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ORIGINAL ARTICLE

Development of a dual-protective live attenuated vaccine against H5N1 and H9N2 avian influenza viruses by modifying the NS1 gene

Eun-hye Choi[1] · Min-Suk Song[1] · Su-Jin Park[1] · Philippe Noriel Q. Pascua[1] · Yun Hee Baek[1] · Hyeok-il Kwon[1] · Eun-Ha Kim[1] · Semi Kim[1] · Hyung-Kwan Jang[2] · Haryoung Poo[3] · Chul-Joong Kim[4] · Young Ki Choi[1]

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Abstract An increasing number of outbreaks of avian influenza H5N1 and H9N2 viruses in poultry have caused serious economic losses and raised concerns for human health due to the risk of zoonotic transmission. However, licensed H5N1 and H9N2 vaccines for animals and humans have not been developed. Thus, to develop a dual H5N1 and H9N2 live-attenuated influenza vaccine (LAIV), the HA and NA genes from a virulent mouse-adapted avian H5N2 (A/WB/Korea/ma81/06) virus and a recently isolated chicken H9N2 (A/CK/Korea/116/06) virus, respectively, were introduced into the A/Puerto Rico/8/34 backbone expressing truncated NS1 proteins (NS1-73, NS1-86, NS1-101, NS1-122) but still possessing a full-length NS gene. Two H5N2/NS1-LAIV viruses (H5N2/NS1-86 and H5N2/NS1-101) were highly attenuated compared with the full-length and remaining H5N2/NS-LAIV viruses in a mouse model. Furthermore, viruses containing NS1 modifications were found to induce more IFN-β activation than viruses with full-length NS1 proteins and were correspondingly attenuated in mice. Intranasal vaccination with a single dose ($10^{4.0}$ PFU/ml) of these viruses completely protected mice from a lethal challenge with the homologous A/WB/Korea/ma81/06 (H5N2), heterologous highly pathogenic A/EM/Korea/W149/06 (H5N1), and heterosubtypic highly virulent mouse-adapted H9N2 viruses. This study clearly demonstrates that the modified H5N2/NS1-LAIV viruses attenuated through the introduction of mutations in the NS1 coding region display characteristics that are desirable for live attenuated vaccines and hold potential as vaccine candidates for mammalian hosts.

Introduction

Avian influenza (AI) is a major respiratory disease of poultry caused by type A influenza viruses. These viruses are commonly found in aquatic wild birds, but they have also been isolated from animals of various species, including pigs, horses, dogs, sea mammals, and humans [54]. In recent years, the threat of a potential pandemic has been posed by highly pathogenic avian influenza (HPAI) H5N1 viruses; these viruses have been repeatedly isolated from wild birds and domestic poultry since 1996 [45]. The H5N1 virus was first identified in Southeast Asia and spread rapidly across Eurasia and Africa [42]. Notably, these viruses have also been isolated from pig populations in China, Indonesia, and Vietnam [5, 33, 44]. The spread of H5N1 is not only confined to animals but has also been found in humans. H5N1 infection has resulted in more than 600 human cases in 15 countries in Asia, Africa, the

E. Choi, M.-S. Song and S.-J. Park contributed equally to this work.

✉ Young Ki Choi
choiki55@chungbuk.ac.kr

[1] Department of Microbiology, College of Medicine and Medical Research Institute, Chungbuk National University, 12 Gaeshin-Dong Heungduk-Ku, Cheongju 361-763, Republic of Korea

[2] Departments of Infectious Diseases and Avian Diseases, College of Veterinary Medicine and Korea Zoonosis Research Institute, Chonbuk National University, Chonbuk, Republic of Korea

[3] Korean Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea

[4] College of Veterinary Medicine, Chungnam National University, 220 Gung-Dong, Yuseoung-Gu, Daejeon 305-764, Republic of Korea

Pacific, Europe, and the Middle East since November 2003, with a mortality rate of approximately 60 %. Although efficient human-to-human transmission of the H5N1 virus has not occurred, recent studies have demonstrated that a few substitutions can confer efficient transmissibility of the virus among ferrets, elevating concerns about the high potential for the next pandemic [18, 20]. The high mortality rate of H5N1 is an important public health concern, prompting the need to develop various approaches for dealing with H5N1 infections in humans. Similar to the H5N1 virus, the avian influenza H9N2 virus has also been considered a potential threat to human health. The H9N2 virus was first reported in the United states in 1966 [19]. Since then, the virus has been detected in species such as wild birds, poultry, pigs, and humans in geographically far-reaching countries [2, 21]. Moreover, the H9N2 virus has been proposed to be a donor virus for humans infected with avian influenza viruses such as H5N1 and H7N9, raising further concerns [14, 55]. Therefore, the H9N2 virus is also a considered to be a human influenza pathogen.

Stockpiling of an effective influenza vaccine is urgently needed in preparation for a possible influenza pandemic in humans. Because pandemics have the potential to spread rapidly through human populations across a large region, it is crucial for a pandemic vaccine candidate to be made available to the public on short notice. Cross-protectiveness of the pandemic vaccine is equally important because influenza viruses readily undergo antigenic drift and shift due to error-prone RNA polymerase activity or reassortment, producing escape mutants that may be resistant to subtype-specific vaccines. Since 2000, more than 10 phylogenetic clades of H5N1 viruses have already evolved based on their H5 HA genes [43, 46, 56]. Thus, the immunity conferred by a vaccine should be effective against heterologous viruses that are antigenically different from the original vaccine strain [15].

Breakthroughs in reverse genetics methods have provided the means to design live-attenuated influenza vaccines (LAIVs). LAIVs can mimic the course of natural infection, potentially providing superior protection by inducing both humoral and cross-reactive cell-mediated antibody responses [12, 13]. Other advantages of live viral vaccines are the potential ease of administration through intranasal application, induction of mucosal immunity, and cost effectiveness [48]. To date, various modified LAIVs have been created by manipulating previously identified molecular markers in different viral genes, resulting in viral attenuation while maintaining immunogenicity [36]. The influenza A virus nonstructural protein 1 (NS1) is a virulence factor with multiple functions in infected cells [16], and the eighth viral gene segment has been targeted for introducing attenuating mutations. NS1 includes two functional domains: an N-terminal (amino acids 1–73) RNA-binding domain that binds double-stranded RNA and a C-terminal (amino acids 74–230/237) effector domain that binds several host proteins. In addition to potentially controlling viral RNA replication [8] and viral protein synthesis [17], one of the major functions of the NS1 protein is the inhibition of host interferon (IFN) responses [10]. This can occur via inhibition of the IRF-3, NF-κB, and c-Jun/ATF-2 transcription factors [16, 48], possibly through prevention of intracellular sensing of viral single-stranded RNA by inhibition of RIG-I activation [9, 39]. The NS1 protein can also block the function of OAS1 and PKR [28, 29], inhibit host mRNA processing and activate the phosphatidylinositol 3-kinase (PI3K) pathway [16]. Thus, NS1 has the potential to influence multiple aspects of innate immune activation and apoptosis in infected host cells.

Accordingly, several human and animal influenza A viruses possessing various forms of modified NS1 proteins have been shown to be greatly attenuated while remaining immunogenic in poultry [47], mice [27, 31, 48], pigs [22, 41, 52], horses [3], macaques [1], and humans [53]. Using the reverse genetics method, we generated a panel of modified H5N2 LAIV vaccine candidates based on the A/Puerto Rico/8/34(H1N1) (PR8) virus containing H5 hemagglutinin (HA) and N2 neuraminidase (NA) genes derived from the A/WB/Korea/ma81/06(H5N2) (WB/ma81/H5N2) and A/CK/Korea/116/06(H9N2) (CK/116/H9N2) viruses, respectively. Additionally, viruses encoded either full-length NS1 (NS1-WT) or NS1 proteins truncated in the C-terminus at positions 73 (NS1-73), 86 (NS1-86), 101 (NS1-101), and 122 (NS1-122). Although the H5N2/NS1-LAIV viruses express truncated forms of NS1 protein, they still possess a full-length NS gene, minimizing loss of viral fitness. All recombinant H5N2/NS1-LAIV viruses grew to high titres in 10-day-old embryonated chicken eggs and in Madin-Darby canine kidney (MDCK) cells. Notably, the H5N2/NS1-86 and H5NS/NS1-101 viruses were significantly attenuated in mice, conferring complete protection in mice from challenge with virulent mouse-adapted H5N2 and H9N2 viruses. The high level of protection also extended to challenge with wild-type highly pathogenic avian influenza virus (HPAI) A/EM/Korea/W149/06(H5N1) (EM/149/H5N1). Therefore, the recombinant H5N2/NS1-LAIV viruses exhibited characteristics desirable for live attenuated vaccines and hold potential as vaccine candidates for mammalian hosts.

Materials and methods

Cell culture

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (EMEM) (Lonza) with 5 % fetal bovine serum (FBS) (Gibco). 293T human

embryonic kidney and A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 5 % FBS. All cells were maintained at 37 °C in 5 % CO_2 conditions.

Generation of recombinant H5N2/NS1 live attenuated influenza vaccine candidates

Recombinant influenza viruses were generated that contained one modified PR8-derived NS segment. The NS segments encoded unmodified nuclear export protein (NEP) and either full-length NS1 protein or C-terminal-truncated NS1 protein products created by adding three serial stop codons comprising amino acids 1-73, 1-86, 1-101, and 1-122 without any nucleotide deletions (Fig. 1A). The surface glycoprotein plasmids encoded the HA from A/WB/Korea/ma81/06 (H5N2) (WB/ma81/H5N2), which had been adapted in mice, and the NA segment from A/CK/Korea/116/03 (H9N2) (CK/116/H9N2). All recombinant viruses were rescued as described previously [37]. All rescued viruses were fully sequenced to ensure the absence of unwanted mutations. Virus stock titres were determined by plaque assay.

Plaque assay

H5N2/NS1-LAIV virus stocks were serially diluted tenfold in appropriate media. MDCK cells were infected with the diluted samples in six-well plates. After infection for 1 h, the cells were washed with PBS and overlaid with a 0.7 % agarose-medium mixture containing L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin. Then, the plates were incubated at 35 °C with 5 % CO_2. After incubation for 72 h, the overlays were removed and the cells were fixed with 10 % neutral buffered formaldehyde for 2 h. Finally, the plates were stained with 0.5 % crystal violet solution for 10 minutes.

Virus replication *in vitro*

MDCK cells were inoculated with recombinant viruses to determine the growth properties of each virus. The cells were grown in Eagle's minimum essential medium (EMEM) (LONZA) with 5 % fetal calf serum (Gibco) and 50 μg of penicillin/streptomycin (Gibco) per ml at 37 °C in 5 % CO_2. The cells were infected with each recombinant virus at a multiplicity of infection (MOI) of 0.001 in the

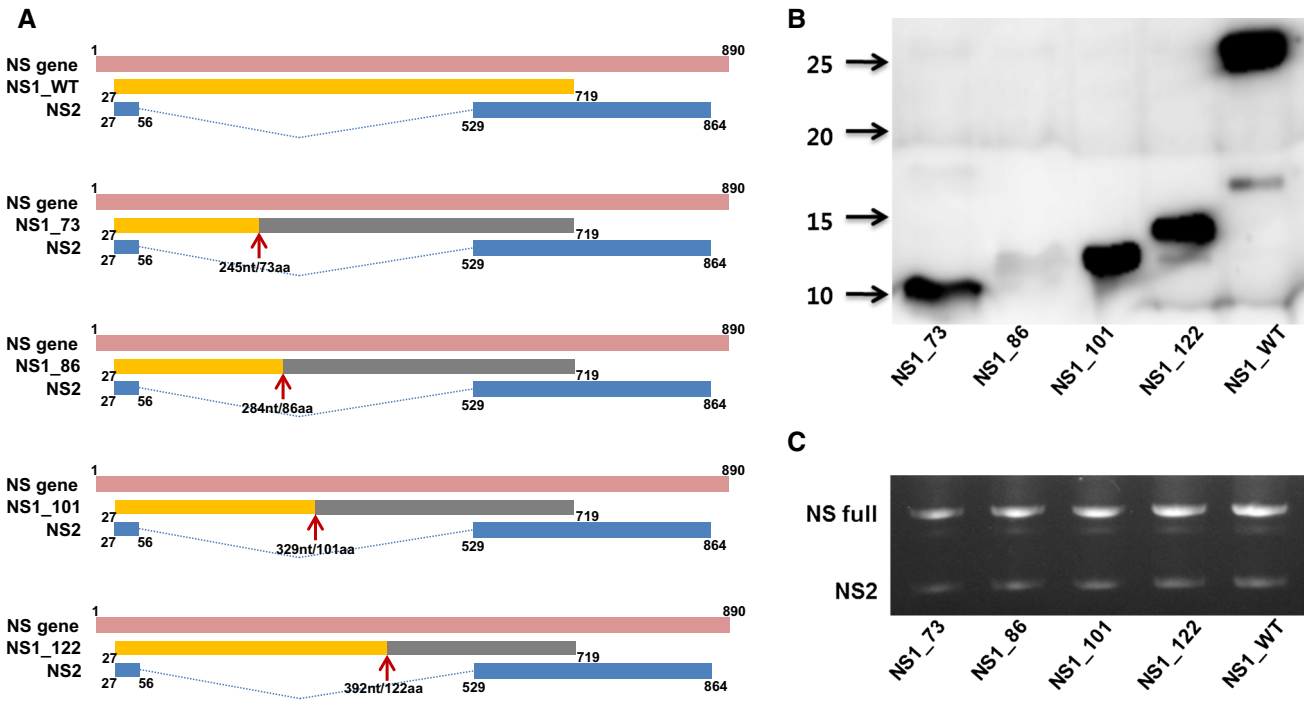

Fig. 1 Engineering H5N2/NS1-LAIV candidates and controls. (A) Schematic diagram of NS antigenomic RNA (positive sense, cRNA) depicting the wild-type RNA and the deletion NS mutants designed to create H5N2/NS1-LAIV candidates. The NS1 protein is directly translated from the full-length mRNA of PR8 shown above the gene; the NEP protein is translated from spliced mRNA and is illustrated below the gene. Selected amino acid positions are labelled for NS1 and NEP. H5N2/NS1-LAIV candidates (NS1 stop mutations) NS1-73, NS1-86, NS1-101, NS1-122 are shown, as well as NS1-full (WT), containing intact NEP. (B) Western blot analysis to confirm expression of the truncated NS1 proteins. (C) An image of RT-PCR bands in an agarose gel showing the full-length NS and NEP genes from the individual viruses

presence of TPCK-treated trypsin (2 μg/ml). Cell culture supernatants were collected at 6, 12, 24, 48 and 72 hours postinfection (hpi), and the $TCID_{50}$ assay was performed in MDCK cells.

Experimental infection of mice with recombinant viruses

To determine the 50 % lethal dose (LD_{50}) of viruses in mice, groups of 5 mice were inoculated intranasally (i.n.) with tenfold serial dilutions containing $10^{2.5}$ to $10^{5.5}$ PFU/ml of virus. The MLD_{50} was expressed as $\log_{10}$PFU/ml. All tissue culture infectious dose 50 ($TCID_{50}$) and MLD_{50} calculations were performed by the method of Reed and Muench [26]. Additionally, thirty 5-week-old female BALB/c mice were infected i.n. with recombinant viruses at a concentration of $10^{5.5}$ PFU/ml. Mice were caged individually by group and were observed for two weeks to compare the pathogenesis and replication capacity of each recombinant virus. The mice were housed in a facility that maintained consistent temperature and humidity. On days 1, 3, 5, and 7 postinfection (dpi), mouse lungs ($n = 5$) from each group were collected antiseptically for titration of viruses. Lung tissues were homogenised and clarified by centrifugation. The supernatants were serially diluted in phosphate-buffered saline (PBS), and the $TCID_{50}$ was performed with cells incubated in a 37 °C CO_2 incubator for 48 h.

Immunization and challenge of mice

Groups of 5-week-old female BALB/c mice ($n = 10$ per group) were vaccinated with H5N2/NS1-wild-type (WT) and H5N2/NS1-LAIV candidates (NS1-73, NS1-86, NS1-101 or NS1-122) at a concentration of $10^{4.0}$ PFU/ml. The vaccines were distributed through the intranasal route, or mice were sham inoculated with PBS. At 17 days after virus immunization, mice from each group were challenged with 100 MLD_{50} of WB/ma81/H5N2, HPAI A/EM/Korea/W149/06 (H5N1) (EM/W149/H5N1) or 10 MLD_{50} of lethally mouse-adapted A/CK/Korea/ma116/06 (H9N2) (CK/ma116/H9N2) using the same routes and volumes described above for the vaccination. All mice were observed daily for clinical signs of disease and mortality. Virus preparation, titration, inoculation, and serologic testing for H5N1 virus were performed in an enhanced biosafety level 3 (BSL-3+) containment facility approved by the Korean Centers for Disease Control and Prevention.

Serological assays

HI assays were performed as described elsewhere [25]. Briefly, serum samples were treated with receptor-destroying enzyme (RDE, Denka Seiken, Japan) to inactivate nonspecific inhibitors, with a final serum dilution of 1:10. RDE-treated sera were serially diluted twofold, and an equal volume of virus (8 HA units/50 μl) was added to each well. The microplates were incubated at room temperature for 30 min, followed by the addition of 0.5 % (v/v) chicken red blood cells (RBCs). The plates were gently mixed and incubated at 37 °C for 30 min. The HI titre was determined as the reciprocal of the last dilution that did not induce agglutination of the chicken RBCs. The detection limit for the HI assay was set to ≥ 40 HI units.

A serum neutralisation (SN) assay was performed as described previously with modifications to determine cross-reactivity of the collected sera from mice and ferrets [25]. Viruses used for the SN assay were diluted from virus stock solutions at a titre between 100 and 300 $TCID_{50}$/0.1 ml. Initial serum dilutions of 1:10 were made using PBS. Twofold serial dilutions of all samples were made to a final serum dilution of 1:10,240. To each serum dilution, 50 μl of 100–300 $TCID_{50}$/0.1 ml of virus was added, and the sample was incubated for 1 h at 37 °C in 5 % CO_2. After incubation, the virus and serum mixtures were added to 96-well tissue culture plates containing confluent MDCK cell monolayers ($\sim 1.5 \times 10^4$ cells/well) and incubated for 48 h at 37 °C in 5 % CO_2. After infection, cultures were monitored for the appearance of a cytopathic effect (CPE). Viral replication in the supernatants of each well was confirmed using the haemagglutination test.

Bioassay for type I IFN stimulation

A549 cells were inoculated with each of the recombinant influenza viruses at an MOI of 3 or mock inoculated. Supernatants were then collected at 6, 12 and 24 hpi. Supernatants were treated with UV irradiation to inactivate viruses and transferred to naive A549 cells. After incubation for 24 h at 37 °C, supernatants were removed, and the cells were inoculated with VSV-GFP virus at an MOI of 2. GFP expression in the cells was examined by fluorescence microscopy at 18 hpi to assess VSV-GFP virus replication.

Results

Generation of experimental live attenuated influenza vaccines (LAIVs)

To develop dual-effective H5N2 live-attenuated vaccines, a panel of five recombinant viruses was created with HA and NA segments derived from the corresponding segments of WB/ma81/H5N2 and CK/116/H9N2, respectively, in the genetic background of the PR8 virus. The coding region of the PR8 NS segment is 693 nucleotide bases long and

encodes an NS1 protein 230 amino acids in length (Fig. 1A). To further understand the capacity of mutant influenza viruses with altered NS1 functions to elicit protective immune responses, the NS segments of four viruses were attenuated by introducing sequential stop codons in the carboxy-terminal region, resulting in four constructs encoding the first 73 (H5N2/NS1-73), 86 (H5N2/NS1-86), 101 (H5N2/NS1-101), and 122 (H5N2/NS1-122) amino acids of the NS1 protein open reading frame (Fig. 1A). To prevent reversion to the wild-type NS1 gene, three consecutive stop codons were incorporated; the nuclear export protein (NEP/NS2) encoded by the viral NS gene remained intact. In contrast to previous studies where recombinant LAIVs either expressed only truncated NS1 proteins or did not express NS1 at all because the entire gene was deleted, the NS1-truncated viruses in this study still contained the remaining portion of the NS1 gene downstream of the engineered stop codons.

To verify that the incorporated mutations produced truncated NS1 proteins, MDCK cells were infected at an MOI of 0.5 with each of the five H5N2 recombinant viruses (H5N2/NS1-WT, H5N2/NS1-73, H5N2/NS1-86, H5N2/NS1-101, and H5N2/NS1-122). At 24 hpi, Western blotting was conducted to examine the individual sizes and expression levels of the modified NS1 proteins. Using a polyclonal antibody against the NS1 protein, analysis of immunoblots demonstrated strong expression of the truncated NS1 proteins from the H5N2/NS1-73 (8 kDa), H5N2/NS1-86 (9.5 kDa), H5N2/NS1-101 (11 kDa), H5N2/NS1-122 (13.5 kDa) and H5N2/NS1-WT (25 kDa) viruses. Interestingly, the band intensity of the target protein in the H5N2/NS1-86 (9.5 kDa) virus was noticeably lower compared with those of the other NS1-mutant viruses. Real-time PCR of the NS segment of the recombinant viruses using in-house-designed primers specific for the NEP/NS2 region revealed amplification of the full NS1 ($\sim$857 nt) and NS2 ($\sim$375 nt) genes, indicating the presence of intact segment 8 sequences despite the introduction of internal stop codons (Fig. 1C). Furthermore, all recombinant viruses were subjected to serial passage in MDCK cells and 10-day-old embryonated chicken eggs. Full sequencing of the NS gene segment did not show any indication of revertant viruses after at least 10 growth cycles of 48 h at 37 °C in any of the media used (data not shown). Overall, these results demonstrated the viability and genetic stability of the NS1-modified viruses.

The H5N2/NS1-LAIV candidates did not suppress IFN induction in human lung cells

The NS1 protein of influenza virus has previously been shown to act as an IFN-α/β antagonist. We investigated the induction of IFN in cells infected with the H5N2/NS1-LAIVs to determine whether the modified NS1 proteins were defective in the ability to inhibit the IFN-α/β system when compared with the intact NS1 expressed by the H5N2/NS1-WT virus. Human A549 cells were inoculated with each of the recombinant viruses or mock inoculated. A multiplicity of infection (MOI) of 3 was used to ensure efficient infection of the A549 cells. Supernatants from the infected A549 cells harvested at 6, 12, and 24 hpi were used to determine the levels of secreted IFN-α/β in a bioassay based on the inhibition of VSV-GFP replication. Freshly prepared A549 cells were pretreated with the supernatants and infected at an MOI of 2 with green fluorescent protein–expressing vesicular stomatitis virus (VSV-GFP). Supernatants from cells infected with the virus with intact NS1 (H5N2/NS1-WT), which is known to antagonize IFN production, did not inhibit GFP expression, allowing the VSV inoculum to replicate. This result indicated an impaired ability to antagonize an antiviral state (Fig. 2, first panel). Similarly, mock infection did not inhibit VSV-GFP virus growth, suggesting that IFN-α/β was not present in those supernatants (Fig. 2, last panel). By contrast, cells that were pretreated with supernatants collected from most of the H5N2/NS1-LAIVs showed a reduction in GFP expression at 24 hpi, suggesting inefficient inhibition of IFN induction. However, the highest degree of evident suppression of VSV-GFP growth was observed following pretreatment with the H5N2/NS1-86 supernatant. Thus, these results demonstrate the ability of the H5N2/NS1-LAIVs to induce IFN expression, which can be used to delineate differences in the host response to the various H5N2/NS1-LAIVs.

Characterization of H5N2/NS1-LAIV candidates in MDCK cells and embryonated eggs

To examine the fitness of each H5N2/NS1-LAIV virus, their growth properties were compared with those of the H5N2/NS1-WT virus in MDCK cells and 10-day-old embryonated chicken eggs (Fig. 3). MDCK cells were infected at an MOI of 0.001, and growth kinetics were monitored at each designated time point. The H5N2/NS1-WT virus reached peak titers (7.3 $\log_{10}TCID_{50}$/ml) at 72 hpi (Fig. 3A). All of the H5N2/NS1-LAIVs also replicated well in culture, producing titers at least 1 $\log_{10}$ lower than H5N2/NS1-WT virus. In particular, H5N2/NS1-86, which exhibited reduced NS1 expression, grew only to a peak titer of 5.2 $\log_{10}TCID_{50}$/ml in collected cell culture supernatants; this titer was significantly lower ($p < 0.05$) than that of the H5N2/NS1-WT virus, implying that replication was attenuated although their plaque morphologies were similar (Fig. 1B and 3). Next, the growth phenotype of each virus was assessed in embryonated chicken eggs, the traditional substrate for the production of influenza vaccines. Ten-day-

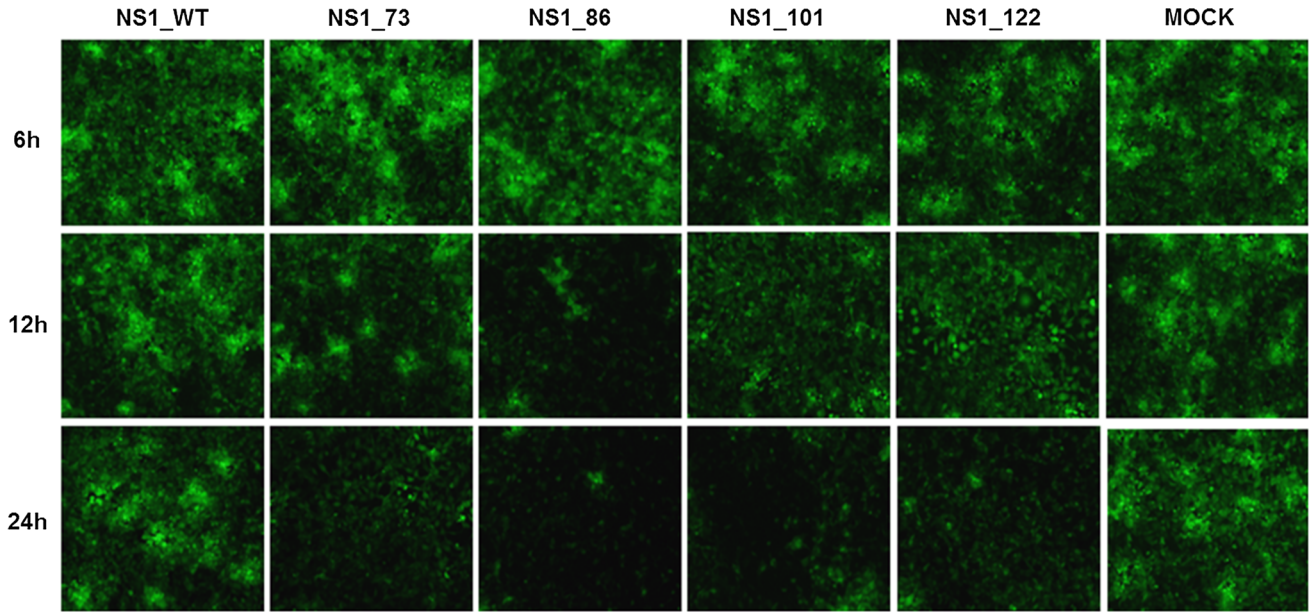

Fig. 2 Induction of type I IFN in A549 cells infected with H5N2/NS1-LAIV viruses. At various time points after infection (indicated on the left) with H5N2/NS1-WT (NS1-WT) virus or H5N2/NS1-LAIVs (NS1-73, NS1-86, NS1-101 or NS1-122), supernatants were harvested from A549 cells infected with the indicated viruses. Following UV inactivation, supernatants were applied to fresh A549 cells and incubated for 24 h, followed by VSV-GFP infection. At 18 hpi, cells expressing GFP were visualized by fluorescence microscopy

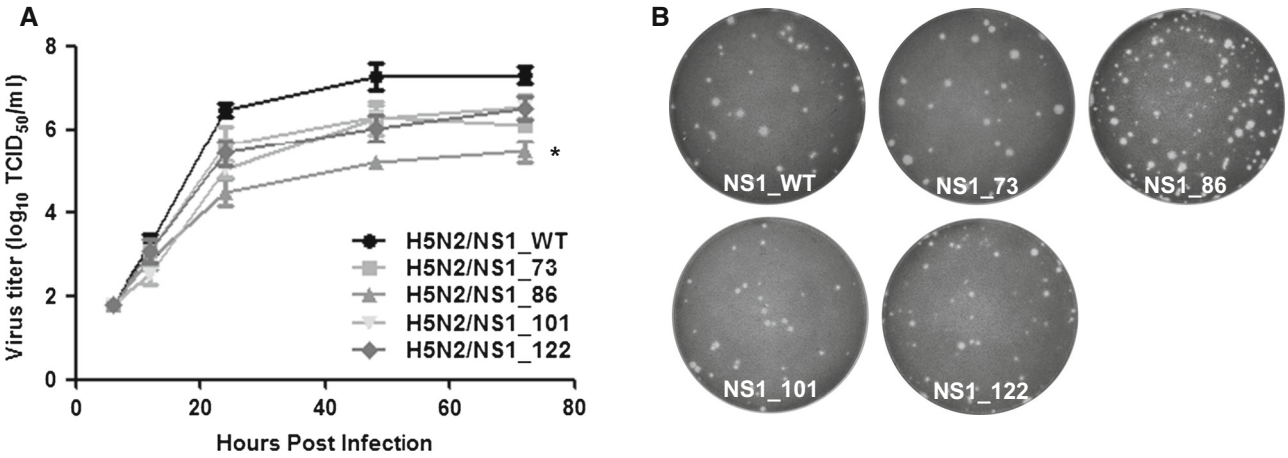

Fig. 3 Viral growth kinetics in MDCK cells. (A) MDCK cells were infected at an MOI of 0.001, and the culture supernatants were collected at 6, 12, 24, 48 and 72 hpi. The viral titer in culture supernatants was determined by $TCID_{50}$ assay using MDCK cells. The error bars represent the standard error of the mean (SEM) of triplicate assays. (B) Plaque morphology of the H5N2/NS1-LAIVs and WT viruses in MDCK cells

old embryonated chicken eggs were inoculated with each of the H5N2/NS1-LAIV viruses. Allantoic fluid was harvested after incubation for 48 h at 37 °C, followed by plaque titration in MDCK cells (Table 1). Surprisingly, in contrast to the observed growth properties in MDCK cells, all five viruses grew efficiently, with titers up to at least 7.0 log_{10}-PFU/ml from 10-day-old embryonated chicken eggs. The H5N2/NS1-WT virus produced the highest viral titer, as observed in MDCK cells (8.2 log_{10}PFU/ml), whereas H5N2/NS1-122 had the lowest titer (7.0 log_{10}PFU/ml) (Table 1). The remaining viruses exhibited titers ranging from 7.5 to 8.0 log_{10}PFU/ml. Overall, these results indicated that the H5N2/NS1-LAIVs could produce high titers in substrates used for vaccine production.

Table 1 H5N2/NS1-LAIV candidates exhibit different levels of attenuation *in vitro* and *in vivo*

Virus	[a]Viral titer (log_{10}PFU/ml ± SEM)	Virulence and growth properties in mice					
		[b]MLD_{50} (log_{10}PFU/ml)	[c]%Weight loss ± SEM	[d]Lung titer ($log_{10}TCID_{50}$/g ± SEM)			
				1 dpi	3 dpi	5 dpi	7 dpi
H5N2/NS1-73	7.60 ± 0.27	5.0	13.1 ± 8.0	4.8 ± 0.0	4.5 ± 0.2	4.6 ± 0.3	2.9 ± 0.1
H5N2/NS1-86	7.51 ± 0.24	>6.0	4.0 ± 1.8	3.8 ± 0.0	4.5 ± 0.2	3.9 ± 0.1	<1.8 ± 0.0
H5N2/NS1-101	8.00 ± 0.23	>6.0	2.0 ± 1.1	3.8 ± 0.0	4.8 ± 0.0	3.6 ± 0.3	<1.8 ± 0.0
H5N2/NS1-122	7.03 ± 0.24	5.4	6.7 ± 8.8	3.8 ± 0.0	5.1 ± 0.3	4.3 ± 0.0	3.0 ± 0.0
H5N2/NS1-WT	8.17 ± 0.16	4.7	>25.0	6.0 ± 0.0	6.1 ± 0.3	6.0 ± 0.3	[e]NA

[a] Plaque-forming units (PFU) in MDCK cells for H5N2/NS1-WT virus and LAIVs grown in 10-day-old embryonated chicken eggs

[b] 50 % mouse lethal dose (MLD_{50}) for H5N2/NS1-WT and LAIVs. Groups of five mice per dose were inoculated intranasally with tenfold serial dilutions containing $10^{2.5}$ to $10^{5.5}$ PFU/ml. Mice that lost 25 % body weight were assumed to be near death and were euthanized. Survival of mice was monitored for 14 days after inoculation

[c] 5-week-old female BALB /c mice were inoculated intranasally with $10^{5.5}$ PFU/ml of the H5N2/NS1-WT virus or H5N2/NS1-LAIVs. Body weight of inoculated mice (n = 10/group) was recorded daily and is given as a percentage of the animal's weight on the day of inoculation

[d] Viral titers in the lung homogenates from each time point from inoculated mice (n = 5/group) were determined for each mouse using the $TCID_{50}$ assay at 1, 3, 5, and 7 dpi

[e] Not applicable, indicating that all virus-inoculated mice were dead or humanely euthanized

SEM, standard error of the mean

In vivo characterization of H5N2/NS1-LAIV candidates in mice

Mouse challenge studies were performed to assess the pathogenicity of the H5N2/NS1-LAIV viruses in a mammalian host. Because NS1-deficient viruses are highly attenuated in IFN-competent hosts, we infected mice with the maximum dose of the mutant viruses. Therefore, groups of 30 mice were inoculated i.n. with $10^{5.5}$ PFU/ml of the H5N2/NS1-WT virus and H5N2/NS1-LAIVs (H5N2/NS1-73, H5N2/NS1-86, H5N2/NS1-101, and H5N2/NS1-122). Lungs from infected mice were harvested at 1, 3, 5, and 7 dpi (5 mice per day) for viral titration via the $TCID_{50}$ assay in MDCK cells. The remaining mice were monitored daily for survival, using body weight as an indicator of morbidity. Mice exhibiting body weight loss of at least 25 % relative to their starting weight were presumed to be near death and were humanely euthanized. The fifty percent lethal dose (MLD_{50}) was determined for each separate group of mice infected with $10^{2.5}$ to $10^{5.5}$ PFU/ml of each virus.

Infection with the H5N2/NS1-WT virus induced severe clinical signs of disease (i.e., ruffled fur and hunched back) as early as 3 dpi and high mean maximum weight losses. Consistently, high viral titers were detected in mouse lungs up to 5 dpi. None of the mice in this group survived infection during the course of the experiment (MLD_{50} = 4.7 log_{10}PFU/ml) (Fig. 4 and Table 1). Among the H5N2/NS1-LAIVs, mice infected with the H5N2/NS1-73 LAIV exhibited 13 % weight reduction and showed 40 % mortality due to infection (MLD_{50} = 5.0 log_{10}PFU/ml). Moderate mean weight loss (6.7 %) was observed in the H5N2/NS1-122 LAIV-infected group, but the mice started to rapidly regain weight at 8 dpi; a total of 80 % of infected mice survived (MLD_{50} = 5.4 log_{10}PFU/ml). Relative to H5N2/NS1-WT virus, lower peak viral titers were recovered from mouse lungs inoculated with the H5N2/NS1-73 and H5N2/NS1-122 LAIVs, which persisted up to 7 dpi (Table 1). By contrast, mice in the H5N2/NS1-86 and H5N2/NS1-101 LAIV-infected groups did not show any indications of severe clinical disease, and all mice survived infection (MLD_{50} > 6.0 log_{10}PFU/ml), although there was a slight reduction in mean body weight (2.0-4.0 %) relative to initial values over the course of the experiment (Fig. 4A). Furthermore, the H5N2/NS1-86 and H5N2/NS1-101 LAIVs induced significantly lower titers in mouse lungs (approximately 100-fold, $P < 0.05$) compared with the H5N2/NS1-WT virus; infections with the H5N2/NS1-86 and H5N2/NS1-101 LAIVs were undetectable in lung tissue homogenates at 7 dpi. Taken together, these results demonstrated that the H5N2/NS1-LAIVs are attenuated (especially H5N2/NS1-86 and H5N2/NS1-101) compared with the H5N2/NS1-WT virus despite the high doses used for inoculation.

H5N2/NS1-LAIV strains are immunogenic and protect mice from lethal challenge

The ability of the generated H5N2/NS1-LAIVs to elicit immunogenic responses was determined by inoculating groups of mice (n = 10/group) i.n. with $10^{4.0}$ PFU/ml of H5N2/NS1-WT virus, H5N2/NS1-LAIV, or PBS as a mock

control. Two weeks after inoculation, blood samples were collected from immunized animals, and the collected sera were subsequently tested for antigenicity against the WB/ma81/H5N2, HPAI EM/W149/H5N1, and CK/ma116/H9N2 viruses using HI assays (Fig. 5A). Serological analysis showed that all modified H5N2/NS1-LAIVs elicited high HI antibody titers (180-250 HI units) against the homologous H5N2 virus (Fig. 5A). Additionally, all of the H5N2/NS1-LAIV candidates demonstrated cross-reactivity against the heterologous HPAI H5N1 virus, although titers (at least 80 HI units) were considerably lower than those observed for homologous virus sero-reactivity. By contrast, none of the sample sera cross-reacted with the heterosubtypic H9N2 virus (Fig. 5A). Serum neutralization assays demonstrated that the H5N2/NS1-LAIVs elicited serum neutralizing antibodies in all of the H5N2/NS1-LAIVs-immunized mice. Mean serum neutralization titers against H5N2 virus were approximately 320-450 MN units, while 320-390 mean MN titers were achieved against EM/W149/H5N1 (Fig. 5B). Interestingly, modest to moderate neutralization (approximately 100-150 MN units) of H9N2 virus was observed despite the lack of HI activity against this virus. These results showed that the H5N2/NS1-LAIVs could elicit neutralizing antibodies against the H5 and H9N2 subtypes.

To demonstrate the protective efficacy of each H5N2/NS1-LAIV candidate, each of the vaccinated groups of mice was challenged with a lethal dose of WB/ma81/H5N2, CK/ma116/H9N2 or HPAI EM/W149/H5N1 at 17 days post-vaccination (dpv) (Fig. 6). All mice immunized with the H5N2/NS1-LAIV candidates survived throughout the 14-day observation period after challenge with 100 MLD_{50} of WB/ma81/H5N2 virus (Fig. 6A). Notably, no remarkable clinical signs of disease manifestation were observed in any of the vaccinated groups. By contrast, ruffled fur, hunched posture, and weight loss were observed in the mock-immunized mice as early as 2 days post-challenge, with the clinical signs progressing to severe disease until all mice succumbed at 4 dpi. When similarly vaccinated groups were experimentally inoculated with the HPAI EM/W149/H5N1 virus at 100 MLD_{50}, approximately 20 % mortality was observed in H5N2/NS1-86 LAIV-immunized mice; no deaths were observed in groups that received the H5N2/NS1-73, H5N2/NS1-101, or

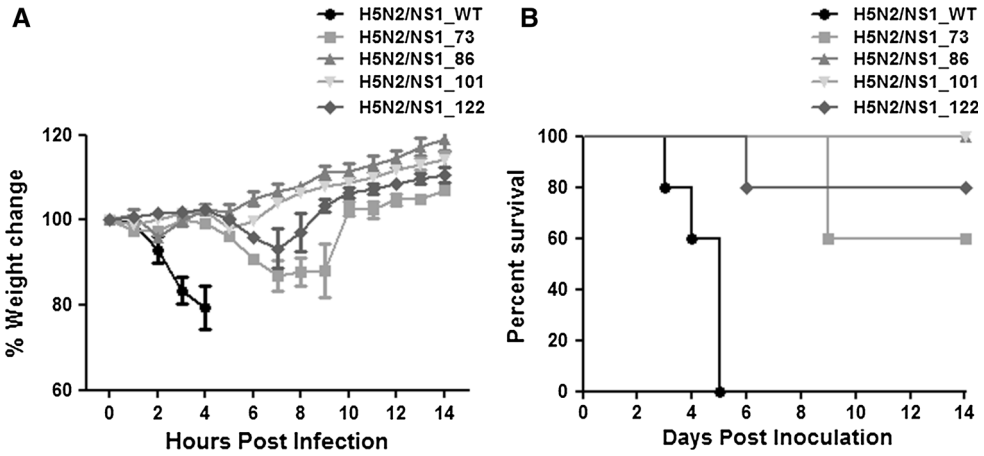

Fig. 4 Virulence properties of H5N2/NS1-LAIVs in mice. In this assay, 5-week-old female BALB/c mice were inoculated intranasally with $10^{5.5}$ PFU/ml of H5N2/NS1-WT virus and LAIVs. Body weight (A) and survival rate (B) of inoculated mice (10 per group) were recorded daily and are represented as the percentage of the animal's weight on the day of inoculation

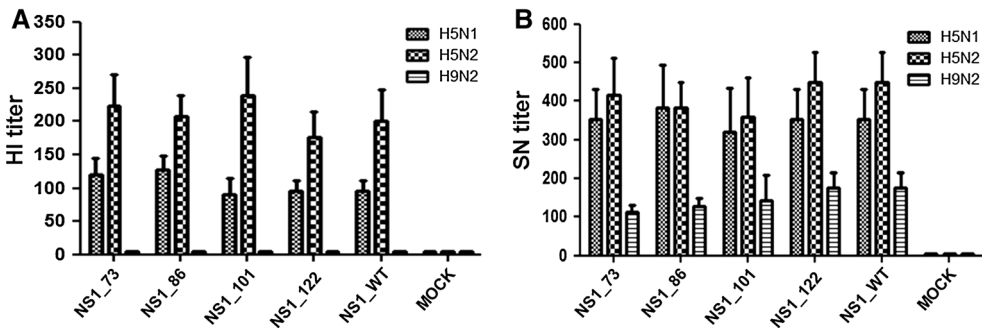

Fig. 5 Mean HI and neutralization titers of sera from mice vaccinated with recombinant viruses. Groups of mice (10 per group) were immunized by intranasal inoculation with the H5N2/NS1-WT virus and LAIVs or inoculation medium alone (Mock). Antibody levels in sera collected 14 days post-immunization were determined by hemagglutination inhibition assay (A) and by microneutralization assay (B) using antigens from the A/WB/Korea/ma81/06 (H5N2), A/EM/Korea/W149/06 (H5N1), and H9N2 viruses

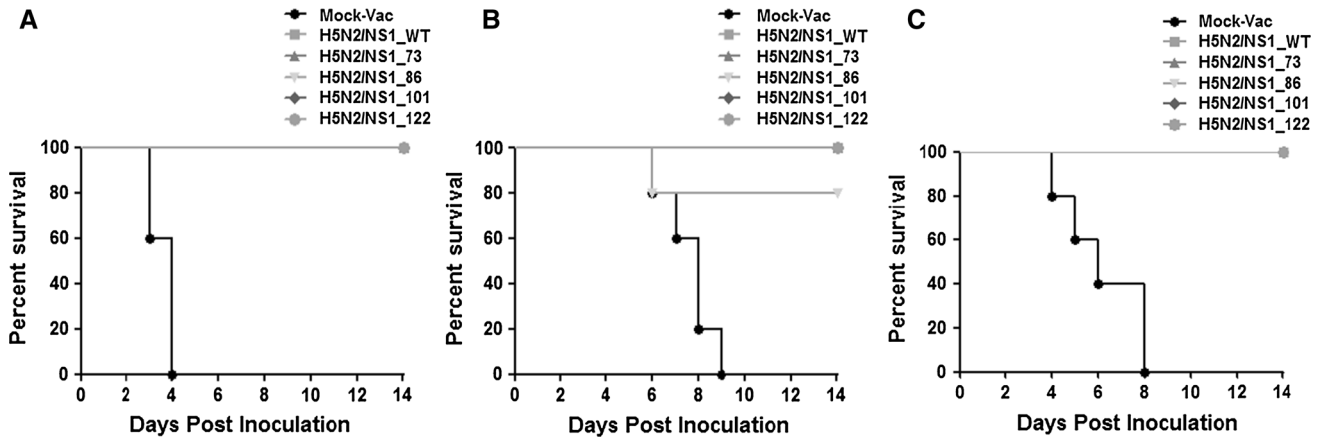

Fig. 6 H5N2/NS1-LAIV candidates protect mice from lethal infection. Groups of mice (10 per group) were immunized by intranasal inoculation with the H5N2/NS1-WT virus and LAIVs (NS1-73, NS1-86, NS1-101 or NS1-122) or inoculation medium alone (Mock). At 17 days post-immunization, each group of mice was challenged intranasally with 100 MLD_{50} of a lethal mouse-adapted variant of A/WB/Korea/ma81/06 (H5N2) (A), HPAI A/EM/Korea/W149/06 (H5N1) (B), or 10 MLD_{50} of a lethal mouse-adapted variant of A/CK/Korea/ma116/06 (H9N2) virus (C), respectively, and their survival rate was monitored for 14 days post-challenge

H5N2/NS1-122 LAIVs. In challenge experiments with the mouse-adapted H9N2 virus, no deaths were observed in any of the vaccinated groups inoculated with 10 MLD_{50} of CK/ma116/H9N2 (Fig. 5C). By contrast, 100 % mortality was observed in the mock-immunized group at 8 dpi (Fig. 6C). These data indicated that the H5N2/NS1 LAIV candidates could be cross-protective against heterologous H5N1 and heterosubtypic H9N2 influenza viruses following a single immunization.

Discussion

In recent years, NS1 deletion or truncation has become an attractive approach for the development of various live-attenuated influenza A vaccine candidates. Such strategies are based on the capacity of the viral NS1 protein to counter the antiviral effects of the type I IFN response during infection. Compared with viruses with full-length NS1 proteins, the length of the protein correlates inversely with viral growth and IFN inhibition; proteins with shorter segments are less able to antagonize the host IFN system [38]. However, viruses carrying deleted NS1 proteins may be too attenuated in animal hosts to constitute a viable live attenuated vaccine. In contrast to deletion of the NS1 gene, more moderate attenuation of influenza viruses can be achieved by incremental truncation of the NS1 protein. This approach has produced effective vaccine viruses as demonstrated in various animal models [41, 47, 48]. However, due to the relatively low viral titers obtained in animals or MDCK cells, additional mutations in other segments (e.g., 627K in PB2) have been used in combination with the NS1 modification [47]. In this study, we generated a panel of five H5N2/NS-LAIV candidates using reverse genetics; four of the candidates are H5N2/NS1-LAIVs. Although several studies have assessed the vaccine potential of NS1-truncation mutants, this is the first demonstration of the vaccine efficacy of modified H5N2 NS1-LAIVs in which the surface glycoproteins were separately derived from influenza viruses endemic in various avian species such as wild birds and poultry in South Korea. Recently, Chou et al. [6] and Noda et al. [34] clearly demonstrated robust selection of each individual segment from pools of whole segments during the packaging of influenza virus genomes. These results led us to hypothesize that proper gene size could also be a determinant for viral packaging and the overall replication of the virus.

To generate H5N2 viruses with attenuated virulence that would allow growth to high titers in 10-day-old embryonated chicken eggs and MDCK cells, vaccine viruses were prepared in the background of PR8, a laboratory strain widely used for vaccine studies, primarily due to its high-growth capacity and safety. Additionally, three consecutive stop codons were introduced in-frame to the NS gene sequence, resulting in the desired truncated NS1 proteins. In contrast to previous work, no nucleotide sequence deletions were introduced, and the NS2 protein remained intact in all of the H5N2/NS1-LAIVs created here. Most of the recombinant viruses used in this study grew to maximal titers ranging from 7.5 to 8.0 $\log_{10}$PFU/ml at 48 h. These titers represent only a modest loss of yield compared with that of the wild-type virus in 10-day-old eggs, which can be conveniently used for the production of the vaccine. With the recent inclusion of MDCK cells as a substrate, the high-yield capacity provides an alternative means for robust vaccine preparation [35]. The

use of three consecutive stop codons to generate the viruses *de novo* without deletions allowed the codon usage of each individual NS1 protein to be altered in a way that should reduce the risk of reversion to its wild-type form even if the full-length nucleotide sequence is present. The stability of the truncated NS1 proteins was demonstrated by Western blotting; no full-length NS1 proteins were detected among the NS1-modified viruses even after multiple rounds of growth in MDCK and embryonated eggs (at least 10 passages for each virus).

Infection of mice with the H5N2/NS1-LAIV viruses demonstrated varying degrees of virulence and viral growth in the lungs. It is noteworthy that most of the recombinant viruses (particularly H5N2/NS1-86 and H5N2/NS1-101) exhibited pronounced attenuation in our mouse models relative to the H5N2/NS1-WT virus despite infection with the normally lethal dose of $10^{5.5}$ PFU/ml. However, the H5N2/NS1-73 LAIV extended the duration of viral replication and remained moderately virulent, killing 40 % of the inoculated animals. When mice were immunized with $10^{4.0}$ PFU_{50}/ml, all H5N2/NS1-LAIVs demonstrated high antigenic and neutralizing titers despite the observed attenuation. Notably, these immunogenic titers were able to provide complete protection of vaccinated mice from lethal challenge with heterologous HPAI EM/W149/ H5N1 and heterosubtypically lethal mouse-adapted CK/ma116/H9N2 viruses in addition to the homologous WB/ma81/H5N2 virus. Taken together, these results show that the modified H5N2 NS1-LAIVs (particularly H5N2/NS1-86 and H5N2/NS1-101) are sufficiently immunogenic, conferring protection against H5N1 and H9N2 avian virus challenges despite evident attenuation compared with the H5N2/NS1-WT virus.

Natural or vaccine-induced immunity to homologous or antigenically related heterologous viruses is thought to be due to antibodies directed against the major surface viral antigen HA [52]. In our animal models, the levels of cross-reactive HI antibodies were relatively low against HPAI H5N1, and they were virtually undetectable against H9N2 viruses. Despite this, it was noteworthy that the H5N2/NS1-LAIVs still demonstrated neutralization and were able to cross-protect against lethal challenge with these viruses. Since live virus vaccines mimic the process of natural infection better than their inactivated counterparts, they are considered to provide better protection against illness and mortality. They may induce humoral, mucosal and cell-mediated immunity [49, 50]. In addition, due to the high similarity of NA genes between vaccine and H9N2 challenge virus (99.4 % homology), the NA-protein-induced antibodies might induce antibodies for protection [4]. Although NA-specific antibodies do not prevent influenza virus infection (infection-permissive) [40], humoral immunity induced by NA can hamper virus replication and consequently modulate disease severity and duration of illness [11, 24, 32]. It has also been shown that antibodies raised against non-HI epitopes or other viral proteins, in combination with the cell-mediated immune responses, can confer substantial levels of heterosubtypic immunity, leading to cross-protection against different influenza virus subtypes [7, 30, 50, 51]. Furthermore, broadly cross-reactive T-cell epitopes located in internal proteins of influenza viruses (e.g., nucleoprotein and matrix segments) appear to be more conserved than the surface HA and NA glycoproteins [23, 50, 57].

In summary, a panel of H5N2/NS1-LAIVs encoding modified NS1 proteins were generated and characterized. Vaccination of mice with these LAIVs resulted in complete protection against lethal challenge with homologous (H5N2) and heterologous (HPAI H5N1) viruses as well as a heterosubtype (H9N2) influenza A virus. Thus, recombinant influenza viruses attenuated through the introduction of mutations in the NS1 coding region display characteristics desirable for live attenuated vaccines and exhibit potential as vaccine candidates in mammalian hosts.

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