Temporal Stability and Molecular Persistence of the Bone Marrow Plasma Cell Antibody Repertoire

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ABSTRACT

Plasma cells in human bone marrow (BM PCs) are thought to be intrinsically long-lived and to be responsible for sustaining lifelong immunity through the constitutive secretion of antibody—but the underlying basis for this serological memory remains controversial. Here, we analyzed the molecular persistence of serological immunity by an examination of BM PC immunoglobulin heavy-chain (IGH) transcripts derived from serial bone marrow specimens obtained during a span of several years. Using high-throughput sequence analysis of the same individual for 6.5 years, we show that the BM PC repertoire is remarkably stable over time. We find that the bias in IGH V, D, and J individual gene usage and also the combinatorial V–D, V–J, D–J, and V-D-J usage across time to be nearly static. When compared to a second donor with time points 2 years apart, these overall patterns are preserved, and surprisingly, we find high correlation of gene usage between the two donors. Lastly, we report the persistence of numerous BM PC clonal clusters (~2%) identifiable across 6.5 years at all time points assayed, supporting a model of serological memory based, at least in part, upon intrinsic longevity of human PCs. We anticipate that this longitudinal study will facilitate the ability to differentiate between healthy and diseased antibody repertoire states, by serving as a point of comparison with future deep-sequencing studies involving immune intervention.

INTRODUCTION

The human bone marrow (BM) is a specialized immune compartment that is responsible for both the initial generation of newly-formed B cells and also the maintenance of terminally differentiated, antibody-secreting plasma cells (PCs). The BM, and the PCs it harbors, is a major site of antibody production and is the major source of all classes and subclasses of human immunoglobulins (Ig) detectable in the serum[1,2]. Ig-secreting BM PCs are generally believed to be "long-lived" and maintained for the lifespan of the organism[3]. In this regard, it has been well established in longitudinal serological studies that antiviral serum antibodies can be remarkably stable, with half-lives ranging from 50 years (e.g. varicella-zoster virus) to 200 years for other viruses (e.g. measles and mumps); however, in contrast, antibody responses to non-replicating antigens (e.g. tetanus and diphtheria bacterial toxins) rapidly decay with much shorter half-lives of only 10-20 years[4]. Not only does this suggest that antigen-specific mechanisms play a substantial role in the establishment and/or maintenance of serological memory, but raises the question of whether the differential stability of antibody responses might reflect differential intrinsic longevity of PCs themselves. This has been previously proposed in the context of vaccinations and infections[4,5], and is also supported by observations of differential stability of autoantibody titers when using B-cell depleting therapies to treat autoimmune diseases[6,7].

The basis underlying lifelong serological memory (antibody responses) remains controversial[3,8,9]. Data supporting a model for intrinsic longevity in PC survival (and hence longevity in serum antibody maintenance) has been posited for the laboratory mouse[10,11], but data for human PCs are absent. Based upon the murine models, it has been assumed that human BM PCs are similarly long-lived and that these long-lived PCs are the major source of serum antibodies; however, only recently have antigen-specific BM PCs been ascertained for their contribution to the pool of serum antibodies in humans[5,12]. Despite these notable advances, the availability of corresponding molecular data (namely, sequence data of BM PC Ig transcripts) and of information regarding PC dynamics *in vivo* are scarce.

Three studies have generated BM PC data using next-generation sequencing techniques, but none of which have examined the temporal changes that occur in the immune repertoire over time[5,13,14]. Here, building upon our prior experiences with the

comprehensive analysis of human cellular and serological antibody repertoires[15–19], we present the first longitudinal study of serially acquired human BM PCs assayed by next-generation deep sequencing. To directly measure the temporal dynamics of BM PCs—and to indirectly gain insight into long-lived serological memory—we sequenced the recombined VHDJH region, which encodes the variable region (V domain) of IGH heavy chains of BM PCs from the same individual over seven timepoints encompassing a total of 6.5 years and from a second individual with two timepoints over 2.3 years. The temporal resolution and duration of sampling provides a method to interrogate the *in vivo* temporal dynamics of BM PCs in a previously uncharacterized way. We provide detailed temporal information on the individual genes (IGH V, D, and J), gene combinations (V-D, V-J, D-J, V-D-J), and persistent CDR-H3 clonotypes. The second individual provides support that our observations are not unique. Overall, our results (i) underscore the temporal stability of the IGH V region repertoire according to multiple metrics (temporally stable IGH molecular phenotypes), and (ii) provide unequivocal sequence-based evidence for the persistence of PC cellular clonotypes spanning 6.5 years.

RESULTS

Serial Bone Marrow Biopsies Followed by Next-Generation Sequencing Demonstrate Temporal Dynamics of the Immune Repertoire.

To investigate the temporal dynamics of the IGH antibody gene repertoire of bone marrow plasma cells (BM PCs), we sampled, sorted, and performed high-throughput sequencing (Fig. 1a). Serial bone marrow biopsies were obtained from two adolescents (10–17 years of age) as part of routine evaluations for non-immuno-hematological disease. BM PCs were isolated using fluorescence-activated cell sorting (FACS). BM PCs were obtained by sorting for CD38++ CD138+ cells within the mononuclear light-scatter gate (Fig. 1b). Additionally, the cells were uniformly positive for the TNF-receptor superfamily member CD27 (Fig. 1b, inset). Importantly, we avoided gating of the pan-B cell marker CD19 since previous characterizations of human BM PCs show heterogeneous expression of CD19[20,21]. Therefore, our method captured all recently described BM PC subpopulations[5,12] with an overall $CD19^{+/-}$ $CD27^{+}$ $CD38^{++}$

CD138$^+$ phenotype. Subsequently, transcripts were amplified from BM PCs expressing IgM, IgG, and IgA using RT-PCR followed by high-throughput sequencing.

In total, 51,200 BM PC were sorted, which generated 503,415 reads after quality-threshold filtering (see Methods and Supplementary Table S1). These data were distributed across seven timepoints spanning 6.5 years (Fig. 1c). A biological replicate, a second frozen ampule derived from the same bone marrow aspiration, was also collected from each donor and analyzed. Multiple sampling from the same donor allows us to accurately identify the active heavy chain genes that compose this donor's antibody repertoire. Specifically, we identify 38 IGHV genes, 21 IGHD genes, and 6 IGHJ genes (4,788 combinations).

IGH V, D, and J Frequency Are Highly Stable Over 6.5 Years.

To determine the stability of individual gene usage, we assessed the frequency of each IGH V, D, and J gene across time (Fig. 2). Surprisingly, we saw stable behavior of these genes, with the most frequently used genes (e.g. IGHV4-34) showing consistently high expression while less frequently used genes (e.g. IGHV3-72) showed consistently low expression. This observation was quantified using the Mann-Kendall Test, which evaluates trends in time series data. We found that 89% of IGHV genes, 95% of IGHD genes and 100% IGHJ genes showed no statistically significant trends (Mann-Kendall test, $p>0.05$), indicating that the IGHV (Fig. 2a), IGHD (Fig. 2b), and IGHJ (Fig. 2c) genes were time stable.

Next, we analyzed population behavior of gene usage. Averaging across all timepoints, we observed a highly skewed distribution of individual gene frequencies, consistent with previous single timepoint observations. Only 6 IGHV genes (16%) accounted for greater than 50% of total IGHV gene usage by frequency (Fig. 2d). IGHD genes that have previously been shown to have biased usage IGHD2-2, IGHD3-3, and IGHD3-22[22] together accounted for 33% of total IGHD usage (Fig. 2b). In addition, known biases in IGHJ usage[23] are recapitulated as IGHJ4, IGHJ6, and IGHJ5 account for 86% of total IGHJ usage. Furthermore, our analysis demonstrated that IGH V, D, and J gene usage were not significantly different from a log-normal distribution (Anderson-Darling, $H=0$, $p>0.05$).

IGH V-D, D-J, V-J, and V-D-J Combinations Are Stable Over Time.

Given the temporal stability of individual genes, we hypothesized that differential intrinsic longevity might be found in gene combinations. Surprisingly, our analysis suggests that gene combinations, like their individual component genes, are time stable as well. We found that 92% V-J (Fig. 4), 97% V-D (Supplementary Fig. 1a), 95% D-J (Supplementary Fig. 1b), and 97% V-D-J (Supplementary Fig. 1c) do not show significant trends (Mann-Kendall, H=0, p>0.05).

To better understand the nature of gene combinations, we analyzed preferential gene pairing biases by comparing the expected versus observed frequency of pairwise gene combinations. The observed frequency of each gene combination was found to be correlated to its expected frequency (Spearman r): V-D (0.74), V-J (0.87), D-J (0.93), and V-D-J (0.65) (Fig. 5a-d). This high level of correlation and lack of significant outliers suggests that there is minimal gene pairing linkage and that the gene pairing is a random process.

Persistent Clonotypes Are Stable Over Time.

To understand how each of these individual genes and gene combinations together might indicate the existence of long lived PCs, we analyzed the behavior of the CDR-H3, the highest resolution possible for a single identifier of an antibody producing cell. To eliminate errors and ambiguities, we clustered CDR-H3s into clonotypes based on previously established criteria (see Methods). On average, we found that 16% of clonotypes are shared between adjacent timepoints (Fig. 6a, top). Interestingly, 23 clonotypes persisted across all timepoints (Fig. 6b). We found that 100% of these persistent clonotypes were time stable (Fig. 6a, bottom, Mann-Kendall test, h=0, p>0.05) and 78% (18/23) were of the IgA isotype. In addition, characteristics of the complete CDR-H3 population, specifically CDR-H3 lengths (Supplementary Fig. 2) and hydropathy index (Supplementary Fig. 3), are unchanged over time. The overall total distribution of CDR-H3 lengths are consistent with previously reported single timepoint values[27]. Also, higher expressing CDR-H3s tended to be neither hydrophobic nor hydrophilic (Supplementary Fig. 3) and no significant trends between hydrophobicity and expression level were found.

A Second Donor Corroborates The Observations From The First Donor.

To verify our longitudinal observations of stability and random gene choices from Donor 1, we analyzed a second donor across two years (Fig. 7). We identified 38 IGHV genes, 22 IGHD genes, and 6 IGHJ genes (5,016 combinations, 6,763 cells, 48,525 reads) (Supplementary Table 1 and Supplementary Fig. 4). Donor 1 and Donor 2 show highly correlated IGHV gene usage (r=0.82). Thus, the trends observed in Donor 1 were also observed in Donor 2. Specifically, individual IGHV, IGHD, and IGHJ gene usages were time stable (Supplementary Fig. 4), as were the gene combinations (Supplementary Fig. 5). Consistent with Donor 1, Donor 2 showed no preferential pairing in gene combinations (Supplementary Fig. 6). These results are highly consistent with the trends observed in Donor 1, and together, they indicate that BM PC antibody gene and gene combination usage show surprisingly minimal variation between individuals and across time.

DISCUSSION

Next-generation sequencing has enabled unprecedented ability to explore the details of the human immune repertoire[28,29]. Whereas previous studies have been able to describe some aspects of immune repertoire at a single point in time, our results harness the power of next-generation sequencing to elucidate the temporal dynamics of BM PCs over 6.5 years. Importantly, our data provide molecular resolution of antibody identity in the form of clonotypes, which is not possible with classic techniques like enzyme-linked immunosorbent assay (ELISA).

In this study, we show that the immune system is naturally polarized in both gene choice and gene combination usage and that the polarization is maintained over time. We also found that the bias is not primarily a result of gene linkage, suggesting there are additional genomic or extrinsic factors that contribute to polarization. For example, there may be an Eμ like enhancer in humans that results in higher than expected IGHJ4 usage[30]. Alternatively, the long arms race between the human immune system and the antigens it faces throughout evolutionary history may have established a preferential gene choice long ago. So higher expressing genes are likely broad-spectrum antibodies that have been useful in fighting particular classes of disease and continue to do so

today. For example, IGHV1-69 is implicated in many viral diseases like influenza and HIV. IGHV4-34 is associated with a range of autoimmune disorders, like cold agglutinin disease and systemic lupus erythematosus (SLE).

Taken together, our results indicate that the BM PC antibody gene and gene combination usage is highly stable over long periods of time. While short term studies over days, weeks or at most a year show that antigen stimulus can result in dramatic immune repertoire changes over weeks[31] our study extends over half a decade and is the longest longitudinal study to date. Given the known resistance we have for a large and diverse array of pathogenic antigens, it is surprising that the overall effect of these changes is not reflected in gene usage. This suggests that (i) either the existing immune gene combinations are established early on or that the mechanism of gene choice results in consistent bias over long periods of time, (ii) the effective size and variability of the immune repertoire is restricted, and (iii) antigen challenges in the form of vaccinations and illness do not have lasting changes at the gene usage level. Therefore, the immune repertoire must defend against a wide array of pathogens with only relatively small changes in immune diversity. On the other hand, intrinsic immunological memory is a well-established concept and BM PCs are thought to persist for long periods of time. An outstanding question remains of how intrinsic longevity is established and maintained. Although our results are unable to verify that any particular PC member persists, we can conclude that the clonotype, which defines binding specificity at the molecular level, does persist for at least 6.5 years. Our results suggest clonotype persistence contributes to the mechanism underlying long term immunological memory.

Moreover, we observe steady usage of gene and gene combinations throughout our experiment. Our observations suggest that there are large resident pools of plasma cells of the same identity, from which we can sample continuously with no loss of relative expression levels. We conclude that a large population of clonally-related PCs is responsible for persistent and stable gene expression of a given antibody, and that any given PC clone does not contribute greatly to the overall expression of that antibody.

In conclusion, we have used high-throughput, next-generation sequencing to definitively identify long-term persistent BM PC clonotypes, which has implications in clinical intervention studies, vaccines, and immunotherapy. Future next-generation sequencing studies can provide an even more detailed picture of the B cell immune repertoire as these studies could include advances in VH:VL native-pair sequencing (paired BCR-seq[19]), the analysis of correlations between BM PC repertoires and serum

immunoglobulin species (Ig-seq[17,18]), and an examination of the connectivity of B cells at various developmental stages (e.g., clonal relationships between circulating memory B cells and sessile BM PCs) . Our study provides a foundation upon which these further studies can be built.

MATERIALS & METHODS

Bone Marrow Specimens

Serially acquired human bone marrow specimens were collected from two donors by aspiration from the ileac crest, and mononuclear cells were enriched by Ficoll hypaque centrifugation. The two adolescent–teenage donors were originally diagnosed with neuroblastoma but had been asymptomatic and disease-free for many years according to routine bone marrow histology. A complete description of the donors' past medical history and ages at the time of the multiple time point collections is included in Supplementary Table 1. All procedures were performed per a standard operating procedure at the Memorial Sloan-Kettering Cancer Center and collected according to a longstanding protocol approved by the MSKCC Institutional Review Board. Aspirates were withdrawn from four sites and combined (total of 8–10 mL from 4 sites, 2–2.5 mL per site) drawn from the following: anterior right iliac crest, anterior left iliac crest, posterior right iliac crest, and posterior left iliac crest. The same attending physicians performed these procedures and usually biopsied through the same surgical site each time. De-identified specimens were shipped overnight on dry ice to the University of Texas at Austin.

Flow Cytometry and Isolation of Plasma Cells (PC)

BM samples were quick-thawed in a 37 °C H_2O bath and slowly diluted into RPMI-1640 complete medium containing DNaseI (Sigma D 4513; 20 U/mL), pelleted, washed and re-suspended in 2 mL FACS buffer (Dulbecco's PBS + 0.5% BSA Fraction V). Cell viability was determined using Trypan Blue exclusion and on average was approximately 90% per specimen. After a one-hour recovery at room temperature, BM cells were stained for 30 minutes at room temperature using empirically-determined optimal titrations of monoclonal antibodies: CD38-FITC (HIT2), CD138-PE (B-B4), CD27-APC

(M-T271), and CD19-v450 (HIB19). $CD19^{+/-}CD38^{++}CD138^{+}$ cells in human BM were collected as plasma cells (PC). PCs were observed to be heterogeneous for expression of the CD19 B-lineage marker; therefore, CD19-gating was avoided. $CD38^{++}CD138^{+}$ PCs were additionally gated by light scatter properties (FSC v. SSC) to exclude debris, apoptotic cells, and remnant granulocytes. All cell sorts were performed on a FACSAria flow cytometer. Cells were sorted directly into TRI Reagent for RNA preservation.

RT-PCR, High Throughput Sequencing of IGH V, D, and J Genes

All methods and reagents were as previously described[15]. Variable genes (recombined VHDJH region, which encodes the V domain) of IGH isotypes IgM, IgG, and IgA were amplified from oligo-dT cDNA and sequenced at high-throughput using the Roche 454 GS FLX technology using titanium long-read chemistry.

Data Processing, Analysis, and Visualization

All sequence data have been deposited to NCBI SRA under BioProject number PRJNA310043. IGHV, IGHD, IGHJ, and CDR-H3 regions for each read was quality filtered, processed and annotated using the VDJFasta utility described previously[32]. Reference IGHV, IGHD, and IGHJ genes from the international ImMunoGeneTics (IMGT) database were used. Mann-Kendall Tests were performed in Matlab, against the null hypothesis of no trend (alpha=0.05). Spearman r non-parametric correlation analysis was performed in python using the scipy library. To perform clonal clustering analysis, CDR-H3 sequences were clustered to form clonotypes, as established previously[33,34], using CD-HIT[35] with the following criteria: (i) Same IGHV and IGHJ, (ii) minimum length of 5, and (iii) 85% similarity threshold on the amino acid level.

Circular visualization plots were created with Circos software v0.67-7[36] where genes were sorted by expression within each timepoint and connected to adjacent timepoints via colored lines showing their expression levels. All other data visualization was performed using Python and matplotlib.

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Authorship contributions

GG and NKC conceived the study. GCW and GCI designed and performed experiments, analyzed data, prepared figures, and wrote the manuscript, under the supervision of EMM. All authors reviewed the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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422

423

FIGURE LEGENDS

Figure 1. Serial sampling, isolation by FACS, and NGS of BM PCs reveal temporal dynamics of antibody repertoire. (a) Overview of antibody repertoire characterization method. Serial sampling of human bone marrow plasma cells (BM PCs) over 6.5 years (left). Analysis of individual genes, gene combinations, and CDR-H3s (center) show temporally stable expression of persistent entities (right). (b) Representative fluorescence-activated cell sorting (FACS) gates of BM PCs (CD138+, CD38++) isolated from bone marrow mononuclear cells (BMMCs). (c) Sample collection timeline and summary of cell counts, sequencing reads, and unique CDR-H3s.

Figure 2. **Individual gene use frequencies for Donor 1 are temporally stable.** (a-c) IGHV (a), IGHD (b), and IGHJ (c) gene usage frequency over time. Plots are sorted by decreasing mean frequency. Only gene calls that appear in all timepoints are shown. (d) Mean frequency of IGHV gene use. Error bars are standard deviation.

Figure 3. Usage frequencies of gene combinations are temporally stable. IGH V-J usage frequencies for Donor 1 are shown. Plots are sorted by decreasing mean frequency. Only gene calls that appear in all timepoints are shown. See Supplementary Figure 1(a-c) for usage frequencies of IGH V-D, D-J, and V-D-J.

Figure 3. Gene combinations do not preferentially associate and are instead randomly assorted. (a-d) Spearman's rank correlation of expected versus observed IGH V-D (a), V-J (b), D-J (c), and V-D-J (d) gene combination frequencies. Expected (by random association) frequencies are calculated as products of the frequencies of the individual component genes. Diagonal lines in red indicate no difference between the expected and observed frequencies.

Figure 5. Usage frequencies of persistent clonotypes are temporally stable. (a) Circos plot of shared clonotypes between adjacent timepoints (top). Circos plot of the persistent clonotypes across all timepoints (bottom). Each band in the outermost perimeter represents the clonotypes found in a given timepoint, sorted by decreasing expression. The inner curved lines indicate the same clonotype shared by two timepoints. Green indicates high expression; purple, low expression; with lighter colors

458 indicating intermediate expression. (b) Gene usage frequency over time of the 23
459 persistent clonotypes (see Methods) found in all timepoints. Plots are sorted by
460 decreasing mean frequency. Gene names (for IGHV and IGHJ), representative amino
461 acid sequences, and isotype are above each plot.
462
463 **Figure 6. Gene and gene combination use frequencies correlate between Donor 1**
464 **and Donor 2.** (a) Spearman's rank correlation of individual gene frequencies between
465 the two donors: IGHV (top), IGHD (center), and IGHJ (bottom). (b-e) Spearman's rank
466 correlation of combination gene frequencies between the two donors: V-D (b), V-J (c), D-
467 J (d), and V-D-J (e). (a-e) Red lines indicate least squares regression.
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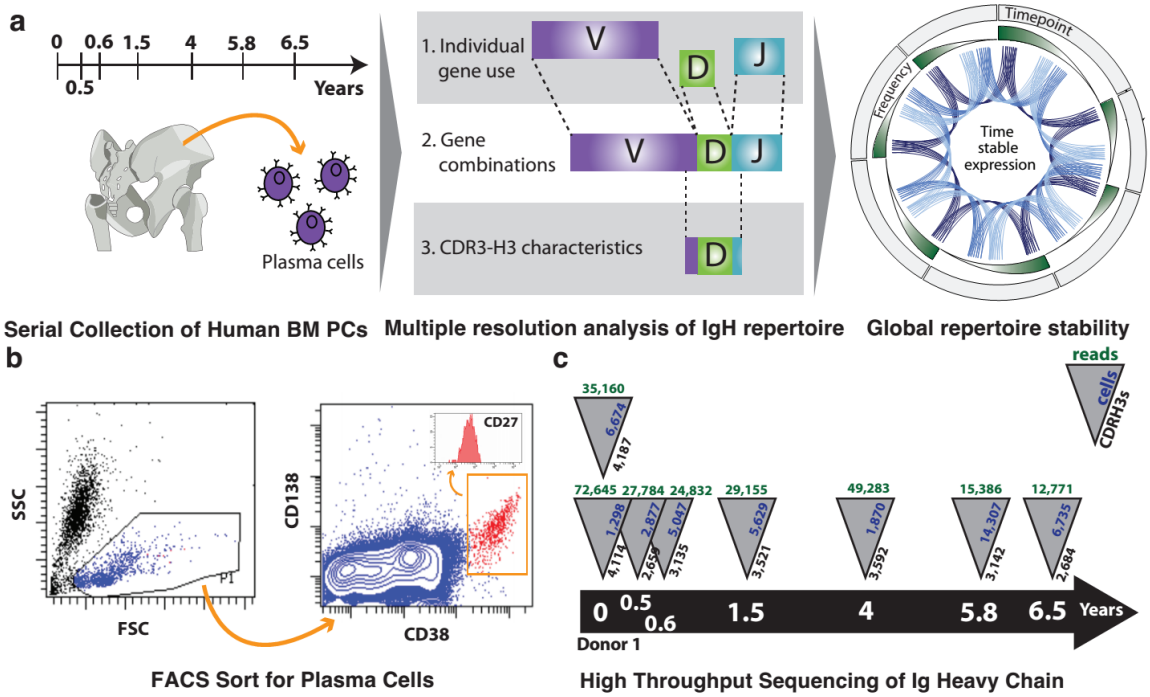
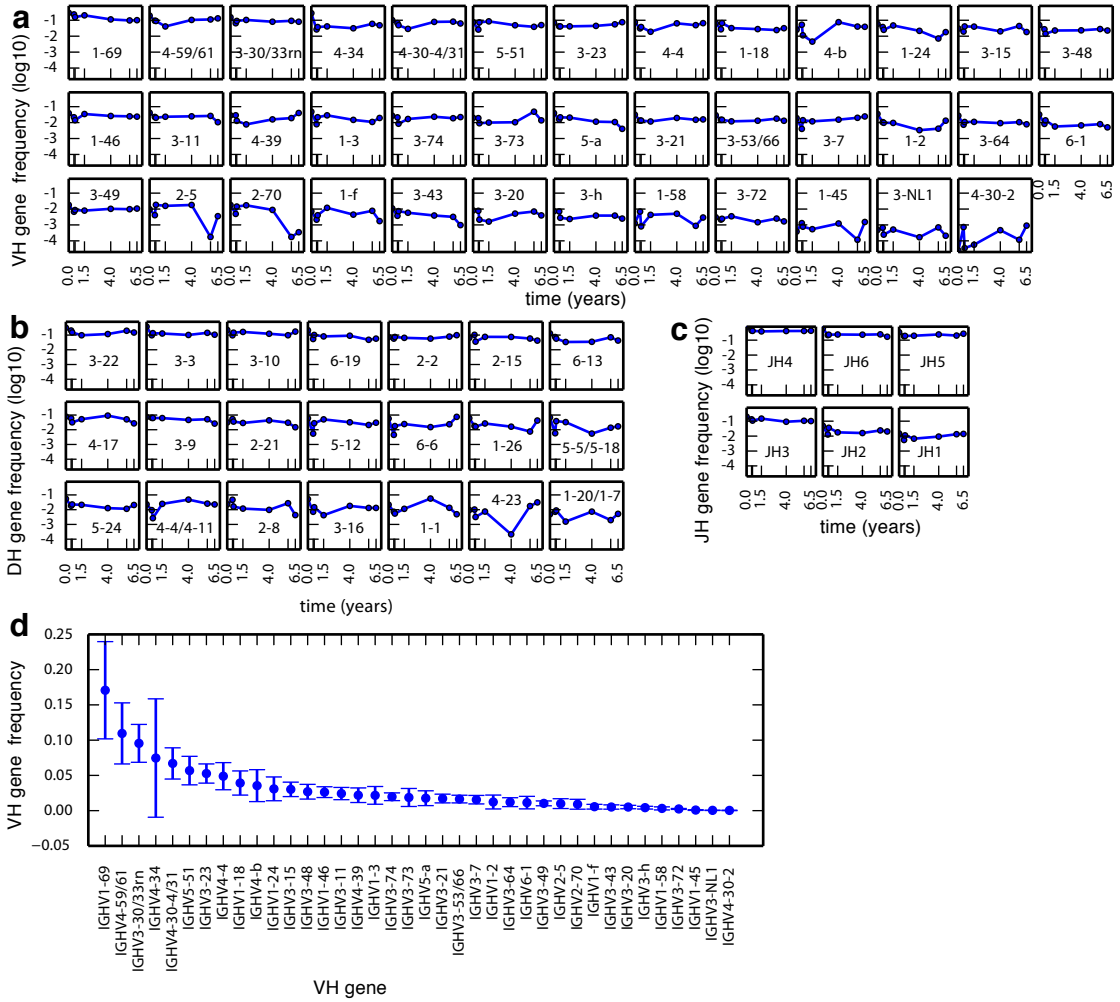


Figure 1



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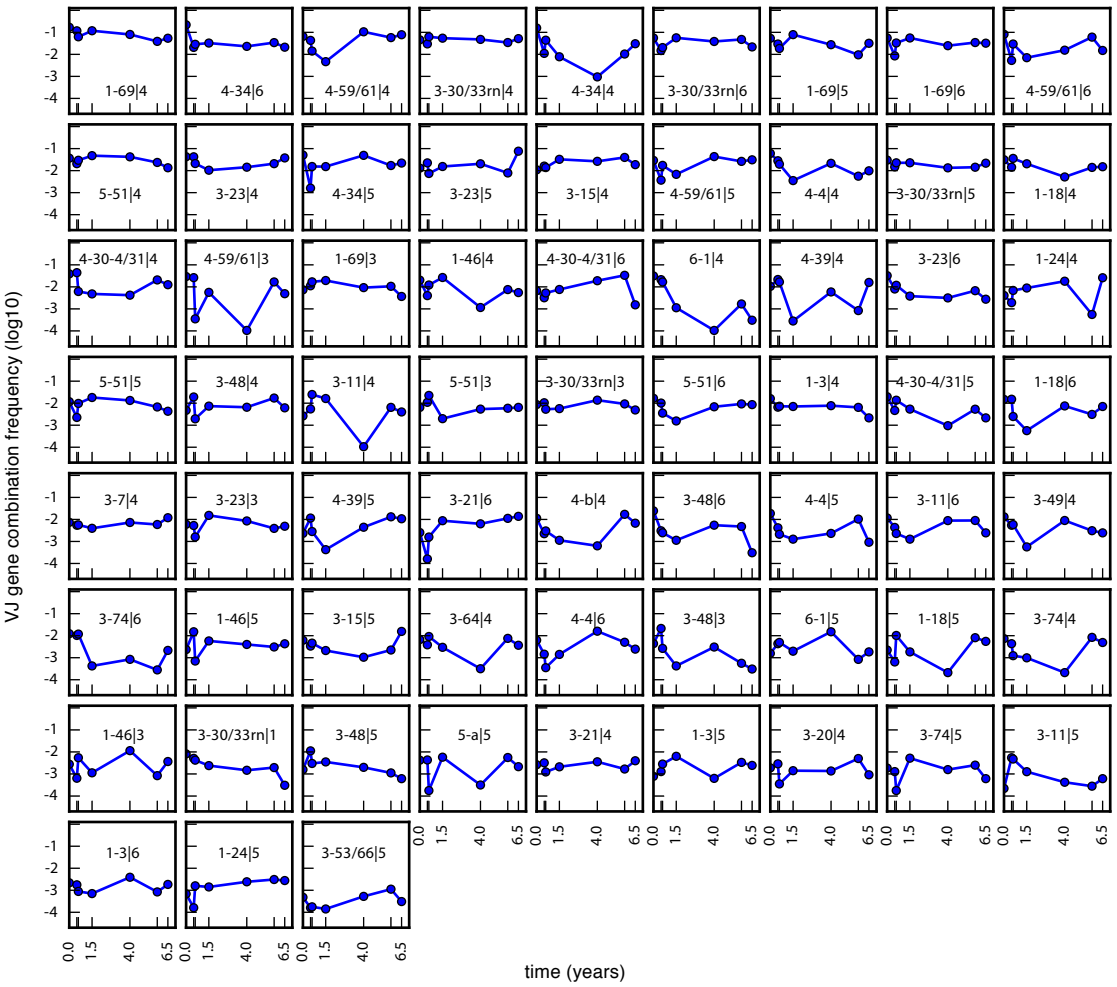

Figure 3

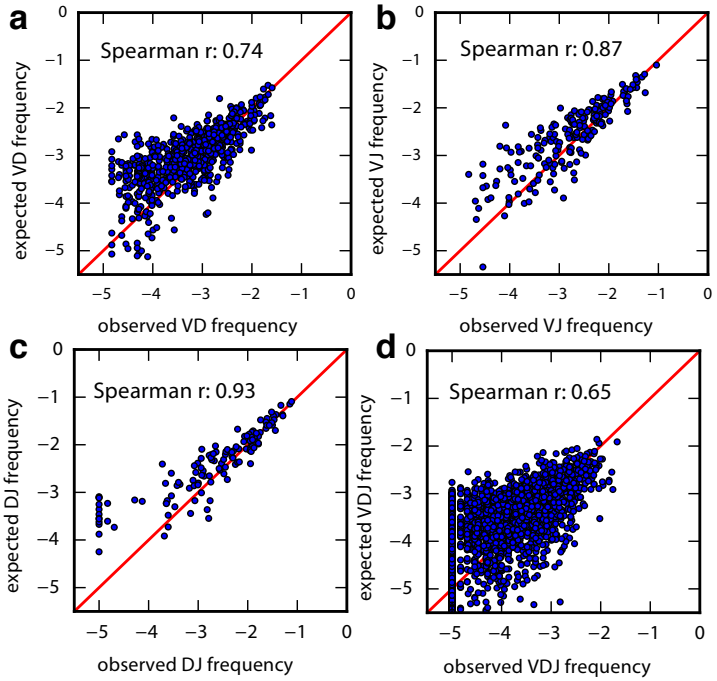

Figure 4

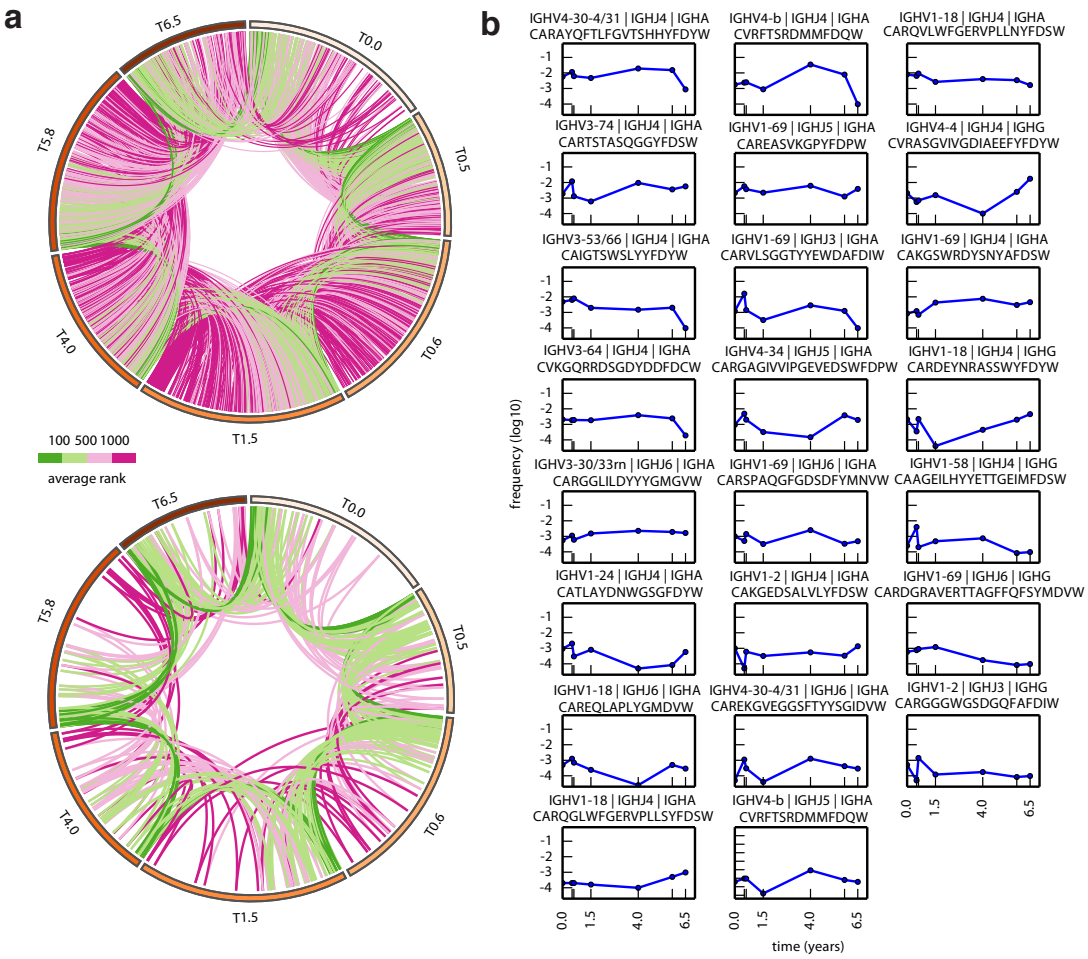

Figure 5

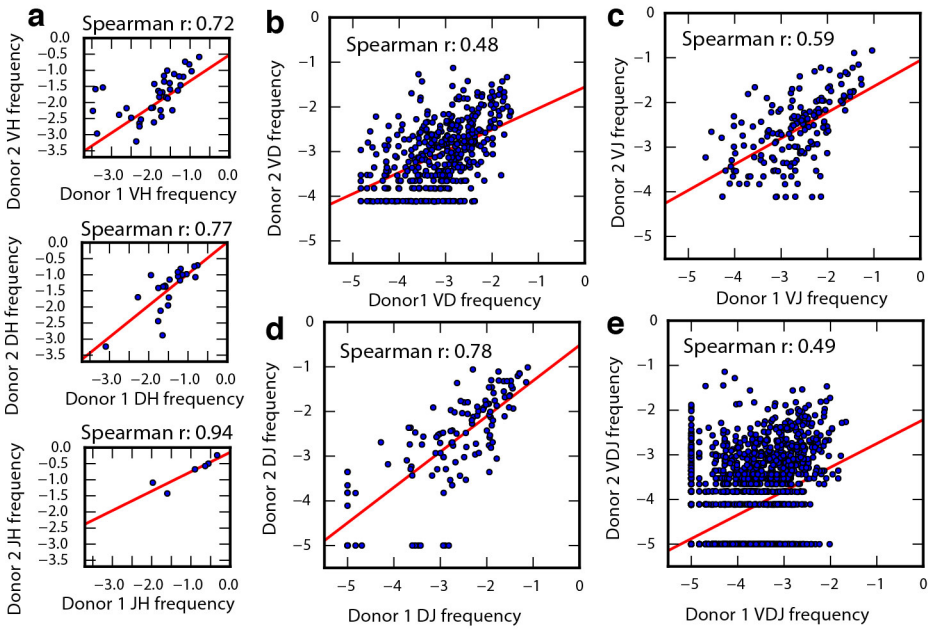

Figure 6