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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/6xSJL)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 *Tnfsf7* (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.

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 auto-antibodies 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

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of SLE still shows female gender preference in children (6:1 female:male ratio) and post-menopausal 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 Kleinfelter'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 ubiquination [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 co-stimulatory 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 overexpression 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 overexpression 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/6JxSJL)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 CD2rtTA 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 oophroctemized 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), PE-Cy5-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 FACS-Calibur 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, 0.72 mg/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