Increased pulmonary pneumococcal clearance after resolution of H9N2 avian influenza virus infection in mice

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Running Title: H9N2 virus infection and pneumococcal clearance
Abstract

H9N2 avian influenza virus has been continuously circulating among poultry and could infect mammals, indicating that this virus is a potential pandemic strain. During influenza pandemics, secondary bacterial (particularly pneumococcal) pneumonia usually contributes to excess mortality. In the present study, we observed the dynamic effect of H9N2 virus infection on host defense against secondary pneumococcal infection in mice. BALB/c mice were intranasally inoculated with $1.2 \times 10^5$ plaque forming units (PFU) of H9N2 virus followed by $1 \times 10^6$ colony forming units of *Streptococcus pneumoniae* on 7, 14 or 28 days post-H9N2 infection (D.P.I.). The bacterial load, histopathology, body weight and survival were assessed after pneumococcal infection. Our results showed that H9N2 virus infection had no significant impact on host resistance to secondary pneumococcal infection on 7 D.P.I. However, H9N2 virus infection increased pulmonary pneumococcal clearance and reduced pneumococcal pneumonia-induced morbidity after secondary pneumococcal infection on 14 or 28 D.P.I., as reflected by significantly decreased bacterial loads, markedly alleviated pulmonary histopathological changes and significantly reduced weight loss in mice infected with H9N2 virus followed by *S. pneumoniae* compared with mice infected only with *S. pneumoniae*. Further, the significantly decreased bacterial loads were observed when mice were previously infected with a higher dose ($1.2 \times 10^6$ PFU) of H9N2 virus. Besides, similar to the results obtained in BALB/c mice, improvement in pulmonary pneumococcal clearance was also observed in C57BL/6 mice. Overall, our results showed that pulmonary pneumococcal clearance
Introduction

H9N2 avian influenza virus has become widespread among poultry in many areas of Eurasia and Africa over the last three decades and is the dominant subtype isolated from chickens in China during 2016–2019 (1, 2). H9N2 virus has also been isolated from pigs, minks and humans, demonstrating that this virus could cross species barriers to infect mammals (3-5). Several serological surveys have showed that 13.7% to 37.2% of people in China might be previously infected with H9N2 virus (6, 7). Moreover, H9N2 virus contributes to the genesis of the novel H7N9, H10N8 and H5N6 viruses, which have been found to cause severe diseases and even fatalities in humans (8-12). The wide prevalence, enlarged mammalian hosts and extensive genetic reassortment underscore the pandemic threat of H9N2 virus to human health (13).

*Streptococcus pneumoniae*, or pneumococcus, is a common inhabitant of the upper respiratory tract of approximately 20% to 90% of healthy children and 5% to 20% of healthy adults (14, 15). Defects in host defense, however, could alter the normal interactions between *S. pneumoniae* and host and enable *S. pneumoniae* to invade the lung causing pneumonia. Pneumococcal pneumonia is still a major health problem worldwide despite interventions by vaccines and antibiotics (16). Influenza virus infection is a well-recognized risk factor for pneumococcal pneumonia. Infections
with influenza virus followed by bacteria, particularly *S. pneumoniae*, are associated with high morbidity and mortality, which is evident from previous influenza pandemics as well as from seasonal influenza epidemics (17-21). For example, the estimates from clinical and autopsy cases have shown that more than 95% and 50% of severe illnesses and deaths that occurred during 1918 pandemic and 2009 pandemic, respectively, are due to secondary bacterial (especially pneumococcal) infections (17-20).

Mechanisms of increased susceptibility to secondary bacterial infections following influenza infection have been widely studied since the 1918 pandemic (22, 23). Data from animal models indicate that influenza virus infections facilitate bacterial transmission (24), colonization and infection by impairing tracheal mucociliary clearance (25), damaging the airway epithelium to expose bacterial attachment sites (26, 27) and suppressing lung innate immunity (28). Defects in lung innate immune response, including the loss and dysfunction of alveolar macrophages (29, 30) and neutrophils (31, 32), and the dysregulation of cytokine productions (33), could play a key role in promoting secondary bacterial pneumonia.

However, secondary bacterial infections were mostly performed after a limited set of mouse-adapted laboratory influenza virus infections in previous studies. Secondary bacterial infections following other influenza virus infections have been less studied. Given the pandemic threat of H9N2 virus to human beings and the fact that secondary pneumococcal pneumonia accounts for excess mortality during influenza pandemics, it is necessary to determine whether H9N2 virus infection...
predisposes host to secondary pneumococcal infection. Thus, the present study was
designed to observe the effect of H9N2 virus infection on the host resistance to
secondary pneumococcal infection at different time points post-H9N2 infection by
utilizing mouse models. Understanding the interplays among H9N2 virus, host and S.

\textit{pneumoniae} may provide better strategies for the H9N2 pandemic preparedness. Here,
our results showed that H9N2 virus infection did not increase the susceptibility of
mice to secondary pneumococcal infection on 7 days post-H9N2 infection, and
improved pulmonary pneumococcal clearance when secondary pneumococcal
infection were performed after resolution of H9N2 virus infection.

\textbf{Results}

\textbf{H9N2 virus infection caused obvious respiratory diseases in BALB/c mice}

After a nonlethal dose (1.2 \times 10^5 PFU) of H9N2 virus infection, BALB/c mice
exhibited slight inactivity, chill, ruffled fur and inappetence on 3 days post-H9N2
infection (D.P.I.), and severer clinical signs from 5 to 7 D.P.I. Besides, H9N2-infected
mice showed a gradual weight loss, reached the peak weight loss on 6 D.P.I., and
recovered gradually thereafter (Fig. 1A). The body weight was not significantly
different between H9N2-infected mice and mock-infected mice on 10 D.P.I. and
afterwards (Fig. 1A). The virus was detected in the lungs of H9N2-infected mice on 3
and 7 D.P.I., but not on 14 or 28 D.P.I. (Fig. 1B). Additionally, pronounced bronchiolitis and alveolitis, characterized by extensive inflammatory cellular infiltration around bronchiole, alveoli and blood vessels, were seen in the lungs of H9N2-infected mice on 7 D.P.I. (Fig. 1C). The overall architecture of the lungs of H9N2-infected mice was similar to those of mock-infected mice on 14 and 28 D.P.I. (Fig. 1C). In line with these observations, the percent of lung areas affected, determined by semi-quantitative measurement of histopathological alterations, was significantly increased in H9N2-infected mice compared with mock-infected mice on 7 D.P.I. but was not significantly different between the two groups on 14 and 28 D.P.I. (Fig. 1D). Altogether, these data showed that H9N2 virus infection caused obvious respiratory diseases in BALB/c mice and mice infected with H9N2 virus had recovered by 14 D.P.I.

**H9N2 virus infection had no significant impact on host resistance to secondary pneumococcal infection on 7 D.P.I. in BALB/c mice**

It is well recognized that 7 days after influenza virus infection is a window of susceptibility to secondary bacterial infections in human and mouse model(34, 35). Therefore, we observed the effect of H9N2 virus infection on the host resistance to secondary pneumococcal infection on 7 D.P.I. in BALB/c mice. As the ability of the lung to clear bacterial pathogens is an important part of host defense against pulmonary bacterial infections, we firstly measured the pulmonary bacterial loads at 6 h and 12 h post 1 × 10^6 CFU of pneumococcal infection on 7 D.P.I. No statistically
significant differences in pulmonary bacterial loads were found between mice infected with H9N2 virus followed by *S. pneumoniae* (dual-infected mice) and mice infected only with *S. pneumoniae* (*S. pneumoniae*-infected mice) (Fig. 2A and 2B, left).

We then assessed lung histopathology at 6 h post pneumococcal infection on 7 D.P.I. Pronounced interstitial pneumonia, characterized by denuded epithelia, intra-alveolar fibrin exudation and extensive inflammatory cell recruitment around bronchiole, alveoli and blood vessels, were seen in the lungs of mice at 6 h after pneumococcal infection (Fig. 3A). The extent of pulmonary histopathological alterations were similar between dual-infected mice and *S. pneumoniae*-infected mice (Fig. 3A and 3B).

The body weight changes and survival were monitored post a 0.6 median lethal dose (1 × 10⁸ CFU) of *S. pneumoniae* infection on 7 D.P.I. The body weight changes after pneumococcal infection were showed in Fig. 4A, and the degree of weight loss was not obviously different between dual-infected mice and *S. pneumoniae*-infected mice (Table 1). The survival after pneumococcal infection was also not different between dual-infected mice and *S. pneumoniae*-infected mice (Fig. 4B). Together, the above results showed that H9N2 virus infection had no significant impact on host resistance to secondary pneumococcal infection on 7 D.P.I. in BALB/c mice.
Preceding H9N2 virus infection increased pulmonary pneumococcal clearance and reduced pneumococcal pneumonia-induced morbidity in BALB/c mice after recovery from influenza.

We also observed the effect of H9N2 virus infection on the host resistance to secondary pneumococcal infection on 14 or 28 D.P.I., when mice that infected with H9N2 virus had recovered from influenza. Bacterial loads at 6 h and 12 h post pneumococcal infection on 14 D.P.I. were both significantly decreased in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice (Fig. 2A and 2B, middle). Similarly, bacterial loads at 6 h and 12 h post pneumococcal infection on 28 D.P.I. were both significantly decreased in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice (Fig. 2A and 2B, right).

Markedly improved lung lesions with reduced inflammatory infiltrates were observed in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice at 6 h post pneumococcal infection on 14 D.P.I. (Fig. 3A). In line with these observations, the percent of lung areas affected was significantly decreased in dual-infected mice compared with *S. pneumoniae*-infected mice at 6 h post pneumococcal infection on 14 D.P.I. (Fig. 3B). Similar results were also observed at 6 h post pneumococcal infection on 28 D.P.I., though a statistical significance decrease in the percent of lung areas affected in dual-infected mice compared with *S. pneumoniae*-infected mice was not obtained (Fig. 3A and 3B).

The body weight changes and survival were also monitored post a 0.6 median lethal dose (1 × 10^8 CFU) of *S. pneumoniae* infection on 14 D.P.I. Weight loss was
significantly reduced on 1 days after pneumococcal infection in dual-infected mice compared with *S. pneumoniae*-infected mice (Fig. 4C). The survival after pneumococcal infection was not different between dual-infected mice and *S. pneumoniae*-infected mice (Fig. 4D). Collectively, the above results demonstrated that prior H9N2 virus infection increased pulmonary pneumococcal clearance and reduced pneumococcal pneumonia-induced morbidity in BALB/c mice after recovery from influenza.

H9N2 virus infection modulated pulmonary chemokine and cytokine responses to subsequent pneumococcal infection

To determine the pulmonary chemokine and cytokine responses to secondary pneumococcal infection following H9N2 virus infection, we measured the levels of chemokines [keratinocyte chemoattractant (KC) and mouse macrophage inflammatory protein-2 (MIP-2)], anti-inflammatory cytokine interleukin-10 (IL-10) and pro-inflammatory cytokines [interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β)] at 6 h after pneumococcal infection on 7, 14 or 28 D.P.I.

After pneumococcal infection on 7 D.P.I., the levels of KC and MIP-2 were similar in the lungs of dual-infected mice and *S. pneumoniae*-infected mice (Fig. 5A and 5B, left). The levels of IL-10, IL-6 and IL-1β were also similar in the lungs of dual-infected mice and *S. pneumoniae*-infected mice (Fig. 6A, 6B and 6D, left). However, the TNF-α levels were significantly decreased in the lungs of dual-infected
mice compared with *S. pneumoniae*-infected mice (Fig. 6C, left). These data showed that H9N2 virus infection reduced TNF-α production after pneumococcal infection on 7 D.P.I.

After pneumococcal infection on 14 D.P.I., the levels of KC and MIP-2 were both significantly decreased in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice (Fig. 5A and 5B, middle). The levels of IL-10, IL-6 and TNF-α were similar in the lungs of dual-infected mice and *S. pneumoniae*-infected mice (Fig. 6A, 6B and 6C, middle). However, the IL-1β levels were significantly increased in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice (Fig. 6D, middle). After pneumococcal infection on 28 D.P.I., no statistically significant differences were found in the levels of these chemokines and cytokines between dual-infected mice and *S. pneumoniae*-infected mice (Fig. 5A, 5B and 6A–6D, right). Taken together, these results suggested that H9N2 virus infection reduced productions of KC and MIP-2 but promoted IL-1β production after pneumococcal infection on 14 D.P.I.

A higher dose of H9N2 virus infection also promoted pulmonary pneumococcal clearance

Different doses of H9N2 virus infection might have different effects on the ability of the lung to clear *S. pneumoniae*. Therefore, we measured the bacterial loads at 12 h post pneumococcal infection following a lower dose (6 × 10⁴ PFU) or a higher dose (1.2 × 10⁶ PFU) of H9N2 virus infection. The significantly decreased bacterial
loads at 12 h after pneumococcal infection on 14 D.P.I. were also observed when mice were previously infected with the higher dose, but not the lower dose, of H9N2 virus (Fig. 7). Thus, these data showed that a higher dose of H9N2 virus infection also promoted pulmonary pneumococcal clearance.

**H9N2 virus infection increased pulmonary pneumococcal clearance in C57BL/6 mice after recovery from influenza**

Previous studies have shown that BALB/c mice and C57BL/6 mice are differed in their susceptibility to pneumococcal infection (36, 37). To determine whether the effect of H9N2 virus infection on pulmonary pneumococcal clearance was mouse strain-specific, we measured the bacterial loads at 6 h post pneumococcal infection following H9N2 virus infection in C57BL/6 mice. Bacterial loads were similar in the lungs of dual-infected mice and *S. pneumoniae*-infected mice after pneumococcal infection on 7 D.P.I. (Fig. 8). Besides, bacterial loads were in a tendency to decrease in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice after pneumococcal infection on 14 D.P.I., though did not achieve statistical significance (Fig. 8). Further, bacterial loads were significantly decreased in the lungs of dual-infected mice compared with *S. pneumoniae*-infected mice after pneumococcal infection on 21 D.P.I. (Fig. 8). The similar results were obtained after pneumococcal infection on 28 and 35 D.P.I. but not on 42 D.P.I. (Fig. 8). Therefore, these results showed that H9N2 virus infection also increased pulmonary pneumococcal clearance in C57BL/6 mice after recovery from influenza.
Discussion

H9N2 virus has been considered as a potential pandemic strain due to its features of the wide prevalence, enlarged mammalian hosts and extensive genetic reassortment (13). Our results showed that a nonlethal dose (1.2 \times 10^5 PFU) of H9N2 virus infection caused obvious signs of illness and significantly decreased body weight from 3 to 7 D.P.I. in BALB/c mice. The virus was detected in the lungs of H9N2-infected mice on 3 and 7 D.P.I., and extensive inflammatory cellular infiltration were observed in the lungs of H9N2-infected mice on 7 D.P.I. Further, there were no significant differences between the H9N2-infected mice and mock-infected mice concerning the clinical signs and body weight on 14 D.P.I. The virus had been completely eliminated from the lungs of H9N2-infected mice by 14 D.P.I., and lung histopathology in H9N2-infected mice was similar to that in mock-infected mice on 14 D.P.I. These results showed that H9N2 virus infection caused obvious respiratory diseases in BALB/c mice and mice infected with H9N2 virus had recovered by 14 D.P.I., which were consistent with our previous and other published research findings (38-40).

Although influenza virus alone could cause a substantial impact on global health, secondary bacterial infections post-influenza are associated with increased morbidity and mortality during both epidemic and pandemic influenza (23). Secondary bacterial
pneumonia, particularly from *S. pneumoniae*, accounts for more than 95% and 50% of severe illnesses and deaths that occurred during 1918 pandemic and 2009 pandemic, respectively (17-20). Therefore, it is important to understand the interactions among influenza virus, host and bacteria. Most previous studies by utilizing mouse models have showed that influenza virus infections could increase host susceptibility to secondary bacterial infections around 7 days post-influenza by decreasing lung defense (29, 30, 41). Results from the study of Ashok K. Chockalingam *et al.* showed that H9N2 virus (A/duck/Hong Kong/702/1979) infection increased susceptibility of BALB/c mice to secondary pneumococcal infection on 7 days post-influenza in terms of pulmonary bacterial loads, the degree of weight loss and survival (42). However, our results revealed that H9N2 virus (A/Chicken/Hebei/4/2008) infection did not increase the susceptibility of BALB/c mice to secondary pneumococcal infection on 7 D.P.I. with respect to bacterial loads, lung histopathology, the degree of weight loss and survival. The disparate results between the study by Ashok K. Chockalingam *et al.* and the present study might be due to the different strains of H9N2 virus being used. It has been proposed that several virulence factors of influenza virus have viral strain-specific effects on the host that contribute to secondary bacterial pneumonia (43). Influenza viruses with functional PB1-F2 proteins or decreased glycosylation of surface proteins are thought to effectively facilitate subsequent bacterial infections (23). Additionally, high-activity neuraminidase of influenza viruses could cleave sialic acid receptors more effectively to expose bacterial attachment receptors and enable bacteria to cause disease (41, 44). Results from the study of Ville
T. Peltola et al. showed that the neuraminidase activity of H9N2 virus from chicken was very low (44), which may help explain that H9N2 virus (isolated from chicken) infection did not promote secondary pneumococcal infection on 7 D.P.I. in our study.

Increased susceptibility of mice to secondary pneumococcal infection are also observed when mice are challenged with *S. pneumoniae* after recovery from influenza in numerous previous studies. For example, H1N1 and H3N2 virus infection had been shown to cause significantly increased pulmonary bacteria loads and mortality post pneumococcal infection on 14 days post-influenza in mice (45-48). Besides, a study performed by Arnaud Didierlaurent et al. demonstrated that H3N2 virus infection could still lead to significantly increased pulmonary bacteria loads and mortality after pneumococcal infection on 42 days post-influenza in mice (46). On the contrary, our result showed that H9N2 virus infection caused significantly decreased bacterial loads after pneumococcal infection on 14 or 28 D.P.I., suggesting that prior H9N2 virus infection increased pulmonary pneumococcal clearance in mice after recovery from influenza. Correlated with decreased bacterial loads, markedly alleviated pulmonary histopathological changes were also observed after pneumococcal infection on 14 D.P.I. Besides, H9N2 virus infection led to significantly reduced weight loss but did not change the mortality after pneumococcal infection on 14 D.P.I. These results implied that the effect of H9N2 virus infection on increasing the host resistance to pneumococcal infection on 14 D.P.I. is limited, which could reduce secondary pneumococcal pneumonia-induced morbidity, but was not sufficient to decrease secondary pneumococcal pneumonia-induced mortality in BALB/c mice.
We also determined whether the dose of H9N2 virus impacts the pulmonary pneumococcal clearance. Our result showed that the significantly decreased bacterial loads after pneumococcal infection were also observed when mice were 14 days previously infected with a higher dose, but not a lower dose, of H9N2 virus. Thus, a higher dose of H9N2 virus infection also promoted pulmonary pneumococcal clearance. Moreover, the beneficial effect of H9N2 virus infection on pulmonary pneumococcal clearance was not dependent on active viral replication, as the virus has been completely eliminated from the lungs of H9N2-infected mice by 14 D.P.I.

It has been reported that C57BL/6 mice are more susceptible to pneumococcal infection than BALB/c mice (36, 37). In the present study, our results showed that, similar to the results obtained in BALB/c mice, H9N2 virus infection could still lead to significantly decreased bacteria loads post pneumococcal infection on 21, 28 and 35 D.P.I. in C57BL/6 mice, suggesting that the beneficial effect of H9N2 virus infection on pulmonary pneumococcal clearance was not mouse strain-specific.

Recently, similar to our results, Helena Aegerter et al. found that H3N2 virus preceding 28-day infection also increased host resistance to secondary pneumococcal infection in terms of bacterial loads and mortality in C57BL/6 mice and this prolonged antibacterial protection was attributed to a population of monocyte-derived alveolar macrophages that produce increased IL-6 (49). During pneumococcal infection, neutrophils also play a key role in eliminating S. pneumoniae (50). The exact roles of macrophages and neutrophils underlying the protection against secondary pneumococcal infection conferred by H9N2 virus infection would need to
be investigated in further study.

It is generally assumed that local productions of chemokines and cytokines, as an important part of the innate immune response against bacterial infections, might play a role in the clearance of bacterial pathogens. However, a study done by Dallaire et al. had shown that the levels of KC, MIP-2, IL-6 and IL-1β in lungs of mice with pneumococcal infection were positively correlate with bacterial load(51). It had been shown that the productions of KC, IL-10, IL-6, TNF-α and IL-1β were significantly enhanced when bacterial loads were also significantly increased after S. pneumoniae on 7 days post H9N2 virus (A/duck/Hong Kong/702/1979) infection(42). Our results showed that the productions of KC, MIP-2, IL-10, IL-6 and IL-1β did not significantly change when bacteria loads were similar in the lungs of dual-infected mice and S. pneumoniae-infected mice after pneumococcal infection on 7 D.P.I. These results might be explained by the fact that productions of these mediators are dependent, at least in part, on the direct stimulation by S. pneumoniae. However, TNF-α production was significantly reduced in the lungs of dual-infected mice compared with S. pneumoniae-infected mice when bacteria loads were similar between the two groups after pneumococcal infection on 7 D.P.I. in our study. Results from the study of Alun C. Kirby et al. showed that TNF-α was mainly produced by alveolar macrophages during pneumococcal pneumonia but was not essential for the pneumococcal clearance(52). Thus, the significantly decreased TNF-α production in the present study might be explained by the fact that H9N2 virus preceding 7-day infection limited the ability of alveolar macrophages to produce TNF-α without
impacting pneumococcal clearance.

When secondary pneumococcal infection were performed on 14 D.P.I., the productions of KC and MIP-2 were significantly reduced when bacteria loads were significantly decreased in the lungs of dual-infected mice compared with S. pneumoniae-infected mice in our study. Conversely, KC production was significantly enhanced when bacteria loads were significantly increased after pneumococcal infection on 14 days post-H1N1 infection or on 14 days post-H3N2 infection (47, 48).

These results also suggested that productions of KC and MIP-2 after secondary pneumococcal infection on 14 days post influenza were associated with the direct stimulation by S. pneumoniae. Besides, the productions of IL-10, IL-6 and TNF-α did not significantly change with the notable exception of IL-1β, which significantly enhanced after pneumococcal infection on 14 D.P.I. in our study. Recently, the enhanced IL-1β production has been shown to be associated with induction of trained immunity (53-55). The concept of trained immunity is proposed to describe the fact that long-term activation of innate immune responses by certain pathogens or live vaccines could confer non-specific protection against subsequent infections of dissimilar pathogens (56). Taking this into account, the beneficial effect of H9N2 virus infection on pulmonary pneumococcal clearance might partially be due to the induction of trained immunity associated with the enhanced IL-1β production. Since bacteria loads were significantly decreased but the IL-1β production did not significantly change after pneumococcal infection on 28 D.P.I., other cellular and soluble mediators might be involved in improving bacterial clearance during
secondary pneumococcal infection following resolution of H9N2 virus infection. Further investigation would be required to clarify the induction of trained immunity by H9N2 virus infection.

In conclusion, our present study showed that H9N2 virus infection did not enhance the susceptibility of mice to secondary pneumococcal infection on 7 days post-H9N2 infection, and increased pulmonary pneumococcal clearance when secondary pneumococcal infection were performed after resolution of H9N2 virus infection. The interactions among influenza virus, host and *S. pneumoniae* are complex and the effects of other influenza virus infections on susceptibility to secondary pneumococcal infection need to be investigated in further study.

Materials and Methods

Mice strains

Specific pathogen-free (SPF) male BALB/c mice and C57BL/6 mice, all of which were between 6 and 8 weeks of age and weighed 18–20 g, were purchased from Beijing Vital River Laboratory Animal Technology Company Limited (China). All mouse experiments were approved by the Laboratory Animal Welfare and Animal Experimental Ethical Committee of China Agricultural University (No. AW12210202-2). All mice were acclimatized for 7 days before experimental treatments and had free access to food and water during the experiments.
Viral and bacterial strains

The H9N2 virus [A/Chicken/Hebei/4/2008(H9N2)] used in this study is one of the representative H9N2 isolates in northern China (57). The complete genome sequences of the virus are available in GenBank under accession numbers FJ499463–FJ499470. Its pathogenicity in mice had been assessed in detail in our previous study and the results showed that this H9N2 virus infection caused severe lung injury with a high mortality without prior adaptation (38). The virus was propagated in the allantoic cavities of 10-day-old embryonated SPF chicken eggs at 37 °C for 72 h, and then the allantoic fluid was centrifuged and stored at −80 °C for use in all of the experiments described herein. For H9N2 viral inoculation, the frozen virus liquid was thawed and diluted in sterile saline. Actual H9N2 virus concentration was determined by plaque assay as described below. Results were expressed as plaque forming units (PFU)/mL.

*S. pneumoniae* (NCTC7466, serotype 2) was grown in Todd-Hewitt broth supplemented with 0.5% yeast extract broth at 37 °C. When cultured at mid-log phase (OD600 = 0.3–0.4), the pneumococcal culture maintained in broth plus 20% glycerol was stored at −80 °C for use in all of the experiments described herein (58). For *S. pneumoniae* inoculation, the frozen stock was thawed and cultured in broth at 37 °C until midlogarithmic phase. Then the culture was centrifuged, washed twice in sterile phosphate-buffered saline (PBS) and subsequently pelleted before diluting to the desired concentration. Actual pneumococcal concentration was determined by plating 0.1 mL of 10-fold serial dilutions on blood agar plates and colonies were counted after
incubating for 24 h at 37 ℃. Results were expressed as colony forming units (CFU)/mL.

**Viral and bacterial inoculation**

Mice were lightly anesthetized by inhalation of isoflurane and then received a volume of 50 μL of viral or bacterial suspension at the tip of the nose and involuntarily inhaled. To facilitate the migration of the inoculum to the lung, mice were held in an upright position for 1 minute. As a control, mice were mock infected with 50 μL of noninfectious allantoic fluid or sterile PBS in an identical manner.

**Experimental protocol**

The present study was designed to observe the effect of H9N2 virus infection on host resistance to secondary pneumococcal infection at different time points post-H9N2 infection (Fig. 9), and was performed as described in the following three parts.

In the first part, two experiments were carried out. The first experiment was to observe the effect of H9N2 virus infection on the bacterial loads, lung histopathology and cytokine levels after pneumococcal infection. BALB/c mice were intranasally inoculated with $1.2 \times 10^5$ PFU of H9N2 virus or with noninfectious allantoic fluid as control. Seven, 14 or 28 days after H9N2 virus infection, mice were intranasally inoculated with $1 \times 10^6$ CFU of *S. pneumoniae* or with sterile PBS as control.
(experimental mouse groups were shown in Table 2). The dose of $1.2 \times 10^5$ PFU of H9N2 virus were chosen in the present study, as a pilot experiment indicated that mice infected with $1.2 \times 10^5$ PFU of H9N2 viruses would be ill but not dead and could be used to conduct secondary pneumococcal inoculation post H9N2 virus infection. After H9N2 virus infection, the clinical signs and body weight, as the measures of morbidity, were monitored daily; viral titers were measured on 3, 7, 14 and 28 D.P.I. and lung histopathology was assessed on 7, 14 and 28 D.P.I. in mock-infected mice and H9N2-infected mice. After pneumococcal infection on 7, 14 or 28 D.P.I., the four groups of mice were sacrificed at 6 h or 12 h and the whole lung tissues were harvested to analyze the lung histopathology, bacterial loads and cytokine levels as described below.

The second experiment was to observe the effect of H9N2 virus infection on the body weight changes and survival after pneumococcal infection. BALB/c mice were also randomized into the four groups as described in Table 2. H9N2 virus infection was performed as described in the first experiment and secondary pneumococcal infection was performed by intranasally inoculating mice with a 0.6 median lethal dose ($1 \times 10^8$ CFU) of *S. pneumoniae* on 7 or 14 D.P.I. Body weight was monitored daily and survival was recorded every 12 h until 7 days after pneumococcal infection when no more death was observed. Mice were euthanized when lost over 25% of their initial weight and appeared moribund (based on inability to move freely and access food and water), and considered to have died on that day (42).

In the second part, we determined whether the dose of H9N2 virus impacts the
pulmonary pneumococcal clearance. BALB/c mice were intranasally inoculated with
6×10^4 PFU (a lower dose), 1.2×10^5 PFU or 1.2×10^6 PFU (a higher dose) of H9N2
virus, and then all intranasally inoculated with 1×10^6 CFU of *S. pneumoniae* on 14
D.P.I. The mice were sacrificed at 12 h after pneumococcal infection and the whole
lung tissues were harvested to analyze the bacterial load as described below.

In the third part, we determine whether the effect of H9N2 virus infection on
pulmonary pneumococcal clearance was mouse strain-specific, as previous studies
have shown that BALB/c mice and C57BL/6 mice are differed in their susceptibility
to pneumococcal infection(36, 37). C57BL/6 mice were intranasally inoculated with
1.2 × 10^5 PFU of H9N2 virus and then intranasally inoculated with 1 × 10^6 CFU of *S.
pneumoniae* on 7, 14, 21, 28, 35 or 42 D.P.I. The mice were sacrificed at 6 h after
pneumococcal infection and the whole lung tissues were harvested to analyze the
bacterial load as described below.

**Plaque assay**

At the indicated time points after H9N2 virus inoculation, mice were sacrificed
by cervical dislocation, and the whole lungs were collected aseptically into sterile
tubes and homogenized in 1 ml of sterile saline. The lung homogenates were
centrifuged and then the supernatants were filtrated using a 0.22 μm filter membrane.
Then H9N2 virus concentrations in lung tissues were determined by plaque assay as
described previously(59). Briefly, adsorption 0.5 mL of 10-fold serial dilutions of
viral samples was performed on Madin-Darby canine kidney monolayers and overlaid
with 1% final concentration of agarose and 1 μg/ml final concentration of TPCK trypsin. After 72 h, cells were fixed with 4% formaldehyde and stained with 2% crystal violet for plaques to be detected. Viral titer (PFU/mL) = plaque counts/(0.5 mL × dilution factor of the sample).

Histopathological examination of lung tissues

At the indicated time points after H9N2 virus or S. pneumoniae inoculation, the left lobes of the lungs were removed immediately after mice were euthanized, fixed in 4% paraformaldehyde, and then embedded in paraffin. Fixed sections (3–5 μm) of paraffin-embedded lungs were stained with hematoxylin-eosin (H&E) for examining histopathological alterations in the lung parenchyma under a light microscope. Based on consideration of the extent of histopathological alterations, including peribronchial inflammation, intraalveolar inflammation, perivascular inflammation, bronchial epithelial shedding and intra-alveolar fibrin exudation, three sections per lung were blindly scored on a scale of 0 (no lung area affected) to 4 (100% of the lung area affected) by an experienced pathologist as described previously (60, 61). Results were expressed as lung area affected (%) = (total scores of three sections/3) × 25%.

Measurement of bacterial loads in lung tissues

At the indicated time points after S. pneumoniae inoculation, mice were sacrificed by cervical dislocation, and the whole lungs were collected aseptically into sterile tubes and homogenized in 1 ml of sterile PBS. Then the volume of the lung
homogenate was increased to 3 mL with sterile PBS. Finally, bacterial loads in lung tissues were determined by plating 0.1 mL of 10-fold serial dilutions on blood agar plates and colonies were counted after incubating for 24 h at 37 °C. Bacterial loads (CFU/lung) = (colony counts × 3 mL)/(0.1 mL × dilution factor of the sample)(58).

Measurement of cytokine levels in lung tissues

Lung homogenates were centrifuged and the supernatants were collected and stored at −80 °C until measuring of cytokine levels. The levels of keratinocyte chemoattractant (KC), mouse macrophage inflammatory protein-2 (MIP-2) and interleukin-1 beta (IL-1β) were measured using mouse Quantikine ELISA Kits (R&D Systems, USA). The levels of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) were measured using mouse Quantikine ELISA Kits (Solarbio, China). All of the above cytokines were measured according to the manufacturer's instructions.

Statistical analysis

All data were presented as mean ± standard error of mean (SEM). Data between two groups were analyzed by using two-tailed unpaired Student’s t test. Data among multiple groups were analyzed by using ordinary one-way ANOVA following by Tukey’s multiple comparisons test. Survival data were analyzed by using Log-rank (Mantel-Cox) test. All statistical analyses were performed using the GraphPad Prism 8.
software (Graph Pad Software, USA). Results with a $P$ values of < 0.05 were considered significant (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

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### Author contributions

Jingyun Li designed and performed most of the experiments, analyzed data and wrote the article. Hongyan Wang, Pengjing Lian, Yu Bai, Zihui Zhang, Lihong Zhao and Tong Xu helped perform experiments. Jian Qiao conceived the project, analyzed data and revised the article. All authors read and approved the final article.
Declaration of Competing Interest

The authors declare no conflict of interest.

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Figure legends

FIG 1 H9N2 virus infection caused obvious respiratory diseases in BALB/c mice. BALB/c mice were intranasally inoculated with $1.2 \times 10^5$ PFU of H9N2 virus or with noninfectious allantoic fluid diluted in sterile saline as control. (A) Body weight changes of mock-infected mice and H9N2-infected mice after H9N2 virus infection (n
(B) Viral titers in the lungs of H9N2-infected mice on 3, 7, 14 and 28 D.P.I. (n = 2 to 3/group). (C) Representative H&E-stained lung sections (magnification, × 400) of mock-infected mice and H9N2-infected mice on 7, 14 and 28 D.P.I. (n = 3/group). Extensive inflammatory cellular infiltration around bronchiole (black arrows), alveoli (blue arrows) and blood vessels (red arrows) were observed in the lungs of H9N2-infected mice on 7 D.P.I. (D) Percent of lung areas affected in mock-infected mice and H9N2-infected mice, calculated from specified histopathological parameters including peribronchial inflammation, intraalveolar inflammation and perivascular inflammation (n = 3/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for two-group comparisons: * = p < 0.05; ** = p < 0.001. N.S., Not significant; PFU, plaque forming units; D.P.I., days post-H9N2 infection.

FIG 2 H9N2 virus infection improved pulmonary pneumococcal clearance in BALB/c mice when secondary pneumococcal infection was performed on 14 or 28 D.P.I. BALB/c mice were intranasally inoculated with 1.2 × 10⁵ PFU of H9N2 virus or with noninfectious allantoic fluid diluted in sterile saline as control; seven, 14 or 28 days after H9N2 virus infection, mice were intranasally inoculated with 1 × 10⁶ CFU of S. pneumoniae. Bacterial loads in the lungs of S. pneumoniae-infected mice and dual-infected mice at (A) 6 h and (B) 12 h post pneumococcal infection on 7, 14 or 28 D.P.I. (n = 3 to 5/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for two-group comparisons: * = p < 0.05; ** =
p < 0.01. N.S., Not significant; CFU, colony forming units; D.P.I., days post-H9N2 infection; Sp, *Streptococcus pneumoniae*.

**FIG 3** H9N2 virus infection alleviated pulmonary histopathological changes induced by pneumococcal infection on 14 D.P.I. in BALB/c mice. (A) Representative H&E-stained lung sections (magnification, × 400) of *S. pneumoniae*-infected mice and dual-infected mice at 6 h post pneumococcal infection on 7, 14 or 28 D.P.I. (n = 3/group). Denuded epithelia (green arrows), intra-alveolar fibrin exudation (yellow arrows) and extensive inflammatory cell recruitment around bronchiole (black arrows), alveoli (blue arrows) and blood vessels (red arrows) were observed at 6 h post pneumococcal infection. (B) Percent of lung areas affected in *S. pneumoniae*-infected mice and dual-infected mice, calculated from specified histopathological parameters including peribronchial inflammation, intraalveolar inflammation, perivascular inflammation, bronchial epithelial shedding and intra-alveolar fibrin exudation (n = 3/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s *t* test were applied for two-group comparisons: * = *p* < 0.05. N.S., Not significant; D.P.I., days post-H9N2 infection; Sp, *Streptococcus pneumoniae*.

**FIG 4** H9N2 virus infection did not change survival but reduced weight loss after secondary pneumococcal infection on 14 D.P.I. in BALB/c mice. BALB/c mice were intranasally inoculated with 1.2 × 10^5* PFU of H9N2 virus or with noninfectious...
allantoic fluid diluted in sterile saline as control; Seven or 14 days after H9N2 virus infection, mice were intranasally inoculated with a 0.6 median lethal dose (1 × 10^8 CFU) of S. pneumoniae. (A and C) Body weight changes and (B and D) survival post pneumococcal infection on (A and B) 7 D.P.I. or (C and D) 14 D.P.I. (mock-infected mice and H9N2-infected mice: n = 5/group; S. pneumoniae-infected mice and dual-infected mice: n = 10/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for body weight changes of two-group comparison and log-rank (Mantel-Cox) test were applied for survival comparison: *** = p < 0.001. D.P.I., days post-H9N2 infection; PBS, phosphate-buffered saline; Sp, Streptococcus pneumoniae.

FIG 5 H9N2 virus infection reduced productions of KC and MIP-2 post pneumococcal infection on 14 D.P.I. Concentrations of (A) KC and (B) MIP-2 in the lungs of mock-infected mice, H9N2-infected mice, S. pneumoniae-infected mice and dual-infected mice at 6 h post pneumococcal infection on 7 D.P.I. (left), 14 D.P.I. (middle) or 28 D.P.I. (right) (n = 3 to 5/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for two-group comparisons: * = p < 0.05; ** = p < 0.01. N.S., Not significant; N.D., Not detectable; KC, keratinocyte chemoattractant; MIP-2, mouse macrophage inflammatory protein-2; D.P.I., days post-H9N2 infection; PBS, phosphate-buffered saline; Sp, Streptococcus pneumoniae.

FIG 6 H9N2 virus infection reduced TNF-α production post pneumococcal
infection on 7 D.P.I. and promoted IL-1β production post pneumococcal infection on 14 D.P.I.. Concentrations of (A) IL-10, (B) IL-6, (C) TNF-α and (D) IL-1β in the lungs of mock-infected mice, H9N2-infected mice, S. pneumoniae-infected mice and dual-infected mice at 6 h post pneumococcal infection on 7 D.P.I. (left), 14 D.P.I. (middle) or 28 D.P.I. (right) (n = 3 to 5/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for two-group comparisons: * = p < 0.05. N.S., Not significant; IL-10, interleukin-10; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; D.P.I., days post-H9N2 infection; PBS, phosphate-buffered saline; Sp, Streptococcus pneumoniae.

FIG 7 A higher dose of H9N2 virus infection also promoted pulmonary pneumococcal clearance. BALB/c mice were intranasally inoculated with 6 × 10^4 PFU (a lower dose), 1.2 × 10^5 PFU or 1.2 × 10^6 PFU (a higher dose) of H9N2 virus or with noninfectious allantoic fluid diluted in sterile saline as control; fourteen days after H9N2 virus infection, all mice were intranasally inoculated with 1 × 10^6 CFU of S. pneumoniae. Pulmonary bacterial loads at 12 h after pneumococcal infection were measured (n = 3 to 4/group). Data were presented as mean ± SEM. Ordinary one-way ANOVA following by Tukey’s multiple comparisons test were applied for four-group comparisons: ** = p < 0.01; *** = p < 0.001. N.S., Not significant; D.P.I., days post-H9N2 infection; CFU, colony forming units; PFU, plaque forming units; Sp, Streptococcus pneumoniae.
FIG 8 H9N2 virus infection increased pulmonary pneumococcal clearance in C57BL/6 mice when secondary pneumococcal infection was performed on 21, 28 or 35 D.P.I. C57BL/6 mice were intranasally inoculated with 1.2 × 10^5 PFU of H9N2 virus or with noninfectious allantoic fluid diluted in sterile saline as control; seven, 14, 21, 28, 35, 42 days after H9N2 virus infection, mice were intranasally inoculated with 1 × 10^6 CFU of S. pneumoniae. Pulmonary bacterial loads at 6 h after pneumococcal infection were measured (n = 2 to 9/group). Data were presented as mean ± SEM. Two-tailed unpaired Student’s t test were applied for two-group comparisons: * = p < 0.05; ** = p < 0.01. N.S., Not significant; D.P.I., days post-H9N2 infection; CFU, colony forming units; Sp, Streptococcus pneumoniae.

FIG 9 Schematic diagram of H9N2 virus infection and secondary pneumococcal infection. In the present study, secondary pneumococcal infection were mainly performed on 7, 14 or 28 days after H9N2 virus infection in mice.
A 7 D.P.I.  14 D.P.I.  28 D.P.I.

Saline/Sp

H9N2/Sp

B

Lung area affected (%%)

Saline/Sp  H9N2/Sp

7 D.P.I.  14 D.P.I.  28 D.P.I.
Figure A: IL-10 concentrations (pg/mL) over time (7 D.P.I., 14 D.P.I., 28 D.P.I.)

Figure B: IL-6 concentrations (pg/mL) over time (7 D.P.I., 14 D.P.I., 28 D.P.I.)

Figure C: TNF-α concentrations (pg/mL) over time (7 D.P.I., 14 D.P.I., 28 D.P.I.)

Figure D: IL-1β concentrations (pg/mL) over time (7 D.P.I., 14 D.P.I., 28 D.P.I.)

Legend:
- Saline/PBS
- H9N2/PBS
- Saline/Sp
- H9N2/Sp

Significance:
- N.S. = Not Significant
- * = Significant difference
TABLE 1. The degree of weight loss (%) after secondary pneumococcal infection on 7 days post-H9N2 infection

| Group                     | Days after secondary pneumococcal infection |
|---------------------------|-------------------------------------------|
|                           | 1  | 2  | 3  | 4  | 5  | 6  | 7  |
| *S. pneumoniae*-infected mice | 8.18 | 12.76 | 14.81 | 13.69 | 15.20 | 3.28 | -1.04 |
| dual-infected mice         | 8.03 | 12.08 | 12.20 | 11.41 | 8.48  | 5.47 | 4.89 |

*The degree of weight loss (%) in *S. pneumoniae*-infected mice = mean percent of body weight in mock-infected mice – mean percent of body weight in *S. pneumoniae*-infected mice. Similarly, the degree of weight loss (%) in dual-infected mice = mean percent of body weight in H9N2-infected mice – mean percent of body weight in dual-infected mice.
TABLE 2. Experimental mouse groups after secondary pneumococcal infection

| Group           | Primary inoculation                  | Secondary inoculation |
|-----------------|--------------------------------------|-----------------------|
| mock-infected   | noninfectious allantoic fluid         | sterile PBS           |
| H9N2-infected   | H9N2 virus                            | sterile PBS           |
| S. pneumoniae-infected | noninfectious allantoic fluid     | S. pneumoniae         |
| dual-infected   | H9N2 virus                            | S. pneumoniae         |