Impaired Receptor Editing in the Primary B Cell Repertoire of BASH-Deficient Mice[1]

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The editing of B cell Ag receptor (BCR) through successive rearrangements of Ig genes has been considered to be a major mechanism for the central B cell tolerance, which precludes appearance of self-reactive B cells, through studies using anti-self-Ig transgenic/knock-in mouse systems. However, contribution of the receptor editing in the development of the normal B cell repertoire remains unclear. In addition, the signaling pathway directing this event is unknown. In this study, we demonstrate that receptor editing in anti-DNA Ig knock-in mice is impaired in the absence of an adaptor protein BASH (BLNK/SLP-65) that is involved in BCR signaling. Remarkably, the supposed hallmarks of receptor editing such as Igλ chain expression, recombination sequence rearrangements at *Igκ* loci, and presence of in-frame VκJκ joins in the *Igκ* loci inactivated by the recombination sequence rearrangements, were all diminished in BASH-deficient mice with unmanipulated *Ig* loci. BCR ligation-induced *Igλ* gene recombination in vitro was also impaired in BASH-deficient B cells. Furthermore, the BASH-deficient mice showed an excessive Ab response to a DNA carrier immunization, suggesting the presence of unedited DNA-reactive B cells in the periphery. These results not only define a signaling pathway required for receptor editing but indicate that the BCR-signaled receptor editing indeed operates in the development of normal B cell repertoire and contributes to establishing the B cell tolerance. ***The Journal of Immunology,*** **2004, 173: 5980–5988.**

Clonal selection theory is an essential concept in immunology, explaining how the immune system avoids generating self-reactive lymphocyte clones while developing a huge Ag receptor repertoire. It is generally believed that B lymphocytes carrying self-reactive Ag receptors are counterselected during development in the bone marrow through clonal deletion, anergy, or receptor editing. These central mechanisms for B cell tolerance have been proposed by the analyses of prerecombined Ig-transgenic/knock-in mice whose B cells express monoclonal self-reactive Ag receptors (1–6). However, there has been little evidence that the proposed mechanisms of central tolerance operate in the development of normal B cell repertoire and contribute to the elimination of self-reactivity and hence to the avoidance of autoimmunity. This is partly due to the lack of animals that are genetically defective for these mechanisms. So far, B cell-mediated autoimmunity developing in several mutant mouse strains has mostly been attributed to nonspecific lymphoproliferation and/or hyperactivation of peripheral B cells and not to a defect in central B cell tolerance (7–9). Recently, it has been reported that protein kinase Cδ-deficient mice develop autoimmune disease with appearance of autoreactive Abs (10, 11). These mice exhibited normal clonal deletion to a self-membrane-bound Ag but impaired anergy to a self-soluble Ag in an anti-hen egg lysozyme Ig-transgenic system, although the latter may indicate a break of the peripheral tolerance (10).

Receptor editing is a process by which the specificity of the B cell Ag receptor (BCR)[5] is modified by further V gene (mainly of L chain) rearrangements when the BCR on newly generated immature B cells is bound with self-Ag. This was first observed in the conventional transgenic mice carrying Ig H and L chain genes which together encode BCR recognizing self-Ag that is expressed as a membrane-bound form. In such mice, bone marrow B cells expressed RAGs and rearranged their endogenous κ and λ L chain genes in the presence of the self-Ag, in contradiction to allelic exclusion theory (1, 6). However, receptor editing was a relatively rare event in these transgenic mice and most of the self-reactive B cells were clonally deleted (1, 3–6). This is because such transgenes are randomly integrated into the genome and therefore hardly silenced by recombination of endogenous *Ig* genes. Then studies using mice carrying targeted *Ig* gene replacements (knock-in) with the rearranged VDJ_H and/or *VJκ* genes encoding variable regions of autoantibodies have indicated that receptor editing plays a major role in the central B cell tolerance. In such mice, secondary rearrangement of the *Ig* gene loci (primarily at the *Igκ*, then at the *Igλ*, and less frequently at the *IgH* loci) silences the knock-in *Ig* genes, which efficiently rescue originally autoreactive B cells from clonal deletion (12–18). Cellular deletion has been proposed to occur only when the secondary rearrangement is precluded by the absence of unrearranged J segments or of RAG proteins (18, 19). In accord with these in vivo data, BCR engagement on bone marrow immature B cells in vitro was shown to induce expression of RAGs (when it was suppressed by transgenic BCR expression) and secondary L chain gene rearrangements, but not apoptosis (20–22). However, receptor editing has also been observed in Ig knock-in mouse strains in which corresponding self-Ags were not

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[5] Abbreviations used in this paper: BCR, B cell Ag receptor; RS, recombination sequence; IRS, intron-recombining sequence; RSS, recombination signal sequence.

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apparently present (12, 23, 24). This raises a possibility that receptor editing might not be related to self-tolerance but to some inherent nature of such artificial systems, at least in some cases. Thus, it remains unclear whether receptor editing indeed operates and contributes to establishing self-tolerance in the development of normal B cell repertoire.

So far, molecular mechanisms that specify the BCR signaling pathway to receptor editing have been unclear. For example, tyrosine kinase Btk, tyrosine phosphatase CD45, and coreceptor CD19, all of which are known to positively regulate BCR signal transduction, have been reported to be unnecessary for receptor editing in anti-self-MHC or anti-hen egg lysozyme Ig-transgenic mouse systems (25–28). However, this conclusion may not be definitive, because the conventional transgenic systems they applied are not optimal to observe receptor editing but dominantly elicit clonal deletion (3, 4, 29). Thus, BCR signaling requirements for the induction of the receptor editing are currently unclear.

A B cell-specific adaptor protein, BASH (also known as BLNK or SLP-65), interacts with signaling proteins such as phospholipase Cγ2, Vav, Grb2, Syk, Btk, and HPK1 upon BCR stimulation and mediates activation of downstream signaling cascades (30–36). BASH-deficient mice display a phenotype reflecting BCR/pre-BCR signaling deficiencies including a partial blocking in early B cell development, severe reduction of peripheral mature (follicular and marginal zone) B cells and peritoneal B-1 cells, defective activation and proliferation of B cells upon BCR ligation in vitro, low serum Ig levels, and no thymus-independent, but largely normal thymus-dependent, immune responses (37–40). Allelic exclusion of *IgH* loci is signaled through pre-BCR, but has been shown to be unaffected in BASH-deficient mice (41, 42). Spleen B cells from BASH-deficient mice showed a higher rate of spontaneous apoptosis in culture than those from normal mice, perhaps because the former are mostly immature B cells (34). In contrast, bone marrow immature B cells are relatively resistant to apoptosis in vitro in the culture with other bone marrow cells, even when their BCR was ligated (20, 22), and showed no significant difference between BASH-deficient and normal mice in a survival rate in such a culture system as well as in vivo in an innocuous BCR knock-in mouse system (43).

In the present study, we sought to clarify the role for BASH in the BCR signaling pathway that leads to receptor editing and analyzed BASH-deficient mice with anti-DNA Ig gene knock-in loci as well as unmanipulated *Ig* loci. The results shown here indicate that BASH is required for signaling the receptor editing and also have provided a genetic evidence for the concept that receptor editing operates in the development of the normal B cell repertoire to restrain its self-reactivity.

Materials and Methods

Experimental mice

BASH-deficient mice with a C57BL/6 background were described previously (42). $IgH^{3H9/+}$ and $Ig\kappa^{V\kappa4/+}$ mice were reported previously (13, 15). These mice were crossed to obtain the mice used in this study. Mice were analyzed at 6–12 wk of age.

Flow cytometry

Cells were stained on ice with the following Abs: PE-conjugated anti-mouse B220/CD45R (RA3-6B2; BD Pharmingen, San Diego, CA); FITC-goat anti-mouse κ, FITC- or PE-goat anti-mouse μH, and PE-goat anti-mouse λ (Southern Biotechnology Associates, Birmingham, AL); biotin-goat anti-mouse μH (Cappel, Durham, NC), followed with streptavidin-CyChrome (BD Pharmingen). The cells within the lymphocyte gate defined by light scatters were analyzed through FACSort with CellQuest software (BD Biosciences, Mountain View, CA). Cell sorting was performed by FACSVantage (BD Biosciences) or MACS (Miltenyi Biotec, Auburn, CA) according to the standard procedures.

PCR analysis of recombination sequence (RS) and Ig rearrangements

Genomic DNAs from sorted cells were used as a template for PCR. Equality of the input genomic DNA amount was roughly evaluated by gel electrophoresis in every assay. RS recombination was detected by amplification of a ~1500-bp product with primers Jκ intron (*a* in Fig. 1*A*, 5′-CTGACT GCAGGTAGCGTGGTCTTCTAG-3′) and RS-637 (*c* in Fig. 1*A*, 5′-TT GACTGTTTGCTACTTCAGCTCACTG-3′). The 3′ region of RSS (294 bp) was amplified with primers RS-341 (*b* in Fig. 1*A*, 5′- CAGTTGAGCT CAGATTTGAGCCCTAATG-3′) and the RS-637. Both amplifications were performed as follows: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 25 cycles. Products were separated by electrophoresis in 1% agarose gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled probes at 42°C. A DNA fragment amplified by PCR using the primers RS-341 and RS-637 was used as a probe. After washing the membrane, the signals were visualized by exposure to radiographic film (BioMax; Kodak, Rochester, NY). *Igκ* gene rearrangements were detected as described elsewhere (44), with a modified amplification protocol (95°C for 30 s, 60°C for 1 min, and 72°C for 1 min for 25 cycles). In Fig. 1*C* (*middle*), a subset of Vκ genes, not including the *Vκ4* gene, was amplified using the primer Vκ1 (*d* in Fig. 1*A*) (16). The products were hybridized with the Jκ1-5 probe generated by PCR with tail DNA as a template and the following primers: Jκ1-5′ (5′-TGGACGTTCGGTGGAGGCAC-3′) and Jκ5-as (5′-CTAACATGAAAACCTGTGTCTTACACA-3′). In Fig. 1*C* (*top*), the targeted *Vκ* locus was amplified with the primers Vκ4 (*e* in Fig. 1*A*, 5′-CCCATTCACGTTCGGCTCGGGG-3′) and 3′ IRS (*g* in Fig. 1*A*, 5′-ACACTGGATAAAGCAGTTTATGCCC-3′). PCR data shown here are the representatives of at least two assays using more than five individuals of each genotype in total, which showed essentially the same results.

Nucleotide sequence analysis of VκJκ joins

To amplify the VκJκ rearrangement upstream of RS recombination, a degenerated primer for Vκ framework region 3 (*h* in Fig. 2*E*, 5′-GGCTGC AGSTTCAGTGGCAGTGGRTCWGGRAC-3′) (45) and a primer just 3′ of the RSS (*i* in Fig. 2*E*: 5′-ACATGGAAGTTTTCCCGGGAGAATATG-3′) were used in the following amplification protocol: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 35 cycles. The major products (~1450 bp) corresponding to the alleles that underwent Vκ–Jκ5 and RS recombinations at the same time were gel isolated, cloned into the pGEM-T easy vector (Promega, Madison, WI), and bacterial colonies were screened by hybridization using the IVS probe (45). Thus, selected clones were sequenced with the dideoxy termination method (Beckman Coulter, Fullerton, CA) using the Jκ5-as primer (see above).

Detection of Vλ1–Jλ1 excision product

Eight- to 10 wk-old mice were gamma irradiated (5 Gy) and sacrificed after 14 days when reconstitution of the bone marrow by newly generated immature B cells reached maximum (Ref. 22 and our unpublished data). The bone marrow cells from three mice of each genotype were combined and first bound on ice with rat anti-mouse Igκ mAb (BioSource International, Camarillo, CA) as a stimulation reagent (as indicated) and then with goat anti-mouse κ polyclonal Ab conjugated with FITC (Southern Biotechnology Associates). Igκ^+ cells were enriched by MACS using anti-FITC MicroBeads (Miltenyi Biotec) and directly sampled or cultured in RPMI1640 with 10% FCS for 48 h at 1×10^5/100 μl per well along with an equal number of bone marrow cells from RAG2$^{-/-}$ mice. Just before the culture, the same anti-Igκ mAb (20 μg/ml) was supplemented in the culture (as indicated) to ensure the stimulation. After the culture, cells were lysed in 90 μl of water, incubated at 95°C for 10 min, followed by incubation with 1 mg/ml protease K (Merck, West Point, PA) at 55°C for 1 h and then at 95°C for 10 min. The lysates were used as templates for PCR (94°C for 1 min, 64°C for 1 min, and 72°C for 2 min for 27 cycles) with primers detecting Vλ1–Jλ1 excision product (P46 and P47) (6). Genomic DNA content in the lysates was standardized according to wild-type RAG2 gene dosage estimated by PCR (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 27 cycles) with primers RAG2–5′ (5′-TGACATAGCCTGCT TATTGTCTCCT-3′) and RAG2–3′ (5′-CTTGTATAGATTAATAGTG GACCTT-3′). The products were verified by Southern blot hybridization with appropriate probes (6).

Single-cell PCR analysis

Single-cell PCR was performed as previously described (46) with a slight modification. Single cells were picked up by glass capillary under a microscope from a cell suspension of splenic B cells purified by anti-B220 Ab-conjugated magnetic beads for MACS. The single cells were directly

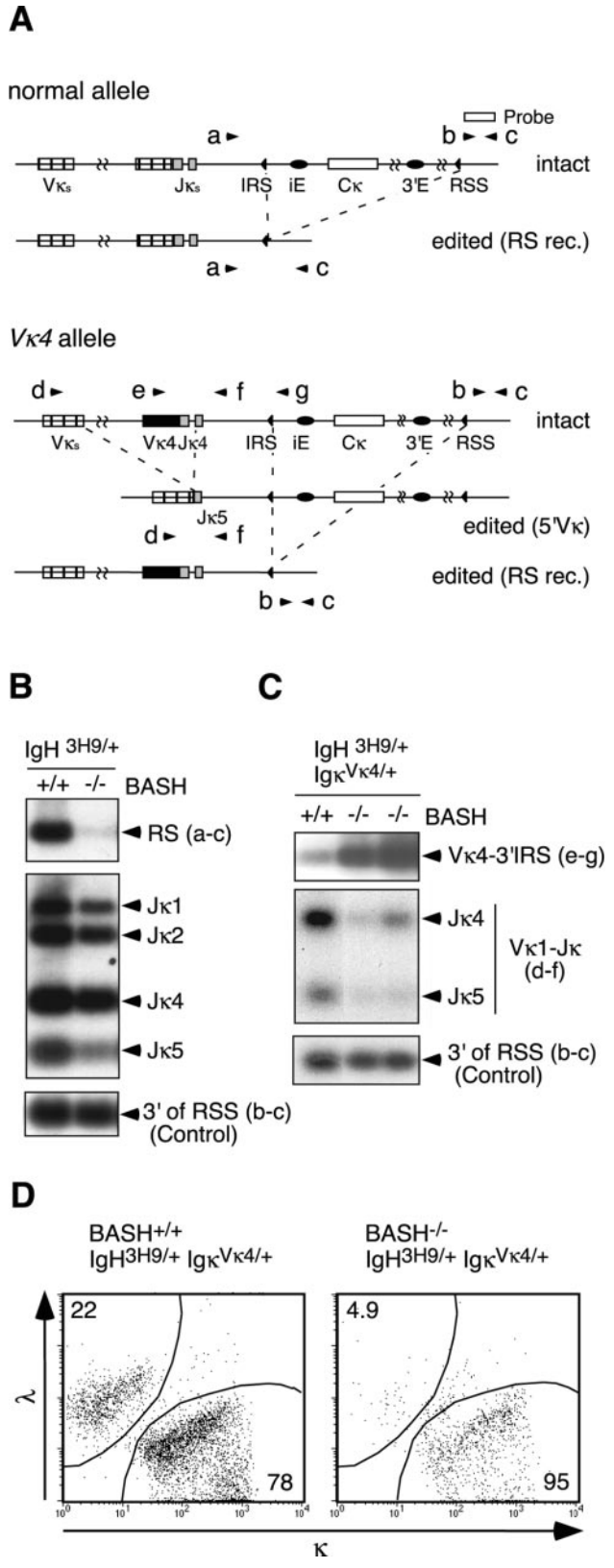

FIGURE 1. Requirement of BASH for receptor editing in anti-DNA Ig knock-in mice. *A,* Schematic representation of RS recombination and Vκ replacement at *Igκ* locus in the normal and *Vκ4* knock-in alleles. Primers (*a–g*) for PCR are indicated by arrowheads. iE, intron enhancer; 3′E, 3′ enhancer. *B,* PCR analysis of RS and VκJκ recombination. Genomic DNA from equal number of sorted immature B cells ($IgM^{high}IgD^{low}$, purity was >90%) from spleens of the indicated mice were subjected to the PCR with primers *a* and *c* in Fig. 1*A*, followed by the hybridization with the probe shown in Fig. 1*A* for the RS recombination (*top*) or to the PCR analysis for VκJκ recombination (*middle*) performed as described previously (44). 3′ region of the RSS was amplified using primers *b* and *c* (Fig. 1*A*) to control genomic DNA input (*bottom*). *C,* PCR detection of an unedited *Vκ4* knock-in locus using the primers *e* and *g* (Fig. 1*A*) in κ^+ splenic B cells of the indicated mice (*top*). Recombination of a subset of Vκ genes, not including the knock-in *Vκ4* gene, and Jκs was also amplified using primers *d* (Vκ1) and *f* shown in Fig. 1*A* (*middle*). Amplification of Vκ-Jκ1/Jκ2 recombination was inefficient probably due to the long distance to be amplified. 3′ region of the RSS was amplified as in *B* to control genomic DNA input (*bottom*). *D,* Bone marrow cells from the indicated mice were stained with anti-κ, anti-λ, and anti-μH Abs and analyzed by flow cytometry. To exclude pre-BCR^+ pre-B cells accumulating in the bone marrow of $BASH^{-/-}$ mice (37, 42), only μH^{high} B cells were electrically gated and analyzed for κ and λ expression. Numbers represent percentages of the μH^{high} B cells.

transferred to tubes containing 25 μl of 1 mg/ml protease K and incubated for 1 h at 55°C, then for 10 min at 95°C to inactivate the protease K. This lysate was subjected to two rounds of PCR. The first round contained the following six primers: 3H9-5′ (5′-CTGTCAGGAACTGCAGGTAAG G-3′), 3H9-3′ (5′-CATAACATAGGAATATTTACTCCTCGC-3′), Vκ1, Vκ2, Vκ3 (16), and the Jκ5-as (see above). Five-microliter aliquots of the product were subjected to the second PCR to amplify the *3H9* gene and Vκ-Jκ rearrangements separately by the following primers: for 3H9, 3H9–5′ inner (5′-GCATTCAGTAGYTCCTGGATGAAC-3′) and 3H9–3′ inner (5′-TCTTGCACAGAAGTAGACCGCAGA-3′); for Vκ-Jκ rearrangements, forward region 3 (see above) and Jκ1-3′- (5′-GCCACAGACATAGA CAACGGAAGA-3′), Jκ2-3′ (5′-AATTTGCTAAATCCCTGAAATC TCCT-3′), Jκ4-3′ (5′-CACACAAGTTACCCAAACAGAACC-3′), or Jκ5-3′ (5′-GTGTACTTACGTTTCAGCTCCAGC-3′).

Immunization and anti-DNA Ab titration by ELISA

Mice (8–10 wk old) were immunized by an i.p. injection of 100 μg of an oligonucleotide-avidin complex prepared as follows: biotinylated oligonucleotide (5′-ACAAGCAGGGAGCAGATACTCGAGCGG-3′, a sequence derived from Rous sarcoma virus long terminal repeat) was mixed with avidin (from egg white; Sigma-Aldrich, St. Louis, MO) at a molecular ratio of 4:1 in PBS and either precipitated in alum or emulsified with CFA (H37 Ra; Difco, Detroit, MI). Pre- and postimmunization sera were analyzed for anti-dsDNA or anti-ssDNA IgG titers by ELISA. Plates were precoated for 1 h at room temperature with 0.001% protamine sulfate (Sigma-Aldrich), washed with water, and coated with calf thymus DNA (1 μg/well; Sigma-Aldrich) in 0.15 M NaCl/0.015 M sodium citrate (pH 8.0) by drying at 37°C. The dsDNA was prepared by shearing and the ssDNA by additional boiling. The plates were masked with 3% BSA in PBS and incubated with serially diluted sera. Bound Abs were detected with HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) and a tetramethylbenzidine peroxidase enzyme immunoassay substrate kit (Bio-Rad, Hercules, CA).

Results

Impaired editing of anti-DNA Ag receptors in Ig knock-in mice lacking BASH

To examine whether BASH is necessary for BCR to signal receptor editing, we analyzed the BASH-deficient mice carrying a targeted IgH locus in which D_{Q52} and all the J_H gene segments had been replaced by a rearranged V_H3H9 gene encoding an H chain of anti-dsDNA Ab ($IgH^{3H9/+}$) (13). In the $IgH^{3H9/+}$ mice, the 3H9 H chain forms dsDNA-binding receptors in combination with ~60% of endogenous mouse κ-chains (47). Such receptors are edited mostly through repeated L chain gene rearrangements and partly through recombination between the V_H3H9 gene and the upstream endogenous V_H or D_H genes as previously shown by analyses of splenic B cell hybridomas (13, 48). The editing at the L chain gene loci often results in a deletion of the responsible κ gene through RS recombination, a recombination between an intron-recombining sequence (IRS) located in the Jκ-Cκ intron and a recombination signal sequence (RSS) located ~25 kb downstream of the Cκ exon (Fig. 1*A*) (16, 49). Therefore, the extent of the RS recombination

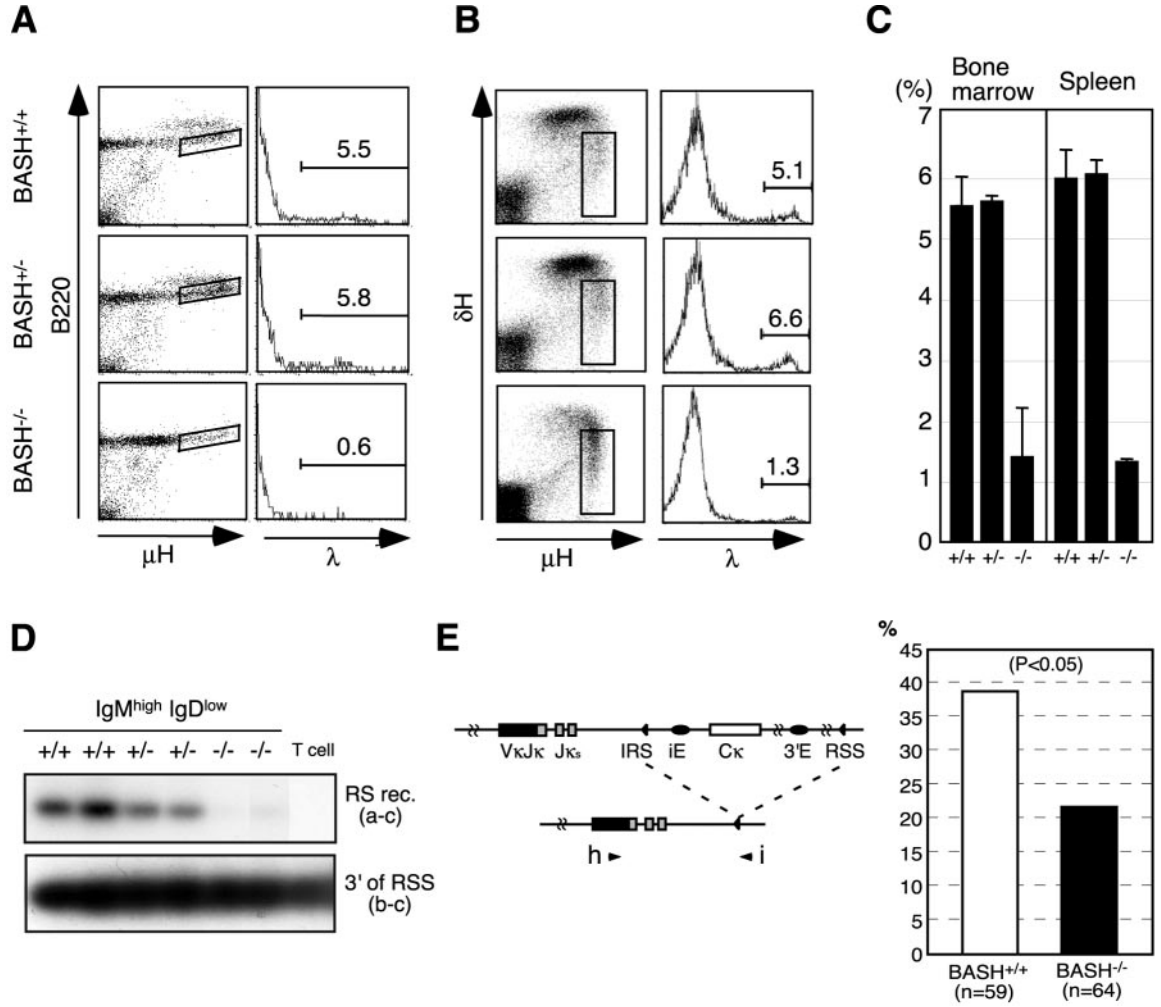

FIGURE 2. Receptor editing in the normal B cell repertoire in wild-type and BASH-deficient mice. *A–C,* Igλ expression (each *right panel*) in bone marrow (*A*) and spleen (*B*) immature B cell population ($\mu H^{high}B220^{middle}$ and $\mu H^{high}\delta H^{low}$, respectively, as indicated by the window in each *left panel*). Numbers indicate $Ig\lambda^+$ cell percentages of the immature B cells, which are summarized in *C* ($n = 6$ for each genotype). *D,* PCR analysis for RS recombination frequency. Genomic DNAs (1 ng/sample) from splenic immature B cells (IgM^{high} IgD^{low}) of the indicated genotypes and normal thymocytes (T cell) were used for the PCR with the primers *a* and *c* shown in Fig. 1*A* for the RS recombination (*top*) or with the primers *b* and *c* (Fig. 1*A*) to control genomic DNA input (*bottom*). *E,* Frequency of in-frame VκJκ joins upstream of RS recombination. Genomic DNAs from $Ig\lambda^+$ B cells sorted from spleens by MACS were subjected to PCR with a universal Vκ primer and a primer 3′ of RSS (*h* and *i* in the *left scheme*, respectively). VκJκ joins of the products were clonally sequenced. Each bar represents the percentage of the number (*n*) of clones sequenced. Statistics was determined using the χ^2 test.

was first assessed by PCR with genomic DNA from splenic immature B cells as a template. Robust amplification of RS recombination at κ loci was observed in splenic immature B cells from $BASH^{+/+}IgH^{3H9/+}$ mice, whereas essentially no RS recombination was detected in the same subset of cells from $BASH^{-/-}IgH^{3H9/+}$ mice (Fig. 1*B*, *top*), as compared with the similar level of overall Vκ-Jκ recombination between the two genotypes (Fig. 1*B*, *middle*). This indicates that the RS recombination due to the editing of DNA-binding BCR is strongly dependent on BASH.

To determine the extent of the V_H gene replacement, the integrity of the knocked-in V_H3H9 locus in single splenic B cells was analyzed by PCR. Vκ-Jκ-rearranged alleles were simultaneously amplified to identify the cells as B cells. Among the cells having undergone Vκ-Jκ rearrangement, the V_H3H9 gene was undetectable in 38% of the $BASH^{+/-}$ B cells, whereas it was undetectable in only 13% of the $BASH^{-/-}$ B cells (Table I), suggesting that receptor editing through V_H gene replacement is also defective in $BASH^{-/-}$ mice. These results combine to suggest that BASH is required for receptor editing caused by self-Ag recognition.

To confirm the latter conclusion, we further introduced a κ allele carrying targeted integration of a rearranged Vκ4Jκ4 gene (*Vκ4*) (15) into mice carrying V_H3H9 and $BASH^-$ alleles. The *Vκ4*-encoded κ-chain forms a specific receptor for dsDNA in combination with the 3H9 H chain and is edited in essentially all B cells in mice

Table I. *Frequency of receptor editing by V_H3H9 replacement*[a]

Genotype	No. of B cells		
	Total[b]	$3H9^+$	$3H9^-$
$IgH^{3H9/+}BASH^{+/-}$	53	33	20 (37.7%)[c]
$IgH^{3H9/+}BASH^{-/-}$	40	35	5 (12.5%)[c]

[a] Single $B220^+$ splenic B cells were subjected to two-step PCR to amplify the targeted V_H3H9 gene as well as endogenous Vκ-Jκ rearranged alleles. The number of B cells retaining the V_H3H9 gene ($3H9^+$) or not retaining the V_H3H9 gene ($3H9^-$) is shown.

[b] The number of single B cells carrying one or two Vκ-Jκ rearrangements.

[c] Difference is significant ($p < 0.005$ using χ^2 test).

Table II. *Number of B cells in the bone marrow or spleen*

Genotype	No. of IgM$^+$ Cells in the Bone Marrow[a] ($\times 10^5$)	No. of IgM$^+$ Cells in Spleen[a] ($\times 10^6$)
BASH$^{+/+}$, *IgH*$^{+/+}$, *Igκ*$^{+/+}$	6.4 ± 1.6 (n = 3)	15.3 ± 7.0 (n = 3)
BASH$^{-/-}$, *IgH*$^{+/+}$, *Igκ*$^{+/+}$	1.6 ± 0.5 (n = 3)	1.7 ± 1.1 (n = 3)
BASH$^{+/+}$, *IgH*$^{3H9/+ \text{ or } 3H9/3H9}$, *Igκ*$^{Vκ4/+}$	3.4 ± 1.2 (n = 3)	13.7 ± 2.8 (n = 2)
BASH$^{-/-}$, *IgH*$^{3H9/+ \text{ or } 3H9/3H9}$, *Ig*$^{Vκ4/+}$	1.0 ± 0.7 (n = 4)	0.9 ± 0.5 (n = 3)

[a] Estimated by a percentage of total lymphocytes as determined by flow cytometry and the total counts of nucleated cells.

(12, 14). In contrast to the innocuous V_H/Vκ gene knock-in mice deficient for BASH, in which the number of immature B cells in the bone marrow was recovered to the level of wild-type mice (43), BASH-deficient *V_H3H9/Vκ4* knock-in mice failed to recover the B cell number (Table II). This suggests that the majority of the dsDNA-binding B cells in the BASH-deficient knock-in mice were deleted in the bone marrow, possibly because of inefficient receptor editing. By contrast, in BASH-sufficient *V_H3H9/Vκ4* knock-in mice, B cells were not rigorously deleted, but presumably edited as efficiently as previously reported (12–18). These assumptions were tested as follows.

In *IgH*$^{3H9/+}$*Igκ*$^{Vκ4/+}$ mice, it was reported that receptor editing at the *Igκ* locus includes a deletion of the inserted *Vκ4* gene by a recombination between upstream Vκ segments and a downstream Jκ5 segment, the RS recombination on the targeted allele, and/or a recombination on the untargeted allele (14). Thus, we assessed the *Vκ4* deletion and the RS recombination on the targeted allele simultaneously by PCR using a primer specific for the *Vκ4* gene (*e* in Fig. 1*A*) and another for the 3′ region of the IRS (3′ IRS, *g* in Fig. 1*A*). Such recombination was greatly reduced; namely, far more intact *Vκ4* knock-in locus was retained in *BASH*$^{-/-}$*IgH*$^{3H9/+}$ *Igκ*$^{Vκ4/+}$ mice as compared with *BASH*$^{+/+}$*IgH*$^{3H9/+}$*Igκ*$^{Vκ4/+}$ mice (Fig. 1*C*, *top*). This was confirmed by PCR using a primer specific for a neomycin-resistant gene located upstream of the *Vκ4* gene and the 3′ IRS primer (data not shown). In addition, recombination of the endogenous Vκ and Jκ segments was less frequent in the former mice than in the latter (Fig. 1*C*, *middle*).

It has been shown previously that, in anti-self IgH/κ transgenic/knock-in mice, receptor editing results in λ-chain gene rearrangement and appearance of λ$^+$ cells, probably due to a failure to produce a functional and innocuous receptor despite repeated rearrangements of κ-chain loci (6, 12, 16, 20). In agreement with this finding, a markedly high proportion (22%) of the bone marrow B cells was λ$^+$ in *IgH*$^{3H9/+}$*Igκ*$^{Vκ4/+}$*BASH*$^{+/+}$ mice. However, the appearance of λ$^+$ B cells was suppressed to 5% in *IgH*$^{3H9/+}$ *Igκ*$^{Vκ4/+}$*BASH*$^{-/-}$ mice (Fig. 1*D*). This indicates that editing of the dsDNA-binding BCR resulting in the replacement of κ-chain with λ was also hampered in *BASH*$^{-/-}$ mice.

Taken together, in *BASH*$^{-/-}$*IgH*$^{3H9/+}$*Igκ*$^{Vκ4/+}$ mice, the dsDNA-binding BCR is not efficiently edited and the unedited B cells are considerably deleted but somewhat remain in the periphery, although direct measurement of the frequency of such cells was hampered by unavailability of a clonotypic Ab. Aside from this quantitative uncertainty, these results collectively indicate that the editing of the anti-dsDNA receptor is impaired in BASH-deficient mice and that BASH is crucial for BCR signaling that directs receptor editing.

Impaired receptor editing in the normal B cell repertoire of BASH-deficient mice

Although appearance of Igλ$^+$ B cells and RS rearrangements have been considered as hallmarks of receptor editing in the *IgH/κ* transgenic/knock-in mouse systems, it remains unclear whether the generation of Igλ$^+$ B cells and RS rearrangement in normal mice results from the editing of self-reactive BCR. Thus, it has not been proven whether receptor editing indeed operates in the development of normal Ig repertoire. Since receptor editing was impaired in the *BASH*$^{-/-}$ mice with the knock-in *Ig* loci as so far described, we next analyzed *BASH*$^{-/-}$ mice with unmanipulated *Ig* loci to

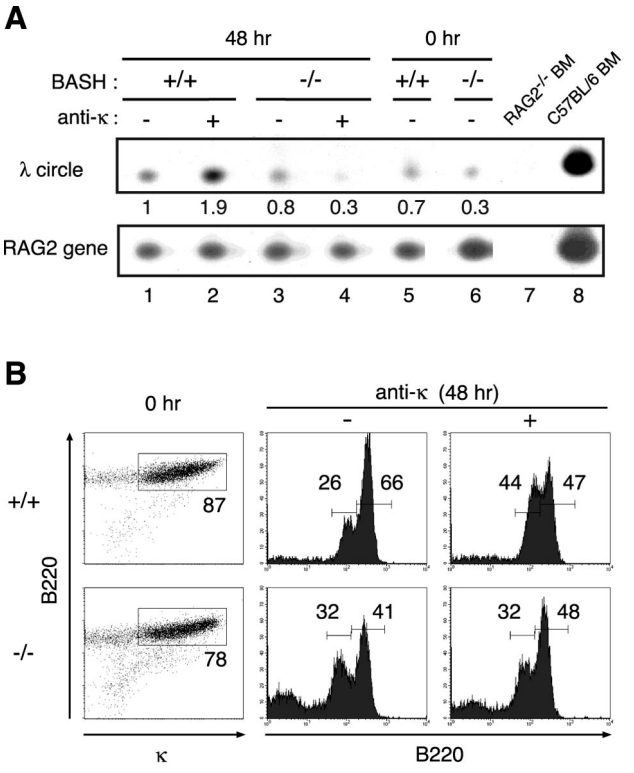

FIGURE 3. Anti-κ Ab-induced λ rearrangement and maturation block in vitro are defective in BASH-deficient mice. *A,* Self-reconstituted bone marrow κ$^+$ immature B cells of the indicated *BASH* genotypes were sampled before culture (0 h) or cultured with (+) or without (−) anti-mouse κ mAbs for 48 h in the presence of bone marrow cells from *RAG2*$^{-/-}$ mice. The cell lysates were subjected to PCR for amplification of Vλ1–Jλ1 excision products (λ circle; *top*) or wild-type *RAG2* gene (*bottom*). The latter does not amplify the targeted *RAG2* gene in the *RAG2*$^{-/-}$ mice, therefore it was used to normalize the number of input B cells in the samples. Bone marrow cells from normal C57BL/6 and *RAG2*$^{-/-}$ mice were used as positive and negative controls for the PCRs, respectively. Numbers reflect the relative intensity of the hybridization bands representing λ circle DNA, which was normalized according to those representing *RAG2* gene. *B,* After the same culture as in *A*, beside that five times more cells were used in the culture, the cells were stained with anti-B220 Ab and analyzed by flow cytometry. Numbers indicate percentages of the cells in the lymphocyte gate (*middle* and *right panels*). Before the culture, the sorted immature B cells were stained for κ L chain and B220 and analyzed by flow cytometry to show the similar homogeneity of the sorted samples (*left panels*).

determine whether such BASH-dependent receptor editing operates in mice with normal Ig repertoire. As known for most experimental mice, ~5–6% of immature B cells in the bone marrow as well as in spleen were λ^+ in *BASH*$^{+/+}$ and *BASH*$^{+/-}$ mice (Fig. 2, *A–C*). In *BASH*$^{-/-}$ mice, the proportion of λ^+ B cells within the comparable immature B cell population was decreased to 1.5 and 1.3% on average in the bone marrow and spleen, respectively. This suggests that BASH is necessary for the generation of a large part of λ-bearing B cells, possibly through receptor editing.

Next, we assessed by PCR the extent of RS recombination of *Igκ* loci in splenic immature B cells from mice of the three genotypes (Fig. 2*D*). The frequency of RS recombination per genome was markedly reduced in *BASH*$^{-/-}$ mice as compared with *BASH*$^{+/+}$ or *BASH*$^{+/-}$ mice, suggesting that the RS recombination in the normal repertoire is induced largely through BASH-mediated BCR signaling. RS recombination could also happen to delete a nonfunctional *Igκ* allele. In such a case, a rearranged VκJκ sequence upstream of the RS recombination would be likely to have an out-of-frame joining, whereas it should be in-frame if RS recombination had inactivated a self-reactive κ-chain gene (45). To distinguish these possibilities for the residual RS recombination in *BASH*$^{-/-}$ B cells, the VκJκ joins upstream of the RS recombination were amplified from splenic λ^+ B cells in which the edited cells should be enriched, and their nucleotide sequences were determined as described previously (45). As shown in Fig. 2*E*, the frequency of in-frame VκJκ joins was 38% of such VκJκ joins in the B cells from wild-type mice. In those from *BASH*$^{-/-}$ mice, it significantly deceased (21%), suggesting RS recombination had inactivated a self-reactive κ-chain gene less frequently than in wild-type mice. The remaining in-frame joins may still include nonfunctional genes such as Vκ pseudogenes or those producing mispairing κ-chains. Taken together, these results indicate that receptor editing, and perhaps the editing of self-reactive BCR, is greatly impaired in a normal B cell repertoire in the absence of BASH. This provides the first genetic evidence, to our knowledge, that BCR-signaled receptor editing indeed operates in the development of the normal B cell repertoire of mice.

To determine a role for BASH in the BCR signaling that directs secondary recombination of L chain loci in vitro, a population enriched for immature κ^+ B cells was obtained from the bone marrow of irradiated, self-reconstituted *BASH*$^{+/+}$ and *BASH*$^{-/-}$ mice, and anti-κ Ab-induced Vλ-Jλ recombination was estimated by detecting excised circle DNA (λ circle) by PCR (6). Lymphocyte-free bone marrow cells from RAG2-deficient mice were added to the culture to prevent BCR-signaled apoptosis of the immature B cells (22). Before culture, the background level of λ circle could be detected in the immature B cells, possibly due to contamination of λ^+ cells having just finished the λ rearrangement, and such cells appeared to be less in *BASH*$^{-/-}$ immature B cells than in *BASH*$^{+/+}$ cells (Fig. 3*A*, *lanes 5* and *6*) in line with the result shown in Fig. 2, *A* and *C*. Anti-κ stimulation during the culture resulted in the increase of the λ circles in the *BASH*$^{+/+}$ cells as compared with unstimulated cells (Fig. 3*A*, *lanes 1* and *2*), but not in the *BASH*$^{-/-}$ cells (*lanes 3* and *4*). In the latter, the λ circles were rather decreased by the anti-κ stimulation, which might be due to a prolonged survival of stimulated κ^+ B cells in this culture condition. Therefore, the 2-fold increase of λ circle in the *BASH*$^{+/+}$ cells in this data might be underestimated. As previously reported (43), the immature B cells differentiated into the mature stage (B220^{high}) during the culture without anti-κ Ab, with some retardation in BASH$^{-/-}$ cells (Fig. 3*B*, *middle*). Anti-κ stimulation suppressed the differentiation in BASH$^{+/+}$ cells, but not in BASH$^{-/-}$ cells (Fig. 3*B*, *right*). Thus, the BCR-signaled delay of differentiation at the immature B cell stage may correlate to the induction of λ recombination. These results indicate that BASH is necessary for BCR ligation-induced recombination of λ-chain gene in vitro and therefore suggest a pivotal role for BASH in the BCR-signaling pathway directing receptor editing through secondary L gene rearrangement.

Enhanced anti-DNA Ab production upon DNA immunization in BASH-deficient mice

Assuming that receptor editing, in cooperation with clonal deletion, is indeed responsible for purging self-reactivity from the repertoire of newly generated B cells, then supposedly remaining, unedited B cells in *BASH*$^{-/-}$ mice would respond to self-Ags if they are functional and T cell help is provided appropriately. To verify whether such self-reactive B cells are present in the periphery of *BASH*$^{-/-}$ mice, we assessed anti-DNA Ab production in the mice upon immunization of DNA. Since *BASH*$^{-/-}$ mice were

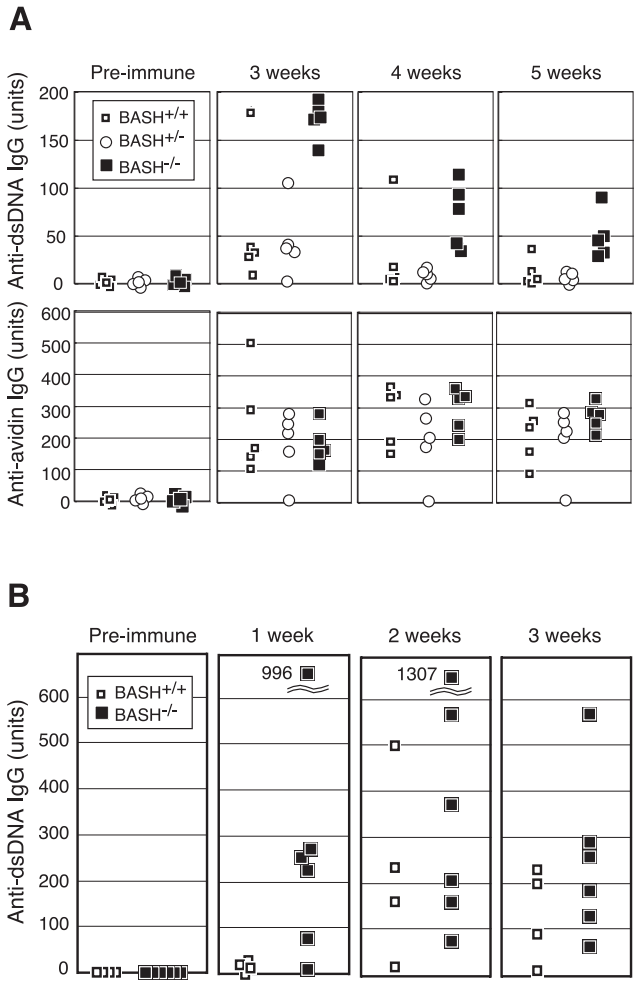

FIGURE 4. Excessive production of anti-dsDNA IgG Ab upon DNA immunization in BASH-deficient mice. *A,* Serum anti-dsDNA IgG (*top*) and anti-avidin IgG (*bottom*) titers 1 wk before (preimmune) or the indicated weeks after immunization with oligonucleotide-avidin complex precipitated in alum. One sample from a wild-type mouse showed high titer for both DNA and avidin for unknown reasons. *B,* Serum anti-dsDNA IgG titers 1 wk before (preimmune) or the indicated weeks after the immunization with oligonucleotide-avidin complex emulsified with CFA. *A* and *B,* Shown are the relative serum IgG concentrations of the individual mice of the indicated genotypes. They are expressed as relative to a value of a standard (set as 1000), namely, an autoimmune serum from a mice suffered from experimental graft-vs-host disease. The titer of a serum from a typical autoimmune NZB/W F_1 mouse was 1771 U when assayed in *B*.

shown to be deficient in a T cell-independent immune response (38, 40), we prepared a novel T cell-dependent DNA Ag, biotinylated oligonucleotides coupled with avidin as a non-self carrier protein. This oligonucleotide itself was not mitogenic for B cells in vitro (data not shown). When this Ag was given in an alum-precipitated form, approximately four times as much anti-dsDNA IgG Ab was produced in $BASH^{-/-}$ mice as in $BASH^{+/+}$ or $BASH^{+/-}$ mice 3 wk after immunization, and this Ab remained at a higher level at a later time course in the former mice than in the latter (Fig. 4*A*). The results for anti-ssDNA IgG Ab production were essentially the same (data not shown). When the Ag was given in CFA, a rapid and strong anti-dsDNA IgG response was observed only in $BASH^{-/-}$ mice at 1 wk after immunization; however, after 2 wk, anti-dsDNA IgG was significantly produced also in wild-type mice, perhaps by low-affinity poly-reactive B cells (Fig. 4*B*). In both experiments, anti-avidin IgG responses were equivalent among the mouse genotypes (Fig. 4*A* and data not shown). These results are consistent with the proposition that DNA-binding BCR are not efficiently edited and functional B cells carrying such BCR remain, if mostly deleted, in the periphery of $BASH^{-/-}$ mice. Interestingly, an anti-dsDNA IgG response did not recur in either of the genotypes upon secondary immunization at 7 wk in the former experiment, or 13 wk in the latter (data not shown), although a memory response to T-dependent Ag is not impaired in $BASH^{-/-}$ mice (50). This suggests that strong tolerance to dsDNA was induced after the primary immune response by some unknown mechanism.

Discussion

In this report, we show that receptor editing in anti-DNA Ig knock-in mice was severely impaired in the absence of BASH, which clarifies that BASH is crucial for the BCR signaling pathway directing receptor editing. Although it has been unclear whether receptor editing operates in the development of the normal B cell repertoire and contributes to the self-tolerance, appearance of the editing-deficient phenotype and augmented anti-DNA Ab response in BASH-deficient mice with unmanipulated Ig loci as shown here strongly suggest that it is indeed the case. At the same time, it has become apparent that a large part of λ^+ B cells and RS recombination in normal mice are generated instructively by BCR signaling through BASH. We cannot formally exclude the possibility that a reduced life span or accelerated rate of export of the newly formed immature B cells in the BASH-deficient mice could allow too little time for the cells to undergo editing. However, we think either is unlikely because BASH-deficient mice possessed as many immature B cells in the bone marrow as wild-type mice when their *Ig* loci had been "knocked-in" along with $V_H/V\kappa$ genes encoding an innocuous BCR (43), which suggests that the scarcity of immature B cells in the bone marrow of $BASH^{-/-}$ mice stems primarily from inefficient L chain gene rearrangement (42) but not from a reduced survival or export rate.

BCR signaling results in a variety of cellular events including activation, cell cycling, survival, differentiation, apoptosis, and induction or suppression of Ig gene rearrangement, depending on the developmental stage or condition of the cells and interaction with surroundings. Signaling pathways from BCR leading to these events may differ from each other. The pathway to receptor editing has not been studied extensively, because of its requirement for a complicated mouse system. So far, Btk-, CD45-, and CD19-positive regulators for BCR signal transduction have been examined in anti-self-Ig-transgenic mouse systems and reported to be unnecessary for receptor editing (25–28). However, conventional Ig-transgenic systems applied in these reports are not appropriate to examine receptor editing, because such transgenes are randomly integrated into the genome and therefore cannot be silenced by recombination of endogenous Ig genes. Thus, direct comparison between these reports and ours is not applicable. Nevertheless, data reported by Dingjan et al. (27) actually implied that editing of the anti-self-MHC Ig was partially impaired in Btk-deficient mice, despite the authors' conclusion. They also demonstrated that enforced expression of an active form of Btk in the pro-B cell stage in mice resulted in an increased λ usage on the descendant B cells, suggesting a role for Btk as a positive regulator of the V-J recombination at *Igλ* loci. Alternatively, this may be interpreted as that the Btk-mediated signal extends the duration in which the recombination at all L gene loci is active, as proposed for the model of receptor editing (12). This would result in exhaustion of recombination substrates and in RS recombination at the *Igκ* loci, which are shown to be more accessible to the recombination machinery than *Igλ* loci (51), and finally in an increased λ gene rearrangement. BASH may also be engaged in the same mechanism. Although the phenotype of BASH-deficient mice closely resembles that of Btk-deficient mice, it is likely that BASH and Btk have independent signaling functions because mice deficient for both BASH and Btk showed a much severer defect in B cell development than either of the single-deficient mice (52, 53). Thus, BASH and Btk might be engaged independently and perhaps cooperatively in the BCR signal extending the duration for the L gene recombination and thus for the receptor editing.

Since it is known that *RAG* expression continues from the pre-B to immature B cell stage in the bone marrow (54–56), it seems unlikely that BCR signaling induces receptor editing by regulating *RAG* expression in the development of the normal B cell repertoire. Then how does BCR signaling induce receptor editing? More specifically, why do the B cells undergo receptor editing when their BCR is bound with self-Ag, but not when unbound, while BCR-null pre-B cells continue L chain gene recombination? Pre-BCR signal is proposed to activate the accessibility of L chain loci for V(D)J recombinases and to induce cell cycling concomitantly (42, 57–61). As pre-BCR is down-regulated, the cell cycle arrests and *RAGs* are up-regulated, V-J recombination at L chain loci starts and continues until the cell succeeds in a functional rearrangement and the expression of innocuous BCR. We speculate that unligated BCR may signal the L chain loci to lose the accessibility and stop further recombination. On the contrary, Ag-bound BCR may signal the loci to sustain its accessibility to the recombinases. BASH would then be required for the latter (Ag-bound) BCR signaling pathway but not the former (unbound). As BASH-deficient pre-B cells show a reduced level of germline κ transcription and κ gene rearrangement (42), BASH may be adjusted to transmit a relatively strong or persistent signal from the Ag-bound BCR as well as self-cross-linking pre-BCR (62) and may not be involved in a weaker or transient signal from unbound BCR, at least in the bone marrow. Btk might also work in the same way as BASH (27). The sustained accessibility of the L chain loci would result in a repeated recombination of both κ- and λ-chain loci, but κ loci would be more frequently recombined in the early period because of their higher accessibility than λ loci (51), and later λ loci more frequently when κ loci become less available for the recombination. Although a weaker signal from unbound BCR is supposed to shut off the L chain locus accessibility, a much weaker signal below a certain threshold does not seem to and causes receptor editing (23, 24, 63), just as pre-BCR/BCR-null small pre-B cells repeat L chain gene recombination until they express functional BCR. Minimal receptor editing occurring in BASH-deficient B cells may be attributable to this mechanism.

By means of loss-of-function mutation in mice, we clearly illustrate the fact that BCR-mediated receptor editing operates in the

development of the normal B cell repertoire and contributes to limit self-reactivity of the B cell repertoire in the peripheral immune system. This result reconfirms importance of the central mechanism of B cell tolerance in the homeostasis of the immune system and in pathogenesis of autoimmune disease. Although BASH-deficient mice appeared to contain self-reactive B cells in the periphery, they did not spontaneously develop autoimmune disease. This is probably because the T-independent response of B cells to Ags is severely impaired (38, 40, 50) and, at the same time, T cell tolerance to self-Ags must be maintained and prevent autoimmunity in these mice (64). In addition, even when T cell help was provided to the self-reactive B cells, autoantibody production occurred only in the primary but not in the secondary response in BASH-deficient as well as wild-type mice as described above, suggesting that another mechanism of tolerance that is induced during the primary response to self-Ag. BASH-deficient mouse mutant will offer itself as a useful model for studying a contribution of such mechanisms for self-tolerance to avoidance of autoimmune disease.

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