Science
AAAS

Supplementary Materials for

A multivalent nucleoside-modified mRNA vaccine against all known influenza virus subtypes

Claudia P. Arevalo, Marcus J. Bolton, Valerie Le Sage, Naiqing Ye, Colleen Furey, Hiromi Muramatsu, Mohamad-Gabriel Alameh, Norbert Pardi, Elizabeth M. Drapeau, Kaela Parkhouse, Tyler Garretson, Jeffrey S. Morris, Louise H. Moncla, Ying K. Tam, Steven H. Y. Fan, Seema S. Lakdawala, Drew Weissman, Scott E. Hensley*

Correspondence to: hensley@pennmedicine.upenn.edu

This PDF file includes:

Other Supplementary Materials for this manuscript includes the following:

Materials and Methods

mRNA production

Sequences of the 20 HAs were codon-optimized, synthesized (GenScript), and cloned into a mRNA production plasmid. After ligation into expression vectors, mRNAs were produced using T7 RNA polymerase (Megascript, Ambion) on linearized plasmids. mRNAs were transcribed to contain 101 nucleotide-long poly(A) tails. m1Ψ-5′-triphosphate (TriLink) instead of UTP was used to generate modified nucleoside-containing mRNA. Capping of the in vitro transcribed mRNAs was performed cotranscriptionally using the trinucleotide cap1 analog, CleanCap (TriLink). mRNA was purified by cellulose purification as previously described (*1*). All mRNAs were analyzed by denaturing or native agarose gel electrophoresis and were stored frozen at -20°C. For HA subtypes currently circulating in human (H1, H3, B/Yam, and B/Vic), we included HAs from contemporary WHO selected vaccine strains. For HA subtypes that have caused infrequent outbreaks or pandemics in humans (H2, H5, and H7), we selected HAs from prototype strains from that outbreak/pandemic. For other subtypes, we chose HAs that have previously been shown to be able to be produced as a recombinant protein (e.g., H4, H8, H9, H11, H12, and H16 (*2*)). mRNAs expressing the following list of HAs were included in this study: A/Michigan/45/2015(H1), A/Japan/305/1957 (H2), A/Singapore/INFIMH-16-0019/2016(H3), A/duck/Czech/1956 (H4), A/Vietnam/1203/2004 (H5), A/Taiwan/2/2013 (H6), A/Shanghai/02/2013 (H7), A/mallard/Sweden/24/2002 (H8), A/Hong Kong/33982/2009 (H9), A/Jiangxi/09037/2014 (H10), A/shoveler/Netherlands/18/1999 (H11), A/mallard/Interior Alaska/7MP0167/2007 (H12), A/shorebird/Delaware/68/2004 (H13), A/mallard/Gurjev/263/1982 (H14), A/shearwater/West Australia/2576/1979 (H15), A/black headed gull/Sweden/5/1999 (H16), A/yellow shouldered bat/Guatemala/06/2010 (H17), A/bat/Peru/33/2010 (H18), B/Colorado/06/2017 (IBV; Victoria-lineage), and B/Phuket/3073/2013 (IBV; Yamagata-lineage).

LNP formulation of the mRNA

Cellulose purified m1Ψ-containing RNAs were encapsulated in LNPs using a self-assembly process as previously described wherein an ethanolic lipid mixture of ionizable cationic lipid, phosphatidylcholine, cholesterol and polyethylene glycol-lipid was rapidly mixed with an aqueous solution containing mRNA at acidic pH (*3*). The RNA-loaded particles were characterized and subsequently stored at -80°C at a concentration of 1 µg/µl. The mean hydrodynamic diameter of these mRNA-LNP was ~80 nm with a polydispersity index of 0.02-0.06 and an encapsulation efficiency of ~95%.

Phylogenetic analyses of HAs in the 20 HA mRNA-LNP vaccine

We downloaded all available full-length HA genes from GISAID (gisaid.org) for the following influenza A HA types: H1N1 (2009-present), H2, H3N2 (2000-present), H4, H5, H6, H7, H8, H9N2, H10, H11, H12, H13, H14, H15, H16, and H17. All available H18 HA sequences were downloaded from the Influenza Research Database (*4*). For influenza B HAs, we downloaded all available full length HA sequences from 1980-present for the Victoria and Yamagata lineages from GISAID. An acknowledgment list for all GISAID sequences used in the phylogenies is available as a Supplementary file. To contextualize the vaccine sequences, we built three phylogenetic trees using the Nextstrain pipeline (*5*): one for the group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18), one for the group 2 HAs (H3, H4, H7, H10, H14, and H15), and one for influenza B HAs (Victoria and Yamagata lineages). For each tree, we

randomly subsampled the data to 10 sequences per HA type (for influenza A sequences) or lineage (for influenza B sequences) per year, excluding duplicate sequences, sequences sampled prior to 1950, and any sequences with incomplete collection dates or containing non-nucleotide characters. Sequences for aligned with MAFFT (*6*), and divergence phylogenies were constructed with IQ-TREE (*7*) under a GTR substitution model. Tree plotting and visualization was performed using Baltic (https://github.com/evogytis/baltic).

Mouse experiments

Female 6-8-week-old C57BL/6 mice were purchased from Charles River Laboratories. mRNA-LNPs (2.5-50 µg) were diluted in 50-100 µl PBS and injected into animals intramuscularly (i.m.) into one or both hind legs. For the 20 HA mRNA-LNP vaccinations, individual HA mRNA-LNPs were pooled before vaccination. After vaccination, blood samples were obtained on different days from the submandibular vein and serum was isolated for antigenic analyses. For some experiments, mice were then anesthetized by inhalation of isoflurane (4-5% in oxygen) and intranasally (i.n.) inoculated with 100,000 $(TCID)_{50}$ A/California/7/2009 H1N1 (5 LD50) or 500 $(TCID)_{50}$ of A/Puerto Rico/8/1934 H1N1 influenza virus (2 LD50) in 50 µl of PBS. Clinical severity scores were calculated by monitoring mice for lethargy, hunched posture, ruffled fur, and labored breathing. Clinical severity was scored by an analyst blinded to the experimental groups. Weight loss and survival were also monitored. For some experiments, we passively transferred 800 µl of serum from mice immunized with the 20 HA mRNA-LNP vaccine into naïve mice 5 hours prior to A/California/7/2009 or A/Puerto Rico/8/1934 infection. For some experiments, $CD4^+$ and $CD8^+$ T cells were depleted from mice simultaneously using 0.2 mg per injection of anti-Thy1.2 (BioXCell, BE0066) as previously described (*8*). Antibody treatments were administered 3 days and 1 day before infection and 2 days and 5 days after infection. Depletion was confirmed by flow cytometric analysis of splenocytes on day 6 post-infection. All murine experiments were approved by the Institutional Animal Care and Use Committees of the Wistar Institute and the University of Pennsylvania. Sample size for each experiment was determined based on similar previous experiments.

Ferret experiments

Ferret experiments were completed at the University of Pittsburgh. Six-month-old male ferrets were purchased from Triple F Farms (Sayre, PA, USA). All ferrets were screened for antibodies against circulating influenza A and B viruses, as determined by hemagglutinin inhibition assays using the following antigens obtained through the International Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA: 2018-2019 WHO Antigen, Influenza A (H3) Control Antigen (A/Singapore/INFIMH-16- 0019/2016), BPL-Inactivated, FR-1606; 2014-2015 WHO Antigen, Influenza A(H1N1)pdm09 Control Antigen (A/California/07/2009 NYMC X-179A), BPL-Inactivated, FR-1184; 2018-2019 WHO Antigen, Influenza B Control Antigen, Victoria Lineage (B/Colorado/06/2017), BPL-Inactivated, FR-1607; 2015-2016 WHO Antigen, Influenza B Control Antigen, Yamagata Lineage (B/Phuket/3073/2013), BPL-Inactivated, FR-1403. Ferrets were vaccinated i.m. with 60 µg mRNA-LNPs (in a total volume of 100 µl) and then boosted with the same vaccine dose 28 days later. Ferrets were infected i.n. with 10^6 $TCID_{50}$ of A/Ruddy turnstone/Delaware/300/2009 H1N1 influenza virus (in a total volume of 500 µl split between nostrils) 28 days after the second vaccination. Clinical signs such as weight loss, temperature, activity, sneezing, coughing,

lethargy, and nasal discharge were recorded during each procedure as previously described (*9*). Animals were provided urgent care diet cat food twice a day to entice eating once they reached 10% weight loss and Ringer's lactated solution was administered subcutaneously up to twice daily upon observed dehydration. Humane endpoints for this study included body weight loss exceeding 20% (relative to weight at challenge) and a prolonged inactivity based on the system described in (*9*). Animals were sedated using isoflurane for all nasal washes (5% isoflurane) and survival blood draws (3% isoflurane). Ketamine (25mg/kg) and xylazine (2mg/kg) were used for sedation for all terminal procedures followed by cardiac administration of euthanasia solution (10mg/kg). All ferret experiments were approved by the Institutional Animal Care and Use Committees of the University of Pittsburgh and the University of Pennsylvania. Sample size for this experiment was determined based on similar previous experiments.

Recombinant HA proteins

Recombinant full-length HA proteins and "headless" HA proteins were used as antigens in ELISAs and absorption assays. For full-length HA proteins, plasmids were created with codon-optimized full-length HA sequences and HA transmembrane domains were replaced with a FoldOn trimerization domain from T4 fibritin, an AviTag site specific biotinylation sequence, and a hexahistidine tag, as previously described (*10*). We obtained plasmids encoding recombinant headless H1 and H3 HA proteins from A. McDermott and B. Graham from the Vaccine Research Center at the National Institutes of Health (*11, 12*). Plasmids were transfected into 293F suspension cells (RRID:CVCL_D603) and supernatants were isolated 4 days later and clarified by centrifugation at 3200xg. HA proteins were purified from supernatants by Ni-NTA affinity chromatography (Qiagen). For some experiments, proteins were biotinylated using the Avidity BirA-500 kit according to the manufacturer's instructions.

ELISAs

Ninety-six well ELISA plates (Immulon) were coated with recombinant proteins and ELISAs were performed as previously described (*13*). For some experiments, we used streptavidin-coated ELISA plates and biotinylated HA proteins as previously described (*13*). Plates were incubated with peroxidase-conjugated goat anti-mouse IgG (Jackson, 115-035-003), or peroxidase-conjugated goat anti-ferret IgG (Abcam, ab112770). Data were analyzed using Prism 8.0 (GraphPad), and the area under the curve (AUC) was calculated. Data were represented as mean+/- SEM.

Focus reduction neutralization test (FRNT)

Serum samples were treated with receptor-destroying enzyme (RDE) (Denka-Seiken) for 2 hours at 37ºC, followed by heat-inactivation for 30 min at 56ºC and FRNTs were performed as previously described (*14*). $FRNT_{50}$ titers are reported as the highest reciprocal serum dilution that inhibited at least 50% of virus in relation to virus only control wells. Serum samples that reduced the amount of virus less than 50% at a 1:20 dilution were assigned an $FRNT_{50}$ titer of 10.

GFP-based neutralization assays

For H5 and H7 neutralization assays, we used influenza viruses that encode GFP within the PB1 segment and cells stably expressing PB1 and performed neutralization assays as previously described (*15*). Viruses were produced using reverse genetics plasmids encoding the PB2, PA, NP, M, and NS segments from A/Puerto Rico/8/1934, the reverse genetics plasmid pHH-

PB1flank-GFP which replaces the PB1 coding sequence with GFP, and reverse genetics plasmids encoding the HA and NA segments from either A/Vietnam/1203/2004 (H5N1) or A/Shanghai/02/2013 (H7N9). GFP fluorescence intensity was measured using an EnVision multi-mode microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence signal was determined by averaging GFP levels in cell-only control wells from each plate. To obtain percent of maximal infectivity, background fluorescence signal was subtracted from all wells and the signal from serum-containing wells was divided by the plate's average signal from virus-only wells. Percent neutralization was determined by subtracting the percent of maximal infectivity from 100. Fifty % neutralization titers are reported as the highest reciprocal serum dilution that reduced GFP levels by at least 50% in relation to virus only control wells. Serum samples that reduced GFP levels by less than 50% at a 1:20 dilution were assigned a 50% neutralization titer of 10. The pHH-PB1flank-GFP plasmid and the 293T-CMV-PB1 and MDCK-SIAT1-TMPRSS2-PB1 cells used for these assays were provided by J. Bloom at Fred Hutchinson Cancer Research Center.

Absorption assays

Streptavidin M-280 Dynabeads (ThermoFisher) were couple to biotinylated recombinant HA proteins according to manufacturer's instructions. Briefly, beads were washed with PBS + 0.1% BSA. Excess biotinylated recombinant HA proteins from A/Michigan/45/2015 (H1) or A/Singapore/INFIMH-16-0019/2016 (H3) were coupled to beads at a concentration of 0.2 ug/ul. Proteins were allowed to incubate with beads while rotating for 30 min at room temperature. Beads were separated from unbound proteins using a magnet, washed, and resuspended in PBS + 0.1% BSA. Serum samples were diluted 1:25 in PBS and incubated with protein coupled beads at a ratio of 1:2. The bead and sample mixtures were incubated for 1 hour at room temperature while shaking at 800 rpm. After this incubation, antibodies bound to beads were removed using a magnet. The remaining unbound antibody fractions were used in serological assays.

Antibody-dependent cellular cytotoxicity (ADCC) reporter assay

Bioluminescent reporter assay kits (Promega) were used according to manufacturer's instructions. 293T cells (RRID:CVCL_0063) were transfected to express membrane bound HA proteins from A/Michigan/45/2015 or A/Puerto Rico/8/1934 using Lipofectamine2000 (Invitrogen) and incubated overnight at 37°C with 5% CO2. The next day, cells were seeded on 96-well flat bottom plates (Corning) and allowed to incubate overnight. On the day of the assay, 4%-ultra-low-IgG serum was added to RPMI 1640 medium. Heat-inactivated serum samples were serially diluted three-fold in assay buffer for a final starting dilution of 1:20. Medium was removed from the HA expressing target 293T cells and 25ul of warm assay buffer was added to each well along with 25ul of diluted serum. ADCC effector cells (Jurkat cell line expressing murine FcγRIV cell-surface receptor; Promega) were thawed and diluted at effector:target ratio of 25:1. The mixture was allowed to incubate for 6 hours at 37ºC. Cells and Bio-Glo Luc substrate (Promega) were adjusted to RT and 75ul of substrate was added to each well. Luminescence was read on an EnVision reader. Fold induction was calculated as RLU (induced-background) / RLU (no antibody control-background).

Quantification of virus in infected lungs from mice

Lungs were harvested from mice 2 or 5 days post-infection. Following harvest, lungs were weighed, and then homogenized in sterile PBS to generate a 20% lung-in-buffer solution.

Homogenates were clarified twice by centrifugation, aliquoted and stored at -80°C until they were tittered by $TCID_{50}$ assay. One day before the assay, MDCK cells (RRID:CVCL_0422) were seeded in 96-well flat-bottom tissue culture plates at 25,000 cells per well. On the day of the assay, media was removed from cells and replaced with $TCID_{50}$ medium (1X Modified Eagle's Medium supplemented with 10 ug/ml of gentamicin sulfate, 5 mM HEPES buffer, and 1 ug/ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin). Clarified lung homogenate samples were serially diluted 10-fold in the plates and each sample was tested in quadruplicate. Plates were incubated for 3 days in a 37°C humidified incubator supplemented with 5% CO_2. Wells were scored for cytopathic effect by an analyst blinded to the experimental groups and $TCID_{50}$/ml titers were determined using a previously published method (*16*).

Statistical methods

Statistical analyses were completed using GraphPad Prism version 8. Results are reported as means ± SEM or geometric means ± 95% CI. Statistical significance for group comparisons were determined using a Welch's *t* test or one-way ANOVA with Tukey's post-test or Dunnett's to compare with control groups as indicated in the figure legends. Longitudinal data were analyzed by mixed model ANOVA with Geisser-Greehouse correction and Sidak's multiple comparisons. Data were log transformed for analysis of neutralization data to engender normality. Log-rank tests were used to compare groups with respect to overall survival time. All *P*-values <0.05 were considered statistically significant.

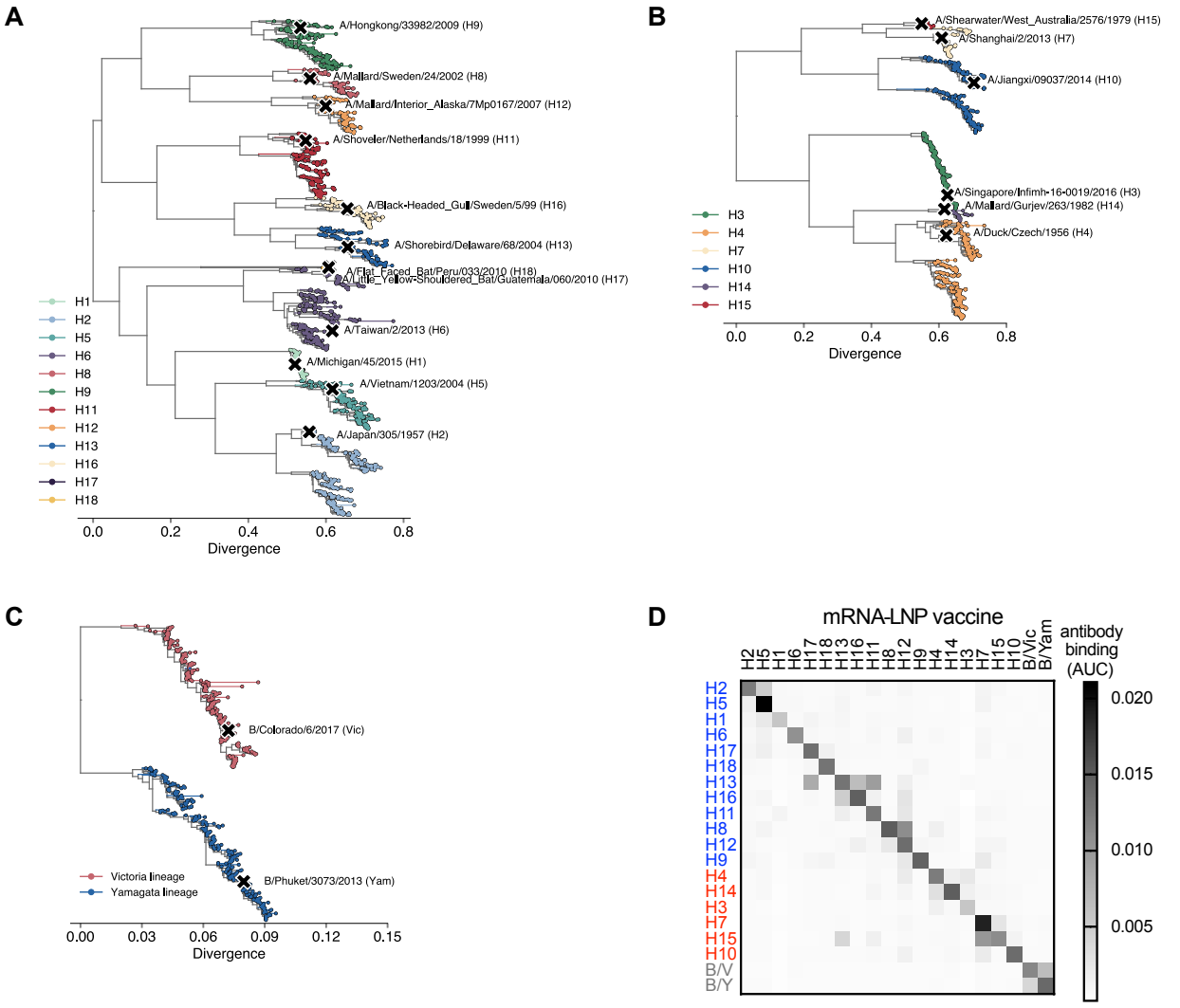

Fig. S1. **20-HA mRNA-LNP antigens.** Phylogenetic trees were created using Nextstrain for group 1 HAs (**A**), group 2 HAs (**B**), and IBV (**C**). mRNA vaccine HAs are indicated with an "X". (**D**) Mice were vaccinated intramuscularly with 3 μg of single HA mRNA-LNPs. Sera were collected 28 days post-vaccination and antibody levels were quantified by ELISA. Group 1 HAs are colored blue and group 2 HAs are colored red. Three animals were included per experimental group. Data are shown as means.

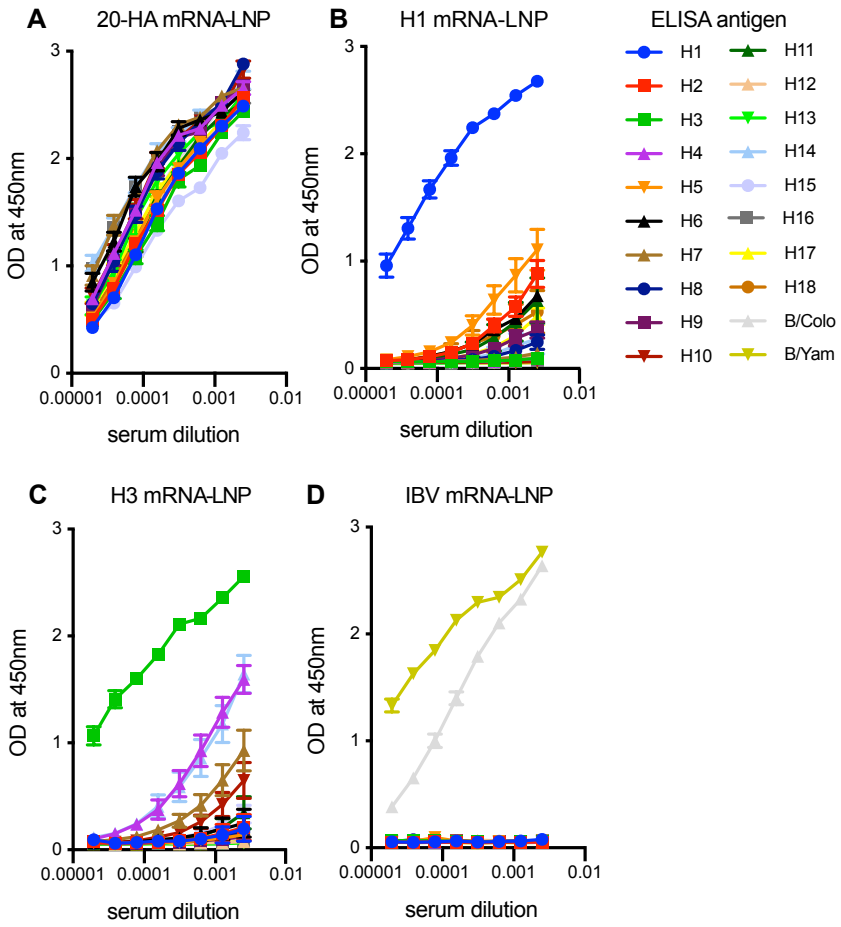

Fig. S2. Representative ELISA curves for Fig. 1. (A) Mice were simultaneously vaccinated i.m. with 20 different HA mRNA-LNPs (a combined total dose of 50 μg mRNA-LNP including 2.5 μg of each individual HA mRNA-LNP). Other groups of mice were vaccinated i.m. with 50 ug of H1 mRNA-LNP (**B**), 50 μg of H3 mRNA-LNP (**C**), or 50 μg of IBV HA mRNA-LNP. Sera were collected 28 days later and antibody reactivity to different HAs were quantified using ELISAs coated with recombinant proteins. Seven or eight animals were included per group. Data are shown as means ± SEM.

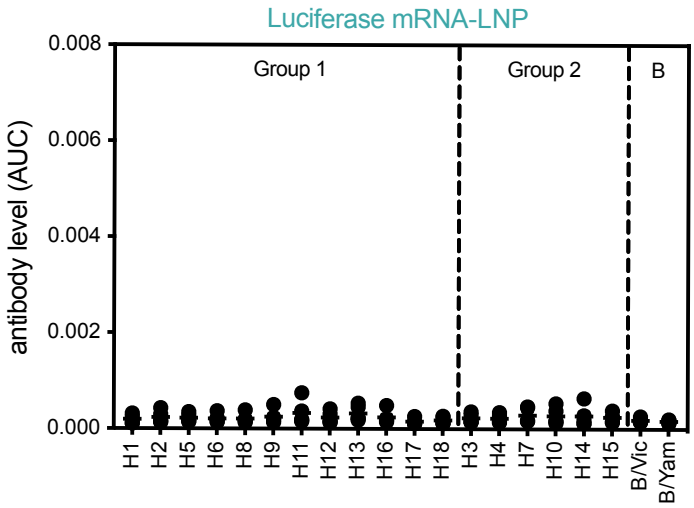

Fig. S3. Mice immunized with mRNA vaccine encoding luciferase do not generate antibody responses to influenza HA. Serum were collected from mice vaccinated with 50 ug of mRNA-LNP encoding a luciferase antigen and ELISAs were completed to measure anti-HA antibodies. Five animals were included. Data points represent titers from individual mice and bars indicate the means ± SEM for each ELISA antigen.

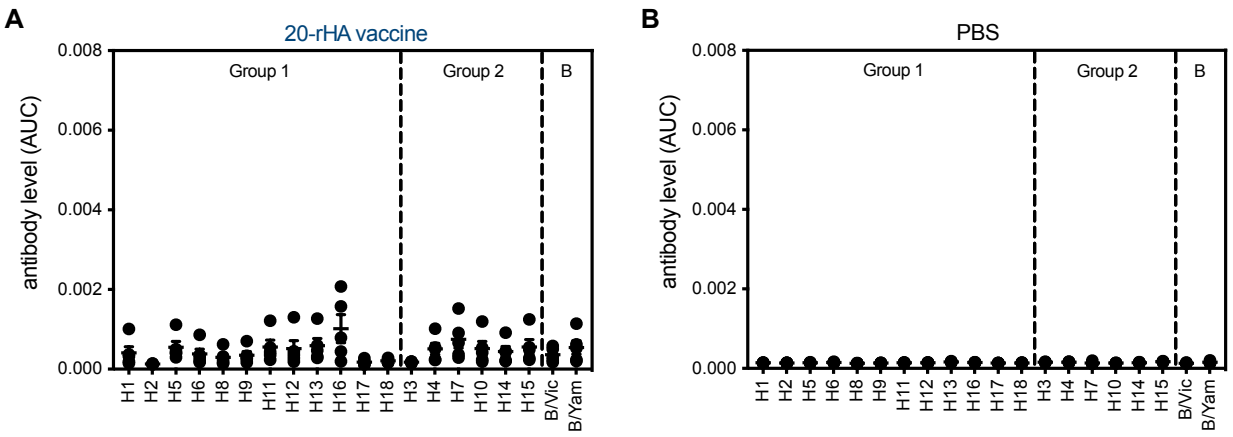

Fig. S4. Multivalent HA recombinant protein vaccine elicits weak antibody responses. Antibody titers were measured by ELISA in mice vaccinated with **(A)** multivalent recombinant HA (rHA) vaccine containing 20 HA proteins or (**B**) PBS. Five animals were included in each group indicated by data points. Bars indicate the means ± SEM for each ELISA antigen.

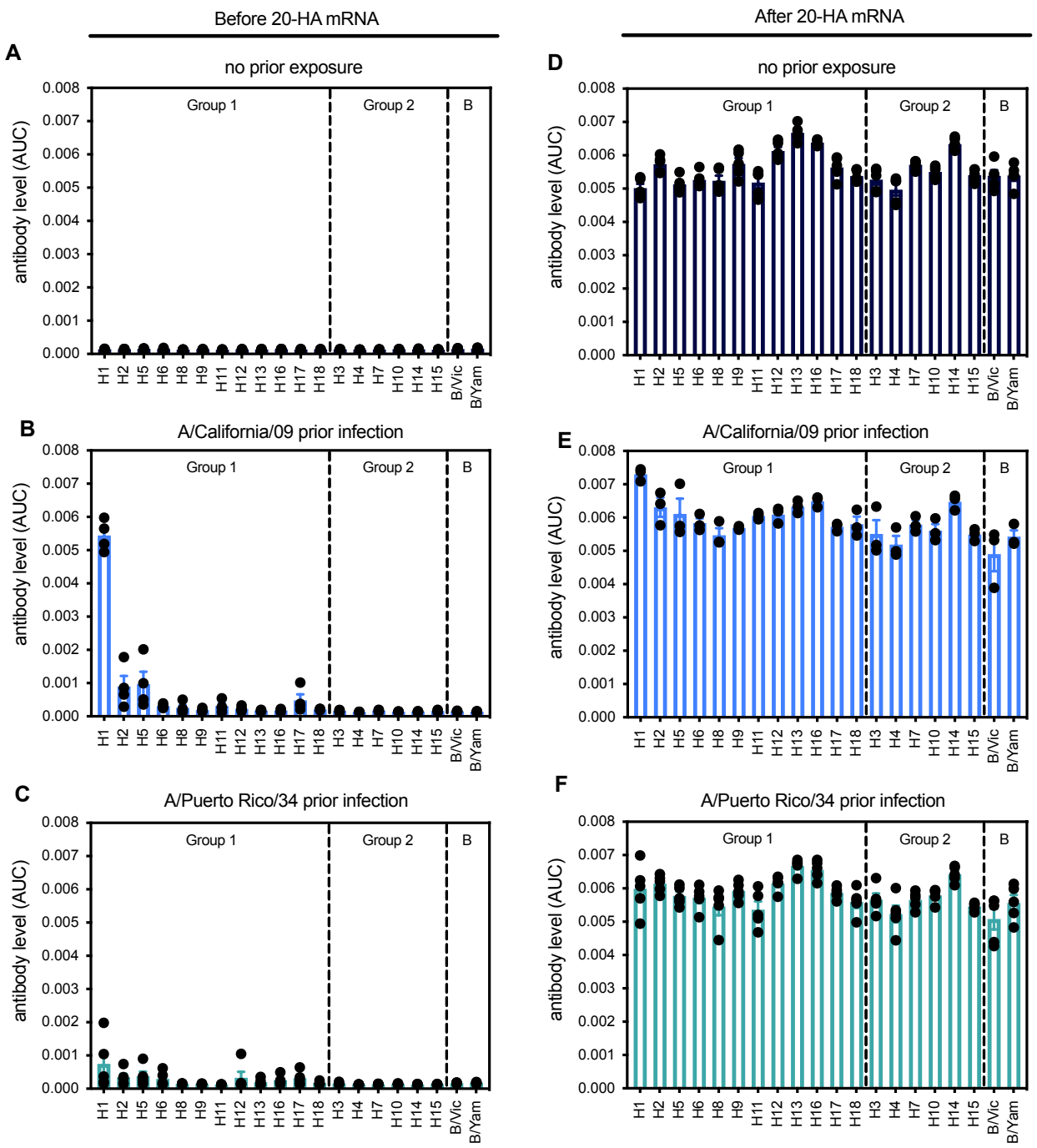

Fig. S5. 20 HA mRNA-LNP vaccination of mice with prior H1N1 exposures. Mice were inoculated i.n. with **(A)** PBS, **(B)** A/California/7/2009 H1N1, or **(C)** A/Puerto Rico/8/1934 H1N1 and allowed to recover. Sera were collected 84 days after inoculation and antibody levels were quantified by ELISA (A to C). Mice were subsequently immunized i.m. with the 20 HA mRNA-LNP vaccine. Sera were collected 28 days post-immunization and antibody levels were quantified by ELISA (**D** to **F**). Each data point represents serum antibodies from an individual animal (n=5 per group) and bars indicate the means ± SEM for each ELISA antigen.

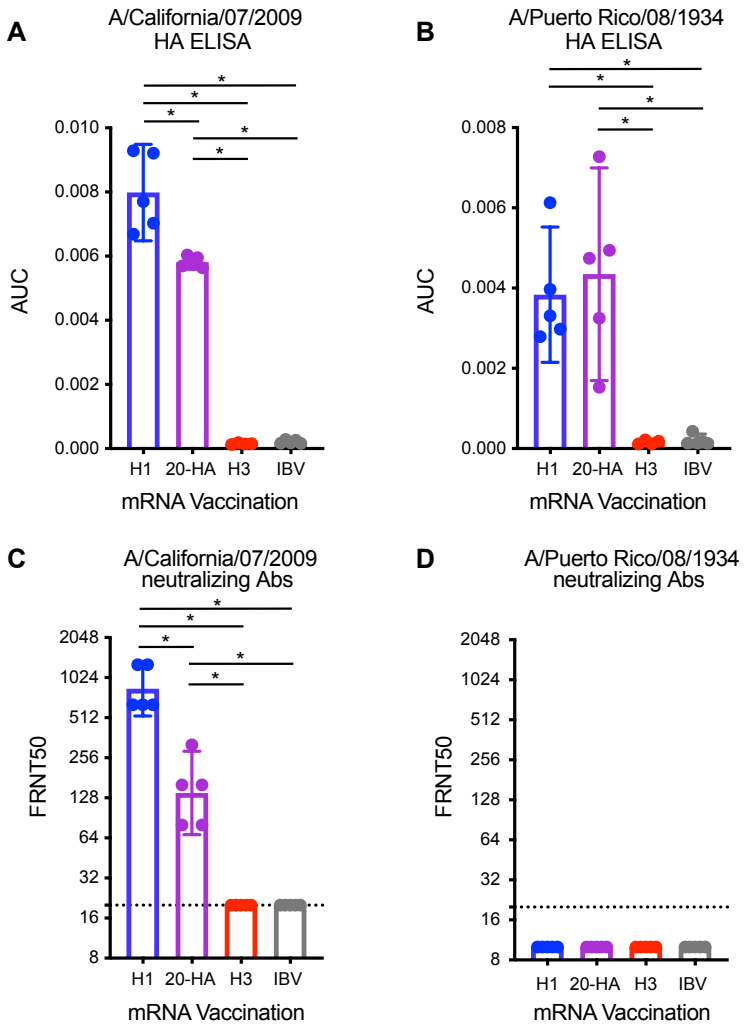

Fig. S6. Antibodies elicited by the 20 HA mRNA-LNP vaccine bind to A/California/07/2009 and A/Puerto Rico/8/1934 HAs, but only neutralize A/California/07/2009. HA reactive antibodies were quantified by ELISA using recombinant HAs from **(A)** A/California/07/2009 and **(B)** A/Puerto Rico/8/1934. Focus reduction neutralization tests (FRNT) were completed using **(C)** A/California/07/2009 and **(D)** A/Puerto Rico/8/1934. Serum amounts required to inhibit 50% of virus infection are reported. AUC; area under the curve. Five mice were included per experimental group and data are representative of two independent experiments. Data in A and B are shown as means ± 95% CI. Data in C and D are shown as geometric mean ± 95% CI. Antibody titers were compared using a one-way ANOVA with Tukey's post-test *P<0.05. Data in panel **C** were log-transformed prior to statistical analysis. Horizontal dotted lines in C and D denote limit of detection.

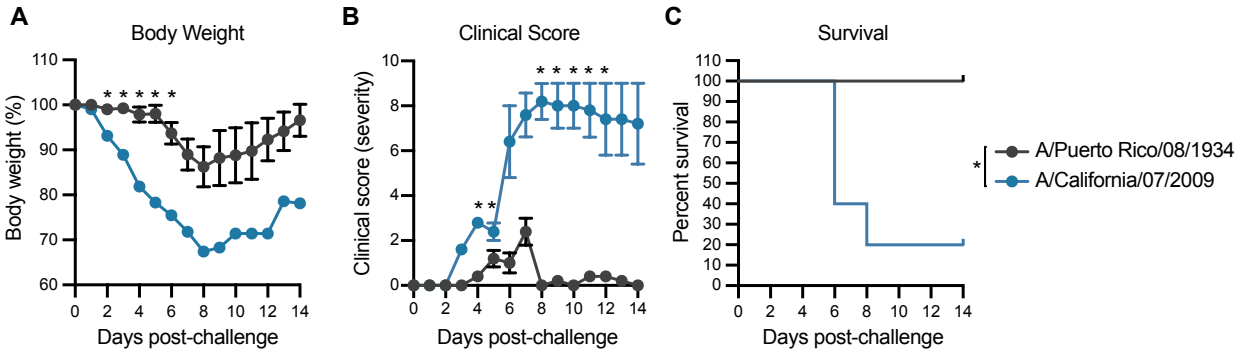

Fig. S7. Evaluation of a multimeric mRNA-LNP vaccine lacking an H1 component. Mice were vaccinated with mRNA-LNPs encoding 19 HAs without including an H1 HA. **(A)** Weight loss, **(B)** clinical scores, and **(C)** survival were monitored for 14 days following A/Puerto Rico/08/1934 (black) or A/California/7/2009 (teal) infection. Five mice were included per group. Data in A and B are shown as means ± SEM and were analyzed by mixed model ANOVA with Geisser-Greehouse correction and Sidak's multiple comparisons; * P <0.05. For animals that died, weight on day prior to death was carried forward for statistical analyses. Data in C were analyzed using a Mantel-Cox log-rank test; *P <0.05.

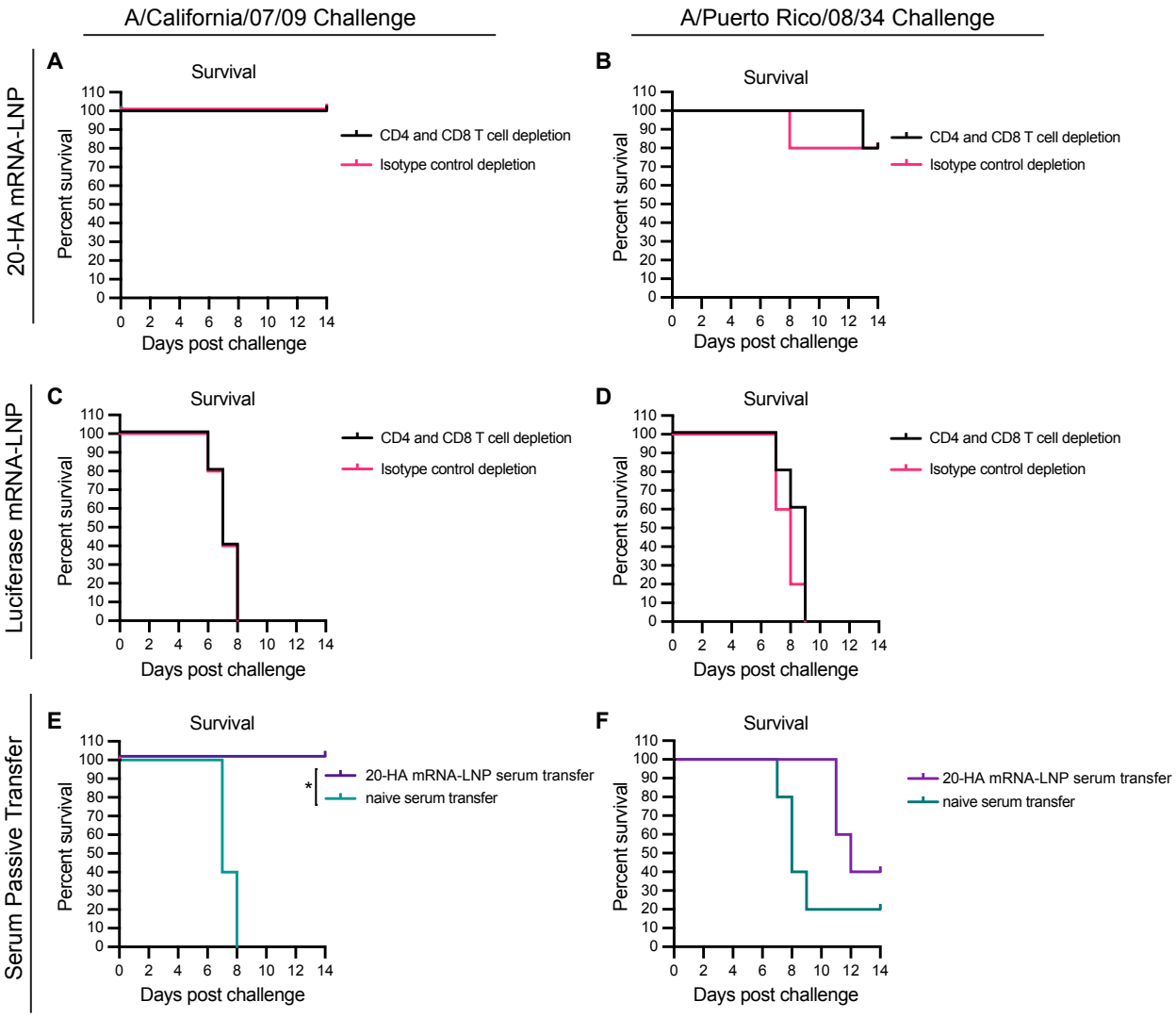

Fig. S8. Mechanisms of protection against matched and mismatched H1N1 viruses. Mice were immunized with 20-HA mRNA-LNPs **(A** and **B)** or luciferase mRNA-LNPs **(C** and **D).** Mice were subsequently infected i.n. 28 days later with A/California/7/2009 (A and C) or A/Puerto Rico/8/1934 (B and D). On days -3, -1, 1, 3, and 5 post-challenge mice were either treated with anti-Thy1.2 to deplete $CD4^+$ and $CD8^+$ T cells or an isotype control antibody. (**E** and **F**) Naïve serum or serum collected from 20 HA mRNA-LNP immunized mice were passively transferred 5 hours prior to challenge with (E) A/California/7/2009 or (F) A/Puerto Rico/8/1934. Survival was monitored for 14 days. Five animals were included per group. Data were analyzed using a Mantel-Cox log-rank test $*P < 0.05$.

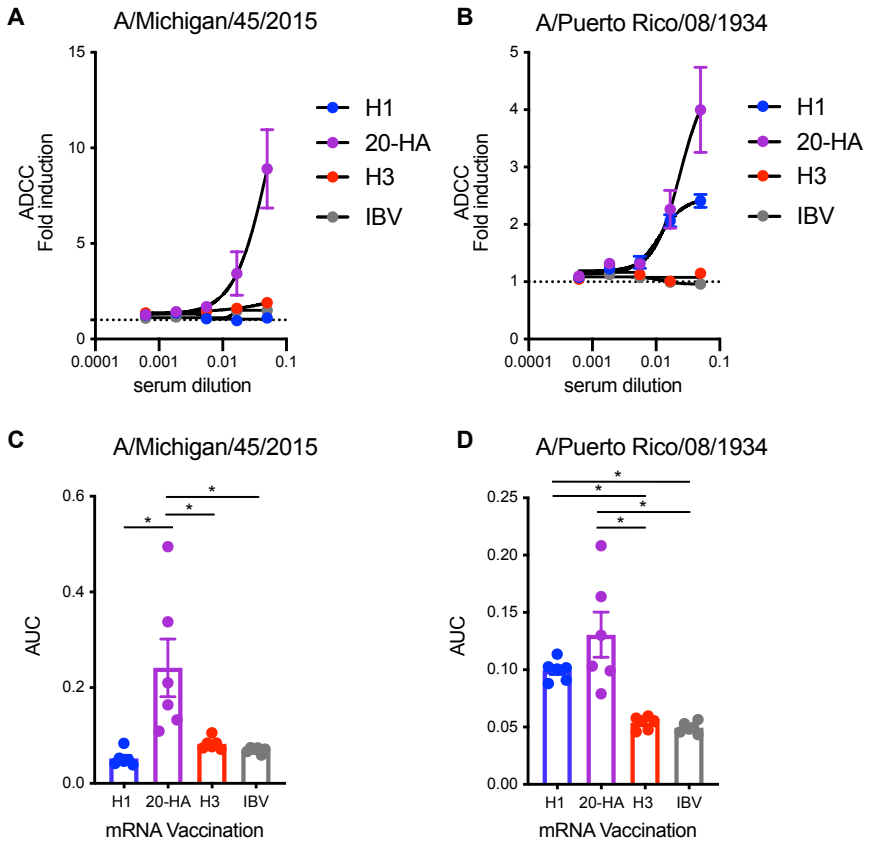

Fig. S9. Antibodies elicited by 20 HA mRNA-LNP vaccination efficiently mediates ADCC activity against matched and mismatched H1 antigens. ADCC activities of sera were quantified using a reporter assay to determine engagement with mouse FcγRIV. Target cells expressing HAs from either **(A** and **C)** A/Michigan/45/2015 or **(B** and **D)** A/Puerto Rico/8/1934 were incubated with serum from mice vaccinated with mRNA-LNPs. Effector cells expressing murine FcγRIV cell-surface receptor were added and ADCC activity was measured (A and B). Five animals were included per group. Data are representative of two independent experiment and are shown as mean ± SEM. Curves in A and B were fit using a nonlinear regression formula (four parameters). AUC from graphs in A and B was calculated, plotted in C and D, and compared using a one-way ANOVA with Tukey's post-test * P <0.05.

References and Notes

1. M. Baiersdorfer *et al.*, A Facile Method for the Removal of dsRNA Contaminant from In Vitro-Transcribed mRNA. *Mol Ther Nucleic Acids* **15**, 26 (2019).
2. R. Nachbagauer *et al.*, Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. *Nat Immunol* **18**, 464 (2017).
3. M. A. Maier *et al.*, Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol Ther* **21**, 1570 (2013).
4. Y. Zhang *et al.*, Influenza Research Database: An integrated bioinformatics resource for influenza virus research. *Nucleic Acids Res* **45**, D466 (2017).
5. J. Hadfield *et al.*, Nextstrain: real-time tracking of pathogen evolution. *Bioinformatics* **34**, 4121 (2018).
6. K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059 (2002).
7. B. Q. Minh *et al.*, IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol* **37**, 1530 (2020).
8. B. J. Laidlaw *et al.*, Cooperativity between CD8+ T cells, non-neutralizing antibodies, and alveolar macrophages is important for heterosubtypic influenza virus immunity. *PLoS Pathog* **9**, e1003207 (2013).
9. K. Mueller Brown *et al.*, Secondary infection with Streptococcus pneumoniae decreases influenza virus replication and is linked to severe disease. *FEMS Microbes* **3**, xtac007 (2022).
10. J. R. Whittle *et al.*, Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. *J Virol* **88**, 4047 (2014).
11. K. S. Corbett *et al.*, Design of Nanoparticulate Group 2 Influenza Virus Hemagglutinin Stem Antigens That Activate Unmutated Ancestor B Cell Receptors of Broadly Neutralizing Antibody Lineages. *mBio* **10**, (2019).
12. H. M. Yassine *et al.*, Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* **21**, 1065 (2015).
13. C. P. Arevalo *et al.*, Original antigenic sin priming of influenza virus hemagglutinin stalk antibodies. *Proceedings of the National Academy of Sciences of the United States of America* **117**, 17221 (2020).
14. S. Gouma *et al.*, Middle-aged individuals may be in a perpetual state of H3N2 influenza virus susceptibility. *Nat Commun* **11**, 4566 (2020).
15. M. B. Doud, S. E. Hensley, J. D. Bloom, Complete mapping of viral escape from neutralizing antibodies. *PLoS Pathog* **13**, e1006271 (2017).
16. L. J. REED, H. MUENCH, A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS12. *American Journal of Epidemiology* **27**, 493 (1938).