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B cell receptor light chain repertoires show signs of selection with differences between groups of healthy individuals and SLE patients

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Abstract

We have developed a microarray to study the expression of L-chain V genes (V_L genes) in healthy and SLE patient peripheral κ - and λ -sorted B cells. In all repertoires tested, one V_L gene accounts for over 10% of all gene V_L expression, consistent with positive selection acting on L-chains. While a few V_L genes were highly expressed in all individuals, most V_L genes were expressed at different levels. Some V_L genes (5 out of a total of 78) were not detected. We attribute their absence from the repertoire to negative selection. Positive selection and negative selection were also found in SLE repertoires, but expression of V_L genes was different; the differences point to less regulation of V_L gene repertoires in SLE. Our data shows that V_L gene expression is variable and supports a model where the L-chain repertoire is generated by both positive and negative selection on L-chains.

Keywords

Receptor editing; Light chain; B cell repertoire; SLE

1. Introduction

B cell receptors are formed by pairing of Ig heavy chain (H-chain) and light chain (L-chain) that together determine the specificity of the receptor. Thus, the initial repertoire diversity is due to combinatorial association of H-chains and L-chains as well as combinatorial joining of V, D and J gene segments. (Kawasaki et al., 2001; Kawasaki et al., 1997; Matsuda et al., 1998). Additional diversity arises at the junction of V, D and J gene segments by N-nucleotide additions, P-nucleotide additions and nibbling (Weigert et al., 1980; Wu and Kabat, 1970). Selection acts on the B cell repertoire through both tolerance mechanisms and antigen selection. Tolerance selects B cells during development when autoreactive receptors are formed and acts on individual B cells through receptor editing, a process which replaces

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the originally rearranged L-chain with a newly-recombined L-chain (Gay et al., 1993; Halverson et al., 2004; Radic et al., 1993; Tiegs et al., 1993). In the case of antigen selection, H-chain and L-chain pairs present in the pre-immune repertoire undergo clonal expansion. We are interested in the L-chain repertoire of B cells that have undergone selection.

Previous repertoire analysis has used a variety of approaches such as isoelectric focusing, Southern blotting and PCR amplification (Bentley, 1984; de Wildt et al., 1997; Klobeck et al., 1984; Mattson et al., 1982; Meffre et al., 2001; Meffre et al., 2000; Walker et al., 1983; Williams et al., 1987); (Dorner et al., 1999; Dorner et al., 2002; Dorner et al., 2001; Farner et al., 1999; Foster et al., 1997; Girschick and Lipsky, 2002; Heimbacher et al., 2001; Jacobi et al., 2002; Kaschner et al., 2001; Lee et al., 2004; Lee et al., 2000; Meffre et al., 2004; Wardemann et al., 2004; Wrammert et al., 2008). However, these studies have limitations, including culture efficiency, PCR biases and cost. To examine L-chain repertoires, we developed a microarray that surveys all human V_L genes. There are several advantages to the microarray method: it is not subject to PCR primer bias; the microarray is highly specific, permitting resolution of members within multi-gene families; and it allows comparison of individual or group repertoires. Because of these features, the microarray allows for complete repertoire analysis on a large scale.

The repertoires of κ and λ B cells from healthy individuals were determined using this method. We also studied L-chain repertoires in patients with SLE, a disease in which tolerance is broken. High expression of V_L genes was observed in each of the repertoires tested. A small, but substantial number of V_L genes could not be detected in the repertoires, individually and in some cases collectively. Within healthy and SLE groups, expression of individual V_L genes varied from one individual to the next, and comparison of healthy and SLE patient L-chain repertoires identified several differences in V_κ and V_λ gene expression.

2. Materials and methods

2.1. Healthy and SLE blood samples

Twelve mL (12 mL) of peripheral blood was collected in BD vacutainer blood collection tubes with EDTA (Becton Dickinson) from 10 healthy donors and 10 SLE patients with evidence of active disease (identified by low complement (C3 or C4) levels or clinical signs of active SLE) after informed consent. The use of human blood was approved by the University of Chicago, Institutional Review Board (protocol 14801B). The blood was immediately processed for cell isolation and subsequent cell sorting.

2.1. FACS sorting

Blood was diluted 1:1 in PBS immediately after collection, and Lymphocyte Separation Medium (Mediatech) was used for isolation of mononuclear cells according to the manufacturer's protocol. Cells were resuspended in RPMI supplemented with 10% FBS at a concentration of 10^8 cells/mL. Cells were stained using the following antibodies for FACS sorting at the concentrations recommended by the manufacturer: anti-CD138 APC (Dako), anti-Ig κ FITC (BD Pharmingen), anti-Ig λ PE (BD Pharmingen), anti-CD20 PE (BD

Pharmingen), anti-CD20 PE (BD Pharmingen), anti-CD27 APC (eBiosciences) and anti-CD38 PE-Cy7 (eBiosciences). Eight samples from each group were phenotyped for CD27 and CD38 levels. Sorting was performed using MoFlo (Dako-Cytomation) and FACSARIA (BD Biosciences) machines using the single cell sort purity mode. Lymphocytes were gated according to forward- and side-scatter profiles and doublets were excluded using forward-scatter pulse width and pulse area profiles. One-thousand events in the CD20+CD138-Igκ+Igλ- and 1,000 events in the CD20+CD138-Igκ-Igλ+ gates were sorted into RNeasy Lysis Buffer (Qiagen) and stored at -20°C. Although rare in peripheral blood, plasma cells were specifically excluded in the sorting strategy based upon the following: forward- and side-scatter gates were set to exclude plasma cells; CD138+ cells were not gated; and plasma cells have absent or low levels of surface CD20, κ and λ.

2.3. cDNA preparation

Total RNA for each sample was isolated using TRIzol (Invitrogen). The RNA concentration and quality was determined using a NanoDrop (Thermo Scientific), and only RNA with 260/280 greater than 1.75 were used for amplification. Twenty ng (20 ng) of total RNA was used for cDNA amplification using Ovation RNA Amplification System V2 (Nugen) and purified using DNA Clean and Concentrator – 25 columns (Zymo research) according to Nugen protocols. If necessary, samples were stored at -80°C until the day of hybridization. Purified cDNA used for the microarray was labeled by adding 4 µg of cDNA in 20 µl to 10 µl of Ulysis Alexa-Fluor 647 (Invitrogen) and heating to 80°C for 20 minutes. Labeled cDNA was purified using Bio-Spin 6 SSC columns (Bio-Rad) after washing the columns 3 times with water. A NanoDrop was used to quantify the cDNA amount and amount of Alexa-Fluor 647.

2.4. Syndecan-1 (CD138) PCR

Primers were designed based on NCBI reference sequence for syndecan (NM_002997.4). The forward primer (AAATGGCAAAGGAAGGTGGATGGC) and reverse primer (ATACACTCCAGGCAGAAAGTCGCA) were synthesized by IDT DNA, and the PCR steps were: 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 50 seconds; and 72°C for 10 minutes. Reagents were used at the recommended concentrations for the polymerase, Taq DNA Polymerase (Invitrogen).

2.5. Microarray probe design

Light chain V region sequences used for probe design were obtained from publications on the human *IGK* and *IGL* loci (Kawasaki et al., 2001; Kawasaki et al., 1997; Schable and Zachau, 1993). Control genes were included on the microarray; sequences for *ACTA* (NCBI reference sequence: NM_001100), *ACTB* (NM_001101), *AKR1B1* (NM_001628.2), *B2M* (NM_004048.2), *CD19* (NM_001770), *GAPDH* (NM_002046), *IGKC* (J00241.1), *LDHA* (NM_005566), *MS4A1* (NM_152866) and *NONO* (NM_001145409) were downloaded from the National Center for Biotechnology Institute gene database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>). Probe sequences were determined by filtering V_L gene sequences for: probe length (65–74 bp) aligning to any region of the V_L gene, uniqueness compared with all V_L genes (BLAST), self-binding (Smith and Waterman,

1981), complexity, melting temperature ($69.0 \pm 1.5^\circ\text{C}$) and distance from 3' end. Probes were named and are reported using the original nomenclature to be consistent with the microarray data files. Seven pairs of V_κ genes were identical or nearly-identical, and probes meeting the design criteria correspond to identical regions in the pair. For these V_κ genes, expression is reported together (e.g. *O8* and *O18* are detected by the probe O8/O18). For the expression levels by cluster or position, estimates of expression for each gene within the pair were assumed to be equal. Appendix Table A.1 lists sequences for the V_L gene probes. Probes were manufactured by Integrated DNA Technologies.

2.6. Reference sample

A reference sample containing the reverse-complement of all V_L gene probe sequences at equal molar concentrations and 0.12 ng of each reference sequences was co-hybridized with every cDNA sample (Integrated DNA Technologies). The reference was labeled using Ulysis Alexa-Fluor 555 (Invitrogen). For the series of experiments used to estimate the amount of each gene present, the reference was labeled using the Ulysis Alexa-Fluor 647 dye (Invitrogen).

2.7. Estimation of expression level

The complementary reverse sequence of two V_κ genes (B2 and O2/O12) and two V_λ genes (2–13 and 1–19) were hybridized using the same techniques and methods as the cDNA samples. The four genes were chosen from different gene families (Figure A.2). The effect of increasing DNA concentration on signal intensity was also determined using these four genes by adding Alexa Fluor 647-labeled V_κ and V_λ targets to Alexa Fluor 647-labeled reference sample at known concentrations (see 2.11 and Figure A.3), which was then hybridized along with the Alexa Fluor 555-labeled reference sample. V_κ B2 was tested at 12.2%, 21.7% and 41.0%; V_κ O2/O12 was tested at 5.2% and 10.0%; V_λ 2–13 was tested at 10.6%, 19.2% and 37.3%; and V_λ 1–19 was tested at 19.2%, 8.7% and 4.6% (percent refers to the molar amount of the gene present in the sample). Each V_L gene and concentration was hybridized two times. The second hybridization of V_λ 1–19 at 4.6% had a scratch across a portion of the array and was not included in the analysis. Normalized expression values for each of these hybridizations (reference subtracted—see section 2.11 for data analysis and normalization) were compared with the concentration of the genes in the hybridized sample. The Curve Fitting Tool in Matlab was used to identify the best-equation for this data. This equation was then used to estimate expression levels for all of the cDNA samples.

2.8. Microarray spotting

Microarrays were spotted using a GeneMachines OmniGrid 100 (Genomic Solutions) onto SuperAmine 2 slides (ArrayIt). Each oligonucleotide probe was spotted twelve times per array, and the print layout was such that these twelve replicates were spotted by four different pins. In addition to the human oligonucleotides, the microarrays also had mouse oligonucleotides spotted on the array. After printing, microarrays were dehydrated following the manufacture's recommendations and stored in the dark at room temperature.

2.9. Microarray hybridization and scanning

Prior to hybridization, microarrays were re-hydrated, washed (Wash Buffers A, B and C, ArrayIt), prehybridized for two hours (BlockIt Blocking Buffer, ArrayIt) and washed again. Hybridizations were performed with 10 ng of the labeled reference sample added to 1.5 µg labeled cDNA and 2 µg human Cot-1 DNA (Invitrogen). DNA was mixed with HybIt 2 hybridization buffer (ArrayIt) at 1x concentration to a final volume of 20 µL, denatured at 80°C for 3 minutes and hybridized overnight at 37.0°C for 14–16 hours. Following hybridization, microarrays were washed with Wash Buffers A, B and C for 2 minutes each, dried and scanned using an Axon GenePix 4000B scanner (Molecular Devices). All amplified and labeled cDNA samples were hybridized two times as technical replicates. cDNA labeling, prehybridization, hybridization, washing and scanning were all performed by the same individual at approximately the same time of the day.

2.10. Data quality

Image analysis was performed using GenePix Pro 6.0 (Molecular Devices). Spot recognition and flags were checked for each spot and manually adjusted when appropriate. Because the reference sample was co-hybridized with all samples, hybridization quality could be readily observed and missing spots could be easily detected. Missing spots were consistent between arrays, and no more than 3 of 12 replicate spots per gene were missing. Therefore, only 9 replicate spots were used for analysis even though the majority of probes were spotted 12 times per array. For genes that had 12 replicate spots that were of adequate quality on all arrays, the 3 spots printed last were not analyzed. In addition, the quality of hybridization was assessed by ensuring that the positive control spots produced signal when appropriate (for example, strong signal being detected for the *ACTA* and *ACTB* probes). Replicate hybridizations were also plotted for comparison and correlation coefficients calculated to ensure consistent data between experiments.

2.11. Data normalization and analysis

Microarray data and processing has been submitted to the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>), entry GSE33544. This reported data the Minimum Information About a Microarray Experiment (MIAME) standard. All analysis was performed using MAANOVA 2.0 software (Xiangqin Cui, Hao Wu, Gary A. Churchill, The Jackson Lab) in Matlab (Mathworks). The median spot log₂ values were used for the analysis and prior to normalization, intensity-based Lowess adjustment of all data was made. Normalization was performed using mixed effects models which included variables for array (A), gene (G), sample (S), and health status (H). Two different models were used for normalization. The first model did not separate individuals into groups (healthy versus SLE) and was used to estimate expression levels in the repertoires; the following equation was used for this model: $\log_2(y_{ikg}) = \mu + A_i + S_k + G_g + (AG)_{ig} + (SG)_{kg} + \varepsilon_{ikg}$. The second model included a term to account for health status [(HG)_{hg}] and used the following equation: $\log_2(y_{hikg}) = \mu + H_h + A_i + S_k + G_g + (AG)_{ig} + (SG)_{kg} + (HG)_{hg} + \varepsilon_{ikg}$. For determination of statistical significance, the (SG)_{kg} terms from the first model (the null model where health status was not included) was compared with the (SG)_{kg} term from the second model (in the second model health status was included in normalization). Differential gene expression was

determined using the MAANOVA package, and results from two different F-tests are presented. κ -sorted and λ -sorted samples were analyzed independently for V_{κ} and V_{λ} expression, respectively. To evaluate inclusion of V_L gene expression, κ -sorted and λ -sorted samples were analyzed together for expression of all V_L genes.

To remove the probe effect and make intra-array comparisons, normalized values for the reference sample (containing equal concentrations of all V_L gene targets) were subtracted from each sample tested. Thus, probes which hybridized well to the reference sample were assumed to also hybridize well to the experimental samples. These reference-adjusted values were then fit to a standard curve generated by spiking in known concentrations of V_L gene targets. Estimates for expression levels were restricted to take on values between 0 and 30%. Expression level estimates were not further manipulated, and the sums of expression level estimates for a sample were allowed to be more or less than 100%. Comparison of biological replicates was performed to evaluate reproducibility of the method (see Appendix A.5).

Unless otherwise noted, two-tailed t-tests were performed to generate p-values, and 0.05 was used as the p-value cut-off.

2.12. NCBI database search for V_L genes identified as absent

The germline V_L nucleotide sequences for those V_L genes identified as not being expressed in either SLE or healthy groups was searched against all entries in the NCBI database (<http://www.ncbi.nlm.nih.gov/igblast/>, search performed on 11/10/2010). Default parameters were used for alignment and scoring. All sequences returned in the search were manually evaluated and excluded if the entry was more closely related to another germline V_L gene or if the entry was from a non-RNA source (i.e. genomic library). The number of sequences meeting the criteria is reported in the tables.

2.13. Spectratyping

Minor modifications were made to the recommended protocol for H-chain spectratype analysis (IGH Gene Clonality Assay, Invivoscribe Technologies). Instead of using genomic DNA for each PCR reaction, 40ng of amplified cDNA was used as the template. Three PCR reactions (Tube A, Tube B and Tube C) were performed on five healthy individuals (both κ - and λ -sorted B cells). Four SLE κ -sorted samples were tested, and five SLE λ -sorted samples were tested. PCR products were run on a 3130XL DNA Analyzer (Applied Biosystems). For quantitative comparison, Tube C data were used. Peak heights for all lengths within the appropriate H-chain size range were measured and each length was normalized according to the highest peak in the sample (thus, the peak had a value of 1.0). For comparison of restriction between the healthy and SLE groups, the normalized polyclonal control peak heights were subtracted from the samples at the corresponding lengths. The standard deviation in these values was determined for each sample and compared using an unpaired t-test.

3. Results

3.1. Peripheral blood B cell phenotypes in healthy individuals and SLE patients

B cells from 10 SLE patients and 10 healthy donors were phenotyped using flow cytometry. Samples were co-stained using reagents to CD27 (memory B cell marker), CD38 (germinal center/transitional cell marker) and the pan-B cell marker, CD20 (Figure 1A). The frequency of CD20+CD27+ cells was the same in healthy and SLE groups (p-value > 0.05), while the percent of CD20+CD38+ B cells was higher in samples from SLE patients (p-value < 0.05). Using a separate stain, surface CD20, light chain (κ and λ) and CD138 (plasma cell marker) levels were determined (Figure 1B). The ratio, $\kappa:\lambda$, of B cells was higher in SLE with a median of 1.9 compared with healthy B cells with a median of 1.4 (p-value < 0.05). Plasma cells were rare in the lymphocyte gated population, comprising less than 0.001% of cells in any of the blood samples collected. Because plasma cells contain large quantities of H- and L-chain RNA that would overshadow the true frequency of a V_L gene in the repertoire, this population was excluded from the sorting gates (Figure 1A). Cells were then sorted into κ samples (CD20+CD138- $\kappa+\lambda$ -) and λ samples (CD20+CD138- $\kappa-\lambda$ +) for microarray analysis with κ and λ B cells tested separately. Prior to microarray hybridization, cDNA from each sample was also tested for plasma cell contamination using a *syndecan-1* (CD138) PCR; contamination was not identified in any samples.

One thousand B cells were sorted for each sample. This number was chosen because it is large enough to include rarely expressed V_L genes. According to the binomial distribution, V_L genes comprising more than 0.45% of the repertoire have a 95% chance of being included in the sample. The sorted samples from healthy and SLE-affected individuals were used to generate cDNA for hybridization on identical light chain microarrays. A reference sample that included equal concentrations of the V_L gene targets was co-hybridized with the experimental samples. The reference sample was also used in a series of experiments to evaluate for cross-reactivity and quantify expression levels. These experiments did not show signs of cross-reactivity, demonstrated high sensitivity and accurately quantified expression levels (see Appendix sections A.2, A.3 and A.4).

3.2. Biased V_L gene expression in healthy repertoires

We evaluated B cell repertoires with the aim of addressing whether V_L genes are expressed at different levels. If all V_L genes were equally expressed, each $V\kappa$ gene would comprise 2.4% (1/42) of the total κ signal and each $V\lambda$ would comprise 2.8% (1/36) of the total λ signal. Most V_L genes in the healthy samples were expressed at levels higher or lower than these values. For example, 5 $V\kappa$ genes in the Healthy01 κ sample were expressed at more than 10% the repertoire (Figure 2, left, bars > 0.1). Other $V\kappa$ genes in different samples were similarly expressed at over 10% of the total repertoire. $V\lambda$ genes were also highly expressed (Figure 2, right; Appendix Table A.2 and Table A.3 contains complete expression level estimates). The probability of observing a single V_L gene at 10% of the repertoire by chance assuming equal expression of V_L genes is extremely small (less than 2×10^{-27}). Some V_L genes were highly expressed in all healthy individuals ($V\kappa$ genes A3/A19, A20 and O8/O18 and the $V\lambda$ genes I-4, I-7, I-2 and I-3) as indicated by low rank-order numbers

(Appendix Figure A.5). Other V_L genes were expressed at different levels between individuals with variable rank-order values.

3.3. Absence of light chain V genes from the repertoires of healthy individuals

Some V_L genes were not detected in any of the repertoires from healthy individuals although they appear to be functional. These included three V_K genes (*A10*, *L14* and *O11a*) and seven V_λ genes (*1-9*, *3-2*, *3-3*, *4-2*, *4-3*, *5-1* and *5-2*). The finding that certain V_L genes were absent from repertoires was consistent with data from the NCBI database. A search of all 93,626 sequences in the NCBI database for evidence of expression of these genes revealed that three had no entries, six had between one and sixteen entries and one had more than sixteen entries (Table 1).

The V_L genes absent from repertoires varied between individuals in both identity and number. For V_K genes this ranged in number from 6 to 18, and the range of V_λ genes not detected was 9 to 17. As described above, V_L genes present at greater than 0.45% of the repertoire are likely to have been included in our sample (95% confidence level). Therefore, these V_L genes are either not expressed or expressed at levels so low they are undetectable in the repertoire.

3.4. Repertoires from SLE patients

Light chain repertoires from SLE patients shared many of the same features as repertoires from healthy individuals but also had some differences. SLE patient repertoires had high expression of V_L genes in both κ and λ samples. Some of the same V_L genes that were highly expressed in all healthy individuals were also highly expressed in all SLE patients, including *A3/A19*, *A20* and *1-4* (Appendix Figure A.6 and Figure A.7). A few other genes that had inconsistent expression in healthy repertoires were highly expressed in all SLE patient repertoires (e.g. *1-19*). The expression of *L6*, *L12*, *1-20* and *2-8* was variable in SLE patient repertoires but consistently high in repertoires from healthy individuals (Appendix Figure A.6 and Figure A.7).

While 10 V_L genes were not expressed in any repertoires from healthy individuals, 7 V_L genes were absent from all repertoires of SLE patients (Table 2). Four of the V_L genes (*A14*, *1-9*, *4-3* and *5-2*) missing from all SLE samples were also not detected in any of the healthy samples. None of these showed evidence of expression in the NCBI database. Other V_L genes were expressed exclusively in healthy individuals or SLE patients. Expression levels indicated that when present, these V_L genes made significant contributions to the repertoire. For example, when *O11a*, *3-3*, *4-2* and *5-1* were expressed in SLE repertoires, their expression levels were 4.3%, 4.3%, 4.8% and 6.7%, respectively. These expression levels were much higher than could be attributed to sampling error (Appendix Figure A.5). In summary, some genes that were not expressed in the healthy samples were expressed in some repertoires from SLE patients, and some V_L genes that were not expressed in repertoires from SLE patients were expressed some healthy samples.

Differences in V_L gene expression between groups were detected using two different F-tests, both of which are valid in determining statistical significance alone but do so with different

methods. F_1 values were calculated using gene-specific standard deviations while F_3 values were calculated using the standard deviation of all V_L genes (Woo et al., 2005). A total of 11 genes (5 V_{κ} s and 6 V_{λ} s) were identified as differentially expressed, eight according to the F_1 test and seven according to the F_3 test (p -value < 0.05 , Table 3). Four V_L genes were identified as differentially expressed by both F -tests. Genes expressed at higher levels in repertoires from healthy individuals than SLE patient repertoires include the V_{κ} s *B3* and *L1* and V_{λ} s *1-2*, *1-3* and *4-1*. Genes expressed at higher levels in SLE include the V_{κ} s *A3/A19*, *A27* and *O11a* and the V_{λ} s *2-8*, *3-2* and *5-1*. As described above, *O11a*, *3-2* and *5-1* were not expressed in any healthy repertoires and had rare occurrences in the NCBI database. However, in some SLE repertoires these V_L genes were expressed. There was intra-group variability in expression levels with overlap in the expression levels of healthy and SLE groups for most of these V_L genes.

3.5. Heavy chain restriction in the B cell repertoire

Clonal selection, both during normal development and resulting from antigen encounter, can play a role in shaping the B cell repertoire and could contribute to the biased V_L gene expression observed in the microarray data. Because clonal selection restricts both a H-chain and a L-chain repertoire by increasing the frequency of a single clone, H-chain CDR3-length restrictions can be used as a marker for clonal selection. Spectratype analysis of H-chain CDR3 lengths was performed using the same cDNA that was used for microarray hybridization. Several spectratypes, such as the κ -sorted Healthy01, showed minimal signs of H-chain CDR3 length restriction as indicated by normally-distributed peak heights (Figure 3A). However, the majority of the 16 spectratype patterns tested showed evidence of restricted CDR3 lengths. Restriction between samples was quantified and compared with a polyclonal control sample. SLE- κ samples had greater H-chain restriction than healthy- κ samples, while there was no significant difference between the SLE- λ and the healthy- λ (Figure 3B). Thus, there is evidence for clonal selection in repertoires from both healthy individuals and SLE patients with a difference in the amount of selection in κ B cells.

3.6. Transcription of V_{κ} genes in λ B cells

A feature of our method is that it allows examination of repertoires at the mRNA level. Hence, we are able to analyze expression of V_L genes that are not necessarily functional at the protein level. Such transcripts include aberrantly rearranged and unrearranged V_L genes (Delpy et al., 2004; Staudt and Lenardo, 1991). B cells were sorted on the basis of κ and λ isotypes (CD19+ κ + λ -CD138- and CD19+ κ - λ +CD138-), and the differences between these two populations are of interest because rearrangement of *IGL* genes and expression of the λ L-chain repertoire occurs after *IGK* rearrangement (Belessi et al., 2005; Brauninger et al., 2001; Hieter et al., 1981; Klein et al., 2005; Korsmeyer et al., 1981; Korsmeyer et al., 1982; van der Burg et al., 2001). This temporal difference could result in different selective forces acting on B cells bearing different isotypes. V_{λ} gene expression was not detected above background in κ -sorted samples (Figure 4 shows one example). However, V_{κ} expression was detected in the λ -sorted samples, albeit significantly lower than V_{λ} expression (p -value less than 0.05). Thus, there is little if any expression of V_{λ} genes in κ + λ - B cells, but there is appreciable expression of V_{κ} genes in κ - λ + B cells.

4.0. Discussion

4.1. Selection on L-chains shapes the B cell repertoire

One-third of V_L genes were not expressed in a given repertoire. Lack of V_L gene expression can be due to several factors. *Cis*-elements can limit recombination, an example of this is the octamer motif bound by OcaB which decreases recombination of some V_K genes when altered or deleted (Casellas et al., 2002; Nadel et al., 1998). V_L genes consistently expressed at low levels or not expressed may be influenced by such *cis*-elements that limit recombination. Differences in the recombination signal sequences also affect V_L gene recombination (Hesse et al., 1989). Although rare, nonsense codons at the end of a V region limit functional rearrangements for these V_L genes. The mouse $V\lambda X$ and two human $V\lambda$ genes, 5-1 and 5-2, are the only V_L genes with nonsense codons at the end of the V region (Kawasaki et al., 1997; Sanchez et al., 1990). Since the microarray was designed to detect only functional V_L genes, the possibility that the V_L genes not expressed are pseudo-genes is excluded. Negative selection on L-chains can also limit V_L gene expression. While all of these mechanisms may decrease expression levels, we think selection on L-chains plays the largest role in shaping the repertoire.

B cells with autoreactive receptors undergo negative selection by receptor editing, and L-chains with intrinsic self-reactivity are negatively selected by this mechanism (Gay et al., 1993; Halverson et al., 2004; Radic et al., 1993; Tiegs et al., 1993). The mouse λX L-chain has been shown to bind the self-antigen myelin basic protein alone or in combination with V_{Hs} (Doyle et al., 2006; Galin et al., 1996). This may account for the low expression of λX observed in wild-type mice (Li et al., 2004). Other L-chains with intrinsic autoreactivity are also expected to have low expression in the repertoire. The human V_L genes 5-2 and 5-1 are similar in protein sequence to the mouse $V\lambda X$, and neither 5-2 nor 5-1 was detected in any of the healthy repertoires. We think their absence is not a coincidence, but rather due to negative selection on 5-1 and 5-2 because of intrinsic autoreactivity. While some V_L genes were not expressed in any repertoire, there was variability in which V_L genes were not expressed between individuals. This variability may reflect inter-individual differences in negative selection.

We also have evidence for positive selection on L-chains based on high expression of V_L genes. A set of V_L genes were uniformly highly expressed without H-chain restriction, and this is consistent with selection of particular L-chains rather than clonal selection. It is not obvious what selective forces might govern expression of these genes, but they may be L-chains that have intrinsic specificity for self-antigens. Such specificities are found at high frequencies in the repertoire and are thought to play an important housekeeping role by clearing immune complexes (Radoux et al., 1986; Shlomchik et al., 1987; Shlomchik et al., 1986). These consistently highly expressed V_L genes may perform a similar function. Among the V_L genes with high expression in all repertoires was $V_K A27$. This is in agreement with other studies that have shown a high frequency of A27 in the repertoire (de Wildt et al., 1999; Foster et al., 1997; Jacobi et al., 2002). However, while A27 was the most frequent V_K in these studies, we found other V_L genes had consistently higher expression than A27. This discrepancy is likely due to differences in approaches and methods. While

these other studies used a limited number of B cells from few individuals, our method sampled a large number of B cells from many individuals. We also avoided potential bias introduced by culture and PCR efficiency. For these reasons, our analysis provides a more comprehensive understanding of positive selection on L-chains.

Positive selection in the form of clonal expansion also contributes to the repertoire. Our repertoire analysis was performed on populations of B cells expressing κ or λ L-chains and included sub-populations of memory and plasmablast B cells. We detected concomitant H-chain restriction by spectratyping and high expression of V_L genes. This pattern is consistent with clonal expansion.

Repertoire selection operates differently in κ and λ B cells, and our data show that the λ repertoire was more restricted than the κ repertoire. This restriction can be attributed to the order and timing of recombination. Initial recombination occurs at *IGK*, and only if recombination of *IGK* fails will *IGL* undergo recombination (Belessi et al., 2005; van der Burg et al., 2001). While B cells have the potential to undergo many recombination events, the actual number of attempts at recombination is limited and exhaustive recombination does not occur (Louzoun et al., 2002; Mehr et al., 1999). Because of these features of B cell development, receptor editing occurs predominantly at *IGK* and may be responsible for the less restricted repertoire observed in κ B cells. Differences in the organization of the *IGK* and *IGL* loci may also contribute to increased restriction in λ B cells. The *IGK* locus can be inactivated by recombination to *IGKDE* (the deletion element) while the *IGL* locus lacks this feature (Delpy et al., 2004; Klobeck and Zachau, 1986; Nemazee and Weigert, 2000; Siminovitch et al., 1985). Thus, recombination at *IGL* may result in a receptor that cannot be deleted. Receptor editing is consequentially different between κ and λ B cells as a result of these features, and the repertoires we analyzed here reflect this difference.

4.2. Transcription of V_k genes in lambda B cells

We detected V_k gene expression in λ B cells but did not detect $V\lambda$ expression in κ B cells. Three types of V_L gene transcripts have been reported: rearranged V_L - J_L mRNA which is in-frame and produces a functional L-chain; rearranged V_L - J_L mRNA which has a premature nonsense codon – either because recombination introduces the nonsense codon or results in the J_L - C_L being out-of-frame; and sterile transcription of unrearranged V_L genes (Delpy et al., 2004; Staudt and Lenardo, 1991). The absence of a surface κ L-chain excludes the possibility that the observed V_k expression in $V\lambda$ -sorted cells is a functional L-chain protein. The nonsense-mediated decay pathway is efficient in degrading transcripts with a nonsense codon at the V-J junction. Over 90% of out-of-frame κ transcripts are degraded by this mechanism (Chemin et al.). The impact of these non-functional messages on the expression level is likely to be negligible for several reasons: 1) in both κ and λ samples, a large number of V_L genes were not expressed, implying that aberrant rearrangement of V_L genes is relatively infrequent; 2) as compared with λ message levels, κ message levels in λ B cells was low; and 3) nonsense-mediated decay is efficient.

The V_k gene *A3/A19* is unique as it is highly expressed in both κ and λ B cells. This pattern of expression is consistent with sterile transcription of *A3/A19*. Sterile transcription is a phenomenon associated with κ locus activation and is a surrogate for recombination. This

observation suggests that the κ locus continues to prepare for recombination despite a B cell having a functional λ L-chain. If the κ -locus is deleted before recombination begins at λ (as is typical of λ -expressing cells) (Belessi et al., 2005; Brauninger et al., 2001; Hieter et al., 1981; Klein et al., 2005; Korsmeyer et al., 1981; Korsmeyer et al., 1982; van der Burg et al., 2001), then expression of any $V\kappa$ would be unexpected. However, deletion at the κ locus is primarily $C\kappa$ deletion. Thus, some $V\kappa$ genes are likely to remain in λ B cells. It should also be remembered that this analysis was performed on a population of B cells, and it would be expected that at least some λ B cells retain the *A3/A19* $V\kappa$ gene. Rearranged and functional L-chains using *A3/A19* have been reported (Bensimon et al., 1994; Dorner et al., 1998; Girschick and Lipsky, 2002; Yurasov et al., 2006; Yurasov et al., 2005). We expect that some of the measured *A3/A19* is functional, although the proportion of functional transcripts is unknown.

4.3. Proximity of V_L to J_L

We asked whether V_L gene position affects expression. $V\kappa$ genes are organized in two clusters, and $V\lambda$ are organized in three clusters in humans (Fripiat et al., 1995; Kawasaki et al., 2001; Kawasaki et al., 1995). We analyzed V_L gene expression according to position and found V_L genes from each cluster were highly expressed. Each cluster also had V_L genes that were not expressed (Figure 5). The median cluster expression of V_L genes was highest for the J-proximal clusters (Figure 5). There was no difference between healthy and SLE groups in expression levels according to clusters. J-proximal V_L genes have been reported to be expressed at higher levels than distal V_L genes (Girschick and Lipsky, 2002; Lee et al., 2004; Richl et al., 2008). However, expression was not dictated by position as high and low expression of V_L genes from all clusters was observed. Therefore positive and negative selection on L-chains appears to be more important in shaping the repertoire than V_L gene proximity to J-genes.

4.4. Repertoire selection in SLE

Previous work in our lab focused on regulation of auto-antibodies in mice and identified several V_L genes important for tolerance in the 56R H-chain transgenic model (Doyle et al., 2006; Li et al., 2001; Li et al., 2004; Sekiguchi et al., 2006). These studies demonstrated that editor V_L genes are expressed differently in lupus-susceptible and lupus-resistance mice. While our present analysis evaluated all V_L genes, we were particularly interested in the expression of V_L genes that are homologous to those identified as editors in the mouse B cell repertoire. $V\lambda$ 5-1, the human counterpart to mouse editor $V\lambda X$, was identified in our screen of the SLE group as over-expressed. These two $V\lambda$ s are more similar to each other than to any other human or mouse V_L genes (Solomon et al., 1997). The consequence of $V\lambda X$ expression is context-dependent and somewhat paradoxical because while it vetoes 56R H-chain's DNA reactivity, it also forms poly-reactive auto-antibodies with this and other H-chains (Doyle et al., 2006). Assuming that 5-1 has similar potential to generate poly-reactive antibodies as does $V\lambda X$, this feature of 5-1 expression is likely to be disadvantageous in establishing tolerance. Expression of 5-1 in SLE repertoires may give rise to incompletely edited or poly-reactive antibodies that are common in SLE (Yurasov et al., 2005).

The V_L gene repertoires in SLE patients were similar to repertoires from healthy individuals by some measures, but subtle differences exist. Repertoires in SLE patients show signs of positive and negative selection on L-chains as indicated by high V_L gene expression and absent expression of other V_L genes, respectively. The number of V_L genes undergoing positive and negative selection was similar in SLE patients and healthy individuals; no global defects in positive or negative selection were seen in the SLE group. We observed considerable inter-individual diversity in V_L gene expression in the SLE group that was also present in the healthy group. However, there are notable differences in the V_L gene repertoires between the healthy and SLE groups. There was greater variation in expression levels of V_K genes in SLE patients than healthy individuals. V_L genes that were not expressed in any healthy individual were expressed in some SLE patient repertoires. This is likely due to differences in negative selection between healthy individuals and SLE patients. A set of V_L genes were expressed at different levels between the healthy and SLE groups. We acknowledge that the differences were modest but conclude that V_L genes are selected differently in those without autoimmune disease than in individuals with autoimmune disease. Small differences in selection may not lead to an overwhelming repertoire shift but may lead to the appearance of disease-causing B cells and antibodies.

We have been able to study V_L gene expression in a large number of individuals and different populations of B cells because of a novel approach to repertoire analysis. The results provide insight into selection on L-chains and identified differences in diseased repertoires. Using the V_L gene microarray to study subsets of SLE patients and additional B cell populations will continue to improve our understanding of selection and the role of L-chains in autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

L-chain	Light chain
H-chain	Heavy chain
V_L gene	Light chain V gene
SLE	Systemic lupus erythematosus

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Highlights

- We developed a microarray to study B cell receptor light chain V gene repertoires
- Negative and positive selection acts on light chains to shape the B cell repertoire
- K and λ B cells are selected differently
- Certain light chain V genes are expressed at different levels in SLE patients

A

B

Figure 1.

FACS plot and phenotype of healthy and SLE B cells. (A) Peripheral blood cells were sorted into κ samples (CD20+CD138- κ + λ -) and λ samples (CD20+CD138- κ - λ +) based on the gating strategy shown at the top from a representative sample (Healthy01). A CD27 versus CD38 plot is also shown. (B) Quantification of FACS data for κ to λ ratios, CD38+ and CD27+ B cells. Both the ratio of κ -to- λ and the percentage of CD38+ were elevated in SLE (p-value <0.05), while there was no difference in the percentage of CD27+ B cells.

Figure 2.

Distribution of V_L gene expression levels in healthy individuals. The range of expression level estimates was split into 1% intervals and the number of V_L genes with expression within each 1% interval was counted. The x-axis indicates the interval (i.e. 0.10 indicates expression at levels between 10% and 11%) and the y-axis indicates that number of V_L genes in a given interval. The κ repertoires are shown on the left and the λ repertoires are shown on the right.

Figure 3.

Heavy chain spectratype analysis. (A) Two different H-chain spectratype patterns, Healthy01-κ (left) and Healthy02-κ (right). (B) The polyclonal control peak heights were subtracted from the sample peak heights at the corresponding lengths. The standard deviation of these differences within a sample was then determined as a measure of restriction (standard deviation is plotted). The only significant difference was between the Healthy-κ and SLE-κ groups ($p < 0.05$).

Figure 4.

Transcription of V_{κ} genes in λ -sorted B cells. B cells sorted on the basis of surface κ or λ protein were compared for expression levels of V_L genes. The V_L genes are grouped according to κ or λ from each sample. The two samples are from Healthy01. One V_{κ} (A3/A19) in the λ B cell population is expressed at a level that is higher than any V_{λ} .

Figure 5.

Expression levels by cluster and position. Average expression of V_L genes per cluster, (A) V_{κ} clusters and (B) V_{λ} clusters, was calculated for each individual and is shown for both healthy and SLE groups. Red line indicates the median for each group. For the κ clusters average expression was different between proximal and distal clusters for both healthy and SLE groups (p-value less than 0.01). There was no difference between groups. The average expression was different for the λ clusters for both healthy and SLE groups (p-value <0.01, tested using one-way ANOVA), but there was no difference between groups. Expression levels for each V_L gene are shown according to position for Healthy01 κ genes (C) and λ genes (D).

Table 1

List of V_L genes not expressed in any healthy repertoires. The number of entries in the NCBI database is also indicated (over 93,000 total entries).

V κ genes		V λ genes	
Name	Evidence of expression in NCBI database	Name	Evidence of expression in NCBI database
A10	1	1-9	0
L14	3	3-2	>100
O11a	0	3-3	2
		4-2	6
		4-3	0
		5-1	11
		5-2	16

Table 2

L-chain V genes not detected in any of the SLE repertoires.

V κ genes		V λ genes	
Name	Number of sequences in NCBI database	Name	Number of sequences in NCBI database
A14	0	1-9	0
L1	8	4-3	0
		4-6	0
		5-2	16

Table 3Differences in V_L gene expression between healthy and SLE groups.

Gene name	κ or λ	Average expression in healthy	Average expression in SLE	F ₁ p-value	F ₃ p-value
Higher expression in healthy repertoires					
B3	κ	4.0%	1.9%	0.067	0.002
L1	κ	0.7%	Not detected	0.047	0.113
1-2	λ	14.5%	9.6%	0.015	0.089
1-3	λ	14.3%	9.2%	0.041	0.082
4-1	λ	5.9%	2.4%	0.049	0.025
Higher expression in SLE repertoires					
A3/A19	κ	23.4%	26.9%	0.067	0.002
A27	κ	8.4%	12.8%	0.083	0.029
O11a	κ	Not detected	0.5%	0.022	0.041
2-8	λ	2.0%	8.3%	0.025	0.001
3-2	λ	Not detected	<0.3%	0.024	0.094
5-1	λ	Not detected	1.1%	0.008	0.001