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BAFF Overexpression Promotes anti-dsDNA B cell Maturation and Antibody Secretion

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Abstract

Overexpression of BAFF is believed to play an important role in Systemic Lupus Erythematosus and elevated levels of serum BAFF have been found in lupus patients. Excess BAFF also leads to overproduction of anti-dsDNA antibodies and a lupus-like syndrome in mice. In the present study, we use mice transgenic for the R4A-C μ (IgM) heavy chain of an anti-dsDNA antibody, to study the effects of BAFF overexpression on anti-dsDNA B-cell regulation. We observe that overexpression of BAFF promotes anti-dsDNA B cell maturation and secretion of antibody and enriches for transgenic anti-dsDNA B cells in the marginal zone and follicular splenic compartments. In addition, our data suggests that BAFF rescues a subset of anti-dsDNA B cells from a regulatory checkpoint in the transitional stage of development.

Keywords

B-cells; autoimmunity; anti-dsDNA antibodies; BAFF; B-cell regulation; tolerance; anergy; Systemic Lupus Erythematosus (SLE)

1. Introduction

The B cell activating factor, BAFF also known as BLyS is a member of the TNF family of cytokines. BAFF is produced as a membrane bound protein that is cleaved and released as a soluble ligand, which is the active form of BAFF. BAFF has been shown to play an indispensable role in B cell survival and maturation [1;2;3]. Mice deficient in BAFF or mice in which the action of BAFF is blocked, have abnormally low numbers of mature peripheral B cells and a severe reduction in total serum immunoglobulin [3;4]. BAFF is predominantly

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produced by dendritic cells, monocytes, macrophages, neutrophils and bone marrow stromal cells [5;6;7]. More recently BAFF production has also been observed by activated T and B cells [8;9]. BAFF can bind and deliver signals through three receptors, BAFF-R, TACI and BCMA, which are differentially expressed during B cell development.

Three independent BAFF transgenic mouse models have been generated and each exhibits a profound increase in peripheral B cell number, hypergammaglobulinemia, elevated titers of anti-dsDNA antibody, and immune complex deposition in the kidneys, characteristic of Systemic lupus erythematosus (SLE) [10;11;12]. In one of these BAFF Tg mouse models, mice also develop sialadenitis, decreased saliva production, and submaxillary gland destruction as they age, resembling the autoimmune disease, Sjögren's syndrome (SS) [13].

Elevated serum levels of BAFF, increased titers of anti-dsDNA antibodies, and proteinuria have also been observed in autoimmune NZB/W F1 and MRL-lpr/lpr mice [10]. Treatment of these lupus prone mice with BAFF blocking agents has been shown to prevent lupus like disease and prolong survival [10;14;15]. Elevated levels of BAFF have also been observed in the sera of patients with SLE, Rheumatoid Arthritis (RA) and SS and these levels are associated with high titers of serum anti-dsDNA antibodies [16;17;18]. The association between increased autoantibody production and BAFF overexpression has led to investigations of whether BAFF overexpression alters B cell tolerance.

The maintenance of B cell tolerance has been shown to occur at several regulatory checkpoints throughout B cell development and maturation. The earliest checkpoint that has been identified occurs in the bone marrow at the immature stage of B cell development. A number of well-established Tg mouse models have been used to study B cell tolerance and have identified three major mechanisms by which autoreactive B cells are regulated in the bone marrow; receptor editing, deletion, and anergy [19;20;21;22;23]. B cell tolerance has also been observed to occur in the periphery at multiple regulatory checkpoints, although the mechanisms of tolerance at these checkpoints are less clearly defined. One peripheral regulatory checkpoint that has been observed occurs as newly emigrant transitional B cells become mature B cells and a second checkpoint has been observed when mature naïve B cells transition to IgM memory B cells [24;25].

Recent studies have begun to address whether BAFF overexpression can rescue autoreactive B cells from central and/or peripheral deletion and anergy [2;26;27;28]. The effects of excess BAFF were first examined in a model in which the neo-self antigen, hen egg lysozyme (HEL) was presented in either membrane-bound (mHEL) or soluble form (sHEL) to HEL specific B cells. It was observed that overexpression of BAFF could not rescue high affinity self-reactive B cells from central deletion but could rescue them from peripheral deletion if there was negligible competition from non-self reactive B cells for BAFF. However, in a more diverse B cell environment, high affinity anti-HEL B cells could not effectively compete with non self-reactive B cells for BAFF and were therefore eliminated [28]. It was further demonstrated that self reactive anti-HEL B cells are more dependent on BAFF for survival than non self-reactive B cells [27].

More recently, we and others have been interested in studying the role of BAFF overexpression on the regulation of more physiologic autoantigens, such as double stranded DNA (dsDNA) and chromatin since these are the hallmark autoantigens in SLE [26]. We previously demonstrated that tolerance is maintained by anergy and deletion in mice transgenic for the germline encoded S107 V₁₁ heavy chain of an anti-dsDNA antibody, designated R4A, regardless of whether the transgene contained an IgG2b (R4A- γ 2b) or an IgM (R4A-C μ) constant region [22;29]. In both R4A-C μ and R4A-C γ 2b transgenic (Tg) mice, the transgenic heavy chain can pair with a variety of light chains to produce a diverse B cell repertoire,

including dsDNA binding B cells with a range of affinities for dsDNA as well as non dsDNA binding B cells. We demonstrated in R4A-C μ Tg mice, that anergic anti-dsDNA B cells display a phenotype characterized by downmodulation of surface IgM, arrest in development, and inability to respond to activation by BCR crosslinking and T cell help. However, we also showed that these B cells could be activated to secrete Tg anti-dsDNA antibody by T cell independent B cell signaling via TLR4 and TLR9 receptors [29]. The R4A Tg mouse model has become a very successful model to study genetic and hormonal influences on anti-dsDNA B cell regulation [30]. Our laboratory recently demonstrated that overexpression of CD19, a molecule involved in lowering the threshold for B cell activation, could lead to a loss of B cell tolerance and the spontaneous secretion of Tg anti-dsDNA antibody [31].

The present study was undertaken to examine the effect of BAFF overexpression on the regulation of anti-dsDNA B cells. We now demonstrate that high levels of BAFF expression in R4A-C μ mice, promotes the maturation of a subset of transgenic anti-dsDNA B cells and the secretion of antibody. In addition, our data suggests that BAFF enables these B cells to escape a regulatory checkpoint in the transitional stage of development.

2. Materials and Methods

2.1 R4A-C μ /BAFF transgenic mice

C57BL/6 BAFF transgenic mice used in this study were a generous gift from Dr. Susan Kalled (Biogen Idec Inc., Cambridge, MA). R4A-C μ transgenic mice heterozygous for the BAFF transgene (R4A-C μ /BAFF^{+/-}), were generated by crossing C57BL/6 mice transgenic for the R4A-C μ (IgM^a) heavy chain gene with mice homozygous for the BAFF transgene (BAFF^{+/+}). R4A-C μ mice homozygous for the BAFF transgene (R4A-C μ /BAFF^{+/+}) were generated by back crossing R4A-C μ /BAFF^{+/-} mice with BAFF^{+/+} mice. The presence of the R4A-C μ and BAFF transgenes were ascertained by PCR using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ). The following primer pair; 5' GGTGAAGCTGGTGGAGTCTGG 3' (FR1 primer) and 5'CATAGGGATCCTATCTCT3' (reverse junctional primer) was used to amplify a 313 bp fragment of the rearranged R4A transgene. PCR conditions for the detection of the rearranged transgenic VDJ were as follows: 94°C for 9 min, followed by 30 cycles of 94°C for 30 sec, 51°C for 30 sec and 72°C for 40 sec, followed by an extension at 72°C for 5 min. The following primer pair was used for detection of the BAFF transgene; 5'GCAGTTTCA CAGCGATGTCCT 3' (BAFF forward primer) and 5'GTCTCCGTTGCGTGAAAT CTG 3' (BAFF reverse primer). PCR conditions for detection of a 719 bp fragment of the BAFF transgene were as described by Mackay et al. [12].

2.2 ELISAs

Anti-dsDNA ELISAs—ELISAs to detect the presence of anti-dsDNA antibody in mouse sera, were performed as previously described using calf thymus dsDNA coated 96 well plates [29]. Transgenic (Tg) (IgM^a), or endogenous (IgM^b) anti-dsDNA antibody was detected by biotinylated goat anti-mouse IgM^a or IgM^b antibody respectively (BD Pharmingen, San Jose, CA) followed by the addition of streptavidin conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL). Endogenous IgG anti-dsDNA antibody was detected by goat anti-mouse IgG conjugated directly to alkaline phosphatase (Southern Biotech). P-nitrophenyl disodium phosphate (Sigma) was added as substrate. Color development was allowed to proceed at 37°C for 1 to 3 hours and plates were read at 405 nm using a Titertek Multiscan ELISA plate reader.

To quantitate Tg IgM^a anti-dsDNA antibody by ELISA, two-fold serial dilutions of mouse serum samples (beginning with a 1:50 dilution), were applied to calf thymus dsDNA coated

plates and incubated for 2 hours at 37°C. The remainder of the anti-dsDNA ELISA was performed as described above. A purified mouse monoclonal IgM^a anti-dsDNA antibody, isolated in our laboratory was also serially diluted, beginning with a concentration of 1000 ng/ml and was applied to wells and used to generate a standard curve. The concentration of serum IgM^a anti-dsDNA antibody was extrapolated from this curve.

BAFF ELISA—Serum BAFF was quantitated using a BAFF ELISA kit (Apotech, Lausen, Switzerland) according to the manufacturer's protocol.

2.3 Flow Cytometry

To identify B cells in the pro and pre B cell stages of development, bone marrow cells from R4A-C μ , R4A-C μ /BAFF^{+/+}, C57BL/6 wild type (WT) and BAFF mice were immunostained with anti-B220-PerCP-CY5.5, anti-CD43-APC and anti-IgM-PE antibodies (BD Pharmingen, San Jose, CA). Splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with the following antibody combinations: anti-B220-APC-Cy7 (BD Pharmingen), anti-IgM-FITC, to detect total IgM B cells; anti-B220-APC-Cy7, anti-CD24-FITC, and either anti-IgM^a-PE or anti-IgM^b-PE (BD Pharmingen) to detect immature/transitional transgenic or endogenous B cells respectively; anti-B220-APC-Cy7, anti-IgM^a-PE, anti-AA4.1-APC (eBiosciences, San Diego, CA), and anti-CD23-FITC (BD Pharmingen) to detect transitional type 1, 2, and 3 B cells (T1, T2, T3), according to the classification of Allman et al, [32]; anti-B220-APC-Cy7, anti-CD21/anti-rat-APC (BD Pharmingen), anti-IgM^a-PE, and anti-CD23-FITC to detect follicular (FO) and marginal zone (MZ) transgenic B cells according to Loder et al [33]. In many instances, splenocytes were pre-incubated with the FcR block, anti Fc γ R III/II (BD Pharmingen) to prevent binding of antibodies to B cells via their Fc receptors. To examine the levels of BR3 and TACI on AA4.1⁺ and mature FO and MZ B cells, splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with the following antibody combination: anti-B220-APC-Cy7, anti-AA4.1-APC, anti-CD23-FITC, anti-IgM^a-PE or anti-IgM^b-PE, and biotinylated antibody to either BR3 or TACI (R&D Systems, Minneapolis, MN) followed by streptavidin-PerCP-Cy5.5 (BD Pharmingen). Peritoneal B cells were immunostained with antibodies to IgM^a or IgM^b, B220 and anti-CD5-APC (BD Pharmingen) in order to detect B1 B cells. Immunostained cells were fixed in PBS/paraformaldehyde and analyzed using a BD LSR II FACS machine with FACS Diva software. Additional analyses were performed using FloJo software.

2.4. Adoptive transfer

Splenocytes from an R4A-C μ mouse were immunostained with antibodies to B220, IgM^a, and AA4.1 and then cell sorted using a BD FACS ARIA to isolate transitional Tg B cells (B220⁺ IgM^a AA4.1⁺). Approximately 1×10^5 transitional B cells were resuspended in 200 μ L of 1% FBS/PBS and were then adoptively transferred retro-orbitally, into either a wild type C57BL/6 or BAFF Tg mouse. Forty eight hours post transfer, recipient mice were sacrificed and their splenocytes immunostained with antibodies to IgM^a, B220, and AA4.1 for detection of mature (AA4.1⁺) and immature/transitional (AA4.1⁺) Tg B cells.

2.5. Histology

Spleens were embedded in OTC compound. Six micrometer sections were transferred onto BondRite slides, fixed in ice cold acetone, blocked with 3% FBS/PBS and immunostained with PE conjugated antibodies to total IgM or IgM^a and biotin conjugated antibody to MOMA-1 followed by streptavidin-FITC. Slides were then treated with ProLong Gold antifade reagent (Molecular Probes, Carlsbad, CA) and examined under a Nikon Eclipse TE2000-5 (Nikon, Melville, NY) fluorescent microscope at 100 to 600 $\times$ magnification.

2.6. In vitro activation and ELISpot

Splenocytes from four R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with fluorochrome conjugated antibodies to B220, CD23, and CD21 and cell sorted using a FACS Aria, to isolate MZ (B220⁺ IgM^{hi} CD21^{hi} CD23^{lo}) and FO (B220⁺ IgM^{int} CD21^{int} CD23⁺) B cells. Splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were also immunostained with fluorochrome conjugated antibodies to B220, IgM^a, and AA4.1 and sorted into B220⁺ IgM^a AA4.1⁺ (transitional) and B220⁺ IgM^a AA4.1⁻ (mature) B cell populations. Following cell sorting, purified B cell populations, at a concentration of 1×10^6 cells per ml, were cultured for 48 hours in RPMI-1640 media (Sigma) supplemented with 10% Fetal Calf Serum (HyClone, Logan, UT), 2mM L-glutamine (Sigma), 100 μ M nonessential amino acids (Sigma), 100U/ml penicillin, 100 μ g/ml streptomycin (Sigma), and 50 μ M β -mercaptoethanol. B cells were incubated in media alone or activated with 1 μ g/ml of stimulatory CpG oligonucleotide sequences (ODN 1826) (Oligos Etc, Wilsonville, OR). After stimulation, B cells were harvested, counted and plated on dsDNA or anti-IgM coated 96-well-plates at a concentration of 10,000-100,000 cells/well. After an overnight incubation, plates were washed and incubated for 3 hours with goat anti mouse IgM^a antibody conjugated to biotin followed by a 3 hour incubation with streptavidin-AP according to Taylor et al [31]. 5-Bromo-4-chloro-3-indoyl phosphate (Sigma) was added as substrate at 1mg/ml. Antibody secreting cells were enumerated under a dissecting microscope using 40 $\times$ magnification.

3. Results

3.1. Overexpression of BAFF promotes the secretion of transgenic anti-dsDNA antibody

We have previously shown that tolerance is maintained in C57BL/6 mice transgenic for the R4A-C μ (IgM^a) heavy chain gene. Because anti-dsDNA B cells are regulated in these mice by anergy and deletion, they do not spontaneously secrete Tg IgM^a anti-dsDNA antibody. Overexpression of the B cell survival factor, BAFF has been shown to lead to the secretion of anti-dsDNA as well as other autoantibodies and to the development of lupus in mice as well as humans. To study the effect of BAFF overexpression on anti-dsDNA B cell tolerance, we therefore bred C57BL/6 R4A-C μ mice to C57BL/6 mice transgenic for BAFF, to generate R4A-C μ mice heterozygous for the BAFF transgene (R4A-C μ /BAFF^{+/-}). We then backcrossed these mice to BAFF Tg mice to generate R4A-C μ mice homozygous for BAFF (R4A-C μ /BAFF^{+/+}). Transgenic IgM antibody can be distinguished from endogenous antibody in these mice, by an IgM allotype specific antibody. The IgM transgene is of the a allotype since it was originally obtained from a BALB/c mouse, while endogenous IgM in C57BL/6 mice is of the b allotype. Mice were screened by ELISA for the presence of Tg (IgM^a) anti-dsDNA antibody in their serum. Serum samples from R4A-C μ mice heterozygous or homozygous for BAFF were considered positive for IgM^a anti-dsDNA antibody if the OD was at least 4 standard deviations above the average OD value obtained from the serum of R4A-C μ mice. We observed that overexpression of BAFF contributed to the secretion of Tg IgM^a anti-dsDNA antibody in greater than 50% of R4A-C μ mice homozygous for BAFF, but only 10% of R4A-C μ mice heterozygous for BAFF (Fig. 1A). The concentration of Tg anti-dsDNA antibody was measured by a quantitative ELISA and we observed that the majority of R4A-C μ /BAFF^{+/+} mice had much higher serum concentrations of IgM^a anti-dsDNA antibody than R4A-C μ /BAFF^{+/-} mice although both mice heterozygous and homozygous for BAFF had significantly higher concentrations of Tg anti-dsDNA antibody than R4A-C μ mice ($p < 0.05$) (Fig. 1B).

We next measured the serum concentration of BAFF in R4A-C μ , R4A-C μ /BAFF^{+/-} and R4A-C μ /BAFF^{+/+} mice to see if elevated serum BAFF concentrations correlated with increased serum concentrations of Tg, IgM^a anti-dsDNA antibody. The first thing we observed was that both mice heterozygous and homozygous for BAFF had variable concentrations of serum BAFF, although BAFF concentrations were generally higher in mice homozygous for BAFF

(Fig. 2A). Variations in the serum concentration of BAFF in BAFF transgenic mice have been observed by others as well [34]. One potential reason for this is that some BAFF Tg mice may develop antibodies to BAFF which either facilitates the clearance of BAFF or masks its detection by anti-BAFF antibodies used in the ELISA [35]. Another reason is that excess BAFF may get excreted in the urine [35]. The second thing we observed was that elevated BAFF concentrations didn't always correlate with high concentrations of Tg anti-dsDNA antibody suggesting that BAFF alone is not sufficient for Tg autoantibody secretion and that other factors may play a role. However, we did observe that a threshold concentration of BAFF (~300 ng/ml) was required for Tg B cells to secrete anti-dsDNA antibody (Fig. 2B). Below this threshold, few R4A-C μ /BAFF^{+/+} mice secreted Tg anti-dsDNA antibody while above it 60% of R4A-C μ /BAFF^{+/+} mice, produced Tg anti-dsDNA antibody. Since not all peripheral B cells in R4A-C μ /BAFF^{+/+} mice expressed the Tg antibody but expressed non Tg IgM instead (paralleling observations in R4A-C μ mice), the effect of BAFF overexpression on the secretion of non Tg anti-dsDNA antibody was also examined. Interestingly, we observed that almost 90% of R4A-C μ /BAFF^{+/+} mice secreted non Tg anti-dsDNA antibodies even at low concentrations of serum BAFF (10-250 ng/ml) (Fig. 2B). It is not clear why the Tg anti-dsDNA B cells required higher levels of BAFF to secrete antibody than the non Tg B cells but differences in the affinity or fine specificity of the Tg and non Tg anti-dsDNA antibodies may play a role. The Tg anti-dsDNA antibodies may have a higher affinity for dsDNA or they may be more broadly cross-reactive with other autoantigens than the non Tg anti-dsDNA B cells. This may cause them to be more stringently regulated and therefore require more BAFF to become antibody secreting cells. In addition, the extent of B cell receptor signaling (BCR) on autoreactive B cells could affect their level of expression of BAFF receptors, which could influence their responsiveness to BAFF signaling.

Since secretion of transgenic anti-dsDNA antibodies was predominantly observed in mice expressing high concentrations of BAFF (>300 ng/ml), we chose to use only R4A-C μ mice homozygous for BAFF for all subsequent experiments in this study.

3.2. Transgenic B cells display a reduced level of expression of BAFF R and TACI relative to endogenous B cells

We were interested in determining whether the Tg, IgM^a B cells expressed altered levels of the BAFF receptors, TACI and BR3, on their surface relative to non Tg, IgM^b B cells, since this could influence their susceptibility to BAFF signals. We therefore examined the levels of TACI and BR3 on IgM^a and IgM^b B cells from R4A-C μ and R4A-C μ /BAFF^{+/+} mice by flow cytometry. There are three BAFF receptors; BR3, TACI, and BCMA which are differentially expressed on B cells as they mature [36;37]. BR3 also known as BAFF-R, is the dominant receptor that promotes B cell survival [38;39]. TACI (transmembrane activator and calcium modulating cyclophilin ligand interacting protein) is believed to have both a positive and negative regulatory role and is believed to be involved in T-cell independent type II antibody responses, isotype switching and B cell homeostasis [39;40]. BCMA (B-cell maturation antigen) promotes plasma cell survival [40]. We did not examine expression levels of BCMA in this study since its expression has been shown to be negligible on transitional and mature peripheral B cells [26]. Figure 3 depicts the levels of BR3 (A) and TACI (B) on transitional (AA4.1⁺), mature follicular (FO) (AA4.1⁻ CD23^{hi} IgM^{int}) and marginal zone (MZ) (AA4.1⁻ CD23^{lo} IgM^{hi}) B cells expressing the IgM^a transgene (solid black line) or endogenous IgM (IgM^b) (grey line) in R4A-C μ /BAFF^{+/+} mice. Consistent with reports by others, we observed that the levels of both BR3 and TACI were highest on MZ B cells compared to FO and transitional B cells [41]. High levels of TACI and BR3 on MZ B cells, could explain why MZ B cells are exceptionally responsive to BAFF survival signals. In addition, we observed that the levels of BR3 (Fig. 3A) and TACI (Fig. 3B) were consistently much lower on transitional B cells and slightly lower on follicular B cells expressing the IgM^a transgene than non Tg

IgM^b B cells, while they were comparable on IgM^a and IgM^b MZ B cells. B cells with a reduced level of BR3 and TACI are likely to have a lower probability of engaging BAFF when they are competing with B cells that express higher levels of BAFF receptors. This could explain why higher levels of BAFF were required for Tg B cells to secrete anti-dsDNA antibody than non Tg B cells. No difference was observed in the level of expression of TACI and BR3 on Tg B cells obtained from R4A-C μ /BAFF^{+/+} and R4A-C μ mice (data not shown).

The relative expression levels of BR3 and TACI have been observed to vary in different peripheral niches during B cell development, regulation, and activation and the ratio of expression of BR3 to TACI has been reported to have important implications in the role of BAFF in B cell survival [39]. Hondowicz et al observed that anti-chromatin B cells in the transitional stage had a lower ratio of BR3 to TACI than non Tg B cells due to an elevated level of TACI expression, implying that TACI levels may be higher on autoreactive B cells [26]. We therefore determined the ratios of the MFIs of BR3 to TACI on transitional and mature IgM^a and IgM^b B cells in both R4A-C μ /BAFF^{+/+} and R4A-C μ mice to see if there was a relative increase in TACI expression particularly on transitional Tg B cells. We did not observe a lower ratio of BR3 to TACI on IgM^a B cells compared to IgM^b B cells in R4A-C μ /BAFF^{+/+} mice (Fig. 3C). In fact the ratio of BR3 to TACI was slightly higher for IgM^a than IgM^b transitional and follicular B cells and there was relatively no difference in the ratio for IgM^a and IgM^b MZ B cells.

3.3. Excess BAFF does not alter development of bone marrow B cells in R4A-C μ /BAFF^{+/+} mice

Several studies have reported that bone marrow (BM) B cells are unresponsive to BAFF [3; 28;42]. Mackay et al observed that there were no significant differences in the frequency and absolute number of pro, pre, and IgM⁺ B cells in the BM of BAFF Tg versus WT type mice [12]. Furthermore, Thien et al observed that excess BAFF failed to rescue BM HEL B cells from deletion following adoptive transfer into mHEL, BAFF Tg mice [28]. We were interested in determining the affect of BAFF overexpression on the development and regulation of anti-dsDNA B cells in the BM of R4A-C μ mice. Using flow cytometry, we examined the frequencies of pro (B220⁺ CD43⁺ IgM⁻), pre (B220⁺ CD43⁻ IgM⁻) and IgM⁺ (B220⁺ CD43⁻ IgM⁺) B cells in the BM of R4A-C μ , R4A-C μ /BAFF^{+/+}, WT, and BAFF mice (Fig. 4A). We observed that the frequencies of pro, pre, and IgM B cells in R4A-C μ /BAFF^{+/+} mice paralleled those in R4A-C μ mice. The higher frequency of pro B cells observed in R4A-C μ and R4A-C μ /BAFF^{+/+} mice compared to WT and BAFF mice is due to the presence of the pre-rearranged VDJ transgene which accelerates entry into this early stage of development. However, the reduced frequency of IgM⁺ B cells in the BM of R4A-C μ and R4A-C μ /BAFF^{+/+} mice relative to WT and BAFF mice suggests that some Tg anti-dsDNA B cells may be undergoing deletion in the BM upon transit from pre B to immature B cells even when BAFF is overexpressed. We also observed that the total number of B220⁺ B cells in the BM of R4A-C μ /BAFF^{+/+} was similar to that in R4A-C μ mice but reduced relative to the number of B220⁺ B cells in C57BL/6 WT and BAFF overexpressing mice (Table 1). The number of Tg IgM^a B cells in the BM of R4A-C μ and R4A-C μ /BAFF^{+/+} mice was similar as well (Table 1).

3.4. Overexpression of BAFF promotes the maturation of Tg B cells in the periphery of R4A-C μ /BAFF^{+/+} mice

We previously reported a reduction in the frequency and absolute number of total B220⁺ and IgM⁺ B cells in the spleens of R4A-C μ mice relative to wild type mice [29]. In the present study, a similar reduction in the frequency and number of splenic B cells was observed in R4A-C μ /BAFF^{+/+} mice (Fig. 4B and Table 1). This was surprising since we assumed that excess BAFF would promote the survival of peripheral B cells and therefore lead to an increase in B cell number. The frequency of total splenic IgM B cells in R4A-C μ /BAFF^{+/+} (17%) mice was

similar to that in R4A-C μ mice (20%) and much less than that of BAFF mice (65%) and WT mice (42%) (Figure 4B). In addition, the frequency of B cells expressing the IgM^a transgene was similar in R4A-C μ (10%) and R4A-C μ /BAFF^{+/+} (10.6%) mice (Fig. 4C).

The total number of B cells was slightly elevated in R4A-C μ /BAFF^{+/+} relative to R4A-C μ mice but this was due to a preferential expansion of the non Tg, IgM^b and IgG B cells rather than the IgM^a population (Table 1).

Although the total number of Tg B cells was not increased in the spleens of R4A-C μ /BAFF^{+/+} mice, we wondered whether the number of Tg B cells with a mature phenotype was increased since BAFF has been reported to promote B cell maturation [2;4]. We previously observed that the majority of Tg B cells present in R4A-C μ mice were arrested in the immature/early transitional stage of development [29]. We used flow cytometry to determine the frequencies of transitional and mature splenic B cells in R4A-C μ /BAFF^{+/+} and R4A-C μ mice and compared this to that in wild type mice. Splenocytes were immunostained with antibodies to IgM^a and B220 and the heat stable antigen (HSA), also known as CD24. High levels of CD24 expression (CD24^{hi}) have been shown to be characteristic of immature and transitional B cells while mature B cells are CD24^{lo} [43]. We observed that the frequency of mature, IgM^a CD24^{lo} Tg B cells was much higher in R4A-C μ /BAFF^{+/+} mice (63%) than in R4A-C μ mice (35%) (Fig. 5A). More recently, immature/transitional B cells have also been shown to express the cell surface type I transmembrane protein, AA4.1 (CD93) [32]. By immunostaining splenocytes with antibody to AA4.1 we demonstrated that R4A-C μ /BAFF^{+/+} mice have a lower frequency of AA4.1⁺ transitional B cells (30%) and a higher frequency of mature, AA4.1⁻ B cells (70%) than R4A-C μ mice (61% and 39% respectively) (Fig. 5B).

While BAFF has been shown to be necessary for normal B cell development, [2;3;4;12;44] it has been difficult to discern whether BAFF directly plays a role in B cell differentiation, or if BAFF simply enhances survival of transitional B cells which subsequently go on to mature. Therefore to demonstrate that BAFF can promote the maturation of transitional Tg B cells, to mature B cells, we isolated AA4.1⁺ IgM^a B cells from R4A-C μ mice by cell sorting and adoptively transferred them into either C57BL/6 WT or C57BL/6 BAFF Tg mice (Fig. 5C). Following the cell sort, the purity of AA4.1⁺ Tg B cells was almost 95%. One hundred thousand cells were then transferred into each recipient mouse and 48 hours later, splenocytes were collected and immunostained with antibodies to AA4.1, IgM^a, and B220. Cells were gated on IgM^a and the frequency of AA4.1⁺ B220⁺ and AA4.1⁻ B220⁺ Tg B cells were determined by flow cytometry. We observed that a higher frequency of IgM^a AA4.1⁺ B cells matured to AA4.1⁻ cells in BAFF recipients (79%) than in wild type recipients (51%) indicating that excess BAFF promoted their maturation (Fig. 5D).

Transitional AA4.1⁺ B cells have been classified into three subgroups, T1, T2 and T3, according to the classification of Allman et al: T1 cells are AA4⁺, IgM^{hi}, CD23⁻. T2 cells are AA4⁺, IgM^{hi}, CD23⁺ and T3 cells are AA4⁺, IgM^{lo}, CD23⁺ [32]. T1 cells are the precursors of T2 cells, but it is unclear whether T3 represents a developmental stage between T2 and mature B cells or an anergic population of B cells as recently described by Merrell et al [32; 45]. A study by Hsu et al suggested that BAFF promotes the survival of late transitional B cells but not T1 B cells [42]. Furthermore, several studies reported that full responsiveness to BAFF is not achieved until B cells reach the T2 stage [3;42;44]. To determine the effect of excess BAFF on transitional Tg B cell populations, splenic B cells from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with antibodies to B220, IgM^a, AA4.1, and CD23 and the frequency of T1, T2, and T3 B cells examined by flow cytometry. Although the overall number of transitional B cells was reduced in R4A-C μ /BAFF^{+/+} mice, the relative frequency of T2 and T3 B cells was increased and the frequency of T1 B cells decreased in R4A-C μ /BAFF^{+/+} mice compared to R4A-C μ mice (Fig. 6A). This is consistent with reports that BAFF promotes the

survival of late, but not early transitional B cells. However, our data cannot exclude the possibility that BAFF may promote the maturation of T1 to T2 B cells or enable autoreactive B cells to escape arrested development in the T1 stage.

3.5. R4A-C μ mice overexpressing BAFF have an enlarged MZ and an increased frequency of Tg B cells displaying a MZ phenotype

Mice overexpressing BAFF have previously been shown to have an enlarged MZ [12]. Several laboratories have demonstrated that the splenic MZ is a reservoir for autoreactive B cells [1; 30;46;47;48]. We were interested in determining whether R4A-C μ /BAFF^{+/+} mice have an enlarged MZ relative to R4A-C μ mice with a higher frequency of Tg B cells residing in this splenic compartment. To determine the frequency of B cells with a MZ phenotype, we immunostained splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice as well as BAFF overexpressing and wild type mice, with antibodies to B220, CD21, CD23, IgM^a, or IgM^b. We then examined by flow cytometry, the frequency of Tg, IgM^a B cells from R4A-C μ and R4A-C μ /BAFF^{+/+} mice, or non Tg, IgM^b B cells from BAFF or WT mice, that displayed a MZ (CD21^{hi}, CD23^{lo}) or FO (CD21^{int}, CD23^{hi}) phenotype. Consistent with reports by Mackay et al, we observed that the frequency of splenic IgM B cells with a MZ phenotype was greater in BAFF overexpressing mice than WT mice (Fig. 6B, bottom panel) [12]. In addition, we observed that R4A-C μ /BAFF^{+/+} mice had an increased frequency of Tg B cells with a MZ phenotype compared to R4A-C μ mice (Fig. 6B, top panel). No significant difference was observed in the frequency of B cells with a FO phenotype in R4A-C μ or R4A-C μ /BAFF^{+/+} mice.

Next, by immunohistochemical staining, we examined the splenic B cell compartments in R4A-C μ /BAFF^{+/+} and R4A-C μ mice for the localization of Tg B cells. Frozen spleen sections were immunostained with PE conjugated antibodies to IgM or IgM^a to visualize total IgM B cells and Tg IgM^a B cells respectively, and biotin labeled anti-MOMA-1 antibody followed by streptavidin FITC, to visualize metallophilic macrophages that appear as a ring in the marginal sinus. The marginal sinus demarcates the boundary between the MZ and the follicle. We observed that the follicles were larger and the MZ expanded in R4A-C μ /BAFF^{+/+} mice compared to R4A-C μ mice (Fig. 7A). We also observed that Tg B cells were distributed in both the FO and MZ of R4A-C μ and R4A-C μ /BAFF^{+/+} mice but that there were significantly more Tg B cells in the MZ and follicular compartments of R4A-C μ /BAFF^{+/+} than R4A-C μ mice (Fig. 7B).

Without an anti-idiotypic antibody specific for Tg anti-dsDNA B cells, we were unable to determine by immunohistochemical staining whether any of the Tg B cells that localized to the MZ and FO compartments were specific for dsDNA. A previous report by Lesley et. al. demonstrated that B cells normally compete for limiting amounts of BAFF in order to gain entry into the follicles and that autoreactive B cells require more BAFF for entry than non self-reactive B cells [27]. However, in the presence of excess BAFF, high affinity autoreactive B cells have been reported to enter follicles when competition with non self-reactive B cells is limited [28]. Excess BAFF has also been shown to rescue autoreactive B cells of intermediate affinity and facilitate their entry into the MZ as well as the follicles [28].

3.6. Follicular and marginal zone B cell populations in R4A-C μ mice overexpressing BAFF are enriched for Tg anti-dsDNA B cells

We were interested in determining whether the frequency of Tg anti-dsDNA B cells with a MZ or follicular phenotype was increased in R4-C μ /BAFF^{+/+} mice relative to R4A-C μ mice. Therefore splenocytes from R4-C μ /BAFF^{+/+} and R4A-C μ mice were sorted by flow cytometry into MZ and FO B cell subsets. They were then cultured for 48 hours in vitro, in media alone or in media supplemented with stimulatory CpG sequences, to induce antibody secretion. The

number of IgM^a anti-dsDNA secreting B cells per 10⁵ cells (Fig. 8A) and the number of total IgM^a secreting B cells per 10⁵ cells (Fig. 8B) were then enumerated by ELISpot. Hypomethylated CpG sequences have been reported to activate dsDNA binding B cells via TLR 9 receptors and we have previously demonstrated that CpG sequences could activate Tg anti-dsDNA B cells isolated from R4A-C μ mice overexpressing CD19 [31;49;50]. In the present study, we observed that the number of IgM^a secreting B cells specific for dsDNA was higher in both the MZ and FO compartments of R4A-C μ /BAFF^{+/+} mice than R4A-C μ mice. Unstimulated Tg B cells were not observed to spontaneously secrete anti-dsDNA antibodies in either R4A-C μ or R4A-C μ /BAFF^{+/+} mice.

3.7. BAFF overexpression promotes the escape of Tg anti-dsDNA B cells from regulatory checkpoints in the transitional stage of development

The increased frequency of Tg anti-dsDNA B cells in the MZ and FO compartments of R4A-C μ /BAFF^{+/+} relative to R4A-C μ mice suggested that BAFF might enable some Tg anti-dsDNA B cells that normally undergo negative selection in the transitional stages of development, to escape regulatory checkpoints and transition to more mature B cells in the periphery. To address this, we decided to sort on AA4.1⁺ (transitional) and AA4.1⁻ (mature) B cells from R4A-C μ and R4A-C μ /BAFF^{+/+} mice and determine the frequency of Tg dsDNA binding B cells in each of these populations by ELISpot. Sorted B cells were activated in vitro with stimulatory CpG sequences to induce antibody secretion. We reasoned that if regulation was intact in the transitional population, then the percent of dsDNA binding B cells in this population would be higher than in the mature B cell population, while if there was a defect in regulation at the transitional stage in R4A-C μ /BAFF^{+/+} mice then the percent of Tg dsDNA binding B cells would not be significantly different in the transitional and mature B cell populations. We observed that in R4A-C μ mice, the percent of Tg anti-dsDNA B cells was significantly higher in the AA4.1⁺ population compared to the AA4.1⁻ population ($p=0.0056$) indicating that at least some Tg anti-dsDNA B cells were unable to progress from the transitional stage to the mature stage of development (Fig. 9). In contrast, in R4A-C μ /BAFF^{+/+} mice, the frequency of Tg anti-dsDNA B cells was not significantly different in the AA4.1⁺ and AA4.1⁻ populations ($p=0.414$), suggesting that BAFF enables some Tg anti-dsDNA B cells to escape a regulatory checkpoint in the transitional stage of development.

3.8. B1 cells are unlikely to be the source of Tg anti-dsDNA B antibody in R4A-C μ /BAFF^{+/+} mice

Mice overexpressing BAFF have been shown to have an expansion of B cells belonging to the B1 subset as well as MZ B cells and antibody produced by B1 cells in these mice has been shown to contribute to autoreactivity and disease [51]. We previously demonstrated that in R4A-C μ mice overexpressing CD19, B cells secreting the Tg anti-dsDNA antibody were not of the B1 lineage [31]. To determine whether B1 B cells are a potential source of secreted Tg anti-dsDNA antibody in R4A-C μ /BAFF^{+/+} mice, we isolated peritoneal B cells from both R4A-C μ /BAFF^{+/+} and R4A-C μ mice by lavage and immunostained these B cells with antibodies to B220, CD5 IgM^a, or IgM^b and measured the frequency of CD5⁺ and CD5⁻ B cells expressing the transgene or endogenous IgM. R4A-C μ /BAFF^{+/+} mice were observed to have an elevated frequency of CD5⁺ B1 cells relative to R4A-C μ mice (Fig. 10A). However, when gates were set on B220, the frequency of IgM^a CD5⁺ and IgM^a CD5⁻ B1 cells was relatively low in R4A-C μ /BAFF^{+/+} mice (Fig. 10B, upper panel). Most of the CD5⁺ or CD5⁻ B1 cells in these mice expressed IgM^b (Fig. 10B, lower panel). Since negligible numbers of B1 B cells expressed the transgene in R4A-C μ /BAFF^{+/+} mice, it is unlikely that these B1 cells were the source of Tg anti-dsDNA antibody. Absolute proof of this would require in vitro activation of peritoneal B cells to see if they secrete Tg anti-dsDNA antibody. However, this is technically very difficult to do since the number of peritoneal B cells expressing the transgene is so low.

4. Discussion

This study demonstrates that BAFF overexpression can alter B cell regulation and promote the maturation of a population of Tg anti-dsDNA B cells and lead to their secretion of antibody. These anti-dsDNA B cells can enter MZ and FO niches and be activated to secrete antibody following CpG stimulation. In contrast to the results in this study, Hondowicz et al observed that Tg anti-dsDNA/chromatin binding B cells in VH3H9 heavy chain Tg mice, failed to enter splenic follicles following daily ip injections of BAFF [26]. Similarly, Lesley et al observed that in a diverse B cells environment in HEL/anti-HEL mice, anti-HEL B cells, failed to enter splenic follicles after being given daily BAFF injections [27]. They concluded that autoreactive B cells have a higher dependence on BAFF for survival and for entry into the follicles than non-self reactive B cells and that in a diverse microenvironment they are at a competitive disadvantage for follicular entry. One possible explanation for the difference in their observations and ours is that exogenous BAFF treatment may not have provided enough BAFF to enable self-reactive B cells, in the context of a heterogeneous B cell repertoire, to enter splenic compartments, while in mice homozygous for the BAFF transgene, sufficient BAFF may have been present for self-reactive B cells to traffic to the follicles as well as the MZ. Another potential reason for the disparate observations is the likely differences in the affinities of the self-reactive B cells in each of these transgenic mouse models. The anti-chromatin/dsDNA B cells and the anti-HEL B cells in the other studies may have been of higher affinity than the R4A-C μ anti-dsDNA B cells in our study. Thien et al observed that while high affinity anti-HEL B cells could not enter follicles in the presence of high intercellular competition in HEL/anti-HEL Tg mice, intermediate affinity B cells could [28]. Furthermore, they observed that excess BAFF enabled intermediate affinity HEL B cells to enter the MZ as well. While we do not know the dsDNA binding affinities of the Tg anti-dsDNA B cells that enter the FO and MZ compartments in R4A-C μ /BAFF^{+/+} mice, we suspect that they have a range of affinities from intermediate to low. A future study is underway to resolve this.

In R4A-C μ /BAFF^{+/+} mice, we observed that the Tg anti-dsDNA B cells require higher levels of serum BAFF for antibody secretion than the non Tg B cells. This may be because they express lower levels of both TACI and BR3, especially in the transitional stage of development, than their non Tg counterparts. In a diverse B cell environment, we hypothesize that B cells with reduced levels of BAFF receptors would have a lower probability of engaging BAFF than B cells with higher levels of BAFF receptors, especially if the amount of BAFF is limiting. However, when serum BAFF levels are high, then B cells with low levels of BAFF receptors would have a better probability of engaging BAFF. Hondowicz et al observed a higher level of TACI expression on immature anti-chromatin Tg B cells compared to immature non Tg B cells although, no difference was found in the levels for BR3. They concluded that TACI is upregulated on autoreactive B cells and plays a negative role in B cell survival and homeostasis. It is unclear what accounts for the difference in TACI levels observed on the Tg B cells in our Tg mice and in the Hondowicz model but differences in the signaling strength of the autoreactive BCRs in both transgenic mouse models may play a role. Another reason why higher concentrations of BAFF may have been required for R4A-IgM^a anti-dsDNA B cells to become antibody secreting cells than IgM^b anti-dsDNA B cells, may be because of differences in their affinity and/or fine specificity for dsDNA and their cross-reactivity with other autoantigens. These factors have been shown to affect the stringency of B cell regulation [29;52]. If the Tg anti-dsDNA B cells are more tightly regulated than the non Tg anti-dsDNA B cells because they cross-react more strongly with other self antigens or because they have a higher affinity for self antigen than the non Tg anti-dsDNA B cells, then they may require more BAFF in order to escape peripheral regulation.

BAFF does not enhance the absolute number of bone marrow B cells in R4A-C μ /BAFF^{+/+} mice relative to R4A-C μ mice. As previously demonstrated, the number of bone marrow B

cells is reduced in R4A-C μ mice relative to WT mice and overexpression of BAFF does not seem to alter this. In addition, the frequency and absolute number of immature IgM B cells is similarly reduced in R4A-C μ /BAFF^{+/+} and R4A/C μ mice relative to WT mice. This is consistent with a report by Thien et al that excess BAFF does not alter central deletion [28].

Surprisingly, R4A-C μ /BAFF^{+/+} mice do not have enlarged spleens, enriched in B cells as observed in BAFF mice. Instead they have a reduced number of total splenic B cells. We previously demonstrated that a subset of high affinity anti-dsDNA B cells present in an autoimmune strain of mouse transgenic for the R4A-C γ 2b heavy chain gene, are deleted from R4A-C γ 2b mice on a wild type background [52]. The reduced number of splenic B cells found in R4A-C μ mice relative to WT mice similarly suggests deletion of a population of anti-dsDNA B cells. The lack of expansion of peripheral B cells in R4A-C μ /BAFF^{+/+} mice may be due to BAFF's inability to rescue high affinity anti-dsDNA B cells targeted to deletion. This is consistent with a study by Thien et al demonstrating that excess BAFF fails to rescue high affinity anti-HEL B cells in sHEL/anti-HEL BAFF Tg mice [28].

If BAFF is unable to rescue high affinity R4A-C μ anti-dsDNA B cells, then what is the source of the Tg anti-dsDNA antibody secretion in R4A-C μ /BAFF^{+/+} mice? B1 cells are unlikely to be the source since the frequency of B1 cells expressing the Tg is negligible in these mice. We speculate that BAFF may rescue intermediate to low affinity anti-dsDNA B cells from regulation in the transitional stages of development. It has recently been reported that the transitional stages of development also function as regulatory checkpoints [24;25]. We observed that in R4A-C μ mice, the percent of Tg anti-dsDNA B cells in the transitional stage is significantly higher than in the mature stage suggesting that many of the autoreactive B cells are undergoing regulation in the transitional stage. However, in R4A-C μ mice overexpressing BAFF, a greater frequency of Tg anti-dsDNA B cells are able to enter the mature B cell stage and the ratio of mature to transitional Tg anti-dsDNA B cells is much higher in R4A-C μ /BAFF^{+/+} mice than in R4A-C μ mice suggesting that BAFF enables some of the autoreactive B cells to escape regulatory checkpoints in the transitional stage. The exact mechanism of regulation at these checkpoints is unclear, however, arrested development and/or deletion have been proposed. In addition, Merrell et al suggests that anergy may play an important role as they have demonstrated that transitional, T3 B cells display an anergic phenotype and that a high frequency of B cells in this population are autoreactive [32;45]. Interestingly, we recently observed that the frequency of Tg anti-dsDNA B cells is significantly higher in the T3 than the T2 and T1 populations in R4A-C μ mice and that these Tg T3 anti-dsDNA B cells appear to have an anergic phenotype (data not shown). While BAFF may rescue autoreactive B cells from regulatory checkpoints in the transitional stages of development, it is not known how these B cells get activated to become antibody secreting cells. We propose that BAFF may promote the survival and maturation, of intermediate affinity anti-dsDNA B cells targeted to anergy as well as low affinity anti-dsDNA B cells in R4A-C μ /BAFF^{+/+} mice and that this increases the opportunity for an antigen independent, environmental trigger such as CpG to activate these autoreactive B cells. Studies are underway to investigate this.

In summary, this study demonstrates that high levels of serum BAFF promotes the survival and maturation of a subset of anti-dsDNA B cells and facilitates their secretion of antibody. Furthermore, our data suggests that BAFF enables anti-dsDNA B cells to escape a peripheral regulatory checkpoint in the transitional stage. These results are important as they illustrate the role of BAFF in altering peripheral B cell tolerance and they emphasize how excess BAFF poses a risk that can lead to the development of anti-dsDNA antibodies that arise in SLE.

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List of abbreviations

MZ	marginal zone
FO	follicular
dsDNA	double stranded DNA
BAFF	B cell activating factor of the TNF family

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Figure 1. R4A-C μ mice homozygous for BAFF secrete elevated levels of IgM^a anti-dsDNA antibody
A. Sera from 13 R4A-C μ mice, 18 R4A-C μ /BAFF^{+/-} mice and 18 R4C μ /BAFF^{+/+} mice were tested by ELISA for Tg, IgM^a anti-dsDNA antibody. Horizontal lines represent average OD at 405 nm. Dotted line represents 4 standard deviation units above the average OD of serum samples from R4A-C μ mice. **B.** IgM^a anti-dsDNA antibody concentrations were measured from the sera of 10 R4A-C μ mice (circles), R4A-C μ /BAFF^{+/-} mice (squares) and R4C μ /BAFF^{+/+} mice (triangles) by a quantitative ELISA.

Figure 2. R4A-C μ anti-dsDNA B cells require high concentration of serum BAFF for the secretion of antibody

A. Variations in serum BAFF concentrations in mice overexpressing BAFF. Serum concentrations of BAFF were measured by ELISA for 11 R4A-C μ mice, 15 R4A-C μ /BAFF^{+/-} and 15 R4A-C μ /BAFF^{+/+} mice. Horizontal bars represent average concentrations of BAFF. **B.** A higher concentration of serum BAFF is required for the secretion of Tg versus non Tg anti-dsDNA antibody. Serum BAFF concentrations were measured for 30 R4A-C μ /BAFF^{+/+} mice. Mice were grouped into those with high serum concentrations of BAFF (300 - 1000 ng/ml) and those with lower concentrations of BAFF (10 - 250 ng/ml). Graph depicts percent of mice in each group secreting either non Tg (IgM^b or IgG) (open bar) or Tg (IgM^a) anti-dsDNA antibody (closed bar).

Figure 3. Expression levels of BR3 and TACI are reduced on R4A-C μ , Tg B cells

Splenocytes from R4A-C μ /BAFF^{+/+} mice were immunostained with antibodies to IgM^a, IgM^b, B220, AA4.1, CD23, TACI, and BAFF-R. Expression levels of BR3 (**A**) or TACI (**B**) were examined on transitional (B220⁺ AA4⁺), FO (AA4⁻, CD23^{hi} IgM^{int}), and MZ (AA4⁻ CD23^{lo} IgM^{hi}) B cells expressing the IgM^a transgene or endogenous IgM^b. Gates were set on IgM^a (solid black line) or IgM^b (solid grey line) B cells with a transitional, FO, or MZ phenotype. Dashed histogram indicates isotype control. Dotted vertical line depicts arbitrary reference point. Histograms are representative of at least 6 experiments. **C**. Ratios of the Mean Fluorescent Intensities (MFIs) of BR3 (in A) to TACI (in B).

Figure 4. BAFF overexpression does not alter B cell development in the bone marrow

A. Bone marrow B cells from R4A-Cμ, R4A-Cμ/BAFF^{+/+}, WT, and BAFF mice were immunostained with antibodies to B220, IgM and CD43 and analyzed by flow cytometry. Gates were set on B220⁺ cells. Insert indicates windows for pro (CD43⁺IgM⁻), pre (CD43⁻IgM⁻), and immature/mature IgM (CD43⁻IgM⁺) B cells. **B.** Splenic B cells from R4A-Cμ, R4A-Cμ/BAFF^{+/+}, WT, and BAFF mice were immunostained with antibodies to B220 and IgM. Gates were set on live lymphocytes. **C.** Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF^{+/+} mice were immunostained with antibodies to B220 and IgM^α. Results in A, B, and C are representative of at least 3 experiments.

Figure 5. Overexpression of BAFF promotes the maturation of R4A-C μ Tg B cells in the periphery Splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with antibodies to IgM^a, B220, CD24, and AA4.1 and analyzed by flow cytometry. **A.** Histogram displays CD24^{hi} and CD24^{lo} IgM^a B cells. Gate set on live, IgM^a, B cells. **B.** Contour graph indicates frequency of AA4.1⁺ and AA4.1⁻ Tg B cells in R4A-C μ and R4A-C μ /BAFF mice. Gate set on live, IgM^a, B cells. **C.** Splenocytes from R4A-C μ mice were sorted to collect AA4.1⁺ Tg B cells, using a BD FACS, ARIA. Purity of the post sort population was 95% (right panel). **D.** Sorted AA4.1⁺ IgM^a cells from C were transferred into wild type or BAFF mice, intravenously. Two days later, the spleens of recipient mice were harvested and immunostained with antibodies to IgM^a and AA4.1 to assess the maturation state of the transferred cells. Results in A and B are representative of 6 experiments and results in C and D are representative of 3 experiments.

Figure 6. Increased frequencies of late transitional and MZ, Tg B cells in R4A-C μ /BAFF^{+/+} mice
 Splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with antibodies to IgM^a, B220, CD23, and AA4.1 and analyzed by flow cytometry. **A.** Contour graph indicates the frequency of AA4.1⁺, T1 (IgM^a^{hi}, CD23⁻), T2 (IgM^a^{hi}, CD23⁺) and T3 (IgM^a^{lo}, CD23⁺) B cells in R4A-C μ and R4A-C μ /BAFF^{+/+} mice. Gates were set on live, IgM^a AA4.1⁺ B cells. **B.** Splenocytes from R4A-C μ and R4A-C μ /BAFF^{+/+} mice (top panel), and WT and BAFF mice (bottom panel) were immunostained with antibodies to B220, CD21, CD23, and antibody to IgM^a (to detect Tg B cells, top panel) or antibody to IgM^b (to detect non Tg B cells from WT and BAFF mice, bottom panel) and analyzed by flow cytometry to determine the frequency of MZ (CD21^{hi} CD23^{lo}) and FO (CD21^{int} CD23^{hi}) B cells. Gates were set on live B220 IgM^a cells for R4A-C μ and R4A-C μ /BAFF^{+/+} mice and live B220 IgM^b cells for WT and BAFF Tg mice. Results are representative of 6 experiments.

Figure 7. MZ and FO compartments are enriched with Tg IgM α B cells in R4A-C μ /BAFF^{+/+} mice
 Frozen spleen sections (6 μ m) from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with antibody to MOMA-1 (marker for metallophilic macrophages) and antibodies to total IgM (A) or IgM α (B). Immunostaining of marginal sinus is indicated by green fluorescence. Immunostaining of total IgM B cells in (A) or IgM α B cells in (B) is depicted by red fluorescence. (PALS, Periaarteriolar lymphatic sheath; MZ, marginal zone; FO, follicle). Crossbars indicate width of the MZ. Sections are representative of 4 experiments.

Figure 8. The number of R4A-Cμ, Tg anti-dsDNA B cells is increased in the MZ and FO of R4A-Cμ/BAFF^{+/+} mice

Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF^{+/+} mice were immunostained with antibodies to B220, CD21, and CD23 and sorted to obtain MZ (B220⁺ CD21^{hi} CD23^{lo}) and FO (B220⁺ CD21^{int} CD23^{hi}) B cells. Cells were then cultured in either media alone or activated in vitro with 1μg/ml of stimulatory CpG oligonucleotides for 48 hours and then IgM^a anti-dsDNA secreting B cells (**A**) and total IgM^a secreting B cells (**B**) were enumerated by ELISpot.

Figure 9. BAFF enables R4A-Cμ, Tg anti-dsDNA B cells to escape the transitional stage of development

Splenic B cells from R4A-Cμ and R4A-Cμ/BAFF^{+/+} mice were immunostained with antibodies to B220 and AA4.1 and sorted into B220⁺ AA4⁺ (transitional) and B220⁺ AA4⁻ (mature) populations. They were then activated in vitro with 1 μg/ml of stimulatory CpG oligonucleotides and the number of IgM^a anti-dsDNA secreting B cells and the total number of IgM^a secreting B cells was determined by ELISpot. Results are represented as the percent of IgM^a secreting B cells that are specific for dsDNA. * indicates p < 0.005.

Figure 10. Negligible frequency of peritoneal B1 cells express the IgM^a transgene in R4A-C μ /BAFF^{+/+} mice

Peritoneal cells isolated by lavage from R4A-C μ and R4A-C μ /BAFF^{+/+} mice were immunostained with antibodies to B220, CD5, and IgM^a to detect the frequency of B1 cells expressing the transgene and IgM^b to detect B1 cells expressing non Tg IgM and examined by flow cytometry. **A.** The frequency of B220⁺ CD5⁺ B cells is >3 fold greater in R4A-C μ /BAFF^{+/+} than R4A-C μ mice. **B.** Very few peritoneal B cells derived from the B1 lineage express the IgM^a transgene in both R4A-C μ and R4A-C μ /BAFF^{+/+} mice (top panel). Most express the non Tg IgM^b (bottom panel). Gate was set on B220⁺ B cells and the frequencies of CD5⁺ and CD5⁻ B cells expressing IgM^a (top panels) or IgM^b (lower panels) are indicated.

Table 1
Absolute number of Tg B cells in R4A-C μ /BAFF^{+/+} mice

Absolute numbers of total B cells and B cells expressing Tg (IgM^a) or non Tg (IgM^b) antibody were determined by flow cytometry. Results are the average of six experiments.

	WT	BAFF	R4A-C μ	R4A-C μ /BAFF ^{+/+}
Bone marrow				
Total B cells ($\times 10^6$)	5.1 $\pm$ 1.4	4.3 $\pm$ 0.9	1.7 $\pm$ 0.7	1.8 $\pm$ 0.8
IgM ^a B cells ($\times 10^6$)	NA	NA	0.3 $\pm$ 0.1	0.4 $\pm$ 0.3
IgM ^b B cells ($\times 10^6$)	1.9 $\pm$ 0.9	2.8 $\pm$ 0.8	0.1 $\pm$ 0.02	0.1 $\pm$ 0.04
Spleen				
Total B cells ($\times 10^6$)	21.6 $\pm$ 9.7	70.3 $\pm$ 26.0	2.8 $\pm$ 1.1	4.2 $\pm$ 2.5
IgM ^a B cells ($\times 10^6$)	NA	NA	1.0 $\pm$ 0.5	0.9 $\pm$ 0.8
IgM ^b B cells ($\times 10^6$)	18.0 $\pm$ 7.6	61.1 $\pm$ 20.6	1.1 $\pm$ 0.5	2.1 $\pm$ 1.3