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Regulation of the B Cell Receptor Repertoire and Self-Reactivity by BAFF

Miyo Ota,* Bao H. Duong,*+ Ali Torkamani,† Colleen M. Doyle,* Amanda L. Gavin,* Takayuki Ota,*+1 and David Nemazee*+1

The TNF-family cytokine BAFF (BlyS) promotes B lymphocyte survival and is overexpressed in individuals with systemic lupus erythematosus and Sjögren’s Syndrome. BAFF can rescue anergic autoreactive B cells from death, but only when competition from nonautoreactive B cells is lacking. Yet, high BAFF levels promote autoantibody formation in individuals possessing diverse B cells. To better understand how excess BAFF promotes autoimmunity in a polyclonal immune system, Ig L chain usage was analyzed in 3H9 site-directed IgH chain transgenic mice, whose B cells recognize DNA and chromatin when they express certain endogenous L chains. BAFF levels were manipulated in 3H9 mice by introducing transgenes expressing either BAFF or its natural inhibitor ΔBAFF. B cells in BAFF/3H9 mice were elevated in number, used a broad L chain repertoire, including L chains generating high-affinity autoreactivity, and produced abundant autoantibodies. Comparison of spleen and lymph node B cells suggested that highly autoreactive B cells were expanded. By contrast, ΔBAFF/3H9 mice had reduced B cell numbers with a repertoire similar to that of 3H9 mice, but lacking usage of a subset of Vc genes. The results show that limiting BAFF signaling only slightly selects against higher affinity autoreactive B cells, whereas its overexpression leads to broad tolerance escape and positive selection of autoreactive cells. The results have positive implications for the clinical use of BAFF-depleting therapy. The Journal of Immunology, 2010, 185: 4128–4136.

The TNF-superfamily cytokine BAFF plays key roles in B cell survival and homeostasis that when dysregulated can lead to symptoms ranging from systemic autoimmunity to immunodeficiency (1). BAFF responsiveness in B cell development begins at the transitional 2 (T2) stage (2, 3). Partial loss of BAFF or BAFF-R function leads to a reduced half-life and steady-state numbers of follicular and marginal zone (MZ) B cells, indicating that BAFF is a homeostatic cytokine for these subsets, but not for B-1 cells (4). Overexpression of BAFF is linked to B cell hyperplasia, hypergammaglobulinemia, and systemic autoimmunity in both mouse models and in patients with systemic lupus erythematosus (SLE) and Sjögrens Syndrome (1, 5–10). Suppression of BAFF levels has been shown to ameliorate or delay lupus symptoms (11–16). Thus, BAFF has both beneficial and potentially toxic effects that must be tightly regulated.

One natural negative regulator of BAFF is the splice isoform ΔBAFF (DBF), which suppresses BAFF bioactivity by consuming potentially functional BAFF mRNA, “poisoning” bioactivity in BAFF/DBF heterotrimerics, and preventing membrane release (17). Transgenic (Tg) mice expressing DBF under the control of the human CD68 promoter have a phenotype indicative of reduced BAFF/BAFF-R bioactivity, including subnormal B cell numbers, particularly in the MZ and lymph nodes (LNs) (18). Companion strains of mice expressing full length BAFF under the same control elements have B cell hyperplasia, elevated serum Ig levels and autoantibodies (18 and this study).

The link between BAFF levels and autoantibody formation has prompted analyses of the effects of BAFF on B cell tolerance. Anergic autoreactive B cells have a heightened BAFF dependency compared with nonautoreactive B cells owing to BCR desensitization (19, 20). This phenomenon leads to rapid, competition-dependent turnover of autoreactive B cells in the context of a polyclonal repertoire of B cells, but allows improved survival of autoreactive cells in monoclonal Tg models and in the context of lymphopenia (19–21). Because in a polyclonal repertoire nonautoreactive B cells are presumably in excess, it is somewhat puzzling that BAFF-overexpressing mice have elevated autoantibody levels and lupus-like disease. One explanation may be that low-affinity autoreactive B cells are selectively spared (20).

Evidence is accumulating that therapeutic targeting of BAFF may be an effective treatment for SLE. A phase II clinical trial of belimumab, a human mAb to BAFF, in a cohort of 449 SLE patients resulted in a response in 46% of patients at 1 y compared with 29% of placebo patients (14, 15). Ongoing phase III trials show promise. One of the speculations regarding the efficacy of this approach is that reduced BAFF levels may gradually render the naive B cell repertoire less autoreactive over time. However, there is little direct evidence that BAFF levels influence the selection of the naive autoreactive B cell repertoire.

In this study, we analyze how BAFF levels alter B cell tolerance by characterizing changes in the B cell repertoire that occur with over or under expression of BAFF. Our strategy involved interbreeding BAFF Tg or DBF Tg mice with mice Tg for the site-
directed 3H9 Ig H chain, whose specificity is derived from a DNA and chromatin-specific hybridoma obtained from a diseased MRL/lpr mouse (22, 23). This strain exhibits excellent H chain allelic exclusion (23, 24), and many of the L chain partners that generate autoantibodies or are nonautoimmune when paired with 3H9 chain are known (23, 25–29), facilitating analysis of alterations in immune repertoire. On a nonautoimmune-prone background, certain L chains generating autoimmune BCRs lead to B cell developmental arrest and receptor editing, whereas others allow B cell maturation but lead to functional anergy by BCR desensitization (23, 30). We reasoned that if BAFF sets the affinity threshold for peripheral B cell tolerance the repertoire of allowed L chains might be expanded in LNs and spleens of BAFF/F3H9 Tg mice compared with 3H9 mice, and might be reduced in the 3H9/DBF Tg background, with cells of borderline autoreactivity showing the greatest relative changes in survival and function.

Materials and Methods

Animals

All mice were bred and maintained in The Scripps Research Institute Animal Resources facility according to the Institutional Animal Care and Use guidelines. C57BL/6J mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and bred in The Scripps Research Institute custom breeding colony. Site-directed 3H9 H chain Tg mice (22, 23) on a C57BL/6 (B6) background were kindly provided by Dr. Martin Weigert (University of Chicago, Chicago, IL). BAFF and DBF Tg mice on a B6 background have been previously described (18). BAFF/F3H9 and DBF/3H9 double Tg mice were generated by crossing homozygous BAFF or DBF Tg with 3H9 mice.

ELISA

Assay for dsDNA autoantibodies was carried out as follows: 2 µg/ml dsDNA from salmon sperm was coated to Nunc Maxisorp 96-well plates in Reacti-Bind DNA coating solution (Pierce, Rockford, IL). After overnight coating, wells were blocked for 1 h in TBS containing 5% BSA. Mouse sera or hybridoma supernatant diluted in blocking solution were applied and incubated for 90 min at room temperature. After extensive washing, bound Abs were detected with 1:3000 diluted HRP-conjugated goat anti-mouse IgG or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with 1-Step-Ultra TMB colorimetric substrate (Pierce). OD450nm was measured with a Versamax plate reader (Molecular Devices, Sunnyvale, CA). For IgA and IgM chain detection we used biotin conjugated rat anti-mouse IgA (C10-1, BD Biosciences, San Jose, CA) or biotin conjugated rat anti-mouse IgM (R73-41, Biolegend, San Diego, CA) for secondary Ab and bound Abs were detected with 1:5000 diluted streptavidin-HRP (BD Biosciences).

Flow cytometry analysis

Cell-surface marker stains for FACS analyses were performed using a standard protocol. All of the following Abs were used at 1:200 dilution in FACS buffer (HBSS containing 0.5% BSA, 1 mM EDTA, and 0.05% NaN3), with 3 × 106 cells/stain: FITC or PE anti-CD4 (500-244, BD Biosciences), PE anti-CD8 (53-6.7, BD Biosciences), PE anti-CD1d (1B1, eBioscience, San Diego, CA), PerCP-Cy5.5 anti-CD8 (53-6.7, BD Biosciences), FITC anti-CD21 (7G6), PE anti-CD1d (1B1, eBioscience, San Diego, CA), PE-Cy5 anti-CD44 (H12F4, BD Biosciences), and allophycocyanin anti-IgG2a (clone 2G11, eBioscience, San Diego, CA). For intracellular staining, cells were stained during surface staining with unlabeled anti-CD4 (R3-3.2, BD Biosciences) and PE-Cy5.5 anti-CD4 (GK1.5, BD Biosciences), PerCP-Cy5.5 anti-CD8 (53-6.7, BD Biosciences). For intracellular IgG and staining, cells were incubated for 1 h at 4°C with PE-Cy5.5 anti-CD44, followed by fixation and permeabilization using a kit (Cytofix/CytoPerm, BD Biosciences) cells were stained with FITC anti-CD4 and allophycocyanin anti-CD8. All flow cytometric data were acquired on a LSR II (BD Biosciences) and were analyzed using the FlowJo program (TreeStar, Ashland, OR).

k-chain V region 5′-RACE (conventional method)

B cells were isolated from spleen or LN cells of 12- to 16-wk-old mice using a “no touch” B cell isolation kit (Miltenyi Biotec, Auburn, CA) supplemented with bio–anti-IgM (AF6-78, BD Biosciences) for samples carrying the 3H9 gene or bio–anti-IgM (DS-1, BD Biosciences) for control B6 cells. Ab-bound cells were identified with PE streptavidin. Selected cells were reverse transcribed and amplified using 5′-RACE kit (FirstChoice RLM-RACE, Ambion, Austin, TX) according to the manufacturer’s protocol. Briefly, mRNA was treated to remove 5′ phosphate present on uncapped transcripts, then treated with tobacco acid pyrophosphatase to remove the 7-methyl-Gpp cap. Subsequent ligation of an RNA “adapter” oligonucleotide to the 5′ end using RNA ligase was then carried out, followed by reverse transcription using oligo dT primer with a Transcriptor High Fidelity cDNA synthesis kit (Roche, Indianapolis, IN), and PCR amplification with oligonucleotides corresponding to adapter and Cx sequences. PCR was performed with inner primer (5′-AAAACGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′) and m Laur (5′-CTGCTCACTGGATGGTGGGAAGATGG-3′) using Phusion High Start polymerase (NEB, Beverly, MA) for 35 cycles with annealing 56°, 30 s; extension 72°, 30 s; denaturation 98°, 15 s. PCR products were purified after agarose gel electrophoresis and cloned using Zero Blunt TOPO PCR Cloning Kits (Invitrogen, San Diego, CA). Ligated product was transformed into chemically competent Escherichia coli (Top1, Invitrogen). Single colonies were directly amplified by PCR with T7 and M13 reverse primer and sequenced. Obtained sequences were analyzed at the ImMunoGeneTics (IMGT) Web site (www.imgt.org) (31).

454 sequencing

B cells were isolated from four 3-mo-old mice, which included two females and two males. Splen and LN cells were harvested, then depleted of erythrocytes using ACK buffer prior to B cell isolation. For splenic B cell isolation, 50 million spleen cells from each gender were combined and IgM B cells isolated using the “no touch” B cells isolation kit (Miltenyi Biotec) supplemented with bio–anti-IgM. Total RNA was obtained from purified B cells using TRIZOL reagents (Invitrogen). k-chain variable sequences from each sample were obtained in a 5′ RACE kit (Ambion) following the manufacturer’s protocol. A total of 1.0 µg of total RNA per sample was used. For RT-PCR, Transcriptor High Fidelity (Roche) and Phusion Hot Start (NEB) was used, respectively. A bar code strategy was used to distinguish female and male samples in later analysis. The following oligonucleotides were used to amplify k-chain sequences: K-R1 (male) 5′-TTGACTGCTTACTGATGGTGGAAGATGG-3′, K-R2 (female) 5′-TTTTACTGCTTACTGATGGTGGAAGATGG-3′. Each PCR product was purified after agarose gel electrophoresis and 2.5 µg each of male and female amplicons were combined and used for 454 sequence analysis. GS FLX Titanium sequencing kit XL R70 (Roche) was used for sample preparation. Data were collected at GeneChip Microarray Core (University of California, San Diego, La Jolla, CA) and analyzed at Scripps Translational Science Institute or our laboratory. Data were analyzed using stand-alone BLAST program (32) downloaded from National Center for Biotechnology Information. k-chain sequences were obtained from the IMGT Web site (www.imgt.org) (31).

Analysis of reads obtained from 454 sequencing

k-chain sequences were analyzed by BLAST (32) downloaded from National Center for Biotechnology Information and compared with germline Vx and Jc sequences obtained from IMGT (31) (listed in Supplemental Table D). Because of the relative lack of diversity of L chains, repeated sequences were not excluded. We analyzed clones without unread bases in two different ways and display data using the first approach, which involved using the BLASTALL program with default settings. Average read length was 318 bp. We analyzed reads >200 bp in length with BLASTALL using “no overlap” Vx and Jc databases. Because of sequencing errors using 454 technology, some Vx sequences were corrected based on germline sequence. All sequences were translated and those which had a stop codon were omitted. If a Jx sequence was detected in a clone, Jx frame and CDR amino acid sequence criteria were analyzed, which required the conserved framework 3 cysteine upstream of a tryptophan. If clones satisfied all the requirements, we used the data for further analysis, a small fraction of which failed the criteria. For each analysis, sequences that lacked Jx were also included. The number of sequences analyzed from each experimental sample set is summarized in Table I. All programs used in this analysis are available on request.

Similar results were obtained using the MEGABLAST algorithm as follows. To match Vx regions we required 90% sequence identity and used a 28-bp word size setting. Reads were assigned to genes based upon a hierarchical BLAST results. Differences in the next parameter hierarchy was considered only if a read matched multiple genes with the previous parameter. The hierarchy, ordered to be more permissive to gaps in the BLAST alignments because of known homopolymer sequencing errors
using 454 technology, was as follows: highest bit score, highest percent identity, longest alignment length, least number of mismatches, and finally least number of gaps. For a small percentage (~3%) of reads for which the score matched multiple V genes exactly, the read was distributed according to the percentage of uniquely mapping reads for each gene. Jk genes were assigned similarly, requiring 80% sequence identity, using a 7-bp word size, and requiring the read to span at least 23 bases of the Jk genes.

Hybridoma generation and analysis

Lymphocytes from spleen and pooled LNs from 12- to 16-wk-old mice were cultured for 72 h in RPMI 1640 supplemented with 10% FCS and LPS (50 µg/ml) and fused with the SP2/0 myeloma line using polyethylene glycol. Cells from each fusion were then distributed into 96-well plates and hybrids selected with hypoxanthine/aminopterin/thymidine medium. Any wells with two distinct colonies were excluded. Cell supernatants were then screened for 3H9 H chain idiotype. Reactivity against dsDNA was measured by ELISA. L chain sequences were amplified and sequenced from the cDNA from hybridomas. The L chain sequence was amplified using mixed 5’ primers for Vκ gene amplification and Cκ primer (5’-CTGCTCCTGGATGGTGGGGAATG-3’) to obtain joined VJ sequences. The PCR products were electrophoresed on 1.5% agarose gels, purified with a kit (Qiagen) and cloned in pSMART-HCKan vector (Lucigen, Middleton, WI), and the insert were sequenced (Retrogen, San Diego, CA). Sequence analysis was carried out with the IMGT/V*QUEST program (http://imgt.cines.fr/).

Results

Regulation of B cell compartment size by BAFF expression level

As BAFF levels regulate B cell survival, we quantified B cell numbers in lymphoid tissues of non-Tg B6, 3H9, BAFF/3H9 and DBF/3H9 mice. Compared with B6 mice, splenic B220+ B cell numbers in 3H9 Tg mice were significantly decreased and DBF/3H9 mice had even fewer B cells. By contrast, BAFF/3H9 mice had abnormally high B cell numbers (Fig. 1A). MZ B cells were increased in number and were similar in all 3H9 strains (Fig. 1B). This finding was surprising because MZ B cell numbers are most strikingly affected by altered BAFF levels in BAFF Tg and DBF Tg mice (which lack the 3H9 Tg) (18). B-1 cell numbers were low in 3H9 mice, and the B-1 cells that did develop lacked expression of 3H9 H chain (not shown). Both κ+ and λ+ B cell numbers were increased in BAFF/3H9 mice even compared with B6 mice (Fig. 1C, 1D). Usually, λ L chains combined with the 3H9 H chain bind to DNA (25, 28, 29, 33). In 3H9 mice λ+ B cell numbers were reduced compared with B6, whereas in BAFF/3H9 mice the proportion of λ+ B cells increased even in the LNs. By contrast, DBF/3H9 LNs had an even lower frequency of λ+ B cells than did 3H9 LNs (Fig. 1D, 1E). We conclude that BAFF levels regulate B cell numbers in 3H9 mice, with preferential rescue of follicular and λ+ cells in BAFF/3H9 mice and their preferential loss in DBF/3H9 mice.

Regulation by BAFF of anti-dsDNA Ab levels and transitional B cells

We tested if serum anti-dsDNA titers were affected by elevated BAFF in BAFF/3H9 mice, compared with 3H9, DBF/3H9, and B6 mice. In other studies, BAFF overexpression causes a lupus-like syndrome that includes activated lymphocytes and anti–self-Ab production, whereas 3H9 mice on the BALB/c or B6 backgrounds lack anti-dsDNA Abs (23, 30, 34, 35). Indeed, BAFF/3H9 mice had significantly higher levels of IgM, IgG, IgA, and λ anti-dsDNA than did 3H9 mice (Fig. 2A). By contrast, DBF/3H9 mice had significantly lower IgM, IgG, and IgA anti-dsDNA levels compared with 3H9 controls (Fig. 2A). Low autoantibody production in DBF/3H9 mice correlated with a higher proportion of splenocytes with a CD93hi phenotype (6.9% of B220+ B cells) and a T3’ phenotype (37.8%: B220+CD93hiIgMloIgAD23hi) (Fig. 2B, 2C). These subsets are reported to be anergic self-reactive B cells (36, 37). In contrast, BAFF/3H9 mice had a lower proportion of CD93hi B cells (2.0%) and 44.9% of these cells had a T2 phenotype (B220+CD93hiIgMhiIgAD23lo) (Fig. 2B, 2C), which is considered to be a stage permissive for further maturation. Clearly, excess BAFF allowed maturation of autoreactive cells that were normally regulated by developmental arrest, whereas developmental arrest was apparently enhanced in DBF/3H9 mice.

Igk repertoire analysis of B cells from 3H9, BAFF/3H9, and DBF/3H9 mice

Consistent with earlier studies (24, 38), >90% of B cells in 3H9, BAFF/3H9, and DBF/3H9 strains on the B6 background express H chain from the targeted allele, allowing us to analyze BAFF-dependent changes in B cell repertoire by comparing κ-chain usage. Accordingly, we sorted nonactivated B cells that lacked expression of IgMκ (yielding a population that was >98% IgMκ+) and PCR amplified their Igκ mRNA transcripts using a 5’-RACE approach with two invariant primers, one in Cκ and the second in a universal 5’ linker (see Materials and Methods). We then carried out conventional plasmid cloning and sequencing (Supplemental Table II, Supplemental Fig. 1) or direct pyrosequencing (454 sequencing) analysis of amplicons. The 454 sequencing generated a total of over 117,000 high- quality sequences carrying identi-
B cells had significantly reduced use of Jκ1 (18–22% compared with 33–34% in non-Tg mice) and increased utilization of Jκ5 (33–42% versus 30–31%) (Supplemental Fig. 2). This skewing from wild type was partly lost in BAFF/3H9 mice. The potential significance of these BAFF-regulated alterations in Jκ usage are considered in the Discussion.

**BAFF strongly affects Vκ repertoire selection, especially in LNs**

BAFF/3H9 B cells had a strikingly altered pattern of Vκ usage compared with 3H9 and DBF/3H9 B cells in spleen and LNs (Supplemental Fig. 3), which was particularly conspicuous when presented as absolute B cell numbers rather than percent usage (Fig. 3). Vκ genes showing the statistically significant increases in usage in BAFF/3H9 are highlighted in Fig. 4A. Of these, all but Vκ4-62 are known to sustain dsDNA binding when paired with 3H9 H chain (Fig. 4A). (“Vκ” substitutes for the official prefix “IGKV”). Vκ4-62 is rarely used in wild-type mice, but was found often in BAFF/3H9 samples, suggesting strong positive selection and autoreactivity. In BAFF/3H9 mice there were also proportionate reductions in usage frequency, but not absolute numbers, of Vκs that veto dsDNA binding (Vκ “editors”: 17–127, 14–100, 1–99, 13–85, and 3–4) (28, 29, 39; and our hybridoma data) (Figs. 3, 4B, 4C). We conclude that changes in BAFF levels in vivo can alter the B cell repertoire and the abundance of autoreactive cells.

There was broad similarity between the dominant Vκ usages in spleen and LNs of 3H9 and DBF/3H9 mice (Figs. 3, 4). Four known editor L chains were used frequently in both strains (Fig. 4C). However, in DBF/3H9 mice a subset of Vκs was significantly under-represented (Fig. 4B). As a result, the overall diversity of Vκ usage among cells present at a reasonable frequency was constricted in DBF/3H9 mice, as was the case for the overall B cell population (Fig. 3, Supplcmental Fig. 3). For example, 3H9 LNs had 41 Vκ genes represented at >0.5% frequency, whereas DBF/3H9 had only 31 (Supplemental Fig. 3).

In some cases Vκs that were used at similar frequencies in all strains showed differential Jκ usage. For example, lower BAFF levels in 3H9 and DBF/3H9 mice correlated with increased Jκ4 and Jκ5 association with Vκ19–93 and Vκ12–46, whereas in BAFF/3H9 B cells these Vκs were more frequently joined to Jκ1 and Jκ2 (Fig. 5A).

**Hybridoma analysis of BCR dsDNA reactivity and sequence**

To analyze in more detail the correlations between Ab specificity and L chain sequence usage, we produced hybridomas with LPS-stimulated spleen and LN cells from BAFF/3H9 and DBF/3H9 mice. We randomly picked ~40 single clones derived from each tissue of each strain for further analysis. To simplify the analysis, we focused on hybridomas clearly expressing the 3H9 H chain that had affinity for dsDNA. Among hybrids from 3H9 H chain Tg mice have been characterized in many publications, we did not generate these for analysis.) Next, we determined the fraction of hybridomas carrying 3H9 H chain and VH idiotype Ab to test Abs secreted into the supernatant. In our samples, ~70% of spleen hybridomas and ~80% of LN hybridomas scored strongly positive in this assay, with no significant difference between strains (Table II). (As hybridomas from 3H9 H chain Tg mice have been characterized in many publications, we did not test these for analysis.) In some cases Vκs that were used at similar frequencies in all strains showed differential Jκ usage. For example, lower BAFF levels in 3H9 and DBF/3H9 mice correlated with increased Jκ4 and Jκ5 association with Vκ19–93 and Vκ12–46, whereas in BAFF/3H9 B cells these Vκs were more frequently joined to Jκ1 and Jκ2 (Fig. 5A).

**FIGURE 2.** Effects of BAFF bioactivity on serum anti-dsDNA Abs and CD93+ splenic B cell subsets. Mice of the indicated genotypes were assessed for serum anti-dsDNA activity by ELISA and their splenocytes were analyzed for markers of B cell maturity by flow cytometry. A: IgM, IgG, IgA, and A anti-dsDNA Ab production in sera from 12- to 16-wk-old mice of the indicated genotypes. Diamonds represent values obtained in in vivo constricted in DBF/3H9 mice, as was the case for the overall B cell population (Fig. 3, Supplemental Fig. 3). For example, 3H9 LNs had 41 Vκ genes represented at >0.5% frequency, whereas DBF/3H9 had only 31 (Supplemental Fig. 3).

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significant frequencies of cells with anti-dsDNA reactivity in spleen and LNs that could be captured as hybridomas by LPS activation prior to fusion (Table II). This result is generally in agreement with previous studies of the 3H9 model bred to nonautoimmune-prone backgrounds (28, 30, 40).

We carried out sequencing of L chains expressed by all 3H9 H chain positive hybridomas to analyze their V\(_k\) usage and to correlate that usage with Ab autoreactivity. BAFF/3H9 hybridomas had higher diversity of V\(_k\) family usage among dsDNA-reactive cells, whereas DBF/3H9 hybrids had more diverse V\(_k\) usages in non-dsDNA reactive cells (Fig. 6). Positive hybridomas from BAFF/3H9 mice had more varied \(\kappa\)-chains compared with those from DBF/3H9 spleen. By contrast, among LN-derived hybridomas eight of 12 dsDNA-reactive ones from DBF/3H9 were paired with V\(_k\)8 (V\(_k\)8–24, –27 and –28) (Fig. 6A) and seven of eight were rearranged to J\(_k\)5, a significant skewing compared with BAFF/3H9 samples \((p, 0.05)\). It has been reported that B cells carrying 3H9 H chain paired with V\(_k\)8 are anergic in the peripheral lymphoid organs and that 3H9/V\(_k\)8 double Tg mice lack anti-dsDNA Abs in their sera (28, 30). These similarities may suggest that DNA-reactive B cells in DBF/3H9 mice are anergic.

Previous studies showed that V\(_k\)21D (V\(_k\)3–4), V\(_k\)20 and V\(_k\)12/13 gene family members render the 3H9 BCR nonautoreactive to chromatin and DNA. Sequence analysis of all anti-dsDNA negative clones confirmed that V\(_k\)17–127 (V\(_k\)20 family), V\(_k\)14–100 (V\(_k\)9/10 family), and V\(_k\)3–4 (V\(_k\)21 family) were used often in all

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** V\(_k\) usage depicted as estimated absolute cell number. Data shown estimate total cells in the indicated tissues that express the indicated V\(_k\) genes. A, SP; B, LNs. Values shown are fractional usage taken from Supplemental Fig. 3 multiplied by total B220\(^+\) B cell number from Fig. 1A.

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Table I. Summary of Igk cDNA 454 pyrosequencing results

<table>
<thead>
<tr>
<th>Tissue</th>
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<th>Mouse Genotype</th>
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<tr>
<td></td>
<td>3H9</td>
<td>BAFF/3H9</td>
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<td>Total reads</td>
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<td>Identifiable V(_k)s</td>
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<tr>
<td>% V(_k) coverage(b)</td>
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<td>92.9</td>
</tr>
</tbody>
</table>

*Theoretical total VJ combinations are calculated as follows: 99 functional V genes × 4 functional J genes = 396.

\(b\)A total of 99 functional V genes including open reading frame genes in B6 mice are listed in the IMGT database (31) (http://imgt.cines.fr/textes/IMGTrepertoire/).
FIGURE 4. Vκ usages significantly skewed by altered BAFF levels and analysis of corresponding hybridoma proteins for DNA reactivity. Vκ genes showing selectively increased usage in LN B cells from the following strains are shown: (A) BAFF/3H9, (B) 3H9, and (C) DBF/3H9. Histogram bars indicate genotypes as follows: 3H9, gray; BAFF/3H9, white; DBF/3H9, black. Data are for four mice per group. The 12- to 16-wk-old mice were used. Asterisks show $p$ values calculated by the χ² test. *** $p < 0.001$. D, Avidity assessment of dsDNA binding measured by ELISA. Supernatants from the indicated hybridomas were adjusted to equalize IgM concentrations before assay. Hybridomas express the indicated L chain genes: Vκ1–117 and Vκ8–24 (solid lines with open symbols), which were increased in BAFF/3H9 samples; Vκ2–109 and Vκ8–19 (dotted lines with solid symbols), selectively increased in 3H9; Vκ17–127 (dashed line with crossmarks) was abundant in DBF/3H9. The experiment was carried out with duplicate assays and was representative of three experiments for each hybrid shown.

Discussion

Data presented in this study suggest that elevated BAFF levels not only promote B cell survival in general, but also affect B cell tolerance, rescuing cells with affinity for self Ags and promoting autoantibody secretion. Excess BAFF led to more striking autoantibody formation than several other genetic modifications that exacerbate defects in the higher affinity 3H9-56R autoantibody Tg allele (35). However, these mutations did not insignificantly skew BAFF/3H9 Tg toward self-reactivity, which allows cells to be efficiently counterselected only under conditions of reduced BAFF. Hybridomas expressing members of this group (Vκ2–109 and Vκ8–19) were captured among BAFF/3H9 hybrids and were found to be weakly DNA reactive. The reduction in BAFF bioactivity in DBF/3H9 mice is likely to be less than that observed in selected 3H9 mice but common in B6 cells unconstrained by the Tg H chain specificity. In BAFF/3H9 mice, there was both broad rescue of many autoantibody specificities and apparent skewing favoring highly autoantibody cells. By contrast, reduction of BAFF levels in DBF/3H9 mice lowered total B cell numbers, among which a higher frequency carried L chains that disallowed dsDNA binding. Vκ genes that were frequently used in 3H9 mice tended to be used even more often in DBF/3H9 mice, with the notable exception of a subset found in 3H9 but not in DBF/3H9 LNs (Fig. 4B). This subset of L chains likely confers on 3H9 Tg B cells a low-affinity self-reactivity, which allows cells to be efficiently counterselected only under conditions of reduced BAFF. Hybridomas expressing members of this group (Vκ2–109 and Vκ8–19) were captured among BAFF/3H9 hybrids and were found to be weakly DNA reactive. The reduction in BAFF bioactivity in DBF/3H9 mice is likely to be less than that observed in...
BAFF heterozygous-deficient mice (18), suggesting that a small reduction in BAFF levels can have profound effects on B cell repertoire. This conclusion is based on the more modest or negligible reductions in B cell numbers and normal serum Ig levels in DBF compared with BAFF heterozygous-deficient mice (18, 44, and our unpublished results). Although it is an attractive notion that limiting BAFF levels might preferentially eliminate the highest affinity autoreactive B cells in the population (20), this model fits well only to our data with DBF/3H9 cells, as excess BAFF in BAFF/3H9 mice appears to lead to escape of both high- and low-affinity autoreactive cells. In any case, our data support the idea that therapeutic reduction of BAFF in the context of its overexpression should also selectively eliminate high-affinity autoreactive B cells.

Despite these selections, even in DBF/3H9 mice many autoreactive B cells were found in the spleen and LNs, as revealed most directly in the specificity of hybridoma Abs. About 30% of DBF/3H9 hybridomas retained some apparent self-reactivity as measured in dsDNA ELISA, but these cells apparently did not contribute to serum Abs, reminiscent of 3H9/Vk8 double Tg B cells that have been shown to be anergic and of relatively low affinity for DNA (23, 30, 42, 45).

The broad escape of autoreactive B cells in BAFF/3H9 mice may be related to the affinity toward or nature of the autoantigens seen by the 3H9 receptor, rather than elevated BAFF alone. BAFF Tg mice bred to mice expressing low levels of a high-affinity Igκ-reactive superantigen (BAFF/pUliklow) undergo competition based peripheral deletion, indicating that BAFF is limiting in this model (21). In BAFF/3H9 mice, the nucleic acid-containing autoantigens are likely to be present in vivo only intermittently and are able to stimulate TLRs (46–48), whereas in BAFF/pUliklow mice “self”-Ag was constantly available and associated with cell surfaces that may be especially tolerogenic (49, 50).

The B cell repertoire of BAFF/3H9 mice is consistent with possible BAFF-regulated positive selection or expansion of certain highly autoreactive clones, such as those expressing 3H9 H with λ-chain or with κ-chains encoded by Vκ4 and Vκ1 family genes. Autoantibody formation in BAFF/3H9 mice indicates that autoreactive B cells are present, unregulated, and presumably expanded prior to Ab secretion. In BAFF/3H9 mice usage of many L

### Table II. Anti-dsDNA specificity and 3H9 H chain analysis of hybridomas from BAFF/3H9 and DBF/3H9 mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genotype</th>
<th>Total Hybridomas</th>
<th>3H9 H id⁺ (%)</th>
<th>dsDNA⁺ (%)</th>
<th>dsDNA⁻ (%)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAFF/3H9</td>
<td>47</td>
<td>33 (70.2)</td>
<td>24 (72.7)</td>
<td>9 (27.3)</td>
<td>0.0017</td>
</tr>
<tr>
<td></td>
<td>DBF/3H9</td>
<td>38</td>
<td>26 (68.4)</td>
<td>8 (30.8)</td>
<td>18 (69.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAFF/3H9</td>
<td>37</td>
<td>29 (78.4)</td>
<td>20 (67.0)</td>
<td>9 (31.3)</td>
<td>0.0114</td>
</tr>
<tr>
<td></td>
<td>DBF/3H9</td>
<td>42</td>
<td>34 (80.9)</td>
<td>12 (35.2)</td>
<td>22 (64.7)</td>
<td></td>
</tr>
</tbody>
</table>

*A 3H9 H chain positive as measured by strong binding to anti-idiotype Ab 1.209 (59).

*bPercent of 3H9 id+ hybridomas.

*cFisher exact test.

**FIGURE 6.** Hybridoma analysis. Shown are Vk usages of hybridomas derived from 3H9 H chain positive BAFF/3H9 and DBF/3H9 B cells, sorted by DNA reactivity. Fusions were carried out from SP and LN cells stimulated for 3 d with LPS. A and B, Vk usages observed for hybridoma proteins (A) reactive to dsDNA, (B) without detectable binding to dsDNA. BAFF/3H9 hybridomas (open bars) and DBF/3H9 hybridomas (filled bars) "p < 0.05," refers to p values calculated by χ² tests comparing Vk8 family usage in LNs from BAFF/3H9 versus DBF/3H9.
chains known to promote high-affinity autoreactivity were not only higher than 3H9 controls, but often elevated beyond even the normal levels found in non-Tg mice. Elevation of λ usage in BAFF/3H9 mice was confirmed by flow cytometry analyses. A BAFF/3H9 hybridoma expressing a Vκ1–117/Jκ4 combination that was often present among LN sequences proved to have high anti-DNA affinity (Fig. 4D). Autoreactive B cells require more BAFF for survival than innocuous cells and in their presence turn over rapidly (19–21). However, in autoimmune disease, autoreactive B cells must survive and produce autoantibody despite an initially polyclonal repertoire. Our data suggest that autoreactive B cells with certain specificities or autoantigen properties might be positively selected if excess BAFF is available.

BAFF/3H9 mice had an increased total B cell number with skewing, not only of Vκ usage, but also of Jκ usage, involving an increase in the percentage of cells carrying Jκ1 and a relative reduction in Jκ5 usage. Although Jκ bias has been interpreted in the context of receptor editing, it is unclear if BAFF can act directly on editing-competent B cells because they respond poorly to BAFF and have little BAFF-R expression (51, 52). Bosma and colleagues (37) working with the 3H9-56R model have suggested that a small splenic B cell subset that they call T3′ might include autoreactive cells in the process of editing. T3′ cells had markers reminiscent of putative natural anergic B cells (T3 cells), although they lacked expression of CD23. T3 cells are believed to be at least in part BAFF resistant (19, 20, 36), but the possible BAFF responsiveness of T3′ cells is unknown. It is therefore not excluded that, in BAFF/3H9 mice, BAFF overexpression might suppress editing in T3′ cells, thus contributing to the Jκ skewing. Eilat and colleagues (53) have also argued for peripheral editing in another anti-DNA Tg model, but in that case it was considered a possible contributor to autoimmunity, not a hindrance. Arguing against a role for suppressed editing in response to excess BAFF in the BAFF/3H9 mice is the fact that λ expression, which is also associated with editing, was enhanced rather than reduced in BAFF/3H9 mice. We favor instead the interpretation that post-editing autoreactive cells of many kinds may be selectively expanded or rescued from cell death in BAFF/3H9 mice. Autoreactive cells that fail to edit but are able to mature to BAFF responsive because of the intermittent presence of cognate autoantigen should be rescued from peripheral deletion in the presence of excess BAFF. These cells may be dominated by usage of Jκ1 and Jκ2 because they rearrange first and most efficiently (54). In the context of reduced BAFF, the B cells may need to have previously reduced autoreactivity through editing to survive BAFF-dependent selection at a later stage.

Two studies have taken an approach with similarities to ours, but with somewhat different results. Erikson and colleagues (55) studied conventional 3H9 μ-chain Tg mice that were given exogenous BAFF for 9–21 d, focusing on λ cells. Although BAFF injection increased total and CD93 λ B cell numbers, λ autoantibodies were not elevated, even when the mice were bred to an Igκ−/− background where there was less competition with B cells carrying innocuous specificities. The quantity, quality, or duration of exposure to excess BAFF may explain the differences between the results of that study and ours. Alternatively, the inability of the H chain transgene to undergo class switch or the analysis of mice on the BALB/c genetic background may have affected the results. The B6 background used in the current study has been suggested to be more autoimmune prone than BALB/c owing to differences in B cell tolerance (39, 41, 56). In a second study, Spatz and colleagues (57) studied BAFF Tg mice carrying a conventional IgM transgene encoding an anti-DNA Ab called R4A. In the R4A model (in the absence of excess BAFF) B cell numbers were reduced ~90% from normal and the mice produce little anti-dsDNA Ab, suggesting strong negative selection and minimal competition from nonautoreactive B cells. BAFF transgene expression promoted autoantibody production in a subset of animals and promoted the development of more B cells from early transitional to later developmental stages, but it was unclear if BAFF had a selective B cell survival effect depending upon self-specificity or affinity.

A significant technical feature of the current study was the use of next generation sequencing of 5′-RACE cDNA libraries for providing a broad repertoire analysis. A major disadvantage of conventional sequencing techniques is that it is laborious to analyze >200 clones per sample. The 454 technology allowed us to generate more mouse Igκ sequences than are presently in the IMGT database (31) by a factor of >20-fold, providing an unprecedented view of the mouse Igκ repertoire. The technology is ideal to study the repertoire of more complex immune systems (58). We are currently characterizing Igκ usage in B6 mice.

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Disclosures
The authors have no financial conflicts of interest.

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