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p-STAT1 regulates the influenza A virus replication and inflammatory response *in vitro* and vivo

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ABSTRACT

Influenza A virus infection activates various intracellular signaling pathways, which is mediated by the transcription factors. Here, a quantitative phosphoproteomic analysis of A549 cells after infection with influenza A virus (H5N1) was performed and we found that the transcription factor STAT1 was highly activated. Unexpectedly, upon inhibition of p-STAT1, titers of progeny virus and viral protein synthesis were both reduced. The STAT1 inhibitor Fludarabine (FLUD) inhibited an early progeny step in viral infection and reduced the levels of influenza virus genomic RNA (vRNA). Concomitantly, there was reduced expression of inflammatory cytokines in p-STAT1 inhibited cells. *In vivo*, suppression of p-STAT1 improved the survival of H5N1 virus-infected mice, reduced the pulmonary inflammatory response and viral burden. Thus, our data demonstrated a critical role for p-STAT1 in influenza virus replication and inflammatory responses. We speculate that STAT1 is an example of a putative antiviral signaling component to support effective replication.

1. Introduction

Influenza A virus (IAV) is one of the most infectious diseases that cause significant morbidity and mortality worldwide. Highly pathogenic IAV H5N1 can cause acute respiratory infections in both poultry and humans; which present a great threat to economic and international public health security. The high mortality associated with H5N1 infection has attracted the interest of many researchers, and the excessive levels of pro-inflammatory cytokine production, also termed a "cytokine storm," has been suggested to be the direct reason for the lethal clinical symptoms (Koutsakos et al., 2019). Additionally, IAV can undergo substantial changes (through recombination or antigenic shift) that make it more difficult to develop protective vaccine and treatment strategies (Cox and Subbarao, 2000; I. Stephenson and M. Zambon, 2002; Loo and Jr, 2007).

Cell signaling pathways, such as TLR/RIG-I, NF-kB, PI3K/Akt, and MAPK pathway are activated by IAV infection (Dai et al., 2017; Ehrhardt et al., 2006; Gaur et al., 2011; Yan et al., 2018). These pathways are important for viral entry, replication, and propagation, and are involved in host antiviral response (Zhang et al., 2018). When these pathways are activated, many cytokines and chemokines are released that can initiate and shape the innate and adaptive immune response. The over production of pro-inflammatory cytokines and chemokines such as IFNs, TNF-α, IL-6 and MCP-1, can act to eliminate the virus and stimulate inflammatory responses, but can also directly contribute to serious lung injury (Peiris et al., 2009; Simmons and Farrar, 2008). The activation of these pathways is commonly meditated by the phosphorylation and dephosphorylation of related kinases or proteins. Among these pathways, certain specific cellular functions essential for virus replication might represent antiviral targets (Ludwig, 2009; Shaw, 2011). Accordingly, characterizing intracellular phosphorylation after virus infection might be extremely useful for identifying new antiviral methods.

Systems-level evaluations of infection-related changes to host phosphoprotein networks are currently available for influenza A virus. The use of stable isotope labeling by amino acids in cell culture (SILAC)

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has led to the identification of 280 host proteins with significant changes in expression levels in A549 cells infected with the A/PR/8 (H1N1) strain (Coombs et al., 2010); 70 differentially expressed host proteins in A549 cells infected with the pandemic influenza A/California/07/2009 (H1N1) virus (Dove et al., 2012); 93 differentially regulated proteins in primary human broncho-tracheal epithelial cells infected with the A/PR/8 (H1N1) strain (Kroeker et al., 2012); and 106 differentially expressed proteins in primary human alveolar macrophages infected with A/PR/8 (H1N1) virus (Liu et al., 2012). The use of isobaric tags for relative and absolute quantitation (iTRAQ) has led to the identification of 1321 differentially expressed proteins in primary human macrophages from buffy coats infected with the A/Udorn/72 (H3N2) strain (Lietzen et al., 2011). Similar to the data set described above, variation in proteomics results have also been observed as a consequence of differences in techniques, cell lines, virus strains, and data analysis methods (Stertz and Shaw, 2011). Most of these previous studies reported the results of large-scale quantitative proteomics experiments that focused on changes in protein abundances in whole cell lysates or individual organelles, while the specific function of these phosphorylation sites during virus infection remains unclear.

The primary signal transduction cascade promoted by type I IFNs is mediated by the Janus family of protein tyrosine kinase 1 (JAK1)-signal transducers and activators of transcription (STAT) pathway (Nacken et al., 2012). The transcription of STAT1 involves alternative splicing that can result in the generation of two isoforms: STAT1α (91 kDa) and STAT1β (84 kDa). The 91-kDa protein can be activated by type I (α/β) and type II IFNs, epidermal growth factor, platelet-derived growth factor, and IL-6 (Najjar and Fagard, 2010). Additionally, Matikainen et al. reported that influenza A virus and Sendai virus activate STAT1 in macrophages. Virus-induced STAT1 phosphorylation is transient compared with that induced by IFNs, indicating that viral proteins can somehow directly interfere with STAT1 or with the production of type I IFN (Matikainen et al., 2000). Additionally, many studies have focused on STAT1 knock-out mice, which lack the STAT1 protein, *in vitro* and *in vivo*, which found that STAT1-deficiency could accelerate the pathogenesis of IAV (Lieberman et al., 2003; MerazJ et al., 1996). Additionally, Dempoya found that poly (I:C) can induce STAT1 phosphorylation in both a type I IFN-dependent and -independent manner, depending on the period of time after the introduction of poly (I:C) into the cells. There is an early phase (< 3 h) of STAT1 phosphorylation and a subsequent late phase (> 8 h) (Dempoya et al., 2012). Cervasi et al. found that during simian immunodeficiency virus (SIV) infection, administering the STAT1-specific inhibitor Fludarabine delayed the rebound of viral replication, suggesting a reduction in the size of SIV reservoirs (Cervasi et al., 2006). All of these studies suggest that activation of STAT1 could play a complex role, instead of only acting as an effector of IFNs.

Previously, we showed that the phosphorylation of c-Jun protein, which is a downstream molecule of the JNK pathway, along with virus infection and replication, could play a crucial role early in the process of H5N1 infection (Xie et al., 2014). Moreover, NF-κB plays a similar role during virus infection, and is essential for viral replication (Kumar et al., 2008; Zhong et al., 2018). Herein, quantitative phosphoproteomic analysis of A549 cells infected with influenza A virus (H5N1) at the very early stage (2 h) was performed. We found that STAT1 was highly activated by H5N1 infection, which was a representative transcription factor that might be involved in the process of influenza virus infection. Thus, we found that impairing STAT1 activation could efficiently reduce IAV replication in the early stage, along with inflammatory cytokine production *in vitro* and *in vivo*, which adds to our existing understanding of the complex regulatory network that controls both viral infection and host responses, and also provides a potential therapeutic target for the control of H5N1 infection.

2. Materials and methods

2.1. Virus

The H5N1 influenza virus (A/chicken/Henan/1/2004) used in this study 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 were harvested, aliquoted, and stored at −80 °C. The PFU and LD50 were determined in MDCK or mice after serial dilution of the stock (8×10^7 PFU/ml and 3×10^6 LD50).

All experiments with the H5N1 virus were conducted in a biosafety level 3 (BSL-3) containment laboratory approved by the Ministry of Agriculture of China.

2.2. Influenza virus infection of A549

The A549 cell line was provided by the Cell Resource Center of Peking Union Medical College. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Beijing, China) containing 10% fetal bovine serum (Hyclone Laboratories), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were washed with phosphate-buffered saline (PBS) and then were pre-incubated with or not with 50 μM fludarabine (FLUD, Selleck Chemicals, S1491; purity > 99%). After 1 h incubation, supernatants were removed and cells were infected with H5N1 influenza A virus at a multiplicity of infection (MOI) of 1. For some experiments, virus was replaced by 50 μg/mL poly (I:C). Cells were shaken gently every 15 min, and the supernatants were changed to DMEM containing 1% fetal bovine serum.

2.3. iTRAQ labeling and mass spectrometry

Cell lysates from mock- and virus-infected cultures were prepared and tryptic protein digests were enriched for phosphopeptides using a Phosphoprotein Enrichment Kit (Pierce, Thermo Fisher Scientific Inc. IL, USA). Enriched phosphopeptides were labeled with iTRAQ according to the manufacturer's protocol (iTRAQ Reagents Multiplex kit; Applied Biosystems, Foster City, CA, USA). A total of 100 μg per sample was precipitated by the addition of 4 × the sample volume of cold (−20 °C) acetone to the tube, incubating the mixture for 2 h, and then carefully decanting the supernatant. Protein pellets were then dissolved in solution buffer and denatured, and cysteines were blocked as described in the iTRAQ protocol (Applied Biosystems). Each sample was digested with 20 μL of 0.25 μg/L sequencing grade modified trypsin (Promega) solution at 37 °C overnight and labeled with iTRAQ tags as follows: control and three independent infected virus groups. The labeled samples were pooled before analysis by tandem mass spectrometry to identify resistance.

2.4. Western blot analysis

Cells or the lungs were lysed in RIPA buffer (Beyotime, Haimen, Jiangsu, China) with 10 mM PMSF (Beyotime) and 20 mM cocktail (Roche, Meylan, France) on ice for 10–20 min. Lysates were centrifuged at 10,000 × *g* for 10 min to remove cell debris. Proteins in the supernatants were quantified using the 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. Antibody binding was detected using a Western Lightning chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA, USA); β-actin served as a loading control.

2.5. Quantitative real-time RT-PCR

Total RNA was extracted and was reverse transcribed into cDNA as

previously described (Liu et al., 2014). Alternatively, CAT-specific primers were used in the same reverse transcription reaction to detect vRNA, cRNA, or mRNA. Primers sequences are shown in sTable 3. For normalization, gene expression levels of the housekeeping genes GAPDH or β-tubulin were determined.

2.6. siRNA transfection and analysis for siRNA transfection efficiency

The target sequences used for the knockdown of STAT1 were 5′-GGCGUUAGGACCAAGAAGC-3′, 5′-GAACCUGACUUCCAUGCGG-3′, and 5′-ACUUCUUGCUACAGCAUAA-3′. The negative control siRNA was purchased from Genepharm Co. (Suzhou, China). The double-stranded siRNA duplex molecules were dissolved in DEPC-treated water. For transfection, 1×10^6 cells were plated into 6-well plates and incubated overnight. A 5 μL aliquot of Lipofectamine 2000) reagent (Invitrogen, Carlsbad, CA, USA) was diluted in 250 μL Opti-MEM I Reduced Serum Medium (Invitrogen) and incubated at room temperature for 5 min. Additionally, 100 pmol siRNA duplex was diluted in 250 μL Opti-MEM I Reduced Serum Medium and mixed with pre-diluted Lipofectamine 2000. The mixture was incubated at room temperature for 20 min, then was added to each well and incubated at 37 °C. At 60 h after transfection, cells were washed with PBS and infected with H5N1 influenza A virus in serum-free DMEM (MOI = 1). Cells were shaken gently every 15 min, then the supernatant was changed to DMEM containing 1% fetal bovine serum. The transfection efficiency was measured by qPCR and Western blot analysis.

2.7. Plaque assay

The procedures of hemagglutination assay and plaque assay were the same as previous described (Huo et al., 2018; Liu et al., 2014). Plaques were counted and photographed; counts were expressed as the mean $\log_{10}$ PFU/ml.

2.8. Viral challenge and sample collection in vivo

Animal experiments were approved by the Animal Ethics Committee of China Agricultural University (approval number 201206078) and were performed in accordance to Regulations of Experimental Animals of Beijing Authority. Besides, experimental protocols conformed to the guidelines of the Beijing Laboratory Animal Welfare and Ethics Committee and were approved by the Beijing Association for Science and Technology (approval number SYXK-2009-0423).

Mice were anesthetized, infected intranasally with 3 × LD50 for influenza virus as previous described (Jin et al., 2011). FLUD was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5μg/μL and stored at −80 °C. FLUD or solvent control DMSO was administrated intranasally to H5N1-infected mice at 0.001 mg/mouse. Body weights were determined every two days.

2.9. Histopathological and immunohistochemical analysis

Mice were sacrificed and lung tissues were collected. The procedures of histopathological and immunohistochemical staining were the same as previous reference published by our team (Huo et al., 2018; Jin et al., 2011).

Pathological changes were evaluated by a veterinary pathologist and scored 0 to 4 in a blinded study. The indications for the scores were as follows: 0 = no microscopic lesions; 1 = extremely mild, characterized by mild interstitial edema and desquamation of rare epithelial cells; 2 = mild, characterized by interstitial edema and inflammatory cell infiltration around the small blood vessels; 3 = moderate, characterized by interstitial edema, inflammatory cell infiltration around the small blood vessels, bronchial and alveolar structural damage and slight inflammatory cells infiltration; 4 = severe, characterized by interstitial edema, inflammatory cell infiltration around the small blood vessels, serious bronchial and alveolar structural damage and more slight inflammatory cells infiltration. The detection of the IAV antigen was scored from 0 to 4 according to the number of positive cells per section. The indications for the scores were as follows: 0 = no positive cells, 1 = 1–10 positive cells, 2 = 11–50 positive cells, 3 = 51–100 positive cells and 4 ≥ 100 positive cells.

2.10. Data and statistical analysis

The false discovery rate and q-value methodology have recently been applied in proteomics studies to estimate the acceptable level of true or false positives for a call of significance. Statistical analyses were performed using the one-way ANOVA Turkey post-hoc test contained in the SPSS (version 12.0; 2003, SPSS Taiwan Corp.) or GraphPad Prism (version 5.0; GraphPad Software, San Diego, CA, USA) software packages. P-values < 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Identification of phosphoproteins in infected A549 cells

Human lung A549 cells were grown in 100 mm cell culture dishes and infected with influenza virus at 1 MOI. A total of three replicate experiments of influenza virus-infected and mock-infected cells were performed. After LC–MS/MS analysis, a total of 348 proteins were identified and quantified from the three runs. Distribution of the z-scores of virus/mock (117/114) iTRAQ protein ratios showed that most proteins (292, 83.9%) were not significantly altered in A549 cells during influenza virus infection (less than 2-fold changes). Z-score analysis identified 34 up regulated proteins showing a ≥2-fold increase ($P < 0.05$; sTable 1). Z-score analysis identified 17 down regulated proteins displaying a ≥2 fold increase (sTable 2). These proteins were classified according to biological function (Fig. 1).

It has been established that H5N1 influenza A virus infection triggers the activation of a series of cellular functions. Accordingly, a clear increase in the expression of the transcription factor STAT1 was observed in influenza A H5N1 virus-infected cells. Transcription factors, such as NF-κB, c-Jun and ATF-2, have been shown to play a central role in driving the expression of numerous genes and to participate in influenza A virus replication (Chaudhuri et al., 2008; Ludwig et al., 2002; Xie et al., 2014). The phosphoproteomic results presented above showed that the expression of the transcription factor STAT1 increased significantly after H5N1 infection, and STAT1 was the transcription factor that was uniquely activated by H5N1 infection. Based on this tantalizing result, we decided to test whether the classical transcription factor STAT1 also participated in influenza virus replication and host inflammation.

3.2. The kinetics of STAT1 phosphorylation in response to influenza a virus infection in A549 cells

To determine whether the STAT1 signaling cascade was activated upon an influenza A virus infection, we first investigated the specific phosphorylation of STAT1 at tyrosine 701, a site that is required for full STAT1 activation and is targeted in a strictly STAT1-dependent manner. Upon infection of A549 cells with influenza A (H5N1) virus, phosphorylation of STAT1 could be detected. STAT1 phosphorylation was not observed in mock-infected cells at any time point (Fig. 2A). STAT1 activation could be observed earlier than 2 h post infection (p.i.), reached a peak value at 4–8 h p. i., and then gradually decreased to zero.

To investigate the association between the kinetic profiles of STAT1 phosphorylation and either IAV replication or IFN induction, we performed real-time PCR using specific hemagglutinin (HA) and IFN-β

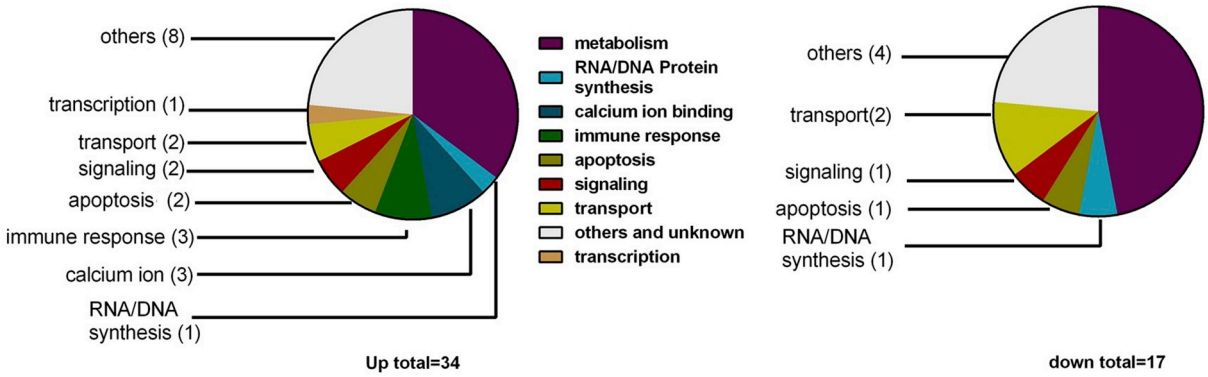

Fig. 1. Function classification of differentially regulated proteins at 2 h post infection. Classification of proteins that are upregulated (fold-change > 2) and downregulated (fold-change > 2) in H5N1 influenza A virus-infected cells.

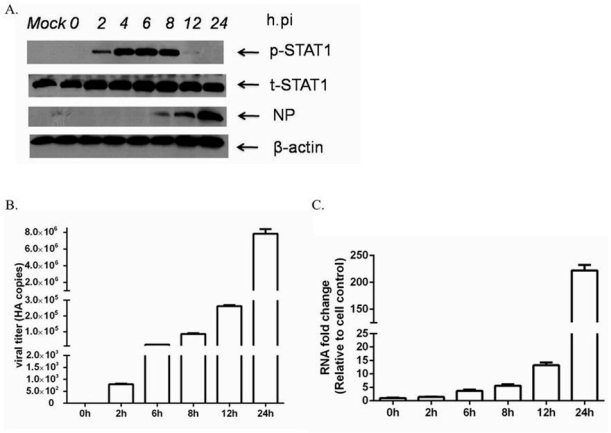

Fig. 2. The kinetic activation of STAT1 upon influenza A virus infection in A549 cells. (A) Western blot analysis of total lysates of A549 cells infected with MOI = 1 influenza A virus H5N1. P-STAT1 (Tyro 701) was detected at the indicated times (h) post-infection (p.i.). Efficient infection was confirmed by immunostaining for viral NP protein (middle panels). Equal loading was confirmed by detecting total STAT1 and β-actin (lower panels). (B) qPCR analysis of the replication of H5N1 virus in A549 cells using primers specific for the HA gene (N = 3). (C) IFN-β expression was analyzed by real-time PCR using specific primers (N = 3).

primers to assess this relationship. Similar to the peak expression of p-STAT1, the viral load in A549 cells exhibited a sustained growth trend until 24 h (Fig. 2B) and IFN-β production showed a trend that was similar to that of viral load (Fig. 2C). These results suggested that along with IAV infection and replication, p-STAT1 might play a crucial role in the initial process of H5N1 infection (4–8 h), independent of its role in IFN activation. Thereafter, activated STAT1 might be less important for sustaining of IAV replication.

3.3. Inhibition of the STAT1 activation results in impaired viral propagation

To determine the effectiveness of Fludarabine and to explore an optimal concentration, we measured the p-STAT1 and total-STAT1 levels in A549 cells stimulated with IFN-α. We found that drug-treated cells markedly downregulated p-STAT1, which showed significant dose-dependence, while having no effect on the total levels of STAT1 expression. This validated Fludarabine as an effective inhibitor of p-STAT1. Accordingly, we chose 50 μM as a working concentration.

Previous studies suggested that JAK/STAT1 signaling is involved in the antiviral response to influenza virus infection. Thus, one would expect that inhibition of STAT1 would lead to increased replication efficiency, which could result in higher amounts of progeny virus. However, we found an opposite result, as STAT1 inhibition in virus-infected cells treated with FLUD resulted in a reduction of virus titers. Western blot assays demonstrated that after drug treatment, p-STAT1 expression was obviously impaired and viral protein production was simultaneously reduced compared with the untreated group (Fig. 3A). We performed qPCR assays on the viral RNA extracted from infected cells and showed that p-STAT1 could significantly inhibit virus replication at 6, 12, and 24 h p. i. (Fig. 3B; $P < 0.01$). A plaque assay was designed to examine infectious virus titers in cell culture supernatants. We found that Fludarabine markedly reduced the production of viral particles compared with the cells without drug treatment at 6, 12, and 24 h p. i. (Fig. 3C; $P < 0.01$). These findings demonstrated that inhibition of p-STAT1 could significantly reduce viral production.

3.4. Fludarabine appears to block the early stages of the influenza virus life cycle by reducing vRNA synthesis

Considering that the first generation of influenza virus particles are released within 12 h of the initiation of infection, we further characterized which stage of the virus life cycle was affected by STAT1 activation. When FLUD was added to cells at different time points, including at 1 h pre-infection (−1), and at 1, 3, 5, 7, 9, and 11 h post-infection (IAV infection was carried out at the time 0), viral replication could be obviously suppressed by the STAT1 inhibitor from −1 h pre-infection to 3 h post-infection (Fig. 4A); however, when applied at or after 5 h p. i., the inhibitor did not affect virus production, suggesting that it was not effective at late stages of the viral life cycle ($P < 0.01$). Most notably, these data demonstrate that this inhibitor blocks an early stage of the influenza virus life cycle that precedes viral gene expression, which occurs between ~3 and 5 h p. i.

Next, we investigated whether the p-STAT1 inhibitors affected viral RNA synthesis. To address this question, we infected A549 cells with influenza virus at an MOI of 1 for 1 h, and before that we treated the cells with a p-STAT1 inhibitor (FLUD) or vehicle control (DMSO) for 1 h. At 5 h p. i., when viral RNA synthesis had already been completed, total RNA from the infected cells was prepared and expression levels of individual segments of influenza virus-specific vRNA, cRNA, and mRNA were determined by quantitative real-time RT-PCR. The levels of each segment of vRNA in the FLUD-treated samples were significantly lower than those in the DMSO-treated ones. Moreover, the differences in cRNA and mRNA expression levels were not statistically significant (Fig. 4B; $P < 0.05$). Collectively, our data suggest that p-STAT1 inhibitors specifically reduce the expression levels of vRNA, but not those of cRNA and mRNA in virus-infected cells.

3.5. Suppression of pro-inflammatory cytokines in influenza virus-infected cells

Highly pathogenic influenza A viruses are known to induce the

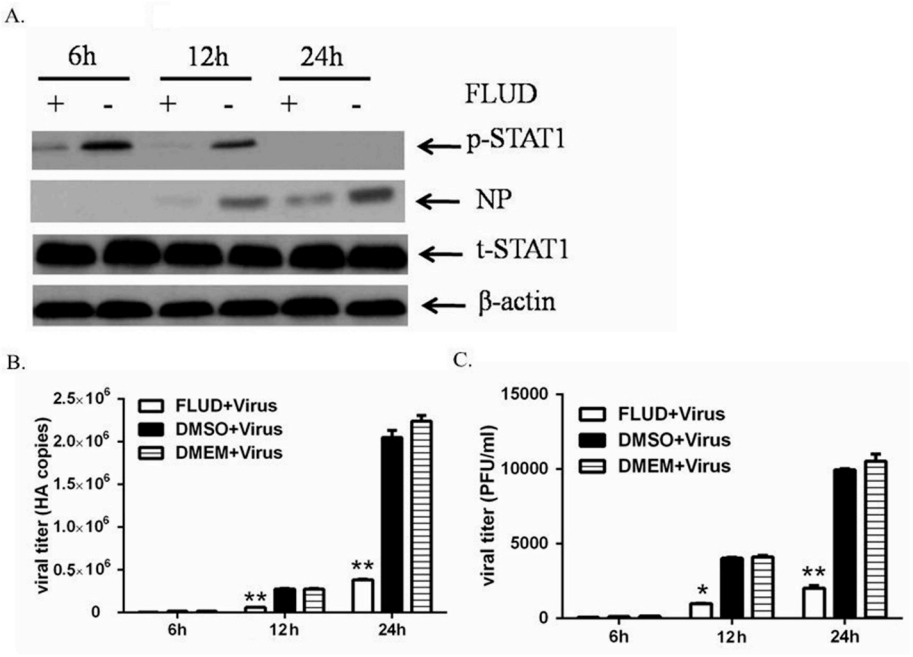

Fig. 3. Downregulation of p-STAT1 suppresses influenza A virus replication. (A) Expression of the viral protein NP was analyzed by western blotting. The A549 cells were lysed at the indicated times after FLUD treatment and then were infected with H5N1 virus. (B) HA gene expression was analyzed by real-time PCR. A549 cells were harvested at the indicated times after FLUD treatment and then were infected with H5N1 virus (N = 3). (C) Viral titers were analyzed by a plaque assay. Virus titers in the supernatants at the indicated times were determined by plaque assays (N = 3); **, P < 0.01.

release of a wide variety of cytokines and chemokines, known as "hypercytokinemia" or "cytokine storm," which is a response associated with acute respiratory syndrome. To determine the immunomodulatory effects of FLUD on cytokines' induction during influenza virus infection, the levels of inflammatory cytokines and chemokines, including IP-10, IFN-α, IFN-β, IL-6, MCP-1, and TNF-α, in the infected A549 cells were measured after treatment with FLUD. The FLUD-treated cells showed a significant inhibition of IP-10, IFN-α/β, TNF-α, and IL-6 induction at 24 h post-infection (P < 0.01). IP-10 levels were markedly reduced both at 12 and 24 h post-infection (P < 0.01). However, no significant differences in the levels of MCP-1 protein were observed compared with the solvent (DMSO) control (Fig. 5A).

To confirm that STAT1 regulates inflammation, rather than simply lowering viral levels, we performed an additional experiment using an inflammatory stimulator, poly (I:C), to mimic IAV. We found that FLUD could directly reduce levels of inflammatory cytokine (IFN-β, TNF-α) expression upon poly (I:C) treatment, indicating that STAT1 is involved in RNA-induced inflammation (Fig. 5B, P < 0.01). These data exclude the possibility that the suppressed inflammatory response was a consequence of impaired viral replication.

3.6. Knockdown of STAT1 reduces the amounts of virus that are produced in cells

The effect of reduced STAT1 activation on viral infection in A549 cells was tested by reducing the levels of activated STAT1 and testing for a reduction of viral progeny. To specifically investigate the role of STAT1 during IAV infection, siRNA-mediated knockdown of STAT1 was performed in IAV-infected A549 cells to address the role of STAT1 in influenza A virus-induced infection. We observed that specific siRNA could inhibit the expression of STAT1 in a time-dependent manner (sFig.1). At 60 h post-transfection, A549 cells were infected

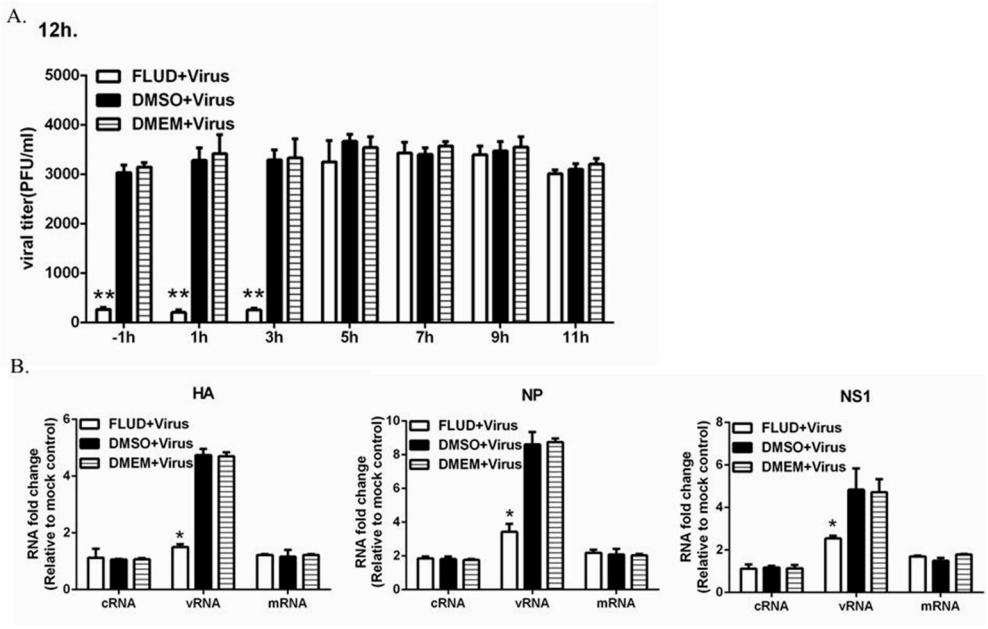

Fig.4. p-STAT1 downregulation blocked influenza A virus production at an early stage, which resulted from the inhibition of viral polymerase activity. (A) Time course of the effect of a p-STAT1 inhibitor on influenza virus production. A549 cells were infected with H5N1 influenza A virus. Fludarabine treatment or a vehicle control (DMEM or DMSO) was applied at different times post-infection [-1 h (1 h before infection), 1, 3, 5, 7, 9, and 11 h post-infection]. Virus titers in the supernatants at 12 h p. i. were determined by plaque assays (N = 3); **, P < 0.01. (B) p-STAT1 downregulation blocked H5N1 virus production because of the inhibition of viral polymerase activity. Levels of the HA, NP, and NS1 segments of vRNA, mRNA, and cRNA in virus-infected cells treated with either DMSO or FLUD. A549 cells were incubated with 50 μM FLUD and infected with H5N1. A549 cells were harvested at 5 h post-infection. RNA levels were normalized to those of GAPDH levels (N = 3); *, P < 0.05.

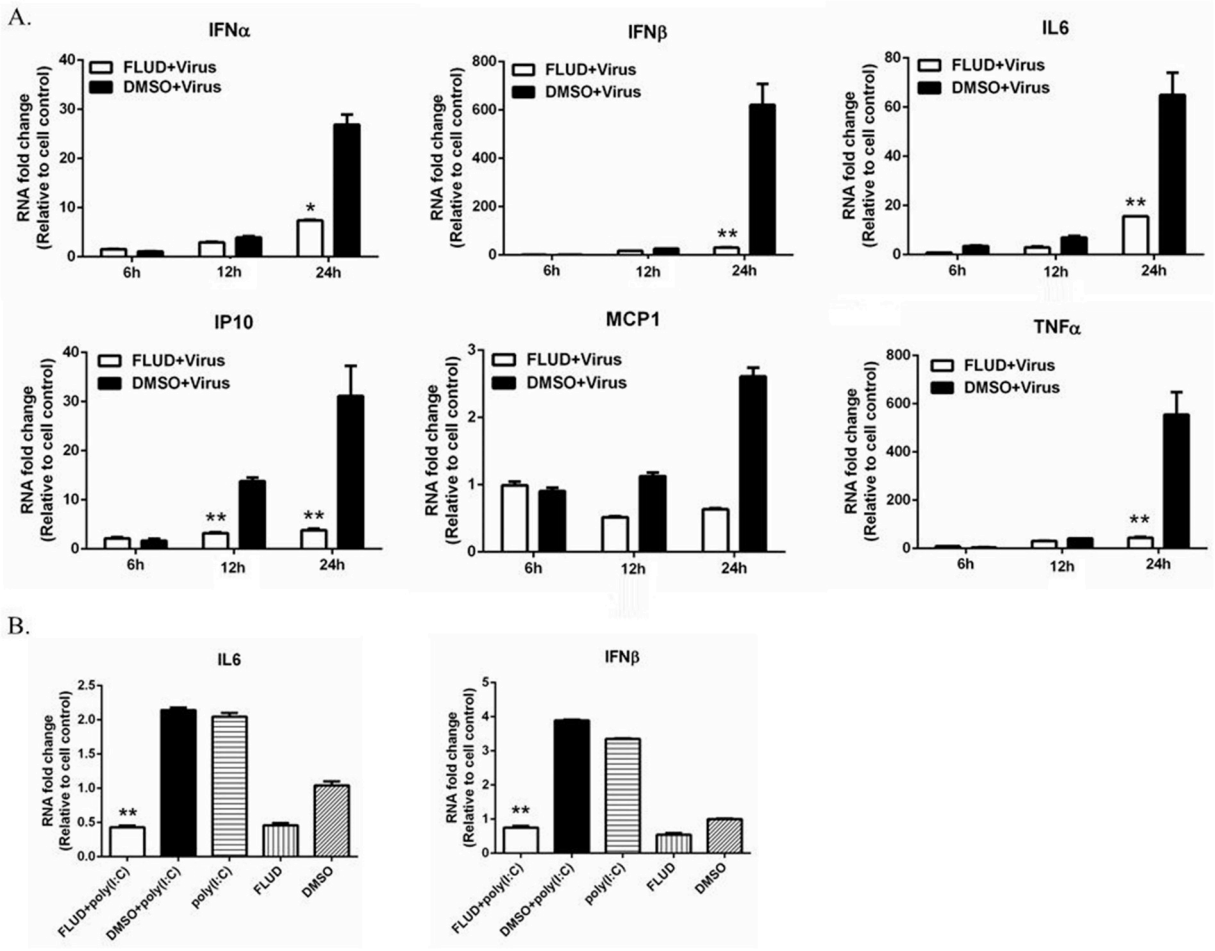

Fig. 5. Inhibition effects of FLUD on cytokine expression in H5N1-infected A549 cells. (A) A549 cells were first pre-treated with 50 μM FLUD for 1 h before infection with H5N1 virus at a MOI = 1. Total mRNA was collected at 6, 12, and 24 h post-infection. The mRNA levels of IFN-α, IFN-β, IL-6, TNF-α, IP-10, and MCP-1 were determined by real-time RT-PCR (N = 3). (B) Levels of IL-6 and IFN-β mRNA in A549 cells were determined by real-time PCR 24 h after treatment with FLUD and 50 μg/mL poly (I:C) (N = 3); **P < 0.01; *, P < 0.05.

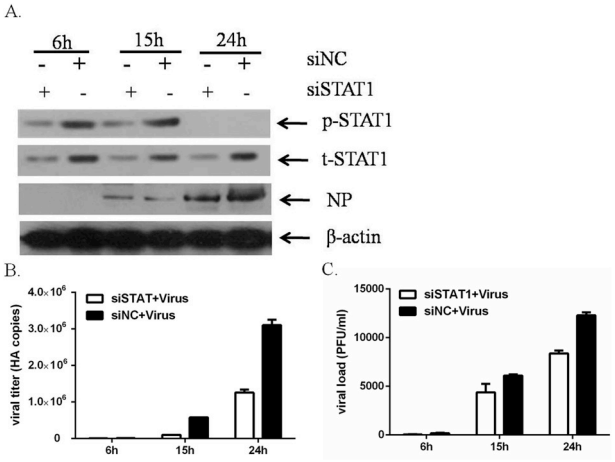

Fig. 6. STAT1 knockdown suppressed H5N1 virus replication. (A) Expression of the viral protein PA was analyzed by western blotting. A549 cells were harvested at the indicated times after siSTAT1 and siNC transfection, and cells were then infected with H5N1 virus (N = 3). (B) HA gene expression was analyzed by real-time PCR. A549 cells were harvested at the indicated times after siSTAT1 and siNC transfection, and cells were then infected with H5N1 virus (N = 3). (C) STAT1 suppression by siSTAT1 attenuated H5N1 propagation *in vitro*. A549 cell supernatants were harvested 6, 12, and 24 h after H5N1 infection, followed by transfection with siSTAT1 or siNC. Viral titers in cell supernatants were tested by plaque assay (N = 3).

with IAV at a MOI of 1. Cells were harvested at the indicated times. After virus infection, our findings showed that knock-down of STAT1 resulted in reduced amounts of viral NP protein (Fig. 6A). We performed qPCR assays on viral RNA extracted from the infected cells and found that siSTAT1 could inhibit virus replication at 6, 12, and 24 h p. i. (Fig. 6B). The plaque assay also showed reduced production of viral particles compared with the negative control at 6, 12, and 24 h post-infection (Fig. 6C). These data were similar to those obtained with FLUD-treated cells that infected with IAV. Considering these data, we can conclude that p-STAT1 participates in virus replication during H5N1 influenza A virus infection of A549 cells.

3.7. Enhanced survival of H5N1-infected animals after the suppression of p-stat1 expression

To investigate the roles of p-STAT1 in IAV H5N1 infection *in vivo*, we challenged BALB/c mice with 3 LD50 H5N1 and administered the inhibitor FLUD at the same time via an intranasal route. Lung tissue samples from three mice per group were collected at days 3 and 6 for real-time PCR, plaque assay, western blotting, and histopathological analyses. Additionally, seven mice per group were used to monitor survival over a period of 14 days.

We found that 57.1% of the FLUD-treated mice survived until termination of the experiments (14 days p. i), while the survival rate DMSO-treated mice was 28.5% (Fig. 7A). The average body weight of mice treated with FLUD was higher than those of the other groups

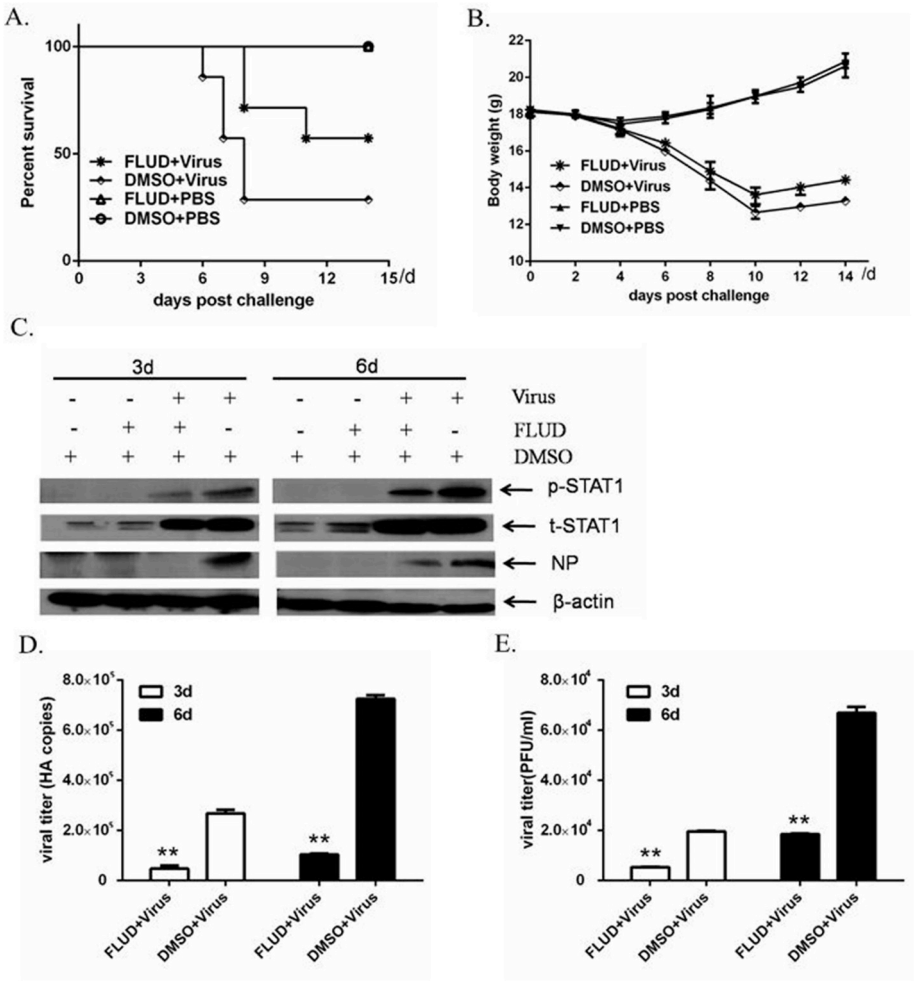

Fig. 7. Suppression of p-STAT1 improved the survival rate and health status of H5N1 virus-infected mice. (A) The rate of survival of mice upon H5N1 challenge and DMSO or FLUD treatment (N = 7); body weights were recorded daily (B). (C) Lung tissues were collected on days 3 and 6 p. i., and total and p-STAT1 and viral protein expression were analyzed by western blotting. Equal loading was verified by detecting β-actin (lower panels). P-STAT1 affected H5N1 propagation *in vivo*. Viral titers in the lungs were determined by real-time PCR (D) and plaque assay (E) (N = 3); *P < 0.05, **P < 0.01.

(Fig. 7B).

When pooled protein samples from the lung tissues of each group (totally four groups) were analyzed by western blotting, the two virus-infected groups exhibited high expression of total STAT1 and p-STAT1, but the levels of these molecules in drug-treated mice were down regulated (Fig. 7C). The levels of viral protein NP in the FLUD-treated group were much lower than those in the solvent-treated positive control group. To investigate the influence of p-STAT1 downregulation on viral replication *in vivo*, we examined the viral load in the lung tissues of virus-infected mice. Plaque assay and real-time qPCR using H5N1-specific primers (HA genes) showed that the pulmonary viral titer in the FLUD + virus group was significantly lower than that in mice treated with DMSO, both on days 3 and 6 p. i. (Fig. 7D and E).

No obvious clinical signs were observed in mice within the two-week observation period in the DMSO and FLUD-only inoculation groups. The lung lesions in the DMSO + virus-infected mice were characterized by interstitial edema and inflammatory cell infiltration around the small blood vessels, exfoliation of the bronchiolar epithelium, thickened alveolar walls, and flooded edema fluid mixed with epithelial cells, erythrocytes, and inflammatory cells in the alveolar lumen on day 6 p. i. (Fig. 8B). However, these symptoms appeared to be mitigated in the FLUD + virus-infected group (Fig. 8D). The lung tissues from mice of the PBS or FLUD-alone treatment groups showed no obvious pathological changes (Fig. 8A and C). By IHC, IAV antigens could be detected throughout cells in the lungs of mice from the DMSO + virus-infected group, while the presence of antigens in the p-STAT1 suppressed group was sporadic (Fig. 8F). No positive signals were detected in the PBS- or FLUD-treated groups. The scores of pathological changes and IAV antigens in lung could further show the significant difference between DMSO + virus-infected group and FLUD + virus-infected group (Fig. 8I and J). These data indicated that downregulation of p-STAT1 *in vivo* could reduce the H5N1 virus-induced pathological damage, thus improving the overall survival of infected animals.

3.8. p-STAT1 regulates cytokine expressions in vivo

To examine the role of p-STAT1 on inflammation in influenza virus-infected mice, analyses of IL-6, TNF-α, IFN-β, and IL-10 expression were performed by real-time PCR on days 3 and 6 p. i. The mRNA levels of IL-1β, IL-6, IFN-β, IL-10, MCP-1, and TNF-α in the FLUD-treated group were significantly reduced compared with the DMSO-treated groups at days 3 and 6 p. i. The expression levels of IFN-β, and MCP-1 mRNA at day 6 p. i. were lower than those at day 3p.i. (Fig. 9). Those findings, together with the observations in cell cultures, indicated the critical role of p-STAT1 in the initiation and regulation of inflammatory responses to IAV H5N1 infection both *in vitro* and *in vivo*.

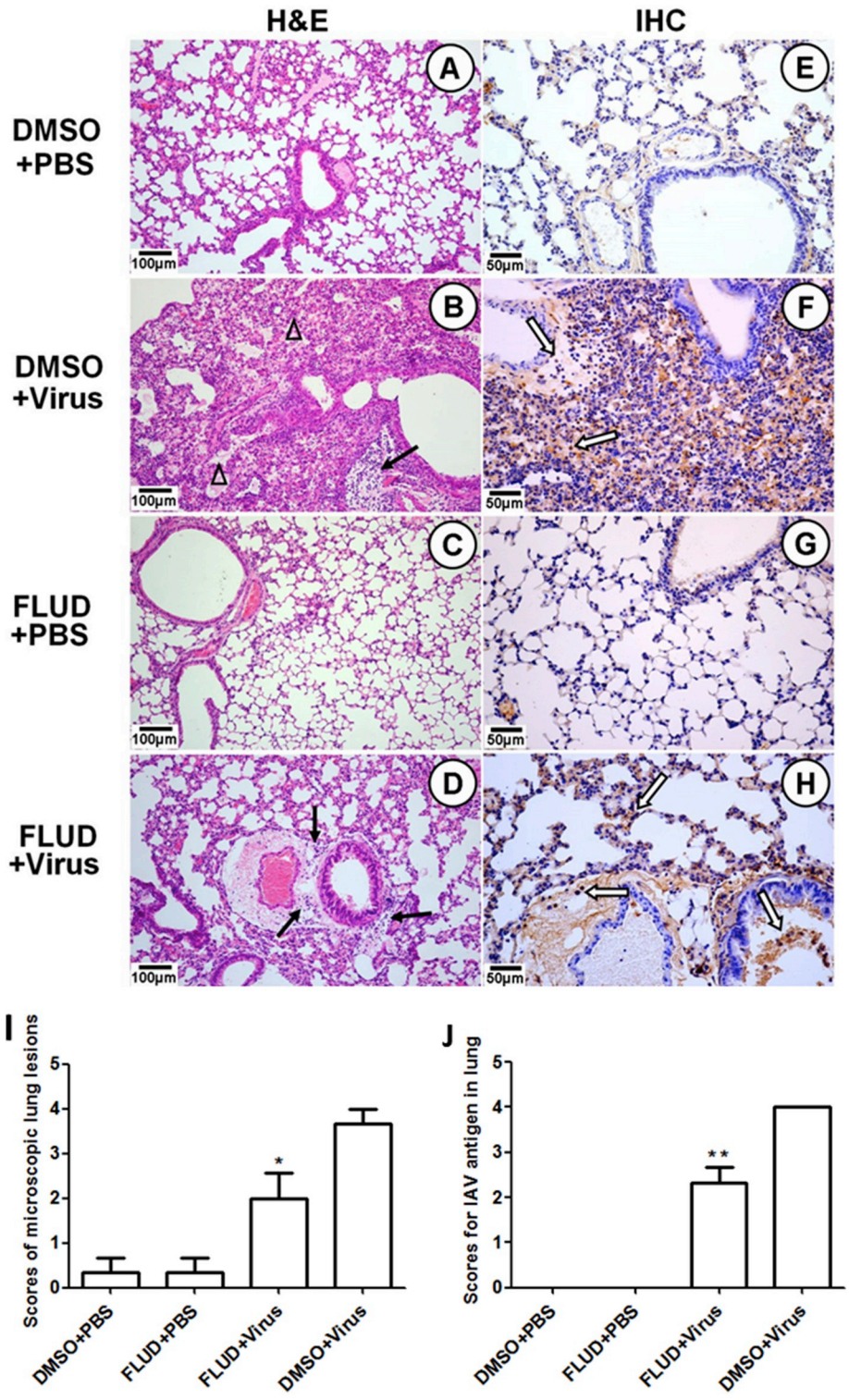

Fig. 8. Lung histopathology at days 3 and 6 p. i. Representative lung sections from each group were subjected to H&E staining and IHC analysis. (A–D) Solid arrows indicate interstitial edema and inflammatory cell infiltration around small blood vessels; triangles indicate alveolar lesions. Scale bar = 100 μm. (E–H) Immunohistochemical detection of H5N1 antigens in the lung after treatment with FLUD and DMSO, followed by infection with influenza A H5N1 virus. Hollow arrows indicate positive signals. Scale bar = 50 μm. (I–J) Pathological changes and detection of the IAV antigens in lung were evaluated by a veterinary pathologist and scored 0 to 4 in a blinded study (N = 3); *P < 0.05, **P < 0.01.

4. Discussion

STAT1, a key transcription factor, plays a role in many cellular events, such as immune regulation, cell differentiation, tumor suppression, cell growth inhibition, and apoptosis. The binding of type I IFN (IFNα/β) to its receptor is the initial step in this signaling process, followed by activation of the JAK family and the subsequent activation of STAT proteins (Kosciuczuk et al., 2019). STAT proteins then enter the nucleus and trigger the transcription of a series of antiviral molecules (Matikainen et al., 2000). Herein, we show that STAT1 becomes activated upon influenza infection during the first replication cycle of the H5N1 virus (2–12 h p. i.). Moreover, for the first time, we demonstrated that STAT1 regulates influenza A virus replication and the inflammatory response. The specific inhibition of STAT1 expression by FLUD or siSTAT1 led to a significant reduction of H5N1 virus replication. However, in addition to this obvious antiviral effect, this transcription factor also exhibits a function that appears to support virus replication at a very early stage. Additionally, this reduction in viral propagation might be induced by impairing the ability of the viral polymerase complex to synthesize vRNA. This finding appears to be

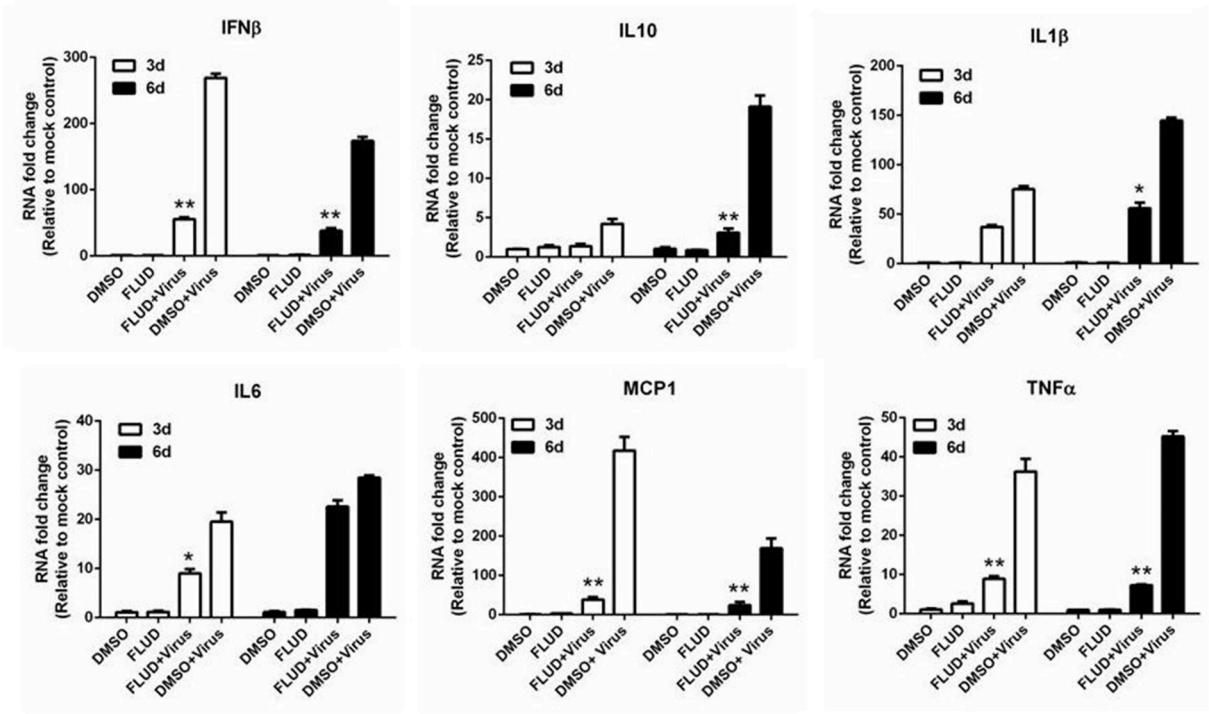

Fig. 9. Inhibition effect of FLUD on cytokines expression in H5N1-infected mice. The mRNA levels of IFN-β, IL-1β, IL-6, TNF-α, MCP-1, and IL-10 in the lung on days 3 and 6 p. i. were determined by real-time PCR (N = 3); *P < 0.05, **P < 0.01.

contradictory as STAT1 is known to be an important anti-viral molecule during IAV infection (Garcia-Sastre et al., 1998; Wei et al., 2014). It is well known that viral infection and host response is a complex regulatory network. Recently, serials of experiments have found that NK-κB p65, c-jun and other transcription factor were playing a bivalent role during influenza virus infection and host cell defense (Ehrhardt et al., 2006; Xie et al., 2014). In addition, our data showed that suppression of STAT1 activation also led to down-regulated IFN-β, which seems to be contradictory. However, this in turn supports our hypothesis that STAT1 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. We speculate that p-STAT1 might support virus replication, in addition to its activity as a downstream effector of IFNs. Thus, STAT1 is a perfect example of a seemingly antiviral signaling component that is misused by the virus to support effective replication.

Our work suggests that p-STAT1 contributes to efficient influenza virus replication via the preferential regulation of vRNA synthesis, but not the synthesis of complementary positive-strand cRNA or mRNA. To date, it represents the first evidence of a p-STAT1 signaling pathway that regulates the synthesis of vRNA during H5N1 viral replication. Influenza virus RNA synthesis consists of three steps: (i) the transcription of vRNA into mRNA, (ii) the replication of vRNA into cRNA, and (iii) the replication of cRNA into vRNA. While we found that cRNA and mRNA synthesis were affected, we speculate that the process of cRNA replication into vRNA was impaired. The mechanisms of how p-STAT1 regulates viral RNA species in virus-infected cells are not well understood, which is a subject that demands further exploration.

High expression of cytokines and chemokines is a hallmark of influenza A virus infection, most notably in highly pathogenic avian influenza viruses or pandemic strains (Huo et al., 2018; Kawai and Akira, 2006; LUDWIG et al., 1999). This cytokine and chemokine deregulation of the immune system leads to higher virus loads and greater lethality. Thus, in addition to controlling virus replication, it is also necessary to control the expression of pro-inflammatory cytokines, particularly in severe cases of influenza virus infection (Mok et al., 2014; Simmons and Farrar, 2008; Sithisarn et al., 2013). Influenza A viruses are known to activate the transcription factor NF-κB, which can enhance the expression of many cytokine and chemokine genes, including IL-6, IP-10, MCP1, and RANTES (Cartwright et al., 2018; PAHL and BAEUERLE, 1995). Viral infections do not appear to directly activate STATs, but instead induce cytokines, especially IFN-α, which can activate multiple STAT proteins. STATs are involved in the activation of IP-10 and MCP-1 gene expression (Majumder et al., 1998; Valente et al., 1998). In this present study, we analyzed cytokine and chemokine gene expression in H5N1-infected A549 cells with or without treatment with a specific inhibitor of p-STAT1. We showed that after treatment with FLUD during virus infection, inflammatory cytokine and chemokine induction was markedly reduced, specifically IFN-β, IP-10, and MCP-1. Furthermore, Wu et al. showed that siSTAT1-treated mice with spinal cord injury had obviously lower cytokine induction (Wu et al., 2014), which was in agreement with our findings, although *in vivo* expression of the anti-inflammatory cytokine IL-10 was clearly reduced. We speculate that Fludarabine is an immunosuppressive drug that can cause broad-spectrum inhibition of the inflammatory response.

Fludarabine can be used as a single agent or in combination with other drugs, and has been extensively used in recent years for the treatment of many hemato-oncologic disorders (Herishanu et al., 2019). Moreover, Fludarabine is a STAT1 activation inhibitor that can significantly reduce STAT1 phosphorylation (FRANK et al., 1999; Torella et al., 2007). Chaudhuri showed that specific STAT1 inhibitor Fludarabine blocked HIV-1-induced STAT1 activation and decreased IL-6 expression and secretion. These data strongly support the notion that the inhibition of STAT1 activation could represent a unique therapeutic strategy to prevent HIV-1-induced breach of the blood-brain barrier (BBB) and, as such, improve clinical outcomes in infected individuals (Chaudhuri et al., 2008). Additionally, Cervasi et al. found that during simian immunodeficiency virus (SIV) infection, use of the STAT1-specific inhibitor Fludarabine delayed the rebound of viral replication, thus suggesting a reduction in the size of the SIV reservoir (Cervasi et al., 2006). Herein, we examined the roles of p-STAT1 in host inflammatory responses and H5N1 virus replication using a specific inhibitor, FLUD.

We found that this inhibitor had the same effect that has been observed HIV-infected humans. Our data presented herein indicate that FLUD could reduce influenza A virus production and reduce cytokine expression levels *in vitro* and *in vivo*. Based on the effects of the drug when used in humans, our data imply that FLUD could be a potential therapeutic method for treating IAV, even though its complete mechanism of action is not yet clear.

Years ago, it was discovered that activation of the Raf/MEK/ERK signaling pathway is a prerequisite for efficient influenza virus replication and that inhibition of this intracellular signaling pathway leads to reduced influenza virus production (Pleschka et al., 2001). Then, a strategy of using intracellular signaling pathway inhibitors as antiviral agents has become a major focus of research in recent years. Recently, the number of intracellular pathways that have been found to be required for efficient influenza virus replication has steadily increased (De Clercq, 2006; Ludwig, 2009; Shaw, 2011; Sithisarn et al., 2013; Song et al., 2005). It has been demonstrated that inhibiting the NF-κB or PI3K signaling pathways could obviously impair virus replication, and the virus also needs the NF-κB signaling pathway for efficient replication (Ehrhardt et al., 2006; Kumar et al., 2008; Zhong et al., 2018). Our group has shown that inhibition of the c-Jun protein, which is downstream of the JNK/MAPK pathway, lead to the reduction of influenza A virus and inflammation *in vitro* and *in vivo* (Xie et al., 2014). When these pathways become activated, they are usually accompanied by protein phosphorylation or dephosphorylation. We have also investigated all of the phosphorylated proteins after influenza A virus infection using phosphoproteomic analysis, which might aid the identification of additional molecules that could participate in virus entry and replication.

In summary, our results for the first time demonstrate that p-STAT1 can carry out a function that regulates influenza A virus replication and the associated inflammatory response. Our work adds to the existing understanding of the complex regulatory networks that control both viral infection and the host inflammatory response.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.08.023.

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Conflicts of interest

All authors declare that they have no conflict of interests.

Author contributions

SPZ, CYH, TF and YXH carried out experiments, analyzed data and wrote the paper. JX, SMZ, PQ, MW, LQS and YXH designed the study and supervised the project. SPZ assisted in the data analysis and discussion. SPZ, CYH and YXH drew the figures. All authors read and approved the final manuscript.

Data availability statement

The data are reliable and all present in our manuscript.

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