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Progesterone Inhibits Activation-Induced Deaminase by Binding to the Promoter

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Regulation of activation-induced deaminase (AID), an essential factor in Ig diversification, can alter not only somatic hypermutation and class switch recombination (CSR), but may also influence oncogenesis. AID deaminates cytosine to uracil in the Ig locus, thereby initiating Ig diversification. Unregulated AID can induce oncogenic DNA alterations in Ig and non-Ig loci, leading to mutations, recombination, and translocations. In this study, we demonstrate that AID mRNA production in activated mouse splenic B cells can be reduced by treatment with the sex hormone progesterone. This down-regulation is independent of translation or splicing and is predominantly achieved by inhibiting transcription. During cell treatment we could detect progesterone receptor bound to the AID promoter in proximity to NF-κB binding. Importantly, the progesterone-induced repression was also extended to the protein level of AID and its activity on somatic hypermutation and class switch recombination. The Journal of Immunology, 2009, 183: 1238–1244.

Humoral immune responses triggered by foreign Ags require B cell activation. The activated B cell undergoes Ab affinity maturation, which in higher vertebrates includes somatic hypermutation (SHM) and gene conversion of Ag binding V regions, as well as class switch recombination (CSR) (1); all of these processes require activation-induced deaminase (AID) (2–5). In contrast, loss of AID regulation can lead to B cell pathogenesis, including cancer (6–11) and autoimmunity (12–14). Importantly, if AID is expressed outside the immune system it can induce cancer in various tissues (15–17). As the regulation of AID expression is critical not only for the immune system, a number of factors and pathways have been implicated in activating AID transcription, such as E box proteins (18), NF-κB (19), and Pax5 (20).

The interplay between the endocrine system and the immune system is well documented, including how immunoreactions and immunopathologies can be attributed to the variable levels of sex hormones (21–23). Although there is extensive literature on the effect of estrogen and its potential to modulate various aspects of the humoral immune response, including alterations and inductions of B cell-derived autoimmunity, publications on progesterone are not as extensive, and a number of hypotheses for the function of progesterone have been inferred from conclusions on estrogen research (23). In general, it is thought that estrogen has a stimulatory effect on the humoral immune response, whereas the effect of progesterone is inhibitory. On the molecular level, this could be due to the alterations in cell proliferation, differentiation, or repression of apoptosis (24). Recently, we have been able to demonstrate that AID can be activated by estrogen in immune as well as nonimmune cells (25). The enhanced AID production also led to a concomitant increase in CSR, SHM, and oncogenic translocations (c-Myc/IgH). Together, this described a novel molecular pathway of how estrogen can alter the immune system and induce oncogenic DNA alterations.

In this study, we show that like estrogen, progesterone can directly alter AID mRNA production. Although progesterone and estrogen can be costimulatory (estrogen activates the progesterone receptor (PR) promoter 26) in the immune system, they seem to counteract each other’s activities (23). In stimulated B cells, progesterone reduced AID mRNA, independent of translation or RNA processing. Progesterone treatment induced binding of the PR to the AID promoter, recruiting an inhibitory transcription complex to the AID promoter. The induced repression was also detected at the AID protein level, and in extension on AID-dependent processes, such as SHM and CSR.

Materials and Methods

Cells and tissue

Part of the methods have been described in detail in our previous publication (25). Mouse tissue samples and splenic B cells were derived from 8- to 12-wk-old female unplugged BALB/c mice. During the procedure, RBCs were removed according to the manufacturer’s protocol (Lympholyte M; Cedarlane Laboratories), and mouse splenic B cells were isolated with Mouse B Cell Negative Isolation Kit (Dynal Biotech) according to the manufacturer’s instructions. RAMOS-HS13, a human Burkitt lymphoma cell line, SiHa, and mouse B cells were cultivated in RPMI 1640 plus glutamax medium (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and grown at 37°C with 5% CO₂. Chicken DT40 cells were maintained in RPMI 1640 plus glutamax medium with 10% FCS, 1% chicken serum, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin at 39°C with 10% CO₂. For the removal of hormones, cells were treated for 72 h in hormone-depleted serum (OptiMem Reduced Serum medium; Invitrogen) supplemented with the following: charcoal-stripped FBS (Invitrogen); 50 μM 2-ME; nonessential amino acids (final concentration and Sigma-Aldrich); L-alanine (8.9 g/ml, product no. A4534); L-glutamic acid (14.7 g/ml, product no. G7126); proline (11.5 μg/ml, product no. P5607); and l-serine (10.5 μg/ml, product no. S2600). B cell stimulation was conducted as previously described (25).
using 25 μg/ml LPS (Sigma-Aldrich), 50 ng/ml mouse IL-4 (R&D Systems), 20 ng/ml human TNF-α (R&D Systems), and 2 ng/ml human TGF-β1 (R&D Systems). The concentrations of progesterone (Sigma-Aldrich) are indicated in each experiment illustrated; the final concentration for cycloheximide (Sigma-Aldrich) was 10 μg/ml.

RT-PCR and quantitative real-time PCR

Analysis of AID mRNA and switch circle transcript by quantitative real-time PCR was previously described (25) (see primer sequences in supplemental material). Gene expression was normalized to the expression of GAPDH.

Promoter analysis

Human AID promoter analysis was conducted in SiHa cells as previously described (25) (see primer sequences in supplemental material). Modifications, 24 h after transfection, include cells treated with indicated concentrations of progesterone or TNF-α for 4 h and analyzed thereafter for luciferase signal using Dual Luciferase Reporter Assay System (Promega) on a GloMax luminometer (Promega). The 1 μg of CMV promoter-driven Renilla luciferase expression vector (Promega) was included in all transfections and used as an internal control for transfection efficiency.

Chromatin immunoprecipitation (ChIP)

RAMOS B cells were treated for 72 h in hormone-depleted serum before the 4 h hormone or TNF-α treatment. ChIP procedure was done as described earlier, but using anti-NF-kBp65 Ab (sc-109; Santa Cruz Biotechnology), anti-PR Ab (ab2764; Abcam), or goat anti-mouse lambda control Ab (Ab-960-01; Southern Biotechnology Associates). All Abs were added to the supernatant at a concentration of 2 μg/assay. The released DNA samples were subjected to quantitative real-time PCR, using forward primer CHIP1 (see primer sequences in supplemental material) and reverse primer CHIP2 for region I, forward primer CHIP3 and reverse primer CHIP4 for region II, forward primer CHIP5 and reverse primer CHIP6 for region III, forward primer CHIP7 and reverse primer CHIP8 for region IV, forward primer CHIP9 and reverse primer CHIP10 for region V.

DT40 analysis

The modified DT40 cell line has been previously described (25). A dual peptide epitope tag (3xFLAG-2xTEV-3xMyc) was fused to the last exon of the AID gene locus. Expression of mRNA was monitored by quantitative real-time PCR and protein by quantitative Western blot analysis using the LICOR system.

Mutation analysis

RAMOS mutation analysis and VH and C region cloning and sequencing are described in Pauklin et al. (25). The human CD95/Fas locus was PCR amplified and sequenced from isolated genomic DNA of RAMOS clones. The human CD95/Fas locus was PCR amplified from the pool of clones. A consensus sequence for comparison was derived from the all the clones, our previous publication (25), and the original publication (30).

Results

Progesterone inhibits AID mRNA production

Our previous work indicated that progesterone down-modulated AID mRNA in activated B cells (25). IL-4- and LPS-activated splenic B cells were treated with progesterone, and AID mRNA was analyzed by quantitative real-time PCR. Progesterone was able to inhibit AID transcription in a dose-dependent manner (Fig. 1A), with maximum inhibition of 5-fold occurring with more than 10 nM (well within the physiological range found in blood). To test for a direct effect of progesterone on the AID transcription unit, we treated activated splenic B cells with translation (cycloheximide) and transcription (α-amanitin/actinomycin D) inhibitors. Cycloheximide addition did not inhibit the repression (Fig. 1B), suggesting a more direct effect of progesterone-induced down-modulation of AID mRNA. At the same time, α-amanitin/actinomycin D treatment did inhibit the activity of progesterone (Fig. 1B), indicating that transcription was directly involved. As quantitative real-time PCR is a measure of steady-state levels of mRNA, we also tested whether progesterone could alter the splicing of AID mRNA. Again, progesterone treatment did not significantly alter the RNA splicing (see supplemental Fig. 1) because each intron was repressed to an equal extent as the mature AID mRNA. These data lead us to conclude that progesterone was most likely to act on the promoter of AID and influence AID mRNA production.

PR binds to the AID promoter

By dissecting the AID promoter in a luciferase-based heterologous transcription assay (Fig. 2A), we identified at least one region of the AID promoter that could act as a repressor of transcription after progesterone treatment (Fig. 2B, fragment C). Because our quantitative real-time PCR data (Fig. 1) was dependent on stimulating splenic B cells with LPS or IL-4 (known to stimulate AID mRNA through the NF-κB activation pathway), we treated the reporter cell line with TNF-α, to elicit a general NF-κB activation. This requirement for activation can limit the identification of progesterone response element (PRE) sites in the AID promoter, as we could only detect those PREs that are near an activating site as well. Bioinformatic analysis of the complete AID promoter identified a potential PRE in fragment C near a published NF-κB binding site (19). To demonstrate that during progesterone stimulation PR is recruited to this region of the AID promoter, we performed ChIP with an anti-PR Ab. Using various quantitative real-time PCR analyses of the ChIP-AID promoter (Fig. 3A), a fragment 1.2 kb upstream of the transcription start site, spanning the proposed progesterone response element and NF-κB binding sites, was preferentially PCR amplified during progesterone stimulation (Fig. 3B, fragment III). Using a control Ab we were unable to detect an

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increase in signal (Fig. 3D), indicating that progesterone did not amplify the DNA content in our sample. This observation is also reflected in the lack of amplification of region V of the AID promoter after progesterone treatment.

PR does not compete with NF-κB for binding to the AID promoter

Because progesterone could inhibit the TNF-α-induced activation in the heterologous promoter analysis, we tested whether this repression would manifest itself on the level of transcription factor binding. ChIP analysis of PR and NF-κB from singly and costimulated cells showed TNF-α alone did not enhance PR binding (Fig. 3, A and D, TNF-α), but activating the NF-κB pathway during progesterone treatment enhanced PR binding above that of the progesterone alone (Fig. 3, B and D, progesterone/TNF-α). Because progesterone was able to inhibit activation through the NF-κB pathway, it was possible that the enhanced PR binding after TNF-α treatment was a result of PR reducing NF-κB binding to the promoter. Surprisingly, using ChIP analysis of NF-κB on the AID promoter showed that TNF-α-induced NF-κB binding was not inhibited during progesterone treatment (Fig. 3E), indicating the presence of two transcriptional regulatory complexes on the promoter.
Progesterone inhibits AID protein production, CSR, and SHM

To determine whether the inhibitory activity of progesterone would also affect AID protein production, we tested a modified DT40 cell line (a chicken B cell lymphoma, that constitutively expresses AID and undergoes Ig diversification) (25), in which one of the endogenous AID alleles was tagged with a FLAG-Myc tag (Fig. 4A, AID-FM). Using this cell line, we were able to use quantitative Western blot analysis to monitor the expression of AID protein. Progesterone inhibited the activation of AID mRNA as well as protein after TNF-α stimulation (Fig. 4, B and C, respectively).

FIGURE 3. PR is recruited to the AID promoter. A, Schematic representation of the 5’ end of the genomic AID locus. The five quantitative real-time PCR products tested in the ChIP are indicated (I–V). B, ChIP of 4 h untreated, TNF-α-, progesterone-, and TNF-α/progesterone-treated RAMOS cells. AID mRNA expression is down-regulated in RAMOS cells after a 4 h of progesterone treatment (see supplemental Fig. 4A). Chromatin fragments are immunoprecipitated with anti-PR Ab, and the decrosslinked DNA is subject to quantitative real-time PCR. Each PCR fragment is 150 bp in length and the center position (in bp upstream of the start site) of each of the fragments is indicated next to the color key. Results are normalized to input DNA. Expression levels are in relation to expression levels of AID in untreated DMSO-treated cells. Results and SD are derived from three independent experiments.

FIGURE 4. The effects of progesterone on AID protein in DT40. A, Schematic representation of the AID locus in DT40 after targeting exon 5 (last) with the peptide tag FLAG-Myc (3xFLAG-2xTEV-3xMyc) generating an AID-FM allele (25). B, Progesterone inhibits AID mRNA expression in DT40. AID-FM-tagged DT40 cells were treated with no or 200 nM progesterone and TNF-α, lysed, and analyzed for AID mRNA expression at various time points (three samples for each treatment). Analysis of mRNA by fold inhibition was based on setting the LPS or IL-4 DMSO-stimulated AID mRNA expression to 1 as in Fig. 1. C, Progesterone inhibits AID-FM fusion protein expression. Tagged DT40 cells were treated with no or 200 nM progesterone and TNF-α, lysed, and analyzed for AID mRNA expression at various time points as in B, but lysates were analyzed for FLAG epitope expression using quantitative Western blot analysis (pooled analysis from three samples per treatment). Western blots (top) and quantitation of bands (bottom) are shown. Time line of treatments is given at bottom.
As indicated by quantitating the Western blot (Fig. 4C), progesterone was able to inhibit AID protein formation by over 3-fold. For the analysis we pooled the cell lysates of the three independent samples from the mRNA analysis to obtain an “average” Western blot of AID expression.

As there was a direct effect on AID protein, we also determined whether progesterone could inhibit the function of AID in CSR using activated mouse splenic B cells. Regardless of the subclass of Ig being assayed for (Fig. 5A), progesterone was able to inhibit the switching activity (as measured by switch circle formation and quantitative real-time PCR) by more than 2.5-fold, suggesting that the effect of progesterone-induced AID repression can be directly responsible for altering CSR. Analysis of CSR by surface Ig expression after 4 days in the presence of progesterone (as measured by FACS analysis) showed a similar trend, in that reduced AID expression could lead to reduced CSR (Fig. 5, B and C) (see supplemental Fig. 3). The difference between the FACS and switch circle analysis could be due to the additional effects progesterone may have on the overall process of switching (cell replication, protein expression, cofactor expression, or others). Therefore, switch circle analysis is likely to be a more direct readout of AID activity on CSR, which was also highlighted in our previous publication (25).

FIGURE 5. Progesterone inhibits CSR and SHM. A, Progesterone inhibits isotype switching. Isolated mouse splenic B cells were stimulated for 48 h with LPS plus IL-4 for switching to IgG1 and IgE, LPS plus TGF-β for switching to IgA, and LPS plus IFN-γ for switching to IgG3. Indicated amounts of progesterone were added to the cells together with cytokines. Relative efficiency of class switching was determined by detecting circle transcripts with quantitative real-time PCR. Data are normalized to the control treatment with DMSO from three independent experiments and error bars indicate SD. B and C, FACS analysis of isotype switching to IgA. Cells were treated as in A (for IgA switching) and analyzed for surface Ig production after 4 days. The percentage of switching was determined from DMSO-treated (B) and progesterone-treated (C) samples that were B220- and IgA-positive. D, Progesterone decreases the mutation frequency. Using a cell culture surface IgM reversion assay (25, 30), human RAMOS cells were continuously cultured (see supplemental Fig. 4B), followed by sequencing of 341 bp from the rearranged V_H (VH186) or 750 bp from human CD95/Fas locus. Mouse splenic B cells were treated for 6 days with LPS and 200 nM progesterone. Genomic DNA was amplified and 750 bp of the Sy3 region sequenced (see supplemental Fig. 4). Mutation frequency is normalized to the control treatments with DMSO.

The Burkitt lymphoma cell line RAMOS has been extensively used to study SHM and how AID is targeted to the Ig locus (30, 31). Experiments in RAMOS showed that AID expression was responsive to progesterone treatment (see supplemental Fig. 4A). We therefore cultured single cell RAMOS clones (sorted surface IgM negative) continuously for 3 wk (~20 doublings) in the presence of progesterone and monitored the surface expression of IgM of each clone (reversion to surface IgM positive is an indication for SHM (see supplemental Fig. 4B)), as well as the change in the mutation frequency (Fig. 5D). Analysis of over 25 clones from each treatment indicated that progesterone could reduce surface IgM production by more than 2.5-fold. Mutation analysis of the rearranged V_H region of RAMOS was used to verify that this effect was also seen at the sequence level. As shown in Fig. 5D, progesterone did inhibit the mutation frequency of the V_H loci. We did not observe a change in mutation spectrum upon progesterone treatment (see supplemental Fig. 4D), and as RAMOS almost exclusively uses phase 1a for diversification (transitions at dC:dG (1)), the data indicates that the reduction is predominantly due to a loss of AID activity. Although RAMOS has provided a number of important breakthroughs in the field of SHM, we also wanted to determine whether SHM in primary B cells could be altered with progesterone. Sequence analysis of ex vivo-treated BALB/c splenic B cells (32) showed that reducing AID production with progesterone has a direct impact on the mutation frequency of a switch region, without affecting the mutation spectrum (Fig. 5D) (and see supplemental Fig. 4F). In the past, there have been reports of SHM (and in extension AID) being active outside the Ig locus (8–10, 33). To test the effect of progesterone on nonphysiological SHM targets, we performed sequence analysis of the tumor suppressor CD95/Fas gene from progesterone-treated RAMOS cultures (Fig. 5D) (and see supplemental Fig. 4E). As with the mutation frequency of the Ig locus, CD95 mutation frequency showed a marked decrease after progesterone treatment.

Discussion
Identifying key molecular mechanisms that link the activity of hormones with that of the humoral immune system is important for understanding the physiological aspects of immunity, as well as identifying targets for treating immune/hormone pathology. Systemic lupus erythematosus is a B cell-derived autoimmunity that is almost exclusively found in women (90%) (21). The strong sex bias has been linked to the levels of hormones, such as estrogen. Furthermore, it has been shown that in mouse models of systemic lupus erythematosus, altering the levels of estrogen can alter the severity of the disease (34, 35). Our data on the ability of estrogen to induce AID protein (25), and the previously published observation that levels of AID protein can influence the severity of autoimmunity (13, 14), led us to investigate the molecular mechanism by which another sex hormone, progesterone, could alter AID activity.
The current data and our work on estrogen (25) indicated that AID expression could be regulated in an antithetical fashion by the sex hormones estrogen and progesterone. This effect is analogous to other classically hormonally regulated genes, such as PR 36, and suggests that AID expression is part of physiological hormonal homeostasis. Furthermore, the identified progesterone response element is in close proximity to one of our currently identified estrogen receptor binding sites (estrogen response element) (25), and the stimulatory effect of estrogen is evolutionarily conserved among the other DNA deaminases (37), such as APOBEC3B, APOBEC3F, and APOBEC3G.

Our work also indicated, that different regulatory pathways, such as hormon and NF-xB, interact leading to altered AID expression. The ChIP data in Fig. 3 suggested that the progesterone and TNF-alpha pathways act through different transcription factor binding, and may even slightly enhance each other for chromatin accessibility. A possible interdependence was also demonstrated when we looked at the effect of progesterone on AID expression in DT40 (a chicken B cell line constitutively expressing AID). Although progesterone treatment of TNF-alpha-activated DT40 cells led to a 2.2-fold reduction in AID mRNA expression (Fig. 4), adding progesterone alone to the constitutively expressed AID in DT40 only reduced AID expression 1.5-fold (see supplemental Fig. 2). This observation indicated that progesterone could inhibit AID expression more substantially, if other costimulatory signals (such as TNF-alpha) were present at the same time. The presence of both transcription complexes on the AID promoter during the progesterone induced repression could indicate 1) that the progesterone complex disrupts the activation potential of NF-xB without affecting the binding, 2) the presence of an inactivating modification on NF-xB, 3) the recruitment of a dominant repressive transcription complex by PR, or 4) PR can act through a yet novel region of the promoter. Recent work from us (25) and other laboratories have shown a direct link between AID levels and downstream function (38–40). This work was based on either estrogen treatment (25), inactivation of a regulatory miRNA (38), removal of the miRNA binding site (40), or production of heterozygous AID mice (39). Our current data are in line with those observations, indicating that natural hormones can have the same effect on SHM and CSR as in vitro experiments; S. Pauklin executed the experiments.

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Although, the role for estrogen in tumor formation is well established, understanding how progesterone causes or inhibits oncogenesis is much less studied. Epidemiological analysis seems to indicate that cotreatment of estrogen and progesterin (a synthetic progesterone like steroid) during hormone-replacement therapy seems to enhance the risk for breast cancer development (41, 42), yet it is not clear whether natural progesterone has the same effect (43). Our data indicating that progesterone can inhibit AID activity, in combination with the previously mentioned reports that reduced levels of AID can also reduce oncogenic translocations, would indicate that there may be antitumorigenic effects of progesterone, at least for those cancers derived from DNA deaminases. Further work will elucidate which other hormones (natural or synthetic derivatives) can influence the activity of AID.

In conclusion, we demonstrated that AID can be regulated by progesterone at the level of transcription, and that the hormonally induced effect on SHM and CSR is primarily due to the alterations of AID protein expression.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

References

**Primers**

For ChIP:

- **CHIP1:** 5'-GCAGGAAGGCAGTTGGGAA-3'
- **CHIP2:** 5'-GCTCCTCTCTTTTTTAAATTCCCATGCAC-3'
- **CHIP3:** 5'-GACAGGCTGGAGGTGCTCAG-3'
- **CHIP4:** 5'-GACAGGCTGGAGGTGCTCAG-3'
- **CHIP5:** 5'-CCACATGTCATGGGAGGGA-3'
- **CHIP6:** 5'-GTGGTAGGCAAGAGAGAGCTCAC-3'
- **CHIP7:** 5'-TCAGGCATGTCTTTATTAGCAGTGT-3'
- **CHIP8:** 5'-GATGCAATGCTCTAAGCAATGCAG-3'
- **CHIP9:** 5'-GATCTTAGCCTGTTTTCCAAATTCAAGC-3'
- **CHIP10:** 5'-TTTTGCATGAGCCATCTTTTTTAG-3'

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**Suppl. Figure 1: Progesterone does not affect AID pre-mRNA processing.**

(A) Schematic depiction of AID locus. Exons (grey) with corresponding numbers and UTRs (yellow) are indicated as boxes. Red lines with capital letters mark the relative positions of the PCR products (A-G) along the AID locus (1). (B) The effects of progesterone on AID pre-mRNA. Mouse spleen B-cells were treated with LPS/IL4 as in Figure 1, followed by DMSO or progesterone addition for a further 4 h. cDNA was synthesized, followed by analysis of AID pre-mRNA by qRT-PCR. Results are normalized to DMSO-treated cells for each PCR product. Data represents results from three independent experiments and error bars indicate standard deviations from the average. Time-lines of cell treatments are indicated below the graph.

**Suppl. Figure 2: Progesterone inhibits constitutive AID expression in DT40.**

DT40 cells were treated with DMSO or 200 nM of progesterone up to 8 h in the absence of any further stimulation. Cells were lysed at the indicated time points, AID mRNA production monitored with qRT-PCR, normalized to GAPDH, and plotted as in Figure 1 (DMSO set to 1).

**Suppl. Figure 3: Progesterone alters CSR.**

Progesterone reduces CSR at the level of surface Ig expression. Isolated mouse splenic B-cells were stimulated with LPS + IL-4 for switching to IgG1 and LPS + IFN-γ for switching to IgG3, and FACS analysed after 4 days. See materials and methods for details. Percent switching was determined from DMSO (A) & (C) treated cells and progesterone (B) & (D) treated cells that were B220 and Ig (IgG1 - (A) & (B); IgG3 - (C) & (D)) positive.
Suppl. Figure 4: The effect of progesterone on SHM in RAMOS HS13 cells, and splenic B cells.

(A) In RAMOS cells, AID mRNA is inhibited by progesterone treatment. RAMOS cells were treated with 200 nM progesterone for 4 h and AID mRNA analyzed by qRT-PCR as in Figure 1. (B) Hormonal effects on slgM expression in RAMOS. Sorted individual slgM negative cells were grown in the presence of indicated amounts of progesterone for 3 weeks. Clones were analyzed for slgM expression by flow cytometry. Each colored dot represents the relative proportion of slgM positive cells per clone. (C) The data from (B) expressed as fold inhibition on the median surface expression (DMSO set to 1). Cell growth analysis indicated that progesterone only had a marginal influence on cell replication, and results analysed on the number of cell duplications were not different (data not shown). (D & E) Progesterone decreases the mutation frequency in the VH locus and the CD95/Fas locus. RAMOS cells were grown in the presence of indicated amounts of progesterone for 3 weeks, followed by PCR amplification, cloning and sequencing of individual human VH (D) or human CD95/Fas (E) loci. To maximize the number of mutations detected, we chose the 2 µM progesterone treatment. During the 3 week culturing, progesterone was only replenished every 48 hrs, possibly reducing progesterone's effectiveness. Furthermore, the data from surface IgM expression (B & C) indicated a slightly more pronounced effect of progesterone treatment at 2 µM. The number of sequences analyzed (Seq), total base pairs (BP), number of mutations (Mut), mutation frequency per base pair (Mut/bp), overall percentage of mutations at C:G base pairs (% C:G), percentage of transitions (% Ts) and pie charts (mutations per sequence) are indicated for each of the treatments. Statistical significance was tested using a two-tailed unpaired T-test. (F) Progesterone inhibits SHM in the Sγ3 of mouse splenic cells. Mouse splenic cells were treated for 6 d with LPS and 200 nM progesterone. Genomic DNA was amplified and 750 bp of the Sγ3 region sequenced. Table was generated as
indicated in (D). Mutation frequencies are normalized to the control treatments with DMSO. A standard unpaired two-tailed T-test showed a significant difference in mutation frequency in the Sy3 loci of DMSO and progesterone treated spleen cells.

**Suppl. Figure 5. Effect of progesterone treatment on splenic B cells in complete serum.**

Splenic B cells from BALB/c mice were isolated as in Figure 1 without hormone depletion, treated with LPS and progesterone, and analysed for AID expression at the indicated time points with qRT-PCR.
A

Fold Inhibition

12 3 4 5

0 h 24 h 20 h

LPS/IL4

B

Fold Inhibition

(LPS/IL4)

DMSO

Progestrone

Pauklin et al. Suppl. Figure 1
Fold Inhibition

Pauklin et al.  Suppl. Figure 2
Pauklin et al. Suppl. Figure 3
**A**

![Bar chart showing Relative Change](image)

**B**

![Scatter plot showing % surface IgM (+) per clone](image)

**C**

![Bar chart showing Relative sIgM Fold Inhibition](image)

**D**

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*p = 0.08

**E**

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*p = 0.05

**F**

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*p = 0.04
Fold Inhibition

DMSO 20 nM
Progesterone 2 μM

0 h 72 h
LPS qRT-PCR

Pauklin et al. Suppl. Figure 5