the surface of stressed cells. Previously, we demonstrated that NKG2D mediates pulmonary clearance of P. aeruginosa. In the present study, we investigated the cellular and molecular mechanisms of NKG2D-mediated clearance of P. aeruginosa using a novel transgenic mouse model of doxycycline-inducible conditional expression of NKG2D ligands (retinoic acid early transcript 1, α) in pulmonary epithelial cells. NKG2D ligand expression in this model increased pulmonary clearance, cellular phagocytosis, and survival following P. aeruginosa respiratory infection. Additionally, NK cell sensitivity to ex vivo LPS stimulation was greater in lung cells isolated from naive transgenic mice administered doxycycline. We also showed that NK cells are the primary source of lymphocyte-derived IFN-γ in response to P. aeruginosa respiratory infection. Significantly, we demonstrated that NKG2D is critical to the nonredundant IFN-γ production by pulmonary NK cells following acute P. aeruginosa infection. These results represent the principal report of NKG2D-mediated activation of lung NK cells following respiratory infection with an opportunistic pathogen and further establish the importance of NKG2D in the host response against P. aeruginosa respiratory infection. The Journal of Immunology, 2008, 181: 5481–5489.

Pseudomonas aeruginosa is a ubiquitous, Gram-negative opportunist pathogen that is a major causative microorganism of nosocomial respiratory infections (1). Importantly, immunocompromised patients are at increased risk for P. aeruginosa infection, and it is the predominant cause of morbidity and mortality in patients with cystic fibrosis (CF) (2–4). P. aeruginosa is a frequently identified pathogen in patients with ventilator-associated pneumonia (a severe complication of intensive care), and has a high mortality rate compared with other pathogens (34–48%) (5). Additionally, P. aeruginosa is associated with exacerbations of chronic obstructive pulmonary disease (6). The pathogenesis of P. aeruginosa lung infection is complex, and because of its ubiquitous nature and ability to develop resistance to antibiotics, P. aeruginosa respiratory infections are problematic and difficult to treat.

The clearance of P. aeruginosa from the airways involves the coordinated effort of multiple cell types, including the respiratory epithelium and both resident and recruited immune cells. NK cells are cytotoxic lymphocytes generally recognized for their crucial role in the innate immune response against viral infections and tumors (7). Persistent bacterial infections occurring in NK cell-deficient patients underscore the clinical importance of these cells in the immune response to bacterial pathogens (8). Although the cytotoxic function of NK cells seems to be minor during bacterial infections, their production of cytokines is significant. In particular, lung NK cell-derived IFN-γ plays an important role in expunging various types of pulmonary bacterial infections (9–11). However, the role of NK cells and NK cell-derived IFN-γ in the eradication of P. aeruginosa respiratory infection is unclear.

NK cell activation is controlled by a balance of signals between activating and inhibitory receptors. The NKG2D-activating receptor is constitutively expressed on the surface of circulating and tissue-resident NK cells and other cytotoxic lymphocytes (12–14), and NKG2D activation stimulates cytotoxic effects of these cells against virally infected, transformed, or stressed cells in vitro and in vivo (15). Importantly, the recognition of NKG2D ligands also induces production of several cytokines, including IFN-γ (16, 17). NKG2D binds distinct, but structurally related ligands based on recognition of structural patterns (18, 19). Several families of NKG2D ligands have been identified in mice and include retinoic acid early transcript 1, α to ε (Rae1a, b, c, d, e), histocompatibility 60, Ulbp1 (20, 21), and MHC I-like leukocyte 1 (22).

Previously, we reported that NKG2D ligands are expressed on stressed airway epithelial cells (23). Our laboratory also provided the first evidence that P. aeruginosa is a potent inducer of NKG2D

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ligands in pulmonary epithelial cells following in vitro and in vivo infection (24). Importantly, we also showed that NKGD2 receptor blockade inhibited pulmonary clearance of *P. aeruginosa* in mice, indicating that NKGD2 effector function is required for a complete host response. In the current study, we investigated the cellular and molecular mechanisms of NKGD2-mediated pulmonary clearance of *P. aeruginosa* using a novel mouse model of doxycycline (DOX)-inducible conditional expression of *Rae1* in pulmonary epithelial cells. Using this model, we determined a role for NKGD2 ligand expression in bacterial clearance, cellular phagocytosis, and survival following acute *P. aeruginosa* respiratory infection. Moreover, we demonstrate that NKGD2 ligand expression increases NK cell sensitivity to bacterial products, and that NKGD2 is critical for IFN-γ production by NK cells following acute *P. aeruginosa* infection.

**Materials and Methods**

**Mice**

*Ccp-rtta* transgenic mice (FVB/NJ background) were previously generated by Tichelaar et al. (25). Mice bearing the target (*tetO*)-CMV-*Rae1* transgene were generated at the University of Cincinnati Medical Center using RAET1a cDNA obtained by PCR cloning, as previously described (25). Bisttransgenic *Ccp-rtta* × (*tetO*)-CMV-*Rae1* mice (hereafter referred to as *Rae1*-*tg* mice) were identified using PCR primers specific for each homogenized in 1 ml of PBS with a VirTis Tenbroeck tissue grinder. Serial dilutions of lung homogenates diluted in PBS were plated onto tryptic soy agar plates and incubated overnight at 37°C, and individual colony counts in terms of log_{10} CFU were determined.

**RAET1 immunohistochemistry**

Naive *Rae1*-*tg* mice were euthanized with an i.p. injection of sodium pentobarbital (Nembutal, 150–200 mg/kg; Henry Schein), followed by exsanguination by severing of the posterior abdominal aorta. Lung tissue was obtained, processed, and immunostained for RAET1, as described previously (24).

**P. aeruginosa inoculation**

The stationary-phase *P. aeruginosa* strain PAO1 (26), a burn wound isolate (strain SBI-N), and a PAO1 strain harboring a GFP-expressing plasmid (PAO1-GFP) were used. Briefly, isolated single colonies grown on tryptic soy agar plates were inoculated in Luria broth, followed by overnight incubation at 37°C on an orbital shaker (60 rpm). The digested lungs were sheared through 19- and 21-gauge needles, and filtered through 40-μm cell strainers (BD Biosciences) to obtain a single-cell suspension. Residual RBCs were lysed with RBC lysis solution (Qiagen), and cells were then centrifuged in 30% Percoll (Sigma-Aldrich). Cells were then washed, resuspended in 100 μl of FACS buffer, and incubated with 1 μg of purified mouse BD Fc block at 4°C for 10 min. The following Abs were used for cell surface staining of lymphocytes: allophycocyanin-conjugated rat anti-mouse NKGD2 (clone CX3; e Bioscience), PE-conjugated rat anti-mouse CD8a (clone 53-6.7; BD Biosciences), PerCP-Cy5.5-conjugated hamster anti-mouse CD3ε (clone 145-2C11; BD Biosciences), FITC-conjugated rat anti-mouse CD4 (clone GK1.5; BD Biosciences), FITC-conjugated TCR γδ (clone GL3; BD Biosciences), and goat anti-mouse NKp46 (AF2225; R&D Systems). For cell surface staining of NKp46, cells were washed, resuspended in 100 μl of FACS buffer, and incubated with 1 μg of allophycocyanin-conjugated donkey anti-goat IgG secondary Ab (F0108; R&D Systems) for 30 min on ice. Additionally, the appropriate isotype-matched control Abs were used, as follows: PE rat IgG1 (12-4301; eBioscience), FITC hamster IgG2 (clone B81-3; BD Biosciences), and PerCP-Cy5.5 hamster IgG1 (clone A19-3; BD Biosciences). After staining, cells were washed and fixed in 500 μl of 4% paraformaldehyde, and fluorescence was measured in the FL1 channel.

**Cellular phagocytosis**

After 2 h of infection with *5 × 10^6* CFU PAO1 constitutively expressing GFP, *Rae1*-*tg* mice were euthanized, as described above, and the lungs were lavaged with two 1-ml aliquots of HBSS without Ca^2+ and Mg^2+ (pH 7.2, 37°C; Invitrogen). Total cell counts were performed with a hemacytometer using trypan blue (Invitrogen). Recovered bronchoalveolar lavage (BAL) fluid samples were then centrifuged (400 × g, 10 min, 4°C), and the cell pellets were washed with 2 ml of FACS buffer (0.5% BSA/0.05% sodium azide in PBS) before flow cytometry. Cells were resuspended in 100 μl of FACS buffer and incubated with 1 μg of purified mouse BD Fc block (rat anti-mouse CD16/CD32 (clone 2.4G2; BD Biosciences)) for 10 min at 4°C. For cell surface staining, cells recovered from the BAL (i.e., BAL cells) were incubated with allophycocyanin-conjugated rat IgG2a isotype control (eBioscience) for 30 min on ice. Cells were washed and resuspended in 500 μl of FACS buffer. Flow cytometry was performed immediately using a BD FACS Calibur system, and the data were analyzed using BD CellQuest Pro software. BAL cells were identified based on autofluorescent (AF) properties in the FL2 channel as well as cell surface expression of F4/80, as follows: F4/80^− (i.e., alveolar macrophages, monocytes) and AF^*F4/80*^− (i.e., neutrophils and lymphocytes). Phagocytosis of PAO1-GFP was detected by increased fluorescence in the FL1 channel.

**Characterization of NKGD2**^+^ lymphocytes in the lung

Naive FVB/NJ mice were euthanized, and the lungs were then voided of blood by perfusion through the right ventricle with 10 ml of PBS containing 0.6 mM EDTA. Lungs were withdrawn aseptically from the chest cavities, homogenized in 5 ml of PBS with a Dounce homogenizer, and digested in 5 ml of RPMI 1640 with 2.05 mM t-glutamine (HyClone) containing 175 U/ml collagenase 1-A, 0.2 U/ml pancreatic elastase, 35 U/ml hyaluronidase, 20 kU/ml Dnase I (Sigma-Aldrich), 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (MP Biomedicals) for 1 h at 37°C on an orbital shaker (60 rpm). The digested lungs were sheared through 19- and 21-gauge needles, and filtered through 40-μm cell strainers (BD Biosciences) to obtain a single-cell suspension. Residual RBCs were lysed with RBC lysis solution (Qiagen), and cells were then centrifuged in 30% Percoll (Sigma-Aldrich). Cells were then washed, resuspended in 100 μl of FACS buffer, and incubated with 1 μg of purified mouse BD Fc block at 4°C for 10 min. The following Abs were used for cell surface staining of lymphocytes: allophycocyanin-conjugated rat anti-mouse NKGD2 (clone CX3; e Bioscience), PE-conjugated rat anti-mouse CD8a (clone 53-6.7; BD Biosciences), PerCP-Cy5.5-conjugated hamster anti-mouse CD3ε (clone 145-2C11; BD Biosciences), FITC-conjugated rat anti-mouse CD4 (clone GK1.5; BD Biosciences), FITC-conjugated TCR γδ (clone GL3; BD Biosciences), and goat anti-mouse NKp46 (AF2225; R&D Systems). For cell surface staining of NKp46, cells were washed, resuspended in 100 μl of FACS buffer, and incubated with 1 μg of allophycocyanin-conjugated donkey anti-goat IgG secondary Ab (F0108; R&D Systems) for 30 min on ice. Additionally, the appropriate isotype-matched control Abs were used, as follows: PE rat IgG1 (12-4301; eBioscience), FITC hamster IgG2 (clone B81-3; BD Biosciences), and PerCP-Cy5.5 hamster IgG1 (clone A19-3; BD Biosciences). After staining, cells were washed and fixed in 500 μl of 2% parafomaldehyde, and flow cytometry was performed, as described above.

**Ex vivo NK cell activation**

Naive *Rae1*-*tg* mice were euthanized; the lungs were harvested and digested; and the RBCs were removed, as described above. The cells were then resuspended in RPMI 1640 with 2.05 mM t-glutamine (HyClone) containing 10% FBS, 1% sodium pyruvate, 100 μg/ml kanamycin, 0.05 mM 2-ME, 1X nonessential amino acids (MP Biomedicals), and 20 U/ml mouse rIL-2, and 5 × 10^6 cells/well were aliquoted into a 96-well round-bottom culture plate (Costar). The cells were stimulated with 1, 10, and 100 ng/ml LPS from *Escherichia coli* O111:B4 (L5293; Sigma-Aldrich) or sterile PBS (unstimulated) for 22 h at 37°C in a humidified incubator containing 5% CO2. Brefeldin A (10 g/ml; eBioscience) was added to all wells at the final 4 h of incubation. The cells were washed, resuspended in 100 μl of FACS buffer, and incubated with 1 μg of purified mouse BD Fc block at 4°C for 10 min. Cell surface staining of NKp46 was performed, as described above. The cells were then fixed in 100 μl of fixation buffer (2% paraformaldehyde) for 20 min at room temperature, and then washed with permeabilization buffer (FACS buffer containing 0.1% saponin (MP Biomedicals)). The cells were resuspended in 100 μl of permeabilization buffer containing 10% mouse serum (MP Biomedicals), and incubated for 15 min at 4°C to block. After washing, cells were again resuspended in 100
performed.

Flow cytometric analysis of NK cell recruitment

Four and 16 h postinfection with $1 \times 10^7$ CFU PAO1 or SBI-N, FVB/NJ mice were euthanized; the lungs were lavaged, harvested, and digested; and the RBCs were removed, as described above. Cells from the BAL and whole lung compartments were stained for NKp46, as described above. The cells were washed and fixed in 500 μl of 2% paraformaldehyde, and flow cytometry was performed.

Intracellular cytokine staining of NK cell-derived IFN-γ

Four hours postinfection with $1 \times 10^7$ CFU PAO1, FVB/NJ mice were euthanized; lungs were harvested and digested; and the RBCs were removed and aliquoted into 96-well round-bottom culture plates, as described above. The cells were maintained at 37°C overnight in a humidified incubator containing 5% CO₂, and brefeldin A (10 μg/ml; eBioscience) was added for the final 4 h of incubation. Lung NK cell-derived IFN-γ was determined by flow cytometry, as described above. Cells were washed and fixed in 500 μl of 2% paraformaldehyde, and flow cytometry was performed.

Anti-NKG2D administration

NKG2D receptor function was blocked by administration of functional grade purified anti-mouse NKG2D (clone CX5; eBioscience), as described previously (24, 27). The CX5 Ab blocks NKG2D ligand binding and inhibits NK cell function in vitro and in vivo (27, 28). Briefly, 16 h before infection with $1 \times 10^7$ CFU PAO1 or SBI-N, FVB/NJ mice were given an i.p. injection of 100 μg of anti-NKG2D or functional grade purified rat IgG1 isotype control (clone A110-1; BD Biosciences) for 30 min on ice. Cells were washed and fixed in 500 μl of 2% paraformaldehyde, and flow cytometry was performed.

Statistical analyses

Significant differences among groups were identified by ANOVA wherever appropriate, and individual comparisons between groups were confirmed by a post hoc Tukey test. For the survival study, a Peto-Peto test was used to assess a significant difference in survival between groups. For all analyses, a p value < 0.05 was considered statistically significant.

Results

Transgenic expression of NKG2D ligands

To characterize the in vivo pulmonary response elicited by overexpression of NKG2D ligands following acute *P. aeruginosa* infection, we generated a transgenic mouse model that allowed for the conditional expression of Raet1a in pulmonary epithelial cells. We expressed Raet1a in pulmonary epithelial cells under the control of DOX administration using the Ccsp-rtta transgenic system previously described (25). We established three separate transgenic Raet1a-tg mouse lines (lines 20, 22, and 32) bearing the target (tetO)-CMV-Raet1a transgene. Line 22 Raet1a-tg mice exhibited robust Raet1a transgene induction, and these mice were used in all of the studies presented in this work. Immunohistochemical staining of lung tissue from line 22 Raet1a-tg mice confirmed RAET1 expression was not evident in mice that did not receive DOX (Fig. 1, A and C), but is induced throughout the airway and alveolar epithelium following DOX administration (Fig. 1, B and D).

NKG2D ligand expression increases pulmonary clearance and survival following *P. aeruginosa* respiratory infection

We have previously shown that NKG2D receptor blockade inhibits pulmonary clearance of *P. aeruginosa* (24). To assess whether overexpression of *Raet1a* ameliorates the eradication of *P. aeruginosa* from the lungs, Raet1a-tg mice treated with or without DOX were intranasally infected with $1 \times 10^7$ CFU PAO1 or SBI-N. At 16-h postinfection, Raet1a-tg mice administered DOX exhibited significantly greater clearance of PAO1 than mice that did not receive DOX (Fig. 2A). Similar to PAO1, Raet1a-tg mice treated with DOX exhibited significantly greater clearance of SBI-N than mice that did not receive DOX (Fig. 2B). For control purposes, single transgenic mice that were positive for the (tetO)-CMV-Raet1a transgene, but did not express the Ccsp-rtta transgene, were also treated with or without DOX. In these studies, no
FIGURE 2. NKG2D ligand expression increases pulmonary clearance and survival following acute P. aeruginosa respiratory infection. Raet1a-tg mice treated with or without DOX were intranasally infected with 1 × 10^9 CFU PAO1 (A) and SBI-N (B), and bacterial CFU in the lungs were assessed 16 h postinfection. Data are presented as means ± SEM (n = 5–7 mice/group). *, Denotes significant difference from PAO1-infected Raet1a-tg mice not administered DOX, p < 0.05.

NK cells constitute the majority of NKG2D^+ pulmonary lymphocytes

NK cells, CD8^+ T cells, and γδ^+ T cells express NKG2D in mice (12–14). To thoroughly survey the abundance of NKG2D-expressing lymphocytes in the lungs of FVB/NJ mice, flow cytometry was performed on isolated lung lymphocytes. NK cells were defined as Nkp46^+ cells in the lymphocyte gate. Although CD4^+ T cells > NK cells > CD8^+ T cells > γδ^+ T cells in terms of the relative abundance of pulmonary lymphocytes (Fig. 5A), only NK cells and γδ^+ T cells were predominantly NKG2D^+ (Fig. 5B). Furthermore, NK cells comprised the greatest percentage of total NKG2D^+ pulmonary lymphocytes (Fig. 5C), with γδ^+ T cells, CD4^+ T cells, and CD8^+ T cells accounting for a small proportion of the NKG2D^+ population.

NKG2D ligand expression increases NK cell-derived IFN-γ following ex vivo LPS stimulation

To determine whether overexpression of Raet1a altered intracellular IFN-γ levels in resident lung lymphocytes, lung cells were isolated from naive Raet1a-tg mice treated with or without DOX and stimulated ex vivo in culture with 1, 10, and 100 ng/ml LPS for 22 h, and flow cytometry was performed. LPS was used because it is a potent TLR ligand known to induce intracellular production of IFN-γ.}

FIGURE 4. NKG2D ligand expression increases phagocytosis of P. aeruginosa in BAL cells. Data are presented as means ± SEM (n = 10 mice/group). *, Denotes significant difference from PAO1-GFP- or PAO1-GFP^+ infected Raet1a-tg mice not administered DOX, p < 0.05.
IFN-γ in NK cells. There was no difference in the number of IFN-γ-producing NK cells (defined as NKp46 IFN-γ+ cells in the lymphocyte gate) between mice administered DOX as compared with mice that were not given DOX at baseline (i.e., unstimulated). After 22 h of LPS stimulation, there was a dose-dependent increase in the number of IFN-γ-producing NK cells from the lungs of mice treated with or without DOX (Fig. 6). However, mice administered DOX had significantly more IFN-γ-producing NK cells compared with mice that did not receive DOX following stimulation with all three doses of LPS, with the greatest difference observed after stimulation with the lowest dose (1 ng/ml). LPS stimulation also induced IFN-γ in non-NK cell lymphocytes (defined as NKp46 IFN-γ+ cells in the lymphocyte gate) in a dose-dependent manner in lung cells from mice treated with DOX and untreated mice. No differences in LPS-induced non-NK cell IFN-γ production were observed between the DOX treatment groups.

Lung NK cell recruitment following P. aeruginosa respiratory infection

FVB/NJ mice were intranasally infected with 1 × 10^7 CFU PAO1 (or left uninfected for control purposes), and the lungs were lavaged and then digested 4 and 16 h postinfection (n = 3 mice/group). Flow cytometry was then performed to determine the presence of NK cells (defined as NKp46+ cells in the lymphocyte gate) in the BAL and whole lung compartments. The total numbers of NK cells in the whole lung compartment were not increased over uninfected control values at either time point postinfection (Fig. 7A). The total number of NK cells in the BAL compartment was

![Figure 5](https://www.jimmunol.org/)

**FIGURE 5.** Characterization of NKG2D expression on pulmonary lymphocytes. Lung cells were harvested from naive FVB/NJ mice, and lymphocytes were isolated and cell surface stained with the following Abs: allophycocyanin-conjugated NKG2D, PE-conjugated CD8a, PerCP-Cy5.5-conjugated CD3e, FITC-conjugated CD4, FITC-conjugated TCR γδ, and unconjugated NKp46 (followed by an allophycocyanin-conjugated secondary Ab to distinguish NK cells), and cells were analyzed by flow cytometry. The percentage of pulmonary lymphocytes (A), the percentage of lymphocyte populations that express NKG2D (B), and the percentage of total NKG2D+ lymphocyte populations (C) are presented as means ± SEM (n = 3 mice).

![Figure 6](https://www.jimmunol.org/)

**FIGURE 6.** NKG2D ligand expression increases intracellular IFN-γ in NK cells following ex vivo stimulation with LPS. Lung cells were harvested from naive Raet1a-tg mice treated with or without DOX and stimulated with 1.0, 10, or 100 ng/ml LPS or sterile PBS (unstimulated) for 22 h, and the presence of IFN-γ-producing NK cells was assessed. For analysis by flow cytometry, cells underwent cell surface staining with an unconjugated NKp46 Ab and an allophycocyanin-conjugated secondary Ab (to distinguish NK cells), followed by intracellular staining with a FITC-conjugated IFN-γ Ab. Numbers in the upper right quadrant are percentage of NK cells that are IFN-γ+ (NKp46 IFN-γ+). Numbers in the lower right quadrant are percentage of non-NK cell lymphocytes that are IFN-γ+ (NKp46 IFN-γ+). Dot plots shown are gated on lymphocytes and are representative of two mice per group from four independent experiments.
not increased over control 4 h postinfection, but was significantly increased 16 h after infection (Fig. 7B). However, NK cells in the BAL compartment at this time point postinfection represent only ~3.7% of the total number of NK cells in the entire lung. P. aeruginosa preferentially induces IFN-γ in NK cells following respiratory infection

NK cell-derived IFN-γ is important in the host response and eradication of many pathogens. To analyze the capacity of lung NK cells to produce IFN-γ following P. aeruginosa respiratory infection, FVB/NJ mice were intranasally infected with 1 × 10^7 CFU PAO1 for 4 h (or left uninfected for control purposes), and total lung cells were isolated and analyzed by flow cytometry to determine the presence of IFN-γ-producing NK cells (defined as NKp46^+ IFN-γ^+ cells in the lymphocyte gate). Significantly more lung NK cells from PAO1-infected mice produced IFN-γ than similar cells from uninfected control mice (Fig. 8). Furthermore, the number of IFN-γ-producing NK cells was five times greater than other IFN-γ-producing non-NK cell lymphocytes (defined as NKp46^+ IFN-γ^+ cells in the lymphocyte gate) following infection.

NKG2D is critical to NK cell-derived IFN-γ production following P. aeruginosa respiratory infection

Because P. aeruginosa induces NK-cell derived IFN-γ following respiratory infection and NKG2D ligand expression increases the number of NK cells producing IFN-γ following ex vivo stimulation with LPS, we hypothesized that blockade of NKG2D can abate IFN-γ production by lung NK cells in P. aeruginosa-infected mice. FVB/NJ mice were administered a NKG2D-blocking Ab 16 h before intranasal infection with 1 × 10^7 CFU PAO1 or SBI-N (or left uninfected for control purposes). Mice pretreated with the anti-NKG2D Ab had significantly fewer IFN-γ-producing lung NK cells compared with mice that did not receive the Ab following respiratory infection with PAO1 (Fig. 9, B and D). Compared with PAO1, mice pretreated with the anti-NKG2D Ab also had fewer IFN-γ-producing lung NK cells compared with mice that did not receive the Ab following respiratory infection with SBI-N (Fig. 9, D and E).

Discussion

Pulmonary epithelial cells are the initial line of defense against exposure to inhaled pathogens. However, our understanding of the role of these cells in signaling to resident mucosal immune cells is limited. We have previously established that NKG2D ligand expression is induced on pulmonary epithelial cells following acute P. aeruginosa respiratory infection, and NKG2D blockade inhibits the eradication of P. aeruginosa from the lungs (24). In the current study, we show that NKG2D-mediated pulmonary epithelial cell-lymphocyte interactions before respiratory infection enhance the host defense against P. aeruginosa. Furthermore, we demonstrate a critical role for NKG2D in the nonredundant production of IFN-γ by resident lung NK cells following acute P. aeruginosa infection.

Pulmonary epithelial cell-specific induction of NKG2D ligands enhanced the antibacterial defense of the lung, as evidenced by...
increased bacterial clearance and cellular phagocytosis in our model of *P. aeruginosa* lung infection, and those outcomes could account for the observed increase in survival in DOX-treated *Raet1a*-tg mice infected with a lethal dose of PAO1. Because NK cells constitute the majority of resident NKG2D+ cells in the pulmonary submucosa and parenchyma of naive FVB/NJ mice (Fig. 5C), we initiated investigations on the role of NKG2D ligand expression on NK cell activation to determine the cellular and molecular mechanisms of NKG2D-mediated host defense against *P. aeruginosa*. In naive *Raet1a*-tg mice, expression of NKG2D ligand by itself did not induce NK cell-derived IFN-γ. Thus, our ex vivo results demonstrate that resident lung NK cells are primed by NKG2D ligand expression to produce greater amounts of IFN-γ in response to pathogenic stress. We hypothesize that the priming of resident NK cell-derived IFN-γ can augment the early microbial effector functions of resident alveolar macrophages (e.g., phagocytosis) following acute *P. aeruginosa* lung infection. Our data implicate that pulmonary epithelial cell-NK cell interactions are vital in directing production of NK cell-derived IFN-γ at the mucosal interface, and these interactions within the local microenvironment are important in the early host defense against acute *P. aeruginosa* respiratory infection. Furthermore, priming of NK cell activation by NKG2D ligand expression before pathogenic stress could also be clinically significant as a potential therapeutic strategy useful in the treatment of *P. aeruginosa* respiratory infection.

In addition to conditional expression of *Raet1a* in pulmonary epithelial cells before infection, there are most likely alternative sources of NKG2D ligand production that are generated following acute *P. aeruginosa* respiratory infection in our *Raet1a*-tg model. Immunohistochemical analysis previously revealed that RAET1A was strongly induced in the conducting airway epithelium, alveolar epithelium, and alveolar macrophages of mice 24 h after *P. aeruginosa* lung infection (24). Thus, it is plausible that *P. aeruginosa*-induced NKG2D ligand expression on alveolar macrophages and epithelial cells may contribute to persistent NKG2D-mediated production of IFN-γ from resident NK cells (and perhaps those that infiltrate into the alveolar space) following respiratory infection. The induction of the NKG2D receptor-ligand system by *P. aeruginosa* could lead to sustained NKG2D-mediated up-regulation of the microbial effector functions of infiltrating inflammatory cells at later time points postinfection, resulting in the facilitation of pulmonary clearance.

The data from our *Raet1a*-tg model suggest that the detection and response of pathogen by lung NK cells may involve interactions between NKG2D and TLRs. Multiple TLRs mediate *P. aeruginosa* recognition and signaling in vivo (29). Hamerman et al. (30) demonstrated that TLR stimulation could increase the expression of NKG2D ligands. Additionally, *P. aeruginosa* can directly induce NKG2D ligand expression on pulmonary epithelial cells in vitro (24). These observations, taken with our finding that NKG2D ligand expression increases NK cell sensitivity to LPS-induced TLR stimulation (as assessed by intracellular IFN-γ), led us to postulate that NKG2D and TLR costimulation creates an amplification loop following recognition of pathogen. In other words, NK cells have the capacity to detect the presence of pathogens indirectly via up-regulation of NKG2D ligands, thus sensing stress via the NKG2D receptor-ligand system. This indirect signaling, in turn, could lower the threshold at which NK cells respond to the presence of pathogen directly through the engagement of TLRs. The activation threshold of non-NK cells was also lowered after LPS treatment, suggesting that the occurrence of
NKG2D and TLR costimulation is not NK cell specific. Our findings indicate that the NKG2D and TLR pathways operate in a coordinated effort in pathogen recognition and eradication. A more complete understanding of these pathways and the mechanisms controlling their communication is necessary.

Early, innate induction of IFN-γ is critical to immunological defense against multiple pathogens, and resident NK cells in the pulmonary submucosa and parenchyma are a prodigious source of IFN-γ. NK cell-derived IFN-γ is induced in the host defense repertoire of several animal models of respiratory bacterial infection, including Bordetella pertussis (9), Mycobacterium tuberculosis (10), and Shigella flexneri (11). However, compared with P. aeruginosa respiratory infection in which NK cell-derived IFN-γ was the primary, nonredundant source of lymphocyte-derived IFN-γ, NK cells are not the principal source of lung lymphocyte-derived IFN-γ following B. pertussis, M. tuberculosis, and S. flexneri infections (e.g., T cells and NKT cells also produce redundant, significant amounts of IFN-γ). Thus, the early, nonredundant production of lung NK cell-derived IFN-γ is a unique feature of P. aeruginosa respiratory infection in our mouse model.

Different strains of P. aeruginosa can vary significantly in their virulence properties. For example, many clinical isolates derived from the lungs of CF patients lack the intercellular signaling system known as quorum sensing (31). Such P. aeruginosa strains are significantly impaired in murine models of burn and airway infection (32). Another significant component of P. aeruginosa virulence that involves direct contact of the bacterium to host cells is the type III secretion system (TTSS). TTSS involves a syringe-like apparatus that injects effector molecules (mostly toxins) into host cells (33). The type III apparatus was shown by Holder et al. (34, 35) to be critical for infection in a mouse burn model. Because of the possibility of strain-specific virulence effects in our mouse model, we also used P. aeruginosa strain SBI-N that is fully capable of both quorum sensing and TTSS to corroborate our findings with those obtained using PAO1. Raet1a-tg mice treated with DOX exhibited greater clearance of SBI-N than untreated mice in a manner similar to PAO1. Likewise, SBI-N-infected FVB/NJ mice pretreated with the anti-NKG2D Ab had fewer IFN-γ-producing lung NK cells compared with mice that did not receive the Ab. Compared with PAO1 respiratory infection and consistent with the increased virulence of strain SBI-N, SBI-N-infected Raet1a-tg mice exhibited a greater bacterial burden, as well as a greater NGK2D-mediated NK cell IFN-γ response in FVB/NJ mice. The findings suggest that NGK2D-regulated bacterial clearance and NK cell activation represent conserved outcomes in the host response to acute respiratory infection with different strains of P. aeruginosa that vary in their virulence characteristics in our mouse model.

NKp46 is selectively expressed by NK cells across multiple species, and creates a centralized phenotypic definition of NK cells based on NKp46 cell surface expression (36). As opposed to measuring IFN-γ in a mixed lymphocyte population or via ELISA in whole lung homogenates, we were able to specifically identify NK cells as the predominant lymphocyte producer of IFN-γ by using the NKp46 cell surface marker in combination with isolated lung cells from infected mice. A great deal of support for IFN-γ in the clearance of P. aeruginosa has been previously proposed. Previously, Moser et al. (37) showed an improved outcome of chronic P. aeruginosa respiratory infection in mice by an IFN-γ, Th1-dominated response. Rat models of P. aeruginosa respiratory infection show that pulmonary clearance is enhanced after preadministration of exogenous IFN-γ via adenoviral vectors (38), and i.p. treatment with rat rIFN-γ diminished the magnitude of pulmonary inflammation following P. aeruginosa infection (39). Of clinical importance, there is also mounting evidence that an IFN-γ, Th1-dominated immune response might improve the prognosis of CF patients with chronic P. aeruginosa lung infection (40, 41). Taken together, these studies support the importance of IFN-γ in protection against P. aeruginosa lung infection.

Several in vivo studies have examined the role of NGK2D in NK cell responses directly against tumors (42), as well as cells infected with an intracellular pathogen (43–45). Thus, the current paradigm for NGK2D signaling in the removal of pathogens is centered on the direct recognition of infected cells that express NGK2D ligands and their subsequent NK cell-mediated removal. In the present study, we demonstrate that NK cell activation (as assessed by IFN-γ production) is critically dependent on NGK2D signaling following acute P. aeruginosa respiratory infection. Our data represent the first observation of NGK2D-mediated activation of lung NK cells following respiratory infection with an extracellular pathogen. Significantly, our findings also expand the current paradigm of the NGK2D receptor-ligand system in that extracellular P. aeruginosa is capable of inducing NGK2D ligands on bystander cells, such as pulmonary epithelial cells or alveolar macrophages, resulting in NGK2D-mediated activation of resident NK cells. In contrast to the current NGK2D-signaling paradigm in which activation of NK cells results in the direct NK cell-mediated removal of NGK2D ligand-expressing infected cells, we postulate that activated NK cells indirectly ameliorate the eradication of extracellular pathogen through IFN-γ-mediated augmentation of antibacterial defense mechanisms. Thus, our data indicate that NGK2D is necessary and sufficient for NK cell activation and clearance of acute P. aeruginosa in our mouse model of respiratory infection.

In the present study, we show that pulmonary epithelial cell-specific NGK2D ligand expression in a transgenic mouse model increases pulmonary clearance, cellular phagocytosis, and survival following P. aeruginosa infection. Additionally, NGK2D ligand expression increases NK cell sensitivity to LPS. We unequivocally demonstrate that the nonredundant production of NK cell-derived IFN-γ following P. aeruginosa respiratory infection is dependent upon NGK2D. These findings advance our understanding of the mechanisms of interactions between the NGK2D receptor and pulmonary epithelial cell-derived ligands in the lung, and their effects on the pulmonary innate immune system. Because of the ubiquitous nature of P. aeruginosa and its ability to develop resistance to antibiotics, therapeutic strategies are limited and respiratory infections are problematic and difficult to treat. Thus, novel approaches that target NK cells, and more specifically NGK2D, may lead to alternative therapeutics designed to improve the outcome of P. aeruginosa respiratory infection.

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References


