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Swift and Strong NK Cell Responses Protect 129 Mice against High-Dose Influenza Virus Infection

Kai Zhou,*† Jing Wang,*† Jie An Li,* Wenming Zhao,* Dongfang Wang,*† Wei Zhang,* Jinghua Yan,* George Fu Gao,* Wenjun Liu,* and Min Fang*

It is generally unclear what roles NK cells play during influenza virus infection with regard to different host genetic backgrounds. In this study, we show that in six inbred mouse strains, NK cells play an important protective role only in 129 mice during high-dose influenza A H1N1 virus infection. Swift and strong NK cell responses efficiently control early pulmonary viral replication in 129 mice, providing survival privilege. In addition, we identified that early activation of TLRs and RIG-I signaling in 129 mice resulted in quick production of type 1 IFNs and inflammatory cytokines, which are important reasons for the swift kinetics of NK cell responses post influenza virus infection. Thus, under different microenvironments, NK cells play differential roles against viral infections. The kinetics and magnitude of NK cell responses correlate with the distinct roles that NK cells play against influenza virus infections. Thus, our works further our understandings about the complex role of NK cells during influenza virus infection. The Journal of Immunology, 2016, 196: 000–000.

In recent years, frequently emerging influenza A virus (IAV) epidemics have become a challenge to both veterinary and public health worldwide, such as the 2009 pandemic influenza A H1N1, avian influenza A H5N1, and H7N9. A variety of host cells, including ciliated epithelial cells, type I and II alveolar cells, and immune cells, are infected by IAV within the respiratory tract (1–4). IAV-infected cells are eliminated through two major mechanisms: viral replication-mediated apoptosis or necrosis (5, 6) and immune system–mediated viral clearance (7, 8). The pathogenesis and outcome of influenza virus infection are the result of the balance between host defense mechanisms and viral pathogenicity. Both innate and adaptive immunity are activated and play important roles in viral clearance.

NK cells are large granular lymphocytes that mediate innate protection from some viral infections and tumor cells (9, 10). In humans, NK cells are essential for resistance to several viral infections, such as EBV, human CMV, varicella-zoster virus, and HSV (11–14). In mice, NK cells are clearly critical to control mouse CMV (15–17) and the agent of mousepox, ectromelia virus (18–20). The roles that NK cells play during influenza virus infection are intricate. Several studies highlight the pivotal role of NK cells in the control of IAV infection in that defects in NK cell activity or depletion of NK cells result in delayed viral clearance and increased morbidity and mortality (21–23). However, there are also examples in which NK cells exacerbate morbidity and pathology during lethal dose influenza virus infection in mice (24, 25), which indicate that NK cells may play dual roles during influenza virus infection in mice, conferring beneficial or deleterious function depending on the viral dose. Furthermore, production of IL-22 by NK and NKT cells has an important role in the repair of epithelial damage caused by IAV (26, 27). Meanwhile, NK cells play important roles in bridging the innate and adaptive immune responses to IAV (28). The major and complex roles of NK cells in IAV infection still require further in-depth investigations.

Previous studies show that host genetic background strongly influences the responses to IAV infections (29, 30). However, the functions of NK cells during IAV infection with regard to different host genetic backgrounds remain unclear. In this study, we demonstrate that NK cells contribute to IAV clearance and recovery in 129 but not in five other mouse strains, suggesting that the genetic background and infecting virus dose strongly influence the importance of NK cells in recovery from IAV infection.

Materials and Methods

Ethics statement

The mouse experimental design and protocols used in this study were approved by the Regulation of the Institute of Microbiology, Chinese Academy of Sciences (permit no. PZIM-CAS2012012008). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China.

Virus, mice, and infections

The mouse-adapted influenza A/Puerto Rico/8/34 (H1N1; PR8) strain was propagated in the chorioallantoic cavities of 10-d-old specific pathogen-free embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology) for 48–72 h at 37°C; then allantoic fluids were harvested and stored in aliquots at −80°C. Virus titers were determined by plaque assays on Madin-Darby canine kidney cells (ATCC, CCL-34). In brief, 10-fold serial dilutions of the virus were used to infect confluent Madin-Darby
canine kidney cells in 12-well plates for 1 h at 37°C. The virus inoculums were removed by washing with PBS. Cell monolayers were then overlaid with agar medium (DMEM supplemented with 1% low melting point agarose and 1 µg/ml N-tosyl-L-phenylalanyl chloromethyl ketone–treated trypsin) and incubated at 37°C for 48–72 h. The plates were first fixed with 4% paraformaldehyde for 1 h; the agarose overlays were carefully removed. Staining buffer (0.1% crystal violet and 20% ethanol in water) was added to the wells and stained for 10 min. The staining buffer was subsequently aspirated, the plates were counted, and virus titers were calculated accordingly.

Inbred mouse strains B6, 129S2/SvPasCrlivr (129), FVB, C3H, BALB/c, and DBA/2 were obtained from Vital River, China. All mice were housed in an animal facility under specific pathogen-free conditions. For infection experiments, mice were transferred to a biosafety level 2 room. Mice (8–10 wk of age) were i.p. anesthetized with trichloroacetaldehyde hydrate (375 mg/kg body weight) and inoculated intranasally with indicated PR8 virus dose in 20 µl sterile PBS. Postinfections, weight loss and survival of infected mice were observed daily for a period of 12–14 d. In addition to mice that were found dead, mice with weight loss of >30% of the starting body weight were euthanized and recorded as dead.

Isolation of lung lymphocytes
The isolation of lung lymphocytes was adapted as described previously (18). In brief, mice were anaesthesized from the orbital cavity to decrease the amount of blood in the lung. The lung was then removed and passed through a cell strainer (BD Falcon) to obtain a single-cell suspension. The cells were resuspended in 35% Percoll solution (in D-Hank’s buffer) and centrifuged at 830 x g for 15 min at room temperature. The upper phase liquid was removed from the tube; the lymphocyte pellet was resuspended in 0.84% NH4Cl solution to lyse the RBCs and then washed twice with medium.

BrdU incorporation assay
Detection of BrdU incorporation in NK cells was performed as described previously (18). In brief, at the indicated times postinfection, mice were injected with 2 mg BrdU i.p. Three hours later, the peripheral blood (PB) was collected. Spleens and lymph nodes (LN)s were removed and made into single-cell suspensions. The lung lymphocytes were obtained as described earlier. The cells were then stained for cell-surface molecules, fixed, permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions, incubated with DNase at 37°C for 1 h, and subsequently stained with FITC-conjugated anti-BrdU mAb (eBioscience).

Flow cytometry
Detection of NK cell responses was performed as previously described (18, 31). In brief, to determine NK cell responses in the PB, LN, spleen, and lung, we incubated single-cell suspensions in 96-well plates at 37°C in medium containing 50 IU/ml IL-2, monensin (BD Biosciences), and FITC-conjugated anti-CD107a Ab for 2 h. Then the cell lysate was added into single-cell suspensions. The lung lymphocytes were obtained as described earlier. The cells were then stained for cell-surface molecules, fixed, permeabilized, and stained for intracellular cytokines using the Cytofix/Cytoperm kit according to the manufacturer’s instructions. The following mAb and staining reagents were used: FITC-labeled anti-mouse CD107a mAb (clone: 1D4B) and allophycocyanin-labeled anti-mouse IFN-γ (clone: XMG1.2) were purchased from BD Biosciences; PE-labeled anti-mouse NKp46 mAb was purchased from eBioscience; and PerCP-labeled anti-mouse CD3e mAb (clone: 145-2C11) was purchased from Sungen Biotech (Tianjin, China). The stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star).

In vitro NK cell stimulation and IFN-γ assay
The ectodomain of influenza virus hemagglutinin (HA) was expressed in Hi5 insect cells as previously described (32). Flat-bottom, high protein–binding plates were coated with anti-NKGD2 Ab CX5 (10 µg/ml) or HA (50 µg/ml) at 4°C overnight. After extensive washing, B6 or 129 splenocytes (105/well) in complete RPMI 1640 medium containing 10 µg/ml brefeldin A and 500 IU/ml recombinant mouse IL-2 (BD Pharmingen) were added to triplicate wells. Cells were incubated for 5 h at 37°C, stained for surface cell markers and intracellular IFN-γ, and analyzed by flow cytometry.

Quantitative real-time PCR
Universal ProbeLibrary probes were purchased from Roche. Primers were synthesized at Sangon, Beijing. The primers used are listed in Table I. Total RNA was extracted from the lungs of infected mice with TRIZol reagent (Invitrogen). The first-strand cDNA was synthesized by using oligonucleotide primers. Quantitative real-time PCR was performed using a LightCycler 480 (Roche). The cycling conditions for real-time PCR were: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. The fold-increase in mRNA expression was determined using the ΔΔCt method relative to the values in mock-treated samples after normalization to GAPDH gene expression.

Cytokine and chemokine analysis
Mice were sacrificed at the indicated times, and pulmonary homogenates were prepared. Supernatants were stored at −80°C. Cytokine and chemokine levels were measured using a Mouse Cytokine Magnetic 20-plex Panel (Invitrogen) kit according to the manufacturer’s instructions and read on a Luminex 100 (Bio-Rad).

In vivo depletion of NK cells
To deplete NK cells in vivo, we injected anti-asialo GM1 antisera (20 µl; Wako Chemicals) i.p. into the mice 1 d before influenza virus infection.

Western blotting
Mice were sacrificed at the indicated times, and pulmonary homogenates were resuspended in cell lysis buffer (98038; Cell Signaling). Then SDS-PAGE loading buffer was added to the samples. The samples were heated to 100°C for 5 min, and cell lysates were analyzed by 10% SDS-PAGE. Proteins were electrotransferred to polyvinylidene fluoride membranes and probed with a monoclonal anti-IRF7 Ab (Santa Cruz) and an anti-β-actin Ab. Bound Abs were detected using an HRP-conjugated anti-mouse IgG secondary Ab (ZSGB-BIO, Beijing, China) and ECL development (Pierce) according to the manufacturer’s protocol.

Histopathology
For histological analysis, lung tissues were removed and fixed with 4% paraformaldehyde for ≥12 h, dehydrated in a gradient ethanol series, and embedded in paraffin. Tissue sections (5 µm) were cut and stained with H&E.

Statistics
All values represent the mean ± SD. Intergroup differences in means and medians were examined by t test or two-way ANOVA. Two-tailed p values <0.05 were considered significant. GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for data analysis and preparation of all graphs.

Results

NK cells protect 129 mice against high-dose PR8 infection
Previous studies show that different inbred mouse strains exhibit large differences in their response to 2 × 106 PFU PR8 infection (29). To determine whether genetic backgrounds affect host responses against a higher-dose PR8 infection, we infected six different inbred mouse strains [C57BL/6 (B6), FVB, BALB/c, 129, DBA/2, and C3H] with 1 × 106 PFU PR8. As shown in Fig. 1A and 1B, we observed large differences in the kinetics of weight loss and survival rates. B6 and FVB mice were more resistant to PR8 infection than other strains, showing less weight loss (Supplemental Table I) and higher survival rates. In contrast, BALB/c, 129, DBA/2, and C3H mice were highly susceptible: all of the mice lost weight very rapidly, and >70% died. These results indicate that inbred mouse strains exhibit different resistance to high-dose PR8 infection.

NK cells are innate effector cells that serve as a first line of defense against certain viral infections and tumors. The protective functions of NK cells during IAV infection have been demonstrated before (21, 23). However, there are also reports indicating that NK cells exacerbate morbidity and pathology during high-dose IAV infection in mice (24, 25). Most of these studies were performed in B6 mice, and the roles of NK cells in other inbred mouse strains during IAV infection are less studied. To determine whether NK cells are required for resistance to IAV infection in other inbred mouse strains, we depleted NK cells in our six experimental mouse strains with anti-asialo GM1 antisera. As shown in
A significant difference in survival rates between B6 and FVB mice. Also, between B6/FVB and DBA/2, FVB, C3H, BALB/c mice. There were no significant differences of survival rates and survival rates were performed with two-way ANOVA and log-rank means.

Weight loss was compared with NK cell–intact B6 mice. In contrast, NK cell depletion in 129 mice did not significantly affect virus replication in the lungs of infected B6 mice, but the extent was less conspicuous than the lungs of 129 mice (Fig. 3). In addition, no significant differences in the inflammation in bronchioles, edema, or alveolitis were noted between NK cell–intact and NK cell–depleted B6 mice (Fig. 3).

**FIGURE 1.** Six inbred mouse strains exhibited different morbidity and mortality post high-dose PR8 infection. B6, DBA/2, FVB, C3H, BALB/c, and 129 mice were infected intranasally with 1 × 10^6 PFU PR8 virus. Weight loss (A) and survival (B) of infected mice were monitored. For all mouse strains, data are from at least three independent experiments with three to five mice per group in each experiment. Data points indicate means ± SD. Statistical analysis of weight loss (see Supplemental Table I) and survival rates were performed with two-way ANOVA and log-rank (Mantel–Cox) test. There were significant differences of survival rates between B6/FVB and DBA/2, FVB, C3H, BALB/c mice. There were no significant differences of survival rates between B6 and FVB mice. Also there were no significant differences of survival rates among DBA/2, C3H, BALB/c, and 129 mice. *p < 0.05.

Fig. 2A and Supplemental Fig. 1, anti-asialo GM1 antiserum treatment efficiently depleted NK cells, but not other cell populations in mice. The intact and NK cell–depleted mice were infected with 1 × 10^6 PFU PR8; at 3 d postinfection (dpi), mice were sacrificed, and the lung homogenates were measured by plaque assay for virus titers. Surprisingly, with the exception of 129 mice, NK cell depletion in B6, BALB/c, C3H, FVB, and DBA/2 mice did not significantly affect virus replication in the lungs (Fig. 2B). In contrast, NK cell depletion resulted in significantly increased virus titers in 129 mice. Because NK cells displayed different functions in the control of virus replication between 129 and other inbred mouse strains, we chose B6 mice and 129 mice for further investigations.

B6 and 129 mice were infected with PR8 and monitored daily for disease and death. As shown in Fig. 2C, even though NK cell–depleted B6 mice displayed a slower recovery of body weight, there was no significant difference in weight loss and mortality rate compared with NK cell–intact B6 mice. In contrast, NK cell depletion in 129 mice resulted in pronounced body weight loss, and 100% of the mice died, indicating that NK cells play an important protective role during high-dose PR8 infection of 129 mice (Fig. 2D).

Histological analysis of infected mice at 4 dpi revealed striking differences between tissue lesions in NK cell–intact and NK cell–depleted 129 mice. The lungs from the NK cell–depleted 129 mice exhibited more inflammatory infiltrates than those from the NK cell–intact 129 mice. The overall lung tissues were more densely consolidated with larger numbers of affected airways in NK cell–depleted 129 mice compared with NK cell–intact 129 mice, and the epithelia of the alveolar and bronchiolar walls were seriously disrupted and thickened because of massive infiltration of leukocytes. In contrast, tissue inflammation was also observed in the lungs of infected B6 mice, but the extent was less conspicuous than the lungs of 129 mice (Fig. 3). In addition, no significant differences in the inflammation in bronchioles, edema, or alveolitis were noted between NK cell–intact and NK cell–depleted B6 mice (Fig. 3).

**Swift kinetics of NK cell responses in 129 mice after PR8 infection**

To determine NK cell responses post PR8 infection, we measured NK cell percent, as well as IFN-γ and surface CD107a expression of NK cells in 129 and B6 mice at different times postinfection. Surface expression of CD107a correlates with CD8+ T cell and NK cell cytotoxicity (33). As shown in Fig. 4A, compared with uninfected mice, the percentage of CD3+ NKp46+ NK cells in the local draining LN significantly increased in 129 mice at 1 dpi, continued to increase at 3 dpi, then decreased at 5 dpi. The total NK cell number also significantly increased at 1 dpi, peaked at 3 dpi, and began to decline at 5 dpi. More importantly, NK cells produced large amounts of IFN-γ at 1 dpi, which began to decrease from 3 dpi (Fig. 4B). Also, NK cell–surface CD107a expression was slightly upregulated in 129 mice at 1 dpi. In B6 mice, in contrast, the NK cell percent in the LN increased at 3 dpi and also started to decrease at 5 dpi. Moreover, NK cells in B6 mice displayed a delayed kinetics of IFN-γ production in the LN with a peak at 3 dpi (Fig. 4B). Aside from the delayed kinetics of NK cell responses in B6 mice, the overall magnitude of NK cell responses as measured by the percent of IFN-γ–producing NK cells was also lower in B6 mice than in 129 mice.

Similarly, NK cell activation in the lung was earlier in 129 mice than in B6 mice (Fig. 4C, 4D). We found a significant increase in IFN-γ production in the lung NK cells in 129 mice at 1 dpi, which peaked at 3 dpi. In B6 mice, the IFN-γ production of lung NK cells was slightly upregulated at 1 dpi and peaked at 3 dpi, but the percentage of IFN-γ–producing NK cells was lower than in 129 mice (Fig. 4D). The production of IFN-γ in the lung NK cells began to decrease at 5 dpi in both 129 and B6 mice. The NK cell responses all peaked at 5 dpi in both mice strains in the spleen, but the responses were much weaker compared with the LN and lung (data not shown). After viral infection, NK cells can migrate to infected organs through the PB (34). Consistently, we found that the NK cell percent was dramatically reduced in the PB of 129 mice at 1 dpi. In B6 mice, this decrease in NK cell percentage in the PB happened at 3 dpi, 2 d later than in 129 mice (Fig. 4E), indicating that after PR8 infection, NK cells in 129 mice may quickly migrate to the LN and lung from the PB and become activated.

**Recruitment, but not proliferation, resulted in early NK cell accumulation in 129 mice**

Our previous results demonstrated an early decrease in the NK cell percent in PB, indicating that NK cells might migrate to the lung.
and LN via the PB. However, the early activation of NK cells might also lead to NK cell proliferation. To determine whether the increase of NK cells in the lung and LN resulted from recruitment and/or proliferation, we inoculated B6 and 129 mice infected with PR8 at different times postinfection with BrdU i.p. and sacrificed the mice 3 h later to determine BrdU incorporation by flow cytometric analysis. As shown in Fig. 5A and 5B, <3% of NK cells incorporated BrdU in the lung, LN, and PB during the first 5 dpi in B6 and 129 mice. Less than 10% of NK cells (6.6 ± 1.7 for 129 mice, 6.8 ± 1.4 for B6 mice) proliferated in the spleen at 3 dpi. Thus, the early increase of NK cells in the lung and LN in 129 mice is mostly due to recruitment rather than proliferation.

Previous studies demonstrate CCR2-dependent NK cell migration during IAV infection (35). Thus, we next assessed the
changes in the mRNA levels of MCP-1 (Table I), a CCR2 ligand, at early times postinfection. As shown in Fig. 5C, transcription of MCP-1 was significantly induced in 129 mice at 6 h postinfection (hpi), but not in B6 mice. A dramatic increase in the MCP-1 concentration in the lung in 129 mice at 24 hpi was also confirmed by cytokine Luminex assays (Fig. 5D). Thus, the high expression level of MCP-1 in 129 mice may be an important reason for the early recruitment of NK cells to infected organs.

Cytokines rather than differential Ag stimulation account for the swift kinetics of NK cell responses in 129 mice

NK cells can be activated by specific ligation of activating receptors or by cytokines (16, 36). Previous studies show that recognition of viral HAs of influenza virus-infected cells by NK cell receptor NKp46 activates NK cells (37). To determine whether the NK cells from 129 or B6 mice respond differently to HA, we incubated splenocytes from naïve 129 or B6 mice for 5 h with plate-immobilized HA or the anti-NKG2D mAb CX5 (as a positive control); then NK cells were analyzed by flow cytometry for intracellular IFN-γ production. We found that CX5 and HA induced IFN-γ production in both 129 and B6 NK cells. In fact, B6 NK cells responded more strongly to both CX5 and HA stimulation than 129 NK cells (Fig. 6A). Thus, the strong NK cell responses in 129 mice are not likely caused by differential stimulation of NK cells through ligation of NKp46 and HA.

We next investigated whether the swift NK cell responses in 129 mice resulted from differential stimulation of cytokines. We used Luminex assays to assess cytokine levels in pulmonary homogenates after PR8 infection. Surprisingly, PR8 infection in 129 mice resulted in high levels of cytokines [TNF-α, IFN-γ, IL-1α, IL-1β, IL-5, IL-6, IL-12, CCL3 (MIP1α), chemokine (C-X-C motif) ligand 9 (MIG), MCP1, chemokine (C-X-C motif) ligand 1 (KC), and GM-CSF] compared with uninfected mice (Fig. 6B) at 1 dpi. In contrast with the early cytokine responses in 129 mice, there was no significant increase of measured cytokines in B6 mice at 1 dpi. A recent study shows that influenza-infected 129 mice produce increased levels of type 1 IFNs compared with B6 mice (30). Indeed, we also found strong and early upregulation of Ifna4 and Ifnb1 transcripts (Table I) in 129 mice (relative to B6 mice) post PR8 infection (Fig. 6C).

The TLR and RIG-I signaling pathways all play important roles in the initiation of anti-influenza virus responses, including the type 1 IFNs and proinflammatory cytokines (38, 39). Thus, we further determined the transcription levels of different TLRs after PR8 infection (Table I). Among the TLRs, we found that TLR3 transcription increased (although not significantly) in both B6 and...
PR8 infection resulted in swift and strong NK cell responses in 129 mice. B6 or 129 mice were infected with $1 \times 10^6$ PFU PR8. Lymphocytes from local draining mediastinal LN (A and B), lung (C and D), or PB (E) were analyzed by flow cytometry. (A) Representative flow cytometry plots with gating strategy and summary graphs showing the NK cell percent in lymphocytes and total NK cell numbers in the draining LN at the indicated time postinfection. (B) Representative flow cytometry plots and column graphs showing the frequency of IFN-γ and CD107a surface expression in gated NKp46⁺CD3⁻ NK cells. (C) As in (A) but showing the lymphocytes from the lung. (D) As in (B) but showing the NK cells from the lung. (E) NK cell percent in the PB after PR8 infection. As labeled in the graph, there were significant decreases in NK cell percent at 1 dpi of infected 129 mice compared with uninfected 129 mice, and at 3 dpi of infected B6 mice compared with uninfected B6 mice. Data correspond to the mean ± SD from four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
129 mice from 6 hpi, which is consistent with a previous study showing that TLR3 is involved in anti-IAV responses (38). Surprisingly, we also found that the mRNA level of TLR9 was significantly increased in 129 mice from 12 hpi but remained unchanged until 24 hpi in B6 mice (Fig. 6D and data not shown). Accordingly, the mRNA levels of the downstream molecule IRF7 were also upregulated in 129 mice early after viral infection (Fig. 6D). In addition, the RIG-I signaling pathways were also activated early in 129 mice as shown that the transcripts of RIG-I, MAVS, and TBK1 all upregulated in 129 mice as early as 6 hpi (Fig. 6E). IRF7 is involved in both TLR and RIG-I signaling pathways, and activation of IRF7 results in the rapid production of type 1 IFNs (40, 41). We thus further measured the protein levels of IRF7 by Western blotting. As shown in Fig. 6F, IRF7 increased dramatically at 24 hpi in 129 mice but not in B6 mice. Thus, swift TLR and RIG-I signaling pathways in 129 mice resulted in early production of type 1 IFNs and inflammatory cytokines, which are important reasons for the initiation of strong and prompt NK cells responses.

FIGURE 5. Recruitment, but not proliferation, resulted in early NK cell accumulation in 129 mice. (A and B) Wild-type 129 and B6 mice were infected with 1 × 10^6 PFU PR8 virus. NK cell proliferation was determined at 1, 3, and 5 dpi by BrdU incorporation assays. (A) Representative flow cytometry plots of gated NKp46^+CD3^- NK cells from lungs are shown. (B) Summary graphs showing the BrdU^+ NK cells in the lung, local draining mediastinal LN, spleen, and PB. (C and D) 129 or B6 mice were infected with 1 × 10^6 PFU PR8 virus. The mice were sacrificed and pulmonary homogenates were prepared to measure MCP-1 levels by real-time quantitative PCR (C) or Luminex assays (D) at 6 or 24 hpi. Data correspond to the mean ± SD of three replicate samples and are representative of at least two independent experiments. **p < 0.01.
Table 1. Oligonucleotides and probes used for real-time quantitative PCR

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**NK cells are not protective against low-dose PR8 infection**

With rapid recruitment from the PB, NK cells in the lung and LN were promptly activated and protected the 129 mice against high-dose PR8 infection. However, previous studies indicate that NK cells may play protective or deleterious roles during IAV infection depending on the infecting virus doses (22). Therefore, we further investigated whether NK cells play a similar protective role in 129 mice during a low-dose PR8 infection. Thus, B6 and 129 mice were infected with 1 × 10^6 PFU PR8. As shown in Fig. 7A and 7B, intact or NK cell–depleted B6 and 129 mice all displayed no significant differences in weight loss or survival rate postinfection. Furthermore, NK cells did not significantly affect virus replication at 3 dpi in either B6 or 129 mice (Fig. 7C). Therefore, in contrast with high-dose PR8 infection, NK cell depletion in B6 or 129 mice did not result in significant differences in viral clearance or mortality compared with the respective NK cell–intact mice after low-dose PR8 infection.

Next, we measured the NK cell responses in 129 or B6 mice after low-dose PR8 infection. As shown in Fig. 8A–D, the kinetics of NK cell responses were similar between 129 and B6 mice, with the NK cell percent and total cell number in the LN and lung all peaked at 3 dpi. Moreover, NK cell expression of IFN-γ also peaked at 3 dpi in both mouse strains. The percentage of IFN-γ–producing NK cells was higher in the lung and LN in 129 mice compared with B6 mice, but the kinetics was similar, that is, all declined at 5 dpi. Consistently with IFN-γ production, NK cell-surface expression of CD107a was also slightly upregulated in the lungs at 3 and 5 dpi in both mouse strains. Further, the NK cell percentage in the PB reached its lowest at 3 dpi in both mouse strains, which is in accordance with the NK cell increase in the lung and LN at 3 dpi (Fig. 8E). Thus, in contrast with the faster kinetics observed in B6 mice after low-dose PR8 infection, we further determined the cytokine profiles in both mouse strains after low-dose PR8 infection. As shown in Supplemental Fig. 2, no early cytokine production was found in 129 or B6 mice at 1 dpi. Thus, different from high-dose PR8 infection, NK cells did not play a distinct protective role during low-dose PR8 infection in 129 mice. With a low-dose infection, the kinetics of NK cell responses were similar between 129 and B6 mice.

**Discussion**

In this study, we demonstrated that NK cells play differential roles during IAV infection, with the distinct functions dependent on the host genetic background and also infecting virus doses. The kinetics and magnitude of NK cell responses correlated with the distinct roles that NK cells play against influenza virus infection. Importantly, we also investigated the possible reasons underlying these differences in NK cell responses.

Three main factors are involved in determining the variability in the severity of influenza virus infection: the intrinsic pathogenicity of the virus, acquired host factors (such as immunity and comorbidity), and intrinsic host susceptibility (42). Among them, determiners of host genetic background are much less well studied (43). Genome-wide RNA interference screening has identified 287 human genes that influence IAV replication (44). Previous reports indicate that inbred laboratory mouse strains exhibit differences in susceptibility to IAV infection (29). It has also been suggested that H5N1 influenza virus-induced pathology is affected by complex and multigenetic host factors of mice, and the lethal dose in these hosts is associated with differences in replication kinetics and increased production of some proinflammatory cytokines (45).

In this study, we confirmed that different host genetic backgrounds result in different resistance to IAV infection. Surprisingly, we found that NK cells only play a significant protective role in 129 mice during 1 × 10^6 PFU PR8 infection. NK cell depletion in 129 mice resulted in significantly increased virus titers and increased mortality, whereas the virus titers and mortality rate of B6 mice remained unchanged (albeit all slightly reduced) in the absence of NK cells. Our results are partially in agreement with recent findings by Zhou et al. (24). They found that depletion of NK cells improves the survival rate of high-dose (5 hemagglutination units) PR8 infection, whereas depletion of NK cells increases morbidity and mortality in medium-dose (0.5 hemagglutination unit) PR8 infection. However, their high dose is higher than the dose we used (1 × 10^6 PFU). These differences in the virus infection dose may account for the different outcomes. Similarly, previous studies also demonstrate that the doses of infecting virus affect NK cell–regulated antiviral T cell responses during lymphocytic choriomeningitis virus infection in mice (46). Thus, the functions of NK cells vary greatly with host genetic backgrounds and infecting viral doses.

NK cells can control viral infections by producing antiviral cytokines, such as IFN-γ, or by perforin-mediated killing of infected cells (47). We analyzed NK cell responses in detail to determine the reasons for the differential roles that NK cells play in 129 and B6 mice. Surprisingly, we found that in 129 mice, PR8 infection results in early NK cell accumulation and activation in the lung and LN. 129 NK cells mounted faster responses 2 d after infection compared with B6 mice during a low-dose PR8 infection. Thus, B6 and 129 mice were infected with 1 × 10^6 PFU PR8. As shown in Fig. 8A–D, the kinetics of NK cell responses were similar between 129 and B6 mice. Therefore, in contrast with high-dose PR8 infection, NK cell depletion in B6 or 129 mice did not result in significant differences in viral clearance or mortality compared with the respective NK cell–intact mice after low-dose PR8 infection.

Next, we measured the NK cell responses in 129 or B6 mice after low-dose PR8 infection. As shown in Fig. 8A–D, the kinetics of NK cell responses were similar between 129 and B6 mice, with the NK cell percent and total cell number in the LN and lung all peaked at 3 dpi. Moreover, NK cell expression of IFN-γ also peaked at 3 dpi in both mouse strains. The percentage of IFN-γ–producing NK cells was higher in the lung and LN in 129 mice compared with B6 mice, but the kinetics was similar, that is, all declined at 5 dpi. Consistently with IFN-γ production, NK cell-surface expression of CD107a was also slightly upregulated in the lungs at 3 and 5 dpi in both mouse strains. Further, the NK cell percentage in the PB reached its lowest at 3 dpi in both mouse strains, which is in accordance with the NK cell increase in the lung and LN at 3 dpi (Fig. 8E). Thus, in contrast with the faster kinetics observed in B6 mice after low-dose PR8 infection, we further determined the cytokine profiles in both mouse strains after low-dose PR8 infection. As shown in Supplemental Fig. 2, no early cytokine production was found in 129 or B6 mice at 1 dpi. Thus, different from high-dose PR8 infection, NK cells did not play a distinct protective role during low-dose PR8 infection in 129 mice. With a low-dose infection, the kinetics of NK cell responses were similar between 129 and B6 mice.

**Discussion**

In this study, we demonstrated that NK cells play differential roles during IAV infection, with the distinct functions dependent on the host genetic background and also infecting virus doses. The kinetics and magnitude of NK cell responses correlated with the distinct roles that NK cells play against influenza virus infection. Importantly, we also investigated the possible reasons underlying these differences in NK cell responses.

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FIGURE 6. Early production of cytokines is an important reason for swift NK cell responses in 129 mice. (A) Splenocytes from wild-type 129 and B6 mice were incubated for 5 h with the indicated bound mAbs in the presence of 500 IU/ml IL-2. Cells were analyzed by flow cytometry. Data showed the proportion of NK cells (NKp46+CD3-IFN-γ) that expressed intracellular IFN-γ. (B) B6 or 129 mice were infected with 1 × 10^6 PFU PR8. The mice were sacrificed, and pulmonary homogenates were prepared to measure the levels of inflammatory cytokines using a Luminex assay at 1 dpi. Each value represents that pulmonary cytokine concentration of infected mice compared with respective uninfected mice. Data correspond to the mean ± SD of three individual mice. (C–E) 129 or B6 mice were infected with 1 × 10^6 PFU PR8 virus. The mice were sacrificed and pulmonary homogenates were prepared to measure the levels of the indicated molecules by real-time quantitative PCR at indicated times postinfection. (F) 129 or B6 mice were infected with 1 × 10^6 PFU PR8 virus. The mice were sacrificed and pulmonary homogenates were prepared to measure the levels of IRF7 by Western blotting at 24 hpi. Data correspond to the mean ± SD of three replicate samples and are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
whether the swift NK cell responses in 129 mice also relate to
different viral strains, we further tested NK cell responses to A/
Jiangxi/262/2005 (H3N2) virus. As shown in Supplemental Fig. 3,
the NK cell responses to H3N2 virus were also stronger in 129
mice compared with that of B6 mice. However, NK cells from B6
mice did not mount obvious responses as measured by IFN-
\(\gamma\) production after H3N2 infection, which is different compared with
the responses to PR8 infection. A possible reason might be that
H3N2 virus is generally less pathogenic than PR8 in mice. Thus,
host genetic background has an important impact on NK cell re-
sponses. The kinetics and magnitude of NK cell responses cor-
relate with the distinct roles that NK cells play against influenza
virus infections.

We further investigated the reasons for the differential NK cell
responses in B6 and 129 mice. One possible reason could be
differential NK cell activation by ligation of the NK cell activating
receptor NKp46 and IAV HA. However, we found that NK cells
from B6 mice tended to be more easily activated by plate-coated
HA or anti-NKG2D mAb. Given that early post PR8 infection, the
amount of HA exposed to the lung or LN NK cells might be at
similar levels, the early NK cell activation in 129 mice does not
likely result from stronger HA stimulation.

NK cells also can be activated by cytokines. We thus determined
the cytokine levels in the lungs of 129 and B6 mice post PR8
infection. Postinfection, influenza viral nucleic acids bind to pattern
recognition receptors within epithelial and immune cells, causing
production of high levels of inflammatory cytokines and chemok-
es. This alters the lung microenvironment and initiates the
trafficking of immune cells (e.g., macrophages, dendritic cells, NK
cells, and neutrophils) to the site of infection, and subsequently,
adaptive immune responses clear the viral infection (5). Among
them, type 1 IFNs play an important role in the protection against
IAV infection, both by direct inhibition of viral replication through
IFN-stimulated, gene-encoded proteins (48) and by enhancing NK
cell activities (49). Indeed, we found that multiple cytokines and
chemokines (or chemokine ligands) were produced in large
amount in the lungs in 129 mice at 1 dpi, whereas there was no
significant increase in cytokines level in the lungs of B6 mice at
Further, we also determined that the mRNA levels of type 1 IFNs were upregulated early in 129 mice, but not in B6 mice. Because TLR and RIG-1 signaling pathways all play important roles in the initiation of type 1 IFNs and proinflammatory cytokines, we further demonstrated that both the TLR9 and the RIG-I signaling pathways were activated in 129 mice early postinfection. Thus, early activation of innate signaling pathways leads to prompt production of type 1 IFNs and inflammatory cytokines.
which are important driving forces for NK cell activation. However, the molecular mechanisms that result in the differential activation of the pattern recognition receptors in 129 and B6 mice are still unknown.

Our data indicate that NK cells were rapidly activated in the LN and lung after high-dose PR8 infection in 129 mice, where they exerted antiviral functions, which is in accordance with the protective role of NK cells in 129 mice. However, we also found that the kinetics of NK cell responses were similar between 129 and B6 mice during low-dose PR8 infection conditions. In contrast with their protective role during high-dose PR8 infection, NK cells did not provide significant protection against low-dose PR8 infection in 129 mice. Furthermore, 129 mice did not mount early cytokine productions, which is different from high-dose infection. Thus, our results link the kinetics and magnitudes of NK cell responses to the distinct roles that NK cells play during viral infection. This may explain why NK cells play distinct functions during viral infection. NK cell responses might be an important indicator for their functions, not only during IAV infections, but also during other viral infections as well.

In summary, our data revealed that NK cell responses vary with different host genetic backgrounds and also are affected by infecting virus doses. We also showed that in 129 mice, the early induction of type I IFNs and inflammatory cytokines are important reasons for the strong and prompt NK cell responses. Our data revealed the complexity and flexibility of NK cell responses during viral infections. Emerging influenza viruses (H1N1, H5N1, and H7N9) pose a serious challenge to global human health; therefore, our findings have implications for developing new therapies to control influenza virus infection. Host and viral factors must be considered to better harness the beneficial effect of NK cells when developing new therapies for fighting lethal viral infections.

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Disclosures
The authors have no financial conflicts of interest.

References


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P-values for significance were calculated for all pairwise comparisons between strains and for the indicated days shown in Figure 1A using Student’s t test.

* represents P value < 0.05, ** represents P value < 0.01, *** represents P value < 0.001. Due to the restriction of table size, the comparison for some days was not shown.
Fig. S1. NK cells, but not other cell populations were efficiently depleted in 129 mice with anti-asialo GM1 antiserum treatment. Anti-asialo GM1 antiserum (20ul; Wako Chemicals) were i.p. injected into 129 mice. Cell populations from lung, spleen, lymph node and peripheral blood were analysed by flow cytometry at 1 day after injection. Summary graphs showing the proportions of NKp46⁺CD3⁻ NK cells, CD8⁺ T cells, CD4⁺ T cells, CD11c⁺/CD11b⁺ dendritic cells, GR1⁺/F480⁻ Neutrophil and CD11b⁺/F480⁺ Macrophage in total lymphocytes of the indicated organs. Data correspond to the mean ± SD from at least three independent experiments. *P < 0.05, ***P < 0.001.
Fig. S2. Low-dose PR8 infection did not induce early inflammatory cytokine production. 129 or B6 mice were infected with $1 \times 10^3$ pfu PR8 virus. The mice were sacrificed, and pulmonary homogenates were prepared to measure the levels of inflammatory cytokines using a Luminex assay at 1 dpi. Each value represents that pulmonary cytokine concentration of infected mice compared to respective uninfected mice. The data correspond to the mean ± SD of three individual mice.
Fig. S3. H3N2 infection resulted in swift NK cell responses in 129 mice.

B6 or 129 mice were infected with 1×10^6 pfu A/Jiangxi/262/2005 (H3N2) virus. Lymphocytes from local draining mediastinal lymph node (LN) (A and B) or lung (C and D) were analysed by flow cytometry. (A) Representative flow cytometry plots with gating strategy and summary graphs showing the NK cell percent in lymphocytes and total NK cell numbers in the draining LN at the indicated time pi. (B) Representative flow cytometry plots and column graphs showing the frequency of IFN-γ and CD107a surface expression in gated NKp46^+, CD3^- NK cells. (C) As in A but showing the lymphocytes from the lung. (D) As in B but showing the NK cells from the lung. Data correspond to the mean ± SD from three independent experiments.

* P < 0.05, **P < 0.01.