# Circulating human B cells that express surrogate light chains and edited receptors

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**Immunoglobulin gene recombination can result in the assembly of self-reactive antibodies. Deletion, anergy or receptor editing normally silence B cells that produce these autoantibodies. Receptor editing is highly efficient in mouse B cells that carry pre-recombined autoantibody transgenes or gene "knock-ins". However, it has been difficult to identify cells that have edited receptors in unmanipulated mice and humans. To try to identify such cells we isolated and characterized B cells that coexpress surrogate and conventional light chains (V-preB$^+$L$^+$) from the blood of normal human donors. V-preB$^+$L$^+$ B cells express RAG mRNA, display an unusual heavy and light chain antibody repertoire consistent with antiself reactivity, and show evidence of receptor editing. These cells accumulate in the joints of patients with rheumatoid arthritis, consistent with a role for V-preB$^+$L$^+$ B cells and receptor editing in autoimmune disease.**



B cell tolerance is maintained by a combination of deletion, anergy and receptor editing[1–3]. In the mouse bone marrow, self-reactive B cells are induced to express recombinase-activating genes (RAGs) and edit their receptors in the late stages of B cell development[4,5]. Receptor editing was discovered in mice that carry autoreactive antibody transgenes[6–8]. In the presence of the autoantigen, secondary V(D)J recombination replaces the antibodies that react with self [9–11].

At the heavy chain locus, incoming $V_H$ genes recombine with pre-existing V(D)$J_H$s by using cryptic recombination signal sequences (RSSs) embedded in the V(D)$J_H$[12–14]. In contrast, incoming $V_\kappa$ genes delete pre-existing $V_\kappa J_\kappa$ genes during light chain editing by recombining with downstream $J_\kappa$s[15]. Therefore, light chain editing results in increased utilization of downstream $J_\kappa$s, and heavy chain editing produces hybrid $V_H$ genes.

In transgenic mice, receptor editing is a highly efficient process that can occur at both the immunoglobulin heavy and light chain loci[9,10,13,14]. Less is known about receptor editing and the mechanisms that prevent the development of self-reactive B cells in normal mice and humans. Hybridoma analysis has shown that editing may occur frequently in the 5% of mouse B cells that express Ig$\lambda$[16]. In humans, evidence for receptor editing at the heavy chain locus is restricted to the finding of rare hybrid $V_H$ genes that could be the products of editing or of gene conversion[17]. Attempts to find receptor editing at the light chain locus in normal human B cells have been either unsuccessful[18], or limited to B cell clones[19,20].

Editing appears to be more frequent in the setting of autoimmunity. There is a higher incidence of editing in MRL-*lpr*/*lpr* mice and this is associated with increased autoantiobody production[21]. In humans the data is limited to a case report of a patient with systemic lupus erythematosis who showed increased $J_\kappa 5$ and $J_\lambda 7$ on both Ig$\kappa$ and Ig$\lambda$ loci[22,23].

A subpopulation of B cells in human tonsil expresses RAG, TdT and surrogate light chains V-preB and $\lambda$-like (also called $\lambda 5$)[19,24,25]. Although the precise nature of the RAG-expressing cells in the tonsil was not determined, these cells are enriched in germinal center (GC) B cell fractions; immunohistochemistry revealed V-preB–expressing B cells in some GCs and in the T cell zone[24].

Here, we report on the initial characterization of circulating human B cells that coexpress V-preB and conventional light chains. We find that these cells have an unusual immunoglobulin repertoire resembling autoantibodies and that they show evidence of receptor editing.

## Results

### RAG mRNA expression in circulating V-pre-B$^+$L$^+$ B cells

We examined human peripheral blood for B cells coexpressing conventional and surrogate light chains using a monoclonal antibody (mAb) to V-preB (anti–V-preB)[26]. Of all peripheral B cells that express conventional light chains 0.5–1% stained with anti–V-preB and there was no significant variation with age (1-day-old to 92-year-old normal donors, $n$=55). To further characterize V-preB$^+$L$^+$ B cells we enriched this subpopulation with anti–V-preB on magnetic beads. V–preB$^+$L$^+$ B cells resembled conventional B cells in that they expressed CD19, CD20, CD40, CD22, CD24 and CD72, and they

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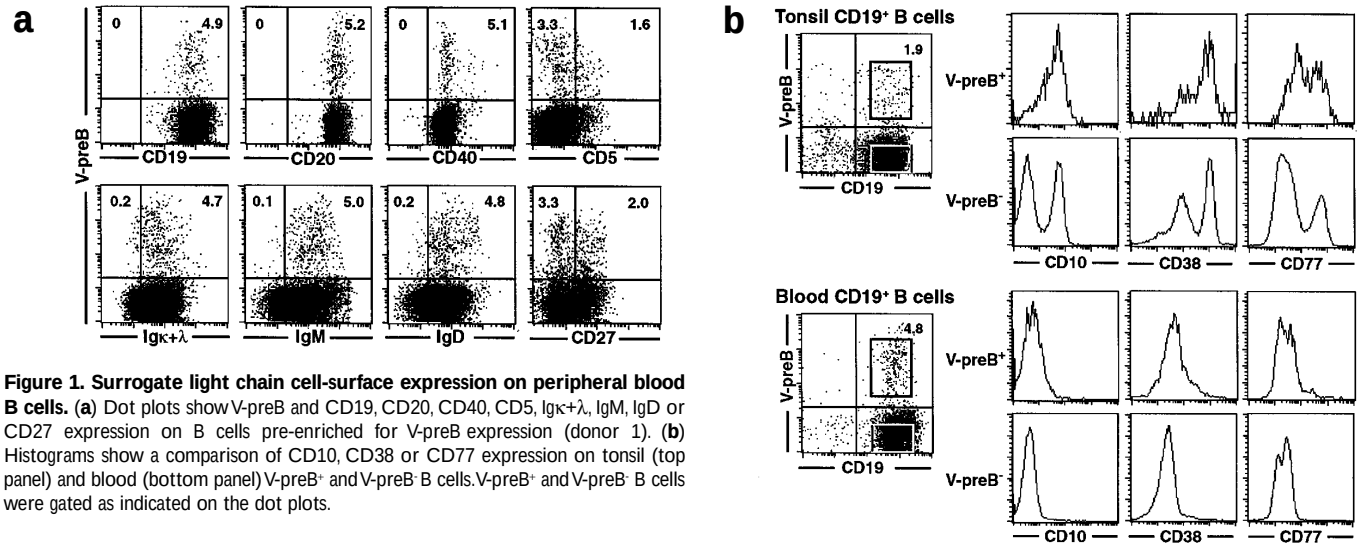


**Figure 1. Surrogate light chain cell-surface expression on peripheral blood B cells.** (**a**) Dot plots show V-preB and CD19, CD20, CD40, CD5, Igκ+λ, IgM, IgD or CD27 expression on B cells pre-enriched for V-preB expression (donor 1). (**b**) Histograms show a comparison of CD10, CD38 or CD77 expression on tonsil (top panel) and blood (bottom panel) V-preB$^+$ and V-preB$^-$ B cells. V-preB$^+$ and V-preB$^-$ B cells were gated as indicated on the dot plots.

were heterogeneous for CD5 and CD27 expression (**Fig. 1a** and also data not shown). The proportion of CD5- and CD27-expressing cells was similar in V-preB$^+$L$^+$ and conventional B cells. V-preB$^+$L$^+$ B cells also expressed IgM and IgD, Igκ or Igλ and their Igκ/λ ratio was similar to conventional B cells (**Fig. 1a** and data not shown). We conclude that V-preB$^+$L$^+$ B cells resemble conventional B cells in their cell surface markers.

V-preB$^+$L$^+$ are found in tonsils, in the T cell zone at the periphery of the B cell follicle, and in some GCs[24]. To determine whether peripheral V-preB$^+$L$^+$ B cells are circulating GC B cells or precursors from the bone marrow, we examined them for expression of CD10 and CD38 (which are found on both GCs and developing B cells), and CD77 (which is uniquely expressed on GC centroblasts)[27,28]. Tonsil V-preB$^+$L$^+$ B cells express CD10, CD38 and CD77 (**Fig. 1b** top, and see[24]). In contrast, blood V-preB$^+$L$^+$ B cells did not express CD10, CD38 or CD77 (**Fig. 1b** bottom). Thus, circulating V-preB$^+$L$^+$ B cells do not display the classical markers of bone marrow precursors or GC B cells and their surface features differ from those of V-preB$^+$L$^+$ B cells found in tonsils.

As RAG and surrogate light chains are frequently coexpressed we examined peripheral V-preB$^+$L$^+$ B cells for RAG mRNA and compared them to other peripheral B cell subpopulations: conventional B cells (CD19$^+$V-preB$^-$L$^+$), IgG$^+$ B cells (CD19$^+$IgM$^-$IgD$^-$IgG$^+$), B cells (CD19$^+$IgM$^-$IgD$^+$IgG$^-$)[29,30] and IgG$^+$ B cells (CD19$^+$IgM$^-$IgD$^-$IgG$^+$) and CD19$^+$IgM$^-$IgD$^-$IgG$^-$ B cells, which include B cell receptor–deficient (BCR$^-$) CD10$^+$CD38$^{high}$-circulating B cell precursors (**Fig. 2**). RAG-1, RAG-2, TdT, λ-like and V-preB were only expressed in V-preB$^+$L$^+$ B cells and in the fraction that contained BCR$^-$ B cell precursors (**Fig. 2b**). Expression of these mRNAs in V-preB$^+$L$^+$ B cells was two orders of magnitude less than that found in bone marrow–derived pro-B CD19$^+$CD34$^+$ or pre-B CD19$^+$CD34$^-$CD10$^+$IgM$^-$ cells, and one order of magnitude less than in immature B cells from bone marrow (**Fig. 2c**). We conclude that circulating V-preB$^+$L$^+$ B cells express low levels of RAG, TdT, λ-like and V-preB.

## RAG expression and transplant and XLA patients

Circulating immature B cells in mice express low levels of RAGs[31,32]. To determine whether V-preB$^+$L$^+$ B cells are recent bone marrow emigrants we analyzed B cells in humans receiving T cell–depleted bone marrow allografts after myeloablation ($n$ = 5). B cells first appeared in the blood of these patients 6–7 weeks after the transplant, and were enriched with anti-Igκ + anti-Igλ (anti-Igκ+λ) on magnetic beads and sorted for CD19 and Igκ+λ expression (**Fig. 3a**). Most of these newly generated B cells coexpressed IgM and CD10,

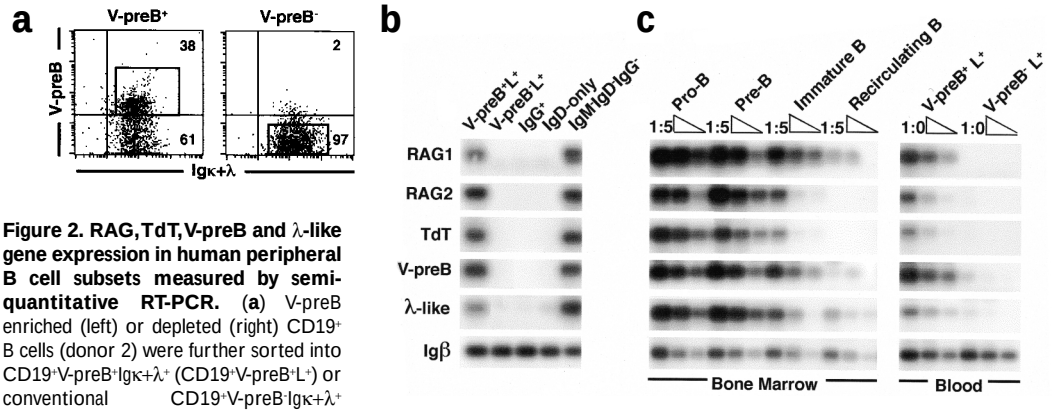


**Figure 2. RAG, TdT, V-preB and λ-like gene expression in human peripheral B cell subsets measured by semi-quantitative RT-PCR.** (**a**) V-preB enriched (left) or depleted (right) CD19$^+$ B cells (donor 2) were further sorted into CD19$^+$V-preB$^+$Igκ+λ$^+$ (CD19$^+$V-preB$^+$L$^+$) or conventional CD19$^+$V-preB$^-$Igκ+λ$^+$ (CD19$^+$V-preB$^-$L$^+$) B cells as indicated by the gated regions. The level of purity in the sorted V-preB$^-$ fraction is >99%. (**b**) Semiquantitative RT-PCR for RAG, TdT, V-preB and λ-like RNA extracted from FACS-sorted V-preB$^+$L$^+$CD19$^+$ (V-preB$^+$L$^+$), conventional V-preB$^-$L$^+$CD19$^+$ (V-preB$^-$L$^+$), IgM$^-$IgD$^-$IgG$^+$CD19$^+$ (IgG$^+$); IgM$^-$IgD$^+$IgG$^-$CD19$^+$(IgD-only); and IgM$^-$IgD$^-$IgG$^-$ CD19$^+$ B lymphocytes. Igβ RT-PCR was used as a B cell specific mRNA loading control. PCR assays were designed to distinguish between genomic DNA and mRNA using primers on different exons. (**c**) Comparison of RAG, TdT, V-preB and λ-like RNA levels in human bone marrow or peripheral B cell subsets. Serial fivefold dilutions of cDNA are shown; the dilutions of cDNA for CD19$^+$CD34$^+$ (pro-B), CD19$^+$CD34$^-$CD10$^+$IgM$^-$ (pre-B), CD19$^+$CD34$^-$CD10$^+$IgM$^+$ (immature B) or CD19$^+$CD34$^-$CD10$^-$IgM$^+$ (recirculating B) bone marrow cell groups were 1:5, 1:25 and 1:125 respectively. The first dilution of all blood samples was 1:0 and serial fivefold dilutions are shown. Igβ PCR is shown as loading control.

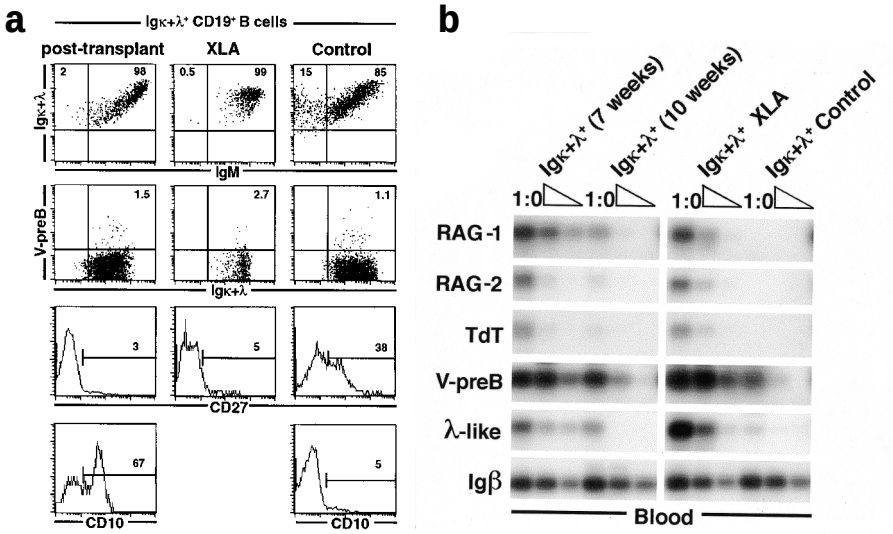


**Figure 3. RAG-1, RAG-2, TdT and surrogate light chain gene expression in newly emigrated B cells.** (**a**) Enriched Igκ+λ+ B cells from 7-week post-transplant patients (left), XLA patients (middle) and controls (right). Dot plots show Igκ+λ and IgM expression (top) and V-preB and Igκ+λ expression (bottom). Histograms show CD27 and CD10 expression on Igκ+λ+CD19+ B cells. (**b**) Semiquantitative RT-PCR for RAG, TdT and surrogate light chain gene expression on Igκ+λ+ CD19+ B cells purified from the blood of patients 7 or 10 weeks post bone marrow transplant, XLA patients and controls.

which are found on developing B cells in the bone marrow. They did not express CD27, which is a marker of memory B cells (**Fig. 3a**)[30,33]. In contrast, 30–50% of the control B cells expressed the CD27 memory B cell marker and they did not express CD10 (Fig. 3a). These newly generated B cells contained RAG-1, RAG-2, TdT and surrogate light chain mRNA whereas control B cells obtained from the blood of normal individuals did not (**Fig. 3b**, $n$=15). The RAG mRNA levels expressed by the newly generated B cells were lower than those found in pro- and pre-B cells and similar to the levels of RAG expression found in V-preB$^+$L$^+$ B cells (**Figs 2c, 3b**). Consistent with a decreasing proportion of immature B cells in blood in later stages of bone marrow reconstitution, RAG, TdT and surrogate light chain mRNA levels decreased 9–10 weeks after transplant, and were not detectable 11–12 weeks after transplant (**Fig. 3b** and data not shown).

To confirm that newly generated human B cells expressed RAG-1, RAG-2, TdT and surrogate light chain mRNA we analyzed B cells from the blood of patients with X-linked agammaglobulinemia (XLA, $n$=4). These patients have few peripheral blood B cells, most of which are thought to be recent bone marrow emigrants[34]. Mature B cells do not accumulate in the periphery in XLA patients and there are no CD27$^+$ memory B cells (**Fig. 3a**, and see[34]). B cells purified from the blood of XLA patients contained levels of RAG, TdT and surrogate light chain mRNA similar to those found in B cells 7 weeks after bone marrow grafting and in V-preB$^+$L$^+$ B cells (**Figs 2c, 3b**). We concluded that human RAG-1, RAG-2, TdT and surrogate light chain expression was not completely terminated in the bone marrow and that these genes were expressed at low levels in newly generated B cells emigrating to the periphery.

Only a small fraction of Igμ-positive pre-B cells that express surrogate light chain mRNA displayed cell surface surrogate light chains[25,35–37]. To determine whether new bone marrow emigrants express cell surface V-preB we examined B cells from bone marrow transplant and XLA patients for V-preB expression (**Fig. 3a**). B cells from bone marrow transplant recipients and XLA patients resembled control B cells in that only 0.5–2.5% of the B cells were V-preB$^+$L$^+$ (**Fig. 3a**). We concluded that cell surface V-preB expression overlaps with, but is not a marker for, new bone marrow emigrants. Further, expression of V-preB in immature B cells resembles V-preB expression in pre-B cells in that mRNA synthesis is not directly correlated with protein expression on the cell surface[25,35–37].

## Distinct V(D)$J_H$ rearrangements in V-preB$^+$L$^+$B cells

To characterize the recombination of antibody genes in V-preB$^+$L$^+$ B cells we cloned and sequenced $V_H$5 gene family rearrangements[38]. With the exception of $J_H$3 and $J_H$6, which are over- and underrepresented respectively, $J_H$ usage by $V_H$5 gene family members was similar to that reported for unfractionated $V_H$S[39,40].

In three donors we found that V(D)$J_H$ rearrangements in V-preB$^+$L$^+$ B cells were skewed to downstream $J_H$s when compared to conventional B cells[39,40]. In the three individuals examined, $J_H$6 usage increased from 7%, 6%, 13% in conventional B cells to 25%, 19%, 41% in V-preB$^+$L$^+$ B cells ($n$=136; $P$<0.001, **Fig. 4a**). Consistent with the increased frequency of tyrosines encoded by $J_H$6, we found that V-

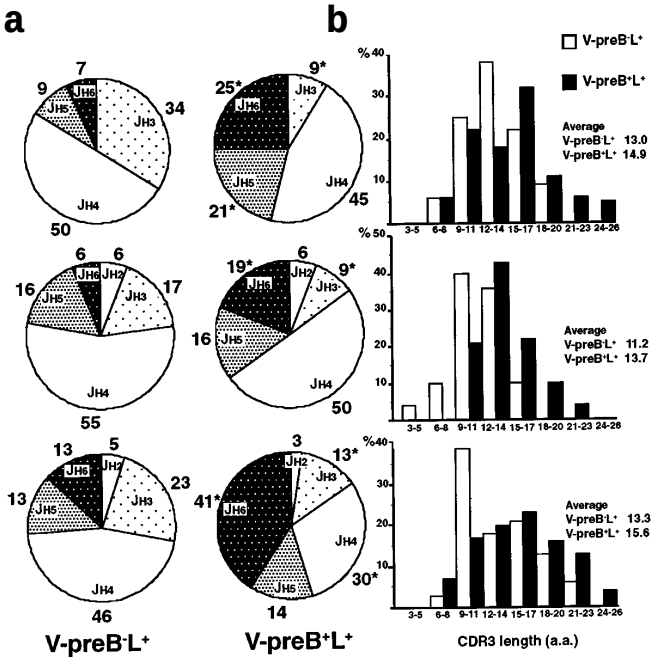


**Figure 4. Characterization of $V_H$5(D)$J_H$ expression in conventional and V-preB$^+$L$^+$ B cells.** (**a**) $J_H$ usage in conventional (V-preB$^-$L$^+$) and V-preB$^+$L$^+$ B cells from three unrelated normal donors. 102 conventional (32 from donor 1, top; 31 from donor 2, middle; and 39 from donor 3, bottom) and 136 V-preB$^+$L$^+$ (67 from donor 1, top; 32 from donor 2, middle; and 37 from donor 3, bottom) $V_H$5(D)$J_H$ sequences were analyzed. Percentage $J^H$ usage is indicated. (*$P$<0.001). (**b**) $V_H$5(D)$J_H$ CDR3 length in circulating conventional and V-preB$^+$L$^+$ B cells. The average CDR3 length for V-preB$^-$ cells and V-preB$^+$ cells was 12.6 aa and 14.8 aa, respectively ($P$=0.01).

preB$^+$L$^+$ B cell CDR3s were enriched in tyrosine residues (17.5% compared to 11.7% in conventional B cells, $P$<0.001). In addition, V-preB$^+$L$^+$ B cells have longer IgH CDR3s than conventional B cells ($P$<0.01; **Fig. 4b**). Again, increased $J_H$6 usage is in part responsible for this phenomenon as $J_H$6 has the longest $J_H$ coding sequence. However, we also found that 14 out of 136 V(D)$J_H$s contained D-D rearrangements, nine of which strictly conformed to the criteria defined by Corbett *et al.* (**Table 1**)[41]. D-D recombination is prohibited by the 12/23 rule, is rarely reported, and was not found in the V(D)$J_H$ sequences obtained from conventional B cells (**Table 1**)[41,42]. Thus, V-preB$^+$L$^+$ B cells display a specific IgH repertoire with an increased frequency of $J_H$6 usage, long CDR3s and frequent D-D rearrangements. These unusual features are characteristic of autoreactive antibodies in humans and mice[43–45] .

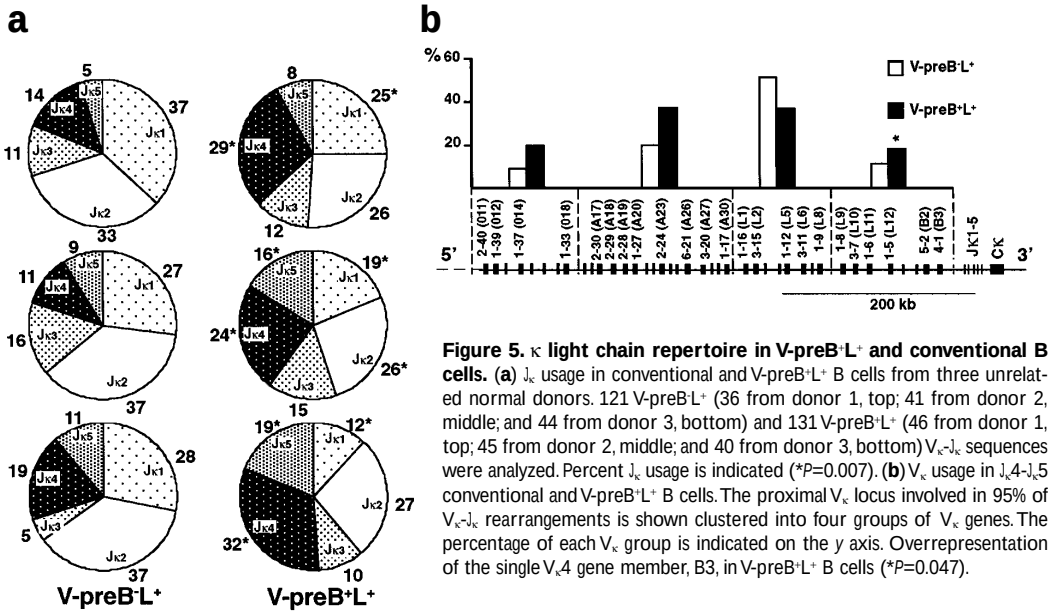


**Figure 5. κ light chain repertoire in V-preB$^+$L$^+$ and conventional B cells.** (**a**) $J_κ$ usage in conventional and V-preB$^+$L$^+$ B cells from three unrelated normal donors. 121 V-preB$^-$L$^+$ (36 from donor 1, top; 41 from donor 2, middle; and 44 from donor 3, bottom) and 131 V-preB$^+$L$^+$ (46 from donor 1, top; 45 from donor 2, middle; and 40 from donor 3, bottom) $V_κ$-$J_κ$ sequences were analyzed. Percent $J_κ$ usage is indicated (*$P$=0.007). (**b**) $V_κ$ usage in $J_κ$4-$J_κ$5 conventional and V-preB$^+$L$^+$ B cells. The proximal $V_κ$ locus involved in 95% of $V_κ$-$J_κ$ rearrangements is shown clustered into four groups of $V_κ$ genes. The percentage of each $V_κ$ group is indicated on the $y$ axis. Overrepresentation of the single $V_κ$4 gene member, B3, in V-preB$^+$L$^+$ B cells (*$P$=0.047).

Although most of the V(D)$J_H$ sequences obtained from V-preB$^+$L$^+$ B cell did not contain somatic mutation, 15–20% of the $V_H$ genes in either the conventional or the V-preB$^+$L$^+$ B cell subpopulations had two or more mutations (data not shown). The mutation frequency in both fractions was similar, with an average of 8.3–11.2 mutations per sequence (2.9–3.7% somatically mutated nucleotides) in conventional B cells *versus* 6.7–8.3 mutations per sequence (2.3–2.8%) in V-preB$^+$L$^+$ B cells (data not shown). We conclude that V-preB surface expression is not restricted to naïve B cells.

## 3′-biased $J_κ$ usage in V-preB$^+$L$^+$ B cells

Continued RAG expression has been associated with secondary V(D)J recombination[4,5,24,46–51]. At the Igκ locus secondary V(D)J recombination involves the replacement of $V_κJ_κ$s by deletion, and therefore editing should bias the repertoire to more downstream $J_κ$s. To determine whether RAG expression in V-preB$^+$L$^+$ B cells has functional consequences for antibody gene recombination, we examined $J_κ$ usage in these cells. Igκ sequences were obtained by amplifying mRNAs by reverse transcriptase polymerase chain reaction (RT-PCR) using a 5′ consensus $V_κ$ primer and a 3′ $C_κ$ primer. Consistent with previous reports, conventional B cells preferentially use $J_κ$1 and $J_κ$2 segments ($n$=121 independent sequences from three normal donors, **Fig. 5a**) [52]. In contrast, V-preB$^+$L$^+$ B cells derived from the same three donors ($n$=131 independent clones) showed decreased $J_κ$1 and $J_κ$2 usage, and increased downstream $J_κ$4 and $J_κ$5 segment usage (**Fig. 5a**). In conventional B cells $J_κ$4-$J_κ$5 rearrangements accounted for 23±5% of all Igκ, whereas in V-preB$^+$L$^+$ B cells, $J_κ$4-$J_κ$5 rearrangements accounted for 43±6% ($P$=0.007). $J_κ$4-$J_κ$5 rearrangements in V-preB$^+$L$^+$ B lymphocytes displayed a bias toward more upstream $V_κ$ gene segments but this was not statistically significant, possibly because inversional recombination would preserve downstream $V_κ$s for further recombina-

**Table 1. D-D junctions in V(D)J rearrangements of V-preB$^+$L$^+$B cells**

| | | CDR3 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Clone | $V_H$ | N/P | D | N/P | D | N/P | $J_H$ | Segments |
| 2H11° | GCGAGA | CGGGAT | GATATTGTAGTAGTACCAGCTGCTAT | ACACAA | CGGGGAG | CG | TGATGCTTTTGATATCTGG | D2-2 D3-10 JH3 |
| 7V10 | GCGAGA | CTGGGGCCCGC | GGTGGTAGCTGC | CGGGTATGCGG | TGACTACAG | AAGGAGAGGG | TGATGCTTTTGATATCTGG | D2-15 D4-17 JH3 |
| 2H16 | GCGAGA | CTCCCAGAG | GCAGCAGCTG | CCTC | AGTGGGAGC | CGAGGGGTTTGAA | GACTACTGG | D6-13 D1-26 JH4 |
| 2HB16 | GCGAGA | CAGGAGGAAAAAG | ATTTTTGGAGTGGTTATT | CCCTC | CACACGGCGCC | AAGCCCGGACCG | CTACTTTGACTACTGG | D3-3 DIR1 JH4 |
| 2H17° | GCGAGA | CGGG | GGGGGAG | GTATTACTATGATAGTAGTGG | | ATCTCGA | TTTGACTACTGG | D3-16 D3-22 JH4 |
| 6V2 | GCGAGA | ACGACCATAAGCAA | TGACTACGGTGACTAC | ACCAA | TGACTACG | CCT | ACTACTTTGACTACTGG | D4-17 D4-23 JH4 |
| 6V48 | GCGAG | GGCACGTGGG | TATGATTATG | GC | AGTGGTAATTA | TCAGGTGTAT | TACTTTGACCAATGG | D3-16 D3-22 JH4 |
| 7V16 | GCGACA | CTTTTT | GGTATAG | | TACAGCTATGG | GAA | AACTGGTTCGACCCCTGG | D6-13 D5-18 JH5 |
| 2H8 | GCGAGA | CAAA | GGTTCGGGGA | T | ATAGCAGCAGC | GGCTA | ACTACTACTACGGTATGGACGTCTGG | D3-10 D6-13 JH6 |
| 1P3 | GCGAGA | CC | GGATATTGTAGTAGTACCAGCTGCT | CCGTG | GGATACAGCTATGG | CCCCGGGTAA | CTACTACTACTAC---ATGGACGTCTGG | D2-2 D5-5 JH6* |
| 6V11° | GCGAGA | | CGGGGAG | | ACGGTGACTAC | GGGGG | CTACTACTACTACGGTATGGACGTCTGG | D3-10 D4-17 JH6 |
| 7V1° | GCGAGA | GCAAGTCGGCCGTA | CTATGGTT | CAGGGGGGAAC | TTCGGGGA | | CTACTACTAC---ATGGACGTCTGG | D5-5 D3-10 JH6* |
| 7V6° | GCGAGA | CATCAGTT | TGACTACAGTAACTAC | | CCCCACCC | CCGAA | ACTACTACTACTACGGTATGGACGTCTGG | D4-4 DIR JH6 |
| 7V45 | GCGAGA | CAGTCATACC | ATTGTACTGGTGGTGTATGCTA | | TTTGGGGGATT | GGGCATAC | TACTACTACTACTAC---ATGGACGTCTGG | D2-8 D3-16 JH6* |

Emboldened nucleotides differ from reported germline sequences. *Indicates $J_H$6c, which shows deletion of three nucleotides. °Marks clones that do not strictly follow the Corbett *et al.* criteria.

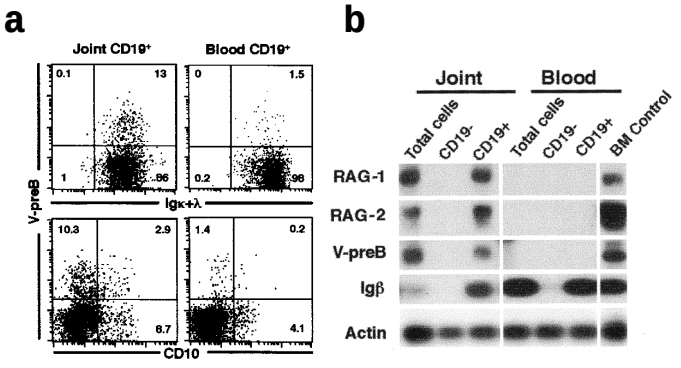


**Figure 6. V-preB$^+$L$^+$ B cells are overrepresented in rheumatoid synovia.** (**a**) Flow cytometric analysis of CD19$^+$ B cells from joint and blood of four patients with rheumatoid arthritis. Dot plots show V-preB, and anti-Igκ+λ, or anti-CD10 staining. Eight other patients showed no enrichment of V-preB$^+$L$^+$ in the joint. (**b**) Semiquatitative RT-PCR for RAG, TdT and V-preB gene expression in CD19$^+$ B cells from joint and blood of the patient depicted in **a**.

tion[53] (**Fig. 5b**). V-preB$^+$L$^+$ B cells also showed overrepresentation of a single member of the V$_\kappa$4 family, B3, in all three donors (**Fig. 5b** and data not shown, $P$=0.047) and a higher proportion of unusually long Igκ CDR3s (**Table 2**, $P$=0.04). We conclude that V-preB$^+$L$^+$ cells have unusually long Igκ CDR3s and a V$_\kappa$J$_\kappa$ repertoire skewed toward upstream V$_\kappa$s and downstream J$_\kappa$s consistent with increased secondary V(D)J recombination.

### V-preB$^+$L$^+$ B cell enrichment in rheumatoid synovia

B cells producing Igκ chains with unusually long CDR3s are frequently found in joints of patients with rheumatoid arthritis[54,55]. To determine whether V-preB$^+$L$^+$ B cells are enriched in rheumatoid synovia we compared blood and joint samples from patients with rheumatoid arthritis undergoing surgical joint replacement ($n$=12). Four of the 12 patients had increased numbers of V-preB$^+$L$^+$ B cells in their joints relative to their blood. In these four patients there was a 10- to 20-fold enrichment of V-preB$^+$L$^+$ B cells in the synovia. Of the CD19$^+$ B cells extracted from these four joints affected by rheumatoid arthritis, 5–15% expressed V-preB whereas only 0.5–1.5% of the peripheral blood B cells from the same patients were V-preB$^+$L$^+$ (**Fig. 6a**). V-preB$^+$L$^+$ B cells from these joints were heterogeneous for CD10 and CD38 expression (**Fig. 6a**, and not shown). Of the V-preB$^+$L$^+$ B cells in these joints, 20–30% were CD10$^+$CD38$^+$, similar to tonsil V-preB$^+$L$^+$ B cells, whereas the remainder were CD10$^-$CD38$^{low/-}$, as seen in blood. The presence of tonsil-like CD10$^+$CD38$^+$ V-preB$^+$L$^+$ B cells in joints affected by rheumatoid arthritis is consistent with the presence of GC-like structures in rheumatoid synovia[56]. B cells from rheumatoid synovia displayed RAG-1, RAG-2, TdT and V-preB mRNA similar to those found in V-preB$^+$L$^+$ B cells in blood and tonsil (**Fig. 6b**). In contrast, these mRNAs were not detected in B cells from joints of the remaining eight patients that did not contain V-preB$^+$L$^+$ B cells. We conclude that V-preB$^+$L$^+$ B lymphocytes are enriched in one-third of rheumatoid synovia in patients undergoing surgical joint replacement.

## Discussion

We have identified a group of human B cells that display features consistent with receptor editing, including persistent RAG expression, 3′-skewed J$_\kappa$ usage and an unusual antibody repertoire associated with self-reactivity. These cells are enriched in the joints of patients with rheumatoid arthritis.

In humans, three subpopulations of peripheral B cells express RAGs. These cells cofractionate with GC centrocytes in the tonsil and probably account for the high levels of RAG mRNA found in these fractions[19,24]. The highest RAG expression is found in a small number of peripheral B cells that are CD19$^+$Igμ$^-$ and Igκ+λ$^-$ and are likely to be circulating B cell progenitors. Two other groups of B cells in human blood express RAG mRNA: immature B cells and V-preB$^+$L$^+$ B cells and these are distinct populations based on CD10 and CD27 cell surface marker expression. In the mouse, a green fluorescent protein reporter gene was used to show that most of the RAG-expressing B cells in the periphery are either immature B cells or B cell progenitors[31,32]. Immature B cells in the mouse express low levels of residual RAG mRNA and do not reactivate RAG expression in the periphery[31,32]. Similar low levels of RAG mRNA were found in immature B cells and V-preB$^+$L$^+$ B cells in the human and RAG could not be induced in human B cells[24].

Why do V-preB$^+$L$^+$ B cells display cell surface V-preB whereas immature B cells that express similar levels of V-preB mRNA do not? Surrogate light chains bind to normal heavy chains at similar affinities[57]. However, when both are present, conventional light chains are expressed at a much higher level and therefore out-bind the heavy chain from surrogate light chains[25,35,37,57]. We propose that cell surface expression of surrogate light chains and conventional light chains in V-preB$^+$L$^+$ B cells is due to the unusual structure

**Table 2. V$_\kappa$ clones containing 11 amino acid–long CDR3 from V-preB$^-$ and V-preB$^+$L$^+$B cells**

| | | CDR3 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clone | V$_\kappa$ | 89 | 90 | 91 | 92 | 93 | 94 | 95 | GL | N/P | GL | 96 | 97 | Jκ |
| **V-preB$^-$** | | | | | | | | | | | | | | |
| 2K9 | L2 | C**C**A | CAC | T**G**T | AAT | AAC | TGG | CCT | CC | G GCA AC | - | --G | ACG | JK1 |
| 2K16 | L2 | CAG | CAG | TAT | AAT | AAC | TGG | CCT | CC | G CG | -G | TAC | ACT | JK2 |
| 2K10 | L2 | CAG | CAG | TAT | AAT | AAC | TGG | CCT | CC | C CT | A | TTC | ACT | JK3 |
| 6K23 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | G GG | G | ATC | ACC | JK5 |
| **V-preB$^+$** | | | | | | | | | | | | | | |
| 2K44 | L12 | CAA | CAG | TAT | AAT | AGT | TAT | TCT | CC | G CGG G | - | -GG | ACG | JK1 |
| 2K41 | B3 | CAG | CAA | TAT | TAT | AGT | ACT | CCT | C- | TC A | TG | TAC | ACT | JK2 |
| 7K13 | L2 | CAG | CAG | TAT | AAT | AAC | TGG | CCT | CC | C CC | -G | TAC | ACT | JK2 |
| 6K28 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | G A | TG | TAC | ACT | JK2 |
| 7K34 | A27 | CAG | CA**C** | TAT | GGT | A**C**C | TCA | C-- | -- | TC CCC A | TG | TAC | ACT | JK2 |
| 7K37 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | C A | TG | TAC | ACT | JK2 |
| 6K46 | 012 | CAA | CAG | AGT | TAC | AGT | ACC | CC- | -- | C CCC TT | A | TTC | ACT | JK3 |
| 7K30 | B3 | CA**A** | CAA | TA**C** | **AGT** | A**C**T | A**GT** | CCT | C- | TG GA | A | TTC | ACT | JK3 |
| 2K12 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | C CTT | - | CTC | ACT | JK4 |
| 2K26 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | G AGG G | - | -TC | ACT | JK4 |
| 2K40 | L6 | CAG | CAG | CGT | AGC | AAC | TGG | CCT | CC | G GGG T | - | -TC | ACT | JK4 |
| 7K46 | L6 | CAG | CAG | CGT | **AAT** | AGC | **G**AC | TGG | CCT | CC | G | CTC | ACT | JK4 |
| 6K12 | A27 | CAG | CAG | TAT | G**A**T | **TCT** | **GAT** | AGC | TCA | CC | G | ATC | ACC | JK5 |
| 6K24 | 018 | CAA | CAG | TAT | **C**AT | AAT | **G**TC | C-- | -- | GG GGA TC | G | ATC | ACC | JK5 |

Dashes indicate nucleotides removed by exonuclease. Emboldened nucleotides differ from reported germline sequences. Clones 7K46 and 6K12 shows an insertion of three and six nucleotides, respectively.

of the antibodies in these cells. The long CDR3s might interfere with normal heavy and light chain pairing, or alternatively favor pairing of surrogate light chains with the heavy chain.

What are the characteristics of circulating V-preB$^+$L$^+$ B cells, and how are they related to other B cells? Circulating V-preB$^+$L$^+$ B cells are found among immature B cells, mature B cells and memory B cells. They express many of the same cell surface markers as conventional B cells. However, these cells do not appear to be either circulating bone marrow precursors or GC cells. Circulating V-preB$^+$L$^+$ B cells may be the precursors of the tonsil V-preB$^+$L$^+$ B cells, which constitute up to 11% of the B cells in the tonsil[24]. But, unlike circulating V-preB$^+$L$^+$ B cells, tonsil V-preB$^+$L$^+$ B cells express CD10, CD38 or CD77 GC markers, which may be a reflection of activation and GC recruitment.

Do V-preB$^+$L$^+$ B cells contribute to antibody responses? Like conventional B cells, circulating V-preB$^+$L$^+$ B cells are heterogeneous for CD27, a memory B cell marker and 15–20% of the $V_H$ sequences obtained from these cells contain somatic mutations[30,33]. Therefore these cells can participate in immune responses and go through GC reactions[30,33]. However, this is a small subpopulation of only 0.5–1% of total peripheral B cells in healthy donors and although they may contain a disproportionate number of autoantibody producers (see below) V-preB$^+$L$^+$ B cells are likely to have only a limited role in shaping the normal peripheral B cell repertoire.

V-preB$^+$L$^+$ B cells are distinct in that in addition to RAG they also express an unusual group of heavy and light chains. Heavy chains expressed in these cells are biased toward downstream $J_H6$ segment. The $J_H6$ bias could result from secondary rearrangements between a cryptic V(D)$J_H$ RSS and $J_H6^{13}$. Alternatively, the $J_H6$ bias might simply be due to selection. Although it is difficult to rule out secondary IgH recombination, the idea that long CDR3s are being selected in the V-preB$^+$L$^+$ B cells is supported by the high frequency of D-D rearrangements and the long N nucleotide additions. These unusual CDR3s are typical of the immunoglobulin heavy chain repertoire in autoimmune prone MRL-*lpr*/*lpr* mice[43] and human polyreactive autoantibodies[44,45]. A second feature of polyreactive antibodies also shared by V-preB$^+$L$^+$ B cell antibodies is an increased number of aromatic residues such as tyrosine in CDR3[44,45]. This increase in aromatic residues is believed to increase the flexibility of the IgH CDR3 loop thereby enhancing antibody binding to a number of different self-antigens[58].

The light chains expressed in V-preB$^+$L$^+$ B cells are also remarkable in that they show increased usage of downstream $J_\kappa4$ and $J_\kappa5$ gene segments, long CDR3s, and overrepresentation of $V_\kappa4$ family member B3. $V_\kappa4$ family member B3 is overrepresented in patients with systemic lupus erythematosus and thought to encode antibodies to DNA[22,59], whereas long Igκ CDR3s are frequently found in B cells from joints of patients with rheumatoid arthritis[54,55]. Thus, the Igκ chains of the antibodies found in rheumatoid synovia resemble those produced by V-preB$^+$L$^+$ B cells and we speculate that the higher incidence of these cells in rheumatoid synovia may account for the increase in long Igκ CDR3s found in rheumatoid arthritis.

Long IgH and Igκ CDR3s in V-preB$^+$L$^+$ B cells may be result from selection, but it is difficult to attribute the shift in $J_\kappa$ usage and continued RAG expression to selection. In mouse models downstream-biased $J_\kappa$ usage and continued RAG expression are found in B cells undergoing receptor editing or receptor revision induced by autoreactive antibodies[5,9,10,15]. V-preB$^+$L$^+$ B cells are a distinct group of human B cells that display these same characteristics. Receptor editing in V-preB$^+$L$^+$ B cells is likely to occur in the bone marrow where RAG mRNA levels are the highest. Receptor revision in V-preB$^+$L$^+$ B cells in the periphery is not excluded, but is probably limited because of the low levels of RAG expression. B cells that bind self-antigen weakly can leave the bone marrow without being deleted and become anergic[9,11,60]. In autoimmune prone MRL-*lpr*/*lpr* mice these B cells escape normal tolerance mechanisms and can produce autoantibodies by deregulated receptor editing[21]. V-preB$^+$L$^+$ B cells appear to be cells with low affinity antiself antibodies that fail to be edited or deleted and survive to enter the peripheral circulation[9,11,60]. Under normal circumstances these B cells are likely to be anergic[9,11,60,61] but in autoimmune prone backgrounds they may escape normal tolerance mechanisms and produce autoantibodies[21].

## Methods

**Patient samples.** Control bone marrow samples were obtained from normal donors undergoing bone marrow collection for allogeneic transplantation. Peripheral blood was collected from recipients of T cell–depleted allogeneic hematopoietic stem cell transplants at serial timepoints after transplantation as indicated. All patients were free of clinical graft-*versus*-host disease. XLA blood samples were collected from patients with defined *BTK* gene mutations. Synovial tissues and peripheral blood were obtained by the RA Registry and Tissue Repository from patients undergoing joint replacement surgery at the Hospital for Special Surgery. All patients met the revised ACR criteria for the diagnosis of rheumatoid arthritis. Freshly isolated synovial specimens were placed in normal saline and used immediately for cell isolation. After collagenase (Sigma) digestion for 2 h at 37 ºC, mononuclear cells from joints affected by rheumatoid arthritis were isolated by Ficoll-Hypaque density gradient centrifugation. Bone marrow, tonsil, blood mononuclear cells were also isolated by Ficoll gradients. All samples were collected after signed informed consent in accordance with IRB-reviewed protocols.

**Cell preparation and sorting.** Bone marrow or blood B cells were stained with human fluorescein isothiocyanate (FITC)–anti-κ and human FITC–anti-λ (Pharmingen) and then enriched by positive selection using MACS mouse CD19 or MACS anti-FITC microbeads (Miltenyi Biotech., Auburn, CA).

V-preB$^+$L$^+$B cells were enriched from peripheral blood mononuclear cells in three steps. First, B cells were isolated by negative selection using a cocktail of tetrameric antibody complexes that recognized both dextran and human determinants of erythrocytes, granulocytes, monocytes, platelets, T cells and NK cells (StemCell Technologies,Vancouver, BC). Second, V-preB$^+$ B cells were stained with phycoerythrin (PE)-conjugated mAb to 4G7 anti-VpreB and enriched by positive selection using MACS anti-PE microbeads (Miltenyi Biotech.)[26]. Third, for transcription and repertoire analysis, V-preB$^+$ B cells were stained with human FITC–anti-κ, human FITC–anti-λ and allophycocyanin–anti-CD19 (Pharmingen), then sorted on a FACSvantage for V-preB, Igκ or Igλ and CD19 expression.

IgM$^-$IgD$^+$IgG$^-$CD19$^+$ (IgD-only B cells), IgM$^-$IgD$^-$IgG$^+$CD19$^+$ (IgG$^+$ B cells) and IgM$^-$IgD$^-$IgG$^-$CD19$^+$ (IgA, IgE and BCR$^-$ B cell precursors) were obtained by removing the IgM$^+$ cells with biotinylated anti-IgM and streptavidin microbeads (Miltenyi Biotech.) and then sorting. For sorting the IgM$^-$ B cells were stained with PE–anti-IgD, biotinylated anti-IgG and allophycocyanin-conjugated anti-CD19 (all from Pharmingen). Biotinylated anti-IgG was visualized by using streptavidin-RED613 (Gibco-BRL, Gaithersburg, MD). B cell phenotyping was done using the following FITC-conjugated antibodies: anti-CD22, anti-CD24 (Immunotech, Marseille France/Coulter, Hialea, FL), anti-CD5, anti-CD10, anti-CD19, anti-CD20, anti-CD34, anti-CD38, anti-CD27, anti-CD72, anti-κ; the following biotinylated antibodies: anti-IgM, anti-IgD, anti-κ or anti-λ (Pharmingen); and uncojugated rat anti-CD77 (Immunotech/Coulter), and visualized using FITC-sheep anti-rat IgM (Serotech, Raleigh, NC). B cell viability was determined by propidium iodide staining.

**RNA and RT-PCR.** Total RNA was extracted from $10^4$–$10^5$ purified cells using TRIzol Reagent (Gibco-BRL). After DNAse I (Boehringer-Manheim/Roche, Indianapolis, IN) treatment, 10 µl RNA was reverse transcribed with Superscript II (Gibco-BRL). For RT-PCR reactions, 1 µl of cDNA was amplified for 25 (actin), 35 (Igβ, $V_\kappa$-$C_\kappa$, $V_H5$-Cµ), 38 (λ–like and V-preB) or 40 (RAG-1, RAG-2 and TdT) cycles of 30 s at 94 ºC, 60 ºC and 72 ºC with a final 10-min extension at 72 ºC using Hot star Taq DNA polymerase (Qiagen, Valencia, CA) and the following primers: $V_\kappa$ consensus sense 5′–ATGACCCAG(A/T)CT CCA(C/G/T)(C/T)C(A/T)CCCTG–3′, $C_\kappa$ sense 5′–CGGGAAGATGAAGACAGATG–3′; V-preB sense 5′–GAGTCAGAGCTCTGCATGTCTG–3′, antisense 5′–GTGAGGCGGAT TGTGGTTCCAAG–3′; RAG-1 sense 5′–TGCAGACATCTCAACACTTTGGCCAG–3′, antisense 5′–TTTCAAAGGATCTCACCCGGAACAGC–3′; RAG-2 sense exon 1A 5′–AGCAGCCCCTCTGGCCTTCAG–3′, exon 1B 5′–CCTTCAGACTGCGGTCT CCAG–3′, antisense 5′–CATGGTTATGCTTTACATCCAGATG–3′; TdT sense 5′–CC TTCTGGAGACACCACCAGATG–3′, antisense 5′–GTTCTCTGCTACAATGTGGGT GACAG–3′. Actin, Igβ, $V_H5$ sense, Cµ antisense, and λ-like specific primers were as previously described[24,62,63]. RT-PCR products were analyzed on 2% agarose gels and hybridized with cognate cDNAs as previously described[24]. Phosphorimager analysis (Molecular Dynamics, Storm, Sunnyvale, CA) was done on samples amplified in the linear range.

**Cloning and sequencing.** PCR products were gel purified (Qiaquick, Qiagen) and cloned into TA vectors (Invitrogen, Carlsbad, CA). Double-stranded DNA sequences were obtained

using T7 and M13 reverse primers and Dye Terminator Cycle Sequencing (PE Applied Biosystems, Foster City, CA). Sequences were analyzed by comparison with GenBank. IgH CDR3 length was determined by counting amino acid residues between position 94 and position 102 (conserved tryptophan in all $J_H$ segments) and D segments were identified following the criteria of Corbett *et al.*[41].

Acknowledgments

We thank the Nussenzweig lab for comments and discussions; G. Michel, H. Chambost and C. Fossat for blood samples; W. Muller and K. Tai for tonsil and joint samples and P. Wilson, J. D. Capra and N. Chiorazzi for sharing unpublished data. Supported by grants from the NIH (to M.C.N. and J.W.Y.) and the Leukemia and Lymphoma Society (to J.W.Y.). M.C.N. is based at the Howard Hughes Medical Institute.

Received 9 May 2000; 4 August 2000.

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