Klebsiella pneumoniae Alleviates Influenza-Induced Acute Lung Injury via Limiting NK Cell Expansion

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**Klebsiella pneumoniae** Alleviates Influenza-Induced Acute Lung Injury via Limiting NK Cell Expansion

Jian Wang,* Fengqi Li,* Rui Sun,**† Xiang Gao,‡ Haiming Wei,*‡ and Zhigang Tian*,†,§

A protective effect induced by bacterial preinfection upon a subsequent lethal influenza virus infection has been observed, but the underlying immune mechanisms have not yet been fully elucidated. In this study, we used a mouse model of *Klebsiella pneumoniae* preinfection to gain insight into how bacterial preinfection influences the subsequent lethal influenza virus infection. We found that *K. pneumoniae* preinfection significantly attenuated lung immune injury and decreased mortality during influenza virus infection, but *K. pneumoniae*-specific immunity was not involved in this cross-protection against influenza virus. *K. pneumoniae* preinfection limited NK cell expansion, which was involved in influenza-induced immune injury and death. Furthermore, *K. pneumoniae* preinfection could not control NK cell expansion and death during influenza virus infection in Rag1−/− mice, but adoptive transfer of T cells from wild-type mice was able to restore this protective effect. Our data suggest that the adaptive immune response activated by bacterial infection limits the excessive innate immune response induced by a subsequent influenza infection, ultimately protecting mice from death. *The Journal of Immunology*, 2014, 193: 000–000.

The immune system is an organism’s safeguard for defending against foreign pathogens. Depending on the class and quantity of the invading pathogens, the extent and type of the induced immune response will be different. In a natural environment, both human and animal organisms are commonly exposed to multiple pathogens simultaneously. Therefore, the immune response generated against one pathogen will inevitably influence the immune response to another (1–3). For example, this often occurs in the immune responses against pathogens in the respiratory tract, which contains many inhaled Ags, including allergens, infectious agents, and particulate debris (4).

As the major source of severe viral respiratory infection, the highly pathogenic influenza virus leads to substantial morbidity and mortality. Annual epidemics commonly affect 5–15% of the population and result in 3–5 million deaths worldwide (5). Influenza virus infection is often accompanied by secondary bacterial infection in humans, because influenza-induced immune suppression favors subsequent bacterial invasion (6, 7). Interestingly, if the reverse occurs, primary infection by some bacteria leads to host resistance to subsequent influenza infection (8, 9). Although several studies suggest that bacterial preinfection protects against influenza virus by dampening inflammation, the underlying cellular and molecular immune mechanisms remain unknown. *Klebsiella pneumoniae*, a Gram-negative bacterium, is one of the most common bacterial causes of respiratory tract infections in human beings. Although *K. pneumoniae* is the main participant in the subsequent secondary bacterial infection induced by influenza virus infection, pretreatment with a glycoprotein extract from *K. pneumoniae* before influenza infection can afford significant protection against the influenza virus–induced death in mice (10).

NK cells are an important innate immune cell type that exert multiple functions during viral infection in terms of recognizing Ag and initiating immune responses (11). In the lung, NK cells make up the highest percentage of resident lymphocytes compared with any other tissue (12, 13), suggesting that quick and effective NK-mediated immune responses are critical for eliminating pathogens and maintaining homeostasis in the lung. Indeed, activation of lung NK cells promotes adaptive immune function and the elimination of viruses during influenza virus infection. However, the immunopathology exacerbated by excessive NK cell responses is also a major contributor to acute death of thehost during a highly pathogenic influenza virus infection (14).

In this study, we built a mouse model of *K. pneumoniae* preinfection to explore the cellular and molecular immune mechanisms underlying how bacterial preinfection dampens the excessive inflammatory response induced by subsequent influenza virus infection. We found that *K. pneumoniae* infection attenuated lung immune injury and protected mice against death induced by subsequent influenza virus infection. Further study showed that T cells activated by *K. pneumoniae* infection were responsible for this protective effect by controlling the excessive NK cell activation induced by a subsequent influenza virus infection. Thus, our study suggests that nonspecific adaptive immune control over the innate immune system may be one way that the immune system uses to inhibit excessive or lethal inflammatory responses.

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Abbreviations used in this article: AsGM-1, asialo–GM-1; BALF, bronchoalveolar lavage fluid; HA, hemagglutination; HI, hemagglutination inhibition; i.n., intranasally; poly(I:C), polyinosinic-polycytidylic acid; TCID₅₀, 50% tissue culture infectious dose; WT, wild-type.

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Materials and Methods

Mice

Male C57BL/6 wild-type (WT) mice were obtained from the Shanghai Experimental Animal Center, Chinese Science Academy (Shanghai, China). Male Rag1−/− mice (C57BL/6 background) were obtained from Model Animal Research Center, Nanjing University (Nanjing, China). BALB/c mice used to prepare antiserum were also obtained from the Shanghai Experimental Animal Center. All mice were housed in an animal facility under specific pathogen-free conditions and were treated at 6–10 wk of age (body weight 20–25 g). Animal care and experimental procedures were performed in accordance with experimental animal guidelines of the University of Science and Technology of China.

Preparation of bacteria and virus

The K. pneumoniae strain (ATCC 700603) was a gift from the Department of Microbiology, Anhui Medical University. K. pneumoniae cultures were grown from frozen stocks to the midexponential phase of growth (OD at 600 nm is 0.75) in tryptic soy broth medium at 37˚C with constant shaking (250 rpm). The bacteria were then washed with chilled pyrogen-free saline, resuspended in pyrogen-free saline at appropriate concentrations, and kept on ice until infection. Mouse-adapted influenza A/PR/8/34 strain (H1N1) was a gift from the Institute of Basic Medicine, Shandong Academy of Medical Sciences. The virus strain was amplified in allantoic cavities of 10-d-old specific pathogen-free embryonated chicken eggs (Merial-Vital Laboratory Animal Technology, Beijing, China). Allantoic fluids were harvested 72 h after inoculation and then stored at −80˚C until needed. The hemagglutination (HA) titer was 10,240 HA U/ml.

Mouse infections

Mice were i.p. anesthetized with sodium pentobarbital (50 µg/g body weight) before i.n. infection. For influenza virus infection, mice were infected intranasally (i.n.) with the PR8 strain (lethal dose, 0.5 HA; sub-lethal dose, 0.1 HA) in 50 µl sterile saline. For K. pneumoniae infection, mice were infected i.n. with K. pneumoniae in 50 µl sterile saline. Control mice received 50 µl sterile saline alone. For coinfection studies, mice were infected with PR8 at different time points postinfection with various doses of K. pneumoniae. After PR8 infection, body weight was recorded daily. In addition, clinical illness scores were estimated using the following values and symptom scores, as described previously (15): 0, healthy; 1, barely ruffled fur; 2, ruffled fur, but active; 3, ruffled fur and inanition; 4, ruffled fur, inactive, hunched, and gaunt; 5, dead. Illness scores were estimated on a daily basis by a blinded observer.

Measurement of total protein, cytokines, and Igs in bronchoalveolar lavage fluid or serum

Bronchoalveolar lavage fluid (BALF) was collected as previously described (16). In brief, the lungs were washed once with 1 ml sterile saline through an intratracheal cannula. BALF samples were then centrifuged at 350 g for 5 min, and the supernatants were collected and kept at −80˚C until the assays were performed. Total protein in BALF was measured using the BCA Protein Assay Kit (Fierce, Rockford, IL). IL-6 and IFN-γ levels in BALF or serum were measured using ELISA kits from Soenixing Biotech (Shanghai, China). IgM and IgG levels in serum were measured using ELISA kits from Cloud-Clone (Houston, TX).

Histopathology and inflammation scores

For histological analysis, lung tissue was removed, fixed in 10% neutral-buffered formalin for >24 h, embedded in paraffin, and cut into 5-µm sections. The sections were then deparaffinized and stained with H&E to determine histological changes. Inflammation scores were recorded from 20 randomly selected fields from the right and left lung sections according to a previously established scoring system (17). In brief, inflammation was scored as follows based on the number and distribution of inflammatory cells within the lung tissues: 0, no inflammation; 1, mild, inflammatory cell infiltrate of perivascular and peribronchial space; 2, moderate, inflammatory cell infiltrate of perivascular and peribronchial space with modest extension into the lung parenchyma; 3, severe, inflammatory cell infiltrate of perivascular and peribronchial space with large inflammatory foci found in the lung parenchyma. Mean scores were calculated independently for each group.

Determination of viral titer

Viral titer in the lungs was determined by 50% tissue culture infective dose (TCID50) assay after PR8 infection. In brief, at various time points after influenza virus infection, lungs were harvested into 1 ml viral maintenance medium (DMEM plus 0.2% BSA and 0.5 µg/ml TPCK-trypsin), homogenized, serially diluted, and added to duplicate wells (n = 5) of 96-well flat-bottom plates containing confluent monolayers of MDCK cells. After 2–3 d of incubation at 37˚C, infected test units in each dilution were recorded. Spearman–Kärber method was used to calculate TCID50.

HA inhibition assay

The PR8 and K. pneumoniae antiserums were prepared in BALB/c mice. For the HA inhibition (HI) assay, the serum was treated as follows: serum, saline, and the KIO4 solution (0.025 M) were mixed in a 1:1:1 volume. After a 2-h incubation at 37˚C to destroy any nonspecific inhibitors, a 5% glucose solution (2-fold volume for serum) was added to remove excess KIO4, making a final 1:5 dilution of the obtained serum. A fixed amount of influenza virus (2 HA) in 50 µl was added to every well of a 96-well plate, and a dilution series (1:5; 1:10, and 1:20) of K. pneumoniae antiserum in 50 µl was added. Serum from normal BALB/c mice and PBS was used as the negative control. All samples were tested in triplicate. Finally, 0.5% chicken RBCs in 100 µl were added to each well. After 30 min, the wells were photographed and evaluated.

Flow cytometry assay

After BALF was collected and centrifuged, the cell pellets were resuspended, and cell numbers were counted. CDRs were blocked with anti-CD16/32, and single-cell suspensions were intraconal labeled with anti-CD8 (clone 53-6.7), CD3 (clone M1/70), K. pneumoniae (clone PK136), CD3e (clone 145-2C11), CD6 (clone RM4-5), CD8α (clone 53-6.7), CD10 (clone M1/70). To stain intracellular IFN-γ, the cells were fixed, permabilized, and then stained with anti-mouse IFN-γ (clone XMG1.2). All Abs and isotype controls were purchased from BD Biosciences.

Cell depletion

To deplete NK cells, we treated mice i.v. with rabbit anti-mouse asialo-GM-1 (AsGM-1) Abs (50 µg/mouse) 1 d before and 2 d and 5 d after PR8 infection. To deplete T cells, mice were treated i.v. with anti-CD4 Ab (100 µg/mouse) 2 d before PR8 infection. To deplete CD4+ or/and CD8+ T cells, we treated mice i.v. with anti-CD4 Ab (100 µg/mouse) or/and anti-CD8 Ab (100 µg/mouse) at day 5 after K. pneumoniae infection and then i.n. infected mice with PR8 at day 5 after K. pneumoniae infection.

T cell isolation and adoptive transfer

Splenic T cells from WT mice were purified by MACS. T cells (5 × 106 cells/mouse) were adoptively transferred i.v. into Rag1−/− mice. Three weeks later, recipient Rag1−/− mice were infected with 1 × 10⁸ CFU K. pneumoniae and infected with 0.5 HA of PR8 7 d later. Survival, body weight changes, and illness scores were evaluated at the indicated time points after PR8 infection.

Polyinosinic-polyctydidylic acid stimulation in vitro

The procedure performed as previously described (18). In brief, T cells in BALF from K. pneumonia-infected WT mice were purified by MACS, and then splenocytes from Rag1−/− mice were stimulated with 100 µg/ml polyinosinic-polycytidylic acid [poly(I:C)] in the presence or absence of purified T cells. After coculture in a 24-well plate in RPMI 1640 medium for 20 h, splenocytes cells were collected for IFN-γ analysis. A Transwell plate system (Costar) was used to prevent contact between splenocytes and T cells.

Statistical analysis

Results were analyzed using two-tailed Student t tests. The data were expressed as the mean ± SEM. The p values <0.05 were considered statistically significant. Significance was denoted as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

K. pneumoniae preinfection protects mice against influenza-induced acute death

To examine the influence of K. pneumoniae infection on a subsequent influenza virus infection, we infected C57BL/6 mice i.n.
with *K. pneumoniae* and subsequently infected them i.n. with a lethal dose of the PR8 influenza strain. *K. pneumoniae* pre-infection markedly decreased the PR8-induced mortality, and this protective effect occurred in a dose- (Fig. 1A) and time-dependent (Fig. 1B) manner. Based on the observed dose- and time-dependent effects on survival rate, we selected $1 \times 10^7$ CFU *K. pneumoniae* 7 d before influenza virus infection as the optimal protocol for further experiments. Compared with control mice, *K. pneumoniae*–preinfected mice displayed lower body-weight loss (Fig. 1C) and illness scores (Fig. 1D) after a lethal dose of PR8 infection using this protocol.

*K. pneumoniae* preinfection attenuates influenza-induced immune injury

As previously reported, immune-mediated pathological injury is responsible for influenza-induced death (19, 20). To explore the underlying mechanism by which *K. pneumoniae* preinfection prevents influenza-induced death and to observe the dynamic effect of *K. pneumoniae* infection on subsequent influenza-mediated pathological injury over time, we infected mice with a sublethal dose of PR8 7 d after *K. pneumoniae* infection. Similar to the results that we obtained using the lethal dose of PR8, *K. pneumoniae*–preinfected mice also displayed lower body-weight loss (Fig. 2A) and illness scores (Fig. 2B) after receiving a sublethal dose of PR8 infection. Compared with control mice, *K. pneumoniae*–preinfected mice exhibited less lymphocyte infiltration and immune injury in their lungs after PR8 infection (Fig. 2C). Furthermore, the levels of total protein and inflammatory cytokines, including IL-6 and IFN-γ, in BALF and serum were also lower in *K. pneumoniae*–preinfected mice after PR8 infection (Fig. 2D, Supplemental Fig. 1A). Therefore, these results suggest that *K. pneumoniae* preinfection can attenuate influenza-induced immune injury.

Because excessive amplification of the influenza virus within the lung has also been shown to be responsible for the death of the infected host (21), we tested viral titer and found that *K. pneumoniae*–preinfected mice exhibited decreased viral titer in their lungs compared with control mice (Fig. 2E). However, the levels of IgM and IgG in serum from *K. pneumoniae*–preinfected mice also were lower when compared with control mice after PR8 infection (Supplemental Fig. 1B), suggesting that the decrease of the viral titer did not depend on augmenting adaptive immune against influenza virus infection. Given that viral titer in *K. pneumoniae*–preinfected mice was lower than in control mice from the first day after PR8 infection (Fig. 2E), the decrease of the viral titer might be caused by the *K. pneumoniae* infection–activated immune response, which inhibited viral amplification in a nonspecific way. Taken together, these data suggest that the *K. pneumoniae* preinfection–mediated protective effect may depend on attenuating lung immune injury and/or inhibiting viral amplification.

K. pneumoniae preinfection–mediated protective effect depends on the adaptive immune system

To test how the immune response generated against *K. pneumoniae* infection influenced the immune response to subsequent influenza virus infection, we first evaluated the overall state of the immune system in the lungs after *K. pneumoniae* infection. Compared with control mice, *K. pneumoniae*–infected mice showed slight body weight loss from days 1–5 postinfection, but then recovered to normal body weight at day 7 (Fig. 3A). The number of total lymphocytes in the lung increased at days 3 and 7 upon *K. pneumoniae* infection (Fig. 3B). As important components of the innate and adaptive immune responses, the respective frequency and number of NK cells and T cells can reflect which type of immune response is dominant at a particular time point (22, 23). At day 3 after *K. pneumoniae* infection, the percentage and number of NK cells in lungs increased together with number of T cells, although the percentage of T cells decreased. At day 7, however, the percentage and number of T cells in lungs increased, whereas the percentage and number of NK cells were sharply reduced (Fig. 3C, 3D). This dominant T cell response at day 7 after *K. pneumoniae* infection coincided with the optimal time point for infecting mice with the influenza virus after *K. pneumoniae* infection according to our optimized experimental protocol, implying that *K. pneumoniae* infection–activated T cells might be the major factor influencing the immune response to the subsequent influenza virus infection. To test this hypothesis, we built the mouse model in T and B cell–

**FIGURE 1.** *K. pneumoniae* preinfection prevents influenza-induced acute death in mice. (A) Seven days after i.n. infection with different doses ($5 \times 10^5, 1 \times 10^6, 5 \times 10^6, 1 \times 10^7$ CFU) of *K. pneumoniae*, C57BL/6 mice were i.n. infected with a lethal dose (0.5 HA) of PR8 and evaluated for survival. (B) At different time points (1, 3, 7, or 14 d) after i.n. infection with $1 \times 10^7$ CFU *K. pneumoniae*, C57BL/6 mice were i.n. infected with 0.5 HA of PR8 and evaluated for survival. (C and D) Seven days after i.n. infection with $1 \times 10^7$ CFU *K. pneumoniae*, C57BL/6 mice were i.n. infected with 0.5 HA of PR8 and evaluated for (C) body weight changes and (D) illness scores. Illness scores were estimated using the following values and symptoms: 0, healthy; 1, barely ruffled fur; 2, ruffled fur, but active; 3, ruffled fur and inactive; 4, ruffled fur, inactive, hunched, and gaunt; 5, dead. Data are expressed as the mean ± SEM. Data represent three independent experiments with at least nine mice/group.

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deficient Rag1−/− mice and found that, unlike WT mice, pre-infection with K. pneumoniae could not protect Rag1−/− mice against influenza-induced death (Fig. 4A). In Rag1−/− mice, K. pneumoniae preinfection could not reduce body weight loss (Fig. 4B) or illness scores (Fig. 4C) compared with control mice. Thus, these results suggest that the protective effect mediated by K. pneumoniae preinfection depends on the adaptive immune system.

To test whether viral amplification was responsible for this effect that occurred in Rag1−/− mice, we detected viral titer in the lungs and found that there was not a significant difference between K. pneumoniae–preinfected WT mice and K. pneumoniae–preinfected Rag1−/− mice at days 1 and 5 after PR8 infection (Fig. 4D), suggesting that the protective effect mediated by K. pneumoniae preinfection did not depend on inhibiting viral amplification. Taken together, these data suggest that K. pneumoniae preinfection–mediated protective effect depends on the presence of the adaptive immune system, and on T cells in particular, which may be to prevent influenza-induced death mainly through downregulating excessive inflammatory response rather than inhibiting viral amplification.

The K. pneumoniae preinfection–mediated protective effect is nonspecific
Because the K. pneumoniae preinfection–mediated protective effect required the adaptive immune system, we wondered whether this protection was due to any cross-reactive Ags between K. pneumoniae and influenza virus. To test this hypothesis, we prepared antisera to K. pneumoniae and the PR8 strain in BALB/c

**FIGURE 2.** K. pneumoniae preinfection attenuates influenza-induced lung injury. Seven days after i.n. infection with 1 × 10⁷ CFU K. pneumoniae, C57BL/6 mice were i.n. infected with a sublethal dose (0.1 HA) of PR8. (A) Body weight changes, (B) illness scores, (C) H&E staining of lung tissue section, inflammation scores, as well as (D) total protein and inflammatory cytokines (IL-6 and IFN-γ) in BALF were detected after PR8 infection. Inflammation scores were estimated from 20 fields randomly selected from the right and left lung sections using the following values and symptoms: 0, no inflammation; 1, mild, inflammatory cell infiltrate of perivascular and peribronchiolar space; 2, moderate, inflammatory cell infiltrate of perivascular and peribronchiolar space with modest extension into the lung parenchyma; 3, severe, inflammatory cell infiltrate of perivascular and peribronchiolar space with large inflammatory foci found in the lung parenchyma. (E) Viral titer in the lungs was detected by TCID₅₀ assay at the indicated time points after PR8 infection. Data are expressed as the mean ± SEM. Scale bar in (C), 200 μm. Data represent two independent experiments with at least three mice/group in (A)–(D) and at least 5 mice/group in (E). Two-tailed Student t tests, *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 3.** T cell responses are dominant in the lungs at day 7 after K. pneumoniae infection. C57BL/6 mice were i.n. infected with 1 × 10⁷ CFU K. pneumoniae. (A) Body weight changes and (B) total lymphocyte numbers in lung were detected at the indicated time points postinfection. (C and D) The percentage and number of NK cells (CD3– NK1.1+) and T cells (CD3⁺NK1.1−) in lung were evaluated by flow cytometry at days 3 and 7 postinfection. Data are expressed as the mean ± SEM. Data represent two independent experiments with at least three mice/group. Two-tailed Student t tests, *p < 0.05, **p < 0.01, ***p < 0.001.
mice and evaluated cross-reactivity by an HI assay. Compared with the antiserum against PR8, the antiserum against *K. pneumoniae* could not inhibit PR8-mediated HA (Fig. 5). Moreover, a BLAST search failed to identify any matching epitopes between *K. pneumoniae* and the H1N1 influenza A virus (data not shown). Therefore, *K. pneumoniae* does not contain any Ags that cross-react with influenza virus, suggesting that the *K. pneumoniae* preinfection-mediated protective effect against influenza virus infection is nonspecific.

**K. pneumoniae** preinfection limits NK cell expansion during influenza virus infection

To further test how the immune response generated against *K. pneumoniae* infection influenced the immune response to subsequent influenza virus infection, we next compared the overall state of the immune system in the lungs between mice preinfected with *K. pneumoniae* or saline control after influenza virus infection. The number of total lymphocytes in BALF was not significantly different between saline- and *K. pneumoniae*-infected mice at days 3 and 7 after PR8 infection (Fig. 6A). However, the percentage and number of NK cells in BALF from *K. pneumoniae*-preinfected mice significantly decreased compared with mice preinfected with saline at days 3 and 7 after PR8 infection (Fig. 6B, 6C). Perhaps because of the presence of T cells activated by *K. pneumoniae* infection, the percentage and number of T cells in BALF from *K. pneumoniae*–preinfected mice were higher than in BALF from saline-preinfected mice (Fig. 6B, 6D). Furthermore, as opposed to findings in WT mice, the percentage and number of NK cells in BALF from *K. pneumoniae*–preinfected Rag1−/− mice significantly increased compared with saline-preinfected Rag1−/− mice at day 3 after PR8 infection (Fig. 6E, 6F). Therefore, these data suggest that the immune state in the lung of WT mice created by *K. pneumoniae* infection can limit NK cell expansion during influenza virus infection.

Because *K. pneumoniae* preinfection could inhibit NK cell expansion during a subsequent influenza virus infection, we wondered whether this was the underlying reason that *K. pneumoniae* preinfection prevented influenza-induced death. To test this hypothesis, we treated WT mice with rabbit anti-mouse AsGM-1 to deplete NK cells in vivo before and during influenza virus infection (Supplemental Fig. 2A). Consistent with previous reports (14), NK cell depletion in PR8-infected mice significantly prolonged survival (Fig. 7A) and reduced illness scores (Fig. 7B) compared with control mice, although no improvement in body weight loss was observed (Fig. 7C). Given that the rabbit anti-mouse AsGM-1 is well recognized to eliminate activated T cells (24), we next treated mice with PK136 to deplete NK cells before influenza virus infection (Supplemental Fig. 2B) and found that PK136 treatment significantly protected mice against influenza-induced death (Fig. 7D). Meanwhile, PK136 treatment decreased the levels of IgM and IgG in serum and increased the viral titer in the lungs after PR8 infection (Supplemental Fig. 3), suggesting that NK cell expansion, but not viral amplification, was the main reason for influenza-induced acute death. In addition, we have found that *K. pneumoniae* preinfection–mediated protective effect occurs in a time-dependent manner, and *K. pneumoniae* treatment 7 d before influenza virus infection is the optimal time point (Fig. 1B). Accordingly, comparing the influence of *K. pneumoniae* treatment at different time points before influenza virus infection on NK cells in BALF after PR8 infection, we found that the percentage and number of NK cells were lower after PR8 infection when *K. pneumoniae* infected 7 d before influenza virus infection than

**FIGURE 4.** The *K. pneumoniae* preinfection–mediated protective effect is abolished in Rag1−/− mice. (A–C) Seven days after i.n. infection with 1 × 10⁷ CFU *K. pneumoniae*, Rag1−/− mice were i.n. infected with 0.5 HA of PR8. (A) Survival, (B) body weight changes, and (C) illness scores were evaluated at the indicated time points after PR8 infection. (D) Seven days after i.n. infection with 1 × 10⁷ CFU *K. pneumoniae*, C57BL/6 and Rag1−/− mice were i.n. infected with 0.1 HA of PR8. Viral titer in the lungs was detected by TCID₅₀ assay at days 1 and 5 after PR8 infection. Data are expressed as the mean ± SEM. Data represent two independent experiments with at least five mice/group in (D) and at least six mice/group in (A)–(C). Two-tailed Student *t* tests.

**FIGURE 5.** There are no cross-reactive Ags between *K. pneumoniae* and the influenza A virus. Cross-reactive Ags between *K. pneumoniae* and the influenza A virus were evaluated by the HI assay. Normal BALB/c serum and PBS were used as the negative control, and PR8 antiserum was used as positive control.

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any other time points (Fig. 7E, 7F). All together, these data suggest that limiting NK cell expansion may be the main reason underlying how *K. pneumoniae* preinfection can prevent influenza-induced death.

**K. pneumoniae** preinfection–activated T cells limit NK cell expansion during influenza infection

Because the *K. pneumoniae* preinfection–mediated protective effect depended on the presence of T cells, and this protective effect was achieved by limiting NK cell expansion during influenza virus infection, we wondered whether T cells activated upon *K. pneumoniae* preinfection limited NK cell expansion and, therefore, protected mice against death during influenza infection. To test this hypothesis, we adoptively transferred splenic T cells from WT mice (Supplemental Fig. 4) into Rag1\(^{-/-}\) mice. Three weeks after transfer, recipient mice were infected with 1 × 10⁷ CFU *K. pneumoniae* and then infected 7 d later with a lethal dose of PR8. Although the total number of immune cells in the BALF was not significantly different between control Rag1\(^{-/-}\) and T cell–transferred Rag1\(^{-/-}\) mice at day 3 after PR8 infection (Fig. 8A), the presence of the adoptively transferred T cells significantly decreased the percentage and number of NK cells in the BALF and increased the survival rate of mice (Fig. 8B–D). Next, to detect whether T cells activated upon *K. pneumoniae* preinfection inhibited NK cells activation, we stimulated splenocyte from Rag1\(^{-/-}\) mice with poly(I:C) and analyzed IFN-γ production by NK cells with or without purified T cells from BALF of *K. pneumoniae*–infected WT mice. The presence of cocultured T cells led to a decrease in IFN-γ production by NK cells after poly(I:C) stimulation. Furthermore, the inhibitory effect was reduced when contact between splenocytes and T cells was prevented by the Transwell plate system (Fig. 8E, 8F). Taken together, these data suggest that adoptive transfer of T cells can restore the protective effect that *K. pneumoniae* preinfection has on subsequent influenza infection in Rag1\(^{-/-}\) mice; this protective effect depends on the inhibitory effect of *K. pneumoniae* preinfection–activated T cells on the influenza-induced excessive NK cell expansion and activation; moreover, T cell–mediated inhibition of NK cells is dependent on inhibitory cytokines and cell–cell contact.

To determine which kind of T cell subset plays a central role in *K. pneumoniae* preinfection–mediated protective effect, we first examined the state of CD4⁰ and CD8⁰ T cells, and found that the number of CD4⁰ T cells was more than the number of CD8⁰ T cells in BALF at day 7 after *K. pneumoniae* infection (Fig. 8G). Next, we treated mice i.v. with anti-CD4 Ab or/and anti-CD8 Ab at day 5 after *K. pneumoniae* infection and then infected mice i.n. with 0.5 HA of PR8 at day 7 after *K. pneumoniae* infection. The results showed that both anti-CD4 Ab treatment and anti-CD8 Ab treatment could reduce the *K. pneumoniae* preinfection–mediated protective effect, and anti-CD4/CD8 Ab treatment could almost completely abolish this protective effect (Fig. 8H). Therefore, these results suggest that both CD4⁰ T cells and CD8⁰ T cells activated by *K. pneumoniae* infection are necessary for *K. pneumoniae* preinfection–mediated protective effect.

**Discussion**

Because the respiratory tract is exposed to many different kinds of pathogens, the immune response to one pathogen will inevitably
influence the immune response to another (7, 25). Secondary bacterial infections are a common phenomenon during an influenza pandemic because influenza-mediated immune suppression promotes pulmonary infection by bacteria (26). Interestingly, some bacterial infections, such as those caused by Propionibacterium acnes (27) and Bordetella pertussis (8), conversely confer resistance to a subsequent influenza infection. Although some studies suggested that this protective effect depended on dampening the inflammatory response, the mechanism underlying how the inflammatory response was inhibited had not yet been fully elucidated. In this study, we chose K. pneumonia, a common bacterium in respiratory tract infections, to build a mouse model of bacterial preinfection. Consistent with previous studies, K. pneumonia preinfection protected mice against influenza-induced death via dampening the inflammation that occurs upon influenza infection. Further study indicated that K. pneumonia preinfection–activated T cells inhibited NK cell expansion and downregulated the inflammatory response during influenza virus infection, and adoptive transfer of T cells was able to control NK cell expansion and restore the protective effect of K. pneumonia preinfection in Rag1−/− mice.

Immune responses require rapid and precise cooperation among different cell subsets. NK cells and T cells are essential components of the immune response, and cross talk between these two cells can provide the necessary cytokines and cytotoxic activity to control infections and avoid pathology. Some studies found that an uncontrolled innate immune response occurred and caused death during viral infection in the absence of T cells, and that an NK cell–dependent “cytokine storm” was involved in this process (18, 28), suggesting that adaptive immune cells had an unexpected role in controlling excessive innate immune responses and preventing death during viral infection. In our study, K. pneumoniae preinfection inhibited NK cell expansion during influenza virus infection. Moreover, adoptive transfer of T cells downregulated the percentage and number of NK cells, and restored the K. pneumoniae–mediated protective effect in Rag1−/− mice. These findings collectively suggest that inhibition of NK cell expansion during influenza virus infection by K. pneumoniae preinfection–activated T cells may be the reason to explain how K. pneumoniae preinfection prevents influenza-induced immune injury and death. As for the molecular mechanism underlying how T cells inhibit NK cells, natural regulatory T cells, CD4+CD25+Foxp3+ T cells, and CD8+ T cells are all potential candidates previously shown to regulate NK cells (18), but understanding the detailed mechanism requires further study.

A common question that arises when trying to understand how the protective effect of K. pneumoniae preinfection on influenza-induced illness could depend on the presence of adaptive immune system is whether K. pneumoniae contains any Ags that cross-react with the influenza virus (29). In our study, we ruled out the possibility that any cross Ags existed between K. pneumoniae and the influenza A virus used in our study by the HI assay. Consistent with our study, the protective effect of attenuated B. pertussis on subsequent infection with influenza A virus by dampening the “cytokine storm” also does not correlate with cross-protection (8). Therefore, the inhibitory effect of bacterial infection–induced adaptive immune cells on innate immune cells during influenza virus infection is nonspecific, and this inhibitory effect is involved during both reinfection of pathogens with the same Ags and coinfection of pathogens with different Ags.

The contribution of immunopathology and viral load on death of the infected host during influenza virus infection is controversial, with some studies supporting that immune injury and death is due mainly to excessive immune responses (21, 30) and others suggesting that death is a direct consequence of a high viral titer (31). To distinguish among these possibilities in our model, we measured viral titer in lungs of influenza-infected mice. K. pneumoniae preinfection decreased the viral titer in WT mice during influenza virus infection; furthermore, the viral titer was not significantly different between WT and Rag1−/− mice after...
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K. pneumoniae and influenza virus coinfection, although previous studies showed that the viral titer in Rag1−/− mice was higher than in WT mice after a single influenza virus infection (32). Therefore, the K. pneumoniae preinfection–mediated protective effect observed in our model was not dependent on the inhibition of viral replication.

In our previous work, we chose Staphylococcus aureus, a common commensal bacterium in the upper respiratory tract, to build a mouse model of bacterial preinfection and found that it dampened the subsequent influenza-induced “cytokine storm” through a mechanism involving the induction of the immune-inhibitory M2 macrophage cells (16). In this S. aureus preinfection model, the most optimal protective effect occurred when mice were infected with S. aureus 3 d before PR8 infection; if mice were infected with S. aureus 7 d before PR8 infection, the protective effect was weakened. In contrast with the S. aureus preinfection model, the most optimal protective effect in the K. pneumoniae preinfection model occurred when mice were infected with K. pneumoniae 7 d before PR8 infection, and this effect was dependent on T cell activation. We think that the discrepancy between these two models may be attributed to the differences in bacterial pathogenicity between S. aureus and K. pneumoniae, which likely induce different types and degrees of immune responses (33, 34).

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