(*28*), whereas A/WySnJ mice have normal T cell–independent responses, but impaired T cell–dependent responses (*25*) and TACI knockout mice are only deficient in the T-independent response (*34*).

We have identified BAFF-R, a previously unknown receptor specific for BAFF that has a role in BAFF signaling which is distinct from the two other known receptors for BAFF, BCMA, and TACI. A functional role for BCMA has not yet been defined, and even though TACI may play a role in T cell–independent responses, BAFF-R appears to be the principal receptor required for BAFF-mediated mature B cell survival and for generating an effective T cell–dependent immune response. Future studies on BAFF-R will more precisely elucidate its role in BAFF signaling.

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22. An enzyme-linked immunosorbent assay (ELISA) plate was coated with 5 μg/ml mouse anti-hIgG in carbonate buffer and then blocked (phosphate-buffered saline, 0.5% Tween-20, 4% nonfat milk). Twenty microliters of supernatant from receptor:Fc–transfected cells expressed in Optimem (Invitrogen, Carlsbad, CA) (about 1 to 10 μg/ml) was added to the plate. Purified protein was used for BAFF-R:Fc (20 μl at 2.7 μg/ml). The BAFF-R:Fc expression construct [amino acids 2 to 71 (aa 2–71)] was generated and protein produced similar to BCMA:Fc (*6*). Incubation with 20 μl of supernatant containing the Flag-tagged TNF ligands followed (*9*), and, after washing, the plates were incubated with 100 μl of M2-biotin (0.5 μg/ml) (Sigma, St. Louis, MO). Then, the plate was washed, incubated with 100-μl horseradish peroxidase (HRP)–streptavidin (1:4000; Jackson ImmunoResearch, West Grove, PA), washed, incubated with o-phenylenediamine (OPD) (Sigma) and read at 490 nm.
23. B cells were isolated from the spleens of C57BL/6 mice (8 weeks old; The Jackson Laboratory, Bar Harbor, ME) with the use of a B cell recovery column (Cedarlane Laboratories, LTD, Hornby, Ontario, Canada). Cells were incubated in 96-well plates [10^5 cells/well in 50 μl RPMI supplemented with 10% fetal bovine serum (FBS)] for 72 hours in the presence of various concentrations of human BAFF, 2 μg/ml of a goat antibody to human μ chain (Fab'2) (Jackson ImmunoResearch), and either no Ig or with control hIgG (10 μg/ml); purified human BAFF-R:Fc; or BCMA:Fc (aa 1–51) (10 μg/ml) (*9*). Cells were pulsed for 18 hours with [^{3}H]thymidine (1 μCi/well) and harvested. Incorporation was monitored by liquid scintillation counting.
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31. Genomic DNA from A/J or A/WySnJ mice was prepared (*28*). A cloned Eco RI fragment containing the murine BAFF-R gene was used as a control. Either 10 ng of the cloned fragment or 100 ng of genomic DNA were used for PCR with *Pfu* buffer (Stratagene, La Jolla, CA), 10% dimethyl sulfoxide (DMSO), 0.2 mM deoxynucleoside triphosphates (dNTPs), 150 ng of each primer, and 1.25 units *Pfu* Turbo polymerase (Stratagene) and then cycled 30 times at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. Primers are as follows: Exon 1, AAAGCGGCCGCCATG GGCGCCAGGAGACTCCG and GTCCAGTGTCCGGCGTGTGGA; Exon 2, GCAGCCTGGAGCCTGGGACAG and GGACTCCTTCTGAAG TGTCTG; Exon 3, GGAAAATGTCTTTGTACCCTC and AAAGCGG CCGCCTATTGCTCT GGGCCAGCTGT.
32. Splenocytes from 6- to 7-week-old A/J and A/WySnJ male mice were prepared by disruption of spleens between glass slides in Hanks' balanced salt solution (HBSS) plus 2% FBS that were strained through 70 μM nylon mesh and depleted of red blood cells (RBCs) by ammonium chloride lysis. After washing, cells were resuspended at 2 to 5 × 10^7 cells/ml in HBSS-FBS plus Fc block (10 μg/ml) (BD PharMingen, San Diego, CA). Cells were stained with biotinylated BAFF (200 ng/ml), anti-murine BAFF-R peptide (aa 139–154) or preimmune serum at a final dilution of 1:2000 for a period of 30 min. CyC-conjugated anti-CD3e and antigen-presenting cell (APC)–conjugated anti-B220 (PharMingen) were included in all staining reactions. After washing the cells, phycoerythrin (PE)–conjugated streptavidin (PharMingen) or PE-conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch) were added for detection. Panels show scatter-gated cells (FACScalibur, Becton Dickinson, San Jose, CA). Data from 10,000 B220-positive cells are shown.
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An Essential Role for BAFF in the Normal Development of B Cells Through a BCMA-Independent Pathway

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The B cell activating factor BAFF (BlyS/TALL-1/zTNF4) is a tumor necrosis factor (TNF)–related ligand that promotes B cell survival and binds to three receptors (BCMA, TACI, and the recently described BAFF-R). Here we report an absolute requirement for BAFF in normal B cell development. Examination of secondary lymphoid organs from BAFF-deficient mice revealed an almost complete loss of follicular and marginal zone B lymphocytes. In contrast, mice lacking BCMA had normal-appearing B lymphocyte compartments. BAFF therefore plays a crucial role in B cell development and can function through receptors other than BCMA.

B cell development is a temporally and spatially regulated process that begins in the bone marrow, where common lymphoid progenitors differentiate into pro–B cells, pre–B cells, and later, B lymphocytes (*1*, *2*). After B cell receptor expression, rearrangement, and deletion of autoreactive clones (*3*, *4*), a fraction of the cells migrate to secondary lymphoid organs. There, they may encounter antigen and undergo clonal selection and deletion in a complex series of steps (*5*, *6*). In the spleen, newly formed ($B220^+$, IgM^{hi}) cells acquire more mature phenotypes with down-regulation of IgM and up-regulation of molecules including CD21, CD23, and IgD.

A new TNF ligand thought to play a

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central role in B cell development is BAFF (*7*), also known as BlyS, TALL-1, THANK, or zTNF4 (*8–11*). BAFF specifically binds to B lymphocytes, costimulates their proliferation, and promotes the survival of splenic B cells in vitro (*12*). Overexpression studies in mice established that BAFF can cause a systemic lupus erythematosis (SLE)–like syndrome (*13*, *14*). Because circulating levels of BAFF are elevated in some lupus patients (*15*, *16*), there has been great interest in further understanding the possible roles of this ligand in human disease and B cell development.

Three TNF receptors are known to specifically bind BAFF. The B cell maturation antigen, BCMA (*17*), and a transmembrane activator and calcium-modulating cyclophilin ligand interacting protein, TACI (*18*), have been previously described. The BAFF receptor, BAFF-R, was only recently identified (*19*). Derivatives of BCMA and TACI have been implicated in reducing peripheral B cell numbers, prolonging survival in mouse SLE models (*11*, *20*), and inhibiting humoral immune responses (*21*). However, their mechanism of action has been unclear for several reasons. (i) In contrast to BAFF-R, both BCMA and TACI have also been identified as receptors for the more broadly expressed TNF ligand APRIL (*22*, *23*). (ii) Only low levels of BCMA on developing B lymphocytes have been demonstrated (*24*). (iii) TACI was originally reported to be present not only on B cells, but also on activated T lymphocytes (*18*).

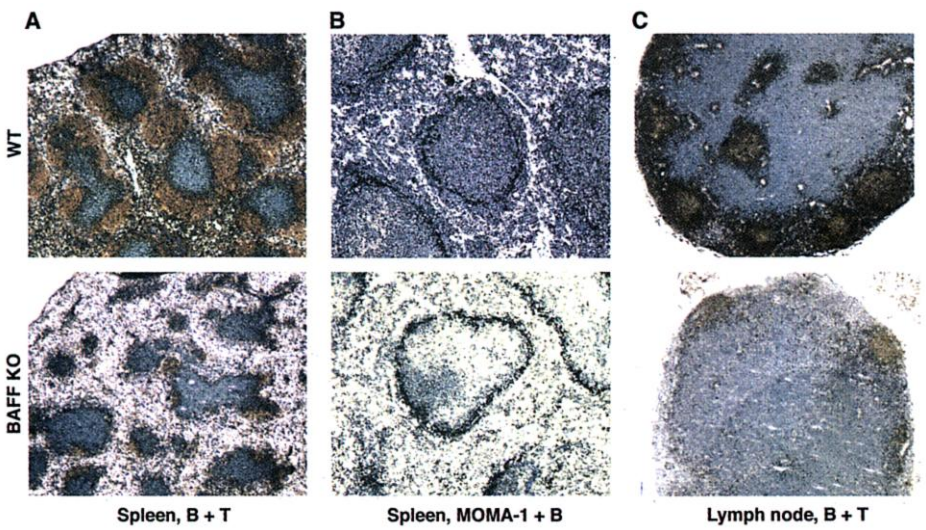

Fig. 1. Immunohistochemistry. Micrographs showing wild-type (WT) or BAFF$^{-/-}$ (BAFF KO) tissue sections, as indicated at left. Antigens detected are shown below the panels (*27*). (**A**) Spleen B cells were stained for B220 (brown); T cells were stained for CD4 and CD8 (blue). (**B**) Spleen metallophilic macrophages were stained for MOMA-1 (brown, ring-shaped structures); B cells were stained for B220 (blue). (**C**) Lymph node B cells were stained for B220 (brown); T cells were stained for CD4 and CD8 (blue).

Table 1. B and T cells from wild-type and BAFF$^{-/-}$ mice. Cell numbers are $\times 10^{-6}$. Cells from six wild-type and six BAFF$^{-/-}$ mice were counted and analyzed by FACS to enumerate the indicated populations of cells. B220^{+} B cells include the following categories: follicular = CD21^{+}, CD23^{+}; marginal zone = CD21^{+}, CD23^{lo}; newly formed = CD21^{-}, CD23^{-}; T1 = IgM^{hi}, CD21^{-}; T2 = IgM^{hi}, CD21^{+}; pro-B = CD43^{+}, IgM^{-}; pre-B = CD43^{-}, IgM^{+}; recirculating B = IgD^{+}, IgM^{lo}; immature B = IgD^{-}, IgM^{+}. *P* values were determined by Student's *t* test; $^{*}P \leq 0.05$.

Cell type	Wild type		BAFF KO		*P*
	Mean	SD	Mean	SD	
		Spleen			
B lymphoid					
B220^{+}	49.0	16.5	6.5	4.8	0.002*
Follicular	39.6	16.9	0.2	0.3	0.002*
Marginal zone	1.7	0.7	0.02	0.02	0.003*
Newly formed	4.9	1.7	5.3	3.8	0.796
T1 transitional	3.8	1.4	2.9	2.1	0.498
T2 transitional	5.9	3.6	0.1	0.1	0.011*
T lymphoid					
CD3e^{+}	28.3	11.0	18.7	7.0	0.103
CD4^{+}	17.5	7.6	11.1	5.3	0.107
CD8^{+}	9.0	3.0	6.3	1.9	0.131
CD69^{+}	3.3	1.4	1.9	0.8	0.063
		Bone marrow			
Total nucleated cells	41.3	15.2	59.6	19.4	0.121
Pro-B	1.9	0.9	1.7	0.7	0.405
Pre-B	5.0	3.1	4.8	2.6	0.447
Recirculating B	1.3	0.9	0.3	0.2	0.050*
Immature B	1.3	1.1	1.7	0.9	0.448

To better determine the importance and mechanism of BAFF function, we generated mice that were deficient either in BAFF or in BCMA (*25*). All were outwardly normal and survived to at least 6 to 8 months of age without unusual morbidity. At necropsy, all major organs including thymus, spleen, and lymph node were present, although average spleen weights of BAFF$^{-/-}$ animals were significantly reduced (*26*).

Immunohistochemical studies of secondary lymphoid tissues from BAFF$^{-/-}$ mice revealed severe losses of B220^{+} cells (Fig. 1A) (*27*). In the spleen, those that remained were in regions directly adjacent to the periarterial T cell zones, which appeared largely normal. Simultaneous detection of MOMA-1 and B220 (Fig. 1B) suggested that BAFF$^{-/-}$ mice have significantly fewer marginal zone and follicular B cells than wild-type animals; this was confirmed by fluorescence-activated cell sorting (FACS) analysis (see below). By contrast, staining for other antigen-presenting cell markers including CD11c, MAdCAM-1, and FDC-M1 demonstrated essentially normal splenic architecture outside the B cell compartment (*26*). In lymph nodes from mutant mice, the few remaining B220^{+} cells tended to localize at the subcapsular locations normally occupied by follicular B cells (Fig. 1C).

FACS analysis revealed very marked reductions in specific populations of peripheral B cells in BAFF$^{-/-}$ animals. Staining for mature follicular (CD21^{hi}, CD23^{hi}) and marginal zone (CD21^{hi}, CD23^{lo}) splenocytes showed almost complete loss of these cells (Fig. 2A). The remaining B lymphocytes mostly exhibited staining similar to that of recently described T1 transitional B cells (Fig. 2B). Although these cells (CD21^{lo}, IgM^{hi}) were normal in number, there were almost no cells of a T2 phenotype (CD21^{hi}, IgM^{hi}) thought to represent the immediate next stage of B cell development (*6*, *28*). Other hematopoietic cell lineages were not affected in BAFF$^{-/-}$ mice. Bone marrow cells were present in nearly normal numbers; only the normal population of recirculating mature B lymphocytes was absent (*26*). In addition, there were no statistically significant abnormalities in the proportions of CD4-, CD8-, and CD69-expressing cells in

thymus (*20*) or spleen (Table 1).

These findings in BAFF-deficient mice bear marked similarities to previous descriptions of the mutant A/WySnJ mouse strain (*29*). These mice were reported to exhibit an ~10-fold loss of splenic B cells (*30*) and were recently shown to be mutant in the newly identified BAFF receptor, BAFF-R (*19*). We therefore directly compared their splenocytes to BAFF$^{-/-}$ splenocytes, using FACS analysis (Fig. 2, A and B). Follicular and marginal zone B cells from homozygous A/WySnJ mice were substantially reduced as compared with either mixed wild-type (129/Sv, C57BL/6) or parental (A/J) animals of identical age, but less severely affected than in mice lacking BAFF. By contrast, cells from BCMA$^{-/-}$ mice were similar in number and in staining to wild-type controls (Fig. 2) (*26*).

Lymph node and peritoneal B cells obtained from BAFF$^{-/-}$ mice exhibited further abnormalities consistent with those observed in their spleens. Although lymph nodes were present and were readily identified, they exhibited an ~10-fold reduction in B cell numbers (*20*). Of note, peritoneal lavage of these animals returned normal-appearing B1 B cells (*31*) but included markedly fewer cells of the B2 lineage (B220^{+}, CD23^{hi}) (Fig. 2C). Again similar to the A/WySnJ strain, mice lacking BAFF exhibited no abnormalities in the ratios of B1a and B1b cells recovered.

To establish the function of the remaining B cells in BAFF$^{-/-}$ mice, we determined their serum antibody levels at baseline and in response to both T-dependent and T-independent antigens (*32*). The results from assays of resting wild-type, heterozygous, and knockout mouse sera are shown in Fig. 3A. Homozygous BAFF knockout mice exhibited a profound reduction (10-fold) in total serum immunoglobulin and in each subclass with the exception of immunoglobulin A (IgA), which was only moderately reduced in knockout animals. Also of note was the finding that heterozygous (BAFF$^{+/-}$) mice exhibited a reproducible, roughly twofold baseline reduction in IgG subclasses and IgM present in their sera (*26*).

Reduced serum antibody responses to intraperitoneal immunization with a T-dependent antigen, NP-KLH (*33*), were also apparent in BAFF$^{-/-}$ mice. Thus, BAFF knockout mice failed to produce a measurable specific antibody response at early time points, although they were able to mount a very small detectable response at later times (Fig. 3B) (*20*). Similar results were obtained after immunization of additional mice with the T-independent antigen TNP-Ficoll (Fig. 3C).

To better understand BAFF-dependent signaling, we also generated mice deficient in BCMA, one of three proposed receptors for BAFF. As for BAFF$^{-/-}$ mice, the bone marrow of BCMA$^{-/-}$ animals contains normal proportions of B cells and their readily identifiable precursors. However, they exhibited neither significant loss of splenic B cells (Fig. 2A) (*26*), nor loss of the T2 transitional B cells that occurred in mice lacking BAFF and in A/WySnJ mice.

Targeted mutation of the BAFF locus established that the encoded protein is necessary to support normal B cell development and function in vivo. Analysis of BAFF$^{-/-}$ spleens by both FACS and immunohistochemistry revealed loss of follicular and marginal zone B cells. Our results are consistent with the suggestion that T2 transitional B cells are a primary target for BAFF action (*12*). Alternatively, T1 (B220^{+}, IgM^{hi}, CD21^{lo}) cells may require BAFF for progres-

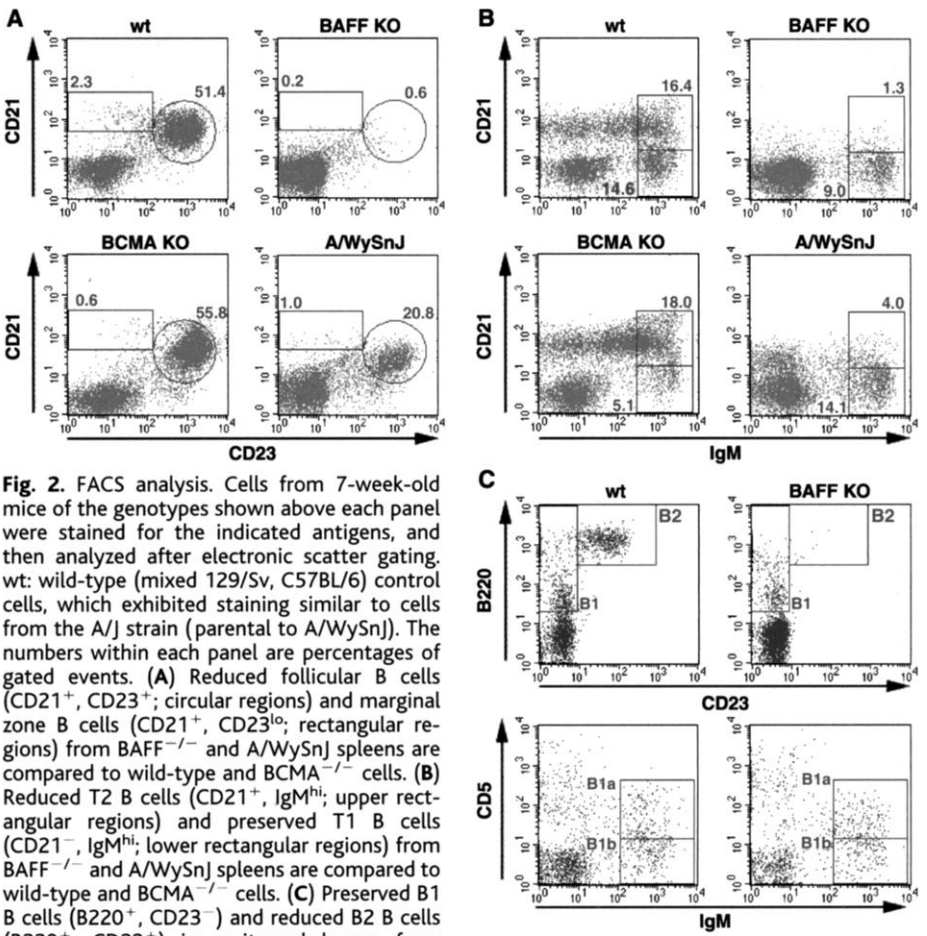

Fig. 2. FACS analysis. Cells from 7-week-old mice of the genotypes shown above each panel were stained for the indicated antigens, and then analyzed after electronic scatter gating. wt: wild-type (mixed 129/Sv, C57BL/6) control cells, which exhibited staining similar to cells from the A/J strain (parental to A/WySnJ). The numbers within each panel are percentages of gated events. (**A**) Reduced follicular B cells (CD21^{+}, CD23^{+}; circular regions) and marginal zone B cells (CD21^{+}, CD23^{lo}; rectangular regions) from BAFF$^{-/-}$ and A/WySnJ spleens are compared to wild-type and BCMA$^{-/-}$ cells. (**B**) Reduced T2 B cells (CD21^{+}, IgM^{hi}; upper rectangular regions) and preserved T1 B cells (CD21^{-}, IgM^{hi}; lower rectangular regions) from BAFF$^{-/-}$ and A/WySnJ spleens are compared to wild-type and BCMA$^{-/-}$ cells. (**C**) Preserved B1 B cells (B220^{+}, CD23^{-}) and reduced B2 B cells (B220^{+}, CD23^{+}) in peritoneal lavage from BAFF$^{-/-}$ mice are compared to wild-type cells. After gating on B1 cells, CD5 and IgM staining reveals B1a and B1b cells, as indicated. Results represent stainings from eight mice of each genotype.

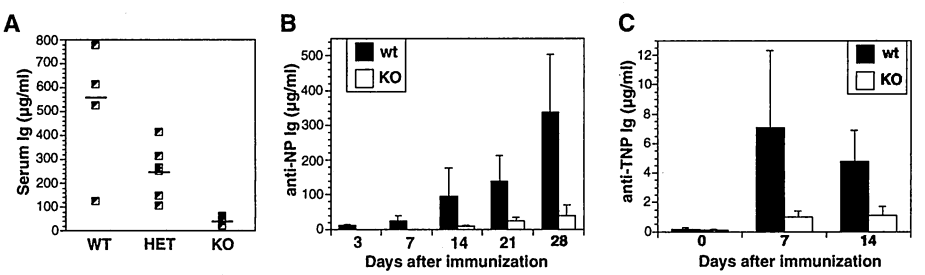

Fig. 3. Serum immunoglobulin (Ig) levels. (**A**) Resting total Ig levels from BAFF$^{-/-}$ (KO), BAFF$^{+/-}$ (HET), and wild-type (WT) mice were determined by enzyme-linked immunosorbent assay (*32*) and are shown for individual mice. Horizontal bars indicate the average for each genotype. (**B**) The NP-specific antibody responses of BAFF$^{-/-}$ and wild-type animals to a T-dependent antigen, NP-KLH, is shown. Bars represent the average of four mice for each genotype, and the error bars indicate +1 SD. (**C**) TNP-specific antibody responses to a T-independent antigen, TNP-Ficoll, are shown as described for (B).

sion to the T2 stage, and then beyond.

A notable feature of BAFF deficiency is its minimal effect on the bone marrow and on peritoneal populations of B1 cells. The bone marrow's nearly normal percentage of immature ($B220^+$, $CD43^{hi}$) cells suggests that marrow B lymphopoiesis proceeds in a BAFF-independent manner. Similarly, the peritoneal population of B1 B cells is far less sensitive to BAFF deficiency than are peritoneal B2 lymphocytes, consistent with both major views of B1 cell development (*26*, *34*, *35*).

These results illuminate several important aspects of the mechanism by which BAFF functions in vivo. The phenotype of BAFF-deficient mice is similar to, but more severe than, that of a previously identified mutant mouse strain, A/WySnJ (Fig. 2) (*29*). A/WySnJ mice express a mutated form of BAFF-R, a recently cloned member of the TNF receptor superfamily of proteins that binds only to BAFF (*19*). The phenotypes of $BAFF^{-/-}$ and A/WySnJ mice, combined with the binding specificity of BAFF-R, strongly suggest that BAFF primarily acts to promote survival and maturation of B2 cells through the newly identified receptor. This hypothesis is consistent with the more subtle B cell phenotypes that we and others have observed in animals lacking BCMA and TACI (*36*, *37*). The persistence of some splenic B cells in A/WySnJ mice might result from strain-specific effects, redundant signaling through TACI and BCMA, or mutated BAFF-R that reaches the surface of the cells but cannot properly signal.

It has been suggested that both ligand (BAFF and APRIL) and receptor (BCMA and TACI) redundancy may support B cell development (*21*). Our data demonstrate that there is no significant functional BAFF redundancy among TNF ligands in supporting B cell survival: APRIL cannot compensate for BAFF by signaling through TACI, BCMA, or any other receptor in BAFF-deficient mice.

Numerous details of the mechanism of BAFF signaling remain to be elucidated (*26*). In addition to a central role in regulating splenic B cell development shown here, BAFF could have further importance to events including secondary immune responses, memory, and mature B cell survival that occur after the T2 developmental stage. Also, crosses of BAFF-deficient mice with animals prone to other B cell disorders (e.g., malignancies) may soon validate clinical applications involving the BAFF pathway beyond those already envisioned in autoimmune diseases.

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25. For the BAFF and BCMA knockouts, mouse 129SvJ genomic DNA contained in separate bacterial artificial chromosomes (Genome Systems) was subcloned into high–copy number plasmids, then targeted by bacterial recombinational cloning to insert a tailless human CD2 reporter at each initiating ATG. The wild-type and knockout sequences for each construct are available on request. For the BAFF knockout, the final construct was deleted in 732 nucleotides of genomic DNA encoding the first 134 amino acids of BAFF; the deletion ends in the second intron downstream of the ATG. The BCMA knockout construct was deleted in the initiating ATG and 1.38 kb of downstream sequence corresponding to nucleotides 145 to 383 of the mouse cDNA (GenBank AF061505). This deletion removes amino acids 1 to 87, including the putative transmembrane domain. Further details are provided in Web fig. 1 (*26*).
26. Supplemental information is available on *Science* Online at www.sciencemag.org/cgi/content/full/1061964/DC1.
27. For (A) and (C), frozen tissue sections were incubated for 45 to 60 min with a mixture of fluorescein isothiocyanate (FITC)–tagged antibodies (each at 1:50 dilution) (Pharmingen) including anti–CD4-FITC (RM4-5), anti–CD8a-FITC (53-6.7), anti–CD8b.2-FITC (53-5.8), and anti–CD45R/B220-biotin (RA3-6B2). After washing, horse radish peroxidase (HRP)–coupled streptavidin (Jackson Immunoresearch, 016-030-084) and anti–FITC-AP (AP, alkaline phosphatase) (Roche Molecular Biochemicals, catalog number 1 426 338) secondary reagents (1:50) were added for 30 to 60 min. After washing, HRP (brown) color reactions were developed with diaminobenzidine substrate (DAKO, K3465); after further washing, AP (blue) color reactions were developed with BCIP-NBT (bromochloroindolyl phosphate–nitro blue tetrazolium) substrate (Vector, SK5400). For (B), the primary antibody mixture included neat anti–MOMA-1 (Serotec, MCA 947) and anti-CD45R/B220, as described above. The secondary reagents were peroxidase-coupled mouse F(ab')2 anti-rat IgG (Jackson, 212-036-082) and avidin-AP complex (DAKO K0376), used and developed as described above.
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