The anti-apoptotic factor Bcl-2 can functionally substitute for the B cell survival but not for the marginal zone B cell differentiation activity of BAFF

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The TNF family ligand B cell-activating factor (BAFF, BLyS, TALL-1) is an essential factor for B cell development. BAFF binds to three receptors, BAFF-R, transmembrane activator and CAML interactor (TACI), and B cell maturation antigen (BCMA), but only BAFF-R is required for successful survival and maturation of splenic B cells. To test whether the effect of BAFF is due to the up-regulation of anti-apoptotic factors, TACI-Ig-transgenic mice, in which BAFF function is inhibited, were crossed with transgenic mice expressing FLICE-inhibitory protein (FLIP) or Bcl-2 in the B cell compartment. FLIP expression did not rescue B cells, while enforced Bcl-2 expression restored peripheral B cells and the ability to mount T-dependent antibody responses. However, many B cells retained immaturity markers and failed to express normal amounts of CD21. Marginal zone B cells were not restored and the T-independent IgG3, but not IgM, response was impaired in the TACI-Ig×Bcl-2 mice. These results suggest that BAFF is required not only to inhibit apoptosis of maturating B cells, but also to promote differentiation events, in particular those leading to the generation of marginal zone B cells.

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1 Introduction

The TNF family ligand B cell-activating factor (BAFF, also known as BLyS or TALL-1) binds three distinct receptors, transmembrane activator and CAML interactor (TACI), B cell maturation antigen (BCMA), and BAFF-R, whereas the related ligand a proliferation-inducing ligand (APRIL) binds only TACI and BCMA [1]. Newly formed B cells in the bone marrow reach the spleen as transitional type-1 B cells that rely on BAFF and BAFF-R, but not TACI, BCMA or APRIL, to further differentiate into transitional type-2, follicular and marginal zone B cells [1]. However, BAFF is not essential for the formation of B1 B cells that populate the peritoneal cavity and develop independently of B2 B cells [2–4]. BAFF has also been implicated in survival of mature B cells, costimulation of BCR-induced proliferation responses, and the CD40 ligand-independent isotype switch [5–7]. TACI is not critically involved in B cell survival, but is required for T-independent type-2 humoral responses against repetitive antigens and also functions as a tumor suppressor in controlling the size of the B cell compartment [8, 9]. In contrast, no function has been assigned yet to BCMA, although its expression pattern suggests a possible role in plasma cells [3, 10].

BAFF-transgenic (Tg) mice experience a dramatic expansion of B2 B cells, in particular the type-2 and marginal zone B cell compartments, and develop autoimmune symptoms resembling human systemic lupus erythematosus and autoimmune Sjögren's syndrome as they age [11–14]. BAFF-blocking agents rapidly decrease the number of mature peripheral B cells *in vivo*

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Abbreviations: BAFF: B cell-activating factor **BCMA:** B cell maturation antigen **TACI:** Transmembrane activator and CAML interactor **APRIL:** A proliferation-inducing ligand **FLIP:** FLICE-inhibitory protein **Tg:** Transgenic **dTg:** Double-transgenic **NP-CGG:** NP^{28}-chicken gamma globulin

and have proved beneficial in treating models of autoimmune disease in the mouse [11, 15, 16].

The type-1 developmental block observed in the absence of BAFF can be interpreted as the lack of a survival signal, or the absence of a differentiation signal, or both. Results from *in vitro* studies rather suggest that BAFF acts as a survival factor on transitional and mature B cells [17, 18]. However, the study of BAFF function on follicular or marginal zone B cells *in vivo* is hampered by the fact that their precursors depend on BAFF.

Expression of cell death inhibitors in B cells should bypass the requirement for BAFF-mediated survival effects *in vivo*. We found that B cell-specific expression of Bcl-2 in a BAFF-low environment rescued peripheral B cells and humoral responses to T-dependent antigens, confirming the anti-apoptotic nature of BAFF. However, marginal zone B cells and T-independent type-2 responses were not or only partially rescued in this model, revealing a hitherto unidentified marginal zone B cell differentiation role for BAFF *in vivo*.

2 Results

To investigate whether anti-apoptotic proteins can substitute for the function of BAFF as a survival and/or maturation factor of B cells *in vivo*, we forced expression of Bcl-2 and FLICE-inhibitory protein (FLIP) in the B cell compartment and investigated their ability to counteract the effects of a BAFF deficiency. Bcl-2 is a potent inhibitor of various pro-apoptotic stimuli converging at mitochondrial-induced cell death, and a Tg mouse line expressing Bcl-2 in B cells has been previously characterized [19]. FLIP is another anti-apoptotic protein that inhibits activity of death receptors by interfering with the activation of pro-caspase-8 [20].

We generated mice expressing FLIP under the B cell-specific promoter of CD19. Expression of the transgene was several-fold higher than that of endogenous FLIP in B cell-rich lymphoid organs such as spleen and lymph nodes (Fig. 1A). In contrast, no Tg FLIP could be detected in the thymus, which mainly contains T cells and stromal cells. Both endogenous and Tg FLIP were detected as full-length molecules and as a processed 43-kDa fragment that is diagnostic of FLIP recruitment into death-inducing signaling complexes of various death receptors [20]. A shorter 22-kDa fragment of the Tg FLIP was also apparent in spleen extracts. According to its size and to the epitope recognized by the antibody, this fragment contains the two death effector domains of FLIP and is also predicted to interfere with apoptosis [20]. Splenic B cells stimulated by LPS *in vitro* are sensitive to cell death triggered by recombinant FasL. As expected, B cell blasts from FLIP-Tg mice were entirely resistant to FasL (Fig. 1B). We conclude that CD19-FLIP-Tg mice express sufficient amounts of FLIP in the B cell compartment to prevent Fas-mediated apoptosis of B cell blasts.

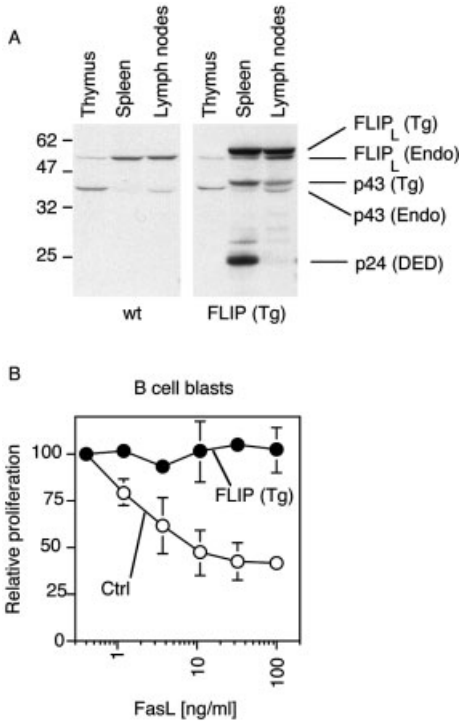

Fig. 1. Generation of B cell-specific FLIP-Tg mice. (A) Western blot analysis of thymus, spleen and lymph node tissue extracts from Tg mice expressing the long form of a Flag-tagged version of FLIP under the control of the CD19 promoter, and from non-Tg littermate (wt). FLIP was detected with the Dave-2 mAb, which recognizes an epitope within the N-terminal portion (death effector domains) of FLIP. DED: death effector domains. Molecular mass standards are in kDa. (B) B cell blasts of FLIP-Tg mice resist deleterious effects of FasL. Two-day-old LPS-induced B cell blast cultures were treated for 24 h with the indicated amounts of recombinant FasL, and proliferation was assessed by thymidine incorporation. Data are normalized to untreated controls. Counts were 19,000 and 19,700 cpm for untreated wt and FLIP-Tg mice, respectively.

The B cell-specific Bcl-2-Tg and FLIP-Tg mice were crossed with TACI-Ig-Tg mice. TACI-Ig mice secrete into their circulation a soluble form of a receptor that binds to both BAFF and APRIL, resulting in a peripheral B cell maturation defect resembling that of BAFF-deficient mice [2–4]. Consequently, lymph nodes of TACI-Ig mice are severely depleted in mature B cells (Fig. 2). Enforced expression of FLIP did neither rescue the deficit of

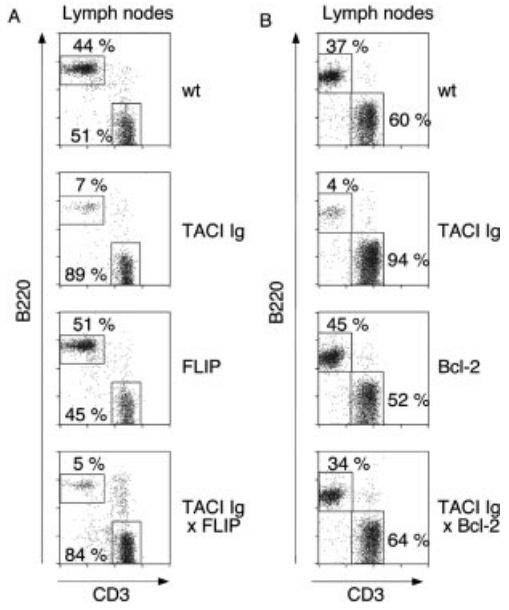

Fig. 2. Enforced expression of Bcl-2 but not FLIP rescues lymph node B cells in TACI-Ig-Tg mice. TACI-Ig-Tg mice were crossed with FLIP-Tg or Bcl-2-Tg mice. B and T cell content of F1 littermate lymph nodes was analyzed by FACS. The indicated percentages refer to gated lymphocytes.

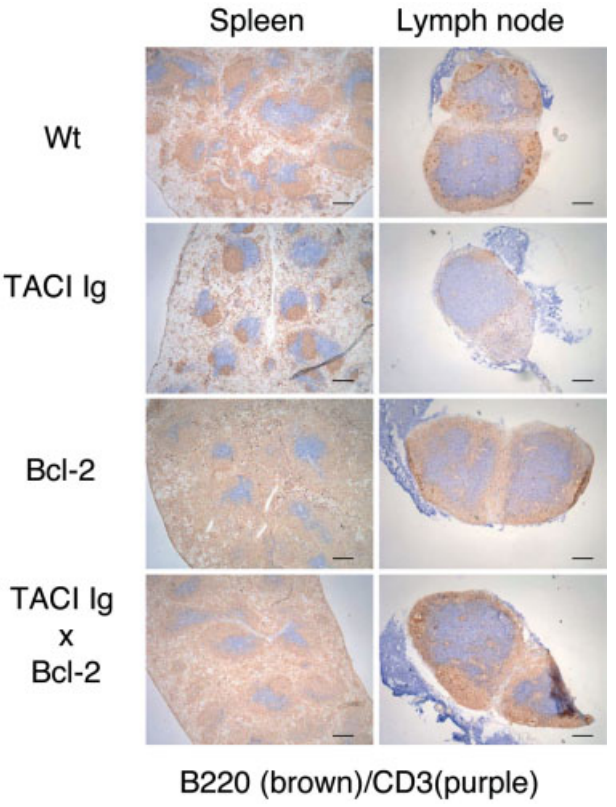

Fig. 3. Immunohistochemistry of frozen spleen and inguinal lymph node sections. Sections were double-stained with B cell (anti-B220, brown) and T cell (anti-CD3ε, purple) markers. Bar = 200 μm.

mature B cells in lymph nodes of TACI-Ig-Tg mice, nor affect its splenic B cell populations (Fig. 2 and data not shown). However, expression of Bcl-2 in B cells restored a close to normal ratio of B to T cells in lymph nodes of TACI-Ig-Tg mice (Fig. 2). These results were corroborated by immunohistochemistry. Lymph nodes of TACI-Ig-Tg mice were practically devoid of B cells, whereas lymph nodes of TACI-Ig×Bcl-2 double-Tg (dTg) mice displayed distinct B cell follicles as in wild-type mice (Fig. 3).

We next assessed whether the B cells that are rescued by Bcl-2 expression in the TACI-Ig environment are functional. TACI-Ig-Tg mice immunized with the T-dependent model antigen NP[28]-chicken gamma globulin (NP-CGG) had a severely impaired anti-NP humoral response, consistent with their deficit in mature B cells. In contrast, TACI-Ig×Bcl-2 dTg mice readily mounted T-dependent humoral responses, indicating that expression of Bcl-2 rescues not only the presence but also the function of B cells (Fig. 4A). We conclude from these experiments that expression of Bcl-2, but not FLIP, can circumvent the need for BAFF in generating lymph node B cells and T-dependent humoral responses.

It is presently unclear whether BAFF acts purely as a survival factor, enabling B cells to live long enough in order to undergo a BAFF-independent differentiation program, or actively in the differentiation of B cells. The TACI-Ig×Bcl-2 dTg mice provide an interesting model to address this question, because it is expected that Bcl-2 mimics the survival function of BAFF, but not its putative differentiation effects.

Bcl-2-Tg, and to a lesser extent TACI-Ig×Bcl-2 dTg mice, have a 2.5- to 5-fold excess of B cells in the spleen and in the blood compared to wild-type mice (Table 1). Spleen sections of these mice also reveal an expanded B cell compartment (Fig. 3). Despite their elevated number of B cells, TACI-Ig×Bcl-2 dTg mice displayed an unusually high proportion of immature splenic B cells (defined as $CD93^+$), which were enriched in the most immature transitional type-1 and type-2 subsets (defined as $CD93^+$, IgM^{high}) (Fig. 5A). Markers of immaturity were also evident in peripheral blood B cells, which were dominated by IgM^{high} cells and contained a higher proportion of L-selectin/CD62L-negative cells (Fig. 5B). A similar observation was made in peritoneal B2 B cells of dTg mice, which mainly displayed an immature phenotype (IgM^{high}, $CD23^-$) (Fig. 5C). Taken together, these

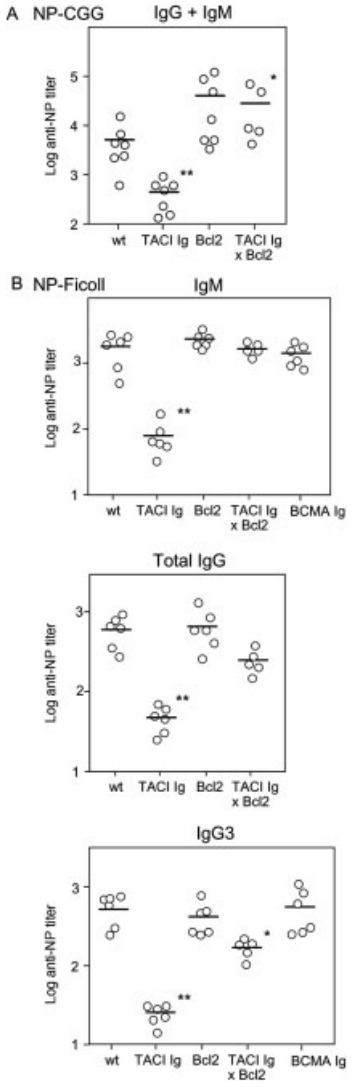

Fig. 4. Enforced expression of Bcl-2 in TACI-Ig-Tg mice rescues T-dependent, but only partially T-independent type-2 antibody responses. Mice were immunized with NP-CGG or NP-Ficoll. Antibody titers (IgG + IgM) were measured after 14 days for NP-CGG and after 8 days for NP-Ficoll (IgM, total IgG, IgG3). *p* values compared to wild type are indicated; $*p<0.05$, $**p<0.005$.

results indicate that although Bcl-2 makes B cells survive, a concomitant depletion of BAFF impairs at least some of the subsequent maturation steps.

In contrast to the B2 B cells, which are strongly affected by the lack of BAFF or by the presence of Bcl-2, the peritoneal B1 B cells ($B220^{int}$, $CD5^{+}$, $CD23^{-}$) remained relatively unaffected by Bcl-2 and TACI-Ig (Fig. 5C; Table 1). Splenic marginal zone B cells ($CD21^{high}$, $CD23^{low}$) displayed yet another pattern of sensitivity to TACI-Ig and Bcl-2. This particular B cell population did not expand in Bcl-2-Tg mice as the absolute number remained comparable to that of wild-type mice (Table 1). In contrast, TACI-Ig mice had virtually no marginal zone B cells, and this population was not rescued in the dTg mice (Fig. 6A). Interestingly, whereas the bulk of wild-type follicular B cells express CD21, this expression is slightly decreased in Bcl-2-Tg mice, and is even more reduced in the spleen, blood and lymph nodes of TACI-Ig-Tg and TACI-Ig×Bcl-2 dTg mice (Fig. 6A and data not shown). Because the identification of marginal zone B cells relies on CD21, a marker that appears to be sensitive to BAFF and Bcl-2 expression levels, we reanalyzed marginal zone B cells in a CD21-independent manner, based on their IgM^{high}, IgD^{low} and $CD1b^{high}$ phenotype [21], and using CD93 to further differentiate these cells from immature B cells. Using these criteria, the absence of marginal zone B cells in TACI-Ig and dTg mice was confirmed (Fig. 6B), indicating that the generation of marginal zone B cells is critically dependent on a differentiation signal provided by BAFF.

As marginal zone B cells are believed to be important for T-independent type-2 responses [22], immunization with NP-Ficoll was performed in the different mice. As expected, antibody titers obtained were comparable in wild-type and Bcl-2-Tg mice, but were much reduced in TACI-Ig-Tg mice (Fig. 4B). dTg mice had a normal IgM response to NP-Ficoll, but yielded significantly lower titers of total IgG, and in particular IgG3 ($p<0.05$) (Fig. 4B). In contrast, no impairment of T-independent type-2 responses was noted in BCMA-Ig-Tg mice, in which only APRIL is blocked (Fig. 4B) [4].

3 Discussion

BAFF is a B cell survival factor for transitional type-2 and other B cells *in vitro* [17, 18, 23]. *In vivo*, absence of BAFF signaling results in a severe deficit of mature B cells in $BAFF^{-/-}$, BAFF-R-deficient and TACI-Ig-Tg mice [2–4, 24, 25]. Therefore we wondered whether the cell death inhibitor FLIP could mediate the survival effects of BAFF. Tg expression of FLIP was sufficient to protect B cell blasts from FasL-induced death but was unable to rescue B cells in the TACI-Ig-Tg mice. We conclude that BAFF is unlikely to mediate its survival function by blocking death receptor-induced apoptosis.

The death receptor Fas plays a role in the homeostatic control of B cells, but most likely acts at a later stage [26, 27]. Consistent with our result, immature splenic B cells are insensitive to Fas triggering [28]. In contrast to FLIP,

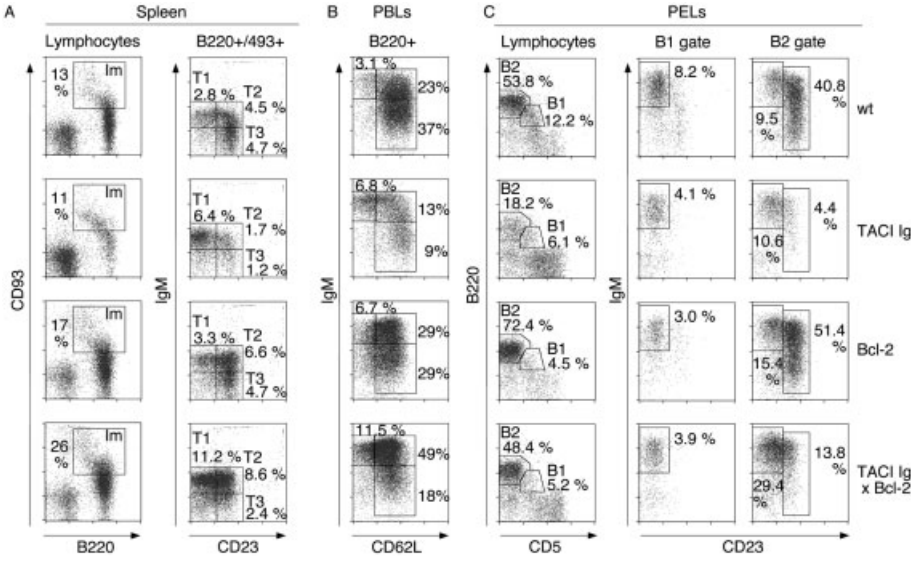

Fig. 5. Enforced expression of Bcl-2 rescues B cells in TACI-Ig-Tg mice, but these cells retain some immature features. (A) Spleen. Four-color FACS analysis of immature splenic B cell populations based on B220, CD93 (C1qRp), CD23 and surface IgM expression. Im: immature B cells; T1: transitional type-1 B cells; T2: transitional type-2 B cells; T3: transitional type-3 B cells. (B) Peripheral blood lymphocytes. Analysis of B cell populations based on the expression of B220, CD62L (L-selectin) and IgM. (C) Peritoneal exudate lymphocytes. Four-color FACS analysis of B cell populations based on B220, CD5, CD23 and surface IgM expression. The indicated percentages always refer to gated lymphocytes.

Tg expression of Bcl-2 restored B cell maturation and function in TACI-Ig-Tg mice, as judged by the rescue of mature lymph node B cells and the ability to mount a T-dependent antibody response. Therefore, in a first approximation, Bcl-2 can functionally replace BAFF. Retroviral expression of Bcl-X_L in BAFF-R-deficient animals was also recently shown to increase B cell numbers in short-term experiments [29]. This is also consistent with the observation that BAFF induces Bcl-2 expression in transitional B cells generated in bone marrow cultures [23].

A straightforward hypothesis is that BAFF induces expression of anti-apoptotic factors of the Bcl-2 family, enabling apoptosis-prone transitional B cells to survive long enough to receive and integrate differentiation signals, such as those leading to CD21 expression. This maturation process is likely coupled to negative-selection events, because BAFF-Tg and Bcl-2-Tg mice, in which B cell survival is increased, develop signs of autoimmunity [12, 13, 19]. Continuous BAFF signaling may be required for maintenance of Bcl-2 family members in mature B cells, as BAFF also promotes survival of these cells *in vitro* [30]. However, this latter issue was not addressed by our experiments.

BAFF may transduce at least some of its survival or differentiation signals via the NF-κB pathway, as B cells with an intrinsic deficiency for both c-Rel and RelA do not mature past the type-1 stage in the spleen. This block can be rescued by enforced Bcl-2 expression [31].

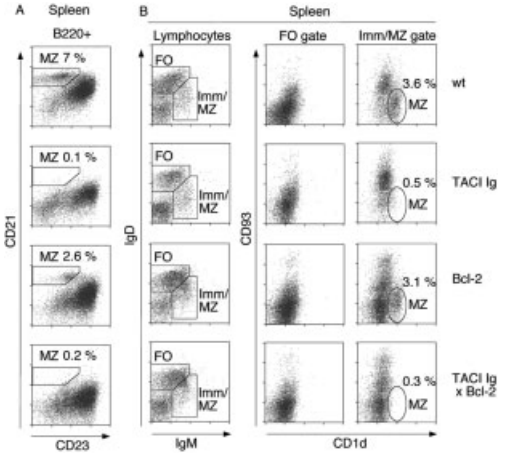

Fig. 6. Marginal zone B cells are not rescued by Bcl-2 expression in TACI-Ig-Tg mice. (A) Spleen. Four-color FACS analysis of marginal zone B cells based on B220, CD21, CD23 and surface IgM expression. Marginal zone B cells were IgM^{bright} (data not shown). (B) Spleen. Four-color FACS analysis of marginal zone B cells based on IgM, IgD, CD1d and CD93 expression. FO: follicular B cells; MZ: marginal zone B cells; MZ/Im: marginal zone and immature B cells. Thirty-week-old mice were used in this experiment. Percentages refer to gated lymphocytes.

Table 1. Lymphocytes counts in wild-type, Bcl-2-, TACI-Ig- and Bcl-2×TACI-Ig-Tg mice

	Wild type		TACI Ig		Bcl-2		TACI Ig x Bcl-2	
	Cells (x10^6) [a]	% [b]	Cells (x10^6)	%	Cells (x10^6)	%	Cells (x10^6)	%
Spleen								
All cells	49.0 ± 11	100	26.0 ± 12	100	193 ± 23	100	112 ± 27	100
T cells	16.3 ± 3.9	33.3 ± 0.6	12.3 ± 3.7	50.0 ± 9.2	25.9 ± 5.6	13.4 ± 2.0	23.0 ± 2.5	21.7 ± 7.9
CD4$^+$	11.9 ± 2.6	24.3 ± 0.5	8.3 ± 2.5	33.5 ± 5.9	18.5 ± 4.6	9.5 ± 1.6	14.5 ± 1.3	13.6 ± 4.3
CD8$^+$	3.9 ± 1.2	7.9 ± 0.9	3.5 ± 1.0	14.4 ± 3.3	6.4 ± 0.9	3.3 ± 0.4	7.5 ± 1.7	7.3 ± 3.5
B cells [c]	30.0 ± 6.7	61.2 ± 0.5	7.6 ± 4.1	28.8 ± 2.5	154 ± 16	79.6 ± 1.3	81.7 ± 28	71.7 ± 7.7
T1	1.4 ± 0.2	3.0 ± 0.5	1.9 ± 1.4	6.7 ± 2.0	5.7 ± 0.2	3.0 ± 0.4	10.6 ± 2.5	9.6 ± 1.5
T2	2.3 ± 0.3	4.9 ± 0.9	0.6 ± 0.6	2.1 ± 1.0	11.8 ± 2.2	6.1 ± 0.7	9.6 ± 2.6	8.5 ± 0.3
T3	2.2 ± 0.4	2.2 ± 0.4	0.4 ± 0.3	1.4 ± 0.5	10.3 ± 4.7	5.2 ± 1.7	2.9 ± 1.0	2.6 ± 0.3
FO	21.6 ± 6.3	43.6 ± 3.7	3.4 ± 1.1	14.0 ± 2.4	109 ± 1.1	57.1 ± 6.6	56.3 ± 16	50.0 ± 5.2
MZ	1.4 ± 0.4	2.8 ± 0.3	0.02 ± 0.02	0.06 ± 0.04	0.9 ± 0.3	0.5 ± 0.2	0.06 ± 0.05	0.06 ± 0.05
Lymph node								
All cells	2.9 ± 1.7	100	3.2 ± 1.3	100	4.6 ± 0.3	100	4.3 ± 1.2	100
T cells	1.7 ± 1.0	60.2 ± 2.7	2.9 ± 1.1	91.2 ± 2.5	2.3 ± 0.2	51.5 ± 4.0	2.8 ± 0.9	65.9 ± 2.0
CD4$^+$	1.1 ± 0.6	38.5 ± 2.5	1.7 ± 0.7	55.4 ± 2.1	1.4 ± 0.1	29.9 ± 3.0	1.6 ± 0.4	38.4 ± 2.3
CD8$^+$	0.6 ± 0.4	20.3 ± 2.6	1.1 ± 0.5	33.9 ± 2.9	0.9 ± 0.1	19.9 ± 1.5	1.1 ± 0.4	25.4 ± 3.8
B cells	1.1 ± 0.6	37.3 ± 2.6	0.2 ± 0.1	6.5 ± 2.0	2.1 ± 0.3	45.9 ± 4.0	1.3 ± 0.3	31.1 ± 2.1
PBLs								
All cells	13.7 ± 2.0	100	11.8 ± 4.4	100	45.2 ± 16.3	100	43.4 ± 5.1	100
T cells	3.7 ± 0.4	27.3 ± 2.4	6.6 ± 2.1	57.2 ± 3.8	3.3 ± 0.3	7.7 ± 1.9	3.8 ± 0.4	8.8 ± 1.8
CD4$^+$	2.9 ± 0.2	21.3 ± 1.8	4.9 ± 1.4	43.1 ± 4.8	1.9 ± 0.1	4.4 ± 1.2	2.2 ± 0.2	5.2 ± 0.9
CD8$^+$	0.7 ± 0.2	5.1 ± 1.3	1.5 ± 0.7	12.2 ± 1.0	1.2 ± 0.3	2.7 ± 0.6	1.3 ± 0.2	3.2 ± 0.8
B cells	9.1 ± 1.7	66.3 ± 3.2	3.5 ± 1.5	29.3 ± 1.4	40.4 ± 16.3	88.6 ± 3.4	37.6 ± 5.2	86.6 ± 2.2
Immat. B	0.4 ± 0.1	2.7 ± 0.7	0.9 ± 0.4	7.6 ± 1.4	4.5 ± 1.8	10.0 ± 0.8	8.0 ± 2.3	18.2 ± 3.3
PEL's								
All cells	3.9 ± 0.7	100	8.7 ± 9	100	8.8 ± 3.9	100	8.6 ± 4.7	100
B1	0.4 ± 0.1	10.2 ± 1.9	0.4 ± 0.3	5.1 ± 1.5	0.4 ± 0.1	5.3 ± 0.9	0.3 ± 0.2	3.9 ± 1.3
B2	1.6 ± 0.3	40.5 ± 9.4	1.0 ± 0.9	13.6 ± 4.1	5.8 ± 3.5	63 ± 15	5.2 ± 3.0	60 ± 11

a) Cell numbers are in million per organ. For LN, two inguinal LN. For PBL, numbers are for 1 ml of blood.

b) % refers to the total lymphocyte population. Three or four mice (9–12-week-old females) were analyzed in each group.

c) Population definition: Spleen: T1 (B220$^+$, 493$^+$, CD23$^-$, IgM^{++}); T2 (B220$^+$, 493$^+$, CD23$^+$, IgM^{++}); T3 (B220$^+$, 493$^+$, CD23$^+$, IgM^{low}); MZ (B220$^+$, CD23$^-$, CD21^{++}, IgM^{++}); FO (B220$^+$, 493$^-$). PBL: Immature B (B220$^+$, CD62L$^-$). PEL: B1 (B220^{int}, CD5$^+$); B2 (B220$^+$, CD5$^-$).

More direct evidences implicate the NEMO-independent, alternative NF-κB pathway downstream of BAFF and BAFF-R, because processing of NF-κB2/p100 to p52 is inducible in immature B cells upon BAFF treatment *in vitro* [32]. In addition, BAFF-induced differentiation of B cells in bone marrow cultures is blocked in NF-κB2$^{-/-}$ cells [23], and BAFF signaling is impaired in cells lacking NIK and IKKα, two components of the alternative NF-κB pathway [23].

The TACI-Ig×Bcl-2 dTg model permits uncoupling of the survival and differentiation functions of BAFF *in vivo*. Our results suggest that BAFF does not solely act as a survival factor, but participates in the maturation of B2 B cells. Indeed, loss of the B cell immaturity marker CD93 is impaired or retarded in TACI-Ig×Bcl-2 dTg mice compared to Bcl-2-Tg mice. In addition, we found that BAFF is required for normal CD21 expression in B cells and for the generation of marginal zone B cells. Importantly, a Bcl-2 transgene rescued the marginal zone B cell deficiency in CD19$^{-/-}$ mice, ruling out the hypothesis that these cells could be unresponsive to Bcl-2 [33].

In B cells, CD21 (complement receptor 2) is part of the B cell coreceptor complex. In marginal zone B cells, its engagement by blood-borne antigens opsonized with C3d is believed to provide the basis for T-independent type-2 antibody responses, in particular of IgG2a and IgG3 isotypes [22, 34]. The impaired IgG3 response to NP-Ficoll in TACI-Ig×Bcl-2 dTg mice is consistent with their lack of marginal zone B cells and is comparable to that observed in the marginal zone B cell-deficient Pyk-2$^{-/-}$ mice [34]. Neither Pyk-2 nor TACI-Ig×Bcl-2 mice have significantly impaired IgM responses to NP-Ficoll, and their IgG3 responses are reduced but not abolished.

This suggests that cells other than marginal zone B cells participate to T-independent type-2 responses.

In our model of TACI-Ig-Tg mice, both BAFF and APRIL are inhibited by TACI-Ig. B cell survival and emergence of marginal zone B cells are probably only dependent on BAFF and BAFF-R, because $TACI^{-/-}$ and $BCMA^{-/-}$ mice have mature B cell and marginal zone B cell populations [3, 9]. T-independent type-2 responses may, however, depend on both BAFF and/or APRIL, because they are mediated, at least in part, through TACI [9, 35] and are enhanced in APRIL-Tg mice [36]. In addition, both BAFF and APRIL induce CD40L-independent isotype switch in human peripheral blood B cells *in vitro* [7]. However, the unimpaired T-independent antibody responses found in BCMA-Ig-Tg mice (which block APRIL but not BAFF) argue against an essential, non-redundant role of APRIL in this respect.

RelB and p50, two subunits of the NF-κB transcription factor, are required for marginal zone B cell formation [37, 38] and may mediate BAFF signaling in that respect. RelB is likely to heterodimerize with NF-κB2/p52 and to participate in the alternative NF-κB pathway. p50 may also play role in this pathway, because the sustained, NEMO-independent NF-κB response induced by the TNF family ligand TWEAK contains not only p52 and RelB, but also a prominent fraction of the NF-κB1/p50 transcription factor [39].

The balance between marginal zone versus follicular B cell development is also regulated by Aiolos and Btk [21], whereas CD19 is important for the survival and differentiation of several B cell populations, including B1 and marginal zone B cells [33]. Inactivation of the repressor activity of RBP-J by Notch signaling promotes marginal zone B cell formation, whereas the Notch-antagonizing protein MINT prevents it [40, 41]. Lack of marginal zone B cells is also observed upon deficiencies of Pyk-2, DOCK-2 and Lsc, three proteins implicated in migratory responses to chemokines [34, 42–44], and of the transcriptional co-activator BOB.1/OBF.1/OCA-B [45]. Interestingly, neither BOB.1/OBF.1/OCA-B, nor RBP-J or BAFF deficiencies affects the B1 B cell population [2, 3, 41, 45]. It will be of interest to assess whether and how the aforementioned proteins are related to BAFF signaling.

In conclusion, our results indicate that BAFF is both a survival and a differentiation factor for B cells, and that these two functions can be distinguished. In particular, BAFF allows differentiation of marginal zone B cells independently of its survival activity.

4 Materials and methods

4.1 Transgenic mice

TACI-Ig- and BCMA-Ig-Tg mice have been described previously [4]. These mice express the transgenes under the control of the human α1 anti-trypsin promoter and display a serum concentration of 5 μg/ml of TACI-Ig and up to 100 μg/ml of BCMA-Ig [4]. The C57BL/6-TgN(BCL2)22Wehi mice were obtained from the Jackson Laboratories. This line expresses Bcl-2 under the control of the Eμ promoter in the B cell compartment [19]. Transgene was detected by PCR on tail genomic DNA using oligonucleotides JT2519 5'-GGTCATGTGTGTGGAGAGCGTCA-3' and JT2520 5'-TCACACCACAGAAGTAAGGTTCC-3'.

The Tg vector, pCD19-FLIP, encoding Flag-muFLIP under the control of the B cell-specific CD19 promoter was constructed in the pBSKS II(–) vector and contained (a) in the NotI site, a 6.4-kb EagI fragment of vector CD19 XS (containing nucleotides –6435 to –15 of hCD19) [46]; (b) in the BamHI/XhoI sites, nucleotides 421–1592 of rabbit β-globin (comprising the end of exon 2, intron 2, exon 3 and the poly A addition signal) [4]; (c) in the blunted EcoRI site of the β-globin sequence, a 1525-nucleotide sequence encoding Flag-mouse $FLIP_L$ (MDYKDDDDKEFGL followed by amino acid residues 2–481 of mouse $FLIP_L$) [20]. Tg mice were generated by microinjection of the BssHII fragment of pCD19-FLIP into fertilized (C57BL/6×DBA/2) F2 oocytes, and screened by PCR using oligonucleotides JT1822 5'-TCAAGAGTGAGGCGGTTTGACC-3' and JT1823 5'-TCCTGATTCCTGGATGGATGTC-3'.

4.2 FACS analysis and monoclonal antibodies

The following antibodies were purchased from BD Biosciences (San Jose, CA): anti-CD8a-PE (53–6.7), anti-CD5-PE (53–7.3), anti-CD21-FITC (7G6), anti-CD23-PE (B3B4), anti-B220-CyChrome (RA3–6B2), anti-CD1d-FITC (1B1) and anti-IgM-biotin (1B4B1). Anti-CD3ε-FITC (17A2), anti-CD4-biotin (GK1.5), anti-B220-Cy5 (RA3.6B2), anti-IgD-Cy5 (11–26c.2a) and anti-IgMb-PE (MB86) were produced and conjugated in our laboratory. Anti-CD62L-PE (MEL-14) was from Caltag (Burlingame, AL). Biotinylated anti-C1qRp (anti-CD93) mAb 493 was used as described [30]. Streptavidin-allophycocyanin (Molecular Probes, Leiden, The Netherlands) was used to reveal biotin conjugates. Cells were treated with anti-CD16/CD32 (as hybridoma supernatants of clone 2.4G2) prior to staining to block FcR binding and analyzed using a four-color FACSCalibur™ flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). Dead cells and debris were gated out using FSC and SSC during acquisition.

4.3 Immunohistochemistry

Acetone-fixed 7-μm frozen sections of spleen and inguinal lymph nodes were stained with anti-CD3ε-biotin (145–2C11) and anti-B220 (RA3–6B2) (both from BD Biosciences) as previously described [4].

4.4 Immunizations

Nine-to-twelve-week-old F1 mice from TACI-Ig-Tg×Bcl-2-Tg crosses, or BCMA-Ig-Tg mice were immunized i.p. with a single injection of 50 μg of NP-CGG or 10 μg of NP^{59}-Ficoll in PBS (Biosearch Technologies). NP-CGG at 1 mg/ml in water was precipitated for 30 min with one volume of 9% alum (Sigma) at pH 8, recovered by centrifugation, washed twice with PBS and resuspended in PBS for injection. Tail vein blood was collected at days 8 and 14 post-immunization and specific anti-NP antibody titers in the serum were determined by ELISA.

For this purpose, plates were coated for 16 h with 10 μg/ml of NP25 BSA (Biosearch Technologies) in 50 mM sodium carbonate buffer pH 9.6, blocked with 4% skimmed milk, 0.5% Tween-20 in PBS, washed and incubated with serum (1:100 and threefold dilutions in block buffer; for TACI-Ig mice, the first serum dilution was 1:50). Bound antibodies were revealed with goat anti-mouse IgG (1:2,000; Jackson ImmunoResearch, West Grove PA), goat anti-mouse IgG+M (1:2,000; Caltag), biotinylated goat anti-mouse IgM (1:500; Caltag), or horseradish peroxidase-coupled goat anti-IgG3 (1:750; Caltag). When required, secondary reagents were horseradish peroxidase-coupled mouse anti-goat and horseradish peroxidase-coupled streptavidin (1:4,000; Jackson ImmunoResearch). Enzymatic activity was measured at 490 nm with o-phenylenediamine reagent (Sigma). Titer was defined as the dilution giving half-maximal signal. *p* values were determined on log of titers using the paired *t*-test.

4.5 Splenocyte survival assay

Spleens of FLIP-Tg and non-Tg littermates were mechanically disrupted using loose-fitting Dounce homogenizer. Splenocytes (triplicates of 150,000 cells/well, 100 μl, 96-well plate) were cultured in RPMI, 10% FCS, 50 μM β-mercaptoethanol in the presence of 10 μg/ml LPS (Sigma). At time 48 h, Flag-hFasL was added at the indicated concentration, in the presence of anti-Flag at 1 μg/ml (Apotech, Lausen, Switzerland) for an additional 24 h. At that time, 0.5 μCi [^{3}H]thymidine was added to each well, and cells were harvested 16 h later. [^{3}H]Thymidine incorporation was determined by scintillation using a TopCount counter (Packard). Values were expressed as mean ± standard deviation and normalized to that of cells without FasL treatment.

4.6 Western blot

Spleen, inguinal lymph nodes and thymus of FLIP-Tg mice and non-Tg littermates were collected. Cell extracts were prepared in lysis buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, Complete™ protease inhibitor mix (Roche)] and 30 μg of extracts were analyzed by Western blotting. FLIP was revealed using the rat mAb Dave-2 (2 μg/ml, 16 h incubation), followed by horseradish peroxidase-coupled goat anti-rat antibodies (1:4,000; Southern Biotechnologies Associate) and ECL (Amersham).

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