A novel antibody discovery platform identifies anti-influenza A broadly neutralizing antibodies from human memory B cells

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ABSTRACT
Monoclonal antibody isolation directly from circulating human B cells is a powerful tool to delineate humoral responses to pathological conditions and discover antibody therapeutics. We have developed a platform aimed at improving the efficiencies of B cell selection and V gene recovery. Here, memory B cells are activated and amplified using Epstein-Barr virus infection, co-cultured with CHO-muCD40L cells, and then assessed by functional screenings. An in vitro transcription and translation (IVTT) approach was used to analyze variable (V) genes recovered from each B cell sample and identify the relevant heavy/light chain pair(s). We achieved efficient amplification and activation of memory B cells, and eliminated the need to: 1) seed B cells at clonal level (≤1 cell/well) or perform limited dilution cloning; 2) immortalize B cells; or 3) assemble V genes into an IgG expression vector to confirm the relevant heavy/light chain pairing. Cross-reactive antibodies targeting a conserved epitope on influenza A hemagglutinin were successfully isolated from a healthy donor. In-depth analysis of the isolated antibodies suggested their potential uses as anti-influenza A antibody therapeutics and uncovered a distinct affinity maturation pathway. Importantly, our results showed that cognate heavy/light chain pairings contributed to both the expression level and binding abilities of our newly isolated VH1-69 family, influenza A neutralizing antibodies, contrasting with previous observations that light chains do not significantly contribute to the function of this group of antibodies. Our results further suggest the potential use of the IVTT as a powerful antibody developability assessment tool.

Introduction
Antibody isolation directly from human B cells has distinct advantages in harnessing rare antibodies with desirable functions. Following the early successes with the isolation of monoclonal antibodies (mAbs) 4E10 and 2F5,1,2 there has been a rapid growth in the number of potent HIV-neutralizing antibodies as a result of the application of B cell-based platforms.3 Potent antibodies targeting severe acute respiratory syndrome coronavirus,4 influenza virus,5–7 respiratory syncytial virus8 as well as other viruses9 have also been isolated. In addition, B cell-derived antibodies against human self-antigens have helped in the understanding of autoimmune diseases.10 These advances highlight the potential of B cell-based antibody discovery platforms, while underscoring the technical challenges in using such a strategy.11 Unlike from rodent B cells, hybridoma generation from human B cells has faced various difficulties, and alternative approaches have been sought after.12

Human antibody discovery using primary B cells faces 2 main obstacles. The first is the ability to maintain and screen the antibody-producing B cells. The primary approaches to overcome this challenge are B-cell immortalization and transient B-cell activation (reviewed in ref. 12). These strategies remain topics of active research because successful studies adopted methods that were often proprietary.13 The second obstacle is the capacity to recover antibody genes from as few as one cell. Technologies have advanced sufficiently to allow such a practice, but recombinant IgG cloning and recombinant expression procedures are labor intensive and time consuming, especially when the number of samples needing V gene rescue is large. This necessitates clonal B cell culture or single B cell sorting prior to V gene recoveries by all current protocols.13 Memory B cell immortalization by Epstein-Barr virus (EBV) infection is a low efficiency process that involves a balancing act among many regulatory elements.14 Clone losses are common prior to achieving the true “immortalization.” However, the initial outgrowth stage after infection is robust and should be long enough for screening B cells of interest. We have found that the number of candidate B cell samples needed for gene recovery could be reduced through a carefully designed screening strategy, and that it is not always necessary to achieve immortalization when EBV is used. Following this strategy, we developed a new platform that allows functional screenings of EBV-activated B cells seeded in non-clonal format, which can then be followed by in vitro transcription and translation (IVTT) Fab expression to quickly identify the functional heavy/light chain pairs. The IVTT Fab expression procedure does not require the assembly of a single operon containing both heavy chain...
(HC) and light chain (LC), an attrition-causing step, or the construction of expression vectors. These attributes should lead to significantly increased efficiencies.

Influenza virus infections continue to be a health threat and economic burden despite decades of vaccine and therapeutics development.\textsuperscript{15,16} B cell-based platforms and phage panning both have contributed to the identification of broadly protective antibodies.\textsuperscript{5,17-20} We attempted to validate our platform by recovering anti-hemagglutinin (HA) neutralizing antibodies from a healthy donor. This effort led to the isolation of several broadly neutralizing antibodies, one of which utilizes a distinct affinity maturation pathway that has not been reported previously. Furthermore, this platform revealed that accurate heavy and light chain pairings may be an important step in the assemblies of selected antibodies. This platform can therefore be used as a unique tool to assess the developabilities for some antibody therapeutics.

**Results**

**Design of the platform**

Clonal B cell culture achieved through immortalization and limited dilution cloning (LDC) preceding V gene recovery poses significant technical challenges due to the high attrition rate, and has been performed routinely by only a limited number of groups.\textsuperscript{4,8} Transient activation of B cells followed by functional screening and V gene rescue protocol yielded the first 2 of a group of broadly neutralizing anti-HIV antibodies, but it demanded “near clonal density” (1.3 cells/well) of B cells at seeding to assist V gene rescue.\textsuperscript{21} To circumvent those restrictions, we adopted 2 critical improvements to develop our platform. The first is the initiation of gene recovery without achieving B cell immortalization or LDC to reduce the cell loss and save time, and the second is the V gene matrix pairings through a novel IVTT platform to allow non-clonal B cell seeding. The experimental procedure is summarized in Fig. 1a. In a typical campaign, a 7-step protocol will yield a sufficient amount of antigen-specific, recombinant IgG for in-depth functional analysis in an estimated 1–1.5 month time frame. The IVTT step (step 6), which is unique to this platform, is illustrated in detail in the subsequent V gene recoveries from sample 1N23.

During IVTT template assembly, a 2-step nested PCR ensures maximum V gene recovery efficiency and the addition of T7 promoter and ribosomal binding site (RBS). The third PCR step allows the attachments of: 1) CH1 to the VHs and CK and C\(\lambda\) to the VLs, and 2) translational terminator to the 3’ of the templates (Fig. 1b). This step also adds c-Myc and Flag affinity tags to the LC and HC, respectively allowing Western blot or ELISA detections. The primers used for PCR amplification of V genes were designed based on the IMGT\textsuperscript{\textregistered} database (www.imgt.org/) with the inclusion of appropriate sequence degeneracies to cover all major V genes and are listed in Table 1. V gene rescue efficiency of 80% or better was achieved consistently on sorted single memory B cells from various healthy donors, and sequencing of randomly selected, rescued VH, VK, and V\(\lambda\) confirmed their uniqueness and diversities (data not shown).

**Establishment of in vitro transcription and translation of Fab**

IVTT was used previously for a-glycosylated Fab and IgG productions.\textsuperscript{22} We optimized the critical parameters for the IVTT platform by using known antibodies so this platform can be suitable for rapid screening of cognate HC and LC pairs (Fig. 2a, b). The amount of template, inclusion of disulfide bond enhancer, and reaction temperature were determined for maximum Fab yield. However, for screening purposes \(~125\) ng of each of the HC and LC templates was used routinely because this amount was easily achievable through the RT-PCR process and the Fab yield was not significantly improved by using more templates (Fig. 2a). The yield of the Fab was typically 1–10 µg/ml in a 25 µl
reaction volume, which is sufficient for several rounds of ELISA-based preliminary screening. Mimicking a higher throughput process, we used the platform first to recover V genes from sorted single B cells in a plate format (Fig. 2c). We then assembled the IVTT in a pairwise fashion between the VH and VL from each B cell directly from the RT-PCR products. 80% of the samples displayed robust Fab expression falling into the range observed with known antibodies (Fig. 2d).

**Memory B cell activation and maintenance for functional screenings**

EBV infection was proposed as a useful tool for human B cell immortalization. Although it has been attempted by many groups, the outcomes have been inconsistent and mostly poor. Modifications are constantly being made to increase the “immortalization” rate. We used irradiated CHO-muCD40L instead of allogeneic peripheral blood mononuclear cell (PBMC) as the feeder layer to: 1) provide consistent CD40L stimulation, 2) prevent a potential T cell response, and 3) eliminate false positive signal from the PBMC feeder layer during ELISA and molecular cloning.

At four memory B cells + 10,000 CHO-muCD40L cells/well seeding density in a 384-well plate, we observed robust outgrowth and B cell cluster formation in 85% of the wells 10 d post seeding, and detected human IgG production in 100% of the wells, including those without cluster formation but live B cells. The supernatant from each well was used in ELISA for HA binding.

From 9 (plates) × 384 (wells/plate) × 4 (cells/well) samples screened (~14,000 memory B cells), 34 HA (H1, CA09) positive wells were identified in the first screening. This represents a ~10^-3 HA-positive rate, which is in line with similar surveys using healthy donors prior to receiving vaccination. Half of the cells were collected for V gene recovery, and the other half was transferred to a 96-well plate for continued culturing and monitoring. We observed a steady decrease in antigen-specific IgG production over time, with 50% of the original wells losing the HA binding activity after 3 months of culture. Well 1E18 displayed robust outgrowth and maintained HA reactivity throughout the process. However, attempts to generate clonal culture through LDC were not successful. These observations underscored the importance of performing gene recovery at early time points and the challenges to generate truly “immortalized” B cell clones by EBV infection.

**V gene recovery from wells of interest**

Influenza A viruses are serologically divided into different HA subtypes, which are subdivided into 2 distinct phylogenetic groups, group 1 and group 2. Currently, H1 and H3 HA subtypes are associated with seasonal human disease, although other more diverse HA subtypes are thought to be of concern for future pandemics. Single-point ELISA screening of the 34 HA (H1, CA09) positive wells against an expanded panel of recombinant HAs indicated that a few of them were broadly reactive against influenza A HA proteins. We failed to observe any sample well that was...
cross reactive to both influenza A and B isolates, confirming the rarity of such B cells. Eight of the 34 were reactive against H5, with 5 of those also recognizing H2 and H6 proteins. Among all samples tested, wells 1E18 and 1N23 displayed the most robust pan group 1 HA activities and well 2H4 was the only one that recognized all group 1 and 2 HAs tested. Based on these observations, wells 1E18, 1N23, 2H4, and a mono-reactive well, 7G16 were chosen as test cases for antibody recovery using the new platform.

Cell pellets collected immediately after the HA binding screenings as described in the “activation and screening” section were applied to the IVTT platform. After confirming the successful HC and LC template assembly (Fig. 3a) and HA binding activities by IVTT products from each well against HA (H1, CA09) (Fig. 3b), we proceeded to identify the original HC/LC pair by performing matrix pairings. Although we set out to seed memory B cells at 4 cells/well, TA cloning and sequencing indicated that between 3–13 VHs had been recovered from the wells of interest due to variations in manual operation. There existed a reverse correlation between the number of V genes recovered and the ELISA signal in the first round of IVTT from each sample well. Wells 7G16, 1E18, and 1N23 each contained 3, 4, and 11 unique VHs, respectively. This might have contributed to the weaker IVTT signal from well 1N23 compared to those of 7G16 and 1E18 (Fig. 3b). V gene recovery from well 1N23 is presented as an example, illustrating in detail how matrix pairings are used in step 6 of the platform (Fig. 1a). We recovered more VHs (11) than VLs (7) from sample well 1N23; this could be attributed to the fact that we did not attempt to rescue lambda LCs due to the lack of contribution to the binding by lambda LCs (Fig. 3b). After identifying the positive groups from reaction # 2 pairing groups B and d (Fig. 3a, right upper panel), we proceeded to pair the individual heavy and light chains from the positive B and d groups in a second step (IVTT2). The exact pairing between HC #5 and LC #3 was resolved in 2 steps involving fewer than 20 IVTT reactions. The increase in specific ELISA signal was significant from step 1 to step 2 as the specific HC/LC pair was identified (Fig. 3a, lower panel). Wells 7G16 and 1E18 were subjected to the new IVTT process, and the HC/LC pairs recapitulating the parental well activities were identified in one step (data not shown).

Well 2H4 recovery was atypical as it had a very poor $K_D$ and 13 unique VHs leading to weak IVTT signals. In order
to recover this rare cross-reactive clone, we applied less stringent screening criteria (e.g., the ELISA signal was counted as positive if it was 50% above negative control instead of the typical 3 folds (300%) in the first step), and succeeded in identifying the functional VH and VL pair in 2 steps involving 14 IVTT reactions. Its recovery process suggested that the B cell number/well should not exceed a limit if high recovery rate is preferred over throughput.

Figure 3. Identification of the original HC/LC pairs through the IVTT. (a) Agarose gels showing the assembled HC and LC templates from the wells of interest (#1–8). Note that from each well both kappa and lambda chains were recovered. (b) IVTT products from 8 HA positive wells were tested in an antigen binding ELISA. Both kappa (red bar) and lambda (blue bar) Fabs were assembled in the IVTT and tested. Wells #1–7 contain kappa functional clones and well #8 has a lambda functional clone. Wells corresponding to 1E18 (well #2), 1N23 (well #3), and 7G16 (well #7) were indicated. 2H4 well was recovered from a separate experiment and not shown here. (c) HC and LC were grouped according to their germline subfamilies and used in the first IVTT experiment (IVTT1) to narrow down the search of the original HC/LC pair to groups B and d. This was followed by a final step of matrix pairing using HCs and LCs from groups B and d (IVTT2) to identify the original HC/LC pair as VHseq5/VLseq3.
**Anti-HA antibodies display cross binding and broadly neutralizing activity by targeting a conserved epitope**

The identified V genes were used to construct IgG1 expression vectors for all 4 new mAbs for further functional analysis. Recombinant IgG1s 1E18 and 1N23 displayed broad binding activity against group 1 HAs, whereas IgG1 2H4 showed activities against all group 1 and 2 HAs tested, albeit with poor affinity (Fig. 4a). Their K_D against the HA from H1 (CA 09) isolate were assessed using a bio-layer interferometry (BLI) assay. Both mAbs 1E18 and 1N23 displayed rapid on-rate and slow off-rate resulting in excellent K_Ds (0.09 and 0.1 nM, respectively). mAb 2H4 had a slow on-rate and a fast off-rate, leading to a very poor K_D (349 nM, Fig. 4b and Table 2). These K_Ds correlated directly with their neutralizing ability in a micro-neutralization assay. Recombinant IgGs 1E18 and 1N23 neutralized all tested group 1 isolates effectively, with activities comparable to that of Fl6v3, a broadly neutralizing antibody isolated previously from a plasma cell after vaccination (Fig. 4c). In contrast, IgG1 mAb 7G16, an antibody that was isolated through the same platform but displayed binding to only one HA (H1 CA 09) recognized a different epitope.

**Sequence analysis revealed distinct antibody features**

Sequence analysis revealed that mAbs 1E18, 1N23, and 2H4 each belonged to a unique group of influenza A neutralizing antibodies. mAbs 1E18 and 1N23 both use a Vh1–69 heavy chain, a VH family preferred by a large number of influenza A, group 1 broadly neutralizing antibodies, including FI10 and CR6261 isolated from phage panning, and a series of others directly from B cells. Unlike mAb 1E18, however, mAb 1N23 does not have 98Y in HCDR3, which is seen in the majority of Vh1–69 stem binding antibodies. 2H4 uses a VH subfamily Vh3–48/3 as compared to Fl6, which is cross reactive to both group 1 and 2 isolates and uses Vh3–30. 2H4 has significantly fewer somatic hypermutation (SHM) compared to Fl6 (Table 3).

Recent studies have highlighted the importance of a tripartite of residues, 98Y (HCDR2), P52aA (HCDR2), and 54F (HCDR2), which represent the respective roles of VDJ recombination, founder mutations, and polymorphism in the development of Vh1–69 using HA stem binding broadly neutralizing antibodies. Notably, mAb 1E18 has a 98Y, but a P52aG mutation instead of a P52aA, which was found to be predominant in all 98Y using influenza A group 1 neutralizing antibodies. Strikingly, mAb 1N23 does not possess 98Y and has a P52aG mutation, a combination that has not previously been described in the maturation process.

**mAb 1N23 represents a new affinity maturation branch**

To understand the significance of 52a mutations in the maturation process of the newly identified antibodies, we created the germline predecessors of both mAbs 1E18 and 1N23 and their variants carrying the mutations (Fig. 5). Both P52aA and P52aG were capable of restoring the germline antibody binding to several HAs tested, confirming their roles as the “founder mutation” (Fig. 6, upper 2 panels). For mAb 1E18, “G” was more effective than “A,” supporting a modeling-based prediction that a smaller residue at position 52a would be beneficial. We then investigated if this finding is true for 98Y anti-HA antibodies identified from another study. In the absence of UCA9 light chain information, we constructed UCA9H/1E18L hybrid germline predecessor, its mutant variants and tested them in the same ELISA assay. We found that “P52aG” was also equally or more effective than “P52aA” in restoring the germline antibody bindings by UCA9H/1E18L (Fig. 6, lower panel). Significantly, P52aA and P52aG both restored germline 1N23 binding effectively. This is an observation that has not been made with any non-98Y antibody. This result necessitates the addition of a new affinity maturation sub-branch typified by the non-98Y usage and a P52aG mutation to those of VH1–69, HA stem binding antibodies proposed in a previous publication.

**The HC/LC pairing is regulated at the Fab assembly level**

It has been reported that broadly influenza neutralizing antibodies arising from the VH1–69 family displayed no LC contributions to their binding activities. However, when identifying the unique LCs for mAbs 1E18 and 1N23 during the matrix pairings, we saw preferential LC pairings. In the first round of IVTT before conducting matrix pairings, LC usages for sample wells 1E18, 1N23, and a few others were either kappa or lambda, but not both (Fig. 3b). While pairing the mAb 1N23 HC with all kappa LCs recovered from the same sample well, we found that the Fab expression level varied significantly, but the functional pair was the highest (Fig. 7a). The dramatic differences in the fully assembled Fab level were not due to the expression levels of individual heavy and light chains, as all the chains were expressed at comparable levels when measured by Western blot (Fig. 7b). Furthermore, all the LCs also paired equally well with another irrelevant HC #6 (VH1–2 subfamily) in assembling into Fab (Fig. 7c). We then tested several pairs in the IgG format. IgG expression retained the same trend even though the differences were more moderate (Fig. 7d). Importantly, the “mismatched” LC also led to a reduction in binding ability, in contrast to previous reports (Fig. 7e). It is worth noting that LCs #3 and #4 are from the same Vk3 germline family (Vk3–20) and only differ in the SHM, whereas LCs #1 and 2 are from the subfamilies Vk1–9 and Vk1–5, respectively. The differences in LCs #3 and 4 did not affect IgG binding, yet contributed to different Fab and IgG productions. The biased Fab expressions for the functional pairs were also observed during mAb 1E18 and 2H4 V gene recoveries (data not shown).

To investigate the contribution of the LC to similar antibodies from other donors, we generated UCASH/1N23L hybrid
germline predecessor and found that it lost completely the reported binding ability of UCA5 to the HA from H1 (CA) isolate (data not shown), supporting our finding that the LC is critical for maintaining the binding activity of this family of antibodies. Therefore, IVTT reduced HC/LC pairing ambiguity during gene recovery through at least 2 mechanisms, i.e., assembly efficiency and binding affinity.

Discussion

We demonstrated in this study the development of a novel IVTT-based antibody discovery platform. The unique characteristics of the isolated antibodies validated the quality and efficiency of the new platform and added new information to the current understandings of anti-influenza humoral responses.

Figure 4. Newly identified mAbs recognize a highly conserved, neutralizing epitope. (a) Recombinant IgGs 1E18, 1N23, and 2H4 were tested in ELISAs against indicated HA antigens. One HA from a B isolate Brisbane was included as a control. (b) The binding kinetics of recombinant IgGs were estimated using a Bio-Layer Interferometry (BLI) assay as described in the methods. (c) mAbs 1E18 and 1N23 displayed potent neutralization against various influenza A, group 1 isolates indicated. (d) They all recognized a similar epitope as mAb FI6v3 as revealed by a BLI analysis described in the methods.
By eliminating the expression plasmid construction step, this platform transforms the process through which V genes are recovered from B cells. Almost all current protocols require near clonal culture to minimize the number of expression plasmids to be constructed for antibody expression and confirmation, a critical attrition-inducing and rate-limiting step. We demonstrated that the matrix pairings in the IVTT platform is sensitive and accurate in identifying the functional pairings against a complex background with more than 10 B cells. This strategy theoretically allows the seeding of B cells in a non-clonal format, ideally with a density of 4–10 cells/well, thus reducing the burden of screening by corresponding folds and eliminating the needs for the stringent and time-consuming single B cell culture. The exact seeding densities can be determined according to the estimated hit rate and the sample size to strike a balance between the B-cell screening and V gene recovering efforts. Furthermore, the experience can be significantly improved if FACS sorting is used to dispense B cells into cell culture plates instead of manual operations, which were used in this study and introduced variations and unnecessary complexities.

Deep sequencing offers the opportunity to gain insight into the genetic repertoire of an individual’s humoral response with unprecedented scale. However, to take full advantage of this information, knowledge must be obtained of the functions of the encoded antibodies. Attempts have been made to construct scFv operons maintaining the original HC/LC pairs before library construction, but the efficiency and quality are relatively low and the attrition rate is high. Our platform provides the foundation to develop a fully automated, high throughput process to functionally probe a large number of B cells. Compatibilities with a plate format and Fabs production in a cell-free environment, the prerequisites for an automation process, have been demonstrated in this study.

The IVTT platform might be a useful tool to study the mechanistic basis for antibody maturation. Of interest are the contributions of light chains to the VH1–69 using influenza A neutralizing antibodies. This is to our knowledge the first attempt to use primary B cells to demonstrate that efficient HC/LC pairing may play a role in B cell maturation. Our findings support a hypothesis that antibody expression level is correlated with thermostability, and may be one critical factor in determining the B cell fate. IVTT represents a sensitive tool to assess the stability of therapeutic antibodies under development. However, it is also apparent from our data that the impact from light chains is antibody dependent (Fig. 7C), and caution should be taken in extrapolating to other antibodies.

High efficiency is critical to a B cell platform because the number of desirable B cells is small. Tremendous efforts were required to generate a single broadly neutralizing antibody against both influenza A group 1 and 2 isolates. In comparison, we were able to isolate the influenza A cross-reactive antibody 2H4 from only 104 memory B cells from a healthy donor. mAb 2H4 recognizes the same highly conserved epitope in the HA2 stem region as antibodies from the above described successful campaigns, but displays only modest neutralizing activity at high concentrations. This is expected as mAb 2H4 has a very poor KD profile, which may reflect its nature as a group 1 and 2 cross-reactive antibody in the early stage of maturation. The repeated immunizations preceding B cell isolation by all the above described campaigns seem to support this hypothesis. An anti-influenza therapeutic may result from mAb 2H4 if affinity is improved through antibody engineering.

Table 2. KD measurement of the newly identified mAbs by Octet.

| mAb | K\(_a\) (×10\(^{-3}\) 1/Ms) | K\(_{off}\) (×10\(^{-6}\) 1/s) | K\(_D\) (×10\(^{-9}\) M) | R\(^2\) | X\(^2\) |
|-----|----------------|-----------------|----------------|-----|------|
| 1E18 | 360.0 | 34.8 | 0.097 | 0.99 | 0.87 |
| 1N23 | 560.0 | 58.5 | 0.105 | 0.99 | 0.47 |
| 2H4  | 0.5  | 172.7 | 349 | 0.98 | 1.55 |

Figure 5. Sequence comparison of parental mAbs 1E18, 1N23 (shaded sequences) with other broadly neutralizing antibodies including mAbs F10, CR6261, UCA1, and 9. mAb 1E18 and 1N23 germlines and germlines with single mutations P52aG and P52aA are also included. SHMs from germlines are color highlighted and P52aG is colored in red.
In addition to mAb 2H4, 2 group 1 neutralizing antibodies with unique features were isolated in the same campaign. Given the moderate starting memory B cell number and the even more restricted HA cross reactive B cell number, this platform displayed high efficiency. We expect this platform to perform superbly if immunized/patient sources are readily accessible.

Previous work has established that the majority of group 1 broadly neutralizing antibodies use VH1-69 heavy chain for binding HA and are further divided into 2 groups distinguished by the 98Y and non-98Y usage. P52aA was found to be a major "founder mutation" for 98Y group, accounting for more than 80% of all members. Surprisingly, mAbs 1E18 and 1N23 both possess a P52aG instead of a P52aA. P52aG was observed with higher frequencies in phage-derived 98Y antibodies, but a P52aG in a non-98Y background has never been reported, which makes mAb 1N23 a very unique finding. This dramatic shift away from a recently published model analyzing nearly 200 mAbs mostly from a single donor raises several important questions. Are these differences attributed to donor difference, infection and vaccination-induced bias, or stochastic choosing of pathways? Given the limits of the small sample size of this study and a general lack of anti-influenza mAbs derived directly from B cells from different donors, we believe that both vertical (following an individual over time in response to different flu strain challenges) and lateral (different donors) studies are needed to answer these questions.

**Materials and methods**

**Donor and HA specific B cell selection by ELISA, recombinant HAs**

Sera from healthy donors were collected with informed consent and used in ELISA-based screening of anti-Influenza HA activities. The blood collection procedure has been approved by the Chesapeake Institutional Review Board (protocol number 2010–001). All ELISAs for donor screenings and subsequent analysis were performed as described, and all secondary antibodies were prepared according to manufacturers’ suggested dilutions. Briefly, Nunc MaxiSorp 96-well plates (Corning, PA) were coated with 2 μg/ml recombinant HA (MedImmune, Gaithersburg, MD) isolated from influenza A/California/09 pandemic strain in phosphate-buffered saline. ELISAs were performed as previously described, and OD values were measured at 450 nm.

**Table 3. Comparison between mAbs 2H4 and FI6.**

|          | Heavy chain | Light chain |
|----------|-------------|-------------|
|          | V gene      | CDR3 length (aa) | N1 nt | D gene | N2 nt | J gene | SHM nt (aa) | V gene | CDR3 length (aa) | N nt | J gene | SHM nt (aa) |
| 2H4      | 3–48’03     | 18           | 2      | 3–22’01 | 31    | 4’02   | 23 (13)  | K1–39’01 | 9      | 0    | 2’01   | 15 (7)  |
| FI6      | 3–30’18     | 20           | 22     | 3–9’01  | 11    | 4’02   | 26 (17) | K4–1’01  | 9      | 0    | 1’01   | 14+6 (9+2) |

**Figure 6.** Contributions to binding by mutations at P52a position. Recombinant mAbs in their parental form (WT), their germlines (GL) and germline mutational variants as indicated were tested in ELISAs against a panel of HAs indicated on top of each of the graphs. The mAb variants are in different colors. In the lower panel, mAb 1E18 parental (WT) was used as the parental control (WT) for hybrid mAb UCA9H/1E18L due to the lack of UCA9 light chain information, and the mutations at P52a were generated against the UCA9H/1E18L background.
saline (PBS). Serially diluted sera were added to each well and horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was used for detection. The blocking and dilution buffer for all steps is PBST (PBS + 0.1% Tween20 + 3% dry milk). TMB (KPL, Gaithersburg, MD) substrate was added and color development was stopped by the addition of 0.1 N HCl.

The HA-specific B cell screening procedure is essentially the same, except that Nunc MaxiSorp 384-well plates (Corning, PA) were used for HA coating. Recombinant HAs from Influenza A isolates including H1 (A/South Dakota/06/2007 H1N1, A/California/7/2009 H1N1), H2 (A/swine/Missouri/42964224/2006 H2N3), H5 (A/Vietnam/1203/2005 H5N1), H6 (A/mallard/Alberta/89/65 H6N2), H3 (A/Perth/2009 H3N2) and H7 (A/mallard/Alberta/89/65 H6N2), and one HA from a B isolate (B/Brisbane/60/2008) were prepared at MedImmune as described previously.

Memory B cell activation and amplification through EBV infection and CD40L stimulation

B cells were isolated from whole blood of donors with good HA titer using a combination of EasySep™ Direct Human B Cell Isolation Kit (Stem Cell technologies, Canada) and Ficoll density gradient centrifugation (Histopaque 1077 – 1, Sigma, St. Louis, MO). IgG+ and IgA+ memory B cells were isolated using a Switched memory B cell isolation kit (Miltenyi Biotech Inc., California) following the manufacturer’s suggested protocol. Memory B cells were then infected with EBV (ATCC VR-1492) as described. A total of 1.2 × 10⁶ memory B cells were infected in RPMI medium supplemented with 10% FBS, 1% PenStrep (Invitrogen, CA) and stimulated using TLR agonist CpG ODN2006 (Invivogen, San Diego, CA). The cells were incubated at 5% CO2 at 37°C for 24 hours. Cells were then seeded at 4 cells per well in 384-well plates with 10,000 irradiated CHO-muCD40L cells per well. CHO-muCD40L stable cell line was generated at MedImmune. CHO-muCD40L cells were irradiated at 8000 rads in a Model 68A irradiator using Caesium-137 as the radiation source before seeding. Outgrowth of B cells was estimated by microscopic observation and human IgG expression detection.

V gene rescue and Fab template assembly for in vitro transcription and translation

For validation experiment using single B cells, memory B cells were sorted based on the IgGκkappa or IgGλlambda phenotypes into 96-well PCR plate containing 5 µl/well lysis buffer from the SuperScript® III One-Step RT-PCR system (Invitrogen, Carlsbad, CA). The plates were used either directly for RT-PCR or stored at -80°C until use. For VH and VL recovery from EBV stimulated B cell samples, cells were collected from the selected wells into Eppendorf tubes, washed once with PBS, and pelleted. The cell
pellets were either lysed directly for RT-PCR or stored at \(-80^\circ\text{C}\) until use. The SuperScrip\textsuperscript{TM} III One-Step RT-PCR system (Invitrogen, Carlsbad, CA) was used for first strand cDNA synthesis.

A two-step nested PCR procedure was used to amplify the VH, VK, and V\(_2\) fragments with T7 promoter and RBS attached to the 5’ end. This was followed by an overlapping PCR to add the CH1, CK, CH2, affinity tags and terminator at the 3’ end. The primers used for each step is summarized in Table 1. IVTT was performed using the PURExpress kit from NEB (New England Biolabs, Ipswich, MA). Each reaction (25 \(\mu\)l) contained \(\sim 250\) ng of total template DNA (HC enhancer (New England BioLabs), and 1 \(\mu\)l of Disulfide Bond Enhancer (New England BioLabs), and 1 \(\mu\)l of RNaseOut (40U/\(\mu\)l, Invitrogen). The reactions were incubated at 37°C for 3 hours. The IVTT products were evaluated by both Fab expression and antigen binding ELISAs. In the capture ELISA to measure the Fab expression, the NUNC Maxisorp plates (Corning, PA) were coated with a Goat anti-Fd at 2 \(\mu\)g/ml in PBS (SouthernBiotec, Birmingham, AL) to capture the HC, the fully assembled Fabs were detected by goat anti-LC antibodies (horse-radish peroxidase (HRP)-conjugated anti-human kappa or anti-human lambda, SouthernBiotec). Purified Kappa and Lambda Fab proteins with known concentrations were used as the standards in the ELISA to determine the Fab expression level. In the antigen binding ELISA, the plates were coated with antigens. Fabs that bound to antigens were detected by HRP-conjugated goat anti-human Fab antibody (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Bio-layer interferometry assays**

A ForteBio Octet QK384 instrument was used to study the kinetics of recombinant IgGs 1E18, 1N23, and 2H4 binding to HA (H1, CA09). All the assays were done at 200 \(\mu\)l/well in ForteBio 10x kinetic buffer (part no. 18–1092) at 25°C. 0.3 \(\mu\)g/ml of biotinylated-HA was loaded on the surface of streptavidin biosensors (SA) for 400 s, reaching capture levels between 1.0 and 1.5 nM, followed by a 300 s washing step. Association of HA on the biosensor to the individual mAbs in solution (0.274–200 nM) was analyzed for 600 s. Dissociation of the interaction was probed for 1200 seconds. All graphs were overlaid and aligned at the baseline.

**Epitope binning**

Biotinylated-HA (H1, CA09) was captured onto streptavidin biosensors and coated with FI6v3 at a saturating concentration of 200 nM for 600 seconds. The epitopes of 1E18, 1N23, and 2H4 were probed in relation to FI6v3 by assaying the biotinylated-HA-FI6v3 coated biosensors in 100 nM each of 1E18, 1N23, 2H4, FI6v3 (control mAb), and 7G16 (control mAb) separately for 1200 seconds. All graphs were overlaid and aligned at the baseline.

**Influenza micro-neutralization assay**

The microneutralization assay was performed as described previously. Briefly, 60 TCID50 of virus was added to 3-fold serial dilutions of recombinant mAb IgGs in a 384-well plate in complete MEM medium containing 0.75 \(\mu\)g/ml L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated trypsin (Worthington) in duplicate wells. After 1 hour incubation at 33°C, 5% CO2, 2 \(\times\) 10\(^4\) Madin-Darby Canine Kidney (MDCK) cells/well were added to the plate. Plates were incubated at 33°C, 5% CO2 incubator for approximately 40 hr, and the NA activity was measured by adding a fluorescently-labeled substrate, methylumbelliferyl-N-acetyl neuraminic acid (MUNANA) (Sigma) to each well and incubated at 37°C for 1 hr. Virus replication represented by NA activity was quantified by reading fluorescence in Fluorometer Envision (PerkinElmer) using the following settings: excitation 355 nm, emission 460 nm; 10 flashes per well. The concentrations of mAbs required for a 50% reduction in viral replication (IC50) was calculated using a non-linear fit algorithm curve fit in Graph Pad Prism. If neutralization curve was not complete, then value was assigned the highest concentration of inhibitor tested. Reported IC50 values are an average of 4 independent experiments.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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