Effectiveness of Whole, Inactivated, Low Pathogenicity Influenza A(H7N9) Vaccine against Antigenically Distinct, Highly Pathogenic H7N9 Virus

Masato Hatta, Gongxun Zhong, Shiho Chiba, Tiago J.S. Lopes, Gabriele Neumann, Yoshihiro Kawaoka

The recent emergence of highly pathogenic influenza A(H7N9) variants poses a great risk to humans. We show that ferrets vaccinated with low pathogenicity H7N9 virus vaccine do not develop severe symptoms after infection with an antigenically distinct, highly pathogenic H7N9 virus. These results demonstrate the protective benefits of this H7N9 vaccine.

Low pathogenicity influenza A(H7N9) viruses, which cause mild or asymptomatic disease in poultry, have caused ≥1,564 human infections since March 2013, with a case-fatality rate of ≈40% (1–5). Recently, highly pathogenic H7N9 viruses, characterized by multiple basic amino acids at the cleavage site of their hemagglutinin (HA) protein, have emerged. More than 750 cases of human H7N9 infections in 2017 (6) and the emergence of highly pathogenic H7N9 viruses emphasize the need for effective vaccines against low pathogenicity and highly pathogenic H7N9 viruses. We examined whether a World Health Organization (WHO) candidate vaccine based on a low pathogenicity WHO-recommended H7N9 influenza virus would protect ferrets against an antigenically distinct, highly pathogenic H7N9 influenza virus.

The Study

We generated a recombinant virus (HK125–HYPR8) that possesses the HA and neuraminidase (NA) genes of a low pathogenicity WHO-recommended H7N9 candidate vaccine virus (A/Hong Kong/125/2017 [7]) and the remaining genes from a high-yield A/Puerto Rico/8/34 (PR8) vaccine backbone virus (8). The HK125–HYPR8 virus was inactivated with β-propiolactone and purified through sucrose gradient ultracentrifugation.

We vaccinated 5-month-old female ferrets (6 per group) that were serologically negative for currently circulating human influenza viruses with 15 µg of HA of inactivated whole HK125–HYPR8 virions without adjuvant (Group 1) or mixed at a 1:1 ratio with AddaVax adjuvant (InvivoGen, San Diego, CA, USA), a squalene-based oil-in-water nanoemulsion similar to MF59 (9) (group 2); control animals received phosphate-buffered saline (group 3) or adjuvant (group 4) (Figure 1, panel A). All animals were vaccinated intramuscularly in both hind legs twice, 28 days apart.

Twenty-eight days after the second immunization, we intranasally challenged ferrets with 10^6 PFUs of highly pathogenic H7N9 rGD/3-NA294R virus (a neuraminidase inhibitor–sensitive subpopulation of highly pathogenic A/Guangdong/17SF003/2016 H7N9 virus) (10). These vaccine and challenge viruses belong to the Yangtze River Delta lineage of H7N9 viruses, which is responsible for recent infections of humans with highly pathogenic H7N9 viruses (6). However, A/Hong Kong/125/2017 and the A/Guangdong/17SF003/2016 challenge virus differ antigenically (11) (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/10/18-0403-Techapp1.pdf).

We monitored clinical signs, body weight, and body temperature daily for 14 days and collected throat and nasal swab specimens every day until day 7 postchallenge. On day 4 postchallenge, we euthanized 3 ferrets from each group and collected organs (lung, trachea, nasal turbinates, olfactory bulbs, and brain tissues pooled from anterior and posterior brain sections) for virus titration. We conducted statistical analysis of hemagglutinin inhibition (HI) titers, virus titers in swab and organ samples, and bodyweight and temperature changes among groups (online Technical Appendix Tables 2–21). We defined statistical significance as p<0.05.

After 1 immunization, HI titers were significantly lower in the ferrets immunized with nonadjuvanted HK125–HYPR8 vaccine than in those immunized with AddaVax-adjuvanted HK125–HYPR8 vaccine (p = 0.038; Figure 1, panel B; online Technical Appendix Table 2); however, after 2 immunizations, ferrets vaccinated with or without adjuvant (groups 1 and 2) developed high HI titers against HK125–HYPR8 virus. Vaccination with HK125–PR8 vaccine did not elicit measurable HI titers against the rGD/3-NA294R challenge virus after the first immunization but elicited reasonably high titers after the second immunization (Figure 1, panel B). After challenge with highly pathogenic H7N9 virus,
nonvaccinated ferrets (groups 3 and 4) became lethargic, experienced diarrhea, and lost appetite and bodyweight on days 2–6 postinfection (online Technical Appendix Figure), whereas vaccinated ferrets showed no noticeable symptoms. In addition, nonvaccinated ferrets demonstrated statistically higher body temperature than vaccinated ferrets on days 1, 2, 3, 5, and 6 postchallenge (online Technical Appendix Figure, Table 5). One ferret in group 3 and 2 ferrets in group 4 had to be euthanized on days 6–8 postinfection (Figure 1, panel C) because of severe symptoms (neurologic signs or inability to remain upright). In contrast, none of the vaccinated ferrets had any symptoms, indicating a protective effect of the low pathogenicity H7N9 vaccine against the challenge virus.

Analysis of throat and nasal swab samples demonstrated replication of highly pathogenic challenge virus in all ferrets (Figure 2, panel A). However, virus titers started to decline in vaccinated ferrets by day 3 postchallenge, and the infection was resolved by day 5 postchallenge; in contrast, nonvaccinated ferrets continued to shed high titers of challenge virus 4–7 days postchallenge. The virus titers in nasal swab samples on days 1, 3, 4, 5, 6, and 7 postchallenge and those in throat swab samples on days 1–7 postchallenge from nonvaccinated ferrets were significantly higher than those in vaccinated ferrets (online Technical Appendix Table 10). Thus, vaccination with HK125–HYPR8 virus led to reduced replication of the challenge virus in the upper respiratory tract of infected ferrets.

On day 4 postinfection, we euthanized 3 animals per group and determined virus titers in organs. We also assessed virus titers in organs of ferrets that were euthanized because of severe disease symptoms. In nonvaccinated ferrets, we detected high titers of virus in respiratory organs; in addition, we recovered virus from the olfactory bulbs or pooled samples from anterior and posterior sections of

Figure 1. Study design, HI titers after vaccination, and survival rates of vaccinated and nonvaccinated ferrets challenged with highly pathogenic influenza A(H7N9) virus. A) Study design. Six ferrets per group were immunized with inactivated whole HK125–HYPR8 virions containing 15 µg of HA protein without (group 1) or with adjuvant (group 2); control animals were vaccinated with PBS (group 3) or adjuvant (group 4). Animals were vaccinated intramuscularly twice 28 days apart. Twenty-eight days after the second immunization, ferrets were challenged with highly pathogenic H7N9 rGD/3-NA294R virus. Throat and nasal swab specimens were collected on days 1–7 postchallenge; 3 animals per group were euthanized on day 4 postchallenge to assess virus titers in organs. B) HI titers after vaccination. HI assays were performed against HK125–HYPR8 (upper panel) and rGD/3-NA294R (lower panel) with ferret sera collected before the second immunization (preboost) and before challenge (prechallenge). Statistical significance was determined as described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/10/18-0403-Techapp1.pdf). C) Survival rates. Survival was monitored for 14 days after challenge. Because 3 ferrets were euthanized on day 4 postchallenge for organ sampling, the survival rate was calculated on the basis of a group size of n = 3 thereafter. HA, hemagglutinin; HI, hemagglutination inhibition; PBS, phosphate-buffered saline.
the brains of 7 of the 9 animals tested (Figure 2, panel B). In vaccinated ferrets, we detected virus in the nasal turbinate of 4 of 6 animals and in the olfactory bulbs of 2 of 6 animals. We recovered no virus from the tracheas, lungs, or pooled samples from anterior and posterior brain sections (Figure 2, panel B), indicating that vaccination with HK125–HYPR8 prevented challenge virus replication in the lower respiratory organs.

Conclusions
We report the effectiveness of a whole, inactivated, low pathogenicity H7N9 vaccine against an antigenically distinct, highly pathogenic H7N9 virus in a ferret model. Vaccination prevented challenge virus replication in the lower respiratory organs, led to faster virus clearance in the upper respiratory organs, and prevented severe disease and death in ferrets, although the HI titers against the rGD/3-NA294R

![Figure 2. Virus titers in throat and nasal swab specimens and in the organs of vaccinated and nonvaccinated ferrets challenged with highly pathogenic influenza A(H7N9) virus. A) Virus titers in swab samples. Throat and nasal swabs were collected on days 1–7 postchallenge. Virus titers were determined based on plaque assays in MDCK cells. Statistical significance was determined as described in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/10/18-0403-Techapp1.pdf). B) Three ferrets from each group were euthanized on day 4 postchallenge for virus titration in the indicated organs. We also assessed virus titers in organs of ferrets that were euthanized because of severe symptoms (*). Virus titers were determined based on plaque assays in MDCK cells. Numbers along baseline indicate animal number. PBS, phosphate-buffered saline.]
challenge virus were lower than those against the HK125–HYPR8 vaccine virus. Statistical analyses demonstrated that HI titers against the HK125–HYPR8 vaccine virus after the first immunization were significantly higher (p = 0.038) in animals immunized with adjuvanted vaccine compared with animals immunized with nonadjuvanted vaccine (Figure 1, panel B; online Technical Appendix Table 2). Bodyweight changes after challenge were significantly milder (p = 0.0132–0.0489 on days 4–10, 12, and 13) in ferrets immunized with adjuvanted vaccine than in those vaccinated with nonadjuvanted vaccine. In addition, virus titers in nasal swabs on days 3 and 4 postchallenge (p = 0.0052 on day 3; p = 0.0163 on day 4) and in throat swabs on days 1, 3, and 4 (p = 0.0047 on day 1; p = 0.0003 on days 3 and 4) in ferrets immunized with nonadjuvanted vaccine were significantly higher than in those ferrets immunized with adjuvanted vaccine (online Technical Appendix Tables 9, 11), suggesting superior efficacy with Addavax.

Previously, WHO selected several low pathogenicity H7N9 candidate vaccine viruses, including A/Hong Kong/125/2017 (7). With the emergence of highly pathogenic H7N9 viruses that are antigenically distinct from previously circulating H7N9 viruses, WHO has updated its recommendations, and a candidate vaccine virus for highly pathogenic H7N9 viruses is now available (12). We tested whether in the event of a large-scale outbreak of highly pathogenic H7N9 viruses, candidate vaccine viruses to antigenically distinct H7N9 viruses might serve as a first line of defense. Our results in ferrets indicate the potential of a whole, inactivated vaccine based on a low pathogenicity H7N9 virus to prevent severe disease with fatal outcome after infection with an antigenically distinct, highly pathogenic H7N9 virus.

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Technical Appendix

Supplementary Methods

Cells

Madin-Darby canine kidney (MDCK) cells (obtained from ATCC) were maintained in Eagle’s minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. Human embryonic kidney 293T cells (obtained from ATCC) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) with antibiotics. All cells were maintained at 37°C with 5% CO₂ unless otherwise stated.

Virus and Reverse Genetics

The sequences of the haemagglutinin (HA) and neuraminidase (NA) genes of a low pathogenic WHO-recommended H7N9 candidate vaccine virus (A/Hong Kong/125/2017, H7N9) (1) were obtained from GenBank (accession numbers: CY235363 and CY235364, respectively). Based on the obtained sequences, the HA and NA genes were oligo-synthesized by SGI-DNA (La Jolla, CA) and cloned into a plasmid for viral RNA production (pPolI vector) (2). Plasmid-based reverse genetics for generating HK125-HYPR8 virus possessing the HA and NA genes of A/Hong Kong/125/2017 and the remaining genes from our high-yield A/Puerto Rico/8/34 (PR8) vaccine backbone virus was performed as previously described (2,3). At 48 h post-transfection, culture supernatants were collected and inoculated to MDCK cells for virus propagation. The virus stock was sequenced to confirm the absence of unwanted mutations.
Vaccine Preparation

The HK125-HYPR8 virus was propagated in 10-day-old embryonated chicken eggs. The viruses in the allantoic fluids were inactivated with 0.1% β-propiolactone (final concentration) at 4°C overnight and then purified through ultracentrifugation by using a linear 20%–50% (w/v) sucrose gradient. The HA amount of purified virus was calculated based on the intensities of the viral protein bands separated on a 4%–12% (wt/vol) NuPAGE Bis-Tris gel (Thermo Fisher Scientific) and the amount of total viral proteins was determined by using a Pierce BCA Protein assay kit (Thermo Fisher Scientific).

Animal Experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Madison, which also approved the protocol used (protocol numbers V00806). The facilities where this research was conducted are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Ferret Vaccine-Challenge Experiment

Five-month-old female ferrets (Triple F Farms), which were serologically negative by hemagglutination inhibition assay for currently circulating human influenza viruses, were used in this study. Six ferrets per group were vaccinated with 15 μg of HA of inactivated whole HK125-HYPR8 virions without adjuvant (Group 1) or mixed at a 1:1 ratio with AddaVax adjuvant (InvivoGen) (Group 2); control animals received PBS (Group 3) or adjuvant (Group 4) (Figure 1, panel A). All animals were vaccinated intramuscularly in both hind legs twice 28 days apart.

Twenty-eight days after the second immunization, ferrets were intranasally challenged with 10⁶ PFUs (PFU) of highly pathogenic H7N9 rGD/3-NA294R virus (a neuraminidase inhibitor-sensitive subpopulation of highly pathogenic A/Guangdong/17SF003/2016 H7N9 virus) (4). Clinical signs, bodyweight, and body temperature were monitored daily for 14 days. Throat and nasal swabs were collected every day until day 7 post-challenge. On day 4 post-challenge, three ferrets from each group were euthanized and organs (lung, trachea, nasal turbinates, olfactory bulbs, and brain tissues pooled from anterior and posterior brain sections) were collected for virus titration.
Hemagglutination Inhibition (HI) Assay

To detect hemagglutination inhibition (HI) activity (https://www.cdc.gov/flu/professionals/laboratory/antigenic.htm), serum samples were treated with receptor-destroying enzyme (RDE; Denka Seiken Co., Ltd) at 37°C for 16–20 h, followed by RDE inactivation at 56°C for 30–60 min. The treated sera were serially diluted 2-fold with PBS in 96-well U-bottom microtiter plates (Thermo Scientific, Rochester, New York, USA) and mixed with the amount of virus equivalent to eight hemagglutination units, followed by incubation at room temperature (25°C) for 30 min. After 50 μL of 0.5% turkey red blood cells was added to the mixtures, they were gently mixed and incubated at room temperature for a further 45 min. HI titers are expressed as the inverse of the highest antibody dilution that inhibited hemagglutination.

Statistical Analysis

Body temperature, bodyweight, nasal, and throat swabs were analyzed using a linear mixed model, with the groups and time as fixed effects, and the animals as random effects.

The commands lmer, lsmeans, and cld were used for the analysis, and all groups were compared to each other (pairwise). The p-values were adjusted using Holm’s method. For the comparison of the HI titers, we used two-tailed unpaired t-tests, and adjusted the p-values using Holm’s method. The virus titers from the organs were analyzed using one-way ANOVA, followed by Tukey’s post-hoc test.

Biosafety and Biosecurity

All recombinant DNA protocols were approved by the University of Wisconsin-Madison’s Institutional Biosafety Committee after risk assessments were conducted by the Office of Biologic Safety. In addition, the University of Wisconsin-Madison Biosecurity Task Force regularly reviews the research program and ongoing activities of the laboratory. The task force has a diverse skill set and provides support in the areas of biosafety, facilities, compliance, security, and health. Members of the Biosecurity Task Force are in frequent contact with the principal investigator and laboratory personnel to provide oversight and assure biosecurity. All experiments with live highly pathogenic H7N9 virus were performed in biosafety level 3 agricultural (BSL-3Ag) laboratories at the University of Wisconsin-Madison approved for such
use by the Centers for Disease Control and Prevention (CDC) and Animal and Plant Health Inspection Service (APHIS). Staff working in BSL-3Ag wear disposable overalls and powered air-purifying respirators.

The BSL-3Ag facility at University of Wisconsin-Madison was designed to exceed the standards outlined in *Biosafety in Microbiological and Biomedical Laboratories* (5th edition; http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf). Features include controlled access, entry/exit through a shower change room, effluent decontamination, negative air-pressure, double-door autoclaves, gas decontamination ports, HEPA-filtered supply and double-HEPA-filtered exhaust air, double-gasketed watertight and airtight seals, and airtight dampers on all ductwork. The structure is pressure-decay tested regularly. The University of Wisconsin-Madison facility has a dedicated alarm system that monitors all building controls (~500 possible alerts). Redundancies and emergency resources are built into the facility, including two air handlers, two compressors, two filters wherever filters are needed, two effluent sterilization tanks, two power feeds to the building, an emergency generator in case of a power failure, and other physical containment measures in the facility that operate without power. Biosecurity monitoring of the facility is ongoing. All personnel undergo Select Agent security risk assessment by the United States Criminal Justice Information Services Division and complete rigorous biosafety, BSL-3, and Select Agent training before participating in BSL-3-level experiments. Refresher training, including drills and review of emergency plans, is scheduled on a regular basis. The principal investigator participates in training sessions and emphasizes compliance to maintain safe operations and a responsible research environment. The laboratory occupational health plan is in compliance with the University of Wisconsin-Madison Occupational Health Program. Select Agent virus inventory, secured behind two physical barriers, is checked monthly and documentation is submitted to the University of Wisconsin-Madison Select Agent Program Manager. Virus inventory is submitted 1–2 times per year to the file holder in the Division of Select Agents and Toxins at the CDC. The research program, procedures, occupational health plan, documentation, security, and facilities are reviewed annually by the University of Wisconsin-Madison Responsible Official and at regular intervals by the CDC and the APHIS as part of the University of Wisconsin-Madison Select Agent Program.
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### Technical Appendix Table 1. Antigenic differences among H7 viruses by hemagglutination inhibition assays

| Virus                      | Monoclonal antibodies against HA from A/seal/Massachusetts/1/80 (H7N7) | Monoclonal antibodies against HA from A/Anhui/1/2013 (H7N9) | Antisera against HA and NA from A/Hong Kong/125/2017 |
|---------------------------|---------------------------------------------------|--------------------------------------------------------|----------------------------------------------------|
|                           | 46/6 55/3 58/2 | 2–20–20 3–7–19 19–17–20 | NR-9226 3,031 |                                             |
| H7N9 HK125-HYPR8 (HA and NA from A/Hong Kong/125/2017) | 6,400 12,800 3,200 | 1,600 1,600 1,600 | 2,560 640 |
| A/Guangdong/17SF003/2016  | 100 3,200 800 | 400 400 400 | 40 80 |
| A/Anhui/1/2013            | 3,200 25,600 3,200 | 6,400 12,800 6,400 | 1,280 640 |
| H7N7 A/seal/Massachusetts/1/1980 | 6,400 12,800 3,200 | 800 800 800 | 640 40 |

*HI titers are described as the inverse of the highest antibody dilution that inhibited hemagglutination. Values obtained with homologous antibodies are shown in bold. Monoclonal antibodies against the HA proteins of A/seal/Massachusetts/1/80 (H7N7) and A/Anhui/1/2013 (H7N9) viruses, and ferret antisera against A/Hong Kong/125/2017 were generated in our laboratory. Goat antiserum against A/Netherlands/219/03 (H7N7) was obtained from BEI Resources.
### Technical Appendix Table 2. Statistical analysis of HI titers of groups 1 and 2 against HK125-HYPR8 in Figure 1, panel B (Upper panel).

| A       | B       | Stage       | \( P \) value |
|---------|---------|-------------|---------------|
| Group 1 | Group 2 | Pre-boost   | 0.0380        |
| Group 1 | Group 2 | Pre-challenge| 0.3381        |

The two groups listed in columns A and B were compared.

Cyan: Values in column B are significantly higher than those in column A.

### Technical Appendix Table 3. Statistical analysis of HI titers of groups 1 and 2 against rGD/3-NA294R in Figure 1, panel B (Lower panel).

| A       | B       | Stage       | \( P \) value |
|---------|---------|-------------|---------------|
| Group 1 | Group 2 | Pre-boost   | N.A.          |
| Group 1 | Group 2 | Pre-challenge| 0.4871        |

The two groups listed in columns A and B were compared.

N.A.: not applicable

### Technical Appendix Table 4. Statistical analyses of body temperature changes in the Technical Appendix Figure (Comparison of the indicated groups)

| A       | B       | Days post-challenge | Estimate | \( t \)-ratio | \( P \) value |
|---------|---------|---------------------|----------|--------------|-------------|
| Group 4 | Group 2 | 0                   | 0.1000   | 0.2670       | 0.7898      |
|         |         | 1                   | 0.4500   | 1.2015       | 0.2314      |
|         |         | 2                   | 1.1667   | 3.1151       | 0.0022      |
|         |         | 3                   | 1.3167   | 3.5156       | 0.0006      |
|         |         | 4                   | -0.0667  | -0.1780      | 0.8590      |
|         |         | 5                   | 1.0333   | 1.9510       | 0.0529      |
|         |         | 6                   | 0.6667   | 1.2587       | 0.2100      |
|         |         | 7                   | -0.4115  | -0.6944      | 0.4885      |
|         |         | 8                   | -0.4777  | -0.6366      | 0.5253      |
|         |         | 9                   | 0.7223   | 0.9624       | 0.3373      |
|         |         | 10                  | 1.5889   | 2.1173       | 0.0358      |
|         |         | 11                  | 0.6556   | 0.8736       | 0.3837      |
|         |         | 12                  | -0.7111  | -0.9475      | 0.3448      |
|         |         | 13                  | 0.3556   | 0.4739       | 0.6363      |
|         |         | 14                  | -0.2444  | -0.3257      | 0.7451      |
|         |         | 0                   | 0.3167   | 0.8455       | 0.3991      |
|         |         | 1                   | 0.9833   | 2.6256       | 0.0095      |
|         |         | 2                   | 1.4833   | 3.9606       | 0.0001      |
|         |         | 3                   | 1.2167   | 3.2486       | 0.0014      |
|         |         | 4                   | 0.5333   | 1.4240       | 0.1565      |
|         |         | 5                   | 1.1333   | 2.1398       | 0.0339      |
|         |         | 6                   | 1.2667   | 2.3915       | 0.0180      |
|         |         | 7                   | 0.3218   | 0.5430       | 0.5879      |
|         |         | 8                   | 0.0889   | 0.1185       | 0.9058      |
|         |         | 9                   | 0.5899   | 0.7848       | 0.4338      |
|         |         | 10                  | 0.6556   | 0.8736       | 0.3837      |
|         |         | 11                  | 1.3233   | 1.7620       | 0.0800      |
|         |         | 12                  | -0.5111  | -0.6810      | 0.4969      |
|         |         | 13                  | -0.1111  | -0.1480      | 0.8825      |
|         |         | 14                  | -0.1777  | -0.2368      | 0.8131      |
| Group 4 | Group 1 | 0                   | 0.1333   | 0.3560       | 0.7223      |
|         |         | 1                   | -0.9333  | -2.4921      | 0.0138      |
|         |         | 2                   | 0.7333   | 1.9581       | 0.0520      |
|         |         | 3                   | -0.0500  | -0.1335      | 0.8940      |
|         |         | 4                   | -0.5833  | -1.5575      | 0.1214      |
|         |         | 5                   | -1.0333  | -1.9510      | 0.0529      |
|         |         | 6                   | -1.0000  | -1.8880      | 0.0609      |
|         |         | 7                   | -0.8782  | -1.4818      | 0.1404      |
|         |         | 8                   | -0.9495  | -1.1937      | 0.2344      |
|         |         | 9                   | 0.2005   | 0.2520       | 0.8014      |
|         |         | 10                  | 1.1005   | 1.3834       | 0.1685      |
|         |         | 11                  | 0.9505   | 1.1949       | 0.2340      |
|         |         | 12                  | -0.5495  | -0.6908      | 0.4907      |
|         |         | 13                  | -0.4995  | -0.6280      | 0.5309      |
|         |         | 14                  | -0.5495  | -0.6908      | 0.4907      |
| Group 2 | Group 1 | 0                   | 0.2167   | 0.5785       | 0.5638      |
|         |         | 1                   | 0.5333   | 1.4240       | 0.1565      |
|         |         | 2                   | 0.3167   | 0.8455       | 0.3991      |
|         |         | 3                   | -0.1000  | -0.2670      | 0.7898      |
|         |         | 4                   | 0.6000   | 1.6020       | 0.1112      |
| A | B | Days post-challenge | Estimate | t-ratio | P value |
|---|---|---------------------|----------|---------|---------|
| 5 | 0.1000 | 0.1888 | 0.8505 |
| 6 | 0.6000 | 1.1328 | 0.2590 |
| 7 | 0.7333 | 1.3846 | 0.1682 |
| 8 | 0.5667 | 1.0699 | 0.2863 |
| 9 | –0.1333 | –0.2517 | 0.8016 |
| 10 | –0.9333 | –1.7622 | 0.0800 |
| 11 | 0.6667 | 1.2587 | 0.2100 |
| 12 | 0.2000 | 0.3776 | 0.7062 |
| 13 | –0.4667 | –0.8811 | 0.2796 |
| 14 | 0.0667 | 0.1259 | 0.9000 |

Group 2

| Group 3 | | Estimate | t-ratio | P value |
|---------|---|----------|---------|---------|
| 1 | –1.3833 | –3.6936 | 0.0003 |
| 2 | –0.4333 | –1.1570 | 0.2490 |
| 3 | –1.3667 | –3.6491 | 0.0004 |
| 4 | –0.5167 | –1.3795 | 0.1697 |
| 5 | –2.0667 | –3.9019 | 0.0001 |
| 6 | –1.6667 | –3.1467 | 0.0020 |

Group 1

| Group 3 | | Estimate | t-ratio | P value |
|---------|---|----------|---------|---------|
| 1 | –1.9167 | –5.1177 | 0.0000 |
| 2 | –0.7500 | –2.0026 | 0.0470 |
| 3 | –1.2667 | –3.8217 | 0.0097 |
| 4 | –1.1167 | –2.9163 | 0.0333 |
| 5 | –2.1667 | –4.9070 | 0.0001 |
| 6 | –2.2667 | –4.7955 | 0.0000 |
| 7 | –1.2000 | –2.2656 | 0.0249 |

The two groups listed in columns A and B were compared.
Orange: Values in columns A are significantly higher than those in column B.
Cyan: Values in columns B are significantly higher than those in column A.

Technical Appendix Table 5. Statistical analyses of body temperature changes in the Technical Appendix Figure [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A | B | Days post-challenge | Estimate | t-ratio | P value |
|---|---|---------------------|----------|---------|---------|
| Non-vaccinated | Vaccinated | 0 | 0.1417 | 0.5182 | 0.6050 |
| Non-vaccinated | Vaccinated | 1 | 1.8333 | 4.3285 | 0.0000 |
| Non-vaccinated | Vaccinated | 2 | 0.9583 | 3.5055 | 0.0006 |
| Non-vaccinated | Vaccinated | 3 | 1.2917 | 4.7248 | 0.0000 |
| Non-vaccinated | Vaccinated | 4 | 0.5250 | 1.9204 | 0.0564 |
| Non-vaccinated | Vaccinated | 5 | 1.8000 | 4.1384 | 0.0001 |
| Non-vaccinated | Vaccinated | 6 | 1.4667 | 3.7936 | 0.0002 |
| Non-vaccinated | Vaccinated | 7 | 0.4690 | 1.1559 | 0.2492 |
| Non-vaccinated | Vaccinated | 8 | 0.4663 | 0.9404 | 0.3483 |
| Non-vaccinated | Vaccinated | 9 | 0.5296 | 1.1160 | 0.2659 |
| Non-vaccinated | Vaccinated | 10 | 0.3963 | 0.8350 | 0.4048 |
| Non-vaccinated | Vaccinated | 11 | 0.3629 | 0.7648 | 0.4454 |
| Non-vaccinated | Vaccinated | 12 | –0.2371 | –0.4995 | 0.6180 |
| Non-vaccinated | Vaccinated | 13 | 0.4629 | 0.9755 | 0.3306 |
| Non-vaccinated | Vaccinated | 14 | 0.1629 | 0.3433 | 0.7317 |

The two groups listed in columns A and B were compared.
Orange: Values in columns A are significantly higher than those in column B.
Technical Appendix Table 6. Statistical analyses of bodyweight changes in the Technical Appendix Figure (Comparison of the indicated groups)

| A         | B         | Days post-challenge | Estimate | t-ratio | P value |
|-----------|-----------|---------------------|----------|---------|---------|
| **Group 4** | **Group 2** |                     |          |         |         |
| 0         | 0         | 0.0000              | 0.0000   | 1.000   |         |
| 1         | 1         | -2.8454             | -1.8434  | 0.0672  |         |
| 2         | 2         | -4.7353             | -3.0678  | 0.0025  |         |
| 3         | 3         | -7.8214             | -5.0671  | 0.0000  |         |
| 4         | 4         | -10.5902            | -6.8609  | 0.0000  |         |
| 5         | 5         | -13.9604            | -6.3952  | 0.0000  |         |
| 6         | 6         | -17.0433            | -7.8075  | 0.0000  |         |
| 7         | 7         | -17.9853            | -7.3576  | 0.0000  |         |
| 8         | 8         | -19.9715            | -8.1702  | 0.0000  |         |
| 9         | 9         | -19.7374            | -6.3707  | 0.0000  |         |
| 10        | 10        | -16.9340            | -5.4658  | 0.0000  |         |
| 11        | 11        | -17.7547            | -5.7307  | 0.0000  |         |
| 12        | 12        | -15.9514            | -5.1487  | 0.0000  |         |
| 13        | 13        | -16.7998            | -5.4225  | 0.0000  |         |
| 14        | 14        | -21.7251            | -7.0123  | 0.0000  |         |
| **Group 4** | **Group 1** |                     |          |         |         |
| 0         | 0         | 0.0000              | 0.0000   | 1.000   |         |
| 1         | 1         | -1.4601             | -0.9459  | 0.3456  |         |
| 2         | 2         | -3.5500             | -2.2999  | 0.0228  |         |
| 3         | 3         | -5.8525             | -3.7915  | 0.0002  |         |
| 4         | 4         | -7.3417             | -4.7563  | 0.0000  |         |
| 5         | 5         | -8.9101             | -4.0817  | 0.0001  |         |
| 6         | 6         | -12.1794            | -5.5793  | 0.0000  |         |
| 7         | 7         | -12.5117            | -5.1184  | 0.0000  |         |
| 8         | 8         | -15.1942            | -6.2158  | 0.0000  |         |
| 9         | 9         | -14.7183            | -4.7507  | 0.0000  |         |
| 10        | 10        | -12.5474            | -4.0500  | 0.0001  |         |
| 11        | 11        | -14.5611            | -4.6999  | 0.0000  |         |
| 12        | 12        | -10.6810            | -3.4475  | 0.0007  |         |
| 13        | 13        | -12.4662            | -4.0237  | 0.0001  |         |
| 14        | 14        | -18.4718            | -5.9622  | 0.0000  |         |
| **Group 4** | **Group 3** |                     |          |         |         |
| 0         | 0         | 0.0000              | 0.0000   | 1.000   |         |
| 1         | 1         | 0.5914              | 0.3831   | 0.7022  |         |
| 2         | 2         | -0.6286             | -0.4072  | 0.6844  |         |
| 3         | 3         | -0.6640             | -0.4302  | 0.6677  |         |
| 4         | 4         | -0.4278             | -0.2772  | 0.7820  |         |
| 5         | 5         | -2.5794             | -1.1816  | 0.2392  |         |
| 6         | 6         | -2.9783             | -1.3643  | 0.1744  |         |
| 7         | 7         | -1.6499             | -0.6750  | 0.5007  |         |
| 8         | 8         | -6.1377             | -2.2957  | 0.0230  |         |
| 9         | 9         | -9.0080             | -2.7447  | 0.0068  |         |
| 10        | 10        | -5.0677             | -1.5441  | 0.1246  |         |
| 11        | 11        | -8.9753             | -2.7348  | 0.0070  |         |
| 12        | 12        | -4.3748             | -1.3329  | 0.1845  |         |
| 13        | 13        | -5.4998             | -1.6758  | 0.0958  |         |
| 14        | 14        | -11.3311            | -3.4526  | 0.0007  |         |
| **Group 2** | **Group 1** |                     |          |         |         |
| 0         | 0         | 0.0000              | 0.0000   | 1.000   |         |
| 1         | 1         | 1.3853              | 0.8975   | 0.3709  |         |
| 2         | 2         | 1.1853              | 0.7679   | 0.4437  |         |
| 3         | 3         | 1.9689              | 1.2755   | 0.2040  |         |
| 4         | 4         | 3.2486              | 2.1046   | 0.0369  |         |
| 5         | 5         | 5.0503              | 2.3135   | 0.0220  |         |
| 6         | 6         | 4.8640              | 2.2282   | 0.0273  |         |
| 7         | 7         | 5.4736              | 2.5075   | 0.0132  |         |
| 8         | 8         | 4.7773              | 2.1885   | 0.0301  |         |
| 9         | 9         | 5.0191              | 2.2992   | 0.0228  |         |
| 10        | 10        | 4.3866              | 2.0095   | 0.0462  |         |
| 11        | 11        | 3.1936              | 1.4630   | 0.1455  |         |
| 12        | 12        | 5.2704              | 2.4143   | 0.0163  |         |
| 13        | 13        | 4.3337              | 1.9852   | 0.0489  |         |
| 14        | 14        | 3.2533              | 1.4903   | 0.1382  |         |
| **Group 2** | **Group 3** |                     |          |         |         |
| 0         | 0         | 0.0000              | 0.0000   | 1.000   |         |
| 1         | 1         | 3.4368              | 2.2265   | 0.0274  |         |
| 2         | 2         | 4.1067              | 2.6605   | 0.0086  |         |
The two groups listed in columns A and B were compared. Orange: Values in columns A are significantly higher than those in column B. Cyan: Values in columns B are significantly higher than those in column A.

### Technical Appendix Table 7. Statistical analyses of bodyweight changes in the Technical Appendix Figure [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A         | B         | Days post-challenge | Estimate | t-ratio | P value |
|-----------|-----------|---------------------|----------|---------|---------|
| Group 1   | Group 3   | 0                   | 0.0000   | 0.0000  | 1.0000  |
|           |           | 1                   | 2.0515   | 1.3290  | 0.1858  |
|           |           | 2                   | 2.9215   | 1.8927  | 0.0603  |
|           |           | 3                   | 5.1885   | 3.3614  | 0.0010  |
|           |           | 4                   | 6.9138   | 4.4791  | 0.0000  |
|           |           | 5                   | 6.3307   | 2.9001  | 0.0043  |
|           |           | 6                   | 9.2011   | 4.2150  | 0.0000  |
|           |           | 7                   | 10.8617  | 4.9757  | 0.0000  |
|           |           | 8                   | 9.0565   | 3.7049  | 0.0003  |
|           |           | 9                   | 5.7103   | 2.3360  | 0.0208  |
|           |           | 10                  | 7.4798   | 3.0599  | 0.0026  |
|           |           | 11                  | 5.5858   | 2.2851  | 0.0237  |
|           |           | 12                  | 6.3065   | 2.5799  | 0.0108  |
|           |           | 13                  | 6.9664   | 2.8499  | 0.0050  |
|           |           | 14                  | 7.1407   | 2.9212  | 0.0040  |

### Technical Appendix Table 8. Statistical analyses of nasal swab titers in Figure 2, panel A (Comparison of the indicated groups)

| A         | B         | Days post-challenge | Estimate | t-ratio | P value |
|-----------|-----------|---------------------|----------|---------|---------|
| Non-vaccinated | Vaccinated | 0                   | 0.0000   | 0.0000  | 1.0000  |
| Non-vaccinated | Vaccinated | 1                   | -2.4485  | -2.2821 | 0.0237  |
| Non-vaccinated | Vaccinated | 2                   | -3.8284  | -3.5683 | 0.0005  |
| Non-vaccinated | Vaccinated | 3                   | -6.5050  | -6.0630 | 0.0000  |
| Non-vaccinated | Vaccinated | 4                   | -8.7520  | -8.1574 | 0.0000  |
| Non-vaccinated | Vaccinated | 5                   | -10.1456 | -9.6866 | 0.0000  |
| Non-vaccinated | Vaccinated | 6                   | -13.1222 | -8.6484 | 0.0000  |
| Non-vaccinated | Vaccinated | 7                   | -13.7356 | -8.6156 | 0.0000  |
| Non-vaccinated | Vaccinated | 8                   | -14.4331 | -8.4747 | 0.0000  |
| Non-vaccinated | Vaccinated | 9                   | -11.5925 | -6.1986 | 0.0000  |
| Non-vaccinated | Vaccinated | 10                  | -11.7323 | -6.2734 | 0.0000  |
| Non-vaccinated | Vaccinated | 11                  | -10.5443 | -5.6382 | 0.0000  |
| Non-vaccinated | Vaccinated | 12                  | -10.7698 | -5.7587 | 0.0000  |
| Non-vaccinated | Vaccinated | 13                  | -11.3965 | -6.0617 | 0.0000  |
| Non-vaccinated | Vaccinated | 14                  | -12.9144 | -6.9055 | 0.0000  |

The two groups listed in columns A and B were compared. Cyan: Values in columns B are significantly higher than those in column A.
The two groups listed in columns A and B were compared.  
Orange: Values in columns A are significantly higher than those in column B.  
Cyan: Values in columns B are significantly higher than those in column A.

### Technical Appendix Table 9. Statistical analyses of nasal swab titers in Figure 2, panel A (Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups)

| A                      | B           | Days post-challenge | Estimate | t-ratio | P value |
|------------------------|-------------|---------------------|----------|---------|---------|
| Non-vaccinated         | Vaccinated  | 1                   | 0.6913   | 2.5238  | 0.0131  |
| Non-vaccinated         | Vaccinated  | 2                   | 0.1423   | 0.5194  | 0.6045  |
| Non-vaccinated         | Vaccinated  | 3                   | 2.4564   | 8.9678  | 0.0000  |
| Non-vaccinated         | Vaccinated  | 4                   | 2.7034   | 9.8697  | 0.0000  |
| Non-vaccinated         | Vaccinated  | 5                   | 4.7185   | 12.1807 | 0.0000  |
| Non-vaccinated         | Vaccinated  | 6                   | 4.5263   | 11.6847 | 0.0000  |
| Non-vaccinated         | Vaccinated  | 7                   | 2.2603   | 5.5538  | 0.0000  |

### Technical Appendix Table 10. Statistical analyses of throat swab titers in Figure 2, panel A (Comparison of the indicated groups)

| A                      | B           | Days post-challenge | Estimate | t-ratio | P value |
|------------------------|-------------|---------------------|----------|---------|---------|
| Group 4                | Group 2     | 1                   | 1.7086   | 4.1442  | 0.0001  |
| Group 4                | Group 2     | 2                   | 1.0755   | 2.6085  | 0.0105  |
| Group 4                | Group 2     | 3                   | 3.8785   | 9.4070  | 0.0000  |
| Group 4                | Group 2     | 4                   | 4.1299   | 10.0168 | 0.0000  |
| Group 4                | Group 2     | 5                   | 5.0419   | 8.6470  | 0.0000  |
| Group 4                | Group 2     | 6                   | 4.8938   | 8.3930  | 0.0000  |
| Group 4                | Group 2     | 7                   | 4.2523   | 6.5030  | 0.0000  |
| Group 4                | Group 1     | 1                   | 0.5150   | 1.2492  | 0.2146  |
| Group 4                | Group 1     | 2                   | 0.7191   | 1.7441  | 0.0843  |
| Group 4                | Group 3     | 1                   | 2.3366   | 5.6872  | 0.0000  |
| Group 4                | Group 3     | 2                   | 2.6004   | 6.3071  | 0.0000  |
| Group 4                | Group 3     | 3                   | 5.0419   | 8.6470  | 0.0000  |
| Group 4                | Group 3     | 4                   | 4.8938   | 8.3930  | 0.0000  |
| Group 4                | Group 3     | 5                   | 4.2523   | 6.5030  | 0.0000  |
| Group 4                | Group 3     | 6                   | 0.2125   | 0.5153  | 0.6075  |
| Group 4                | Group 3     | 7                   | -0.1370  | -0.3324 | 0.7403  |
| Group 4                | Group 3     | 8                   | 0.2038   | 0.4942  | 0.6223  |
| Group 4                | Group 3     | 9                   | -0.1453  | -0.3525 | 0.7252  |
| A     | B     | Days post-challenge | Estimate | t-ratio | P value |
|-------|-------|--------------------|----------|---------|---------|
| 2     | Group 1 | 1                  | -1.1936  | -2.8950 | 0.0047  |
| 2     | Group 1 | 2                  | -0.3564  | -0.8644 | 0.3985  |
| 4     | Group 1 | 3                  | -1.5419  | -3.7398 | 0.0003  |
| 4     | Group 1 | 4                  | -1.5295  | -3.7097 | 0.0003  |
| 5     | Group 2 | 1                  | 0.0000   | 0.0000  | 1.0000  |
| 6     | Group 2 | 1                  | 0.0000   | 0.0000  | 1.0000  |
| 7     | Group 2 | 1                  | 0.0000   | 0.0000  | 1.0000  |
| 2     | Group 3 | 1                  | -1.4985  | -3.6289 | 0.0005  |
| 2     | Group 3 | 2                  | -1.2125  | -2.9409 | 0.0041  |
| 3     | Group 3 | 3                  | -3.6747  | -8.9128 | 0.0000  |
| 4     | Group 3 | 4                  | -4.2752  | -10.3693| 0.0000  |
| 5     | Group 3 | 5                  | -4.7254  | -8.1041 | 0.0000  |
| 6     | Group 3 | 6                  | -4.1531  | -7.1227 | 0.0000  |
| 7     | Group 3 | 7                  | -3.1449  | -5.3935 | 0.0000  |

The two groups listed in columns A and B were compared. Orange: Values in columns A are significantly higher than those in column B. Cyan: Values in columns B are significantly higher than those in column A.

**Technical Appendix Table 11.** Statistical analyses of throat swab titers in Figure 2, panel A [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A     | B     | Days post-challenge | Estimate | t-ratio | P value |
|-------|-------|--------------------|----------|---------|---------|
| Non-vaccinated | Vaccinated | 1                  | 1.0056   | 3.1990  | 0.0018  |
| Non-vaccinated | Vaccinated | 2                  | 0.9658   | 3.0723  | 0.0027  |
| Non-vaccinated | Vaccinated | 3                  | 3.0057   | 9.5613  | 0.0000  |
| Non-vaccinated | Vaccinated | 4                  | 3.4378   | 10.9361 | 0.0000  |
| Non-vaccinated | Vaccinated | 5                  | 4.8836   | 10.9852 | 0.0000  |
| Non-vaccinated | Vaccinated | 6                  | 4.5235   | 10.1750 | 0.0000  |
| Non-vaccinated | Vaccinated | 7                  | 3.5427   | 7.5786  | 0.0000  |

The two groups listed in columns A and B were compared. Orange: Values in columns A are significantly higher than those in column B.

**Technical Appendix Table 12.** Statistical analyses of brain titers in Figure 2, panel B (Comparison of the indicated groups).

| A     | B     | LWR | UPR | Difference | Adjusted P value |
|-------|-------|-----|-----|------------|------------------|
| Group 2 | Group 4 | -3.8181 | 1.7667 | -1.0257 | 0.6569 |
| Group 1 | Group 4 | -3.8181 | 1.7667 | -1.0257 | 0.6569 |
| Group 3 | Group 4 | -3.1334 | 2.4514 | -0.3410 | 0.9783 |
| Group 1 | Group 2 | -2.7924 | 2.7924 | 0.0000 | 1.0000 |
| Group 3 | Group 2 | -2.1078 | 3.4770 | 0.6846 | 0.8592 |
| Group 3 | Group 1 | -2.1078 | 3.4770 | 0.6846 | 0.8592 |

The two groups listed in columns A and B were compared. LWR: Lower confidence interval UPR: Upper confidence interval

**Technical Appendix Table 13.** Statistical analyses of brain titers in Figure 2, panel B [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A     | B     | LWR | UPR | Difference | Adjusted P value |
|-------|-------|-----|-----|------------|------------------|
| Vaccinated | Non-vaccinated | -2.0956 | 0.3853 | -0.8551 | 0.1556 |

The two groups listed in columns A and B were compared. LWR: Lower confidence interval UPR: Upper confidence interval

**Technical Appendix Table 14.** Statistical analyses of lung titers in Figure 2, panel B (Comparison of the indicated groups).

| A     | B     | LWR | UPR | Difference | Adjusted P value |
|-------|-------|-----|-----|------------|------------------|
| Group 2 | Group 4 | -7.3317 | -3.7885 | -3.5601 | 0.0000 |
| Group 1 | Group 4 | -7.3317 | -3.7885 | -3.5601 | 0.0000 |
| Group 3 | Group 4 | -1.3220 | 2.2212 | 0.4496 | 0.8469 |
| Group 1 | Group 2 | -1.7716 | 1.7716 | 0.0000 | 1.0000 |
| Group 3 | Group 2 | 4.2381 | 7.7813 | 6.0097 | 0.0000 |
### Technical Appendix Table 15. Statistical analyses of lung titers in Figure 2, panel B [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Vaccinated   | Non-vaccinated | –6.5960 | –4.9737 | –5.7849 | 0.0000 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval

### Technical Appendix Table 16. Statistical analyses of nasal turbinate titers in Figure 2, panel B (Comparison of the indicated groups).

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Group 2      | Group 4      | –11.8235 | 2.0552 | –4.8841 | 0.1885 |
| Group 1      | Group 4      | –9.8570  | 4.0217 | –2.9177 | 0.5621 |
| Group 3      | Group 4      | –7.0473  | 6.8314 | –0.1079 | 1.0000 |
| Group 1      | Group 2      | –4.9729  | 8.9058 | 1.9664  | 0.8017 |
| Group 3      | Group 2      | –2.1632  | 11.7155 | 4.7762 | 0.2017 |
| Group 3      | Group 1      | –4.1296  | 9.7491 | 2.8097  | 0.5896 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval

### Technical Appendix Table 17. Statistical analyses of olfactory bulb titers in Figure 2, panel B (Comparison of the indicated groups).

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Group 2      | Group 4      | –2.5603 | 3.1929 | 0.3163   | 0.9839 |
| Group 1      | Group 4      | –2.8465 | 2.9067 | 0.0301   | 1.0000 |
| Group 3      | Group 4      | 0.0017  | 5.7549 | 2.8783   | 0.0499 |
| Group 1      | Group 2      | –3.1628 | 2.5904 | –0.2682  | 0.9880 |
| Group 3      | Group 2      | –0.3146 | 5.4386 | 2.5620   | 0.0820 |
| Group 3      | Group 1      | –0.0284 | 5.7248 | 2.8482   | 0.0523 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval

### Technical Appendix Table 18. Statistical analyses of olfactory bulb titers in Figure 2, panel B [Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups].

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Vaccinated   | Non-vaccinated | –7.0544 | –0.6395 | –3.8469 | 0.0234 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval

### Technical Appendix Table 19. Statistical analyses of olfactory bulb titers in Figure 2, panel B (Comparison of the indicated groups).

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Group 2      | Group 4      | –8.1933 | –5.3690 | –6.7811 | 0.0000 |
| Group 1      | Group 4      | –8.1933 | –5.3690 | –6.7811 | 0.0000 |
| Group 3      | Group 4      | –1.7448 | 1.0795 | –0.3326 | 0.8724 |
| Group 1      | Group 2      | –1.4121 | 1.4121 | 0.0000   | 1.0000 |
| Group 3      | Group 2      | 5.0363  | 7.8606 | 6.4485   | 0.0000 |
| Group 3      | Group 1      | 5.0363  | 7.8606 | 6.4485   | 0.0000 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval

### Technical Appendix Table 20. Statistical analyses of tracheal titers in Figure 2, panel B (Comparison of the indicated groups).

| A            | B            | LWR   | UPR   | Difference | Adjusted P value |
|--------------|--------------|-------|-------|------------|-----------------|
| Group 2      | Group 4      | –8.1933 | –5.3690 | –6.7811 | 0.0000 |
| Group 1      | Group 4      | –8.1933 | –5.3690 | –6.7811 | 0.0000 |
| Group 3      | Group 4      | –1.7448 | 1.0795 | –0.3326 | 0.8724 |
| Group 1      | Group 2      | –1.4121 | 1.4121 | 0.0000   | 1.0000 |
| Group 3      | Group 2      | 5.0363  | 7.8606 | 6.4485   | 0.0000 |
| Group 3      | Group 1      | 5.0363  | 7.8606 | 6.4485   | 0.0000 |

The two groups listed in columns A and B were compared. Cyan: Values in column B are significantly higher than those in column A. LWR: Lower confidence interval UPR: Upper confidence interval
### Technical Appendix Table 21

Statistical analyses of tracheal titers in Figure 2, panel B (Comparison of vaccinated (groups 1 and 2 merged) and unvaccinated (groups 3 and 4 merged) groups).

|       | B     | LWR   | UPR   | Difference | Adjusted P value |
|-------|-------|-------|-------|------------|-----------------|
| Vaccinated | Non-vaccinated | -7.2579 | -5.9717 | -6.6148    | 0.0000          |

*UPR: Upper confidence interval*

The two groups listed in columns A and B were compared.

Cyan: Values in columns B are significantly higher than those in column A.

LWR: Lower confidence interval

UPR: Upper confidence interval
Technical Appendix Figure. Bodyweight and temperature changes in vaccinated and non-vaccinated ferrets challenged with highly pathogenic H7N9 virus. Six ferrets per group were challenged intranasally with $10^6$ PFU of highly pathogenic H7N9 rGD/3-NA294R virus; bodyweight and temperature were monitored daily for 14 days. Ferrets #4 – #6 in each group were euthanized on day 4 post-challenge for organ sampling. Ferret #1 in group 3, and ferrets #1 and #2 in group 4 were euthanized on days 7, 6, and 8 post-challenge, respectively, due to severe symptoms. Statistically significant differences in bodyweight changes between ferrets in Groups 1 and 2 are marked (*); *, p<0.05.