Tolerance to DNA in (NZB × NZW)F₁ Mice That Inherit an Anti-DNA V_H Chain Transgene but Not as a V_H Knock-in Transgene

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Tolerance to DNA in (NZB × NZW)F1 Mice That Inherit an Anti-DNA V_H as a Conventional μ H Chain Transgene but Not as a V_H Knock-in Transgene

Meredith A. Steeves and Tony N. Marion

Lupus-prone (NZB × NZW)F1 (BWF1) mice were made transgenic (Tg) for an anti-DNA Ab inherited either as a conventional V_H3H9-μ H chain Tg (3H9-μ) with or without a conventional V_L8-k Tg, or a V_H3H9 V_L knock-in Tg allele (3H9R) with or without a V_L4 V_L knock-in Tg allele (V_L4R). V_H3H9 yields an anti-DNA Ab with most L chains including an anti-ssDNA with the V_L8 Tg and an anti-dsDNA with the V_L4 Tg. BWF1 mice that inherited the conventional 3H9-μ had normal serum IgM, little to none of which was encoded by 3H9-μ, and only a small percentage of those mice had serum anti-DNA, none of which was transgene encoded. B cells expressing the conventional 3H9-μ Tg were anergic. BWF1 mice that inherited the knock-in 3H9R Tg allele also had normal serum IgM, one-half of which was encoded by 3H9R, and produced anti-DNA encoded by the Tg allele. Most B cells expressing the knock-in 3H9R Tg also had an anergic phenotype. The results indicate that autoimmune-prone BWF1 mice initially had normal serum IgM, one-half of which was encoded by 3H9R, and produced anti-DNA encoded by the Tg allele. Most B cells expressing the knock-in 3H9R Tg also had an anergic phenotype. The results indicate that autoimmune-prone BWF1 mice initially develop effective B cell tolerance to DNA through anergy, and anergy was sustained in 3H9-μ Tg peripheral B cells but not in 3H9R Tg B cells. B cells expressing the 3H9R knock-in Tg allele were able to achieve an activation threshold that B cells expressing the 3H9-μ conventional Tg could not. The maintenance of B cell tolerance to DNA in autoimmune-prone BWF1 mice appears to differ from both normal mice and autoimmune-prone MRL<sup>Im</sup>/Jerpr<sup>pr</sup> mice.

Systemic lupus erythematosus (SLE) is characterized in part by autoimmunity to DNA (1), as well as other nuclear autoantigens (2). A consistently elevated anti-DNA Ab titer is a major diagnostic criterion in clinical assessment of potential SLE patients. In accordance, IgG anti-DNA Abs have been found deposited in the glomeruli of diseased human kidneys (3, 4) and, when eluted, exhibit specificity for dsDNA. (NZB × NZW)F1 (BWF1) mice are the classic animal model for SLE and closely mimic the human autoimmune disease with respect to autoantibody production, sex distribution, and the pathology of lupus nephritis (5, 6). BWF1 mice spontaneously develop Abs to dsDNA, as well as other nuclear autoantigens, that contribute to severe glomerulonephritis and premature death at 8–10 mo. Autoimmune anti-DNA B cells are present in the periphery of both autoimmune and nonautoimmune mice (7) and can be activated to produce anti-DNA Ab (8); however, in BWF1 autoimmune mice, the spontaneous activation of anti-DNA B cells is sustained (9, 10). The autoimmune response to DNA in BWF1 mice has all of the characteristics of an Ag-driven, clonally selected, secondary immune response (11). The relevant immunogen for the Ag-driven autoimmune response must be DNA, structures that contain DNA, or, alternatively, structures on apoptotic cells or blebs that bind anti-DNA (12). Anti-DNA Abs with the same structural and pathogenic characteristics as autoimmune anti-DNA can be induced by deliberate immunization (13) or virus inoculation (14) but only transiently. Induced autoimmunity to DNA in nonautoimmune mice is characterized by low autoantibody titers and lack of a true memory B cell response (15). Thus, it is apparent that self-reactive B cells in normal mice are under active, negative regulation. The high titers of anti-DNA, as well as other autoantigens seen in BWF1 mice, indicate that a breakdown in normal immunological tolerance to self has occurred. Several regulatory mechanisms control self-reactive B cells including deletion (16, 17), anergy (18, 19), and receptor editing (20–23).

To better understand how tolerance to DNA is lost in autoimmune-prone BWF1 mice and more closely dissect the development of the autoimmune anti-DNA response, NZW and NZB mice transgenic (Tg) for different rearranged anti-DNA V_H and V_L genes, respectively, were generated by backcross breeding. NZW mice inherited either the rearranged V_H gene from the 3H9 anti-DNA hybridoma (V_H3H9) (24) or the rearranged V_H gene from an anti-(4-hydroxy-3-nitrophenyl)acyetyl (NP) Ab, H50G (V_HNP) (25). V_H3H9 was inherited as either a site-directed J_H knock-in (3H9R) (26) or a conventional, randomly inserted J_H chain transgene (3H9-μ) (27). V_HNP was inherited as a μ H chain conventional Tg (V_HNP-μ) (28). NZB mice inherited either the rearranged V_L8-J_L5 of an anti-influenza hemagglutinin Ab as a conventional κ Tg (28) (V_k8-κ) or the rearranged V_L4-J_L4 from 3H9 as a J_κ knock-in (22) (V_κ4R). Although the 3H9R V_H Tg can be class-switched and the 3H9R and V_L4R Tgs can be somatically mutated and replaced by receptor editing, the conventional 3H9-μ and V_k8-κ Tgs cannot be class-switched or edited. V_H3H9 with Vk8 produces an anti-DNA with ssDNA specificity (29), V_H3H9 with Vk4 produces an anti-DNA with dsDNA specificity, and V_HNP-Vk8 yields an Ab with unknown specificity. Previous studies have used BWF1 mice Tg only for the H chain of an anti-DNA Ab (30, 31) or a knock-in of the V_H for an anti-DNA (32), and each of the previous studies used a different anti-DNA V_H Tg. The present study is the first to compare B

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; Tg, transgene or transgenic; ANA, anti-nuclear Ab; NP, (4-hydroxy-3-nitrophenyl)acyetyl; HEL, hen egg lysozyme.

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cell development and anti-DNA Ab production in autoimmune-prone BWF1 mice. Tg for the V\_H of an anti-DNA Ab inherited either as a \( \lambda_2 \) knock-in or as a conventional H chain Tg.

BWF1 mice Tg for 3H9-\( \mu \) or 3H9-\( \mu \)-V\_k-8-K produced no anti-DNA even though mice from both Tg groups had normal total serum IgM and IgG anti-nuclear Ab (ANA). Very little of the serum Ig was derived from the 3H9-\( \mu \)-Tg, and B cells expressing Tg-encoded IgM\_k appeared to be anergic. Serum IgKs and IgG anti-DNA in V\_k-8-K and Tg\_K littersmates were similar to those in normal BWF1 mice. The majority of B cells expressing IgM\_k encoded by the 3H9R knock-in, with or without a V\_4R encoded L chain, had an anergic phenotype; nevertheless, the respective BWF1 mice had serum IgM and IgG anti-DNA similar to that in normal, non-Tg BWF1 mice. The differences in B cell phenotype and Ab production between conventional and knock-in Tg BWF1 mice offer interesting insight into tolerance and autoimmunity to DNA in these autoimmune-prone mice.

Materials and Methods

Mice

Female NZB and male NZW mice were obtained from Harlan (Indianapolis, IN) and housed in a specific pathogen-free facility at the University of Tennessee Health Science Center, Memphis, TN. V\_H 3H9-\( \mu \) (19) and V\_L 8-\( \kappa \) Tg (26) BALB/c mice were gifts from Dr. J. Erikson (Wistar Institute, Philadelphia, PA). V\_H 3H9R (26) and V\_H 4R (22) Tg BALB/c mice were gifts from Dr. M. Weigert (University of Chicago, Chicago, IL). C.B-17 mice Tg for a conventional H chain Tg (V\_L NP-\( \mu \)) associated with an anti-NP Ab (27) were generously provided by Dr. G. Kelsoe (Duke University, Durham, NC). Mice Tg for the V\_H 3H9R Tg which has a VDJ with VH 3H9R combines with many different L chains to produce an anti-DNA that has 95\% specificity for DNA anti-DNA. Pooled normal BALB/c mouse serum was used as a negative control. Data were statistically analyzed by ANOVA with Kruskal-Wallis post test for nonparametric and Student-Newman-Keuls multiple comparisons test for parametric data using GraphPad InStat version 3.05 (GraphPad Software, San Diego, www.graphpad.com).

Flow cytometry

Murine spleen and bone marrow cells were collected, blocked with goat and rat IgG (Sigma-Aldrich), both at a final concentration of 0.2 mg/ml, and incubated with one to three fluorochrome-conjugated Abs. Stained cells were analyzed on a BD FACScan (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). The following Abs were used: FITC, PE, or biotin-conjugated anti-CD45R/B220 (0.5 \( \mu \)g/10\(^6\) cells) (SBA); PE or biotin-conjugated anti-IgD (0.05 or 0.5 \( \mu \)g/10\(^6\) cells) (SBA); FITC-conjugated anti-IgM (0.5 \( \mu \)g/10\(^6\) cells) (SBA); biotin-conjugated anti-CD24/HA (1 \( \mu \)g/10\(^6\) cells); PE-anti-IgM (0.125 \( \mu \)g/10\(^6\) cells; BD PharMingen, San Diego, CA), or FITC-IgM (1.0 \( \mu \)g/10\(^6\) cells) (BD PharMingen). Biotin-conjugated Abs were detected with streptavidin-PerCP (BD Biosciences). A minimum of three individual adult mice per genotype were analyzed. Ages were between 6 wk and 9 mo.

Results

Longevity and absence of autoimmune disease in Tg BWF1 mice

B cell development and autoantibody production were analyzed in Tg BWF1 mice that inherited anti-DNA Ab production as a conventional H chain Tg with or without a conventional L chain Tg or a knock-in V\_H Tg allele with or without a knock-in V\_L Tg allele (Table I). As noted above, B cell receptors and Abs encoded by conventional Tgs cannot be edited or isotype switched, but they can be eliminated, presumably as a consequence of attempted editing. B cell receptors and Abs encoded by V\_H and V\_k knock-in Tgs can be edited, somatically mutated, and isotype switched. V\_H 3H9R combines with many different L chains to produce an anti-DNA Ab (35). Mice that inherited both the conventional 3H9-\( \mu \) and the V\_k-8-\( \kappa \) L Tgs were monospecific for an anti-DNA that has samples as an internal control for DNA quality with sense 5’-CAAGTA TACTCACGCCACC-3’ and antisense 5’-CCACAGATGGCAGTC CAG-3’ primers. All Tgs except V\_4R were amplified using 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min for 39 cycles. V\_4R was amplified with 95°C for 1 min, 66°C for 30 s, and 72°C for 1 min for 35 cycles.

Table 1. V\_H and V\_L transgenes inherited by BWF1 mice

<table>
<thead>
<tr>
<th>Transgene Pairs(^a)</th>
<th>V_H (NZW)</th>
<th>V_L (NZB)</th>
<th>Transgene Insertion</th>
<th>VH-VL Specificity(^b)</th>
<th>Transgenic CH Isotype(^c)</th>
<th>V Gene Editing</th>
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<td>3H9-( \mu )</td>
<td>V_8-( \kappa )</td>
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<td>3H9R</td>
<td>V_4R</td>
<td>J_\alpha/\beta site directed</td>
<td>dsDNA</td>
<td>All</td>
<td>Yes</td>
<td></td>
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<tr>
<td>V_L NP-( \mu )</td>
<td>V_8-( \kappa )</td>
<td>Random</td>
<td>NP/unknown</td>
<td>IgM</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \)BWF1 progeny of the NZB \times NZW matings inherited both, either, or neither of the indicated transgenes.

\( ^b \)The V\_H 3H9 yields a DNA-binding Ab with many different L chains. The DNA specificity for mAbs with V\_H 3H9-V\_8 and V\_L NP-\_8-\( \kappa \) are indicated. The V\_L NP is from the rearranged and HSOG mutated V\_H 186.2 V gene that encodes a low affinity NP-specific mAb with V\_L 1. The specificity of Abs with V\_L NP-\( \mu \) is unknown.

\( ^c \)The 3H9-\( \mu \) and V\_L NP-\( \mu \) transgenes were inherited as \( \mu \) H chains, IgM-6a allele, and cannot be isotype switched or expressed with IgD. 3H9R was inherited as a site-directed V gene replacement and can be expressed with all isotypes.
relatively low affinity for DNA and binds ssDNA but not dsDNA. Mice that inherited both the knock-in 3H9R V_H and the V_4R V_K were monospecific for an anti-DNA that binds dsDNA with relatively high affinity. The experiments also analyzed B cell development and autoantibody production in BWF_1 that inherited a conventional H and/or L chain Tg for an Ab with no known DNA or other autoactivity.

All BWF_1, Tg^- littermates developed autoimmune disease and had life spans similar to those of BWF_1 mice derived from matings of inbred NZB and NZW mice (data not shown). The Tg^- mice produced IgG anti-DNA by 6 mo of age and developed glomerulonephritis with proteinuria and elevated blood urea nitrogen and died by ~10 mo of age. V_8-k and V_4R Tg BWF_1 mice had delayed development of autoimmune disease and prolonged life compared with Tg^- BWF_1 mice. V_8-k and V_4R Tg BWF_1 mice lived as long as 18 mo unless terminated for experiments. 3H9R-4R Tg BWF_1 mice had delayed development of autoimmune disease and died by 10 mo of age. V_8-k Tg BWF_1 mice, up to 15 mo of age still had no signs of autoimmune disease. Although V_10NP-9-k and V_9NP-9 Tg BWF_1 mice had no signs of glomerulonephritis detected as proteinuria or elevated blood urea nitrogen up to 1 year of age at which time most were terminated. 3H9R-4R Tg BWF_1 mice had no signs of autoimmune disease. Although V_10NP-9-k and V_9NP-9 Tg BWF_1 mice had no signs of autoimmune disease, they were highly susceptible to the development of eye infections after retro-orbital puncture for blood collection. The infections were resolved with antibiotics. Susceptibility to infection in V_10NP-9 and V_10NP-9-k Tg BWF_1 mice was likely due in part to the absence of isotype switched Ig, e.g., IgA, in those mice (see below).

Tolerance to DNA in conventional 3H9R-4R and knock-in 3H9R-V_4R Tg mice

Conventional 3H9R-4R and 3H9R-4R Tg BWF_1 mice differed remarkably from 3H9R-V_4R and 3H9R knock-in Tg BWF_1 mice with respect to the production of Tg-encoded IgM^a anti-DNA. Only 2 of 26 3H9R-V_8-k and 4 of 16 3H9R Tg BWF_1 mice developed detectable serum IgM or IgG anti-DNA even after 9–12 mo of age (Fig. 1, B and D). By 14 mo of age, three of five 3H9R-V_8-k and four of four 3H9R Tg BWF_1 mice were producing IgG anti-DNA (geometric mean titers (SD), 238 ± 1.73 and 448 ± 3.12, respectively), but even after 14 mo of age, none of the mice produced Tg-encoded IgM^a anti-DNA (not shown). Conventional 3H9R-4R Tg BWF_1 mice were autoimmune because most of the sera assayed from 3H9R-4R and 3H9R Tg BWF_1 mice had IgG ANA with nucleolar and/or speckled nuclear staining patterns on Hep-2 cells (Fig. 2 and Table II). Knock-in 3H9R-V_4R and 3H9R Tg BWF_1 mice, in contrast, developed autoimmune IgM and IgG anti-DNA and a homogeneous ANA (Fig. 2 and not shown) similarly to non-Tg littermate BWF_1 mice (Fig. 3, B and D) (p < 0.05). Most of the IgM anti-DNA in 3H9R-V_4R and 3H9R BWF_1 mice was Tg-encoded IgM^a (p < 0.01, IgM^a vs IgM anti-DNA). Both conventional L chain only V_8-k and V_8-k Tg BWF_1 mice (Fig. 3, B and D) and knock-in L chain only V_4R Tg BWF_1 mice (Fig. 3, B and D) had serum IgM and IgG anti-DNA titers similar to those in Tg^- littermates (p < 0.05).

Anergy in IgM^a B cells in conventional 3H9R-4R and knock-in 3H9R but not V_10NP-9 conventional Tg BWF_1

There was no significant difference in total serum IgM and total serum IgG among conventional 3H9R-4R and 3H9R Tg BWF_1 mice and 3H9R-4R and V_8-k Tg and Tg^- littermates (p > 0.05; Fig. 1, A and C); however, little to none of the IgM was Tg-encoded IgM^a. Most of the serum IgM and all of the IgG in mice that inherited the conventional 3H9R-4R Tg, alone or together with V_8-k, were derived from endogenous IgM^b H chain alleles. Tg-encoded IgM^a was consistently 50- to 80-fold lower (p < 0.001) than IgM^a in

FIGURE 1. Total serum Ig and anti-DNA in 3H9R-4R and 3H9R-4R Tg^- littermate BWF_1 mice. A, Total serum IgM; B, serum IgM anti-DNA; C, total serum IgG; D, serum IgG anti-DNA. Titers for total IgM and IgG and for IgM and IgG anti-DNA are indicated. Titers were measured as reciprocal serum dilutions that yielded 50% maximum binding by ELISA. Each point represents an individual mouse. Ages were ~6 mo. ×, IgM^a levels in pooled normal BALB/c sera; +, IgM^b levels in pooled unimmunized NZW sera.
Serum levels of IgMα and IgMβ were similar in 3H9R-Vγ8-κ Tg BWF1 mice (Fig. 1, A and C). Serum levels of IgMα and IgMβ were similar in 3H9-μ-Vκ8-κ and 3H9-μ Tg BWF1 mice (p > 0.05). IgMα titers in 3H9-μ-Vκ8-κ Tg BWF1 mice and the 3H9-μ and Vκ8-κ Tg and Tgβ littermates were consistently higher than those in NZW sera in keeping with less than that in Vγ8-κ. IgMβ was detected in Vκ8-κ NP-3H9R-Vγ4R Tg BWF1 mice during development. Total IgG was similar in 3H9R-Vγ4R Tg BWF1 mice and Vκ8-κ Tg BWF1 mice (not shown). There was no statistically significant difference (p > 0.05) in the percentage of B cells identified as surface μα cells in 3H9-μ-Vκ8-κ, 3H9R-Vκ4R, Vκ4NP-μ-Vκ8-κ, 3H9R, Vκ8-κ, and Vκ4R Tg and Tgβ BWF1 spleens. The percentage of B cells in 3H9-μ Tg BWF1 was reduced by 40% compared with Tgβ mice (p < 0.01). Approximately 85% of splenic B cells in 3H9-μ-Vκ8-κ Tg BWF1 mice expressed surface IgM derived from the Tg-encoded H chain, 8% expressed surface IgM derived from rearrangements of endogenous H chain alleles, and a small percentage expressed both IgMα and IgMβ (Fig. 5B). The percentages of IgMα+ and IgMβ+ IgMβ+ splenic B cells increased with age. 3H9-μ-Vκ8-κ Tg BWF1 mice ≥10 mo old often had >20% IgMα+ and ~12–15% IgMα+ IgMβ+ B cells (not shown), but 3H9-μ-Vκ8-κ Tg BWF1 mice 6 mo old had ~1% IgMα+ and ~1% IgMα+ IgMβ+ B cells (not shown). Surface IgM expression was 2- to 3-fold reduced on IgMα B cells compared with IgMβ B cells in 3H9-μ-Vκ8-κ Tg BWF1 mice and in 40% of the Vκ8-κ Tg BWF1 mice by 6 mo of age (Fig. 4B). Neither IgMγ anti-DNA nor IgG anti-DNA was detected in Vκ8NP-μ-Vκ8-κ or Vκ4NP-μ Tg BWF1 mice (Fig. 4B and data not shown).

Total serum IgG in 3H9R-Vκ4R and 3H9R Tg BWF1 mice was less than that in Vκ4R Tg and Tgβ littermates (p < 0.05; Fig. 3A), and serum levels of IgMα-encoded and endogenously encoded IgMβ were similar in 3H9R-Vκ4R Tg BWF1 (p > 0.05). This no doubt reflects the ability of 3H9R to pair with L chains that do not yield an anti-DNA. Serum levels of IgMα were 6-fold higher than IgMβ in 3H9R Tg BWF1 mice (p < 0.01). The different VH expressed as IgMβ could be expected to yield a lower percentage of DNA-reactive B cells and be less susceptible to inactivation during development. Total IgG was similar in 3H9R-Vκ4R, 3H9R, and Vκ4R Tg BWF1 mice and Tgβ littermates (Fig. 3C) although IgG encoded by the knock-in Tg allele could not be distinguished from that encoded by an endogenous allele.

Flow cytometric analysis of bone marrow and splenic B cells in Tg mice

There was no statistically significant difference (p > 0.05) in the percentage of B cells identified as surface μα cells in 3H9-μ-Vκ8-κ, 3H9R-Vκ4R, Vκ4NP-μ-Vκ8-κ, 3H9R, Vκ8-κ, and Vκ4R Tg and Tgβ BWF1 spleens. The percentage of B cells in 3H9-μ Tg BWF1 was reduced by 40% compared with Tgβ mice (p < 0.01). Approximately 85% of splenic B cells in 3H9-μ-Vκ8-κ Tg BWF1 mice expressed surface IgM derived from the Tg-encoded H chain, 8% expressed surface IgM derived from rearrangements of endogenous H chain alleles, and a small percentage expressed both IgMα and IgMβ (Fig. 5B). The percentages of IgMα+ and IgMβ+ IgMβ+ splenic B cells increased with age. 3H9-μ-Vκ8-κ Tg BWF1 mice ≥10 mo old often had >20% IgMα+ and ~12–15% IgMα+ IgMβ+ B cells (not shown), but 3H9-μ-Vκ8-κ Tg BWF1 mice 6 mo old had ~1% IgMα+ and ~1% IgMα+ IgMβ+ B cells (not shown). Surface IgM expression was 2- to 3-fold reduced on IgMα B cells compared with IgMβ B cells in 3H9-μ-Vκ8-κ Tg BWF1 mice and in 40% of the Vκ8-κ Tg BWF1 mice (Fig. 6). The narrow range of reduced surface IgMα expression on B cells from 3H9-μ-Vκ8-κ Tg BWF1 would be consistent with expression of a monospecific, self-reactive receptor on the majority of B cells (Fig. 5Bb). These results also suggest that relatively few of the 3H9-μ-Vκ8-κ B cells were able to eliminate or edit the DNA reactivity of the 3H9-μ-Vκ8-κ Tg receptor. Most splenic B cells from Vκ4NP-μ-Vκ8-κ Tg BWF1 mice expressed relatively high levels of Tg-encoded surface IgMα, consistent with expression of a monospecific, nonautoreactive receptor (Fig. 7Bc). Unlike B cells from 3H9-μ-Vκ8-κ Tg BWF1 mice (Fig. 5Bd), there were almost no IgMα-expressing B cells in Vκ4NP-μ-Vκ8-κ Tg BWF1 mice (Figs. 7Bd). Surface IgM expression on splenic B cells from Vκ4NP-μ Tg BWF1 mice was similar to that for B cells from Vκ4NP-μ-Vκ8-κ Tg BWF1 mice (not shown).

Surface IgMα expression on B cells from 3H9R-Vκ4R, 3H9R (not shown) and 3H9-μ (Fig. 8) Tg BWF1 mice was different from that on B cells from both Vκ4NP-μ-Vκ8-κ and 3H9-μ-Vκ8-κ Tg BWF1 mice. Surface IgMα expression on B cells from 3H9R-Vκ4R (Fig. 9) and 3H9R (not shown) were identical. Surface IgMγ+ splenic B cells from 3H9R-Vκ4R, 3H9R, and 3H9-μ Tg BWF1 mice formed three distinct subpopulations (Figs. 8 and 9B). Surface IgMα expression on the c subpopulation of splenic B cells was similar to surface IgMα expression on the majority of B cells in Vκ4NP-μ-Vκ8-κ Tg BWF1 mice and the highest level of expression on B cells in Tgβ BWF1 (Fig. 9Bc). The b subpopulation of splenic IgMγ+ B cells in 3H9R-Vκ4R, 3H9R, and 3H9-μ Tg BWF1 mice had surface IgMα expression similar to that for splenic B cells from 3H9-μ-Vκ8-κ Tg BWF1 mice and 2- to 3-fold less than that for B cells from Vκ4NP-μ-Vκ8-κ Tg BWF1 mice (Fig. 7Bb vs Figs. 8b and 9Bb). Surface IgMα expression on the “a” subpopulation of splenic B cells in 3H9R-Vκ4R, 3H9R, and 3H9-μ Tg BWF1 was

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<th>Age (mo)</th>
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<tr>
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* Tg− BWF1 mice inherited neither H nor L Tg.

† Number of mice serologically positive for ANA/total number of sera tested for the given age group.

‡ Number of mice serologically positive for a homogeneous nuclear ANA/total number of sera tested for the given age group.
even more reduced, 10-fold or more compared with surface IgM expression on \( \text{V}_{\mu}\text{NP}-\mu \)-splenic B cells (Fig. 7Bc vs 8a and 9Ba). The percentage of IgM\(^{a-}\) splenic B cells in 3H9R-V\(\kappa\)4R Tg BWF1 was approximately one-fourth that in 3H9-\(\mu\)-V\(\kappa\)-\(\kappa\) Tg BWF1.

L chain expression in both site-directed 3H9R and conventional 3H9-\(\mu\) B cells was determined by rearranged endogenous alleles. L chains that vetoed the DNA specificity of 3H9R or 3H9-\(\mu\) (35, 36) would be expected to yield the B cells detected in subpopulation c.

FIGURE 3. Total serum Ig and anti-DNA in 3H9R-V\(\kappa\)4R and 3H9R, V\(\kappa\)4R, and Tg\(^{−}\) littermate BWF1 mice. For details, see legend to Fig. 1.

FIGURE 4. Total serum Ig and anti-DNA in V\(\mu\)NP-\(\mu\)-V\(\kappa\)8-\(\kappa\) and V\(\mu\)NP-\(\mu\), V\(\kappa\)8-\(\kappa\), and Tg\(^{−}\) littermate BWF1 mice. For details, see legend to Fig. 1.
Subpopulation “c” in 3H9R and 3H9R-Ve4R Tg BWF1 mice would also contain B cells that edited their DNA-reactive IgMα receptors to eliminate autoreactivity. IgMα B cells with L chains that could not effectively veto the specificity of 3H9R or 3H9-μ would be expected to be in subpopulations a and b. The difference between the a and b subpopulations probably relates to DNA reactivity with the a subpopulation having higher relative affinity for DNA, such as with VH3H9-Ve4. B cells in the b subpopulation are more likely to have lower relative affinity for DNA, such as with VH3H9-Ve8. As expected, IgMα B cells from 3H9R and 3H9R-Ve4R Tg BWF1 mice also expressed surface IgD, but IgMα B cells from 3H9-μ, 3H9-μ-VH NP-Ve8, and VH NP-Ve8-Tg BWF1 mice did not have surface IgD (not shown).

The reduced surface IgM expression on IgMα splenic B cells in 3H9-μ-VH8-κ Tg BWF1 mice was also apparent in bone marrow (Fig. 5A), and the percentage of B220/CD45R+ surface IgM+ cells in bone marrow from 3H9-μ-VH8-κ Tg BWF1 mice was much higher than the percentage of B220/CD45R+ surface IgM+ cells in either VH NP-VH8-κ Tg or non-Tg BWF1 mice. More than 90% of B220/CD45R+ bone marrow cells were surface IgMα vs

FIGURE 5. Flow cytometric analysis of IgMα and IgMβ expression on bone marrow and splenic B cells from 3H9-μ-VH8-κ (A and B) and age-matched littermate Tg- (C and D) BWF1 mice. Cells from bone marrow and spleen were gated on B220/CD45R+ mononuclear cells. a–d, Percentages of cells. The results are representative of at least three adult mice 3–9 mo old.

FIGURE 6. Relative levels of surface IgMα vs IgMβ on splenic B cells from representative 3H9-μ-VH8-κ and VH8-κ and Tg- littermate BWF1 mice. Splenic B cells from 9-mo-old 3H9-μ-VH8-κ BWF1 mice were stained with anti-IgMα (dotted line) or anti-IgM (solid line). The anti-IgM reagent binds both IgMα and IgMβ. The results are representative of mice 3–9 mo old.

FIGURE 7. Flow cytometric analysis of IgMα and IgMβ expression on mice bone marrow (A) and splenic (B) B cells from a VH NP-μ-VH8-κ Tg BWF1 mice. Cells from both bone marrow and spleen were gated on B220/CD45R+ mononuclear cells. a–d, Percentages of cells. The results are representative of two adult mice 3–6 mo old.

FIGURE 8. Flow cytometric analysis of IgMα and IgMβ expression on splenic B cells from 3H9-μ BWF1 mice. Cells were gated on B220/CD45R+ mononuclear cells. a–d, Percentages of cells. The results are representative of at least three adult mice 3–7 mo old.
80% IgM<sup>a</sup> in V<sub>μ</sub>NP-µ-V<sub>κ</sub>4R Tg (Fig. 7A) and only 39% IgM<sup>b</sup> in non-Tg BWF1 (Fig. 5C). Similarly to splenic B cells, surface IgM<sup>a</sup> expression on bone marrow B cells in 3H9-µ-V<sub>κ</sub>8-κ Tg BWF1 mice was also reduced compared with bone marrow B cells in V<sub>μ</sub>NP-µ-V<sub>κ</sub>8-κ and non-Tg BWF1 mice. These results are consistent with the hypothesis that 3H9-µ-V<sub>κ</sub>8-κ B cells encountered autoantigen in the bone marrow and were arrested in their development. Surface expression of IgM<sup>a</sup> on bone marrow B cells in 3H9R-V<sub>κ</sub>4R Tg BWF1 mice was reduced compared with IgM expression on non-Tg B cells (Fig. 5C and Fig. 9, A and C) and IgM<sup>b</sup> expression on B cells from V<sub>μ</sub>NP-µ-V<sub>κ</sub>8-κ BWF1 mice (Fig. 7A). The percentage of IgM<sup>a</sup>, B220/CD45R<sup>+</sup> was similar for bone marrow from 3H9R-V<sub>κ</sub>4R and non-Tg BWF1 mice.

**Discussion**

Anti-DNA Tg BWF1 mice were used to analyze DNA-specific B cell development and activation in an autoimmune background. Mice monospecific for an anti-DNA Ab formed by the 3H9V<sub>H</sub> and either V<sub>κ</sub>8 or V<sub>κ</sub>4 were compared with mice that inherited only 3H9V<sub>H</sub>, V<sub>κ</sub>8, or V<sub>κ</sub>4 or were Tg<sup>−</sup>. Mice inherited the 3H9V<sub>H</sub> as either a conventional 3H9V<sub>H</sub>-µ Tg or the 3H9RV<sub>H</sub> knock-in Tg targeted to J<sub>κ</sub>8. V<sub>κ</sub>8 was inherited as a conventional V<sub>κ</sub>8-κ Tg, and V<sub>κ</sub>4, as the V<sub>κ</sub>4R V<sub>κ</sub> knock-in targeted to J<sub>κ</sub>. The results indicate that B cell development and autoantibody production differ remarkably in BWF1 mice that inherited the conventional 3H9-µ H chain compared with the 3H9R V<sub>H</sub> knock-in. When B cells expressed V<sub>μ</sub>3H9 as a knock-in Tg, the respective BWF1 mice developed autoimmune anti-DNA similarly to Tg<sup>−</sup> BWF1 mice. When B cells expressed V<sub>μ</sub>3H9 as a conventional Tg, only a small percentage of the respective BWF1 mice produced relatively low serum anti-DNA titers detected only late in life compared with Tg<sup>−</sup> BWF1 mice. Most serum IgM and all of the IgM anti-DNA produced in the conventional Tg BWF1 mice was IgM<sup>b</sup> derived from endogenous V gene rearrangements rather than the V<sub>μ</sub>3H9-µ Tg. In contrast, much of the serum IgM and most of the IgM anti-DNA produced in the site-directed Tg BWF1 mice were products of the 3H9R Tg. We expect that the IgG produced in site-directed 3H9R Tg BWF1 mice was also encoded by the 3H9R Tg. The majority of B cells expressing V<sub>µ</sub>3H9 as either the conventional or knock-in Tg had a phenotype consistent with Ag exposure during development in the bone marrow and subsequent inactivation and anergy. All of the IgM produced in BWF1 mice that inherited the conventional V<sub>μ</sub>NP-µ-Tg with or without the V<sub>κ</sub>8-κ Tg was IgM<sup>a</sup>. B cells in the V<sub>μ</sub>NP-µ Tg BWF1 mice did not have an anergic cell surface phenotype. Overall, the results indicate that central tolerance is effective in BWF1 mice, and that developmental arrest of IgM<sup>a</sup> B cells was sustained in the periphery in the conventional 3H9-µ but not knock-in 3H9R Tg BWF1 mice.

Several functional differences between the conventional 3H9-µ and the knock-in 3H9R Tgs could contribute to the phenotypic differences between 3H9-µ and 3H9R Tg BWF1 mice and the respective 3H9-µ-V<sub>κ</sub>8-κ and 3H9R-V<sub>κ</sub>4R double Tg BWF1 mice. 1) The 3H9R and V<sub>κ</sub>4R Tgs can be edited (22, 26); however, the 3H9-µ and V<sub>κ</sub>8-κ Tgs cannot. 2) Mature IgM<sup>a</sup> B cells in 3H9R, but not 3H9-µ BWF1, express surface IgD. 3) Mature IgM<sup>b</sup> B cells in 3H9R but not 3H9-µ BWF1 mice can switch the Tg V<sub>μ</sub>3H9 to IgG or other constant regions. The potential for both 3H9R and V<sub>κ</sub>4R to be edited may explain to some extent why the knock-in Tg BWF1 mice had higher levels of serum IgM<sup>a</sup> than those detected in the sera of conventional Tg BWF1 mice. The difference in editing potential between conventional and site-directed Tgs is less likely to explain why 3H9R Tg BWF1 mice produce IgM<sup>a</sup>, including IgM<sup>a</sup>-anti-DNA, but 3H9-µ Tg BWF1 mice did not. The Tg<sup>−</sup> H chains in both 3H9-µ and 3H9R Tg B cells could be paired with any L chain, and surface IgM expression was similar on B cells from 3H9-µ and 3H9R Tg BWF1 mice. These results suggest that the difference in production of Tg-encoded IgM<sup>a</sup> and IgM<sup>a</sup>-anti-DNA between 3H9-µ and 3H9R Tg BWF1 mice was more likely determined by the potential for activation than by susceptibility to induction of anergy, particularly for IgM<sup>a</sup> B cells in the c subpopulation (Fig. 8). Potential Ags for IgM<sup>a</sup> B cells should be no different between 3H9-µ and 3H9R Tg BWF1 mice; however, IgM<sup>b</sup> B cells in 3H9R but not 3H9-µ Tg BWF1 mice expressed IgD and could switch V<sub>μ</sub>3H9 or a different V<sub>μ</sub> generated by editing on the Tg allele to IgG or other constant regions. Recently reported results have indicated that IgD may be important for B cell activation and germinal center formation (37, 38). Likewise, signaling through B cell surface IgE is more effective for signaling activation, and consequently, the induction of more clonal expansion than signaling through IgM (39). An inability to class switch should not prevent activation to IgM production, however, and does not explain why 3H9-µ Tg BWF1 mice produced little IgM<sup>a</sup> and no IgM<sup>a</sup>-anti-DNA. Even though IgM<sup>b</sup> B cells did not appear to be anergic in either V<sub>μ</sub>NP-µ or V<sub>μ</sub>NP-µ-V<sub>κ</sub>8-κ Tg BWF1 mice, total serum IgM was markedly reduced in both compared with Tg<sup>−</sup> BWF1 mice. Reduced serum IgM in V<sub>μ</sub>NP-µ Tg BWF1 mice was most likely due to limited B cell activation and IgM Ab production caused by the absence of an appropriate immune stimulus, the absence of IgD, or both. The V<sub>μ</sub>NP-µ Tg did not include C<sub>α</sub> or C<sub>λ</sub>. Assuming that attempts to edit the V<sub>μ</sub>NP-µ Tg would have resulted in its elimination and IgM<sup>a</sup> production, there appeared to be little attempt to edit the Tg H chain in V<sub>μ</sub>NP-µ-V<sub>κ</sub>8-κ and V<sub>μ</sub>NP-µ Tg BWF1 mice, probably because the respective receptors did not have autoimmune specificity. The IgM<sup>a</sup>-anti-DNA detected in some of the V<sub>μ</sub>NP-µ and V<sub>μ</sub>NP-µ-V<sub>κ</sub>8-κ Tg BWF1 mice was most likely produced by B cells expressing a V<sub>κ</sub> other than V<sub>κ</sub>8, yielding an anti-DNA that did not induce anergy. Analyses of
IgMα and IgMβ B cells sorted by flow cytometry and IgMα- and IgMβ-producing hybridomas derived from 3H9-μ and 3H9R BWF1 mice and IgMα-producing hybridomas from V3HNP-μ and V3HNP-μ-V8.8-κ Tg BWF1 are currently under way. The results will provide insight about the effects of the V3HNP-μ, 3H9-μ, and 3H9R Tgs on the functional status of B cells in Tg BWF1 mice.

The results from studies of BWF1 mice Tg for the conventional 3-32-μ anti-DNA H chain are consistent with the results and conclusions described above for 3H9-μ (31), 3-32-μ BWF1 mice also had very low serum levels of IgMα and IgMβ anti-DNA titers no higher than those detected in normal BALB/c mice. The 3-32-μ Tg also did not include Cμ and could not be switched to Cκ. B cells in R4A-γ2b conventional Tg BWF1, in contrast, were not anergic and produced Tg-encoded serum anti-DNA at higher titers than in normal, non-Tg BWF1 mice (30). Although it is not clear why expression of the R4A-γ2b Tg in BWF1 mice would have a different influence on B cell development and activation than 3H9-μ or 3-32-μ, the effect may be due to the effects of expression of Cκ vs Cμ during B cell development (40). The more effective activation signaling through Cκ (39) noted above may determine the difference in anti-DNA production from the R4A-γ2b vs 3H9-μ or 3-32-μ Tgs. Alternatives, differences in specificity between R4A-γ2b and 3H9-μ or 3-32-μ-encoded Abs could have contributed to differences in B cell development and activation in R4A-γ2b vs 3H9-μ or 3-32-μ Tg BWF1 mice. BWF1 mice Tg for the D42 anti-DNA Vκ knock-in (Tg (32) had a phenotype similar to that of 3H9R-V4R and 3H9R knock-in Tg BWF1 mice. D24 Vκ knock-in BWF1 mice produced anti-DNA encoded by the Tg. The D24 Vκ knock-in Tg could be expressed with Cκ and isotype switched to Cκ or other Cκ similarly to the 3H9R knock-in Tg. 3H9-μ and V3HNP-μ Tg BWF1 mice are similar in many respects to 2–12H Tg Cκ-17 mice (41) although Cκ and Cκ-17 mice are not autoimmune prone. The 2–12H Tg is a conventional μ, anti-Sm Tg H chain. 2–12H Tg mice produce very little Tg serum IgM, whether specific for Sm or not, even though 2–12H expressing B cell seems to be fully functional. In 2–12H Tg mice in which B cell receptor signaling was enhanced by a human CD19 Tg, serum IgM production was restored to levels greater than that in normal, non-Tg mice (42). The absence of 2–12H Tg-encoded Ab is thought to be due to clonal ignorance and differentiation of 2–12H Tg B cells to B1 which have a higher signaling threshold for activation.

Because there were IgMα B cells that did not express IgMα in 3H9-μ-V8.8-κ and 3H9-μ Tg BWF1 mice, the 3H9-μ Tg must have been inactivated or eliminated in the respective B cells, likely as an attempt to edit autoreactivity. Assuming that rearrangements of endogenous H and L chain alleles are random, rearrangement of endogenous H and/or L chain alleles should have included Vκ and Vκ combinations that encoded anti-DNA. Yet, fewer than 10% of 3H9-μ-V8.8-κ BWF1 mice and 25% of 3H9-μ BWF1 mice produced detectable IgMα or IgG anti-DNA when 1 year old or less. B cells from 3H9-μ-V8.8-κ Tg BWF1 spleen and bone marrow also included a subpopulation of B cells that expressed both IgMα and IgMκ on the cell surface. If rearrangement and expression of an endogenous H chain allele generated an IgMα that bound DNA, the respective B cell may have expressed, at least temporarily, two cell surface anti-DNA antibodies, likely with different epitope specificity. Expression of two different anti-DNA Abs could in turn have yielded even stronger receptor cross-linking on the respective B cells and thus stronger signaling leading to deletion or more profound anergy of the respective B cells in the bone marrow. IgMα Abs that did not bind DNA or other relevant autoantigens could have diluted the negative signaling resulting in survival and escape from anergy, especially if the Tg IgMα loci in the respective B cells were inactivated or eliminated. In fact, μ expression from a rearranged endogenous allele could have disrupted the DNA specificity of 3H9-μ Ab receptors. Likewise, expression of L chains that vetoed DNA reactivity would have also disrupted the DNA specificity of 3H9-μ Ab receptors. Expression of both 3H9-μ and μ derived from an endogenous rearrangement was detected in many hybridomas from 3H9-μ Tg MRL+/+ (43). Secrecy IgM from hybridomas that expressed both 3H9-μ and μ from an endogenous allele either did not bind to DNA or did so with an ANA pattern different from 3H9. The relatively small amount of IgMα detected in sera of 3H9-μ-V8.8-κ and 3H9-μ Tg BWF1 mice could have been produced by B cells expressing both IgMα and IgMκ. Because either or both H and L loci containing 3H9R and V4R, respectively, could be edited to eliminate and/or disrupt DNA specificity, IgMκ B cells in 3H9R-V4R and 3H9R Tg BWF1 mice would have had more opportunity to generate non-DNA-reactive receptors. B cells in 3H9R-V4R and 3H9R Tg BWF1 that edited either 3H9R or V4R to generate another anti-DNA Ab would also have expressed two different anti-DNA antibodies. Such B cells would have been susceptible to the same tolerogenic signaling as B cells in 3H9-μ-V8.8-κ and 3H9-μ BWF1 mice. The two subpopulations of 3H9R-V4R spleen B cells with low and very low level expression of surface IgMα (Fig. 9A, a and b) likely included B cells that either did not edit the high affinity 3H9-μ-V4R receptor or those that did edit the receptor but generated another anti-DNA or other autoreactive receptor. The production of IgMα anti-DNA in 3H9R-V4R and 3H9R Tg BWF1 mice is evidence that editing could lead to generation of a second anti-DNA receptor; however, IgMα anti-DNA production was generally only 12% or less of the IgMα anti-DNA in the same mice. Recent results obtained from 3H9 and 3H9/Sl6R knock-in Tg MRL+/+ mice indicate that Vκ gene editing can generate B cells that express two different cell surface receptors (44, 45). When only one of the receptors was specific for DNA, the respective B cells were rescued from anergy or deletion. The IgMα and IgG anti-DNA in 3H9R-V4R and 3H9R Tg BWF1 mice may have been produced by 3H9R Tg B cells that escaped tolerance induction or by B cells in which receptor editing generated new anti-DNA receptors that did not induce tolerance.

Raff et al. (46) were the first to describe the effects of Ig receptor engagement on immature bone marrow B cells. Prolonged exposure of immature B cells to anti-Ig in vitro resulted in the irreversible inactivation of the respective B cells. The effects of Ig receptor engagement on immature bone marrow B cells are similar when the engagement is by specific Ag in vivo (16, 18). When nonautoimmune-prone mice were made Tg for production of soluble lysozyme (hen egg lysozyme; HEL) and IgM and IgD that bind lysozyme with high affinity (HEL-Ig), B cells in the double-Tg mice were anergic (18). HEL-specific B cells in mice Tg for HEL and for the same high affinity anti-HEL as a knock-in Vκ with the conventional Vκ were also anergic (47). Anergy of HEL-specific B cells was dependent on the presence of soluble HEL, and as long as soluble HEL was present, T cell help could not stimulate B cell differentiation to Ab production (48). HEL-specific B cells were excluded from follicles in HEL-Tg mice (49). The greatly increased percentage of B cells with reduced IgM expression in the bone marrow of conventional 3H9-μ and knock-in 3H9R Tg BWF1 mice, with or without V8.8-κ or V4R, respectively, indicates that immature, DNA-specific B cells must have encountered relevant Ag in the bone marrow and were developmentally arrested. DNA-specific B cells were also developmentally arrested in 3H9-μ Tg BALB/c and MRL+/+ mice (19, 50), and like 3H9-μ Tg
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BWF1 mice, the developmental arrest was sustained in the periphery. Developmental arrest of DNA-reactive B cells was not sustained in 3H9-\text{\textmu} \text{V}{\text{H}} MRL\text{\textnumero}lpr/lpr (50) and BALB/\text{\textnumero}lpr/lpr (51) mice. When anergic, DNA-specific B cells in 3H9-\text{\textmu} \text{V}{\text{H}} Tg BALB/c mice were provided a source of T cell help, the developmentally arrested, DNA-specific B cells not only entered follicles but were also activated to produce anti-DNA Ab (51). Follicular inclusion of DNA-reactive B cells and anti-DNA autoantibody production in 3H9-\text{\textmu} \text{V}{\text{H}} Tg MRllpr/lpr (50) and BALB/lpr/lpr (52) mice were also Th cell dependent (51). When CD4+CD25+ T-regulatory cells were provided along with Th cells, DNA-specific B cells were still follicularly included but did not differentiate to anti-DNA Ab-producing cells. Even though anergic B cells may affect the development of activated Th cells in BWF1 mice (53), reduced T cell help or a difference in CD4+CD25+ T-regulatory cells seems unlikely to account for the difference in IgM+ B cell activation between 3H9-\text{\textmu} and 3H9R Tg BWF1 mice. 3H9-\text{\textmu} and 3H9R Tg BWF1 had similar percentages of anergic B cells and similar total serum IgG, and both 3H9-\text{\textmu} and 3H9-\text{\textmu}-V\text{\textnu}8-k Tg BWF1 mice produced IgG ANA. What is unknown at present is whether 3H9-\text{\textmu} and 3H9R Tg B cells differ in their potential for Ag presentation to Th cells. Experiments are under way to determine whether anti-DNA B cells in 3H9-\text{\textmu} and 3H9R Tg BWF1 mice are follicularly included and how 3H9-\text{\textmu} and 3H9R Tg B cells may differ in their ability to present Ag to Th cells and respond to T cell help.

In the end, tolerance to DNA in 3H9-\text{\textmu} and 3H9-\text{\textmu}-V\text{\textnu}8-k Tg BWF1 mice must be due to the inability of the autoimmune stimulation normally present in the lymphoid periphery of BWF1 mice to achieve the threshold of activation needed to overcome the developmental arrest imposed on 3H9-\text{\textmu} Tg-expressing B cells in the bone marrow. A combination of factors including but not limited to the specificity of 3H9, the inability of 3H9-\text{\textmu} and V\text{\textnu}8-k to be edited, the lack of IgD on B cells expressing only IgM, and their affinities. Allergic B cells have been provided with T cell help, the developmentally arrested, DNA-specific B cells not only entered follicles but were also activated to produce anti-DNA Ab (51). Follicular inclusion of DNA-reactive B cells and anti-DNA autoantibody production in 3H9-\text{\textmu} \text{V}{\text{H}} Tg MRllpr/lpr (50) and BALB/lpr/lpr (52) mice were also Th cell dependent (51). When CD4+CD25+ T-regulatory cells were provided along with Th cells, DNA-specific B cells were still follicularly included but did not differentiate to anti-DNA Ab-producing cells. Even though anergic B cells may affect the development of activated Th cells in BWF1 mice (53), reduced T cell help or a difference in CD4+CD25+ T-regulatory cells seems unlikely to account for the difference in IgM+ B cell activation between 3H9-\text{\textmu} and 3H9R Tg BWF1 mice. 3H9-\text{\textmu} and 3H9R Tg BWF1 had similar percentages of anergic B cells and similar total serum IgG, and both 3H9-\text{\textmu} and 3H9-\text{\textmu}-V\text{\textnu}8-k Tg BWF1 mice produced IgG ANA. What is unknown at present is whether 3H9-\text{\textmu} and 3H9R Tg B cells differ in their potential for Ag presentation to Th cells. Experiments are under way to determine whether anti-DNA B cells in 3H9-\text{\textmu} and 3H9R Tg BWF1 mice are follicularly included and how 3H9-\text{\textmu} and 3H9R Tg B cells may differ in their ability to present Ag to Th cells and respond to T cell help.

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