BAFF Mediates Survival of Peripheral Immature B Lymphocytes

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Abstract

B cell maturation is a very selective process that requires finely tuned differentiation and survival signals. B cell activation factor from the TNF family (BAFF) is a TNF family member that binds to B cells and potentiates B cell receptor (BCR)-mediated proliferation. A role for BAFF in B cell survival was suggested by the observation of reduced peripheral B cell numbers in mice treated with reagents blocking BAFF, and high Bcl-2 levels detected in B cells from BAFF transgenic (Tg) mice. We tested in vitro the survival effect of BAFF on lymphocytes derived from primary and secondary lymphoid organs. BAFF induced survival of a subset of splenic immature B cells, referred to as transitional type 2 (T2) B cells. BAFF treatment allowed T2 B cells to survive and differentiate into mature B cells in response to signals through the BCR. The T2 and the marginal zone (MZ) B cell compartments were particularly enlarged in BAFF Tg mice. Immature transitional B cells are targets for negative selection, a feature thought to promote self-tolerance. These findings support a model in which excessive BAFF-mediated survival of peripheral immature B cells contributes to the emergence and maturation of autoreactive B cells, skewed towards the MZ compartment. This work provides new clues on mechanisms regulating B cell maturation and tolerance.

Key words: B cell maturation • autoimmunity • transitional B lymphocyte • spleen • antigen receptor

Introduction

Over the past 30 years, research on B lymphocyte development has concentrated on the early stages, in the bone marrow (BM),[1] where lymphoid progenitor cells give rise to fully committed B lymphocytes after a process of sequential recombination and assembly of Ig gene components (1). The BM is also an important site for induction of immune tolerance, where self-reactive B lymphocytes are eliminated if they encounter membrane-bound self-antigen during their development (2–4).

In contrast, little is known about the signals driving the maturation of peripheral immature IgM^+ B cells into mature, recirculating B cells. Several studies have shown that immature B cells leaving the BM, in contrast to mature B cells, do not proliferate in response to stimulation of their B cell receptor (BCR), but rather undergo death by apoptosis (5). This feature is thought to allow negative selection of emerging autoreactive B cells after encounter with self-antigen in the periphery (6, 7).

Immature B cells found in the periphery are referred to as immature transitional B cells to distinguish them from their BM counterparts (6). The phenotype of transitional B cells varies from study to study but is generally thought to feature high levels of surface IgM (IgM^{hi}) and CD24 (heat stable antigen [HSA]; references 5, 6, 8–10). The lack of a clear definition of immature transitional B cells may have

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[1]*Abbreviations used in this paper:* ANOVA, analysis of variance; BAFF, B cell activation factor from the TNF family; BCMA, B cell maturation antigen; BCR, B cell receptor; BM, bone marrow; FSC, forward light scatter; HSA, heat stable antigen; MLN, mesenteric LN; MZ, marginal zone; PI, propidium iodine; PLN, peripheral LN; SSC, side light scatter; T1, transitional immature B cell type 1; T2, transitional immature B cell type 2; TACI, transmembrane activator and calcium-modulating and cyclophilin ligand interactor; Tg, transgenic.

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arisen from possible heterogeneity in this population. This idea is supported by a recent study demonstrating the existence of two transitional B cell stages in the spleen. Transitional type 1 (T1) B cells are IgM^{hi} and negative for IgD (IgD^-), L-selectin (L-$selectin^-$), CD23 ($CD23^-$), and CD21 low ($CD21^{lo}$), and transitional type 2 (T2) B cells are IgM^{hi}, IgD^{hi}, $CD21^{hi}$, $CD23^+$, and L-$selectin^+$ (11). Immature B cells leaving the BM are thought to differentiate into T1 B cells before reaching the spleen. B cell maturation is an active process that appears to take place in the spleen, as T1 B cells do not express L-selectin, preventing them from homing to LNs, and T2 B cells are only found in the spleen (11). Adoptive transfer experiments in recombination activating gene (RAG)-$2^{-/-}$ mice using purified T1 or T2 B cell subsets have shown that T1 are the precursors for T2 and mature B cells and T2 B cells are the direct precursors of mature B cells (11). It is still unclear whether T1 B cells can differentiate directly into mature B cells. The maturation of B cells through the T1 and T2 stages depends on signals through the BCR (11–13).

B cell activation factor from the TNF family (BAFF; also called TNF and apoptosis ligand–related leukocyte-expressed ligand 1 [TALL-1], TNF homologue that activates apoptosis, nuclear factor κB, and c-Jun NH_2-terminal kinase [THANK], B lymphocyte stimulator [BlyS], and zTNF4) is a member of the TNF family expressed by T cells, monocytes/macrophages, and dendritic cells (14–18). BAFF can be found both as a membrane-bound factor and as a soluble agent (14, 17). BAFF specifically binds to B cells and promotes their proliferation in the presence of anti-μ (14–17). Recently, two known orphan TNF receptor–like molecules, B cell maturation antigen (BCMA [19, 20]) and transmembrane activator and calcium-modulating and cyclophilin ligand (CAML) interactor (TACI [21]), were identified as specific receptors for BAFF (18, 22, 23). Expression of BCMA is B cell specific (19, 20, 24, 25), whereas that of TACI can be found on B cells as well as on subsets of activated T cells (21). Injection of BAFF in normal mice leads to the disruption of the splenic B and T cell structures and results in elevated levels of IgM in the serum (17). BAFF transgenic (Tg) mice have an elevated number of B lymphocytes in the periphery, secrete various autoantibodies, and develop an SLE-like condition leading to severe glomerulonephritis (18, 26, 27). The number of marginal zone (MZ) B cells is increased in BAFF Tg mice (26). In contrast, B cell development in the BM is not affected by the BAFF transgene (18, 26, 27). Treatment of normal mice with blocking soluble BCMA-Ig fusion protein led to a marked reduction in B cell numbers in the periphery (22). Treatment of mice with TACI-Ig inhibits T cell–dependent and –independent immune responses (23), abolishes germinal center formation (28), and was shown to prevent proteinuria and prolong the life of mice in a model of SLE (18).

Current information clearly demonstrates that BAFF is a very important factor controlling several aspects of B cell biology with the potential to break immune tolerance when overexpressed. Here we show that, in vitro, BAFF preferentially supports the survival of a subset of immature (T2) and some MZ B cells found solely in the spleen of normal mice. The T2 and MZ B cell subsets are enlarged in BAFF Tg mice, and some survive 72 h in vitro without exogenous stimuli. Our results suggest that excess survival signals provided to peripheral immature B cells in BAFF Tg mice reduce sensitivity of autoreactive B cells to BCR-mediated negative selection, resulting in autoimmunity. Our work underscores the importance of BAFF for B cell maturation, immune tolerance, and maintenance of B cell homeostasis.

Materials and Methods

Mice. Full-length murine BAFF was expressed in Tg mice using the liver-specific α1 antitrypsin promoter with the apolipoprotein (Apo) E enhancer as described previously (26). C57BL/6 and C3H/HeJ mice were purchased from Animal Resource Center. BAFF Tg mice are maintained as heterozygotes for the transgene by backcrossing onto C57BL/6 mice. BAFF Tg mice are screened for the presence of the transgene, both by PCR and Southern blot analysis using genomic DNA isolated from 2–3-mm long tail snips. We used mice from two separate lines of BAFF Tg mice issued after 8–10 backcrossings to C57BL/6. In experiments including BAFF Tg mice, negative littermates were used as control. Animals between 6 and 12 mo old were used, except for experiments including histological procedures for which younger 3–4-mo-old animals were selected. Animals were housed under conventional barrier protection and handled in accordance with the Animal Experimentation and Ethic Committee, which complies with the Australian code of practice for the care and use of animals for scientific purposes.

Reagents. Flag-tagged soluble human BAFF (amino acids 83–285) was expressed by *Escherichia coli* and purified as described previously (14, 22). Human recombinant BAFF was shown to efficiently stimulate mouse B cells (17, 22). We found that the optimal concentration for BAFF in our assays was 2 μg/ml (data not shown). BAFF was also inactivated by boiling for 30–45 min and used as control. Polymyxin B and LPS from *E. coli* were obtained from Sigma-Aldrich and used at a final concentration of 5 and 10 μg/ml, respectively. Polyclonal rabbit anti-BAFF serum was obtained from rabbits immunized with human flag-tagged BAFF (amino acids 83–285) as described previously (14). The serum collected from bleeds made before immunization of these rabbits with BAFF was used as control.

Preparation and Culture of Lymphocytes. Mice were killed by cervical dislocation and lymphoid organs were collected under sterile conditions. Spleen, thymus, and LNs were dissociated by grinding between frosted glass slides (Menzel-Glaser). Cells from the BM were collected after flushing mouse femurs with RPMI. PBLs were isolated by density gradient centrifugation of EDTA-treated mouse blood over Ficoll-Paque™ PLUS (Amersham Pharmacia Biotech). Cells were filtered though a 70-μm nylon cell strainer (Falcon; Becton Dickinson), and erythrocytes were removed by osmotic lysis with red blood cell lysis solution (8.34 g/liter ammonium chloride, 0.84 g/liter sodium bicarbonate, and 1 mM EDTA, pH 8.0). Cultures were conducted in glutamine-containing RPMI 1640 supplemented with 10% FCS and 100 U/ml penicillin/streptomycin (Life Technologies). Lymphocytes (3×10^6/ml) were routinely stimulated for 72 h in culture with 2 μg/ml recombinant soluble human flag-tagged BAFF. Polyclonal rabbit anti-BAFF and rabbit control sera were used at the final

concentration of 5%. Specific goat anti–mouse μ chain antibody was purchased from Southern Biotechnology Associates, Inc. and was used in culture at 10 μg/ml.

Flow Cytometry and Cell Sorting. Freshly prepared or cultured lymphocytes were resuspended in FACS® buffer (1% BSA, 0.05% sodium azide in PBS) at a concentration of 5×10^6 cells/ml. Three- to four-color fluorescence surface staining was done using various combinations of FITC-, PE-, Cy5-, and Cychrome™-labeled antibodies. Fluorescent-labeled anti–mouse antibodies anti-CD4 (L3T4), anti-CD8α (Ly-2), anti-CD45R/B220 (RA3-6B2), anti-CD1 (1B1), anti-IgD (11-26c.2a), anti-IgM R6-60.2), anti-CD69 (H1.2F3), anti-CD62L (L-selectin), anti-CD5, anti-Fas, anti-CD23 (IgE Fc receptor), anti-CD24 (HSA, 30F1), and anti-CD21 were supplied by BD PharMingen. Cy5-conjugated anti-IgM antibody was purchased from Jackson ImmunoResearch Laboratories. FITC-labeled antibodies were used diluted 1:100, whereas other fluorochrome-labeled antibodies were used at a 1:200 final dilution. Annexin V-FITC Apoptosis Detection Kit 1 (BD PharMingen) was used according to the manufacturer's instructions. For flow cytometry we acquired 30,000 events per sample.

For BAFF binding experiments, sorted T1 and T2 B cells were incubated with 2 μg/ml of flag-tagged human BAFF for 1 h on ice in FACS® buffer. Cells were washed twice and stained with a biotinylated anti-Flag antibody M2 (Sigma-Aldrich) diluted 1:500. Cells were washed twice and the signal was detected using PE-labeled streptavidin (Jackson ImmunoResearch Laboratories).

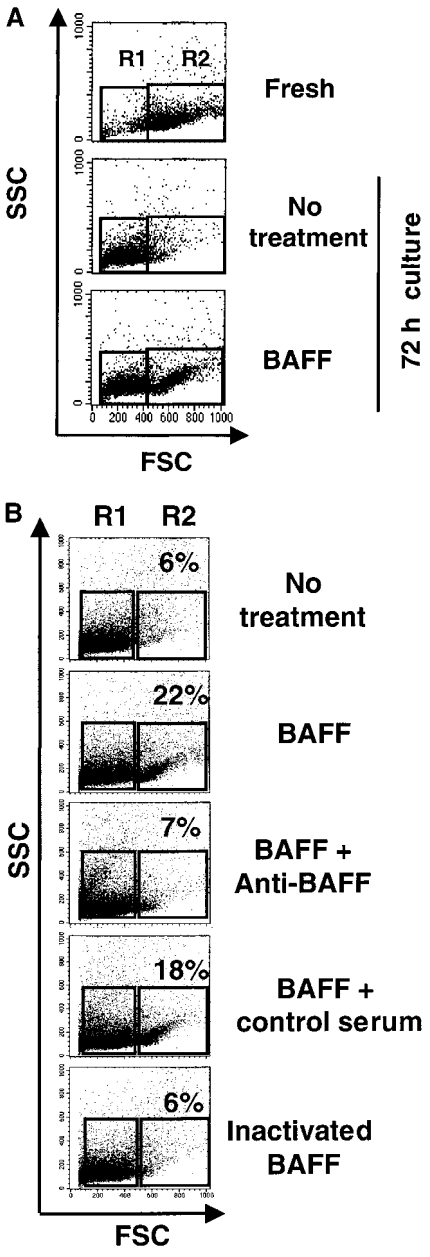


Figure 1. Survival of BAFF-treated splenocytes in vitro. C57BL/6-derived splenocytes were cultured for 72 h with or without BAFF and analyzed by flow cytometry on FSC/SSC plots. Gates R1 (low FSC, dead cells) and R2 (high FSC, live cells) are indicated in each plot. (A) R1 and R2 populations in fresh and cultured splenocytes 72 h with or without BAFF. (B) The effect of rabbit anti-BAFF, control rabbit serum, or 2 μg/ml inactivated (boiled) BAFF on the size of the R2 population as indicated. The slight inhibition observed with control rabbit serum is due to the higher serum concentration in the culture and was similar to that of four other control rabbit sera tested in this assay (data not shown). These plots are representative of at least five experiments.

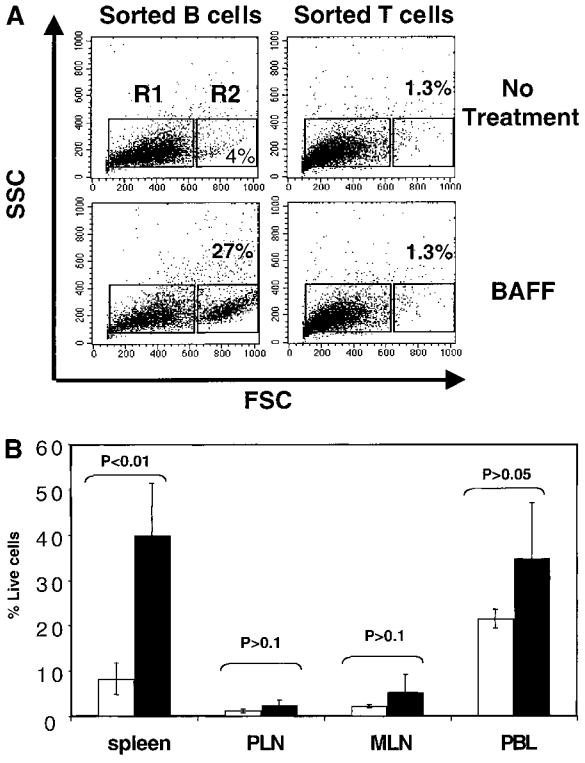


Figure 2. BAFF specifically promotes the survival of splenic B cells. (A) Splenocytes from C57BL/6 mice were stained with antibodies to B220 and CD3. $B220^+$ B cells and $CD3^+$ T cells were purified by cell sorting and incubated for 72 h with BAFF (bottom) or without (top). The R1 (dead cells) and R2 (live cells) gates for sorted B cells (left) and sorted T cells (right) are drawn. (B) Lymphocytes were prepared from spleen, PLNs (inguinal and brachial LNs), MLNs, and PBLs, and incubated with BAFF (black bars) or without (white bars) for 72 h. Staining with annexin V/PI was analyzed by flow cytometry and percentages of annexin V/PI double-negative R2-gated cells (live cells) are shown for each lymphocyte preparation. The plots in A are a representation of three separate experiments and the graph in B represents the mean and standard deviation of six separate lymphocyte preparations. *P* values were obtained using analysis of variance (ANOVA).

Data were collected on a FACSCalibur™ flow cytometer and analyzed using CELLQuest™ software (Becton Dickinson).

For cell sorting of splenic B and T cells, 5–10 × 10^8 freshly isolated splenocytes were stained with PE-labeled anti-B220 and FITC-labeled anti-CD3 in PBS plus 10% FCS. Staining with biotin-labeled anti-HSA (revealed using Cychrome™-labeled streptavidin) and FITC-labeled anti-CD21 was used to sort T1 and T2 B cells instead of anti-IgM or anti-IgD to prevent potential activation signals through the BCR. The Mo-Flo cell sorter (Cytomation) at the Microbiology and Immunology Department of the University of New South Wales (New South Wales, Australia) was used to sort gated $B220^+$ B cells, $CD4^+$ or $CD8^+$ T cells, $HSA^{hi}CD21^{lo/-}$ T1 B cells, and $HSA^{hi}CD21^{hi}$ T2 B cells. Reanalysis of sorted B or T cell populations demonstrated >98% purity. The purity for sorted populations of T1 and T2 B cells was 90 and 95%, respectively.

Immunohistochemistry. Frozen sections of spleen were subjected to immunohistochemical analysis as described previously (29). Biotin-labeled goat anti–mouse IgM antibodies and horseradish peroxidase–streptavidin were purchased from Jackson ImmunoResearch Laboratories.

Results

BAFF Specifically Induces Survival of Splenic B Lymphocytes In Vitro. Previous studies (17, 18, 22, 26, 27) have suggested a potential role for BAFF in B cell survival. We

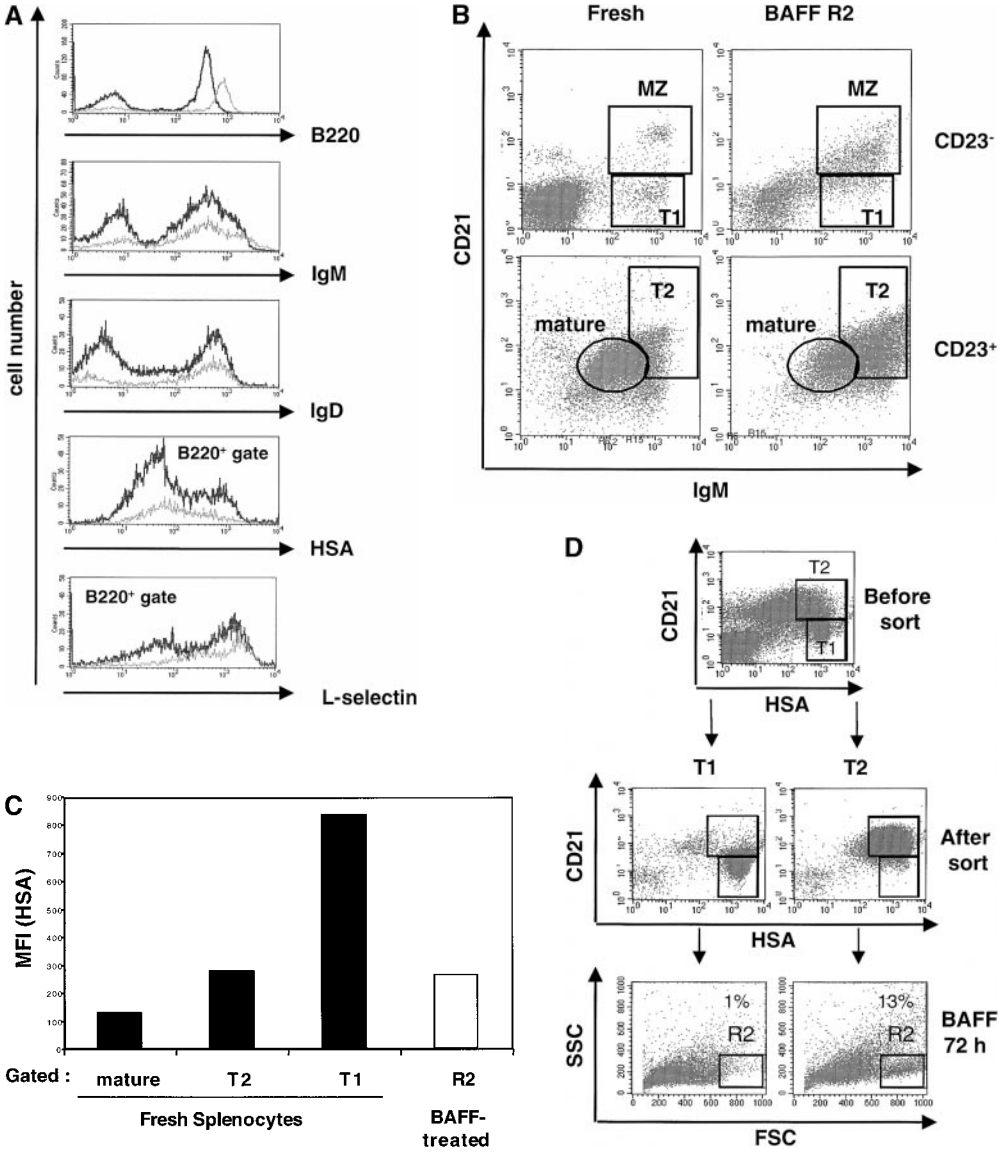


Figure 3. BAFF induces survival of T2 B cells. (A) C57BL/6-derived splenocytes were incubated for 72 h with BAFF, stained with antibodies to B220, IgM, IgD, HSA, and L-selectin, and analyzed by flow cytometry. Freshly prepared C57BL/6 splenocytes were stained in parallel. For HSA and L-selectin expression, $B220^+$ gated B cells are shown. Histograms for fresh splenocytes (black line) and R2-gated BAFF-treated splenocytes (gray line) are overlayed for comparison. (B) Three-color flow cytometric analysis of fresh and R2-gated BAFF-stimulated splenocytes from A. Cells were stained with antibodies to IgM, CD21, and CD23 and were separated into $CD23^-$ and $CD23^+$ cells. $CD23^-$ cells include $CD21^{lo}IgM^{hi}$ T1 cells and $CD21^{hi}IgM^{hi}$ MZ B cells. $CD23^+$ B cells include $CD21^{int}IgM^{dull}$ mature B cells and $CD21^{hi}IgM^{hi}$ T2 B cells. T1, T2, mature, and MZ B cell populations are indicated. (C) Four-color flow cytometric analysis of fresh and R2-gated BAFF-stimulated splenocytes from A. Cells were stained with antibodies to IgM, CD21, CD23, and HSA. Freshly prepared splenocytes were gated on the T1, T2, and mature B cell populations as described in B and analyzed for the expression of HSA. The mean fluorescence intensity (MFI) for HSA on gated T1, T2, and mature B cells (black bars) is compared with that of R2-gated BAFF-stimulated splenocytes (white bar). Experiments in A, B, and C are representative of at least 10 animals and cultures analyzed. (D) Freshly prepared splenocytes were stained with antibodies to CD21 and HSA. $CD21^{lo}HSA^{hi}$ T1 and $CD21^{hi}HSA^{hi}$ T2 B cells were identified (Before sort, top), sorted (After sort, middle), and cultured for 72 h with BAFF (bottom). Cells were then analyzed by flow cytometry for the presence of surviving cells in the R2 gate on FSC/SSC plots as indicated (bottom). R1 and R2 populations are shown. Percentages of cells in R2 are indicated. This figure is representative of six different cell sorting experiments analyzed.

tested the survival effect of BAFF in vitro by incubating mouse splenocytes for 72 h with or without BAFF, and made a first assessment by flow cytometry using forward light scatter (FSC)/side light scatter (SSC) plots (Fig. 1 A). Freshly prepared splenocytes displayed higher FSC levels and are shown in the R2 population (Fig. 1 A, top). After 72 h in culture, untreated splenocytes died and display lower FSC levels, shown as the R1 population (Fig. 1 A, middle). Interestingly, many cells were detected in the live R2 gate in 72-h cultures of BAFF-stimulated splenocytes (Fig. 1 A, bottom). The FSC level of cells in R2 was similar to that of fresh splenocytes analyzed at the same time using the same instrument settings (Fig. 1 A, top and bottom). The R2 cell population was markedly reduced when BAFF was blocked using a blocking polyclonal rabbit anti-serum, but not when a control rabbit serum or BAFF inactivated by boiling was used (Fig. 1 B). We showed previously that blocking BAFF with BCMA-Ig, but not a control Ig fusion protein, was also able to reproduce this result (22). This phenomenon was not blocked by polymyxin B at concentrations which in parallel inhibited the effect of 10 μg/ml *E. coli*–derived LPS on splenocytes (data not shown). Moreover, the R2 population was also seen in BAFF-stimulated LPS-hyporesponsive splenocytes from C3H/HeJ mice (data not shown). These control experiments allowed us to rule out the possibility of endotoxin contamination from the *E. coli*–derived BAFF preparation as the factor responsible for the presence of the R2 population. BAFF alone at the concentrations used in our assays does not promote B cell proliferation (14, 15). In conclusion, treatment of splenocytes for 72 h with BAFF specifically leads to the presence of cells in the R2 gate, with FSC levels suggesting that these cells might be surviving. We confirmed the viability of BAFF-stimulated cells present in the R2 population after 72 h in culture by showing that these cells were negative for annexin V binding and propidium iodine (PI) staining by flow cytometry (data not shown).

Cultured splenocytes were also double stained with anti-B220 and anti-CD4, or CD8 antibodies to detect B and T lymphocytes, respectively, by flow cytometry. The staining showed that 80% of cells in R2 are B cells. Surviving B cells represent 20–30% of the total BAFF-stimulated splenocyte culture, whereas only 2–3% of B cells survived in 72-h cultures of nonstimulated splenocytes. Some T cells (6.1 ± 1.8 vs. 1.2 ± 0.5% in controls), mainly $CD4^+$ T cells, survived in this assay; however, this effect might result from some secondary support from surviving B cells. Evidence for that lies in the fact that T cells, highly purified by cell sorting, do not survive after 72-h stimulation with BAFF (Fig. 2 A). In contrast, 27% of purified B cells survive upon BAFF stimulation in the same assay (Fig. 2 A). Viability of the R2 population was always confirmed in parallel using the annexin V/PI staining for each experiment (data not shown). In conclusion, BAFF specifically induces the survival of B lymphocytes independently of the presence of T cells in vitro.

To test the potential survival effect of BAFF on B cells from other lymphoid organs, we purified lymphocytes from mouse blood (PBLs), peripheral LN (PLNs), mesenteric LNs (MLNs), and spleen, and incubated these cells with or without BAFF for 72 h. Significant BAFF-induced cell survival, as assessed by flow cytometric analysis of the annexin V/PI staining, was observed for cultured splenocytes (Fig. 2 B). Little cell survival was detected with BAFF-treated lymphocytes from LNs compared with untreated controls (Fig. 2 B). Unstimulated PBLs survived better in culture compared with lymphocytes from other sources, and the effect of BAFF on survival of these cells was variable but overall not highly significant (Fig. 2 B). No BAFF-induced survival was seen in mouse BM, thymus, or PBLs from human blood (data not shown). This result indicates that BAFF induces the survival of predominantly spleen-resident B cell subpopulations in vitro.

BAFF Preferentially Induces the Survival of Splenic Immature T2 B Cells. The spleen contains various subpopulations of B cells such as immature transitional B cells, which are divided into the T1 and T2 subgroups (11). The mature B cell subset is predominant, expressing low levels of IgM (IgM^{dull}), high levels of IgD (IgD^{hi}), intermediate levels of CD21 ($CD21^{int}$), and low to intermediate levels of HSA ($HSA^{lo/int}$; reference 1). Finally, MZ B cells, localized in the MZ, a structure delineating the red pulp from the white pulp, like T2 B cells are IgM^{hi} and $CD21^{hi}$ but do not express either CD23 or IgD (30).

To define which of these B cell subpopulations survived after stimulation with BAFF, BAFF-treated splenocytes were stained with anti-IgM, anti-IgD, anti-B220, anti-HSA, anti-CD23, anti-CD21, and anti–L-selectin antibod-

Table I. *Proportion of T1, T2, Mature, and MZ B Cells in Freshly Prepared or Cultured BAFF-treated Surviving Splenocytes*

	Fresh splenocytes (percentage of live gate)	BAFF-treated splenocytes (percentage of living pool)	*P* values
Mature	36 ± 1.5	20 ± 0.1*	<0.05
MZ	10 ± 1.3	2.2 ± 0.6	<0.01
T1	3.2 ± 0.4	0.7 ± 0.1	<0.01
T2	3.5 ± 0.3	53 ± 1.4	<0.01

Splenocytes were isolated from C57BL/6 mice and cultured with BAFF for 72 h. These cells as well as freshly prepared splenocytes from C57BL/6 mice were stained in parallel with a cocktail of anti-IgM, anti-IgD, and anti-CD21 antibodies and analyzed by flow cytometry. Various B cell subsets were gated according to their respective phenotype as in the legend to Fig. 3 B. The FSC versus SSC profile was obtained for each gated B cell subset and the percentage of surviving cells in the R2 gate is indicated for BAFF-treated splenocytes. For fresh control splenocytes, percentage within the live gate is indicated.

*These cells are falling into the mature B cell gate based on their $IgM^{duller}IgD^+CD21^{int}$ phenotype; however, they express on average higher levels of HSA and IgM compared with control mature B cells and may not be considered strictly as mature (see Fig. 3, A and B, and data not shown). Values are given as mean ± SD and are representative of at least 10 splenocyte cultures analyzed. *P* values were obtained using ANOVA.

ies. By flow cytometry, cells were gated on the R2 surviving population (using FSC/SSC plots) and analyzed for expression of IgM, IgD, B220, HSA, and L-selectin in comparison with normal fresh splenic B cells, which were analyzed at the same time using the same instrument settings (Fig. 3 A). The surviving R2 B cell population contained IgM^{hi} and IgD^{+} cells (Fig. 3 A). These cells expressed very high levels of B220, suggesting that they were naive B cells (Fig. 3 A). R2 surviving B cells also expressed high levels of L-selectin, indicating that they were not T1 B cells (Fig. 3 A). Staining of control fresh splenocytes with HSA gave rise to two separate populations (Fig. 3 A), $HSA^{lo/int}$ and HSA^{hi} cells, corresponding to mature and immature B cells, respectively. Interestingly, the expression of HSA on R2 surviving B cells was higher than that of mature B cells from fresh splenocytes, a sign of immaturity indicating that surviving B cells might be immature transitional B cells (Fig. 3 A).

To test this hypothesis, BAFF-treated and fresh splenocytes were stained with anti-IgM, anti-CD21, and anti-CD23 antibodies, and T1, T2, mature, and MZ B cell subpopulations were analyzed as described previously (11). BAFF-stimulated splenocytes were gated on the R2 surviving population (Fig. 3 B). Both fresh and R2 B cells were gated on the $CD23^{+}$ and $CD23^{-}$ subpopulations to distinguish T2/mature B cells from T1/MZ B cells, respectively. The staining showed that a large proportion of the surviving cells were T2 B cells. Almost no T1 B cells were present after 72-h culture with BAFF (Fig. 3 B). Some survival was observed in the MZ and mature B cell compartment (Fig. 3 B and Table I); however, cells falling into the mature B cell gate also expressed higher levels of HSA and IgM compared with control mature B cells (Fig. 3 A and see Fig. 4 B, and Table I). Interestingly, the pattern of IgM/CD21/CD23 expression of cells in R2 was identical to that seen with splenocytes from CBA/N and $CD45^{-/-}$ mice, in which B cell maturation is arrested at the T2 B cell stage (11). Therefore, some or all of the mature-like B cells may in fact be T2 cells expressing slightly lower levels of IgM. The results indicate that T2 B cells account for most of the surviving cells in R2 when compared with fresh splenocytes (Fig. 3 B and Table I). The same results were

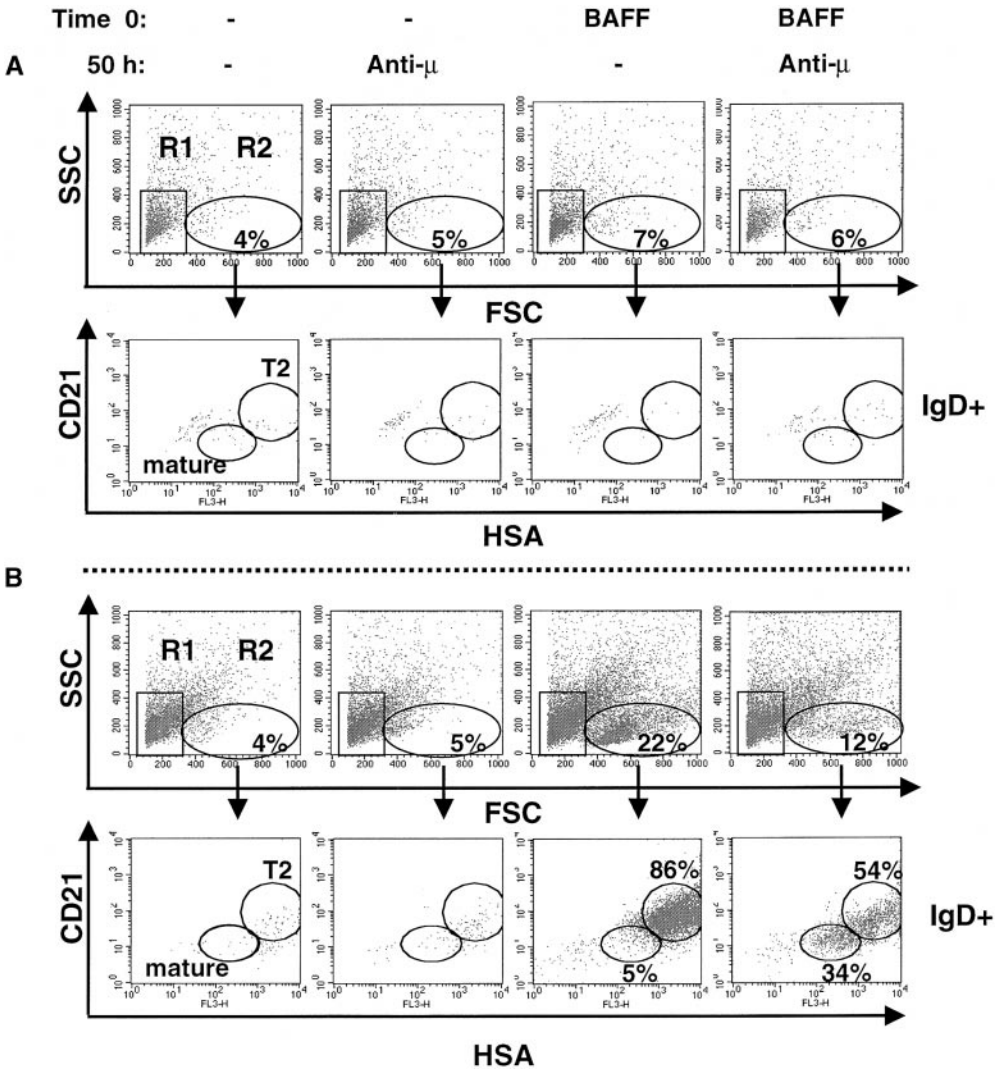


Figure 4. BAFF and anti-μ antibodies promote the maturation of T2 B cells in vitro. (A) $CD21^{int}HSA^{lo/int}$ mature and (B) $CD21^{hi}HSA^{hi}$ T2 B cells from C57BL/6-derived splenocytes were sorted and incubated with or without BAFF at time 0 as indicated. After 50 h, anti-μ antibodies were added to the cultures, which were carried out for an additional 22 h. Cells were stained with antibodies to HSA, CD21, and IgD. Anti-IgM antibody could not be used for staining due to interferences with anti-μ antibodies used for stimulation. Mature B cells were defined as $HSA^{int/lo}CD21^{int}IgD^{+}$; T2 cells as $HSA^{hi}CD21^{hi}IgD^{+}$; T1 cells as $HSA^{hi}CD21^{lo}IgD^{-}$; and MZ B cells as $HSA^{lo}CD21^{hi}IgD^{-}$. The top panel for each B cell subset (A and B) shows FSC/SSC plots indicating the R1 and R2 regions; the bottom panel shows analysis of HSA versus CD21 on cells from the R2 surviving population subgated on the IgD^{+} subpopulation. Boxes for T2 and mature B cells are drawn. This experiment is representative of three separate cell sorting experiments.

obtained when the IgD^+ and IgD^- subpopulations were gated instead of the $CD23^+$ and $CD23^-$ B cells (data not shown). Moreover, the level of HSA expression on R2 surviving B cells is similar to that of gated T2 B cells from fresh splenocytes (Fig. 3 C).

T1 and T2 B cells were purified from normal splenocytes by cell sorting and incubated with BAFF for 72 h. Only the T2, but not the T1, B cell subset survived (Fig. 3 D) and kept their original T2 B cell phenotype (data not shown). We analyzed by flow cytometry the level of flag-tagged BAFF binding on the surface of sorted T1 and T2 B cells. We observed a more intense binding of BAFF to T1 B cells compared with that to T2 B cells, yet T1 B cells do not survive in vitro after BAFF treatment (Fig. 3 D). These results indicate that BAFF, in vitro, supports survival of T2 and some MZ B cells but not of T1 or mature B cells

BAFF and Anti-μ Antibodies Promote Differentiation of T2 B Cells into Mature B Cells In Vitro. To investigate whether BAFF, together with signals through the BCR, may be required for maturation of T2 B cells, we designed an in vitro maturation assay. We purified by cell sorting $HSA^{hi}CD21^{hi}$ T2 and $HSA^{lo/int}CD21^{int}$ mature B cells and incubated them for 72 h with BAFF. Due to technical limitations, ~2% of mature B cells inevitably contaminate preparations of sorted T2 cells. Anti-μ antibodies can promote proliferation of mature B cells in the presence of BAFF and can interfere with analysis of our maturation assay (14–17). To overcome this problem, we added anti-μ antibodies 50 h after T2 and mature B cell cultures were started, when most mature B cells had already died. Cells were stained with antibodies to HSA, CD21, and IgD to delineate populations of mature and T2 B cells. BAFF-treated mature splenic B cells did not survive and the addition of anti-μ after 50 h of culture did not rescue these cells and did not promote their proliferation in the presence of BAFF (Fig. 4 A). This control was important, as it indicates that the inevitable low level of contaminating mature B cells found in the sorted T2 B cells will not interfere with our maturation assay using this particular experimental format. Untreated sorted T2 B cells died after 72 h in culture (Fig. 4 B). No cell survival was detected in cultures of T2 cells treated with anti-μ, in contrast to treatment with BAFF, which allowed T2 cells to survive (Fig. 4 B). Interestingly, in cultures of sorted T2 cells incubated for 72 h with BAFF and treated with anti-μ after 50 h in culture, we detected the presence of $HSA^{int/lo}$, $CD21^{int}$, and IgD^+ cells corresponding to the phenotype of mature B cells (Fig. 4 B). However, fewer cells were detected in the surviving R2 population (Fig. 4 B, top) compared with BAFF treatment alone. This possibly indicates that many T2 B cells died after anti-μ plus BAFF stimulation and only a few of them were able to respond to the differentiating signals promoted by these reagents. No differentiation into MZ B cells was detected in these assays and T1 B cells did not survive in any of the culture conditions described here (data not shown). These results indicate that, in vitro, BAFF can induce B cell maturation when signals are triggered through the BCR of some T2 B cells.

Elevated Numbers of T2 and MZ B Cells in the Spleen of BAFF Tg Mice. As BAFF preferentially induces the survival of T2 and some MZ B cells from normal splenocytes in vitro, we analyzed whether these populations were affected in Tg mice overexpressing BAFF. Freshly prepared control and BAFF Tg mice–derived splenocytes were stained with anti-IgM, anti-CD21, and anti-CD23 antibodies as described above, and subpopulations of mature, T1, T2, and MZ B cells were analyzed by flow cytometry. Absolute cell numbers for each B cell subset were higher in spleens from BAFF Tg mice than from control littermates, and reflected the splenomegaly observed in these animals (26; Table II). However, comparison of the proportion for each B cell subsets revealed a dominant expansion of the T2 and MZ B cells in the spleen of BAFF Tg mice compared with that of control mice (Fig. 5 A and Table II). Expansion of the MZ was also evident through histological analysis of spleen sections stained with anti-IgM (Fig. 5 B). In contrast, the proportion of typical mature B cells decreased (Table II). The proportion of T1 B cells in BAFF Tg mice remains essentially similar to that of control splenocytes (Fig. 5 A and Table II). These results were reproduced in another laboratory (Woodcock, S.A., Biogen Incorporated, personal communication). Interestingly, the level of CD21 expression on T1 B cells from BAFF Tg mice is higher and that of IgM lower compared with levels on T1 B cells from control spleen. Although not conclusive, this observation suggests faster kinetics of B cell maturation in BAFF Tg mice (Fig. 5 A).

$CD1^{hi}$ B cells have been shown to be the main source of autoantibodies in a murine model of SLE (31). We ana-

Table II. *Proportion of T1, T2, Mature, and MZ B Cells in the Spleen of Control Versus BAFF Tg Mice*

	T1	T2	Mature	MZ
Percentage from gated cells				
Control	13 ± 1.8	23 ± 7.6	67 ± 9.2	7.8 ± 1.75
BAFF Tg	20 ± 7	57 ± 13	29 ± 11.6	17 ± 2.8
P values	>0.05	<0.01	<0.01	<0.01
Absolute numbers ($\times 10^6$)				
Control	2.1	5	17	1.6
BAFF Tg	24	75	55	54
P values	<0.01	<0.01	<0.05	<0.01

Splenocytes isolated from control littermates and BAFF Tg mice were stained in parallel with a cocktail of anti-IgM, anti-IgD, and anti-CD21 antibodies and analyzed by flow cytometry. Various B cell subsets were gated according to their respective phenotype as in the legend to Fig. 3 B. Percentage from gated $CD23^+$ cells is shown for T2 and mature B cells and from gated $CD23^-$ cells for T1 and MZ B cells. Corresponding absolute numbers per spleen are also shown. Results are representative of at least 10 animals analyzed per group. *P* values were obtained using ANOVA.

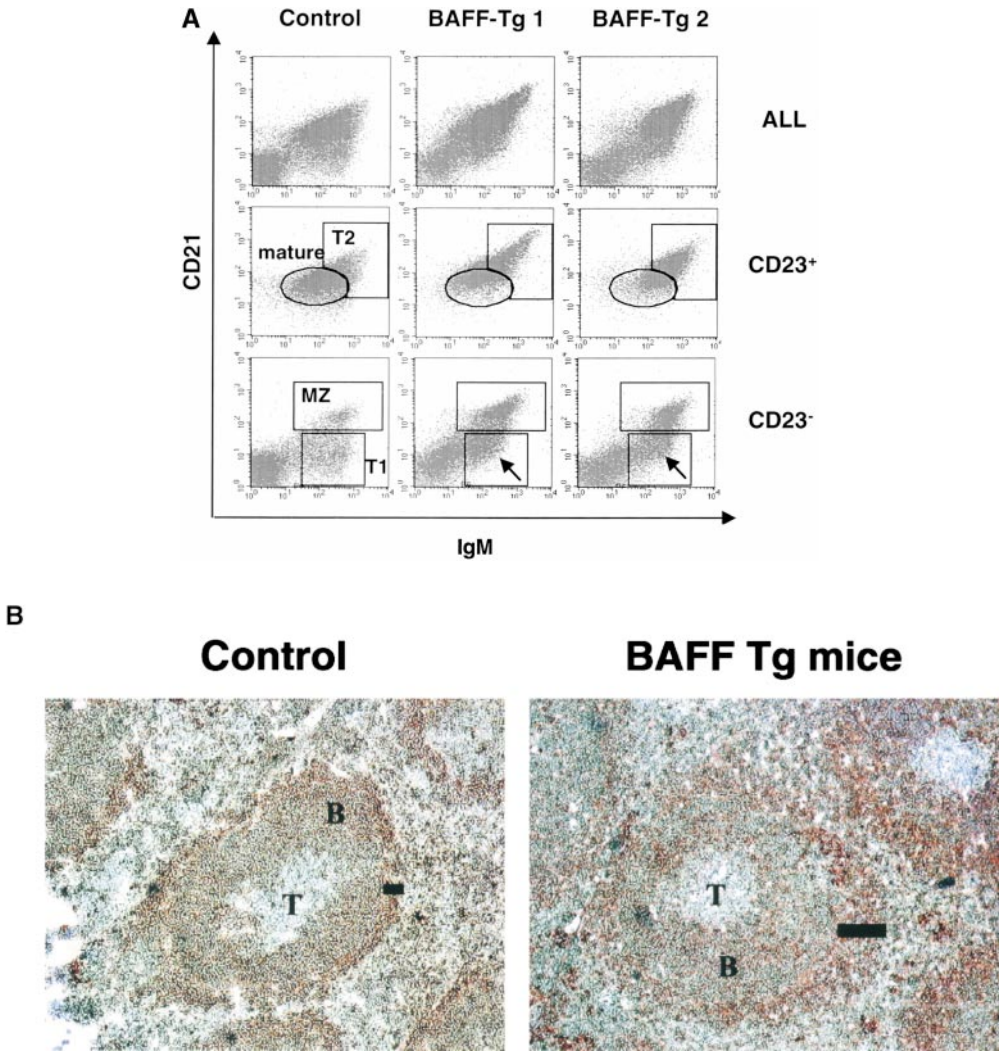
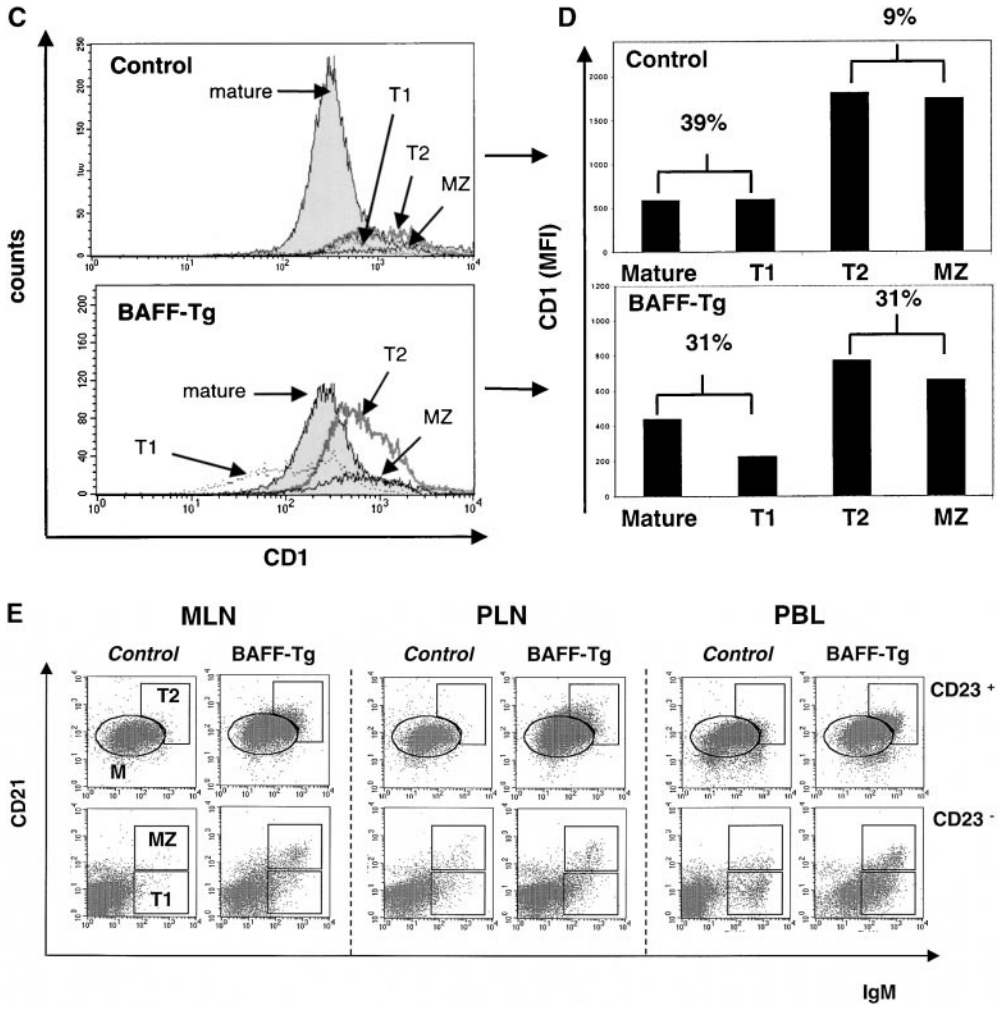


Figure 5. Expansion of the T2 and MZ B cell compartment in BAFF Tg mice. (A) Splenocytes freshly isolated from control littermates (Control) and BAFF Tg mice were stained with antibodies to IgM, CD21, and CD23, and were separated into $CD23^-$ and $CD23^+$ cells. T1, T2, mature, and MZ B cell regions were drawn as shown in Fig. 3 B. The top three histograms show all cells. Note the difference of phenotype of the T1 B cells in BAFF Tg mice (shown with arrows), which consistently show higher CD21 levels and slightly lower IgM levels compared with control T1 splenocytes. These plots are representative of 14 control littermates and 11 BAFF Tg mice analyzed. (B) Frozen tissue sections of spleens from a control littermate (left) and a BAFF Tg mouse (right) were stained with biotin-labeled anti-mouse IgM and revealed using horseradish peroxidase–labeled streptavidin (brown staining). Periarteriolar lymphoid sheath (PALS) or T cell zone (T) and B cell follicle (B) areas are indicated. The width of the MZ, showing a stronger IgM expression,

lyzed each splenic B cell subset for the presence of $CD1^{hi}$ B cells in control and BAFF Tg mice. Levels of CD1 expression were generally slightly lower on B cells from BAFF Tg mice compared with control splenocytes (Fig. 5). Interestingly, in both control and BAFF Tg mice, T2 and MZ B cells express high levels of CD1 (Fig. 5 C). However, the $CD1^{hi}$ B cell compartment accounts for >30% of all splenocytes in BAFF Tg mice versus only 9% in control mice (Fig. 5 D).

We also analyzed B cells in PLNs, MLNs, and blood from BAFF Tg mice. Few MZ-like cells could be detected in the PBLs and PLNs of control littermates (Fig. 5 E). Surprisingly, we found increased numbers of B cells displaying a T2 and MZ phenotype in these lymphocyte preparations (Fig. 5 E). Immaturity of the T2-like cells found in these preparations was confirmed by their expression of HSA, which was higher than that of gated mature B cells (data not shown). Expression levels of L-selectin on freshly prepared B cells from BAFF Tg and control mice were similar (data not shown) and do not account for aberrant homing of T2 and MZ B cells to the LNs. In conclusion, overexpression of BAFF in vivo leads to augmented numbers of all B cells in the spleen with the preferential expansion of the $CD1^{hi}$ T2 and MZ B cell compartments but also expansion of T2 and MZ-like B cells in the blood and LNs.

BAFF Tg Mice–derived Splenocytes Survive for 72 h in Normal Medium in the Absence of Exogenous Factors. Splenocytes from control littermates were incubated in medium supplemented or not with BAFF. Splenocytes from BAFF Tg mice were cultured in nonsupplemented medium. As



is indicated with a black bar. These pictures are representative of at least six animals analyzed in each group. (C) Splenocytes freshly isolated from control littermates and BAFF Tg mice were stained with antibodies to IgM, CD1, CD21, and CD23, and were separated into $CD23^-$ and $CD23^+$ cells. T1, T2, mature, and MZ B cell regions were gated as shown in A and expression of CD1 for each B cells subset was overlayed on histogram plots. (D) A corresponding graph for each histogram plot in C shows the mean fluorescence intensity (MFI) of CD1 expression on mature, T1, T2, and MZ B cells. These results are representative of three animals analyzed per group. (E) Lymphocytes isolated as in the legend to Fig. 2 B from PLNs, MLNs, and PBLs of control littermates and BAFF Tg mice were stained and analyzed by flow cytometry for the presence of T1, T2, mature, and MZ B cells as described above in A. Boxes indicating each B cell subset have been drawn. These plots are representative of at least six animals analyzed per group.

shown previously, survival in control splenocyte cultures is only apparent (R2 population) if BAFF is added to the culture (Fig. 6 A). In contrast, cultures of BAFF Tg–derived splenocytes in nonsupplemented medium gave rise after 72 h to a surviving population of cells in the R2 gate (Fig. 6 A). Surviving cells were mainly B cells with the same phenotype as surviving B cells after 72-h BAFF treatment of C57BL/6-derived splenic cells (Fig. 3 A), displaying IgM^{hi}, IgD^{hi}, $B220^{hi}$, and HSA^{hi} levels (Fig. 6 B), and therefore were mainly T2 B cells. These surviving cultured cells also displayed higher levels of L-selectin compared with fresh splenocytes from BAFF Tg mice (data not shown). T2 B cells from BAFF Tg mice, isolated by cell sorting, also survived for 72 h in vitro, indicating that the survival status of these cells is intrinsic rather than promoted by BAFF produced by splenic T cells in mixed cultures (data not shown). These results suggest that T2 B cells were "programmed/sensitized" by BAFF in vivo leading to extended, stimulation-independent survival of these cells ex vivo.

Discussion

The mechanisms responsible for the differentiation of immature B cells emerging from the BM into mature B cells found in the periphery are poorly understood. It is, for instance, still unclear why only 10% of newly formed IgM^+ B cells reach the spleen, and why only a very small proportion of these achieve maturation (32). Many reports indicate that selection of specific transitional B cells takes place

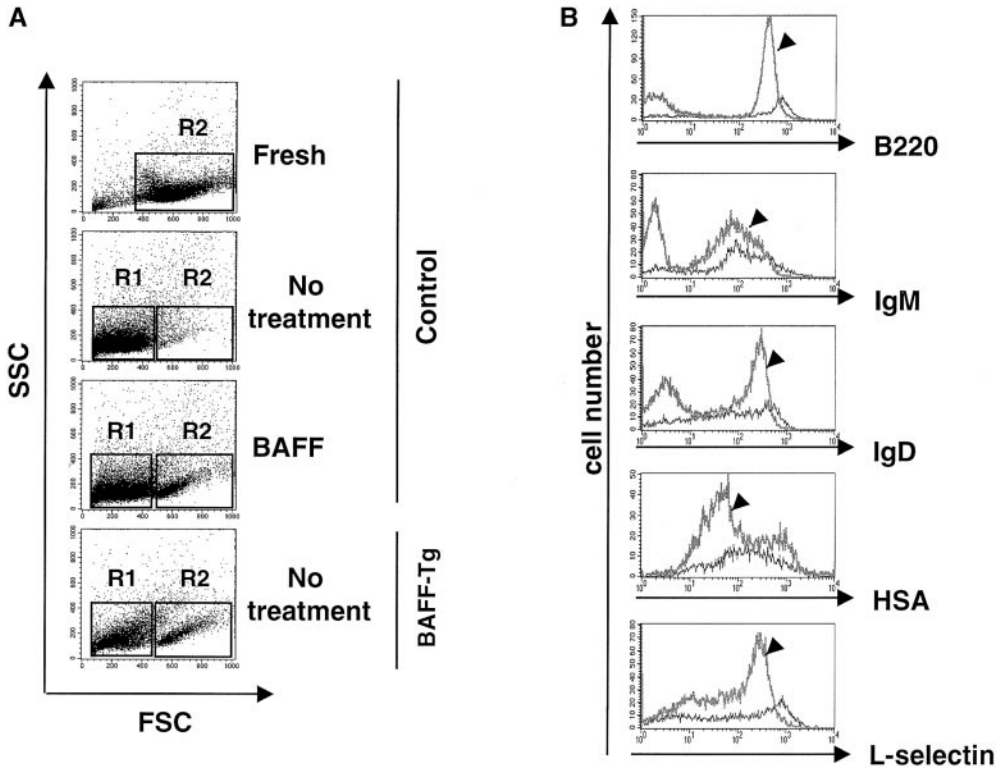


Figure 6. Immature B cells from splenocytes isolated from BAFF Tg mice survive in vitro in the absence of exogenous stimuli. Splenocytes isolated from control littermates were cultured for 72 h with or without BAFF as indicated. Splenocytes from BAFF Tg mice were plated in parallel in normal nonsupplemented medium. The R1 and R2 populations were analyzed by flow cytometry on FSC/SSC plots. The R1 and R2 gates are drawn on the plots. As in the legend to Fig. 3 A, expression of B220, IgM, IgD, HSA and L-selectin was analyzed on control freshly prepared splenocytes and BAFF Tg mice–derived splenocytes cultured for 72 h in normal medium. Histograms for fresh control splenocytes (gray line indicated with an arrowhead) and R2-gated BAFF Tg mice–cultured splenocytes (black line) are overlayed for comparison.

during B cell maturation in the periphery, and signaling through the BCR is a critical event in this process (11, 12, 33). Paradoxically, it is also clear that most immature transitional B cells die after stimulation of their BCR, a mechanism thought to eliminate autoreactive B cells (5, 6, 8, 9). The exact nature of the positive signal driving maturation of B cells, although unknown, is either highly specific and/or requires concomitant signals apart from those through the BCR to allow transitional B cells to survive and differentiate into mature B cells.

We tested in vitro whether BAFF could be this extra factor needed to push immature B cells towards survival and differentiation. In our in vitro assays, we have been able to demonstrate that BAFF promotes the survival of immature T2 B cells and the differentiation of some of them into mature B cells when anti-μ antibodies were added to trigger signals through the BCR. Therefore, in vitro, BAFF synergizes with signals through the BCR to promote maturation of T2 B cells and as such is a critical new element for our understanding of this process.

The general expansion of the B cell compartment in the spleen of BAFF Tg mice, which is characterized by increased B cell numbers in all subsets, suggested that BAFF might act on all peripheral B cells. The discrepancy of this result with our in vitro results may be explained by environmental/tissue-specific signals acting in association with BAFF in vivo which the in vitro system could not mimic. However, the vastly unequal expansion of these subsets, favoring strongly the T2 and MZ B cell compartments, indicated that overexpression of BAFF directly or indirectly modified the dynamics of the entire B cell maturation and differentiation pathway in BAFF Tg mice. These results tend to indicate that BAFF might be a general survival factor for all B cells in vivo, with the specific potential to alter the process of B cell maturation.

Experiments in which BCMA-Ig was used to block BAFF in normal mice led to a marked and general reduction in B cell numbers in the periphery (22). Similarly, previous studies have shown that shutting off the expression of the BCR on the surface of B cells led rapidly to a reduced number of mature B cells in the periphery (34). The exact nature of the BCR-mediated survival signal in mature B cells is unknown. It is therefore possible that, in vivo, constant signals from BAFF and through the BCR are needed to keep the mature B cells alive. The dynamics of B cell homeostasis are very complex, and other studies have suggested that immature B cells may also compete with recirculating B cells for survival signals (35). We believe that normal recirculating B cells are probably not self-renewing and can only be replaced through the differentiation of immature B cells recruited in the periphery. Therefore, inhibition of BAFF, like the suppression of BCR signaling, could also affect B cell maturation and prevent the renewal of the mature B cell pool. 11-d treatment with BCMA-Ig markedly (although still incompletely) reduced B cell numbers in the periphery (22). A longer treatment with BCMA-Ig, reflecting the life span of recirculating B cells, might allow most mature B cells to naturally disappear from the periphery without being replaced. Supporting this model, BAFF does not promote survival of naive mature B cells, in vitro, despite expression of both BAFF receptors on these cells (18, 22, 23). BAFF might only be an impor-

tant cofactor for proliferation once mature B cells have been activated (14–18). Nevertheless, we cannot formally exclude the possibility that, in vivo, BAFF directly supports survival of mature B cells. Adoptive transfer of B cell subsets in mice deficient for BAFF is the next strategy to obtain a final confirmation on whether BAFF is a general survival factor for all B cells or a specific survival and maturation factor for peripheral immature B cells. IL-4 and CD40 ligand are also factors involved in B cell survival; however, gene targeting experiments have shown that both factors are not required for B cell maturation (36, 37). TACI and BCMA are also the receptors for another member of the TNF family, a proliferation-inducing ligand (APRIL), and it is at this point unclear whether or not this factor may also play a role in B cell survival (38).

Other intriguing features of BAFF Tg mice are the dramatic expansion of the MZ B cell compartment, its possible relation with the parallel expansion of the T2 B cells compartment, and the autoimmune phenotype displayed in these mice. The MZ B cell population is an obscure subset, which contains memory as well as virgin B cells (39, 40). Previous studies have shown that both CBA/N and $CD45^{-/-}$ mice have their B cell development blocked at the T2 B cell stage, yet these mice have a perfectly normal population of MZ B cells (11, 41, 42). This observation led to the hypothesis that some T2 B cells, under specific conditions, may directly differentiate into MZ B cells without going through a mature B cell stage. It is therefore conceivable that in BAFF Tg mice, B cell maturation is aberrantly skewed toward the MZ compartment. Recently, studies using various Ig heavy chain Tg mice have shown that, depending on their BCR composition, some newly formed mature B cells could be positively selected into the MZ compartment (40). Moreover, some B cells selected to migrate to the MZ are suspected to be potentially autoreactive (43). In addition, recent work showed that $CD1^{hi}$ B cells in the spleen of NZB × NZW mice, a model of SLE, are the major source of autoreactive B cells (31). We showed that in normal mice, T2 and MZ B cells express high levels of CD1 compared with mature and T1 B cells, confirming previous reports (44). We also showed that the population of $CD1^{hi}$ B cells is dramatically enlarged in BAFF Tg mice. Our findings led us to a model described in Fig. 7. BAFF promotes the survival of T2 B cells, which in normal conditions will lead to the maturation of highly selected cells into the mature follicular B cell compartment. In contrast, in BAFF Tg mice, overexpression of BAFF may trigger excessive survival signals in T2 B cells, including autoreactive cells, which may fail to respond to censoring death signals. The autoreactive nature of their receptors might also force their massive assignment to the MZ compartment and subsequent expansion of this compartment, as observed in BAFF Tg mice. These B cells may become activated by autoantigens at this site, contribute to the formation of numerous germinal centers observed in these mice (26), and differentiate into memory B cells, some of which may also localize in the MZ (Fig. 7). BAFF may also be important for the survival of germinal center B cells (28), and we do not exclude the possibility that autoreactive B cells may also emerge at this stage, due to aberrant survival of self-reactive B cells created after errors during affinity maturation.

Recent work described the selective absence of MZ B cells in PyK-2–deficient mice and a possible defect in MZ-specific chemotaxis (45). It is also possible that BAFF stimulates the production of an MZ-specific chemokine or a chemokine receptor on B cells driving most of these cells into the MZ of BAFF Tg mice. It is unclear whether the presence of T2 B cells in LNs of BAFF Tg mice also reflects an aberrant homing process or possible BAFF-enhanced B cell maturation in LNs. L-selectin expression was normal on freshly isolated splenocytes from BAFF Tg mice and cannot be evoked to explain this result. Previous studies indicated that B cell maturation takes place in the spleen (6, 11), yet immature B cells have also been detected in LNs (46). B cell maturation outside of the spleen has not been investigated, but the relatively well-sustained numbers of B lymphocytes in splenectomized individuals raises the question of potential alternative sites for B cell maturation.

BAFF is a pivotal survival factor during B cell maturation, a process which is as yet poorly understood. Our re-

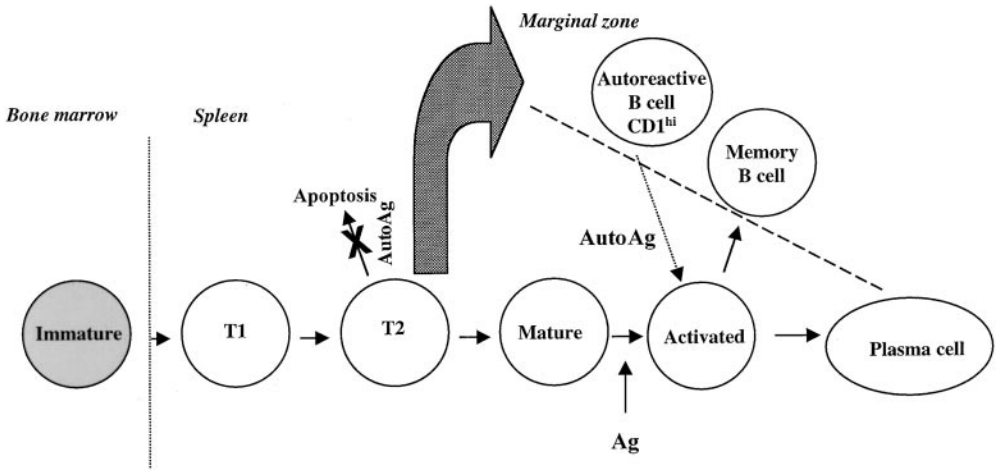


Figure 7. Proposed mechanism for one origin of autoimmune B cells in the spleen of BAFF Tg mice. BAFF induces excessive survival of all B cells in the spleen with a preferential role on immature T2 B cells. Autoreactive T2 B cells fail to respond to censoring death signals and massively escape to differentiate into $CD1^{hi}$ autoreactive MZ B cells. In contact with autoantigens (AutoAg), autoreactive MZ B cells are activated and become plasma cells or undergo affinity maturation in a germinal center to further differentiate into memory B cells, some of which will relocate into the MZ.

sults suggest that excessive BAFF-induced survival might induce the breakdown of immune tolerance at a critical stage of peripheral B cell maturation. Therefore, the highly selective recruitment of immature B cells into the mature B cell pool might be the result of active mechanisms of self-tolerance, possibly controlled by BAFF.

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