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18. Neutrophils (10^5) were treated with TNF+CHX or antibody to Fas, rinsed with PBS, and incubated in 50 mM tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin at 37°C for 10 min. After centrifugation at 15,000*g* for 10 min, the lysate was incubated in DEVD-AFC (Enzyme Systems Products) for 30 min. Released AFC was measured by spectrofluorimetry with excitation at a wavelength of 380 nm and emission at 460 nm.
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26. Rat embryo fibroblasts (REF 52) [T. Joneson, M. A. White, M. H. Wigler, D. Bar-Sagi, *Science* **271**, 810 (1996)] were seeded on acid-treated glass cover slips, and 70% density cells were microinjected with plasmid DNA pGG-NT (encoding residues 1 through 352 of gelsolin and tagged with EYMPME epitope at the COOH-terminus) or pGG-CT (encoding residues 353 through 731 of gelsolin and tagged with EYMPME epitope at the COOH-terminus) at 0.1 μg/ml in 100 mM Hepes (pH 7.4). A plasmid encoding green fluorescent protein (GFP) (pEGFPN1, Promega) at 1 ng/ml was coinjected as a marker. Cells were fixed in 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, and stained for the expression of tagged proteins, using a mouse monoclonal antibody to EYMPME (50 μg/ml) and a secondary goat anti-mouse immunoglobulin G coupled to Texas Red (Amersham). Actin filaments were visualized with tetramethyl rhodamine isothiocyanate (TRITC)–Phalloidin (Sigma).
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V(D)J Recombination in Mature B Cells: A Mechanism for Altering Antibody Responses

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The clonal selection theory states that B lymphocytes producing high-affinity immunoglobulins are selected from a pool of cells undergoing antibody gene mutation. Somatic hypermutation is a well-documented mechanism for achieving diversification of immune responses in mature B cells. Antibody genes were also found to be modified in such cells in germinal centers by recombination of the variable (V), diversity (D), and joining (J) segments. The ability to alter immunoglobulin expression by V(D)J recombination in the selective environment of the germinal center may be an additional mechanism for inactivation or diversification of immune responses.

One of the key features of adaptive immunity is the ability to respond to an enormous number of different antigens. To account for this diversity the clonal selection theory proposes selective expansion of cells with antibody gene mutations during specific immune responses (*1*). Antibody diversity is initially generated in the bone marrow by a lymphocyte-specific V(D)J recombinase that assembles antigen receptor genes (*2*). Each B cell has only one antibody receptor, a phenomenon known as allelic exclusion (*3*). Exclusion is essential for clonal selection and is established by a feedback mechanism from the membrane-bound B cell receptor (BCR) that extinguishes the expression of the recombinase-activating genes (*RAG1* and *RAG2*) (*4*). Self-reactive BCRs generated during this random gene recombination process can be eliminated in the bone marrow by continued recombination (receptor editing) or by deletion (*5*). B cells leaving the bone marrow are hypothesized to have fixed receptors that can only be altered by somatic hypermutation, a process that would maintain allelic exclusion (*6*). Thus, somatic hypermutation is thought to be the mechanism of antibody gene mutation predicted in the clonal selection theory (*1*).

RAG1 and RAG2 are the recombination signal sequence (RSS)–specific endonucleases that activate V(D)J recombination (*7*). In addition to being found in developing lymphocytes, RAGs are transcribed in germinal centers (*8*), which are the foci of hypermutation (*9*), switch recombination (*10*), and B cell clonal expansion in response to antigen (*11*). The finding that RAGs are expressed in germinal centers suggested that they might mediate antigen receptor diversification in mature B cells that are responding to antigenic stimulation. However, expression of the RAG genes does not necessarily translate into immune receptor gene recombination: *RAG1* is transcribed in the brain, with no known function to date (*12*), and *RAG1*

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and *RAG2* coexpression does not result in antibody gene assembly in T cells or T cell receptor gene recombination in B cells.

To determine whether RAGs induced in mature B cells were active, we assayed for de novo, RAG-specific DNA double-stranded breaks by the ligation-mediated polymerase chain reaction (LM-PCR) (*13*, *14*). There are few potential RSS targets for the RAGs in mature B cells because they have extensive V(D)J gene rearrangements on both alleles of heavy and light chain loci. To increase the sensitivity of the assay, we used mice with targeted $V_{B1\text{-}8}DJ_H2$ and $V_{3\text{-}83}J_\kappa2$ replacement alleles [$\mu^{i/+}\kappa^{i/+}$ mice (where the superscript i stands for inactivated); Fig. 1A] (*15*, *16*). The rearranged receptors in these mice allelically exclude the endogenous heavy and light chain alleles, which should therefore remain unrecombined (*15*, *16*) and available for cleavage by RAGs (*17*).

RAG genes were induced in spleen cells from $\mu^{i/+}\kappa^{i/+}$ mice and wild-type controls by stimulation with bacterial lipopolysaccharide (LPS) and interleukin-4 (IL-4) (*8*). As a control, B cells were stimulated with LPS alone, which does not induce the RAGs but does produce B cell activation and proliferation (*8*). J_H and J_κ signal breaks were either absent or difficult to detect in unstimulated cells, whereas both J_H and J_κ signal breaks were found in the $\mu^{i/+}\kappa^{i/+}$ B cells cultured in LPS and IL-4 for 2 days (Fig. 1B). In contrast, $\mu^{i/+}\kappa^{i/+}$ B cells that had been cultured in LPS alone had no signal breaks. Wild-type B cells responded to LPS and IL-4 stimulation somewhat similarly to $\mu^{i/+}\kappa^{i/+}$ B cells, though the amount of J_H and J_κ signal breaking induced was only 10 to 20% of that found in the $\mu^{i/+}\kappa^{i/+}$ mice, as measured by phosphorimager and dilution analysis (Fig. 1B). The low frequency of signal breaks found in the wild type could be explained simply on the basis of reduced availability of RSSs.

In the bone marrow, after RSS-directed, RAG-mediated DNA cleavage, joining reactions lead to antigen receptor gene assembly. We used PCR to determine whether VDJ_H and VJ_κ joints accumulated on the allelically excluded alleles in mature $\mu^{i/+}\kappa^{i/+}$ B cells. The $V_{3\text{-}83}J_\kappa$ replacement (*15*) deleted the $J_\kappa1$ segment (Fig. 1A); therefore, any joining that uses the $J_\kappa1$ segment must take place on the wild-type allele. Few $VJ_\kappa1$ joints were found in wild-type bone marrow and mature B cell controls, and VJ_κ joints were virtually undetectable in unstimulated $\mu^{i/+}\kappa^{i/+}$ B cells (Fig. 2A). In contrast, $VJ_\kappa1$ joints were abundant in $\mu^{i/+}\kappa^{i/+}$ B cells that had been cultured for 48 hours with LPS and IL-4, but not with LPS alone (Fig. 2A). Heavy chain gene rearrangements were assessed in the same samples with the use of V_{J558L} primers (*18*). The $V_{B1\text{-}8}DJ_H$ replacement uses J_H2 and deletes J_H1, J_H3, J_H4, and J_H5 (Fig. 1A); thus, any joining that involves any of those four J_H segments must be on the wild-type allele (*16*). $V_{J558L}DJ_H1$ joints were found in the wild-type B cells but were virtually undetectable in $\mu^{i/+}\kappa^{i/+}$ B cells (Fig. 2B). The near absence of these joints in the $\mu^{i/+}\kappa^{i/+}$ B cells was expected because endogenous VDJ_H rearrangements are inhibited by the $V_{B1\text{-}8}DJ_H$ replacement (*16*). In vitro stimulation with LPS and IL-4, but not with LPS alone, resulted in significant accumulation of $V_{J558L}DJ_H1$ joints in the $\mu^{i/+}\kappa^{i/+}$ B cells (Fig. 2B). These joints are suggestive of new V(D)J rearrangements at the previously excluded wild-type allele.

To determine whether B cells stimulated with LPS and IL-4 change the specific immunoglobulin M (IgM) they express, we stained $\mu^{i/+}\kappa^{i/+}$ B cells and controls with the Ac146, an antibody that recognizes the idiotype produced by the $V_{B1\text{-}8}DJ_H$– $V_{3\text{-}83}J_\kappa$ replacement combination (*19*). As expected, 90% of the unstimulated B220^{hi} $\mu^{i/+}\kappa^{i/+}$

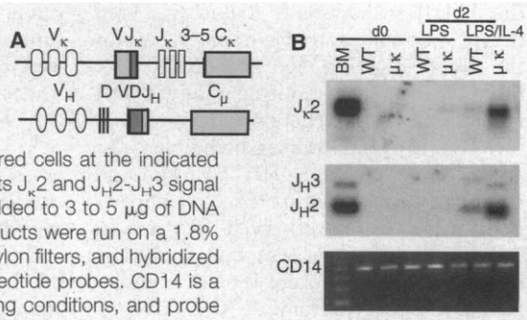

Fig. 1. J_κ and J_H signal breaks in B cells stimulated with LPS and IL-4. (**A**) Diagrammatic representation of the κ ($V_{3\text{-}83}$) and μ ($V_{B1\text{-}8}$) Ig replacement loci. (**B**) LM-PCR used to detect signal breaks was performed on high molecular weight DNA (*27*) isolated from cultured cells at the indicated times, according to (*13*). The assay detects $J_\kappa2$ and J_H2-J_H3 signal breaks as indicated. Briefly, linker was added to 3 to 5 μg of DNA and ligated for 24 to 36 hours. PCR products were run on a 1.8% agarose gel, Southern (DNA) blotted on nylon filters, and hybridized with appropriate kinase-labeled oligonucleotide probes. CD14 is a DNA loading control. PCR primers, cycling conditions, and probe sequences were as previously published (*13*). Symbols: V, variable region coding exons; D, diversity segments; J, joining segments; C, constant region coding exons; VJ_κ, rearranged $V_{3\text{-}83}$; VDJ_H, rearranged $V_{B1\text{-}8}$; d0, day zero; d2, day 2; μ κ, $\mu^{i/+}\kappa^{i/+}$ mice; WT, wild-type mice; BM, bone marrow cells from WT mice.

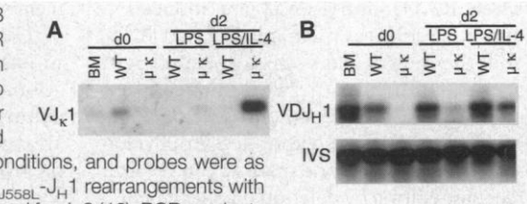

Fig. 2. VJ_κ and VDJ_H rearrangements in B cells stimulated with LPS and IL-4. (**A**) PCR amplification of $VJ_\kappa1$ rearrangements with a V_κ degenerate primer, which anneals to 80% of all mouse V_κ genes, and a primer for $J_\kappa1$ (*28*). PCR products were analyzed as in Fig. 1B. Primer sequences, PCR conditions, and probes were as described (*28*). (**B**) PCR amplification of V_{J558L}-J_H1 rearrangements with primers for the VJ558L family of V_H genes and for J_H2 (*18*). PCR products were visualized with an Apa I–Xba I mouse J region probe (*18*) that detects J_H1 and J_H2. J_H2 amplification was uniform in all samples. Symbols: $VJ_\kappa1$, amplified $VJ_\kappa1$ rearrangements; VDJ_H1, amplified DNA from the VJ558L V gene family; IVS, amplified DNA from intervening sequences (loading control); other symbols as in Fig. 1.

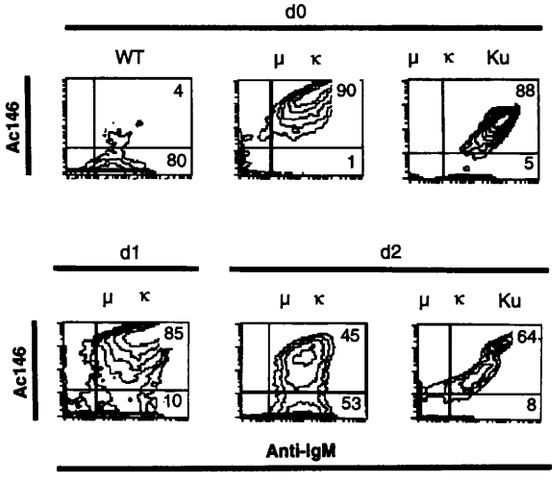

Fig. 3. Fluorescence-activated cell sorting (FACS) analysis of B cells stimulated with LPS and IL-4. Uninduced cells (d0) or cells cultured with LPS and IL-4 for 1 or 2 days (d1 and d2) were stained with biotinylated Ac146 antibody (*15*, *19*) and with fluoroscein isothiocyanate–labeled antibody to mouse IgM (anti-mouse IgM) (Pharmingen). Lymphocytes were gated on B220^{hi} cells. The numbers in each quadrant represent percentages of gated lymphocytes. All data acquisition and analysis were done on a FACScan with CellQuest software (Becton Dickinson). Symbols: μ κ Ku, $\mu^{i/+}\kappa^{i/+}$ Ku80$^{-/-}$ mice; Ac146, Ac146 antibody staining; anti-IgM, anti-mouse IgM staining; other symbols as in previous figures.

lymphocytes were positive for both Ac146 and membrane IgM expression ($Ac146^+$ $mIgM^+$), and only 1% of the cells were Ac146 negative and mIgM positive ($Ac146^-mIgM^+$) (Fig. 3). After 24 hours in culture with LPS and IL-4 the number of $\mu^{i/+}\kappa^{i/+}$ B cells that were idiotype negative had increased from 1 to 10%, and by 48 hours this number had increased to 53% (Fig. 3). In contrast, during the same period of time the percentage of B cells that expressed the idiotype had decreased by half (90 to 45%) (Fig. 3).

To verify that the observed conversion was specifically due to the induction of the V(D)J recombinase system, we bred the $\mu^{i/+}\kappa^{i/+}$ mice to be homozygous for the Ku80 deletion ($\mu^{i/+}\kappa^{i/+}$ $Ku80^{-/-}$). $Ku80^{-/-}$ animals cannot complete V(D)J recombination and therefore have no mature B cells (*20*). However, breeding of the $\mu^{i/+}\kappa^{i/+}$ mice to the $Ku80^{-/-}$ background reconstitutes the mature B cell compartment (*21*). If the observed increase in $Ac146^-mIgM^+$ cells was due to V(D)J recombination in mature B cells, stimulation of splenocytes from $\mu^{i/+}\kappa^{i/+}$ $Ku80^{-/-}$ mice with LPS and IL-4 should not lead to an increase in idiotype-negative cells. The percentage of $\mu^{i/+}\kappa^{i/+}$ $Ku80^{-/-}$ B cells that were $Ac146^-mIgM^+$ was unaltered after 48 hours of culture Fig. 3 (*22*). Thus, when RAG1 and RAG2 are induced in mature B cells they can cut DNA at RSSs, and the new breaks are associated with accumulation of otherwise allelically excluded gene rearrangements.

Continued RAG expression in developing B cells in the bone marrow can result in receptor editing by deletional replacement of a prerearranged Ig gene (*5*). To determine whether a prerearranged Ig gene could also be replaced in mature B cells, we bred mice to be homozygous for the $V_{3\text{-}83}J_\kappa$ replacement and heterozygous for the $V_{B1\text{-}8}DJ_H$ replacement ($\mu^{i/+}\kappa^{i/i}$) (*15, 16*). Because the $J_\kappa3$, $J_\kappa4$, and $J_\kappa5$ alleles remain in the germline configuration, further VJ_κ recombination in $\mu^{i/+}\kappa^{i/i}$ B cells could only be accomplished by looping out the $V_\kappa3\text{-}83\text{-}J_\kappa2$ replacement. Although $VJ_\kappa4$ rearrangements were difficult to detect in maure $\mu^{i/+}\kappa^{i/i}$ B cells, $J_\kappa4$ signal breaks and $VJ_\kappa4$ recombination products were abundant in $\mu^{i/+}\kappa^{i/i}$ B cells after they were cultured for 2 days in LPS and IL-4 (Fig. 4A). The relative increase in $VJ_\kappa4$ rearrangements was by a factor of 10 to 20, as measured by semiquantitative dilution PCR and phosphorimaging. The limited cell division and cell death in the first 48 hours of culture with LPS and IL-4 made it likely that these $VJ_\kappa4$ joints represent new rearrangements that "edit" out the preexisting receptors (*22*).

To determine whether RAGs expressed in germinal centers in vivo were active, we examined B cells from $\mu^{i/+}\kappa^{i/+}$ mice immunized with trinitrophenol coupled to keyhole limpet hemocyanin (TNP-KLH) for signal breaks (*13, 14*). Germinal center B cells were enriched by density centrifugation as measured by GL-7 staining. J_κ breaks were found 7 days after immunization in the activated but not in the resting B cell fractions (Fig. 4B), and the breaks were associated with a loss of Ac146 staining. This time course of break induction correlates with the reported expression of RAG1 in germinal centers 5 days after immunization (*8, 23*). Thus, antigen-driven activation of the RAGs in germinal center B cells was associated with de novo J_κ breaks and perhaps the expression of previously excluded membrane Igs.

Does V(D)J recombination really occur in mature B cells in more physiologic situations? We would predict that light chain editing is not uncommon, because $V\text{-}J_\kappa$ rearrangements could occur on any rearranged allele that still contains $J_\kappa5$ or more 5′ J_κ genes. In contrast, heavy chain gene editing may be a less frequent event. Any heavy chain allele that has a functional or nonfunctional V_H gene would not be available for standard V(D)J recombination because all D_H-associated RSSs would have been deleted. Nevertheless, heavy chain gene replacement using internal cryptic RSSs in VDJ_H genes has been documented and would remain possible (*24*).

Active gene rearrangement in mature B cells that express membrane μ suggests that allelic exclusion may be violated in germinal centers and conflicts with the generally accepted idea that membrane μ regulates allelic exclusion (*4, 25*). However, three mechanisms are likely to reduce the number of "allelically included" B cells that can emerge from the selective environment of the germinal centers: (i) somatic mutation and Ig gene inactivation; (ii) elimination by selection of mixed- or low-affinity receptors (*26*); and (iii) incompatibility between heavy and light chain pairs. Support for the idea that most replacements would be selected against comes from the immunochemical observation that RAG1 is abundant in clusters of apoptotic B cells in the germinal centers (*23*).

In conclusion, affinity maturation, which is thought to be driven by somatic mutation, might also involve V(D)J recombination and receptor editing. Edited receptors would usually comprise the original heavy chain with an altered light chain. Because a disproportionately large part of the antigen-binding pocket is frequently contributed by the heavy chain, changes in the light chain might be expected to yield antibodies with various affinities for the original immunogen.

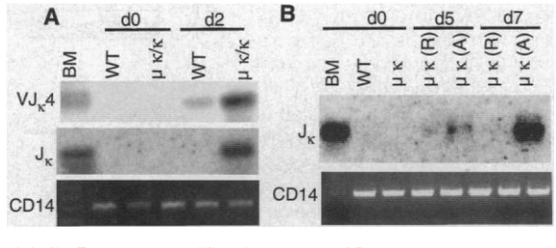

Fig. 4. Molecular substitution of the $V_{3\text{-}83}J_\kappa2$ replacement allele by $VJ_\kappa4$ and signal breaks in vivo. (**A**) Top, PCR amplification of $VJ_\kappa4$ rearrangements (*29*). Middle, induction of DNA breaks in splenic $\mu^{i/i}\kappa^{i/i}$ B cells cultured with LPS and IL-4. LM-PCR was performed as described in Fig. 1B. PCR primers, cycling conditions, and probe sequences were as previously published (*13*). Bottom, amplification of the CD14 locus is the DNA loading control. (**B**) J_κ signal breaks induced in GL-7^+ B cells in vivo. T cell–depleted, activated (A), and resting (R) B cells were used to make high molecular weight DNA for LM-PCR (see Fig. 1B). CD14 is the DNA loading control. Symbols: μ κ/κ, $\mu^{i/+}\kappa^{i/i}$ homozygous kappa-inactivated mice; $VJ_\kappa4$, $VJ_\kappa4$ rearrangements; J_κ, J_κ signal breaks; other symbols as in previous figures.

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22. It takes 36 hours for $\mu^{V+}\kappa^{V+}$ cells to enter the cell cycle, and only 15 to 20% of the cells are in the S or G_2 phase at 48 hours. After 48 hours 15 to 20% of the cells die, and there is no net increase in the total number of live B cells. Therefore, even if we assume a 6-hour cell cycle, selective expansion of small subpopulations of cells does not account for the loss of idiotype expression.
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29. Primer sequences, PCR conditions, and probes were as follows: V_κ degenerate, as in (*28*); $J_\kappa 4$: 5′-GGATCTTCTATTGATGCACAGGTTGCCAGG-3′; $J_\kappa 4$ probe: 5′-GGCTCGGGGACAAAGTTGGAA-ATAAAACGTAAG-3′; 24 cycles of 30 min at 94°C, 30 min at 60°C, and 30 min at 72°C.
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V(D)J Recombinase Activity in a Subset of Germinal Center B Lymphocytes

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Reexpression of the V(D)J recombinase-activating genes *RAG1* and *RAG2* in germinal center B cells creates the potential for immunoglobulin gene rearrangement and the generation of new antigen receptor specificities. Intermediate products of V(D)J recombination are abundant in a subset of germinal center B cells, demonstrating that the κ immunoglobulin light-chain locus becomes a substrate for renewed V(D)J recombinase activity. This recombinationally active cell compartment contains many heavy-chain VDJ rearrangements that encode low-affinity or nonfunctional antibody. In germinal centers, secondary V(D)J recombination may be induced by diminished binding to antigen ligands, thereby limiting abrupt changes in receptor specificity to B cells that are usually eliminated from the germinal center reaction. This restriction preserves efficient antigen-driven selection in germinal centers while allowing for saltations in the somatic evolution of B cells.

The primary antibody repertoire is created during the pre-B and immature stages of B lymphogenesis by V-to-J recombination in the κ and λ light-chain (L-chain) loci (*1*) and by tolerance mechanisms that either purge newly generated, autoreactive B cells by apoptosis (*2*) or render them harmless by a process of secondary V(D)J rearrangements known as receptor editing (*3*). Remarkably, during the immune response known as the germinal center (GC) reaction, some B cells begin to transcribe λ5, a component of the pre-B cell receptor complex (*4, 5*), express the RAG1 and RAG2 V(D)J recombinase proteins (*5*), and become highly sensitive to receptor-induced apoptosis (*6*). This broad recapitulation of lymphocyte development and the availability of V(D)J recombinase suggests that at least some GC B cells might also undergo secondary immunoglobulin (Ig) gene rearrangements.

GCs are established by the focal proliferation of mature, antigen-reactive lymphocytes in the B cell zones of secondary lymphoid tissues (*7*). They are crucial for immunological memory in the B cell compartment and are primary sites for the V(D)J hypermutation and cellular selection necessary for affinity maturation of antibody responses (*8*). B cells that enter nascent GCs bear IgM and IgD on their surface and express the low-affinity IgE Fc receptor, CD23 ($CD23^+$). With time, GC B cells cease to express membrane IgD and CD23 and acquire a characteristic phenotype that includes avid binding of peanut agglutinin and the GL-7 antibody (PNA^{hi}, $GL\text{-}7^+$), upregulation of the Fas death trigger ($CD95^+$), and increased expression of CD24 ($CD24^{hi}$) (*7, 9*). This core phenotype remains stable during the primary GC reaction.

Although the majority of splenic GC B cells ($GL\text{-}7^+$, $CD95^+$, $CD24^{hi}$) express amounts of B220 (CD45R) typical of mature B lymphocytes ($B220^{hi}$), a population of GC B lymphocytes that expresses B220 in amounts similar to pre-B cells ($B220^{lo}$) appears by day 10 to 12 after immunization (*10*) and expands, eventually constituting as much as half (25 to 53%) of all $GL\text{-}7^+$ B lymphocytes (Fig. 1). Proliferation, as measured by incorporation of the nucleotide analog, 5-bromo-2′-deoxyuridine (BrdU), is equivalent in $B220^{lo}$ and $B220^{hi}$ GC cells and consistent with the rapid proliferation of GC B lymphocytes (*7, 11*).

The delayed appearance of the $B220^{lo}$ population of GC B cells is similar to the kinetics of local RAG expression (*5*). To compare the distribution of recombinase and λ5 expression in $B220^{lo}$ and $B220^{hi}$ GC cell populations, we purified 1×10^4 follicular ($GL\text{-}7^-$, $B220^+$), GC ($GL\text{-}7^+$, $B220^+$), and $B220^{lo}$ and $B220^{hi}$ GC ($GL\text{-}7^+$) cells from the splenocytes of immunized C57BL/6 mice by fluorescence-activated cell sorting (FACS) (*12*). A reverse transcriptase–dependent polymerase chain reaction (RT-PCR) was then used to determine the presence of RAG1, RAG2, λ5, and hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA in aliquots of each sorted population (*13*). Whereas equivalent amounts of HPRT mRNA were present in all cell samples (Fig. 2D), recombinase and λ5 message (Fig. 2, A to C) were present in $B220^+$ GC cells but could not be detected in follicular B lymphocytes. Among GC cells, RAG and λ5 transcripts were largely confined to the $B220^{lo}$ compartment. Transcription of the *RAG* and λ5 genes is correlated with the $B220^{lo}$ GC cell phenotype.

To determine if V(D)J recombination was active in GC B cells, we designed a locus-specific, ligation-mediated PCR (LM-PCR) assay to detect double-stranded (ds) recombination signal sequence (RSS) breaks in the Ig κ locus (Fig. 3A). These blunt, 5′-phosphorylated, ds breaks at RSSs are intermediates of V(D)J recombination and depend on the unique enzymatic activity of RAG1 and RAG2 (*14*). Purified DNA samples were ligated to an unphosphorylated linker oligonucleotide (BW) containing one blunt and one overhanging end. RSS breaks 3′ to a prior V-to-J_κ rearrangement were then amplified by PCR, using a linker-specific primer (BW-H) and a primer, $V_\kappa B$ or $V_\kappa S$, that hybridizes to most V_κ gene segments (*15, 16*). A fraction of this initial PCR product was then amplified in a second round of PCR using BW-H

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