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Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors

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The TNF family member protein BAFF/BLyS is essential for B cell survival and plays an important role in regulating class switch recombination as well as in the selection of autoreactive B cells. In humans, increased concentrations of soluble BAFF are found in different pathological conditions, which may be as diverse as autoimmune diseases, B cell malignancies, and primary Ab deficiencies (PAD). Because the mechanisms that regulate BAFF levels are not well understood, we newly developed a set of mAbs against human BAFF to study the parameters that determine the concentrations of soluble BAFF in circulation. Patients with PAD, including severe functional B cell defects such as BTK, BAFF-R, or TACI deficiency, were found to have higher BAFF levels than asplenic individuals, patients after anti-CD20 B cell depletion, chronic lymphocytic leukemia patients, or healthy donors. In a comparable manner, mice constitutively expressing human BAFF were found to have higher concentrations of BAFF in the absence than in the presence of B cells. Therefore, our data strongly suggest that BAFF steady-state concentrations mainly depend on the number of B cells as well as on the expression of BAFF-binding receptors. Because most patients with PAD have high levels of circulating BAFF, the increase in BAFF concentrations cannot compensate defects in B cell development and function. *The Journal of Immunology*, 2012, 188: 497–503.

The B cell-activating factor of the TNF family (BAFF, BLyS) binds with different affinities to three different TNFR-like proteins expressed by B cells termed BAFF-R, TACI, and BCMA (1). Binding to BAFF-R activates the classical and the noncanonical NF-κB signaling pathways, resulting in the expression of a series of downstream genes that are essential for B cell survival (2–4). In humans, homozygous deletion of the

BAFF-R gene results in severe B lymphopenia caused by the arrest of B cell development at the stage of transitional B cells (5). As a consequence, more mature B cell subsets, including follicular, marginal zone, and memory B cells, are present only in very small numbers and T-independent immune responses are severely impaired, proving that BAFF-R is one of the central survival receptors for human B cells (5). In mice, homozygous deletion of the BAFF-encoding Tnfsf13b gene interrupts B cell development at the stage of transitional B cells (6), whereas overexpression of BAFF results in a phenotype sharing some of the autoimmune manifestations found in human systemic lupus erythematosus (SLE) (7). Elevated BAFF levels have also been reported in patients suffering from different autoimmune diseases, including Sjögren's syndrome, rheumatoid arthritis, and SLE (8–10). Disruption of BAFF/BAFF-R interactions by BAFF-blocking Abs depletes naive IgM$^+$IgD$^+$CD27$^-$ B cells in humans as well as in mice (11–13), and in a subgroup of SLE patients, the BAFF-blocking mAb belimumab has been found to be effective in reducing disease activity (12). Therefore, anti-BAFF therapy has recently been approved for SLE treatment.

Mutations in the BAFF-encoding TNFSF13B gene have not been found so far in patients with primary antibody deficiencies (PAD). Analysis of BAFF serum concentrations in patients suffering from common variable immunodeficiency (CVID)—the most frequent PAD syndrome characterized by low levels of serum IgG and IgA and/or IgM concentrations, impaired in vivo B cell function, and lack of plasma cells (14, 15)—showed markedly increased BAFF levels but no relationship to clinical parameters, B cell numbers, and subsets (16). In a different study (17), an age-related increase in BAFF serum concentrations was reported in healthy donors and CVID patients, but the regulatory circuits of these apparently paradox findings (high BAFF levels and impaired or absent B cell function) remained unclear.

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Abbreviations used in this article: CLL, chronic lymphocytic leukemia; CRP, C-reactive protein; CVID, common variable immunodeficiency; PAD, primary antibody deficiency; SLE, systemic lupus erythematosus.

To gain further insight into the relationships between steady-state BAFF serum levels and maintenance of B cell homeostasis in humans, we systematically studied BAFF serum concentrations, B cell subsets, and numbers in relationship to clinical parameters in 309 CVID patients. To address whether the steady-state BAFF concentrations are mainly regulated by BAFF binding to receptor-bearing B cells or by other parameters such as inflammation or monocyte activation, we included other primary immunodeficiencies affecting B cell function such as BAFF-R, TACI, BTK, AID, CD40L, and ICOS deficiency. Samples from patients receiving anti-CD20 treatment and after splenectomy were included as a comparison for severe changes affecting B cell numbers or critical organs for B cell maturation, whereas samples from healthy donors as well as from cord blood and from the corresponding mothers served as controls. In this study we report that long-term steady-state BAFF serum concentrations in patients with PAD depend primarily on B cell numbers and on the presence of BAFF-R but not on inflammatory parameters, splenomegaly, or autoimmunity.

Materials and Methods

Animals

Female Lewis rats were purchased from RCC (Fuelinsdorf, Switzerland). Rats were used at 8 wk age. BALB/c, BALB/c Rag2-deficient, BALB/c human BAFF transgenic, and BALB/c Rag2-deficient human BAFF transgenic mice were maintained and bred in our animal facility under specific pathogen-free conditions. Human BAFF transgenic mice were generated in our laboratory using the β-actin promoter to drive the expression of the transgene. All animal experiments were carried out in accordance with institutional guidelines (permission nos. 1887 and 1888).

Patients

In total, 421 serum and plasma samples from patients between 1 and 79 y of age from centers in the Czech Republic, Germany, Italy, and in the United Kingdom were analyzed. The cohort included 2 AID (HIGM2)-deficient patients, 7 patients with CD40L deficiency (HIGM1), 2 with BAFF-R deficiency, 14 with homo- or heterozygous BAFF-R mutations, 3 TACI-deficient patients, 5 patients with ICOS deficiency, 1 patient with Wiskott-Aldrich syndrome, 20 patients with X-linked agammaglobulinemia (BTK deficiency), 3 patients with myotonic dystrophy type 2 caused by expansion of CCTG repeats in intron 1 of the Znf9 gene, 5 patients with Good's syndrome, 8 with IgA deficiency, 7 patients with selective IgG deficiency, 1 patient with IgM deficiency, and 347 patients with CVID who were between 2 and 79 y old with a median age of 42 y. The female/male ratio of CVID patients was 1:1. Sera from 23 immunocompetent but splenectomized patients, from 11 anti-CD20 (rituximab)–treated SLE patients, and from 21 chronic lymphocytic leukemia (CLL) patients were used for comparison, and sera from 50 adult healthy individuals and of 12 newborns and of their mothers were used as controls. The study was approved by the Ethics Committee of the Albert-Ludwigs-University Freiburg (no. 78/2001), and all patients, parents, and control persons agreed by written informed consent.

Generation of anti-BAFF mAbs

Anti-BAFF mAbs were generated by immunizing Lewis rats with recombinant Fc-tagged human BAFF generated in our laboratory (18). Upon fusion with the myeloma cell line Sp2/0, hybridomas were tested for the production of anti-BAFF mAbs by ELISA using plates coated with Fc-BAFF or FLT3L-Fc. Binding was revealed by mouse anti-rat IgG alkaline phosphatase (Jackson ImmunoResearch Laboratories). Those hybridomas that produced IgG binding to Fc-BAFF but not to FLT3L-Fc were subsequently tested for the binding to commercial human BAFF (PeproTech) by ELISA. This analysis revealed 49 hybridomas producing anti-human BAFF mAbs that were either of the rat IgG2A or IgG2B isotype. Of these, 15 hybridomas produced mAbs that were able to block the binding of BAFF to its receptor. Different combinations of blocking and non-blocking anti-BAFF mAbs were used to establish the ELISA showing the highest sensitivity to soluble human BAFF.

BAFF ELISA

BAFF concentrations were measured by a sandwich ELISA using two different rat mAbs recognizing different BAFF epitopes. Serum or plasma was diluted in PBS containing 4% BSA and 0.2% Tween 20 (ELISA buffer) and applied to ELISA plates coated with 10 μg/ml mAb 2.81 in PBS. Plates were incubated for 2 h at room temperature and washed five times with H_2O containing 0.2% Tween 20. After adding biotinylated mAb 4.62 at 2 μg/ml in ELISA buffer, plates were incubated for 2 h at room temperature, washed five times, incubated with streptavidin-conjugated alkaline phosphatase (Amersham Biosciences, Buckinghamshire, U.K.) in ELISA buffer for 1 h at room temperature, washed five times, and developed with DNP-phosphate (1 mg/ml) in diethanolamine substrate buffer at pH 9.8. After 30 min the reaction was stopped by adding 1 M NaOH and the OD at 405 nm was determined. The standard curve using recombinant BAFF (PeproTech) is shown in Fig. 1. The detection limit was ~0.1 ng/ml BAFF, and differences between soluble BAFF concentrations in serum or plasma samples collected from the same individuals were not found.

Statistical analysis

Correlations between BAFF concentrations and other parameters were calculated with GraphPad Prism using the two-sided t test within a 95% confidence interval or the Spearman correlation.

Results

BAFF levels in serum of adult healthy controls and newborns

To determine reference values for soluble BAFF, we first analyzed BAFF concentrations in sera of 52 adult healthy controls. As shown in Fig. 2A, the median BAFF level found in 52 adults was 0.6 ng/ml, ranging from 0.3 to 2.25 ng/ml. Sera from cord blood (Fig. 2B) contained more BAFF (2.1 ng/ml; range, 0.6–4.5 ng/ml) than the sera from the blood of the corresponding mothers (0.6 ng/ml; range, 0.3–2.25 ng/ml), suggesting that BAFF concentrations are higher in individuals when the immune system and the B cell repertoire are not yet fully developed.

BAFF concentrations in patients with PAD

Soluble BAFF was detected in all patient samples except for three CVID patients who had BAFF concentrations at background levels (Fig. 2C). Because very low BAFF concentrations might result from mutations in the TNFSF13B gene, the coding regions of the alleles were sequenced but found to be normal (data not shown). Most of the samples from non-CVID patients contained elevated BAFF levels except for the AID-deficient individuals and for one sample from a patient with IgAD, two patients with persistent polyclonal B lymphocytosis, and two patients with mytonic dystrophy due to mutations in ZNF9. From the patients of the CVID cohort, 48 of 347 analyzed samples (13.8%) had normal BAFF concentrations (mean ± SD: 0.6 ± 0.2 ng/ml; HD, 0.6 ± 0.3 ng/ml), whereas all others had increased BAFF concentrations with peak levels at 90 ng/ml (mean, 9.8 ± 15 ng/ml), and 44 (12.6%) samples contained BAFF at concentrations >20 ng/ml. In CLL patients, BAFF serum concentrations were very similar to healthy controls (t test, $p = 0.8445$).
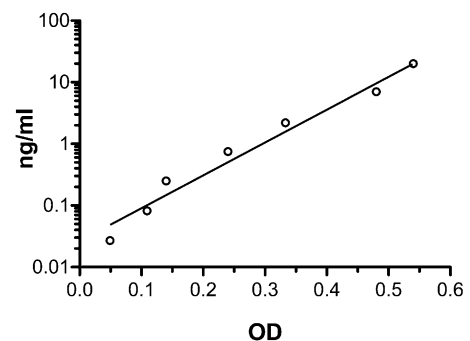
FIGURE 1. Standard curve and detection limits. The ELISA was standardized using recombinant human BAFF. The detection limit is at 0.1 ng/ml soluble human BAFF.
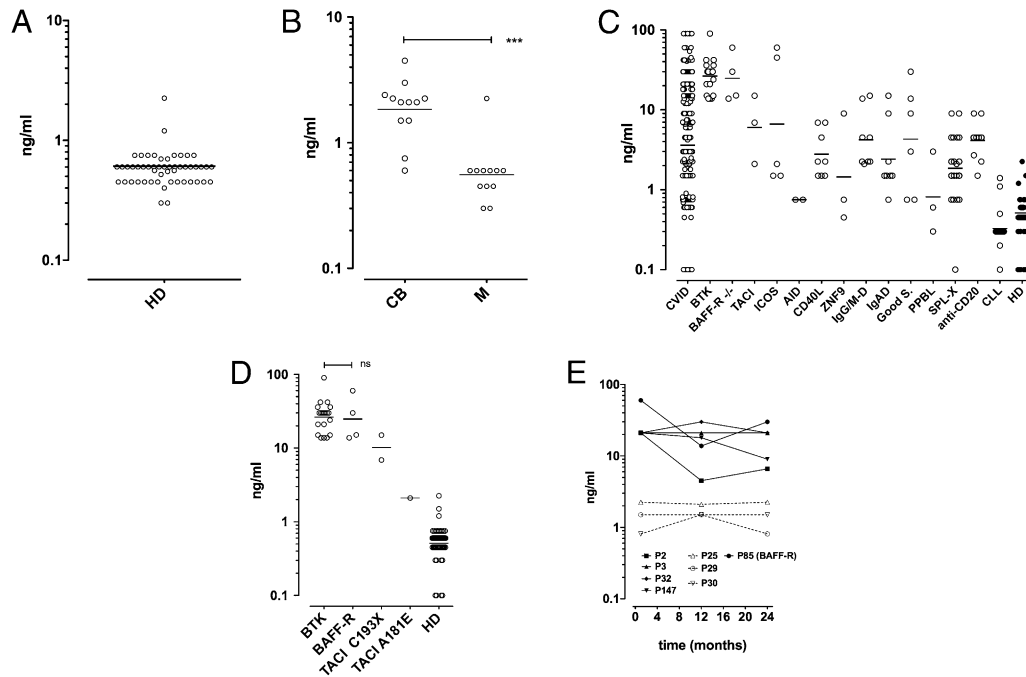
FIGURE 2. Soluble BAFF concentrations in healthy donors and patients with PAD. In healthy donors (*A*), BAFF concentrations are generally very low with a mean of 0.61 ± 0.27 ng/ml (SD; *n* = 52). Soluble BAFF concentrations are significantly higher (*p* = 0.0003) in the cord blood of newborns (*n* = 12) than in the blood of the corresponding mothers (*B*). Patients with PAD show elevated BAFF concentrations (*C*). The plot shows BAFF serum concentrations in 347 CVID samples, in 20 samples from patients with BTK, 4 with BAFF-R, 8 with CD40L, 2 with AID, 5 with ICOS, 8 with IgA (IgAD), 8 with selective IgM/G, 3 with TACI, 3 with ZNF9 deficiency, 3 with persistent polyclonal B lymphocytosis (PPBL), 23 samples from patients after splenectomy (SPL-X), 21 patients with CLL, and 11 patients under anti-CD20 (rituximab) treatment. Sixty samples from healthy donors served as controls. BAFF levels in BTK-, BAFF-R–, and TACI-deficient patients are shown in *D*; changes in BAFF concentration for eight CVID- and one BAFF-R–deficient patient within a period of 2 y are shown in *E*. Statistic differences were determined by the two-sided *t* test for unpaired samples in a 95% confidence interval. Geometric means are shown as horizontal lines.

Very high BAFF concentrations (≥10 ng/ml) were detected in samples of BTK patients, in patients with a homozygous BAFF-R deletion, in patients carrying heterozygous C193X TACI mutations, and in two ICOS patients with very low (≤2%) percentage of B cells (Fig. 2*C*, 2*D*). Intermediate levels (2–10 ng/ml) were found in patients with CD40L deficiency, myotonic dystrophy type 2, Good's syndrome, selective IgG, IgA, or IgM deficiency, in splenectomized patients, and in anti-CD20 treated patients. Healthy controls and CLL patients had significantly lower BAFF concentrations (0.1–2 ng/ml) (Fig. 2*A*, 2*C*). As reported before (19–21), short-term depletion of B cells by anti-CD20 treatment correlated with an increase of BAFF levels in patients under treatment, which, however, did not reach the concentrations found in BTK and CVID patients. Long-term asplenism after splenectomy also led to an increase of BAFF, which was comparable to anti-CD20 treatment (Fig. 2*C*). These data suggest that cells residing in the human spleen are not the major source of soluble BAFF found in circulation.

Because soluble BAFF concentrations may change during aging (17), in response to infection (22), or with monocyte activation (23, 24) we compared BAFF concentrations of seven CVID patients during a 2-y period (Fig. 2*E*). Although up to 2-fold changes in BAFF levels were observed for four CVID patients, those who started with BAFF levels >10 ng/ml retained high levels and those who had intermediate BAFF concentrations ranging from 0.8 to 2.3 ng/ml remained at lower levels, indicating that in different individuals BAFF concentrations remain remarkably stable over long periods of time.

It was reported before that elevated BAFF serum concentrations associate with autoimmunity as well as with primary and acquired immunodeficiencies (5, 16, 17, 25). It was also proposed that in CVID, elevated BAFF levels result from increased constitutive production and/or underlying immunoregulatory or inflammatory conditions (16).

We therefore analyzed whether BAFF levels change with clinical complications frequently associated with PAD such as autoimmunity, splenomegaly, granuloma, and lymphoproliferation, but we failed to detect differences between patients with or without these complications (Fig. 3*A*). The comparison between BAFF concentrations and two parameters changing during infection or inflammatory, namely the percentage of neutrophils and the concentration of C-reactive protein (CRP) (Fig. 3*B*), only revealed a weak association with CRP (*p* = 0.0243, *r* = 0.198). Analyzing the data of 270 CVID patients, a weak inverse correlation was found between BAFF levels and age (Fig. 3*B*; *p* = 0.036, *r* = −0.128), although there were no differences in the distribution of BAFF concentrations comparing very young patients at ages <15 y (*n* = 22) and patients at ages >64 y (*n* = 23, data not shown).

Because we noted that BAFF concentrations were high in all BTK-deficient patients, who have extremely few B cells, but were very low in CLL patients, who have highly increased numbers of circulating and BAFF-R– and TACI-expressing cells (Fig. 2*C*, 2*D*), we analyzed whether BAFF levels in CVID patients with B cell pools of different size would correlate to the percentage and numbers of B cells (Fig. 4*A*, 4*B*). As suspected, an inverse, nonlinear correlation was found between soluble BAFF concentrations and the percentage (*p* < 0.0001, *r* = −0.5228) and number (*p* < 0.0001, *r* = −0.224) of circulating B cells. In particular, from the group of 50 CVID patients with ≤50 B cells/μl (normal range, 80–450 B cells/μl), only 13 (26%) had BAFF concentrations <10 ng/ml, whereas 28 (56%) patients had soluble BAFF concentrations >20 ng/ml. For patients carrying mutations affecting either directly or indirectly B cell development or dif-
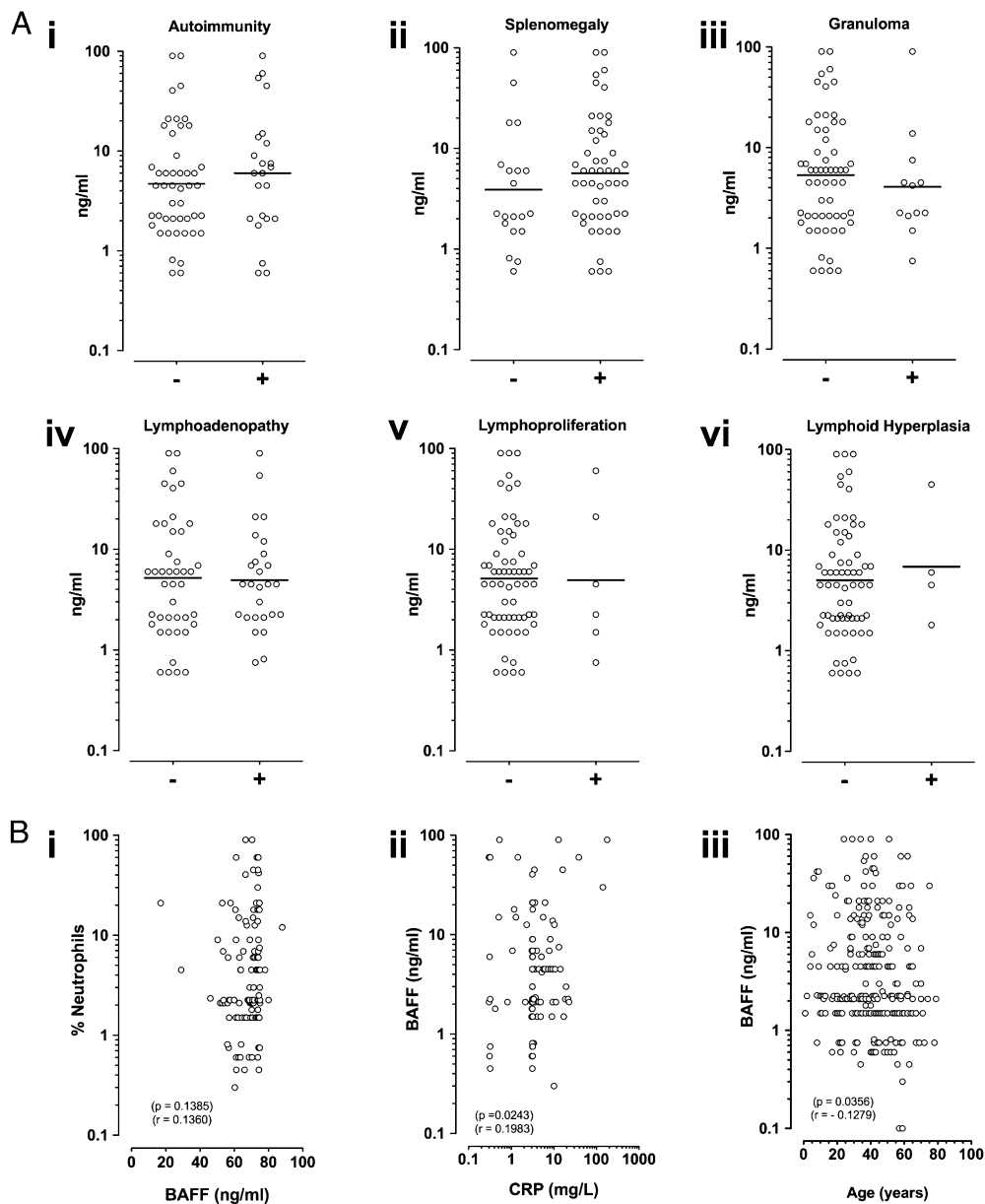
FIGURE 3. Differences in BAFF concentrations between patients with or without clinical complications. *A*, Differences in BAFF concentrations (ng/ml) in a cohort of 70 CVID patients with a complete clinical and immunological record between individuals with or without autoimmunity (*i*), splenomegaly (*ii*), granuloma (*iii*), lymphoadenopathy (*iv*), lymphoproliferation (*v*), and lymphoid hyperplasia (*vi*). Significant statistical differences between groups of patients without or with these complications were not found by the two-tailed *t* test within a 95% confidence interval for unpaired samples with different variances. *B*, Correlations between BAFF concentration, the percentage of circulating neutrophils (*i*), the concentration of CRP (*ii*), or age (*iii*) determined by the Spearman correlation within a 95% confidence interval (*n* = 70). A weak but significant correlation was found between BAFF serum levels and CRP (*ii*; $p = 0.0243$, $r = 0.1983$) or age (*iii*; $p = 0.0356$, $r = 20.1279$). Horizontal lines represent geometric means.

ferentiation (BTK, CD40L, ICOS) and BAFF binding (BAFF-R, TACI), the correlation between BAFF concentrations and the percentage (Fig. 4*C*; $p < 0.0001$, $r = -0.8$) or numbers of B cells (Fig. 4*D*; $p = 0.0001$, $r = -0.7$) was even stronger, and in BTK deficiency, similar BAFF levels were found in young (5 y) as in old (47 y) patients. These data suggest that the number of B cells, and thus the number of available BAFF-binding sites, is one of the most critical parameters in regulating the steady-state concentrations of soluble BAFF.

BAFF concentration in the serum of wild-type and Rag2-deficient human BAFF transgenic mice

The results described above strongly indicated that soluble BAFF concentrations in humans are to a large extent determined by the

number of B cells, in that individuals with low B cell numbers have higher circulating BAFF levels than do those with normal B cell numbers. The most simple explanation for this finding would be that "consumption" of BAFF by B cells regulates the concentrations of soluble BAFF. To test this hypothesis in a system where BAFF production is constant but the number of B cells is varying, we determined BAFF levels in the sera of wild-type (BALB/c) and Rag2/Il2rg double-knockout mice on a BALB/c background expressing a human BAFF transgene under the control of the β-actin promoter. Clearly, human BAFF was undetectable in wild-type and Rag2/Il2rg-deficient mice lacking the human BAFF transgene (Fig. 5), whereas human BAFF transgenic wild-type mice had soluble BAFF concentrations at ~10 ng/ml. In marked contrast, ~5-fold higher BAFF levels (55 ng/ml) were measured in
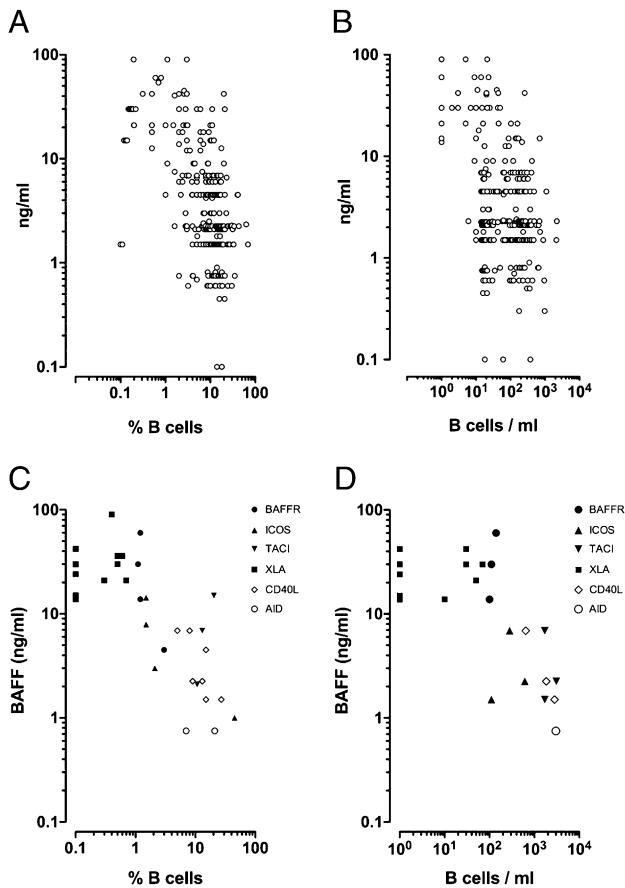
FIGURE 4. Correlation between BAFF levels and the percentage and numbers of B cells in patients with PAD. The relationships between BAFF levels (ng/ml) and the percentage of circulating B cells (A), the number of B cells/ml (B) in CVID patients, and the percentage (C) and numbers (D) of B cells in patients with genetically caused PAD were calculated by the Spearman correlation. An inverse correlation between BAFF concentrations was found for the percentage ($p < 0.0001$, $r = -0.5228$) and the number of B cells ($p < 0.0001$, $r = -0.2224$) in CVID patients and in patients with genetically defined PAD ($p < 0.0001$, $r = -0.7966$ for the percentage and $p = 0.0001$, $r = -0.6768$ for B cell numbers).
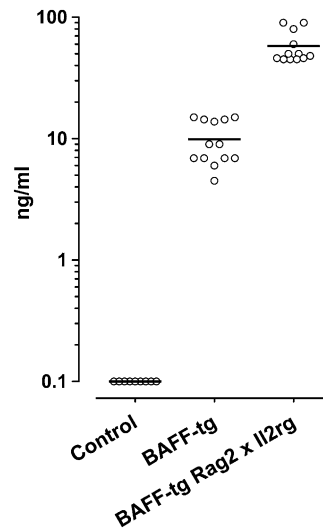

FIGURE 5. Soluble BAFF levels in human BAFF transgenic mice in the presence and absence of B cells. Human BAFF transgenic mice have ~10 ng/ml BAFF on a BALB/c background with normal B cell numbers and 55 ng/ml on the Rag2/Il2rg background, which completely lacks B cells.

transgenic human BAFF Rag2/Il2rg-deficient mice. Because human BAFF binds to murine BAFF-R and TACI expressed by the B cells of wild-type mice, and because the Rag2/Il2rg-deficient mice completely lack B lymphocytes, this result strongly supports our hypothesis that steady-state BAFF levels are to a large extent regulated by its consumption by B cells.

Although the concentrations of human BAFF in wild-type human BAFF transgenic mice were ~4- to 30-fold higher than in healthy controls (0.3–2.25 ng /ml), we did not observe any signs of autoimmunity. However, increased BAFF concentrations led to the 2- to 3-fold expansion of the B cell pool (data not shown), which is consistent with the survival promoting function of BAFF (26).

Discussion

BAFF-induced signals are essential for the development of a functional B cell compartment (1), and the treatment of patients with autoimmune diseases such as SLE with the BAFF-neutralizing Ab belimumab showed that transitional and naive IgD$^+$CD27$^-$ B cells are much more dependent on BAFF-induced survival signals than are IgD$^-$CD27$^+$ switched memory or IgD$^+$CD27$^+$ marginal zone-like B cells (11).

BAFF exists as membrane-bound protein and in a soluble form (27), which has been shown to aggregate into 3-mers or 60-mers

depending on the primary structure of the protein (27–33). The cytokine is produced by hematopoietic (23) as well as by non-hematopoietic cells (34), and its expression has been found to be upregulated by proinflammatory responses (23, 27, 34, 35), during viral infections (25, 36, 37), and in various autoimmune conditions (8, 10, 38–40).

From studies of small cohorts of patients suffering either from common variable immunodeficiency, selective IgA deficiency, or X-linked agammaglobulinemia, increased levels of soluble BAFF have been reported in adolescent as well as in adult patients (16, 17). However, it remained unclear whether the increase in BAFF levels correlated to the size of the B cell pool, the developmental potential of B cells, or to immunodeficiency-related inflammatory conditions (16). BAFF-supported survival of transformed B cells and increased BAFF concentrations have also been reported for B lymphomas and leukemia (41–47), but the direct comparison of these results remains difficult since different BAFF-specific Abs and standards were used.

In this study we report the generation of a new set of mAbs against human BAFF, which we used to study factors regulating soluble BAFF concentrations in humans. To reveal whether the developmental stage of the immune system, the size of the B cell pool, and the availability of BAFF-binding receptors regulate soluble BAFF levels we tested samples from healthy newborns and adults, from patients with severe functional B cell defects such as BTK, BAFF-R, TACI, and AID deficiency, from patients with reduced B cell numbers after splenectomy or during anti-CD20–mediated B cell depletion, as well as in CLL patients who have a highly expanded, clonal B cell population.

By analyzing cord blood, we show that BAFF is present from birth on to support the maturation and survival of human B cells. The vast majority of B cells in newborns, who have in general ~2-fold higher B cell numbers per blood volume unit than do adults, are transitional B cells, which express less BAFF-R than do mature B cells and are more susceptible to apoptosis. Thus, higher BAFF levels in newborns and small children (17) may be a physiological response to ensure the survival of transitional B cells and to support the expansion of a growing B cell compartment.

In healthy adults, soluble BAFF levels are very low (0.6 ± 0.3 ng/ml) or even below the lowest sensitivity limit of our assay,

suggesting that only as much BAFF is produced as is needed to provide sufficient survival signals to maintain and limit the size of the steady-state pool of B lymphocytes. This finding implies that feedback mechanisms exist that adjust BAFF production to the requirements of BAFF-dependent B cell subsets. One of the critical feedback loops seems to be the presence of BAFF-binding receptors. This interpretation is strongly supported by the >5-fold decrease in BAFF levels in human BAFF transgenic mice with a normal B cell compartment compared with their Rag2/Il2rg$^{-/-}$ counterparts. In BTK-deficient patients, who lack mature BAFF-R$^+$ B cells from birth on, BAFF concentrations are up to 150-fold higher than in healthy controls. In BAFF-R–deficient patients, who have up to 10-fold less circulating B cells lacking the receptor with the highest affinity for BAFF, BAFF levels are increased up to 100-fold, whereas in patients with heterozygous TACI C193X mutations, who show normal BAFF-R but reduced TACI expression and ligand binding (48), soluble BAFF levels are increased only up to 25-fold. In contrast, BAFF concentrations are close to normal in the patient carrying a heterozygous TACI A181E mutation, which allows almost normal TACI expression and ligand binding.

The regulation of soluble BAFF levels by steady-state B cell numbers is further supported by the results obtained from analyzing other genetically caused PAD. In this study, BAFF levels were inversely correlated with the percentage of circulating B cells but were independent of the mutations in AID, CD40L, or ICOS affecting different cells and functions required for the generation of switched memory B and plasma cells. Similar observations were made for other, genetically not yet defined primary immunodeficiencies such as Good's syndrome and selective IgM, IgG, and IgA deficiencies. Again, BAFF levels inversely correlated to the numbers and the percentage of circulating B cells.

For CVID patients, a similar relationship between low numbers and percentage of circulating B cells and high BAFF levels was found, although it was not as pronounced as for the genetically defined primary immunodeficiencies since some patients showed both increased BAFF concentrations and normal or even increased B cell numbers. Elevated BAFF concentrations in splenectomized individuals and the lack of correlation between BAFF levels and splenomegaly, which is a sign of splenic dysfunction, indicate that spleen function is not required to maintain BAFF serum concentrations.

In none of the analyzed cohorts of PAD did BAFF concentrations correlate to autoimmunity, splenomegaly, lymphadenopathy, or acute inflammation, as reflected by CRP levels or increased percentage of neutrophils. Therefore, the size of the B cell pool and the availability of BAFF receptors seem to be the primary factors regulating steady-state concentrations of soluble BAFF, although a long-term increase in BAFF levels in response to chronic infections and inflammation cannot be excluded. The differences between our human BAFF transgenic mice and other BAFF transgenic mouse lines in developing autoimmunity may be due to different genetic (7) backgrounds (BALB/c versus C57BL/6) and to differences in BAFF concentrations, which were 10- to 75-fold higher (49) than in our transgenic mice.

In conclusion, our results suggest that the number of B cells and the presence of BAFF-binding receptors determine the concentrations of soluble BAFF. Although multiple parameters that are not well understood act on BAFF-producing cells, the number of B cells and thus of available BAFF receptors seems to be one of the most important regulatory factors adjusting BAFF levels and the size of the B cell pool in adults. Many patients with PAD have low numbers of circulating B cells that are blocked in differentiating into switched memory B cells or plasma cells. Our study shows that in these patients defects in B cell development and function cannot be compensated by increased BAFF concentrations.

References

1. Mackay, F., and P. Schneider. 2009. Cracking the BAFF code. *Nat. Rev. Immunol.* 9: 491–502.
2. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nat. Immunol.* 3: 958–965.
3. Enzler, T., G. Bonizzi, G. J. Silverman, D. C. Otero, G. F. Widhopf, A. Anzelon-Mills, R. C. Rickert, and M. Karin. 2006. Alternative and classical NF-κB signaling retain autoreactive B cells in the splenic marginal zone and result in lupus-like disease. *Immunity* 25: 403–415.
4. Sasaki, Y., E. Derudder, E. Hobeika, R. Pelanda, M. Reth, K. Rajewsky, and M. Schmidt-Supprian. 2006. Canonical NF-κB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity* 24: 729–739.
5. Warnatz, K., U. Salzer, M. Rizzi, B. Fischer, S. Gutenberger, J. Böhm, A. K. Kienzler, Q. Pan-Hammarström, L. Hammarström, M. Rakhmanov, et al. 2009. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proc. Natl. Acad. Sci. USA* 106: 13945–13950.
6. Schiemann, B., J. L. Gommerman, K. Vora, T. G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M. L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111–2114.
7. Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J. Exp. Med.* 190: 1697–1710.
8. Cheema, G. S., V. Roschke, D. M. Hilbert, and W. Stohl. 2001. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum.* 44: 1313–1319.
9. Zhang, J., V. Roschke, K. P. Baker, Z. Wang, G. S. Alarcón, B. J. Fessler, H. Bastian, R. P. Kimberly, and T. Zhou. 2001. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J. Immunol.* 166: 6–10.
10. Groom, J., S. L. Kalled, A. H. Cutler, C. Olson, S. A. Woodcock, P. Schneider, J. Tschopp, T. G. Cachero, M. Batten, J. Wheway, et al. 2002. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjögren's syndrome. *J. Clin. Invest.* 109: 59–68.
11. Jacobi, A. M., W. Huang, T. Wang, W. Freimuth, I. Sanz, R. Furie, M. Mackay, C. Aranow, B. Diamond, and A. Davidson. 2010. Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: extension of a phase II, double-blind, placebo-controlled, dose-ranging study. *Arthritis Rheum.* 62: 201–210.
12. Wallace, D. J., W. Stohl, R. A. Furie, J. R. Lisse, J. D. McKay, J. T. Merrill, M. A. Petri, E. M. Ginzler, W. W. Chatham, W. J. McCune, et al. 2009. A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active systemic lupus erythematosus. *Arthritis Rheum.* 61: 1168–1178.
13. Rauch, M., R. Tussiwand, N. Bosco, and A. G. Rolink. 2009. Crucial role for BAFF-BAFF-R signaling in the survival and maintenance of mature B cells. *PLoS ONE* 4: e5456.
14. Chapel, H., R. Geha, F. Rosen; IUIS PID (Primary Immunodeficiencies) Classification Committee. 2003. Primary immunodeficiency diseases: an update. *Clin. Exp. Immunol.* 132: 9–15.
15. Cunningham-Rundles, C., and C. Bodian. 1999. Common variable immunodeficiency: clinical and immunological features of 248 patients. *Clin. Immunol.* 92: 34–48.
16. Knight, A. K., L. Radigan, T. Marron, A. Langs, L. Zhang, and C. Cunningham-Rundles. 2007. High serum levels of BAFF, APRIL, and TACI in common variable immunodeficiency. *Clin. Immunol.* 124: 182–189.
17. Jin, R., H. Kaneko, H. Suzuki, T. Arai, T. Teramoto, T. Fukao, and N. Kondo. 2008. Age-related changes in BAFF and APRIL profiles and upregulation of BAFF and APRIL expression in patients with primary antibody deficiency. *Int. J. Mol. Med.* 21: 233–238.
18. Swee, L. K., A. Tardivel, P. Schneider, and A. Rolink. 2010. Rescue of the mature B cell compartment in BAFF-deficient mice by treatment with recombinant Fc-BAFF. *Immunol. Lett.* 131: 40–48.
19. Cambridge, G., W. Stohl, M. J. Leandro, T. S. Migone, D. M. Hilbert, and J. C. Edwards. 2006. Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B cell depletion, circulating antibodies, and clinical relapse. *Arthritis Rheum.* 54: 723–732.
20. Lavie, F., C. Miceli-Richard, M. Ittah, J. Sellam, J. E. Gottenberg, and X. Mariette. 2007. Increase of B cell-activating factor of the TNF family (BAFF)

after rituximab treatment: insights into a new regulating system of BAFF production. *Ann. Rheum. Dis.* 66: 700–703.

21. Pers, J. O., V. Devauchelle, C. Daridon, B. Bendaoud, R. Le Berre, A. Bordron, P. Hutin, Y. Renaudineau, M. Dueymes, S. Loisel, et al. 2007. BAFF-modulated repopulation of B lymphocytes in the blood and salivary glands of rituximab-treated patients with Sjögren's syndrome. *Arthritis Rheum.* 56: 1464–1477.

22. Fabris, M., L. Quartuccio, S. Sacco, G. De Marchi, G. Pozzato, C. Mazzaro, G. Ferraccioli, T. S. Migone, and S. De Vita. 2007. B-Lymphocyte stimulator (BLyS) up-regulation in mixed cryoglobulinaemia syndrome and hepatitis-C virus infection. *Rheumatology (Oxford)* 46: 37–43.

23. Moore, P. A., O. Belvedere, A. Orr, K. Pieri, D. W. LaFleur, P. Feng, D. Soppet, M. Charters, R. Gentz, D. Parmelee, et al. 1999. BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* 285: 260–263.

24. Scapini, P., F. Bazzoni, and M. A. Cassatella. 2008. Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunol. Lett.* 116: 1–6.

25. Fontaine, J., J. Chagnon-Choquet, H. S. Valcke, J. Poudrier, M. Roger; Montreal Primary HIV Infection and Long-Term Non-Progressor Study Groups. 2011. High expression levels of B lymphocyte stimulator (BLyS) by dendritic cells correlate with HIV-related B-cell disease progression in humans. *Blood* 117: 145–155.

26. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, et al. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189: 1747–1756.

27. Nardelli, B., O. Belvedere, V. Roschke, P. A. Moore, H. S. Olsen, T. S. Migone, S. Sosnovtseva, J. A. Carrell, P. Feng, J. G. Giri, and D. M. Hilbert. 2001. Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood* 97: 198–204.

28. Bossen, C., T. G. Cachero, A. Tardivel, K. Ingold, L. Willen, M. Dobles, M. L. Scott, A. Maquelin, E. Belnoue, C. A. Siegrist, et al. 2008. TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts. *Blood* 111: 1004–1012.

29. Bossen, C., A. Tardivel, L. Willen, C. A. Fletcher, M. Perroud, F. Beermann, A. G. Rolink, M. L. Scott, F. Mackay, and P. Schneider. 2011. Mutation of the BAFF furin cleavage site impairs B-cell homeostasis and antibody responses. *Eur. J. Immunol.* 41: 787–797.

30. Kim, H. M., K. S. Yu, M. E. Lee, D. R. Shin, Y. S. Kim, S. G. Paik, O. J. Yoo, H. Lee, and J. O. Lee. 2003. Crystal structure of the BAFF-BAFF-R complex and its implications for receptor activation. *Nat. Struct. Biol.* 10: 342–348.

31. Liu, Y., X. Hong, J. Kappler, L. Jiang, R. Zhang, L. Xu, C. H. Pan, W. E. Martin, R. C. Murphy, H. B. Shu, et al. 2003. Ligand-receptor binding revealed by the TNF family member TALL-1. *Nature* 423: 49–56.

32. Liu, Y., L. Xu, N. Opalka, J. Kappler, H. B. Shu, and G. Zhang. 2002. Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. *Cell* 108: 383–394.

33. Zhukovsky, E. A., J. O. Lee, M. Villegas, C. Chan, S. Chu, and C. Mroske. 2004. TNF ligands: is TALL-1 a trimer or a virus-like cluster? *Nature* 427: 413–414, discussion 414.

34. Gorelik, L., K. Gilbride, M. Dobles, S. L. Kalled, D. Zandman, and M. L. Scott. 2003. Normal B cell homeostasis requires B cell activation factor production by radiation-resistant cells. *J. Exp. Med.* 198: 937–945.

35. Scapini, P., B. Nardelli, G. Nadali, F. Calzetti, G. Pizzolo, C. Montecucco, and M. A. Cassatella. 2003. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. *J. Exp. Med.* 197: 297–302.

36. Toubi, E., S. Gordon, A. Kessel, I. Rosner, M. Rozenbaum, Y. Shoenfeld, and E. Zuckerman. 2006. Elevated serum B-lymphocyte activating factor (BAFF) in chronic hepatitis C virus infection: association with autoimmunity. *J. Autoimmun.* 27: 134–139.

37. Ittah, M., C. Miceli-Richard, P. Lebon, C. Pallier, C. Lepajolec, and X. Mariette. 2011. Induction of B cell-activating factor by viral infection is a general phenomenon, but the types of viruses and mechanisms depend on cell type. *J. Innate Immun.* 3: 200–207.

38. Thangarajh, M., A. Gomes, T. Masterman, J. Hillert, and P. Hjelmström. 2004. Expression of B-cell-activating factor of the TNF family (BAFF) and its receptors in multiple sclerosis. *J. Neuroimmunol.* 152: 183–190.

39. Krumbholz, M., U. Specks, M. Wick, S. L. Kalled, D. Jenne, and E. Meinl. 2005. BAFF is elevated in serum of patients with Wegener's granulomatosis. *J. Autoimmun.* 25: 298–302.

40. Krumbholz, M., D. Theil, T. Derfuss, A. Rosenwald, F. Schrader, C. M. Monoranu, S. L. Kalled, D. M. Hess, B. Serafini, F. Aloisi, et al. 2005. BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma. *J. Exp. Med.* 201: 195–200.

41. Novak, A. J., R. J. Bram, N. E. Kay, and D. F. Jelinek. 2002. Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. *Blood* 100: 2973–2979.

42. He, B., A. Chadburn, E. Jou, E. J. Schattner, D. M. Knowles, and A. Cerutti. 2004. Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. *J. Immunol.* 172: 3268–3279.

43. Nishio, M., T. Endo, N. Tsukada, J. Ohata, S. Kitada, J. C. Reed, N. J. Zvaifler, and T. J. Kipps. 2005. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1α. *Blood* 106: 1012–1020.

44. Novak, A. J., D. M. Grote, S. C. Ziesmer, M. P. Kline, M. K. Manske, S. Slager, T. E. Witzig, T. Shanafelt, T. G. Call, N. E. Kay, et al. 2006. Elevated serum B-lymphocyte stimulator levels in patients with familial lymphoproliferative disorders. *J. Clin. Oncol.* 24: 983–987.

45. Bojarska-Junak, A., I. Hus, S. Chocholska, E. Wasik-Szczepanek, M. Sieklucka, A. Dmoszyńska, and J. Roliński. 2009. BAFF and APRIL expression in B-cell chronic lymphocytic leukemia: correlation with biological and clinical features. *Leuk. Res.* 33: 1319–1327.

46. Molica, S., G. Digiesi, F. Mauro, R. Mirabelli, G. Cutrona, G. Vitelli, F. Morabito, F. Iuliano, R. Foà, and M. Ferrarini. 2009. Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukemia. *Leuk. Res.* 33: 162–165.

47. Sawicka-Powierza, J., E. Jablonska, J. Kloczko, J. Piszcz, M. Garley, and W. Ratajczk-Wrona. 2011. Evaluation of TNF superfamily molecules release by neutrophils and B leukemic cells of patients with chronic B-cell lymphocytic leukemia. *Neoplasma* 58: 45–50.

48. Mohammadi, J., C. Liu, A. Aghamohammadi, A. Bergbreiter, L. Du, J. Lu, N. Rezaei, A. A. Amirzargar, M. Moin, U. Salzer, et al. 2009. Novel mutations in TACI (TNFRSF13B) causing common variable immunodeficiency. *J. Clin. Immunol.* 29: 777–785.

49. Batten, M., C. Fletcher, L. G. Ng, J. Groom, J. Wheway, Y. Laâbi, X. Xin, P. Schneider, J. Tschopp, C. R. Mackay, and F. Mackay. 2004. TNF deficiency fails to protect BAFF transgenic mice against autoimmunity and reveals a predisposition to B cell lymphoma. *J. Immunol.* 172: 812–822.