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BAFF/APRIL Inhibition Decreases Selection of Naive but Not Antigen-Induced Autoreactive B Cells in Murine Systemic Lupus Erythematosus

Weiqing Huang,*1 Ioana Moisini,*1 Ramalingam Bethuniaickan,* Ranjit Sahu,* Meredith Akerman,† Dan Eilat,‡ Martin Lesser,‡ and Anne Davidson*†

BAFF inhibition is a new B cell-directed therapeutic strategy for autoimmune disease. Our purpose was to analyze the effect of BAFF/APRIL availability on the naive and Ag-activated B cell repertoires in systemic lupus erythematosus, using the autoreactive germline D42 H chain (glD42H) site-directed transgenic NZB/W mouse. In this article, we show that the naive Vλ repertoire in both young and diseased glD42H NZB/W mice is dominated by five L chains that confer no or low-affinity polyreactivity. In contrast, glD42H B cells expressing L chains that confer high-affinity autoreactivity are mostly deleted before the mature B cell stage, but are positively selected and expanded in the germinal centers (GCs) as the mice age. Of these, the most abundant is VκRF (Vκ16-104*01), which is expressed by almost all IgG anti-dsDNA hybridomas derived from the glD42H mouse. Competition with nonautoreactive B cells or BAFF/APRIL inhibition significantly inhibited selection of glD42H B cells at the late transitional stage, with only subtle effects on the glD42H-associated L chain repertoire. However, glD42H/VκRF-encoding B cells were only slightly overrepresented in the GC, and serum IgG anti-dsDNA Abs arose with only a slight delay. Thus, although BAFF/APRIL inhibition increases the stringency of negative selection of the naive autoreactive B cell repertoire in NZB/W mice, it does not correct the major breach in B cell tolerance that occurs at the GC checkpoint. The Journal of Immunology, 2011, 187: 6571–6580.

S
ystemic lupus erythematosus (SLE) is an autoimmune disorder in which pathogenic autoantibodies directed to nuclear material initiate inflammation in target tissues. SLE patients have defects in negative selection of autoreactive B cells at the immature and transitional checkpoints (1) and also fail to restrain pathogenic effector B cells that arise in the germinal center (GC) (2, 3). Understanding how these defects contribute to the production of pathogenic autoantibodies will allow therapy for SLE to be directed to the appropriate B cell developmental stage.

Signals transduced by interaction of the B cell homeostatic cytokine BAFF with its receptor, BAFF-R, regulate B cell selection at the late transitional stage and act as a rheostat for the size of the mature B cell compartment (4, 5). Autoreactive B cells that escape negative selection in the bone marrow often downregulate both cell surface IgM and BAFF-R and therefore do not compete well for BAFF in the periphery. Conversely, when BAFF levels are high, autoreactive B cells may be rescued (6, 7). The monoclonal anti-BAFF Ab belimumab has recently been approved for the treatment of SLE in humans (8), but it is unclear whether its therapeutic effect is actually due to alterations in B cell selection, nor is it known whether BAFF or APRIL inhibition alters the selection of effector autoreactive B cells.

The purpose of our experiments was to use germline D42 H chain (glD42H) NZB/W mice bearing a site-directed anti-dsDNA Ab-derived VH11 transgene to determine how BAFF availability and BAFF/APRIL blockade influence the selection of naive and Ag-activated autoreactive B cells during the evolution of SLE. Nonautoimmune glD42H mice have a low frequency of anti-dsDNA producing B cells as a result of clonal deletion, anergy, and receptor editing (9, 10). When glD42H is introduced into lupus-prone NZB/W mice, high-affinity IgG anti-dsDNA Abs appear in the serum by 6–7 mo of age, and nearly all spontaneous IgG anti-dsDNA Ab–producing hybridomas use the original D42 L chain VκRF (Vκ16-104*01)/Jκ5, which confers high-affinity anti-dsDNA reactivity (11, 12).

In this article, we show that B cells expressing a repertoire of L chains that confer no or low-affinity autoreactivity are positively selected into the naive B cell pool of glD42H NZB/W mice. In contrast, B cells with high-affinity autoreactivity are mostly deleted in the bone marrow and at the early transitional B cell stage, but are preferentially selected and expanded in the GCs of diseased mice. Competition with a diverse repertoire markedly inhibits selection of glD42H-expressing B cells into the naive B cell repertoire, and selection of these cells is further inhibited by BAFF/APRIL blockade with TACI-Ig. However, TACI-Ig does not prevent selection or expansion of high-affinity autoreactive B cells in the GCs. These findings show that BAFF/APRIL inhibition does not prevent the major breach in B cell tolerance that occurs during the GC response in NZB/W mice.

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The online version of this article contains supplemental material.
Abbreviations used in this article: FO, follicular; GC, germinal center; glD42H, germline D42 H chain; MZ, marginal zone; PC, plasma cell; SLE, systemic lupus erythematosus; TI, transitional 1; T2, transitional 2; wt, wild-type.
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Materials and Methods

Mice

Female NZB/W glD42H mice were bred with NZW males (The Jackson Laboratory, Bar Harbor, ME). Transgenic (IgDβ allotope-positive) female offspring were tested for proteinuria and anti-dsDNA Abs every 4 wk, as previously described (13). Groups of mice were sacrificed for analysis at 8 and 32 wk of age.

Bone marrow chimeras were generated by transfer of either 30/70 or 50/50% glD42H/wild-type (wt) bone marrow into totally irradiated 8- to 12-wk-old NZB/W F1 females. Groups of four or five chimeric mice were given 100 μg TAC Ig (13) three times per week continuously, starting at day 3 after transplantation or no treatment. Mice were sacrificed 12–16 wk after transplant.

Flow cytometry

Spleen and bone marrow B cells from 8- and 32-wk-old glD42H mice and from chimeric mice harvested 12–16 wk after bone marrow transplant were gated using anti-CD19 (spleno) or anti-B220 (bone marrow), as previously described (13, 14) (Fig. 1). Transitional 1 (T1) cells were CD23hi/IgMhi/CD21−, transitional 2 (T2) MZP cells were CD23hi/IgMhi/CD21+, marginal zone (MZ) cells were CD23hi/IgMhi/CD21+, follicular (FO) cells were IgD−/IgM−, GC cells were CD19+/IgM−/IgD−/PDCA/Fas−, and class-switched cells were IgD+/IgM−. Plasma cells (PCs) were B220+/IgD−/CD138hi (15). Bone marrow B cells were separated into B220−/IgD−/IgM− (pro- or pre-B cells), B220+/IgD−/IgM− (immature) and; B220+/IgD−/CD138hi (PC) subsets. For the bone marrow chimeras, anti-IgD was used to sort the FO subset.

Single-cell sorting and PCR

Single cells were sorted from B cell subsets from three to five individual mice from the groups of mice described above, and cDNA was synthesized as previously described (16). cDNA was used as template for a 20-μL PCR reaction containing 2 μL cDNA, 10 μL FastStart PCR master (Roche), and 62.5 nm of each primer (Supplemental Table I). The PCR program was as follows: 4 min at 94°C; 50 cycles of 30 s at 94°C, 30 s at 60°C, and 55 s at 72°C, followed by 10 min at 72°C. A second round was then performed using primers specific for CDR1 and CDR3 of glD42H. Wells that yielded a product of the correct size contained transgenic B cells, and these were then subjected to PCR for Vκ as previously described (17). PCR products were sequenced by Genewiz (South Plainfield, NJ), and sequences were identified using the International ImMunoGeneTics database.

Real-time PCR was performed as previously described (18). Primers are listed in Supplemental Table I. The average of the raw data for each sample (Ct value) was normalized to the internal control (housekeeping gene β-actin). Ratios of D42/CD19, Vκ/CD19, and Vκ/RF4/CD19, and Vκ/RF4/RF4 were then calculated.

Expression studies

The glD42H H chain and selected germline L chain variable regions (Supplemental Table II; synthesized by Genescript, New Piscataway, NJ) were cloned into mouse IgG2a and κ expression vectors, respectively (InvivoGen, San Diego, CA). Using the manufacturer’s instructions, H and L chains were cotransfected into 293T cells, using the LyoVec Kit (InvivoGen). Then 48-h supernatants were normalized to a concentration of 1 μg/ml and tested by ELISA for reactivity to cardiolipin, dsDNA, histones, phosphatidyl serine, phosphatidyl choline, insulin, chymotrypsinogen A, cytochrome C, and keyhole limpet hemocyanin, all plated at 10 μg/ml. To increase the relative avidity of the Abs for dsDNA, we also preincubated the supernatant with the secondary Ab to generate preformed complexes.

Statistical analysis of repertoire data

The data on the L chain repertoire in the various B cell subsets come in the form of very large and sparse r × c contingency tables (Supplemental Table II). Of the columns of the table represent the B cell subsets (e.g., pro- or pre-B, immature B, T1, T2, MZ cells, FO cells, GC cells, PCs) in each group of mice (8-wk-old glD42H, 32-wk-old glD42H, untreated and treated bone marrow chimeras), and the rows represent the many possible L chains. The table entries were the frequency of L chains of a given Vκ genotype within B cell subsets. Columns totals were considered “fixed” (i.e., column sampling), so that L chain “percentages” were computed as so-called column percentages. Thus, the column totals were denoted by n1, n2, ..., nC, where C is the total number of B cell subsets being considered in the analysis. By convention, r, the number of rows, corresponded to the number of L chains that have representation in the table. In other words, an L chain must have had at least one nonzero frequency count to be considered in the table and the calculations cited below.

Owing to the typically small sample sizes of single cells, the usual Pearson χ² statistic is not valid for determining whether differences exist in the distributions of L chains across B cell subsets. Instead, the Fisher exact test is applicable. However, the computing time required for running the exact test on large, sparse tables is prohibitive and, moreover, the power of the test is low. Accordingly, identifying L chains of interest without using formal inferential methods (i.e., without using p values or confidence intervals) is needed. This exploratory approach is frequently used in identifying interesting genes in gene expression microarray studies (19–21).

To this end, we used a novel method for “screening” the r × c table for cell frequencies that appear to be inconsistent, with the null hypothesis that the B cell subset and L chain distributions are independent. This approach was founded on a method advocated by Snedecor and Cochran (22), which is based on identifying table cells that contribute large values to the overall χ² statistic (even though the χ² is not being used for inference). The contribution of the jth cell is $X^2_{ij} = (O_{ij} - E_{ij})^2/E_{ij}$, where $O_{ij}$ is the observed cell frequency, computed in the usual way. The values of $X^2_{ij}$, for $i = 1, 2, ...$ and $j = 1, 2, ...$ were computed. Then, $P_{ij} = 100*X^2_{ij}/\sum X^2_{ij}$ the percentage of overall $X^2$ contributed by the jth cell, was calculated.

The values of $P_{ij}$ were ordered from largest to smallest, and the order statistics, $P_{(1)} \leq P_{(2)} \leq ..., \leq P_{(rc)}$ were enumerated. $P_{ij}$ was plotted against i to form a scree plot, as is commonly done in principal components analysis (23). Typically, one would look for the “elbow,” or turning point, of the scree plot to determine a cut-off point of interest. In effect, this cut-off value would aid in identifying L chains whose contributions to $X^2$ are clearly deviant from the null hypothesis of independence (see Supplemental Fig. 1 for examples). Because visualizing the elbow is a subjective determination, a more objective analytical approach was used, whereby the cumulative order statistics were computed [i.e., $P_{(1)} + P_{(2)} + ..., + P_{(j)}$, and so on]. When a cumulative sum of 50% was reached, then the L chains that contributed to that sum were targeted for more in-depth investigation. By this, we mean further investigation in the laboratory, using other methods, but not by conducting a usual Pearson $\chi^2$ test for proportions because performing the $\chi^2$ test at the usual significance levels (e.g., $p < 0.05$ or 0.01) is invalid when the decision to carry out the comparison is based on preselection of results that appeared to be interesting based on the scree plot methodology. With the arbitrary cumulative sum of 50% as a guide, 49 of the 84 identified Vκ genes were represented within the top 50% or contributed >5% to the $X^2$ in any of the comparisons performed (Table I, Supplemental Tables II, III). Of these 49, 40 genes contributed >2% of the total Vκ repertoire in any of the subsets.

Other statistical analyses

Comparisons in Figs. 2, 4, 5, and 6 were performed using the Mann–Whitney U test. The p values ≤ 0.05 were considered significant.

Results

B cell phenotype of glD42H mice

The 8-wk-old glD42H mice have mild B cell lymphopenia with an expanded MZ (Fig. 2A, 2B). The percentage of CD19-positive cells and of class-switched B cells increases as the mice age to 32 wk (Fig. 2A). We have previously reported that in nonautoimmune glD42H mice surface levels of IgM are decreased and a large proportion of B cells are anergic (9, 11). In contrast, in the autoimmune glD42H NZB/W mice we observed only a modest downregulation of surface IgM and IgD in the FO population compared with nontransgenic NZB/W littermates (Fig. 2C–F). As previously reported (11), IgM anti-dsDNA Abs were first detected in the serum of glD42H NZB/W mice at 9–11 wk of age and IgG anti-dsDNA Abs at 16–18 wk (data not shown).

Single-cell PCR analysis of the B cell repertoire in glD42H mice

We performed single-cell PCR of splenic B cell subpopulations (Fig. 1A) from 8-wk-old and 32-wk-old glD42H mice (three to five per group) and analyzed the L chain repertoire associated with use of the glD42H H. A total of 84 Vκ genes from the Interna-
Phenotype of glD42H transgenic mice.

**FIGURE 2.** Phenotype of glD42H transgenic mice. A, glD42H mice have mild B cell lymphopenia, with an increase in MZ B cells and a decrease in FO B cells, compared with wt NZB/W. These defects are corrected in bone marrow chimeras (mean ± 1 SD; *p < 0.05, †p < 0.01 compared with 8-wk-old glD42H). B, Increased IgM expression on B cells of glD42H (green) compared with wt NZB/W littermates (pink) reflects the enlarged IgMhi MZ subset in young mice (Supplemental Table II); these 40 genes are shown in Fig. 3. Mice (Fig. 3 B old mice but accounted for 65% of the GC repertoire in 32-wk-old mice (Supplemental Tables II, III). V

**FIGURE 1.** Sorting strategy for spleen and bone marrow cells. A, Spleen: After exclusion of clumps and gating on lymphocytes, CD19+ cells were gated into CD23+ and CD23– (a), IgD+/IgM into (FO) (b) and IgM–/IgD– (class-switched) (c) fractions. d, T2-MZP cells are CD23+IgMhi/CD21hi. e, T1 cells are CD23–/IgM+IgD+CD21hi. MZ cells are CD23–/IgMhi/CD21hi. f, GC cells are IgM–/IgD–/Fas+/PNAhi. g, Lymphocytes were gated into the B220hi/IgDhi population. h, PCs are B220hi/IgDhi/CD138hi. B, Bone marrow: B220+ B cells were gated into R1 (immature and recirculating) and R2 (pro- or pre-) B cell fractions. Upper panels show that the R1 population is CD43–. IgMhi/IgDhi immature B cells were sorted from R1 after gating for the B220hi population. The lower panels show that 60% of the R2 gate consists of pre-B cells (CD43+/IgM–/IgD–). Pro-B cells do not express Vκ L chains.

D42/VκRF ratios

The expression of D42 and VκRF in each B cell subset was measured by real-time PCR. We found a high ratio of VκRF/D42 in bone marrow pre-B and immature cells, with a decrease in the transitional (T1 and T2-MZP), MZ, and FO compartments and an expansion in the GC and in bone marrow PCs (Fig. 4A). Together, our single-cell PCR and real-time PCR data show that B cells expressing glD42/VκRF are generated in the bone marrow but are mostly deleted after the immature stage.

RAG expression

To determine whether peripheral receptor editing might account for the increased expression of VκRF/Jκ in the GCs, we measured RAG-1 and RAG-2 expression in sorted bone marrow and spleen B cells, using real-time PCR. RAG was expressed by the bone marrow pre- or pre-B cell fraction as expected, but not by FO or GC populations (Fig. 4B). Similarly, we did not detect an

*Immunogenetics database* was represented, indicating good coverage by our methodology. Of these, 40 contributed greater than 5% of the χ2 value in any of the comparisons we performed (Table I, Supplemental Table II); these 40 genes are shown in Fig. 3.

Both the MZ and FO B cell repertoires in young mice and old mice were dominated by three L chain genes: Vκ4–55*01, Vκ6–15*01, and Vκ13–8–19*01 (Fig. 3 k family members and Vκ4–55*01, Vκ6–15*01, and Vκ8–19*01 in both compartments; Jκ5 was the most frequent Jκ associated with Vκ4–55*01 and Vκ8–19*01 in both compartments; Jκ5 was associated with Vκ6–15*01 in the MZ compartment, whereas Jκ2 and Jκ4 were used in the FO compartment. In old mice, Vκ1 family members and Vκ12–46*01 and Vκ13–85*01 appeared in the FO repertoire; together, these genes constituted 32% of the FO repertoire in old mice compared with 5% in young mice (Supplemental Tables II, III). VκRF was not detected in the naive B cell repertoire of either 8-wk-old or 32-wk-old mice but accounted for 65% of the GC repertoire in 32-wk-old mice (Fig. 3 B).

**FIGURE 3.** Phenotype of glD42H transgenic mice. A, glD42H mice have mild B cell lymphopenia, with an increase in MZ B cells and a decrease in FO B cells, compared with wt NZB/W mice. These defects are corrected in bone marrow chimeras (mean ± 1 SD; *p < 0.05, †p < 0.01 compared with 8-wk-old glD42H). B, Increased IgM expression on B cells of glD42H (green) compared with wt NZB/W littermates (pink) reflects the enlarged IgMhi MZ subset in young mice (Supplemental Table II); these 40 genes are shown in Fig. 3. Mice (Fig. 3 B old mice but accounted for 65% of the GC repertoire in 32-wk-old mice (Supplemental Tables II, III). VκRF was not detected in the naive B cell repertoire of either 8-wk-old or 32-wk-old mice but accounted for 65% of the GC repertoire in 32-wk-old mice (Fig. 3 B).
Table I. Comparison of V<sub>k</sub> repertoires between subsets

<table>
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<tr>
<th>Mouse Group</th>
<th>B Cell Subset Comparison</th>
<th>No. V&lt;sub&gt;k&lt;/sub&gt; Genes</th>
<th>Top Gene Represented</th>
<th>Top 5 Genes</th>
<th>Top 10 Genes</th>
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<td>No. Genes</td>
<td>No. Genes</td>
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Bone marrow chimeras

To examine the effect of BAFF/APRIL availability on selection of the B cell repertoire, we generated bone marrow chimeras, thus providing an environment in which immature autoreactive B cells were subject to normal competition for survival (24, 25). In some of these mice we depleted BAFF/APRIL by administering TACI-Ig, starting immediately after bone marrow transplant to ensure that all B cells matured in the low BAFF/APRIL environment.

The B cell phenotype in the control chimeric mice was restored to that of wt NZB/W mice, with normal numbers of B cells and normal distribution of the MZ and FO populations (Fig. 2A). However, reconstitution of transgenic B cells was poor even when the ratio of transgenic/wt cells was increased from 30:70 to 50:50 (Fig. 2G). Furthermore, IgM was no longer downregulated on the transgenic B cells (Fig. 2F), suggesting that surviving cells were not anergic. Thus, when competition from nonautoreactive cells is provided, glD42H confers a high enough affinity for autoantigen to result in deletion of the majority of transgenic B cells. To further explore this possibility, we analyzed sorted bone marrow B cells (Fig. 1B) of 50:50 wt/glD42H chimeras for expression of the glD42H transgene, using single-cell PCR. Although some mouse to mouse variability was present, we found that >90% of the B cells from the pre- + pre-B cell fraction and a variable percentage, up to 90%, from the immature bone marrow fraction expressed the glD42H, indicating a survival advantage of B cells with an already rearranged H chain. In the FO repertoire, however, only 4% of B cells expressed the glD42H (Fig. 2G, 2F), indicating that deletion of most glD42H-expressing B cells occurred after the immature B cell stage.

We confirmed these results by measuring the ratio of glD42H and VxRF/Jk5 to CD19. The ratio of glD42H/CD19 decreased after the bone marrow immature stage and before the T2 stage. Similarly, although VxRF/Jk5 was detected in the bone marrow and in the T1 subset, most B cells expressing glD42H/VxRF/Jk5 were deleted by the T2 stage (Fig. 5). Importantly, the frequency of B cells bearing the major specificities Vxk5–48*01, Vxk6–15*01, Vxk8–19*01, Vxk12–46*01, and Vxk13–85*01 in the chimeric mice was similar to that in control glD42H mice (Fig. 6A), indicating that most naive glD42H B cells did not compete effectively for survival when a diverse B cell repertoire was present. Only subtle changes in the FO repertoire occurred in the chimeric mice (Fig. 6B). As in control glD42H mice, VxRF was vastly overrepresented in the GCs and PCs of the chimeric mice (Fig. 6C).

To analyze the DNA-binding characteristics of the various glD42H-encoded Abs, we cotransfected glD42H/IgG2a with 13 individual L chains, including VxRF/Jk5 was no longer downregulated on the transgenic B cells (Fig. 2F), suggesting that surviving cells were not anergic. Thus, when competition from nonautoreactive cells is provided, glD42H confers a high enough affinity for autoantigen to result in deletion of the majority of transgenic B cells. To further explore this possibility, we analyzed sorted bone marrow B cells (Fig. 1B) of 50:50 wt/glD42H chimeras for expression of the glD42H transgene, using single-cell PCR. Although some mouse to mouse variability was present, we found that >90% of the B cells from the pre- + pre-B cell fraction and a variable percentage, up to 90%, from the immature bone marrow fraction expressed the glD42H, indicating a survival advantage of B cells with an already rearranged H chain. In the FO repertoire, however, only 4% of B cells expressed the glD42H (Fig. 2G, 2F), indicating that deletion of most glD42H-expressing B cells occurred after the immature B cell stage.

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To analyze the DNA-binding characteristics of the various glD42H-encoded Abs, we cotransfected glD42H/IgG2a with 13 individual L chains, including VxRF (bolded in Supplemental Table II), into 293T cells. For some L chains, more than one Jk region was used (Supplemental Table II). Binding was observed only to dsDNA, cardiolipin, and histones, with no binding to any of the other Ags in the panel. Apart from VxRF, we identified seven other autoreactive L chains with variable binding to DNA, cardiolipin, or histones (Fig. 7). Importantly, of the five dominant L chains in the naive repertoire, only Vxk12–46*01 conferred weak reactivity with histones alone. Thus these L chains have no or low autoreactivity because they do not bind to autoantigens when they are expressed with glD42H as a dimeric IgG2a. The other six autoreactive L chains, Vxk3–12*01, Vxk4–57*01, Vxk4–63*01, Vxk5–45*01, Vxk5–48*01, and Vxk10–94*01, like VxRF, are expressed in the bone marrow, are infrequent in the FO repertoire, and appear in the GCs or among PCs.
The effect of BAFF/APRIL inhibition on the B cell repertoire

We next analyzed chimeric mice that had been treated with continuous TACI-Ig at a dose sufficient to deplete 50–60% of naive splenic B cells, an amount similar to that observed in primates treated with belimumab (26). TACI-Ig further depleted D42-positive B cells after the T1 stage (Fig. 8), with a 60% decrease in mature transgenic B cells (1.2 ± 0.7% of CD19+ B cells versus 3.3 ± 0.8% in untreated chimeras, p < 0.01; Figs. 2H, 2I, and 8A). To determine whether there was preferential deletion of B cells expressing particular L chains, we compared the FO repertoires of untreated and TACI-Ig–treated chimeric mice. The frequency of the five most common L chain genes, Vk4–55*01, Vk6–15*01, Vk8–19*01, Vk12–46*01, and Vk13–85*01, was not substantially different in TACI-Ig–treated mice than in control glD42H or untreated chimeras. Thus, although TACI-Ig significantly impairs survival of naive glD42H B cells that escape from the bone marrow, it causes only subtle alterations in the accompanying L chain repertoire (Fig 6B, Supplemental Table III).

We next determined whether TACI-Ig altered selection of the GC repertoire in chimeric mice. The percentage of GC, class-switched cells, and PCs as a proportion of CD19+ cells was increased in the spleens of TACI-Ig–treated mice (data not shown). A modest decrease in the total number of GC B cells was noted in the spleens of TACI-Ig–treated chimeras, compared with untreated chimeras, reflecting the decrease in total numbers of CD19+ cells per spleen. However, the total number of class-switched cells and PCs in the spleens was not significantly decreased by TACI-Ig treatment, despite the modest decrease in spleen size and marked FO B cell depletion (Fig. 8A). In accord with these data, TACI-Ig–treated mice developed high-titer IgG anti-DNA Abs with only a 2-wk delay (Fig. 8I). As in the control glD42H transgenics, VxRF/Jk5 was the dominant L chain specificity in the GC repertoire, and VxRF/Jk5–expressing PCs were dominant in the spleens of both treated and untreated chimeras (Fig. 8C).

Discussion

B cell tolerance to self-antigen is maintained at a series of “checkpoints” that purge high-affinity self-reactive B cells from the naive B cell repertoire and prevent the emergence of auto-reactivity during protective responses to foreign Ags (27, 28). Newly formed autoreactive B cells may be deleted, anergized, or subject to receptor editing in the bone marrow. Large numbers of autoreactive B cells are subsequently deleted or anergized in the periphery before reaching the mature stage (16). BCR signal strength is the major determinant of B cell fate in the bone marrow and in early transitional (T1) cells. In the late transitional stage, BCR signaling cooperates with BAFF signaling to promote B cell survival by upregulating expression of BAFF-R, by producing antiapoptotic molecules, and by generating p100, a substrate for the nonclassic NF-κB signaling pathway used by BAFF-R (29). The interaction of BAFF with BAFF-R is required for the survival of most B cells past the T1 stage, and complete absence of BAFF results in depletion of ≥90% of splenic B cells, even in autoimmune-prone mice. Conversely, excess BAFF increases the number of MZ and FO B cells in the spleen. In contrast, the homologous molecule APRIL, which does not bind to BAFF-R (29), Studies in transgenic mice have shown that BAFF levels may influence the selection of the peripheral B cell repertoire. These studies in sum have shown that supraphysiologic BAFF excess does not alter the negative selection that occurs before or at the T1 stage, but rescues autoreactive cells that are anergized after the T1 stage.
and promotes their maturation into FO and or MZ cells (30). Because autoreactive B cells may have downregulated their BCR as a consequence of Ag stimulation at the early transitional stage, they signal less well through the classic NF-κB pathway and provide less of the p100 substrate needed for BAFF-R signaling. A lower BCR signal also results in lower cell surface expression of BAFF-R. Because BCR and BAFF-R signals synergize in promoting B cell survival, autoreactive B cells may therefore compete less well for survival as they progress to the T2 stage (6, 30, 31).

When B cell numbers and BAFF levels are normal, deletion of many autoreactive B cells occurs; conversely, an increase in serum BAFF levels may result in relaxation of B cell selection (5, 7, 32). Increased serum levels of BAFF and overrepresentation of autoreactive specificities in the naive repertoire have been reported in human SLE patients (33–36). Mature germline-encoded autoreactive B cells are usually excluded from the GC (27) or fail to progress past the early GC stage (37) and are therefore unlikely to undergo the class switching and somatic hypermutation that may yield pathogenic high-affinity self-reactivity. This checkpoint is defective in SLE patients (3, 38). Autoreactivity acquired by somatic mutation triggers a poorly defined deletion mechanism in GC B cells that purges the emerging memory B cell and PC repertoire of high-affinity auto-reactivity (28). This checkpoint is defective in human SLE and in MRL/lpr and Nba2 mice (3, 39). In SLE, attenuation of B cell expression of the inhibitory receptor FcRIIB also contributes to the failure to delete autoreactive B cells in GCs (40, 41).

Although supraphysiologic excess of BAFF alters naive B cell selection in nonautoimmune strains of mice (5–7, 32), and BAFF inhibition depletes transitional and naive B cells in humans (42), it is not known whether limiting BAFF reverses the described abnormalities of naive B cell selection in SLE patients or whether either BAFF or APRIL inhibition has any impact on the selection of autoreactive B cells that arise after Ag activation. These questions need to be answered so that BAFF/APRIL-inhibiting drugs are used appropriately in SLE. Importantly, it has been shown in transgenic models that BAFF excess has much less effect on B cell selection if competition is provided by nonautoreactive B cells (reviewed in Ref. 30). The factors that contribute to the deletion of autoreactive B cells under conditions of normal competition are not fully understood, but, in addition to the availability of BAFF, could include intrinsic BCR signal strength, BCR cell surface levels, autoantigen availability and composition, ability of internalized autoantigens to stimulate TLRs or other innate receptors, other exogenous factors such as sex hormones, and availability of Ags that drive positive selection into the naive

![FIGURE 5](http://www.jimmunol.org/) 

Quantitative analysis of H and L chains from each B cell subset from glD42H mice and 50:50 wt/glD42H chimeric mice. Ratio of V\(\kappa\)RF and glD42H to CD19 expression in B cell subsets. Negative selection of glD42H/V\(\kappa\)RF-expressing B cells occurs at the T1 stage, and positive selection occurs at the GC stage. Pellets from three individual mice were used per group, and experiments were repeated once. *Not done, †\(p < 0.01\), ‡\(p < 0.05\) compared with previous B cell developmental stage.

![FIGURE 6](http://www.jimmunol.org/) 

Effect of TACI-Ig on the L chain repertoire in 50:50 wt/glD42H chimeric mice. A–C, Repertoire analysis of glD42H-associated V\(\kappa\) chains in control chimeric and TACI-Ig–treated mice harvested 12–16 wk after transplant. Genes that contribute >5% of the \(x^2\) value in any of the comparisons are shown. The y-axis shows percentage of total repertoire. A, A total of 42–137 sequences from 10 mice per group sorted as individuals (4 mice per group, Experiment 1) or in pools of 3 (6 mice per group, Experiment 2) were analyzed per subset. B, A total of 40–137 sequences were analyzed per subset from 4 individual and 3 pools of chimeric mice and 4 individual wt mice in each group. C, A total of 29–53 sequences were analyzed per subset from 4 individual and 3 pools of control chimeric mice and 4 individual TACI-Ig–treated mice. The complete dataset is shown in Supplemental Table III.
It is therefore important, when using transgenic models, to perform studies of BAFF inhibition in model systems such as the one we describe in this article, in which competition is provided.

The studies we present have addressed the fate of B cells expressing either a germline-encoded low-affinity polyreactive autoantibody (gID42H/Vκ4-55) or a germline-encoded high-affinity anti-dsDNA autoantibody (gID42H/VκRF) under conditions of relative BAFF excess, of normal competition with nonautoreactive B cells, and of BAFF/APRIL inhibition in autoimmune NZB/W mice. The fates of these two types of B cells are quite different.

In normal humans as well as SLE patients, autoreactive specificities are very common in newly arising bone marrow B cells, but most of these are removed before the mature FO B cell stage (16); the defect in tolerance in SLE patients occurs both in the bone marrow and during peripheral deletion of autoreactive transitional cells (33, 34). In gID42H NZB/W mice, we found that B cells expressing the low-affinity polyreactive receptor are not deleted in the bone marrow and are positively selected into the T1 compartment of NZB/W mice, perhaps reflecting the abnormalities of immature B cell selection in SLE that occur in the bone marrow and transitional compartment and can be present even during periods of remission (34). In nonautoimmune gID42H mice, LPS-induced anti-dsDNA IgM hybridomas use a diverse L chain repertoire (11). In contrast, 67% of LPS-induced IgM hybridomas elicited from D42/Vκ1-Jκ1 NZB/W mice receptor edit to Vκ4–55*01 (12). IgM D42/Vκ4–55*01 binds DNA with only low affinity (12); we were not able to demonstrate dsDNA, cardiolipin, or histone binding of the germline-encoded but class-switched gID42H/Vκ4–55*01 Ab. Apart from Vκ4–55*01, we found only two other L chain genes, Vκ6–15*01 and Vκ8–19*01, in the MZs of young mice; these three L chains also constitute almost 50% of the FO repertoire. Selection of these L chains into both the FO and MZ repertoires may be due to differences in J region usage as well as variations in BCR expression.

Positive selection is a major contributor to the shape of the mature B cell repertoire (43, 44), and as shown by our single-cell PCR experiments, the highly skewed naive repertoire is positively selected before the BAFF-dependent T2 stage. Some diversification of the naive repertoire occurs as the mice age, with the recruitment of Vκ12-46*01, which confers low-affinity antihistone reactivity, into the FO repertoire, and of a more diverse MZ repertoire, suggesting a shift in the stringency for selection. Of importance, however, the dominant L chains in the naive repertoire have no or low autoreactivity because they are not autoreactive when expressed as dimeric IgG2a. Furthermore, the B cells most commonly selected into the naive repertoire are uncommon among the GC and PC repertoires, especially when the repertoire is diverse, suggesting that they may not be the appropriate target for therapeutic intervention.

In this article, we show that the GC entry checkpoint is defective in gID42H NZB/W mice and this defect is the major reason for loss of tolerance to dsDNA in these mice. In particular, the VκRF/Jκ2 L chain, which confers high-affinity anti-DNA reactivity and is used by nearly all IgG hybridomas from this mouse (12), is rare in the naive repertoire but constitutes >60% of the GC repertoire in old mice. This vast overrepresentation of VκRF in the GCs does not appear to be due to peripheral receptor editing, as we were unable to detect an increase in RAG expression in sorted GC B cells.
compared with FO B cells, using quantitative PCR, nor were we able to detect RAG proteins in the spleen, using immunohistochemical methods. Our data suggest that rare VxRF-expressing B cells are preferentially selected into and expand in the GCs. Furthermore, the checkpoint that prevents clonal expansion and differentiation of autoreactive GC B cells into effector cells is also defective in glD42H mice, as PCs also express VxRF. Importantly, our repertoire data also show failure of exclusion of several other germline-encoded autoreactive B cells from the GCs. A similar defect in GC tolerance has been reported in human SLE patients (3, 38, 45, 46).

Within the GC microenvironment, B cells compete both for Ag, based on BCR affinity and availability of Ag presented by follicular dendritic cells, and for growth and costimulatory factors provided by T cells, resulting in selection of those high-affinity B cells that receive help, but in active death of B cells that receive low-affinity BCR signals or BCR signals without cognate T cell help (47). Downregulation of BCR expression on GC B cells, low levels of BAFF, and high expression of Fas all help ensure that only those high-affinity B cells that receive additional help from T cells are selected. Three alternative hypotheses could explain the preferential selection of VxRF-expressing B cells into the GCs of NZB/W mice. First, a loss of T cell tolerance may occur. Second, the DNA-binding activity of VxRF-expressing B cells may result in both a strong BCR signal and increased signaling through nucleic acid-recognizing TLRs; this could drive preferential selection and survival of these cells even if partial downregulation of BCR expression is present and cognate T cell help is absent (48). TACI-Ig might be expected to affect this mechanism because BAFF and APRIL amplify TLR signals (49). Alternatively, BCR downregulation resulting from the high affinity of glD42H/VxRF for DNA may result in insufficient BCR signals to induce tolerance. This mechanism for tolerance loss has been postulated in Sle1 mice (2).

Inhibition of BAFF is the first successful biologic therapy for human SLE (8). Because the naive and Ag-activated repertoires in NZB/W F1 glD42H mice are distinct, we were able to study the effects of BAFF/APRIL inhibition on B cell selection at two different B cell developmental checkpoints, using TACI-Ig. The effects of TACI-Ig on selection of the naive repertoire are due to BAFF inhibition, as APRIL plays no role in promoting naive B cell survival (50). Provision of B cells expressing a diverse B cell repertoire was sufficient to impair the survival of most glD42H B cells after the immature stage. The L chain repertoire of these surviving B cells was similar to that of control glD42H mice, indicating that most glD42H-bearing B cells compete inefficiently for survival under conditions of normal B cell competition. The disappearance of glD42H B cells that had lower levels of surface IgM and IgD in the chimeric mice indicates that these B cells are preferentially susceptible to deletion under conditions of normal competition.

Few studies have addressed the fate of autoreactive B cells within a diverse repertoire under conditions of either BAFF excess or BAFF inhibition. Studies in HEL/anti-HEL transgenic mice have shown that BAFF excess does not rescue anergic high-affinity autoreactive transitional B cells but alters the localization of intermediate-affinity cells from the FO region to the MZ. Similarly, in the IgK-reactive macroself Ag transgenic mouse, excess BAFF does not rescue anergic transitional B cells once competition is provided. In the 3H9 model, excess BAFF increases the maturity of transgenic B cells but does not rescue them into B cell follicles or induce ANA production. In contrast, in the R4A H chain transgenic mouse, excess BAFF induces the production of anti-DNA Abs. In this mouse, BAFF inhibition decreases autoreactivity in the reconstituting B cell repertoire after B cell depletion. A recent study using two H/L transgenic B cells encoded by highly related Ig genes that confer differential affinity for autoantigen showed that competition from nonautoreactive B cells significantly decreased selection of both types of transgenic autoreactive B cells, but neither a moderate excess of BAFF nor BAFF inhibition further altered their selection, despite BCR downregulation in one case and high affinity for autoantigen in the other (51). In our study, in which only the H chain was constrained by a transgene, we show clearly that BAFF/APRIL inhibition with TACI-Ig induces a significant decrease in the survival of glD42H-expressing B cells in the periphery in chimeric mice, but with only subtle alterations to the accompanying L chain repertoire. These findings in sum suggest that the effect of excess BAFF on naive B cell selection can be quite variable, and that not all autoreactive B cells are equally susceptible to BAFF inhibition at the transitional B cell checkpoint. It will be very important to further dissect what factors determine BAFF responsiveness of autoreactive B cells and to find a means of determining which individuals are responsive to BAFF inhibition.

The role of either BAFF or APRIL in regulating selection of the Ag-experienced repertoire is not clearly defined. BAFF-deficient mice fail to form a mature GC follicular dendritic cell network, resulting in premature termination of the GC response (52, 53). Although somatic mutation and affinity maturation still occur in BAFF-deficient mice, the magnitude of the Ab response to T cell-dependent Ags is significantly attenuated (53). In contrast, APRIL appears to have a minimal role in GCs (52). Neither BAFF nor APRIL is required for the development of memory B cells but either is sufficient to support PCs, especially IgM PCs (14).

We have reported that when NZB/W mice are treated with TACI-Ig, they still produce IgG anti-DNA Abs that are capable of depositing in the glomeruli, but with less glomerular damage than in untreated controls (7, 10). We therefore wished to know whether differences in the class-switched repertoire exist between TACI-Ig treated mice and untreated chimeras. We found that despite the ability of TACI-Ig to modulate the naive B cell repertoire, it did not prevent the selection of VxRF/Jk5 into the GC or the terminal differentiation of VxRF/Jk5-expressing B cells to PCs or the secretion of IgG anti-DNA Abs. These data show that TACI-Ig treatment fails to completely eliminate D42/VxRF/Jk5-expressing B cells that escape negative selection from the naive repertoire, confirming that not all germline-encoded autoreactive B cells are susceptible to BAFF inhibition. Our findings are also consistent with the observed failure of BAFF/APRIL inhibition (13, 14) or even complete BAFF (54) or BAFF-R (55) deficiency to prevent an anti-DNA response in autoimmune mice. Because the autoreactive GC repertoire in glD42H mice is highly restricted to B cells that are already autoreactive in their germline state, we were not able to address the effect of BAFF/APRIL inhibition on autoreactive effector B cells generated de novo in the GC by somatic mutation. This endeavor will require a different transgenic system. Nevertheless, our studies suggest that regulation of GC entry and expansion should be an important therapeutic goal to prevent autoantibody production in SLE and that such an approach may synergize with BAFF/APRIL inhibition.

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