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Effects of Aging, Cytomegalovirus Infection, and EBV Infection on Human B Cell Repertoires

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Elderly humans show decreased humoral immunity to pathogens and vaccines, yet the effects of aging on B cells are not fully known. Chronic viral infection by CMV is implicated as a driver of clonal T cell proliferations in some aging humans, but whether CMV or EBV infection contributes to alterations in the B cell repertoire with age is unclear. We have used high-throughput DNA sequencing of IGH gene rearrangements to study the BCR repertoires over two successive years in 27 individuals ranging in age from 20 to 89 y. Some features of the B cell repertoire remain stable with age, but elderly subjects show increased numbers of B cells with long CDR3 regions, a trend toward accumulation of more highly mutated IgM and IgG Ig genes, and persistent clonal B cell populations in the blood. Seropositivity for CMV or EBV infection alters B cell repertoires, regardless of the individual’s age: EBV infection correlates with the presence of persistent clonal B cell expansions, whereas CMV infection correlates with the proportion of highly mutated Ab genes. These findings isolate effects of aging from those of chronic viral infection on B cell repertoires and provide a baseline for understanding human B cell responses to vaccination or infectious stimuli. The Journal of Immunology, 2014, 192: 603–611.

Many elderly individuals have a compromised immune system, leading to increased susceptibility to infectious diseases and decreased responses to vaccination (1). Aging has been reported to impair innate immunity, T cells, and Ab-producing B cells (1–5). Humoral responses are critical for responding to pathogens such as Streptococcus pneumoniae and influenza viruses that cause increased morbidity and mortality in the elderly, but age-related changes in human B cells and Ig repertoires are only beginning to be understood (6–8).

Advanced age has been reported to lead to increased or decreased B cell counts in the peripheral blood, increased, decreased or unchanged proportions of naive B cells, and increased CD5+ B cell populations (3, 5, 9–13). Changes in serum Ab production, including decreases in vaccine-specific Abs, and decreased isotype switching associated with lower expression of activation-induced cytidine deaminase in B cells also have been described previously (8, 10, 14). Understanding the effects of aging on B cell function is further complicated by the common chronic viral infections seen at higher rates in the aging population, such as CMV and EBV. CMV infection is correlated with increased counts of LFA-1hiCD8* memory T cells and reduced naive CD8* T cells, whereas total B cell counts in the blood are reportedly increased in CMV-seropositive individuals (15–17).

Following V(D)J rearrangement to generate functional Ig genes in B cells, the Ig repertoire during a human’s life span is further shaped by negative selection against self-Ags, clonal expansion of B cells stimulated by Ag, activation-induced mutation of Ig genes, and receptor editing, among other processes. Ineffective Ab responses in the elderly have been attributed to decreased diversity of Ab repertoires with accumulation of memory B cells and decrease of naive B cell populations (18). Influenza vaccination responses in the elderly are associated with decreased numbers of vaccine-stimulated B cells (8), and a recent study that included four elderly subjects show decreased diversity of influenza vaccine-stimulated B cells (19). However, there is also evidence of relatively preserved Ig repertoire diversity in tonsillar tissue of aged humans and increased proportions of naive B cells in some elderly individuals (20). Mutation of IGHV in B cell populations reportedly changes with aging, with one study reporting modestly increased mutation in IgG but not memory IgM B cell populations in the blood, whereas data from tonsillar B cells indicate increased mutation in memory IgM B cells but not other subsets (20, 21). Most prior studies of IGHV gene rearrangements in young versus elderly subjects have been limited to examination of tens to hundreds of sequences, from small numbers of individuals, or have not assessed the potentially confounding effects of chronic herpesvirus infections (20–23). Seropositivity for CMV, in particular, increases with age in human populations and should be controlled for in studies of the effects of aging on the immune system (24).
In this study, we characterize peripheral blood IgH repertoires measured with >500,000 sequences from a cohort of healthy young (n = 10) and older (n = 17) people over two consecutive years and analyze features that change with age, CMV, or EBV infection. Some B cell repertoire features are stable with age, but we find that elderly individuals show increased numbers of B cells expressing long IgH CDR3 regions and that the proportion of highly mutated B cells, particularly in IgM and IgG populations, shows a trend toward increasing with age and is increased in subjects infected with CMV. Unusual large persistent clonal populations of B cells are common in the oldest individuals in our data set and are absent from younger individuals; notably, the contribution of both large and small persistent B cell clones over the yearlong time course is correlated with EBV seropositivity, regardless of age. Taken together, these findings isolate age-specific and CMV- or EBV-associated alterations in the B cell repertoire and provide a baseline for further study of impaired Ag-specific responses and increased autoreactivity in the elderly.

Materials and Methods

Specimen collection

Human peripheral blood was collected from a cohort of 27 healthy participants aged 20–89 y during each of two consecutive years: Y2 (2008) and Y3 (2009). Participant ID, age, and other demographic information is described in Supplemental Table I. The participants were grouped into three age categories: 20–31 y (n = 10), 61–69 y (n = 7), and 72–89 y (n = 10). Recruitment of patients, documentation of informed consent, collection of blood specimens, and experimental measurements were carried out with Institutional Review Board approval at Stanford University.

CMV and EBV serotyping

Serum was separated by centrifugation of clotted blood and stored at −80°C before use. After all samples were collected, specimens were thawed at room temperature and assayed for the presence of CMV and EBV Abs (both IgG and IgM) using the CMV or EBV ELISA Kit from Calbiotech (Spring Valley, CA), as recommended by the manufacturer. No specimens were positive for virus-specific IgM.

Genomic DNA and cDNA template preparation and PCR amplification

Isolation of PBMCs was performed as described previously, and isolation of genomic DNA (gDNA) and RNA was performed using Allprep kits (Qiagen) (22). PCR of IgH V region rearrangements from each sample was carried out with six independent 100 ng gDNA aliquots to generate six independent bar-coded libraries per sample. Multiplexed primer sets hybridizing to framework regions (FR1 and FR2), and a J primer were based on the BIOMED-2 design (25). For isotype-specific Ig libraries, mRNA was reverse-transcribed to cDNA using random hexamer primers, and cDNA corresponding to 100 ng RNA was used for PCR using the same 5′-FR1 and FR2 multiplex primers and 3′-isotype-specific primers located in the first exon of the C region. Sample identity and replicate library identity were encoded by 10-nucleotide “bar code” sequences in the primers. PCR was carried out with AmpliTag Gold (Roche) following the manufacturer’s instructions and used a program of 94°C 5 min, 35 cycles of (94°C 30 s, 60°C 95 s, and 72°C 90 s), and final extension at 72°C for 10 min. PCR products from each replicate library were quantitated, pooled in equimolar amounts, and then gel-purified and gel extracted (Qiagen). High-throughput sequencing was performed on the 454 (Roche) platform using Titanium chemistry.

Sequence quality assessment and filtering

Sequences with matching bar codes were assigned to the appropriate replicates and samples and then were trimmed of bar codes and IGHV primer sequences. The V, D, and J regions and V-D (N1) and D-J (N2) junctions were identified by alignment with germline IGH sequences from ImMunoGeneTics and National Center for Biotechnology Information databases, using the alignment program iHMmune-align (26, 27). Amino acid sequences and location of CDR3 were defined by the conserved cysteine-104 and tryptophan-118 based on the ImMunoGeneTics numbering system (28). Sequences were filtered to remove non-germline sequences with V-gene insertion or deletions, and chimeric sequences. Samples with <100 sequences were excluded from further analysis (two samples from Y3). A total of 323,285 gDNA and 189,915 cDNA sequences were analyzed. The sequences can be accessed via the National Center for Biotechnology Information dbGAP online archive (http://www.ncbi.nlm.nih.gov/gap) with accession number phs000666.v1.p1.

V, D, and J usage, junctional features, and V-mutation analysis

Sequences from all replicates of each sample were pooled, and unique clones were defined by the same V and J gene usage and identical CDR3 as a sequence. To analyze V, D, and J usage frequencies, CDR3 features, and mutations in V, reads belonging to each unique clone were collapsed to a single read. Hydrophobicity of CDR3 peptides was calculated using the Kyte-Doolittle scale (29) and net charge was calculated using the Henderson–Hasselbalch equation (30). CDR3 segments from out-of-frame sequences were excluded from CDR3 feature analysis. Distribution of mutation along the V-region was obtained by alignment with gapped ImMunoGeneTics germ-line sequences and counting the mutation frequency per nucleotide position.

Clonal expansion analysis

To summarize the contribution of clonally expanded B cells to the repertoire while normalizing for sequencing depth obtained for each sample, we calculated a “clonality score,” adapted from a coincidence index used in cryptanalysis, sum(fij), (where i is the frequency of component i) (31). To compensate for the effect of PCR amplification on sequence counts, we calculated the clonality score as follows: sum of Nij X Njk (j!=k) over i,j,k, divided by the sum of Tj X Tk (j!=k) over j,k, where Nij and Njk are the copy numbers of clone i observed in independent replicate PCR libraries j and k generated from independent aliquots of template DNA, and Tj and Tk are the total read numbers in the corresponding replicate libraries. To quantitate the contribution of persistent B cell clonal populations detected in the two time points i apart in the study, we calculated a “persistent clonality score” exactly as above, except that only replicate libraries from different years were compared with each other. Large clones were defined as those observed in more than four of the six replicate libraries from a sample and comprising >1% of total reads from the sample.

Correlation and regression analysis for clonality

We tested for correlation between clonality score, persistent clonality score, IgCJV mutation frequency, age, gender, and seropositivity for EBV and CMV using multiple linear regression, including all subjects in the analysis. Laplace smoothing was applied to avoid occurrence of zeros prior to calculating the logarithm of the clonality score.

Phylogenetic visualization of large clones

ImmuInniter is a dedicated algorithm and implementation for building immune receptor sequence trees from high-throughput DNA sequencing data (32). The following briefly describes the conceptual basis for calculation in Immunitree, although additional details of the model-building and computation will be presented elsewhere. A variety of broadly applied phylogenetic algorithms deal with sequences derived from species that are presumed to have evolved from common ancestors but where the intermediate shared ancestors are no longer present to be observed and must therefore be inferred. In contrast, ImmuniTree takes into account the possibility that common ancestor B cells may still be present in the members of a clonal lineage that are observed.

In the ImmuniTree model of somatic hypermutation (SHM) of Ig gene rearrangements, potential nucleotides at each position on sequences representing parent and child subclones are modeled using a conditional multinomial distribution. Sequencing error is modeled similarly: the mutations from subclones to reads are also modeled using a conditional multinomial distribution. These distributions are subject to different priors: the SHM prior penalizes indels severely, whereas the sequencing error prior treats indels more permissively. This reflects the fact that true SHM is under selective pressure to produce in-frame Abs, whereas the sequencing error prior treats indels more permissively. It also provides the opportunity to handle SHM events that occur in multiple subclones (as in the case where the SHM event is a germline event) while normalizing for sequencing error. In the model, the SHM prior penalizes indels severely, whereas the sequencing error prior treats indels more permissively. ImmuniTree thus builds on and extends existing phylogenetic algorithms, implementing the aforementioned fine-tuning designed to account for the complex realities of Ig repertoire sequencing, and uses a single sampling and optimization approach to compute a lineage that optimizes the overall likelihood. Any specific lineage has a likelihood consisting of the following parameters: SHM rates from parent subclones (nodes) to children subclones, sequencing error of reads corresponding to a given subclone, the best V and J reference segments, the parent subclone of each subclone, and subclone birth and death rates. The parameters are sampled using a Metropolis–Hastings Markov Chain Monte Carlo approach. After running for

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Results

V, D, and J usage varies among individuals but is comparable between young and older people

We first examined whether there were discernable age-related changes in the usage of V, D, or J gene in IGH rearrangements. Fig. 1 shows that the proportion of different genes was comparable across three age groups of samples in 2008 (Y2), with gene frequencies consistent with literature reports (33). V, D, and J gene usage from 2009 (Y3) follows a similar pattern and bears a high correlation with that from Y2 (data not shown). V gene segment usage in humans shows a different distribution in mutated versus nonmutated Ab gene rearrangements, so we separated these sequence subsets in our analysis (33). To be conservative in our mutation frequency estimates, given that 454 sequencing can demonstrate error rates of ∼0.3–1% per base (22), we consider sequences with <1% V-mutation frequency as unmutated and those with >1% as mutated. The V, D, and J gene usage did not show a significant difference between younger and older groups within either mutated and unmutated categories. Variation between individuals in gene usage patterns was seen, similar to that previously reported (34), consistent with individual differences in the intrinsic mechanisms that generate VDJ rearrangements, including copy-number variation in IGH germline sequences, or recombination signal sequence features, or individual variation in the B cell selection process.

CDR3 length in both mutated and unmutated Abs increases with age

We further analyzed biochemical features of the IgH CDR3 aa sequences, given the prominent role of this sequence region in determining Ab binding specificity, and the effects of B cell selection on the length of IgH CDR3 regions present in the B cell repertoire. It has been reported that B cell development in the bone marrow is accompanied by preferential removal of B cells expressing Abs with long CDR3s, potentially because of the increased levels of autoreactivity demonstrated by such Abs (35). Evidence of additional selection against B cells expressing Abs with long CDR3s, or in favor of sequences with shorter CDR3s during Ag-driven peripheral responses, is seen in the decreased IgH CDR3s present in B cells expressing mutated Ig genes compared with unmutated ones (36). To look for age-associated alterations in these selection processes, we analyzed the CDR3 features of mutated and unmutated sequences separately in our data set. Confirming prior observations, the unmutated sequences in our data set Fig. 2, middle panel, have longer CDR3s than mutated sequences Fig. 2, lower panel. Interestingly, the CDR3 regions of both unmutated and mutated sequences are significantly longer in older people compared with young individuals, especially within mutated sequences (Fig. 2, middle and lower panel), whereas there is no obvious change in hydrophobicity or net charge with age. The presence of increased numbers of B cells with longer CDR3 segments in both unmutated and mutated Ab H chain rearrangements in elderly individuals suggests an overall decreased level of selection against Abs with long CDR3s in the aging immune system, both in the generation of naive B cells, as well as during Ag-driven responses.

IgM and IgG show an age- and CMV-associated increase in mutation

We initially evaluated the levels of IGHV mutation regardless of Ab isotype in participants of various ages. We grouped sequences into three groups: <1% mutation frequency (unmutated), 1–10% mutation frequency (moderate), and >10% mutation frequency (high).
EBV correlate with the extent of IGHV upper panel individuals aged (20–31), (61–69), and (72–89), respectively (Fig. 3B, comprises, on average, 68, 68, and 75% of the repertoire in individuals' total B cell repertoire. Fig. 3B shows that highly mutated Ig showed that CMV has a stronger effect than that regression analysis of factors contributing to the proportion of IgM and IgG mutation rates within each age group did not permit rejection of the ANOVA testing null hypothesis of there being no significant difference between age groups in the proportion of sequences with >10% mutation (p = 0.38 for IgM and p = 0.19 for IgG). As with the overall B cell repertoire measured with gDNA, none of the isotypes show IGHV mutation frequencies significantly correlated with gender or EBV infection. As an incidental finding in the data set, we noticed occasional subjects showing IgM sequences that had accumulated unusually increased mutational loads. One of these participants, p65, also had an increase in their proportion of IgG sequences with low levels of mutation. These observations will require further investigation, but the subjects were healthy at the time of blood draws, without known infections or autoimmune disorders. The data indicate that phenotypic variation in IGHV mutational frequencies in human populations may be wider than has been previously reported.

IGHV gene usage differs between naive and mutated IGH but is comparable in mutated IgM, IgG, and IgA

Previous studies have reported evidence that memory IgM and isotype switched memory B cell populations use IGHV families with differing frequencies, with IgM memory cells having a lower frequency of IGHV1 gene usage and higher frequency of IGHV3 gene usage than IgG and IgA isotype–switched memory B cells (37). We considered IgM or IgD sequences with 1% or greater mutation frequency to be derived from IgM memory B cells, whereas those with <1% mutation were from naive cells (38). Supplemental Fig. 2 shows that naive IgM and IgD sequences have significantly higher IGHV1 and lower IGHV3 usage than mutated IgM, IgD, IgG, or IgA. However, we saw little difference in IGHV gene family usage between mutated IgM, IgD, IgG, or IgA (Supplemental Fig. 2). Similarly, age, CMV, or EBV infection had minimal impact on IGHV gene usage (Supplemental Fig. 2).

Age and chronic EBV infections are correlated with persistent B cell clonal expansion

We quantified clonally expanded B cells in each individual, using a conservative clone definition of sharing the same V and J genes shown in Fig. 3A, the proportion of reads falling in these groups in each individual is highly correlated between two consecutive years, showing both the variation between individuals, and the stability of this feature of the B cell repertoire over time. Unmutated IGHV comprises, on average, 68, 68, and 75% of the repertoire in individuals aged (20–31), (61–69), and (72–89), respectively (Fig. 3B, upper panel).

We then asked whether gender or seropositivity for CMV or EBV correlate with the extent of IGHV mutation in each individual’s total B cell repertoire. Fig. 3B shows that highly mutated IGHV is increased in CMV-positive versus CMV-negative participants (p < 0.05). No significant differences in mutation rates were seen with regard to gender or EBV infection status. Multiple regression analysis of factors contributing to the proportion of highly mutated Ig showed that CMV has a stronger effect than that of age, gender, or EBV infection, with p = 0.11 in the model.

To evaluate IGHV mutational frequencies in greater detail, we examined sequences from Abs of particular isotypes to look for changes associated with aging or chronic viral infection. Fig. 4A shows that highly mutated IgM and IgG sequences show a trend toward being increased in the oldest individuals (72–89 y) compared with younger individuals, although one individual in the age 21–30 group (p65, described further below) had a proportion of >10% mutated IgM sequences that was unusually high for their age; this individual proved to be EBV and CMV seropositive. CMV infection is associated with an increased proportion of highly mutated sequences of IgM and IgG isotypes (Fig. 4B). No significant age- or CMV-related increase in mutation frequencies was seen for other isotypes including IgA or IgD (Supplemental Fig. 1). There were too few IgE sequences obtained for this analysis. The association between CMV seropositivity and elevated IgM and IgG mutation frequencies is stronger than the correlation between mutational frequencies and age, because the presence of variability of IgM and IgG mutation rates within each age group did not permit rejection of the ANOVA testing null hypothesis of there being no significant difference between age groups in the proportion of sequences with >10% mutation (p = 0.38 for IgM and p = 0.19 for IgG).
and having identical CDR3 amino acid sequences. Six independent IGH libraries amplified from gDNA template were examined from each sample, to distinguish between PCR amplification bias and true clonal expansion of B cells, by requiring that the same rearrangement be detected in at least two independent libraries from the sample to be considered clonally expanded. We calculated a “clonality score,” as described in Materials and Methods, which can be thought of as the probability that two B cells selected at random from the patient’s peripheral blood will belong to the same clonal lineage. Fig. 5A and 5B show that most samples from healthy individuals sequenced at this depth show low levels of B cell clonality, indicating that almost all of the sequences observed in each replicate library from a given individual are unique. Significantly increased clonality scores are seen in some elderly individuals, and correlate with CMV infection regardless of age. (A) Frequencies of V-mutation grouped by level and age, gender, CMV, or EBV status. Sequences are pooled from two years for each participant. The p values are calculated by two-sided Student t test, including all data points for each category. *p < 0.05.

We evaluated the correlation between B cell repertoire clonality and an individual’s age, gender, and serological status for EBV and CMV infection. To combine sequences from 2 y, we took the average of the clonality score from the 2 y. The heat map of Fig. 5C shows that age is correlated with B cell clonal expansions detected within each year of the study, with a correlation of 0.46 and two-sided p value of 0.016. We used multiple linear regression to address potential confounding variables, using gender, CMV status, and EBV status as covariates, correcting for multiple hypotheses. Age remained a significant factor, with a Benjamini–Hochberg corrected false-discovery rate of 0.061, and EBV infection status showed significance with a false-discovery rate of 0.083. Persistent clonality scores normalized for sequencing depth were calculated as described in Materials and Methods to quantify B cell clones persisting in an individual through the yearlong time course of the study. Correlation coefficients (Fig. 5D) showed that the persistent clonality score (i.e., the levels of B cell clones that were found to persist in a particular individual and were detected in both year Y2 and year Y3) was correlated with age and EBV status. Multiple linear regression analysis demonstrated that the effects of age and EBV infection on the persistent clonality score are significant after multiple hypothe-
Persistent B cell clonal expansion has been described in aged population, but the Ab gene sequences of expanded clones have not been fully characterized. The sequencing depth in our study enabled us to examine the IGH sequences of the expanded clones and infer the lineage relationships of clone members in detail. We searched for the biggest clones in the sequence data from each individual, focusing on clones that appeared in at least four of six replicate sublibraries and comprised 1% of total sequence reads. One large persistent B cell clone in elderly individuals could contribute by expanded clonal populations, normalized for the depth of sequencing carried out in each sample, are presented in Materials and Methods.

FIGURE 5. Age and chronic EBV infections are correlated with persistent B cell clonal expansion. (A and B) Clonality scores of the Ig repertoire of each sample are grouped by age for time point Y2 (A, n = 27) and Y3 (B, n = 25). The pairwise comparison between different age groups is performed with two-sided Wilcoxon tests. **p < 0.01. (C) Correlation heat map of Y2-Y3 average clonality score (log) for each participant’s B cell repertoire, according to age, gender, CMV seropositivity, and EBV seropositivity in 27 individuals. (D) Correlation heat map of persistent clonality score (log) for clones detected in Y2 and Y3 of the study, according to age, gender, CMV, EBV, and frequency of highly mutated sequences (V-mut. freq.), in 25 individuals. The colored scale bar represents the strength of correlation for each variable pair. Details of the clonality score calculations, which yield a measure of the proportion of the B cell repertoire contributed by expanded clonal populations, normalized for the depth of sequencing carried out in each sample, are presented in Materials and Methods.

Discussion

We have analyzed >500,000 rearranged IGH sequences derived from the peripheral blood of young adults and elderly individuals in serial samples taken from each subject at two time points separated by ~1 y. Our data indicate that overall V, D, and J gene usage are similar in young and older individuals but that the aging B cell repertoire shows prominent changes in the lengths of IGH CDR3 regions that are present in B cells and in the levels of somatic mutation in Ab sequences. Importantly, the individuals studied in this paper were of known seropositivity status for CMV and EBV chronic infection, and there were sufficient numbers of seropositive and seronegative individuals to allow analysis of the impact of these viral infections on the B cell repertoire, independent of patient age. Our findings highlight an association between CMV seropositivity and levels of Ab gene somatic mutation, whereas EBV seropositivity is correlated with the contribution of persistent clonal populations to the B cell repertoire.

Increased levels of B cells expressing long IGH CDR3 regions in the elderly are seen in our data both in unmutated sequences, and in mutated sequences. Selection against B cells expressing Abs with long CDR3s during the process of generating the naive B cell repertoire has previously been correlated with the higher frequency of autoreactivity in Abs with long CDR3 regions. The effects of this selection process can be detected in the difference between the longer CDR3 regions present in unproductively rearranged IGH genes compared with productive expressed IGH sequences. The presence of longer CDR3 regions in mutated IGH sequences in the elderly in our data may simply reflect the fact that mutated sequences derive from the initially unmutated pool or could reflect additional age-related impairment in positive selection for B cells expressing Abs with shorter CDR3s in Ag-driven responses. Prior literature has documented increased levels of autoantibodies in the sera of elderly individuals compared with younger individuals. The results presented here may indicate that increased autoimmunity in the elderly stems from two different defects: impaired selection against Abs with long CDR3 regions in generation of the naive B cell repertoire, and decreased selection against long CDR3 Abs in B cell responses associated with Ab mutation and isotype switching. Other CDR3 features that have been associated with autoreactivity, such as hydrophobicity, did not show age-related changes in our data.

Both IgM and IgG mutated memory B cell pools in our data set show a trend toward age-associated accumulation of highly mutated sequences, although there is considerable variability between individuals in all age groups examined. These findings could
suggest that progressive antigenic exposure stimulating B cell proliferation and targeted Ig gene mutation can lead to the accumulation of increasingly highly mutated IGHV genes over the course of a human lifespan. Overall, there is considerable overlap in the levels of IGHV mutation seen in the B cell repertoires of young and elderly individuals. Implications of the latter result are that most long-lived memory B cells may be formed primarily from the relatively less-mutated members of a clonal proliferation or that memory B cells formed from more highly mutated clone members may have a shorter lifetime in the host. Indeed, very long-lasting memory B cells, such as those specific for the 1918 pandemic influenza strain in nonagenarian or centenarian survivors of the pandemic have shown IGHV mutation levels of $\sim 10\%$, within the range of mutation frequencies that can be seen in memory B cells in younger individuals (47). The origin of IgM memory B cells is a subject of debate, with some reports proposing novel developmental pathways that are independent of germinal center stimulation, or independent of Ag stimulation entirely, whereas other interpretations describe these cells as the output of immune responses associated with decreased germinal center function or simply a subset of memory B cells typically produced in T cell–dependent immune responses (38, 48). If the generation of IgM memory cells is a consequence of impaired germinal center function, then age-associated increases in IgM mutation frequencies could be a consequence of decreased germinal center activity in the elderly, leading to greater reliance on B cell clonal lineages that show some Ig mutation, but have not been able to isotype switch.

We identified an independent correlation between CMV infection and the mutation levels in both IgM and IgG-expressing B cells in individuals regardless of age. This could be a direct effect of some B cells being specific for CMV, or alternately, could reflect the reported superantigen-like effects reported for CMV phosphoprotein pUL32 or other CMV proteins (49). Arguing against the latter possibility, the described superantigen-like activity of pUL32 was for B cells expressing IGHV1-69 or IGHV3-21 gene
segments, and we did not see such segments overrepresented among highly mutated sequences in our data.

We identified large B cell clones that persisted over the yearlong period of the study only in individuals >70 y old (3 of 10), although one younger subject showed comparably large clones at a single time point. The mechanisms leading to persistent B cell clones are of great interest and tie our findings to reports in the hematolo–oncology and infectious disease literatures. Current classification of B cell lymphomas and leukemias defines numerous subtypes of overt malignancy, but also includes the category "monoclonal B cell lymphocytosis" (MBL) an age-associated condition in which abnormal persistent clones of B cells with the immunophenotype of chronic lymphocytic leukemia (CLL) are identified, but at lower cell counts than the minimum required for a CLL diagnosis (5 × 10^3 cells/μL) (40–42). Although flow cytometry data to test for such abnormal B cell populations are not available for the subjects in this study, it is likely that some of the persistent large clones identified by IGH sequencing correspond to MBL populations. Indeed, the clonal lineage tree analysis showed that several of the large persistent clones were mutated but were fixed with all members showing essentially identical mutation patterns, a characteristic feature of mutated CLL clones (42).

Epidemiological studies report correlations between infectious disease exposure and the development of MBL or CLL, and stereotypic IGHV rearrangements in CLL have been proposed as evidence of Ag drive contributing to oncogenesis (50–52). The IGH CDR3 sequences of the clones in our study differed from known stereotyped CLL CDR3 sequences, but we find a correlation between the levels of persistent B cell clones and EBV seropositivity, suggesting that EBV infection may contribute to age-associated B cell clonal expansions. Large clonal proliferations of CD8+ T cells associated with CMV infection are well described, but the effects of CMV or EBV on B cell populations have been less clear (2, 15). Although the elderly participants who have the largest clonal expansions in our study are all positive for both CMV and EBV infection, our multiple regression analysis found that age and EBV status were the most significant contributors to the overall levels of clonally expanded B cells when all participants were considered.

These measurements of peripheral blood IGH sequences from young and elderly individuals highlight effects of aging and chronic CMV or EBV infection on the B cell repertoire. Increased age is associated with the development of large persistent clonal B cell populations and a trend toward increased IGHV mutation levels in B cells expressing IgM or IgG, whereas features of the repertoire such as V, D, and J segment usage are relatively age invariant. B cell repertoires in the elderly show decreased selection against Abs with long CDR3 regions, both in unmutated Abs likely associated with naive B cells as well as in presumably Ag-experienced B cells expressing mutated Abs. CMV infection is correlated with high mutational levels in IgM- and IgG-expressing B cells, whereas EBV infection correlates with the proportion of persistent B cell clones in the blood over a year's time, revealing distinct and important shaping of the B cell repertoire by these common chronic viral infections. Studies of Ag-specific responses stimulated by vaccination or infection in the elderly hold the promise of revealing further age- or chronic viral infection-associated deficiencies.

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Disclosures
S.D.B. has consulted for and has stock interest in Immumetrix Co., a private company, but there is no overlap presenting a conflict of interest with the subject of this manuscript. Similarly, S.D.B., A.Z.F., E.L.M., and K.S. have a patent application pending related to immunological sequencing, but this does not represent a conflict of interest with the subject of this manuscript.

References