

Supporting Information for

Influenza A Virus H7 nanobody recognizes a conserved immunodominant epitope on hemagglutinin head and confers heterosubtypic protection

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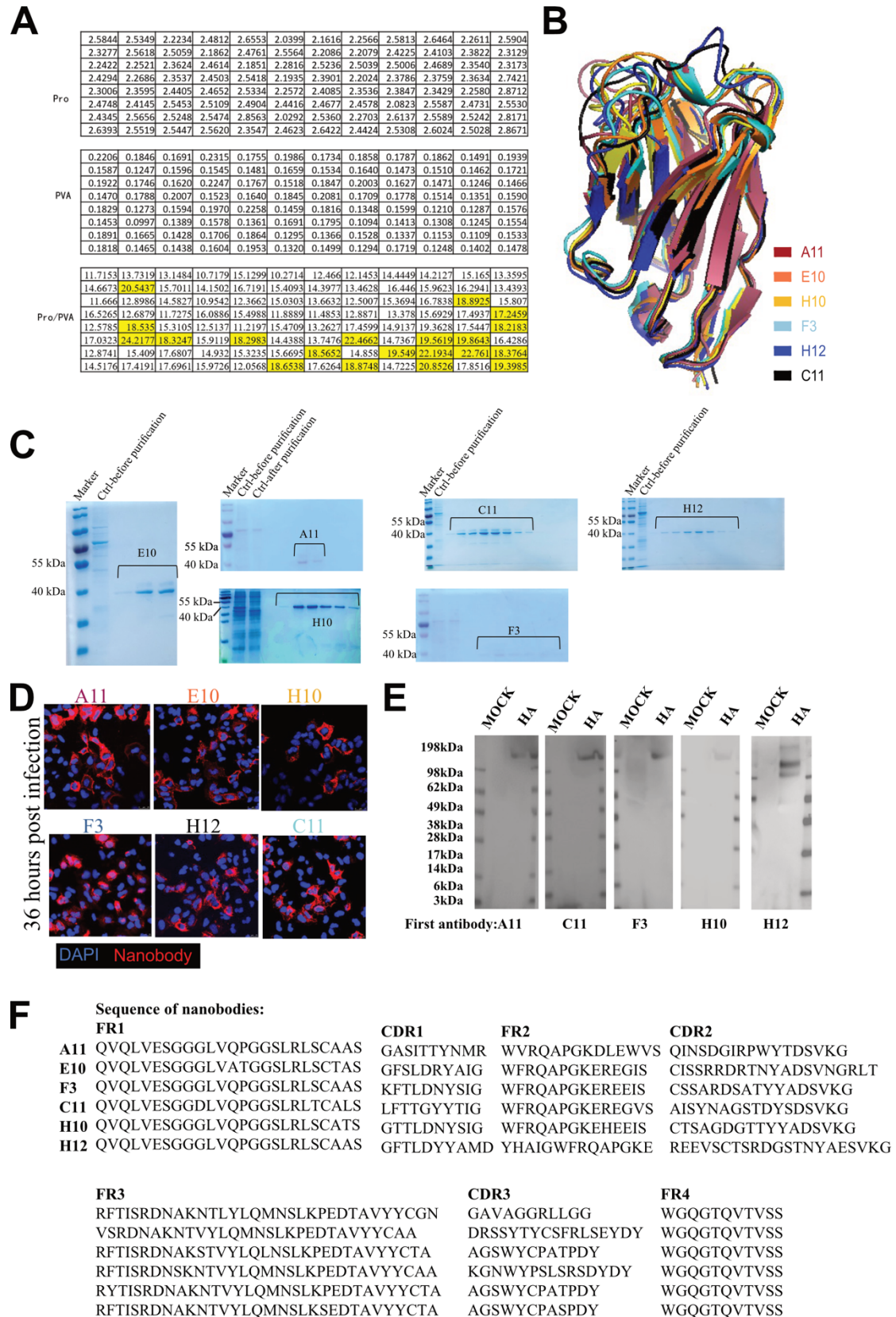
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Supplementary Table 1. PCR primers used in this study.

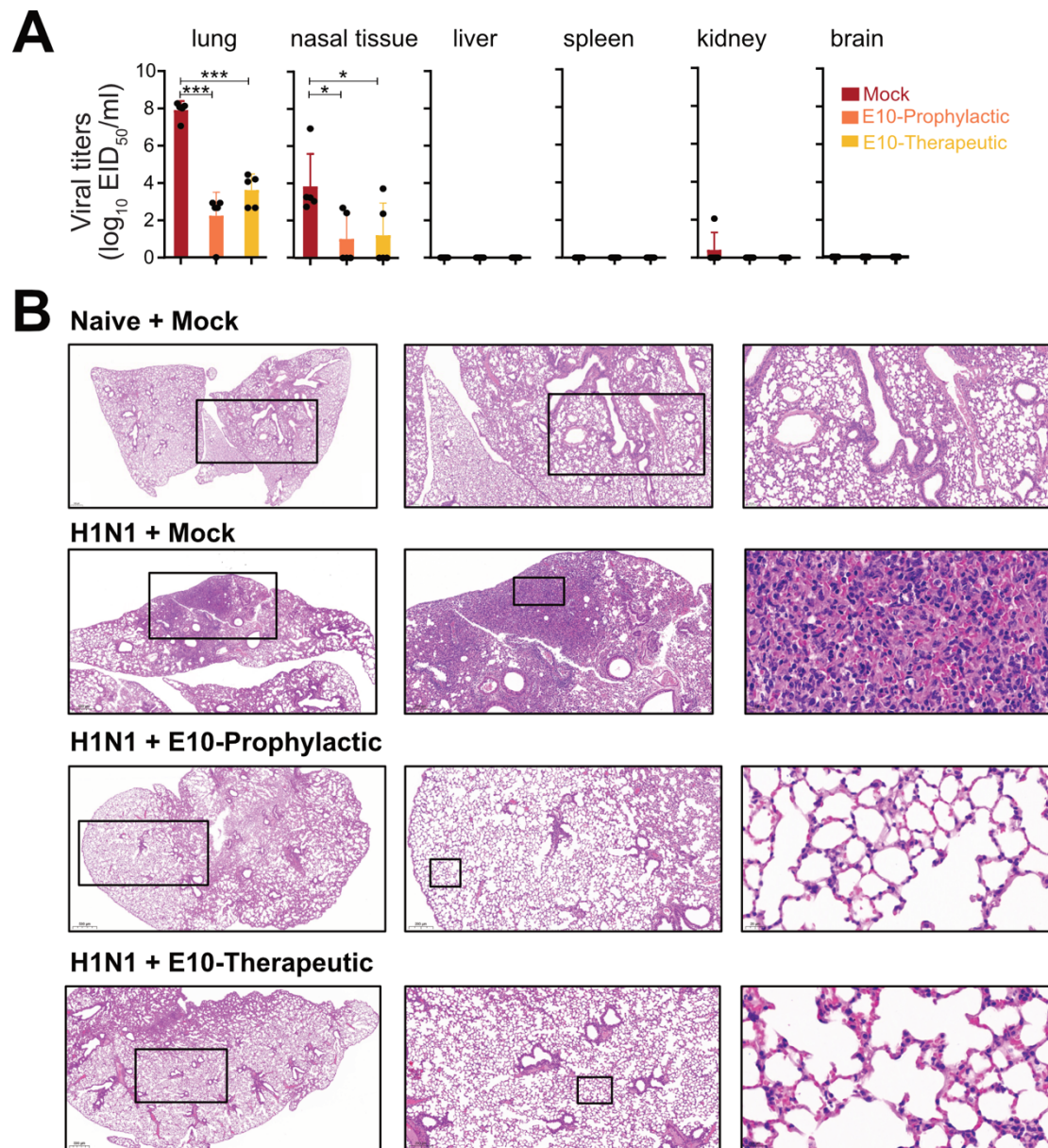
Primer name	Primer sequence
Fragment A-Forward	ATCATTTTGGCAAAGGAATTCGAGCTCGGTACCCGG
Fragment A-Reverse	GAGTGTTTATTATGTTTTTGTCCACCCTGCTTTTGCTCCCC
Fragment B-Forward	CAAAAACATAATGAACACTCAAATCCTGGTATTTCG
Fragment B-Reverse	TTTTTTAGTATTATATACAAATAGTGCACCGCATGTTTCCA
Fragment C-Forward	TTGTATATAATACTAAAAAACACCCTTGTTTCTACTAATAACCCG
Fragment C-Reverse	AAAAAGATCTGCTAGCTCGAGCATGCCCGGGTACCAT
IgG-Forward	GTCCTGGCTGCTCTTCT
IgG-Reverse	GGTACGTGCTGTTGA
VHH-Forward	CTACAAATGCCTATGCATCCCAGGTGCAGCTCGTGGAGTC.
VHH-Reverse	AAACAACCTTTCAACAGTGGAGGGGTCTTCGCTGTGGTGCG
pComb-Forward	AAAGAATATCGCATTTCTTCTTGCATCT
pComb-Reverse	AGGAGACGGTGACCTGGGTCCCCTG
Nb-Forward	TCCAGTGTGGTGGGAATTCGCCACCAAAAAGAATATCGCATTTCTTC TTGCATCT
Nb-Reverse	TCTAGACTCGAGTCAGTGATGGTGGTGGTGGTGGTGGTGGTGTGAGG AGACGGTGACCTGGG
GAPDH-Forward	GTCATTGAGAGCAATGCCAG
GAPDH-Reverse	GTGTTGCTACCCCAATGTG
HA-Forward	ATGAACACTCAAATCCTGGT
HA-Reverse	TTATATACAAATAGTGCACC



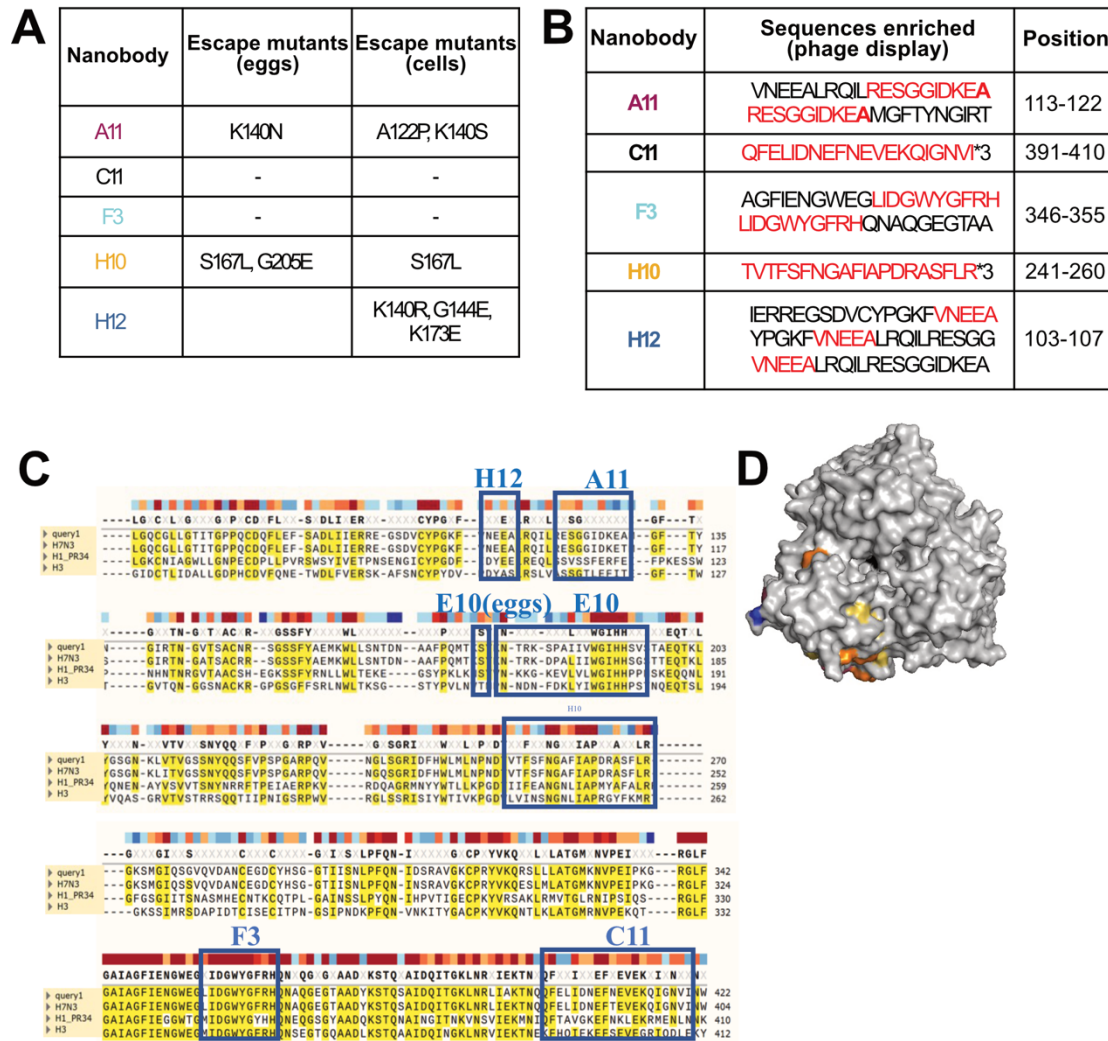
Supplementary Figure 1. Generation and characterization of H7-specific nanobodies.

(A) Binding ability of 96 selected phage colonies as detected by Indirect-ELISA using H7. (20 high binding colonies: yellow). (B) Nanobodies structural model created using

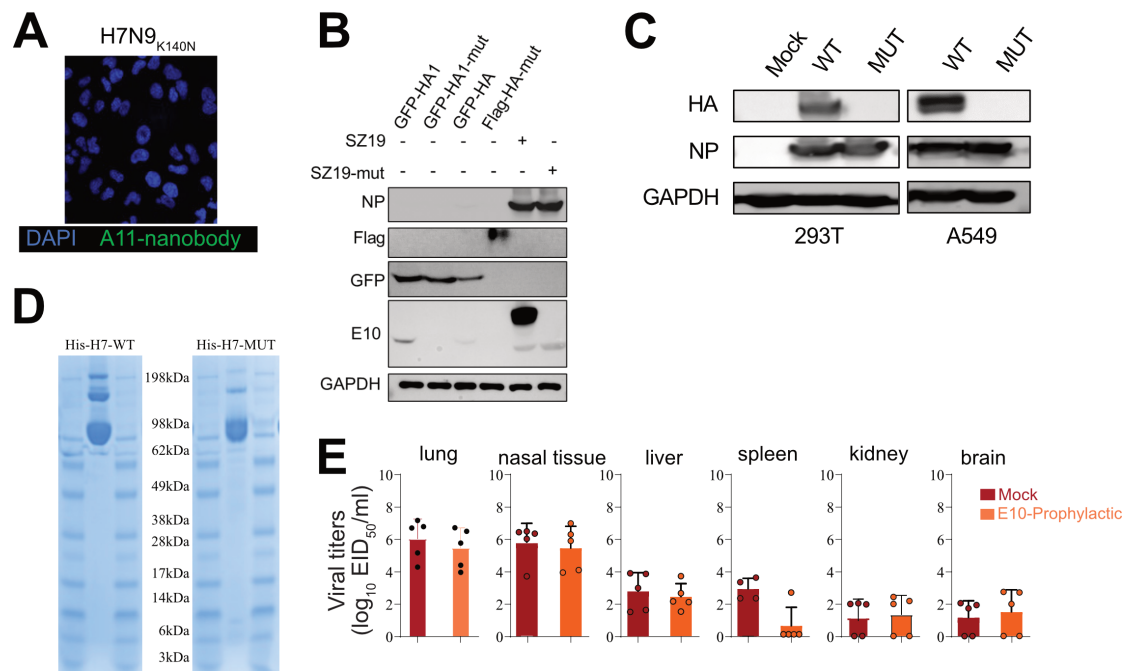
ImmuneBuilder and AlphaFold 2. (Different colors represent different nanobody: A11: red, C11: black, E10: orange, F3: blue, H10: yellow, H12: dark blue.) (C) Coomassie Brilliant Blue Staining detection of the expression of different nanobodies. (D) Immunofluorescence assay (IFA) showing the recognition of the SZ19 virus by different nanobodies. A549 cells were infected with SZ19 H7 IAV (MOI=0.1) for 36h and stained with distinct primary nanobodies, followed by secondary goat anti-human IgG Fc Alexa Fluor™ 488 (A11: red, E10: orange, H10: yellow, F3: blue, H12: dark blue, C11: black.). Scale bar, 250 μ m (E) Western blot analysis of A549 cells infected with SZ19 H7 IAV (MOI=1) for 24h. Cell lysate was probed with respective nanobodies and detected using goat anti-human IgG-Fc HRP. Data is representative of at least two independent experiments. (F) Sequence of six selected nanobodies.



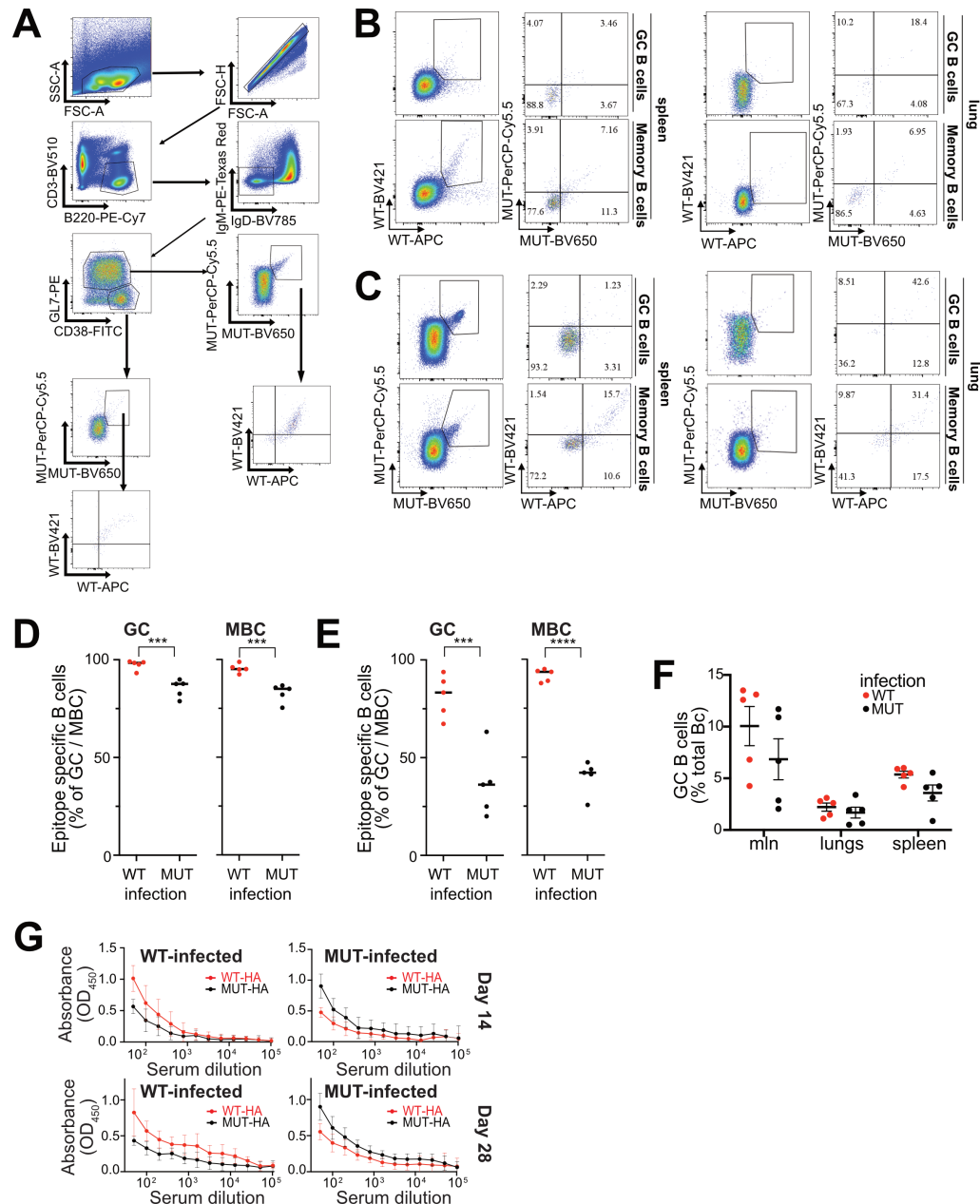
Supplementary Figure 2. E10-Fc treatment protects mice against homo- and heterosubtypic IAV challenge. (A) Viral titer, measured by EID₅₀, in six different organs (lung, nasal, liver, spleen, kidney, brain) on day 3 after H1N1 PR8 infection with or without E10 administration, as outlined in 2E. Graphs shows mean $\pm$ SD. (B) Representative histopathological analysis of lungs from mice on day 3 after H1N1 PR8 infection with or without E10 administration, and of MOCK-infected mice, as outlined 2E. Scar bar, 500 μ m in the left row, 200 μ m in the middle row, 20 μ m in the right row.



Supplementary Figure 3. E10 recognizes a conserved epitope located on HA-head lateral patch. (A) Nanobody escape mutations in the H7N9 virus were identified by selection in SPF eggs and MDCK cells. Variations at key residues involved in nanobody escape are highlighted, showing the sites where resistance developed. (B) Phage display selection of other 5 nanobody identified specific peptides. The red region depicts an overlapping area among selected peptides. (C) HA subtype numbering conversion of H7N9-SZ19- HA protein with H1N1-PR8-HA and H3N2-HA. Alignment performed on the website of NIAID Bioinformatics Resource Centers (<https://www.bv-brc.org/>). The blue boxes show the different nanobodies binding regions, as identified by phage display or eescape selection. (D) Epitope mapping of the nanobodies on SZ19 H7N9 HA protein showing the binding sites of six different nanobodies, each labeled with distinct colors (A11: red, E10: orange, H10: yellow, F3: blue, H12: dark blue, C11: black). Complementary view to Fig 4C. The head domain of SZ19 H7 HA was modeled using Swiss-Model. Images were generated using Open-Source PyMOL version 2.5.0.



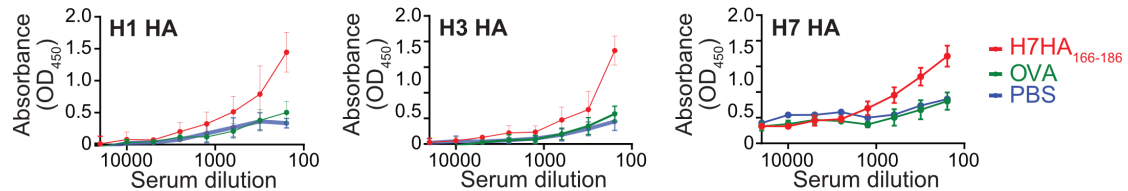
Supplementary Figure 4. H7-HA_{K166T, S167L} double mutant escapes E10 recognition but has lower viral fitness. (A) IFA of A549 infected with H7N9_{K140N} virus (MOI=0.1) at 24h post-infection. Cell was stained with A11 as the primary antibody, followed by secondary goat anti-human IgG Fc Alexa Fluor™ 488. Nuclei were counterstained with DAPI (blue), and NP detection is shown in green. Data is representative of at least two independent experiments. (B) Western blot (WB) analysis of A549 cells infected with WT or MUT virus or transfected with GFP-HA and GFP-HA-MUT with or without E10 pre-incubation, showing detection of NP and E10. Data are representative of at least two independent experiments. Shown are the mean values of three technical replicates. (C) Western blot (WB) showing the infection of A549 or 293T cells 24h post infection with WT and MUT virus. Data are representative of at least two independent experiments. Shown are the mean values of three technical replicates. (D) Coomassie Brilliant Blue Staining detect the express and size of recombinant WT-HA and MUT-HA protein. (E) Viral titer, measured by EID₅₀, in six organs (lungs, nasal tissue, liver, spleen, kidney, brain) on day 3 post-MUT infection, with or without E10 treatment, as described in 5E. Data are representative of three independent experiments and shown as mean values from three technical replicates. Statistical analysis was performed using unpaired t test. *p < 0.05; **p < 0.01; ****p < 0.0001; ns: not significant.



Supplementary Figure 5. E10-epitope is immunodominant upon H7-IAV infection..

(A) Flow cytometry gating for B cell characterization and analysis. (B) Representative flow cytometry gating of GC and MBC and epitope identification in spleen and lung of 14 days infected WT mice, gated GC as live CD3⁻ B220⁺ IgD⁻ IgM⁻ GL7⁺ CD38⁻ WT⁺ MUT⁺, MBC as live CD3⁻ B220⁺ IgD⁻ IgM⁻ GL7⁻ CD38⁺ WT⁺ MUT⁺. (C) Same as in B but for MUT infected mice, gated GC as live CD3⁻ B220⁺ IgD⁻ IgM⁻ GL7⁺ CD38⁻ MUT⁺ WT⁺, MBC as live CD3⁻ B220⁺ IgD⁻ IgM⁻ GL7⁻ CD38⁺ MUT⁺ WT⁺. (B, C) Quantification of epitope-specific MBC and GC B cells in lung and spleen of 14 days infected WT or MUT mice. (D, E) Percentage of HA-specific B cells at spleen/lung after infected with WT or MUT virus 14 days. Two independent experiments with 5 mice each. Bars represent SEM; statistical analysis was performed using two-sided unpaired t test. (F) Percentage of B cells at mln/spleen/lung after infected with WT or MUT virus 14 days. Two independent experiments with 5 mice

each. Bars represent SEM.(G) ELISA curves showing the detection of WT-HA and MUT-HA in serum of WT or MUT infected mice, at 14, 28 days post infection. Data represent four independent experiments with 5 mice each (n = 20). Bars represent mean $\pm$ SEM



Supplementary Figure 6. H7HA₁₆₆₋₁₈₆ peptide immunization confers partial protection from lethal H7N9 infection. ELISA test showing binding of serum of mice immunized with H7-HA₁₆₆₋₁₈₆ peptide (red) or relevant controls (OVA-immunized mice: green; PBS immunized mice: blue) to H1N1/H3N2/H7N9 HA proteins. Representative of two independent experiments with 4-5 mice per group. Bars represent mean $\pm$ SEM