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Genetic Determination of T Cell Help in Loss of Tolerance to Nuclear Antigens

Yifang Chen, Carla Cuda, and Laurence Morel

Sle1 is a major lupus susceptibility locus in NZM2410 lupus model that is associated with a loss of tolerance to nuclear Ags. At least three genes, Sle1a, Sle1b, and Sle1c contribute to Sle1, and their relative role in lupus pathogenesis is unknown. We show here that Sle1-expressing CD4+ T cells present an activated phenotype associated with increased proliferation and cytokine production. In addition, Sle1 CD4+ T cells provide help to anti-chromatin B cells to produce anti-nuclear antibodies, whether or not these B cells express Sle1. The Slela locus alone accounts for all these Sle1 phenotypes, implying that a specific genetic defect in Slela is necessary and sufficient to produce autoreactive T cells. However, Slelc induces intermediate T cell activation and only provides help to Sle1-expressing anti-chromatin-producing B cells, demonstrating the synergic interactions between Sle1c T and Sle1 B cells. Moreover, Slela and Sle1c were associated with a significantly reduced level of CD4+CD25+ regulatory T cells that precedes autoantibody production, suggesting a causal relationship with the generation of autoreactive T cells. Our study identifies for the first time that a specific genetic defect is responsible for lupus pathogenesis by inducing autoreactive T cells to break self-tolerance and that this genetic defect is also associated with a decreased number of regulatory T cells. The Journal of Immunology, 2005, 174: 7692–7702.

Systemic lupus erythematosus (SLE) is an autoimmune disease that results from interactions between several genetic loci that cannot, by themselves, induce clinical disease (1). We have used the NZM2410 strain derived from (New Zealand black (NZB) × New Zealand white (NZW))F1 mice (2) to identify by linkage analysis the major genetic loci responsible for SLE susceptibility (3, 4). The strongest of these loci was Sle1 on telomeric chromosome 1, a region that has been independently linked to SLE in several mouse models (5) and human patients (6). Functional analysis of the B6.Sle1 congenic mice carrying Sle1 on a C57BL/6 (B6) background (7) showed that this locus induced a loss of tolerance to nuclear Ag (8, 9). Genetic experiments have demonstrated that Sle1 was necessary for the development of nephritis in the NZM2410 model (10, 11). Moreover, coexpression of Sle1 with a number of single mutations or other Sle-susceptibility locus produced a highly penetrant clinical pathology (11–14).

Based on these results, we have proposed a model in which SLE pathogenesis is the consequence of a multistep process rooted on Sle1 (15). Sle1 congenic recombinants have shown that the production of anti-nuclear Abs corresponds to at least three independent loci, Sle1a, Sle1b, and Sle1c (16). The penetrance of anti-chromatin IgG Ab was the strongest for Sle1b. Mice carrying either Sle1a or Sle1c showed only a 30% penetrance of anti-chromatin Abs, with an enhanced response to the histone 2A/histone 2B/dsDNA (H2A/H2B/dsDNA) subnucleosomal particle only found for Sle1a (16). Ccr2, which encodes for the complement receptors type 1 and 2 (CR1/CR2), is a strong candidate gene for Sle1c, due to a mutation in the NZM2410 allele that introduces a novel glycosylation site impairing the function of CR1/CR2 as BCR coreceptor (17). We have also proposed that Sle1b is allelic with a cluster of seven genes from the SLAM/CD2 family, and the strongest candidate among them is Ly108 (18). No candidate gene has been identified for Sle1a. Overall, these results suggest that the three Sle1 loci may contribute the loss of tolerance to nuclear Ag through different, but unknown, pathways.

A number of studies have shown that both B and T cell abnormalities contribute to the production of anti-nuclear Abs (19, 20). Autoantigen-specific T cell help is required for disease induction in MRL/lpr mice (21), and nucleosome-specific T cells provide help to chromatin-specific B cells (22). Mixed bone marrow chimeras have shown that Sle1 was functionally expressed in T cells, where it resulted in an up-regulation of activation markers, cytokine production, and generation of histone-specific T cells (8, 23). The increased activation in Sle1-expressed CD4+ T cells was independent from the presence of B cells, indicating that there is an intrinsic defect in Sle1-expressed T cells. The respective contribution of each of the three Sle1 loci to these T cell phenotypes is unknown.

In this study, we show that Slela T cells were similar in activation and cytokine production to T cells expressing the entire Sle1 region. Moreover, adoptively transferred T cells from either Slel or Slela provided help to chromatin-specific B cells, whether or not these B cells expressed Sle1. Sle1c T cells provided help only to Sle1-expressing but not to B6-derived chromatin-specific B cells. In contrast, Sle1b T cells were not different from B6 controls. These results demonstrate that Slela is necessary and sufficient to induce nuclear Ag-specific autoreactive T cells, and these T cells are able to provide help to anti-chromatin-specific B cells to produce anti-nuclear Abs. Finally, we showed that Slela and Sle1c were associated with a reduced level of CD4+CD25+ regulatory T cells (Treg) that precedes autoantibody production, suggesting a...
causal relationship with the generation of autoreactive T cells. Loss of regulatory T cells has been associated with a number of organ-specific autoimmune diseases (24). Double transgenic models expressing a TCR and its cognate Ag have recently concurred in showing that Treg play a role in preventing systemic autoimmunity (25–27). Tolerance induction to nuclear Ag with histone peptides in the (SWR × NZB)F1 model was dependent on the generation of Ag-specific Treg (28). Results have been less clear in spontaneous models. Neonatal thymectomies have shown mixed results in lupus models (29, 30), but no spontaneous global deficiency has been found in at least one lupus model, NZM2410 (30). Our results demonstrate for the first time that the specific genetic defect down-regulates the number of Treg, which is associated with the generation of autoreactive T cells and autonuclear Abs.

Materials and Methods

Mice

C57BL/6J (B6) and C57BL/6-J-Tcra<sup>tm1</sup> Molm (B6.Tcra<sup>−/+</sup>) mice were obtained from The Jackson Laboratory and bred at University of Florida. The B6.Sle1 congenic strain contains a 37-cM NZM2410-derived interval defined by the D1MIT101 and D1MIT155 markers (7). The subcongenic lines containing Slela, Slelb, or Slelc have been described previously (16) and each represent a 4.4, 2.5, and 3.7 cM interval, respectively (Fig. 1). B6.Sle1.Tcra<sup>−/+</sup> mice do not carry conventional αβ TCR<sup>+</sup> T cells, and their CD3<sup>+</sup> lymphocytes are γδ or ββ TCR<sup>+</sup> (23), as shown for other Tcra<sup>−/−</sup> mice (31, 32). All mice, except B6.Sle1.Tcra<sup>−/−</sup> and B6.Tcra<sup>−/−</sup>, which were kept in specific pathogen-free conditions, were housed in conventional housing and both males and females were used. Unless specified, experiments were conducted with 6- to 8-month-old congenic mice and B6 controls. This age corresponds to the induction of antinuclear Ab in most B6.Sle1 and the subcongenic mice (15, 16). All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Cell preparation and culture

Single cell suspensions from spleen, iliac lymph node, or thymus were used for flow cytometric analysis or cultured with or without stimuli 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 µM 2-ME (Invitrogen Life Technologies) in a 37°C, 5% CO2 incubator. CD4<sup>+</sup> T cells were purified from spleenocytes by negative selection with Miltenyi Biotec reagents. Naive CD62L<sup>−</sup> CD4<sup>+</sup> T cells were further purified by positive selection with a biotinylated anti-CD262 (MEL-14) Ab and streptavidin microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. For cytoplasmic CD154 staining, splenocytes were activated with PMA and ionomycin (leukocyte activation mixture; BD Pharmingen) for 4 h at density of 10<sup>6</sup> cells/ml. For proliferation assays, 10<sup>5</sup> lymphocytes were cultured in triplicate on anti-CD3 (17A2) (BD Pharmingen) or anti-CD3 (145–2C11), anti-CD28 (90.3) (BD Pharmingen) and incubated at 37°C for 5 h. ASCs were detected with HRP-conjugated anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), and anti-IL-4 (BVD4-1D11) Abs were used as recommended by the manufacturer (BD Pharmingen).

Flow cytometry

Briefly, cells were first blocked on ice with staining buffer (PBS, 5% horse serum, 0.05% sodium azide) supplemented with 10% normal rabbit serum and anti-CD16/32 (2.4G2). Cells were then stained with pretitrated amounts of the following: FITC, PE, allophycocyanin, or biotin-conjugated Abs: CD2e (145–2C11), CD4 (RM4–5), CD8 (Ly-2), CD49d (H1.L23), CD25 (7D4), CD62L (MEL-14), CD134 (OX-86), B220 (RA3–6B2), CD138 (syndecan-1), TCRβ (H57–597), or annexin V (BD Pharmingen). Biotin-conjugated Abs were revealed using streptavidin-Quantum red (Sigma-Aldrich). Intracellular expression of CD154 (MR1) was analyzed in fixed permeabilized cells with Cytotox/Cytoperm Plus kit (BD Pharmingen). Cell staining was analyzed using a FACS Calibur (BD Biosciences). At least 10,000 events were acquired per sample, and dead cells were excluded based on scatter characteristics. Positive staining was determined relative to isotype controls. For intracellular cytokine detection, PE-conjugated anti-IL-2 (JES6-5H4), anti-IFN-γ (XMG1.2), and anti-IL-4 (BVD4-1D11) Abs were used as recommended by the manufacturer (BD Pharmingen).

T cell adoptive transfers

T cells were enriched from splenocytes by negative selection with anti-CD3-PE Dynabead magnetic beads (Dynal) according to the manufacturer’s directions. Flow cytometry analysis showed that, on average, 90% of the selected cells were CD3<sup>+</sup> and 0.5% were B220<sup>+</sup>. The 10<sup>7</sup> donor T cells from 6- to 8-month-old mice were tail vein injected into 2- to 3-month-old sex-matched B6.Tcra<sup>−/−</sup> or B6.Sle1.Tcra<sup>−/−</sup> hosts. Serum was collected monthly after transfer up to 3–4 mo when the chimeric mice were sacrificed for cellular analysis. Two to three independent transfer experiments were performed with 3–5 chimeric mice in each group. Anti-chromatin, anti-mouse thyroglobulin and anti-keyhole limpet hemocyanin (KLH) IgG serum levels were measured by ELISA as previously described (32). Anti-chromatin Ab-secreting cells (ASC) were quantified by ELISPOT, using a modified manufacturer’s protocol (Millipore). Briefly, MultiScreen hemagglutination plates were coated with 5 µg/ml dsDNA (Sigma-Aldrich) and 1 µg/ml total histone (Roche Diagnostics) in 0.1 M NaHCO<sub>3</sub>, pH 9.8. Serially titrated normalized numbers of splenocytes were plated in duplicate and incubated at 37°C for 5 h. ASCs were detected with HRP-conjugated goat anti-mouse IgG (Southern Biotech) diluted 1:1000 in PBS, 0.1% Tween 20, and 0.5% FBS in 4°C overnight. ASC were visualized with the chromogen substrate 3-amin-9-ethylcarbazole and normalized to 10<sup>6</sup> splenocytes.

Chromatin-specific T cell assay

T cell proliferation assays in response to histone were adapted from previous work (8). T cells were enriched from 9-month-old splenocytes by negative selection with anti-B220-coated Dynabead magnetic beads (Dynal), leading T cells with >90% purity. Irradiated (2000 Rads) RDC-depleted B6 splenocytes were pulsed with total histone (Roche Diagnostics) and dsDNA (Sigma-Aldrich) at 1 µg/ml each in PBS for 1 h at 37°C. After washing, the irradiated splenocytes were cocultured in quadruplicate with T cells (2.5 × 10<sup>5</sup>/ml each) in serum-free HL-1 medium (Biowhittaker) for 48 h. Negative controls consisted of T cells stimulated with unpulsed APCs, and with APCs pulsed with mouse thyroglobulin (Sigma-Aldrich) at 1 µg/ml. Anti-CD3 (17A2) (BD Pharmingen) -stimulated T cells provided positive controls. Duplicate wells were used for proliferation measurements after the addition of 10 µCi/ml [3H]thymidine (MP Biomedicals) for the last 18 h of culture. Stimulation indexes were calculated as the ratio between Ag-pulsed and unpulsed cultures. Supernatants from the remaining duplicate wells were assayed for IFN-γ, IL-2, and IL-4 with OptiEIA kits (BD Pharmingen) according to the manufacturer’s instructions.

FIGURE 1. Genetic map of the Slela, Slelb, and Slelc intervals. The upper part represents the telomeric end of chromosome 1 with the position (cM) of D1MIT markers and genes relevant to this study. The positions of the NZM2410-derived congenic intervals for B6.Slela, B6.Slelb, B6.Slelc, and B6.Sle1 are shown as boxes linked to the flanking homozygous NZM2410 markers indicated on the chromosome. Horizontal lines extended on each side of these boxes indicate the area of recombination between the NZM2410 and B6 markers. For clarity, the centromeric end of the Sle1 interval is not shown, and a break between D1MIT541 and D1MIT407 has been introduced.
**Foxp3 gene expression**

CD4⁺CD25⁺ cells were isolated with the CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec) from splenocytes or thymocytes pooled from 5 age-matched mice per strain. The purity of the selected fraction reached 90% of CD4⁺CD25⁺ cells, as determined by flow cytometry. The CD4⁺ and CD4⁺CD25⁺ fractions were also collected as negative controls. Total RNA was extracted from 10⁶ cells from each of these fractions with the RNasea Mini kit (Qiagen) and reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad). RNA samples used for the semiquantitative RT-PCR and real-time PCR were obtained independently from different pools of mice. Semiquantitative PCR primer sequences were as follows: Foxp3, CAG CTG CCT ACA GTG CCC CTA G and CAT TTG CCA GCA GTG TTT C amplifying a 388-bp product; β-actin, GTG GGC CGC TCT AGG CAC CAA and CTT TGA TGT CAC GCA CGA TTT C amplifying a 538-bp product. The relative quantity of Foxp3 cDNA normalized to β-actin was measured from ethidium bromide-stained agarose gels with an Eagle-Eye densitometer (Stratagene). Furthermore, Foxp3 mRNA was quantified by real-time PCR using the DNA Engine Opticon Thermal Cycler (MJ Research) with TaqMan reagents from Applied Biosystems, including Assays-on-Demand primers for Foxp3 and GAPDH control. Similar amplification efficiencies were obtained for Foxp3 and GAPDH, as evaluated on a standard curve. All samples were run in duplicate. Normalized values for Foxp3 mRNA expression in each sample were calculated using the comparative Ct method.

**Statistical analysis**

Comparisons were made with two-tailed Student’s t tests after verification that the data was normally distributed with the GraphPad Prism 4 software package; p < 0.05 was used as the threshold for statistical significance.

**Results**

*Sle1* CD4⁺ T lymphocyte activation maps to *Sle1a* and *Sle1c*

*Sle1* results in a significantly increased number of activated CD4⁺ T cells (8, 23). To determine which of the three *Sle1* loci was responsible for this phenotype, the number and percentage of T cells expressing various activation markers was compared between subcongenic strains. A significantly higher percentage (Fig. 2) or number (data not shown) of *Sle1* and *Sle1a* CD4⁺ T cells expressed CD69 than B6 CD4⁺ T cells, and there was a trend in the same direction in B6.*Sle1c* mice. No significant difference was obtained between B6 and B6.*Sle1b* mice (Fig. 2A, left panel). After stimulation with anti-CD3 and anti-CD28, the difference between B6.*Sle1a* and B6 increased, and became significant for B6.*Sle1c* mice (Fig. 2A, right panel). However, CD69 expression in B6.*Sle1b* CD4⁺ T cells remained similar to B6. The highest CD69 expression was reached after 12–24 h of stimulation and was maintained in *Sle1*, *Sle1a*, and *Sle1c* CD4⁺ T cells up to 72 h, whereas it gradually decreased in B6 and B6.*Sle1b* T cells (data not shown). Similar results were obtained with 2- to 3-mo-old mice, although the overall level of CD69 expression was lower (data not shown).

The intracellular expression of CD154 was analyzed with or without PMA and calcium ionophore stimulation, which maximizes CD154 expression (33). The percentage of CD154⁺CD4⁺ T cells and the intensity of CD154 expression were significantly higher in B6.*Sle1*, B6.*Sle1a*, and B6.*Sle1c* than in B6 and B6.*Sle1b*, with or without stimulation (Fig. 2B). Similar results were obtained with membrane CD154 at a lower level (data not shown). Increased CD4⁺ T cell activation level in B6.*Sle1*, B6.*Sle1a*, and, to a lesser extent, B6.*Sle1c* mice was confirmed by decreased CD62L and increased CD134 (data not shown).

**Increased proliferation and decreased apoptosis in T cells**

Significantly higher numbers of lymphocytes isolated from B6.*Sle1*, *Sle1a*, or *Sle1c* lymph nodes (Fig. 3A) or spleen (data not shown) were obtained in response to anti-CD3 and anti-CD28 than lymphocytes isolated from B6.*Sle1b* or B6 (Fig. 3A). Lymphoblasts could be clearly seen in B6.*Sle1*, B6.*Sle1a*, and B6.*Sle1c*, but not in B6 and B6.*Sle1b* lymphocytes cultured for 72 h with IL-2 alone. In addition, lymphocytes expressing *Sle1*, *Sle1a*, or *Sle1c* could proliferate until 96 h in the presence of IL-2 alone, whereas B6 and B6.*Sle1b* lymphocytes did not survive after 72 h (data not shown). CD4⁺ T cell proliferation was directly compared among the subcongenic strains by MTT cleavage (Fig. 3B) and CFSE labeling (Fig. 3, C–E) in splenocytes cultured with anti-CD3 and anti-CD28 for 48 h. Total T cells (Fig. 3B), CD4⁺ T cells (Fig. 3, C and D) and activated CD69⁺CD4⁺ T cells (Fig. 3E) cells proliferated significantly more in B6.*Sle1*, B6.*Sle1a*, and B6.*Sle1c* than in B6 and B6.*Sle1b* mice. As expected, the majority of the proliferation occurred in CD69⁺ cells. However, increased proliferation was also observed in CD69⁻CD4⁺ *Sle1*, *Sle1a*, and *Sle1c* cells (Fig. 3E). In addition, activation-induced apoptosis of CD4⁺CD69⁻ cells, as shown by annexin V binding, was significantly reduced in B6.*Sle1*, B6.*Sle1a*, and B6.*Sle1c* as compared with B6 and B6.*Sle1b* mice (Fig. 3, F and G). These results were confirmed with purified CD4⁺ T cells obtained from younger or older mice (Fig. 4). B6.*Sle1a* CD4⁺ T cells and, to a lesser extent, B6.*Sle1c*
CD4+ T cells showed a decreased apoptosis (Fig. 4A), increased proliferation (Fig. 4B), activation (Fig. 4D), and cell size (Fig. 4E). Young mice showed the same trend as older mice, although the differences were smaller, consistent with the age-dependent amplification seen for most lupus phenotypes. For Sle1a and Sle1c, greater differences with B6 were obtained with CD4+ T cells stimulated in the presence of other cell populations than with purified CD4+ T cells, suggesting that accessory cells may play a role in the expression of these T cell phenotypes. This was not true for Sle1b, for which purified CD4+ T cells showed some elevated CD69 expression and increased in blast numbers. Additional experiments will be necessary to clarify this question. Finally, comparison of the CD62L+ and CD62L− subsets showed that increased proliferation occurred in the naive CD4+ T cells (Fig. 4F). Overall, Sle1 CD4+ T cells have a higher proliferation rate and lower apoptosis, either spontaneously or in response to TCR and CD28 stimulation. Moreover, Sle1 alone induced a proliferation, and apoptosis phenotype in Th cells was indistinguishable from the entire
FIGURE 4. *Sle1a* and *Sle1c* CD4⁺ T cell phenotypes. 10⁵ CD4⁺ T cells from 6- to 8-mo-old mice were stimulated with anti-CD3 and anti-CD28. *A*, Annexin-V⁺ CD4⁺ staining and the percentage of CD4⁺ annexin-V⁻ AAD⁻ live cells were compared after 12 h of culture. Cell division, as percentage of CFSE⁺⁺⁺ corresponding to the last two divisions (*B*), CD69 expression (*D*), and cell size (*E*) were measured in CD4⁺ gated cells after 36 h of culture of CD4⁺ sorted cells from old (6–8 mo) or young (2 mo) mice. In panels *A*, *D*, and *E*, the gray histograms represent B6, the black lines, *Sle1a*, and the gray lines, *Sle1c*. The light gray histogram in panel *D* represents the anti-CD69 isotype control. *C*, Proliferation of CD4⁺ CD62L⁺ or CD62L⁻ obtained from B6 and B6.Sle1a mice after 36 h of stimulation with anti-CD3 and anti-CD28, as measured by MTT binding. Means and SDs of three samples per strain.
Sle1. Because we found that a significantly increased proliferation occurred in CD4+ CD69+ cells and in naive CD4+ CD62L+ but not activated CD62L− cells, the mere increased percentage of activated T cells is unlikely to account for the increased proliferation associated with the Sle1 loci.

Sle1, Sle1a, and Sle1c are associated with a higher cytokine production

To further assess the impact of the Sle1 loci on effector T cells, we measured intracellular cytokine levels in activated CD4+ T cells. A greater number of Sle1 CD4+ T cells produced IL-2, IL-4 and IFN-γ compared with B6 (Fig. 5). No significant bias toward a higher production of IL-4 compared with IFN-γ was observed. The cytokine levels were similar between B6.Sle1a and B6.Sle1, whereas it was intermediate in B6.Sle1c mice. Cytokine production in B6.Sle1b was undistinguishable from B6. Overall, cytokine production by CD4+ T cells followed the same pattern among the Sle1 loci as activation and proliferation.

Sle1 and Sle1a T cells induce activation of B cells from non-autoimmune mice

By breeding the Sle1 locus with the Igh6−/− mutation, we have previously shown that Sle1 resulted in T cell intrinsic defects, which culminated with the production of histone-reactive CD4+ IFN-γ-secreting cells even in the absence of B cells (23). However, this experiment did not address whether Sle1 T cells could activate B cells from normal mice and induce them to produce autoantibodies and, conversely, whether Sle1 B cells could get activated and help to produce IgG autoantibodies by T cells from normal mice. To answer these questions and to assess the respective contributions of the Sle1 loci, we performed T cell adoptive transfers from the Sle1 congenics into B6.Sle1.Tcrα−/− or B6.Tcrα−/− mice (Table I). Because all the results presented above indicated that Sle1a and Sle1c, but not Sle1b, directly impact T cell phenotypes, we limited the transfers to Sle1, Sle1a, and Sle1c expressing T cells. In this experimental model, Tcrα−/− recipients mice do not carry any conventional αβ TCR+ T cells, but they present a small number of unconventional ββ TCR+ CD3+ cells (31, 32) that we have found at similar levels in both B6.Tcrα−/− and B6.Sle1.Tcrα−/− mice (23). B6 T cells were transferred to B6.Tcrα−/− and Sle1 T cells were transferred to B6.Sle1.Tcrα−/− mice as negative and positive controls, respectively.

Three to four months after transfer of 10⁷ cells, Tcrβ+ CD4+ T cells constituted ~5−15% of the splenocytes in chimeric mice, contrasting with intact Tcrα−/− mice, in which <0.3% of the splenocytes were Tcrβ+ CD4+ (Fig. 6A). This result confirmed that T cells were successfully transferred and survived in Tcrα−/− spleens, as previously shown in a similar model (34). A significantly higher number of Sle1 Tcrβ+ CD4+ T cells were recovered after transfer into either B6.Sle1.Tcrα−/− or B6.Tcrα−/− mice, compared with transfers from the other strains, reflecting a higher proliferation and survival of these cells in the chimeric mice (Table I and Fig. 6A). We cannot exclude at this point that the transferred T cells from Sle1 but not from B6 induced the expansion of the recipient Tcrα− CD4+ T cells. However, if that were the case, it would still indicate that Sle1 T cells are phenotypically different from B6 T cells. Significantly higher numbers of Sle1 and Sle1a CD4+ T cells expressed CD69 after transfer into either B6.Sle1.Tcrα−/− or B6.Tcrα−/− mice, compared with T cells donated from the other strains, which confirms the CD4+ T cell activation induced by Sle1 and Sle1a (Table I and Fig. 6B).

Transfers of Sle1 and Sle1a T cells resulted in a significantly higher expression of MHC class II I-Aα molecules by B cells in B6.Sle1.Tcrα−/− or B6.Tcrα−/− mice, compared with transfer of B6 T cells in the same mice (Table I and Fig. 6C). It is to be noted that the level of I-Aα expression on B cells is always higher in B6.Sle1.Tcrα−/− than in B6.Tcrα−/− mice (23), but the addition of Sle1 or Sle1a, but not B6 T cells increased this level by ~40% in both recipient strains. T cells from B6.Sle1c mice were able to induce a significantly higher I-Aα expression only in Sle1 B cells (Table I). The small amount of B cells (< 0.5%) transferred along with the T cells is unlikely to account for the observed differences. Finally, transfers of Sle1 and Sle1a T cells resulted in significantly higher numbers of B220+ CD138+ plasmablasts in the spleens of either B6.Sle1.Tcrα−/− or B6.Tcrα−/− mice, compared with transfer of B6 T cells (Table I and Fig. 6C). No difference was obtained for the small number of B220− mature splenic plasma cells (data...
not shown). Similar to the B cell activation markers, transfers of Sle1c T cells induced a significantly higher number of plasmablasts only in B6.Sle1.Tcr"−/−" spleens.

**Sle1a and Sle1c generate histone-specific T cells that induce anti-chromatin IgG Abs production**

Transfers of Sle1 T cells into B6.Tcr"−/−" mice resulted in a significantly higher production of anti-chromatin IgG Abs compared with transfers of B6 T cells (Fig. 7A, left panel). In the latter group, anti-chromatin Abs levels were comparable with that of normal B6 mice (data not shown), indicating that the result obtained with Sle1 T cells transfer was not due to autoimmune B cells generated in the absence of αβ+ T cells. Moreover, Sle1a- but not Sle1c-expressed T cells were able to induce a significant level of anti-chromatin IgG in B6.Tcr"−/−" mice. Anti-chromatin IgG Abs were detectable the first month after transfer of Sle1 and Sle1a T cells in most mice, increased the second month, peaked the third month, and leveled during the fourth month (data not shown). The isotope distribution of total IgG or anti-chromatin Abs were similar between all transfer groups (data not shown), confirming the above results (Fig. 5) showing an absence of significant cytokine bias in Sle1 T cells. The same results were obtained by quantifying the number of splenic chromatin-specific ASC (Fig. 7B, left panel). No difference was obtained between the levels of anti-chromatin Abs or ASC induced by Sle1 or Sle1a T cells, indicating that Sle1a accounts for the entire Sle1 phenotype.

B cells from B6.Sle1.Tcr"−/−" mice were induced to produce anti-chromatin IgG Abs by T cells expressing Sle1, Sle1a, or Sle1c, but not by T cells obtained from B6 mice (Fig. 7, A and B, right panels). Interestingly, significantly higher amounts of anti-chromatin IgG Abs and ASCs were obtained with transfers of Sle1, Sle1a, or Sle1c T cells to B6.Sle1.Tcr"−/−" mice than the same transfers to B6.Tcr"−/−" mice (p < 0.01). This clearly demonstrates that interactions between Sle1 T and B cells are responsible for the high level of anti-chromatin IgG Abs produced by B6.Sle1 mice. Finally, Sle1 B cells, which contain a high number of autoreactive anti-nuclear clones (23), cannot be induced to produce anti-chromatin IgG Abs by B6 T cells. Indeed, there was no statistical difference between the anti-chromatin IgG production after B6 T cells transferred to B6.Tcr"−/−" or to B6.Sle1.Tcr"−/−" mice (Fig. 7, A and B).

To confirm that Sle1a and Sle1c resulted in the production of chromatin-specific T cells, we evaluated their in vitro response to a mixture of histone/dsDNA. As we have previously shown for

### Table I. Lymphocyte subsets in the B6.Tcrα−/− and B6.Sle1.Tcrα−/− spleens three to 4 mo after transfer of T cells isolated from B6 or the Sle1 congenics (mean ± SD)

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<td>66.8 ± 7.4</td>
<td>60.4 ± 10.8</td>
<td>60.3 ± 11.7</td>
<td>69.8 ± 10.3</td>
<td>60.5 ± 10.7</td>
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<tr>
<td>Tcrβ</td>
<td>4.4 ± 1.1</td>
<td>5.0 ± 1.4</td>
<td>7.3 ± 1.0d</td>
<td>7.2 ± 1.2d</td>
<td>4.9 ± 0.7</td>
<td>5.0 ± 0.9</td>
<td>4.2 ± 1.1</td>
<td>4.6 ± 0.7</td>
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<tr>
<td>CD69</td>
<td>28.0 ± 4.4</td>
<td>28.0 ± 4.4</td>
<td>38.9 ± 2.7c</td>
<td>37.3 ± 2.7d</td>
<td>36.4 ± 4.0c</td>
<td>39.3 ± 4.6d</td>
<td>27.0 ± 2.2</td>
<td>27.0 ± 2.8</td>
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<tr>
<td>CD4</td>
<td>86.8 ± 10.4</td>
<td>116.0 ± 27.4</td>
<td>121.3 ± 16.9</td>
<td>161.6 ± 27.6</td>
<td>127.5 ± 10.6c</td>
<td>157.7 ± 21.1c</td>
<td>98.9 ± 13.9</td>
<td>155.1 ± 22.8c</td>
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<tr>
<td>% B220+</td>
<td>8.03 ± 0.94</td>
<td>8.13 ± 0.96</td>
<td>10.34 ± 1.44c</td>
<td>11.66 ± 2.24d</td>
<td>10.00 ± 1.79c</td>
<td>12.16 ± 2.26d</td>
<td>8.57 ± 0.57</td>
<td>10.61 ± 1.35d</td>
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MF, mean fluorescence intensity.

a Number of splenocytes (×10⁶).

b Number of splenic Tcrβ+ CD4+ cells (×10⁶).

c p < 0.05 compared with B6 → B6.Sle1.Tcrα−/−.

d p < 0.05 compared with B6 → B6.Sle1a.Tcrα−/−.
Sle1, Sle1a, and Sle1c T cells proliferated significantly more (Fig. 7C) and produced significantly more IFN-γ (Fig. 7D), IL-2, and IL-4 (data not shown) than B6 T cells in response to histone/DNA. Sle1a and Sle1c responses were equivalent and both lower than that of the whole Sle1 interval. No difference in the proliferative or cytokine responses was obtained with mouse thyroglobulin (data not shown), confirming the specificity of the Sle1 T cells response for nuclear Ag. These results demonstrate that the production of autoreactive T cells associated with Sle1a and, to a lesser extent, Sle1c, is an essential component of the production of class-switched anti-nuclear Abs in the NZM2410 model.

Sle1, Sle1a, and Sle1c are associated with decreased numbers of CD4⁺CD25⁺ Treg

We determined whether the CD4⁺ T cell phenotypes conferred by Sle1, Sle1a, and Sle1c were associated with a decreased CD4⁺CD25⁺ Treg cells. The percentage of CD4⁺CD25⁺ splenocytes was significantly decreased in B6.Sle1, B6.Sle1a, and B6.Sle1c mice compared with B6.Sle1b or B6 (Fig. 8A). To verify that the decrease in the CD4⁺CD25⁺ population does not involve activated cells, we compared the CD4⁺CD25⁺CD62L⁻ subset, which has been shown by adoptive transfers to delay the onset of
diabetes (35). Similar results were obtained between the CD4 CD25+ and CD4 CD25+CD62L− subset (Fig. 7B), confirming that Sle1, Slela, and Sle1c affect the Treg subset. In addition, we evaluated the CD4 CD25+CD62L− population in 1- to 2-mo-old mice, before any anti-chromatin Ab is produced. The same results were obtained as in older mice (Fig. 8B), indicating that the Slela- and Sle1c-associated decrease in Treg population precedes autoimmune manifestations and does not correspond to a secondary effect of the autoimmune process.

To further confirm the impact of Sle on Treg cells, we compared Foxp3 gene expression between the congenic strains. This transcription factor is necessary and sufficient to confer a Treg phenotype to CD4+ T cells (36). Semiquantitative RT-PCR analysis of Foxp3 expression showed a decreased expression of Foxp3 in B6.Sle1 CD4+CD25+ spleenocytes compared with B6 (Fig. 8C). Foxp3 expression was also decreased, but to a lesser extent, in B6.Slela and B6.Slelc but not in B6.Slelb CD4+CD25+ cells. The same pattern was found in thyocytes, indicating that Slela and Sle1c are not simply involved in the peripheral maintenance of Treg but may control their production from the thymus. Using TaqMan real-time PCR, we determined that Foxp3 expression was reduced by 4-fold in B6.Sle1 and B6.Slela and by 2.5-fold in B6.Sle1c CD4+CD25+ cells, compared with B6.Slelb and B6 (Fig. 8D). Overall, these results demonstrate that Slela and, to a lesser extent, Sle1c down-regulate CD4+CD25+ regulatory T cells.

Discussion

A number of studies that autoreactive T cells are required for the development of systemic autoimmunity (20). Specifically, nucleosome-specific T cells provide help to anti-DNA-specific B cells in murine models (22) and human lupus (37), and help from cognate αβ T cells is required for disease induction in the MRL/lpr mouse (21). The genetic analysis of the NZM2410 lupus model identified Sle1 as its strongest susceptibility locus (3). Congenic analysis showed that Sle1 promotes T cell activation, and the production of nucleosome-specific T cells (8). At the initiation of the present study, we have further characterized the phenotype of Sle1 T cells. A larger panel of activation markers confirmed the activation status of Sle1 T cells and showed that it was enhanced by TCR and CD28 stimulation. The Sle1-induced up-regulation of CD154 is of particular interest. Abnormal expression of CD154 has been associated with lupus (37, 38). Furthermore, blocking of the CD40/CD154 interactions significantly delayed disease onset and severity in murine lupus (39). Our results suggest that up-regulation of CD154 might be one of the mechanisms by which Sle1 T cells participate in SLE pathogenesis. We also showed that Sle1 was associated with a greater proliferation and a lower activation-induced cell death of CD4+ T cells. A lower activation threshold has been demonstrated in MRL helper T cells (40). Our results show that this phenotype also exists in the NZM2410 under the genetic control of Sle1.

We have shown here that transfer of Sle1-expressing T cells was sufficient to induce increased activation, plasma cell differentiation, and anti-chromatin Abs production in B6 B cells. Sle1 T cells activation is independent of expression of Sle1 in B cells, as we have shown in the B6.Sle1.lgh6−/− mice (23). However, the transferred T cells developed in the presence of other lymphocytes expressing Sle1. Consequently, we cannot formally exclude at this point the role of B cells or other types of accessory cells in inducing autoreactivity in the Sle1 T cells. Indeed, an small nuclear ribonucleoprotein BCR transgenic MRL/lpr model has suggested that autoantigen presentation by B cells was necessary to induce autoreactive T cells (41). Breeding of the Igh6−/− mutation on Slela and Sle1c is ongoing to answer this question.

Transfers of syngeneic T cells in ββ T cell-deficient B6 mice induced a strong but transient anti-nuclear Ab response that peaked at 28 days posttransfer (34). We did not observe such a response in the B6.Tcrα−/− hosts after transfer of B6 T cells, possibly due to tolerogenic effects from the y6 T cells. Instead, anti-chromatin Abs production by B6.Tcrα−/− hosts required the expression of Sle1 in the transferred T cells, peaked at 3 mo, and was maintained up to 4 mo postransfer. Similar results have been obtained in a transgenic system in which cognate h-LA specific T cell help was sufficient to trigger anti-nuclear autoantibodies in h-LA transgenic naïve B cells (41). The existence of autoreactive B cells has been shown in T cell-deficient mice (31, 34), but also in normal mice (42) and humans (43). Our data show that Sle1, Slela, or Sle1c T cells but not B6 T cells can provide help in vivo to anti-chromatin-specific autoreactive B cells. The presence of chromatin-specific autoreactive T cells associated with these loci was further confirmed by intra assays. Expression of Sle1 in the B cells greatly enhanced the anti-chromatin Ab response, illustrating the synergistic effect of Sle1 expression in B and T cells. Our results suggest that Sle1 mediates a loss of tolerance that is mostly targeted at nuclear Ags because transfer of Sle1 T cells did not induce the production ofAbs against foreign Ags such as keyhole limpet hemocyanin and only limited production of nonnuclear autoantigens, such as thyroglobulin. Sle1 expression in T cells was required to induce autoantibody production by Sle1 B cells, indicating that cognate help is required for Sle1 B cells to produce autoantibodies. It also showed that tolerization of anti-chromatin T cells is effective in B6 mice, although small nuclear ribonucleoprotein autoreactive T cells have been found in that strain (44).

CD4+CD25+ Treg have been clearly shown to play a role in organ-specific autoimmune diseases such as type 1 diabetes (45) or experimental autoimmune encephalomyelitis (46). Evidence for a role of Treg in lupus is less clear (28, 29), although it has been strongly suggested in transgenic models of systemic autoimmunity (25–27). Here we show for the first time a significant reduction of Treg associated with the generation of autoreactive T cells in a spontaneous model of lupus. A significant reduction in Foxp3 expression, a gene that is necessary and sufficient to confer the suppressive phenotype to CD4+ T cells (36), was also found in Sle1 CD4+CD25+ T cells, confirming the overall reduced number of regulatory T cells when Sle1 is expressed. This reduction was not secondary to the autoimmune process and overall immune dysregulation, because significant differences were found in very young mice before the appearance of anti-chromatin Abs. A similar decreased Foxp3 expression was found in both Sle1 spleen and thymus, suggesting that Sle1 may impact the generation of Treg. It is not known at this point whether Sle1 affects only nuclear Ag-specific Treg or the entire regulatory subset. This issue will be resolved by characterizing the specificity of Sle1 T cell clones and the effect of Sle1 on T cells with defined specificities.

Sle1 corresponds to at least three loci that independently induce the production of anti-chromatin Abs (16). Initial characterization of these loci (16) and analysis of their interactions with other SLE-susceptibility loci (47) suggested that they were not functionally equivalent. This study shows that all the phenotypes that we have described for the Sle1 T cells, including activation, proliferation, cytokine production, help to anti-chromatin B cells, and Treg numbers, were accounted for by Slela, except for the limited Ag spreading seen in Sle1 but not Slela T cells. It has been proposed that epitope spreading in lupus was the consequence of interactions between autoreactive B and T cells (19, 20). Our data suggest that interactions between autoreactive Slela T cells and autoreactive B
cells generated by the expression of another Sle1 locus, such as Sle1b, are necessary to initiate epitope spreading. Sle1a represents a 2.5-Mb segment that is relatively gene-poor, with 14 reported known genes or predicted transcripts (www.ensembl.org/Mus_musculus). Future identification of the gene corresponding to Sle1a presents a unique opportunity to gain insights into the genetic mechanisms that regulate T cell tolerance to nuclear Ags. Sle1b does not contribute to any of the T cell phenotypes that we have examined. This result was unexpected because Sle1b is allelic to a cluster of seven genes from the CD150/CD2 superfamily (18), which are expressed on T cells and APC (48), and Sle1b T cells showed increased calcium flux in response to receptor cross-linking. Our data does not exclude an impact of Sle1b on T cell phenotypes, but shows that, when compared side by side, Slela and Slelc have a stronger effect on CD4 T cell proliferation, activation, and cytokine production than Sle1b. Slela and Sle1c interact synergistically to increase anti-nuclear Abs production and activation markers on both B and T cells (47). It is tempting to speculate that this synergism results from Slela expression in T cells and Slelc expression in B cells. The low pen- etrant low titers anti-chromatin Abs produced by the B6.Slela strain (16) are consistent with these autoantibodies resulting from Slela T cells providing help to B6-like Slela B cells. Slelc expression also contributes to an abnormal T cell phenotype, but not as pronounced as Slela. This was best illustrated in the adoptive transfer experiments in which Sle1c T cells provided help to Sle1b but not B6 B cells. Both Slela and Slelc result in a reduction of Treg cells, but with different effects on the ensuing autoimmune phenotypes. We cannot determine at this point whether it is due to quantitative or qualitative differences. We have proposed that Slela was allelic with Cr2 and we have shown an abnormal phenotype in Sle1c B cells (17). Expression of CR1/CR2 results on activated T cells in A/J mice (49), a result that we have not been able to confirm in B6 mice. However, several lines of evidence suggest that Cr2 may indirectly effect T cell phenotypes, either through Ag presentation or B cell regulation (50). Alternatively, another gene in the Sle1c interval besides Cr2 may contribute to the T cell phenotype. Slelc recombinants are being analyzed to resolve this issue.

In conclusion, we have shown that two genetic loci initially identified through their induction of anti-chromatin Abs are responsible for the production of anti-nuclear autoreactive T cells, and that this loss of tolerance is associated with a significant reduction in Treg. Apart from mutations in the Fosp3 gene itself (51) or Stat1 deficiency (52), our results also associate for the first time genetic defects, the Slela and Slelc loci, with Treg production. These two loci do not contain any obvious candidate gene that has been associated with T cell regulation. Consequently, identification of the genes corresponding to Slela and Sle1c has the potential to reveal new mechanisms controlling Treg numbers and provide new insights on the mechanisms of tolerance to nuclear Ags that prevent the development of systemic autoimmunity.

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Disclosures
The authors have no financial conflict of interest.

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