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CUTTING EDGE

Cutting Edge: Expansion and Activation of A Population of Autoreactive Marginal Zone B Cells in a Model of Estrogen-Induced Lupus

Christine M. Grimaldi,* Daniel J. Michael,* and Betty Diamond[2]*†

We have demonstrated previously that 17 β-estradiol (E2) treatment of BALB/c mice transgenic for the heavy chain of a pathogenic anti-DNA Ab induces a lupus-like phenotype with expansion of anti-DNA B cells, elevation of anti-DNA Ab titers, and glomerular immunoglobulin deposition. To understand this loss of B cell tolerance, the effects of E2 on B cell development and activation were examined. A sustained increase in E2 resulted in an altered distribution of B cell subsets, with a diminished transitional population and an increase in marginal zone B cells. Depletion of CD4$^+$ T cells did not abrogate these effects. Furthermore, the B cells that spontaneously secreted anti-DNA Abs displayed a marginal zone phenotype. Thus, a sustained increase in E2 alters B cell development, leading to the survival, expansion, and activation of a population of autoreactive marginal zone B cells implicating this B cell subset in autoimmunity. ***The Journal of Immunology,*** **2001, 167: 1886–1890.**

The autoimmune disease systemic lupus erythematosus is characterized by the production of Abs specific for numerous self-Ags, expansion of autoreactive B cells, and glomerular deposition of immunoglobulin complexes that contribute to kidney damage (1). Aspects of the human disease are observed in naturally occurring mouse models of systemic lupus erythematosus, such as the NZB/ZNW F_1 and MRL/*lpr* mouse strains. These mice spontaneously develop pathogenic autoantibodies that cause glomerulonephritis and have served to dissect the molecular pathways that regulate autoreactive B cells. Much information has also been learned from the lupus-like phenotype exhibited in nonautoimmune mice that are deficient in or overexpress key regulatory models that modulate B cell tolerance, selection, and activation.

We have shown that treatment with exogenous 17 β-estradiol (E2)[3] abrogates B cell tolerance in anti-DNA H chain transgenic BALB/c mice (R4A-IgG2b), which normally display effective regulation of high affinity transgene (Tg)-expressing anti-DNA B cells (2–5). The lupus-like phenotype induced by E2 treatment of R4A-IgG2b BALB/c mice includes an expansion of the Tg-positive B cell population, a significant rise in IgG2b anti-dsDNA Ab titers, an increase in the number of B cells spontaneously secreting IgG2b anti-dsDNA Ab, and the presence of IgG glomerular deposits (2, 3).

In this study, we demonstrate that E2 alters the maturation of splenic B cell precursors and enhances development and activation of autoreactive marginal zone B cells. Little is known about the role of marginal zone B cells in autoimmunity, but emerging evidence suggests that marginal zone B cells contribute to the pathogenic autoantibody responses generated in NZB/NZW F_1 mice (6–8). We now provide evidence that the anti-DNA Abs induced in E2-treated nonspontaneously autoimmune mice may be secreted by autoreactive marginal zone B cells.

Materials and Methods

E2 treatment of mice

BALB/c mice (2–3 mo) were purchased from The Jackson Laboratory (Bar Harbor, ME). Transgenic R4A-IgG2b BALB/c mice (2–3 mo) were bred in a specific pathogen-free barrier facility. Mice were ovariectomized and treated with E2 time-release or placebo (P) pellets (Innovative Research of America, Saratoga, FL) (2).

Flow cytometry

Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Tree Star, San Carlos, CA). Single cell suspensions from bone marrow or spleen were stained with mixtures of Abs specific for the following markers: B220, CD1, CD19, CD21, CD23, CD138, heat-stable Ag (HSA), IgD, IgM, B7.2, MHC class II (MHC II), CD44, and IgG2b. All Abs were purchased from BD PharMingen (San Diego, CA), except CD19 (Caltag Laboratories, Burlingame, CA).

CD4$^+$ T cell depletion

Mice received i.p. injections (500 μg) of purified anti-CD4 GK1.5 mAb for 2 consecutive days. On day 3, E2 or P pellets were implanted in the mice. This protocol was repeated every 3 wk and was sufficient to deplete >98% of CD4$^+$ T cells during the 5- to 7-wk E2 treatment period.

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[3] Abbreviations used in this paper: E2, 17 β-estradiol; P, placebo; Tg, transgene; MFI, mean fluorescence intensity; HSA, heat-stable Ag; MHC II, MHC class II; T1, type 1; T2, type 2.

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Immunohistochemistry

Frozen splenic sections (5 μm) were fixed in acetone. After blocking with 3% BSA/PBS, the slides were incubated with a 1/200 dilution of 7-amino-4-methylcoumarin-3-acetic acid-labeled anti-IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) and Texas Red-labeled anti-IgG2b (BD PharMingen).

ELISPOT assay

Splenocytes from three E2-treated or three P-treated R4A-IgG2b BALB/c mice were pooled. B cells were isolated by depletion with biotin-labeled anti-CD43 and streptavidin-labeled Dynabeads (Dynal, Lake Success, NY) and stained with FITC-labeled anti-CD21 and PE-labeled anti-CD23. Follicular B cells ($CD21^{int}/CD23^{+}$) and marginal zone B cells ($CD21^{high}/CD23^{neg\text{-}low}$) were isolated using a FACSVantage cell sorter (BD Biosciences) with >90% purity. Two-fold serial dilutions of cells starting from 1×10^5 follicular B cells or 4×10^4 marginal zone B cells were added in duplicate to microtiter wells coated with dsDNA (100 μg/ml) and incubated for 16 h at 37°C. IgG2b anti-DNA B cells were revealed by the addition of biotin-labeled anti-IgG2b and alkaline phosphatase-labeled streptavidin as previously described (2).

Results

E2 treatment activates a small population of B cells

To understand how a sustained increase in E2 abrogates B cell tolerance in R4A-IgG2b BALB/c mice, we asked whether E2-treated mice contain a large population of activated Tg^{+} B cells consistent with an E2-induced polyclonal activation. Flow cytometry was performed on splenocytes with Abs to B7.2, CD44, and MHC II and IgG2b to distinguish Tg^{+} B cells from the endogenous B cell population. The Tg^{+} B cell population of E2-treated mice displayed an increase in both the level of B7.2 and the percentage of $B7.2^{+}$ B cells (Table I). However, there was only a modest increase in the level of CD44 expression and no change in the level of MHC II expression. Furthermore, there was no apparent increase in B cell size based on forward scatter flow cytometric profiles (data not shown). E2-treated mice displayed a 2- to 3-fold increase in Tg^{+} cells that were positive for CD138, a marker for plasma cells (Table I). Although the increase in B7.2 and $CD138^{+}$ cells suggests that some of the Tg^{+} B cells were activated, the majority of splenic Tg^{+} B cells were not, arguing against the hypothesis that a sustained increase in E2 results in polyclonal B cell activation (9).

E2 treatment alters splenic B cell development

We examined the impact of a sustained increase of E2 on the distribution of splenic B cell subsets in E2-treated nontransgenic BALB/c mice, because it has been shown that transgenic expression of rearranged Ig genes may alter the normal distribution of B cell subsets (10). Furthermore, E2-treated BALB/c mice also exhibit a modest increase in anti-DNA Ab titers (Ref. 11, and data not shown), suggesting that similar cellular activation occurs in R4A-IgG2b and nontransgenic mice. In agreement with previous reports of decreased lymphopoiesis with E2 treatment (12–14), flow cytometry revealed a reduction in bone marrow $B220^{+}$ B cells in E2-treated BALB/c mice (Table II). The percentage of splenic B cells was unchanged; however, there was a shift in the immature and mature B cell compartments with reduced numbers of $B220^{+}/HSA^{high}$ transitional B cells and increased numbers of $B220^{+}/HSA^{low\text{-}int}$ mature B cells (Fig. 1*A*). The reduction of $B220^{+}/HSA^{high}$ B cells was due to a decrease in the percentage of $CD21^{neg}/HSA^{high}$ transitional type 1 (T1) B cells, which represent the most immature splenic B cell and give rise to transitional type 2 (T2) B cells (15, 16). The $CD21^{high}/HSA^{high}$ transitional T2 population, which differentiates into the mature splenic B cell populations, was slightly reduced in E2-treated mice. In fact, in E2-treated mice, there are similar numbers of T1 and T2 B cells, whereas in untreated mice, there are twice as many T1 as T2 B cells (Fig. 1*B* and Table II). The increased number of $B220^{+}/HSA^{low\text{-}int}$ mature splenic B cells in E2-treated mice was largely due to the 3- to 5-fold increase in $CD21^{high}/HSA^{int}$ marginal zone B cells (Fig. 1*B* and Table II).

To confirm that the altered distribution of splenic B cell subsets observed in E2-treated BALB/c mice reflected a shift in the number of B cells belonging to each subset and was not the result of E2-induced changes in levels of CD21 or HSA, additional markers were examined. E2-treated BALB/c mice displayed a significant decrease in $CD1^{neg}/CD23^{neg}$ B cells, which includes the transitional T1 B cell population, relatively similar numbers of $CD1^{high}/CD23^{high}$ transitional T2 B cells, and an increase in $CD1^{high}/CD23^{neg\text{-}low}$ marginal zone B cells (Fig. 1*C*). Finally, as has been reported (15, 16), analysis of IgD expression confirmed that the expanded population of $CD1^{high}/CD23^{+}$ was IgD^{+} transitional T2 B cells and that the $CD1^{high}/CD23^{neg\text{-}low}$ population was $IgD^{neg\text{-}low}$ marginal zone B cells (Fig. 1*D*).

The Tg^{+} transitional T1 B cell population was significantly increased following E2 treatment from ~25 to 50% ($p < 0.02$; data not shown), suggesting that the expansion of Tg^{+} B cells occurs before the development of immature B cells into transitional T1 B cells and may reflect impaired negative selection in the bone marrow. In addition, a significant increase in the number of Tg-expressing $CD1^{high}/CD21^{high}$ transitional T2 and marginal zone B cells, and $CD1^{int}/CD21^{int}$ follicular B cells, was observed in E2-treated mice (Fig. 2). The expansion of Tg^{+} B cell subsets was not dependent on cognate $CD4^{+}$ T cell interactions or $CD4^{+}$ T cell-derived cytokines because depletion of $CD4^{+}$ T cells did not block this expansion (Fig. 2). Thus, the rescued population of Tg^{+} B cells in E2-treated R4A-IgG2b mice differentiated into distinct B cell compartments before a stage at which cognate T cell help or T cell-derived cytokines are required.

Table I. *Expression of activation markers on Tg^{+} B cells*

	P	E2	*p* Value[a]
$B7.2^{b}$ (MFI)	137.0 (±9.6)	168.5 (±5.0)	<0.001
MHC II^{b} (MFI)	422.2 (±43.9)	399.3 (±22.1)	NS[c]
$CD44^{b}$ (MFI)	350.6 (±85.8)	464.4 (±63.0)	NS
$B7.2^{b}$ (%)	24.3 (±5.7)	42.2 (±5.7)	<0.002
$CD138^{d}$ (%)	0.9 (±0.6)	2.8 (±0.4)	<0.002

[a] An unpaired Student's *t* test was performed to determine statistical significance.
[b] Values were determined from $B220^{+}/IgG2b^{+}$ gated B cells from P- or E2-treated R4A-IgG2b mice ($n = 4$). Data are presented as the mean ± SD; MFI, mean fluorescence intensity.
[c] NS, Not statistically significant.
[d] Values were determined as the percentage of $CD138^{+}/IgG2b^{+}$ lymphoid gated cells.

Table II. *B cell maturation in E2-treated BALB/c mice*

$B220^{+}$ B Cell Population	P	E2	*p* Value[a]
Bone marrow-total[b]	26.7 (±2.8)	19.4 (±4.7)	<0.03
Splenic-total[b]	44.1 (±4.7)	43.8 (±4.7)	NS
Splenic-transitional[c]			
Type 1	21.1 (±2.3)	5.4 (±2.5)	<0.000001
Type 2	10.5 (±2.2)	6.7 (±3.7)	<0.03
Splenic-mature[c]			
Follicular	62.7 (±7.2)	68.7 (±6.4)	NS[d]
Marginal zone	5.8 (±1.3)	19.9 (±2.5)	<0.000001

[a] An unpaired Student's *t* test was performed to determine statistical significance of P ($n = 5$)- and E2 ($n = 7$)-treated BALB/c mice.
[b] Data are presented as a percentage of lymphoid gated cells.
[c] $B220^{+}$ subsets were distinguished by CD21 and HSA staining.
[d] NS, Not statistically significant.

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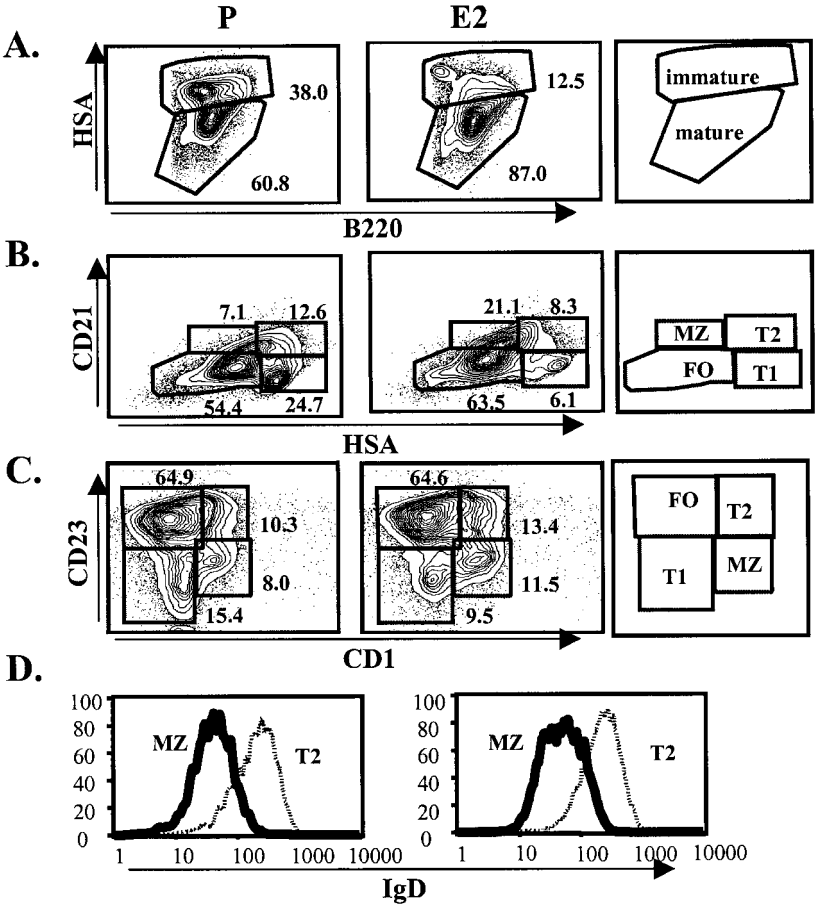

FIGURE 1. Altered distribution of B cell subsets in E2-treated mice. Splenocytes from nontransgenic BALB/c mice treated with E2 or P pellets (n = 3–7) were stained for B220, CD21, CD23, and HSA (*A* and *B*) or B220, CD1, CD23, and IgD (*C* and *D*). The immature HSA^{high} population was reduced and the mature HSA$^{low\text{-}int}$ population was increased in E2-treated mice. *B*, Analysis of CD21 and HSA revealed that the marginal zone (MZ) B cells were expanded, the transitional T1 and transitional T2 B cells were reduced, and the follicular cells (FO) were slightly increased in E2-treated mice. *C*, Staining with CD23 and CD1 revealed a similar distribution of B subsets in E2-treated mice as CD21 and HSA. *D*, Gating on CD23^{+}/CD1^{high} (dashed line) and CD23$^{neg\text{-}low}$/CD1^{high} cells (heavy line) to assess IgD expression confirmed that these populations correspond to transitional T2 (IgD^{+}) and marginal zone (IgD$^{neg\text{-}low}$) B cells, respectively.

The distribution of mature splenic Tg^{+} B cells in E2-treated R4A-IgG2b BALB/c mice was confirmed by immunohistochemistry. In P-treated R4A-IgG2b BALB/c mice, the Tg^{+} B cells were localized predominantly in the T cell/B cell interface and the marginal sinuses (Fig. 3). The spleens of E2-treated R4A-IgG2b mice exhibited an increase in Tg^{+} B cells in both follicular regions and marginal zones. Tg^{+} B cells were expanded in the red pulp areas as well, and may represent Tg^{+} plasma cells, consistent with the increase in Tg^{+}/CD138^{+} cells observed by flow cytometry.

Anti-DNA-secreting Tg^{+} B cell subsets in E2-treated mice

To determine which mature subsets were activated by E2 in vivo to secrete anti-DNA Ab, an ELISPOT assay was performed with isolated follicular and marginal zone B cells. Increases in both Tg^{+} follicular and marginal zone anti-DNA B cells from E2-treated R4A-IgG2b mice were observed (Fig. 3). However, the frequency of anti-DNA-secreting Tg^{+} marginal zone B cells was 10-fold higher than anti-DNA Tg^{+} follicular B cells. Thus, it appears that the increase in anti-dsDNA Ab titers in E2-treated R4A-IgG2b mice is largely due to the activation of autoreactive Tg^{+} marginal zone B cells.

Discussion

A sustained increase in E2 has been shown to accelerate the onset of disease and decrease the life span of mouse models of lupus (17–19) and to induce a lupus-like phenotype in some nonautoimmune-prone mouse strains (9, 11). We have been studying the impact of E2 administration on the regulation of autoreactive B cells. R4A-IgG2b BALB/c transgenic mice treated with E2 demonstrate survival and activation of B cells that have high affinity for DNA and unmutated light chain genes (2, 3), showing that E2 alters the selection of the naive repertoire of B cells exiting the bone marrow, and leads to the activation of these cells.

We found an altered maturation of transitional B cells with either rapid transit from the T1 to the T2 stage and/or a lack of deletion at the T2 stage. During the transitional stage, negative selection of autoreactive B cells that have not encountered self-Ag in the bone marrow is believed to occur (20, 21). Transitional T1 B cells are thought to arise from bone marrow immature B cells and to be direct precursors of transitional T2 B cells (15). Cross-linking of the B cell receptor mediates signal transduction events that direct the differentiation of transitional B cells into the mature marginal zone or follicular B cell subset (10, 15). The expansion of marginal zone B cells occurs in both R4A-IgG2b and nontransgenic BALB/c mice treated with E2. Therefore, it is possible that E2 alters signaling pathways to skew B cell development toward a marginal zone phenotype. Interestingly, mice transgenic for B cell activation factor from the TNF family display a lupus phenotype and exhibit a decrease in transitional T1 B cells and an expansion of transitional T2 and marginal zone B cells (16). This appears to be the result of impaired negative selection of autoreactive B cells and leads to a disproportionate number of marginal zone B cells in these mice (16).

The rescued autoreactive B cell population in R4A-IgG2b mice consists predominantly of marginal zone B cells. The role of marginal zone B cells in autoimmunity is not well understood. Anti-DNA Abs associated with lupus in both humans and mouse models display somatic mutations and have undergone isotype class switching, hallmarks of a T cell-dependent immune response (22, 23). Furthermore, the T cell-dependent follicular B cell subset is

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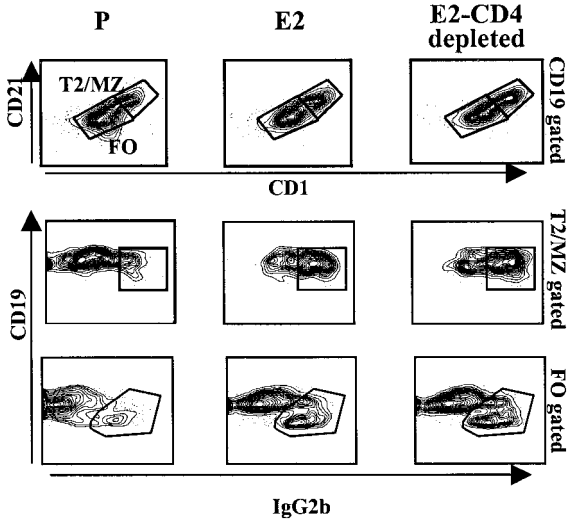

FIGURE 2. Expansion of Tg^+ B cells in the absence of $CD4^+$ T cells. Splenocytes from P ($n = 7$), E2 ($n = 7$) and E2-$CD4^+$ T cell-depleted ($n = 5$) transgenic mice were stained for CD19, CD1, CD21, and IgG2b. Expansion of the Tg^+ B cells in both $CD19^+/CD1^{high}/CD21^{high}$ (T2/MZ populations) and $CD1^{int}/CD21^{int}$ (FO) gated populations was observed in E2-treated mice. Depletion of $CD4^+$ T cells before E2 treatment did not block expansion of mature Tg^+ subsets. Representative profiles are shown. Percentage of Tg^+ TZ/MZ B cells in P-treated mice = 25.6 ± 9.3%, E2-treated mice = 50.0 ± 8.5%, and E2-treated/$CD4^+$-depleted = 55.9 ± 11.2%; the percentage of Tg^+ FO B cells in P-treated mice = 12.9 ± 5.0%, E2-treated mice = 25.2 ± 6.2%, and E2-treated/$CD4^+$-depleted = 27.4 ± 8.1%. A two-tailed Student's *t* test revealed that the differences between the values of P and E2 or E2-$CD4^+$ T cell-depleted-treated mice are significant ($p < 0.005$).

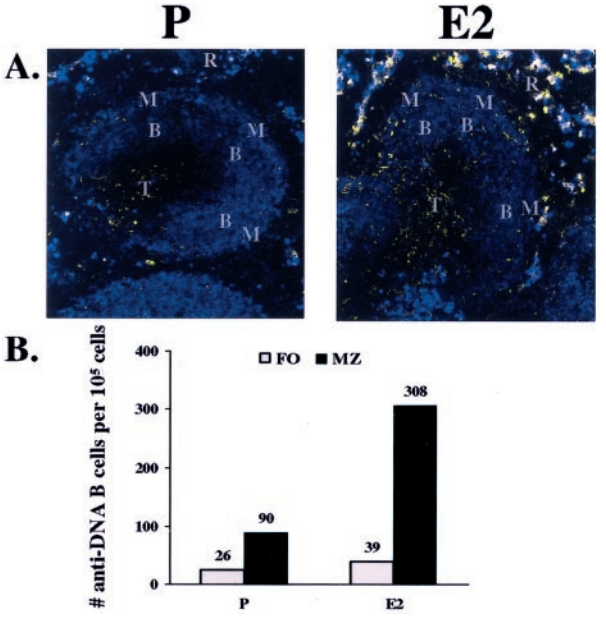

FIGURE 3. Localization of Tg^+ anti-DNA B cells. *A*, Frozen splenic sections were stained for IgM (blue) to identify B cell regions and for IgG2b (yellow) to localize Tg^+ B cells. The Tg^+ B cells of P-treated mice were localized predominantly to the T/B cell interface (T). The spleens of E2-treated mice exhibited an increased number of Tg^+ B cells that were found in B cell follicles (B), around the marginal sinus (M), and in the red pulp (R). Magnification, ×10. *B*, ELISPOT assay was performed using cell-sorted marginal zone and follicular B cells. Cells were incubated in duplicate in dsDNA-coated microtiter wells. Both the marginal zone and follicular B cell populations isolated from E2-treated mice displayed an increase in the number of anti-DNA-secreting cells as compared with cells from P-treated mice; however, the increase in the number of anti-DNA marginal zone B cells was most prominent.

clearly a source of pathogenic autoantibodies, because $CD4^+$ T cell depletion (24) or blockade of T cell costimulation (25) reduces autoantibody titers and prolongs the life of NZB/NZW F_1 mice. However, autoantibody production may not be limited to the follicular B cell subset, and the T cell-independent marginal zone B cell subset may also contribute to an autoimmune response. Marginal zone B cells are increased in young NZB/NZW F_1 mice (8). This increase is linked to the *Nba2* locus that associates with nephritis (7). Furthermore, $CD1^{high}$ B cells produce large amounts of anti-dsDNA IgM Ab in NZB/NZW F_1 mice (6), although it still remains to be established that marginal zone B cells secrete Abs that are pathogenic. It has also been suggested that T cell-independent B cells assist in the development of T cell-dependent B cell immune responses by providing a source of Ag-Ab complexes that are deposited on follicular dendritic cells (26). Marginal zone B cells may act as APCs (27), potentially triggering a T cell-dependent follicular B cell response. It has also been shown that CD40-CD40 ligand interactions activate marginal zone B cells (28). It is interesting to note that in the absence of $CD4^+$ T cells, the E2-induced expansion of the Tg^+ marginal zone B cell population still occurred, but did not result in elevated serum IgG2b anti-DNA Ab titers (data not shown).

An expansion of Tg^+ follicular B cells and a modest increase in the number of anti-dsDNA follicular B cells occur in E2-treated R4A-IgG2b mice. These cells may also contribute to the serum anti-dsDNA response. We propose that E2 rescues autoreactive transitional B cells from deletion; most differentiate to marginal zone B cells, and some to follicular B cells. Activation of Tg^+ follicular B cells in E2-treated mice would require the presence of activated autoreactive T cells because it has been shown that anti-dsDNA B cells are routinely generated in BALB/c mice (29, 30), but are not typically activated due to an absence of Ag-specific T cells (31, 32). We hypothesize that there is a small number of anti-DNA follicular B cells that are rescued but not activated by E2. Perhaps these cells differ in fine specificity from anti-DNA marginal zone B cells, as there is evidence that the antigenic specificity of B cells contributes to the determination of their subset phenotype. Whether the antigenic specificities of the Tg^+ marginal zone actually differ from those of follicular B cells needs further study.

In conclusion, E2 treatment of BALB/c mice alters B cell development and expands the population of marginal zone B cells. The increased maturation and activation of autoreactive marginal zone B cells in E2-treated mice, as well as in B cell activation factor for the TNF family mice and NZB/NZW F_1 mice, may be mediated by a common defect in the tolerance induction pathway. Continued analysis of autoreactive marginal zone B cells should provide insight into the role of T cell-independent B cell responses in the development of B cell-mediated autoimmune diseases.

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