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ORIGINAL ARTICLE

Protective effects of SP600125 on mice infected with H1N1 influenza A virus

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Abstract
Influenza A virus (IAV) can cause high morbidity and mortality globally every year. Myriad host kinases and their related signaling pathways are involved in IAV infection, and the important role of the c-Jun N-terminal kinase signaling pathway during infection has been demonstrated. SP600125, an inhibitor of c-Jun N-terminal kinase, was found in our previous study to suppress IAV replication *in vitro*. In this study, we established a mouse model of H1N1 IAV infection and treated the mice with SP600125 to study its protective effect. The results showed that SP600125 treatment reduced the pulmonary inflammatory response, lung injury, and pulmonary viral load and increased the survival rate of H1N1-infected mice. Our data confirm the crucial role of c-Jun N terminal kinase in H1N1 virus replication and inflammatory responses *in vivo*. Hence, we speculate that SP600125 has a potential antiviral therapeutic benefit against IAV infection.

Introduction

Influenza A virus (IAV) is a globally important respiratory pathogen that is characterized by internal gene reassortments. It causes huge economic losses and poses great health risks to humans [1, 2]. Epidemics of influenza A virus are reported every year. In the past 100 years, there have been four influenza pandemics, two of which were caused by the H1N1 subtype. The Spanish flu of 1918 caused nearly 50 million deaths [3]. Currently, vaccination is the primary and most effective method of fighting against IAV infection. However, vaccination is still recommended each year because of antigenic drift and because the optimal timing for vaccinations is still unknown [4, 5]. Meanwhile, antiviral drug resistance remains a potential risk. H1N1 subtype influenza virus has been shown to be able to develop resistance to the neuraminidase (NA) inhibitor oseltamivir and the adamantane class of membrane protein (M2) ion channel inhibitors [6]. Thus, novel and effective antiviral approaches against multiple IAV strains are urgently needed. The better we understand the cellular mechanisms associated with IAV infection, the more likely it is that we can develop a novel prophylaxis and therapy strategy by regulating the host antiviral response.

A series of robust inflammatory responses known as 'cytokine storm' are activated in the host after influenza virus infection [7]. The cytokine storm has been confirmed to be a direct cause of death, but the mechanisms involved are complicated and still unclear. c-Jun terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) family. JNK is activated by mitogen-activated protein kinase kinases (MKKs) 4 and 7, which are regulated by several mitogen-activated protein kinase kinase kinases (MAP3Ks) [8]. Phosphorylated JNK triggers the Raf/MEK/ERK pathway and mediates the expression of cytokines and chemokines, including interleukin, tumor necrosis factor, and interferon [9]. JNK has three isoforms, and JNK 1 and 2 have been shown to be involved in viral RNA (vRNA) synthesis and cytokine regulation during influenza virus replication [10]. In addition, recent studies have shown that nonstructural protein 1 (NS1) of IAV activates JNK, which in turn increases IAV replication by regulating autophagy

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[11, 12]. Thus, the JNK pathway plays an important role in influenza virus infection.

SP600125 is a reversible ATP competitive inhibitor of JNK that is widely utilized in studies of the JNK pathway [13]. Our previous studies have demonstrated that c-Jun, which is downstream of JNK, suppresses the replication of H5N1 virus at an early stage and that SP600125 impairs the process of synthesis of vRNA from cRNA, while cRNA and mRNA synthesis are not affected *in vitro* [10, 14]. In the present study, we investigated whether SP600125, as an inhibitor of the JNK signaling pathway, has potential value in clinical therapy of H1N1 infection *in vivo*. We found that SP600125 treatment may be a useful strategy for alleviating lung lesions and balancing the pro-inflammatory and anti-inflammatory responses.

Materials and methods

Animals

Female BALB/c mice aged 6-7 weeks were used in the experiments. The mice were housed with a separate ventilation system and given sterile food and water. Experiments were carried out after all of the mice were acclimatized for at least one week.

Virus and challenge

The H1N1 influenza virus (A/WSN/33) used in the experiment was described in our previous publication [15]. The half lethal dose (LD_{50}) in mice was determined previously [16]. The mice were anesthetized with 50 μL of Zoletil (Virbac, Carros, France) by intramuscular injection and infected intranasally with a dose of 3 LD_{50} of H1N1 virus [16].

Reagents

SP600125 (Selleck Chemicals, USA) was dissolved at a concentration of 3 mg/mL at 37℃ in a solvent composed of 10% ethanol, 15% polyoxyethylene castor oil, 30% polyethylene glycol, 20% propylene glycol, and 25% physiological saline and stored at -20℃.

Treatment trial

Mice were randomly assigned to the H1N1 virus challenge group (H1N1-virus-infected mice treated with solvent), the SP600125 treatment group (H1N1-virus-infected mice treated with SP600125), or the control group (mice without virus infection or treatment). SP600125 was administered intraperitoneally to anaesthetized mice at a dose of 30 mg/kg body weight [13, 17], starting two days before viral challenge, five days in all. In the H1N1 virus challenge group, SP60025 was replaced by solvent, and there was no viral challenge or treatment in the control group. Survival, body weight, and clinical symptoms were observed daily for at least 14 continuous days. On day 3 and day 6 postinfection, lung tissues of three randomly selected mice from each group were collected in sterile tubes and stored in liquid nitrogen or in 4% formalin until required. The details of the experimental procedure are shown in Figure 1.

Histology and Immunochemistry

Lung tissues were collected from euthanized mice, rapidly transferred to 4% formalin, and stored for at least 48 h. Preparation of paraffin sections, staining with hematoxylin and eosin (H&E), immunohistochemistry (IHC), and evaluation of pathological changes were carried out by previously published methods [18].

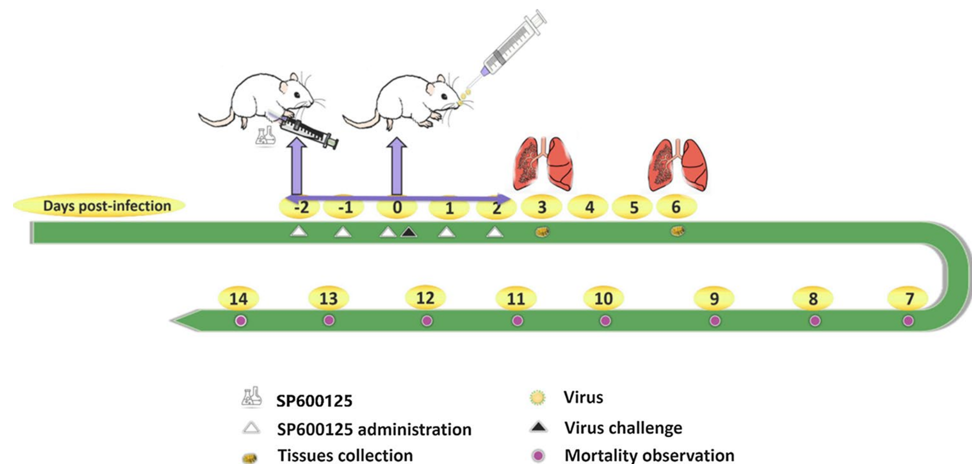

Fig. 1 Treatment and viral challenge of mice

Real-time quantitative PCR analysis

TRIzol Reagent (Invitrogen, UT, USA) was used to extract total RNA from lung tissue (approximately 10 mg) according to the manufacturer's introductions. The procedures for analysis of gene expression were described previously [18]. The expression level of the NS1 gene of IAV was calculated using the relative quantification method. The constitutively expressed housekeeping gene β-actin was used as an internal control to determine the fold change in the NS1 gene. Expression of interferon (IFN)-γ, IFN-β, interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α was measured by the relative quantification method. Changes in gene expression were normalized to the control using the $2^{-\Delta\Delta ct}$ method with β-actin as an internal standard. The primers used are listed in Supplementary Table 1.

Western blot analysis

Lung tissues were collected and lysed in RIPA buffer with 10 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 20 min. The procedures for determination of protein concentration and analysis of proteins were the same as in our previous publication [19]. Anti-phosphorylated-JNK (p-JNK) antibody (cat. no. 612540) was purchased from BD Biosciences, and anti-total-JNK (t-JNK) antibody (cat. no. 9252) was purchased from Cell Signaling Technology. β-actin served as an internal standard to determine the amount of protein loaded.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Differential expression was determined using one-way or two-way ANOVA with GraphPad Prism 8. $P < 0.05$ was considered statistically significant, $P < 0.01$ was considered highly significant, and $P < 0.001$ was considered extremely significant.

Results

SP600125 alleviates clinical signs and improves survival rates in H1N1-virus-infected mice

To examine the protective effects of SP600125 on H1N1-virus-infected mice, we observed and recorded survival rate, body weight, and clinical signs. On day 3 postinfection, mice in the H1N1 virus challenge group developed significant clinical signs characterized by ruffled fur and depression, with squinting, huddling, and reduced activity. Mice in the SP600125 treatment group had no obvious clinical signs. On day 6 postinfection, mice in the H1N1 virus challenge group became thinner and had slower reactions. The clinical changes in the mice in the SP600125 treatment group were milder than those in the H1N1 virus challenge group. As shown in Figure 2A, the weight loss trends in the H1N1 virus challenge group and the SP600125 treatment group were essentially identical. Compared with the H1N1 virus challenge group, there was slightly less body weight loss in the SP600125 treatment group from day 2 postinfection to day 10 postinfection. After day 10 postinfection, an evident difference in body weight loss was observed between the H1N1 virus challenge group and the SP600125 treatment group as the surviving SP600125-treated mice began to gain weight. The survival rate observed on day 9 postinfection was 50.0% in the H1N1 virus challenge group and 83.3% in the SP600125 treatment group. The mice in the control group had no obvious clinical signs, weight loss, or death. These results suggest that SP600125 treatment can partially improve the clinical signs and increase the survival rate of H1N1-virus-infected mice.

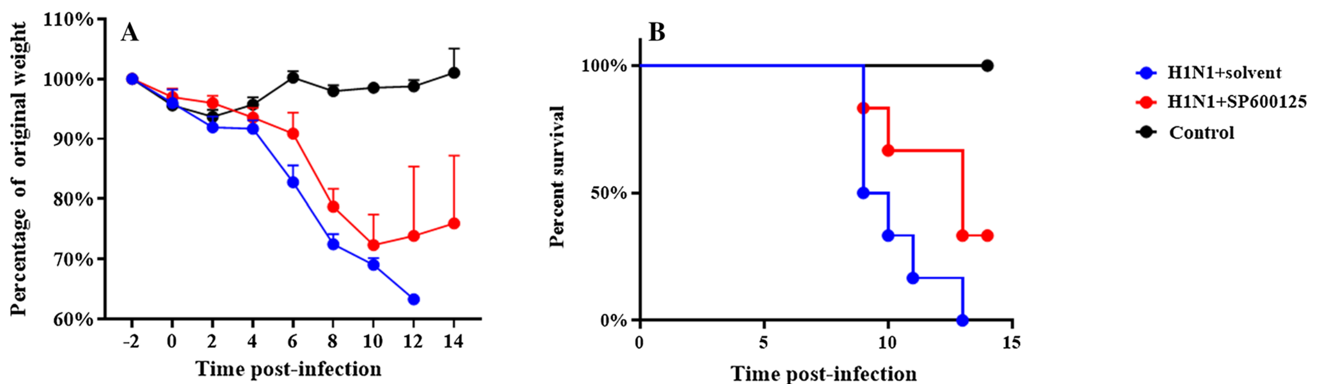

Fig. 2 SP600125 increases the survival rate and alleviates clinical symptoms in H1N1-virus-infected mice. (A) The percentage of the body weight after infection relative to the original weight (N = 6 per group). (B) The survival rates of H1N1-virus-infected mice with or without SP600125 treatment (N = 6 per group). Data are expressed as the mean ± SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

SP600102 reduces pathological injury in the lungs of H1N1-virus-infected mice

Lung lesions were observed in mice on day 3 and day 6 postinfection. There were dramatic differences in the histopathological changes in lung tissue between the H1N1 virus challenge and SP600125 treatment groups (Fig. 3A). Edema of blood vessels around the bronchioles, congestion of alveolar septal capillaries, and inflammatory cells were observed in the lungs of mice in the H1N1 virus challenge group on day 3 postinfection, and on day 6 postinfection, the normal structure of the lung was severely damaged, with exudates in the blood vessels, exfoliated alveolar epithelial cells, thickened alveolar walls, and decreased alveolar space. The pathological damage appeared to be less severe in mice treated with SP600125, in which only slight edema and inflammatory cellular infiltration around the vessels and the bronchioles were observed. There were no obvious lesions in the lungs of the mice in the control group. Scores for pathological lesions in H1N1-virus-infected mice with or without SP600125 treatment on days 3 and 6 are shown in Figure 3B. The results indicate that SP600125 treatment can effectively reduce the severity of pathological lesions in the lungs of H1N1-virus-infected mice.

SP600125 reduces the pulmonary viral load of H1N1-virus-infected mice

We found significant differences in the distribution and expression of the influenza virus nucleoprotein (NP) in lungs of mice of the different groups (Fig. 4A). NP antigen was observed in bronchial epithelial cells, alveolar cells,

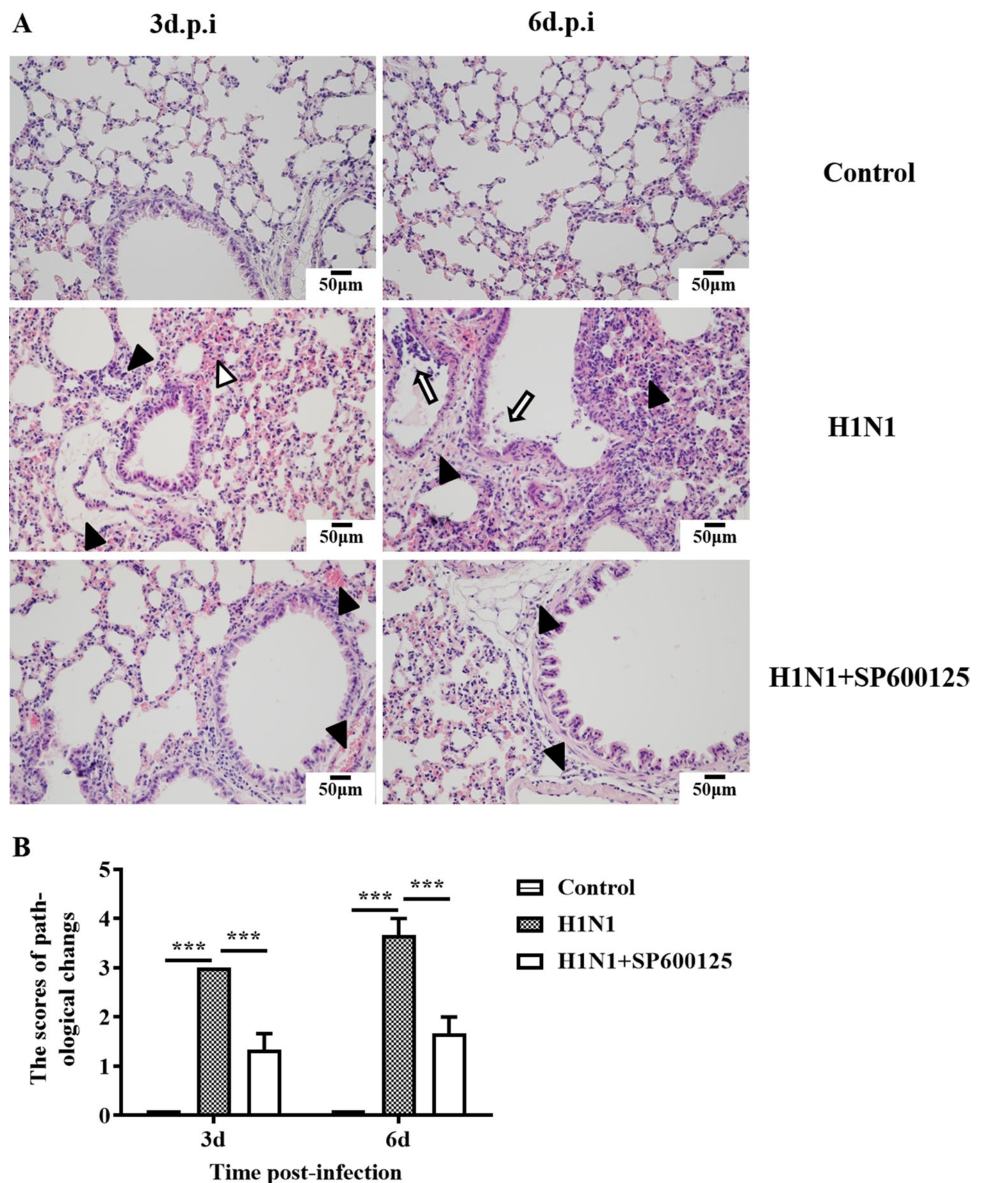

Fig. 3 Representative images and scores of pathological changes in the lung tissues of mice. (A) Lung histopathology on days 3 and 6 postinfection. Representative lung sections were subjected to H&E staining. Open triangles indicate alveolar lesions. Black triangles indicate edema and inflammatory cell infiltration around the vessels and bronchioles. Arrows indicate lesions of bronchial mucosa and vessels. Scale bar, 50 μm. (B) Scores of pathological changes in lungs of mice on day 3 and 6 postinfection. Pathological changes in lung tissue were evaluated by a veterinary pathologist and scored from 0 to 4 in a blinded study (N = 3). Data are expressed as the mean ± SD. ***, $P < 0.001$ when H1N1 was compared with the control and H1N1 + SP600125 by two-way ANOVA multiple comparisons.

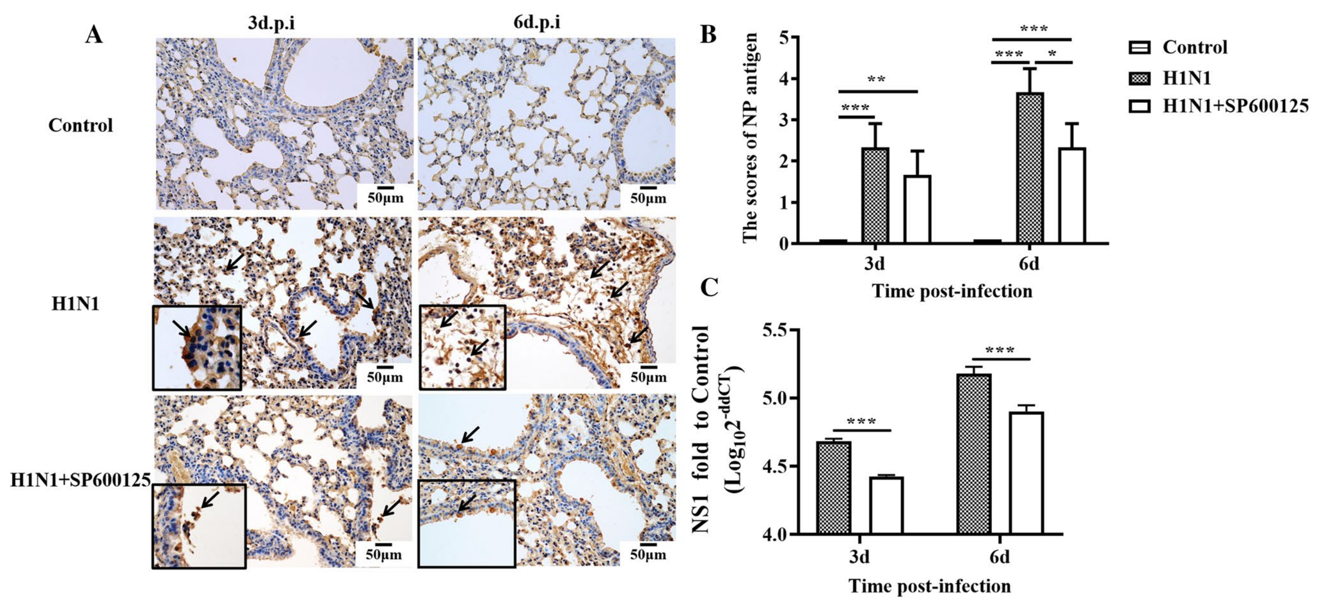

Fig. 4 SP600125 reduces the viral load in the lungs of H1N1-virus-infected mice. Data are expressed as the mean ± SD. Differential expression was evaluated using two-way tests of variance in GraphPad Prism 8. (A) Distribution of nucleoprotein antigen in the lungs of mice on days 3 and 6 postinfection, as determined by immunohistochemistry. Scale bar, 50 μm. (B) Scores for nucleoprotein antigen in the lungs of mice on days 3 and 6 postinfection. IAV antigens in the lungs were evaluated by a veterinary pathologist and scored from 0 to 4 in a blinded study (N = 3); *, $P < 0.05$ when H1N1 was compared with H1N1 + SP600125 on day 6 postinfection. **, $P < 0.01$ when H1N1 + SP600125 was compared with the control on day 3 postinfection. ***, $P < 0.001$ when the control was compared with H1N1 and H1N1 + SP600125 on day 6 postinfection and with H1N1 on day 3 postinfection. (C) Viral load in the lungs of mice in the H1N1 virus challenge group and the SP600125 treatment group on days 3 and 6 postinfection, estimated using real-time PCR (N = 3). *, $P < 0.05$ when H1N1 + SP600125 was compared with H1N1 on day 3 postinfection. ***, $P < 0.001$ when H1N1 + SP600125 was compared with H1N1 on day 6 postinfection

and inflammatory cells in the lungs of H1N1-virus-infected mice, while few NP-antigen-positive signals were found in the bronchial epithelial cells of the SP600125 treatment group. No positive signals were observed in lungs of mice in the control group. The scores of NP antigens in the lungs of mice are shown in Figure 4B. There was a significant difference in the number of NP-positive signals in the lungs of mice in each group on day 6 postinfection ($p < 0.05$). To further investigate the effect of SP600125 on H1N1 virus replication *in vivo*, the viral load in lungs of mice was examined using real-time quantitative PCR. As shown in Figure 4C, the fold change in the level of the NS1 gene relative to the control in the lungs of mice treated with SP600125 was significantly lower than in the H1N1 virus challenge group on days 3 and 6 postinfection. Overall, our data suggest that SP600125 may play a key role in inhibiting the replication of H1N1 virus in the lungs.

SP600125 treatment reduces the expression level of inflammatory cytokines and p-JNK protein

To investigate the mechanism by which SP600125 inhibits infection, we measured the expression levels of IFN-β, IFN-γ, IL-6, IL-1β, and TNF-α by qPCR (Fig. 5) and the expression of phosphorylated JNK protein (p-JNK) and total JNK protein (t-JNK) by western blot in the lungs of H1N1-virus-infected mice and SP600125-treated mice (Fig. 6A and B). The expression levels of all five of these cytokines in lungs of SP600125-treated mice were lower than those in untreated mice on day 3 postinfection, and the differences in IFN-β, IL-1β, and IL-6 expression levels were statistically significant. The expression levels of IL-6, IFN-γ, IFN-β, and IL-1β in the lungs of the SP600125 treatment group mice were lower those that in the H1N1 virus challenge group on day 6 postinfection, and the IL-1β expression levels differed significantly. There was no obvious difference in the level of t-JNK expression, while the level of p-JNK expression in the lungs of the H1N1 virus challenge group was higher than in the SP600125 treatment group on days 3 and 6 postinfection. These data are consistent with the pulmonary histopathologic changes described above. Our results suggest that SP600125 treatment can significantly reduce the inflammatory response in the lungs of H1N1-virus-infected mice and downregulate the expression of cytokines and p-JNK.

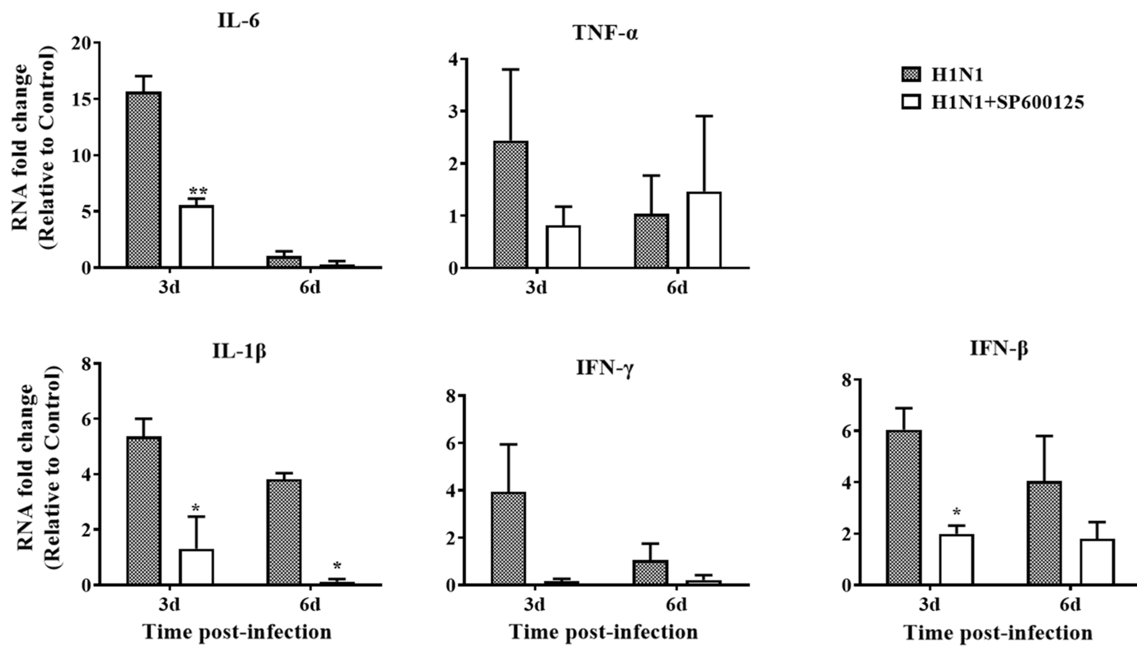

Fig. 5 SP600125 reduces the expression level of inflammatory cytokines in the lungs of H1N1-virus-infected mice on days 3 and 6 postinfection. The expression of IL-6, IFN-β, IL-1β, IFN-γ, and TNF-α in the lungs of mice in the different treatment groups on days 3 and 6 postinfection was measured using real-time PCR. The results are presented as the mean ± SD of three independent replicates. *, $P < 0.05$ when H1N1 + SP600125 was compared with H1N1. **, $p < 0.01$ when H1N1 + SP600125 was compared with H1N1

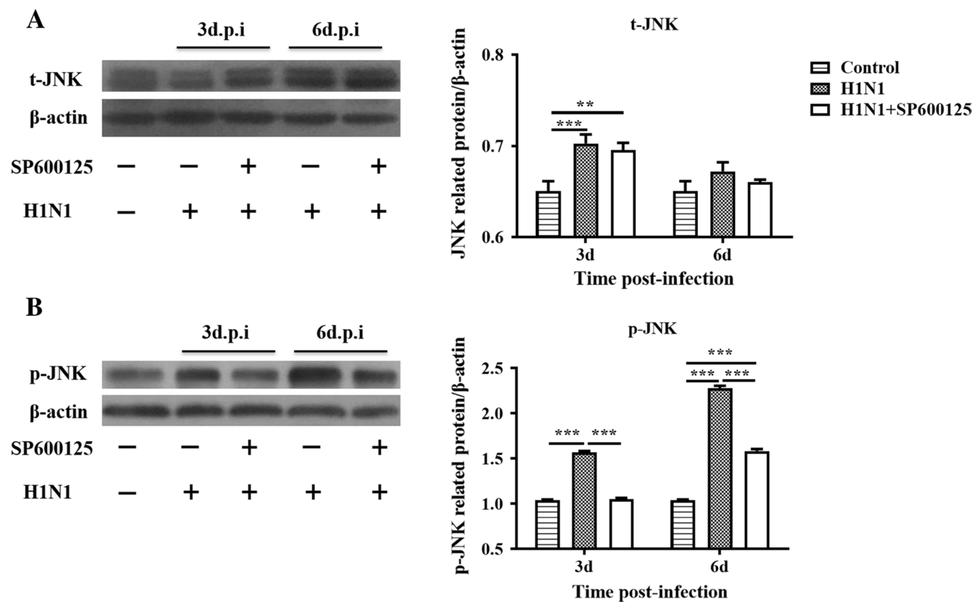

Fig. 6 SP600125 reduces the expression level of p-JNK protein in the lungs of H1N1-virus-infected mice on days 3 and 6 postinfection. The relative band intensity was determined digitally using Quantity One 4.1.1 software and was expressed as the ratio of c-Jun to β-actin. (A) Total-JNK (t-JNK) expression in lung tissue on days 3 and 6 postinfection analyzed by Western blot. **, $p < 0.01$ when H1N1 + SP600125 was compared with the control on day 3 postinfection. ***, $p < 0.001$ when H1N1 was compared with the control on day 3 postinfection. (B) Phosphorylated JNK (p-JNK) expression in lung tissue on days 3 and 6 postinfection, analyzed by Western blot. ***, $p < 0.001$ when H1N1 was compared with H1N1 + SP600125 and control on day 3 postinfection and on day 6 postinfection, and when H1N1 + SP600125 was compared with control on day 6 postinfection

Discussion

Aberrant kinase activity is associated with IAV infection, but no small-molecule kinase inhibitors (SMKIs) are being tested in clinical trials of IAV infection [20]. Our group has shown that inhibition of the activation of c-Jun, which acts downstream of the JNK/MAPK pathway, can lead to a reduction in IAV replication, inflammation, and impaired vRNA synthesis *in vitro* [10, 14]. In this study, we explored the protective role of the JNK inhibitor SP600125 in pulmonary inflammatory responses induced by H1N1 virus infection in a mouse model.

JNK3 is expressed in the brain, while JNK1 and JNK2, which are more biologically active, are widely distributed in cells and tissues [21, 22]. Therefore, JNK is presumed to be a potential treatment target for various diseases. Small-molecule inhibitors of JNK such as SP600125 have been demonstrated to have potential as new therapeutic drugs for asthma [23]. The IAV titer is enhanced by the accumulation of reactive oxygen species (ROS), which is correlated with the JNK pathway [24]. In the present study, we showed that SP600125, an inhibitor of c-Jun, could reduce the death rate, decrease the severity of illness, and balance the inflammatory response in the lungs of H1N1-virus-infected mice.

IAV infection can result in a "cytokine storm", in which high expression levels of inflammatory cytokines are released and recruited to the infection site to trigger viral pneumonia [7]. The uncontrolled release of cytokines has been suggested to be associated with high morbidity and mortality in IAV infection. Studies have shown that JNK activation is associated with the cytokine storm phenomenon [9]. During SARS coronavirus (SARS-CoV) infection, activated protein 1 (AP-1) activity increases due to triggering of the ERK and JNK cascades by the SARS-CoV 3b protein [9], and AP-1 regulates the transcription of many pro-inflammatory and antiviral cytokines, including IL-6, IL-8, and IFN-β [25–27]. H7N7 and H2N2 IAV infection also induces JNK- and AP-1-dependent gene expression in infected cells and triggers expression of antiviral cytokines [28]. Our data show that expression of IFN-γ, IFN-β, IL-6, IL-1β, and TNF-α was elevated on day 3 postinfection. However, SP600125 treatment led to significant downregulation of these cytokines, suggesting that inhibition of N-terminal c-Jun phosphorylation may have a strong influence on cytokine expression. In our previous study, c-Jun was shown to be involved in RNA-induced inflammation and to play a critical role in the initiation and regulation of inflammatory responses to H5N1 virus infection *in vitro* [14]. The level of expression of IFN-β on day 6 postinfection was still high in the H1N1 virus challenge group, which is consistent with its pro-inflammatory activity in the early stage of infection and its antiviral properties [29]. We also found that SP600125 treatment inhibited virus replication in the lungs of H1N1-virus-infected mice, and these results were consistent with our previous *in vitro* experiments [14]. In addition, we demonstrated systematically that SP600125 treatment reduced the amount of lung injury caused by H1N1 virus infection *in vivo*. Our previous study showed that a c-Jun-targeting DNAzyme (Dz13) directly decreased the level of inflammatory cytokines during H5N1 virus infection [14], and we therefore assumed that the suppressed inflammatory response observed in the present study was not due to impaired viral replication. Together, both balanced cytokine expression and suppressed viral replication ultimately regulated lung inflammation and increased the rate of survival of the mice. Our data are in line with the expectation that inhibiting the JNK pathway can reduce lung injury in IAV-infected mice. Combined with our previous research on SP600125 *in vitro* [10], we conclude that SP600125 treatment targeting the JNK pathway may provide a novel antiviral treatment option against IAV infection. Our findings link IAV infection to the activation of an important signaling pathway *in vivo* that seems to be required for the antiviral response to infection.

Since all of the mice used in our study were healthy adults, more animal models need to be established to simulate the effects of treatment of elderly and immunosuppressed patients. The specific mechanisms by which the JNK pathway is involved in ameliorating inflammatory damage in IAV infection also warrant further study.

In conclusion, SP600125 treatment partially protected H1N1-virus-infected mice from death by alleviating lung lesions and inhibiting viral replication. Thus, it will be of interest to explore the potential therapeutic effects of SP600125 in combating IAV infection.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05103-0.

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Declarations

Conflict of interest The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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