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**Supplementary Material**

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Single-Cell and Deep Sequencing of IgG-Switched Macaque B Cells Reveal a Diverse Ig Repertoire following Immunization

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The nonhuman primate model is important for preclinical evaluation of prophylactic and therapeutic intervention strategies. The recent description of the rhesus macaque germline Ig loci and establishment of a database of germline gene segments offer improved opportunities to delineate Ig gene usage in the overall B cell repertoire as well as in response to vaccination. We applied 454-pyrosequencing and single-cell RT-PCR of bulk and sorted memory B cells, respectively, to investigate IGHV gene segment expression in rhesus macaques. The two methods gave remarkably concordant results and identified groups of gene segments that are frequently or rarely used. We further examined the VH repertoire of Ag-specific memory B cells induced by immunization with recombinant HIV-1 envelope glycoproteins, an important vaccine component. We demonstrate that HIV-1 envelope glycoprotein immunization activates a highly polyclonal response composed of most of the expressed VH gene segments, illustrating the considerable genetic diversity of responding B cells following vaccination. The Journal of Immunology, 2014, 192: 3637–3644.

The ability of the naive B cell repertoire to recognize almost any Ag is dependent on the process of V(D)J recombination, whereby V, D, and J gene segments generate a large number of unique B cell clones. In addition, diversity is generated in the recombining D-J and V-D junctions owing to trimming and addition of nontemplated nucleotides. The resulting highly variable domain spanning the V-D-J junction corresponds to the CDR3 of the Ab H chains. A similar process occurs in V-J recombination of the Ig L chain gene segments to form its CDR3. The CDR3s, together with the V-gene-encoded CDR1 and 2 regions of both the H and L chains, usually constitute most Ab contacts with the Ag (1). Additional variation is generated through random pairing of Ig H and L chains in the developing pro–B cell. The resulting B cell diversity is a major component of protective immunity to pathogens following re-encounter or vaccination. Following Ag-specific BCR activation of naive B cells, Ab affinity maturation occurs through somatic hypermutation (SHM) of the Ig genes of B cells recruited into germinal centers within B cell follicles, eventually resulting in T cell–dependent class switching from IgM to IgG isotype–bearing Abs (2).

In any given individual, at any given moment, the circulating B cell repertoire comprises naive B cells poised to respond to new Ags and IgG-switched memory B cells generated from prior exposure to pathogens, environmental Ags, or vaccine Ags (3). Ag-specific memory B cells have the capacity to rapidly differentiate into Ab-producing cells upon Ag re-encounter (4, 5). Examination of Ag-specific memory B cell repertoires therefore comprehensively surveys the B cell clones engaged by a specific Ag following infection or immunization. Single-cell sorting of HIV-1 envelope glycoprotein (Env)–specific memory B cells from chronically HIV-1–infected individuals indicates a limited-memory B cell repertoire size of ~50 clonotypes, with a bias toward the use of IGHV1 family gene segments (6). Vaccination with tetanus toxoid, in contrast, was shown to yield a repertoire size of ~100 clonotypes, which was not diversified further by boosting (7, 8). So far, little is known about V-gene segment usage and clonality of B cell responses elicited by other vaccine Ags. Yet, interest has increased in understanding germline VH gene activation and Ab maturation in response to immunization, not the least in the HIV-1 vaccine field because it is known that several highly potent, broadly neutralizing Abs against the Env from HIV-1–infected individuals use the same IGHV1 family gene segment (9, 10).

To establish a baseline of VH usage in rhesus macaques, we investigated the contribution of individual Ab H chain V-gene segments in total IgG-switched rhesus macaque B cells. Next,
we similarly analyzed the Ag-specific B cells isolated from non-human primates (NHPs) immunized with soluble HIV-1 Env trimers in adjuvant. For total IgG-switched B cells, we used two independent methods: ultradepth 454-pyrosequencing of V(D)J transcripts generated from mRNA isolated from PBMCs and single-cell sorting of IgG-switched memory B cells followed by nested PCR of V(D)J sequences. We observed highly congruent results with the two methods, allowing us to identify a large number of genetically distinct VH gene segments that were frequently or less frequently used. Furthermore, when we examined the gene segment use of Env-specific IgG+ memory B cells obtained from highly specific flow cytometric sorting (11), we observed a similar broad pattern of VH usage. These data demonstrate that the polyclonal B cell response to the HIV-1 trimers used in this study is genetically highly diverse, providing a basis for studies aimed to activate selected VH gene segments in a more specific manner. These results represent a first comprehensive analysis of Ig VH gene usage in IgG-switched rhesus macaque memory B cells, as well as in the Ag-specific B cell response to HIV-1 Env protein immunization.

Materials and Methods

**Samples and ethics statement**

Immunizations and blood samples from the rhesus macaques used in the current study are described elsewhere (12). Briefly, Chinese rhesus macaques were immunized five times in a monthly interval with recombinant gp140-F trimers based on the HIV-1 YU2 isolate (13) in AbISCO-100 and CpG-C adjuvant. Peripheral bleeds were taken while the macaques were under ketamine sedation, 1 and 2 wk after each immunization, and the PBMCs were isolated via density-gradient centrifugation with Ficoll-Hypaque. After extensive washing in PBS, the cells were frozen in FCS supplemented with 10% DMSO. During the study the macaques were housed at the Astrid Fagraeus Laboratory animal facility at Karolinska Institutet (Stockholm, Sweden). All procedures were approved by the Local Ethical Committee on Animal Experiments. Some Ig sequences from macaques F125, F126, F128, F129, and F130 were described previously (11, 14), whereas new memory B cell isolations were performed from frozen PBMCs for macaque F124, following the fourth immunization, and macaques F125 and F128, following the second and fifth immunizations.

**Cell preparation and single-cell flow cytometric sorting**

Frozen PBMCs were thawed and resuspended in 10 ml RPMI 1640 media supplemented with 10% FBS and 10,000 U/mg DNase I. After washing, the pellets were resuspended in 50 µl PBS and 5 µl Aqua Dead Cell stain and incubated for 20 min at 4°C. The cells were then stained essentially as previously described (11), using a mixture of Abs for human CD3 (allophycocyanin-Cy7; SP34-2), CD8 (Pacific blue; RPA-T8), CD14 (Qdot 550; BD), and 0.0625 M HEPES, 10 mM 2'-deoxyadenosine, 0.4 mM 2'-deoxyguanosine 5'-triphosphate, 1 µl each of 10 mM Mg2+ and 5' cloning primers, 2 µl nested PCR product, and water to 25 µl. The PCR had an initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 55°C (10 cycles) and 60°C (25 cycles) for 30 s, and 72°C for 30 s. There was a final elongation at 72°C for 7 min before evaluation on 1% agarose gel. Positive bands (∼400 bp for the H chain V(D)J and ∼350 bp for the κ and λ chain VJ) were then gel extracted (Thermo Scientific).

**Restriction enzyme digestion of PCR products and cloning into eukaryotic expression vectors containing human IgY1 H, IgK2, or IgL L chain Ab expression cassettes were performed as previously described (15), with some modifications. Restriction digestion products were purified using FastDigest enzymes (Thermo Scientific) according to the manufacturer’s instructions. Following digestion, products were purified (Thermo Scientific) and ligated into linearized, shrimp alkaline phosphatase–treated expression vectors using T4-DNA ligase (Thermo Scientific). XL10-Gold ultracompotent bacteria were transformed according to the manufacturer’s instructions (Agilent Technologies). Colonies were screened for positive insert by PCR, and plasmids containing products of the correct size were expanded and sequenced (GATC Biotech).

**Ab expression and specificity mapping**

H and L chain vectors with functional inserts were transfected at equal ratio into 293F cells, cultured to a density of 1.2 million cells per milliliter, using FreeStyle MAX Reagent (Life Technologies) according to the manufacturer’s instructions. The cell culture supernatants were tested for the presence of secreted IgG and Ag binding via ELISA 4–5 d following transfection and then harvested and purified by Protein G Sepharose columns (GE Healthcare) 7 d following transfection. The purified Abs were further tested for sensitivity and specificity by ELISA. Neutralizing Abs were coated with 1–2 µg/ml Goat–anti-IgG<sub>γ</sub> (Jackson Immunoresearch), gp140-F (the Ag used for immunizations), and influenza hemagglutinin 1, included as a control protein, in PBS overnight at 4°C. The samples were added in a 5-fold dilution series, starting from 5 µg/ml purified mAb or 1:10 diluted culture supernatant, and incubated for 1.5 h at room temperature. HRP-conjugated goat–anti-rhesus IgG (Nordic MBL) was then added at 1:2000 dilution and 3,3′,5,5′-tetramethylbenzidine+ (Life Technologies) for 5 min before stopping the reaction with an equal volume of 1 M H<sub>2</sub>SO<sub>4</sub>. The OD was measured at 450 nm. Between each step the ELISA plates were washed six times with PBS supplemented with 0.05% Tween 20.

**Library preparation for 454-pyrosequencing**

The 454-library preparation was largely performed as described before (16), with some modifications. Total RNA was extracted from 12 million PBMCs from NHP F128 following the fifth immunization. mRNA was isolated from the purified total RNA, using the Oligoex Kit (QIAGEN). Then cDNA synthesis was performed in three reactions at 40 µl, using SuperScript III and Oligo(dT)15 primers according to the manufacturer’s instructions (Life Technologies). The cDNA was then pooled and PCR purified, followed by elution with 30 µl 10× Tris-KCI (pH 8.5), making each microtiter correspond to ∼0.4 million input PBMCs.

Separate PCR reactions were set up for the IGHV1, 3, and 4 families, using mixes of primers covering the rhesus germline genes (Supplementary Table I). Library amplification was performed on 5 µl cDNA, corresponding to 2 million input PBMCs, in 50-µl reactions using the Phusion High-Fidelity PCR Kit with the GC Buffer according to instructions. The PCR samples were incubated for 2 min at 98°C, followed by 10 cycles of 98°C for 10 s, 51°C (for VH3) or 55°C (for VH1 and 4) for 30 s, and 72°C for 30 s and 15 cycles of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s. Following cycling there was a 10-min elongation at 72°C and,
finally, cooling to 4°C. PCR products were evaluated on a 1% agarose gel, the bands were then excised, and the DNA was extracted. To remove potential gel residues, the PCR products were further purified by phenol/ chloroform extraction followed by ethanol precipitation. The DNA was quantified using Qubit (Life Technologies) before sequencing in a GS FLX instrument (Roche 454 Life Sciences), using the manufacturer’s suggested methods and reagents. Image collection and subsequent signal processing, quality filtering, and generation of nucleotide sequence quality scores were performed as described previously (10). The sequences are available under accession number SRP033611 at the National Center for Biotechnology Information SRA database (http://www.ncbi.nlm.nih.gov/sra).

Sequence analysis

The 454-pyrosequencing and single-cell RT-PCR–generated sequences were first filtered for read lengths of 300–600 bp. Germline V-genes for each sequence were then assigned using a BLAST procedure with parameters set to mimic IgBLAST (17). V region sequences were extracted based on the ClustalW2 global alignment of each sequence to single-cell RT-PCR–generated Ab sequences. Incomplete sequences and problematic sequences (sequences with internal stop codons, frameshifts, or loss of any of the conserved CDRH3-flanking motifs C/X/R and W/GX) were removed from downstream analysis. The clustering program CD-HIT (18) was next used with 100% sequence coverage and identity to remove redundant reads. Finally, the divergence (percent nucleotide mutation from the assigned germline V-gene) was calculated based on ClustalW2 global alignment to the V region of the most homologous germline V-gene segment in a germline database constructed from previously annotated rhesus macaque Ig genes (11, 14). V, D, and J assignments were additionally performed by IMGT/HighV-Quest (19) (version 1.1.3, reference directory release 201338-1) using the rhesus macaque option and further analyzed in the recently published Ig Analysis Tool (IgAT, version 1.1.4) (20) program. However, individual V-gene assignments could not be performed using the IMGT tools, as their database is still limited compared to published rhesus germlines (11).

Clonality within the sorted populations (total and Env–specific memory B cells) was determined by IgAT with the criteria that clonally related sequences 1) use the same VH and JH genes, 2) have the same CDRH3 length, and 3) have a CDRH3 nucleotide sequence homology of $\geq 90\%$.

Statistical analysis

The p values for comparing total and Env–specific V(D)J-family distributions were calculated with the $\chi^2$ test. Comparisons of specific V-gene segments were made with two-way ANOVA followed by the Bonferroni multiple comparison posttest. Correlation was determined with the nonparametric Spearman correlation test, with a two-tailed p value calculated for significance.

Results

Ab sequence isolation and purification

To investigate the representation of the recently described H chain Ig V-gene segments in the rhesus macaque B cell repertoire (11, 14), IgG-switched B cells were interrogated using two independent approaches: 454-pyrosequencing of expressed V(D)J sequences from bulk PBMCs of NHP F128 (12) (Fig. 1A) and flow cytometric sorting of single memory B cells from five different NHPs—F124, F126, F128, F129, and F130 (12)—followed by RT-PCR of expressed H chain V(D)J sequences (Fig. 1B). The 454-pyrosequencing was performed for the major IGHV families (IGHV1, 3, and 4), constituting 53 of 62 described germline V-genes (11). Each IGHV1, 3, and 4 library was prepared from cDNA corresponding to 2 million PBMCs and sequenced separately to reduce PCR amplification bias. For single-cell analysis, IgG specificity was achieved by sorting memory B cells based on positive surface expression of CD20, CD27, and IgG and using 3′ primers specific for rhesus IgGl-4. Single-cell RT-PCR for IgH families 1–7 was performed using recently described rhesus–specific VH primers (14).

To analyze the generated Ab sequences, we implemented a bioinformatics pipeline for NHP H chain sequences (Fig. 1C). Briefly, sequence reads were first filtered for length, whereby only sequences between 300 and 600 bp were included. The sequences were then mapped to the current rhesus germline VH database and retained only if they included the conserved amino acid C/X/R and W/GX motifs present at the beginning and end of the CDRH3 region. Sequences with premature stop codons were discarded, as were sequences with amino acid/nucleotide ratios of $\leq 0.7$ for identity to the assigned germline sequence, owing to the likelihood that insertions or deletions resulted in a change of reading frame. In addition, to reduce potential PCR bias of specific amplicons, only nonredundant sequences were included in the analysis. After application of these filters, the 454-pyrosequencing yielded a large number of sequences for each V-gene family: IGHV1 (20,166), IGHV3 (51,320), and IGHV4 (85,645). Single-cell sorting and PCR amplification of total memory B cells generated 480 raw sequences that, following bioinformatic processing, resulted in 416 unique sequences.

Ig gene contribution to the IgG-switched B cell repertoire

The combination of deep sequencing and single-cell RT-PCR allows for qualitative and quantitative assessment of the Ab V(D)J
gene contributions. Deep sequencing provides the capacity to ascertain, with both depth and high accuracy, genes that are expressed or not, whereas single-cell RT-PCR provides a quantitative measurement of how often specific gene segments are used. The latter means of analysis provides nonbiased PCR amplification from a single cell in which a unique V(D)J recombination event has occurred. The V-, D-, and J-gene contribution of known annotated germline genes in IGHV families 1, 3, and 4 was analyzed for sequences generated by 454-pyrosequencing and single-cell RT-PCR (Fig. 2A–C). Owing to the challenge of correctly assigning D-gene segments, the D-gene contribution was determined at the family level. Overall, the expression of Ig genes varied greatly; some were abundantly used, whereas others contributed to a lesser extent to the expressed IgG-switched B cell repertoire. In addition, for the V-gene segments, some were not detected at all in our data set, despite having high homology to the PCR primers used for library preparation or single-cell RT-PCR. Generally, a very high concordance was found between the 454-pyrosequencing and the single-cell RT-PCR, resulting in a highly correlated pattern of gene segment contribution (Spearman $r = 0.92$, $p < 0.0001$; Fig. 2D). This finding suggests that the PCR protocol used for 454-pyrosequencing had no major bias and that both data sets therefore can be used for quantitative assessments and qualitative observations.

To further assess the germline VH gene segments contributing to the functional IgG-switched B cell repertoire, the 454-pyrosequencing–derived sequences corresponding to unique V(D)J recombination events were plotted from highest to lowest frequency of contribution within each of the IGHV1, 3, and 4 families, respectively. The percentage SHM of each contributing sequence was calculated relative to the assigned germline sequence, and the average SHM levels were determined (Fig. 3A–C). These analyses show that unmutated variants, or variants very close to the assigned germline, suggesting that these sequences may have derived from a V-gene that deviates from the published VH1.61 germline sequence. Overall, 42 of the possible 53 annotated V-gene segments were found to contribute to the circulating IgG-switched B cell repertoire, although usage of some gene segments was clearly preferred relative to others, closely resembling the pattern of V-gene usage observed in humans (21).

Highly diverse V-gene usage in the HIV-1 Env-specific IgG-switched memory B cell repertoire

We next investigated the range of V(D)J gene segments used in vaccine-induced responses, using samples from rhesus macaques inoculated with well-described recombinant HIV-1Env protein–based trimers (12, 13). Both total and Env-specific memory B cells were sorted to allow comparisons within individual animals. To isolate the Env-specific memory repertoire, B cells were sorted at single-cell density based on expression of cell-surface CD20, CD27, and IgG, as well as binding to a fluorochrome-conjugated Env trimer probe, as previously described (11). The Env-specific population was found to be $\sim 4\%$ of the total memory population (Fig. 4A). Next, H chain V(D)J sequences from sorted Env-specific memory B cells were rescued with nested RT-PCR, and

![FIGURE 2. High concordance in Ig VDJ gene usage by single-cell RT-PCR and deep sequencing. The frequency of H chain VDJ gene contribution (Y-axis) was determined for the individual V-gene segments (A) or D- and J-families (B and C) (X-axis) within the three major V-gene families, IGHV1, 3, and 4, for 454-pyrosequencing–generated sequences (open bars) and single-cell flow-sorted total memory B cells (filled bars). The number of sequences included in the V-gene analysis (A) were for 454-pyrosequencing: VH1, $n = 20,226$; VH3, $n = 51,320$; VH4, $n = 85,645$; and for single-cell RT-PCR: VH1, $n = 41$; VH3, $n = 152$; VH4, $n = 188$. Individual n-family and J-gene (B, C) contribution to all analyzed sequences was determined following pooling of the sequences (454-pyrosequencing, $n = 148,105$; single-cell RT-PCR, $n = 379$). (D) The correlation of the V-gene, n-family, and J-gene contribution to the analyzed sequences (A–C), as determined by 454-pyrosequencing (Y-axis) and single-cell RT-PCR (X-axis), was calculated by determining the nonparametric Spearman correlation coefficient ($r$) and the $p$ value.](http://www.jimmunol.org/)
a total of 638 VH sequences were generated. After processing in the bioinformatics pipeline (Fig. 1) for annotation of individual V-gene segments and IMGT/HighV-Quest, followed by analysis in IgAT for V(D)J-gene family assignment, 563 and 570 unique Ab sequences were obtained, respectively. Of these, 33 H chain V(D)J sequences from the same cell to allow expression of the complete mAbs to verify the specificity of the Ag-specific sort. The resulting mAbs were evaluated for binding to HIV-1 Env by ELISA and were all confirmed positive, indicating a high diversity of Ab specificities elicited toward the trimeric Env Ag.

**Discussion**

Rhesus macaques are frequently used as models of human immunology and are important for the evaluation of primate responses to infection and vaccination (22–24). Until recently, systems to investigate elicited B cell responses in NHPs at the cellular and genetic level were poorly developed. With the publishing of the rhesus macaque genome (25), the annotation of rhesus Ig germline gene segments, and the description of methods to phenotype and sort rhesus macaque B cell populations (11), the ability to dissect vaccine- and infection-induced B cell responses in NHPs is now available. This capability, coupled with methods to clone and express Ag-specific human mAbs (6, 15), recently adapted to the rhesus macaque system (14), and recent developments in deep sequencing approaches to study human Ig repertoires (16, 21, 26–39), motivated us to investigate VH gene usage in IgG-switched and Ag-specific repertoires in rhesus macaques. Although isolated studies of VH gene segment use in rhesus macaque B cells were reported (40–43), to our knowledge, this is the first comprehensive report describing the IgG-switched B cell repertoire in NHPs at a level of high resolution to more specifically elucidate the genetic diversity of rhesus macaque memory B cells.

Another unique aspect of our study is that we use both 454-pyrosequencing and single-cell sorting for genetic characterization of IgG-switched memory B cells. Despite performing the PCR under very different conditions by these two distinct means of analysis, we obtained remarkably similar results with the two methods, demonstrating VH gene segments that were frequently used, rarely used, or not detectably used. Most notable was the lack of representation of several gene segments of the VH3 family, the largest VH family in both humans and macaques, in agreement with results previously obtained from 454-pyrosequencing of the human Ab repertoire (21). It is possible that a greater sequencing depth, or the analysis of more animals, would reveal the use of additional gene segments, as we observed some variation in the five different macaques analyzed in this study (Supplemental Figs. 2, 3). Although isolated studies of VH gene segment use in rhesus macaque B cells were reported (40–43), to our knowledge, this is the first comprehensive report describing the IgG-switched B cell repertoire in NHPs at a level of high resolution to more specifically elucidate the genetic diversity of rhesus macaque memory B cells.

**FIGURE 3.** Differential usage of germline Ig V-gene segments. Frequencies of the individual V-gene segment contribution to the IGHV1 (A), IGHV3 (B), and IGHV4 (C) families, as determined by 454-pyrosequencing, are plotted in descending order. The number of sequences (n) and mean SHM, based on nucleotide alignment to closest germline sequence, of each V-gene segment are indicated in the table. The scatter plot (right) shows SHM for each V-gene segment with mean values indicated by the gray line.
mAbs. This is particularly important for the analyses of Ag-specific repertoires in which it is critical to confirm the specificity of the flow cytometry–based sort by evaluating the binding capacity and specificity of the resulting mAbs. In the current study, we observed 100% specificity of a panel of 33 cloned mAbs, strongly supporting the conclusions in regard to the Env-specific VH repertoire presented in this article.

In the past few years a number of studies were performed using 454-pyrosequencing to investigate the Ab repertoires in HIV-1–infected individuals, with the aim to trace the evolution of broadly neutralizing Abs in these individuals (16, 27–31). These analyses provide valuable information about the antibodyome induced and shaped by chronic HIV-1 infection and highlight the extreme levels of Ab affinity maturation that can develop under these conditions (44), likely a common feature of, and a consequence of, chronic infection (45). Deep sequencing approaches were also recently used to investigate human Ab responses to vaccination (32, 46), but, so far, such studies have not been reported from immunization trials in NHPs. Our results obtained by deep sequencing of rhesus macaque B cell responses therefore provide timely information, which will greatly facilitate future studies of B cell/Ab repertoires in the NHP model.

In parallel, we generated 1000 sequences from single-cell sorted total and Ag-specific memory B cells from animals inoculated with soluble HIV-1 Env trimers (12). In contrast to studies of infection-induced B cell responses, in which the Env Ag is highly variable owing to the continuously changing virus population and therefore not easily definable (6, 47), we used a probe for FACS sorting that was identical to the protein used for immunization, allowing isolation of all potential Env-elicited subspecificities. Clonal lineage analysis of the isolated sequences estimated that 502 unique Ab clonotypes were present in 606 Env-specific sequences, suggesting that vaccination results in a highly polyclonal response. In addition, the overall VDJ gene distribution of the Env-specific sequences was highly similar to that observed in the IgG-switched total memory B cell repertoire, with the exception of an increase of VH5-using sequences, especially of VH5.46, in Env-immunized macaques, potentially signifying that Env-specific Abs using this V-gene segment might have a selective advantage following immunization, a finding that needs further investigation.

Table I. Clonal lineage analysis of Env-specific memory B cells

<table>
<thead>
<tr>
<th>Donor</th>
<th>Functional a</th>
<th>Unique b</th>
<th>Clonotypes c</th>
</tr>
</thead>
<tbody>
<tr>
<td>F124</td>
<td>217</td>
<td>200</td>
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<td>F125</td>
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<td>F128</td>
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<td>195</td>
<td>180</td>
</tr>
<tr>
<td>Total</td>
<td>606</td>
<td>570</td>
<td>502</td>
</tr>
</tbody>
</table>

Env refers to CD20+CD27+IgG+gp140-F+ B cells.

aNumber of functional sequences as determined by IMGT/V-Quest and IgAT.

bNumber of unique sequences as determined by IMGT/V-Quest and IgAT.

cDefined as 1) use the same VH and JH genes, 2) identical CDR3 length, 3) 90% CDR3 nucleotide homology.


