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BAFF Receptor Signaling Aids the Differentiation of Immature B Cells into Transitional B Cells following Tonic BCR Signaling

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BAFF is an important prosurvival cytokine for mature B cells. However, previous studies have shown that BAFRR is already expressed at the immature B cell stage, and that the prosurvival protein Bc-12 does not completely complement the B cell defects resulting from the absence of BAFFR or BAFF. Thus, we hypothesized that BAFF also functions to aid the differentiation of nonautoreactive immature B cells into transitional B cells and to promote their positive selection. We found that BAFFR is expressed at higher levels on nonautoreactive than on autoreactive immature B cells and that its expression correlates with that of surface IgM and with tonic BCR signaling. Our data indicate that BAFFR signaling enhances the generation of transitional CD23<sup>+</sup> B cells in vitro by increasing cell survival. In vivo, however, BAFFR signaling is dispensable for the generation of CD23<sup>+</sup> transitional B cells in the bone marrow, but it is important for the development of transitional CD23<sup>+</sup> T1 B cells in the spleen. Additionally, we show that BAFF is essential for the differentiation of CD23<sup>+</sup> into CD23<sup>+</sup> transitional B cells both in vitro and in vivo through a mechanism distinct from that mediating cell survival, but requiring tonic BCR signaling. In summary, our data indicate that BAFFR and tonic BCR signals cooperate to enable nonautoreactive immature B cells to differentiate into transitional B cells and to be positively selected into the naïve B cell repertoire. The Journal of Immunology, 2010, 185: 4570–4581.

Cytokines act on cells of the immune system to regulate and coordinate their survival, differentiation, and activity. During the last decade, the cytokine BAFF (also known as BlyS) has been defined as a critically important and specific factor that promotes the survival of transitional T2, follicular, and marginal zone B cells (1–6). The ability of BAFF to promote B cell survival is mediated specifically through its binding to BAFFR (also known as BR3), as indicated by the similar phenotypes of mutant mice lacking BAFFR or BAFF, as well as the differences from those lacking other BAFFRs, such as transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation Ag (5, 7–9). Moreover, evidence indicates that BAFFR signaling mediates B cell survival by preventing TRAF2/TRAF3 from inhibiting the alternative NF-κB pathway (10–12).

In recent years it has been established that the BCR generates a ligand-independent tonic signal that is also important for the survival of B cells (13–15). This signal, moreover, synergizes with those of cytokine receptors such as IL-7R and BAFFR to promote the survival of B lymphocytes at different stages of differentiation.

Specifically, during early B cell development the pre-BCR and the IL-7R synergize to promote survival and proliferation of pre-B cells (16, 17). In naive mature B cells both tonic BCR and BAFFR signals are necessary for B cell survival, as demonstrated by the lack of mature B cells in the absence of either signal (9, 13). Immature B cells are the first developing B cells to express a mature BCR, in the form of IgM, on the cell surface. Whereas immature B cells still express the IL-7R, they appear to respond minimally to IL-7 (18). In the bone marrow, immature B cells undergo a selection process that eliminates self-reactive specificities and generates the naive mature B cell repertoire in the periphery (reviewed in Refs. 19–21). Interestingly, no cytokine so far has been found to be necessary for the survival of immature B cells during these selection events, nor for aiding their differentiation into transitional and mature B cells.

Newly developed IgM<sup>+</sup>IgD<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> bone marrow immature B cells undergo differentiation into mature peripheral B cells through an intermediate step in development called the transitional stage (22). Transitional B cells are found in both bone marrow and spleen and retain high levels of CD24 and CD93 expression seen on immature B cells, but they also display variable expression of IgD, CD21, and CD23 (22–25). In fact, splenic transitional B cells have been further classified into three subpopulations based on differential levels of expression of these markers (26, 27): T1 (IgM<sup>high</sup>IgD<sup>low</sup>CD21<sup>+/−</sup>CD23<sup>−/−</sup>), T2 (IgM<sup>low</sup>IgD<sup>−</sup>CD21<sup>−</sup>CD23<sup>+</sup>), and T3 (IgM<sup>+</sup>IgD<sup>+</sup>CD21<sup>−</sup>CD23<sup>+</sup>), with T1 cells being developmental precursors of both T2 and T3 cells. Although a similarly precise definition of transitional B cells in the bone marrow is lacking, cells resembling splenic transitional T1 and T2 B cells are present also in this tissue (22, 25, 26).

B cell dependency on BAFF is currently considered to begin at the transitional T2 B cell stage of development in the spleen (4, 5, 9), a stage that displays a noteworthy increase in the expression of BAFFR (28). However, development of BAFFR-deficient B cells has been only minimally assessed in competition with wild-type B cells (29), which is a more stringent test of
B cell development (30, 31). In fact, some evidence suggests a potential role for BAFF and BAFFR at B cell stages earlier than T2. For instance, a small reduction in the number of transitional T1 B cells was observed in the spleen of BAFF and BAFFR mutant mice, although this difference was not statistically significant (5, 9, 32). Additionally, BrdU incorporation studies have demonstrated a reduced export of bone marrow immature B cells into the spleen of BAFFR-deficient mice (7). BAFF was also suggested to promote either the in vitro differentiation of bone marrow immature B cells or to increase the survival of newly generated transitional B cells (10). Overall, these data suggest that BAFF may also function at the immature and transitional T1 B cell stages.

It has been shown both in mice and humans that 30–50% of the newly generated immature B cells are nonautoreactive and presumably undergo positive selection into the spleen, while as many as 50–70% are autoreactive and potentially subjected to mechanisms of negative selection (33, 34). Interestingly, other studies have shown that only approximately half of wild-type mature bone marrow B cells express BAFFR and bind BAFF (28, 35), suggesting that BAFF may play a role in the process of immature B cell selection. In the studies presented in this paper, we tested the hypothesis that BAFFR is expressed by nonautoreactive, but not autoreactive, immature B cells. Additionally, we evaluated whether BAFFR expression and signaling promote the differentiation of nonautoreactive immature B cells into transitional B cells, thus contributing to the positive selection process.

We found that BAFFR is indeed expressed at higher levels on nonautoreactive than on autoreactive immature B cells, and that BAFF aids the positive selection and differentiation of nonautoreactive immature B cells only when these cells also receive sufficient tonic BCR signals. Moreover, we show that BAFFR is important for the differentiation of immature B cells into CD23+ as well as CD23- transitional B cells, but potentially via different molecular pathways.

Materials and Methods

Mice

The 3-83Igi.H-2b (IgM, IgG2a, IgG2b) (36, 37), 3-83Igi.Rag1−/−, H-2b (IgG2a, IgG2b) (37, 38), B1-8/3-83Igi.H-2b or H-2a (IgG2a, IgG2b) (38), 3-83Igi-low (IgG1, IgG2b) (39), and 3-83Igi-mb1−/− (IgG1, IgG2b, IgG2a) (39) mice have been previously described and were all on a BALB/c genetic background. BAFFR−/− (tnfrsf13c−/−) mice on a C57BL/6 genetic background have been previously described (9) and were obtained from The Jackson Laboratory (Bar Harbor, ME). A/WySn, C57BL/6, C57BL/6.Ly5.1 (C57BL/6 mice congenic for Ly5.1, also known as B6SIL), BALB/c, and CB17 mice were purchased from The Jackson Laboratory and either used immediately or bred in our facility. All mice were bred and maintained in specific pathogen-free rooms at the Biological Research Center at National Jewish Health (Denver, CO). All animal experiments were approved by the Institutional Animal Care and Use Committee.

Flow cytometry and Abs

Single-cell suspensions were stained with fluorescent or biotinylated mAbs against B220 (RA3-6B2), CD19 (1D3) (42), and CD23+ (7G6), CD21, and their CD23+ isotype controls (BD Biosciences), or a CyAn analyzer (Beckman Coulter, Fullerton, CA). Flow cytometric analyses were performed on live B220+ or CD19+ lymphocytes based on incorporation of propidium iodide and/or forward and side scatter with FlowJo software (Tree Star, Ashland, OR).

Cell sorting and microarray analysis

Bone marrow cells were isolated from femurs, tibias, and pelvises of 3-83Igi, H-2b and 3-83Igi.Rag1−/−H-2b groups of mice (eight mice per group), pooled, and stained with anti-CD43, anti-IgD, anti-CD23, and anti-B220 Abs. Stained cells were sorted for B220+CD23−CD43+ IgD+ (immature B cells) at ~7000 events/s using a MoFlo cell sorter (Beckman Coulter, Fullerton, CA). Sorted cells (~1–5 × 10⁶) were analyzed for purity by comparing to unsorted samples using a FACSCalibur (BD Biosciences). The purity was >95% in all sorted cell samples. Cell pellets were flash frozen in liquid nitrogen for 15 s and stored at −80°C until Miltenyi Biotec (Auburn, CA). RNA preparation, amplification, and hybridization to Agilent Whole Mouse Genome Oligo Microarrays 4 × 4K were performed by Miltenyi Biotec. The microarray data have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (43) and are accessible through the series accession no. GSE22802 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22802).

Retroviral constructs and production of retroviral particles

The murine tnfrsf13c cDNA (encoding BAFFR) was amplified with primers NotIATAATTTCGACTGATGAGAAGCTTTACTGGGGCG (coding for BAFFR-5-NotI) and SalITCTAGAGCGGCCTTGCCGCTCG (coding for BAFFR-3-SalI) (5′- TATGCGGCCGCTGTCCCAGCTGCATGAG-3′ and Accu-Taq polymerase (Sigma-Aldrich, St. Louis, MO) from cDNA synthesized from BALB/c RNA. The PCR product was cloned between NotI and SalI in the retroviral pMSCV-ires-GFP vector (39) to generate the pMSCV-BAFFR-IRES-GFP plasmid. The pMSCV-Flag-BcI2-ires-Thyl.1 (44) and pMSCV-GFP-ires-Ih-RasG12D (39) retroviral vectors were described before. All retroviral vectors were produced in replication-defective viruses. Retroviruses were produced in Phoenix cells as previously described (39).

Generation of bone marrow chimera and retrogenic mice

Recipient mice were irradiated as described (45). For nonretrogene bone marrow chimera donors, bone marrow cells depleted of RBCs were mixed at the indicated ratios and a total of 1–2 × 10⁶ cells in 100 μl of PBS were injected in a tail vein of each recipient. When generating retrogenic mice, donor mice were injected i.p. with 3.75 mg of 5-fluorouracil 4 d prior to bone marrow harvest. Bone marrow cells isolated from 5-fluorouracil–treated donor mice were cultured overnight at 1 × 10⁶ cells/ml in complete IMDM (Invitrogen, Carlsbad, CA) (100 μg/ml Streptomycin, 100 μg/ml penicillin, 0.05 mM β-mercaptoethanol, 2 nM Glutamax, 0.1 mM nonessential amino acids, 10% heat-inactivated FBS) supplemented with recombinant cytokines (IL-3, IL-6, and stem cell factor, a gift of Dr. Yosef Refaeli, National Jewish Health, Denver, CO). After 24 h, the cells were resuspended in 0.5 ml of complete IMDM, 1 ml of Phoenix cell supernatant containing retrovirus particles, 3.2 μg (2.1 μg/ml final) of polybrene, and recombinant cytokines and centrifuged at 1124 × g for 1.5 h at room temperature. Cells were cultured overnight in fresh complete medium with recombinant cytokines and transduced a second time as described above. Following transduction, recipient mice received a total of 1–5 × 10⁶ cells in 100 μl of PBS via tail vein injection. Mice were analyzed 5–8 wk later.

In vitro B cell differentiation and immature B cell transduction

Bone marrow cells were cultured in complete IMDM in the presence of IL-7 for 3–4 d to enrich for IgM+ immature B cells. Cells were then washed twice with PBS to remove IL-7 and plated at 2–4 × 10⁶ cells/ml with 10 ng/ml recombinant murine BAFF (R&D Systems) or as otherwise indicated. On subsequent days, cells were stained with Abs against B220, CD21, CD23, IgM, and IgD to determine differentiation state. For immature B cell transduction, bone marrow cells were cultured in IL-7 as above for 2–3 d and subsequently resuspended at 4 × 10⁶–10⁷ cells/ml in a mixture consisting of 0.5 ml of complete IMDM, 1 ml of retroviral supernatant, 3.2 μg of polybrene (2.1 μg/ml final), and IL-7. Cells were transfected at 1124 × g for 1.5 h at room temperature and cultured overnight in fresh complete medium containing IL-7.

Statistical analysis

Statistical significance was calculated with Graphpad Prism software using a one-tailed Student’s t test with equal variance (using Welch’s correction when appropriate). A value of p < 0.05 was considered significant. Data are represented as means ± SD.

Results

BAFFR is differentially expressed in autoreactive and nonautoreactive immature B cells

To test the hypothesis that BAFFR is differentially expressed by autoreactive and nonautoreactive developing B cells, we analyzed
immature B cells from mouse models that generate a monoclonal population of either nonautoimmune or autoreactive immature B cells (Supplemental Table 1). The 3-83Igi mice have been previously established as a model to analyze development and selection of immature B cells with predefined Ag reactivity (37, 46). In these H and L chain gene-targeted mice, all B cells develop expressing the 3-83 Ig. When maintained on an H-2d genetic background, newly developed 3-83Ig+ (3-83H/H-2k) immature B cells are nonautoreactive and develop into mature B cells that largely (>90%) retain the 3-83 specificity (36, 37, 47). In contrast, newly developed 3-83Ig+ B cells from 3-83Igi,H-2b mice on a Rag1−/− genetic background (3-83Igi,Rag1−/−,H-2b) represent a uniform population of autoreactive immature B cells because of the high-avidity binding of 3-83 Ig to the MHC class I protein Kb (37, 46) and the absence of receptor editing due to the deficiency of Rag1 (37, 38).

Bone marrow cells from nonautoreactive 3-83Igi,H-2d and autoreactive 3-83Igi,Rag1−/−,H-2b mice were analyzed for B220, IgD, and BAFFR expression. Fig. 1A shows BAFFR expression on B220+IgD−-gated lymphocytes, which mostly represent immature B cells in 3-83Igi mice, as pro-B cells are relatively few, and Ig H and L chain gene-targeted mice do not generate pre-B cells (46). As shown in the histogram representation, nonautoreactive immature B cells uniformly expressed BAFFR on the cell surface. The specificity of the anti-BAFFR Abs was indicated by the absence of staining on B220− non-B lineage cells (Fig. 1A) and BAFFR-deficient B cells (data not shown). In accordance with our hypothesis, we found that BAFFR was not detected, or was detected only at very low levels, on the surface of autoreactive immature B cells (Fig. 1A, histogram). On average, the expression of BAFFR on autoreactive immature B cells was ∼3.8-fold (±1.1, n = 3) lower than that detected on nonautoreactive immature B cells, and only slightly above the background level observed on non-B lineage cells.

Differential expression of BAFFR by immature B cells was also confirmed at the gene transcription level. Nonautoreactive and autoreactive immature B cells were sorted from bone marrow of 3-83Igi,H-2d and 3-83Igi,Rag1−/−,H-2b mice, respectively, as B220+CD23−CD43−IgD+ cells. After RNA isolation and cDNA preparation, their cDNAs were hybridized to Agilent whole mouse genome microarrays. Transcript levels of BAFFR, were found to be ∼4-fold higher in nonautoreactive immature B cells from 3-83Igi,H-2d mice, respectively, as compared to those of nonautoreactive immature B cells from 3-83Igi,Rag1−/−,H-2b mice (Fig. 2A). Importantly, BAFFR levels were higher on dual BCR-expressing nonautoreactive immature B cells from two groups each of non-auto 3-83Igi,H-2d (non-aut, intact line) and 3-83Igi,Rag1−/−,H-2b (aut, dashed line) mice, and on B220− non-B cells (filled histogram). Data are representative of three mice per group analyzed in three independent experiments. B, Bone marrow B220+IgD+ CD23− CD43− immature B cells from two groups each of non-auto 3-83Igi,H-2d (black bar) and aut 3-83Igi,Rag1−/−,H-2b (gray bar) mice were sorted via MoFlo to >95% purity and subjected to whole genome microarray analyses. The relative BAFFR mRNA levels (arithmetic mean of normalized processed signals from two groups of mice) in the immature B cell samples are shown. Differences between the signals had values of p < 10−12. aut, autoreactive; non-aut, nonautoreactive.

FIGURE 1. BAFFR is differentially expressed by nonautoreactive and autoreactive immature B cells. A, Bone marrow cells from non-aut 3-83Igi,H-2d and aut 3-83Igi,Rag1−/−,H-2b mice were analyzed by flow cytometry for expression of B220, IgD, and BAFFR. Dot plots show expression of B220 and IgD on live lymphoid cells. Histograms display BAFFR levels on gated B220+IgD− cells (representing mostly immature B cells) from 3-83Igi,H-2d (non-aut, intact line) and 3-83Igi,Rag1−/−,H-2b (aut, dashed line) mice, and on B220− non-B cells (filled histogram). Data are representative of three mice per group analyzed in three independent experiments. B, Bone marrow B220+IgD+ CD23− CD43− immature B cells from two groups each of non-auto 3-83Igi,H-2d (black bar) and aut 3-83Igi,Rag1−/−,H-2b (gray bar) mice were sorted via MoFlo to >95% purity and subjected to whole genome microarray analyses. The relative BAFFR mRNA levels (arithmetic mean of normalized processed signals from two groups of mice) in the immature B cell samples are shown. Differences between the signals had values of p < 10−12. aut, autoreactive; non-aut, nonautoreactive.

BAFFR expression correlates with IgM expression and tonic BCR signaling in immature B cells

The low expression of BAFFR observed in autoreactive immature B cells could indicate suppression by autoantigen-mediated BCR signaling, or lack of induction in the absence of tonic BCR signals. To assess these two possibilities we analyzed BAFFR levels on immature B cells from additional mouse strains. The B1-8/3-83Igi, H-2d mouse strain generates immature B cells, with each cell coexpressing autoreactive (3-83H/H-2k) and nonautoreactive (B1-8/H-2k/H-2b) BCRs (Supplemental Table 1). As a consequence, these dual BCR-expressing B cells receive both autoantigen-mediated and tonic BCR signals (38). In contrast, when the B1-8/3-83Igi mice are on an H-2d background, both BCRs are nonautoreactive and B cells receive only tonic BCR signals (Supplemental Table 1). When assessed by flow cytometry we found that IgD+ immature B cells that coexpress autoreactive and nonautoreactive BCRs (B1-8/3-83Igi,H-2d) displayed surface BAFFR at levels that were 2.3-fold (±0.6, n = 3) reduced relative to those of nonautoreactive B1-8/3-83Igi,H-2d cells (Fig. 2A, bottom panel). Importantly, BAFFR levels were higher on dual BCR-expressing (B1-8/3-83Igi/H-2d) than on single BCR-expressing (3-83Igi,Rag1−/−,H-2b) autoreactive immature B cells, but nevertheless they were lower than those on nonautoreactive (3-83Igi,H-2d) and B1-8/3-83Igi,H-2b) immature B cells. Also of significance, IgD− B1-8/3-83Igi,H-2b immature B cells express, on average, half the amount of surface IgM when compared with dual BCR-expressing nonautoreactive immature B cells from B1-8/3-83Igi,H-2d mice (Fig. 2A, top panel, and Ref. 38), a level potentially resulting in diminished tonic BCR signaling (39). These data suggest that tonic, and not autoantigen-mediated, BCR signals regulate BAFFR levels on immature B cells. To test this idea further, we used another mouse strain, 3-83Igi-low (Supplemental Table 1), that is a hypomorphic Ig-α strain in which 3-83Ig+ nonautoreactive immature B cells express reduced amounts of
mature B cells that express normal levels (3-83Igi,H-2d mice, Normal, intact line) or low levels (3-83Igi-low mice, Low, dashed line) of nonautoreactive BCRs (B1-8/3-83Igi,H-2d mice, dual-non-aut, SP, gray filled histogram) is shown as a negative control. BAFFR expression on spleen CD19+ B cells from B1-8/3-83Igi,H-2d mice (dual–non-aut,SP, gray filled histogram) and BAFFR expression on B220+IgD-GFP control-transduced (Low+GFP, dotted line) and N-RasD12–transduced (Low+RasD12, black line) cells from 3-83Igi-low mice. Immature B cells were transduced or not. Shown are BAFFR levels on BCR-normal nontransduced cells from 3-83Igi,H-2d mice (Normal, filled histogram) and on BCR-low GFP control-transduced (Low-GFP, dotted line) and N-RasD12–transduced (Low+RasD12, black line) cells from 3-83Igi-low mice. Immature B cells were gated as B220+IgD− (Normal) or B220+IgD+ GFP+ (Low+GFP and Low+RasD12).

FIGURE 2. Expression of BAFFR correlates with that of surface IgM and tonic BCR signaling. A, Flow cytometric analysis of IgM (top) and BAFFR (bottom) expression on B220+IgD− bone marrow immature B cells that carry two types of nonautoreactive BCRs (B1-8/3-83Igi,H-2d mice, dual-non-aut, black intact line) or autoreactive and nonautoreactive BCRs (B1-8/3-83Igi,H-2d mice, dual-aut, black dashed line). Expression on B220− non-B cells (non-B, filled histograms) is shown as a negative control. Expression of BAFFR on spleen CD19+ B cells from B1-8/3-83Igi,H-2d mice (dual–non-aut,SP, gray line) is shown as reference (spine B cells from BALB/c mice had identical levels; data not shown). Data are representative of three mice per group collected in three independent experiments. B, Flow cytometric analysis of IgM (top) and BAFFR (bottom) expression on B220+IgD− bone marrow immature B cells that express normal levels (3-83Igi,H-2d mice, Normal, intact line) or low levels (3-83Igi-low mice, Low, dashed line) of nonautoreactive BCRs. Expression on B220− non-B cells (non-B, filled histograms) is shown as a negative control. Data are representative of four mice per group collected in three independent experiments. C, Representative flow cytometric analysis of BAFFR expression on immature B cells generated in culture with IL-7 and thereafter. We found that expression of N-RasD12 augmented BAFFR levels on the surface of BCR-low IgD− immature B cells, to a level similar to that of BCR-normal (3-83Igi,H-2d) immature B cells, whereas gfp control transduction did not (Fig. 2C, 2D).

Overall, these data indicate that BAFFR is expressed on bone marrow IgM+IgD− immature B cells and its expression level correlates with that of surface IgM and tonic BCR signaling.

BAFF augments the generation of transitional B cells in vitro

It is known that cytokine and AgR signaling can synergize to guide lymphocyte survival and differentiation. To test whether BAFFR has a function in immature B cell development, we investigated its role in the differentiation of immature B cells into transitional B cells using an in vitro system that we have recently developed (Ref. 39 and Fig. 3A). In this system, bone marrow IgM+IgD− CD21−CD23− immature B cells are generated in IL-7 cultures and then recultured in the absence of IL-7 and in the presence of BAFF where they differentiate into cells that retain high levels of CD24 and variably express IgD, CD21, and CD23 (Ref. 39 and data not shown). Although it was proposed that BAFF regulates expression of CD21 and CD23 (28), most data indicate that it does not control CD23 (or IgD) expression (9, 32, 48), and it only slightly affects that of CD21 (49). Thus, IgD, CD21, and CD23 can still be used as developmental markers in these analyses. Transitional B cells in the bone marrow have yet to be characterized, but these markers can provide important information about the mechanisms of B cell development. Therefore, we examined the effect of BAFF on the generation of transitional B cells in our in vitro system. Immature B cells were cultured with IL-7 and then transduced or not. Shown are BAFF levels on BCR-normal nontransduced cells from 3-83Igi,H-2d mice (Normal, filled histogram) and on BCR-low GFP control-transduced (Low-GFP, dotted line) and N-RasD12–transduced (Low+RasD12, black line) cells from 3-83Igi-low mice. Immature B cells were gated as B220+IgD− (Normal) or B220+IgD+ GFP+ (Low+GFP and Low+RasD12). D, Geometric MFI (±SD, n = 4, from four independent experiments) of BAFFR expression on immature B cells analyzed as described in C. *p < 0.05; **p < 0.005. MFI, mean fluorescence intensity.

Recent studies have shown that BAFFR is expressed on immature B cells and that its expression is regulated by cytokine and AgR signaling. In this report, we investigated the role of BAFFR in the differentiation of immature B cells into transitional B cells using an in vitro system that we have recently developed (Ref. 39 and Fig. 3A). In this system, bone marrow IgM+IgD− CD21−CD23− immature B cells are generated in IL-7 cultures and then recultured in the absence of IL-7 and in the presence of BAFF where they differentiate into cells that retain high levels of CD24 and variably express IgD, CD21, and CD23 (Ref. 39 and data not shown). Although it was proposed that BAFF regulates expression of CD21 and CD23 (28), most data indicate that it does not control CD23 (or IgD) expression (9, 32, 48), and it only slightly affects that of CD21 (49). Thus, IgD, CD21, and CD23 can still be used as developmental markers in these analyses. Transitional B cells in the bone marrow have yet to be characterized, but these markers can provide important information about the mechanisms of B cell development. Therefore, we examined the effect of BAFF on the generation of transitional B cells in our in vitro system. Immature B cells were cultured with IL-7 and then transduced or not. Shown are BAFF levels on BCR-normal nontransduced cells from 3-83Igi,H-2d mice (Normal, filled histogram) and on BCR-low GFP control-transduced (Low-GFP, dotted line) and N-RasD12–transduced (Low+RasD12, black line) cells from 3-83Igi-low mice. Immature B cells were gated as B220+IgD− (Normal) or B220+IgD+ GFP+ (Low+GFP and Low+RasD12). D, Geometric MFI (±SD, n = 4, from four independent experiments) of BAFFR expression on immature B cells analyzed as described in C. *p < 0.05; **p < 0.005. MFI, mean fluorescence intensity.

Recent studies have shown that BAFFR is expressed on immature B cells and that its expression is regulated by cytokine and AgR signaling. In this report, we investigated the role of BAFFR in the differentiation of immature B cells into transitional B cells using an in vitro system that we have recently developed (Ref. 39 and Fig. 3A). In this system, bone marrow IgM+IgD− CD21−CD23− immature B cells are generated in IL-7 cultures and then recultured in the absence of IL-7 and in the presence of BAFF where they differentiate into cells that retain high levels of CD24 and variably express IgD, CD21, and CD23 (Ref. 39 and data not shown). Although it was proposed that BAFF regulates expression of CD21 and CD23 (28), most data indicate that it does not control CD23 (or IgD) expression (9, 32, 48), and it only slightly affects that of CD21 (49). Thus, IgD, CD21, and CD23 can still be used as developmental markers in these analyses. Transitional B cells in the bone marrow have yet to be characterized, but these markers can provide important information about the mechanisms of B cell development. Therefore, we examined the effect of BAFF on the generation of transitional B cells in our in vitro system. Immature B cells were cultured with IL-7 and then transduced or not. Shown are BAFF levels on BCR-normal nontransduced cells from 3-83Igi,H-2d mice (Normal, filled histogram) and on BCR-low GFP control-transduced (Low-GFP, dotted line) and N-RasD12–transduced (Low+RasD12, black line) cells from 3-83Igi-low mice. Immature B cells were gated as B220+IgD− (Normal) or B220+IgD+ GFP+ (Low+GFP and Low+RasD12). D, Geometric MFI (±SD, n = 4, from four independent experiments) of BAFFR expression on immature B cells analyzed as described in C. *p < 0.05; **p < 0.005. MFI, mean fluorescence intensity.
FIGURE 3. BAFF augments the differentiation of immature B cells into transitional B cells in vitro. A, Schematic of the system to study the differentiation of immature B cells in vitro. Briefly, bone marrow cells are cultured with IL-7 for 3–4 d and then transferred in cultures with or without BAFF for 2 d. B, Representative flow cytometric analysis of CD21, CD23 (dot plots), and IgD (histograms) expression on 3-83Igi,H-2d nonautoreactive immature B cells at days 0 and 2 of culture with or without BAFF. C, Absolute cell numbers (±SD, n = 5 mice from two independent experiments) of CD21⁺CD23⁻ (immature), CD21⁺CD23⁺ (T1-like), and CD21⁺CD23⁺ (T2-like) B cells at days 0 and 2 of culture with or without BAFF as described in B. D, Absolute cell numbers (±SD, n = 5 mice from two independent experiments) of CD21⁺CD23⁻ (immature), CD21⁺CD23⁺ (T1-like), and CD21⁺CD23⁺ (T2-like) B cells as described in B. *p < 0.05; **p < 0.005; ***p < 0.001.

To further explore whether BAFF aids the maturation of immature B cells in addition to mediating B cell survival, 3-83Igi,H-2d nonautoreactive immature B cells generated in vitro in the presence of IL-7 were transduced with Bcl-2 or control Thy1.1 encoding vectors, and transduced B cells were assessed for differentiation.

BAFF mediates its effects by activating Akt and the alternative NF-κB pathways, leading to higher expression of prosurvival Bcl-2 family members, among other changes (50). In fact, a Bcl-2 transgene appeared to genetically complement BAFFR mutant mice to restore normal frequencies of follicular mature B cells (9, 49). In TACI-Ig transgenic mice, however, a Bcl-2 transgene only partially restored T2 and follicular B cell numbers, whereas it did not restore T3 and marginal zone B cell subsets, suggesting that BAFF does not act solely as a survival factor, but that it also participates in B cell maturation (51). Indeed, these previous analyses did not discriminate between the survival and the accumulation of mature B cells as a result of Bcl-2 expression, and it remains possible that the absence of BAFFR signaling hinders B cell differentiation.

To further explore whether BAFF aids the maturation of immature B cells in addition to mediating B cell survival, 3-83Igi,H-2d nonautoreactive immature B cells generated in vitro in the presence of IL-7 were transduced with Bcl-2 or control Thy1.1 encoding vectors, and transduced B cells were assessed for dif-
ferentiation in cell cultures that contained BAFF or not. As shown in Fig. 4, Bcl-2 expression promoted the generation of transitional CD21\(^{+}\)CD23\(^{+}\) T1-like B cells in the absence of BAFF at a frequency similar to that induced by BAFF in control cells. However, CD21 levels were lower in the absence of BAFF (1.36 ± 0.17-fold lower, on average) even upon expression of Bcl-2 (1.29 ± 0.23-fold lower, on average), and in accordance with previous studies indicating that BAFF is required for optimal CD21 expression (9, 49, 51). In contrast to T1-like B cells, the generation of transitional CD21\(^{+}\)CD23\(^{+}\) and IgD\(^{\text{high}}\) T2-like B cells only occurred in the presence of BAFF, which could not be substituted by Bcl-2 (Fig. 4 and data not shown).

Overall, these data indicate that BAFF functions to promote both B cell survival and differentiation. Moreover, they indicate that differentiation of CD21\(^{-}\)CD23\(^{-}\) immature B cells into transitional CD21\(^{+}\)CD23\(^{+}\) T1-like B cells largely depends on the prosurvival effect of BAFF, whereas that of transitional CD21\(^{+}\)CD23\(^{-}\) T1-like B cells into transitional CD21\(^{+}\)CD23\(^{+}\) T2-like B cells relies on a promaturation function of this cytokine.

**Increased expression of BAFFR does not rescue differentiation of BCR-low immature B cells**

Recently we have shown that BCR-low immature B cells are strongly impaired in their differentiation into transitional B cells both in vitro and in vivo (39). As shown above (Fig. 2B), BCR-low immature B cells express subnormal levels of BAFFR, in addition to low levels of surface IgM. Thus, we asked whether the reduced differentiation of BCR-low immature B cells was in part due to the low expression of BAFFR and, consequently, low response to BAFF.

To test this hypothesis, we generated a retroviral construct that encodes mouse BAFFR. The functionality of the BAFFR retroviral construct was confirmed by its ability to partially restore mature B cell generation in A/WySnJ mice, which express a codominant natural mutant form of BAFFR (8, 49) (Supplemental Fig. 2). We next transduced BCR-low immature B cells from 3-83Ig\(^{-}\)low mice with retroviruses encoding either BAFFR or GFP only and tested the ability of transduced immature B cells to differentiate in vitro in the presence of BAFF. Importantly, transduction with the BAFFR-encoding retrovirus significantly increased levels of BAFFR on BCR-low IgD\(^{-}\) immature B cells relative to GFP control transduction (Fig. 5A). In accordance with our previous studies (39), we found that most BCR-low immature B cells were arrested in differentiation at the IgD\(^{-}\)CD21\(^{+}\)CD23\(^{-}\) stage, and only a very small frequency (0.5–5%) of CD21\(^{+}\)CD23\(^{-}\) T1-like and CD21\(^{+}\)CD23\(^{+}\) T2-like transitional B cells were generated after 2 d of culture with BAFF (Fig. 5B, 5C, GFP, and data not shown).

However, increased expression of BAFFR did not promote normal generation of transitional BCR-low B cells (as compared with 3-83Ig\(^{-}\),H-2\(^{b}\) B cells in Fig. 3), but only slightly increased their frequency to 1–8% (Fig. 5B, 5C, BAFFR). Increased BAFFR expression was also unable to restore normal generation of BCR-low transitional and mature B cells in vivo (Supplemental Fig. 3), and similar results were obtained when BAFFR expression was enforced on autoreactive 3-83Ig\(^{-}\),Rag1\(^{-}\) mice (Supplemental Fig. 4).

As mentioned above, expression of N-RasD12 was able to complement suboptimal tonic BCR signaling in BCR-low immature B cells, promoting higher levels of BAFFR expression (Fig. 2C, 2D), and differentiation into transitional and mature B cells (39). However, it remained unclear whether the differentiation process mediated by N-RasD12 was still dependent on, or rather independent of, the presence of BAFF. Therefore, we transduced BCR-low immature B cells with N-RasD12 or GFP only encoding retroviruses and assessed their differentiation in vitro in the presence or absence of BAFF. In contrast to the poor differentiation attained with increasing only BAFFR expression (Fig. 5C), optimal in vitro differentiation of BCR-low CD21\(^{-}\)CD23\(^{-}\) immature B cells into CD21\(^{+}\)CD23\(^{-}\)/CD23\(^{+}\) transitional B cells was achieved upon reinstatement of tonic BCR signaling via expression of N-RasD12 (Fig. 5D, 5E), in accordance with what we have previously reported (39). In addition to these data, we found that generation of transitional CD21\(^{+}\)CD23\(^{+}\) T2-like B cells in N-rasD12–transduced BCR-low cell cultures was further increased by the presence of BAFF (Fig. 5D, 5E), suggesting that the signaling pathway activated by N-RasD12 could not completely substitute for the absence of BAFFR signaling.

Overall, these data indicate that reduced tonic BCR signaling is responsible for the reduced differentiation of BCR-low CD21\(^{-}\)CD23\(^{-}\) immature B cells into CD21\(^{+}\)CD23\(^{-}\)/CD23\(^{+}\) transitional B cells, and that the role of BAFFR signaling in transitional B cell generation is secondary to that of tonic BCR signaling.

**BAFFR expression aids the development of transitional T1 B cells in the spleen but not T1-like B cells in the bone marrow**

Studies of BAFF- and BAFFR-deficient mice have clearly shown a requirement for this cytokine for the survival and maintenance of spleen transitional T2 and mature B cells, while minimal and

**FIGURE 4.** BAFF is required for the differentiation of CD23\(^{-}\) into CD23\(^{+}\) transitional B cells via a mechanism distinct from that mediating cell survival. A, Representative flow cytometric analysis of CD21 and CD23 expression on Thy1.1 control and Bcl-2–transduced nonautoreactive BCR-normal immature B cells from 3-83Ig\(^{-}\),H-2\(^{b}\) mice at the beginning (day 0) and end (day 2) of culture with or without BAFF. Numbers represent frequencies of live B220\(^{+}\) Thy1.1\(^{+}\) B cells in indicated gates. B, Mean frequency (±SD, n = 2 pooled mice in each of four independent experiments) of CD21\(^{+}\)CD23\(^{+}\) (T1-like) and CD21\(^{+}\)CD23\(^{+}\) (T2-like) transitional B cells in the Thy1.1\(^{+}\) population of Thy1.1 control (open bars) and Bcl-2–transduced 3-83Ig\(^{-}\),H-2\(^{b}\) B cells at day 2 of culture with or without BAFF, as described in A. *p < 0.05.
statistically insignificant effects have been observed at the transitional T1 B cell stage in vivo (4, 5, 9). Our in vitro data, however, suggest that BAFF operates also on IgD<sup>2</sup>CD21<sup>2</sup>CD23<sup>2</sup> immature B cells and IgD<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> T1 B cells to augment their differentiation into transitional IgD<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup> (T2-like) and IgD<sup>high</sup>CD21<sup>+</sup>CD23<sup>+</sup> T2-like B cells, respectively. Thus, we hypothesized that a significant role for BAFF in the generation of transitional T1 B cells could be found in vivo in a competitive environment.

To test whether BAFF functions in the generation of transitional T1 B cells in vivo, we established mixed bone marrow chimeras by combining either C57BL/6 Ly5.2 BAFFR-deficient or -sufficient bone marrow cells with C57BL/6 Ly5.1 congenic wild-type bone marrow cells (Fig. 6A and Supplemental Table 1). These chimeric mice allowed us to compare the differentiation ability of BAFFR-deficient B cells to that of BAFFR-sufficient B cells side by side, thus representing a more stringent test for the function of BAFFR in B cell development. Mixed bone marrow chimeras were analyzed by flow cytometry 6 wk after their reconstitution to determine the frequency of BAFFR-deficient and -sufficient Ly5.2<sup>+</sup> donor cells in pro-B, pre-B, immature B, transitional B, and follicular B cell populations in bone marrow and spleen tissues (gated as shown in Supplemental Fig. 5A). In the spleen, transitional B cells were gated based on high levels of CD24 expression, which discriminates them from more mature B cells (22). The CD24<sup>high</sup> B cell population of the spleen also contains some IgM<sup>+</sup>IgD<sup>2</sup>CD21<sup>2</sup>CD23<sup>2</sup> immature B cells, but these cells represent only a small fraction (<20%, data not shown). In the bone marrow, instead, transitional B cells were gated based on high IgM and low IgD expression because CD24 is also expressed by IgM<sup>2</sup>pro-B and pre-B cells (52). As shown in Fig. 6B (filled bars), the frequency of BAFFR-deficient B cells remained fairly stable throughout B cell development, as indicated by the fact that similar frequencies of Ly5.2<sup>+</sup> wild-type B cells were observed at all B cell stages. In contrast, the frequency of BAFFR-deficient B cells was significantly decreased at the CD24<sup>high</sup>CD23<sup>+</sup> T2/T3 transitional B cell and CD24<sup>low</sup>CD23<sup>+</sup> mature follicular B cell stages in the spleen of bone marrow chimeric mice relative to their precursor B cell populations in the same animals (Fig. 6B, open bars). These data indicate that BAFFR-sufficient B cells remained fairly stable throughout B cell development, as indicated by the fact that similar frequencies of Ly5.2<sup>+</sup> wild-type B cells were observed at all B cell stages. In contrast, the frequency of BAFFR-deficient B cells was significantly decreased at the CD24<sup>high</sup>CD23<sup>+</sup> T2/T3 transitional B cell and CD24<sup>low</sup>CD23<sup>+</sup> mature follicular B cell stages in the spleen of bone marrow chimeric mice relative to their precursor B cell populations in the same animals (Fig. 6B, open bars). These data indicate that BAFFR plays a significant role in both the generation and maintenance of T2, T3, and mature B cells, confirming previous observations (9). In addition to these anticipated results, we also found that the frequency of BAFFR-deficient transitional CD24<sup>high</sup>CD23<sup>+</sup> T1 (and immature) B cells in the spleen was significantly reduced to half of that of their precursor bone marrow immature B cell population in the same mice (Fig. 6B, open bars). As shown through multiparametric flow cytometric analysis (Supplemental Fig. 5B), spleen CD24<sup>high</sup>
CD23− T1 B cells also express low to high levels of CD21 and IgD, markers that distinguish them from newly generated IgD−CD21+CD23− immature B cells. Thus, these data support the conclusion of our in vitro data that BAFF contributes to the differentiation of immature B cells into transitional T1 B cells.

Cells displaying characteristics generally similar to those of splenic transitional T1 and T2 cells are also found in the bone marrow (Refs. 25, 26 and Supplemental Fig. 1), and recent studies have suggested that immature B cells can differentiate into transitional B cells in both bone marrow and spleen tissues (25, 26). We used a multiparameter flow cytometric analysis to distinguish donor-derived bone marrow T1-like (B220+IgM−IgD−CD23+; T2-like (B220+IgM−IgD−CD23+) B cells, as well as spleen immature/T1 (B220+IgM−IgD−CD23+) and T2/T3 (B220+IgM+IgD−CD23+) B cells in BAFFR-deficient and -sufficient mixed chimeras (Supplemental Fig. 5). These analyses indicated that whereas the size of the transitional CD23+ B cell population was reduced in both spleen and bone marrow in the absence of BAFFR, that of the transitional CD23− B cell population was significantly decreased only in the spleen (Fig. 6C). In fact, the frequency of B cells within the bone marrow IgMIgD−CD23− T1-like population was similar to that of those within the IgM−IgD− immature B cell population, regardless of BAFF expression (Fig. 6C).

These results indicate that BAFF is critical for the generation of transitional T1 B cells in the spleen, but not for that of transitional T1-like B cells in the bone marrow.

**IL-7 cannot replace BAFF for the in vitro generation of transitional B cells**

We have shown that optimal generation of transitional IgD+CD21+CD23− T1-like B cells in vitro requires the prosurvival function of BAFF, which can be substituted by constitutive Bcl-2 expression (Fig. 4). Moreover, we have found that only spleen T1, but not bone marrow T1-like, CD23− transitional B cells are affected by the absence of BAFFR in vivo (Fig. 6). Thus, we questioned whether a different prosurvival cytokine could promote the generation of transitional T1-like B cells in the bone marrow.

The cytokine IL-7 has both prosurvival and differentiation functions in early B cell development (18). However, whether IL-7 operates on immature B cells is controversial (18), despite the fact that these cells still express IL-7R (53). Therefore, we decided to test whether IL-7 could replace BAFF, at least partially, in the differentiation of immature B cells into transitional B cells in vitro.

For this experiment, nonautoreactive (3-83Ig,H-2d) immature B cells generated in vitro in an IL-7 culture were washed and recultured in the presence or absence of IL-7, or with BAFF as control, and analyzed for differentiation after 2 d. Neither low (pg/ml) nor high (ng/ml) concentrations of IL-7 promoted differentiation of IgD+CD21+CD23− immature B cells into IgD+CD21−CD23−/− transitional-like B cells, as shown by the absence of significant CD21, CD23, and IgD expression (Fig. 7A, 7C and data not shown). In light of this result, and because it has been previously suggested that IL-7 may inhibit B cell differentiation (18), we asked whether IL-7 could actually suppress the differentiation of immature B cells mediated by BAFF. Thus, we compared differentiation of 3-83Ig,H-2d immature B cells in the presence of BAFF and with or without IL-7. We found that IL-7 did not prevent BAFF-mediated differentiation of IgD+CD21+CD23− immature B cells into transitional IgD+CD21−CD23− immature B cells in transitional-like CD21+CD23− T1-like and IgD−CD21+CD23− T2-like B cells, as indicated by significant expression of CD21, CD23, and IgD in cultures containing both BAFF and IL-7 (Fig. 7B and data not shown). A small but significant reduction was observed in the frequency of T1-like and T2-like B cells in cultures containing both BAFF and IL-7 (Fig. 7C), but these differences were caused by increased survival of nondifferentiated immature B cells in response to IL-7 (Fig. 7D). In fact, cell counting indicated that T1-like and T2-like B cells were present in similar numbers in cultures containing BAFF regardless of IL-7 (Fig. 7D).

Thus, our data indicate that IL-7 cannot replace BAFF in the generation of CD21+CD23−/− transitional B cells in vitro. Importantly, however, IL-7 does not inhibit BAFF activity in this process. Moreover, other bone marrow factors that could potentially affect newly generated immature B cells in the bone marrow, such as a proliferation-inducing ligand (34, 55), hemokinin-1 (56), and macrophage migration inhibitory factor (57), were unable to promote differentiation of IgD+CD21+CD23− immature B cells into IgD+CD21−CD23−/− transitional B cells in vitro (Supplemental Fig. 6), suggesting that BAFF may be unique in this capacity.
Discussion

The studies presented in this work were developed to test whether BAFFR is differentially expressed in autoreactive and nonautoreactive immature B cells, and whether BAFFR expression and signaling contribute to immature B cell selection. We found that BAFFR is expressed at levels 4-fold higher by nonautoreactive than autoreactive immature B cells, both at the RNA and surface protein levels. Moreover, our data indicate that BAFF contributes to the selection of immature B cells by promoting the differentiation of immature B cells into transitional B cells upon normal tonic BCR signaling.

Previous studies have shown that expression of BAFFR coincides with that of IgM during B cell development (28, 35). Our data extend those findings by indicating that BAFFR expression is dependent on tonic BCR signaling and its level correlates with that of surface IgM. To support these conclusions, we show that immature B cells displaying low levels of IgM and tonic BCR signaling manifest low levels of BAFFR expression. Moreover, reinstatement of tonic BCR signaling by expression of the constitutive active N-RasD12 protein leads to higher BAFFR levels on immature B cells, displaying low levels of IgM and tonic BCR signaling. These data suggest that tonic BCR signaling leads to changes in the expression of nuclear proteins that promote transcription of the mtnfs13c gene. A likely candidate for modulating mtnfs13c gene transcription downstream of tonic BCR signaling is the NF-κB pathway, which may be activated by tonic BCR signaling and whose inhibition leads to reduced mtnfs13c mRNA expression (58). Additionally, previous studies have shown that tonic BCR signaling generates NF-κBp100, which is a substrate of the BAFFR signaling pathway (59). Thus, it appears that immature B cells would be capable of responding to BAFF only upon optimal tonic BCR signaling, which increases both BAFFR expression and function.

Numerous publications have indicated that BAFF is an important factor for the survival of mature follicular and marginal zone B cells. Some reports, however, have suggested that BAFF may promote B cell differentiation as well. For instance, a reduced export of B cells from the bone marrow has been observed in BAFFR mutant A/WySnJ mice (7). Moreover, the B cell defects seen in the absence of BAFF or BAFFR cannot completely be corrected by constitutive expression of the prosurvival protein Bcl-2. Constitutive Bcl-2 expression does not rescue marginal zone B cells and only partially reinstates numbers of follicular B cells in TACI-Ig transgenic mice (51), and it does not restore normal CD21 expression in BAFFR mutant mice (9, 49), suggesting an underlying defect in B cell generation. On the other hand, constitutive Bcl-2 was able to functionally substitute for BAFF in vivo, allowing a T cell-dependent immune response to a standard Ag, but one can argue that immature/transitional B cells could be drawn into an immune response in the absence of competition with mature B cells and in the presence of the antiapoptotic Bcl-2 pathway (51). Our data, in fact, support a role for BAFF in the differentiation of B cells, and more explicitly in the differentiation of IgM+IgD+CD24(lo)CD21(lo)CD23+ immature B cells into IgM+IgD+CD24(lo)CD21+CD23−/- transitional B cells.

FIGURE 7. IL-7 neither promotes nor blocks the differentiation of immature B cells into transitional B cells in vitro. A, Bone marrow cells from 3-83Igi, H-2d mice were cultured in the presence of IL-7 for 3 d to generate BCR-normal nonautoreactive immature B cells. Immature B cells were then washed with PBS and recultured with or without BAFF or IL-7, as indicated. Dot plots are representative flow cytometric analyses of CD21 and CD23 expression on B cells at the beginning (day 0) and end (day 2) of culture with the indicated cytokines. Numbers represent frequencies of live B220+ B cells in indicated gates. IL-7 was used at ∼250 pg/ml (low IL-7) and ∼10 ng/ml (high IL-7). B, Immature B cells from 3-83Igi, H-2d mice were generated in the presence of IL-7 as described in A and then recultured in the presence of IL-7 and BAFF, as indicated. Cells were analyzed as described in A. C, Mean frequency (±SD, n = 3 mice, one in each of three independent experiments) of CD21+CD23− (T1-like) and CD21+CD23+ (T2-like) 3-83Igi,H-2d transitional B cells at days 0 and 2 of culture with indicated cytokines, as described in A and B. D, Absolute cell numbers (±SD, n = 3 mice from one experiment) of CD21+CD23− (immature), CD21+CD23− (T1-like), and CD21+CD23+ (T2-like) B cells at days 0 and 2 of culture with indicated cytokines as described in A and B. *p < 0.05; ***p < 0.005; ****p < 0.001.
Specifically, in our in vitro data show that the addition of BAFF to bone marrow-derived immature B cells enhances and promotes their differentiation into transitional-like B cells that express IgD, CD21, and, for some, CD23. A role for BAFF in the differentiation of immature B cells into transitional B cells is also supported by our in vivo data showing a significant reduction of BAFFR-deficient CD24<sup>hi</sup>CD23<sup>-</sup> immature/T1 and CD24<sup>hi</sup>CD23<sup>+</sup> T2/T3 transitional B cell numbers in the spleen of mice in which BAFFR-deficient B cells developed in competition with BAFFR-sufficient B cells.

It has been proposed that BAFF regulates CD21 and CD23 expression (28). Thus, a justified question can be raised of whether our results indicate that BAFF promotes the differentiation of immature B cells or increases the expression of the CD21 and CD23 markers that we used for transitional B cell classification. We are confident that our analyses appropriately show that BAFF promotes the differentiation of immature into transitional B cells because of the following arguments. First, more than one study has challenged the idea that BAFF regulates CD23 expression (9, 32, 48). Thus, the use of CD23 to distinguish transitional T2 and T2-like B cells from transitional T1 and T1-like B cells in our studies seems appropriate both in the absence and in the presence of BAFF. Second, although CD21 expression is indeed influenced by BAFF, CD21 levels are only slightly (2-fold) reduced in the absence of BAFF (49). In our studies we used CD21 to characterize the presence of CD21<sup>+</sup> transitional B cells developing from CD21<sup>-</sup> immature B cells only in vitro. In these studies we were careful to gate CD21<sup>+</sup> cells based on CD21<sup>-</sup> cell controls, such as BCR-negative (3-383g/mb1<sup>−/−</sup>) and BCR-low (3-383g/low) immature B cells. Thus, these analyses were able to distinguish CD21<sup>low</sup> (transitional) from CD21<sup>+</sup> (immature) B cells both in the presence and in the absence of BAFF. Third, IgD can also be used to distinguish transitional from immature B cells (22), and BAFF does not appear to affect IgD expression. In our studies we observed higher levels of IgD expression and increased frequency of IgD<sup>+</sup> B cells in cultures containing BAFF, indicating an increase in B cell differentiation. Fourth, transitional T1 B cells in the spleen were gated based on high CD24 expression and the absence of CD23, markers that are not affected by BAFF. The fact that we observed a strong and significant reduction of BAFFR-deficient transitional T1 B cells in the spleen of mixed bone marrow chimeras indicates that BAFF is important for the generation of these cells, and that BAFFR signaling has an effect preceding the T2 B cell stage.

Splenic transitional B cells are classified as T1 when they are CD23<sup>-</sup>, and T2 or T3 when they express CD23 and depending on levels of IgM (23). Additionally, all transitional B cells express variable amounts of IgD and CD21 (27). Although transitional B cells are also present in bone marrow (22, 25, 26), they are still not fully characterized in this tissue. Nevertheless, it was necessary in our studies to distinguish immature from transitional B cells in both bone marrow and spleen. Thus, we classified as transitional those cells that also upregulated expression of IgD and CD21 in bone marrow cultures and bone marrow tissues, discriminating transitional T1-like and T2-like B cells on the basis of CD23 expression. Our data indicate that BAFFR and BAFF play a larger role in T2 and T3 than in T1 B cell differentiation. In fact, differentiation of immature B cells into transitional CD23<sup>-</sup> B cells in bone marrow cultures, in bone marrow tissues, and in spleen occurred to some extent even in the absence of BAFF, although these processes were enhanced by the addition of BAFF. On the other side, we show that the differentiation of transitional CD23<sup>-</sup> B cells required BAFF both in vitro and in vivo. The differential dependency on BAFF among transitional B cell subsets may be due to the fact that BAFFR expression and BAFF binding increase with their differentiation (28) and, in fact, T1 B cells are considered the developmental precursors of both T2 and T3 B cells (27).

An important finding supporting a role for BAFF in the differentiation of immature into transitional B cells is the inability of Bcl-2 to fully substitute for BAFF in this function. In fact, enforced expression of Bcl-2 in immature B cells replaced BAFF for the development of transitional CD23<sup>-</sup> T1-like, but not CD23<sup>+</sup> T2-like, B cells in vitro. Moreover, addition of BAFF to bcl-2-transduced immature B cells further increased their differentiation into T1-like transitional B cells. Because enforced Bcl-2 expression does not reconstitute CD23<sup>+</sup> transitional B cell generation in the absence of BAFF in vitro, these data strongly suggest that BAFF mediates the differentiation of CD23<sup>-</sup> transitional T1 B cells into CD23<sup>+</sup> transitional T2 B cells via a promaturation function. Development of CD23<sup>+</sup> B cells has been observed in vivo in BAFF- and BAFFR-deficient mice reconstituted with Bcl-2 transgenes (9, 49, 51). To reconcile our findings with those previous observations, we argue that Bcl-2 may have enhanced the survival and accumulation of the few transitional T2 and follicular CD23<sup>+</sup> B cells that develop even in the absence of BAFF (9, 49).

In particular, the results of our in vivo competition studies highlight the possibility that the differentiation of BAFF- and BAFFR-deficient CD23<sup>+</sup> B cells observed with and without Bcl-2 transgenes (9, 49, 51) may be the product, at least in part, of the lack of competition with normal B cells. Overall, our data support the idea that BAFF-mediated signals lead to enhanced differentiation of immature B cells into transitional T1 B cells and are necessary for the differentiation of transitional T1 B cells into T2 B cells. However, it appears that the role of BAFF in these events is secondary to that of tonic BCR signaling. We have previously shown that tonic BCR signaling is required for the generation of both CD23<sup>-</sup> and CD23<sup>+</sup> transitional B cells (39). Moreover, our present data indicate that increasing BAFFR expression in immature B cells with insufficient tonic BCR signaling does not reconstitute normal differentiation in response to BAFF, and that optimal BCR expression and function are essential for normal BAFFR expression. Thus, our data support a model in which optimal tonic BCR signaling induces the differentiation of immature B cells into early transitional T1 B cells, by initiating the expression of CD21 and IgD, as well as by increasing that of BAFFR. BAFF binding, then, triggers BAFFR signaling, causing further differentiation into cells that express higher levels of IgD and CD21 (late T1) and of CD23 (T2). Thus, a synergy between BAFFR and tonic BCR signaling appears necessary for optimal generation of transitional T1 B cells, for the differentiation of transitional T1 into transitional T2 B cells, and ultimately, therefore, for the production of mature B cells.

Immature B cells generated in the bone marrow can either migrate to the spleen for further differentiation, or first differentiate into transitional B cells within the bone marrow before migrating to the spleen (26). Our studies show that during in vivo B cell development BAFFR signaling affects the generation of transitional T1 B cells in the spleen, but not that of T1-like B cells in the bone marrow. There are different potential explanations for this finding. For instance, it is possible that the B cells we have classified as transitional T1-like B cells in bone marrow do not belong to the same developmental stage as T1 B cells in the spleen. In support of this idea, we noticed that CD21 expression is higher on splenic T1 than on bone marrow T1-like B cells (data not shown). If so, tonic BCR signaling may be sufficient for the differentiation of immature B cells into transitional T1-like B cells in the bone marrow, while a cooperation of tonic BCR and BAFFR signals...
may be required for the differentiation of immature and/or T1-like B cells into the splenic transitional T1 B cell population. Another possible explanation is one related to the environment. There may be a cytokine distinct from BAFF in the bone marrow that is able to promote the differentiation of immature B cells into T1-like B cells in cooperation with tonic BCR signaling. Our data show that increased cell survival, such as that mediated by constitutive Bcl-2 expression, can substitute for BAFF to enhance the differentiation of immature B cells into transitional T1-like B cells in vitro. Thus, another prosurvival cytokine in the bone marrow could potentially relieve the need for BAFF. We tested the cytokine IL-7 in this context because IL-7 permeates the bone marrow environment and promotes early B cell survival and differentiation. Our data show, however, that IL-7 does not enhance the generation of IgD⁺CD21⁻CD23⁻ T1-like B cells in vitro. Thus, a different bone marrow cytokine may be involved in this process. In preliminary studies we tested whether a proliferation-inducing ligand, hemokin-1, or macrophage migration inhibitor factor may be able to substitute for BAFF in promoting the differentiation of immature B cells into transitional B cells in vitro, as these cytokines have been shown to affect B cells and are present in the bone marrow tissue (54–57). However, none of these cytokines had any effect on the upregulation of IgD, CD21, and CD23 in culture and, thus, the differentiation of immature into transitional B cells, suggesting that BAFF may be unique in this function.

It has been reported that IL-7 hampers the differentiation of immature and mature B cells from their progenitors, although these data are controversial (18). Consequently, we assessed whether IL-7 could actually counteract the effects of tonic BCR and BAFFR signaling in the differentiation of immature B cells into transitional B cells in culture. We found that tonic BCR and BAFFR signaling were both still equally capable of promoting immature B cell differentiation in the presence or absence of IL-7. The absence of an inhibitory effect by IL-7 may be related to the fact that IL-7R expression and function are low on immature B cells (18, 53), although we have not tested whether this is the case in our system.

The differentiation of immature B cells into transitional B cells is a critical event for the proper generation of the naive B cell repertoire. More than 50% of newly generated immature B cells are autoreactive (33, 34), and their further differentiation is unwarranted. However, nature has evolved a checkpoint that prevents positive selection of autoreactive immature B cells. Autoreactive immature B cells display low levels of surface IgM, as IgM is internalized following binding to autoantigen. Extrapolating from our analyses of BCR-low immature B cell development, the low IgM expression on autoreactive immature B cells would translate into low tonic BCR signaling that is insufficient to promote differentiation into transitional B cells, as also suggested by other studies (60). In addition to displaying low tonic BCR signaling, we show in this study that autoreactive immature B cells are unable to upregulate expression of BAFFR. Therefore, we suggest that the absence of both optimal tonic BCR and BAFFR signaling prevents autoreactive immature B cells from further differentiation and thus contributes to their negative selection. We also envision that autoreactive immature B cells with different degrees of autoreactivity may progress to different stages of differentiation depending on levels of tonic BCR signaling and BAFFR expression. Thus, low-avidity autoreactive immature B cells, such as the prototypical anergic B cells, may progress into transitional B cells because they receive low levels of both tonic BCR and BAFFR signaling, but they fail to reach the mature B cell stage because these signals are still reduced compared with those of nonauto- reactive B cells. In contrast, high-avidity autoreactive immature B cells, such as the 3-83 Ig⁺ B cells we studied in this paper, are absolutely blocked in differentiation because they do not receive any tonic BCR signaling.

Our study indicates that tonic BCR and BAFFR signals cooperate for the differentiation of normal IgM-expressing nonauto- reactive immature B cells, by promoting their differentiation into transitional B cells. Therefore, tonic BCR and BAFFR signaling pathways together guide the positive selection of nonautoactive immature B cells into the peripheral mature B cell compartment, and they are critical for shaping the naive B cell pool such that it can mount a protective humoral immune response in the absence of autoreactivity.

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Disclosures
The authors have no financial conflicts of interest.

References


Table S1: Characteristics of mouse strains used in the Rowland et al. study.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>$Igh^{a or b}$</th>
<th>$Igk$</th>
<th>MHC</th>
<th>$rag1$ (Ig-α)</th>
<th>$tnfsf13c$ (BAFF-R)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-83Igi,H-2$^d$</td>
<td>3-83/3-83$^a$</td>
<td></td>
<td>d</td>
<td>+/+</td>
<td>WT</td>
<td>Generates non-autoreactive B cells with normal BCR expression</td>
</tr>
<tr>
<td>3-83Igi,Rag1$^{-/-}$,H-2$^b$</td>
<td>3-83/3-83$^a$</td>
<td></td>
<td>b</td>
<td>-/-</td>
<td>WT</td>
<td>Generates autoreactive immature B cells</td>
</tr>
<tr>
<td>B1-8/3-83Igi,H-2$^d$</td>
<td>B1-8/3-83$^a$</td>
<td></td>
<td>d</td>
<td>+/+</td>
<td>WT</td>
<td>Generates dual reactive B cells that co-express two types of non-autoreactive BCRs</td>
</tr>
<tr>
<td>B1-8/3-83Igi,H-2$^b$</td>
<td>B1-8/3-83$^a$</td>
<td></td>
<td>b</td>
<td>+/+</td>
<td>WT</td>
<td>Generates dual reactive B cells that co-express a non-autoreactive and an autoreactive BCRs</td>
</tr>
<tr>
<td>3-83Igi-low</td>
<td>3-83/3-83$^a$</td>
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<td>d</td>
<td>+/+</td>
<td>Hypomorphic</td>
<td>Generates non-autoreactive B cells that display low surface BCR expression</td>
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<tr>
<td>3-83Igi-mb1$^{-/-}$</td>
<td>3-83/3-83$^a$</td>
<td></td>
<td>d</td>
<td>+/+</td>
<td>+/-</td>
<td>Generates pro-B cells that are blocked in differentiation (immature B cells are absent)</td>
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<td>BAFF-R$^{-/-}$</td>
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<td>b</td>
<td>+/+</td>
<td>WT</td>
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<tr>
<td>A/WySnJ</td>
<td>+/-</td>
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<td>a</td>
<td>+/+</td>
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<td>Expresses truncated non-functional BAFF-R protein</td>
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<tr>
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<td>+/-</td>
<td>+/-</td>
<td>d</td>
<td>+/+</td>
<td>WT</td>
<td>Igh congenic BALB/c wild-type strain</td>
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</table>
Figure S1. Flow cytometric characterization of bone marrow transitional B cells. Bone marrow cells from wild-type mice were analyzed by flow cytometry for the expression of B220, IgM, IgD, CD21, and CD23. (A) Left panel shows representative expression of IgM and IgD on $B_{220}^+IgM^+$ live lymphocytes. Mature ($IgM^{low}IgD^{high}$), transitional ($IgM^{high}IgD^+$), and immature ($IgM^+IgD^-$) B cells were gated as shown and each population was analyzed for the expression of CD21 and CD23 (right panels). T1-like (CD23$^-$) and T2-like (CD23$^+$) populations are indicated within the transitional B cell population (middle right panel). (B) CD21 expression on bone marrow immature (filled histogram), T1-like (dashed line), T2-like (gray line), and mature (black line) B cell populations, as defined in (A).
Figure S2. A BAFF-R encoding retrovirus improves A/WySnJ B cell generation. Bone marrow hematopoietic cells from A/WySnJ mice were transduced with either GFP control or BAFF-R encoding retroviruses and injected into lethally irradiated recipient mice to generate retrogenic bone marrow chimeras. The distribution of non-transduced (GFP⁻) and transduced (GFP⁺) CD19⁺ spleen B cells in the imm/T1 (CD24<sup>high</sup>CD23⁻), T2/T3 (CD24<sup>high</sup>CD23⁺) and follicular (Fo., CD24<sup>low</sup>CD23⁺) subsets of retrogenic mice are shown. Data are representative of 5 retrogenic mice per group from one experiment.
Figure S3. Increased expression of BAFF-R does not rescue differentiation of BCR-low immature B cells in vivo.

(A) Scheme for the generation of 3-83Igi-low (BAFF-R-transduced) and CB17 (intact) mixed bone marrow retrogenic mice. (B) Representative flow cytometric analysis of BAFF-R expression on BAFF-R transduced 3-83Igi-low (GFP⁺, black lines), non-transduced 3-83Igi-low (GFP⁻, gray lines), and CB17 (black dashed lines) B220⁺ cells from bone marrow (top) and spleen (bottom) of mixed retrogenic mice generated as described in (A). B220⁻ non-B lineage cells (filled histograms) are shown as a negative control. (C) Changes in the frequency of CB17 (white bars) and BAFF-R transduced (GFP⁺, gray bars) or non-transduced (GFP⁻, black bars) 3-83Igi-low B cells in developmentally progressive B cell populations of mixed retrogenic mice generated as described in (A). Donor B cells were discriminated by the expression of IgMa on 3-83Igi-low, IgMb on CB17, and GFP on transduced cells. The bar graphs represent average fold changes in frequencies of immature/T1, T2/T3 and mature follicular B cells over that of the corresponding bone marrow immature B cell populations (± SD, n = 6 mice). The following surface markers were used to discriminate B cell subsets within the total IgM⁺ (IgMa⁺ + IgMb⁺) lymphoid population in bone marrow (BM) and spleen of retrogenic mice: immature (BM): IgD⁻; immature/T1 (spleen): CD24highCD23⁻; T2/T3 (spleen): CD24highCD23⁺; follicular (Fo.) (spleen): CD24lowCD23⁺. P-values of <0.05 (*) and <0.001 (***)) are indicated. N.S. = not significant.
Figure S4. Increased expression of BAFF-R does not rescue the differentiation of autoreactive immature B cells in vivo.

(A) Scheme for the generation of GFP (EMPTY) control and BAFF-R autoreactive 3-83Igi, Rag1\(^{-/-}\),H-2\(^{b}\) retrogenic mice, and expression of BAFF-R and GFP on bone marrow cells after *in vitro* cell transduction.  (B) Expression of BAFF-R on B220\(^*\)GFP\(^*\) bone marrow autoreactive (3-83Igi,Rag1\(^{-/-}\),H-2\(^{b}\)) B cells in representative control and BAFF-R retrogenic mice.  (C) Average distribution (arithmetic mean ± SD) of B220\(^*\)GFP\(^*\) B cells in the CD23\(^{-}\) (pro-B, pre-B, immature B and T1-like) cell subset of the bone marrow, and CD24\(^{\text{high}}\) (immature and transitional), CD24\(^{\text{low}}\) (mature) and CD23\(^*\) (follicular) B cell subsets of the spleen.  Frequencies of B220\(^*\) B cells in wild-type mice are shown to represent normal distribution.  Data are representative of 4 mice per group.  (D) Scheme for the generation of mice carrying adoptively transferred transduced immature B cells.  (E) Bone marrow immature B cells from non-autoreactive (B1-8Hi/3-83ki, (46)) and autoreactive (3-83Igi,Rag1\(^{-/-}\),H-2\(^{b}\)) mice were transduced with either empty control (GFP only) or BAFF-R encoding retroviruses *in vitro* in the presence of IL-7.  After transduction, cells were adoptively transferred into sub-lethally irradiated syngeneic recipient mice.  Spleen cells from recipient mice were analyzed at day 3 (top panels) and day 10 (bottom panels) to identify the presence of immature/transitional (B220\(^*\)CD24\(^{\text{high}}\)) and mature (B220\(^*\)CD24\(^{\text{low}}\)) B cells in the B220\(^*\)GFP\(^*\) transferred B cell populations.  Intact non-transduced wild-type mice were analyzed in parallel for gating controls.  Contour plots (2% probability) show B220\(^*\) or B220\(^*\)GFP\(^*\) gated cells.  Data are representative of two similar independent experiments.
Figure S5. Flow cytometric analysis of BAFF-R deficient and sufficient mixed bone marrow chimeras.

(A) Gating strategy used during analysis of BAFF-R-deficient and -sufficient mixed bone marrow chimera mice described in Fig. 6. B cell subsets within the total B220+ population in bone marrow (top and middle panels) and spleen (bottom panels) were distinguished as indicated in this figure. Cells within each stage of B cell development were analyzed for the frequency of Ly5.2+ (5.2) derived donor cells as shown. (B) Bone marrow and spleen cells from one C57BL/6 mouse were analyzed for expression of IgM, IgD, CD23 and CD24 expression. IgD expression on bone marrow IgM+IgD- immature B cells (filled histogram), and on spleen IgM+CD24\textsuperscript{high}CD23- immature/T1 (dashed gray line) and IgM+CD24\textsuperscript{high}CD23+ T2/T3 (black line) B cells is shown. Immature B cells represent <30% of the CD24\textsuperscript{high}CD23- B cell population (data not shown).
Figure S6. APRIL, HK-1, and MIF do not promote the differentiation of immature B cells into transitional B cells in vitro.
Bone marrow cells from one 3-83Igi,H-2^d mouse were cultured in the presence of IL-7 for 3 days to generate immature B cells. Immature B cells were then washed with PBS and re-cultured in the absence or presence of BAFF, APRIL, HK-1, or MIF as indicated. Dot plots show flow cytometric analyses of CD21 and CD23 expression on B cells at the beginning (day 0) and end (day 2) of culture with the indicated cytokines. Histograms show IgD expression at the same time points. Numbers represent frequencies of live B220^+ B cells in indicated gates. Cytokines were used at the following concentrations: BAFF, 10 ng/ml; APRIL (Antigenix America, Inc.), 50 ng/ml; HK-1 (Tocris Biosciences), 40 μM; MIF (R&D Systems), 50 ng/ml.