Direct Reduction of Antigen Receptor Expression in Polyclonal B Cell Populations: Developing In Vivo Results in Light Chain Receptor Editing

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*J Immunol* 2012; 188:47-56; Prepublished online 30 November 2011;
doi: 10.4049/jimmunol.1102109
http://www.jimmunol.org/content/188/1/47

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/11/30/jimmunol.1102109.DC1

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Direct Reduction of Antigen Receptor Expression in Polyclonal B Cell Populations Developing In Vivo Results in Light Chain Receptor Editing

Shixue Shen and Tim Manser

Secondary Ab V region gene segment rearrangement, termed receptor editing, is a major mechanism contributing to B lymphocyte self-tolerance. However, the parameters that determine whether a B cell undergoes editing are a current subject of debate. We tested the role that the level of BCR expression plays in the regulation of receptor editing in a polyclonal population of B cells differentiating in vivo. Expression of a short hairpin RNA for \( \kappa \) L chain RNA in B cells resulted in reduction in levels of this RNA and surface BCRs. Strikingly, fully mature and functional B cells that developed in vivo and efficiently expressed the short hairpin RNA predominantly expressed BCRs containing \( \lambda \) light chains. This shift in L chain repertoire was accompanied by inhibition of development, increased Rag gene expression, and increased \( \lambda \) V gene segment-cleavage events at the immature B cell stage. These data demonstrated that reducing the translation of BCRs that are members of the natural repertoire at the immature B cell stage is sufficient to promote editing. The Journal of Immunology, 2012, 188: 47–56.

Antigen receptors play pivotal roles in regulating lymphocyte activation, proliferation, and developmental progression (1–3). Early in B cell development, expression of an IgM H chain and formation of the pre-BCR result in suppression of the expression of the Rag-1 and -2 proteins and, thus, VDJ recombination at the H chain loci (3–5). Pre-BCR signaling also promotes a phase of proliferation leading to the stage in which L chain V gene segment recombination is initiated (5–7). Expression of a functional L chain leads to expression of cell surface IgM (sIgM), suppression of Rag gene expression, and, thus, L chain VJ rearrangement, as well as promotion of further development (8).

Superimposed on these processes is the tolerance mechanism termed “receptor editing” or revision (9–12). Development of B cells expressing sIgM with certain autoreactivities is blocked at an early immature stage, and the expression of the Rag proteins is sustained. This leads to ongoing VJ recombination at the \( \kappa \) and then \( \lambda \) L chain loci. A developing B cell whose autoreactivity is altered or reduced via editing then proceeds in development (11).

The role that BCR and pre-BCR ligands play in regulation of primary B cell development and receptor editing is a current subject of controversy. Although ligand(s) for the pre-BCR have been found, their engagement appears unnecessary for functional pre-BCR signaling (13, 14). This suggests that formation of a membrane-bound pre-BCR complex is sufficient to provide the “tonic” or “basal” signaling required to suppress Rag expression and promote development.

That the level of BCR expression on developing B cells and hence, the amount of basal BCR signaling, might regulate receptor editing was first suggested from the analysis of mice hemizygous for Ig transgenes encoding an anti-class I MHC BCR. In the apparent absence of BCR ligands, the B cells in these mice displayed extensive receptor editing, but B cells in mice homozygous for the Ig transgene locus, resulting in an ∼2-fold increase in sIgM expression, did not (15, 16). Additional studies supportive of this concept showed that perturbation of the BCR-signaling pathways, either by deletion of coreceptors or downstream effectors or treatment with pharmacologic agents targeting these pathways, could influence editing (16–18). More recent studies implicated modulation of the Syk–P(3)K–Akt–Foxo pathway by basal BCR signaling as being crucial in the control of receptor editing (19, 20).

More direct evidence of a role for BCR expression and basal signaling in the regulation of receptor editing has been obtained by analysis of the behavior of immature B cells developing in in vitro cultures after expression of their transgenic BCRs are genetically ablated (21, 22). This results in induction of Rag gene expression, endogenous VJ rearrangement, and “back” differentiation to an immature phenotype. This led to a model for the regulation of receptor editing in which basal signaling from sIgM played a central, suppressive role and that auto-Ag engagement by the BCR induced receptor editing not directly via BCR signaling but due to stimulation of BCR internalization via endocytosis. This, in turn, led to reduced basal signaling from cell surface BCRs.

However, as has been pointed out, all of the above studies are subject to caveats because of the experimental systems used (16, 23). For example, expression of Ig transgenes begins early in development, abnormally accelerating or preventing subsequent stages of differentiation. IL-7–supported bone marrow (BM) cultures undoubtedly do not accurately mimic the microenvironmental niches in which developing B cell reside in vivo. Addition of exogenous antagonists or agonists of BCR-signaling pathways to such cultures may result in additional nonphysiological respon-
ses. As such, we investigated the effects of direct reduction of BCR expression levels in developing B cells in vivo using a κ L chain RNA “knockdown” approach. This approach differs fundamentally from those of previously published studies in which expression of the κ locus was precluded by the introduction of germline deletions (24, 25), because developing B cells would first have to productively rearrange and express a κ allele to become susceptible to reduction of BCR levels via κ RNA knockdown.

Materials and Methods

Ligation-mediated PCR

Genomic DNA for ligation-mediated PCR (LM-PCR) was prepared from sorted cells, according to an established protocol (26). After ligation of oligonucleotide linkers, DNAs were amplified with either κ or λ locus-specific primers to detect particular dsDNA breaks in these loci, as described (27). The λ-specific signal end break primer (5'-GGAGATGTAG-CACCCTGTTAAG-3') was kindly provided by Dr. Pamela Nakajima.

RNA isolation, real-time RT-PCR, and sequence analysis

Total mRNA was purified from sorted cell populations using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. First-strand cDNA was synthesized using TaqMan reverse-transcription reagents (Applied Biosystems) with random hexamer primers. Relative quantitative real-time RT-PCR was performed using TaqMan gene-expression assays on an ABI Prism 7000 sequence-detection system (Applied Biosystems). The following primers were obtained from Applied Biosystems: κ L chain (Mm01611305_g1), Rag-2 (Mm00501300_m1), and GAPDH (Mm99999915_g1, used as endogenous control). Real-time RT-PCR reactions were performed in triplicate, and ΔΔCT values were normalized to those obtained from GAPDH RNA amplification. The mean and standard deviations were calculated using Microsoft Excel software. The synthesized cDNA was also used for normal PCR reactions. Variable regions of λ L chain cDNA were amplified with the primer pair 5'-TGGAG-GACAAGGCCTCCCTCACCATACAG-3' and 5'-GAGGCTCTCAGG-GAAATGGAACABGGT-3' (28). Purified PCR products were cloned into plasmid vectors using the pGEM-T Easy Vector System (Promega, Madison, WI). Sequencing reactions were performed on the plasmid inserts in the Kimmel Cancer Center genomics facility, and the CLUSTAL W multiple sequence alignment program was used to align the V and J regions of λ light chains.

Hybridomas

Total splenocytes from chimeric mice were stimulated with LPS (20 μg/ml) and IL-4 (50 ng/ml) for 3 d. Hybridomas were constructed using the SP2/0 fusion partner, as previously described (29).

Mice

C57BL/6 (CD45.2) and C57BL/6SJL-Pprrca Pechb/BoyJ (CD45.1) congenic mice were purchased from The Jackson Laboratory, maintained under specific pathogen-free conditions, and provided autoclaved food and water. Mice were 6–12 wk old at initiation of the experiments. The use of mice in these studies was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University under protocol 344A.

Abl

Biotin-, allylphycocyanin-, and Pacific Blue–anti-β220 (RA3-6B2), PE-Cy7–anti-CD38 (H1/11), PE–anti-TCRβ (H57–597), alliophycocyanin–anti-CD34 (S7, Ly48), biotin- and PE-Cy7–anti-CD23 (B3B4), alliophycocyanin–anti-C14R (AA4.1), biotin–anti-GL7 (Ly7), PerCP-Cy5.5–SA, biotin–anti-CD45.1 (A20), and PE–anti-CD45.2 (104) were from eBioscience. PE–anti-BP-1 (6C3), alliophycocyanin–anti-CD21/CD35 (7G6), biotin–anti-HSA (30-F1), biotin–anti-CD5 (Ly-1), and biotin–anti-CD138 (281-2) were from BD Biosciences. Biotin–anti-IgD (11-26), biotin–anti-CD5 (Ly-1), and biotin–anti-κ were from Southern Biotechnology Associates. PE–anti-IgM was from Jackson ImmunoResearch Laboratories, and SA-Alexa 633 was from Molecular Probes.

B lymphocyte cultures

B lymphocyte cultures were as previously described (29). Briefly, BM cultures were performed with murine IL-7 (32 ng/ml; R&D Systems). Naive splenic B lymphocytes were enriched by negative selection with anti-CD43 MACS microbeads (Miltenyi Biotec) and cultured in RPMI 1640 medium (10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μM 2-ME), LPS (20 μg/ml), and murine IL-4 (50 ng/ml, PeproTech).

Retrovirus constructs

The following short hairpin RNA (shRNA)-encoding 97mer oligonucleotide was designed using the online program RNAi Central-RNAi Oligo Retriever (http://katahdin.cshl.org/html/scripts/resources.pl) to target the C region of κ L chain mRNA and synthesized by Integrated DNA Technologies: 5'-TGCTGTGACAGTGACACTGCTGTGGTCCTT-GGATAGTGAACACAGATGTTATCTCAAGAAGCACAGCTGAGTG-TCGCCCTGCTCGGA3'. The region in bold is homologous to the Coding region, beginning at nucleotide 486. This region was cloned into the MSCV/LTR/miR-30-PIG (LMP) retrovector vector (30) (from Open Biosystems), according to the manufacturer’s protocol. Retroviral supernatants were harvested from 293T cells transfected using Fugene 6 (Roche), according to the manufacturer’s protocol. Cultured BM or splenic cells were infected with retroviral supernatant in six-well plates containing 4 μg/ml polybrene (Sigma-Aldrich) by spinning at 2500 rpm at room temperature for 1.5 h on each of the next 2 d.

BM transduction and mixed BM chimeras

BM cells from CD45.2 C57BL/6 (b6) mice treated with i.p. 5-fluorouracil (American Pharmaceutical Partners) were cultured overnight in complete RPMI 1640 medium supplemented with stem cell factor (100 ng/ml), IL-3 (10 ng/ml) and IL-6 (5 ng/ml) (PeproTech), according to an established protocol (31). The cells were then spin-infected, as described above, on each of the next 2 d. After washing in PBS, a 1:1 mixture of the infected cells and freshly isolated BM cells from CD45.2 B6 mice were adoptively transferred i.v. into irradiated (11 Gy) CD45.1 congenic recipients. This BM cell mixture was used to improve the level of T cell reconstitution in the resulting chimeric mice. Chimeric mice were given antibiotics and analyzed or immunized 8–9 wk later.

Immunizations

Chimeric mice were immunized with 100 μg 4-hydroxy-3-nitrophenylacetyl-chicken γ globulin (Biosearch Technologies) in alum i.p. and were sacrificed at day 9 for flow cytometric and histological analysis, as previously described (32).

Flow cytometry and cell sorting

Up to six-color flow cytometric analyses were performed using Becton Dickinson LSRII or FACSCalibur flow cytometers, and data were analyzed using FlowJo software (Tree Star). Cell sorting was performed on a MoFlo high-performance cell sorter (DakoCytomation).

Immunofluorescence

Spleens were isolated and fixed in 4% paraformaldehyde and 10% sucrose for 2 h at 4°C and processed by snap-freezing, cryosectioning, and immunostaining, as previously described (29, 33). Stained sections were analyzed on a Leica DM5000B digital microscope using related software (Leica Microsystems).

Statistical analyses

Statistical significance was performed with the Microsoft Excel program using a two-tailed, unpaired Student’s t test.

Results

We designed five shRNAs targeting the C region exon of κ L chain mRNA. DNA encoding these shRNAs was introduced into the MSCV/LTR/miR-30 PIG (LMP) retrovector vector that contains a gene encoding eGFP (30). B6 splenic B cells were activated with LPS in vitro and transduced with the κ shRNA vectors or the control LMP vector. One day later, surface expression of κ L chains was assayed by flow cytometry, and levels of κ RNA transcripts were evaluated by quantitative RT-PCR in FACs-purified B220+ GFP+ cells. One shRNA construct, termed KKD68, reduced surface κ expression ~2-fold and κ RNA to 40% of control levels (Fig. 1A, 1B).
Immature B cells developing in vitro that express the anti-κ

shRNA reveal evidence of receptor editing

To initially determine whether the reduction of κ expression mediated by the KKD68 shRNA might induce receptor editing, an IL-7 BM culture system was used. B6 BM cultures were infected with the KKD68 retrovirus, a κ knockdown retroviral vector that did not result in reduction in surface κ expression on LPS-activated splenic B cells (KKD59), and the control vector for 2 d. Two days later, the frequency of B cells expressing surface λ and κ L chains was evaluated. KKD68-transduced, immature B cells contained three to four times the frequency of λ-expressing cells as controls. In addition, a major subpopulation of KKD68-transduced cells that expressed both λ and κ L chains was detected (Fig. 1C). The cultures transduced with the control, KKD59, and KKD68 vectors revealed an increased frequency of B cells expressing very low to undetectable levels of L chains, perhaps due to nonspecific effects of retroviral gene expression on development in vitro. FACS-purified B220⁺, GFP⁺ cells from KKD68-transduced BM cultures expressed >2-fold more Rag-2 RNA than did controls (Fig. 1D). These data indicated that B cells in BM cultures that express the KKD68 shRNA undergo an increased frequency of L chain receptor editing.

ShRNA-expressing B cells developing in BM chimeras display altered primary development

To assess the effects of KKD68 shRNA expression on B cell development in vivo, we transduced B6 BM cells with the KKD68 or control vectors and then reconstituted lethally irradiated B6. CD45.1 recipients with a mixture of this BM and freshly isolated, untreated B6 BM. Ten to twelve weeks later, donor-derived lymphocyte development was assayed in the chimeric mice by histology and flow cytometry. The percentages of KKD68-transduced cells in the pro-B and early pre-B stages of development (B220low, CD43⁺) appeared normal, but increases in the percentage of late pre-B cells (B220low, CD43⁺, sIgM⁺/IgMlow) were observed relative to controls (Fig. 2A). Also, the frequency of immature B cells (B220low, CD43⁺, sIgM⁺) was increased in the KKD68-transduced compartment, these cells expressed lower levels of sIgM than controls, and the frequency of BM mature B cells (B220high) transduced with the KKD68 vector was reduced.

Although the majority of BM B220low pro, pre-B, and immature B cells transduced with either the KKD68 or control viruses expressed high levels of GFP, many transduced B220high cells expressed intermediate to low levels of GFP (Fig. 2B). Because this phenomenon was observed in both vector- and KKD68-transduced B cell populations, it could not be attributed only to κ knockdown and may have resulted from dilution and silencing of retroviral genomes during pre-BCR-driven proliferation and subsequent differentiation. In agreement with the data in Fig. 2A, the KKD68 shRNA had inhibitory effects on immature BM B cell development, because the frequency of B220high, IgMlow, GFP⁺, mature B cells was reduced, and the frequency of B220low, sIgM⁻, GFP⁺ B cells was increased compared with controls (Fig. 2B). In total, these data suggested that the developmental progression of many KKD68-transduced B cells is inhibited at the pre-B to immature B cell transition, but a subpopulation escapes this fate and enters the mature B cell pool expressing normal sIgM levels.

KKD68 shRNA vector gene expression results in decreased levels of κ RNA, increased levels of Rag gene and λ L chain expression, and increased frequencies of λ V gene segment cleavage events in vivo

The above data suggested that a subpopulation of KKD68-transduced B cells might escape a developmental block at the immature B cell stage by undergoing L chain receptor editing.
resulting in expression of \( \lambda \) L chains. To test this idea, we assessed levels of \( \kappa \) and \( \lambda \) L chain surface expression in the B220\(^{\text{low}}\), GFP\(^{\text{high}}\), and GFP\(^{-}\) BM subpopulations of the two types of chimeras. Although \( \kappa \)-expressing cells were abundant and \( \lambda \)-expressing cells were barely detectable in both compartments in control chimeras, few \( \kappa \) expressing cells could be detected in the GFP\(^{+}\) compartment of KKD68 chimeras, and a small subpopulation of B cells that expressed both surface \( \kappa \) and \( \lambda \) L chains was present (Fig. 3A).

Next, we FACs purified surface B220\(^{\text{low}}\), \( \kappa \) -BM B cells from various types of chimeric and control mice and measured \( \kappa \) and Rag-2 RNA levels, as well as the frequency of cleavage events at \( V \) gene segments in the \( \kappa \) and \( \lambda \) loci in these subpopulations. Rag-2 RNA levels were elevated \( \sim \) 8-fold and \( \kappa \) RNA levels were decreased \( \sim \) 8-fold in the KKD68-transduced subpopulation compared with all control subpopulations (Fig. 3B). Much reduced and dramatically increased frequencies of DNA breaks at \( J \) gene segments in \( \kappa \) loci (Supplemental Fig. 1) and \( \lambda \) loci (Fig. 3B, Supplemental Fig. 1), respectively, were detected by LM-PCR in the KKD68-transduced subpopulation compared with controls. These latter data indicated that most developing B cells that undergo only \( \kappa \) editing or rearrange and express both \( \kappa \) alleles are lost as a result of KKD68 shRNA knockdown. Although alterations in development due to expression of the KKD68 shRNA might have influenced the data obtained in these PCR assays, it seems unlikely that this could account for the \( \sim \) 8-fold decrease and 100-fold increase in \( \kappa \) RNA and recombination signal-\( \lambda \) DNA breaks, respectively, in the KKD68-transduced B220\(^{\text{low}}\), \( \kappa \) -BM B cells versus controls.

Among GFP\(^{+}\) B cells in the spleen, lymph nodes, and peritoneal cavity (PTC), those transduced with the KKD68 vector contained a dramatically increased frequency of surface \( \lambda^{+} \) cells relative to GFP\(^{-}\) B cells. Smaller subpopulations of GFP\(^{+}\) B cells in the spleen and lymph nodes of KKD68 chimeras were also present that did not detectably express \( \lambda \) L chains and that expressed both surface \( \kappa \) and \( \lambda \) L chains (Fig. 4A).

Although Fig. 2 shows that the percentage of mature B cells in the BM within the KKD68-expressing lymphocyte subpopulation was somewhat reduced, Fig. 4B illustrates that the spleens in all of the chimeric mice had normal lymphoid architecture, with KKD68-transduced cells (GFP\(^{+}\)) uniformly distributed in the red pulp, white pulp, and marginal zone areas. In agreement with the flow cytometric analyses, KKD68 chimeras contained a substantially increased frequency of \( \lambda \)-expressing cells in B cell folicles. As expected, many of these cells appear yellow in the immunofluorescent image shown in Fig. 4B (middle right panel), as a result of the staining of GFP\(^{+}\) (green) cells with anti-\( \lambda \)-PE (red).

KKD68-transduced B cells that develop in chimeric mice via the L chain-editing pathway contribute to peripheral B cell compartments in a manner similar to \( \lambda \)-expressing B cells in normal mice

We next determined whether developing B cells that expressed the \( \kappa \) shRNA contributed normally to the peripheral B cell compartment and its subsets. The percentage of KKD68-transduced splenic B cells that expressed high levels of the complement receptor C1qRp, detected by the AA4.1 mAb, was increased relative to controls (Fig. 5, left panels). Although high C1qRp expression is associated with the transitional stages of B cell development (34, 35), the frequency of AA4.1\(^{+}\) cells among normal B6 mice was found to be elevated relative to \( \kappa \)-expressing cells (Fig. 5, middle panels), as previously reported (34). Moreover, the frequency of \( \lambda \)-expressing B cells in the T1 and T2 stages of development was reduced and increased, respectively, in normal B6, control, and KKD68 chimeras compared with \( \lambda^{-} \) B cells in the same mice (Fig. 5, middle and right panels). This may be a manifestation of developmental selection of B cells with particular specificities predominantly conferred by \( \lambda \) L chains.

This notion was reinforced by the results of analysis of mature B cell subsets in the spleens and PTC of these mice. Analysis of levels of CD21 and CD23 expression indicated similar ratios of follicular and marginal zone B cells among \( \lambda \)-expressing splenic B cells in B6 mice and \( \lambda \)-expressing GFP\(^{\text{high}}\) B cells in vector control and KKD68 chimeric mice. However, in all three of these
subpopulations, a greater percentage of B cells are sIgM high, sIgDlow compared with \( \lambda^2 \) B cells (Supplemental Fig. 2). An increased frequency of sIgM high, \( \lambda^+ \) B cells was also observed in the PTC. In addition, in the PTC, a greater percentage of \( \lambda^- \)-expressing B cells in B6 mice, as well as GFP high, \( \lambda^+ \) B cells in both types of chimeric mice, were CD23 low, sIgM high, CD5+ (i.e., members of the B1a subset) (Supplemental Fig. 3). This is consistent with previous observations that \( \lambda^- \)-expressing B cells are enriched in the B1a compartment (36). This biased development of \( \lambda^+ \) B cells to a B1a phenotype took place in the control and KKD68 chimeras, despite the fact that normal adult BM does not efficiently reconstitute this B cell subset (37).

To determine whether \( \lambda \) V gene diversity in control and KKD68-transduced peripheral B cell populations was similar, rearranged V\( \lambda \)1 and V\( \lambda \)2 genes were RT-PCR amplified from FACS-purified GFP+ splenic B cells from KKD68 and control chimeric mice, and the products were cloned and sequenced. Among 23 V\( \lambda \) gene control sequences, 6 were unique, with a predominant V\( \lambda \)1-J\( \lambda \)1 representation (18 clones with three different junction sequences). Similarly, among 27 such V\( \lambda \) genes sequenced from KKD68 chimeras, 6 were unique, with a predominance of V\( \lambda \)1-J\( \lambda \)1 genes (21 clones with three different junction sequences, Table I).

**Frequency and level of surface \( \lambda \) L chain expression among peripheral B cells correlates with level of KKD68 vector expression**

The above observations suggested that the fate of KKD68-transduced immature B cells originally expressing \( \kappa \) L chains and that expressed high levels of the \( \kappa \) shRNA was either elimination or rearrangement and expression of \( \lambda \) L chain genes resulting in expression of normal levels of \( \lambda \)-containing sIgM, allowing developmental progression. If recovery of normal levels of sIgM expression was the driving force behind this pathway, developing B cell subpopulations expressing lower levels of the \( \kappa \) shRNA might follow alternative pathways. To test this idea, mature B cells in the PBL and spleens of chimeric mice that expressed low, intermediate, and high levels of GFP, as an indicator of general retroviral gene-expression level, were analyzed for frequency of surface \( \lambda^+ \) cells. Among GFP+ and the three subpopulations of GFP+ splenic B cells in control chimeras, all contained similarly low frequencies of surface \( \lambda^+ \) cells. In con-

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**FIGURE 3.** Knockdown of \( \kappa \) L chain in BM B cells in BM chimeric mice is associated with increased levels of \( \lambda \) L chain and Rag-2 gene expression and \( \lambda \) locus recombination. A. Surface \( \kappa \) and \( \lambda \) L chain levels were evaluated in immature B220low subsets in BM chimeras created with KKD68 or control vector retrovirus-transduced BM. Percentages of B cells in each gate in the lower panels are shown. B. BM B cells from B6 and BM chimeric mice created with KKD68 or control vector retrovirus-transduced BM were stained with anti-B220 and anti-\( \kappa \) L chain Abs, then B220low, surface \( \kappa^- \) cells that were either GFP+ or GFP− were purified by FACS (upper panel). A representative flow cytometric plot is shown. Plots obtained from all BM samples were similar in terms of subset distribution. The relative expression levels of Rag-2 and \( \kappa \) L chain RNA were quantified by real-time RT-PCR (upper and middle panels, respectively) in these fractions, and the frequency of double-stranded breaks in the \( \lambda \) locus was quantified by LM-PCR (lower panel) in the GFP+ fractions. Figures are representative of data obtained from at least three independent experiments.
Contrast, these three GFP+ subpopulations in KKD68 chimeras all contained increased frequencies of surface λ+ B cells compared with GFP− B cells, with these frequencies correlating with level of GFP expression (Fig. 6A). Analogous results were obtained from PBL (data not shown).

As mentioned above, among the λ-expressing B cells in KKD68 chimeras, two subpopulations were observed: one exclusively expressing λ L chains and one that appeared to express both κ and λ L chains. The former cells constituted the predominant subpopulation of all λ-expressing cells among GFP high KKD68-transduced B cells (Fig. 6A) and expressed levels of sIgM and cell surface IgD (sIgD) similar to λ+ B cells in B6 mice (Supplemental Fig. 2). The latter cells made up a greater percentage of all λ-expressing B cells in the GFP low and intermediate compartments compared with the GFP high compartment. These data indicated that in cells expressing high levels of the κ shRNA, the major developmental pathway that is followed is receptor editing, resulting in expression of levels of λ L chain compatible with normal levels of expression of surface BCR (sBCR). However, when levels of κ shRNA are lower, other pathways may result in acquisition of normal levels of sBCR and developmental progression, such as coexpression of κ and λ L chains. Expression of both κ alleles by a developing B cell may also represent one of these pathways. However, as mentioned above, the fact that we did not detect an increased frequency of κ locus rearrangements among KKD68-transduced B220low, κ− BM B cells (Supplemental Fig. 1) suggests that if a cell embarks on this pathway it may be rapidly lost because of κ knockdown.

**FIGURE 4.** Knockdown of κ L chain results in increased λ L chain expression among peripheral B cells. A, Cells from peripheral lymphoid tissues (spleen [SP], lymph node [LN], and peritoneal cavity [PTC]: upper, middle, and lower panels, respectively) from BM chimeras generated with KKD68 or control vector retrovirus-transduced BM were analyzed by flow cytometry. Percentages of cells in each fraction are shown in the gates. B, Spleen cryosections from BM chimeric mice were analyzed by immunohistology and fluorescence microscopy. Upper panels, Sections were stained with anti-TCRB (red) and anti-B220 (blue). Middle panels, Sections were stained with anti-λ (red) and anti-κ (blue) L chain. Lower panels, Sections were stained with anti-IgM (red) and anti-IgD (blue). Original magnification ×200. The data shown are representative of those obtained from at least three mice from each type of chimera.

**FIGURE 5.** Similar phenotypes of λ-expressing splenic B cells in C57BL/6 and KKD68 BM chimeric mice. Splenic B cells from B6 and BM chimeras generated with KKD68 or control vector retrovirus-transduced BM were stained with Abs specific for the indicated markers and evaluated by flow cytometry. IgM and CD23 expression levels were analyzed on AA4.1+ B cells, and gates were set to show percentages of T1 (IgM−/CD23−), T2 (IgM+/CD23−), and T3 (IgM+/CD23+) transitional subsets. Upper and lower panels show data from λ+ and λ− gated cells, respectively. Figures are representative of data obtained from at least two independent experiments.
chains (Fig. 6), a frequency in good agreement with the analysis of splenic B cells by flow cytometry (Fig. 6). A screened for GFP expression. GFP + hybridomas were produced BM. The percentages of cells in each subset are shown next to the gates. Figures are representative of data obtained from at least three independent experiments.

KKD68-transduced splenic B cells indeed expressed both κ and λ L chains (i.e., was isotypically included), splenic B cells from a KKD68 chimeric mouse were polyclonally activated in vitro, and hybridomas were generated. GFP-expressing hybridomas were then analyzed for expression of κ and λ L chains. Of eight GFP+ hybridomas analyzed, two were found to express κ and λ L chains (Fig. 6B), a frequency in good agreement with the analysis of splenic B cells by flow cytometry (Fig. 6A).

GFPhigh KKD68-transduced B cells also contain a subpopulation that did not expressably detectably express λ L chains and expressed surface levels of κ L chain that appeared ~3-fold reduced (mean fluorescence intensity) compared with controls. Nonetheless, this subpopulation was found to express levels of slgM and slgD similar to λ-expressing B cells (Supplemental Fig. 2). The nature of this subpopulation is currently unclear. We observed a greater percentage of B cells that stained with a mAb specific for the usually infrequently expressed Vλ L chain in the KKD68 GFPhigh B cell compartment compared with this compartment in control mice (Supplemental Fig. 4). However, this increase could not account for all of the κlow, λ−, GFPhigh B cells in KKD68 chimeras.

To more rigorously determine whether a subpopulation of KKD68-transduced splenic B cells indeed expressed both κ and λ L chains (i.e., was isotypically included), splenic B cells from a KKD68 chimeric mouse were polyclonally activated in vitro, and hybridomas were generated. GFP-expressing hybridomas were then analyzed for expression of κ and λ L chains. Of eight GFP+ hybridomas analyzed, two were found to express κ and λ L chains (Fig. 6B), a frequency in good agreement with the analysis of splenic B cells by flow cytometry (Fig. 6A).

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λ L chain, KKD68-expressing B cells can mount normal T cell-dependent immune responses

To further examine the differentiative capabilities of λ-expressing, KKD68-transduced B cells, we exploited the fact that the primary T cell-dependent (TD) immune B cell response to 4-hydroxy-3-nitrophenylacetyl in B6 mice contains a predominant λ-expressing component (38). BM chimeric mice were immunized with 4-hydroxy-3-nitrophenylacetyl–chicken γ globulin; 9 d later, the frequency of λ-expressing B cells in the GFP+ and GFPhigh responding germinal center (GC) and Ab-forming cell (AFC)

Table I. Combinational and junctional diversity of Vλ genes

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The number of Vλ genes with each type of gene segment combination and sequence at the Vλ–Jκ junction are indicated. Junctional sequences are shown with contributions from Vλ and Jκ segments separated. Nucleotides in these junction regions that are not encoded in germline Vλ or Jκ segments are shown in bold type.

FIGURE 6. Frequency and level of λ L chain and both κ and λ L chain expression on splenic B cells in KKD68 BM chimeras is correlated with level of KKD68 retroviral gene expression. A. Splenic B cells from naive C57BL/6 mice served as a control. Vector-1 and vector-2 are two BM chimeras created with control vector retrovirus-transduced BM and KKD68-1 and KKD68-2 are two BM chimeras generated with KKD68 retrovirus-transduced BM. The percentages of cells in each subset are shown next to the gates. Figures are representative of data obtained from at least three independent experiments. B. Hybridomas were generated from the mitogen-activated splenic B cells of a KKD68 chimeric mouse and a vector control chimeric mouse and screened for GFP expression. GFP+ hybridomas were then assayed for expression of κ and λ L chains by flow cytometry. All hybridomas from the vector control mouse expressed only κ L chain (example in left panel). Of eight GFP+ hybridomas from the KKD68 chimeric mouse, six expressed reduced levels of κ L chain only (example in middle left panel), and two expressed both κ and λ L chains (two right panels).
compartments was evaluated. As expected, among both non-transduced and transduced B cells in control chimeras, as well as nontransduced cells in KKD68 chimeras, λα cells represented a major fraction of both GC B cells and AFCs. However, such cells made up >90% of both the GC B cell and AFC subpopulations in the GFPα high compartment of KKD68 chimeras (Fig. 7). Histological analysis of the spleens of immunized KKD68 chimeric mice corroborated these results, showing that GFPα, λα B cells were prevalent within GCs in follicles and AFC foci located in bridging channels (data not shown). These data demonstrated that B cells that have developed via the KKD68 shRNA-induced λ L chain-editing pathway are fully capable of participating in a primary TD immune response.

Discussion

Our data demonstrate that direct inhibition of BCR expression promotes L chain receptor editing in developing polyclonal B cell populations in vivo and that B cells that emerge from this pathway contribute normally to peripheral subsets and are functional. Studies on 3-83Igi anti-MHC I Ig knockin mice first suggested that the level of BCR expression influenced receptor editing (15, 16). In mice hemizygous for the BCR transgenes and lacking expression of the cognate MHC I allele, endogenous L chains, including λα, were predominantly expressed among peripheral B cells. In contrast, such cells represented a minor fraction of peripheral B cells in mice homozygous for the BCR transgenes. Recent studies in this system showed that an activated form of N-Ras can rescue the development of 3-83Igi B cells expressing low levels of BCR (39). Caveats in these studies included Ig transgene-driven perturbation of development and lack of clonal competition that might alter the BCR-signaling requirements for regulation of editing and that the transgenic BCR is cross-reactive with a non-MHC ligand. In addition, these studies used a BCR derived from the memory, not the naive, B cell compartment.

We previously described anti-nuclear Ag Ig knockin transgenic mice, termed HKIR, in which developing transgenic BCR-expressing B cells do not undergo receptor editing and develop to mature follicular B cells, but express low levels of sBCR (40, 41). We proposed that these cells avoid the editing pathway by downregulating levels of sIgM early in development and, thus, avidity for auto-Ag. In support of this idea, in mice in which the transgenic HKIR H chain locus was homozygous, extensive L chain editing took place, resulting in emergence of a major population of B cells expressing BCRs with reduced autoreactivity encoded by the transgenic H chain and endogenous λ L chains (29). These data were interpreted to support the idea that the avidity of developing HKIR B cells for endogenous auto-Ag(s) directly influenced levels of auto-Ag-induced BCR signaling, with low levels of such signaling being compatible with developmental progression and high levels inducing L chain editing.

Apparently disparate data like these have continued to fuel the debate on the pathways that regulate receptor editing. Behrens and colleagues attempted to reconcile such data by proposing that the role of auto-Ag and BCR auto-Ag avidity in regulating editing is via the stimulation of endocytic removal of BCR from the cell surface, thus reducing levels of basal BCR signaling (21). Data supporting this hypothesis were obtained using a system allowing inducible deletion of an Igh-targeted transgenic H chain, resulting in ablation of BCR expression on immature B cells in BM cultures. This led to increased Rag expression and an overall gene-expression profile consistent with “back” differentiation to a more immature stage (22). Subsequent studies using conventional Ig-transgenic B cells developing in vitro BM cultures also supported this hypothesis (21). However, it is unlikely that these experimental systems recapitulate the immature B cell developmental pathway in normal mice, because BM culture conditions are optimized to promote growth of B cell precursors, likely promoting Rag expression and reducing interclonal competition. In addition, as discussed above, the constitutive expression of transgenic Ig chains starting very early in B cell development accelerates or even precludes subsequent steps in this pathway.

Our experimental approach did not use BCR-transgenic B cells and so is not subject to the caveats mentioned above. However, this approach requires that developing, immature B cells express an endogenous κ L chain gene to become susceptible to KKD68 shRNA-mediated reduction of BCR levels. Although κ recombination and expression precedes or precludes λ recombination and expression in most developing mouse B cells (42), the former is not a prerequisite for the latter (24, 25, 43). In combination with the fact that many immature B cells expressing high levels of the KKD68 shRNA appear to be lost, this raises the possibility that preferential outgrowth of B cells that first rearranged and expressed the λ locus might explain our results.
We view this interpretation as unlikely for the following reasons. Among KKD68-transduced B cells expressing different amounts of GFP, but similar levels of surface IgM and IgD, there was a clear correlation among the frequencies of λ-expressing cells, levels of surface λ and κ-chain, and GFP expression. This suggests that there are at least two “developmental solutions” of how to acquire levels of BCR expression compatible with developmental progression in the face of different extents of κ RNA reduction. In one, substantial elimination of κ RNA precludes developmental progression, unless L chain editing takes place and the resulting B cell expresses only λ L chain. If early rearrangement and expression of λ L chains, followed by preferential expansion, accounted for the presence of this compartment, then exclusive expression of λ should not correlate directly with high levels of KKD68 vector expression. In addition, such preferential expansion might be expected to result in an oligoclonal λ-expressing B cell compartment, yet we found that V λ gene diversity in KKD68-transduced and control B cell compartments was similar.

Second, lower levels of KKD68 vector expression results in L chain isotype inclusion that, by definition, requires that both κ and λ loci undergo productive rearrangement and are expressed. Indeed, we isolated hybridomas derived from KKD68-transduced splenic B cells that coexpressed κ and λ L chains. The presence of a third population of KKD68-transduced B cells with undetectable and reduced levels of surface λ and κ L chains, respectively, indicated that a third pathway of developmental compensation for reduced levels of κ expression may also be taking place, as suggested by our previous studies of the HKIR knockin mice (40, 41). However, it is also possible that these cells express BCRs with particular H:L pairs that reduce or preclude detection with the anti-L chain reagents used in our studies. Indeed, using a mAb that detects normally rarely used VxL chains (44), we found an increased frequency of Vx λ cells among KKD68, but not control vector-transduced, B cells.

Because we do not know the specificities of BCRs initially expressed by the members of the polyclonal population of immature B cells that subsequently gave rise to the λ-expressing mature B cells observed in our studies, we cannot formally rule out that auto-Ag–induced BCR signaling is necessary, but not sufficient, for induction of L chain editing. However, given the diversity of BCR V regions in the normal repertoire, this scenario would require that an equally diverse collection of auto-Ag specificities and avidities all fulfill this requirement. Thus, our data support the conclusion that the level of BCR expression is the dominant parameter influencing receptor editing at the immature B cell stage. Future studies will be required to determine whether this reduced expression, as expected, results in reduced levels of basal BCR signaling, as well as whether B cells that regain normal levels of BCR expression via receptor editing, resulting in expression of λ light chains, regain normal levels of basal signaling.

Acknowledgments

We thank Scot Fenn for technical support, the Kimmel Cancer Center Flow Cytometry Facility, and all members of the Manser laboratory for indirect contributions.

Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure 1

A

M  KKD68 GFP+  KKD68 GFP-  Vector GFP+  Vector GFP-

1000bp
800bp
650bp
500bp
400bp

Jk3
Jk2
Jk1

800bp
650bp

B

M  Vector GFP+  KKD68 GFP+

400bp
300bp
200bp
100bp

RS-κ
RS-λ

800bp
650bp

β2M

β2M
Supplemental Figure 3

CD23

B6

B220⁺κ⁺

43.7

35.1

B220⁺λ⁺

49.3

47.2

Vector

B220⁺GFP⁺κ⁺

51.2

37.9

B220⁺GFP⁺λ⁺

45.9

49.2

KKD68

B220⁺GFP⁺κ⁺

13.4

B220⁺GFP⁺λ⁺

31.4

CD5

IgM

IgMhigh

CD23low

B2

B1

36.8

62.3

8.5

34.0

17.6

44.9
Supplemental Figure 4

Vector  B220⁺IgM⁺  KKD68

GFP⁻  5.94  0.2  92.6  1.29

GFP⁺  5.59  0.293  92.7  1.38

GFP⁻  5.39  0.118  93.2  1.28

GFP⁺  5.39  0.118  93.2  1.28
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Low frequency of \( \kappa \), and high frequency of \( \lambda \) locus recombination breaks in the KKD68-transduced pro/pre BM B cell compartment

A and B: BM B cells that were \( {B220}^{\text{low}} \), surface \( \kappa^{\text{negative}} \) and either GFP+ or GFP- were FACS purified from KKD68 and vector control chimeric mice and DNA prepared from these cells and subjected to LM-PCR analysis as described (27). PCR of the \( \beta 2M \) gene in these DNAs was used as a standard. A: A genomic primer hybridizing to a region 5’ of J\( \kappa 1 \) was used to detect dsDNA breaks at the J\( \kappa 1, 2 \) and 3 gene segments. B, upper panel: a genomic primer hybridizing to a region 5’ of the 3’ RS element in the \( \kappa \) locus was used to detect breaks at this location. B, middle panel: a \( \lambda \) RS specific primer was used to detect breaks in the \( \lambda \) locus.

Supplemental Figure 2. KKD68-transduced splenic B cells display a phenotype similar to \( \lambda^+ \) splenic B cells in C57BL/6 mice

Splenic B cells from B6 and BM chimeras generated with KKD68 or control vector retrovirus-transduced BM were stained with Abs specific for the indicated markers and evaluated by flow cytometry. Left panels: The frequency of follicular B cells (FO, \( \text{CD21}^{\text{low}} \text{CD23}^{\text{high}} \)) and marginal zone B cells (MZ, \( \text{CD21}^{\text{high}} \text{CD23}^{\text{low}} \)) were evaluated in different gated subsets (\( \text{B220}^+ \text{GFP}^+ \), \( \text{B220}^+ \), \( \text{B220}^+ \lambda^- \) and \( \text{B220}^+ \lambda^+ \)). Right panels: Surface IgM and IgD levels were evaluated on these same subsets. Percentages of cells in each fraction are shown next to the gates. Figures are representative of data obtained from at least two independent experiments.
Supplemental Figure 3. Similar phenotypes of \( \lambda \)-expressing peritoneal cavity B cells in C57BL/6 and KKD68 BM chimeric mice

Peritoneal cavity cells from B6 and BM chimeras created with KKD68 or control vector retrovirus-transduced BM were stained with Abs specific for the indicated markers and evaluated by flow cytometry. Upper panels: Gates are set to show the percentages of B1 (CD23\(^{\text{high}}\)IgM\(^{\text{high}}\)) and B2 (CD23\(^{\text{high}}\)IgM\(^{\text{low}}\)) B cells. Lower panels: Gates are set to show the percentages of B1a (CD5\(^{+}\)IgM\(^{\text{high}}\)) and B1b (CD5\(^{+}\)IgM\(^{\text{high}}\)) B cells. The percentages of cells in each fraction are shown next to the gates. Figures are representative of data obtained from at least two independent experiments.

Supplemental Figure 4. Increased frequency of B cells expressing V\(\lambda\)x in the KKD68 transduced subpopulation in chimeric mice.

Spleen cells from the indicated types of chimeric mice were stained with antibodies specific for the indicated markers and analyzed by flow cytometry. Cells gated as being B220\(^{+}\), IgM\(^{+}\) and either GFP\(^{+}\) or GFP\(^{-}\) were further analyzed for expression of \( \lambda \) and V\(\lambda\)x.