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14. ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease that occurs preferentially in women. In murine models of SLE, it is clear that increased or sustained high physiologic levels of estradiol can accelerate onset of disease and exacerbate disease severity. We have shown that estradiol alters B cell maturation in vivo but does so in a genetically restricted fashion. We have also shown that estradiol can act directly on B cells to alter B cell receptor (BCR) signalling strength. This proposal is to understand which estrogen receptors mediate the effects of estradiol on B cell survival, maturation and activation in order to assess whether hormonal manipulation has a potential therapeutic role in SLE. The proposal is further designed to ask why estradiol affects B cell function in mice of one genetic background but not another.

15. SUBJECT TERMS
Systemic Lupus, Estrogen, BCR Signaling, B Cell Maturation, B Cell Selection

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Introduction:

Progress has been made in all aims of the proposal and two manuscripts are being prepared for submission to journals

Body:

1) Determine which estrogen receptor is responsible for estrogen-induced alterations in BCR signaling.

We have demonstrated that ERα is responsible for the reduction in transitional B cells in estradiol-treated mice in studies employing ERα or ERβ deficient mice and have confirmed this using specific agonists in wildtype mice (Fig 1a & 1b). Similarly, ERα is responsible for an expansion of marginal zone B cells (Fig 2a and Fig 2b). Neither ERα nor ERβ alone can effect a reduction in the strength of BCR signaling (Fig 3a and 3b). In wildtype mice, estradiol causes a reduction in calcium flux mediated by cross-linking of membrane Ig in immature, but not mature B cells. In $ER\alpha^{-/-}$ mice which express only ERβ there is no comparable reduction in BCR signaling strength. Similarly, in $ER\beta^{-/-}$ mice which express only ERα there is no estrogen-induced alteration in BCR signaling strength. We hypothesize that these observations signify that ERα/β heterodimers are critical to the reduced BCR signal. It has been shown that ER heterodimers can alter gene expression in a unique fashion.

We had previously hypothesized that the alteration in BCR signaling strength was a consequence of enhanced expression of CD22, an inhibitory regulator of the BCR. We now know this conjecture is incorrect as estrogen causes an upregulation of CD22 in $ER\alpha^{-/-}$ and $ER\beta^{-/-}$ mice (Fig 4a) but there is no associated estrogen-induced reduction of BCR signalling in these mice. We have addressed this question data using ERα or ERβ specific agonists in wildtype mice. Only the ERα agonist appears to enhance CD22 expression (Fig 4b). We believe the discrepancy between the analysis of genetically manipulated mice given estradiol and wildtype mice given specific ER agonists may reflect an upregulation of ERβ in $ER\alpha^{-/-}$ mice. We also believe that the reason for the lack of a diminished BCR signal may be that more than CD22 upregulation is required, or, more interestingly, estrogen appears to down-regulate the glycosyl transferase that produces the CD22 ligand. We are exploring this possibility.

2) Analyze B cell maturation and selection in placebo or estrogen-treated C57Bl/6 mice.

We previously demonstrated that estradiol abrogate B cell tolerance in BALB/c mice but not C57Bl/6 mice transgenic for the R4A-γ2b heavy chain. In order to understand this disparity in the response to estradiol in these two strains, we first analyzed B cell maturation during continuous exposure to 75 – 100pg/ml of estradiol in both strains. In each strain, there was a decrease in B cell lymphopoiesis with a concomitant reversal in the ratio of splenic transitional (T) 1 cells to T2 cells. Furthermore, there was an expansion of marginal zone B cells and of transgene-expressing B cell in each strain. We further detected an estradiol-induced

upregulation of CD22 in B cells in each strain. The critical difference between the strains was that there was no estradiol-mediated diminution in the strength of B cell receptor signalling in

C57Bl/6 mice while a diminution in the strength of BCR signalling was seen in transitional B cells from estradiol-treated BALB/c mice. Furthermore, we detected an increase in RAG expression in B cell of estradiol-treated C57Bl/6 mice and an increase in lambda light chain expression which we believe to be a consequence of the increased RAG expression.

We have described the effect of 17-β-estradiol, present at a serum concentration of 100 pg/ml, on B cell selection and maturation in BALB/c mice. After exposure to estradiol, BALB/c mice transgenic for the γ2b heavy chain of the R4A anti-DNA antibody display positive selection of high affinity DNA-reactive B cells, negative selection of low affinity (potentially protective) DNA-reactive B cells and an expansion of marginal zone B cells with the DNA-reactive B cells preferentially acquiring a marginal zone phenotype. We have also determined that C57Bl/6 mice transgenic for the same anti-DNA heavy chain fail to break B cell tolerance following exposure to estradiol (Fig 5), however, B cells expressing the R4A transgene were expanded in C57Bl/6 mice as well as in BALB/c mice after estrogen treatment. We proposed the hypothesis that the transgenic autoreactive B cells in BALB/c mice were mostly deleted and anergic while the transgenic autoreactive B cells in C57Bl/6 mice underwent receptor editing. This hypothesis suggests that estrogen influences the mechanism of tolerance at the level of negative selection differentially in BALB/c and C57Bl/6 mice and that receptor editing rather than deletion occurs in C57Bl/6 mice and would explain an expansion of transgene-expressing B cells with no increase in serum anti-DNA activity.

Micro-array analysis of gene expression in BALB/c and C57BL6 splenic B cells treated with estrogen or placebo revealed differential expression of Pax5 and Foxp1 transcription factors, which focused our attention to the expression of their regulated genes, RAG1 and RAG2. Estrogen treatment induces a decrease of RAG expression, which affects both mouse strains to the same extent but interestingly the basal level of RAG expression is 2.5 to 3 fold higher in C57Bl/6 mice than in BALB/c mice. These differences in RAG1 and RAG2 levels of expression may underlie the differential ability to undergo receptor editing in C57Bl/6 mice compared to BALB/c mice. Consistent with this hypothesis, we observe more lambda light chain associated with the transgenic heavy chain in C57Bl/6 than in BALB/c mice.

Negative selection is known to occur at an immature stage of B cell development and to depend on BCR engagement. We observed that estrogen treatment induces a diminished calcium flux in BALB/c but not C57Bl/6 mice (Fig 6) and a resistance of immature B cells to undergo apoptosis triggered by BCR engagement. This anti-apoptotic effect is higher in BALB/c mice than in C57Bl/6 mice (Fig 7). It could be due to a direct action of estrogen on B cells through the estrogen receptors ERα and/or ERβ, which are both expressed in B cells at the transitional T1 and T2 stage.

We propose to continue to analyze functionally and by gene expression the differences in the B cell response to estrogen in BALB/c and C57Bl/6 mice in order to determine the molecular players in the estrogen response in BALB/c mice. This study could provide insight into the heterogeneity of lupus patients in their response to change in estrogen levels and might identify new therapeutic targets in SLE.

We analyzed calcium flux triggered by BCR engagement in mature and transitional B cells of estrogen and placebo treated BALB/c and C57BL6 mice observed a specific decrease of calcium spike for transitional T2 B cells of BALB/c mice treated with estrogen. As the expression level of membrane IgM (BCR) is not influenced by estrogen treatment, these data suggest that estrogen causes a strain-specific desensitizing of the downstream BCR signaling events. As we believe that these differences are B cell autonomous, we will perform *"in vitro"* experiments with transitional B cells of BALB/c and C57BL6 mice treated for 6 hours with 17-β-estradiol (10^{-8} M) or with the estrogen inhibitor ICI 182,780 (10^{-7} M).

These data are of interest for several reasons. First, we found that C57Bl/6 mice, given estradiol, demonstrated an expansion of marginal zone B cells in the absence of altered BCR signaling strength. This is strikingly similar to what we observed in ERβ$^{-/-}$ BALB/c mice given estradiol, suggesting perhaps that C57Bl/6 mice have an increased expression of ERα homodimers. We believe the reason there is no abrogation of B cell tolerance in C57Bl/6 mice is that the upregulation of RAG that we detect in C57Bl/6 but not BALB/c mice permits receptor editing to maintain tolerance. We are currently performing an analysis of the light chains associated with the R4A transgene in both C57Bl/6 and BALB/c mice to determine if we can detect the molecular footprint of receptor editing, an increased expression of lambda light chains associating with the R4A heavy chain in this analysis.

3) Determine the genetic basis for an estrogen unresponsive B cell compartment in C57Bl/6 mice. We have, as described above shown that in many ways C57Bl/6 B cells are susceptible to estradiol. We have begun analyzing this in 2 ways. We have as proposes in our application, begun an analysis of F1 mice backcrossed to BALB/c mice to identify BALB/c loci that are responsible for tolerance abrogation. We have treated approximately 90 mice with estradiol and are hoping to obtain at least 40 mice with a clear serologic phenotype for genetic analysis. We have also performed microarray analyses on C57Bl/6 mice and BALB/c mice treated with either placebo or estradiol. We are currently confirming differential expression of several genes using real time PCR.

Key Research Accomplishments:

- We have demonstrated that the maturational changes in B cells induced by estradiol are mediated through ERα.
- We have demonstrated that ERα engagement also breaks B cell tolerance.
- We have shown that estradiol alters B cell development in C57Bl/6 mice.
- We have further shown that estradiol increases RAG expression in C57Bl/6 mice, suggesting that receptor revision may be an estradiol-mediated tolerance mechanism.

Reportable outcomes:

PhD anticipated September 2008 – Latia Hill

Fellowship Award SLE Foundation - Joel Cohen-Solal

Invitation to speak at 2009 ACR symposium hormones and the immune system – Betty Diamond

Invitation to speak at 2008 Merinoff Symposium on SLE – Christine Grimaldi

Two manuscripts in preparation

Conclusion:

We have now clearly shown that ERα is responsible for the estrogen-induced alterations in B cell maturation. It will, therefore, be important to test ERα antagonists in murine studies of B cell development and in murine models of lupus. This approach to therapy might provide clinical benefit without immunosuppression or intolerable masculinization in women.

The continued studies of BCR signaling and estrogen may identify other process that are critical in lupus pathogenesis and can be modulated by sex hormones. These studies may have implications for many diseases the phenotype of which is altered by hormone exposure.

References: NA

Appendices: NA

Supporting Data: See following pages

Fig. 1a

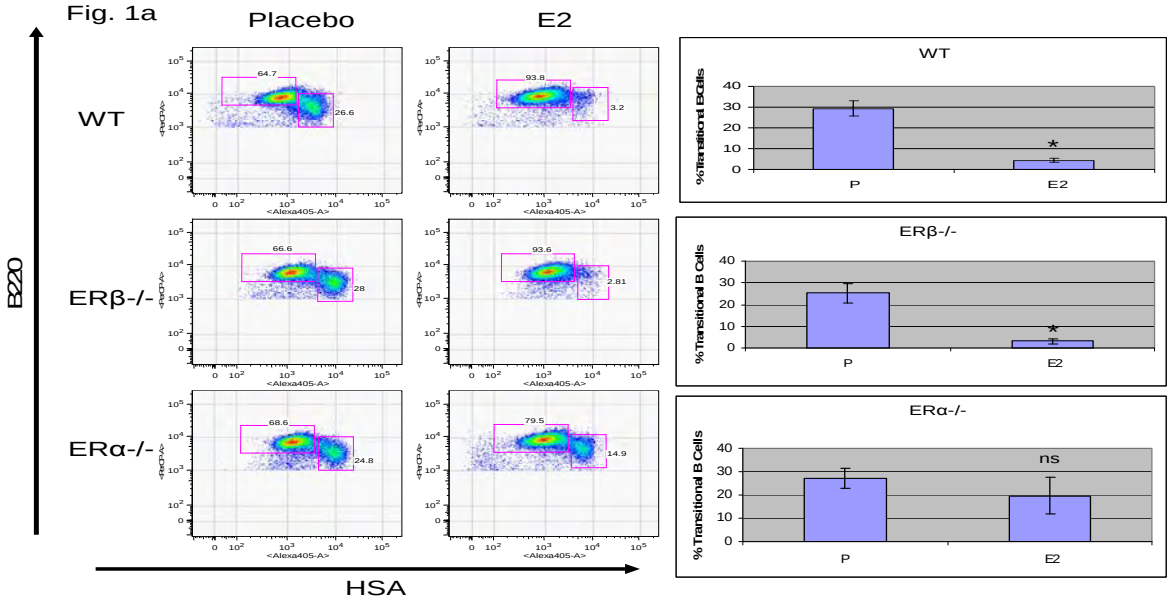


Fig. 1b

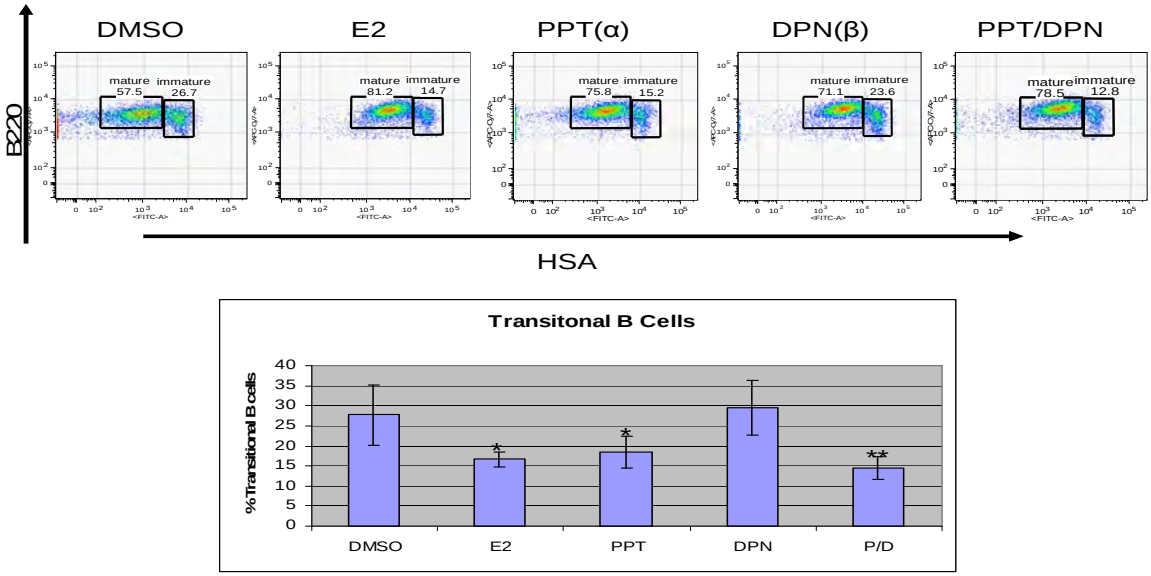


Figure 1: Maturation of B cells

a) WT, ERα $^{-/-}$ and ERβ$^{-/-}$ were implanted with placebo (P) or estradiol (E2) pellets and splenic B cell subsets were analyzed after 2-3 weeks. HSA^{hi} B220^{+} cells were identified as transitional B cells. B) WT mice were given daily injections of DMSO (vehicle), E2, PPT, an ERα agonist) DPN (an ERβ agonist) or PPT and DPN. After 2-3 weeks splenic B cells were analyzed. HSA^{hi} B220^{+} cells were identified as transitional B cells.

Fig. 2a

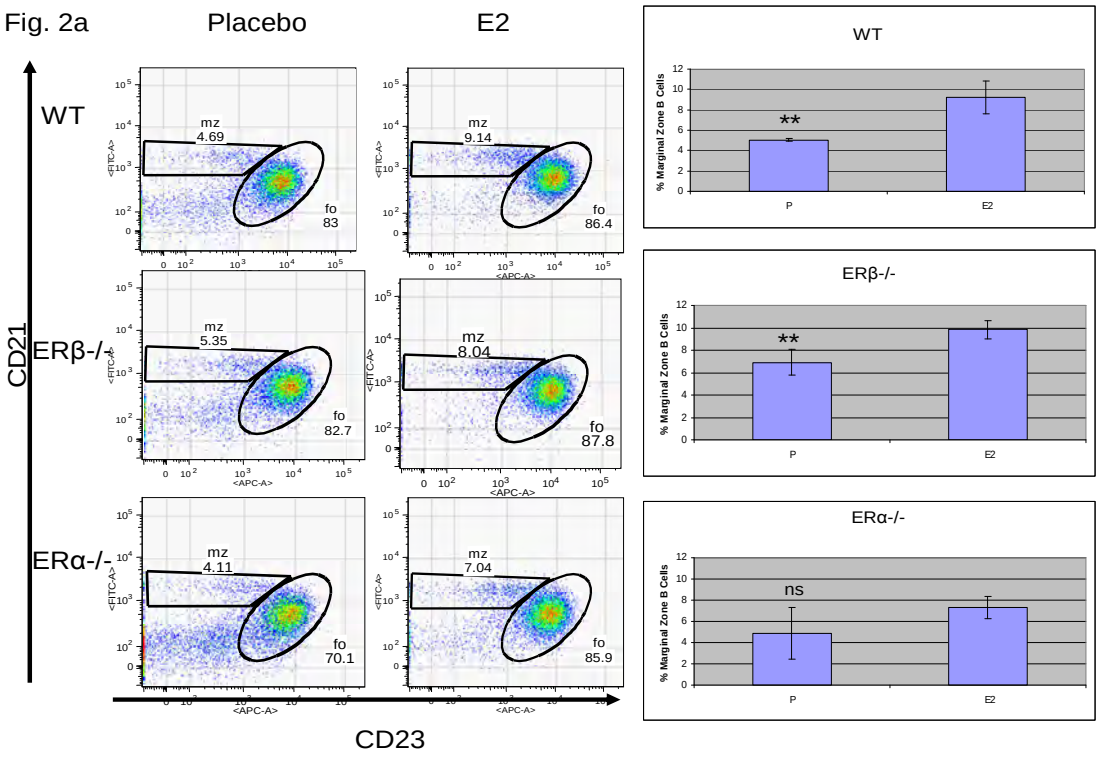


Fig. 2b

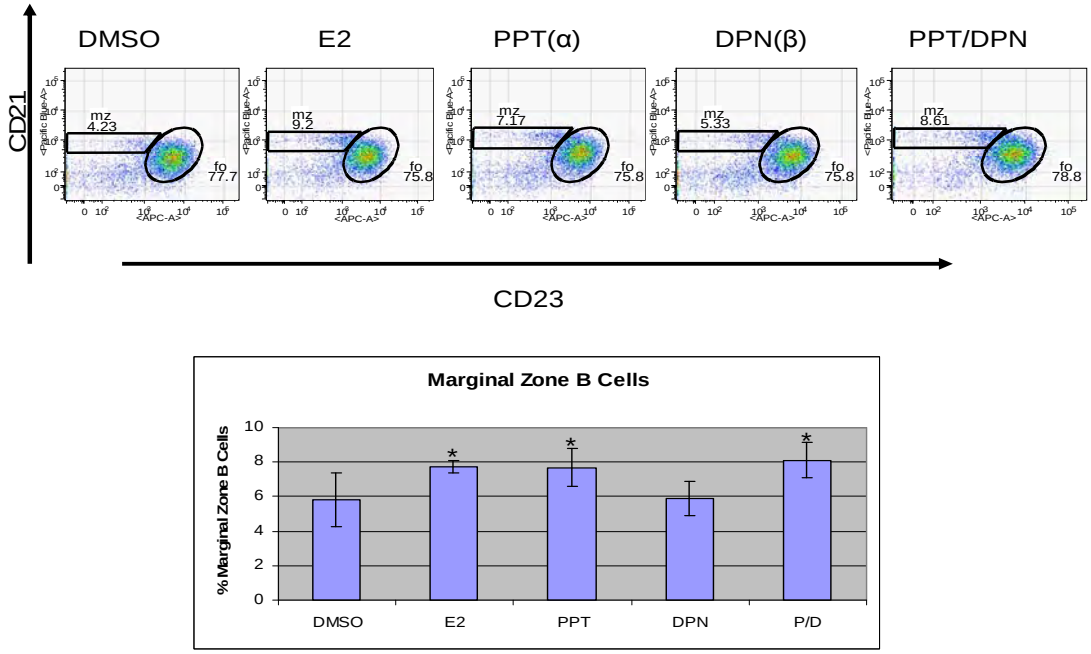


Figure 2: Marginal zone B cells
a) WT ERα$^{-/-}$ and ERβ$^{-/-}$ were treated as in Fig 1a. B cells were gated on the AA4^{-} population. CD21^{hi}, CD23^{lo} cells were identified as MZ B cells. b) WT mice were treated as in Fig1b. Cells were analyzed as in Fig 2a.

Fig. 3a

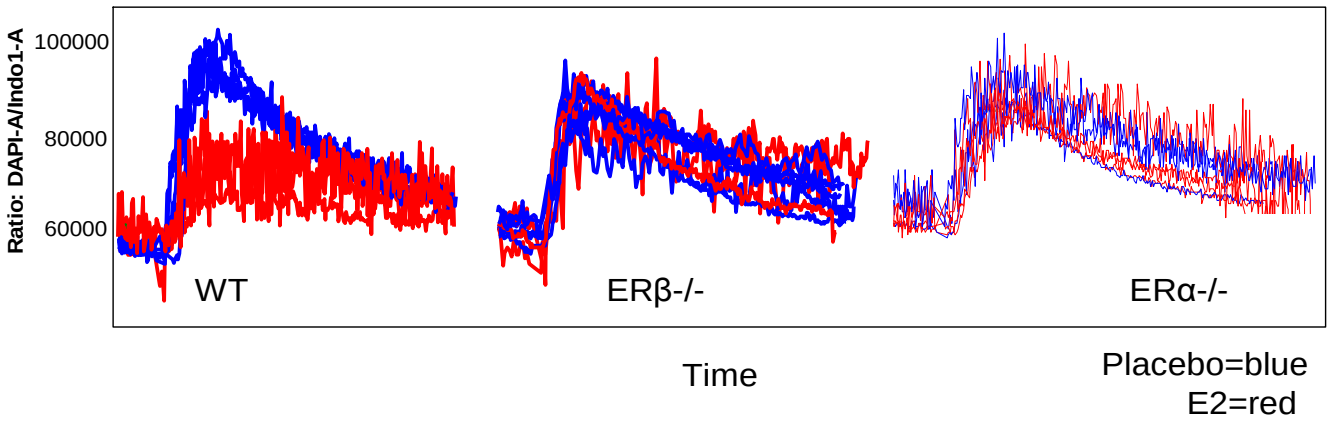


Fig 3b

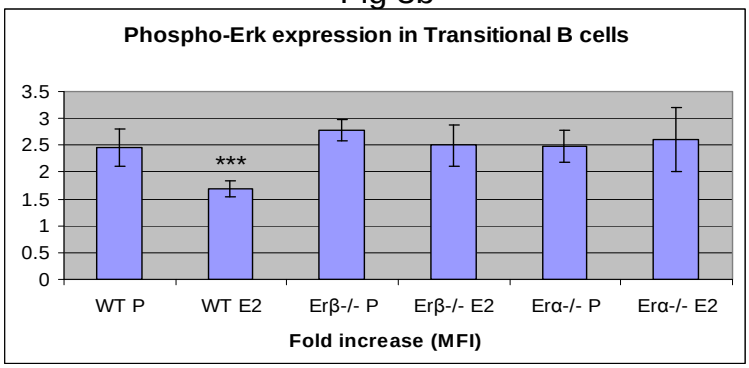


Figure 3: B cell receptor signalling

a) Mice were treated as in Fig 1a and $B220^{+}$ HSA^{hi} cells were analyzed for calcium flux after exposure to Fab^{1}_{2} anti-IgM. b) p-Erk was analyzed by intracellular staining by flow cytometry.

Fig. 4a

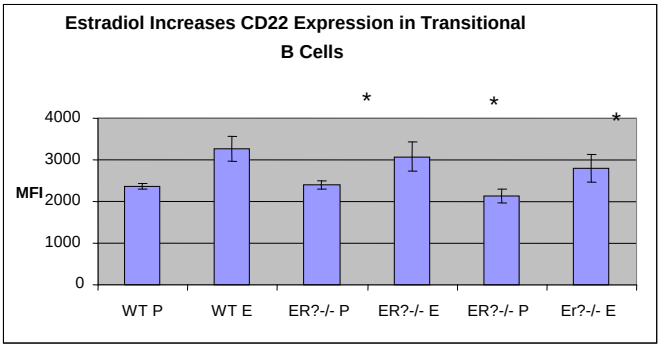


Fig. 4b

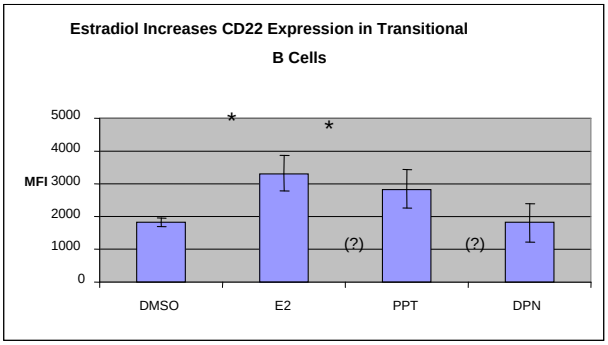
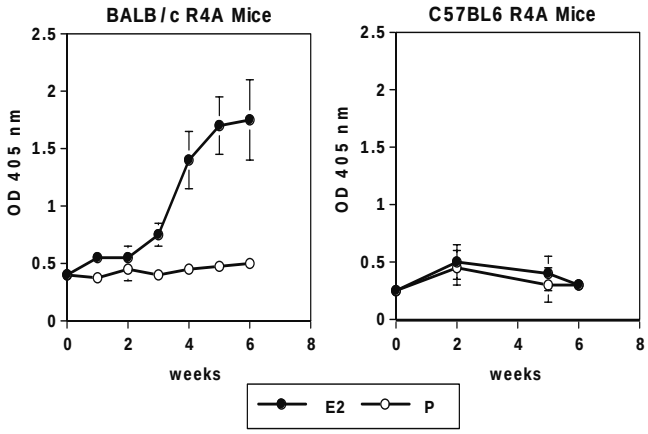


Figure 4: CD22 expression
a) CD22 was assessed by flow cytometry on B220^{+}, HSA^{hi} B cells of mice treated as in Fig 1a.
b) CD22 was assessed by flow cytometry on B220^{+}, HSA^{hi} B cells B cells from mice treated as in Fig 1b.



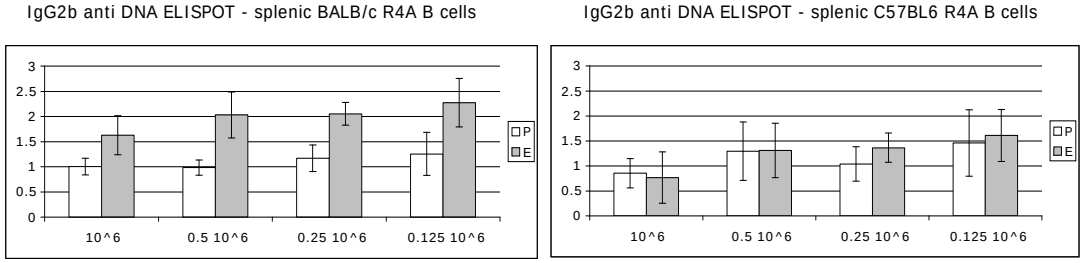


Figure 5 : A-ELISA for the detection of IgG2b anti DNA Antibodies
ovariectomized BALB/c R4A versus C57BL6 R4A mice were treated either by placebo or estradiol pellets for 6 weeks and bled repeatedly. Sera have been tested for IgG2b anti DNA auto-antibodies.

B-ELISPOT for the detection of splenic B cells secreting IgG2b anti DNA Antibodies
splenic B cells from ovariectomized BALB/c (A) versus C57BL6 (B) mice treated with placebo (P) or estradiol (E) pellets for 6 weeks have been tested for their ability to secrete anti-DNA autoantibodies encoded by the IgG2b R4A heavy chain transgene

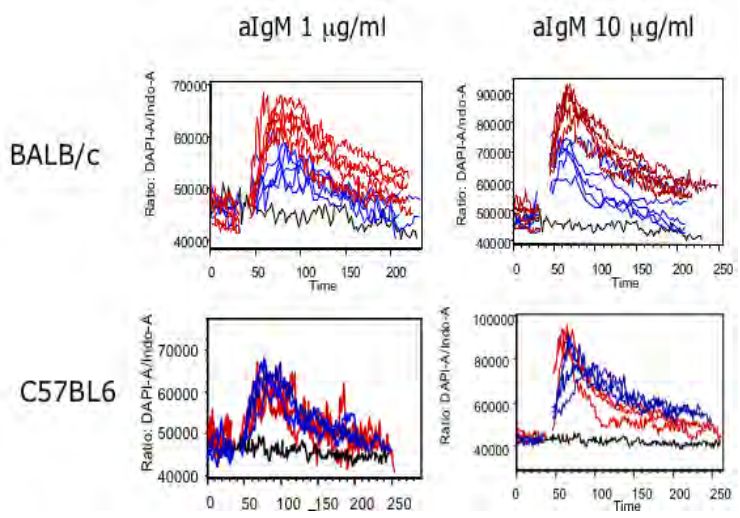


Figure 6: The lower calcium flux induced by estrogen treatment is strain specific
Calcium flux triggered by BCR stimulation with increasing doses of anti IgM GaM Fab'2 on splenic T2 B cells from ovariectomized BALB/c or C57BL6 mice treated 4 weeks with placebo (red) versus estradiol (blue) pellets.
BALB/c T2 (B220pos AA4.1high CD23pos CD21pos) B cells from estradiol treated mice have a lower calcium spike upon non saturating BCR aggregation than B cells from placebo treated mice.

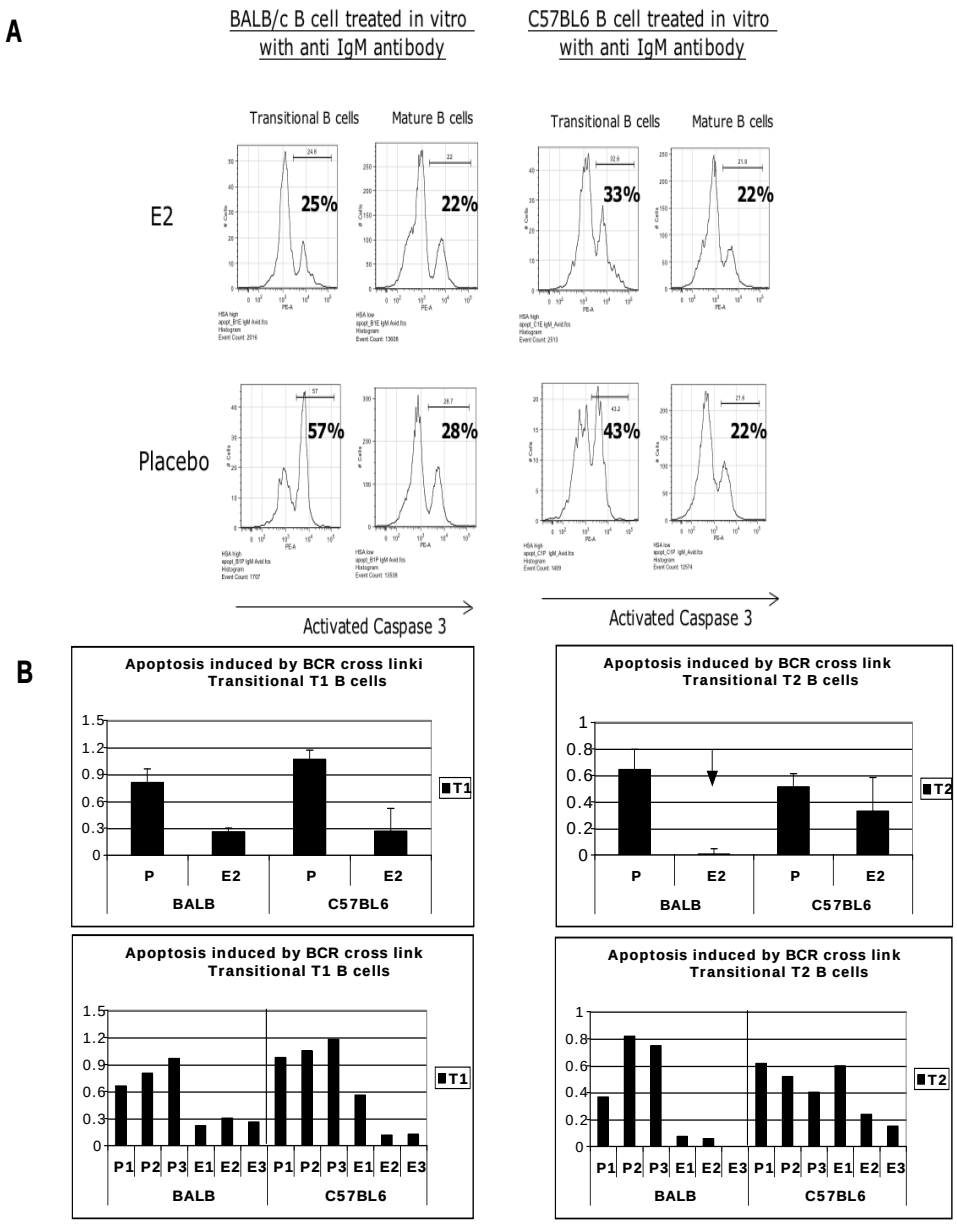


Figure 7: Apoptosis of splenic B cells triggered by BCR aggregation
A-Transitional BALB/c B cells from estradiol treated mice are resistant to apoptosis while transitional C57BL6 B cells are less resistant.
B- Increase in apoptosis in transitional B cell subtypes. T1 B cells of both strains are equally sensitive to apoptosis while BALB/c T2 B cells are specifically resistant.