The combination of two Sle2 lupus-susceptibility loci and Cdkn2c deficiency leads to T-cell-mediated pathology in B6.Faslpr mice

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INTRODUCTION
Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by production of autoantibodies (autoAb) including dsDNA and chromatin, followed by inflammation and damage in multiple organs, such as the kidneys and the skin.1 In spite of striking advances in the last few years, the identification of lupus-susceptibility genes and their causative variants still constitutes a great challenge, although it represents a critical step to fully understand SLE pathogenesis.2 Murine models have led to the discovery of a large number of genomic intervals associated with lupus susceptibility or SLE-related phenotypes.3 Only a few murine candidate genes have been proposed with a reasonable degree of confidence for causative etiology, and they have been implicated in immune tolerance, autoimmune amplification or end-organ damage.3

We have identified by linkage analysis three susceptibility loci, Sle1, Sle2 and Sle3, that collectively represent the genomic variants that are necessary and sufficient for lupus-prone NZM2410 mice to develop clinical disease.4,5 The autoimmune phenotypes resulting from Sle2 expression on a C57BL/6 (B6) background have been largely linked to B cells.6-9 A congenic dissection of the Sle2 locus based on the expansion of the peritoneal B1a cell compartment has identified three sub loci, Sle2a, Sle2b and Sle2c.10 Sle2a and Sle2b, but not Sle2c, mediated autoimmune disease in combination with the Sle1 and Sle3 loci.10 We have further shown that the lack of contribution of Sle2c to autoimmune pathogenesis was due to the presence of a suppressive locus at the telomeric end of Sle2c, termed Sle2c1.11 The centromeric portion of Sle2c, Sle2c1, is the strongest contributor to B1a cell expansion.12 Within the Sle2c1 interval, a polymorphism in the promoter of the Cdkn2c gene results in a fivefold decreased expression of the cyclin-dependent kinase inhibitor P18INK4c (p18).13 By comparing the phenotypes of B6.Sle2c1 to those of B6.p18<sup>−/−</sup> mice, we have shown that p18 deficiency is the principal cause of B1a cell expansion.14 The Sle2c1 phenotypes are however not limited to B1a cells. Recently, we have bred CD95 deficiency (Faslpr allele) onto Sle2c1 to characterize its contribution to SLE pathogenesis using a similar strategy as described for Sle1.15 We found that by 4–6 months of age, Sle2c1.lpr mice showed a significantly enhanced lymphadenopathy as compared with B6.lpr, and developed dermatitis and T-cell-mediated glomerulonephritis (GN), a fully novel phenotype for the Sle2 locus.

The analysis of B6.p18<sup>−/−</sup>.lpr mice revealed that p18 deficiency contributed only partially to the autoimmune pathogenesis in Sle2c1.lpr mice.16 The present study was designed to map which additional loci within Sle2c1 interacted with CD95 deficiency to produce autoimmune pathogenesis. We have produced and fine-mapped five new strains combining CD95 deficiency with Sle2c1 recombinant intervals. A comprehensive phenotypic analysis of these congenic strains indicated that three interacting subloci were responsive for inducing autoimmune pathogenesis in B6.lpr mice. One of these subloci corresponds most likely to p18 deficiency. Another major locus mapping to a 2-Mb region at the telomeric end of Sle2c1 is necessary to both renal and skin pathology. Finally, a third locus centromeric to p18 enhances the severity of lupus nephritis. These results provide new insights into the genetic interactions leading to systemic lupus erythematosus disease presentation, and represent a major step towards the identification of novel susceptibility genes involved in T-cell-mediated organ damage.

RESULTS
Production and fine-mapping of Sle2c1.lpr recombinant strains In order to map the phenotypes of the Sle2c1.lpr strain,12 we have bred the lpr mutation onto three existing Sle2c1 recombinants.10

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Sle2c1.rec1, Sle2c1.rec1a and Sle2c1.rec1b. In addition, we have produced two new recombinants, Sle2c1.rec1c.lpr and Sle2c1.rec1d.lpr, totaling five Sle2c1.lpr substrains (Figure 1). The Sle2c1 interval represents ~13 Mb of the chromosome 4 NZB-derived genome between D4Mit119 and D4Mit11.10 We have fine-mapped the five recombinant intervals using all possible markers that are polymorphic between the NZB and B6 genomes, including microsatellites and single-nucleotide polymorphisms (SNPs), collected from the Mouse Genome Informatics (MGI) and the National Center for Biotechnology Information (NCBI), or identified through our own genomic sequencing (Table 1). Sle2c1.rec1 corresponds to an interval ~10 Mb between the rs28166289 and rs27480282 markers. Sle2c1.rec1b and Sle2c1.rec1c correspond to nonoverlapping intervals at the proximal and distal ends of Sle2c1.rec1, respectively. The relatively longer Sle2c1.rec1a and Sle2c1.rec1d intervals cover the Sle2c1.rec1b and Sle2c1.rec1c regions, respectively, and share an ~5-Mb overlap at the middle of Sle2c1.rec1 that includes the lpr gene. We have therefore produced five overlapping recombinant intervals covering the entire Sle2c1 locus that should allow the genetic analysis of its interaction with CD95 deficiency. The phenotypes of B6/p18−/−lpr, some of which have already been reported,12,14 will also be included in the analysis to map which of the Sle2c1.lpr phenotypes map to the Cdkn2c gene itself.

Production of autoAb and lymphoid expansion

We have previously shown that Sle2c1.lpr but not B6/p18−/−lpr mice produced significantly more anti-dsDNA and anti-chromatin IgG than B6/lpr mice.12,14 The recombinant strains map anti-dsDNA IgG production to the Sle2c1.rec1d interval (Figure 2a). Anti-chromatin IgG was significantly higher in Sle2c1.rec1d/lpr than in B6/lpr mice (Figure 2b). The difference did not reach significance for Sle2c1.rec1d/lpr mice although the trend was in the same direction as for anti-dsDNA. Anti-nuclear IgG production in the rec1a, rec1b and rec1d intervals combined to lpr was similar to B6/lpr.

Lymphoid expansion, measured as spleen and lymph node weights, was significantly higher in Sle2c1lpr and B6/p18−/−lpr than in B6/lpr mice.12,14 Larger spleens and lymph nodes were found in Sle2c1.rec1d/lpr and Sle2c1.rec1d/lpr mice (Figures 2c and d). Similar results were obtained with total spleenocyte and lymph node (LN) cell numbers, and affected both B cells and CD3+ T cells (data not shown). These results showed that the rec1d interval is involved in both anti-nuclear Ab production and lymphoid expansion, while the rec1a interval and p18 deficiency contribute to lymphoid expansion.

Alterations in T-cell subpopulations and activation

Sle2c1 preferentially expanded CD3+ T cells to the expense of B cells, and this phenotype was enhanced in LNs as compared with the spleen.12 Therefore, we examined the relative proportion

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Table 1. Fine mapping to the Sle2c1.rec1 loci

| SNPs and markers | position | rec1 | rec1a | rec1b | rec1c | rec1d |
|------------------|----------|------|-------|-------|-------|-------|
| D4Mit321         | 103575801| B    | B     | B     | B     | B     |
| rs32095460       | 103755131| B    | B     | B     | B     | B     |
| rs28154332       | 103913531| B    | B     | B     | B     | B     |
| rs33205840       | 104257942| B    | B     | B     | B     | B     |
| rs28166289       | 105596268| N    | N     | N     | N     | B     |
| rs3664701        | 105891733| N    | N     | N     | N     | B     |
| rs32327171       | 106685675| N    | N     | N     | N     | B     |
| rs32014382       | 107076284| N    | N     | N     | N     | B     |
| rs28162653       | 107669976| N    | N     | N     | N     | B     |
| rs33011000       | 107720590| N    | N     | N     | N     | B     |
| rs28150412       | 108531685| N    | N     | N     | N     | B     |
| rs22270804       | 108549143| N    | N     | N     | N     | B     |
| rs28132605       | 108551173| N    | N     | N     | N     | B     |
| rs28132547       | 108567192| N    | N     | N     | N     | B     |
| p18              | 109337862| N    | N     | B     | N     | N     |
| promoter         |          |      |       |       |       |       |
| rs13477908       | 109098297| N    | N     | B     | N     | N     |
| rs32194443       | 111068240| N    | N     | B     | N     | N     |
| rs47071801       | 111648032| N    | N     | B     | N     | N     |
| novel3           | 112653568| N    | N     | B     | N     | N     |
| novel5           | 113479088| N    | N     | B     | N     | N     |
| D4Mit278         | 114846185| N    | B     | N     | N     | N     |
| rs27495341       | 115561566| N    | B     | N     | N     | N     |
| rs32080914       | 115575513| N    | B     | N     | N     | N     |
| rs4224727        | 115975049| N    | B     | N     | N     | N     |
| rs27480282       | 116237764| N    | B     | N     | N     | N     |
| rs27513842       | 116476573| B    | B     | B     | B     | B     |
| D4Mit37          | 116887379| B    | B     | B     | B     | B     |

*NCBI m37 release. B = B6 allele. N = NZB allele.

Figure 1. Physical map of the Sle2c1.rec1 locus and its derived subloci. The gray rectangles show the NZB homozygous intervals corresponding to Sle2c1.rec1 (rec1) and its derived recombinants, rec1a, rec1b, rec1c and rec1d, on the B6 genetic background. The white rectangles indicate the areas of recombination between the two genomes. The fine mapping and boundaries of these intervals were determined using a combination of markers that are polymorphic between the NZB and B6 genomes, including microsatellites as well as SNPs obtained from public databases and our own genomic sequencing, such as Novel5. For clarity purposes, only the terminal markers are displayed on the top panel. The entire list of markers used is provided in Table 1. The lower panel reveals the positions of the markers according to Ensembl NCBI m37.
of T and B cells in each recombinant interval in these two tissues. The percentage of T cells showed a significant increase in Sle2c1.rec1a.lpr, Sle2c1.rec1d.lpr and Sle2c1.rec1.lpr spleens and LN as compared with B6.lpr (Figure 3a). In addition, T-cell expansion was greater in the LN than in the spleens of these mice, as illustrated by a 14-fold increase in the average T-cell number in the Sle2c1.rec1d.lpr vs B6.lpr LN as opposed to a 4-fold increase in the spleens from the same strains. As previously reported, the percentage of T cells in B6.p18/C0/C0.lpr was significantly higher than in B6.lpr LN but not spleens. The percentage of T cells in Sle2c1.rec1b.lpr and Sle2c1.rec1c.lpr mice was similar to B6.lpr. The T-cell expansion was mirrored by a corresponding decreased percentage of B cells in all concerned strains, including in the B6.p18^-/-lpr spleens (Figure 3b). These results showed that the preferential T-cell expansion that we have reported in Sle2c1.lpr mice maps to the Sle2c1.rec1a and Sle2c1.rec1d intervals, as well as to p18 deficiency.

T-cell expansion in Sle2c1.lpr mice disproportionally affected CD3^+ CD4^- CD8^- T cells (double negative, DN) and CD3^+ B220^+ T cells, which are greatly expanded by Fas deficiency.16,17

Figure 2. Sle2c1.rec1.lpr anti-nuclear autoantibody production and lymphoid expansion map to Sle1c1.rec1d and Sle1c1.rec1a. Serum levels of anti-dsDNA IgG (a) and anti-chromatin IgG (b). The spleen (c) and pooled lymph nodes (d) weight. Results are expressed as units and are relative to a standard curve derived from a serum pool of aged B6.Sle1.Sle2.Sle3 mice. Statistical analysis was performed using Dunn’s multiple comparison tests with B6.lpr values. ***P < 0.001.

Figure 3. Sle2c1.rec1.lpr T cell expansion maps to two loci. Percentages of CD3^+ T cells (a), CD19^- B cells (b), CD3^- B220^- T cells (c) and DN T cells (d) in the spleens (top row) and LNs (bottom row). Statistical analysis was performed using Dunn’s multiple comparison tests with B6.lpr values. In addition, the comparison between rec1 and rec1d is shown in d; ***P < 0.001.
The percentage of CD3⁺ B220⁺ and DN T cells was significantly increased in Sle2c1.rec1.lpr, Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr as compared with B6.lpr spleens and LN (Figures 3c and d). In addition, the percentage of DN T cells was significantly higher in Sle2c1.rec1lpr than in B6.lpr LN, and there was a trend for the percentage of CD3⁺ B220⁺ cells in the same strain (two-sided t test: *P = 0.023). The percentage of DN T cells was similar in the Sle2c1.rec1.lpr and Sle2c1.rec1a.lpr spleens, but markedly lower than in Sle2c1.rec1d.lpr (Figure 3d). Finally, the percentages of CD3⁺ B220⁺ and DN T cells were similar between B6.p18⁻/⁻.lpr and Sle2c1.rec1a.lpr mice, except for DN T cells, which were lower in B6.p18⁻/⁻.lpr spleens (Figure 3d).

The interaction between Sle2c1 and Fas deficiency had an impact on CD4⁺ T cell functions, with an increased percentage of CD4⁺ CD44⁺ effector T cells (Teff) and a decreased percentage of CD4⁺ FoxP3⁺ regulatory T cells (Treg). Therefore, we evaluated changes in effector T cell subsets from the LN of the five recombinant congenic mice. Sle2c1.rec1.lpr, Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice showed a significantly higher percentage of CD69⁺ activated CD4⁺ T cells than B6.lpr controls (Figure 4a). The percentages of activated CD4⁺ T cells was similar between Sle2c1.rec1a.lpr and Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice, and significantly lower than in Sle2c1.rec1d.lpr mice (Figure 4a). Similarly, the percentage of Teff was significantly increased in Sle2c1.rec1.lpr, Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice when compared with B6.lpr controls (Figure 4b). Interestingly, Sle2c1.rec1a.lpr mice also displayed a significantly higher percentage of Teff than B6.lpr mice, but it was lower than in the three other recombinant strains. In contrast, the percentage of Treg was significantly reduced and to the same extent in Sle2c1.rec1a.lpr, Sle2c1.rec1a.lpr, Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice as compared with B6.lpr control. In addition, we detected a mild increase in the percentage of Tregs in Sle2c1.rec1b.lpr mice as compared with B6.lpr (Figure 4c). We confirmed, as previously reported, that the percentage of Teff was significantly higher in B6.p18⁻/⁻.lpr than in B6.lpr LN, but there was no difference for Tregs. In addition, we now report that p18 deficiency does not affect CD69 expression on T cells (Figure 4a). Overall, these data suggest that the expansion of DN and B220⁺ T cells, as well as that of activated and CD4⁺ Teff cells, mapped to both the Sle2c1.rec1a and Sle2c1.rec1d intervals, but these two intervals were not equivalent. Within Sle2c1.rec1d, Sle2c1.rec1c provided a significant contribution to T-cell activation. In addition, there is some evidence that the phenotypes of the Sle2c1.rec1a interval were not completely accounted for by p18 deficiency, with a potential additional locus within Sle2c1.rec1a.

Renal and skin pathology map to the Sle2c1.rec1d locus

Proteinuria at 4–6 months of age was negligible (<30 mg ml⁻¹) and similar between all congenic lines and B6.lpr controls. Differences were however found in renal pathology scores. The renal pathology of 4–6-month-old B6.lpr mice was limited to a mild mesangial expansion. We found significantly increased GN scores in Sle2c1.rec1a.lpr, Sle2c1.rec1d.lpr and Sle2c1.rec1p.lpr as compared with B6.lpr mice, and each of these congenic Sle2c1 recombinant strain presented a different renal pathology (Figures 5a and b). There was no difference in GN scores between Sle2c1.rec1a.lpr and B6.p18⁻/⁻.lpr mice. Their pathology was characterized by a mesangial expansion with increased cellularity. Sle2c1.rec1d.lpr mice presented a significantly more severe phenotype than Sle2c1.rec1a.lpr mice, and the type of lesions was qualitatively different, with the Sle2c1.rec1d.lpr kidneys being characterized by glomerular cell proliferation, as well as cellular infiltrates of a mixed population of lymphocytes and monocytes. Finally, GN scores were equivalent between Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice, corresponding to class IV lupus nephritis, and there was no significant difference between Sle2c1.rec1a.lpr mice (Figure 5b). However, only the whole Sle2c1.rec1 interval conferred a high penetrance of subendothelial hyaline glomerular deposits (Figure 5b), indicating a predominant role of autoantibodies and immune complexes in these lesions.

We have previously reported that about 20% of Sle2c1.lpr mice develop dermatitis, but no skin lesions were observed in either B6.p18⁻/⁻.lpr or B6.lpr mice.12,14 Dermatitis was observed in some of the recombinant mice, particularly in the Sle2c1.rec1d.lpr strain. The mice typically experienced hair loss, skin ulceration and scab formation on the interscapular and neck regions, and the ears (Figure 6a), as described in B6.Sle2c1.lpr mice.12 The histology assessment of the lesions showed inflammatory cell infiltration, epidermal hyperplasia and ulceration in Sle2c1.rec1d.lpr skins (Figure 6b). B6.lpr as well as Sle2c1.rec1a.lpr and Sle2c1.rec1c.lpr mice never developed dermatitis (Figure 6c and data not shown), and showed a normal skin architecture and morphology (Figure 6b). Among the three other strains, Sle2c1.rec1d.lpr mice showed the highest incidence of dermatitis (39%, 23/59), which was significantly higher than in either Sle2c1.rec1a.lpr (12%, 9/85) or Sle2c1.rec1a.lpr (5%, 2/38) mice (Figure 6c), and was even higher than in Sle2c1.lpr mice (13/56, P = 0.034). The major components of the cellular infiltrates in the skin lesions of Sle2c1.rec1d.lpr mice were CD3⁺ T cells, the majority of which were DN, and Ly6G⁺ neutrophils (Figures 6d and e). In addition, the Sle2c1.rec1d.lpr skin lesions contained smaller but significantly higher amounts of DCS (Figure 6f), CD4⁺ and CD8⁺ T cells as compared with B6.lpr.

These results show that the rec1d interval contains genetic variation supporting the development of T-cell-driven autoimmune pathology in both the skin and kidney. The rec1a interval supports only a mild induction of renal pathology in a manner that is consistent with p18 deficiency being the main contributor to the phenotype. There is evidence, however, of an additional locus in the Sle2c1.rec1a interval that, when co-expressed with the other loci, is required for hyaline deposits in addition to the proliferative GN pathology. This third locus does not seem to be involved in skin pathology, and it may actually be protective.

DISCUSSION

We have previously reported that Sle2 and Sle2c1 expression regulates B-cell differentiation and homeostasis.6,7,10 In addition,
Figure 5. p18 deficiency and the rec1d locus contribute to renal pathology in the Sle2c1.rec1.lpr mice. (a) Representative PAS-stained kidney sections from B6.lpr, Sle2c1.rec1a.lpr, Sle2c1.rec1d.lpr and Sle2c1.rec1.lpr mice. All images in this figure were photographed at the same magnification (×100 oil immersion objective) and maintained through preparation. Therefore, the sizes of the glomeruli are representative and are a marker of inflammation. B6.lpr (score Mm2) shows macula densa at 6 o’clock. There is minimal deviation from normal with a mild increase in the mesangial matrix in a minority of glomeruli. Sle2c1.rec1a.lpr (score Mc4) shows afferent arteriole at 4–5 o’clock, with further increased mesangium and mesangial hypercellularity in a larger percentage of glomeruli. The capillary walls and lumens are generally preserved. Sle2c1.rec1d.lpr (score P global 4) shows stalk region at 4–5 o’clock. There is additional hypercellularity with involvement of the capillary walls and lumens. Small lymphocytes (white arrows) are prominent. Sle1c2.rec1.lpr (score Pq4) shows glomerular vascular pole at 10 o’clock. There are many activated cells with large, open, vesicular nuclei. Capillaries are compromised by cells and contain large subendothelial immune complex hyaline deposits (wire loops, arrow heads). (b) Quantitation of renal pathology scores from at least 15 mice per strain. The pie charts show the distribution (percentages) of renal pathology scores grouped as N (white): negative; M1–2 (blue): mild mesangial expansion; M3–4 (green): severe mesangial expansion; P1–2 (yellow): mild proliferative; P3–4 (red): severe proliferative. The black and white ‘hyaline’ pie chart shows the percentage of mice presenting with heavy glomerular hyaline deposits for each strain. No incidence of this phenotype was observed in B6.lpr, Sle1c1.rec1a.lpr and B6.p18/C0/C0.lpr mice. Statistical comparisons between strains were performed with two-tailed Fisher exact tests comparing grouped mild GN scores (M1–2 + M3–4 + P1–2) with severe GN scores (P3–4). *P < 0.05, ns: not significant.

Figure 6. The rec1d locus contributes to skin pathology. (a) Representative Sle1c1.rec1d.lpr mice showing a typical interscapular dermatitis. (b) Representative H&E-stained interscapular skin sections from an unaffected B6.lpr mouse (top) and from a lesion in a Sle1c1.rec1d.lpr mouse. (c) Frequency of dermatitis B6.lpr: N = 50; Sle1c1.rec1a.lpr: N = 38; B6.p18−/−.lpr: N = 50; Sle1c1.rec1d.lpr: N = 59; and Sle2c1.rec1.lpr: N = 85. Absolute numbers of DN T cells (d), Gr1+ granulocytes (e) and CD11c+ dendritic cells (f) collected from 1 cm2 of interscapular skin from B6.lpr, unaffected (normal) or affected (derm) Sle1c1.rec1d.lpr mice. Statistical comparisons were performed with two-tailed Mann–Whitney tests; **P < 0.01, ***P < 0.001.
Sle2c1 greatly enhances the lymphadenopathy and accelerates autoimmune pathology associated with CD95 deficiency.\textsuperscript{12} We have showed that p18 deficiency, the genetic variation responsible for the altered B-cell homeostasis in Sle2c1, was not sufficient to account for the autoimmune phenotypes of Sle2c1.lpr mice.\textsuperscript{14} The current study used five new recombinant intervals within Sle2c1 to map these phenotypes and established the existence of three nonredundant loci. One of these loci interacting with CD95 deficiency is located in the middle of the interval and is likely the Cdkn2c gene, or a gene closely linked to it. The two other ones, a telomeric interval of ~2 Mb and a larger centromeric interval, each enhance the autoimmune phenotype of the Cdkn2c-linked middle locus.

The middle locus corresponding to the overlapping region between rec1a and rec1d in the middle of the interval exerted the strongest effects on the development of autoimmune pathology, as shown by several phenotypes. First, the Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice showed a remarkable T-cell expansion, reflected by a significant increase in both percentages and absolute numbers in the spleen and LNs as compared with B6.lpr controls. This phenotype suggests that the susceptibility gene in the rec1a/rec1d overlap affects T-cell proliferation. As T cells were also significantly expanded in B6.p18\textsuperscript{–/–}.lpr mice, our results are consistent with the combination of p18 and CD95 deficiencies resulting in the preferential proliferation of T cells. We have also detected a marked decrease in the percentage of B cells in Sle2c1.rec1a.lpr, Sle2c1.rec1d.lpr and Sle2c1.lpr mice. This raises the question why T cells behave differently from B cells in these strains, with the possibility that the increase in absolute B-cell numbers is secondary to the T-cell expansion. Second, the presence of the rec1a/rec1d overlap was required for all phenotypes, except for the expansion of DN T cells and Teffs in LN for which Sle2c1.rec1a expression was sufficient. It is well recognized that T cells have a critical role in the lupus.\textsuperscript{18,19} The massively expanded T cells, specifically CD3\textsuperscript{+}B220\textsuperscript{−}T, DN-T, activated CD4\textsuperscript{+}T and CD4\textsuperscript{−} Teff, as well as a striking reduction in percentage of Treg cells in the Sle2c1.rec1p mice, concomitive with CD95 deficiency in B6.lpr mice. This likely participate in autoimmune pathogenesis, as T cells represent a large fraction of infiltrates in their kidneys,\textsuperscript{12} and skin (this report). Finally, significant enhancement of anti-nuclear autoAb (ANA) and/or deposition of large immune complexes. We have not found any differences in cellular phenotypes between Sle2c1.rec1a.lpr and Sle2c1.rec1d.lpr mice, and it is not clear why the presence of the Sle2c1.rec1b would exacerbate renal pathology but ameliorate skin pathology. A recent study showed that MyD88 signaling in dendritic cells was critical for dermatitis but not nephritis in MRL/lpr mice,\textsuperscript{27} clearly indicating a different mechanism for pathology in these respective target organs. Sle2c1.rec1b is still a relatively large (~6 Mbp) interval with 43 known protein-coding genes, none of which is an obvious candidate for the reported phenotypes.

In summary, we have shown the existence of two loci within Sle2c1 that synergize with p18 deficiency to greatly accelerate T-cell-mediated autoimmune kidney and skin pathology in CD95 deficient mice. These two loci are not redundant and differentially affect skin and renal pathology. The identification of these genes within the intervals that we have defined will advance our understanding of the complex genetic architecture of lupus susceptibility.

\section{Materials and Methods}

\subsection{Mice}

The Sle2c1.lpr and B6.p18\textsuperscript{–/–}.lpr strains have been previously described.\textsuperscript{12,14} The recombinant congenic intervals Sle2c1.rec1a, Sle2c1.rec1a1 and Sle2c1.rec1b, also previously described,\textsuperscript{19} were crossed to homozygosity to B6.Fas\textsuperscript{−/−} (B6.lpr), which were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). In addition, two new recombinant strains, Sle2c1.rec1a1p1 and Sle2c1.rec1d1p1, were produced from Sle2c1.lpr mice by repeated backcrossing with B6.lpr mice and intercrossing to homozygosity. Both males and females were analyzed at 4–6 months of age.

\subsection{Autoantibody Measurements}

Anti-dsDNA and anti-chromatin IgG were measured by ELISA as previously described.\textsuperscript{28} Briefly, mBSA-precoated plates were coated overnight with 50 mg ml\textsuperscript{–1} dsDNA, with the addition of 10 mg ml\textsuperscript{–1} of histone H1, H2A, H2B, H3 and H4 overnight for anti-chromatin IgG measurement. Test sera diluted 1:100 were added to the plates and bound autoantibodies were detected using alkaline phosphate-conjugated goat anti-mouse IgG and PNP substrate. Raw optical densities were converted to units per milliliter, using a standard curve derived from pooled B6.Sle1.Sle2.Sle3 sera, arbitrarily setting the reactivity of a 1:100 dilution of this serum to 100 U ml\textsuperscript{–1}.

\subsection{Flow Cytometry}

Cell subset distribution and activation in the spleen and LN were determined by flow cytometry as previously described.\textsuperscript{12} In brief, single-cell suspensions were prepared by lysing red blood cells with 0.83% NH\textsubscript{4}Cl.
Afterwards, cells were blocked with saturating amounts of anti-CD16/CD32 (2.4G2) and then stained with fluorochrome-conjugated antibodies: CD3e (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD19 (1D3), CD44(17M), CD69(H1.2F3), B220 (RA3-6B2). Intracellular staining for FoxP3 (FJK-16s) was performed after fixation/permeabilization of all antibodies. All antibodies were purchased from BD PharMingen (Franklin Lakes, NJ, USA) or eBioscience (Arlington, VA, USA). At least 30,000 events were acquired per sample using a FACS monday cytometer (BD Biosciences, San Jose, CA, USA).

Kidney and skin pathology

Proteinuria was determined with Albustix strips (Bayer). Kidneys from 4–5-month-old mice were fixed and stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Renal lesions were scored in a blinded manner as previously described. Briefly, glomerular lesions were classified as negative (N), mesangial matrix (PAS +) or mesangial cellularity (Mc) expansion, or proliferative glomerulonephritis (glomerular hypercellularity, P). Extent of involvement was graded on a 1–4 scale in both M and P categories. In addition, the presence of heavy hyaline PAS + deposits was noted.

The incidence of dermatitis was recorded during daily checks. Formalin-fixed skin specimens were embedded in paraffin and sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). Dermatitis incidence was recorded during daily checks.

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 software (La Jolla, CA, USA) with the statistical tests indicated in the text. Non-parametric tests were used when data were not distributed normally. Multiple comparison tests were performed using either Bonferroni or Dunns’ tests. Means, s.e.m. and the levels of statistical significance are shown in the figures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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