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Mutation of the BAFF furin cleavage site impairs B-cell homeostasis and antibody responses

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B-cell-activating factor of the TNF family (BAFF)/BLyS contributes to B-cell homeostasis and function in the periphery. BAFF is expressed as a membrane-bound protein or released by proteolytic cleavage, but the functional importance of this processing event is poorly understood. Mice expressing BAFF with a mutated furin consensus cleavage site, i.e. furin-mutant BAFF (fmBAFF), were not different from BAFF-deficient mice with regard to their B-cell populations and responses to immunization. It is however noteworthy that an alternative processing event releases some soluble BAFF in fmBAFF mice. Mild overexpression (~5-fold) of fmBAFF alone generated intermediate levels of B cells without improving humoral responses to immunization. Processed BAFF was however important for B-cell homeostasis, as peripheral B-cell populations and antibody responses were readily restored by administration of soluble BAFF trimers in BAFF-deficient mice. However, the rescue of CD23 expression in B cells of BAFF-deficient mice required both soluble BAFF trimers and fmBAFF, or a polymeric form of soluble BAFF (BAFF 60-mer). These results point to a predominant role of processed BAFF for B-cell homeostasis and function, and indicate possible accessory roles for membrane-bound BAFF.

Keywords: Antibodies · B-cell development · Cytokines

Supporting Information available online

Introduction

B-cell-activating factor of the TNF family (BAFF, also known as BLyS) and a proliferation-inducing ligand (APRIL) are related members of the TNF family. Both bind to transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor (TACI) and B-cell maturation antigen (BCMA). In addition, BAFF binds to BAFF receptor (BAFFR, also known as BR3) with high affinity. In the absence of BAFF, B-cell maturation proceeds normally in the bone marrow, but splenic B-cell numbers are reduced, especially transitional T2, mature and marginal zone (MZ) B cells [1, 2]. Humoral responses to both T-dependent (TD) and T-independent (TI) antigens are markedly reduced, but not entirely abrogated in BAFF$^{-/-}$ mice [2]. BAFF acts mainly as a survival factor and can be functionally

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replaced by enforced expression of the anti-apoptotic factor Bcl-2 in B cells [3, 4]. In contrast to BAFF, APRIL acts later in B-cell development [5]. Two strains of APRIL-deficient mice presented elevated TD humoral responses, low IgA serum levels, impaired mucosal IgA responses and reduced IgA production in response to TI antigen [6, 7]. BAFFR deficiency recapitulates most of the phenotype of BAFF-deficient mice, but affects TD and especially TI humoral responses less severely [3, 8], suggesting that although BAFFR transmits important BAFF signals, there are other functional receptors for BAFF in vivo. Interestingly, BAFFR deficiency in two patients with common variable immunodeficiency (CVID) strongly decreased B-cell numbers and immunoglobulin levels, except for IgA [9].

The role of TACI is less well understood. It is a likely negative regulator of B cells because TACI$^{-/-}$ mice have an enlarged B-cell pool that may be linked to autoimmune manifestations [10, 11]. But in spite of their elevated B-cell numbers, TACI-deficient mice are also characterized by reduced antibody responses to TI antigens [11], suggesting that the engagement of TACI by BAFF or APRIL is important for this response. In humans, about 8% of CVID patients and 2% of the control population have mutations in TACI, suggesting a role for TACI in the production of antibodies of various isotypes [12].

The third receptor for BAFF, BCMA, is apparently dispensable for early B-cell differentiation stages, but likely plays a role in the long-term maintenance of bone marrow plasma cells [13]. BAFF and APRIL play a redundant role for the maintenance of these cells, suggesting that both ligands can activate BCMA [14].

BAFF is expressed by myeloid cells and by radiation-resistant cells, possibly stromal cells of secondary lymphoid organs [15–17]. BAFF can be expressed at the cell surface or released into a soluble form after cleavage at a consensus furin cleavage site [18–20]. Processed human BAFF can either remain as a trimer, which is usual for TNF family ligands or assemble into 60-mer composed of 20 trimers [21]. Mouse BAFF 60-mer has also been identified in the serum of BAFF transgenic mice [22]. Oligomerization of BAFF 3-mer into 60-mer in human BAFF is prevented by mutation of His218, a residue critical for 3-mer-to-3-mer interactions, but not for receptor binding [21]. Recombinant soluble BAFF is an active protein both in vitro and in vivo, but the function of membrane-bound BAFF remains unknown. In this study, we show that the expression of BAFF with a mutated furin cleavage site in BAFF$^{-/-}$ mice failed to efficiently rescue B-cell development and TI type 2 humoral responses, despite residual BAFF processing, indicating that efficient release of soluble BAFF is necessary for its action. In line with this conclusion, administration of soluble recombinant BAFF in BAFF$^{-/-}$ mice rapidly restored functional B cells. Membrane-bound BAFF may however play other roles in B cells such as the upregulation of CD23.

Results

Generation of mice expressing a mutated furin cleavage site

When transfected into a variety of cell types, human BAFF is cleaved after arginine 133 (R133), which corresponds to R126 in the mouse [19, 20]. R125 and R126 of mouse BAFF were mutated to alanines in order to generate a non-cleavable furin-mutant BAFF (fmBAFF) (Fig. 1A). In a second BAFF construct, silent mutations were introduced in the WT BAFF (wtBAFF) sequence close to the furin-processing site to generate an AscI restriction site (wtBAFF(Asc)). A soluble form of mouse BAFF (sBAFF) starting at Ala 127 and preceded by the signal peptide of viral heamaglutinin was also generated (Fig. 1A).

Upon transient transfection into 293T cells, full-length forms of wtBAFF, wtBAFF(Asc) and fmBAFF (~32 kDa) were expressed at similar levels in cell lysates (Fig. 1B). As a likely consequence of overexpression, sBAFF was also detected in cell lysates. BAFF processing was tested by immunoprecipitation of cell supernatants with recombinant BCMA-Fc. As expected, shorter forms (~17 kDa) of soluble BAFF were readily detected for soluble BAFF, full-length wtBAFF and wtBAFF(Asc), but not fmBAFF (Fig. 1B). Relatively low levels of BAFF were also detected at the cell surface by staining with BAFFR-Fc, BCMA-Fc or an anti-mouse BAFF monoclonal antibody, but not TNFR1-Fc, indicating proper folding and surface expression (Fig. 1C). As anticipated, surface levels of fmBAFF were higher than those of wtBAFF (Fig. 1C). Taken together, these results indicate that fmBAFF is not processed to a soluble form but accumulates at the cell surface, and that all forms of BAFF are competent for receptor binding.

The corresponding mutations of wtBAFF(Asc), fmBAFF and sBAFF were introduced into a bacterial artificial chromosome (BAC) of BAFF for the generation of transgenic mouse lines (Supporting Information Fig. S1). Six lines were generated and subsequently backcrossed into the BAFF$^{-/-}$ background. For unknown reasons, sBAFF transgenic lines did not express any BAFF protein despite expression of sBAFF mRNA with the expected nucleotide sequence (data not shown) and were not analyzed further. For the purpose of clarity, the BAFF$^{-/-}$ × wtBAFF(Asc) and BAFF$^{-/-}$ × fmBAFF transgenic lines are called wtBAFF♯1, wtBAFF♯2, fmBAFF-low and fmBAFF-high in the remaining of the study.

Mutation of the furin cleavage site reduces BAFF release in vivo

Splenocytes of transgenic lines expressed BAFF mRNA at levels comparable to those of BAFF$^{+/-}$ mice, except fmBAFF-high mice that expressed about five times more, in agreement with our finding that it incorporated five to six copies of the transgene (Fig. 2A and data not shown). Circulating BAFF levels in sera of wtBAFF♯1 and wtBAFF♯2 lines were comparable to those of BAFF$^{+/-}$ mice for one of the lines and enhanced by about 50% in the other, although the difference between wtBAFF♯1 and wtBAFF♯2 was not statistically significant (Fig. 2B). Unexpectedly, BAFF was also detected in sera of fmBAFF lines, although at lower levels (Fig. 2B). We named this unanticipated form of BAFF sfmBAFF (soluble form of fmBAFF). sfmBAFF could still bind to BCMA and was depleted from serum by BCMA-Fc as efficiently as soluble wtBAFF (Fig. 2C). The fraction of wtBAFF and sfmBAFF

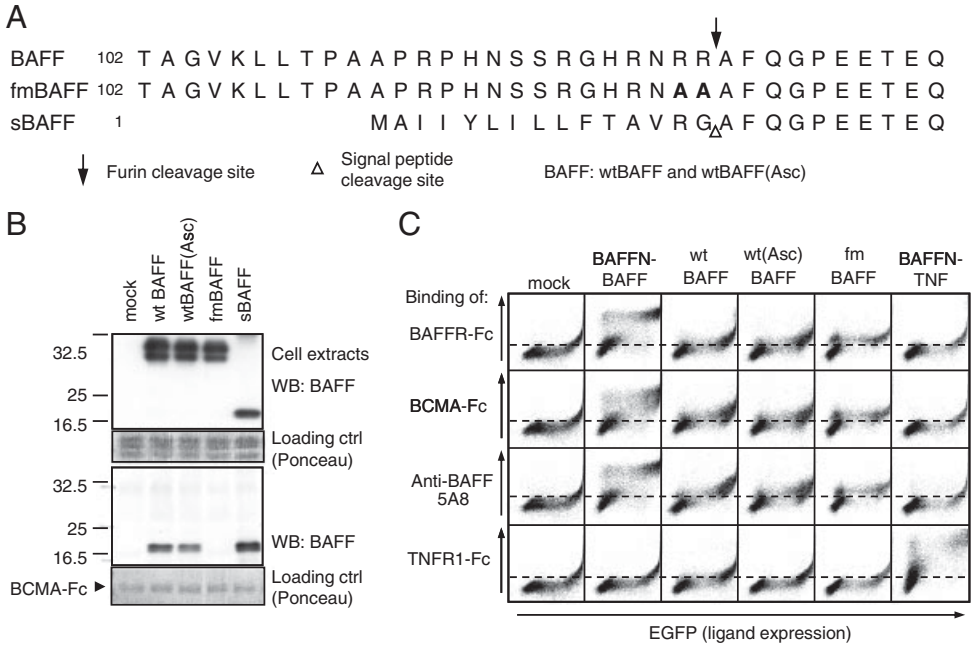

Figure 1. Characterization of fmBAFF. (A) Amino acid sequences of BAFF, fmBAFF (R125A, R126A) and sBAFF in the vicinity of the furin consensus cleavage site. sBAFF starts at A127, after cleavage of the signal peptide. (B) Western blot analysis of cell extracts and supernatants of transfected 293T cells using anti-mouse BAFF antibody (ProSci). BAFF in supernatant was immunoprecipitated with BCMA-Fc. In cell extracts, multimeric forms of BAFF (data not shown) were also detected in addition to the monomeric bands. (C) 293T cells co-transfected with constructs indicated at the top of the figure and an EGFP tracer plasmid were stained with reagents indicated on the left hand side, and analyzed by FACS. BAFFN constructs, which express well at the cell surface, were used as positive controls. They contain the N-terminus of hBAFF (intracellular, transmembrane and stalk domains) fused to the TNF homology domain of muBAFF or hTNF. Data shown are representative of three experiments with similar results.

that could not be depleted may either reflect the presence of inactive forms of BAFF or a limitation of the method. sfmBAFF in culture supernatants of DCs was produced in much smaller amounts than sBAFF and displayed a slightly higher molecular weight, suggestive of an inefficient cleavage event occurring close to the consensus furin site (Fig. 2D). sfmBAFF was under the detection limit in fmBAFF-low DCs (Fig. 2D). Unfortunately, we failed to detect surface expression of BAFF on DCs using different commercial antibodies, even after LPS stimulation (data not shown).

In summary, BAFF mRNA is present at comparable levels in $BAFF^{+/-}$, wtBAFF♯1, wtBAFF♯2 and fmBAFF-low mice, but overexpressed in fmBAFF-high mice. Production of soluble BAFF protein is comparable in $BAFF^{+/-}$, wtBAFF♯1, wtBAFF♯2, but is reduced in fmBAFF-low and fmBAFF-high mice that use an alternative and less-efficient BAFF cleavage site.

B-cell development is impaired in mice expressing BAFF with a mutated furin site

We analyzed how different forms of BAFF rescued peripheral B-cell development in $BAFF^{-/-}$ mice. Splenic transitional type 1 (T1), T2, follicular and MZ B cells as well as peritoneal B1 and B2 B cells were completely restored in both percentages and numbers in wtBAFF♯1 and wtBAFF♯2 mice (Fig. 3 and Table 1). Migration of mature B cells was also apparently normal, as inguinal LNs contained about 20% of B cells in both $BAFF^{+/-}$ and wtBAFF transgenic lines compared to only 2% in $BAFF^{-/-}$ mice (Fig. 3G).

B cells were however not rescued in spleens and LNs of fmBAFF-low mice (Fig. 3), except for the percentage of B2 B cells in the peritoneal cavity that was slightly higher than in $BAFF^{-/-}$ mice (Fig. 3I and Table 1). In the fmBAFF-high line, some mature B cells were present in spleen and LNs, but without reaching levels of $BAFF^{+/-}$ mice (Fig. 3 and Table 1). The ratio of B1 to B2 cells in the peritoneal cavity was however normal (Fig. 3I and Table 1).

Reduced basal levels of serum immunoglobulins in fmBAFF mice

Both fmBAFF-low and fmBAFF-high lines had reduced levels of serum immunoglobulins like in $BAFF^{-/-}$ mice (Fig. 4A). The only differences were observed for IgG1 and IgA that were higher in fmBAFF-high than in $BAFF^{-/-}$ mice.

B-cell function is impaired in mice expressing furin-mutated BAFF

$BAFFR^{-/-}$ and $BAFF^{-/-}$ mice both show a similar block in B-cell development, but TI-II responses are less impaired in $BAFFR^{-/-}$

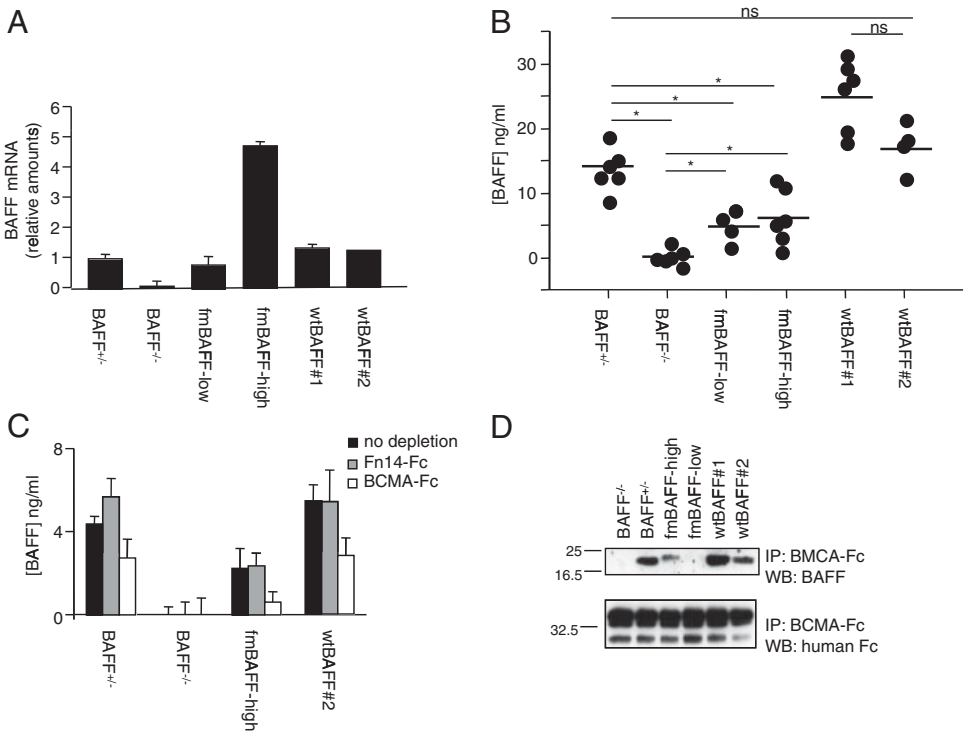

Figure 2. BAFF expression in transgenic lines. All mice (except BAFF$^{+/-}$) were in the BAFF$^{-/-}$ background. (A) Splenic BAFF mRNA levels were determined by qPCR and normalized to the housekeeping gene TBP. Data are presented as means+SD, $n = 3$. (B) Levels of circulating BAFF were determined by ELISA in sera of 9–12 wk-old females of the indicated genotype. ns: $p>0.01$. * $p<0.01$. Paired t test. (C) Sera of the indicated mouse lines were pre-incubated on plates coated with or without Fn14-Fc, a control receptor that does not bind to BAFF, or with BCMA-Fc to allow for the depletion of receptor binding-competent BAFF, and then quantified by ELISA. Data are presented as means+SD, $n = 3$. (D) Soluble BAFF in supernatants of bone marrow-derived DCs of the indicated genotypes was immunoprecipitated with BCMA-Fc and analyzed by Western blotting using R&D anti-mouse BAFF. BCMA-Fc used for the precipitation is shown as a control. Data shown are representative of two experiments with similar results.

mice [8], in line with the observation that TACI expression is required for TI-II responses [11]. We thought that although B-cell development was not rescued in fmBAFF-low mice, membrane-bound BAFF could act as a TACI ligand to generate TI-II humoral responses. This was however not the case: wtBAFF♯1 and wtBAFF♯2 lines immunized with the TI-II antigen NP-Ficoll responded like BAFF$^{+/-}$ mice, whereas fmBAFF-low and fmBAFF-high mice behaved as BAFF$^{-/-}$ mice (Fig. 4B). In addition, fmBAFF mice, like BAFF$^{-/-}$ mice, did not mount efficient T-cell-dependent responses (data not shown).

Soluble BAFF is sufficient for B-cell development and TI-II responses

To address the role of soluble BAFF in B-cell maturation, BAFF$^{-/-}$ mice were treated with purified human BAFF 3-mer (with mutation H218A) or 60-mer (wt) [21] (Supporting Information Fig. S2). These proteins differ in their signalling abilities in vitro [22]. Half-lives of BAFF 60-mer and BAFF 3-mer administered i.v. in wt C57BL/6 mice were 70 and 150 min, respectively (data not shown). When administered to BAFF$^{-/-}$ or fmBAFF-high mice, recombinant BAFF rescued development of T2, follicular and MZ B cells and even induced a slight hypertrophy of the B-cell compartment, with a bias towards MZ B cells overproduction in fmBAFF-high mice (Fig. 5A and B). TI-II responses to NP-Ficoll were efficiently restored (Fig. 5C), with similar amounts of NP-specific antibodies produced in treated BAFF$^{-/-}$ or treated fmBAFF-high mice, indicating that fmBAFF does not contribute significantly to TI-II response under these conditions. However, there was a trend towards stronger TI-II responses in BAFF$^{-/-}$ mice treated with BAFF 60-mer compared to those treated with BAFF 3-mer, suggesting that BAFF 60-mer is more potent in that respect than BAFF 3-mer, despite its shorter half-life (Fig. 5C).

Taken together, these results indicate that soluble BAFF 3-mer is sufficient to drive B-cell maturation and expansion in the absence of membrane-bound BAFF, and that B cells elicited in this environment can mount TI-II responses.

Membrane-bound BAFF may regulate CD23 expression in collaboration with soluble BAFF

Expression of CD21 and CD23 is low in mature B cells of BAFF$^{-/-}$ mice [23]. CD21 expression was readily restored in follicular B cells of wtBAFF♯1 and wtBAFF♯2 mice, and was even higher than normal in BAFF$^{-/-}$ mice treated with soluble BAFF

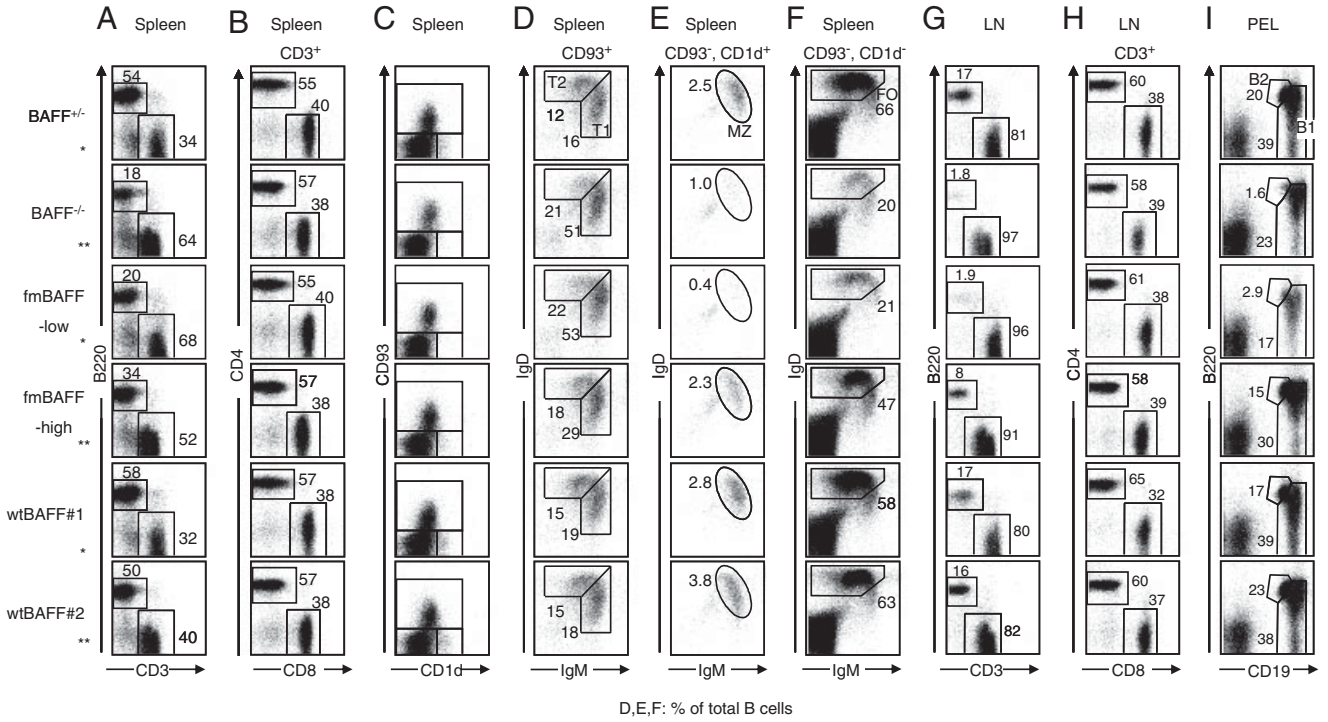

Figure 3. B-cell rescue in the different transgenic lines. B-cell subtypes were analyzed by FACS in 9–12 wk-old female mice. All mice (except BAFF$^{+/-}$) were in the BAFF$^{-/-}$ background. Plots are representative of at least three mice. Percentages in panels A, B, G, H and I refer to gated lymphocytes and in panels D, E and F to gated B cells. Results were generated from two experiments, with BAFF$^{-/-}$, BAFF$^{+/-}$, wtBAFF#1, fmBAFF-low in one experiment (*) and BAFF$^{-/-}$, BAFF$^{+/-}$, wtBAFF#2, fmBAFF-high in the other (**). Results for BAFF$^{-/-}$ and BAFF$^{+/-}$ included in both experiments were comparable. (A–F) Spleen. (A) Splenic B (B220) and T (CD3) cell populations. (B) CD4$^+$ and CD8$^+$ T-cell populations. (C) Splenic B-cell populations were separated into CD93$^+$, CD93$^-$CD1d$^-$ and CD93$^-$CD1d$^+$ cells. Based on IgM and IgD expression, these populations were further resolved into (D) T1 and T2 B cells, (E) MZ B cells and (F) follicular (FO) B cells. (G and H) Inguinal LNs. (G) B (B220) and T (CD3) cell populations. (H) CD4$^+$ and CD8$^+$ T-cell populations. (I) Peritoneal exudates lymphocytes. Peritoneal B lymphocytes are classified into B1 (CD19^{hi}B220$^{+/low}$) and B2 (CD19$^+$B220^{hi}) cells. Data shown are representative of two experiments with similar results. For gating schemes, see Supporting Information Fig S5.

(Fig. 5B). However, CD23 expression remained low in all BAC transgenic lines, even those expressing wtBAFF (Fig. 5B). This was the only phenotype we have looked at that was not improved in wtBAFF#1 and wtBAFF#2 mice compared to BAFF$^{-/-}$ mice. CD23 also remained low in BAFF$^{-/-}$ mice treated with BAFF 3-mer, despite the rescue of B-cell maturation and antibody responses (Fig. 5B and C). However, treatment with BAFF 60-mer restored normal CD23 expression on follicular B cells, and higher than normal levels of CD23 expression was obtained in fmBAFF-high mice treated with BAFF 3-mer (Fig. 5B). BAFF 60-mer also upregulated CD23 expression on purified B cells. In culture, about 60% of B splenocytes died within 48 h, while surviving cells gradually decreased CD23 expression (Fig. 6A and B). BAFF 3-mer and BAFF 60-mer reduced B-cell death to about 30% and also counteracted loss of CD23 (Fig. 6A and B). BAFF 60-mer even increased CD23 expression to roughly twice the initial levels (Fig. 6B), an effect that correlated with an increase of CD23 mRNA at 24 h (Supporting Information Fig. S3).

Taken together, these results indicate that CD21 and CD23 have distinct requirements for their expression, and that the generation of CD23$^+$ B cells in vitro and in vivo may require a multimeric form of BAFF like BAFF 60-mer or the concerted action of membrane-bound and soluble BAFF.

Discussion

BAFF exists as membrane-bound and soluble forms [16, 24]. The rescue of B cells in BAFF$^{-/-}$ mice treated with soluble BAFF 3-mer and the severely impaired B-cell development and function observed in fmBAFF mice indicate that soluble BAFF is both sufficient and necessary for B-cell homeostasis in vivo. Interestingly, mutations of the furin cleavage site of ectodysplasin A, another TNF family member, also abrogate its function in the development of ectodermal appendages [25, 26]. Although mutation of the furin site of mouse BAFF strongly reduced processing (over 100 times in vitro, data not shown) some cleavage still occurred close to R126 to generate a slightly bigger protein. As this form of BAFF was undetectable in wt DC supernatants, it is unlikely to be functionally relevant.

Despite the predominant functional role of processed BAFF in vivo, membrane-bound BAFF might also play a role. Indeed, soluble BAFF (3-mer) can trigger BAFFR but not TACI, whereas oligomeric forms of BAFF (BAFF 60-mer), which may mimic membrane-bound BAFF, activate both receptors [22]. Soluble and membrane-bound BAFF could therefore differentially activate TACI-dependent responses and, in particular, TI-II responses [11]. However, TI-II responses remained very low in

Table 1. Lymphocyte counts in BAC transgenic lines[a)]

	BAFF$^{+/-}$	BAFF$^{-/-}$	fmBAFF-low	fmBAFF-high	wtBAFF#1	wtBAFF#2
Spleen (million)						
Total cells	47.1±5.5	27.7±4.6	29.9±3.8	36.9±11.4	49.4±6.3	37.7±5.9
T cells	12.0±2.3	10.0±2.0	10.1±1.0	11.0±2.0	8.3±0.9	11.6±1.8
CD4$^+$ T cells	6.7±1.3	5.2±1.2	5.5±0.8	6.2±1.1	5.0±0.6	6.2±1.5
CD8$^+$ T cells	4.6±0.9	4.3±1.0	4.1±0.1	4.3±0.8	2.9±0.3	4.2±1.5
B cells (B220)	14.2±1.8	2.4±0.9	3.3±1.8	7.1±1.5	16.0±1.7	12.1±4.9
B cells (IgM/IgD)	11.2±2.0	1.8±0.7	2.3±1.2	5.9±1.1	13.4±1.7	11.5±2.6
T1 B cells	2.0±0.4	0.9±0.5	1.1±0.7	1.9±0.4	2.2±0.3	2.4±1.0
T2 B cells	1.4±0.3	0.4±0.1	0.4±0.2	1.0±0.3	1.5±0.3	1.5±0.4
Follicular B cells	6.7±1.6	0.4±0.1	0.6±0.2	2.6±0.3	8.5±1.1	6.7±2.1
MZ B cells	0.66±0.19	0.04±0.01	0.06±0.02	0.26±0.04	0.84±0.20	0.64±0.17
LN (million)						
Total cells	1.35±0.37	1.11±0.36	0.9±0.1	1.55±0.56	1.4±0.27	1.26±0.13
T cells	1.08±0.29	1.08±0.35	0.85±0.1	1.42±0.50	1.09±0.22	1.07±0.11
CD4$^+$ T cells	0.62±0.15	0.65±0.2	0.54±0.04	0.78±0.33	0.71±0.13	0.59±0.04
CD8$^+$ T cells	0.42±0.15	0.39±0.14	0.29±0.06	0.59±0.18	0.36±0.08	0.45±0.07
B cells	0.24±0.12	0.02±0.01	0.03±0.01	0.10±0.06	0.28±0.05	0.17±0.02
PELs						
%B1 cells	37.9±7.4	21.5±7.6	18.3±2.6	26.0±4.7	36.8±3.4	37.4±9.6
%B2 cells	22.0±4.2	1.8±0.5	4.4±2.0	16.8±1.7	23.1±8.7	24.4±2.4
B1/B2	1.8±0.4	12.4±3.0	4.5±1.4	1.6±0.3	1.7±0.6	1.5±0.6

[a)] Three to six mice (9–12 wk-old females) were analyzed in each group. All mice (except BAFF$^{+/-}$) were in the BAFF$^{-/-}$ background. Results were pooled from two experiments (see legend to Fig. 3). Population definition: Spleen: B cells (IgM$^+$ and/or IgD$^+$), T1 B cells (IgM^{hi}IgD^{lo}CD93$^+$), T2 B cells (IgM^{hi}IgD^{hi}CD93$^+$), mature B cells (IgM$^+$IgD$^+$CD93$^-$CD1d$^-$), MZ B cells (IgM$^+$IgD$^+$CD93$^-$CD1d$^+$). Peritoneal exudates B lymphocytes: B2 (B220^{hi}CD19$^+$), B1 (B220$^{+/low}$CD19^{hi}).

fmBAFF mice, which can readily be explained if effector cells require processed BAFF for their generation. It was more surprising to find that BAFF$^{-/-}$ mice treated with trimeric BAFF generated good antibody responses to NP-Ficoll, although these mice lacked membrane-bound or multimeric forms of BAFF. Several hypotheses may explain this result: potential aggregation of recombinant BAFF 3-mers in vivo, boost of the residual TACI-independent TI-II responses by an excess of BAFF and/or TACI stimulation by endogenous APRIL. If the latter is true, APRIL and membrane-bound BAFF could be redundant for TACI activation, as APRIL$^{-/-}$ mice display normal TI-II responses [5, 6]. Finally, it cannot be excluded that TACI can respond to trimeric BAFF in vivo. It should be noted, nevertheless, that BAFF$^{-/-}$ mice treated with BAFF 60-mer induced a more robust TI-II response than mice (both BAFF$^{-/-}$ and fmBAFF-high) reconstituted with BAFF 3-mer, despite the shorter half-life of BAFF 60-mer. This effect of BAFF 60-mer must be independent of enhanced MZ B-cell rescue as fmBAFF-high mice treated with BAFF 3-mer had more MZ B cells than BAFF$^{-/-}$ mice treated with BAFF 60-mer. It will be of interest to monitor TI-II responses in TACI$^{-/-}$, BAFF$^{-/-}$ × TACI$^{-/-}$ or BAFF$^{-/-}$ × APRIL$^{-/-}$ mice treated with BAFF 3-mer to distinguish between some of these hypotheses.

Our results suggest that the effects of membrane-bound BAFF may potentially include (i) the production or survival of peritoneal B2 B cells, (ii) the production of basal levels of IgA, (iii) the differentiation of MZ B cells and/or (iv) the upregulation of CD23 expression. These effects are not seen with fmBAFF alone, suggesting that membrane-bound BAFF mainly acts on B cells previously elicited by the action of processed BAFF. Some of these effects are relatively weak, but the upregulation of CD23 was rather clear-cut. CD23 is a low-affinity IgE receptor expressed in all B cells post T1 stage, and subsequently downregulated in MZ B cells [27]. Regulation of CD23 may affect the production of specialized immunoglobulin classes, as CD23-deficient mice produce more IgE [28]. It was previously shown that BAFF$^{-/-}$ B cells fail to upregulate CD23 and the complement receptor CD21/CD35 [23]. One study challenged a direct link between BAFF and CD23 expression, arguing that the slight induction of CD23 by BAFF paralleled that of other B-cell markers such as B220 [29]. In the present study, the expression of a wt BAC BAFF transgene in BAFF$^{-/-}$ mice restored B-cell maturation and CD21 expression, but not CD23 expression, indicating that the expression pattern and/or the expression level of the transgene must differ slightly from that of the natural BAFF gene. Interestingly, CD23 was not upregulated by fmBAFF or by recombinant BAFF 3-mer taken alone, but was induced by the combination of both and also by BAFF 60-mer. This is unlikely the result of a general upregulation of surface markers as B220 was instead slightly reduced (data not shown). CD23 upregulation may be at least partially dependent on BAFFR, as BAFFR$^{-/-}$ mice present reduced expression of CD23 on mature B cells [8], whereas TACI$^{-/-}$ have normal CD23 expression [11]. CD23

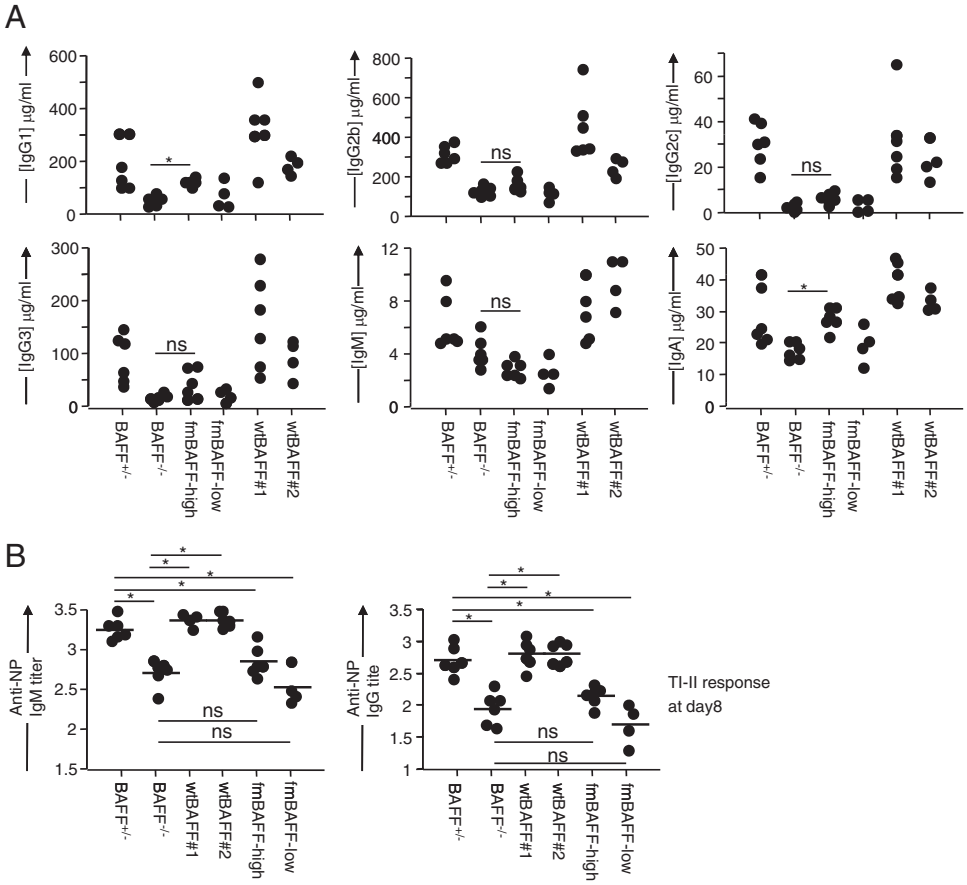

Figure 4. Basal antibody levels and TI-II humoral responses in transgenic lines. (A) Immunoglobulin levels quantified by ELISA in sera of 9–12 wk-old female mice. (B) Mice were immunized with NP-Ficoll. NP-specific antibody titers (IgG, IgM) in serum were measured after 8 days. All mice (except BAFF$^{+/-}$) were in the BAFF$^{-/-}$ background. ns: $p>0.01$. *$p<0.01$. Paired *t* test. Data shown are representative of two experiments with similar results.

upregulation might therefore be mediated through BAFFR by BAFF 60-mer or membrane-bound BAFF.

In summary, we have shown that an optimal release of processed BAFF is essential for B-cell development and B-cell function and that most of BAFF effects can be attributed to soluble BAFF. We also propose that membrane-bound BAFF may play fine-tuning functions such as the upregulation of CD23.

Materials and methods

Mice

Mice were handled according to Swiss Federal Office guidelines, under the authorization of the Office vétérinaire cantonal du canton de Vaud (authorization 1370.3 to PS). BAFF$^{-/-}$ mice have been described before [2, 30]. Transgenic mice were obtained by microinjection of modified BAC constructs into fertilized C57BL/6 oocytes. Founder mice were backcrossed into the C57BL/6 BAFF$^{-/-}$ background. Details on the generation of the transgenic mice are provided in the Supporting Information.

Plasmids, recombinant proteins production and injection

Plasmids were constructed according to standard molecular biology techniques (Supporting Information Fig. S4). hBCMA-Fc, hFn14-Fc, hBAFFR-Fc and hTNFR1-Fc have been produced and purified as described previously [31]. His-tagged BAFF 3-mer and 60-mer were purified from bacteria (Supporting Information Fig. S2). Proteins were tested as endotoxin-free (< 0.1 EU/µg). Mice were administered 40 µg of BAFF i.p. every second day for 10 days. At day 10, they were immunized with NP-Ficoll (see section *Immunisation*). BAFF was then injected every day. At day 18, tail vein blood was collected and mice sacrificed. Six mice were used per group.

Cells

HEK 293T cells were grown in DMEM, 10% fetal calf serum and 5 µg/mL each penicillin and streptomycin. They were transfected by the calcium phosphate method and grown for 48 h in serum-free Opti-MEM medium (Invitrogen)

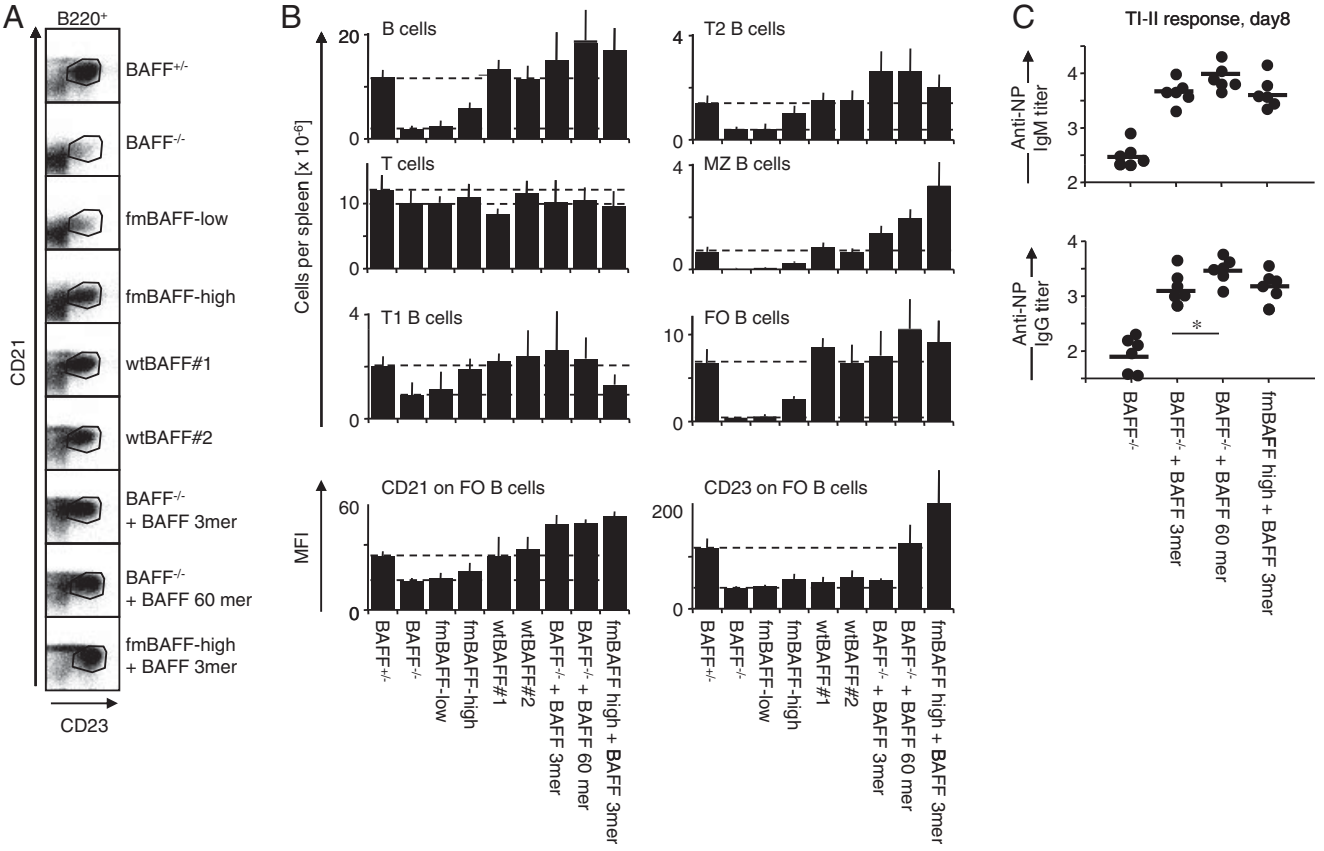

Figure 5. Restoration of the mature B-cell pool, TI-II humoral responses and CD23 expression in BAFF$^{-/-}$ mice treated with soluble BAFF. All mice (except BAFF$^{+/-}$) were in the BAFF$^{-/-}$ background. BAFF$^{-/-}$ and fmBAFF-high mice were treated for 18 days with i.p. injections of the indicated BAFF protein or with PBS. At day 10, mice were immunized with NP-Ficoll (six mice per group). (A and B) B-cell development in the spleen was determined at day 18 (three mice per group). Untreated mice of various phenotypes are shown for comparison. (A) Expression of CD21 and CD23 in B220^{+} cells. (B) Mean number+SD (n = 3–10) of various lymphocyte populations, and mean fluorescence intensity of CD21 and CD23 in follicular B cells (population gated in panel (A)). At least three mice were analyzed per group (six, ten and four mice per group for BAFF$^{+/-}$, BAFF$^{-/-}$ and fmBAFF-high, respectively). (C) NP-specific antibody titers were measured at day 18 (day 8 post-immunization; six mice per group). *p<0.01. Paired t test. Data shown are representative of two experiments (one experiment only for BAFF injections) with similar results.

(for immunoprecipitatons) or in complete medium (for FACS analysis).

Bone marrow cells (3×10^6) obtained from tibias and femurs were differentiated into DCs by incubation for 10 days in 10 mL of RPMI, 10% fetal calf serum, 5 μg/mL each penicillin and streptomycin, 50 μM 2-mercaptoethanol and 20 ng/mL GM-CSF (Immunotools, Friesoythe, Germany).

Immunoprecipitations and Western blot

293T cells were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton-X100, Cømplete™ protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). Soluble BAFF in 1 mL supernatant of transfected 293T cells or in 16 mL of bone marrow-derived DCs culture supernatants were incubated with an excess (1 g) of hBCMA-Fc and 10 μL of Protein A-Sepharose beads (GE Healthcare) for 16 h at 4°C on a rotating wheel. Beads were washed with PBS and eluted with 15 μL of 100 mM Na-citrate, pH 2.7. The eluate was neutralized with 5 μL of 1 M Tris-HCl, pH 9. BAFF was detected by Western blotting using rabbit anti-BAFF (CT) antibody (ProSci, for 293T cells) or goat anti-BAFF antibody (R&D, for DCs). It is noteworthy that hBCMA cross-reacts with mouse BAFF and APRIL, and that although the affinity of monomeric BCMA for BAFF (1600 nM) is lower than that for APRIL (16 nM), the avidity of dimeric BCMA-Fc for BAFF (0.63 nM) is as good as for its high-affinity ligand APRIL (0.2 nM) [1, 2].

PCR

Quantitative RT-PCR was performed with a Light Cycler (Roche), using the following mouse primers, with F and R indicating forward and reverse primers, respectively: TATA-binding protein (TBP) F, 5′-ACT TCG TGC AAG AAA TGC TGA A-3′; TBP R, 5′-TGT CCG TGG CTC TCT TAT TCT CA-3′; BAFF F, 5′-AGG CTG GAA GAA GGA GAT GAG-3′; BAFF R, 5′-CAG AGA AGA CGA GGG AAG GG-3′; CD23 F 5′-TGG CAA AGC TGT GGA TAG AG-3′; CD23 R 5′-GGA GCC CTT GCC AAA ATA GT-3′.

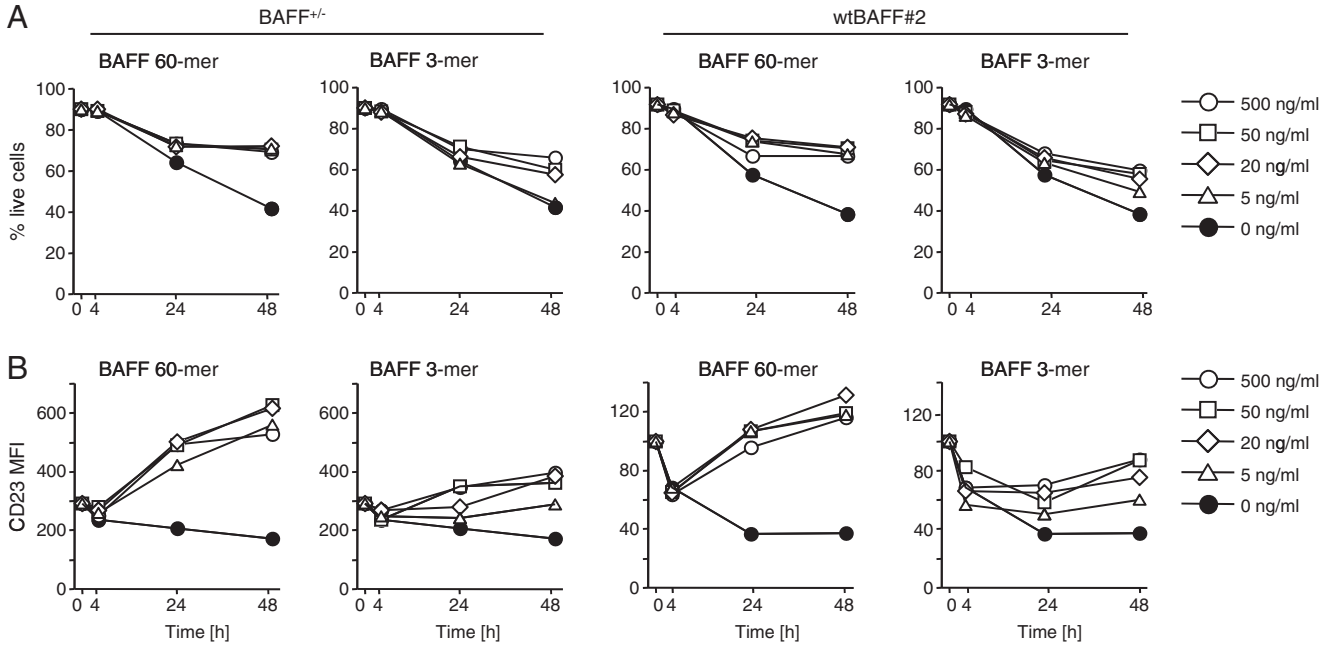

Figure 6. BAFF induces CD23 upregulation in B cells *ex vivo*. Purified B splenocytes from BAFF$^{+/-}$ or wtBAFF#2 (in the BAFF$^{-/-}$ background) were cultured for 0, 4, 24 or 48 h in the presence of graded concentrations of BAFF 3-mer or 60-mer, and analyzed by FACS for B-cell survival (A) and CD23 expression (B). BAFF 60-mer promoted B-cell survival and CD23 expression. Data shown are representative of three experiments with similar results.

ELISA

For the detection of mouse BAFF, ELISA plates were coated overnight with 100 μL of mAbs 5A8 (Alexis) at 3 μg/mL, blocked, incubated for 3 h with 100 μL of mouse sera at 1/10 dilution and revealed for 2 h with 100 μL of biotinylated mAb 1C9 (Alexis) at 2 μg/mL, followed by horseradish peroxidase-coupled streptavidin.

For the depletion of receptor binding-competent mouse BAFF, ELISA plates were coated overnight with 100 μL of hBCMA-Fc or hFn14-Fc at 1 μg/mL or with 100 μL of PBS, blocked and incubated for 2 h with mouse sera at 1/10 dilution in incubation buffer. The depletion was repeated twice by transferring the content to a new receptor-Fc-coated well. The resulting supernatants were analyzed with the mouse BAFF ELISA.

For the detection of NP-specific antibodies, ELISA plates were coated for 16 h with 10 μg/mL of NP_{25} BSA (Biosearch Technologies) in 50 mM sodium carbonate buffer, pH 9.6, blocked with blocking buffer, washed and incubated with serum (1:100 in incubation buffer and three-fold dilutions). Bound antibodies were revealed with biotinylated goat anti-mouse IgG (1:2000; Jackson ImmunoResearch) or biotinylated goat anti-mouse IgM (1:2000; Jackson ImmunoResearch) followed by horseradish peroxidase-coupled streptavidin (1:4000; Jackson Immuno Research). Enzymatic activity was measured at 490 nm with *o*-phenylenediamine reagent (Sigma).

For the detection of basal serum immunoglobulin levels, ELISA plates were coated with goat anti-mouse IgG plus IgM (2 μg/mL; Jackson ImmunoResearch) and serial dilutions of sera or of purified mouse IgG1, 2b, 2c, 3, M and A standards were added. Captured antibodies were revealed with biotinylated anti-IgG1 (1:2000; Caltag), anti-IgG2b, anti-IgG2c, anti-IgG3, anti-IgM (all at 1:2000; Jackson ImmunoResearch), and anti-IgA (1:2000; Caltag), respectively, followed by horseradish peroxidase-coupled streptavidin (1:4000). Concentrations were inferred from the IC_{50} values.

FACS analyses

The following antibodies were used: anti-B220-PECy5.5 (RA3.6B2), anti-CD4-PE (L3T4), anti-CD8-APC (Ly-2), anti-CD1d-FITC (1B1), anti-IgM-PE (II/41), biotinylated anti-IgD (11–26c), anti-CD93-APC (AA4.1), streptavidin-PECy5.5 and streptavidin-APC (all from eBioscience), anti-CD3-FITC (17A2) and biotinylated anti-CD19 (1D3) (BD Biosciences), biotinylated anti-IgM (1B4B1) (Southern Biotechnology Associates). Cells of the peritoneal cavity were collected by washing the cavity with 5 mL of PBS supplemented with 2% FCS and 50 μL of heparin (Liquemin, 5000 IU/mL, Roche Pharma). Inguinal LNs were collected and disrupted in 2 mL of RPMI 10% FCS using a 2 mL loose-fitting Dounce homogenizer. Cells were filtered on restrainers-caped tubes and counted. Spleens were disrupted using a 10 mL loose-fitting Dounce homogenizer. Erythrocytes were lysed, cells were filtered and counted. Following staining, cells were analyzed using a four-color FACSCalibur™ flow cytometer and FlowJo software (TreeStar, Ashland, OR).

Cell viability was assessed based on gates of live and dead cells in the forward scatter/side scatter (FSC/SSC) scatergrams. For CD23 upregulation experiments, splenic B cells were isolated with anti-B220 magnetic beads (Miltenyi Biotech) and cultured in vitro ±BAFF. Transfected 293T cells were stained with BAFFR-Fc, BCMA-Fc, TNFR1-Fc or anti-BAFF 5A8 (all at about 4 μg/mL), followed by appropriate PE-coupled secondary reagents.

Immunizations

Nine to 12-wk-old mice were immunized i.p. with a single injection of 10 μg of NP_{59}-Ficoll in PBS (Biosearch Technologies). Tail vein blood was collected at day 8 post-immunization and specific anti-NP antibody titers in the serum were determined by ELISA. Titer was defined as the dilution giving half-maximal signal. *p*-Values were determined on log of titers using the paired *t* test.

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Abbreviations: **APRIL:** a proliferation-inducing ligand · **BAC:** bacterial artificial chromosome · **BAFF:** B-cell-activating factor of the TNF family · **BAFFR:** BAFF receptor · **BCMA:** B-cell maturation antigen · **CVID:** common variable immunodeficiency · **fmBAFF:** furin-mutant BAFF · **MZ:** marginal zone · **sBAFF:** soluble form of mouse BAFF · **sfmBAFF:** soluble form of fmBAFF · **TACI:** transmembrane activator and CAML interactor · **TBP:** TATA-binding protein · **TD:** T-dependent · **TI:** T-independent

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