





# Design of Nanoparticulate Group 2 Influenza Virus Hemagglutinin Stem Antigens That Activate Unmutated Ancestor B Cell Receptors of Broadly Neutralizing Antibody Lineages

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**ABSTRACT** Influenza vaccines targeting the highly conserved stem of the hemagglutinin (HA) surface glycoprotein have the potential to protect against pandemic and drifted seasonal influenza viruses not covered by current vaccines. While HA stem-based immunogens derived from group 1 influenza A viruses have been shown to induce intragroup heterosubtypic protection, HA stem-specific antibody lineages originating from group 2 may be more likely to possess broad cross-group reactivity. We report the structure-guided development of mammalian-cell-expressed candidate vaccine immunogens based on influenza A virus group 2 H3 and H7 HA stem trimers displayed on self-assembling ferritin nanoparticles using an iterative, multipronged approach involving helix stabilization, loop optimization, disulfide bond addition, and side-chain repacking. These immunogens were thermostable, formed uniform and symmetric nanoparticles, were recognized by cross-group-reactive broadly neutralizing antibodies (bNAbs) with nanomolar affinity, and elicited protective, homosubtypic antibodies in mice. Importantly, several immunogens were able to activate B cells expressing inferred unmutated common ancestor (UCA) versions of cross-group-reactive human bNAbs from two multidonor classes, suggesting they could initiate elicitation of these bNAbs in humans.

**IMPORTANCE** Current influenza vaccines are primarily strain specific, requiring annual updates, and offer minimal protection against drifted seasonal or pandemic strains. The highly conserved stem region of hemagglutinin (HA) of group 2 influenza A virus subtypes is a promising target for vaccine elicitation of broad cross-group protection against divergent strains. We used structure-guided protein engineering employing multiple protein stabilization methods simultaneously to develop group 2 HA stem-based candidate influenza A virus immunogens displayed as trimers on self-assembling nanoparticles. Characterization of antigenicity, thermostability, and particle formation confirmed structural integrity. Group 2 HA stem antigen designs were identified that, when displayed on ferritin nanoparticles, activated B cells expressing inferred unmutated common ancestor (UCA) versions of human antibody lineages associated with cross-group-reactive, broadly neutralizing antibodies (bNAbs). Immunization of mice led to protection against a lethal homosubtypic influenza virus challenge. These candidate vaccines are now being manufactured for clinical evaluation.

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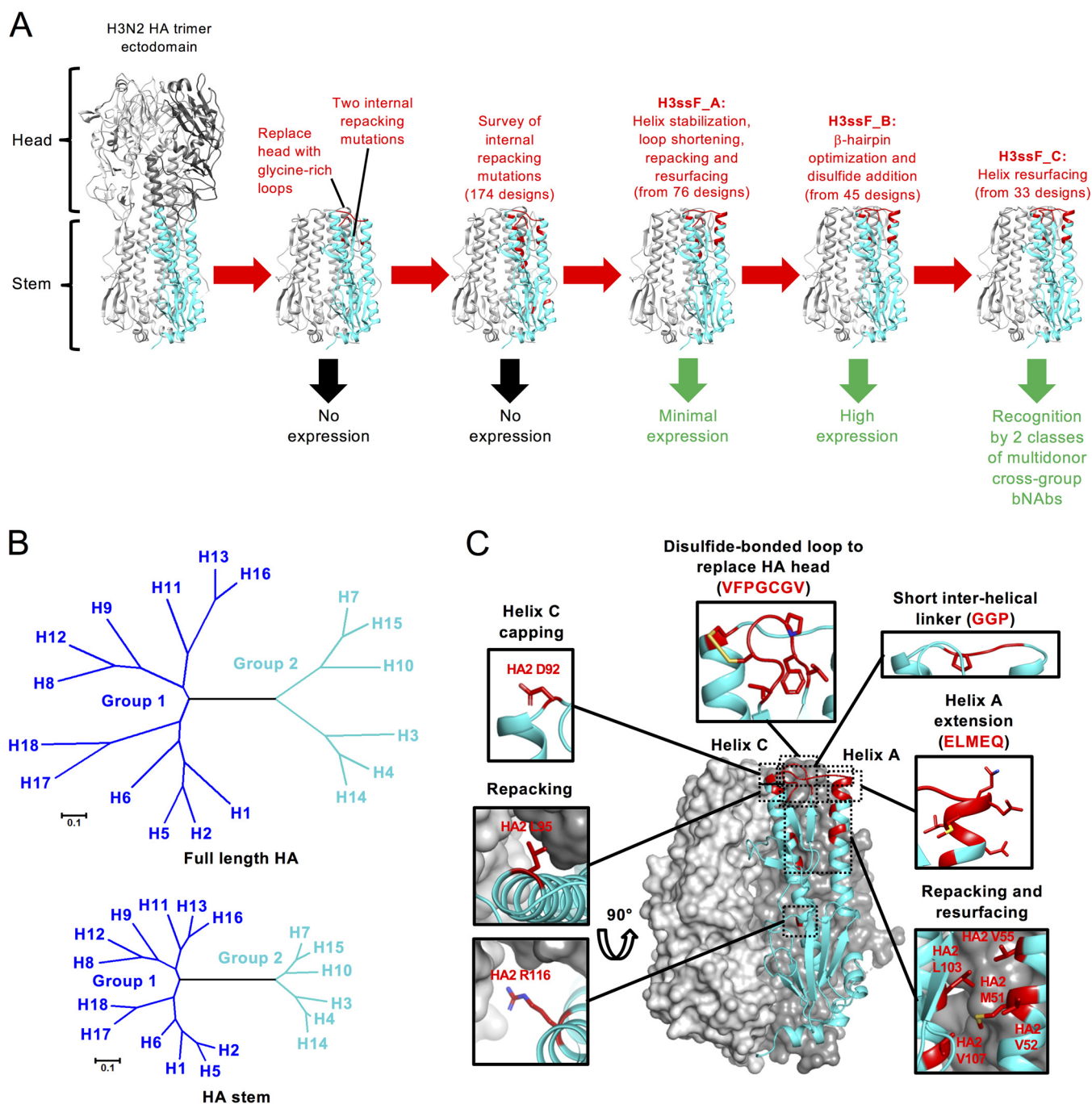
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Influenza continues to be a significant global health burden, typically resulting in about 500,000 deaths worldwide annually (1), even though the technology for conventional egg-grown, whole inactivated influenza virus vaccines was developed more than 70 years ago. Constant antigenic drift of the influenza virus hemagglutinin (HA) coupled with immunodominant strain-specific antibody responses directed to the variable HA head domain results in conventional vaccine effectiveness ranging from 10 to 60% (2) and the need for seasonal updates of virus strains included in licensed vaccines. Furthermore, current vaccine approaches provide minimal protection against pandemic influenza virus strains (3, 4). Improved influenza vaccines would not be produced in eggs, would be designed to induce broad immunity against future drifted and pandemic strains without seasonal reformulation, and would elicit durable immune responses, avoiding the need for annual vaccination (4). One approach for achieving broadly cross-reactive influenza virus-specific antibodies is to target highly conserved neutralization-sensitive epitopes in the stem region of the influenza A virus HA surface glycoprotein (5). Monoclonal antibodies (MAb) have been identified that bind conserved HA stem epitopes and possess broad neutralizing activity across diverse HA subtypes within an influenza A virus group, as well as some that have cross-group neutralizing activity (6–16). We and others have recently developed influenza A virus group 1 HA stem trimer antigens (17, 18). One candidate displaying H1-based HA stem trimers on self-assembling ferritin nanoparticles protected mice and ferrets from a lethal heterosubtypic H5N1 influenza challenge (17). However, these group 1 HA stem immunogens did not provide cross-group protection against group 2 viruses, such as H3N2 and H7N9. The need for a better group 2 vaccine is particularly acute, as vaccine effectiveness against H3N2 over the past decade has averaged only 33% (19), recent H3N2 strains have exhibited increased virulence (20), and H7 viruses represent one of the greatest pandemic threats from nonseasonal strains. H7N9 immunization was recently shown to induce multiple HA stem-directed antibody lineages in humans that recognized both group 1 and group 2 HA molecules, whereas H5N1 immunization induced primarily HV1-69 antibody lineages that cross-reacted primarily within group 1 influenza A virus subtypes (13). This suggests that group 2 HA stem immunogens may be more effective at inducing cross-group responses than group 1 immunogens in humans with preexisting influenza immunity or at priming multidonor antibody lineages with the capacity for broad neutralizing activity in influenza-naïve infants. In our studies, we used iterative structure-based design to develop headless group 2 HA stabilized stem trimers displayed on ferritin nanoparticles based on hemagglutinin sequences from H3N2 (H3ssF) and H7N9 (H7ssF) viruses as candidate vaccines. Notably, structural subtleties ultimately required the use of a set of design strategies to stabilize group 2 HA stem immunogens that was completely different than the simpler hydrophobic repacking used to stabilize group 1 immunogens. Ultimately, H3ssF and H7ssF immunogen designs were identified that could activate B cells expressing inferred unmutated common ancestor (UCA) of HA stem-directed human broadly neutralizing antibodies (bNAbs), suggesting the potential for inducing cross-reactive antibody lineages with the capacity to develop broad influenza virus neutralizing activity in humans.

## RESULTS

**Design of group 2 stabilized HA stem nanoparticle immunogens.** The initial design of group 2 stabilized HA stem nanoparticles incorporated some features of group 1 H1-stabilized HA stem nanoparticle immunogens (17) (referred to as H1ssF herein). This comprised replacing the HA head region (HA1 residues 43 to 313, H3 numbering [21]) with a GSG loop, replacing the HA2 interhelical region (residues 60 to 92) with a 6-residue glycine-rich loop, including two repacking substitutions in the HA2



**FIG 1** Structure-based design of group 2 HA stem nanoparticles. (A) Ribbon models depicting the HA stem nanoparticle design pathway for stabilizing the HA stem starting from an H3N2 HA trimer ectodomain. Two of the three protomers are gray, and one protomer is cyan with the design modifications in red. Positive or negative outcomes are indicated by green or black arrows, respectively, below each step. bNAB, broadly neutralizing antibody. (B) Maximum-likelihood phylogenetic trees for protein sequences from representative full-length HAs (top) and HA stems (bottom) of the 18 different subtypes. Group 1 subtypes are dark blue, and group 2 subtypes are cyan. (C) A molecular model of the HA stem of an H3ssF\_C design is shown colored as described for panel A. Two of the three protomers are depicted as surface representations, and one protomer is displayed as a ribbon diagram. Insets show close-ups of the modified regions with stick representations for side chains of modified residues. Surface representations are removed from some insets for clarity. PDB entry 2YP2 was used as an H3N2 model template.

hydrophobic core (replacing K with M at position 103 [K103M] and E with L at position 51 [E51L]), and connecting the C-terminal HA2 residue 174 to the N-terminal residue 5 of bacterial ferritin with a short SGG linker (Fig. 1A; Fig. S1 and S2 in the supplemental material). In the group 1 context, this strategy was sufficient for displaying antigenically correct H1, H2, and H5 HA stem trimers on ferritin nanoparticles (17). However, multiple

additional steps were needed to achieve the expression of group 2 H3 and H7 HA stem nanoparticles in mammalian HEK293 cells (Fig. 1). Although there is structural similarity between H1 and H3 HA stem regions (e.g., root mean square deviation [RMSD] of 1.0 Å for 134 stem residues between A/California/04/2009 [H1N1; PDB identification code [ID] 3UBQ] and A/Victoria/361/2011 [H3N2; PDB ID 4O5N]) and sequence identity of approximately 55%, phylogenetic analysis indicates groups 1 and 2 comprise two distinct branches with consistent and predictable amino acid differences even for the stem region of HA (Fig. 1B). We hypothesized that further structural stabilization was required for expression, and therefore, an iterative structure-based design effort was employed to stabilize the HA stem region of H3ssF immunogens, using the 1.9-Å-resolution crystal structure of HA for A/Finland/486/2004 (H3N2; PDB ID 2YP2) (22) as a template. To screen for expression and correct antigenicity, designs were expressed in HEK293T cells using a high-throughput 96-well format (23). Supernatants containing HA stem trimers genetically fused to ferritin nanoparticles were assessed directly for antigenic recognition either by an immunoprecipitation assay using the human cross-group HA stem-specific bNAb Fl6v3 or by an enzyme-linked immunosorbent assay (ELISA) using the bNAbs CR9114, Fl6v3, and CT149. CT149 was specifically included to assess trimer integrity because its epitope incorporates two adjacent protomers of the HA stem and, thus, may be sensitive to quaternary structure (15).

Since internal hydrophobic repacking mutations were critical for the stabilization of the group 1 HA stem immunogens (17) and the burying of just a single  $-CH_2-$  group in a protein interior can add approximately 1.1 kcal/mol of free energy to protein stability (24, 25), we explored additional repacking mutations selected computationally using Rosetta (26). In combination with some of the repacking mutations, we also explored limited residue swapping between H1N1 and H3N2 subtypes (Table S1). Surprisingly, none of these 174 mutants could be expressed and recognized by the bNAb CR9114 or Fl6v3 (Table S1), indicating that novel approaches for structural stabilization would be required.

Upon further investigation of multiple protein stabilization strategies involving another 76 designs (Table S1), a lead design was successfully expressed (H3ssF\_A) that was recognized by bNAb HA stem antibody Fl6v3 by immunoprecipitation and self-assembled into nanoparticles (Table 1; Table S1, Fig. S2 and S3). H3ssF\_A included the initial H1ssF-based mutations in addition to (i) helix stabilization, (ii) loop optimization, and (iii) point mutations of select H3 residues to improve internal packing (Fig. 1A and C; Fig. S2A). Each of the successful alterations was focused on the membrane distal end of the two central helices (A and C) of the HA2 fold. The outer helix A in H3N2 HA is approximately 5 residues shorter at its C terminus than in H1 HA, and it is known that helix length is related to stability (27). Therefore, helix A was extended at its C terminus by 5 residues (ALMAQ) with high helix-forming propensities (28). Likewise, the N terminus of the adjacent helix C was stabilized by adding an N-terminal Asp (HA2 92) as a helix-capping residue (29, 30), and two other mutations (HA2 S93A and N95L) were made at the N terminus of helix C to increase its helix-forming propensity and improve interprotomer packing (Fig. 1C). Biophysical studies have shown that excess loop length can destabilize protein folds by up to several kcal/mol (31), and in the initial H1ssF and H3ssF designs, helices A and C were connected by a 6-residue linker even though they are separated by only 12 Å. Therefore, the glycine-rich linker was shortened to a 4-residue GGP linker to more closely match the interhelical distance. Finally, 4 residues (HA2 52, 55, 107, and 116) on helices A and C were mutated to improve side-chain packing (Fig. 1C; Fig. S2A). Three of these (HA2 52, 55, and 116) comprised mutations to H1 residues.

To further improve expression and stability, another 45 designs were explored, focusing on the short  $\beta$ -hairpin connected to a 3-residue loop which replaced the HA head in HA1 (Fig. 1). Since the stability of short, two-stranded  $\beta$ -sheets has been observed to increase with length (32), we replaced the GSG loop with longer loops of 6 or 7 residues designed to double the hairpin length. Residues with high strand-forming propensities (28), such as Val, Ile, and Phe, were used for strand extension, and



**TABLE 1** Physical characteristics of the H3ssF and H7ssF nanoparticles

Subtype	Design	Design characteristic						EM characterization <sup>g</sup>		
		HA1 loop <sup>a</sup>	Helix A extension <sup>b</sup>	Interhelix loop <sup>c</sup>	Helix C mutation <sup>d</sup>	Predicted disulfide(s) <sup>e</sup>	Relative yield <sup>f</sup>	<i>T<sub>m</sub></i> (°C)	Ferritin core size (Å)	HA stem size (Å)
H3	H3ssF_A	--GSG--	ALMAQ	GGP	DAYL	NA	–	ND <sup>h</sup>	ND	ND
H3	H3ssF_B	VFPGCGV	ALMAQ	GGP	DCYL	DS1	+++	66.2	124.0 ± 7.0	82.0 ± 9.0
H7	H7ssF_B						+++	58.6	126.9 ± 6.8	71.6 ± 6.5
H3	H3ssF_C	VFPGCGV	ELMEQ	GGP	DCYL	DS1	++	66.6	123.2 ± 3.4	65.0 ± 5.2
H7	H7ssF_C						+++	61.6	124.4 ± 4.4	66.9 ± 6.4
H3	H3ssF_D	VFPGC-V	ALMAQ	GGP	DCYL	DS1	++	66.3	125.3 ± 5.1	67.7 ± 12.4
H7	H7ssF_D						+++	61.2	139.7 ± 4.8	64.8 ± 3.4
H3	H3ssF_E	VFP-CGV	ALMAQ	GGP	DCYL	DS1	++	63.0	130.1 ± 5.3	66.9 ± 7.2
H7	H7ssF_E						+	56.7	124.7 ± 6.5	68.1 ± 2.7
H3	H3ssF_F	VFPGCGV	ALMEE	GGP	DCYL	DS1	+	65.6	127.1 ± 6.0	67.0 ± 4.1
H7	H7ssF_F						+++	59.9	130.2 ± 6.3	65.1 ± 5.5
H3	H3ssF_G	CFNGIC-	ALMAQ	GGP	DAYL	DS2	+++	59.9	126.3 ± 4.0	68.5 ± 3.3
H3	H3ssF_H	VFPGCGV	ELMEQ	GGP	DCYL	DS1, DS3	++	56.6	126.9 ± 3.5	67.8 ± 4.5
H7	H7ssF_I	VFPNCGV	ALMAQ	GGP	DCYL	DS1	+++	58.7	124.3 ± 4.1	68.4 ± 6.6
H7	H7ssF_J	VFPGCGV	ALMAQ	GPP	DCYL	DS1	+++	57.0	131.4 ± 5.1	62.6 ± 7.6

<sup>a</sup>Between HA1 residues 42 and 314 (H3 numbering).

<sup>b</sup>Directly following HA1 56.

<sup>c</sup>Directly following the helix A extension.

<sup>d</sup>Region between the interhelix loop and HA2 96.

<sup>e</sup>DS1, HA1 C145-HA2 C93; DS2, HA1 C141-C146; DS3, HA1 C23-C322; NA, not applicable.

<sup>f</sup>Based on gel electrophoresis analysis of unpurified supernatants following transient transfection.

<sup>g</sup>EM, electron microscopy.

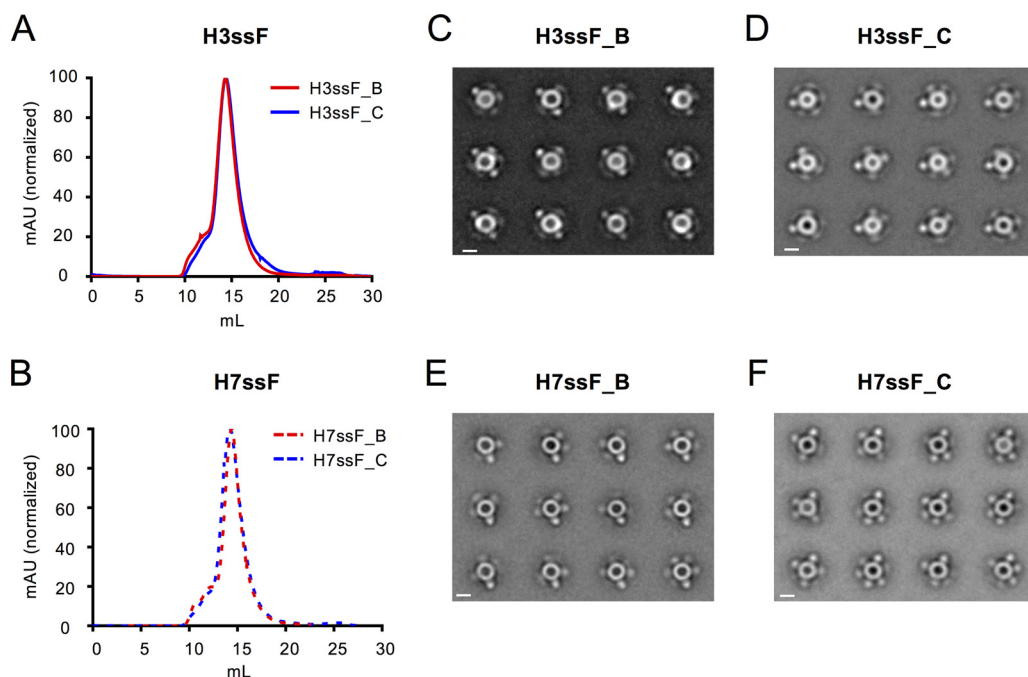
<sup>h</sup>ND, not determined.

dipeptides with propensities to form  $\beta$ -turns (33, 34), such as PG and NG, were used to connect them. Disulfides were also designed into the hairpin loops for added stability (35). From an initial survey of loop extensions, H3ssF\_B, designed with a 7-residue hairpin loop extension disulfide bonded to the N terminus of helix C, resulted in expression levels of more than 5 mg/liter and recognition by CT149 (Fig. 1, Table 1; Table S1).

Further loop optimization of the H3ssF\_B design included removing a glycine from either side of the cysteine in the  $\beta$ -hairpin loop (H3ssF\_D and H3ssF\_E) and incorporating a different  $\beta$ -hairpin sequence with an internal disulfide bond engineered to link both strands together (H3ssF\_G). Additional H3ssF\_B variants incorporated hydrophilic, helix-preferring Glu into the relatively hydrophobic helix A extension motif, for improved solubility (H3ssF\_C, H3ssF\_F, and H3ssF\_H), and an engineered intraprotomer disulfide between HA1 residues 23 and 322 (H3ssF\_H). All six variants resulted in nanoparticles recognized by CT149 in ELISAs but did not improve overall expression beyond that of H3ssF\_B. Based on the successful expression and antigenic recognition of multiple H3ssF variants, we transferred mutations from constructs H3ssF\_B, H3ssF\_C, H3ssF\_D, H3ssF\_E, and H3ssF\_F into analogous H7ssF nanoparticles using sequences from the human H7N9 strain (A/Shanghai/2/2013) and also created two additional loop variants (H7ssF\_I and H7ssF\_J) (Table 1; Fig. S2B). All seven of these H7ssF designs expressed milligram-per-liter quantities of nanoparticles recognized by CT149 (Table S1).

**Physical characterization of H3ssF and H7ssF nanoparticles.** Fourteen nanoparticles were selected for further characterization. These were purified by lectin chromatography followed by size exclusion chromatography, which resulted in distinct peaks consistent with approximately 1.2-MDa particles (Fig. 2A and B; Fig. S4). Negative-stain electron microscopy of purified nanoparticles followed by reference-free 2-dimensional (2-D) classification and averaging revealed spherical particles with five or six visible, regularly spaced, protruding spikes (Fig. 2C to F; Fig. S4). All nanoparticles contained ferritin cores consistent with the expected 12- to 13-nm diameter and HA stems with the expected length of approximately 7 nm (Table 1).

**Thermostability of H3ssF and H7ssF nanoparticles.** We next investigated immunogen thermostability using differential scanning calorimetry (DSC). DSC measure-



**FIG 2** Physical characterization of group 2 HA stem nanoparticles. Gel filtration chromatograms for lectin-purified H3ssF (A) and H7ssF (B) nanoparticles reveal single peaks. (C to F) Negative-stain electron microscopy 2-D class averages for H3ssF (C, D) and H7ssF (E, F) B and C variants demonstrate the formation of particles with visible arrangements of HA stem trimers projecting from hollow spheres. White bars represent 10-nm size markers.

ments revealed thermal melting temperatures ( $T_m$ ) for the earliest transitions ranging from 56.6 to 66.6°C for H3ssF variants and 56.7 to 61.6°C for H7ssF variants (Table 1; Fig. S5A and B). Three other, higher temperature transitions at approximately 78, 96, and 106°C were consistently observed for all the immunogens tested. In contrast, the ferritin nanoparticle alone had a primary transition at 105.7°C and a minor one at 84°C. Since the earliest transitions were the most variable, we hypothesized that these were related to the HA stem structures.

The three H3ssF designs with the highest  $T_m$ s, ranging from 66.2 to 66.6°C, had either the 7-residue  $\beta$ -hairpin extension disulfide bonded to helix C (H3ssF\_B and H3ssF\_C) or the same loop with one glycine removed just after the cysteine (H3ssF\_D). Glycine removal just prior to the cysteine (H3ssF\_E) was less well tolerated thermodynamically. Curiously, H3ssF\_H with the same hairpin loop as H3ssF\_B and H3ssF\_C but designed to contain an additional disulfide bond to stabilize the HA1 region of the stem had the lowest  $T_m$ , 56.6°C, indicating that this variant had less than optimal stability. Design H3ssF\_G, engineered to have an internal disulfide bond in the  $\beta$ -hairpin extension, had a  $T_m$  6°C lower than those of H3ssF\_B and H3ssF\_C. Incorporation of Glu into the two Ala positions in the 5-residue helix A extension had little effect on stability (H3ssF\_C), but Glu incorporation to replace the last two residues decreased the  $T_m$  by 1°C (H3ssF\_F). For the five immunogen designs (B to F) made in both H3 and H7 formats, the  $T_m$  values were on average 5.9°C higher for the H3ssF than for the H7ssF designs, and similar trends were observed between designs. Finally, two additional H7ssF variants not represented in H3ssF, with an Asn incorporated into the HA1  $\beta$ -hairpin extension (H7ssF\_H) and a Pro incorporated into the HA2 GGP loop (H7ssF\_I), did not significantly alter the  $T_m$ . While improved thermostability will facilitate vaccine transport and storage, increasing rigidity and stability of antigens can sometimes be counterproductive for immunogenicity (36). Therefore, multiple designs with various levels of DSC-determined stability were advanced for further evaluation.

**Antigenicity of H3ssF and H7ssF nanoparticles.** Biolayer interferometry (BLI) was used to measure the binding kinetics and affinities of nanoparticles for antigen-binding

**TABLE 2** Antigenicity of the H3ssF and H7ssF nanoparticles for Fab forms of bNAbs as determined by BLI<sup>a</sup>

Design	CT149			MEDI8852			CR8020			CR9114		
	$K_D$ (nM)	$K_{on}$ ( $M^{-1}s^{-1}$ )	$K_{off}$ ( $s^{-1}$ )	$K_D$ (nM)	$K_{on}$ ( $M^{-1}s^{-1}$ )	$K_{off}$ ( $s^{-1}$ )	$K_D$ (nM)	$K_{on}$ ( $M^{-1}s^{-1}$ )	$K_{off}$ ( $s^{-1}$ )	$K_D$ (nM)	$K_{on}$ ( $M^{-1}s^{-1}$ )	$K_{off}$ ( $s^{-1}$ )
H3ssF_A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H3ssF_B	NB	NB	NB	0.7	$2.9 \times 10^5$	$2.2 \times 10^{-4}$	20.9	$2.8 \times 10^5$	$5.8 \times 10^{-3}$	ND	ND	ND
H7ssF_B	7.0	$4.2 \times 10^5$	$3.0 \times 10^{-3}$	1.0	$2.6 \times 10^5$	$2.6 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H3ssF_C	21.3	$8.1 \times 10^5$	$1.7 \times 10^{-2}$	0.8	$3.0 \times 10^5$	$2.4 \times 10^{-4}$	23.3	$2.4 \times 10^5$	$5.5 \times 10^{-3}$	93.6	$7.0 \times 10^4$	$6.5 \times 10^{-3}$
H7ssF_C	0.9	$6.0 \times 10^5$	$5.3 \times 10^{-4}$	1.0	$2.7 \times 10^5$	$2.6 \times 10^{-4}$	NB	NB	NB	47.9	$1.0 \times 10^5$	$4.8 \times 10^{-3}$
H3ssF_D	NB	NB	NB	1.1	$2.9 \times 10^5$	$3.2 \times 10^{-4}$	21.7	$2.8 \times 10^5$	$6.0 \times 10^{-3}$	ND	ND	ND
H7ssF_D	9.0	$3.7 \times 10^5$	$3.3 \times 10^{-3}$	0.9	$2.7 \times 10^5$	$2.3 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H3ssF_E	NB	NB	NB	1.2	$3.2 \times 10^5$	$3.7 \times 10^{-4}$	18.7	$3.0 \times 10^5$	$5.7 \times 10^{-3}$	ND	ND	ND
H7ssF_E	8.2	$3.7 \times 10^5$	$3.0 \times 10^{-3}$	0.8	$2.9 \times 10^5$	$2.4 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H3ssF_F	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H7ssF_F	2.7	$6.7 \times 10^5$	$1.8 \times 10^{-3}$	0.9	$2.9 \times 10^5$	$2.4 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H3ssF_G	NB	NB	NB	0.9	$3.0 \times 10^5$	$2.6 \times 10^{-4}$	17.9	$2.7 \times 10^5$	$4.8 \times 10^{-3}$	ND	ND	ND
H3ssF_H	22.2	$8.2 \times 10^5$	$1.8 \times 10^{-2}$	ND	ND	ND	ND	ND	ND	ND	ND	ND
H7ssF_I	7.2	$4.1 \times 10^5$	$2.9 \times 10^{-3}$	1.0	$2.6 \times 10^5$	$2.7 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H7ssF_J	9.8	$3.5 \times 10^5$	$3.4 \times 10^{-3}$	1.9	$2.5 \times 10^5$	$4.9 \times 10^{-4}$	NB	NB	NB	ND	ND	ND
H3 HA	42.2	$7.5 \times 10^4$	$3.2 \times 10^{-3}$	1.2	$1.3 \times 10^5$	$1.5 \times 10^{-4}$	ND	ND	ND	ND	ND	ND
H7 HA	3.7	$3.6 \times 10^5$	$3.1 \times 10^{-3}$	1.2	$1.1 \times 10^5$	$1.3 \times 10^{-4}$	ND	ND	ND	ND	ND	ND

<sup>a</sup> $K_D$ , equilibrium dissociation constant;  $K_{on}$ , association rate constant;  $K_{off}$ , dissociation rate constant; ND, not determined; NB, no detectable binding.

fragments (Fabs) of bNAbs MEDI8852, CT149, and CR8020 (Table 2; Fig. S6 and S7). To avoid avidity effects, the nanoparticles were immobilized to anti-Fc-decorated sensors using the bNAb CR9114 IgG and monovalent Fabs were used as the analyte. CR9114 Fab was observed to recognize H3ssF and H7ssF immunogens with affinities of 94 and 48 nM, respectively (Table 2; Fig. S6E). However, given the bivalent nature of the CR9114 IgG and the 24 epitope sites on each nanoparticle, the avidity of this complex was more than enough to stably immobilize nanoparticles to the sensor for measurements of high-affinity bNAb Fabs.

The first two antibodies (MEDI8852 and CT149) recognize similar HA stem epitopes centered on the HA2 helix A and fusion peptide loop supersite and represent two multidonor classes of cross-group bNAbs (HV6-1+HD3-3 class and HV1-18 QXXV class, respectively) recently identified in healthy human adults vaccinated with H5N1 (12, 14, 15). CR8020 is a group 2-specific bNAb that recognizes a less conserved, more membrane-proximal HA stem epitope (11). The HV6-1+HD3-3 class MAb MEDI8852 recognized all H3ssF and H7ssF nanoparticles, as well as full-length H3 and H7 HA trimers, with similar nanomolar affinities and kinetics (Table 2; Fig. S6A and B). The HV1-18 QXXV class MAb CT149 also recognized all H7ssF nanoparticles with approximately 1 to 10 nanomolar affinity. Of these, H7ssF\_C was recognized with approximately 10-fold-greater affinity (0.9 nM) and a 10-fold-slower off rate ( $5.3 \times 10^{-4} s^{-1}$ ) than the other H7ssF nanoparticles. However, among the H3ssF nanoparticles, only H3ssF\_C and H3ssF\_H were recognized by CT149, with affinities of 21.3 to 22.2 nM; the other four H3ssF immunogens showed no measurable affinity to CT149 (Table 2; Fig. S6). This was surprising, since each of these designs was initially chosen based on strong binding to CT149 in ELISAs. Furthermore, ELISAs performed on purified H3 and H7 immunogens for designs B and C also indicated a high affinity to each of the group 2-reactive HA stem antibodies tested (Fig. S7A). This apparent disparity between BLI and ELISA was potentially due to utilization of bivalent IgGs in ELISAs as opposed to monovalent Fabs in BLI assays, which provides an avidity advantage for apparent binding affinity of up to 2 orders of magnitude (37–39). Consistent with this idea, we observed in ELISAs that the Fab form of CT149 did not bind to H3ssF\_B or H3ssF\_C and bound only modestly to H7ssF\_B and H7ssF\_C (Fig. S7B). Moreover, ELISAs used higher densities of immobilized antigen than BLI, and there are inherent differences in the methodologies of the two assays. Full-length H3 and H7 HA trimers were observed to bind CT149 with affinities and kinetics consistent with those of the C and H versions of the H3ssF and H7ssF nanoparticles (Fig. S6C to D). Interestingly, the nanoparticles with

greatest affinity for CT149, H3ssF\_C, H3ssF\_H, and H7ssF\_C were each designed to have two outwardly facing Glu residues at the C-terminal end of helix A, whereas the other designs had Ala at these positions.

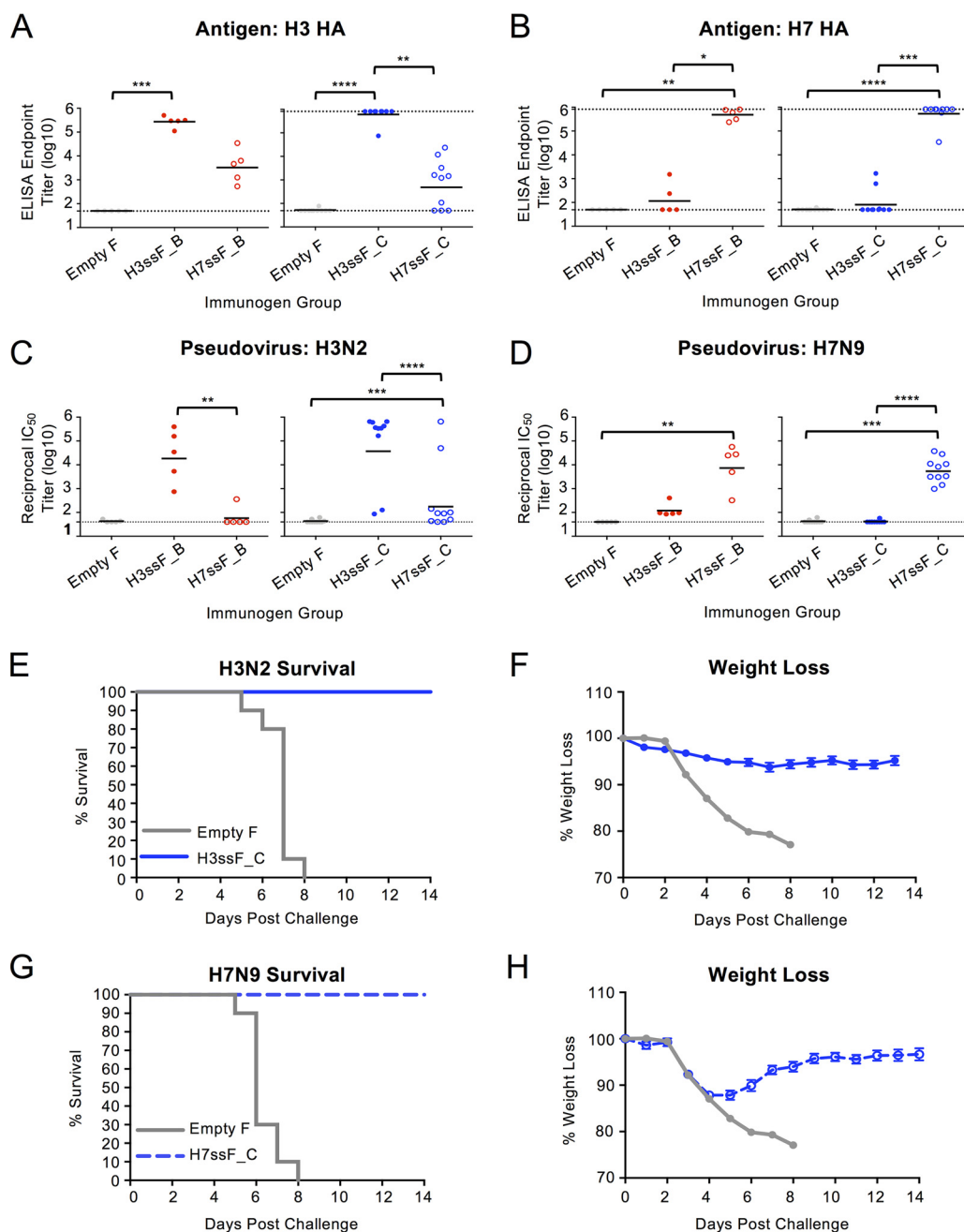
The group 2-specific HA stem bNAb CR8020 recognized all of the H3ssF nanoparticles tested with affinities of 17.9 to 23.3 nM but did not recognize any of the H7ssF nanoparticles (Table 2; Fig. S7C). This is consistent with the previously reported lack of CR8020 binding to full-length A/Shanghai/2/2013 (H7N9) HA, the same strain used in the H7ssF designs (40). The CR8020 epitope is not completely conserved even among group 2 HAs, as there are six epitope mutations between the A/Hong Kong/1/1968 (H3N2) HA and the A/Shanghai/2/2013 (H7N9) HA.

Newly recombined, naive B cell receptors (BCRs) need to first recognize an immunogen to initiate affinity maturation and B cell proliferation (41). Therefore, we also used BLI to assess the relative affinities of three sets of mature stem bNAbs and their inferred UCAs to HA stem nanoparticle immunogens (Fig. S7D). The UCAs were each inferred phylogenetically from multiple clonal family members and include unmutated CDRH3 loops (Fig. S8). Two of these (54-1G07 and 09-1B12) were from the human multidonor HV6-1+HD3-3 class and HV1-18 QXXV class, respectively (13). The third antibody, 04-1D02, was from an HV3-53+HD3-3 lineage. Measurable H3ssF and H7ssF immunogen recognition by inferred UCAs for each of the three antibodies was not considerably different from the binding of mature forms of the antibodies or the CR9114 positive control (Fig. S7D). This is interesting, considering that there are 2 to 5 unmutated CDRH3 residues in these UCAs (Fig. S8) and crystal structures of MAb complexes with HA show considerable CDRH3 binding for members of the HV1-18 QXXV and HV6-1 HD3-3 multidonor antibody classes (12, 14, 15).

**Immunogenicity of H3ssF and H7ssF nanoparticles.** To evaluate the immunogenicity of the group 2 HA stem nanoparticles, we immunized mice three times intramuscularly at 4-week intervals with the B and C designs of H3ssF and H7ssF. Both H3ssF and H7ssF elicited robust homosubtypic antibody responses against H3 and H7, respectively (Fig. 3A and B). Similarly, strong homotypic H3ssF and H7ssF neutralizing antibody responses were elicited against H3N2 A/Wisconsin/67/2005 and H7N9 A/Anhui/1/2013 pseudoviruses, respectively (Fig. 3C and D). For both H3 and H7, B and C designs elicited similar levels of antibody responses (Fig. 3A to D). Following homosubtypic challenges, H3ssF-vaccinated mice were fully protected against H3N2 A/Philippines/2/1982 influenza virus (Fig. 3E, H3ssF\_C); likewise, H7ssF vaccination fully protected mice from H7N9 A/Anhui/1/2013 (Fig. 3G, H7ssF\_C). Overall, these animal studies demonstrated robust immunogenicity and protective efficacy of our group 2 HA stem nanoparticles against homotypic viruses, with limited cross-recognition of heterosubtypic strains by elicited sera. Although the stabilized group 2 HA stem antigens are immunogenic and protective, the murine species tested does not have the appropriate B cell repertoire to evaluate the potential for eliciting bNAbs with specific characteristics similar to those of known classes of human MAbs with cross-subtype binding (12, 42, 43).

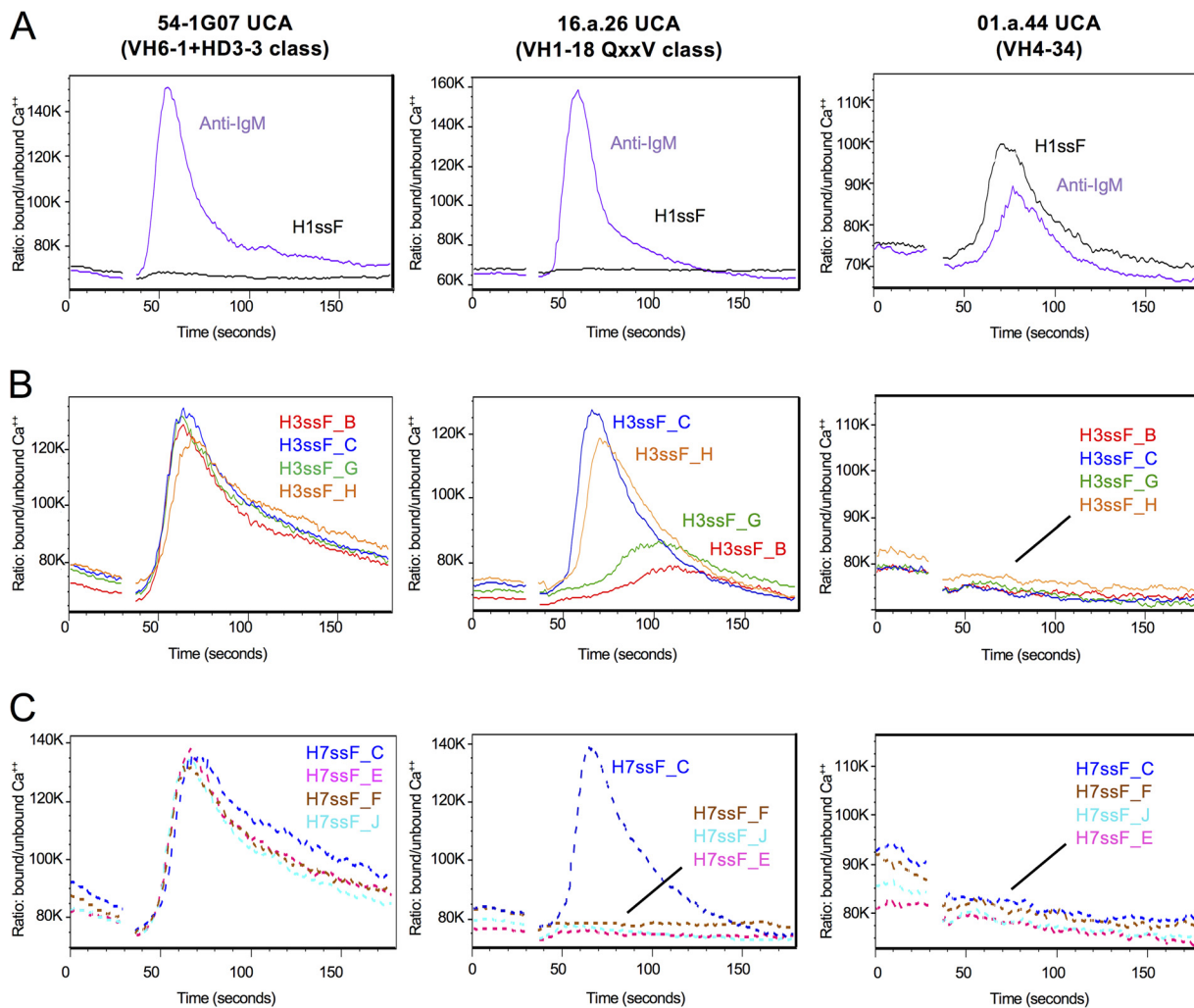
**B cell activation by H3ssF and H7ssF nanoparticles.** Recent studies have indicated that characterization of *in vitro* engagement of V gene-reverted BCRs by antigen can be used to assess and assign rank order to vaccine immunogens (43). Therefore, to better understand how the immunogens may be able to engage elements of the human antibody repertoire, Ca<sup>++</sup> flux assays were used to measure activation of Ramos B cells expressing IgM versions of the inferred UCAs of the human antibodies 16.a.26 and 54-1G07, representing two multidonor classes of cross-group human bNAbs (HV1-18 QXXV class and HV6-1+HD3-3 class, respectively) (12, 13, 43). All eight H3ssF and H7ssF designs tested strongly activated the 54-1G07 UCA BCRs, with magnitudes comparable to that of the anti-IgM control, whereas a group 1 H1ssF negative control resulted in no activation (Fig. 4, left column). In contrast, only two H3ssF immunogens (H3ssF\_C and H3ssF\_H) and one H7ssF immunogen (H7ssF\_C) were observed to elicit a strong activation of the 16.a.26 UCA BCR (Fig. 4, middle column). No





**FIG 3** Immunogenicity of group 2 HA stem nanoparticles in mice. BALB/c mice were immunized with H3ssF or H7ssF. B and C design groups were tested in separate experiments. (A to D) Serum was assessed for binding antibodies to H3 A/Hong Kong/1/1968 HA (A) and H7 A/Anhui/1/2013 HA (B) and neutralizing antibodies against H3N2 A/Wisconsin/67/2005 (C) or H7N9 A/Anhui/1/2013 (D) pseudovirus. (E to H) H3ssF\_C- and H7ssF\_C-immunized mice were challenged with lethal doses of H3N2 A/Philippines/2/1982 or H7N9 A/Anhui/1/2013, respectively. Survival (E, G) and weight loss (F, H) were recorded postchallenge. Dotted lines in panels A to D represent assay limits of detection. One-way ANOVA with Kruskal-Wallis posttest was used to compare mean ELISA and IC<sub>50</sub> titers between groups. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

activation of the HV4-34 group 1-specific 01.a.44 UCA BCR was observed (Fig. 4, right column) (12). Notably, these results were consistent with the BLI binding data. In both sets of experiments, the representatives of the multidonor HV6-1+HD3-3 class bNAbs engaged all of the H3ssF and H7ssF immunogens tested and the representatives of the multidonor HV1-18 QXXV class bNAbs engaged only the C and H design immunogens that contain added Glu in the helix A C-terminal extension. Our results suggest that



**FIG 4** Group 2 HA stem immunogens activate B cells expressing the inferred UCA versions of multidonor cross-group-reactive human BCRs. The results from Ca<sup>++</sup> flux assays indicate B cell activation by the IgM positive control and a group 1 H1ssF (A), H3ssF variants (B), and H7ssF variants (C). BCR activation is displayed for the group 2-specific 54-1G07 UCA, group 2-specific 16.a.26 UCA activation, and group 1-specific 01.a.44 control BCRs. Ca<sup>++</sup> flux was measured by flow cytometry as the ratio (y axis) of the Ca<sup>++</sup>-bound/-unbound states of the Ca<sup>++</sup>-sensitive dye fura red over time in seconds (x axis). Each experiment was repeated three times, and representative curves from one of the repeats are shown for each cell line.

these C and H designs could potentially activate nascent B cells expressing unmutated versions of multidonor HV6-1+HD3-3 or HV1-18 QXXV class bNABs to initiate B cell proliferation and affinity maturation, thereby eliciting cross-group-reactive bNAb responses.

## DISCUSSION

To address the need for influenza virus immunogens that can elicit broad hetero-subtypic responses in humans, particularly against group 2 influenza A virus, we used an iterative, structure-based design to develop headless group 2 HA stem-only nanoparticle immunogens from both H3 and H7 influenza virus subtype sequences. Stabilizing the group 2 HA stem was unexpectedly difficult compared to doing so for group 1 HA stem antigens. In addition to modifications used to achieve antigenically correct and immunogenic H1ssF candidate vaccines (17), multiple combined protein engineering strategies, including helix stabilization, loop optimization, and side-chain repacking, were required to achieve the first lead design candidate. Without all three stabilization strategies, the HA stem trimers were not stable enough to be expressed and displayed on self-assembling ferritin nanoparticles. This suggests that screening multiple stabili-

zation methods concurrently may be necessary to design vaccine antigens from metastable proteins or proteins that have had significant portions removed.

Ultimately, H3ssF and H7ssF immunogen designs were identified that provided uniform, thermostable nanoparticles recognized by HA stem-directed bNAbs with subnanomolar to nanomolar affinity. Additionally, H3ssF and H7ssF were immunogenic in mice, eliciting robust homotypic neutralizing antibody responses that fully protected mice against homosubtypic lethal challenges.

The unique antibody repertoire of humans makes it difficult to predict or model the human immune response of candidate influenza vaccines using currently available animal models like mice (42, 44) or ferrets, particularly given the specific classes of human V, D, or J gene combinations associated with broadly cross-reactive immune responses across multiple individuals (12). Therefore, instead of relying on a mouse model to assess the potential breadth of antibody response, we utilized *in vitro* Ca<sup>++</sup> flux assays (43) to evaluate the ability of group 2 HA stem immunogens to activate B cells expressing IgM BCRs comprising inferred UCA forms of the human, cross-group-reactive, HA stem-directed bNAbs from two multidonor classes (12). Germline targeting with vaccines designed to initiate specific antibody lineages is a new concept being tested with HIV antigens and not yet proven as a way of achieving broad immunity in humans. Even when transgenic animals are used for preclinical evaluation, the artificial constraints imposed may lessen correlations with the human immune system. Furthermore, since this type of strategy is intended to enhance *de novo* antibody responses, it may be particularly difficult to evaluate in preimmune adults and may require clinical evaluation in naive children to demonstrate proof of concept.

Consistency with the BLI binding results informed the choice to focus on immunogens H3ssF\_C, H3ssF\_H, and H7ssF\_C, each of which was designed to have two outwardly facing surface Glu's (at positions *i* and *i* + 3) at the C terminus of helix A. The first Glu occurs at a position (HA2 57) that naturally has a Glu in most human H3 strains from before 2002 and the majority of human H7 and H10 strains, although it is not in a helical conformation in full-length HA. Modeling with CT149 suggests the two Glu residues might interact with light-chain CDRL1 and CDRL2 loops (Fig. S9).

The observation that H3ssF and H7ssF variants were able to activate inferred UCA versions of cross-reactive BCRs suggests that these immunogens could potentially induce broad cross-group multidonor antibodies in humans. Due to their broad heterosubtypic breadth (12, 14, 15), such antibodies could potentially protect against pandemic strains, in addition to drifted seasonal strains, and preclude the need for annual vaccine reformulations. Furthermore, group 2 HA immunogens based on conventional H7N9 monovalent split product formulations have been shown to induce more diverse lineages of cross-group-reactive HA stem antibodies than conventional group 1 H5N1 immunogens in humans (13). Immunological imprinting from earlier or first exposures has also been suggested to play a significant role in shaping the subsequent immune response to influenza virus (45, 46). Thus, in influenza virus-naive individuals, the order of antigen priming may influence the composition of the influenza virus-specific B cell repertoire. Current vaccine approaches combine multiple strains of influenza virus in a single formulation. While this may be an effective strategy in adults with preexisting immunity from prior infections and vaccinations, it is possible that using an influenza A virus group 2 HA stem-based vaccine as the primary antigen exposure in children could potentially imprint or prime for broader cross-group responses later in life. Moreover, since titers of anti-HA stem antibodies increase with age and are inversely correlated with influenza virus infection rates, any early robust induction of broad, cross-reactive HA stem antibodies has the potential to mitigate the relatively high incidence of severe influenza disease in children, in addition to augmenting protection from influenza later in life (47). For adults and the elderly who have likely already encountered influenza virus, the group 2 stem immunogens could selectively boost antibody responses with greater breadth by not engaging otherwise predominant group 1 HA stem-specific HV1-69 antibodies (48, 49). These group 2 immunogens complement our earlier development of group 1 HA stem immunogens,

which were demonstrated to elicit within-group heterosubtypic protection in mice and ferrets (17). Group 1 HA stem immunogens could potentially be used as a boost for the group 2 HA stem immunogens to better focus the antibody response to the neutralization-sensitive epitope centered on helix A. Finally, group 1 and 2 HA stem immunogens could also be given together in a manner analogous to the current multivalent inactivated influenza vaccines (IIV).

Another approach for inducing an HA stem antibody response involves immunizing sequentially with heterologous chimeric HA immunogens comprising two or more HAs with the same stem but head regions from different subtypes. This strategy has elicited heterosubtypic group 1 and group 2 protection in animal models (50–53). However, the exotic HA heads may still be immunodominant and potentially interfere with or limit HA stem-specific responses, as it has been shown in H5 vaccine trials in humans that a transient vaccine-induced boost of the stem-directed antibody responses can be replaced by immunodominant head-directed responses over time (49, 54). From a practical clinical perspective, careful records would need to be maintained to ensure the correct order of administration of heterologous vaccine products.

Although bacterially produced HA stem-only immunogens from H1, H5, H3, and H7 subtypes have also been reported to protect mice from H3 or H7 influenza virus, complete protection has only been observed for some homotypic strains (55–57), indicating that the breadth of response is limited. The immunogens in our study more closely mimic the native H3N2 and H7N9 HA stems in that they include native N-linked glycans as well as the membrane-proximal region of the HA stem that are both missing in the bacterially expressed designs. Eukaryotic expression of HA stem immunogens may be of particular importance because each native HA stem trimer has nine glycans and glycans have been observed to guide antibody binding by either masking certain epitopes (58, 59) or participating as part of an epitope surface (60). Indeed, many cross-group-reactive HA stem-directed bNAbs are immediately proximal to the HA2 N38 glycan when recognizing group 2 HA (12). Interestingly, the HA stem-directed bNAbs most frequently elicited from group 1 influenza virus, which does not have N38 glycans, are of the HV1-69 class (48), which is generally incompatible with the N38 glycan, and therefore, they are not cross-group reactive. Thus, group 2 HA stem immunogens bearing native glycans may provide a selective advantage for eliciting broad influenza virus antibody responses. However, to date, no experimental data have shown glycosylated hemagglutinin immunogens to be more effective than nonglycosylated versions. A recently reported particulate vaccine comprising nonglycosylated HA stem cross-linked to desolvated tandem M2e segments has been shown to elicit broad cross-group protection in mice (61). However, these particles comprised proteins derived from multiple host species, strains, and subtypes of influenza virus with the goal of accumulating breadth, as opposed to the approach used herein of attempting to achieve breadth through targeting conserved antigenic supersites.

In summary, we have designed group 2 headless HA stem trimers with conformational and antigenic fidelity to native HA. These antigens were immunogenic in mice and provided protection against a lethal homosubtypic challenge. Since mice lack critical genetic elements found in human bNAbs targeting the HA stem, we used a B cell receptor activation assay to demonstrate the potential for eliciting broadly reactive human antibody responses. Based on activation of B cells expressing inferred UCA versions of human antibodies associated with broad cross-neutralizing activity, candidate vaccines with antigens displayed on self-assembling ferritin nanoparticles are now being manufactured for clinical evaluation. Additional animal studies will be performed in parallel, including testing in mice expressing specific human immunoglobulin genes and in nonhuman primates. These immunogens represent a step toward achieving the goal of a vaccine that can elicit protective immunity against drifted seasonal and pandemic strains of influenza A virus.

## MATERIALS AND METHODS

**Structure-based design.** The crystal structure of A/Finland/486/2004 (H3N2) HA (PDB ID 2YP2) was used as a template for subtype H3, and the crystal structure of A/Shanghai/2/2013 (H7N9) HA (PDB ID 4LN6) was used as a template for subtype H7. The complete sequences for A/Finland/486/2004 HA and A/Shanghai/2/2013 (H7N9) HA, including the signal sequence regions not included in the crystal structures, were obtained from the database records with GISAID accession number EPI397685 and GenBank accession number YP\_009118475.1, respectively. The changes in free energy from point mutations were estimated using the program DDG\_MONOMER (26) from the ROSETTA suite. Loops were modeled with LOOPY (62), superpositions were performed using UCSF Chimera (63) or LSQMAN (64), and energy minimization of final models was performed using RELAX (65) from the ROSETTA suite. The graphics programs PyMOL (66) and UCSF Chimera (63) were used for detailed visual inspection of structures and the generation of structural figures.

**Phylogenetic tree generation for HA sequences.** Maximum-likelihood trees were created using the program MEGA7.0 (67) from 18 representative HA sequences (13) aligned with MUSCLE (68). The stem regions of HA sequences were defined as residues HA1 10 to 42 and 314 to 329 and HA2 1 to 59 and 93 to 174 (H3 numbering [21]).

**Antigenic screening of nanoparticle immunogen designs.** Most designs were initially screened in a high-throughput ELISA format as described previously (23). However, the contributions of individual mutations within multiple-mutation designs were also assessed by expressing transmembrane versions of H3 HA stem containing the native C-terminal HA sequence starting with HA2 175 (SVELKSGYKDWILWISFAISCFLLCVALLGFMWACQKGNIRCNICI) on the surface of HEK293F cells and measuring recognition of bNAbs Fl6v3 and CR8020 using flow cytometry. Since HA stem was more readily expressed in this format, with less stabilization required, this strategy enabled a more sensitive evaluation of HA stem mutations than the nanoparticle format. Promising designs that exhibited greater Fl6v3 and CR8020 binding were subsequently expressed in the nanoparticle format for final evaluation.

**Expression and purification of immunogens and antibodies.** All H3ssF and H7ssF nanoparticles and Fl6v3, CT149, CR8020, CR6261, MEDI8852, 315-53-1F12, 54-1G07, 04-1D02, and 09-1B12 MAbs were expressed in 293 Expi cells (Life Technologies) and purified by affinity chromatography (*Galanthus nivalis* lectin for the nanoparticles and protein A for MAbs), using previously described methods (13, 17). Assembly of nanoparticles was assessed by gel filtration with a Superose 6 increase 10/300 GL column (GE Healthcare). To make MEDI8852 and CR8020 Fabs, IgG was digested with endoproteinase Lys-C (New England Biolabs) overnight at room temperature (RT). To make CT149 Fab, CT149 IgG containing an HRV-3C cleavage site engineered in the heavy-chain hinge region was digested using HRV-3C enzyme overnight at RT. The reactions were assessed by SDS-PAGE and, upon completion, were quenched by the addition of protease inhibitor cocktail (MilliporeSigma), and Fc and Fab antibodies were separated by protein A affinity chromatography.

**ELISA binding assays.** ELISAs were done as previously described (17). Briefly, ELISA plates were coated with 100 ng/well H1ssF, H3ssF, or H7ssF antigens overnight at 4°C. The plates were then incubated with IgG or Fab forms of MAbs with dilution series starting at 10 µg/ml. For Fab, anti-CH1 MAb was then bound at 10 µg/ml. For testing of mouse sera, H3 HA and H7 HA antigens represented A/Hong Kong/1/1968 HA and A/Anhui/1/2013, respectively. Mouse serum serial dilutions started at 1:50. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were used to detect primary antibody, followed by a colorimetric detection assay. Linear regression analysis of the absorbance values (optical density at 450 nm [OD<sub>450</sub>]) was completed using GraphPad Prism software. Serum endpoint titers were determined to be the serum dilution that resulted in a 4-fold increase in OD value above the background level. One-way analysis of variance (ANOVA) with Kruskal-Wallis posttest was used to compare mean endpoint titers between groups.

**Immunoprecipitation assay.** Magnetic protein G Dynobeads (Thermo Fisher) were used to capture Fl6v3 MAb according to the manufacturer's protocol and allowed to incubate for 1 h at RT. Fl6v3-bound Dynobeads were washed 2 times with 1× phosphate-buffered saline (PBS) and incubated with supernatants from H3ssF or H7ssF expressed in HEK293 cells or purified control protein for 1 h at RT. Beads were washed 2 times with 1× PBS and heated to 95°C for 10 min to detach bound protein. Proteins were then analyzed by SDS-PAGE.

**Biolayer interferometry binding assays.** All biosensors were hydrated in PBS prior to use. CR9114 IgG (10 µg/ml diluted in 1% bovine serum albumin [BSA]-PBS) was immobilized on AHC biosensors through conjugated anti-human Fc antibody (fortéBio). After briefly dipping in assay buffer (1% BSA-PBS), the biosensors were dipped in various H3ssF and H7ssF constructs (10 µg/ml) to capture nanoparticles. The biosensors were then equilibrated in assay buffer for 1 min before being dipped in a 2-fold dilution series of CR8020, CT149, or MEDI8852 Fab for 5 min, followed by dipping in assay buffer to allow Fab to dissociate from nanoparticles for 5 min. All assay steps were performed at 30°C with agitation set at 1,000 rpm in the Octet HTX instrument (fortéBio). Correction to subtract a baseline drift was carried out by subtracting the measurements recorded for a sensor incubated with no Fab. Data analysis and curve fitting were carried out using Octet analysis software (version 9.0) as previously described (69, 70). Experimental data were fitted with the binding equations describing a 1:1 interaction. Global analyses of the complete data sets assuming binding was reversible (full dissociation) were carried out using nonlinear least-squares fitting, allowing a single set of binding parameters to be obtained simultaneously for all concentrations used in each experiment.

**Electron microscopy analysis.** Samples were diluted to ~0.02 mg/ml with buffer containing 10 mM HEPES, pH 7.0, and 150 mM NaCl and adsorbed to freshly glow-discharged carbon film grids for 15 s. The grids were washed with the same buffer and stained with 0.7% uranyl formate. Images were collected



at a nominal magnification of 50,000 semiautomatically using SerialEM (71) on an FEI Tecnai T20 transmission electron microscope operated at 200 kV and equipped with a 2,048- by 2,048-pixel Eagle charge-coupled-device (CCD) camera. The pixel size was 0.44 nm. Particles were picked automatically using software developed in-house (Y.T., unpublished data). Particle alignment and reference-free 2-D classification were performed with SPIDER (72) and Relion 1.4 (73).

**DSC.** Thermal melting points were determined using a MicroCal VP-capillary differential scanning calorimeter (DSC; Malvern Instruments). All protein samples were in the range of 0.2 to 0.5 mg/ml and were extensively dialyzed against PBS. Thermal denaturation was probed at a scan rate of 60°C/h from 30 to 120°C. Buffer correction, normalization, and baseline subtraction procedures were applied before the data were analyzed using the VP-capillary DSC automated data analysis software.

**Mouse immunizations.** Animal experiments were carried out in compliance with all pertinent U.S. National Institutes of Health regulations and policies. The Animal Care and Use Committee of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, reviewed and approved all animal experiments. Female BALB/cJ mice aged 6 to 8 weeks (Jackson Laboratory) were immunized ( $n = 10$ ) with 2  $\mu$ g H3ssF or H7ssF and adjuvanted with the Sigma adjuvant system at 0, 4, and 8 weeks. Mice were inoculated with 100  $\mu$ l intramuscularly, given as 50  $\mu$ l into each hind leg. Two weeks after the final immunization, sera were collected for measurement of antibody responses. When appropriate, challenges were performed at 4 to 8 weeks postimmunization. Mice were inoculated intranasally with a  $10 \times 50\%$  lethal dose ( $LD_{50}$ ) of H3N2 A/Philippines/2/1982 or a  $20 \times LD_{50}$  of H7N9 A/Anhui/1/2013. Weight loss was recorded daily for 14 days postchallenge.

**Pseudovirus neutralization assays.** Lentivirus-based pseudoviruses displaying influenza HA and NA were produced as previously described (17). Mouse serum was treated with receptor-destroying enzyme (Denka Seiken). Serial dilutions of mouse sera (4-fold, starting point 1:40, 8 dilutions) were mixed with either H3N2 A/Wisconsin/67/2005 or H7N9 A/Anhui/1/2013 pseudovirus (whose titers to target 50,000 relative luciferase units [RLU] were previously determined) for 30 min at RT. The virus-pseudovirus mixture was then added to previously plated 293A cells (ATCC) in triplicate. After 2 h at 37°C and 5%  $CO_2$ , fresh Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin-streptomycin was added. Cells were lysed at 72 h, and luciferase substrate (Promega) was added. Luciferase activity was measured as RLU at 570 nm on a SpectramaxL (Molecular Devices). Sigmoidal curves, taking averages of triplicates at each dilution, were generated from RLU readings; 50% neutralization (50% inhibitory concentration [ $IC_{50}$ ]) titers were calculated considering uninfected cells as 100% neutralization and cells transduced with only virus as 0% neutralization. One-way ANOVA with Kruskal-Wallis posttest was used to compare mean  $IC_{50}$  titers between groups.

**Determination of inferred UCAs for HA stem antibodies.** Paired heavy- and light-chain DNA sequences for 58 members of the 54-1G07 clonal family (315-54-1G07) were isolated from single  $IgG^+$  B cells, along with 6 additional heavy-chain DNA sequences from cells for which light chains could not be amplified (13). A maximum-likelihood phylogenetic tree was constructed from concatenated heavy- and light-chain sequences and the most recent common ancestor (MRCA) of the lineage was inferred using DNAML in SONAR (74). This MRCA had 5 residual nucleotide mutations each in the heavy and light chain (resulting in 2 and 5 amino acid changes, respectively) compared to the sequences of the assigned germ line VH and VK genes. To construct the final UCA, these changes were reverted to the presumed germ line sequence. The same procedure was used for 57 paired heavy- and light-chain DNA sequences and 8 unpaired heavy-chain DNA sequences from the 09-1B12 clonal family. This MRCA had 1 residual nucleotide mutation (1 amino acid change) in the heavy chain and no residual mutations in the light chain. For the 04-1D02 clonal family, 29 paired heavy- and light-chain DNA sequences and 1 unpaired heavy-chain DNA sequence were used. The inferred MRCA contained 4 residual nucleotide mutations in the heavy chain and 6 nucleotide mutations in the light chain (4 and 5 amino acid changes, respectively). For this clonal family, only changes in VH genes were reverted; the lambda chain of 04-1D02-MRCA was used with the residual mutations included.

Ninety-three heavy-chain and 83 light-chain sequences were identified for the 16.a.26 clonal family and 51 heavy-chain and 49 light-chain sequences for the 01.a.44 clonal family from single sorted  $IgG^+$  B cells. For the heavy chain, a maximum-likelihood phylogenetic tree based upon the V gene sequence alone was constructed using Geneious (75), with each tree rooted to the respective germ line reference sequence as listed in the IMGT database. The VDJ junction of clonal members with the lowest mutation loads was inferred using IMGT V-quest, allowing identification of residues within the V, D, and J gene segments most likely subject to somatic mutation. To construct the heavy-chain UCA, these changes were reverted to the presumed germ line sequences. Due to the lack of precision in estimating N-nucleotide addition, mutations falling at V/D or D/J junctions were not reverted. For the light chains, an analogous process was used for V and J gene segments.

**Generation of B cell lines.** B cell lines expressing inferred UCA versions of monoclonal antibodies were generated by transduction of surface-IgM-negative Ramos cells as previously described (43). Briefly, DNA encoding VDJ (heavy-chain) and VJ (light-chain) immunoglobulin regions was synthesized by GenScript and cloned, along with the consensus human IgM C region, into the pLVX-ZsGreen and the pLVX-mCherry expression vector (both from Clontech), respectively. Each of these vectors was then cotransfected with the lentivirus packaging plasmid psPAX2 and the vesicular stomatitis virus envelope protein G (VSV-G)-expressing plasmid pMD2.G (both from Addgene) into HEK293T cells for formation of lentiviral particles using Lipofectamine 2000 (ThermoFisher). Supernatant was harvested 3 days after transfection and cleared by centrifugation before IgM-negative Ramos cells were cotransduced for both heavy and light chains. After 5 days, transduced Ramos cells were enriched by cell sorting for the expression of ZsGreen and mCherry using a FACSAria II interfaced to the FACSDiva software (BD

Biosciences). This procedure was repeated until a pure double-positive population could be selected. Cell lines were further enriched for high IgM surface expression by using a fluorescently labeled anti-human IgM monoclonal antibody.

**B cell activation assays.** The ability of the different particles to induce calcium flux upon BCR engagement was measured *in vitro* using inferred UCA monoclonal antibodies expressed as surface IgM in Ramos cells as previously described (76). Briefly, 1 million cells (per test) were stained with 0.35  $\mu$ l fura red (Thermo Fisher Scientific) in 100  $\mu$ l serum-free medium at ambient temperature in the dark for 30 min. After washing, cells were resuspended in 300  $\mu$ l serum-free medium and heated to 37°C for 3 to 5 min in a heated water bath before being acquired on a FACSymphony interfaced to the FACSDiva software (BD Biosciences). Cells were first acquired for 30 s in the absence of antigen to record baseline intracellular calcium levels, and then tubes were removed (leaving the acquisition in progress) to add the antigen and quickly vortexed before being placed back on acquisition for a total time of 180 s. Nanoparticles were used at the final concentration of 50 nM. An anti-human unlabeled mouse F(ab')<sub>2</sub> anti-IgM (Southern Biotech) was used (1.5  $\mu$ g/test) to determine the maximal induction of calcium flux measured by the change between the emission of fura red bound to calcium and that of unbound fura red (ratio) over time. The inferred UCA for 01.a.44 was chosen as a negative control since 01.a.44 is observed to bind only to group 1 HAs and not to group 2 HAs (not shown). Likewise, the inferred UCAs for 54-1D07 and 16.a.26 are observed to recognize group 2 HAs only (data not shown).

**Accession number(s).** The sequences for the inferred UCA versions of the following HA stem antibodies have been deposited into GenBank under the accession numbers in parentheses: 54-1G07 (MH631450 and MH631451), 04-1D02 (MH997406 and MH997407), 09-1B12 (MH997404 and MH997405), 01.a.44 (MK291363 and MK291365), and 16.a.26 (MK291364 and MK291366).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02810-18>.

**FIG S1**, PDF file, 2.3 MB.

**FIG S2**, PDF file, 0.04 MB.

**FIG S3**, PDF file, 0.2 MB.

**FIG S4**, PDF file, 1.0 MB.

**FIG S5**, PDF file, 0.9 MB.

**FIG S6**, PDF file, 0.8 MB.

**FIG S7**, PDF file, 0.4 MB.

**FIG S8**, PDF file, 0.04 MB.

**FIG S9**, PDF file, 0.7 MB.

**TABLE S1**, XLSX file, 0.04 MB.

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J.C.B., K.S.C., S.M.M., H.M.Y., M.K., L.W., J.R.M., and B.S.G. are named inventors on pending patent applications involving influenza vaccines.

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