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Plasmablast antibody repertoires in elderly influenza vaccine responders exhibit restricted diversity but increased breadth of binding across influenza strains

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Abstract

Seasonal influenza vaccines elicit antibody responses that can prevent infection, but their efficacy is reduced in the elderly. While a subset of elderly individuals can still mount sufficient vaccine-induced antibody responses, little is known about the properties of the vaccine-induced antibody repertoires in elderly as compared to young responders. To gain insights into the effects of aging on influenza vaccine-induced antibody responses, we used flow cytometry and a cell-barcoding method to sequence antibody heavy and light chain gene pairs expressed by individual blood plasmablasts generated in response to influenza vaccination in elderly (aged 70 – 89) and young (aged 20 – 29) responders. We found similar blood plasmablast levels in the elderly and young responders seven days post vaccination. Informatics analysis revealed increased clonality, but similar heavy chain V(D)J gene usage in the elderly as compared to young vaccine responders.

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Potential conflicts of interest

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Although the elderly responders exhibited decreased antibody sequence diversity and fewer consequential mutations relative to young responders, recombinant antibodies from elderly responders bound a broader range of influenza strain HAs. Thus elderly influenza vaccine responders mount plasmablast responses with restricted diversity but with an increased breadth of binding across influenza strains. Our results suggest that the ability to generate plasmablast responses encoding cross-strain binding antibodies likely represents a mechanism important to vaccine responses in the elderly.

Introduction

Influenza infections are a common cause of morbidity and mortality, especially in adults 65 years and older [1]. It is estimated that 5–15% of the worldwide population is infected each year, resulting in 3–5 million cases of severe illness and up to 500,000 deaths [2]. Annual vaccination is recommended by the WHO to prevent infection via induction of anti-influenza antibodies [3]. However, the efficacy of vaccination declines with age [4–6]. Influenza vaccination is less effective in the elderly, and the reduced efficacy correlates with lower vaccine-induced antibody responses [7]. Reduced effectiveness in the elderly has been attributed to age-associated differences in innate and adaptive immune responses, arising from factors including dysregulation of TLR function in dendritic cells [8, 9], decreased cytotoxicity of natural killer cells [10], and decreased numbers and function of T and B cells [11–14].

The primary mechanism for influenza vaccine-mediated protection is induction of anti-influenza antibody responses that block viral entry [15, 16]. In adults, the B cell response to the influenza vaccine is predominantly a recall response of memory B cells, rather than a primary response of naïve B cells [17]. It was demonstrated that poor vaccine-induced antibody responses in the elderly are associated with overall reduced quantities of vaccine-specific neutralizing antibodies in the blood, rather than reduced production of IgG antibodies by individual B cells [7]. Nevertheless, our understanding of how aging impacts the B cell response to influenza vaccination remains incomplete. Despite the general inferiority of the antibody response in the elderly, the influenza vaccine is effective in a subset of elderly recipients. Here, we sequenced plasmablast antibody repertoires in and characterized recombinant plasmablast clonal family antibodies from young and elderly influenza vaccine responders to gain insights into the B cell and antibody responses elicited by effective vaccination.

Methods

Human participants

Samples from 14 individuals that were responders to a seasonal trivalent inactivated influenza vaccine study (TIV, 2010/2011) were analyzed. All participants were enrolled in a Stanford University influenza vaccine study, and received the 2010/2011 seasonal trivalent influenza vaccine Fluzone (Sanofi Pasteur) which consists of three strains of inactivated influenza: an A/California/07/2009 X-179A (H1N1), A/Victoria/210/2009 X-187 (an A/Perth/16/2009 – like virus) (H3N2), and B/Brisbane/60/2008. PMBC and serum samples

were collected from each participant at three time points: day 0 before vaccination, day 7 for PBMC collection, and day 28. This study was approved by the Stanford University Institutional Review Board and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Hemagglutination inhibition (HAI) assays were used to identify subjects that were “good responders” to the influenza vaccine. HAI assays measure the antibodies produced *in vivo* against viral hemagglutinin (HA) proteins in response to vaccination, and are generally accepted as the standard for evaluating the effectiveness of influenza vaccines. In this study, only “good responders” (referred to herein as responders) to seasonal influenza vaccine were analyzed, as poor responders tend not to have sufficient vaccine-elicited plasmablast responses. Based on previously defined criteria[18], responders were designated based on exhibiting a ≥ 4 -fold increase in serum HAI titers against two or three vaccine strains 28 days following vaccination, or exhibiting seroprotection based on possessing a preexisting serum neutralizing serum titer of $>1:40$. The patient cohort, including HAI titers and CMV status are presented in Supplementary Table 1.

Flow cytometry isolation of plasmablasts

PBMCs were stained with anti-human CD3-PE-CF594 (BD #562280), anti-human IgA-FITC (Milenyi #130-093-071), anti-human IgM-APC-Cy7 (Biolegend #314520), anti-human IgD-PE-CF594 (BD #562540), anti-human CD14-PE-CF594 (BD #562335), anti-human CD20-PerCP-Cy5.5 (BD #340955), CD38-PE-Cy7 (BD #335790), anti-human CD19-Brilliant Violet 421 (Biolegend #302234), and anti-human CD27-APC (BD #302810). Plasmablasts were sorted based on the phenotype of $CD19^+CD20^-CD27^{hi}CD38^{hi}IgA^-IgM^-$. Cells were first bulk sorted with a BD FACS Aria II, and then single-cell sorted into 96-well PCR plates containing lysis buffer (10mM Tris-HCl pH 7.6) comprised of 2 mM dNTPs (New England Biolabs), 5 μ M oligo(dT)20VN, and 1 unit/ μ l of RiboLock RNase Inhibitor (ThermoFisher Scientific). Single-cell sorted plates were stored at -80°C until use for reverse transcription (RT).

Reverse transcription and single-cell cDNA barcoding

RT was performed at 42°C for 120 minutes in a buffer containing 3 mM MgCl_2 , 1 unit/ μ l RiboLock RNase inhibitor, $1\times$ M-MuLV buffer (New England Biolabs), and 1 unit/ μ l Maxima reverse transcriptase (ThermoFisher Scientific). The unique well-ID barcode containing adapter sequences at a final concentration of 1 μ M were added to the RT reaction in each well. Single-cell barcodes were added on to the cDNA ends based on the template switching mechanism [19]. Following RT, cDNA products from all wells on a single plate were pooled.

PCR with well-ID and plate-ID adaptors, and 454 sequencing

Phusion Hot Start II High-Fidelity DNA polymerase (ThermoFisher Scientific) was used for both the first PCR (PCR1) and the nested PCR (PCR2). Cocktail primers for that amplified the heavy and light chains were used as previously described [20]. Forward primers used in PCR1 contained different plate-IDs to different samples. Gene-specific reverse primers, lambda, and gamma were used to amplify the constant regions of kappa, lambda, and

gamma chains, respectively. Final PCR products containing the full-length V(D)J region (~600 bp for light chain and ~650 bp for heavy chain) were visualized on agarose gels and quantified using the MatLab software. For 454 sequencing, equal amounts of the amplified DNA was pooled from each gene and gel purified, and Lib-L sequencing adapters added. DNA QC was determined by the Agilent 2100 Bioanalyzer and the final library was sent for GS FLX+ Titanium sequencing (Roche).

Sequencing data analysis

Filtered passed, high-quality sequence data were analyzed using 454 GS FLX data analysis software (Roche). Sff output files were read into Python scripts by using the Biopython package, and sequences were grouped and parsed into separate sff files on the basis of their plate-ID and well-ID combinations. Consensus sequences corresponding to plasmablast heavy and light chain sequences were generated using the Newbler 2.6 software. Where multiple assemblies occurred per well, an assembly was accepted if it contained >50% of all reads per well. Otherwise, we assumed that the well contained more than one plasmablast or sequencing errors were introduced during pyrosequencing, and sequence reads from that well were disregarded. To assign V(D)J families for consensus sequences, we analyzed heavy and light chain sequences with the IMGT HighV-QUEST (www.imgt.org/HighV-QUEST) database to predict germline allele usage, germline sequence recombination, and mutations relative to the germline sequence. To generate dendrograms, heavy and light chain sequences were binned according to their V-gene usage and aligned with Muscle. They were clustered with PhyML maximum-likelihood clustering and rooted by their germline V gene. Each V-gene phylogenetic tree was then arranged by heavy chain V-gene families and drawn by using ETE30. Clonal family antibodies were defined as antibodies with shared germline heavy chain V(D)J and light chain VJ gene usage. Chord diagrams were generated using VDJviz tools [21].

Cloning and expression of monoclonal antibodies

For the selection of antibodies representative of clonal families, sequences were bioinformatically aligned, compared, and selected for the most frequent member and/or heavy and light chain pair most representative of the consensus sequence of the clonal family. Using direct gene fragment synthesis (Integrated DNA Technologies), kappa and lambda light chains were cloned into pFUSE2-CLIg-hK, pFUSE2-CLIg-hL2, and pFUSE-CHIg-hG1 vectors (InvivoGen), respectively. Paired light and heavy chain-containing plasmid constructs were transiently transfected into Expi293 suspension cells using the ExpiFectamine293 transfection kit (ThermoFisher Scientific). Expressed antibodies from the culture supernatants were purified using the Protein A Plus agarose beads according to the manufacturer's instructions (Pierce).

HA peptide microarrays

Customized HA peptide microarrays were printed and probed, and datasets were analyzed, as previously described [20].

Results

The seasonal influenza vaccine induced similar plasmablast levels in young and elderly responders

We characterized the plasmablast response and antibody repertoires in a total of 14 young (age 20-29) or elderly (age 70-89) subjects enrolled in the seasonal trivalent inactivated influenza vaccine study (TIV, 2010/2011). To identify influenza vaccine responders, HAI titers were measured in serum samples collected before vaccination (day 0) and on day 28 after vaccination. Subjects were designated as responders based on exhibiting a ≥ 4 fold increase in HAI titer against two or three vaccine strains, or by exhibiting seroprotection based on possessing a $> 1:40$ preexisting neutralizing serum titer (Fig. 1A and Supplementary Table 1). While not all individuals responded to all three vaccine strains, the elderly responders exhibited good responses to the A/California/07/2009 strain based on exhibiting a ≥ 4 fold increase in HAI titers (Fig. 1A).

Day 7 post-vaccination PBMC samples from these subjects were analyzed by flow cytometry for their plasmablast levels. IgG-producing CD19⁺CD20⁻CD27^{hi}CD38^{hi}IgA-IgM⁻ plasmablasts were identified based on immunophenotyping markers. Although the elderly individuals exhibited lower quantities of B cells in the peripheral blood (Fig. 1B; $p < 0.05$), no difference in the relative levels of IgG-producing plasmablasts elicited by the vaccine was detected between the young and elderly responders (Fig. 1C). These data indicate that among the responders, the young and elderly groups demonstrated similar plasmablast levels in response to vaccination despite lower B cell numbers in the elderly group.

Higher clonality in the plasmablast repertoires of elderly responders

To further characterize the B-cell responses induced by vaccination in the young and elderly responders, we next sequenced the blood plasmablast antibody repertoires from PBMC samples collected 7 days following vaccination. We sorted individual blood plasmablasts into 96-well plates and used oligonucleotide cell-specific barcoding to sequence the endogenously paired heavy and light chain antibody genes. To visualize clonal expansions of plasmablasts, we generated phylogenetic trees of the antibody sequences using maximum likelihood clustering that included heavy chain-weighted clustering of the paired heavy- and light-chain sequences. We considered sequences derived from the same germline heavy chain V(D)J and light chain VJ gene segments (Fig. 2A) as clonal families. By this definition, 27% of the antibodies in the young responder repertoires and 55% of the antibodies in the elderly responder repertoires were clonal (Fig. 2B), representing 37 and 30 clonal families, respectively. When we compared the sizes of these clonal families, the elderly responders possessed larger clonal expansions versus the younger cohort (Fig. 2C; young: 2.76 ± 0.29 , elderly: 5.2 ± 0.96 , $p < 0.05$), consistent with the higher clonality observed in their plasmablast repertoires. In contrast, young responders exhibited smaller and more diverse plasmablast clonal families.

Similar germline V(D)J gene usage in the immunoglobulin heavy chain sequences between the young and elderly responders

We also analyzed the heavy chain V(D)J and light chain VJ gene usage in the young and elderly responders. The frequencies of dominant V gene usage were comparable between the young and elderly (Fig. 3A). Specifically both the young and elderly showed a high-frequency usage of IGHV3-23 and IGHV4-39 of the V gene and IGHJ4 of the J gene segments. However, the IGHD gene usage varied: the young responders exhibited greater usage of the IGHD3-22 and IGHD3-10 gene segments, while the elderly responders exhibited greater usage of the IGHD5-12, IGHD3-3, and IGHD5-18 gene segments. In the light chain, V and J gene usage in the elderly responders was skewed towards IGLV4-69 (18.4%), IGLJ3 (31.1%), IGKV1-39 (12.4%), and IGKJ2 (19.4%), whereas the young responders showed more diverse V gene usage, with IGKV3-15 (11%) and IGKJ2 (17.2%) being most commonly used. In clonally expanded plasmablasts following vaccination, the IGHV and IGHD genes used were similar between the young and elderly groups, with a dominant usage of IGHV3-23 and IGHJ3 combination in the young group, and IGHV3-23 and IGHJ4 combination in the elderly group (Fig. 3B, 3C). In the identified clonal families, the light chain V gene usage of the clonal expanded plasmablasts was represented by the IGKV1-33, IGKV3-15, IGKJ4, and IGLJ2 in the young responders, in contrast to IGLV4-69 and IGLJ3 in the elderly responders. Together, our data shows no major differences in the heavy chain V, D and J gene usage and some differences in light chain V and J gene usage between the young and elderly responders.

Decreased somatic hypermutation rates and shorter CDR3 lengths in the elderly responders

We further analyzed the rates of somatic hypermutation in the young and elderly plasmablast repertoires. Percentages of mutated nucleotides in the variable region of the heavy or light chains relative to the predicted germline sequences were compared between the two groups. We found that the young responders exhibited higher mutation rates in the heavy chain ($8 \pm 0.16\%$) as compared to the elderly responders ($7 \pm 0.18\%$, $p < 0.05$), while no differences were observed in mutation rates in the light chains (Fig. 4A). The higher mutation rates in the young responders were associated with higher numbers of amino acid substitutions (non-silent mutations, Fig. 4B; $p < 0.05$). No difference in mutation rates was detected between the clonal and non-clonal family antibodies ($p > 0.05$). Consistent with previous findings [15, 16], we observed higher mutation frequencies in the heavy chains of the clonally expanded antibodies from cytomegalovirus (CMV) seropositive individuals ($p < 0.001$) regardless of age (Fig. 4C).

We next compared the average CDR3 amino acid length between the young and elderly repertoires. We found that in the young responders, the heavy chain CDR3 lengths were significantly longer in antibodies that belong to clonal families (clonal: 16.7 ± 0.19 vs. non-clonal 15.5 ± 0.24 , $p < 0.05$). In contrast, in elderly responders the clonal family heavy chain CDR3 lengths were shorter than those for non-clonal antibodies (clonal: 14.5 ± 0.16 vs non-clonal: 15.43 ± 0.27 , $p < 0.05$; Fig. 4D). Antibodies that belong to clonal families from the young responders had significantly longer CDR3 lengths than those from the elderly responders (young: 16.7 ± 0.19 , elderly: 14.5 ± 0.16 , $p < 0.05$), while no differences were

observed in CDR3 lengths between non-clonal antibodies in the young and elderly responders (young: 15.49 ± 0.24 , elderly: 15.43 ± 0.27 , $p > 0.05$).

Plasmablast clonal family antibodies from elderly responders cross react with HA antigens derived from multiple influenza strains

We next examined the binding specificity of recombinant antibodies representing the plasmablast clonal families identified in young and elderly responders. Representative antibodies from clonal families were selected, their variable regions recreated by DNA synthesis and cloned into an expression vector containing the human IgG1 Fc, and antibody protein was produced by recombinant expression in Expi293 cells *in vitro*. The recombinant antibodies were tested for their binding specificity on influenza HA antigen arrays containing HA proteins derived from a variety of prior seasonal influenza strains. HA antigen array analysis showed that the recombinant clonal family antibodies from elderly responders bound predominantly to HA1 antigens and exhibited cross-reactivity against HA1 antigens derived from multiple influenza strains (Fig. 5). This is in agreement with the high HA1 seroconversion rates observed in the elderly responders (Fig. 1A). In contrast, recombinant clonal family antibodies expressed from the young responders showed some cross-reactivity against HA1 or HA3, but less cross-reactivity as compared to the antibodies from the elderly responders (Fig. 5). Together, our data suggest that in elderly responders, the seasonal influenza vaccine activates plasmablasts encoding antibodies that bind epitopes conserved across multiple influenza strains.

Discussion

In this study, we sequenced the paired, full-length, heavy and light chain immunoglobulin mRNAs of the IgG-producing plasmablasts from young and elderly individuals who responded well to the influenza vaccine. Our data revealed similar plasmablast levels and germline immunoglobulin V and J gene usage between the young and elderly responders, but increased clonality, lower somatic hypermutation rates, and shorter CDR3 lengths in the elderly responders. Additionally, the elderly responder plasmablast clonal families encoded antibodies that exhibited greater cross-reactivity to HAs derived from multiple influenza strains.

It is intriguing that elderly responder plasmablast clonal families encode antibodies that bind HA antigens representing a broader range of influenza strains. This observation suggests that elderly responders positively select and preserve B cell clones that are more cross-reactive to HA epitopes shared across influenza strains, and that these B cells are effectively recalled and clonally expand during effective vaccination. The ability of elderly influenza vaccine responders to generate plasmablast responses encoding cross-strain binding antibodies represents a potentially key protective mechanism. Moving forward, it will be important to determine if specific immunogens, adjuvants, vaccine regimens, or other factors can promote the generation and re-activation of B cells encoding these cross-strain reactive antibodies, thereby conferring protection against influenza infection.

Influenza vaccines confer protection by stimulating B cells to produce neutralizing antibodies. Antibody repertoire sequencing provides a powerful approach to further define

vaccine-induced antibody repertoires, however the majority of studies to date have utilized methodologies that provide limited information due to (i) failure to accurately pair the native heavy and light chains, (ii) lack of analysis of the full-length variable regions, and (iii) insufficient process error correction [22]. In this study, we utilize a cell barcoding method [20, 23] to sequence the full-length, natively paired, immunoglobulin heavy and light chain variable regions from plasmablasts derived from young and elderly influenza vaccine responders. Our cell barcoding method enables determination of a consensus sequence for each variable region, thereby providing robust process error correction [20, 23]. We further expressed and characterized recombinant antibodies representing the identified plasmablast clonal families, thereby linking the plasmablast antibody repertoire sequence data to the binding characteristics of the encoded antibodies [20, 23].

Our results found that despite the lower numbers of B cells in elderly responders, the vaccine-induced relative plasmablast levels were similar in the elderly and young responders. However, repertoire diversity and clonality differed significantly between the two age groups. Consistent with previous findings [24], we found that the elderly group responded to the seasonal influenza vaccine with less diverse but more clonally expanded plasmablast repertoires. It has previously been demonstrated that in adults the B cell response to influenza vaccine or infection involves the activation of memory B cells that are further affinity-matured [25, 26]. We found that the Ig gene segment usage in the heavy chains was comparable between the young and elderly responders, but that the frequencies of non-silent mutations were increased in the young responders. The lower somatic hypermutation rates that we observed in the elderly responders is in agreement with several reports [16, 27, 28], although other studies described increased baseline mutation levels in the elderly [15, 24]. Together, our findings suggest that elderly responders activate memory B cells encoding HA cross-reactive antibodies to undergo clonal expansion with minimal additional affinity maturation, in contrast to young responders whose plasmablast responses undergo additional affinity maturation presumably to increase their antibody affinity to the vaccine-encoded antigens.

The IgH CDR3 forms the core of the antigen-binding site and is frequently affected by the affinity maturation process [29, 30]. A prior study reported that in the case of CMV infection, elderly CMV infected subjects exhibit increased numbers of B cells with long CDR3 regions, suggesting impaired selection of B cells expressing antibodies with shorter CDR3 regions [15]. In this study, we observed a shorter mean CDR3 length of antibody clonal families in the repertoires of CMV-negative elderly influenza vaccine responders, suggesting that CMV infection did not confound analysis of CDR3 length in this study. Several studies have indicated that a shorter CDR3 length may provide an advantage in effective vaccine responses [15, 31, 32], possibly because B cells expressing antibodies with shorter CDR3 lengths may more effectively bind their target antigens [33].

Limitations of this study include the relatively small number of patients characterized, the limited depth of plasmablast sequencing, and the lack of analysis of an absence of data regarding non-responders. Influenza vaccine non-responders generally fail to mount sufficient plasmablast responses for repertoire sequencing, and other approaches will be necessary to further define the mechanisms underlying the insufficient B cell and adaptive

immune responses that characterize the majority of elderly influenza vaccine non-responders. Together, our findings showed that the quantity of B cells is affected by aging, but the quality of B cell response and antigen recognition is not compromised in elderly responders.

Aging of the human population is a rising challenge in effective vaccination, and significant work is needed to improve immunization strategies for the elderly. Our findings in this study further elucidate the characteristics of the B cell response in elderly influenza vaccine responders. We found that elderly influenza vaccine responders mount plasmablast responses with restricted diversity but increased breadth of binding across influenza strains. The ability to generate plasmablast responses encoding cross-strain binding antibodies could be important to vaccine response in the elderly, and further work is needed to develop vaccines and immunization strategies that enhance both the generation and activation of such B cell responses in the elderly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of the blood plasmablast response in young and elderly responders to the seasonal influenza vaccine. Samples from 14 young (age 20-29) or elderly (age 70-89) responders enrolled in a seasonal trivalent inactivated influenza vaccine study (TIV, 2010/2011) were selected for analysis. **(A)** Seroconversion rates of the selected young or elderly vaccine responders to the 2010 seasonal influenza vaccine. All analyzed individuals were defined as influenza vaccine responders based on a ≥ 4 fold increase in HAI titers against 2 or 3 vaccine strains 28 days following vaccination. **(B)** Percent B cells in the young and elderly responders 7 days following vaccination. **(C)** Relative plasmablast numbers in the young and elderly responders 7 days following vaccination. Data are the mean $\pm$ SEM. *, $p < 0.05$ by t test.

Figure 2.

Analysis of the IgG plasmablast repertoire in young and elderly influenza vaccine responders. **(A)** Clustering of paired heavy chain and light chain dendrograms based on the heavy chain V region usage of individual plasmablasts. Each node at the end of a branch of a phylogenetic tree represents one plasmablast. Antibodies in clonal families are colored red. **(B)** Clonal vs non-clonal plasmablasts in the antibody repertoires of the young and elderly responders. **(C)** Increased clonal family size in the elderly responders. Each blue circle denotes the size of a clonal family in a young subject, and each red square denotes the size of a clonal family in an elderly subject. The mean $\pm$ SEM of each group are indicated. *, $p < 0.05$ by t test.

Figure 3.

Similar V and J gene usage in the heavy chains of plasmablast antibodies from the young and elderly responders. The germline V gene usage plots for the entire antibody repertoire (A) and for antibodies that are in clonal families (B) from young and elderly responders. (C) Chord diagram illustrates the heavy chain usage of the major V and J gene combination. Each chord is scaled to the frequency of antibodies by the corresponding gene, and each arc is scaled to the number of antibodies by gene segment combination.

Figure 4.

Analysis of somatic hypermutations and CDR3 lengths of the plasmablast antibody repertoires in young and elderly influenza vaccine responders. Mutation rates in the heavy chain are higher (A), and are predominantly non-silent (B), in the young group. (C) CMV seropositive individuals exhibited higher mutation frequencies in their heavy chains of clonally expanded antibodies as compared to CMV seronegative individuals. (D) The length of the CDR3 region in the heavy (left) and light (right) chains of clonal families is longer in the young responders. Each dot represents data from an individual. The mean $\pm$ SEM of each group are also indicated. *, $p < 0.05$ by t test.