Genetic Dissection of SLE: SLE1 and FAS Impact Alternate Pathways Leading to Lymphoproliferative Autoimmunity

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Abstract
Genetic dissection of lupus pathogenesis in the NZM2410 strain has recently revealed that Sle1 is a potent locus that triggers the formation of IgG anti-histone/DNA antibodies, when expressed on the B6 background as a congenic interval. B6.lpr mice, in contrast, exhibit distinctly different cellular and serological phenotypes. Both strains, however, do not usually exhibit pathogenic autoantibodies, or succumb to lupus nephritis. In this study, we show that the epistatic interaction of Sle1 (in particular, Sle1/Sle1) with FASlpr leads to massive lymphospleno-megaly (with elevated numbers of activated CD4 T cells, CD4+CD8+ double negative (DN) T cells, and B1a cells), high levels of IgG and IgM antinuclear (including anti-ssDNA, anti-dsDNA, and anti-histone/DNA), and antiglomerular autoantibodies, histological, and clinical evidence of glomerulonephritis, and >80% mortality by 5–6 mo of age. Whereas FASlpr functions as a recessive gene, Sle1 exhibits a gene dosage effect. These studies indicate that Sle1 and FASlpr must be impacting alternate pathways leading to lymphoproliferative autoimmunity.

Key words: lupus • genetics • apoptosis • ALPS • anti-DNA

Introduction
Lupus is a truly polygenic disease where several distinct genetic aberrations and pathogenic events collectively orchestrate antinuclear antibody (ANA)* mediated systemic end-organ damage (for a review, see references 1 and 2). In particular, Sle1, Sle2, and Sle3 are three major lupus susceptibility loci in the NZM2410 murine lupus model (3). When introgressed onto the normal C57BL/6 (B6) background, the NZM2410 alleles of these loci lead to very different immunophenotypes (4–13). Sle1 triggers the formation of anti-histone/DNA Abs that react poorly with naked dsDNA (7). Sle2 effects generalized B cell hyperactivity, leading to an expansion of B1 cells, and increased serum polyclonal/polyreactive Ig (6, 8). In contrast, Sle3 leads to reduced activation-induced T cell death (AICD), increased CD4:CD8 ratios, and low-grade antinuclear seroreactivity (9). Although none of these loci engender severe glomerulonephritis in isolation, the epistatic interaction of these loci leads to high-titred anti-dsDNA ANAs, severe GN, and mortality (10, 13).

The B6.Sle1 strain has a rather peculiar seroprofile, compared with most other lupus-prone strains. In the BWF1 and MRL/lpr lupus models, as well as in lupus patients, anti-histone/DNA ANAs appear early in disease, and precede the emergence of anti-dsDNA ANAs (14–16). In contrast, this transition to anti-dsDNA (and nephrophilic) ANAs does not routinely occur in the B6.Sle1 strain. Thus, <30% of these mice progress to develop anti-dsDNA ANA (7). This phenotype is reminiscent of that seen in patients with drug-induced lupus, who also develop anti-histone/DNA ANAs, but not renal disease (17). These observations suggest that anti-histone/DNA ANAs may not be pathogenic. More interestingly, these studies lend credence to the notion that anti-histone/DNA B cells may be regulated differently from anti-dsDNA B cells. Although Sle1 may be sufficient to breach tolerance of anti-histone/DNA B cells, B-cell-depletion, additional mechanisms may be in place to check the emergence and evolution of anti-dsDNA B cells, and any consequent pathology.

Since Sle3 has the potential to augment anti-dsDNA production, in epistasis with Sle1, Sle3 may be representative of loci that regulate tolerance of anti-dsDNA B cells.

*Abbreviations used in this paper: ANA, antinuclear antibody; AICD, activation-induced T cell death; ALPS, autoimmune lymphoproliferative syndrome; BUN, blood urea nitrogen; DN, double negative; PerC, peritoneal cavity.
Although the culprit gene(s) within the Sle3 locus remains unknown, we already know that Sle3 has the potential to impair AICD, leading to an accumulation of activated T and B cells, with increasing age (9). Indeed, in many respects these phenotypes parallel those seen in FAS<sup>+/−</sup> mice. The lpr mutation of FAS also impairs activation-induced lymphocyte apoptosis, leading to an accumulation of activated lymphocytes, serum anti-dsDNA ANAs, and clinical autoimmunity on certain genetic backgrounds (18, 19).

Although loci such as Sle1 may be critical in modulating immune tolerance of anti-histone/DNA B cells, the regulation of anti-dsDNA specificities (and hence the development of end-organ disease) may require additional checkpoints, such as those modulated by Sle3 and FAS. In this study we test this hypothesis by generating B6.Sle1<sup>+/+</sup> mice, bearing both Sle1 and FAS<sup>+/+</sup> and FAS<sup>−/−</sup> controls. Interestingly, these mice have greatly elevated levels of nephrophilic, anti-dsDNA ANAs, prominent lymphopenopathy and splenomegaly, and accelerated mortality, compared with B6, B6.Sle1, and B6.lpr controls. These observations suggest that the more pathogenic specificities (such as anti-dsDNA and nephritic Abs) must have been effectively tolerized in B6.Sle1 mice through FAS/FASL-dependent mechanisms. Thus, Sle1 and FAS appear to be impacting two alternate, nonredundant, pathways leading to lymphoproliferative autoimmunity.

**Materials and Methods**

**Mice.** C57BL/6 (B6) mice were obtained from The Jackson Laboratory and subsequently bred in our animal colony. The derivation of B6 congenic mice bearing NZM2410 derived lupus-susceptibility intervals has been detailed previously (4). B6.Sle1 (<i>i.e., B6.Sle1<sup>NZM2410</sup></i>) are C57BL/6 mice congenic homozygotes for a 37 centimorgan interval on murine chromosome 1, spanning the 95% confidence interval flanking Sle1, derived from NZM2410, with termini at D1MIT101 and D1MIT155. The immunological phenotypes of this strain have been reported previously (7). B6 mice bearing homozygous FAS<sup>+/−</sup> mutations were obtained from The Jackson Laboratory, and are referred to as B6.lpr, for simplicity (18). B6.Sle1<sup>+/+</sup> mice expressing Sle1 and FAS<sup>+/−</sup>, both homozygous, were derived by breeding B6.Sle1 with B6.lpr mice for two generations, and then selecting F2 progeny that were homozygous at both loci. In addition, where indicated, mice bearing single copies of these loci were also studied. Thus, B6.Sle1<sup>+/−</sup> or FAS<sup>−/−</sup> or Sle1 mice are homozygous for FAS<sup>+/−</sup>, but heterozygous at Sle1. Likewise, B6.Sle1<sup>+/−</sup> mice are homozygous at Sle1, but heterozygous for FAS<sup>−/−</sup>. The primers used to identify the Sle1 interval and FAS<sup>−/−</sup> have been detailed previously (4, 7, 20). All mice used for this study were bred and housed in a specific pathogen free colony at UT Southwestern Medical Center Department of Animal Resources in Dallas, TX. Equal numbers of male and female mice were used for all experiments, and any observed sex differences are indicated.

**Cell Preparation and Culture.** Spleenocytes were depleted of red blood cells using Tris Ammonium Chloride, and single-cell suspensions were prepared for culture or flow cytometric analysis (described below). LN cells were obtained from the inguinal sites, and crushed to obtain single cell suspensions, for FACS® analysis. Peritoneal cavity (PerC) cells were obtained by flushing the peritoneal cavities with fresh media. For the in vitro ANA production assays, red cell–depleted splenocytes were cultured (10<sup>6</sup> cells per well) with or without LPS (20 μg/ml; Sigma-Aldrich) at 37°C. Supernatants were harvested 5 d after culture, diluted 1:2, and assayed for total IgG or IgG ANAs by ELISA, as described below.

**Flow Cytometric Analysis and Antibodies.** FACS® was performed as described previously (6–10). In brief, cells were first blocked with staining medium (PBS, 5% horse serum, 0.05% azide) containing 10% normal rabbit serum. Cells were then stained on ice with optimal amounts of FITC, phycocerythrin, or biotin-conjugated primary Abs diluted in staining medium for 30 min. The following dye- or biotin–coupled Abs were obtained from BD PharMingen: CD4 (RM4–5); CD5 (53–7.3); CD8 (Ly–2); CD23 (B3B4); CD24 (M1/69); CD25 (7D4); CD43 (S7); CD44 (IM7); CD45R/B220 (RA3–6B2); CD62L (MEL14); CD69 (H1.2F3); CD80/B7–1 (16–10A1); CD86/B7–2 (GL1); and used at pretitrated dilutions. After two washes, the biotin-conjugated Abs were revealed using streptavidin–Tricolor (Caltag), or streptavidin–Quantum Red (Sigma–Aldrich). Cell staining was analyzed using a FACScan™ (Becton Dickinson). Dead cells were excluded on the basis of scatter characteristics, and 10,000 events were acquired per sample. The mean linear units on the forward scatter channel were used as indicators of cell size. CD3<sup>−</sup> T cells that did not express CD4 or CD8, were classified as double negative (DN) T cells. Cell sorting was conducted using a FACStar™ machine (Becton Dickinson). B1a (B220<sup>hi</sup>), CD23<sup>hi</sup>, CD5<sup>−</sup> or B2 cells (B220<sup>hi</sup>, CD23<sup>−</sup>, CD5<sup>−</sup>) were sorted after staining for B220, CD23, and CD5. Sorted cells were typically >95% pure.

**ELISA for Total Ig.** Total serum IgM and IgG levels were assayed using a sandwich ELISA. In brief, goat anti–mouse IgM or IgG (Boehringer Mannheim) was first coated onto Immulon 1 plates and blocked. Sera were diluted serially and added to the plates for 2 h at room temperature. Bound Ig was revealed with alkaline-phosphatase conjugated goat anti–mouse IgM or IgG Abs (Boehringer Mannheim), using pNPP as a substrate. Serial dilutions of isotype-specific Ig standards were also added to each plate, for quantitation and interplate standardization.

**ELISA for Autoantibodies.** The anti-dsDNA, anti-histone, and anti-histone/DNA ELISAs were performed as described previously (21). For the anti-dsDNA ELISA, Immulon II plates (Dynatech) precoated with methylated BSA (mBSA), were coated overnight with 50 μg/ml dsDNA (Sigma–Aldrich, dissolved in PBS, and filtered through cellulose acetate before use). For the anti-histone/DNA ELISA, the dsDNA-coated plates were then postcoated with 10 μg/ml of “total” histones (a mixture of all histones, purchased from Boehringer Mannheim) overnight at 4°C. After blocking with PBS/3% BSA/0.1% gelatin/3 mM EDTA, 1:100 (starting) dilutions of the test-sera, or 1:2 dilutions of culture supernatants, were incubated in duplicate for 2 h at room temperature. Bound IgG was detected with alkaline-phosphatase-conjugated anti–mouse IgG (Jackson Immunoresearch Laboratory), using pNPP as a substrate. Raw optical density was converted to U/ml, using a positive control mAb derived from a NZM2410 mouse, arbitrarily setting the reactivity of a 1:100 dilution of this serum to 100 U/ml. This control mAb showed equally strong (OD) reactivities to dsDNA and histone/DNA. Sera with reactivities stronger than the test standard were diluted further and reassayed. The glucmerular-binding ELISA was performed as described previously (10), using sonicated rat glomeruli as substrate.
Histopathology. Mice were killed at 3 or 6 mo of age (or earlier, if evidently in distress), and kidneys, spleens, and other internal organs were fixed, sectioned, and stained with H&E, and periodic acid Schiff. For the analysis of kidney sections, at least 100 glomeruli were examined per section, by light microscopy, for evidence of inflammation, and/or tissue damage, as described previously (22), in a blinded fashion. In brief, the severity of GN was graded on 0 to 4 scale, in which the grades 1, 2, 3, and 4, were accorded when 1–10%, 11–25%, 26–50%, and >50% of the glomeruli were affected, respectively. GN of grades 3–4 is referred to in this communication as “severe GN.” The occurrence of any mesangioapathic, capillary hyaline, proliferative, membranous, or crescentic, glomerular changes was also noted. In addition, kidneys were also tested for evidence of Ig deposits by indirect immunofluorescence. Spleens and nodes were examined for their architecture, and any evidence of atypical hyperplasia, lymphomas, etc. In addition, the lungs, hearts, thymus, alimentary tracts, salivary glands, pancreas, liver, and skin of these mice were also sectioned, stained with H&E, and screened for any evidence of pathology.

Clinical Nephritis. Mice were monitored at 3 and 6 mo of age for evidence of clinical nephritis. The total amount of urinary protein was assayed using metabolic cages for urine collection, and a Coomassie-based assay (Pierce Chemical Co.). Typically, normal B6 mice do not excrete >1 mg urinary protein per day. In addition the levels of blood urea nitrogen (BUN) were measured using a commercially available kit (Sigma-Aldrich). Typically, normal B6 mice do not exhibit >30 mg/dl BUN.

Statistics. Where the samples studied were normally distributed, statistical comparisons were performed using the Students’ t test. Where the samples deviated from normality, a nonparametric, Mann Whitney Rank-Sum test was used for comparisons. Statistical analyses were performed using SigmaStat software. For all experiments, the mean and the SEM are also depicted.

Results

B6.Sle1/lpr Mice Exhibit Lymphosplenomegaly, with Altered Lymhocyte Subsets. One of the earliest and most impressive phenotypes that is apparent in B6.Sle1/lpr mice is the massive lymphadenopathy and splenomegaly, evident as early as 2–3 mo of age, as illustrated in Fig. 1. At the age of 3 mo, B6.Sle1/lpr mice (n = 6) have mean spleen weights of 413 mg, which are significantly heavier than age-matched B6 (mean = 74 mg, n = 8, P < 0.002), B6.Sle1 (mean = 90 mg, n = 6, P < 0.001), and B6.lpr (mean = 105 mg, n = 6, P < 0.006), as summarized in Table I. Likewise, all LN in this strain are also enlarged. For in-

Figure 1. B6.Sle1/lpr mice exhibit prominent splenomegaly and lymphadenopathy, as early as 3 mo of age. (A) The enlarged cervical LN are visible superficially as early as 3 mo of age. (B) The cervical LN are grossly enlarged and matted together, abutting onto the submandibular glands. Shown pictures are representative of at least 12 B6.Sle1/lpr mice, at the age of 3 mo. (C) In addition to the cervical LN, the spleen and inguinal LN are also enlarged (see Table I for quantitation and statistical analysis). The histology of these enlarged organs are depicted in Fig. 8.
Evidently, the increased size of the secondary lymphoid organs is partly due to the increased cell numbers, and partly due to the increased size of individual lymphocytes. In particular, B6.Sle1/lpr spleens exhibit significantly higher numbers of CD4⁺, and CD4⁺CD8⁻ DN, but not CD8⁺, T cells. On the average, each B6.Sle1/lpr spleen

**Table I. FACS® Analysis of 3-mo-old (mean ± SEM)**

|                  | B6  | B6.Sle1 | B6.lpr | B6.Sle1.lpr | P value¹ |
|------------------|-----|---------|--------|-------------|----------|
|                  | n = 8 | n = 6  | n = 6  | n = 6       | B6       | B6.Sle1 | B6.lpr |
| **Spleen**       |       |         |        |             |          |         |
| Weight (mg)      | 74.2 ± 10.6 | 89.6 ± 5.6 | 105.0 ± 9.3 | 413.2 ± 97.2 | ** ** *** ** |
| Cell number (×10⁶) | 83.1 ± 13.7 | 107.2 ± 10.1 | 127.2 ± 29.1 | 224.6 ± 21.8 | ** ** ** * |
| % CD4⁺ T cell    | 15.4 ± 1.0  | 15.1 ± 0.8  | 16.7 ± 1.1  | 27.5 ± 0.6  | *** *** *** |
| % CD8⁺ T cell    | 10.2 ± 0.7  | 10.9 ± 0.5  | 9.6 ± 1.9   | 6.6 ± 0.6   | * ** NS   |
| % CD3⁺CD4⁺CD8⁻ (DN) | 4.8 ± 1.1  | 8.2 ± 1.2   | 15.1 ± 2.2  | 15.9 ± 2.1  | *** * NS   |
| CD4⁺ T cell: size (mean FSC) | 46.1 ± 0.8  | 45.2 ± 0.9  | 50.2 ± 1.1  | 50.6 ± 1.0  | ** ** NS   |
| % CD69⁺         | 27.1 ± 1.3  | 27.8 ± 0.6  | 35.1 ± 1.2  | 52.5 ± 4.6  | *** ** *   |
| % CD25⁺         | 25.4 ± 2.8  | 28.2 ± 0.3  | 24.6 ± 2.0  | 42.4 ± 6.9  | * NS NS    |
| % CD62L⁻/⁻/⁻   | 51.9 ± 3.7  | 44.0 ± 3.8  | 72.2 ± 3.4  | 79.0 ± 0.7  | ** ** NS   |
| % CD45RB⁻⁻⁻⁻   | 31.8 ± 4.3  | 38.2 ± 3.3  | 50.5 ± 3.6  | 81.4 ± 1.1  | *** ** **  |
| CD8⁺ T cell: % CD69⁺ | 18.8 ± 2.6 | 18.6 ± 1.6 | 17.2 ± 0.5 | 47.5 ± 5.2 | *** ** |
| % CD25⁺         | 12.1 ± 2.0  | 14.8 ± 2.1  | 15.1 ± 3.9  | 37.0 ± 7.2  | ** NS      |
| % CD62L⁻⁻⁻⁻   | 52.4 ± 3.7  | 34.9 ± 4.5  | 65.6 ± 3.5  | 84.9 ± 2.7  | *** * **   |
| % CD45RB⁻⁻⁻⁻   | 10.6 ± 1.9  | 7.1 ± 0.3   | 20.7 ± 7.4  | 38.9 ± 6.4  | ** ** NS   |
| % B1a cells     | 4.9 ± 0.8   | 10.0 ± 4.7  | 19.9 ± 3.8  | 23.7 ± 4.0  | *** ** *   |
| % B2 cells      | 53.8 ± 3.3  | 52.1 ± 2.2  | 33.6 ± 6.8  | 16.8 ± 3.4  | *** *** **  |
| B cell: size (mean FSC) | 46.2 ± 0.8 | 48.2 ± 0.8 | 49.1 ± 0.7 | 51.5 ± 0.7 | * NS |
| I-A² (MFI)³     | 1,000      | 1,154 ± 55 | 1,163 ± 53 | 2,735 ± 125 | *** *** *** |
| B7-1 (MFI)³     | 50         | 53.6 ± 9.4 | 57.7 ± 4.3 | 78.2 ± 3.9 | * *       |
| B7-2 (MFI)³     | 50         | 59.8 ± 5.3 | 74.3 ± 14.6| 93.0 ± 12.5| ** NS     |
| CD44 (MFI)³     | 300        | 303.3 ± 9.5| 360.9 ± 32.6| 570.9 ± 61.9| *** * **   |
| **LN**          |           |         |        |             |          |         |
| Weight (mg)      | 7.5 ± 1.1  | 13.6 ± 2.0 | 14.6 ± 2.8 | 111.0 ± 47.1| * ** ** |
| Cell number (×10⁶) | 2.7 ± 0.4 | 4.3 ± 1.5 | 4.7 ± 1.2 | 17.3 ± 3.1 | * ** ** |
| % CD4⁺ T cell    | 24.1 ± 2.2 | 22.5 ± 2.3 | 18.7 ± 2.2 | 17.7 ± 1.7 | *** NS NS |
| % CD8⁺ T cell    | 21.4 ± 2.0 | 23.4 ± 2.9 | 15.2 ± 2.2 | 8.7 ± 1.3  | *** * ** |
| % CD3⁺CD4⁺CD8⁻ (DN) | 10.9 ± 2.9 | 8.6 ± 1.4  | 13.4 ± 1.7 | 27.5 ± 3.2 | *** * |
| CD4⁺ T cell: mean FSC | 46.7 ± 1.7 | 45.4 ± 2.0 | 48.2 ± 1.4 | 52.4 ± 0.8 | * * |
| CD8⁺ T cell: % CD69⁺ | 23.8 ± 2.2 | 21.3 ± 1.2 | 35.0 ± 5.0 | 43.0 ± 2.9 | *** NS |
| CD4⁺ T cell: % CD69⁺ | 15.2 ± 3.6 | 8.0 ± 1.1  | 23.3 ± 9.2 | 29.2 ± 2.9 | * ** |
| % B1a cells      | 4.5 ± 0.4  | 4.7 ± 0.8  | 15.5 ± 5.1 | 35.1 ± 4.8 | *** ** |
| % B2 cells       | 34.4 ± 4.0 | 31.0 ± 2.2 | 37.0 ± 7.8 | 15.1 ± 3.2 | ** NS NS |
| Bcell size (mean FSC) | 44.7 ± 1.7 | 45.5 ± 1.3 | 48.1 ± 1.3 | 53.5 ± 0.6 | *** * |
| **PerC**         |           |         |        |             |          |         |
| B1a cells        | 37.6 ± 1.7 | 34.5 ± 3.4 | 38.1 ± 6.1 | 61.1 ± 4.8 | ** ** |
| B2 cells         | 61.7 ± 0.8 | 56.4 ± 1.3 | 55.6 ± 5.3 | 22.3 ± 0.9 | ** ** |

Cellular composition of B6.Sle1/lpr and control spleens, LNs, and PerC. Six to eight mice of each strain were examined at 3 mo of age. Shown values represent mean ± SEM. For the spleen and LNs, the shown percentages of CD4⁺, CD8⁺, DN, B1a, and B2 cells represent percentages of all lymphocytes, whereas the percentages of B1a and B2 cells in the PerC represent percentages of all PerC B cells.

¹Student’s t test was performed to compare all three control strains with B6.Sle1/lpr mice. For the comparison of LN weights, Mann-Whitney Rank Sum test was employed, as these data sets failed normality tests. *P < 0.05; **P < 0.01; and ***P < 0.001. NS, not significant (P > 0.05).

²Each FACS® staining experiment was conducted with B6 controls and the experimental strains in parallel. Within each experiment, the level of expression of I-A², B7-1, B7-2, and CD44 on B6 B cells was arbitrarily set at 1,000, 50, 50, and 300 MFI units, respectively, by adjusting the fluorescence channel voltage. This facilitated the comparison of staining experiments done on different days. MFI, mean fluorescence intensity.

³Shown data was obtained from individual inguinal LNs isolated from the different strains.
bears $61.3 \pm 6.3 \times 10^6$ CD4 T cells ($n = 6$, aged 3 mo), compared with age-matched B6 (14.1 $\pm 2.5 \times 10^6$, $P < 10^{-5}$), B6.Sle1 (16.1 $\pm 0.5 \times 10^6$, $P < 0.0002$), and B6.lpr (21.2 $\pm 2.2 \times 10^6$, $P < 0.02$) spleens, as can be deduced from Table I. Since the absolute numbers of CD8 T cells are not significantly changed, B6.Sle1/lpr spleens also exhibit increased CD4:CD8 ratios (mean of six 3-mo-old mice is 4.17), compared with age-matched B6 (mean = 1.5, $n = 8$, $P < 10^{-6}$). B6.Sle1 (mean = 1.4, $n = 6$, $P < 10^{-9}$), B6.lpr (mean = 1.7, $n = 6$, $P < 0.01$), as can be deduced from Table I. In addition, significantly more B6.Sle1/lpr T cells exhibit an activated/memory phenotype, as gauged by the surface levels of CD69, CD25, CD45RB, and CD45RB, compared with all three control strains. Nearly twice as many 3-mo-old B6.Sle1/lpr splenic CD4 T cells bear CD69, compared with the control strains, with a similar trend being noted for CD25. Also, $\sim$ 80% of 3-mo-old B6.Sle1/lpr CD4$^+$ T cells have phenotype as CD62L$^+$ and CD45RB$^+$, and a similar pattern is seen with B6.Sle1/lpr CD8$^+$ T cells (Table I). Thus, compared with the control strains, B6.Sle1/lpr T cells have undergone a massive shift to an activated/memory phenotype.

The FAS$^{lo}$ defect is well known to expand the numbers of DN T cells, which are CD3$^+$ve, but negative for CD4 and CD8. These represent CD8 T cells that have failed to undergo activation induced T cell death, and have subsequently downregulated their surface CD8 (23, 24). As reported by others, B6.lpr spleens bear significantly higher levels of DN T cells (nearly 40% of all T cells), compared with B6 and B6.Sle1 mice (Table I). This unusual subset of T cells is further expanded in B6.Sle1/lpr spleens (54.1 $\pm 7.8 \times 10^6$ cells per spleen, $n = 6$), this being significantly higher than the corresponding numbers in B6 (11.3 $\pm 3.2 \times 10^6$ DN T cells per spleen, $P < 0.0008$) and B6.Sle1 mice (16.3 $\pm 3.5 \times 10^6$ DN T cells per spleen, $P < 0.0008$). Although the numbers of DN T cells in B6.Sle1/lpr spleens are $\sim$ 2–3-fold higher than the corresponding numbers in B6.lpr (17.9 $\pm 6.5 \times 10^6$), these do not reach statistical significance, owing perhaps to the large variations from mouse to mouse, within both strains. Fig. 2 illustrates the expansion of these T cell subsets in B6.Sle1/lpr mice.

B6.Sle1/lpr spleens also show significant changes in the B cell compartment. 3-mo-old B6.Sle1/lpr spleens have an average of $41.4 \pm 7.0 \times 10^6$ B2 cells each. This is not significantly different from the absolute numbers of B2 cells in B6, B6.Sle1, and B6.lpr spleens, as can be deduced from Table I. However, B6.Sle1/lpr spleens exhibit significantly elevated numbers of B1 cells, mostly B1a cells, expressing CD5. Thus, the absolute numbers of B1a cells in 3-mo-old B6.Sle1/lpr spleens (62.8 $\pm 9.8 \times 10^6$) are significantly higher than in the spleens of B6 (5.6 $\pm 1.9 \times 10^6$, $P < 0.0001$), B6.Sle1 (11.1 $\pm 5.1 \times 10^6$, $P < 0.0003$), and B6.lpr (21.1 $\pm 5.1 \times 10^6$, $P < 0.02$). Fig. 2 illustrates the expansion of B1a cells in B6.Sle1/lpr mice. All strains exhibited minimal levels of B1b cells. B6.Sle1/lpr B2 cells also exhibit several features of being already activated in vivo, as demonstrated by their significantly increased size, and heightened (about twofold) surface levels of I-A$^\alpha$, CD80, CD86, and CD44, compared with the control strains (Table I). Within the data sets examined, both male and female mice appear to be affected equally, with respect to all of the T and B cell phenotypes detailed in Table I.

Similar patterns are also seen in the LNs. B6.Sle1/lpr inguinal LNs are $\sim$ 10-fold larger, and fourfold more hypercellular than the control LNs (Table I). In particular, DN T cells comprise nearly half the lymphocytes in B6.Sle1/lpr LNs. Generalized lymphocyte activation, increased CD4:CD8 ratios, and B1a cell expansion are also seen in B6.Sle1/lpr LNs (Table I). In the PerC, the combined action of Sle1 and FAS$^{lo}$ leads to a significant expansion of B1a cells, accounting for nearly two-thirds of all the B cells at this site (Table I). Of note, B1b (B220$^+$, CD23$^+$, CD5$^+$ve) cells are not prominently expanded in any of the secondary lymphoid organs of the study strains. Finally, similar changes are also noted in the peripheral blood of these mice.
As noted for the cellular phenotypes, age accentuates these phenotypes. Thus, 6-mo-old B6.Sle1|lpr spleens are twice as large (546.8 ± 37.7 mg versus 286 ± 32.5 mg, n = 6, P < 0.002), compared with 3-mo-old B6.Sle1|lpr spleens. The activation status of B and T cells also exhibit similar trends of accentuation. However, it should be pointed out that the 6-mo data are likely to be “underestimates,” as >80% of B6.Sle1|lpr mice are already dead by this age (see below).

**B2 Cells, but not B1a Cells, Are the Dominant Producers of IgG ANAs.** Given the prominent changes in the B cell compartment of B6.Sle1|lpr mice (B1a expansion, and activated B2 cells), we next examined if these B cells might be functionally important for disease. Since both the B6.Sle1 and B6.lpr strains are known to have different spectrum of ANAs, we asked if the B6.Sle1|lpr splenic B cells secreted any ANAs. As diagramed in Fig. 3, A and B, 3-mo-old B6.Sle1|lpr splenic B cells secrete more IgG anti-ssDNA (P < 0.0004, P < 0.02, and P < 0.005, respectively), IgG anti-dsDNA (P < 0.008, P < 0.036, and P < 0.008, respectively), IgG anti-histone/DNA (P < 0.032, P < 0.036, and P < 0.032, respectively), and IgG anti-histone ANAs (P < 0.008, P < 0.036, and P < 0.008, respectively), compared with age-matched B6, B6.Sle1, and B6.lpr splenic B cells. Importantly, these differences become very much more pronounced with age. Thus, 6-mo-old B6.Sle1|lpr splenic B cells secrete 40–700-fold higher levels of IgG ANAs, compared with splenic B cells from age-matched control mice (P < 0.001). Given the observation that B6.Sle1|lpr spleens have increased numbers B1a cells, and activated B2, we next investigated which of these two populations were actually producing the ANAs. To determine this, B2 and B1a cells were FACSort®-sorted from 3-mo-old B6.Sle1|lpr spleens, and cultured in vitro for 5 d, with LPS. As depicted in Fig. 3 C, IgG ANAs arise predominantly from splenic B2 cells, rather than B1a cells.

**B6.Sle1|lpr Mice Exhibit a Wide Spectrum of Serum Autoantibodies.** As one might have extrapolated from the above findings, the epistatic interaction of Sle1 with FAS<sup>bw/lpr</sup> also leads to high titres of serum IgM and IgG autoantibodies, as early as 3 mo of age (Fig. 4). This includes anti-nuclear specificities, as well as Abs that acquire affinity for glomerular antigens. Compared with 3-mo-old B6 mice, B6.Sle1 mice exhibit ∼1.5–2-fold higher levels of total IgG (P < 0.009); however, this monogenic strain does not exhibit high levels of ANAs at this early age, but only when much older (7). Compared with 3-mo-old B6 mice, B6.lpr sera exhibit 1–3-fold higher levels of total IgG (P < 0.02), IgG anti-histone (P < 0.001), IgG anti-ssDNA (P < 0.001), and several specificities of IgM ANAs. B6.Sle1|lpr mice, as early as 3 mo of age, exhibit significantly elevated total serum IgM and IgG (P < 0.001), and an impressive array of IgG and IgM ANAs, targeting ssDNA, dsDNA, histone/DNA, and histones. In addition to ANAs, it is clear from Fig. 4, that B6.Sle1|lpr mice also develop significantly (100–1,000-fold) higher levels of IgM and IgG anti-glomerular Abs (P < 0.001, compared with the control strains). As noted for the cellular phenotypes, age accentuates the ANA serotitres, but the observed levels are likely to be “underestimates,” as >80% of B6.Sle1|lpr mice are dead by this age (see below). It is interesting to note that 3-mo-old female B6.Sle1|lpr mice have significantly higher IgG autoantibody levels to dsDNA (P < 0.024), and histone/DNA (P < 0.026), and IgM autoantibodies to dsDNA (P < 0.002), and ssDNA (P < 0.008), compared with their male counterparts (unpublished data). Indeed, this is the only phenotype where we could demonstrate any statistically significant sex differences.

**Sle1 Exerts a Gene-Dosage Effect.** To ascertain the necessity for homozygosity at either of the two loci studied (Sle1, FAS<sup>bw</sup>), mice that are heterozygous at these loci were also examined. As depicted in Fig. 5, B6.Sle1<sup>+/−</sup>|lpr

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**Figure 3.** In vitro IgG ANA production of B6.Sle1|lpr splenic B cells. Total splenocytes from 3-mo-old (A) and 6-mo-old (B) mice were cultured (10<sup>6</sup> cells per well) for 5 d with LPS. For each strain, splenocytes from 3–6 individual mice were cultured. Shown values represent mean (± SEM) levels of ANAs secreted by the 3–6 independent cultures from each strain. B6.Sle1|lpr splenocytes produce significantly more IgG ANAs, compared with the control strains, both at the 3-mo age (P < 0.036), and at the 6-mo age (P < 0.001), as detailed in the text. (C) B220<sup>hi</sup>, CD23<sup>hi</sup>, CD5<sup>ve</sup> (B1a) and B220<sup>hi</sup>, CD23<sup>hi</sup>, CD5<sup>ve</sup> (B2) cells were FACSort® sorted from B6.Sle1|lpr spleens, and cultured (10<sup>6</sup> cells per well), for 5 d, with LPS. The culture supernatants were assayed for IgG anti-dsDNA and anti-histone/DNA levels, by ELISA. Shown ELISA ODs represent three independent FACSort® sorting experiments. Within each experiment, the ANA levels produced by B1a and B2 cells from the same B6.Sle1|lpr spleen are shown connected by lines.
Figure 4. Impact of Sle1/lpr on serum Ig and ANAs. 3-mo-old B6 (n = 14), B6.Sle1 (n = 14), B6.lpr (n = 6), and B6.Sle1/lpr (n = 19) mice were examined for their serum levels of total IgM and IgG, as well as IgM and IgG autoantibodies to ssDNA, dsDNA, histone/DNA, total histones, and renal glomeruli. Sera that exhibited autoantibody levels beyond the linear range of the assay were diluted further, and reassayed. Shown bars depict the mean autoantibody levels in each strain. Depicted below each strain are the P values, compared with the autoantibody levels in B6.Sle1/lpr mice.

These mice have significantly larger spleens (P < 0.025), increased CD69+ CD4 T cells (P < 0.01), and elevated serum IgG anti-ssDNA and anti-histone/DNA ANAs (P < 0.02), compared with B6.lpr mice, as early as 3 mo of age.

However, most of their phenotypes are less dramatic than those seen in B6.Sle1/lpr mice. Thus, the B6.Sle1/lpr spleens weights (P < 0.02), CD4:CD8 ratios (P < 0.004), mean B cell size (P < 0.002), B1a cell expansion (P < 0.025), and IgG anti-dsDNA levels (P < 0.05), are all significantly less than that seen in B6.Sle1/lpr mice (Fig. 5).

Thus, in the absence of FAS function (lpr/lpr genotype), Sle1 appears to exert a gene dosage effect. In contrast, B6.Sle1/lpr mice do not differ significantly from B6.Sle1 mice, phenotypically. Thus, in the context of the Sle1/Sle1 genotype, having a single allele of FAS negated does not significantly accentuate any of the Sle1-triggered phenotypes (Fig. 5).

B6.Sle1/lpr Mice Exhibit Severe Clinical and Histological GN. Since the B6.Sle1/lpr mice exhibit high titres of potentially pathogenic ANAs, it is important to evaluate the extent of renal pathology and disease in this strain. As depicted in Fig. 6 A, B6.Sle1/lpr mice exhibit significantly higher levels of urinary protein, compared with B6 (P < 0.0003), and B6.lpr mice (P < 0.02), as early as 3 mo of age. At this age, B6.Sle1/lpr mice also exhibit significantly higher levels of BUN (Fig. 6 B), compared with B6 (P < 0.001), B6.Sle1 (P < 0.025), and B6.lpr (P < 0.024). Both, male and female B6.Sle1/lpr mice show equally high 24-h urinary protein (P = 0.37) and BUN (P = 0.24). Interestingly, there was also a fairly good correlation between IgG anti-dsDNA levels and 24-h urinary protein (n = 12 mice, aged 6 mo, correlation coefficient = 0.86). As one would predict, B6.Sle1/lpr kidneys demonstrate several features of chronic GN, which become pronounced with age. These include Grade 4 proliferative glomerulopathy (noted in four out of six kidney sections examined) with hyaline mesangial deposits, evidence of interstitial infiltrates, (as illustrated in Fig. 6 C), pertinacious deposits in the tubules, and glomerular Ig deposits (unpublished data). However, no significant vasculitis or tubular changes were seen.

B6.Sle1/lpr Mice Exhibit Accelerated Mortality, most likely from Renal Failure. As one might have predicted from the serology and pathology, B6.Sle1/lpr mice suffer significantly increased mortality, as diagramed in Fig. 7. At 3 mo of age, none of the B6, B6.Sle1, and B6.lpr mice are dead,
whereas \( \sim 14\% \) of male B6.Sle1.lpr, and 28\% of female B6.Sle1.lpr mice are dead by this age. The cumulative 6-mo mortality rate in this strain is 70\% among males, and 90\% among females. Although the females tend to have worse mortality, the observed differences are not statistically different. Although these mice have clear evidence of clinical GN (with raised BUN, and increased proteinuria), we wondered if additional pathology elsewhere might also be contributing to death.

**Discussion**

We have recently reported that the epistatic interaction of the lupus susceptibility loci, Sle1 and Sle3 (on the B6 background), is sufficient to recreate most of the lupus phenotypes seen in lupus-prone NZM2410 mice, from which strain these loci originate (10). Whereas Sle1 appears to be critical in breaching tolerance to chromatin, Sle3 reduces activation induced T cell death, accompanied by increased CD4:CD8 ratios, and low titres of ANAs. Indeed, in several respects, B6.Sle3 mice resemble B6.lpr mice, phenotypically (9). Thus, we reasoned that epistatic interactions of Sle1 with FAS\(^{lp} \) mice might also lead to phenotypes that are similar to those seen in B6.Sle1 SLE mice.

As predicted, the epistatic interaction of Sle1 with FAS\(^{lp} \) precipitates several autoimmune features with a
very early age of onset. The extent of splenomegaly and generalized lymphadenopathy, the degree of lymphocyte activation, the levels of serum ANAs, and the extent of renal disease are far more severe and acute in onset, compared with the B6.Sle1/Sle3 bicongenic strain (10). Indeed, in many respects, these mice resemble PTEN haploinsufficient mice (25–27). The expansion in absolute numbers of activated CD4+ T cells and B cells is remarkable. The increased CD4:CD8 ratios in B6.Sle1/lpr mice are similar to those seen in the NZM2410 lupus prone mice. However, the expansion of DN T cells is not a feature of the NZM2410 strain, or the B6.Sle congenics. It is very likely that the DN T cells have arisen from CD8+ T cells that have failed to undergo apoptosis, as has been demonstrated in B6.lpr mice (23, 24). The expansion of DN T cells in B6.Sle1/lpr mice (relative to the levels seen in B6.lpr mice) suggests that Sle1 itself is facilitating the activation of CD8 (and CD4) T cells, that then resist AICD, hence leading to an accumulation of these cells as DN T cells. These observations strongly suggest that Sle1 is also impacting the T cell compartment, in addition to the previously documented intrinsic impact on B cells (12).

B1a cells are not prominent in B6.Sle1 mice (7). It has also been reported that this population of B cells is not prominent in MRL/lpr mice, as well (28). However, in our colony, B6.lpr mice themselves reveal a respectable expansion of B1a cells, constituting ~37% of all splenic B-cells, as presented in Table I. In contrast, the epistatic interaction of Sle1 and FAS(lpr) leads to a massive expansion of B1a cells, which comprise ~60% of splenic B cells in these mice (Table I). The finding that the CD5 molecule becomes expressed on anti-self B-cells entering an anergic state after chronic antigenic stimulation (29), and the reports that CD5 transmits inhibitory signals in B cells (30), are consistent with the following model. The epistatic interaction of Sle1 with FAS(lpr) is sufficient to impede lymphocyte apoptosis strongly enough, so that activated B2 cells accumulate over time. The ongoing incessant autoimmune signaling then drives more and more of these B cells to express CD5, in an “attempt” to downregulate the autoimmune response. Consistent with this model, and the reports from other lupus strains (31–33), most of the IgG ANAs appear to be produced by the B2 cells, rather than the B1a cells (Fig. 2 C). Finally, it is tempting to draw a parallel between the DN T cells and the B1a cells, in these mice: both are believed to represent lymphocytes that have been chronically activated, but had resisted subsequent purging. It is interesting to note that both these unusual populations of lymphocytes are somewhat elevated in B6.lpr mice, and both become significantly expanded in B6.Sle1/lpr mice. The serological and renal phenotypes in B6.Sle1/lpr mice resemble those seen in B6.Sle1/Sle2/Sle3 (13), NZM2410, and other lupus models, and do not appear to be unique in any fashion. However, it’s worth stressing the point that the epistatic interaction of Sle1 and FAS(lpr) is sufficient to engender nephropathic, anti-dsDNA ANAs, and grade 4 GN with increased proteinuria and BUN, all being phenotypes not prominent in either the B6.Sle1 or B6.lpr strain. Thus, it appears that whereas Sle1 by itself is sufficient to breach the tolerance of anti-histone/DNA B-cells, the combined defects of Sle1 and FAS(lpr) are potent enough.
to rescue even the nephrophilic and dsDNA-reactive B cells from the tolerance checkpoints. These conclusions are presently being verified using Ig Tg models of tolerance. It is also important to note the fairly good correlation between these phenotypes: the correlation coefficient between IgG anti-dsDNA ANAs and 24-h urinary protein in 6-mo-old B6.Sle1/lpr mice was 0.86. The mortality rate in this strain parallels that in the B6.Sle1/Sle2/Sle3 triple congenic mice (13), and is far quicker than in NZM2410 mice. Given the absence of other potential causes of death (based on the pathological survey), and the clear evidence of histological and clinical nephritis, it appears most likely these mice are dying from renal failure.

There are several literature reports of mouse strains with lymphoproliferative autoimmunity on the normal (B6 or B6/129) background with phenotypes close to those seen in B6.Sle1/lpr strain. B6.lpr/B129 mice also develop massive lymphadenopathy with increased numbers of DN T cells (34). However, these mice do not exhibit elevated IgG ANAs, B1 cells, GN, or early mortality. Thus, this strain is clearly different from B6.Sle1/lpr mice, except for the massive lymphadenopathy. Perhaps the closest resemblance to our strain is the B6/129.PTEN+/− strain (25). PTEN encodes a phosphatase that is homozygously mutated in a high percentage of human tumors (35, 36). Although homozygous deficiency leads to embryonic lethality, B6/129.PTEN+/− mice develop severe lymphadenopathy prominently affecting the submandibular, axillary, and inguinal LNs, similar to B6.Sle1/lpr mice. These mice also develop high titres of IgG ANAs, and die of GN with a time course that is similar to B6.Sle1/lpr mice. Importantly, B6/129.PTEN+/− mice also exhibit an expanded B1a cell population (25), akin to that observed in the B6.Sle1/lpr strain. Likewise, mice that hyperexpress PI3 kinase also exhibit similar phenotypes (37). Ongoing studies are aimed at determining how FASp and Sle1 may be differentially impacting specific apoptotic/survival pathways. Collectively, the above reports establish that the infringement of key apoptotic/survival pathways has the potential to trigger lymphoproliferative autoimmunity (38–40).

These studies also have important implications for our understanding of autoimmune lymphoproliferative syndrome (ALPS). Patients with ALPS also develop massive lymphoplasenomegalgy with concomitant autoimmunity. As extensively reviewed (41–44), most of these patients exhibit mutations in FAS/FASL. Nevertheless, family studies clearly indicate that FAS/FASL defects alone may not be sufficient for ALPS to develop – a “second hit” appears to be required (45, 46). Based on the findings in B6.Sle1/lpr mice, we post that Sle1 (and other players in the same pathway) might constitute this “second hit.” It is now clear that Sle1 is not only a murine lupus/ALPS susceptibility locus, the syntenic region on human chromosome 1 also appears to confer lupus susceptibility (47–49). Thus, this locus on human and murine chromosome 1 potentially holds important clues for our understanding of not only lupus, but also ALPS. Decoding Sle1 and defining the molecular pathways impacted by FAS and Sle1 will lay out the molecular blueprints for lymphoproliferative lupus.

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