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Functional Consequences of the Developmental Arrest and Follicular Exclusion of Anti-Double-Stranded DNA B Cells

Laura Mandik-Nayak,* Su-jean Seo,* Ashlyn Eaton-Bassiri,* David Allman, † Richard R. Hardy, † and Jan Erikson**

Anti-dsDNA B cells are actively tolerized in nonautoimmune BALB/c mice, as manifested by their developmental arrest, follicular exclusion, and rapid turnover rate. Previously, we have documented changes in the maturation status and follicular localization of anti-dsDNA B cells in autoimmune-prone MRL (+/+ and lpr/lpr) mice. To determine whether these differences in developmental status and follicular localization affect the functional capacity of anti-dsDNA B cells, we have now compared their in vivo life spans and their responses to in vitro stimuli. Our study shows that although anti-dsDNA B cells from both BALB/c and MRL-+/+ mice are localized to the T/B interface, only those in BALB/c mice have a rapid turnover rate. Therefore, the immature status and not the exclusion from the B cell follicle correlates with a shortened life span. Interestingly, apoptotic anti-dsDNA B cells were not detected at the T/B interface in BALB/c mice, suggesting that they are not dying there. This study also demonstrates that anti-dsDNA B cells, regardless of maturation status or follicular localization, are able to proliferate and up-regulate the costimulatory molecule B7-2 in response to CD40 ligand and IL-4. Therefore, one of the critical in vivo differences between anti-dsDNA B cells in BALB/c and MRL-+/+ mice compared with MRL-lpr/lpr mice may be the availability of T cell help. The Journal of Immunology, 2000, 164: 1161–1168.

B cell tolerance has been shown to be maintained by deletion, receptor editing, and anergy (1–5). The ultimate fate of an autoreactive B cell depends upon many factors, such as the form and location of Ag, the available T cell help, and the surrounding B cell repertoire (1–4). B cell anergy has been most extensively studied in the hen egg lysozyme (HEL)6 tolerance model. In this case, anergic B cells are excluded from the B cell follicle and have a rapid turnover rate, suggesting that localization to the T/B interface correlates with a reduced life span (6, 7). Additionally, although anergic anti-HEL B cells can proliferate to LPS and CD40 ligand (CD40L), they are unable to do so to anti-Ig (8). Upon encountering T cells in vivo, anergic anti-HEL B cells die via a Fas-dependent mechanism (9). Whether this accounts for their reduced life span in vivo and whether anergic B cells are actually regulated in a Fas-mediated manner are not known. However, a breakdown in tolerance to HEL was not found in mice deficient in Fas (lpr/lpr mice) (10).

Our studies have focused on understanding how the rules for tolerance that are evolving from model Ag systems apply to those Ags, such as dsDNA, that are typical of autoimmune disease. To do this, we have used the VH3H9 Ig transgenic (Tg) model (11). VH3H9 Tg mice allow us to track anti-dsDNA B cells in a diverse repertoire and compare their fate in both nonautoimmune (BALB/c) and autoimmune-prone (MRL-+/+ and MRL-lpr/lpr) mice. Similar to the HEL model, anergic anti-dsDNA B cells in nonautoimmune mice are excluded from the B cell follicle and have a rapid in vivo turnover rate. Additionally, and unlike the HEL model, anti-dsDNA B cells are developmentally arrested (12). Another striking difference from the HEL model is seen in the autoimmune background; lpr/lpr mice produce anti-dsDNA Abs (13). The VH3H9 Ig Tg model has been useful in that it allows us to follow anti-dsDNA B cells both in an environment where they are actively tolerized (BALB/c) and in one where their specificity is present in the serum (MRL-lpr/lpr). Using this approach, we have found that the manifestations of anergy seen in BALB/c mice are altered in MRL-+/+ and MRL-lpr/lpr mice, including changes in maturation status and follicular localization (12, 13).

In this study, we use a combination of in vivo and in vitro assays to determine the functional consequences of differences in developmental status and follicular localization. A comparison of the in vivo life span and proliferative status of anti-dsDNA B cells from BALB/c, MRL-+/+, and MRL-lpr/lpr mice indicates that the developmental arrest of a B cell influences its turnover rate. In vitro assays demonstrate that anti-dsDNA B cells from all three backgrounds are able to proliferate and up-regulate the costimulatory molecule B7-2 in response to CD40L and IL-4, suggesting their potential to respond to T cell help. In light of this, CD40L + IL-4 stimulation was used to generate hybridomas from a VH3H9 BALB/c mouse. In contrast to previous reports suggesting that anti-dsDNA B cells are deleted in the bone marrow (BM) (14, 15), we find multiple examples of anti-dsDNA B cells that use distinct Ig genes. Interestingly, the same Igs are used in anti-dsDNA 7 B cell hybridomas generated from VH3H9 MRL-lpr/lpr mice (25).

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3 Abbreviations used in this paper: HEL, hen-egg lysozyme; CD40L, CD40 ligand; Tg, transgenic; BM, bone marrow; AP, alkaline phosphatase; ANA, anti-nuclear Ab; BrdU, 5-bromo-2'-deoxyuridine; CFSE, carboxyfluorescein diacetate succinimidyl ester; HN, homogenous nuclear.

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Materials and Methods

**Mice**

BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). MRL-lpr/lpr and MRL-+/+ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). VH3H9 Tg mice have been described previously (16). The VH3H9 Tg mice have been backcrossed onto the BALB/c and MRL backgrounds for at least 9 and 17 generations, respectively, and have been bred and maintained in a specific pathogen-free room at The Wistar Institute animal facility. BALB/c and MRL-+/+ mice were 2–12 mo old, and MRL-lpr/lpr mice were 6–20 wk old. In all cases, age-matched BALB/c mice and/or Tg® littermates were used as controls. The presence of the VH3H9 Tg was determined by PCR amplification of tail DNA with primers specific for VH3H9 (16).

**Flow cytometry analysis**

Cells (5 × 10^6) were surface stained according to standard protocols (17). The following Abs were used: 1D3-FITC or -biotin (anti-CD19), R-46-FTTC or biotin (anti-Igα total), RK-140-PE (anti-Igκ), GL1-PE (anti-CD86, B7.2; Pharmingen, San Diego, CA), LS136-biotin (anti-IgA1, gift of G. Kelsoe, Duke University, Durham, NC), JC5.1-PE (anti-Igα total, gift of J. Kearney, University of Alabama, Birmingham, AL); and streptavidin-Red670 (Life Technologies, Gaithersburg, MD). For B7-2 staining, FcRs were blocked with 2.4G2 (anti-FcR; grown as ascites) before staining. As we have shown previously, the majority of IgA B cells are IgA1; therefore, we use anti-IgA reagents to identify VH3H9/A1 B cells (12, 13).

All samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. A total of 60,000 events, gated for live lymphocytes based on forward and side scatter, were collected for each sample.

**Cell cycle analysis**

Spleen cells were sorted into CD19^+ Igα and CD19^+ Igα subsets using a FACStar®流 (Becton Dickinson), and the sorted cells were fixed in 95% EtOH at 4°C overnight. The cells were then stained in a PBS containing 1% glucose, propidium iodide (PI) (10 g/ml), and RNase A (50 μg/ml). Cell-cycle status was determined by evaluating the DNA content of propidium iodide-labeled B cells by flow cytometry.

**Detection of apoptotic cells in situ**

Spleens were suspended in OCT, frozen in 2-Methyl-Butane cooled with liquid nitrogen, sectioned, and fixed with acetone before fixing in 4% paraformaldehyde to optimize surface Ag detection. Asparagine deaminase (ADA) positive (anti-ADA, grown as ascites) before staining. As we have shown previously, the majority of IgA B cells are IgA1; therefore, we use anti-IgA reagents to identify VH3H9/A1 B cells (12, 13).

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**Signal sequence analysis**

The VH3H9 H-chain Tg pairs with endogenous L-chains to generate both anti-DNA and non-DNA B cells (27). The specificity of the Igκ-bearing cells in VH3H9 mice is variable: hybridoma analysis has identified Igκ B cells that are ANA” and others that are ANA” (25). ANAs are found in a high frequency of systemic lupus erythematosus serum and correlate with the presence of anti-

**In vitro proliferation assay**

Spleen cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) as described (19). CFSE-labeled spleen cells (2 × 10^9/ml) were then cultured in either media alone (RPMI 1640/10% FCS/5 × 10^-3 M 2-ME), LPS (10 μg/ml; Sigma), or a combination of CD40L-CD8 fusion protein (gift of P. Lane, Basel Institute for Immunology, Basel, Switzerland) (20); and cross-linked with anti-CD8 (2.5 μg/ml) and baculovirus IL-4 (equivalent to 2 ng/ml recombinant protein; gift of E. Pure, The Wistar Institute, Philadelphia, PA). Cells were removed from culture after 1, 2, and 4 days to determine viability, as assessed by trypan blue exclusion under a light microscope, and proliferation. Proliferation was measured by flow cytometry as a decrease in CFSE intensity relative to unstimulated cells. Each cell division is accompanied by a decrease in CFSE intensity by one-half (21).

**5-Bromo-2’-deoxyuridine (BrdU) labeling**

**Long-term labeling.** Mice were injected i.p. with 200 μl of 3 mg/ml BrdU (Sigma) in PBS every 12 h for 4, 8, or 11 days. BrdU staining was performed essentially as described (22), with the exception that the cells were not fixed in ethanol. Briefly, spleen and BM cells from mice were isolated and surface stained to identify B cells 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 14 Nase I. The incorporated BrdU was then detected using an anti-BrdU-FITC Ab (B44) from Becton Dickinson (Mountain View, CA).

**Four-hour pulse.** Mice were injected i.p. with 200 μl of 3 mg/ml BrdU in PBS. The spleen and BM cells were isolated 4 h later and stained by flow cytometry as described above. Proliferating B cells in the BM were used as a positive control for BrdU labeling.

**Hybridoma generation**

Spleen cells from a VH3H9 Tg mouse were cultured overnight in media (DMEM/10% FCS/10^-1 M 2-ME) containing CD40L-CD8 fusion protein, cross-linked with anti-CD8 (2.5 μg/ml), and rIL-4 (2 ng/ml) (Genzyme Diagnostics, Cambridge, MA). The cells were then fused to the Igκ myeloma, Sp2/0. Cells were plated at limiting dilution and wells bearing single colonies were expanded for analysis.

**Sequence analysis**

The H- and L-chain V regions of the hybridomas were sequenced from mRNA according to the protocol described (23). Briefly, cytoplasmic RNA was isolated and constant region-specific primers were used to direct synthesis of cDNA copies of the H- (Cα1) and L- (Cα1 and Cε) chain V regions. The DNA was then amplified using the constant region primers in conjunction with VH51 for H-chains and A1L or L5 primers for L-chains (23). Amplification products were sequenced by automated analysis (The Wistar Institute Nucleic Acid Facility). All hybridomas shown were found to express a single L-chain. Sequence translation and comparison was conducted using the Sequencer program (Gene Codes, Ann Arbor, MI) and by searching EMBL/GenBank/DDBJ databases. The lowercase letter designation was used to distinguish among individual genes as was previously done for hybridoma Igks from VH3H9 Tg BALB/c (24) and MRL-lpr/lpr (25) mice. A sequence was designated the same gene and given the same lowercase letter suffix if there were seven or fewer nucleotide differences (97.5% nucleotide similarity), with no more than four of these in framework regions.

**Anti-nuclear Ab (ANA) assay**

The presence of ANAs in hybridoma supernatants was detected using permeabilized HEp-2 cells as the substrate following manufacturer’s instructions (Antibodies, Inc., Davis, CA). Supernatants giving a homogeneous nuclear (HN) staining pattern were defined as ANA⁺. ANA binding was detected using an anti-mouse Igκ + Igλ or Igκ-FITC secondary Ab (Southern Biotechnologies Associates). The samples were visualized under a fluorescent microscope and scored in a blind fashion.

**Crithidia lucilieae assay**

The presence of anti-dsDNA Abs in hybridoma supernatants was detected using permeabilized C. lucilieae protozoans as the substrate following manufacturer’s instructions (Antibodies, Inc., Davis, CA). Supernatants giving a homogeneous nuclear (HN) staining pattern were defined as ANA⁺. ANA binding was detected using an anti-mouse Igκ + Igλ or Igκ-FITC secondary Ab (Southern Biotechnologies Associates). The samples were visualized under a fluorescent microscope and scored in a blind fashion.

**Statistical significance**

Statistical significance was determined using an unpaired Student’s t test and Instat software (GraphPad Software, San Diego, CA).

**Results**

**The VH3H9/κ anti-dsDNA model**

The VH3H9 H-chain Tg pairs with endogenous L-chains to generate both anti-DNA and non-DNA B cells (27). The specificity of the Igκ-bearing cells in VH3H9 mice is variable: hybridoma analysis has identified Igκ B cells that are ANA⁺ and others that are ANA⁻ (25). ANAs are found in a high frequency of systemic lupus erythematosus serum and correlate with the presence of anti-dsDNA, anti-histone, and/or anti-chromatin Abs (28). By flow cytometry, looking at cell-surface phenotype as well as turnover rate,
70–80% of the Igκ B cells in VH3H9 Tg mice are analogous to Tgκ B cells (12). To analyze a defined population of anti-dsDNA B cells, we rely on the fact that VH3H9 pairs with the endogenous IgA1 L-chain to generate an anti-dsDNA Ab (25, 27). Therefore, we can use anti-Igλ-specific reagents to follow anti-dsDNA B cells in a diverse repertoire.

Previously, we have shown that the fate of VH3H9/anti-dsDNA B cells is different in nonautoimmune (BALB/c) vs autoimmune (MRL-+/+ and MRL-lpr/lpr) mice (summarized in Table I). Note that in all strains the level of surface Igκ is low, suggesting that in all backgrounds the anti-dsDNA B cells have encountered Ag. In BALB/c mice, the anti-dsDNA B cells are phenotypically immature, they are located at the T/B interface in the splenic follicle, and their Igκ is absent from the serum (12). In MRL-+/+ mice, anti-dsDNA B cells continue to localize to the T/B interface and their Igκ is still not found in the serum, but in this case they are developmentally mature (13). In MRL-lpr/lpr mice, anti-dsDNA Ab is present in the serum after 10 wk of age. However, even before this the anti-dsDNA B cells are phenotypically mature and localize to the B cell follicle (13).

In vivo turnover rate and proliferation status
To determine whether differences in developmental status and splenic localization affect the turnover rate of anti-dsDNA B cells, their in vivo life span was estimated. BALB/c, MRL-+/+, and MRL-lpr/lpr mice were continuously labeled for 11 days with the thymidine analogue BrdU. Mice were sacrificed on days 4, 8, and 11 of labeling and the incorporated BrdU in Igκ vs Igκ splenic B cells was measured. Cells that are turning over more rapidly will be replaced more quickly by their labeled precursors from the BM and, therefore, as a population will have a higher percentage of BrdU+ cells. Cells that are actively proliferating in the spleen will also be labeled. To distinguish between these possibilities, the in vivo proliferative status of the B cells has also been assessed.

After 4, 8, and 11 days of labeling, a higher percentage of Igκ B cells in VH3H9 BALB/c mice is BrdU+ compared with the Igκ subset or B cells from Tgκ BALB/c mice, consistent with previous data (Fig. 1, A and B and Ref. 12). Furthermore, this increase in BrdU label was shown to be due to an increased turnover of the Igκ B cells and not due to their proliferation because no BrdU label was detected in a short-term BrdU pulse (12) and <5% of the Igκ B cells were in cycle (Table II). In contrast to VH3H9 BALB/c mice, the percentage of BrdU+ Igκ cells in VH3H9 MRL-+/+ mice is more similar to that in Tgκ MRL-+/+ mice (Fig. 1A). Therefore, although VH3H9/Ab cells in both BALB/c and MRL-+/+ mice are located at the T/B interface, the turnover rate for the MRL-+/+ anti-dsDNA B cells is not as rapid as that in BALB/c mice. The reduced life span of the VH3H9/Ab B cells appears to be a feature of their immature status and not of their restricted localization. Immature B cells from wild-type mice have also been shown to have an increased turnover (22, 29).

In the MRL-lpr/lpr background, VH3H9/Ab B cells are both mature and localize to the B cell follicle (13), a location that has been correlated with a long-lived B cell (29). However, in VH3H9 MRL-lpr/lpr mice, the Igκ B cells have a higher frequency of BrdU+ cells than in Tgκ MRL-lpr/lpr mice or VH3H9 MRL-+/+ mice (Fig. 1A). One possibility is that the anti-dsDNA B cells are turning over faster in MRL-lpr/lpr mice than in MRL-+/+ mice, even though the anti-dsDNA B cells are mature in both cases. Another possibility is that the VH3H9/Ab B cells are proliferating.

To determine whether the increased frequency of BrdU label in the MRL-lpr/lpr splenic B cells was due to proliferation, two approaches were taken. First, mice were pulsed with BrdU for 4 h. This time period is long enough to detect cells that are undergoing DNA synthesis but short enough to exclude a contribution from exported BM precursors. After a 4-h pulse, <5% of the anti-dsDNA B cells were labeled in VH3H9 splenic B cells from BALB/c, MRL-+/+, and MRL-lpr/lpr mice (Ref. 12; and data not shown). Because BrdU will only label cells that are in S-phase, another approach was taken to determine the percentage of cells in cycle. VH3H9/Ab B cells were sorted and then analyzed for DNA content by propidium iodide incorporation using flow cytometry. Again, very few (<5%) of the B cells were found to be cycling (Table II). This shows that the vast majority of anti-dsDNA B cells in BALB/c, MRL-+/+, and MRL-lpr/lpr mice are not cycling. It is notable that a higher percentage of Igκ B cells in MRL-lpr/lpr mice than in MRL-+/+ or BALB/c mice are cycling (Table II). Therefore, this makes life span measurements by long-term BrdU labeling studies difficult to interpret in MRL-lpr/lpr mice. The increased BrdU label of MRL-lpr/lpr compared with MRL-+/+ Igκ B cells could be accounted for by this seemingly small difference in proliferation or by a higher frequency of newly generated B cells from the BM. We are able to compare the turnover rates of anti-dsDNA B cells from MRL-lpr/lpr mice with those in BALB/c mice. Igκ B cells in MRL-lpr/lpr mice have a higher percentage in cycle and yet have a decreased level of BrdU label compared with Igκ B cells in BALB/c mice. This indicates that the Igκ B cells in MRL-lpr/lpr mice are not turning over as rapidly as in BALB/c mice.

Anti-dsDNA B cells do not die at the T/B interface
The increased turnover rate of anti-dsDNA B cells in BALB/c mice suggests that they are rapidly dying in vivo, whereas Igκ B cells in VH3H9 MRL-+/+ mice do not show this accelerated turnover. In both cases, the Igκ B cells are located at the T/B interface. To determine whether the anti-dsDNA B cells are dying at this location, spleen sections were stained for apoptotic cells using the TUNEL method. TUNEL+ cells were detected in the spleens of both BALB/c and MRL-+/+ mice; however, the VH3H9/Ab B cells found at the T/B interface were not TUNEL+ (data not shown). This suggests that, although the anti-dsDNA B cells in BALB/c mice have a decreased life span, they are not undergoing apoptosis at the T/B interface. It is possible that the apoptotic anti-dsDNA B cells are engulfed and cleared from this site rapidly and, therefore, at any given time there are too few to be detected. Alternatively, they may die at another

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| Table I. Summary of VH3H9/anti-dsDNA B cell phenotype in nonautoimmune and autoimmune-prone mice* |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Serum           | BALB/c          | MRL-+/+         | MRL-lpr/lpr     | MRL-lpr/lpr     |
| Surface Ig      | Low             | Low             | Low             | Low             |
| Developmental status | Immature       | Mature          | Mature          | Mature          |
| Splenic localization | T/B interface  | T/B interface   | Follicle        | Follicle        |

*The following markers were used to assess B cell developmental status: B220, HSA, CD21, CD22, CD23, and CD44 (12, 13).
There is no significant difference between VH3H9 BALB/c and VH3H9 MRL-lpr/lpr compared to BALB/c mice (p by propidium iodide incorporation. A similar percentage of Ig

Percentage of B cells in cycle a

Table II.

Table II. Percentage of B cells in cycleb

<table>
<thead>
<tr>
<th>Mice</th>
<th>% Igλ B Cells in Cycle</th>
<th>% Igκ B Cells in Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH3H9 BALB/c</td>
<td>4.16 ± 0.56</td>
<td>3.68 ± 0.72</td>
</tr>
<tr>
<td>VH3H9 MRL-+/+</td>
<td>3.33 ± 0.63</td>
<td>2.15 ± 0.60</td>
</tr>
<tr>
<td>VH3H9 MRL-lpr/lpr</td>
<td>5.94 ± 1.26</td>
<td>3.41 ± 0.64</td>
</tr>
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b Spleen cells were stained with anti-CD19 and anti-Igλ and then were sorted by flow cytometry into CD19+Igλ+ (Igλ) and CD19+Igλ- (Igκ) sets. The sorted cells were then fixed in ethanol and stained with propidium iodide. The percentages of cells in cycle (S, G2, and M) were determined by analyzing DNA content, as determined by propidium iodide incorporation. A similar percentage of Igλ B cells from VH3H9 BALB/c, VH3H9 MRL-+/+, and VH3H9 MRL-lpr/lpr mice are in cycle (p > 0.1). There is no significant difference between VH3H9 BALB/c and VH3H9 MRL-+/+ Igλ B cells (p = 0.16). A significantly greater percentage of Igλ B cells in VH3H9 MRL-lpr/lpr than in VH3H9 MRL-+/+ (p = 0.02) and a slightly greater percentage compared to BALB/c mice (p = 0.08) are in cycle. n = 3 mice of each genetic background.

After 4 days in culture, Igκ and Igλ B cells from Tg− mice proliferate to both LPS and CD40L + IL-4 (Fig. 2B). Little proliferation is apparent in media alone (data not shown). On day 2 of the LPS culture, B cells in MRL-+/+ and MRL-lpr/lpr mice but not in BALB/c mice begin to proliferate (Fig. 2A). This may be an indication that there is a population of B cells in MRL mice that is hyperresponsive to LPS. In this regard, marginal-zone B cells have been shown to have an increased proliferative response to LPS (31), and MRL-+/+ and MRL-lpr/lpr mice have an exaggerated population of CD21/35highCD23lowIgMhighIgDlow B cells (13), a phenotype that is reminiscent of marginal-zone B cells (32). On day 3, B cells from Tg− BALB/c mice also begin to proliferate to LPS, and by day 4 they resemble those from MRL mice (Fig. 2B and data not shown). Likewise, in VH3H9 Tg mice, the majority of Igκ B cells proliferate to both LPS and CD40L + IL-4, consistent with their normal B cell phenotype (Fig. 2B).

In contrast to Tg− B cells and most of the Igκ B cells in VH3H9 mice, the anti-dsDNA VH3H9/A B cells do not proliferate to LPS (Fig. 2B). This is true of VH3H9/A B cells in BALB/c, MRL-+/+, and MRL-lpr/lpr mice. Therefore, VH3H9/A B cells are functionally distinguished from naive B cells by their inability to respond to LPS. It has been suggested that a previous signal through Ig may dampen future responsiveness to mitogens such as LPS (33). Therefore, this may be another indication that anti-dsDNA B cells have previously encountered Ag. In contrast to their lack of response to LPS, VH3H9/A anti-dsDNA B cells do proliferate to CD40L + IL-4 (Fig. 2B). This suggests that anti-dsDNA B cells may be able to be rescued by T cell help.

FIGURE 1. In vivo turnover rate of VH3H9/λ anti-dsDNA B cells. Tg− (open symbols) and VH3H9 (filled symbols) BALB/c (left), MRL-+/+ (middle), and MRL-lpr/lpr (right) mice were labeled with BrdU for 11 days. On days 4, 8, and 11 after labeling, mice were sacrificed. Spleen cells were stained with anti-CD19 and anti-Igλ, fixed, and permeabilized, and then incorporated BrdU was detected with anti-BrdU Ab. Graphs show percentage of BrdU+ Igλ (A) and Igκ (B) (defined as CD19+Igκ−) B cells. Symbols represent individual mice. A minimum of two mice of each genotype as analyzed at each timepoint.
FIGURE 2. Proliferation to in vitro stimuli. Spleen cells from Tg−/− and VH3H9 BALB/c (left), MRL−+/+ (middle), and MRL−lpr/lpr (right) were labeled with CFSE and then cultured for 2 (A) or 4 (B) days in either LPS or CD40L-CD8, cross-linked with anti-CD8, and IL-4. Cells were then harvested and stained with anti-CD19 and anti-IgAbs. Histograms show CFSE intensity of CD19+IgM+ (bold histograms) and CD19+IgM− (thin histograms) B cells overlaying the nonproliferating T cells (dotted histograms). The underlaid histograms (thin and dotted histograms) were scaled down to allow for comparison to the IgM B cells (which comprise ~10% of the total B cell population before culture). The overlaid histograms (bold histograms) are unscaled. Note: For the LPS cultures, very few VH3H9/α B cells remain (n = 5 mice of each genotype).
B cell survival to in vitro stimuli

On day 1 of culture, ~50% of the Igλ B cells survived, regardless of genotype or culture stimulus (Fig. 3A). By day 4, very few B cells of any genotype survived in media alone. For the Igκ B cells, the addition of either LPS or CD40L + IL-4 promoted cell survival, consistent with the proliferation data (data not shown). In Tg− mice, the Igκ B cells survived as well as the Igλ B cells to all stimuli. However, by day 4, <20% of Igλ B cells from VH3H9 Tg mice survived in LPS (Fig. 3B). Therefore, LPS does not foster proliferation or survival of VH3H9/λ B cells. Furthermore, even though the CD40L + IL-4-induced proliferation of the VH3H9/λ B cells, it rescued them to varying degrees. Igλ B cells from MRL−/− and MRL−lpr/lpr mice survived better in CD40L + IL-4 (percent survival: 62.5 ± 25.5 and 67.5 ± 11.7, respectively) than did Igλ B cells from BALB/c mice (33.8 ± 24.6; p = 0.04) (Fig. 3B). This suggests that being developmentally mature gives a survival advantage to the anti-dsDNA MRL B cells.

Up-regulation of B7-2

Signals through CD28 on T cells from B7-2 on APCs, such as B cells, are required for productive T/B collaboration (34–36). B7-2 is up-regulated shortly after activation through the B cell receptor and is only transiently expressed (37–39). To determine whether there is a difference in the ability of anti-dsDNA B cells from BALB/c, MRL−/+, and MRL−lpr/lpr mice to up-regulate B7-2, spleen cells were cultured in vitro with CD40L + IL-4 for 24 h. Before culture, the Igλ B cells were primarily small by forward and side scatter and expressed undetectable levels of B7-2 (Fig. 4). After culture, the Igλ B cells appeared blasted and increased their expression of B7-2 by 10- to 15-fold (Fig. 4). Importantly, the ability of anti-dsDNA B cells from all three backgrounds to up-regulate the costimulatory molecule B7-2 documents their potential to elicit productive T cell help.

Anti-dsDNA B cells persist in the periphery of nonautoimmune mice

The fate of anti-dsDNA B cells in nonautoimmune mice has been controversial. Although it has been reported, using an IgH1/L Tg (VH3H9/Vκ4), that anti-dsDNA B cells are deleted in the BM...
have previously been retrieved from VH3H9 MRL-1pr/1pr/ nonautoimmune mice of VH3H9/V12/13d L-chain in combination solely with the Ig and generated splenic hybridoma panels. Hybridomas that use the CD40L in LPS cultures. To determine whether additional anti-dsDNA B cells are present from hybridoma panels from VH3H9 mice, generated after LPS stimulation (16). The data presented in Figs. 2 and 3 may account for this; anti-dsDNA B cells do not proliferate or survive in LPS cultures. To determine whether additional anti-dsDNA B cells persist in the periphery, we took advantage of the observation that CD40L + IL-4 induces proliferation of anti-dsDNA B cells, and generated splenic hybridoma panels. Hybridomas that use the VH3H9 Tg H-chain in combination solely with the IgL, were retrieved after CD40L + IL-4 stimulation (Ref. 12 and Table III). Importantly, ANA-/Crithidia+/ hybridomas that use IgL L-chains were also recovered (Table III), thus confirming that other anti-dsDNA B cells are also present in the periphery of BALB/c mice. Two hybrids (7942-H26 and 7942-H46) using the VH3H9 Tg H-chain in combination with the V12/13d L-chain were isolated (Fig. 5). Although these hybrids have similar staining patterns in the ANA and C. luciliae assays, their use of distinct CDR3 (complementarity-determining regions) and Jk segments indicates that the two are not clonally related (Fig. 5 and Table III). Anti-dsDNA B cells using the VH3H9 Tg H-chain in combination with the V12/13d L-chain have previously been retrieved from VH3H9 MRL-lpr/lpr-derived hybridomas (25). What accounts for the deletion in nonautoimmune mice of VH3H9/V12/13d anti-dsDNA B cells but not of the anti-dsDNA B cells described here has not been established. The ANA and Crithidia binding patterns of these Abs are similar (Table III). Possibly there are differences in specificity or affinity that the ANA and Crithidia assays do not reveal or differences in when the autoreactive Tg receptors are first expressed that dictate the fate of the B cells.

Discussion

Anti-dsDNA B cells are actively regulated in nonautoimmune BALB/c mice as manifested by their developmental arrest, localization to the T/B interface, rapid turnover rate, and absence of their Ig in the serum (12). In contrast, in MRL-lpr/lpr mice, anti-dsDNA B cells are phenotypically mature, localize in the B cell follicle, and by 10 wk of age their Ig is present in the serum. MRL-+/+ anti-dsDNA B cells have an intermediate phenotype; they are developmentally mature but are retained at the T/B interface, and their Ig is not in the serum (13). We have previously suggested that the follicular localization of anti-dsDNA B cells in MRL-lpr/lpr mice may be due to the absence of a Fas/FasL interaction that restricts their entry into the B cell follicle (13). One obvious way Fas could exert its effect is by inducing apoptosis of anergic B cells at the T/B interface. This scenario is attractive in that anergic anti-HEL (6, 7) and anti-dsDNA B cells in nonautoimmune mice (12) localize to the T/B interface and have a rapid turnover rate. However, we see no evidence of apoptotic cells at the T/B interface. Furthermore, anti-dsDNA B cells in MRL-+/+ mice also localize to the T/B interface (13). However, in this case BrdU labeling studies show that they do not turn over as rapidly as they do in BALB/c mice, and again there is no evidence of apoptosis at this site. Together, these data do not support the model that autoreactive B cells undergo apoptosis in situ in the T cell zone (40).

The location of the VH3H9/1 anti-dsDNA B cells in MRL-lpr/lpr mice is reminiscent of Ag-activated Ig Tg anti-HEL cells given cognate T cell help (41). In the first days of an immune response, the anti-HEL B cells were reported to have undergone a proliferative burst and were scattered throughout the B cell follicle. However, the anti-dsDNA B cells in this study differ from the anti-HEL Ig Tg B cells in that the vast majority of anti-dsDNA B cells are not proliferating.

Importantly, this study shows that anti-dsDNA B cells from BALB/c, MRL-+/+, and MRL-lpr/lpr mice all proliferate and up-regulate the critical costimulatory molecule B7-2 in response to CD40L + IL-4. In the anti-HEL B cell tolerance model, anergic anti-HEL B cells are impaired in their ability to up-regulate B7-2 in response to Ag and it is hypothesized that this prevents them from eliciting T cell help (42, 43). However, in another study they have been shown to up-regulate B7 molecules after anti-CD40 Ab treatment (44). Restoring B7-2 expression by a B7-2 Tg restored the ability of anergic anti-HEL B cells to respond to T cell help, emphasizing the importance of the block in B7-2 to the maintenance of B cell anergy (45). The ability of anti-dsDNA B cells to express B7-2 indicates that they may be able to elicit T cell help.

We suggest, based on the responsiveness of anti-dsDNA B cells to CD40L + IL-4, that one of the critical in vivo differences between anti-dsDNA B cells in BALB/c and MRL-+/+ vs MRL-lpr/lpr mice may be the availability of T cell help. We have previously documented a CD4 T cell infiltration into the B cell follicle that accompanies the follicular localization of anti-dsDNA B cells in MRL-lpr/lpr mice (13). Additionally, when VH3H9 MRL-lpr/lpr mice are treated with anti-CD4 Abs, the anti-dsDNA B cells now localize to the T/B interface, suggesting a requirement for CD4 T cells in anti-dsDNA follicular localization (S. Seo, manuscript in preparation). Possibly these are autoreactive T cells, which are tolerated in Fas-sufficient mice but escape this fate in

**FIGURE 5.** Comparison of IgL-chain gene usage of ANA+/Crithidia+ hybridomas. MRL2-154 has been published previously (25) and is shown here for comparison. Gene segment usage was assigned as defined in Materials and Methods. Framework (FW) and complementarity-determining regions (CDRs) are shown. Sequence comparison reveals that identical genes are used by ANA+/Crithidia+ hybrids from VH3H9 MRL-lpr/lpr (MRL2-154) and VH3H9 BALB/c (7942-H26 and 7942-H46) mice. Nucleotide sequence for these L-chains are available from EMBL/GenBank/DDBJ under accession numbers AF193816 and AF193817.

**Table III.** Ig gene usage of anti-dsDNA hybridomas (dsDNA binding was determined by the Crithidia luciliae assay).

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>H-Chains</th>
<th>L-Chains</th>
<th>ANA</th>
<th>dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H9a</td>
<td>VH3H9</td>
<td>V4e5d5a-Jc4</td>
<td>HN</td>
<td>+</td>
</tr>
<tr>
<td>7942-L9</td>
<td>VH3H9</td>
<td>V4a1-JA1b</td>
<td>HN</td>
<td>+</td>
</tr>
<tr>
<td>7942-H26</td>
<td>VH3H9</td>
<td>V4c12/13d-Jc2</td>
<td>HN</td>
<td>+</td>
</tr>
<tr>
<td>7942-H46</td>
<td>VH3H9</td>
<td>V4c12/13d-Je5</td>
<td>HN</td>
<td>+</td>
</tr>
</tbody>
</table>

a The 3H9 hybridoma has been previously described (11).

b Of 44 hybrids isolated from a VH3H9 Tg BALB/c mouse, four were anti-dsDNA V4a1/JA1 hybrids (12).
lpr/lpr mice. Experiments to characterize these T cells are underway.

In conclusion, this study has shown that alterations in developmental maturity and splenic localization translate into differences in the survival of anti-dsDNA B cells. Anti-dsDNA B cells from both BALB/c and MRL-+/+ mice localize to the T/B interface; however, only those in BALB/c mice have an increased turnover. This allows us to assign the rapid turnover rate to their immature status and not to their localization. Furthermore, because we do not detect apoptotic IgA cells at the T/B interface, the anti-dsDNA B cells likely die at another location. Importantly, we demonstrate that anti-dsDNA B cells from all three backgrounds proliferate and up-regulate B7-2 in response to CD40L and IL-4, suggesting that in vivo the anti-dsDNA B cells may be reactivatable by bona fide T cell help. Finally, the isolation of additional anti-dsDNA hybridomas from BALB/c mice that use the same Igαs as those found in MRL-lpr/lpr mice suggests that the tolerance mechanisms we have described for VH3H9/A1 may also apply to other anti-dsDNA B cells.

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References