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The Transcription Factor Bright Plays a Role in Marginal Zone B Lymphocyte Development and Autoantibody Production

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Abstract

Previous data suggested that constitutive expression of the transcription factor Bright (B cell regulator of immunoglobulin heavy chain transcription), normally tightly regulated during B cell differentiation, was associated with autoantibody production. Here we show that constitutive Bright expression results in skewing of mature B lineage subpopulations toward marginal zone cells at the expense of the follicular subpopulation. C57Bl/6 transgenic mice constitutively expressing Bright in B lineage cells generated autoantibodies that were not the result of global increases in immunoglobulin or of breaches in key tolerance checkpoints typically defective in other autoimmune mouse models. Rather, autoimmunity correlated with increased numbers of marginal zone B cells and alterations in the phenotype and gene expression profiles of lymphocytes within the follicular B cell compartment. These data suggest a novel role for Bright in the normal development of mature B cell subsets and in autoantibody production.

Keywords

Bright transcription factor; B lymphocyte; autoantibodies; marginal zone development

1. Introduction

Autoimmunity results from a loss of tolerance to self-antigen. Due to the complexity of this disease, its cause is still largely unknown and treatment options are limited (Fairhurst *et al.*, 2006). Mouse models have been instrumental in identifying normal B cell tolerance checkpoints (Gay *et al.*, 1993; Goodnow *et al.*, 1988; Hartley *et al.*, 1993; Nemazee and Burki, 1989; Okamoto *et al.*, 1992). Receptor editing is the process of continued light chain rearrangement which utilizes other gene segments within the kappa (κ) light chain loci and

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Disclosures

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resorts to the lambda (λ) light chain loci if necessary to change the antigen receptor specificity (Gay *et al.*, 1993; Nemazee and Weigert, 2000). Anergy is a property of low-affinity autoreactive cells that persist in the periphery and demonstrate a reduced capacity to participate in immune responses (Merrell *et al.*, 2006). At the cellular level they do not mobilize Ca^{2+} , initiate tyrosine phosphorylation or proliferate (Merrell *et al.*, 2006; Teague *et al.*, 2007). These anergic cells are commonly referred to as transitional type 3 (T3) immature B cells, and this subset is selected away from the T1→T2→mature B cell developmental pathway (Teague *et al.*, 2007). In multiple lupus-prone strains, this subset is decreased (Teague *et al.*, 2007). BCR stimulation can also lead to clonal deletion and occurs predominantly at the immature T1 B cell stage (Carsetti *et al.*, 1995; Petro *et al.*, 2002; Su and Rawlings, 2002). Furthermore, others have shown that intrinsic hyperactivity and expansions in mature marginal zone (MZ) B cells, which are enriched for autoreactive clones (Martin and Kearney, 2002), are associated with breaches in tolerance. Therefore, defects in multiple pathways and at multiple stages of B cell development contribute to autoimmunity.

Bright (for B cell regulator of Ig heavy chain transcription) is a transcription factor that belongs to the ARID (AT-rich interaction domain) family (Herrscher *et al.*, 1995), which consists of 15 proteins divided into seven subfamilies. Bright and two ARID orthologs in the human and yeast have been associated with chromatin remodeling (Ayoub *et al.*, 2003; Lai *et al.*, 2001; Lin *et al.*, 2007), suggesting a possible function in epigenetic regulation. However, the first Bright binding sites discovered were in the Ig loci and the best characterized function of Bright is its ability to increase immunoglobulin (Ig) production (Webb *et al.*, 1989; Webb, 2001). Predicted Bright-binding sites are present upstream of over half of the variable region genes and within the intronic E μ enhancer necessary for expression of all Igs (Herrscher *et al.*, 1995; Johnston *et al.*, 2006). Bright Ig enhancing function requires a multi-protein complex containing Bright dimers (Nixon *et al.*, 2004a) and both Bruton's tyrosine kinase (Btk) and its substrate, TFII-I (Rajaiya *et al.*, 2006; Rajaiya *et al.*, 2005). More recent data suggest that Bright may also affect genes outside of the Ig locus.

Bright is expressed in multiple embryonic tissues where its function is unlikely to be associated with Ig expression. Although transgenic mice that expressed a dominant negative form of Bright specifically in the B cell lineage showed defects in B1 B cell Ig secretion and decreased levels of serum IgM (Nixon *et al.*, 2008), Bright knockout mice died between days 12 and 14 of gestation due to defects in their ability to generate sufficient red blood cells (Webb *et al.*, 2011). In addition, Bright deficient cells from multiple tissues including spleen, bone marrow, and lymph node became developmentally plastic with the ability to differentiate into cells expressing markers from all three germ lines (An *et al.*, 2010). These data demonstrate that Bright plays a role in non-Ig related functions and suggest that it regulates genes other than the immunoglobulin loci.

Bright expression in the adult is restricted to discrete stages during B cell development in both the mouse and human. Bright mRNA expression occurs in large pro-B, pre-B and recirculating B cells in the bone marrow and in germinal center B cells in the spleen, but it is actively down regulated in the majority of peripheral B cells in the spleen (Nixon *et al.*, 2004b; Webb *et al.*, 1998). Normal follicular B cells do not express Bright mRNA transcripts, while marginal zone B cells maintain low levels of expression. These two phenotypically and functionally distinct mature B cell subsets can terminally differentiate into memory or plasma B cells once they have initiated an antibody response. Follicular (FO) B cells represent the largest B cell subset in the periphery, are defined as B220⁺CD93⁻CD23⁺IgM⁺ (Allman *et al.*, 2001; Allman and Pillai, 2008), and re-circulate between various lymphoid sites where they come into contact with activated T cells and undergo germinal center reactions. In contrast, MZ B cells are a minor mature subset

accounting for roughly 5% of the total B cell population, are defined as B220⁺CD93⁻CD23⁻IgM^{hi} (Allman *et al.*, 2001; Allman and Pillai, 2008), and produce natural IgM antibody rapidly to blood-borne antigens. Relative to FO B cells, MZ B cells are partially activated, expressing higher basal levels of co-stimulatory molecules and developing into plasma cells more rapidly following activation (Oliver *et al.*, 1999; Oliver *et al.*, 1997). MZ B cells have a relatively restricted B cell repertoire biased towards common bacterial and self-antigen that tends to be polyreactive, and thus capable of being autoreactive (Reviewed in (Loder *et al.*, 1999)). Generally, autoreactive cells are excluded from the FO compartment, whereas the MZ compartment is enriched for autoreactive clones (Reviewed in (Martin and Kearney, 2002)).

Previously, we demonstrated that over-expression of the transcription factor Bright throughout B cell development was associated with ANA production, proteinuria and kidney glomerulonephritis as transgenic FVB/N mice aged (Shankar *et al.*, 2007). However, other studies suggest that normal FVB/N mice exhibit some traits commonly associated with autoimmunity (Maier *et al.*, 2007), suggesting that the genetic background could have influenced the phenotype of the Bright transgenic mice. Here, we sought to analyze how Bright over-expression resulted in autoantibody production. Therefore, it was important to backcross the FVB/N Bright transgenic lines onto the non-autoimmune strain C57Bl/6 (Bygrave *et al.*, 2004; Vidal *et al.*, 1998) for those analyses. Bright expression was also sufficient to generate autoantibodies on this background in mice only four weeks old, and these mice were analyzed to determine if losses in tolerance were consistent with mechanisms observed in other autoimmune models. Intriguingly, the data presented here suggest new roles for Bright in both B cell development and autoimmunity.

2. Material and Methods

2.1. Mice

FVB/N mice that express the Bright transgene driven by the B lineage restricted CD19 promoter were described previously (Shankar *et al.*, 2007). Two independent transgenic lines were backcrossed onto the C57Bl/6 background for more than 10 generations and were genotyped as described (Shankar *et al.*, 2007). All BrTg mice were maintained as heterozygotes. C57Bl/6 and Bcl2 E μ transgenic mice on the C57Bl/6 background were purchased from Jackson ImmunResearch Laboratories. Btk^{-/-} mice, also on the C57Bl/6 background, were a gift from Wasif Khan (Vanderbilt University School of Medicine, TN). C57Bl/6 (SJL Ly5.1) congenic mice were a gift from Paul W. Kincade (Oklahoma Medical Research Foundation) and were lethally irradiated (1300 rad) before retro-orbital injection with 2 $\times$ 10⁶ whole bone marrow cells from Bright transgenic and/or C57Bl/6 donors (both Ly5.2). 5-weeks post injections, recipients were sacrificed and analyzed. All mice were bred and maintained in the OMRF animal care facility according to Institutional Animal Care and Use Committee approved protocols. Male and female mice were analyzed between 4 and 12 weeks of age.

2.2. ANA

Mouse sera were tested at multiple dilutions for the presence of anti-nuclear antibodies using the NOVA Lite HEP-2 ANA Kit (INOVA diagnostics) as previously described (Shankar *et al.*, 2007). ANA positive control sera from adult mice typically showed strong staining compared to sera from negative littermate controls even at dilutions of 1:360 (Maier *et al.*, 2007). However, for younger mice and mice with low serum Ig levels, higher dilutions (1:45) were also assayed. Antibodies to nuclear antigens were detected using a secondary FITC-conjugated anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) and

visualized using a Zeiss Axioplan 2i inverted microscope with an AxioCam HRm camera (Carl Zeiss).

2.3. ELISA

Serum Ig levels were determined using the clonotyping system-AP kit (Southern biotech) and a Biotech EL800 plate reader with GEN5 Biotech software as previously described (Shankar *et al.*, 2007). Briefly, duplicate serum samples were serially diluted from 1:400 to 1:51,200 and concentrations were determined using standard curves generated with appropriate Ig isotype controls of known concentration. Ig secretion from purified B cells plated at 1×10^6 cells/ml and stimulated with 10 μ g/ml goat F(ab')₂ anti-mouse IgM or LPS for 5 days was measured similarly from supernatants by ELISA using anti-kappa light chain reagents as previously described (Nixon *et al.*, 2008).

2.4. Flow cytometry

Cells from spleens, bone marrow, peritoneal cavity and thymuses were isolated and prepared, and B cell staining was performed as described (Shankar *et al.*, 2007). Data were collected using an LSR II (BD Biosciences) and DIVA software ver. 4.1. A minimum of 100,000 events were recorded. Data were analyzed using Flojo (Treestar) software ver. 8.3. Fluorescence conjugated antibodies phycoerythrin-cyanin 5 (PE-Cy5)-B220 (Ra3-6B2), peridinin chlorophyll-a protein (PerCP)-B220 (RA3-6B2), phycoerythrin (PE)-CD23 (B3B4), and -CD43 (S7) were purchased from BD Biosciences; allophycocyanin (APC)-CD93/ClqRp (AA4.1), biotin-CD86/B7.2 (GL1), -TLR4/MD2 (MTS510), and -RP105 (RP/14), fluorescein isothiocyanate (FITC)-CD21/35 (7G6), -CD19 (MB19-1), -IgM (II/41), and PE-CD24 (30-F1) from eBiosciences; streptavidin-PE-TEXAS RED from Invitrogen; FITC-CD23 (01234D), -lambda light chain (R24-46), PE-kappa light chain (187.1), PerCP Cy5.5-CD45.2 (104), biotin-CD80/B7 (16-10A1), -CD45.1, and -CD25(7D4) from Pharmingen; and APC-CD23 and biotin-IgM from Southern biotech. Appropriate isotype controls (BD Biosciences and ebiosciences) were used.

2.5. Proliferation and viability assays

Resting B cells for *in vitro* stimulation were obtained by CD43 depletion from whole spleens according to the manufacturer's protocol (Miltenyi Biotech). B220⁺ B cell subsets were defined and sorted using CD24 and CD21 (Su *et al.*, 2004; Loder *et al.*, 1999; Batten *et al.*, 2000) to avoid inadvertent stimulation through surface IgM. Cells were sorted as previously described (Shankar *et al.*, 2007) using a FACS Aria (BD Biosciences) and DIVA software ver. 4.1 or DakoCytomation MLS3042 (Dako) using Summit software ver. 4.3.

Isolated B cells were maintained in RPMI 1640 containing 7% FCS (Webb *et al.*, 1989) and were treated with 0.5 to 20 μ g/ml LPS (*Escherichia coli* 0111:1B4 (Sigma Aldrich) or anti-mouse IgM (Thermo Scientific). Viabilities were measured by flow cytometry using forward/side scatter properties and 7-amino actinomycin D (7AAD) (eBiosciences) staining (Batten *et al.*, 2000).

Cell proliferation was measured after 48 hours from quadruplicate cultures of 1×10^6 cells/ml pulsed with 1 μ Ci [³H] thymidine 8 hours prior to harvest and harvested using a PHD cell harvester.

2.6. Ca²⁺ measurements

Intracellular Ca²⁺ was monitored as described (Cooney *et al.*, 2001; Malhotra *et al.*, 2009). Briefly, cells were loaded with 5 μ M INDO-1 acetoxymethyl ester (Invitrogen) with 0.5% pluronic acid (Invitrogen) in HBSS (supplemented with 1mM Ca²⁺ and 1mg/ml BSA) for 30 min at 37°C. Cells were washed with HBSS, resuspended at 1×10^6 cells/ml, and loaded into

a quartz cuvette placed into a 37°C LS50B spectrofluorometer (Perkin Elmer). Samples were excited at 355nm and emission at 380nm was recorded over time using FLWinlab software. A base line was collected for 200 seconds and intracellular Ca²⁺-binding (emission at 380) was monitored in response to 10µg/ml anti-mouse IgM for at least 5 minutes. To monitor efficient cell loading at the end of the response, cells were lysed with 0.5% Triton X 100 to release the INDO-1 and the signal was quenched with 0.5µM EDTA.

2.7. EMSA and western blotting

Nuclear cell extracts were prepared as described (Dignam *et al.*, 1983) and EMSAs were performed according to (Webb *et al.*, 2000). Double stranded oligonucleotides corresponding to NF-κB binding sites from the IL-2 receptor promoter (5'-CAACGGCAGGGGAATTCCCCTCTCCTT-3') (Andrews and Rawlings, 2009) were labeled on one strand, hybridized with the complementary strand by heating to 95°C for 5 minutes and cooling to room temperature and used for reactions. For whole cell lysates, cells were pelleted, washed once with cold PBS and lysed in TN1 buffer (50mM Tris-HCL, pH 8.0, 125mM NaCl, 10mM EDTA, 1% Triton supplemented with 10µg/ml leupeptin, 10µg/ml aprotinin, 1mM DTT, 3mM Na₃VO₄ and PMSF). Protein concentrations were determined using the Bradford assay according to manufacturer's protocol (Biorad). Samples were run on a 10% SDS-PAGE gel and western blots were probed for pTyrosine (PY99) (Santa Cruz) and actin (c-11) (Santa Cruz) and developed with AP conjugated anti-mouse and anti-goat antibodies (Southern Biotech), respectively.

2.8. QRT-PCR

RNA was isolated from 1×10⁶ sort-purified cells using TRI REAGENT according to the manufacturer's protocol (Molecular research center, Inc.). RNA was reverse transcribed with M-MLV Reverse transcriptase (Promega) and treated with RNasin and RNase Ribonuclease (Promega) according to the manufacturers' protocols. Quantitative RT-PCR was performed using the RT² SYBR Green qPCR Master Mix (SA Biosciences) and ABI Prism 7500 thermocycler. Specific genes were amplified using the following primer sets: KLF4: 5' GCGAACTCACACAGGCGAGAAACC and 3' TCGCTTCCTCTTCTCCGACACA; Notch2: 5' CCGTCGATTTCGCTCGAGT and 3' TGGGCAATGCTGCGTTTT; Hes1: 5' CAGCGGAATCCCCTGTCTAC and 3' GAGCAGTTGAAGGTTTATTATGTCTTAGG, Belx1: 5' CTGGGACACTTTTGTGGATCTCT and 3' GAAGCGCTCCTGGCCTTT, and Actin: 5' GGTCAGAAGGACTCCTATGTG and 3'AGAGCAACATAGCACAGCTTC. Non-template controls were included for each reaction and dissociation analyses were performed to confirm the specificity of the reaction. Relative expression levels were determined using the ΔΔC_T method. Briefly, the C_T value of the actin house-keeping gene was subtracted from that of the target gene to get the ΔC_T = (C_T Test gene - C_T Actin). The ΔΔC_T was calculated by subtracting the ΔC_T of the control population from that of the test population ΔΔC_T = (ΔC_T - ΔC_T FO control). Two raised to the negative of this value = 2^(-ΔΔT) gave the relative fold change.

2.9. Statistical analyses

All data were subjected to statistical analyses using the unpaired Student's T test. P values less than 0.05 were considered to be significant.

3. Results

3.1. Bright transgenic mice exhibit defects in B cell maturation

Previous studies suggest that Bright over-expression in B lineage cells resulted in autoantibody production (Shankar *et al.*, 2007), but the FVB/N background used was suggested to contribute to autoimmunity and precluded performing additional studies to determine how Bright dysregulation resulted in breaches in B cell tolerance. Therefore, we examined the effect of Bright over-expression on ANA production and B cell development in C57Bl/6 transgenic mice derived from more than 10 backcrosses. Bone marrow and splenic B cell subsets were enumerated and compared in Bright transgenic (BrTg) and controls using flow cytometry and gating as described (Shankar *et al.*, 2007). The frequencies and total numbers of B cells from each bone marrow subset were not different from controls (Table I). Importantly, the size of the mature recirculating B cell compartment, which contains a subset of the animal's antibody secreting cells, was not enlarged compared to controls. Therefore, constitutive Bright expression did not alter early B cell development.

Similar analyses were performed on the immature splenic B cell subsets defined by Allman (Allman *et al.*, 2001) as previously described (Shankar *et al.*, 2007). The most recent emigrant population from the bone marrow, the immature T1 B cell subset, was slightly increased in relative proportions compared to littermate controls (Figure 1A), although absolute T1 cell numbers were not different (Figure 1B and Table I). Alternatively, the absolute numbers of BrTg T2 B cells were decreased by >35% compared to littermate controls, suggesting a partial block in differentiation from the T1→T2 B cell stage. However, the size of the T3 B cell pool, which largely consists of anergic B cells and is decreased in other lupus prone mouse models (Teague *et al.*, 2007), did not differ between transgenics and controls (Figure 1B and Table I). Thus, ANA production in the BrTg mice does not appear to be due to defective production of anergic B cells.

Unexpectedly, mature splenic B cell subsets in the BrTg mice were dramatically altered. Total splenic B cell numbers were reduced in the transgenic mice, but BrTg MZ B cell numbers were increased slightly relative to the littermate controls (Figure 1B). In contrast, FO B cells were reduced by nearly 50% in the BrTg mice versus controls (Figure 1B). This resulted in a ratio of one MZ for every three FO cells in the BrTg mice, compared to a 1:10 ratio of MZ to FO cells in littermate controls. Consistent with the decreases observed in T2 cell numbers, these data suggest that constitutive expression of Bright negatively impacts FO B cell maturation.

Mature peritoneal B cell numbers were not significantly altered in the BrTg mice compared to littermate controls (Figure 1C). Numbers and proportions of conventional B2 B cells, B1a B cells and B1b B cells were equivalent in all mice tested, indicating that over-expression of Bright did not affect the ability of mature B cells to fill this compartment.

3.2. Bright transgenic mice produce normal levels of circulating Ig, but maintain autoantibody production

Enumeration of CD138⁺ plasma cells in the spleens of BrTg mice showed slight increases relative to wild type controls (0.28±0.02% versus 0.18±0.02% of total spleen cells, respectively). Therefore, we asked if serum Ig levels were altered in the BrTg mice. Ig isotype levels were measured by ELISA in sera from 6 week-old, non-immunized BrTg mice and littermate controls (Figure 2A). IgM and all IgG isotypes measured were comparable between controls and transgenics. Therefore, levels of serum Ig were not adversely affected by Bright over-expression in these mice.

Sera from 4 to 8 week-old mice from each of the resulting lines were assayed for antinuclear antibody (ANA) production. Littermate controls were assayed in parallel and representative results from one line are shown in Figure 2B. 100% (11 of 11) of the transgenic mice produced ANAs in mice as young as 4 weeks old, while none of the littermate controls (0 of 10) showed positive nuclear reactivity, consistent with results from FVB/N Bright transgenics (Shankar *et al.*, 2007). Nuclear staining patterns differed from punctuate to homogeneous among different animals of the same line (Figure 2B). The variability in staining patterns suggests that the antibody response was polyclonal, rather than specific for only one type of nuclear antigen, and western blotting confirmed reactivity of individual sera with more than one protein (not shown). These data demonstrate that Bright over-expression in the B lineage was sufficient for ANA production and indicate that their production was not simply a result of global increases in all Igs.

3.3. Early tolerance checkpoints were not affected by Bright transgene over-expression

To begin to explore how Bright over-expression resulted in breaches in B cell tolerance, we asked if Bright transgenic mice might show increases in receptor editing suggesting it affected early events in B cell development. In polyclonal B cell populations, the ratio of λ to κ light chain usage is sufficient to measure alterations in levels of receptor editing (Diamant *et al.*, 2005). Therefore, we compared the λ : κ ratio of BrTg B cells with those from littermate controls. Immature and mature B cells were analyzed using flow cytometry and average ratios of λ to κ were 1:25 in the BrTg and 1:29 in the controls (Table II). Likewise, average mature B cell λ : κ ratios were 1:14 in the transgenics versus 1:16 in the controls. Cell numbers expressing λ or κ light chains did not differ significantly between BrTgs and control mice. These data suggest that BrTg mice exhibit normal levels of receptor editing.

Negative selection of autoreactive B cells also occurs at the T1 B cell stage where these cells undergo rapid apoptosis in response to signals through the B cell receptor (BCR) (Carsetti *et al.*, 1995; Petro *et al.*, 2002; Su and Rawlings, 2002). Because T1 B cells were slightly increased in the BrTg mice, we hypothesized that those cells might allow breaches in tolerance at this checkpoint and show increased viability relative to controls. T1 B cells were sorted using markers for CD21 and CD24 to avoid signaling through the Ig receptor (Petro *et al.*, 2002; Su and Rawlings, 2002) and were subsequently stimulated with anti-IgM (Figure 3A and B). Viabilities assessed over time showed no apparent differences in survival between BrTg T1 cells and normal controls, either with or without stimulation (Figure 3B). As expected, the T1 cells from both transgenic and littermate controls were less viable than FO B cells in both cases (Figure. 3C). Nor was there significant incorporation of H^3 -thymidine in response to BCR signals in either control or BrTg T1 B cells (Figure 3D), as was previously reported by Su, *et al.* (Su and Rawlings, 2002), which might have suggested increased cell division of the BrTg cells. These data suggest that Bright over-expression does not increase the survival of T1 B cells or alter their responses to BCR signaling.

3.4. Over-expression of Bright does not appear to cause clonal deletion of follicular B cells

Elimination of autoimmune cells can occur by apoptosis and deletion of those cells, as shown in mouse models expressing clonally related autoantibodies. Our previous data suggested that Bright over-expression allows production of B cells with multiple antigen specificities (Shankar *et al.*, 2007). Because FO B cell numbers were greatly reduced in the Bright transgenic mice, we hypothesized that these cells might have been eliminated as an ineffective attempt to eliminate autoreactive cells from this compartment. This would suggest defects in this tolerance checkpoint. In other autoimmune models where clonal deletion occurs, the anti-apoptotic protein Bcl2 has been used to rescue autoreactive cells that would normally be deleted and allow their survival (Lang *et al.*, 1997; Nisitani *et al.*,

1993). Therefore, we asked if we could restore normal numbers of FO B cells in the BrTg mice if they also over-expressed Bcl2. BrTg mice were bred with mice that over-express Bcl2 from the E μ enhancer. Bcl2 expressing littermate controls also showed expansion of B cell compartments, such that they showed approximately 3–5-fold increases in their total B cell numbers compared to non-transgenic control mice. Therefore, we examined relative increases in the BrTg x Bcl2Tg double transgenic B cell subsets directly to those obtained in the Bcl2 transgenics. FO B cell numbers in mice that expressed both BCL2 and Bright transgenes were decreased compared to the FO B cell numbers in the BCL2 transgenic mice suggesting that BCL2 did not rescue these cells. However, the double transgenic mice showed more than 5-fold increases MZ B cells compared to BCL2 transgenic MZ cell numbers (Table III). These data are consistent with the possibility that the MZ B cell compartment of BrTg mice may be dramatically enriched in clonally deleting (i.e. autoimmune) B cells. The ability of the Bcl2 transgene to hyper-expand the BrTg B cell subsets was also evident at the T1 B cell stage, albeit to a lesser degree, but not at the T3 B cell stage (Table III). Together, these data suggest that reduced numbers of FO cells in the BrTg mice are not the result of deletion of autoreactive cells.

We also considered that deletion of the FO cells might be the result of inappropriate sequestering of Bright with Btk because Btk deficient mice also exhibit defects in FO cell development (Khan *et al.*, 1995), and Bright forms a transcription factor complex that requires Btk (Rajaiya *et al.*, 2006; Rajaiya *et al.*, 2005). However, Btk-deficient Bright transgenic mice did not produce ANAs (not shown), indicating that the phenotype of the BrTg mice could not be explained solely by defects resulting from inappropriate sequestering of Btk.

3.5. Constitutive Bright expression predisposes B lineage development of MZ B cells

MZ B cell numbers were increased relative to FO cell numbers in both BrTg mice and Br Tg x Bcl2-Tg double transgenic mice relative to controls. Therefore, we asked if Bright over-expression resulted in B lineage cells which were preferentially predisposed to become MZ B cells. To test this hypothesis, bone marrow chimeras were generated in which whole bone marrow cells from BrTg and/or C57Bl/6 (both Ly5.2⁺) mice were transferred at differing ratios into lethally irradiated Ly5.1⁺ congenic recipients. Splenic development of Ly5.2⁺ donor cells was analyzed after five weeks (Figure 4, Table IV). Engraftment of Ly5.2⁺ cells was equivalent in mice receiving either BrTg or control donor bone marrow, and accounted for 75% of the total cells in the recipient spleens (Figure 4A). Analyses of the engrafted donor cells from recipients which received 100% wild type control cells showed a ratio of FO to MZ B cells of 6 to 1 (Figure 4B and C), whereas recipients of 100% BrTg cells showed a FO/MZ ratio of 2 to 1. Consistent with these findings, mice which underwent competitive reconstitution with mixed numbers of BrTg and wild type cells showed intermediate ratios of FO to MZ cell development which correlated with the percent of BrTg B cells used. Increased percentages of BrTg donor B cells also resulted in decreases in total FO B cell numbers as was observed in the original BrTg lines. These data show a clear bias towards MZ B cell versus FO development in B cells over-expressing Bright, suggesting that Bright over-expression may drive B cells toward a MZ cell fate.

To determine if autoantibody production correlated with numbers of MZ B cells in the reconstituted mice, we analyzed sera from each of these mice for ANA levels. Indeed, in all but one of the twelve animals, ANA staining intensity correlated with the numbers of MZ B cells present in the mice (Figure 4D). This suggests a strong correlation between MZ B cell numbers and ANA production in the BrTg mice.

3.6. BrTg FO B cells are partially hyperactive

We next asked whether the existing BrTg FO B cells were functionally normal. B cells from some lupus-like models demonstrate increased hyperactivity including hyper-proliferation, spontaneous Ig secretion or increased basal activation (Atencio *et al.*, 2004; Nijnik *et al.*, 2006; Wu *et al.*, 2007). Levels of surface markers typically associated with activation were similar on both BrTg and control FO cells (Figure 5A). Additionally, BCR and TLR4 receptor levels did not differ between control and BrTg B cells (Figure 5B). However, isolated FO B cells from BrTg mice were hyper-responsive to stimuli through both the BCR and TLR4 receptors and proliferation was significantly increased in comparison to controls (Figure 5C and D). Isolated MZ B cells from BrTg and littermate control mice proliferated equivalently when stimulated in culture (Figure 5E). Although BrTg FO cells were hyper-proliferative, total secreted Ig levels did not differ substantially from levels obtained from stimulated control cells in either MZ or FO cultured cells (Figure 5F). Secreted Ig from neither the MZ nor the FO cultured cells contained detectable ANA antibodies (not shown). These data suggest that constitutive expression of Bright in FO cells affects some aspects of their activation through antigen receptors.

To determine if proximal signaling responses were also affected by Bright expression, resting B cells were loaded with INDO-1, and Ca^{2+} influx was measured in response to BCR stimulation. Relative to baseline Ca^{2+} levels, transgenic B cells showed similar increases in magnitude of intracellular Ca^{2+} compared to control B cells (Figure 5G), suggesting that early membrane signals were not impaired or amplified in the transgenic B cells. Likewise, BrTg and control FO cells exhibited similar global tyrosine phosphorylation kinetics (Figure 5H). NF- κ B is a more distal signaling intermediate common to both the BCR and TLR4 signaling pathways and was shown to be hyperactive in other autoimmune models (Wu *et al.*, 2007). We measured NF- κ B binding activity 18 hour post-stimulation by EMSA. Basal NF- κ B levels were consistently 3-fold lower in FO B cells from BrTg mice than in controls (Figure 5I) compare lanes 1 and 4). However, antigen stimulation led to equivalent increases in levels of NF- κ B DNA-binding activity, as assessed using ImageJ software, in both transgenic and control B cells (Figure 5I compare lanes 2 and 3 to 5 and 6). Together, these data demonstrate minor differences in some properties of FO BrTg B cells versus controls, but suggest that the transgenic FO B cells are capable of normal responses through the BCR.

3.7. BrTg FO B cells exhibit MZ-like gene expression profiles

Kin, et al. (Kin *et al.*, 2008), showed that a subset of genes were differentially expressed in FO and MZ B cells from C57Bl/6 mice such that those genes expressed at high levels in MZ cells were expressed at very low levels in FO cells, and vice versa. Because constitutive Bright expression favored MZ development, we asked if the gene expression profile of Bright transgenic B cells compared to control B cells would also indicate a predisposition toward MZ gene expression. Preliminary array data (not shown) obtained from total BrTg versus littermate control, CD43-depleted splenic B cells showed differential expression of 25 of the 29 genes described by Kin et al., to be consistently over-expressed in MZ versus FO cells in multiple mouse strains (Kin *et al.*, 2008). To control for differences in MZ versus FO cell numbers in the BrTg versus control mice, QRT-PCR analyses were performed using RNA obtained from sorted BrTg and littermate control FO and MZ B cells. Relative expression levels of five representative genes are shown in Figure 6. All data were standardized against actin levels, and then are presented compared to the expression level obtained for FO control cells which was given a value of 100% or 1.0. Although expression levels of KLF4, Notch2, Hes1 and BclxL were similar in MZ cells from both BrTg and control mice, expression of these same genes in BrTg FO cells was much lower than in control FO cells, and resembled levels observed in MZ cells from both transgenic and control mice. The exception was cMyc which showed lower than normal expression levels

in BrTg MZ B cells compared to control MZ B cells (Figure 6). These data demonstrate that over-expression of Bright in FO cells (which do not normally express this protein) alters the gene expression profile of the transgenic FO cells to more closely resemble that of control MZ B cells.

4. Discussion

4.1. Constitutive Bright expression during B cell maturation preferentially leads to marginal zone B cells

In this study, transgenic mice that failed to down-regulate Bright expression in T2 and FO splenic B cells showed skewed development toward marginal zone rather than follicular B cell development. Constitutive Bright expression was sufficient to cause autoantibody production on the non-autoimmune, C57Bl/6 background, and ANA production was directly associated with increases in the MZ B cell subset. Intriguingly, our data also demonstrate that transgenic Bright resulted in alterations in transcription of genes typically differentially expressed in FO versus MZ cells such that many genes in the transgenic FO B cells were expressed at levels that more closely resembled control MZ cells rather than control FO B cells. Together, these data show that Bright plays a critical role in developmental pathways leading to maturation of splenic B cells, and suggest that these Bright-mediated effects may be the result of alterations in gene expression profiles.

MZ versus FO cell differentiation has been shown to be influenced in part by B cell receptor (BCR) signaling strength, where strong signals are required for FO development and weaker signals are permissive for MZ B cell development (reviewed in (Pillai and Cariappa, 2009)). BCR surface levels did not differ appreciably between BrTg and control spleen cells at any stage of B cell development (Figures 1 and 5). Moreover, BrTg FO cells exhibited normal signaling responses as measured by Ca^{2+} influx and tyrosine phosphorylation of downstream mediators (Figure 5). Carey et al, suggested that MZ cells could be derived directly from the T1 immature B cell subset based on similarities in surface marker expression and CDR3 characteristics that preclude cells at the T2 developmental stage (Carey *et al.*, 2008). Indeed, the BrTg mice exhibit slightly decreased numbers of T2 cells (Table I) which might be consistent with the idea that Bright over-expression shunts cells towards the MZ compartment early during differentiation, thus accounting for the depletion in both T2 and FO cells. Because transgenic FO cells exhibit altered gene expression patterns, it is also possible that these cells could also directly differentiate into FO cells, accounting for the decreased FO numbers, but we consider this to be less likely.

Several other proteins have been shown to contribute directly to the MZ versus FO developmental pathway. Notch-2 is a critical player in this lineage decision (reviewed in (Pillai and Cariappa, 2009)). However, over- or under-expression of these proteins does not reproduce the full phenotype of the BrTg mice. For example, c-fos over-expression increases numbers of MZ cells, but not FO cells (Yamashita *et al.*, 2005). Mzb1 protein is expressed specifically in MZ cells rather than FO cells and contributes directly to signaling strength and antibody production (Flach *et al.*, 2010). FO B cell generation is dependent on expression of the ets family of transcription factors, PU.1, Spi-B and Spi-C (DeKoter *et al.*, 2010), but this correlates with direct effects on the surface marker CD23. Intriguingly, KLF-2 deficiency results in increased numbers of MZ cells and FO cells that exhibit some characteristics of normal MZ cells, much like our BrTg B cells (Hart *et al.*, 2011; Winkelmann *et al.*, 2011). It is not yet clear how Bright regulation is linked to these other proteins.

4.2. Bright transgenic mice show normal functions at tolerance checkpoints

Although we originally hypothesized that ANA production in the BrTg mice would correlate with defects in known tolerance checkpoints, this was not the case. Numbers of anergic T3 B cells were normal (Table I), suggesting Bright over-expression and ANA production did not occur from failure to produce this subpopulation. In the NZB/W lupus model, inappropriate responses to BCR stimulation led to the aberrant survival of T1 B cells (Roy *et al.*, 2005), but BrTg T1 B cell numbers were not increased and these cells demonstrated normal viability and proliferative responses to BCR stimulation *in vitro* (Figure 3). MRL/*lpr* lupus prone mice show decreased levels of receptor editing (i.e. decreased proportion of λ^+ expressing cells), which likely contributes to the breaches in tolerance seen in this model (Lamoureux *et al.*, 2007). However, λ : κ ratios were similar between BrTg and control B cells (Table II), implying that ANA production was not the result of gross defects in receptor editing. Therefore, Bright expression does not result in ANA production through obvious breaches in these well-characterized tolerance checkpoints.

4.3. Autoantibody production correlates with numbers of marginal zone B cells

Reconstitution experiments suggested that ANA production correlated with numbers of BrTg MZ B cells (Figure 4). Several lupus-prone mouse strains, including the NZB/W F1 hybrid and MRL/*lpr*, have increased MZ B cells (Atencio *et al.*, 2004; Nijnik *et al.*, 2006), and autoreactive B cells were shown previously to preferentially populate the MZ (Martin and Kearney, 2002; O'Reilly *et al.*, 1997). Other studies also suggest that MZ B cells are enriched for autoreactive clones compared to FO B cells (Cyster *et al.*, 1994; Martin and Kearney, 2000). Chromosome 13 transgenic mice show skewing of splenic B cell subpopulations similar to our BrTg mice and also produce autoantibodies (Loh *et al.*, 2011). However, the BrTg mice did not show altered surface activation markers as was found in the congenic mice. The murine lupus susceptibility locus Sle2a was also shown to recruit B cells to the MZ compartment (Zeumer *et al.*, 2011). Consistent with these observations, over-expression of both Bright and Bcl2 in MZ B cells resulted in a >5-fold expansion of cells compared to Bcl2-only expressing MZ B cells (Table III). This result could be due to increased numbers of autoreactive cells that were rescued from apoptosis within the MZ subpopulation of BrTg mice, or it could be the result of undefined synergistic actions between Bright and Bcl2 specific to the MZ cell subpopulation. In any case, our results strongly suggest that Bright-induced ANA production is strongly associated with a MZ cell phenotype.

Secreted antibodies from either sorted MZ and FO B cells stimulated with LPS *in vitro* failed to show consistent ANA staining in any of four experiments, making it difficult to exclude the possibility that ANAs are also derived from activated BrTg FO cells. Several lines of evidence suggest that BrTg FO cells differ functionally and phenotypically from control FO cells. BrTg FO cells were consistently hyperproliferative to both BCR and TLR4 signals when compared to control FO cells, although the differences were less than two-fold (Figure 5). MZ B cells typically respond more robustly to LPS stimulation *in vitro* than do FO cells (Oliver *et al.*, 1997). However, the BrTg MZ B cells were not hyperproliferative compared to their control MZ counterparts. Other autoimmune transgenic models show increased B cell proliferation, including the c-Myc Tg (Refaeli *et al.*, 2005) and Fli-1 Tg (Bradshaw *et al.*, 2008) models, and it is possible that Bright contributes to shared pathways with these transgenes. More importantly, BrTg FO B cells exhibit altered gene expression patterns that suggest that the reduced numbers of FO cells that develop in the BrTg show broad similarities at the transcription level to normal MZ B cells with some similarities to KLF-2 deficient FO cells (Hart *et al.*, 2011; Winkelmann *et al.*, 2011). KLF4 is a negative regulator of B cell proliferation and is normally expressed at lower levels in MZ B cells compared to the FO cells (Kin *et al.*, 2008). Over-expression of Bright caused decreased

KLF4 levels in BrTg FO cells that resembled levels found in both control and BrTg MZ cells. Nonetheless, BrTg FO cells also differ substantially from control and BrTg MZ cells. For example, the surface markers which define FO versus MZ B cells allow designation of the BrTg cells as FO cells. Furthermore, it seems likely that specific environmental niches and limiting accessory cell types, such as MZ macrophages, may affect gene expression patterns of the BrTg FO cells so that they will also be functionally different from typical MZ cells. Intriguingly, the *Sle2* locus, which has been linked to MZ development in lupus models, contains the *KLF4* gene (Zeumer *et al.*, 2011). It will be important to examine earlier stages of B cell development, such as the T1 and T2 stages, for expression of this gene, to determine if Bright mediated gene expression patterns that may ultimately affect MZ versus FO development occur there as well.

4.4. Bright over-expression does not mimic a Btk-deficient phenotype

The most striking effect of Bright over-expression in B lineage cells was the skewing of the MZ/FO ratio. Although MZ cell numbers were only increased 1.5-fold over control cell numbers in BrTg spleens, BrTg FO cell numbers were decreased by half (Table I). Because Bright associates with Btk and Btk-deficient mice also develop approximately half the normal number of FO spleen cells, we considered the possibility that decreases in FO cell development in the BrTg mice could be the result of inappropriate sequestration of Btk which then resulted in blocks in development at the FO cell stage. Several arguments can be made that this was not the case. B cells from Btk deficient mice exhibit reduced Ca^{2+} mobilization (Fluckiger *et al.*, 1998) and proliferation (Satterthwaite *et al.*, 1997) in response to BCR signaling, whereas BrTg B cells mobilize Ca^{2+} similarly to normal cells (Figure 5). Secondly, serum Ig levels are reduced in Btk deficient mice, presumably due to decreases in recirculating Ig-secreting B cells in the bone marrow (Khan *et al.*, 1995). Serum Ig levels were normal in the BrTg mice (Figure 2), as were numbers of recirculating B cells (Table I). Moreover, all of the BrTg mice produced ANAs, a phenotype not observed in Btk deficient mice at any dilution of serum. Therefore, the decrease in FO B cells likely results from a mechanism independent of Btk depletion.

4.5. Bright affects expression of multiple genes which may be associated with autoimmunity

We have recently shown that inhibition of Bright function alone results in alterations in global gene expression patterns such that the resulting cells develop pluripotent tendencies (An *et al.*, 2010), and suggesting that alterations in Bright levels may broadly impact gene expression and development of specific cell types. Intriguingly, Bright deficiency results in increased KLF4 levels, which are necessary for reprogramming cells to a pluripotent state. These data suggest that Bright may function together, or with other associated proteins, to produce epigenetic changes in gene expression patterns. Lupus is clearly a multigenic disease and recent papers suggest that epigenetic mediators may play a role in its pathology. Interestingly, we found that the human orthologue of Bright, ARID3a, is expressed at abnormally high levels in approximately half of lupus patients we examined (our unpublished data). Thus, Bright/ARID3a may also contribute to ANA production in human autoimmunity.

5. Conclusions

These data confirm that over-expression of Bright in B lymphocytes leads to ANA production on the non-autoimmune C57Bl/6 background. However, constitutive Bright expression in these mice had additional effects on the development of mature B cell subsets, not observed in FVB/N BrTg mice (Shankar *et al.*, 2007), suggesting that appropriate regulation of the transcription factor Bright is critical for normal development of mature B

cell subsets. Moreover, constitutive Bright expression in B lineage cells is associated with autoantibody production that correlates with increased numbers of MZ cells rather than with noticeable breaches in earlier tolerance checkpoints. Understanding which genes this transcription factor regulates during B cell differentiation may suggest new targets for interfering with autoantibody production.

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Abbreviations

Bright	B cell regulator of immunoglobulin heavy chain transcription
MZ	marginal zone
ARID	AT-rich interaction domain
Ig	immunoglobulin
Btk	Bruton's tyrosine kinase
FO	follicular
BrTg	Bright transgenic
ANA	antinuclear antibody
BCR	B cell receptor

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Highlights

- The transcription factor Bright was over-expressed in murine B lineage cells.
- Bright over-expression skewed mature B cell development toward marginal zone cells.
- Bright transgenic mice produced autoantibodies.
- Autoantibody production was associated with numbers of marginal zone B cells.
- Transgenic follicular B cell gene expression resembled marginal zone B cells.

Figure 1. Splenic B cell subsets are skewed in BrTg mice

Splenic B cells from littermate controls and BrTg mice were analyzed using flow cytometry and representative gated populations are shown in (A) for immature transitional type 1 (T1 = CD93⁺IgM^{hi}CD23⁻), transitional type 2 (T2 = CD93⁺IgM^{hi}CD23⁺), and transitional type 3 (T3 = CD93⁺IgM^{lo}CD23⁺), and mature marginal zone (MZ = CD93⁻IgM^{hi}CD23⁻) and follicular (FO = CD93⁻IgM⁺CD23⁺) B cell subsets. Percentages of individual subpopulations are given. (B) Graphic representation of splenic B cell subsets from 9 control and 10 BrTg mice. Standard error bars are shown. (C) Peritoneal lavage cells from 11 control and BrTg mice were gated for B220⁺ B cells and average percentages of B1a (CD5⁺ IgM⁺), B1b (CD5⁻ IgM^{hi}) and B2 (CD5⁻ IgM^{lo}) cells are shown with standard error bars.

Figure 2. Bright over-expression results in ANA production without altering serum Ig levels
 (A) Sera from non-immunized 6 to 8 week old mice were analyzed for IgM, IgG1, IgG2a, IgG2b and IgG3 levels by ELISA. Concentrations were determined from standard curves generated for each isotype from serially diluted duplicate samples. Each symbol represents the average of triplicate values from a single mouse. Bars represent the MEAN $\pm$ SE for each group (n=7 littermate controls, n=7 BrTg mice). (B) Representative nuclear antigen staining of HEp-2 cells with sera from one of ten control, and two of eleven BrTg mice are shown. All controls were negative for ANA staining, while all 11 BrTg sera were positive. Nuclear antigen reactivity was detected using anti-mouse IgG-FITC. Magnification was at 400x.

Figure 3. BrTg and control T1 B cells exhibit similar viabilities and responses to BCR signaling
 (A) Representative sort and post-sort data for isolating T1 and FO cells are shown. Immature T1 B cells were B220⁺CD24^{hi}CD21^{-/lo} and mature FO B cells were B220⁺CD24⁺CD21⁺.
 (B) Purified T1 or (C). FO cells (1×10^6 cells/ml) were stimulated with 10 $\mu\text{g/ml}$ anti-IgM, or with medium alone and viabilities were assessed over time using forward/side scatter properties and 7AAD dye exclusion. Each symbol represents the mean value of quadruplicate samples from pooled cells obtained from two or more mice. Data are representative of two separate experiments. (D) Anti-IgM-treated T1 cells from littermate controls (filled bars) and BrTg mice (empty bars) were cultured for 48 hours and pulsed with [³H] thymidine 8 hours prior to harvest. Results are representative of two independent experiments.

Figure 4. Constitutive Bright expression enhances development of MZ B cells

(A) Four groups of three lethally irradiated mice (CD45.1⁺) each received 2×10^6 bone marrow cells from C57Bl/6 or BrTg mice (CD45.2⁺) in the ratios of donor cells shown and engraftment of donor cells (CD45.2⁺) was analyzed by flow cytometry. Representative plots of one mouse from each group are shown and exhibit similar levels of engraftment as indicated. (B) Donor spleen cells were gated and analyzed for MZ vs FO cell numbers as shown by representative data for mice receiving 100% control or BrTg cells. (C) The average ratios of donor-derived FO to MZ B cells per spleen for mice from each donor group are shown with standard error bars. (D) ANA staining of sera from positive and negative controls, and sera at identical, representative dilutions from mice receiving the indicated mixtures of BrTg B donor cells are shown.

Figure 5. BrTg FO B cells exhibit normal BCR signaling responses, but are mildly hyperproliferative

(A) FO B cells from control and BrTg mice were analyzed for surface activation markers CD25, CD86 and CD80, and (B) BCR and LPS receptors and representative histograms are shown. Staining with isotype controls are presented as gray filled histograms. Data are representative of 4 mice each. (C) Isolated BrTg and control FO cells were stimulated for 48 hours at 1×10^6 cells/ml with the indicated concentrations of anti-IgM or LPS, (D) Proliferation was measured after an 8 hour pulse with [^3H] thymidine. Bars represent the MEAN $\pm$ SE of cells isolated from two mice and assayed in quadruplicate. Data are representative of three individual experiments. P values were determined by students T-test. (E) Sorted MZ cells were stimulated with 10 $\mu\text{g/ml}$ LPS and proliferation was measured as in D. (F) Sorted FO and MZ cells from three BrTg and three littermate controls were stimulated with 10 $\mu\text{g/ml}$ anti-IgM and secreted Ig kappa was measured by ELISA after 5 days. Bars represent MEAN $\pm$ SE values. (G) Calcium influx after BCR signaling of CD43 depleted splenic control (gray line) and BrTg (dark black line) B cells is shown. Data are representative of four independent experiments. (H) Whole cell lysates from B cells stimulated with 10 $\mu\text{g/ml}$ anti-IgM for the times indicated were subjected to western blotting and developed with anti-pTyrosine and anti-actin. Data are representative of three independent experiments. (I) NF- κ B activity was assessed by EMSA from nuclear extracts prepared from 1×10^6 sorted FO B cells stimulated with 10 $\mu\text{g/ml}$ anti-IgM or LPS for 18 hours. Western blots of duplicate samples were developed for actin as a loading control (bottom panel).

Figure 6. Bright expression in FO B cells alters gene expression profiles

QRT-PCR was performed on RNA isolated from sorted control (open bars) and BrTg (filled bars) FO and MZ B cells. Values were standardized to actin levels from individual samples. For comparison, values of control FO cells were arbitrarily set at 1.0 in each case, and the other data are presented relative to that value. Reactions were performed in triplicate and MEAN $\pm$ SEM are shown. P values were determined by students T test.

Table I

Numbers of splenic B cells within individual B cell subsets differed between BrTg mice and littermate controls^a

	Control	BrTg	<i>P</i> value
Bone marrow B cells ($\times 10^6$)			
Total cells per femur	21.6 $\pm$ 2.89 ^b	24.5 $\pm$ 2.19	0.443 ^c
Pro-B (B220 ^{lo} CD43 ⁺ IgM ⁻)	0.21 $\pm$ 0.04	0.27 $\pm$ 0.04	0.317
Pre-B (B220 ^{lo} CD43 ⁻ IgM ⁻)	1.94 $\pm$ 0.22	2.40 $\pm$ 0.24	0.190
Imm (B220 ^{lo} CD43 ⁻ IgM ⁺)	0.69 $\pm$ 0.10	0.90 $\pm$ 0.11	0.175
Recirc (B220 ^{hi} CD43 ⁻ IgM ⁺)	1.02 $\pm$ 0.15	1.28 $\pm$ 0.15	0.220
Splenic B cells ($\times 10^6$)			
Total cells	85.7 $\pm$ 9.07	73.8 $\pm$ 8.16	0.343
CD19 ⁺ B cells	43.9 $\pm$ 3.94	35.3 $\pm$ 2.68	0.089
T1 (B220 ⁺ IgM ^{hi} CD93 ⁺ CD23 ⁻)	5.64 $\pm$ 0.80	6.47 $\pm$ 0.37	0.360
T2 (B220 ⁺ IgM ^{hi} CD93 ⁺ CD23 ⁺)	7.34 $\pm$ 0.96	4.80 $\pm$ 0.37	0.024
T3 (B220 ⁺ IgM ^{lo} CD93 ⁺ CD23 ⁻)	1.82 $\pm$ 0.21	1.75 $\pm$ 0.39	0.871
MZ (B220 ⁺ IgM ^{hi} CD93 ⁻ CD23 ⁻)	2.29 $\pm$ 0.29	3.38 $\pm$ 0.25	0.009
FO (B220 ⁺ IgM ⁺ CD93 ⁻ CD23 ⁺)	23.9 $\pm$ 2.32	13.0 $\pm$ 1.02	0.001

^a Male and female mice (n = 9 littermates and n = 10 BrTg mice) were analyzed at 6 weeks of age.

^b Total cell numbers (MEAN $\pm$ SEM) per femur or spleen were determined by multiplying the frequency of live gated cells (Figure 2) by the number of total cells per femur or spleen and are shown $\times 10^6$.

^c Student's t test

Table II

The average proportion of kappa and lambda light chain expressing cells was not appreciably different between BrTg and control mice^a

	Control	BrTg	P value
Immature B cells (%)			
Lambda ⁺ (B220 ⁺ CD93 ⁺)	3.13±0.13 ^b	3.60±0.12	0.093 ^c
Kappa ⁺ (B220 ⁺ CD93 ⁺)	90.8±0.55	87.8±0.46	0.060
λ: κratio	1:29	1:25	
Mature B cells (%)			
Lambda ⁺ (B220 ⁺ CD93 ⁻)	5.19±0.11	6.09±0.21	0.100
Kappa ⁺ (B220 ⁺ CD93 ⁻)	85.5±0.73	82.9±1.05	0.107
λ: κratio	1:16	1:14	

^a Male mice (n = 4 littermates and n = 5 BrTg mice) were analyzed at 12 weeks of age.

^b Frequency (MEAN±SEM) of B220⁺ cells

^c Student's t test

Table III

Bcl2 over-expression preferentially expands T1, T2 and MZ B cells in BrTg mice^a

	C57Bl/6 control	BrTg	Bcl2	BrTg x Bcl2	Average fold- increase
Splenic cell numbers (× 10 ⁶)					
Total cells	109.6±18.5 ^c	90.5±13.2	533.3±18.9	575.4±63.9	
CD19 ⁺ B cells	47.5±4.48	38.2.6±2.8	264.6±21.9	267.5±24.9	
T1 ^b	5.63±0.81	7.39±0.76	14.4±2.45	35.7±4.22	2.5 ^d
T2	8.33±1.15	5.12±0.47	34.2±6.02	28.0±4.68	0.82
T3	2.94±0.83	2.41±0.49	20.2±4.88	12.1±1.65	0.60
MZ	2.25±0.27	3.69±0.32	7.08±2.22	40.1±7.17	5.7
FO	26.2±2.98	15.3±1.30	144.0±10.5	75.3±9.99	0.52

^a Male and female mice (n = 7 to 11 mice per group) were analyzed at 8 to 12 weeks of age.

^b Splenic B cell subsets were defined as in Fig 1.

^c Total cell numbers (MEAN±SEM) per spleen were determined by multiplying the frequency of live gated cells by total cells and are shown ×10⁶.

^d Average fold increase in BrTg x Bcl2 double transgenic mice compared to Bcl2 transgenic mice was calculated by dividing the average total cell numbers for each subset.

Table IV

Bone marrow B cells over-expressing Bright preferentially develop into MZ cells^a.

	FO	MZ	Ratio
CD45.2⁺ B cells splenic B cells ($\times 10^6$)			
0% BrTg (100% Control)	35.40 $\pm$ 8.70 ^b	5.66 $\pm$ 0.94	6.18
50% BrTg (50% Control)	39.23 $\pm$ 1.62	8.47 $\pm$ 0.27	4.63
75% BrTg (25% Control)	27.77 $\pm$ 2.23	6.99 $\pm$ 0.82	4.01
100% BrTg (0% Control)	22.90 $\pm$ 3.46	10.65 $\pm$ 1.14	2.14

^a Recipient mice (n = 3 per group) were analyzed after 5 weeks.

^b Total cell numbers (MEAN $\pm$ SEM) per spleen were determined by multiplying the frequency of live cells gated as in Figure by the number of total cells per spleen and are shown $\times 10^6$.