1   **Design of nanoparticulate group 2 influenza hemagglutinin stem antigens that activate**

2   **unmutated ancestor B cell receptors of broadly neutralizing antibody lineages**

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17   Running Head: Group 2 influenza HA stem antigen design

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29

30 **Abstract**

31 Influenza vaccines targeting the highly-conserved stem of the hemagglutinin (HA) surface

32 glycoprotein have the potential to protect against pandemic and drifted seasonal influenza viruses

33 not covered by current vaccines. While HA stem-based immunogens derived from group 1

34 influenza A have been shown to induce intra-group heterosubtypic protection, HA stem-specific

35 antibody lineages originating from group 2 may be more likely to possess broad cross-group

36 reactivity. We report the structure-guided development of mammalian cell-expressed candidate

37 vaccine immunogens based on influenza A group 2 H3 and H7 HA stem trimers displayed on

38 self-assembling ferritin nanoparticles using an iterative, multipronged approach involving helix

39 stabilization, loop optimization, disulfide bond addition, and side chain repacking. These

40 immunogens were thermostable, formed uniform and symmetric nanoparticles, were recognized

41 by cross-group-reactive broadly neutralizing antibodies (bNAbs) with nanomolar affinity, and

42 elicited protective, homosubtypic antibodies in mice. Importantly, several immunogens were

43 able to activate B cells expressing inferred unmutated common ancestor (UCA) versions of

44 cross-group-reactive human bNAbs from two multi-donor classes, suggesting they could initiate

45 elicitation of these bNAbs in humans.

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49 **Importance**

50 Current influenza vaccines are primarily strain specific, requiring annual updates and offer

51 minimal protection against drifted seasonal or pandemic strains. The highly conserved stem

52 region of hemagglutinin (HA) of group 2 influenza A subtypes is a promising target for vaccine

53 elicitation of broad cross-group protection against divergent strains. We used structure-guided

54 protein engineering employing multiple protein stabilization methods simultaneously to develop

55 group 2 HA stem-based candidate influenza A immunogens displayed as trimers on self-

56 assembling nanoparticles. Characterization of antigenicity, thermostability, and particle

57 formation confirmed structural integrity. Group 2 HA stem antigen designs were identified, that

58 when displayed on ferritin nanoparticles activated B cells expressing inferred unmutated

59 common ancestor (UCA) versions of human antibody lineages associated with cross-group

60 reactive, broadly neutralizing antibodies (bNAbs). Immunization of mice led to protection

61 against a lethal homosubtypic influenza challenge. These candidate vaccines are now being

62 manufactured for clinical evaluation.

63

64 **INTRODUCTION**

65   Influenza continues to be a significant global health burden, typically resulting in about

66 500,000 deaths worldwide annually (1), even though the technology for conventional egg-grown,

67 whole inactivated influenza virus vaccines was developed more than 70 years ago. Constant

68 antigenic drift of the influenza virus hemagglutinin (HA) coupled with immunodominant strain-

69 specific antibody responses directed to the variable HA head domain results in conventional

70 vaccine effectiveness ranging from 10-60% (2) and the need for seasonal updates of virus strains

71 included in licensed vaccines. Furthermore, current vaccine approaches provide minimal

72 protection against pandemic influenza strains (3, 4). Improved influenza vaccines would not be

73 produced in eggs, would be designed to induce broad immunity against future drifted and

74 pandemic strains without seasonal reformulation, and elicit durable immune responses avoiding

75 the need for annual vaccination (4). One approach for achieving broadly cross-reactive

76 influenza-specific antibodies is to target highly conserved neutralization-sensitive epitopes in the

77 stem region of the influenza A HA surface glycoprotein (5). Monoclonal antibodies have been

78 identified that bind conserved HA stem epitopes and possess broad neutralizing activity across

79 diverse HA subtypes within an influenza A group, and some that have cross-group neutralizing

80 activity (6-16). We and others have recently developed influenza A group 1 HA stem trimer

81 antigens (17, 18). One candidate displaying H1-based HA stem trimers on self-assembling

82 ferritin nanoparticles protected mice and ferrets from a lethal heterosubtypic H5N1 influenza

83 challenge (17). However, these group 1 HA stem immunogens did not provide cross-group

84 protection against group 2 viruses such as H3N2 and H7N9. The need for a better group 2

85 vaccine is particularly acute as vaccine effectiveness against H3N2 over the past decade has

86 averaged only 33% (19), recent H3N2 strains have exhibited increased virulence (20), and H7

5

87   viruses represent one of the greatest pandemic threats from non-seasonal strains. H7N9

88   immunization was recently shown to induce multiple HA stem-directed antibody lineages in

89   humans that recognized both group 1 and group 2 HA molecules, whereas H5N1 immunization

90   induced primarily HV1-69 antibody lineages that cross-reacted primarily within group 1

91   influenza A subtypes (13). This suggests that group 2 HA stem immunogens may be more

92   effective at inducing cross-group responses than group 1 immunogens in humans with pre-

93   existing influenza immunity, or priming multi-donor antibody lineages with the capacity for

94   broad neutralizing activity in influenza-naïve infants. In our studies, we used iterative structure-

95   based design to develop headless group 2 HA stabilized stem trimers displayed on ferritin

96   nanoparticles based on hemagglutinin sequences from H3N2 (H3ssF) and H7N9 (H7ssF) viruses

97   as candidate vaccines. Notably, structural subtleties ultimately required the use of a completely

98   different set of design strategies to stabilize group 2 HA stem immunogens relative to simpler

99   hydrophobic repacking used to stabilize group 1 immunogens. Ultimately, H3ssF and H7ssF

100   immunogen designs were identified that could activate B cells expressing inferred unmutated

101   common ancestor (UCA) HA stem-directed human broadly neutralizing antibodies (bNAbs),

102   suggesting the potential for inducing cross-reactive antibody lineages with the capacity to

103   develop broad influenza neutralizing activity in humans.

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105   **RESULTS**

106   **Design of group 2 stabilized HA stem nanoparticle immunogens**

107   Designing group 2 stabilized HA stem nanoparticles incorporated some features of group

108   1 H1-stabilized HA stem nanoparticle immunogens (17) (referred to as H1ssF herein). This

109   comprised replacing the HA head region (residues HA1 43-313, H3 numbering (21)) with a GSG

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110   loop, replacing the HA2 interhelical region (residues 60-92) with a six-residue glycine-rich loop,

111   including two repacking substitutions in the HA2 hydrophobic core (K103M/E51L), and

112   connecting the C-terminal HA2 residue 174 to the N-terminal residue 5 of bacterial ferritin with

113   a short linker (Fig. 1A and Figs. S1-2). In the group 1 context this strategy was sufficient for

114   displaying antigenically correct H1, H2 and H5 HA stem trimers on ferritin nanoparticles (17).

115   However, multiple additional steps were needed to achieve expression of group 2 H3 and H7 HA

116   stem nanoparticles in mammalian HEK293 cells (Fig. 1). Although there is structural similarity

117   between H1 and H3 HA stem regions (e.g. root mean square deviation (RMSD) of 1.0 Å for 134

118   stem residues between A/California/04/2009 (H1N1), PDB 3UBQ and A/Victoria/361/2011

119   (H3N2), PDB 4O5N) and sequence identity of approximately 55%, phylogenetic analysis

120   indicates groups 1 and 2 comprise two distinct branches with consistent and predictable amino

121   acid differences even for the stem region of HA (Fig. 1B). We hypothesized that further

122   structural stabilization was required for expression and therefore, an iterative structure-based

123   design effort was employed to stabilize the HA stem region of H3ssF immunogens using the 1.9

124   Å resolution crystal structure of HA for A/Finland/486/2004 (H3N2), (PDB 2YP2) (22) as a

125   template. To screen for expression and correct antigenicity, designs were expressed in HEK

126   293T cells using a high-throughput 96-well format (23). Supernatants containing HA stem

127   trimers genetically fused to ferritin nanoparticles were assessed directly for antigenic recognition

128   by either an immunoprecipitation assay using the human cross-group, HA stem-specific bNAb

129   FI6v3 or by an ELISA using the bNAbs CR9114, FI6v3, and CT149. CT149 was specifically

130   included to assess trimer integrity because its epitope incorporates two adjacent protomers of the

131   HA stem and thus may be sensitive to quaternary structure (15).

132   Since internal hydrophobic repacking mutations were critical for the stabilization of the

133   group 1 HA stem immunogens (17), and the burying of just a single -CH_2- group in a protein

134   interior can add approximately 1.1 kcal/mol of free energy to protein stability (24, 25), we

135   explored additional repacking mutations selected computationally using Rosetta (26). In

136   combination with some of the repacking mutations we also explored limited residue swapping

137   between H1N1 and H3N2 subtypes (Table S1). Surprisingly, none of these 174 mutants could be

138   expressed and recognized by bNAbs CR9114 or FI6v3 (Table S1), indicating novel approaches

139   for structural stabilization would be required.

140   Upon further investigation of multiple protein stabilization strategies involving another

141   76 designs (Table S1), a lead design was successfully expressed (H3ssF_A), that was recognized

142   by bNAb HA stem antibody FI6v3 by immunoprecipitation, and self-assembled into

143   nanoparticles (Table 1, Table S1, and Figs. S2-3). H3ssF_A included the initial H1ssF-based

144   mutations in addition to: i) helix stabilization, ii) loop optimization, and iii) point mutations of

145   select H3 residues to improve internal packing (Figs. 1A and 1C and Fig. S2A). Each of the

146   successful alterations were focused on the membrane distal end of the two central helices (A and

147   C) of the HA2 fold. The outer helix A in H3N2 HA is approximately five residues shorter at its

148   C-terminus than in H1 HA and it is known that helix length is related to stability (27). Therefore,

149   helix A was extended at its C-terminus by five residues (ALMAQ) with high helix-forming

150   propensities (28). Likewise, the N-terminus of the adjacent helix C was stabilized by adding an

151   N-terminal Asp (HA2 92) as a helix-capping residue (29, 30) and two other mutations (HA2

152   S93A and N95L) at the N-terminus of helix C were made to increase its helix-forming

153   propensity and improve interprotomer packing (Fig. 1C). Biophysical studies have shown that

154   excess loop length can destabilize protein folds by up to several kcal/mol (31), and in the initial

155   H1ssF and H3ssF designs, helices A and C were connected by a six-residue linker even though

156   they are separated by only 12 Å. Therefore, the glycine-rich linker was shortened to a four

157   residue GGPD linker to more closely match the interhelical distance. Lastly, four residues (HA2

158   52, 55, 107 and 116) on helices A and C respectively, were mutated to improve side chain

159   packing (Fig. 1C and Fig. S2A). Three of these (HA2 52, 55 and 116) comprised mutations to

160   H1 residues.

161   To further improve expression and stability, another 45 designs were explored, focusing

162   on the short β-hairpin connected to a three-residue loop which replaced the HA head in HA1

163   (Fig. 1). Since the stability of short, two-stranded β-sheets has been observed to increase with

164   length (32), we replaced the GSG loop with longer loops of 6-7 residues designed to double the

165   hairpin length. Residues with high strand-forming propensities (28) such as Val, Ile and Phe

166   were used for strand extension and dipeptides with propensities for form β-turns (33, 34) such as

167   PG and NG were used to connect them. Disulfides were also designed into the hairpin loops for

168   added stability (35). From an initial survey of loop extensions, H3ssF_B, designed as a 15-

169   residue hairpin loop disulfide-bonded to the N-terminus of helix C resulted in expression levels

170   of greater than 5 mg/L and recognition by CT149 (Fig. 1, Table 1 and Table S1).

171   Further loop optimization of the H3ssF_B design included removing a glycine from

172   either side of the cysteine in the β-hairpin loop (H3ssF_D and H3ssF_E) and incorporating a

173   different β-hairpin sequence with an internal disulfide bond engineered to link both strands

174   together (H3ssF_G). Additional H3ssF_B variants incorporated hydrophilic, helix preferring Glu

175   into the relatively hydrophobic helix A extension motif for improved solubility (H3ssF_C,

176   H3ssF_F and H3ssF_H), and an engineered intra-protomer disulfide between HA1 residues 23

177   and 332 (H3ssF_H). All six variants resulted in nanoparticles recognized by CT149 in ELISA

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178   assays, but did not improve overall expression beyond that of H3ssF_B. Based on the successful

179   expression and antigenic recognition of multiple H3ssF variants, we transferred mutations from

180   constructs H3ssF_B, H3ssF_C, H3ssF_D, H3ssF_E, and H3ssF_F into analogous H7ssF

181   nanoparticles using sequences from the human H7N9 strain (A/Shanghai/2/2013) and also

182   created two additional loop variants (H7ssF_I and H7ssF_J) (Fig. S2B). All seven of these H7ssF

183   designs expressed milligram per liter quantities of nanoparticles recognized by CT149 (Table 1).

184

185   **Physical characterization of H3ssF and H7ssF nanoparticles**

186   Fourteen nanoparticles were selected for further characterization. These were purified by

187   lectin chromatography followed by size exclusion chromatography, which resulted in distinct

188   peaks consistent with approximately 1.2 MDa particles (Fig. 2A-B and Figs. S4). Negative stain

189   electron microscopy of purified nanoparticles followed by reference-free 2D classification and

190   averaging revealed spherical particles with five to six visible regularly-spaced protruding spikes

191   (Figs. 2C-D, 2E-F and Figs. S4). All nanoparticles contained ferritin cores consistent with the

192   expected 12-13 nm diameter, and HA stems with the expected length of approximately 7 nm

193   (Table 1).

194

195   **Thermostability of H3ssF and H7ssF nanoparticles**

196   We next investigated immunogen thermostability using differential scanning calorimetry

197   (DSC). DSC measurements revealed thermal melting temperatures (T_m) for the earliest

198   transitions ranging from 56.6 to 66.6°C for H3ssF variants and 56.7 to 61.6°C for H7ssF variants

199   (Table 1 and Fig. S5A-B). Three other higher temperature transitions were consistently observed

200   at approximately 78, 96, and 106°C for all the immunogens tested. In contrast, the ferritin

201   nanoparticle alone had a primary transition at 105.7°C and a minor one at 84°C. Since the

202   earliest transitions were most variable, we hypothesized that these were related the HA stem

203   structures.

204   The three H3ssF designs with the highest T_ms of 66.2-66.6°C had either the seven residue

205   β-hairpin extension disulfide-bonded to helix C (H3ssF_B and H3ssF_C) or the same loop with

206   one glycine removed just after the cysteine (H3ssF_D). Glycine removal just prior to the cysteine

207   (H3ssF_E) was less well tolerated thermodynamically. Curiously, H3ssF_H with the same

208   hairpin loop as H3ssF_B and H3ssF_C, but designed to contain an additional disulfide bond to

209   stabilize the HA1 region of the stem had the lowest T_m of 56.6°C, indicating this variant had less

210   than optimal stability. Design H3ssF_G, engineered to have an internal disulfide bond in the β-

211   hairpin extension had a T_m temperature 6°C lower than H3ssF_B and H3ssF_C. Incorporation of

212   Glu into the two Ala positions in the five-residue helix A extension had little effect on stability

213   (H3ssF_C), but Glu incorporation to replace the last two residues decreased the T_m by 1°C

214   (H3ssF_F). For the five immunogen designs (B-F) made in both H3 and H7 formats, the T_m

215   values were on average 5.9°C higher for the H3ssF relative to H7ssF designs and similar trends

216   were observed between designs. Finally, two additional H7ssF variants not represented in H3ssF,

217   with an Asn incorporated in the HA1 β-hairpin extension (H7ssF_H) and a Pro incorporated into

218   the HA2 GGP loop (H7ssF_I), did not significantly alter the T_m. While improved thermostability

219   will facilitate vaccine transport and storage, increasing rigidity and stability of antigens can

220   sometimes be counterproductive for immunogenicity (36). Therefore, multiple designs with

221   varying levels of DSC-determined stability were advanced for further evaluation.

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223   **Antigenicity of H3ssF and H7ssF nanoparticles**

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224   Biolayer interferometry (BLI) was used to measure binding kinetics and affinities of

225   nanoparticles to antigen-binding fragments (Fabs) of bNAbs MEDI8852, CT149 and CR8020

226   (Table 2 and Figs. S6-S7). To avoid avidity effects, the nanoparticles were immobilized to anti-

227   Fc-decorated sensors using the bNAb CR9114 IgG and monovalent Fabs were used as the

228   analyte. CR9114 Fab was observed to recognize H3ssF and H7ssF immunogens with affinities of

229   94 and 48 nM respectively (Table 2 and Fig. S6E). However, given the bivalent nature of the

230   CR9114 IgG and the 24 epitope sites on each nanoparticle, the avidity of this was complex was

231   more than enough to stably immobilize nanoparticles to the sensor for measurements of high

232   affinity bNAb Fabs.

233   The first two antibodies (MEDI8852 and CT149) recognize similar HA stem epitopes

234   centered on the HA2 helix A and fusion peptide loop supersite and represent two multi-donor

235   classes of cross-group bNAbs (HV6-1+HD3-3 class and HV1-18 QxxV class, respectively)

236   recently identified in healthy human adults vaccinated with H5N1 (12, 14, 15). CR8020 is a

237   group 2 specific bNAb that recognizes a less conserved, more membrane proximal HA stem

238   epitope (11). The HV6-1+HD3-3 class monoclonal antibody (mAb) MEDI8852 recognized all

239   H3ssF and H7ssF nanoparticles as well as full length H3 and H7 HA trimers with similar

240   nanomolar affinities and kinetics (Table 2 and Fig. S6A-B). The HV1-18 QxxV class mAb

241   CT149 also recognized all H7ssF nanoparticles with approximately 1-10 nanomolar affinity. Of

242   these, H7ssF_C was recognized with approximately 10-fold greater (0.9 nM) and a 10-fold

243   slower off rate ($5.3 \times 10^{-4} s^{-1}$) than the other H7ssF nanoparticles. However, among the H3ssF

244   nanoparticles, only H3ssF_C and H3ssF_H were recognized by CT149, with affinities of 21.3-

245   22.2 nM; the other four H3ssF immunogens showed no measurable affinity to CT149 (Table 2

246   and Fig. S6). This was surprising since each of these designs were initially chosen based on

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247   strong binding to CT149 in ELISA assays. Furthermore, ELISAs performed on purified H3 and

248   H7 immunogens for designs B and C also indicated high affinity to each of the group 2-reactive

249   HA stem antibodies tested (Fig. S7A). This apparent disparity between BLI and ELISA was

250   potentially due to utilization of bivalent IgGs in ELISAs as opposed to monovalent Fabs in BLI

251   assays, which provides an avidity advantage for apparent binding affinity of up to two orders of

252   magnitude (37-39). Consistent with this idea, we observed in ELISAs that the Fab form of

253   CT149 did not bind to H3ssF_B or H3ssF_C and bound only modestly to H7ssF_B and H7ssF_C

254   (Fig. S7B). Moreover, ELISAs used a higher density of immobilized antigen than BLI, and there

255   are inherent differences in the methodologies of the two assays. Full length H3 and H7 HA

256   trimers were observed to bind CT149 with affinities and kinetics consistent with the C and H

257   versions of the H3ssF and H7ssF nanoparticles (Fig. S6C-D). Interestingly, the nanoparticles

258   with greatest affinity for CT149, H3ssF_C, H3ssF_H and H7ssF_C, were each designed to have

259   two outwardly facing Glu residues at the C-terminal end of helix A whereas the other designs

260   had Ala at these positions.

261   The group 2-specific HA stem bNAb CR8020 recognized all of the tested H3ssF

262   nanoparticles with affinities of 17.9-23.3 nM, but did not recognize any of the H7ssF

263   nanoparticles (Table 2 and Fig. S7C). This is consistent with the previously reported lack of

264   CR8020 binding to full length A/Shanghai/2/2013 (H7N9) HA, the same strain used in the

265   H7ssF designs (40). The CR8020 epitope is not completely conserved even within group 2 HAs

266   as there are six epitope mutations between the A/Hong Kong/1/1968 (H3N2) HA and

267   A/Shanghai/2/2013 (H7N9) HA.

268   Newly recombined, naïve B cell receptors (BCRs) need to first recognize an immunogen

269   to initiate affinity maturation and B cell proliferation (41). Therefore, we also used BLI to assess

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270   the relative affinities of three sets of mature stem bNAbs and their inferred UCAs to HA stem

271   nanoparticle immunogens (Fig. S7D). The UCAs were each inferred phylogenetically from

272   multiple clonal family members and include unmutated CDRH3 loops (Fig. S8). Two of these

273   (54-1G07 and 09-1B12) were from the human multi-donor HV6-1+HD3-3 class and HV1-18

274   QxxV class, respectively (13). The third antibody, 04-1D02 was from an HV3-53+HD3-3

275   lineage. Measurable H3ssF and H7ssF immunogen recognition by inferred UCAs for each of the

276   three antibodies was not considerably different from the binding of mature forms of the

277   antibodies or the CR9114 positive control (Fig. S7D). This is interesting considering that there

278   are 2-5 unmutated CDRH3 residues in these UCAs (Fig. S8) and crystal structures of mAb

279   complexes with HA show considerable CDRH3 binding for members of the HV1-18 QxxV and

280   HV6-1 HD3-3 multi-donor antibody classes (12, 14, 15).

281   **Immunogenicity of H3ssF and H7ssF nanoparticles**

282   To evaluate the immunogenicity of the group 2 HA stem nanoparticles, we immunized

283   mice three times intramuscularly at four week intervals with the B and C designs of H3ssF and

284   H7ssF. Both H3ssF and H7ssF elicited robust homosubtypic antibody responses against H3 and

285   H7, respectively (Fig. 3A-B). Similarly, strong homotypic H3ssF and H7ssF neutralizing

286   antibody responses were elicited against H3N2 A/Wisconsin/67/2005 and H7N9

287   A/Anhui/1/2013 pseudoviruses, respectively (Fig. 3C-D). For both H3 and H7, B and C designs

288   elicited similar levels of antibody responses (Fig. A-D). Following homosubtypic challenges,

289   H3ssF-vaccinated mice were fully protected against H3N2 A/Philippines/2/1982 influenza

290   (shown with H3ssF_C); likewise, H7ssF vaccination fully protected mice from H7N9

291   A/Anhui/1/2013 (shown with H7ssF_C). Overall, these animal studies demonstrated robust

292   immunogenicity and protective efficacy of our group 2 HA stem nanoparticles against homotypic

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293   viruses, with limited cross-recognition of heterosubtypic strains by elicited sera. Although the

294   stabilized group 2 HA stem antigens are immunogenic and protective, murine species tested do

295   not have the appropriate B cell repertoire to evaluate the potential for eliciting bNAbs with

296   specific characteristics similar to known classes of human mAbs with cross-subtype binding (12,

297   42, 43).

298   **B cell activation by H3ssF and H7ssF nanoparticles**

299   Recent studies have indicated that characterization of *in vitro* engagement of V-gene-

300   reverted BCRs by antigen can be used to assess and rank order vaccine immunogens (43).

301   Therefore, to better understand how the immunogens may be able to engage elements of the

302   human antibody repertoire, Ca^{++} flux assays were used to measure activation of Ramos B cells

303   expressing IgM versions of the inferred UCA of the human antibodies 16.a.26 and 54-1G07

304   representing two multi-donor classes of cross-group human bNAbs (HV1-18 QxxV class and

305   HV6-1+HD3-3 class, respectively) (12, 13, 43). All eight tested H3ssF and H7ssF designs

306   strongly activated the 54-1G07 UCA BCRs with magnitudes comparable to the anti-IgM control,

307   whereas a group 1 H1ssF negative control resulted in no activation (Fig. 4, left panels). In

308   contrast, only two H3ssF immunogens (H3ssF_C and H3ssF_H) and one H7ssF immunogen

309   (H7ssF_C) were observed to elicit a strong activation of the 16.a.26 UCA BCR (Fig. 4, middle

310   panels). No activation was observed of the HV4-34 group 1-specific 01.a.44 UCA BCR (12)

311   (Fig. 4, right panels). Notably, these results were consistent with the BLI binding data. In both

312   sets of experiments, representatives of the multi-donor HV6-1+HD3-3 class bNAbs engaged all

313   of the tested H3ssF and H7ssF immunogens and the representatives of the multi-donor HV1-18

314   QxxV class bNAbs engaged only the C and H design immunogens that contain added Glu in the

315   helix A C-terminal extension. Our results suggest that these design C and H designs could

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316   potentially activate nascent B cells expressing unmutated versions of multi-donor HV6-1+HD3-3

317   or HV1-18 QxxV class bNAbs to initiate B cell proliferation and affinity maturation, thereby

318   eliciting cross-group reactive bNAb responses.

319

320   **DISCUSSION**

321   To address the need for influenza immunogens that can elicit broad heterosubtypic

322   responses in humans, particularly against group 2 influenza A, we used an iterative, structure-

323   based design to develop headless group 2 HA stem-only nanoparticle immunogens from both H3

324   and H7 influenza subtype sequences. Stabilizing the group 2 HA stem was unexpectedly difficult

325   compared to group 1 HA stem antigens. In addition to modifications used to achieve

326   antigenically correct and immunogenic H1ssF candidate vaccines (17), multiple combined

327   protein engineering strategies including helix stabilization, loop optimization, and side chain

328   repacking, were required to achieve the first lead design candidate. Without all three stabilization

329   strategies, the HA stem trimers were not stable enough to be expressed and displayed on self-

330   assembling ferritin nanoparticles. This suggests that screening multiple stabilization methods

331   concurrently may be necessary to design vaccine antigens from metastable proteins or proteins

332   that have had significant portions removed.

333   Ultimately, H3ssF and H7ssF immunogen designs were identified that provided uniform,

334   thermostable nanoparticles recognized by HA stem-directed bNAbs with subnanomolar to

335   nanomolar affinity. Additionally, H3ssF and H7ssF were immunogenic in mice, eliciting robust

336   homotypic neutralizing antibody responses that fully protected mice against homosubtypic lethal

337   challenges.

338   The unique antibody repertoire of humans makes it difficult to predict or model the

339   human immune response of candidate influenza vaccines using currently available animal

340   models such as mice (42, 44) or ferrets, particularly given the specific classes of human V-, D- or

341   J-gene combinations associated with broadly cross-reactive immune responses across multiple

342   individuals (12).Therefore, instead of relying on a mouse model to assess potential breadth of

343   antibody response, we utilized *in vitro* Ca^{++} flux assays (43) to evaluate the ability of group 2

344   HA stem immunogens to activate B cells expressing IgM BCRs comprising inferred UCA forms

345   of the human, cross-group-reactive, HA stem-directed bNAbs from two multi-donor classes (12).

346   Germline targeting with vaccines designed to initiate specific antibody lineages is a new concept

347   being tested with HIV antigens, and not yet proven as a way of achieving broad immunity in

348   humans. Even when transgenic animals are used for preclinical evaluation, the artificial

349   constrains imposed may mitigate correlations with human immune system. Furthermore, since

350   this type of strategy is intended to enhance *de novo* antibody responses it may be particularly

351   difficult to evaluate in pre-immune adults and may require clinical evaluation in naïve children to

352   demonstrate proof-of-concept.

353   Consistency with the BLI binding results informed the choice to focus on immunogens

354   H3ssF_C, H3ssF_H and H7ssF_C, each of which were designed to have two outwardly facing

355   surface Glu (at positions *i* and *i*+3) at the C-terminus of helix A. The first Glu occurs at a

356   position (HA2 57) that naturally has a Glu in most human H3 strains before 2002 and the

357   majority of human H7 and H10 strains, though it is not in a helical conformation in full length

358   HA. Modeling with CT149 suggests the two Glu residues might interact with light chain CDRL1

359   and CDRL2 loops (Fig. S9).

17

360   The observation that H3ssF and H7ssF variants were able to activate inferred UCA

361   versions of cross-reactive BCRs suggest these immunogens could potentially induce broad cross-

362   group multi-donor antibodies in humans. Due to their broad heterosubtypic breadth (12, 14, 15),

363   such antibodies could potentially protect against pandemic strains in addition to drifted seasonal

364   strains and preclude the need for annual vaccine reformulations. Furthermore, group 2 HA

365   immunogens based on conventional H7N9 monovalent split product formulations have been

366   shown to induce more diverse lineages of cross-group-reactive HA stem antibodies than

367   conventional group 1 H5N1 immunogens in humans (13). Immunological imprinting from earlier

368   or first exposures has also been suggested to play a significant role in shaping the subsequent

369   immune response to influenza (45, 46). Thus, in influenza-naïve individuals, the order of antigen

370   priming may influence the composition of the influenza-specific B cell repertoire. Current

371   vaccine approaches combine multiple strains of influenza in a single formulation. While this may

372   be an effective strategy in adults with pre-existing immunity from prior infections and

373   vaccinations, it is possible that using an influenza A group 2 HA stem-based vaccine as the

374   primary antigen exposure in children could potentially imprint or prime for broader cross-group

375   responses later in life. Moreover, since titers of anti-HA stem antibodies increase with age and

376   are inversely correlated with influenza infection rates, any early robust induction of broad, cross-

377   reactive HA stem antibodies has the potential to mitigate the relative high incidence of severe

378   influenza disease in children in addition to augmenting protection from influenza later in life

379   (47). For adults and elderly who have likely already encountered influenza, the group 2 stem

380   immunogens could selectively boost antibody responses with greater breadth by not engaging

381   otherwise predominant group 1 HA stem-specific HV1-69 antibodies (48, 49). These group 2

382   immunogens complement our earlier development of group 1 HA stem immunogens which were

18

383   demonstrated to elicit within-group heterosubtypic protection in mice and ferrets (17). Group 1

384   HA stem could potentially be used as a boost for the group 2 HA stem immunogens to better

385   focus the antibody response to the neutralization-sensitive epitope centered on helix A. Finally,

386   group 1 and 2 HA stem immunogens could also be given together in a manner analogous to the

387   current multivalent inactivated influenza vaccines (IIV).

388   Another approach for inducing an HA stem antibody response involves immunizing

389   sequentially with heterologous chimeric HA immunogens comprising two or more HAs with the

390   same stem, but head regions from different subtypes. This strategy has elicited heterosubtypic

391   group 1 and group 2 protection in animal models (50-53). However, the exotic HA heads may

392   still be immunodominant and potentially interfere with or limit HA stem-specific responses as it

393   has been shown that transient vaccine-induced boost of the stem-directed antibody responses can

394   be replaced by immunodominant head-directed responses over time in H5 vaccine trials in

395   humans (49, 54). From a practical clinical perspective, careful records would need be maintained

396   to assure the correct order of administration of heterologous vaccine products.

397   Although bacterially-produced HA stem-only immunogens from H1, H5, H3 and H7

398   subtypes have also been reported to protect mice from H3 or H7 influenza, complete protection

399   has only been observed for some homotypic strains (55-57), indicating that the breadth of

400   response is limited. The immunogens in our study more closely mimic the native H3N2 and

401   H7N9 HA stems in that they include native N-linked glycans as well as the membrane proximal

402   region of the HA stem that are both missing in the bacterially-expressed designs. Eukaryotic

403   expression of HA stem immunogens may be of particular importance because each native HA

404   stem trimer has nine glycans and glycans have been observed to guide antibody binding by either

405   masking certain epitopes (58, 59) or participating as part of an epitope surface (60). Indeed,

19

406   many cross-group-reactive HA stem-directed bNAbs are immediately proximal to the HA2 N38

407   glycan when recognizing group 2 HA (12). Interestingly, the HA stem-directed bNAbs most

408   frequently elicited from group 1 influenza, which does not have N38 glycans, are of the HV1-69

409   class (48), that is generally incompatible with the N38 glycan, and therefore not cross-group-

410   reactive. Thus, group 2 HA stem immunogens bearing native glycans may provide a selective

411   advantage for eliciting broad influenza antibody responses. However, to date no experimental

412   data has shown glycosylated hemagglutinin immunogens to be more effective than

413   nonglycosylated versions. A recently reported particulate vaccine comprising nonglycosylated,

414   HA stem crosslinked to desolvated tandem M2e segments has been shown to elicit broad cross-

415   group protection in mice (61). However, these particles comprised proteins derived from

416   multiple host species, strains and subtypes of influenza with the goal of accumulating breadth as

417   opposed to the approach herein of attempting to achieve breadth through targeting conserved

418   antigenic supersites.

419   In summary, we have designed group 2 headless HA stem trimers with conformational

420   and antigenic fidelity to native HA. These antigens were immunogenic in mice and provided

421   protection against a lethal homosubtypic challenge. Since mice lack critical genetic elements

422   found in human bNAbs targeting the HA stem, we used a B-cell receptor activation assay to

423   demonstrate the potential for eliciting broadly reactive human antibody responses. Based on

424   activation of B cells expressing inferred UCA versions of human antibodies associated with

425   broad cross-neutralizing activity, candidate vaccines with antigens displayed on self-assembling

426   ferritin nanoparticles are now being manufactured for clinical evaluation. Additional animal

427   studies will be performed in parallel, including testing in mice expressing specific human

428   immunoglobulin genes and in nonhuman primates. These immunogens represent a step towards

429   achieving the goal of a vaccine that can elicit protective immunity against drifted seasonal and

430   pandemic strains of influenza A virus.

431

432   **MATERIALS AND METHODS**

433   **Structure-based design**

434   The crystal structure of A/Finland/486/2004 (H3N2) HA (PDB IB 2YP2) was used as a template

435   for subtype H3 and the crystal structure of A/Shanghai/2/2013 (H7N9) HA (PDB ID 4LN6) was

436   used as a template for subtype H7. The complete sequences for A/Finland/486/2004 HA and

437   A/Shanghai/2/2013 (H7N9) HA including the signal sequence regions not included in the crystal

438   structures were obtained from GISAID accession EPI397685 and Genbank accession

439   YP_009118475.1 respectively. The change in free energy from point mutations were estimated

440   using the program DDG_MONOMER (26) from the ROSETTA suite. Loops were modeled with

441   LOOPY (62), superpositions were performed using UCSF Chimera (63) or LSQMAN (64), and

442   energy minimization of final models was performed using RELAX (65) from the ROSETTA

443   suite. The graphics programs PyMOL (66) and UCSF Chimera (63) were used for detailed visual

444   inspection of structures and the generation of structural figures.

445   **Phylogenetic tree generation for HA sequences**

446   Maximum likelihood trees were created using the program MEGA7.0 (ref (67)) from 18

447   representative HA sequences (13) aligned with Muscle (68). The stem regions of HA sequences

448   were defined as residues HA1 10-42, 314-329 and HA2 1-59 and 93-174 (H3 numbering (21)).

449   **Antigenic screening of nanoparticle immunogen designs**

450   Most designs were initially screened in a high throughput ELISA format as described previously

451   (23). However, the contributions of individual mutations within multiple-mutation designs were

21

452   also assessed by expressing transmembrane versions of H3 HA stem containing the native C-

453   terminal HA sequence starting with HA2 175 (SVELKSGYKDWILWISFAISCFLLCVALLGF

454   IMWACQKGNIRCNICI) on the surface of HEK293F cells and measuring recognition of bNAbs

455   FI6v3 and CR8020 using flow cytometry. Since HA stem was more readily expressed in this

456   format with less stabilization required, this strategy enabled a more sensitive evaluation of HA

457   stem mutations than the nanoparticle format. Promising designs that exhibited greater FI6v3 and

458   CR8020 binding were subsequently expressed in the nanoparticle format for final evaluation.

459   **Expression and purification of immunogens and antibodies**

460   All H3ssF and H7ssF nanoparticles and FI6v3, CT149, CR8020, CR6261, MEDI8852, 315-

461   53-1F12, 54-1G07, 04-1D02 and 09-1B12 mAbs were expressed in 293 Expi cells (Life

462   Technologies) and purified by affinity chromatography (*Galanthus nivalis* lectin for the

463   nanoparticles and protein A for mAbs), using previously described methods (13, 17).

464   Assembly of nanoparticles was assessed by gel filtration with a Superose 6 Increase 10/300

465   GL column (GE Healthcare). To make MEDI8852 and CR8020 Fabs, IgG was digested with

466   endoproteinase Lys-C (New England Biolabs) overnight at room temperature (RT). To make

467   CT149 Fab, CT149 IgG containing an HRV-3C cleavage site engineered in the heavy chain

468   hinge region was digested using HRV-3C enzyme overnight at RT. The reactions were assessed

469   by SDS-PAGE and upon completion, the reactions were quenched by addition of protease

470   inhibitor cocktail (Millipore-Sigma) and the Fc and Fab were separated by Protein A affinity

471   chromatography.

472   **ELISA binding assays**

473   ELISA assays were done as previously described (17). Briefly, ELISA plates were coated with

474   100 ng/well H1ssF, H3ssF, or H7ssF antigens overnight at 4°C. The plates were then incubated

22

475   with IgG or Fab forms of mAbs with dilutions starting at 10 μg/ml. For Fab, anti-CH1 mAb was

476   then bound at 10 μg/mL. For testing of mouse sera, H3 HA and H7 HA antigens represented

477   A/Hong Kong/1/1968 HA and A/Anhui/1/2013, respectively. Mouse sera serial dilutions started

478   at 1:50. Appropriate HRP-conjugated secondary antibodies were used to detect primary antibody

479   followed by a colorimetric detection assay. Linear regression analysis of the absorbance values

480   (OD 450 nm) was completed using GraphPad Prism software. Serum endpoint titers were

481   determined to be the serum dilution that resulted in four-fold increase in OD value above

482   background. One-way ANOVA with Kruskal-Wallis post-test was used to compare mean

483   endpoint titers between groups. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

484   **Immunoprecipitation assay**

485   Magnetic protein G Dynobeads™ (Thermo Fisher) were used to capture FI6v3 mAb, per

486   manufacturer's protocol, and allowed to incubate for 1 hour at RT. FI6v3-bound Dynobeads™

487   were washed 2 times with 1X PBS and incubated with supernatants from H3ssF or H7ssF

488   expressed in HEK293 cells or purified control protein, for 1 hour at RT. Beads were washed 2

489   times with 1X PBS and heated to 95 °C for 10 minutes to detach bound protein. Proteins were

490   then analyzed by SDS-PAGE.

491   **Biolayer interferometry binding assays**

492   All biosensors were hydrated in PBS prior to use. CR9114 IgG (10 μg/ml diluted in 1% BSA-

493   PBS) were immobilized on AHC biosensors through conjugated anti-human Fc antibody

494   (fortéBio). After briefly dipping in assay buffer (1% BSA-PBS), the biosensors were dipped in

495   various H3ssF and H7ssF constructs (10 μg/ml) to capture nanoparticles. The biosensors were

496   then equilibrated in assay buffer for 1 min before dipped in a 2-fold dilution series of CR8020,

497   CT149 or MEDI8852 Fab for 5 min followed by dipping in assay buffer to allow Fab to

23

498   dissociate from nanoparticles for 5 min. All assay steps were performed at 30ºC with agitation

499   set at 1,000 rpm in the Octet HTX instrument (fortéBio). Correction to subtract a baseline drift

500   was carried out by subtracting the measurements recorded for a sensor incubated with no Fab.

501   Data analysis and curve fitting were carried out using Octet analysis software (version 9.0) as

502   previously described (69, 70). Experimental data were fitted with the binding equations

503   describing a 1:1 interaction. Global analyses of the complete data sets assuming binding was

504   reversible (full dissociation) were carried out using nonlinear least-squares fitting allowing a

505   single set of binding parameters to be obtained simultaneously for all concentrations used in each

506   experiment.

507   **Electron microscopy analysis**

508   Samples were diluted to ~0.02 mg/ml with buffer containing 10 mM HEPES, pH 7.0, and 150

509   mM NaCl and adsorbed to freshly glow-discharged carbon-film grids for 15 seconds. The grids

510   were washed with the same buffer and stained with 0.7% uranyl formate. Images were collected

511   at a nominal magnification of 50,000 semi-automatically using SerialEM (71) on an FEI Tecnai

512   T20 microscope operated at 200 kV and equipped with a 2k x 2k Eagle CCD camera. The pixel

513   size was 0.44 nm. Particles were picked automatically using in-house developed software (YT,

514   *unpublished*). Particle alignment and reference-free 2D classification were performed with

515   SPIDER (72) and Relion 1.4 (ref (73)).

516   **Differential scanning calorimetry (DSC)**

517   Thermal melting points were determined using a MicroCal VP-Capillary differential scanning

518   calorimeter (Malvern Instruments). All protein samples were in the range of 0.2-0.5 mg/ml and

519   extensively dialyzed against phosphate-buffered saline (PBS). Thermal denaturation was probed

520   at a scan rate of 60°C/h from 30 to 120°C. Buffer correction, normalization, and baseline

24

521   subtraction procedures were applied before the data were analyzed using the VP-Capillary DSC

522   Automated data analysis software.

Mouse immunizations

524   Animal experiments were carried out in compliance with all pertinent US National Institutes of

525   Health regulations and policies. The National Institutes of Health, National Institute of Allergy

526   and Infectious Diseases, Vaccine Research Center Animal Care and Use Committee reviewed

527   and approved all animal experiments. Female BALB/cJ mice aged 6–8 weeks (Jackson

528   Laboratory) were immunized with 2 μg H3ssF or H7ssF, adjuvanted with Sigma Adjuvant

529   System, at 0, 4, and 8 weeks. Mice were inoculated with 100 μL intramuscularly, given as 50 μL

530   into each hind leg. Two weeks after the final immunization, sera were collected for measurement

531   of antibody responses. When appropriate, challenges were performed at 4-8 weeks post-

532   immunization. Mice were inoculated intranasally with a $10xLD_{50}$ dose H3N2

533   A/Philippines/2/1982, or a $20xLD_{50}$ dose H7N9 A/Anhui/1/2013. Weight loss was recorded daily

534   for 14 days post-challenge.

Pseudovirus neutralization assays

536   Lentivirus-based pseudoviruses displaying influenza HA and NA were produced as previously

537   described (17). Mouse serum was treated with receptor destroying enzyme (Denka Seiken).

538   Serial dilutions of mouse sera (1:40, four-fold, eight dilutions) were mixed with either H3N2

539   A/Wisconsin/67/2005 or H7N9 A/Anhui/1/2013 pseudovirus (which were previously titered to

540   target 50,000 RLU), for 30 minutes at RT. The virus/pseudovirus mixture was then added to

541   previously-plated 293A cells (ATCC), in triplicate. After 2 hours, at 37 °C and 5% CO_2, fresh

542   DMEM supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin was

543   added. Cells were lysed at 72 hours, and luciferase substrate (Promega) was added. Luciferase

544   activity was measured as relative luciferase units (RLU) at 570 nm on a SpectramaxL (Molecular

545   Devices). Sigmoidal curves, taking averages of triplicates at each dilution, were generated from

546   RLU readings; 50% neutralization (IC_{50}) titers were calculated considering uninfected cells as

547   100% neutralization and cells transduced with only virus as 0% neutralization. One-way

548   ANOVA with Kruskal-Wallis post-test was used to compare mean IC_{50} titers between groups. *

549    $= p < 0.05$, ** $= p < 0.01$, *** $= p < 0.001$, **** $= p < 0.0001$.

550   **Determination of inferred UCAs for HA stem antibodies.**

551   Paired heavy and light chain DNA sequences for 58 members of the 54-1G07 clonal family

552   (315-54-1G07) were isolated from single IgG^+ B cells, along with 6 additional heavy chain DNA

553   sequences from cells for which light chains could not be amplified (13). A maximum likelihood

554   phylogenetic tree was constructed from concatenated heavy and light chain sequences and the

555   most recent common ancestor (MRCA) of the lineage was inferred using DNAML in SONAR

556   (74). This MRCA had 5 residual nucleotide mutations each in heavy and light chain (resulting in

557   2 and 5 amino acid changes, respectively) compared to the assigned germline VH and VK genes.

558   To construct the final UCA, these changes were reverted to the presumed germline sequence.

559   The same procedure was used for 57 paired heavy and light chain DNA sequences and 8

560   unpaired heavy chain DNA sequences from the 09-1B12 clonal family. This MRCA had 1

561   residual nucleotide mutation (1 amino acid change) in the heavy chain and no residual mutations

562   in the light chain. For the 04-1D02 clonal family, 29 paired heavy and light chain DNA

563   sequences and 1 unpaired heavy chain DNA sequence were used. The inferred MRCA contained

564   4 residual nucleotide mutations in the heavy chain and 6 nucleotide mutations in the light chains

565   (4 and 5 amino acid changes, respectively). For this clonal family, only changes in VH genes

566   were reverted; the lambda chain of 04-1D02-MRCA was used with the residual mutations

567   included.

568

569   For the 16.a.26 clonal family, 93 heavy chain, and 83 light chain sequences and for the 01.a.44

570   clonal families, 51 heavy chain and 49 light chain sequences were identified from single sorted

571   IgG$^+$ B cells. For the heavy chain, a maximum likelihood phylogenetic tree based upon V-gene

572   sequence alone was constructed using Geneious (75), with each tree rooted to the respective

573   germline reference sequence as listed in the IMGT database. The VDJ junction of clonal

574   members with the lowest mutation load was inferred using IMGT V-quest, allowing

575   identification of residues within the V and D and J gene segments most likely subject to somatic

576   mutation. To construct the heavy chain UCA, these changes were reverted to the presumed

577   germline sequences. Due to the lack of precision in estimating N-nucleotide addition, mutations

578   falling at V/D or D/J junctions were not reverted. For the light chains, an analogous process was

579   used for V and J gene segments.

580   **Generation of B cell lines**

581   B cell lines expressing inferred UCA versions of monoclonal antibodies were generated by

582   transduction of surface IgM-negative Ramos cells as previously described (43). Briefly, DNA

583   encoding for VDJ (heavy chain) and VJ (light chain) immunoglobulin regions was synthesized

584   by GenScript and cloned, along with the consensus human IgM C region, into the pLVX-

585   ZsGreen and pLVX-mCherry expression vectors (both from Clontech) respectively. Each of

586   these vectors was then co-transfected with the lentivirus packaging plasmid psPAX2 and with

587   the VSV-G envelope expressing plasmid pMD2.G (both from Addgene) into HEK293 T cells for

588   formation of lentiviral particles using Lipofectamine 2000 (Thermofisher). Supernatant was

27

589   harvested 3 days after transfection and cleared by centrifugation before IgM-negative Ramos

590   cells were co-transduced for both heavy and light chains. After 5 days, transduced Ramos cells

591   were enriched by cell sorting for expression of ZsGreen and mCherry using a FACS AriaII

592   interfaced to the FacsDiva software (BD Biosciences). This procedure was repeated until a pure

593   double positive population could be selected. Cell lines were further enriched for high IgM

594   surface expression by using a fluorescently labeled anti-human IgM monoclonal antibody.

595   **B cell activation assays**

596   The ability of the different particles to induce calcium flux upon BCR engagement was measured

597   in vitro using inferred UCA monoclonal antibodies expressed as surface IgM in Ramos cells as

598   previously described (76). Briefly, 1 million cells (per test) were stained with 0.35 μL Fura Red

599   (Thermo Fisher Scientific) in 100 μL serum-free medium at ambient temperature in the dark for

600   30 min. After washing, cells were resuspended in 300 μL serum-free medium and heated to 37°C

601   for 3-5 min in a heated water bath before they were acquired on a FACS Symphony interfaced to

602   the FacsDiva software (BD Biosciences). Cells were first acquired for 30 s in the absence of

603   antigen to record baseline intracellular calcium levels and then tubes were removed (leaving the

604   acquisition in progress) to add the antigen and quickly vortexed before placed back on

605   acquisition for a total time of 180 seconds. Nanoparticles were used at the final concentration of

606   50 nM. An anti-human unlabeled Mouse F(ab')2 Anti IgM (Southern Biotech) was used (1.5

607   μg/test) to determine the maximal induction of calcium flux measured by the change between the

608   emission of Fura Red bound to calcium and the one unbound (ratio) over time. The inferred

609   UCA for 01.a.44 was chosen as a negative control since it is observed to bind only to group 1

610   HAs and not group 2 (not shown). Likewise, the inferred UCAs for 54-1D07 and 16.a.26 are

611   observed to recognize group 2 HAs only (not shown).

612 **Accession numbers for inferred antibody UCA sequences**

613 The sequences for the inferred UCA versions of the following HA stem antibodies have been

614 deposited into GenBank: 54-1G07 (MH631450 and MH631451), 04-1D02 (MH997406 and

615 MH997407), 09-1B12 (MH997404 and MH997405), 01.a.44 (MK291363 and MK291365) and

616 16.a.26 (MK291364and MK291366).

617

618 **ACKNOWLEDGEMENTS**

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890   **FIGURE LEGENDS**

891   **Fig. 1.** Structure-based design of group 2 HA stem nanoparticles. (A) Ribbon models depicting

892   the HA stem nanoparticle design pathway for stabilizing the HA stem starting from an H3N2 HA

893   trimer ectodomain. Two of the three protomers are gray and one protomer is cyan with the

894   design modifications in red. Positive or negative outcomes are indicated by green or black

895   arrows, respectively, below each step. bNAb, broadly neutralizing antibody. (B) Maximum

896   likelihood phylogenetic tree for protein sequences from representative full-length HAs (top

897   panel) and HA stem (bottom panel) of the 18 different subtypes. Group 1 subtypes are navy blue

898   and group 2 subtypes are cyan. (C) A molecular model of the HA stem of an H3ssF_C design is

899   shown colored as in (A). Two of the three protomers are depicted as surface representations and

900   one protomer is displayed as a ribbon diagram. Insets show close-ups of the modified regions

901   with stick representations for side chains of modified residues. Surface representations are

902   removed from some insets for clarity. PDB entry 2YP2 was used an H3N2 model template.

903

904   **Fig. 2.** Physical characterization of group 2 HA stem nanoparticles. Gel filtration chromatograms

905   for lectin-purified H3ssF (A) and H7ssF (B) nanoparticles reveal single peaks. Negative stain

906   electron microscopy 2D class averages for H3ssF (C-D) and H7ssF (E-F) B and C variants

907   demonstrate the formation of particles with a visible arrangement of HA stem trimers projecting

908   from hollow spheres. White bars represent 10 nm size markers.

909

910   **Fig. 3.** Immunogenicity of group 2 HA stem nanoparticles in mice. BALB/c mice were

911   immunized with H3ssF or H7ssF. B and C design groups were tested in separate experiments.

912   Serum was assessed for binding antibodies to H3 A/Hong Kong/1/1968 HA (A) and H7

42

913   A/Anhui/1/2013 HA (B) and neutralizing antibodies against H3N2 A/Wisconsin/67/2005 (C) or

914   H7N9 A/Anhui/1/2013 pseudovirus (D). H3ssF_C and H7ssF_C immunized mice were

915   challenged with lethal doses of H3N2 A/Philippines/2/1982 or H7N9 A/Anhui/1/2013,

916   respectively. Survival (E,G) and weight loss (F,H) were recorded post-challenge. Dotted lines in

917   A-D represent assay limits of detection. One-way ANOVA with Kruskal-Wallis post-test was

918   used to compare mean ELISA and IC_{50} titers between groups. * = p < 0.05, ** = p < 0.01, *** =

919   p < 0.001, **** = p < 0.0001.

920

921   **Fig. 4.** Group 2 HA stem immunogens activate B cells expressing the inferred UCA versions of

922   multi-donor cross-group-reactive human BCRs. The results from Ca^{++} flux assays indicate B cell

923   activation by IgM positive control and a group 1 H1ssF (A), H3ssF variants (B), and H7ssF

924   variants (C). BCR activation is displayed for the group 2-specific 54-1G07 UCA in the left

925   panels, group 2-specific 16.a.26 UCA activation in the middle panels, and the group 1-specific

926   01.a.44 control BCR in the right panels. Ca^{++} flux was measured by flow cytometry as the ratio

927   (y-axis) of the Ca^{++} bound/unbound states of the Ca^{++} sensitive dye Fura Red over time in

928   seconds (x-axis). Each experiment was repeated three times, and representative curves from one

929   of the repeats are shown for each cell line.

930

931   **SUPPLEMENTARY FIGURE LEGENDS**

932   **Fig. S1.** Design of group 1 HA stem nanoparticle immunogens. Ribbon diagrams depict the

933   design of HA stem-ferritin nanoparticles by 1) removing the head region of HA and replacing

934   with glycine-rich linkers, 2) two internal packing mutations and 3) genetically fusing to a ferritin

935   nanoparticle by a three-residue linker. Structures are shown by ribbon diagrams. One monomer

936   of each HA and HA stem trimer is colored blue and mutated regions or residues are colored red.

937   The nanoparticle core is brown.

938

939   **Fig. S2**. Sequence alignments of select H3ssF (A) and H7ssF (B) variants with the full length

940   HA ectodomains for A/Finland/486/2004 (H3N2) (H3_FI04) and A/Shanghai/2/2013

941   (H7_SH13) respectively. Mutations are in red. Residues identical to the full length sequence are

942   represented by dots and deletions represented by dashes. H3N2 numbering is shown above each

943   alignment. For clarity, the HA head regions and the ferritin sequence is not shown. In each

944   construct, the C-terminal HA2 174 residue is connected to ferritin residue Asp5 by a short SGG

945   linker (not shown).

946

947   **Fig. S3.** Characterization of H3ssF_A. (A) Superose 6 gel filtration chromatograms for and

948   H3ssF_A (magenta) and H3ssF_B (red) nanoparticles. (B) SDS PAGE analysis of the results

949   from an FI6v3 immunoprecipitation of the supernatants from H3ssF_A expressed in HEK293

950   cells. Molecular weight standards (MW) are designated as kDa. The band indicative of H3ssF_A

951   is boxed in magenta. H3ssF_null is previous design iteration that did not express. H1ssF is

952   shown as a positive control. (C) Negative stain electron microscopy 2D class averages of gel

953   filtration purified H3ssF_A. The white bar represents a 10nm size marker.

954

Fig. S4. Physical characterization of H3ssF (A-E) and H7ssF (F-J) nanoparticles. Superose 6 gel

filtration chromatograms (left panels) for lectin-purified nanoparticles reveal single peaks.

Negative stains electron microscopy 2D class averages (right panels) demonstrate the formation

of particles with a visible arrangement of HA stem trimers projecting from hollow spheres.

White bars represent 10nm size markers for right panels.

960

Fig. S5. Differential scanning calorimetry (DSC) plots for group 2 HA stem immunogens. (A)

H3ssF. (B) H7ssF. (C) Ferritin alone. Plots of heat capacity (Cp) versus temperature depicts

melting transitions for each protein. The Cp values on the Y-axis are shown with an arbitrary

scale.

965

Fig. S6. BLI binding curves for MEDI8852 (A and B) and CT149 (C and D) Fab recognition of

H3ssF and H7ssF immunogens respectively. Binding constants and kinetic parameters for each

plot are shown in Table 2. (E) Binding of H3ssF_C and H7ssF_C to CR9114 Fab. Nanoparticles

were immobilized to the sensor tip by binding to CR9114 IgG coupled by human anti-Fc

antibody and HA trimers were immobilized on HIS1K sensors through C-terminal His tags. Data

curves are in red; fitting for a 1 to 1 interaction are in black.

972

Fig. S7. Antigenic recognition of group 2 HA stem nanoparticles. (A) ELISA binding of H3ssF

and H7ssF designs B and C by six broadly neutralizing HA stem antibodies. (B) Relative

recognition of H3ssF (top) and H7ssF (bottom) by IgG and Fab forms of CT149 as measured by

ELISA. In both (A) and (B), the nanoparticles were immobilized on the plate. (C) BLI binding

45

977   curves for CR8020 Fab recognition of H3ssF (left) and H7ssF (right) immunogens. Binding

978   constants and kinetic parameters for each plot are shown in Table 2. (D) Relative antigenic

979   recognition of H3ssF and H7ssF by mature and inferred UCAforms of HA stem human

980   antibodies as measured by BLI. CR9114 and the HA head antibody CH65 served as positive and

981   negative controls respectively. Antibody recognition was determined by Octet and binding

982   relative to CR9114 was plotted on the y axis as a percentage. In (C) and (D), all nanoparticles

983   were immobilized to the sensor tip by binding to CR9114 IgG coupled by human anti-Fc

984   antibody.

985

986   **Fig. S8.** Sequence alignments of mature and inferred UCA for mAbs 09-1B12, 16.a.26, 54-

987   1G07, 04-1D02 and 01.a.44. Dots indicate identical residues. CDR loops are boxed according to

988   Kabat numbering and convention. Shading indicates the antibody classes labeled in the upper left

989   of each box.

990

991   **Fig. S9.** Model of CT149 interaction with helix A of H3ssF_C. The left panel shows a ribbon

992   diagram model of CT149 (green and brown) bound to H3ssF_C (blue and gray). Helix A from

993   one protomer of the H3ssF_C model was superimposed onto HA helix A (chain B) of the

994   CT149/H3N2 HA crystal structure (PDB entry 4UBD) using PyMOL. For clarity, the H3N2 HA

995   is not shown and only one CT149 Fab (chains C and D) is depicted. The middle panel shows a

996   close-up of the light chain interaction with HA helix A and the right panel shows the same close-

997   up view rotated 90° along the x-axis. For clarity, the middle and right panels show only helix A

998   from one chain of the H3ssF_C model. The two Glu side chains at the C-terminal end of

999   H3ssF_C helix A are shown as stick models.

46

Table 1. Physical characteristics of the H3ssF and H7ssF nanoparticles

Subtype	Design	Design characteristics					Relative Yield[f]	Tm (°C)	EM characterization	
		HA1 loop[a]	Helix A extension[b]	Inter-helix loop[c]	Helix C mutations[d]	Predicted disulfides[e]			Ferritin core size (Å)	HA stem size (Å)
H3	H3ssF_A	--GSG--	ALMAQ	GGP	DAYL	NA	-	ND	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

[a] Between HA1 residues 42 and 314 (H3 numbering)

[b] Directly following HA1 56

[c] Directly following the helix A extension

[d] Region between the interhelix loop and HA2 96

[e] DS1, HA1 C145-HA2 C93; DS2, HA1 C141-C146; DS3, HA1 C23-C322; NA, not applicable.

[f] Based on gel electrophoresis analysis of unpurified supernatants following transient transfection.

1007 **Table 2. Antigenicity of the H3ssF and H7ssF nanoparticles as determined by BLI**
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Design	CT149[a]			MEDI8852[a]			CR8020[a]			CR9114[a]		
	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[b]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
H3ssF_B	NB[c]	NB	NB	0.7	2.9×10^5	2.2×10^{-4}	20.9	2.8×10^5	5.8×10^{-3}	ND	ND	ND
H7ssF_B	7.0	4.2×10^5	3.0×10^{-3}	1.0	2.6×10^5	2.6×10^{-4}	NB	NB	NB	ND	ND	ND
H3ssF_C	21.3	8.1×10^5	1.7×10^{-2}	0.8	3.0×10^5	2.4×10^{-4}	23.3	2.4×10^5	5.5×10^{-3}	93.6	7.0×10^4	6.5×10^{-3}
H7ssF_C	0.9	6.0×10^5	5.3×10^{-4}	1.0	2.7×10^5	2.6×10^{-4}	NB	NB	NB	47.9	1.0×10^5	4.8×10^{-3}
H3ssF_D	NB	NB	NB	1.1	2.9×10^5	3.2×10^{-4}	21.7	2.8×10^5	6.0×10^{-3}	ND	ND	ND
H7ssF_D	9.0	3.7×10^5	3.3×10^{-3}	0.9	2.7×10^5	2.3×10^{-4}	NB	NB	NB	ND	ND	ND
H3ssF_E	NB	NB	NB	1.2	3.2×10^5	3.7×10^{-4}	18.7	3.0×10^5	5.7×10^{-3}	ND	ND	ND
H7ssF_E	8.2	3.7×10^5	3.0×10^{-3}	0.8	2.9×10^5	2.4×10^{-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	6.7×10^5	1.8×10^{-3}	0.9	2.9×10^5	2.4×10^{-4}	NB	NB	NB	ND	ND	ND
H3ssF_G	NB	NB	NB	0.9	3.0×10^5	2.6×10^{-4}	17.9	2.7×10^5	4.8×10^{-3}	ND	ND	ND
H3ssF_H	22.2	8.2×10^5	1.8×10^{-2}	ND	ND	ND	ND	ND	ND	ND	ND	ND
H7ssF_I	7.2	4.1×10^5	2.9×10^{-3}	1.0	2.6×10^5	2.7×10^{-4}	NB	NB	NB	ND	ND	ND
H7ssF_J	9.8	3.5×10^5	3.4×10^{-3}	1.9	2.5×10^5	4.9×10^{-4}	NB	NB	NB	ND	ND	ND
H3 HA	42.2	7.5×10^4	3.2×10^{-3}	1.2	1.3×10^5	1.5×10^{-4}	ND	ND	ND	ND	ND	ND
H7 HA	3.7	3.6×10^5	3.1×10^{-3}	1.2	1.1×10^5	1.3×10^{-4}	ND	ND	ND	ND	ND	ND

1009 [a]Fab

1010 [b]ND, not determined

1011 [c]NB, no detectable binding

Figure 1

Figure 2

A
H3ssF

C
H3ssF_B

D
H3ssF_C

B
H7ssF

E
H7ssF_B

F
H7ssF_C

Figure 3

Figure 4