Original antigenic sin priming of influenza virus hemagglutinin stalk antibodies

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Immunity to influenza viruses can be long-lived, but reinfections with antigenically distinct viral strains and subtypes are common. Reinfections can boost antibody responses against viral strains first encountered in childhood through a process termed "original antigenic sin." It is unknown how initial childhood exposures affect the induction of antibodies against the hemagglutinin (HA) stalk domain of influenza viruses. This is an important consideration since broadly reactive HA stalk antibodies can protect against infection, and universal vaccine platforms are being developed to induce these antibodies. Here we show that experimentally infected ferrets and naturally infected humans establish strong "immunological imprints" against HA stalk antigens first encountered during primary influenza virus infections. We found that HA stalk antibodies are surprisingly boosted upon subsequent infections with antigenically distinct influenza A virus subtypes. Paradoxically, these heterosubtypic-boosted HA stalk antibodies do not bind efficiently to the boosting influenza virus strain. Our results demonstrate that an individual's HA stalk antibody response is dependent on the specific subtype of influenza virus that they first encounter early in life. We propose that humans are susceptible to heterosubtypic influenza virus infections later in life since these viruses boost HA stalk antibodies that do not bind efficiently to the boosting antigen.

influenza virus | hemagglutinin | original antigenic sin

Most humans are infected with influenza viruses early in life (1) and then subsequently reinfected with antigenically distinct viral strains every 5 to 10 y (2). In a classic essay published in 1960, Thomas Francis Jr. reported that humans typically have high antibody titers against influenza viruses that they first encountered in childhood and that these antibodies are boosted upon subsequent exposures to antigenically drifted influenza virus strains (3). Francis coined the term "original antigenic sin" to describe this phenomenon. In 1966, Robert Webster and Stephen Fazekas de St. Groth demonstrated that original antigenic sin can be recapitulated in rabbits and ferrets that are sequentially infected with different H1N1 strains (4, 5). Recent human (6–9) and animal (9–12) serological studies have confirmed and extended these findings. Most studies of original antigenic sin have analyzed immune responses elicited by sequential exposures with drifted versions of the same influenza virus subtype. Much less is known about how prior exposures affect immune responses against heterosubtypic influenza virus infections. This is relevant since three different influenza A virus subtypes (H1N1, H2N2, and H3N2) have circulated in humans over the past century, and most humans alive today have been infected with more than one of these subtypes at some point in their lives (13).

Different influenza A virus subtypes can be broadly split into two phylogenetic groups ("group 1" and "group 2") based on conservation in the hemagglutinin (HA) protein (14). The globular head of HA differs substantially among these viruses, but there are more conserved epitopes in the HA stalk domain. Antibodies that recognize the HA stalk of group 1 or group 2 viruses have been well characterized, but antibodies that recognize the HA stalk of both group 1 and group 2 viruses are rare (15, 16). Interestingly, childhood influenza A virus exposures are associated with heterosubtypic protection against distinct emerging influenza virus subtypes later in life (13). For example, early childhood exposures to H1N1 and H2N2 (both group 1 viruses) are associated with protection from H5N1 (also a group 1 virus), whereas childhood exposures to H3N2 (a group 2 virus) are associated with protection from H7N9 (a group 2 virus) (13). This apparent cross-subtype protection is possibly mediated by antibodies targeting conserved epitopes in the HA stalk; however, it is unclear how initial childhood influenza exposures affect the development of HA stalk antibody responses.

Here, we set out to determine how initial childhood influenza virus infections impact antibody responses against subsequent heterosubtypic influenza virus infections. We first examined antibody responses elicited in ferrets sequentially exposed to different influenza virus subtypes. We then examined antibodies elicited in children who had different prior influenza virus exposures.

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Significance

Humans are typically infected with influenza viruses in childhood and then continuously exposed to antigenically distinct influenza virus strains throughout life. Antibody responses elicited by initial influenza virus infections can be boosted upon subsequent exposures with antigenically drifted influenza virus strains. Here, we examined how initial influenza virus infections affect antibody responses against subsequent infections with an unrelated influenza virus subtype. We show that heterosubtypic infections in ferrets and humans boost hemagglutinin stalk antibodies that paradoxically do not bind effectively to the boosting influenza virus strain. We propose that hemagglutinin stalk antibody repertoires are shaped by the specific subtype of influenza virus that an individual encounters early in life, and that this affects susceptibility to heterosubtypic infections later in life.

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Results

HA Stalk Antibodies Elicited by Initial Influenza Virus Infections in Ferrets Are Boosted upon Subsequent Heterosubtypic Infections. To better understand how initial influenza virus exposures shape antibody responses against subsequent infections with distinct influenza virus subtypes, we completed a series of infection experiments in ferrets that had never previously encountered influenza virus. We infected ferrets with H1N1 (A/California/07/2009) and then reinfected the same ferrets 84 d later with H3N2 (A/Perth/16/2009). We infected other ferrets with H3N2 and then reinfected them with H1N1 84 d later. As controls, we infected two additional groups of ferrets with either H1N1 or H3N2 (both influenza A viruses) and then reinfected with influenza B virus (B/Brisbane/60/2008). We quantified total serum HA antibody levels using ELISAs coated with full-length HA proteins, serum HA head-specific antibodies by hemagglutination inhibition (HAI) assays, and serum HA stalk-reactive antibodies using ELISAs coated with "headless" HA proteins.

As expected, animals initially infected with H1N1 produced H1-reactive antibodies (Fig. 1*A*) that targeted both the H1 head (Fig. 1*B*) and H1 stalk (Fig. 1*C*), but did not produce antibodies that recognized H3 antigens (Fig. 1 *D*–*F*). Similarly, animals initially infected with H3N2 produced H3-reactive antibodies (Fig. 1*D*) that targeted both the H3 head (Fig. 1*E*) and H3 stalk (Fig. 1*F*), but did not produce antibodies that recognized H1 antigens (Fig. 1 *A*–*C*). For H1N1 and H3N2 primary infections, total HA antibodies (Fig. 1 *A* and *D*) and HA stalk antibodies (Fig. 1 *C* and *F*) remained at similar levels between 28 and 84 d after infection, while HA head antibodies peaked 14 d after infection and then gradually declined over time (Fig. 1 *B* and *E*).

Animals initially infected with H1N1 and then subsequently infected with H3N2 mounted H3 antibody responses that were similar to those elicited by primary H3N2 infections (Fig. 1 *D*–*F*). Surprisingly, we found that secondary infections with H3N2 boosted H1 stalk-reactive antibodies in animals that were initially infected with H1N1 (4.2 μg/mL before and 81.0 μg/mL 14 d after secondary H3N2 infection; $P = 0.0013$ comparing titers; Fig. 1*C*). We found similar results when the order of H1N1 and H3N2 infections was reversed. Animals initially infected with H3N2 and subsequently infected with H1N1 mounted H1 antibody responses that were comparable to those elicited by primary H1N1 infections (Fig. 1 *B* and *C*); however, we found that

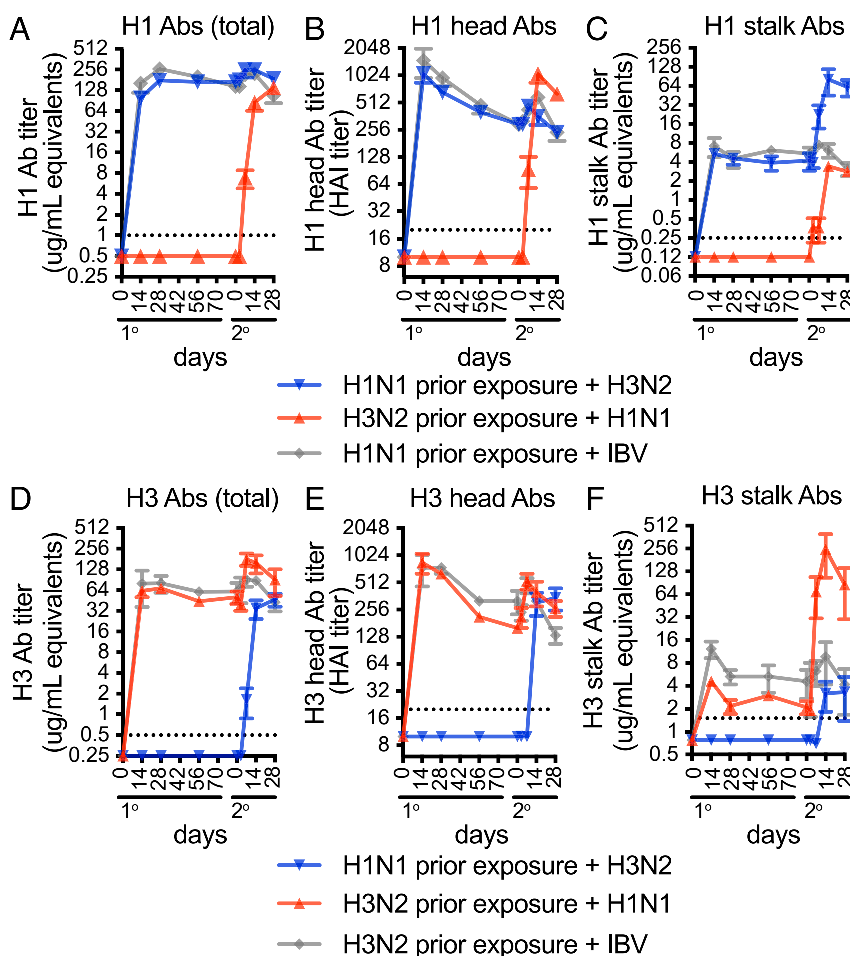

Fig. 1. Heterosubtypic boosting of HA stalk antibodies in ferrets. Ferrets were infected with H1N1 or H3N2 and then reinfected with H1N1, H3N2, or influenza B virus (IBV) 79 to 84 d later. Sera were collected at different times after the primary (1°) and secondary (2°) infections, and serological assays were completed. (*A*–*C*) Animals were infected with H1N1 and then reinfected with H3N2 (blue), infected with H3N2, and then reinfected with H1N1 (red) or infected with H1N1 and reinfected with IBV (gray). (*D*–*F*) Animals were infected with H1N1 and then reinfected with H3N2 (blue), infected with H3N2 and then reinfected with H1N1 (red), or infected with H1N1 and reinfected with IBV (gray). (*A*) Total H1 antibodies were quantified by ELISA, (*B*) H1 head-reactive antibodies were quantified by HAI assays, (*C*) H1 stalk-reactive antibodies were quantified by ELISA, (*D*) total H3 antibodies were quantified by ELISA, (*E*) H3 head-reactive antibodies were quantified by HAI assays, and (*F*) H3 stalk-reactive antibodies were quantified by ELISA. Data are presented as endpoint titrations or μg/mL equivalents based on a standard monoclonal antibody. Three to six ferrets were included for each experimental group. Data are shown as mean ± SEM.

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secondary H1N1 infections boosted H3 stalk-reactive antibodies in these animals (1.9 μg/mL before and 256.1 μg/mL 14 d after secondary H1N1 infection; $P = 0.049$ comparing titers; Fig. 1*F*). HA stalk Ab boosting against the first infecting strain did not occur when secondary infections were with an influenza B virus (Fig. 1 *C* and *F*). Furthermore, the HA antibodies boosted by heterosubtypic H3N2 or H1N1 infection bound to diverse HA subtypes (*SI Appendix*, Fig. S1 *A* and *B*); however, there was no boost in antibody binding to other HA subtypes after influenza B virus infections (*SI Appendix*, Fig. S1 *C* and *D*).

We completed similar experiments where we sequentially infected ferrets with the same influenza A virus strain. We found that homologous influenza A virus infections boosted HA stalk antibody responses (*SI Appendix*, Fig. S2), but to a lesser extent compared to heterosubtypic influenza A virus infections (Fig. 1 *C* and *F*). It is possible that secondary heterosubtypic influenza A virus infections are more efficient at boosting HA stalk antibodies relative to homologous infections because there is more virus replication and antigen during heterosubtypic infections compared to homologous infections (*SI Appendix*, Fig. S3).

HA Stalk Antibodies Elicited in Ferrets by Sequential Heterosubtypic Infections Have Original Antigenic Sin Properties. The HA stalks of H1 and H3 are antigenically distinct; however, there are some conserved regions between the stalks of these HAs (*SI Appendix*, Fig. S4), and rare human monoclonal antibodies that cross-react to H1 and H3 stalks have been identified (17–23). We therefore completed absorption experiments to determine if the heterosubtypic-boosted HA stalk antibodies in our ferret model were cross-reactive to H1 and H3 (Fig. 2). We incubated sera with cells expressing either H1 or H3 HA antigens and then quantified levels of HA stalk antibodies that remained in the unabsorbed sera fractions. As expected, H1 stalk antibodies were completely absorbed after incubation with H1 antigen (Fig. 2*A*) and H3 stalk antibodies were completely absorbed after incubation with H3 antigen (Fig. 2*B*). Even though H3N2 infection boosted H1 stalk antibodies, we found that the H3 antigen was unable to efficiently absorb H1 stalk antibodies (Fig. 2*A*). Similarly, H3 stalk antibodies boosted by H1N1 infection were not absorbed by H1 antigen (Fig. 2*B*).

Given the surprising result that boosting HAs could not absorb boosted antibodies, we completed several absorption experiments to confirm our results. First, we repeated absorption assays with different antibody/HA cell antigen ratios. Similar results were obtained when we used four times the amount of HA-expressing cells (*SI Appendix*, Fig. S5*A*) and when we decreased antibody/HA cell antigen ratios by decreasing serum concentrations (*SI Appendix*, Fig. S5*B*). It is possible that other isotypes like IgM and IgA could potentially inhibit IgG binding in our absorption assays. To account for this, we repeated absorption assays using purified IgG and obtained similar results (*SI Appendix*, Fig. S5 *C* and *D*) compared to experiments with total serum antibodies (Fig. 2). Finally, we completed experiments to determine if the disparities between ELISA and absorption assay data were due to differences in bivalent antibody binding to antigens in the two different assays. It is possible that antigens are in closer proximity to each other on ELISA plates and further apart on transfected cells. To account for this, we repeated absorption assays with serum Fabs (*SI Appendix*, Fig. S5 *E* and *F*) and obtained similar results compared to experiments with intact serum antibodies (Fig. 2).

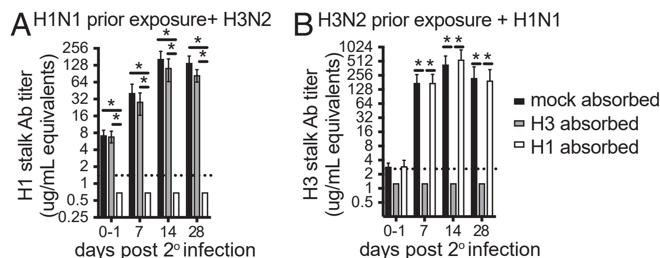

Fig. 2. Heterosubtypic-boosted HA stalk antibodies from ferrets do not bind efficiently to the boosting antigen. Serum samples were collected from ferrets before and 7, 14, and 28 d after secondary (2°) heterosubtypic influenza virus infection. Samples were incubated with 293F cells expressing H1 HA (white), H3 HA (gray), or no HA (black), and (*A*) H1 stalk and (*B*) H3 stalk antibody levels remaining in the unabsorbed fraction were quantified by ELISA. Serum from three to six ferrets was included for each experimental group. Data are shown as mean ± SEM. Titers were compared using a one-way ANOVA with Tukey's posttest at each time point on log-transformed data (*$P < 0.05$).

Taken together, these data suggest that the HA stalks of H1 and H3 are similar enough for heterosubtypic boosting of B cell responses, but that most soluble HA stalk antibodies produced from such boosting paradoxically do not bind efficiently to the boosting antigen. These data are consistent with the principles of original antigenic sin first proposed ∼60 y ago (3).

Original Antigenic Sin Boosting of HA Stalk Antibodies in Children. Cross-subtype boosting of HA antibodies has been previously reported in humans (24), although the specificity of antibodies involved in this process remains unknown. To determine if original antigenic sin heterosubtypic boosting of HA stalk antibodies occurs in humans, we characterized antibodies in the sera of children (ages 2 to 6 y old) before and after PCR-confirmed H3N2 infection in 2017. We stratified children into different experimental groups based on their prior H1 and H3 exposure histories, which we inferred based on H1 and H3 serum antibody reactivity prior to H3N2 infection (*SI Appendix*, Fig. S6). We excluded children with evidence of both H1N1 and H3N2 prior exposures since we could not determine which infection occurred first. We proceeded using serum from five children with evidence of only H1N1 prior exposure, six children with evidence of only H3N2 prior exposure, and seven children with no evidence of H1N1 or H3N2 prior exposure (*SI Appendix*, Table S1). We quantified H1 and H3 stalk-reactive antibodies in serum before and after H3N2 infection in this group of 18 children (Fig. 3). As expected, antibodies reactive to full-length H3 increased in nearly every child after PCR-confirmed H3N2 infection (*SI Appendix*, Fig. S6 *A*–*C*), whereas antibodies reactive to full-length H1 did not significantly change in most of these children (*SI Appendix*, Fig. S6 *D*–*F*). None of the children, including those with evidence of prior H3N2 infection, possessed detectable levels of H3 stalk-reactive antibodies prior to PCR-confirmed H3N2 infection (Fig. 3 *A*–*C*). This suggests that HA stalk antibodies make up a small fraction of total HA antibodies elicited by primary influenza virus exposures in humans, which is consistent with our ferret studies (Fig. 1). We were able to detect low levels of H1 stalk-reactive antibodies in children with evidence of prior H1N1 infections (Fig. 3*F*), and these antibodies were not present in children without evidence of prior H1N1 infections (Fig. 3 *D* and *E*). Consistent with our ferret experiments (Fig. 1), H1 stalk-reactive antibodies in children with prior H1N1 exposures were boosted >10-fold following heterosubtypic H3N2 infection (Fig. 3*F*). Only 2 of the 13 H3N2-infected children without a prior H1N1 exposure had detectable H1 stalk antibodies (Fig. 3 *D* and *E*), which confirms that H3N2 viruses do not inherently elicit high levels of H1 stalk-reactive antibodies in individuals without prior H1N1 exposures.

We completed absorption assays to determine if heterosubtypic boosted HA stalk antibodies in children are H1/H3 cross-reactive. As expected, H3 stalk-reactive antibodies elicited by H3N2 infection were absorbed after incubation with H3 antigen but not H1

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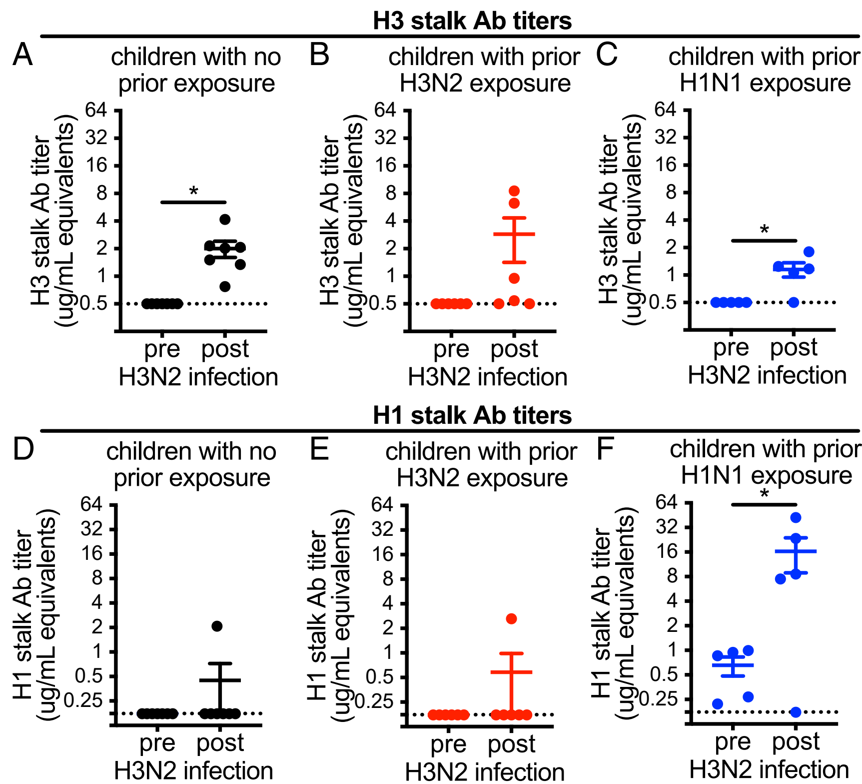

Fig. 3. H3N2 influenza infection boosts H1 stalk antibodies in children with evidence of prior H1N1 exposure. Serum samples were collected from 18 children before and after PCR-confirmed H3N2 infection in 2017. Samples were stratified into different experimental groups based on prior H1 and H3 exposure history, which we inferred based on H1 and H3 antibody reactivity prior to H3N2 infection in 2017 (*SI Appendix*, Fig. S3). We quantified (*A*–*C*) H3 stalk and (*D*–*F*) H1 stalk antibodies in serum from children with no evidence of prior exposure ($n = 7$; *A* and *D*), H3N2 prior exposure ($n = 6$; *B* and *E*), and H1N1 prior exposure ($n = 5$; *C* and *F*). Each circle represents a sample from one individual. Data are shown as mean ± SEM. Pre- and postinfection antibody titers were compared using a paired *t* test on log-transformed data ($^*P < 0.05$).

antigen (*SI Appendix*, Fig. S7 *A*–*C*). H1 stalk-reactive antibodies that were boosted by H3N2 infection were absorbed after incubation with H1 antigen, but these antibodies were not efficiently absorbed by the boosting H3 antigen (Fig. 4 *A*–*C*). Therefore, HA stalk antibody responses elicited in children mirror those that were elicited in our ferret model. In both cases, we found that HA stalk antibody responses elicited by previously encountered influenza viruses are boosted by heterosubtypic infections; however, these antibodies bind poorly to the boosting HA antigen.

Discussion

Our studies suggest that early life encounters with influenza viruses establish immunological memory B cell responses that can be recalled upon subsequent exposures with distinct influenza virus subtypes later in life. We propose that human HA stalk antibody specificities are critically dependent on the specific influenza virus subtype that an individual encounters in childhood. We demonstrate that antibody responses against the HA stalk of viral strains initially encountered in childhood are boosted by subsequent homologous and heterosubtypic influenza virus infections.

It is unclear why heterosubtypic influenza A virus infections efficiently boost HA stalk antibodies that inefficiently bind to the boosting antigen. While our absorption experiments demonstrate that heterosubtypic boosted HA stalk antibodies are not fully cross-reactive (Figs. 2 and 4), it is possible that these antibodies partially cross-react to H1 and H3 when expressed as BCRs. A typical B cell expresses over 10^5 B cell receptors (BCRs) (25). Heterosubtypic influenza viruses potentially engage HA stalk memory B cells through multiple low-affinity interactions with thousands of identical BCRs on individual B cells. Memory B cells stimulated in this manner would be expected to produce soluble antibodies that bind poorly to the boosting antigen. It is possible that original antigenic sin simply occurs because mismatched antigens engage and restimulate B cells through multiple low-affinity interactions, yet soluble antibodies produced through this process cannot efficiently bind to the same recalling antigen.

Our findings might partially explain why different-aged individuals have different susceptibilities to H5N1 and H7N9 infections (13). H1N1, H2N2, and H5N1 are all group 1 influenza A viruses with similar HA stalks, whereas H3N2 and H7N9 are group 2 influenza A viruses with similar HA stalks (15, 16). H1N1, H2N2, and H3N2 have circulated at different times over the past 100 y, and therefore an individual's birth year largely predicts which of these viruses they were infected with early in childhood (26). Our findings suggest that individuals exposed early in childhood to H1N1 might have strong immunological memory against group 1 HA stalks, whereas individuals exposed early in childhood to H3N2 might have strong immunological memory against group 2 HA stalks. Heterosubtypic infections with viruses that belong to a different antigenic group relative to viruses encountered early in childhood might lead to the recall of HA stalk antibodies that fail to bind and protect against the recalling antigen. For example, it is possible that H5 recalls protective group 1 HA stalk antibodies in individuals primed early in childhood with group 1 HA viruses and nonprotective group 2 HA stalk antibodies in individuals primed early in childhood with group

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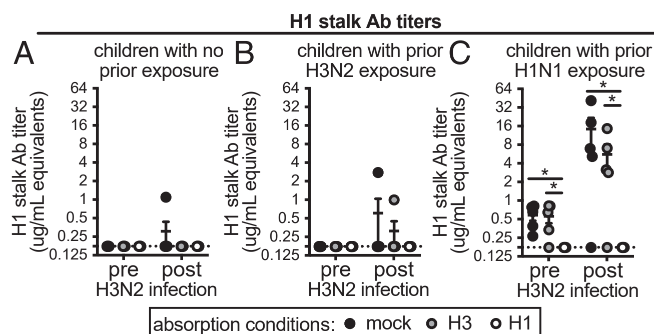

Fig. 4. Heterosubtypic-boosted HA stalk antibodies from children do not bind efficiently to the boosting antigen. Serum samples were collected from 18 children before and after PCR-confirmed H3N2 infection in 2017. Samples were stratified into different experimental groups based on prior H1 and H3 exposure history, which we inferred based on H1 and H3 antibody reactivity prior to H3N2 infection in 2017 (*SI Appendix*, Fig. S3). We collected samples from individuals with (*A*) no evidence of prior exposure ($n = 7$), (*B*) evidence of prior H3N2 exposure ($n = 6$), and (*C*) evidence of prior H1N1 exposure ($n = 5$). Samples were incubated with 293F cells expressing H1 HA (white), H3 HA (gray), or no HA (black), and H1 stalk antibody levels remaining in the unabsorbed fraction were quantified by ELISA. Each circle represents a sample from one individual. Data are shown as mean ± SEM. Antibody titers were compared using a one-way ANOVA with Tukey's posttest on log-transformed data (*$P < 0.05$).

2 HA viruses. Similarly, H7 potentially recalls protective group 2 HA stalk antibodies in individuals primed early in childhood with group 2 HA viruses and nonprotective group 1 HA stalk antibodies in individuals primed early in life with group 1 HA viruses. While we focused on HA stalk antibodies in this study, it is possible that antibodies against other HA epitopes and other viral proteins such as neuraminidase (27–29) are boosted in a similar manner.

There are some limitations to our ferret experiments. We only tested a limited number of viral strains in ferrets, all of which have been isolated since 2008. Additional ferret experiments should be completed in the future to verify that our findings can be recapitulated with additional viral strains. For example, additional studies should be completed to determine how initial H2N2 priming affects responses against H1N1 and H3N2 viral strains. It may also be worthwhile to repeat these experiments in younger ferrets since very young ferrets have different immune responses and clinical outcomes to influenza virus infections compared to older ferrets (30–32). Although this is a possible caveat, we found that antibody responses elicited in 6-mo-old ferrets (Figs. 1 and 2) were remarkably similar to antibody responses elicited in children (Figs. 3 and 4), suggesting that initial viral infections, rather than unique properties of infant immune systems, are important for the phenotypes observed in this manuscript. Finally, we quantified levels of HA stalk antibodies in our study but we did not determine if these antibodies prevent subsequent infections. Additional studies should directly test the functionality of heterosubtypic boosted HA stalk antibodies.

The real "sin" of original antigenic sin (3) might be that humans typically encounter only a limited number of viruses early in childhood and therefore have somewhat narrow immune memory skewed toward viral strains encountered early in life. Influenza vaccines have recently been approved for use in children in some parts of the world (33), and therefore many children now first encounter antigens from multiple influenza virus strains at the same time through vaccination rather than antigens from a single influenza virus strain through infection. It remains to be seen if seasonal influenza vaccines elicit broader immunological memory compared to influenza infections in childhood and how childhood influenza vaccinations impact the development of memory responses against the HA stalk and other viral epitopes. Moving forward, special attention should be given to new vaccines that efficiently establish long-lived memory against the HA stalk of diverse influenza virus strains, which could provide universal influenza virus immunity later in life (14).

Materials and Methods

Ferret Infections. Six-month-old ferrets (*Mustela putorius*) were obtained from Triple F farms. Ferrets were confirmed to be seronegative for influenza A and B viruses prior to the initiation of experiments. Ferrets were sequentially infected with influenza viruses as described in the main text. A/California/07/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008 viruses were used for ferret infections. Previous studies demonstrated that these viruses effectively infect ferrets (34–36). Secondary infections took place 79 to 84 d following primary infections, which allows time for primary responses to subside (37, 38). For each infection, ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 $TCID_{50}$/mL virus in 500 μL MEM. Nasal wash samples were collected at different time points postinfection using 1 mL of PBS as previously described (27). Virus titers in nasal wash samples were determined by $TCID_{50}$ assays using MDCK cells. Animals were bled via the vena cava at different time points before and after primary and secondary infections, and serological analyses were completed as described later. Samples indicated as "day 0" following a secondary infection were collected before or within 24 h after the secondary infection. Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility. The University of Pittsburgh Institutional Animal Use Committee (IACUC) approved all animal experiments described in this manuscript (protocol no. 16077170).

Human Samples. Samples were collected as part of a household cohort study in Nicaragua in 2017 (28). Households with at least one child aged 12 or under were enrolled with the intention to prospectively follow the household members for influenza virus infection for 5 y and to investigate household influenza virus transmission. Blood samples were collected from all participants at enrollment. Upon detection of laboratory-confirmed influenza virus infection in a household member, the household entered an intensive monitoring phase where a blood sample was collected from all household members, up to five nasal/oropharyngeal swab specimens were collected over a 9- to 14-d period, and a follow-up blood sample was collected 3 to 5 wk later. We examined samples from children who had PCR-confirmed H3N2 infections and detectable H3N2 FRNT titers post-H3N2 infection ($n = 69$). Using sera collected prior to the PCR-confirmed infection, we completed ELISAs coated with full-length H1 and H3 proteins to infer immune histories prior to PCR-confirmed H3N2 infection in 2017. We identified many samples with evidence of both H1 and H3 prior exposure ($n = 51$), but we did not complete additional serological analyses with these samples since the prior order of exposure in these individuals could not be determined. We identified five children with evidence of only H1N1 prior exposure, six children with evidence of only H3N2 prior exposure, and seven children with no evidence of H1N1 or H3N2 exposure (*SI Appendix*, Table S1). We completed additional serological analyses on sera collected after PCR-confirmed H3N2 infection with these samples as described later.

This human study was approved by the institutional review boards of the University of Michigan (HUM 00119145) and the Ministry of Health, Nicaragua (CIRE05/04/12–080), and serological assays were completed with deidentified samples with approval from the institutional review board of the University of Pennsylvania. Written or proxy written informed consent was obtained for all individuals. Assent was obtained from children 6 y of age or older.

Recombinant HA Proteins. Recombinant full-length HA proteins for A/California/07/2009 WT, A/Perth/16/2009 WT, A/Colorado/15/2014 WT, A/Japan/305/1957 (H2N2), A/duck/Czech/1956 (H4N6), A/Vietnam/1203/2004(H5N1), A/mallard/Sweden/24/2002 (H8N4), A/Jiangxi/09037/2014 (H10N8), or A/shearwater/West Australia/2576/1979 (H15N9) were produced for ELISAs. Plasmids were created with codon-optimized full-length HA sequences, and the HA transmembrane domain was replaced with a FoldOn trimerization domain from T4 fibritin, an AviTag site-specific biotinylation sequence, and a hexahistidine tag, as previously described (29). We transfected these plasmids into 293F suspension cells and isolated recombinant HA proteins 4 d later. Supernatants were isolated from the cells and clarified by centrifugation. HA proteins were purified by Ni-NTA affinity chromatography (Qiagen). We also produced recombinant headless HA proteins. For this, plasmids encoding

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recombinant headless H1 and H3 HA proteins were provided by Adrian McDermott and Barney Graham from the Vaccine Research Center at the National Institutes of Health (30, 31). Headless HA proteins were expressed in 293F cells and purified using Ni-NTA affinity chromatography (Qiagen) as described earlier. Following purification, the headless HA stalk proteins were biotinylated using the Avidity BirA-500 kit.

Full-Length HA ELISAs. ELISA plates (96-well; Immulon) were coated with 50 μL of recombinant proteins in coating buffer (KPL) at 2 μg/mL and incubated overnight at 4 °C. The day of the experiment, plates were blocked with 150 μL of PBS–0.1% Tween 20, 3% normal goat serum, and 0.5% milk powder and incubated for 1 h at room temperature. Twofold serial dilutions of samples in blocking buffer were added to the plates and allowed to incubate for 2 h at room temperature. As coating controls and to determine relative serum titers, human mAbs were added to each plate. Plates were then incubated with peroxidase-conjugated goat anti-human IgG (Jackson) or peroxidase-conjugated goat anti-ferret IgG (Abcam). SureBlue TMB peroxidase substrate (KPL) was added to each well, and the reaction was then stopped with the addition of 250 mM HCl solution. Absorbance was read at 450 nm using a plate reader (Molecular Devices). Plates were washed three times with PBS–0.1% Tween 20 between each step. Background signals at each dilution were subtracted for each sample, and one-site specific binding curves were fit to the data using GraphPad Prism.

Headless HA ELISAs. Streptavidin-coated 96-well ELISA plates (Pierce) were washed and then incubated for 1 h at room temperature with 50 μL of 0.5 μg/mL biotinylated stalk protein in TBS–0.01% Tween 20 and 0.1% BSA. Plates were then blocked with TBS–0.01% Tween 20 and 1% BSA for 1 h at room temperature. Twofold serial dilutions of samples were added to the plates and incubated for 1 h at room temperature. As coating controls and to determine relative serum titers, human mAbs were added to each plate. Plates were then incubated with peroxidase-conjugated goat anti-human IgG (Jackson) or peroxidase-conjugated goat anti-ferret IgG (Abcam). SureBlue TMB peroxidase substrate (KPL) was added to each well, and the reaction was then stopped with the addition of 250 mM HCl solution. Absorbance was read at 450 nm using a plate reader (Molecular Devices). Plates were washed three times with PBS–0.1% Tween 20 between each step. Background signal at each dilution was subtracted for each sample, and one-site specific binding curves were fit to the data using GraphPad Prism.

HAI Assays. Sera samples were pretreated with receptor-destroying enzyme (Denka Seiken) followed by hemadsorption. HAI titrations were performed in 96-well round plates (Corning). Sera were serially diluted twofold and added to four agglutinating doses of influenza A virus in a total volume of 100 μL. Next, 12.5 μL of a 2% (vol/vol) turkey erythrocyte solution was added. The sera, virus, and erythrocytes were gently mixed, and the assay was read out after incubating for 60 min at room temperature. HAI titers were recorded as the inverse of the highest antibody dilution that inhibited four agglutinating doses of virus.

Absorption Assays. Two days before the experiments, 293F suspension cells were transfected using 293fectin (Gibco) with plasmids expressing A/California/07/2009 WT HA, A/Perth/16/2009 WT HA, A/Colorado/15/2014 WT HA, or a mock transfection control containing no plasmid or transfection reagent. On the day of the experiment, transfected cells were pelleted by centrifugation, washed, and resuspended at the desired volume. Serum samples were diluted 1:25 in PBS and incubated with $\sim 1 \times 10^7$ cells per absorption condition. The cell and sample mixtures were incubated for 1 h at room temperature while shaking at 800 rpm. After this incubation, serum samples were centrifuged in order to remove antibodies bound to cells. The remaining unbound antibody fraction was used in serological assays.

Serum IgG Purification. IgG was isolated using Protein G magnetic beads (Millipore) according to manufacturer's instructions. Briefly, 100 μL of beads were added to a magnetic stand, storage solution was discarded, and beads were washed with PBS. Samples were diluted 1:20 in PBS and incubated with beads at room temperature for 1 h with constant mixing. IgG-depleted samples were removed from the beads using the magnetic stand, and beads were washed with PBS. Following discard of the wash fraction, bound IgG was eluted with 100 μL of 0.2 M glycine, pH 2.5, and neutralized with 10 μL of 1.0 M Tris, pH 8.8.

Ferret Serum Fab Production and ELISA. Ferret serum samples were digested to produce Fab fragments with papain agarose (Pierce) according to manufacturer's instructions. Briefly, serum samples were diluted 1:10 in digestion buffer supplemented with 20 mM cysteine-HCl (pH 7) and incubated with 130 μL of equilibrated papain agarose for 24 h at 37 °C with constant mixing. Digested samples were separated from papain agarose and then diluted in PBS for use in absorption experiments (described earlier) and ELISAs. ELISAs were conducted as described earlier with the following variation to the secondary detection step: Fabs bound to antigen were detected using anti-light chain antibodies, mouse anti-ferret Igκ (Kerafast mAb 4-B10), or mouse anti-ferret Igλ (Kerafast mAb 8-H9), both diluted to 1 μg/mL. Peroxidase-conjugated goat anti-mouse IgG (Jackson) was then used to detect the mouse anti-ferret Igκ and Igλ.

Statistical Methods. Paired *t* test or ANOVA with Tukey's posttest were performed on log-transformed data where indicated. Statistical analyses were performed using GraphPad Prism 7.0.

Data Availability. All data needed to support the findings of this manuscript are included in the main text and *SI Appendix*.

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1. R. Bodewes *et al*., Prevalence of antibodies against seasonal influenza A and B viruses in children in Netherlands. *Clin. Vaccine Immunol.* **18**, 469–476 (2011).
2. A. J. Kucharski *et al*., Estimating the life course of influenza A(H3N2) antibody responses from cross-sectional data. *PLoS Biol.* **13**, e1002082 (2015).
3. T. Francis, On the doctrine of original antigenic sin. *Proc. Am. Philos. Soc.* **104**, 572 (1960).
4. R. G. Webster, Original antigenic sin in ferrets: The response to sequential infections with influenza viruses. *J. Immunol.* **97**, 177–183 (1966).
5. S. Fazekas de St Groth, R. G. Webster, Disquisitions on original antigenic sin. II. Proof in lower creatures. *J. Exp. Med.* **124**, 347–361 (1966).
6. J. M. Fonville *et al*., Antibody landscapes after influenza virus infection or vaccination. *Science* **346**, 996–1000 (2014).
7. M. S. Miller *et al*., Neutralizing antibodies against previously encountered influenza virus strains increase over time: A longitudinal analysis. *Sci. Transl. Med.* **5**, 198ra107 (2013).
8. J. Lessler *et al*., Evidence for antigenic seniority in influenza A (H3N2) antibody responses in southern China. *PLoS Pathog.* **8**, e1002802 (2012).
9. Y. Li *et al*., Immune history shapes specificity of pandemic H1N1 influenza antibody responses. *J. Exp. Med.* **210**, 1493–1500 (2013).
10. J. H. Kim, I. Skountzou, R. Compans, J. Jacob, Original antigenic sin responses to influenza viruses. *J. Immunol.* **183**, 3294–3301 (2009).
11. S. L. Linderman, S. E. Hensley, Antibodies with "original antigenic sin" properties are valuable components of secondary immune responses to influenza viruses. *PLoS Pathog.* **12**, e1005806 (2016).
12. D. M. Carter *et al*., Sequential seasonal H1N1 influenza virus infections protect ferrets against novel 2009 H1N1 influenza virus. *J. Virol.* **87**, 1400–1410 (2013).
13. K. M. Gostic, M. Ambrose, M. Worobey, J. O. Lloyd-Smith, Potent protection against H5N1 and H7N9 influenza via childhood hemagglutinin imprinting. *Science* **354**, 722–726 (2016).
14. M. Worobey, S. Plotkin, S. E. Hensley, Influenza vaccines delivered in early childhood could turn antigenic sin into antigenic blessings. *Cold Spring Harb. Perspect. Med.*, 10.1101/cshperspect.a038471 (2020).
15. S. F. Andrews, B. S. Graham, J. R. Mascola, A. B. McDermott, Is it possible to develop a "Universal" influenza virus vaccine? Immunogenetic considerations underlying B-cell biology in the development of a pan-subtype influenza A vaccine targeting the hemagglutinin stem. *Cold Spring Harb. Perspect. Biol.* **10**, a029413 (2018).
16. F. Krammer, P. Palese, Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr. Opin. Virol.* **3**, 521–530 (2013).
17. C. Dreyfus *et al*., Highly conserved protective epitopes on influenza B viruses. *Science* **337**, 1343–1348 (2012).
18. D. Corti *et al*., A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* **333**, 850–856 (2011).
19. S. Lang *et al*., Antibody 27F3 broadly targets influenza A group 1 and 2 hemagglutinins through a further variation in V_H1-69 antibody orientation on the HA stem. *Cell Rep.* **20**, 2935–2943 (2017).
20. N. L. Kallewaard *et al*., Structure and function analysis of an antibody recognizing all influenza A subtypes. *Cell* **166**, 596–608 (2016).
21. Y. Fu *et al*., A broadly neutralizing anti-influenza antibody reveals ongoing capacity of haemagglutinin-specific memory B cells to evolve. *Nat. Commun.* **7**, 12780 (2016).
22. M. G. Joyce *et al*.; NISC Comparative Sequencing Program, Vaccine-induced antibodies that neutralize group 1 and group 2 influenza A viruses. *Cell* **166**, 609–623 (2016).

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23. S. F. Andrews *et al*., Preferential induction of cross-group influenza A hemagglutinin stem-specific memory B cells after H7N9 immunization in humans. *Sci. Immunol.* **2**, eaan2676 (2017).
24. R. Nachbagauer *et al*., Defining the antibody cross-reactome directed against the influenza virus surface glycoproteins. *Nat. Immunol.* **18**, 464–473 (2017).
25. L. A. Casten, S. K. Pierce, Receptor-mediated B cell antigen processing. Increased antigenicity of a globular protein covalently coupled to antibodies specific for B cell surface structures. *J. Immunol.* **140**, 404–410 (1988).
26. S. Cobey, S. E. Hensley, Immune history and influenza virus susceptibility. *Curr. Opin. Virol.* **22**, 105–111 (2017).
27. F. Krammer *et al*., NAction! How can neuraminidase-based immunity contribute to better influenza virus vaccines? *MBio* **9**, e02332-17 (2018).
28. M. C. Eichelberger, D. M. Morens, J. K. Taubenberger, Neuraminidase as an influenza vaccine antigen: A low hanging fruit, ready for picking to improve vaccine effectiveness. *Curr. Opin. Immunol.* **53**, 38–44 (2018).
29. A. J. Kucharski, W. J. Edmunds, Cross-immunity and age patterns of influenza A(H5N1) infection. *Epidemiol. Infect.* **143**, 1119–1124 (2015).
30. S. S. Huang *et al*., Differential pathological and immune responses in newly weaned ferrets are associated with a mild clinical outcome of pandemic 2009 H1N1 infection. *J. Virol.* **86**, 13187–13201 (2012).
31. S. G. Paquette *et al*., Influenza transmission in the mother-infant dyad leads to severe disease, mammary gland infection, and pathogenesis by regulating host responses. *PLoS Pathog.* **11**, e1005173 (2015).
32. S. J. Bissel *et al*., Age-related pathology associated with H1N1 A/California/07/2009 influenza virus infection. *Am. J. Pathol.* **189**, 2389–2399 (2019).
33. American Academy of Pediatrics Committee on Infectious Diseases, Recommendations for influenza immunization of children. *Pediatrics* **113**, 1441–1447 (2004).
34. S. S. Lakdawala *et al*., The soft palate is an important site of adaptation for transmissible influenza viruses. *Nature* **526**, 122–125 (2015).
35. S. S. H. Huang *et al*., Pathogenic influenza B virus in the ferret model establishes lower respiratory tract infection. *J. Gen. Virol.* **95**, 2127–2139 (2014).
36. S. S. Huang *et al*., Comparative analyses of pandemic H1N1 and seasonal H1N1, H3N2, and influenza B infections depict distinct clinical pictures in ferrets. *PLoS One* **6**, e27512 (2011).
37. G. A. Kirchenbaum, D. M. Carter, T. M. Ross, Sequential infection in ferrets with antigenically distinct seasonal H1N1 influenza viruses boosts hemagglutinin stalk-specific antibodies. *J. Virol.* **90**, 1116–1128 (2015).
38. M. E. Francis *et al*., Historical H1N1 influenza virus imprinting increases vaccine protection by influencing the activity and sustained production of antibodies elicited at vaccination in ferrets. *Vaccines (Basel)* **7**, 133 (2019).

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