

# Nonreplicating Influenza A Virus Vaccines Confer Broad Protection against Lethal Challenge

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**ABSTRACT** New vaccine technologies are being investigated for their ability to elicit broadly cross-protective immunity against a range of influenza viruses. We compared the efficacies of two intranasally delivered nonreplicating influenza virus vaccines (H1 and H5 S-FLU) that are based on the suppression of the hemagglutinin signal sequence, with the corresponding H1N1 and H5N1 cold-adapted (*ca*) live attenuated influenza virus vaccines in mice and ferrets. Administration of two doses of H1 or H5 S-FLU vaccines protected mice and ferrets from lethal challenge with homologous, heterologous, and heterosubtypic influenza viruses, and two doses of S-FLU and *ca* vaccines yielded comparable effects. Importantly, when ferrets immunized with one dose of H1 S-FLU or *ca* vaccine were challenged with the homologous H1N1 virus, the challenge virus failed to transmit to naive ferrets by the airborne route. S-FLU technology can be rapidly applied to any emerging influenza virus, and the promising preclinical data support further evaluation in humans.

**IMPORTANCE** Influenza viruses continue to represent a global public health threat, and cross-protective vaccines are needed to prevent seasonal and pandemic influenza. Currently licensed influenza vaccines are based on immunity to the hemagglutinin protein that is highly variable. However, T cell responses directed against highly conserved viral proteins contribute to clearance of the virus and confer broadly cross-reactive and protective immune responses against a range of influenza viruses. In this study, two nonreplicating pseudotyped influenza virus vaccines were compared with their corresponding live attenuated influenza virus vaccines, and both elicited robust protection against homologous and heterosubtypic challenge in mice and ferrets, making them promising candidates for further evaluation in humans.

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Seasonal epidemics and sporadic pandemics of influenza A viruses (IAV) pose a global public health burden. Hemagglutinin (HA)-specific antibodies directly bind the virus and prevent its entry into host cells, providing narrow immunity from reinfection by closely related strains (1). CD8 T cell responses to IAV generated against highly conserved viral proteins/epitopes contribute to clearance of virus during primary IAV infection and also confer broad heterosubtypic protection in animal models (2–4). Recent evidence links the cross-reactive CD8 T cell response in humans to reduced viral replication and protection from severe illness in pandemic H1N1 infections in European populations (5, 6) and H7N9 infections in China (7, 8). Because preexisting T cell immunity, independent of baseline antibodies, protects against symptoms and viral shedding associated with influenza, influenza vaccines that stimulate broadly reactive CD8 T cell responses may have the capacity to protect against any pandemic influenza A virus.

Two types of seasonal influenza vaccines are widely available: (i) inactivated influenza virus vaccines (IIV) which mediate protection primarily by a neutralizing serum antibody response against the immunodominant head region of the HA protein and

(ii) live attenuated influenza virus vaccines (LAIV) which are based on a temperature-sensitive and attenuated influenza virus backbone; both types of vaccines contain the HA and neuraminidase (NA) from strains anticipated to circulate in the next season (9). LAIV elicit anti-HA antibodies in young children, but the vaccines are efficacious even in the absence of a detectable antibody response (10). Intranasally (i.n.) administered LAIV elicit a humoral and cellular immune response that resembles natural immunity (1). LAIV can boost virus-specific cytotoxic CD8 T lymphocytes (CTL) and mucosal and serum antibodies and provide broad cross-protection against heterologous IAV, including avian viruses (1, 11).

Human infections with H5N1 and H7N9 avian IAV and the 2009 H1N1 pandemic have spurred an interest in the development of vaccines against IAV with pandemic potential. Major challenges to this effort include our inability to predict which virus will emerge and rapid production and deployment of vaccine if the virus spreads rapidly and vaccine yield is not optimal. In addition, the number of doses of vaccine required depends on whether the population is immunologically naive. Therefore, vaccine technologies that elicit broadly cross-reactive and protective

immune responses against a range of influenza viruses and that can be scaled up are desirable. These efforts have included the development of pandemic LAIV. However, the potential for LAIV to reassort with circulating viruses and introduce a novel HA into the human population will likely limit their use prior to a pandemic. S-FLU is designed to remove this risk (12). The S-FLU particles are coated with HA protein but do not contain a viral RNA (vRNA) that could reassort with seasonal IAV during coinfection. In addition, S-FLU can infect cells for only one round, and thus, the likelihood of coinfection with a seasonal influenza virus is greatly reduced.

We developed a pseudotyped influenza A/Puerto Rico/8/34 (PR8) virus that was replication incompetent (12) due to suppression of the HA signal sequence (S-FLU); this virus can infect cells and express the NA and the conserved internal viral proteins but can replicate only in cell lines expressing HA to provide the HA protein on pseudotyped budding virus particles; the expressed HA can be derived from any subtype. This vaccine protected mice from challenge with homologous virus and a heterologous H3N2 virus (12). Protection was associated with a robust influenza virus-specific pulmonary CD8 T cell response in the absence of neutralizing antibodies (NtAb), because although the virus particles are pseudotyped with HA, no further amplification of the antigen occurs (12). On the basis of these promising data, we generated two S-FLU vaccine candidates, an H1 S-FLU expressing the HA and NA of A/England/195/2009 (pandemic H1N1 [H1N1pdm]) and internal protein genes of PR8 and an H5 S-FLU expressing the HA of the highly pathogenic avian influenza virus (HPAI) A/Vietnam/1203/2004 (VN/04) (H5N1 clade) with the NA and internal protein genes of PR8. The H5 HA cDNA was codon optimized, and the polybasic site was replaced with a trypsin cleavage site.

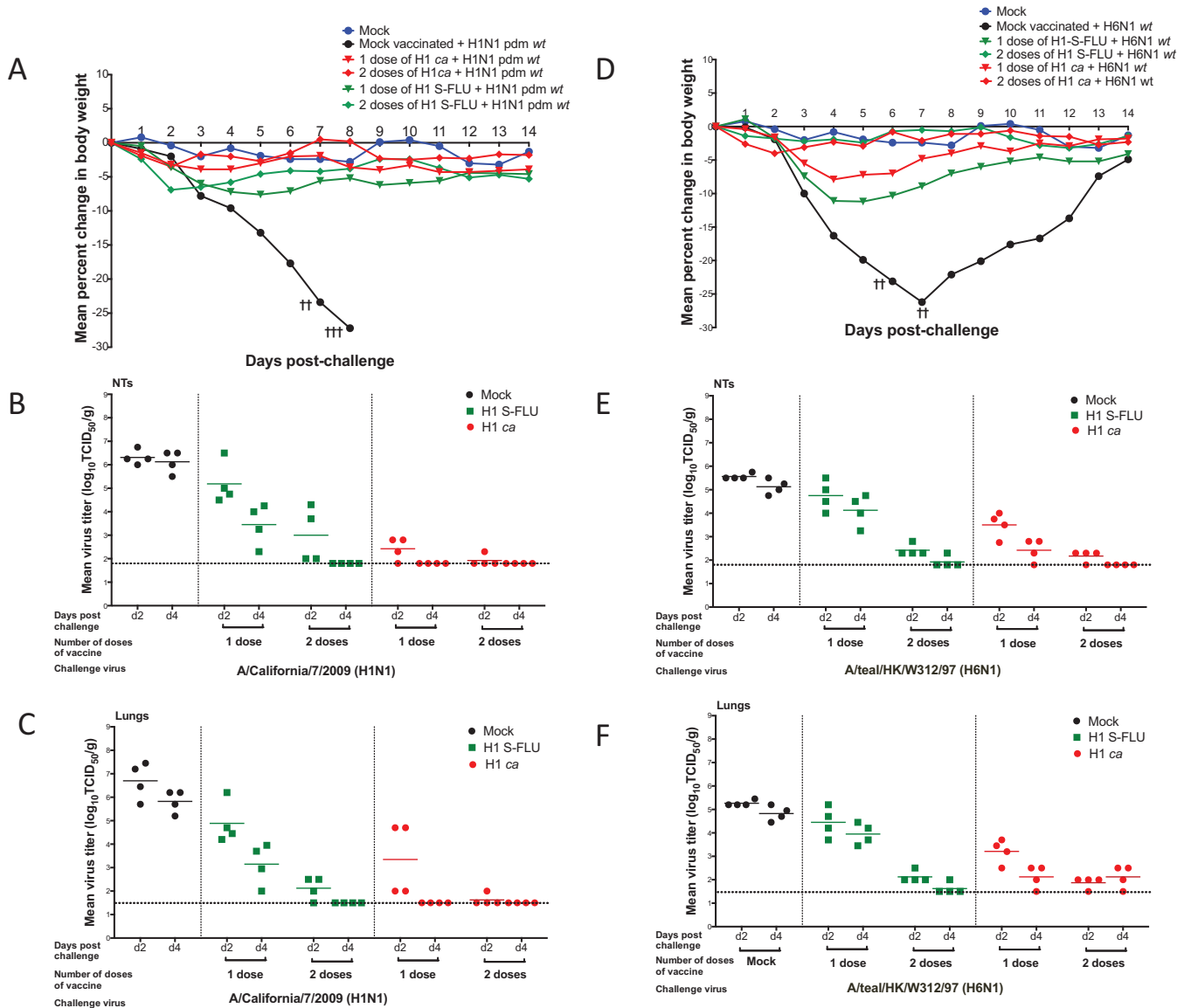
Mice are widely used to evaluate the humoral and cellular immune response and efficacy of candidate vaccines. Ferrets are outbred and the favored model for influenza research, because they are susceptible to a range of influenza viruses, they display clinical signs resembling those in humans, and viruses that transmit well from person to person transmit between ferrets. However, evaluation of T cell responses in ferrets is difficult (13). Thus, we evaluated the efficacy of the two pseudotyped vaccine candidates, H1 S-FLU and H5 S-FLU in mice and ferrets. In order to determine how nonreplicating virus vaccines would compare with replicating but highly attenuated virus vaccines, we compared them with the corresponding H1N1pdm and H5N1 cold-adapted (*ca*) LAIV (H1 *ca* and H5 *ca*) (14, 15). Since broad cross-protection is an important attribute for a pandemic vaccine, we evaluated protection against homologous and heterologous influenza viruses. We selected A/teal/HK/W312/97 (tl/97) (H6N1) as the heterosubtypic challenge virus for the H1 vaccines. Because H5N1 viruses have evolved since 1997 into many clades and subclades, we evaluated cross-clade protection of the H5 vaccines with A/Indonesia/5/2005 (Indo/05) (H5N1, clade 2.1) as well as heterosubtypic challenge with A/Anhui/13 (H7N9) from the ongoing outbreak in China.

## RESULTS

**H1 S-FLU vaccine confers robust protection from replication of homologous and heterologous challenge viruses in mice and ferrets.** First, we immunized mice i.n. with one or two doses of H1 S-FLU or H1 *ca* viruses and collected sera on day 28 after each

vaccine dose. Mice were challenged i.n. with the homologous A/California/07/2009 (CA/09) or heterologous tl/97 (H6N1) virus 28 days after vaccination and were monitored for weight loss for 14 days. Another group of mice was challenged, and virus titers in the nasal turbinates (NTs) and lungs were measured on days 2 and 4 postchallenge (p.c.) (Fig. 1A to F). There were no deaths in the mice immunized with one dose of H1 S-FLU vaccine, while mock-vaccinated mice challenged with CA/09 and tl/97 showed mortality rates of 100 and 80%, respectively (Fig. 1A and D). Mice that received a single dose of H1 S-FLU vaccine had transient weight loss and ruffled fur after homologous or heterologous virus challenge, but they recovered rapidly. Although one dose of H1 *ca* vaccine was more efficacious than one dose of H1 S-FLU vaccine, two doses of either vaccine were highly effective in controlling replication of the homologous or heterologous challenge viruses in the respiratory tract; virus titers in the NTs and lungs were very low or undetectable by day 4 p.c. (Fig. 1B, C, E, and F). Because S-FLU is a pseudotyped vaccine virus that does not synthesize HA, the only HA-specific antibody response is due to the HA coating the particles in the viral inoculum. Therefore, as expected, the H1 S-FLU vaccine did not elicit an antibody response against the homologous virus or cross-reactive antibodies against the heterologous virus (see Fig. S2A in the supplemental material). To investigate the cellular immune response to the H1 S-FLU vaccine, the induction of conserved nucleoprotein (NP<sub>147</sub>) CTL epitope-specific CD8<sup>+</sup> CTLs in the lungs of mice was examined by flow cytometry (Fig. 2). Eight days after the first dose of H1 S-FLU, 4.8% of pulmonary CD8<sup>+</sup> T cells were NP<sub>147</sub> specific, and this percentage increased to 30.3% ( $P = 0.008$ ) after the second dose of vaccine (see Fig. S1 for gating strategy). The immunogenicity and efficacy of the H1 S-FLU vaccine validated our earlier findings with PR8 S-FLU (12), and the nonreplicating pseudotyped H1 S-FLU virus compared well with the corresponding LAIV in mice.

Therefore, we evaluated the H1 S-FLU vaccine in ferrets by immunizing groups of ferrets i.n. with one or two doses of H1 S-FLU or H1 *ca* vaccine and challenging them i.n. 28 days later with CA/09 virus (Fig. 3A and B) or tl/97 virus (Fig. 3C and D). One dose of either H1 S-FLU or H1 *ca* vaccine provided robust protection from pulmonary replication of the homologous and heterologous challenge viruses (Fig. 3B and D). In the NTs, one dose of H1 *ca* vaccine conferred better protection against homologous challenge than one dose of H1 S-FLU vaccine, although ferrets that received the S-FLU vaccine had significantly reduced ( $P < 0.006$ ) titers of the CA/09 virus on day 4 p.c. than the mock-vaccinated animals did (Fig. 3A). Ferrets that received two doses of H1 S-FLU had significantly reduced tl/97 virus titers in the NTs ( $P < 0.022$ ) by day 4 p.c., while the H1 *ca* vaccine conferred near-complete protection (Fig. 3C). We detected a modest homologous NtAb response after one dose of H1 S-FLU vaccine (geometric mean titer [GMT], 90), with a slight increase after the second dose (GMT, 158), but not against the heterologous H6N1 virus. The H1 *ca* vaccine elicited a robust homologous NtAb response with GMTs of 273 and 974 after the first and second dose, respectively, but again, cross-reactive antibodies against the H6N1 virus were not detected (see Fig. S2B in the supplemental material). Because the NAs of the tl/97 and CA/09 viruses are both of the N1 subtype and NA-inhibiting antibodies (NAI) are associated with protection against influenza and correlate with reduced viral shedding and disease symptoms (16, 17), we hypothesized that cross-reactive NA immunity could contribute to protection conferred

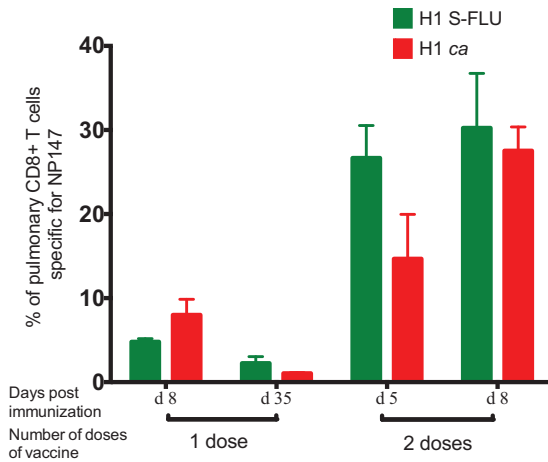


**FIG 1** Protective efficacy of H1 S-FLU and H1 *ca* vaccines against lethal homologous and heterologous challenge in BALB/c mice. (A and D) Weight loss in mice (five mice in each group) inoculated intranasally with one or two doses of 50  $\mu$ l of VGM containing  $3.42 \times 10^5$  TCID<sub>50</sub> of H1 S-FLU vaccine or  $10^6$  TCID<sub>50</sub> of H5 *ca* vaccine and challenged with  $10^6$  TCID<sub>50</sub>/50  $\mu$ l of CA/09 (H1N1) or  $10^5$  TCID<sub>50</sub>/50  $\mu$ l of the tl/97 (H6N1) virus. Animals were monitored daily for weight loss, and mortality was recorded over a period of 14 days. Mice were euthanized when they lost 25% of their original body weight. A † symbol indicates that a mouse died on the specified day. (B, C, E, and F) Protection conferred by the H1 S-FLU and H1 *ca* vaccines against homologous virus (B and C) and heterologous virus (E and F) challenge. Animals (four mice in each group) were i.n. inoculated with either VGM (mock) or one or two doses of  $3.42 \times 10^5$  TCID<sub>50</sub> of H1 S-FLU or  $10^6$  TCID<sub>50</sub> of H1 *ca* vaccines and challenged 28 days following the last vaccine dose with  $10^6$  TCID<sub>50</sub>/50  $\mu$ l of CA/09 virus or  $10^5$  TCID<sub>50</sub>/50  $\mu$ l of the tl/97 virus. Virus titers were determined on day 2 (d2) and day 4 (d4) postchallenge in the nasal turbinates (NTs) or lungs of mice. In panels B, C, E, and F, each symbol represents the titer for an individual mouse, and each horizontal bar depicts the mean titer for a group of mice. The dotted horizontal line shows the lower limit of detection ( $10^{1.8}$  TCID<sub>50</sub>/g for NTs and  $10^{1.5}$  TCID<sub>50</sub>/g for lungs).

by the H1 S-FLU vaccine. We measured NAI titers by enzyme-linked lectin assay (ELLA) and observed that ferrets immunized with two doses of H1 S-FLU vaccine had detectable, although lower, homologous NAI titers than those immunized with H1 *ca* vaccine, with GMTs of 50 and 113, respectively. However, NAI titers against the heterologous tl/97 virus were barely detected (GMT, 6.3) after two doses of H1 S-FLU vaccine, and a titer of 44 was observed after two doses of H1 *ca* vaccine (see Table S1 in the supplemental material). Thus, NAI antibodies are not likely to have played a role in H1 S-FLU-mediated heterosubtypic protec-

tion, and we infer that protection was conferred by cellular immunity. In summary, the H1 S-FLU vaccine conferred robust protection against pulmonary replication of homologous and heterologous challenge viruses, and one dose of H1 *ca* was more effective than one dose of H1 S-FLU. However, that may not be surprising, because *ca* vaccines induce antibodies as well as T cell responses.

**H1 S-FLU-vaccinated ferrets do not transmit challenge virus via the airborne route.** Since ferrets immunized with one dose of H1 S-FLU vaccine had high titers of homologous challenge virus



**FIG 2** H1 S-FLU and H1 *ca* vaccines prime mice for robust recall of pulmonary CD8<sup>+</sup> CTL responses. To evaluate the recall of cellular responses following vaccination with H1 S-FLU, groups of mice (four mice in each group) were immunized with H1 S-FLU or H1 *ca* vaccine. Mice received one or two doses of H1 S-FLU or H1 *ca* vaccine, and on days 8 and 35 after the first dose or days 5 and 8 after the second dose, lungs were harvested, and isolated cells from the tissue were stained with CD3-PerCP, CD8-APC, CD19-FITC, and NP147-155 pentamer-PE. The number of CD8<sup>+</sup> NP147-155 pentamer plus T cells was enumerated by flow cytometry, and the percentage was determined. A minimum of 50,000 lymphocytes were analyzed for lung samples.

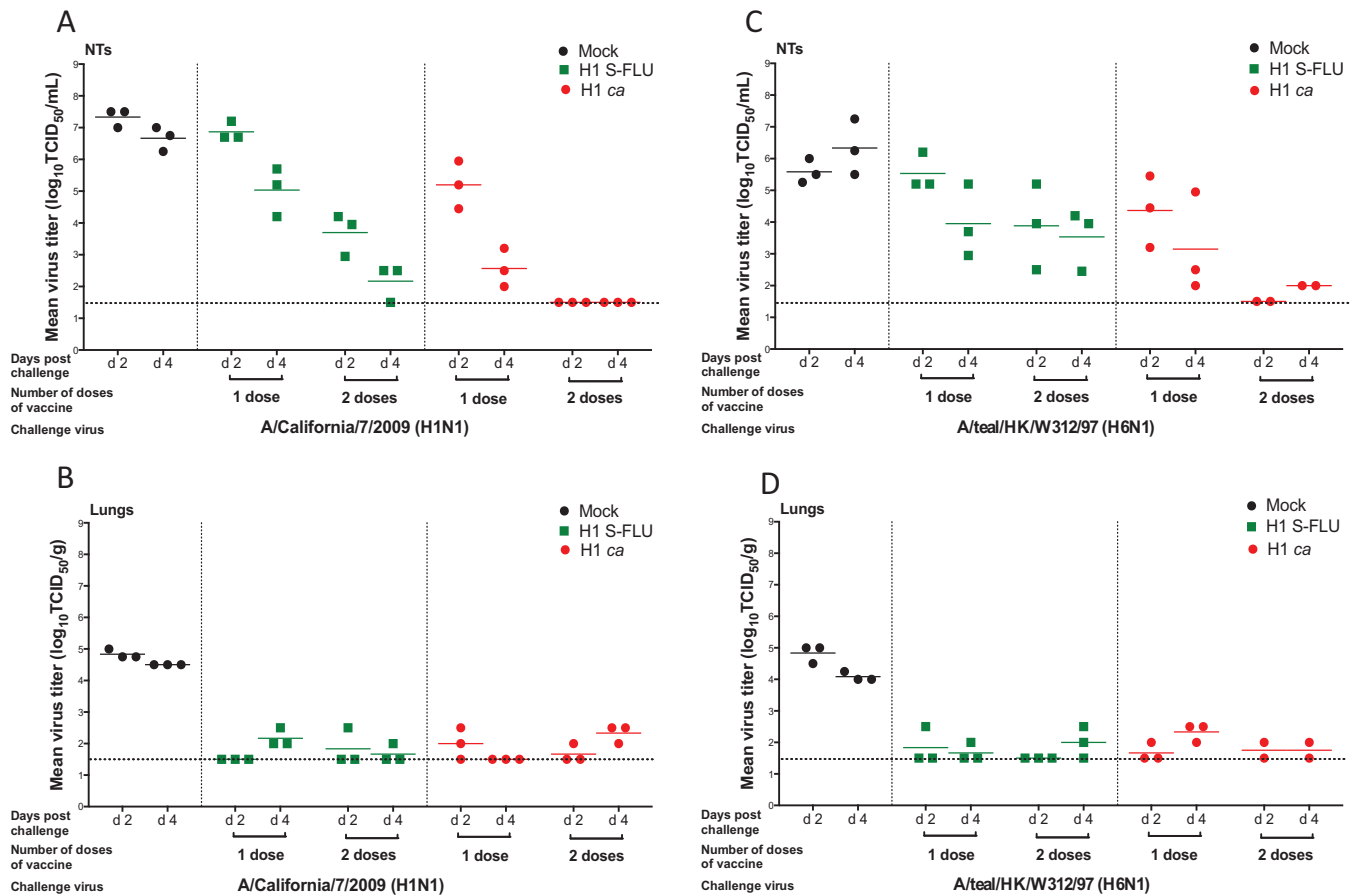
in the NTs but robust protection in the lungs, we asked whether the S-FLU vaccine would prevent airborne transmission of the challenge virus to naive ferrets housed in an adjacent cage that were monitored for virus replication in nasal secretions and seroconversion on day 14 postexposure. We compared transmission in these ferret pairs with ferrets that were either mock vaccinated or vaccinated with one dose of LAIV. All three airborne-contact ferrets in the mock-vaccinated group shed virus in the nasal secretions, but only two seroconverted. The airborne-contact ferret that did not seroconvert likely had insufficient time to mount an antibody response, because virus was detected only on days 10 and 13 postexposure and sera were collected on day 14 (Fig. 3E, panel iv; see Table S2 in the supplemental material). However, one dose of the H1 S-FLU or LAIV vaccines (Fig. 3E, panels i and iii) prevented airborne transmission of the challenge virus (Fig. 3E and Table S2).

**H5 S-FLU vaccine protects mice and ferrets from homologous and heterosubtypic virus challenge.** Because H1 S-FLU vaccine was promising in mice and ferrets, we developed an H5 S-FLU vaccine candidate to evaluate a more virulent challenge and to assess protection against multiple clades within a subtype and across subtypes. In preliminary experiments (data not shown), we established that the H5 S-FLU vaccine protected BALB/c mice from challenge with the highly virulent mouse-adapted PR8 Cambridge strain (H1N1) and X31 (H3N2). We now compared H5 S-FLU and H5 *ca* vaccines employing a more rigorous challenge with wild-type H5N1 and H7N9 IAV. In general, the titers of the three challenge viruses were much higher in the lungs than in the NTs (Fig. 4). Mock-immunized mice and mice immunized with one dose of H5 S-FLU vaccine that were challenged with the homologous virus (VN/04, H5N1 clade 1) died on day 5 or 6 p.c. In contrast, 80% of the mice immunized with two doses of H5 S-FLU vaccine recovered from 14% weight loss that peaked on day 3 and

survived (Fig. 4A). One dose of H5 S-FLU conferred near-complete protection in the NTs (Fig. 4B) and prevented dissemination of the virus to the brain (data not shown) but was not sufficient to prevent infection in the lungs (Fig. 4C); in contrast, mice immunized with one dose of the H5 *ca* vaccine completely cleared the virus in the NTs and lungs by day 5 p.c. Two doses of the H5 S-FLU vaccine also led to early pulmonary clearance of the homologous challenge virus (Fig. 4B and C).

Mock-immunized mice and mice immunized with one dose of H5 S-FLU vaccine that were challenged with the mismatched Indo/05 (H5N1, clade 2.1) virus died, while 60% of the mice that received two doses of H5 S-FLU vaccine survived, and mice that received two doses of the H5 *ca* vaccine experienced minimal weight loss and survived (Fig. 4D). Notably, we observed a statistically significant reduction in the challenge virus titer in the NTs ( $P < 0.0016$ ) but not in the lungs ( $P = 0.87$ ) after one dose of H5 S-FLU vaccine on day 5 p.c. However, in mice that received two doses of H5 S-FLU vaccine, nearly complete clearance or significant reduction ( $P < 0.0001$ ) of the virus was observed on day 5 p.c. in the NTs and lungs, respectively (Fig. 4E and F). Mice immunized with one dose of H5 *ca* vaccine completely cleared the challenge virus by day 5 p.c. in both the upper and lower respiratory tracts (Fig. 4E and F). Dissemination of the challenge virus to the brain was not observed in vaccinated mice (data not shown). Interestingly, one dose of H5 S-FLU vaccine was sufficient to prevent any deaths in mice following heterosubtypic challenge with the Anhui/13 (H7N9) virus (Fig. 4G); this corresponded with a statistically significant decrease in H7N9 virus replication in the NTs and lungs ( $P = 0.006$  and  $P = 0.016$ , respectively) on day 3, with a further reduction in titers on day 5 p.c. (Fig. 4H and I). Two doses of either vaccine conferred robust and equivalent protection in the NTs and early clearance from the lungs of mice. The H5 S-FLU vaccine did not elicit an antibody response against the homologous virus or two heterologous challenge viruses in mice (see Fig. S2C in the supplemental material).

We then evaluated whether two doses of the H5 S-FLU or H5 *ca* vaccines would protect ferrets from challenge with the homologous VN/04 virus or mismatched Indo/05 (H5N1) and Anhui/13 (H7N9) viruses. We did not evaluate a single dose of the vaccines, because we had previously demonstrated that two doses of H5 *ca* vaccine were necessary for protection from pulmonary replication in ferrets (15). Two doses of H5 S-FLU vaccine conferred robust protection from replication of the homologous VN/04 virus in the NTs, lungs (Fig. 5A and B), and brain (data not shown) with complete clearance by day 5 p.c. With cross-clade challenge, Indo/05 (H5N1) virus titers in the NTs and lungs of H5 S-FLU-immunized ferrets were not significantly lower than those of mock-immunized ferrets on day 3 p.c. (Fig. 5A and B). However, virus was not detected in the lungs of ferrets immunized with H5 *ca* vaccine (Fig. 5B). Because we examined only the early time point (day 3) after cross-clade challenge, we do not know whether the challenge virus would have been cleared by a later time point, e.g., day 5, in H5 S-FLU-vaccinated ferrets. Virus was detected at a low titer ( $10^2$  TCID<sub>50</sub> [50% tissue culture infective doses]/g of body weight) in the brain of only one of three ferrets immunized with H5 S-FLU vaccine, compared to all mock-vaccinated ferrets ( $10^{2.7}$ ,  $10^{4.2}$ , and  $10^{4.2}$  TCID<sub>50</sub>/g) (data not shown). Interestingly, both vaccines provided robust heterosubtypic protection against the Anhui/13 (H7N9) virus, with near-complete clearance in the NTs and undetectable virus in the lungs of ferrets on day 5 p.c. In



**FIG 3** H1 S-FLU and H1 *ca* vaccines protect ferrets against homologous and heterologous virus challenge and prevent airborne transmission of the homologous challenge virus to naive ferrets housed in an adjacent cage. (A to D) Twelve-week-old ferrets were anesthetized with intramuscular injection of a ketamine-xylazine mixture prior to intranasal virus inoculation with one or two doses (28 days apart) of  $3.42 \times 10^6$  or  $10^7$  TCID<sub>50</sub> of H1 S-FLU or H1 *ca* vaccine, respectively. Mock-inoculated controls received virus growth medium (VGM) (DMEM with penicillin, streptomycin, and 0.1% BSA) alone. (A to D) Animals were challenged with  $10^6$  TCID<sub>50</sub> of CA/09 (H1N1) virus (A and B) or tl/97 (H6N1) virus (C and D), and virus titers in the nasal turbinates (NTs) (A and C) and lungs (B and D) of the ferrets (three ferrets in each group) sacrificed on 2 and 4 days postinfection (dpi) are expressed as log<sub>10</sub> TCID<sub>50</sub>/gram of tissue. Two animals immunized with the H1 *ca* vaccine did not recover from anesthesia after bleeding on day 28; therefore, there are only four ferrets in the group after dose 2. Each symbol represents the titer for an individual ferret, and each horizontal bar represents the mean titer for a group of ferret. The dotted horizontal line indicates the lower limit of detection,  $10^{1.5}$  TCID<sub>50</sub> per gram for the NT and lungs. (E, i–iv) Three 5- to 8-month-old male adult ferrets were anesthetized as described above for panels A to D and immunized with one or two doses of  $3.42 \times 10^6$  TCID<sub>50</sub> of H1 S-FLU vaccine or one dose of  $10^7$  TCID<sub>50</sub> H1 *ca* vaccine in 500  $\mu$ l of VGM or VGM alone (mock immunized). Twenty-eight days after the first or second dose, ferrets were challenged with  $10^6$  TCID<sub>50</sub> of CA/09 virus. Challenged ferrets were placed in the section of the cage closest to the air inlet the day of challenge. One day later, a naive ferret was placed into the cage on the other side of the divider. Nasal washes were collected every other day for 14 days, and virus titers in the experimentally infected ferrets and ferrets with airborne contact are presented. Each bar represents a single ferret.

general, the efficacies of two doses of H5 S-FLU and H5 *ca* vaccines were comparable in ferrets (Fig. 5A and B). Unlike the H1 S-FLU vaccine, the H5 S-FLU vaccine did not elicit a detectable NtAb response in ferrets, likely because the H5 HA is a poor immunogen. In contrast, the H5 *ca* vaccine induced NtAb against the homologous VN/04 virus with a GMT of 248, but cross-reactivity against the Indo/05 H5N1 (GMT, 31) was poor, and cross-reactivity against Anhui/13 (H7N9) was not detected (see Fig. S2D in the supplemental material). In the absence of antibody, T cell responses to well-conserved peptide epitopes, such as those in the NP (nucleoprotein), M1 (matrix protein 1), PB1 (polymerase basic protein 1), PB2, and PA (polymerase acidic protein) proteins (Table S3), play an important role in promoting viral clearance and reducing disease severity. The pattern of early clearance of challenge virus and more effective protection in the lower respi-

ratory tract than in the upper respiratory tract of ferrets after S-FLU vaccines is consistent with T cell-mediated protection. It is likely that mucosal antibody responses contribute to the protection in the upper respiratory tract.

## DISCUSSION

Vaccines that are broadly cross-protective are needed for seasonal and pandemic influenza. T cell responses to highly conserved internal proteins of IAV that confer broad cross-protection can be induced by live virus vaccines. Nonreplicating S-FLU vaccines performed well in this regard.

The S-FLU vaccines are comparable to vaccines such as recombinant adenovirus (rAd) (18–20) and modified vaccinia virus Ankara (MVA) vectored vaccines expressing NP and M2 proteins (21–23) in mediating protection via T cells. However, preexisting

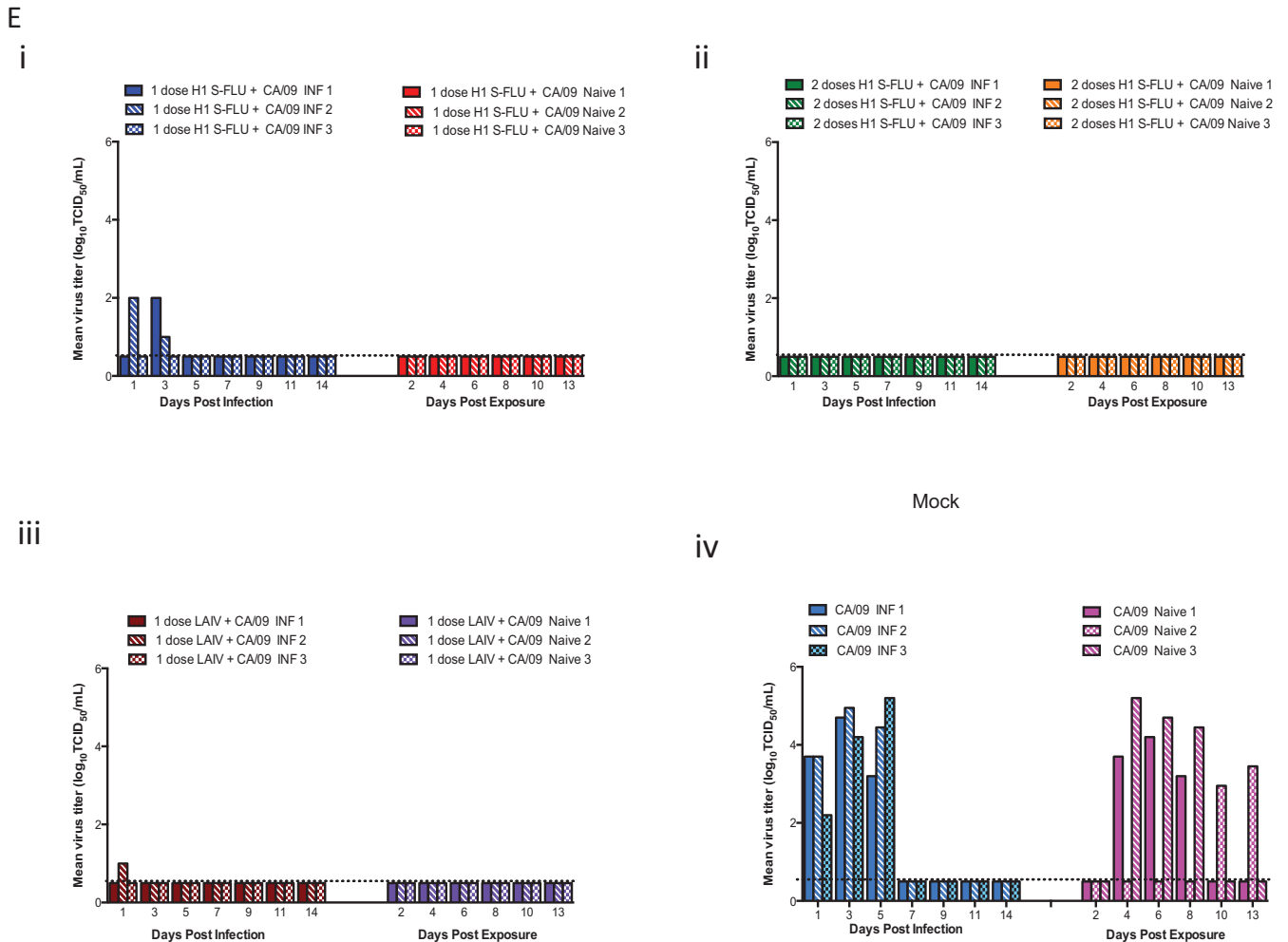


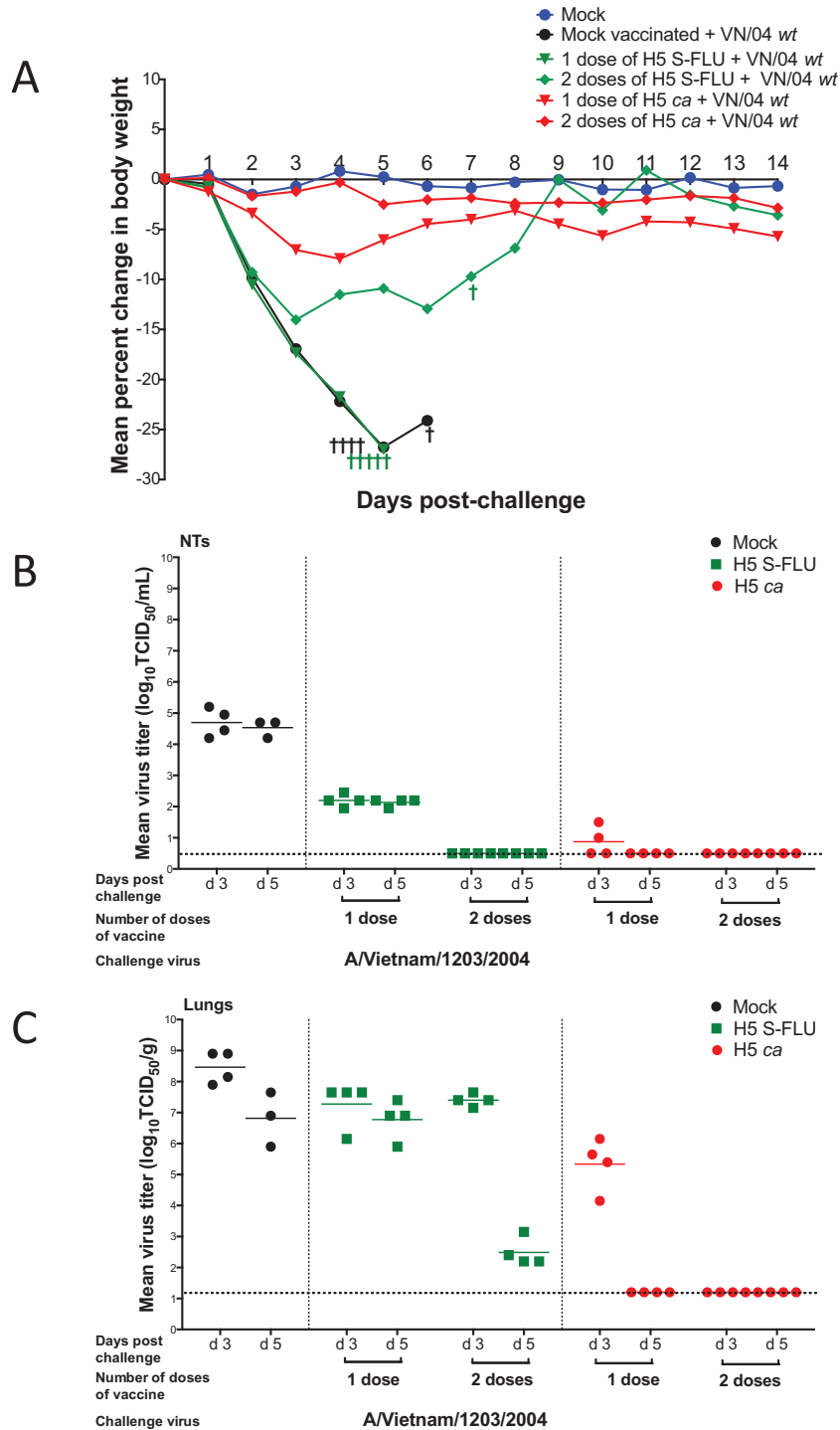
FIG 3 continued

antibodies (Abs) to Ad in humans may limit the use of rAd vectors, and a dose of  $\sim 1 \times 10^{10}$  particles of each rAd is needed to prevent weight loss in vaccinated animals (18). The MVA vaccine showed only weak protection from mild upper respiratory infection in human challenge studies with an H3N2 virus (24), and the combination of Ad and MVA was unable to prevent death after challenge with low doses of A/PR/8/34 virus in mice (25). By comparison, S-FLU vaccine can protect against lethal challenge with a 1,000-fold-greater dose of the same virus (12). In addition, other vaccine candidates based on influenza viruses that are highly attenuated (26) or restricted to a single cycle of replication (27) elicited heterosubtypic immunity (mostly in the absence of neutralizing antibodies), suggesting that T cell-based protection was induced. However, none of these preparations have been evaluated head to head with a live attenuated influenza virus vaccine as in the present study. The potential advantages of S-FLU vaccines are as follows. (i) They can infect but not replicate in nontransfected cells; therefore, immune responses can be induced with a rigorously controlled infection in the lungs. (ii) They are based on inactivation of the vRNA encoding HA and thus do not contain a viable HA vRNA that could reassort with seasonal influenza viruses, as in the case of LAIV. (iii) They can be pseudotyped with

any HA in the envelope. (iv) They express all of the conserved viral proteins and NA that can induce protective immune responses. (v) They can be made by standard techniques (12, 28, 29). (vi) They are self-replicating *in vitro* for bulk production. (vii) They could be administered by small droplet aerosol to the lung because they are replication incompetent. In addition, several safeguards were engineered into the HA to minimize the probability of reversion of the signal-minus HA segment to a competent HA, including a mutated start codon, a single-base deletion at the end of the signal sequence, and replacement of arginine at the cleavage site.

The repeated incursions of avian IAV into humans highlights the need for influenza vaccines that provide broad protection against multiple IAV strains and subtypes. In the present study, we demonstrate that nonreplicating S-FLU vaccines confer protection from homologous and heterologous virus challenge in mice and ferrets. This protection is associated with a vigorous CD8 T cell response in the lung and occurs in the absence of or with a low-titer NtAb response. Importantly, despite robust replication of the challenge virus in the upper respiratory tract, airborne transmission from ferrets immunized with one dose of S-FLU vaccine to naive recipient ferrets was abolished.

In summary, S-FLU vaccines represent an important and



**FIG 4** Protective efficacy of H5 S-FLU and H5 *ca* vaccines against lethal homologous and heterologous virus challenge in BALB/c mice. (A to I) Weight loss in mice (five mice in each group) inoculated intranasally with one or two doses of  $10^6$  TCID<sub>50</sub> of H5 S-FLU or H5 *ca* vaccine and challenged with  $10^5$  TCID<sub>50</sub> of VN/04 (H5N1, clade 1), Indo/05 (H5N1, clade 2.1), or Anhui/13 (H7N9). (A, D, and G) Animals were monitored daily for weight loss, and mortality was recorded over a period of 14 days. Mice were euthanized when they lost 25% of their original body weight. A † symbol indicates that a mouse died on the specified day. (B, C, E, F, H, and I) Protection conferred by the H1 S-FLU and H1 *ca* vaccines against homologous (B and C), cross-clade (E and F), and heterosubtypic (H and I) virus challenge was assessed in the NTs or lungs of mice (four mice in each group). Animals were i.n. inoculated with either VGM (mock) or with one or two doses of  $10^6$  TCID<sub>50</sub> of H5 S-FLU or H5 *ca* vaccine and challenged 28 days following the last vaccine dose with  $10^5$  TCID<sub>50</sub> of VN/04, Indo/05, or Anhui/13. Virus titers were determined on days 3 and 5 postchallenge.

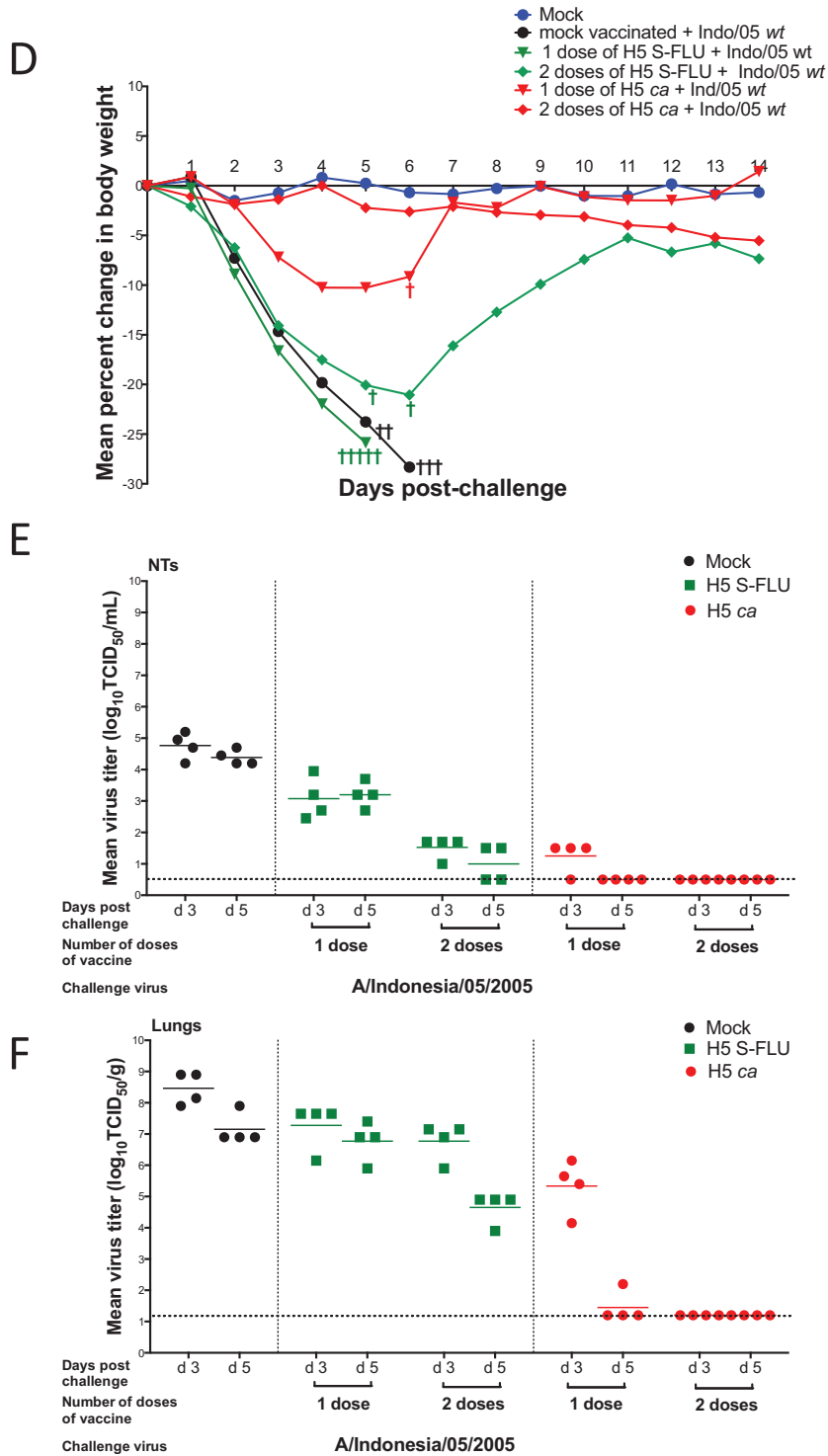


FIG 4 continued

promising advance in vaccine development; the S-FLU vaccine viruses compare well with live attenuated influenza virus vaccines and have the advantage of being engineered with several safeguards. Our results suggest that pseudotyped nonreplicating influenza viruses show great promise for eliciting broad protection against homologous and heterosubtypic viruses, and their safety and immunogenicity should be evaluated in humans.

**MATERIALS AND METHODS**

**Viruses.** The A/California/07/2009 (CA/09) (H1N1pdm), A/Vietnam/1203/2004 (VN/04) (H5N1, clade 1), A/Indonesia/05/2005 (Indo/05) (H5N1, clade 2.1) and A/Anhui/1/2013 (Anhui/13) (H7N9) viruses were provided by the Centers for Disease Control and Prevention (CDC). A/teal/HK/W312/97 (tl/97) (H6N1) was obtained from the influenza virus repository at St. Jude Children’s Research Hospital, Memphis, TN.



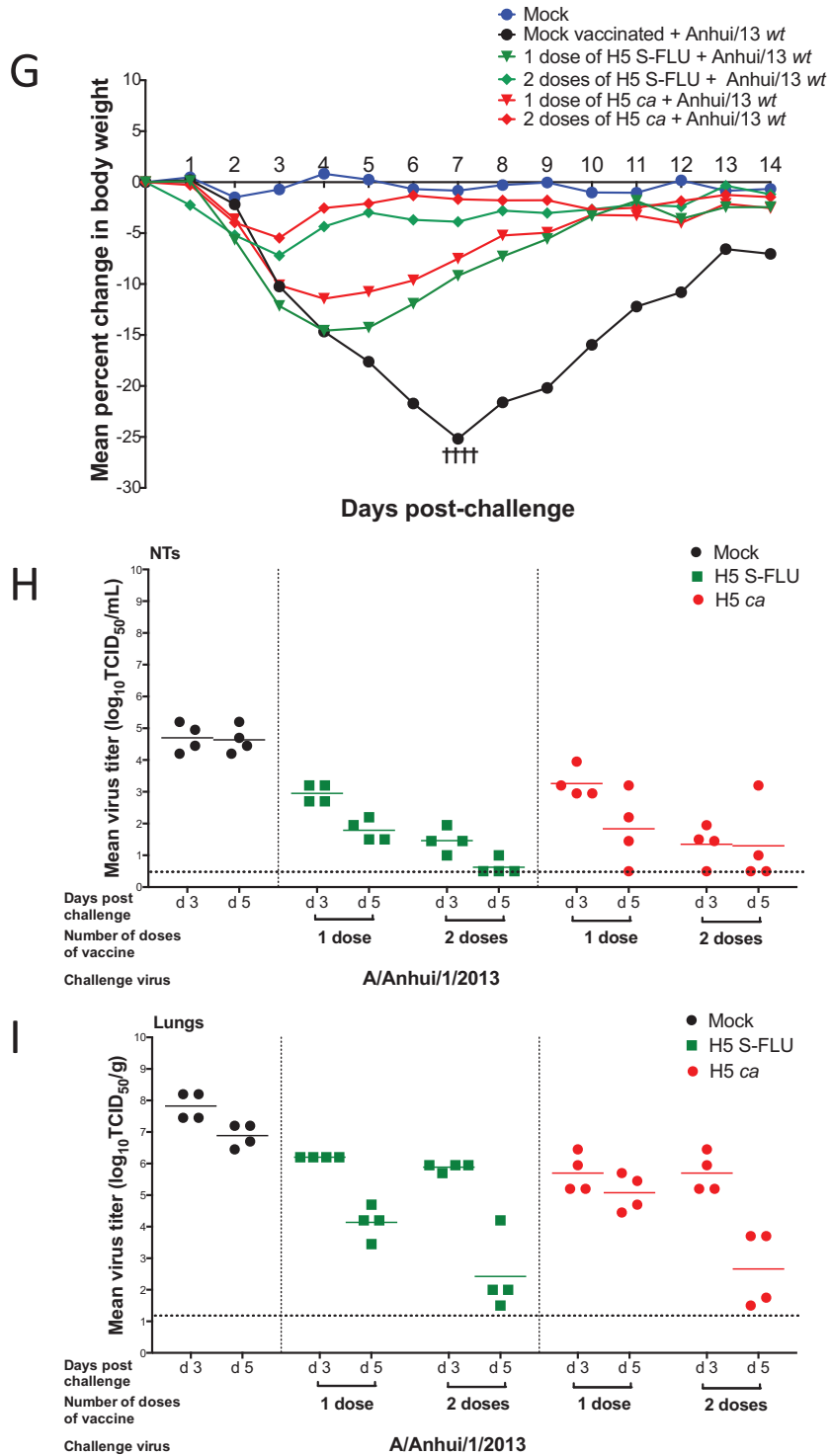
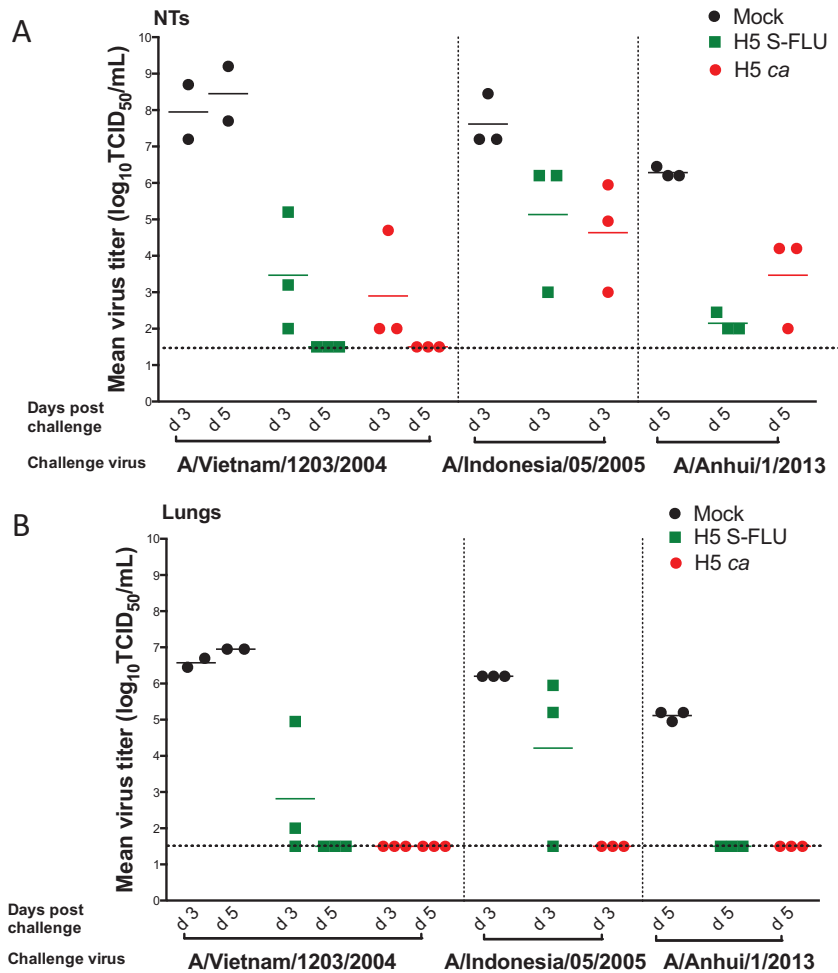


FIG 4 continued

The live attenuated cold-adapted (*ca*) reassortant, A/California/7/2009 (H1 *ca*) H1N1pdm and A/Vietnam/1203/2004 (H5 *ca*) viruses, kindly provided by Hong Jin (MedImmune, Mountain View, CA), were generated by reverse genetics as previously described (15, 30). Virus stocks were propagated in the allantoic cavities of 9- to 11-day-old embryonated specific-pathogen-free chicken eggs (Charles River Laboratories, North Franklin, CT) at 35°C. Allantoic fluid was harvested at 72 h postinocula-

tion (p.i.) or 24 p.i. for the H5N1 viruses, tested for hemagglutinating activity using 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA), pooled, aliquoted, and stored at -80°C until use. Virus titers were determined in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) and calculated using the Reed and Muench method (31). The HA amino acid sequence identity of the NP and M1 genes from PR8 and A/Ann Arbor/6/60 *ca* virus versus each of the chal-



**FIG 5** H5 S-FLU and H5 *ca* vaccines protect ferrets against homologous and heterologous virus challenge. (A and B) Twelve-week-old ferrets were anesthetized with intramuscular injection of a ketamine-xylazine mixture prior to intranasal virus inoculation with one or two doses (28 days apart) of  $10^7$  TCID<sub>50</sub> of H5 S-FLU or H5 *ca* vaccine. Mock-inoculated controls received VGM. Animals were challenged with  $10^7$  TCID<sub>50</sub> of VN/04 (H5N1, clade 1), Indo/05 (H5N1, clade 2.1), or Anhui/13 (H5N1) wt viruses in 500  $\mu$ l of VGM, and virus titers in the NTs and lungs of ferrets (three ferrets in each group) sacrificed on days 3 and 5 postinfection (p.i.) (VN/04), day 3 p.i. (Indo/05), and day 5 p.i. (Anhui/13) were determined. Titters are expressed as log<sub>10</sub> TCID<sub>50</sub>/gram of tissue. Each symbol represents the titer for an individual ferret, and each horizontal bar represents the mean titer for a group of ferrets. Two mock-vaccinated ferrets challenged with VN/04 showed clinical signs (lethargy, labored breathing, and uncontrolled movements) of severe infection and were sacrificed on days 3 and 4 postchallenge. The dotted horizontal line indicates the lower limit of detection,  $10^{1.5}$  TCID<sub>50</sub> per gram for the NT and lungs.

challenge viruses (CA/09, tl/97, VN/04, Indo/05, and Anhui/13) is represented in Table S3 in the supplemental material.

**Generation of S-FLU vaccine candidates.** The construction of H1 (A/England/195/2009 [A/Eng/195/2009]) S-FLU has been described by Powell et al. (12). Its formal name was [S-eGFP/N1(Eng195)].H1(Eng195), signifying that it contained a signal inactivated HA gene with coding sequence replaced with enhanced green fluorescent protein (eGFP), the NA gene from A/Eng/195/2009, and all other genes from A/PR/8/34 and was coated in H1 protein from A/Eng/195/2009. The H5 S-FLU was constructed similarly. The homologous N1 NA from the A/Vietnam/1203/2004 (H5N1) virus was not incorporated into the H5 S-FLU to alleviate concerns that complete vRNAs from pandemic strains in S-FLU might present a risk of reassortment with seasonal influenza viruses. Briefly, MDCK-SIAT1 cells (29) were transduced to stably express a codon-optimized cDNA (lacking 3' and 5' untranslated regions [UTRs]) encoding the A/Vietnam/1203/2004 sequence (GenBank accession no. EF541403.1) with the polybasic cleavage site removed and replaced with a trypsin site (H5\*t). An H1 (PR8) pseudotyped S-FLU [S-eGFP/N1(PR8)].H1(PR8) produced earlier (12) was seeded onto the H5\*t-SIAT

cells, and virus was grown in the presence of trypsin as described previously (12) to produce [S-eGFP/N1(PR8)].H5\*t (VN1203) with a titer of  $2 \times 10^8$  TCID<sub>50</sub>/ml.

**Serologic assays. (i) Microneutralization (MN) assay.** Serial twofold dilutions of heat-inactivated serum were prepared starting from a 1:20 dilution. Equal volumes of serum and virus were mixed and incubated for 60 min at room temperature. The residual infectivity of the virus-serum mixture was determined in MDCK cells using four wells for each dilution of serum. Neutralizing antibody (NtAb) titer was defined as the reciprocal of the serum dilution that completely neutralized the infectivity of 100 TCID<sub>50</sub> of the virus as determined by the absence of cytopathic effect on MDCK cells at day 4.

**(ii) Hemagglutination inhibition.** Antibody titers in postinfection ferret sera were determined by hemagglutination inhibition (HAI) assays according to standard protocols (32). Nonspecific inhibitors were removed from serum by overnight treatment with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan). The HAI titer was recorded as the reciprocal of the highest dilution of serum that completely inhibited agglutination of turkey red blood cells.

(iii) **Neuraminidase inhibition assay.** An enzyme-linked lectin assay (ELLA) was performed to measure NA inhibiting antibodies in sera from ferrets immunized with one or two doses of H1 LAIV or H1 S-FLU vaccine. To measure neuraminidase inhibiting (NAI) antibody titers, reassortant viruses containing the N1 of the CA/09 or tl/97, HA from A/Udorn/307/72 (H3N2), and the remaining gene segments from A/Ann Arbor/6/60 *ca* (H2N2) were created using plasmid-based reverse genetics (33). The NAI assay was performed as described previously using fetuin as the substrate (34), and the NAI titer was defined as the inverse of the highest dilution that gave at least 50% inhibition of NA activity.

**Flow cytometry.** A FACSCalibur instrument (BD Biosciences) was used to evaluate the cellular immune response in mice. Animals were lightly anesthetized and inoculated intranasally (i.n.) with one or two doses (28 days apart) of  $3.42 \times 10^5$  TCID<sub>50</sub> of H1 S-FLU (maximum dose possible) or  $10^6$  TCID<sub>50</sub> of the H1 *ca* vaccines in 50  $\mu$ l of growth medium (the growth medium was virus growth medium [VGM], which was Dulbecco modified Eagle medium [DMEM] with penicillin, streptomycin, and 0.1% bovine serum albumin [BSA]). To examine the primary responses induced by a single dose of each vaccine, mice were killed on day 8 or 35 after the first dose. Memory responses were evaluated on day 5 or 8 after the second dose with the same vaccines. The lungs were collected, and isolated cells from the tissue were stained with CD3-PerCP (CD3 labeled with peridinin chlorophyll protein [PerCP]) (clone 145-2C11), CD8-APC (CD8 labeled with allophycocyanin [APC]) (clone 53-6.7), CD19-FITC (CD19 labeled with fluorescein isothiocyanate [FITC]) (clone 1D3), and NP147-155 pentamer (NP147-155 pentamer labeled with PE, H-K2d from ProImmune Inc.). The percentage of CD8<sup>+</sup> NP147-155 pentamer plus T cells was enumerated by flow cytometry. A minimum of 50,000 lymphocytes were analyzed for lung samples.

**Mouse and ferret vaccination protocols.** Six- to 8-week-old female BALB/c mice (Taconic Farms, Inc., Germantown, NY) and 10- to 12-week-old ferrets (Triple F Farms, Sayre, PA) were used in all mouse and ferret experiments. For mouse immunization, animals were lightly anesthetized and inoculated i.n. with one or two doses (28 days apart) of  $10^6$  TCID<sub>50</sub> of the H1 or H5 *ca* or H5 S-FLU vaccine and  $3.42 \times 10^5$  TCID<sub>50</sub> of H1 S-FLU vaccine in 50  $\mu$ l of VGM. Mock-inoculated controls received VGM alone. The doses of *ca* vaccine viruses and challenge viruses were based on our previous studies (14, 15, 35).

Serum NtAb responses to homologous (CA/09 and VN/04) and heterologous (tl/97, Indo/05, and Anhui/13) viruses were determined prior to inoculation (prebleed) and 28 days after the first or second dose of vaccine by MN assay as previously described. On day 28 after one or two doses of vaccine, groups of eight mice immunized with H1 *ca* or H1 S-FLU vaccine were challenged i.n. with  $10^6$  TCID<sub>50</sub>/50  $\mu$ l of CA/09 virus or  $10^5$  TCID<sub>50</sub>/50  $\mu$ l of the tl/97 virus and sacrificed on days 2 and 4 postchallenge (p.c.), as previously described (14, 35), and lungs and nasal turbinates (NTs) were harvested and stored at  $-80^{\circ}\text{C}$ .

Mice immunized with H5 *ca* or H5 S-FLU vaccine were challenged with  $10^5$  TCID<sub>50</sub>/50  $\mu$ l of wild-type (wt) VN/04, Indo/05, or Anhui/13 virus and sacrificed on day 3 or 5 p.c. to harvest lungs and NTs. We chose these time points based on our previous observations of the kinetics of replication of these wt viruses. Lungs and NTs were weighed and homogenized in Leibovitz 15 (L15) medium containing 2 $\times$  antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B) (Invitrogen-Gibco) to make 10% and 5% (wt/vol) tissue homogenates, respectively. Tissue homogenates clarified by centrifugation were titrated on MDCK cell monolayers, and infectivity was determined by recording the presence of cytopathic effect (CPE). The dilution at which 50% of the wells were infected (50% tissue culture infective dose [TCID<sub>50</sub>]) was computed using the Reed and Muench method (31), and titers were expressed as log<sub>10</sub> TCID<sub>50</sub>/gram of tissue.

Morbidity was monitored by body weight loss after immunization, as well as by clinical signs of infection (hunching, ruffling of fur, malaise, or respiratory distress). Each day, percent body weight was determined relative to the starting weight prior to manipulation. Mice losing greater than

25% body weight were euthanized humanely, and some mice were found dead.

For ferret (12-week-old) immunization, animals were seronegative for HAI antibodies to circulating H3N2, H1N1, and B human influenza viruses. Ferrets were anesthetized by intramuscular (i.m.) injection of a ketamine-xylazine mixture prior to i.n. virus inoculation with one or two doses (28 days apart) of  $10^7$  TCID<sub>50</sub> of the H1 or H5 *ca* or H5 S-FLU vaccine and  $3.42 \times 10^6$  TCID<sub>50</sub> (maximum dose possible) of the H1 S-FLU vaccine in 500  $\mu$ l of VGM. Mock-inoculated controls received L15 medium alone. Serum NtAb responses to homologous (CA/09 and VN/04) and heterologous (tl/97, Indo/05, and Anhui/13) wt viruses were determined prior to inoculation (prebleed) and 28 days after the first or second dose of vaccine by MN assays. On day 28 after one or two doses of vaccine, groups of three ferrets immunized with H1 *ca* or H1 S-FLU vaccine were challenged i.n. with  $10^6$  TCID<sub>50</sub> of CA/09 or tl/97 virus and sacrificed on days 2 and 4 p.c., and their lungs and NTs were harvested and stored at  $-80^{\circ}\text{C}$ . Ferrets immunized with H5 *ca* or H5 S-FLU vaccine were challenged with  $10^7$  TCID<sub>50</sub> of VN/04, Indo/05, or Anhui/13 virus and sacrificed on days 3 and 5 p.c. to harvest lungs and NTs. We chose these time points based on our previous observations of the kinetics of replication of these viruses. Animals that showed signs of severe disease were sacrificed. Tissues were homogenized in a 10% (wt/vol) suspension that was clarified by centrifugation at 1,500 rpm for 10 min and titrated in 24- and 96-well tissue culture plates containing MDCK cell monolayers. Infectivity was determined as described above, and titers are expressed as log<sub>10</sub> TCID<sub>50</sub>/gram of tissue.

Animal studies were conducted in biosafety level 2 laboratories (BSL-2) or BSL-3 (for H5N1 and H7N9 challenge) at the National Institutes of Health (NIH), and protocols were approved by the National Institutes of Health Animal Care and Use Committee and in accordance with the Select Agent guidelines of the NIH, CDC, and U.S. Department of Agriculture (USDA).

**Transmission studies in ferrets.** We performed airborne transmission studies using a caging system developed in our lab (36). Briefly, three 5- to 8-month-old male adult ferrets obtained from Triple F farms (Sayre, PA) that were seronegative by HAI assay for seasonal influenza H1N1, H3N2, and B viruses were anesthetized by i.m. injection of a ketamine-xylazine mixture prior to i.n. immunization with one or two doses of  $3.42 \times 10^6$  TCID<sub>50</sub> of H1 S-FLU vaccine or with one dose of  $10^7$  TCID<sub>50</sub> H1 *ca* vaccine in 500  $\mu$ l of VGM or with VGM alone (mock immunized). Twenty-eight days after the first or second dose, ferrets were challenged with  $10^6$  TCID<sub>50</sub> of A/California/07/2009 virus. Challenged ferrets were placed into the section of the cage closest to the air inlet the day of challenge. One day later, a naive ferret was placed into the cage on the other side of the divider. Environmental conditions inside the laboratory were monitored daily; the temperature was consistently  $19^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ , and the relative humidity was  $60\% \pm 2.2\%$ . The transmission experiments were conducted in the same room to minimize any effects of caging and airflow differences on aerobiology. The naive ferret was always handled before the infected ferret. Animals were carefully handled during nasal wash collections and husbandry in order to ensure that no direct contact occurred between the ferrets. Nasal washes were collected every other day for 14 days by washing the right nostril of an anesthetized ferret with sterile phosphate-buffered saline (PBS), and 1,000 to 500  $\mu$ l of liquid was expelled from the left nostril. These nasal secretions were analyzed for the presence and titer of infectious viruses and expressed as 50% tissue culture infectious doses (TCID<sub>50</sub>) per ml. On day 14 postinfection, blood was collected from each animal, and serology was performed by HAI and MN assays.

**Statistics.** The significance of differences between different groups was assessed with two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test using Prism 5 (GraphPad Software, Inc.). A *P* value of less than 0.05 was considered significant.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01487-15/-/DCSupplemental>.

- Figure S1, TIF file, 1.5 MB.
- Figure S2, TIF file, 1.5 MB.
- Table S1, DOCX file, 0.1 MB.
- Table S2, DOCX file, 0.1 MB.
- Table S3, DOCX file, 0.1 MB.

## ACKNOWLEDGMENTS

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