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Murine Lupus Susceptibility Locus *Sle1a* Requires the Expression of two Subloci to Induce Inflammatory T Cells

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Abstract

The NZM2410-derived *Sle1a* lupus susceptibility locus induces activated autoreactive $CD4^+$ T cells and reduces the number and function of $Foxp3^+$ regulatory T cells. In this study, we first showed that *Sle1a* contributes to autoimmunity by increasing anti-nuclear antibody production when expressed on either NZB or NZW heterozygous genomes, and by enhancing the chronic graft vs. host disease response indicating an expansion of the autoreactive B cell pool. Screening two non-overlapping recombinants, the *Sle1a.1* and *Sle1a.2* intervals that cover the entire *Sle1a* locus, revealed that both *Sle1a.1* and *Sle1a.2* were necessary for the full *Sle1a* phenotype. *Sle1a.1*, and to a lesser extent *Sle1a.2*, significantly affected $CD4^+$ T cell activation as well as Treg differentiation and function. *Sle1a.2* also increased the production of autoreactive B cells. Since the *Sle1a.1* and *Sle1a.2* intervals contain only one and 15 known genes, respectively, this study considerably reduces the number of candidate genes responsible for the production of autoreactive T cells. These results also demonstrate that the *Sle1* locus is an excellent model for the genetic architecture of lupus, in which a major obligate phenotype results from the co-expression of multiple genetic variants with individual weak effects.

Keywords

Lupus; T cells; Treg; autoantibodies; genetics

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Introduction

Genetic analyses conducted with systemic lupus erythematosus (SLE) patients and murine models have revealed that SLE susceptibility results from a large number of polymorphisms, each of which results in a weak phenotypic effect.1 Among the murine quantitative traits loci (QTLs) associated with SLE,2 *Sle1* on telomeric chromosome 1 provided the strongest linkage to disease in the NZM2410 model.3 This NZW-derived locus overlaps with several other lupus susceptibility QTLs: NZM2328-derived *Fcgnz1*,4 NZB-derived *Nba2*,5 BXSB-derived *Bxs3*,6 129-derived *Sle16*,7 and MRL-derived *Mag*.8 Furthermore, this region is syntenic to 1q23, a region of confirmed linkage with human SLE.9 Overall, this region has the strongest non-MHC concordance among lupus models and humans, and it is very likely that at least some of the genetic polymorphisms responsible for these linkages are shared among them. A congenic analysis has shown that either $Sle1^{NZW}$ or $Sle16^{129}$ expression on a C57BL/6 (B6) background induced the production of anti-nuclear auto-antibodies (ANA), 10,11 and that the *Sle1* phenotype was mediated though intrinsic defects in both B and T cells.12 *Sle1* congenic recombinants have revealed that the production of anti-chromatin antibodies (Abs) was mediated by at least 3 independent loci, *Sle1a*, *Sle1b*, and *Sle1c*.13 *Sle1b* results from polymorphisms in the SLAM family gene cluster.14 Polymorphisms in *Cr2* are associated with some of the *Sle1c* phenotypes.15 Based on this work, an association between *Cr2* polymorphisms and disease has been shown in lupus patients.16,17 *Sle1a*, as well as the centromeric portion of *Sle1c*, are associated with the production of activated autoreactive $CD4^+$ T cells that provided help to B cells to secrete anti-chromatin IgG.18,19 Further analysis showed that *Sle1a* regulates the number and function of regulatory $CD4^+$ T cells (Treg) not only through the Tregs themselves, but also by decreasing the effector T cells (Teff) response to Tregs, and by impairing the ability of dendritic cells to support suppression though the production of IL-6.20,21 Here we report the analysis of congenic recombinants showing that the *Sle1a* $CD4^+$ T cells phenotypes map to two independent loci, *Sle1a.1* and *Sle1a.2*, each contributing to overlapping but distinct autoimmune alterations. The *Sle1a.1* interval contains only one gene, and the *Sle1a.2* interval contains 15 known genes, which puts us considerably closer to the identification of the genetic variants responsible for the associated autoimmune phenotypes.

Results

Sle1a contributes to autoantibody production

Sle1 induces the production of anti-chromatin IgG and triggers autoimmune pathology when expressed on an NZW heterozygous genome.22 *Sle1c* by itself leads to a very modest autoAb production, but significantly increased autoAb production and renal pathology when expressed on a NZB heterozygous genome.23 Using the same strategy for *Sle1a*, we compared (NZB X B6.*Sle1a*)F1 and (NZW X B6.*Sle1a*)F1 to (NZB X B6)F1 and (NZW X B6)F1 mice, respectively, up to 12 months of age. (NZW X B6.*Sle1a*)F1 mice produced significantly more anti-chromatin IgG than (NZW X B6)F1 controls (Fig. 1A), and the amount of anti-dsDNA IgG was significantly greater in both (NZB X B6.*Sle1a*)F1 and (NZW X B6.*Sle1a*)F1 compared to controls (Fig. 1B). Interestingly, (NZB X B6.*Sle1a*)F1 mice produced significantly more anti-dsDNA IgG than the (NZW X B6.*Sle1a*)F1, while the

reverse was observed for anti-chromatin IgG (Fig. 1C and D). This difference could be due to the differential effect of NZW/NZW vs. NZW/NZB *Sle1a* alleles or to differences between the NZB and NZW alleles on the rest of the genome. No significant renal pathology was observed in either (NZB X B6.*Sle1a*)F1 or (NZW X B6.*Sle1a*)F1. Nonetheless, this experiment shows that *Sle1a* expression significantly enhances anti-nuclear autoAb production.

To better define the role of *Sle1a* in autoimmunity, we used the chronic graph versus host disease (cGVHD) model in which I-Aβ-mismatched $CD4^+$ T cells transferred from B6.bm12 mice provide bystander help to resident autoreactive B cells that are otherwise tolerized.24 B6.bm12 splenocytes induced significantly more anti-chromatin and anti-dsDNA IgG, lymphoid expansion, expansion of T1 B cells to the expense of follicular B cells, and T and B cell activation in B6.*Sle1a* than in B6 mice (Fig. 2A–F). C3 and IgG deposition was also significantly increased in B6.*Sle1a* as compared to B6 glomeruli (Fig. 2G and J). These results indicate that *Sle1a* expression expands the pool of autoreactive B cells, which, in this model, require interactions with $CD4^+$ T cells during their development.25 To determine whether *Sle1a* enhanced cGVHD through T or B cells, we induced cGVHD in mixed bone-marrow (BM) chimeras reconstituted with a mixture of BM from two of the B6.*Sle1a.Tcrb*$^{-/-}$.*Tcrd*$^{-/-}$, B6.*Sle1a.Igh6*, B6.*Tcrb*$^{-/-}$.*Tcrd*$^{-/-}$ or B6.*Igh6* strains in such a way that the T and B cell origin was either from *Sle1a* or B6. Activation of either $CD4^+$ T cells or B cells in these chimeras tracked with the *Sle1a* origin of each of these cell types (data not shown). The chimeras in which both T and B cells expressed *Sle1a* produced significantly more anti-dsDNA IgG (Fig. 2H) and showed an increase in spleen weight (data not shown) compared to chimeras in which both T and B cells were of B6 origin. The expression of *Sle1a* in B cells only, but not in T cells, showed a similar trend. A significant difference was obtained when all chimeras were grouped according to the origin of their B cells, either *Sle1a* or B6, but not their T cells (Fig. 2I), suggesting an intrinsic B cell effect of *Sle1a* expression. Taken together, these data showed that *Sle1a* increases the number of autoreactive B cells that respond to alloreactive T cell help, at least partially in an intrinsic B cell manner.

More than one gene contributes to *Sle1a* ANA production

The entire *Sle1a* interval is covered by the combination of the *Sle1a.1* and *Sle1a.2* intervals (Fig. 3). The *Sle1a.1* interval contains only one gene, *Pbx1*, while the *Sle1a.2* interval contains 15 known genes and 3 ESTs or predicted peptides, 7 of them expressed in $CD4^+$ T cells, 9 expressed in B cells, and 11 expressed in myeloid cells (Table 1). The *Sle1a.1* and *Sle1a.2* intervals potentially overlap between rs31413434 and rs30711102 (< 100 Kb) upstream of the *Pbx1* transcriptional start site, in a region that does not contain any gene. The *Sle1a.2* interval extends on the telomeric end beyond the *Sle1a* interval, resulting in the *Fcgr2b* gene having the B6 and NZW alleles in B6.*Sle1a* and B6.*Sle1a.2*, respectively.26

The whole *Sle1a* interval is associated with a low but significant production of anti-chromatin IgG.13 Both 9–12 month old B6.*Sle1a.1* and B6.*Sle1a.2* mice, however, produced anti-chromatin IgG to the same level as aged-matched B6 controls (data not shown). A robust ANA staining was observed in 80% of the 9–12 month old B6.*Sle1a* sera,

while only 25% on the B6.*Sle1a*.2 sera and no B6.*Sle1a.1* sera showed nuclear staining (Fig. 2K). The ANA titers reflected the same pattern with the only significant difference existing between B6.*Sle1a* and B6 (median titer: 160 vs. 40, $p < 0.01$). We observed the same result for spleen weight, which was significantly higher in B6.*Sle1a* than B6 mice, but similar between B6.*Sle1a.1*, B6.*Sle1a.2* and B6 (Table 2). Peripheral differentiation and activation of B cells was affected by *Sle1a* expression, with significant accumulation of T1 transitional B cells and increased expression of CD86 and CD22. Neither the increased T1/Follicular B cell ratio nor increased CD86 expression mapped to either *Sle1a.1* or *Sle1a.2*, while the increased CD22 expression mapped to both *Sle1a.1* and *Sle1a.2* (Table 2). When cGVHD was induced in the *Sle1a* sub-congenic strains, the B6.*Sle1a.1* phenotypes were equivalent to B6, while the B6.*Sle1a.2* phenotypes were similar to that of B6.*Sle1a* (Fig. 2A–F). This result indicates that the amplification of autoreactive B cells revealed in cGVHD maps to *Sle1a.2*. Overall, ANA production and enhanced cGVHD response observed in B6.*Sle1a* mice map to *Sle1a.2*, but the weaker phenotype of B6.*Sle1a.2* mice suggests an indirect contribution of *Sle1a.1*.

Sle1a increased $CD4^+$ T cell proliferation and activation maps to both *Sle1a.1* and *Sle1a.2*

Sle1a expression significantly increases $CD4^+$ T cell proliferation and activation,18 including ICOS expression.20 Expression of either *Sle1a.1* or *Sle1a.2* significantly increased spontaneous *in vivo* proliferation of the $CD4^+$ T cells (Fig. 4A and B). This difference was observed as early as 2–3 month old, which most likely indicates a primary defect. Moreover, no difference was observed for B cell proliferation (data not shown), indicating that it does not correspond to a generalized lymphoproliferation. Increased activation was also detected in both *Sle1a.1* and *Sle1a.2* $CD4^+$ T cells, with increased CD69 expression at either 2–3 month old (Fig. 4C) or 8–12 month old (data not shown), and expanded $CD44^{hi}$ $CD62L^-$ memory over $CD44^{lo}$ $CD62L^+$ naïve $CD4^+$ T cells in the older mice (Fig. 4D). For each of these phenotypes, the results obtained with *Sle1a* were similar to either that of *Sle1a.1* or *Sle1a.2*, indicating that the two sub-loci did not have an additive effect. Increased ICOS expression however only mapped to *Sle1a.1* (Fig. 4E). These results clearly show that both sub-loci contribute to increased $CD4^+$ T cell proliferation and general activation, and that in addition, *Sle1a.1* up-regulates ICOS expression.

Both *Sle1a.1* and *Sle1a.2* expression leads to decreased Treg suppression

Sle1a reduces the number of splenic Tregs,18,20 and we found a comparable decrease induced by either *Sle1a.1* or *Sle1a.2* (Fig. 5A and B). Tregs are $CD4^+$ $CD25^+$ $CD62L^+$, a T cell subset that is significantly decreased in B6.*Sle1a* mice, indicating that *Sle1a* is associated with a higher proportion of recently activated $CD4^+$ $CD25^+$ T cells.20 This phenotype also mapped to both *Sle1a.1* and *Sle1a.2* (Fig. 5C). Overall these results indicate that both *Sle1a.1* and *Sle1a.2* contribute to a reduced Treg compartment.

We have previously reported that *Sle1a* Tregs are no longer suppressive at low Treg:Teff ratios, indicating that the B6.*Sle1a* $CD4^+$ $CD25^+$ population contains a smaller proportion of functional Tregs than B6 mice.20 Here we assessed the suppressive capacity of B6.*Sle1a.1* and B6.*Sle1a.2* Tregs in assays in which the only variable was the Treg origin (B6, B6.*Sle1a.1* or B6.*Sle1a.2*), while all other cells came from B6 mice. As shown in Fig. 5E,

there was only a small reduction of the inhibitory capacity of B6.*Sle1a.1* and B6.*Sle1a.2* Tregs at the 1:16 and 1:4 Treg:Teff ratio. *Sle1a* expression in Teffs and APCs also significantly impacted the ability of Tregs to suppress Teff proliferation at all Treg:Teff ratios tested.20 Therefore, we performed the same suppression assay to test the consequences of *Sle1a.1* or *Sle1a.2* expression in Tregs, Teffs, and APCs (Fig. 5F and G). Expression of either *Sle1a.1* (Fig. 5F) or *Sle1a.2* (Fig. 5G) in Teffs or APCs resulted in a significantly hindered Treg suppression at the lower Treg:Teff ratios. Taken together, expression of both *Sle1a.1* and *Sle1a.2* is necessary for *Sle1a* expression to significantly impact the ability of Tregs to suppress Teff proliferation *in vitro*. Expression of either *Sle1a.1* or *Sle1a.2* alone, however, affects the ability of Teffs to resist Treg suppression and that of APCs to mediate Treg suppression, although to a lower level than that of the entire *Sle1a* interval.

Using the experimental colitis model,27 we have shown that *Sle1a* Tregs did not suppress tissue inflammation induced by either B6 or B6.*Sle1a* Teffs, and that *Sle1a* Teffs were resistant to suppression.20 We repeated this experiment to assess the *in vivo* effect of *Sle1a.1* or *Sle1a.2* expression on Teff and Treg functions, with B6.Ragmice receiving $CD4^+$ $CD25^-$ Teffs from B6 or congenic (B6.*Sle1a*, B6.*Sle1a.1* or B6.*Sle1a.2*) mice in the presence or absence of $CD4^+$ $CD25^+$ Tregs from these mice. As expected, the B6 Teff-induced colitis, defined by a 15% body weight loss and by histology, was completely abrogated by B6 Tregs (Fig. 6A), and the most severe colitis was induced by *Sle1a* Teff alone (Fig. 6B). A spectrum of severity was observed in mice receiving *Sle1a.1* or *Sle1a.2* Teffs and Tregs in combination with B6 T cells. The lesser involved colons showed inflammation limited to the crypts (cryptitis) and lamina propria. Increasing inflammation involved the muscularis mucosa and inner submucosal connective tissue stroma. The most severe inflammation involved the muscularis propria, submucosal stroma and serosa. The capillaries and venules in each layer with inflammation showed margination and congestion with mononuclear cells and swelling. Cryptitis was primarily lymphocytic but higher-grade specimens also had focal neutrophilic crypt abscesses. More epithelioid inflammation was also present in the higher-grade lesions in the lamina propria and deeper layers with nodules and occasional multinucleated epithelioid giant cells (Fig. 6B). Increasing inflammation was associated with increasing wall thickness up to three-fold normal (data not shown). *Sle1a* or *Sle1a.1* Tregs did not suppress B6 Teff-induced colitis as well as B6 Tregs (Fig. 6G). Impaired *Sle1a.2* Tregs were detected clinically with body weight loss (data not shown), but not by histology (Fig. 6G), indicating that this functional impairment was not as severe as for *Sle1a.1*. *Sle1a.1* Teffs were resistant to suppression by B6 Tregs to a similar level as *Sle1a* Teffs (Fig. 6G). In contrast, mice reconstituted with B6 Tregs:*Sle1a.2* Teffs or B6 Tregs:B6 Teffs had equivalent scores, indicating that *Sle1a.2* does not increase Teff inflammatory functions *in vivo*.

In addition to colitis, B6.Rag mice injected with *Sle1a* Teffs develop nephritis,20 which was characterized by up to 30 interstitial inflammatory foci per kidney (Fig. 6D). The foci were oval with histologically normal cortex between foci, and generally specimens with more foci also had larger individual foci. The character of the renal inflammation was remarkably similar to the colon mucosa in three respects: 1. The interstitium was expanded by primarily

lymphocytes and monocytes and edema. This involved both the fibrous interstitial space and the peritubular capillaries (capillaritis). 2. The tubular epithelial cells were infiltrated with lymphocytes (tubulitis) with characteristic halos (Fig. 6F).28 3. The larger foci had epithelioid character with occasional multinucleated epithelioid giant cells (Fig. 6D). Furthermore, colitis severity and the number of renal foci were highly correlated (R = 0.80, p = 0.0002). The B6.Rag kidneys from mice reconstituted with B6 Teff: B6 Treg were similar to that of unmanipulated B6 mice (Fig 6C and E). *Sle1a* Teffs induced a significantly higher number of renal foci as compared to B6 Teffs (Fig. 6D and F), but neither *Sle1a.1* nor *Sle1a.2* Teffs were able to reproduce this phenotype (Fig. 6H). *Sle1a*, and to a lesser extent *Sle1a.1* and *Sle1a.2* Tregs, were defective in preventing renal infiltrates induced by B6 Teffs (Fig. 6I). Finally, renal infiltrates induced by *Sle1a* Teffs were only partially suppressed by B6 Tregs, while those induced by either *Sle1a.1* or *Sle1a.2* Teffs were completely suppressed by B6 Tregs (Fig. 6I). Overall, these *in vivo* results determined that expression of both *Sle1a.1* and *Sle1a.2* is necessary to achieve *Sle1a* Treg and Teff defects, with *Sle1a.1* providing the greater contribution. It also showed that *Sle1a* T cells induce a significant level of renal inflammation, which may play a significant role in the disease process of lupus nephritis.

Sle1a.1 or *Sle1a.2* expression intrinsically affects $CD4^+$ T cell phenotypes

Sle1a expression affects multiple hematopoeitic cell compartments, but it results in intrinsically activated $CD4^+$ T cells that do not require *Sle1a* expression in other cell types. 20 The same mixed BM-chimera approach for *Sle1a.1* and *Sle1a.2* produced similar results (Fig. 7). The increased activation of $CD4^+$ T cells (Fig. 7A) and the decreased percentage of $CD4^+$ $CD25^+$ $CD62L^+$ Tregs (Fig. 7B) were again completely reproduced by *Sle1a.1* or *Sle1a.2* BM-derived cells, and in the mixed chimeras containing both congenic and normal $CD4^+$ T cells, only those T cells expressing *Sle1a.1* or *Sle1a.2* displayed enhanced activation and a decreased proportion of $CD62L^+$ Tregs. Interestingly, the increased level of activation and decreased level of Tregs associated with the *Sle1a* sub-loci were exaggerated when assessed in the lymphopenic environment of the mixed BM-chimera as compared to unmanipulated mice as shown in Fig. 4 and 5. Taken together, we conclude that expression of either *Sle1a.1* or *Sle1a.2* results in T cell-intrinsic phenotypes. The abnormal phenotypes are not transferable to bystander normal T cells, excluding *Sle1a.1* or *Sle1a.2* being mediated through a soluble factor.

Discussion

Genome-wide linkage and association studies have shown that the genetic architecture of lupus is based on a large number of susceptibility genes, each of them with a modest size effect.29 The results reported here showed that lupus susceptibility in the NZM2410 mouse fits this genetic model. *Sle1*, initially identified as the strongest QTL linked to lupus nephritis,3 is necessary for pathogenesis.30 A congenic dissection revealed that *Sle1* primary effect was a loss of tolerance to chromatin, which is considered to be an initiating event in the progression to autoimmune pathogenesis.10,31 Screening of *Sle1* congenic recombinants for the production of anti-chromatin IgG initially identified three independent loci, *Sle1a*, *Sle1b*, and *Sle1c*.13 *Sle1b* corresponds to polymorphisms in four genes of the

SLAM family, *Slamf1*, *Slamf2*, *Slamf5*, and *Slamf6* (Ly108).14 The differential expression of two splice isoforms in the $Ly108^{NZW}$ allele results in the production of autoreactive B cells.32 Further recombinant analyses revealed that *Sle1c* corresponds to at least two loci.19 The present study reveals that the *Sle1a* phenotypes also correspond to genetic variations in more than one gene. Overall, these results showed that *Sle1* results from the combined effects of at least 5 genes.

As predicted by the model, each of the *Sle1* sub-loci presents a weaker phenotype as compared to the entire locus, especially for *Sle1a* and *Sle1c*, for which the penetrance of anti-chromatin IgG was only about 30%.13 An outcross with NZB has however shown that *Sle1c* contributed to autoimmune pathology.23 Here, the same strategy showed that *Sle1a* augments the production of anti-chromatin IgG in (NZW × B6.*Sle1a*)F1 mice and anti-dsDNA IgG in both (NZW × B6.*Sle1a*)F1 and (NZB × B6.*Sle1a*)F1 mice. Furthermore, the cGVHD model showed that *Sle1a* expands the pool of ANA-autoreactive B cells, and this was largely due to intrinsic *Sle1a* expression in B cells. This was matched by a high penetrance of ANA in B6.*Sle1a* mice. These results show that *Sle1a*, which has by itself a small individual autoimmune effect, contributes to the overall lupus phenotype.

We performed a systematic assessment of the *Sle1a* phenotypes in the two *Sle1a.1* and *Sle1a.2* recombinants intervals that cover the entire *Sle1a* locus. The small potential overlap between *Sle1a.1* and *Sle1a.2* is unlikely to play a role since it does not contain any gene, and the message level of *Pbx1*, which is located downstream of the overlap, is similar between the NZW and B6 alleles (Cuda et al., unpublished). The NZW allele of *Fcgr2b* in B6.*Sle1a.2* affects germinal center formation,26 but it is not involved in the phenotypes considered in this study since *Sle1a* expresses $Fcgr2b^{B6}$. The increased cGVHD response, as well as the increased in T1/Follicular B cell ratio mapped to *Sle1a.2* and not *Sle1a.1*. This was not sufficient however to confer a highly penetrant ANA production, suggesting that *Sle1a.2* directly increases the number of autoreactive B cells, but *Sle1a.1* is also required for ANA production.

Mixed BM-chimeras showed that both *Sle1a.1* and *Sle1a.2* increase $CD4^+$ T cells activation and decreased Tregs levels in an intrinsic manner, as we have previously shown for *Sle1a*.18 Both loci also resulted in an increased spontaneous proliferation and activation of the $CD4^+$ T cells. Only *Sle1a.1*, however, increased ICOS expression, which is critical for $CD4^+$ T cells to provide help to B cells, as well as for regulating T cell tolerance.33 A recent study has shown that ICOS controls $CD4^+$ T cell inflammatory functions in the MRL/lpr model of lupus.34 Our results suggest that *Sle1a.1* plays an important role in this process. Both *Sle1a.1* and *Sle1a.2* reduced the number of peripheral Tregs, but contrary to *Sle1a*, the *in vitro* suppressive functions of *Sle1a.1* and *Sle1a.2* Tregs were similar to that of B6 Tregs, except for a small reduction at low Treg:Teff ratios. The experimental colitis model showed that *Sle1a.1*, but not *Sle1a.2*, Tregs have diminished functions as compared to B6 Tregs, and that also *Sle1a.1*, but not *Sle1a.2*, Teffs were resistant to Treg suppression as compared to B6 Teffs. In fact, the effect of *Sle1a.1* seemed greater on Teffs than on Tregs. Overall, both *Sle1a.1* and *Sle1a.2* expression affect Treg numbers and functions and contribute to the overall phenotype that we have reported for *Sle1a*. But if *in vivo* assays are a better

indicators a physiological functions, *Sle1a.1* has a stronger effect on Treg functions and on the ability of Teff to respond to Tregs.

We also showed that renal inflammation paralleled the colon inflammation in the experimental colitis model, strongly suggesting that the immunologic and inflammatory pathway involved in colitis and interstitial nephritis are similar and modified by the inherent differences in organ anatomy and functions. The histopathology and experimental design suggest a Type IV hypersensitivity reaction for those responses. This further supported the contribution of T cell-mediated hypersensitivity response to the pathogenesis of lupus nephritis in some strains of lupus mice35 and some sub-sets of clinical disease.36 Our results show that *Sle1a* $CD4^+$ T cells display an enhanced capacity to induce renal injury, and suggest this might be an additional pathway by which this locus contributes to lupus pathogenesis,

The *Sle1a.1* interval contains only one gene, *Pbx1*, whose expression has been reported in B cells and macrophages,37 but not in normal $CD4^+$ T cells.38 Gene arrays have shown however *Pbx1* expression in murine and human T cells (Table 2). *Sle1a.2* contains 15 known genes (Table 2). We cannot predict at this time whether the *Sle1a.2* B cell and CD 4^+ T cell phenotypes result from polymorphisms in the same gene or two different genes. Among the *Sle1a.2* genes, *Sh2d1b* encodes for Eat-2, a SAP family adaptor of the SLAM genes, whose function is best understood in NK cells,39 but is also expressed in B and T cells, and dendritic cells, in which it recruits Src kinases.40 Given the strong epistasis between *Sle1b*, which corresponds to polymorphisms in SLAM family genes,14 and *Sle1a*, 41 *Sh2d1b* should be considered as a strong candidate for *Sle1a.2*.

In summary, both *Sle1a.1* and *Sle1a.2* are necessary for the full expression of *Sle1a* phenotypes. *Sle1a.1*, and to a lesser extent *Sle1a.2*, significantly affects $CD4^+$ T cell activation as well as Treg differentiation and function. *Sle1a.2* also results in an increased production of autoreactive B cells. We did not observe major epistatic effects between *Sle1a.1* and *Sle1a.2*, as many of their phenotypes were similar between *Sle1a* and at least one of the sub-loci. Interestingly, none of the genome-wide association studies in human lupus has identified so far any evidence of epistasis, but rather these studies have provided evidence that each genetic variant acts independently to increase disease susceptibility.29 Our data suggest that *Sle1a.1* and *Sle1a.2* fits this model.

Materials and Methods

Mice

B6.*Sle1a*$^{NZW/NZW}$ (B6.*Sle1a*) mice were described as B6.*Sle1a*(15–353)13 and B6.*Sle1a.2*$^{NZW/NZW}$ (B6.*Sle1a.2*) mice as B6.*Sle1*(111–148).42 The *Sle1a.1*$^{NZW/NZW}$ (*Sle1a.1*) interval was obtained as a *Sle1a* recombinant (Fig. 3). Genotyping with microsatellite markers was performed as previously described,13 and SNP genotyping was performed by direct sequencing. B6, NZB/BinJ (NZB), NZW/J, B6.129S7-*Rag1*^{tm1Mom}/J (B6.Rag), B6.129P2-*Tcrb*^{tm1Mom}.*Tcrd*^{tm1Mom}/J (B6.*Tcrb*$^{-/-}$.*Tcrd*$^{-/-}$), B6.129S2-*Igh-6*^{tm1Cgn}/J (B6.*Igh6*), B6.C-*H2*^{bm12}/KhEg (B6.bm12) mice were originally obtained from The Jackson Laboratory. Cohorts of female (NZB X B6.*Sle1a*)F1, (NZW X B6.*Sle1a*)F1, (NZB X B6)F1 and (NZW

X B6)F1 hybrids were aged to one year. Female congenic mice and B6 matched controls were used at the age specified for each experiment. All mice were bred and maintained at the University of Florida in specific pathogen-free conditions. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

Antibody measurements

Anti-dsDNA and anti-chromatin ELISAs were carried out as previously described.31 Sera were tested in duplicate at a 1:100 dilution. Relative units were standardized using a positive serum from an NZM2410 mouse, arbitrarily setting the reactivity of a 1:100 dilution of this control serum to 100 units. ANA were measured by applying 1:100 diluted sera to fixed Hep-2 cells (Inova, San Diego, CA) and revealed with anti-mouse IgG-FITC (Invitrogen, Carlsbad, CA).

Flow cytometry

Briefly, cells were first blocked on ice with anti-CD16/CD32 (2.4G2). Cells were then stained with pre-titrated amounts of the following FITC-, PE-, allophycocyanin-, or biotin-conjugated Abs: CD4 (RM4–5), CD69 (H1.2F3), CD25 (7D4), CD62L (MEL-14), ICOS (7E.17G9), B220 (RA3-6B2), CD86 (GL1), IgM (Igh-6b), CD21 (7G6), CD23 (B3B4), CD22.2 (Cy34.1), CD90.1 (Thy1a), CD90.2 (Thy1b), or isotype controls, all from BD Biosciences (San Diego, CA). Biotin-conjugated Abs were revealed by streptavidin-PercP-Cy5a. Foxp3 expression was determined with eBioscience reagents (San Diego, CA). I*n vivo* cell proliferation was measured in mice injected i.p. with 2 mg BrdU 18 h prior to sacrifice, and BrdU incorporation was then measured with the BD Biosciences Flow kit. Cell staining was analyzed using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ). At least 30,000 events were acquired per sample, and dead cells were excluded based on scatter characteristics.

cGVHD induction

cGVHD was induced according to an established protocol.43 Briefly, 50–80 × 10^6 B6.bm12 splenocytes were injected intra-peritoneally into 9 month old B6.*Sle1a*, B6.*Sle1a.1*, B6.*Sle1a.2* or B6 mice (4 – 6 mice per strain). Serum was collected weekly up to 5 weeks after injection, when the mice were sacrificed and flow cytometry was performed on splenocytes. The presence of immune complexes in the kidneys was evaluated on frozen sections stained with FITC-conjugated anti-C3 (Cappel, Durham, NC) and anti-IgG H + L chains (Jackson Immunoresearch, West Grove, PA). Staining intensity was evaluated in a blind fashion on a semi-quantitative 0–3 scale and averaged on at least 10 glomeruli per section. The experiments were performed twice. We also induced cGVHD in lethally irradiated B6 mice reconstituted with mixed BM from B6.*Sle1a.Tcrb*$^{-/-}$.*Tcrd*$^{-/-}$, B6.*Sle1a.Igh6*, B6.*Tcrb*$^{-/-}$.*Tcrd*$^{-/-}$ or B6.*Igh6* (1:1 ratio) in such a way that the T cell and B cell origin was either from *Sle1a* or B6. B6.bm12 splenocytes were injected 2 months after BM reconstitution.

In vitro and in vivo assessment of Teff and Treg functions

$CD4^-$ (used as APC), $CD4^+$ $CD25^-$ Teff, and $CD4^+$ $CD25^+$ Treg splenocytes were purified using the $CD4^+$ $CD25^+$ cell kit (Miltenyi Biotec, Auburn, CA) to > 90% purity. *In vitro* suppression assays were performed as previously described in the presence of 1μg/ml anti-CD3.20 The number of Teffs was kept constant at 5×10^5 cells/well, while the number of Tregs was titrated in 4-fold increments. To assess the suppressive function of T cells *in vivo*, 4×10^5 $CD4^+$ $CD25^-$Teff from 6-mo-old donors were transferred into 2-month-old B6.Rag mice in the presence or absence of 1×10^5 Tregs as previously described.20 The body weight of the injected mice was monitored weekly for up to 8 wks. Mice that lost 15% or more of their body weight were sacrificed. The colon was processed and longitudinal sections were stained with hematoxylin and eosin (H&E) and periodic acid Shiff (PAS) stains. The degree of inflammation was evaluated in two dimensions, one from the mucosa (lamina propria, epithelial crypts and muscularis mucosa) through the submucosa, muscularis propria (externa) and serosa and two, along the longitudinal axis. The colons were assigned a rank score from least to most inflammation (one through n) where n was the total number of samples. Each kidney divided transversly at the midsection was processed and stained as for the colon. Interstitial inflammation was limited to the cortex with minimum extension into the outer stripe of the outer medulla. Inflammation was assessed by counting the number of inflammatory foci per cross-section of kidney. An inflammatory focus was defined as continuous inflammation involving 2 or more contiguous tubules and adjacent structures (glomeruli, vessels) if present.

BM chimeras

Chimeras were prepared as previously described.12 In brief, 6- to 8-week-old lethally irradiated B6 mice were reconstituted with BM from B6.*Thy1a* and B6.*Sle1a.1* or B6.*Sle1a.2* mice at a 1:1 ratio. Flow cytometry was performed 8 weeks after reconstitution to evaluate T cell activation and Treg levels. CD69 levels were measured 12 h after stimulation with anti-CD3 (1 μg/ml) and anti-CD28 (0.5 μg/ml).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 4 Software. Unless indicated, graphs show median values for each group and non-parametric tests and multiple-test corrections were used as appropriate. Comparisons for BM chimeras were made with paired two-tailed Student's *t* tests. Each *in vitro* experiment was performed at least twice with reproducible results.

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Abbreviations in this paper

SLE	Systemic lupus erythematosus

B6	C57BL/6
ANA	anti-nuclear autoantibodies
Ab	antibodies
Treg	regulatory T cell
Teff	effector T cell
cGVHD	chronic graph versus host disease
BM	bone marrow

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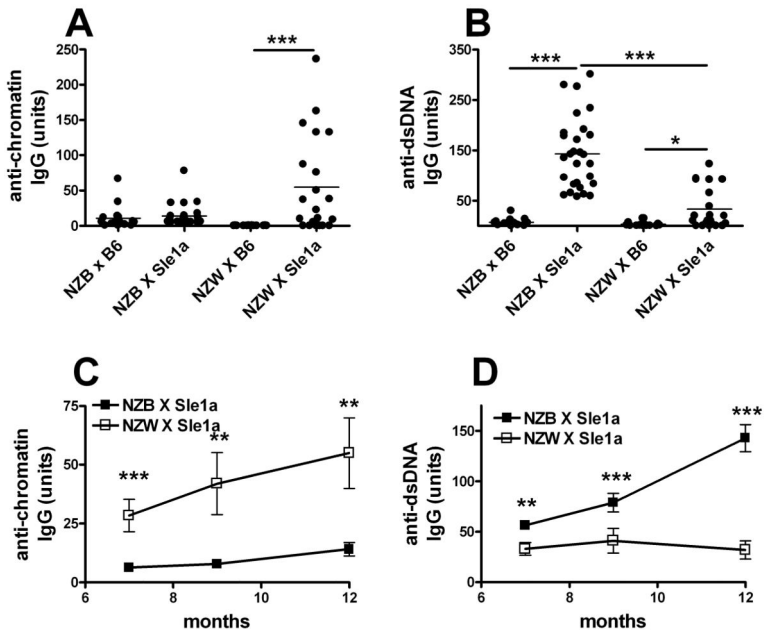

FIGURE 1.

Sle1a expression increased autoAb production on NZB or NZW heterozygous genomes. Anti-chromatin (**A)** and anti-dsDNA (**B)** IgG in 12 month old mice. Time course production of anti-chromatin (**C**) and anti-dsDNA (**D**) IgG in (NZB X B6.*Sle1a*)F1 (black symbols) and (NZW × B6.*Sle1a*)F1 (white symbols). For **E** and **F**, means and standard errors are shown and statistical significance is indicated for *t* tests between the cohorts at each time point. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

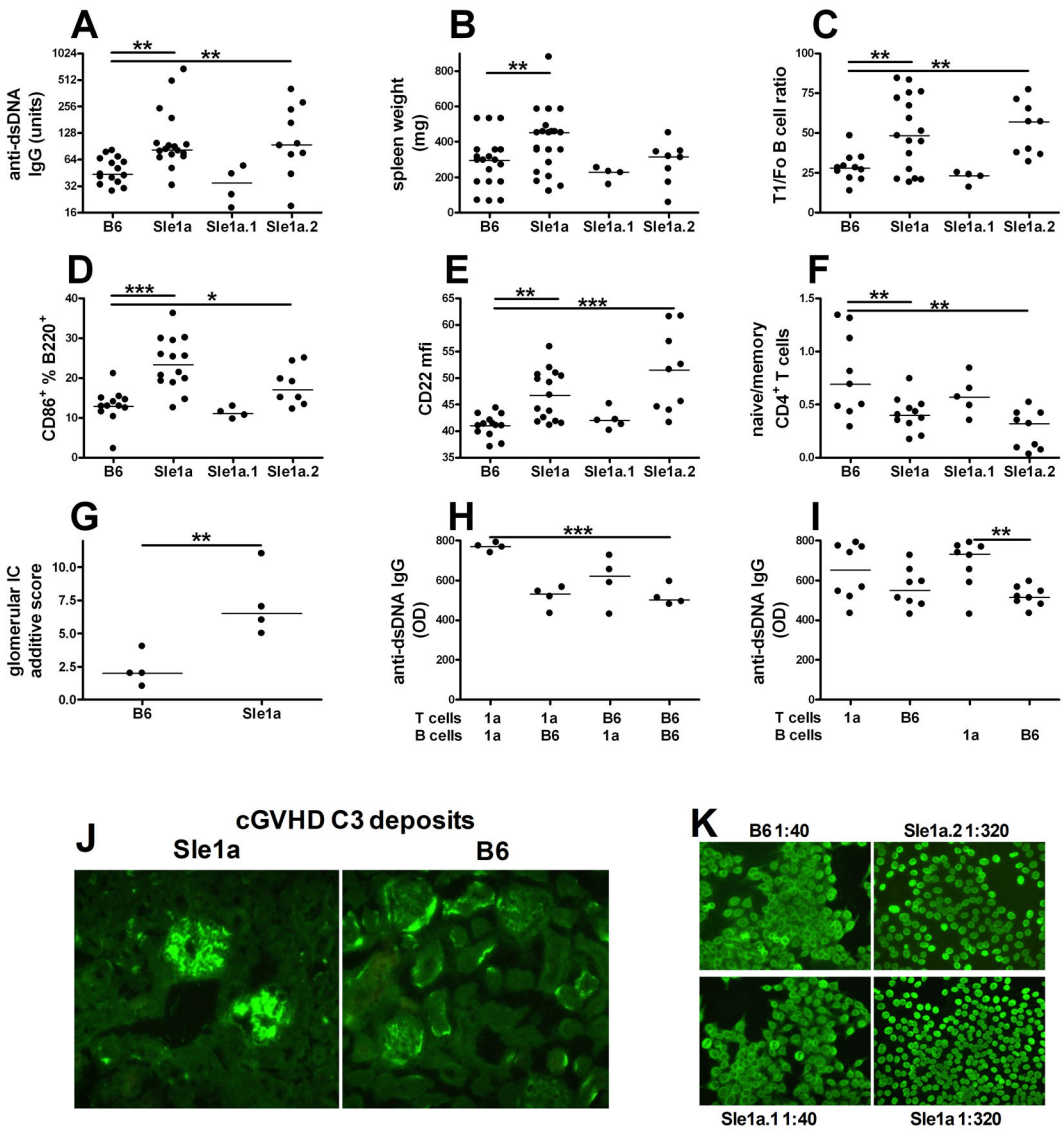

FIGURE 2.

Sle1a or *Sle1a.2* expression increased the cGVHD response. Anti-dsDNA IgG (**A**); spleen weight (**B**); ratio of AA4.1^{+} IgM^{+} CD21^{lo} CD23^{lo} T1 over AA4.1^{-} IgM^{+} CD21^{int} CD23^{+} follicular B cells; percentage of CD86^{+} (**D**) and CD22 expression (**E**) in B220^{+} gated cells; and ratio of CD62L^{+} CD44^{-} naïve over CD62L^{-} CD44^{+} memory CD4^{+} T cells five weeks after cGVHD induction in B6, B6.*Sle1a* and B6.*Sle1a* sub-congenic mice. **G**. Additive semi-quantitative scores of glomerular C3 and IgG deposits in B6.*Sle1a* and B6 mice five weeks after cGVHD induction. **H**. Anti-dsDNA IgG production in BM chimeras expressing *Sle1a* in both B and T cells (1a T + 1a B), in T cells only (1a T + B6 B), in B cells only (B6 T + 1a B), or in neither B nor T cells (B6 T + B6 B) one week after cGVHD induction. **I**. Anti-dsDNA IgG production in BM chimeras grouped according to the *Sle1a* or B6 origin of their T cells (1a T or B6 T), or B cells (1a B or B6 B). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. **J**. Representative C3 immunofluorescence staining in B6.*Sle1a* and B6 mice five weeks after

cGVHD induction (100x). **K**. Representative ANA stain and corresponding titer in 9–12 month old mice.

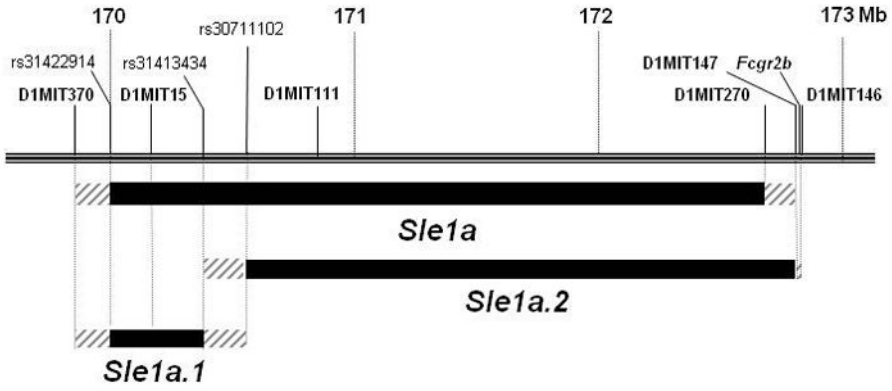

FIGURE 3.

Map of *Sle1a* and its two recombinant intervals. From top to bottom are shown: a scale in Mb, the location of the microsatellite markers or SNPs mapping the interval termini, and the *Sle1a*, *Sle1a.1* and *Sle1a.2* intervals, in which the black rectangles show the regions of known NZW allelic derivation, and the hatched rectangles on each side indicate the regions of recombination between the NZW and B6 genomes.

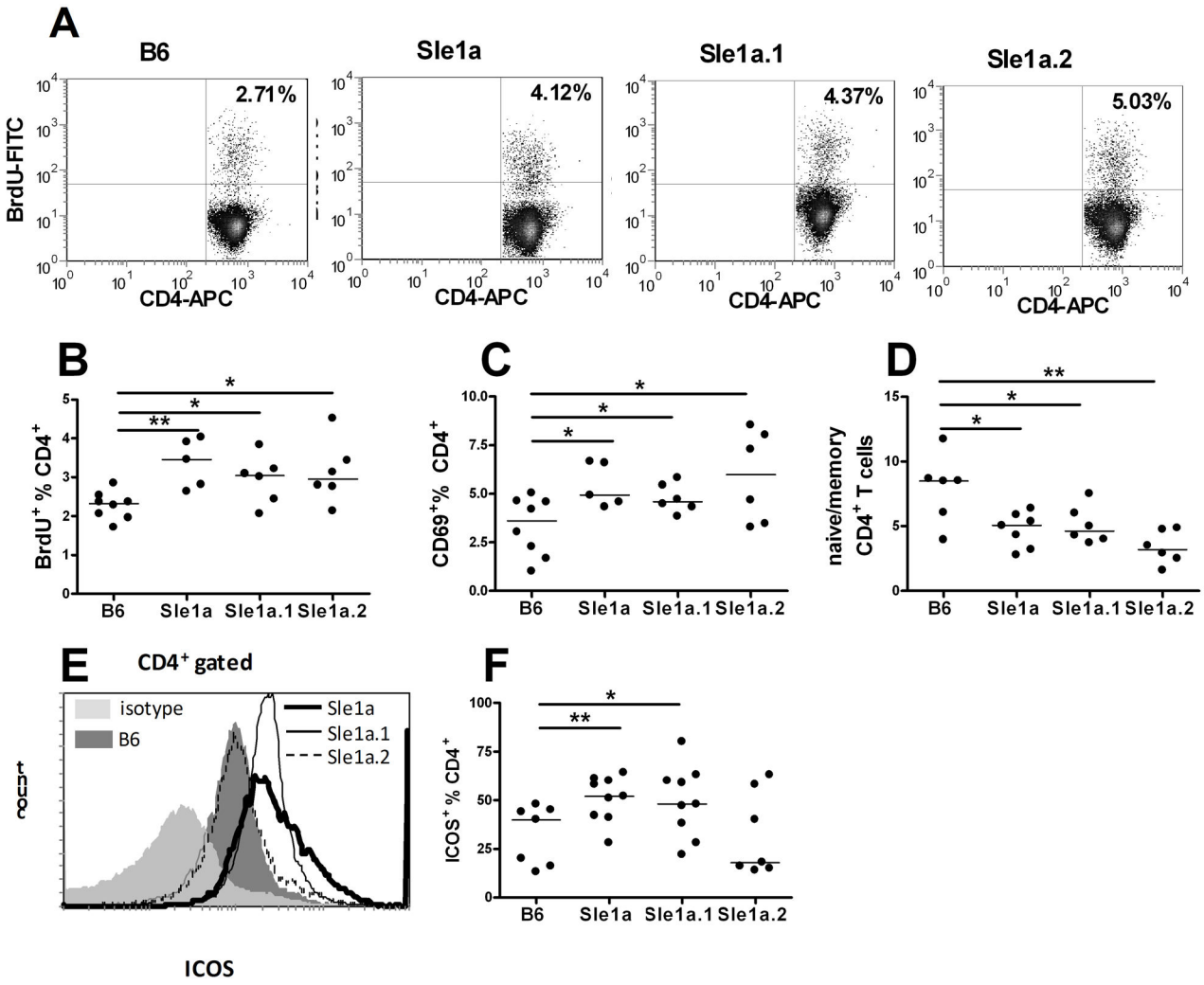

FIGURE 4.

Both *Sle1a.1* and *Sle1a.2* induced a higher spontaneous proliferation and activation in $CD4^+$ T cells, but only *Sle1a.1* was associated with higher ICOS expression. **A**–**B.** *In vivo* BrdU incorporation shown in representative FACS plots and their quantitation. **C**. CD69 expression. **D**. $CD44^{lo}$ $CD62L^+$ naïve/$CD44^{hi}$ $CD62L^-$ memory $CD4^+$ ratio. **E**. ICOS expression. **A**–**C** shows 2–3 month old mice and **D**–**E** 8–12 month-old mice. *: $p < 0.05$; **: $p < 0.01$.

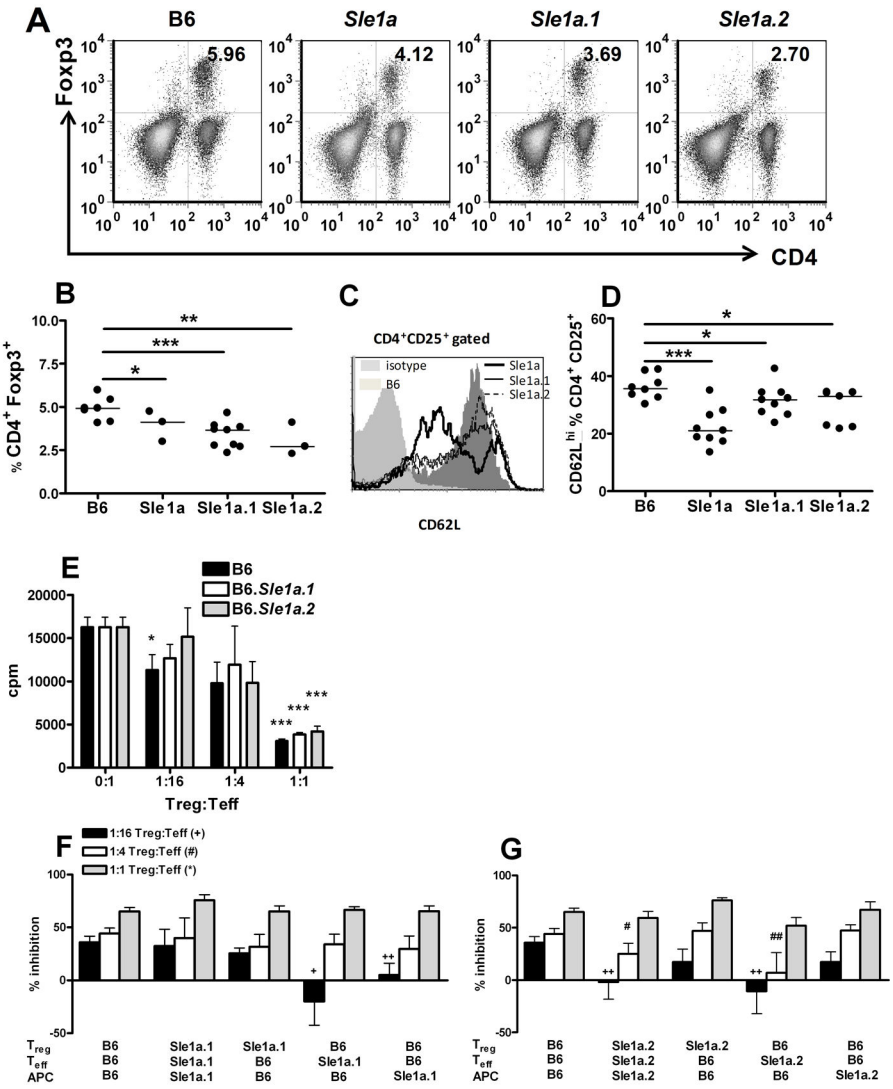

FIGURE 5.

Both *Sle1a.1* and *Sle1a.2* decreased *in vitro* Treg suppression. **A**–**B**. Representative FACS plots and quantitation of $CD4^+$ $Foxp3^+$ splenocytes. **C**–**D**. Representative histograms of CD62L staining in $CD4^+$ $CD25^+$ gated splenocytes and corresponding quantitation. The light gray filled histogram shows the isotype control, the dark gray filled histogram shows B6, while thick, thin and dashed black lines represent B6.*Sle1a*, B6.*Sle1a.1* and B6.*Sle1a.2*, respectively. **E**. B6 (black), *Sle1a.1* (white) and *Sle1a.2* (grey) Treg suppression of B6 Teff proliferation at the indicated ratios in the presence of B6 APCs. For each strain, proliferation in the presence of Treg at various ratios was compared to proliferation in the absence of Tregs. *Sle1a.1* (**F**) or *Sle1a.2* (**G**) expression in Tregs, Teffs, or APCs affects the extent of the inhibition of Teff proliferation. The inhibition of Teff proliferation in presence of 1:1, 1:4, or 1:16 Treg:Teff ratios is expressed as a percentage of the proliferation induced in the absence of Tregs for each condition. Data were collected from 5–7 month old mice. E–G graphs are representative of 2 independent assays each with 3–4 mice per strain. All comparisons were performed with B6 values. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

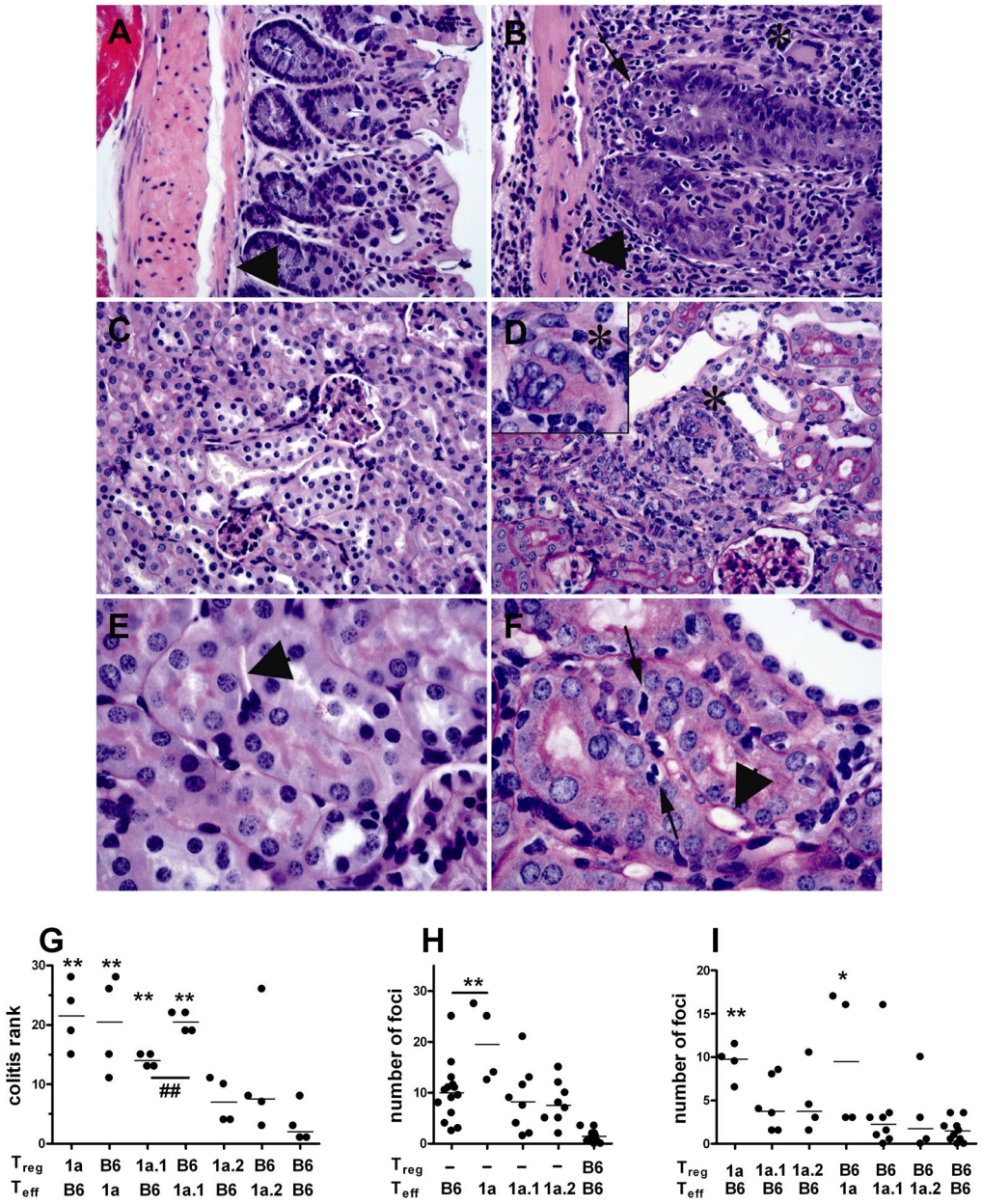

FIGURE 6.

Sle1a Tregs and Teffs induce colon and renal inflammatory infiltrates. **A**. Colon from a B6.Rag mouse reconstituted with B6 Teff: B6 Treg, which is indistinguishable from unmanipulated B6. Lumen is toward the right of figure and the arrow head marks muscularis mucosa. **B**. High grade colitis in a mouse reconstituted with *Sle1a* Teff alone. Arrow head points to muscularis mucosa as in **A**. The wall is markedly expanded with lymphocytic cryptitis (thin arrow), inflammation of the lamina propria with occasional giant cells (upper right, asterisk), inflammation of the muscularis mucosa, and interstitial submucosa on left of picture. **C**. Kidney cortex from a B6.Rag mouse reconstituted with B6 Teff: B6 Treg, which is indistinguishable from unmanipulated B6. **D**. Kidney from a mouse reconstituted with

Sle1a Teff alone, with a moderate size focus of interstitial inflammation (center between glomerulus and vein in upper center). Epithelioid giant cell (asterisk) is shown in set (1000x magnification). **E**. Normal tubules a mouse reconstituted with B6 Teff: B6 Treg, with open round nuclei in epithelial cells and peritubular capillary (arrowhead). **F**. Tubules from same animal as in D, showing tubulitis with lymphocytic infiltration containing dark, dense oval nuclei (arrows and others) and capillaritis in peritubular capillaries congested with lymphocytes (between tubules with arrows). Dilated but otherwise empty capillaries are also present (e.g. arrowhead). **A**–**D**. 400x original magnification, **E**–**F**. 1000x original magnification. **A**–**B**. H&E stain; **C**–**F**. PAS stain. **G**. Histological assessment of colitis ranked among the 6 groups with Teffs:Tregs of mixed origin. **: $p<0.01$ indicate the significance of comparisons with the B6:B6 group in a Dunnett's Multiple Comparison Test. ##: $p < 0.0004$, t test between *Sle1a1* Tregs:B6 Teffs and B6 Tregs:*Sle1a.1* Teffs. **H**. Number of renal foci induced by Teffs as compared to controls B6:B6. The values of Teffs alone are significantly higher than that of B6:B6 for all strains. **: $p<0.01$ indicate the significance of the comparison between B6 and *Sle1a* Teffs. **I**. Number of renal foci induced Teffs:Tregs of mixed origin *: $p<0.05$, **: $p<0.01$ indicate the significance of comparisons with the B6:B6 group in a Dunnett's Multiple Comparison Test.

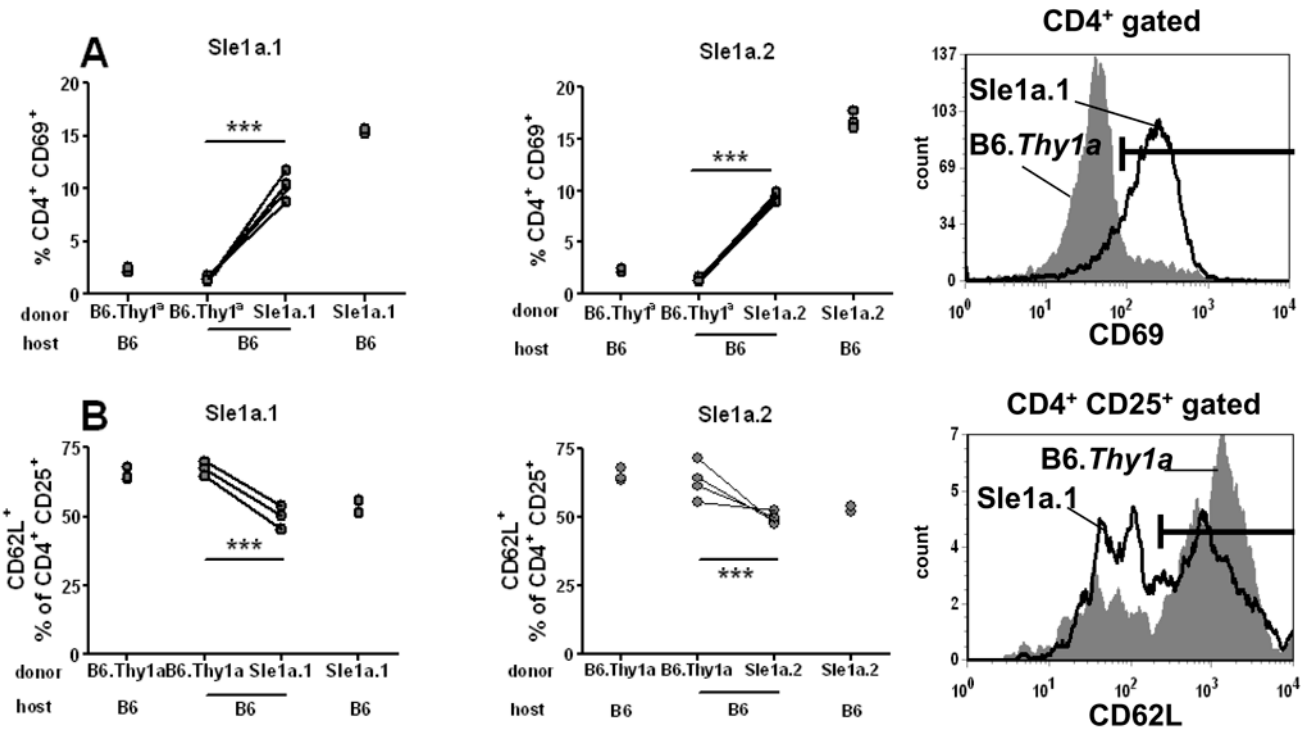

FIGURE 7.

Expression of either *Sle1a.1* or *Sle1a.2* affects $CD4^+$ functions in a cell-intrinsic manner. B6 hosts were reconstituted with B6.*Thy1a* and/or B6.*Sle1a* sub-loci (*Thy1b* allele) BM. Connected symbols indicate values for $CD4^+$ T cells expressing the *Thy1a* (CD90.1-gated) or *Thy1b* (CD90.2-gated) alleles within the same mouse. Controls are represented by B6.*Thy1a* → B6 and B6.*Sle1a.1* or B6.*Sle1a.2*→ B6 single-strain BM transfers. **A**. Percentage of $CD4^+$ $CD69^+$ splenocytes after stimulation with anti-CD3 and anti-CD28 for 12 h. **B**. Percentage of $CD4^+$ $CD25^+$ $CD62L^+$ Treg splenocytes. Representative histograms showing CD69 and CD62L expression for *Sle1a.1* chimeras are shown on the right. Two-tailed paired *t* tests: *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Table 1

Genes in the *Sle1a* interval based on Ensembl Release 40

Symbol	Description	CD4$^+$ T [1]	B cells [1]	CD11b[1]
Pbx1	pre B-cell leukemia transcription factor 1	+	+	+
XP_922544.2	RefSeq peptide predicted			
Cdca1	cell division cycle associated 1	+	+	+
Rsg5	regulator of G-protein signaling 5			
Rsg4	regulator of G-protein signaling 4			
1700084C01Rik	RIKEN cDNA			
Hsd17b7	hydroxysteroid (17-beta) dehydrogenase 7	−	−	+
Ddr2	discoidin domain receptor family, member 2	−	−	−
Uap1	UDP-N-acetylglucosamine pyrophosphorylase 1	+	+	+
Uhmk1	U2AF homology motif (UHM) kinase 1	+	+	+
Sh2d1c	EAT-2b	−	−	−
Sh2d1b	EAT-2a	+	+	+
1700015E13Rik	RIKEN cDNA			
Nos1ap	nitric oxide synthase 1 adaptor protein	+	+	+
Olfml2b	olfactomedin-like 2B	−	−	−
Atf6	activating transcription factor 6	−	+	+
Dusp12	dual specificity phosphatase 12	+	+	+
Fcrlb	Fc receptor-like B	+[2]	+[2]	+[2]
Fcrla	Fc receptor-like A	−	+	+

[1] Expression in CD4$^+$ T cells, B cells, or myeloid cells as reported for the mouse in biogps.gnf.org, expect for

[2] human expression (no data available for mouse)

Table 2

Selected phenotypes of B6.*Sle1a* mice and its sub-.congenics that showed significant differences with B6. Cohorts of 9–12 month old mice, 5–10 per strain.

	B6	**B6.*Sle1a***	**B6.*Sle1a.1***	**B6.*Sle1a.2***
Spleen weight (mg)	78.67 ± 4.92	117.20 ± 7.98**	91.67 ± 5.20	85.00 ± 6.51
T1/Follicular B cells	19.28 ± 0.41	35.04 ± 2.83**	16.68 ± 1.53	30.32 ± 3.40*
$CD86^+$ % $B220^+$	10.41 ± 0.59	13.13 ± 0.70*	11.19 ± 1.45	11.51 ± 0.62
CD22 MFI (B220hi $CD22^+$ gated)	29.07 ± 0.52	33.00 ± 0.67**	33.58 ± 0.96**	34.62 ±0.95**

* Means and SEM, with: $p < 0.05$;

** : $p < 0.01$ indicating the level of significance of t tests with B6 values.