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Environmental Exposure, Estrogen and Two X Chromosomes are Required for Disease Development in an Epigenetic Model of Lupus

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease primarily afflicting women. The reason for the gender bias is unclear, but genetic susceptibility, estrogen and environmental agents appear to play significant roles in SLE pathogenesis. Environmental agents can contribute to lupus susceptibility through epigenetic mechanisms. We used (C57BL/6 × SJL)F1 mice transgenic for a dominant-negative MEK (dnMEK) that was previously shown to be inducibly and selectively expressed in T cells. In this model, induction of the dnMEK by doxycycline treatment suppresses T cell ERK signaling, decreasing DNA methyltransferase expression and resulting in DNA demethylation, overexpression of immune genes *Itgal* (CD11a) and *Tnfrsf7* (CD70), and anti-dsDNA antibody. To examine the role of gender and estrogen in this model, male and female transgenic mice were neutered and implanted with time-release pellets delivering placebo or estrogen. Doxycycline induced IgG anti-dsDNA antibodies in intact and neutered, placebo-treated control female but not male transgenic mice. Glomerular IgG deposits were also found in the kidneys of female but not male transgenic mice, and not in the absence of doxycycline. Estrogen enhanced anti-dsDNA IgG antibodies only in transgenic, ERK-impaired female mice. Decreased ERK activation also resulted in overexpression and demethylation of the X-linked methylation-sensitive gene *CD40lg* in female but not male mice, consistent with demethylation of the second X chromosome in the females. The results show that both estrogen and female gender contribute to the female predisposition in lupus susceptibility through hormonal and epigenetic X chromosome effects and through suppression of ERK signaling by environmental agents.

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Keywords

Extracellular Receptor Kinase (ERK); Systemic Lupus erythematosus (SLE); Mouse

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, relapsing autoimmune disease afflicting 1.5 million Americans, 90% of whom are women [1]. SLE affects many organs among which are joints, skin, kidneys, heart, lungs, blood vessels and the brain. Disease ensues when abnormally functioning B and T lymphocytes form autoantibodies to DNA and nuclear proteins, resulting in immune complexes that cause inflammation and tissue damage. While the cause(s) of SLE are unknown, its etiology involves genes that confer susceptibility, as well as hormones and environmental factors [2, 3]. Evidence for a genetic contribution comes from familial aggregation in 20% of lupus cases, a higher concordance rate in monozygotic twins (~25%) compared to dizygotic twins (2%), and known lupus-associated polymorphisms in genes encoding HLA molecules, complement components, cytokines and programmed cell death proteins as well as others [4, 5]. Of the genetic factors predisposing to SLE, female gender is the strongest. The reason this autoimmune disease primarily affects women is poorly understood. Estrogen is thought to be one explanation for the gender dimorphism in lupus and is supported by data from animal models showing that disease is ameliorated by oophorectomy and exacerbated by estrogen administration [6]. However, estrogen is probably more important in disease severity [7] since the incidence of SLE still shows female gender preference in children (6:1 female: male ratio) and postmenopausal women (4:1) compared with men of the same age [8].

Another explanation for the female predominance in SLE may be the aberrant activation of immune response genes on the inactive X chromosome [9, 10]. Men with Klinefelter's Syndrome (47, XXY) have a higher incidence of lupus than men in the general population [11] while there is a striking absence of SLE in women with Turner's Syndrome (45, XO) suggesting that two X chromosomes may predispose to SLE [12]. Because males have one X chromosome while females have two, most of the genes on the second X chromosome in females are silenced by epigenetic mechanisms that include DNA methylation as well as histone deacetylation, trimethylation and ubiquitination [13, 14]. Inappropriate activation of immune genes on the normally silenced X chromosome, caused by DNA demethylation, may thus contribute to increased prevalence of SLE in women [10, 15]. One such X chromosome gene, *CD40LG*, encodes a B cell costimulatory molecule transiently expressed on the surface of activated T cells and is demethylated and overexpressed on T cells from women but not men with SLE [9, 10, 16]. In mice, CD40L plays an important role in promoting lupus-like pathogenic IgG auto-antibodies and kidney disease [17, 18].

Environmental agents can alter T cell gene expression through effects on DNA methylation, resulting in autoreactive T cells that promote autoimmunity. Evidence for an environmental component in SLE arises from observations that the majority of lupus cases are idiopathic, drugs such as procainamide, hydralazine and others as well as UV light trigger lupus-like autoimmunity [19], and the incomplete concordance between genetically identical twins [20]. The way environmental agents interact with the various genetic loci to induce lupus is unclear. However, work for our group showed that 5-azacytidine, an inhibitor of DNA-methyltransferase (DNMT1) activity, causes hypomethylation and over-expression of immune genes *ITGAL* (CD11a), *TNFSF7* (CD70), *KIR* genes and *CD40LG* in T lymphocytes ([9, 21–25]. In mice, adoptive transfer of experimentally demethylated murine T cells caused anti-dsDNA antibodies and lupus-like disease in the recipients [26, 27]. Furthermore, ERK pathway signaling is an important regulator of DNMT1 and is decreased

in hydralazine-treated T cells and in T cells from patients with idiopathic lupus [19]. Therefore, environmental agents that inhibit ERK signaling, its upstream regulator PKC- δ , or other conditions such as diet and aging, that impact DNMT1 activity may increase methylation-sensitive gene expression through epigenetic mechanisms to cause a lupus-like disease in genetically predisposed individuals [3, 28, 29].

The mechanism by which genes, hormones and environmental factors interact to cause lupus is unknown. Animal models of SLE have revealed a wealth of information about specific genes that can contribute to development of a spontaneous, lupus-like disease and the influence hormones have on disease development [30]. However, they cannot be used to address gene-environment interactions in SLE because in the existing animal models, the disease develops spontaneously and once begun, continues to progress without environmental input. We previously developed a transgenic mouse model with an inducible ERK pathway signaling defect that is sufficient to decrease DNMT1 expression, cause over-expression of methylation-sensitive genes in mature T cells and induce anti-dsDNA IgG antibody in C57BL/6J mice, a non-autoimmune prone mouse strain [31]. In the present study, we used a transgenic hybrid (C57BL/6J $\times$ SJL)F1 mouse strain, with the same inducible T cell DNA methylation defect but which also has lupus-susceptibility genes and develops a more severe lupus-like disease only with exogenously-induced transgene activation. We used this model to clarify the interaction of genes, gender, hormones, and environmental influences on SLE induction and female prevalence.

2 Materials and Methods

2.1. Animals

SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice bearing the TRE2-dnMEK and CD2-rtTA transgenes [31] were bred and maintained in a specific pathogen-free facility by the Unit for Laboratory Animal Medicine at the University of Michigan in accordance with National Institutes of Health and American Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Guidelines. The animals were housed in filter-protected cages and provided with standard irradiated PicoLab Rodent Diet 20 (LabDiet, Brentwood, MO) and water ad libitum. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

C57BL/6.dnMEK⁺.CD2rtTA⁺ mice were bred with SJL animals and the F1 progeny screened by PCR for the presence of both transgenes. Protein and hemoglobin in mouse urine was measured by Chemstrip 6 dipstick (Roche, Madison, WI). Four mg/ml doxycycline (DOX) (Sigma, St. Louis, MO)/5% glucose was administered in the drinking water of selected groups of mice. Where indicated, 6–8 week old female mice were oophorectomized and males were orchiectomized. The animals were allowed to recover from the surgery (approximately 4 weeks), before being used in an experiment.

2.2. Antibodies and Flow Cytometry

The following antibodies were used in this study: PE-Hamster anti-mouse CD154 (CD40L), PECy5-rat anti-mouse CD4, anti-CD11a (BD Pharmingen, Fullerton, CA), HRP-Goat anti-mouse IgG-Fc-specific (Bethyl Labs, Montgomery, TX), HRP-goat anti-mouse Ig (H+L) (Southern Biotech, Birmingham, AL) and mouse monoclonal anti-dsDNA (Chemicon Intl, Temecula, CA). The cells were stained, fixed in 2% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) as previously described [22].

2.3. ELISA

Mouse anti-dsDNA IgG antibodies were measured by ELISA as follows. Costar (Corning, NY) 96 well microtiter plates were coated overnight at 4° C with 10 µg plasmid dsDNA per ml PBS, pH 7.2. Two to five microliters of mouse sera or murine monoclonal anti-dsDNA antibody were added to each well and incubated overnight at 4° C. Bound anti-dsDNA antibody was detected using HRP-goat anti-mouse IgG and OneStep Ultra TMB substrate (Thermo, Rockford, IL) and measured at 450 nm in a SpectraMax Pro spectrophotometer equipped with Softmax Pro software (Molecular Devices, Sunnyvale, CA). Mouse anti-dsDNA IgG antibody levels were confirmed using Alpha Diagnostic ELISA kits (San Antonio, TX).

2.4. Estrogen

One mm pellets containing β-Estradiol (90 day release, 0.72mg/pellet) or placebo (Innovative Research of America, Sarasota, FL) were implanted using a trocar, under the skin on the left side of the necks of anesthetized, double transgenic male and female mice that had been surgically neutered. Ninety days later a second identical β-estradiol or placebo-containing pellet was implanted on the right side of the neck. At the end of the experiment, estrogen levels were measured in murine plasma using an Estradiol EIA Kit (Caymen Chemical Co, Ann Arbor, MI) according to the manufacturer's instructions.

2.5. RNA and DNA Isolation and Real-Time Quantitative RT-PCR

Total RNA and DNA were simultaneously isolated from bead-purified (Miltenyi, Auburn, CA) CD4⁺ T cells using a Qiagen All-Prep RNA/DNA/Protein Mini Kit (Qiagen, Valencia, CA). The RNA and DNA were quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Wilmington, DE). One microgram of total RNA per sample was used to synthesize cDNA using a Transcriptor First Strand cDNA Synthesis Kit and anchored oligo(dT)₁₈ primers (Roche, Indianapolis, IN) according to the manufacturer's instructions. Primers for murine mRNA were as follows: *CD11a*, F 5'-CGGGACGATTTTGTAACATAGGTC-3', R 5'-GCCCTGCTAAAACATTGTATCCAG-3'; *dnmt1*: F. 5'-GGAAGGCTACCT GGCTAAAGTCAAG-3', R. 5'-ACTGAAAGGGTGTCACTGTCCGAC; *CD70*: F. 5'-TGGCTGTGGGCATCTGCT-3', R. 5'-ACATCTCCGTGGACCAGGTATG-3'; *CD40lg*: F. 5'-ATACCCACAGTTCCTCCAGCTTT-3', R. 5'-TAGGACAGCGCACTGTTTCAAGT-3'; *kir11*: F. 5'-AGACTTTGTTCTGGCTCTGCTCCT-3, R. 5'-TTCCCAAGTTGTCTGACATC CTCT-3'; *β-actin*: F. 5'-TTGCTGACAGGATGCAGAAGGAGA-3, R. 5'-ACTCCTGCTTGC TGATCCACATCT-3. The primers were obtained from Integrated DNA Technologies (Coralville, IA). RT-PCR reactions to quantitate gene expression were carried out using QuantiTect Fast Start SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and Light-Cycler, 0.25U Uracil-DNA Glycolase (Roche) with 1µl template cDNA, 0.5µM forward and reverse primers in a total volume of 20 µl and amplified under the following conditions: 50° C 2 min., 95° C 10 min (94° C 15 s, 56° C 30 s, 72° C 30 s) 36 cycles, using a Rotor-Gene 3000 (Corbett Robotics, San Francisco, CA). Mice were genotyped for the presence of the dnMEK and CD2rtTA transgenes using genomic DNA isolated from tail snips (Qiagen Blood & Tissue Kit) using primers and PCR methods previously described [31]. Product formation was verified using melting curve analysis and by 2% agarose gel electrophoresis.

2.6. Bisulfite conversion and pyrosequencing CD40lg

Genomic DNA was isolated from CD4⁺ T cells using the DNeasy blood and tissue kit (QIAGEN), and then bisulfite treated using the EZ DNA Methylation-Gold kit (ZYMO Research, Irvine, CA). The pyrosequencing primers were designed using PSQ Assay Design

software (Biotage, Uppsala, Sweden). PCR was performed using PyroMark PCR kit (QIAGEN) under the following conditions: initial incubation 95° C for 15 min, then 55 cycles of 95° C for 15 s, 60° C for 15 s and 72° C for 30 s for the CD40L promoter. Biotinylated PCR products were immobilized on streptavidin-coated sepharose beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). 40 µl of binding buffer including 2 µl streptavidin-coated sepharose beads was added to 40 µl of PCR product and mixed using a shaker, incubating at room temperature for 10 min while agitating constantly to keep the beads dispersed. The biotinylated amplicon was purified and denatured using a PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden). The DNA was resuspended in 14 µl annealing buffer containing 0.5 µM sequencing primer in a 96-well PSQ96 plate (Biotage). The sequencing primers were allowed to anneal on a heat plate set to 85° C for 2 min then the samples were cooled to room temperature. After completion of primer annealing, sequencing was run on the Pyro Mark MD system (Biotage) with Pyro Gold Reagents (Biotage) according to the manufacturer's instructions. Data were analyzed using Pyro Q-CpG Software (Biotage). Statistical analysis was performed using Student's t-test.

CD40lg pyro-PCR primers: F: 5'-GGGAAAGTTTGGGAAGTGAATGATA-3', R: Biotin-5'-CAACAAAAACACAAATCCAATCA-3', sequencing primer 1: 5'-TTTGTGGGATAGAAGATTA-3', sequencing primer 2: 5'-AGTTTTTAGTTAGTATGATAGA-3'.

2.7. Cell Culture

Spleen cells were isolated and cultured for 24 hr with 5 µg/ml concanavalin A (Sigma Chem Co, St. Louis, MO) in RPMI 1640 medium containing 10% fetal bovine serum, 1% Pen strep (GIBCO, Grand Island, NY), 100 mM HEPES, 4 mM glutamine at 37° C in 5% CO₂ balanced air. For *CD40lg* expression, the cells were washed and cultured for an additional 6 hr with fresh medium containing 5 ng/ml phorbol-13-myristate 12-acetate (PMA) and 500 ng/ml ionomycin. In some experiments, spleen cells were stimulated overnight with Con-A and treated with 5µM 5-azacytidine (5-azaC) for 4 days. CD4⁺ T cells were isolated using magnetic bead technology (Miltenyi Inc., Auburn, CA). Cell pellets were stored at -80° C for later RNA and DNA extraction.

2.8. Histopathology

Female and male (C57BL/6 × SJL)F1 mice with the dnMEK and CD2rtTA transgenes were treated for 16 wk with 4mg/ml DOX/5% sucrose in their drinking water. Control groups included double transgenic littermates treated with 5% sucrose alone and littermates lacking transgenes but treated with the DOX/sucrose or sucrose alone. At the end of an experiment, the animals were terminated and their kidneys bisected. Half of the tissue was embedded in O.C.T. (Thermo Fisher) and snap frozen in liquid nitrogen while the other half of the kidney was fixed in 10% formalin, paraffin embedded and stained with H&E. Five micron sections were cut from the frozen tissue and fixed for 10 minutes in ice cold acetone. Non-specific sites were blocked with 10% horse serum/PBS and the sections were stained for mouse IgG deposits using a 1:50 dilution of biotin-Goat anti-mouse IgG(Fc specific) antibody (US Biologicals)/FITC-Streptavidin (BD Pharmingen).

2.9. Statistical analysis

Student's *t*-test, linear regression or ANOVA as appropriate, were used to determine the significance of differences between groups using SYSTAT software on a Dell PC Optiplex 745 microcomputer.

3. Results

3.1. Influence of gender on serum anti-dsDNA antibody

The treatment protocols and results for our study are summarized in Table S1. Male and female dnMEK⁺CD2rtTA⁺(C57BL/6 × SJL)F1 mice were given water with DOX /5% glucose or 5% glucose for 4 – 24 weeks. Control groups included littermates lacking both transgenes but treated with the DOX/sucrose or sucrose alone. DOX treatment induced anti-dsDNA IgG antibody in double transgenic female animals compared to non-DOX treated controls ($p=0.01$, Fig. 1A). In contrast, DOX treatment failed to induce anti-dsDNA antibody in double transgenic male mice ($p>0.05$ male DOX⁺ vs. DOX⁻, Fig 1B). In the absence of the transgenes, no anti-dsDNA antibody was induced by DOX treatment of male or female littermate controls (Fig 1B). The increase in anti-dsDNA IgG antibody in female versus male DOX⁻treated mice ($p<0.005$ by regression analysis) was not due to polyclonal Ig elevation since there was no difference found in the amount of total IgG in the sera of any of the groups (not shown).

3.2. Pathology

The effect of impaired ERK signaling on lupus-associated glomerulonephritis was investigated. Kidneys from dnMEK⁺CD2rtTA⁺(C57BL/6 × SJL)F1 mice from Fig. 1 were bisected, half snap frozen and stained for IgG and half paraffin embedded and stained with H & E. In the absence of DOX treatment no IgG was found in the glomeruli of female double transgenic mice (Fig 2A) and no glomerulonephritis was found by light microscopic examination (Fig 2B). No IgG was present in the kidneys of double transgenic male, DOX-treated mice or any non-transgenic littermate (not shown), consistent with the lack of anti-dsDNA antibodies in these animals. In contrast, IgG was found in the glomeruli of all 4 DOX-treated female double transgenic mice (Fig. 2C and D) ($p=0.001$ DOX⁺ female vs. male). The kidneys of these animals exhibited a glomerulonephritis with glomerular hypercellularity including leukocyte infiltration and evidence of nuclear karyorrhexis (Fig 2E, arrows). Hemoglobin (50–100 erythrocytes/ μ l) was present in the urine of 4 of 7 female but only 1 of 5 male double transgenic, DOX-treated mice, but the differences were not statistically significant ($p>0.05$, data not shown). In the absence of the transgenes or DOX treatment, no hematuria was observed. Taken together, the data indicate that impaired ERK signaling induced lupus-like disease in genetically susceptible female but not male animals. Furthermore, neither antibody nor disease developed in animals that possessed susceptibility genes in the absence of impaired ERK signaling.

3.3. Gene Expression

We next tested whether impaired ERK signaling decreased DNMT1 gene expression with downstream effects on the expression of methylation-sensitive genes. Spleen cells from the mice shown in Figure 1 were stimulated with Con-A, cultured, then restimulated with PMA and ionomycin as described in materials and methods. CD4⁺ T cells were then isolated and gene expression measured by quantitative real-time PCR. CD4⁺ T cells from both male and female double transgenic mice made large amounts of dnMEK mRNA relative to actin (Fig 3A) in response to DOX treatment. Activation of dnMEK gene expression significantly ($p=0.03$) reduced DNMT1 mRNA (Fig 3B) compared to their untreated controls. Reduced DNMT1 levels resulted in overexpression of the methylation-sensitive autosomal genes encoding CD70 (Fig 3C) and CD11a (Fig 3D) in these same T cells from both female and male transgenic mice (Fig 3). Since male and female CD4⁺ T cells showed comparable patterns of gene expression in response to DOX treatment, we hypothesized that other, gender-related factors were responsible for the female restricted anti-dsDNA antibody and glomerular nephritis. Therefore, we further investigated the role of sex hormones and X chromosome genes in the female predominance of SLE.

3.4. Estrogen

To examine the role of estrogen in our model, male and female double transgenic mice were neutered, implanted with time-release pellets delivering placebo or 17 β -estradiol and given DOX/5% sucrose or 5% sucrose alone in their drinking water. Blood and urine were collected at 4 week intervals for 20 wks. Anti-dsDNA IgG antibody results are shown in Fig. 4. Estrogen levels were measured in serum at final (20 wk) time point. Normal estradiol levels in mouse serum range from 14 – 104 pg/ml depending on the estrus cycle and age of the mouse [32]. Sham operated, control (hormonally intact) female mice had 15 pg estrogen/ml serum. No estrogen was detectable in the sera of male controls. Similarly no estrogen was detectable in the sera of neutered female or male mice implanted with placebo-containing pellets. Oophorectomized females implanted with estrogen pellets had 144 $\pm$ 40.8 pg/ml (mean $\pm$ S.D., n=5) serum estrogen. Orchiectomized males implanted 17 β -estradiol pellets had comparable levels of estrogen (126.8 $\pm$ 31.2 pg/ml, n=3) in their serum. DOX-treated female mice exhibited glomerular nephritis but the kidneys of both neutered female and male, estrogen-treated mice were badly damaged by severe hydronephrosis (not shown). Both neutered female and male estrogen-treated mice developed bladder outlet obstruction and hydronephrosis when treated with high dose estrogen for prolonged periods of time [33–35]. The pathologic effects of high dose estrogen is unique to mice as other species do not develop these conditions [36] and may be due to regulation of neuronal nitric oxide synthase [37].

DOX induced high levels of anti-dsDNA IgG antibodies in neutered, estrogen-supplemented female but not male mice ($P < 0.001$, Fig. 4). No anti-dsDNA antibody was observed in DOX-treated intact or neutered, placebo-treated male transgenic mice. In the absence of DOX treatment, high levels of estrogen had a slight but significant effect on anti-dsDNA antibody levels in female mice compared to either DOX-treated ($p = 0.003$) or untreated ($p = 0.048$) male mice. These results, taken together, demonstrate that estrogen and environmental factors that impair ERK signaling act synergistically to enhance anti-dsDNA antibody. However, female gender, independent of estrogen, is also required for SLE in this model.

3.5. X-chromosome gene expression and methylation

Since females have two X chromosomes and demethylation of genes on the second X may be responsible for the predominance of lupus in women, we examined the expression of the X-linked, methylation-sensitive *CD40lg* gene in our mouse model of SLE [9, 38]. DOX-treated female mice had twice the level of CD40L protein on their CD4⁺ T cells compared with male animals (MFI 30.85 $\pm$ 5.65 vs. 14.72 $\pm$ 0.94, $p = 0.02$, n=4/group), consistent with demethylation of the second X chromosome in the females (Fig. 5A). We therefore examined the methylation status of the *CD40lg* gene in these T cells using pyrosequencing. Methylation patterns of the *CD40lg* promoter were compared in CD4⁺ T cells from male and female mice treated for 20 wk with DOX or vehicle. The genomic organization of the human and murine CD40L genes are identical [38] and the region of the CD40L gene from –250 to +405 bases relative to the transcription start site (TSS) was 87% homologous between mice and humans. We examined seven CG pairs in the murine *CD40lg* promoter within 150 bases of the transcription start site. Three of these CG pairs, –35, –43, and –46 5' of the TSS are homologous to CG pairs in the human *CD40LG* promoter and are demethylated in T cells from female lupus patients and 5-azaC treated cells from healthy women [9]. The first 4 CG pairs 5' to the *CD40lg* TSS, which included all three human homologs, were demethylated in the female double transgenic mice treated with DOX (74.2 $\pm$ 0.86% vs. 65.2 $\pm$ 1.4% methylated, n=4/group, $p = 0.003$ by regression), Fig 5B. *In vitro* treatment of CD4⁺ T cells with 5-azaC confirmed that the four CG pairs 5' to the TSS were methylation-sensitive in female (Fig. 5C) but not male (Fig 5D) T cells. *CD40lg* was

constitutively less methylated in the males ($38.2 \pm 2.7\%$, $n=3/\text{group}$, $p=1.3 \times 10^{-6}$ vs. females) and did not demethylate further with 5-aza-C or DOX treatment (not shown). We previously reported that demethylation of the human *CD40LG* promoter in this region increases *CD40LG* gene expression and protein in CD4^+ T cells from women [9]. Thus *CD40lg* in mice, like humans, appears to be methylation-sensitive and may increase gene expression exclusively in females through DNA hypomethylation.

We further explored the activation of genes on the inactive X chromosome in the female lupus mice by examining the expression of a second gene, *Kir1l*, the mouse homolog to the human *KIR2DL2* gene [39]. Human *KIR* genes are located on autosomes and are not expressed on normal CD4^+ T cells, but their expression is upregulated in CD4^+ T cells from healthy women by the irreversible DNA methyltransferase inhibitor 5-azaC, and in women with SLE [21, 40]. However, *kir1l* is located on the X chromosome in mice [39]. We found that *kir1l* mRNA was overexpressed in CD4^+ T cells from ERK-impaired double transgenic female mice (DOX^+ 1.96 ± 0.4 vs DOX^- , 0.95 ± 0.18 , mean $\pm$ SEM, $n=4-8/\text{group}$, $p=0.02$). However, *kir1l* expression was not upregulated in male CD4^+ T cells (DOX^+ 0.5 ± 0.4 vs DOX^- 0.26 ± 0.17 , $p=0.16$), but the difference in *kir1l* expression between male and female mice receiving DOX was significant ($p=0.02$). The aberrant expression in mice of *kir1l* mRNA in female but not male CD4^+ T cells further supports our hypothesis that X chromosome genes play a role in determining female gender susceptibility to SLE.

4. Discussion

T cell DNA methylation is impaired in lupus, causing aberrant overexpression of genes that make the cells pathogenic, both in animal models and in human SLE [19]. DNA methylation patterns are maintained by DNMT1, which is regulated in part by ERK signal transduction [41–43]. We therefore used a transgenic C57BL/6 mouse model in which ERK signaling was inducibly impaired. The dnMEK transgene contains three mutations - substitutions of serines 218, 222 and lysine 97 to alanine - and a tetracycline response element. A second transgene, CD2rt-TA, containing a reverse tetracycline transactivator under the control of a CD2 promoter restricts the dnMEK activity to T cells. In the presence of DOX, the dnMEK causes a ~60% reduction in phosphorylation of its substrate ERK, only in T cells [31]. Using this model we previously reported that reduced ERK signaling lowered DNMT1 mRNA levels, resulting in increased expression of the methylation-sensitive genes encoding *CD11a* and *CD70* and the formation of anti-dsDNA IgG antibodies in female mice. However, no kidney disease was observed because the C57BL/6 mice carrying the transgenes appear to lack genes that contribute to organ-specific disease observed in human lupus. Clinical manifestations of lupus-like disease in mice are strain-dependent, requiring additional genes for full disease development [30]. This situation is similar to hydralazine-induced autoimmunity in humans [44]. SJL mice possess lupus-susceptibility genes making them susceptible to chemically induced lupus but they do not spontaneously develop the disease [45, 46]. Therefore, we made dnMEK⁺CD2rtTA⁺(C57BL/6 $\times$ SJL)F1 hybrid animals and used them in the present study to investigate the role of gender on lupus disease when ERK was similarly impaired. Our results confirm that impaired ERK signaling resulted in increased IgG anti-dsDNA antibody in female double transgenic mice. They further showed that male mice failed to make anti-dsDNA antibody even when both transgenes were present and activated by DOX treatment (Figs. 1, 3). The presence of IgG anti-dsDNA antibody correlated with kidney disease, since no disease was observed in kidneys from the females in the absence of DOX, or in male mice either in the presence or absence of DOX. DOX activated dnMEK, decreased DNMT1 mRNA and enhanced *CD70* and *CD11a* gene expression in males comparable to female mice. Therefore the failure in males to produce pathogenic antibody must result from sex hormones or other gender-specific differences.

Sex hormones are thought to be responsible for the female predominance observed in SLE. The role of estrogen, which influences both humoral and cellular immunity, has been widely studied and particularly in the NZB/W F1 mouse model of spontaneous SLE [47]. Female NZB/W F1 females die of SLE more rapidly than do males [6, 48]. Autoantibody formation is accelerated by estradiol treatment of the females [6, 49] while tamoxifen, an estrogen blocker, ameliorates disease progression [50]. However, estrogen may be more important in mediating disease severity than incidence. In people, lupus tends to flare during pregnancy [51] and estrogen supplementation is associated with mild lupus flares [52]. Our observation that estrogen greatly enhanced anti-dsDNA antibody only in female mice when ERK was impaired supports this hypothesis.

While estrogen may increase the frequency and severity of lupus flares, sex hormones do not completely explain female predisposition to SLE. Lupus primarily affects women of child-bearing age (15–44 years), at a female to male ratio of 9:1. However, female prevalence is still seen in pre-pubescent girls [53] and post-menopausal women [8] indicating that other factors besides estrogen contribute to SLE [54, 55]. DNA demethylation may affect women more than men because women have two X chromosomes, one of which is silenced by epigenetic mechanisms including DNA methylation [13, 14]. Inappropriate activation of immune genes on the X chromosome may thus contribute to increased prevalence of SLE in women [10, 15]. One such X chromosome gene, *CD40LG*, encodes a B cell co-stimulatory molecule transiently expressed on the surface of activated T cells and is overexpressed on T cells from women but not men with SLE [9, 10, 16]. In mice, CD40L was shown to play an important role in promoting pathogenic IgG auto-antibodies and kidney disease [17, 18]. We found that ERK impairment resulted in overexpression of CD40L protein on CD4⁺ T cells in female but not male mice and that the level of CD40L protein was twice the amount in females compared to males. This finding is consistent with re-expression of the CD40L gene on the inactive X chromosome and similar to what is observed in 5-azaC treated T human cells from healthy donors and in CD4⁺ T cells from lupus patients [9, 10]. Pyrosequencing of DNA from our ERK-impaired murine CD4⁺ T cells revealed that the promoter region of the mouse *CD40lg*, which is highly homologous with human *CD40LG* [9, 38, 56, 57], was largely demethylated in male mice while the gene was only partly demethylated in females. Suppression of ERK activity or inhibition of DNA-methyltransferases resulted in further demethylation of sequences on the inactive X chromosome only in females. Because one chromosome is inactivated by epigenetic processes including DNA methylation, the methylated gene is from the inactive X. Three of the seven CG pairs analyzed in the murine promoter were homologous with human sequences and in humans these CG pairs were among those that regulated human *CD40LG* function [9]. Thus, changes in methylation of these residues suggest that they are transcriptionally relevant in mice as well as humans. *CD40LG* regulatory sequences demethylated by 5-azaC treatment in mice at the same CG sites as those demethylated in women and in women with lupus. In human CD4⁺ T cells, *CD40LG* function is also affected by methylation of CG pairs in an enhancer located 5' (–600 to –1000 TSS) of the TSS, CG pairs in the promoter, and a downstream (+11311 to +12959) enhancer [9, 57]. Murine sequences are highly (75%) homologous with the human DNA in the 3' enhancer region, however none of the eleven CG pairs in the human gene in this region were present in mice, so no further examination of this region was performed. After our initial report on methylation of the human *CD40LG* regulatory sequences, a second enhancer approximately 5' of the TSS in humans was reported [58] in a region highly homologous between mice and humans. These sequences may be important regulatory regions for future study.

Demethylation and activation of other genes besides *CD40LG* on the inactive X chromosome may also contribute to female predominance of lupus. We have previously identified the *KIR* gene family as methylation-sensitive genes whose inappropriate

expression in human CD4⁺ T cells is associated with SLE [21, 22]. *KIR* genes constitute a polymorphic family whose products are normally expressed on NK cells, but are aberrantly expressed on T cells by experimental demethylation or in lupus [21]. Expression of KIR2DL4 and KIR3DL1 proteins on T cells contributes to IFN- γ release and macrophage killing in human lupus. While *KIR* genes are located on autosomal chromosomes in humans, the murine homolog of human *KIR3DL1*- the *KIR*-like gene (*kirl1*) – is located on the X chromosome in mice [59, 60] [39]. *Kirl1* mRNA was upregulated in female but not male mice when their ERK signaling was impaired (Fig 2E). Therefore, increased expression of lupus-associated immune genes can contribute to development of the disease and the presence of the genes on the X chromosome would cause overexpression in females alone.

Abnormal X chromosome gene dosage has been linked to autoimmune disease pathogenesis ([61, 62]. Some autoimmune diseases, such as systemic sclerosis, autoimmune thyroid disease and primary biliary cirrhosis are characterized by haploinsufficiency of X chromosome genes particularly in T and B lymphocytes [63, 64]. However, haploinsufficiency was not observed in SLE [65]. Rather, the opposite occurs in that women with Turner's syndrome (45, X0) show increased susceptibility to autoimmune disease, in particular autoimmune thyroiditis but have a strikingly decreased susceptibility to SLE [12]. In contrast, men with Klinefelter's syndrome possess an extra X chromosome (47, XXY) and have a similar risk as women for developing SLE [12]. Together, these reports support our hypothesis that epigenetic activation of genes on the inactive X chromosome is a major mechanism for female predominance of lupus autoimmunity.

The incidence of many autoimmune diseases including SLE, increases with age [62], while at the same time the percent of methylated genes declines [66]. Early studies showed that patients with SLE and RA exhibit globally hypomethylated DNA ([67]. Javierre et al. [20] recently reported the use of high through-put genome-wide sequencing techniques to study difference in DNA methylation between lupus affected and unaffected monozygous twins. They clearly showed that numerous genes are hypomethylated in SLE affected twins compared to their unaffected twin controls. Our data in mice support a role for environmental factors in lupus development. They further support the role of DNA demethylation as a mechanism in initiating SLE. Unfortunately analysis performed by Javierre et al. [20] did not include any of the genes described in our studies. An earlier study of SLE in monozygous twins by Huang et al. [68] failed to find differential methylation of the androgen receptor gene, located on the X chromosome using methylation-sensitive restriction enzymes. Although the subjects in this study exhibited some of the symptoms associated with lupus, four of the five monozygous affected twins failed to meet the full criteria of SLE diagnosis. Javierre et al. [20] found evidence of differential methylation of the androgen receptor gene between SLE and unaffected monozygous twins but the methylation levels of this gene varied greatly between pairs of twins. Their subjects met the full criteria of SLE but differences in methylation of the gene may reflect differences in disease activity. Another potential source of variability is the cell type studied. Monosomy in X chromosome genes was more frequently observed in T and B lymphocytes than in monocytes and cells of the innate immune system in both systemic sclerosis and autoimmune thyroiditis [64]. The studies by Huang et al. [68] and Javierre et al. [20] used unseparated PBMC. We restricted our investigations to T lymphocytes because they are necessary and sufficient to cause lupus-like disease in mice. None-the-less, we observed heterogeneity in T cell subsets and their expression of methylation-sensitive genes [21, 69]. Together, these observations highlight the importance of factors such as diagnosis, disease activity, the selection of tissue, and heterogeneity of cells in the tissue in understanding the role of genes and their expression in autoimmunity.

In conclusion, our results show that SLE requires a complex interaction between genes, hormones, and the environment. Like human SLE, development of autoimmunity in this model requires 2 X chromosomes, estrogen and reduced ERK signaling induced by environmental agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

1. We examined how genes, environment, and hormones influence female lupus susceptibility.
2. Environmental agents suppress ERK signaling, epigenetically activating immune genes.
3. Transgenic female but not male mice with an inducible ERK defect developed lupus.
4. Suppressing ERK epigenetically activated X chromosome CD40L and Kirl1 genes in females.
5. Female predisposition required estrogen, two X chromosomes and ERK suppression.

Fig 1.

Effects of decreased ERK signaling and gender on anti-dsDNA antibody. A. IgG anti-dsDNA antibody was measured in male (M) and female (F) (C57BL/6 × SJL)F1 mice bearing the dnMEK and CD2rtTA transgenes (+/+) and given 4 mg/ml DOX/5% glucose (DOX+) or 5% glucose alone (DOX-) in their drinking water for 4–24 weeks. B. Comparison between groups after 24 weeks of treatment. (*) $p=0.01$ Female (+/+) DOX+ vs. DOX-; (**) $p<0.005$ DOX-treated Female vs. Male (+/+). Mean $\pm$ S.E.M. 3–5 mice per group. Data are representative of 2 independent experiments.

Fig. 2.

Glomerular IgG immune complex deposition in transgenic mice. Kidneys from female and male (C57BL/6 $\times$ SJL)F1 mice from Fig. 1 were bisected and half of the tissue snap frozen, the remaining half fixed in 10% formalin and paraffin embedded. Five micron sections of frozen tissue were stained with biotin-goat anti-mouse IgG (Fc specific) and FITC-streptavidin. Sections from formalin-fixed, paraffin embedded tissue were stained with H&E and examined by light microscopy. (A), Immunofluorescence (B) light microscopy of glomeruli from DOX-transgenic female mice. (C) 10 $\times$ magnification of a section from a representative kidney of DOX+ female mice, (D) 40 $\times$ view of area in box from (C), (E) light micrograph showing histopathology of a glomerulus from a DOX+ female mouse. Arrows indicate areas of abnormal histopathology. Sections shown are representatives of 5 mice per group.

Fig. 3.

Effect of reduced ERK signaling on gene expression in male and female CD4⁺ T cells. Male and female transgenic mice were treated for 1 week with DOX or vehicle. Their spleen cells were isolated, treated for 24hr with Con-A, CD4⁺ T cells isolated then (A) dnMEK, (B) DNMT1, (C) CD70, and (D) CD11a, were measured by quantitative RT-PCR relative to β -actin. Mean $\pm$ S.E.M. from 5 mice per group.

Fig. 4.

Estrogen and two X chromosomes are required to make anti-dsDNA antibody in response to impaired ERK pathway signaling. Neutered and estrogen or placebo supplemented dnMEK⁺CD2rtTA⁺ transgenic mice were given DOX/5% glucose (DOX+) or 5% glucose alone (DOX-) in their drinking water for 20 wks. $P < 0.001$ female, estrogen treated DOX+ vs. DOX-. Mean $\pm$ S.D. of 3-5 mice per group.

Fig. 5.

X chromosome gene CD40L expression and demethylation in CD4⁺ T cells. (A) CD4⁺ T cells from dnMEK⁺CD2rtTA⁺ male and female DOX-treated (DOX+) or control (DOX-) mice were stained with FITC-CD40L antibody then mean fluorescence intensity was analyzed by FACS and normalized to the males ($p=0.02$ female vs male in the DOX-treated cells). (B) The promoter region of the CD40L gene from female DOX+ transgenic mice. (C) spleen cells from female or male (D) mice were stimulated with Con-A, treated with 5Azacytadine, and 4 days later CD4⁺ T cells isolated and their DNA pyrosequenced to confirm demethylation of the *CD40lg* promoter region. Data shown are from a representative of 3 experiments of 3–5 mice per experiment. Values are Mean $\pm$ S.E.M. of 3–5 mice per group. CG pairs are numbered relative to the transcription start site described by Tsitsikov et al. [38].

Murine C57BL/6 *CD40lg* accession number VERSION NT_039706.7 GI:149271867 from the NCBI GenBank <http://www.ncbi.nlm.nih.gov/nucleotide/149271867>.