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Several Genes Contribute to the Production of Autoreactive B and T Cells in the Murine Lupus Susceptibility Locus Sle1c

Yifang Chen,* Daniel Perry,* Susan A. Boackle,‡ Eric S. Sobel,† Hector Molina,§ Byron P. Croker,* and Laurence Morel²*

The systemic lupus erythematosus 1 (Sle1) locus mediates the loss of tolerance to nuclear Ags in the NZM2410 mouse model of lupus through intrinsic defects in both B and T cells. Congenic analysis has shown that Sle1 corresponds to at least three genetic loci, Slea, Sleb, and Slec. Telomeric Slec is associated with abnormal B cell responses to subthreshold stimulation with anti-IgM and C3d and with decreased T-dependent humoral immune responses. We have proposed that these phenotypes resulted from polymorphisms in the C3 complement receptor Cr2 gene. We have also found that Slec was associated with the production of histone-specific autoreactive CD4⁺ T cells, which correlated with higher activation and proliferative responses, and a reduction in the CD4⁺CD25⁺CD62L⁻forkhead/winged helix transcription factor gene (Foxp3⁺) compartment. In this study we showed, using congenic recombinants, that the decreased humoral immune response and impaired GC formation map to the NZM2410 Cr2 allele. A chronic graft-vs-host disease model also showed that Slec produces significantly more autoreactive B cells than B6 controls, and that this phenotype maps to two regions excluding the Cr2 gene. Mixed bone marrow chimera demonstrated that the increased activation, proliferative response, and reduced regulatory T cell compartment were intrinsic to Slec-expressing CD4⁺ T cells. These phenotypes mapped to the same two loci identified with the chronic graft-vs-host disease model, excluding the Cr2 region. Overall, these results show that Slec results in the production of autoreactive B and T cells through the expression of three different genes, one of which is consistent with Cr2, based on the phenotypes of the Cr2-deficient mice, and the other two corresponding to as yet unidentified genes. The Journal of Immunology, 2005, 175: 1080–1089.

Systemic lupus erythematosus 1 (Sle1) is a major susceptibility locus in the NZM2410 lupus model, an inbred mouse strain derived from the New Zealand Black (NZB) and New Zealand White (NZW) F₁ model (1, 2). Congenic C57BL/6 (B6) mice carrying the NZM2410-derived Slel interval (B6.Sle1) are characterized by the production of high titers of anti-nuclear Abs, specifically directed to the H2a/H2b/DNA subnucleosome, the presence of histone-reactive T cells, as well as an increased lymphocyte activation (3). Sle1 is functionally expressed in B and T cells, and it affects independently the break in tolerance to nuclear Ags and increased activation in both lymphocyte subsets (4, 5), two phenotypes that are the hallmark of systemic autoimmunity (6). The comparison of combinations of multiple susceptibility intervals has shown that Sle1 is necessary for the development of clinical disease in the NZM2410 model (7). We have also identified an NZW-derived potent suppressor locus, H-2⁻-linked Sle1, that is sufficient to abrogate the autoimmune phenotypes initiated in the presence of Sle1 (8). Finally, the Sle1 region on telomeric chromosome 1 and the corresponding syntenic human 1q23–42 have been linked and associated with lupus susceptibility in multiple murine and human studies (9, 10). These results concur to establish that the identification of the gene(s) corresponding to Sle1 and the elucidation of the mechanisms by which their Sle1 allele contribute to systemic autoimmunity represent essential steps for understanding the genetic basis of SLE.

By screening B6.Sle1 congenic recombinants for anti-nuclear Abs, we have established that Sle1 corresponds to at least three independent loci that we have named Slea, Sleb, and Slec (11). The identity of the gene corresponding to Slea is currently unknown. Sleb, which by itself produces the most robust levels of anti-nuclear Abs, corresponds to mutations in genes of the SLAM/CD2 family, which form a seven-gene cluster contained in the Sleb critical interval (12). Slec induces anti-nuclear Abs in ~30% B6.Slec by 9 mo of age (11). Despite this modest autoantibody contribution, we have recently shown a significant contribution of Slec to pathogenesis by comparing anti-dsDNA IgG production and renal pathology between (NZB × B6).Slecf₁ and (NZB × B6)F₁ mice (13). We have proposed Cr2, which yields complement receptor 1 (CR1; CD35) and 2 (CR2 or CD21) through alternative splicing, as a candidiate gene for Slec based on the presence of multiple polymorphisms between the B6 and NZW alleles (14). One of these polymorphisms introduces a glycosylation site in the short consensus repeat 7 (SCR7) that is predicted to impair binding of C3d, the CR1/CR2 ligand. CR1/CR2 functions as a B cell coreceptor and increases germinal center (GC) efficiency through Ag trapping by the follicular dendritic cells (FDC) (15). Cr2 deficiency has been associated with loss of...
B cell tolerance and autoantibody production, especially in the absence of Fas expression (16, 17). Finally, CR1/CR2 levels are significantly decreased in lupus patients (18, 19) and in certain cases are inversely related to disease activity (20). Similarly, decreased CR2 expression has been reported on B cells before the onset of disease in the MRL/lpr (21) and chronic graft-vs-host disease (cGVHD) models of SLE (22).

We have shown the same altered B cell phenotypes in B6.Sle1c mice, although to a lesser degree, as those described for CR2-deficient mice: decreased C3d binding, decreased Ca2+ flux in response to subthreshold levels of BCR and CR1/CR2 cross-linking, and impaired Ab response to T-dependent (TD) Ag (14). Furthermore, as in Cr2 deficiency, the lpr mutation significantly enhanced the Sle1c autoimmune phenotypes (23). A recent examination of the effect of the individual Slec loci on T cells revealed that Sle1c resulted in increased proliferation and activation of CD4+ T cells and the production of autoreactive histone-specific T cells (56). Forthmore, B6.Sle1c mice harbored a significantly decreased number of CD4+CD25+CD62Lhigh forkhead/winged helix transcription factor gene (Foxp3+) cells, which correspond to regulatory T cells (24). Finally, Sle1c CD4+ T cells were able to induce anti-chromatin Ab production in Sle1-expressing B cells in vivo, although B6 T cells could not. The altered T cell phenotypes in B6.Sle1c mice could be the direct or indirect consequences of the same Cr2 mutations that produce B cell alterations. CR1/CR2 expression has been reported on activated T cells in A/J mice (25). It suppression has been reported on activated T cells in A/J mice (25). It has also been proposed that CR1/CR2 could indirectly influence T cell phenotypes through Ag presentation (26, 27). Alternatively, the Sle1c locus may correspond to more than one gene, including Cr2 inducing the previously described B cell phenotypes and another gene affecting T cells, both contributing to the overall autoimmune phenotype of this locus.

In this study we examined the effects of Sle1c on B and T cells in more detail to test these hypotheses, and we produced Sle1c recombinants to map these phenotypes relative to Cr2. We show in this study that Cr2 deficiency and Sle1c produce similar alterations on B cells and Gcs, and that these phenotypes segregate with the Cr2w+ allele. Using the cGVHD model, we show that Sle1c results in the production of autoreactive B cells, but this phenotype maps centromerically to Cr2. Finally, we show that the activated Cd4+ T phenotypes are intrinsic to Sle1c-expressing cells, and that these phenotypes map to two loci centromeric to Cr2. Overall, these results strongly suggest that Sle1c corresponds to at least three independent genes. One gene, most likely Cr2, alters the B cell response to Ag and GC structure. Two centromeric genes result in a break of B and T cell tolerance and alter CD4 + T cell activation.

### Materials and Methods

#### Mice

The congenic strains B6.Sle1 and B6.Sle1c have been described previously (11, 28). The entire Sle1 region is derived from the NZW genome (29). B6.Sle1c recombinants were generated from a (B6.Sle1c × B6) × B6 cross and then bred to homozygosity. Recombination breakpoints were mapped using Massachusetts Institute of Technology (MIT) microsatellite markers that are polymorphic between NZW and B6. The identity of the Cr2 allele was determined using the restriction fragment length polymorphism-PCR specific for the C1342A mutation in the SCR7/SCR1 domain as previously described (14). B6, B6.Sle1, B6.Sle1c, and the B6.Sle1c recombinant mice were bred and maintained at the University of Florida. B6.129.Cr2w−/− mice have been described previously (30) and were maintained along with B6.129.Cr2w−/− controls at the University of Colorado Health Sciences Center. B6, B6.SLE-Pprcre+Pep−/−BoyJ (B6, Ly5+), and B6.C-H2m12/KHeg (B6.bm12) mice were obtained from The Jackson Laboratory. Unless specified, experiments were conducted with 6- to 8-mo-old congenic mice and B6 controls. Both females and males were used, because no significant difference was noted between the two sexes. All experiments were conducted according to protocols approved by the University of Florida and the University of Colorado institutional animal care and use committees.

#### Bone marrow (BM) chimeras

Chimeras were prepared as previously described (5). In brief, 6- to 8-wk-old mice were lethally irradiated with two doses of 525 rad gamma irradiation (3 h apart) in a Gammacell 40 137Cs apparatus. Donor BM cells were depleted of mature T and B cells by a mixture of anti-CD4, anti-CD8, anti-Thy-1.2 (mMT1), and anti-I-Ak (clone D3-137.5), followed by complement lysis (Rabbit Lo-Tox; Jackson Immunoresearch Laboratories). Production of mixed BM chimeras was performed at a 1:1 ratio for the two donors. Ten million cells were given to each mouse by tail vein injection. Chimeric mice were maintained for 8 wk before evaluation to allow full development of the hemopoietic system from the transferred BM.

#### Immunization

The response to T-dependent Ags was measured by injecting mice i.p. with 100 µg of DNP-keyhole limpet hemocyanin (DNP-KLH; Calbiochem) in 100 µl of sterile PBS mixed with 100 µl of Imject alun (Pierce). Unmanipulated mice were immunized at 8 wk of age. The reciprocal (B6: B6.Sle1c) chimeras were immunized 8 wk after BM transfer. Serum titers of IgM and IgG3 anti-DNP Ab were determined 21 days after immunizations by ELISA in duplicate on DNP-BSA-coated (Calbiochem) microtiter plates as previously described (14).

#### Immunohistochemistry

Mice were killed 10 days after immunization with DNP-KLH. Acetone-fixed, 7-µm sections were obtained from spleens frozen in OCT (Miles) and 2-methylbutane. For the immunofluorescence study, the following Abs were used: CD4-allophycocyanin (RM4-5), B220-allophycocyanin (RA3-6B2), GL7-FITC, CD16/CD32-biotin (2.4G2; all from BD Pharmingen) and CD21/CD35-biotin (7E9 and 8C12). Biotinylated Abs were revealed with streptavidin-rhodamine (Molecular Probes). Immune complex (IC) trapping was measured as previously described (31). Briefly, spleen sections were incubated with saline buffer containing 20% fresh mouse serum (as a source of complement) and 10% rabbit HRP/rabbit HRP Ab complexes (DakoCytomation). HRP was developed with 3-amin-9-ethyl-carbazole, and the sections were counterstained with 1% methyl green (Sigma-Aldrich).

#### Chronic GVHD induction

Chronic GVHD was induced in B6.Sle1c mice with allogeneic T cell help from B6.bm12 mice, according to the protocol established by Eisenberg et al. (32, 33). Briefly, 80 × 106 RBC-depleted B6.bm12 splenocytes were injected i.p. in 200 µl of sterile saline buffer into B6.Sle1c or B6 mice. Recipient mice (four to six mice per group) were either 3-4 or 9 mo old. Serum was collected weekly for 3 wk, and the mice were killed on day 21 after injection. The experiment was performed twice. Anti-chromatin and anti-dsDNA IgG serum levels were quantified by ELISA as previously described (3). The presence of immune complexes in the kidneys was evaluated on 7-µm frozen sections stained with FITC-conjugated anti-C3 (ICN Biomedicals), anti-IgG γ chain (Jackson Immunoresearch Laboratories), or IgM (Igh-6b; BD Pharmingen).

#### Flow cytometry

Briefly, cells were first blocked on ice with staining buffer (PBS, 5% horse serum, and 0.05% sodium azide) supplemented with 10% normal rabbit serum and anti-CD16/CD32 (2.4G2). Cells were then stained with pretitrated amounts of the following FITC-, PE-, allophycocyanin-, or biotin-conjugated Abs: CD4-allophycocyanin (RM4-5), B220-allophycocyanin (RA3-6B2), GL7-FITC, CD16/CD32-biotin (2.4G2; all from BD Pharmingen) and CD21/CD35-biotin (7E9 and 8C12). Biotinylated Abs were revealed with streptavidin-rhodamine (Molecular Probes). Immune complex (IC) trapping was measured as previously described (31). Briefly, spleen sections were incubated with saline buffer containing 20% fresh mouse serum (as a source of complement) and 10% rabbit HRP/rabbit HRP Ab complexes (DakoCytomation). HRP was developed with 3-amin-9-ethyl-carbazole, and the sections were counterstained with 1% methyl green (Sigma-Aldrich).
Camk1g (52), NZM2410 genomes are shown by thin lines. black lines, and the area of recombination between the B6 and the three recombinant congenic intervals are represented by horizontal thick top line). The bottom line shows the MIT microsatellite markers that are polymorphic between B6 and NZM2410/NZW. Below are shown candidate genes selected based on their known or putative function in the immune system: Sle1c, Sle1c.Cr2b-1, Sle1c.Cr2w-1, Cr2b, Cr2w, and Cr2. The Sle1c and the three recombinant congenic intervals are represented by horizontal thick black lines, and the area of recombination between the B6 and the NZM2410 genomes are shown by thin lines.

### Proliferation assays

Splenic single-cell suspensions were labeled with 2.5 μM CFSE (Molecular Probes). Lymphocytes were then cultured in triplicate on anti-CD3-coated 96-well plates (BD Pharmingen) with anti-CD28 (1 μg/ml; PeproTech) in complete RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 100 U/ml penicillin plus 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 μg/ml M 2-ME (Invitrogen Life Technologies) in a 37°C, 5% CO2 incubator for 48 h. In vivo proliferation was assessed by exposing mice to BrdU (Sigma-Aldrich) in drinking water (0.8 mg/ml) for 9 days. Fixed and permeabilized splenocytes were then treated with DNase and analyzed with a FITC-conjugated anti-BrdU Ab (BD Pharmingen).

### Results

**Map of Sle1c interval and Sle1c recombinants**

The Sle1c congenic interval corresponds to an ~7-Mb genomic segment derived from NZM2410 chromosome 1, starting between the D1MIT459 and D1MIT274 markers on the centromeric side and extending all the way to the telomere (Fig. 1). The recently published physical map ((www.ensembl.org/Mus_musculus/)), rather than the genetic map ((www.informatics.jax.org/)), was used for the Sle1c map. Indeed, we found that this map corresponds better to the marker positions that we have established with a panel of 493 meioses between the NZM2410 and B6 strains (11). Eleven known genes located in the Sle1c interval have known or putative functions in the immune system (Fig. 1). In addition, a number of novel genes are located in this region and should also be considered as candidate genes. To map the genes responsible for the Sle1c phenotypes and specifically address the role of Cr2, we have generated three overlapping recombinants; two centromeric recombinants, B6.Sle1c.Cr2w-1 and -2, have the B6 allele at Cr2, although the telomeric recombinant, B6.Sle1c.Cr2w-1, has the NZW allele at Cr2. The recombination breakpoints of these recombinants were mapped using all MIT markers that are polymorphic between NZW and B6, as indicated in Fig. 1, and the allele of Cr2 with an restriction fragment length polymorphism in the SCR1/SCR7 repeat as previously described (14).

**FIGURE 1.** Physical map of the Sle1c locus. Positions (in megabases; top line) are shown according to (www.ensembl.org/Mus_musculus/). The bottom line shows the MIT microsatellite markers that are polymorphic between B6 and NZM2410/NZW. Below are shown candidate genes selected based on their known or putative function in the immune system: Sle1c, Sle1c.Cr2b-1, Sle1c.Cr2w-1, Cr2b, Cr2w, and Cr2. The Sle1c and the three recombinant congenic intervals are represented by horizontal thick black lines, and the area of recombination between the B6 and the NZM2410 genomes are shown by thin lines.

**FIGURE 2.** A decreased humoral response in B6.Sle1c mice is produced by both radiosensitive and radioresistant cells and maps to the Cr2w allele. The anti-DNP IgM (A) and IgG3 (B) responses to DNP-KLH immunization were evaluated in reciprocal B6 and B6.Sle1c BM chimeras. Responses were compared among the four groups indicated on the x-axis (host/donor) or between groups of B6 and B6.Sle1c donors regardless of the recipients. C, Immunization of B6.Sle1c recombinants (Cr2w, B6.Sle1c.Cr2w-1 and -2; Cr2w; B6.Sle1c.Cr2w-1) showed that the reduced IgM and IgG3 responses segregate with the Cr2w allele. No significant difference was obtained between B6.Sle1c.Cr2w-1 and -2. Values are the mean and SE for five mice per group/strain. Statistical significance of t tests is shown between the indicated groups in A and B and between the recombinants and B6 in C. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Statistics**

Unless indicated, unpaired one-tailed t test statistics were used to compare the phenotype of the B6.Sle1c strain or its recombinants with that of B6. Nonparametric Mann-Whitney tests were used when the data were not normally distributed.
Decreased humoral response in B6.Sle1c mice is contributed by both radiosensitive and radioresistant cells and maps to the Cr2<sup>−/−</sup> allele

We have previously shown that Sle1c was associated with decreased IgM and IgG3 responses to TD Ag (14), a phenotype that has also been described for Cr2-deficient mice (30, 34). Reciprocal BM chimeras have shown that CR1/CR2 deficiency on both B cells (radiosensitive) and FDC (radioresistant) contributed to this phenotype (35, 36). Similar reciprocal BM chimeras were performed between B6 and B6.Sle1c, providing four groups of mice segregating Sle1c expression on B cells, FDC, or both. Chimeric mice were immunized 8 wk after transfer with DNP-KLH, and the anti-DNP Ab response was measured 21 days later. Mice reconstituted with Sle1c BM produced significantly less anti-DNP IgM and IgG3 Abs than mice reconstituted with B6 BM regardless of the genotypes of the hosts (Fig. 2, A and B). No significant difference was observed when the results were grouped according to the host genotype (data not shown). A significant difference was obtained, however, between the anti-DNP IgM Abs produced by B6 and Sle1c recipients reconstituted with Sle1c BM (Fig. 2A). These results indicate that Sle1c on BM-derived cells is primarily responsible for the decreased humoral response, and that an additional decrease results from Sle1c expression on radiosensitive cells, presumably FDCs. This latter effect, however, is overridden by B6 BM, suggesting that the expression of Sle1c has a greater effect on hemopoietic cells than on stromal cells.

The three Sle1c recombinants were immunized to map the TD response phenotype. A decreased Ab response to DNP-KLH was found in the B6.Sle1c.Cr2<sup>−/−</sup>-1, but not in the B6.Sle1c.Cr2<sup>−/−</sup>-1 and -2, recombinants (Fig. 2C), consistent with this phenotype resulting from the Cr2<sup>−/−</sup> allele in B6.Sle1c mice.

Abnormal GCs are associated with Sle1c and the Cr2<sup>−/−</sup> allele

It is well established that Cr2 deficiency affects GC structure and function (31, 35, 36). GC morphology in B6.Sle1c spleens 10 days after DNP-KLH immunization was similar to that in Cr2-deficient spleens (Fig. 3, A and B). The FDC networks were poorly developed, and the overall GC size was smaller, although there was no difference in GC numbers. Conversely, Sle1c FDC networks within the GCs were also less efficient at retaining immune complexes (Fig. 3C), although, contrary to what was observed in Cr2-deficient mice (31), some IC binding was maintained. Abnormal GC morphology segregated with the Cr2<sup>−/−</sup> allele in the Sle1c recombinants, although less consistently than in the intact Sle1c congenics (Fig. 3, A and B). Overall, these results are congruent with an altered function of the Cr2<sup>−/−</sup> allele affecting GC structure and their capacity to retain Ag in an IC form.

Sle1c is associated with enhanced cGVHD responses

In the cGVHD model, transferred MHC-mismatched CD4<sup>+</sup> T cells provide cognate help to autoreactive B cells, leading to a set of serological, cellular, and renal phenotypes that have been well characterized (32, 33). We used this model to test whether Sle1c was associated with an increased production of autoreactive B cells. Young (3- to 4-mo-old) and old (9-mo-old) B6.Sle1c and B6.Cr2<sup>−/−</sup>-1 mice were injected with B6.bm12 splenocytes. The presence of anti-chromatin and anti-dsDNA Ab was monitored for 3 wk after transfer, when the distribution and activation of splenic splenocytes and kidney pathology were evaluated. Old B6.Sle1c mice showed a significant enhancement of cGVHD responses compared with age-matched B6 mice. Spleen weight increased in both strains, but it was significantly greater in B6.Sle1c than in B6 mice (337.8 ± 13.5 vs 251.4 ± 26.9 mg; p < 0.02). This splenocyte expansion was accompanied by a significant redistribution of the B cell subsets, with significant expansion of the marginal zone (MZ) and reduction of the follicular B cell compartments (Fig. 4, A–C). Similar results were obtained whether the B cell subsets were defined by differential expression of CD21 and CD23 (as shown in Fig. 4, A and B) or IgM and IgD (data not shown). Anti-chromatin and anti-dsDNA IgG Abs were produced in significantly greater amounts by B6.Sle1c mice as early as 1 wk after transfer for anti-dsDNA (Fig. 4D), and this coincided with a greater percentage of plasma cells in the B6.Sle1c spleens (Fig. 4, E and F). B cell activation was significantly increased in B6.Sle1c mice, as illustrated in Fig. 4, G and H, by an increased expression of B7-1 (CD80) and MHC-II 1-α as well as an increased expression of B7-2 and CD69 and decreased expression of CD22 and B220 (data not shown). Host CD4<sup>+</sup> T cells are necessary for cGVHD induction, although their role is as yet unknown (37). CD4<sup>+</sup> T cell activation was significantly higher in B6.Sle1c than in B6 hosts after cGVHD induction, as exemplified by the proportion of CD4<sup>+</sup> CD62L<sup>low</sup>CD44<sup>+</sup> memory T cells in spleens (48.2 ± 3.0 vs 37.1 ± 1.3% of CD4<sup>+</sup> T cells; p < 0.003). This result suggests an increased response of Sle1c T cells to bm12 cells, which is consistent with the increased activation of Sle1c T cells that we have previously described (56). The experimental design, however, did not discriminate between the donor and host origins of these T cells, and we cannot formally exclude an increased response of bm12 T cells to Sle1c Ags. Previous reciprocal BM transfers between B6 and B6.Sle1 did not show any evidence of minor histocompatibility activation between these two strains (4, 5), which makes the latter hypothesis unlikely. Finally, we observed a marked increased in the size and number of GCs in B6.Sle1c spleens after cGVHD induction (Fig. 5A), which illustrates the enhanced response to bm12 allogeneic help. In addition, we found an increased amount of C3 (Fig. 5B) and IgG, but not IgM, deposits (data not shown) in B6.Sle1c kidneys compared with those in B6 mice, which correlates with the increased production of nuclear Abs noted above. This IC deposition was not
sufficient, however, to induce renal pathology in either strain, as determined on H&E- and periodic acid-Schiff-stained sections. It should be noted that the differences observed between B6.Sle1c and B6 mice for all these phenotypes, except anti-chromatin Ab, were cGVHD induced, because no difference exists between unmanipulated B6.Sle1c and B6 mice (Fig. 4 and data not shown). Similar trends were observed for cGVHD induced in young mice, although the differences were not significant. These results indicate that Sle1c mediates an age-dependent accumulation of autoreactive B cells.

B6.Sle1c recombinants were used to map the enhanced cGVHD response. Most of the phenotypes described above, including anti-chromatin and anti-dsDNA Abs, increased T1 and decreased follicular B cells, glomerular C3 deposition (Fig. 6), increased expression of B7-1 and B7-2, and decreased expression of CD22 and B220 on B cells (data not shown), mapped to the B6.Sle1c.Cr2b-1. Intense IgG deposits were observed, however, in the glomeruli from all three recombinant strains (Fig. 6 C). Moreover, increased MHC-II I-a expression on B cells and expansion of the MZ B cell compartment clearly mapped to both B6.Sle1c.Cr2b-2 and B6.Sle1c.Cr2w1, but not B6.Sle1c.Cr2b-1. The simplest interpretation of these results is that two genes are involved in the Sle1c cGVHD response. The major gene is located between D1MIT459 and D1Mit362, and a separate gene resides between D1MIT274 and D1MIT117 in the overlapping region between B6.Sle1c.Cr2b-2 and B6.Sle1c.Cr2w-1, but not B6.Sle1c.Cr2b-1.

Sle1c results in T cell-intrinsic phenotypes

We have previously shown that Sle1c-expressing CD4+ T cells proliferate significantly more, are more activated, and contain a
significantly decreased subset of CD4\(^+\)CD25\(^+\)CD62L\(^-\)Foxp3\(^+\) cells (56). We used mixed chimeras in which BM from either normal B6.Ly5\(^a\) or B6.Sle1c (Ly5\(^b\)) mice reconstituted lethally irradiated B6 hosts to investigate whether these phenotypes are intrinsic to Sle1c-expressing T cells. As shown in Fig. 7, Sle1c-expressing T cells transferred into a lethally irradiated host displayed the same phenotypes as CD4\(^+\) T cells from unmanipulated B6.Sle1c mice. Furthermore, in mixed chimeras containing both Sle1c and normal CD4\(^+\) T cells, only Sle1c-expressing T cells displayed enhanced proliferation, as measured by in vitro CFSE dilution (Fig. 7A) or in vivo BrdU incorporation (Fig. 7D), and activation, as measured by CD69 expression (Fig. 7B). The CD4\(^+\)CD25\(^+\) subset of B6.Sle1c origin expressed significantly lower levels of CD62L (Fig. 7C), strongly suggesting, along with our previous Foxp3 expression results (56), that these cells were recently activated and do not have regulatory activity. These results show unambiguously that Sle1c results in intrinsic activated phenotypes in T cells, that these phenotypes do not require Sle1c expression in nonhemopoietic cells, and that these phenotypes are not transferable to bystander normal T cells.

We used the three congenic recombinants to map the activated T cell phenotypes within the Sle1c interval. Proliferation in response to in vitro stimulation with anti-CD3 and anti-CD28 was significantly increased in both B6.Sle1c.Cr2\(^b\)-2 and B6.Sle1c.Cr2\(^b\)-1 to the same level as in B6.Sle1c CD4\(^+\) T cells (Fig. 8A). Ex vivo CD69 expression on CD4\(^+\) T cells and the size of the CD4\(^+\)CD25\(^+\)CD62L\(^-\) compartment were equivalent in all three recombinants and B6.Sle1c and were significantly different from those in B6 CD4\(^+\) T cells (Fig. 8, B and C). The simplest interpretation of these results is that again two genes control these phenotypes; one between D1MIT459 and D1Mit362 affects proliferation, activation, and Treg reduction, and a separate gene between D1MIT274 and D1MIT117 affects only proliferation.

**Cr2 expression on T cells**

Based on the results presented above, a condition for the Cr2\(^b\) allele to account for the activated T cell phenotypes would be for Cr1/CR2 to be expressed on T cells. CD21/CD35 expression on activated CD4\(^+\) T cells has been reported in a model of myocarditis (25). Using three different clones of anti-CD21/CD35 Abs (7E9, 8C12, and 7G6), we showed that a small, but consistent, number (between 1 and 5\%) of CD4\(^+\) T cells express CR2 (Fig. 9). We confirmed that these cells were T cells with anti-CD3 Ab (data not shown). The CD21\(^+\) CD4\(^+\) T cells expressed higher levels of CD69, confirming earlier report of CD21 expression as an activation marker on T cells. This correlates with a trend for greater CD21 expression by B6.Sle1c T cells compared with B6. It is notable that only ~20–25\% of CD69\(^+\)CD4\(^+\) T cells express CD21 (data not shown), and it is not clear at this point whether there are functional differences associated with CD21 expression in activated T cells.

**Discussion**

The major SLE locus Sle1 was divided into three subloci based on anti-chromatin Ab production (11). Strong quantitative trait loci encompassing clusters of weaker additive loci have also been demonstrated in the NOD murine model of type 1 diabetes (38), and this feature is most likely common to many autoimmune diseases. This additional layer of complexity on the path leading to the identification of the underlying genetic defects requires additional functional characterization and genetic mapping of these subloci. The Sle1c locus was defined as a 7-Mb segment at the very telomeric end of chromosome 1 associated with anti-chromatin IgG Ab in ~30\% of the B6.Sle1c congenic mice (11). We subsequently showed that Sle1c was associated with decreased C3d binding, Ca\(^2+\) flux in response to subthreshold IgM cross-linking, and Ab response to TD Ags (14) and increased pathogenicity in Fas-deficient mice (23). Furthermore, we have identified polymorphisms in the NZW Cr2 allele, one of which is predicted to alter CR2 binding of its C3d ligand (14). All these results were consistent with Sle1c being allelic with Cr2, a gene whose expression is altered in SLE patients and some murine lupus models (18, 19, 21, 22) and which has been implicated in B cell tolerance (16). By crossing B6.Sle1c to NZB and comparing these mice to (NZB × B6)F\(_1\) controls, we have recently established that this locus significantly contributes to anti-dsDNA Ab production and aggravation of renal lesions (13). This provided an ultimate validation of Sle1c as a lupus susceptibility locus.

Using both mixed BM chimera and genetic manipulations, we have shown that Sle1 affects B and T cells independently (4, 5). The next step was to assess which of the Sle1 subloci affected these two lymphocyte lineages. We have recently shown that CD4\(^+\) T cell proliferation, activation, and cytokine production were significantly increased by Sle1c (56). Moreover, Sle1c was associated with a significant decrease in CD4\(^+\)CD25\(^+\)CD62L\(^-\)Foxp3\(^+\) cells, a population that has been associated with dominant regulation. Finally, we have shown that Sle1c T cells could provide help to Sle1 B cells to produce anti-chromatin IgG Ab, suggesting that this locus induced a loss of tolerance in T cells. To reassess Cr2 as a candidate gene for these phenotypes, we conducted a more detailed characterization of the functional defects associated with Sle1c and analyzed the phenotypes of three Sle1c recombinants, two carrying the Cr2\(^b\) allele and one carrying the Cr2\(^a\) allele.

We show in this study that the reduced Ab response to TD Ag that we have previously associated with Sle1c (13) is primarily

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**FIGURE 6.** An enhanced eGVHD response maps to the B6.Sle1c.Cr2\(^b\)-1 congenic recombinant. A, Anti-chromatin IgG Abs. B, T1/Fo follicular B cell ratio. C, Representative anti-C3, anti-IgG, and anti-IgM staining in B6.Sle1c recombinant kidneys (magnification, ×100). *, p < 0.05.
associated with Sle1c expression on radiosensitive cells, most likely B cells. An effect of Sle1c-radioresistant cells, most likely FDCs, was also observed in combination with Sle1c-BM-derived cells. These results are reminiscent of similar experiments performed in Cr2-deficient mice (35, 36), which found that Cr2 expression on both B cells and FDCs was necessary for an optimal response to TD Ag. Furthermore, a reduced Ab response to DNP-KLH was observed in the Sle1c recombinant with the Cr2w allele, but not in the two recombinants with the Cr2b allele. Overall these results are consistent with the Cr2w allele being responsible for the reduced Ab response to TD Ag through alterations of both B cell and FDC functions. Equally consistent with these results, Sle1c is associated with abnormal GCs, which were comparable to GCs in Cr2-deficient mice, specifically in their reduced FDC network. In addition, Sle1c affected GCs in their ability to sequester immune complexes, although some binding activity was retained compared with Cr2−/− GCs (36). Finally, the abnormal GC structure segregated with the Cr2w allele, and a logical interpretation of these results is that the Cr2w allele leads to defective GC formation.

A logical question regarding the contribution of Sle1c to autoimmune pathogenesis was whether this locus induces a break in B cell tolerance. A classical approach to address this question has been to use BCR transgenic mice, as was the case for Cr2−/− mice (16), and this approach is ongoing for Sle1c. A faster experimental approach has been cGVHD, which mimics much of SLE pathogenesis in a rapid and predictable fashion and depends on the presence of autoreactive B cells responding to MHC-mismatched alloreactive T cell help (32). In this study we compared the cGVHD responses in B6 and B6.Sle1c mice. Sle1c B cells showed a greater increase in activation and production of anti-dsDNA IgG Abs, leading to a greater IgG IC deposition in the kidneys. Furthermore, a redistribution of the splenic B cell subsets was observed in Sle1c cGVHD, with an increase in MZ B cells and in the T1 to follicular B cell ratio. This phenotype has not been reported in the cGVHD model. However, negative selection at the T1 stage is an important checkpoint for autoreactive B cells (39). Therefore, an increased T1 compartment in cGVHD may indicate that Sle1c-autoreactive B cells are not deleted at this stage as efficiently as in B6 mice. An
increased MZ B cell subset has also been associated with autoreactivity in several murine models of autoimmune disease (40, 41). Importantly, the distribution of B cell subsets is normal in unmanipulated B6.Sle1c mice. This suggests that the expansion of T1 and MZ B cells and the corresponding increase in autoreactive B cells observed in the cGVHD reaction in B6.Sle1c mice are T cell dependent. Finally, a difference between Sle1c and B6 B cells was significant only in old mice, which indicates that Sle1c results in an age-dependent accumulation of autoreactive B cells. Most of the cGVHD phenotypes mapped to a <2-Mb region (lc-1) in the centromeric portion of the Sle1c interval corresponding to the B6.Sle1c.Cr2w-1 congenic. Two additional phenotypes mapped to an ~1-Mb overlap region (lc-2) between B6.Sle1c.Cr2b-1 and B6.Sle1c.Cr2w-1. These results most likely exclude Cr2 as being responsible for the production of Sle1c-autoreactive B cells in this model.

Using mixed BM chimeras, we have shown that Sle1c expression is necessary for CD4+ T cells to display an activated phenotype, which includes increased proliferation and a decreased regulatory T cell subset. This activated phenotype is therefore intrinsic to T cells, and it excludes soluble or cell-to-cell contact factors as possible candidate genes. In this study we have confirmed a previous report (25) that a small number of CD4+ T cells express CR1/CR2, and that these cells tend to be more activated than their CR1/CR2 counterparts. The functional significance of CR1/CR2 expression on T cells at this point is unknown, and we cannot exclude that it plays a role in the activated phenotype that we have described in Sle1c T cells. Examination of the T cell phenotypes in Sle1c recombinants, however, excluded CR2 from playing a major role in this phenotype. Instead, the same two centromeric loci, lc-1 and lc-2, which were revealed with the cGVHD model, are most likely to account for the Sle1c-activated T cell phenotypes. Both lc-1 and lc-2 play a role in increased activation and a reduced Treg population, and lc-2 plays an additional role in increased proliferation.

Overall, these results reveal a complex situation in which at least three genes are responsible for the Sle1c phenotypes. In addition to Cr2, which is associated with an impaired humoral immune response to TD Ag and impaired GC formation, two centromeric regions, lc-1 and lc-2, are associated with the generation of autoreactive B and T cells. Additional recombinants isolating these two regions from each other and lc-2 from Cr2 will be necessary to address their relative contributions to pathogenesis. Additional recombinants will also be necessary for fine-mapping and to eliminate candidate genes. A number of genes with known or potential roles in the immune system that are located in the nonoverlapping portion of the Sle1c.Cr2w-1 recombinant (Fig. 1) cannot be formally eliminated at this stage, although their involvement would invoke a more complex model than the three-gene model that we propose in this study.

Both lc-1 and lc-2 are relatively gene poor, but contain a number of novel genes or poorly characterized genes. Among them, lc-1 contains a cluster of four novel genes termed A8 with fibronectin type III domain fragment (ENSF0000002192). Interestingly, a large number of ILR chains contain such a domain, indicating that these novel genes may play a role in the immune system. In lc-2, Pptm14, which codes for protein tyrosine phosphatase, nonreceptor-type 14, (also known as PTP36), is a gene...
that has been isolated from a thymus cDNA library and is expressed in T cells (42). Although its role as a tyrosine phosphatase remains elusive, it has been shown to regulate cell adhesion and growth through its effect on the cytoskeleton (43). Interestingly, a polymorphism in another nonreceptor protein tyrosine phosphatase, PTPN22, has been recently shown to be associated with rheumatoid arthritis (44), type 1 diabetes (45), and lupus (46) in human populations, increasing the potential of Ptpn14 as a candidate gene. Finally, although some of the Sle1c phenotypes parallel phenotypes described in the C2r-deficient mice, we cannot exclude two other related genes, Crry and Mcp. Because of their very close linkage with Cr2, it would be nearly impossible to exclude these two genes by recombination. Functional and genomic analyses of these genes in B6.Sle1c mice are currently being performed to address this question.

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Disclosures

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