

1 Broadly-reactive IgG responses to 2 heterologous H5 prime-boost influenza 3 vaccination are shaped by antigenic 4 relatedness to priming strains

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13 ABSTRACT

14 Prime-boost vaccinations of humans with different H5 strains have generated
15 broadly protective antibody levels. However, the effect of an individual's H5 exposure
16 history on antibody responses to subsequent H5 vaccination is poorly understood. To
17 investigate this, we analyzed the IgG response to H5 A/Indonesia/5/2005 (Ind05) vacci-
18 nation in three cohorts: (1) a double primed group that received two H5 vaccinations:
19 A/Vietnam/203/2004 (Vie04) 5 years ago and A/Hong Kong/156/1997 (HK97) 11 years
20 ago, (2) a single primed group that received Vie04 5 years ago, and (3) an H5-naïve
21 group that received two doses of the Ind05 vaccine 28 days apart. Hemagglutinin
22 (HA)-reactive IgG levels were estimated by multiplex assay against an HA panel that
23 included 21 H5 strains and 9 other strains representing H1, H3, H7, and H9 subtypes.
24 Relative HA antibody landscapes were generated to quantitatively analyze the magni-
25 tude and breadth of antibody binding after vaccination. We found that short-interval
26 prime-boosting with the Ind05 in the naïve group generated a low anti-H5 response.
27 Both primed groups generated robust antibody responses reactive to a broad range
28 of H5 strains after boosting with Ind05; IgG antibody levels persisted longer in subjects
29 who had been double primed years ago. Notably, the IgG responses were strongest
30 against the first priming H5 strain, that reflecting influenza virus immune imprinting.
31 Finally, the broad anti-H5 IgG response was stronger against strains having a small
32 antigenic distance to the initial priming strain.

33
34 **IMPORTANCE** The antigenic shift and drift of hemagglutinin (HA) in influenza viruses
35 is accepted as one of the major reasons for immune evasion. The analysis of B cell
36 immune responses to influenza infection and vaccination is complicated by the im-
37 pact of exposure history and antibody cross-reaction between antigenically similar in-
38 fluenza strains. To assist in such analyses, the influenza "antibody landscape" method
39 has been used to analyze and visualize the relationship of antibody mediated immu-
40 nity to the antigenic distance between influenza strains. In this study, we describe a
41 "relative antibody landscape" method, calculating the antigenic distance between the
42 vaccine influenza strain and other H5 strains, and using this relative antigenic distance
43 to plot with the anti-H5 IgG levels post-vaccination. This new method quantitatively

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44 estimates and visualizes the correlation between the humoral response to a particular
45 influenza strain, and the antigenic distance to other strains. Our findings demonstrate
46 the effect of H5 exposure history on H5 vaccine responses quantified by the relative
47 antibody landscape method.

48 **KEYWORDS:** H5 monovalent influenza vaccine (MIV), hemagglutinin (HA) antigenic
49 distance, influenza virus antibody landscape, Original antigenic sin (OAS), HA
50 imprinting.

51 INTRODUCTION

52 A number of highly pathogenic avian influenza (HPAI) A viruses, such as the H5, H7,
53 and H9 strains, pose a significant threat to cause human pandemics as a result of
54 their fast mutation rate and high pathogenicity (1, 2). To date, there is no evidence
55 of sustained human-to-human transmission of these strains, despite repeated docu-
56 mentation that humans can contract these viruses from infected poultry (3). The first
57 known human H5N1 infection was reported in 1997 during a poultry H5 outbreak in
58 Hong Kong (4). From 2003 to January 2015, a total of 694 laboratory-confirmed hu-
59 man H5 cases were reported across 16 countries and 58% of those people have died
60 as a result (5). Vaccination against future pandemic strains is the most viable path
61 towards mitigating potential outbreaks. However, current H5 non-adjuvanted mono-
62 valent influenza vaccine (MIV) formulations are poorly immunogenic (6, 7, 8, 9, 10),
63 and generally require a prime and boost strategy in order to achieve protective levels
64 of immunity (11, 12). Interestingly, boosting with non-adjuvanted MIV, even in sub-
65 jects who had been primed several years prior lead to robust and broad antibody
66 responses to variant H5 MIV vaccine(11). Such prime and boost strategies also ap-
67 pear to be needed for recent RNA vaccines(13) to other non-influenza viruses; and
68 understanding the immunobiology of this phenomenon remains highly relevant.

69 It has been generally accepted that the immunological protection against influenza
70 infection is predominately due to antibodies directed against the viral surface hemag-
71 glutinin (HA) protein, which is thus the major target of most influenza vaccines(14). A
72 specific language has evolved to describe the potential confounding effects of such ex-
73 posure on the development of subsequent immunity to influenza. HA imprinting is the
74 initial exposure to an influenza strain, first described in childhood H1 influenza, which
75 emerging evidence suggests may protect from subsequent H5 infection (2). However,
76 when a person is sequentially exposed to two related virus strains, they tend to elicit
77 an immune response dominated by antibodies against the first strain they were ex-
78 posed to(15, 16). This is true even following a secondary infection or vaccination. This
79 phenomenon has been variously referred to as "original antigenic sin" (OAS), HA se-
80 niority, or negative antigenic interaction (17, 18, 19).Thus, the immune response to
81 a new influenza viral infection or vaccination is at least partially shaped by preexist-
82 ing influenza immunity. Because there is still antigenic overlap between even mostly
83 dissimilar influenza strains, it is critical to understand the antibody response against
84 antigenically similar virus stains, for vaccine development, especially within the con-
85 text to OAS.

86 The HA protein is composed of two domains, the highly plastic globular HA1 head
87 domain and the conserved HA2 stalk domain. The hypervariable head domain is be-
88 lieved to be immunodominant and virus infection or/and vaccination elicits strain-
89 specific neutralizing antibodies primarily targeting this domain, resulting in limited
90 cross-reactivity to divergent virus strains that vary significantly in HA1 head domain

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91 sequence(20). In contrast, antibodies targeting the conserved stalk HA2-reactive do-
92 main have been shown to broadly cross-react with multiple influenza viral strains (21).
93 The viruses themselves can be categorized based on the phylogenetic distance of HA
94 sequences. Ten clades of H5 HA (clade 0-9) have been identified within the H5N1
95 virus subtype (22). H5N1 viruses from clades 0, 1, 2, and 7 have the capacity to in-
96 fect humans (23). These scatter into three distinct antigenic clusters, as determined
97 by antigenic cartography generated by analyzing neutralizing serum antibody levels
98 elicited in mice vaccinated against single influenza strains (1). As such, an effective H5
99 influenza vaccine would ideally induce broad cross-reactivity that against all three H5
100 clades. However, as discussed above, HA imprinting or OAS may impede generation
101 of broadly cross-reactive H5N1 antibodies if the prime and boost H5N1 vaccine strains
102 reside in different antigenic clusters.

103 To address this issue, we re-analysed serum samples from a previous H5 human
104 vaccine study (DMID 08-0059)(24) using our mPlex-Flu assay multiplex assay(25) to
105 measure the anti-HA IgG antibody against all 10 clades (subclades) of H5 influenza
106 virus. During this study, longitudinal samples were collected prior and post-vaccination
107 with inactivated A/Indonesia/5/05 (Ind05) MIV from subjects: a) who had received
108 two primed H5 MIV vaccinations (A/Hong Kong/156/97 (HK97) in 1997-1998 and
109 A/Vietnam/1203/04 (Vie04) in 2005-2006 (DL-boost group); b) who had only received
110 one Vie04 prime vaccination in 2005-2006 (L-boost group); and c) H5 influenza virus
111 naive group, who were also boosted by Ind05 28 days after the prime event (S-boost
112 group). The mPlex-Flu assay(25) enables us to simultaneously evaluate the magni-
113 tude and breadth of the IgG repertoire directed against HAs from 21 H5 influenza
114 virus strains and 9 other IAV strains (H1, H3, H7, H9). We also introduced a novel mul-
115 tiple dimensional data analysis method: relative antibody landscapes, which enables
116 quantitative analysis of the antibody response to influenza virus antigenic similarity
117 strains related to vaccine strains. The relative antibody landscape enables analysis
118 of antibody-mediated immunity to a spectrum of HAs after H5 vaccine priming and
119 boosting. This report demonstrates that as the relative antigenic distance between
120 the original priming and the new H5 boosting vaccine strain becomes smaller (i.e. the
121 strains are more antigenically similar), the greater the increase in the anti-HA IgG re-
122 sponse to original H5 MIV strain. Thus, in a vaccine response, the original HA im-
123 printing influences vaccine responses occurring significantly later. We discuss the rel-
124 evance of these findings to the development of influenza vaccines designed to induce
125 broad antibody-mediated protection.

126 RESULTS

127 **Characteristics of subjects.** Prior exposure to the predominant seasonal H1 or
128 H3 influenza strain circulating close to a subject's birth year can alter H5 or H7 in-
129 fection and death rates (2, 26). Thus, we first tested tested for differences in age,
130 as a surrogate for circulating strains, that could alter the antibody levels between
131 the H5 vaccine groups. To assess the birth year related influenza virus exposure
132 history, we regrouped the study cohorts based on two key birth years: 1968 and
133 1977, when H3 and H1, respectively, became the dominant circulating influenza A
134 virus strains (Table 1) (2). Subjects without baseline (pre-vaccination) serum samples
135 were excluded, leaving a total of 55 subjects. The H5 naive subjects (Naive, $n = 12$)
136 and primed subjects (L-boost, $n = 30$) previously received an inactivated subvirion
137 influenza A/Vietnam/1203/04 (Vie04) vaccine in 2005-2006(11). The double primed
138 group (DL-boost, $n = 13$) received the recombinant influenza A/Hong Kong/156/97
139 vaccine (A/HK97) in 1997 - 1998 (6) and the Vie04 vaccine in 2005 - 2006. We found no

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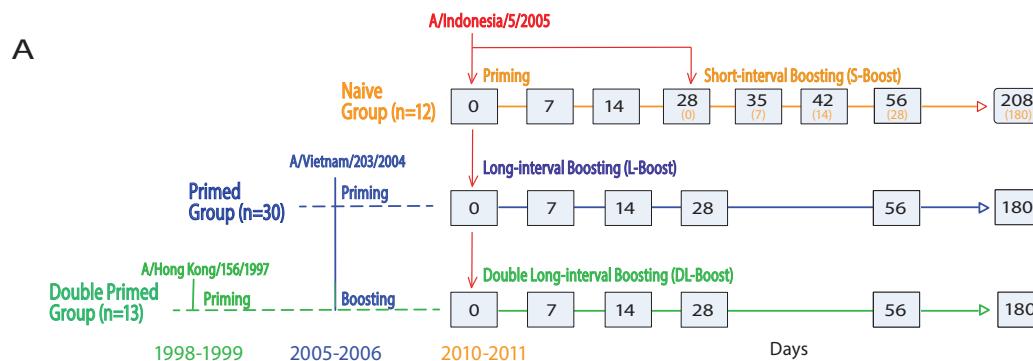


FIG 1 Vaccination strategy. (A) Trial and sampling design: All subjects in the DMID 08-0059 study cohorts were vaccinated with inactivated A/Indonesia/5/05 (Ind05) intramuscular influenza vaccine. The **Naïve group**; **S-boost** received the Ind05 vaccine on day 0, and short interval boosting on day 28. The **primed long-interval boost (L-boost)** group had previously received the inactivated subvirion influenza A/Vietnam/1203/04 (Vie04) vaccine in 2005–2006; and the **double primed long interval boost (DL-boost)** group additionally received the baculovirus expressed recombinant influenza A/Hong Kong/156/97 vaccine (HK97) in 1997–1998. Both L-boost and DL-boost groups also received long-interval vaccination with Ind05 on the day 0. Grey boxes indicate serum sampling.

A) Summary of prime and boost strains and groups.

140 significant difference in birth year distributions between the cohorts ($P > 0.05$; Fisher's
 141 exact test), suggesting that the effects of flu exposure history on the H5 MIV vaccine
 142 response should be similar across the three groups.

TABLE 1 The number of subjects stratified by birth year in each cohort of the DMID 08-0059 study. Subjects were grouped by birth year based on key years when either H3 or H1 representing the predominant circulating seasonal flu strains, as prior exposure history might influence the antibody responses to the H5 vaccines.

Group	Birth Year			Total
	< 1968	1968 to 1977	> 1977	
Circulating strains	H1 or H2	H3	H3 and H1	
Naïve (Short-Interval Boost (S-Boost))	10 (83)	1 (8)	1 (8)	12
Long-Interval Boost (L-boost)	24 (80)	3 (10)	3 (10)	30
Double primed long-Interval Boost (DL-boost)	11 (85)	2 (15)	0 (0)	13

143 **High anti-H5 IgG responses after long-interval boosting are shaped by the**
 144 **priming vaccine strain.** Using a 48-HA mPLEX-Flu assay panel, we observed that IgG
 145 levels against the HA of A/Indonesia/5/05 (Ind05), Vie04 and HK97 were very low in the
 146 naïve group, and about two-fold higher in the short interval boosting (S-boost) group

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147 who were boosted after 28 days (FIG 2 A, B). In both primed groups (L-boost and
148 DL-boost), however, inactivated Ind05 MIV induced ~5-fold higher vaccine-specific an-
149 tibody levels by 14 days post-vaccination. Anti-Vie04 and HK97 IgG levels increased
150 ~7-8 fold, also peaking at 14 days in both primed groups (FIG 2). While both primed
151 groups had higher pre-existing (day 0) anti-H5 IgG levels, their IgG response kinetic
152 curves against the vaccine strains were similar. These differences result in a relative
153 increase in the DL-boost group's anti-HA antibody levels peaking at 3.5-fold (FIG2, D),
154 even though the post-boosting IgG levels are similar in the S- and DL-boost groups. In
155 both groups, anti-H5 HA antibodies levels remained high for over six months. These
156 results are consistent with the previous finding that non-adjuvanted MIVs are poorly
157 immunogenic in naive subjects (6, 7, 8, 9, 10), and long-interval boosting with H5 anti-
158 genic variant MIVs elicits significant and robust antibody responses (11, 24). However,
159 this is the first report to show differences in antibody response induced by single vs.
160 double long-interval MIV boosting.

161 Importantly, we also found that the Ind05 MIV elicited robust antibody responses
162 against the two previous priming H5 strains (Vie04, HK97) in both vaccine groups, and
163 that the anti-HA IgG responses shared similar kinetic patterns. Interestingly, Ind05
164 MIV elicited higher levels of IgG antibodies to Vie04 and HK97 than to Ind05. In order
165 to directly compare the effects of the priming virus strain, we plotted the concen-
166 trations of anti-H5 HA by groups, shown in FIG2 C, and the fold change of antibody
167 concentrations against three vaccine strains of the different groups (FIG2 D). The re-
168 sults revealed higher antibody levels against the HA of Vie04 in the L-boost group,
169 and HK97 in the DL-boost group, which were the first H5 viral strains subjects were
170 respectively vaccinated against. These results could be interpreted as indicative of HA
171 imprinting (16, 15), in which subjects generate a robust antibody response against the
172 H5 influenza virus strain they were first exposed to, by infection or vaccination, and
173 maintain this response over their entire lifetime (29).

174 To confirm the protective activities of the higher level of long-lasting antibodies
175 in the L-boost and DL-boost groups we re-analyzed the HAI and MN data from the
176 DMID 08-0059 study using generalized linear mixed effects models with identity link
177 functions, as we have previously described (27, 28). The results confirmed that all
178 three H5 MIV strain vaccines induced serum with viral neutralizing capacity that could
179 protect cells from viral infection (FIG 2 D and FIG S8).

180 **Relative antigenic response landscapes of H5 MIV HAs.** Our results also raised
181 another fundamental question: Does the magnitude of the imprinted recall response
182 to the primordial H5 HA correlate with the antigenic distance between the HAs of
183 the prime and boost strains? We hypothesized that the antigenic distance between
184 the vaccine strain and a target H5 HA is inversely correlated with the cross-relativity of
185 antibody response induced by the H5 MIV. In other words, smaller antigenic distances
186 from the first influenza virus strain (imprinting strain) produce larger IgG responses.
187 To answer this question, we performed antigenic cartography to quantitatively eval-
188 uate the antigenic distances between H5 clades and subclades.

189 Recombinant H5 HA proteins were expressed and purified. Strains were chosen
190 to cover all 10 H5 clades (0-9) and subclades, and 4 new H5 avian strains (Cl4.4.4.3) iso-
191 lated in the US (TABLE S1, and FIG S1). Antibody reactivity to these strains was plotted
192 against mouse anti-H5 HA IgG serum reactivity generated utilizing a monovalent DNA
193 vaccination approach (FIG S2 A). We thus generated a comprehensive antigenic dis-
194 tance matrix between 17 H5 influenza virus strains and each of 21 H5 and 9 other in-
195 fluenza virus strains using the mPlex-Flu assay. The individual antibody levels against
196 H5 viruses are shown as MFI units at specific dilutions, with the dilution factors be-

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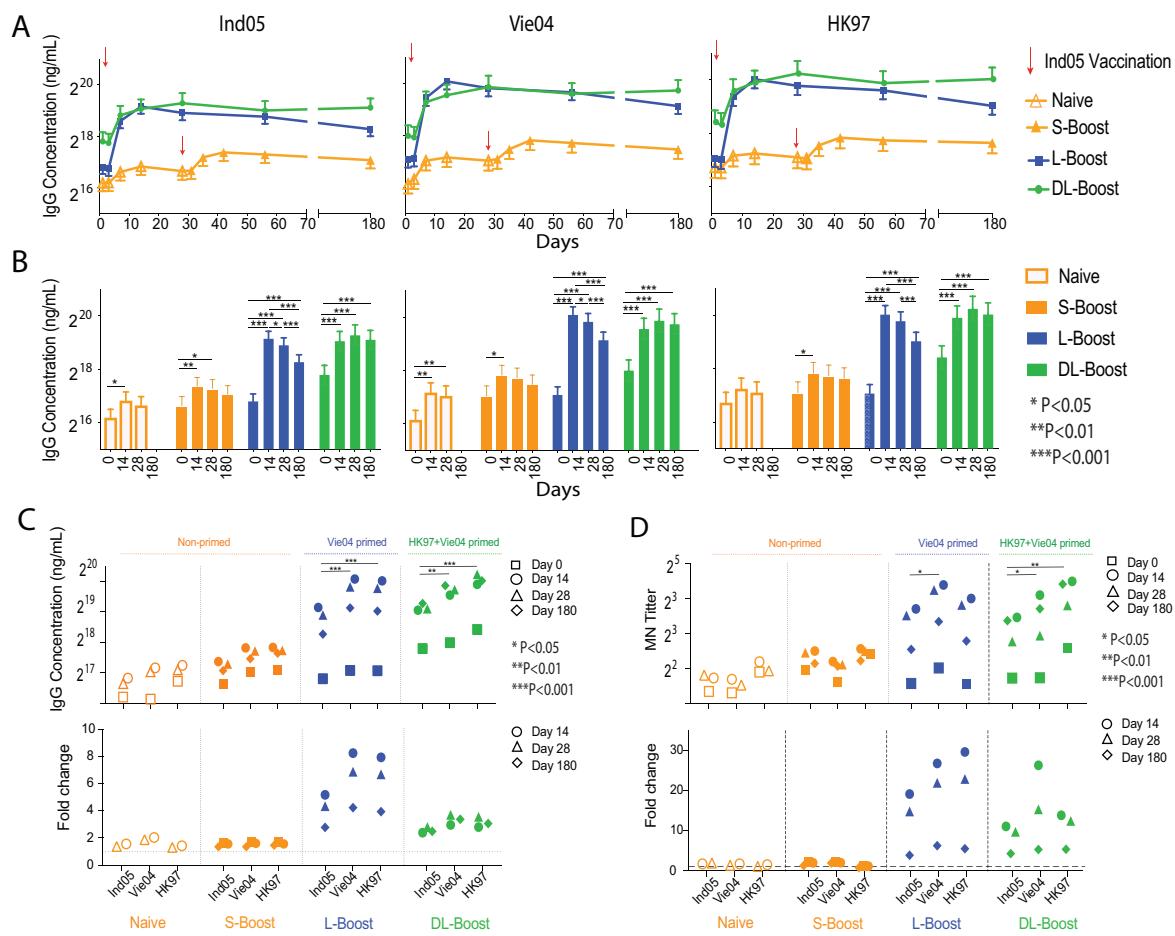


FIG 2 The effects of prior vaccination with H5 monovalent influenza vaccine (MIV) on multiplex HA antibody responses against three different H5 virus boosting vaccine strains. The mean and standard deviation of IgG concentration for each group were estimated by the mPlex-Flu assay. Antibody concentrations were adjusted within the linear mixed effects models using age at enrollment, gender, ethnicity (Caucasian vs. non-Caucasian), dose (two dose levels: 15 and 90 μ g), and assay batch (five batches) (27, 28). A. The H5 kinetic antibody levels against three vaccine strains after MIV H5 vaccination with A/Indonesia/05/2005 (Ind05; clade 2). The prime response (Naive, unfilled symbols) and short-interval boost response (S-boost, filled symbols) of naive subjects; the long-interval boost response (L-boost) after one dose of Ind05 MIV in subjects primed by Vie04 MIV the 5 years previously, and in the subjects who were double primed with Vie04 (5 years previously) and A/Hong Kong/156/1997 (HK97; clade 0) HK97 (12-13 years previously), as the double long-interval boost response (DL-boost), against Ind05, A/Vietnam/1203/2004 (Vie04; clade 1) and A/Hong Kong/156/1997 (HK97; clade 0), three vaccine H5 strains. B. Comparison of antibody responses between time points in the same groups for each vaccine strain. C. The antibody concentrations against each vaccination strain and fold changes as compared to day 0, grouped by study cohort. D. The antibody titers for micro-neutralization (MN) against each vaccination strain and fold changes, as compared to day 0, grouped by study cohort. The original MN assay data was been re-analysed with linear mixed effects modeling, as above. Shown are the geometric mean of titers. * P<0.05, **P<0.01, ***P<0.001 Linear contrasts within the linear mixed effects model framework were used to conduct the statistical comparisons.

ing normalized using a generalized linear model with an identity link function for the sera samples. We used classical multidimensional scaling (MDS) (30) to project relative distances between strains into 2 dimensions, and the matrix data was created by calculating a Euclidean distance matrix from two-dimensional coordinates. Finally, we used a modification of the approach of Smith, et al. (31) to visualize the antigenic distance between influenza virus HAs (31, 1) (FIG S2, C). This approach accounts for the continuous nature of the mPlex-Flu assay data and the consistent range of estimated strain-specific binding (27, 28), yielding the same results as antigenic cartography. The

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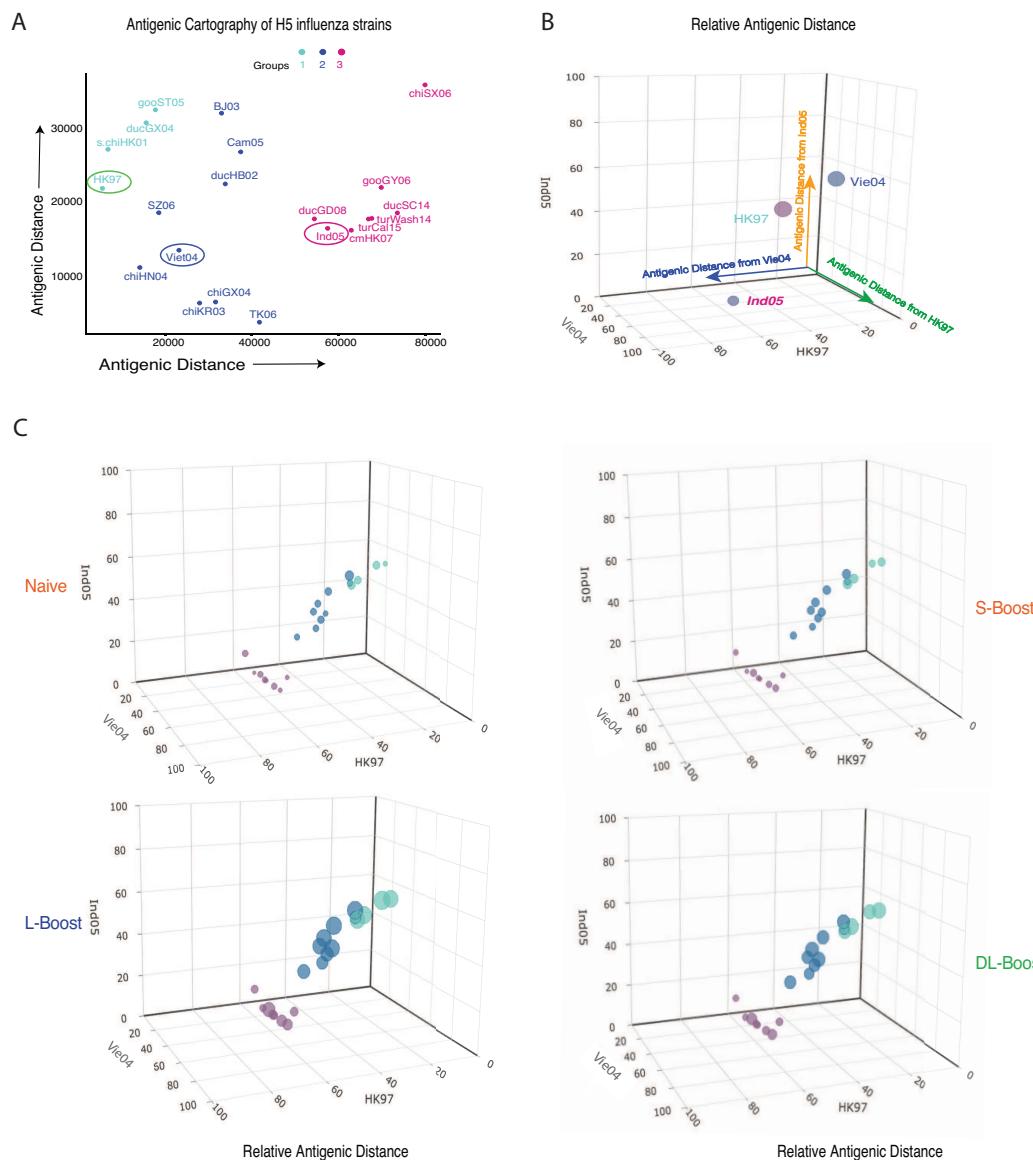


FIG 3 HA antibody responses were plotted against the related antigenic distances to each monovalent H5 vaccination (MIV) strain of different study cohorts. **A.** Antigenic cartography of 21 H5 influenza virus strains generated by mPlex-Flu assay of antisera against 17 anti-H5 influenza viruses, and plotted using using classical multi-dimensional scaling (MDS; see Methods). The three vaccine strains are circled. **B.** We then used three dimensional plots to show the relative antigenic distance of all mPLEX-Flu target HAs to the three vaccine strains A/Hong Kong/97 (HK97, clade 0), A/Vietnam/1203/2004 (Vie04; clade 1), A/Indonesia/05/2005 (Ind05; clade 2). **C.** The IgG response of subjects in the DMID 08-0059 study to 21 H5 strains plotted in 3D bubble plots. The relative antigenic distances of the 21 H5 strains assayed were plotted against their antigenic distance to each of the three MIV strains to determine giving 3D-antigenic cartography. The bubble size represents the concentration (10^4 ng/mL) of IgG against an H5 influenza virus at day 14 post MIV boosting. (A) Using unsupervised hierarchical clustering, three H5 antigenic groups were identified. Interactive 3D bubble plots can be accessed through the following links: Prime group (<http://rpubs.com/DongmeiLi/565996>); S-boost group(<http://rpubs.com/DongmeiLi/565998>); L-boost: (<http://rpubs.com/DongmeiLi/565989>); DL-boost: (<http://rpubs.com/DongmeiLi/565994>).

205 antigenic distance matrix was also generated from the above multiplex data of mPlex-
206 Flu assay using the single virus DNA vaccine anti-sera. (FIG S3).

207 In order to show the relative antigenic distance between individual HAs and the
208 H5 MIV strains (FIG 3 B), we plotted the distance of each H5 HA relative to the 3 vac-
209 cine strains: HK97 (X-axis), Vie04 (Y-axis) and Ind05 (Z-axis). Each marker diameter

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represents the magnitude of the IgG concentration 14 days after MIV boosting. This allowed visualization of the magnitude of the antibody response against specific H5 HAs, associated with the antigenic distances with respect to both prime and boost vaccine strains in the different cohort groups. The same diagram allowed visualization of H5 strain vaccine strain relative distances from other H5 strains. Naive subjects had low anti-HA IgG levels against all H5 strains after priming and short-interval boosting with MIV. However, the L-boost and DL-boost groups had significantly enhanced antibody responses after 14 days, with higher IgG responses to H5 strains in the Vie04 and HK97 cluster groups than to the viruses in the MIV Ind05 cluster group, which are antigenically similar to the strain of the more recent MIV (FIG 3 C). These data more clearly show the relationship between the anti-HA IgG antibody response and the antigenic distances to the reference strains: higher cross-reactive antibody levels are elicited against the HAs from strains in the same cluster group with the first priming virus strain.

Long-interval boosting (L-boost) of MIV elicited heterogeneous IgG responses against all H5 clade/subclades, which were correlated with the antigenic distance to the first primed virus strains. We next generated antigenic landscape plots (26) to visualize the magnitude of serological responses in relation to the antigenic distance between the vaccine strain HA and the H5 HAs in the mPlex-Flu panel. We first focused on the relationship between the magnitude of boosted IgG response and the antigenic distance between the boost HA and the three H5 vaccine strains. To this end, IgG antibody concentrations against 21 H5 strains were measured by mPlex-Flu assay for each cohort on days 9, 14, and 28, which were plotted against their relative antigenic distances to Ind05 (FIG 4A, B), Viet04 (FIG 4C, D), and HK97 (FIG 4E, F). Correlation test results are given in the figure inset, and all data are presented in FIG S4, S5, S6).

We found that the immune response in the Naive and S-boost groups were very weak, and since subjects in these groups were only exposed to the Ind05 MIV strain, we made antigenic landscapes (26) using Ind05 as the reference influenza virus strain. The relative antigenic landscapes for these two groups at days 0, 14 and 180 are shown in FIG4 A and B. Similarly, the serological responses of the L-boost and D-boost groups after boosting were plotted against the antigenic distance relative to Vie04 and HK97, shown in FIG4 C and D. Note that the antigenic distance between the cognate vaccine strain and itself is zero (e.g. Vie04 - Vie04 = 0). The Ind05 MIV showed very low antigenicity in both naive subject groups. Changes in IgG concentration ($\Delta IgG = [IgG_t] - [IgG_{day0}]$) were not correlated with antigenic distance ($P = 0.014$ and 0.020). However, Ind05 MIV boosting showed higher antibody responses to HAs from strains with a smaller antigenic distance in both L-boost ($R^2 = 0.57$) and DL-boost groups ($R^2 = 0.73$). These results support our hypothesis that that the imprinting of primed individuals is highly correlated with the related antigenic distance to the priming strains for long-interval H5 vaccination. FIG 4.

Long-interval boosting with H5 MIV induced broadly heterosubtypic antibody responses against Group 1 influenza viruses. To assess the breadth of heterosubtypic immunity generated by the H5 MIV prime and boost strategy, including IgG reactive against other influenza virus HAs, we estimated antibody cross-reactivity to select group 1 (H1, H2, H5, H6, and H9) and group 2 (H3, H4, H7) HAs (Table S1) using the mPlex-Flu assay (FIG 5). In all subjects, we detected high pre-existing anti-H1 HA subtype IgG levels against older (A/South Carolina/1/18 (SC18), A/Puerto Rico/8/1934 (PR8)) and newer (A/New Caledonia/20/1999 (NewCall99), A/California/07/2009 (Cali09)) strains. However, these anti-HA levels were not significantly affected by H5 MIV vac-

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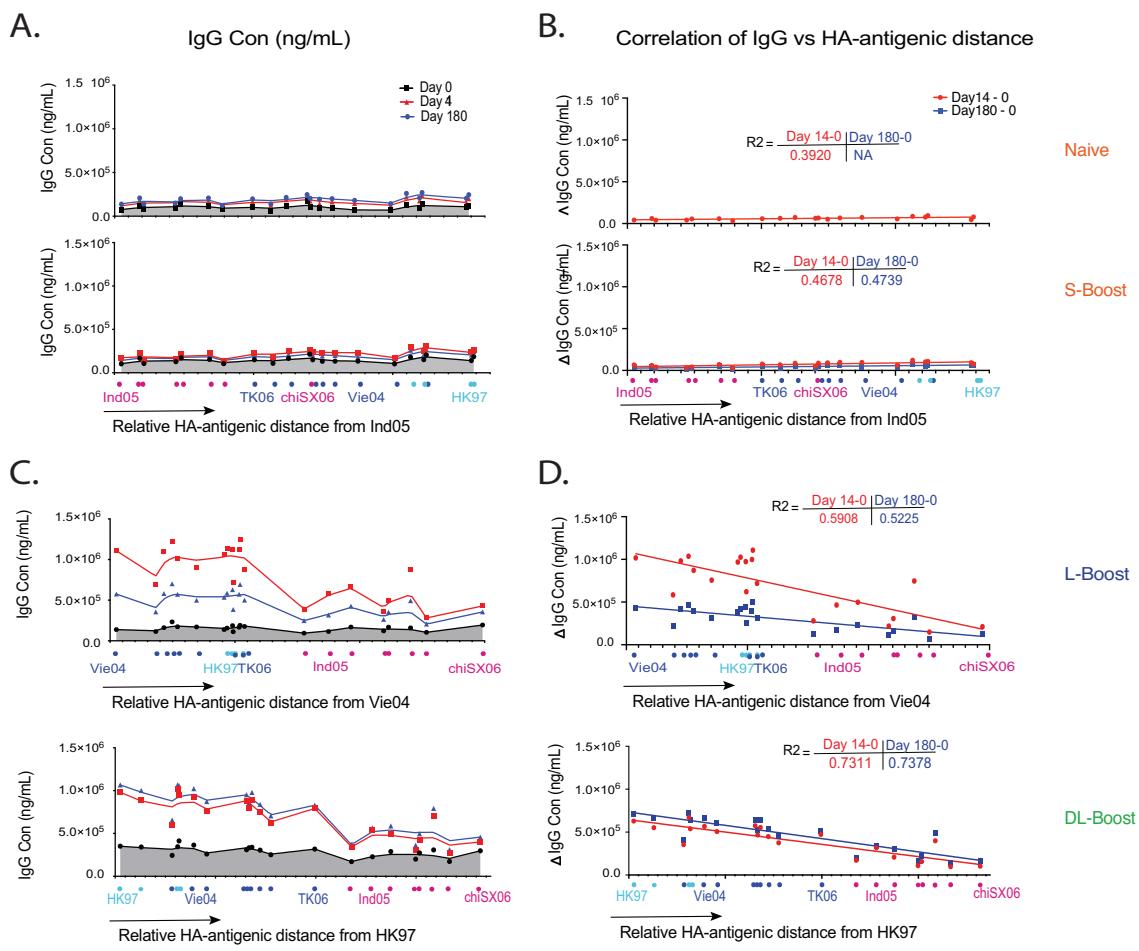


FIG 4 Relative HA antibody landscapes, anti-HA IgG levels and relative antigenic distances from vaccine strains. **A.** The relative HA antibody landscapes of H5 virus strains as a function of the relative HA antigenic similarity distance from the vaccination strain Ind05 for the Naive group and short interval boost (S-boost) group (see Materials and Methods). **B.** Correlation of the HA antibody response to the HA-antigenic distance from the vaccine strain HAs of the Naive and S-boost groups. The coordinates of each H5 strain result represent the relative antigenic distance of H5 HA_i to the vaccine strain HA on each axis. **C.** Relative HA antibody landscapes for each group using the relative HA antigenic distance from the H5 reference strains A/Vietnam/1203/2004 (Vie04; clade 1), or A/Hong Kong/97 (HK97, clade 0). **D.** The correlation between the HA antibody response and the HA-antigenic distance to the imprinting (first exposure) H5 strain: Vie04 for the long-interval boost group (L-boost) or HK97 for the double long-interval boost group (DL-boost). The change of IgG concentration (ΔIgG_{conc}) is the difference between the anti-HA antibody concentration of past-vaccination from that of prior vaccination. The R^2 values were calculated from linear regression fitting.

cination (FIG S7 A). In addition, we found dramatic increases in anti-HA IgG levels targeting other group 1 influenza viruses (e.g. H2, H6) that had lower baseline levels compared to those against influenza group 2 (H1, H3) subtype virus HAs.

Further analysis demonstrated that post-H5 vaccination IgG reactivity across influenza virus strains was inversely correlated to both phylogenetic and antigenic distance between the strains, especially the stalk regions. Based on phylogenetic distance, the gene sequence of H6 is closer to H5 than H9 (20). Similarly, the gene sequence of H2 is closer to H5 than H6 and H1 (FIG S1 A). In addition, we found that IgG responses induced by H5 MIV against HA of A/Japan/305/1957 (Jap57, H2) were significantly higher than that against A/Taiwan/2/2013 (TW13, H6) and A/guinea fowl/Hong Kong/WF10/1999 (gfHK99, H9) (FIG5, FIG S7 A), the latter two strains have stalk regions phylogenetically and antigenically distant from the H5 clade stalk. We

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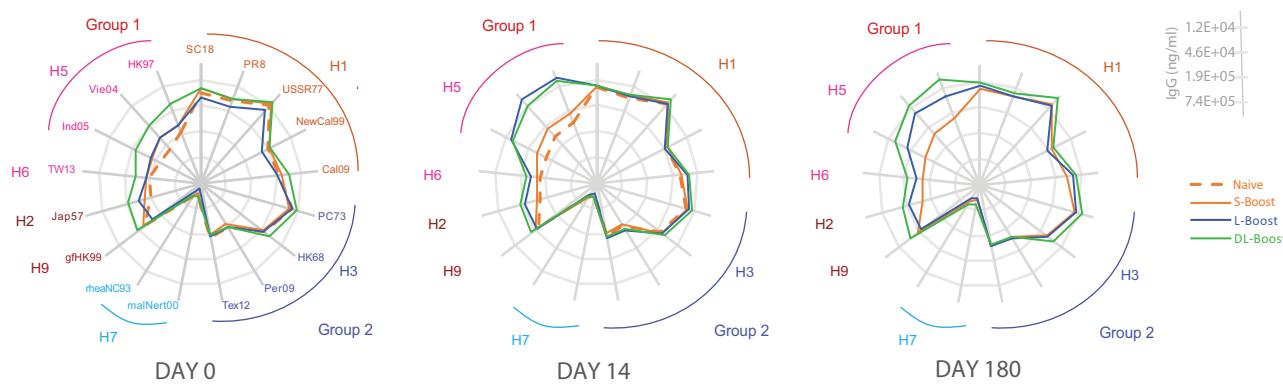


FIG 5 The human heterosubtypic IgG antibody response elicited by H5 MIV. The IgG antibody response induced by H5 influenza vaccine against previously circulating or vaccine virus strains (H1, H2, H3, H6, H7, H9), were measured by mPlex-Flu assay pre- (day 0) and post- vaccination (days 14, 180).

272 also found that, in both primed groups, H5 MIV elicited cross-reactive anti-H2 IgG re-
273 sponses in naive subjects, with a higher peak and a sustained duration than in the
274 Naive subjects. Those responses were stronger than those against H6 and H9 HAs.
275 No significant changes were detected in IgG levels against H3 and other group 2 in-
276 fluenza viruses (FIG S7 B). Together, these findings also support the hypothesis that
277 cross-strain, anti-HA antibody responses are highly correlated with phylogenetic simi-
278 larity, and inversely correlated with antigenic distance, to the vaccine strain.

279 **Long-interval boosting elicited IgG antibodies against the HA head domain.**
280 The HA stalk domain is highly conserved within influenza virus phylogenetic groups,
281 and stalk-reactive antibodies have been hypothesized to be the major contributors
282 mediating cross-reactivity of anti-HA IgG antibodies across group 1(32) strains. How-
283 ever, broadly cross-reactive neutralizing antibodies against the HA head domain have
284 recently been identified, and could also contribute to this phenomenon (reviewed in
285 (33)). Thus, we next measured the change in the relative proportions of head versus
286 stalk reactive IgG within H5 boosting group.

287 H5 head (HA1) specific IgG levels were measured using beads coupled with the
288 Ind05 head domain only. Anti-stalk IgG was measured using chimeric cH9/1 and
289 cH4/7 proteins to estimate, respectively, group 1 and group 2 stalk-reactive antibod-
290 ies (34, 35, 36). The results demonstrate that short-interval boosting can induce an
291 ~2 fold increase in anti-H5 head IgG levels in naive subjects (FIG 6). In addition, sig-
292 nificant increases in head-specific IgG were also detected in the L-boost group: 27
293 fold (14d), 20 fold (28d), and 10 fold (180d). Examining the DL-boost group, ~7-8 fold
294 increases were observed at 14, 28, 180 days after vaccination. High levels of group
295 1 stalk-reactive IgG were found in both boosting groups. However, these increases
296 accounted for less than a 2-fold overall change in IgG levels, primarily because these
297 stalk-reactive IgG antibodies were present at relatively high levels prior to vaccination.
298 We did not observe any significant post-vaccination increases in group 2 stalk-reactive
299 antibody levels regardless of test groups. Overall, our results suggest that broadly
300 cross-reactive IgG against H5 influenza virus HAs or the phylogenetic group 1 are most
301 likely mediated by conserved epitopes on the head domain of HA as opposed to the
302 stalk domain.

Cross-reactive IgG response to H5 vaccination

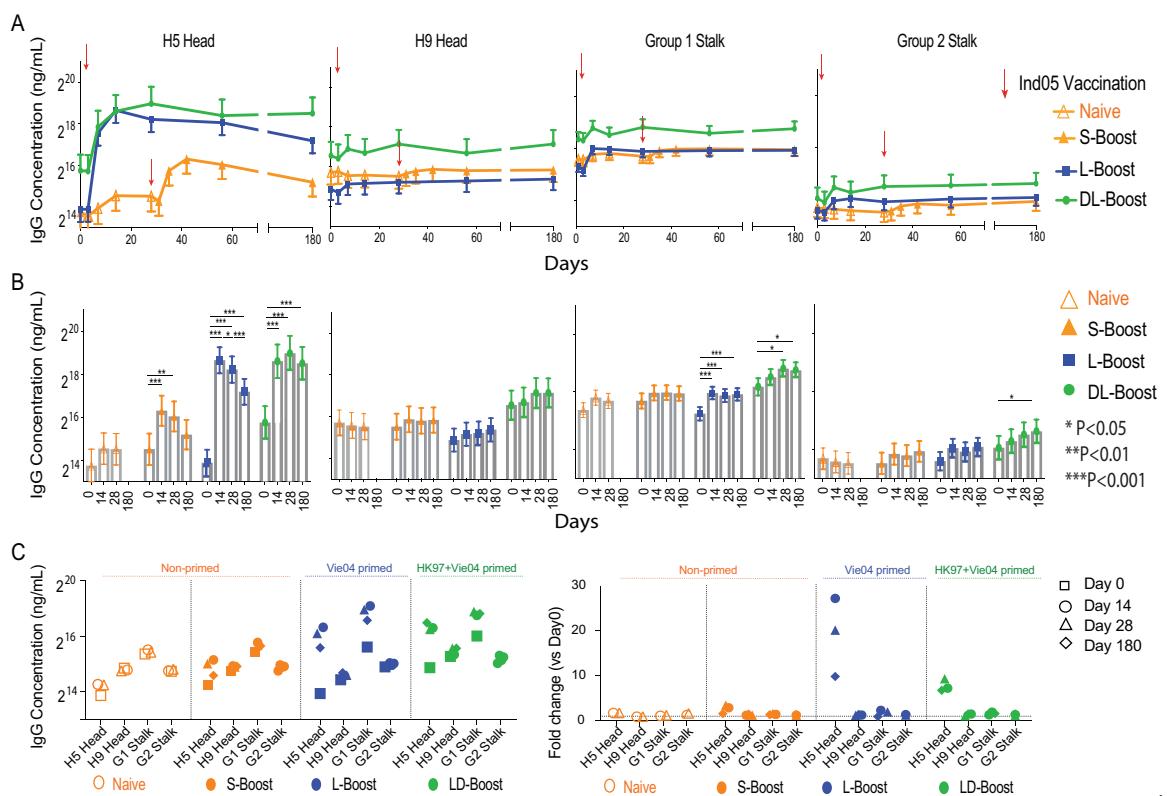


FIG 6 The head and stalk-reactive IgG response induced by the human MIV H5 vaccine. **A.** The kinetic profile of the IgG response against the HA head or stalk domain estimated by mPlex-Flu assay. **B.** Comparison of concentrations of each H5 HA specific antibody pre- (day 0) and post-vaccination (14, 28 and 180 days). Linear contrasts within the linear mixed effects models framework were used for statistic testing (* P<0.05, **P<0.01, ***P<0.001). **C.** Comparison of anti-HA IgG concentrations between HAs, including antibodies against chimeric ch9/1 HA (termed group 1 stalk-reactive antibodies; G1 Stalk), and ch4/7 HA (termed group 2 stalk-reactive antibodies; G2 Stalk).

DISCUSSION

Two major impediments to universal flu vaccine development are the constant antigenic changes of influenza viruses, and that the human antibody response is shaped by prior influenza virus exposure history (37). In addition, vaccination strategies for emergent influenza viruses need to take into account both the vaccination schedule, and the ability of HA imprinting to can hinder immune responses to new antigens. Antibody mediated immune responses to new HA antigens are generally weak after the priming vaccination, and require further boosting to elicit adequate titers for infection prevention. This phenomenon can be leveraged if the subject has been primed by exposure to HA antigens, by prior infection or vaccination of H1 or H3 influenza virus, that are antigenically distance from emergent strain HAs (heterosubtypic immunity).

The antigenic distance between two virus strain HAs can be calculated empirically or experimentally. Empirically, antigenic distance is correlated with the difference between surface protein sequences of HA (e.g. edit distance, Damerau-Levenshtein distance). Experimentally it can be derived by calculating the n-dimensional distance between immune reactivity of sera from a subject vaccinated with a single virus against a panel of other HAs from disparate virus strains (37). As we have previously shown (35), the smaller the antigenic distance between the prime and boost HAs, the stronger the post-boost vaccination increase in vaccine specific anti-HA IgG levels.

In this study, we also analyzed changes in multi-dimensional anti-H5 HA IgG re-

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sponses after vaccination and boosting using a modification of the antibody landscape method (29), a variant of antigenic cartography (31). We initially analyzed anti-HA IgG antibody levels against a comprehensive panel of H5 clade/subclade HAs as a function of the relative antigenic distance to the reference vaccine HA. We call this multi-dimensional measure the *relative antibody landscape* (Fig 4 A and C). This novel method, combined with multiplex serum IgG measurements, allows an analysis of the breadth of the antibody response as a function of the antigenic distance from the vaccine strain. Our results using the relative antibody landscape method show that the anti-H5 HA IgG responses elicited by boosting in both primed groups are highly correlated with the antigenic distance between the priming and boosting H5 vaccine strains. These findings provide further evidence of for the HA antigenic imprinting in H5 influenza vaccination. Most significantly, we demonstrate that relative antibody landscape methods can be used to analyze the effects of previous HA antigen exposure on vaccine responses, allowing for quantitative analysis of antigenic imprinting.

Our work also demonstrates that long-interval boosting augments H5 vaccine-induced immunity. Studies using variants of the H5 MIVs have shown that long-interval prime-boost strategies, on the order of 4-8 years between vaccinations, result in robust and durable antibody responses (11) to what are relatively poorly immunogenic vaccine components (6, 7, 24). Intermediate intervals of 6-12 months between priming and boosting with H5 variants significantly increases antibody responses (38, 39), compared to 8 weeks or less. One potential mechanism for these results is a time-dependent increase in long-lived memory B cells, which may take 2-4 months after vaccine priming (40). These memory B cells can then respond rapidly to long interval boosting (41). Studies showed that adjuvanted H5 MIV used in short-interval boosting also significantly increased the immunogenicity of vaccines (42, 43, 44, 45), and indicated that prime-boost vaccination induced the monoclonal antibodies largely recognized the HA head region of the H5 MIV strain(46). Significant additional work is necessary to define the optimum prime-boost interval for robust responses.

Our results also support the hypothesis that long-interval boosting increases antibody responses targeting the HA head domain, rather than the stalk. Recently, several broadly neutralizing antibodies (bnAbs) have been identified from both infected or vaccinated human subjects that target the hypervariable HA head domain, including C05 (47), 5J8 (48), CH65 (49) and CH67. These bnAbs exhibit considerable neutralizing breadth within the H1 (47, 48, 49) and H3 (50) influenza virus subtypes. Such bnAbs are thought to bind highly conserved regions on the sialic acid receptor binding site (RBS) in the HA head domain, explaining their ability to broadly neutralize viral binding from different subtypes (49, 51). As the head domain is known to be immunodominant in the induction of strong antibody responses, broadly head-reactive antibodies could be the major mediator of cross-reactive immunity across subtypes or hetero-subtypes. Our results are also consistent with recent work that found rapid activation and expansion of pre-existing memory B cell responses to the conserved epitopes on the HA stalk and head domains after long interval prime-boost vaccination with H7N9 (40).

Finally, our results contribute further to a framework for thinking about influenza vaccine development strategies. The aspirational goal of a influenza vaccine is to create long-lasting protective immunity to a wide spectrum of influenza viruses. In such cases, future exposure, via infection or vaccination may occur years after the initial priming and imprinting event. Our work demonstrates that the long interval prime-boost strategy for H5 vaccination induces long-lasting cross-reactive antibodies against conserved regions on the HA1 head domain. This may help in universal

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373 influenza vaccine development not as a single vaccine, but as a long-interval boost
374 strategy to generate cross-reactive antibodies to recognize the conserved sites on HA1
375 head domain.

376 In conclusion, we used a multiplex antibody assay and a novel antibody landscape
377 method to analyze antibody mediated immunity to various HAs after H5 vaccine prim-
378 ing and boosting. These methods quantitatively account for the antigenic distances
379 between the vaccine and other strain HAs. This new approach demonstrated that anti-
380 H5 IgG antibody responses elicited by boosting are highly correlated to the antigenic
381 similarity between the priming and boosting H5 vaccine strains, providing evidence
382 for OAS and HA imprinting within the context of H5 vaccination.

383 MATERIALS AND METHODS

384 **Human Subjects Ethics Statement** This sub-analysis study was approved by the
385 Research Subjects Review Board at the University of Rochester Medical Center (RSRB
386 approval number RSRB00012232). Samples were analyzed under secondary use con-
387 sent obtained previously as part of prior clinical trial (24). All research data were coded
388 by sample IDs in compliance with the Department of Health and Human Services' Reg-
389 uulations for the Protection of Human Subjects (45 CFR 46.101(b)(4)).

390 **Samples and data** Serum samples for the multiplex assay were obtained from
391 a prior clinical trial, DMID 08-0059 (Figure 1)(24). Subjects without pre-vaccination
392 serum samples (Day 0 baseline) were excluded. All subjects in the three cohorts were
393 inoculated with inactivated A/Indonesia/5/05 (A/Ind05) vaccine. H5 naive subjects
394 ($n = 12$), who were healthy adults, not at risk for H5 exposure and with no H5 vaccina-
395 tion history, received 2 identical A/Ind05 vaccinations separated by 28 days. Primed
396 subjects ($n = 30$) previously received the inactivated subvirion A/Vietnam/1203/04
397 (A/Vie04) vaccine in 2005-2006 (11). The double primed group ($n = 13$) had received
398 both the recombinant A/Hong Kong/156/97 vaccine (A/HK97) in 1997-1998 (6) and the
399 influenza A/Vie04 vaccine in 2005-2006. Serum samples were collected before vacci-
400 nation (Day 0) and on days 7, 14, 28, 56, and 180 after vaccination. Serum samples
401 were collected from the naive group subjects on days 7, 14, and 28 days after the sec-
402 ond immunization. All data from the mPlex-Flu, HAI, and MN assays were adjusted for
403 dose difference using linear mixed effects models, as previously described (27, 28).

404 **mPLEX-Flu Analysis** We estimated the concentrations of anti-HA IgG antibodies
405 against a 45 HA antigen panel of influenza viruses using the mPLEX-Flu assay, as de-
406 scribed previously(25, 34). All influenza HA sequence identifiers used are listed in the
407 TABLE S1 and the HA genetic distance (phylogenetic tree) is shown in FIG S1 A . The
408 panel recombinant HA proteins were expressed by baculovirus system and purified
409 Ni⁺ affinity column selection as previously described (34) and verified (FIG S1 B).

410 The calculation of individual IgG concentrations for each influenza strain anti-
411 HA IgG was performed using standard curves generated from five-parameter logis-
412 tic regression models (27, 28). All IgG concentration results from the mPlex-Flu as-
413 say was adjusted using linear mixed effects models accounting for the group, day,
414 and group-day interactions for each H5 vaccine strain. Covariates adjusted in the lin-
415 ear mixed effects models included age at enrollment, gender, ethnicity (Caucasian vs.
416 non-Caucasian), dose (two dose levels: 15 and 90 μ g), and analytic batch (five batches)
417 factors (27, 28).

418 **Antigenic cartography of H5 influenza viruses generated by mPlex-Flu assay**
419 **data.** In order to estimate the antigenic distance of HA antigens of H5 influenza virus
420 strains, we adopted the 17 H5 HA genes that covered all 10 clades/subclades strains
421 of H5 from Dr. Paul Zhou from Institute Pasteur of Shanghai, Chinese Academy of Sci-

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422 ences, Shanghai, China (1). The 17 individual antisera against each H5 influenza virus
423 strain were generated with mouse DNA vaccination as previously described (1), and
424 shown in **FIG S2 A**. Using the mPlex-Flu assay, we evaluated the 17 anti-sera against a
425 panel of 36 HA antigens to create a multi-dimensional matrix, after normalizing the di-
426 lution factors and subtracting the background levels, using generalized linear models
427 with identity link functions (**FIG S2 B**). Classical multidimensional scaling was used to
428 project multi-dimensional distances into two-dimensional antigenic cartography plots
429 plots(30, 25). The coordinates for two-dimension antigenic cartography were further
430 used to calculate the Euclidean distance between H5 influenza viruses to obtain the
431 antigenic distance matrix(**FIG S3**).

432 **Relative antigenic landscapes of antibody response.** Based on the antigenic
433 distances generated above, and using the three vaccine strains as reference: A/Hong
434 Kong/156/97 vaccine (HK97, clade 0) A/Vietnam/1203/04 (Vie04, clade 1) A/Indonesia/5/05
435 (Ind05, clade 2) a vaccine-strain relative antigenic distance matrix was selected. Next,
436 relative antigenic antibody landscape-like figures were created by using the relative
437 antigenic distance as the X-axis and the Y-axis is IgG antibody response. Data points
438 were linked by LOWESS fit spline curves (Prism 8 software). A set of antibody response
439 landscape-like plots were generated for each vaccination strain.

440 **H5 head and stalk specific antibody response.** We used the mPlex-Flu assay
441 to simultaneously assess the antibodies to the head and stalk domains of HA. We
442 coupled Luminex beads with the head region of HA, which are purified recombinant
443 proteins of HA1 domain of H5/Ind05 and H9/A/guinea fowl/Hong Kong/WF10/1999
444 (gfHK99, H9). To detect the group 1 stalk-reactive antibodies, we used the chimeric
445 cH5/H1 (head/stalk) and cH9/H1 proteins. For group 2 stalk-reactive antibodies, we
446 used the cH5/H3 and cH7/H4 proteins kindly provided by Dr. Florian Krammer(52, 32,
447 34, 35).

448 **Reanalyses of HAI and MN data** Primary HAI and MN data were generated pre-
449 viously during the vaccine trial as described (24). Serum antibody responses to the
450 homologous A/Indonesia/05/2005 PR8-IBCDC-RG2 virus were measured at the South-
451 ern Research Institute (6). We reanalyzed these data using linear mixed effects models,
452 with correlations from repeated measurements within the same subject considered.
453 The same predictors and covariates were used in the linear mixed effects models for
454 the HAI and MN data analysis as for the mPLEX-Flu data analysis (27).

455 **Availability of data and materials.** All data generated in this study are included
456 in this published article and in the Supplementary Material.

457 **SUPPLEMENTAL MATERIAL**

458 **Supplementary Material Main Text**

459 **Supplementary Table 1:** The mPlex-Flu assay panel of seasonal influenza viruses,
460 H5 clades and subclades.

461 **Supplementary Figure 1:** HA protein characters of 35 influenza virus A strains
462 in mPlex-Flu assay. A. The phylogenetic tree was generated using HA amino acid se-
463 quences of the 35 influenza A virus strains obtained from the phylogenetic tree maker
464 on the Influenza Research Database Website (<https://www.fludb.org/brc/home.spg?decorator=influenza>).
465 B. SDS-PAGE gel image of purified HA proteins of H5 influenza viral strains. C. HPLC
466 analysis results of four representative HA proteins flowing through the Biosep-SEC-
467 S4000 columns with the Bio-rad protein standards.

468 **Supplementary Figure 2:** Antigenic cartography is generated with a mouse DNA
469 vaccination model. A. Mouse DNA vaccination strategy. B. Heat map of the multiple
470 dimensional antibody data generated by the mPlex-Flu assay. Each mouse polyclonal

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471 antiserum was induced by DNA vaccination with a DNA plasmid encoding HA proteins,
472 and the antibody levels in the sera were estimated by mPlex-Flu assay. C. Antigenic
473 cartography of 36 influenza A strains assessed by mPlex-Flu assay with the Multiple
474 Dimensional Scaling (MDS) method.

475 **Supplementary Figure 3:** The heat-map matrix of the antigenic distance be-
476 tween the 21 H5 influenza virus strains. The three vaccination strains are highlighted
477 with red arrows.

478 **Supplementary Figure 4:** The correlation between the HA antibody response
479 and HA antigenic similarity of A/Hong Kong/156/97 (HK97) to 21 H5 influenza virus
480 strains. A. The HA antibody response landscape-like plots of each group using the rel-
481 ative HA antigenic distance of A/Hong Kong/156/97 (HK97, clade 0) as the reference
482 strains (see material and methods). X-axis is relative antigenic distance; Y-axis is IgG
483 antibody response; the spots were linked by LOWESS fit spline curve (Prism 8 soft-
484 ware). B. The correlation of the HA antibody response to the HA-antigenic distance.
485 The Δ change of antibody concentration of pre- and post- vaccination versus the rela-
486 tive HA antigenic distance of Vie04. The R squared values were calculated with simple
487 linear regression analysis (Prism 8 software).

488 **Supplementary Figure 5:** The correlation between the HA antibody response
489 and HA antigenic similarity between A/Vietnam/1203/2004 (Vie04) and 21 H5 influenza
490 virus strains. A. The HA antibody response landscape-like plots of each group using
491 the relative HA antigenic distance of A/Vietnam/1203/2004 (Vie04, clade 1) as the ref-
492 erence strains (see material and methods). X-axis is relative antigenic distance; Y-axis
493 is IgG antibody response; the spots were linked by LOWESS fit spline curve (Prism 8
494 software). B. The correlation of the HA antibody response to the HA-antigenic dis-
495 tance. The Δ change of antibody concentration of pre- and post- vaccination versus
496 the relative HA antigenic distance of Vie04. The R squared values were calculated with
497 simple linear regression analysis (Prism 8 software).

498 **Supplementary Figure 6:** The correlation between the HA antibody response
499 and HA antigenic similarity of A/Indonesia/5/05 (Ind05) to 21 H5 influenza virus strains.
500 A. The HA antibody response landscape-like plots of each group using the relative HA
501 antigenic distance of A/Indonesia/5/05 (Ind05, clade 1) as the reference strains (see
502 material and methods). X-axis is relative antigenic distance; Y-axis is IgG antibody
503 response; the spots were linked by LOWESS fit spline curve (Prism 8 software). B. The
504 correlation of the HA antibody response to the HA-antigenic distance. The Δ change
505 of antibody concentration of pre- and post- vaccination verse the relative HA antigenic
506 distance of Vie04. The R squared values were calculated with simple linear regression
507 analysis (Prism 8 software).

508 **Supplementary Figure 7:** The IgG concentration of group 1 and 2 influenza virus
509 strains was estimated by mPlex-Flu assay in the DMID 08-0059 study. The mPlex-Flu
510 assay estimated the mean and standard deviation of IgG concentration for each group.
511 Then the antibody concentrations were adjusted within the linear mixed-effects mod-
512 els, which included the following: age at enrollment, gender, ethnicity (Caucasian vs.
513 non-Caucasian), dose (two dose levels: 15 and 90 μ g), and batch (five batches). A.
514 The mPlex-Flu assay estimated the antibody concentrations of group 1 influenza virus
515 strains (including five human H1, one of each H2, H6, and H9). B. The antibody con-
516 centrations to group 2 influenza A virus strains (including four H3, and two H7 strains)
517 were estimated by the mPlex-Flu assay.

518 **Supplementary Figure 8:** Prior vaccination with a monovalent influenza vac-
519 cine (MIV) increased the serum titers of hemagglutination-inhibition (HAI) and micro-
520 neutralization (MN) antibody responses against three antigenically drifted virus vac-

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521 cine strains, including new vaccine strain A/Indonesia/05/2005 (Ind05; clade 2), previous
522 MIV strains A/Vietnam/1203/2004 (Vie04; clade 1), A/Hong Kong/156/1997 (HK97;
523 clade 0). Naive subjects (**Unprimed**) received the MIV Ind05 strain and were subse-
524 quently boosted at day 28 with the same strain. A previous primed group, received
525 the MIV Vie04 5 years prior, (**Primed**) then received a single dose of Ind05. The previ-
526 ous double primed MIV Vie04 and HK97 (**Multiple**). The mean and standard deviation
527 of IgG concentration for each group were estimated by linear mixed effects models
528 with group, day, and group-day interaction used to fit the data for each H5 vaccine
529 strain. Covariates adjusted in the linear mixed effects models included the following:
530 age at enrollment, gender, ethnicity (Caucasian vs. non-Caucasian), dose (two dose
531 levels: 15 and 90 μ g), and batch (five batches). * P<0.05, **P<0.01, ***P<0.001 Lin-
532 ear contrasts within the linear mixed effects models framework were used to do the
533 statistical testing.

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547 J.W. and M.Z. conceived of the project, designed and oversaw the experiments
548 the experiments and analysis, and wrote the paper. J.T. provided the data and sam-
549 ples from the prior DMID 08-0059 study, and contributed to the study design. D.L.
550 performed the statistical analyses and modeling. J.W. S.P. and A.W performed the ex-
551 periments. S.P.H. contributed to the experimental design and wrote the paper. M.S.
552 contributed substantially to the design and analysis of the imprinting experiments. All
553 authors read and approved the manuscript.

554 We declare no competing interests.

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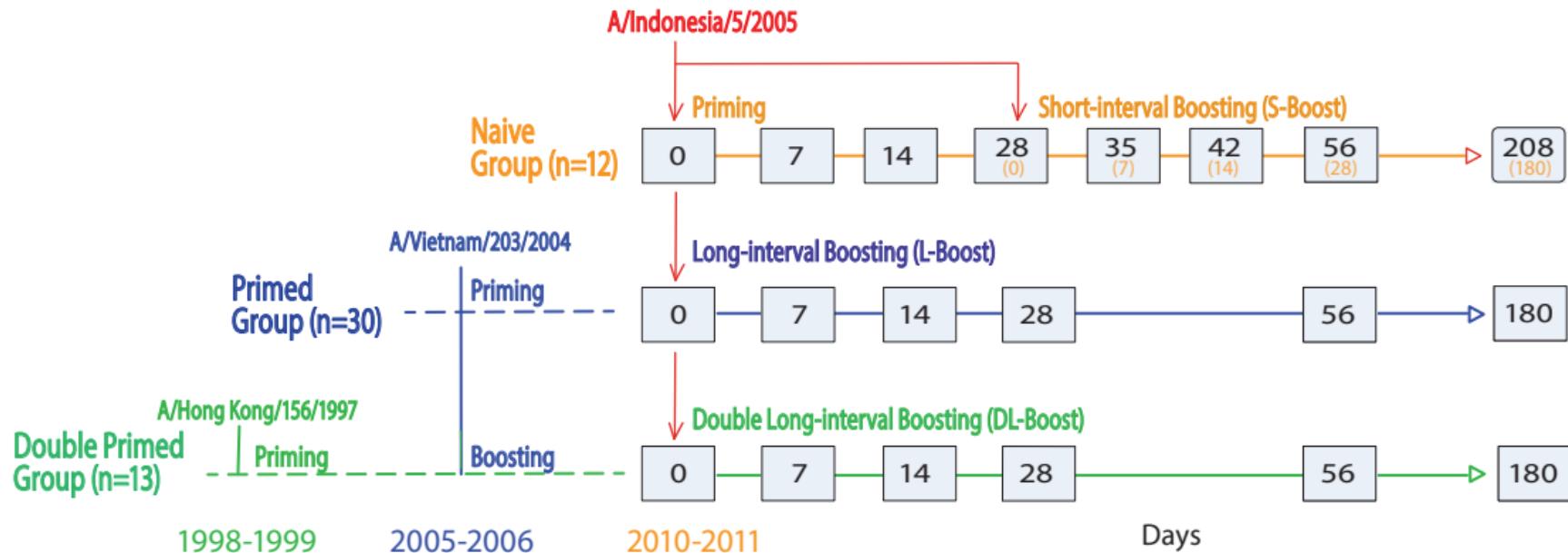
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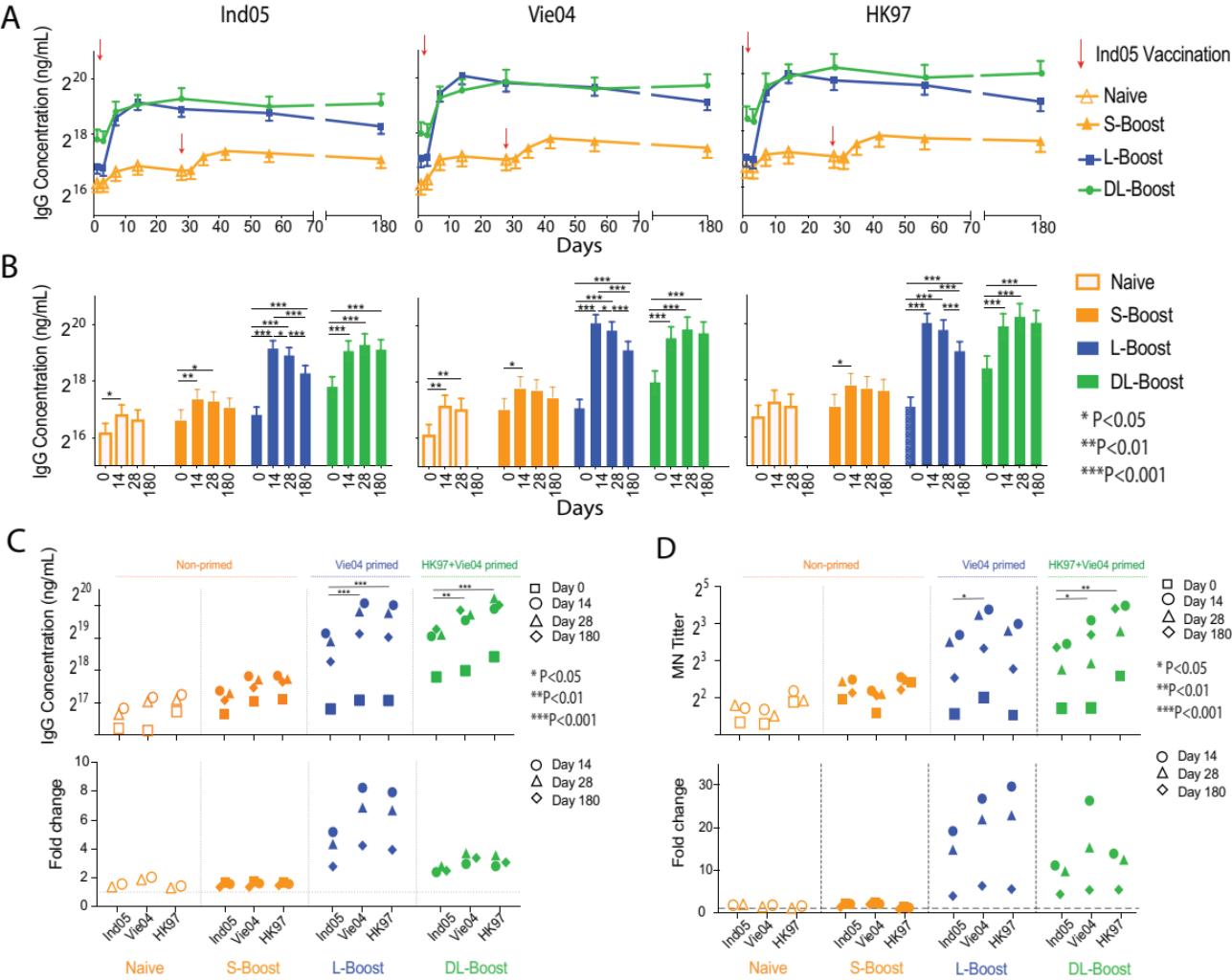
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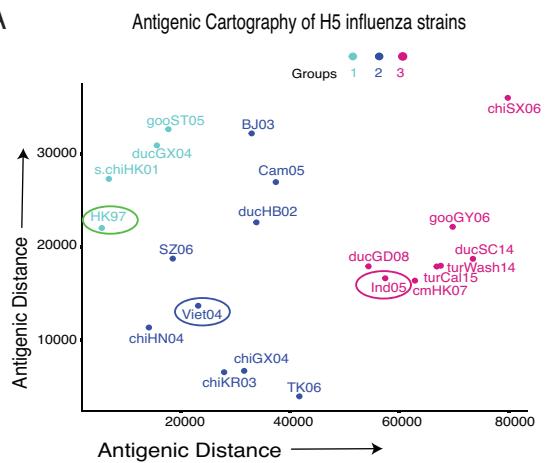
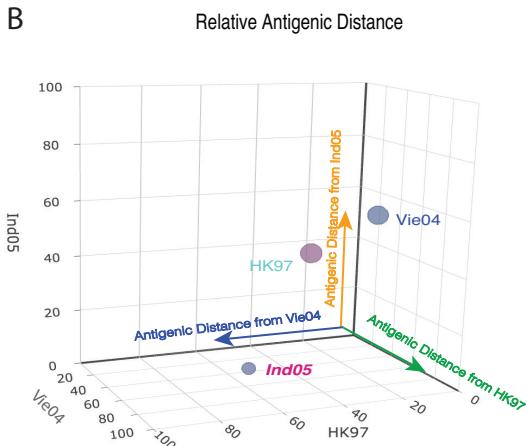
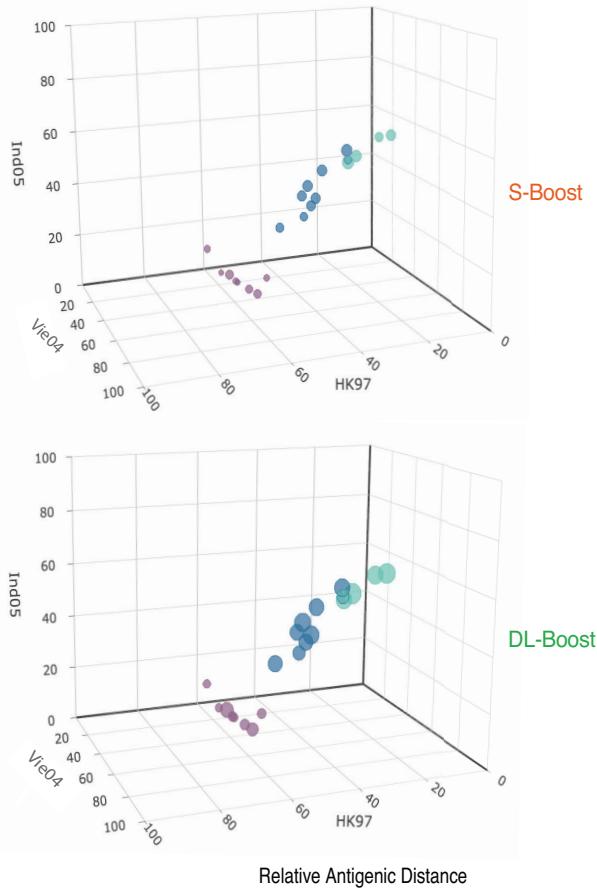
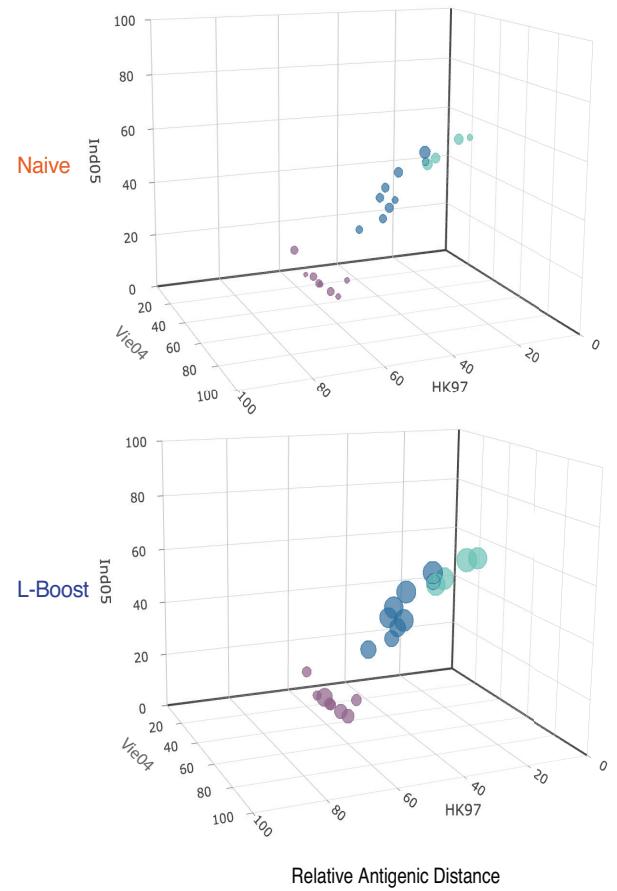
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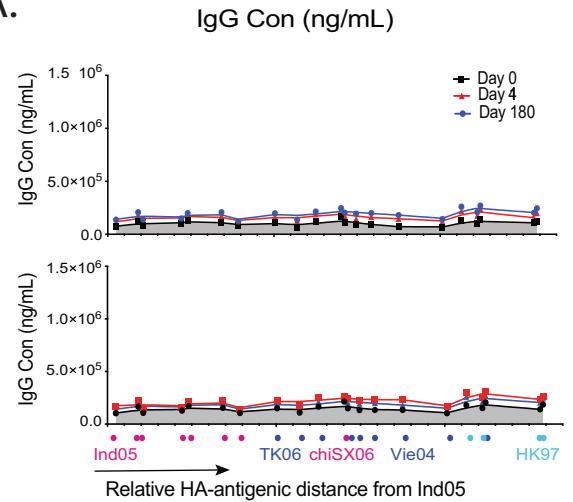
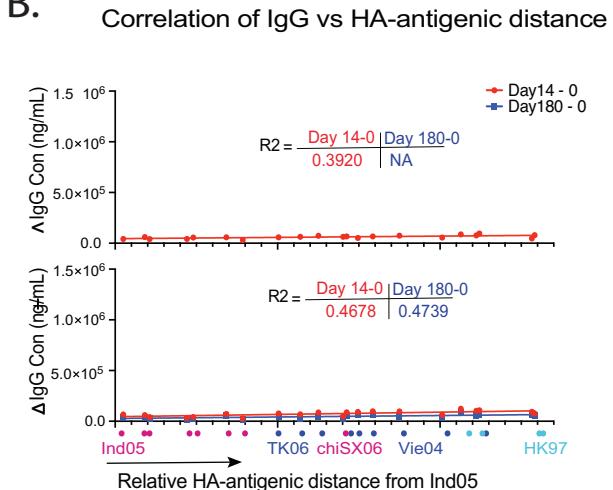
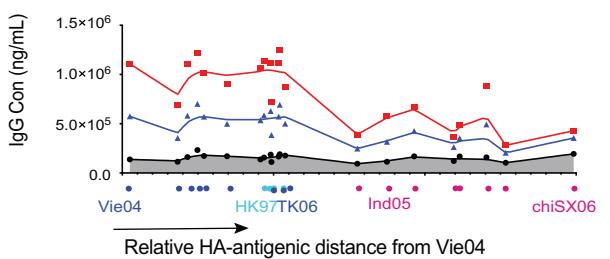


B

Priming Type	Boost Type	Boost Interval(s)	Imprinting/Priming Strain	Boost Strain
Naive (none)	Short Interval (S-Boost)	28 d	A/Indonesia/5/2005	A/Indonesia/5/2005
Primed	Long Interval (L-Boost)	5 yr	A/Vietnam/203/2004	A/Indonesia/5/2005
Double Primed	Double Long-Interval (DL-Boost)	7 & 12 yr	A/Hong Kong/156/1997 x 2	A/Indonesia/5/2005



A**B****C**

A.**B.****C.****D.**