Pandemic, Epidemic, Endemic: B Cell Repertoire Analysis Reveals Unique Anti-Viral Responses to SARS-CoV-2, Ebola and Respiratory Syncytial Virus

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Immunoglobulin gene heterogeneity reflects the diversity and focus of the humoral immune response towards different infections, enabling inference of B cell development processes. Detailed compositional and lineage analysis of long read IGH repertoire sequencing, combining examples of pandemic, epidemic and endemic viral infections with control and vaccination samples, demonstrates general responses including increased use of IGHV4-39 in both Zaire Ebolavirus (EBOV) and COVID-19 patient cohorts. We also show unique characteristics absent in Respiratory Syncytial Virus or yellow fever vaccine samples: EBOV survivors show unprecedented high levels of class switching events while COVID-19 repertoires from acute disease appear underdeveloped. Despite the high levels of clonal expansion in COVID-19 IgG1 repertoires there is a striking lack of evidence of germinal centre mutation and selection. Given the differences in COVID-19 morbidity and mortality with age, it is also pertinent that we find significant differences in repertoire characteristics between young and old patients. Our data supports the hypothesis that a primary viral challenge can result in a strong but immature humoral response where failures in selection of the repertoire risk off-target effects.

Keywords: B cell, COVID-19, ebola, RSV (respiratory syncytial virus), immunity, repertoire, class-switch recombination (CSR)
INTRODUCTION

The emergence of SARS-CoV-2 in 2019, the ensuing pandemic and evolution of novel variants continues to make COVID-19 a matter of global public health significance. The recent SARS, MERS, Zika and Ebola outbreaks have also highlighted a need to better understand how the human immune system responds to novel infections, develop better treatments and control their emergence and spread. Initial reports from the COVID-19 pandemic, relying heavily on serum antibody titres, saw rapid emergence and spread. Initial reports from the COVID-19 novel infections, develop better treatments and control their better understand how the human immune system responds to MERS, Zika and Ebola outbreaks have also highlighted a need to

IMMUNOGLOBULINS (Ig), both as secreted antibodies and as B Cell Receptors (BCRs), mediate immunity against multiple pathogens through their vast variability in antigen binding. This variability is produced by V-D-J recombination (4), where V, D and J genes are recombined from a pool of diverse genes. B cells with Ig genes encoding disease-specific antibodies are expanded upon challenge, causing a skewing of the repertoire towards greater use of antigen-specific genes associated with the challenge in question. Furthermore, the imprecise joining of gene segments, together with the action of terminal deoxynucleotidyl transferase (TdT) creates a highly diverse complementarity determining region (CDR)3, which is important for antigen binding, and can be used to identify "clones" of B cells within a repertoire. These clonal assignments allow us to track lineages and follow the progress of the post-activation diversification events of somatic hypermutation (SHM) and Class Switch Recombination (CSR) as the B cell response develops. Thus, repertoire analyses can help to characterise changes in the memory/effector B cell compartments and identify individual genes of interest for possible antibody therapeutics.

Both SHM and CSR are mediated by the enzyme Activation-Induced cytidine Deaminase (AID) and have traditionally been associated with germinal centre events in secondary lymphoid tissue, involving T cell help (5–7). There is, however, also evidence that CSR may occur outside of the germinal centre environment (8–11) and may not require direct T cell help. The ability of a B cell to mount a directed effector response prior to the formation of a germinal centre allows a more rapid immune response but with lower affinity.

Immune responses are often impaired in older people, which has been of particular concern in COVID-19 patients. The older immune system has shown reduced responses to vaccination, frequently with higher numbers of autoreactive antibodies and inflammatory cytokines (12–14). In B cells we, and others, have shown that particular subsets of B cells are altered with age: IgM memory cells (CD19+CD27+IgD+) are decreased in older people while the Double Negative (CD19+CD27-IgD-) are increased (15, 16). Since IgM memory cells are often associated with a T-independent response, the decrease in IgM memory in older people could have severe consequences in infections where a rapid extrafollicular response is required (17, 18). It has also been shown that the B cell repertoire is skewed towards sequences with longer, more hydrophobic CDR3 regions as we age (16, 19). As an immune response can result in a shift towards lower, less hydrophobic CDR3 regions (14, 20), and higher hydrophobicity has previously been correlated with increased polyspecificity (21–23), the older immune repertoire seems to be disadvantaged in this respect.

In this study we took a long-read repertoire amplification approach that allowed us to track the V-D-J clonal lineages in the context of antibody subclass to better understand, compare and contrast B cell responses to emerging or endemic viruses. Samples were taken from COVID-19 patients during and after infection, Ebola virus disease (EBOV) survivors from West Africa and the UK, volunteers challenged with Respiratory Syncytial Virus and compared with samples from healthy donors. We report the variation of repertoire between disease states in novel virus infection, with a focus on elderly who are known to respond less well to infection, particularly in SARS-CoV-2.

METHODS

Sample Collection

Whole blood samples (RSV, COVID-19, Healthy) were collected into Tempus™ Blood RNA tubes, kept at 4°C, and frozen down to -20°C within 12 hours. Ebola samples were cone filters from plasmapheresis, dissolved in Tri reagent. RNA was extracted using Tempus™ kits according to instructions. Healthy samples taken after SARS-CoV-2 emergence were all confirmed negative for anti-SARS-CoV-2 antibodies by SureScreen lateral flow test and by ELISA (24). Ebola RNA blood samples were collected from convalescent patients with viral RNA negative PCR tests in the 2014-2016 West African outbreak, three patients were Caucasian treated in the UK, and the remaining were convalescent plasma donor participants from a trial in Sierra Leone (25) (consented under the Sierra Leone Ethics and Scientific Review Committee ISRCTN139990511 and PACTR201602001355272 and authorised by Pharmacy Board of Sierra Leone, #PBSL/CTAN/MOHSCST001). COVID-19 samples were collected from SARS-CoV-2 positive patients at Frimley and Wexham Park hospitals during 2020 (consented under UK London REC 14/LO/1221). Each participant was attributed a “severity score” in relation to their fitness observations at the time of hospital admission using the metadata collected. This score used the “mortality scoring” approach of SR Knight et al. (26) adapted to disregard age, sex at birth and comorbidities, and ranged from 0 to 6; patients scoring 0 to 3 were attributed low severity and patients scoring 4 to 6 were attributed high severity (25). Convalescent COVID-19 patients, from hospital sampling, were contacted for further donations and sample taken 2-3 months post hospital discharge. RSV samples were collected from participants who took part in a human challenge study and were monitored for infection by viral PCR tests (consented under UK London REC 11/LO/1826). Briefly, healthy participants were challenged intranasally with 10⁶ plaque-forming units of the M37 strain of RSV and monitored for up to 6 months as previously described (27).
Reproductive Library Generation

Tempus™ tube samples were defrosted at room temperature and RNA was extracted using the Tempus™ RNA extraction kit according to the manufacturer’s instructions. RNA samples were template switch reverse transcribed using SMARTScribe™ reverse transcriptase (Clonetech) according to manufacturer’s instructions using the SmartNNN TSO Primer (Supplementary Methods Table 1) with a minimum of 170 ng of RNA input. The samples were then treated with 0.5 units/µl of Uracil-DNA Glycosylase (NEB) for 60 min at 37°C to reduce UMI interference, then incubated at 95°C for 10 min to inactivate the enzyme. Samples were amplified using Q5 polymerase (NEB) according to manufacturer’s instructions with an annealing step of 65°C for 20s and extension step of 72°C for 50 s for 21 cycles. Round one of PCR was performed with forward primer Smart20 and mixed heavy chain (IG[M, G, A]-R1) reverse primers (Supplementary Methods Table 1). For PCR1 8 x 20 µl reactions were performed with 1 µl of RNA input per reaction. A semi-nested 2nd PCR was performed with forward primer PID-Step and reverse primers IG[M/G/A]-R2 (Supplementary Methods Table 1); 16 reactions of 20µl each was performed for each isotype with the same thermal cycling conditions as PCR1 but with 12 cycles, with 1µl of template. The primers in PCR2 also contain Patient Identifier (PID) sequences to allow multiplexing on PacBio (Supplementary Methods Table 2). Samples were run on a bioanalyzer (Agilent 7500), isotypes from patients were pooled at equal concentrations and concentrated using Wizard PCR Clean-up kits (Promega) from patients were pooled at equal concentrations and concentrated using Wizard PCR Clean-up kits (Promega) according to manufacturer’s instructions with 30 µl of elute. Each isotype was then purified using a PippinPrep™ with Marker K reagents (Sage Biosciences) used as an external ladder reference (IgM/G/A 600-100bp). The concentration was checked using a DNA quantification kit on the Qubit according to manufacturer’s protocol, the different isotype samples were pooled at equal concentrations and purified with SPRIselect beads (Beckman Coulter) at X0.8 sample volume with elution in 30µl of TE buffer. Sequencing was performed on either the PacBio RSII or Sequel platforms (See Supplementary Methods Table 2).

Quality control, data cleaning and removal of multiplicated UMIs was carried out as published (16, 28). Immunoglobulin V-D-J gene usage and CDRH3 was determined using IMGT/High V-quest. Clonotype clustering was carried out as per (16, 28), in brief: a Levenshtein distance matrix was generated on the CDRH3, hierarchically clustered and branches cut at 0.05 to generate clones. Physicochemical properties were calculated using the R Peptides package (29).

Analysis of Clonal Diversity

We sought for methods to qualitatively (visualising clone size distribution) and quantitatively compare clonal diversity (calculating metrics which summarise clonal diversity). We first noted, as one would expect, that sequencing depth (i.e., number of sequences sampled per repertoire) was a strong predictor of the number of clones (Supplementary Figure S1). For all repertoires considered here, a wide range of sequencing depth was observed (number of sequences range from 836 to 105,323, median = 12,040). We therefore adopted the following procedure in this analysis: first, to quantify the extent of clonal diversity we used the Gini coefficient which measures the evenness in the distribution of clone size across clones; application of this metric to quantify BCR clonal diversity has been well documented (30–32). For a given repertoire, clones were ordered by their clone sizes, and the cumulative distributions of clone sizes (in terms of percentage of sequences in repertoire) and its percentile distribution were compared for evenness. As such, the resulting metric was independent from the absolute numbers of sequences and clones, thereby allowing fair comparison across repertoires of different sequencing depths. As Gini coefficient is an indicator of evenness, we took (1 – Gini coefficient) as the metric of clonal diversity. To qualitatively compare clonal diversity, we generated visualisation using the following procedure to minimise the impact of sequencing depth differences: we first sampled 12,000 sequences (= median sequencing depth; see above) from each repertoire; for repertoires with less than 12,000 unique observations, this number of sequences was sampled with replacement. We then sampled up to 100 clones with probability scaled by clone sizes to generate bubble plots where each bubble represents a clone and bubble sizes are scaled with clone sizes. Genotypic features like V gene usage can be represented as colours. Such plots were included in Figure 2C.

Analysis of BCR Clone Lineage Trees

Lineage trees were reconstructed using the maximum parsimony method implemented in the dnaps executable in the phylip package (33). All clones with at least 3 sequences were considered; IMGT-gapped, V-gene nucleotide sequences of all observations in the clone together with the annotated germline V-gene sequence (included to root the tree) were included as input to dnaps. Functionalities implemented in the alakazam R package (34) were used to call dnaps and reformat the output into text-based tree files (newick format) and directed graphs (igraph objects manipulated in R). The directed graphs were further parsed using functions in alakazam and igraph to obtain, for each observed sequence in the given clone, its distance D from the given germline gene g (denoted here as $D_g$), as estimated by the dnaps-reconstructed lineage tree: the closer this distance is to 0, the closer the sequence is to germline and therefore a lower mutational level.

We sought to summarise, for a given clone, the distribution of $D_g$; this distribution would indicate the overall mutational level of sequences within the clone (summarised using conventional statistics like the median of $D_g$) and the evenness of mutational level (i.e. whether the clone consists of expansion of sequences with a similar mutational level, or it comprises sequences with a wide range of mutational levels). This can be visualised as a heatmap (clones [vertical axis] versus $D_g$ [horizontal axis], with colours scaling with density of the distribution; see Figure 5B), or as a curve (clones [vertical axis] versus the median of $D_g$ [horizontal axis], See Figure 5C). The curve representation allows calculation of area-under-curve (AUC) as a metric which we termed “Germline Likeness”, to quantify mutational
levels across clones. This is similar to quantifying sequence similarity to germline, except that here Germline Likeness quantifies the tendency to which all clones from the BCR repertoire of a given individual have high similarity to the germline (and therefore lower mutational levels).

Detecting Class-Switch Recombination Events From Lineage Trees
Since the lineage trees were constructed using only V-gene sequences (see above), in theory antibody sequences of different subclasses could be ordered in the tree in a way that imply class-switch recombination (CSR) events which are mechanistically impossible. We therefore pruned the dnapars-reconstructed tree to remove edges which imply CSR events that violate the physical order of constant region genes in the human IGH locus. This was performed using a Python implementation of the Edmond’s algorithm to construct a minimum spanning arborescence tree with the given germline V gene sequence as root. With this arborescence tree the type of CSR (subclass switched from/to) and the distance-from-germline at which the CSR event occurred (estimated as the median distance-from-germline of the two observations relevant to the given event) were obtained.

We noticed that the quantification of CSR events is dependent on the number of sequences sampled in the repertoire. To eliminate this confounding factor, we followed the sampling protocol for the clone size visualisation (Figure 2C): briefly, we sampled 12,000 sequences (=median sequencing depth across all samples) from each repertoire; for repertoires with less than 12,000 unique observations, sampling was performed with replacement. The analysis presented here (Figure 6) is the result after this subsampling analysis, therefore corrected for difference in sequencing depth.

Convergent Network
Productive heavy chain sequences with CDRH3 of length shorter than 30 amino acids were considered in the construction of a convergent network. Sequences were connected if they meet the following criteria (a) same V and J gene usage; (b) from different individuals; (c) same CDRH3 amino acid length, and (d) ≥85% CDRH3 amino acid identity. The same criteria have been applied in published studies investigating convergent clonotypes across SARS-CoV-2 B-cell repertoires (35, 36). To allow interpretation of possible targets of sequences in convergent network clusters, known binders were included in constructing the network. Known binders were taken from the following sources: first experimentally determined antigen-antibody structural complexes deposited in the Protein Data Bank (PDB); PDBe was queried on 19 May 2021 with the search term ‘Organism: Severe acute respiratory syndrome coronavirus 2’. The resulting list of PDB entries was overlapped with entries in the SAbDab structural antibody databases (37) to obtain list of PDB complexes of antibodies and SARS-CoV-2 proteins. A total of 215 heavy chains from 186 structures were considered. Second, known binders validated in experiments where antibody variable regions were cloned and assessed for SARS-CoV-2 protein binding were taken from published work (38–43). All known binder sequences were annotated for V/J gene usage using either IMGT/High-VQuest (if DNA sequences were provided) or IMGT/DomainGapAlign (if only amino acid sequences were provided). Information regarding specificity (i.e. SARS-CoV-2 protein targets) was obtained from either Supplementary Data Files in the cited publications or by visual inspection (for PDB structures). Supplementary Table S2 contains all known binder sequences included in this analysis. To construct the network, known binders were connected to one another and to repertoire sequences using the identical criteria mentioned above. In total 809 unique CDRH3 sequences were considered in constructing the convergence network. The resulting network contains 7500 sequences (7370 from repertoire, 130 known binders).

We note that the method of constructing convergent networks is comparable to single-linkage clustering. We compared these results with complete-linkage clustering (applied in R using the command ’hclust(method = ”complete”)’) and found that using the same amino-acid identity cutoff, it produced clusters which are notably smaller (Supplementary Figure S6A). Since in the binders list we have SARS-CoV-2 binders for which crystal structures are available, we reason that those antibody structures which bind the antigen in the same way should be grouped together. Manual inspection of these structures found that they are grouped together in our networks, but are separated under complete-linkage clustering (Supplementary Figure S6A). We confirm this observation quantitatively, by comparing the antibody-antigen structures by calculating the TM-score (44) between every pair of binder structures, which confirm that our convergent networks group together binders with similar interface at the three-dimensional structural level (Supplementary Figure S6B, see figure legend therein). We therefore reason that complete linkage is too stringent, and our networks are likely to group sequences which engage with the antigen in the same way.

Analogous convergent networks were constructed using the EBOV and RSV repertoire data, separately considered with respective known binders and Healthy individuals’ repertoire; the majority of clusters were formed mainly of sequences from Healthy donors absent of known binders (45–48), although we were able to identify two convergent clusters of RSV-infected individuals with similar CDRH3 to known binders of the RSV fusion glycoprotein (Supplementary Figure S7). To investigate whether clusters shared across disease conditions exist, convergent networks were also constructed considering CV19, RSV and EBOV repertoire and binder sequences altogether (Supplementary Figure S8). Supplementary Table S3 contains all convergent networks constructed, presented as list of pairwise sequences.

Statistical Analysis and Data Visualisation
V-D-J gene usage for each patient was turned into a proportion to normalise for different numbers of sequences and allow for comparison. Gene usage analysis was performed in GraphPad Prism 8.4.3 using a two-way ANOVA with a Dunnett’s *post hoc* test. P-values have been corrected using the Benjamini-Hochberg method wherever applicable. All other statistical analyses were performed in the R statistical computing environment (version 4.0.2). Data visualisation was performed using the ggplot2 package and the following specialised R packages: visNetwork
(for visualising convergent CDRH3 network clusters) and ggseqlogo (for visualising CDRH3 sequence logos). PDB structures were visualised using PyMOL (version 2.3.0). Histograms of CDRH3 length and hydrophobicity, as measured by Kidera factor 4, were constructed on the Brepertoire website (49).

RESULTS

Patient Cohorts

IGH sequences, of total V-D-J plus ~150-200 bp of C regions, were obtained from pandemic, epidemic and endemic diseases and stages along with 24 healthy controls across multiple age ranges (Figure 1 and Table 1). This included: 16 hospitalised COVID-19 patients (CV19), 5 of these patients had follow-up convalescent samples (CV19-Recovered, hereafter CV19R), 12 Ebola convalescent plasma donors (EBOV), 12 participants challenged with RSV, 6 of whom became infected (RSV-I) and 6 of whom did not (RSV-U). Healthy Samples (Healthy) were a grouping of YFVD0, RSVD0 and samples taken as controls during the (COVID-19) pandemic (n=24). Numbers of sequences varied from 836 to 105,323, median = 12,040 per sample, IGH gene usage for each patient was expressed as a proportion of the total in order to normalise for differences in sequence numbers between different samples.

IGH Gene Repertoire Changes in Response to Viral Infection

Although the humoral immune response is varied, with different subclasses of antibody having different effector functions (50), many methods of repertoire analysis have hitherto not distinguished between antibody subclasses. We have used PacBio methods to obtain full V-D-J sequence in the context of subclass usage to investigate class switching events during immune responses to infection. We also distinguish between mutated versus unmutated IgM sequences, as a proxy for identifying IgM memory responses. Comparisons of subclass distribution, in relation to healthy controls, revealed a significant increase in the proportion of IgA1 compared to IgA2 in CV19, and RSV-I and the proportion of IgG1 relative to IgG2 in CV19.

Unusually, we also saw a decrease in diversity of unmutated IgM sequences, indicative of clonal expansion prior to SHM and CSR (Figure 2D). These values returned to normal in the CV19R samples. In comparison, IGHV1 family was expanded in RSV-I IgG1 partition (Figure 2C), particularly of IGHV1-18 (Supplementary Figure S4). Active infection with RSV, as well as samples taken 28 days after yellow fever vaccination, showed an increase in diversity of IgA2. Interestingly, EBOV memory B cell populations were more diverse than healthy controls in all the main switched subclasses (IgG1, IgG2, IgA1, IgA2) (Figure 2D and Supplementary Figure S3).

Large clone sizes can mask whole repertoire changes, so we analysed the frequency of gene use after reducing the data to one representative sequence per clone. We found increased use of IGHV3-30 in IgM mutated sequences in CV19 patients (Figure 2E), and also of IgM-mutated/IgA1/IgG1 in CV19R; this was unique to CV19. An increase in use of IGHV4-39 was found in CV19 IgA1 sequences and was also found increased across the board in EBOV and RSV-U samples (Figure 2E). IGHV3-23 was found to be reduced in ongoing infection (likely an offset as a result of relative increases in usage of other genes) but exceeded the healthy levels in CV19R. IGHV1-69, which has previously been associated with viral infections (51, 52), was increased in RSV-I but not EBOV or CV19. The YF day 28 vaccine samples increased use of IGHV3-7 in IgM-mutated only (Figure 2E; comparisons for other V genes in Supplementary Figure S4).

Complementarity Determining Region 3 (CDRH3) Immaturity in COVID-19

Given the importance of CDRH3 in antibody recognition, and the contribution to CDRH3 from the IGHD and IGHJ genes, we analysed these also. In CV19 samples there was a significant increase in use of IGHD2-2, IGHD3-3 and IGHJ6 (Figure 2E). These genes tend to be more hydrophobic (IGHJ6 being the exception) and all have among the longest amino acid lengths with only IGHD3-16 being 2 AA longer. This contribution can be seen in the overall CV19 repertoire which skews towards longer amino acid sequences and increased hydrophobicity, indicative of early response as affinity maturation causes shorter less hydrophobic CDRs (Supplementary Figure S5). A clustering analysis of peptide physicochemical properties of CDRH3 regions generally results in a difference between IgM sequences and memory sequences (Figure 2F), presumably reflecting biases in antigen selection during post-challenge development. We can see that healthy and CV19 subclass sequences mostly have similar CDRH3 properties to each other, however, in the case of CV19 IgG1 and IgG3 cluster closer to IgM sequences from healthy and EBOV rather than healthy IgG1 and IgG3 sequences implying a more ‘naive’, unselected, repertoire.

Convergent Antibody Clusters Reveal Distinct VDJ Preferences

To assess the functional importance of the skewed patterns of V, D and J gene usage in CV19 we created networks connecting sequences observed in our CV19 and control repertoire data...
in our total cohort analysis (Figure 2E), is significantly increased in the under 50s IgM-mutated partition (Figure 4C). We also found that IGHV4-39, IGHD2-2, IGHD3-3 and IGHJ6, which we find are expanded in CV19 across multiple B cell partitions, only have significantly increased expression in the under 50s and not the over 60s; IGHV4-34 appeared increased in both age groups in the IgG1 partition, but did not reach statistical significance (Figure 4C).

IgG1 Is Immature in Response to COVID-19

Beyond the scope of gene usage, our BCR repertoire data also enabled reconstruction of individual BCR trees to make inferences about the evolution of a particular clone. Using the annotated germline V allele as the root of the tree, we estimate, for each sequence in the lineage, its distance from the root (Figure 5A); this distance being directly proportional to mutational level. We visualise the distribution of this germline distance across all clonotypes observed in each given individual, and observe that the repertoire is dominated by clonotypes with very low mutational levels for a subset of CV19 patients, whilst the predominance of such clones is broadly absent in healthy controls (Figure 5B, C). Interestingly, in repertoires from convalescent individuals (both EBOV and CV19), we instead observe dominance of clonotypes with higher mutational levels, although the pattern is less striking than the CV19 patients during hospitalisation (Figure 5C). These curves allow for quantification of the Area Under the Curve (AUC), which constitutes a metric we term “Germline Likeness”: a higher Germline Likeness corresponds to a lower level of mutation across all clones (Figure 5D); this is akin to quantifying sequence similarity to the germline, except that Germline Likeness here quantifies such phenomenon for a given repertoire in general, rather than a specific sequence. Using this metric we confirm that CV19 repertoires were dominated by clones that were largely unmutated, while EBOV samples carried the greatest mutation rate (Figure 5E). As might be expected, with time to generate a germinal centre response, Germline Likeness in CV19 faded with time (Figure 5F), to the point where the CV19R repertoires have similar level of mutations compared to the EBOV-convalescent and healthy control repertoires. Partitioning the analysis by isotype, RSV and healthy controls demonstrate the expected trend where an increased level of mutations can be found in both IgG and IgA compared to IgM (Figure 5G). However, in CV19 only IgA showed a significant change in Germline Likeness from IgM, albeit with a similar level of Germline Likeness to the healthy IgM (Figure 5G).

Ongoing Class Switch Recombination (CSR) Is Detectable in PBMCs of COVID-19 and EBOV Patients

Our lineage trees were further analysed for CSR events: respecting the sequential order of CSR in the genome, we identify CSR events where sequences of different antibody classes/subclasses are directly connected in the lineage tree. This enables us to trace the timing of CSR events (distance from the germline), the direction of class switching (e.g. from IgM to IgG1) and frequency of observation. Many clones have

VDJ Selection Differs Between Ages in COVID-19

The disparity in COVID-19 severity and mortality between age groups is striking, so we looked for age-related differences in our B cell repertoire data, grouping by <50 and >60 based on this mortality disparity. The difference in IgA1/IgA2 ratio is less in older people, not reaching statistical significance. (Figure 4A).

On the other hand, the increase usage of IgG1 in CV19 is robust across age (Figure 4B). Considering Ig gene usage, we observe the intriguing case of IGHV3-30 which is only preferentially used by the over 60s during infection (Figure 4C). Conversely, IGHV3-53, which appears relatively frequently in known binder data in combination with IGHJ4/6 but did not appear

(Figure 3A), using criteria previously employed in discovering ‘convergent’ antibody sequences shared between patients (53). By also including known SARS-CoV-2 binders (see Methods), we determined that this method is superior in comparison with complete-linkage clustering, in grouping together antibody sequences which bind the antigen in similar manner (Supplementary Figure S6). We obtain clusters of CDR3 sequences found in both CV19 patients and healthy controls, some of which converge towards known binders of SARS-CoV-2 proteins such as those targeting the receptor binding domain (RBD) of the spike protein (Figure 3B). Many of these large convergent clusters did not, however, include a known binder in the network (Figure 3C). Interestingly, we observe that some of these convergent clusters also contain sequences from Healthy controls, suggesting that our baseline repertoire contains B cells capable of recognising SARS-CoV-2 proteins, as reported by others (36, 53). Overall, convergent clusters use a diverse set of V genes, but most of our larger convergent clusters contain IGHV3 or IGHV4 families and demonstrate increased IGHJ6 usage as well as the more commonly used IGHJ4 (Figure 3C). A comparison exclusively of the known binders to date reveals distinct combinations of V and J gene preferences (Figure 3D).

We do find clusters of sequences using IGHV3-53 and IGHV1-58 such as those used in anti-RBD antibodies (e.g. Figure 3B). We find that sequences from convergent clusters tend to be found in larger clonal expansions than those without evidence of convergence (Figure 3E), possibly implying that specific clonal expansions in response to challenge are shared across patients. We note that half of the larger clusters have substantial contributions from healthy control sequences, so there may be some IGH genes, such as IGHV3-33/IGHJ5 found also in RSV-I and EBOV convergent networks (Figure 3C and Supplementary Figures 6A, B), which have increased versatility such that they are often seen in response to multiple different challenges.

Similar analyses of RSV and EBOV were limited by the paucity of information on antibody binders, however it was notable that only RSV-I, and not RSV-U, showed evidence of convergence. IGHV1-18 appears in a large cluster with a known RSV F-protein binder and although the large IGHV3-23 cluster does not contain a known binder it forms part of the larger expansion of IGHV3-23 genes in mutated IgM genes from this cohort (Figure 2E, Supplementary Figure S7B).

### References

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evidence of CSR, particularly in EBOV, even after correcting for clone sizes (Figure 6A). In particular, CV19 patients were more likely to switch early to IgG1 from IgM, with little mutation (Figures 6B–D) and to IgA1 from IgG1 later in the lineage with more mutation (Figures 6B, D). This agrees with the lack of CDRH3 “maturity” in IgG1 (Figure 2F) and the overall increased use of IgG1 and IgA1 seen in CV19 (Figures 2A, B).

The evidence of increased CSR in convalescent EBOV patients is striking and occurs across the board with the exception of IgM switching to IgG1 (Figure 6D). We noticed

See Supplementary Table S1 for a detailed summary of metadata per donor.
that although there is a similar pattern of CSR preferences in White and West Africans individuals, the overall distance from germline is longer before CSR occurs in West Africans (Figure 6B). This may suggest that the ethnic bias in existing immunoglobulin sequence databases has resulted in misassignment of germline alleles. No CSR differences were seen in the RSV data.

**DISCUSSION**

We compared immunoglobulin gene sequences from pandemic (SARS-CoV-2), epidemic (Ebola) and endemic (Respiratory Syncytial Virus) patients in order to discover features that might distinguish newly emergent and endemic infections. We notice that although the sequencing depth varies between sample types, metrics related to gene usage and mutational levels are typically highly stable at different sequencing depths (Supplementary Note), allowing us to perform robust comparisons across different immune challenges. The ability of B cells to generate a highly diverse immunoglobulin repertoire that might bind any antigen, and the diverse functionality of the antibodies produced, is critical for an effective immune response. Repertoire studies aim to identify specific antibodies by looking for biased usage of particular Ig genes, and have been useful in the past (16, 28). However, not all expanded genes encode
specific binders (49) and we need to consider the possibility that expansions found in the midst of an acute response may be a side effect of the disease involving inappropriate expansion of B cells carrying antibodies with off-target effects rather than a specific targeting to the challenge. Repertoire selection is normally a delicate balance between tolerance versus immune response to a pathogen and the inflammatory state of acute disease can upset the balance. Serological studies have shown an increase in autoreactive antibodies, particularly to interferons, during acute COVID-19 for example (54, 55).

Looking across different viral diseases, we found a general increase of IGHV4-39 in the repertoire of two different viral diseases (COVID-19 and Ebola). Despite this, only one of our convergent clusters, dominated by COVID-19 sequences, uses IGHV4-39 (Figure 3C); it is possible that there are unannotated IGHV4-39 SAR-CoV-2 binders. One single cluster does not, however, explain the larger expansion in IGHV4-39 use across the COVID-19 or Ebola repertoire. IGHV4-39 may therefore be involved in the pathogenesis of the disease by promiscuous binding to self-proteins. Alternatively, IGHV4-39 may simply support a wide range of specific binding properties, supported by the lack of convergence and given it has also been dominant in cancer, bacterial infection, influenza and HIV responses (56–59).

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3** CDR3 regions from different CV19 patient repertoires converge, some with known SARS-CoV-2 binders. (A) CDR3 known binder networks were created using same V, J and CDR3 length with at least 85% amino acid (AA) identity. (B) Convergent clusters from healthy and CV19 repertoire with known PDB structures. IGHV and IGHJ use and the CDR3 AA sequence were noted. (C) Clusters containing at least 10 sequences were visualized, with breakdown of repertoire origin (stacked bar plots), and the IGHV and IGHJ gene usage of each cluster aligned beneath. The number of donors with sequences in each depicted cluster are shown as bar graphs (bottom panel, C), broken down into subsets with age ≤50 (light grey) and ≥60 (dark grey). (D) All known binders were analysed for similarity of IGHV/J gene use to specific SAR-CoV-2 antibody targets. Dots coloured by enrichment (log-odds ratio, logOR). For each V/J combination only the target with the top logOR metric was shown. (E) Comparison of clonal expansion of convergent (split by clone size; ≥10 or <10 sequences) and non-convergent clusters in healthy and CV19 repertoires. Statistical significance evaluated using a Wilcoxon rank-sum test, ****: FDR < 0.0001. See Supplementary Figure S7 for analogous analyses on RSV and EBOV repertoires.
dominated by CV19/CV19-R sequences, yet only 5 matched known binders, suggesting previously unknown SARS-CoV-2 specific binders. As more studies on both naturally infected and vaccinated individuals identify and validate new SARS-CoV-2 binders, we expect more experimental validation to ascertain the relevance of these clonotypes in the immune response. Further data integration exercise incorporating more repertoires (from both healthy and infected/vaccinated individuals) will discover more convergent clusters which richer annotations.

In addition to expansion of gene use as an indicator of activation, we can infer biological information from assessment of the AID-mediated activities of CSR and SHM. These have long been associated with germinal centre formation (60, 61). However, there is mounting evidence that CSR can occur prior to the germinal centre formation (8–11, 36, 53). T-independent activation has been shown to be driven by CD40-independent TLR/TACI activation (62). Our data indicates an early switching to IgG1 without extensive hypermutation. This data is consistent with Woodruff et al. (63) who also found high germline similarity in IgA1, and (42) where COVID-19 samples were found to have more naïve-like characteristics. Our COVID-19 IgA1 sequences also indicate a lower level of hypermutation than the control group, albeit higher than the COVID-19 IgG1, likely reflecting their distance along the CSR hierarchy. Uniquely, our diversity analyses also indicate expansion of unmutated IgM clones.

Alongside these data we see that CDRH3 region maturation of IgG1 and IgG3 genes in the COVID-19 patients is less removed from the IgM state than healthy IgG1 and IgG3, or any other class switched repertoires. Together with the lineage analysis of CSR timing, the whole picture in COVID-19 is of an early immature response of IgM, switching to IgG1 but without much SHM such as might occur in the absence of T cell help in a GC reaction, and then to IgA1. Whether these responses are unique to a live infection or because the virus is so novel is difficult to ascertain, with future vaccine and comparative studies likely to shed further light on this phenomenon. IgG1 is known for its antiviral properties (50, 64) so is expected in this data. The majority of rapid immunological protection assays for COVID-19 focus on IgM or IgG (65–68). Since switching to IgA1 is notable in our data it would be useful for future serology assays to include IgA. Euroimmun’s IgA on LFA had one of the highest sensitivities at 87.8% compared to IgM and IgG from other assays (range 43.8-93%, mean 72.5%, median 76%) (67, 69).

It is known that healthy older people generally have more antibodies capable of binding self-proteins (70). The balance between antibodies with positive versus negative/bystander activity may be changed in older patients. We cannot tell this from our data except that we see a higher frequency of known spike binders clustering with COVID-19 repertoires in the younger cohort. Significant age-related differences occur in the dominant IGH COVID-19 genes: the increased use of IGHV3-30 is only seen in older COVID-19 patients and that of IGHV4-39
only in the younger group. We also see selection for IGHD2-2, IGHD3-3 and IGHJ6 only in the young, with IGHJ6 occurring frequently in known binder networks, given the importance of IGHD and IGHJ genes to the CDRH3 region it is striking that the differences seen here are solely in the younger age group. We note that by separating samples by age group the small sample size limits the potential to discover more gene usage differences with strong statistical significance. In the future integration with other repertoire datasets, including those in response to SARS-CoV-2 vaccination, would yield more insights into age-related differences in the humoral response against this virus.

In comparison to our CV19 data our EBOV data paints an unusual picture where, even 2-3 months post-recovery with viral negative PCR tests, there are abnormally high proportions of class switched clones with little or no direction towards a particular subclass. Given CSR is largely understudied, as far as we can tell such high rates of class switching, particularly so long after recovery, are entirely unique to this infection. Another unusual observation was that EBOV
survivors’ memory B cell populations were more diverse than healthy controls suggesting stimulation with more diverse antigens, or a less structured and directed immune response. A ‘decay-stimulation-decay’ pattern resulting in the peak of antibody response being some 200 days after infection has previously been reported (71) and cytokine storms during infection may also be contributing to this phenomenon (72, 73). It was not possible to collect blood samples from unrecovered patients, so we do not know if these observations were a requirement of patient recovery or a phenomenon unique to Ebola infection in general. In comparison to other sample types, our EBOV samples were only sequenced to modest depth (median number of sequences for EBOV = 5,346; CV19 = 38020.5; Healthy = 12,486.5). We note that the detection of CSR events within lineages is dependent upon sequencing depth (Supplementary Note); it is possible that the estimates of CSR frequencies for EBOV in Figure 6 only represent the lower bound, further highlighting the importance of CSR in the response against EBOV.

By comparing examples of pandemic, epidemic and endemic viral disease responses our results show that while aspects of B cell responses are unique to particular infections, the human immunoglobulin gene repertoire can show similarities of response across two very different diseases. There are many questions to be answered about the balance of beneficial versus bystander responses in acute inflammatory diseases, where the initial class switched responses seem to be immature (COVID-19) and possibly unregulated (EBOV infection). Coupled with the finding of genes such as IGHV4-39 appearing in two
completely different diseases, these data add weight to the hypothesis that an emergency humoral immune response to primary challenge can bypass normal stringent regulation and thus allow the production of autoimmune antibodies.

**CODE AVAILABILITY**

Functions implemented to generate and analyse lineage trees were included in a R package BrepPhylo, which is available at https://github.com/Fraternalilab/BrepPhylo. All other code snippets used in analysing data presented in this work are available as R markdown files available at https://github.com/Fraternalilab/BrepPhyloAnalysis.

**DATA AVAILABILITY STATEMENT**

IMGT/High-VQuest-annotated immunoglobulin sequence data file is available at Zenodo (https://dx.doi.org/10.5281/zenodo.5146019).

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by UK London REC 14/LO/1221 for COVID-19 samples, UK London REC 11/LO/1826 for RSV samples, and Sierra Leone Ethics and Scientific Review Committee ISRCTN13990511 and PACTR201602001355272 and authorised by Pharmacy Board of Sierra Leone, #PBSL/CTAN/MOHS-CST001 for Ebola samples. The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

DD-W, AS, JSO’H, and MB designed study and protocols to collect and analyse COVID-19 and YFV samples under REC 14/LO/1221. AS, MB, NK, ES, KL, CF, CCo, HL and FO acquired and biobanked COVID-19 samples. CCh and PO provided RSV samples. MS, JS, and JB provided Ebola samples. ES, AS, IS, JSO’H, AP, and BW devised repertoire protocol and performed repertoire data generation. JN, AS, ES, DK, CM, DD-W, and FF performed bioinformatic analysis and data interpretation. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.807104/full#supplementary-material

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