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Christine M. Grimaldi, Venkatesh Jeganathan and Betty Diamond

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Hormonal Regulation of B Cell Development: 17β-Estradiol Impairs Negative Selection of High-Affinity DNA-Reactive B Cells at More Than One Developmental Checkpoint

Christine M. Grimaldi, Venkatesh Jeganathan, and Betty Diamond

There are increasing data suggesting that sex hormones, such as estrogen, have immunomodulatory effects and play a role in disease progression and pathogenesis in patients with the autoimmune disorder systemic lupus erythematosus. We have shown previously that treatment with 17β-estradiol (E2) induces a lupus phenotype in BALB/c mice that express a transgene-encoded H chain of an anti-DNA Ab. Because E2 treatment interferes with normal tolerance of naive DNA-reactive B cells, we elected to study the effects of hormonal modulation on the regulation of autoreactive B cells at early developmental checkpoints. Single-cell PCR was performed to study the repertoire of DNA-reactive B cell subsets. High-affinity DNA-reactive B cells were rescued at both the immature and transitional B cell stage in E2-treated mice. Interestingly, although low-affinity DNA-reactive B cells survive negative selection in control mice, the frequency of these cells was significantly reduced in the mature pool of E2-treated mice, suggesting that the high-affinity DNA-reactive cells that mature to immunocompetence out-compete the low-affinity population for survival as mature B cells. These data provide evidence that an elevation in serum levels of E2 facilitates the maturation of a pathogenic naive autoreactive B cell repertoire and hampers the maturation of a potentially protective autoreactive B cell repertoire. Furthermore, these data show that both positive and negative selection occur within the transitional B cell stage. The Journal of Immunology, 2006, 176: 2703–2710.

Systemic lupus erythematosus (SLE) is an autoimmune disorder that occurs almost 10 times more frequently in women than in men (1, 2) demonstrating that gender, along with genetic and environmental factors, contribute to disease susceptibility and pathogenesis. We and others have previously established that mice given 17β-estradiol (E2) time-release pellets, which elevate serum E2 levels to peak physiologic levels, produce elevated titers of anti-DNA Ab and develop glomerular immune complex deposition (3, 4). Anti-DNA B cells that would normally be deleted from the naive repertoire are rescued by E2 treatment and enter into the mature B cell pool (3, 5). These data demonstrated that an elevation in E2 was sufficient to alter negative selection of autoreactive B cells. Thus, the need is apparent for gaining a better understanding of the effects of estrogen on the regulation of autoreactive B cells.

During the generation of the naive B cell repertoire in the bone marrow, numerous antigenic specificities are generated, including specificities for self-Ags. Autoreactive B cells are subject to negative selection at multiple developmental checkpoints. The earliest stage of negative selection of autoreactive B cells occurs at the immature stage following acquisition of surface Ig, and is mediated by engagement of the BCR with self-Ag (6). How the B cell interprets the signals mediated by autoantigen engagement of the BCR is not fully understood, but the degree of BCR cross-linking is known to play an important role in determining the fate of autoreactive B cells. This is underscored by observations made in several different autoantibody transgenic models that demonstrate that high-affinity autoreactive B cells are subject to negative selection, whereas lower affinity autoreactive B cells are ignored. There are data that demonstrate that immature B cells that survive the selection process in the bone marrow develop into transitional type 1 (T1) B cells (7). T1 B cells migrate from the bone marrow and home to the spleen, where they take up residence in the periarteriolar lymphoid sheath (8). T1 cells undergo additional maturation to become transitional type 2 (T2) B cells (8, 9), then transitional type 3 (T3) B cells (10), before becoming fully mature, immunocompetent follicular or marginal zone B cells.

It has been clearly established in transgenic mouse models that negative selection can occur in the bone marrow, but less is known about the regulation of autoreactive B cells that escape negative selection at the immature B cell stage. There is increasing evidence that autoreactive B cells in mice and in humans that escape tolerance at the immature B cell stage may be regulated at the transitional stage of B cell development (7, 11–16); however, the events that govern selection at the transitional B cell stage are not completely understood. It has been reported that T1 cells are susceptible to anti-IgM-mediated apoptosis in vitro, whereas T2 cells are more resistant (9, 17). However, it has also been reported that both T1 and T2 are susceptible in vitro to anti-IgM-induced apoptosis, but T2 cells can be rescued by CD40L on T cells or by IL-4 (18, 19). The different properties of T1 and T2 B cells are not yet fully understood.
estrogen alters both negative and positive B cell selection

elucidated, but there is strong circumstantial evidence that autoreactive cells can be blocked from entry into the mature B cells pool at the transitional B cell stage by encounter with Ag. There is also evidence that T2 B cells may proliferate in response to BCR engagement (9), suggesting that there may be a stage at which positive selection of B cells can occur.

To gain a better understanding of how alterations in sex hormones affect B cell selection and exacerbate SLE, we have been studying the negative selection of DNA-reactive B cells in E2-treated R4A transgenic mice. Our previous data suggest that an elevation in E2 alters the negative selection of DNA-reactive B cells derived from the naive B cell repertoire (3, 5). Thus, it appears that E2’s effects on B cell tolerance may be due to alterations in early B cell developmental checkpoints. In this study, we examined the DNA-reactive repertoires at early and mature stages of B cell development. We show that high-affinity DNA-reactive B cells usually undergo negative selection in both the bone marrow and the spleen, and that E2 impairs selection at both sites. Low-affinity DNA-reactive B cells may be negatively regulated in the bone marrow, but are positively regulated in the spleen. When tolerance is perturbed by E2, the high-affinity, and not the low-affinity, DNA-reactive B cells are preferentially selected into the mature B cell pool. These alterations in selection demonstrate the critical importance of the transitional B cell stage as one in which positive and negative selection both occur, and show that defects in both can contribute to disease pathogenesis.

Materials and Methods

Mice and E2 treatment

Six- to 8-wk-old female mice were used in these studies. R4A BALB/c mice have been described previously (20). BALB/c mice and CD4-deficient BALB/c mice were obtained from The Jackson Laboratories. Mice were housed in a specific pathogen-free facility, and the experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee. Sixty-day time-release pellets containing E2 (0.18 mg/pellet) or placebo pellets (vehicle control) were obtained from Innovative Research of America and implanted underneath the skin. The E2 implant elevates serum E2 levels to 75–100 pg/ml (3). Mice were treated for 5–6 wk unless stated otherwise.

Flow cytometry and Abs

Fluorochrome-labeled Abs specific for B220 (RA3-6B2), CD19 (1D3), CD21/CD35 (7G6), CD23 (B34), and heat-stable Ag (HSA; M1/69), and biotin-labeled IgG2b (R12-3) and CD23 (B34), and fluorochrome-labeled streptavidin were purchased from BD Biosciences Pharmingen. Fluorochrome-labeled AA4.1 was obtained from eBioscience. Fluorochrome-labeled anti-IgG2b was obtained from Molecular Probes. Four- and five-color flow cytometry was performed using an LSR II instrument (BD Biosciences), and analysis was performed using the FlowJo software package (Tree Star). At least 5000 B cell-gated events were analyzed per mouse.

Single-cell PCR

To determine the frequency of high- and low-affinity DNA-reactive B cells, single-cell PCR was performed. Splenocytes from three placebo- or E2-treated R4A mice were stained for CD19, IgG2b, HSA, and CD21. Transitional (CD19+/IgG2b+/HSAhigh/CD21low/+), and mature (CD19+/IgG2b+/HSAmin/CD21high/-) cells were isolated using a MoFlow cell sorter (Cytomation). Single cells were collected into 96-well microtiter plates containing PCR buffer (Roche Applied Science) and proteinase K (Boehringer Mannheim). Amplification of κ-chain genes using Vs and Jκ primers was performed as previously described (21) by two rounds of PCR using GeneAmp PCR systems 9700 PCR machine (Applied Biosystems). The PCR products were either cloned into the TOPO TA cloning vector (Invitrogen Life Technologies) or directly purified from the agarose gels, and nucleotide sequences were determined by Genewiz. The Basic Local Alignment Search Tool program was used for sequence analysis.

BrdU analysis

Mice were treated with E2 for at least 2 wk before receiving a single i.p. injection of BrdU (1 mg/ml; BD Biosciences Pharmingen) and water containing BrdU (800 μg/ml) for 3–7 days. Splenocytes were stained with fluorochrome-labeled Abs, and BrdU incorporation was determined using the BrdU Flow Kit (BD Biosciences Pharmingen) according to the manufacturer’s protocol.

Autoreconstitution studies

Mice were treated with E2 for at least 2 days before receiving 600 rads of gamma irradiation (12). Mice were sacrificed 14 days later, and splenocytes were analyzed by flow cytometry.

Statistical analysis

Statistical significance was determined using the Student’s paired t test for flow cytometry studies and the χ2 test for Vκ-Jκ analysis. A p value of <0.05 was considered to be significant.

Results

The regulation of anti-DNA B cells normally occurs at the T2 stage and is diminished by E2 treatment

We have shown previously that the R4A BALB/c mouse, which expresses a transgene (Tg) encoding the H chain of an anti-DNA Ab (22), is an established model for the regulation of DNA-reactive B cells (20, 23–25). The R4A transgenic H chain pairs with a wide array of endogenously expressed L chains to generate Tgpos B cell populations with apparent affinities for dsDNA ranging from undetectable to 10−13 M (3, 23, 25). In untreated R4A BALB/c mice, Tgpos B cells with a low apparent affinity for DNA can survive to immunocompetence, whereas those that exhibit high affinity for DNA are regulated by deletion or anergy (23, 25). Based on our previous studies of E2-treated R4A mice, we have observed that an elevation in E2 blocks deletion of high-affinity DNA-reactive B cells that arise from the naive repertoire, because the vast majority of the light of these B cells are unmutated (3).

Because <1% of the B cells in age-matched, naive nontransgenic BALB/c mice are surface IgG2bpos, Tgpos B cells can be identified with an Ab that recognizes the IgG2b constant region present on the Tg-encoded H chain (3, 20). We have shown that these B cells, despite expressing an IgG H chain express normal maturation markers (Refs. 5, 20, and 24; C. M. Grimaldi, unpublished observations; and this report). As shown in Fig. 1, and in agreement with our previous studies, E2 treatment resulted in expansion of the Tgpos population (3). Tgpos populations of both placebo- and E2-treated mice typically display a range of staining intensities, which may be reflective of Ag-induced down-regulation of the autoreactive BCR (26).

We first examined whether there was any evidence for regulation of autoreactivity in E2-treated mice or whether all tolerance checkpoints were breached. Because we have shown previously that E2 treatment of nontransgenic BALB/c mice expands the transitional T2 population relative to the T1 population (5), we anticipated that autoreactive R4A B cells would normally be regulated at the T1 B cell stage. The marker C1qRp (identified by the AA4.1 Ab) was used to distinguish between the transitional and mature Tgpos B cell pools of placebo and E2-treated mice (Fig. 1a). The Tgpos B cell population of placebo-treated mice had a higher percentage of transitional (AA4.1pos) B cells than the Tgpos population (Fig. 1b), presumably due to their high frequency of autoreactivity. The Tgpos B cell population of E2-treated mice also had a higher percentage of transitional B cells than the Tgpos population, suggesting that there is some regulation of autoreactive B cells even in E2-treated mice. Yet, a significantly higher percentage of Tgpos cells progress to the mature B cell pool (AA4.1neg) in
E2-treated mice than in placebo-treated mice (\( p < 0.002 \)), suggesting that an elevation in E2 can impair the deletion of autoreactive B cells at the transitional stage. Thus, incomplete selection occurs in E2-treated transitional B cells.

The transitional B cell compartment was also assessed using the markers CD21 and HSA (8). Although we had anticipated that selection occurs at the T1/T2 interface, we found an accumulation of Tgpos B cells in the T2 compartment. As the cells matured from the T1 stage to T2 stage, both placebo- and E2-treated mice showed no significant decrease in the percentage of Tgpos B cells (Table I). This suggests that autoreactive B cells that bind DNA were, therefore, not arrested at the transitional T1 stage, but can mature to the T2 compartment; in placebo-treated mice they may be subject to negative selection as T2 cells.

An increased frequency of DNA-reactive B cells is present in the repertoire of R4A BALB/c mice

Affinity for autoantigen is an important parameter dictating whether an autoreactive B cell will be eliminated or ignored, because it is a critical determinant of BCR signaling strength. Because we have previously determined the relative affinities for some V\( \kappa \)-J\( \kappa \) genes that generate either high- or low-affinity anti-dsDNA B cells (3, 23, 25), we reasoned that we could determine how maturation and selection of DNA-reactive B cells occurs based on the frequencies of particular V\( \kappa \)-J\( \kappa \) rearrangements. Single-cell PCR was used to identify the L chains of Tgpos B cells. For these studies, transitional B cells were identified as CD19pos/IgG2bpos/HSA\(_{\text{high}}\)/CD21intneg and mature as CD19pos/IgG2bpos/HSA\(_{\text{int}}\)/CD21int. Although we cannot rule out the possibility that some of the CD19pos/IgG2bpos/HSA\(_{\text{high}}\)/CD21intneg cells may include marginal zone B cells, we have shown previously that marginal zone B cells express detectably lower levels of HSA than T2 B cells (Ref. 5; C. M. Grimaldi, unpublished observations). Single-cell PCR was performed using primers that amplify all V\( \kappa \) and J\( \kappa \) family members with no known V\( \kappa \) bias (Ref. 21; V. Jeiganathan, unpublished observations).

The L chain sequences for transitional or mature cells isolated from three placebo- and E2-treated mice were determined. Of the 23 different V\( \kappa \)-J\( \kappa \) combinations identified in this study (data not shown), nine were previously determined to exhibit binding to DNA in combination with the R4A H chain (3, 23, 25) and are summarized in Table II. Tgpos B cells that use the V\( \kappa \)1A-J\( \kappa \)1, V\( \kappa \)\( \kappa \)1A-J\( \kappa \)4, and V\( \kappa \)\( \kappa \)1A-J\( \kappa \)5 L chains generate high-affinity anti-dsDNA Abs with apparent affinities of 10\(^{-8}\) to 10\(^{-9}\) M (3, 23), whereas the V\( \kappa \)\( \kappa \)1A-J\( \kappa \)5, V\( \kappa \)\( \kappa \)21-J\( \kappa \)1, and V\( \kappa \)\( \kappa \)21-J\( \kappa \)2 L chains generate low-affinity anti-DNA Abs with apparent affinities of 10\(^{-6}\) to 10\(^{-7}\) M (25). We compared the frequency of cells in placebo- and E2-treated mice that used V\( \kappa \)\( \kappa \)1A-J\( \kappa \)1, V\( \kappa \)\( \kappa \)1A-J\( \kappa \)4, and V\( \kappa \)\( \kappa \)10-J\( \kappa \)5 or V\( \kappa \)\( \kappa \)1A-J\( \kappa \)5, V\( \kappa \)\( \kappa \)21-J\( \kappa \)1, and V\( \kappa \)\( \kappa \)21-J\( \kappa \)2 L chains, because it is reasonable to speculate that cells that bear these L chains are representative of high- and low-affinity DNA-reactive B cells. The total frequency of Tgpos splenic B cells that use these L chains, and therefore produce anti-DNA Ab, was significantly higher in E2-treated mice than in placebo-treated mice (Table III), confirming

### Table I. Distribution of Tgpos and Tgneg B cells in E2-treated R4A mice

<table>
<thead>
<tr>
<th>Placebo</th>
<th>E2</th>
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<tr>
<td>Tgpos</td>
<td>Tgneg</td>
</tr>
<tr>
<td>T1</td>
<td>10.2 ± 5.8</td>
</tr>
<tr>
<td>T2</td>
<td>7.0 ± 3.1</td>
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</table>

\( ^{a} \) Gates were set on Tgpos (CD19pos/IgG2bpos) or Tgneg (CD19pos/IgG2bpos) cells, and the percentages of transitional T1 (CD21\(_{\text{int}}\)/HSA\(_{\text{high}}\)) and T2 (CD21\(_{\text{int}}\)/HSA\(_{\text{high}}\)) B cell populations were determined. Placebo = 9 mice; E2 = 8 mice.

\( ^{b} \) Data are presented as the percentage of cells belonging to each gate.
our previous observations that E2 treatment breaks tolerance of DNA-reactive B cells.

Regulation of high-affinity DNA-reactive B cells occurs in the transitional B cell compartment

DNA-reactive B cells in the transitional pool represent B cells that survive negative selection at the immature B cell stage in the bone marrow. Both high- and low-affinity anti-DNA B cells were present in the transitional compartment of placebo-treated mice, indicating that DNA-reactive B cells are not exclusively regulated at the immature B cell stage. There was no decrease in the frequency of low-affinity DNA-reactive B cells as they moved from the transitional to mature B cell stages (Table III; 23 vs 22%, respectively), demonstrating that low-affinity DNA-reactive B cells are not normally regulated at this checkpoint. In contrast, high-affinity DNA-reactive B cells were present at a decreased frequency in the mature B cell compartment of placebo-treated mice compared with the transitional compartment (Table III; 9 vs 23%, respectively), indicating that these autoreactive B cells undergo negative selection at the transitional B cell stage.

Escape of high-affinity DNA-reactive B cells in E2-treated R4A mice occurs in the immature and transitional compartments

The percentage of high-affinity DNA-reactive transitional B cells was higher in E2-treated mice than in placebo-treated mice (Table III; 40 vs 23%, respectively; p < 0.04), suggesting that E2 impairs negative selection at the immature B cell checkpoint. Although the analysis of DNA-reactive B cells in placebo-treated (described above) mice suggests that some high-affinity DNA-reactive B cells escape negative selection in the bone marrow and can mature to the transitional B cell stage, we interpret the increase of high-affinity DNA-reactive B cells in E2-treated mice as evidence that at least some of these B cells can normally be eliminated in the bone marrow. The percentage of high-affinity DNA-reactive B cells in E2-treated mice did not decrease as the cells progressed from the transitional to the mature stage, (Table III; 40 vs 38%, respectively), indicating that E2 treatment totally prevented the elimination of high-affinity DNA-reactive cells at the transitional B cell stage. Thus, it appears that an elevation in E2 impairs negative selection of high-affinity DNA-reactive B cells at both immature and transitional stages of development.

E2 treatment impairs the selection of low-affinity DNA-reactive B cells into the mature B cell pool

There was an increase in the frequency of low-affinity DNA-reactive transitional B cells in E2-treated mice compared with placebo-treated mice (Table III; 35 vs 23%, respectively), suggesting that, like high-affinity DNA-reactive B cells, they are also normally regulated at the immature B cell stage in the bone marrow. Because there was no change in the frequency of low-affinity DNA-reactive B cells at the transitional/mature checkpoint in placebo-treated mice, low-affinity anti-DNA B cells are regulated primarily in the bone marrow. Of particular interest, however, was the finding that the frequency of low-affinity DNA-reactive B cells was significantly reduced in the mature pool compared with the transitional pool of E2-treated mice (Table III; 17 vs 35%, respectively; p < 0.03). Because these low-affinity DNA-reactive B cells are not routinely regulated at this stage, we speculate that they must fail to compete for survival factors when high-affinity DNA-reactive B cells are present. The fact that high-affinity DNA-reactive B cells displayed a selective advantage over lower affinity DNA-reactive B cells for entry into the mature B cell pool demonstrates that positive selection as well as negative selection operates on transitional B cells to shape the mature B cell repertoire.

Transitional B cells of E2-treated mice exhibit a longer lifespan

Transitional B cells have a renewal rate of 2–3 days (10, 27). During this time, some transitional B cells are selected to undergo further differentiation into mature B cell subsets, whereas the majority of transitional B cells are fated to undergo programmed cell death, presumably to purge the B cell repertoire of autoreactive B cells that were not eliminated at the immature B cell stage in the bone marrow. To confirm the increased survival of transitional B cells in E2-treated mice, BrdU-labeling studies were performed in nontransgenic E2-treated mice to determine the lifespan of transitional B cells without bias that may be introduced by the Tg-encoded BCR.

BrdU was administered to mice 14 days after treatment with placebo or E2 pellets, because we have determined thus far that this is the earliest time point for E2-induced changes in the percentages of transitional T1 and T2 B cells (C. M. Grimaldi, unpublished observations). BrdU incorporation was studied on day 3, which has been shown to be the optimal time point for the analysis of labeled cells that enter the transitional B cell compartment (10, 27). Following 3 days of incorporation, 45% of HSAhigh transitional splenic B cells from placebo-treated mice were BrdUpos, compared with only 26% of the HSAhigh B cells from E2-treated mice (Fig. 2a). By day 7, 84% of the HSAhigh cells of placebo-treated mice were BrdUpos compared with 67% from E2-treated mice (Fig. 2a). Analysis of the transitional T1 and T2 B cell subsets with the Ab AA4.1 and with the markers CD21 and CD23 revealed similar results. On day 3, 50% of T1 (AA4.1pos/CD21pos/CD23neg) and 35% of T2 (AA4.1pos/CD21pos/CD23pos) B cells of placebo mice were BrdUpos, compared with 39% of T1 and 17% of T2 B cells of E2-treated mice (Fig. 2b). The slower rate of BrdU

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<tr>
<th>Table II. Relative affinities of DNA-reactive B cells</th>
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<tr>
<th>V_J usage</th>
<th>Relative Affinity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>V_1A-Jc1</td>
<td>9.1 \times 10^{-8}</td>
<td>(3, 25)</td>
</tr>
<tr>
<td>V_1A-Jc4</td>
<td>4.5 \times 10^{-9}</td>
<td>(3)</td>
</tr>
<tr>
<td>V_1A-Jc5</td>
<td>4.2 \times 10^{-6}</td>
<td>(25)</td>
</tr>
<tr>
<td>V_10-Jc5</td>
<td>6.6 \times 10^{-8}</td>
<td>(23)</td>
</tr>
<tr>
<td>V_21-Jc1</td>
<td>2.2 \times 10^{-6}</td>
<td>(3)</td>
</tr>
<tr>
<td>V_21-Jc2</td>
<td>9 \times 10^{-6}</td>
<td>(3)</td>
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<tr>
<td>V_45-Jc5</td>
<td>ND</td>
<td>(23)</td>
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<tr>
<td>V_19-Jc5</td>
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<tr>
<td>V_24/25-Jc2</td>
<td>ND</td>
<td>(23)</td>
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| The relative affinity of the R4A H chain paired with each L chain was determined in previous studies by inhibition ELISA (3, 23, 25). |

<table>
<thead>
<tr>
<th>Table III. Analysis of Tgpos anti-DNA V_x-Jc repertoire</th>
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<table>
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<tr>
<th>Placebo</th>
<th>E2</th>
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<tbody>
<tr>
<td>Frequency</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>----</td>
</tr>
<tr>
<td>Translational</td>
<td>Mature</td>
</tr>
<tr>
<td>High affinity</td>
<td>17/74 (23%)</td>
</tr>
<tr>
<td>Low affinity</td>
<td>17/74 (23%)</td>
</tr>
<tr>
<td>Total</td>
<td>34/74 (46%)</td>
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* a Splenocytes were stained with Abs specific for CD19, HSA, and CD21 to discriminate between transitional (CD19high/HSAlow/CD21low/high) and mature (CD19high/ HSAmid/low/CD21high/low) B cells and with IgG2b to identify the Tgpos B cells of R4A mice. The cells were sorted into 96-well microtiter plates, and single-cell PCR was performed using primers to amplify mouse V\_x and Jc genes (21). Data were compiled from the analysis of three mice from each group. The data are presented as the number of cells that use a given V\_x-Jc gene of the total number of cells that were sequenced for each B cell population.
incorporation in T1 and T2 B cells of E2-treated mice is strongly suggestive of a longer lifespan. These data suggest that the transitional B cell subsets of E2-treated mice, in general, have a survival advantage. This is consistent with the observation that autoreactive B cells that would normally be eliminated at this stage are better able to gain entry into the mature B cell pool. Although it is also possible that E2 treatment affects BrdU uptake or the time required to transit to the mature B cell stage, the data suggest that these alternative explanations are not likely because the percentage of mature BrdUpos B cells is similar for both placebo-treated and E2-treated mice. Transitional B cell subsets were determined with Abs specific for CD19, CD21, CD23, and the AA4.1 Ab to identify T1 (CD19pos/CD21int/CD23neg/AA4.1pos) and T2 B cells (CD19pos/CD21int/CD23pos/AA4.1pos). Both transitional T1 and T2 B cell subsets of E2-treated mice displayed a lower percentage of BrdUpos cells compared with placebo-treated mice (p < 0.02 and p < 0.004, respectively).

Enhanced maturation of splenic B cell subsets occurs in E2-treated mice

To delineate more precisely the effects of E2 on B cell maturation, autoreconstitution studies were performed as described by Monroe and colleagues (12). Mice were treated with placebo or E2 pellets for 2 days, and then were exposed to 600 rads of gamma irradiation. Splenocytes were examined 14 days later because it has been demonstrated previously that most B cells in autoreconstituting mice at that time are transitional cells (12, 28). As expected on day 14, ~92% of the spleen cells in placebo-treated mice were AA4.1pos transitional B cells (Fig. 3a and Table IV). In contrast, only 72% of spleen cells in E2-treated mice were AA4.1pos transitional B cells, and there was a concomitant increase in AA4.1neg mature B cells (Fig. 3a and Table IV). The percentages of cells with a marginal zone (CD21pos/CD23neg) and follicular B cell (CD21int/CD23pos) phenotype were significantly increased in E2-treated autoreconstituting mice compared with placebo-treated mice (Fig. 3b and Table III). In placebo-treated mice, the majority of the CD21int/CD23pos cells were AA4.1pos, T3 transitional B cells (Fig. 3b). There was, however, a significantly higher percentage of CD21int/CD23pos/AA4.1neg follicular B cells in E2-treated mice (Fig. 3b). These data confirm that the loss of B cell tolerance in E2-treated mice associates with a higher percentage of transitional B cells that are able to differentiate into mature follicular and marginal zone B cells.

In autoreconstituting placebo-treated mice, there was a 2:1 ratio of T1:T2 B cells, whereas in E2-treated mice, the ratio was ~1:1 (Figs. 3c and Table IV). This result confirms our previous observation that maturation from the T1 to the T2 B cell stage is enhanced in E2-treated BABL/c mice. These data are in agreement with the BrdU studies and with our previous data, suggesting that E2 treatment blocks the elimination of B cells at the T1 to T2 and mature B cell junctions (5). The enhanced maturation from the transitional to mature stages is particularly noteworthy based on the studies of Kincade and colleagues (29 –32), which have demonstrated that an elevation in E2 decreases B cell lymphopoiesis. Although not reported, the E2-induced effects on lymphopoiesis is presumably responsible for the reduced number of transitional B cells that we observe in E2-treated mice (5). Despite this reduction, however, the transitional B cells of E2-treated mice are better able to reach immunocompetence than the B cells of placebo-treated mice.

There is evidence that T cell-derived factors, such as CD40L and IL-4, can rescue transitional T2 B cells, but not transitional T1 B cells, from deletion (18, 19). To assess the role of helper T cells in the peripheral maturation of B cells in placebo- or E2-treated autoreconstituting mice, mice with a targeted deletion in the cd4 gene were analyzed. The percentage of transitional and mature B cells was assessed on day 14 of autoreconstitution as described above (Fig. 4). CD4 T cell-deficient placebo- and E2-treated mice displayed the same distribution of B cell subsets as T cell sufficient mice, suggesting that helper T cells do not play an important role in the enhanced B cell maturation mediated by E2. In addition, the percentage of transitional and mature B cell subsets in placebo and E2-treated autoreconstituting mice given anti-CD4 Ab to deplete CD4pos T cells was similar to those observed in the CD4-deficient mice (data not shown).
higher percentage of AA4.1neg/CD21int/CD23pos follicular B cells. To distinguish between T3 and follicular B cells, which are both CD21int/CD23pos, AA4.1 reactivity was analyzed. E2-treated mice contained a significantly increased (\(p < 0.005\)) percentage of mature CD23pos/CD21hi cells that were AA4.1neg.

Analysis of the transitional AA4.1pos gated cells revealed that cells with a T1 phenotype are significantly decreased in E2-treated mice. There was no significant difference in the percentage of T2 B cells in E2-treated mice.

E2 treatment enhances B cell maturation in autoreconstituting mice. Placebo- and E2-treated mice were irradiated and analyzed on day 14 with Abs specific for CD19, CD21, CD23, and the AA4.1 Ab to identify B cell subsets. Representative dot plots are shown, and the percentages of each subset, the number of mice studied, and the statistical analysis are presented in Table IV. CD19 gated events are shown. \(\ast, p < 0.05\) value was determined by Student’s t test. a, The percentage of mature AA4.1pos B cells is increased in E2-treated autoreconstituting mice. b, Analysis of mature B cell populations revealed that cells with a follicular (FO) and marginal zone (MZ) B cell phenotype are significantly increased. To distinguish between T3 and follicular B cells, which are both CD21hi/CD23hi. AA4.1 reactivity was analyzed. E2-treated mice contained a higher percentage of AA4.1pos/CD21hi/CD23hi follicular B cells. c, Analysis of the transitional AA4.1pos gated cells revealed that cells with a T1 phenotype are significantly decreased in E2-treated mice. There was no significant difference in the percentage of T2 B cells in E2-treated mice.

**Discussion**

Increased levels of E2 have been associated with disease progression in patients with SLE (33–36). Data from several clinical studies support the hypothesis that an elevation in estrogen can exacerbate disease in some patients and raise concerns about the use of estrogenic substances in patients with SLE. A sustained elevation of E2 is clearly sufficient to alter negative selection and can induce a lupus phenotype in nonspontaneous autoimmune mice (3) or can exacerbate a lupus phenotype autoimmune mice (4). In this study, we attempted to discriminate which regulatory checkpoints are abolished by E2.

The data presented in this study demonstrate several novel findings. The first is that high-affinity DNA-reactive B cells are normally regulated at both the immature and the transitional stage, whereas low-affinity DNA-reactive B cells are normally regulated only at the immature B cell stage. To our knowledge, this is the first study to demonstrate the elimination of DNA-reactive B cells in both the bone marrow and the spleen. E2 is also the first soluble factor to be identified that is able to rescue high-affinity autoreactive B cells at the transitional B cell stage. Although administration of soluble BAFF (B cell-activating factor belonging to the TNF family) was found to rescue HEL-reactive transitional B cells in HEL-expressing mice and promote their development to the mature B cell stage, BAFF could only rescue lower affinity HEL-reactive B cells when competitor B cells were also present (14).

Other studies of anti-DNA transgenic mice, such as D42, 3H9, and 56R, have demonstrated that these cells are regulated by either receptor editing or deletion in the bone marrow of nonautoimmune mice (37–39). In contrast, our results show that in nonautoimmune mice, high-affinity DNA-reactive B cells are not exclusively eliminated in the bone marrow, but are subject to selection at the transitional B cell stage also. Studies of the 2-12H Tg mice revealed that the majority of the splenic Tgpos Smith antigen-reactive B cells exhibit an immature phenotype. Like the anti-dsDNA B cells in our model, these cells survive negative selection in the bone marrow and are regulated at the transitional stage before reaching immunocompetence (13). This raises the likelihood that differential regulation of naive autoreactive B cells is, in part, due to antigenic fine specificity and affinity for self Ag, and potential unknown cross-reactivities.

A second novel finding is that low-affinity DNA-reactive B cells are normally regulated in the bone marrow. It has been suggested that the susceptibility to negative selection of immature B cells is maintained as they progress to the T1 stage. Our data suggest that there is a difference in vulnerability to the BCR signaling between

**Table IV. Distribution of B cell subsets in autoreconstituting mice**

| Subset            | Placebo | E2    | p Value 
|-------------------|---------|-------|---------
| Transitionala     | 91.9 ± 4.6 | 71.5 ± 14.4 | <0.005 |
| T1                | 63.8 ± 11.2 | 37.5 ± 11.0 | <0.0005 |
| T2                | 25.4 ± 12.5 | 33.0 ± 12.2 | NS      |
| Marginal zoneb    | 0.3 ± 0.2 | 3.4 ± 2.7 | <0.005 |
| Folliculerc       | 1.9 ± 1.1 | 7.3 ± 3.6 | <0.0005 |

a The percentages of CD19 gated events are shown. Transitional B cells were determined by staining with the AA4.1 Ab, and T1 and T2 B cells were defined as AA4.1pos/CD23pos or AA4.1pos/CD23hi, respectively, and marginal zone and follicular B cells were identified as CD21hi/CD23pos and CD21hi/CD23hi, respectively.

b A two-tailed Student’s t test was performed to determine statistical significance between placebo- and E2-treated mice.

c Placebo = 17 mice; E2 = 14 mice.

d Placebo = 14 mice; E2 = 11 mice.

e The follicular B cell population was determined from the percentage of CD23pos/CD21hi cells that were AA4.1neg.

**Figure 3.** E2 treatment enhances B cell maturation in autoreconstituting mice. Placebo- and E2-treated mice were irradiated and analyzed on day 14 with Abs specific for CD19, CD21, CD23, and the AA4.1 Ab to identify B cell subsets. Representative dot plots are shown, and the percentages of each subset, the number of mice studied, and the statistical analysis are presented in Table IV. CD19 gated events are shown. \(\ast, p < 0.05\) value was determined by Student’s t test. a, The percentage of mature AA4.1pos B cells is increased in E2-treated autoreconstituting mice. b, Analysis of mature B cell populations revealed that cells with a follicular (FO) and marginal zone (MZ) B cell phenotype are significantly increased. To distinguish between T3 and follicular B cells, which are both CD21hi/CD23hi. AA4.1 reactivity was analyzed. E2-treated mice contained a higher percentage of AA4.1pos/CD21hi/CD23hi follicular B cells. c, Analysis of the transitional AA4.1pos gated cells revealed that cells with a T1 phenotype are significantly decreased in E2-treated mice. There was no significant difference in the percentage of T2 B cells in E2-treated mice.

**Figure 4.** CD4pos T cells are not required for enhanced maturation observed in E2-treated mice. Five placebo- and E2-treated CD4-deficient mice were irradiated and analyzed as in Fig. 3. \(\ast, p < 0.05\) value determined by Student’s t test. Follicular and marginal zone B cells were significantly increased \((p < 0.01 \text{ and } p < 0.02, \text{ respectively})\), and transitional T1 and T2 B cells were significantly reduced \((p < 0.03 \text{ and } p < 0.02, \text{ respectively})\) in E2-treated mice.
the two stages, with T1 cells requiring a stronger signal to affect deletion. Alternatively, it may be normally possible to rescue low-affinity autoreactive B cells in the transitional stage. Thus, those that escape negative selection in the bone marrow receive rescue survival signals once they migrate to the spleen.

Finally, our data also demonstrate that both positive and negative selection operate in the transitional B cell pool. When high-affinity DNA-reactive B cells are rescued by E2 treatment, there is a decline in the frequency of low-affinity B cells that mature to immunocompetence. These same low-affinity DNA-reactive B cells are clearly found in the mature B cell compartment of untreated mice (Ref. 25 and this study). High-affinity autoreactive B cells in the transitional pool are spared elimination and may compete most effectively for survival factors such as BAFF if they also receive signaling through the BCR (40, 41). When high-affinity anti-DNA B cells escape apoptosis, they may out-compete low-affinity autoreactive B cells for BAFF or other survival factors and, thus, for entry into the mature B cell pool.

The data presented in this study have important clinical implications for patients with B cell-mediated autoimmune disorders such as SLE. In patients with SLE, the higher affinity autoreactive B cells may not only be a source of pathogenic autoantibody, but may also exclude low-affinity protective autoreactive B cells from entry into the mature B cell pool. In healthy individuals, low-affinity autoreactive B cells are believed to be an important source of Ab to facilitate the clearance and lack of immunogenicity of apoptotic debris (42, 43). In addition, low-affinity autoreactive B cells are thought to be precursors to protective antimicrobial B cells (43). The loss of these cells may mediate the enhanced susceptibility to infectious diseases that has been observed in patients with SLE (44, 45).

How E2 alters selection is only partly understood. We have shown that E2 treatment results in the increased expression of molecules Src homology phosphatase-1 and CD22 (46), which are known to regulate the strength of BCR signaling. It also appears that an elevation in E2 increases levels of soluble BAFF (V. Jeganathan and B. Diamond, unpublished data), which has been shown to rescue transitional B cells from deletion (14, 40, 47). It will be important to identify more extensively the pathways regulated by E2 that impair negative selection of autoreactive B cells, because these may represent important therapeutic targets in SLE.

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Disclosures
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