Spontaneous Class Switch Recombination in B Cell Lymphopoiesis Generates Aberrant Switch Junctions and Is Increased after VDJ Rearrangement[1]

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Mature B cells replace the μ constant region of the H chain with a downstream isotype in a process of class switch recombination (CSR). Studies suggest that CSR induction is limited to activated mature B cells in the periphery. Recently, we have shown that CSR spontaneously occur in B lymphopoiesis. However, the mechanism and regulation of it have not been defined. In this study, we show that spontaneous CSR occurs at all stages of B cell development and generates aberrant joining of the switch junctions as revealed by: 1) increased load of somatic mutations around the CSR break points, 2) reduced sequence overlaps at the junctions, and 3) excessive switch region deletion. In addition, we found that incidence of spontaneous CSR is increased in cells carrying VDJ rearrangements. Our results reveal major differences between spontaneous CSR in developing B cells and CSR induced in mature B cells upon activation. These differences can be explained by deregulated expression or function of activation-induced cytidine deaminase early in B cell development. *The Journal of Immunology,* **2007, 179: 6555–6560.**

During lymphopoiesis in the bone marrow (BM),[3] B lymphocytes rearrange Ig V, D, and J gene segments to construct the BCR variable domains (1, 2). Signaling through the newly generated BCR is necessary for positive and negative selection of the immature B cells (3–6). Upon Ag encounter in the secondary lymphoid organs, B lymphocytes undergo further genomic modifications: class switch recombination (CSR) and somatic hypermutation (SHM). CSR directs Ab production toward the synthesis of effector Abs (IgG, IgA, and IgE), whereas the introduction of SHM into the Ig V region genes is the basis of Ab-affinity maturation. Both processes utilize a similar enzymatic machinery that includes activation-induced cytidine deaminase (AID) and uracil-DNA glycosylase (reviewed in Ref. 7).

During BM development, B cells utilize IgM receptors for the generation of signals necessary to promote developmental progression. Studies using Ig-transgenic (Ig-Tg) mice suggest that IgM receptors are more efficient than IgG receptors in promoting B cell development (8–11). In contrast, other studies suggest that the IgG membrane tail is a molecular determinant of memory response (12) and is required for the generation and survival of IgG memory B cells (13). Hence, it is generally thought that IgM receptors are required for development and tolerance while non-IgM receptors have effector function in memory responses. For this reason, it is also thought that CSR occurs exclusively in activated mature B cells in the periphery. Early studies by Rolink et al. (14) have shown that treatment with anti-CD40 Abs and IL-4 stimulates CSR in RAG2$^{-/-}$ and normal developing B lymphocytes (14). However, B cell precursors in this study were first induced to differentiate as revealed by the expression of the mature B cell markers CD23 and MHC class II. Accordingly, CSR that was induced in these experiments could only be found in cells that express the mature phenotype (14), thereby supporting a model that limits CSR to mature B cells.

Recently, several studies that are inconsistent with this paradigm have been published. In an earlier study, we have shown that AID is expressed during B lymphopoiesis and that a low number of IgG-expressing B cells are produced in the BM and can exit into the periphery (15–17). It was later shown that immature B cells can also acquire SHM (18). Recently, low levels of AID were found in sorted BM B cell subsets (19). Lastly, using mice where the H chain locus allows only γ1 H chain (γ1H) expression, we showed that γ1H expression is compatible with B cell development and maturation (20). These findings suggest that CSR spontaneously occurs during BM development, and that IgG receptors can efficiently promote B cell lymphopoiesis. However, the mechanism and the regulation of this CSR are unclear. In this study, we show that spontaneous CSR occurs throughout B lymphopoiesis and generates aberrant switch junctions. Furthermore, we find that spontaneous CSR occurs at higher frequency in cells carrying a VDJ rearrangement. Our results suggest that spontaneous CSR may be distinct from CSR induced in mature B cells in the regulation or function of the CSR enzymatic machinery.

Materials and Methods

Experimental mice

Mice used for these experiments were normal, unimmunized B10.D2nSn/J, 3-83Tg B10.D2nSn/J- expressing IgM/IgD BCR specific to class I MHC Ags K^k and K^b (21), 3-83HKI B10.D2nSn/J (22). Mice were housed and bred at the pathogen-free animal facility of the Technion, Faculty of Medicine, and used for the experiments at 4–8 wk old. Mice deficient of RAG-2 that carry or do not carry the $D23^{stop}$ knock-in allele (23) were

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[3] Abbreviations used in this paper: BM, bone marrow; CSR, class switch recombination; AID, activation-induced cytidine deaminase; SHM, somatic hypermutation; BM, bone marrow; GLT, germline transcript; PST, postswitch transcript; DIG, digoxigenin; Tg, transgenic; CT, circular transcript.

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littermates and were on the 129 × C57/BL6 mixed background. The mice used were bred and maintained at the animal facility of the Infectious Disease Institute, Institute for Biomedical Research. All mice studies were approved by the institutional committee for the supervision of animal experiments.

Cell culture

B cell precursors were grown in vitro as previously described (24). Briefly, BM cells were depleted of erythrocytes and cultured in IMDM (Biological Industries) with 50–100 U/ml rIL-7 for 5 days. Cells grown in these primary cultures (>95% $B220^+$) were used directly for cellular and molecular analysis.

Flow cytometry and magnetic cell sorting

Single-cell suspensions from BM specimens, BM cultures, or spleens were stained for surface markers using FITC-, PE-, and biotin-conjugated Abs. The following Abs were used: anti-B220 RA3-6B2; goat anti-mouse γH-specific (all from Southern Biotechnology Associates); anti-CD43 Ly-48, leukosialin; anti-IgD JA12.5 (all from BD Biosciences); anti-c-kit ACK2; anti-CD25 PC61.5 (all from eBioscience); anti-IgM, $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratories); and anti-IgM (Zymed Laboratories). Data were collected on a FACSCalibur and analyzed using CellQuest software (BD Biosciences). Cultured BM cells or ex vivo BM preparations were fractionated using magnetic cell sorting (Miltenyi Biotec). In some experiments, B cell precursors from BM specimens were sorted ex vivo using CD19 MACS microbeads (Miltenyi Biotec). Splenic B cells were also purified using MACS microbeads and stimulated with LPS (50 μg/ml) and IL-4 (10 ng/ml) for 72 h. Cells were collected for DNA and RNA analysis.

RT-PCR and Southern blotting analysis

Total RNA was extracted from cells using RNA-Bee (Tel-Test) according to the manufacturer's instructions. One microgram of RNA samples was reversed transcribed into cDNA using Moloney murine leukemia virus (Promega) in a 20-μl reaction. Expression of AID, Cγ1 germline transcript (GLT), Cγ1 postswitch transcript (PST), and Cγ1 circular transcript (CT) was determined by PCR as previously described (16, 25, 26). For each PCR, we used 2 μl of the cDNA solution. For detection of $3\text{-}83_{VDJ}$-γH chain expression, we used 5′ oligonucleotide specific for $3\text{-}83_{VDJ}$ 5′-TG GAGTGCCAACATATGC-3′ and γCH1 5′-CGTGTCAGGCTAGCGGGT GTTGTTTTGGC-3′. PCR conditions for $3\text{-}83_{VDJ}$-γH chain were: 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C for 34 cycles. PCR products were fractionated by electrophoresis on 2% agarose gels and transferred to nylon membranes (Schleicher & Schüll), fixed, and cross-linked with UV. Southern blotting was performed using nonradioactive methodology according to the manufacturer's instructions (Roche Diagnostics). The DNA probes were synthesized by incorporation of digoxigenin (DIG) 11-dUTP during PCR using specific primers that synthesize a DNA fragment corresponding to the amplified product. Membranes were prehybridized for 30 min in DIG Easy Hyb solution. The prehybridization solution was replaced with prewarmed DIG Easy Hyb solution containing 60–100 ng/ml heat-denatured DIG-labeled DNA probe and incubated overnight at 45°C. Membranes were washed, blocked with blocking buffer, and incubated at room temperature for 1 h with anti-DIG-alkaline phosphatase conjugate diluted 1/20,000 in 1× blocking buffer. Bound probes were visualized using a chemiluminescent substrate and quantified using an UVIdoc gel documentation system and UVIdoc software (UVItec) as we have previously described (27).

Analysis of switch recombination junctions

Analysis of CSR junctions was performed as described elsewhere (28). Briefly, genomic DNA was extracted from IgG-expressing BM culture B cell precursors or from 4-day LPS/IL-4-stimulated splenic B cells. The PCR conditions and primer sequences were as described previously (28). Nested PCR amplification of Sμ/Sγ1 was done using the Sμ1 and Sγ1.1 primers for the first round. The second PCR round was done using Sμ2 and Sγ1.2 primers (28). PCR products were purified, cloned into pGEM, and sequenced. CSR junctions were analyzed by using GenBank and Ig Blast database with the low-complexity filter disabled. Mutations were determined at the ±50-bp vicinity of the switch junction as described elsewhere (29).

Real-time PCR

Total RNA was prepared using TriReagent (Sigma-Aldrich) according to the manufacturer's protocol. First-strand cDNA was synthesized with Moloney murine leukemia virus (Promega). Real-time quantitative PCR for AID expression was performed using SYBR Green PCR Master Mix (AB gene) in an Applied Biosystems Prism 7000 sequence detection system. The primer sequences used for AID amplification were: sense: 5′-GG GAAAGTGGCATTCACCTA-3′ and antisense: 5′-GAACCCAATTCTGG CTGTGT-3′. We normalized samples with β_2-microglobulin which was amplified using the following primers: sense, 5′-TTCTGGTGCTTGTCTC ACTGA-3′ and antisense, 5′-CAGATGTTCGGCTTCCCATTC-3′. PCR product specificity was confirmed by agarose gel electrophoresis and melting curve analysis. The crossover point (threshold cycle) values were used to calculate the gene-specific input mRNA amount for each sample according to the calibration curve method. Data were analyzed using SDS software version 1.9.1 (Applied Biosystems).

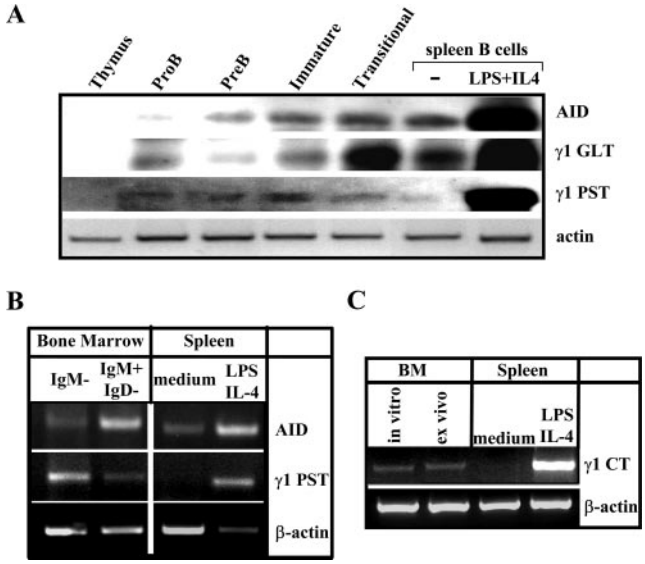

FIGURE 1. Spontaneous CSR in developing B lymphocytes. *A,* BM cells from normal mice were grown in BM cultures and sorted to proB ($B220^+CD43^+IgM^-$), preB ($B220^+CD43^-IgM^-$), immature ($B220^+IgM^+IgD^-$), and transitional ($B220^+IgM^+IgD^+$) populations. Normal splenic B cells either unstimulated or stimulated with LPS and IL-4, as well as thymus cells were used as controls. mRNA samples from the sorted populations were subjected to RT-PCR amplification and Southern blot analysis for detection of IgG1 GLT and PST and for AID expression. Blot shown is from an individual mouse and is a representative of three mice. *B,* BM cells from normal mice were sorted ex vivo to pro/preB ($B220^+IgM^-$) and immature (IgM^+IgD^-). Normal splenic B cells either unstimulated or stimulated with LPS and IL-4 were used as controls. mRNA samples from the sorted populations were subjected to RT-PCR amplification for IgG1 PST for AID expression. *C,* IgG-depleted, BM culture B cells, or ex vivo-isolated $B220^+$ cells were sorted and analyzed by RT-PCR for detection of IgG1 CT. Normal splenic B cells either unstimulated or stimulated with LPS and IL-4 were used as controls. Gel images shown in *B* and *C* are from individual mice and are representative of three mice.

Results

Spontaneous CSR occurs throughout B cell lymphopoiesis

We have previously shown that spontaneous CSR in BM cultures generates a population of 1–2% IgG-expressing cells (16). To study the process of CSR in B lymphopoiesis, we sorted B cells grown in BM cultures into proB ($B220^+CD43^+IgM^-$), preB ($B220^+CD43^-IgM^-$), immature ($B220^+IgM^+IgD^-$), and transitional populations ($B220^+IgM^+IgD^+$) as previously described (27). Sorted cells were analyzed for the following markers of CSR: induction of AID, expression of IgG1 GLTs (Iμ-Cμ), and expression of IgG1 PSTs (Iμ-Cγ1) (26). We found low levels of AID as well as IgG1 GLTs and PSTs in all cell fractions (Fig. 1*A*). In parallel cultures that contained serum-free medium, we found an equivalent frequency of IgG-expressing cells (1–2%; data not shown), indicating that the detected CSR is not induced by any stimulatory agent that may be present in the supplemented serum. In addition, we found

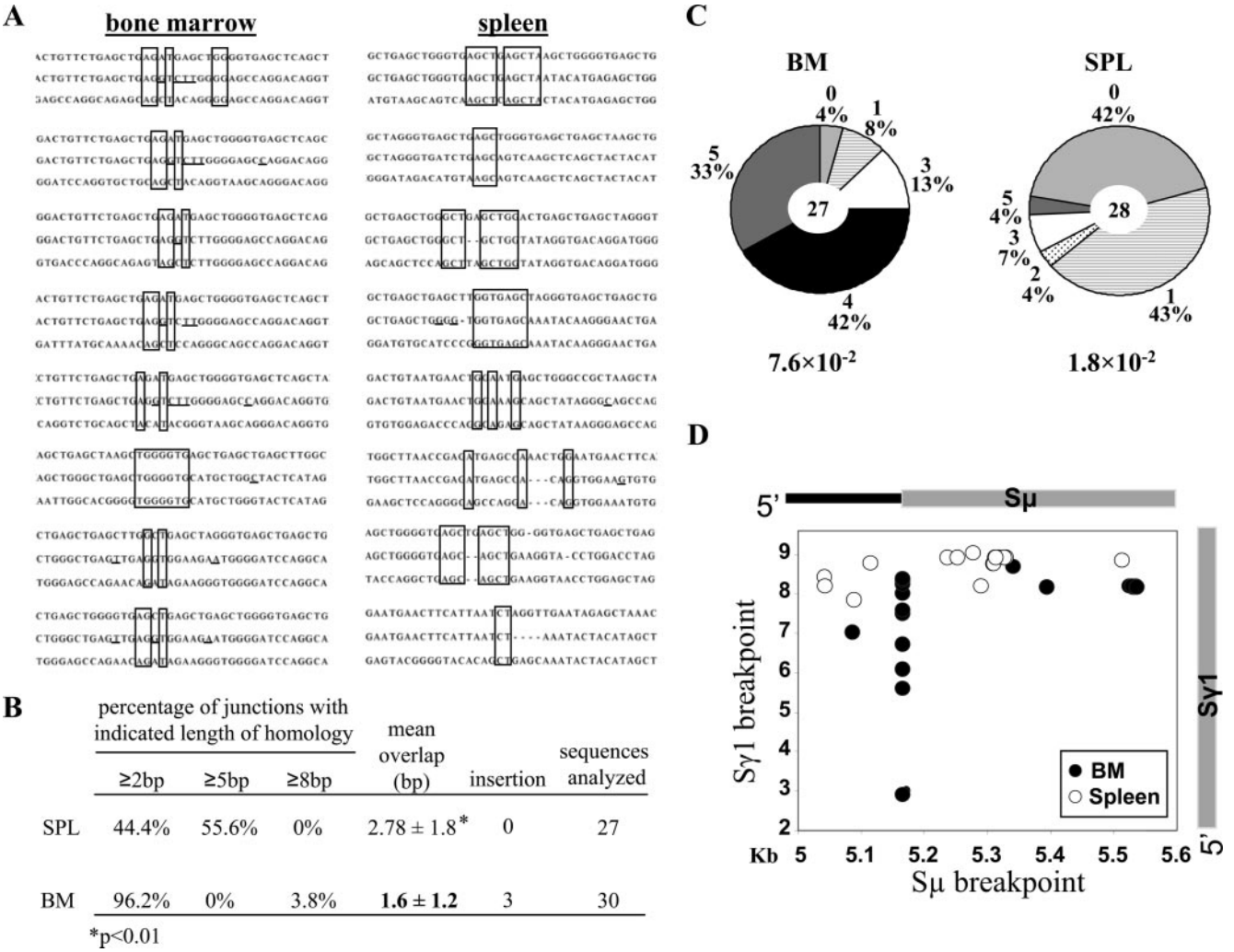

FIGURE 2. Analysis of Sμ/Sγ1 junctions in spontaneous CSR during B lymphopoiesis. IgG-expressing cells were sorted from 5-day BM culture of a normal mouse. For control, we sorted IgG-expressing cells from purified normal splenic B cells that were stimulated with LPS and IL-4. DNA was extracted from the purified cells and subjected to CSR junction analysis. *A,* Nucleotide sequences surrounding Sμ/Sγ1 break points. Overlap was determined by identifying the longest region of perfect uninterrupted donor/acceptor identity at the switch junction. The Sμ and the Sγ1 germline sequences are shown above and below the clone sequence, respectively. Mutations at switch junctions are underlined and homology at the junction is boxed. Shown are eight representative sequences from each group. *B,* Length of microhomologies at Sμ/Sγ1 junctions in BM and spleen (SPL) B cells. *C,* Mutation frequency at the ±50-bp vicinity of the Sμ/Sγ1 switch junction. *D,* Scatter analysis of the μ/γ1 break points. The *x*-axis indicates the position of the Sμ break point and the *y*-axis indicates the Sγ1 break point.

the CSR indicators in immature (IgM^+IgD^-) and in pro/preB ($B220^+IgM^-$) cell fractions that were sorted ex vivo from unimmunized wild-type mice (Fig. 1*B*). To further confirm occurrence of CSR, BM culture cells or ex vivo-sorted, IgG-depleted B cells were analyzed for expression of Cγ1 CTs (Iγ1-Cμ), which are synthesized from the excised circular DNA (25). The results in Fig. 1*C* reveal a significant expression of Cγ1 CT in both in vitro- and ex vivo-isolated precursor B cells. From this, we concluded that spontaneous CSR occurs at low levels throughout B lymphopoiesis.

Spontaneous CSR generates aberrant switch junctions

To study the spontaneous CSR in developing B cells, we sequenced the Sμ-Sγ1 joining region. To do so, we sorted IgG-expressing cells that were spontaneously generated in BM cultures from a wild-type mouse and cloned the Sμ-Sγ1 junctions. As a control, we used IgG-expressing cells generated from splenic B cells stimulated with LPS plus IL-4, whose Sμ-Sγ1 junctions are well defined (29). The junctional sequences that were obtained are presented in Fig. 2*A*. Sequence analysis revealed significant differences in the extent of donor/acceptor homology at the junctions (1.6 bp in BM B cells relative to 2.78 in the control-stimulated splenic B cells; $p < 0.01$, Fig. 2*B*). We also found that the mutation frequency in the ±50-bp vicinity of the Sμ-Sγ1 junctions was significantly increased (7.6×10^{-2} in BM B cells relative to 1.8×10^{-2} in the controls; $p < 0.001$, Fig. 2*C*). In addition, the breakpoint distribution in Sμ and Sγ1 measured by scatter analysis revealed excessive deletion of both switch regions in spontaneous CSR (Fig. 2*D*). Thus, we found that spontaneous CSR break points accumulate at the 5′ end of the Sμ and are distributed throughout the 9-kb Sγ1 region. This is in contrast to stimulated splenic B cells whose CSR breaks are distributed along the Sμ region and only within the first 2 kb of the Sγ1 region (Fig. 2*D*). Notably, in three clones generated by spontaneous CSR, we found at the break point a DNA fragment of 60–120 bp, which was not part of the original sequence at this site. By comparing the sequences of these fragments to the whole Sγ1 region, we found that they are highly repeated (>50 times) in it. The insertions thus likely reflect recombination/duplication events within the Sγ1 switch region.

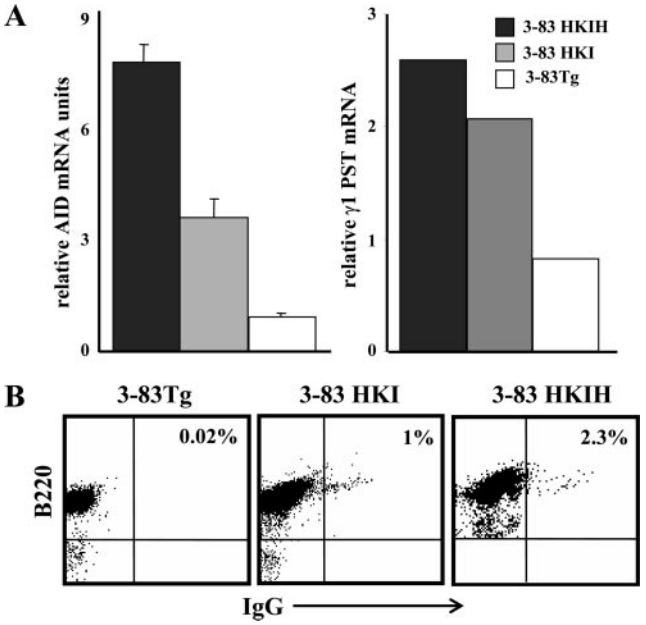

FIGURE 3. Spontaneous CSR in immature B cells was increased after VDJ assembly. Immature B cells were sorted ex vivo from BM of 3-83Tg, 3-83HKI, and 3-83HKIH mice and were analyzed to quantify spontaneous CSR. *A,* Levels of AID mRNA were determined by real-time quantitative PCR as detailed in *Materials and Methods* (*left*). Levels of γ1 PST were determined by semiquantitative RT-PCR and normalized to those of actin (*right*). Results are mean ± SE of four individual mice. *B,* BM cultures from 3-83Tg (*left*), 3-83 HKI (*center*), and 3-83Tg HKIH (*right*) mice were prepared and cells were stained for B220 and IgG and analyzed by FACS. The plots shown are from individual mice and are representative of three to four mice in each group.

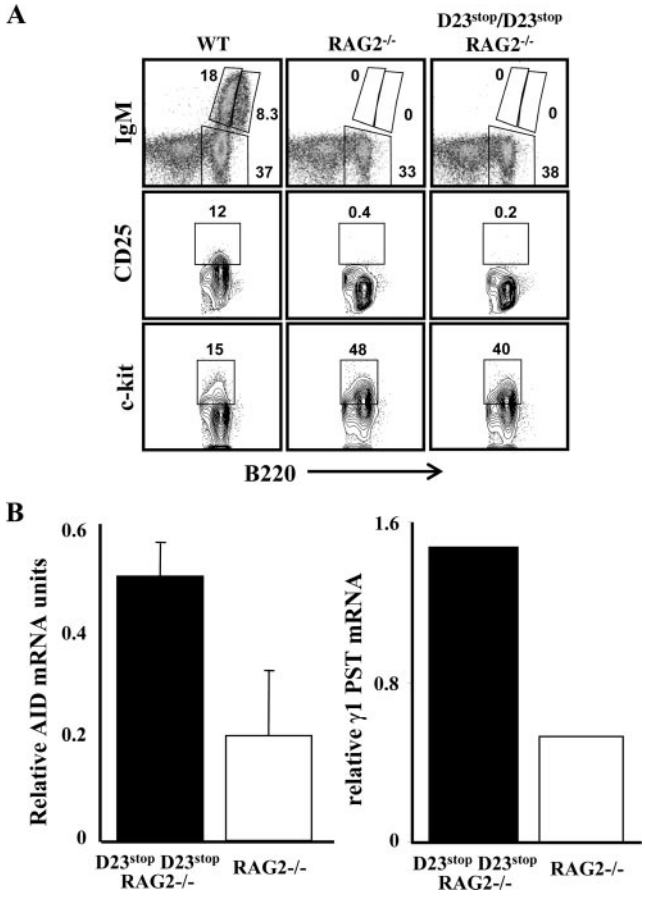

FIGURE 4. Spontaneous CSR in early proB cells was increased after VDJ assembly. *A,* BM cells from mice with the indicated genetic background were stained for B220, IgM, CD25, and c-kit and analyzed by FACS. *B,* $CD19^+$ cells were sorted ex vivo from BM of the indicated mice and analyzed to quantify spontaneous CSR. Levels of AID mRNA were determined by real-time quantitative PCR (*left*). Levels of γ1 PST were determined by semiquantitative PCR and normalized to those of actin (*right*). Results are mean ± SE of three individual mice.

Thus, we conclude that spontaneous CSR during B lymphopoiesis generates aberrant Sμ-Sγ1 junctions.

Increased spontaneous CSR in B cells that carry VDJ rearranged alleles

The V-D-J recombination initiates at the H chain locus at the proB stage. Since we found spontaneous CSR in proB cells (Fig. 1), we studied whether VDJ assembly affects the occurrence of spontaneous CSR. To address this, we used two independent experimental settings. First, we addressed this question in immature B cells that are derived from Ig-Tg mice. To do so, we sorted immature B cells from BM of a conventional Ig-Tg mouse line (3-83Tg) that carries genes encoding the IgM and IgD forms of 3-83 (21). The transgene in this model is incorporated outside the Ig locus, but because of efficient allelic exclusion no V-to-DJ rearrangements were detected (30–32), leaving the endogenous locus upstream of DFL16 in germline configuration. We compared spontaneous CSR in these 3-83Tg immature B cells to spontaneous CSR in immature B cells sorted from BM of heterozygous (3-83HKI) or homozygous (3-83HKIH) mice bearing a site-targeted insertion of the 3-83 VDJ segment in the IgH locus, placing it in the physiological genomic context (22). The sorted cells were analyzed for indicators of CSR using quantitative PCR. Spontaneous CSR was observed in immature B cells from all mice (Fig. 3*A*). However, we found that AID expression increases 2-fold in the presence of a VDJ rearrangement in one of the two IgH loci (3-83 HKI relative to the 3-83Tg) and four-fold when two rearranged VDJ knock-in alleles are present (3-83HKI relative to the 3-83Tg; Fig. 3*A, left*). Similarly, IgG1 PSTs were elevated 2.5-fold in the presence of one knock-in allele and 3.2-fold when two 3-83 HKI alleles were present (Fig. 3*A, right*). Importantly, since the 3-83Tg VDJ is incorporated outside of its physiological context, no IgG-expressing cells could be detected in BM cultures (Fig. 3*B, left*). Such cells, however, could be found in BM cultures from 3-83HKI (Fig. 3*B, center*) and 3-83HKIH (Fig. 3*B, left*) mice.

In the second experimental setting, we addressed this question in proB cells. To do so, we sorted proB cells from BM of RAG2-deficient mice, which are devoid of VDJ rearrangements. In comparison, we sorted proB cells from mice carrying a site-directed insertion of a nonproductive $V_HD_HJ_H$ into its physiological position in the H chain locus (the $D23^{stop}$ allele (33)). However, since secondary H chain gene rearrangements rescue B cell development in this mouse (33), the $D23^{stop}$ mice were bred to a RAG2-deficient background, thus aborting B cell development at the early proB cell stage (Fig. 4*A*). The sorted cells were analyzed by quantitative PCR for levels of CSR markers. We found that the levels of AID in proB cells that are homozygous for the $D23^{stop}$ allele ($D23^{stop}D23^{stop}$ $RAG2^{-/-}$) were 2- to 2.5-fold higher than those in the $RAG2^{-/-}$ proB cells (Fig. 4*B, left*). Similarly, the levels of IgG1 PSTs were increased 2.8-fold in the $D23^{stop}D23^{stop}$ $RAG2^{-/-}$ proB cells relative to those of the $RAG2^{-/-}$ proB cells (Fig. 4*B, right*). Thus, we conclude that spontaneous CSR is increased in cells with a VDJ-rearranged IgH locus.

Discussion

Spontaneous CSR occurs during B cell lymphopoiesis, but its mechanism and regulation are unknown. Sequence analysis of the switch junctions from B cells that underwent spontaneous CSR revealed that they are significantly different from those generated in mature B cells that are stimulated to undergo CSR. This suggests that there are differences in the enzymatic machinery that carry out the process of spontaneous CSR or that the enzymatic machinery is the same, but the expression levels of its components differ in developing vs mature B cells. Such differences of expression levels have been shown to exist for BCR signaling intermediaries, such as B cell linker protein, Bruton's tyrosine kinase, and phospholipase Cγ2, and in the response to BCR signaling (34).

Because CSR is a deletional reaction, it is thought that dsDNA breaks are repaired by the nonhomologous end joining pathway, where base-excision repair or the mismatch repair machinery operate (7). The lack of proteins of the nonhomologous end joining or the DNA repair machineries, such as Msh-2 H2AX and 53BP1 (29, 35, 36), leads to a significant reduction in CSR efficiency. Their absence has also been shown to result in abnormalities at the switch junctions such as increased or decreased microhomology and increased frequency of short (35) and large (36) insertions (reviewed in (7, 35)). Similarly, we found here that spontaneous CSR occurs at low frequency and forms aberrant switch junctions. We found a decrease in microhomology at the Sμ-Sγ1 junctions in spontaneous CSR, which may suggest inappropriate processing of the DNA ends, as described for B cells deficient of the mismatch repair enzyme Msh-2 (35). Nevertheless, RT-PCR analysis revealed that Msh-2 is expressed in progenitor B cells that undergo spontaneous CSR (data not shown). We also found few Sμ-Sγ1 junctions with an inserted DNA fragment, although the sequences of these fragments corresponded to different sites than what was reported for 53BP1$^{-/-}$ cells. Hence, it is possible that another enzymatic component of the repair machinery is not expressed during B lymphopoiesis, leading to the formation of the aberrant switch junctions. However, according to the current understanding of the CSR process, this possibility does not provide a sufficient explanation for the excessive deletion of the switch regions that we found in spontaneous CSR during B cell development.

As an alternative hypothesis, we propose that the aberrant switch junctions in spontaneous CSR are formed as a consequence of deregulation of the CSR machinery or one of its components. It is possible that in B cells undergoing CSR early in development, AID expression is deregulated, resulting in higher or prolonged AID activity. Since AID activity initiates DNA lesions that are processed by other CSR enzymes into DNA breaks (37, 38), prolonged AID activity may result in an increased load of somatic mutations and excessive loss of the switch regions. Our sequencing analysis of spontaneous CSR revealed increased frequency of mutations in and excessive loss of both the Sμ and Sγ1 switch regions, which is in agreement with the expected outcome of deregulated AID expression. Furthermore, our data revealed that most of the breaks occurred at the 5′ end of the Sμ (Fig. 2*D*), where the most upstream S region repeat element that contains the G-rich consensus sites for AID is found. Thus, it is possible that higher or prolonged activity of AID causes DNA breaks at several tandem repeats along Sμ, but, ultimately, the most upstream one will be utilized for CSR. Our sequencing analysis also revealed a significantly reduced sequence overlap at the recombination break points in BM B cells that have undergone spontaneous CSR, which may also be the result of increased AID activity. In mature B cells, AID generates nicks at the nontemplate strand and this, combined with a closely spaced nick on the opposite strand, can generate double-strand breaks (7). It is possible that enhanced or prolonged AID activity increases the frequency of cytidine deamination and the subsequent formation of nicks, leading to double-strand DNA breaks with shorter single-stranded sticky ends, thereby forming joints with shorter donor/acceptor microhomology. Interestingly, we found that most of the breaks in spontaneous CSR (>95%) occur within the G-rich consensus sequence, relative to only 30% of the CSR induced in splenic B cells. Considering that cytidine deamination initiates nick formation also on the template strand (7), this finding may also be explained by increased activity of AID, since the CSR tandem repeats are G rich at the nontemplate strand and are therefore enriched in cytidine residues at the template strand along these regions. Eventually, this may increase the probability of cytidine deamination and nick formation within the CSR consensus repeats as we found here. Expression of AID is restricted to B cells in the germinal centers of the lymphoid organs (39). Studies by Muto et al. (40) have shown that in B cells transgenic for AID the accumulated AID protein is inactive. These findings propose a mechanism by which activated mature B cells are protected from increased AID activity that may result in aberrant translocations and tumorigenesis. It is possible that BM B cells fail to inactivate the AID protein, thus resulting in excessive activity. Hence, deregulated, prolonged activity of AID in developing B cells undergoing spontaneous CSR may be the cause of the aberrant switch junctions in the progenitor cells.

We have previously shown that spontaneous CSR in B lymphopoiesis is T cell independent (16, 41). Spontaneous CSR has also been found in transformed B cell lines (42, 43). In resting human B cells, a significant level of γ1, γ3, and ε H chain GLTs are found, but in the absence of stimulatory signals no CSR was observed (44). We found spontaneous γ1H (this study) and μH chain (data not shown) GLTs before and after VDJ rearrangements of the IgH genes, eventually resulting in CSR. However, the frequency of spontaneous CSR increases when a VDJ segment was assembled in the cells. It is possible that before VDJ assembly the accessibility of the CH genes at the IgH locus was limited (45). This is supported by the fact that germline transcription of the constant μH chain (Cμ), initiation of DJ and VDJ rearrangements, expression of μH, and opening of the Sμ region to CSR rely on regulatory elements such as the transcriptional enhancer elements (iEμ), which are located in the JH-Cμ intron (reviewed in Refs. 7 and 46). In this case, assembly of VDJ and transcription through the locus may render the CH region more amenable to spontaneous CSR. We do not know what may stimulate AID expression in B lymphopoiesis and why its level increases in cells carrying VDJ rearrangements. In a normal mouse, AID is mainly expressed in germinal center B cells (39). Studies have shown that AID is regulated by protein kinase A phosphorylation (47, 48), a pathway that also regulates V(D)J recombination and RAG gene expression (49, 50). Also, AID expression levels increase with successive divisions (51), and assembly of a productive VDJ segment in proB cells is known to promote cell division (52). However, proB cells that carry a nonproductive VDJ (D23^{stop}) should not have any proliferative advantage relative to proB cells whose IgH loci retain their germline configuration (RAG$^{-/-}$). It has also been shown that CSR frequency is positively correlated with the expression levels of AID (51). This is in agreement with our findings showing that proB cells or immature B cells carrying an assembled VDJ segment in their IgH loci (D23^{stop}, RAG2$^{-/-}$, and 3-83HKI, respectively) express higher levels of AID and IgG1 PSTs relative to proB and immature B cells whose IgH loci retain germline configuration (RAG2$^{-/-}$ and 3-83Tg, respectively; Figs. 3 and 4). A recent study has shown that AID expression levels in BM B cells are 500- to 1000-fold lower compared with AID levels in germinal

center B cells (19). This is in agreement with our studies showing low levels of AID in the overall population of developing B cells. However, it is possible that AID is expressed only in rare cells in the population. In these cells, AID may be expressed at a higher level or persist for a longer time than in germinal center B cells, thus resulting in the formation of aberrant switch junctions. Taken together, spontaneous CSR is a rare event in B cell development. Nevertheless, it generates a B cell population whose development is driven by non-IgM receptors.

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Disclosures

The authors have no financial conflict of interest.

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