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Impaired Light Chain Allelic Exclusion and Lack of Positive Selection in Immature B Cells Expressing Incompetent Receptor Deficient of CD19

Shoham Shivieli,* Nira Leider,* Orit Sadeh,† Zaki Kraiem,*† and Doron Melamed†‡

Positive signaling is now thought to be important for B cell maturation, although the nature of such signals has not yet been defined. We are studying the regulatory role of B cell Ag receptor (BCR) signaling in mediating positive selection of immature B cells. To do so, we use Ig transgenic mice (3-83Tg) that are deficient in CD19, thus generating a monoclonal immature B cell population expressing signaling-incompetent BCR. Immature 3-83Tg CD19<sup>−/−</sup> B cells undergo developmental arrest in the bone marrow, allowing maturation only to cells that effectively compensate for the compromised receptor by elevated levels of BCR. We find that developmentally arrested 3-83Tg CD19<sup>−/−</sup> B cells fail to impose L chain allelic exclusion and undergo intensive V(D)J recombination to edit their BCR. Furthermore, immature 3-83Tg CD19<sup>−/−</sup> B cells, which were grown in vitro, failed to undergo positive selection and to survive when adaptively transferred into normal recipients. However, elevation of BCR expression levels, obtained by transgene homozygosity, effectively compensated for the compromised BCR and completely restored BCR-mediated Ca<sup>2+</sup> influx, allelic exclusion, and positive selection. Our results suggest that the BCR signaling threshold mediates positive selection of developing B cells, and that a receptor-editing mechanism has an important role in rescuing cells that fail positive selection because of incompetent receptors.


B cell receptor (BCR)<sup>3</sup> expression and signaling are critically involved in promoting B cell development. B cell tolerance, survival, and response to Ag (1, 2). It is randomly rearranged from Ig gene segments during B cell development (2–5). B lymphocytes are monospecific, resulting from halting V(D)J recombinase and Ig gene assembly once an Ag receptor is expressed (1, 6, 7). This allelic exclusion occurs at both the H and L chain loci and is thought to be important for the prevention of autoimmunity (7, 8). Mutagenic studies showed that Ig gene rearrangement, receptor expression, and functional signaling capabilities are all required for normal B cell development (9–17) and for the establishment of allelic exclusion (reviewed in Ref. 18). Signaling of pre-BCR is essential for positive selection of proB cells and progression into a preB stage concomitantly with establishing H chain allelic exclusion and directing the V(D)J recombination machinery to the L chain loci (1, 2, 18, 19). In contrast, incompetent preBCR expression results in developmental arrest and activation of V(D)J recombination (18, 20–22).

The CD19 molecule is a positive regulator of the BCR, which lowers the signaling threshold required for B cell activation upon BCR ligation (23, 24). Through highly conserved nine tyrosine residues in its cytoplasmic domain, CD19 is thought to act as an adapter protein that recruits Src homology-2 domain-containing signaling molecules such as Lyn, Vav, phosphoinositol 3-kinase, and phospholipase Cγ2 (reviewed in Ref. 25). Expression of CD19 is also required for maximal BCR-mediated phosphotidylinositol 3-kinase activation and Ca<sup>2+</sup> mobilization (26), implicating that deficiency of CD19 results in incompetent BCR (27). CD19 is expressed from the earliest stages of B cell development and regulates pre-BCR signaling (28) and recombinase gene expression in pro-B cells (29). CD19-deficient mice have relatively normal B cell development in the bone marrow (BM) (30, 31). However, these mice have 50–60% reduction in peripheral B cells, which are hyporesponsive to most transmembrane signals (30, 31). Interestingly, CD19-deficient mice have a severe loss of B-1 B cells, whereas CD19-overexpressing mice have increased B-1 cell compartment (30–32). These data imply a critical role for CD19 in balancing the BCR signaling threshold required for mature B cell activation, while its function in promoting B cell development is still not clear.

Signaling competence of the BCR is important to promote B cell maturation, as revealed by developmental stage-dependent differences in BCR signaling (33, 34), and in mice deficient of Btk, SH2-domain-containing leukocyte protein (SLP)-65, and Lyn (14, 16, 35). Impaired B maturation (36) and failure to populate the marginal zone compartment in spleen (37) are described in different Ig transgenic (Tg) mouse models deficient of CD19. In a recent study Reichlin et al. (13) used mice lacking a functional Igβ cytoplasmic domain to show that immature B cells expressing signaling-incompetent BCR are developmentally arrested. We have previously shown that developmental arrest is also imposed in immature B cells encountering self-Ag in the BM (38, 39). These cells however, elevate V(D)J recombination machinery and undergo receptor editing to alter the BCR specificity (39, 40). Therefore, it is possible that developmentally arrested immature B cells expressing signaling-incompetent receptors undergo secondary V(D)J recombination to express new receptors. This hypothesis is

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Abbreviations used in this paper: BCR, B cell receptor for Ag; BM, bone marrow; HSA, heat-stable Ag; LN, lymph node; MFI, mean fluorescence intensity; RAG, recombinase-activating gene; Tg, transgenic; SLP, SH2-domain-containing leukocyte protein.
tested in the present study using the 3-83Tg mice (that manifest excellent allelic exclusion in vivo and in vitro) (41, 42) deficient in CD19 (31). Our results propose that immature 3-83Tg B cells expressing signaling-incompetent receptors, deficient in CD19, fail positive selection and are developmentally arrested. These B cells undergo intensive secondary L chain V(D)J recombination to edit their BCR. Therefore, we suggest that CD19 regulates the BCR signaling threshold required for the establishment of L chain allelic exclusion, positive selection, and maturation.

Materials and Methods

Experimental mice

Mice used were 3-83Tg B10.D2SnJ, encoding a BCR reactive to the mouse class I MHC Ags K\(^+\) and K\(^-\) (a gift from Dr. D. Nemazee, Scripps Institute, La Jolla, CA (43)) or non-Tg littersmates. CD19-deficient mice were a gift from Dr. R. Rickert, University of California (San Diego, CA) (31). The 3-83Tg CD19\(^{+/−}\) mice were generated and backcrossed >10 generations on an H-2d-selected B10.D2 background to ensure the absence of the 3-83 cognate Ag as revealed by both tissue staining and analysis of B cell development. Mice were housed and bred at the animal facility of Technion, Faculty of Medicine (Haifa, Israel), and used at 4–10 wk of age. For some experiments, homozygous 3-83Tg CD19\(^{+/−}\) (3-83Tg/12 F1) CD19\(^{+/−}\) mice were generated and typed by genomic Southern blot (39). To do so, genomic DNA was digested with HindIII, electrophoresed on agarose gel, blotched to a \(\alpha\) probe membrane (Bio-Rad, Richmond, CA), hybridized with a \(\alpha\)[\(32\)P]Jk1–5 DNA probe, and subjected to phosphor imager analysis (FUJIFILM FLA 2000; Fuji, Stamford, CT) for quantitation. The ratio of transgenic fragment (at 3.2 kb) and the germline fragment (at 2.8 kb) was calculated and used to distinguish 3-83 homozygous from heterozygous mice. A restriction map of the relevant DNA fragment was presented previously (39).

Cell culture

BM cultures for B cell precursors were prepared as we have previously described (39, 42). Briefly, BM cells, RBC depleted, were cultured for 5 days in the presence of 50–100 U/ml rIL-7. Cells grown in these primary cultures (>95% B220\(^+\)) were used directly for cellular and molecular analysis or for adoptive transfer experiments. In some experiments B cells were stained using Fab goat anti-mouse IgM FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) and anti-mouse IgD J1A2.5-biotin (42) and fractioned to IgM\(^{+}\)/IgD\(^{−}\) and IgM\(^{−}\)/IgD\(^{+}\) using anti FITC and/or streptavidin MACS microbeads (Miltenyi Biotec, Auburn, CA). For some experiments intracellular levels of cAMP were determined by RIA as described previously (44).

RNA and DNA analyses

RNA and DNA from a single-cell suspension of BM, spleen, or lymph nodes (LN) or from BM cultures were prepared as we have previously described (39, 42). Quantitation of endogenous V\(\kappa\)-Jk1 DNA rearrangements was determined by PCR assay using genomic DNA template and normalized to \(\alpha\)-actin. PCR conditions and primer sequences were described in detail previously (42). Levels of recombination-activating gene-2 (RAG-2) and B220 (CD45R) control gene mRNA were determined by RT-PCR assay. PCR conditions and primer sequences for RAG-2 were previously described (42). PCR conditions for B220 are 30 s at 94°C, 30 s at 64°C, and 1.5 min at 72°C for 22 cycles. Primer sequences for B220 are: sense, 5′-ctgagcacaagagattcctc-3′; and antisense, 5′-ctgaaagatctatgggtgctc-3′. PCR products were run on gel, transferred to membrane, and probed using \(32\)P-labeled specific probes for RAG-2 and B220 as previously described (42). Blots were exposed to x-ray film and scanned with a phosphorimager. To obtain a semiquantitative estimate of gene expression, the signal intensity of RAG-2 was normalized to the B220 signal as a phosphorimager. To obtain a semiquantitative estimate of gene expression, the signal intensity of RAG-2 was normalized to the B220 signal as a phosphorimager.

Flow cytometry

Single-cell suspensions from BM, spleen, LN, or BM cultures were stained for surface marker expression using FITC-, PE-, and biotin-conjugated mAbs, visualized with streptavidin TrueColor (Caltag Laboratories, San Francisco, CA). Abs used for cell staining were goat anti-mouse IgM (Caltag Laboratories); goat anti-mouse \(\lambda\) (Southern BioTechnology Associates, Birmingham, AL); mAbs to B220 and RA3-6B2 (Caltag Laboratories); IgD\(^{+}\), AMS 9.1, heat-stable Ag (HSA), and 30-F1 (BD PharMingen, San Diego, CA); and IgM\(^{+}\), R53, and anti-3-83 idiotype 54.1.4 (42). Data for three-color analysis were collected on a FACSCalibur and analyzed using CellQuest software (BD PharMingen).

Calcium flux

Kinetic variations of intracellular Ca\(^{2+}\) were determined using the Ca\(^{2+}\) indicator Fluo-3. This fluoroscein derivative undergoes a dramatic increase in fluorescence intensity upon Ca\(^{2+}\) binding (45) and has emission/excitation spectra similar to those of fluorescein (46). The use of Fluo-3 to analyze Ca\(^{2+}\) in hemopoietic cells upon stimulation has been described (46). To study Ca\(^{2+}\) influx, 2 × 10⁶ B cell precursors were washed in MGB buffer (5 mM KCl, 147 mM NaCl, 0.22 mM KH\(_2\)PO\(_4\), 1.1 mM Na\(_3\)HPO\(_4\), 5.5 mM glucose, 0.3 mM MgSO\(_4\), 1 mM MgCl\(_2\), 10 mM HEPES (pH 7.4), and 0.1% BSA) and incubated in MGB buffer and 0.5 mg/ml Fluo-3/AM for 30 min. After washing, B cells were resuspended in MGB buffer and 1 mM CaCl\(_2\) and warmed to 37°C. Cells were stimulated with monoclonal anti-IgM Ab B.7.6 (10–20 μg/ml) (40), and calcium flux was measured by the fluorescence intensity of Fluo-3/AM using the green channel (525 nm) on a FACS Calibur. Data were acquired for 60 s before BCR cross-linking.

Serum Ab detection

Detection of allotype-specific IgM\(^{+}\) Abs in serum was performed by standard ELISA as previously described (47). Plates were coated with RS3 anti-IgM mAbs, and bound Abs were revealed by anti-3-83 idiotype (54.1) or with polyclonal goat anti-IgM or goat anti-mouse \(\lambda\) (Southern Bio-technology Associates). Amounts of Abs in serum were calculated using a reference standard curve of purified IgM\(^{+}\) Ab.

BM chimera and adoptive transfer

BM chimera were prepared as previously described (48). Donor BM cells, T and B cells depleted (confirmed by FACS), were i.v. injected into lethally irradiated (800 rad) recipients of the indicated genetic background (5 × 10⁵ cells/mouse). Four weeks after reconstitution spleen and LN were inspected by flow cytometry for the presence of donor-derived, idiotype "\(\lambda\)" B cells. For adoptive transfer experiments B cell precursors were grown in BM cultures, washed, and i.v. injected into sublethally irradiated (400 rad) B10.D2 recipients (5 × 10⁶ cells/mouse). Five days after cell transfer spleens of recipient mice were inspected by flow cytometry for the presence of transferred cells using anti-idiotypic staining.

Statistical analysis

The statistical significance of differences between experimental groups was determined using unpaired two-tailed Student’s t test or one-way multiple range ANOVA, with differences considered significant at \(p < 0.05\).

Results

Immature 3-83Tg CD19\(^{−/−}\) B cells are developmentally arrested and fail to impose L chain allelic exclusion in vivo

In 3-83Tg mice B cell development and maturation occur rapidly (42, 49). Nonetheless, 3-83Tg mice deficient in CD19 manifest a severe reduction (60–80%) in mature IgM\(^{+}\) B cells in spleen (36) (Fig. 1A, A and B, respectively) and accumulation of immature cells in the BM (36) (data not shown), implying that immature 3-83Tg CD19\(^{−/−}\) B cells are developmentally arrested. To determine whether developmentally arrested 3-83Tg CD19\(^{−/−}\) B cells undergo receptor editing, we studied the induction of V(D)J recombination and the establishment of allelic exclusion in 3-83Tg CD19\(^{−/−}\) mice (Fig. 1). Although all splenic IgM\(^{+}\) B cells in 3-83Tg CD19\(^{−/−}\) mice express the Tg receptor, a large population of cells coexpress an endogenous \(\lambda\) L chain (1.6%; Fig. 1A). This population reflects >5% of the total IgM\(^{+}\) B cells in the 3-83Tg CD19\(^{−/−}\) spleen (Fig. 1B), a value that is not different from that in a non-Tg mouse (5–8%; Fig. 1B). We also found many endogenous V\(\kappa\)-Jk1 DNA rearrangements in purified IgM\(^{+}\) 3-83Tg CD19\(^{−/−}\) splenic B cells, but not in 3-83Tg splenic B cells (not shown). H chain allotypic staining revealed that in both mice all splenic B cells express the transgenic encoded IgM\(^{+}\) receptor, and no IgM\(^{−}\)-positive B cells were found (not shown). Similar results were found in LN (not shown). Furthermore, serum Ab analysis revealed that 3-83Tg CD19\(^{−/−}\) mice produce large amounts of...
significantly (fi/H11002)

nation to edit their receptors.

endogenous L chain (fi/H9261) in CD19, are developmentally arrested and undergo V(D)J recombin-

ition to yield 30,000 cells. B, A table summarizing absolute numbers of IgM+ and λ+ B cells in spleens of 3-83Tg, 3-83Tg CD19−/− and B10.D2 mice, as analyzed by FACS and gated on all viable cells. Absolute numbers were calculated by multiplying frequencies of IgM+ and λ+ by the total number of viable spleen cells. The results are group means of five mice from each group ± SEM. The percent λ+ values are the ratio of total λ+ divided by total IgM+ × 100. a and b, statistically different values of percentage of λ+ cells (p < 0.05). C, Serum Abs from the indicated mice were analyzed by ELISA to detect IgM+, idiootype−, and IgM+/λ Abs, as described in Materials and Methods. Ab concentrations were determined using the appropriate IgM+ standard curve. The results shown are from five to seven mice in each group ± SEM. Differences in titers of idiootype (middle panel) and IgM+/λ (lower panel) Abs are statistically significant (p < 0.05). D, RT-PCR analysis of mRNA expression of RAG-2 in BM spleen and LN of B10.D2, 3-83Tg, and 3-83Tg CD19−/− mice. B220 signal serves as internal control. Data shown represent five to seven mice in each group ± SEM. Differences in RAG-2 expression levels in BM samples are statistically significant (p < 0.05). E, A representative RT-PCR blot for detection of RAG-2 expression in BM of the indicated mice. Samples of cDNA in serial dilution were amplified for RAG-2 and for B220 internal control as described in Materials and Methods.

IgM+ Abs, composed of the transgenic H chain (μH+) and the endogenous L chain (λ; Fig. 1C, lower panel). This is accompanied by a marked decrease in the total amount of idiootype−/IgM+ Abs (middle panel). In contrast, essentially no IgM+/λ were found in serum of 3-83Tg mice.

BM analysis of 3-83Tg CD19−/− mice revealed a significantly elevated levels of RAG-2, achieving 50–60% relative to non-Tg BM (Fig. 1, D and E). In contrast, no RAG-2 expression was found in spleen or LN, suggesting that lack of allelic exclusion and in-

duction of V(D)J recombination in 3-83Tg CD19−/− mice are restricted to developing B cells in the BM. We conclude that immature 3-83Tg B cells expressing signaling-competent BCR, deficient in CD19, are developmentally arrested and undergo V(D)J recombin-

ation to edit their receptors.

3-83Tg CD19−/− B cell precursors do not maintain allelic exclusion in vitro

To further assess the development of immature 3-83Tg CD19−/− B cells we used our in vitro BM culture system (42). After 5 days of culture 3-83Tg and 3-83Tg CD19−/− grow equally well, with

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<td>0.6 ± 0.1</td>
<td>5.3 ± 0.8b</td>
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<td>B10.D2</td>
<td>28 ± 3</td>
<td>2.1 ± 0.4</td>
<td>2.7 ± 0.4 b</td>
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Differences in RAG-2 expression levels in BM samples are statistically significant (p < 0.05). E, A representative RT-PCR blot for detection of RAG-2 expression in BM of the indicated mice. Samples of cDNA in serial dilution were amplified for RAG-2 and for B220 internal control as described in Materials and Methods.

3-83Tg CD19−/− B cell precursors do not maintain allelic exclusion in vitro

To further assess the development of immature 3-83Tg CD19−/− B cells we used our in vitro BM culture system (42). After 5 days of culture 3-83Tg and 3-83Tg CD19−/− grow equally well, with no differences in cell recovery (not shown), purity (>95% B220+; not shown), or phenotype (IgM and IgD expression; Fig. 2A, left). However, immature 3-83Tg CD19−/− B cells did not maintain allelic exclusion in culture relative to the control 3-83Tg cells, as reflected by a significant population of B cells coexpressing endogenous λ L chain and the transgenic 3-83 receptor (4%; Fig. 2A, right). This is supported by the findings of a significant 10-fold elevation in RAG-2 gene expression (Fig. 2B, upper panel) and a significant 10- to 15-fold increase in endogenous Vc-Jκ1 DNA rearrangements (Fig. 2B, lower panel) in 3-83Tg CD19−/− cultures. Furthermore, RAG expression was detected in both early (IgM+IgD−) and late (IgM+IgD−; transitional) immature 3-83Tg CD19−/− B cells grown in culture (Fig. 2C), perhaps suggesting that acquisition of sigD is not sufficient to turn off DNA recombination in these cells. Thus, lack of allelic exclusion in 3-83Tg CD19−/− B cells was shown both in vivo and in vitro at the levels of DNA, RNA, and protein. Moreover, we found a 2- to 3-fold increase in intracellular cAMP levels in 3-83Tg CD19−/− B cell precursors relative to 3-83Tg precursors (Fig. 2D). These observations are in agreement with earlier studies showing that
V(D)J recombination and RAG gene expression are increased upon induction of intracellular second messengers such as protein kinase A and cAMP (50, 51).

Mature 3-83Tg CD19\(^+\)B cells express elevated levels of BCR

Earlier studies showed that the expression level of BCR is important to promote B cell development and to establish allelic exclusion (52–55). Fig. 3 shows that splenic 3-83Tg CD19\(^+\)B cells express significant 1.5- to 2-fold higher levels of sIgM receptors relative to their 3-83Tg counterparts (left). In contrast, 3-83Tg and 3-83Tg CD19\(^−/−\)developing B cells in BM in vivo (Fig. 3, middle) and in cultures (Fig. 3, right) express indistinguishable levels of sIgM. We conclude that the difference in the BCR level of expression in mature B cells may reflect a selection process in vivo rather than a differential level of BCR synthesis between the two mice. Thus, immature 3-83Tg CD19\(^−/−\)B cells that fail selection because of incompetent BCR are developmentally arrested and undergo intensive V(D)J recombination to edit their receptors.

3-83Tg CD19\(^−/−\) B cells fail to impose allelic exclusion in BM chimera

To test whether the induction of V(D)J recombination in 3-83Tg CD19\(^−/−\) B cells reflects an intrinsic B cell defect, we generated short term BM chimera. BM cells from 3-83Tg or 3-83Tg CD19\(^−/−\)donor mice were used to reconstitute lethally irradiated B10.D2 or non-Tg CD19\(^+\)/H11002/CD19\(^+\)/H11002 mice. Spleen cells of reconstituted mice were stained after 4 wk to detect idiotype/H11001/H9261 double-expressing cells as an indication of the lack of allelic exclusion (Fig. 4). The results show that in contrast to 3-83Tg BM chimera, mice reconstituted with 3-83Tg CD19\(^−/−\)BM produce significant idiotype'/\lambda' double-expressing cells regardless of the genotype of recipient mice (Fig. 4A). We conclude that lack of allelic exclusion in 3-83Tg CD19\(^−/−\)B cells is an intrinsic B cell defect and does not reflect environmental changes resulting from CD19 deficiency. Importantly, because 3-83Tg cells develop equally well in B10.D2 and CD19\(^−/−\)chimera (Fig. 4), it is...
Analysis of surface IgM expression was performed on 10,000 gated B220 cells grown in culture were stained for surface B220 and IgM expression. Analysis of surface IgM expression was performed on 10,000 gated B220 cells. The results are representative of five different experiments. Note that for spleen cell samples of 3-83Tg CD19–/– mice approximately five times more lymphocytes were analyzed.

 unlikely that V(D)J recombination in 3-83Tg CD19–/– mice is driven by self-Ag. Additional phenotypic analyses revealed that mature 3-83Tg CD19–/– B cells, developing in either B10.D2 or CD19–/– chimera, had significantly 1.5- to 2-fold higher levels of BCR expression (Fig. 4B), but a 60–70% reduction in frequencies (Fig. 4). This supports our hypothesis that the BCR level of expression is important in promoting developmental progression of 3-83Tg B cells expressing signaling-incompetent BCR, deficient in CD19.

**Homologous 3-83Tg CD19–/– mice restore establishment of allelic exclusion in vivo and in vitro**

To test the hypothesis that high expression levels of BCR can compensate for compromised signaling in promoting allelic exclusion, selection, and maturation, we generated homologous 3-83Tg CD19–/– mice (3-83TgHz CD19–/–). In these experiments, we adopted a previously shown experimental maneuver, in which homozgyosity of the transgene loci enhances the level of transgene expression compared with heterozygosity (52, 53). Bone marrow analysis of early immature B220low/HSA low B cells showed that transgene homozygosity resulted in 10- to 15% elevated levels of BCR expression (Fig. 5A; IgM mean fluorescence intensity (MFI) in 3-83TgHz CD19–/– is 69 relative to 58–60 in the 3-83Tg and 3-83Tg CD19–/–). In later developmental stages in the BM (B220low/HSA high; Fig. 5A) no significant difference in BCR expression levels was found, probably reflecting the fact the BCR expression levels in the 3-83Tg mouse model are developmentally regulated (40). The results in Fig. 5B (table) support our hypothesis by showing that allelic exclusion was completely restored in the 3-83TgHz CD19–/–. In a striking contrast to 3-83Tg CD19–/– mice, RAG-2 gene expression in BM of 3-83TgHz CD19–/– mice was undetectable, and essentially no idiotype /A+ double-expressing B cells were found in their spleens (Fig. 5B). Similar results were found in BM cultures in vitro (Fig. 5B). As expected, we found that 3-83TgHz CD19–/– B cells expressed high levels of BCR relative to 3-83Tg B cells, but not significantly higher then heterozygous 3-83Tg CD19–/– B cells (Fig. 5C, left), and had a reduced immature B cell compartment in the BM relative to 3-83Tg CD19–/– (not shown). Also, analysis of HSA expression revealed that splenic B cells from 3-83TgHz CD19–/– and control 3-83Tg mice acquired the mature HSA high phenotype (MFI, 390 and 362, respectively), whereas more splenic 3-83Tg CD19–/– B cells expressed the immature HSA low phenotype (MFI, 550; Fig. 5C, right). Hence, our results suggest that high levels of BCR expression can compensate for incompetent BCR, deficient in CD19, in signaling for establishment of allelic exclusion matura-


**FIGURE 3.** BCR expression levels in mature and immature B cells. 3-83Tg and 3-83Tg CD19–/– spleen and BM cells as well as immature B cells grown in culture were stained for surface B220 and IgM expression. Analysis of surface IgM expression was performed on 10,000 gated B220 cells. The results are representative of five different experiments. Note that for spleen cell samples of 3-83Tg CD19–/– mice approximately five times more lymphocytes were analyzed.

**FIGURE 4.** Analysis of allelic exclusion and selection in BM chimera. A, BM cells, RBC T and B cell-depleted, from 3-83Tg or 3-83Tg CD19–/– donor mice were used to reconstitute lethally irradiated B10.D2 or CD19–/– recipients. Four weeks after reconstitution spleen cells were stained for IgM, idiotype, and B220 chain. Absolute numbers of transgenic-infused cells and of idiotype /A+ cells were calculated as described in Fig. 1. Values of percent idiotype /A+ B cells shown are the ratio of total idiotype /A+ divided by total IgM. Data shown are mean ± SEM from three or four mice in each group. In B10.D2 and CD19–/– chimeric mice injected with 3-83Tg BM cells, we found a total of 5–6 × 107 IgM+ cells, whereas in B10.D2 and CD19–/– chimeric mice injected with 3-83Tg CD19–/– BM cells, we found only 2–3 × 107 cells. Differences between 3-83Tg and 3-83Tg CD19–/– groups are statistically significant (p < 0.05). B, The IgM+ level of expression of donor-derived B-cells was determined in spleens from B10.D2 (left) or CD19–/– (right) chimeric mice. Analysis was performed on 10,000 idiotype+ gated cells. The data shown are representative of three or four mice from each group.

**FIGURE 5.** Analysis of selection in BM chimera. A, BM cells, RBC T and B cell-depleted, from 3-83Tg or 3-83Tg CD19–/– donor mice were used to reconstitute lethally irradiated B10.D2 or CD19–/– recipients. Four weeks after reconstitution spleen cells were stained for IgM, idiotype, and a chain. Absolute numbers of transgenic-derived IgM+ expressing cells and of idiotype /A+ cells were calculated as described in Fig. 1. Values of percent idiotype /A+ B cells shown are the ratio of total idiotype /A+ divided by total IgM+ cells. Data shown are mean ± SEM from three or four mice in each group. In B10.D2 and CD19–/– chimeric mice injected with 3-83Tg BM cells, we found a total of 5–6 × 107 IgM+ cells, whereas in B10.D2 and CD19–/– chimeric mice injected with 3-83Tg CD19–/– BM cells, we found only 2–3 × 107 cells. Differences between 3-83Tg and 3-83Tg CD19–/– groups are statistically significant (p < 0.05). B, The IgM + level of expression of donor-derived B-cells was determined in spleens from B10.D2 (left) or CD19–/– (right) chimeric mice. Analysis was performed on 10,000 idiotype+ gated cells. The data shown are representative of three or four mice from each group.
**FIGURE 5.** Homozygous 3-83Tg CD19\textsuperscript{−/−} mice restore allelic exclusion in vivo and in vitro. A, MFI of surface IgM in BM immature B cells. BM cells from the indicated mice were stained for B220, IgM, IgD, and HSA. Using the HSA marker, immature cells (B220\textsuperscript{−}/IgD\textsuperscript{−}) were divided into early (HSA\textsuperscript{low}) and late (HSA\textsuperscript{high}) as we have previously described (40), and the MFI of IgM was determined in each population. B, A table summarizing RAG-2 mRNA expression in BM and B cell cultures (normalized to the B220 control) and percentage of λ\textsuperscript{+} B cells in spleen and in B cell cultures (%λ\textsuperscript{+} B cells was determined as described in Fig. 1B). B cell cultures derived from BM of 3-38Tg, 3-83Tg CD19\textsuperscript{−/−}, and 3-83TgHz CD19\textsuperscript{−/−} were prepared as described in Materials and Methods. Numbers shown in bold are statistically different from their respective values in 3-83TgHz CD19\textsuperscript{−/−} B cells. The results are representative of five different experiments.

**FIGURE 6.** Ca\textsuperscript{2+} flux response of immature 3-83Tg, 3-83Tg CD19\textsuperscript{−/−}, and 3-83TgHz CD19\textsuperscript{−/−} B cells upon BCR cross-linking. Immature B cells were grown in vitro from BM of the indicated mice, loaded with Fluo-3, and stimulated with 10 μg/ml monoclonal anti-IgM B-7-6. The kinetics of Ca\textsuperscript{2+} mobilization were determined by flow cytometry on the green channel (525 nm). Dot plots represent Ca\textsuperscript{2+} flux in stimulated cells over 300 s. Baseline was acquired for 60 s. The results shown are representative of three different experiments from the indicated mice stimulated with the same Ab concentration.

**Discussion**

The present study focuses on a narrow window in the life span of B cells, in which newly generated, immature B cells are selected to mature and survive in the periphery. At this time of development B cells expressing a non-self-BCR suppress V(D)J recombination and mature, a process often referred to as positive selection (1, 2, 59). It has been clearly shown that this developmental progression is promoted by the expression and signaling of functional BCR (reviewed in Refs. 1, 2, and 18). In this study, we show data suggesting that the CD19 coreceptor regulates the BCR signaling threshold required to promote positive selection and maturation and to establish L chain allelic exclusion.

Because of signaling incompetence, B cell maturation is impaired, and B cells are developmentally arrested at the immature stage (13, 14, 16, 35). In immature 3-83Tg CD19\textsuperscript{−/−} B cells this developmental arrest induces intensive V(D)J recombination attempts to edit the BCR, thereby abrogating the strict allelic exclusion imposed by the 3-83Tg receptor (Figs. 1 and 2). Similarly, developmental arrest is imposed in immature autoreactive B cells encountering self-Ag, and in proB cells expressing incompetent preBCR (18, 20–22, 39, 40, 60). Both have been shown to undergo secondary V(D)J recombination. Thus, receptor editing is a salvage mechanism for self-reactive immature B cells and for developing B cells expressing incompetent receptors. The possibility that V(D)J recombination in 3-83Tg CD19\textsuperscript{−/−} B cells is induced by self-Ag is unlikely (Fig. 3). Other explanations for secondary V(D)J recombination, which may account for normal-developing B cells such as poor H and L pairing (61), out-of-frame DNA rearrangements (4), and preferences/favoring of specific Vκ segments (19), are irrelevant for the 3-83Tg model (42, 43). We therefore suggest that in 3-83Tg CD19\textsuperscript{−/−} mice receptor competence plays a critical role in limiting B cell maturation and imposing developmental arrest at the immature stage. Interestingly, when

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**Expression (Figs. 2A and 3), we conclude that the poor Ca\textsuperscript{2+} mobilization is due to the expression of signaling-incompetent BCR, deficient in CD19. This is supported by the finding that immature 3-83TgHz CD19\textsuperscript{−/−} B cells completely restored their Ca\textsuperscript{2+} mobilization response upon anti-IgM stimulation (Fig. 6). Thus, a high BCR level of expression effectively compensates for a compromised receptor in Ca\textsuperscript{2+} mobilization upon BCR stimulation.**

**Impaired selection and survival of immature 3-83Tg CD19\textsuperscript{−/−} B cells in vivo**

To test the possibility that immature 3-83Tg CD19\textsuperscript{−/−} B cells are developmentally arrested and undergo receptor editing because they fail positive selection, we used an adoptive cell transfer approach in which cell survival is recapitulated as positive selection (56–58). In these experiments, immature 3-83Tg, 3-83Tg CD19\textsuperscript{−/−}, and 3-83TgHz CD19\textsuperscript{−/−} B cells grown in vitro were adoptively transferred into non-Tg B10.D2 recipients. Five days after cell transfer the spleen and LN of reconstituted mice were analyzed for the presence of Tg B cells. The results presented in Fig. 7 clearly show that the selection and survival of immature 3-83Tg CD19\textsuperscript{−/−} B cells in vivo were substantially reduced. The survival of immature 3-83Tg CD19\textsuperscript{−/−} B cells in the reconstituted mice was 2- to 3-fold lower than that in control immature 3-83Tg B cells in both percentages (Fig. 7A) and absolute numbers (Fig. 7B). Careful analysis and comparison of the MFI of sIgM revealed that selected 3-83Tg CD19\textsuperscript{−/−} B cells had significantly elevated (30–40%) BCR expression levels compared with the selected 3-83Tg B cells (Fig. 7B). Similar results were found in the LN (not shown). In contrast, immature 3-83TgHz CD19\textsuperscript{−/−} B cells had survival and selection rates similar to those of control immature 3-83Tg B cells (Fig. 7), and both had also acquired a high level of IgD (not shown). We conclude that high levels of BCR expression can compensate for a signaling-incompetent receptor, deficient in CD19, in signaling not only for allelic exclusion, but also to promote maturation and positive selection.
Selection and allelic exclusion. The ability of 3-83Hz CD19
This suggests that the BCR signaling threshold regulates positive
cent in CD19 (37).
failure of the receptor- editing mechanism has been described previously (27). That lack of
compensate for the compromised BCR by coexpression of endogenous
expression levels were elevated (52, 53). A model proposing a role for BCR
signaling in establishing L chain allelic exclusion has been recently
described, although no direct evidence has been presented (65). The data described in this study strongly support the proposed model. Thus, functional, non-self-BCR can signal in immature
B cells for positive selection and developmental progression, concomitantly with establishment of L chain allelic exclusion. The
data we show in this study suggest that the CD19 molecule is an important regulator in determining the BCR signaling threshold
required for these processes. It also proposes that immature B
cells that fail positive selection because of receptor signaling in-
competence can activate the receptor-editing mechanism to alter
the BCR.

Interestingly, only L chain allelic exclusion was impaired in
3-83Tg CD19<sup>−/−</sup> mice, whereas endogenous H chain gene recombina-
tion was efficiently suppressed. This finding supports the hypo-
thesis that allelic exclusion establishment at the H and L chain
loci are independent (1, 3, 18, 19). It was previously shown that
pre-BCR signaling and positive selection of pro-B cells are fol-
lowed by changes in locus-specific V(DJ) recombinase activity
from the H chain locus to the L chain locus, indicating that this
activity is developmentally regulated (3, 19). Thus, pro-B cells that
are developmentally arrested continue to rearrange H chain genes
(20–22), whereas developmentally arrested immature B cells con-
tinue to rearrange L chain genes (39, 40). Because 3-83Tg
CD19<sup>−/−</sup> B cells are arrested at the immature stage, V(DJ) re-
combination is directed to the L chain locus rather than to the H
chain locus. This situation resembles tolerance process in B cell
development, where self-reactive immature B cells are develop-
mentally arrested upon self-Ag binding, and secondary DNA re-
arrangements are directed to the L chain loci rather then to the H
chain locus (42, 64).

The functional competence of the BCR complex determines not
only developmental progression, but also survival and activation of
B cells (1, 59). Earlier studies showed that mature 3-83Tg
CD19−/− B cells had a diminished Ca2+ response when stimulated with Ag, and many of the cells did not respond at all (36). In contrast, the signaling capacity (Ca2+ influx and phosphodiesterase 3-kinase activation) of mature non-Tg CD19−/− B cells is controversial, and it appears that different BCR stimuli account for the different results (26, 36, 66). We show in this study that immature B cells expressing signaling-incompetent 3-8Tg receptor, deficient in CD19, have poor Ca2+ response upon BCR ligation (Fig. 6). However, Ca2+ mobilization is completely restored in 3-8TgHz CD19−/−, suggesting that a high level of BCR expression can effectively circumvent and compensate for CD19 deficiency. It is now important to extend these studies and to exclude the possibility that Fc receptor signaling is altered in the absence of CD19 and contributes to the obtained results. In such experiments anti-IgM F(ab′)2 (36) or a 3-8-specific mimetic peptide (33) should be used to stimulate the cells. Interestingly, a high BCR expression level directs B cell development to the B1 compartment (53–55), which is completely absent in CD19−/− mice and in mice deficient in Btk or SLP-65 (14, 16, 35). This proposes that the BCR signaling threshold, which is regulated by CD19, Btk, and SLP-65 molecules, not only determines developmental progression, but is also involved in directing the B cell lineage.

The conclusions of this study were reached by taking advantage of a well-described IgTg mouse model and an IL-7 BM culture system. Because both experimental maneuvers perturb B cell development in several ways, as we have described (40, 42), it is important to confirm these results in a more physiological system. This system, however, should confound the difficulties of distinguishing V(D)J recombination normally expressed in developing B cells and recombination processes associated with the lack of positive selection. It will also be important to address whether the positive selection process we found in the adoptive transfer experiments also apply to normal immature BM B cells expressing nontransgenic BCR.

Finally, if BCR signaling is required to establish L chain allelic exclusion and to promote maturation, it probably involves intracellular second messengers, such as cAMP. Those aim to transduce signals to the nucleus and to activate transcription factors regulating V(D)J recombination (67, 68). Several studies have shown that agents activating protein kinase A or increasing cAMP also elevate V(D)J recombination in B cells (50, 51). How BCR or pre-BCR signal for allelic exclusion is not known, nor have the ligands, if any, that transduce these signals been identified. In developing T cells, a high TCR level and suppression of RAG gene expression is required to establish immunoglobulin β chain gene rearrangement (68, 85). The signaling capacity (or the basal level of signaling) with V(D)J recombination is required.

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