

Estrogen Upregulates Cyclic AMP Response Element Modulator α Expression and Downregulates Interleukin-2 Production by Human T Lymphocytes

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Systemic lupus erythematosus (SLE) is an autoimmune disease with a complex multifactorial pathogenesis. T lymphocytes play a critical role in disease pathogenesis and display abnormal gene expression and poor interleukin (IL)-2 production. We previously showed that the expression of the transcriptional repressor cyclic AMP response element modulator α (CREM α) is increased in SLE T cells and contributes to reduced IL-2 production. Although estrogen is implicated in the onset and exacerbation of SLE, the precise nature of molecular events regulated by estrogen in immune cell function is not well understood. Here, we asked whether estrogen regulates the expression of CREM α in human T lymphocytes. We show that exposure of human T cells to 17- β -estradiol leads to a dose-dependent increase in CREM α mRNA expression, and this increase appears to be mediated through the estrogen receptors α and β . We show that the increased expression of CREM α is due to increased transcriptional activity of the *CREM* promoter and is mediated by increased expression and binding of the Sp1 transcriptional activator. We further show that estrogen treatment leads to a dose-dependent decrease in IL-2 mRNA and cytokine production by T cells. Finally, the effect of β -estradiol on CREM α is observed more frequently in T cells from women than from men. We conclude that estrogen can modulate the expression of CREM α and lead to IL-2 suppression in human T lymphocytes, thus revealing a molecular link between hormones and the immune system in SLE.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune multiorgan disease that afflicts women and follows an unpredictable clinical course marked by flares and remissions. The disease is characterized by autoantibody production and immune complex deposition in target organs such as the joints, kidneys and brain resulting in disease pathology. Abnormal T cells orchestrate responses of multiple cellular components of the immune system either directly or through

secretion of cytokines. Additionally, T-cell infiltration into target organs contributes to inflammatory damage of the tissues (1,2). Thus, understanding the molecular events that contribute to the aberrant SLE T-cell behavior is of utmost importance in understanding disease pathophysiology.

SLE patients experience disease flares during phases of hormonal change such as puberty, menstruation (3), pregnancy (4) and reduced disease activity after menopause (5). Estrogen treatment of

lupus-prone mice accelerates disease onset and severity (6). Patients with SLE have altered estrogen metabolism with increased levels of estrogen metabolites 16-hydroxy estrone and estriol and decreased testosterone levels (7), and hormone replacement therapy increases the risk of mild-to-moderate flare-ups in menopausal women with lupus (8). These studies indicate that estrogen plays a role in SLE disease onset, progression and exacerbation. However, little is known about the precise nature of molecular events regulated by estrogen in the immune system. Estrogen has been reported to increase the expression and activity of calcineurin phosphatase (9) and CD40L expression (10) in SLE T cells. Mice treated with synthetic estrogen were susceptible to *L. monocytogenes* infection, and their splenocytes showed reduced IL-2 production (11). On the other hand, splenocytes from estrogen-

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treated mice showed increased IL-17 production (12).

We and others have shown that SLE T cells display decreased capacity to produce IL-2, a cytokine crucial to the development of cytotoxic responses, regulatory T-cell function and activation-induced cell death (13). IL-2 levels are determined at the transcription level and limited *IL-2* promoter activity was demonstrated to account for deficient IL-2 production in SLE (14). The *IL-2* promoter defines a consensus CRE site at position -180, which can bind the cyclic AMP response element binding (CREB) and cyclic AMP response element modulator (CREM) transcription factors. The phosphorylated (p) form of the CREB protein activates *IL-2* gene transcription, whereas pCREM α represses *IL-2* transcription. Thus, a balance between the expressions of these two transcription factors determines the occupancy of the *IL-2* promoter and thus transcription (15). Increased expression of the protein phosphatase 2A (PP2A) enzyme in SLE T cells leads to dephosphorylation of CREB (16) and thus a tilting of the balance in favor of the CREM α repressor. Increased binding of CREM α to the *IL-2* promoter is enabled by calcium-calmodulin kinase IV in SLE T cells (17). More recently, we presented evidence that the increased levels of CREM α in SLE T cells (18) can be attributed to the increased binding of the specificity protein (Sp)-1 transcription factor to the *CREM* promoter (19).

17- β -Estradiol is the predominant form of estrogen in nonpregnant females and regulates gene expression through binding to the intracellular estrogen receptor α (ER α) and ER β and to the recently identified membrane G protein-coupled receptor (GPR30) (20,21). On ligand binding, the ER homo- or heterodimerizes and binds directly to estrogen response elements defined by promoters of target genes. Alternatively, the ER may recruit other transcription factors such as Sp1, activator protein (AP)-1 and nuclear factor (NF)- κ B, or other cofactors to regulate gene transcription (22). Human T lymphocytes were recognized to express both

ER α and ER β . Moreover, putative membrane receptors were reported and are demonstrated to modulate T-cell receptor-induced responses (23).

It is known that CREM α is differentially expressed in nonpregnant versus pregnant myometrium, such that the highest levels are expressed in pregnant and laboring myometrium (24). It is also known that estrogen levels rise steadily throughout pregnancy, increasing up to 100-fold over physiological levels. Furthermore, estrogen mediates effects on lymphocytes to modulate cytokine production (23) through transcriptional regulation. On the basis of these concepts, we hypothesized that estrogen regulates CREM α expression in T lymphocytes. Our results show that 17- β -estradiol induces increased expression of CREM α in human T cells, which appears to be ER dependent, and this increase is due to an increased transcriptional activity of the *CREM* promoter. We show that 17- β -estradiol induces increased expression of the Sp1 transcription factor and that Sp1 binds to the *CREM* promoter. We further show that estrogen suppresses IL-2 production in a dose-dependent manner. Finally, we show that, whereas estrogen induces CREM α expression in both sexes, the response was observed more frequently in T cells from women than men. Our data present a novel link between hormones and the immune system, which may be of importance in the pathogenesis of SLE.

MATERIALS AND METHODS

Materials

Anti-Sp1 and mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CREM antibody was custom synthesized by the laboratory as described previously (17). The β -actin antibody, 17- β -estradiol, 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), diarylpropionitrile (DPN) and methyl-piperidino-pyrazole dihydrochloride (MPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD3 and anti-

CD28 antibodies were purchased from Biologend (Raritan, NJ, USA), and the goat anti-mouse cross-linker antibody was purchased from Millipore (The Woodlands, TX, USA). All primers were purchased from Eurofins MWG Operon (Huntsville, AL, USA).

T-Cell Isolation and β -Estradiol Treatment

De-identified peripheral blood samples from healthy volunteers after platelet-pheresis obtained from the Kraft donor center (Dana Farber Cancer Institute) were used in this study (for experiments in Figures 1–5). For some experiments (Figure 6), peripheral blood samples from healthy adult men and women (25–45 years of age) were used. All women had regular menstrual cycles and were not on oral contraceptives. The study protocol was approved by the institutional review board. Written informed consent was obtained from all participating subjects. Peripheral blood was collected by venipuncture and T cells purified using the RosetteSep T cell kit (STEMCELL Technologies, Vancouver, BC, Canada) and Lymphocyte Separation Medium (Mediatech, Manassas, VA, USA). T cells ($3\text{--}5 \times 10^6$ cells per 1.5 mL per well) were resuspended in RPMI medium without phenol red (Invitrogen, Carlsbad, CA, USA), supplemented with charcoal-dextran-stripped fetal bovine serum (Hyclone; Fisher, Agawam, MA, USA) and treated with the indicated concentrations of β -estradiol and cultured at 37°C in a 5% CO₂ incubator. In some cases, T cells were treated with 10^{-6} mol/L β -estradiol and 10^{-5} mol/L PPT, DPN or MPP as indicated.

mRNA Expression Studies

Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA (200 ng) was reverse-transcribed into single-stranded cDNA using the AMV reverse transcriptase kit (Promega Madison, WI, USA). The sequences were amplified by the following primers: CREM α : forward 5'-GAA ACA GTT GAA TCC CAG CAT GAT GGA

AGT-3' and reverse 5'-TGC CCC GTG CTA GTC TGA TAT ATG-3' (25); house-keeping gene cyclophilin A: forward 5'-TTC ATC TGC ACT GCC AAG AC-3' and reverse 5'-TCG AGT TGT CCA CAG TCA GC-3'; Sp1: forward 5'-CAC CAC TCT CAC ACC CAT TG-3' and reverse: 5'-TCC ACC TGC TGT GTC ATC AT-3'; IL-2: forward 5'-CAC TAC TCA CAG TAA CCT CAA CTC CT-3' and reverse: 5'-GTG GGA AGC ACT TAA TTA TCA AGT CAG TG-3'. Real-time polymerase chain reaction (PCR) amplification was carried out with SYBR Green I by using a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) with the following program: initial denaturation at 95°C for 5 min, 40 cycles of amplification: denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 30 s; one cycle of melting curves: 95°C for 15 s, 65°C for 2 min and 97°C (continuous) and a final cooling at 37°C. All PCRs were performed in triplicate. Threshold cycle (Ct) values were used to calculate relative mRNA expression by the $\Delta\Delta Ct$ relative quantification method.

Immunoblotting

Cells were pelleted and lysed with RIPA buffer (Boston Bioproducts, Boston, MA, USA). Lysates were resolved on 4–12% Bis-Tris gels and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h, incubated with primary antibody (1:1,000) for 1 h (for β -actin) or overnight (for CREM α and Sp1), washed three times with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h, washed three times with TBS-T, developed with ECL reagents (GE Healthcare, Piscataway, NJ, USA) and visualized by the Fujifilm LAS-4000 imager (GE Healthcare, Piscataway, NJ, USA).

Cytokine Production Analysis

IL-2 production was measured using a human cytometric bead array kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Briefly, culture supernatants were col-

lected from β -estradiol-treated cells stimulated with and without anti-CD3 antibody (10 μ g/mL), anti-CD28 antibody (5 μ g/mL) and goat anti-mouse cross-linker (5 μ g/mL). The samples were incubated with capture beads conjugated to the cytokine-specific antibody and stained with a phycoerythrin (PE)-detection reagent. After washing, the samples were analyzed on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and cytokine concentrations calculated from the standard curves.

Reporter Gene Constructs and Luciferase Assays

The luciferase reporter plasmid driven by the CREM promoter was generated in the laboratory as described before (19). T cells were transfected by electroporation with a nucleofector and a human T-cell nucleofector kit (Lonza, Cologne, Germany). Briefly, 4×10^6 cells were resuspended in 100 μ L nucleofector solution. Plasmid DNA (1 μ g per 10^6 cells) and transfection efficiency control plasmid pRTK (10 ng per 10^6 cells) were added and cells were transfected using the nucleofector V-024 program. Cells were rescued in prewarmed medium and immediately incubated with indicated concentrations of β -estradiol. Luciferase activity was determined using the Dual Luciferase assay system (Promega) following the manufacturer's protocol. Luciferase activity was normalized for transfection efficiency by obtaining the ratio of *Firefly* to *Renilla* luciferase readings. The data are presented as the fold-increase in luciferase activity.

Chromatin Immunoprecipitation (ChIP) Assays

Four to ten million T cells were used per investigated antibody. After β -estradiol treatment, the cells were collected and treated with 37% formaldehyde (1% final concentration) for 10 min, washed, lysed and sonicated. The DNA protein complexes were immunoprecipitated with the indicated antibody bound to Dynabeads Protein A/G (MAGnify Chromatin Immunoprecipitation System; Invitrogen, Carlsbad, CA, USA) and

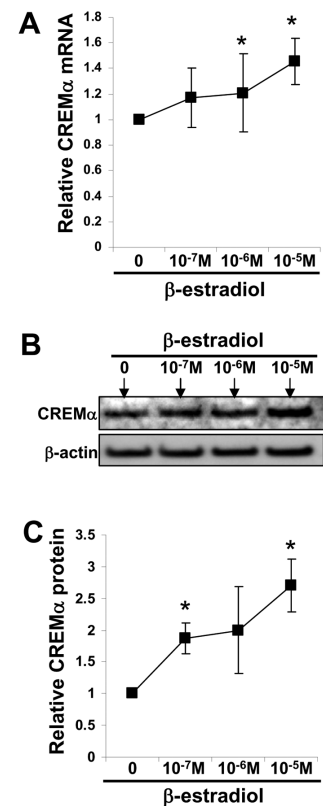


Figure 1. Estrogen induces the expression of CREM α in T cells. (A) Peripheral blood T cells from de-identified donors were treated with increasing concentrations (10^{-7} to 10^{-5} mol/L) of β -estradiol or solvent DMSO control for 18–24 h. Cells were collected, total RNA extracted and quantitative real-time RT-PCR performed using specific primers for CREM α and housekeeping gene cyclophilin A. Graph shows relative expression of CREM α mRNA normalized to cyclophilin A. Results show average values from $n = 14$ (responders) of a total of $n = 24$ donors assessed. Error bars represent SD. (B) Peripheral blood T cells from de-identified donors were treated without and with increasing concentrations (10^{-7} to 10^{-5} mol/L) of β -estradiol for 18–24 h. Cells were collected, lysed and immunoblotted for CREM α and β -actin. A representative blot is shown. (C) Average CREM α protein expression normalized to β -actin from $n = 8$ (responders) of a total of $n = 13$ donors examined. Error bars represent standard error of the mean (SEM). * $P < 0.05$.

extracted. After several washing steps, the cross-link between DNA and protein was reversed and protein digestion with

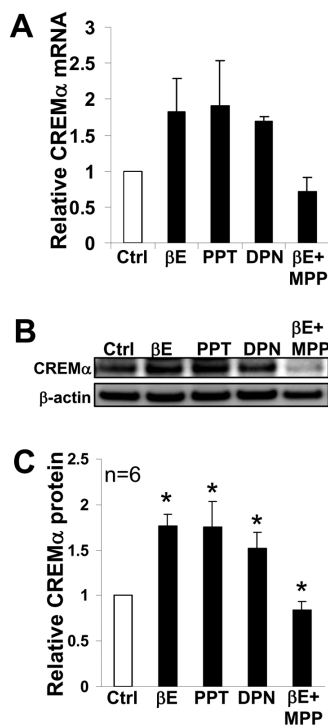


Figure 2. The estrogen-mediated effect on CREM α is ER dependent. T cells were treated for 24 h with solvent DMSO control or 10^{-6} mol/L β -estradiol, or 10^{-5} mol/L of the selective ER α and ER β agonists PPT and DPN, respectively, and 10^{-5} mol/L of the selective ER α antagonist MPP. (A) Cells were collected, total RNA extracted and quantitative real-time RT-PCR performed using specific primers for CREM α and cyclophilin A. Graph shows relative expression of CREM α mRNA normalized to cyclophilin A. Results show average values of PCR triplicates from one representative experiment of three independent experiments ($n = 3$) of a total of $n = 8$ donors examined. Error bars represent SEM. (B) Cells were lysed and immunoblotted for CREM α and β -actin. A representative blot is shown. (C) Graph shows an average of CREM α protein expression normalized to β -actin from $n = 6$ donors and error bars represent SEM. * $P < 0.05$.

proteinase K was performed at 55°C for 20 min, followed by a 95°C incubation, and the DNA was then eluted. The DNA was amplified with primers flanking the CREM promoter. The sequences were as follows: forward 5'-GGG AGA TAG AGG TTG CAG AG-3' and reverse 5'-GAC

CAA AAG TAG CGC TGC AG-3'. Semi-quantitative PCR was performed using the following program: initial denaturation at 95°C for 5 min, 30 cycles of amplification: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, final extension at 72°C for 5 min and a final cooling at 4°C; PCR products were run on 1.8% agarose gels in 1 \times Tris acetate EDTA buffer, stained with ethidium bromide and imaged using a Bio-Rad gel doc system. Densitometric quantitation of bands was performed using the Quantity1 software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Student *t* test and the two-way repeated-measures analysis of variance (ANOVA) were used for statistical analysis. Statistical significance was defined as $P < 0.05$.

RESULTS

Estrogen Exposure Leads to Increased CREM α Expression in Human T Lymphocytes

We have previously shown that SLE T cells produce decreased amounts of IL-2 on stimulation *in vitro* and that increased expression of the CREM α transcriptional repressor contributes to this deficiency (26). To understand the mechanisms of CREM α regulation, we asked whether hormones may contribute to its aberrant expression. Because SLE is an autoimmune disease seen predominantly in women, with estrogen implicated in disease development and acceleration, we chose to examine the role of estrogen in the expression of CREM α . To determine whether β -estradiol affects CREM α expression, we treated peripheral blood T lymphocytes with increasing 10^{-7} to 10^{-5} mol/L (100 nmol/L to 10 μ mol/L) concentrations of β -estradiol for 24 h and assessed CREM α mRNA expression by quantitative real-time reverse-transcriptase (RT)-PCR. On the basis of initial dose and time response optimization experiments, we found the maximal responses to β -estradiol to be within this range, which we used subsequently. A total of 24 donors were examined; 14 of them displayed in-

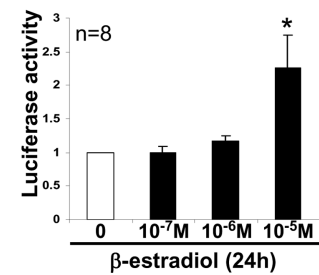


Figure 3. Estrogen induces CREM α transcription in T cells. Peripheral blood T cells were transfected with a CREM α promoter-luciferase reporter construct using a nucleofector. Cells were rescued in pre-warmed medium and incubated immediately with increasing concentrations (10^{-7} to 10^{-5} mol/L) of β -estradiol or solvent DMSO control for 24 h. Cells were lysed and luciferase activity was measured using the dual luciferase assay system. Luciferase activity was normalized for transfection efficiency (firefly luciferase readings normalized to renilla luciferase readings). Data from β -estradiol treated samples were then normalized to data from control DMSO-treated sample. Results are average values from $n = 8$ donors of a total of $n = 13$ donors examined and error bars represent SEM. * $P < 0.05$.

creased responses (designated as an increase in CREM α mRNA expression if ≥ 1.2 -fold of control treatment). As shown in Figure 1A, induction of CREM α mRNA expression was heterogeneous, but increased in a dose-dependent manner with increasing concentrations of β -estradiol. To assess whether β -estradiol induces the expression of CREM α at the protein level, we performed similar experiments and immunoblotted cell lysates for CREM α (Figures 1B, C). In these experiments, an increase (if ≥ 1.2 -fold of control treatment) in CREM α expression was observed in 8 of 13 donors examined. We found that estrogen induced a dose-dependent increase in CREM α protein expression levels. These results show that estrogen induces CREM α expression in human T cells.

Estrogen-Mediated Effect on CREM α Expression Is ER Dependent

To examine whether the β -estradiol-induced increase in CREM α expression

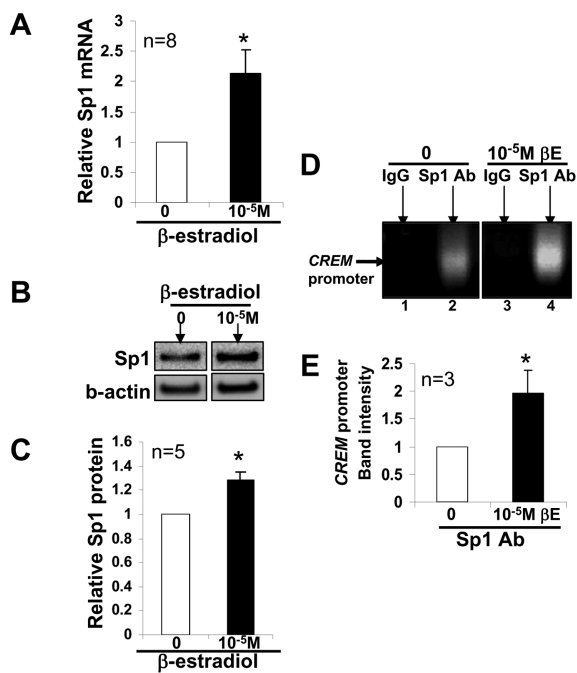


Figure 4. Estrogen exposure leads to increased Sp1 expression and binding to the *CREM* promoter in T cells. Peripheral blood T cells were treated with 10^{-5} mol/L β -estradiol or solvent DMSO control for 18–24 h. (A) Cells were lysed, total RNA extracted and quantitative real-time RT-PCR was performed using specific primers for Sp1 and cyclophilin A. The graph shows the relative expression of Sp1 mRNA normalized to cyclophilin A. Results show average values from $n = 8$ (responders) of a total of $n = 14$ donors examined. Error bars represent SEM. (B) Cells were lysed and immunoblotted for Sp1 and β -actin. A representative blot is shown. (C) Graph shows average Sp1 protein expression normalized to β -actin from $n = 5$ (responders) of a total of $n = 10$ donors examined. Error bars represent SEM. (D) Peripheral blood T cells were treated with 10^{-5} mol/L β -estradiol or solvent DMSO control (β E) for 18 h. Cells were formalin fixed, lysed, washed and sonicated according to the ChIP protocol outlined in Materials and Methods. The anti-Sp1 and anti-IgG antibody immunoprecipitates were subjected to semiquantitative PCR using specific primers for the *CREM* promoter, and PCR products run on a 1.8% agarose gel stained with ethidium bromide. (E) Densitometric quantification and background subtraction of the PCR bands from the anti-Sp1 immunoprecipitates (lanes 2 and 4) was performed and expressed as relative units. Results are the average values from $n = 3$ of a total of $n = 7$ donors examined. Error bars represent SEM. * $P < 0.05$.

was mediated through ER α and/or ER β , we used pharmacological agonists and antagonists of these receptors. PPT and DPN are selective agonists of ER α and ER β , respectively. MPP is a selective ER α antagonist. We treated T cells with solvent dimethyl sulfoxide (DMSO) control or 10^{-6} mol/L β -estradiol or 10-fold molar excess of the selective ER α and ER β agonists or ER α antagonist. Treatment of T cells with either ER α or ER β agonist led to increased CREM α expression similar to that seen with β -estradiol. Blocking the

ER α using selective antagonist MPP was sufficient to block the effect of β -estradiol on CREM α mRNA expression (Figure 2A). We further determined the effect of the ER agonists and antagonist on the CREM α protein expression and found that PPT and DPN increased CREM α protein expression in a manner similar to that recorded with β -estradiol, whereas the ER α antagonist MPP blocked this increase (Figures 2B, C). These results indicate that the effect of estrogen on CREM α expression is ER dependent.

Estrogen Exposure Leads to Increased CREM Promoter Transcriptional Activity

Because estrogen mediated an increase in the mRNA expression of CREM α and because estrogen regulates gene expression at the transcription level, we asked whether estrogen regulates the transcription of CREM α . We used a *CREM* promoter–luciferase vector construct to transfect T cells using nucleoporation. Transfected cells were treated immediately with increasing concentrations of β -estradiol or solvent DMSO control, and cells were collected 24 h later. Luciferase activity was measured and normalized for transfection efficiency. Luciferase activity was expressed as fold change over control (DMSO)-treated cells. Significantly increased transcriptional activity was seen at the 10^{-5} mol/L β -estradiol concentration, with little to no increase seen at the 10^{-7} and 10^{-6} mol/L concentrations (Figure 3). These results show that high concentrations of β -estradiol leads to increased transcription of CREM α .

Estrogen Exposure Leads to Increased Sp1 Expression and Binding to the CREM Promoter in Human T Lymphocytes

Estrogen mediates its effect on gene transcription through several modes, including direct binding of the ER to the estrogen response elements within target genes, or by recruitment and binding of other transcriptional activators such as Sp1 and AP-1 (22). We scanned the *CREM* promoter sequence, and although estrogen response elements or AP-1 consensus sites were not identified within this region, it defines seven core binding sites for the transcription factor Sp1. Previously, we have shown that Sp1 binds to and activates transcription of *CREM* (19). Because the increase in *CREM* promoter activity was observed with the 10^{-5} mol/L concentration of β -estradiol, we chose this concentration in the following experiments. We analyzed the mRNA expression levels of Sp1 in β -estradiol–treated cells at 18 and 24 h after treatment and noted a significant

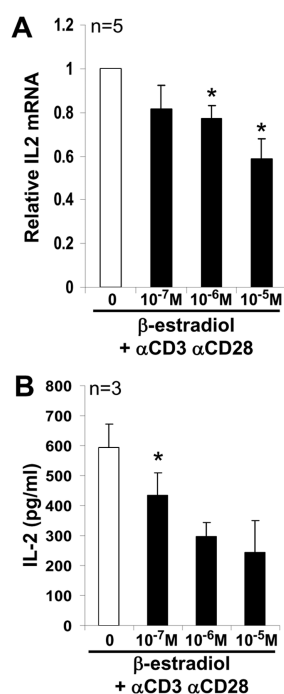


Figure 5. Estrogen suppresses IL-2 production in T cells. (A) T cells were incubated with increasing concentrations (10^{-7} to 10^{-5} mol/L) of β -estradiol or solvent DMSO control for 24 h. Anti-CD3, anti-CD28 and goat anti-mouse cross-linker antibodies were added to the cell cultures for another 24 h. β -Estradiol was kept in culture throughout activation. Supernatants were collected and IL-2 production was measured using a flow cytometry-based cytokine bead array. Graph shows averages from $n = 3$ of a total of $n = 8$ donors examined. Error bars represent SEM. (B) T cells were incubated with increasing concentrations (10^{-7} to 10^{-5} mol/L) of β -estradiol or solvent DMSO control for 24 h. Anti-CD3, anti-CD28 and goat anti-mouse cross-linker antibodies were added to the cell cultures for another 5 h. β -Estradiol was kept in cultures throughout the activation. Cells were lysed, total RNA extracted and quantitative real-time RT-PCR was performed using specific primers for Sp1 and cyclophilin A. The graph shows the relative expression of Sp1 mRNA normalized to cyclophilin A. Results show average values from $n = 5$ of a total of $n = 9$ donors examined. Error bars represent SEM. * $P < 0.05$.

increase in Sp1 expression at 18 h of β -estradiol treatment (Figure 4A), whereas Sp1 expression either remained

high or decreased at 24 h of treatment (data not shown). We further examined the effect of β -estradiol on the induction of Sp1 protein expression and found that Sp1 protein levels increased in response to β -estradiol (Figures 4B, C).

The observation that β -estradiol treatment increases Sp1 expression led us to question whether Sp1 bound to the CREM promoter on β -estradiol treatment. Accordingly, we treated T cells with 10^{-5} mol/L of β -estradiol for 18 h and then performed chromatin immunoprecipitation (ChIP) assays using an anti-Sp1 antibody to determine Sp1 binding to the CREM promoter. We found that β -estradiol treatment led to an increased Sp1 binding to the CREM promoter, as seen in Figures 4D and E. An isotype control IgG antibody did not identify any protein binding to the CREM promoter (Figure 4D, lanes 1 and 3). A basal level of Sp1 binding was seen in untreated cells (Figure 4D, lane 2), which increased in the β -estradiol-treated cells (Figure 4D, lane 4, and Figure 4E). These data show that β -estradiol treatment leads to increased expression of Sp1 and increased Sp1 binding to the target CREM promoter.

Estrogen Exposure Suppresses IL-2 Production by Human T Lymphocytes

We have previously shown that CREM α represses IL-2 transcription and cytokine production, and we show herein that β -estradiol treatment induces CREM α expression (Figure 1). Therefore, we wished to assess whether β -estradiol treatment affected IL-2 production. We treated T cells with increasing concentrations of β -estradiol for 24 h and then stimulated the cells with anti-CD3 and anti-CD28 antibodies for another 5 h, with β -estradiol present in the cultures throughout. IL-2 mRNA expression was measured by quantitative real-time RT-PCR. We observed a dose-dependent decrease in IL-2 mRNA levels with increasing concentrations of β -estradiol (Figure 5A). In parallel, cells were treated with β -estradiol for 24 h and then stimulating antibodies were added for another

24 h, with β -estradiol kept in the cultures throughout. Cell culture supernatants were collected, and IL-2 levels were measured using a flow cytometry-based cytokine bead array. We found that increasing concentrations of β -estradiol treatment led to gradually decreasing amounts of IL-2 production (Figure 5B), suggesting that the effect of β -estradiol on the CREM α expression may lead to downstream suppression of IL-2 production.

Estrogen Increases CREM α Expression in T Cells from Men and Women

The above experiments (Figures 1–5) were performed in T cells obtained from de-identified healthy donors for whom information on age or sex gender was not available. To assess whether the estrogen-mediated increase in CREM α expression was sex specific, we obtained blood samples from 10 healthy men and 9 healthy women donors. In women, estrogen levels vary during the menstrual cycle such that they are the lowest in the follicular phase, increase steadily thereafter and peak just before ovulation to decrease during the luteal phase. Thus, the widest difference in estrogen levels occurs between the follicular (lowest) and peri-ovulatory (highest) phases. Accordingly, in female donors, blood was drawn twice during the menstrual cycle (once during the follicular phase [about d 6–8] and once during the peri-ovulatory phase [about d 13–17]). Peripheral blood T cells were exposed to increasing concentrations of β -estradiol and CREM α mRNA, and protein expression levels were assessed. In these experiments, β -estradiol concentrations from 10^{-8} to 10^{-5} (10 nmol/L to 10 μ mol/L) were included. Results from these experiments showed that T cells from 3 of the 10 male donors showed an increase in CREM α mRNA (Figure 6A) and protein (Figure 6B) expression levels. In the T cells from all nine women, we observed a dose-dependent increase in CREM α mRNA expression (Figure 6C) in response to β -estradiol (two-way repeated-measures ANOVA [phase, con-

centration], concentration $F(3) = 5.835$, $P = 0.002$). However, responses were not significantly different between the follicular and ovulatory phases (two-way repeated-measures ANOVA [phase, concentration], phase $F(1) = 1.048$, $P = 0.32$). To assess whether β -estradiol also induced CREM α protein expression, we performed immunoblots for CREM α . We obtained sufficient amounts of lysates from six of the nine samples. We found a significant dose-dependent increase in CREM α protein expression (Figures 6D, E) during both the follicular and ovulatory phases (two-way repeated-measures ANOVA [phase, concentration], concentration $F(3) = 2.959$, $P = 0.048$). Again, the responses were not significantly different between the follicular and ovulatory phases (two-way repeated-measures ANOVA [phase, concentration], phase $F(1) = 0.632$, $P = 0.44$). These results show that the estrogen-mediated CREM α induction in T lymphocytes is observed in both men and women, although more frequently in women than men.

DISCUSSION

In this study, we show for the first time that β -estradiol treatment induces an ER-dependent increase in CREM α mRNA expression in human peripheral blood lymphocytes, and this increase is due to an induction of transcriptional activity. We show that estrogen treatment increases the expression of the transcription factor Sp1 and also increases Sp1 binding to the CREM promoter. We further show that estrogen treatment suppresses IL-2 production in a dose-dependent manner. Finally, we show that estrogen mediates increased CREM α expression in female rather than male human T lymphocytes.

Increase in the CREM promoter activity was seen most strikingly at the 10^{-5} mol/L concentration (Figure 3), whereas increases in CREM α mRNA expression were observed even at the 10^{-6} and 10^{-7} mol/L concentrations (Figure 1). This discrepancy may be explained by a differential regulation at different concentrations. At lower concentrations of es-

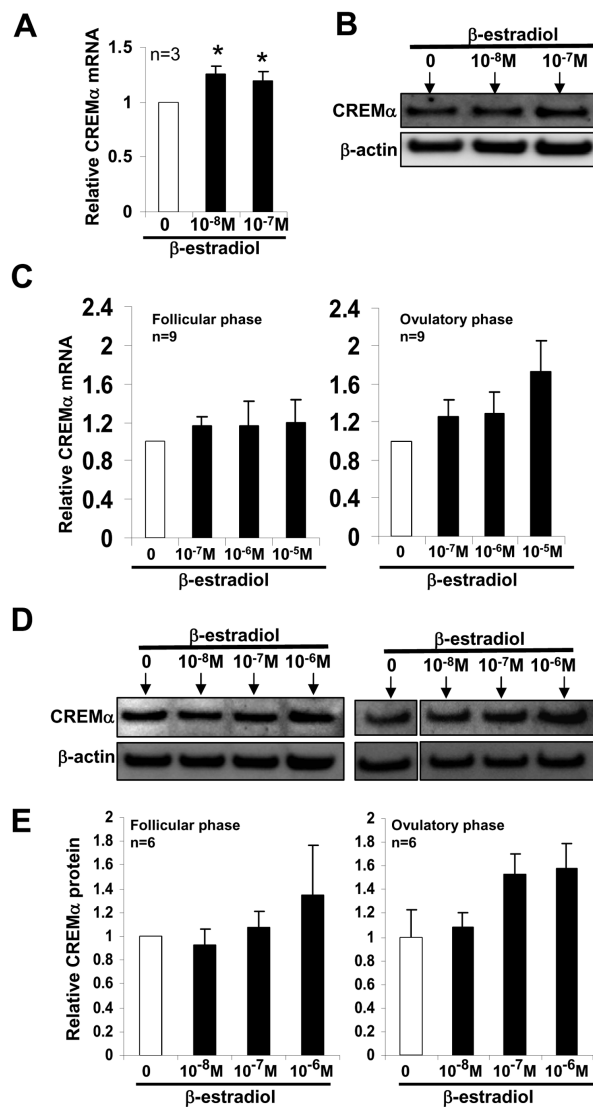


Figure 6. Estrogen induces CREM α expression in T cells from men and women. (A–C) T cells from peripheral blood from 10 healthy male donors were treated with increasing (10^{-8} to 10^{-7} mol/L) concentrations of β -estradiol or solvent DMSO control for 18–24 h. (A) Cells were collected, total RNA extracted and quantitative real-time RT-PCR performed using specific primers for CREM α and cyclophilin A. Graph shows relative expression of CREM α mRNA normalized to cyclophilin A. Results show average values from $n = 3$ of a total of $n = 10$ donors examined. Error bars represent SEM. $*P < 0.05$. (B) Cells were collected, lysed and immunoblotted for CREM α and β -actin. A representative blot (one representative of $n = 3$) is shown. (C–E) T cells from peripheral blood (collected at follicular and ovulatory phases of menstrual cycle) from nine healthy female donors were treated with increasing concentrations (10^{-8} to 10^{-5} mol/L) of β -estradiol or solvent DMSO control for 18–24 h. (C) Cells were collected, total RNA extracted and quantitative real-time RT-PCR performed using specific primers for CREM α and cyclophilin A. Graphs show relative expression of CREM α mRNA normalized to cyclophilin A. Results show average values from $n = 9$ donors. Error bars represent SEM (two-way repeated-measures ANOVA, $P = 0.002$). (D) Cells were collected, lysed and immunoblotted for CREM α and β -actin. A representative blot is shown (one representative of $n = 6$). (E) Graphs show average CREM α protein expression normalized to β -actin from $n = 6$ donors. Error bars represent SEM. Two-way repeated-measures ANOVA, $P = 0.048$.

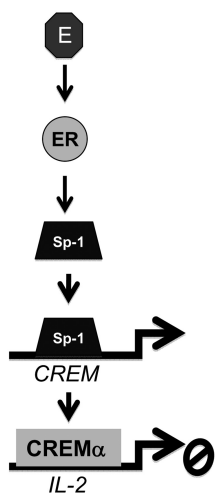


Figure 7. Molecular events that link estrogen to decreased IL-2 production. Estrogen binds to the ER, increases Sp1 expression (which binds to the *CREM* promoter) and enhances the production of CREM α (which may suppress the production of IL-2).

trogen, there may be a preferential increase in CREM α due to posttranscriptional regulatory mechanisms such as alternative splicing or mRNA stabilization. Indeed, a preferential expression of CREM α over the alternatively spliced isoform CREMtau2 α is seen progressively within the myometrium from non-pregnant to pregnant and laboring stages, and this switch is mediated by the splicing factor SRp40 (24). β -Estradiol has also been shown to increase ER expression by stabilizing its mRNA (27).

The estrogen-initiated suppression of IL-2 production (Figure 5) may contribute to the pathogenesis of SLE, since it is clear that IL-2 is needed for the generation of cytotoxic responses, the generation of T regulatory cells and activation-induced cell death. In patients with SLE, various forms of cytotoxic responses have been described ineffective and may account for the increased rate of infection-associated morbidity and mortality (28,29). Regulatory T-cell function and numbers have been reported to be decreased in SLE patients, albeit the reports are not definitive (30). Activation-induced cell death is defective in SLE patients (31), and this may account for the

failure to eliminate activated autoreactive T cells in a timely manner. Estrogen-initiated suppression of IL-2 production may not represent the only mechanism whereby estrogens contribute to the aberrant immunoregulation in SLE patients. Estrogen has been claimed to increase calcineurin expression in SLE T cells (9), and this may lead to dephosphorylation and activation of nuclear factor of activated T cells (NFAT), which in turn accounts for the increased expression of CD40 ligand in SLE T cells (32,33). In addition, treatment of T cells with estrogen leads to increased expression of CD40 ligand on T cells (10). β -Estradiol is known to dampen the B-cell receptor-initiated B-cell signaling and to impair negative selection of high-affinity anti-DNA-producing B cells (34). In addition, estrogen alters the expression of apoptosis-related molecules and renders B cells resistant to apoptosis (35).

While estrogen levels have not been found to be significantly different in women with SLE compared with healthy women, expression of estrogen metabolites is increased and testosterone levels are decreased in SLE patients. Furthermore, it is thought that the sensitivity to estrogen due to differential expression of estrogen receptors may differ in patient SLE compared with healthy individuals. While it was shown that the ER β expression was not significantly different in patients with SLE compared with healthy women, there was a wide variation in the expression of ER α in SLE patients, suggesting its potential role in the sensitivity to estrogen in SLE T cells (36). In our studies, it appears that the effect of β -estradiol on CREM α is mediated through the ER, since inducing either ER α or ER β , with specific agonists, mimicked this effect. However, blocking ER α alone significantly decreased the estrogen-induced CREM α expression (Figure 2), suggesting its potential importance in this mechanism.

Physiological estrogen levels in serum range from 10^{-10} mol/L (100 pmol/L) to 10^{-7} mol/L (100 nmol/L). Although the 10^{-6} mol/L (1 μ mol/L) and 10^{-5} mol/L

(10 μ mol/L) concentrations we have used in this study are above the physiological range, the 10^{-7} mol/L (100 nmol/L) concentration is routinely used for *in vitro* experiments with human peripheral blood T cells. We have also included the 10^{-8} mol/L (10 nmol/L) concentration in some experiments (Figures 6A, B, D, and E; and data not shown). Furthermore, the T cells we studied are from healthy donors and hence may not respond to physiological hormone levels.

In our studies, we find that CREM α expression increased in response to β -estradiol in T cells from both men and women, albeit from a smaller percentage of men. In studies with T cells from women, whereas we noticed a trend toward greater variability and higher magnitude of responses in the ovulatory phase compared with the follicular phase, the responses were not significantly different between the two phases of the menstrual cycle (Figure 6). Accordingly, while estrogen is known to upregulate expression of the ER in various cell types (37), ER expression in T lymphocytes is not different between the follicular and luteal phases of the menstrual cycle (36).

CONCLUSION

In conclusion, we provide evidence for a new molecular link between hormones and the immune system (Figure 7). Specifically, β -estradiol increases Sp1 expression in human T cells, which binds to the *CREM* promoter and enhances its activity. CREM α in turn binds to the *IL-2* promoter and suppresses the production of IL-2, a central cytokine in the regulation of the immune system.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecu-*

lar Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- Crispin JC, et al. (2010) Pathogenesis of human systemic lupus erythematosus: recent advances. *Trends Mol. Med.* 16:47–57.
- Davidson A, Diamond B. (2001) Autoimmune diseases. *N. Engl. J. Med.* 345:340–50.
- Jungers P, et al. (1985) Hormonal modulation in systemic lupus erythematosus: preliminary clinical and hormonal results with cyproterone acetate. *Arthritis Rheum.* 28:1243–50.
- Mund A, Simson J, Rothfield N. (1963) Effect of pregnancy on course of systemic lupus erythematosus. *JAMA.* 183:917–20.
- Mok CC, Lau CS, Ho CT, Wong RW. (1999) Do flares of systemic lupus erythematosus decline after menopause? *Scand. J. Rheumatol.* 28:357–62.
- Cohen-Solal JF, Jeganathan V, Grimaldi CM, Peeva E, Diamond B. (2006) Sex hormones and SLE: influencing the fate of autoreactive B cells. *Curr. Top. Microbiol. Immunol.* 305:67–88.
- Lahita RG, Bradlow HL, Kunkel HG, Fishman J. (1979) Alterations of estrogen metabolism in systemic lupus erythematosus. *Arthritis Rheum.* 22:1195–8.
- Buyon JP, et al. (2005) The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial. *Ann. Intern. Med.* 142:953–62.
- Rider V, Jones SR, Evans M, Abdou NI. (2000) Molecular mechanisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus T cells. *Clin. Immunol.* 95:124–34.
- Rider V, et al. (2001) Estrogen increases CD40 ligand expression in T cells from women with systemic lupus erythematosus. *J. Rheumatol.* 28:2644–9.
- Pung OJ, Tucker AN, Vore SJ, Luster MI. (1985) Influence of estrogen on host resistance: increased susceptibility of mice to *Listeria monocytogenes* correlates with depressed production of interleukin 2. *Infect. Immun.* 50:91–6.
- Khan D, Dai R, Karpuzoglu E, Ahmed SA. (2010) Estrogen increases, whereas IL-27 and IFN- γ decrease, splenocyte IL-17 production in WT mice. *Eur. J. Immunol.* 40:2549–56.
- Malek TR, Bayer AL. (2004) Tolerance, not immunity, crucially depends on IL-2. *Nat. Rev. Immunol.* 4:665–74.
- Crispin JC, Tsokos GC. (2009) Transcriptional regulation of IL-2 in health and autoimmunity. *Autoimmun. Rev.* 8:190–5.
- Katsiari CG, Tsokos GC. (2006) Transcriptional repression of interleukin-2 in human systemic lupus erythematosus. *Autoimmun. Rev.* 5:118–21.
- Katsiari CG, Kyttaris VC, Juang YT, Tsokos GC. (2005) Protein phosphatase 2A is a negative regulator of IL-2 production in patients with systemic lupus erythematosus. *J. Clin. Invest.* 115:3193–204.
- Juang YT, et al. (2005) Systemic lupus erythematosus serum IgG increases CREM binding to the IL-2 promoter and suppresses IL-2 production through CaMKIV. *J. Clin. Invest.* 115:996–1005.
- Kyttaris VC, Wang Y, Juang YT, Weinstein A, Tsokos GC. (2006) CAMP response element modulator expression in patients with systemic lupus erythematosus. *Lupus.* 15:840–4.
- Juang YT, et al. (2011) Transcriptional activation of the cAMP-responsive modulator promoter in human T cells is regulated by protein phosphatase 2A-mediated dephosphorylation of SP-1 and reflects disease activity in patients with systemic lupus erythematosus. *J. Biol. Chem.* 286:1795–801.
- Thomas P, Pang Y, Filardo EJ, Dong J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology.* 146:624–32.
- Revankar CM, Cimino DE, Sklar LA, Arterburn JB, Prossnitz ER. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science.* 307:1625–30.
- Nilsson S, et al. (2001) Mechanisms of estrogen action. *Physiol. Rev.* 81:1535–65.
- Pernis AB. (2007) Estrogen and CD4+ T cells. *Curr. Opin. Rheumatol.* 19:414–20.
- Tyson-Capper AJ, Bailey J, Krainer AR, Robson SC, Europe-Finner GN. (2005) The switch in alternative splicing of cyclic AMP-response element modulator protein CREM τ 2 α (activator) to CREM α (repressor) in human myometrial cells is mediated by SRp40. *J. Biol. Chem.* 280:34521–9.
- Tenbrock K, Juang YT, Tolnay M, Tsokos GC. (2003) The cyclic adenosine 5'-monophosphate response element modulator suppresses IL-2 production in stimulated T cells by a chromatin-dependent mechanism. *J. Immunol.* 170:2971–6.
- Tenbrock K, Juang YT, Gourley MF, Nambiar MP, Tsokos GC. (2002) Antisense cyclic adenosine 5'-monophosphate response element modulator up-regulates IL-2 in T cells from patients with systemic lupus erythematosus. *J. Immunol.* 169:4147–52.
- Robertson JA, Farnell Y, Lindahl LS, Ing NH. (2002) Estradiol up-regulates estrogen receptor messenger ribonucleic acid in endometrial carcinoma (Ishikawa) cells by stabilizing the message. *J. Mol. Endocrinol.* 29:125–35.
- Lieberman LA, Tsokos GC. (2010) The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. *J. Biomed. Biotechnol.* 2010:740619.
- Iliopoulos AG, Tsokos GC. (1996) Immunopathogenesis and spectrum of infections in systemic lupus erythematosus. *Semin. Arthritis Rheum.* 25:318–36.
- La Cava A. (2011) Regulatory immune cell subsets in autoimmunity. *Autoimmunity.* 44:1–2.
- Kovacs B, Vassilopoulos D, Vogelgesang SA, Tsokos GC. (1996) Defective CD3-mediated cell death in activated T cells from patients with systemic lupus erythematosus: role of decreased intracellular TNF- α . *Clin. Immunol. Immunopathol.* 81:293.
- Kyttaris VC, Wang Y, Juang YT, Weinstein A, Tsokos GC. (2007) Increased levels of NF-ATc2 differentially regulate CD154 and IL-2 genes in T cells from patients with systemic lupus erythematosus. *J. Immunol.* 178:1960–6.
- Cron RQ. (2003) CD154 transcriptional regulation in primary human CD4 T cells. *Immunol. Res.* 27:185–202.
- Grimaldi CM, Jeganathan V, Diamond B. (2006) Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint. *J. Immunol.* 176:2703–10.
- Grimaldi CM, Cleary J, Dagtas AS, Moussai D, Diamond B. (2002) Estrogen alters thresholds for B cell apoptosis and activation. *J. Clin. Invest.* 109:1625–33.
- Rider V, et al. (2006) Differential expression of estrogen receptors in women with systemic lupus erythematosus. *J. Rheumatol.* 33:1093–101.
- Murphy AJ, Guyre PM, Wira CR, Pioli PA. (2009) Estradiol regulates expression of estrogen receptor ER α 46 in human macrophages. *PLoS One.* 4:e5539.