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An Allelic Variant of Crry in the Murine *Sle1c* Lupus Susceptibility Interval Is Not Impaired in Its Ability To Regulate Complement Activation

Svetlana N. Tchepeleva,*,† Joshua M. Thurman,*,† Katherine Ruff,*,† Stephen J. Perkins,‡ Laurence Morel,§ and Susan A. Boackle*,†

The *Sle1c* subinterval on distal murine chromosome 1 confers loss of tolerance to chromatin. *Cr2*, which encodes complement receptors 1 and 2 (CR1/CR2; CD35/CD21), is a strong candidate gene for lupus susceptibility within this interval based on structural and functional alterations in its protein products. CR1-related protein/gene Y (*Crry*) lies 10 kb from *Cr2* and encodes a ubiquitously expressed complement regulatory protein that could also play a role in the pathogenesis of systemic lupus erythematosus. Crry derived from B6.*Sle1c* congenic mice migrated at a higher m.w. by SDS-PAGE compared with B6 Crry, as a result of differential glycosylation. A single-nucleotide polymorphism in the first short consensus repeat of *Sle1c* Crry introduced a novel N-linked glycosylation site likely responsible for this structural alteration. Five additional single-nucleotide polymorphisms in the signal peptide and short consensus repeat 1 of *Sle1c* Crry were identified. However, the cellular expression of B6 and B6.*Sle1c* Crry and their ability to regulate the classical pathway of complement were not significantly different. Although soluble *Sle1c* Crry regulated the alternative pathway of complement more efficiently than B6 Crry, as a membrane protein, it regulated the alternative pathway equivalently to B6 Crry. These data fail to provide evidence for a functional effect of the structural alterations in *Sle1c* Crry and suggest that the role of *Cr2* in the *Sle1c* autoimmune phenotypes can be isolated in recombinant congenic mice containing both genes. ***The Journal of Immunology*****, 2010, 185: 2331–2339.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies to nuclear Ags and the involvement of multiple organ systems (1). To identify the genes involved in SLE pathogenesis, multiple mouse models developing lupus-like disease have been used. NZM2410 is a lupus-prone recombinant inbred mouse strain derived from NZB and NZW mice (2) that contains three recessive loci strongly associated with lupus susceptibility (*Sle1*, *Sle2*, and *Sle3*, located on chromosomes 1, 4, and 7, respectively) (3–6). The *Sle1* region, derived from NZW and associated with SLE susceptibility in multiple human ethnic groups and mouse strains (7, 8), has been divided into at least three subintervals: *Sle1a*, *Sle1b*, and *Sle1c* (9). B6.*Sle1c* congenic mice have a mild disease phenotype characterized by the production of anti-chromatin Abs with a 30% penetrance by 9 mo of age without the development of overt renal disease. Compared with B6 mice, they have lower levels of serum IgM, impaired humoral immune responses to T-dependent Ags, and increased numbers of $CD4^+CD62L^{lo}CD44^{hi}$ T cells and $CD4^+CD25^+FoxP3^+$ T regulatory cells (9, 10). *Cr2*, which encodes complement receptor (CR)1 and CR2 in the mouse by alternative splicing of a single mRNA transcript, is a strong candidate gene for lupus susceptibility in the ~7.5-Mb *Sle1c* interval based on alterations in its structure and function (11).

Approximately 60 known or novel protein-coding or noncoding RNA genes lie within the *Sle1c* interval. Mice with an ~1-Mb recombinant subinterval containing the very centromeric region of *Sle1c* expressed the T cell phenotypes attributed to *Sle1c* (10), whereas mice with the more telomeric ~6-Mb subinterval containing *Cr2* had decreased humoral immune responses and impaired germinal center formation (10) also seen in *Cr2*-deficient mice (12–16), suggesting that the effects of each subinterval can be attributed to distinct genes. Whether genes in both intervals contribute to the loss of tolerance to chromatin present in B6.*Sle1c* mice is not known, nor is it known whether other genes in addition to *Cr2* contribute to the effects demonstrated by the larger subinterval. *Cr2* is the most telomeric gene on chromosome 1, but in the 0.5-Mb region directly 3′ of it there are four protein-coding genes (*Plxna2*, *Cd34*, *Cd46*, and *Crry*), two noncoding RNA genes (mmu-mir-29b2 and mmu-mir-29c), and one pseudogene (AC162692.2) (www.ensembl.org). Because a 1.1-Mb gene desert lies upstream of this region, generation of a ≤0.5-Mb recombinant of *Sle1c* containing *Cr2* is feasible; however, specific effects of the recombinant interval cannot be attributed to *Cr2* until the potential contributions of other genes in this interval, especially those lying most proximal to it, are determined.

*Department of Medicine and †Department of Immunology, University of Colorado School of Medicine, Aurora, CO 80045; ‡Department of Structural and Molecular Biology, University College London, London, United Kingdom; and §Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL 32610

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Address correspondence and reprint requests to Dr. Susan A. Boackle, Division of Rheumatology, University of Colorado School of Medicine, 1775 Aurora Court, Room 3102C, Mail Stop B-115, Aurora, CO 80045. E-mail address: susan.boackle@ucdenver.edu

Abbreviations used in this paper: anti-fB, anti-factor B; AP, alternative pathway; CP, classical pathway; CR, complement receptor; Crry, complement receptor 1-related gene/protein Y; DAF, decay-accelerating factor; MCP, membrane cofactor protein; MFI, mean fluorescence intensity; SCR, short consensus repeat; SLE, systemic lupus erythematosus; SNP, single-nucleotide polymorphism; SS, signal sequence; SUN, serum urea nitrogen.

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Complement activation is one of the major effector mechanisms resulting in end organ damage in SLE, and complement deficiency is a major lupus susceptibility factor (17), likely due to altered handling of apoptotic cells, a known source of lupus autoantigens (18). Complement regulatory proteins can inhibit complement-mediated injury during the effector phase of autoimmunity and can also affect handling of apoptotic cells, which are partially cleared through complement opsonization (19, 20); thus, the genes encoding them are key candidates for lupus susceptibility. Lying within 10 kb of *Cr2* (21), CR1-related gene/protein Y (*Crry*) encodes a 65-kDa transmembrane glycoprotein composed of five extracellular 60–70-aa repeating subunits, termed short consensus repeats (SCRs); a transmembrane region; and a 50-aa cytoplasmic tail (22, 23). Expressed only on rodent cells, it is structurally homologous to human CR1 (24–26) and functionally homologous to human decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP; CD46) (27, 28). Because murine MCP is expressed only in the testis, Crry may substitute for it in most mouse tissues. Although Crry and DAF are often coexpressed in mouse tissues, their functions are not entirely redundant because Crry regulates the alternative pathway (AP) more effectively than DAF, whereas DAF is a better regulator of the classical pathway (CP) (29, 30). Crry may also specifically prevent spontaneous complement activation based on studies of GPI-DAF–deficient erythrocytes (31) and the finding that Crry-deficient mice undergo complement-mediated embryonic lethality (32). Transgenic expression of Crry or its administration as a soluble protein diminishes renal injury in mice with either spontaneous or induced glomerulonephritis, supporting its protective role against complement-mediated kidney damage in vivo (33–35) and validating its further study as a candidate gene for lupus-associated autoimmune phenotypes.

Taking into account the proximity of *Crry* to *Cr2* and its potential role in autoimmune disease, we analyzed the *Sle1c* allele of *Crry* in B6.*Sle1c* mice for alterations in its expression, structure, and function. We identified six nonsynonymous single-nucleotide polymorphisms (SNPs) in the *Sle1c Crry* sequence, one of which introduced a new N-linked glycosylation site and two of which were located in a potentially important functional region of the protein. Although in vitro studies demonstrated that *Sle1c* Crry regulated activation of the CP of complement equivalently to B6 Crry as a membrane protein, it was found to regulate activation of the AP more efficiently than B6 Crry as a soluble protein. However, superior regulatory function of *Sle1c* Crry was not observed when the membrane protein was studied using an AP-dependent in vivo model of acute renal injury. These data fail to support the hypothesis that the structurally altered *Sle1c* Crry contributes to the *Sle1c* autoimmune phenotypes and suggest that the contribution of *Cr2* to these phenotypes can be studied effectively in recombinant mice also containing *Crry.*

Materials and Methods

Experimental animals

The generation of the B6.*Sle1c* subcongenic strain from B6.*Sle1* has been described (9). C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Crry- and factor B-deficient mice were generated, as previously described (32, 36), and bred together to generate mice homozygous deficient in Crry and factor B, because homozygous Crry deficiency is lethal in the presence of a functional complement system (32). Mice were housed and maintained at the Center for Laboratory Animal Care at the University of Colorado School of Medicine. All animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The University of Colorado School of Medicine Institutional Animal Care and Use Committee approved all experimental procedures performed on the animals.

Immunoprecipitation and SDS-PAGE analysis

Splenic single-cell suspensions were depleted of RBCs with Lympholyte (Cedarlane Laboratories, Burlington, NC) and surface biotinylated with EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL). Cells were lysed with radioimmunoprecipitation assay buffer, and lysates were incubated with rat anti-mouse Crry (5D5) for 30 min on ice, followed by the addition of Protein G Sepharose (GE Healthcare Life Sciences, Buckinghamshire, U.K.) and incubation for 1 h at 4˚C. After several washes, bound protein was treated with 0.6 U PNGaseF (New England Biolabs, Ipswich, MA) for 2 h at 37˚C. Nonreduced samples were analyzed by 10% SDS-PAGE, transferred to a nitrocellulose filter, probed with HRP-streptavidin (Invitrogen, Carlsbad, CA), and visualized using a chemiluminescence detection kit (GE Healthcare Life Sciences).

Sequencing of Crry

TRIzol (Invitrogen) was used to extract RNA from spleens from the following mouse strains: B6, B6.*Sle1c*, NZW, NZB, MRL, NOD, SWR, BALB/c, and DBA/1. cDNA was generated by reverse transcription using random hexamers. Genomic DNA was obtained from The Jackson Laboratory for the following mouse strains: DBA/2, CBA, AKR, BXSB, C3H, FVB, SJL, and 129. A PCR product spanning the signal sequence (SS) of Crry was amplified from cDNA or genomic DNA using the following primer set: Crry 1F 5′-GGAGTTCAGAGCCTCTGGATCC-3′ and Crry 1R 5′-GTGTCCGTGCTTCCCCAG-3′. A PCR product spanning SCR1 of Crry was amplified from cDNA or genomic DNA using the following primer set: Crry 2F 5′-CCAATGTAATGTTGCTGTGTCTC-3′ and Crry 2R 5′-GTATACACTTATCTTCAGCACTCGTC-3′. For cDNA, cycling parameters were 94˚C for 2 min, 35 cycles of 94˚C for 1 min, 54˚C for 1 min, 72˚C for 1 min, and then 72˚C for 7 min. For genomic DNA, cycling parameters were 94˚C for 2 min, 20 cycles of 94˚C for 30 s, 65˚C → 55˚C for 1 min, 72˚C for 1 min, 15 cycles of 94˚C for 30 s, 55˚C for 1 min, 72˚C for 1 min, and then 72˚C for 10 min. PCR products were purified with the QIAquick PCR Product Purification Kit (QIAGEN, Valencia, CA) and sequenced with the above primers by the University of Colorado Cancer Center DNA Sequencing and Analysis Core Facility.

Flow cytometry

Splenic cell suspensions prepared using glass slides were depleted of RBCs with Gey's solution. Cells were incubated with 2.4G2 (American Type Culture Collection, Manassas, VA) for 5 min at 4˚C to block FcγRIIRs and then incubated with FITC anti-B220 and biotinylated rat anti-mouse Crry (1F2) (BD Biosciences, San Jose, CA), followed by incubation with PE-streptavidin (Southern Biotechnology Associates, Birmingham, AL). Thymus-cell suspensions prepared using glass slides and erythrocytes in peripheral blood collected in heparin were stained with biotinylated 1F2 and PE-streptavidin alone. Cells were fixed with 0.5% formaldehyde and analyzed by flow cytometry using a BD FACScan. Data were analyzed using FlowJo software.

Molecular modeling

Molecular views of Crry were prepared using INSIGHT 98 graphics software (Accelrys, San Diego, CA) with the homology model used in the solution structure determinations of Crry and its fusion protein Crry-Ig (Brookhaven Protein Data Bank codes 1ntj and 1ntl) (37).

Generation of Flp-In CHO transfectants expressing B6 Crry and Sle1c *Crry*

Full-length B6 or *Sle1c* Crry cDNA was amplified from the TOPO-TA vectors generated above using the following primers: 5′FRT Crry 5′-CACCGGAATGGAGGTCTCTTCTC-3′ and Crry 4R 5′-GTGATACAGACAGAGCATGTTCTC-3′. The following PCR cycling parameters were used: 95˚C for 2 min, 32 cycles of 95˚C for 30 s, 50˚C for 30 s, 72˚C for 1 min 30 s, and then 72˚C for 10 min. PCR products were directionally cloned into the pEF5/FRT/V5-D-TOPO vector (Invitrogen) and cotransfected with pOG44 (which transcribes the Flp-In recombinase) into Flp-In CHO cells (Invitrogen) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Stable transfectants were selected using cloning disks (Bel-Art, Pequannock, NJ) after growth in 1200 μg/ml hygromycin B. Transfectants expressing equivalent levels of Crry were selected for functional studies.

Assays for regulation of CP complement activation

For CHO C3-deposition experiments, 10^6 Flp-In CHO cells expressing B6 Crry or *Sle1c* Crry were incubated with anti-hamster lymphocyte serum (Sigma-Aldrich, St. Louis, MO) (final concentration 7.8 mg/ml), 10%

BALB/c serum, 250 mM $MgCl_2$, and 30 mM $CaCl_2$ for 20 min at 37°C to initiate CP complement activation. To block the activation of AP of complement, BALB/c serum was preincubated with the anti-factor B (anti-fB) mAb 1379 (final concentration 0.01 μg/μl) for 10 min and then added to cells. Untransfected Flp-In CHO cells were used as a positive control, and untransfected Flp-In CHO cells without serum added were used as a negative control. Cells were stained with FITC-conjugated goat anti-mouse C3 Ab (MP Biomedicals, Solon, OH), and the amount of C3 deposited on their surface was analyzed by flow cytometry using a BD FACScan.

For erythrocyte C3-deposition experiments, a 2% suspension of mouse erythrocytes was sensitized with the 34-3C IgG2a anti-mouse erythrocyte mAb (38) (kind gift of Wen-Chao Song, University of Pennsylvania School of Medicine, Philadelphia, PA with the permission of Dr. Shozo Izui, University of Geneva, Geneva, Switzerland) for 30 min at 4°C. After washing to remove free Abs, sensitized erythrocytes were incubated with C6-deficient mouse serum (kind gift of Dr. Lihua Bao and Dr. Richard Quigg, University of Chicago, Chicago, IL) diluted 5% in PBS for 30 min at 37°C. The cells were washed with PBS and stained with FITC-conjugated goat anti-mouse C3 Ab, and C3 deposition was determined by flow cytometry using a BD FACSCalibur.

Generation of soluble Crry-IgG1 fusion proteins

cDNA was generated from B6.*Sle1*c splenic RNA by reverse transcription using random hexamers (Applied Biosystems, Foster City, CA). A PCR product spanning the full-length cDNA for *Sle1c* Crry was amplified using the following primers: Crry 1F 5′-CTGGGTGGGACTGCTTTCTAC-3′ and Crry 4R 5′-GTGATACAGACAGAGCATGTTCTC-3′. The following PCR cycling parameters were used: 94°C for 3 min, 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 3 min, and then 72°C for 7 min. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced to ensure that no mutations had been introduced. The extracytoplasmic domain of *Sle1c* Crry was amplified using the following primers to introduce NheI sites at each end of the PCR product: sCrryF 5′-CATTGCTAGCGGATCAGTGCCCAGCCCCATCACAG-3′ and sCrryR 5′-CATTGCTAGCCGAGATACACATTTGGCCAGAAGAGG-3′. The following PCR cycling parameters were used: 94°C for 1 min, 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and then 72°C for 10 min. The PCR product was cloned into the pCR 2.1-TOPO vector, digested with NheI, and ligated into the NheI site of the p118-mIgG_1 vector, which contains exons encoding the hinge, CH2 and CH3 domains of mouse IgG_1 (33). The vector was sequenced to ensure the correct sequence and orientation of the cloned product. To generate the p118-mIgG_1-Crry plasmid containing the extracellular domain of B6 Crry, site-directed mutagenesis (Quick Change II XL Site-Directed Mutagenesis kit; Agilent Technologies, Santa Clara, CA) was performed on a Crry plasmid previously generated using BALB/c cDNA (kind gift of Dr. V. Michael Holers, University of Colorado School of Medicine, Aurora, CO) (33). Both vectors were transiently transfected into Crry-negative 293F embryonic human kidney cells using the FreeStyle 293 Expression System (Invitrogen), according to the manufacturer's protocol. Crry-Ig proteins were purified using affinity chromatography over an anti-mouse IgG_1 (H chain specific) agarose column (Sigma-Aldrich). Eluted fractions were concentrated and tested for purity by SDS-PAGE. Amino-terminal sequence analysis was performed on purified proteins after transfer to the Pro-Blot Membrane (Applied Biosystems). Protein concentrations were determined by spectrophotometry and confirmed by bicinchoninic acid analysis and Coomassie staining. The molar concentrations were determined based on the m.w. of each protein calculated using Dual Color Precision Plus Protein Standards (Bio-Rad, Hercules, CA), which allows the m.w. of unknown proteins to be measured with ≥96% accuracy.

In vitro assay for regulation of AP complement activation

Zymosan particles (Sigma-Aldrich) in 0.15 M NaCl were activated by boiling for 60 min and washed twice in PBS. A total of 10^7 zymosan particles were incubated with 10% BALB/c serum, 250 mM $MgCl_2$, and 250 mM EGTA at 37°C for 10 min to induce activation of the AP of complement. Increasing concentrations of B6 Crry-Ig or *Sle1c* Crry-Ig (10.6–220.9 nM) were added to inhibit complement activation. Zymosan particles without added serum were used as a negative control, and zymosan particles without added Crry-Ig were used as a positive control. Zymosan particles were stained with FITC-conjugated goat anti-mouse C3 (MP Biomedicals), and the amount of C3 deposited on their surface was analyzed by flow cytometry using a BD FACScan. The percentage of inhibition of C3 deposition on zymosan particles was calculated using the following formula: % inhibition = [(mean fluorescence intensity [MFI] zymosan particles without Crry Ig − MFI zymosan particles plus EDTA) − (MFI zymosan particles with Crry-Ig − MFI zymosan particles plus EDTA)]/(MFI zymosan particles without Crry-Ig − MFI zymosan particles plus EDTA) × 100%.

Induction of ischemic acute renal failure

Mice weighing 20–25 g were anesthetized with 300 μl 2,2,2-tribromoethanol (Sigma-Aldrich) injected i.p. After the mice were anesthetized, they were placed on a heating pad to maintain their temperature during surgery. Laparotomies were performed, and the renal pedicles were isolated by blunt dissection and clamped with surgical clips (Miltex, York, PA). Occlusion of blood flow was confirmed by visual inspection of the kidneys. The clamps were left in place for 24 min and then released. This time of ischemia was chosen to obtain a reversible model of ischemic acute renal failure with a minimum of vascular thrombosis and to avoid animal mortality. The kidneys were observed for ~1 min to ensure blood reflow and then fascia and skin were sutured with 4-0 silk (U.S. Surgical/Covidien, Norwalk, CT). The mice were volume resuscitated with 0.5 ml normal saline and kept in an incubator at 29°C to maintain body temperature. After 24 h of reperfusion, blood was obtained by tail bleed to determine levels of serum urea nitrogen (SUN) as a measure of renal function using a Beckman Autoanalyzer (Beckman Coulter, Brea, CA).

Statistics

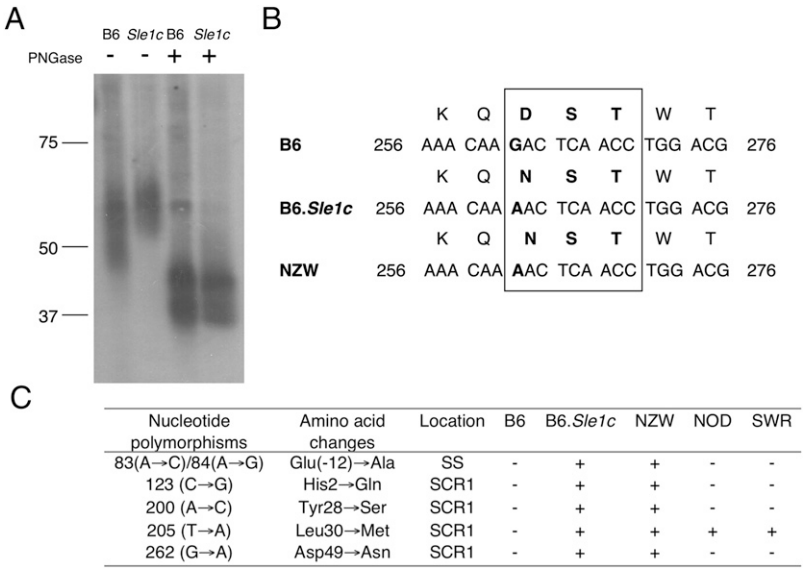
Statistical analyses were performed using GraphPad InStat and GraphPad Prism Software (GraphPad, La Jolla, CA). Multiple-group comparisons were performed using ANOVA with the Tukey posttest if values were sampled from Gaussian distributions or with the Dunn posttest if values were not. Comparisons between two groups were performed using the unpaired *t* test. Results are reported as mean ± SEM. A four-parameter logistic-regression analysis was used to compare the ability of B6 and *Sle1c* Crry-Ig to regulate the AP of complement. For the ischemia–reperfusion experiment, the Mann–Whitney *U* test was used to compare groups, and results are reported as median ± interquartile range. After removal of the outlier from the B6.*Sle1c* group, an unpaired *t* test was used, and results are reported as mean ± SD. A p value <0.05 was considered statistically significant. StatMate (GraphPad) was used to estimate the power of the ischemia–reperfusion experiment to detect a difference between the groups.

Results

The NZW allele of Crry encodes a protein that is structurally different from its B6 counterpart

To determine whether the *Crry* gene product was altered in B6.*Sle1c* mice, Crry was immunoprecipitated from surface biotinylated splenic cell suspensions using a mAb to Crry (5D5). After SDS-PAGE and immunoblotting with HRP-streptavidin, Crry was found to migrate as a broad band (Fig. 1*A*), which may represent multiple isoforms, as has been described in the kidney (39). *Sle1c* Crry migrated at a higher m.w. compared with B6 Crry. This observation was reminiscent of that seen with *Sle1c* CR1 and CR2, which migrated at a higher m.w. than the B6 proteins as the result of the presence of an SNP in the *Cr2* gene that introduced a novel N-linked glycosylation site into SCR1 of CR2 (11). Accordingly, treatment with PNGaseF reduced the Crry proteins from B6 and B6.*Sle1c* mice to equivalent m.w. (Fig. 1*A*). The differential glycosylation of *Sle1c* Crry was explained by a base change of G to A at nucleotide 262, which resulted in an amino acid change of Asp49Asn and the introduction of a new N-linked glycosylation site into SCR1 of *Sle1c* Crry (Fig. 1*B*).

Eight nucleotide sequence differences were identified in the *Sle1c Crry* allele compared with B6. In addition to the SNP at nucleotide 262, there were five nonsynonymous SNPs in the *Sle1c Crry* sequence, including two in the SS and three in SCR1 (Fig. 1*C*), as well as two synonymous SNPs (C > T at nucleotide 516 in SCR2 and A > G at nucleotide 939 in SCR4). No other glycosylation sites introduced by unique polymorphisms in the NZW sequence were identified, suggesting that the SNP at nucleotide 262 was responsible for the demonstrated change in glycosylation. The SS and SCR1 were sequenced in seven other autoimmune strains (NOD, SWR, NZB, MRL, BXSB, SJL, and 129), as well as seven non-autoimmune strains (BALB/c, DBA/1, DBA/2, CBA, AKR, C3H, and FVB), to determine whether any of the nonsynonymous SNPs were shared with other strains. One of these SNPs, the T to A



C

Nucleotide polymorphisms	Amino acid changes	Location	B6	B6.*Sle1c*	NZW	NOD	SWR
83(A→C)/84(A→G)	Glu(-12)→Ala	SS	-	+	+	-	-
123 (C→G)	His2→Gln	SCR1	-	+	+	-	-
200 (A→C)	Tyr28→Ser	SCR1	-	+	+	-	-
205 (T→A)	Leu30→Met	SCR1	-	+	+	+	+
262 (G→A)	Asp49→Asn	SCR1	-	+	+	-	-

FIGURE 1. Structural alteration in *Sle1c* Crry due to an SNP in SCR1. *A*, Immunoprecipitates of Crry from surface biotinylated splenic cells were treated (+) or not (−) with PNGase prior to SDS-PAGE and detection with HRP-streptavidin. Crry from B6.*Sle1c* mice was increased in m.w. compared with Crry from B6 mice due to differential glycosylation. *B*, Sequence of SCR1 of *Crry* in B6.*Sle1c* mice demonstrating a polymorphism at position 262, which introduces a novel N-linked glycosylation site, seen also in NZW mice. *C*, Presence (+) or absence (−) of nonsynonymous *Crry* polymorphisms determined by direct sequencing of PCR products amplified from cDNA.

transversion located at nucleotide 205, was found in two other strains in addition to NZW: NOD, which develops autoimmune diabetes, and SWR, which develops severe lupus-like disease when crossed with NZB (Fig. 1*C*). These two strains also shared the SNP in SCR1 of NZW CR2, which introduces the novel glycosylation site to that domain (11).

Approximately 10% of Crry transcripts have been reported to contain 129 additional bp immediately 3′ of the SS (22). These transcripts are thought to represent alternative splice variants of Crry, although their functional relevance is not known. To assess whether this region was polymorphic between B6 and B6.*Sle1c* mice, the genomic DNA sequence for this region was analyzed in these strains. Although some nucleotide polymorphisms were identified, the genomic sequence for this region of *Crry* in both strains was found to contain 128 bp rather than 129 bp. In the absence of the extra nucleotide, located at position 82 in the original sequence (22), a premature stop codon is introduced into the SS. Therefore, it is likely that splice variants containing this region would generate truncated and nonfunctional proteins and that nucleotide polymorphisms in this region of *Sle1c Crry* would not have a functional effect.

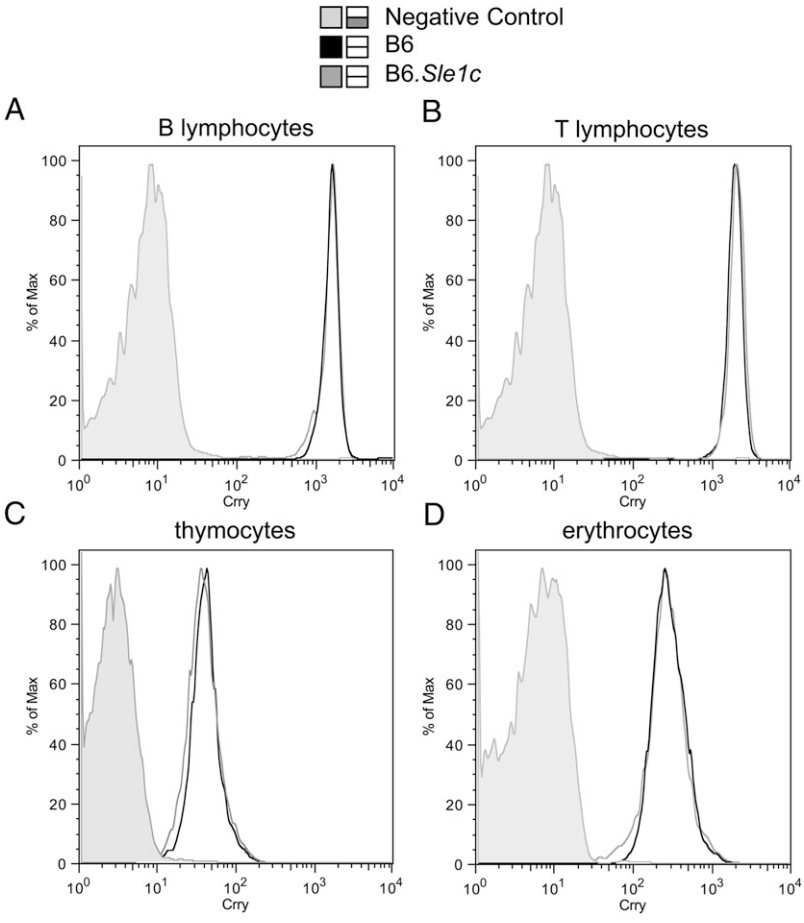


FIGURE 2. Expression of Crry in B6 and B6.*Sle1c* mice. Splenic cells, thymocytes, or erythrocytes from B6 or B6.*Sle1c* mice were stained with the mAb to Crry, 1F2. Equivalent expression of Crry on B lymphocytes (*A*), T lymphocytes (*B*), thymocytes (*C*), and erythrocytes (*D*) from B6 and B6.*Sle1c* mice suggests that the polymorphisms identified in the SS of Crry do not alter Crry expression.

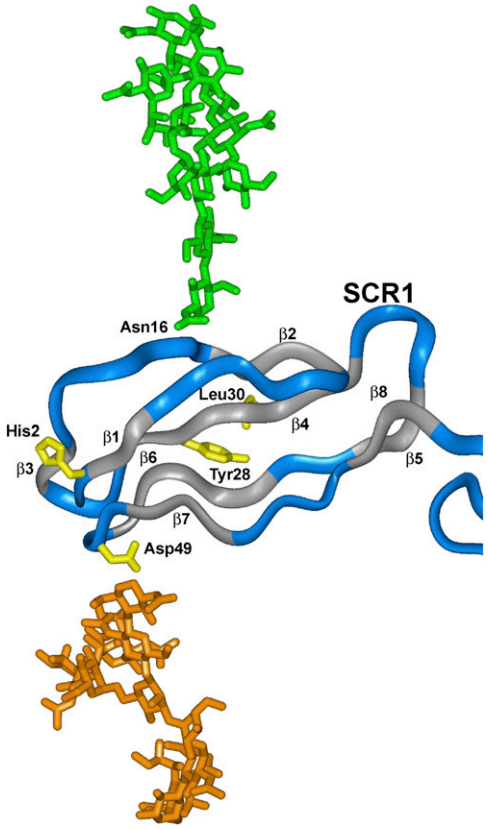


FIGURE 3. Structural location of nonsynonymous SNPs in SCR1 of Crry. SCR1 of B6 Crry is shown as a gray and blue ribbon, with the gray denoting the β-strands (β1–β8) and the blue denoting the loops. The putative carbohydrate group at Asn16 on the hypervariable loop of SCR1 is shown in green, and the side chains of His2, Tyr28, Leu30, and Asp49 are shown in yellow. The new N-linked glycosylation site resulting from the Asp49Asn residue change in *Sle1c* Crry would introduce a second carbohydrate group, shown in orange, on the opposite face from that at Asn16. The Tyr28Ser and Leu30Met residue changes on strand β4 are located in a region likely to be near the binding site of C3b and C4b in Crry.

Expression of Crry is equivalent in B6 and B6.Sle1c mice

The SS is essential for directing nascent proteins to the rough endoplasmic reticulum, where they can complete protein synthesis, fold correctly, and undergo posttranslational modifications. To evaluate whether the polymorphic nucleotide changes in the SS of *Sle1c* Crry alter its expression, levels of Crry were measured on several different cell types. Crry expression was equivalent on splenic B cells (Fig. 2*A*), splenic T cells (Fig. 2*B*), thymocytes (Fig. 2*C*), and erythrocytes (Fig. 2*D*) derived from B6 and B6.*Sle1c* mice as determined by flow cytometry using the anti-Crry mAb, 1F2. Results were similar when expression of Crry on splenic B cells from B6 and B6.*Sle1c* mice was examined using two additional anti-Crry mAbs 5D5 and 7E8 (40) (data not shown). These data suggest that the SNPs in the SS of *Sle1c Crry* are not likely to be functionally relevant.

The SNPs in SCR1 of Sle1c Crry are located in potentially functional domains

We next analyzed the positions of the amino acids affected by the four SNPs in SCR1 of *Sle1c* Crry using molecular modeling based on the solution structure of Crry and its fusion protein Crry-Ig (37). SCR domains typically contain six to eight β-strands with a functionally important hypervariable sequence loop between strands β2 and β3 (41). There are eight putative N-glycosylation sites in the protein, one of which is located in SCR1 at Asn16 at this hypervariable loop. The polymorphism at nucleotide 262 causes an Asp49Asn residue change in the *Sle1c* sequence (Fig. 1*C*) and introduces an N-linked glycosylation site into *Sle1c* Crry at a surface loop between β6 and β7 on the opposite face of SCR1 from Asn16 (Fig. 3). Fig. 3 shows that the extra carbohydrate chain will not block access to strand β4. The polymorphism at nucleotide 123 corresponds to a His2Gln residue change near the N terminus of SCR1, whereas the two polymorphisms at nucleotides 200 and 205, which convert Tyr28 and Leu30 in the B6 sequence to Ser28 and Met30, respectively, in the *Sle1c* sequence, correspond to predicted surface-exposed positions on the β4 strand of SCR1 (Fig. 3). Therefore, they are available as a potential direct interaction site for ligand binding based on their highly exposed nature. The surface accessible locations of the latter two SNPs prompted further assessment of the *Sle1c* Crry protein for functional alterations associated with these sequence changes.

Regulation of complement deposition on cells expressing Sle1c Crry

To test the ability of Crry to regulate complement activation initiated by the CP, we first used a system in which CHO cells are incubated with antiserum to hamster lymphocytes in the presence of normal mouse serum as a source of complement to initiate activation of the CP (42). Activation of the CP results in activation of the AP via the amplification loop (23). To isolate the CP, we used serum that was preincubated with a neutralizing Ab to factor B, which blocks 98% of AP activation by binding the cleavage site of factor B (43). Insignificant cell lysis occurs during the 30 min that the cells are exposed to serum (data not shown). The amount of complement deposited on the surface of the cells was assessed by flow cytometry using a FITC-labeled polyclonal Ab to C3. C3 was deposited at high levels on the surface of CHO cells in this system (thick gray line, Fig. 4*A*). This was dependent on the initiation of complement activation by the CP, because C3 deposition was markedly reduced when EGTA and $MgCl_2$, which selectively block the CP, were added to the reaction (thin gray line, Fig. 4*A*). C3 deposition was also reduced when anti-fB was added to the reaction (thick black line, Fig. 4*A*), demonstrating the role of the AP in the amplification of complement activation in this system. To confirm that the C3 deposited on the cells was not due to binding of activation products preformed in the serum, EDTA was added to the sample to prevent activation of the CP and AP (thin black line, Fig. 4*A*). There was no C3 bound to the cells under this condition, comparable to the results obtained when no serum was present in the reaction (shaded graph, Fig. 4*A*), indicating that the deposited C3 represents activation products generated during the course of the experiment.

Next, we examined C3 deposition on CHO cells that had been transfected with the full-length cDNA for B6 or *Sle1c* Crry. Equivalent expression of Crry on the two transfectants was confirmed by flow cytometry (Fig. 4*B*). Although inhibition of complement activation by either allele of Crry was not complete in this system, *Sle1c* Crry inhibited complement deposition on CHO cells as effectively as B6 Crry (normalized MFI C3 deposition on *Sle1c* Crry CHO versus B6 Crry CHO: 40.0 ± 7.5 and 45.5 ± 7.3, respectively; $p = 0.61$; Fig. 4*C*). *Sle1c* Crry also inhibited C3 deposition as effectively as B6 Crry when activation of the AP was blocked by anti-fB (normalized MFI C3 deposition on *Sle1c* Crry CHO versus B6 Crry CHO: 35.1 ± 6.1 and 39.9 ± 5.7, respectively; $p = 0.58$; Fig. 4*D*). Therefore, these data show that the *Sle1c* allele of Crry regulates complement activation initiated by the CP as effectively as the B6 allele.

As another measure of the ability of *Sle1c* Crry to regulate CP activation in the context of other membrane complement regulatory

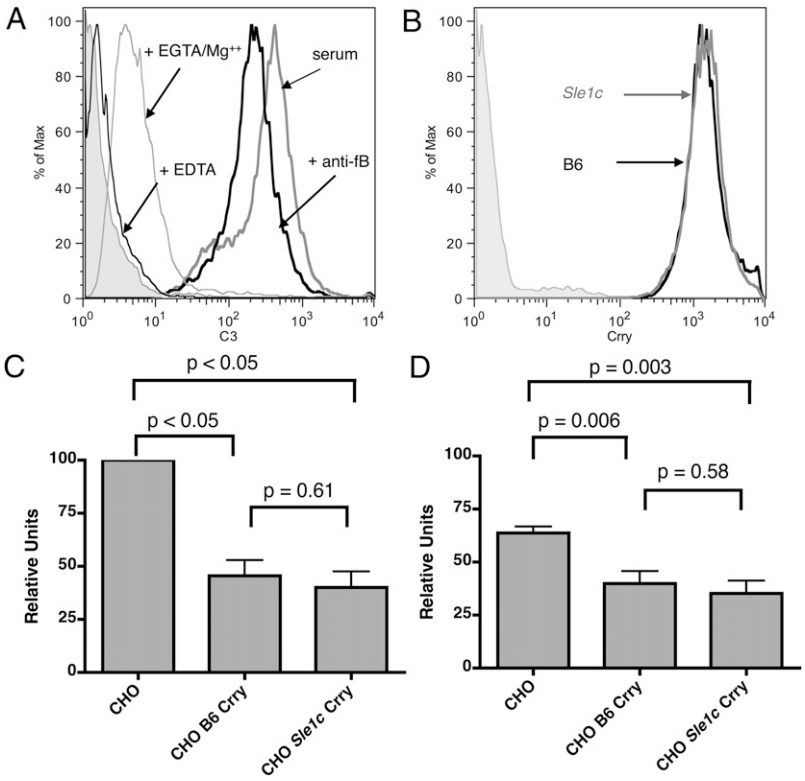


FIGURE 4. *Sle1c* Crry regulates complement deposited on CHO cells via CP activation equivalently to B6 Crry. *A*, Flow cytometric analysis of C3 deposition on untransfected CHO cells incubated with anti-hamster lymphocyte serum in the presence (thick gray line) or absence (shaded graph) of normal mouse serum. Anti-fB (thick black line), EGTA and $MgCl_2$ (thin gray line), or EDTA (thin black line) was added to inhibit the AP, the CP, or both. *B*, Crry expression on CHO cells transfected with B6 Crry (black line) or *Sle1c* Crry (gray line). Shaded graph represents staining with an isotype control Ab. *Sle1c* Crry transfectants inhibited C3 deposition equivalently to B6 Crry transfectants in the presence (*C*) or absence (*D*) of AP activation. In each of five separate experiments, the sample with the maximal MFI (untransfected CHO cells incubated with serum in the absence of anti-fB mAb) was set at a relative value of 100, and the other samples were normalized to this value. The mean and SEM of the normalized values for MFI are demonstrated.

proteins, protection of Ab-sensitized erythrocytes from C3 opsonization was assessed. Ab-sensitized B6 and B6.*Sle1c* erythrocytes were incubated with 5% C6-deficient mouse serum, and C3 deposition was measured by flow cytometry. Erythrocytes from Crry-deficient mice were used as a control. C3 deposition was increased 10-fold on Crry-deficient erythrocytes compared with Crry-sufficient erythrocytes, but there were no significant differences in C3 deposition on erythrocytes from B6 and B6.*Sle1c* mice as measured by MFI (33.7 $\pm$ 4.1 and 31.5 $\pm$ 3.8, respectively; p = 0.71, Fig. 5).

Soluble Sle1c *Crry regulates complement activation initiated by the AP more efficiently than B6 Crry*

To study the ability of *Sle1c* Crry to regulate the AP, we generated rIgG1 fusion proteins containing the five extracellular SCRs of B6 or *Sle1c* Crry (33) (Fig. 6A). Analysis of the purified proteins by SDS-PAGE revealed that *Sle1c* Crry-Ig migrated at a higher m.w. compared with B6 Crry-Ig (Fig. 6*B*), indicating that the proteins were appropriately glycosylated in this system. The N-terminal sequence analysis of the two proteins demonstrated the appropriate sequence, indicating that proper signal peptide cleavage had occurred.

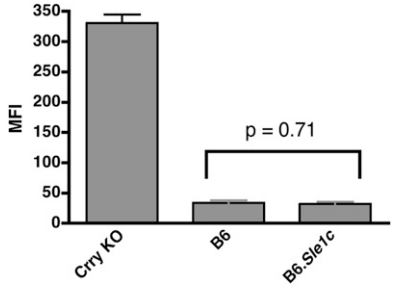


FIGURE 5. *Sle1c* Crry regulates complement deposited on erythrocytes via CP activation equivalently to B6 Crry. Flow cytometric analysis of C3 deposition on Ab-sensitized erythrocytes from Crry-deficient (n = 1), B6 (n = 3), or B6.*Sle1c* (n = 3) mice after incubation with C6-deficient mouse serum. Triplicate samples from each mouse were analyzed.

The ability of the different Crry-Ig fusion proteins to regulate complement activation initiated by the AP was tested using normal mouse serum as a source of complement and zymosan particles as an activator of the AP. EGTA and $MgCl_2$ were added to inhibit activation of the CP, thereby isolating the effects of Crry on the AP. Taking into account the different m.w. of B6 Crry-Ig and *Sle1c* Crry-Ig, equivalent molar concentrations of each, ranging from 10.6–220.9 nM, were prepared and added to the reaction. The amount of complement deposited on the surface of the zymosan particles was assessed by flow cytometry using a FITC-labeled polyclonal Ab to C3. The percentage of inhibition of C3 deposition at each concentration of Crry-Ig was determined using the equation described in *Materials and Methods*. Although both proteins inhibited C3 deposition throughout the range of concentrations, *Sle1c* Crry-Ig inhibited complement deposition more effectively than did B6 Crry-Ig (IC_{50}: 32.90 and 46.84, respectively; Fig. 6*C*). The results of a four-parameter logistic regression of the percentage of inhibition on $\log_{10}$[dose] show that it was significantly greater for *Sle1c* Crry-Ig than for B6 Crry-Ig at all doses: $\log IC_{50}$ (95% CI): 1.517 (1.478–1.557) and 1.671 (1.619–1.722), respectively; $p < 0.0001$; and Hill slope (95% CI): 1.452 (1.252–1.652) and 1.799 (1.476–2.122), respectively; p = 0.046) (Fig. 6*D*).

*Regulation of complement deposition in kidneys of B6.*Sle1c *mice*

We used the ischemia–reperfusion mouse model of acute renal injury to determine whether *Sle1c* Crry functions in vivo to regulate complement activation initiated by the AP more efficiently. In this model, complement activation in the kidney after ischemia–reperfusion occurs exclusively via the AP (44), and heterozygous gene-targeted mice expressing lower amounts of Crry are more sensitive to ischemic injury (45). Renal ischemia was induced in B6 and B6.*Sle1c* mice for 24 min, and blood was collected 24 h later for determination of SUN levels as a measure of renal function.

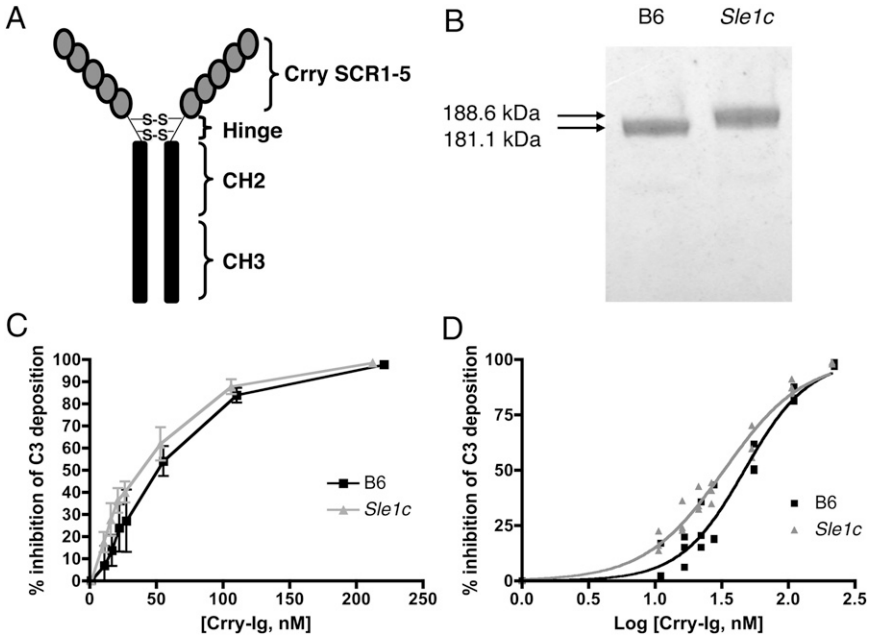


FIGURE 6. Soluble *Sle1c* Crry is a more effective regulator of the AP. *A*, Structure of the Crry-Ig fusion protein. Circles represent SCR1-5 of Crry, which are fused to the hinge, CH2, and CH3 region of mouse IgG1. *B*, Four to 20% Tris-HCl gel of purified B6 and *Sle1c* Crry-Ig recombinant fusion proteins. *Sle1c* Crry-Ig protein has a higher m.w. (188.6 kDa) compared with B6 Crry-Ig (181.1 kDa) due to enhanced glycosylation. *C*, Regulation of the deposition of the C3 component of complement on zymosan particles was assessed by flow cytometry. The MFI of C3 deposited at each dose of Crry-Ig, in the absence of Crry-Ig (0% inhibition), and in the presence of EDTA (100% inhibition) was determined in duplicate and averaged to allow the percentage of inhibition of C3 deposition to be calculated for B6 Crry-Ig and *Sle1c* Crry-Ig, as described in *Materials and Methods*. Data shown are mean ± SD for values calculated from three independent experiments. *D*, Four-parameter logistic regression of transformed data from three independent experiments. $LogIC_{50}$ and Hill slope for the *Sle1c* Crry-Ig dose-response curve were significantly different from those of B6 Crry-Ig ($p < 0.0001$ for $logIC_{50}$ and $p = 0.0461$ for Hill slope).

The median SUN in B6 and B6.*Sle1c* mice was not significantly different, indicating equivalent tubular damage due to alternative complement pathway activation in both groups (167.5 ± 50.5 mg/dl versus 200.5 ± 32.5 mg/dl; $p = 0.50$; Fig. 7). Even after removing the outlier in the B6.*Sle1c* group, there was no significant difference in the mean ± SD between the groups (182.3 ± 31.7 mg/dl versus 199.7 ± 15.8 mg/dl; $p = 0.20$). Using the selected sample sizes, this experiment had 80% power to detect a difference between the means of 40.2 (~20%) at a significance level of 0.05, two-tailed.

Discussion

The purpose of this study was to dissect the role of *Crry* as a possible candidate gene for lupus susceptibility in the *Sle1c* interval. The *Sle1c* allele of *Crry* was structurally altered, because it migrated at a higher m.w. than its B6 counterpart. This was due to a polymorphism in the first SCR of *Sle1c* Crry, which introduced an N-linked glycosylation site. Five other SNPs in the *Sle1c Crry* sequence were identified, introducing one amino acid change in the signal peptide sequence and three additional amino acid changes in the first SCR. These alterations in the protein sequence of *Sle1c* Crry did not impair the ability of the membrane protein to regulate the CP of complement activation, but it seemed to confer on the soluble protein an enhanced ability to regulate the AP. However, in an in vivo model of acute renal injury that is driven by alternative complement pathway activation (44), this apparent hyperfunction of *Sle1c* Crry could not be demonstrated, suggesting that *Sle1c* Crry functions equivalently to B6 Crry with respect to its ability to regulate complement and that structural alterations in the protein products of this gene do not contribute to the *Sle1c* autoimmune phenotypes.

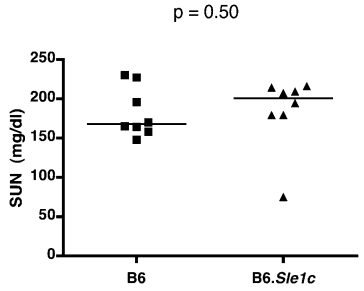


FIGURE 7. Renal tubular injury from ischemia–reperfusion is induced equivalently in B6 and B6.*Sle1c* mice. SUN levels were measured in serum collected from B6 ($n = 8$) and B6.*Sle1c* ($n = 8$) mice 24 h after induction of ischemic acute renal failure by occlusion of the renal pedicles for 24 min.

There are several possible mechanisms by which the polymorphisms in *Sle1c* Crry could enhance the regulation of the alternative complement pathway. First, the additional carbohydrate group resulting from the SNP at position 262 could enable *Sle1c* Crry to more effectively dissociate the AP C3 convertase through steric hindrance. Second, this nucleotide change may increase the affinity of Crry for C3b, by inducing a structural alteration due to the additional carbohydrate group or by removing a negative charge from the protein as a result of the conversion of the aspartic acid to an asparagine. In either case, a higher affinity interaction would be expected to result in more efficient proteolytic inactivation of C3b, thus reducing the formation of the AP C3 convertase. The other SCR1 polymorphisms in *Sle1c* Crry, Tyr28Ser and Leu30Met, could also contribute because they are located at the center of SCR1 on a well-defined β-strand that may contain amino acids that directly interact with ligands. Mutagenesis studies support this hypothesis, because the amino acids that they encode in the homologous domains in human CR1 specifically alter binding of iC3b and C4b when manipulated, resulting in increased cofactor activity (46).

However, when we examined the function of *Sle1c* Crry in its native membrane form using a well-characterized model for acute renal injury in which damage is dependent on the alternative complement pathway (44) and protection is dependent on Crry (45), we found no difference in its ability to regulate alternative complement pathway activation compared with B6 Crry. There are several potential explanations for these conflicting results. First, the configuration of soluble Crry may vary from that of the membrane protein to allow better access to and higher affinity interactions with C3b. Alternatively, *Sle1c* Crry may function more efficiently than B6 Crry as a membrane protein; however, in an in vivo system in which complement-mediated damage is ongoing, this difference may not be physiologically relevant. However, we cannot rule out the possibility that a more efficient AP regulator may have different effects in a chronic disease process, such as SLE, than in an acute model of injury due to ischemia–reperfusion.

Although regulation of complement activation is the primary function of Crry, this protein also has effects on T cell function (47–49) and potentially could contribute to the *Sle1c* autoimmune phenotypes through this mechanism. Crry acts as a costimulatory molecule in $CD4^+$ T cell activation, modifying the pattern of cytokine secretion toward a Th2 profile via an increase in early TCR-dependent activation signals (47, 48). It may also be involved in the selection of T cells in the thymus via enhancement of cell adhesiveness and activation of thymocytes through LFA-1 (49). MCP, the human analog of Crry, controls self-reactive T lymphocytes via the induction of T regulatory 1 cell differentiation (50), although this function has not been shown for Crry. However, because the previously identified *Sle1c* T cell phenotypes were shown to segregate with loci centromeric to *Crry* (10), it seems unlikely that these phenotypes are related to alterations in this gene.

This report emphasizes the difficulties inherent in analyzing potential susceptibility genes for complex diseases. Variations in nucleotide sequence in candidate genes are not uncommon, and although these may be located in potentially functional domains and cause dramatic structural effects in the case of hyperglycosylation of the protein, they may not substantially alter the known functions of the protein. Furthermore, functional changes identified using in vitro methods or artificial systems may not be physiologically relevant in vivo. Our findings provide no definitive evidence for a role for Crry in the autoimmune phenotypes attributed to the *Sle1c* lupus susceptibility interval, suggesting that future studies focusing on *Cr2* as the responsible autoimmunity gene in this very telomeric region of chromosome 1 are justified. However, it will be important to keep in mind that the relevance of minor alterations in gene function on chronic disease may not be apparent in the acute settings in which many gene effects are studied or in the absence of functional variants of other susceptibility genes. The importance of the AP of complement in disease pathogenesis has become increasingly apparent, as demonstrated in animal models of lupus (51, 52), rheumatoid arthritis (53), and antiphospholipid Ab syndrome (43, 54), as well as in human diseases like atypical hemolytic-uremic syndrome (55–61) and age-related macular degeneration (62–64). That polymorphisms in murine Crry resulted in a soluble protein with increased complement regulatory function that could dampen the alternative complement pathway, in contrast to the polymorphic variants that have been associated with human disease (55–61), which enhance activation of this pathway, was a novel and intriguing finding. Because B6.*Sle1c* mice do not develop glomerulonephritis despite developing autoantibodies to chromatin, a hyperfunctional Crry could be hypothesized to protect from disease. Alternatively, hyperfunction of Crry resulting in decreased complement deposition on apoptotic cells could result in impaired clearance of cellular debris that could drive autoimmune responses. Perhaps only by the genetic manipulation of candidate genes in murine lupus susceptibility intervals and the study of the epistatic interactions of these candidates in the development of chronic disease manifestations will we be able to definitively prove, or disprove, their association with disease.

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Disclosures

The authors have no financial conflicts of interest.

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