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Supplemental Information

A Multi-Targeting, Nucleoside-Modified mRNA Influenza Virus Vaccine Provides Broad Protection in Mice

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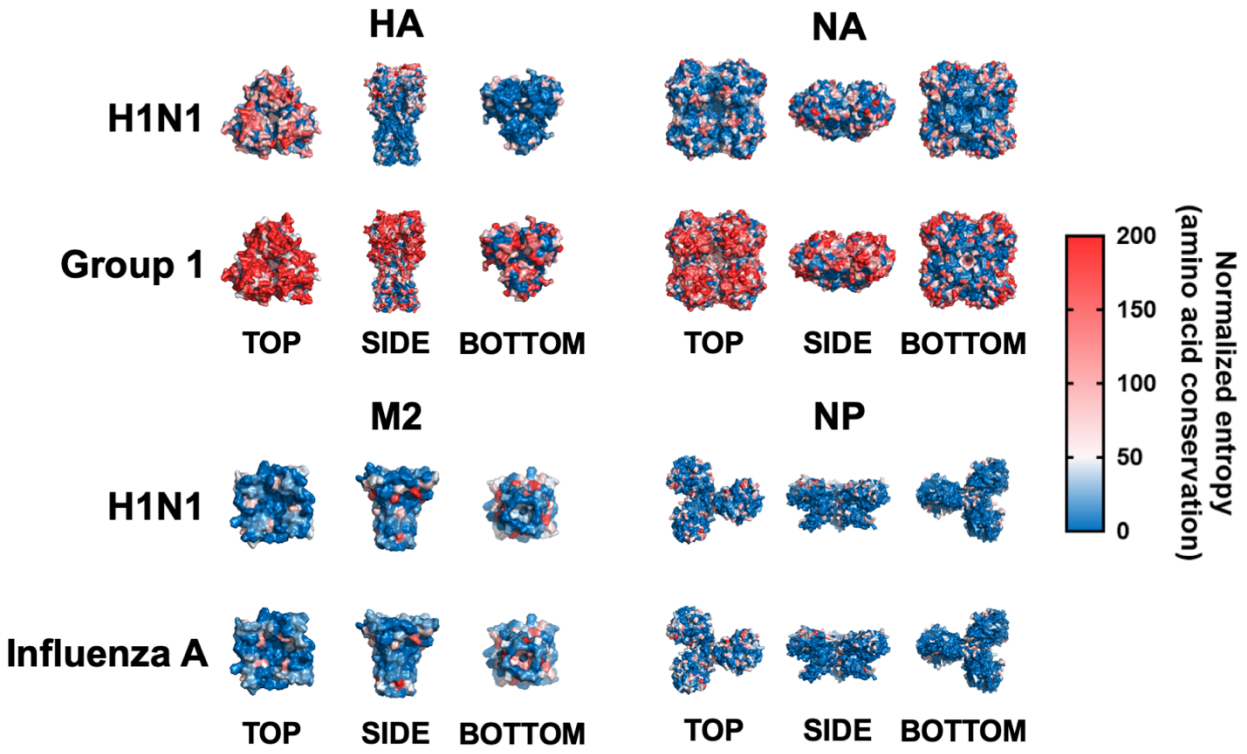
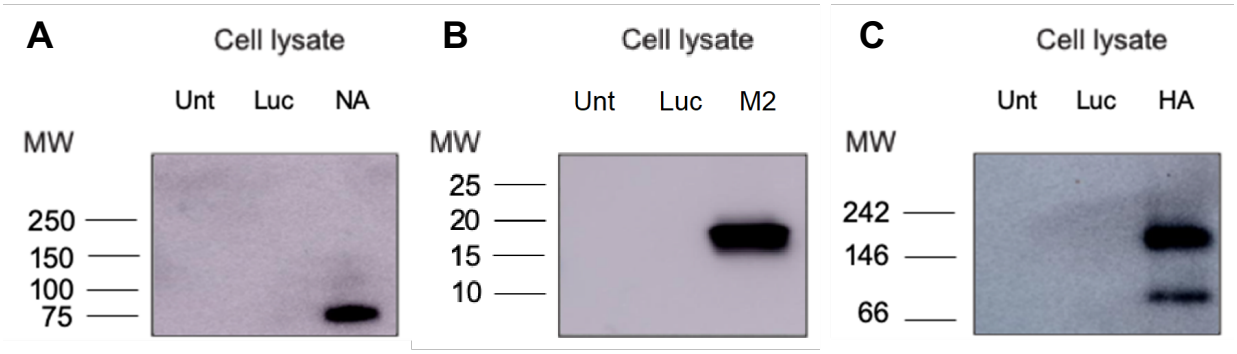
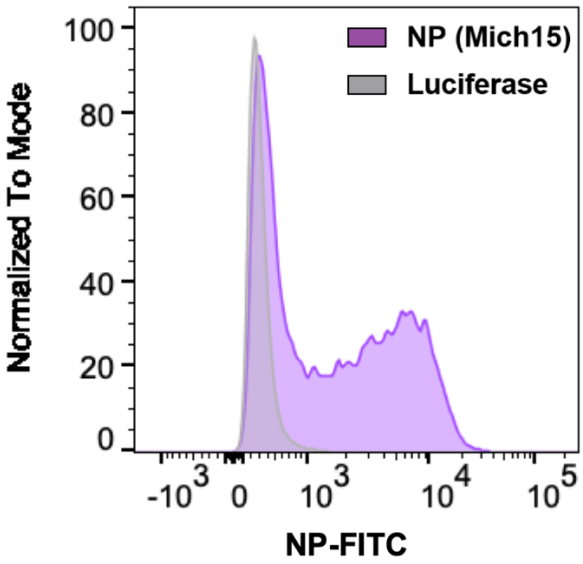


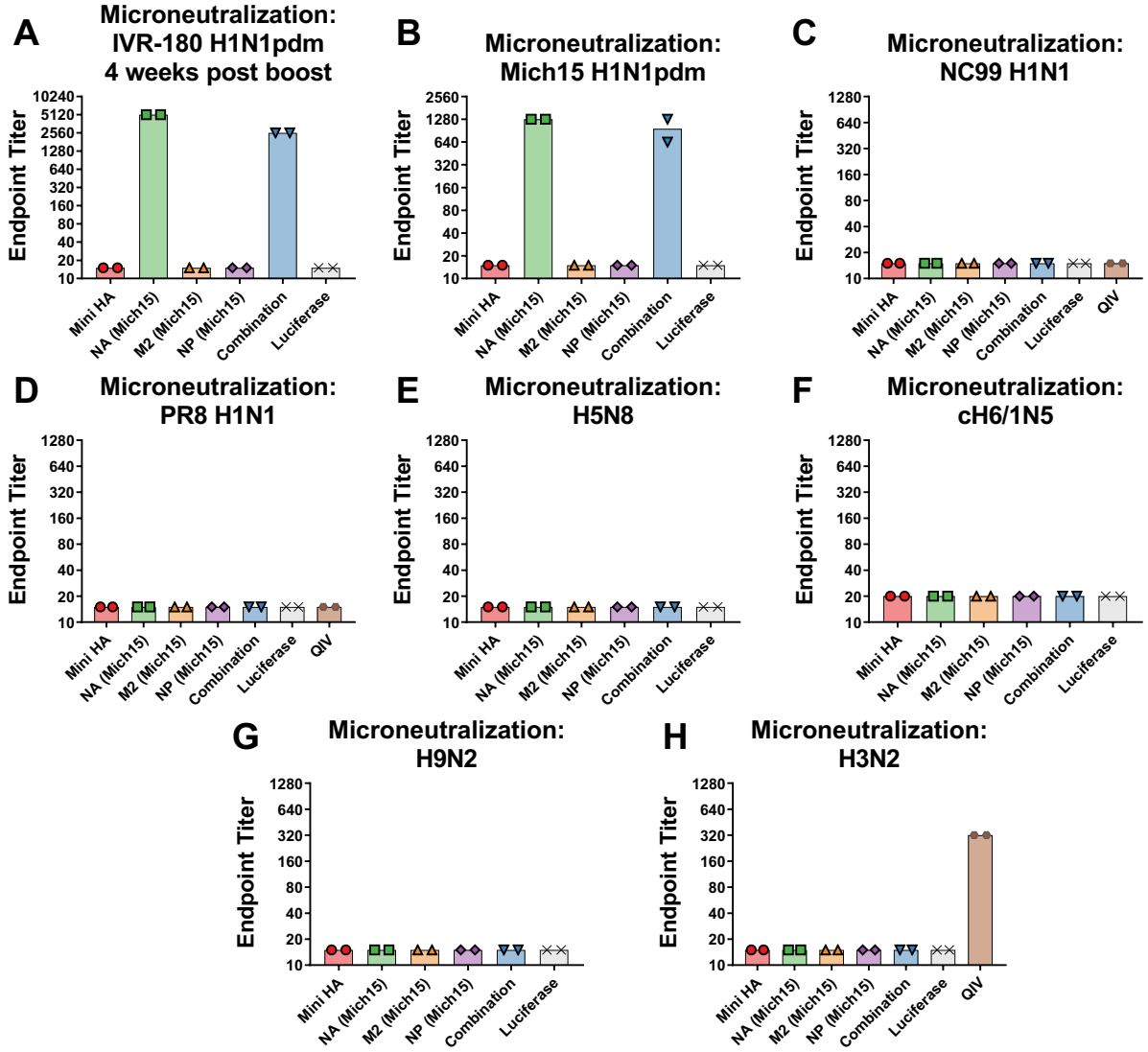
Supplemental Figure 1. Influenza viruses display conserved epitopes which can be targeted in the development of a universal influenza virus vaccine. Single amino acid polymorphism of proteins from a representative set of influenza virus strains was scored using a formula derived by Crooks *et al.*[1] Scores were mapped to corresponding amino acid residues and represented as a heat map; blue residues show no variation and red residues show substantial variation. H1N1 strains were selected for each year available dating back to 1918 (n = 49-52/group). Influenza virus strains were selected to evenly distribute between influenza A group one HAs, group one NAs, or influenza A human, avian, and swine strains for M2 and NP (n = 50/group). Angles are shown for top, side, and bottom views for all antigens: A/Puerto Rico/8/1934 H1 trimer (PDB: 1RU7),[2] A/Brevig Mission/1/1918 N1 tetramer (PDB: 3B7E),[3] A/Udorn/307/1972 M2 tetramer (PDB: 2L0J),[4] and A/Wilson-Smith/1933 NP trimer (PDB: 2IQH).[5] Proteins are not rendered to scale.



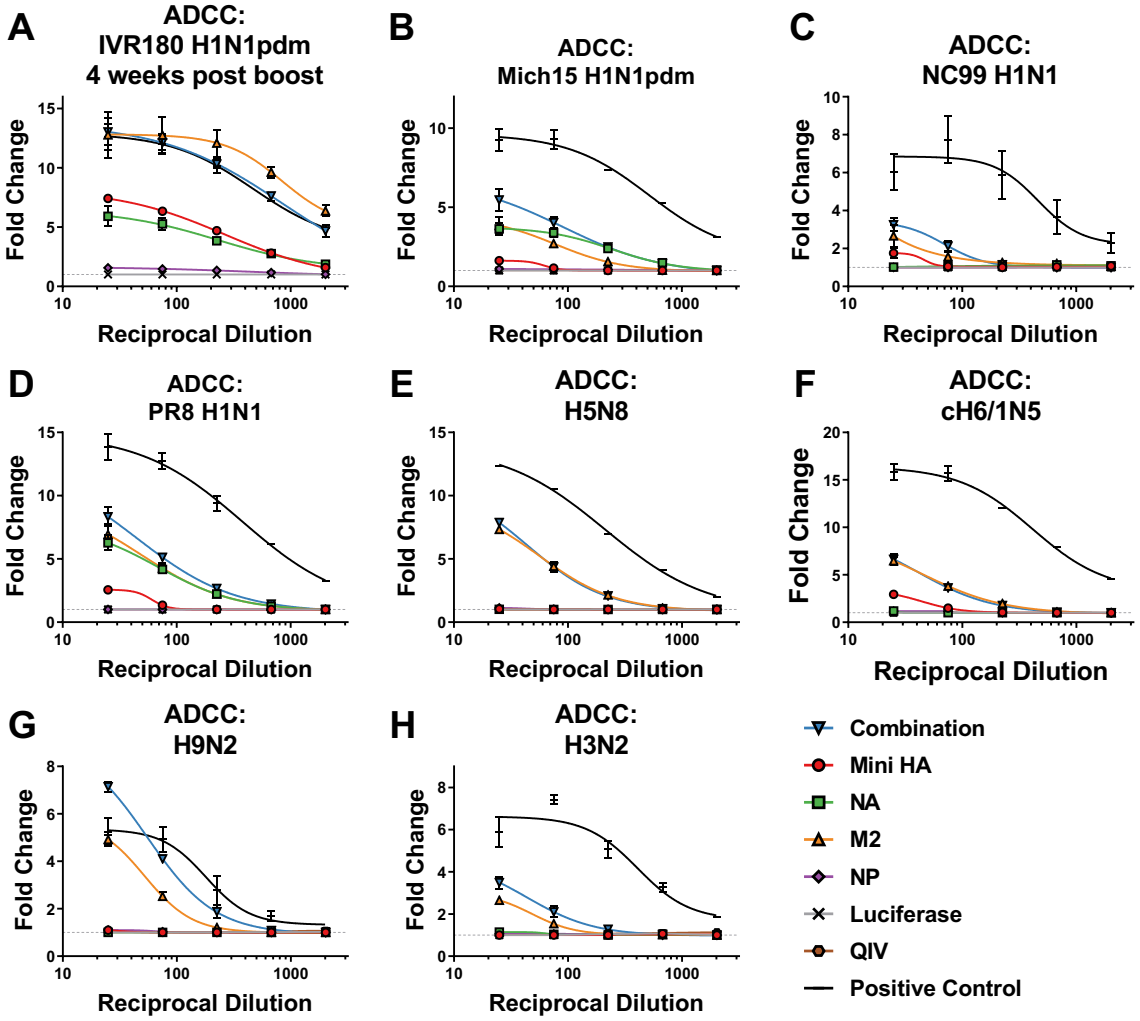
Supplemental Figure 2. Characterization of neuraminidase (NA), matrix-2 (M2), and mini-HA encoding mRNAs by Western blot analyses. mRNAs were transfected into NIH/3T3 cells. (A) NA, (B) M2, and (C) mini-HA protein expression in cell lysates was probed by Western blot, using firefly luciferase (Luc)-encoding mRNA-transfected cells and untransfected (unt) cells as negative controls.



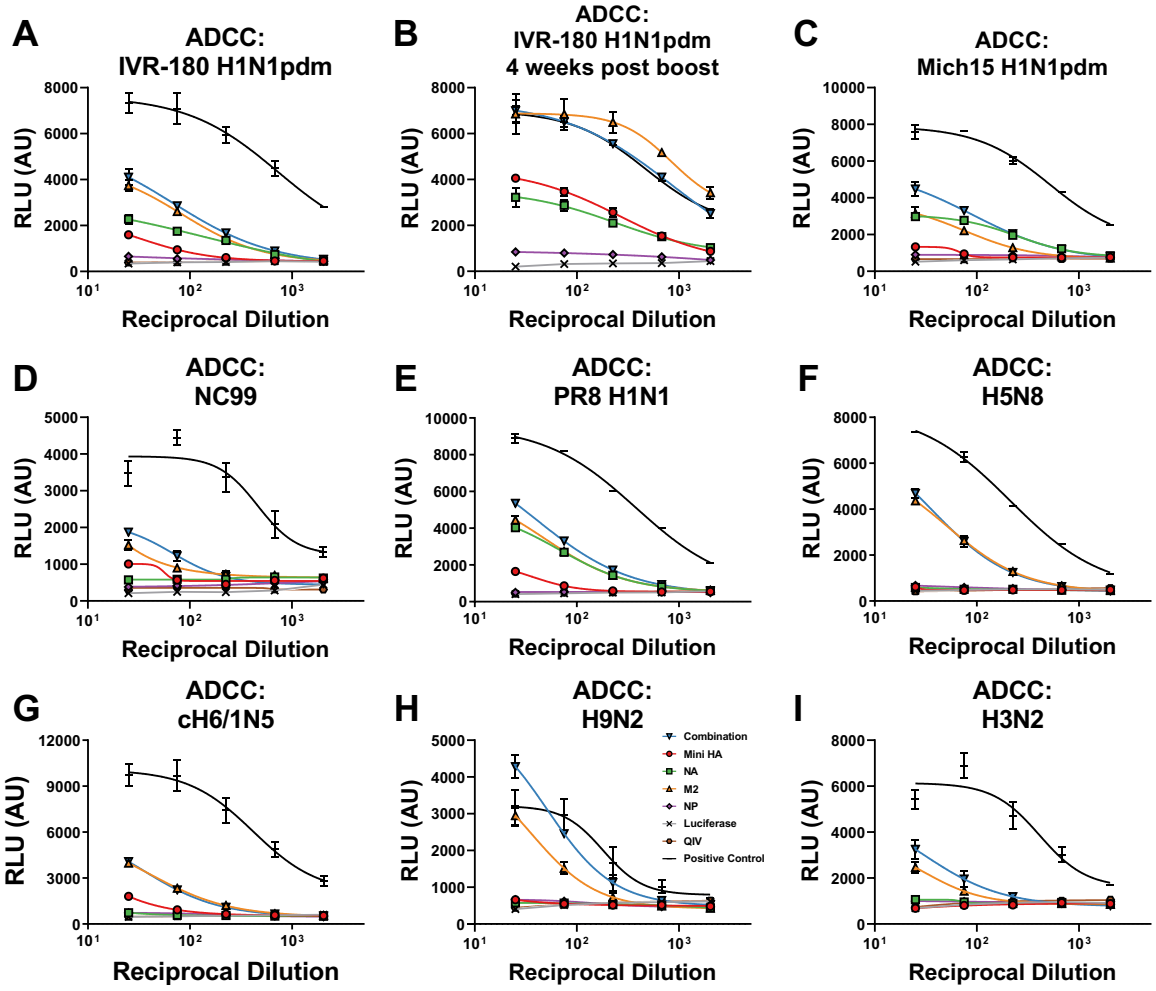
Supplemental Figure 3. Characterization of nucleoprotein (NP) encoding mRNA by flow cytometry. NP mRNA was transfected into NIH/3T3 cells. Positive binding of the anti-NP antibody to NP-transfected cells (purple) relative to luciferase transfected cells (gray). Two independent experiments were performed with similar results.



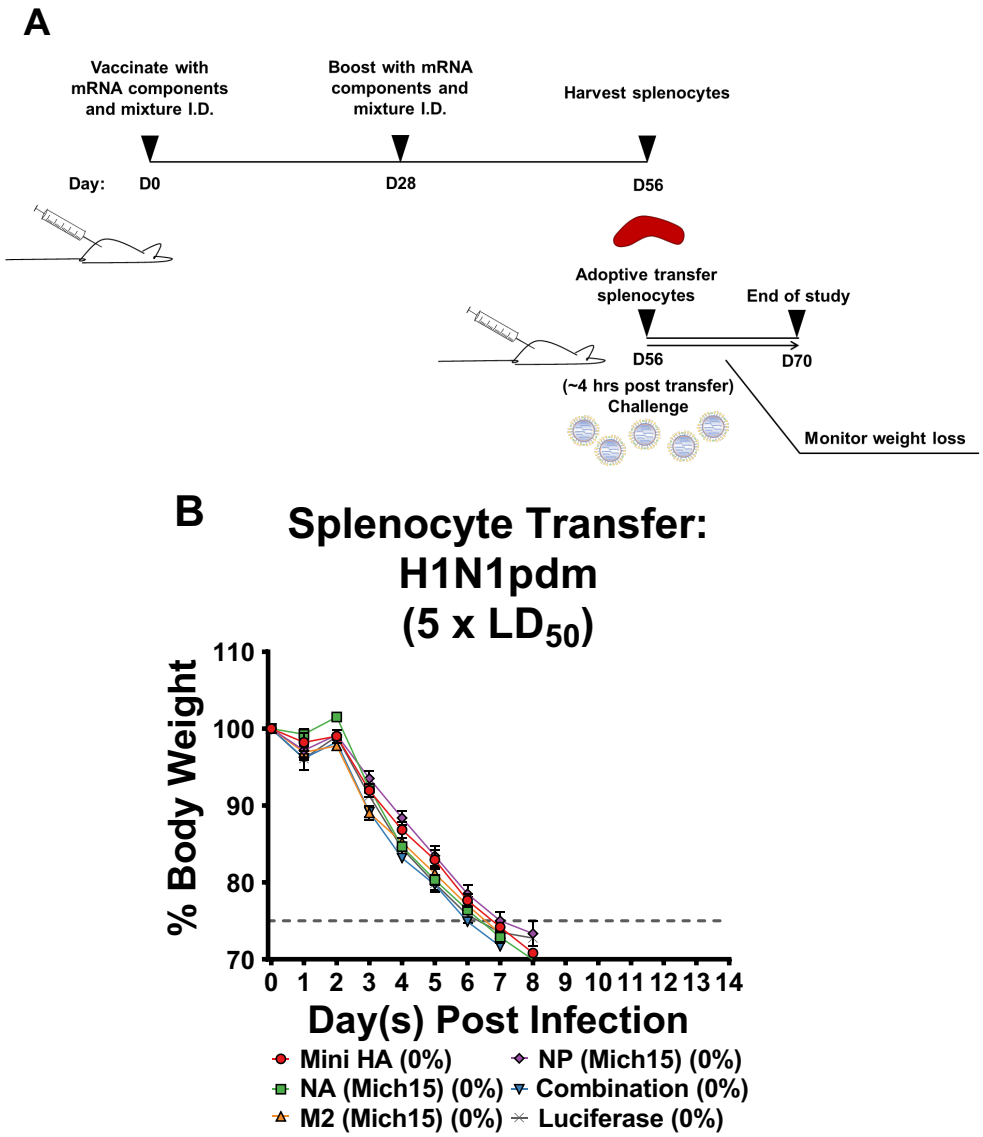
Supplemental Figure 4. Microneutralization assays using sera from vaccinated mice shows limited neutralization breadth. Mice were vaccinated in either a prime-boost regimen with 10 µg given twice with three weeks between doses and four weeks between the final boost and serum harvest or in a prime-only regimen with 20 µg delivered and serum harvested four weeks later. (A) Endpoint titers of a multi-cycle microneutralization assay to determine the potential of antibodies elicited by the prime-boost vaccination regimen to neutralize IVR-180 H1N1pdm virus. (B-H) Endpoint titers of a multi-cycle microneutralization assay to determine the potential of antibodies elicited by the prime-only vaccination regimen to neutralize listed viruses. (C, D, H) Sera from mice taken 4 weeks after vaccination with 1.5 µg of the 2018-2019 quadrivalent influenza virus vaccine were included in this assay. Only limited volumes of QIV serum were available and therefore not included in all assays. Sera were pooled and run in duplicate.



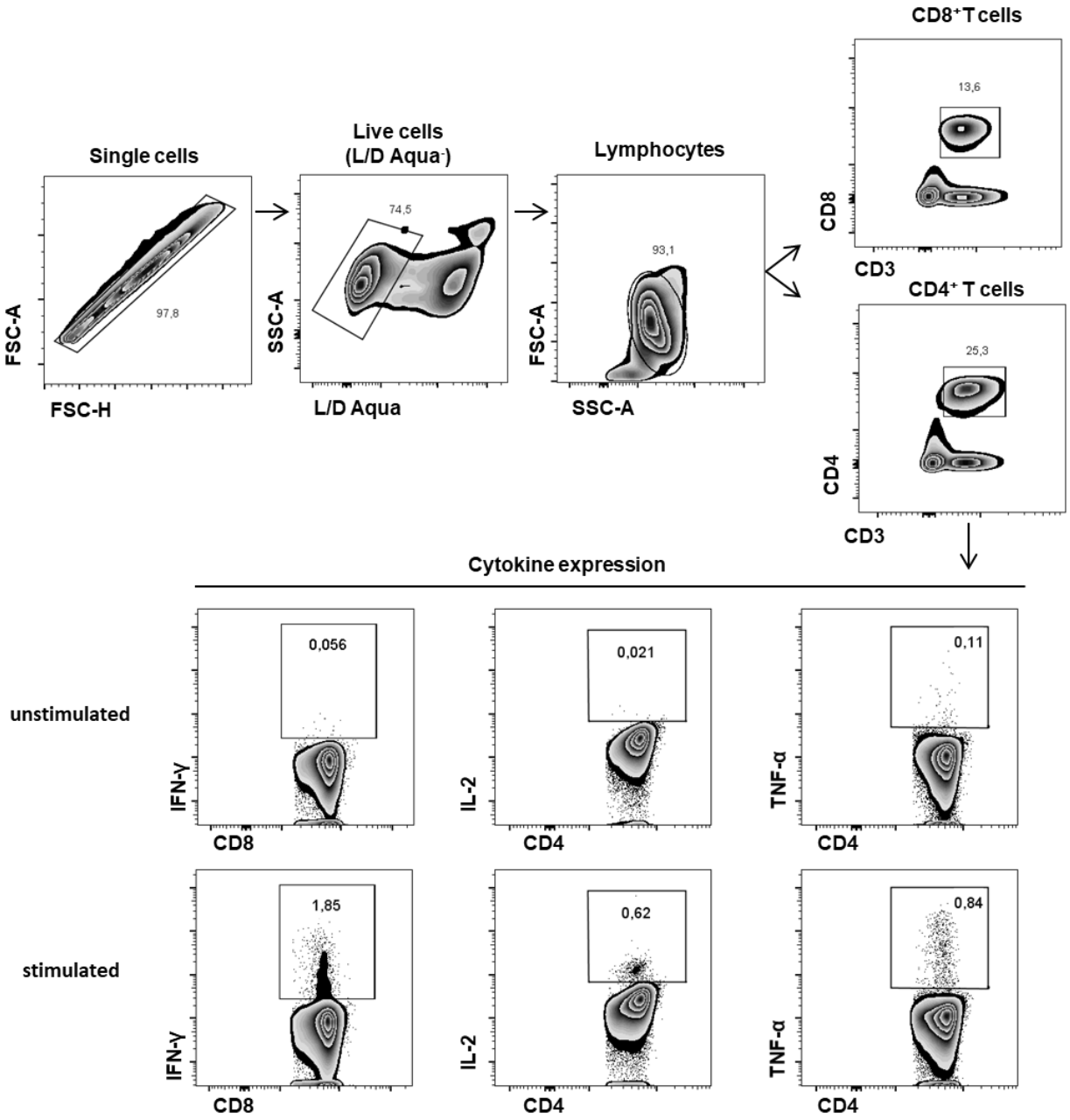
Supplemental Figure 5. Antibody-dependent cell-mediated cytotoxicity assays show breadth of Fc-mediated effector functions elicited by mRNA-LNP vaccination. Twenty-eight days after a (A) 10 μg prime-boost vaccination regimen or (B-H) a single 20 μg dose of mRNA-LNPs for the corresponding influenza virus antigen, sera were harvested and pooled from vaccinated mice. Sera from mice taken 4 weeks after vaccination with 1.5 μg of the 2018-2019 quadrivalent influenza virus vaccine were included in this assay. A reporter-based ADCC assay was performed on MDCK cells infected with the corresponding influenza virus at a multiplicity of infection (MOI) of five. Firefly luciferase protein expression (luminescence) was determined, and fold change is reported by dividing by the average of background wells plus three times the standard deviation within those wells. Data are represented as mean with SD. (A-F) Pooled sera were run in triplicate. (G and H) Pooled sera were run in duplicate. Positive control: (A-F) KB2, (G) CR9114, (H) 9H10. Curves were fit using a nonlinear regression formula log(agonist) vs. response – Variable slope (four parameters).



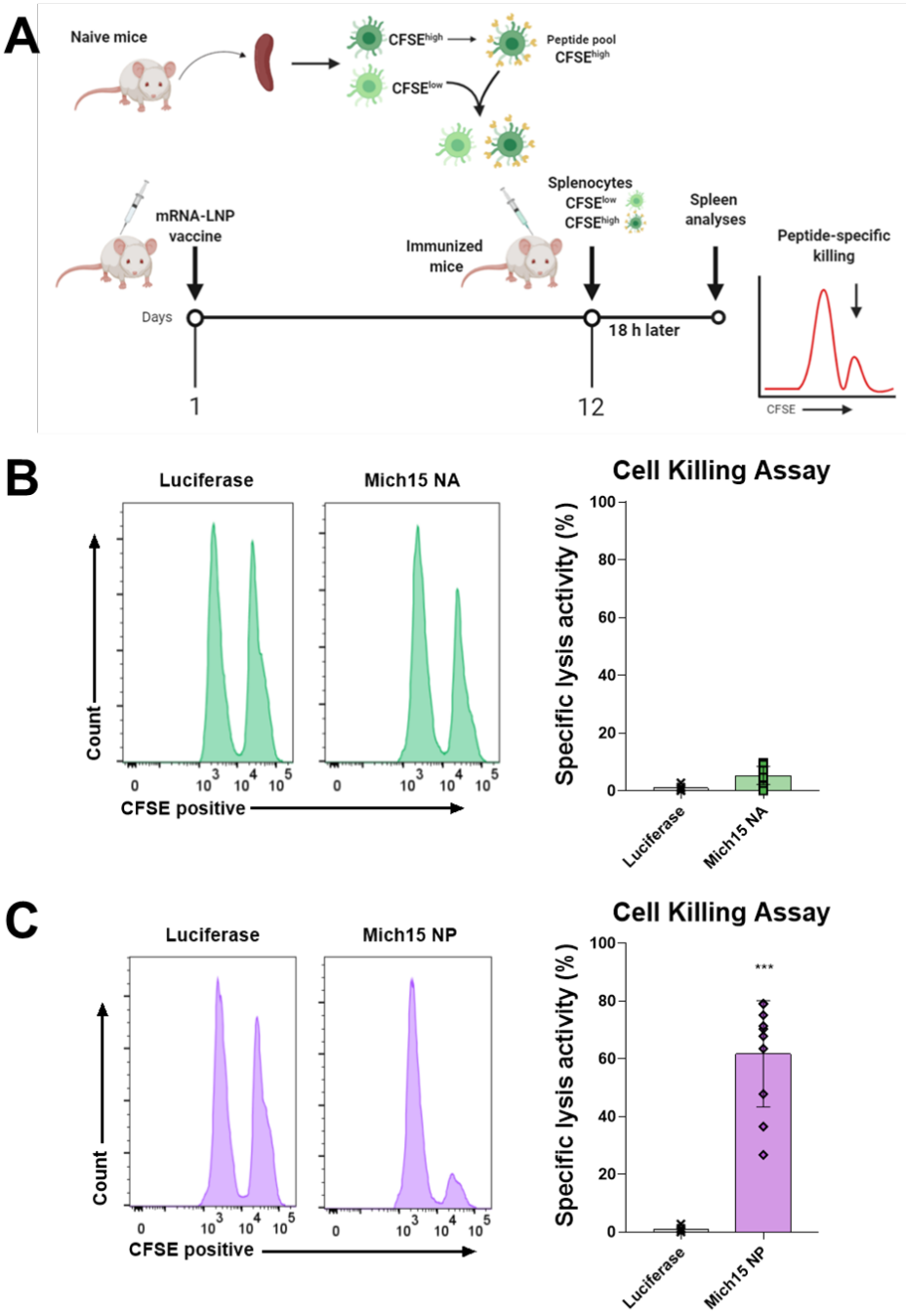
Supplemental Figure 6. Raw (non-normalized) antibody-dependent cell-mediated cytotoxicity luminescence reads of sera from mRNA-LNP vaccinated mice . Twenty-eight days after a (A) 10 µg prime-boost vaccination regimen or (B-H) a single 20 µg dose of mRNA-lipid nanoparticles for the corresponding influenza virus antigen, sera were harvested and pooled from vaccinated mice. A reporter-based ADCC assay was performed on MDCK cells infected with the corresponding influenza virus at a multiplicity of infection (MOI) of five. Luciferase expression is reported as relative luminescent units. Data is same as in Supplementary Figure 5 and are represented as mean with SD. (A-F) Pooled sera were run in triplicate. (G and H) Pooled sera were run in duplicate. Positive control: (A-F) KB2, (G) CR9114, (H) 9H10. Curves were fit using a nonlinear regression formula log(agonist) vs. response – Variable slope (four parameters).



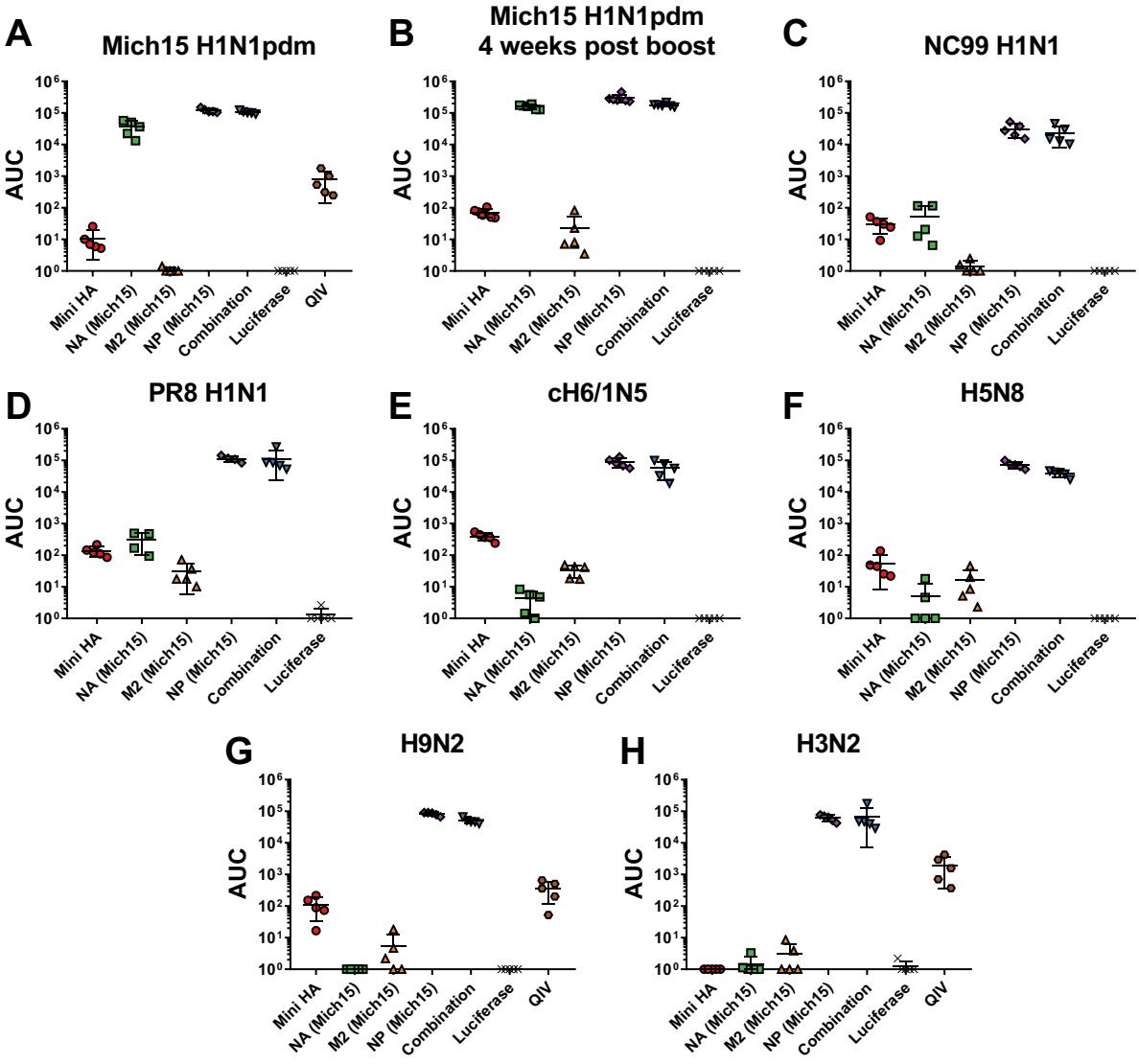
Supplemental Figure 7. Adoptive transfer of splenocytes from nucleoside-modified mRNA-LNP immunized mice does not provide protection from influenza virus challenge. (A) Mice were vaccinated intradermally with 10 µg of mRNA-LNPs in four-week intervals. Animals were euthanized on day 56 post initial vaccination and splenocytes were harvested, pooled, and transferred into naïve mice. (B) 2 hours post-transfer, recipient mice were infected with 5 x LD_{50} of H1N1pdm and weight loss was monitored for 14 days. Weight loss curves of mice adoptively transferred 80 million splenocytes from hyper-immune mice (n = 5). Average weight loss with SEM is plotted. Mortality is reported as the % of surviving mice for each group.



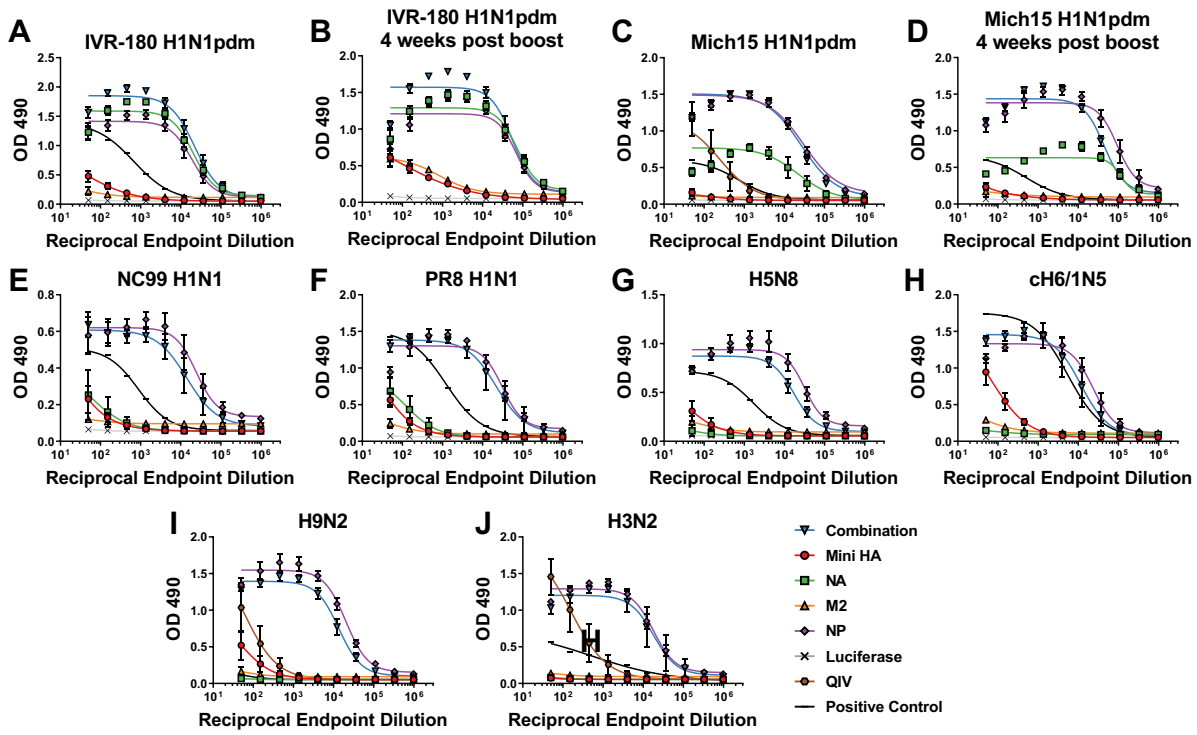
Supplemental Figure 8. Flow cytometric gating strategy for the investigation of T cell responses in neuraminidase and nucleoprotein mRNA-LNP-immunized mice. Representative flow cytometry plots for unstimulated and peptide-stimulated samples are shown.



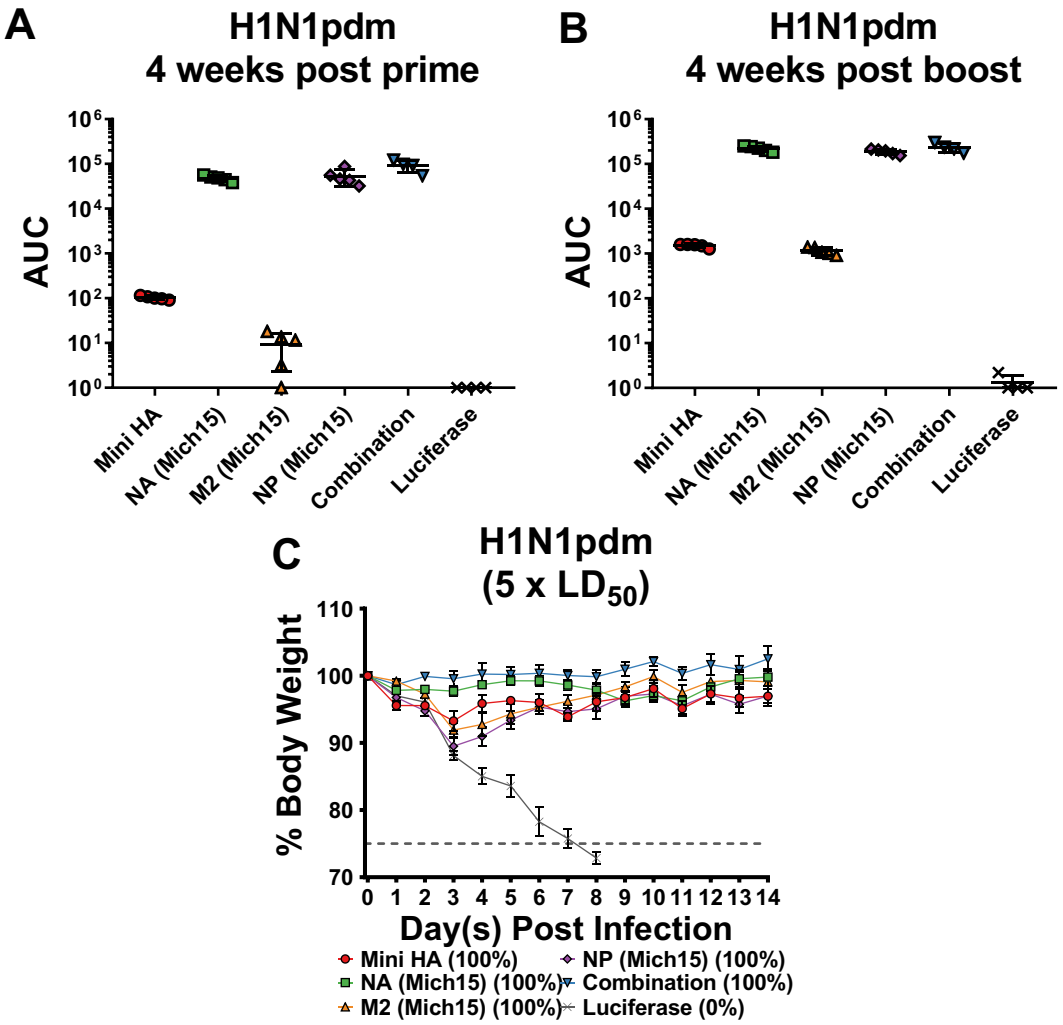
Supplemental Figure 9. Nucleoside-modified NP mRNA-LNP vaccination elicits strong antigen-specific *in vivo* cellular killing activity. Mice were vaccinated intradermally with a single dose of 20 µg of NA, NP, or Luc mRNA-LNPs and NA and NP-specific killing activity was determined. (A) Schematic illustration of the *in vivo* cytotoxicity assay. *In vivo* antigen-specific killing activity in mice immunized with (B) NA or (C) NP mRNA-LNPs. Each symbol represents one animal and error is shown as SEM (n = 10 mice per group). Data from 2 independent experiments are shown. Statistical analysis: Mann-Whitney test, *** $P < 0.001$.



Supplementary Figure 10. Serological analysis of mice vaccinated with nucleoside-modified mRNA-LNPs show breadth of binding to influenza A viruses. Twenty-eight days after intradermal vaccination with 20 µg of mRNA-LNPs, mice were bled to perform serological analysis. Sera from mice taken 4 weeks after vaccination with 1.5 µg of the 2018-2019 quadrivalent influenza virus vaccine were included in this assay. Only limited volumes of QIV serum were available and therefore not included in all assays. ELISAs were ran against purified virus (250 ng per well) for the following strains: (A) Mich15 H1N1pdm (n = 5 per group), (B) Mich15 H1N1pdm using sera from mice taken 4 weeks after a prime-boost vaccination course (n = 5 per group), (C) A/New Caledonia/20/1999 H1N1 (n = 5 per group), (D) A/Puerto Rico/8/1934 H1N1 virus (n = 4-5 per group), (E) cH6/1N5 reassortant virus (n = 5 per group), (F) H5N8 reassortant virus (n = 5 per group), (G) H9N2 reassortant virus (n = 5 per group), (H) A/Hong Kong/4801/2015 H3N2 virus (n = 5 per group). Area under the curve was calculated and shown as mean with SD.



Supplemental Figure 11. Raw ELISA curves for nucleoside-modified mRNA-LNP vaccine-induced responses to purified influenza A virus preparations. Twenty-eight days after intradermal vaccination with 20 μg of mRNA-LNPs, mice were bled to perform serological analysis. Sera from mice taken 4 weeks after vaccination with 1.5 μg of the 2018-2019 quadrivalent influenza virus vaccine were included in this assay. Only limited volumes of QIV serum were available and therefore not included in all assays. ELISAs were ran against purified virus (250 ng per well) for the following strains: (A) IVR-180 (n = 5 per group), (B) IVR-180 using sera from mice taken 4 weeks after a prime-boost vaccination course (n = 5 per group), (C) A/Michigan/45/2015 H1N1pdm (n = 5 per group), (D) A/Michigan/45/2015 H1N1pdm using sera from mice taken 4 weeks after a prime-boost vaccination course (n = 5 per group), (E) A/New Caledonia/20/1999 H1N1 virus (n = 5 per group), (F) A/Puerto Rico/8/1934 H1N1 virus (n = 4-5 per group), (G) cH6/1N5 reassortant virus (n = 5 per group), (H) H5N8 reassortant virus (n = 5 per group), (I) H9N2 reassortant virus (n = 5 per group), (J) A/Hong Kong/4801/2015 H3N2 virus (n = 5 per group). Data is identical to Supplementary Figure 10 with mean of OD490 values for individual mouse sera dilutions plus SD shown. Curves were fit using a nonlinear regression formula log(agonist) vs. response – Variable slope (four parameters). Positive controls: (A-I) KB2, (J) 9H10.



Supplemental Figure 12. Nucleoside-modified mRNA-LNP vaccines administered as a prime-boost regimen increases serum antibody responses with a modest increase in protection. To determine the effect of a prime-boost vaccination regimen on immune responses, 10 μg of vaccine was delivered twice, four weeks apart. ELISAs were ran against purified H1N1pdm virus using serum from animals (A) four weeks after prime and (B) four weeks after boost. (C) Mice were challenged with 5 x LD_{50} of H1N1pdm virus and weight loss was monitored for 14 days (n = 5 per group). Average weight loss with SEM is plotted. Mortality is reported as the % of surviving mice for each group.

Supplemental Methods

Conservation diagrams

To determine the amino acid conservation of influenza virus proteins, data sets were established containing full length, complete influenza virus isolates by searching fludb.org. For H1N1 subtype variation, human isolates were chosen randomly to select one strain per year (n = 49-52). Additionally, influenza virus isolates were chosen randomly to fairly spread the strains across the HA group 1 subtypes (H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16), NA group 1 subtypes (N1, N4, N5, and N8), or across human, avian, and swine influenza A isolates for M2 and NP (n = 50). Single nucleotide polymorphism scoring was performed based on a formula modified from Crooks *et al.*[1] In brief, a consensus sequence is produced based on the protein sequences analyzed and variation from consensus is scored based on the number and abundance of alleles or indels. These scores were used to color amino acid residues using PyMOL (Schrödinger).

mRNA Transfection

Transfection of NIH/3T3 cells was performed utilizing TransIT-mRNA (Mirus Bio), according to the manufacturer's instructions: mRNA (0.3 µg) was combined with TransIT-mRNA Reagent (0.34 µl) and Boost Reagent (0.22 µl) in 17 µl serum-free medium, and the complex was added to 3×10^4 cells in 183 µl complete medium. After overnight incubation at 37°C, NA and M2 mRNA-transfected cells were lysed for 30 minutes on ice in radio immunoprecipitation assay (RIPA) buffer (Sigma), Mini HA mRNA-transfected cells were lysed with 1X NativePAGE Sample Buffer (Invitrogen), NP-transfected cells were collected for staining and flow cytometry analysis, all at 18 hours post transfection.

Western blot analyses of NA, M2, and Mini HA protein expression

Whole-cell lysates obtained from 6×10^4 NA and M2 mRNA-transfected cells were assayed for NA and M2 protein by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blot. Samples were combined with 4X Laemmli buffer (Bio-Rad) and incubated at 95°C for 5 min, then separated on a 4%–15% precast polyacrylamide Mini-Protean TGX gel (Bio-Rad) for 1 hour at 120 V. Whole-cell lysates obtained from 6×10^4 Mini HA mRNA-transfected cells were assayed for HA protein by Western blot under non-denaturing conditions. Samples were combined with 4X NativePAGE Sample Buffer, then separated on a NativePAGE 4-16% Bis-Tris Protein Gel (both from Invitrogen) for 1 hour at 150V, followed by 30 minutes at 250 V, all on ice. Transfer to polyvinylidene fluoride membrane was completed utilizing a Horizontal Semi-Dry Electro Blotter (Ellard Instrumentation) at 10 V for 1 hour. For NA and M2, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline buffer containing 0.1% Tween-20 (TBS-T). For HA, the membrane was incubated in 8% acetic acid for 15 minutes to fix the proteins, followed by a 5 minutes rinse with methanol to remove background dye, before blocking in the same manner as with NA and M2. NA, M2, and HA proteins were probed by incubating with a 1:2,000 dilution of 4A5 (anti-NA),[6] E10 (anti-M2),[7] and KB2 (anti-HA)[8] mouse monoclonal antibodies at 1 mg ml^{-1} overnight at 4°C, followed by incubation with a 1:5,000 dilution of donkey anti-mouse horseradish peroxidase (HRP)-IgG (Jackson ImmunoResearch Laboratories) secondary antibody for 1 hour at room temperature, all antibodies diluted in 5% non-fat dry milk in TBS-T. Blots were developed using Amersham ECL Western Blotting Detection Reagent on an Amersham Imager 600 (both from GE Healthcare) system.

In vivo cell killing assay

Groups of mice were injected with 20 µg of NA, NP, or Luc mRNA-LNPs intradermally. 12 days after immunization, single cell splenocyte suspensions from naïve mice were generated and divided into two populations and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) at a final concentration of 7 µM ($CFSE^{high}$) or 0.5 µM ($CFSE^{low}$). $CFSE^{high}$ cells were pulsed with overlapping NA (BEI Resources; NR-19249) or NP (JPT peptides; PM-INFA_NPH2N2) peptide pools at 2.5 µg/mL per peptide in complete medium at 37 °C for 40 minutes. Equal numbers of pulsed and unpulsed cells from naïve mice were injected intravenously (a total of 2×10^7 cells /mouse) into mice immunized with NA, NP, or Luc mRNA-LNPs 12 days earlier. Splenocyte single cell suspensions from immunized mice were generated 18 h later and were analyzed for CFSE expression by flow cytometry using a modified LSR II flow cytometer (BD Biosciences). The numbers of $CFSE^{high}$ and $CFSE^{low}$ cells were used to calculate the percentage of peptide-pulsed target cell killing, determined by using the formula: [1 − (% $CFSE^{high}$ immunized/% $CFSE^{low}$ immunized)/(% $CFSE^{high}$ naive/% $CFSE^{low}$ naïve)] × 100, as previously described.[9]

Optimized sequences

Mini HA:
ATGAAGGTGAAGCTGCTGGTGCTGCTGTGCACCTTCACCGCCACCTACGCCGACACCATCTGCATCGG

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GAACCGCGAGAAGATCGACGGCGTGAAGCTGGAGTCCATGGGCGTGTACCAGATCGAGGGCCGCtaa

Michigan NA:
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CCTGCAGATCGGCAACATCATCTCCATCTGGGTGTCCCACTCCATCCAGATCGGCAACCAGTCCCAGA
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Michigan NP:
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CAACtaa

Michigan M2i:
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TGCGCGAGGAGTACCAGCAGGAGCAGCAGTCCGCCGTGGACGTGGACGACGGCCACTTCGTGAACAT
CGAGCTGGAGtaa

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