The origin of anti-nuclear antibodies in bcl-2 transgenic mice

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Abstract

bcl-2 transgenic mice develop anti-double-stranded (ds) DNA antibodies similar to those present in systemic lupus erythematosus. To begin to understand where a breakdown in the regulation of autoreactive lymphocytes is occurring, we have used a bcl-2 transgene (Tg) in conjunction with an Ig Tg that allows us to identify and track anti-dsDNA B cells. Previously, we have shown that anti-dsDNA B cells are actively tolerized in BALB/c mice as manifested by their developmental arrest, follicular exclusion, increased in vivo turnover rate and lack of their antibody in the serum. The bcl-2 Tg mice increased the lifespan of anti-dsDNA B cells, but did not alter the other features of tolerance, indicating that the anergy of the anti-dsDNA B cells is independent of their reduced lifespan. Furthermore, these data suggest that the serum anti-dsDNA antibodies in bcl-2 transgenic mice are not due to a breakdown in the induction or maintenance of B cell anergy; rather they may originate from B cells that have transited through a germinal center.

Introduction

One characteristic of many autoimmune diseases such as systemic lupus erythematosus (SLE) is the presence of specific autoantibodies that are absent in non-autoimmune individuals. A breakdown in the regulation of autoreactive B lymphocytes could occur at multiple levels, including in the generation of the initial repertoire in the bone marrow (BM) (by blocking deletion or anergy) or in the formation of the modified repertoire during germinal center (GC) maturation. In the latter case, autoantibodies may arise as a result of somatic mutation of non-autoreactive B cells. Generally these autoreactive B cells would die in the absence of T help to a new antigen (reviewed in 1). Death due to lack of a positive signal is often referred to as death by neglect (2). This would predict then, if death by neglect is blocked, autoantibodies would be produced.

Bcl-2 has been shown to protect lymphocytes from some forms of death by neglect, including growth factor withdrawal (3). Furthermore, Bcl-2 is implicated in B lymphocyte repertoire selection by its tightly regulated pattern of expression: Bcl-2 is not expressed during times of B cell selection, including pre-B and immature B cells in the BM and GC B cells (4–6). Consistent with this, some bcl-2 transgenic mice (bcl-2-22) develop a fatal autoimmune disease. This disease was reported to resemble SLE in that the mice developed serum antibody to nuclear antigens such as snRNPs, histones and double-stranded (ds) DNA, and their kidneys showed evidence of immune complex glomerulonephritis (7). It should be noted that another bcl-2 lineage (M23) did not show this phenomenon (8). It has been suggested that the relevant parameter distinguishing these two bcl-2 transgenics was their genetic background (C57BL/6 for bcl-2-22 and C57BL/6 for M23) (9).

To address the role that altered Bcl-2 expression could have on B cell tolerance, several groups have used these bcl-2 transgenic mice in conjunction with Ig transgenic mice. In the anti-erythrocyte and anti-MHC class I tolerance models, Bcl-2 was shown to block peripheral deletion of autoreactive B cells, while leaving deletion in the BM intact (10,11). In contrast, in the anti-hen egg lysozyme (HEL)/mHEL model, Bcl-2 has been shown to protect lymphocytes from some forms of death by neglect, including growth factor withdrawal (3). Furthermore, Bcl-2 is implicated in B lymphocyte repertoire selection by its tightly regulated pattern of expression: Bcl-2 is not expressed during times of B cell selection, including pre-B and immature B cells in the BM and GC B cells (4–6). Consistent with this, some bcl-2 transgenic mice (bcl-2-22) develop a fatal autoimmune disease. This disease was reported to resemble SLE in that the mice developed
in the BM. None of these experiments, however, addressed the fate of B cells with specificities that arise spontaneously in bcl-2 transgenic mice, i.e. anti-nuclear antibodies (ANA).

To determine where B cell tolerance to nuclear antigens breaks down in bcl-2 transgenic mice, we have used VH3H9 Ig transgenic mice (14). These mice allow us to identify and track anti-dsDNA B cells in the context of a bcl-2 Tg (bcl-2 line 6 transgenic mice) (15). The bcl-2 Tg is expressed in B cells at all developmental stages, including those when endogenous Bcl-2 is absent: pre-B and immature B cell stages in the BM and in GC B cells (16). Previously, using

VH3H9 transgenic mice, we have shown that in the non-autoimmune BALB/c background, anti-dsDNA B cells are actively tolerized as manifested by their developmental arrest, localization to the T/B interface in the spleen, increased in vivo turnover rate and lack of serum autoantibody (17). In autoimmune-prone MRL-lpr/lpr mice, this tolerance breaks down: anti-dsDNA B cells are no longer developmentally arrested nor held at the T/B interface and by 10 weeks of age their antibody is in the serum (18). Here, we show that Bcl-2 extends the lifespan of anti-dsDNA B cells in VH3H9 transgenic mice, but does not inhibit other manifestations of B cell tolerance. Therefore, increasing a B cell’s lifespan is not enough to break tolerance. With age, bcl-2 transgenic mice do develop serum anti-dsDNA antibodies. These antibodies do not originate from the anti-dsDNA B cells rendered tolerant in the BM, rather we hypothesize that they may originate from B cells that have transited through a GC.

**Methods**

**Mice**

BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). MRL-lpr/lpr and C57BL/6-lpr/lpr mice were purchased from Jackson Laboratory (Bar Harbor, ME). To generate the bcl-2 transgenic mice used in this study (bcl-2 line 6), human bcl-2 was placed under control of the IgH chain intron enhancer, with the intention of directing expression to lymphocytes (15). bcl-2 line 6 transgenic mice, originally on the SWR background, and VH3H9 transgenic mice (14) have been backcrossed for four to 10 generations onto the BALB/c background. No difference was detected between early and late backcross mice for the parameters tested. The mice have been bred and maintained in a separate specific pathogen-free room at The Wistar Institute animal facility. In all cases, age-matched BALB/c mice or non-transgenic littermates, housed similarly, were used as controls. The presence of the bcl-2 and VH3H9 Tgs was determined by PCR amplification of tail DNA with primers specific for human bcl-2 (15) and VH3H9 (14) respectively.

**Identification of VH3H9/λ1 anti-dsDNA B cells**

The VH3H9 H chain transgenic has been shown to be a good excluder of endogenous H chain rearrangement in the BALB/c background (14), and the lack of endogenous H chain in the IgM only VH3H9 transgenic mice was confirmed here by the lack of staining for IgD and IgG (data not shown). VH3H9 when paired with Igλ1 generates an anti-dsDNA Ig and we have followed the fate of anti-dsDNA B cells in VH3H9 transgenic mice using anti-Igλ1-specific reagents (17). Several different reagents were used to track Igλ1+ and Igλ1+ B cells (LS136, R11-153, JCS and R26-46). Using these reagents and flow cytometry we have shown that the majority (79 ± 19%) of Igλ1+ B cells in VH3H9 and VH3H9/bcl-2 transgenic mice are Igλ1+, as they are from Tg(-) mice (17 and data not shown). Therefore, we are able to follow VH3H9/λ1 B cells in bcl-2 transgenic mice using anti-pan Igλ reagents. Previously we have shown the vast majority of Igλ1+ B cells in bcl-2 transgenic mice, by flow cytometry (91.9 ± 3.5%) and hybridoma analysis (88.9%) co-expressed an Igκ L chain,
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Fig. 3. Bcl-2 prolongs lifespan of VH3H9/λ B cells. Tg(–) (top), VH3H9 (middle) and VH3H9/bcl-2 (bottom) mice were continuously labeled with BrdU for 8 days. Spleen cells were stained with antibodies to Igλ and B220, fixed, permeabilized, and then incorporated BrdU was detected with an anti-BrdU antibody. Dot-plots show B220 versus Igλ and histograms show BrdU label within the B220^+ Igλ^+ gate. Percentages are given for the BrdU^+ cells. These are representative plots for n = 4 mice of each genotype.

indicating that multiple L chain rearrangements had occurred (16). Unlike the Igλ^+ B cells in bcl-2 transgenic mice, the majority of Igλ^+ B cells in VH3H9/bcl-2 transgenic mice do not co-express an Igκ L chain as assessed by flow cytometry (5.2 ± 2.5% co-express Igκ and Igλ). Furthermore, isotype analysis of hybridomas generated from VH3H9/bcl-2 BALB/c mice confirmed the presence of Igλ^+ B cells that exclusively express an Igκ L chain (data not shown). The fact that the majority of Igλ^+ B cells in VH3H9 transgenic mice do not co-express Igκ is what allows us to use Igλ-specific reagents to identify anti-dsDNA B cells in VH3H9 transgenic mice without the concern that we are following VH3H9/λ/κ^+ cells which may have an altered specificity.

Flow cytometry analysis

Cells (5×10^5) were surface stained according to standard protocols (19). The following antibodies were used: RA3-6B2–phycoerythrin (PE) or −biotin (anti-B220), R11-153–FITC (anti-Igλ1), R26-46–FITC or −biotin (anti-Igκ total), R8-140–PE (anti-Igκ), 1D3–FITC (anti-CD19), 7G6–FITC (anti-CD21/35), Cy3-4.1–FITC (anti-CD22), B3B4–FITC (anti-CD23), and M1/69–FITC (anti-HSA) (PharMingen, San Diego, CA); LS136–biotin (anti-Igλ1) and JC5.1–PE (anti-Igκ total) (gifts from J. Kearney; University of Alabama, Birmingham); polyclonal anti-IgG–FITC (Sigma, St Louis, MO), polyclonal anti-IgM–PE and SBA-1–PE (anti-IgD) (Southern Biotechnology Associates, Birmingham, AL); and streptavidin–Red670 (Gibco/BRL, Gaithersburg, MD).

All samples were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. In total, 40,000 events, gated for live lymphocytes based on forward and side scatter, were collected for each sample.

BrdU labeling

Mice were injected i.p. with 200 µl of 3 mg/ml BrdU (Sigma) in PBS every 12 h for 8 days. BrdU staining was performed essentially as described (20), with the exception that the cells were not fixed in ethanol. Briefly, spleen and BM cells from mice were isolated and surface stained as described above. The cells were then fixed and permeabilized with 1% paraformaldehyde containing 0.1% Tween 20. The DNA was denatured using 10 µM HCl and 100 U/ml DNase I. The incorporated BrdU was then detected using an anti-BrdU–FITC antibody (B44) from Becton Dickinson and cells analyzed by flow cytometry as described above.

Immunohistochemistry

Spleens were suspended in OCT, frozen in 2-methyl-butane cooled with liquid nitrogen, sectioned and fixed with acetone. The spleen sections were stored at –20°C and then stained according to the protocol described (21). Briefly, the sections were blocked using PBS/5% normal goat serum/0.1% Tween
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20, and then stained with GK1.5–biotin (anti-CD4), 53-6.7-biotin (anti-CD8), RA3-6B2–biotin (anti-\( bcl-2 \)) (grown as supernatants) and/or anti-Ig\( \lambda \)–alkaline phosphatase (AP; Southern Biotechnology Associates). Apoptosis was determined using an \textit{in situ} cell death detection kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer’s instructions, with the exception that the slides were fixed with acetone prior to fixing in 4% paraformaldehyde to optimize surface antigen staining. The sections were then stained with peanut agglutinin (PNA)–biotin (Vector, Burlingame, CA). TUNEL staining (dUTP–FITC) was detected with anti-FITC–AP (Sigma). Streptavidin–horseradish peroxidase (HRP) or –AP (Southern Biotechnology Associates) was used as a secondary antibody with the biotinylated reagents. HRP and AP were developed using the substrates 3-amino-9-ethylcarbazole and Fast-Blue BB base (Sigma) respectively.

Quantification of apoptotic GC B cells in situ

TUNEL-stained spleen sections from influenza-immunized mice were analyzed for the number of apoptotic cells per GC. Sections were counted in a blind fashion by three separate investigators. The ratio of apoptotic cells in Tg(–) and \( bcl-2 \)-transgenic GC was consistent among the three readers. At least 40 GC were counted per spleen and were separated based on GC size: small (10–29 cells in diameter) and large (>30 cells in diameter) to equalize for the frequency of non-apoptotic cells. No difference was detected in the number of small GC versus large GC for Tg(–) and \( bcl-2 \) transgenic mice.

Hybridoma generation and Ig sequence analysis

Unmanipulated spleen cells from a \( bcl-2 \) transgenic mouse (age 7.5 months, serum ANA\(^+\)) and two \( VH3H9/bcl-2 \) transgenic mice (ages 7 weeks and 5.5 months, both serum ANA\(^+\)) were fused to the Ig\( ^\gamma \) myeloma, Sp2/0. To ensure that any clones detected were not due to expansion \textit{in vitro}, cells were immediately plated at limiting dilution and wells bearing single colonies were expanded for analysis. The H and L chain V regions were sequenced from mRNA according to the protocol described (22). Briefly, cytoplasmic RNA was isolated and constant region-specific primers were used to direct synthesis of cDNA copies of the H (C\( \alpha \)) and L (C\( \kappa \)) chain V regions. The cDNA was then amplified using the constant region primers in conjunction with VH5/1 and L5 primers that hybridize to the 5’ ends of H and L chain V region genes respectively (22). Amplification products were sequenced by automated analysis (Wistar Institute Nucleic Acid Facility). Sequence translation and comparison was carried out using the Sequencer program and by searching EMBL/GenBank/ DDBJ databases. To control for PCR errors, H and L chain sequencing was repeated on PCR products generated from two independent cDNA reactions and identical results were obtained. Family classification for both H and L chains was made based on homology (H chain 89%; L chain 96% identity at the nucleotide level) to GenBank published sequences.

\textbf{ELISA assay}

The Ig isotype of hybridomas was determined via an indirect solid-phase ELISA assay, using anti-IgH + L (Southern Biotechnology Associates) as the primary antibody and developing with AP-labeled anti-IgM, -IgG, -IgX or -Ig\( \lambda \) antibodies (Southern Biotechnology Associates).

\textbf{Southern blots}

DNA was prepared from hybridoma cell cultures using proteinase K digestion followed by phenol extraction. Then 10 \( \mu \)g of DNA was digested with either EcoRI or BamHI, size separated, and blotted onto Zeta-probe blotting membrane (BioRad, Richmond, CA). DNA rearrangements at the H chain locus were detected by probing EcoRI-digested DNA with pJ11 (23) and rearrangements at the L chain locus were analyzed by probing BamHI-digested DNA using the pECK probe (24).

\textbf{ANA assay}

The presence of ANA in serum and supernatants was detected using permeabilized HEP-2 cells as the substrate following the manufacturer’s instructions (Antibodies Incorporated, Davis, CA). Serum giving a homogeneous nuclear (HN) staining pattern was defined as ANA\(^+\). This pattern is found in a high frequency of SLE serum, and correlates with the presence of anti-dsDNA, anti-histone and/or anti-chromatin antibodies (25). Serum ANA titers were determined for a subset of the...
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Fig. 5. Phenotypic analysis of VH3H9/λ B cells. (A) VH3H9/λ anti-dsDNA B cells are present with a reduced Ig density. BM (left), spleen (middle) and LN (right) cells from Tg(-) (top), VH3H9 (middle) and VH3H9/bcl-2 (bottom) mice were stained with antibodies to B220 and Igλ. MFI is given for the Igλ+ cells in the boxed region. (B) Bcl-2 does not alter the developmental arrest of VH3H9/λ B cells in the spleen. Spleen cells were stained with antibodies to B220, Igλ and either HSA, CD21/35, CD22 or CD23. Histograms are gated on B220+Igλ+ cells from the indicated mouse (bold line) overlaid B220+ cells from the Tg(-) BALB/c mouse (thin line). The underlaid histograms were scaled down to allow for the comparison the Igλ+ B cells (which are present at ~10% the frequency of total B cells). These are representative plots from a minimum of n = 4 mice of each genotype.

ANA+ mice. The serum samples were tested at an initial 1:100 dilution and at serial 10-fold dilutions. The serum titer was defined as the reciprocal of the last dilution at which positive staining was seen (data not shown). B cell hybridoma supernatants were used undiluted. ANA binding was detected using anti-mouse IgM + IgG or Igλ-FITC secondary antibodies (Southern Biotechnology Associates). The samples were visualized under a fluorescent microscope and scored
Fig. 6. Bcl-2 does not alter the localization of VH3H9/λ B cells in the spleen. Spleen sections from Tg(–), VH3H9, bcl-2 and VH3H9/bcl-2 BALB/c mice were stained with antibodies to Igλ in blue and either B220 (top) or CD4 (bottom) in red. These are representative sections from n = 15 mice of each genotype. Original magnification ×100.

Fig. 7. Bcl-2 protects against GC B cell death in situ. Tg(–) and bcl-2 transgenic BALB/c mice were immunized with influenza virus PR8 and sacrificed 8 days later. Spleen sections from immunized mice were stained for apoptotic cells (blue) using the TUNEL method, followed by PNA (red) to mark GC. Figure shows a representative GC from 3 mice of each genotype. Original magnification ×100.

Table 1. Numbers of apoptotic cells per GC.

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<th>Small GC</th>
<th>Large GC</th>
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<tr>
<td>bcl-2 transgenic</td>
<td>18.7 ± 14.3</td>
<td>48.7 ± 29.0</td>
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<tr>
<td>Tg(–)</td>
<td>38.8 ± 28.8</td>
<td>89.8 ± 52.8</td>
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Bcl-2 protects against GC death in situ. Tg(–) BALB/c and bcl-2 BALB/c mice were immunized with influenza virus PR8 and sacrificed 8 days later. Spleens from immunized mice were sectioned and stained for apoptotic cells using the TUNEL method, followed by PNA to mark GC. bcl-2 transgenic GC have fewer TUNEL+ cells than Tg(–) GC in both small GC (defined as 10–29 cells in diameter, P < 0.0001) and large GC (defined as >30 cells in diameter, P = 0.0009). A minimum of 40 GC each from three mice of each genotype were counted.

Analysis of nephritis

Kidneys from age-matched Tg(–), VH3H9, bcl-2 and VH3H9/bcl-2 BALB/c mice, and a cohort of 15- to 20-week-old MRL-lpr/lpr mice, were fixed in 10% formalin, embedded in paraffin and then sectioned. Kidney sections were stained with hematoxylin & eosin and visualized under light microscopy. Kidneys were graded on a scale from 0–4 for three components (26): (i) glomerulonephritis: cellularity, proliferation, thickness of glomerular basement membranes, and expanded mesangial ratio, presence of cellular crescents; (ii) interstitial nephritis: inflammatory cell infiltration, tubular necrosis, disruption of tubointerstitial architecture; and (iii) vasculitis: perivascular infiltrates and intimal hyperplasia. Grades for the three para-
cell lines were washed once in PBS, normal media added, and were immunized i.v. with 1000 HAU of PR8 virus in 200 µL of infected supernatant, and therefore media was used in most experiments. To examine for differences, cells were collected, cleared by centrifugation at 1000 g for 10 min and stored at -70°C prior to use.

**Apoptosis of GC B cells**

Influenza virus immunization was performed as described (22). Briefly, Tg(-) BALB/c and bcl-2 transgenic BALB/c mice were immunized i.v. with 1000 HA units of PR8 virus in 200 µL volume of PBS. Eight days later spleens and thymus were removed and cultured for 4 h in either media alone, FasL-transfected supernatant or mock-transfected supernatant. No difference was detected between media and mock transfected supernatants, and therefore media was used in most experiments. Thymocytes from lpr/lpr mice, which are deficient in Fas (28,29), were used as a negative control for Fas-mediated killing. After culture, the cells were then harvested and stained for the presence of apoptotic cells using Annexin V-FITC (PharMingen). The spleen cells were then stained with PNA-biotin (Vector) and B220-PE (PharMingen) to mark GC B cells and analyzed by flow cytometry as described above.

**Statistical analysis**

Statistical significance was determined using an unpaired non-parametric test or a Student's t-test and Instat Software.

### Results

**ANA production and nephritis in bcl-2 transgenic mice**

To determine if the bcl-2 line 6 transgenic mice expressed ANA, serum from age-matched Tg(-) and bcl-2 transgenic BALB/c mice were evaluated. In bcl-2 transgenic mice, the frequency of mice expressing serum ANA is increased compared to Tg(-) BALB/c mice and also increases with the age of the mice (Fig. 1). This is similar to what has been reported for bcl-2-22 transgenic mice (7), but different from another bcl-2 transgenic line (M23), which was reported to be ANA- (8). Whether unique aspects of the constructs and hence the expression pattern of the bcl-2 Tgs, or the genetic background on which the Tgs were studied, accounts for these differences has not been determined.

Kidneys from 2- to 8-month-old Tg(-) and bcl-2 line 6 BALB/c mice exhibited no significant kidney pathology (data not shown). In older mice (9–15 months old), however, bcl-2 transgenic mice exhibited significant nephritis compared to Tg(-) BALB/c mice (Fig. 2). This is similar to the results using another bcl-2 transgenic model (bcl-2-22) which also developed ANA and kidney pathology (7). Notably, the extent of pathology observed in the bcl-2 transgenic mice is lower than in diseased MRL-lpr/lpr mice (Fig. 2).

To directly assess the impact of aberrant Bcl-2 expression on anti-dsDNA B cells, we took advantage of the VH3H9 Ig transgenic model. As a Tg, VH3H9 can pair with a variety of different L chains to generate both anti-DNA and non-DNA antibodies (30,31). In particular, VH3H9 pairs with the Igα L chain to generate an anti-dsDNA antibody (30,32), thus enabling us to use anti-Igα-specific reagents to follow anti-dsDNA B cells in a diverse repertoire. Previously we have shown for another mouse model of SLE, the MRL-lpr/lpr mouse, that the presence of the VH3H9 Tg does not alter the kinetics of seroconversion. Furthermore, VH3H9/λ anti-dsDNA antibodies are detected in the serum when the MRL-lpr/lpr mice become ANA+ (18). In contrast, the VH3H9 Tg reduces ANA production in bcl-2 transgenic mice (Fig. 1). Even in those mice that were ANA+, generally Igλ- antibody did not contribute to the serum ANA; Igλ ANA staining was not detected in the serum of >98% of VH3H9/bcl-2 mice (n = 58, ages 2–15 months). Consistent with the reduction in serum ANA, VH3H9/bcl-2 mice exhibit less nephritis than bcl-2 Tg alone (Fig. 2).

**bcl-2 Tg increases the lifespan of anti-dsDNA B cells**

To determine if the bcl-2 Tg increased the in vivo lifespan of VH3H9/λ B cells, Tg(-), VH3H9 and VH3H9/bcl-2 BALB/c mice were labeled with BrdU for 8 days, and the incorporation of BrdU into their splenic B cell populations was measured. To assess the lifespan of the anti-dsDNA population of cells, BrdU incorporation in the Igλ subset was evaluated. Among the Igλ- cells, the frequency of BrdU+ cells is higher in the VH3H9 transgenic mice than in the Tg(-) mice (63.1 ± 5.6 versus 9.5 ± 4.0%), suggesting that the anti-dsDNA B cells have a decreased in vivo lifespan (Fig. 3) (17). However, in the presence of the bcl-2 Tg, only 13.1 ± 1.3% of the VH3H9/λ B cells were labeled. This indicates that the bcl-2 Tg does increase the lifespan of VH3H9/λ B cells. Consistent with this increase in lifespan, Bcl-2 increases the frequency of Igλ+ B cells in the BM, spleen and lymph node (LN) compared to that in VH3H9 transgenic mice without the bcl-2 Tg (Fig. 4). In VH3H9 transgenic mice without the bcl-2 Tg, the frequency of Igλ+ B cells decreases between the spleen and LN. In contrast, in VH3H9/bcl-2 transgenic mice, this decrease is not apparent (Figs 3 and 4), suggesting that an increased lifespan allows the anti-dsDNA B cells to circulate to the LN.

**Bcl-2 does not alter the surface phenotype or splenic localization of anti-dsDNA B cells**

To determine if an increased lifespan has an effect on the phenotype of the anti-dsDNA VH3H9/λ B cells, we used flow cytometry and an anti-Igλ reagent to characterize the anti-dsDNA B cells. The level of Ig on the surface of VH3H9/λ B cells is down-regulated in the periphery compared to the Igλ+ B cells from Tg(-) mice and Bcl-2 does not change this expression (Fig. 5a). Interestingly, the Ig level is also decreased on the Igλ+ B cells from the BM. This decreased Ig density suggests that these cells have encountered their antigen initially in the BM and that Bcl-2 does not affect the timing of this encounter.
To assess if aberrant expression of Bcl-2 translates into other changes in surface phenotype, we compared the levels of a panel of developmental markers (B220, HSA, CD21/35, CD22 and CD23) (19,20,33–35) on splenic VH3H9/λ B cells with and without the bcl-2 transgenic. In the spleen, VH3H9/λ B cells in non-bcl-2 transgenic mice are developmentally arrested as shown by decreased levels of B220 (2-fold), CD21/35 (3-fold), CD22 (2-fold), CD23 (2-fold) and increased level of HSA (2-fold) (17) (Fig. 5b). As is shown in Fig. 5(b), the presence of the bcl-2 Tgs does not alter the developmental arrest of the VH3H9/λ B cells; they still express decreased levels of B220, CD21/35, CD22 and CD23, and an increased level of HSA.

We next addressed whether increasing the lifespan of the anti-dsDNA B cells altered their splenic localization. In Tg(−) BALB/c mice, Igλ+ B cells are located primarily in the B cell follicle with some cells in the red pulp (Fig. 6). Like Tg(−) BALB/c mice, the Igλ+ B cells in bcl-2 transgenic mice are located with the remainder of the B cells, in the B cell follicle, with few to no B cells in the periarteriolar lymphoid sheath. A striking feature of bcl-2 Tg mice is a dramatic increase, compared to Tg(−) mice, in B cells (of both L chain
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isotypes) in the red pulp (Fig. 6). Previously, we have shown that without the bcl-2 Tg, VH3H9/λ B cells localize to the T/B interface (17). In the presence of the bcl-2 Tg, VH3H9/λ B cells are also primarily located at the T/B interface and in the red pulp (Fig. 6). The localization of VH3H9/bcl-2 λ B cells is similar to that found in the HEL model where in the context of a bcl-2 Tg (bcl-2-22) the autoreactive anti-HEL B cells persisted longer in the recipient spleens, but their localization to the T/B interface was not altered (13). Therefore, although Bcl-2 increases the lifespan of anti-dsDNA B cells, it does not alter their developmental arrest or accumulation at the T/B interface in the spleen.

Bcl-2 does not protect GC B cells against Fas-mediated death, but does protect against death-by-neglect

The analysis of the VH3H9/λ anti-dsDNA B cells suggests that central tolerance mechanisms to curtail anti-dsDNA B cells are intact in the bcl-2 transgenic mice. Tolerance can also be broken in the generation of the modified repertoire, e.g. during GC maturation. If the bcl-2 transgenic is providing

Fig. 9. Model of anti-dsDNA antibody production in bcl-2 transgenic mice. In VH3H9 transgenic mice, anti-dsDNA B cells encounter antigen in the BM. These B cells are not deleted, but enter the periphery in a functionally compromised state and have a shortened lifespan. The bcl-2 transgenic is able to protect the anti-dsDNA B cells from death, but does not affect their anergy. Non-DNA B cells in both Tg(-) and VH3H9 transgenic mice do not encounter their antigen in the BM. These cells exit to the periphery as functionally capable B cells. During an immune response, these B cells will encounter their antigen, receive T cell help and enter GC where they can undergo rounds of somatic mutation, thus altering their affinity for antigen. Some of these mutations result in antibodies that have an increased affinity for the desired antigen and these B cells receive positive signals to survive. Other mutations will lead to B cells with decreased affinity for antigen; the lack of a positive signal leads to the death of the B cells. However, a proportion of these mutations will result in B cells with reactivity to self antigens, such as dsDNA. These autoreactive B cells would normally die due to lack of a positive signal (death by neglect). In this model, Bcl-2 is able to protect these cells against death by neglect and thus allows them to survive and produce autoantibodies.

Fig. 8. Bcl-2 protects GC B cells from death by neglect but not from Fas-mediated death. Tg(-) BALB/c and bcl-2 transgenic BALB/c mice were immunized i.v. with influenza virus (PR8) to induce GC. (A) Spleen cells were stained with anti-B220 and PNA to identify follicular (B220+/PNA<sup>low</sup>, gate R4) and GC (B220+/PNA<sup>high</sup>, gate R3) B cells. PNA<sup>high</sup> B cells were detected only in immunized and not naive mice, indicating that they formed as a consequence of immunization (data not shown). Histograms show Fas levels on PNA<sup>low</sup> (bold line) overlaid PNA<sup>low</sup> (thin line) B cells. As has been previously reported, Fas is expressed at low levels on follicular (PNA<sup>low</sup>) B cells (MFI: BALB/c 12.9, bcl-2 transgenic 13.1 and lpr/lpr 2.51) (38). Fas levels are increased on GC (PNA<sup>high</sup>) B cells (MFI: BALB/c 188.6, bcl-2 transgenic 194.4 and lpr/lpr 3.2). (B) Fas wild-type (BALB/c) but not lpr/lpr thymocytes are killed by FasL supernatant. (C) Mean percentages of death from Tg(-) BALB/c (open bars), bcl-2 transgenic (solid bars) GC B cells in media (left) and FasL supernatant (right). Percentages of GC B cell death were determined by adding the number of Annexin<sup>+</sup> B220<sup>-</sup>PNA<sup>high</sup> B cells recovered after culture in either media (left) or FasL supernatant (right) to the number of B220<sup>-</sup>PNA<sup>high</sup> B cells lost during culture, dividing this quantity by the number of B220<sup>-</sup>PNA<sup>high</sup> B cells<sup>ex vivo</sup> and multiplying by 100%. Bcl-2 protects GC B cells from death in media alone (%). Bcl-2 does not protect against Fas-mediated death (media: 29.50 ± 5.52 versus FasL: 69.03 ± 8.06%; P = 0.0022). There is a slight increase in death over that in media alone for Tg(-) BALB/c GC B cells cultured in FasL (media: 61.87 ± 7.52; FasL: 69.03 ± 8.06%; P = 0.1095). n = 3 mice of each genotype.
a survival advantage for GC B cells, then this predicts that there will be less death in GC from bcl-2 Tg mice than from Tg(-) mice. To increase the frequency of GC B cells, Tg(-) and bcl-2 transgenic mice were immunized with influenza virus (PR8) and sacrificed at day 8. Spleen sections were stained by TUNEL to detect apoptotic cells within GC in situ. GC from bcl-2 transgenic mice have 50% less apoptotic cells than Tg(-) GC (Fig. 7 and Table 1). This is consistent with a previously published report using p-azophenylarsonate-immunized bcl-2 transgenic mice (36). Together, these data suggest that the bcl-2 Tg may be protecting GC B cells against some triggers of cell death in the GC.

GC B cells, which are normally Bcl-2+ (6.37), express a high level of Fas (Fig. 8a) (38) and have been shown to be susceptible to death triggered through Fas (39). Because the bcl-2 Tg used in this study is abnormally expressed in the GC (16), we looked to see if Bcl-2 was able to protect these GC B cells against Fas-mediated death. The frequency of PNAhigh GC B cells from mice immunized 8 days earlier with influenza was increased from 1.0 ± 0.31% in naive mice to 4.6 ± 1.4% after immunization (P = 0.0001). As has been previously reported, these PNAhigh B cells from Tg(-) and bcl-2 transgenic mice are Fas−high (38) (Fig. 8a). Note, however, PNAlow B cells express a low, but detectable, level of Fas compared to lpr/lpr B cells. The spleen cells from immunized mice were cultured in vitro for 4 h with either supernatant derived from FasL-transfected cells or mock-transfected cells. Thymocytes from Tg(-) BALB/c mice were used as a positive control and C57BL/6/lpr/lpr mice as a negative control for Fas killing. As is shown in Fig. 8(b), thymocytes from Fas wild-type, but not lpr/lpr mice die when cultured in FasL, showing specificity of the FasL. GC B cells from Tg(-) BALB/c mice die in media alone and, because there is so much death in media alone (Figure 8C), it is hard to detect a significant increase in death when cultured with FasL. Importantly, the presence of the bcl-2 Tg protects the GC B cells against death in media alone. However, Bcl-2 does not protect against death triggered by FasL (Fig. 8c). The ability of Bcl-2 to block Fas-mediated death has been highly controversial (40-45). Here, we show that Bcl-2 has no protective effect for GC B cells against killing through Fas. These data suggest that the role of Bcl-2 may be to protect GC B cells against death resulting from the absence of a positively selecting signal that is missing in the 'media alone' condition, such as T cell help (46).

Clonal expansion of ANA B cells

One characteristic of cells that have transited a GC is their clonal expansion as well as the acquisition of somatic mutations (47,48). To determine if the ANA in bcl-2 transgenic mice arise by this mechanism, a hybridoma panel was generated from the unmanipulated spleen of a bcl-2 transgenic mouse that was producing serum ANA. Twenty percent of the hybridomas recovered were ANA+, consistent with the serum data. All of the hybridomas, regardless of ANA status (n = 43), were analyzed by Southern blot to identify potential clonally related sets. Identical Southern blot rearrangement patterns of H and L chain loci were seen in two of the ANA+ hybridomas, suggesting that they were members of a clone (data not shown). To establish that these two hybrids were indeed clonally related and to identify potential somatic mutations, their H and L chain genes were sequenced. Clonal relatedness was established by identical CDR3 regions (data not shown). The two hybrids expressed the same SM7/JH5 H chain and Vκ4/5/Jκ5 L chain gene segment combinations (22,49). While these H and L gene segment combinations have not been previously reported, the H chain resembles that used by a DNA-binding hybrid from an immunized bcl-2 transgenic mouse (95% identity) (50), and the L chain resembles those previously published for anti-DNA (97% identity) and anti-cardiolipin (96% identity) autoantibodies from lupus-prone mice (51,52). When the sequences of the H and L chains used by the two hybridomas were compared, there was no evidence of somatic mutation. The possibility remains, however, that the two hybrids are somatically mutated from their germline counterpart. To definitively assign the contribution of somatic mutation in the generation of ANA, the germline sequence of these H and L chain genes would need to be identified, and the antibody re-expressed to show that they differ from the Ig of the retrieved hybridomas and that they do not generate anti-dsDNA antibodies.

Summary

bcl-2 transgenic mice develop anti-dsDNA autoantibodies that resemble those present in SLE. To better understand the origin of these autoantibodies, we have used a bcl-2 Tg in association with an Ig Tg that allows us to identify and track anti-dsDNA B cells. Previously, we have shown that one population of anti-dsDNA B cells (VH3H9A1) generated in the BM are actively tolerized in BALB/c mice as manifested by their developmental arrest, localization to the T/B interface in the spleen, increased in vivo turnover rate and absence of their antibody in the serum (17). The expression of a bcl-2 Tg increased the lifespan of these anti-dsDNA B cells, but did not alter the other features of tolerance. Importantly, their antibody remained undetectable in the serum. These data suggest that the anergy of anti-dsDNA B cells is independent of their reduced lifespan. Additionally, the effect of Bcl-2 in the induction of ANA does not occur at the level of breaking B cell anergy, consistent with the effect of bcl-2 Tg on tolerance to model antigens (10–13). Weigert’s group has identified another population of anti-dsDNA B cells (VH3H9/ Vκ4) that, rather than being anergized, are deleted in the BM (53). The fate of these B cells in the context of a bcl-2 Tg has not been determined.

Instead of a breakdown in central tolerance, ANA may arise from modifications of the primary repertoire through somatic mutation in the periphery. While the bcl-2 Tg is aberrantly expressed in pre-B, immature B and GC B cells, studies from other groups have suggested that Bcl-2 primarily affects the survival of GC B cells (36,50). Normally, B cells that have mutated toward self specificities during a GC reaction would not survive in the absence of appropriate T cell help and/or antigen (1,54). Aberrant Bcl-2 expression in the bcl-2 transgenic mice may protect these somatic variants from death by neglect. While we obtained direct evidence of clonal expansion of ANA+ B cells, we did not identify somatic mutations of their Ig to formally demonstrate GC transit. Manser’s group has recovered, in hybridoma panels

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generated from immunized bcl-2 transgenic mice, B cells that show evidence of somatic mutation that leads to DNA binding (36). We have shown that GC-derived B cells that expressed a bcl-2 Tg were protected against death in vitro in media alone and, as previously reported, less apoptotic cells were detected in situ in GC from bcl-2 transgenic mice than from Tg(-) mice (Table 1) (36), suggesting that the bcl-2 Tg does affect the survival of GC B cells.

VH3H9/bcl-2 transgenic mice develop serum ANA at a decreased frequency compared to bcl-2 transgenic mice without the VH3H9 Tg. This is consistent with a model where ANA B cells can arise by two different mechanisms (Fig. 9). The first is by rearrangement in the BM, as is the case for VH3H9/A1. These B cells, as we have detailed in this paper, are rendered tolerant as manifested by developmental arrest and retention at the T/B interface, both with and without the bcl-2 Tg. A second mechanism for generating ANA B cells is by somatic mutation in a GC. In this case, the decreased frequency of ANA in VH3H9 mice may be attributed to the limited diversity of their B cell repertoire that is still capable of participating in a GC reaction. While variable from mouse to mouse, the autoreactive B cells (Igα1 and many Igκ B cells) make up a large fraction of the B cell pool. These B cells are rendered anergic and the bcl-2 Tg does not alter this. This would leave a smaller fraction of naive B cells left to be substrates for somatic mutation during a GC reaction.

Finally, this study suggests that ANA in bcl-2 transgenic mice arise via a different mechanism than in MRL-lpr/lpr mice, another murine model for SLE. By 10 weeks of age, MRL-lpr/lpr mice develop a similar profile of autoantibodies to SLE patients. Previously, we have shown that there is a breakdown in the tolerance of anti-dsDNA B cells in MRL-lpr/lpr mice: anti-dsDNA B cells appear in the spleen with no evidence of developmental arrest and they are not excluded from the B cell follicle, even prior to seroconversion. Additionally, the VH3H9/A anti-dsDNA specificity is expressed in the serum of MRL-lpr/lpr mice at the same time as total ANA appear (18). This is different from bcl-2 transgenic mice where the anti-dsDNA B cells remain developmentally arrested and localized to the T/B interface and the VH3H9/A antibody is not in the serum. Therefore, the origin of ANA in bcl-2 transgenic mice is different from that in MRL-lpr/lpr mice. Instead, the bcl-2 Tg may lead to a breakdown of tolerance, possibly in GC during the formation of the modified repertoire. Which scenario more accurately reflects the etiology of autoantibodies in SLE remains to be determined.

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Abbreviations

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<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>ANA</td>
<td>anti-nuclear antibodies</td>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<td>BM</td>
<td>bone marrow</td>
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<td>ds</td>
<td>double-stranded</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>GC</td>
<td>germinal center</td>
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<td>HEL</td>
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<td>HN</td>
<td>homogeneous nuclear</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HSA</td>
<td>heat stable antigen</td>
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<td>LN</td>
<td>lymph node</td>
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<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>phycoerythrin</td>
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<td>PNA</td>
<td>peanut agglutinin</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<td>ss</td>
<td>single-stranded</td>
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<td>Tg</td>
<td>transgene</td>
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