Enforced $bcl-x_L$ Gene Expression Restored Splenic B Lymphocyte Development in BAFF-R Mutant Mice

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*J Immunol* 2003; 170:4593-4600; doi: 10.4049/jimmunol.170.9.4593

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Enforced bcl-x<sub>L</sub> Gene Expression Restored Splenic B Lymphocyte Development in BAFF-R Mutant Mice<sup>1</sup>

Ian J. Amanna,* Jennifer P. Dingwall,† and Colleen E. Hayes<sup>2✉</sup>

The TNFR family member BAFF-R facilitates peripheral B cell development, although it is unclear whether it promotes survival of B cells, or also initiates a differentiation program. We show that disruption of the BAFF-R encoding gene Tnfrsf13c in strain A/WySnJ mice causes a progressive decline in peripheral B cell numbers, beginning at the transitional 1 developmental stage and continuing through the mature peripheral B cell stage. Bcl-x<sub>L</sub> overexpression in A/WySnJ B cells decreased the turnover of transitional B cells, as determined by 5-bromo-2′-deoxyuridine labeling, and restored follicular B cell development. We conclude that the mutant A/WySnJ allele of Tnfrsf13c can be complemented through the survival signal provided by Bcl-x<sub>L</sub>. The Journal of Immunology, 2003, 170: 4593–4600.

During peripheral B cell replenishment, homeostatic mechanisms must select a diverse B cell receptor repertoire, yet guard against seeding self-reactive B cells into the periphery (1). Disrupted B cell homeostasis underlies diseases such as systemic lupus erythematosus (SLE)<sup>3</sup> (2), X-linked agammaglobulinemia (3), and follicular (FO) B cell lymphoma (4). However, much remains unknown regarding B cell homeostatic mechanisms.

Based on our analysis of the B cell-deficient A/WySnJ mice (5–8), we predicted that a B cell-specific survival factor would provide essential homeostatic controls (7). That factor has now been identified as the TNF family member BAFF (also termed BlyS, TALL-1, zTNF4, and THANK) (9). Patients with the B cell-mediated autoimmune diseases SLE (10) and Sjogren’s syndrome (11) had excessive amounts of BAFF, suggesting a possible cause and effect relationship between increased BAFF and disease. Similarly, mice expressing transgenic BAFF had SLE-like and Sjogren’s-like syndromes (10–12). In contrast, genetically engineered BAFF-null mice completely lacked FO and marginal zone B cell lymphoma (4). However, much remains unknown regarding B cell homeostatic mechanisms.

There is now compelling evidence that the Bcmd-1 gene we characterized as causing B cell deficiency in A/WySnJ mice encodes a mutant form of the TNFR family member, BAFF-R (also BR3), which mediates BAFF’s homeostatic functions. The A/WySnJ mice and the BAFF-null mice have similar B cell deficiencies, but the A/WySnJ mice have some splenic B cells and make IgM responses to T-dependent and T-independent Ags (5, 6), whereas the BAFF-null mice have no B cells and make no Ab responses (13, 14). We found that the Bcmd-1 gene on mouse chromosome 15 (15, 16) controlled a B cell-intrinsic life span defect in A/WySnJ B-2 B cells, but not B-1 B cells (7, 8). Thompson et al (17) cloned the Tnfrsf13c gene encoding BAFF-R, mapped it to the human chromosome 22q13.1 region that is syntenic to the Bcmd-1 region of mouse chromosome 15, and found that the A/WySnJ Tnfrsf13c transcript had an aberrant third exon. We and our collaborators showed that the A/WySnJ B cells did not respond to BAFF in vivo (18). Together, the evidence indicates that Tnfrsf13c is Bcmd-1, the A/WySnJ mice have a loss-of-function mutation in Tnfrsf13c, and BAFF-R mediates BAFF’s essential B lymphocyte homeostatic functions.

A major challenge now is to determine the precise nature of the BAFF-R signals, and how those signals regulate peripheral B lymphocyte development (19). Newly formed (NF) B cells transit via the blood from the adult bone marrow to the marginal sinus of the spleen, where they begin a process of selection, maturation, and relocation according to their Ag receptor specificity (20), B cell-specific chemokines (21), and B cell survival signals (7, 22). Surface markers have been used to distinguish three sequential, shortlived, transitional B cell stages, transitional (T) 1, T2, and T3 (23, 24). In BAFF-null mice, splenic B cell development appeared to be blocked at the T1 to T2 transition (13, 14). Moreover, BAFF-mediated signals appeared to support T2 B cell survival (25). These results led to the suggestion that BAFF-R-mediated differentiation signals drive the T1 to T2 transition, and/or the BAFF-R-mediated survival signals maintain the T2 B cells (26). In contrast, we previously suggested that the BAFF-R-mediated survival signals maintain splenic B-2 B cells of all developmental stages, because turnover studies showed that all A/WySnJ splenic B-2 B cells had shortened life spans (7). Additionally, a recent report shows that BAFF-R can signal NF-κB activation in T1 splenic B cells (27). Thus, it is not yet certain whether BAFF-R provides survival signals to all splenic B-2 B cells, or only to B cells from the T2 stage onward, and there are differing views as to whether this receptor transmits differentiation signals driving the T1-T2 transition.

We sought to address these uncertainties concerning BAFF-R function. We cloned and sequenced the previously uncharacterized 3′ untranslated region of the A/WySnJ Tnfrsf13c transcript to determine the source of the discordant sequence. We also used the
most current method of transitional B cell analysis to compare the T1, T2, and T3 B cells in the spleens from the BAFFR-mutant A/WySnJ and wild-type A/J strains, to learn whether the Tnfrsf13c mutation blocked a particular transition. Most importantly, we investigated whether provision of a survival signal could complement the Tnfrsf13c mutation and restore B-2 B cell development. The data show that enforced in vivo expression of the bcl-\(x_l\) survival gene in A/WySnJ cells complemented the Tnfrsf13c mutation and restored B-2 B cell development. These data are discussed in the context of a model for BAFFR homeostatic function.

Materials and Methods

**Mice**

Male and female A/J and A/WySnJ were from our specific pathogen-free mouse colony in the Department of Biochemistry, University of Wisconsin. The mice were maintained at 23°C with 40–60% humidity and 12-h light-dark cycles and used at age 7–10 wk. The protocols were approved by the Institutional Animal Care and Use Committee (protocol A00847-4-08-99).

**Immunofluorescence analysis of spleen sections**

Frozen sections were prepared, stained, and analyzed, as described (28). In brief, spleens embedded in OCT compound (Lab-Tek Products, Division of Miles Laboratories, Naperville, IL) were flash frozen, sectioned, air dried, acetone fixed, and blocked with normal horse serum. The splenic metalloplastic macrophages were stained with rat Abs to MOMA-1, and developed with biotinylated goat Ab to rat IgG, followed by streptavidin 7-ami-no-4-methylcoumarin-3-acetic acid (Vector Laboratories, Burlingame, CA). The sections were washed and then stained with FITC-rat Ab to mouse CD5 (clone 53-7.3; BD Pharmingen, San Diego, CA) and rhodamine isothiocyanate-coupled goat Ab to mouse IgM (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL). The stained sections were washed, mounted in Fluormount G (Southern Biotechnology Associates, Birmingham, AL).

**B cell staining and analysis**

For immunofluorescence analysis, spleenocytes were dissociated into ice-cold staining buffer (5% heat-inactivated FBS and 0.1% sodium azide in PBS, pH 7.3) and depleted for RBCs. Duplicate samples (\(10^6\) cells/sample) were stained (30 min on ice) with optimal amounts of the mAb conjugates. Reference samples from each strain were stained with fluorochrome-coupled isotype control mAb, or single-color specific mAb stains, which were used to select fluorescence gates. The B cell turnover study and the splenocyte samples (2–4×10^7 cells/sample) were stained as described (7). Stained samples were analyzed on a FACSCalibur using CellQuest software (BD Biosciences, Franklin Lakes, NJ). The streptavidin-fluorescein isothiocyanate protocol (Amersham Pharmacia Biotech, Piscataway, NJ) was performed at the Biotechnology Center's DNA Sequence Laboratory (University of Wisconsin).

**Immunoblotting**

For Bcl-\(x_l\) protein expression analysis, cells were lysed in buffer (0.1% w/v SDS, 0.5% w/v deoxycholate, 1% v/v Nonidet P-40, 0.1 mg/ml PMSF, and 20 μg/ml aprotinin) on ice for 30 min and centrifuged, and the cleared lysates were stored at −70°C. The protein concentration was determined by the bicinchoninic acid protein assay (32). Lysates (17 μg of protein per sample) were electrophoresed in 12% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with mAbs to murine Bcl-\(x_l\) (clone H-5; Santa Cruz Biotechnology, Santa Cruz, CA). Bound mAb was visualized using the ECL Western Blotting Detection System, according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was then stripped with 0.2 M NaOH and immunoblotted for green fluorescent protein (GFP) (clone B-2; Santa Cruz Biotechnology), as above.

Production and characterization of infectious retrovirus

The infectious MigRI.BX retroviral particles were produced, and A/WySnJ bone marrow stem cells were infected, as previously described (29). Briefly, purified MigRI.BX vector DNA (Wizard Plus Maxiprep; Promega) was CaPO_4 precipitated onto ~80~90% confluent monolayers of the Bosc23 packaging cells grown in IMDM (supplemented as above, but without 2-ME). Retroviral supernatants were collected 48–72 h later, stored at −70°C, and titered on NIH/3T3 cells. The NIH/3T3 cells (2×10^5) were grown 24 h in 60-mm dishes, before the supplemented IMDM (without 2-ME) was replaced with 100 μl of thawed retroviral supernatant diluted in 1 ml of medium containing Polybrene (4 μg/ml; Sigma-Aldrich). A 2-ml aliquot of fresh medium was added 24 h postinfection, and flow cytometric analysis for GFP expression was performed 48 h postinfection. Retroviral supernatants that yielded >40% GFP^* NIH/3T3 cells were used for the bone marrow stem cell infections.

Production and characterization of retrovirus-infected mice

The A/WySnJ donor mice were injected i.v. with 5-fluorouracil (5 mg in 400 μl PBS; Sigma-Aldrich). Five days later, bone marrow cells were collected and cultured (10–20×10^5 cells) in IMDM (4 ml) supplemented with 15% heat-inactivated FBS, 5% WEHI-3B-conditioned medium, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, plus mouse rIL-3 (6 ng/ml), mouse rIL-6 (10 ng/ml), and recombinant mouse stem cell factor (100 ng/ml; all from Preprotech, Rocky Hill, NJ). After 24 h of culture, the medium was removed, and two sequential retroviral infections separated by 24 h were performed by spinoculation exactly as described (29). The infected cells were collected 4 h after the second inoculation and suspended in PBS, and 0.5×10^6 to 1×10^6 cells were injected i.v. into each lethally irradiated A/WySnJ mouse. Lethal irradiation (2 doses of 600 rad, separated by 4 h) was done just before bone marrow cell transfer. Splenocytes were collected and analyzed after 14–16 wk of reconstitution.
Results

A retrotransposon insertion disrupted the A/WySnJ Tnfrsf13c gene

The nature of the Tnfrsf13c mutation in strain A/WySnJ mice is not completely known. The A/WySnJ Tnfrsf13c gene had a 4.7-kb insertion disrupting exon 3, which was predicted to replace the final 8 aa of BAFF-R with 21 aa of unknown origin (33). We sequenced the 3′ end of the A/WySnJ Tnfrsf13c transcript to identify the source of the insertion. A 3′ RACE was performed. The 3′ RACE upper primer encompassed the exon 3 insertion site (Fig. 1A). The results showed that beginning at base 548, the 3′ end has been replaced with 299 exogenous bases, including a stop codon and an AU-rich polyadenylation site (Fig. 1B). The predicted amino acid sequence of the mutated BAFF-R (Fig. 1C) was consistent with the published sequence (33). A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) revealed >90% homology between the exogenous bases and an intracisternal type A particle (GenBank ID 4378062; data not shown). We conclude that a retrotransposon insertion disrupted the Tnfrsf13c gene.

The BAFF-R mutation progressively decreased each transitional B cell subset

Our previous histological studies showed that the Tnfrsf13c loss-of-function mutation severely disrupted splenic architecture (7). In those studies, it was clear that well-developed follicles were largely absent in the A/WySnJ spleen, but it was not clear whether these follicles had a marginal zone, nor where the A/WySnJ T and B lymphocytes were to be found. To better understand the function of BAFF-R in B cell development, more detailed immunohistology was performed. The BAFF-R-mutant A/WySnJ spleen and the control A/J spleen show well-defined marginal zones, as judged by the metallophilic macrophages, and well-developed T cell zones (Fig. 2). However, the A/WySnJ follicles show clear deficits in the B cell zones compared with the A/J follicles. Possible differences in MZ B cells cannot be clearly discerned from the immunohistology (J. Kearney, personal communication).

A flow cytometric analysis of the splenocytes supported and extended these results (Fig. 3). Surface expression of IgM, CD21, and CD23 defined the NF (IgM+/CD21+/CD23+), FO (IgM+/CD21high/CD23low), and MZ (IgM+/CD21+high/CD23−) B cell subsets. As shown in Fig. 3 (28, 34), the percentages and numbers of these B cell subsets are summarized in Table I. The A/WySnJ splenocytes showed an 83% decrease in FO B cells (p < 0.01), and a 77% decrease in MZ B cells (p < 0.01) compared with A/J splenocytes. Consequently, we conclude that the BAFF-R mutation had a negative impact on both FO and MZ B cells. The decline in FO B cells can be attributed to a decrease in the number of precursors entering this compartment and a reduced life span of the cells within the compartment (7). The decline in MZ B cells is most likely due to the presence of a retrotransposon insertion.

Materials and Methods

B lymphocytes were to be found. To better understand the function of these follicles had a marginal zone, nor where the A/WySnJ T and B lymphocytes were to be found. To better understand the function of BAFF-R in B cell development, more detailed immunohistology was performed. The BAFF-R-mutant A/WySnJ spleen and the control A/J spleen show well-defined marginal zones, as judged by the metallophilic macrophages, and well-developed T cell zones (Fig. 2). However, the A/WySnJ follicles show clear deficits in the B cell zones compared with the A/J follicles. Possible differences in MZ B cells cannot be clearly discerned from the immunohistology (J. Kearney, personal communication).

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the same two factors, but the life span of A/WySnJ MZ B cells has not been measured so far, and so a possible contribution of BAFF-R to MZ B cell life span is uncertain. It was not possible to draw firm conclusions about transitional B-2 B cells from this analysis, because the T1 B cells and the B-1 B cells were not separated by this surface marker gating scheme (1).

To detect a possible maturation defect at a particular transition, as has been suggested for the BAFF-R-mutant A/WySnJ mice (19), we used the most current method of transitional B cell analysis to compare the T1, T2, and T3 B cells in the A/WySnJ and A/J spleens. Transitional B cells express the C1qRp molecule (previously termed 493 and AA4), whereas B-1 B cells and mature B cells (MB) do not (19, 24). Therefore, the C1qRp+ transitional B cells were gated and subdivided by surface IgM density (which decreases with increasing maturity) and CD23 expression (which characterizes T2 and T3, but not T1 B cells), as shown in Fig. 4. The numbers of T1, T2, T3, and MB cells obtained from this analysis of A/J mice (Table I) were in good agreement with published results for BALB/c mice (24). Compared with the A/J splenocytes, the A/WySnJ splenocytes showed a 39% decrease in T1 B cells, a 77% decrease in T2 B cells (p < 0.01), an 82% decrease in T3 B cells (p < 0.01), and an 88% decrease in MB cells (p < 0.01). These results showed that when the B-1 B cells were rigorously excluded from the transitional B cell analysis, it was clear that the BAFF-R mutation caused a progressive decline in splenic B cell development, beginning with the earliest T1 B cell stage.

**Construction and characterization of a retroviral vector expressing the bcl-xL gene**

It is widely accepted that a survival signal derives from BAFF-R, but there is controversy as to whether this receptor also transmits a differentiation signal. To address this important issue, we used retroviral gene transfer to provide a survival signal to the BAFF-R mutant B cells, and analyzed whether FO B cell development had been restored. We reasoned that if the mutant BAFF-R lacks an essential differentiation signal, then provision of a survival signal would extend the transitional B cell life span, yet would not enhance FO B cell development. We selected the bcl-xL gene for these experiments, because bcl-xL transgenic mice show enhanced FO B cell survival (35), bcl-xL is up-regulated in response to BAFF treatment in vitro (36), bcl-xL has well-documented antiapoptotic function (37), and bcl-xL is the most potent antagonist of the proapoptotic Bik gene (38) that we reported is overexpressed in A/WySnJ B cells (39).

The bcl-xL gene was subcloned into the MigRI retroviral vector (29) to produce the MigRI.BX vector (Fig. 5A). This vector expresses the GFP as a marker of successful gene transfer. To test the ability of MigRI.BX to support cell survival, MigRI.BX was used to infect IL-3-dependent FL5.12 cells. High level Bcl-xL expression can overcome the apoptosis that is initiated in these cells upon IL-3 withdrawal (30). Initially, ~10–15% of the FL5.12 cells became MigRI.BX infected and expressed GFP (Fig. 5B). When IL-3 was withdrawn, the percentage of GFP+ cells increased significantly in the MigRI.BX-infected group, but not in the control group.

### Table 1. Splenic B cell subsets in A/J, A/WySnJ, and AW.bcl-xL mice

<table>
<thead>
<tr>
<th>B Cell Subset</th>
<th>A/J</th>
<th>A/WySnJ</th>
<th>AW.bcl-xL, GFP+ (%)</th>
<th>AW.bcl-xL, GFPhigh (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM, CD23, CD21 staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>16.2 ± 3.0</td>
<td>6.5 ± 2.4</td>
<td>43.7 ± 1.2**</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>FO</td>
<td>72.6 ± 5.6</td>
<td>28.5 ± 8.0</td>
<td>44.8 ± 1.9**</td>
<td>4.8 ± 1.2**</td>
</tr>
<tr>
<td>MZ</td>
<td>8.8 ± 3.2</td>
<td>3.5 ± 1.8</td>
<td>7.2 ± 1.1</td>
<td>0.8 ± 0.2**</td>
</tr>
<tr>
<td>IgM, CD23, C1qRp staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>8.9 ± 1.6</td>
<td>3.6 ± 1.4</td>
<td>21.8 ± 2.9**</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>T2</td>
<td>10.7 ± 1.0</td>
<td>4.3 ± 1.3</td>
<td>10.2 ± 1.7</td>
<td>1.0 ± 0.2**</td>
</tr>
<tr>
<td>T3</td>
<td>12.5 ± 2.1</td>
<td>5.0 ± 1.7</td>
<td>9.3 ± 1.8</td>
<td>0.9 ± 0.2**</td>
</tr>
<tr>
<td>MB</td>
<td>52.1 ± 3.8</td>
<td>21.5 ± 6.7</td>
<td>25.7 ± 2.9**</td>
<td>2.5 ± 0.4**</td>
</tr>
</tbody>
</table>

* IgM+ B cells were divided into subsets using the IgM/CD23/CD21 gating scheme of Oliver et al. (28), as shown in Figs. 3 and 6, or the IgM/CD23/C1qRp gating scheme of Allman et al. (24), as shown in Figs. 4 and 6.

**For the A/J and A/WySnJ mice, the absolute number of each B cell subset was calculated by multiplying the subset percentage by the number of splenic B cells in each individual.

**The Mann-Whitney test (50) gave p < 0.05 (+) or p < 0.01 (++) for comparisons between A/WySnJ and A/J B cell subsets.

**A two-tailed Student’s t test (assuming unequal variances) gave p < 0.05 (+) or p < 0.01 (++) for comparisons between the AW.bcl-xL, GFP+ and GFPhigh B cell subsets.

**FIGURE 4.** The BAFF-R mutation progressively decreased the number of cells in each B cell subset. A, The IgM+ live lymphocytes were gated as shown to enumerate the MB cells. The experiment was done as described in the Fig. 3 legend, except that the C1qRp marker replaced the CD21 marker. B, The C1qRp+ cells were analyzed for CD23 and IgM, and the T1, T2, and T3 B cell subsets were gated as shown. Numbers indicate the percentage for each subset of the total IgM+ gated cells. Representative data from one of three experiments are presented. Six mice in total were analyzed for each strain.
of Bcl-xL and GFP protein was tested by immunoblotting. The experiment served as a positive control for Bcl-xL protein; infected cells grown without IL-3 for 6 days; lanes 4
MigRI.BX-infected cells grown with IL-3; lanes 8

Comparisons made with the two-tailed Student’s t test (assuming unequal variances) gave

Several days. Duplicate samples from each cell culture were analyzed for

pendent FL5.12 cells in vitro. Duplicate FL5.12 cell cultures were infected

EcoRI and SalI identified clones with the proper bcl-xL gene orientation. B. Expression of functional Bcl-xL protein was tested in IL-3-dependent FL5.12 cells in vitro. Duplicate FL5.12 cell cultures were infected with MigRI or MigRI.BX retroviral particles, passed without IL-3 for 0, 4, or 6 days, and allowed to recover in IL-3-supplemented medium for several days. Duplicate samples from each cell culture were analyzed for

GFP expression by flow cytometry, and a mean was calculated. The data presented represent the mean ± SD for the duplicate cell cultures for each time point. Comparisons made with the two-tailed Student’s t test (assuming unequal variances) gave p < 0.05 (+) or p < 0.01 (+*). C. Expression of Bcl-xL and GFP protein was tested by immunoblotting. The experiment was done, as described above, except that cell lysates were collected and immunoblotting was performed. Equivalent amounts of cell lysate (17 μg) were loaded for each sample. Lane 1, Uninfected FL5.12 cells; lanes 2–3, MigRI.BX-infected cells grown with IL-3; lanes 4 and 5, MigRI.BX-infected cells grown without IL-3 for 0, 4, or 6 days; lanes 6 and 7, MigRI.BX-infected cells grown without IL-3 for 4 days; lane 8, BJAB cell lysate as a positive control for Bcl-xL protein; lanes 9 and 10, MigRI-infected cells grown with IL-3; lanes 11 and 12, MigRI-infected cells grown without IL-3 for 6 days; lanes 13 and 14, MigRI-infected cells grown without IL-3 for 6 days.

MigRI-infected group. This result indicated that MigRI.BX-mediated bcl-xL gene expression had enabled the infected FL5.12 cells to survive IL-3 withdrawal. Immunoblotting for GFP and Bcl-xL protein expression in FL5.12 cell lysates supported this interpretation, showing enrichment of these proteins after IL-3 withdrawal, and a good correlation between GFP and Bcl-xL protein expression (Fig. 5C).

Enforced bcl-xL gene expression restored FO B lymphocyte development

The MigRLBX vector was used to generate AW.bcl-xL mice. MigRLBX-infected A/WySnJ bone marrow stem cells were injected into lethally irradiated A/WySnJ mice, and after reconsti-

tution, the splenocytes were analyzed. The transfection efficiencies ranged from 15 to 50%, as judged by the GFP+ splenocyte percentage. When cell lysates from freshly isolated splenocytes were immunoblotted, there was a good correlation between GFP and Bcl-xL protein expression for individual AW.bcl-xL mice (data not shown). In addition, when freshly isolated splenocytes were cultured 2–4 days, the percentage of GFP+ splenocytes increased 2- to 3-fold, indicating that these GFP+ cells had greater longevity than the GFP− splenocytes (data not shown).

We next analyzed whether the provision of a survival signal had extended the transitional B cell life span. Continuous BrdU-labeling studies were done in vivo, as we described (7). A short 5-day labeling period was used to specifically detect an effect of bcl-xL gene expression on transitional B cell longevity (24). Because the MB cell life span is >80 days, the 5-day labeling period did not measure the longevity of the MB cells (40). The results of the BrdU-labeling study showed that in the AW.bcl-xL mice, the B220+ GFP− B cells incorporated ~50% less BrdU than the B220+ GFP− B cells (Fig. 6A). These results indicated that Bcl-xL expression had significantly increased the life span of the transitional GFP− B cells compared with the control GFP− B cells. The increase in longevity was not likely due to the MigRI retroviral vector, because we found no significant effect of the empty MigRI retroviral vector on peripheral B cells (data not shown).

Because the provision of a survival signal had increased transitional B cell longevity, a flow cytometric analysis was done to learn whether bcl-xL gene expression had complemented the Tnfrsf13c mutation and restored FO B cell development. A four-color flow cytometric analysis of IgM, CD21, and CD23 surface marker expression on GFP− and GFPhigh splenocytes from AW.bcl-xL mice was performed (Fig. 6B). It was noteworthy that among the GFPhigh splenocytes, the IgM− B cells made up a larger percentage of the cells than among the GFP− splenocytes. This result indicated that the bcl-xL gene expression was more advantageous to the B cells, which we showed were subject to premature apoptosis (39), than to other hematopoietic lineages that were not subject to premature apoptosis. Because the GFP+ splenocyte percentage varied significantly from mouse to mouse due to varying retroviral infection efficiencies, it was not possible to calculate and compare absolute B cell numbers. Therefore, each B cell subset was expressed as a percentage of the total GFP− IgM− or GFPhigh IgM− B cell pool (Table I). There were no significant differences between the NF and FO B cell subset proportions when the GFP− B cells were compared with the A/WySnJ B cells. The finding that these GFP− subsets were distributed exactly as in A/WySnJ mice indicated that retroviral infection of other hematopoietic lineages had not altered B cell development. In sharp contrast, the GFPhigh B cell subset proportions in the AW.bcl-xL mice were significantly different from the GFP− B cell proportions (p < 0.01), and from the A/WySnJ B cell subset proportions (p < 0.01). Instead the GFPhigh B cell subset proportions in the AW.bcl-xL mice were similar to the A/J B cell subset proportions. These data strongly support the conclusion that bcl-xL gene expression complemented the Tnfrsf13c mutation and restored FO B cell development.

This conclusion was confirmed by a four-color flow cytometric analysis of IgM, CD23, and ClqRp surface marker expression on GFP− and GFPhigh splenocytes from AW.bcl-xL mice (Fig. 6C). In these mice, the proportion of GFP− MB cells was similar to the A/WySnJ MB cell proportion, while the proportion of GFPhigh MB cells was increased to a level that was comparable to A/J mice (Table I). We next compared the GFP− and GFPhigh transitional B cell subsets. We reasoned that if bcl-xL gene expression simply allowed MB cells to accumulate, then all transitional subsets would be expected to decrease proportionately as the MB pool
The number of BrdU/H11001 F2 intercross progeny with and without the GFP transgene remained comparable between A/WySnJ and A/J mice, and A/WySnJ spleens (Table I). The T2 and T3 B cell subset proportions decreased significantly compared with the GFP high T1 B cell subset percentage. This was similar to the decreased proportion of T1 B cells in A/J spleens compared with the T1 B cell proportion in the human BCL2 gene. The AW.bcl-xL mice were created by infecting A/WySnJ stem cells with the MigR1.BX retrovirus, and transferring the infected stem cells into irradiated A/WySnJ mice, as described in Materials and Methods. After 14–16 wk of reconstitution, the splenocytes were analyzed by flow cytometry, as described in the Fig. 3 legend, except that GFP high and GFP low gates were established before IgM gating. The percentages of IgM+ cells within the GFP and GFP low gates are shown. C, The AW.bcl-xL spleenocytes were further analyzed by flow cytometry, as described in the Fig. 4 legend, except that GFP high and GFP low gates were established first. Numbers indicate the percentage for each subset of the total GFP IgM+ or GFP low IgM+ gated cells. The results shown are representative of four AW.bcl-xL mice that were produced and analyzed in two separate experiments.

Discussion
The evidence presented in this work provides important new insights regarding how BAFF-R-mediated signals regulate peripheral B cell homeostasis. We established that a retrotransposon insertion event generated a loss-of-function mutation in the bcl-xL gene of strain A/WySnJ mice. Furthermore, we found that BAFF-R function was essential at every stage of FO B cell development (and MZ B cell development) beginning with the earliest T1 B cell stage. Lastly and most importantly, we found unequivocal evidence that providing Bcl-xL-mediated survival signals to the developing A/WySnJ B cells complemented the Tnfrsf13c mutation and restored FO B cell development. Thus, this mutant BAFF-R protein apparently lacks a survival signaling motif.

The retrotransposon insertion event that generated the Tnfrsf13c mutation in strain A/WySnJ mice is a new example of an insertion that has generated genetic diversity in the mouse genome. The intracisternal type A particles (IAP) are noninfectious retrovirus-like structures that accumulate in the endoplasmic reticulum of rodent cells (42). The inserted IAP proviral genomes are present at ~1000 copies per haploid genome, although only a few appear to be transcriptionally active in any particular cell type. Inserted IAP proviruses can profoundly affect gene function through transcriptional regulation or formation of chimeric proteins (42). The chimeric proteins can have altered function, giving rise to important phenotypic changes, as was seen previously for the Hermansky-Pudlak syndrome protein in the pale ear (ep) mutant mouse strain (43), and in this study with the BAFF-R protein in the Tnfrsf13c mutant A/WySnJ mouse strain.

Others reported that BAFF-R had no function in T1 B cells, but was essential for the T1 to T2 cell developmental transition (13, 14, 44). This view has been interpreted to mean either that BAFF-R transmits a differentiation signal driving this transition, or it transmits a survival signal to the T2 B cells (19, 22). However,
these previous T1 B cell analyses did not exclude the B-1 B cells that populate the A/WySnJ spleen. The inclusion of the B-1 B cells may have led to an overestimation of the T1 B cells, and consequently, to a misinterpretation of BAFF-R function. When the B-1 B cells were rigorously excluded, a substantial negative impact of the BAFF-R mutation on the T1 B cells was apparent. It is also important to note that BAFF-R was expressed on all splenic B220+ cells (17) and that BAFF-R-stimulated activation of NF-κB was seen in T1 B cells (27). The BAFF-R expression and signaling data along with our transitional B cell data support the interpretation that BAFF-R function is essential at every stage of splenic FO B cell development.

There are important and poorly understood phenotypic and functional differences between the BAFF-R-mutant A/WySnJ and the BAFF-null mice that require further investigation to precisely define the homeostatic functions of BAFF. The BAFF-null spleens had less than 0.5 million FO B cells (13, 14), whereas the A/WySnJ spleens had 3–4 million FO B cells (5, 7). In addition, the BAFF-null mice had 80% less serum IgM than their wild-type counterparts (14), whereas the A/WySnJ mice had a serum IgM concentration that was equal to their wild-type A/J counterparts (6). Finally, the BAFF-null mice did not produce an Ab response to trinitrophenyl-Ficoll (13), whereas the A/WySnJ mice produced a robust Ab response to this T-independent Ag, equal to the A/J response (6). One possible explanation for these substantial differences is to suggest that a distinct BAFF-binding receptor such as BCMA (45), TACI (12), or a novel receptor, whose function is retained in the A/WySnJ strain, supports primary IgM responses and contributes to FO B cell development. Alternatively, the mutant BAFF-R in the A/WySnJ may not be a true null receptor and may retain some homeostatic functions.

Very recent reports have begun to document the biochemical pathways that mediate the B cell homeostatic functions of the BAFF-R. BAFF binding to the BAFF-R induces the processing of NF-κB2, which was implicated in the survival of the splenic B cells (27, 46). It was intriguing that this processing occurred through a noncanonical NF-κB activation pathway, setting BAFF-R apart from many other TNFR family members (27). Both the T1 B cells and the combined T2/MB cells showed this NF-κB2 activation, consistent with our interpretation that the BAFF-R has an essential function in promoting the survival of all FO B cells, including T1 B cells.

The severely B cell-deficient phenotype of the A/WySnJ mice is not surprising, given the disruption in BAFF-R structure that was imposed by the retrotransposon insertion. The 8 C-terminal aa residues of the BAFF-R were replaced with 21 randomly encoded residues (33), generating a form of the BAFF-R that is unresponsive to BAFF (18). The 8 truncated residues are part of a 25-aa region that is completely conserved between human and murine BAFF-R, whereas the rest of the receptor shows much less conservation (17). Intriguingly, we noted that part of this conserved BAFF-R region is highly homologous to human and murine CD40. CD40 is also a TNFR family member, and it is known to transmit survival signals to FO B lymphocytes (47). The sequence (T)-(A)-(G/A)-(P)-(E/V)-(Q) occurs in the C-terminal region of CD40 and BAFF-R. This conserved sequence includes a portion of the (P)-(G/A)-(P)-(E/V)-(Q) motif that formed the TRAF2/3 binding site of CD40 (48) and was essential for CD40-induced processing of NF-κB2 (49). It is highly significant that the A/WySnJ BAFF-R lacks the (T)-(A)-(G/A)-(P)-(E/V)-(Q) sequence and also fails to induce NF-κB2 activation (27, 46). We hypothesize that the (T)-(A)-(G/A)-(P)-(E/V)-(Q) sequence of the BAFF-R will be essential for coupling the receptor to the noncanonical NF-κB activation pathway, perhaps via a TRAF adapter protein, and ultimately to induction of a survival gene and/or repression of a proapoptotic gene.

The simplest model to explain how the BAFF-BAFF-R complex regulates B cell homeostasis is to suggest that this complex transmits a survival signal, enabling the cell to complete a differentiation step induced by an intrinsic developmental program or an extrinsic signal such as an Ag encounter. Our evidence that provision of a survival signal in vivo complemented the BAFF-R mutation and restored B cell development strongly supports this model. This model is also consistent with previous studies showing that BAFF provided a survival signal and led to up-regulation of some antiapoptotic Bcl-2 family members (9, 36, 40). At present, it is not yet clear whether the BAFF-R transmits a differentiation signal in addition to a survival signal. The A/WySnJ mutant BAFF-R retains much of its cytoplasmic domain, and thus may retain some BAFF-R functions, for example a differentiation signal. Indeed, the retention of partial function may begin to explain the substantial differences noted above between BAFF-null mice and A/WySnJ mice. The CD40 and other TNFR family members have distinct cytoplasmic domains that interact with specific adapter proteins (48). Perhaps the most C-terminal portion of the receptor is critical for B cell survival, whereas another cytoplasmic domain promotes B cell differentiation. Further studies dissecting the signaling capabilities of the BAFF-R will be needed to address these important questions.

Acknowledgments

We thank Fayre Nashold for technical assistance and mouse husbandry; Dr. Karen Clise-Dwyer for discussions on experimental design and analytical methods; the University of Wisconsin Comprehensive Cancer Center Flow lab for flow cytometry assistance; Drs. John Kearney, John Cymbler, Anthony Rolink, and Warren Pear for reagents; Drs. Mike Cancro and Ben Hsu for providing insight into the retroviral complementation system; and Drs. Shigeki Miyamoto, Demin Wang, and Matthias Wabl for providing critical comments on this manuscript.

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