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Targeted disruption of influenza A virus hemagglutinin in genetically modified mice reduces viral replication and improves disease outcome

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Influenza A virus can cause acute respiratory infection in animals and humans around the globe, and is still a major threat to animal husbandry and public health. Due to antigenic drift and antigenic shift of the virus, development of novel anti-influenza strategies has become an urgent task. Here we generated transgenic (TG) mice stably expressing a short-hairpin RNA specifically targeting hemagglutinin (HA) of influenza A virus, and investigated the susceptibility of the mice to influenza virus infection. We found that HA expression was dramatically disrupted in TG mice infected with WSN or PR8 virus. Importantly, the animals showed reduced virus production in lungs, slower weight loss, attenuated acute organ injury and consequently increased survival rates as compared to wild type (WT) mice after the viral infection. Moreover, TG mice exhibited a normal level of white blood cells following the virus infection, whereas the number of these cells was significantly decreased in WT mice with same challenge. Together, these experiments demonstrate that the TG mice are less permissive for influenza virus replication, and suggest that shRNA-based efficient disruption of viral gene expression in animals may be a useful strategy for prevention and control of a viral zoonosis.

Influenza viruses are negative-sense, single-stranded, segmented RNA viruses, and can be categorized into three types: A, B, and C¹. Influenza A viruses are further divided into subtypes based on surface proteins called hemagglutinin (HA) and neuraminidase (NA). There are 16 known HA and 9 known NA subtypes². The diversity of influenza A viruses make them have multiple hosts including humans and various animals, such as pigs, chickens, quail, ducks, and turkeys. Influenza A virus can cause highly pathogenic respiratory diseases in animals and humans, leading to enormous morbidity and economic loss annually in the world. In addition, the viruses undergo gradual, continuous change (antigenic drift) and dramatic, abrupt change (antigenic shift), which result in broad epidemic and occasionally pandemic occurrence^{3–5}.

To control influenza epidemic and pandemic, various vaccines and antiviral drugs have been developed and recognized for their role in mitigating the health impact of the viral infection⁶. However, despite intensive efforts, there are still restrictions for both strategies to prevent and control influenza A virus^{7,8}. The presence of frequent mutation and occasional reassortment significantly increases the difficulty in control of these viruses. For example, antibodies produced to influenza A virus as a result of infection or vaccination with earlier strains may not be protective against viruses circulating in later years. These necessitate frequent updating of influenza vaccine components to ensure that the vaccine is matched to circulating viruses. However, antigenic alterations of the virus due to evolutionary shift and drift are notoriously unpredictable. Thus, vaccines are unable to provide immediate protection against outbreaks of unexpected influenza virus strains⁹. The protective capability of currently available influenza vaccines is therefore substantially limited.

Development of antiviral drugs with activity against influenza A viruses is another important strategy in the control of the viral infection. Several antiviral drugs in two classes are currently approved for clinical use: the

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adamantanes-amantadine and rimantadine, and the neuraminidase inhibitors-oseltamivir and zanamivir^{10,11}. However, only drugs in second class such as oseltamivir and zanamivir are currently recommended for clinical rational use due to high levels of influenza virus resistance to adamantanes among circulating influenza A virus strains^{12,13}. Dismayingly, more and more studies revealed that resistance of influenza viruses to oseltamivir and zanamivir is being enhanced^{14–17}. Therefore, novel strategies are needed for preventing ongoing influenza infection.

RNA interference (RNAi) is a process by which double-stranded RNA directs selective silencing of genes with homology to the double strand¹⁸. Small interfering RNAs (siRNAs) containing 21–26 nucleotide (nt) are mediators of RNAi¹⁹. Numerous studies have shown that siRNA can significantly suppress viral gene expression and thus protect the host from their infection when delivered into cells *in vitro* or mice *in vivo*^{20–27}. Importantly, there is compelling evidence indicating that RNAi is critical in curtailing influenza virus infection, which implies a promising approach to treat infection by influenza A virus^{28,29}. However, this approach has currently limited applications because it can not create a long-term silence of viral genes *in vivo*³⁰.

In this study, we constructed an expression vector capable of stably expressing short hairpin RNA (shRNA) that specifically targets conserved sequences of influenza A virus HA, a critical component for the viral infection and replication. The vector was then integrated into mouse genome, which generated the genetically modified mice that stably express the specific shRNA. We have found that HA expression was dramatically disrupted in the transgenic (TG) mice after influenza A virus infection. Furthermore, the resulting progeny of TG mice exhibited stable capacity to reduce influenza A virus replication and disease severity. These results establish an effective strategy for prophylaxis of influenza virus infection. This approach overcomes many of the shortcomings previously experienced, and may be applicable to farm animals that are hosts of influenza virus.

Results

Construction of shRNA-based efficient disruption of influenza A virus HA expression *in vitro*.

Influenza A virus contains a segmented RNA genome. Two of the eight RNA segments encode the major surface glycoproteins: HA and NA. HA is responsible for virus attachment to the receptor of host cells to initiate an infection and entry of the viral genome into the target cell, which are critical for virus replication cycle³¹. Thus, we selected HA as a target gene. To effectively silence different subtypes of HA, we sought to design shRNAs targeting conserved sequences of influenza A virus HA. As shown in Supplementary Tables S1 and S2, although there are variations in the highlighted sequences among HA subtypes, they are highly conserved in H1 and H5 subtypes. Therefore, these sequences were selected as targets of shRNAs. Two shRNAs were synthesized and cloned into pSIH-H1-GFP vector respectively. To test the interference efficiency, A549 cell lines stably expressing shRNAs targeting HA (sh-HA-1 or sh-HA-2) or luciferase control (sh-Luc) were generated. These cell lines were then infected with WSN virus and harvested at 8h and 16h post infection, followed by analysis of Western blot. We found that A549 cells expressing either HA-specific shRNA exhibited a significantly reduced HA expression as compared with that of control cells. Remarkably, sh-HA-1 had better interference efficiency than sh-HA-2 (Fig. 1a,b). To further determine the effect of sh-HA-1 on HA expression, the cell lines were infected with PR8 virus. Similarly, expression of PR8 virus HA was dramatically disrupted by the shRNA (Fig. 1c,d). We also found that viral NP expression was clearly reduced in A549 cells expressing HA-specific shRNA when they were infected with low multiplicity of infection (MOI) of the virus, while NP levels were only slightly decreased when infected with high virus MOI (Fig. S1 and Fig. 1a). These experiments demonstrated that shRNA sh-HA-1 could effectively silence both WSN HA and PR8 HA *in vitro*, and thus was used in further studies.

Generation of TG mice stably expressing shRNA that effectively silences HA of influenza A virus.

Since the shRNA sh-HA-1 could deplete HA of influenza A virus *in vitro*, we next generated TG mice using shRNA expression vector that carries sh-HA-1 sequences located downstream of an H1 promoter (Fig. 2a). Three lines of transgenic BALB/c mice that express the specific shRNA were developed. The progeny of these TG mice were genotyped by the PCR with 2 pairs of primers, which exhibited different bands of 350 bp (Primer pair 1) and 373 bp (Primer pair 2) (Fig. 2b). Statistical analysis showed that the ratio of positive generations were about 50%, which was consistent with the expected Mendelian frequency. To test the interference efficiency of shRNA targeting influenza A virus HA, six week-old TG mice and WT littermates were intranasally infected by WSN virus or PR8 virus. Then the viral HA expression in lungs were examined by Western blotting. Consistent with the *in vitro* studies, HA expression was markedly knocked down in TG mice as compared with that of WT mice after infection with either WSN virus (Fig. 2c,d) or PR8 virus (Fig. 2e,f). These experiments provide strong evidence that the expression of influenza A virus HA can be effectively disrupted in the genetically modified mice.

Targeted disruption of influenza virus HA in TG mice causes significantly enhanced resistance of the animals to the virus infection.

Since the generated TG mice could significantly decrease the viral HA expression after the infection, we next determined whether silencing HA by the shRNA could protect the TG mice against challenge with influenza A virus. To this end, TG mice and WT littermates were infected with WSN virus, and their resistance to the virus was compared. Interestingly, we found that WT mice infected with WSN virus had severe flu symptoms, as evidenced by reduced activity, ruffled fur and difficult breathing, whereas only mild flu symptoms were observed in TG mice (Fig. 3a). In addition, WT mice displayed a faster body weight loss than TG littermates during the viral infection (Fig. 3b). Moreover, the survival rate of infected TG mice was increased as compared to WT animals. As shown in Fig. 3c, all WT mice were died within 10 days after infection, whereas approximately 33% of TG littermates remained alive, and about 20% of the TG mice finally survived. Statistical analysis showed that TG mice survived significantly longer than WT mice after WSN infection ($P < 0.05$). Furthermore, viral loads were evaluated in the lungs of the infected mice. As expected, the viral titers in lungs of TG mice were significantly reduced as compared to that of WT mice (Fig. 3d and Fig. S2).

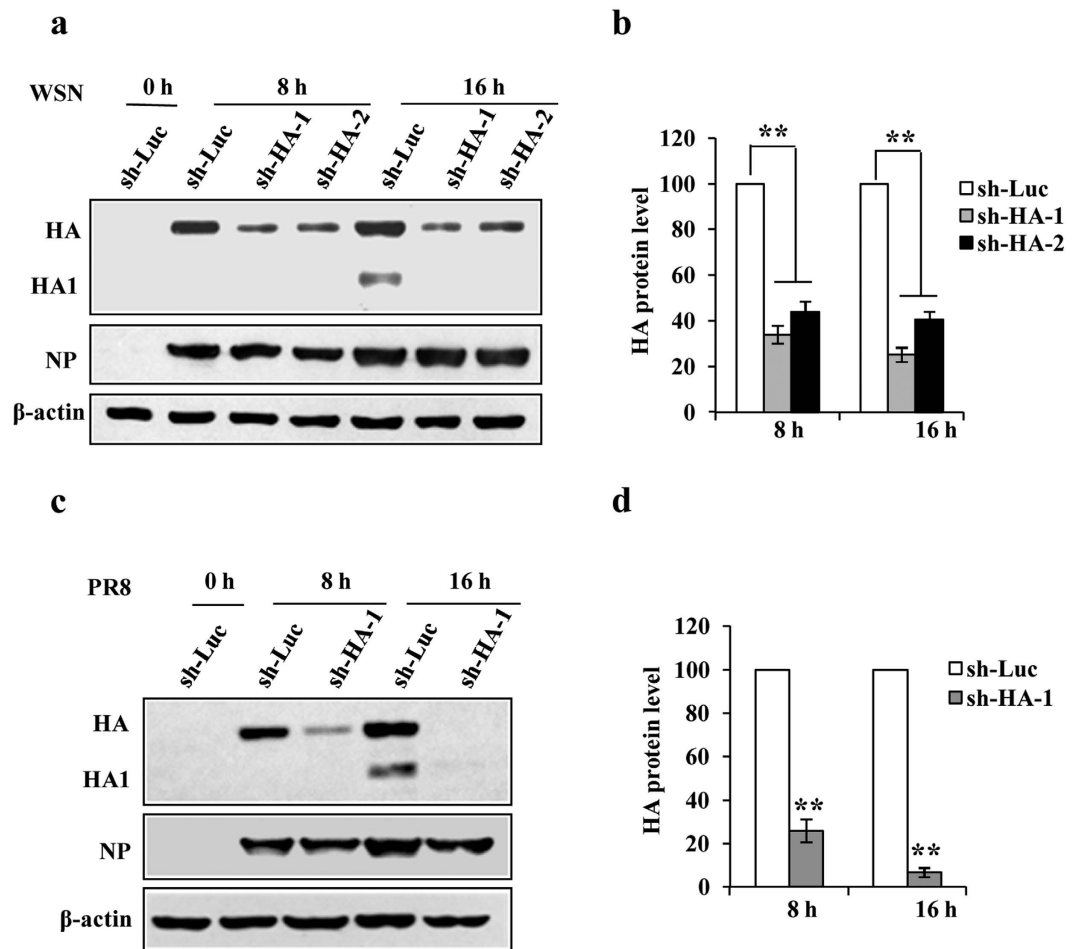


Figure 1. shRNA-based efficient disruption of influenza A virus HA expression in A549 cells. (a) A549 cells stably expressing shRNAs targeting either HA (sh-HA-1 and sh-HA-2) or luciferase control (sh-Luc) were infected with WSN virus (MOI = 1) and harvested at the indicated times, followed by Western blotting using the indicated antibodies. These data are representative of three independent experiments with similar results. (b) The HA levels shown in panel (a) were quantitated by densitometry and normalized to β -actin levels. In each experiment, the HA level in A549 cells expressing sh-Luc is 100. Plotted are the average levels from three independent experiments. ** $P = 0.001275$, sh-Luc vs. sh-HA-1 at 8 h; ** $P = 0.003938$, sh-Luc vs. sh-HA-2 at 8 h; ** $P = 0.001094$, sh-Luc vs. sh-HA-1 at 16 h; ** $P = 0.002148$, sh-Luc vs. sh-HA-2 at 16 h. (c) A549 cells stably expressing sh-HA-1 or sh-Luc were infected with PR8 virus (MOI = 1) and harvested at the indicated times, followed by Western blotting with the indicated antibodies. Shown are representative immunoblots from three independent experiments with similar results. (d) The HA levels shown in panel (c) were quantitated as described in (b). In each experiment, the HA level in A549 cells expressing sh-Luc is 100. ** $P = 0.002921$, sh-Luc vs. sh-HA-1 at 8 h; ** $P = 0.000326$, sh-Luc vs. sh-HA-1 at 16 h.

To further confirm that disruption of HA expression in TG mice has significant effect on susceptibility of the animals to infection with influenza virus, we used PR8 virus to repeat the experiments presented above. Consistent with the observations in experiments using WSN virus, TG mice showed enhanced resistance to PR8 virus infection, as indicated by mild flu symptoms (Fig. 4a), lower weight loss (Fig. 4b), increased survival rate (Fig. 4c), and significantly reduced viral loads in lungs as compared with those of WT littermates under same experimental condition (Fig. 4d). Together, these data indicate that targeted disruption of the HA in the animals provides a protection against lethal infection with the H1N1 influenza virus.

Targeted disruption of influenza virus HA in TG mice causes attenuated acute organ injury and reduced pathological changes of organs during the viral infection.

Previous studies have suggested that destructive effects of highly virulent influenza A virus on host organs may be one crucial factor that contributes to the fatal diseases in mammals³². To further verify the protective efficacy of silencing HA in TG mice against influenza virus, the pathological changes of mouse organs were therefore examined after infection with WSN or PR8 virus. Strikingly, infection of mice with influenza A virus resulted in acute organ injury, including severe lung lesions and severe spleen and thymus atrophy observed in WT mice, whereas these changes were mild in TG mice (Fig. S3). Moreover, haematoxylin and eosin (HE) staining of the lungs showed that abundant

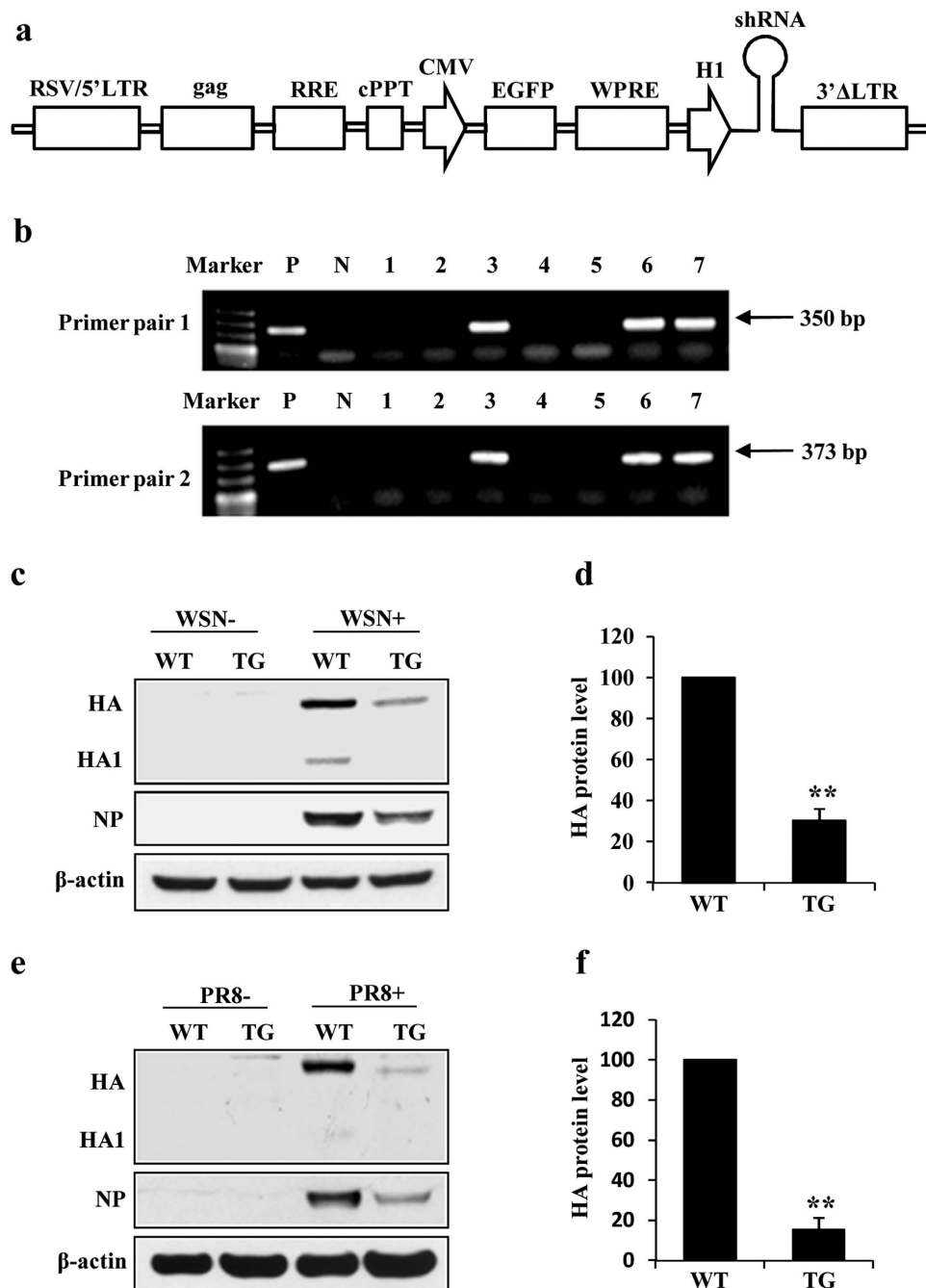


Figure 2. Transgenic (TG) mice expressing shRNA specific for HA can remarkably inhibit HA expression *in vivo*. (a) Schematic diagram of shRNA expression vector (pSIH-H1-GFP) used in this study. Transcription of shRNA is driven by the H1 promoter. EGFP expression is driven by the cytomegalovirus (CMV) promoter. RSV/5'LTR, gag, RRE, cPPT, WPRE and 3'ΔLTR are lentivirus components. (b) The TG mice expressing shRNA targeting HA were genotyped by PCR using mouse tail DNA as a template and two primer pairs. Shown is representative genotyping of TG and wild type (WT) mice. Numbers 1–7, representative TG mice and WT littermates; P, positive control; N, negative control. (c) WT and TG mice intranasally infected with WSN virus for 4 days were sacrificed, and the lungs were homogenized, followed by Western blotting with the indicated antibodies. Shown are representative immunoblots from three experiments with similar results. (d) The HA levels shown in panel (c) were quantitated by densitometry and normalized to β-actin levels. In each experiment, the HA level in WSN infected WT mice is 100. **P = 0.003894. (e) WT and TG mice infected with PR8 virus were treated as described in (c). Western blotting was performed using the indicated antibodies. The results are representative of three independent experiments. (f) The HA levels shown in panel (e) were quantitated as described in (d). In each experiment, the HA level in PR8 infected WT mice is 100. **P = 0.002828.

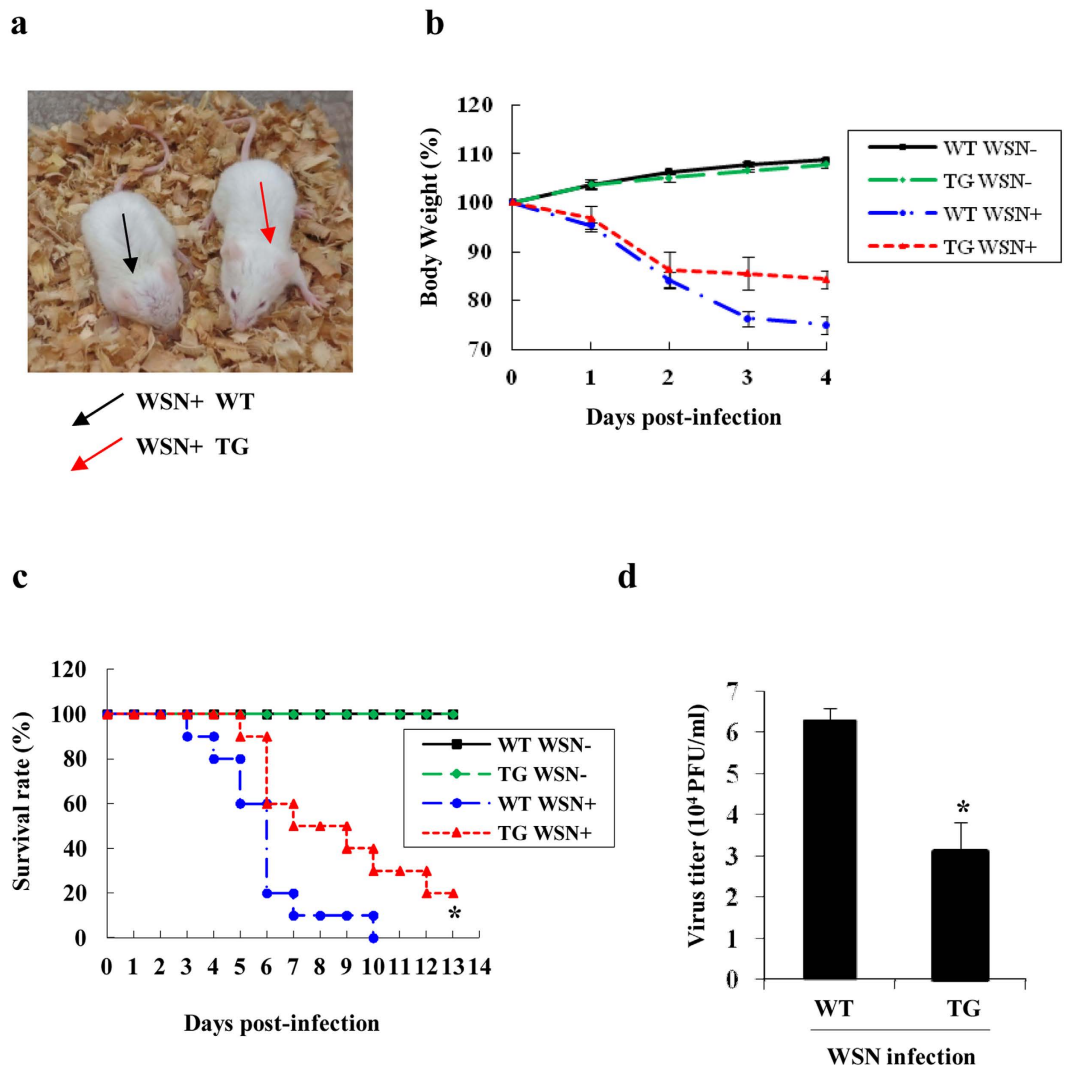


Figure 3. TG mice are resistant to infection with WSN virus as compared to WT mice. (a) WT and TG mice were intranasally infected with WSN virus (5×10^4 PFU/mouse) for 4 days. Shown is a representative photograph of the general appearance of the virus infected mice. (b) Shown is the body weight changes of WT and TG mice mock infected or infected intranasally with WSN virus. The results are shown as mean percentage weight changes from three independent experiments. (c) Survival rate of WT mice (n = 15) and TG mice (n = 15) infected intranasally with WSN virus. Mice were monitored for up to 13 days. During this period, mice were sacrificed when they displayed severe unrelieved distress, hind limb paralysis or excessive weight loss (25% weight loss from initial body weight). Survival curves were compared using a log-rank test (GraphPad Prism 5). * $P = 0.0225$, WSN infected WT mice vs. WSN infected TG mice. (d) Viral loads in the lungs of WT and TG mice infected with WSN virus for 4 days were measured by plaque assay. * $P = 0.015885$.

inflammatory cells were present in the alveoli and diffuse in the peribronchiolar and perivascular regions in the lungs of infected WT mice, whereas only a small number of inflammatory cells were seen in the alveoli of TG mice after same infection (Fig. 5). Spleens of infected WT mice showed a dramatic decrease in lymphoid nodule size of white pulps, while this pathological change in infected TG mice was mild (Fig. S4a,b). Likewise, there was a more severe cortical atrophy in the thymus of WT mice than that of TG mice (Fig. S4c,d), which was likely due to a marked loss of lymphocytes during the viral infection³³.

Because previous studies have shown that low lung index, high spleen index and thymus index correlated well with strong protection against the virus infection³⁴, we next evaluated the lung index, spleen index and thymus index. As shown in Fig. 6, TG mice exhibited obviously lower lung index (Fig. 6a,b), higher spleen index (Fig. 6c,d) and thymus index (Fig. 6e,f) than WT mice following a challenge with either WSN virus or PR8 virus. These data demonstrate that silencing influenza virus HA in genetically modified mice leads to attenuated acute organ injury and reduced their pathological changes during the viral infection.

TG mice can maintain normal levels of white blood cells after challenge with influenza A virus. The innate and adaptive immune responses to viral infection mediated by immune cells are rapid and

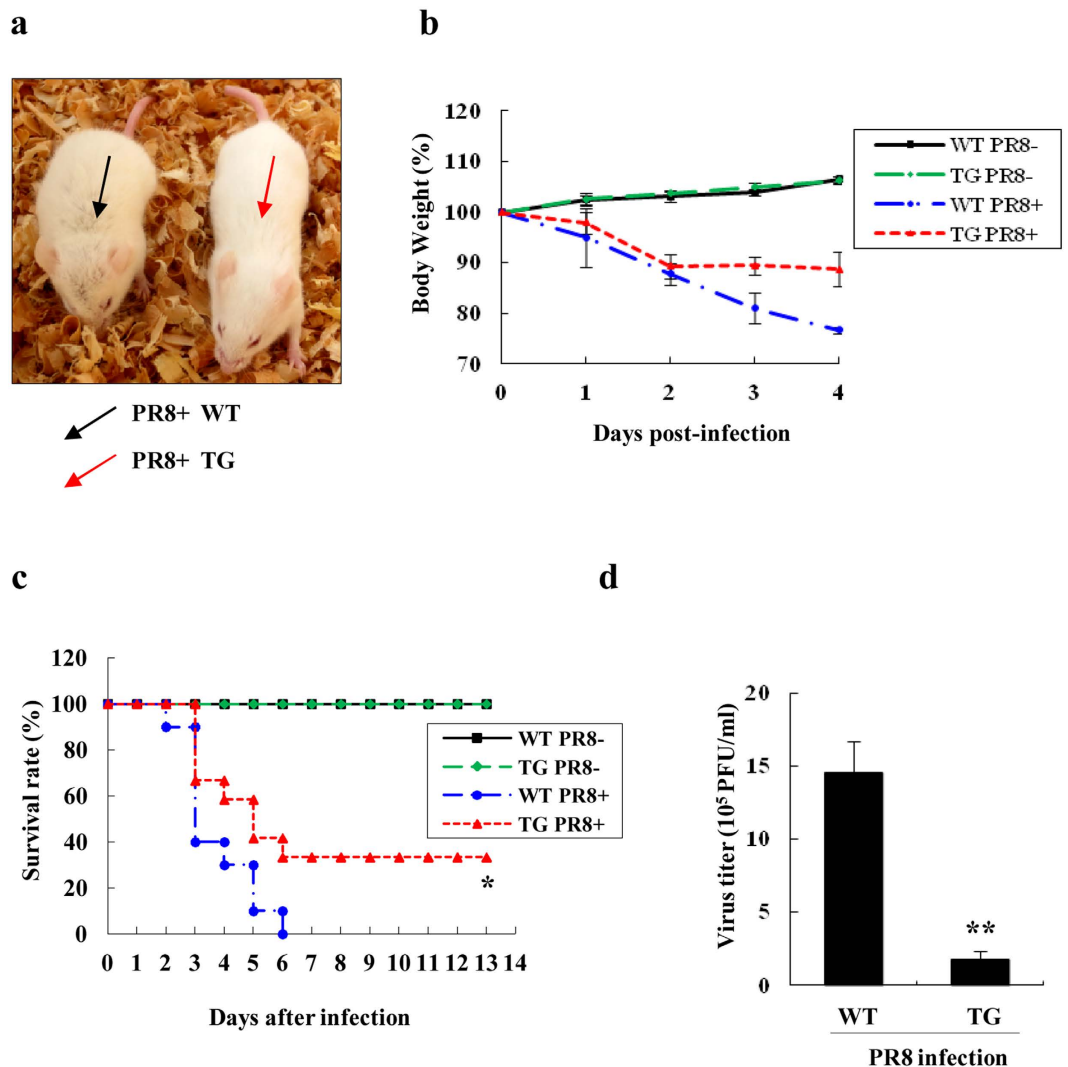


Figure 4. TG mice display resistant to PR8 virus infection compared with WT mice. (a) WT and TG mice were intranasally infected with PR8 virus as described in Fig. 3a. Shown is a representative photograph of the general appearance of the virus infected mice. (b) Shown is the body weight changes of WT and TG mice mock infected or infected intranasally with PR8 virus. The results are shown as mean percentage weight changes from three independent experiments. (c) Survival rate of WT mice (n = 15) and TG mice (n = 15) infected intranasally with PR8 virus was tested as described in Fig. 3c. Survival curves were compared using a log-rank test (GraphPad Prism 5). *P = 0.0356, PR8 infected WT mice vs. PR8 infected TG mice. (d) Viral loads in the lungs of WT and TG mice infected with PR8 virus were measured as described in Fig. 3d. **P = 0.007122.

specific, resulting in viral clearance and establishment of immune memory^{35,36}. To circumvent the host immunity, influenza A viruses have evolved multiple strategies, such as inducing a severe inflammatory response with immune-related complications including significantly reduced numbers of immune cells^{32,33}. Therefore, we determined whether stable expression of shRNA targeting influenza virus HA in mice had any effect on the numbers of white blood cells in mouse peripheral blood after the virus infection. Indeed, we observed that infection with either WSN virus or PR8 virus resulted in a significant decrease in white blood cells of WT mice (Fig. 7). However, white blood cells in TG mice were close to the normal level after same challenge with the viruses (Fig. 7). Taken together, these results reveal that targeted silence of influenza virus HA in genetically modified mice can effectively mitigate the reduction of white blood cells normally occurring during viral infection.

Discussion

Influenza virus is still considered to be a global threat and will continue to pose challenges to public health because of its easy transmission, antigenic shift and drift, and the limited efficacy of current vaccines and antiviral drugs³⁷. Therefore, development of novel anti-influenza strategies is critically required for counteracting the emergence of highly virulent influenza A virus. Previous studies have shown that shRNA expressed in transgenic chickens can function as a decoy, inhibit influenza virus polymerase and hence interfere with virus propagation³⁸. Other experiments have also demonstrated that influenza viral mRNA is direct target of siRNA-mediated

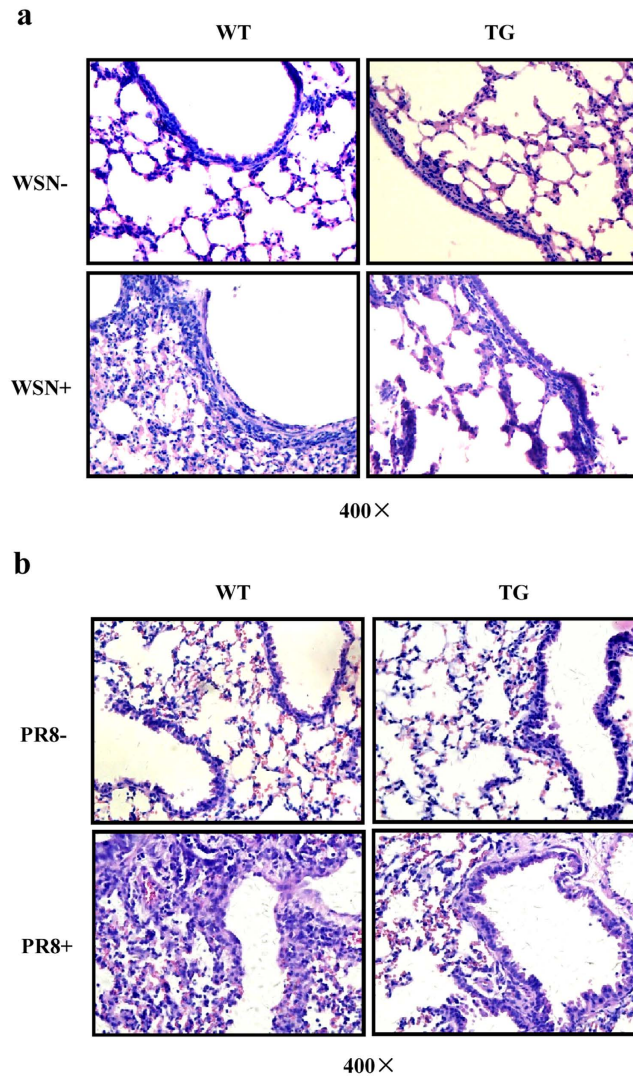


Figure 5. The pathological changes in lungs of influenza virus infected WT and TG mice. (a,b) WT and TG mice were intranasally infected with WSN (a) or PR8 (b) virus. Shown are representative micrographs (magnification, $\times 400$) of the mouse lungs stained with hematoxylin and eosin (HE).

interference, and viral genomic RNA (vRNA) and complementary RNA (cRNA) accumulation can be inhibited indirectly²². In this study, we generated TG mice stably expressing shRNA that causes the animals reduced susceptibility to influenza virus infection through disrupting expression of viral HA. Moreover, we also determined ability of the genetically engineered mice to pass influenza virus to other mice. We observed that mice housed with infected WT mice showed clearly loss of body weight, while the mice housed with infected TG mice showed an increase of body weight during 5 days housing (Fig. S5), suggesting a reduced ability of TG mice in transmission of influenza virus. This property could have a major impact on susceptibility and propagation of infection at the flock level, even though the survival rate of mice primarily challenged with a high dose of influenza virus was less than 40%. Importantly, the three lines of TG mice in our study exhibited similar capacity to reduce influenza A virus replication and disease severity. The results presented above are representative of those obtained from three lines. Together, these findings have significant implications that genetic modification resistant to influenza virus infection can be developed not only in avian, but also in mammals.

Previous studies have raised some concerns on the safety of genetic modification^{39–43}. For example, adverse effects derived from RNA interference were observed *in vivo*, such as progressive weight loss, motor dysfunction and animal demise⁴⁰. Therefore, it is important to examine any potential hazards derived from genetic modification. In this study, at least three founder lines of the TG mice expressing shRNA targeting influenza virus HA have been generated. There are no apparent ill-effects on uninfected transgenic animals as compared with the WT littermates. All these mice developed normally and phenotypically normal and fertile without affecting other genetic properties of these lines, even though they have been maintained on a BALB/c background for more than 3 years. In addition, HE staining showed that there was no significant histological difference between WT and TG mice without virus infection (Fig. S2). One possible explanation is that the designed shRNA targeting HA in this study did not share identity with any host genes and the genomic location of the shRNA integrated into

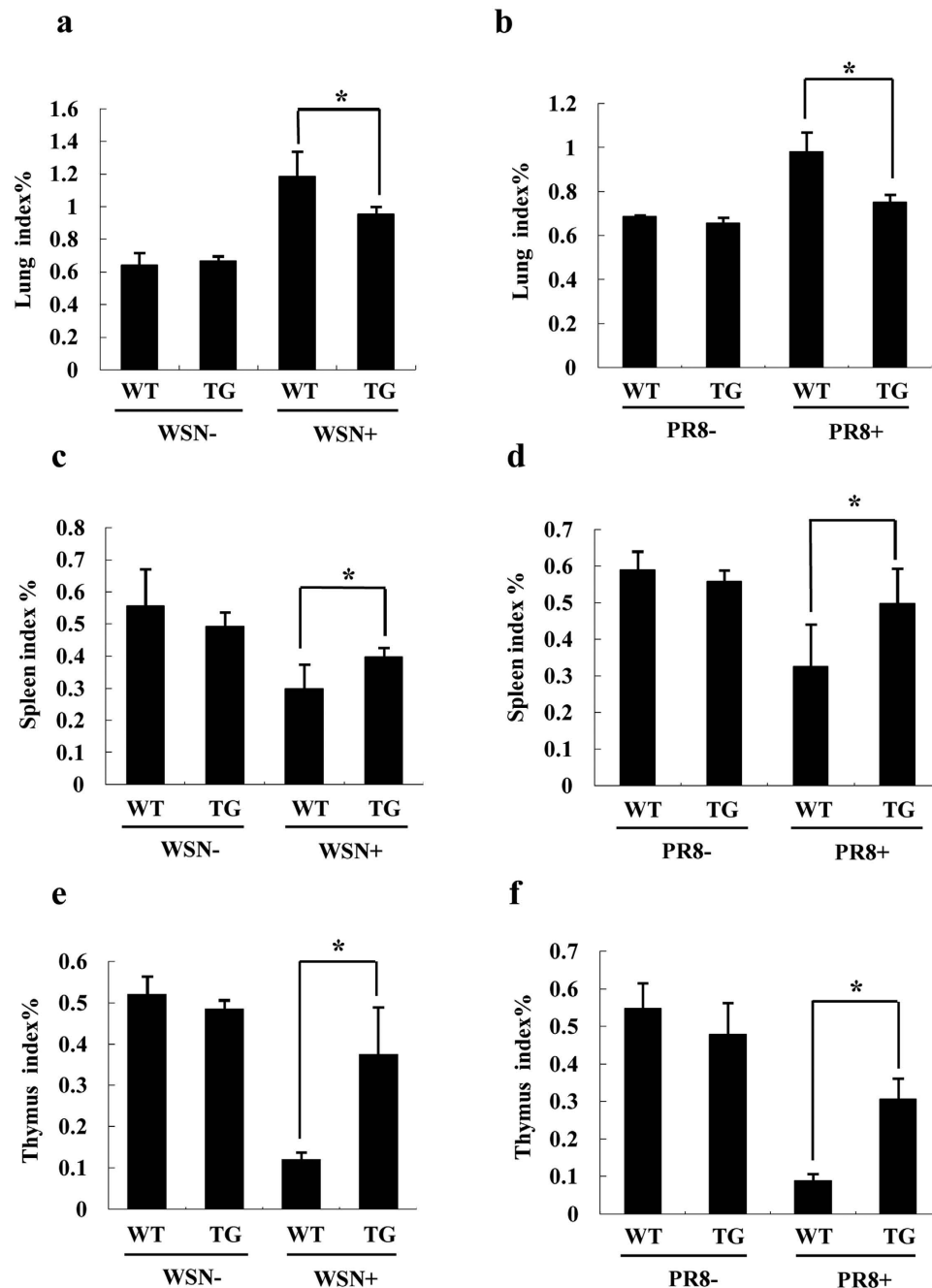


Figure 6. The lung, spleen and thymus index of WT and TG mice infected with influenza virus. (a,b) WT and TG mice were infected with WSN (a) or PR8 (b) virus (5×10^4 PFU/mouse) for 4 days. Then lung index was calculated. Lung index = lung weight (g)/body weight (g) \times 100%. (a) * $P = 0.032695$; (b) * $P = 0.017735$. (c,d) WT and TG mice were infected with WSN (c) or PR8 (d) virus (5×10^4 PFU/mouse) as described in (a,b). Then spleen index was calculated. Spleen index = spleen weight (g)/body weight (g) \times 100%. (c) * $P = 0.021756$; (d) * $P = 0.020536$. (e,f) WT and TG mice were infected with WSN (e) or PR8 (f) virus (5×10^4 PFU/mouse) as described in (a,b). Then thymus index was calculated. Thymus index = thymus weight (g)/body weight (g) \times 100%. (e) * $P = 0.037937$; (f) * $P = 0.013413$.

mouse chromosome was unlikely to present a risk to the host. However, whether the transgene has any effect on susceptibility of the TG animals to other pathogens requires further studies.

Influenza virus HA, the principal antigen on the viral surface, is the primary target for neutralizing antibodies and is responsible for viral binding to host receptors, and subsequent membrane fusion^{44,45}. Now it is generally accepted that HA of human influenza viruses prefer to bind to α 2-6 sialic acids and HA of avian influenza viruses prefer to bind to α 2-3 sialic acids^{46,47}. The α 2-6 sialic acids and α 2-3 sialic acids receptors are dominantly expressed in humans and in avian respectively⁴⁸, while both of the receptors are reported to be present in pigs⁴⁹.

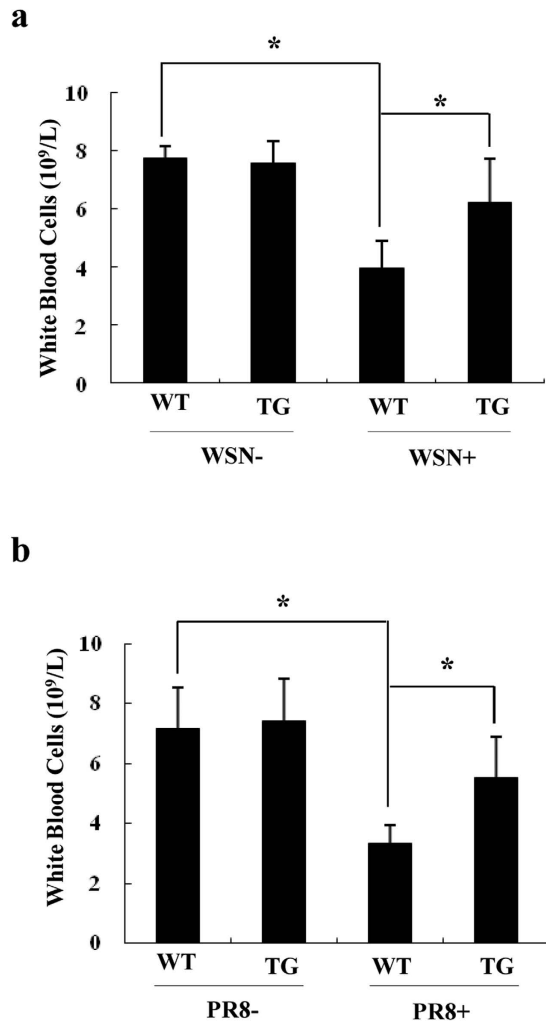


Figure 7. TG mice maintain normal levels of white blood cells after influenza virus infection.

(a,b) WT and TG mice were infected with WSN (a) or PR8 (b) virus (5×10^4 PFU/mouse) for 4 days. Then mice were sacrificed, and number of white blood cells in mouse peripheral blood was analyzed by blood routine examination. (a) * $P = 0.019339$, uninfected WT mice vs. infected WT mice; * $P = 0.044106$, infected WT mice vs. infected TG mice. (b) * $P = 0.020983$, uninfected WT mice vs. infected WT mice; * $P = 0.044035$, infected WT mice vs. infected TG mice.

Based on this, pigs are often considered as mixing vessels for the recombination of human and avian influenza viruses, providing the conditions for gene recombination of influenza viruses to create a new subtype⁵⁰. Therefore, if we could use the technology to develop transgenic pigs that have decreased susceptibility to influenza virus infection, the ability of influenza viruses to cross host species barrier from avian to humans can likely be inhibited to a certain extent. However, this issue remains to be further addressed.

Our approach is also technically applicable to other farm animals that are hosts of influenza A virus, such as chickens, quail, ducks, and turkeys. This approach causes TG animals reduced susceptibility to influenza virus infection and no adverse effects are seen in the host. Even though whether the produced food derived from transgenic farm animals is safe to consumers is still a controversial topic, the application of this technology will very likely bring benefits for the global breeding industry as well as public health, including prevention and control of viral zoonosis. In addition, genetically modified animals generated by using our approach would be useful models for studies of virus-host interaction. In particular, our approach is applicable to generation of animal models for determining function of key viral genes *in vivo* and understanding mechanisms underlying pathogenesis of threatening viruses.

Materials and Methods

Ethics statement. The animal protocol used in this study was approved by “the Regulation of College of Animal Sciences, Fujian Agriculture and Forestry University of Research Ethics Committee” (Permit Number PZCASFAFU2014001). All mouse experiments were carried out according to the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China.

Virus and Antibodies. Influenza virus strains A/WSN/33 (H1N1) and A/PR/8/34 (H1N1) were generated and propagated in specific-pathogen-free (SPF) chicken embryo as previously described⁵¹. The following antibodies were used in this study: anti- β -actin (Abcam, Cambridge, UK), anti-influenza A virus HA was kindly provided by Dr George F. Gao (Institute of Microbiology, CAS), and anti-influenza A virus NP was obtained as previously described^{51,52}.

Cell culture and virus infection. 293T, A549 and MDCK cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/ml streptomycin. For virus infection, cells were washed with phosphate-buffered saline (PBS) and infected with influenza virus at the indicated multiplicity of infection (MOI). After adsorption for 1 h at 37 °C, the cells were washed with PBS and cultured in DMEM containing 2 μ g/ml trypsin. *In vitro* cell infection experiments with WSN or PR8 viruses were performed under biosafety level 2 (BSL-2) laboratory conditions.

Cell extracts and Western blotting. Cell extracts were lysed in RIPA lysis buffer (Cell Signaling Technology; Beverly, MA, USA), according to the manufacturer's protocols. After adding 2 \times loading buffer, lysates were boiled for 5 min. Western blotting was performed as previously described^{51,52}. Briefly, samples were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with antibodies as indicated. Where indicated, immunoblotting signals were quantified by densitometry.

RNA interference and generation of stable cell lines. Short hairpin RNA (shRNA)-based knockdown cell lines were generated as described previously⁵³. The sequences used in the shRNAs targeting HA genes were as follows: HA shRNA-1: 5'-GGGAGGATGAACTATTACT-3' and HA shRNA-2: 5'-CATGGAAAGTGTAAGAAAT-3', and luciferase control shRNA as previously described⁵⁴.

Generation of HA-knockdown transgenic mice. HA-knockdown transgenic mice were generated by the microinjection method as previously described⁵⁵. The sequences used in the shRNA targeting HA were cloned into an expression plasmid under the H1 promoter. Then the plasmid was linearized by single enzyme digestion of Sca I, separated on 1% agarose gel in 1 \times TAE, and purified using the Qiaquick gel extraction kit (Qiagen). The resulting DNA preparations were injected into the pronucleus of fertilized zygotes harvested from BALB/c mice. Genotyping of transgenic mice was performed by the PCR using two pairs of primers: primer 1 forward, 5'-AAATCCTGGTTGCTGTCTCTTTATG-3', primer 1 reverse, 5'-GGAAGTCCGCTGGATTGA-3'; primer 2 forward, CGTCCAGGAGCGCACCATCTTCTT, primer 2 reverse, ATCGCGTCTCGTTGGGTCTTT. The HA-knockdown transgenic mice were bred in SPF conditions and maintained in a BALB/c background.

Mouse experiments. Female BALB/c mice (5–6 weeks old, 18–20 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). HA-knockdown transgenic mice were maintained in our animal facility. For virus infection, mice were anaesthetized and inoculated intranasally with 5×10^4 PFU of WSN or PR8 virus. At the indicated time, mice were euthanized and the lungs, spleens and thymuses were removed for further analysis by hematoxylin and eosin (H&E) staining, plaque assay, and Western blotting. Influenza virus infection of mice was carried out under enhanced BSL-2 (BSL-2+) conditions.

Histopathological analysis. Histopathological analysis was performed as described previously³². Mouse tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Thereafter, 5 μ m thick sections were cut from each block, affixed firmly to clean microscope slides, deparaffinized and stained with H&E using an H&E staining kit (Solarbio, Beijing, China). Sections were examined under an Olympus BH-2 microscope (Tokyo, Japan).

Plaque assay. MDCK cells were infected with serial dilutions of the viruses. After an incubation period, cells were washed with PBS and overlaid with DMEM containing 1.5% low melting point agarose (Promega, Madison, WI) and 2 μ g/ml TPCK (tolylsulfonil phenylalanyl chloromethyl ketone)-treated trypsin (Sigma-Aldrich, St. Louis, MO). After 72 h of incubation at 37 °C, plaques were stained with 0.1605 mg/ml neutral red and number of plaques was counted.

Examination of lung virus titers. Lungs harvested from sacrificed mice were homogenized in 1 ml of ice cold PBS and frozen at -80 °C for 14 h. Thawed samples were centrifuged at 2,000 \times gravity for 10 min, and the supernatants were titrated by plaque assay as described above.

Statistical analysis. All data are presented as means \pm standard errors [SE]. Survival curves were analyzed using the log-rank test (GraphPad Prism 5). Other statistical analysis was performed by Student's *t* test. A level of $P < 0.05$ was considered statistically significant.

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Author Contributions

S.W., C.C. and J.L.C. conceived and designed the experiments. S.W., C.C., Z.Y., X.J.C. and J.Z. performed the experiments. S.W., X.J.C. and J.L.C. contributed to the writing of the manuscript. All authors reviewed the manuscript.

Additional Information

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