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Diet Influences Expression of Autoimmune-Associated Genes and Disease Severity by Epigenetic Mechanisms in a Transgenic Mouse Model of Lupus

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Objective. **Lupus flares occur when genetically predisposed individuals encounter appropriate environmental agents. Current evidence indicates that the environment contributes by inhibiting T cell DNA methylation, causing overexpression of normally silenced genes. DNA methylation depends on both dietary transmethylation micronutrients and ERK-regulated DNA methyltransferase 1 (DNMT-1) levels. We used transgenic mice to study the effect of interactions between diet, DNMT-1 levels, and genetic predisposition on the development and severity of lupus.**

Methods. **A doxycycline-inducible ERK defect was bred into lupus-resistant (C57BL/6) and lupus-susceptible (C57BL/6 × SJL) mouse strains. Doxycycline-treated mice were fed a standard commercial diet for 18 weeks and then switched to a transmethylation micronutrient–supplemented (MS) or –restricted (MR) diet. Disease severity was assessed by examining anti–double-stranded DNA (anti-dsDNA) antibody levels, the presence of proteinuria and hematuria, and by histopathologic analysis of kidney tissues. Pyrosequencing was used to determine micronutrient effects on DNA methylation.**

Results. **Doxycycline induced modest levels of anti-dsDNA antibodies in C57BL/6 mice and higher levels in C57BL/6 × SJL mice. Doxycycline-treated C57BL/6 × SJL mice developed hematuria and glomerulonephritis on the MR and standard diets but not the MS diet. In contrast, C57BL/6 mice developed kidney disease only on the MR diet. Decreasing ERK signaling and methyl donors also caused demethylation and overexpression of the *CD40lg* gene in female mice, consistent with demethylation of the second X chromosome. Both the dietary methyl donor content and the duration of treatment influenced methylation and expression of the *CD40lg* gene.**

Conclusion. **Dietary micronutrients that affect DNA methylation can exacerbate or ameliorate disease in this transgenic murine lupus model, and contribute to lupus susceptibility and severity through genetic–epigenetic interactions.**

Systemic lupus erythematosus (SLE) affects ~1.5 million Americans, 90% of whom are women (1). Lupus involves many organs, including the joints, skin, kidneys, heart, lungs, blood vessels, and brain. Disease ensues when abnormally functioning B and T lymphocytes form autoantibodies to DNA and nuclear proteins, resulting

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in immune complex deposition that causes 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 (1,2). Evidence for a genetic contribution comes from familial clustering of lupus cases in which siblings of lupus patients have a 10–20-fold higher risk than the general population of developing SLE, a higher concordance rate in monozygotic twins (20%) than in dizygotic twins (2%), and known lupus-associated polymorphisms in genes, including those encoding HLA molecules, complement components, cytokines, and programmed cell death proteins (3).

The discordance of SLE between monozygotic twins suggests that nongenetic factors may influence gene expression, triggering lupus (4). However, what these agents are and how they interact with the various predisposing genetic loci to induce lupus are unclear. DNA methylation and histone modifications regulate gene expression through epigenetic mechanisms (5). Drugs such as 5-azacytidine, procainamide, and hydralazine, as well as ultraviolet light, trigger lupus-like autoimmunity through their effects on DNA methylation, resulting in autoreactive T cells that promote autoimmunity (2). Reduced DNA methyltransferase 1 (DNMT-1) activity causes hypomethylation and overexpression of the immune genes, including *ITGAL* (CD11a), *TNFSF7* (CD70), *KIR* genes, and *CD40LG* in T lymphocytes (2,6).

ERK pathway signaling is an important DNMT-1 regulator, and ERK signaling is inhibited in T cells by hydralazine, and in T cells from patients with idiopathic lupus (2,7,8). Therefore, environmental agents that inhibit ERK signaling or its upstream regulator protein kinase Cδ, or other conditions, such as diet and aging, that can decrease DNMT-1 enzymatic activity may increase methylation-sensitive gene expression through epigenetic mechanisms to cause lupus-like disease in genetically predisposed individuals (2,9,10).

Diet is an important environmental component and influences gene expression in vivo. When administered to pregnant mice, diets rich in methyl donors can alter DNA methylation patterns and gene expression in developing embryos (11,12). Furthermore, dietary methyl donor supplementation can increase total genomic d^mC content in leukocyte DNA (13), while dietary restriction of methyl donors leads to DNA hypomethylation in vivo (14). Lupus patients have significantly reduced levels of methylation-associated micronutrients (15,16). We therefore tested the hypothesis that dietary micronutrients necessary for transmethylation influence lupus disease severity. We have previously developed a transgenic mouse model with an inducible T cell ERK pathway signaling defect that results in demethylation and overexpression of methylation-sensitive genes, causing the development of lupus-like autoimmunity in female mice (17). In the present study we used this model to examine the interaction of genes and micronutrients as a potential environmental influence on SLE disease activity and severity. We examined the effect of transmethylation micronutrient–restricted (MR) and transmethylation micronutrient–supplemented (MS) diets on the expression of methylation-sensitive T cell genes and lupus disease, using mice with the inducible T cell DNA methylation defect on a lupus-resistant (C57BL/6) or lupus-susceptible (C57BL/6 x SJL) hybrid genetic background.

MATERIALS AND METHODS

Animals. SJL/J mice were purchased from The Jackson Laboratory. C57BL/6 mice bearing the TRE-containing dominant-negative MEK (dnMEK) transgene were bred with C57BL/6 mice containing the reverse tetracycline *trans*-activator under the control of the CD2 promoter (CD2-rtTA). Double-transgenic (dnMEK+CD2rtTA+) mice inducibly express a dominant-negative MEK selectively in T lymphocytes in the presence of doxycycline, leading to ~60% reduction in ERK phosphorylation (17). In the absence of either transgene, doxycycline administration fails to reduce ERK phosphorylation.

Female double-transgenic mice with the following genetic backgrounds and characteristics were generated for the present study. The dnMEK+CD2rtTA+ parental (P0) strain C57BL/6 was anti–double-stranded DNA (anti-dsDNA) positive and lupus nephritis negative (17). The dnMEK+CD2rtTA+ F1 strain (C57BL/6 × SJL)F1 was anti-dsDNA positive and lupus nephritis positive (17,18). The dnMEK+/CD2rtTA+ F2 strain was (F1 × SJL)F2 (present study).

The animals were housed in filter-protected cages and provided with standard, irradiated rodent diet 5053 (Lab Diet; PMI Nutrition International) and water ad libitum. Selected groups of mice received 4 mg/ml doxycycline (Sigma)/5% sucrose in their drinking water. Protein and hemoglobin in mouse urine were measured using a Chemstrip 7 dipstick (Roche). All mice 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 the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care International Guidelines. All procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

Diets. Diets were selected to represent a range of DNA transmethylation micronutrient concentrations. The concentrations of methyl donors and cofactors were based on the micronutrient content of the diets used by Hollingsworth et al

Table 1. Transmethylation micronutrient concentrations in mouse diets*

	MS diet #06690	MR diet #06688	Standard diet 5053
Methyl donors, gm/kg			
Betaine	15	0	Unknown
Methionine	11.8	1.5	7
Choline	16.5	1.15	2
Methyl cofactors, mg/kg			
Zinc	200	36	87
Folic acid	16.5	3	3
Vitamin B_2	9	9	8
Vitamin B_6	8.6	8.6	9.6
Vitamin B_{12}	1.5625	0.0625	0.051

* MS = transmethylation micronutrient supplemented; MR = transmethylation micronutrient restricted.

(19) and Delaney et al (20). Amino acid–defined MR (TD.06688) and MS (TD.06690) containing the transmethylation micronutrients and cofactors listed in Table 1 were provided by Harlan Laboratories. Mineral and vitamin premixes were AIN-93M and AIN-93, respectively. The MR diet has low methionine (0.15%), moderate cysteine (0.25%), and levels of methyl-related nutrients (choline, folate, vitamin B_{12}, and vitamin B_6) that are within ranges typically found in other purified or standard diets, including 5053. The MS diet has higher methionine than standard diets (1.18%), moderate cysteine (0.25%), and is supplemented with the methyl donors choline and betaine. It also contains specific increases in vitamin B_{12}, folic acid, and zinc content compared to the standard 5053 and MR diets. The MS diet was ~8-fold higher in methionine, 14-fold higher in choline, 5 times higher in folic acid, and 25 times higher in vitamin B_{12} than the MR diet. The standard natural ingredient rodent diet 5053 had intermediate levels of methionine but was similar to the MR diet with regard to levels of other methylation-associated micronutrients.

Flow cytometric analysis. Mouse spleen cells were washed twice in standard buffer (phosphate buffered saline [PBS] containing 1% horse serum and 1 mg/ml sodium azide) at 4°C. Nonspecific binding was blocked by incubating the cells for 1 hour on ice in standard buffer containing 10% horse serum. The cells were then stained in the dark for 1 hour with phycoerythrin (PE)–conjugated hamster anti-mouse CD154 (CD40L), PE-Cy5–conjugated rat anti-mouse CD4, or anti-CD11a (BD PharMingen), washed, then fixed in 2% paraformaldehyde and stored in the dark at 4°C. The cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) as previously described (21).

Enzyme-linked immunosorbent assay (ELISA). Mouse IgG anti-dsDNA antibodies were measured by ELISA as previously described (18). Briefly, 96-well flat-bottomed microtiter plates (Costar) were coated overnight at 4°C with 10 μg/ml of dsDNA in PBS, pH 7.2. Mouse sera or murine monoclonal IgG anti-dsDNA antibody standard (Clone BV16-13; Millipore) were added in various dilutions and incubated overnight at 4°C. Bound anti-dsDNA antibody was detected using horseradish peroxidase–conjugated goat anti-mouse IgG-Fc–specific antibody (Bethyl Laboratories) and OneStep Ultra tetramethylbenzidine substrate (Thermo) and measured at 450 nm.

Bisulfite conversion and pyrosequencing. Genomic DNA was isolated from CD4+ T cells using a DNeasy blood and tissue kit (Qiagen), and then bisulfite treated using an EZ DNA Methylation Gold kit according to the recommendations of the manufacturer (Zymo Research). Pyrosequencing primers for murine *CD40lg* were designed using PSQ Assay Design software (Biotage). We have previously described their sequences and the polymerase chain reaction cycling parameters for this gene (18).

Statistical analysis. Student's *t*-test, chi-square tests, a 2-tailed Fisher's exact test, and linear regression were used as appropriate to determine the significance of differences between groups, using Systat software on a Dell PC Optiplex 745 microcomputer.

RESULTS

Influence of genetic background on anti-dsDNA antibody levels. Inducing dnMEK expression by doxycycline treatment in the double-transgenic mice decreased ERK phosphorylation and DNMT-1 messenger RNA, resulting in increased expression of the methylation-sensitive genes *Cd11a, Cd40lg,* and *Cd70,* and induced anti-dsDNA antibodies (17,18). The genetic background of the mice confers additional factors that influence lupus disease susceptibility and severity (22). We therefore compared the contribution of C57BL/6 ($H2^b$) and SJL ($H2^s$) genetic backgrounds in influencing IgG anti-dsDNA antibody titers in mice with the dnMEK and CD2rtTA transgenes. Female C576BL/6, (B6 × SJL)F1, and (F1 × SJL)F2 mice that were hemizygous for the dnMEK and CD2rtTA transgenes were given drinking water containing 4 mg/ml doxycycline/5% sucrose or 5% sucrose alone. IgG anti-dsDNA antibody responses after 18 weeks of treatment are shown in Figure 1. Transgenic (B6 × SJL)F1 mice had significantly higher anti-dsDNA antibody levels than mice with the transgenes on the pure C57BL/6 background ($P = 0.03$ by Student's *t*-test). Further increasing the SJL contribution to the genetic background by a second backcross onto SJL significantly increased the levels of IgG anti-dsDNA antibody produced ($P = 0.04$ for F2 versus F1 and $P = 0.001$ for F2 versus the pure C57BL/6 background). In the absence of doxycycline treatment, no differences in anti-dsDNA antibody levels were observed.

Although doxycycline-treated transgenic C57BL/6 mice produce anti-dsDNA antibody, they fail to develop lupus-like organ damage and disease (17). SJL mice possess genes that contribute to lupus-like disease when ERK activity is impaired, but they do not spontaneously develop lupus in the absence of doxycycline activation of

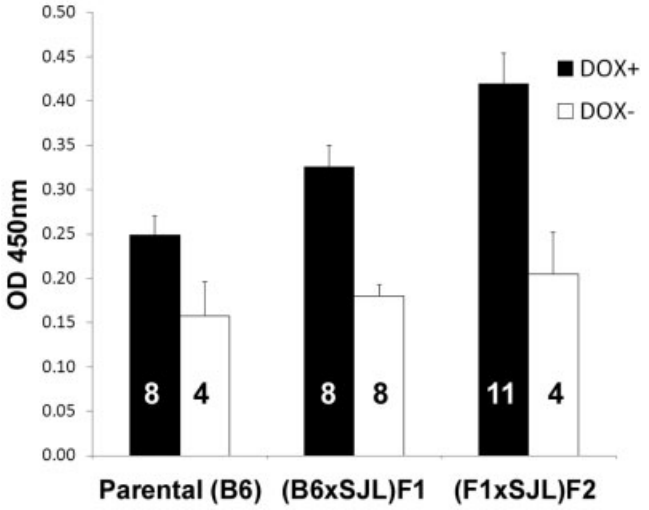

Figure 1. Effect of genetic background on IgG anti–double-stranded DNA (anti-dsDNA) antibody levels in mice. Mice bearing the dominant-negative MEK and CD2 reverse tetracycline *trans*-activator (CD2rt-TA) transgenes on the indicated genetic backgrounds were treated for 18 weeks with doxycycline (DOX) in their drinking water or did not receive doxycycline. Values are the mean ± SEM; n values are shown within the bars. $P = 0.03$, F1 versus parental; $P = 0.042$, F1 versus F2; $P = 0.001$, F2 versus parental, by Student's *t*-test. No significant differences in anti-dsDNA antibody levels were observed between groups in the absence of doxycycline treatment ($P > 0.05$).

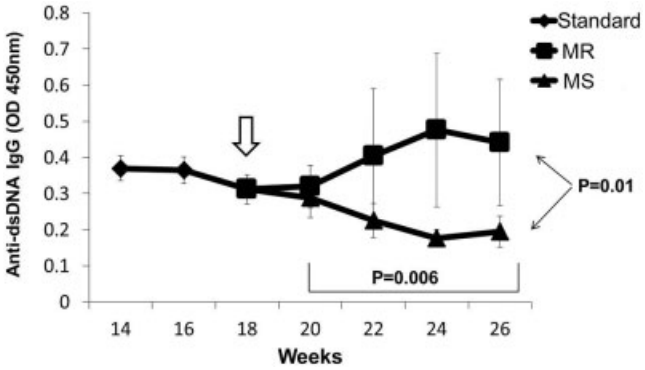

Figure 2. Decline in IgG anti–double-stranded DNA (anti-dsDNA) antibody levels in doxycycline-treated transgenic (F1 × SJL)F2 female mice fed a transmethylation micronutrient–supplemented (MS) diet. The **arrow** indicates the time point (18 weeks) when mice were switched from a standard diet (rodent diet 5053) to either an MS diet or a transmethylation micronutrient–restricted (MR) diet. Values are the mean ± SEM (n = 5–10 mice per group). $P = 0.006$, standard diet (weeks 14–18) versus MS diet (weeks 20–26) by linear regression; $P = 0.368$, standard diet (weeks 14–18) versus MR diet (weeks 20–26) by linear regression; $P = 0.01$, MS diet versus MR diet at week 26 by Student's *t*-test.

the transgene (17,18). We therefore investigated the effect of dietary transmethylation micronutrients on the epigenetic regulation of lupus susceptibility genes and disease in these strains. Double-transgenic C57BL/6 and (F1 × SJL)F2 mice were fed the standard natural ingredient rodent diet 5053 and given doxycycline in their drinking water, as previously described (18). After 18 weeks, half of the mice were fed an MR diet while the remaining animals were fed an MS diet, and all animals continued to receive doxycycline. Anti-dsDNA antibody levels significantly declined in (F1 × SJL)F2 mice switched to the MS diet ($P = 0.006$ by linear regression) and were near background levels 8 weeks later (Figure 2). In contrast (F1 × SJL)F2 mice fed the MR diet showed an increase in anti-dsDNA antibody levels, although the change was not statistically significant ($P > 0.05$).

Diet and hematuria. The effect of diet on the development of kidney disease in mice was investigated. We previously observed glomerulonephritis and hematuria in this transgenic mouse model of lupus (18). As expected, doxycycline-treated transgenic C57BL/6 mice failed to develop hematuria when fed the standard rodent diet 5053 (Figure 3). They also failed to develop hematuria when fed the MS diet. However, 6 of 18 doxycycline-treated, transgenic C57BL/6 mice developed hematuria when maintained on the MR diet. Five of the 6 mice that developed hematuria had 250 erythrocytes/μl. Four of the 6 animals that developed

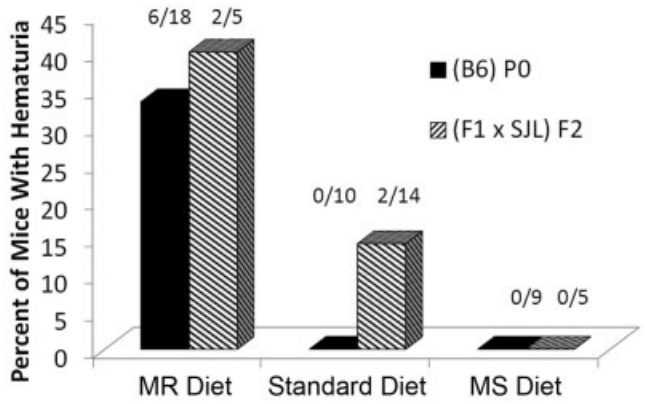

Figure 3. Influence of diet on kidney function in mice. C57BL/6 (B6) or (F1 × SJL)F2 mice bearing the dominant-negative MEK and CD2 reverse tetracycline *trans*-activator transgenes were treated with doxycycline in their drinking water and fed either a transmethylation micronutrient–restricted (MR) diet, as standard rodent diet, or a transmethylation micronutrient–supplemented (MS) diet for up to 18 weeks. Values above the bars are the number of mice with hematuria/number of mice per group. The effect of diet on hematuria was significant ($P = 0.01$, MR diet versus both standard diet and MS diet for both strains of mice, by chi-square test for trend in proportions).

hematuria also had 30–50 mg/dl protein in their urine, and 1 had a trace amount of protein in the urine. One mouse had 100 erythrocytes/μl (minimum detectable level <50 erythrocytes/μl) and a trace amount of protein in the urine. The effect of doxycycline treatment and the MR diet on hematuria in the transgenic C57BL/6 mice was statistically significant ($P = 0.01$).

Two of 14 doxycycline-treated transgenic (F1 × SJL)F2 mice that were fed a standard diet developed hematuria, with 50 erythrocytes/μl and a trace amount of urinary protein in one, and 500 erythrocytes/μl and 30 mg/dl of urinary protein in the other. However, none of the (F1 × SJL)F2 mice fed the MS diet developed hematuria. Of the 5 transgenic (F1 × SJL)F2, doxycycline-treated mice that were fed the MR diet, 1 had 100 erythrocytes/μl and 30 mg/dl urinary protein, and 1 had 500 erythrocytes/μl and 100 mg/dl urinary protein. In the absence of doxycycline, no hematuria developed in either strain with any of the diets used (data not shown). Increasing the dietary methyl donor content reduced the development of hematuria significantly in transgenic mice with SJL genes ($P = 0.01$ by chi-square test for trend in proportions).

The semiquantitative scoring system described by Austin et al (23) to measure renal disease in lupus patients was used to assess kidney damage in the mice. Light microscopic examination of paraffin-embedded sections of kidneys from doxycycline-treated mice confirmed that the transgenic C57BL/6 mice did not develop kidney disease when fed the standard diet (mean ± SEM score 0 ± 0) (Figure 4A). The glomeruli of these animals were normal and exhibited open capillary loops and no increase in cellularity. C57BL/6 animals fed the MR diet had focal glomerular hypercellularity with an increase in mesangial matrix (mean ± SEM score 4.5 ± 0.5 [range 4–5]).

The SJL background contains lupus susceptibility genes which contribute to both the development and the severity of glomerular inflammation. In (F1 × SJL)F2 animals that were fed the standard diet, there was mild focal glomerulonephritis with hypercellularity and increased mesangial matrix (mean ± SEM score 3.6 ± 1.5 [range 1–7]). (F1 × SJL)F2 animals fed the MR diet had more severe glomerulonephritis than the C57BL/6 mice, with a marked increase in diffuse glomerular hypercellularity and matrix deposition with karyorrhectic nuclear debris, and thickening of the glomerular capillary loops consistent with subendothelial deposits (mean ± SEM score 5.8 ± 2.0 [range 2–12]).

In the absence of doxycycline treatment, no animals developed kidney disease (results not shown).

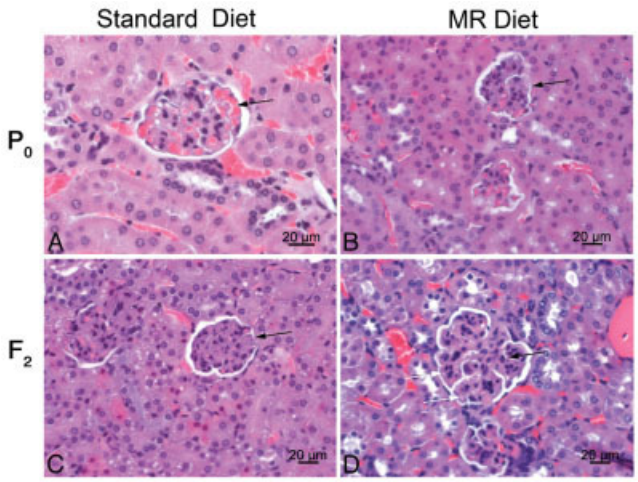

Figure 4. Diet and glomerulonephritis in mice. Formalin-fixed kidneys from transgenic doxycycline-treated C57BL/6 (P0) and (F1 × SJL)F2 mice were processed routinely, stained with hematoxylin and eosin, and examined by light microscopy for evidence of disease. **A**, Section from a C57BL/6 mouse fed a standard commercial diet (rodent diet 5053), showing normal glomeruli with no increase in cellularity and open capillary loops (**arrow**) (group mean ± SEM lupus renal disease score 0 ± 0). **B**, Section from a C57BL/6 mouse fed a transmethylation micronutrient–restricted (MR) diet. Note the increased mesangial matrix (**arrow**). The lupus renal disease score was 4 for the section shown (group mean ± SEM 4.5 ± 0.5 [range 4–5]). **C**, Section from an (F1 × SJL)F2 mouse fed a standard commercial diet (rodent diet 5053). Note the increased mesangial matrix (**arrow**). The lupus renal disease score was 4 for the section shown (group mean ± SEM 3.6 ± 1.5 [range 1–7]). **D**, Section from an (F1 × SJL)F2 mouse fed an MR diet. Karyorrhectic nuclear debris (**black arrow**) and thickening of the glomerular capillary loops consistent with subendothelial deposits (**white arrows**) are seen, and are more severe than in the C57BL/6 mice. The lupus renal disease score was 12 for the section shown (group mean ± SEM 5.8 ± 2.0 [range 2–12]).

Taken together, our data indicate that transmethylation micronutrients, particularly methyl donors such as methionine and betaine, can influence lupus-like disease symptoms such as anti-dsDNA antibody levels and hematuria in a transgenic murine model of SLE.

***CD40lg* gene expression and methylation.** CD40L protein on T cells is elevated in women with lupus and in our transgenic mouse model of lupus, and contributes to disease pathogenesis by stimulating B cell antibody production (24,25). The CD40L gene (*CD40LG*) is on the X chromosome in both humans and mice, and its aberrant demethylation on the inactive X may thus be a determinant of lupus predominance in females compared to males. Increased *CD40LG* expression with decreased methylation in females but not males has been demonstrated in both species (1,18,25). Therefore, the effect of diet on murine *CD40lg* expression and

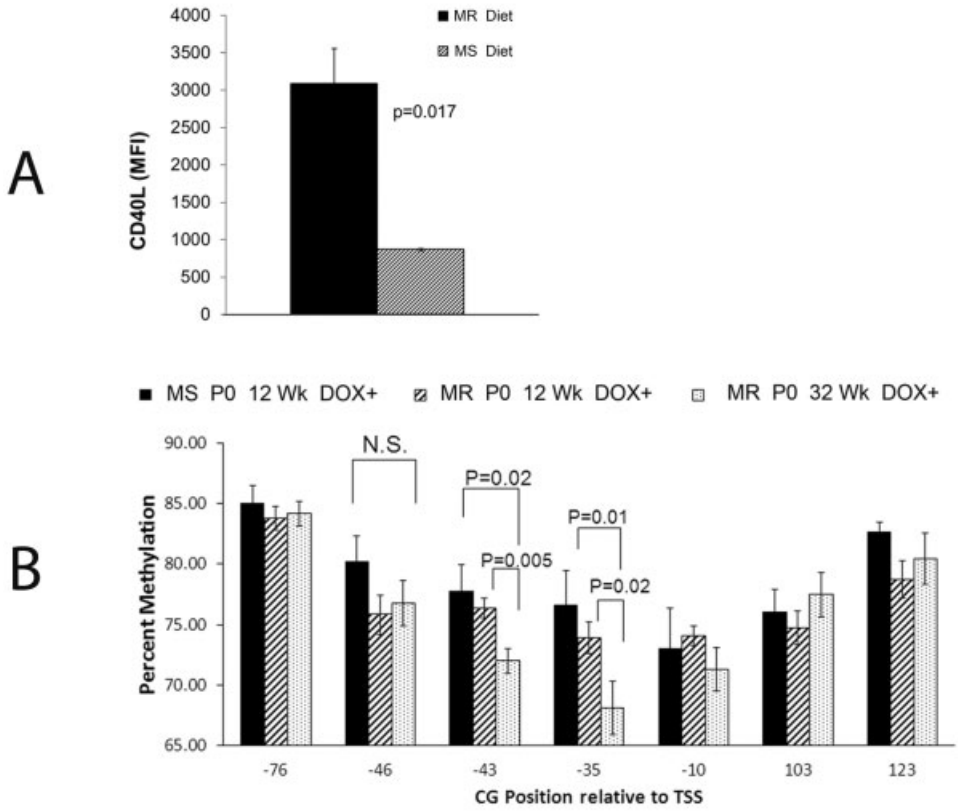

Figure 5. Effect of transmethylation micronutrient levels on CD40L protein expression and DNA methylation in CD4+ T cells from female mice. **A**, CD40L expression in spleen cells from dominant-negative MEK/CD2 reverse tetracycline *trans*-activator transgenic C57BL/6 female mice treated with doxycycline (DOX) and fed either a transmethylation micronutrient–restricted (MR) diet or a transmethylation micronutrient–supplemented (MS) diet for 18 weeks. Mouse spleen cells were stained for CD4+ T cells and CD40L and analyzed by flow cytometry. Values are the mean ± SEM (n = 8) for the MR group and the mean and range (n = 2) for the MS group. Significance was determined by chi-square test. **B**, Methylation of CG pairs in the *Cd40lg* promoter in dnMEK+/CD2rtTA+ transgenic C57BL/6 mice treated with doxycycline and fed an MS diet for 12 weeks, an MR diet for 12 weeks, or an MR diet for 32 weeks. Values are the mean ± SEM (n = 5–9 mice per group). Significance was determined by Student's *t*-test. MFI = mean fluorescence intensity; NS = not significant; TSS = transcription start site.

methylation was examined. CD4+ T lymphocytes from female transgenic, doxycycline-treated C57BL/6 mice that were fed the MR diet overexpressed CD40L protein relative to mice that were fed the MS diet (mean ± SEM mean fluorescence intensity 3,089 ± 466 for the MR diet versus mean 864 [range 848–880] for the MS diet) (P = 0.017) (Figure 5A).

Impaired ERK signaling or inhibition of DNMT-1 contributed to increased CD40L protein on CD4+ T cells from female mice by inhibiting the methylation of CG pairs in the promoter region near the transcription start site (18,25). Therefore, the methylation of CG pairs in this region was measured. After 12 weeks of doxycycline treatment, transgenic C57BL/6 mice that were fed the MR diet had reduced methylation of CG pairs located −46, −43, and −35 bp 5′ of the transcription start site of the *CD40lg* promoter compared with mice that were fed the MS diet, although the reduction was not statistically significant (Figure 5B). The methylation levels at positions −43 and −35 (but not at position −46) continued to decline in mice that were fed the MR diet, and at 32 weeks this decline was statistically significant compared to both the 12-week MS diet group and the 12-week MR diet group. The CG pairs located −35 and −43 bp 5′ relative to the murine transcription start site are homologous to regulatory CG pairs in the human *CD40LG* promoter, and these are demethylated in T cells from female lupus patients and 5-azacytidine–treated T cells from healthy women (25). Demethylation required doxycycline treatment since no decline in methylation levels in residues −76 through −35 was observed in the absence of doxycycline (data not shown).

This experiment confirms that diet and decreased DNMT-1 synergize to inhibit DNA methylation in these mice.

DISCUSSION

The development of SLE involves genes that confer disease susceptibility, hormones, and environmental factors (1,2). The present study investigated the gene–environment interaction in lupus development and severity using a double-transgenic lupus model with an inducible ERK pathway signaling defect bred onto the lupus-resistant C57BL/6 background or a lupus-susceptible C57BL/6 × SJL hybrid genetic background (17). These mice express a dominant-negative MEK uniquely in CD2+ cells when doxycycline is administered in their drinking water. Activation of the transgenes suppresses ERK signaling and subsequent DNMT-1 expression, leading to DNA hypomethylation and overexpression of methylation-sensitive genes (17,18). This is similar to the ERK signaling defect that contributes to human lupus (26).

The results of the present study confirm previous findings in this model that activation of the transgenes in mice of the lupus-resistant C57BL/6 strain, fed a standard commercial laboratory diet, induced low levels of anti-dsDNA antibody and DNA hypomethylation, and that higher anti-dsDNA antibody levels and active kidney disease required the presence of other lupus susceptibility genes present in the SJL mouse strain (17). Increasing the SJL genetic contribution by an additional backcross further increased the levels of anti-dsDNA antibody and disease severity, thus supporting our previous findings (1) correlating lupus disease severity and the number of lupus-associated single-nucleotide polymorphisms in men and women with lupus.

The incomplete concordance of lupus between genetically identical twins strongly supports the notion that nongenetic factors are involved in the etiology of SLE (4,27). Drugs such as hydralazine and procainamide can trigger a lupus-like disease in genetically susceptible individuals through their effects on mechanisms, such as DNA methylation, histone modification, and signal transduction, that control gene expression (8,28). DNA methylation depends on DNMT-1 activity and S-adenosyl methionine (SAM) levels, the latter of which are regulated by methionine, choline, zinc, vitamins B_2, B_6, and B_{12}, and folate from dietary sources. Therefore, we tested the hypothesis that dietary micronutrients involved in transmethylation reactions can epigenetically modify lupus gene expression and disease severity, in experiments in which we continued to treat the transgenic mice with doxycycline following the induction of SLE, but changed the diets to either a low methionine formulation or a diet rich in methyl donors and cofactors. Our results showed that reducing the methionine and choline content of the diet increased lupus disease severity in genetically susceptible hybrid mice and also caused a milder kidney disease in mice with a lupus-resistant C57BL/6 genetic background. In contrast, a diet enriched in methyl donors and the cofactors zinc, folic acid, and vitamin B_{12} ameliorated both the anti-DNA antibody response and kidney disease. Taken together, our results demonstrate that the onset and severity of lupus disease can be influenced by both lupus susceptibility genes and nongenetic factors that affect DNA methylation.

It is tempting to speculate that the onset and/or severity of human lupus may be modulated by dietary intervention. Dietary modification has been used successfully in rodent and human studies to influence disease outcome and epigenetically alter the heritable gene expression profile. For example, dietary folate levels modulate hepatocyte DNA methylation in rats (29). A low-folate diet caused DNA hypomethylation in the lymphocytes of healthy postmenopausal women, which could be reversed with a folate-supplemented diet (14). There are limited data from studies of SLE patients showing that supplementation with vitamin B_6, vitamin B_{12}, and folate ameliorates lupus symptoms (30). However, Wu et al reported that >100 metabolites, many of which contribute to energy metabolism, are significantly altered in SLE patients (16). They further found that methionine and other methyl donors, including cysteine, choline, and cofactors such as vitamin B_6, were significantly reduced in SLE patients compared to healthy matched controls. Folate depletion increases homocysteine (Hcy) levels, which decreases SAM production, resulting in DNA hypomethylation (6,10).

Maintenance of T cell DNA methylation patterns is more sensitive to low folate and methionine levels in older individuals due to a decline in DNMT-1 levels that occurs with age (10). DNMT-1 levels are also inversely proportional to lupus disease activity (31), potentially rendering people with active SLE more sensitive to low levels of micronutrients required for DNA methylation and potentially exacerbating immune dysregulation and contributing to disease activity. Similarly, elevated levels of folate or vitamin B_6 suppressed expression of the methylation-sensitive perforin gene in T cells from lupus patients in vitro (15), supporting the notion that dietary supplementation may have beneficial effects on SLE.

Thus, attention to proper nutrition may be particularly important in the elderly and in lupus patients.

The results of the present study show that diets with low levels of methyl donors and cofactors, together with impaired ERK signaling, cause progressive hypomethylation in *CD40lg* regulatory regions of CD4+ T cells, and that a methyl-supplemented diet prevents DNA demethylation of this region. CD40L protein on T cells plays an important role in stimulating autoantibody production and is overexpressed in women, but not men, with SLE due to activation of the *CD40L* gene on the inactive X chromosome (18,25,32). Thus, dietary approaches that target a specific metabolic pathway such as transmethylation, a key to lupus pathogenesis, are rational and potentially useful approaches to prevention and perhaps therapy.

Successful dietary approaches to treat SLE will probably require simultaneous targeting of multiple elements of 1-carbon metabolism as well as other factors that impact methylation pathways. Methionine participates as a methyl donor in SAM biosynthesis. The key reaction in DNA methylation is the DNMT-catalyzed transfer of methyl groups from SAM to dC bases at CpG pairs in DNA, producing methylated (d^mC) DNA and S-adenosyl Hcy. Intracellular SAM pools are crucially dependent on a number of dietary factors, including folic acid, methionine, choline, betaine, zinc, and vitamins B_6 and B_{12}. While B vitamins and enzyme cofactors are essential for methionine metabolism, they cannot substitute for the required methyl group substrates. The metabolism of methionine to Hcy and remethylation of Hcy back to methionine is tightly controlled, such that intracellular SAM levels tend to be maintained during normal variations in dietary methionine content (33). Betaine provides methyl groups for SAM by the action of betaine-Hcy methyltransferases and 1-carbon units via the folate system (34), and was included in the MS diet. Betaine supplementation lowers Hcy levels and elevates methionine levels, has been used as a safe and effective treatment for 3 different forms of homocystinuria (33), and has been proposed as a therapy for neural tube defects due to its ability to stimulate cellular methylation reactions (33).

Selection of the methyl donor and cofactor concentrations used in the present study was based on several studies by others in which diet was used to modify DNA methylation and biologic responses (12,36–38). The methionine concentration in the MR diet represented the lowest methionine concentration that could be tolerated over an extended period of time (35). The MS diet was based on the formulation used by Hollingsworth et al (19) and the amino acid–defined MS diet used by Delaney et al (20) to study epigenetic changes acquired in utero. Betaine and increased dietary methionine, choline, and folate were used to boost the concentration of methyl donors and cofactors and resulted in hypermethylation of genes in embryos (12,19,20). The MS diet used in the present study reduced anti-dsDNA antibody levels to near background levels in transgenic mice with defective ERK–triggered lupus-like disease, suggesting that micronutrients that enhance transmethylation reactions may ameliorate lupus disease via epigenetic mechanisms. Similarly, the reduced methionine content of the MR diet, together with reduced ERK pathway activity, could have exacerbated lupus disease via epigenetic mechanisms by causing DNA hypomethylation and enhanced immune gene expression.

The terms "low" and "high" used in reference to the methyl donor and cofactor content of the diets used in the present study are relative only to one another and not to "natural" diets. Compared to the MR diet, the MS diet had ~8 times the amount of methionine, ~14 times the amount of choline, ~5 times the amount of folic acid, ~25 times the amount of vitamin B_{12}, and 15 gm/kg betaine. The methyl donor levels in the MS diet are greater than those typically found in commercial rodent diets. The MR diet has lower methionine levels than the common purified diets AIN93G, 5053, and NIH-31 used in other studies of DNA methylation in vivo (12,19,20,36). Levels of the other micronutrients, such as folate, vitamin B_6, and vitamin B_{12}, are similar to those in standard rodent diets.

While our murine study suggests that diets rich in methyl donors may be beneficial for lupus patients, due to size, metabolic rates, and differences in nutrient requirements, extrapolating micronutrient levels from rodents to humans is not straightforward. By expressing dietary nutrient levels based on energy, which takes into account the size and metabolic rates of the two species, a rough comparison of dietary micronutrient levels may be performed. Normal Western diets supply ~2–4 gm sulfur amino acids (methionine plus cysteine) per day, 147 μg/kcal choline, 0.3 μg/kcal folate, and 2.5 ng/kcal vitamin B_{12} (37,38). Men 31–50 years old have an average intake of 2.3 gm of methionine per day, while the average for women is 1.6 gm/day. In a 2,000-kcal diet, methionine represents 0.8–1.15 mg/kcal (38). Supplementation with 5 gm of methionine per day is the maximum dose tolerated in humans (39). The MS diet used in the present study includes 3.1 mg methionine/kcal, ~3 times the typical human intake. The MS diet

consists of 29 times the amount of choline, 14 times the amount of folate, and 160 times the levels of vitamin B_{12} found in a Western diet. Betaine is included in the MS diet at 3.9 mg/kcal, which is 37.5 times the amount found in the human diet (40). The amount of methionine in the MR diet is 0.375 mg/kcal, which is less than half the amount of methionine consumed by adults who eat a Western diet. The MR diet contains twice the amount of choline and folate and 6 times the amount of vitamin B_{12} found in the Western diet.

In conclusion, our results indicate that it is possible to modulate autoantibody levels and kidney disease severity by dietary manipulation in a mouse model of SLE. These data suggest that dietary modification might be a candidate therapeutic approach for future studies in lupus patients.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Strickland had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Strickland, Sawalha, Delaney, Hoeltzel, Yung, Johnson, Mickelson, Richardson.

Acquisition of data. Strickland, Wu, Johnson.

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ADDITIONAL DISCLOSURES

Author Mickelson is an employee of Harlan Laboratories.

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