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An influenza mRNA vaccine protects ferrets from lethal infection with highly pathogenic avian influenza A(H5N1) virus

Masato Hatta^{1,†}, Yasuko Hatta^{1,†}, Angela Choi^{2,†}, Jaber Hossain¹, Chenchen Feng¹, Matthew W. Keller¹, Jana M. Ritter³, Ying Huang¹, Emma Fang^{1,4}, Elizabeth A. Pusch¹, Thomas Rowe¹, Juan A. De La Cruz¹, Monique C. Johnson¹, Jimma Liddell¹, Nannan Jiang¹, Daniel Stadlbauer², Li Liu², Arindam K. Bhattacharjee², Joseph R. Rouse¹, Michael Currier¹, Li Wang¹, Min Z. Levine¹, Marie K. Kirby¹, John Steel¹, Han Di¹, John R. Barnes¹, Carole Henry², C. Todd Davis¹, Raffael Nachbagauer², David E. Wentworth¹, Bin Zhou^{1,*}

¹Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30329, USA.

²Moderna Inc., Cambridge, MA 02142, USA.

³Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30329, USA.

⁴Decatur High School, Decatur, GA 30030, USA.

Abstract

The global spread of the highly pathogenic avian influenza (HPAI) A(H5N1) virus poses a serious pandemic threat, necessitating the swift development of effective vaccines. The success of messenger RNA (mRNA) vaccine technology in the COVID-19 pandemic, marked by its rapid development and scalability, demonstrates its potential for addressing other infectious threats, such as HPAI A(H5N1). We therefore evaluated mRNA vaccine candidates targeting panzootic influenza A(H5) clade 2.3.4.4b viruses, which have been shown to infect a range of mammalian species, including most recently being detected in dairy cattle. Ferrets were immunized with mRNA vaccines encoding either hemagglutinin alone or hemagglutinin and

*Corresponding author. bzhou@cdc.gov.

†These authors contributed equally to this work.

Author contributions: R.N., D.E.W., and B.Z. conceptualized and supervised the study. M.H., Y. Hatta, J.H., C.F., Y. Huang, E.A.P., T.R., J.A.D.L.C., M.C.J., and B.Z. performed in vivo animal studies; M.H., Y. Hatta, C.F., Y. Huang, J.R.R., M.C., and L.W. performed viral titrations and antibody titrations. M.W.K., M.C.J., and J.L. performed RNA quantification. J.M.R. performed pathological analysis. D.S., L.L., and A.K.B. performed the NAI assay. A.C. led the production of mRNA-LNP vaccines. C.F. and N.J. produced recombinant proteins. M.H., E.F., and B.Z. performed data analysis. M.H. and B.Z. wrote the original draft of the manuscript. M.H., Y. Hatta, A.C., M.W.K., J.M.R., Y. Huang, E.F., M.Z.L., M.K.K., J.S., H.D., J.R.B., C.H., C.T.D., R.N., D.E.W., and B.Z. reviewed and edited the manuscript.

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neuraminidase, derived from a 2.3.4.4b prototype vaccine virus recommended by the World Health Organization. Kinetics of the immune responses, as well as protection against a lethal challenge dose of A(H5N1) virus, were assessed. Two doses of mRNA vaccination elicited robust neutralizing antibody titers against a 2022 avian isolate and a 2024 human isolate. Further, mRNA vaccination conferred protection from lethal challenge, whereas all unvaccinated ferrets succumbed to infection. It also reduced viral titers in the upper and lower respiratory tracts of infected ferrets. These results underscore the effectiveness of mRNA vaccines against HPAI A(H5N1), showcasing their potential as a vaccine platform for future influenza pandemics.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) A(H5N1) virus poses a crucial threat to both wild and domestic bird populations, with considerable potential for cross-species transmission to mammals, including humans. The first human case of HPAI A(H5N1) infection was identified in Hong Kong in 1997 (1). Since its resurgence in 2003, the World Health Organization (WHO) has reported 889 confirmed human cases of HPAI A(H5N1) infection from 23 countries, resulting in 463 fatalities (2). A clade of HPAI A(H5), identified as 2.3.4.4b, emerged around 2013, leading eventually to a widespread outbreak in 2021 that resulted in an unprecedented number of deaths among wild birds and poultry (3, 4). By the end of 2021, the 2.3.4.4b A(H5N1) virus had become the predominant clade circulating in Asia, Africa, Europe, the Middle East, and North America (5–8). Since 8 February 2022, the United States alone has reported the detection of HPAI A(H5) in 1171 commercial and backyard poultry flocks across 48 states, culminating in the loss of more than 100 million birds (9). Furthermore, the 2.3.4.4b clade of the HPAI A(H5N1) virus has shown an increased propensity for spillover to a diverse range of mammalian species, including wild terrestrial mammals (10), marine mammals (11, 12), minks (13), cats (14, 15), and, most recently, dairy cattle (16–18). This elevates concerns about the virus's potential for further adaptation in mammals (19). Although the current risk of HPAI A(H5N1) to the general public is considered low, the enhanced infectivity and unprecedented scope of transmission to mammals heighten the risk of the virus gaining the capability to infect humans more efficiently, potentially leading to human-to-human transmission.

Vaccination is the most crucial and effective intervention to prevent and control influenza virus infections and illness. Now, the US Food and Drug Administration (FDA) has approved three A(H5N1) vaccines developed by Sanofi-Pasteur, CSL Seqirus, and GlaxoSmithKline (ID Biomedical Corporation of Quebec), which target earlier strains of the A(H5N1) virus (20–22). These vaccines, derived from strains identified 2 decades ago within the A(H5) clades 1, 2.1.3.2, and 2.2.1, are administered in either high-dose formulations or with adjuvants to enhance the immune response because of the general population's lack of immunity to A(H5N1) and the inherently low immunogenicity of the A(H5) hemagglutinin (HA) (23–25). However, the rapid evolution of the A(H5) virus, particularly the emergence of the 2.3.4.4b clade, has diminished the expected effectiveness of stockpiled vaccines. In response, the US Centers for Disease Control and Prevention (CDC) and WHO Collaborating Centers have developed several candidate vaccine viruses (CVVs) targeting this clade (26). Vaccine manufacturers have adopted these CVVs to

produce Good Manufacturing Practice–compliant vaccines, with clinical trials underway. Despite the anticipation that these vaccines will offer enhanced protection against the 2.3.4.4b A(H5) virus compared with their predecessors, the rapid and large-scale production of these vaccines during an HPAI pandemic may face major hurdles such as limitations in propagating and inactivating infectious virus and subsequent purifying of antigens, potential shortages in egg supply due to HPAI outbreaks in poultry, and the risk of continued antigenic drift, which can lead to reduced vaccine efficacy. Consequently, there is an urgent need for the development of alternative vaccine strategies against the 2.3.4.4b A(H5) clade that bypass the challenges of large-scale virus cultivation and allow for rapid updating of vaccine strains to keep pace with the virus's evolution.

The mRNA vaccine platform, lauded for its pivotal role during the COVID-19 pandemic, offers a promising alternative to traditional vaccine methods because of its swift development cycle, robust production capabilities, and favorable safety and efficacy records (27, 28). After its success, numerous mRNA vaccines for a variety of diseases entered clinical trials, including phase III trials by both Moderna and Pfizer for seasonal influenza (29–35). However, given the greater severity of HPAI A(H5N1) virus infections in humans compared with seasonal influenza, specific vaccine effectiveness data for A(H5N1) vaccines are essential. Considering the rare incidence of A(H5N1) in humans and the high risk associated with human challenge studies, preclinical evaluations of mRNA vaccine protection against A(H5N1) virus are needed to serve as an indicator of potential effectiveness in humans and inform the design of clinical trials. In this study, we used the Moderna mRNA vaccine platform to formulate various HPAI A(H5) mRNA vaccines and tested their immunogenicity and protectiveness in a ferret infection model, providing insights into their development and application to address the ongoing challenges of the A(H5) 2.3.4.4b clade.

RESULTS

H5 mRNA vaccine formulations induced antibody responses in ferrets

To determine the optimal A(H5N1) mRNA formulation for a vaccine protection study in ferrets, we performed a pilot experiment (experiment 1) with multiple mRNA–lipid nanoparticle (LNP) formulations containing different HA designs and doses, with or without the neuraminidase (NA) component. By doing this multilayer comparison, we sought to determine (i) the number of doses (injections) needed for elicitation of immunity, (ii) the effect of removing the polybasic cleavage site from the wild-type HA protein, (iii) the benefit of adding the NA component, and (iv) whether the use of a higher dose could enhance the titer and durability of protective antibodies. We vaccinated ferrets ($n = 3$) with (i) phosphate-buffered saline (PBS) as a control group (Mock, group 1); (ii) 50 µg of mRNA-LNP encoding wild-type HA protein of the HPAI A(H5N1) A/American wigeon/South Carolina/22–000345–001/2021 virus (AW/SC/21, WHO-recommended prototype virus for clade 2.3.4.4b CVV) (H5-WT, group 2); (iii) 50 µg of mRNA-LNP encoding a mutant A(H5) HA protein of AW/SC/21 with the polybasic cleavage site deleted by mutating the REKRRKR residues to RETR (H5-del, group 3); (iv) 50 µg of H5-del mRNA-LNP together with 50 µg of mRNA-LNP encoding AW/SC/21 NA protein (N1) (50 µg

H5-del + N1, group 4); (v) 100 µg of H5-del mRNA-LNP together with 100 µg of N1 mRNA-LNP (100 µg H5-del + N1, group 5); or (vi) 50 µg of recombinant H5-del protein formulated with AddaVax adjuvant (H5-del protein, group 6) (Fig. 1A). Animals were boosted twice at 5 and 28 weeks after prime vaccination, with the same vaccines respective to each group.

We collected serum samples from the ferrets 1 week before vaccination; every week for the first 12 weeks; and at 16, 20, 24, 27, 29, 30, 31, 32, and 33 weeks after prime vaccination and determined hemagglutination inhibition (HI) titers (Fig. 1B). Higher HI titers were detected against the AW/SC/21 vaccine virus when horse red blood cells (HRBCs) were used instead of turkey red blood cells (TRBCs; fig. S1), suggesting that the HI assay with HRBCs is more sensitive in assessing HI titers against 2.3.4.4b A(H5N1). Therefore, we primarily used HRBCs to monitor the kinetics of HI titers in the ferrets over the 33-week period (Fig. 1B).

Overall, all vaccinated ferrets produced HI antibodies. The antibodies reached an average titer of 62 (geometric mean of all ferrets in vaccinated groups) within 3 weeks after the prime vaccination and markedly increased to 1265 1 week after the second vaccination (booster 1), representing a 20-fold increase. HI titers then gradually declined, maintaining an average titer of 62 at week 27 (22 weeks after booster 1), with all vaccinated groups exhibiting titers above 40 except for group 3 (H5-del), which approached the lower limit of detection (HI titer of 10). One week after the third vaccination (booster 2), the average HI titer rose to 2776, a 44-fold increase from the week 27 titer, although only 2.2 times higher than the peak after booster 1. These results suggest that a two-dose regimen of mRNA-LNP vaccines is both necessary and sufficient to induce robust HI titers. Although the titers dropped in the initial weeks, they remained relatively stable for up to 5 months after the second dose, at which point the third dose was administered.

We hypothesized that, by removing the polybasic cleavage site from the H5 HA protein, the H5 HA antigen could be more efficiently or stably expressed on the surface of host cells, inducing a more robust antibody response. However, the H5-del mRNA-LNP induced overall HI titers that were fivefold lower [based on the area under the curve (AUC) analysis] than those of the H5-WT mRNA-LNP (group 2 versus group 3 in Fig. 1B, $P < 0.05$). We also evaluated the benefit of including NA, finding that coadministering the H5-del mRNA-LNP and NA mRNA-LNP resulted in HI titers comparable to the H5-WT mRNA-LNP group (group 2 versus group 4, $P > 0.05$). Moreover, increasing the vaccine dose from 50 to 100 µg also did not improve HI titers (group 4 versus group 5, $P > 0.05$). These results suggest that 50 µg of H5-WT mRNA-LNP is sufficient to induce high HI titers in ferrets.

Three-dose vaccination mitigated disease in ferrets after lethal A(H5N1) challenge

We next used the same ferrets in a pilot challenge study. Despite the small number of animals in each group, this study provided valuable insight and served as a foundation for a more targeted subsequent challenge study. The ferrets were intranasally inoculated with 10^4 50% egg infectious dose (EID₅₀) of an HPAI A(H5N1) strain, A/bald eagle/Florida/22–006544–004/2022 (BE/FL/22), 7 weeks after the third vaccination (Fig. 1). The BE/FL/22 virus was chosen because it is also a 2.3.4.4b clade virus that is antigenically similar

to vaccine strain AW/SC/21, and it is lethal to ferrets, as described by Kandeil *et al.* (36). Clinical signs, body weight, and body temperature were monitored daily for 14 days after challenge. One ferret in the unvaccinated group (group 1) succumbed to infection on day 7 after challenge, and two remaining animals continued to lose body weight and were humanely euthanized when they reached euthanasia criteria (20% loss of initial body weight) on days 7 and 9 after challenge (Fig. 1C). One ferret in the H5-WT mRNA-LNP group (group 2) showed ruffled coat, abnormal breathing, dehydration, and anemia and was euthanized on day 9 after challenge. All ferrets in other groups showed no disease symptoms and survived the lethal challenge. No significant body temperature change among the groups was observed (fig. S2) ($P > 0.05$). Virus titers in nasal wash samples of vaccinated ferrets were significantly lower than those of unvaccinated ferrets on day 1 ($P < 0.05$), and virus was undetectable in vaccinated ferrets at day 9 after challenge (Fig. 1D), indicating more rapid clearance of virus in nasal cavities in vaccinated ferrets. There were no statistical differences in virus titers between vaccinated and unvaccinated groups on days 3 and 5 after challenge. On day 7 after challenge, the virus titers of vaccinated ferrets in group 2 (H5-WT) and group 5 (100 μ g H5-del + N1) were significantly lower than those of the unvaccinated ferrets ($P < 0.05$). Overall, these results demonstrated that mRNA vaccines elicited high HI titers after two doses of vaccination, and a three-dose vaccination regimen protected ferrets from a lethal challenge.

Selected vaccine formulations for in-depth characterization

On the basis of the results obtained in the first experiment described above, we conducted a second experiment with more ferrets ($n = 10$) in each group. Control animals received no vaccination (group 1), and the other groups were vaccinated as follows: Group 2 received 50 μ g of H5-WT mRNA-LNP, group 3 received 50 μ g of H5-WT mRNA-LNP and 50 μ g of N1 mRNA-LNP (H5-WT + N1), and group 4 received 50 μ g of adjuvanted recombinant H5-del protein (Fig. 2A). At week 5 after prime vaccination, ferrets were boosted with the same vaccines. We chose these two mRNA-LNP formulations because (i) the H5-WT mRNA-LNP was noninferior to the other groups tested, and (ii) the N1 mRNA-LNP increased HI titers when coadministered with H5-del mRNA-LNP. Given that the N1 mRNA-LNP likely elicits NA antibodies, providing additional protection independent of HA antibodies, we coadministered it with the H5-WT mRNA-LNP to evaluate its potential to enhance HI titers and overall protection.

Serum samples were collected from the vaccinated ferrets before and at 3, 5, 6, 7, and 9 weeks after prime vaccination. We measured the titers of all the serum samples with three assays: the HI assay with TRBCs (Fig. 2B, left), the HI assay with HRBCs (Fig. 2B, right), and the focus reduction assay (FRA) for neutralizing antibody titers (Fig. 2C). The TRBC HI assay was insufficiently sensitive to quantify antibodies after the prime vaccination, whereas the HRBC HI assay readily detected antibodies elicited by the two mRNA vaccines, and the FRA assay detected antibodies elicited by all three vaccines. Overall, from week 0 to week 9 (based on AUC analysis), antibody responses were similar between the two mRNA vaccines across all three assays. The protein vaccine elicited significantly lower HI antibody titers ($P < 0.05$) but comparable neutralizing antibody titers ($P < 0.05$) relative to the mRNA vaccines.

To determine whether the N1 mRNA-LNP component elicited NA-specific antibodies, we performed an NA inhibition (NAI) assay using serum samples collected at weeks 6 and 7 (1 and 2 weeks after booster). Compared with the H5-WT mRNA-LNP group, which did not elicit detectable NAI antibodies, the inclusion of the NA mRNA-LNP component in the vaccine induced high NAI antibody titers in all ferrets, reaching an average titer of 4159 at week 6. This titer then declined to 1631 at week 7, decreasing at a rate comparable to the HA-specific antibody titers (Fig. 2D). Overall, two doses of 50 µg of H5-WT mRNA-LNP alone elicited high HI and neutralizing titers. The addition of the N1 mRNA-LNP in the vaccine resulted in high NAI titers but did not enhance HI or neutralizing antibody titers.

A two-dose vaccination regimen protected ferrets from A(H5N1) lethal challenge

Four weeks after booster, the vaccinated ferrets and unvaccinated control ferrets were challenged intranasally with 10^4 EID₅₀ of HPAI BE/FL/22 virus. One ferret in the H5-WT mRNA-LNP group (group 2) was eliminated because of a study-unrelated reason before challenge. Therefore, 9 ferrets of this group and 10 ferrets in each of the other three groups were used in the challenge study. Clinical signs, body weight, and body temperature were monitored daily for up to 14 days. One ferret in the unvaccinated group (group 1) and one in the H5-WT + N1 mRNA-LNP group (group 3) demonstrated neurological symptoms on days 5 and 7 after challenge, respectively, and were euthanized (fig. S3). The remaining ferrets in the vaccinated groups did not show significant weight loss ($P > 0.05$, Fig. 2E), whereas the remaining ferrets in the unvaccinated group continued to lose body weight, and all reached the humane endpoint on day 7 after challenge (Fig. 2E). No significant body temperature change among the groups was observed ($P > 0.05$, fig. S4).

For the two ferrets euthanized because of neurological symptoms on day 5 (group 1, unvaccinated ferret) and day 7 (group 3, H5-WT + N1 mRNA-LNP–vaccinated ferret), we collected various tissues and performed influenza gene-specific digital polymerase chain reaction (dPCR) for viral genome detection and quantification. Viral RNA was detected in all collected tissues of the unvaccinated ferret, whereas viral RNA was detected mainly in the nasal turbinates and brain of the vaccinated ferret (fig. S5). Because of the proximity of the nasal turbinates to the olfactory bulbs, the high viral titer in nasal turbinates may have spread to the brain through the olfactory bulbs. Virus replication in the brain was likely responsible for the neurological symptoms of both ferrets. The ferret that succumbed in group 3 (H5-WT + N1) had normal antibody titers (TRBC HI titer of 40, HRBC titer of 320, and FRA titer of 2051) in the serum sample collected on the day of challenge, indicating that HPAI can occasionally overcome the protection provided by humoral immunity.

Vaccination diminished HPAI BE/FL/22 viral titers across organs

Nasal wash samples were collected from all the remaining ferrets on days 1, 3, 5, 7, 9, and 12 after challenge, and infectious viral titers were determined. Because all ferrets in the unvaccinated group needed to be euthanized for humane reasons by day 7 after challenge, no samples were collected from that group beyond day 7. The virus titers in nasal wash samples of vaccinated ferrets were significantly lower than those of unvaccinated ferrets from days 1 to 7 ($P < 0.05$), except for day three samples from the protein vaccine group (Fig. 2F). All of the vaccinated ferrets were cleared of virus by day 9 after challenge (Fig. 2F).

We collected tissue samples from three ferrets in each group on days 3 and 6 after challenge and determined viral RNA copies by dPCR (Fig. 3) and infectious virus titers by 50% tissue culture infection dose (TCID₅₀) assay (fig. S6 and table S1). In nasal turbinates, the copy numbers of viral RNA in the vaccinated ferrets were similar to those in unvaccinated ferrets on days 3 and 6. However, the infectious virus titers in vaccinated ferrets were significantly lower ($P < 0.05$) than those in unvaccinated ferrets on day 6. In the trachea, the numbers of viral RNA copies and viral titers were significantly lower ($P < 0.05$) in vaccinated ferrets than in unvaccinated ferrets on days 3 and 6 (Fig. 3 and fig. S6). In the bronchus and adjacent lung tissues, the numbers of viral RNA copies were significantly lower ($P < 0.05$) in vaccinated ferrets than those in unvaccinated ferrets both on days 3 and 6. In cranial and caudal lobes of the lungs, the numbers of viral RNA copies were similar among unvaccinated and vaccinated groups on day 3 after challenge, whereas those in caudal lobes of vaccinated ferrets were significantly lower ($P < 0.05$) than those in unvaccinated ferrets on day 6 after challenge. In accessory and middle lobes, the copy numbers were significantly lower ($P < 0.05$) in vaccinated animals on days 3 and 6 (Fig. 3). The virus titers in all lung lobes were mostly similar among all groups on day 3, whereas vaccinated ferrets showed significantly lower ($P < 0.05$) titers than unvaccinated ferrets on day 6 (fig. S6). These results demonstrate that mRNA and protein vaccines efficiently reduced viral load throughout the respiratory tract.

Histopathologic evaluation of the trachea and available lung tissues from vaccinated and unvaccinated animals was also performed. Tracheas showed no appreciable pathology and no differences among groups; only minimal subepithelial lymphocytic infiltrates were seen in one or two animals per group. On day 3 after challenge, one unvaccinated animal had severe bronchointerstitial pneumonia with necrotizing and suppurative bronchiolitis and perivascular edema (fig. S7). Similar findings were present in the lung from the unvaccinated animal that was euthanized on day 5 after challenge because of neurologic signs. Lungs from ferrets vaccinated with H5-WT mRNA-LNP (group 2) or H5-del protein (group 4) had no pathology, mild interstitial infiltrates, or perivascular inflammation, whereas lungs from all H5-WT + N1 mRNA-LNP-vaccinated ferrets (group 3) had focal bronchointerstitial changes and perivascular inflammation. On day 6, lungs from all three unvaccinated animals showed some degree of interstitial inflammation, bronchiolitis, and perivascular inflammation and edema. One of the two H5-WT mRNA-LNP-vaccinated animals (group 2) with lung tissue available and one of the three recombinant protein-vaccinated animals (group 4) animals had focal bronchointerstitial inflammation, whereas lungs from the H5-WT + N1 mRNA-LNP vaccine group (group 3) had only minimal, focal inflammatory infiltrates (fig. S7).

Viral RNA measurements in the brains were highest on day 6 in the unvaccinated ferrets (Fig. 3), with viral RNA detected in all four parts of the brains measured. The mRNA vaccines largely prevented virus replication in the brain so that viral RNA copy numbers in most of the vaccinated ferrets were reduced to the order of 10 copies/mg in all four parts of the brain (Fig. 3). The lower copy numbers of viral RNA in brains of vaccinated ferrets were corroborated by similar reductions in virus titer of the samples of the same brain regions (table S1). Among unvaccinated ferrets, infectious virus titers were detected at day 6 after infection in the olfactory bulbs of all three animals, the brainstem and hippocampus

of one animal, and the parietal and frontal lobes of one animal (table S1). In contrast, among vaccinated ferrets, infectious virus was detected only in the olfactory bulbs of one ferret in the H5-WT + N1 mRNA-LNP group and of one ferret of the H5-del protein group (table S1). A substantial amount of viral RNA was detected in the intestines of unvaccinated ferrets on both days 3 and 6 after challenge, whereas viral RNA detection was negligible in the vaccinated ferrets (Fig. 3). Infectious virus was also detected in the intestines of the unvaccinated ferrets on both days 3 (two of the three ferrets) and 6 (three of the three ferrets) after challenge but was undetectable in the vaccinated ferrets (table S1). Together, these results demonstrate that the mRNA vaccines not only effectively protected vaccinated ferrets from a lethal challenge with an HPAI A(H5N1) virus but also reduced virus replication in respiratory tissues and prevented viral spread to other organs.

Serum samples from vaccinated ferrets neutralized a human isolate closely related to 2024 dairy cattle outbreaks

After our challenge study, a human case of A(H5N1) virus closely related to the recent dairy cattle outbreak in Texas, USA, was identified. The virus, A/Texas/37/2024 (TX/37), was isolated for further analysis. Serum samples collected from ferrets 2 weeks after booster vaccination (corresponding to the week 7 sera in Fig. 2B) were tested for their ability to neutralize AW/SC/21 CVV, the challenge virus BE/FL/22, and the 2024 human isolate (TX/37) (Fig. 4). The results demonstrated that the serum samples from mRNA vaccinated ferrets neutralized both the BE/FL/22 and TX/37 viruses, with neutralization efficiency comparable to that against the vaccine virus. This suggests that the mRNA vaccine used in this study remains effective in protecting ferrets from lethal challenges posed by human isolates related to dairy cattle outbreaks.

DISCUSSION

The influenza virus, a critical human and animal pathogen, has been the focus of various mRNA vaccine studies. More than a decade ago, a key study demonstrated that mRNA vaccines could protect animals against A(H1N1), A(H3N2), and A(H5N1) subtypes. However, these vaccines used unmodified nucleotides (37). Subsequent studies have predominantly focused on seasonal influenza viruses or aimed to develop broadly protective vaccines that target the HA stalk region or other conserved viral proteins or that encompass a diverse array of HA mRNAs (31, 38–48). More recently, a self-amplifying RNA vaccine targeting a 2005 isolate of the A(H5N1) virus demonstrated substantial protection in mice (49). Additionally, Furey *et al.* (50) recently reported on an mRNA vaccine developed on the basis of the A(H5N8) strain A/Astrakhan/3212/2020, another clade 2.3.4.4b virus. These studies highlight the promise of mRNA vaccine technology for combating HPAI viruses but underscore the need for further exploration of vaccines specifically targeting contemporary A(H5N1) clades, such as 2.3.4.4b.

In response to the rapid spread of the clade 2.3.4.4b influenza A(H5) virus, the CDC's Influenza Division began collaborating with mRNA vaccine manufacturers to explore whether mRNA vaccine technologies based on COVID-19 vaccines could protect against lethal infection of the HPAI A(H5N1) virus. An advantage of this approach is the use of

a LNP formulation that has already been administered in millions of doses of Moderna's COVID-19 vaccine and has demonstrated safety and efficacy in phase 1, 2, and 3 clinical trials for seasonal influenza vaccines. However, given the markedly greater lethality of HPAI compared with contemporary human epidemic influenza viruses, it remained to be confirmed whether mRNA vaccines could offer adequate protection against the lethal challenge posed by clade 2.3.4.4b A(H5N1) virus.

Like the ferrets used in this study, humans are immunologically naïve to A(H5) influenza virus. Additionally, low immunogenicity has been observed for some earlier A(H5) vaccines in both humans (24, 51–53) and animal models (54, 55). Therefore, we speculated that multiple vaccinations might be necessary to elicit high antibody titers capable of inhibiting the 2.3.4.4b virus. We initially evaluated three doses of mRNA vaccines and found that a second dose increased HI titers, whereas the enhancement provided by a third dose was less pronounced. Despite a decline in HI titers after the booster dose, which is expected with most influenza vaccines regardless of the platform, the titers stabilized for several months during the study, mirroring observations from COVID-19 mRNA vaccines in humans.

We also evaluated various mRNA-LNP compositions and found that the HA-del mRNA-LNP elicited lower HI titers compared with the HA-WT mRNA-LNP; however, this reduction was compensated by the inclusion of the NA component in the formulation. Our first experiment included only three ferrets per group, and further research is necessary to confirm these differences as well as to explore the underlying mechanisms. Nonetheless, given the urgency of the current 2.3.4.4b A(H5N1) situation, we concluded that HA-WT is at least noninferior to HA-del and proceeded with a larger-scale immunization and challenge study using HA-WT. In the second experiment, we included a vaccine group combining both HA-WT and NA to evaluate the potential added benefits of the NA antigen. However, under our experimental conditions, HA-WT alone provided equal protection against lethal challenges compared to the combination group. On the basis of these findings, we propose that, in the early phase of an A(H5N1) pandemic, when vaccine production capacity is limited, prioritizing the inclusion of only well-matched HA mRNA will optimize production and maximize vaccine availability. The addition of the NA could be considered in later stages, particularly if benefits become evident in mitigating antigenic drift in HA or reducing virus transmission.

Our study has several limitations. First, we did not investigate the molecular mechanisms underlying the reduced HI titers elicited by the H5-del mRNA-LNP compared with the H5-WT mRNA-LNP nor the compensatory effect observed with the inclusion of the N1 mRNA-LNP in the formulation. Second, we did not assess cellular immunity, which may play a crucial role in mitigating disease severity. Third, additional animal studies are needed to evaluate vaccine effectiveness against heterologous challenges or antigenically drifted strains, with respect to both pathogenicity and transmission. Last, although the mRNA vaccines tested in this study provided substantial protection against disease in ferrets, they did not prevent infection. Further improvements in antigen design, LNP formulation, and administration methods, among other factors, should be pursued to enhance the performance of mRNA vaccines.

In conclusion, our study highlights the utility of an HPAI A(H5) mRNA vaccine in reducing the impact of the newly emerged 2.3.4.4b HPAI A(H5N1) influenza virus, including the B3.13 genotype responsible for current outbreaks in dairy cattle (56). Clinical trials evaluating Moderna's mRNA-based A(H5N1) candidate vaccines are ongoing and are expected to provide additional information about these vaccines, including the optimal dosage for humans. It is critical to pursue these clinical trials, along with additional preclinical studies, to improve vaccine preparedness and mitigate the potential pandemic risks posed by emerging HPAI A(H5) viruses and other zoonotic influenza viruses.

MATERIALS AND METHODS

Study design

This preclinical study was designed to evaluate the performance of mRNA vaccines against the highly pathogenic avian (HPAI) A(H5N1) virus of the 2.3.4.4b clade. Ferrets, recognized as a relevant small animal model for studying influenza pathogenicity, transmission, and vaccine efficacy, were used in this study. The study was conducted in two phases. The objective of the first experiment was to test multiple mRNA formulations and doses, monitoring the kinetics of the elicited antibodies after the initial vaccination (week 0) and subsequent boosters (weeks 5 and 28) over an 8-month period. This phase aimed to identify the formulation(s) that generated the highest HI antibody titers over time. A challenge component was introduced 7 weeks after the second booster (week 35) to maximize the use of the animals and gain additional insights to inform the design of the second experiment. However, the primary focus of the first experiment was on immunogenicity. In the second experiment, only two mRNA formulations were selected for testing, with an increased sample size of 10 ferrets per group (compared with 3 in the first experiment) to assess postvaccination antibody responses more accurately and enable the collection of tissue samples at multiple time points. This phase focused on evaluating the protective effects of the vaccines against a lethal challenge. In both experiments, a recombinant A(H5) protein group was included as a comparator. However, the study was not designed to determine the superiority between the mRNA vaccine and the protein vaccine.

Group sizes were determined with the smallest effect size to obtain sufficient statistical power. The animals were assigned randomly to the experimental groups. Blinding was not applied to the animal experiments. In the second experiment, one ferret in the H5-WT mRNA-LNP group (group 2) was eliminated because of a study-unrelated reason before challenge. Therefore, the data of this ferret are not available after challenge. All procedures using cell cultures and for serological assays followed CDC's standard operating procedures with an appropriate number of repeats and proper controls to obtain enough statistical power.

Biosafety and animal welfare

All research involving HPAI was conducted within Biosafety Level 3–enhanced (BSL-3E) or Animal Biosafety Level 3 (ABSL-3) facilities at the CDC. All personnel engaged in virus-related activities underwent comprehensive training in relevant safety protocols and specific procedural techniques. Their competence to perform tasks within BSL-3E/ABSL-3 laboratories was rigorously assessed. The use of animals in this study received approval

from the CDC's Institutional Animal Care and Use Committee (IACUC). We adhered strictly to the principle of using the minimum number of animals required to achieve scientific validity. We implemented procedures designed to minimize animal discomfort and pain.

Viruses

H5N1 virus A/bald eagle/Florida/22–006544–004/2022 [A(H5N1) clade 2.3.4.4b, BE/FL/22] was propagated in embryonated chicken eggs and titrated by both EID₅₀ assay and TCID₅₀ assay with standard procedures (57, 58). A/Texas/37/2024 (TX/37) was propagated in Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, CCL-34) and titrated by TCID₅₀ assay. The virus stock was sequenced to confirm the absence of unwanted mutations.

Vaccine preparation

For preclinical mRNA-LNP vaccines, sequence-optimized mRNAs encoding (i) a full-length HA protein of H5N1 A/American wigeon/South Carolina/22–000345–001/2021 [A(H5N1) clade 2.3.4.4b, AW/SC/21, H5-WT], (ii) a full-length AW/SC/21 HA protein with the polybasic amino acids at HA cleavage site mutated from “REKRRKR” to “RETR” (H5-del), and (iii) a full-length AW/SC/21 NA protein with a D151G mutation (N1) were synthesized in vitro as previously described (59). The H5-WT sequence matches Global Initiative on Sharing All Influenza Data (GISAID) EPI2709137, the H5-del sequence matches GenBank WHV02513, and the N1 sequence has a D151G substitution relative to GenBank WHV02512. The mRNAs were formulated in a mixture of four lipids [SM-102 (an ionizable lipid), polyethylene glycol 2000–dimethylglyoxime, 1,2-distearoyl-sn-glycero-3-phosphocholine, and cholesterol] at a total lipid content of 9.7 mg/ml, in 20 mM trometamol (tris) buffer containing sucrose (87 mg/ml) and 10.7 mM sodium acetate at a dosage strength of 0.2 mg/ml, at pH 7.5. The mRNA-LNPs were stored at –60° to –90°C. The product underwent analytical characterization and was deemed acceptable for in vivo preclinical studies.

To produce the recombinant H5-del protein, ectodomain of AW/SC/21 HA, with its polybasic cleavage site mutated from REKRRKR to RETR, was flanked by the C-terminal domain of the bacteriophage T4 fibrin (foldon) and a polyhistidine tag at the C-terminus. The DNA fragment was inserted in the pCI-Neo mammalian expression vector to generate an expression plasmid for transfection into Expi293F human cells (Thermo Fisher Scientific, A14527). On day 4 after transfection, the supernatant was collected, and the recombinant protein was purified using a Ni²⁺ HisTrap FF 5-ml column (Cytiva) on fast protein liquid chromatography (ÄKTA avant 25, Cytiva), followed by size exclusion chromatography on a Superose 6 Increase column (Cytiva) with a running buffer of 20 mM tris (pH 8) and 200 mM NaCl. The quality of the collected soluble trimeric HA proteins was assessed by electrophoresis, using SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE gels.

Ferret vaccination and challenge

All ferret experiments were conducted with approval of the CDC's IACUC and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited ABSL-3 animal facility with enhancements as required by the US Department of Agriculture (60). Male ferrets (Triple F Farms), which were serologically negative to seasonal influenza A and B viruses, were used in this study. The animals were housed in Duo-Flow Mobile units (Lab Products LLC.) throughout the experiments. The mRNA-LNP vaccines were diluted in PBS to a final volume of 300 μ l for each dose. For the HA + NA vaccine groups, the HA mRNA-LNP and NA mRNA-LNP were produced individually at Moderna and mixed and diluted in PBS at CDC. Recombinant H5-del protein was diluted in PBS and mixed at a 1:1 ratio with AddaVax adjuvant (InvivoGen) to a final volume of 300 μ l. All animals were vaccinated with 300 μ l (600 μ l used for Fig. 1A, group 5) of vaccine or PBS intramuscularly in a thigh; both thighs were used for Fig. 1A, group 5. Serum samples were collected at the indicated time points from the cranial venae cavae of ferrets while under anesthesia.

Vaccinated ferrets were intranasally inoculated with 10^4 EID₅₀ of BE/FL/22 virus on the weeks after vaccination as specified in Figs. 1A and 2A. Clinical signs, body weight, and body temperature were monitored daily for 14 days. Nasal wash samples were collected from all remaining animals in each group on days 1, 3, 5, 7, 9, and 12 after challenge. Organ samples [lung sections (cranial, accessory and middle, and caudal lobes), trachea, nasal turbinates, small and large intestines, and brain tissues (cerebellum, stem and hippocampus, parietal and frontal lobes, and olfactory bulb)] were collected for virus titration and pathological analysis on days 3 and 6 after challenge. Organs were not collected in the pilot study.

HI assay

HI assays were performed against the CVV of AW/SC/21 with TRBCs or HRBCs. The CVV was developed per the WHO recommendation and is antigenically similar to the wild-type AW/SC/21 (26, 61). Briefly, ferret serum samples were treated with receptor-destroying enzyme (Denka Seiken Co. Ltd.) at 37°C for 16 to 20 hours followed by inactivation at 56°C for 30 min. After adsorption of nonspecific agglutinins with TRBCs or HRBCs, the treated serum samples were serially diluted twofold with PBS and mixed with the diluted virus of eight hemagglutination units, followed by incubation at room temperature for 30 min. TRBCs (0.5%) or HRBCs (1%) were then added to the mixtures and further incubated at room temperature for 30 min (TRBCs) or 60 min (HRBCs). HI titers are expressed as the highest reciprocal dilution that resulted in inhibition of agglutination.

Focus reduction assay

The FRA for virus neutralization was performed as previously described with slight modification (62). Briefly, serially diluted serum samples and 600 focus-forming units of virus were added to 96-well plates containing confluent MDCK cells and incubated at 37°C for 2 hours. An equal volume of overlay, consisting of 1.2% Avicel RC/CL in 2 $\times$ minimum essential medium with *N*-*p*-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (1 μ g/ml), 0.1% bovine serum albumin (BSA) fraction V, and antibiotics, was then added.

After an overnight incubation, the cells were fixed with 4% cold formalin at 4°C for 30 min. After fixation, the cells were permeabilized with 5% Triton X-100 in PBS/glycine at room temperature for 20 min. Instead of staining the foci with labeled antibodies, the foci were visualized with the fluorogenic NA substrate 2-(benzothiazole-2-yl)-4-bromophenyl 5-acetamido-3,5-dideoxy- α -D-glycero-D-galacto-2-nonulopyranosidonic acid (63).

NAI assay

Ferret serum was heat inactivated at 56°C to remove nonspecific inhibitors of NA activity. Fetuin solution (100 μ l per well) was added to 96-well microplates at 25 μ g/ml in 1 $\times$ Kirkegaard & Perry Laboratories (KPL) coating buffer [SeraCare, catalog no. 5150–0 014 (50–84–00)] and incubated at 2° to 8°C for at least 18 hours. After washing, pretreated serum samples were added to wells and incubated overnight at 37°C with two times the half-maximal effective concentration of recombinant AW/SC/21 NA. Peanut agglutinin conjugated with horseradish peroxidase (100 μ l per well) was added, and plates were incubated for 2 hours before washing and detection with 3,3',5,5'-tetramethylbenzidine (TMB; 100 μ l per well). After 10 min, the reaction was stopped by adding 100 μ l per well TMB Stop Solution. The plates were read at a wavelength of 450 nm, and the half-maximal inhibitory concentration values for each serum sample were calculated.

Virus titration in ferret nasal wash and organ samples

For nasal wash collection, 2 ml of PBS containing 1% BSA fraction V, penicillin/streptomycin (100 U/ml), and gentamicin (10 μ g/ml) was instilled into the nostrils of the ferrets under anesthesia, and the secretions from the nostrils were captured in a sterile specimen cup (Medline). The nasal wash samples were centrifuged, and the cleared supernatants were titrated by TCID₅₀ assay on MDCK cells and expressed as TCID₅₀ units per milliliter. The organ tissue samples were collected from the animals and homogenized in 1 ml of PBS containing 1% BSA fraction V, penicillin/streptomycin (100 U/ml), and gentamicin (10 μ g/ml) by MagNA Lyser (Roche). After clarification by centrifugation, the virus titers in the samples were determined by TCID₅₀ assay on MDCK cells and converted to TCID₅₀ units per gram of the homogenized tissue samples.

Quantification of viral RNA in ferret organ samples by dPCR

RNA was extracted from 100 μ l of organ homogenate in 300 μ l of MagNA Pure LC RNA isolation tissue lysis buffer (Roche) with a MagNA Pure Cellular RNA Large Volume Kit (Roche) on the MagNA Pure 96 system (Roche). The extraction protocol was RNA FF Tissue with a sample volume of 350 μ l and an elution volume of 50 μ l. Each plate of samples included a mock control of 400 μ l MagNA Pure LC RNA isolation tissue lysis buffer (Roche). RNA was stored at –80°C before downstream processes.

For absolute RNA quantification, we used the QIAcuity digital PCR System (Qiagen). We prepared reactions using the QIAcuity OneStep Advanced Probe Kit with InfA primer/probes from CDC rRT-PCR Flu Panel: Influenza A/B Typing Kit [VER 2; 510 (k) number K200370]. Four microliters of each RNA sample was loaded on QIAcuity 8.5k $\times$ 96-well plates with default parameters for priming and cycling conditions as follows: 50°C for 30 min, 95°C for 2 min, and 45 cycles of 95°C for 15 s and 55°C for 30 s. A single channel,

green, was used for imaging. Samples with fewer than 10 negative partitions were diluted and repeated. Raw copy per microliter numbers for the reaction mixture were converted to copy per microgram of tissue by factoring in the weight of the tissue that was homogenized, the volume of that homogenate, the fraction of homogenate carried into extraction, the volume of RNA eluted, any necessary dilution of the RNA, and the dilution of the RNA when added to the reaction mixture. A stock virus with a known titer was used as a positive control, and a buffer-only negative control was included during RNA extraction and carried through the quantification process.

Pathological analyses

Tissues were fixed in 10% neutral buffered formalin for 14 days before being processing for paraffin embedding. Briefly, 4- μ m transverse sections were mounted in Apex superior adhesive slides (Leica biosystem Inc.). Sections were then deparaffinized in xylene and hydrated in different concentrations of ethanol (100, 95, 80, and 75%) for 2 min each. Deparaffinized and hydrated lung sections were stained with hematoxylin (MilliporeSigma) for 7 min, differentiated in 1% acid alcohol for 12 s, and followed by counterstaining with eosin (MilliporeSigma) for 30 s. Slides were then dehydrated with 95 and 100% ethanol twice for 2 min each, cleared by xylene twice for 2 min each, and mounted by Permount mounting medium (Thermo Fisher Scientific). Slides were evaluated qualitatively in a blinded manner by light microscopy by a board-certified veterinary pathologist using a Zeiss Axio Imager M2m microscope.

Statistical analyses

Individual-level data are presented in data file S1. Statistical analysis of the HI and neutralizing antibody responses to the vaccines was based on AUC analysis. The AUC of antibody response was calculated over 33 weeks (Fig. 1B) or 9 weeks (Fig. 2, B and C), representing the cumulative antibody response over the entire vaccination period per animal. Differences in AUCs were tested using a one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical analysis of bodyweight change was also based on AUC analysis. Briefly, the weight per ferret at day 0 was used as a baseline, and weight change was determined relative to baseline. If a ferret died during the follow-up of the study, then the last observed bodyweight was carried forward. The AUC of bodyweight was calculated over the intervals from the day of challenge to 14 days after challenge, representing the cumulative bodyweight change over the entire follow up period. Differences in AUCs were tested using a one-way ANOVA with Tukey's post hoc test. All data presented in bar graphs were statistically analyzed by pairwise *t* test with Tukey-Kramer's adjustment for multiple comparison on the log titers. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc.). Statistical significance cutoff was set at $\alpha = 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability:

All data associated with this study are in the paper or the Supplementary Materials. Requests for viruses and serum samples should be directed to the CDC (bzhou@cdc.gov), whereas requests for mRNA-LNP should be directed to Moderna (raffael.nachbagauer@modernatx.com). Please note that material transfer agreements or other applicable legal agreements may be required for materials sharing.

REFERENCES AND NOTES

1. Chan PK, Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. *Clin. Infect. Dis.* 34, S58–S64 (2002). [PubMed: 11938498]
2. World Health Organization Regional Office for the Western Pacific, “Human infection with avian influenza A(H5) viruses,” *Avian Influenza Wkly. Update* (no. 931) (26 January 2024).
3. Nagy A, Černíková L, Stará M, A new clade 2.3.4.4b H5N1 highly pathogenic avian influenza genotype detected in Europe in 2021. *Arch. Virol.* 167, 1455–1459 (2022). [PubMed: 35469095]
4. Liang Y, Nissen JN, Krog JS, Breum SO, Trebbien R, Larsen LE, Hjulsager CK, Novel Clade 2.3.4.4b highly pathogenic avian influenza A H5N8 and H5N5 viruses in Denmark, 2020. *Viruses* 13, 886 (2021). [PubMed: 34065033]
5. Bevins SN, Shriner SA, Cumbee JC Jr., K. E. Dilione, K. E. Douglass, J. W. Ellis, M. L. Killian, M. K. Torchetti, J. B. Lenocho, Intercontinental movement of highly pathogenic avian influenza A(H5N1) clade 2.3.4.4 virus to the United States, 2021. *Emerg. Infect. Dis.* 28, 1006–1011 (2022). [PubMed: 35302933]
6. Miller BJ, Why unprecedented bird flu outbreaks sweeping the world are concerning scientists. *Nature* 606, 18–19 (2022). [PubMed: 35618804]
7. Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H, Sun Y, Kawaoka Y, Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. *J. Virol.* 80, 5976–5983 (2006). [PubMed: 16731936]
8. Stokstad E, Deadly flu spreads through North American birds. *Science* 376, 441–442 (2022). [PubMed: 35482856]
9. US Department of Agriculture, Animal and Plant Health Inspection Service, 2022–2024 Confirmations of highly pathogenic avian influenza in commercial and backyard flocks (20 June 2024); www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/avian/avian-influenza/hpai-2022/2022-hpai-commercial-backyard-flocks.
10. Elsmo EJ, Wunschmann A, Beckmen KB, Broughton-Neiswanger LE, Buckles EL, Ellis J, Fitzgerald SD, Gerlach R, Hawkins S, Ip HS, Lankton JS, Lemley EM, Lenocho JB, Killian ML, Lantz K, Long L, Maes R, Mainenti M, Melotti J, Moriarty ME, Nakagun S, Ruden RM, Shearn-Bochsler V, Thompson D, Torchetti MK, Van Wettene AJ, Wise AG, Lim AL, Highly pathogenic avian influenza A(H5N1) virus clade 2.3.4.4b infections in wild terrestrial mammals, United States, 2022. *Emerg. Infect. Dis.* 29, 2451–2460 (2023). [PubMed: 37987580]

11. Leguia M, Garcia-Glaessner A, Munoz-Saavedra B, Juarez D, Barrera P, Calvo-Mac C, Jara J, Silva W, Ploog K, Amaro L, Colchao-Claux P, Johnson CK, Uhart MM, Nelson MI, Lescano J, Highly pathogenic avian influenza A (H5N1) in marine mammals and seabirds in Peru. *Nat. Commun.* 14, 5489 (2023). [PubMed: 37679333]
12. Puryear W, Sawatzki K, Hill N, Foss A, Stone JJ, Doughty L, Walk D, Gilbert K, Murray M, Cox E, Patel P, Mertz Z, Ellis S, Taylor J, Fauquier D, Smith A, DiGiovanni RA Jr., A. van de Guchte, A. S. Gonzalez-Reiche, Z. Khalil, H. van Bakel, M. K. Torchetti, K. Lantz, J. B. Lenocho, J. Runstadler, Highly pathogenic avian influenza A(H5N1) virus outbreak in New England Seals, United States. *Emerg. Infect. Dis.* 29, 786–791 (2023). [PubMed: 36958010]
13. Agüero M, Monne I, Sanchez A, Zecchin B, Fusaro A, Ruano MJ, Del Valle Arrojo M, Fernandez-Antonio R, Souto AM, Tordable P, Canas J, Bonfante F, Giussani E, Terregino C, Orejas JJ, Highly pathogenic avian influenza A(H5N1) virus infection in farmed minks, Spain, October 2022. *Euro Surveill.* 28, 2300001 (2023). [PubMed: 36695488]
14. Domańska-Blicharz K, Świętoń E, Świątalska A, Monne I, Fusaro A, Tarasiuk K, Wyrostek K, Styś-Fijoł N, Giza A, Pietruk M, Zecchin B, Pastori A, Adaszek Ł, Pomorska-Mól M, Tomczyk G, Terregino C, Winiarczyk S, Outbreak of highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b virus in cats, Poland, June to July 2023. *Euro Surveill.* 28, 2300366 (2023). [PubMed: 37535474]
15. Briand F-X, Souchaud F, Pierre I, Beven V, Hirchaud E, Hérault F, Planel R, Rigauadeau A, Bernard-Stoecklin S, Van der Werf S, Lina B, Gerbier G, Etteradossi N, Schmitz A, Niqueux E, Grasland B, Highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b virus in domestic cat, France, 2022. *Emerg. Infect. Dis.* 29, 1696–1698 (2023). [PubMed: 37379514]
16. Burrough ER, Magstadt DR, Petersen B, Timmermans SJ, Gauger PC, Zhang J, Siepker C, Mainenti M, Li G, Thompson AC, Gorden PJ, Plummer PJ, Main R, Highly pathogenic avian influenza A(H5N1) clade 2.3.4.4b virus infection in domestic dairy cattle and cats, United States, 2024. *Emerg. Infect. Dis.* 30, 1335–1343 (2024). [PubMed: 38683888]
17. Hu X, Saxena A, Magstadt DR, Gauger PC, Burrough E, Zhang J, Siepker C, Mainenti M, Gorden PJ, Plummer P, Li G, Highly pathogenic avian influenza A (H5N1) clade 2.3.4.4b virus detected in dairy cattle. *bioRxiv* 588916 [Preprint] (2024). 10.1101/2024.04.16.588916.
18. Nguyen T-Q, Hutter C, Markin A, Thomas M, Lantz K, Killian ML, Janzen GM, Vijendran S, Wagle S, Inderski B, Magstadt DR, Li G, Diel DG, Frye EA, Dimitrov KM, Swinford AK, Thompson AC, Snevik KR, Suarez DL, Spackman E, Lakin SM, Ahola SC, Johnson KR, Baker AL, Robbe-Austerman S, Torchetti MK, Anderson TK, Emergence and interstate spread of highly pathogenic avian influenza A(H5N1) in dairy cattle. *bioRxiv* 591751 [Preprint] (2024). 10.1101/2024.05.01.591751.
19. Alkie TN, Cox S, Embury-Hyatt C, Stevens B, Pople N, Pybus MJ, Xu W, Hisanaga T, Suderman M, Koziuk J, Kruczkiewicz P, Nguyen HH, Fisher M, Lung O, Erdelyan CNG, Hochman O, Ojkic D, Yason C, Bravo-Araya M, Bourque L, Bollinger TK, Soos C, Giacinti J, Provencher J, Ogilvie S, Clark A, MacPhee R, Parsons GJ, Eaglesome H, Gilbert S, Saboraki K, Davis R, Jerao A, Ginn M, Jones MEB, Berhane Y, Characterization of neurotropic HPAI H5N1 viruses with novel genome constellations and mammalian adaptive mutations in free-living mesocarnivores in Canada. *Emerg. Microbes. Infect.* 12, 2186608 (2023). [PubMed: 36880345]
20. US Food and Drug Administration, Influenza virus vaccine, H5N1 (for national stockpile) (2018); www.fda.gov/vaccines-blood-biologics/vaccines/influenza-virus-vaccine-h5n1-national-stockpile.
21. US Food and Drug Administration, AUDENZ, Influenza A (H5N1) monovalent vaccine, adjuvanted (2021); www.fda.gov/vaccines-blood-biologics/audenz.
22. US Food and Drug Administration, Influenza A (H5N1) virus monovalent vaccine, adjuvanted (2019); www.fda.gov/vaccines-blood-biologics/vaccines/influenza-h5n1-virus-monovalent-vaccine-adjuvanted.
23. Wong SS, DeBeauchamp J, Zanin M, Sun Y, Tang L, Webby R, H5N1 influenza vaccine induces a less robust neutralizing antibody response than seasonal trivalent and H7N9 influenza vaccines. *NPJ Vaccines* 2, 16 (2017). [PubMed: 29263872]
24. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC, Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3)

- vaccine: A randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 357, 1937–1943 (2001). [PubMed: 11425416]
25. Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M, Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population. *Vaccine* 21, 1687–1693 (2003). [PubMed: 12639491]
 26. World Health Organization, Zoonotic influenza: Candidate vaccine viruses and potency testing reagents (2024); www.who.int/teams/global-influenza-programme/vaccines/who-recommendations/zoonotic-influenza-viruses-and-candidate-vaccine-viruses.
 27. Verbeke R, Lentacker I, De Smedt SC, Dewitte H, The dawn of mRNA vaccines: The COVID-19 case. *J. Control. Release* 333, 511–520 (2021). [PubMed: 33798667]
 28. Pardi N, Hogan MJ, Porter FW, Weissman D, mRNA vaccines - A new era in vaccinology. *Nat. Rev. Drug Discov.* 17, 261–279 (2018). [PubMed: 29326426]
 29. ModernaTX Inc., A study of mRNA-1010 seasonal influenza vaccine in adults (clinicaltrials.gov, 2022); <https://clinicaltrials.gov/study/NCT05827978?rank=1>.
 30. Pfizer, A study to evaluate a modified RNA vaccine against influenza in adults 18 years of age or older (clinicaltrials.gov, 2022); <https://clinicaltrials.gov/study/NCT05827926>.
 31. Kackos CM, DeBeauchamp J, Davitt CJH, Lonzaric J, Sealy RE, Hurwitz JL, Samsa MM, Webby RJ, Seasonal quadrivalent mRNA vaccine prevents and mitigates influenza infection. *NPJ Vaccines* 8, 157 (2023). [PubMed: 37828126]
 32. Lee IT, Nachbagauer R, Ensz D, Schwartz H, Carmona L, Schaefer K, Avanesov A, Stadlbauer D, Henry C, Chen R, Huang W, Schrempp DR, Ananworanich J, Paris R, Safety and immunogenicity of a phase 1/2 randomized clinical trial of a quadrivalent, mRNA-based seasonal influenza vaccine (mRNA-1010) in healthy adults: Interim analysis. *Nat. Commun.* 14, 3631 (2023). [PubMed: 37336877]
 33. ModernaTX Inc., A study of mRNA-based Influenza and SARS-CoV-2 (COVID-19) multi-component vaccines in healthy adults (clinicaltrials.gov, 2023); <https://clinicaltrials.gov/study/NCT05827926>.
 34. Pfizer, A study to evaluate a modified RNA vaccine against influenza in adults 18 years of age or older (clinicaltrials.gov, 2022); www.clinicaltrials.gov/study/NCT05540522?term=NCT05540522&rank=1&tab=history.
 35. BioNTech SE, A study to evaluate the safety, tolerability, and immunogenicity of combined modified RNA vaccine candidates against COVID-19 and influenza (clinicaltrials.gov, 2022); www.clinicaltrials.gov/study/NCT05596734?term=NCT05596734&rank=1&tab=history.
 36. Kandeil A, Patton C, Jones JC, Jeevan T, Harrington WN, Trifkovic S, Seiler JP, Fabrizio T, Woodard K, Turner JC, Crumpton JC, Miller L, Rubrum A, DeBeauchamp J, Russell CJ, Govorkova EA, Vogel P, Kim-Torchetti M, Berhane Y, Stallknecht D, Poulson R, Kercher L, Webby RJ, Rapid evolution of A(H5N1) influenza viruses after intercontinental spread to North America. *Nat. Commun.* 14, 3082 (2023). [PubMed: 37248261]
 37. Petsch B, Schnee M, Vogel AB, Lange E, Hoffmann B, Voss D, Schlake T, Thess A, Kallen KJ, Stitz L, Kramps T, Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. *Nat. Biotechnol.* 30, 1210–1216 (2012). [PubMed: 23159882]
 38. Pardi N, Parkhouse K, Kirkpatrick E, McMahon M, Zost SJ, Mui BL, Tam YK, Kariko K, Barbosa CJ, Madden TD, Hope MJ, Krammer F, Hensley SE, Weissman D, Nucleoside-modified mRNA immunization elicits influenza virus hemagglutinin stalk-specific antibodies. *Nat. Commun.* 9, 3361 (2018). [PubMed: 30135514]
 39. Freyn AW, Ramos da Silva J, Rosado VC, Bliss CM, Pine M, Mui BL, Tam YK, Madden TD, de Souza Ferreira LC, Weissman D, Krammer F, Coughlan L, Palese P, Pardi N, Nachbagauer R, A multi-targeting, nucleoside-modified mRNA influenza virus vaccine provides broad protection in mice. *Mol. Ther.* 28, 1569–1584 (2020). [PubMed: 32359470]
 40. Freyn AW, Pine M, Rosado VC, Benz M, Muramatsu H, Beattie M, Tam YK, Krammer F, Palese P, Nachbagauer R, McMahon M, Pardi N, Antigen modifications improve nucleoside-modified mRNA-based influenza virus vaccines in mice. *Mol. Ther. Methods Clin. Dev.* 22, 84–95 (2021). [PubMed: 34485597]

41. Chivukula S, Plitnik T, Tibbitts T, Karve S, Dias A, Zhang D, Goldman R, Gopani H, Khanmohammed A, Sarode A, Cooper D, Yoon H, Kim Y, Yan Y, Mundle ST, Groppo R, Beauvais A, Zhang J, Anosova NG, Lai C, Li L, Ulinski G, Piepenhagen P, DiNapoli J, Kalnin KV, Landolfi V, Swearingen R, Fu TM, DeRosa F, Casimiro D, Development of multivalent mRNA vaccine candidates for seasonal or pandemic influenza. *NPJ Vaccines* 6, 153 (2021). [PubMed: 34916519]
42. McMahon M, O'Dell G, Tan J, Sarkozy A, Vadovics M, Carreno JM, Puente-Massaguer E, Muramatsu H, Bajusz C, Rijnink W, Beattie M, Tam YK, Kirkpatrick Roubidoux E, Francisco I, Strohmeier S, Kanekiyo M, Graham BS, Krammer F, Pardi N, Assessment of a quadrivalent nucleoside-modified mRNA vaccine that protects against group 2 influenza viruses. *Proc. Natl. Acad. Sci. U.S.A.* 119, e2206333119 (2022). [PubMed: 36322769]
43. Pardi N, Carreño JM, O'Dell G, Tan J, Bajusz C, Muramatsu H, Rijnink W, Strohmeier S, Loganathan M, Bielak D, Sung MMH, Tam YK, Krammer F, McMahon M, Development of a pentavalent broadly protective nucleoside-modified mRNA vaccine against influenza B viruses. *Nat. Commun.* 13, 4677 (2022). [PubMed: 35945226]
44. Xiong F, Zhang C, Shang B, Zheng M, Wang Q, Ding Y, Luo J, Li X, An mRNA-based broad-spectrum vaccine candidate confers cross-protection against heterosubtypic influenza A viruses. *Emerg. Microbes Infect.* 12, 2256422 (2023). [PubMed: 37671994]
45. Ma N, Xia ZW, Zhang ZG, Nian XX, Li XD, Gong Z, Zhang GM, Le Y, Zhou R, Zhang JY, Yang XM, Development of an mRNA vaccine against a panel of heterologous H1N1 seasonal influenza viruses using a consensus hemagglutinin sequence. *Emerg. Microbes Infect.* 12, 2202278 (2023). [PubMed: 37067355]
46. Gouma S, Furey C, Santos JJS, Parkhouse K, Weirick M, Muramatsu H, Pardi N, Fan SHY, Weissman D, Hensley SE, Nucleoside-modified mRNA-based influenza vaccines circumvent problems associated with H3N2 vaccine strain egg adaptation. *J. Virol.* 97, e0172322 (2023). [PubMed: 36533954]
47. Arevalo CP, Bolton MJ, Le Sage V, Ye N, Furey C, Muramatsu H, Alameh MG, Pardi N, Drapeau EM, Parkhouse K, Garretson T, Morris JS, Moncla LH, Tam YK, Fan SHY, Lakdawala SS, Weissman D, Hensley SE, A multivalent nucleoside-modified mRNA vaccine against all known influenza virus subtypes. *Science* 378, 899–904 (2022). [PubMed: 36423275]
48. van de Ven K, Lanfermeijer J, van Dijken H, Muramatsu H, Vilas Boas C, de Melo S, Lenz, Peters F, Beattie MB, Lin PJC, Ferreira JA, van den Brand J, van Baarle D, Pardi N, de Jonge J, A universal influenza mRNA vaccine candidate boosts T cell responses and reduces zoonotic influenza virus disease in ferrets. *Sci. Adv.* 8, eadc9937 (2022). [PubMed: 36516261]
49. Chang C, Music N, Cheung M, Rossignol E, Bedi S, Patel H, Safari M, Lee C, Otten GR, Settembre EC, Palladino G, Wen Y, Self-amplifying mRNA bicistronic influenza vaccines raise cross-reactive immune responses in mice and prevent infection in ferrets. *Mol. Ther. Methods Clin. Dev.* 27, 195–205 (2022). [PubMed: 36320414]
50. Furey C, Scher G, Ye N, Kercher L, DeBeauchamp J, Crumpton JC, Jeevan T, Patton C, Franks J, Rubrum A, Alameh MG, Fan SHY, Phan AT, Hunter CA, Webby RJ, Weissman D, Hensley SE, Development of a nucleoside-modified mRNA vaccine against clade 2.3.4.4b H5 highly pathogenic avian influenza virus. *Nat. Commun.* 15, 4350 (2024). [PubMed: 38782954]
51. Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Höschler K, Zambon MC, Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: Phase I randomised trial. *Lancet* 367, 1657–1664 (2006). [PubMed: 16714186]
52. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M, Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N. Engl. J. Med.* 354, 1343–1351 (2006). [PubMed: 16571878]
53. Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, Wolff M, Rabinovich G, Blackwelder W, Katz JM, Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. *Vaccine* 19, 1732–1737 (2001). [PubMed: 11166898]
54. Rimmelzwaan GF, Claas EC, van Amerongen G, de Jong JC, Osterhaus AD, ISCOM vaccine induced protection against a lethal challenge with a human H5N1 influenza virus. *Vaccine* 17, 1355–1358 (1999). [PubMed: 10195770]

55. Middleton D, Rockman S, Pearse M, Barr I, Lowther S, Klippel J, Ryan D, Brown L, Evaluation of vaccines for H5N1 influenza virus in ferrets reveals the potential for protective single-shot immunization. *J. Virol.* 83, 7770–7778 (2009). [PubMed: 19457991]
56. Peacock T, Moncla L, Dudas G, VanInsberghe D, Sukhova K, Lloyd-Smith JO, Worobey M, Lowen AC, Nelson MI, The global H5N1 influenza panzootic in mammals. *Nature*, (2024); 10.1038/s41586-024-08054-z.
57. World Health Organization, “WHO manual on animal influenza diagnosis and surveillance” (WHO/CDS/CSR/NCS/2002.5, WHO, 2002); <https://iris.who.int/handle/10665/68026>.
58. Balish AL, Katz JM, Klimov AI, Influenza: Propagation, quantification, and storage. *Curr. Protoc. Microbiol.* 29, 15G.1.1–15G.1.24 (2013).
59. Nelson J, Sorensen EW, Mintri S, Rabideau AE, Zheng W, Besin G, Khatwani N, Su SV, Miracco EJ, Issa WJ, Hoge S, Stanton MG, Joyal JL, Impact of mRNA chemistry and manufacturing process on innate immune activation. *Sci. Adv.* 6, eaaz6893 (2020). [PubMed: 32637598]
60. Chosewood LC, Wilson DE, US Centers for Disease Control and Prevention, US National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (HHS Publications, US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health, ed. 5, 2009), pp. xxii–415.
61. World Health Organization, “Summary of status of development and availability of A(H5N1) candidate vaccine viruses and potency testing reagents” (WHO, 23 February 2024).
62. Rowe T, Fletcher A, Svoboda P, Pohl J, Hatta Y, Jasso G, Wentworth DE, Ross TM, Interferon as an immunoadjuvant to enhance antibodies following influenza B infection and vaccination in ferrets. *NPJ Vaccines* 9, 199 (2024). [PubMed: 39448628]
63. Kato D, Kurebayashi Y, Takahashi T, Otsubo T, Otake H, Yamazaki M, Tamoto C, Minami A, Ikeda K, Suzuki T, An easy, rapid, and sensitive method for detection of drug-resistant influenza virus by using a sialidase fluorescent imaging probe, BTP3-Neu5Ac. *PLOS ONE* 13, e0200761 (2018). [PubMed: 30001430]

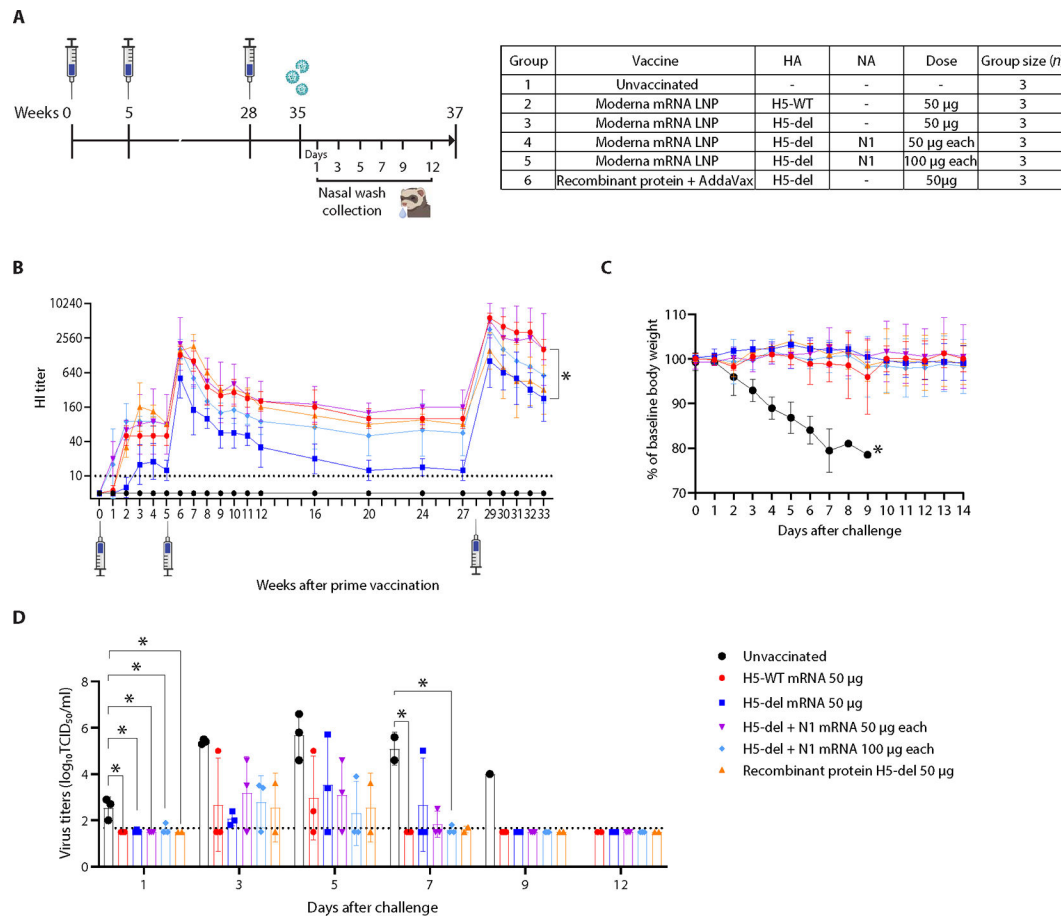


Fig. 1. H5 mRNA vaccines elicited protective immunity in ferrets.

(A) Study design. Three ferrets per group were immunized with PBS mock (group 1), 50 µg of mRNA vaccine encoding H5-WT (group 2) or H5-del (group 3), 50 and 100 µg of H5-del mRNA vaccine together with the same amount of mRNA vaccine encoding N1 NA (groups 4 and 5, respectively), or recombinant H5-del protein adjuvanted with AddaVax (group 6). animals were vaccinated intramuscularly three times. Seven weeks after the third immunization, ferrets were challenged with HPAI BE/FL/22 virus. nasal wash samples were collected on days 1, 3, 5, 7, 9, and 12 after challenge. one of the ferrets in group 6 had an experiment-unrelated issue and was euthanized in week 2 after prime vaccination. (B) HI titers were measured after vaccination using AW/SC/21 CVV antigen with 1% HRBC. (C) The body weights of ferrets were monitored for 14 days after challenge. all animals in the unvaccinated group succumbed by day 9 after infection because of severe disease progression. (D) Virus titers in nasal wash samples were determined on MDCK cells by TCID₅₀ assay. Because all animals in the unvaccinated group succumbed by day 9 after infection, no nasal wash titers on day 12 after infection are shown for this group. Data are presented as geometric mean and geometric SD [(B) and (D)] or arithmetic mean and SD (C). Dotted lines indicate the lower limit of detection. AUC analysis was used for (B) and (C), and differences were tested using a one-way ANOVA with Tukey's post hoc test. Viral titers were analyzed by pairwise *t* tests with Tukey-Kramer's adjustment for multiple comparisons on the log titers (D). Significant differences are indicated by **P* < 0.05.

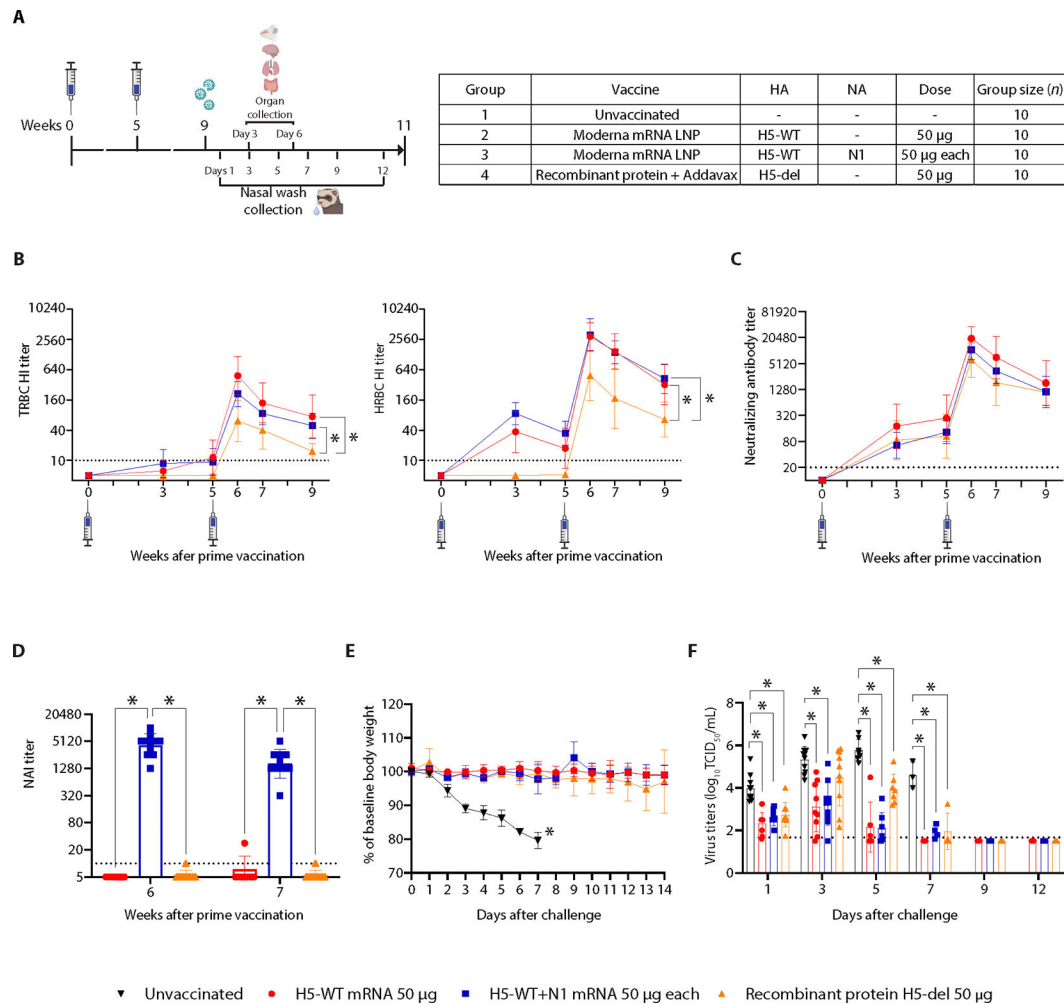


Fig. 2. Selected mRNA vaccines protected ferrets from lethal challenge.

(A) Study design. Ten ferrets per group were not vaccinated (group 1) or vaccinated with 50 µg of mRNA vaccine encoding H5-WT with or without 50 µg of mRNA vaccine encoding N1 NA (groups 2 and 3) or 50 µg of recombinant H5-del protein mixed with AddaVax (group 4). One of the ferrets in group 2 was eliminated for study-unrelated reasons before virus challenge. Four weeks after the second immunization, ferrets were challenged with BE/FL/22 virus. nasal wash samples were collected on days 1, 3, 5, 7, 9, and 12 after challenge. Three animals per group were euthanized on days 3 and 6 after challenge to collect the organs for viral load quantification and histopathology analysis. (B) HI titers were measured after vaccination using AW/SC/21 CVV antigen with 0.5% TRBC (left) or 1% HRBC (right). (C) Virus neutralization antibody titers were determined by Fra using the AW/SC/21 CVV. (D) NAI titers were measured after vaccination using the recombinant NA protein of AW/SC/21. (E) Body weights were monitored for 14 days after challenge. One ferret in group 1 and one in group 3 demonstrated neurological symptoms on days 5 and 7 after challenge, respectively, and were euthanized. The averages of body weight and the SDs were calculated on the remaining three or four ferrets, excluding the ferrets used for organ sampling. (F) Virus titers in nasal wash samples were determined on MDCK cells by TciD₅₀ assay. Because all animals in the unvaccinated group succumbed by day 7 after infection,

no nasal wash titers on days 9 and 12 after infection are shown for this group. Data are presented as the geometric mean with the geometric SD [(B) to (D) and (F)] or arithmetic mean with SD (E). Dotted lines indicate the lower limit of detection. AUC analysis was used for (B), (C), and (E), and differences were tested in a one-way ANOVA with Tukey's post hoc test. Neutralizing antibody titers and viral titers were analyzed by pairwise t tests with Tukey-Kramer's adjustment for multiple comparisons on the log titers in (D) and (F). Significant differences are indicated by $*P < 0.05$.

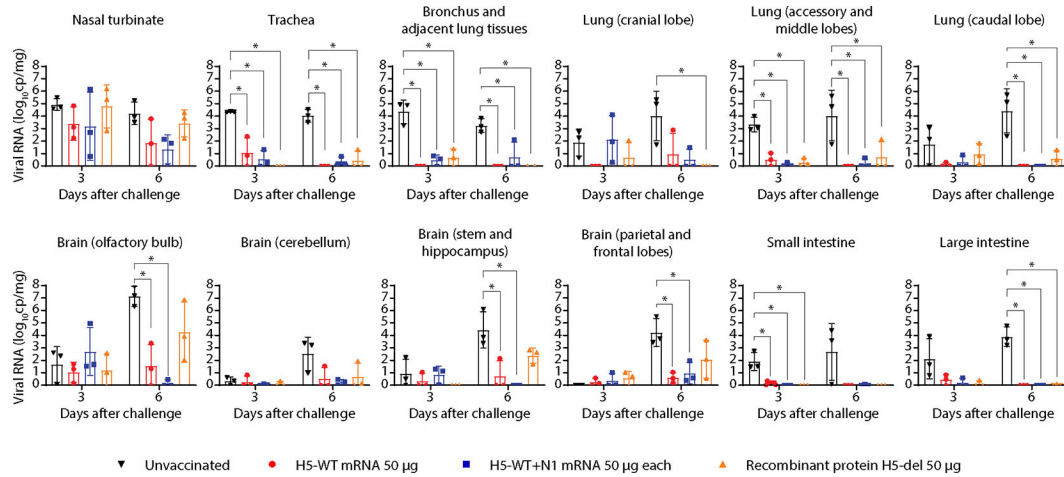


Fig. 3. H5-based mRNA vaccines reduced viral RNA load in organs of challenged ferrets.

Organ samples were collected from three ferrets in each group on days 3 and 6 after challenge and homogenized for quantification of viral RNA. Raw copy per microliter numbers for the reaction mixture were converted to copy (cp) per microgram of tissue. Data are presented as geometric mean with geometric SD. Viral RNA copy numbers were analyzed by pairwise *t* tests with Tukey-Kramer's adjustment for multiple comparisons on the log titers. Significance values between vaccinated groups and the unvaccinated groups are indicated by **P* < 0.05.

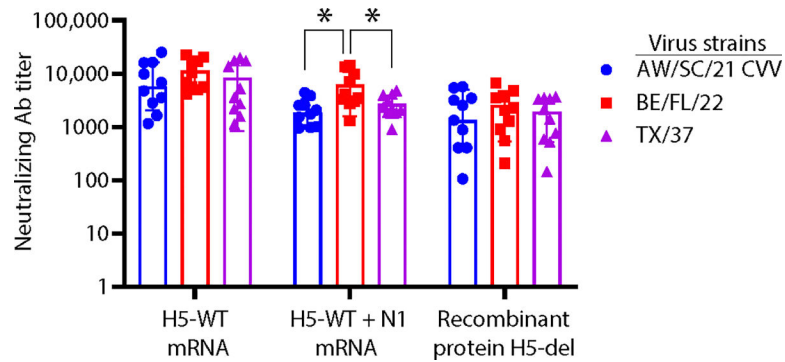


Fig. 4. Serum samples from vaccinated ferrets neutralized a human isolate related to the 2024 H5N1 outbreak in dairy cattle.

Serum samples were collected from ferrets 2 weeks after booster, corresponding to week 7 serum (Fig. 2B). Neutralizing antibody (Ab) titers were measured against the vaccine virus A/American wigeon/South Carolina/22–000345–001/2021 (AW/SC/21 CVV), the challenge virus A/bald eagle/Florida/22–006544–004/2022 (BE/FL/22), and the human isolate A/Texas/37/2024 (TX/37), using an FRA. Data are presented as geometric mean with geometric SD. Neutralizing titers were analyzed by pairwise *t* tests with Tukey-Kramer's adjustment for multiple comparisons on the log titers. Significance values between the different viruses are indicated by * $P < 0.05$.