Estrogen Increases CD40 Ligand Expression in T Cells from Women with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective***. To examine the *in vitro* effects of estrogen on CD40 ligand (CD40L) expression in peripheral blood T cells isolated from patients with systemic lupus erythematosus (SLE) and normal controls.
Methods. T cells from female patients with SLE and controls were cultured in serum-free medium without and with 2-fluoroestradiol. Some T cells were activated by further culture on anti-CD3 coated plates. Calcineurin was activated in some T cells by culture in ionomycin. Cell surface CD40L was quantitated by FACS analysis. mRNA expression was measured using semiquantitative PCR.
Results. Lupus T cells cultured in medium containing 2-fluoroestradiol showed a significant ($p = 0.04$) increase in the amount of CD40L on the cell surface, but not in the number of positive cells, compared to the same T cells cultured without estradiol. Estradiol did not significantly change CD40L expression on the surface of T cells from normal women. In addition, the difference in cell surface CD40L between T cells cultured without and with estradiol was significantly greater ($p = 0.048$) on SLE than on normal T cells. Culture of SLE T cells in medium containing 2-fluoroestradiol followed by T cell receptor (TCR) activation for 2 h using anti-CD3 resulted in a significant ($p = 0.04$) estrogen dependent increase in CD40L mRNA. The estrogen dependent increases in SLE T cell CD40L mRNA and cell surface protein were blocked by the estrogen receptor antagonist ICI 182,780. SLE and normal T cells pretreated with estradiol and cultured with ionomycin for 2 h to activate calcineurin showed no significant differences in CD40L mRNA.
Conclusion. These results suggest that estradiol, working through the estrogen receptor, stimulates the expression of CD40L in unstimulated and activated SLE T cells. Estradiol effects may be exerted on multiple regulatory steps that control CD40L expression. The estrogen dependent increase in CD40L expression could hyperstimulate SLE T cells and thereby contribute to the pathogenesis of SLE. (J Rheumatol 2001;28:2644–9)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS ESTROGEN AUTOIMMUNITY T CELLS

Systemic lupus erythematosus (SLE) predominantly affects women during their reproductive years[1]. In certain animal models of lupus, female mice have more aggressive disease than males and die at a younger age[2]. Ovariectomy of these mice improves their disease symptoms, while estrogen administration aggravates the disease[2]. Although some progress in understanding the role of estrogen in lupus has been reported[3-6], the gene targets and downstream consequences of estrogen action in immune cells are not completely understood[7,8]. The development of lupus has been postulated to involve abnormal regulation of T cell function[9,10] that may be influenced by ligand bound estrogen receptor stimulation of T cell signal transduction molecules[3-6]. T cell hyperactivity in autoimmune disease can be induced by the overexpression of signaling molecules such as CD40 ligand (CD40L) on helper T cells[11], thereby contributing to the pathogenesis of lupus.

CD40L is a transmembrane protein that is transiently expressed on activated T cells[12] during the contact dependent help for antibody production. CD40L binds to CD40 on antigen-presenting cells transducing a second signal that is essential for B cell growth and differentiation[13,14]. Recent data indicate that CD40L upregulates the expression of the B7 family of proteins that promote T cell differentiation by interacting with CD28 on T cells and initiating the immune response[12,15]. Interactions between CD40 and CD40L are required to sustain the immune response[16].

The importance of CD40L in contributing to pathogenesis in lupus has been suggested from several recent studies. CD40L is excessively expressed on human SLE T cells[17,18], and increased levels of soluble CD40L in the sera of patients with lupus has been reported[19]. The levels of CD40L in lupus patients in remission is low, but levels can be readily induced

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to active disease levels by *in vitro* stimulation. During lupus disease activity CD40L is upregulated, resulting in costimulation of CD40 on B cells and excessive production of anti-DNA antibodies of specific idiotypes[17]. B cells under the influence of CD40L both proliferate and switch their immunoglobulin (Ig) from IgM to IgG class[18]. Recombinant CD40L or sera from patients with lupus induced CD95 or CD86 on B cells. This effect on B cells was inhibited by prior treatment *in vitro* with anti-CD40L monoclonal antibody[19,20]. B cells from patients with SLE, but not those from normal controls, increased production of anti-dsDNA autoantibodies in response to stimulation with recombinant CD40L leucine zipper fusion protein[20].

CD40L is integral in producing disease in 2 murine models of lupus specifically stimulating B cells to produce pathogenic autoantibodies[21]. Both SWR × NZB and NZB × NZW F1 mice have female predominant expression of lupus with a renal pathology similar to that found in human SLE. In these F1 mice, CD40L is expressed early and excessively on activated helper T cells, well before the disease manifests itself, implying an intrinsic rather than induced phenomenon. Moreover, treatment of these mice with anti-CD40L antibody delays disease onset and in some cases reverses or prevents the disease process[21,22]. Taken together, these observations suggest that increased levels of CD40L on the surface of SLE T cells, and in the sera of patients with lupus, could stimulate prolonged or excessive signaling via CD40, thereby promoting B cell activation and autoantibody production contributing to human SLE.

We investigated if culture of SLE T cells in estradiol increased CD40L expression. Data from our laboratory showed that T cells from women with SLE displayed a sensitivity to estradiol, with increased expression of calcineurin mRNA and phosphatase activity[3,4,23]. Increased calcineurin expression in SLE T cells was not due to detectable differences in estrogen receptor primary structure[5] or to the absence of estrogen receptor subtypes[4]. In human T cells, the binding of antigen to the T cell receptor (TCR) complex stimulates a signal transduction cascade that is calcium dependent and leads to activation of protein kinase C, changes in the phosphorylation status of regulatory proteins, and the synthesis and secretion of cytokines including CD40L. Calcineurin is a key regulator in this signal transduction pathway because it stimulates the activation of nuclear factor of activated T cells (NFAT), a transcription factor involved in the regulation of CD40L expression. Therefore it was important to test if the estrogen dependent increase in lupus T cell calcineurin would also stimulate an increase in the expression of CD40L.

MATERIALS AND METHODS

Study participants. Participants in this study included 20 female patients with lupus and 12 age matched normal control women. Power analysis of estrogen dependent effects on calcineurin expression showed that a sample size of n = 10 would achieve a power of 0.95, $p < 0.05$ for each test. Therefore, T cell samples from a minimum of 10 patients were tested in each experiment and samples from each subject enrolled in this study were not tested in all of the assays. Sixteen of the patients were Caucasian, 3 were African-American, and one was Hispanic. Normal controls comprised 10 Caucasian and 2 African-American women. The women enrolled in this study had regular menstrual cycles and were between the ages of 26 and 48. No patient or control was taking hormone replacement therapy or oral contraceptives. Patients with SLE met at least 4 of the criteria of the American College of Rheumatology for classification of SLE[24]. Duration of lupus in the patients was 3–17 years, mean 6.3 years. At the time of study anti-dsDNA antibody was 0–59 IU/dl, mean 23.7 IU/dl; 5 patients with SLE had negative anti-dsDNA. Two of the 20 patients had nephritis. Disease activity of patients ranged from none to active, with SLE Disease Activity Index scores between 0 and 22 (mean 7.8). Seventeen lupus patients received prednisone (mean 7.1 mg/day ± 2.3 SEM) and 3 patients were taking no medication. Two patients were taking azathioprine (100 and 150 mg/day), and 2 patients were taking CellCept (2 g daily). Controls were taking no medication during this study and had no history of collagen vascular disease.

Collection and culture of T cell enriched peripheral blood mononuclear cells. T cell enriched mononuclear cells were isolated from controls and patients. Blood samples were collected and immediately separated by density gradient (Histopaque, Sigma, St. Louis, MO, USA). Mononuclear cells were separated further into CD2+ cells via sheep red blood cell rosetting and density gradient[3]. Greater than 95% of the isolated cells expressed the CD3 T cell marker when tested by flow cytometry. T cell enriched mononuclear cells were cultured in serum-free medium (QBSF 56 Serum-Free Medium; Sigma) without and with 2-fluoroestradiol (Steraloid, Wilton, NH, USA) for 18 h as described[3,4]. We chose an 18 h incubation period for this study because previous experiments using lupus T cells showed this time of culture in medium containing estradiol increased calcineurin and phosphatase activity[3,4].

Measurement of cell surface CD40L. T cells (1×10^6/ml) were cultured for 18 h in medium without and with 2-fluoroestradiol (10^{-7} M). This dose was selected because it was found optimal for upregulation of calcineurin expression[3,4]. To analyze CD40L cell surface expression, nonspecific sites were blocked by incubating the cells in human IgG for 5 min. Samples were then incubated with anti-CD40L (Ancell, Bayport, MN, USA) conjugated to R-phycoerythrin for 45 min at 4°C. Cells were washed twice after incubation with cold phosphate buffered saline (PBS), and suspended in PBS (1 ml) for flow cytometric analysis. At least 30,000 cells per sample were counted using FACS analysis with gate settings for T cells and to eliminate cellular debris. The results from each sample were reported as the peak shift of mean intensity of orange fluorescence[17].

Measurement of CD40L mRNA. Total RNA was isolated from T cells after appropriate treatments. cDNA were synthesized in 20 μl from 2 μg of total T cell RNA using an oligo (dT) primer (0.5 μg/μl) and MMLV reverse transcriptase (RT) as described[25,26]. The primers selected for G3PDH (sense, 5'-GAGTCAACGGATTTGGTCGT-3', antisense, 5'-TTCCCGTTCTCAGCCTTGAC-3') and CD40L (sense, 5'-ACATACAACCAAACTTCTCC-3' and antisense, 5'-AGATGTTGTTTTACTGCTGGC-3') included at least one intron to produce different size, or no products if any residual genomic DNA was present[26]. cDNA were amplified by polymerase chain reaction (PCR) using 1 μl (G3PDH) and 2 μl (CD40L) of template. Initial experiments determined that amplification was within the linear range under these conditions for 28–30 cycles. PCR reactions were denatured at 94°C for 30 s, annealed at 60°C for 30 s, and elongated at 72°C for 1 min for 24 cycles. The amplified products were separated on 2% (wt/vol) agarose gels and stained with ethidium bromide. G3PDH and CD40L PCR products from each T cell sample were analyzed on the same gel by scanning densitometry. The relative amount of CD40L was divided by the relative amount of G3PDH in the same reaction to adjust for assay variability. To verify authenticity as CD40L, the PCR product was eluted from the gel and subcloned into the pGEM T vector (Promega). Three independent clones were sequenced using methods standard in our laboratory[3,25]. All 3 clones had identical sequence to CD40L. CD40L mRNA was measured in T cells cultured for 18 h without and with estradiol and the differences between the T cells from the same individuals

were compared. Since CD40L in lupus and normal resting T cells is low[17,18], we did not measure CD40L in untreated T cells at baseline.

Estrogen receptor (ER) antagonism. SLE T cells were cultured for 18 h in medium without or with 2-fluoroestradiol (10^{-7} M) and with 2-fluoroestradiol (10^{-7} M) plus 10-fold molar excess (10^{-6} M) of ICI 182,780 as described[3,4].

Statistical analyses. Mean differences among treatments were determined by Student's t and paired t tests as appropriate. A p value < 0.05 was considered significant.

RESULTS

Estrogen increases CD40L density on T cells of female lupus patients. Lupus T cells (n = 13) cultured in medium containing estradiol showed a significant (p = 0.04) increase in the density (peak shift of fluorescence intensity) of CD40L on their cell surface compared to the same lupus T cells cultured without estrogen (Figure 1). Estrogen did not significantly (p = 0.70) increase the density (peak shift) of CD40L on the surface of normal female T cells (n = 6) cultured in estrogen compared to the same T cells cultured without the hormone. Cell viability of lupus and normal T cells after culture without and with estradiol was similar by trypan blue analysis, confirming our earlier observations[3,4]. Estradiol did not increase the number of CD40L positive T cells in either the patients or controls (data not shown).

The relative amount of CD40L mRNA after 18 h of culture in estradiol was determined by semiquantitative PCR (Figure 2). No apparent difference in the amount of CD40L mRNA was evident in normal female T cells after culture in estrogen (lanes 1 and 2). However, an apparent increase in CD40L mRNA was suggested in the lupus female T cells cultured in

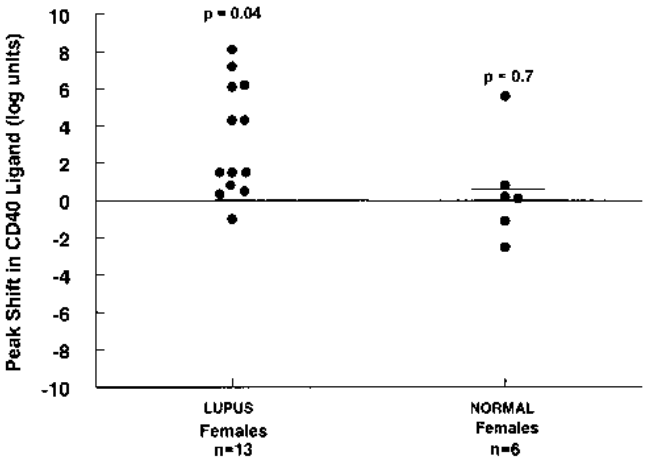

Figure 1. Estrogen increases the density of CD40 ligand (CD40L) on the surface of SLE T cells. T cells were cultured without and with 2-fluoroestradiol for 18 h. The difference in cell surface CD40L expression was compared by FACS analysis. Data shown are the differences in CD40L on the surface of the same T cells after culture without and with estrogen. There was significantly (p = 0.040, paired t test) more CD40L on SLE T cells cultured with estradiol compared to the same T cells cultured without estradiol. The differences in the amount of CD40L on the surface of SLE T cells after culture without and with estradiol was significantly greater (p = 0.048, Student's t test) than those differences on normal female T cells. Horizontal lines indicate the mean peak shift of fluorescence intensity for each group.

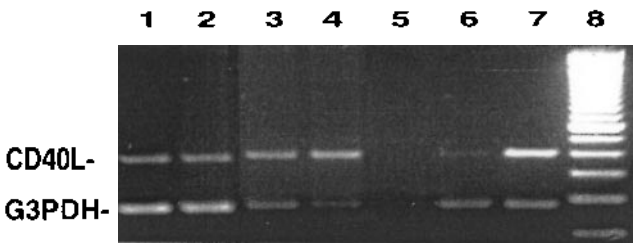

Figure 2. The amount of CD40L mRNA in T cells cultured for 18 h without and with 2-fluoroestradiol. The relative amount of CD40L mRNA in human T cells was determined by semiquantitative PCR. PCR products for CD40L and G3PDH from the same template were analyzed on agarose gel stained with ethidium bromide. A representative gel is shown. Lane 1, normal female; Lane 2, normal female plus estradiol; Lane 3, lupus female; Lane 4, lupus female plus estradiol; Lane 5, no template (negative control); Lane 6, normal female resting T cell; Lane 7, normal female activated (PMA plus ionomycin) T cell (positive control); Lane 8, 100 base pair size standard (Gibco-BRL).

estradiol (compare lanes 3 and 4). To provide more quantitative information about estrogen effects on CD40L expression, the relative amount of CD40L mRNA was measured from multiple samples (Figure 3). Although most lupus T cells showed a positive trend towards increased CD40L mRNA in response to estradiol, the differences were not statistically significant (p = 0.30). In the controls, estradiol did not significantly (p = 0.66) increase CD40L mRNA.

Estrogen increases CD40L mRNA in SLE T cells activated by anti-CD3. CD40L is only transiently expressed on activated T cells. We therefore reasoned that estrogen dependent differences in CD40L mRNA may occur earlier than cell surface expression we measured at 18 h. We also postulated that an activation stimulus would be required to maximize changes in CD40L expression. To test these postulates, T cells were cultured for 18 h in medium without and with estradiol to preload the cells with calcineurin[3,4]. The T cells were then activated by further culture in the respective media on anti-CD3 coated plates. RNA was isolated 2 h after the start of anti-CD3 stimulation. Activated SLE T cells had significantly (p = 0.046) more CD40L mRNA after culture in medium containing estradiol than did T cells from the same patients cultured without estradiol (Figure 4). Estradiol did not significantly (p = 0.6) alter the amount of CD40L mRNA in activated T cells from controls.

The estrogen dependent increase in CD40L expression is mediated by the estrogen receptor. T cells from patients with lupus cultured in medium containing 2-fluoroestradiol plus 10-fold molar excess of the estrogen receptor antagonist ICI 182,780 did not show the estrogen dependent increase in the density of CD40L protein on the cell surface as measured by flow cytometry (mean peak fluorescence). The mean percentage increase in the density of CD40L in the presence of estrogen was 15% and in the presence of estrogen plus ICI 182,780 was 2.5%. The estrogen dependent increase of CD40L mRNA was also blocked in SLE T cells cultured with estradiol plus ICI 182,780 for 18 h and then stimulated with anti-CD3 for 2 h. Mean relative values of CD40L/G3PDH without estrogen were 0.848, with estradiol 0.879, and with estradiol plus ICI 0.840.

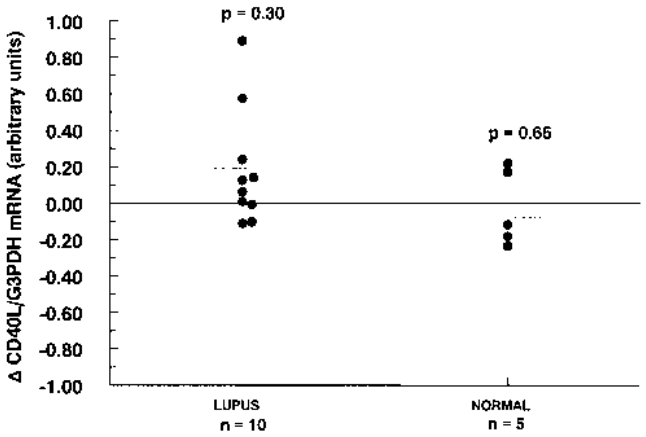

Figure 3. Relative CD40L messenger RNA (mRNA) levels in T cells cultured for 18 h in medium without and with 2-fluoroestradiol. The differences in CD40L mRNA in the same T cells cultured without and with estradiol were not statistically significant (p = 0.3, SLE T cells; p = 0.66 normal T cells, paired t tests). Differences between CD40L mRNA in T cells cultured without and with estradiol were not significantly different between SLE and normal females (p = 0.60, Student's t test). Data are the adjusted values for each sample. Horizontal lines indicate the mean values for each group.

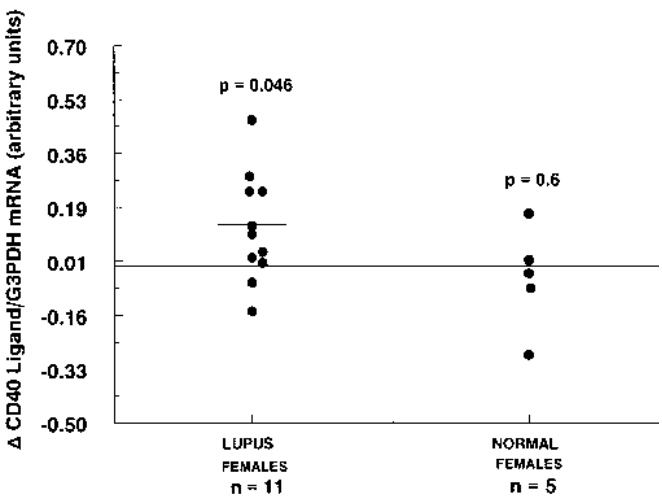

Figure 4. Estrogen increases CD40L mRNA in anti-CD3 activated female SLE T cells. T cells were cultured 18 h in medium without and with estradiol. Cells were activated for 2 h by culture on anti-CD3 coated plates and differences were compared. There was significantly (p = 0.046, paired t test) more CD40L mRNA in SLE T cells after culture with estradiol than the same T cells cultured without estradiol. Differences in T cells cultured without and with estradiol between SLE and normal T cells were not significant (p = 0.11, Student's t test). Data shown are the adjusted values for CD40L mRNA from each sample. Horizontal lines indicate the mean values within each group.

Activation of the estrogen sensitive target calcineurin in SLE T cells is not sufficient to increase CD40L mRNA. Calcineurin is a calcium and calmodulin dependent protein phosphatase of the PP2B class[23]. Since we had described calcineurin as an estrogen sensitive target in SLE T cells[3,4], we wanted to test if an increase in intracellular calcium by exposure to ionomycin was sufficient to activate calcineurin and stimulate expression

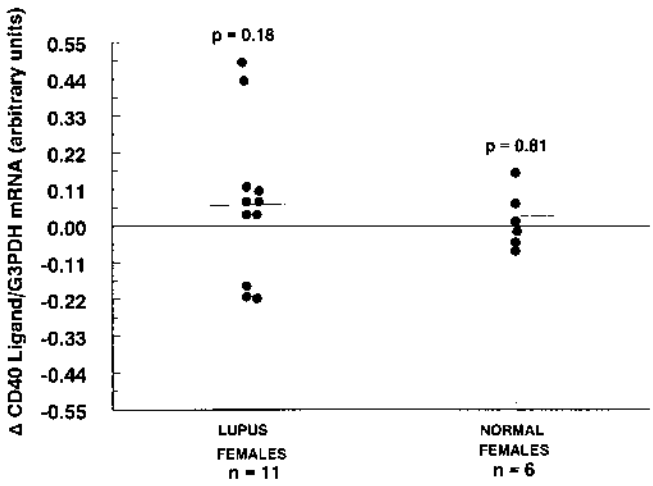

Figure 5. Relative amount of CD40L messenger RNA (mRNA) in T cells cultured without and with estradiol for 18 h and then stimulated with ionomycin (50 ng/ml) for 2 h. Data shown are the adjusted values for CD40L mRNA from each sample. There were no significant differences in CD40L mRNA within (SLE T cells, p = 0.18; normal T cells, p = 0.81, paired t tests) or between (p = 0.31, Student's t test) groups. Horizontal bars indicate mean values within each group.

of CD40L mRNA. T cells were cultured for 18 h without and with estradiol to preload the cells with calcineurin and cultured further in ionomycin for 2 h. There were no statistically significant differences in CD40L mRNA in the T cells from SLE patients (p = 0.18) and controls (p = 0.81) cultured under these conditions (Figure 5). Moreover, the estrogen dependent differences in the amount of CD40L mRNA between SLE and normal T cells after ionomycin stimulation for 2 h was not statistically significant (p = 0.31).

DISCUSSION

We have shown that estradiol, acting through the estrogen receptor, increases the expression of CD40L mRNA and protein density on the surface of SLE T cells. Estradiol does not significantly alter the number of SLE T cells expressing CD40L protein on the T cell surface. Estrogen also does not change the amount of CD40L mRNA and protein expressed by normal T cells. This study identifies CD40L as an estrogen dependent target in SLE T cells that serves as a marker for activation and, therefore, may play a role in the production of autoantibodies from B cells. Upregulation of CD40L on T helper cells contributes to B cell activation through interaction with CD40 on the B cell surface. Interaction of CD40-CD40L induces B cell proliferation, differentiation, and Ig and autoantibody production[13,14]. In addition, CD40-CD40L interactions rescue B cells from apoptosis[27] and enhance the T helper-1 proinflammatory response[28]. Owing to the importance of CD40L in regulating the immune responses, our results may contribute to understanding of the hormone dependent mechanisms that influence autoimmune diseases with female predominance.

Female SLE T cells cultured for 18 h in medium contain-

ing estradiol showed a significant increase in the density of CD40L on the cell surface but not in the cellular levels of CD40L mRNA. This is probably due to a lag time between mRNA synthesis and transport of the CD40L protein to the cell surface. Our interpretation is consistent with a report[29] showing low correlation between transcript levels and cell surface expression in patients with SLE. Since CD40L is transiently expressed on activated T cells, it was important to determine if differential estradiol action on SLE T cells could contribute to increased CD40L expression reported in these cells[17,18]. Engagement of the TCR using anti-CD3 coated plates resulted in a significant estrogen dependent increase in CD40L mRNA at 2 h in SLE but not in normal T cells. We presumed that this increase in CD40L expression occurred through the calcineurin-NFAT-CD40L pathway. However, increasing the amount of free calcium, which should activate calcineurin, did not significantly increase CD40L mRNA under the same experimental conditions that were effective using anti-CD3 coated plates. These data suggest that activation of the calcineurin-NFAT-CD40L pathway may not be sufficient to fully stimulate CD40L expression. Sustained stimulation through TCR engagement may be required to evoke the increased CD40L response, since previous experiments showed that plastic-bound stimuli induced a relatively stable expression of CD40L protein that was apparently dependent on the continuous delivery of the stimulus[30].

This study shows a modest, but statistically significant estrogen dependent increase in SLE T cell CD40L mRNA 2 h after activation. However, by 18 h, estradiol substantially increased the density of CD40L on the surface of SLE T cells. These results suggest that additional signal transduction molecules[31], or secondary signal co-activator molecules such as CD28, may be necessary for estradiol to exert full stimulatory effects on CD40L expression. In normal activated T cells, the stability of CD40L mRNA has been shown to contribute to the overall expression of CD40L protein[32,33]. Estradiol enhancement of CD40L mRNA stability would be consistent with the known post-transcriptional regulatory effects of estrogen in numerous other cell types through mechanisms that increase the stability of target gene mRNA[34]. However, since the estrogen dependent effects on CD40L expression are not simply mediated through the calcineurin-NFAT pathway, additional mechanisms may be involved. Recently, Yi, *et al*[35] showed that in SLE T cell lines, unlike in T cell lines from normal individuals, extracellular signal-related kinase (ERK) was not downregulated during anergy. Continued phosphorylation of ERK, but not other proteins in the mitogen activated protein kinase (MAPK) family of kinases, was postulated to cause anergy resistance and prolonged expression of CD40L in lupus T cells, perhaps by increasing CD40L mRNA stability[35]. It is now important to determine if estrogen action contributes to, or is a target of, prolonged ERK phosphorylation in SLE T cells.

Additional possibilities must also be considered as sites for estrogen dependent regulation of CD40L expression. In tissues well characterized as estrogen targets, we[36] and others[37] have shown that estrogen can upregulate the expression of the AP-1 transcription factors Fos/Jun. If Fos/Jun is an estrogen sensitive target in SLE T cells, then increased MAPK activity upon TCR engagement could potentially stimulate AP-1 dependent transcription, leading to increased or prolonged transcription of CD40L and other cytokine genes. In addition, while estrogen may exert transcriptional or post-transcriptional effects on the CD40L gene, this steroid could also enhance the transport of CD40L protein to the cell surface. Estrogen has been shown to stimulate the secretion of other cytokines such as interferon-γ from lymphocytes[38,39]. If estrogen increases the rate of CD40L transport to the surface of SLE T cells, then the effects of estrogen on CD40L expression would be further amplified. Studies to test these possibilities are now warranted.

Our results suggest a possible role for estrogen in upregulating CD40L in SLE, thereby sustaining the autoimmune process[16]. This observation may be relevant for understanding the role of estrogen in contributing to the gender bias in multiple autoimmune diseases[40,41]. Several fundamental questions remain to be answered from our studies[3–5] (and this report) and others[1,6,42]. We know that one difference between normal women and women with SLE is the hyper-responsiveness of lupus T cells to exogenous estradiol. This altered sensitivity does not appear to be due to differences in the primary structure or to altered ligand binding characteristics of the estrogen receptor-α[5]. It is possible, therefore, that this sensitivity arises from unopposed effects of estrogen by other physiologic regulators or hormones. Alternatively, there may be certain "autoimmune" related changes such as aberrant protein phosphorylation[35,43] that alter the cellular milieu and provide the opportunity for SLE T cells to be upregulated by estrogen. It is also likely that genetic[44] and exogenous environmental factors[45] act in concert with estrogen and facilitate alterations in the T cell signal transduction pathways. It is now possible to delineate the molecular basis for estrogen sensitivity in SLE T cells. Such information is essential to increase our understanding of the gender differences in human autoimmune diseases.

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