



Regulatory roles of c-jun in H5N1 influenza virus replication and host inflammation



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ABSTRACT

The cytokine storm which is a great burden on humanity in highly pathogenic influenza virus infections requires activation of multiple signaling pathways. These pathways, such as MAPK and JNK, are important for viral replication and host inflammatory response. Here we examined the roles of JNK downstream molecule c-jun in host inflammatory responses and H5N1 virus replication using a c-jun targeted DNAzyme (Dz13). Transfection of Dz13 significantly reduced H5N1 influenza virus replication in human lung epithelial cells. Concomitantly, there was a decreased expression of pro-inflammatory cytokines (tumor necrosis factor (TNF)- α , interferon (IFN)- β and interleukin (IL)-6) in c-jun suppressed cells, while the expression of anti-inflammatory cytokines, such as IL-10, was increased. In vivo, compared with control groups, suppression of c-jun improved the survival rate of mice infected with H5N1 virus (55.5% in Dz13 treated mice versus $\leq 11\%$ of control mice) and decreased the CD8⁺ T cell proliferation. Simultaneously, the pulmonary inflammatory response and viral burden also decreased in the Dz13 treated group. Thus, our data demonstrated a critical role for c-jun in the establishment of H5N1 infection and subsequent inflammatory reactions, which suggest that c-jun may be a potential therapeutic target for viral pneumonia.

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1. Introduction

Influenza A virus (IAV) remained to be a common pathogen causing high morbidity and significant mortality worldwide [1]. Highly pathogenic IAV H5N1 can cause acute respiratory infections in both poultry and humans; the mortality rate of human infected H5N1 is about 60% [2,3]. Furthermore, the influenza A virus including H5N1 may cause

pandemics because of its antigenic drift or antigenic shift [1], and thus is a major burden for public health worldwide.

Numerous studies have attempted to determine the reasons for high mortality of H5N1 infection. High viral load and the resulting intense inflammatory response were reported to be responsible for disease severity [4]. H5N1 virus infection induces high viral load in pharyngeal and frequent viral RNA detection in blood, leading to over-expression of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ , IFN- α/β , interleukin (IL)-6, IL-1, and so on. The excessive levels of pro-inflammatory cytokine production also named “cytokine storm”, has been suggested as the direct reason for the lethal clinical symptoms, for example, extensive pulmonary edema, acute bronchopneumonia and alveolar hemorrhage [5,6]. Therefore, the development of a therapeutic agent to inhibit IAV replication and limit host inflammation without having deleterious side effects on innate immunity is urgently required.

Vaccination against influenza remains the most effective preventive measure, but may become less effective because of the antigenic variation of IAV [7]. Alternatively, antiviral drugs such as zanamivir (Relenza) and oseltamivir (Tamiflu) have proven to be efficient, but

Abbreviations: IAV, Influenza A virus; Dz13, a c-jun targeted DNAzyme; ATF, activating transcription factor family; MAF, musculoaponeurotic fibrosarcoma family; MDCK, Madin–Darby canine kidney cell line

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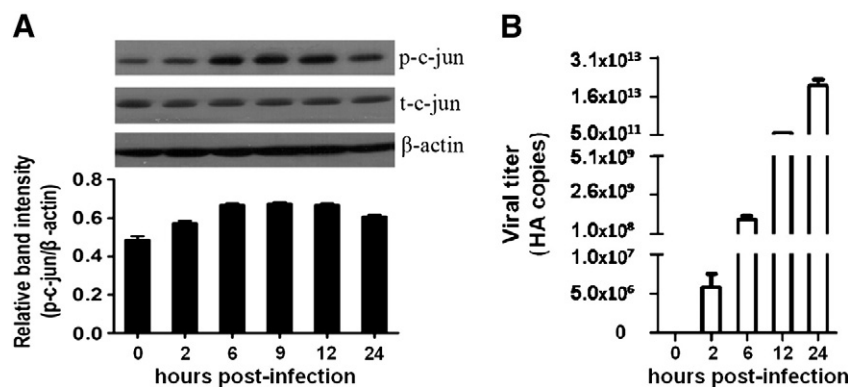


Fig. 1. c-jun activation in the early stage of H5N1 infection in A549 cells. (A) c-jun is phosphorylated upon H5N1 infection in A549 cells. A549 cells were infected with H5N1 and cell lysates were analyzed by Western blotting. Relative band intensity was determined digitally using QuantityOne 4.1.1 software and is expressed as a ratio of c-Jun to β-actin. (B) Replication of H5N1 virus in A549 cells. A549 cells were infected with H5N1 and the viral titer was analyzed by real-time PCR using primers specific for the HA gene. p-c-jun: phosphorylated c-jun; t-c-jun: total c-jun (N = 3).

the gradual emergence of drug-resistant strains will eventually restrict their therapeutic application [8,9].

It has been suggested that NF-κB signaling pathway could differentially regulate influenza A virus replication besides acting on viral initial infections [10–12]. Similar to NF-κB, the C-jun N-terminal Kinases (JNKs) signaling pathway has also been reported to be involved in influenza A virus infections and replications [13,14]. Blockade of JNK signaling pathway resulted in inhibition of IAV-induced JNK and AP-1 (activating protein-1) activation, and impaired the IFN-β expression [14]. On the other hand, inhibition of JNK leads to prevention of host cell apoptosis, an inevitable process for influenza virus infection [15,16]. It was also demonstrated that SP600125, an inhibitor of JNK, may interfere with IAV viral replication via NS1 [13].

C-jun is downstream of JNK and is a prototypical member of the basic region-leucine zipper protein family, it forms homo- and/or hetero-dimers, named AP-1, through the bZIP domain with other proteins, such as Jun family, Fos family, activating transcription factor (ATF) family, and musculoaponeurotic fibrosarcoma (MAF) family [17,18]. C-jun is the most extensively studied protein of the AP-1 complex [19]. The steady elevation of c-jun expression is closely associated with AP-1 activity [20]. C-jun is involved in numerous cell activities, such as proliferation, apoptosis, survival and tumorigenesis [19]. It became evident that c-jun/AP-1 can be activated by many extracellular stimulators including viral infections, such as IAV [14,21]. In response to IAV infection, c-jun is activated (phosphorylated) at a very early stage [14] and can initiate and enhance the transcription of antiviral agents such as IFN-β, and RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) [22], which can cause significant inflammation. In addition, it was reported that the gene promoter of human foamy virus (HFV) has a binding site for c-jun/AP-1 [23]. C-jun therefore appears to have an important role in virus infections and replications.

DNAzymes are synthetic, single-stranded DNA catalysts that can be engineered to bind to their complementary sequence contained in target messenger RNA (mRNA) through Watson–Crick base pairing, and cleave the mRNA at predetermined phosphodiester linkages [24]. Dz13, a catalytic DNA molecule cleaving c-jun mRNA [25], can inhibit tumor growth and is a strong inhibitor of endothelial cell proliferation, migration and tubule formation [26]. It was also reported that it could abrogate monocyte-endothelial cell adhesion in vitro and abolish leukocyte rolling, adhesion and extravasation in a rat model of inflammation. Dz13 also suppressed neutrophil infiltration in the lungs of mice challenged with endotoxin. All these findings implicate c-jun as a useful target for anti-inflammatory therapies.

In the present study, we investigated whether inhibiting c-jun activation (phosphorylation) could block the synthesis of inflammatory cytokines and reduce virus replication, as a potential therapy strategy

for IAV infection. We demonstrated that suppression of c-jun in cells and mice could efficiently reduce IAV replication and restore the balance of pro- and anti-inflammation induced by IAV infection both in vitro and in vivo.

2. Methods

2.1. Ethics statement

Experimentation with animals was approved by the Animal Ethics Committee of China Agricultural University (Approval number 201206078) and followed the Regulations of Experimental Animals of Beijing Authority.

2.2. DNAzymes

DNAzymes were synthesized with an inverted thymidine at the 3' position (Tri-link Biotechnologies, San Diego, CA, USA) and purified by high-pressure liquid chromatography. Sequences of Dz13 (a c-jun-targeting DNAzyme bearing 9 + 9 nucleotide arms; cleavage junction in c-jun mRNA is G¹³¹¹U) and Dz13scr (sequence-scrambled counterpart of Dz13) are 5'-CGGGAGGAAGgtagctacaacgaGAGGCGTTG-Ti-3', and 5'-GCGACGTGAggtagctacaacgaGTGGAGGAG-Ti-3'. Nucleotides in lower-case represent the 10–23 catalytic domain. Ti represents a 3'-3'-linked inverted thymidine.

2.3. Mice

Female BALB/c mice, 6–8 weeks old (Vital River Laboratories, Beijing, China) were housed in independent ventilated cages and received pathogen-free food and water.

2.4. Virus

The H5N1 influenza virus (A/chicken/Henan/1/2004) used in this study, which has the same receptor binding sites as A/Hong Kong/156/97 (H5N1), was isolated from infected chicken flocks. The virus was propagated in Madin–Darby canine kidney (MDCK) cells at 37 °C for 48 h, and then the viral supernatant was harvested, aliquoted, and stored at –80 °C. The PFU and LD₅₀ were determined in MDCK or mice after serial dilution of the stock (8 × 10⁷ PFU/ml and 3 × 10⁶ LD₅₀ respectively).

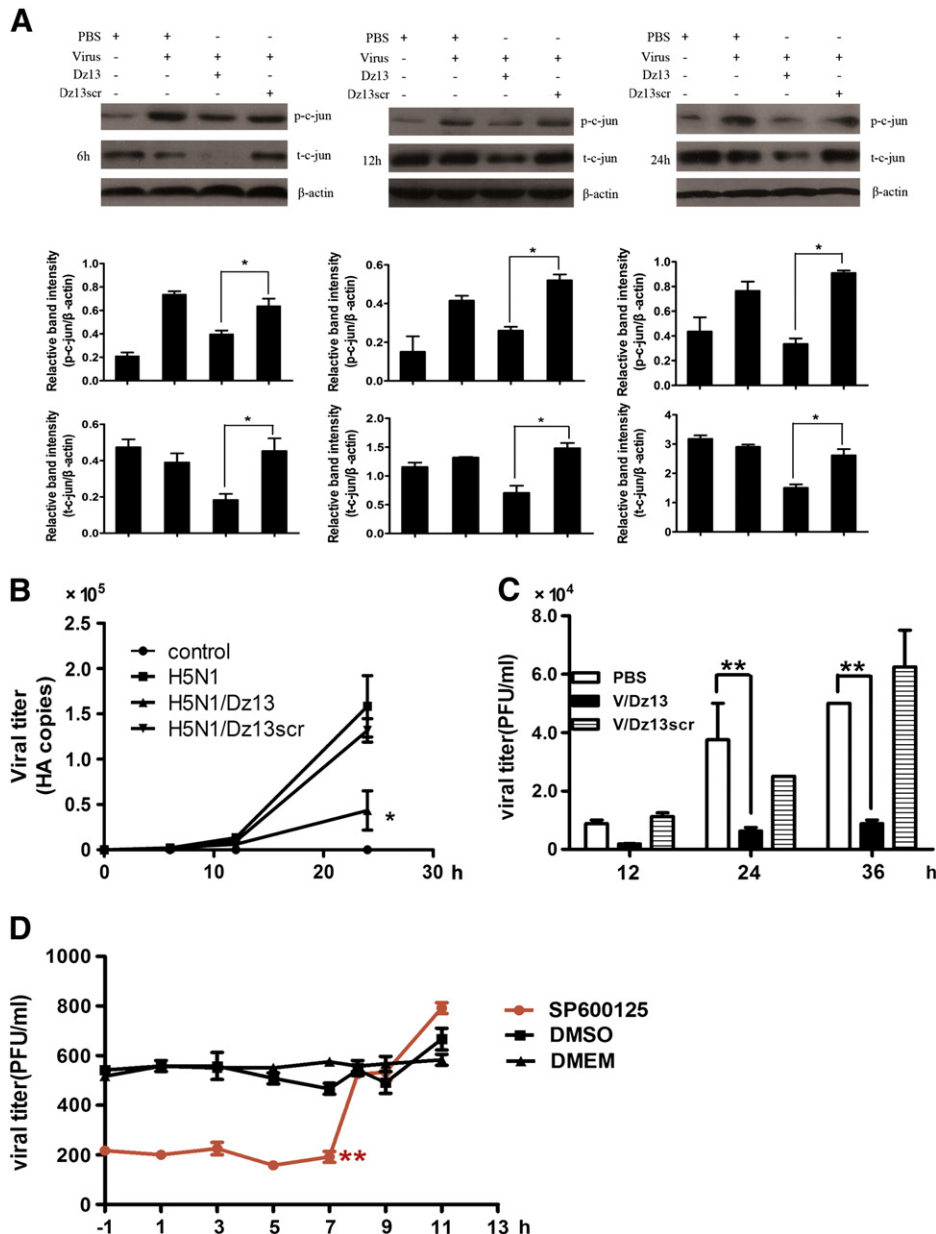


Fig. 2. c-jun activation was inhibited by Dz13 and c-jun downregulation suppressed H5N1 virus replication. (A) Activation of c-jun is attenuated by Dz13 in H5N1 infected A549 cells. A549 cells were transfected twice with Dz13 and Dz13scr and infected with H5N1. Cell lysates were harvested at 6 h, 12 h, and 24 h p.i. and analyzed by Western blotting. (B) HA gene expression was analyzed by real-time PCR. A549 cells were harvested at the indicated times after the second Dz13 and Dz13scr transfection and the followed H5N1 infection ($N = 3$). (C) c-jun suppression by Dz13 attenuated H5N1 propagation in vitro. A549 cell supernatants were harvested 12 h, 24 h, and 36 h after H5N1 infection followed by two transfections with Dz13 and Dz13scr. Viral titers in cell supernatant were tested by plaque assay ($N = 3$), **, $P < 0.01$. (D) Time course of the effect of c-jun inhibitor on H5N1 virus production. A549 cells were infected with H5N1 influenza A virus. Pharmacological treatment or a vehicle control (DMEM or DMSO) was applied at different times post-infection [–1 h, (1 h before infection); 1, 2, 3, 5, 7, 8, 9 and 11 h post-infection]. The virus titers in the supernatants at 12 h p.i. were determined by plaque assays, ($N = 3$), **, $P < 0.01$.

2.5. Cell transfection and viral infection

Human lung adenocarcinoma epithelial A549 cell line was provided by Cell Resource Center of Peking Union Medical College. A549 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Beijing, China) containing 10% fetal bovine serum (Hyclone Laboratories), 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were placed in serum-free medium to initiate growth arrest for 12 h, then we used FuGENE6 reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions to transfect cells (at 60–75% confluence) with Dz13 and its scrambled counterparts Dz13scr. The transfected cells were incubated for 18 h in serum-free medium and then performed second round of transfection in the same way. At 6 h after transfection, cells were washed with phosphate-buffered

saline (PBS) and infected with H5N1 influenza A virus (A/chicken/Henan/1/2004) in serum-free DMEM (moi = 0.1). For some experiments, virus was replaced for polyIC (50 μ g/ml). The cells were shaken gently every 10 min, then the supernatant was changed to DMEM containing 1% fetal bovine serum.

2.6. Administration of Dz13 to animals

The Dz13 were dissolved in PBS in a concentration of 200 μ M, and stored at -20°C . Dz13 or its scrambled control at 2.5 mg/kg (in 50 μ l PBS containing 2.5 μ l Fugene 6 and 1 mM MgCl_2) was administrated intranasally to H5N1 infected mice at day 0 (20 min after viral challenge) and day 2 (48 h after viral challenge).

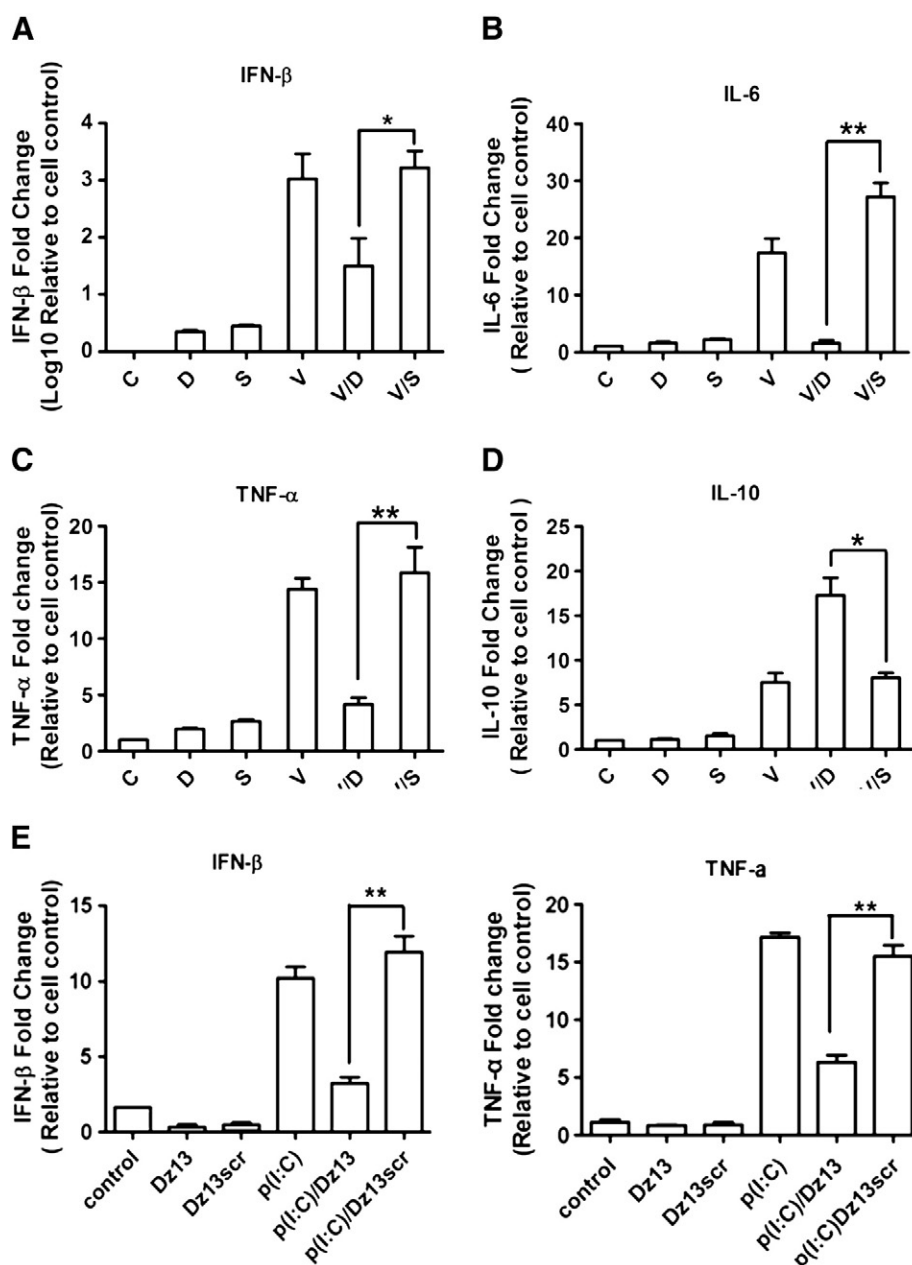


Fig. 3. c-jun suppression by Dz13 alters inflammatory cytokine expression in vitro. The levels of (A) IFN- β , (B) IL-6, (C) TNF- α , and (D) IL-10 mRNA in A549 cells were detected by real-time PCR 24 h after two times transfections of Dz13 and Dz13scr and H5N1 infection (MOI = 0.1). (E) IFN- β , TNF- α mRNA in A549 cells were detected by real-time PCR 24 h after two times transfections of Dz13 and Dz13scr and 50 μ g/ml poly(I:C) (N = 3), * P < 0.05, ** P < 0.01. C: control, D: Dz13, S: Dz13scr, V: H5N1, V/D: H5N1 plus Dz13, V/S: H5N1 plus Dz13scr.

2.7. Administration of SP600125

SP600125 (Beyotime, Haimen, Jiangsu, China) (molecular weight = 220) was dissolved in 100% DMSO (Dimethyl sulfoxide) at the concentration of 20 mM for stock solutions. When administration, the A549 cells were incubated in DMEM medium containing 20 μ M SP600125 for 1.5 h.

2.8. Viral challenge and sample collection

H5N1 virus stocks were diluted in PBS when used for infection. Mice were anesthetized with intramuscular administration of Zoletil (Virbac, Carros, France) and infected intranasally with 3LD₅₀ in 50 μ l. They were sacrificed at various time-points and the lung tissues were stored in liquid nitrogen. Lung tissue from each mouse was homogenized in

1 ml PBS and centrifuged for 10 min at 1500 \times g and the supernatants were stored at -80°C for PFU assay.

2.9. PFU assay

Madin–Darby canine kidney (MDCK) cell line was provided by Cell Resource Center of Peking Union Medical College and was cultured in the same conditions as A549 cells. The supernatants of mouse lung tissues were diluted 10-fold and added to a monolayer of MDCK cells in semi-solid agar that contained 0.5 μ g/ml trypsin TPCK (Sigma-Aldrich, St. Louis, MO, USA). Cultures were incubated at 37°C , 5% CO₂ for 60–72 h, then fixed and stained with 1% crystal violet. Plaques were counted, photographed and expressed as the mean log of 10 PFU/ml.

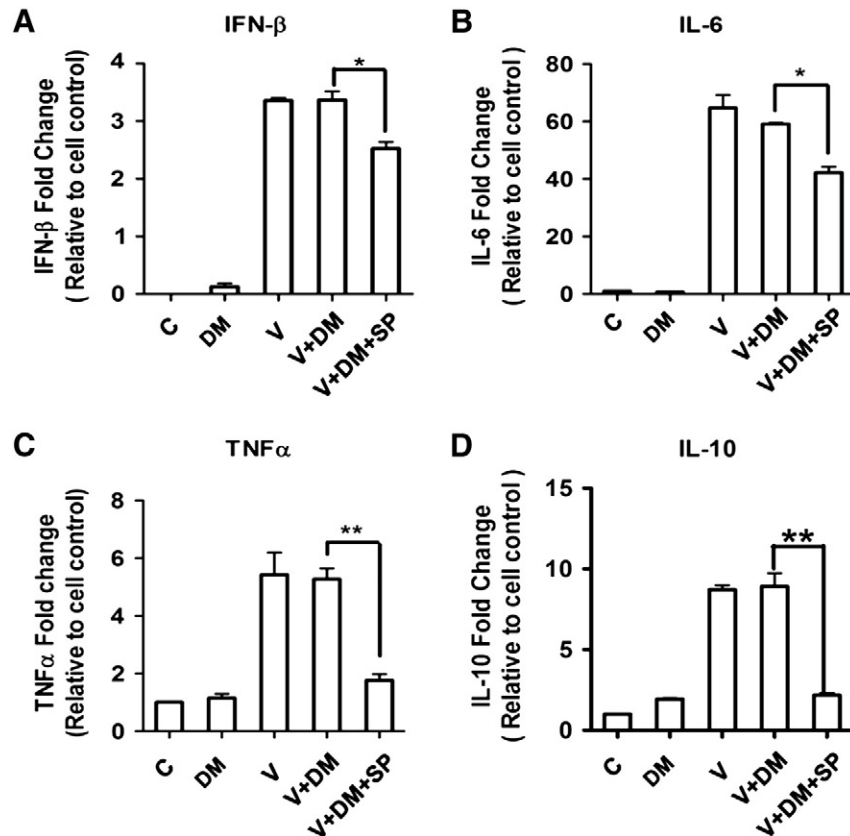


Fig. 4. C-jun suppression by SP600125 alters inflammatory cytokine expression in vitro. The levels of (A) IFN- β , (B) IL-6, (C) TNF- α , and (D) IL-10 mRNA in A549 cells were detected by real-time PCR 24 h after SP600125 treatment and H5N1 infection (N = 3). * P < 0.05, ** P < 0.01. C: control, DM: DMSO, V + DM: H5N1 plus DMSO, V + DM + SP: H5N1 plus DMSO plus SP600125.

2.10. Western blot analysis

Cells or lung tissues were lysed in RIPA buffer (Beyotime, Haimen, Jiangsu, China) with 10 mM PMSF (Beyotime) and 20 mM Cocktail (Roche) on ice for 10–20 min. Lysis of tissues was assisted by grinding with a grinding rod. Lysates were then centrifuged at 10,000 $\times g$ for 10 min to remove cell debris. Proteins in the supernatants were quantitated by BCA protein assay (Applygen, Beijing, China) and resolved on 12% polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) nylon membrane (Millipore, Bedford, MA, USA). The membrane was then incubated with antibodies (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 final dilution. The antibody binding was detected with a Western Lightning chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA, USA).

2.11. RNA extraction and qRT-PCR

RNA was prepared from 10 mg of lung tissue or 1×10^6 cells homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. DNase I-treated RNA (0.2 μg) was reverse transcribed into cDNA using EasyScript First-Strand cDNA Synthesis SuperMix (TransGene, Beijing, China). Real-time PCR (qPCR) was performed to amplify the genes using the Power SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). Primers used are shown in sTable 1. The reactions were run on an ABI 7500 (Applied Biosystems) with the following steps: 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 30 s and extension at 72 °C for 40 s. Data analysis was performed using ABI 7500 Software, version 2.0 (Applied Biosystems), which was provided with the instrument.

2.12. T cell analysis

Three mice were sacrificed 6 days after infection, while spleen and peripheral blood lymphocyte (PBL) were harvested. The tissues and blood were washed and grinded, and subsequently the erythrocytes were lysed by treatment with erythrocytes lysis buffer (M&C GENE, Beijing, China). Single-cell suspensions were prepared by passing through a nylon screen and washed in fluorescence -activated cell sorting diluent (PBS with 0.1% sodium azide and 2.0% fetal bovine serum). Next, 200 μl of cell suspensions containing 10^6 cells was incubated on ice for 1 h with combinations of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled antibodies (eBioscience, San Diego, CA). Accordingly, lymphocyte populations were dual stained with either FITC anti-mouse CD4 and PE anti-mouse CD8a or Cy5.5 anti-mouse CD3. The cells were then washed, resuspended in 1 ml of 2% paraformaldehyde, and analyzed on an Accuri C6 flow cytometer (Accuri Cytometers, Inc., Ann Arbor, MI). A total of 10,000 events, gated for lymphocytes were performed in three independent experiments.

2.13. Histopathological analysis

Nose, trachea and lung tissues of mice were removed at the indicated days and fixed with 4% neutral formalin at room temperature for 48 h. After embedding in paraffin, serial tissue sections were cut to 5 μm thickness, stained with hematoxylin and eosin (H&E). Histopathological changes were observed and scored under on Olympus microscope (Olympus Optical Co. Ltd) by an independent pathologist. Criteria for grading lung histopathological changes were as follows: Grade 0 = no obvious pathological changes; Grades 1–3 = light

inflammatory cells infiltration, light hemorrhage, vasculitis or bronchiolitis; Grade 4–5 = inflammatory cells infiltration, hemorrhage, vasculitis or bronchiolitis, cell apoptosis and necrosis, microthrombus; Grade 6–10 = severe inflammatory cells infiltration, severe hemorrhage, vasculitis or bronchiolitis, obvious edema, cell apoptosis and necrosis, microthrombus, multinucleated giant cell.

2.14. Statistical analysis

Statistical analysis was performed by one-way ANOVA Tukey post hoc test contained in the SPSS software or GraphPad Prism Software (version 12.0; 2003, SPSS Taiwan Corp., Taiwan) (version 5.0, GraphPad Software, San Diego, CA) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Acute response of c-jun expression upon IAV infection

To examine whether c-jun was involved in H5N1 virus infection, we studied the c-jun expression profile upon H5N1 infection. Cells were lysed at indicated times post-infection (p.i.) and Western blot analysis was performed to detect activation of c-jun (phosphorylated). C-jun activation kinetics started earlier than 2 h p.i. and reached a peak value at 6–12 h p.i., there after returning to near basal levels at 24 h (Fig. 1A).

To investigate the association between the kinetic profiles of c-jun phosphorylation and IAV replication, we performed real-time PCR using specific hemagglutinin (HA) gene primers to detect virus

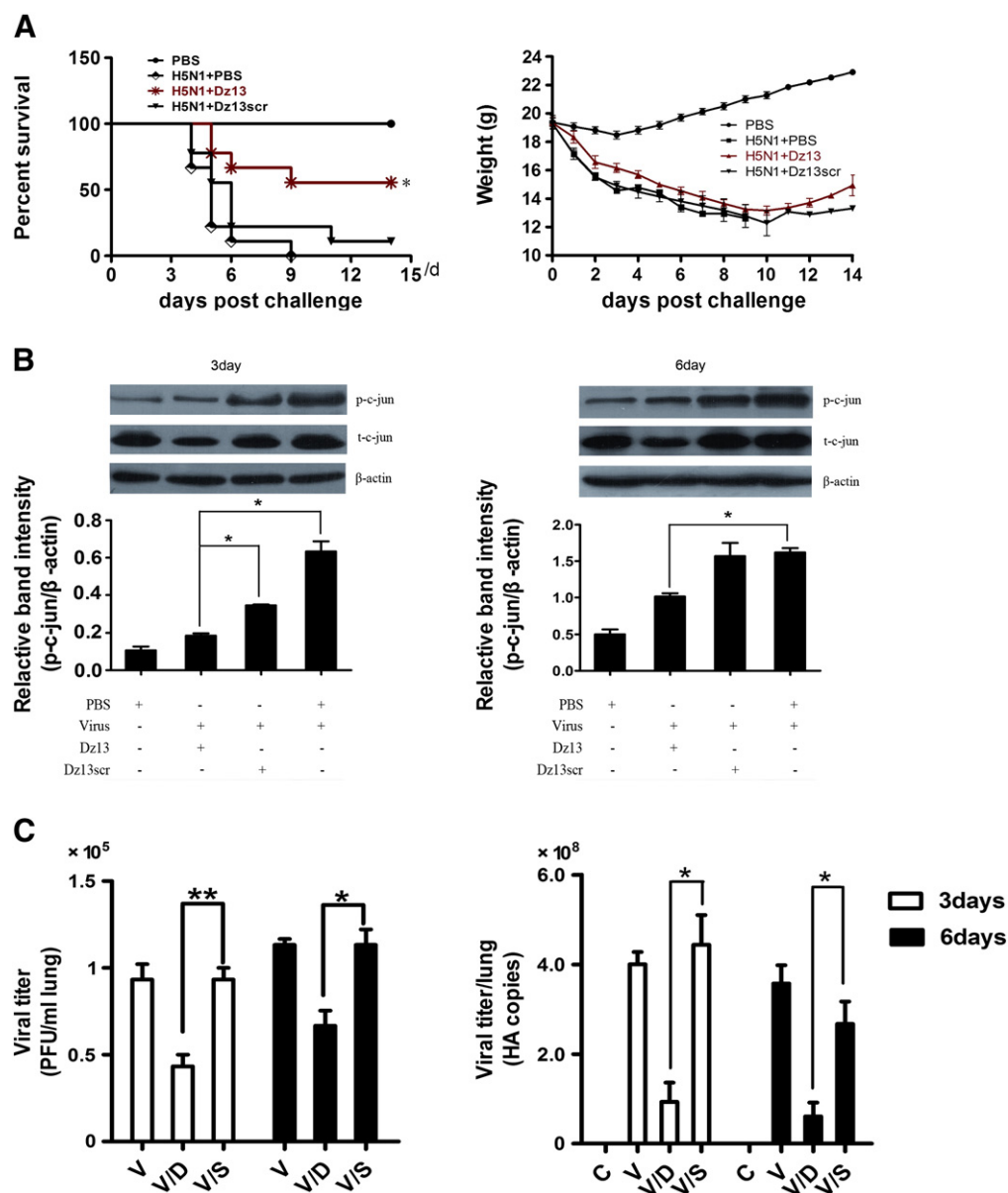


Fig. 5. Suppression of c-jun improved the survival rate and health status of H5N1 virus infected mice. (A) Surviving rates of mice upon H5N1 challenge and Dz13/Dz13scr treatment (N = 9; *, $P < 0.05$ by log rank analysis) and bodyweights were recorded daily. (B) Lung tissues were collected on days 3 and 6 p.i., total and phospho-c-jun expression was analyzed by Western blotting. Relative band intensity was determined digitally using QuantityOne 4.1.1 Software and is expressed as a ratio of c-jun to β -actin. (C) c-jun affected H5N1 propagation in vivo. The viral titers in lungs were determined by plaque assay and real-time PCR (N = 5). * $P < 0.05$, ** $P < 0.01$. C: control, V: H5N1, V/D: H5N1 plus Dz13, V/S: H5N1 plus Dz13scr (D) Lung histopathology at day 6 p.i. Representative nose, trachea and lung sections from each group were subjected to H&E staining. Nose: solid arrows indicate inflammatory cell infiltration and the open arrows indicate extravagated blood. Trachea: solid arrows indicate infiltration of inflammatory cells, erythrocytes and/or dropout of epithelium. Lung: Solid arrows indicate interstitial edema and inflammatory cell infiltration around small blood vessels; open arrows indicate abundant inflammatory cells, erythrocyte infiltration and drop of mucous epithelium in bronchia. (E) The pathological scores of the tissues of infected mice. The pathological lesions were scored according to the method described in Materials and methods.

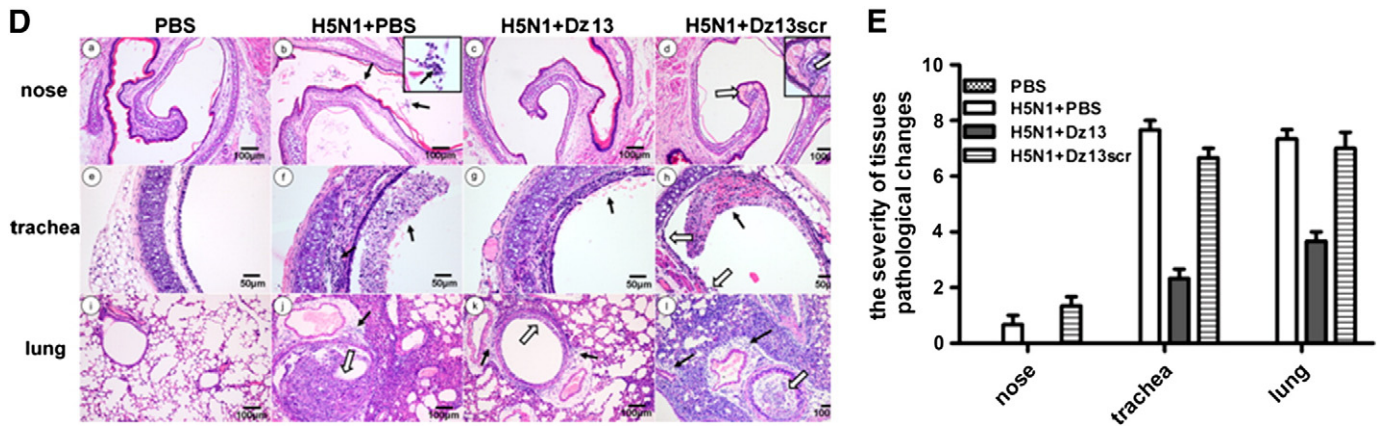


Fig. 5 (continued).

production. As shown in Fig. 1B, concomitant to the peak expression of phosphor-c-jun, the viral load in A549 cells exhibited a sustained growth trend until 24 h. These results suggested that along with IAV infection and replication, phosphor-c-jun may play a crucial part in the initial process of H5N1 infection (6–12 h). Afterwards, activated c-jun may be less involved in the sustaining of IAV replication.

3.2. Effect of c-jun down-regulation on H5N1 replication in A549 cells

To determine the effectiveness of Dz13, we measured the phosphor-c-jun and total-c-jun levels in A549 cells infected H5N1 virus at 6 h 12 h and 24 h post infection with or without Dz13 transfection. As shown in Fig. 2A, at all the time points, Dz13 transfection of IAV-infected A549 cells markedly reduced the levels of both total and phosphorylated c-jun expression. This validated Dz13 as an effective inhibitor of c-jun.

qPCR assays were further performed on the viral RNA extracted from the infected cells and shown that Dz13 could significantly inhibit the H5N1 virus replication at 12 h and 24 h p.i. ($P < 0.05$) (Fig. 2B). A plaque assay for 12 h, 24 h, and 36 h p.i. was designed to further confirm the Dz13 suppressive effect on H5N1 virus replication. (Fig. 2C) ($P < 0.05$) Results demonstrated that inhibition of c-jun could significantly impact on viral production.

Considering that the first generation of influenza virus particles release within 12 h after the initiation of infection [27], we further determined which stage of the H5N1 virus life cycle was affected by c-jun activation by using a small molecule inhibitor of JNK SP600125. The inhibitor could effectively inhibit c-jun activation and reduce H5N1 virus replication (Supplement Fig. 1B), but had no impact on the level of total c-jun protein (Supplement Fig. 1A). When SP600125 was added to the cells at different time points including 1 h pre-infection (–1), and 1, 2, 3, 5, 7, 8, 9, 11 h post infection and IAV infection was carried out at the time 0, the viral replication could be obviously suppressed by the JNK inhibitor from –1 h pre-infection to 7 h post infection ($P < 0.05$) (Fig. 2D), indicating that the c-jun activation was critically involved in the process of the IAV replication and production.

3.3. Effect of c-jun suppression on multiple pro-inflammatory cytokines expression following H5N1 infection in A549 cells

H5N1 virus is a strong inducer of various pro-inflammatory cytokines associated with acute respiratory syndrome [28]. As a crucial factor of AP-1, c-jun is critical for the expression of IFN- β [5]. To investigate the role of phosphor-c-jun suppression on inflammatory cytokine expression in H5N1 infected cells, we analyzed IFN- β RNA expression in A549 cells after two rounds of transfection of Dz13, followed by H5N1 infection. Though the phosphor-c-jun level reached the peak

value at 6–12 h after infection, we chose 24 h p.i. as a measurement to allow the infected cells having sufficient time to express cytokines. As shown in Fig. 3A, the expression of IFN- β mRNA was significantly decreased in Dz13 transfected cells in comparison with the scramble Dz13 control ($P < 0.05$) [6]. In addition, we also found that suppression of c-jun by Dz13 could strongly inhibit IL-6 and TNF- α expression (Fig. 3B, C) ($P < 0.05$). In contrast, IL-10, an anti-inflammatory cytokine, showed increased expression following c-jun inhibition (Fig. 3D) ($P < 0.05$). These data suggested that inhibition of c-jun expression could differentially regulate and balance the expression of both pro- and anti-inflammatory cytokines.

To further explore whether the regulation of the cytokine expression by c-jun upon IAV infection was dependent on c-jun phosphorylation, we employed the JNK inhibitor SP600125 to investigate its impact on the cytokine expression in IAV-infected cells. Interestingly, inhibition of c-jun activation by SP600125 could suppress both pro- and anti-inflammatory cytokines including IFN- β , IL-6, TNF- α and IL-10 (Fig. 4) ($P < 0.05$). This indicated that c-jun (SP600125) phosphorylation could globally affect cytokine expression [22].

To confirm that c-jun indeed regulates inflammation, instead of less viral stimulus, we performed an additional experiment by using an inflammatory stimulator polyIC to mimic IAV. The result demonstrated the Dz13 could directly decrease the inflammatory cytokines (IFN- β , TNF- α) expression upon the polyIC treatment (Fig. 3E), indicating that c-jun is involved in RNA induced inflammation. These data ruled out the possibility that the suppressed inflammatory response was due to impaired viral replication.

3.4. Improved survival of H5N1-infected animals by suppressing c-jun expression

To investigate the roles of c-jun in H5N1 virus infection in vivo, we challenged BALB/c mice with 3 LD₅₀ of H5N1, and Dz13 was administered twice at day 0 (20 min after viral challenge) and day 2 via intranasal route. Lung tissue samples of five mice per group were collected at day 3 and day 6 for real-time PCR, plaque assay, and Western blotting analyses. Tissue samples of the infected animals (nose, trachea and lung) were collected on day 6 for histopathological analysis. Nine mice per group were used to monitor the survival rate over 14 days. Results showed that 55.5% of the Dz13 treated mice survived at the termination of the experiments (14 days p.i.), while PBS treated mice all died by day 9 and 11% of Dz13scr treated mice survived. The body weights of the mice treated with Dz13 was higher than that of other groups (Fig. 5A). When the mixed protein samples from the lung tissues of Dz13-treated mice were analyzed by Western blotting, both total and phosphorylated c-jun levels were shown to be down-regulated,

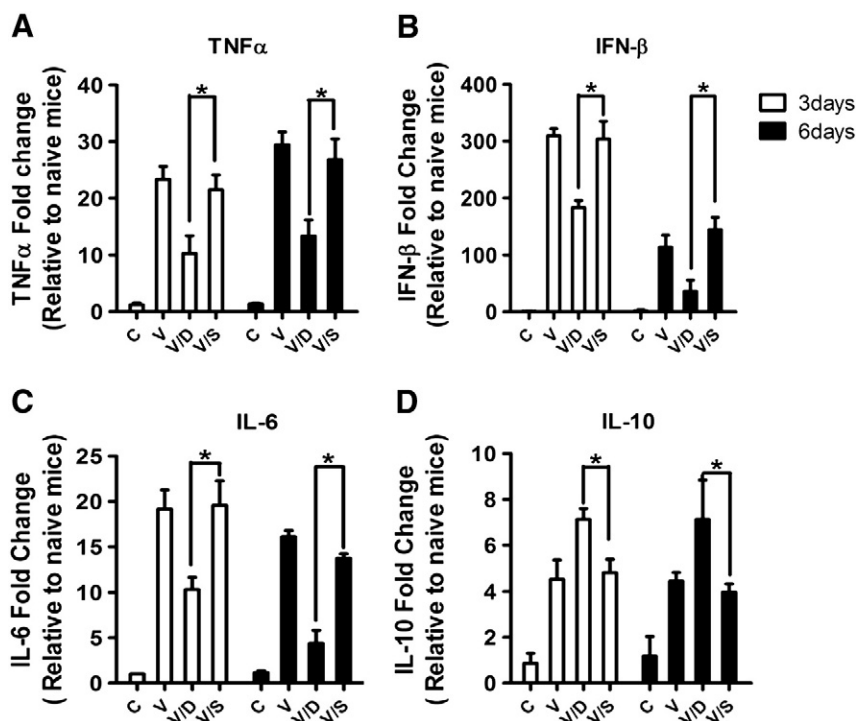


Fig. 6. c-jun suppression alters inflammatory cytokine expression in vivo. The mRNA levels of (A) TNF- α , (B) IFN- β , (C) IL-6 and (D) IL-10 in the lung on days 3 and 6 p.i. were determined by real-time PCR (N = 3). *P < 0.05. C: control, V: H5N1, V/D: H5N1 plus Dz13, V/S: H5N1 plus Dz13scr.

compared with control groups (Fig. 5B). To investigate the influence of c-jun down-regulation on viral replication in vivo, we examined the viral load in lung tissues of H5N1 infected mice. Plaque assay and real-time qPCR using H5N1 specific primers (HA genes) showed that the pulmonary viral titer in H5N1 virus infected mice receiving Dz13 was significantly lower than that in mice treated with Dz13scr or PBS, both on days 3 and 6 p.i. ($P < 0.05$) (Fig. 5C).

The pathological features of the respiratory tract in H5N1 infected and c-jun suppressed mice are shown in Fig. 5D. Gross observation of H5N1 infected mice treated with PBS (Fig. 5D, f) and Dz13scr (Fig. 5D, h) demonstrated that the tracheal cavities were infiltrated with inflammatory cells, erythrocytes and epithelial cells, and there was an obvious loss of tracheal epithelium in H5N1 + Dz13scr treated mice. In contrast, the trachea of Dz13 treated mice (Fig. 5D, j) was characterized by only a small amount of erythrocyte infiltration. The lung lesions in different groups of mice, especially the H5N1 + PBS (Fig. 5D, i) and H5N1 + Dz13scr (Fig. 5D, l) treated groups were characterized by interstitial edema and infiltration of inflammatory cells around small blood vessels, exfoliation of bronchiolar epithelium and epithelial cells, with erythrocytes and inflammatory cells in the alveolar lumen mixed with flooded edema fluid. However, the symptoms were apparently mitigated in the Dz13 treated group (Fig. 5D, k). Different from trachea and lung, the nasal lesions were slight, with minor inflammatory cell infiltration and extravasated blood only in the H5N1 + PBS (Fig. 5D, b) and

H5N1 + Dz13scr (Fig. 5D, d) treated mice. Severity scores for lung histopathological lesions are given in Fig. 5F [29]. These data indicated that down-regulation of c-jun in vivo could lessen the H5N1 virus-induced pathological damage, thus improved overall survival of the infected animals.

3.5. IAV activated CD8⁺ T cell proliferation was affected by suppression of c-jun

To determine whether c-jun could impact the CD8⁺ T cells proliferation, spleen tissue and PBL samples of three mice per group were collected at day 3 and day 6 for flow cytometry assay. Reduced levels of CD8⁺ T cells were observed in H5N1 + Dz13 group compared with PBS or Dz13scr treated mice both in spleen and PBL (Fig. 7).

3.6. c-jun regulation of cytokine expressions in vivo

To examine the role of c-jun on inflammation in H5N1 infected mice, analyses of IL-6, TNF- α , IFN- β , and IL-10 cytokine expression were performed by real-time PCR on days 3 and 6 infection p.i. The mRNA levels of IL-6, IFN- β and TNF- α in the Dz13 treated group were significantly reduced compared with the Dz13scr or PBS treated groups at days 3 and 6 p.i. ($P < 0.05$). The expression of IFN- β and IL-6 mRNA at day 6 p.i. was lower than that at day 3 p.i. (Fig. 6A–C). In contrast to the proinflammatory cytokines, IL-10 showed a higher expression level in Dz13 treated mice than that in other groups on both days 3 and 6 p.i. (Fig. 6D) ($P < 0.05$). Those, together with the observations in cells, demonstrated the critical role of c-jun in initiation and regulation of inflammatory responses to H5N1 virus infection both in vitro and in vivo.

4. Discussion

JNK, a classic pathway involved in numerous cell activities, such as proliferation, apoptosis and inflammation [30], is recognized as an antiviral pathway in influenza infection, but under certain conditions

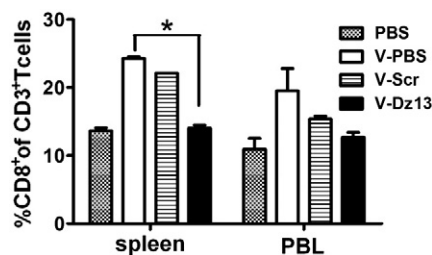


Fig. 7. c-jun suppression decreased the CD8⁺ T cells proliferation in vivo. T cell profiles in spleen and PBLs by flow cytometry (N = 3). *P < 0.05.

it was shown to support influenza virus replication [13]. C-jun as a downstream molecule of JNK and a crucial factor of activator protein AP-1, may participate in the establishment of viral infection; however, its role in H5N1 virus-induced inflammation remains to be elucidated.

Previous studies showed that c-jun/AP-1 was a key transcription factor involved in cell proliferation, transformation, death and differentiation [31]. An AP-1 binding site has been identified on retrovirus promoters, which exerts an effect on the promoter activity of the virus [23,32]. Although the IAV replication cycle does not contain a DNA stage and transcription factors did not act directly on the IAV promoter, it was demonstrated that NF- κ B, a transcription factor with similar functions to AP-1, could differentially activate influenza virus RNA transcription from the cRNA promoter [11]. In the present study, we used A549 cell line and a mouse model to examine whether c-jun plays a critical role in H5N1 virus infection and replication. We showed that c-jun was activated upon H5N1 infection in the first replication cycle of H5N1 virus (6 h to 12 h p.i.). Specific inhibition of c-jun expression by Dz13 led to significant reduction of H5N1 virus replication. Pharmacological inhibition of c-jun phosphorylation showed that c-jun activation occurred at very early stage of the viral replication [27,33], suggesting that c-jun participates in the establishment of H5N1 influenza A virus replication. However, whether c-jun activation is needed for viral entry is yet to be determined.

Inflammatory deregulation accompanying IAV infection has been suggested to associate with the lethal dissemination of H5N1 influenza virus [34]. The imbalance between pro-inflammatory and anti-inflammatory cytokine expression could lead to host immunologic injury [35]. We showed that once the viral infection was established, a series of inflammatory responses were initiated in parallel accompany with the elevated c-jun activation and expression. Down-regulation of c-jun not only significantly suppressed viral replication but also mitigated the subsequent expression of inflammatory cytokines both in vitro and in vivo. Intriguingly, we found that Dz13 down-regulation of c-jun led to decreased expression of pro-inflammatory cytokines (IL-6, IFN- β and TNF- α), and increased expression of anti-inflammatory cytokine (IL-10). However, unlike Dz13, SP600125 inhibition of c-jun phosphorylation caused the decreased expression of both pro- and anti-inflammatory cytokines. Considering that Dz13 is an mRNA sequence-specific inhibitor of c-jun and only impacts on the level of expression while SP600125 acts by inhibiting c-jun phosphorylation, we suggest that the level of total c-jun protein might drive balance of pro- and anti-inflammatory cytokine expression in the context of IAV infection, while c-jun activation (phosphorylation) may exert global impact on cytokine expression. However, the data from the present study could not rule out the possibility that SP600125 may have weak impact on other pathways in addition to JNK [36], which may be involved in the regulation of IL-10 expression. Also, our data showed that suppression of c-jun activation led to improved viral clearance and down-regulated IFN- β , which seems to be contradictory. However, this in turn supports our hypothesis that c-jun is an active regulator for both viral replication and host inflammatory responses, in which IFN- β could act as both antiviral and pro-inflammatory factor. Together, we describe findings that indicate the novel dual functions of c-jun in both establishing viral replication and initiating inflammatory reaction in response to H5N1 virus infection.

Upon infection with influenza virus, CD4⁺ T cells, CD8⁺ T cells and regulatory T cells are induced. CD8⁺ T cells differentiate into cytotoxic cells to defend the host and secrete high levels of cytokines during the cell-mediated immune response. The previously studies demonstrated that JNK1 knock-down or use of the JNK inhibitor SP600125 markedly reduced CD8⁺ T cell proliferation in mice, which is consistent with our findings in the present study. However, in another study, the WT and JNK1 $-/-$ mice showed the approximate T cell response to influenza virus [37–39]. The discrepancy between these observations may be due to the different genetic background of the mice used in the studies, which could lead to different host response to the viral infection.

Use of the nucleic acid-based agents for antiviral therapeutics has been explored for decades, which includes antisense oligonucleotides, siRNA and DNazymes [40,41]. Due to its unique features in stability and lower cost in manufacturing, DNzyme has shown its potential in down-regulation of the disease genes [42]. Recently, Dz13, a c-jun targeted DNzyme, has been successfully trialed in human and was shown to be safe and efficacious in the human skin cancer patients [43]. Thus, it will be valued to explore the therapeutic potentials of Dz13 in combating influenza infection. Indeed the use of anti-c-jun DNzyme may serve as a valuable and novel therapeutic strategy in anti-influenza viral infection as shown in the present study. Our work adds to the existing understanding of the complex regulatory network that controls both the viral infection and host responses and promotes anti-c-jun DNzymes as a potential therapeutic target to control H5N1 infection and its sequelae.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbdis.2014.04.017>.

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