H3N2 Influenza Infection Elicits More Cross-Reactive and Less Clonally Expanded Anti-Hemagglutinin Antibodies Than Influenza Vaccination

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Abstract

Background: During the recent H1N1 influenza pandemic, excess morbidity and mortality was seen in young but not older adults suggesting that prior infection with influenza strains may have protected older subjects. In contrast, a history of recent seasonal trivalent vaccine in younger adults was not associated with protection.

Methods and Findings: To study hemagglutinin (HA) antibody responses in influenza immunization and infection, we have studied the day 7 plasma cell repertoires of subjects immunized with seasonal trivalent inactivated influenza vaccine (TIV) and compared them to the plasma cell repertoires of subjects experimentally infected (EI) with influenza H3N2 A/Wisconsin/67/2005. The majority of circulating plasma cells after TIV produced influenza-specific antibodies, while most plasma cells after EI produced antibodies that did not react with influenza HA. While anti-HA antibodies from TIV subjects were primarily reactive with single or few HA strains, anti-HA antibodies from EI subjects were isolated that reacted with multiple HA strains. Plasma cell-derived anti-HA antibodies from TIV subjects showed more evidence of clonal expansion compared with antibodies from EI subjects. From an H3N2-infected subject, we isolated a 4-member clonal lineage of broadly cross-reactive antibodies that bound to multiple HA subtypes and neutralized both H1N1 and H3N2 viruses. This broad reactivity was not detected in post-infection plasma suggesting this broadly reactive clonal lineage was not immunodominant in this subject.

Conclusion: The presence of broadly reactive subdominant antibody responses in some EI subjects suggests that improved vaccine designs that make broadly reactive antibody responses immunodominant could protect against novel influenza strains.


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Competing Interests: EW is a speaker for Merck & Co. Inc. and sanofi pasteur; is a consultant and advisor to Merck & Co. Inc.; has been an advisor to Novartis Vaccines; and has conducted clinical trials for GlaxoSmithKlein, MedImmune, Merck & Co. Inc., Novartis Vaccines, Pfizer Vaccines, and sanofi pasteur. MM, H-XL, and BH have filed patent applications related to this technology. This does not alter the authors’ adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

Influenza is a persistent threat to public health with seasonal influenza causing >200,000 hospitalizations and >35,000 deaths in the US annually [1,2]. While the most recent pandemic strain did not appear to be significantly more pathogenic than the seasonal strain of influenza that it replaced [3], prior pandemics, such as the 1918 H1N1 influenza pandemic, have been associated with severe mortality [4].

Immunization of susceptible populations is one of the primary methods for preventing influenza-associated morbidity and mortality [5]. In humans, boosting immunizations with trivalent inactivated influenza vaccine (TIV) are associated with the transient appearance of influenza-specific plasma cells/plasmablasts (hereafter termed plasma cells) in peripheral blood [6]. The majority of these plasma cells produce antibodies that bind HA and are both strain-specific and neutralizing [6]. Protective humoral responses to influenza are mediated by antibodies that prevent infection of target cells, and these antibodies are largely directed against variable regions of the HA globular head leading to subtype- and strain-specific antibody responses [7,8]. Broadly neutralizing antibodies reactive with multiple influenza subtypes have been isolated from phage-displayed libraries from uninfected subjects [9], those recovering from H5N1 influenza [10], and those vaccinated against seasonal influenza [11], but such antibodies are not immunodominant and generally are not found in plasma [12].

In order to perform a direct comparison between the antibody repertoires following influenza immunization and infection, we isolated plasma cells from human peripheral blood at seven days following TIV or experimental influenza infection (EI) with H3N2 A/Wisconsin/67/2005 by using single cell sorting. PCR-based amplification of V(D)J gene rearrangements of Ig heavy- and light-chain members from single plasma cells was used for analysis and gene rearrangement for subsequent mAb expression. We found that plasma-cell-derived mAbs from EI were more polyclonal but anti-HA mAbs from EI were more cross-reactive compared to mAbs derived from TIV subjects. The anti-HA response in TIV showed more evidence of clonal expansion and was more strain-specific compared to the response in EI. The largest clonal lineage identified from an EI subject contained anti-HA mAbs that reacted with most HAs tested and neutralized both H1N1 and H3N2 influenza A strains.

Results

Similar Frequencies of Circulating Plasma Cells Following TIV and EI

We studied a group of five subjects immunized with TIV and six subjects enrolled in a protocol of EI with influenza H3N2 A/Wisconsin/67/2005 [13] (Table 1). At 21 days after immunization, all TIV subjects showed a >4-fold rise in antibody titer for HA binding for those components in the vaccine (Fig. S1 online) and a rise in influenza neutralization titer vs. H1N1 A/Solomon Islands/03/2006 or H3N2 A/Wisconsin/67/2005 (Table 1). At 28 days after experimental infection, 5/6 EI subjects had a >4-fold rise in antibody titer against the infecting strain H3N2 A/Wisconsin/67/2005 (Fig. S1 online). For one subject, EI03, no convalescent sample was available; testing of the day 7 sample showed a 3.7-fold rise in titer against the infecting strain (Fig. S1 online). Neutralization titers rose for all EI subjects [2-fold to 16-fold rise; Table 1]. Symptom severity did not correlate with infecting dose (Table 1).

As described [14] we analyzed PBMC for the presence of plasma cells (CD3+/14+/CD27hi CD19+ CD20+76m CD38hi) seven days after TIV or EI. There was no difference in plasma cell frequencies between five TIV subjects and six EI subjects as a percentage of the total B cell population (CD3/14/16/235a CD19+) in PBMC [TIV mean 2.75% ± 0.90%; EI mean 2.26 ± 0.74%; two-tailed t test, p = 0.68] (Fig. 1A; Fig. S2 online).

Higher Frequencies of Influenza-Specific Plasma Cells Following TIV

We isolated single plasma cells from both TIV and EI subjects for the generation of recombinant (r) mAb [14], recovering 404 mAbs from five TIV subjects and 451 mAbs from six EI subjects (Figs. 1B, 1C; Fig. S3 online). The characteristics of all recovered rmAbs are displayed in Tables S3, S4, S5, S6, S7, S8, S9, S10 and Figures S6, S7 online. All 855 rmAbs were tested in ELISA and Luminex®-based assays for reactivity with a panel of nine baculovirus-expressed rHAs and split-virus vaccine preparations (TIV for 2007–2008 or 2008–2009 seasons; see Methods). Split-virus vaccines contain immunogenic HA as well as neuraminidase, nucleoprotein, matrix proteins, and membrane fragments [15]. Almost two-thirds (252/404 rmAbs; 62.4%) of TIV rmAbs reacted with TIV or rHA while fewer than 10% (37/451 rmAbs; 8.2%) of rmAbs from EI subjects did (χ2 = 279.5, p < 0.0001) (Fig. 1B).

Thus, while TIV and EI were associated with a similar degree of plasmacytosis, the circulating plasma cells at 7 days in EI were less frequently HA-specific.

Increased Clonal Relatedness After TIV

We next analyzed the Ig heavy chain (HC) and light chain (LC) gene sequences from all 855 mAbs for clonal relatedness and found that mAbs from TIV subjects were more likely to fit in clonal lineages than mAbs from EI subjects (Fig. 1C; Table S1, Table S2, Fig. S4 online). From TIV subjects, 175/404 (43.3%) mAbs could be arranged into 46 clonal lineages containing 2–19 unique members (Table S1 online). In contrast, 28/451 (6.2%) mAbs from EI subjects could be arranged into one of 12 clonal lineages containing 2–4 unique members (Table S2 online) (Fig. 1C) (χ2 = 162.1, p < 0.0001). Clonal expansion was detected in most subjects [4/5 (80%) of TIV subjects; 4/6 (67%) of EI subjects]; TIV14, EI02, and EI07 lacked evidence for clonal expansion.

Of the 46 clonal lineages identified from TIV subjects, 44 (96%) had at least one rmAb that reacted with influenza antigens; clonal lineages containing rmAbs reactive with influenza antigens were found in all four TIV subjects with identified clonal lineages (Table S1 online). In contrast, of the 12 clonal lineages recovered from EI subjects, only 2 (17%) contained members reactive with rHAs and split-virus vaccine preparations (Table S2 online). Both influenza-reactive lineages were from one subject (EI13) and all rmAbs from these two lineages reacted with rHA. Ninety-one percent (159/175) of rmAbs in TIV clonal lineages were reactive with influenza antigens while only 21% (6/28) of clonal lineages containing rmAbs from EI subjects contained members reactive with influenza antigens (Table S1 online) (χ2 = 76.5, p < 0.0001). When viewed as a proportion of all rHA-specific mAbs, antibodies from TIV were more likely to be in a clonal lineage (159/232; 63.1%) compared to mAbs from EI subjects (6/37; 16.2%) (Fig. 2A, Fig. S4 online) (χ2 = 20.9, p < 0.0001). Thus, while TIV and EI were associated with a similar degree of plasmacytosis, TIV was associated with more clonal expansion of plasma cells making anti-HA antibodies.

Cross-reactive rmAbs Recovered from TIV and EI

Using a panel of 9 rHAs representing diverse temporal (1994–2009) and antigenic (influenza A H1, H3, H5 and influenza B) strains, we tested all 855 rmAbs for their ability to bind rHAs in
ELISA and Luminex® assays. In addition we tested mAbs for binding to split-virus TIV preparations from the 2007–2008 and 2008–2009 seasons. The majority of influenza-specific mAbs isolated from TIV subjects were strain-specific (159/252; 63%) while rmAbs reactive with single influenza HA strains were less common in EI subjects (16/37; 43%) (χ² = 7.74, p = 0.0054) (Fig. 2B). In contrast, rmAbs cross-reactive with 3 or more influenza HA strains were much less common in TIV compared to EI subjects (16/37; 43%) while rmAbs reactive with single influenza HA strains were less common in EI subjects (16/37; 43%).

**Table 1. Subject Characteristics.**

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<tr>
<th>Subject ID</th>
<th>Age</th>
<th>Gender</th>
<th>Immunogen</th>
<th>Peak Sx Score¹</th>
<th>Change in Neutralization Titer from d0 to d21</th>
<th>Change in Neutralization Titer from d0 to d28</th>
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<td>0 to 1:320</td>
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<tr>
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<td>Male</td>
<td>TIV 2008*</td>
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<td>1:80 to 1:80 (no Δ)</td>
<td>0 to 1:40</td>
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<tr>
<td>TIV21</td>
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<td>TIV 2008</td>
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<td>0 to 1:160</td>
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<tr>
<td>TIV24</td>
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<td>Female</td>
<td>TIV 2008</td>
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<td>1:80 to &gt;1:1280</td>
<td>1:10 to 1:640</td>
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<tr>
<td>mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<table>
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<th>Gender</th>
<th>Infecting Strain</th>
<th>Peak Sx Score¹</th>
<th>Change in Neutralization Titer from d0 to d21</th>
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<td>1:10 to 1:40</td>
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<td>H3 Wisc 10^5 TCID₅₀</td>
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</tr>
<tr>
<td>EI05</td>
<td>25</td>
<td>Female</td>
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<td>12</td>
<td>1:10 to 1:10 (no Δ)</td>
<td>1:20 to 1:320</td>
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<tr>
<td>EI07</td>
<td>35</td>
<td>Male</td>
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<td>12</td>
<td>0 to 0 (no Δ)</td>
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<tr>
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<td>Male</td>
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<td>4</td>
<td>1:40 to 1:40 (no Δ)</td>
<td>1:10 to 1:80</td>
</tr>
<tr>
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<tr>
<td>mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.0</td>
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**TIV rmAb Reactivity Is Similar to Plasma Antibody Specificities**

The antibody response in TIV subjects was largely restricted to the subtypes present in the administered vaccine and for most subjects was strain-specific (Text S1, Figs. S1, S8 online). The strain specificity of the response was most striking in the rmAb-binding pattern against rHAs (Text S1, Figs. S1, S8 online). In EI subjects plasma antibody rose more modestly (Fig. S1 online) and recovered rmAbs were less strain specific (Fig. S8 online). The most broadly cross-reactive anti-HA rmAbs were recovered from subject EI13 (Fig. 3; Fig. S8, Table S11 online); comparison of plasma antibody at time zero and 28 days after infection demonstrated a 13-fold increase in rHA H3 A/Wisconsin/67/2005 binding (Fig. S1 online) but only a 2-fold rise in virus neutralization titer to that strain (Table 1). Thus, the broad neutralizing rmAbs isolated from EI13 (Fig. 3) did not contribute significantly to plasma antibody 28 days following EI.

**Table 1. Subject Characteristics.**


¹Modified Jackson score [48]. N/A = not applicable.

Higher Frequency of Original Antigenic Sin (OAS) Antibodies in EI

OAS antibodies are defined as rmAbs that react with rHAs to which the subject was exposed before the current vaccine or infection [12,16,17,18,19]. In this study, OAS rmAbs reacted with rHA strains not contained in the administered vaccine for TIV subjects or with the infecting H3N2 A/Wisconsin/67/2005 strain for EI subjects. From TIV subjects, 5/252 (2%) rmAbs were consistent with OAS (Fig. 2C, Table 2). In contrast, from EI subjects, 7/37 (19%) influenza-specific rmAbs had rHA binding consistent with OAS (Fig. 2C, Table 2); those rmAbs reacted with influenza A H3 rHAs, influenza B rHA, and trivalent vaccine from 2008–2009 (Table 2). Thus, OAS rmAbs from EI subjects comprised a greater proportion of influenza-reactive rmAbs compared to those from TIV subjects (χ² = 19.2, p<0.0001) (Fig. 2C).

Cross-reactive rmAbs (Table S11 online) did not have characteristics of OAS; in fact, no OAS rmAb reacted with more than two rHAs (Table 2). The most common specificity of OAS rmAbs was to rHA H3 A/Johannesburg/33/1994 (TIV 3/5, EI 5/10), a strain isolated more than a decade before the current study was performed. Comparison of the infecting strain with other H3 rHAs tested showed that the Johannesburg strain was least similar (88% amino acid identity vs. 96–98% for other tested strains; Fig. S12 online) suggesting that rHA sequence homology was not responsible for eliciting these antibodies. OAS rmAbs were more frequently recovered from the same EI subjects from whom the most broadly cross-reactive rmAbs derived, suggesting that these populations were stimulated in parallel during EI. Thus, TIV is characterized by few OAS mAbs, clonal expansion of anti-HA responses, and seroconversion to the vaccine; while EI is characterized by more OAS mAbs, less clonal expansion of anti-HA responses, and weaker seroconversion to the infecting strain.
Presence of Strain-specific and Cross-reactive Anti-HA rmAbs in a Single Clonal Lineage From an Influenza Vaccinated Subject

Clonal lineages from TIV subjects were commonly recovered and largely influenza strain-specific (Table S1 online). Analysis of lineage 641 recovered from TIV01 showed remarkable divergence in rHA reactivity within the clonal lineage (Fig. 4). This lineage is composed of IgG1 and IgA1 members [10/18 and 8/18, respectively], and rmAbs in the upper portion of the lineage reacted primarily with rHA H1 A/Solomon Islands/03/2006 (15/18, 83%). For example, rmAb 1270 displayed high affinity for H1 A/Solomon Islands/03/2006 and much lower affinity for H1 A/Brisbane/59/2007 (Fig. 4). These data were consistent with the neutralization pattern of rmAb 1270 which inhibited H1N1 A/Solomon Islands/03/2006 at 0.02 μg/mL but did not inhibit H3N2 A/Wisconsin/67/2005.

One of the IgA1 branches of lineage 641 was composed of six members (rmAbs 692, 1190, 1203, 1218, 1232, and 2249), two of which (1205, 1218) did not bind to any rHA tested (Fig. 4). To study affinity maturation in this lineage branch, we produced inferred intermediate antibodies #5 and #5 (Fig. 4) and these bound with high affinity to H1 A/Solomon Islands/03/2006 but did not bind to other rHAs. The more distantly related branch point intermediate #15 also bound only to H1 A/Solomon Islands/03/2006 but with lower affinity (Fig. 4). These data are consistent with affinity maturation occurring within this branch of clone 641.

Remarkably, IgG1 clonal lineage members in the bottom portion of the 641 tree displayed both an increased affinity for rHA H1 A/Solomon Islands/03/2006 and to other rHAs (Fig. 4). In particular, intermediate #9 bound with high affinity to H1 A/Solomon Islands/03/2006, with moderate affinity to both H1 A/Brasilia/59/2007 and pandemic H1 A/California/04/2009, and with weak affinity to H3 A/Wisconsin/67/2005 (Fig. 4). However, this rHA binding breadth was lost in some more distant members of the lineage (e.g., rmAb 2258; Fig. 4). Intermediate #11 did not bind rHA H3 A/Wisconsin/67/2005 and showed weak affinity for novel H1 A/California/04/2009; the two rmAbs derived from this intermediate (rmAbs 1261 and 676) retained similar breadth and affinity of rHA binding as intermediate #11 (Fig. 4). Subject TIV01 from whom this lineage was derived showed a greater rise in plasma H1N1 A/Solomon Islands/03/2006 neutralization (Table 1) and binding titer (Fig. S1 online) compared to the rise against H3N2 A/Wisconsin/67/2005; and the rmAbs in lineage 641 recapitulate this finding.

Overall, nine lineages from TIV subjects showed evidence of class switching with IgG1 to IgA1 class switching being most common (7/9 lineages; Table S1 online). Additional clonal lineages from subjects TIV01 and TIV21 are presented in the supporting materials online (Text S1, Figs. S10 and S11 online).

Broadly Reactive Clonal Lineage 2569 from H3N2 Infected Subject EI13

As noted, clonal lineages from EI subjects were detected less frequently than from TIV subjects, and only two clonal lineages had rmAbs reactive with influenza antigens (Table S2 online). The largest clonal lineage from subject EI13 consisted of four highly cross-reactive mAbs (Fig. 3; Table 3; Table S11 online). This lineage had one IgG1 and three IgM members; each member of this lineage had a VDJ mutation frequency higher than the average for influenza-specific mAbs from EI (range 8.7–14.4%; EI overall mean 8.6%; Fig. S5 online). Members of this lineage bound rHAs of H1N1 A/Solomon Islands/03/2006 and H3N2 A/Wisconsin/67/2005 in surface plasmon resonance (Fig. S9B, S9C online) and blocked hemagglutination of these two strains (Table 3). These rmAbs also neutralized five temporally distinct H3N2 viruses as well as H1N1 A/Solomon Islands/03/2006 (Table 3). High affinity binding to H3 rHAs, lower affinity for H5 rHAs and even lower affinity for rHA of the pandemic H1N1 A/California/04/2009 strain paralleled the observed pattern of neutralizing activity (Table 3, Fig. 3). The highest affinity binding in clonal lineage 2569 was observed to a temporally remote strain, H3 A/Johannesburg/33/1994 (Fig. 3) that has lower sequence homology to the infecting strain than other tested rHAs (Fig. S12 online). As noted above, broadly HA-reactive antibodies did not boost (Fig. S1 online) nor did neutralizing antibodies persist as a high-titered response in this subject (Table 1). These data suggest that in this subject, H3N2 A/Wisconsin/67/2005 stimulated a subdominant clone of B cells capable of making broadly neutralizing antibodies but that those
Figure 2. Characterization of influenza-specific mAbs from TIV or EI subjects. A. Clonal lineages. From TIV subjects, 159/252 (63.1%) of influenza-specific rmAbs were members of 44 clonal lineages (purple wedge); from EI subjects, 6/37 (16.2%) were members of 2 clonal lineages ($\chi^2 = 28.9$, $p < 0.0001$). B. Multiple reactivity to influenza antigens. From TIV subjects, 159/252 (63%) of rmAbs were strain-specific (blue wedge); multiply reactive rmAbs were less common [two antigens 79/252 (31.4%) (yellow wedge); three antigens 11/252 (4.4%) (orange wedge); four antigens 2/252 (0.8%) (red wedge); five antigens 1/252 (0.4%) (black wedge)]. From EI subjects, 16/37 (43.2%) were strain-specific ($\chi^2 = 7.74$, $p = 0.0054$); multiply reactive mAbs were more common [two antigens 8/37 (21.6%); three antigens 8/37 (21.6%); four antigens 3/37 (8.1%); five antigens 1/37 (2.7%); six antigens 1/37 (2.7%) (white wedge)]. C. Original antigenic sin rmAbs. From TIV subjects, 5/252 (2%) of influenza-specific rmAbs did not react with strains contained in the administered vaccine but only with previously circulating influenza antigens (striped wedge). From EI subjects, 7/37 (19%) of influenza-specific rmAbs did not react with the infecting strain but only with previously circulating antigens ($\chi^2 = 19.2$, $p < 0.0001$).

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Figure 3. Clonal lineage 2569 from EI13. Three of 4 members (75%) derived from IgM-expressing plasma cells, 1/4 (25%) derived from an IgA1 plasma cell. The highest affinity binding for all members was to H3 Jobg; high affinity binding to other H3 rHAs and H1 Bris was also observed. Three members were tested for HAI and neutralization and displayed similar breadth (Table 3). H1 SI = H1N1 A/Solomon Islands/03/2006; H1 Bris = H1N1 A/Brisbane/59/2007; H1 Cal = H1N1 A/California/04/2009; H3 Wisc = H3N2 A/Wisconsin/67/2005; H3 Bris = H3N2 A/Brisbane/10/2007; H3 Jobg = H3N2 A/Johannesburg/33/1994; H5 Indo = H5N1 A/Indonesia/05/2005; H5 Viet = H5N1 A/Vietnam/1203/2004.

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antibodies did not significantly contribute to the plasma antibody pool at day 28 after infection (Table 1; Fig. S1 online).

**Discussion**

In this study, we compared the plasma cell repertoires present at 7 days following TIV and EI. Previous studies have shown that influenza-specific plasma cells appear transiently in the blood with a peak at 7 days after immunization following which they rapidly decline [6]. Studies of infection, however, have been limited to natural infection where timing must be estimated based on exposure and symptoms [20], or where the infection occurred months to years before antibodies were isolated [10,21,22]. This study of vaccination and experimental infection allowed us to compare the two stimuli at exactly the same point after challenge. We found a similar degree of plasmacytosis in the two conditions but found that the frequency of HA-specific plasma cells and the degree of clonality of those cells was lower in the EI group. These findings differ from studies of natural infection where both a higher frequency and clonality of influenza-specific antibodies were found [20]. Trafficking of B cells after infection may have a different kinetic pattern than that following vaccination. This EI study stopped collecting cell samples at 7 days post-infection and so future studies of later time points will be required to determine if the polyclonality of the plasma cell response observed is specific for the period early after infection, specific for H3N2 A/ Wisconsin/67/2005 infection, or if infection with novel pandemic strains consistently stimulates B cell clonal expansion.

For each subject there was an increase in rHA binding and neutralization titers 3–4 weeks after antigen stimulation. The magnitude of rise was greater in the TIV cohort although all EI subjects showed a rise in titer against the infecting strain that was similar to that observed in outbreak situations [23]. The presence of asymptomatic but productive infection is common and has been observed for seasonal influenza [24], pandemic H1N1 [23], and human infection with avian H5N1 [25]. Thus, we found it interesting that similar frequencies of rHA-specific antibodies were recovered from subjects with the highest and lowest symptom scores (Fig. S3 online), and that the most broadly cross-reactive rmAbs were recovered from a subject with mild symptoms and a modest rise in binding and neutralization titer. Whether this response contributed to the mild symptoms experienced by this subject is unknown and future studies will have to address the potential therapeutic role for this group of rmAbs.

The plasma-cell-derived rmAbs from TIV subjects were more frequently rHA-specific and showed evidence of clonal expansion while rmAbs derived from EI subjects were less frequently rHA-specific and had less evidence of clonal expansion. In all subjects, anti-HA responses were primarily strain-specific, but rmAbs derived from EI subjects were more frequently cross-reactive for multiple influenza strains or consistent with OAS compared with rmAbs derived from TIV subjects. In the EI cohort, responses to influenza proteins induced by infection that were not detected in our assays may have been present. Although split-virus vaccines like that we used for screening contains antigens other than HA [15], the presence of antibodies reactive with antigens not tested by our assays cannot be excluded. Isolation of rmAbs from both EI and TIV subjects reactive only with split-virus vaccine preparations (Fig. S8 online) suggests that some recovered rmAbs were reactive with antigens other than HA.

Vaccination is the primary means to prevent seasonal [5] and pandemic [26] influenza infection, however, antibody responses to TIV are generally not broadly-neutralizing but rather strain-specific and directed at highly variable domains on HA [7,8]. Such responses are not absolute—one rmAb isolated from TIV01 was recently shown to have cross-reactivity related to its ability to recognize the sialic acid receptor-binding pocket of HA [27]. Vaccines that bypass regions of diversity by targeting other influenza proteins [e.g., the virus-associated proton-channel M2 [28]] have not been successful in clinical trials [29]. Targeting conserved regions on HA could provide a route to a “universal” influenza vaccine [8,30,31], but seasonal vaccines have not consistently induced these specificities of antibodies as shown in our study. Influenza vaccination strategies using novel adjuvants may induce a greater antibody breadth against HA [32] and it will be of interest to compare mAbs derived from newer vaccines with those currently in use.

**Table 2.** Characteristics of OAS rmAbs.

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>rmAb ID</th>
<th>Lineage*</th>
<th>Isotype</th>
<th>HC Usage</th>
<th>HC CDR3 Length</th>
<th>HC Mutation</th>
<th>LC Usage</th>
<th>LC CDR3 Length</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIV01</td>
<td>1318</td>
<td>A1</td>
<td>V, 4-30 J, 6</td>
<td>18</td>
<td>2.7%</td>
<td>V, 3-20 J, 2</td>
<td>10</td>
<td>6</td>
<td>B Fl, TIV 2008*</td>
</tr>
<tr>
<td>TIV24</td>
<td>2516</td>
<td>N/A</td>
<td>V, 3-15 J, 6</td>
<td>16</td>
<td>10.2%</td>
<td>V, 3-21 J, 1</td>
<td>11</td>
<td>6</td>
<td>H3 Wisc, H3 Jobg</td>
</tr>
<tr>
<td>TIV24</td>
<td>2523</td>
<td>N/A</td>
<td>V, 3-15 J, 6</td>
<td>16</td>
<td>8.4%</td>
<td>V, 1-33 J, 5</td>
<td>9</td>
<td>H3 Jobg</td>
<td></td>
</tr>
<tr>
<td>TIV24</td>
<td>2549</td>
<td>N/A</td>
<td>V, 3-13 J, 6</td>
<td>21</td>
<td>10.5%</td>
<td>V, 1-39 J, 9</td>
<td>4</td>
<td>H1 SI</td>
<td></td>
</tr>
<tr>
<td>TIV24</td>
<td>2575</td>
<td>N/A</td>
<td>V, 3-15 J, 6</td>
<td>16</td>
<td>4.7%</td>
<td>V, 1-39 J, 4</td>
<td>9</td>
<td>H3 Jobg</td>
<td></td>
</tr>
<tr>
<td>EI03</td>
<td>1938</td>
<td>N/A</td>
<td>V, 4-31 J, 6</td>
<td>19</td>
<td>10.3%</td>
<td>V, 1-39 J, 5</td>
<td>9</td>
<td>H3 Jobg</td>
<td></td>
</tr>
<tr>
<td>EI03</td>
<td>1974</td>
<td>N/A</td>
<td>V, 3-74 J, 4</td>
<td>17</td>
<td>10.3%</td>
<td>V, 3-11 J, 2</td>
<td>9</td>
<td>H3 Jobg</td>
<td></td>
</tr>
<tr>
<td>EI03</td>
<td>1975</td>
<td>N/A</td>
<td>V, 2-70 J, 1</td>
<td>17</td>
<td>5.5%</td>
<td>V, 1-40 J, 3</td>
<td>11</td>
<td>H3 Jobg</td>
<td></td>
</tr>
<tr>
<td>EI03</td>
<td>1997</td>
<td>N/A</td>
<td>V, 1-69 J, 5</td>
<td>14</td>
<td>10.8%</td>
<td>V, 1-13 J, 4</td>
<td>9</td>
<td>H3 Jobg, H3 Bris</td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>2318</td>
<td>N/A</td>
<td>V, 1-39 J, 4</td>
<td>16</td>
<td>10.1%</td>
<td>V, 1-51 J, 2</td>
<td>11</td>
<td>B Fl</td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>2423</td>
<td>N/A</td>
<td>V, 3-9 J, 4</td>
<td>18</td>
<td>4.8%</td>
<td>V, 1-39 J, 9</td>
<td>9</td>
<td>H3 Jobg, H3 Bris</td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>2431</td>
<td>N/A</td>
<td>V, 3-13 J, 6</td>
<td>14</td>
<td>9.4%</td>
<td>V, 1-44 J, 3</td>
<td>11</td>
<td>H3 Jobg</td>
<td></td>
</tr>
</tbody>
</table>

*Lineage ID from Table S1 (available online). N/A = not applicable.


2 Reactivity to TIV01 could represent cross-reactivity to the B/Florida/04/2006 component.

doi:10.1371/journal.pone.0025797.0002

Influenza Ab Cross-Reactivity/Clonal Expansion
By producing inferred intermediate antibodies of one clonal lineage, we found evidence of induction and modulation of antibody breadth stimulated by TIV (clonal lineage 641; Fig. 4). This evidence of affinity maturation in humans is similar to findings in mice [33,34] that were used as evidence of V(D)J mutation as the source of affinity maturation [35]. Clonal lineage 641 was remarkable in that some inferred intermediate rmAbs showed a greater breadth of rHA binding than the recovered rmAbs, suggesting that continued affinity maturation might have eliminated rHA cross-reactivity. Our novel observation of a branch displaying cross-reactivity in an otherwise strain-specific clonal lineage implies that making such cross-reactive branches immunodominant through novel vaccine strategies could lead to improved cross-strain protection.

In contrast to the findings in TIV subjects, we found that EI subjects showed less evidence of clonal expansion seven days after infection despite having plasma cell antibodies with a higher frequency of V(D)J mutations. Our observation of polyclonal activation after EI is similar to that seen in animals after γ-herpesvirus infection [36]. This suggests that in our EI subjects either B cell clonal expansion was not required for circulation of B cells at seven days after infection expressing affinity matured cross-reactive antibodies, and/or that the circulation dynamics of HA-specific plasma cells seven days after infection was different than that observed after vaccination.

The human mAb response to influenza infection has been studied in survivors of H5N1 avian influenza [10,22] and in isolation of mAbs from survivors of the 1918 H1N1 pandemic [21]; in these studies some mAbs were cross-reactive with related strains [21] and some mAbs also displayed cross-protection for novel influenza strains [22,37]. While broadly cross-reactive neutralizing antibodies against influenza can be induced with TIV, these responses are not sufficiently immunodominant to result in high-titer neutralizing antibodies that provide protection against infection with highly divergent strains. Our data demonstrate this phenomenon directly, in that cross-reactive rmAbs and inferred intermediates were found in the plasma cell repertoires of both TIV and EI subjects while such cross-reactive antibodies were less prominent or not detected in day 21 or day 28 plasma samples.

Work in mice suggested that some anti-influenza responses can be associated with restriction of Ig gene usage [38], and recent work has suggested that human antibodies with broad cross-reactivity may preferentially derive from restricted Ig gene pools [10,11,36]. Phage-displayed antibody libraries screened for binding to H5 HA were enriched for Vh1-69 usage [11] and
broadly reactive anti-HA stem mAbs using V$_H$1-69 and V$_H$3-21 have been produced from IgM memory B cells [39]. Corti et al. recently reported a series of TIV-induced cross-reactive antibodies using primarily V$_H$1-69 demonstrating that cells expressing those antibodies can be isolated following seasonal vaccination [12]; a strict requirement for V$_H$1-69 was not shown as there were additional antibodies using V$_H$3-23, V$_H$3-30, V$_H$3-53 and V$_H$4-39. It was interesting that none of our clonal lineages used V$_H$1-69 and only three cross-reactive antibodies in this study used that V$_H$ gene. This could be due to a lack of V$_H$1-69 gene usage in our subject population as the frequency of V$_H$1-69 expressing B cells is related to copy number variation [40], and whether this is the case for our current subject groups is not known. In our study, other Ig genes were found to make cross-reactive antibodies including V$_H$3-23, V$_H$3-74, and V$_H$1-46. One group of these antibodies, clonal lineage 2569, was remarkable both in the degree of mutation and that its members were IgM and IgA1. These findings are consistent with the recall of an IgM$^*$ memory B cell lineage expressing a broadly cross-reactive antibody response that appeared transiently following infection but that did not predominate in plasma four weeks after infection. A similar phenomenon has been observed following TIV with isolation of broadly reactive subdominant antibodies that did not predominate in convalescent plasma [12]. In our EI subject, stimulation of a subdominant response may have contributed to the mild symptoms experienced by this subject and could reflect one mechanism by which antibody responses contribute to control of a virus infection without resulting in long-lasting antibody titers. These findings are also consistent with Pape et al. who reported that class-switched memory B cells have different circulation kinetics compared to IgM memory B cells, with the latter persisting for longer periods but also being subject to suppression in the presence of cross-reactive plasma antibodies [41].

Finally, OAS antibodies were more frequent among mAbs derived from EI subjects compared with TIV subjects. Prior studies have shown that OAS occurs following both influenza vaccination [18] and infection [16,19], although work by Wrammert et al. suggested that OAS in humans following influenza vaccination was uncommon; our study of TIV subjects is consistent with Wrammert et al. [6]. That the majority of OAS mAbs were reactive with HA from the H3 A/Johannesburg/33/1994 strain is consistent with both prior exposure to that strain and recall by immunization with H3N2-containing vaccine or by infection with H3N2 influenza virus.

Regardless, the presence of both OAS and cross-reactivity in mAbs from EI subjects suggests that both kinds of antibodies were stimulated in parallel during infection. Whether these two processes can be decoupled to only stimulate broadly cross-reactive antibodies by a vaccine remains unknown. Corti et al. showed that the H5 anti-HA antibody response following TIV was detectable but generally weak, both for serum antibodies and memory B cell frequency [12]. Recent reports have described vaccine designs that may stimulate more broadly reactive anti-HA antibodies. Khurana et al. showed increased anti-HA antibody breadth using an adjuvanted vaccine [32]. Wei et al. [31] and Wang et al. [42] have demonstrated induction of broad of plasma antibody responses by sequential immunization with heterologous HAs, perhaps replicating the cross-protective response that can occur after multiple seasonal influenza infections in ferrets [43]. The fact that both seasonal influenza vaccination and infection involve exposure to heterologous HAs on an annual basis, and that these exposures do not lead to high levels of influenza resistance in the general population, suggests that sequential exposure to heterologous HAs by itself is insufficient. Targeting of the conserved HA stalk to induce antibodies in mice reactive across influenza subtypes has recently been demonstrated through the use of synthetic peptides [44] and rHA subunits [45]. It remains to be seen whether such vaccine strategies will work in humans.

In summary, we have shown that while the anti-HA response in vaccination and H3N2 A/Wisconsin/67/2005 infection differs in the degree of clonal expansion present at seven days, broadly reactive antibodies are induced in both settings. One strategy for vaccine design to expand B cell clonal lineages of broadly cross-reactive antibodies may be to create novel HA molecules with enhanced binding to germline B cell receptors of HA-responsive naive B cells [46,47]. Thus, the study of clonal lineages of antibodies with HA binding and breadth of influenza neutralization in both vaccination and infection could provide guidance for design of influenza vaccines capable of inducing immunodominant broadly cross-reactive antibody responses.

### Methods

#### Ethics Statement

All subject recruitment was performed using written informed consent. For studies performed at Duke University, the Duke University Health System Institutional Review Board for Clinical Investigations approved the protocols. For studies performed at Retroscreen Virology, LTD (Brentwood, UK), the protocol was approved by the East London and City Research Ethics Committee 1 (London, UK), an independent institutional review board (WIRB: Western Institutional Review Board; Olympia, WA), the Duke University Health System Institutional Review Board for Clinical Investigations (Durham, NC), and the SCC-SD IRB (US Department of Defense; Washington, DC). Subjects recruited at Retroscreen Virology, LTD also consented to unspecified future use of their specimens; these specimens were kept at Duke University in the Clinico-Molecular Predictors of Presymptomatic Infectious Disease – Biorepository. The research...
performed was approved by the Duke University Health System Institutional Review Board for Clinical Investigations either as a part of the original study or as an exempt study via the biorepository.

Subjects

Subjects in the TIV group were recruited at Duke University and were given trivalent inactivated seasonal influenza vaccine (Sanofi Pasteur, Swiftwater PA); 2007–2008 Fluzone® vaccine containing A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004 was given to TIV01 and TIV04; 2008–2009 Fluzone® vaccine containing A/Brussels/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2) (classified as a A/Brussels/10/2007-like strain), and B/Florida/04/2006 was given to TIV14, TIV21, and TIV24. Blood was drawn from immunized subjects on day 0 before vaccination and on days 7 and 21 after challenge.

The EI protocol was performed at Retroscreen Virology, LTD (Brentwood, UK) as previously described [13]. Subjects were prescreened and provided informed consent before being given an intranasal challenge. The influenza challenge stock was A/Wisconsin/67/2005 (H3N2) manufactured from a human isolate passed three times in Primary Chick cells, then twice in eggs, before two final passages in GMP Vero cells. The latter stage of passing in GMP Vero cells was accomplished under GMP conditions at Baxter Bioscience (Vienna, Austria). The final product underwent quality testing for identity, appearance, sterility, infectivity and adventitious contaminants, and was assessed according to pre-determined specifications. On the day of inoculation, volunteers received \(10^6.41\) (subjects EI02, EI03), \(10^6.25\) (subjects EI05, EI07), \(10^6.41\) (subject EI12), and \(10^6.08\) (subject EI13) TCID\(_{50}\) of the challenge stock. In this protocol, blood was drawn before challenge, then daily on days 0–7, and on day 28 after challenge. Cells were only collected on samples through day 7. Symptoms were recorded twice daily using a modified Jackson scoring system [48]. Infection was confirmed in all subjects by the presence of viral shedding in nasal washings, seroconversion in day 28 serum samples, or both.

Single-Cell Sorting

PBMC were isolated from blood on the day of draw and cryopreserved using standard techniques until thawed for sorting. Cryopreservation of PBMC was necessary as one protocol was carried out in the UK; in order to harmonize the two protocols all samples were studied after cryopreservation. Single-cell sorting was performed as previously described [14] using a panel of antibodies reactive with the following cell surface molecules: CD138 (FITC), surface IgD (PE), CD3 (PE-Cy5), CD16 (PE-Cy5), CD235a (PE-Cy5), CD20 (PE-Cy7), CD19 (APC-Cy7), CD27 (PacificBlue®) (BD Biosciences, San Jose, CA); CD38 (APC-Cy5.3) (eBioscience, San Diego, CA); CD14 (PE-Cy5), CD38 (APC-Cy5.5) (Invitrogen, Carlsbad, CA); CD38 (APC-AlexaFluor® 647) (Beckman Coulter, Brea, CA). Plasma cells/plasmablasts were sorted using a BD FACSAriaTM or a BD FACSAriaTM II (BD Biosciences, San Jose, CA) by gating on CD3+CD14-CD16-CD235a-CD19+CD20+CD27hiCD38hi cells. Flow cytometry data was analyzed using FlowJo (Treestar, Ashland, OR). The fraction of plasma cells isolated from peripheral blood is lower than the level reported by Wrammert et al. [mean 6.4%] [6]; this is likely due to the use of cryopreserved PBMC in the present study.

Single cells were directly sorted into 96-well plates containing 20 \(\mu\)L per well of RT reaction buffer [5 \(\mu\)L of 5X first strand cDNA buffer, 0.5 \(\mu\)L RNaseOUT™ (Invitrogen, Carlsbad, CA), 1.25 \(\mu\)L dithiothreitol, 0.0625 \(\mu\)L IGEPAL® CA-630 (Sigma, St. Louis, MO), 13.25 \(\mu\)L of dH\(_2\)O (Invitrogen, Carlsbad, CA)]; plates were stored at \(-80^\circ\)C until use and again stored at \(-80^\circ\)C until PCR was performed.

PCR Isolation of Ig V\(_{H}\) V\(_{K}\) and V\(_{L}\) Genes

Single cell PCR was achieved as described [6,14,49]. Briefly, reverse transcription (RT) was performed using 50 units per reaction SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and 0.5 \(\mu\)M human constant region primers (IgG, IgA1, IgA2, IgM, IgD, Igk, Ig\(_\lambda\)) at 37 \(^\circ\)C for 1 h. Separate reactions were used to amplify individual families of V\(_{H}\), V\(_{K}\) and V\(_{L}\) genes from the cDNA template; this was performed using two rounds of PCR [first round: 5 \(\mu\)L of RT reaction product, 5 units HotStar Taq Plus (QIAGEN, Valencia, CA), 0.2 mM dNTPs, 0.5 \(\mu\)M nested constant region primers (IgH consisting of IgM, IgD, IgG, IgA1, IgA2; Igk; or Ig\(_\lambda\)) and matched variable region primers; second round: 2.5 \(\mu\)L of first round reaction product, 5 units HotStar Taq Plus (QIAGEN, Valencia, CA), 0.2 mM dNTPs, 0.5 \(\mu\)M nested constant region and nested variable region primers]. First round PCR was cycled as follows: 95 \(^\circ\)C x 5 min, 35 cycles of [95 \(^\circ\)C x 30 s, 55 \(^\circ\)C (V\(_{H}\) and V\(_{L}\) or 50 \(^\circ\)C (V\(_{L}\)) x 60 s, 72 \(^\circ\)C x 90 s] and one cycle 72 \(^\circ\)C x 7 min. Second round PCR was similar except the extension step was performed at 58 \(^\circ\)C (V\(_{H}\)), 60 \(^\circ\)C (V\(_{L}\)), or 64 \(^\circ\)C (V\(_{k}\)). Products were analyzed using agarose gels (1.2%) and purified using PCR purification kits (QIAGEN, Valencia, CA).

Products were sequenced in forward and reverse directions using a BigDye® sequencing kit on an ABI 3700 (Applied Biosystems, Foster City, CA). Sequence base calling was performed using Phred [50,51]; forward and reverse strands were assembled using an assembly algorithm based on the quality scores at each position [52]. The estimated PCR artifact rate was 0.28 or approximately one PCR artifact per five genes amplified. Ig isotype was determined by local alignment with genes of known isotype [53]; V, D, and J region genes, CD3R loop lengths, and mutation frequencies were identified using SoDA [54]. All data was annotated so that matching subject data and sort information was linked to the cDNA sequence and analysis results.

For this analysis, when two LC were paired with a single HC (55 from TIV, 60 from EI) the LC with the higher estimated frequency of mutation as calculated by SoDA was selected for inclusion in the dataset. This resulted in unambiguous selection in 114 cases. In one case both HC had an estimated rate of mutation of 0%; in this case the LC was selected at random. Both possible members of this pair were tested for binding and neither pairing was found to be reactive with any antigen tested.

Clonal lineage determination

Antibody gene sequences from individual subjects were grouped and analyzed using the following criteria: 1) matching of variable and joining region gene segments; 2) matching of CD3R loop lengths; and 3) ≥70% homology in CD3R nucleotide sequence. Clonal lineages were only identified if both heavy and light chains for a given group satisfied all three criteria. For heavy chain alignments, D region usage was not considered; i.e., heavy chain genes could be considered part of a clonal lineage if they satisfied the above three criteria but had different predicted D region gene usage. Maximum likelihood trees for clonal lineages were generated using V(D)J regions (excluding constant region sequences); trees were constructed (dnapl), reorganized (retree), and plotted (drawgram) with the PHYLIP 3.69 package [55]. Inferred intermediate V\(_{H}\) and V\(_{L}\) sequences generated by dnapl were synthesized (GeneScript, Piscataway, NJ) and expressed as IgG1 rmAbs as described below.
Expression of \( V_H \) and \( V_L \) as full-length IgG1 recombinant mAbs

Isolated Ig \( V_H \) and Ig \( V_L \) gene pairs were assembled by PCR into linear full-length Ig heavy- and light-chain gene expression cassettes using methods as described [14]. Human embryonic kidney cell line 293T (ATCC, Manassas, VA) was grown to near confluence in 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and transfected with 2 \( \mu \)g per well of both IgH and IgL purified PCR-produced cassettes using PolyFect or Effectene (QIAGEN, Valencia, CA). For PolyFect transfected cells, six to eight hours after transfection cells were fed with fresh culture medium supplemented with 2% fetal bovine serum and were incubated at 37°C in a 5% \( CO_2 \) incubator. For Effectene transfected cells, this wash step was omitted. Culture supernatants were harvested three days after transfection and concentrated four-fold using centrifugal concentrators; expressed IgG was transfected cells, this wash step was omitted. Culture supernatants were harvested three days after transfection and concentrated four-fold using centrifugal concentrators; expressed IgG was transfected with 2 \( \mu \)g per well of both IgH and IgL gene constructs were cloned into pcDNA 3.3 using standard molecular protocols. Antibody binding by ELISA, Luminex® and indirect immunofluorescence

Plasma samples from both subject cohorts were evaluated by ELISA [57] against a panel of baculovirus-expressed purified hemagglutinins (H1 A/Solomon Islands/03/2006, H1 A/Brisbane/59/2007, H1 A/California/04/2009, H3 A/Wisconsin/67/2005, H3 A/Brisbane/10/2007, H3 A/Johannesburg/33/1994, H5 A/Vietnam/1203/2004, H5 A/Indonesia/05/2005, B/Florida/04/2006) (Protein Sciences, Meriden, CT) and split virus vaccine preparations (2007–2008 Fluzone® vaccine containing A/Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Malaysia/2506/2004; 2008-2009 Fluzone® vaccine containing A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2) (classified as a A/Brisbane/10/2007-like strain), and B/Florida/04/2006) (Sanofi Pasteur, Swiftwater PA). Split-virus vaccine products have been previously shown to contain HA, neuraminidase, nucleoprotein, matrix protein, and membrane fragments [15]. Samples were diluted serially for the analysis and data were analyzed using 5-parameter curve fitting; endpoint titers were calculated as 3-fold above assay background. The assay cutoff was a 1:25 dilution. Expressed mAbs were tested in the same ELISA system. ELISA testing for antigens was considered positive if the optical density reading was above 0.13 units, except in the case of split virus influenza vaccine ELISA which was considered positive if the optical density reading was above 0.20 units. Affinity of mAbs was determined using 4-parameter curve fit of ELISA titer data.

Reactivity to influenza antigens was also studied using a standardized custom Luminex® assay [38]. Luminex® assays were considered positive if the blank-bead-subtracted value was greater than 20 units and greater than 10 divided by IgG concentration in \( \mu \)g/mL.

Transiently expressed mAbs with low IgG concentration were re-transfected and re-assayed. In cases where multiple tests of the same antibody were available, the final dataset contained data from the assay with the highest concentration of mAbs. In order to account for antigen overlap between influenza vaccines and purified HAs, antigen reactivities were condensed if reactivity was found to certain antigen pairs (e.g., mAb reactivity to both influenza vaccine 2007–2008 and H3 A/Wisconsin/67/2005 was counted singly). Antigen pairs treated this way were as follows: 2007–2008 vaccine & H1 A/Solomon Islands/03/2006; 2007–2008 vaccine & H3 A/Wisconsin/67/2005; 2008–2009 vaccine & H1 A/Brisbane/59/2007; 2009–2010 vaccine & H3 A/Brisbane/10/2007; 2008–2009 vaccine & B/Florida/04/2006.

Surface Plasmon resonance (SPR) analysis of antibody reactivity

SPR binding assays were performed on a BLAcore 3000 (BLAcore Inc, Piscataway, NJ) maintained at 20°C. HA recombinant protein was immobilized on a CM5 sensor chip by standard amine coupling as described [57,59]. Additional tests were performed by capturing human mAbs on anti-human Fc antibody-coupled surfaces; each human mAb was captured to about 200–500 RU. Specific binding responses of mAb binding were obtained following subtraction of non-specific binding on control surfaces. Rate constants were measured using the bivalent analyte model (to account for the avidity of bivalent Ig molecules) and global curve fitting to binding curves obtained from mAb titrations. Antibodies were injected at 30 \( \mu \)L/min for 2–6 min; glassine-HCl pH 2.0 was used as the regeneration buffer.

Hemagglutination Inhibition (HAI) Assay and Influenza Microneutralization Assay

The hemagglutination inhibition (HAI) assay and microneutralization assays were based on standard published protocols [60]. Influenza stocks were grown in embryonated eggs and were titered for hemagglutination units on turkey red blood cells. To perform HAI assays, serial dilutions in PBS of plasma or transfected cell supernatants were placed into 96-well plates and were mixed with an equal volume of washed turkey red blood cells (0.5%) and incubated at room temperature for 30 min before hemagglutination was read directly from the wells.

For microneutralization, MDCK cells were cultured as monolayers in 96-well culture plates at 37°C in 5% \( CO_2 \), followed by the addition of mixtures of serial dilutions of plasma or purified mAbs and 100 TCID50 of the appropriate infectious influenza virus stock. Plates were incubated at 37°C in 5% \( CO_2 \) for 18 h prior to assaying. Final assay of the infectious cultures was performed by using fluorescence (for virus constructs containing fluorescent reporter genes) by ELISA using mouse mAb anti-Influenza A nucleoprotein (BEI resources, Manassas, VA) at 1:4000 dilution, followed by HRP-conjugated Goat anti-mouse IgG (KPL, Gaithersberg, MD) at 1:2,500 dilution (according to CDC SOP); or by testing of the resulting virus cultures in HAI as described above.

Statistical analysis

Statistical tests were performed in SAS v9.2 (SAS Institute, Cary, NC). Comparisons for multiple groups (i.e., CDR3 length, mutation rate) were performed using multiple degree of freedom F-tests using PROC GLM in SAS v9.2 with subsequent pairwise comparisons. For data consisting of two groups only, t-tests using the Satterthwaite correction (for continuous variables), the Kolmogorov-Smirnov test (for comparison of probability distributions), and Pearson’s chi squared tests (for 2×2 category tables) were performed using the appropriate SAS PROC in SAS v9.2; the statistical test used is noted when \( p \)-values are presented. Graphs of the data were created using GraphPad Prism (GraphPad Software, La Jolla, CA) with layout in Illustrator CS4 (Adobe, San Jose, CA).

Supporting Information

Text S1 Additional description of results presented in Supplemental Information.
Influenza Ab Cross-Reactivity/Clonal Expansion

Figure S1  Influenza antigen binding titers of plasma from TIV and EI subjects. Plasma samples from day 0 and from day 21 (TIV) or day 28 (EI) were tested by ELISA for binding to split virus vaccine preparations and to purified recombinant hemagglutinins. For subject EI03, no day 28 plasma sample was available; a day 7 plasma sample was substituted for this analysis. ELISA was performed using serial dilutions and optical density readings were fitted to a 3-parameter curve; endpoint titers were determined as three fold over the background of the assay for each run. Data are plotted as reciprocal titer values and are pre-

Figure S2  Plasmacytosis following TIV or EI. Plots shown are of total B cell populations (CD3/14/16/CD19\(^{+}\)) and are normalized to 10000 events per panel. Ellipse in upper right corner of each panel is homologous to the sorting gate used for the final corner of each panel is homologous to the sorting gate used for the

Figure S3  Reactivities of human rmAbs recovered from TIV and EI. A. Antibodies from TIV subjects. We recovered plasma cells producing rmAbs against influenza antigens from all TIV subjects. In two subjects (TIV01 and TIV21) the majority of recovered rmAbs were reactive with influenza [179/245 (73%) and 39/41 (95%), respectively]. In the other three subjects, less than half of recovered rmAbs were influenza-specific [TIV04 8/17 (47%), TIV14 1/16 (6%), TIV24 35/85 (41%)]. B. Antibodies from EI subjects. We recovered plasma cells producing rmAbs against influenza antigens from five of six EI subjects; from one subject (EI12) we recovered 51 rmAbs that were not reactive for any antigen tested. None of the other EI subjects had more than 25% of rmAbs reactive with influenza [EI02 4/31 (13%), EI03 11/100 (10%), EI05 3/141 (2.1%), EI07 5/34 (9%), EI13 10/66 (21%).

Figure S4  Relationship between influenza reactivity and clonal lineages from TIV and EI subjects. Influenza-specific rmAbs recovered from TIV were more likely to be in a clonal lineage compared with EI. In TIV, 93/404 (23%) of rmAbs were influenza-specific but not a member of a clonal lineage (blue wedge), 159/404 (39%) were both influenza-specific and members of clonal lineages (purple wedge), while only 16/404 (4%) were members of clonal lineages but not reactive with influenza antigens (red wedge). In EI, 31/451 (6.9%) were influenza-specific but not part of a clonal lineage, 6/451 (1.3%) were both influenza-

Figure S5  VDJ mutation rate of influenza-specific mAbs from TIV and EI subjects. VDJ mutation rates in rmAbs from TIV subjects (range 0.9–30.3%, mean 5.8%±0.2%) were lower on average than rmAbs from EI subjects (range 0.4–15.8%, mean 8.6%±0.6%) (two-tailed t-test, \(p<0.0001\)).

Figure S6  VDJ mutation rate of influenza-specific mAbs from TIV and EI subjects by isotype of mAb. A. HC isotype of influenza-specific rmAbs from TIV subjects were found to be predominantly IgG1 (201/252, 80%), followed by IgA1 (36/252, 14%) and IgM (9/252, 3.6%). VDJ mutation rates for these three isotypes were as follows: IgG1 5.9%±0.2%, IgA1 6.1%±0.8%, IgM 3.9%±0.4%. Comparison of mutation rates between isotypes within the TIV group did not show any significant differences. B. HC isotype of influenza-specific rmAbs from EI subjects were also found to be predominantly IgG1 (23/ 37, 62%), followed by IgA1 (7/37, 19%) and IgM (9/37, 24%). VDJ mutation rates for these three isotypes were as follows: IgG1 9.1%±0.7%, IgA1 7.2%±1.7%, IgM 8.2%±2.1%. Comparison of mutation rates between isotypes within the EI group did not show any significant differences. When compared between the TIV and EI groups, however, mutation rates for both IgG1 and IgM were found to be higher in rmAbs derived from EI subjects vs. those derived from TIV subjects (two-tailed t-test, \(p<0.0001\) for IgG1, \(p=0.033\) for IgM).

Figure S7  HC CDR3 length distribution of mAbs from TIV and EI subjects. A. HC CDR3 length distribution of all mAbs isolated from TIV subjects showed a predominance of mAbs with length 19; as with influenza-specific mAbs (Fig. S7C online) a large portion of this was contributed by 13 clonal lineages from one subject (TIV01) that had similar heavy chain rearrangements (\(\text{V}H4-59-\text{J}H6\)) but that did not share light chains. These clonal lineages contributed 68 mAbs (gray portion of bar). B. HC CDR3 length distribution of all mAbs isolated from EI subjects showed a distribution similar to that of the influenza-specific rmAbs (Fig. S7D online). As with influenza-specific mAbs, HC CDR3 lengths of 16 were the most common overall. Kolmogorov-Smirnov test of the distributions in A and B showed a difference in distribution (test statistic = 4.67, \(p<0.0001\)). C. HC
CDR3 length distribution of influenza-specific rmAbs from TIV subjects. The number of aas in HC CDR3 was most commonly 19; 13 clonal lineages from subject TIV01 with similar rearrangements (N14-59-Jb6) contributed 65 rmAbs to this peak (gray portion of bar). D. HC CDR3 length distribution of influenza-specific rmAbs from EI subjects. HC CDR3 lengths of 16 were most common. Kolmogorov-Smirnov test of the distributions in C and D showed a difference in distribution (test statistic = 2.25, p<0.0001).

Figure S8 Distribution of rmAb reactivities among influenza-specific rmAbs from TIV and EI subjects. For each tested antigen, influenza-specific rmAbs were counted to determine how many rmAbs reacted with that antigen. In contrast to the other analyses, each reactivity was counted separately (e.g., rmAb reactive with 2007–2008 influenza vaccine and with H3 A/Wisconsin/67/2005 was counted as positive for both columns). Multiply reactive rmAbs were counted as positive for each antigen with which they reacted; bars do not sum to 100% for this analysis. For each TIV subject, rmAb reactivity was found to be primarily specific for individual strains. TIV01 (173 influenza-specific rmAbs): 171/173 (99.9%) reacted with 2007–2008 influenza vaccine, 149/173 (86%) reacted with H1 A/Solomon Islands/03/2006 while only 1/173 (0.6%) reacted with H3 A/Wisconsin/67/2005. SPR testing of rmAbs derived from subject TIV01 bound to rHA H1 A/Solomon Islands/03/2006 but not to H3 A/Wisconsin/67/2005 (Figs. S9A, S9B, S9C online). TIV04 (8 influenza-specific rmAbs): 8/8 (100%) reacted with 2007–2008 influenza vaccine, 2/8 (25%) reacted with H1 A/Solomon Islands/03/2006; no other reactivities detected. TIV14 omitted from this analysis as there was only one influenza-specific rmAb isolated (this rmAb reacted with 2007–2008 influenza vaccine, H1 A/Solomon Islands/03/2006, and H1 A/Brisbane/59/2007). TIV21 (39 influenza-specific rmAbs): 39/39 (100%) reacted with 2008–2009 influenza vaccine, 28/39 (72%) reacted with HA B/Florida/04/2006, while only 5/39 (13%) reacted with H1 A/Brisbane/59/2007 and 2/39 (5%) reacted with H3 A/Brisbane/10/2007. TIV24 (31 influenza-specific rmAbs): 26/31 (84%) reacted with 2008–2009 influenza vaccine, 18/31 (58%) reacted with H1 A/Brisbane/59/2007, while only 2/31 (6%) reacted with H3 A/Brisbane/10/2007 and 1/31 (3%) reacted with HA B/Florida/04/2006. Additionally, 18/31 (58%) reacted with H1 A/Solomon Islands/03/2006, however, only 4/31 (13%) reacted with 2007–2008 influenza vaccine. For EI subjects, rmAbs were more commonly cross-reactive/less dominantly directed against single influenza strains. EI02 (4 influenza-specific rmAbs): 3/4 (75%) reacted with 2007–2008 influenza vaccine, 2/4 (50%) reacted with H3 A/Wisconsin/67/2005, 2/4 (50%) reacted with H3 A/Brisbane/10/2007, and 3/4 (75%) reacted with H3 A/Johannesburg/33/1994. No rmAbs reacted with H1, H5, or B strain HAs. EI03 (10 influenza-specific rmAbs): 4/10 (40%) reacted with 2007–2008 influenza vaccine, 3/10 (30%) rmAbs reacted with 2008–2009 influenza vaccine, 4/10 (40%) reacted with H3 A/Wisconsin/67/2005, 5/10 (50%) reacted with H3 A/Brisbane/10/2007, and 8/10 (80%) reacted with H3 A/Johannesburg/33/1994. Additionally, 1/10 (10%) reacted with H1 A/California/04/2009; no binding was found to other H1, H5, or B strain HAs. EI05 (3 influenza-specific rmAbs): 1/3 (33%) reacted with 2007–2008 influenza vaccine, 2/3 (67%) reacted with H3 A/Wisconsin/67/2005. No binding was found to other H1, H3, H5, or B strain HAs. EI07 (3 influenza-specific rmAbs): 1/3 (33%) reacted with 2007–2008 influenza vaccine, 1/3 (33%) rmAbs reacted with 2008–2009 influenza vaccine, 2/3 (67%) reacted with H3 A/Wisconsin/67/2005, 1/3 (33%) reacted with H3 A/Brisbane/10/2007, and 2/3 (67%) reacted with H3 A/Wisconsin/67/2005. No binding was found to other H1, H5, or B strain HAs. EI12 was omitted as there were no influenza-specific rmAbs isolated. EI13 (17 influenza-specific rmAbs): 8/17 (47%) reacted with 2007–2008 influenza vaccine, 8/17 (47%) rmAbs reacted with 2008–2009 influenza vaccine, 7/17 (41%) reacted with H5 A/Wisconsin/67/2005, 9/17 (53%) reacted with H3 A/Brisbane/10/2007, and 9/17 (53%) reacted with H3 A/Johannesburg/33/1994. Additionally, 1/17 (6%) reacted with H1 A/Solomon Islands/03/2006, 2/17 (12%) reacted with H1 A/Brisbane/59/2007, 1/17 (6%) reacted with H1 A/California/04/2009, and 4/17 (24%) reacted with B/Florida/04/2006. No rmAbs reacted with H5 HAs. SPR testing of selected rmAbs from EI13 showed binding to rHAs from both H1 A/Solomon Islands/03/2006 and H3 A/Wisconsin/67/2005 (Figs. S9B, S9C online).

Figure S9 Surface plasmon resonance analysis of rmAbs recovered from TIV and EI subjects. A. Human rmAbs 1248, 1258, and 1270 from subject TIV01 bound to H1 A/Solomon Islands/03/2006 bound to an SPR chip. B. Human rmAbs 1210 and 1267 from subject TIV01 and rmAbs 2210 and 2217 from subject EI13 bound to H1 A/Solomon Islands/03/2006 bound to an SPR chip. C. Human rmAbs 1267 and 1210 from subject TIV01 did not bind to H3 A/Wisconsin/67/2005 bound to an SPR chip while rmAbs 2210 and 2217 from subject EI13 did bind. D. Human rmAbs from subjects TIV01 (1210 and 1267) and EI13 (2210 and 2217) showed essentially no interaction with phosphatidylcholine-cardiolipin liposomes. Anti-HIV-1 mAb 4E10 is shown as a positive control and anti-HIV-1 mAbs 2F3 and 13H11 are shown as negative controls. E. Human rmAb 1258 from subject TIV01 shows some degree of binding to phosphatidylcholine-cardiolipin liposomes while rmAb 1270 from subject TIV01 shows a lesser degree of binding. Anti-HIV-1 mAb 4E10 is shown as a positive control. No binding of rmAbs to apoferritin (control protein) or to phosphatidylcholine-phosphatidylethanolamine liposomes was seen (data not shown).

Figure S10 Additional representative clonal lineage of rmAbs from subject TIV01. Data for starred rmAbs appear in Table S12 online. Clonal lineage 643 from subject TIV01. Sixteen of 17 rmAbs (94%) bound one antigen, 1/17 (6%) was not influenza-specific. Four rmAbs were tested in additional assays. Affinity for rHA binding was measured for three rmAbs and all had sub-nanomolar affinity for rHA H1 A/Solomon Islands/03/2006 and H3 A/Wisconsin/67/2005. Neutralization assays for one rmAb (1267 IgG1) showed potent neutralization of H1N1 A/Solomon Islands/03/2006, weak neutralization of H1N1 A/Brasile/59/2007 and no neutralization to H3 N2 A/Wisconsin/67/2005. Two other rmAbs were tested only against H1N1 A/Brasile/59/2007 and neither neutralized. All four tested rmAbs showed potent HA1 against H1N1 A/Solomon Islands/03/2006; two rmAbs showed weak HA1 activity against H3N2 A/Wisconsin/67/2005.

Figure S11 Additional representative clonal lineages of rmAbs from TIV subjects. A. Clonal lineage 690 from subject TIV01. Three of 12 rmAbs (25%) bound one antigen, 8/12 (67%) bound two antigens, 1/12 (8%) bound none. B. Clonal lineage 2737 from subject TIV21. Five of 8 rmAbs (63%) bound one antigen, 3/8 (37%) bound two.

Figure S12 Sequence alignment of H3 HAs used in this study. Amino acid sequences for H3 HA strains used in this study were downloaded from PubMed and aligned to the H3 A/
Wisconsin/67/2005 sequence. Differences are highlighted in colors for each aligned sequence. The H3 A/Johannesburg/33/1994 strain was the least similar with 98.8% identity. Only the HA1 sequence for the Johannesburg strain was available; all sequences were aligned in this region only.

Table S1 Clonal lineages of antibodies from TIV subjects.

Table S2 Clonal lineages of antibodies from EI subjects.

Table S3 Isotypes of isolated influenza-specific rmAbs.

Table S4 Isotypes of isolated rmAbs not specific for influenza antigens.

Table S5 Heavy chain family usage of influenza-specific rmAbs.

Table S6 Heavy chain family usage of isolated influenza-specific rmAbs not specific for influenza antigens.

Table S7 Kappa chain family usage of isolated influenza-specific rmAbs.

Table S8 Kappa chain family usage of isolated rmAbs not specific for influenza antigens.

Table S9 Lambda chain family usage of isolated influenza-specific rmAbs.

Table S10 Lambda chain family usage of isolated rmAbs not specific for influenza antigens.

Table S11 Characteristics of cross-reactive rmAbs.

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References


