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Evaluation of Autoreactivity in VH4-34-Containing Antibodies from Human B-1 VS B-2 Cells

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EVALUATION OF AUTOREACTIVITY IN VH4-34-CONTAINING ANTIBODIES FROM HUMAN B-1 VS B-2 CELLS

by

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B-1 cells are a unique population of lymphocytes, with distinct phenotypic and functional characteristics in comparison to conventional B-2 cells. B-1 cells are thought to be more closely related to the innate immune system than the adaptive, and B-1 cells constitutively secrete antibody that is recognized as broadly reactive with low levels of autoreactivity. The antibody heavy chain segment, VH4-34, is overutilized by the human B-1 cell population. VH4-34 antibodies are associated with autoreactivity, although not all VH4-34 antibodies are autoreactive. The primary objective of this study was to determine whether autoreactive forms of VH4-34 antibodies are localized only to the B-1 cell population, or whether VH4-34 antibodies are autoreactive wherever they are found. We obtained cells from healthy human donors, sorted cells individually, PCR amplified antibodies, and then cloned and expressed the antibodies. The monoclonal antibodies were then tested against a series of autoantigens to determine if B-1 or B-2 cells produced more autoreactive VH4-34-containing antibodies. Results suggested that B-1 cells did produce more autoreactive/polyreactive VH4-34-containing antibodies than memory B-2 cells, though the population observed was small and more samples are required in order to provide statistical significance.

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Introduction

B-1 Cells

B-1 cells are a distinct subpopulation of B lymphocytes that behave differently from, and appear phenotypically different than, conventional B-2 cells. They are unique from B-2 cells in that they more closely resemble the innate immune system than the adaptive. They do not need foreign antigen for antibody stimulation and thus spontaneously secrete natural antibody, predominantly IgM, which provides a baseline level of protection before a pathogen even enters the body (Montecino-Rodriguez & Dorshkind, 2012) (Rothstein et al., 2013) (Baumgarth, 2016).

B-1 cell antibodies (Abs) are known for their broad specificity to many antigens found naturally in the body (Montecino-Rodriguez & Dorshkind, 2012). B-1 cells also act as housekeepers in that they clean up cellular debris and molecular toxins from the break-down of cells, thereby providing a homeostatic balance to the body. They can also help defend against microbial pathogens and foreign invaders prior to the body's adaptive immune response, as these B-1 cells are present prior to birth and can secrete antibodies, coined "natural antibodies", spontaneously and very early in development (Rothstein & Quach, 2015) (Rodriguez-Zhurbenko et al., 2019).

Often times mice or other non-human organisms are used as models to study biological systems, however, observations in other species do not always correlate directly to *Homo sapiens.* In the murine system, B-1 cells are known to be derived from a separate progenitor than B-2 cells, resulting in a separate lineage (Rothstein et al., 2013). Also, in the murine system, the

key identifying phenotypic marker for the B-1 population is CD5, a marker that is typically present on T cells (there are actually two subpopulations of B-1 cells in the murine system: B-1a that are $CD5^+$ and B-1b that are $CD5^-$). Mice also have germ line-like antibodies that arise from B-1 cells with little somatic mutation or N-addition (Rothstein & Quach, 2015).

Not all of these things can be said for the human system. There have been a few theories as to whether the B-1 cell population arises separate from the B-2 cell population via different lineages or if they share a lineage and one cell population is likely converted to another through maturation. Although there has been a separate progenitor found in the mouse, recent results in the last few years have suggested that in humans, these populations of cells share a progenitor as human $Lin^-CD34^+CD38^{lo}$ stem cells reconstituted both cell populations (Quách et al., 2016). Yet, this area still remains a highly debated and controversial subject.

The CD5 surface marker used to be the essential in distinguishing B-1 cells from other subpopulations until it was noted that human $CD5^+$ cells were found in human chronic lymphocytic leukemia (CLL), as well as representing 15-30% of circulating B cells and up to nearly 75% of umbilical cord blood B cells in healthy individuals, which was remarkably high in comparison to the number of cells that were $CD5^+$ in mice (Rothstein et al., 2013) (Rothstein & Quach, 2015). In addition to an excess number of $CD5^+$ cells present, $CD5^-$ B cells were also secreting autoreactive antibodies much like their $CD5^+$ counterparts, further indicating that the presence of the marker was not indicative of a separate cell population (Rothstein & Quach, 2015). Furthermore, CD5 was also found on human B-2 cell populations (transitional, pre-naive,

and activated B cells) indicating that it was not a key identifying marker for human B-1 cell populations alone (Griffin et al., 2011).

It was also discovered that human CD5+ B cells differ in additional ways from the murine system, in that human CD5+ B cells have higher levels of somatic hypermutation (SHM) and junctional diversity. The increased levels of junctional diversity could be due to the presence of terminal deoxynucleotidyl transferase (TdT) in humans that is missing in mice in earlier stages of hematopoietic cell development (Mrczek et al., 2014). CD5 was clearly not a good marker to use to distinguish B-1 cells from conventional B-2 cells in the human B cell repertoire.

The inability to easily identify the B-1 cell population phenotypically in humans led to questions regarding the existence in humans of this population. However, phenotype is only one way to define a cell type. By using a set of distinctive functional features of mouse B-1 cells (spontaneous secretion of IgM, T cell stimulation, and chronic intracellular signaling), a series of experiments were performed to “reverse engineer” the phenotypical profile of the human B-1 cell population which helped serve as the template for which cells were classified as B-1 cells for the single cell sort in this work (Rothstein et al., 2013).

Based upon the results of these experiments, B-1 cells are phenotypically described as $CD20^{+}CD27^{+}CD38^{low}CD43^{+}$ which differs from the memory ($CD27^{+}CD43^{-}$) and naïve ($CD27^{-}CD43^{-}$) profiles. The B-1 cell profile is also different from the plasma cell or plasmablast profile as human B-1 cells express CD20 which is lost during differentiation to the plasmablast stage, and human

B-1 cells do not express CD138 which is acquired by these differentiated plasma cells. Also excluded from the B-1 cell profile are $CD19^{+}CD20^{+}CD27^{+}CD38^{+}$ "pre-plasmablast" cells that share a similarity to plasmablasts in CD27 and CD38 expression, to eradicate the possible confusion between the B-1 cell and plasmablast/pre-plasmablast populations (refer to figure 1 for flow cytometry gating strategy for single cell sorting) (Griffin et al., 2011) (Rothstein & Quach, 2015) .

Antibodies

Antibodies are the proteins that are secreted from B cells to protect the body from foreign invaders or pathogens. Typically, these proteins arise once B cells have been previously exposed to an antigen of some sort, whether it be bacterial (i.e. *Streptococcus pneumoniae)* or viral (i.e. influenza), but in the case of B-1 cells, these proteins can be produced spontaneously without prior exposure to foreign antigens.

Each antibody has a basic "Y" shape in which two identical heavy chains (HC) (composed of variable (V), diversity (D), and joining (J) segments) and two identical light chains (LC) (composed of variable (V) and joining (J) segments) are joined. Each one of these chains has a constant and a variable region. The variable regions of the two chains come together to form the antigen binding site that determines the specificity of the antibody. This antigen binding site consists of 6 hypervariable loops (complementarity determining regions – CDRs), 3 of which come from each of the heavy and light chains (Almagro et al., 1997) (Foreman et al., 2007).

At the center of this antigen binding site is the CDR3 region, in which the specific CDR3 region from the HC (CDR-H3) is the most variable component of the antigen binding region as it is the product of V(D)J recombination and N-region addition (Mroczek et al., 2014). It plays a central role in specifying antigen binding. This characteristic region has been well studied and many distinctive patterns have been elucidated to help predict binding patterns of specific antibodies. Beyond specifying recognition of foreign antigens, some of these CDR3 patterns are associated with autoreactivity. An example includes identifying that arginine is often found in CDR-H3 regions of dsDNA autoantibodies (antibodies that attack self-antigens) (Silva-Sanchez et al., 2015).

Other features of CDR3 regions have been characterized in that the average CDR3 length differs in disease (Foreman et al., 2007). While the CDR3 length alone cannot predict if the antibody will be autoreactive, longer CDR3 regions have been associated with autoreactive or polyreactive antibodies (average length consists of approximately 15.5 ± 3.2 amino acids) (Shi et al., 2014). In addition to length, the presence of positively charged amino acids in the CDR-H3 region has also been associated with autoreactive or polyreactive antibodies (Wardemann et al., 2003) (Huang et al., 2018). It is also key to note that as the cells mature or are exposed to antigen, they typically produce antibodies that have decreased or shortened CDR3 lengths that increase in charge (Silva-Sanchez et al., 2015) (DeKosky et al., 2016). This would lead one to believe, by previous observations, that antibodies produced from memory cells or other B-2 cells, are more likely to have shorter CDR3s than antibodies that are produced from B-1 cells that have not been exposed to antigens previously.

The Human Antibody Repertoire

The human antibody repertoire is diverse, and for good reason. The body is pelted with all sorts of foreign matter, be it viruses or bacteria, that the system needs to be able to defend itself against. In addition to simply being able to protect us from the these threats, the body needs to be able to adapt to these new exposures when they happen, as there is no way our body would be able to predict every single antigen that exists that we could be exposed to before it happens.

There are a few main mechanisms by which the repertoire continues to expand. The first is through V(D)J recombination that occurs in early stages of B cell development, through the random rejoining of different gene segments in the V-D-J regions (Wardemann & Nussenzweig, 2007). Due to the many possible combinations in which these segments could join together, the body has the ability to form an antibody that could react with virtually anything (Andris et al., 1995). On the other hand, that also means that with all of these possible combinations, it is highly likely that a great deal of the antibodies that are produced are also autoreactive. Many of them actually recognize nuclear self-antigens and are polyreactive at this stage (Tiller et al., 2007).

The second mechanism that expands the body's antibody repertoire is through sequence variation via random, non-template-encoded nucleotide addition (N-addition) at the V-D and D-J junctions typically of the heavy chain. Terminal deoxynucleotidyl transferase (TdT) can add up to 20 nucleotides to the single-stranded ends of the coding DNA significantly altering the genetic sequence of the produced antibody (Kumar, Abbas, & Aster, 2015).

Another mechanism that contributes to the body's ever-growing antibody repertoire is somatic hypermutation (SHM) when mature B cells are responding to antigen challenge. These random point mutations during SHM create polyreactive and self-reactive antibodies as well (Tiller et al., 2007). While antibodies are theoretically only supposed to bind to one antigen, according to the lock and key theory, in reality, some antibodies react to many antigens resulting in polyreactivity (Casali & Notkins, 1989). Polyreactive antibodies have the ability to recognize several epitopes, although normally with decreased affinity compared to standard non-polyreactive antibodies. In addition to causing polyreactivity, SHM can also cause an increase in affinity of autoreactive antibodies (Foreman et al., 2007).

Autoreactivity

Autoreactivity, the break in tolerance in a body's immune system, has been implicated in several immune diseases and disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). This occurs when B cells that recognize self-epitopes produce antibodies against antigens located naturally in the body. These cells typically produce antibodies at a high rate and have high affinities for these self-epitopes (Corsiero et al., 2014; Richardson et al., 2013; Malkiel et al., 2016). These antibodies are often directed against other cell surface markers or against other naturally occurring antigens present in the body. In the cases of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), and scleroderma, systemic autoantibodies are present throughout the system that react to DNA, RNA, histone, ribonucleoprotein particles, antinuclear antibodies (ANAs) and other antigens (Stollar, 1991).

It is important to note that autoreactive antibodies are present in low levels in healthy serum (Stollar, 1991). The mere presence of autoreactive antibodies does not result in clinical significance. It is the high abundance and the type of antibody that determines association with clinical illness. The production of the pathogenic antibody alone may not be that significant for the disease to even develop but may in turn provide an indication of underlying disease, not necessarily be the cause of it (Foreman et al., 2007).

The body must have a means to control the number of autoreactive cells that are present in the body. While it is unknown for certain the percentage of cells that are eradicated through B cell maturation, observations indicate that a large number of the self-reactive B cells are lost through the process. It is estimated that 55-75% of all antibodies emerging in the bone marrow are autoreactive which can be attributed to the failure of the body's natural checkpoints to eliminate or anergize the cells, or to the random N-additions or SHM in the CDR-H3 region that allow autoreactive antibodies to be reintroduced into the system (Wardemann et al., 2003).

To prevent the escape of autoreactive antibodies into the system, the body has 3 natural mechanisms that account for the silencing of most autoreactive B cells during development: Receptor editing, clonal deletion, and anergy.

Receptor editing includes a secondary rearrangement of light chains with heavy chains in an attempt to alter the antigen-binding site's specificity therefore preventing the persistence of

autoreactive receptors (Foreman et al., 2007). This process contributes to the exclusion of the majority of autoreactive clones (20%-50%)(Wardemann & Nussenzweig, 2007) (Hoffman et al., 2016)

Clonal deletion is only a secondary line of defense for the cells that are not successfully rearranged from their autoreactive ways during receptor editing. If a B cell receptor recognizes self-antigen with a strong enough signal, the cell triggers apoptosis (negative selection) to prevent the cell from producing antibodies against itself (Wardemann & Nussenzweig, 2007) (Hoffman et al., 2016).

Anergy is the state of hypo-responsiveness where the cells typically survive for a shortened duration of 1-5 days (Hoffman, Lakkis, & Chalasani, 2016). The cells become unresponsive to antigen stimulation so that no antibody production can occur, and they are often prone to apoptosis as they are essentially useless cells. Their numbers are then rapidly lost from the cell repertoire (Wardemann & Nussenzweig, 2007).

Most often when autoantibodies are present in an autoimmune disease they are generally IgG (Foreman et al., 2007). They are not highly cross-reactive, react with high affinity, and are encoded by genes that have undergone mutations from the germline through V(D)J recombination or somatic hypermutation, all of which is typically quite different from natural antibodies (Stollar, 1991), at least as delineated in the mouse system. Patients that have these autoimmune diseases often frequently produce many autoreactive antibodies that react to

various antigens, although not all of the antibodies produced may have the same specificity or are equally as pathogenic (Foreman et al., 2007).

VH4-34

VH4-34 is a gene that codes for a variable region of the heavy chains of immunoglobulins. The VH4-34-derived immunoglobulins constitute less than 1% of total serum immunoglobulins in humans but can increase in response to infections (Pugh-Bernard et al., 2001)(Cambridge et al., 2014). While it is known that VH4-34-derived antibodies have a higher chance of being autoreactive than those that are derived from other variable heavy chain genes, it is unknown which types of cells predominantly produce these autoreactive antibodies and in particular, if they are segregated into a single cell subpopulation, such as the B-1 cell subpopulation. (Milner et al., 2005).

The development of systemic lupus erythematosus (SLE) has been studied greatly and has been largely attributed to the autoreactivity of antibodies produced in the body. VH4-34-derived antibodies have been specifically implicated in this development of SLE as a high proportion of the autoreactive antibodies are VH4-34 (Mockridge et al., 1998) (Milner et al., 2005). VH4-34-derived antibodies are also known to be autoreactive towards the I/i of red blood cells (RBCs) that are an antigenic target of pathogenic autoantibodies in cold-agglutinin (CA) disease. The many additional targets of VH4-34-containing Abs include bacterial LPS, chromatin, cardiolipin, and DNA (Alcéna et al.,2013) (Cambridge et al., 2014) (Sanz, 2014).

Recent results have shown that the VH4-34 gene is overrepresented in the normal adult B-1 cell antibody repertoire as compared to other B cell populations, where it is present in lesser numbers (Rodriguez-Zhurbenko, et al., 2019). Considering that VH4-34 is known to encode autoreactive antibody, and that B-1 cells are thought to generate autoantibodies, the question is raised as to whether VH4-34 antibodies are autoreactive in every cell population in which they appear, or whether they are autoreactive only in the B-1 cell population. We believe that B-1 cells are responsible for producing more VH4-34-containing autoreactive antibodies than other B cell populations.

It is for these reasons that we focused on identifying which cells produced the VH4-34-derived autoreactive antibodies. We know that not all VH4-34-containing antibodies are autoreactive, and we know that naturally occurring autoreactive antibodies are present in healthy human serum, but we do not know if significantly more autoreactive VH4-34 antibodies come from B-1 cells than from B-2 cells. We obtained cells from healthy human donors, sorted the cells individually (figure 1), and cloned and expressed the antibodies. The monoclonal antibodies were then tested against a series of antigens to determine if B-1 or B-2 cells produced more autoreactive VH4-34-containing antibodies. We were able to identify that B-1 cells produced more antibodies that were autoreactive than B-2 antibodies (although not significantly so).

Methods

Acquisition of blood and single cells was performed by other members of the lab. Their procedures are outlined in the sections entitled “B Cell Isolation” through “Single Cell Sorting.”

B Cell Isolation

Human blood samples were collected at Western Michigan University Homer Stryker M.D. School of Medicine from healthy donors and delivered to the lab where the samples were processed immediately upon arrival to prevent damage to the lymphocytes in the sample. Human Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using standard density gradient separation in Lymphocyte Separation Medium (Corning, Ref 25-072-CV).

Fresh blood was placed in heparin-coated collection tubes (Green Cap BD Vacutainer™, BD 367874) at 10 mL/vial. These tubes were spun at 1,800 RPM for 10 minutes at room temperature. The majority of the plasma was removed from the sample and the first Buffy Coat (thin white layer between the red blood cell and plasma fractions) was collected and placed into a 50 mL conical tube. Equal volume of standard wash medium (RPMI with penicilin/streptomycin, and L-glutamine) was added to the Buffy Coat fraction then underlayed with an equal volume of Lymphocyte Separation Medium. The sample was centrifuged at 2,000 RPM for 20 minutes (with the break off) at room temperature. The second Buffy Coat was transferred to a new 50 mL conical tube and centrifuged for 10 minutes at 1,800 RPM at room temperature. The supernatant was aspirated from the sample and the pellet was resuspended in 50 mL of standard wash medium. The sample was counted and immediately followed by B cell enrichment or frozen for

future additional processing.

B Cell Enrichment

B cells were enriched by magnetic bead selection using the EasySep Human CD19 Positive Selection Kit according to the manufacturer's instructions.

Cell Staining

CD19 enriched B lymphocytes were washed with 3 mL of sort/wash/staining buffer (PBS, 0.5% BSA, and 2 mM EDTA). The cells were centrifuged and the pellet was resuspended in 2.5 µL of normal mouse serum per 1 x 10^6 cells, to block Fc receptors on the cell surface and the cells were incubated for 5 minutes on ice.

The antibody staining cocktail was freshly prepared using optimized antibody concentrations. Components of the staining cocktail include the following anti-human mouse monoclonal antibodies: anti-CD3-BV605 (Clone HIT3a; BD Biosciences 564712), anti-CD4-BV605 (Clone SK3; BD Biosciences 565998), anti-CD19-APC-Alexa700 (Clone J3-119; Beckman Coulter A78837), anti-CD20-Pacific Blue (Clone B9E9; Beckman Coulter A74777), anti-CD27-PE (Clone M-T271; BD Biosciences 557330), anti CD38-PC5.5 (Clone LS198-4-3; Beckman Coulter A70205), anti-CD43-FITC (Clone DFT1; Beckman Coulter IM3264U), and Aqua LIVE/DEAD™ viability stain.

The cocktail was then added to the concentrated cell suspension and incubated on ice for 30 minutes, protected from light. The cells were washed with sorting buffer and separated via

centrifugation for 10 minutes at 1,200 RPM at 4°C. The cell pellet was resuspended with sorting buffer and the wash repeated. Washed cells were resuspended at a final concentration of 10 x 10^6 cells/mL. Stained cells were acquired on the BD INFLUX cell sorter. Gates were created around populations of cells based on the antibody staining profile of the sample according to the gating strategy shown below in figure 1.

Single Cell Sorting

B-1 and Memory cells were sorted 1 cell per well into 96 well plates that contained lysis buffer (IgePAL, RNAsa Out, Carrier RNA, DTT, and 5x First Strand/ RT III Buffer). The plates were sealed and then flash-frozen on dry ice and stored at -80^{o}C until the samples were ready to be processed.

Figure 1 illustrates the gating strategy that was used during one of the single cell sorts to obtain a single donor's healthy B cells. Each consecutive plot indicates a more restricted set of criteria to identify single cells of interest (B-1 and Memory).

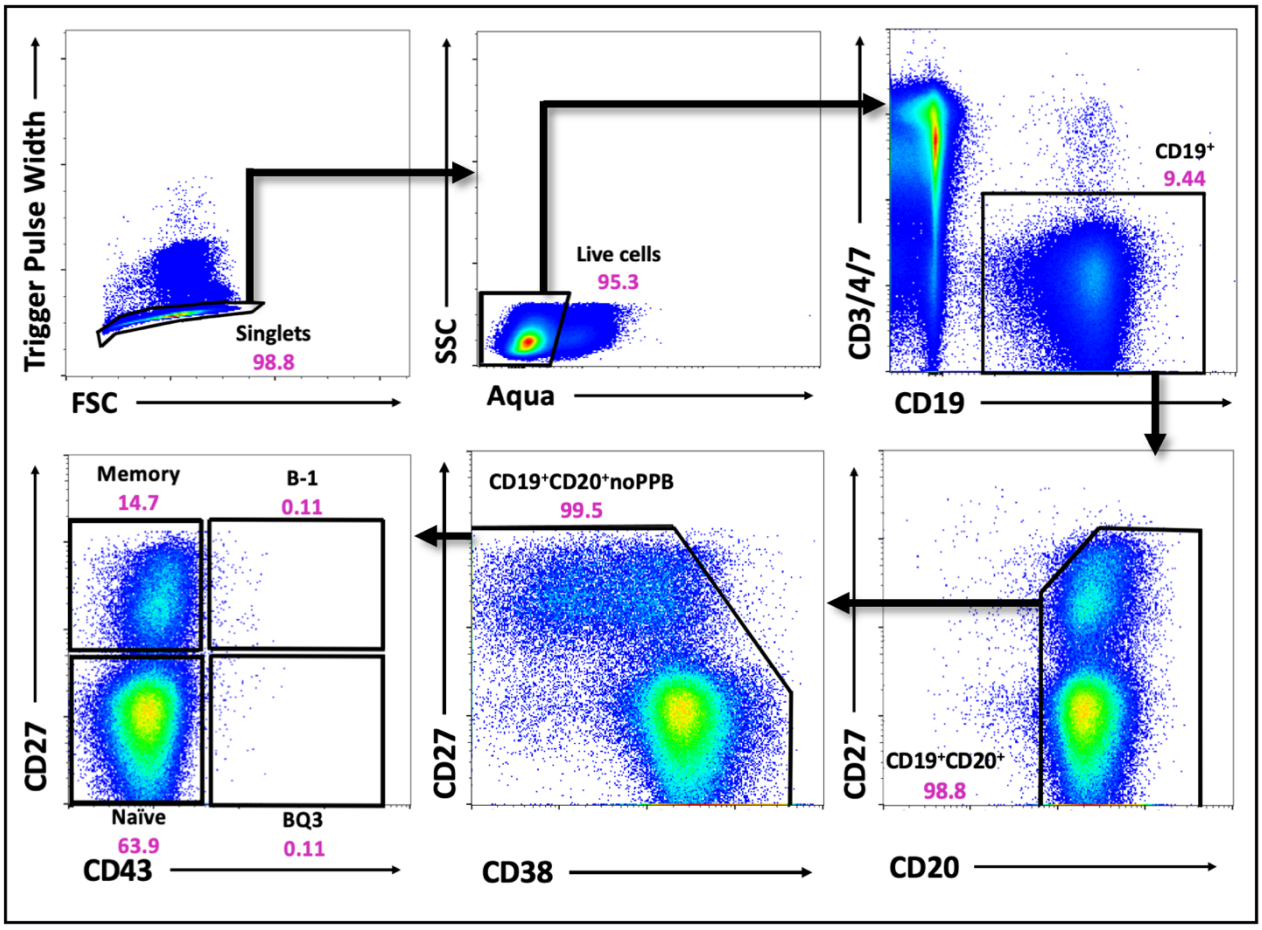

Figure 1. Novel gating strategy designed and implemented by our lab to separate human B-1 cells, memory B cells, and mature (naïve) B cells from whole blood. The trigger pulse width and forward scatter allows for separation of singlets from doublets. Side scatter vs Aqua LIVE/DEAD dye is used to obtain only living cells. CD3/4/7 are used as a dump channel for T cells vs $CD19^+$ B cells. $CD19^+CD20^+$ are next sorted to exclude plasmablasts that are $CD19^+CD20^-$. $CD19^+CD20^+CD27^{hi}CD38^{hi}$ (preplasmablasts) are next excluded. Among $CD19^+CD20^+CD38^{mod}$ B cells, naïve, memory and B-1 cell populations were single cell sorted based upon expression of CD27 and CD43, as indicated.

cDNA Synthesis

Once cells were sorted into the 96 well plates, a master mix of random hexamers, 10mM dNTP mix, and SuperScript III RT was added to the cell lysates to generate cDNA. The plates were placed in an Applied Biosystems ProFlex PCR System thermocycler with the following run time protocol: 42°C for 10 mins, 25°C for 10 mins, 50°C for 10 mins, 94°C for 5 mins, and held at 4°C.

Single Cell RT-PCR and Gene Amplification

There are two rounds of semi-nested PCR required for each chain amplified (IgG, IgM, Igκ and Igλ) (Tiller et al., 2008). For the first-round of semi-nested PCR, a master mix of 10x PCR buffer, 10 mM dNTP mix, HotStarTaq Plus, PCR grade water, 25 µM of chain-specific forward primer(s), and 25 µM of chain-specific reverse primer(s) (table 1) was prepared on ice while the cDNA was thawed on ice, if it was frozen. A volume of 23.75 µL of master mix was added to each well of a new 96 well plate, on ice, and 1.25 µL of cDNA was then added to the plate. The plates were immediately sealed and spun briefly at 1,200 rpm. The plate was then placed in the thermocycler with the following run time protocol: 94°C for 5 mins followed by 50 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 55 seconds at 72°C, then followed by 7 minutes at 72°C and held at 4°C.

For the second-round of semi-nested PCR, a master mix of 10x PCR buffer, 10 mM dNTP mix, HotStarTaq Plus, PCR grade water, 12.5 µM of chain-specific forward primer(s), and 25 µM of chain-specific reverse primer(s) (table 1) was prepared on ice while the first-round PCR products were thawed on ice, if they were frozen. 23 µL of master mix was added to each well of a new 96 well plate, on ice, and 2 µL of the first-round PCR products were then added to the plate. The plates were immediately sealed and spun briefly at 1,200 rpm. The plate was then placed in the thermocycler with the following run time protocol: 94°C for 5 mins followed by 50 cycles of 30 seconds at 94°C, 30 seconds at 57°C, and 45 seconds at 72°C, then followed by 7 minutes at 72°C and held at 4°C.

To specifically examine VH4-34-containing antibodies, a specific VH4-34 primer was used as a forward primer instead of the originally published 13-primer mix (Tiller et al., 2008), after determining through a series of experiments that only using the one primer would not skew the number of VH4-34 amplifications that occurred which in turn significantly streamlined the PCR process greatly.

After second-round amplification of heavy chains, the plates were analyzed on the Qiagen QIAxcell Advanced system using the QIAxcel DNA Screenig Kit (2400) cartridge to determine which wells amplified. The wells that amplified were sequenced using the VH4-34 primer either in house using our SeqStudio Sanger sequencer or sent to Genewiz to confirm the samples were VH4-34.

Igκ amplification required an additional series of steps in order to add proper restriction enzyme-specific cut sites during amplification. After the initial second-round of semi-nested PCR with PanVK as the forward primer, the plates were analyzed on the QIAxcell to determine which wells amplified. These samples were sequenced using the PanVK primer either in house using our sequencer or sent to Genewiz.

Once sequencing information was obtained for Igκ, the sequences were aligned with the restriction enzyme-specific primers using Serial Cloner software to determine the best primers to use to reamplify the second-round of semi-nested PCR. When the best-aligned primers were identified (figure 2), the second round of amplification was performed again using the newly

identified forward and reverse primers. After amplification, samples were once again analyzed on the QIAxcell to identify which samples amplified with the new primers.

Figure 2. Example of an Igκ sequence alignment to restriction enzyme-specific primer.

Table 1. Primer Sequences

Forward Primer	5' - 3' sequence
5´ L-VH 1	ACAGGTGCCCACTCCCAGGTGCAG
5´ L-VH 3	AAGGTGTCCAGTGTGARGTGCAG
5´ L-VH 4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5´ L-VH 5	CAAGGAGTCTGTTCCGAGGTGCAG
5´ AgeI VH1 (incl.: 1-46)	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG
5´ AgeI VH1/5 (incl.: 5-51)	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
5´ AgeI VH 1-18	CTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
5´ AgeI VH 1-24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
5´ AgeI VH3 (incl.: 3-49, 3-7, 3-74, 3-53, 3-21)	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
5´ AgeI VH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
5´AgeI VH3-33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
5´ AgeI VH 3-9	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
5´ AgeI VH4 (incl.: 4-59, 4-31)	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
5´ AgeI VH 4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
5´AgeI VH4-39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
5´ AgeI VH 6-1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
5´ L Vk 1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5´ L Vk 3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5´ L Vk 4	ATTTCTCTGTTGCTCTGGATCTCTG
5` Pan Vk	ATGACCCAGWCTCCABYCWCCCTG
5´ AgeI Vk 1-5 (incl.: 1-6,1-8, 1-12, 1-16, 1-17, 1-27, 1-33, 1-39)	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
5´ AgeI Vk 1-9	TTGTGCTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
5´ AgeI Vk 1D-43	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
5´ AgeI Vk 2-24 (incl.: 2-29, 2-40)	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
5´ AgeI Vk 2-28	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC
5´ AgeI Vk 2-30	CTGCAACCGGTGTACATGGGGATGTTGTGATGACTCAGTC
5´ Age Vk 3-11	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
5´ Age Vk 3-15	CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
5´ Age Vk 3-20	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
5´ Age Vk 4-1	CTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
5´ Ab-sense	GCTTCGTTAGAACGCGGCTAC

Reverse Primer	5' - 3' sequence
3´ Cμ CH1 (mu)	GGGAATTCTCACAGGAGACGA
3´ Cg CH1 (gamma)	GGAAGGTGTGCACGCCGCTGGTC
3´ IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC
3´ SalI JH 1/2	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG
3´ SalI JH 3	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG
3´ SalI JH 4/5	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG
3´ SalI JH 6	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG
3´ Ck 543	GTTTCTCGTAGTCTGCTTTGCTCA
3´ Ck 494	GTGCTGTCCTTGCTGTCCTGCT
3´ BsiWI Jk 1/4	GCCACCGTACGTTTGATYTCCACCTTGGTC
3´ BsiWI Jk 2	GCCACCGTACGTTTGATCTCCAGCTTGGTC
3´ BsiWI Jk 3	GCCACCGTACGTTTGATATCCACTTTGGTC
3´ BsiWI Jk 5	GCCACCGTACGTTTAATCTCCAGTCGTGTC

Vector Cloning

The amplified samples were purified using the Takara PCR/Gel purification kit and double digested with their respective restriction enzymes for 2 hours at 37°C with a 20-minute inactivation at 65°C. Heavy chains were digested with AgeI-HF and SalI-HF, Igκ was digested with AgeI-HF and BsiWi-HF, and Igλ was digested with AgeI-HF and XhoI.

In addition to the samples having to be digested, the plasmid backbones that they were inserted into had to be digested as well using the same chain-specific restriction enzymes. Each chain also had specific backbones or vectors that were used: heavy chain (HC) was AbVec2.0-IGHG1, Igκ was AbVec1.1-IGKC, Igλ was AbVec1.1-IGLC2-XhoI.

Digested samples and plasmids were run on 1% agarose gels made with 1x TAE at 120 mV for approximately 40 minutes or until the samples were about 0.5" away from the edge of the gel. The gel was then stained for 20 mins with 20 mL of 1x GelRed in a dark location and then photographed on a ChemiDoc. Once the gel had been photographed, the purified bands near 400 base pairs were excised and purified using the Takara PCR/Gel purification kit. The OD $A_{260/280}$ nm was measured to obtain the DNA concentration of the samples via the BioTek Epoch2 microplate reader.

Purified digested samples and vectors were ligated using the Takara ligation system. 20 ng of vector and 16 ng of insert were incubated together at 65°C for 2 minutes to disrupt the insert-insert and plasmid-plasmid interactions. The solution was cooled to 4°C on ice and then

Takara solution was added. After, the temperature was raised and the samples were then ligated for 2 hours at 16°C.

OneShot competent *E. coli* cells were transformed with ligated samples via heat shock. 2 µL of ligation mix was added to 20 µL of *E. coli* cells and left on ice for 30 minutes. The samples were heat shocked at 42°C for 30 seconds using a water bath and then immediately placed on ice again for at least 5 minutes. 180 µL of SOC medium was added to the samples and they were horizontally shaken at 37°C in a shaker/incubator at 225 rpm for exactly 1 hour. 200 µL of sample was spread on warmed agar plates that contained ampicillin to ensure only transformed cells grew and the plates were placed, inverted, overnight in an incubator at 37°C.

A negative control with an empty cut plasmid backbone was used, as well as a positive control with uncut plasmid. A single colony per sample was used to inoculate 25 mL of Terrific Broth with ampicillin and placed overnight in a horizontal shaker incubator at 37°C in order to expand the culture.

A glycerol stock was made of each sample consisting of 500 µL of 50% glycerol with 500 µL of bacterial culture. After the glycerol stock was made, the bacterial pellet was harvested from the culture by centrifuging the sample at 6,000 x g for 15 minutes at 4°C. The plasmid DNA was recovered from the bacterial pellet using the Qiagen MIDI prep kit. The OD $A_{260/280}$ nm was measured to obtain the DNA concentration of the samples via the Epoch2.

The purified plasmid DNA was sent for sequencing to ensure the correct orientation of the insert in the vector and to ensure the presence of VH4-34 in the heavy chain plasmids by using the SP6 universal primer from Genewiz.

Transfection

Human embryonic kidney cell (HEK293T) cultures were prepared in advance to transfections. Cells were grown in complete DMEM (Dulbecco's Modified Eagle Medium) 1x (with phenol red) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, Ref. 10-013-CV) supplemented with 10% heat inactivated and filtered Fetal Bovine Serum (FBS) and 1x penicillin/streptomycin in 10 cm Falcon Tissue Culture-Treated Dishes.

The day before a transfection, the cells were split, and the media changed accordingly. Transfection media consisted of phenol red and antibiotic free DMEM supplemented with 4% FBS to ensure efficient purification of the collected supernatant via FPLC. Cells were plated at 100,000 cells/cm^2 (approximately 1.5 x 10^6 cells/mL) to ensure the cells were not overconfluent the following day.

On the day of transfection, cells were observed under a microscope to ensure proper growth and health before transfection. Cells were co-transfected with IgH (heavy chain) and IgL (light chain) chain-specific plasmids using Lipofectamine 3000 following the manufacturer's protocols for optimal transfection efficiency. Transfected plates were carefully placed in the CO_2 at 5% incubator at 37°C.

The first collection of supernatant occurred two days post transfection (day 3 of the procedure) and collection continued every 2 days until either the cells died or 11 days post transfection transpired. The supernatant was carefully removed from the plates using a syringe and filtered through a 0.22-micron filter to ensure any cells that were possibly removed from the plate with the supernatant did not contaminate the collected supernatant. Cells that were potentially in the supernatant were removed to protect the antibodies from potential degrading enzymes that would be released after cell death. The transfection media was carefully replaced without detaching the cells from the plates and the plates were placed back into the CO_2 incubator between each collection. No wash step was performed between collections to increase survival rates of cells by preventing accidental detachment from cell culture plates during the process. A small amount of supernatant, approximately 2 mL, remained on the plate between each collection day to prevent negative reactions from cells to entirely new media as well as preventing additional cells from being removed during collections. The collected supernatant from each day was pooled into one 50 mL falcon tube per sample and kept at 4°C until all collections were completed.

Concentrating the Samples

Dialysis tubing with a 10kDa MWCO was used to concentrate the collected supernatant samples. Supernatant from all collection days for a single sample was added to the dialysis tubing which was covered with polyethylene glycol (PEG) 20,000. The samples were left in the PEG until there was approximately 10 mL of sample remaining in the tubing. The sample was removed from

the tubing using a needle and syringe and stored in the syringe sealed with parafilm at 4°C until ready to be purified (no longer than 2 days).

Sample Purification

Samples were purified via FPLC and a Bio Rad Bio-Scale Mini UNOsphere SUPrA Cartridge protein A column with 0.02 M sodium phosphate pH 7.5 as the wash buffer and 0.1 M glycine pH 2.5 as the elution buffer. Purified samples were collected in test tubes containing 100 µL of 1.0 M TRIS pH 8.8 with a fractionator assembly connected to the FPLC. The fractions containing the purified antibodies were placed in a Thermo Scientific Slide-A-Lyzer Dialysis Cassette G2 (Prod# 87735) and put in a container filled with excess 1x PBS overnight at 4°C while being stirred to exchange the buffer the antibodies are stored in. The final samples were transferred to a Pierce Protein Concentrator (ref 88517) and spun in a centrifuge until the sample volume is approximately 1 mL. The OD A_{280} nm was measured to obtain the concentration of the antibody via the Epoch2.

Determining Reactivity and Polyreactivity

The ELISA kits from ORGENTEC Diagnostika GmbH (anti-dsDNA, ORG 604; anti-insulin ORG 520) and Abnova (anti-cardiolipin, KA0941; ANA Profile, KA1079) were used for basic qualitative binding of the cloned and purified monoclonal antibodies to determine autoreactivity to dsDNA, insulin, cardiolipin, and additional ANA proteins. The manufacturer's instructions were followed initially and additional adjustments in concentrations of sample added to the pre-coated plates were made to optimize the procedure for purified antibodies compared to serum or plasma.

Absorbances were collected via Epoch2 and data analysis was performed using GraphPad Prism version 8 using the recommended 4-Parameter-Fit (4PL) with lin-log coordinates for the ORGENTEC anti-dsDNA and anti-insulin kits and Excel was used for the simple calculations required for the Abnova anti-cardiolipin and ANA Profile kits.

CDR3 Analysis

CDR-H3 analysis was performed using IMGT/HighV-QUEST and NCBI IgGBlast to gather information from genetic sequences. Gathered information was then analyzed using GraphPad Prism version 8.

Determining Hydrophobicity

The hydrophobicity of the samples was determined in conjunction with materials provided from Harry W. Schroeder Jr, MD and Andre M. Vale. Gathered information was then analyzed using GraphPad Prism version 8.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8. Levels of statistical significance were determined via unpaired Mann-Whitney tests with the significance taken as $p \leq 0.05$.

Results

Primer Optimization

The original method for the heavy chain semi-nested PCR amplification required the use of a 13-primer mixture that was designed to amplify all human VH segments. As we were only interested in amplifying 1 particular gene segment, VH4-34, the original method was modified slightly to include the use of a single VH4-34-specific forward primer during the second-round of amplification instead of the 13-primer mix. By eliminating the use of multiple primers, multiple days were eliminated from the workflow as the sequencing confirmation of amplified samples was no longer required.

The alteration to the method was tested several times, in which many samples were amplified using both the original and the modified protocol, and sequences were compared for any variation to the results in amplification. After examining the sequences, it was noted that the single VH4-34 primer method resulted in no false positive, nor false negative amplifications in comparison to the original 13-primer mix method (figure 3).

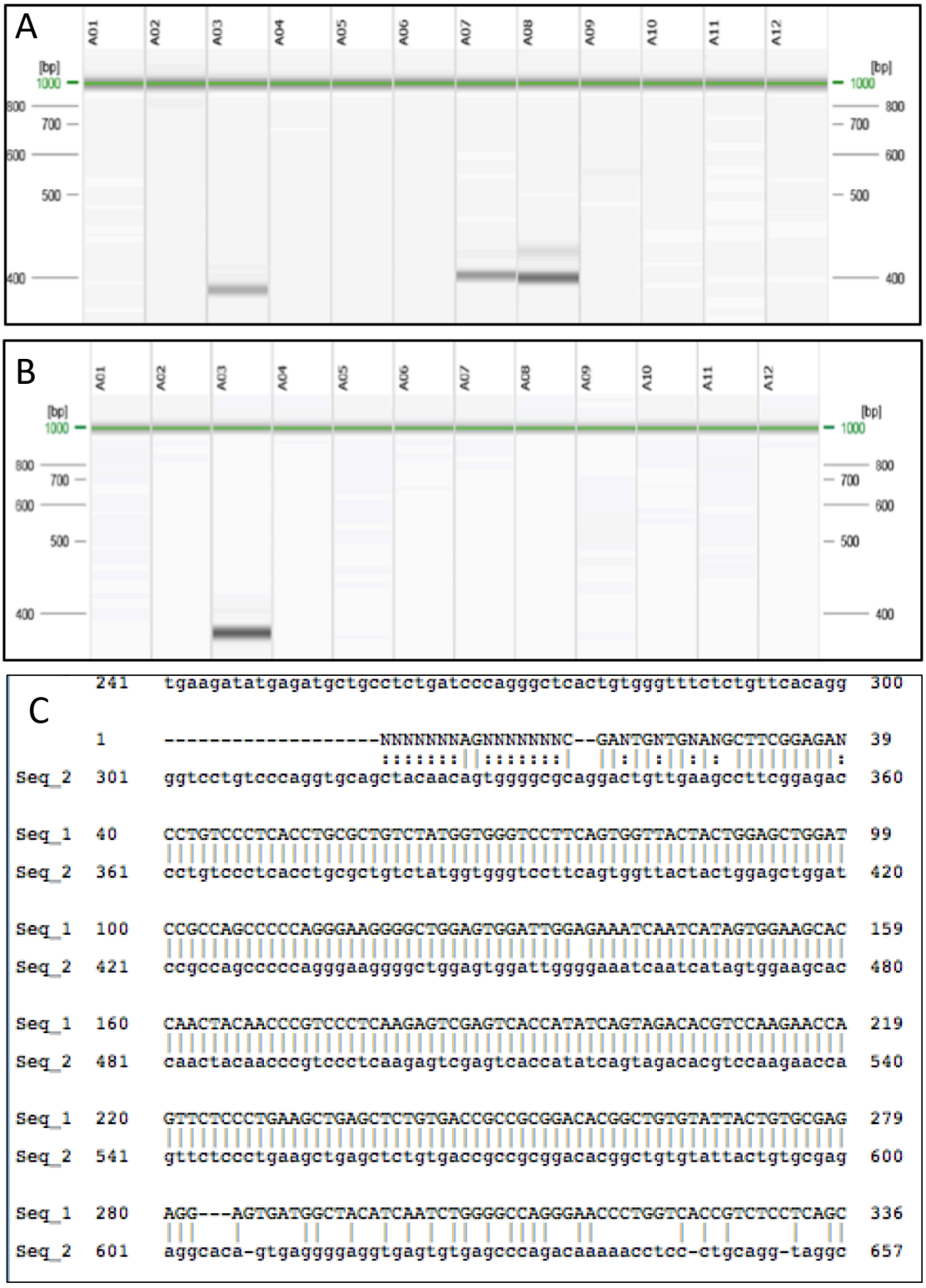

Figure 3. Comparison of original primer method to modified single-primer method. A) One row of IgM amplification of B1 cells that utilized the multiple primer mix. B) Same row of IgM amplification of B1 cells but using only VH4-34 primer as forward primer. C) Sequence alignment to VH4-34 confirming the identity of the sample.

Serum Optimization

The original protocol called for 10% serum in the transfection media which resulted in very difficult working conditions once the collected supernatant was concentrated with dialysis tubing/PEG and needed to be inserted into the FPLC for purification. In addition to this difficult working situation, an optimized length of time in which supernatant containing antibodies should be collected from the transfected cells had not been determined. These factors were evaluated by varying the percentage of FBS supplemented (0%, 0.5%, 1%, 2%, 3%, and 4%) into the transfection media and multiple samples were transfected, supernatants collected every two days post transfection, and antibody levels monitored via ELISA.

FBS concentrations below 2% were lethal to the HEK293T cells and thus not recorded in figure 4. As noted in this trial, samples with 4% of FBS in the transfection media produced the most antibodies as determined by ELISA. The amount of antibody production after 2 weeks post transfection significantly decreased to the point that little to no antibody was being produced which led to the modification of the original protocol that called for 22 days of collection post transfection to end after 12 days post transfection.

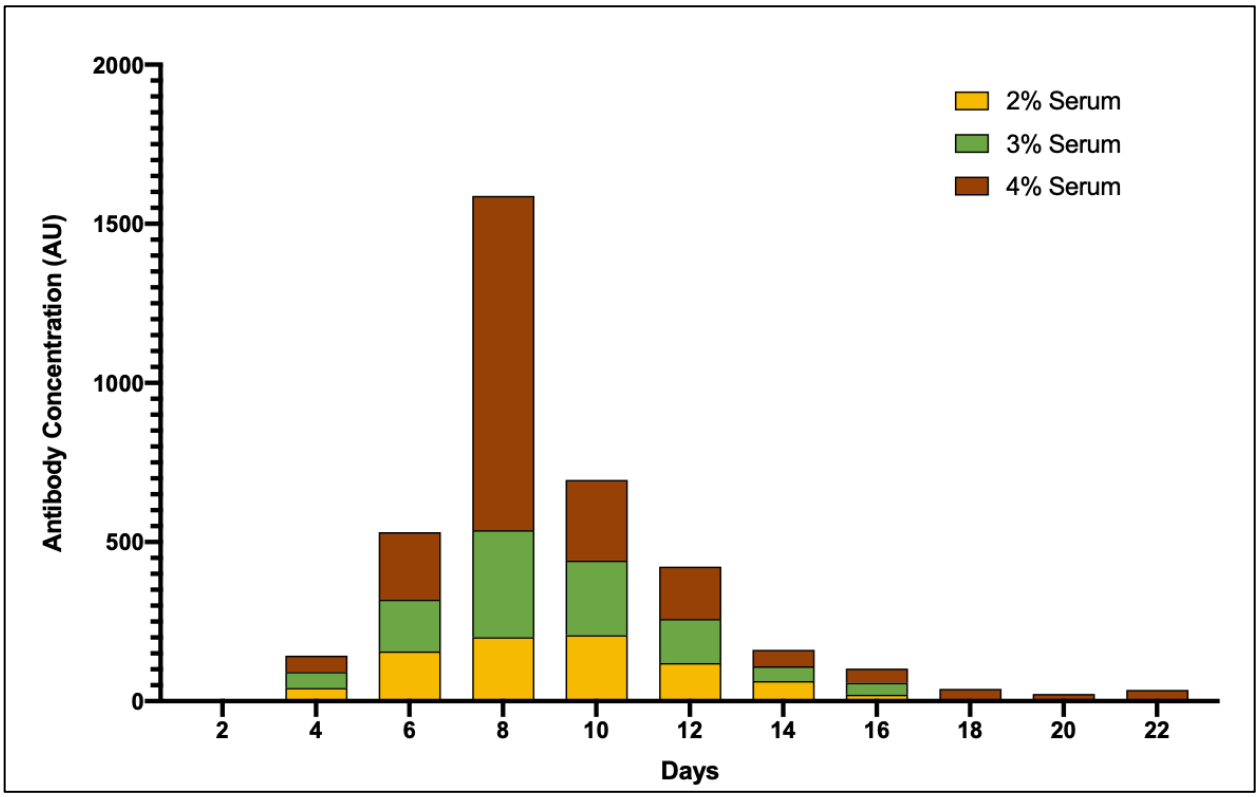

Figure 4. The results of one transfection time trial and serum optimization with a single sample transfected multiple times with different concentrations of FBS in the transfection media.

VH4-34 Utilization

An intermediate step to producing monoclonal antibodies is sending VH4-34 amplified PCR products for sequencing for each of memory and B-1 samples. While not all of these samples were cloned, sequencing information was still collected and analyzed. Figure 5 shows a clear overutilization of VH4-34 in B-1 cells compared to memory cells.

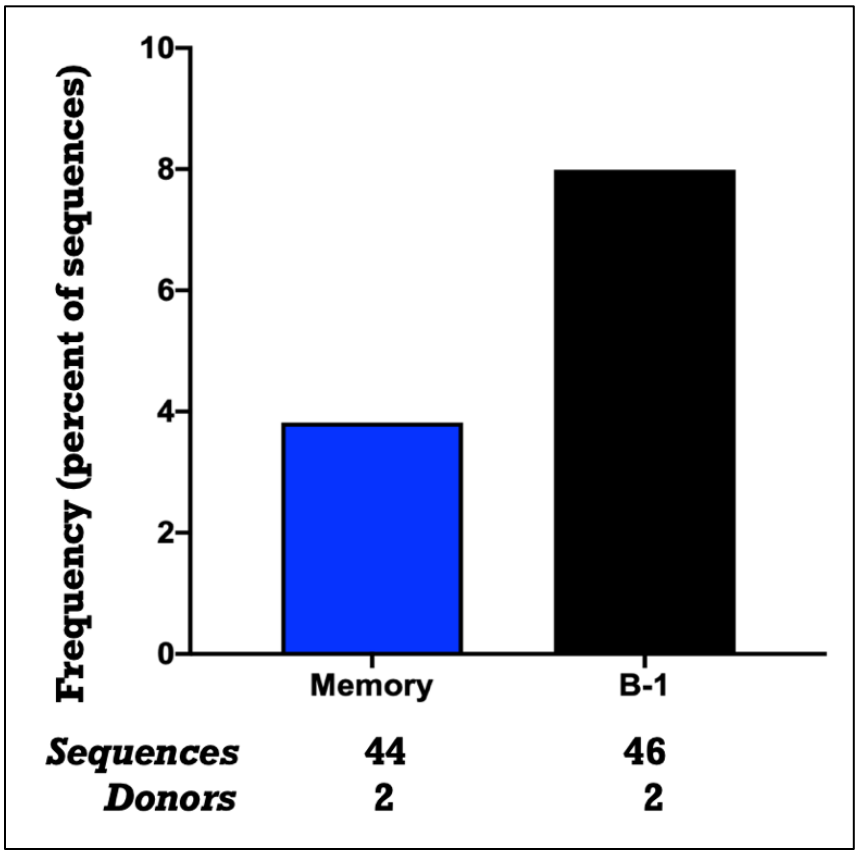

Figure 5. The utilization of VH4-34 in memory and B-1 samples.

Sequencing, CDR-H3, and Mutations

22 monoclonal antibodies were cloned from single cells, 12 originating from B-1 cells and 10 originating from B-2 memory cells. Genetic sequencing via Sanger sequencing was performed to obtain information about their diversity and joining regions, however, no significant pattern was able to be discerned from the information gathered. Regardless of the groupings, either via isotypes of origin, cell of origin, D regions, or J regions, no critical information was gleamed about their potential binding capabilities nor were any correlations made to their CDR-H3 regions (table 2).

In addition to not being able to draw conclusions based upon the V(D)J information gained from sequencing, no patterns were able to be recognized from the mutational analysis either as the lengths of the CDR-H3 did not seem to be impacted by the number of nucleotide mutations (table 2). The cell of origination or isotype of origination did not seem to be an influencing factor on the length of the CDR-H3 nor the number of mutations either (table 2).

Table 2. VDJ and CDR3 characteristics of cloned antibodies determined by Sanger sequencing

Sample Names	Cell of Origin	Isotype of Origin	VH	DH	JH
B1_1_A6_IgG	B1	IgG	4-34	3-3	6-3
B1_1_G8_IgG	B1	IgG	4-34	3-3	6-3
B1_1_C6_IgM	B1	IgM	4-34	3-6	5-1
B1_2_A3_IgM	B1	IgM	4-34	5-24	4-2
B1_2_C1_IgM	B1	IgM	4-34	3-22	4-2
B1_2_D2_IgM	B1	IgM	4-34	3-22	4-2
Mem_5_F2_IgG	Memory	IgG	4-34	6-6	4-2
Mem_5_H1_IgG	Memory	IgG	4-34	3-16	5-1
Mem_5_H8_IgG	Memory	IgG	4-34	5-18	6-2
Mem_6_A4_IgG	Memory	IgG	4-34	6-6	4-2
Mem_6_A10_IgG	Memory	IgG	4-34	3-3	6-3
Mem_6_C12_IgG	Memory	IgG	4-34	6-6	4-2
Mem_6_D6_IgG	Memory	IgG	4-34	6-6	4-2
Mem_6_E6_IgG	Memory	IgG	4-34	3-3	6-3
B1_1_A12_IgG_D2	B1	IgG	4-34	2-2	5-2
B1_2_A8_IgM_D2	B1	IgM	4-34	2-2	6-2
B1_3_A7_IgM_D2	B1	IgM	4-34	2-15	6-2
B1_3_C11_IgG_D2	B1	IgG	4-34	-	-
B1_4_G10_IgG_D2	B1	IgG	4-34	6-19	4-4
B1_4_E1_IgM_D2	B1	IgM	4-34	2-15	6-2
Mem_3_F4_IgM_D2	Memory	IgM	4-34	5-12	5-2
Mem_4_D9_IgM_D2	Memory	IgM	4-34	3-9	4-2

Table 2 – continued

Sample Names	CDR3	CDR3 length	Total V Mutations	Hydrophobicity
B1_1_A6_IgG	ARGGVYDFWSGYNDYYYYMDV	21	1	-0.129
B1_1_G8_IgG	ARGGVYDFWSGYNDYYYYMDV	21	0	-0.129
B1_1_C6_IgM	ASNTDYVWGSYRPRGLL	17	0	-0.349
B1_2_A3_IgM	ARGVMATSI	9	1	0.800
B1_2_C1_IgM	ARGGGGSSGYYVDY	14	2	-0.066
B1_2_D2_IgM	ARGPSGYYPFDS	12	9	-0.189
Mem_5_F2_IgG	ASPRQPGAARPYVR	14	9	-0.349
Mem_5_H1_IgG	ARGGVYDFWSGYNDYYYYMDV	17	0	-0.129
Mem_5_H8_IgG	ARGGVYDFWSGYNDYYYYMDV	17	9	-0.129
Mem_6_A4_IgG	ASPRQPGAARPYVR	14	6	-0.349
Mem_6_A10_IgG	ARGGVYDFWSGYNDYYYYMDV	21	0	-0.129
Mem_6_C12_IgG	ASPRQPGAARPYVR	14	0	-0.349
Mem_6_D6_IgG	ASPRQPGAARPYVR	14	8	-0.349
Mem_6_E6_IgG	ARGVPFTVSGPIYRGHPKFGAAGFDP	21	0	0.187
B1_1_A12_IgG_D2	ARALSGRYQLLYKRRAGYNWFDP	23	0	-0.125
B1_2_A8_IgM_D2	ARGTGCSSTSCHIRRYYYYGMDV	23	0	-0.069
B1_3_A7_IgM_D2	ARGVGYCSGGSCYSRYYYYYGMDV	24	0	0.014
B1_3_C11_IgG_D2	ARSLLRE	7	0	0.650
B1_4_G10_IgG_D2	ARRYSSGWYYFDQ	13	19	-0.303
B1_4_E1_IgM_D2	ARGPSYCSGGSCYSPLRGVWYYGMDV	26	1	0.071
Mem_3_F4_IgM_D2	AGSRQWIQSLQSWFTRFDP	19	16	-0.146
Mem_4_D9_IgM_D2	AREVMVAYYDILTGYYKSGYFDY	23	0	0.184

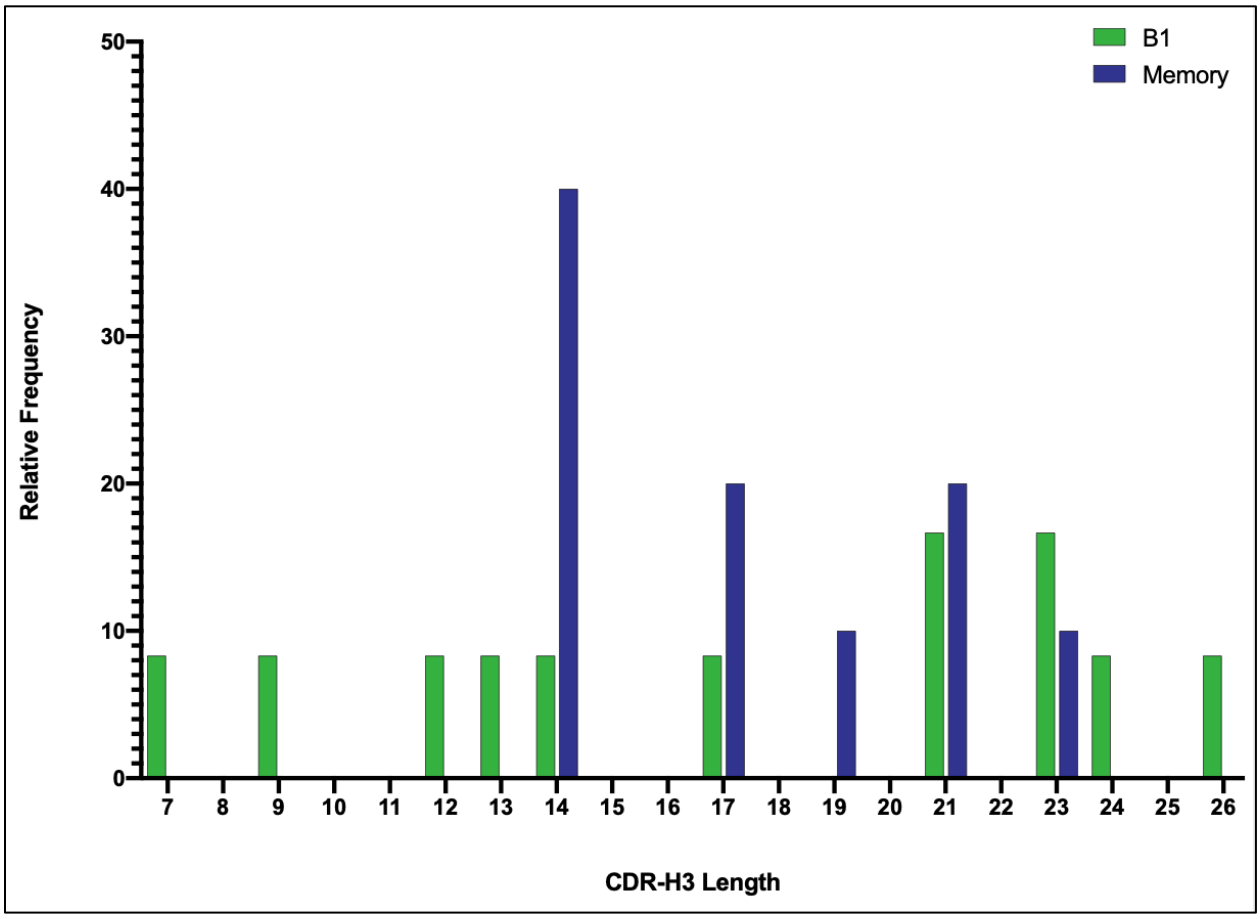

Figure 6. Length of CDR-H3 region for cloned antibodies.

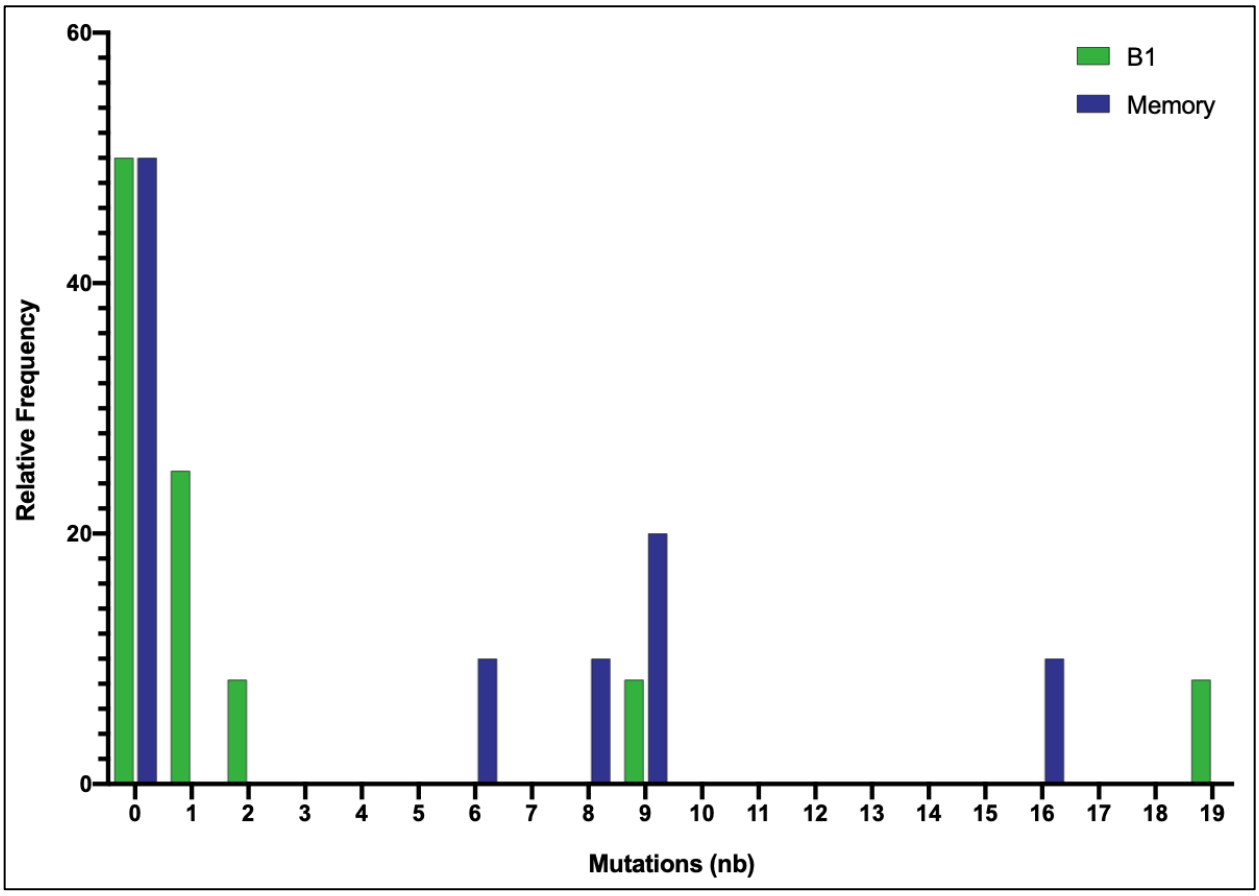

Figure 7. Nucleotide mutations for variable gene of all cloned antibodies.

Hydrophobicity

While levels of mutation and the length of the CDR-H3 regions of the clones were not significantly different between VH4-34-containing antibodies that originated from B-1 cells compared to those that originated from conventional B-2 memory cells, the hydrophobicity did show a difference and there was a level of significance between the two subpopulations. While there does not seem to be a key indicative pattern between each sample's hydrophobicity and it's CDR-H3 length, number of mutations, binding strength to any antigen tested, or original antibody isotype, antibodies that came from B-1 cells do seem to be more positively charged than those that originated from memory samples. It is interesting to note, as indicated in figure

7, that the polyclonal B-1 cell antibody does not follow this trend, but actually appears to be slightly negatively charged sharing the similar charge with two other B-1 samples (all charges listed by sample in table 2) that do not share in the same type of binding patterns.

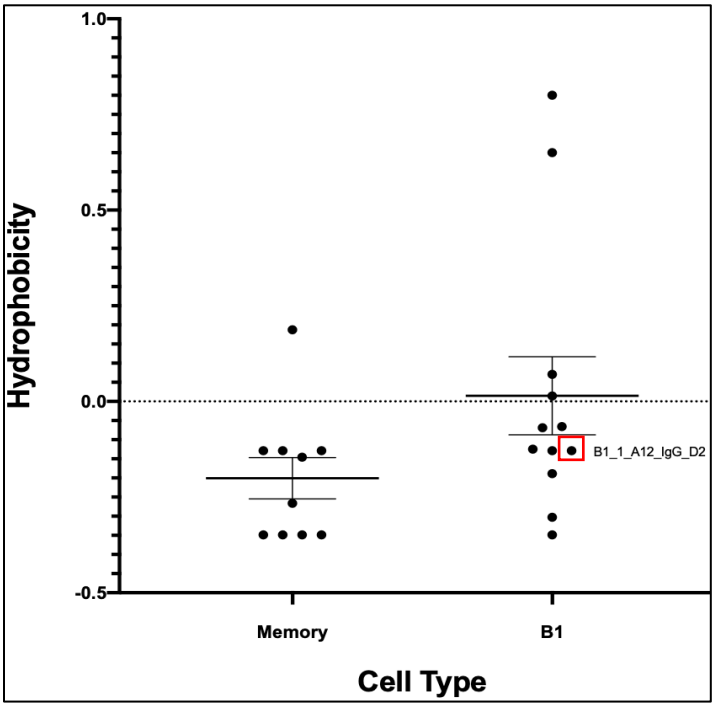

Figure 8. Hydrophobicity of all cloned antibodies with emphasis on which cell type originally produced the antibodies. Note: the labeled sample is the positive binding, polyreactive antibody.

Reactivity Summary

To observe the potential autoreactivity/polyreactivity of VH4-34-containing antibodies produced from B-1 and B-2 cells, we tested cloned and expressed antibodies for binding towards dsDNA, insulin, cardiolipin, and several nuclear antigens. The presence of these potentially autoreactive antibodies are implicated in several autoimmune disorders/diseases.

B1_1_A12_IgG_D2 bound to every antigen tested in all ELISA assays (figures 9-12). Other samples did show minor binding activity and were thus considered positive for low-level binding, except for the dsDNA assay where 2 other B-1 samples (B1_2_A8_IgM_D2 and B1_3_C11_IgG_D2) and a single memory sample (Mem_6_C12_IgG) (figure 9a) were considered positive for moderate-level binding. However, the polyreactive B1_1_A12_IgG_D2 bound to dsDNA strongly enough to be considered strongly autoreactive. Table 3 provides a summary of the reactivity of all cloned antibodies.

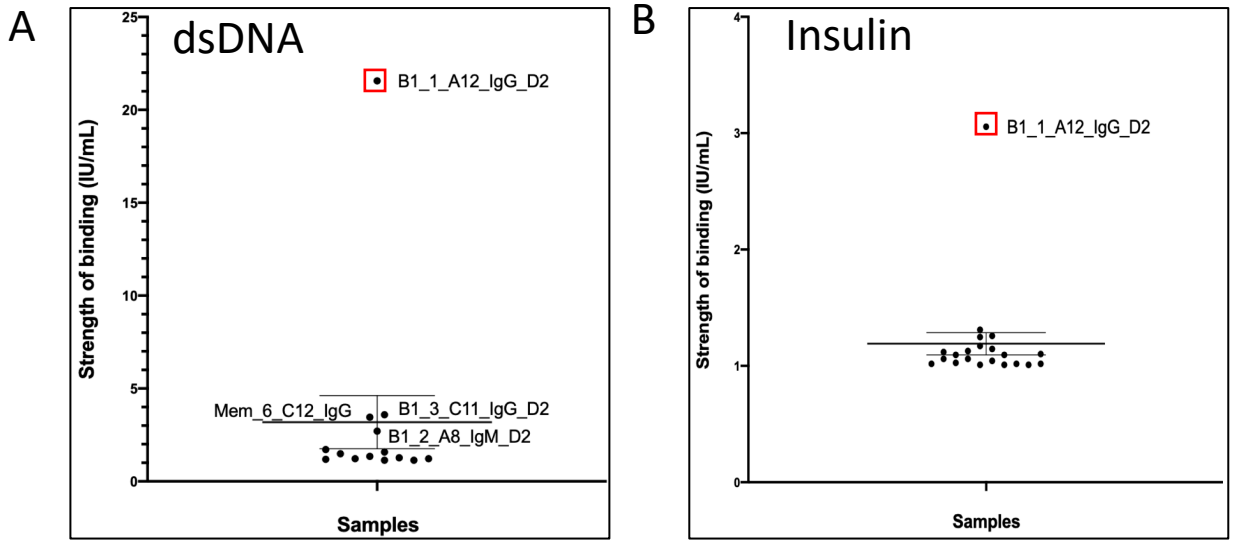

Figure 9. Anti-dsDNA and Anti-Insulin ELISA results. A) Anti-dsDNA; B) Anti-Insulin Note: the labeled sample is the positive binding, polyreactive antibody.

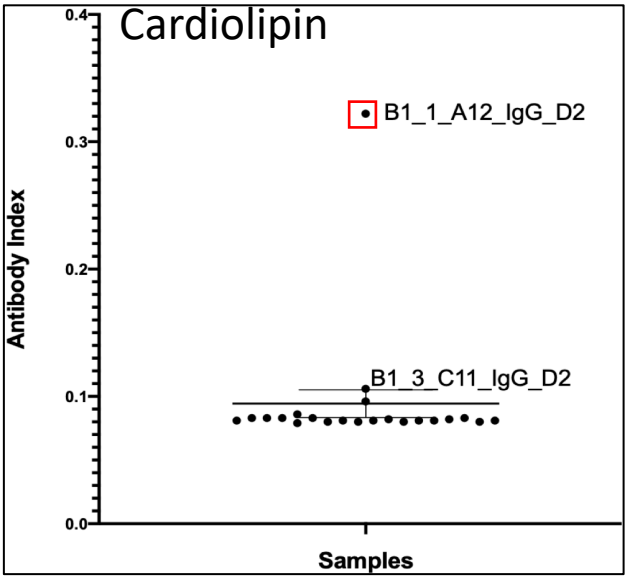

Figure 10. Anti-cardiolipin ELISA results. Note: the labeled sample is the positive binding, polyreactive antibody.

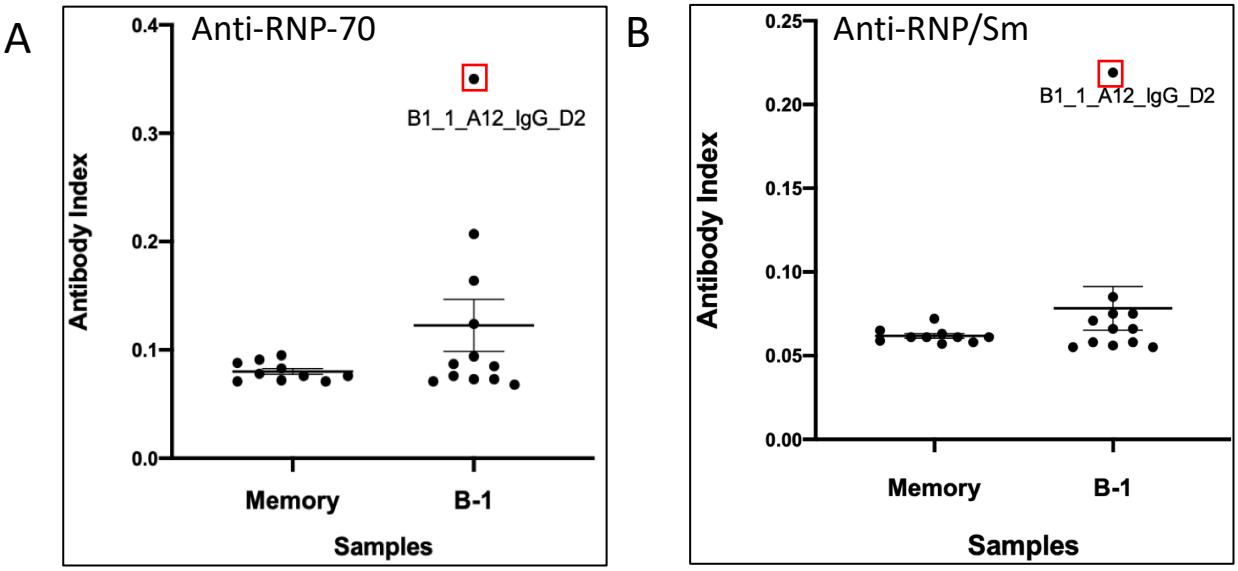

Figure 11. Anti-nuclear Antibodies (ANA) ELISA results. A) Anti-RNP-70; B) Anti-RNP/Sm. Note: the labeled sample is the positive binding, polyreactive antibody.

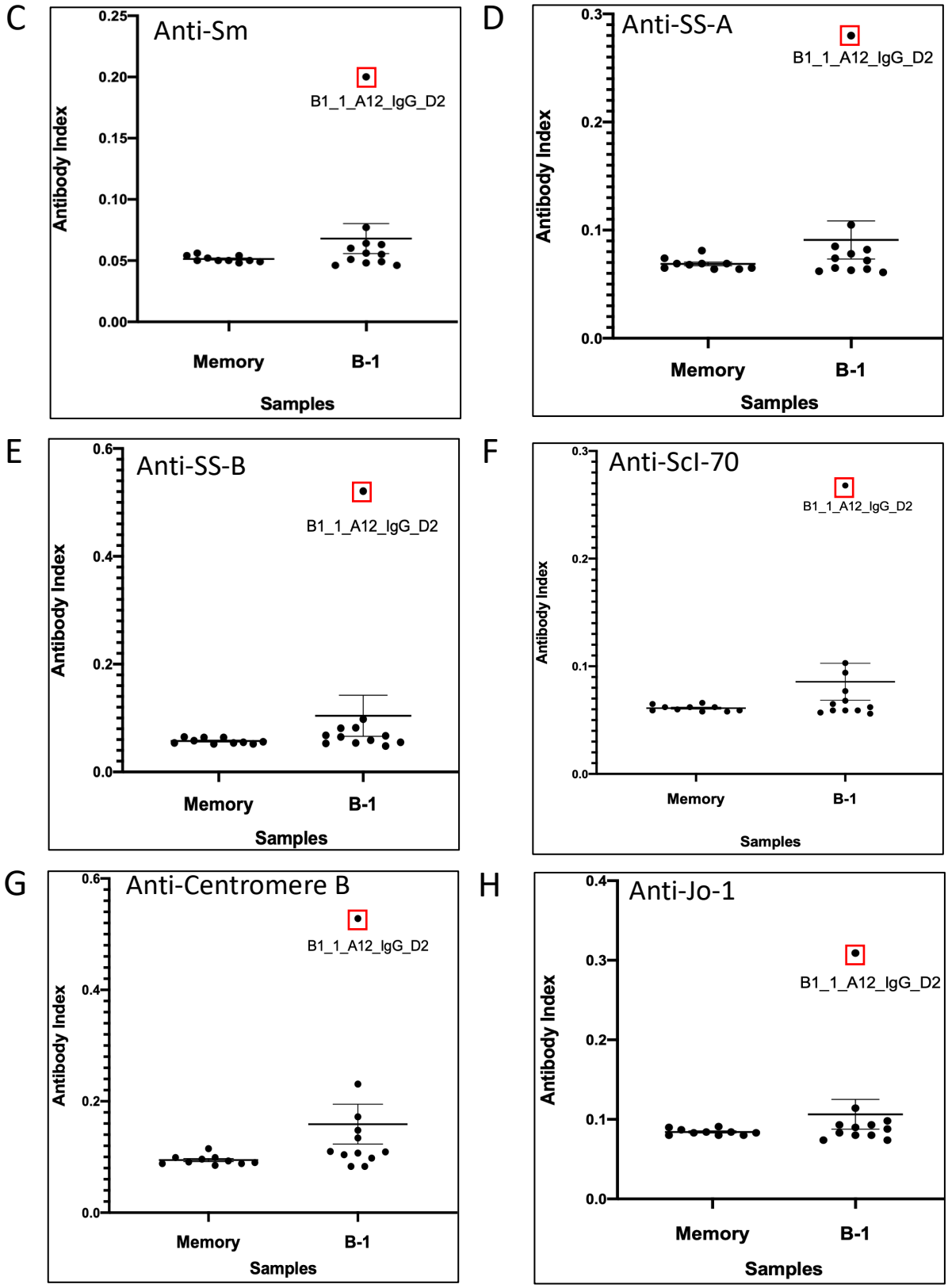

Figure 12. ANA ELISA results (cont.); C) Anti-Sm; D) Anti-SS-A; E) Anti-SS-B; F) Anti-ScI-70; G) Anti-Centromere B; H) Anti-Jo-1. Note: the labeled sample is the positive binding, polyreactive antibody.

Table 3. Summary of antibody binding strength against all antigens tested

Sample Names	dsDNA binding strength	Insulin binding strength	Cardiolipin binding strength	Anti-RNP-70†	Anti-RNP/Sm†	Anti-Sm†	Anti-SS-A†	Anti-SS-B†	Anti-Scl-70†	Anti-Cemtromer B†	Anti-Jo-1†
B1_1_A6_IgG	-	-	-	-	-	-	-	-	-	-	-
B1_1_G8_IgG	-	-	-	-	-	-	-	-	-	-	-
B1_1_C6_IgM	-	-	-	-	-	-	-	-	-	-	-
B1_2_A3_IgM	-	-	-	-	-	-	-	-	-	-	-
B1_2_C1_IgM	-	-	-	-	-	-	-	-	-	-	-
B1_2_D2_IgM	-	-	-	-	-	-	-	-	-	-	-
B1_1_A12_IgG_D2	+++	++	++	++	++	++	++	++	++	++	++
B1_2_A8_IgM_D2	++	-	-	+	-	-	-	-	+	-	-
B1_3_A7_IgM_D2	-	-	-	-	-	-	-	-	-	-	-
B1_3_C11_IgG_D2	++	-	-	+	+	+	+	-	+	+	-
B1_4_G10_IgG_D2	-	-	-	-	-	-	-	-	-	-	-
B1_4_E1_IgM_D2	-	-	-	-	-	-	-	-	-	-	-
Mem_3_F4_IgM_D2	-	-	-	+	-	-	-	-	-	-	-
Mem_4_D9_IgM_D2	-	-	-	-	-	-	-	-	-	-	-
Mem_5_F2_IgG	-	-	-	-	-	-	-	-	-	-	-
Mem_5_H1_IgG	-	-	-	-	-	-	-	-	-	-	-
Mem_5_H8_IgG	-	-	-	-	-	-	-	-	-	-	-
Mem_6_A4_IgG	-	-	-	-	-	-	-	-	-	-	-
Mem_6_A10_IgG	-	-	-	+	+	-	+	-	-	-	-
Mem_6_C12_IgG	++	-	-	-	-	-	-	-	-	+	-
Mem_6_D6_IgG	-	-	-	-	-	-	-	-	-	-	-
Mem_6_E6_IgG	-	-	-	-	-	-	-	-	-	-	-

No binding or negative (-) strength of binding (IU/mL) index cutoff for all tests was determined by the internal standards of the assays adjusted for antibody only testing; + indicates low level binding for samples above the mean; ++ for moderate level of binding above the mean; +++ for samples demonstrating high or strong levels of binding above the mean.

Discussion

VH4-34 Utilization

Based on previous literature, we know that VH4-34 is over utilized in B-1 cells to approximately 8% which I have also been able to replicate based off of my results (figure 5). This previous literature also compared the VH4-34 usage in memory cells to approximately 2% (Quách et al., 2016). While my numbers are a bit high, nearing 4%, there is still a clear distinction that VH4-34 is overutilized B-1 cells compared to memory cells.

Sequencing, CDR-H3, and Mutations

Much of what is known about the CDR-H3 region in terms of pattern has been elucidated from mice. However, it has been proven incredibly difficult to obtain comparative data from human specimens as there is enhanced variability of the human repertoire, especially when compared to the CDR-H3 region. This increased variability can be attributed to the greater diversity of the germline sequence in the DH gene segments and an increase in N-additions (Mroczek et al., 2014).

While some mice antibodies do have nucleotide additions (N-additions), the cells that produce these additions, along with somatic hypermutation, are the more mature cells, such as the conventional B-2 memory cells. Murine system B-1 cells are mainly germline-like and contain little to no somatic hypermutation or N-additions. N-additions are often thought to be missing from early B cell development in mice as the required enzyme terminal deoxynucleotidyl transferase (TdT) is not present during that developmental period. TdT adds a random number

of N-nucleotides to the end of the heavy chain during V(D)J recombination resulting in non-templated nucleotide additions and a further variation away from the original germ-like template (Wardemann & Nussenzweig, 2007) (Rothstein et al., 2013). This is quite different in the human system, however, as TdT is present early in cell development indicating that human B-1 cells may not be as germline-like as their murine counterparts (Pugh-Bernard et al., 2001).

Typically, with CDR-H3 there is a unique pattern in amino acid composition, length, and charge distribution as B cells mature, even in the human system (Mroczek et al., 2014). Stereotypically, length decreases and there is an increase in highly charged amino acids located in the CDR3 loop, which would have led to the antibodies produced from the memory population having a shorter CDR-H3 length with more highly charged amino acids. As memory cells leave the bone marrow having completed their VDJ recombination and N-additions stages, the only way for memory cells to alter their CDR-H3 regions is through somatic hypermutation (SHM) from antigen exposure. However, there was no significant difference identified between the lengths of the antibodies produced from the different subpopulations (figure 6) and there was no significant difference observed between the amino acid composition of the CDR3 loops of the antibodies produced from the different subpopulations (not pictured).

In addition to the length of the CDR-H3 region and the amino acid composition of the CDR3 loop, the average number of mutations from the germline sequence of an antibody should increase through maturation. As a cell develops and continues through the process of SHM, the genetic variability of the antibody repertoire should continue to expand. What should have been

observed was an increase in mutations from germline sequences for the memory samples compared to the B-1 samples that have not been exposed to external antigens or gone through somatic hypermutation. However, we observed no statistically significant difference between the subpopulations in terms of which population had more total variable chain mutations than the other (figure 7).

Hydrophobicity

The hydrophobicity of an antibody is determined by the amino acid composition near the antigen binding site. Each amino acid plays an important role in determining the reactivity of the antibody as well as determining the overall charge. Somatic hypermutation is a good example of how each amino acid plays a key role in determining the antigenic binding capacity of the antibody. As random point mutations occur due to antigen challenge, antibodies become more diversified and the body's repertoire expands (Wardemann & Nussenzweig, 2007). These random point mutations can alter the amino acid composition of the antibody and therefore alter the binding capability.

As mentioned before, generalized patterns in antibody structure and composition are conserved evolutionarily through species, including hydrophobicity. Theoretically, memory cells, or conventional B-2 cells that have been exposed to antigen, should have shorter CDR-H3 regions, but they should also be composed of more positively charged amino acids. However, this was not what was observed. The majority of the cloned memory antibodies had negative hydrophobicity's with only one being positively charged. The B-1 cells, however, did have a level

of significant difference in hydrophobicity compared to memory B cells. There were a few samples that were near the lower negatives much like the lower memory samples, but the mean hydrophobicity of the B-1 cell antibodies was higher at just about neutral in comparison to the generally negative memory B cell antibodies. However, there were two B-1 antibodies that were significantly more positive, above +0.5, that stood out. There was nothing that was easily able to be identified that would indicate why these samples were so significantly different than the remaining B-1 samples, or any of the memory samples, in terms of reactivity, CDR-H3 length, mutations, or original antibody isotype.

Reactivity

Based on previous explanations, predicted observations included a significant increase of reactivity of the B-1 antibodies compared to the memory antibodies. As the original B cells all came from healthy donors, the B-2 cells should have all undergone check points as they matured to ensure self-reactive cells either did not survive, or produce autoreactive antibodies, resulting in a decrease in the number of cloned antibodies that should react to the self-antigens that coated the plates in the ELISAs.

Each of the antigens that coated the different plates are known to be commonly associated with autoreactivity in different autoimmune disorders/diseases, such as systemic lupus erythematosus (SLE), scleroderma, Sjögren's Syndrome, mixed connective tissue disease, and idiopathic inflammatory myopathies (table 4). (Stollar, 1991). Many of these antigens are also specifically targeted in the human system when it is afflicted with SLE and these tests would

provide a baseline level of autoreactivity produced by B-1 and B-2 antibodies which could be judged against the typically elevated levels of autoreactive antibodies detected in SLE patients.

Table 4. Antigens coating the ELISA plates

Autoantigen	Source of antibody	Additional notes	References
dsDNA	Systemic Lupus Erythematosus		Stollar, 1991
Insulin	Hirata's Disease	Abs made to endogenous insulin; rare; associated with other autoimmune diseases, especially Graves's disease	Greenfield et al., 2009
RNP-70	Systemic Lupus Erythematosus	Ribonuclear protein particles of 70 kDa; RNA-protein complexes often located in the nuclei of eukarotic cells	Stollar, 1991; Migliorini et al., 2005
RNP/Sm	Systemic Lupus Erythematosus; Mixed Connective Tissue Disease	often found in 5-30% of SLE patients	Stollar, 1991; Migliorini et al., 2005
Sm	Systemic Lupus Erythematosus		Stollar, 1991
SS-A	Systemic Lupus Erythematosus; Sjögren's Syndrome	Nuclear antigen	Franceschini & Cavazzana, 2005
SS-B	Sjögren's Syndrome	Nuclear antigen	Stollar, 1991; Franceschini & Cavazzana, 2005
Scl-70	Scleroderma	Also known as Anti-Topoisomerase I	Stollar, 1991
Centromere B	Scleroderma		Stollar, 1991
Jo-1	Idiopathic Inflammatory Myopathies	directed against histidyl-tRNA synthetase	Zampieri et al., 2005

While some believe that immunological autoreactivity predominantly occurs due to antibodies that arise from conventional B-2 cells, that is not the picture that seems to be drawn from the results of the different ELISA results. While 22 cloned antibodies, 12 from B-1 and 10 from memory, may not provide statistical significance, we can see that only 1 antibody sample,

a B-1 sample, of 22 was considerably polyreactive. This B-1 antibody was originally IgG. We can also see that only 1 memory sample that was originally IgG, of the 10 cloned, showed moderate reactivity to any test, whereas 3 out of 12 B-1 samples (2 were IgG and 1 was IgM) had at least minor reactivity to at least 1 test. Again, it does not provide statistical significance, but it does hint in the direction that VH4-34-derived antibodies produced from B-1 cells may indeed be more autoreactive in healthy donors than those that are derived from conventional B-2 cells.

Future Directions

We know that VH4-34 is overutilized in B-1 cells compared to other cell populations and that it is often associated with autoreactivity and autoimmunity. We also know that B-1 cell antibodies are known for being broadly reactive with low levels of autoreactivity. These observations led us to further study VH4-34-containing antibodies and which B cell population predominantly produced them. We sought to find out if significantly more B-1 cells produced more autoreactive VH4-34 Abs than other B cell populations and if VH4-34-containing Abs were only autoreactive when derived from B-1 cells.

We did observe that autoreactivity was mainly found in B-1 population, though the population observed was small and requires more samples in order to provide statistical significance. However, we also noticed that there seems to be no obvious correlations of autoreactivity with the other antibody characteristics including D-usage, J-usage, CDR-H3 length, hydrophobicity, isotype origination, mutational analysis, or specific CDR-H3 motif that could be observed.

Overall, more donors are required to obtain true statistical significance for these tests. Some tests, such as the hydrophobicity and the obvious color change in the ELISAs, gave an idea of whether or not memory samples or B-1 samples followed our basic ideas and hypothesis. However, in order to further cement or ground our ideas on whether or not VH4-34-derived antibodies that arise from B-1 cells are more autoreactive than those that arise from memory cells, more samples are required. Once more samples are obtained, we can then seek to confirm our initial finding that although there are VH4-34-containing antibodies in both the B-1 and B-2 cell populations, the autoreactive VH4-34-containing antibodies are only found in the B-1 cell population.

In addition to obtaining more samples, testing additional subpopulations, such as the naïve subpopulation, would give additional insight into whether or not autoreactive VH4-34 antibodies are segregated into B-1 or B-2 subpopulations. Once additional subpopulations and more samples are tested to obtain a solid baseline for autoreactive antibodies in healthy donors, testing can be expanded to SLE patients to identify differences between baseline autoreactivity and diseased levels of VH4-34 autoreactivity in B-1, memory, and additional subpopulations.

Besides additional subpopulation testing, an additional immunofluorescence assay utilizing HEp-2 cells could be performed to characterize the reactivity of the antibodies. This indirect immunofluorescence assay (IIFA) is an important and well recognized diagnostic tool for many autoimmune diseases (Damoiseaux et al., 2019). The assay is based on identifying the varieties of antinuclear antibodies (ANAs) that are present in a sample based upon the staining

pattern of the antibodies. Different staining patterns indicate antibodies reacting with different antigens (dsDNA, nucleosomes, histones, Sm, RNPs, centromere, etc.). By identifying the staining patterns, clinicians can rule out certain diseases as many of the diseases that have elevated levels of ANAs have particular patterns, such as the homogenous staining pattern observed in SLE samples where antibodies react with dsDNA, nucleosomes, and histones (Kumar, Abbas, & Aster, 2015, p. 220). The simple presence of ANAs (a large category of antibodies that are occur in various autoimmune diseases) is not enough to classify antibodies as pathogenic or indicate what disease they may or may not result in.

Conclusion

Identification of the human B-1 cell subpopulation is important for the study of autoreactivity in people. The B-1 cell population has been studied extensively in mice, and for various reasons, using mouse B-1 cells as a representative population does not accurately reflect how human B-1 cells behave, function, and appear naturally in the system.

Throughout recent years, there have been many differing opinions to what human B-1 cells are and or whether human B-1 cells really exist. Yet, in recent years, the development of phenotypic criteria that were defined by identifying human B-1 cells that displayed functions similar to those of mouse B-1 cells has strengthened the case for the existence of B-1 cells. Now, we are able to elucidate more clearly key information these cells may play on autoimmunity/autoimmune disorders as well as other maladies in the human system.

However, the classification of these cells is far from complete. Perhaps, with the better characterization of human B-1 cells, and in turn their functions and their potential pathogenic idiosyncrasies, we will be able to further contribute to the development of therapeutic advances that could prevent the progression of autoimmune disorders or treat those that currently suffer from symptoms (Griffin et al., 2011).

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