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Murine Lupus Susceptibility Locus Sle1c2 Mediates CD4+ T Cell Activation and Maps to Estrogen-Related Receptor γ

Daniel J. Perry,* Yiming Yin,* Tiffany Telarico,†‡§ Henry V. Baker,¶ Igor Dozmorov,‖ Andras Perl,†‡§ and Laurence Morel*

Sle1c is a sublocus of the NZM2410-derived Sle1 major lupus susceptibility locus. We have shown previously that Sle1c contributes to lupus pathogenesis by conferring increased CD4+ T cell activation and increased susceptibility to chronic graft-versus-host disease (cGVHD), which mapped to the centromeric portion of the locus. In this study, we have refined the centromeric sublocus to a 675-kb interval, termed Sle1c2. Mice from recombinant congenic strains expressing Sle1c2 exhibited increased CD4+ T cell intrinsic activation and cGVHD susceptibility, similar to mice with the parental Sle1c. In addition, B6.Sle1c2 mice displayed a robust expansion of IFN-γ–expressing T cells. NZB complementation studies showed that Sle1c2 expression exacerbated B cell activation, autoantibody production, and renal pathology, verifying that Sle1c2 contributes to lupus pathogenesis. The Sle1c2 interval contains two genes, only one of which, Esrrg, is expressed in T cells. B6.Sle1c2 CD4+ T cells expressed less Esrrg than B6 CD4+ T cells, and Esrrg expression was correlated negatively with CD4+ T cell activation. Esrrg encodes an orphan nuclear receptor that regulates oxidative metabolism and mitochondrial functions. In accordance with reduced Esrrg expression, B6. Sle1c2 CD4+ T cells present reduced mitochondrial mass and altered mitochondrial functions as well as altered metabolic pathway utilization when compared with B6 CD4+ T cells. Taken together, we propose Esrrg as a novel lupus susceptibility gene regulating CD4+ T cell function through their mitochondrial metabolism. The Journal of Immunology, 2012, 189: 793–803.

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Abbreviations used in this article: atRA, all-trans retinoic acid; BM, bone marrow; cGVHD, chronic graft-versus-host disease; DAR-4M, diaminorhodamine-4M; ERR, estrogen-related receptor; Esrrg, estrogen-related receptor γ; Fluo-3AM, fluo-3-acetoxyethyl ester; GN, glomerulonephritis; HE, hydroethidine; MRI, macromembrane reactivity; MTG, MitoTracker Green-FM; PI, propidium iodide; ROL, reactive oxygen intermediate; SLAM, signaling lymphocytic activation molecule; SLE, systemic lupus erythematosus; SNP, single-nucleotide polymorphism; Tm, T effector memory cell; TMEM, tetramethylrhodamine methyl ester; Treg, regulatory T cell; VDAC, voltage-dependent anion channel.

This region overlaps with syntenic human SLE quantitative trait loci, 1q22–23 and 1q41–42, suggesting that similar genetic factors may mediate pathogenesis in both species (3). Subsequent studies using congenic mice demonstrated distinct functional requirements that Sle1 imparted in the induction of murine lupus. Specifically, B6.Sle1c mice display B and T cell intrinsic loss of tolerance to chromatin (4–6). Furthermore, complementation analyses with the other NZM2410-derived SLE susceptibility loci (7) and with the NZW genome (8) demonstrated that Sle1 expression was necessary for disease to develop in this model. Still, the identification of the underlying genetic determinants of SLE pathogenesis in this 62-Mb region, which contains an estimated 350 genes, remained a daunting task.

Three Sle1 subloci, Sle1a, Sle1b, and Sle1c, contribute to the production anti-chromatin autoantibodies, revealing the complexity of this locus (9). Further phenotypic characterization of these subloci revealed increased activation of CD4+ T cells by Sle1a and Sle1c and defective B cell tolerance by Sle1b (9–11). Through the use of congenic recombinants, Sle1c was determined to correspond to at least two subloci, Sle1c1 and Sle1c2 (12). Complement receptor 2 (Cr2) was identified as a candidate gene for Sle1c (3) and subsequently found to cosegregate with telomeric Sle1c1 (12). Ensuing human association studies validated these findings by identifying a haplotype that alters Cr2 splicing that was associated with SLE (13, 14). In addition, Sle1b has been attributed to polymorphisms in the signaling lymphocytic activation molecule (SLAM) gene cluster, with direct evidence for one SLAM family member Slamf6 (11, 15, 16). More recently, evidence has shown that expression of Slamf1, Slamf2, and Slamf4, other members of the SLAM family located within Sle1b, also is involved in controlling anti-nuclear autoantibody production (17, 18). Finally, Sle1a1 corresponds to a novel splice isoform of Pbx1, which is found more frequently in SLE patients than in healthy controls (19). Thus, a variety of novel lupus susceptibility genes have been identified so far in the Sle1 locus that affect both B and T cell functions.
We have reported previously that Sle1c2 is associated with increased activation and proliferation of CD4^+ T cells (12). In the current study, we mapped Sle1c2 to estrogen-related receptor γ (Esrrg), which encodes the orphan nuclear receptor ERR-γ. The expression of this gene, which regulates oxidative metabolism (20), has not been reported previously in T cells. We showed that the NZW allele is associated with reduced Esrrg expression in CD4^+ T cells, which strongly correlates with increased cell activation, and the expansion of IFN-γ–secreting T cells. In addition, B6.Sle1c2 CD4^+ T cells showed reduced mitochondrial mass and hyperpolarization consistent with their reduced Esrrg expression. Finally, we demonstrated that Sle1c2 contributes to lupus phenomenotypes in two disease models. These results suggest that Esrrg is a novel lupus susceptibility gene that regulates CD4^+ T cell function and activation through their mitochondrial metabolism.

Materials and Methods

Mice

B6.Sle1c mice that contain an NZW-derived interval at the telomeric end of chromosome 1 have been described previously (9). The loci previously referred to as Sle1c.Cr2-1 on the telomeric end and Sle1c.Cr2-2 on the centromeric end (12) have been renamed Sle1c and Sle1c2, respectively, to be more consistent with the terminology for the other loci. To generate additional recombinant subcongenic strains, (B6 × B6.Sle1c2/F1) × B6 backcross progeny were genotyped for recombination in the Sle1c interval with microsatellites that are polymorphic between NZW and B6 mice. Recombinants were bred to B6, and the progeny of this expansion backcross were bred to homozygosity. To fine-map the ends of the recombinant congenic intervals, single-nucleotide polymorphisms (SNPs) that are polymorphic for B6 and NZW mice were selected from the Mouse Phenome Database (http://phenome.jax.org/SNP), and alleles were determined by sequencing. C57BL/6 (B6), B6.Cg-Tg(TeraTerb)425BnuJ (B6. OTII), B6(C)-H2-Ab1m1/KehlJ (B6m12), B6.Cg-Igh-Thyl1Gip11/1 (B6.Thyl1), B6.129P2-Terb-Terb-1-Terd-68 (B6.Terb3), and NZB mice were purchased from The Jackson Laboratory. B6.Foxp3EGFP mice (21) were a kind gift from Dr. Vijay Kuchroo (Harvard Medical School). All of the mice were bred and maintained at the University of Florida under specific pathogen-free conditions, and experiments were performed using age- and sex-matched cohorts at the ages indicated in the text. All of the experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Cell isolation and culture

Single-cell suspensions were prepared from spleens, and RBCs were lysed. Cells then were washed in ice-cold 5% FCS in PBS and passed through 30-μm nylon mesh to remove debris. Splenocyte suspensions were enriched for CD4^+ T cells by negative selection with magnetic beads (Miltenyi Biotec). RPMI 1640 medium supplemented with 10% FCS, HEPES, 2-ME, and antibiotics was used as the culture medium. Cells were stimulated either with plate-bound 5 μg/ml anti-CD3ε (145-2C11) and 2.5 μg/ml anti-CD28, 2.5 μg/ml PMA and 1 μM ionomycin (Sigma-Aldrich). For Ag-specific proliferation assays, irradiated B6.OTII or B6.C3H mice in triplicate. [3H]Thymidine was added at 1 μCi/200 μl for the last 18 h of 72-h cultures to measure proliferation. Cells then were harvested onto glass filter paper to measure thymidine incorporation. For Th1 polarization, CD4^+ T cells were cultured with anti-CD28 and anti-CD2, 2.5 mg/ml TGF-β (Peprotech), and 25 μg/ml IL-6 (Peprotech) for 48 h. For CD4^+Foxp3^ regulatory T cell (Treg) induction, FACS-sorted CD4^+ Foxp3^ T cells from B6.OTII or B6.Sle1c2 mice were cultured with anti-CD3ε and anti-CD28 and 2.5 mg/ml TGF-β with or without 10 nM all-trans retinoic acid (Sigma-Aldrich).

Flow cytometry

Cell suspensions were blocked with 10% normal rabbit serum and anti-CD16/32 (2.4G2) in staining buffer (5% FCS and 0.05% sodium azide in PBS) and incubated on ice for 30 min. Biotinylated or fluorescein-conjugated Abs specific for CD3 Molecular Complex (17A2), CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), CD62L (MEL-14), CD25 (7D4), CD69 (H1.2F3), CD90.1 (OX-7), CD90.2 (53-2.1), B220 (RA3-6B2), CD19 (1D3), IgM (II/41), CD80 (16-10A1), CD86 (GL1), I-Ab (AF6-120.1), and isotype controls were used in predetermined amounts. SA-PerCP-Cy5.5 (BD Biosciences) was used to detect biotinylated Abs. IFN-γ (XMG-1.2), IL-4 (11B11), IL-17A (TC11-18H10), and Foxp3 (FJK-16s) were detected using Fixation/Permeabilization kits (eBioscience) according to the manufacturer’s protocol. When cytokine profiles were analyzed, cells were treated with leukocyte activation mixture (BD Biosciences) before staining. All of the Abs were from BD Biosciences except for anti-Foxp3 which came from eBiosciences. Analysis was performed on a FACSCalibur cytometer (BD Biosciences) with at least 50,000 events per sample collected, and lymphocyte populations were gated based on forward and side scatter characteristics.

For metabolic labeling, splenocytes derived from 2- to 3-mo-old B6, B6. Thy1^+, and B6.Sle1c2 mice were stained in RPMI 1640 medium at a density of 1 × 10^6 cells per milliliter with cell-permeable metabolic dyes at 37°C for 30–120 min, followed by surface staining with PE-Cy7–conjugated CD3 (17A2), PerCP-conjugated CD4 (GK1.5), allophycocyanin-Cy7–conjugated CD8a (53-6.7), PE-conjugated CD11b (M1/70), allophycocyanin-conjugated CD11c (N418), and Alexa Fluor 700-conjugated CD19 (6DS) Abs for 30 min at 4°C. All of the Abs for this experiment were obtained from Biologend. Metabolic indicators were used for the measurement of NO, mitochondrial transmembrane potential, mitochondrial mass, Ca^2+ stores, superoxide production, apoptosis, and necrosis (22, 23). Diaminohydroxamine-4M (DAR-4M) was used to evaluate peroxynitrite production, a by-product of increased NO production. Mitochondrial transmembrane potential was measured using the potentiometric indicator tetramethylrhodamine methyl ester (TMRM). Mitochondrial mass was measured by fluorescence of Mitotracker Green (Molecular Probes). Calcium stores were measured by fluorescence of fluo-5-acetoxymethyl ester (Fluo-3AM) (1 μM). Hydroethidine (HE), dihydrorhodamine 123 (DHR), and 2',7'-dichlorofluorescin were used as indicators for superoxide production. Apoptosis and necrosis were evaluated by co-staining with FITC-conjugated Annexin V and propidium iodide (PI). TMRM, MTG, HE, Fluo-3AM, and PI were obtained from Invitrogen-Molecular Probes, DAR-4M from Calbiochem, and FITC-conjugated Annexin V from BD Biosciences. The metabolic profiling was evaluated on a LSR-II flow cytometer (BD Biosciences), with 50,000 events per sample collected. Data were analyzed using FlowJo cytometry analysis software (Tree Star, Ashland, OR). Mean fluorescence intensity (MFI) values of B6.Sle1c2 mice were normalized to the means obtained for B6 mice studied in parallel and set at 1.0.

Gene expression analyses

Total RNA was isolated using RNeasy mini kits, QIAshredders, and RNAase-free DNase sets (Qiagen). cDNA then was synthesized using the Primer3 software (http://frodo.wi.mit.edu/primer3/) and used in Sybr Green (Applied Biosystems)-based quantitative RT-PCR. Taqman Gene Expression Assays (Applied Biosystems) were used to measure Esrrg (Mm00516269_mH), spans to measure Gadd45g and Gadd45a transcriptions, and 4 of 4 transcriptions were measured by fluorescence of gene expression was calculated using the comparative Ct method (RQ = 2^-DDCt) normalized to the average DCt of the B6 samples. Global gene expression was compared between negatively bead-selected CD4^+ T cells from 6- to 8-mo-old B6 and B6.Sle1c2 mice (n = 5 per strain). Their cDNA was synthesized, fragmented, and biotin-labeled using the Ovation Biotin RNA Amplification and Labeling System (NuGEN Technologies), then hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Data analysis was based on the use of “internal standards” and generalization of the “Error Model” (24) as presented elsewhere (25).

ELISA

Anti-dsDNA and anti-chromatin IgG were measured by ELISAs as described previously (4). Sera were tested in duplicate in a 1:100 dilution. Relative units were standardized using serial dilutions of a positive serum from a B6.I.A.Sle2.Sle2.Ste11.B6/Nalo mouse, arbitrarily setting the 1:100 dilution reactivity to 100 U.

Western blot analysis

Cell lysates prepared from splenic CD4^+ T cells purified by negative selection were probed with rabbit polyclonal ERR-γ Ab (Z-21; Santa Cruz Biotechnology) and revealed with HRP-conjugated anti-rabbit IgG (Sigma Aldrich). HRP-conjugated anti-GAPDH Ab (Sigma Aldrich) was used as a control. The membranes were developed by enhanced SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL). The bands were quantified using AlphaVew software (Alpha Innotech).
mouse T cell negative isolation kit (Invitrogen) through lysis in radioimmunoprecipitation assay buffer [150 mM NaCl, 2% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM NaF, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, and 10 mM sodium pyrophosphate] at a density of 4 × 10^6 cells per milliliter on ice, followed by the addition of equal volumes of Laemmli buffer and were heated to 95°C for 5 min before separation via SDS-PAGE and transferred to 0.45-μm nitrocellulose membranes. VDAC/porin was detected with rabbit polyclonal Ab (Abcam, #ab34726), and β-actin was detected with mouse mAb (Millipore, #MAB1501R). Western blots were imaged using a Kodak Image Station 440CF and quantified by automated densitometry using Kodak1D software (Eastman Kodak, Rochester, NY).

**Mixed bone marrow chimera**

Chimeras were prepared as described previously (5). In brief, 4- to 5-month-old B6.Tce1b/- recipients were lethally irradiated with two doses of 252 rad 4–6 h apart the day before reconstitution. Bone marrow (BM) cell suspensions from B6.Thp1/R and B6.Sle1c2 were mixed 1:1 after depleting T cells using anti-CD5 magnetic beads (Miltenyi Biotec). Recipients received 10^7 BM cells from sex-matched donors by i.v. injections, and grafts were allowed to reconstitute for 8 wk.

**Chronic graft-versus-host disease**

Chronic graft-versus-host disease (cGVHD) was induced as described previously (26). In brief, B6 and B6.Sle1c2 hosts received 8 × 10^7 B6, bml2 splenocytes via i.p. injections. Sera were collected weekly after induction and screened for anti-dsDNA and anti-chromatin IgG. Hosts were sacrificed 3 wk after transfer, kidneys were prepared for histology, and splenocytes were analyzed by flow cytometry. The presence of the splenomegaly phenotype, which is age-dependent, CD4+ T cell increased activation was detectable as early as 2–3 mo of age (Supplemental Fig. 1A), suggesting that the strain with the shortest interval necessary for increased activation is the REC5 subcongenic and REC1, REC2, REC3, and REC8 were B6-like, Sle1c2 then was defined as the 675-kb region between SNPs rs30920616 and rs32528185 (Fig. 1). Except where noted, the REC5 strain was used as B6.Sle1c2 for the remainder of this study.

**Experimental autoimmune encephalomyelitis**

Experimental autoimmune encephalomyelitis was induced in 4-mo-old male B6 or B6.Sle1c2 mice. On day 0, mice received an emulsion of 50 μg myelin oligodendrocyte glycoprotein peptide sequence 35–55 and 500 μg Mycobacterium tuberculosis (Difco) in IFA (Sigma-Aldrich) via s.c. injections at the base of the tail. In addition, 500 ng pertussis toxin (List Biologicals) was administered i.p. on days 0 and 2. Daily clinical scores were assessed by the following criteria: 0, no disease, 1, flaccid tail, 2, hind limb paraparesis, 3, hind limb paralysis, 4, quadriplegia. Mice were euthanized at a score of 4 or 40 d after induction.

**Results**

In order to narrow the number of candidate genes responsible for the increased CD4+ T cell activation displayed by Sle1c congenic mice, six subcongenic strains were generated in which recombinant screening was targeted to the centromeric end of the interval (Fig. 1A). Phenotypic screening showed that two strains, REC2b and REC5, displayed increased spleen weight and CD4/CD8 T cell ratio in aged mice as compared with B6 (Fig. 2A). In addition, these two strains displayed increased CD4+ T cell activation with a significantly higher percentage of CD69+ T cells and of CD44hiCD62L+ T effector memory cells (Tem). Because the strain with the shortest interval necessary for increased activation is the REC5 subcongenic and REC1, REC2, REC3, and REC8 were B6-like, Sle1c2 then was defined as the 675-kb region between SNPs rs30920616 and rs32528185 (Fig. 1). Except where noted, the REC5 strain was used as B6.Sle1c2 for the remainder of this study.

### Supplemental Material

#### Supplemental Figures

- **Figure 1.** Physical map of Sle1c. (A) Sle1c and its recombinant intervals (REC1 to REC8) are shown on the telomeric end of chromosome 1 with NZW-derived regions in white and B6-derived regions in black. All of the known polymorphic Massachusetts Institute of Technology microsatellite markers as well as SNPs that define recombination intervals are depicted. (B) Sle1c2 is defined by the nonoverlapping centromeric end of Sle1c that carries the NZW alleles in REC5 and REC2b (Sle1c2B) and the B6 alleles in the other strains (Sle1c2A). The SNPs defining the areas of recombination on both ends are shown, along with the ex-intron structure of the two known protein-coding genes located in Sle1c2. Scale is in Mb, and all of the positions are current with Ensemble release 67 (http://www.ensembl.org/Mus_musculus/), which is based on National Center for Biotechnology Information m37. **Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5 software. Unless indicated, graphs show mean and SEM for each group. For comparisons between two groups, two-tailed Mann–Whitney tests were used when n ≥ 5 and Student t tests were used either when n < 5 or when data sets passed D’Agostino and Pearson omnibus normality tests as indicated. ANOVA with Dunn’s posttest and two-way ANOVA with Bonferroni posttest corrections were used for multiple comparisons as appropriate. Each in vitro experiment was performed at least twice.

- **Figure 2.**...
TCR that is specific for the OVA323–339 peptide. Increased proliferation was observed with Ag-specific (B), polyclonal (C), and PMA-induced (D) proliferation of CD4+ T cells from 3- to 5-mo-old mice was measured by [3H]thymidine incorporation, and stimulation indexes were calculated relative to cultures with medium only. Both REC2b OTII and REC5 OTII strains were used as Sle1c2 OTII. Two-way ANOVA (‡‡) was used to measure strain effect on Ag-specific proliferation with Bonferroni’s posttest indicating significance at each concentration in (B). The Mann–Whitney test was used in (C), and the t test was used in (D). (E and F) Absolute numbers of splenic CD4+ T cells (E) and of B and CD8+ T cells (F) were compared between B6 and B6.Sle1c2 in 5- to 8-mo-old and 10- to 14-mo-old cohorts. Naive T cells were defined as CD4+CD62L+CD25−, and Tems were defined as CD4+CD44hiCD62L−. Significance levels indicate Mann–Whitney comparisons to B6 mice of the same age group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ‡‡p ≤ 0.001.

**FIGURE 2.** Phenotypic mapping of Sle1c2. (A) Comparison of spleen weights, CD4/CD8 ratios, CD69 expression, and Tem percentages of CD4+ T cells from spleens of 10- to 14-mo-old mice. Significance levels indicate ANOVA with Dunn’s multiple comparison analysis to B6. (B–D) Ag-specific (B), polyclonal (C), and PMA-induced (D) proliferation of CD4+ T cells from 3- to 5-mo-old mice was measured by [3H]thymidine incorporation, and stimulation indexes were calculated relative to cultures with medium only. Both REC2b OTII and REC5 OTII strains were used as Sle1c2 OTII. Two-way ANOVA (‡‡) was used to measure strain effect on Ag-specific proliferation with Bonferroni’s posttest indicating significance at each concentration in (B). The Mann–Whitney test was used in (C), and the t test was used in (D). (E and F) Absolute numbers of splenic CD4+ T cells (E) and of B and CD8+ T cells (F) were compared between B6 and B6.Sle1c2 in 5- to 8-mo-old and 10- to 14-mo-old cohorts. Naive T cells were defined as CD4+CD62L+CD25−, and Tems were defined as CD4+CD44hiCD62L−. Significance levels indicate Mann–Whitney comparisons to B6 mice of the same age group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ‡‡p ≤ 0.001.

B6.Sle1c2 CD4+ T cells exhibit marked Th1 skewing
A gene expression and pathway analysis of CD4+ splenocytes revealed that a large number of genes related to IFN-γ expression were upregulated in B6.Sle1c2 mice, suggesting Th1 skewing (Supplemental Fig. 2A, 2B). Intracellular staining confirmed that a larger percentage of Sle1c2 CD4+ splenocytes expressed IFN-γ as compared with B6 (Fig. 3A). Importantly, CD4+ T cells from the parental B6.Sle1c strain showed a similar percentage of IFN-γ+ T cells as the B6.Sle1c2 subcongenic strain. No difference was observed for IL-4 production (data not shown).

The microarray analysis also revealed that several genes involved in Th17/Treg homeostasis were upregulated in B6.Sle1c2 CD4+ T cells (Supplemental Fig. 2C). These included Foxp3, Il2ra, and IL10 for Tregs and Irf4, Rora, Il17a, Il21, and Il22 for Th17 cells. In addition, the Ahr and Hif1a pathways, which have been implicated in Th17/Treg homeostasis (22, 27, 28), were also upregulated (Supplemental Fig. 1B, 2C). Given this altered gene expression profile, we examined whether Sle1c2 expression augmented Treg and Th17 differentiation. Using Foxp3GFP reporter mice, we showed that TGF-β with or without all-trans retinoic acid equally induced Foxp3 expression in B6 and B6.Sle1c2 CD4+ GFP+ T cells (Supplemental Fig. 1C). Moreover, contrary to our previous reports that described a decrease in the percentage of Tregs defined as CD4+CD62L+CD25+ (10, 12), the size of the
CD4+ Foxp3+ Treg compartment was similar between B6 and B6. Sle1c2 mice, with an expansion of Foxp3+ Tregs in older B6. Sle1c2 mice (Supplemental Fig. 1D). Interestingly, in vitro Treg polarization produced significantly more Foxp3+ IFN-γ+ cells in B6. Sle1c2 mice at 8–10 mo of age, but not 1–3 mo of age (Fig. 3B, 3D). This not only confirms the strong Th1 skewing associated with Sle1c2 but also suggests that B6. Sle1c2 mice may accumulate Tregs corresponding to a plastic population of IFN-γ+ adaptive Tregs that have been linked to autoimmunity (23).

Th17 in vitro polarization did not reveal differences in IL-17 production between the two strains (Fig. 3C, 3D). However, as with Treg polarization, IFN-γ production was increased in the B6. Sle1c2 Th17-polarized CD4+ T cells (Fig. 3C, 3D), highlighting again the Th1 skewing induced by Sle1c2 expression. Finally, experimental autoimmune encephalomyelitis was used as a model to test for differences in Th17/Treg homeostasis in vivo, and no differences were observed in day of onset or severity between the two strains (Supplemental Fig. 1E). Hence, although the global gene expression analysis predicted increased commitment to Th1, Treg, and Th17 lineages, cellular assays only revealed strong Th1 skewing.

Sle1c2 exacerbates lupus in the induced cGVHD model and via epistatic interactions with the NZB genome

To determine whether the CD4+ T cell phenotypes that segregate with Sle1c2 are relevant to lupus pathogenesis, we used the cGVHD-induced lupus model (29). Preliminary results mapped increased
cGVHD susceptibility to the centromeric portion of Sle1c that includes Sle1c2 (12). In this study, we showed that CD4+ T cells were significantly more activated in B6.Sle1c2 recipients of B6. bm12 splenocytes as compared with B6 recipients (Fig. 4A). In addition, B6.Sle1c2 CD11c+ dendritic cells and B cells displayed altered activation (Fig. 4B, 4C) that was concurrent with increased serum autoantibody production (Fig. 4D). Finally, the kidneys of cGVHD-induced B6.Sle1c2 mice showed increased deposition of IgG2a immune complexes as compared with those of B6 mice (Fig. 4E). These results demonstrate that the activated phenotype of Sle1c2 CD4+ T cells contributes to induced humoral autoimmunity.

We have shown previously that Sle1c expression increases lupus phenotypes presented by (NZB × B6)F1 hybrids (30). Using the same model, we compared the phenotypes of (NZB × B6)F1 and (NZB × B6.Sle1c2)F1 mice, in which the NZB hemigenome interacts with either the B6 or the NZW Sle1c2 alleles, respectively. Importantly, the NZB and NZW strains share the same haplotype at the chromosome 1 189.80–190.050 Mb interval that includes the Sle1c2 portion of Esrrg, as determined for all 228 published SNPs that are polymorphic between B6 and NZW, with an NZB allele known or imputed with confidence ≥0.9 (data not shown). Therefore, the Sle1c locus is predicted to be homozygous for the NZB/NZW allele in (NZB × B6.Sle1c2)F1 mice and heterozygous in (NZB × B6)F1 mice. This is relevant because the Sle1c2 CD4+ T cell phenotype is recessive (data not shown). Our findings showed that, in 12-mo-old NZB hybrids, splenomegaly, CD4/CD8 T cell ratio, percentage of Tems, and B7-2 expression on B cells were increased significantly in mice with the homozygous NZW Sle1c2 allele (Fig. 5A). Moreover, the expression of Sle1c2 significantly enhanced the production of anti-dsDNA IgG triggered by the NZB hemigenome as the mice aged (Fig. 5B). Finally, IgG immune complex deposition was exacerbated greatly by Sle1c2 (Fig. 5C, 5D) but did not result in clinical disease. Taken together, these data establish that Sle1c2 can contribute to lupus through interactions with the NZB genome.

Esrrg expression is downregulated in B6.Sle1c2 CD4+ T cells

The 675-kb Sle1c2 interval contains two protein-coding genes, Esrrg and Ush2a (Fig. 1B), with the latter having no detectable expression in CD4+ cells (data not shown). Esrrg message expression was detected in CD4+ splenocytes and at a lower level in CD4+ splenocytes and in thymocytes (Fig. 6A). Furthermore, Esrrg expression was significantly lower in the B6.Sle1c2 CD4+ T cell fraction as compared with that in the B6 CD4+ T cell fraction (40.6 ± 11.4% less). No difference between strains was observed in CD42 splenocytes. Esrrg expression in CD4+ T cells is low compared with that in liver, heart, brain, and kidney, highly metabolic tissues in which Esrrg expression has been reported.
Esrrg negatively regulates CD4+ T cell activation. In 12-mo-old (NZB x B6)F1 and (NZB x B6.Sle1c2)F1 mice (n = 14 and 25, respectively). (B) Serum anti-dsDNA IgG is shown as unit values at 5 and 12 mo of age and as relative individual increase between these two ages for each strain. (C and D) Glomerular IgG and C3 deposition was detected in frozen kidney sections with FITC-conjugated anti-C3 and anti-IgG Abs. Sections were scored separately for each Ab, and additive values (IgG score + C3 score) are shown in (C). Representative sections (original magnification ×100) are shown in (D). Mann–Whitney tests: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

FIGURE 5. Sle1c2 exacerbates spontaneous lupus phenotypes induced by the NZB genome. (A) Spleen weight, CD4/CD8 ratios, and percentage of CD62L−CD44hiCD4+ and B7-2+B220+ splenocytes in 12-mo-old (NZB × B6)F1 and (NZB × B6.Sle1c2)F1 mice (n = 14 and 25, respectively). (B) Serum anti-dsDNA IgG is shown as unit values at 5 and 12 mo of age and as relative individual increase between these two ages for each strain. (C and D) Glomerular IgG and C3 deposition was detected in frozen kidney sections with FITC-conjugated anti-C3 and anti-IgG Abs. Sections were scored separately for each Ab, and additive values (IgG score + C3 score) are shown in (C). Representative sections (original magnification ×100) are shown in (D). Mann–Whitney tests: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

(31–33), going from ~4 times lower than that in liver to ~50 times lower than that in kidney (Supplemental Fig. 3A). In our microarray analysis, five probes were located within the Esrrg locus (with only one actually probing the ERR-γ coding sequence). However, the signal for all five probes was below the detectable threshold. CD4+ T cell ERR-γ protein expression was detected in CD4+ T cells (Fig. 6B). We were not able, however, to show a difference between B6.Sle1c2 and B6. Esrrg overexpression in cell lines showed that messages and protein levels were not tightly correlated, suggesting important posttranscriptional regulation (data not shown). Regardless of age or strain, Esrrg message expression in splenic CD4+ T cells showed strong negative correlation with both CD69+ T cell and Tem percentages (Fig. 6C), suggesting that Esrrg negatively regulates CD4+ T cell activation.

Esrrg expression was compared between B6.Sle1c2 and B6 adult brain, heart, kidney, and liver. Esrrg expression was decreased significantly in B6.Sle1c2 brain, and a trend for decreased expression was observed in kidney and liver (Supplemental Fig. 3B). Interestingly, an opposite trend was observed in heart. Esrrg-deficient mice die postnatally due to a defective switch from glycolytic to oxidative metabolism (34). Consistent with this result and reduced Esrrg expression in B6.Sle2c2 mice, we have observed high mortality in B6.Sle1c2 neonates (69% total litter loss, corresponding to 34 litters lost out of 49 born) as compared with that in B6 neonates (18% litter loss corresponding to 13 out 71 born during the same period of time and in the same room). It is possible that, in response to reduced Esrrg expression, a compensatory mechanism may increase Esrrg expression in the myocardium of a percentage of B6.Sle1c2 neonates, resulting in their survival.

To determine if decreased Esrrg expression in CD4+ T cells had transcriptional consequences on its putative targets, we selected 12 genes (Supplemental Table I) that were differentially expressed by B6.Sle1c2 CD4+ T cells in the microarray analysis and that also were known to have ERR-γ bound to their promoters by chip-on-chip analyses (34, 35). The expression of five of these genes, transcriptional regulators c-myc binding protein (Mycbp) and retinoic acid receptor α (Rara), a subunit of electron transport complex I (Ndufs1), a mitochondrial protein modifier (Ppif), and a mitochondrial oxidoreductase (Rtn4ip1), was decreased significantly in Sle1c2 CD4+ T cells (Supplemental Fig. 3C). These data suggest that the decreased Esrrg expression induced by Sle1c2 affects Esrrg target gene expression.

Because Esrrg is known to regulate glycolytic and oxidative metabolic programs (34), we analyzed the expression of several genes involved in these pathways by quantitative PCR. Hif1α and Slc16a3, encoding monocarboxylic acid transporter 4, which are both mediators of glycolysis (22), were expressed at a higher level in B6.Sle1c2 CD4+ T cells (Fig. 6D). In addition to glycolysis, a microarray pathway analysis revealed an influence of Sle1c2 in several other metabolic pathways, including glutaminolysis, glycogenesis, and fatty acid oxidation (Fig. 6E). Furthermore, genes regulating the electron transport chain also were differentially regulated, indicating altered mitochondrial function. Finally, out of the seven metabolic genes that were analyzed in both the Esrrg−/− heart (34) and the Sle1c2 CD4+ T cells in this study, five of them (Eno2, Gsk3, Cox6b2, Ndufa10, and Ndufa1) showed an expression change in the same direction. Overall, these data provide evidence that Sle1c2 results in an altered...
metabolic profile in CD4+ T cells, due to decreased Esrrg expression.

**B6.Sle1c2 CD4+ T cells exhibit mitochondrial dysfunction**

Esrrg expression has been reported to directly correlate with mitochondrial biogenesis and functions (33). Accordingly, decreased Esrrg expression is predicted to reduce mitochondrial mass and function in Sle1c2 CD4+ T cells. This indeed was verified in the spleens of 2- to 3-mo-old mice, in which the difference in T cell activation is still minimal and therefore not likely to induce secondary metabolic changes. The mitochondrial membrane potential and mass were decreased significantly in Sle1c2 CD4+ T cells as compared with those in B6 T cells (Fig. 7A, 7B), and this was associated with decreased Ca2+ and NO levels (Fig. 7D, 7E). In turn, the production of reactive oxygen intermediates (ROIs), as measured by HE fluorescence, was increased in Sle1c2 CD4+ T cells. However, no difference in ROIs was found between the two strains when measured with the 2′,7′-dichlorofluorescin or dihydrodorhodamine 123 dyes (data not shown). These findings are suggestive of mitochondrial dysfunction, characterized by an inability to maintain transmembrane potential while leaking ROIs. Consistent with impaired mitochondrial function, a significant increase of necrosis was observed in Sle1c2 splenocytes (Fig. 7F). This corresponds to a predisposition to proinflammatory death by necrosis that has been reported previously in lupus T cells (36).

Interestingly, VDAC levels were increased significantly in Sle1c2 CD4+ T cells (Fig. 7G), which may correspond to a compensatory mechanism to maintain mitochondrial membrane potential despite reduced Esrrg expression. A reduced mitochondrial mass was not observed in other Sle1c2 immune cell populations, except CD11c+ cells (Table I). This may be related to the fact that Esrrg expression is very low in CD4- splenocytes (Fig. 6A), therefore not affecting mitochondria in these cell types. Other parameters showed significant differences between the two strains in non-CD4+ cell types, such as NO levels, which were reduced significantly in CD8+ T cells, B cells, and CD11b+ and CD11c+ splenocytes (Table I), indicating that the metabolic consequences of Esrrg deficiency may not be confined to T cells.

**Discussion**

Within the major NZM2410 lupus susceptibility Sle1 locus, increased CD4+ T cell activation mapped to both Sle1a and Sle1c (10), with each of these loci corresponding to two subloci (12, 37). The current study focused on the functional characterization and mapping of the CD4+ T cell phenotypes associated with Sle1c. Originally defined as the 7.72 Mb at the telomeric end of chromosome 1, Sle1c contains 48 protein-coding genes and 4 microRNAs.
Previous analyses have identified C2r2 on the telomeric end of Sle1c as the candidate gene associated with impaired B cell responses (3). In this study, we confirmed that centromeric Sle1c2 enhances CD4+ T cell activation and have established that Sle1c2 CD4+ T cells are characterized by strong Th1 skewing. In addition, we have demonstrated the contribution of Sle1c2 to autoimmune pathology by showing that its expression enhances both induced and spontaneous lupus. Furthermore, congenic mapping and gene expression analysis have identified Esrrg, one of the two genes located in the critical interval, as the candidate gene for Sle1c2. Finally, consistent with published works that have established that Esrrg is a potent regulator of mitochondrial metabolism (33), the decreased expression of Esrrg in Sle1c2 CD4+ T cells is associated with decreased mitochondrial mass and with significant alterations of mitochondrial function.

ERR-γ belongs to a family of nuclear receptors that are transcription factors whose activities are mediated by endogenous ligands and other coregulatory proteins. Several nuclear receptors already have been implicated in T cell biology, including retinoic acid receptor α, retinoic acid-related orphan receptor γ, PPARγ, the vitamin D and the glucocorticoid receptors, and the estrogen receptors (38–40). ERR-γ, belonging to the ERR family, is expressed at low levels in human spleen and thymus (41) but does not yet have an established role in T cell biology. ERRs are structurally related to estrogen receptors but do not bind natural estrogens. In fact, ERR endogenous ligands have not been identified, and they thus are referred to as orphan nuclear receptors. It has been shown, however, that ERRs are constitutively active due to the properties of their ligand-binding domains (42). Regardless, several synthetic compounds augment ERR-γ-mediated transactivation of its target genes, demonstrating its potential as a therapeutic target (43). Interestingly, bisphenol A, an industrial component known to be an endocrine disruptor, binds to and alters the activity of ERR-γ (44). Bisphenol A exposure has been linked to autoimmunity, and relevant to this study, it has been shown to strongly downregulate IFN-γ production (45). This raises the possibility that ERR-γ may mediate environmental triggers of autoimmunity.

ERRs are important regulators of metabolism and energy homeostasis, and Esrrg expression is highly restricted to metabolically active tissues (42). Esrrg transactivates genes involved in mitochondrial biogenesis, lipid transport and metabolism, tricarboxylic acid cycle, electron transport chain, and oxidative phosphorylation, allowing for energy production by efficient fatty acid oxidation. This vital function is demonstrated in Esrrg null mice, where the lack of a critical switch from glycolytic to lipid-based metabolism in the myocardium results in perinatal lethality (34). Conversely, Esrrg overexpression in skeletal muscle resulted in increased mitochondrial biogenesis and function as well as exercise capacity (33). Activation and proliferation by T cells also is metabolically demanding (46). However, contrary to other metabolically demanding tissues, activated T cells use aerobic glycolysis to meet energy requirements. Known as the Warburg effect, this form of metabolism generates glucose metabolites at the expense of ATP production (47). Remarkably, when ERR-γ expression is suppressed by microRNA, the Warburg effect is induced (48). The rate of glycolysis has direct consequences on CD4+ T cell activation and differentiation. GLUT1 transgenic mice, which have increased glucose uptake in their T cells, show T cell activation phenotypes very similar to B6.Sle1c2 mice, namely, age-dependent accumulation of CD69+ T cells and Tems, increased proliferation, and increased IFN-γ production (49). Consequently, aged mice suffered from hypergammaglobulinemia and GN, exhibiting a direct connection between T cell metabolism and autoimmunity. Conversely, as reported recently in Myc-deficient mice, T cells display impaired proliferation with an altered activation phenotype when the switch to aerobic glycolysis is obstructed (50).
We hypothesize that \textit{Sle1c2} contributes to the Warburg effect in CD4\(^+\) T cells because decreased \textit{Esrrg} expression would limit the transcription of target genes that regulate oxidative lipid-based metabolism, skewing toward glucose-based programs. This hypothesis is supported first by the decreased expression of \textit{Esrrg} target genes, \textit{Ndufs1}, \textit{Ppif}, and \textit{Rin4ip1}, which regulate metabolism and mitochondrial function (51–53), and second by the decreased mitochondrial biogenesis and functions in \textit{Sle1c2} CD4\(^+\) T cells. Recently, Michalek et al. (54) reported that ERR-\(\gamma\) functions as a regulator of T cell metabolism. This isoform, encoded by a separate gene, \textit{Esrra}, binds to the same consensus DNA sequence as ERR-\(\gamma\) and shares the same general function as a metabolic regulator. However, contrary to the increased T cell activation observed in B6.\textit{Sle1c2} mice, which have reduced \textit{Esrrg} expression in CD4\(^+\) T cells, \textit{Esrra}\(^{-/-}\) mice lacked an expansion of Tems as they aged. Interestingly, \textit{Esrra}\(^{-/-}\) CD4\(^+\) T cells were not defective in their proliferative and glycolytic capacities in response to TCR activation, whereas wild-type T cells in which ERR-\(\gamma\) was acutely inhibited were deficient in these capacities. This suggested that a developmental compensatory mechanism was offsetting the effect of \textit{Esrra} deficiency, and indeed the authors observed alterations in mTOR and AMPK pathways. At this point, it is not clear why chronic ERR-\(\gamma\) deficiency leads to increased activation, whereas ERR-\(\alpha\) deficiency results in a more naive phenotype. It is conceivable that these two isoforms use different cofactors to dictate their function. Overall, these findings validate our observations of deficiencies in ERR-\(\gamma\) cause globally augmented metabolism in CD4\(^+\) T cells and result in altered T cell function.

Dysregulated T cells are a major contributor to SLE pathogenesis, and targeting them is an important focus for therapeutic intervention (55). Mitochondrial dysfunctions have been identified specifically as a characteristic of human lupus T cells, which may offer additional therapeutic targets (56). Recent studies have highlighted the regulation of metabolism as an important checkpoint of T cell activation and effector functions. Our study associates, for the first time, to our knowledge, a gene known as a major regulator of metabolism, mitochondrial dysfunction, and CD4\(^+\) T cell activation Th1 polarization in a lupus model. A better understanding of how metabolism regulates immune functions in the context of autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

References


Table I. Comparison of mitochondrial function in the major splenic cell subsets between B6.\textit{Sle1c2} and B6 mice

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MitoFun</th>
<th>B6 (\pm 0.06)</th>
<th>B6 (\pm 0.06)</th>
<th>(0.98 \pm 0.09)</th>
<th>(0.98 \pm 0.09)</th>
<th>(0.98 \pm 0.09)</th>
<th>(0.98 \pm 0.09)</th>
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<td>CD4(^+) T</td>
<td>(1.04 \pm 0.09)</td>
<td>(0.97 \pm 0.09)</td>
<td>(0.98 \pm 0.09)</td>
<td>(0.98 \pm 0.09)</td>
<td>(0.98 \pm 0.09)</td>
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</tr>
<tr>
<td>CD8(^+) T</td>
<td>(1.04 \pm 0.10)</td>
<td>(0.97 \pm 0.09)</td>
<td>(0.98 \pm 0.09)</td>
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<tr>
<td>CD11b(^+)</td>
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<tr>
<td>CD11c(^+)</td>
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<td>(0.70 \pm 0.08)</td>
<td>(0.70 \pm 0.08)</td>
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HE, spleen; Prio-3SM, thymus; DAn-3SM, lymph node. MitoFun values are normalized to B6 means set at 1.0. The values indicate mean \(\pm\) SDM for five mice per strain. Values were compared between the strains for each parameter. Bold indicates \(p < 0.05\), underline indicates \(p < 0.01\), and italic \(p < 0.001\).


