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DNAzymes Dz13 target the c-jun possess antiviral activity against influenza A viruses

Zhaopei Zhang [a, 1], Shouping Zhang [a, b, c, 1], Sanhu Wang [a, *]

[a] Henan Institute of Science and Technology, Xinxiang 453003, China
[b] Postdoctoral Research Base, Henan Institute of Science and Technology, Xinxiang 453003, China
[c] Post-doctoral Research Station, Henan Agriculture University, Zhengzhou 450002, China

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ABSTRACT

The emergence of anti-influenza A virus drugs resistant strain highlights the need for more effective therapy. Our earlier study demonstrated that c-jun, a downstream molecule of JNK, might be important in viral infections and inflammatory responses. In the present study, we explored the function of DNAzymes Dz13 that target c-jun in influenza A virus infected mice. Dz13 displayed non-toxic side effects on A549 cells and BALB/c mice. Moreover, Dz13-treated mice had enhanced survival after influenza compared with untreated mice. Simultaneously, the pulmonary inflammatory responses and viral burden were decreased in Dz13 treated mice. Furthermore, proliferation levels of infection-induced $CD4^+$ and $CD8^+$ T cells were impaired. These data demonstrated that Dz13 could reduce viral replication and inflammatory response in vivo, suggesting that Dz13 may potentially be used to treat influenza A viral infection.

1. Introduction

Influenza A virus (IAV) is one of the most common respiratory pathogens that cause seasonal infections, with high morbidity in humans and animals [1,2]. Moreover, its prevalence threatens the breeding industry and human health. Thus, its pathogenesis has attracted the numerous researchers' attention. High viral load and intense inflammatory response known as the “cytokine storm”, are responsible for its high mortality [3–6]. Therefore, developing a therapeutic agent for both viral replication and host inflammation is urgent.

According to the recent studies, several phosphorylation-regulated signaling cascades, were activated during influenza A virus infection such as NF-κB signaling, PI3K/Akt pathway, MAPK pathway, PKC/PKR signaling. These signaling process are involved in viral entry, replication, inflammation upon altered the activity of relative mediator [7,8]. Moreover, these specific signaling processes initiated during virus replication may be potential targets for developing novel antiviral strategies. The MEK inhibitors U0126 could block Raf/MEK/ERK pathway and markedly impair influenza A virus propagation [9,10]. Additionally, titers of progeny influenza A virus has been reduced upon inhibition of PI3K/Akt activity [11]. Furthermore, viral replication was impaired after decreasing NF-κB and PKC expression [12–14]. After being incubated with JNK inhibitor SP600125, H5N1 virus propagation in cells was restrained [15,16]. Together with U1026, Bay11-7082 (NF-κB inhibitor), LY294002 or wortmannin (PI3K inhibitor) and SP600125 or other inhibitor could effectively target virus replication at different steps; however, none of these has been clinically approved.

As one subgroup of the MAPK (mitogen-activated protein kinase) family, JNK is immediately activated upon influenza A virus infection [8]. C-jun is a member of the basic region-leucine zipper protein family and, together with activating-transcription-factor-2 (ATF-2), make up the transcription factor AP-1, whose transcriptional activity is enhanced by JNKs [17,18]. Moreover, c-jun is involved in numerous cell activities, such as proliferation, apoptosis, survival and tumorigenesis. In our earlier investigation, c-jun is activated (phosphorylated) at a very early stage and involved in H5N1 virus replication and inflammation in vitro and vivo [15]. Therefore, c-jun is important in viral infections and virus-induced inflammatory response. Dz13, a catalytic DNA molecule cleaving c-jun mRNA, can inhibit tumor growth and endothelial cell proliferation or migration. Therefore the DNAzymes of Dz13 viewed

* Corresponding author. College of Animal Science, Henan Institute of Science and Technology, Eastern HuaLan Avenue, Xinxiang 453003, China.
E-mail address: wangsanhu@hist.edu.cn (S. Wang).
[1] These authors contributed equally to this study.

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as attractive candidates for the development of targeted therapies of inflammation [19–22]. The suitability for anti-influenza infection of DNAzymes that have been applied to cancer therapy is present here.

Therefore, in the present study we explored the function of DNAzymes Dz13 in influenza A virus infected mice. We investigated whether DNAzymes of Dz13 would effectively inhibit influenza A virus replication and inflammatory responses, and be a potential therapy for influenza A virus infection.

2. Materials and methods

2.1. Virus and cells

Two subtypes influenza A virus H1N1 (A/WSN/33) and H7N2 (A/Chicken/Hebei/2/02) were used. Viruses were propagated in MDCK cells at 37 °C for 48 h, and viral supernatant was harvested and stored at −80 °C. MDCK and A549 cells were provided by the Cell Resource Center of Peking Union Medical College and conventionally cultured.

2.2. Administration of Dz13 and virus to animals

Female BALB/c mice aged 6–8 weeks were obtained from Vital River Laboratories (Zhengzhou, China). Mice were raised in independently ventilated cages and received food and water freely. Experimentation with animals was carried out according to Regulations for the Administration of Affairs Concerning.

H1N1 virus was diluted in PBS when used for infection. Mice were anesthetized with intramuscular administration of Zoletil (Virbac, Carros, France) and infected intranasal with 200 PFU in 50 μl. The Dz13 and its scrambled (Dz13scr) were dissolved in PBS in a concentration of 5 mg/mL, and then stored at −20 °C. The dose of 2.5 mg/kg (in 50 μl PBS containing 2.5 μl Fugene 6 and 1 mM MgCl2) was administrated intranasally to infected mice at day 0 (20 min after viral challenge) and day 2 (48 h after viral challenge).

2.3. MTT assay

The vitro MTT assay followed the regulation of the instructions. Cells were cultured with Dz13 or Dz13scr at varying concentrations for 48 h, and then medium was removed and washed with PBS before being replenished with 1 mL of 0.5 mg/mL MTT solution. After 4 h incubation, MTT solution was removed, and added 150 μL DMSO to each well. The absorbance at a wavelength of 570 nm was measured using an ELISA plate reader.

2.4. PFU assay

Lung tissue from each mouse was homogenized in 1 mL PBS and centrifuged for 10 min at 1500×*g* and the supernatants were stored at −80 °C for PFU assay. The mouse lung tissue supernatants were diluted 10-fold and added to a monolayer of MDCK cells in semi-solid agar that contained 0.5 μg/ml trypsin TPCK (Sigma-Aldrich, St Louis, MO, USA). At the appointed time, plaques were stained, counted, photographed and expressed as the mean log10 PFU/ml.

2.5. Western blot analysis

Cells 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. 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 using the first and second antibodies (Cell Signalling Technology, Danvers, MA, USA) at 1:1000 final dilution. The antibody binding was detected using a Western Lightning chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA, USA).

2.6. 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), according to 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).

2.7. T cell analysis

On day 6, five mice were sacrificed to harvested spleen and peripheral blood lymphocyte (PBL). The tissues and blood were grinded then erythrocytes were lysed using erythrocytes lysis buffer (M&C GENE, Beijing, China). Single-cell suspensions were prepared by passing through a nylon screen and then washed in PBS. Next, lymphocyte populations (containing 10^6 cells) were dual stained with either FITC anti-mouse CD4 and PE anti-mouse CD8a or Cy5.5 anti-mouse CD3 for 1 h (eBioscience, SanDiego, CA). The cells were resuspended 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.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA Tukey post hoc test in the SPSS software (version 12.0; 2003, SPSS Taiwan Corp., Taiwan) or GraphPad Prism Software (version5.0, GraphPad Software, San Diego, Calif.) and $P < 0.05$ was considered statistically significant.

3. Results

3.1. DNAzymes treatment does not affect the metabolic activity of A549 cells and the animals

To determine the DNAzymes Dz13 efficacy and choose an optimal concentration, A549 cells were cultured with different concentrations (0.05, 0.1, 0.3 and 0.5 μM) of Dz13 and Dz13scr, and total c-jun (t-c-jun) levels were measured. As shown in Fig. 1A, the drug-treated cells markedly down regulated c-jun at the concentration of 0.1 and 0.3 μM. Accordingly, we chose 0.1 μM as a working concentration in further in vitro experiments.

Based on this working concentration, MTT assays were performed to determine cytotoxicity. As shown in Fig. 1B, these two DNAzymes did not cause cytotoxicity at the concentrations of 0.1 μM in A549 cells compared with the control without the drug treatment. Mice treat with 200 μM Dz13 for five consecutive days did not display adverse effects in vivo, as demonstrated by liver and spleen weights (Fig.1C). These results showed that DNAzymes Dz13 treatment does not affect the metabolic activity of A549 cells and the animals.

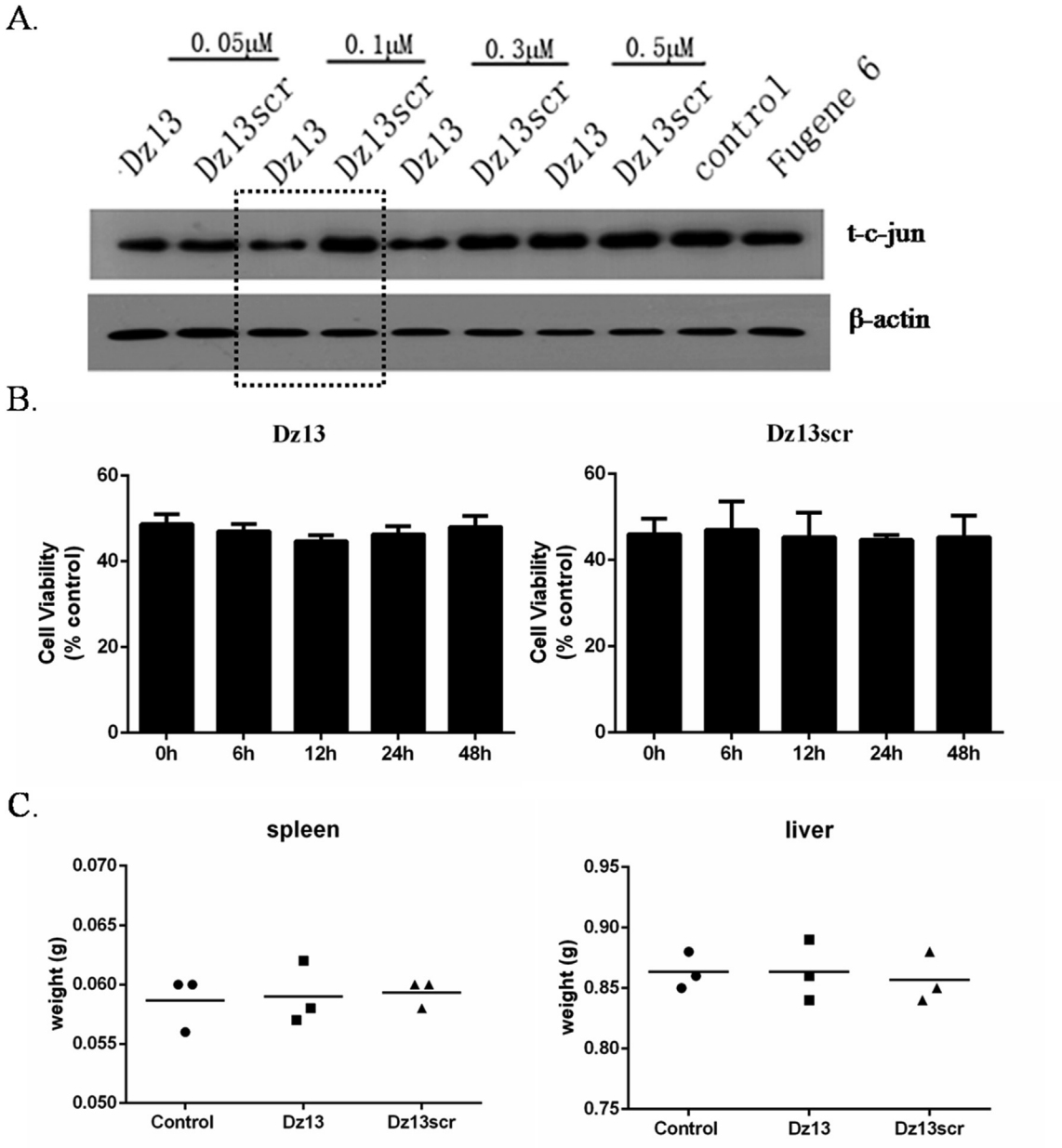

Fig. 1. Assessment of potential toxic effects of Dz13 in vitro and vivo. (A) A549 cells were treated with different Dz13 concentrations. After 12 h, cells were harvested and analyzed by western blotting using specific antibodies. Equal loading was verified using detecting β-actin (lower panels). (B) Cell viability of A549 cells at the indicated concentration. Cells absorbance at 570nm was measured using an ELISA plate reader. The results are expressed in percentage (%) after normalizing all the absorbance data to the value obtained with mock-treated cells. Values represent the mean (%) ± SD from three independent determinations. (C) Weight of spleens and livers of Dz13, Dz13scr and solvent treated mice after a 5-day application period. (N = 3); *,P < 0.05; **, P < 0.01.

3.2. DNAzymes Dz13 reduce IAV replication

To evaluate the potential affects of DNAzymes on viral amplification, we transfected Dz13 and its scramble Dz13scr before IAV-infected A549 cells. A plaque assay was performed to detect virus titer at 12 h, 24 h and 36hp.i.The DNAzymes downregulated c-jun expression and inhibited IAV-induced c-jun activation (data not show). As shown in Fig. 2A and B, the DNAzymes markedly reduced H1N1 and H7N2 viral titers in infected cells ($P < 0.01$). However, in Dz13scr-treated cells, IAV propagation was unaffected. These data indicated that DNAzymes of c-jun Dz13 reduce virus replication and are unrestricted by IAV subtypes.

3.3. Increased survival of the IAV-infected animals by Dz13 treatment

To confirm whether Dz13 treatment would prolong survival, BALB/c mice were infected with 200 PFU of H1N1. Survival and body weight were monitored for 14 days. Results showed that 71.4% of Dz13 treated mice survived at the termination of the experiments (14 days p.i), while PBS and Dz13scr treated mice died by day 8 (Fig. 3A). As shown in Fig. 3B, body weights dropped during infection in all treatment groups, reaching a nadir on day 9–11. However, the extent of weight loss at nadir was not as great and/or the rebound of weight was more rapid in the Dz13-treated group.

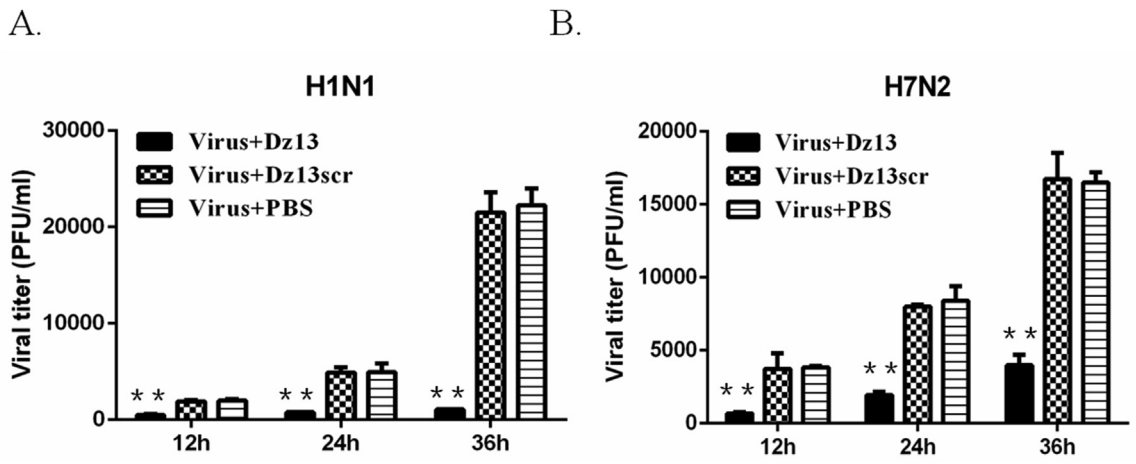

Fig. 2. Suppression of c-jun protein reduced influenza A virus viral replication. Viral titers were analyzed via plaque assay. The virus titers (A) H1N1 and (B) H7N2 in the supernatants at the indicated times (12 h, 24 h and 36 h p.i) were determined by plaque assays. (N = 3); *,P < 0.05; **, P < 0.01.

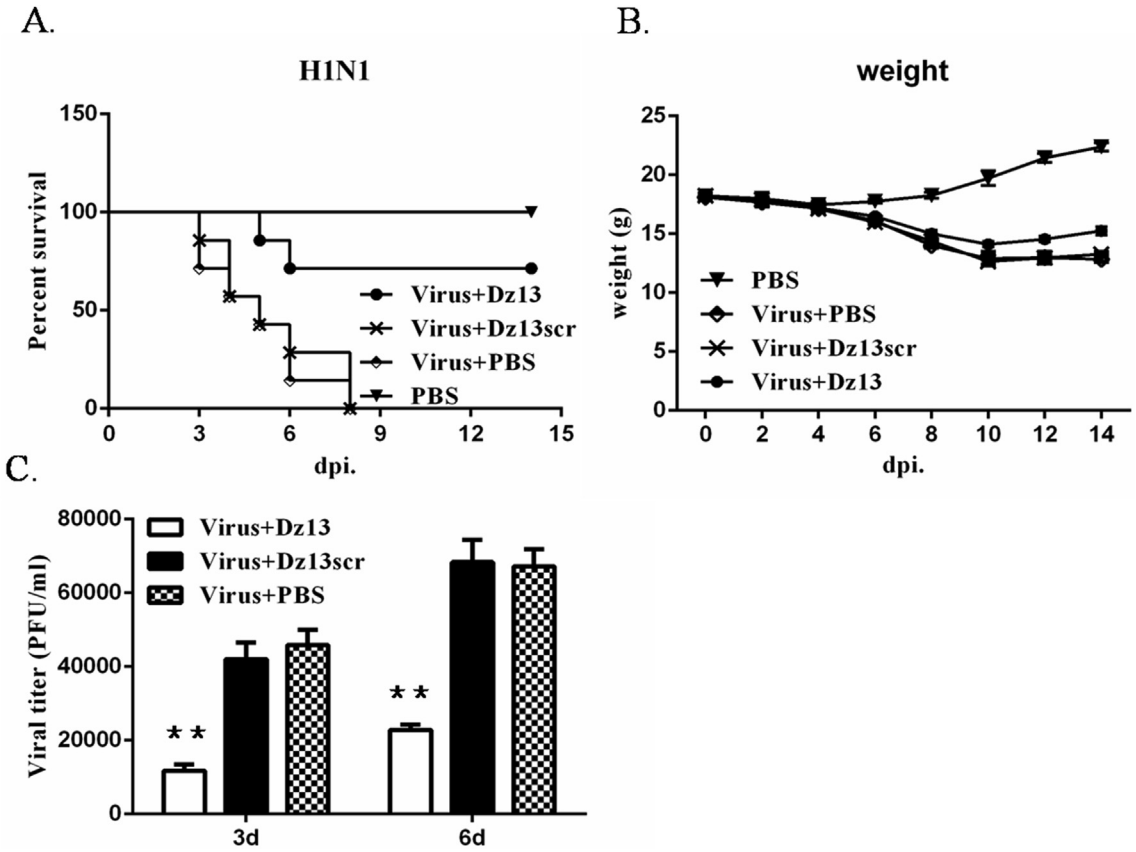

Fig. 3. Suppression of c-jun improved the survival of H1N1-infected mice. (A) Mouse survival rates upon H1N1 challenge and Dz13/Dz13scr treatment and body weights were recorded daily (B) (N = 7). (C) C-jun-infected H1N1 propagation in vivo. Lung tissues were collected on days 3 and 6 p.i. The viral titers in lungs were determined via plaque assay. (N = 3); *,P < 0.05; **, P < 0.01.

To investigate the Dz13 influence on viral replication in vivo, five mice per group were sacrificed on day 3 and 6 to determine lung viral titers using plaque assay in MDCK cells. The data showed that the pulmonary viral titer in Dz13-treated infected mice were significantly lower than those in mice treated with Dz13scr or PBS, both on days 3 and 6 p.i.($P < 0.01$). These data displayed that DNAzymes Dz13 treatment increases the survival of virus infected mice.

3.4. Dz13 treatment reduces cytokine release induced IAV infection

It is well known that highly pathogenic avian influenza A viruses induce the release of a wide pattern of cytokines and chemokines called the "cytokine storm". To investigate whether Dz13 treatment influenced the cytokine and chemokine release, lung samples were collected at day 3 and 6 after infection. Analyses of IL-1β, TNF-α, IFN-β, and IL-10 cytokine expression were performed using real-time PCR. These cytokines induced by H1N1 influenza A virus

infection could be significantly reduced after Dz13 treatment (Fig. 4A–C). In contrast to the pro-inflammatory cytokines, IL-10 showed a higher expression trend in Dz13 treated mice than in other groups on both days 3 and 6 p.i. (Fig. 4D). It demonstrated the regulatroy role of c-jun in inflammatory responses in virus infection mice.

3.5. Dz13 treatment reduces IAV activated $CD4^+$ and $CD8^+$ T cell proliferation

$CD4^+$ and $CD8^+$T cells are two subsets of peripheral T cells that exhibit important and distinct roles in influenza A virus-induced immune response. To determine whether c-jun could affect $CD4^+$ and $CD8^+$ T cell proliferation, spleen tissue and PBL samples of five mice per group were collected on day 6 for flow cytometry assay. As shown in Fig. 5, $CD4^+$ T cells in PBLs and $CD8^+$ T cells in spleen and PBLs were markedly proliferated after virus infection, which may accompany immune response. However, the peripheral T cell proliferation induced by virus was reduced to a normal level in Dz13 treated mice compared with PBS or Dz13scr treated mice. These data indicated that DNAzymes Dz13 inhibits virus-induced peripheral T cells proliferation.

4. Discussion

To date, the approved anti-influenza virus mechanism are M2 inhibitor and NA inhibitor, which block the viral ion channel and inhibit the viral NA activity and prevent release of novel virus particles respectively [23]. However, more influenza A viruses, as well as influenza B viruses, are becoming resistant to the drug (oseltamivir) [24,25]. Additionally, researcher found that oseltamivir-resistant variant H1N1 causing a epidemic disease in the northern hemisphere [26]. Notably, the emergence of these drugs resistance strain highlights the need for more effective therapeutic. Moreover, infection by influenza viruses activate various intracellular signaling pathways. Several inhibitors that target for these pathways was found could impaired IAV viral replication [6,12,27]. However, these components have to be developed further to be approved for clinical evaluations.

In the case of infection, the cytokine storm is suspected to cause severe inflammation in the lung epithelial cells and possibly lead to death. As a member of the transcription factor AP-1, c-jun is crucial for IFN-β and antiviral cytokine expression [17]. In this study, our data demonstrated that once the viral infection was established, a series of inflammatory responses that express cytokines are developed and markedly increased. Because DNAzymes Dz13 damaged the c-jun expression, the cytokine expression was reduced, and our data further conformed this. Thus, the JNK pathway appears to be a crucial mediator of the inflammatory response upon the virus infection.

As two subsets of peripheral T cells, $CD4^+$ and $CD8^+$ T cells present distinct roles during immune response. After virus

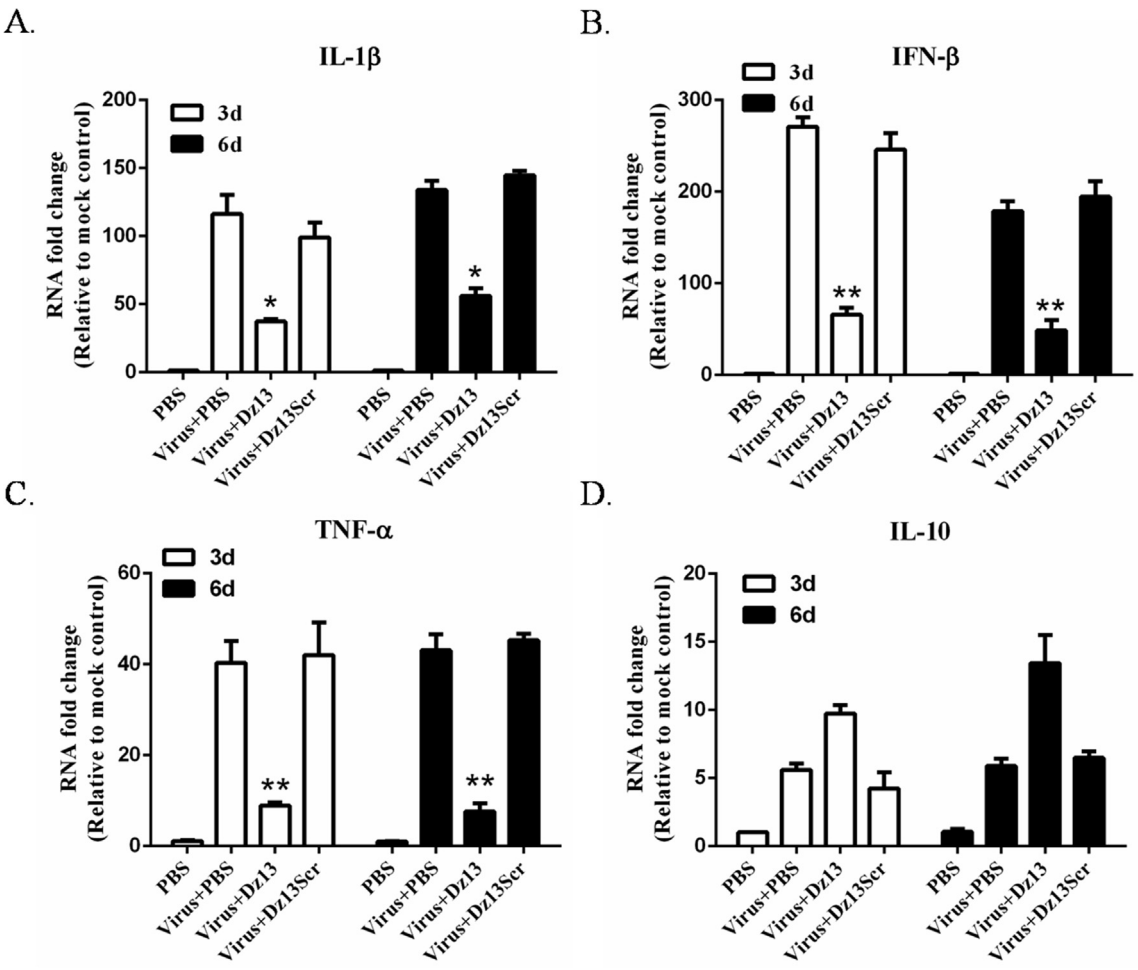

Fig. 4. C-jun suppression altered inflammatory cytokine expression in vivo after H1N1 infection. The mRNA levels of (A) IL-1β, (B) IFN-β, (C) TNF-α and (D) IL-10 in the lungs on days 3 and 6 p.i.were determined by real-time PCR. (N = 5); *,P < 0.05; **, P < 0.01.

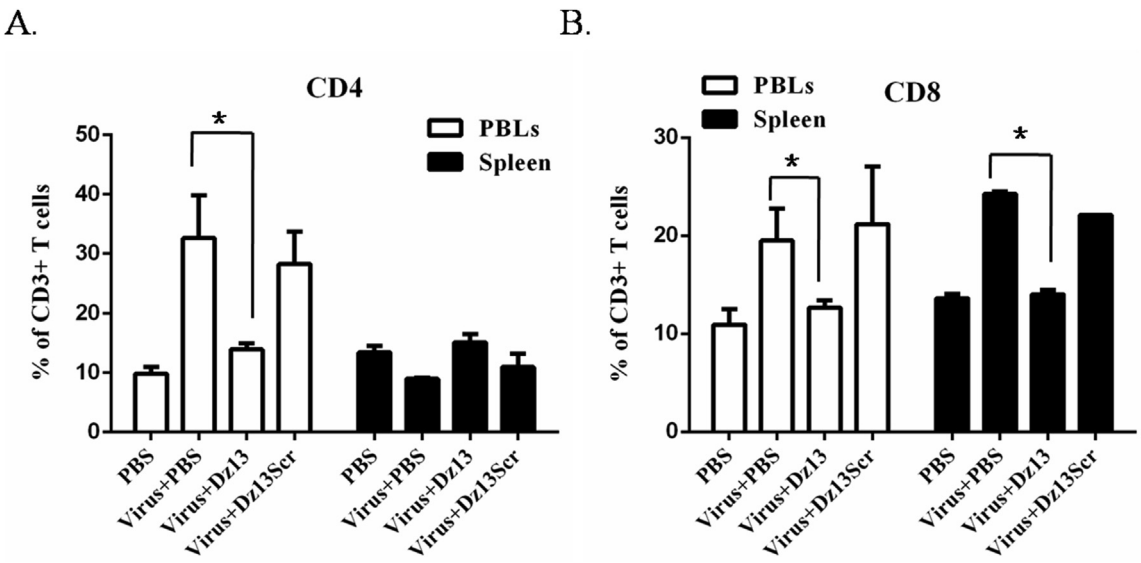

Fig. 5. C-jun suppression decreased the $CD4^+$ and $CD8^+$ T cells proliferation in vivo after H1N1 infection. The T cell proliferation in the spleen and PBLs was detected using flow cytometry. (N = 5); *,P < 0.05; **, P < 0.01.

stimulation, $CD8^+$ T cells differentiate into cytotoxic cells and secrete high levels that have antiviral activities [28,29]. Previous studies demonstrated that blocking the JNK pathway via JNK inhibitor SP600125 markedly reduced $CD8^+$ T cell proliferation in mice, and this is consistent with our present findings. Moreover, the reduced $CD8^+$ T cell proliferation may partly explain the impaired inflammatory response.

In summary, this study demonstrates that DNAmyzes Dz13 might be able to inhibit influenza virus replication and inflammatory cytokines induction. Moreover, these results suggest that Dz13 is a novel therapeutic intervention that might be considered in severe influenza virus infection.

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References

[1] I. Stephenson, M. Zambon, The epidemiology of influenza, Occup. Med. 52 (2002) 241–247.
[2] Y.M. Loo, M. Gale Jr., Influenza: fatal immunity and the 1918 virus, Nature 445 (2007) 267–268.
[3] J. McGill, J.W. Heusel, K.L. Legge, Innate immune control and regulation of influenza virus infections, J. Leukoc. Biol. 86 (2009) 803–812.
[4] C. Ehrhardt, R. Seyer, E.R. Hrincius, T. Eierhoff, T. Wolff, S. Ludwig, Interplay between influenza A virus and the innate immune signaling, Microbes Infect./Institut Pasteur 12 (2010) 81–87.
[5] C. Simmons, J. Farrar, Insights into inflammation and influenza, N. Engl. J. Med. 359 (2008) 1621–1623.
[6] J.M. Song, K.H. Lee, B.L. Seong, Antiviral effect of catechins in green tea on influenza virus, Antivir. Res. 68 (2005) 66–74.
[7] P. Gaur, A. Munjhal, S.K. Lal, Influenza virus and cell signaling pathways. Medical science monitor, Int. Med. J. Exp. Clin. Res. 17 (2011). RA148–54.
[8] Ludwig, S. Influenza viruses and MAP kinase cascades – novel targets for an antiviral intervention ? Signal Transduct. 7 (2007) 81–88.
[9] E. Haasbach, C. Hartmayer, O. Planz, Combination of MEK inhibitors and oseltamivir leads to synergistic antiviral effects after influenza A virus infection in vitro, Antivir. Res. 98 (2013) 319–324.
[10] K. Droebner, S. Pleschka, S. Ludwig, O. Planz, Antiviral activity of the MEK-inhibitor U0126 against pandemic H1N1v and highly pathogenic avian influenza virus in vitro and in vivo, Antivir. Res. 92 (2011) 195–203.
[11] C. Ehrhardt, H. Marjuki, T. Wolff, B. Nurnberg, O. Planz, S. Pleschka, et al., Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence, Cell. Microbiol. 8 (2006) 1336–1348.
[12] E. Haasbach, E.K. Pauli, R. Spranger, D. Mitzner, U. Schubert, R. Kircheis, et al., Antiviral activity of the proteasome inhibitor VL-01 against influenza A viruses, Antivir. Res. 91 (2011) 304–313.
[13] N. Kumar, Z.T. Xin, Y. Liang, H. Ly, Y. Liang, NF-kappaB signaling differentially regulates influenza virus RNA synthesis, J. Virol. 82 (2008) 9880–9889.
[14] S. Ludwig, O. Planz, S. Pleschka, T. Wolff, Influenza-virus-induced signaling cascades: targets for antiviral therapy? Trends Mol. Med. 9 (2003) 46–52.
[15] J. Xie, S. Zhang, Y. Hu, D. Li, J. Cui, J. Xue, et al., Regulatory roles of c-jun in H5N1 influenza virus replication and host inflammation, Biochimica et biophysica acta 1842 (2014) 2479–2488.
[16] S. Zhang, H. Tian, J. Cui, J. Xiao, M. Wang, Y. Hu, The c-Jun N-terminal kinase (JNK) is involved in H5N1 influenza A virus RNA and protein synthesis, Arhiv. Virol. 161 (2016) 345–351.
[17] S. Ludwig, C. Ehrhardt, E.R. Neumeier, M. Kracht, U.R. Rapp, S. Pleschka, Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway, J. Biol. Chem. 276 (2001) 10990–10998.
[18] B. Maurer, E. Serfling, V. ter Meulen, A. Rethwilm, Transcription factor AP-1 modulates the activity of the human foamy virus long terminal repeat, J. Virol. 65 (1991) 6353–6357.
[19] R.G. Fahmy, A. Waldman, G. Zhang, A. Mitchell, N. Tedla, H. Cai, et al., Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun, Nat. Biotechnol. 24 (2006) 856–863.
[20] M.A. Rameix-Welti, V. Enouf, F. Cuvelier, P. Jeannin, S. van der Werf, Enzymatic properties of the neuraminidase of seasonal H1N1 influenza viruses provide insights for the emergence of natural resistance to oseltamivir, PLoS Pathog. 4 (2008) e1000103.
[21] S. Schubert, D.C. Gul, H.P. Grunert, H. Zeichhardt, V.A. Erdmann, J. Kurreck, RNA cleaving '10-23' DNAzymes with enhanced stability and activity, Nucleic acids Res. 31 (2003) 5982–5992.
[22] G. Zhang, C.R. Dass, E. Sumithran, N. Di Girolamo, L.Q. Sun, L.M. Khachigian, Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents, J. Natl. Cancer Inst. 96 (2004) 683–696.
[23] W.B. Dreitlein, J. Maratos, J. Brocavich, Zanamivir and oseltamivir: two new options for the treatment and prevention of influenza, Clin. Ther. 23 (2001) 327–355.
[24] J.L. McKimm-Breschkin, P.W. Selleck, T.B. Usman, M.A. Johnson, Reduced sensitivity of influenza A (H5N1) to oseltamivir, Emerg. Infect. Dis. 13 (2007) 1354–1357.
[25] T.G. Sheu, V.M. Deyde, M. Okomo-Adhiambo, R.J. Garten, X. Xu, R.A. Bright, et al., Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008, Antimicrob. agents Chemother. 52 (2008) 3284–3292.
[26] A. Meijer, A. Lackenby, O. Hungnes, B. Lina, S. van-der-Werf, B. Schweiger, et al., Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007-08 season,

Emerg. Infect. Dis. 15 (2009) 552–560.
[27] N. Kumar, Y. Liang, T.G. Parslow, Y. Liang, Receptor tyrosine kinase inhibitors block multiple steps of influenza a virus replication, J. Virol. 85 (2011) 2818–2827.
[28] D. Conze, T. Krahl, N. Kennedy, L. Weiss, J. Lumsden, P. Hess, et al., c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation, J. Exp. Med. 195 (2002) 811–823.
[29] K. Sabapathy, T. Kallunki, J.P. David, I. Graef, M. Karin, E.F. Wagner, c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation, J. Exp. Med. 193 (2001) 317–328.