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Identification and Characterization of a Lupus Suppressor 129 Locus on Chromosome 3

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The 129-derived Sle16 is a susceptibility locus for systemic autoimmunity when present on the C57BL/6 (B6) background. Genetic analysis of a (129×B6)F2 cross identified a region from the B6 chromosome 3 (Sle18) with positive linkage to antinuclear Abs. In this study, we have generated a B6 congenic strain harboring the 129 allele of Sle18 and intercrossed this line with the lupus-prone B6.129-Sle16 strain. The presence of the 129-Sle18 allele in the B6.129-Sle16Sle18 double congenic mice suppressed the development of Sle16-mediated autoantibody production and ameliorated the renal pathology. The 129-Sle18 locus rectified the B cell abnormalities detected in the B6.129-Sle16 mice, such as the reduction in the percentage of marginal zone B and B1a cells and the increased number of germinal centers. The B6.129-Sle16Sle18 spleens still displayed an increased percentage of activated T and B cells. However, in the B6.129-Sle16Sle18 strain the percentage of naive T cells was equivalent to that in B6.129-Sle18 and B6 mice and these cells showed a reduced proliferative response to anti-CD3 stimulation compared with B6.129-Sle16 T cells. There was a significant increase in the percentage of CD4+FoxP3+regulatory T cells in all congenic strains. These cells had normal regulatory function when tested in vitro. Thus, 129-Sle18 represents a novel, non-MHC lupus-suppressor locus probably operating as a functional modifier of B cells that, in combination with other factors, leads to lupus resistance. Further characterization of this locus will help to uncover the immune mechanism(s) conferring protection against lupus. The Journal of Immunology, 2010, 184: 000–000.

The spontaneous occurrence of autoimmunity is the product of genetic, environmental and stochastic events most likely occurring concurrently. In the autoimmune disorder systemic lupus erythematosus (SLE), numerous disease susceptibility loci have been identified both in human and animal studies (1, 2). Findings from lupus-prone mice and congenic lines have proved that genetic predisposition to SLE is caused by epistatic interactions between several genes spread throughout the genome and organized in susceptibility and modifier/suppressor loci (3). These genes may regulate different pathogenic aspects of SLE, influence the severity of the clinical manifestations and affect the outcome of the disease.

Congenic mouse models have facilitated the dissection of the intricate pathogenic pathways leading to SLE. The genetic analysis of these strains remains a strategy of great value especially for the identification of loci, and the genes within, with a “suppressive” role that are still perhaps beyond the feasibility of the current genome wide association studies performed on SLE patients. Extensive analysis of the genetic and immunological traits in the B6.NZM2410 collection of congenic substrains has demonstrated that the severe lupus-like disease developed by the NZM2410 parental strain is the result of a complex epistatic interaction among the individual phenotypes driven by each susceptibility allele (4). Of note, when two potent NZW loci Sle1 and Sle3 were combined on the C57BL/6 (B6) background (bicongenic strain), a lethal autoimmune phenotype resulted. This feature was absent in the NZW strain, suggesting the existence of other chromosomal regions in the parental genome, distinct from the susceptibility loci, capable of suppressing the spontaneous development of autoimmunity. These genomic regions have been defined and named SLE suppressor loci (3, 5).

We have previously presented the results of an extensive linkage trait analysis in the (B6×129) hybrid strain that unveiled four main susceptibility loci linked to autoimmune traits (6, 7). The most powerful locus, called Sle16, is a 7.4 Mbp 129 interval on chromosome 1, which overlaps with many other SLE susceptibility loci both in humans and animal models (8). This locus is capable of driving the production of high levels of autoantibodies and the development of mild glomerulonephritis (GN) when transferred on a B6 background, as observed in the B6.129-Sle16 congenic strain previously named B6.129chr1b. However, the lack of a comparable autoimmune phenotype in the 129 strain indicates that the same strain contains modifier loci, most likely outside the chromosome 1 fragment, with protective effect(s). Likewise, the data imply that the B6 strain is permissive to the development of autoimmunity, also suggested by the observation that a large number of genetically modified models present an obvious disease only on a B6 background (9). Consistent with these hypotheses, linkage analysis performed on the (B6×129) hybrid strain identified a B6 lupus locus on chromosome 3, termed Sle18, contributing to antinuclear Ab (ANA) production. Experiments from our group have recently
established that the B6 Sle18 fragment when transferred onto a 129 background, the 129.B6-Sle18 congenic model, can promote auto-antibody production, increase T and B cell activation, and mediate mild GN (10). To further demonstrate that B6 Sle18 locus is a key lupus modifier that in epistatic interaction with Sle16 allows the development of autoantibodies in the B6.Sle16 congenic mice, we adopted a reciprocal approach and transferred the protective 129 region onto the B6 strain. This novel congenic strain, named B6.Sle16-Sle18, was then crossed with the B6.Sle16-Sle18 line and the serological and cellular features of the bicongenic mice, B6.Sle16-Sle16Sle18, were compared with the two single congenic strains and the B6 controls. As predicted by genetic analysis, the presence of the 129 allele at the Sle18 locus significantly modified the autoantibody profile of the B6.Sle16-Sle16 mice. However, even though the B6.Sle16-Sle18 bicongenic mice developed markedly less serological features of autoimmunity, signs of splenic B and T cell activation similar to those described in the B6.Sle16-Sle16 autoimmune mice were still detectable, indicating that the Sle18 suppressor locus acted downstream of these molecular pathways. In keeping with this observation, the number of splenic germinal centers (GCs) in the B6.Sle16-Sle16Sle18 mice was significantly reduced. Taken together, these data emphasize further the polygenic nature of the etiology and pathogenesis of SLE.

Materials and Methods

Mice

The generation of B6.Sle16-Sle16 mice has been previously described (8). A new B6 congenic line, carrying a chromosome 3 interval of 129 origin (Sle18), was generated using microsatellite markers polymorphic between 129 and B6 mice as previously described (6, 7, 10). After eight generations of backcrosses, >99% of the genome in these mice was of B6 origin (referred to as B6.Sle16-Sle18) as was created. At the end of the backcrossing, additional microsatellite analyses were carried out in the congenic mice to exclude the presence of unselected 129 genomic regions. The B6.Sle16-Sle16Sle18 line was then derived by a B6.Sle16-Sle16 × B6.Sle16-Sle18 cross (Fig. 1). Only female and homozygous mice entered the study. Twenty-eight B6.Sle16-Sle16, 29 B6.Sle16-Sle16Sle18, 26 B6.Sle16-Sle18 mice along with 25 sex-matched B6 controls were followed up to 1 yo age, when all mice were sacrificed and organs collected. Animals were kept under specific pathogen-free conditions and all animal care and procedures were conducted according to institutional guidelines.

Serology

All mice were bled at regular intervals starting from 4 mo of age and the assays described below were conducted on serum samples:

1) Titers of IgG ANA were measured by indirect immunofluorescence using Hep-2 cells as previously illustrated (8).

2) Anti-ssDNA, anti-dsDNA, and anti-chromatin Abs were detected by capture ELISA. The results were expressed in arbitrary ELISA units (AU) relative to a standard positive sample derived from a serum pool from MRL/Mp.

3) For the detection of anti-histone Abs by ELISA, microtiter plates were coated with histone (Roche, Hertfordshire, U.K.) at 5 μg/ml, Alexa Fluor 488. The plates were incubated overnight at 4 °C. The plates were washed and biotinylated anti-mouse GL7 diluted 1/50 (Dako, Glostrup, Denmark) were added, followed by streptavidin-phosphatase (AP)-conjugated goat anti-mouse IgG (γ-chain specific) (Sigma-Aldrich, Dorset, U.K.) and the results were presented in AU relative to a standard positive sample derived from a serum pool from B6 or 129.B6-Sle16-Sle18B6.Sle16-Sle18 mouse.

Renal assessment

Overnight urine specimens were collected from 1-yl-old mice and proteinuria assessed using Haema-comistix (Bayer Diagnostics, Newbury, U.K.) (8). Kidneys were fixed in Bouin’s solution for at least 2 h, transferred into 70% v/v ethanol, and processed into paraffin. Periodic acid-Schiff stained 3 μm thick sections were scored for GN. Glomerular cellularularity was ranked in a blinded fashion as follows: grade 0, normal; grade 1, hypercellularity involving >50% of the glomerular tuft in 25–50% of glomeruli; grade 2, hypercellularity involving >50% of the glomerular tuft in 50–75% of glomeruli; grade 3, hypercellularity involving >75% of the glomeruli or crescents in >25% of glomeruli; grade 4, severe proliferative GN in >90% of glomeruli. Additional morphological characteristics, such as crescent formation and periglomerular fibrosis, were also noted.

Flow cytometry

Cells were harvested from spleens of at least seven mice from each group at 2, 6, and 12 mo of age. After depletion of RBCs using RBC lysis buffer (Tris base plus NH4Cl), spleen cells were counted, and flow cytometry was performed using a three- or four-color staining protocol and analyzed with a FACSCalibur (Becton-Dickinson, Mountain View, CA). The following Abs were used: anti-CD90.2 (53-2.1), anti-B220 (RA3-6B2), anti-CD21 (7G6), anti-CD23, anti-CD25 (B3B4), anti-CD138 (281-2), anti-CD11c (HL3), anti-IgM (H4/1), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD4 (RM4-5), and anti-CD8 (53-6.7). All Abs were purchased from BD Biosciences Pharmingen (San Diego, CA) with the exception of anti-FoxP3 (FJK-16s) (eBioscience, San Diego, CA). Bio- tylabeled Abs were detected using an aliphophycocyanin-conjugated streptavidin Ab (BD Biosciences Pharmingen). Staining was performed in the presence of a saturating concentration of 2.4G2 mAb (anti-FcγRIII). For the FoxP3 intracellular staining, cells were fixed and lyzed with the reagents and the protocol provided by the company (eBioscience). Data were analyzed using FlowJo software, version 6.4 (TreeStar, Ashland, OR).

Spleen histology

Frozen spleen sections (5 μm thick), pretreated with biotin-blocking system as instructed by the manufacturer (Dako, Glostrup, Denmark), were incubated overnight with anti-mouse CD19 (1D3), anti-CD23 (B3B4), anti-CD138 (281-2), anti-CD5 (53-7.3), anti-CD19 (1D3), anti-CD25 (PC61), anti-CD69 (H1.2F3), anti-CD11c (HL3), anti-IgM (H4/1), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD4 (RM4-5), and anti-CD8 (53-6.7). All Abs were purchased from BD Biosciences Pharmingen (San Diego, CA). Staining was performed using with a Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, U.K.) and drawing around the profile of the spleen section using the Image ProPlus software (version 4.5, Media Cybernetics, USA). Further immunohistochemical analysis of spleen sections was performed combining the GL7 staining with a rat anti-mouse FITC-labeled IgG Ab (1/100) (BD Biosciences Pharmingen). Slides were viewed using an Olympus BX4 fluorescence microscope (Olympus Optical, London, U.K.) and images were acquired with a Photonic Science Color Coolview digital camera (Photonic Science).

In vitro B and T cell assays

B cell assays. Single-cell suspension of splenocytes (10^6 cells/ml) were cultured with either LPS (0.5 and 0.05 μg/ml, Sigma-Aldrich) or goat anti-mouse IgM (Fab')2 (10 and 5 μg/ml, Pierce Biotechnology, Rockford, IL) or anti-mouse CD40 (2.5 μg/ml, Alexis Biochemicals, Lausen, Switzerland) in complete medium as illustrated by the manufacturer’s instructions. CD4+CD25+ T cells were isolated from spleens and selected lymph nodes from B6, B6.129-Sle16-Sle16 mice and then activated with plate-bound anti-CD3 (from 1–30 μg/ml) or PHA (from 1–10 μg/ml) for 3 d. Trinitiated thymidine ([3H]TdR) was added 16 h prior to harvesting and incorporation was measured by liquid scintillation. Each assay was repeated at least three times. A sandwich ELISA for IL-2 was performed using purified capture and biotinylated detection Abs from BD Biosciences Pharmingen.

T cell suppression assays. This assay was performed as previously reported (11). Negative magnetic selection of CD4+ T cells from spleens and selected lymph nodes was carried out using DynaBeads (Dynam, Oslo, Norway) according to the manufacturer’s instructions. CD4+CD25+ regulatory T cells (Tregs) were positively selected on MiniMACS columns (Miltenyi, Bergish Gladbach, Germany), using anti-CD25-PE Abs and anti-PE-MicroBeads (Miltenyi). The cell purity was found to be >90% as determined by flow cytometry. Purified CD4+CD25+ T cells were cultured, in 96-well round-bottomed plates, with 2.5 μg/ml, responder T cells (Tresps), in the presence of Epoxy DynaBeads (1 bead/5 cells; Dynal) coated with anti-CD3 and anti-CD28 mAbs. CD4+CD25+ T cells purified from either the B6 or 129.B6.Sle16 or B6.129-Sle16Sle18 mouse were added to B6 mice.
CD25+ T cells at different Treg/Tresp ratios ranging from 1:1–1:32. Irradiated CD25+ T cells were used as control. After 3 d, the incorporation of [3H]TdR (Amersham Biosciences, Roosendaal, The Netherlands) over 12 h was measured. In addition, CD25+ T cells were prelabeled with CFSE (0.5 µM; Molecular Probes, Invitrogen) and cultured in the presence of Epoxy DynaBeads (1 bead/5 cells; Dynal, Invitrogen) coated with anti-CD3 and anti-CD28 mAb either alone or with Treg at different ratios. The Tresp:Treg cocultures were incubated for 96 h, after which the proliferation of T cells was analyzed by flow cytometry. All assays were performed with RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin (Life Technologies, Paisley, U.K.), 2 mM L-glutamine, 10 mM HEPES, and 10% (v/v) heat-inactivated FCS.

**Immunization**

The primary immune response to a T dependent Ag was assessed after a s.c. injection with 50 µg/mouse of (4-hydroxy-3-nitrophenoxy)acetil conjugated to chicken γ globulin (NP-CGG) (Biosearch Technologies, Novato, CA) in CFA (Sigma-Aldrich). Sera were tested by ELISA every week for 5 consecutive weeks after the injection. Briefly, plates were coated with 5 µg/ml NP-BSA (Biosearch Technologies) and sera were assayed at 1/100 dilution. Bound Abs were detected with AP-conjugated Abs to specific mouse isotypes (Southern Biotechnology Associates, Birmingham, AL). For secondary immunization, mice were boosted with the same dose of NP-CGG in IFA 28 d after the first injection and Ab titers were measured 1 week later.

Responses to a T independent Ag mice were assessed following i.p. injections with 500 µl Pneumovax II (Sanofi Pasteur MSD, Berkshire, U.K.) containing 1 µg of each of the 23 main polysaccharides (PS). Serum samples were obtained weekly and the immune response against PS was measured by ELISA. Microtiter plates were coated with 2 µg/ml of Pneumovax II, sera diluted 1/100 (for IgG3) and 1/1000 (for total IgM) and bound Abs were detected with AP-conjugated Abs to specific mouse isotypes (Southern Biotechnology Associates). Five to seven mice per group were used for each time point.

**Statistics**

The data are presented as median with respective range of values in parentheses unless otherwise stated. The nonparametric Kruskal-Wallis test with Dunn’s multiple comparison test or the Mann-Whitney U test were applied throughout, with differences being considered significant for p values <0.05. Data from in vitro assays were analyzed by two-tailed Student t test for unpaired samples. Statistics were calculated using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA).

**Results**

**The 129 allele of Sle18 suppresses autoantibody production driven by Sle16**

Our previous work (10) demonstrated the presence of a B6-derived epistatic modifier on chromosome 3 (Sle18), which was permissive for the break in tolerance mediated by Sle16. Consequently, the corresponding 129 allele was hypothesized to be a lupus suppressor locus. To test this hypothesis, the central portion of the 129 chromosome 3 was transferred onto the B6 genetic background to create a congenic strain using a marker-assisted selection protocol as described previously. The 129 interval from 71.4 Mb to 129.8 Mb in the B6.129-Sle16 congenic line was defined as the 129 allele of the Sle18 locus (Fig. 1). Autoimmune traits in B6.129-Sle18 mice and in B6.129-Sle16Sle18 congenics were monitored over a 1-y period and compared with the titers present in B6.129-Sle16 mice, known to develop high titer of lupus-associated autoantibodies (8).

At 4 mo of age, the B6.129-Sle16 mice already displayed high titers of anti-ssDNA IgG Abs compared with the B6 controls (B6.129-Sle16: 32.43 AEU, range 3.63–228.9; B6: 4.8 AEU, range 1.6–9.42, p < 0.01, Kruskal-Wallis test with Dunn’s multiple comparison test). More importantly, the B6.129-Sle16Sle18 bicongenic mice had similar titers to those detected in B6 mice and were significantly less autoimmune than the B6.129-Sle16 line (B6.129-Sle16Sle18: 12.11 AEU, range 0–188.5, p < 0.01 versus B6.129-Sle16, Kruskal-Wallis test with Dunn’s multiple comparison test). These initial observations were confirmed at all subsequent time points (6, 9, and 12 mo). At the 1-y final time point, when a more extensive study of the autoimmune profile was performed, the B6.129-Sle16Sle18 bicongenic still showed autoantibody titers (ANA, anti-dsDNA, anti-ssDNA, and anti-histone Abs) equivalent to those measured in B6 mice and statistically lower (p < 0.001, Kruskal-Wallis test with Dunn’s multiple comparison test) than the values detected in B6.129-Sle16 animals (Fig. 2A–F). Notably, B6.129-Sle18 mice had even lower anti-ssDNA and anti-dsDNA Abs values compared with those in B6 controls (B6.129-Sle18 versus B6: anti-ssDNA Abs p < 0.05; anti-dsDNA Abs p < 0.01, Mann-Whitney U test with Dunn’s multiple comparison test) (Fig. 2B, 2C). In addition, the analysis of a separate cohort of 6-mo-old mice heterozygous at the Sle18 locus showed anti-ssDNA and anti-chromatin Ab levels significantly lower compared with those present in the B6.129-Sle16 animals (anti-ssDNA Abs: B6.129-Sle16 = 80.5 AEU, range 20–3398, n = 16 versus B6.129-Sle16Sle18het = 27 AEU, range 10–320, n = 18 < 0.05, Mann-Whitney U test; anti-chromatin Abs: B6.129-Sle16 = 13.2 AEU, range 0–76.94, n = 18 versus B6.129-Sle16Sle18het = 0 AEU, range 0–44.91, n = 14 < 0.05, Mann-Whitney U test). The anti-dsDNA Ab levels showed the same trend but the difference did not reach statistical significance (B6.129-Sle16 8.32 AEU, range 1.3–57.8, n = 10 versus B6.129-Sle16Sle18het 2.18, range 1.5–3.4, n = 8, p = 0.76, Mann-Whitney U test). These findings suggest that the presence of a single 129-derived allele on chromosome 3 is sufficient to alter the serological phenotype driven by the Sle16 locus and indicate that 129-Sle18 locus may operate in a dominant fashion.

The 129 Sle18 locus ameliorates the renal pathology present in B6.129-Sle16 mice

All mice were sacrificed at 12 mo of age and organ and urine collections were performed. Kidneys were processed for light microscopy analysis. No signs of abnormal renal function were found in any of the congenic groups or in the control mice (data not shown).

Periodic acid-Schiff–stained kidney sections were graded in a blinded fashion from 0–4 as described in Materials and Methods. As previously reported (8), B6.129-Sle16 mice developed mild GN (median grade 2, range 0–4), statistically more severe (p < 0.001, Kruskal-Wallis test with Dunn’s multiple comparison test) than B6 mice (median grade 1, range 0–3). In the same analysis, B6.129-Sle16Sle18 kidney sections presented a GN grade (median grade 1, range 0–4) lower than that of B6.129-Sle16 mice (p < 0.001, Kruskal-Wallis test with Dunn’s multiple comparison test) and similar to that in wild-type control animals (Fig. 2F).

Lupus-resistant and lupus-prone mice display similar splenic changes

To investigate the impact of the 129-Sle18 locus at the cellular level, we performed a comprehensive flow cytometric analysis of spleenocytes from all three (B6.129-Sle16, B6.129-Sle16Sle18, and B6.129-Sle18) congenic lines at 2, 6, and 12 mo of age and compared the findings with age-matched B6 controls. As previously reported at 2 mo of age, the only splenic alteration detectable was a small but significant decrease in the percentage of marginal zone (MZ) CD21highCD23low B cells in the B6.129-Sle16 congenics compared with the controls (B6.129-Sle16: 4.29 ± 0.4, n = 13 versus B6: 6.54 ± 0.4, n = 12, p < 0.01, Mann-Whitney U test), associated with a parallel trend toward a reduction in the absolute number.

At the 6 mo time point, the most striking abnormality was the marked expansion of the percentage of Tregs, determined by FoxP3...
expression, in all three congenic lines compared with the B6 controls (Table I). This was associated with a significant increase in the absolute number of Tregs only in the B6.129-Sle16 congenic mice most likely as a result of the tendency of these mice to develop hypercellularity. The expansion of the FoxP3+ T population persisted until the end point (Table I) and was not accompanied by a variation in the intensity of the FoxP3 expression (data not shown). Another interesting finding at 6 mo of age was the marked increase in the percentage of activated T cells (CD69+) in the B6.129-Sle16 and B6.129-Sle16Sle18 congenic animals. Although this finding was predictable for the lupus-prone strain, the detection of a similar change in the nonautoimmune B6.129-Sle16Sle18 strain was intriguing and indicated that lupus resistance was not referable to a block in the mechanism controlling T cell activation.

The final analysis performed on samples from 12-mo-old mice corroborated the numerous cellular differences between B6.129-Sle16 and B6 mice already described (8) and provided some novel findings. Splenomegaly was detected only in the lupus-prone B6.129-Sle16 strain, indicating that 129-Sle18 maintained normal splenic cellularity. As the B6.129-Sle16 spleens were markedly hypercellular, only the results expressed as percentages will be considered significant for $p$ values $<0.05$. 

**FIGURE 1.** Genetic map of congenic mice bearing the Sle16 and Sle18 regions. Loci position (shown in Mbp) has been defined by microsatellite and SNP genotyping (list on request). White, 129 genome; black, B6 genome; hatched, recombination regions. SNP, single-nucleotide polymorphism.

**FIGURE 2.** Serological and histological profiles at one year of age. Each symbol represents one mouse. A, ANA titers. Serum samples were screened at 1/80 and positives titrated to endpoint, (B) anti-ssDNA Ab, (C) anti-dsDNA Ab, (D) anti-chromatin Ab, and (E) anti-histone Ab levels in the B6.129 congenic lines are shown. The Ab levels are expressed in AEU related to a standard positive sample. F, GN graded from 0–4 as described in Materials and Methods. Horizontal bars indicate median. The nonparametric Kruskal-Wallis test with Dunn’s multiple comparison test was applied throughout with differences being considered significant for $p$ values $<0.05$. 

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Table I. Flow cytometric analysis of splenic cell populations at 6 and 12 mo of age

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>FACS Staining</th>
<th>6 mo</th>
<th>12 mo</th>
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<tr>
<td></td>
<td></td>
<td>Sle16</td>
<td>Sle16 vs Sle18</td>
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<tr>
<td>Cells (10^6/ml)</td>
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<td>No. 7</td>
<td>No. 8</td>
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<tr>
<td>% B cells (No. × 10^5)</td>
<td>CD19+</td>
<td>0.43</td>
<td>0.37</td>
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<tr>
<td></td>
<td>CD19+, CD5+</td>
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<td>0.37</td>
</tr>
<tr>
<td>% Plasma cells (No. × 10^6)</td>
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<td>0.43</td>
<td>0.37</td>
</tr>
<tr>
<td>% MZ B cells (No. × 10^5)</td>
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<td>0.43</td>
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<tr>
<td>% FZ B cells (No. × 10^5)</td>
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<td>% B1a cells (No. × 10^5)</td>
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<tr>
<td>% Activated B cells (No. × 10^5)</td>
<td>CD19-</td>
<td>0.43</td>
<td>0.37</td>
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<tr>
<td>% Activated T cells (No. × 10^5)</td>
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<td>0.43</td>
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<tr>
<td>% Naive T cells (No. × 10^5)</td>
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<tr>
<td>% Memory T cells (No. × 10^5)</td>
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<td>0.43</td>
<td>0.37</td>
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<tr>
<td>% Tregs (No. × 10^5)</td>
<td>CD19-</td>
<td>0.43</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Results are shown as mean ± SEM. The p values were considered significant when <0.05 according to the Kruskal-Wallis test with Dunn’s multiple comparison test. Statistical analysis as follows:

* Sle16 versus Sle16 vs Sle18.

† Any congenic versus B6.

‡ Sle16 vs Sle18.

§ Sle16 vs Sle18.

Any congenic versus B6.  
† Any congenic versus B6.  
‡ Sle16 vs Sle18.

One symbol: <0.05; two symbols: <0.01; three symbols: <0.001.

FZ, follicular zone.
discussed, although the absolute numbers reflected these differences in a more exaggerated manner. As expected, the B6.129-Sle16 mice displayed a more "active" phenotype with a statistically significant increase in activated (CD69+) B and T cells compared with the B6 mice. As observed at the earlier time point, the lupus-resistant B6.129-Sle16Sle18 bicongenics showed comparable percentages of activated (CD69+) lymphocytes (Fig. 3, Table I). Consistent with the increase observed in T cell activation status, the CD4+ T cells in aged B6.129-Sle16 mice were skewed toward an activated memory phenotype with an increased percentage of CD4+CD25+CD44high T cells (50.19 ± 1.78 versus 33.4 ± 1.49 in B6 mice; p < 0.001) and a corresponding decrease in the percentage of naive (CD62L+) T cells compared with the B6 controls (28.76% ± 2.23 versus 50.03 ± 2.03 in B6 mice, p < 0.01, Kruskal-Wallis test with Dunn’s multiple comparison test) (Table I). Interestingly, whereas the increase in the memory T cells was comparable between the lupus-prone and the lupus-resistant strains (50.19 ± 1.78 versus 48.46 ± 1.86; p > 0.05), the decrease in the percentage of T cells expressing a naive phenotype was not observed in the B6.129-Sle16Sle18 mice (41.27% ± 2.43 versus 50.03 ± 2.03 in B6 mice) (Fig. 3, Table I).

A more detailed examination of the splenic B cell populations revealed that the B6.129-Sle16 animals had a small but significant decrease in the percentage of CD19+ B cells, accompanied by a reduction in the MZ B cell population and an increase in the follicular B cells (Table I). We also investigated the percentage of splenic CD5+ B1a cells and found that this was decreased-in the B6.129-Sle16 mice, a defect that was not present in the B6.129-Sle16Sle18 bicongenic mice (Fig. 3, Table I). The percentage of plasma cells in the B6.129-Sle16 animals was markedly augmented compared with the B6 controls and this increase, but to a much lesser degree, was also observed in the B6.129-Sle16Sle18 bicongenic mice (Table I).

To substantiate the observations made by flow cytometry, we performed histological analysis of splenic sections in 12-mo-old mice. In the spleens from the B6.129-Sle16Sle18 bicongenic animals we detected a remarkably reduced number of GCs compared with the B6.129-Sle16 mice (Fig. 4). These findings indicate that the 129-Sle18 locus was able to suppress the formation of GCs.

**Impact of Sle18 on B and T cells**

To begin to identify the cellular components contributing to the protection from autoimmunity in B6.129-Sle16Sle18 mice, we then performed in vitro experiments to assess B and T cell responses. We found no differences in the proliferation or Ig production from splenocytes after stimulation with anti-CD40 or anti-IgM Abs or LPS (data not shown). However, we did observe an increased proliferation of naive T cells from young B6.129-Sle16 mice when stimulated in vitro with anti-CD3 or PHA and this was accompanied by an enhanced production of IL-2 (Fig. 5). The presence of the 129-Sle18 locus in the double congenic mice restrained the proliferation of the T cells to levels equivalent to those present in the B6 control mice, indicating that the Sle18 lupus suppressor locus may operate by controlling the proliferative response of naive T cells.

Surprised by the observed expansion of FoxP3+ T cells in the congenic strains, we then compared the functional competency of the Tregs using an in vitro suppressor assay previously described (11). CD4+ T cells were purified from 129.B6-Sle16Sle18, 129. B6-Sle16 and B6 mice and further separated into CD25+ (Treg) and CD25- (Tresp) populations. The freshly isolated Tregs were added to cultures containing a fixed number of Tresps at the indicated Treg/Tresp ratios. The cultures were stimulated with beads coated with anti-CD3 and anti-CD28 Abs and proliferation responses were determined (Fig. 6). To compare the suppressive function of the 129.B6-Sle16Sle18 and 129.B6-Sle16 Tregs with...
that of B6 Tregs, we used the same Tresp (B6 Tresp) (Fig. 6A). As previous reports demonstrated that Tresp from lupus-prone mice have a decreased susceptibility to suppression by Tregs (11), we performed experiments to compare the sensitivity of the different Tresps to suppression mediated by the same B6 Tregs (Fig. 6B). Furthermore, we carried out cross-over experiments, in which the suppressive ability of Tregs from 129.B6-Sle16 or B6 mice to suppress the proliferation of 129.B6-Sle16 Tresp was interrogated (Fig. 6C). Similarly, the response of Tresps from 129.B6-Sle16 or B6 mice to Tregs from 129.B6-Sle16 was compared (Fig. 6D). These experiments demonstrated that the 129.B6-Sle16 and 129.B6-Sle16Sle18 Tregs were fully competent in suppressing B6 Tresp proliferation (Fig. 6) in a dose-dependent manner. In addition, Tresp from 129.B6-Sle16 and 129.B6Sle16Sle18 mice were similarly suppressible by B6 or 129.B6-Sle16 Tregs, indicating no reduced sensitivity to suppression in the congenic animals. Similar results were obtained when suppressor assays using CFSE-labeled Tresp were performed (data not shown).

FIGURE 5. Stimulation of naive T cells. CD4+CD25− naive T cells were purified from 2- to 3-mo-old mice (for details see Materials and Methods). Cells were cultured alone (w/o) or in the presence of plated-bound anti-CD3 Ab (aCD3) (A) or PHA (B) at different concentrations. Cell proliferation was determined by thymidine incorporation after 3 d of stimulation ([3H]thymidine added for the last 16 h). C, Serum IL-2 levels were determined in the culture supernatants after stimulation with plated-bound anti-CD3 Ab (1 μg/ml) using ELISA. Shown are data (mean ± SD) representative of three independent experiments. The data were analyzed by two-tailed Student t test. The genotypes of the mice are shown.
Immune responses to TD and TI Ags

We next investigated whether the presence of the Sle loci (Sle16 and Sle18) could alter the immune responses to exogenous Ags. We administered a T dependent (NP-CGG) and a T independent (Pneumovax II) Ag to cohorts of age and sex-matched B6, B6.129-Sle16, B6.129-Sle16 and B6.129-Sle16Sle18 mice. The congenic strains did not substantially differ from the controls in the levels of (4-hydroxy-3-nitrophenyl)lacetyl (NP)-specific IgG Abs measured at different time points after primary and secondary immunizations. Likewise, anti-NP IgG subclass titers did not differ among the congenic cohorts, suggesting a normal class switching process in the congenic mice (Fig. 7A). When we assessed the TI immune response, we observed that the strains carrying the Sle16 locus showed a trend toward higher IgG3 anti-PS levels, but the values never reached statistical significance at any of the time points analyzed. Interestingly, the nonlupus prone strain B6.129-Sle18 displayed a strong IgM anti-PS response (day 14: B6.129-Sle18 1161 ± 208.8 U/ml versus B6.129-Sle16 326.8 ± 42.71; p < 0.01, Kruskal-Wallis test with Dunn’s multiple comparison test), but IgG3 titers similar to those detected in the other strains (Fig. 7B).

Discussion

The congenic dissection approach allows identification and analysis of chromosome loci modulating autoimmunity in an effort to determine the underlying genes and the human homologs. This article extends our previous work characterizing the suppressor locus mapped on chromosome 3. This locus, named 129-Sle18, encompasses the central region of chromosome 3 spanning from 71.4–129.8 Mb and was able to confer lupus resistance when transferred onto a lupus-prone congenic line, the B6.129-Sle16 strain (8). The 129-Sle18-mediated protection, measured as absence of autoantibodies, was particularly effective in homozygous mice, although the heterozygous 129-Sle18 allele also resulted in partial protection (Fig. 2, data not shown). These results suggest that the 129-Sle18 suppressor effect is additive and most likely due to gain of function. In addition, the presence of the 129-Sle18 locus did not alter the Ab responses to exogenous Ags (T dependent and T independent Ags), indicating that in our model different pathways are used by foreign and self-Ags.

Previous studies on different mouse strains have identified genomic regions with an epistatic “suppressive” effect on disease susceptibility regions or on the phenotype of gene-targeted mice. More specifically, from the genetic dissection of bi and triple congenic models carrying the NZW SLE susceptibility loci Sle1, Sle2, and Sle3, four suppressor regions have been characterized. These NZW loci, named SLE suppressors (Slex) 1–4, have been mapped to chromosome 17, 3, 4, and 9, respectively, and their presence have been shown to abrogate, to a different degree, both the humoral autoimmunity and the nephritis developed by the lupus-prone B6.129 congenic strains (3, 4). Of particular relevance for the data presented in this report is Sles3 that peaks at 78.6 Mb on chromosome 3 (3) and thus partially overlays with the centromeric end of the 129-Sle18 allele. Two other suppressive loci have been mapped on chromosome 3: one in the BXS/long-lived recombinant inbred strain and one in the MRL/lpr model (12, 13). Thus suppressor loci in different strains have been mapped to this chromosomal location indicating that at least some of the molecular mechanisms underlying the lupus-resistance may be shared. In this context it is worth mentioning that, although our linkage analysis in (129 × B6) F2 mice failed to identify a strong lupus modifier locus on chromosome 17 (6, 7), the 129-allele of Sle1 has been shown to complement the NZW Sles1 allele in preventing the development of autoimmune features (5). Therefore,
it appears that the 129 mice may harbor several Sle suppressor loci most likely operating at different molecular levels.

Lupus modifiers act at key steps in the disease pathogenesis and thus their characterization can be very informative. For example, Wakeland et al. elegantly demonstrated that the introgression of Sle1 onto the B6.Sle1lyaa model, carrying the TLR7 translocation and developing a severe lupus disease, inhibited the phenotype without suppressing the TLR7 hyperresponsiveness linked to the yaa locus (14). A lupus-suppressor BALB/c locus, named sbb2<sup>a</sup> and located on chromosome 12, has been shown to reduce the autoimmune pathology of the B6.FcγR2<sup>b−/−</sup> model. The authors demonstrated that the sbb2<sup>a</sup> locus effect is likely to be B cell extrinsic, probably T cell dependent, and to act in trans affecting the IgG2 production by B cells (15). These findings, in agreement with observations in other models (16–18), support the hypothesis that T cells play a critical role in the development of autoimmunity thanks to their physiological function in shaping B cell activation (23). Given that the 129-Sle18 locus partially overlaps with the Sle3 these data together indicate that some of the underlying mechanisms in these two models might be shared.

MZ B cells are not only the major responders to T independent Ags but they have been shown to respond more rapidly than follicular B cells to T dependent Ags and to be more potent T cell activators (24). Thus, to gain insight into the immune response competence of the congenic strains we challenged the animals with a T independent and a T dependent Ag. The congenic lines did not differ from the wild-type mice in their Ab responses in either of two immunization protocols. These data appear to contradict previous observations in B6.Sle models, in which blunted Ab responses have been described (25). However, hyperresponsiveness to exogenous Ags have also been reported in lupus models (26) and these discrepancies may be related to differences in the immunization protocols applied as well as diverse genetic backgrounds.
Both the lupus-prone and the resistant mice displayed a marked expansion of the Treg compartment compared with the B6 controls, as shown by the increased percentage of splenic CD4+CD25+FoxP3+ T cells. This was an intriguing observation given that previous reports have provided evidence of Treg deficiencies (number and/or function) in lupus mice (11, 27–29). In addition, congenic CD4+ CD25high Tregs, purified from young mice, were functionally competent and just as potent as the B6 Tregs in suppressing the proliferation of normal Tregs. Of note, an age-dependent biphasic change of Treg frequency has been described in the MRL/lpr mice, with an early increase, followed by a subsequent decline mirroring disease progression (30). Therefore, one could speculate that the observed enlargement of the Treg compartment in our model may just reflect a failed attempt of the immune system to control the autoimmune diathesis. However, Treg expansion was also observed in the nonautoimmune B6.129-Sle18 strain, indicating that other genetic or environmental factors might have contributed to this phenotype.

In summary, in this report we have described a novel 129 lupus-suppressor locus and provided preliminary insights into the mechanisms by which 129-Sle18 may control the occurrence of spontaneous autoimmunity driven by Sle16. Further characterization of the lupus-resistant B6.129-Sle16Sle18 strain will lead to a better understanding of the mechanisms involved in autoimmune pathogenesis and to useful insights for the development of strategies toward therapeutic interventions.

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Disclosures
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