THE UNIVERSITY OF CHICAGO

INFLUENZA HEMAGGLUTININ STALK-REACTIVE MEMORY B CELL RESPONSES TO A CHIMERIC-BASED UNIVERSAL INFLUENZA VIRUS VACCINE CANDIDATE

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Abstract

Influenza viruses remain a severe threat to public health. Since currently licensed vaccines mediate insufficient protection, novel vaccine approaches that elicit broad protection are being developed. A candidate universal vaccine based on chimeric hemagglutinins (HA) was recently tested in a phase I trial. To dissect the B cell immune responses elicited by this vaccine, we used a “Total-seq HA array”, which revealed the transcriptome and antibody specificity of individual HA-reactive memory B cells after vaccination. Our analyses revealed previously unknown transcriptional heterogeneity within the antigen-specific memory B cell population in response to chimeric HA vaccination, representing seemingly functionally distinct cell subsets. In addition, we provide evidence that the chimeric HA-based universal influenza vaccine induces HA stalk-reactive memory B cells, with B cell clones shared by multiple subjects (public clones). Furthermore, we identified a surprising antigen-specific B cell subset expressing IgD and IgM with low mutation rates. In summary, this study provides an in-depth analysis of the B cell responses elicited by a novel universal influenza virus vaccine candidate and could inform strategies to further optimize future vaccine designs.

1 Introduction

Long-term B cell memory is the protagonist in the quest for vaccination. Memory is sustained by long-lived antigen-specific B cells that are induced by the initial antigen exposure. These cells respond to secondary exposure to antigens by increasing antibody levels and affinity after their initial priming in the primary immune response. This involves both clonal expansion and clonal differentiation.

How B cells mediate immunological memory is an important topic in immunology. Work on human influenza virus infection and/or immunization has advanced the understandings of various memory B cell populations and affinity maturation. In this introduction, I describe recent studies of B cell responses to complex antigens and heterogeneity in memory B cells that mediate the recall humoral response.

1.1 The humoral immune response

The immune system is the body's protection from infectious disease. It consists of both innate and adaptive immune responses. Immunology as a science began with Edward Jenner's work using cowpox (vaccina) to protect against smallpox. This scientific strategy was first proven to be effective in 1796. It was subsequently called vaccination(Riedel, 2005). An effective vaccine that inoculates heathy individuals with attenuated or inactivated strains of disease-causing agents to protect from diseases required both the innate and the adaptive immune system. Innate immune response initiates upon exposure to an infectious organism, whereas the adaptive immune system takes days, rather than hours, to eliminate infection. This is because the

adaptive immune system's antigen-specific lymphocytes, T cells and B cells, recognize antigens via variable antigen receptors. These are T cell receptors (TCRs) and B cell receptors (BCRs), and they recognize antigens on the surface of the lymphocytes. TCRs and BCRs present on the surface of each lymphocyte mature bearing unique antigen receptors. Thus, the T cell and B cell repertoires are diverse. It is estimated that each individual's circulating T cell and B cell repertoires consist of approximately 10-20 million clonotypes(Soto et al., 2019). TCR consist of two chains, either a TCRα and a β chain or a γ and a δ chain. The BCR is produced by the same genes that encode antibodies. These are known as immunoglobulins (Ig).

Antibodies are the secretory form of the BCR, estimated to have a molecular weight of 150kDa. Their structure is composed of two identical heavy chains and two identical light chains. Both include constant and variable regions. The heavy chain is linked to other heavy chains by disulfide bonds. Each heavy chain also links to a light chain via a disulfide bond. There are two types of light chains, kappa (κ) and lambda (λ). Antibodies comprise one or the other. The antigen-binding specificity is determined by the antigen-binding sites via the combination of varying regions of heavy and light chains. Each antibody has two identical antigen binding sites, as they have two identical variable regions, that are the ends of the two arms in a Y shape–the V region of the heavy and light chain (V_H and V_L, respectively). Therefore, an antibody can bind to two antigens simultaneously. This is called avidity. Some antibody subtypes such as dimeric IgA and pentameric IgM form larger multimeric structures. The strength of the bond to a particular antigen is called affinity. The constant region, the stem of the Y, is called the

C region of the heavy and light chains (C_H and C_L, respectively). Five classes of Ig are defined based on the construction of the C region—IgM (denoted by $C\mu$), IgD ($C\delta$), IgG ($C\gamma$), IgA ($C\alpha$) and IgE ($C\varepsilon$), called isotypes. The antibody can be further cleaved through the hinge region into Fab fragments which are responsible for antigen binding. The other fragment is called the Fc fragment. Although the Fc fragment is far less variable than Fab fragments, it interacts with the effector immune cells and the effector molecules, such as the C1q and Fc receptors.

The V region of an antibody is the binding site that determines the antigen's specificity. It consists of the hypervariable region and the framework region. While the four framework regions: FR1, FR2, FR3 and FR4 provide the framework structure for the Ig domain, the hypervariable region: VH1, VH2 and VH3 of the heavy and light chains are paired as a total of 6 hypervariable loops. These are commonly known as the complementarity-determining regions (CDRs). They contribute to determining antigen specificity. Each heavy and light chain contain 3 CDRs—CDR1, CDR2 and CDR3. In most cases, the CDR3 from the heavy chain variable region (V_H) and the light chain variable region (V_L) domains contribute to the final antigen specificity.

During the development of each lymphocyte, gene segments are rearranged to form a variable region. The mature activated B cells then increase their diversity by somatic hypermutation. However, unlike most genes, the variable region is not encoded by a complete DNA sequence of the variable-region exon. Instead, it is encoded by distinct gene segments—variable (V) gene segments, diversity (D) gene segments and joining (J) gene segments. For the light chain, the whole V_L region is encoded by the V

and J gene segments, while the heavy chain is encoded by all V, D and J segments. For the CDR1 and CDR2 loops, both the light chain and the heavy chain are encoded by V gene segments, whereas CDR3 light chain loops are encoded by the V and J segments. The heavy chain's CDR3 is formed by joined V, D and J segments. Therefore, the CDR3 region adds diversity to the antibody repertoire(Kenneth Murphy, 2018; Schroeder and Cavacini, 2010).

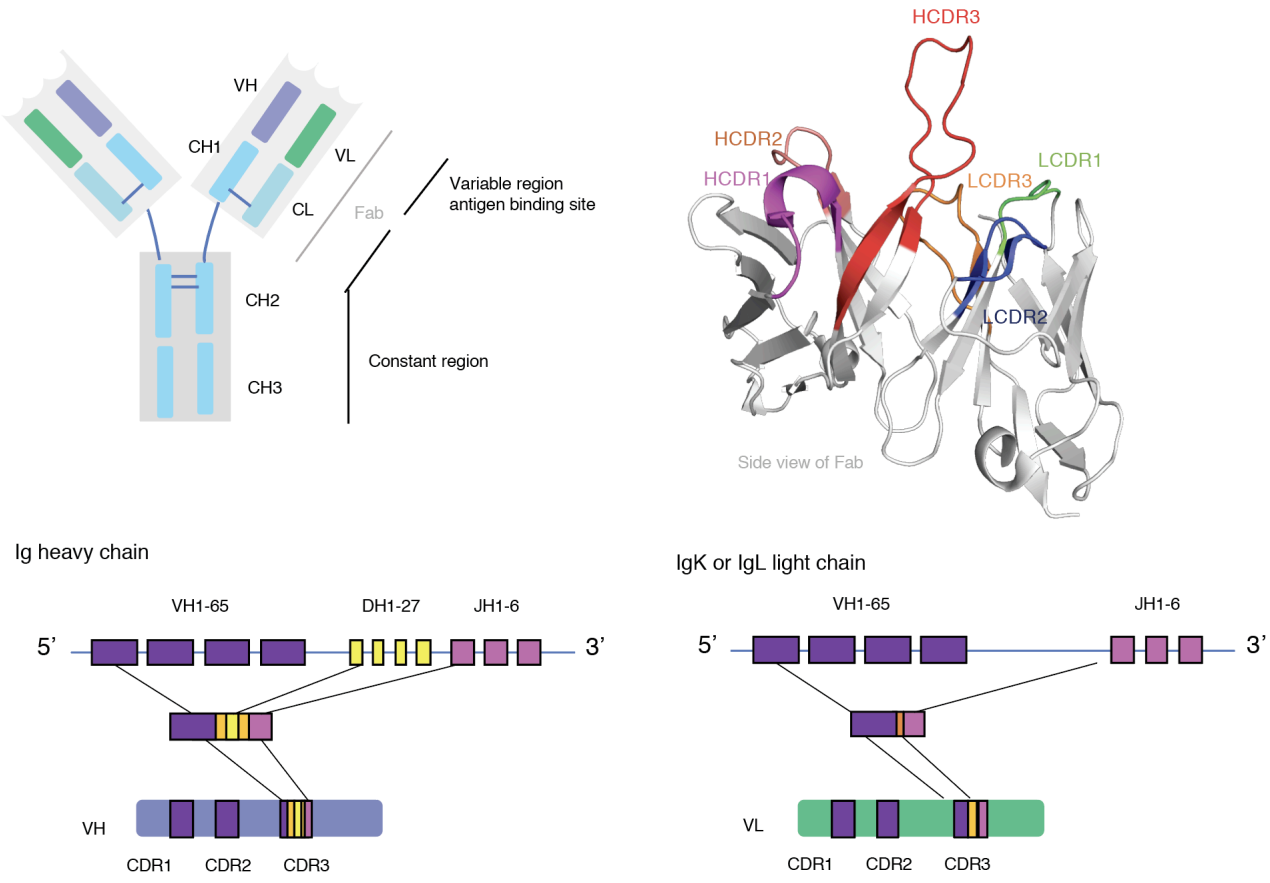


Figure 1-1 Genetics and structure of antibodies.

B cells develop through several steps of immunoglobulin gene rearrangement and expression. These are early pro-B cells, late pro-B cells, large pre-B cells, small pre-B cells, immature B cells (expressing high levels of IgM but little IgD), and mature B cells (expressing low levels of IgM and high levels of IgD). The immature B cells leave the bone marrow and then enter the periphery. The majority of the peripheral B cells are resident in the spleen or other secondary lymphoid organs. They are known as follicular B cells (also referred to as B2 B cells). The follicular B cells are T cell-dependent. There is also another minor population of B cells—marginal zone B cells. While marginal zone and B1 B cells account for early adaptive immune responses by producing T cell-independent antibodies, the follicular B cells are T cell-dependent(2018; Cyster and Allen, 2019; Kenneth Murphy, 2018).

Upon encountering antigen, naïve B cells are activated by antigen through BCR signaling and by presenting peptide antigens bound to MHC class II. The peptide:MHC class II complexes are recognized by T follicular helper (T_{FH}) cells. The T_{FH} facilitates B cell proliferation and differentiation. Later, the activated B cells migrate into follicles and form a germinal center, a specialized microenvironment with three distinct areas: a light zone, a dark zone and a recently identified gray zone(Kennedy et al., 2020). Before entering the germinal center or for extrafollicular B cell differentiation, class-switch recombination takes place(Flemming, 2019). B cell class switches from IgM and IgD to express another subtype such as IgG, IgA or IgE. Class-switching is induced by different cytokines. In the germinal center, B cells proliferate, differentiate, and mutate their V region of antibody genes to achieve higher affinity. The class-switching is

induced by various cytokines to change the effector function of the antibody in response to various pathogens.

The B cells that survive the germinal center reaction egress from the light zone and differentiate into plasma cells, long-lived plasma cells migrating to the bone marrow, and memory B cells(Mesin et al., 2016). Memory B cells become long-lived after infection or vaccination. They persist in the absence of the antigen that originally induced them, and they secrete little or no antibodies. However, they are poised for a rapid response, and have higher affinity for antigen upon recall than do naïve B cells.

Memory B cells can arise from the germinal center; they inherit somatic hypermutation and class-switching. However, memory B cells can also rise in a germinal center-independent manner(Kaji et al., 2012), from plasma cells produced in the primary response(Obukhanych and Nussenzweig, 2006; Pape et al., 2018; Takemori et al., 2014; Taylor et al., 2012; Weisel et al., 2016). They can also re-enter germinal centers and undergo additional somatic hypermutation, class-switching, and affinity maturation during a secondary immune response, though the default appears to be biased for naïve cells to form GCs(Mesin et al., 2020). Finally, there appear to be different types of cells in the memory B cell compartment. Thus, understanding the subsets of memory B cells could allow for the modulation of vaccines to induce their generation.

1.2 Memory B cell heterogeneity

Memory B cells (MBCs) derive from naïve B cells that could be induced by T cell-dependent or T cell-independent antigens. T cell-dependent antigens elicit memory B cells undergo germinal center (GC)-dependent or GC-independent mechanisms(Akkaya et al., 2020; Bortnick and Allman, 2013; Obukhanych and Nussenzweig 2006). In T cell-dependent and GC dependent immune responses, naïve B cells circulate through the follicles of secondary lymphoid organs where they encounter foreign proteins, some proliferating B cells with their associated T follicular helper cells (Tfh) from a primary focus, and some migrate into the follicle to form a GC. Plasmablasts, GC B cells, and early memory B cells generated from primary foci as early as first 4-5 days during a primary immune response. While through GC reaction, B cell undergo several process include somatic hypermutation which enables affinity maturation(Kenneth Murphy, 2018). In addition, class switching occurs during the initial T-B cell interaction prior to GC reaction allows B cells to produce antibodies with variety of effector functions.(Roco et al., 2019) Therefore, GC are critical for B cells differentiate into long-lived plasma cells and memory B cells(Allie et al., 2019; Chong and Ansari, 2018; De Silva and Klein, 2015; McHeyzer-Williams et al., 2011; Mlynarczyk et al., 2019).

Considerable heterogeneity has been described within the MBC population. These memory B cell subsets may get activated at different stages of development and have been identified from different tissues such as peripheral blood, spleen, tonsil, bone marrow and lung(Allie et al., 2019; Lam and Baumgarth, 2019). It has been recognized that distinct MBC populations exhibit different effector functions.

In humans, memory B cells were historically identified by expression of CD27 and loss of IgD expression(Klein et al., 1998; Pascual et al., 1994; Tangye and Good, 2007). However, recent reports have characterized a sub-population of memory B cells that do not express CD27 or mutated IgV region genes. Rather, they express CD80 and CD86. This implies that they may be generated in a T-independent manner, or in earlier GC reactions(Tangye and Good, 2007). Additionally, HA^+ $CD21^{low}$ $T\text{-}bet^{hi}$ memory B cells that are segregated from classical HA^+ $CD21^{hi}T\text{-}bet^{low}$ memory B cells have been identified in peripheral blood. They are induced during the peak of GC activity after seasonal influenza vaccination. These cells have been predicted to be precursors to long-lived plasma cell differentiation(Lau et al., 2017). The differential expression of the transcription factor T-bet for memory B cells has been studied extensively. $T\text{-}bet^{hi}$ and $T\text{-}bet^{low}$ memory B cell subsets have been identified in humans and in mice(Johnson et al., 2020). In addition, CD71 was reported to serve as a B cell activation marker. An activated ($CD71^+$) B cell population that arises at day 7 after influenza vaccination resembles memory B cells(Ellebedy et al., 2016a).

In mice, MBC subsets have been shown to be distinct in terms of both BCR isotype (IgD^+, IgM^+, and IgG^+) and the expression of surface markers such as CD73, CD80, and PD-L2(Good-Jacobson and Shlomchik, 2010; Good-Jacobson et al., 2010). The lack of CD73, CD80, and PD-L2 expression implies that IgD^+ MBCs develop independent of a GC. IgM^+ MBCs and IgG^+ MBCs can be reactivated after a secondary antigen encounter, and thus become IgM^+ and IgG^+ plasmablasts. IgD^+ MBCs' function is unclear, but these cells may re-enter the GC reaction to form secondary GCs(Harms

Pritchard and Pepper, 2018). Moreover, recent studies have revealed additional heterogeneity within the isotype-unswitched MBCs (predominantly comprised of IgD^+ cells, but also including a small population of IgM^+ cells). These cells have increased longevity, compared to isotype-switched (IgG^+) MBCs(Dogan et al., 2009; Harms Pritchard and Pepper, 2018; Weisel and Shlomchik, 2017). While heterogeneity within the memory B cell population has been characterized, it remains unclear whether the antigen-specific memory B cells' response to influenza immunization is also composed of distinct subsets that play unique functional roles in mediating protective immunity.

1.3 Human B cell repertoire diversity

B cell receptors (BCRs), as secreted antibodies form a vast diverse antibody repertoire by undergoing gene rearrangement with non-templated junction additions. In addition to the inherited germlines, the antibodies further diversify by recombining germlines, causing imprecision during the V(D)J recombination, somatic mutation, and class-switch. However, antibodies can show bias from using V_H gene, and there is a long discourse on innate-like recognition characteristics using particular gene-encoded elements(Avnir et al., 2017; Avnir et al., 2016a; Watson and Breden, 2012).

Recently, high-throughput DNA sequencing has provided insight into human B cell repertoires and assessment of the unique (or private) repertoires or shared (or public) repertoires. Each individuals' circulating repertoire consists of approximately 9-17 million clonotypes. Studies have shown that 3 individuals will share 0.3% of their clonotypes(Soto et al., 2019). The shared lineages may be shaped by previous

exposure to common antigens. They may also be induced by foreign antigens. The understanding of private and shared clonotypes enables the study of vaccine response.

The public lineages observed in different individuals may share the same genetic elements and modes of recognition. These B cells may have the same maturation and BCR recombination pathways, in response to immune stimulation(Henry Dunand and Wilson, 2015). For example, shared convergent antibodies have been identified in response to influenza vaccination and infection. Examples include H1N1 2009 and H5N1 influenza vaccination. In response to influenza antigen, these induced public B cell clonotypes are encoded by the VH1-69 and VH1-18 genes (Henry Dunand and Wilson, 2015; Jackson et al., 2014; Joyce et al., 2016; Wheatley et al., 2015). It is hypothesized that the stereotyped B cell clone in 2009 occurred through the recall of memory B cells to the past strains which targeted the functional conserved epitope(Henry Dunand and Wilson, 2015). The response to vaccination in the 2009 pandemic H1N1 influenza strain reveal a convergent feature of antibody clonotype that can be generated for other pathogens and guide vaccine design(Jackson et al., 2014). That is, identifying these reproducible classes and mechanisms, and designing immunogens to induce public clones, is beneficial to a genetically diverse population(Andrews and McDermott, 2018; Lanzavecchia et al., 2016a; Setliff et al., 2018; Wu and Wilson, 2018; Yuan et al., 2020).

1.4 Toward a universal influenza virus vaccine

1.4.1 Current influenza virus vaccines

Annual influenza epidemics cause up to 650,000 deaths every year worldwide(WHO, Date accessed: May 16, 2020). Based on historical precedent, occasional influenza pandemics with potentially devastating effects on human health will continue to occur. Influenza virus vaccines are the best prophylaxis, but recent studies have shown that current vaccines still provide suboptimal. For example. the overall vaccine effectiveness of the influenza virus vaccine during the 2019/2020 season was estimated to be 45%(Dawood FS, 2020).

Vaccine failure typically results from antigenic drift variants that differ from the vaccine strains so that the vaccine needs to be updated annually, requiring approximately 6 months to reformulate. It provides even less protection against influenza A H3N2 viruses, which rapidly spread into antigenically distinct co-circulating clades(Flannery et al., 2018; Kucharski et al., 2015; Skowronski et al., 2019). An additional two lineages of influenza B viruses, the B/Victoria/2/87-like and the B/Yamagata/16/88-like lineages, are diversifying(Virk et al., 2020). Current seasonal vaccines also provide little to no protection against antigenically novel influenza strains that have pandemic potential. Therefore, developing a vaccine that provides broader or universal protection is a public health priority(Erbelding et al., 2018; Nabel and Fauci, 2010; Nachbagauer et al., 2017; Wei et al., 2020).

1.4.2 Influenza virus

There are 4 types of seasonal influenza viruses. Influenza A and B viruses circulate and cause seasonal epidemics of disease. Influenza viruses possess eight segmented, negative-stranded RNAs as their genome. There are 11 genes on these eight RNA segments, encoding proteins including hemagglutinin (HA) and neuraminidase (NA), nucleoprotein (NP), matrix (M1 and M2), non-structural protein (NS1 and NS2/or called nuclear export protein, NEP), as well as viral replicase complex (PA, PB1, PB1-F2 and PB2)(Liu et al., 2018). The influenza virus surface glycoprotein hemagglutinin (HA) is the primary target of the humoral immune response. It consists of a membrane-distal globular head and a membrane-proximal stalk region, which are involved in receptor binding and membrane fusion, respectively. Unlike the HA head domain, the HA stalk domain is highly conserved across influenza virus subtypes. Stalk-reactive antibodies have been shown to be broadly cross-reactive and to neutralize a variety of influenza virus strains(Atsmon et al., 2014; Flannery et al., 2018; Thomson et al., 2012). However, due to the immunodominance of the HA head, current influenza vaccines induce minimal stalk-directed humoral immunity.

1.4.3 Universal influenza vaccine strategies

Currently, there are multiple strategies to develop a universal flu vaccine(Sautto et al., 2018). The computationally optimized broadly reactive antigens (COBRA) method based on consensus HA protein sequences over years, aims to elicit a broad spectrum immune response against the highly variable HA globular head(Crevar et al., 2015;

Wong et al., 2017). It remains to be determined whether COBRA HA is able to protect against future circulating influenza virus strains, especially pandemic strains. In addition, it requires a combination of antigens to achieve the protection against different subtypes. The epitope-based Multimeric-001 (M-001) candidate vaccine is composed of nine conserved B and T cell epitopes from the HA, NP and M1 proteins to induce both B and T cell-specific immune responses. M-001 is not able to elicit HAI antibodies by itself, only when the administration of M-001 is followed by a boosting with seasonal or pandemic strain specific vaccines(Atsmon et al., 2014).

Previous studies have shown that primary exposure to the pandemic H1N1 strain in 2009 preferentially activated B cells specific to highly conserved epitopes on the HA-stalk and the HA-head regions(Andrews et al., 2015a; Krammer and Palese, 2013; Rambaut et al., 2008; Thomson et al., 2012; Wrammert et al., 2011). Stalk-based vaccine approaches have been developed, given the conserved nature of the stalk region. These include immunization with 'headless' HA stalk-fragment immunogen (Impagliazzo et al., 2015; Mallajosyula et al., 2014; Valkenburg et al., 2016; Yassine et al., 2015), stem nanoparticles(Boyoglu-Barnum et al., 2020; Corbett et al., 2019), hyperglycosylated HA head domain(Eggink et al., 2014), as well as sequential immunization with influenza virus-expressed chimeric HA proteins(Andrews et al., 2015b; Bernstein et al., 2020; De Jong et al., 2020; Krammer et al., 2018; Krammer and Palese, 2013; Nachbagauer et al., 2017; Pica and Palese, 2013; Steel et al., 2010).

1.5 Aims and significance

This study has focused on a universal influenza virus vaccine clinical trial, a vaccine that utilizes influenza viruses expressing chimeric HA proteins. Previous studies have shown that progressive immunization of mice and ferrets with this chimeric HA construct directs humoral immunity towards the stalk domain(Krammer and Palese, 2013). Based on these pre-clinical findings, a chimeric HA vaccine strategy progressed into a phase 1 clinical trial. This trial vaccine consisted of an influenza virus expressing a chimeric HA with a 2009 pandemic H1 stalk domain and an exotic avian HA head domain (chimeric H8-head/H1-stalk, or cH8/1) followed by a boost with a different chimeric HA protein (chimeric H5-head/H1-stalk, or cH5/1). Given the phylogenetic distance between the two types of influenza A virus HAs, group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15) and known conservation of HA stalk epitopes(Corti et al., 2011; Dreyfus et al., 2012; Ekiert et al., 2009), the HA stalk-specific antibodies based on the chimeric vaccine are intended to induce broadly cross-reactive antibodies targeting the stalk regions of all of the haemagglutinins in group 1, with the potential to cross-react with the group 2 Has (Figure 1) . The interim results from this trial demonstrated that the vaccine-activated B cells targeting the HA stalk domain induced stalk cross-reactive serum IgG antibodies, as well as peripheral blood plasmablast responses(Bernstein et al., 2020).

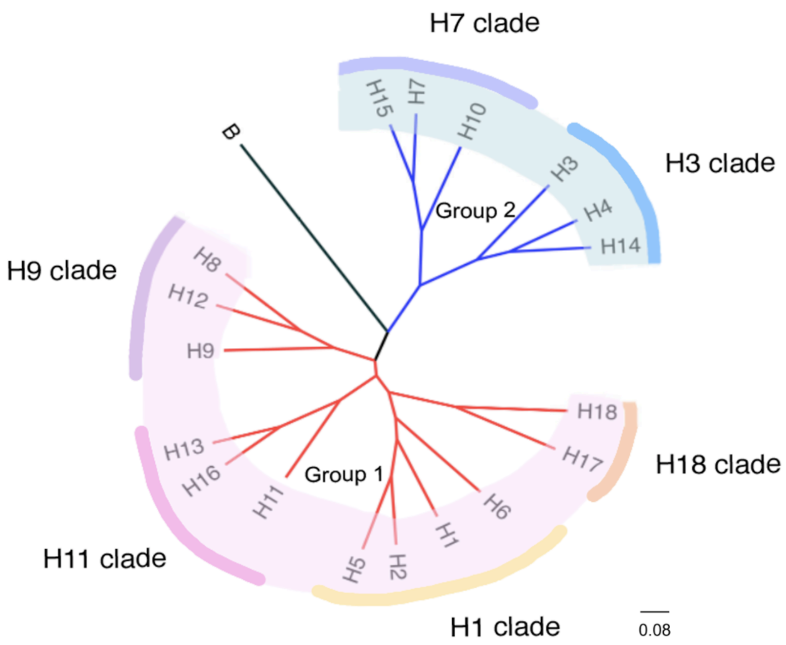


Figure 1-2 Influenza A and B virus HA phylogenetic tree.
The reactivity breadth of the mAbs collected in this trial crosses groups 1 and 2 of influenza A virus HAs. The tree was built using amino acid sequences in Mega and visualized in FigTree. The scale bar represents an 8% change in amino acid identity.

The main question at hand is whether the human immune response to influenza viruses can be re-programmed to target universally conserved stalk epitopes, and whether it can be fixed into the memory B cell pool. In addition, although memory B cell subsets have been characterized, it remains unclear whether the antigen-specific memory B cells' response to influenza immunization is also composed of distinct subsets that play unique functional roles in mediating protective immunity.

To enhance the understanding of B cell biology underlying broad protection from influenza, I present this study to analyze the HA-specific memory B cell repertoire and

transcriptional profile using single-cell RNA sequencing (scRNA-seq). I pursue the following two aims:

Aim 1: Investigate antigen-specific memory B cell differentiation in the context of the cHA-based universal influenza virus vaccine. For this aim, I characterize the memory B cell response to influenza following vaccination with the cHA-based universal influenza virus vaccine. I hypothesize distinct B cell subsets that play unique functional roles in mediating protective immunity.

Aim 2: Provide evidence that the cHA-based universal influenza vaccine induces HA stalk-reactive B cells. I hypothesize that the cHA-based universal influenza virus vaccine should elicit broadly neutralizing antibody responses targeting highly conserved epitopes shared by various influenza strains. Therefore, I will analyze the antigen-specific memory B cell repertoire and identify clonal expansion and differentiation. This will provide insight for the development of a universal influenza virus vaccine.

Moreover, we identify a distinct subset within the HA^+ memory B cell pool that possesses IgD^+ or IgM^+ isotypes with few mutations that likely represents a de novo response. We show that within the HA^+ memory B cell population, this transcriptional heterogeneity reflects previously unknown and functionally distinct memory subsets. Stalk-targeting memory B cells included clonal expansion across multiple individuals

(referred to as public clones) and germline-derived (unmutated) mAbs. These antibodies demonstrate neutralizing capability, and binding breadth to divergent influenza strains. A “Total-seq HA array” was used to validate the antibody specificities of antigen-specific memory B cells using barcoded HA tetramers, cite-seq antibodies, and hashtags followed by single cell RNA-seq. This approach allows simultaneous assessment of the BCR binding affinity/specificity, the whole transcriptome, as well as cell-surface phenotype from each single B cell. These findings should inform future studies aimed at improving understanding of antigen-specific memory B cell subsets and function, as well as future vaccine design efforts(Andrews et al., 2018a).

2 Memory B cell heterogeneity

2.1 Introduction

It is becoming more appreciated that heterogeneous MBC populations exhibit unique functional attributes including survival, response kinetics, and subsequent differentiation potential(Akkaya et al., 2020; Dogan et al., 2009; Harms Pritchard and Pepper, 2018; Kurosaki et al., 2015; Lau et al., 2017; Pauli et al., 2011; Shah et al., 2018; Weisel and Shlomchik, 2017). The highly unique nature of the universal vaccine trial and the robust sampling provide an ideal sample set to analyze human memory B cell heterogeneity and function. Although there is greater appreciation of heterogeneity and function within the memory B cell compartment, influenza-induced memory B cell heterogeneity has never been studied at the single-cell level. Thus, it is of great interest to investigate antigen-specific memory B cell subsets with distinct transcriptional signatures and their potential functions. Here, I investigate HA stalk-reactive memory B cell subsets in the context of the cHA-based universal virus vaccine trial, utilizing scRNA-seq. Analyses reveal previously unknown transcriptional heterogeneity within HA-specific memory B cell populations. Analyses reveal previously undefined HA-specific memory B cell subsets. These data reveled the potential function of memory B cell subsets for their ability to mediate humoral immune responses.

2.2 Results

2.2.1 HA stalk-reactive B cells were selected at day 28 post-boost vaccination

Participants in the phase I trial received a prime with virus expressing a cH8/1 followed by a boost with cH5/1. The vaccine recipients were divided into three groups of 15 subjects, including: prime with inactivated influenza vaccine (IIV) or live attenuated influenza virus vaccine (LAIV), followed by an IIV boost at 90 days, with or without AS03A adjuvant (Fig. 2.1 A). Peripheral blood mononuclear cells were collected at day 0 before vaccination, at day 28 post boost-vaccination, and at 1-year post boost. At 28 days after boost, HA-reactive memory B cells (CD19+IgMlowCD27+CD38low) were bait-sorted with a fluorescently labelled cH5/1 chimeric HA protein from 10 of the LAIV primes, IIV/AS03 boost subjects, 3 of the LAIV primes, IIV boost subjects, 6 of the IIV primes, and IIV/AS03 subjects (Figs. 2.1B-C, Table S1). The cells were then bulk sorted by flow cytometry and processed with the 10x genomics chromium platform. Both single-cell transcriptome and the antibody heavy/light chain pairs were identified. From the paired heavy/light chain sequencing, we were able to recover 2,228 variable genes pairs from 19 subjects.

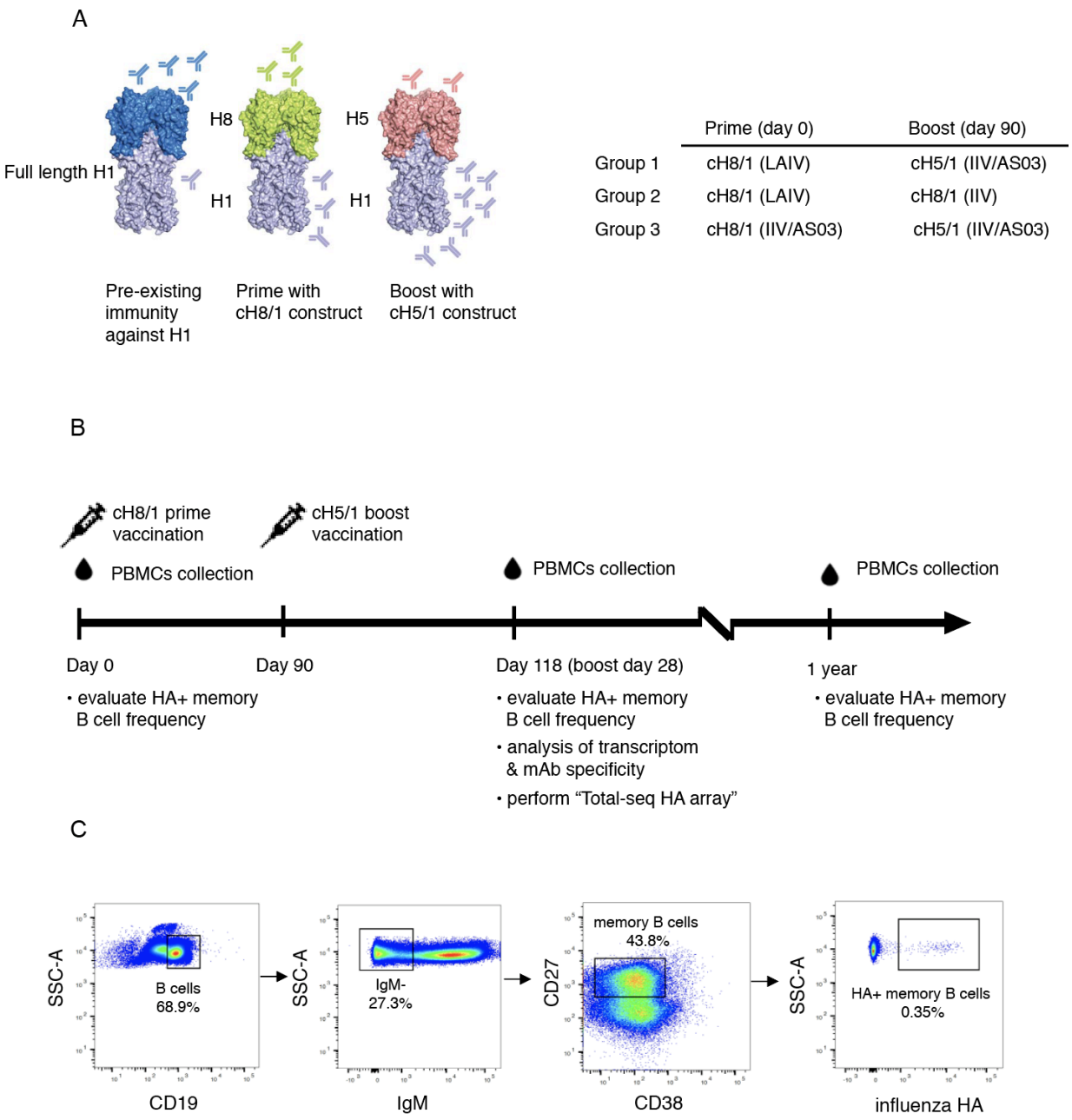


Figure 2-1 Overview of the universal influenza virus vaccine trial.
(A) The chimeric HAs are composed of a seasonal stalk domain (from a pH1 HA) paired with an exotic head domain from a strain to which humans are naive (avian H5 or H8). Through the sequential vaccination strategy, the immunosubdominant stalk-reactive antibodies were selectively recalled and boosted based on prior exposure to H1N1 infection/immunization. (B) Schematic of the sampling time point and analysis. (C) Representative FLOW plot after cH5/1 HA tetramer was used to bait the memory B cell population (CD19+IgMlowCD27+CD38lowHA+) at D0, D28 (boost), and 1 year post-vaccination from donor PBMCs.

Table 1 Information of subjects

#	subject	group	prime	boost	number of mAbs	number of cells
1	308	1	LAIV	IIV/AS03A	80	81
2	310	1	LAIV	IIV/AS03A	47	55
3	317	1	LAIV	IIV/AS03A	101	112
4	319	1	LAIV	IIV/AS03A	356	428
5	327	1	LAIV	IIV/AS03A	34	45
6	334	1	LAIV	IIV/AS03A	128	137
7	336	1	LAIV	IIV/AS03A	68	84
8	337	1	LAIV	IIV/AS03A	125	148
9	347	1	LAIV	IIV/AS03A	316	422
10	350	1	LAIV	IIV/AS03A	305	400
11	326	2	LAIV	IIV	33	29
12	342	2	LAIV	IIV	19	29
13	346	2	LAIV	IIV	43	71
14	301	3	IIV/AS03A	IIV/AS03A	239	355
15	311	3	IIV/AS03A	IIV/AS03A	22	38
16	322	3	IIV/AS03A	IIV/AS03A	219	195
17	324	3	IIV/AS03A	IIV/AS03A	169	196
18	343	3	IIV/AS03A	IIV/AS03A	34	43
19	351	3	IIV/AS03A	IIV/AS03A	113	155

I also analyzed the frequency of chimeric HA+ memory B cells, and found that the fcH5/1+ memory B cells' frequency increased after the day 28 post-boost, compared to day 0. This increase persisted over the next year (Fig. 2.2 A). CH5/1+ memory B cells (CD19+IgMlowCD27+CD38low) were then sorted and partitioned into nanoliter-scale Gel Bead-In-EMulsions (GEMs) to achieve single cell resolution, according to the manufacturer's instructions (10X Genomics). This was done to evaluate whether there was an expansion in HA-reactive cells in the memory B cell compartment at day 28 after the boost, compared to day 0 and 1-year time points. The sorted single cells were processed according to 5' gene expression and B cell immunoglobulin (Ig) enrichment instructions to prepare the libraries (10X Genomics) (Fig 2.2 B). The Cell Ranger

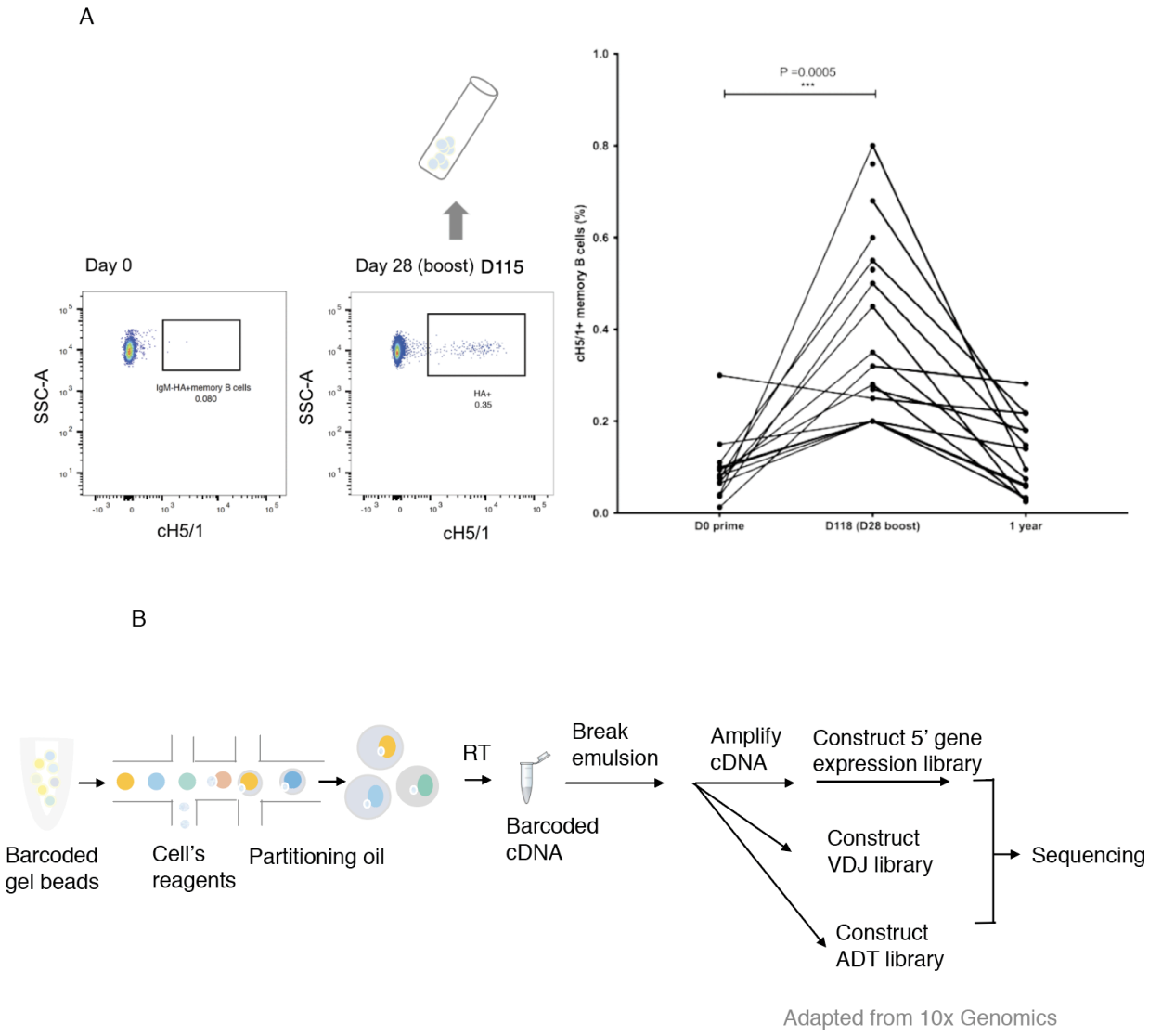


Figure 2-2 Chimeric H5/1+ memory B cells are enriched at D28 boost from the universal influenza virus vaccine trial.
(A) The frequency of CD19^{+}IgM^{low}CD27^{+}CD38^{low} cH5/1^{+} memory B cells significantly increased in size after the day 28 post-boost compare to day 0 and 1year time points. (B) scRNA-seq workflow on 10x Genomic platform.

Single-Cell Software Suite (version 3.1) 62 was used to perform sample de-multiplexing, barcode processing, and to generate a single-cell 5' gene expression matrix and assemble V(D)J pairs of the antibodies. The Seurat analysis tool (v3) was

used to further analyze the 5' gene expression dataset for quality control (QC), integrated analysis, and exploration (Fig. 2.3 A).

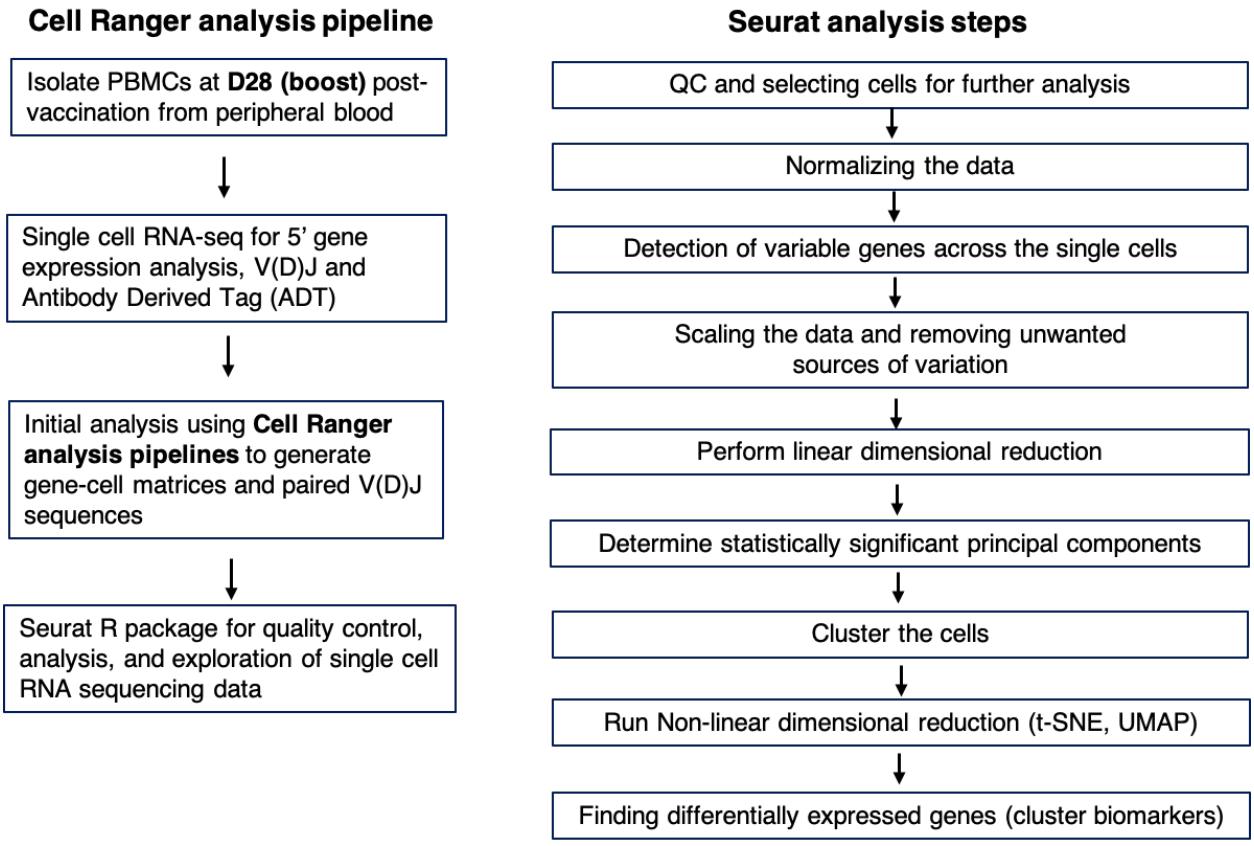


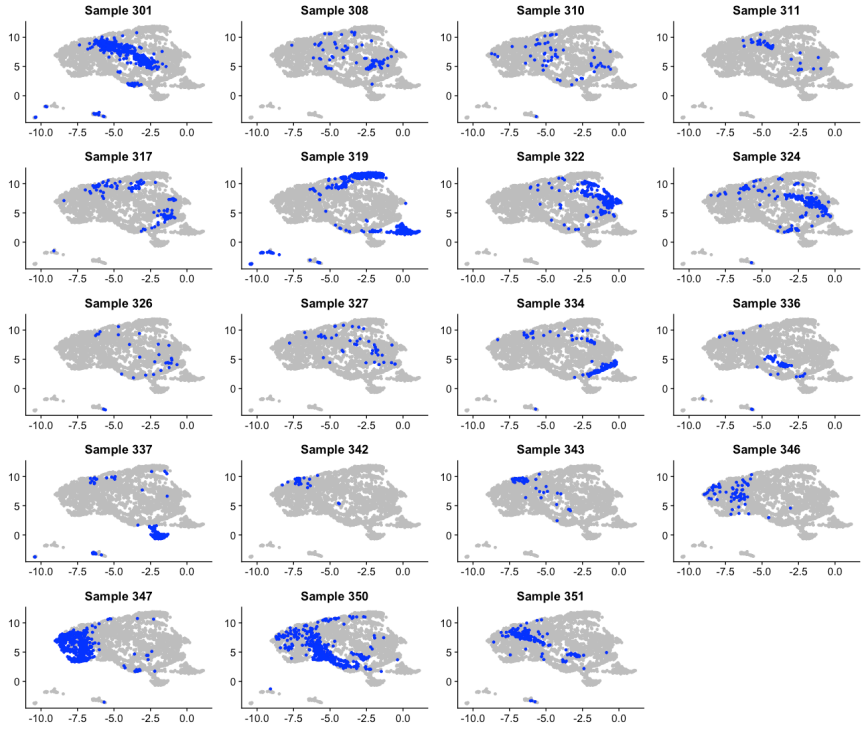
Figure 2-3 Single cell RNA-seq analysis workflow.
(A) Gene-barcode matrix and paired BCR sequences are outputs of the Cell Ranger pipeline. The Seurat analysis pipeline is used for further single-cell transcriptional profiles analyses.

As the memory B cell pool is heterogeneous, it varies in B cell specificity, states, and location(Lam and Baumgarth, 2019). The single-cell RNA sequencing (scRNA-seq) advanced the understanding of various memory B cell subsets by measuring the genes' expression levels in each cell. However, this can be sensitive to 'unwanted' sources of variation. This could include technical noise, batch effects, or even biological sources of

variation such as cycling and dying cells. To regress these signals out of the analysis can improve downstream dimensionality reduction and clustering(Buettner et al., 2015; Stubbington et al., 2017; Svensson et al., 2017).

Recent advances, such as droplet-based protocols combined with cell hashing, enable multiplexed samples in each batch. However, batch effects remain a challenge to data integration. They have been identified as masking biological signals in the integrated analysis, as samples prepared in a single batch may demonstrate confounding biological variation rather than identical cell types from batches(Niebler et al., 2020; Tran et al., 2020). Therefore, I first assessed whether there were any batch effects which could have originated from different sorting dates, library preparation, sequencing, or flow-cells. The batch effects can cause the data to artificially cluster by batch, thus overshadowing the true biological signals. Therefore, I integrated HA+ memory B cells sorted from each subject's cells and determined whether there were batch effects. The subject-specific batch is clearly visible from the plots; most of the cells from the same subject tended to group together and were distinct from the cells coming from other subjects (Figs. 2-4A). After correcting the batch effect with the Seurat Canonical Correlation Analysis (CCA) technique(Butler et al., 2018), the data from each subject were projected into a subspace by maximizing covariance between the subjects' separated datasets. Then, the dataset was aligned for downstream integrated analysis. After applying the batch effects correction, cells from the datasets of different individuals revealed homogenous distribution.

A



B

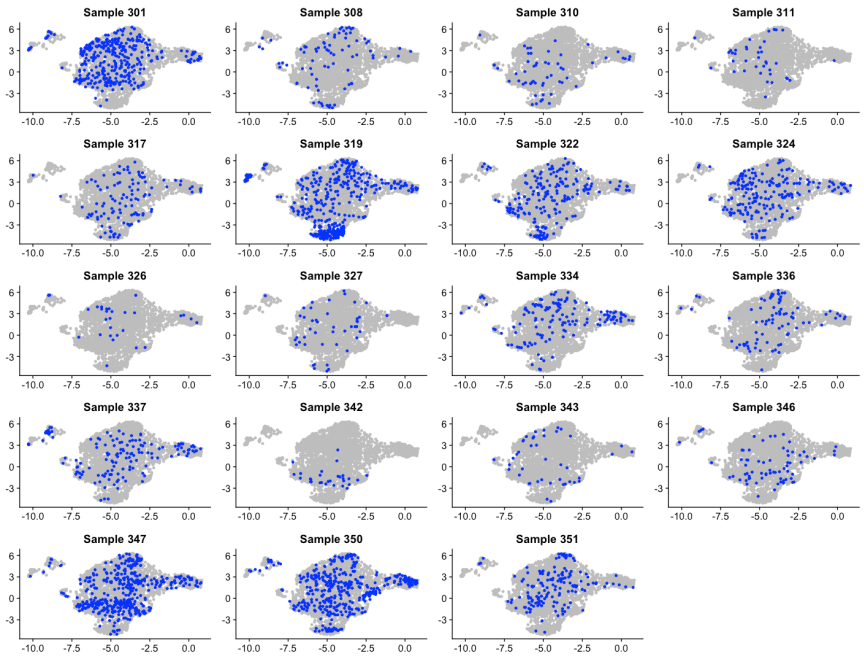
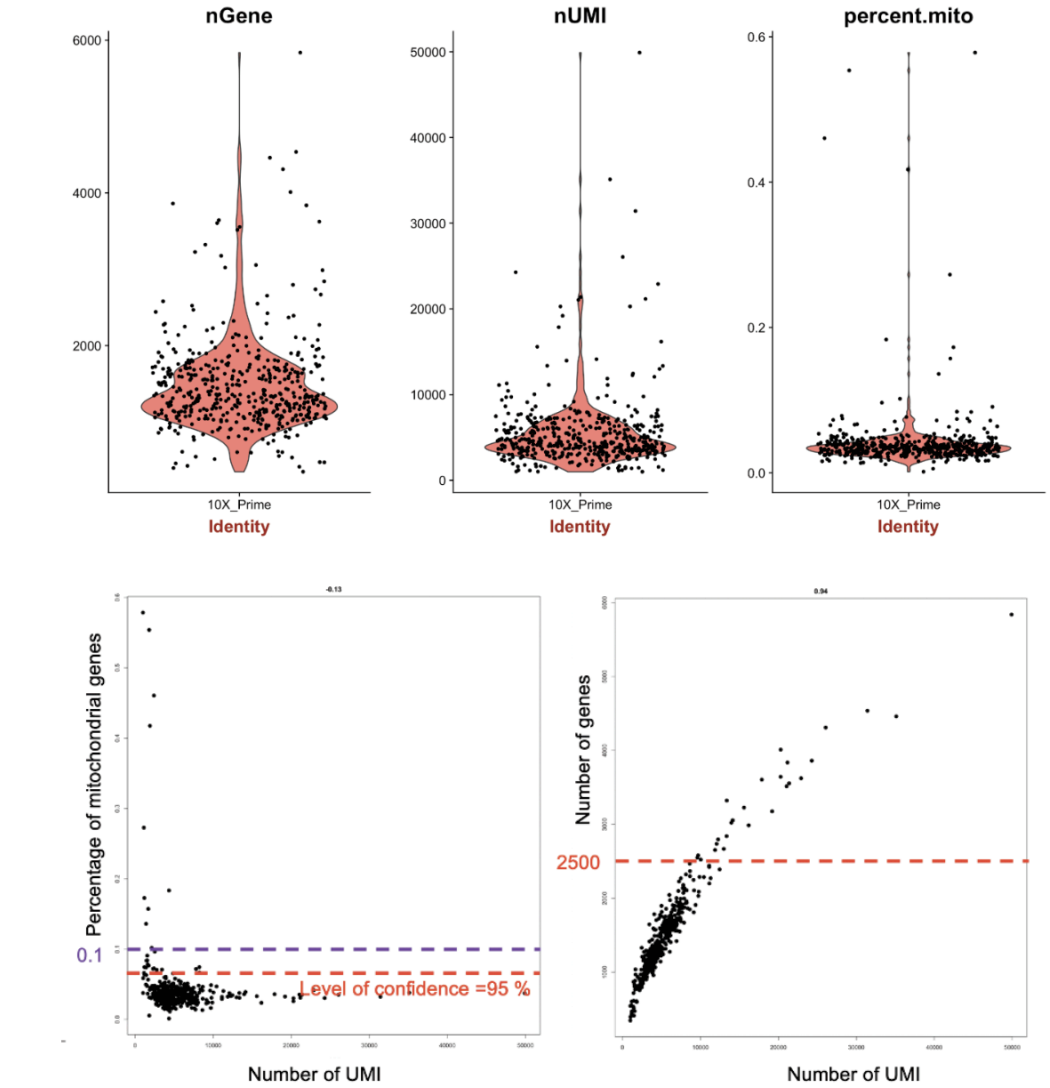


Figure 2-4 Batch effect identification and correction.
(A) UMAP projection of data without batch correction labeled by subject ID shows a strong experimental batch effect. (B) The UMAP plot labeled by subject ID after Seurat integration is used to remove the variability associated with different subjects. The corrected data set revealed homogenous distribution across 19 subjects.

Quality control was performed with the Seurat package (version 3.0)(Butler et al., 2018; Stuart et al., 2019) to further analyze differential gene expression for integrated data. Cells with detected gene counts either greater than 2,500 or less than 200 were excluded. The gene and molecular counts (nUMI) were visualized and their relationships were plotted to exclude any clear gene count outliers, which would have indicated potential multiplets. Cells were also excluded based on the percentage of mitochondrial genes. This is because low-quality, cycling or dying cells often exhibit extensive mitochondrial contamination. Cells were excluded as potential multiplets or cycling cells if the mitochondrial fraction was outside of the 95% confidence interval, or above 0.1% of all detected genes. Expression measurements for each cell were normalized by total expression, and then scaled to 10,000 after log normalization. After detecting variable genes across the single cells and removing unwanted sources (e.g. technical noise, batch effects, and biological sources of variation such as cell cycle stage), statistically significant principal components were determined via linear dimensional reduction (PCA). Cell populations were then predicted using Uniform Manifold Approximation and Projection (UMAP). This preserves much of the local, and more of the global data structure, thus providing more meaningful cell clusters than t-Distributed Stochastic Neighbor Embedding (t-SNE)(Becht et al., 2019).



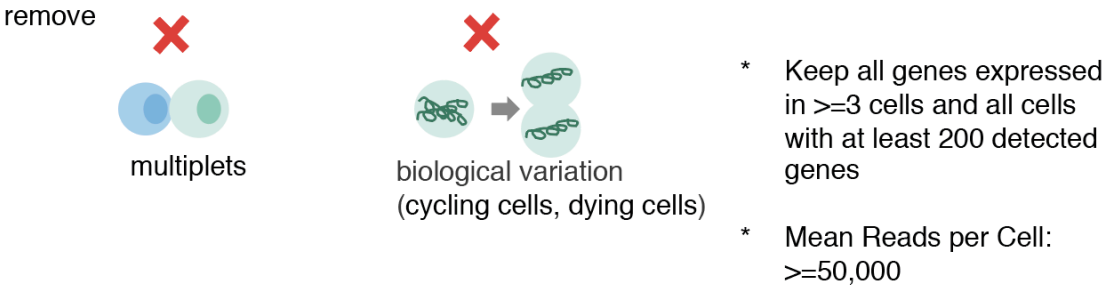
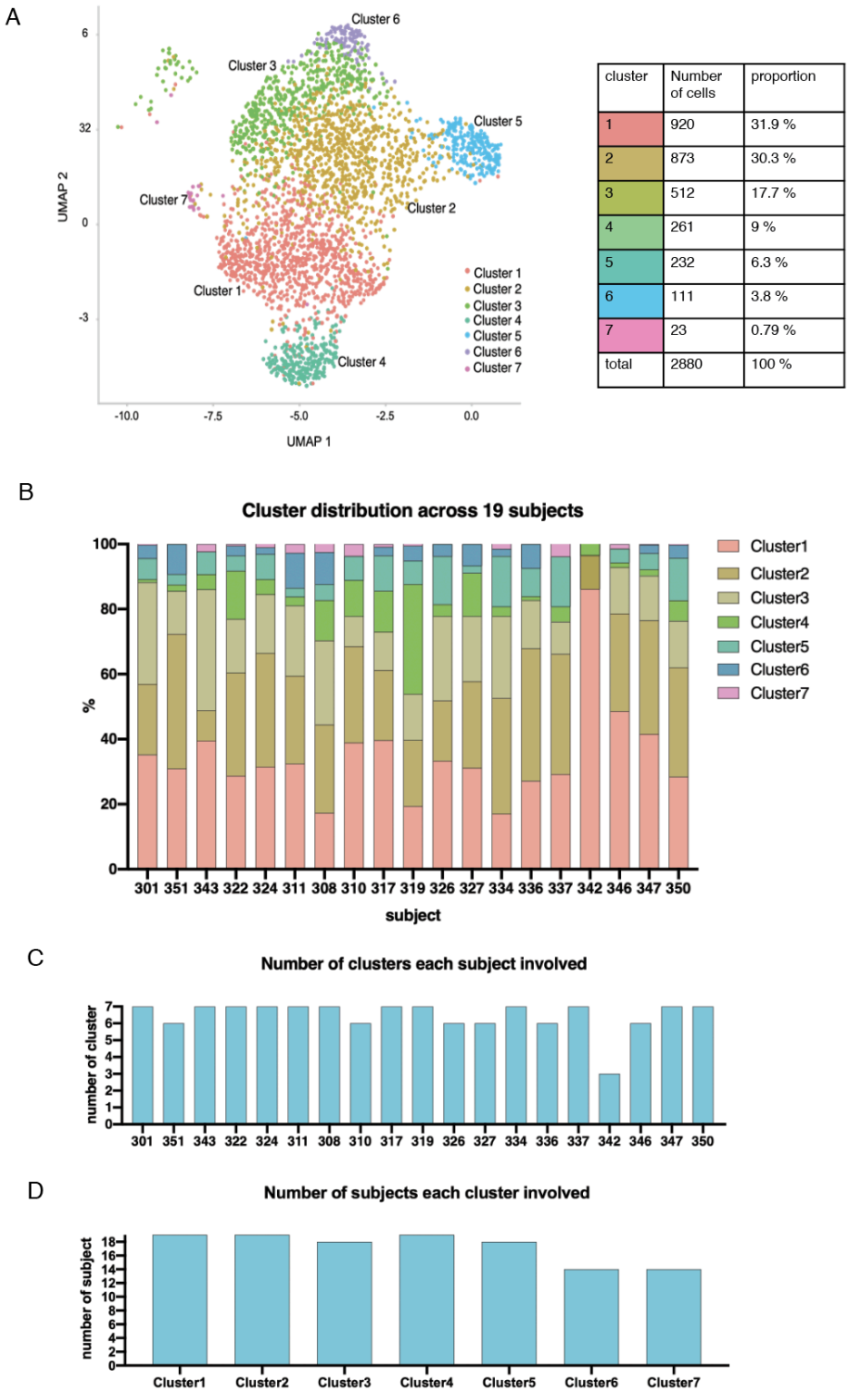


Figure 2-5 QC and filtering out "unwanted" cells.
Workflow of QC and cell filtration criteria using Seurat scRAN-seq analysis pipeline to remove potential multiplets, cycling cells, dying cells and low quality cells.

2.2.2 Distinct memory B cell subsets arise in response to the chimeric HA-based universal influenza virus vaccine

To assess the function of MBC subsets post immunization, we sorted cH5/1+ memory B cells at day 28 post boost. From the single-cell transcriptome information obtained with the 10X genomics platform, we integrated the data from 19 subjects and performed 2-dimensioanl clustering for transcriptional similarity such as unsupervised uniform manifold approximation and projection (UMAP) analysis(Stuart et al., 2019). Based on their transcriptional profile, single HA+ memory B cells segregated into 7 clusters at day 28 post-boost (Figure 2-6A). The HA-specific memory B cell clusters were observed across all individuals in varying proportions suggesting that distinct subsets exist within the circulating chimeric HA-specific memory population (Figure 2-6B-D).



cluster	Number of cells	proportion
1	920	31.9 %
2	873	30.3 %
3	512	17.7 %
4	261	9 %
5	232	6.3 %
6	111	3.8 %
7	23	0.79 %
total	2880	100 %

Figure 2-6 Single-cell RNA-seq analysis of circulating antigen-specific memory B cells responding to chimeric HA-stalk vaccination.

Figure 2-6, continued.
(A) UMAP visualization of 7 clusters of the antigen-specific memory pool after universal influenza virus vaccination (n=3,133 cells). The naïve-like population results in 9% of all chimeric HA+ memory B cells in this trial. (B) Cluster distribution across 19 subjects. (C) Analyses of number of clusters each subject involved (D) Number of subjects each cluster contains.

Most of the B cells from cluster 4 were IgD^+ and IgM^+ B cells with low but detectable frequencies of somatic mutations. These cells also expressed CD27 (Figure 2-2A), a cell surface marker that distinguishes memory from naïve IgM^+ B cells in humans(Shah et al., 2018; Weisel et al., 2016). This B cell cluster highly expressed B cell activation-associated genes (such as IL4R(Ferrer et al., 2014)) and proliferation-associated genes including the *BACH2* gene, which is a Blimp-1 repressor that is required for B cell proliferation in response to BCR signaling and for the formation of germinal center (GC) reactions(Miura et al., 2018). Thus, we defined this cluster as “naïve-like” memory B cells. This naïve-like memory B cell subset also used a more diverse heavy chain V locus repertoire with reduced usage of VH1-69/18 heavy chains, suggestive of less selection to the HA stalk than the other clusters, which contained class-switched B cells with higher somatic mutations and common use of VH1-69/18 (Figure 2-7A, 2-8). The naïve-like memory population was present in 18 of the 19 subjects analyzed and resulted in 9 % of all chimeric HA^+ memory B cells (Figure 2-6A). The low mutation frequency, more diverse repertoire, and gene expression tendencies suggest that these cells were recently activated and that the chimeric HA-based vaccine induced *de novo* B cell responses after immunization. All other clusters expressed highly mutated Ig variable genes that were mostly class switched away from IgM/D. As these cell clusters also had increased VH1-

69/18 usage it appears that a large fraction was either expanded from or consisted of pre-existing HA-stalk reactive memory B cells.

Transcripts for the chemokine receptor CXCR4 were found to be upregulated in clusters 1, 2, 3, 4, and 7. CXCR4 is a chemotaxis receptor that responds to CXCL12 and plays an essential role in both recruiting germinal center B cells into the dark zone of germinal center(Allen et al., 2004; Stein and Nombela-Arrieta, 2005) and in establishing the bone marrow micro-niche for long-lived antibody secreting cells(Khodadadi et al., 2019). The *FCRL5* gene is upregulated in cluster 5 and is involved in regulating B cell activation and is most often associated with atypical B cell subsets(Franco et al., 2018; Kim et al., 2019; Lau et al., 2017; Li et al., 2016; Sullivan et al., 2015). The gene for integrin *ITGB7* is also upregulated in cluster 5 and in cluster 6. ITGB7 can heterodimerize with integrin $\alpha 4$ to make $\alpha 4\beta 7$, which together with the CCR9/CCL25 interaction, strengthen mucosal homing signals(Cyster and Allen, 2019). Thus, we proposed that these two subsets may represent “tissue-like memory B cells” (Figure 2-7B). In total, the expression of different transcriptional profiles in conjunction with unique Ig-repertoire features show that distinct chimeric-HA reactive memory B cells subsets are detectable after administration of the experimental vaccines.

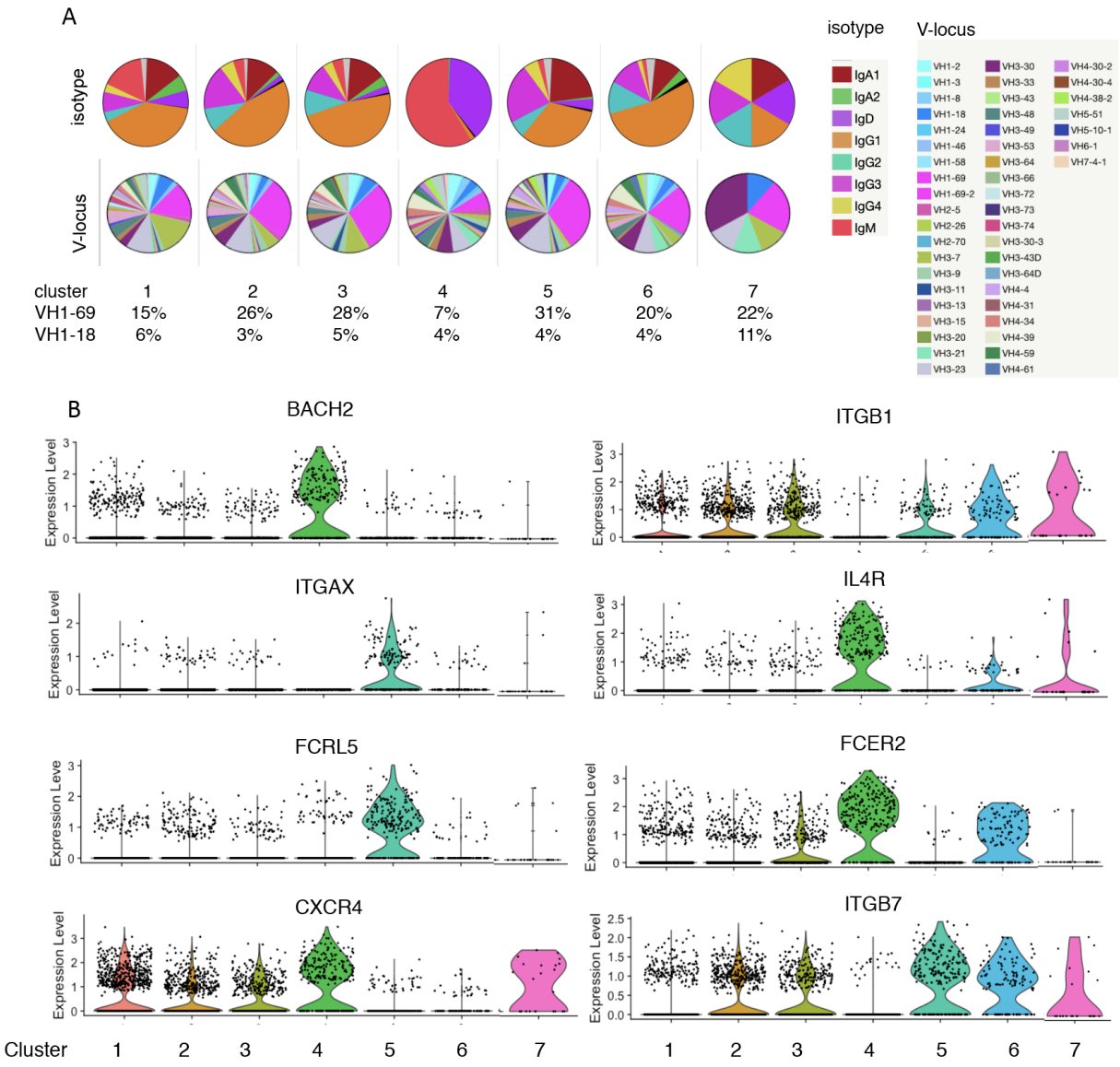


Figure 2-7 Single-cell RNA-seq analysis reveals 7 distinct subsets of circulating antigen-specific memory B cells responding to chimeric HA-stalk vaccination.
(A) Analysis of the antibody variable genes showed that while cluster 4 had few or no somatic hypermutations, other clusters were highly mutated. This is similar to naïve B cells, although express CD27+, cluster 4 consisted of most of the IgD+ and IgM+ B cells (B) Each antigen-specific memory B cell subset has a distinct transcriptional signature. Violin plots were constructed using data obtained from 3,133 sampled cells from 19 subjects. The differential gene expression identified from each population was visualized by testing the p-values between subsets with normalized expression levels.

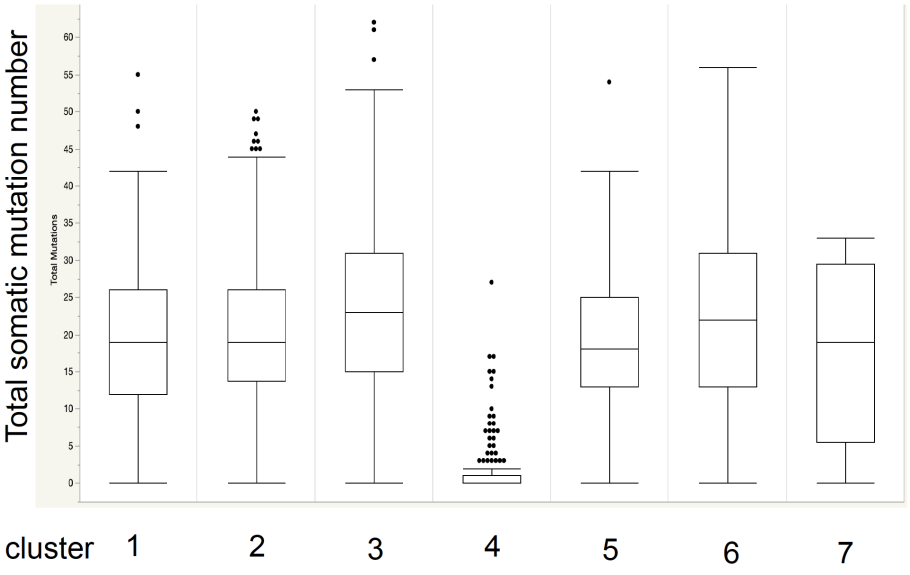


Figure 2-8 Analysis of the antibody variable genes.
Cluster 4 had few or no somatic hypermutations, other clusters were highly mutated. This is similar to naïve B cells, although express $CD27^{+}$, cluster 4 consisted of most of the IgD+ and IgM+ B cells (the mutation rate analysis for each cluster).

2.3 Discussion

The analyses revealed transcriptional heterogeneity within the HA stalk-reactive memory B cell population in response to this novel universal influenza virus vaccine candidate. This observation suggests that this vaccine elicits functionally distinct antigen-specific memory B cell subsets. Importantly, we provide evidence that the cHA-based universal influenza virus vaccine drives an increased frequency of HA stalk-reactive memory B cells even at a year out after vaccination. The detection of B cell clones expressing neutralizing antibodies that are shared by multiple subjects (public clones) demonstrates that a commonality of response can be elicited by this vaccine, improving predicted protection for a larger fraction of people. Finally, we note that a subset of B cells is detectable after vaccination that express IgD/M isotype antibodies and with low variable gene mutation rates but that otherwise have memory phenotypes and preferentially target HA stalk epitopes. The presence of this subset after vaccination provides evidence that the primer-boost series with chimeric HA antigens, each with distinct globular heads but shared stalk regions, can drive *de novo* HA-stalk responses even in influenza-naïve individuals such as infants or young children.

The scRNA-seq data revealed 7 distinct subsets of HA+ memory B cell responses to vaccination. For example, we observed that naïve-like HA+ memory B cells with low somatic mutations exhibited increased levels of transcripts associated with activation and proliferation. This implies that the vaccine may induce a *de novo* response. Moreover, we observed two distinct subsets of “tissue-like memory B cells” that exhibited sustained

expression of genes encoding a homing receptor for migration to gut-associated lymphoid tissues (GALTs) such as *Itgb7*, consistent with prior reports(Gorfu et al., 2010). The *FCRL5* in the “tissue-like memory B cell” subset was upregulated even at day 28 post immunization. This suggests that this subset may be transcriptionally poised for rapid response to BCR stimulation. This analysis has revealed previously unknown transcriptional heterogeneity within HA+ specific memory B cell populations post vaccination. This represents functionally distinct antigen-specific memory B cell subsets, and two antigen-specific tissue-like memory B cell subsets in circulation. Further investigation into specific memory B cell subsets that mirror broad cross-reactivity and neutralization, and how the transcriptional networks that shape these phenotypes vary, will be important for future vaccine design efforts in which one subset is desired over another. The B cell and antibody responses to influenza viruses, in terms of the subsets involved, may also correlate with binding breadth.

3 Establish a "Total-seq HA array" to characterize B cell specificity

3.1 Introduction

In order to comprehensively characterize the B cell response to influenza following vaccination with a universal influenza virus vaccine candidate, I want to establish a "Total-seq HA array" in this context to investigate the antibody specificities of antigen-specific memory B cells. The barcoded HA tetramers, cite-seq antibodies and hashtags are utilized to bait and stain memory B cells, followed by single cell RNA-seq (using 10x genomic platform). With this method, the BCRs' binding affinity/specificity, and each cell's transcriptome as well as full length of immunoglobulin (Ig) gene sequence, are identified. Furthermore, these monoclonal antibodies can be generated from the sequenced Ig genes of the same B cells.

3.2 Results

3.2.1 Increased HA-stalk-reactive B cells at day28 boost in the IIV/AS03-IIV/AS03 universal influenza virus vaccine cohort to the seasonal influenza vaccine cohort

To determine whether a cHA-based vaccine drives a more stalk-reactive memory B cell response than seasonal vaccination, I used two different HA-probe based cytometry assays. With some variability in sampling available by subject, peripheral blood mononuclear cells that were bio-banked were analyzed from day 0 before vaccination, day 28 post boost-vaccination, and at 1 year after vaccination. At 28 days post boost, HA-reactive memory B cells ($CD19^{+}IgM^{low}CD27^{+}CD38^{low}$) were

baited/sorted with a fluorescently labelled chimeric HA protein consisting of the H5-head and the H1-stalk (data shown in Figure 2-2A). This analysis demonstrated a significant peak in cHA (HA-stalk) reactive memory B cells at both peak of the boost response (D28-post) and that persisted at increased levels over the next year. To further characterize frequencies and cross-reactivity between HA types, I used a novel assay termed “Total-seq HA array” to investigate the antigen-specific memory B cells’ antibody specificities. For this, a panel of recombinant HA antigens were generated using streptavidin as tetramers that were fluorescently labelled with PE and each oligonucleotide-barcoded to generate probes that could be uniquely identified for binding to each single B cell after 10x Genomics drop-seq processing and Illumina sequencing, similar to the recently published LIBRA-seq method(Setliff et al., 2019). The HA array consisted of cH5/1, cH8/1, a recent H3 strain HA, plus divergent avian H9 HA (group 1) and H7 strains (group 2). These probes were then utilized to stain and bait/sort all HA-array+ memory B cells, followed by single cell RNA-seq. This method identifies the BCRs’ binding affinity/specificity and each cell’s transcriptome and immunoglobulin gene expression (Figure 3-1A).

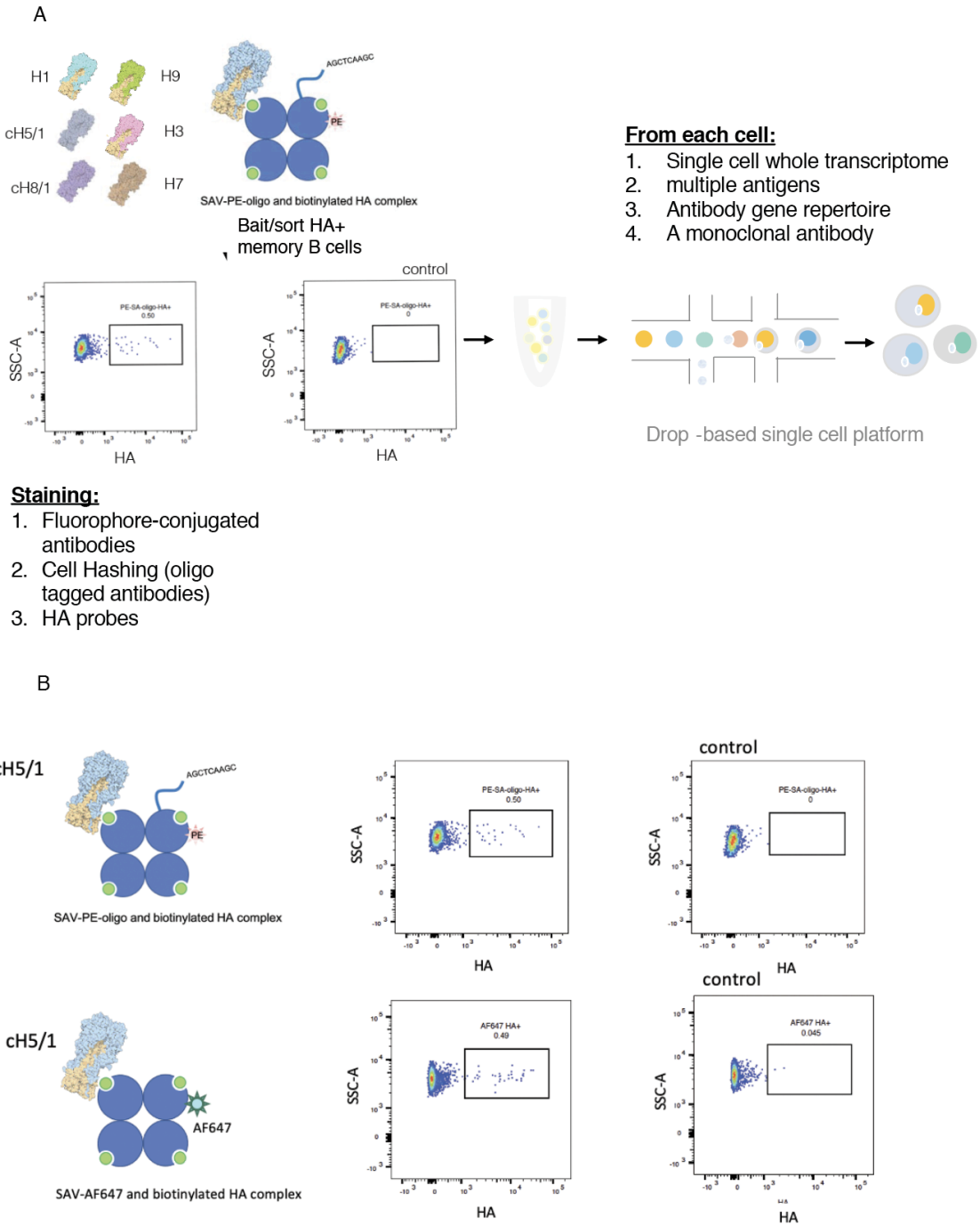


Figure 3-1 Establishing the "Total-Seq HA Arrays" to isolate cross-reactive HA-specific B cells.

(A) "Total-Seq HA Arrays" consisted of cH5/1, cH8/1, H3 strain HAs, H9 and H7 HAs as barcoded probes followed by single cell RNA-seq, allowing for simultaneous quantification of B cell binding to HA antigens across strains, as well as transcriptomic/V(D)J profiling of the same single cells. With the combination of hashtag staining, each cell can be distinguished by unique barcodes.

To decrease experiment costs, identify batch effects, and remove the multiplets, the “Cell hashing technique” was applied in the “total-seq HA array” for multiplexing. Cell hashing is a method that uses oligo-tagged β2M and CD298 (Na+/K+ ATPase) antibodies that contain a unique 12-bp barcode, both of which are ubiquitously expressed in surface proteins(Stoeckius et al., 2018). These oligo-tagged antibodies are used to stain/sort HA+ memory B cells from distinct subjects. HA+ memory B cells from various subjects were subsequently pooled and loaded into the droplet-based systems. Each cell was connected to its original subjects via a unique hashtag oligos (HTOs) by sequencing these tags alongside the cellular transcriptome (Fig. 3-2).

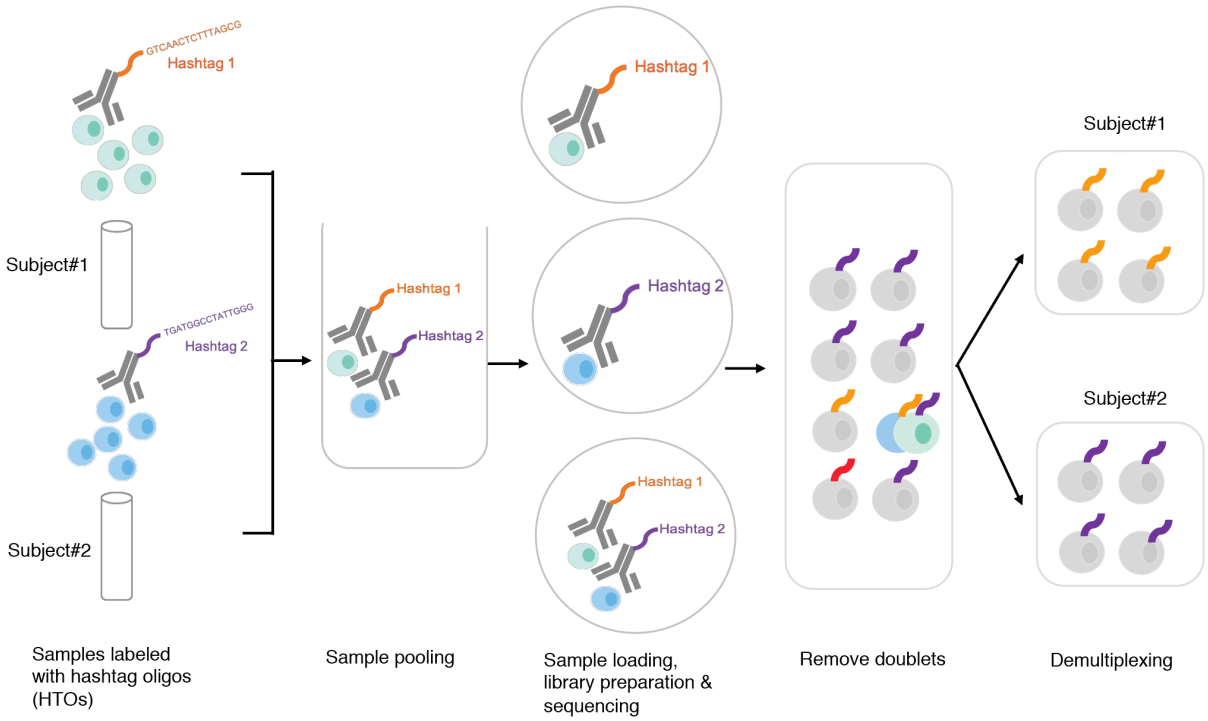


Figure 3-2 Schematic of sample multiplexing by hashtag oligos (HTOs). HA+ memory B cells from distinct subjects are stained by oligo-tagged antibodies, and recognized via the ubiquitous cell surface proteins β2M and CD298. Subjects' cells are identified after de-multiplexing distinct barcodes. This approach allows for the pooling of sorted HA+ memory B cells from multiple subjects into one scRNA-seq experiment. After partitioning to single cells via chromium controller and sequencing along with transcriptome, cells can be connected with their original subjects based on the barcodes.

This approach not only allows for a decrease in per-cell library preparation costs, but also provides the benefit of removing cell multiplets and identifying batch effects. Cells are removed if they are positive for multiple HTOs, as annotated as doublets or multiplets (Fig. 3-2B). In addition to only keeping singlet cells, HTO also allows for the examination of the presence of batch effects. This is done by clustering and visualizing cells using the standard scRNA-seq workflow(Stoeckius et al., 2018).

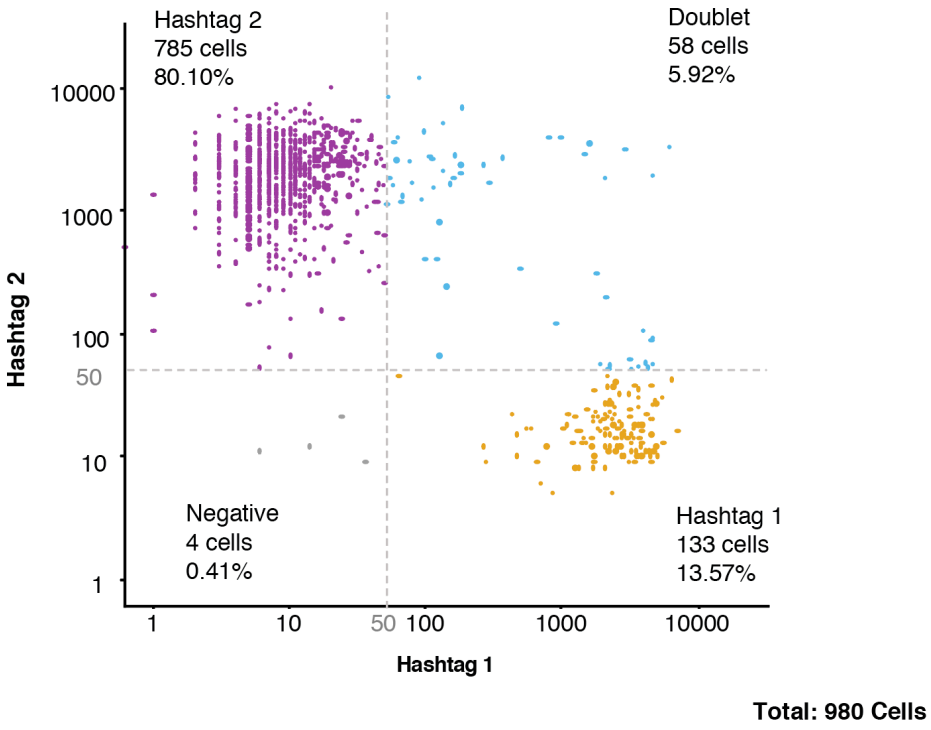


Figure 3-3 Subjects' cells are multiplexed using antibodies with hashtag oligos (HTOs).
Representative scatter plot showing raw counts for Hashtag 1 and Hashtag 2. 5.92% of the cells were excluded, as they were positive for both hashtags, indicating that they were doublets.

Predictably, B cells binding cH5/1 and cH8/1 also bind to H1 from the 2009 H1N1 pandemic influenza strain, but rarely overlap with the H7 strain. Consistent with this notion, more HA stalk-reactive B cells and B cells targeting H1, cH5/1, and cH8/1 were induced by cHA-based vaccination than by the seasonal vaccine. (Figure 3-4).

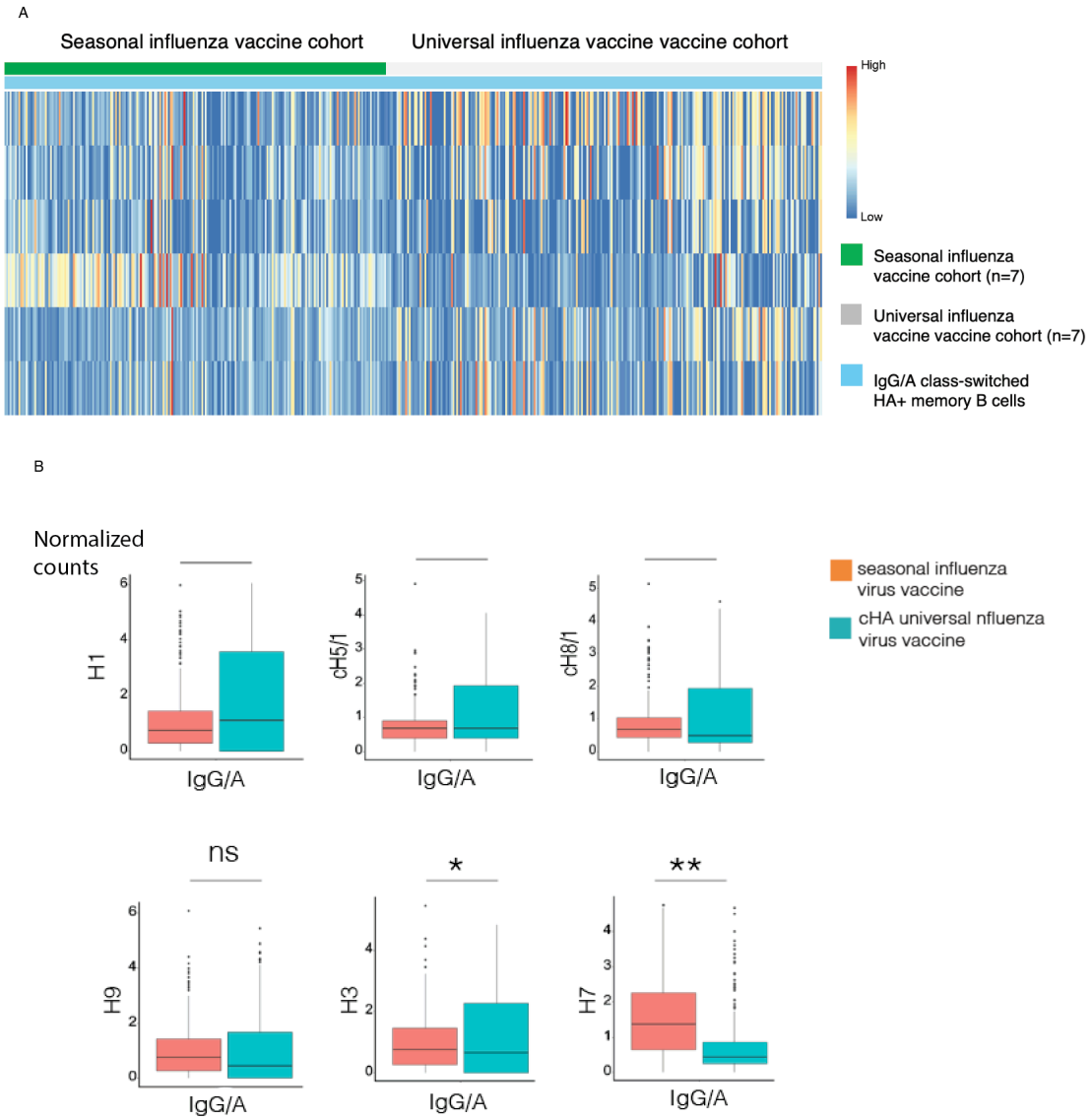


Figure 3-4 cH5/1 specific memory B cells following chimeric HA-stalk vaccination and seasonal vaccine immunization.

(A) Heat map of the "Total-Seq HA Arrays" probe analysis for IgG/A class-switched antigen-specific memory B cells. Subjects are from the seasonal influenza virus vaccine immunization (2018-2019, 2019-2020, quadrivalent influenza virus vaccine, QIV) (n=6) and universal influenza virus vaccine immunization groups (n=7). The heat map shows normalized barcode readout indicated binding intensity. Color corresponds to normalized counts that is computed from log10. (B) Comparison of stalk-reactive class switched (IgG/A) memory B cells of the IIV8/AS03-IIV5/AS03 group and current seasonal vaccination (2018-19, 2019-2020). The Y-axis is the normalized probe binding count; it indicates binding intensity ($P<0.05$ ***, $P<0.01$ ****).

To note the specificity of the assay for staining stalk-reactive B cells, we noted that the CH5/1 probe preferentially bound to stereotypical VH1-69 and VH1-18 B cells, particularly after chimeric vaccine immunization (Figure 3-4A). Additionally, as expected, the isotype-switched memory B cells showed higher binding affinity by this assay than the non-isotype switched IgM/D memory B cells, further demonstrating the quantitative nature of this assay (Figure 3-4B).

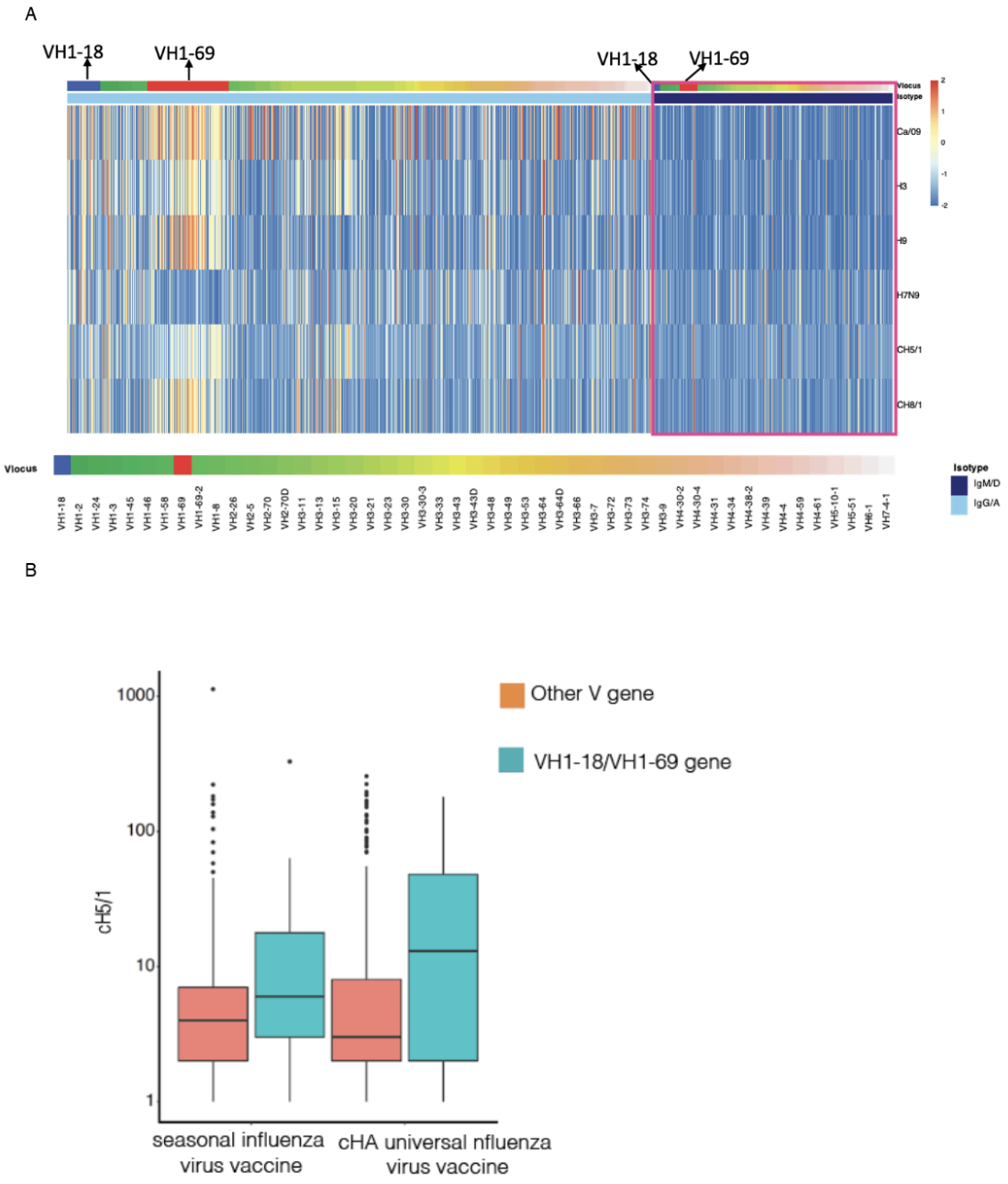
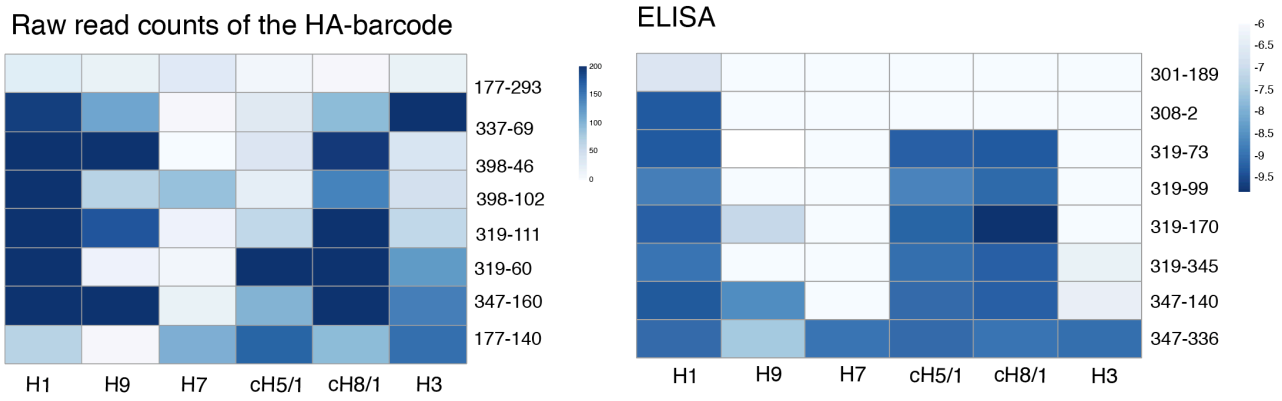


Figure 3-5 Binding specificity and intensity of HA reactive memory B cells by "Total-seq HA array".

(A) Raw HA probe counts for each subjects from seasonal vaccine cohort (n=6) and the universal influenza virus vaccine cohort (n=7) (Table S2). (B) CH5/1 probes preferentially bind to VH1-69+ B cells, and this is even more evident after chimeric vaccine immunization.

Further, by generating mAbs from the same B cell clonotypes that were bait-sorted with fluorescent cH5/1 we could show similar binding-specificity to 6 HA proteins: H1, H9, H7, cH5/1, cH8/1 and H3 by ELISA (Figure 3-5). This analysis demonstrated enhanced binding breath after chimeric HA vaccination and specificity toward H1, cHAs (cH5/1 and cH8/1), H3 and H9 as compared to seasonal vaccination (Table 2).



Same B cell clones: 177-293 & 301-189; 337-69 & 308-2; 398-46 & 319-73; 398-102 & 319-99
319-111 & 319-170; 319-60 & 319-345; 347-160 & 347-140; 177-140 & 347-336

Figure 3-6 Same clone of B cells are expressed and validated the binding specificity by ELISA.

Table 2 Information of subject for Total-seq HA array.

universal influenza virus vaccine cohort	prime	boost	sampling time
301	IIV/AS03A	IIV/AS03A	Day 28 post boost
311	IIV/AS03A	IIV/AS03A	Day 28 post boost
322	IIV/AS03A	IIV/AS03A	Day 28 post boost
324	IIV/AS03A	IIV/AS03A	Day 28 post boost
325	IIV/AS03A	IIV/AS03A	Day 28 post boost
343	IIV/AS03A	IIV/AS03A	Day 28 post boost
351	IIV/AS03A	IIV/AS03A	Day 28 post boost
seasonal influenza vaccine cohort	**Vaccine type**	**season**	**sampling time**
279	QIV	2018-2019	Day 28
290	QIV	2018-2019	Day 28
458	QIV	2019-2020	Day 28
463	QIV	2019-2020	Day 28
469	QIV	2019-2020	Day 28
441	QIV	2019-2020	Day 28
479	QIV	2019-2020	Day 28

3.3 Discussion

The “Total-seq HA array” showed that the cHA+ memory B cells had increased binding affinity and cross-reactivity toward group 1 influenza virus strains (H1, H9, cH5/1, cH8/1) as well as fairly common cross-reactivity to group 2 (H3 and H7) HAs. Notably, the stalk-reactive B cells expanded after vaccination were able to bind to HA mutational variants that had been identified from natural viral evolution or by escape mutant studies. These findings suggest that the chimeric-based influenza virus vaccine would provide long-term protection against influenza A virus strains, including seasonal, zoonotic and potential novel pandemic influenza viruses.

4 The chimeric-HA based universal influenza virus vaccine elicits broadly binding antibody responses that target highly conserved epitopes

4.1 Introduction

We then endeavored to characterize these stalk-reactive B cells baited/sorted with cH5/1 HA probe at day 28 post-boost time point. All antibodies were first screened by ELISA for binding to the H1 (from A/California/7/2009 H1N1) antigen and to divergent influenza viruses or recombinant HA proteins. Antibodies were also screened in a high-throughput fashion for binding to panels of HA in a protein microarray(Meade et al., 2017). For this, we used a large panel of recombinant HA molecules consisting of all known subtypes (H1-H18, N1-1N11, and influenza B HAs). Binding affinities have been estimated from ELISA binding by Scatchard plots and the expected low affinity germline-derived mAbs were measured by biolayer interferometry. Hemagglutinin inhibition (HI) assay was performed to distinguish HA head-reactive mAbs(Ohmit et al., 2011). Antibodies that lack HI activity may bind epitopes such as the side of the HA head or the stalk domain and can also neutralize and protect from influenza infection. Therefore, all mAbs also were screened by in vitro plaque assay to obtain IC50 measurements of neutralization titer(Asthagiri Arunkumar et al., 2019) against the A/California/7/2009 H1N1 virus, which the chimeric stalk domain comes from.

These assays aim for epitope binning and mapping, based on the following assumptions: 1) All HI+ antibodies bind to the HA globular head near the receptor-binding domain 2) HA-protein-binding mAbs that are not HI+ but neutralize in vitro often

bind the HA-stalk. All antibodies will be binned according to the above properties, as well as according to their competition status with a known stalk antibody (CR9114) and other known stalk epitope-specific mAbs (as determined by competition ELISA and biolayer interferometry). HA stalk-reactive antibodies may mediate additional mechanisms of protection, such as antibody-dependent cell-mediated cytotoxicity (ADCC), which engages innate immune cells by Fc–FcγR interactions and contributes to increased efficacy in vivo(Von Holle and Moody, 2019).

4.2 Results

4.2.1 Antibody targeting to highly conserved epitopes

After determining that there was expansion of vaccine-induced and stalk-reactive B cells to the chimeric-HA immunogens, we next sought to determine at the monoclonal antibody level if these cells targeted known protective epitopes and exhibited a wide-breadth of reactivity to various influenza strains. Antibodies representing the most abundant clonal expansions sampled across the various clusters were expressed using the identified variable region genes in a human IgG1 backbone (Figure 4-1).

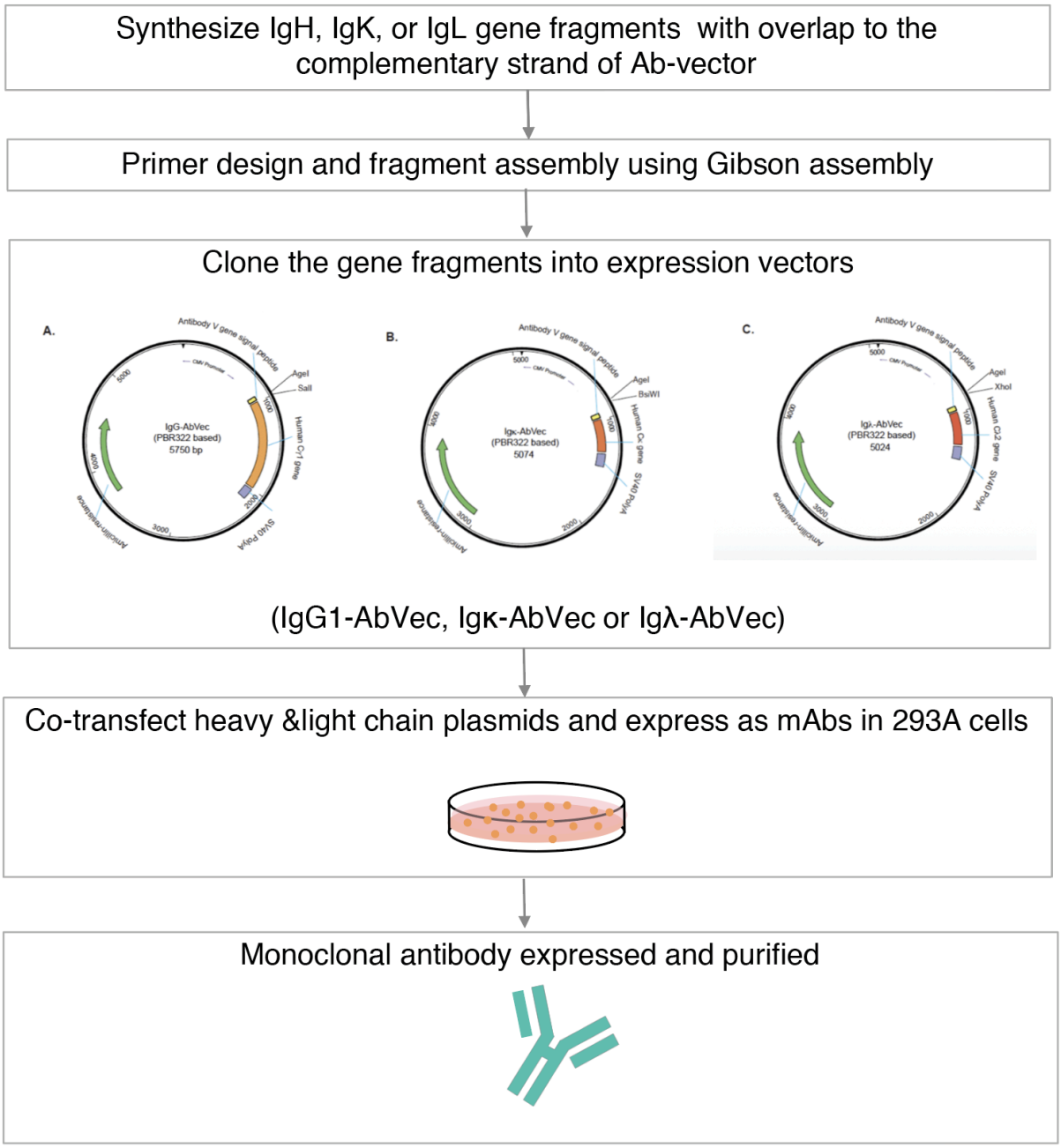


Figure 4-1 Cloning monoclonal antibody from IgH, IgK or IgL sequences.
The IgH, IgL or IgK gene fragments were cloned into expression vectors in the IgG1 backbone. Heavy and light chain plasmids were co-transfected into human kidney epithelial cells (the HEK293 cell line). The secretory form of monoclonal antibodies could then be purified for downstream characterization.

Considering the representation of the biggest clonal expansions in the total data, this sampling covered 7.3% percent of the Ig gene pairs detected from 19 of the subjects in this study. In addition to these representative mAbs, we also expressed germline-derived mAbs (unmutated) to verify HA-probe binding and validate specificity of the naïve-like B cell cluster (cluster 4). MAbs were then tested for binding to HA proteins by ELISA, competition with antibodies that bound known epitopes, and tested for *in vitro* neutralization by plaque assay. We found that 53% of mAbs cloned from HA+ memory B cells bound to known-neutralizing epitopes on the HA stalk domain, 17.9% bound to non-neutralizing stalk epitopes, and 28.6% bound to other epitopes on the H1 HA protein (pH1N1, A/California/04/2009) (Figure 4-2).

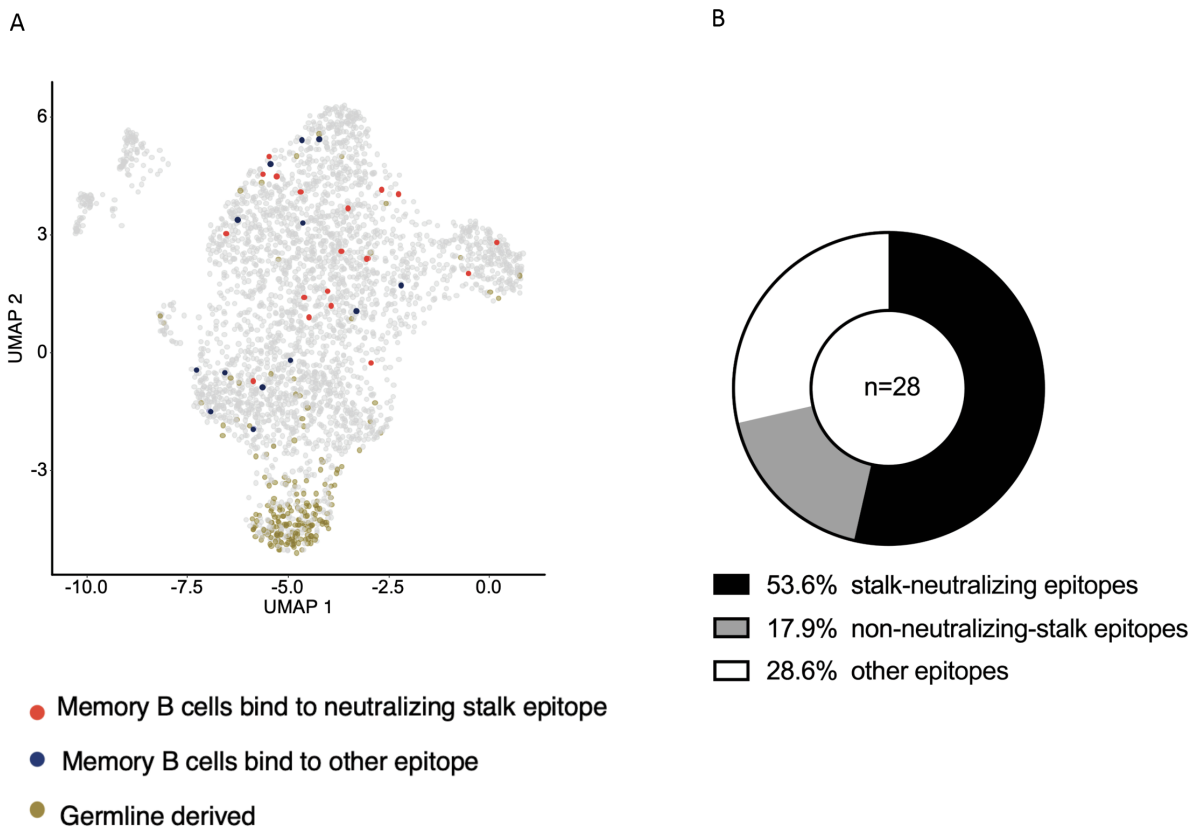


Figure 4-2 MAbs' binding breadth and potency.
7.2% of total monoclonal antibodies were expressed across 6 clusters (except from naïve-like population) and tested the binding to each known neutralizing epitopes from stalk-reactive memory B cells. By test the binding with H1 protein (pH1N1, A/California/04/2009), 53% of mAbs cloned from HA+ memory B cells were found to bind to known stalk-neutralizing epitopes, 17.9% mAbs bind to stalk region but without neutralizing capability to A/California/04/2009 virus, and 28.6% are bind to other epitopes on the H1 protein.

We observed that 58% of mAbs neutralized the pH1N1 influenza virus with high potency below the median PRNT50 of 3.33 µg/mL (Figure 4-3).

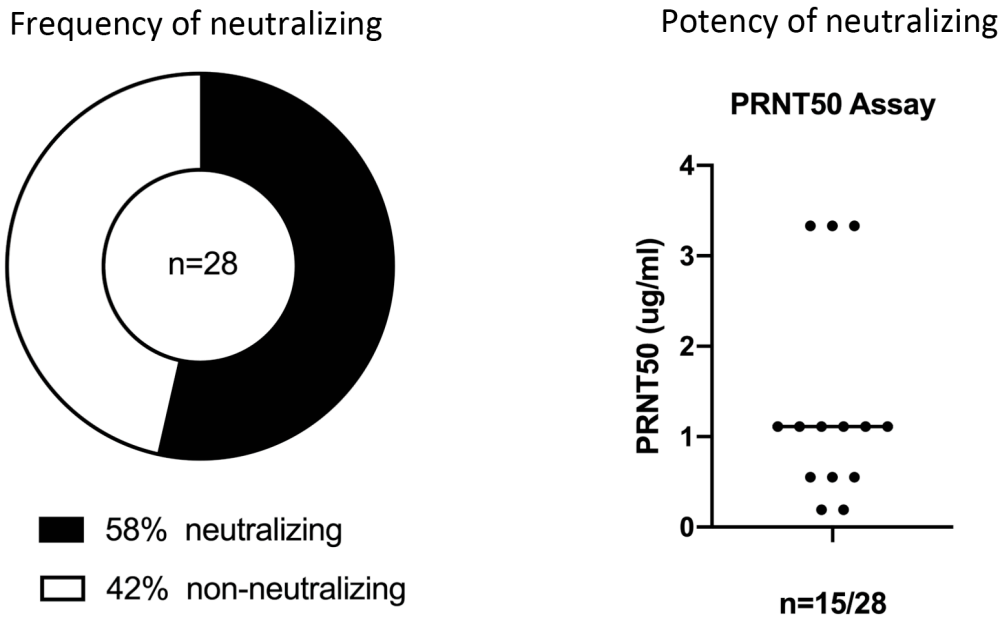


Figure 4-3 Neutralizing frequency and potency of mAbs.
Potency of neutralizing depicted as IC50 values with plaque assay.

In addition, we tested a subset of 11 mAbs for binding to rHA protein using an influenza virus protein microarray and found that the majority of the tested mAbs demonstrated enhanced binding breadth across almost 100 years of antigenic drift and H1N1 virus shifts. Additionally, the mAbs induced by this chimeric HA-based vaccine

also showed cross-reactivity to group 2 influenza HAs such as H3, H14, and H4 (Figure 4-4).

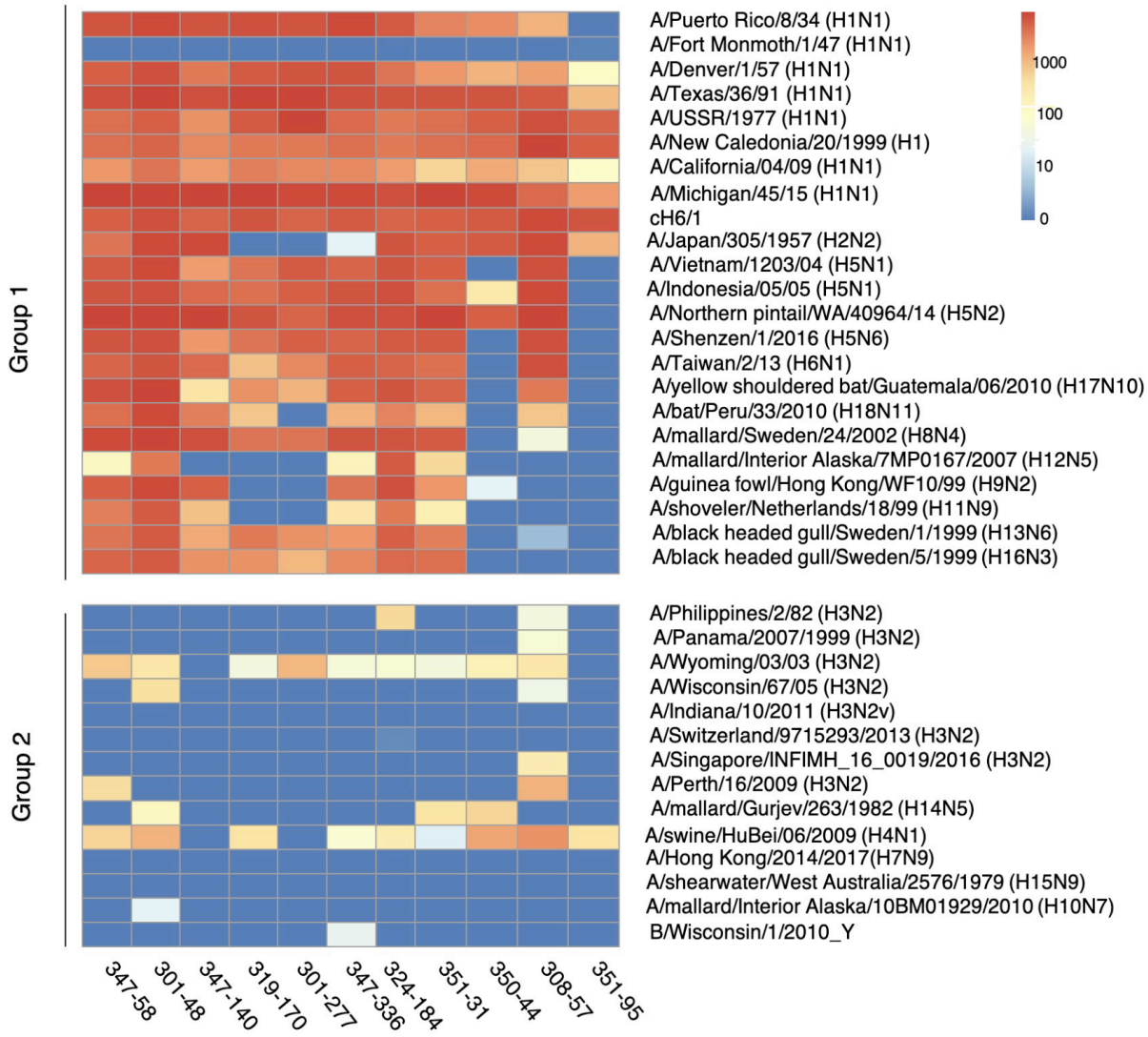


Figure 4-4 MAbs bind to seasonal, zoonotic and pandemic influenza virus proteins (influenza virus protein microarray analysis).
Area under the curve (AUC) was calculated from the median spot fluorescence.

In addition to the major function of preventing the fusion of the virus with endosomal membranes, *in vivo* protection by broadly neutralizing HA stalk-antibodies has been shown to be dependent on Fc-Fc gamma receptor (FcγR) interactions. To

assess FcγR-mediated immune activity we performed an *in vitro* antibody-dependent cell-mediated cytotoxicity (ADCC) assay to evaluate stalk-reactive mAbs, with a focus on those shown to be non-neutralizing by plaque assay. We found 54% (7/13) non-neutralizing stalk-reactive mAbs exhibited ADCC activity (Figure 4-5). These analyses demonstrate that the most common B cell clones present after chimeric-HA vaccination have high reactivity to the HA-stalk, are commonly neutralizing and ADCC-inducing, and they are broadly-reactive to a variety of influenza strains (Table S3).

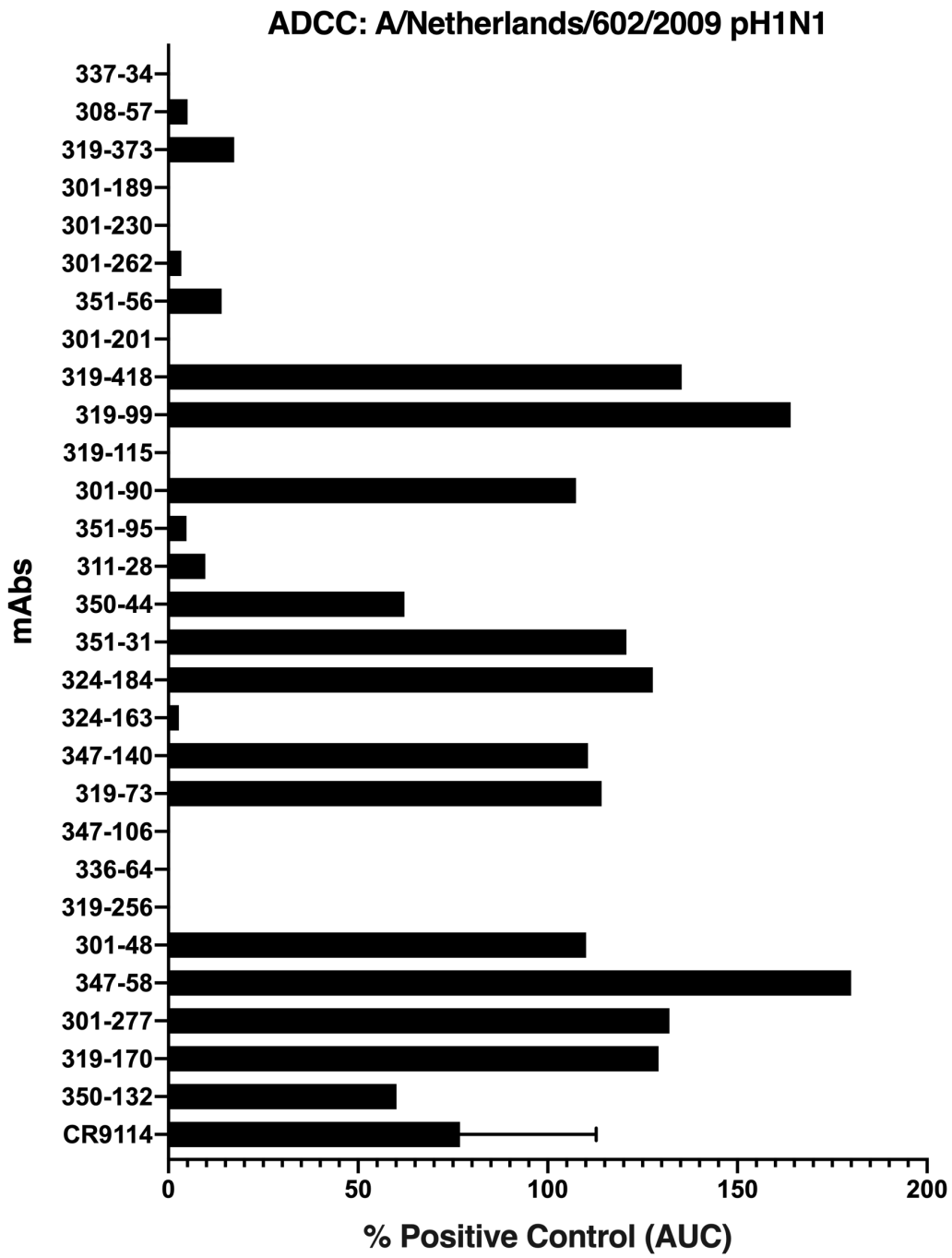


Figure 4-5 ADCC activities of stalk-reactive mAbs .
Stalk-reactive mAbs were measured using a reporter assay to determine engagement with the human FcγRIIIa expressing Jurkat effector T cells. The positive control used was anti-influenza A group 1 and 2 monoclonal antibody CR911477(Laursen and Wilson, 2013). EC50 values were calculated based on fold change of background wells. All data plotted are mean ± SD of triplicate wells for each mAb.

Table 3 Information of mAbs.

mAbs	V gene	Isotype	CDR3 length	private clone	public clone	Neutralizing to A/California/07/2009 influenza virus (μg/mL)	ADCC activity
301-48	VH1-69	IgG	15	Y		0.19	Y
301-90	VH1-69	IgG	15	Y		0.19	Y
319-170	VH1-69	IgG	17	Y		0.55	Y
351-31	VH1-69	IgG	13	Y	Y	0.55	Y
324-184	VH1-69	IgG	13	Y	Y	0.55	Y
319-418	VH1-69	IgG	13			1.11	Y
347-140	VH1-69	IgG	15	Y		1.11	Y
319-73	VH1-69	IgG	13	Y		1.11	Y
301-277	VH1-69	IgG	14	Y		1.11	Y
319-373	VH3-23	IgG	16	Y		1.11	Y
350-132	VH3-48	IgG	16	Y		1.11	Y
319-256	VH1-69	IgG	17	Y		3.33	N
319-99	VH1-18	IgG	15			3.33	Y
347-58	VH1-69	IgG	16	Y		3.33	Y
301-189	VH3-7	IgG	17		Y	30	N
351-56	VH4-59	IgD	13	Y		N	Y
311-28	VH5-51	IgG	19		Y	N	Y
301-262	VH3-7	IgA	17		Y	N	Y
301-201	VH3-7	IgG	9	Y		N	N
350-44	VH5-51	IgG	10		Y	N	Y
324-163	VH3-9	IgA	13		Y	N	Y
351-95	VH3-9	IgD	13		Y	N	Y
301-230	VH3-53	IgA	8			N	N
319-115	VH7-4-1	IgG	21			N	N
337-34	VH3-7	IgM	12	Y		N	N
347-106	VH3-23	IgG	13	Y		N	N
336-64	VH4-4	IgA	20	Y		N	N
308-57	VH4-61	IgG	14		Y	N	Y

4.2.2 Stalk-reactive antibodies induced by the universal influenza virus vaccine candidate are capable of binding to HA-stalk mutational variants.

Since influenza viruses can rapidly mutate through antigenic drift, the virus can escape antibody binding and neutralization, even with just a single mutation. Although stalk-reactive antibodies are less common, they can neutralize various influenza virus strains and it is unclear how easily influenza virus might escape these antibodies if there is strong enough selective pressure to do so(Chai et al., 2016; Clementi et al., 2011; Cobey, 2014; Doud et al., 2018; Lipsitch and O'Hagan, 2007; Park et al., 2020). To gain some insight into this issue, we assessed naturally acquired mutations and escape mutations by generating single amino acid mutations on HA from the A/California/04/09 H1N1 strain. We then examined the change in binding of the neutralizing mAbs that bound to known-stalk epitopes by ELISA. The results showed that the majority of the neutralizing stalk-reactive mAbs were able to bind the influenza virus variants (Figure 4-6). This implies that the mAbs induced by the chimeric HA-based universal vaccine candidate

bound various epitopes on the HA stalk domain and a combination of these mAbs may provide stable protection and resistance to influenza virus antigenic drift.

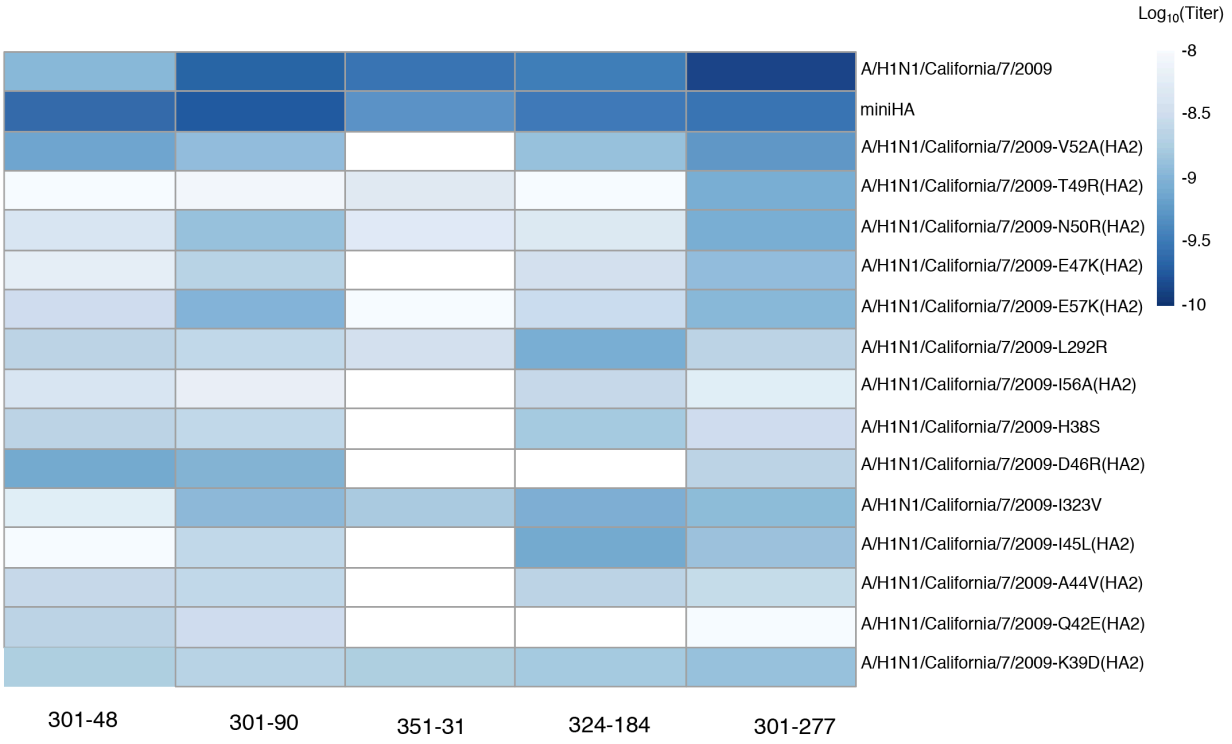


Figure 4-6 MAbs' binding breadth to divergent HAs and HA variants of A/California/04/09.
Single amino acid mutations on A/California/04/09's HA are generated with naturally acquired mutations or escape mutations. These neutralizing stalk mAbs binding pattern toward influenza virus variants.

4.2.3 Identifying public B cell clones that react to chimeric H5/1 HA protein

Because of the highly-targeted nature of the chimeric-HA vaccine, we predicted that there would be biased selection for particular multi-donor class antibodies or public clones shared by multiple individuals. These types of targeted responses have been seen in past studies on antibodies induced by vaccination with zoonotic influenza strains that share only the most conserved epitopes(Henry Dunand and Wilson, 2015; Joyce et al., 2016). In total, 2,228 paired heavy/light chain antibody sequences were

identified across 19 subjects from memory B cells isolated using cH5/1 protein as bait by flow cytometry. As previously defined(Andrews et al., 2015b; Henry et al., 2019; Wrammert et al., 2008), B cells clones were designated as antibodies encoded by the same heavy/light chain variable and joining genes with the same length of CDR3 but with CDR3 nucleotide differences not exceeding the frequency of somatic mutations in CDR1 and CDR2. In this study we identified 18 B cell clones shared by at least 2 different subjects from a total of 212 B cell clonal expansions noted for all subjects. For example, the public clonotype 56 was found the most often and is shared by five subjects (Figure 4-7A). Notably, only three of the public clones were previously reported in the literature and known to bind to the group 1 influenza stains(Joyce et al., 2016). Thus, we identified an additional 15 novel multi-donor classes of antibody that were present at 28 days after boost with the chimeric-HA vaccine (Figure 4-7B). Examples of these public clones were found in 16/19 subjects in the study and represented 8.7% (public clones/all clones) of all of the detected clonal expansions (Table 4).

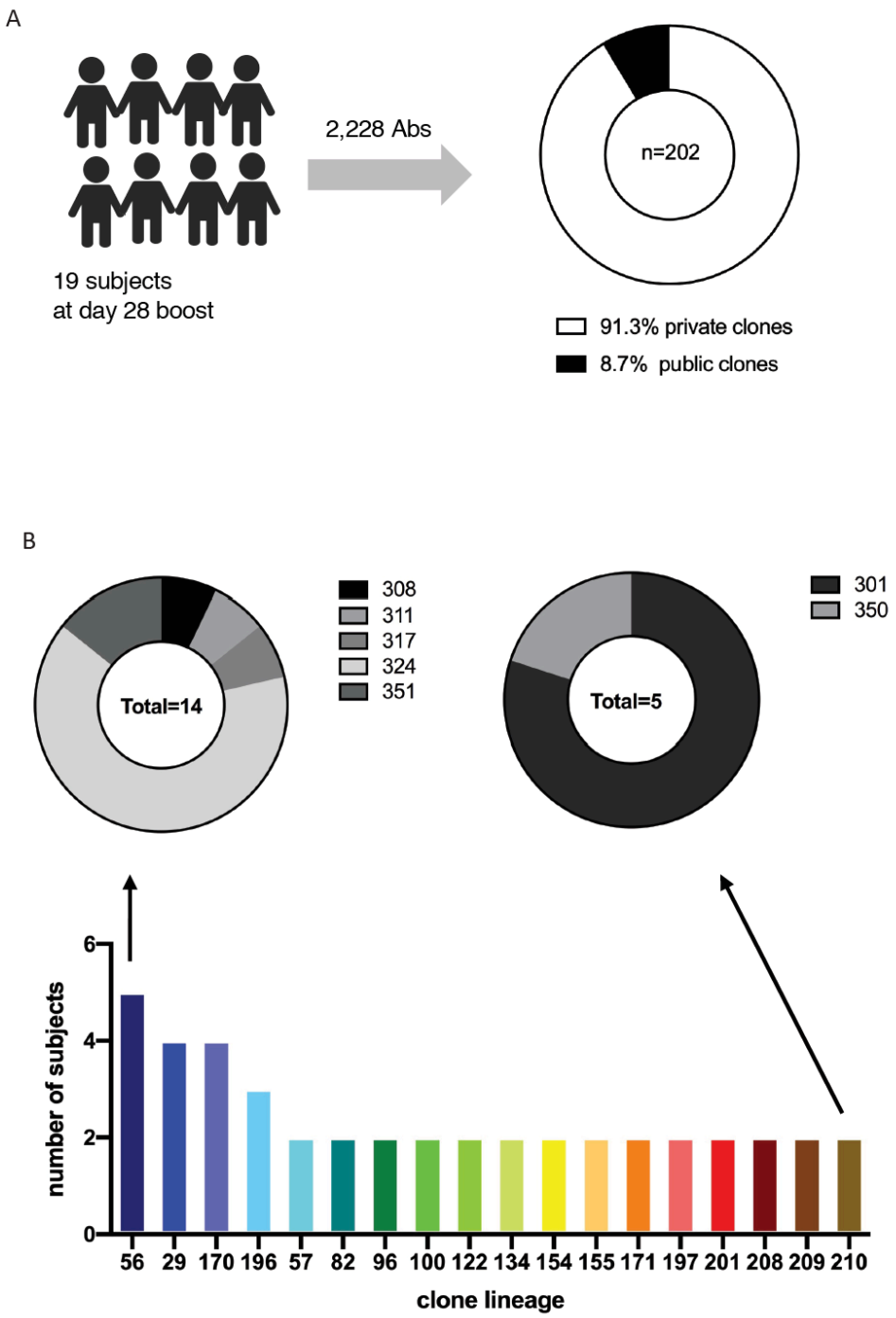


Figure 4-7 Figure 5. Public antibodies are broadly binding and protect against divergent influenza strains.
(A) The identification of 19 public (shared by multiple people) stalk-reactive B cell clones out of 208 clones (8.7% of all clones), across 19 subjects in the universal vaccine cohort. (B) Public clone also overlap with private clonal expansion.

Table 4 Public clones identified from the universal influenza virus vaccine trial.

Clone name	Number of subjects shared	VH	DH	JH	VL	JL	Length of CDR3
170	4	VH3-9	DH3-10	JH4	VK3-15	JK2	13
197	2	VH4-59	DH3-10	JH3	VK1-39	JK1	15
82	2	VH3-20	DH5-18	JH4	VK1-9	JK3	14
210	2	VH5-51	DH1-1	JH6	VK3-11	JK5	11
100	2	VH3-23	DH2-21	JH4	VL1-44	JL3	16
96	2	VH3-23	DH1-26	JH4	VK3-15	JK5	16
122	2	VH3-33	DH1-26	JH4	VK3-15	JK1	13
134	2	VH3-48	DH4-17	JH6	VK3-15	JK4	14
154	2	VH3-7	DH3-22	JH4	VK4-1	JK1	17
171	2	VH3-9	DH3-10	JH5	VK3-15	JK1	12
196	3	VH4-59	DH1-1	JH3	VK1-39	JK1	15
201	2	VH4-61	DH1-26	JH4	VK1-39	JK3	14
209	2	VH5-51	DH3-9	JH6	VK3-11	JK2	10
208	2	VH5-51	DH2-15	JH5	VK2-28	JK3	19
155	2	VH3-7	DH5-12	JH4	VK1-5	JK2	9
29 •	4	VH1-69	DH3-22	JH4	VK3-20	JK2	9
56 •	5	VH1-69	DH3-22	JH6	VK3-20	n.d.	9
57 •	2	VH1-69	DH5-18	JH4	VK4-1	JK3	9

• previously published public clones(Joyce et al., 2016).

Expression of mAbs from examples of these public clones demonstrated that public clones had broader reactivity to divergent influenza virus HA compared to private clone mAbs (Figure 4-8). As indicated in Figure 4-9, the public clones were found mostly in the transcriptional clusters associated with the most differentiated memory. Evidence for common public antibody clone induction suggests that the chimeric vaccine can induce similar protective B cells from multiple individuals.

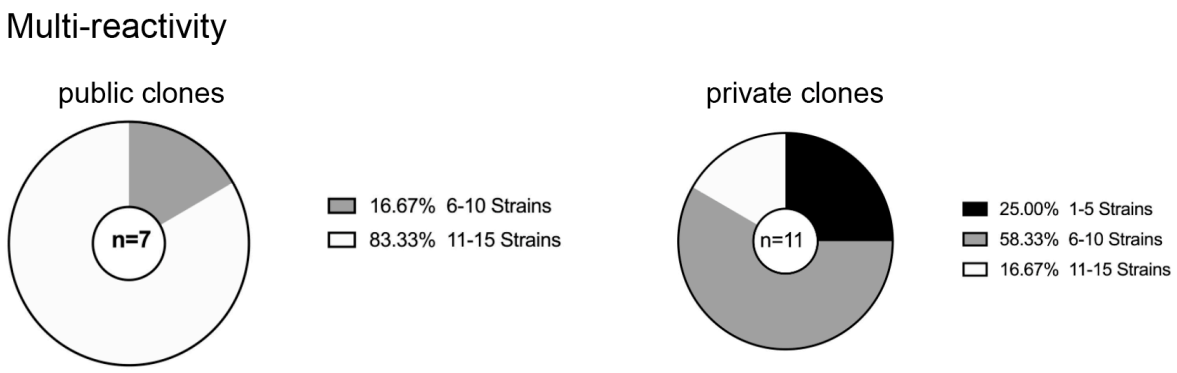


Figure 4-8 Public clones tend to be more multi-reactive than non-public clones.

As indicated in Figure 4.9, the public clones were found mostly in the transcriptional clusters associated with the most differentiated memory. Evidence for common public antibody clone induction suggests that the chimeric vaccine can induce similar protective B cells from multiple individuals.

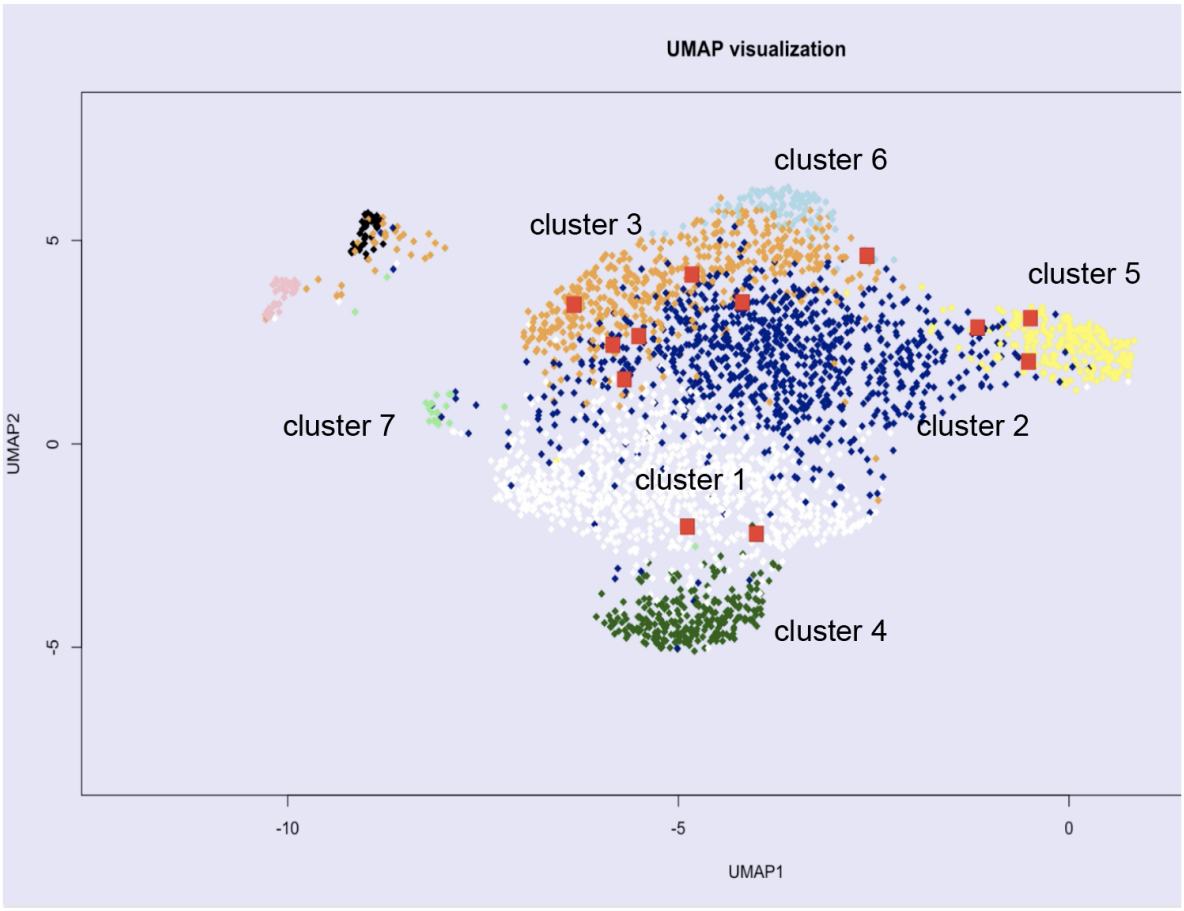


Figure 4-9 Public clonal expansion.
Examples of a public B cell clone expanded within an individual.

4.2.4 Germline (unmutated) B cell clones to chimeric HA protein

While the chimeric-HA vaccine was conceived as a means to boost pre-existing memory B cells to the H1 stalk region, it is also important to consider the potential to drive an HA-stalk response in influenza-naïve infants and small children. Within the naïve-like B cell cluster (Figure 4-10, cluster 4) there were a majority of B cell clones with germline (unmutated) variable genes, allowing assessment the specificity of a sampling of germline (unmutated) naïve-like B cells to the chimeric vaccine.

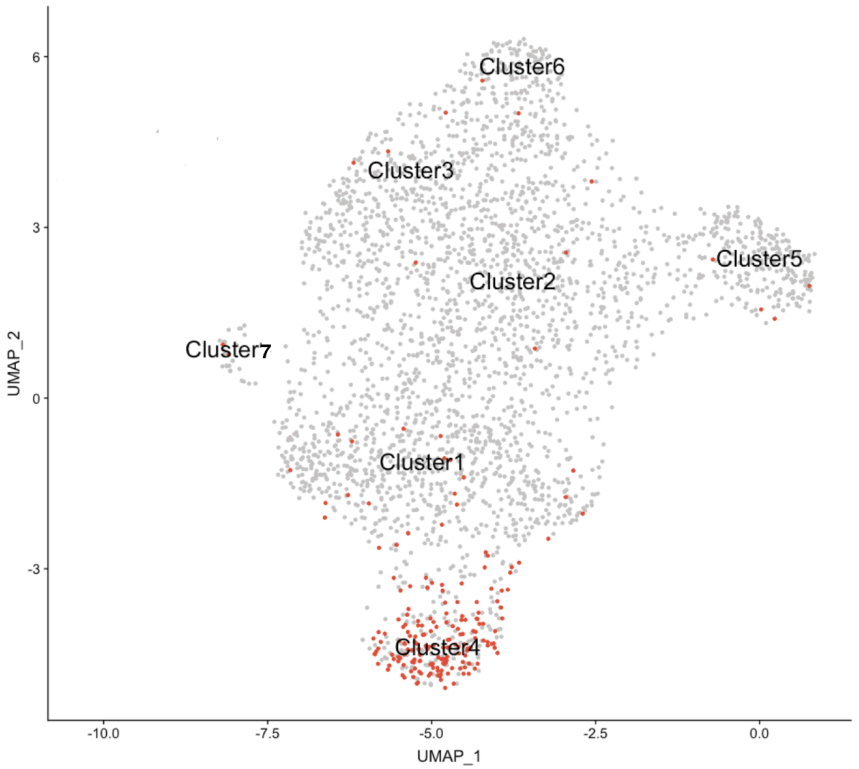


Figure 4-10 Identification of germline (unmutated) stalk-reactive mAbs following cHA-based influenza virus vaccination.
The naïve-like population results in 9% of all chimeric HA+ memory B cells in this trial, majorly in cluster 4. This cluster is similar to naïve B cells, it consisted of nearly all IgD+ and IgM+ B cells. The germline mAbs were mostly identified from this naïve-like B cell subset. Germline (unmutated) mAbs were labeled in red.

This naïve-like subset of HA+ memory B cells accounted 9% of all cH5/1 baited/sorted HA+ memory B cells in this trial (Figure 4-11A). While these germline-derived mAbs were mostly of too low affinity for detectable binding by ELISA, they bound to recombinant H1 HA protein (A/California/04/09) when tested by bio-layer interferometry (Figure 4-11B). Competition for binding by first loading the HA protein with the prototypical anti-stalk antibody (CR9114 mAb) inhibited the binding of 10/13 (78%) of these germline

antibodies, demonstrating that the majority of these cells were binding HA-stalk epitopes (Figure 4-11C).

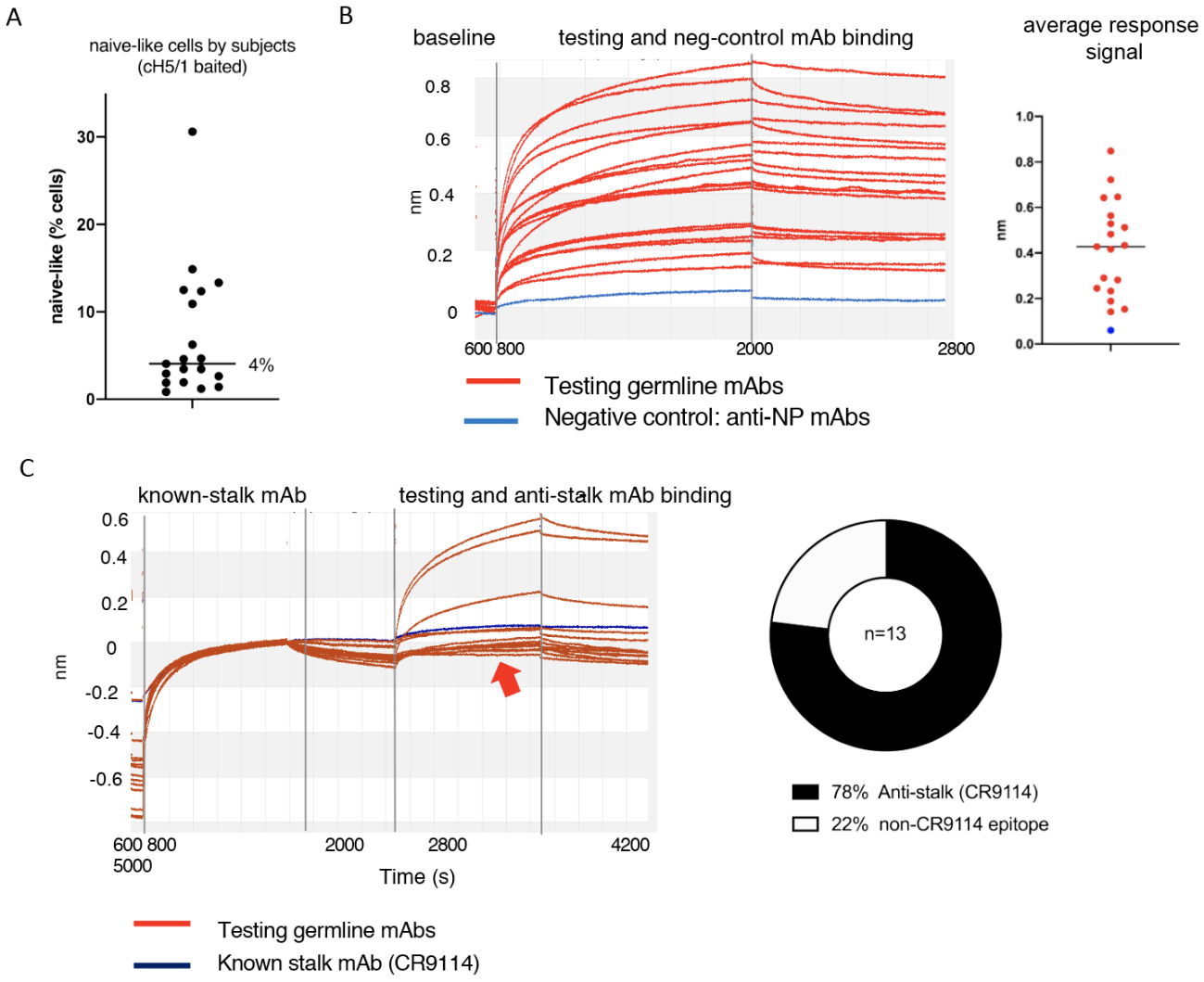


Figure 4-11 Germline (unmutated) mAbs binding to Cal/09 H1 and the frequency of binding to CR9114 epitope.
(A) The naïve like population results in 4% of all cH5/1 baited/sorted HA+ memory B cells in this trial. (B) Germline (unmutated) antibodies generated from cluster 4 and bind to Cal/09 H1 tested by biolayer interferometry. (C) The frequency of germline derived mAbs that bind to neutralizing-stalk epitope (same epitope as CR9114).

Analysis of the fraction of germline-derived mAbs that did have detectable binding by either ELISA (Figure 4-11) or influenza virus protein microarray (Figure 4-12) showed broad binding to divergent group 1 influenza strains, with occasional cross-group binding to particular group 2 strains as well. These data suggest that HA stalk-reactive naïve B cells can be recruited by the chimeric-HA vaccine approach and tend to have low-affinity but broadly adaptive binding. The memory phenotype (i.e., CD27+, some somatic mutations) of these isolated germline B cells in the naïve-like transitional cluster (cluster 4) and tendency to bind the HA-stalk by a majority of these clones suggests that indeed, prime and boost with the H1-stalk but chimeric and divergent globular head regions can drive stalk-biased immunity even in influenza naïve individuals.

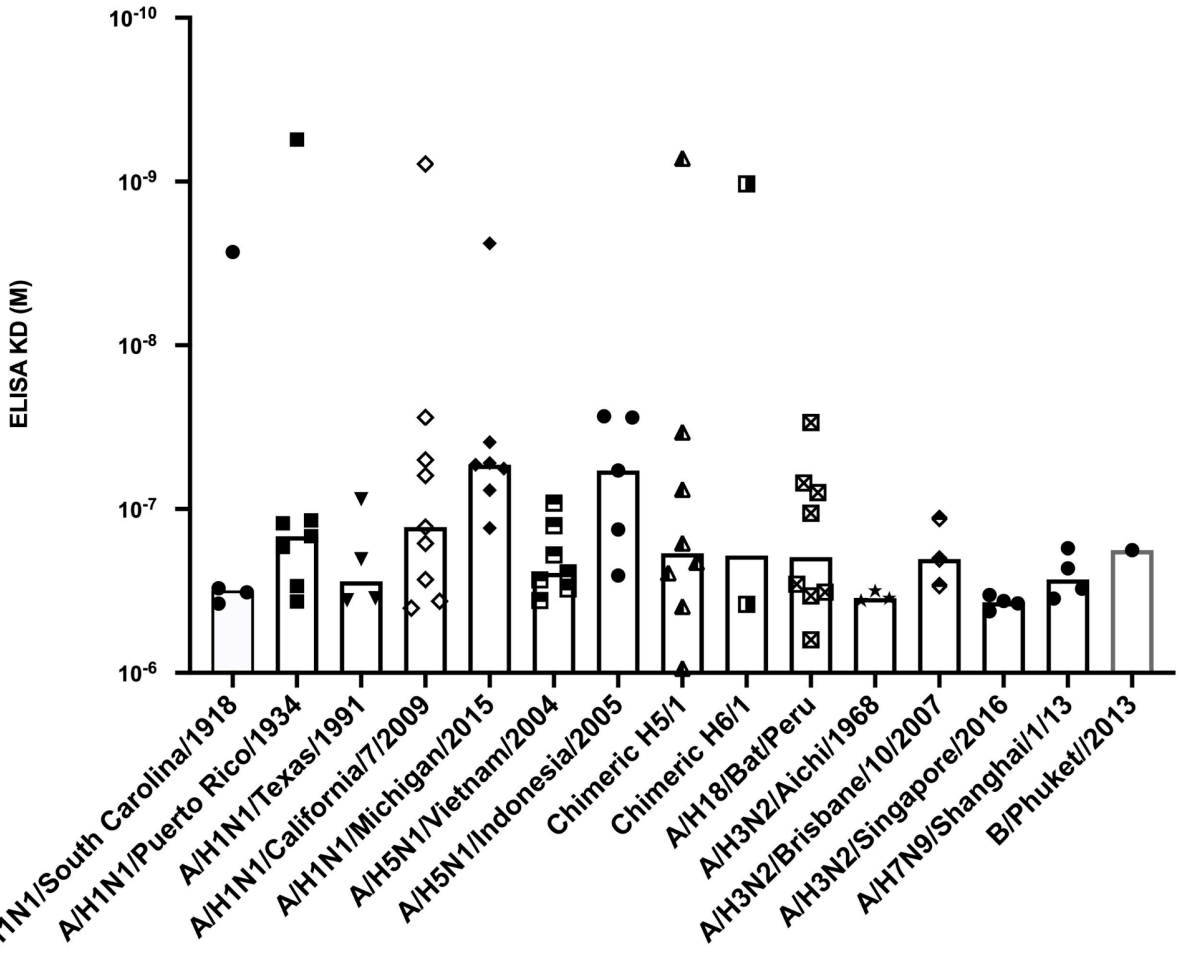


Figure 4-12 Germline derived mAbs' binding breadth by ELISA.
Data are depicted as ELISA KD values for each individual mAb tested against the influenza viruses or HAs. Experiments are performed in duplicate.

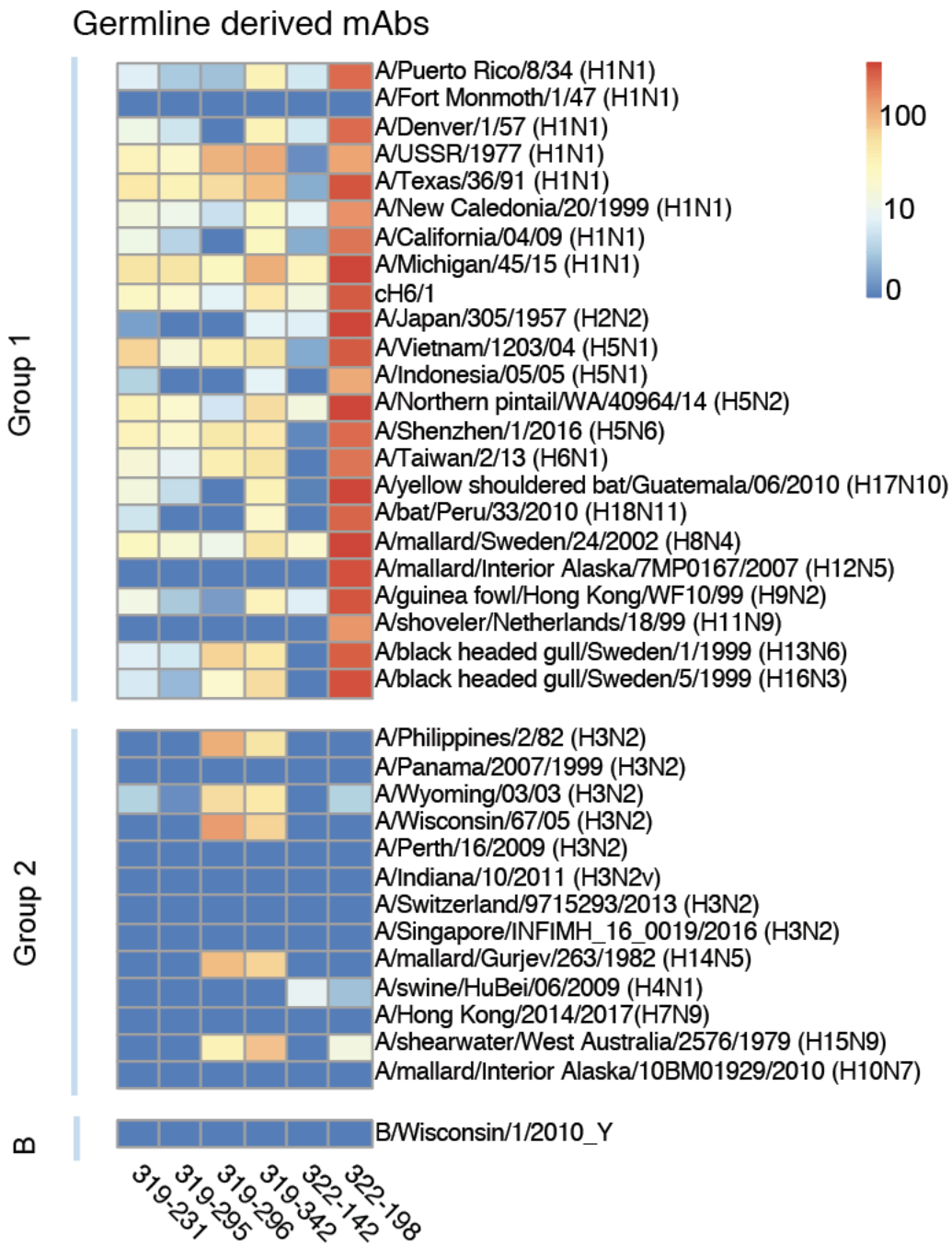


Figure 4-13 Binding breadth and affinity of repetitive germline derived mAbs by influenza virus protein microarray.
Area under curve (AUC) was calculated from the median spot fluorescence. Data are representative of 3 independent spots.

5 Discussion

Current influenza virus vaccines induce strain-specific antibodies which provide minimal short-lived efficacy and narrow protection. Thus, recent efforts have begun to develop a universal influenza virus vaccine. The amino acid sequence diversity and immunodominance of the highly variable HA head domain are key contributors inhibiting the development of a universal influenza virus vaccine. The chimeric-HA universal influenza virus vaccine candidate fused the stalk region of pandemic H1N1, which is present in human pre-existing immunity, with the variable globular head of divergent avian influenza strains. Through sequential vaccination with the same stalk antigens, it aimed to induce antibodies that target this subdominant, functionally conserved stalk region. Interim serological results of this clinical phase 1 trial have shown that this cHA-based influenza virus vaccine elicits cross-reactive serum IgG antibodies that target the stalk domain(Bernstein et al., 2020).

The scRNA-seq analysis revealed transcriptional heterogeneity within the HA stalk-reactive memory B cell population in response to this novel universal influenza virus vaccine candidate. This observation suggests that this vaccine elicits functionally distinct antigen-specific memory B cell subsets. Importantly, we provide evidence that the cHA-based universal influenza vaccine drives an increased frequency of HA stalk-reactive memory B cells present even a year out after vaccination. The detection of B cell clones expressing neutralizing antibodies that are shared by multiple subjects (public clones) demonstrates that a commonality of response can be elicited by this vaccine, improving predicted protection for a larger fraction of people. Finally, we note

that a subset of B cells is detectable after vaccination that express IgD/M isotype antibodies and with low variable gene mutation rates but that otherwise have memory phenotypes and preferentially target HA stalk epitopes. The presence of this subset after vaccination provides evidence that the prime-boost series with chimeric HA antigens, each with distinct globular heads but shared stalk regions, can drive *de novo* HA-stalk responses even in influenza naïve individuals such as infants or young children.

In this study, we also identified B cells with germline (unmutated) B cell receptors that could provide valuable information on the base untrained antibody repertoire induced against influenza, and for identifying HA antigens capable of activating naïve B cells. Previous studies have identified HIV-specific germline B cells in the human naïve B cell repertoire(Havenar-Daughton et al., 2018), which were valuable in the design of step-wise vaccine approaches to first elicit classes of B cells that could then be trained through booster vaccine series for particular activities (Table 4). Numerous germline mAbs have been identified with varied activity to different strains of influenza. These antibodies can be used to template immunogens designed to initiate de novo broadly neutralizing antibody responses(McGuire et al., 2016; Peterhoff and Wagner, 2017). Further investigation of the naïve human B cell repertoire specific to immunogens of influenza will also provide fundamental insight into the starting population of naïve B cells to target(Sangesland et al., 2019).

For the stereotyped B cell clonotype, many questions remain about the B cell clonotype's complexity. First, this cohort was conducted using a small number of

subjects, and consequently it had limited genetic, racial, age and geographical diversity; B cell clonal lineages with diverse genetic backgrounds may exhibit both genetic convergence and divergence to achieve recurring motifs for the recognition of viral protein antigens. Secondly, this study has only addressed circulating blood cells; many B cell populations reside in tissue in which the repertoire differs from that of the blood.

The stalk-reactive antibodies are selected from this cHA-based influenza vaccine trial. One of the major functions of stalk antibodies is to inhibit endosome fusion and viral entry(Krammer and Palese, 2015). This class of antibody also inhibits neuraminidase enzymatic activity via steric hindrance to prohibit viral egress(Chen et al., 2019; Kosik et al., 2019), and also uses Fc-dependent mechanisms, including antibody-mediated cell cytotoxicity (ADCC)(Yassine et al., 2015), complement-dependent cytotoxicity (CDC)(Yassine et al., 2018), antibody-dependent cell phagocytosis (ADCP)(Mullarkey et al., 2016), and antibody-dependent complement-mediated lysis (ADCL)(Winarski et al., 2019).

This study provides evidence that the HA stalk-reactive B cells are selected into long-term memory after vaccination. It also identifies clonal expansions, both within and across multiple individuals (public clones) supporting a commonality of outcome by chimeric HA vaccination. We also report preferential HA-stalk binding in IgM/IgD B cells with only few mutations but that also express the memory marker CD27 and other evidence of selection. Induction of these naïve-like cells suggests that in addition to expanding memory B cells, the chimeric-HA prime-boost vaccine strategy can also drive

de novo antibody responses to the conserved HA-stalk. This work thus paves the way for further development of a universal influenza virus vaccine.

6 Overall implications of this work

This is a study of a universal influenza virus vaccine trial that utilizes influenza viruses engineered to express chimeric hemagglutinin (HA) proteins. Given the conserved nature of the HA-stalk region, broadly neutralizing antibodies that target this region would protect against a wide range of influenza strains. The chimeric HAs present in the vaccine are composed of a typical stalk domain (from an H1 HA) paired with an exotic head from a strain foreign to humans (avian H5 or H8). The study aimed to provide evidence to address an important biological question: can this strategy effectively re-program the human immune response from targeting the immuno-dominant HA head, towards targeting the immuno-subdominant but conserved HA stalk epitopes instead. The generated data suggest that HA stalk-reactive B cells are selected after day 28 post boost immunization, and persist at increased levels over the subsequent year.

A universal influenza virus vaccine should aim to generate B and T cell memory, to provide long-lasting and protective immunity. Memory B cells are generated from antigen specific naïve B cells during an infection or vaccination. They clonally expand and survive as sentinels to guard against future infections. Upon subsequent antigen exposure, memory B cells differentiate into new antibody-secreting plasma cells to provide immune responses that are more rapid and of higher magnitude than primary responses(Sallusto et al., 2010; Shah et al., 2019). Earlier studies have revealed various subsets of memory B cells(Kurosaki et al., 2015; Shah et al., 2019). However, the distinct subsets and functions of HA-specific memory B cells remain

unclear(Kurosaki et al., 2015; Sallusto et al., 2010; Shah et al., 2019; Weisel and Shlomchik, 2017). In this study, 7 antigen-specific memory B cell subsets were found to have distinct transcriptional signatures at day 28 post boost. This may reflect their developmental history, homing, and activation potential. Differential genes, Ig isotypes, and BCR repertoire were analyzed at day 28 post boot vaccination. In this way, in additional to classical memory B cells, atypical antigen specific B subsets such as naïve-like and tissue-like memory B-cells were identified in response to the chimeric based universal influenza virus vaccine candidate.

A “Total-seq HA array” was applied to quantitatively investigate the BCR specificities for antigen-specific memory B cells. The data showed increased HA stalk-reactive B cells at day 28 post boost in the universal influenza virus vaccine trial, relative to the seasonal influenza vaccine cohort. A panel of recombinant HA antigens, including cH5/1, cH8/1, a recent H3 strain HA, plus divergent avian H9 HA (group 1) and H7 strains (group 2) was utilized to detect BCR specificity followed by scRNA-seq. More HA stalk-reactive B cells targeting H1, cH5/1, and cH8/1 were induced by cHA-based universal influenza virus vaccination than by the seasonal vaccine. In addition, the cH5/1 probe preferentially bound to stereotypical VH1-69 and VH1-18 B cells(Avnir et al., 2014; Avnir et al., 2016b; Pappas et al., 2014; Sangesland et al., 2019), particularly after chimeric universal influenza virus vaccine immunization.

To determine the quality of the stalk-reactive B cells elicited from the universal influenza virus vaccine, monoclonal antibodies were expressed based on the heavy and light chain V(D)J sequences. These antibodies exhibit a breadth of reactivity to various

influenza strains. At day 28 after vaccination, the identification of clonal expansions, both within an individual (private clones) and across multiple individuals (public clones) provided the novel mAb reagents to template immunogens for an improved vaccine. From 2,228 stalk-reactive mAbs collected from 19 subjects at day 28 after boost, 15 new public clones were identified from this cohort. These public clones were identified within the most differentiated memory B cell subsets. In addition, these clones demonstrated broader reactivity to divergent influenza virus HA than to private clone mAbs. Evidence for common public antibody clone induction suggests that the chimeric vaccine can induce similar protective B cells from multiple individuals. This indicates that the chimeric-based universal influenza virus vaccine could be applied to the general population to induce similar “herd immunity” to conserved epitopes.

The prime-boost strategy using chimeric HA molecules carrying the 2009 H1N1 pandemic influenza strain (pH1N1) HA stalk, combined with exotic HA heads, is based on the classical principle of “original antigenic sin (OAS)”. “Antigenic seniority” or “antigen imprinting” are better terms for describing this concept. These terms refer to the propensity of immune systems to preferentially utilize immunological memory based on previous exposure to influenza virus strains (Cobey and Hensley, 2017; Francis, 1960; Henry et al., 2018; Paules et al., 2019). The cHAs were designed to replace the usually immuno-dominant head epitopes of seasonal influenza virus HAs with avian head domains which are generally foreign to humans, whereas the conserved stalk epitopes are the same as those of pH1N1. Through the sequential exposure to the same stalk region, pre-existing immunity serves as a natural immuno-focusing

mechanism to the conserved region of the HA stalk. Cross-reactive memory B cells targeting the HA stalk are re-activated and may undergo convergent selection in GC, as evidence by identified public clones.

Although pre-existing immunity influences the induction of broadly cross-reactive antibodies targeting the conserved stalk epitopes, it may also suppress the response. This may occur through reduction of antigen levels through epitope masking and/or Fc-mediated mechanisms (such as phagocytosis). In this case, abundance of antigens could be reduced, which could favor recall of memory B cells over the *de novo* activation of naïve B cells. Since the diversity of the memory pool of B cells targeting the HA stalk domain may already be limited due to its immuno-subdominance, this mechanism could further decrease the diversity of the overall clonotypes. Although our findings indicate such preferential activation of memory B cells after sequential exposure to the same pH1N1 stalk, we also found evidence for *de novo* responses by identifying a naïve-like population and germline-derived mAbs. This naïve-like memory B cell subset expressed CD27 with a handful of mutations of BCRs, composed primarily of IgM and IgD B cells. As expected, the variable genes with more diversity are used for the heavy chain of antibodies. Additionally, it upregulated the IL4R gene which is associated with B cell activation, and the BACH2 gene implied a GC reaction. A majority of B cell clones with germline (unmutated) variable genes was also identified from this population. Analysis of the fraction of germline-derived mAbs showed broad binding to divergent group 1 influenza strains, with occasional cross-group binding to particular group 2 strains. These data suggested that the chimeric-based universal vaccine may

also induce a *de novo* response and overcome the “imprinting”. In addition, this implies that the strategy of sequential immunization with chimeric HAs can drive an HA-stalk response in influenza-naïve infants and small children (Figure6-1).

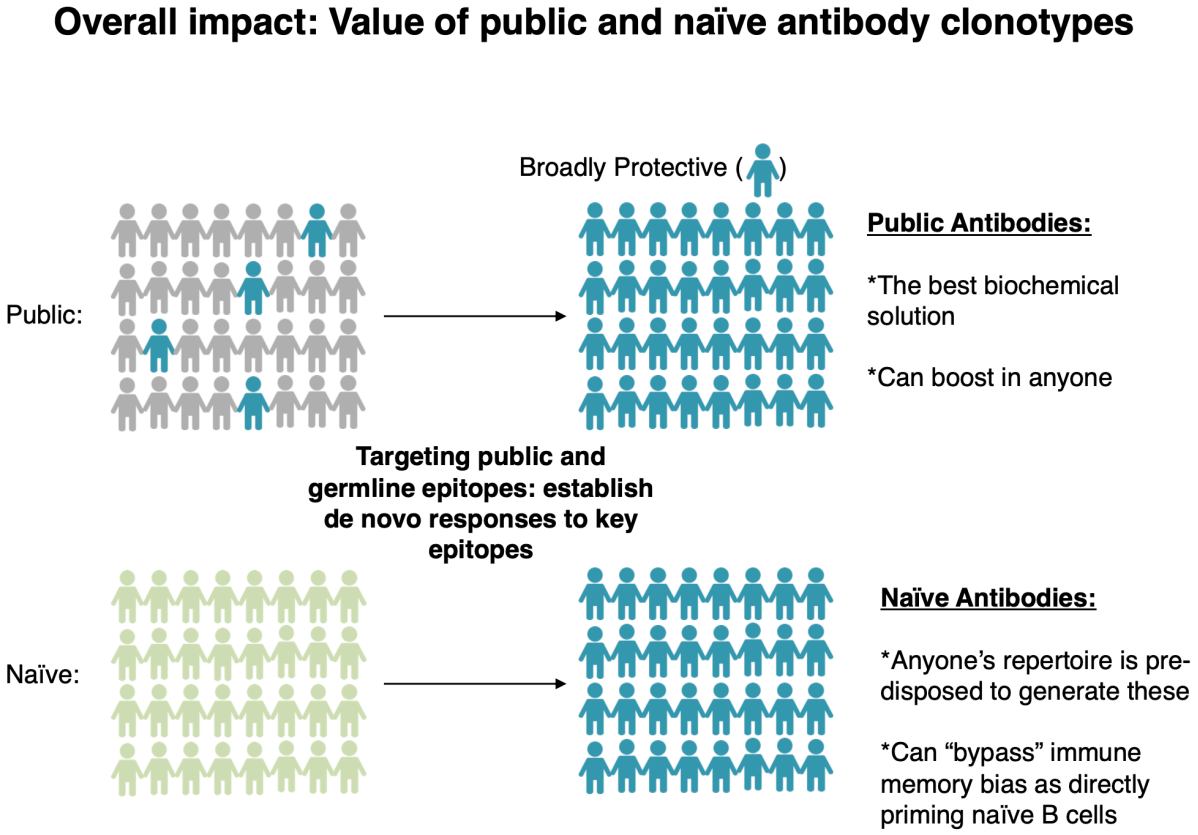


Figure 6-1 Using public and naïve antibodies to establish a *de novo*, base repertoire of B cells that are broadly protective against influenza infection. Public and naïve antibody clonotypes are useful for assessing the most immunogenic strains to use to activate new B cells in adults, avoiding antigenic sin, and to elicit protective immunity in infants and children with limited influenza virus exposure.

7 Current and Future directions

The “Total-seq HA array” approach established in this project allows for comprehensive characterization of B cell responses. It also expedites the therapeutic mAb selection process. The usage of a panel of barcoded HA tetramers as probes to bait/sort the antigen specific B cells followed by scRNA-seq, the BCRs’ binding affinity/specificity, and each cell’s transcriptome and phenotype, can be quantified. Furthermore, these monoclonal antibodies can be generated from the sequenced immunoglobulin genes of the same B cells. In the context of post-infection or vaccination, this approach will facilitate cell type identification, and isolation of high affinity and broadly binding mAbs which might be ideal targets for use as therapeutic mAbs. This method is expandable to various other diseases, including infectious diseases, cancer, and autoimmune disorders.

In this study, the “Total-seq HA array” analysis provides new insight into the burgeoning study of B cell functional heterogeneity. For example, previous work has identified $CD21^{low}$ memory B cells that are distinct from classical memory B cells, and are induced during the peak of GC activity after seasonal influenza vaccination(Lau et al., 2017). These cells are resistant to further GC differentiation and have been predicted to be precursors to long-lived plasma cell differentiation. The identification of an activated ($CD71^{+}$) B cell population that arises at day 7 after influenza vaccination is similar(Ellebedy et al., 2016b). On a wider scale, a surprising variety of functionally distinct B cell subsets have been described in both mice and humans. In the context of the cHA-based universal influenza vaccine trial, 7 antigen-specific memory B cell

subsets have been found with distinct transcriptional signatures that may reflect their developmental history, homing, and specialization potential. Further investigation into specific memory B cell subsets that mirror broad cross-reactivity and neutralization, and how the transcription networks that shape these phenotypes vary, will be critical to vaccine design in which one subset is more desirable than another(Kurosaki et al., 2015). Also, the B cell and antibody responses to influenza viruses, in terms of the compartments and subsets involved, may correlate with binding breadth. In addition to identifying the BCR specificity, transcriptome, phenotype and Ig repertoires on the same cell, the “Total-seq HA array” approach also offers a new way to pursue the comprehensive characterization of antibody responses in influenza vaccinology, and various aspects of B cell biology.

Several transcriptionally distinct antigen-specific memory B cell subsets were identified in this study. It is of great interest to further investigate the chromatin landscape linked to transcriptional changes in immune cell differentiation. This will facilitate an understanding of the underlying stimulation history mechanisms during the emergence of memory B cell subsets. I have hypothesized that different groups of individuals (i.e., elderly adults versus children, with or without allergic or autoimmune disease, gut microbiome variation) undergo different pathways to shape their immune responses. For example, they may have distinct memory cell subsets generated in response to vaccination and infection. What regulatory elements affect the transcriptional outcomes and alter individuals’ cell differentiation? When, where, and how many genes are turned on or off in a state of energy, exhaustion and clonal

expansion of B cells in different populations? While the heterogeneity within the memory B cell population is identified based on distinct transcriptomes, it remains unclear which regulatory elements direct their differentiation so that the varied subsets play unique functional roles in mediating protective immunity.

Germline derived VH1-69 and VH1-18 mAbs were identified in this study, and many of them are validated as binding neutralizing stalk epitopes with broad binding capability. This indicates that most individuals have stalk-reactive precursor germline B cells, including influenza-naïve children. Thus, these germline encoded mAbs from 18 of the 19 subjects within this cohort will allow for templating immunogens that can induce naïve human B cells to target HA molecules' stalk regions. The immunogens that induce high affinity germline stalk-reactive mAbs can be further tested using a transgenic mouse platform that can express a human germline stalk-reactive B cell receptor(Sangesland et al., 2019; Xu and Davis, 2000). These data will reveal binding patterns of germline mAbs on various influenza stains. These antibodies will be useful for assessing the most immunogenic strains for activating new B cells in adults, while preventing antigenic sin, and eliciting protective immunity in infants and children with limited influenza virus exposure.

Public clonotypes are described as the incidence of T or B cells utilizing nearly identical V(D)J gene rearrangements and CDR3 segments across multiple individuals. More research on public clones has been conducted in the context of T cells than B cells(Galson et al., 2015; Greiff et al., 2017; Jackson et al., 2013). This study has identified many unprecedented public stalk-reactive antibody lineages. Exploring the

underlying mechanisms of BCR “public lineages/clonotypes” in response to highly conserved epitopes such as HA stalk region would be a worthwhile future research direction. To further analyze Ig sequence properties, such as convergent amino acids and CDR3 length, heavy-light chain pairings will be invaluable in understanding why these clonotypes emerge and the overall affinity maturation pattern occurring in response to conserved epitopes. To further reconstruct the development pathways of several high affinity and neutralizing public clones, study of these antibodies can guide vaccine design via the identification of protective epitopes. These studies will also allow identification of more protective clonotypes targeting conserved sites of influenza HA across multiple individuals(Andrews et al., 2018b; Lanzavecchia et al., 2016b) (Figure 6-1).

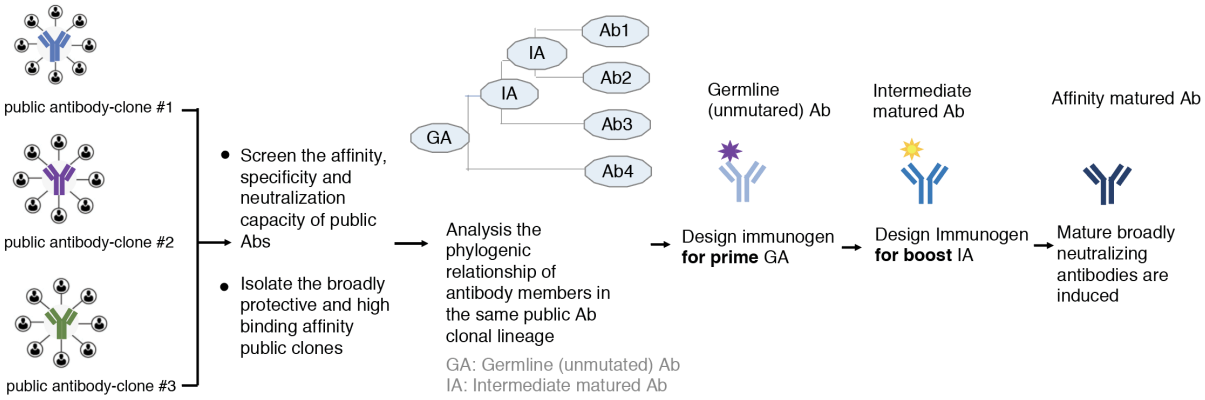


Figure 7-1 Scheme of antibody lineage-based vaccine designs and/or epitope-based vaccine design.
Using the protective public stalk-reactive B cell precursors (by inverted public mAbs’ sequence to germline version) as a template to design immunogens that would drive public stalk-reactive B cells to make broad neutralizing Abs.

In addition to the similarities in the Ig sequences of public clones, analysis of binding to various influenza strains shows that the public clones tend to be more multi-reactive than the non-public clones. Examining the selection of public clones is crucial; Are they selected to bind to epitopes which are more conserved? or do they augment binding by increasing flexibility and becoming poly-reactive? is there potential autoreactivity? Elucidating these potential mechanisms driving multi-reactivity will improve our capacity to select immunogenic antigens to generate broadly neutralizing Ab.

A question of broad impact remains unanswered by this study: Even if the chimeric influenza virus vaccine can induce stalk-reactive antibodies at the population level, does it pressure the virus to mutate over time? The spectrum of stalk-reactive mAbs within this trial will allow for the screening of natural and escape antigenic variants of influenza HA protein to evaluate the long-term protective potential of this and similar vaccine candidates against the HA-stalk.

8 Materials and methods

Study approvals and cohort

Peripheral blood mononuclear cells were collected from the randomized, placebo-controlled observer-blind phase 1 clinical study conducted at Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA) and Duke Early Phase Clinical Center (Durham, NC, USA). 65 participants who received LAIV8-IIV5/AS03, LAIV8-IIV5, IIV8/AS03-IIV5/AS03, or Placebo (SALINE-PBS or PBS-PBS) were block-randomized. Eligible participants needed to be healthy and of non-childbearing potential, including men and women between the ages of 18-39 years old. Eligibility criteria were fulfilled as previously described in Bernstein, et al. This trial is registered with ClinicalTrials.gov, number NCT03300050(Bernstein et al., 2020).

HA-specific B cell sorting and single cell processing

Antigen-specific memory B cells (CD19+IgMlowCD27+CD38low HA+) were bulk sorted and partitioned into nanoliter-scale Gel Bead-In-EMulsions (GEMs) to achieve single cell resolution according to the manufacturer's instruction (10X Genomics). The sorted single cells were processed according to 5' gene expression and B cell Immunoglobulin (Ig) enrichment instruction to prepare the libraries (10X Genomics). The Cellranger Single-Cell Software Suite (version 3.0) was used to perform sample de-multiplexing, barcode processing, and single-cell 5' and V(D)J counting. Cellranger mkfastq was used to de-multiplex raw base call (BCL) files from the HiSeq 4000 or Nextseq sequencer into sample-specific fastq files. Subsequently, reads were aligned to

the GRCh38 human genome, the Cellranger counts and Cellranger vdj package were used to identify gene expression and assemble V(D)J pairs of antibodies.

The Seurat package (version 3.0) was used to further analyze 5' gene expression and integrated data. We utilized Seurat v3 to perform integrated analyses, including identifying cell population that present in all datasets, anchors identification and identification of common cell population allowing comparative analyses. For quality control, we kept all genes expressed in more than 0.1% of the data and all cells with at least 200 detected genes. The fraction of mitochondrial genes was calculated for every cell, and cells with a mitochondrial fraction out of 95% 95% confidence interval were filtered out. Two sources of unwanted variation: UMI counts and fraction of mitochondrial reads were removed with RegressOut function, aiming to exclude cells with a clear outlier number of genes detected as potential multiplets as well as dead/dying/unhealthy cells. Expression measurements for each cell were normalized by total expression and then scaled to 10,000 after log normalization. After detection of variable genes across the single cells and removing unwanted sources including technical noise, batch effects, biological sources of variation such as cell cycle stage, linear dimensional reduction (PCA) was performed to determine statistically significant principal components. Populations of cells was then predicted using analytical tools such as UMAP. The differential gene expression was testing for each subset and combines the p-values using meta-analysis methods from the MetaDE R package(Butler et al., 2018; Stuart et al., 2019). B cell clonal diversity and expansion within and/or across subjects were analyzed by using HCD3 (third complementarity determining region of the rearranged heavy chain variable region

gene) similarity. The software Vgenes used within the lab permits the detection and distinction of numerically expanded B cell clones from distinct cell subsets.

HA probes

Each HA recombinant protein with a mutation (Y98F) in the receptor binding site (RBS) was biotinylated (Thermo, cat# A39259, EZ-Link™ NHS-PEG4-Biotin). A unique barcoded streptavidin labeled either with or without fluorophore phycoerythrin (PE) (Biolegend, TotalSeq™-C 0951 PE Streptavidin-cat#405261, TotalSeq™-C 0952 PE Streptavidin-cat#405263, TotalSeq™-C 0953 PE Streptavidin-cat#405265, TotalSeq™-C 0954 PE Streptavidin-cat#405267, TotalSeq™-C 0955 PE Streptavidin-405269, TotalSeq™-C 0971 Streptavidin-cat#405271,TotalSeq™-C 0972 Streptavidin-cat#405273) was mixed with biotinylated HA. Total volume of biotinylated HA is 0.5ug and streptavidin-barcode with PE or without PE is 2ug in 20 ul. 1/5 of the barcoded streptavidin was added to the HA antigen every 20 minutes at 4°C for 5 times. Next, free biotin was added to saturate the streptavidin (Thermo Scientific, Pierce™ Biotin, cat#29129), producing a final concentration of 0.1 ug/ul for the HA protein. 0.5 ug of the final conjugated HA was used as probe to stain $1x10^6$ cells in 100 ul suspension buffer.

Monoclonal antibody expressing and purification

Monoclonal antibodies were generated per the procedure that is detailed in Guthmiller et al., 2019(Guthmiller et al., 2019) and Wrammert et al., 2008, Smith et al., 2009(Smith et al., 2009). Once peripheral blood was obtained from each subject, a cH5/1 chimeric

protein was used to bait-sort memory B cells which were collected 28+ days after vaccination. The lymphocytes were enriched and isolated using RosetteSep. B cells that bound chimeric H5/1 were sorted from the subjects 28 days after vaccination with cH5/1. The heavy and light chain sequences of the monoclonal antibodies were generated using single cell RNA sequencing data collected from CHA-baited B cells (IDT). After generation, they are cloned into either the human kappa chain, the human lambda expression vectors, or the human IgG1. HEK293T cells were co transfected with polyethylenimine (PEI, Polysciences). After 4 to 5 days, the supernatant liquid containing the secreted antibody was collected. After the supernatant liquid was collected, it was purified with Protein A beads (Pierce). Multiple generations of the same monoclonal antibody were produced in order to ensure that data gathered using these antibodies could be reproduced with accuracy.

Enzyme linked immunosorbent assay (ELISA)

Monoclonal antibodies were generated per the procedure that is detailed in Guthmiller et al., 2019(Guthmiller et al., 2019) and Wrammert et al., 2008, Smith et al., 2009(Smith et al., 2009). Once peripheral blood was obtained from each subject, a cH5/1 chimeric protein was used to bait-sort memory B cells which were collected 28+ days after vaccination. The lymphocytes were enriched and isolated using RosetteSep. B cells that bound chimeric H5/1 were sorted from the subjects 28 days after vaccination with cH5/1. The heavy and light chain sequences of the monoclonal antibodies were generated using single cell RNA sequencing data collected from CHA-baited B cells (IDT). After generation,

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Plaque neutralization assay

The PRNT50 Assays were performed as described in Wrammert et al., 2011(Wrammert et al., 2011), with minor alterations. The plates were coated with MDCK London Line cells. The cells were incubated for 48hours once the agar gel was inserted into the well. After developing with crystal violet solution, the plaques were tallied. The concentration of the antibody that was recorded was the concentration was produced a 50% reduction in plaques compared to the negative control. The results were analyzed using Graphpad Prism software.

Antibody-dependent cellular cytotoxicity (ADCC) assay

Madin-Darby Canine Kidney cells in complete Dulbecco's modified Eagle's medium (DMEM with 10 mM HEPES, 100 units ml-1 Penicillin, 100 µg ml-1 Streptomycin, and 10% FBS; Gibco) were plated at a concentration of 2.5 x 10^6 cells/ml in a 96-well white-walled dish (Costar). Sixteen hours after plating, cells were rinsed with PBS and infected

with an MOI of 5 of A/Netherlands/602/2009 H1N1pdm virus in the absence of TPCK trypsin in Ultra-MDCK media (Lonza). Infection was allowed to proceed for 24 hours before removing media and adding 25 µl of assay buffer (RPMI 1640 with 4% Ultra-Low IgG FBS; Gibco). Monoclonal antibodies were diluted to 30 µg/ml in assay buffer and serial-diluted 3-fold. 25 µl of antibody dilution was added to each corresponding well on the assay plate, including a positive control antibody (CR9114). Human FcγRIIIa expressing Jurkat effector cells (Promega) were diluted to 3 x 10^6 cells/ml and 25 µl was added to each well. The plate was allowed to incubate for 6 hours at 37°C before equilibrating to room temperature. BioGlo luciferase (Promega) was brought to room temperature before adding 75 µl to each well and measuring luminescence using a Synergy H1 hybrid multimode microplate reader (BioTek). EC50 values were calculated after fitting curves using the [Agonist] vs. response -- Variable slope (four parameters) formula using Prism v8.0 (Graphpad).

Influenza protein microarray assay

Influenza virus protein microarray (IVPN) was measured per the procedure that is described in Meade et al., 2017(Meade et al., 2017). Recombinant influenza virus HAs were spotted onto epoxysilane-coated glass slides (Schott, Mainz, Germany) to create influenza virus protein microarrays (IVPM). Each slide contains 24 identical arrays comprised of 13 HAs diluted in 0.1% milk PBS and spotted in triplicate at a volume of 30nl per spot and a concentration of 100 ug/ml. Arrays were vacuum-packed and stored at -80°C until use. On the day of the assay, slides were allowed to warm to room temperature

before being incubated in a humidity chamber at 95-98% relative humidity for 2 hours to bind proteins to the slide and inactivate expoxysilane residues not in contact with recombinant HAs. Slides were then inserted into 96-well microarray gaskets (Arrayit, Sunnyvale, CA, USA), physically dividing each slide into 24 arrays, which were blocked with 220 ul 3% milk PBS containing 0.1% Tween 20 (PBS-T) for 2 hours. After the blocking solution was removed from the arrays, mAbs diluted in 1% milk PBS-T were incubated with the arrays at a volume of 100 ul, and diluted 1:10 across three arrays. Germline derived mAbs were started at a concentration of 20 mg/ml, and mAbs from memory B cells were started at 1 mg/ml. The arrays were then washed 3 times with 220 ul PBS-T, and 50 ul of Cy5-labeled anti-human IgG secondary antibody diluted 1:3000 in 1% milk PBS-T was added to each array and incubated for 1 hour. After the secondary antibody solution was removed, each array was washed 3 times with 220 ul PBS-T and slides were removed from their gaskets. After being rinsed with PBS-T and deionized water, slides were dried with an air compressor. Arrays were then imaged with a Vidia microarray scanner (Indevt, Boulder, CO, USA) at an exposure time of 1000 ms. Area under the curve was calculated from the median spot fluorescence, as total peak area with a minimum threshold of 0.04.

Statistical analysis

All statistical analyses were performed with R or Prism software (Graphpad Version 8.0). P values less than 0.05 were considered significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$. The sample sizes for each subject from the universal influenza virus vaccine and seasonal

influenza virus vaccine can be found in Supplementary Fig 7. The number of monoclonal antibodies are indicated in the corresponding figure legends or in the figures.

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