TOLL-like receptor ligands stimulate aberrant class switch recombination in early B cell precursors

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Keywords: antibodies, B cells, gene rearrangement, hematopoiesis

Abstract

TOLL-like receptor (TLR) ligands stimulate class switch recombination (CSR) in mature B cells. We showed earlier that developing B cells in the bone marrow (BM) express TLR9 and are responsive to CpG DNA. Since CSR is a critical process for synthesis of effector antibodies, we studied the competence of precursor B cells to undergo CSR in response to TLR ligands, and the regulation of these cells. We found that CSR is induced throughout B lymphopoiesis in response to CpG and to LPS. However, sequencing analysis revealed aberrant joining of the switch junctions. In addition, we found that this CSR is independent of IgM expression and/or VDJ assembly and is directed to a specific isotype by cytokines. Finally, we found that activation of the switched precursor B cells is regulated by Fas. Thus, BM B cells can be activated by TLR ligands to undergo CSR and to secrete non-IgM antibodies. However, the effector potential of these cells is regulated by the Fas pathway.

Introduction

B lymphocytes utilize IgM receptors during bone marrow (BM) lymphopoiesis to signal for positive and negative selection and to promote development (1, 2). Upon activation in the periphery, mature B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) to replace the IgM receptors with a downstream immunoglobulin heavy chain isotype (IgH), in order to produce high-affinity effector antibody (3). Both processes critically depend on the expression and function of activation-induced cytidine deaminase (AID) [reviewed in (4)].

CSR occurs between two switch (S) regions located upstream to each constant heavy-chain (CH) gene (except to Cd), and results in a deletion of the intervening DNA fragment (4). Transcription through the S region is the initial step of CSR (4), and cytokines play a role in targeting the CSR to a specific CH isotype (5). Switch regions are guanine-cytosine (GC)-rich, vary in size and are the targets of AID, which is the only B cell-specific factor required for CSR and SHM (4, 6). AID is expressed in germinal center B cells in vivo or in B cells undergoing CSR in vitro (7).

Mature B lymphocytes express TOLL-like receptors (TLRs) and respond to microbial products such as LPS and CpG-enriched DNA. These T-independent antigens stimulate polyclonal B cell proliferation and differentiation to antibody-producing cells (8, 9). Both LPS and CpG stimulate AID expression and CSR in mature B cells (10, 11). A recent study has shown that extensive proliferation of mature naive B cells, isotype switching and differentiation to antibody-secreting cells critically depend on signals transduced through TLRs (12). Hence, TLRs are important regulatory molecules in balancing the immune response of mature B cells to antigens.

In contrast, the role of TLRs during early stages of B lymphopoiesis in the BM is less clear. We and others have shown that developing B cells express TLR4 and RP105 and respond to LPS (13–15). Recently, we have shown that TLR9 is expressed in all stages of B lymphopoiesis, and the cells proliferate and differentiate to IgM-secreting cells in response to stimulation with CpG DNA (16). Thus, developing B cells are responsive to TLR ligands, but their competence to undergo CSR in response to these immunological stimuli is unknown. Earlier studies have shown the induction of CSR in A-murine leukemia virus (MuLV)-transformed pre-B cell lines (17), and in RAG2⁻/⁻ and normal developing B lymphocytes that are treated with anti-CD40 antibodies and IL-4 (18). In the later study, however, cells were first induced to differentiate as revealed by the expression of mature B cell markers (18). We and others have shown that CSR spontaneously occurs at low frequency during B lymphopoiesis (19–21), which is consistent with a recent study showing that γH1 expression is compatible with B cell development and maturation (22) and that BM B cells acquire SHM (21).

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Received 11 June 2008, accepted 26 September 2008

Advance Access publication 29 October 2008
However, spontaneous CSR in BM B cells generates aberrant switch junction, which may result from de-regulation of the CSR enzymatic machinery (20). Here we show that CpG DNA and LPS stimulate CSR in all stages of B cell development, which are independent of BCR expression and/or VDJ assembly. Similar to the spontaneous CSR, we found that CSR induced in BM B cells by mitogenic stimuli generates aberrant switch junctions and that isotype-switched B cells are negatively regulated by the Fas pathway. Thus, BM B cells can be activated to produce effector non-IgM antibodies, but this potential is regulated by Fas.

Methods

Experimental mice

Mice used for this study were normal B10.D2SnJ, 3-83Tg B10.D2Sn/J expressing IgM/IgD BCR specific to class I MHC antigens K\(^{a}\) and K\(^{b}\) (23), 3-83HkJ B10.D2Sn/J (24) and C57BL/6-\(\mu\)MT/\(\mu\)MT (\(\mu\)MT) (25). Mice were housed and bred at the animal facility of the Technion, Faculty of Medicine, and used for the experiments at 4–8 weeks old. All mice studies were approved by institutional committee for the supervision of animal experiments.

BM B cell culture

B cell precursors were grown in vitro as previously described (26). Briefly, BM cells were depleted of erythrocytes and Ig+ cells on day 0 and were cultured in iscove modified dulbecco medium (Biological Industries, Israel) with 50–100 U ml\(^{-1}\) of rIL-7 for 5 days. The cells grown in these primary cultures are highly enriched for the B lineage (>95% B220\(^{+}\)) and C57BL/6-\(\mu\)MT/\(\mu\)MT (\(\mu\)MT) (25). Mice were housed and bred at the animal facility of the Technion, Faculty of Medicine, and used for the experiments at 4–8 weeks old. All mice studies were approved by institutional committee for the supervision of animal experiments.

Flow cytometry and magnetic cell sorting

Single-cell suspensions were stained for surface markers using FITC-, PE- and biotin-conjugated antibodies. The following antibodies were used: anti-B220 RA3-6B2; goat anti-mouse \(\gamma\)H-specific (all from Southern Biotechnology Associates, Inc.); anti-CD43 Ly-48, Leukosialin; anti-IgD JA12.5, anti-Fas (CD95) Jo2 (all from BD Bioscience). Data were collected on a FACS Calibur\textsuperscript{TM} and analyzed using CELLQuest\textsuperscript{TM} software (Becton Dickinson). Cells grown in BM cultures were first depleted of erythrocytes and Ig+ cells (27) and then sorted into pro-/pre-B (B220+/CD43+/IgM-/IgD-/IgG-) and immature B (B220+/CD43+/IgM+/IgD+/IgG-) and transitional B (B220+/CD43+/IgM+/IgD+/IgG-) for cellular and molecular analyses. The sorting procedure was as described (20). The detailed phenotypic analysis of the cultured cells has been shown (20).

B cell activation and analysis of antibodies

B cell precursors from BM cultures or from ex vivo BM specimens that were depleted of IgG-expressing cells and then sorted into specific subsets as detailed above were stimulated with the synthetic oligonucleotides 1826 (CpG ODN) and 1982 (non-CpG ODM) (27) (InvivoGen, San Diego, CA, USA) at 100 nM, or with LPS (50 \(\mu\)g ml\(^{-1}\)) or LPS and IL-4 (10 ng ml\(^{-1}\)) for 72 h at 2 \(\times\) 10\(^6\) cells ml\(^{-1}\). Concentrations of total IgG or specific IgG isotypes in the supernatants were determined by sandwich ELISA using specific goat anti-mouse polyclonal reagents (Southern Biotechnology Associates, Inc.). Antibody concentrations were calculated using a reference standard curve of purified IgG antibodies. In some experiments, cells were stimulated with CpG DNA in the presence of activating hamster anti-Fas antibodies (clone Jo2, BD PharMingen) at a concentration of 5 \(\mu\)g ml\(^{-1}\) as described (19). To determine the frequency of antibody-producing cells, the stimulated cells were collected, washed and placed over anti-IgG-coated membranes, as we have described (ELISPot) (19).

Gene expression by reverse transcription–PCR

Total RNA was extracted from cells using RNA-Bee (Tel-Test, Inc., Friendswood, TX, USA) according to manufacturer’s instructions and reversed transcribed into cDNA using M-MuLV (Promega). Expression of AID; Cy1 germ line transcripts (GLTs) and PSTDs; Cy2b GLTs and PSTDs; Fas and actin was determined by reverse transcription (RT)–PCR in serial dilutions of the cDNA as previously described (19, 28). PCR products were fractionated by electrophoresis on 2% agarose gels. Real-time quantitative PCR for AID expression was performed using SYBR Green PCR Master Mix (AB gene) in an ABI-Prism 7000 sequence detection system (Applied Biosystems, Nieuwkerk aan den IJssel, the Netherlands). The AID levels were normalized with \(\beta\)2-microglobulin. The primer sequences used for AID and \(\beta\)2-microglobulin amplification, the conditions and the analysis were as we have described (20).

Analysis of switch recombination junctions

Analysis of CSR junctions was performed as described (29). Briefly, BM culture B cell precursors were depleted of IgG and stimulated with CpG DNA for 72 h. The IgG-expressing cells were sorted by magnetic beads and genomic DNA was extracted. The PCR conditions and primer sequences were as described (29). Nested PCR amplification of \(\text{Sl}/\text{Sl}2b2\) was done using the \(\text{Sl}1\) and \(\text{Sl}2b.1\) primers for the first round. The second PCR round was done using \(\text{Sl}2b2\) and \(\text{Sl}2b.2\) primers (29). PCR products were purified, cloned into pGEM and sequenced. CSR junctions were analyzed by using BLAST with the low-complexity filter disabled. Mutations were determined at the \(\pm 50\) bp vicinity of the switch junction as described (30).

Fluorescence in situ hybridization

B cell precursors from 3-83Tg BM cultures were incubated in the presence or absence of CpG (100nM) for 72 h. Cells were then collected and treated with hypotonic KCl solution,
fixed in methanol:acetic acid (3:1) and dropped on poly-L-lysine slides (Sigma). Fluorescence in situ hybridization (FISH) was performed as described previously (31). Briefly, denaturation of DNA was done by incubation in 70% deionized formamide, followed by incubation with 2× SSC at 68°C for 2 min. The slides were then dehydrated by a series of ice-cold ethanol washes (70, 90 and 100% for 5 min each). Plasmid DNA containing the probe fragment was labeled by nick-translation kit (Amersham), substituting deoxythymidine triphosphate with biotin–16-dUTP or with digoxigenin–11-dUTP (Roche). Unincorporated nucleotides were separated from the probe solution by centrifugation through 1-ml Sephadex G-50 columns (Amersham). Probe DNA (10–50 ng) was mixed with cot-1 DNA (Life Technologies) (3 μg) and salmon sperm DNA (Sigma) to obtain a total of 10 μg of DNA in a 10-μl hybridization solution. The probe mixture was denatured (80°C for 5 min) and pre-annealing of repetitive DNA sequences was done for 10 min at 37°C. The probe solution was then added to the analyzed specimens for hybridization at 37°C for 14 h. Subsequently, slides were washed (50% formamide, 2× SSC and 2× SSC) and samples were blocked (3% BSA, 4 SSC) for 30 min at 37°C. Bound biotin-labeled probes were detected with Cye-3-conjugated avidin (1:200 dilution) (Sigma) and bound digoxigenin-labeled probes were detected with an anti-digoxigenin antibody conjugated to FITC (Roche) (1:100 dilution). Slides were counterstained using diamidinophenylidole(200 ng ml−1) in Vector antifade solution (Vector Laboratories) and inspected by fluorescence microscope. For these experiments, the following probes were used: pGLRICμ, containing the 10.7-kb EcoRI fragment harboring the Cμ gene and pDFL3p8 (containing a 5’ 3.8-kb fragment upstream of DFL16.1 at the heavy-chain locus) (31).

**Statistical analysis**

Statistical significance in CSR junction length of homology and mutations frequency compared with controls was determined by a two-tailed t-test assuming unequal variance. An unpaired two-tailed Student’s t-test was used to determine significance in the frequencies of IgG-expressing cells. Values of fold of induction were used for the analysis and P < 0.05 was considered significant.

**Results**

*CpG DNA stimulates CSR throughout B lymphopoiesis*

In an earlier study we showed that TLR9 is expressed in all stages of B lymphopoiesis and that the cells proliferate and differentiate to IgM-producing cells in response to CpG DNA (16). To test whether CpG DNA stimulates CSR in developing B cells, we have first used our BM culture system (32). However, we have recently shown that IgG-expressing B cells are generated de-novo from early precursors in BM cultures of normal mice (19). The formation of this cell population, which reaches to ~2%, is due to a process of spontaneous CSR ((19, 20) (see also Fig. 1A). Hence, in all the experiments utilizing the BM culture cells, we have first depleted the IgG-expressing cells that were generated by spontaneous CSR. Normal mouse BM cells, which were depleted of all Ig+ cells on day 0 were cultured for 5 days. The B cell precursors that were grown (Fig. 1A, top) were first depleted of the IgG-expressing B cells and then sorted into pro/pre-B (B220+/CD43+/IgM−/IgG−), immature B (B220+/CD43−/IgM+IgD+/IgG−) and transitional B (B220+/CD43−/IgM+IgD+/IgG−). Sorted cells were cultured in the absence or presence of CpG DNA (100 nM) for 72 h, stained for B220 and IgG and analyzed by FACS. The initial 5-day BM culture prior to the depletion of IgG-expressing cells is shown on top. The plots for each sorted B cell subset stimulated, CpG-stimulated and control are shown below. The results shown are from an individual mouse and are representative of five experiments. (B) Ex vivo BM specimens were first depleted of IgG- and IgD-expressing cells and then sorted into pro-pre-B (B220+/IgM−/IgD−/IgG−) and immature (B220+/IgM+/IgD+/IgG−) B cell subsets. Sorted cells were cultured in the absence or presence of CpG DNA (100 nM) for 72 h, stained for B220 and IgG and analyzed by FACS. Shown are plots for each sorted B cell subset stimulated with CpG DNA and the control. The results shown are from an individual mouse and are representative of three experiments.
marker AA4.1 and were IgG− (data not shown), thus confirming the lack of contaminating mature and/or memory B cells and IgG-expressing cells that were generated in vitro by spontaneous CSR [for cell phenotype and sorting, see also (16, 19, 20)]. Upon stimulation with CpG DNA for 72 h, a substantial population of IgG-expressing B cells was detected in all sorted fractions. The size of the switched population was in correlation with the stage of development and reached >2% in the pro-/pre-B fraction, >4% at the immature fraction and >7% at the transitional fraction. In contrast, in the control unstimulated cells, or stimulated with non-CpG oligo, only low frequencies of IgG-expressing cells were detected, owing to the ongoing process of spontaneous CSR (Fig. 2A). Statistical analysis revealed that this induction is specific in all subsets (in pro-/pre-B fold of induction was 2.6 ± 0.4, in immature fold of induction was 3.6 ± 0.9, in transitional fold of induction was 2.1 ± 0.5, P < 0.05). To confirm the biological relevance of these findings, we sorted pro-/pre- and immature B cells from ex vivo isolated BM cells. Figure 1(B) shows that CpG DNA stimulates CSR in each sorted fraction as revealed by a substantial population of IgG-expressing cells (2.8 and 7.1% at the pro-/pre- and immature, respectively). This induction was statistically significant (in pro-/pre-B fold of induction was 2.4 ± 0.4, in immature fold of induction was 3.2 ± 1, P < 0.05).

As mentioned earlier, in the control unstimulated cells only low frequency of spontaneous switching was detected (~1%).

Expression of AID and production of IgG germ line and post-switch transcripts (PSTs) are indicators for ongoing CSR (28). Figure 2(A) shows that CpG DNA stimulates a profound expression of AID and production of IgG2b GLTs (Igμ-Cμ) and expression of IgG2b PSTs (Igμ-Cγ2b) in all sorted B cell subsets. In contrast, very low levels of these markers are evident in the control unstimulated cells, or stimulated with non-CpG oligos, owing to spontaneous CSR. Quantitative analysis revealed a significant 2.5- to 3.5-fold increase in AID expression upon stimulation with CpG DNA over the level that is spontaneously expressed in the developing BM cells, with no difference between B cell fractions (P < 0.05, Fig. 2B). Lastly, we show that CpG DNA stimulates the production and secretion of IgG antibodies in all stimulated fractions. The amount of the secreted IgG, however, is correlated with the stage of development (Fig. 2C). The analysis of ex vivo isolated pro-/pre- and immature B cells revealed similar results both for AID expression (Fig. 2D) and IgG secretion (Fig. 2E). AID was specifically induced, and IgG was specifically secreted, upon the stimulation with CpG DNA, but not in the respective ex vivo isolated cells that were unstimulated or stimulated with non-CpG oligo. Thus, although spontaneous CSR occurs in all stages of B lymphopoiesis, the

![Fig. 2. CpG DNA stimulates CSR throughout B lymphopoiesis. (A–C) The indicated B cell fractions were sorted from BM cultures and stimulated with CpG DNA or with non-CpG DNA (100 nM) as described in Fig. 1(A). Stimulated cells, as well as unstimulated BM culture cells, were harvested and analyzed for expression of AID and the presence of γ2b GLTs and PSTs by RT-PCR. Normal splenic B cells either unstimulated or stimulated with CpG DNA (100 nM) were used as controls for induction of the CSR markers (A). The levels of AID mRNA in each B cell subset (stimulated with CpG DNA, non-CpG DNA or unstimulated) were determined by real-time quantitative PCR as detailed in the Method (B). Supernatants of stimulated and unstimulated cells were collected and assayed for IgG by ELISA. IgG concentrations were determined using a reference IgG standard curve and are expressed as ng/ml (C). The results shown in (A–C) are from an individual mouse and are representative of five experiments. (D–E) The indicated B cell fractions were sorted ex vivo from BM specimens and stimulated with CpG DNA or with non-CpG DNA as described in Fig. 1(B). The stimulated and the unstimulated controls were then collected and analyzed for AID expression by RT-PCR (D), and the supernatants were assayed for IgG by ELISA (E). The results shown in (D–E) are from an individual mouse and are representative of three experiments.](https://academic.oup.com/intimm/article-abstract/20/12/1575/685155)
stimulation of precursor B cells with CpG DNA significantly enhances the CSR process and the consequential formation of IgG-expressing cells.

CpG-induced CSR in developing B cells generates aberrant switch junctions

To further study the CSR induced in developing B cells by CpG, we sequenced the Sl-S2b joining region and compared it to Sl-S2b junctions that are generated in mature splenic B cells stimulated by CpG. To do so, BM culture B cells (that were depleted of IgG-expressing cells) or purified splenic B cells (that were depleted of IgG-expressing cells) were stimulated with CpG DNA. The IgG-expressing cells were sorted and the Sl-S2b junctions were cloned and sequenced (Fig. 3A). Sequence analysis revealed significantly reduced sequence overlaps at the recombination breakpoint, (1.6 bp in BM B cells relative to 2.8 in the control stimulated splenic B cells; \( P < 0.02 \), Fig. 3B), and a significant increase in mutation frequency (4.8 \( \times 10^{-2} \) in BM B cells relative to 1.2 \( \times 10^{-2} \) in the controls; \( P < 0.002 \), Fig. 3C). The breakpoint distribution in Sl and S2b measured by scatter analysis revealed that in many of the BM clones there was an excessive deletion of the S2b S region (Fig. 3D). Interestingly, we found that CSR breaks in BM B cells were distributed throughout the Sl and S2b regions whereas those of the splenic B cells accumulated at two sites (Fig. 3D). We conclude that CSR induced by CpG DNA in developing B cells generates aberrant Sl-S2b junctions.

CSR induction is independent of IgM expression

Because CSR analysis in our experiments was conducted 72 h after stimulation of the B cell precursors, it was possible that IgM- cells have undergone developmental progression to an IgM+ stage and acquired increased CSR responsiveness to CpG. To test this possibility, we analyzed the induction of CSR in B cell precursors derived from mice lacking the IgM-transmembrane tail exons (\( \mu \)MT mice) (25). In these mice, B cell development is aborted at the pro-B stage due to the failure to express surface \( \mu \)H and the pre-BCR. The occurrence of spontaneous CSR generates few IgG-expressing cells which can be detected in vitro (19), but such cells fail to mature in vivo in C57Bl6 mice. The BM culture cells, which were prepared from these \( \mu \)MT mice, were first depleted of IgG-expressing cells that were generated by spontaneous CSR (19), and then were either unstimulated or stimulated with CpG DNA. The results in Fig. 4 show that CpG DNA stimulated significant expression of AID and production of \( \gamma \)2b GLTs and PSTs in \( \mu \)MT proB cells, relative

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**Fig. 3.** Analysis of Sl/S2b junctions in CpG-stimulated B cell precursors. BM culture cells from a normal mouse were depleted of IgG-expressing cells and stimulated for 72 h with CpG (100 nM). After stimulation, IgG-expressing cells were collected for analysis of the CSR. For control, we sorted IgG-expressing cells from purified normal splenic B cells that were stimulated with CpG. DNA was extracted from the purified cells and subjected to CSR junction analysis. (A) Nucleotide sequences surrounding Sl/S2b breakpoints. Overlap was determined by identifying the longest region of perfect uninterrupted donor/acceptor identity at the switch junction. The Sl and S2b germ line 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 the BM cells. In the splenic cells, we found that all CSSRs occur at two different sites and two sequences of representative clones shown. (B) Length of microhomologies at Sl/S2b junctions in BM and SPL B cells. (C) Mutation frequency at the +50-bp vicinity of the Sl/S2b switch junction. (D) Scatter analysis of the \( \mu \)2b breakpoints. The x-axis indicates the position of the Sl breakpoint and the y-axis indicates S2b breakpoint.
to the levels of spontaneous CSR in the control BM cells (Fig. 4A). This stimulation resulted in the generation of a substantial population (7.5%) of IgG-expressing cells, whereas only ~2.5% were generated by spontaneous CSR in the control unstimulated cultures (Fig. 4B). This induction was statistically significant (fold of induction was 2.8 ± 0.6, P < 0.05). We concluded that CpG-induced CSR in early B cell precursors is independent of IgM expression.

Inducible CSR in immature B cells occurs at heavy-chain loci that carry or do not carry VDJ rearrangement

Stimulation of mature B cells results in germ line transcription on both the functional and the non-functional alleles of the heavy chain (33). This prompts us to test whether developing B cells can undergo CpG-induced CSR at IgH loci which do not carry an assembled VDJ. To do so, we used a conventional Ig transgenic mouse line (3-83Tg) that carries genes encoding the heavy and light chains of the IgM and the IgD forms of 3-83 (23). The transgene in this model is incorporated outside the Ig locus, but because of efficient allelic exclusion no V-to-DJ rearrangements are detected (34–36), leaving the endogenous IgH locus upstream of DFL16 unarranged in a germ line configuration. Results in Fig. 5(A) show a significant expression of AID and synthesis of γ2b GLTs and PSTs in immature and transitional 3-83Tg B cells that are stimulated with CpG DNA, relative to unstimulated cells (Fig. 5A, first left lane). However, since the 3-83 transgene includes only μH and δH regions, and is not inserted in its physiological gene context (23), no IgG-expressing cells (Fig. 5B, left) or secreted IgG (Fig. 5C) are detected. For this reason, there are no IgG-expressing cells in the unstimulated cultures despite the occurrence of spontaneous CSR [data not shown, see also (20)]. To confirm that the induced CSR occurred on the non-rearranged allele, we analyzed the configuration of the IgH loci by a FISH assay. To do so, we used probes corresponding to the Cμ gene and to a fragment upstream of DFL16.1 (Fig. 5D). The probe containing a fragment upstream of DFL16.1 binds to the unrearranged Dμi region in the absence of assembled VDJ (green fluorescence), a configuration present in 3-83Tg B cells (36). In the absence of CSR, the Cμ region is visualized using the Cμ probe (red fluorescence). Thus, germ line configuration is visualized with green and red fluorescent dots (Fig. 5D, marked with yellow arrows). The CSR process (in the absence of assembled VDJ, such as in the 3-83Tg cells) deletes the Cμ region, thus leaving only the probe upstream of DFL16.1 bound to the switched allele and yielding only one green fluorescent dot (Fig. 5D, marked with a yellow arrow). Importantly, the Cμ probe also binds to the transgenic 3-83 sequence, yielding an additional fluorescently red dot in each B cell, which was conveniently referred as a control (Fig. 5D, marked with a yellow arrow). FISH analysis of 3-83Tg B cell precursors that were stimulated with CpG DNA revealed that a substantial proportion of the cells (~14%, Fig. 5D) carry at least one switched allele, but maintain the V-D-J region in germ line configuration. In the unstimulated cultured only unswitched alleles were detected by the FISH assay (data not shown). To confirm that the 3-83 VμiDμiJμi segment can undergo CSR, we used mice carrying a site-targeted insertion of the 3-83 VHDJH into the Ig-H locus, placing it in its physiological genomic context (24) (3-83HKI). We found that the stimulation of 3-83HKI B cell precursors with CpG DNA resulted in a significant expression of AID (data not shown) and the appearance of a statistically significant population of IgG-expressing cells (2.5%, Fig. 5C). We have previously shown, ~1% of IgG-expressing cells are found in the unstimulated 3-83HKI BM cell culture B cells by spontaneous CSR [data not shown, see also (20)]. Unlike the 3-83Tg cells, we found that stimulated 3-83HKI cells secreted high amount of IgG [fold of induction 2.45 ± 0.2, P < 0.05, Fig. 5C]. RT-PCR analysis revealed an mRNA product corresponding to the Cμ gene that is specific to the targeted allele (detected by primers specific for the 3-83 Vμi-Dμi-Jμi region) (Fig. 5D). The probe corresponding to the Cμ region is visualized using the Cμ probe (red fluorescence), a configuration present in 3-83Tg B cells (36). In the absence of CSR, the Cμ region is visualized using the Cμ probe (red fluorescence). Thus, germ line configuration is visualized with green and red fluorescent dots (Fig. 5D, marked with yellow arrows). The CSR process (in the absence of assembled VDJ, such as in the 3-83Tg cells) deletes the Cμ region, thus leaving only the probe upstream of DFL16.1 bound to the switched allele and yielding only one green fluorescent dot (Fig. 5D, marked with a yellow arrow). Importantly, the Cμ probe also binds to the transgenic 3-83 sequence, yielding an additional fluorescently red dot in each B cell, which was conveniently referred as a control (Fig. 5D, marked with a yellow arrow). 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IL-4 directs LPS-induced CSR in B cell precursors

Cytokines direct CSR in mature B cells towards T1,1 (IgG2a and IgG2b) or T1,2 (IgG1 and IgE) isotypes (5). Hence, we have tested whether cytokines can also direct the CSR induced in precursor B cells. Because CpG DNA stimulates a T1,1 response and inhibits switching to IgG1 (37, 38), we used LPS to stimulate normal B cell precursors in these experiments, as we have described (13). BM culture cells...
were first depleted of IgG- and IgD-expressing cells and then sorted to pro-/pre-B (IgM⁺/C0) and immature (IgM⁺IgD⁻) populations and cultured in the presence or absence of CpG DNA (100 nM) for 72 h. Expression of AID and presence of γ2b GLTs and PSTs was determined by RT-PCR. Normal splenic B cells either unstimulated or stimulated with CpG were used as controls for CSR markers. (B) BM culture cells from 3-83Tg or from 3-83HKI (IgG-depleted) were stimulated with CpG DNA for 72 h and stained for B220 and IgG and analyzed by FACS. (C) BM culture cells from normal 3-83HKI and 3-83Tg mice were collected, depleted of IgG and stimulated with 100 nM CpG DNA for 72 h. Supernatants were collected and analyzed for the presence of IgG by ELISA. IgG concentrations were determined using a reference IgG standard curve and are expressed as ng/ml. (D) FISH analysis of VDJ assembly and μH CSR. Schematic presentation of the genomic locus analyzed by the FISH assay before and after CSR and the location of probes is shown on top. B cell precursors from 3-83Tg BM cultures were incubated in the presence or absence of 100 nM CpG for 72 h and analyzed by FISH for VDJ assembly and μ heavy-chain CSR. Germ line loci (indicated by arrows) are visualized by green and red dots. Loci that have undergone CSR (switched alleles, indicated by an arrow) do not bind the red probe and are visualized by green dot. In each cell, an additional red dot is present, which corresponds to the transgenic 3-83 heavy chain (in multiple copies). The results shown are from individual mice and represent three to five experiments.

Fig. 5. Inducible CSR occurs at heavy-chain loci that carry or do not carry VDJ rearrangement. (A) 3-83Tg B cell precursors that were grown in BM cultures were sorted to immature (IgM⁺IgD⁻) and transitional (IgM⁺IgD⁻) populations and cultured in the presence or absence of CpG DNA (100 nM) for 72 h. Expression of AID and presence of γ2b GLTs and PSTs was determined by RT-PCR. Normal splenic B cells either unstimulated or stimulated with CpG were used as controls for CSR markers. (B) BM culture cells from 3-83Tg or from 3-83HKI (IgG-depleted) were stimulated with CpG DNA for 72 h and stained for B220 and IgG and analyzed by FACS. (C) BM culture cells from normal 3-83HKI and 3-83Tg mice were collected, depleted of IgG and stimulated with 100 nM CpG DNA for 72 h. Supernatants were collected and analyzed for the presence of IgG by ELISA. IgG concentrations were determined using a reference IgG standard curve and are expressed as ng/ml. (D) FISH analysis of VDJ assembly and μH CSR. Schematic presentation of the genomic locus analyzed by the FISH assay before and after CSR and the location of probes is shown on top. B cell precursors from 3-83Tg BM cultures were incubated in the presence or absence of 100 nM CpG for 72 h and analyzed by FISH for VDJ assembly and μ heavy-chain CSR. Germ line loci (indicated by arrows) are visualized by green and red dots. Loci that have undergone CSR (switched alleles, indicated by an arrow) do not bind the red probe and are visualized by green dot. In each cell, an additional red dot is present, which corresponds to the transgenic 3-83 heavy chain (in multiple copies). The results shown are from individual mice and represent three to five experiments.

Regulation of CpG-induced IgG production in B cell precursors by Fas–FasL pathway

We have previously shown that IgG-expressing BM B cells, which are generated by spontaneous CSR, are eliminated through the Fas–FasL pathway (19). Figure 7 shows that CpG DNA stimulates a significant increase in the expression of Fas, as revealed by surface expression in BM culture and in ex vivo-sorted BM B cells (Fig. 7A) and in mRNA analysis (Fig. 7B). We found that treatment of B cell precursors with activating anti-Fas antibodies (Jo2) significantly suppressed the amount of secreted IgG (Fig. 7C) and the frequency of IgG-secreting cells (Fig. 7D) in response to CpG DNA. This suggests that B cell precursors that undergo CSR
Discussion
Early B cell precursors express TLRs and respond to TLR ligands (13–16). We show here that developing B cells undergo CSR in response to these mitogenic signals and differentiate to IgG-producing cells. Furthermore, we found many similarities between the CSR induced in BM B cells and the CSR induced in peripheral B cells, suggesting that developing B cells have the potential to participate in an immune response. Sequencing analysis of the switch regions, however, revealed significant differences relative to those induced in mature B cells. This suggests that the CSR enzymatic machinery may be differentially regulated in BM and mature B cells that are stimulated by TLR ligands.

Our results suggest that CpG DNA and LPS stimulate CSR in all stages of B cell development. This is consistent with earlier studies showing that developing B cells express TLR9 (16) and TLR4 (14) and respond to CpG DNA and LPS. It is also in agreement with a recent study showing that immature and transitional B cells elevate AID expression and undergo CSR in vivo in response to bacterial vaccine (39). In contrast, treatment with anti-CD40 antibodies and IL-4 failed to stimulate CSR in developing B cells that are not expressing a mature phenotype (18). This, however, may reflect the low level of CD40 expression in these cells, which is significantly increased upon maturation (18). An alternative explanation is that developing B cells may have a different CSR enzymatic machinery than mature B cells that is regulated by different signals.

in response to CpG DNA are sensitive to Fas-mediated regulation.
possibility is that the competence to undergo CSR in response to treatment with anti-CD40 antibodies and IL-4 is developmentally regulated, as has been shown for the responsiveness to BCR ligation (40).

We found that CSR induced in BM B cells has many similarities to CSR induced in mature B cells. CSR in B lymphocytes is initiated upon stimulation or cytokine treatment, which activate an activation/cytokine-responsive promoter element upstream of an initiation exon (I exon) (4). In mature B cells, these inducible germ line transcripts are synthesized from both of the functional and the non-functional alleles (33), resulting in the occurrence of CSR at the excluded allele in up to 75% of switched cells (41). Similar to mature B cells, we found that CSR induced in 3-83Tg immature B cells by CpG DNA occurs at the non-functional IgH alleles that retain the V, D and J regions in germ line configuration. This CSR was found in relatively high frequency (14% of the cells), suggesting that upon an appropriate stimulus a non-rearranged IgH locus in BM B cells is also amendable for CSR. We also found that CSR induced by CpG DNA is independent of IgM expression as revealed using μMT pro-B cells. Similarly, CSR to IgA has been shown to occur in gut-associated lymphoid tissue in μMT mice, which was dependent on commensal bacteria in the gut (42). These observations are consistent with the findings showing that CSR is induced in pro-B cells from wild-type mouse (this study) and in RAG-deficient and SCID B cell precursors that were induced to differentiate ex vivo (18). Also, DNA recombination occurs in non-B cells that artificially express AID (43), thereby supporting the notion that the induced CSR may not be restricted to alleles that carry an assembled VDJ. We further show that CSR induced in BM B cells by LPS is directed by IL-4 from Cγ2b to Cγ1. Similarly, IL-4 directs CSR in mature B cells to Cγ1 and Cε whereas IFNγ directs it to Cγ2a and Cγ2b (5). In mature B cells, the competence to direct CSR into a specific CH isotype in response to cytokines reflects the regulatory function of the respective TR, cells (5). Our findings, therefore, suggest that CSR induced in BM B cells may also be regulated by TR, cytokines.

Because of their increased autoreactivity (44, 45), developing BM B cells are thought not to participate in an immune response. This dogma, however, was challenged by several studies showing that inflammation causes recruitment of BM B cells into secondary lymphoid organs (46-48) and that precursor B cells express TLRs and respond to TLR ligands by proliferation and differentiation to IgM-producing cells (13-16). However, this activation is not sufficient for the development of autoimmune disease such as lupus, as low-affinity IgM antibodies are not pathogenic (49) and that AID is required for the generation of high-affinity IgG antibodies and the onset of lupus in MRL/lpr mice (50). The findings that developing B cells acquire SHM (21, 51), and can be induced to undergo CSR (as we show here), suggest that these cells have the potential to produce effector, self-reactive antibodies. From physiological perspectives, activation of these cells may not be limited to the BM as early B cell precursors have been shown to migrate from the BM to the periphery upon immunologic challenge or chronic inflammation (46-48). Thus, precursor B cells can be activated by microbial products in secondary lymphoid organs. However, this autoimmune potential, as we show here is regulated by the Fas–FasL pathway. Similarly, we found that the Fas pathway is also important for elimination of developing B cells that have undergone spontaneous CSR (19), as lack of Fas results in the production of high titers of oligo monoclonal autoreactive antibodies in μMT mice (52, 53). In contrast, CpG DNA protects mature B cells from Fas-mediated apoptosis (54), which is in agreement with studies showing that mitogenic signals promote survival and efficient activation of non-tolerant mature B cells (12).

In a previous study, we and others have shown that CSR occurs spontaneously at a very low level during all stages of B lymphopoiesis (19–21). However, sequencing analysis revealed that spontaneous CSR generates aberrant Cγ2-Cγ1 switch junctions, which are characterized by reduced sequence overlaps at the junctions, increased mutation frequency and excessive deletion of the switch region. We show here that the Cγ2-Cγ1b switch junctions which are induced in BM cells by CpG DNA have similar aberrancies as the Cγ2-Cγ1 switch junctions that are generated spontaneously. A possible explanation for the formation of such joints is deregulated expression of AID early in B lymphopoiesis, which may result in enhanced or prolonged activity. It is thought that excessive amounts of AID proteins are inactivated in mature B cells to avoid the occurrence of aberrant translocations and tumorigenesis (43). This suggests that BM B cells fail to inactivate or to properly control AID activity, which results in the formation of aberrant junctions. Hence, deregulated, prolonged AID activity may explain the aberrant switch junctions obtained in BM B cells by spontaneous CSR (20) and by CSR induced upon stimulation with CpG DNA that we show here. It is currently difficult to assess the effect of the defective CSR junctions on the process of B cell selection, BCR signaling and on the antibody function. Nevertheless, the fact that these cells are regulated by the Fas pathway may suggest that these aberrant cells are negatively selected by the immune system.

Funding
Israel Science Foundation; United States–Israel Binational Science Foundation; Elias Fund for Medical Research

Abbreviations
AID activation-induced cytidine deaminase
BM bone marrow
CSR class switch recombination
FISH fluorescence in situ hybridization
GLT germ line transcript
MullV murine leukemia virus
PST post-switch transcript
RT reverse transcription
SHM somatic hypermutation
TLR Toll-like receptor

References
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