Association of Extensive Polymorphisms in the
SLAM/CD2 Gene Cluster with Murine Lupus

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Summary
Susceptibility to autoimmunity in B6.Sle1b mice is associated with extensive polymorphisms between two divergent haplotypes of the SLAM/CD2 family of genes. The B6.Sle1b-derived SLAM/CD2 family haplotype is found in many other laboratory mouse strains but only causes autoimmunity in the context of the C57Bl/6 (B6) genome. Phenotypic analyses have revealed variations in the structure and expression of several members of the SLAM/CD2 family in T and B lymphocytes from B6.Sle1b mice. T lymphocytes from B6.Sle1b mice have modified signaling responses to stimulation at 4–6 weeks of age. While autoimmunity may be mediated by a combination of genes in the SLAM/CD2 family cluster, the strongest candidate is Lyt108, a specific isoform of which is constitutively upregulated in B6.Sle1b lymphocytes.

Introduction
Genetic predisposition is a central element in susceptibility to many common autoimmune diseases, including systemic lupus erythematosus (SLE) (Wandstrat and Wakeland, 2001; Wakeland et al., 2001; Vyse and Todd, 1996). We have used congenic dissection to characterize the susceptibility genes in the lupus-prone NZM2410 mouse (Morel et al., 1994, 2000; Mohan et al., 1998). These analyses identified Sle1, on murine chromosome 1, as causal for a loss in immune tolerance that leads to antinuclear autoantibody (ANA) production, on the B6 background. Interestingly, Sle1 is syntenic with genomic intervals that are associated with susceptibility to SLE in human linkage studies (Tsao et al., 1997; Moser et al., 1998).

Fine mapping of Sle1 revealed a cluster of four loci (designated Sle1a–Sle1d) within the B6.Sle1 congenic interval, each contributing some of the phenotypes originally associated with “Sle1” (Morel et al., 2001). Sle1b is the most potent member of this cluster, mediating gender-biased and highly penetrant ANA production in the B6.Sle1b congenic strain. Sle1b leads to fatal lupus nephritis when combined with the autoimmune-accelerating mutations lpr or yaa, illustrating the importance of this locus to lupus pathogenesis (Croker et al., 2003). Analyses of Sle1 expression in mixed bone marrow chimeras indicate that genes in the Sle1 cluster are cell intrinsic and mediate activation phenotypes in both B and T cell lineages developed from B6.Sle1-derived bone marrow stem cells (Sobel et al., 1999, 2002). These results suggest that all of the susceptibility alleles in the Sle1 cluster are expressed in lymphocytic lineages, although we have not repeated this experiment with bone marrow that varies at individual Sle1 cluster loci.

Here, we describe the genomic characterization of the Sle1b locus, which identifies a highly polymorphic seven-member cluster of SLAM/CD2 family genes in the middle of the Sle1b critical interval. Analyses of this family reveal extensive sequence and expression-level differences between B6 and B6.Sle1b and associate a subset of SLAM/CD2 family members with autoimmunity. Given the well-established role of this family in the modulation of cellular activation and signaling in the immune system, they are ideal candidates for mediating the Sle1b phenotype (for reviews, see Veillette and Tour, 2003; Veillette, 2004; Sidorenko and Clark, 2003; Tangye et al., 2000; Engel et al., 2003).

Results
Mapping and Sequencing the Sle1b Congenic Interval
The location of Sle1b was determined by analyzing a series of congenic strains carrying truncated intervals, and the final two congenic truncations defining Sle1b are presented in Figure 1A. Fine mapping of the congenic ends of these two strains found that the telomeric end of B6.FcR overlaps with the centromeric end of B6.Sle1b. As shown in Figure 1B, B6.Sle1b spontaneously produces ANA in a highly penetrant, gender-biased manner, while B6.FcR does not. This localizes Sle1b to the genomic segment defined by the telomeric end of B6.FcR and the centromeric end of B6.Sle1b (Figure 1A).

A BAC contig spanning the Sle1b interval was constructed by using a C57Bl/6J-derived BAC library produced by de Jong and coworkers (Osoegawa et al., 2000) and the C3H/129Sv-derived CalTech library (Shizuya et al., 1992) (Supplemental Figure S1). Polymorphic markers derived from this contig positioned the telomeric end of B6.FcR to an ~10 kb interval between ApoA2 and B4galnt3, and that of B6.Sle1b to an ~15 kb interval between Copa and Pxf (Figure 2A). This positions Sle1b within a genomic segment that is spanned by a tiling path of seven C57Bl/6J-derived BACs from the RPCI-23 library (194d6, 48c11, 171k8, 145f9, 77a8, 438k9, and 462j8) (Supplemental Figure S1).

More than 8700 high-quality sequence reads were
assessed for the analysis of the BAC inserts in this tiling path, which, when combined with sequence available in public databases (www.genome.ou.edu), provided between 9- and 19-fold coverage for the entire interval and produced a C57Bl/6J consensus sequence composed of 10 ordered contigs (GenBank accession number AY819658). This sequence was analyzed by using a variety of gene-finding software, including PANORAMA (Pertsemlidis et al., 2000) (http://atlas.swmed.edu). GeneScanII (Burge and Karlin, 1997) (http://genes.mit.edu/GENSCAN.html), and HMMgene (Krogh, 1997) (http://www.cbs.dtu.dk/services/HMMGene). As shown in Figure 2A, the Sle1b region is located between 171.3 Mb and 172.2 Mb along chromosome 1. This is a gene-dense 900 kb genomic segment that contains 2 pseudogenes and 24 expressed genes, 19 of which are expressed in the spleen (bolded in Figure 2A). Most notably, seven members of the SLAM/CD2 gene family (Cd244, Cd229, Cs1, Cd48, Cd150, Ly108, Cd84) are clustered in the center of the Sle1b critical interval. These genes encode cell surface molecules that mediate stimulatory and/or inhibitory signaling during cell-cell interactions between several hematopoietic cell lineages and are the only genes located within the Sle1b critical interval with obvious immunological functions (Engel et al., 2003; Sidorenko and Clark, 2003).

Extensive Polymorphisms Distinguish the SLAM/CD2 Family Genes in B6 and B6.Sle1b

Two features of Sle1b were used to evaluate the candidacy of genes within the critical interval: (1) Sle1b may be expressed in many tissues, but is expressed in the spleen; and (2) Sle1b must be functionally polymorphic between B6 and B6.Sle1b. Quantitative real-time PCR (RT-PCR) and Northern blot analysis determined that 19 genes within the critical interval are expressed in splenocytes, and 16 of these are expressed in B and T cells (Figure 2D). Functional polymorphisms in these 19 candidate genes were identified via structural and regulatory comparisons of the B6 and B6.Sle1b alleles. Structural polymorphisms were found by comparing allelic sequences from B6 and B6.Sle1b for each exon, together with relevant surrounding intronic material and 5′ and 3′ UTRs. Transcriptional variations in these genes were assessed by real-time quantitative RT-PCR analysis in B and T lymphocytes from age- and gender-matched B6 and B6.Sle1b mice.

These analyses revealed extensive polymorphisms between B6 and B6.Sle1b, predominantly occurring in genes of the SLAM/CD2 family. As summarized in Figure 2 and Table 1, five members of the SLAM/CD2 family cluster (Cd244, Cd229, Cs1, Cd48, Cd84) have transcriptional and structural polymorphisms with potential functional consequences. Cd229, Cd84, and Cd48 contain a total of seven nonsynonymous mutations in exons encoding their ligand binding domains. The potential functional consequence of these sequence changes on the structure of the binding domains of these alleles were assessed by aligning their sequences with rat and human CD2, whose molecular structures have been solved (Figures 2B and 2C; Cn3D alignment) (Somoza et al., 1993; Jining et al., 2004). The polymorphism located in Cd84 was found to be just proximal to the location of the A β sheet of the N-terminal V-Ig-like domain (Figure 2B). Of the four polymorphisms in the extracellular region of Cd229 (Figure 2C), two (aa 58 and 70) occur in the C and C′ β sheet of the N-terminal V-Ig-like domain, respectively. Polymorphisms at amino acid position 109 are in the F β sheet of the N-terminal C-Ig-like domain, while position 118 is just proximal to the G β sheet. Two recent studies implicate segments of the binding site in ligand interactions. As shown in Figure 2C, two of the polymorphisms located in the Cd229 molecule (aa 58 and 118) align with interface contact residues in the hCD2-hCD58 structure (Jining et al., 2004). Furthermore, a recent analysis of Cd229 mutants implicated residues located in the C β sheet of
Figure 2. Candidate Gene Analysis of the Sle1b Congenic Interval A

(A) Molecular map of Sle1b. The peak linkage marker D1Mit113 resides within the CD150 gene. The gray bar denotes the area in the Sle1b region by congenic breakpoint mapping. Hatched bars denote the area outside the Sle1b interval. Arrows denote the transcriptional direction of genes. Lines without arrows denote pseudogenes. Spleen-expressed genes are indicated in bold.

(B and C) Sequence alignment of rat and human CD2 with B6 and NZW versions of (B) Cd84 and (C) Ly9 extracellular regions. j strand positions were determined from rat CD2 structure and marked by an overline. Boxed residues denote polymorphisms between the murine strains. Interface contact residues in the hCD2-hCD58 structure; *, residues in the interface.

(D) Gene expression in B and CD4 T cells of candidate genes in 6- to 8-week-old female B6 and B6.Sle1b mice (n = 5–7) by real-time PCR. Data are expressed as relative fold differences compared to B6. The gray hatched box represents a 2-fold difference, which is used as the minimal cutoff for identifying genes that are differentially expressed.

(E) Splenic suspensions were stained with anti-B220, -CD4, -CD8, and -CD48. There were significant differences (p < 0.05) in the Median Fluorescent Intensities (MFIs) of CD48 on B220 B cells, CD4 T cells, and CD8 T cells.

the N-terminal V-Ig-like domain (such as residue 58) in the homophilic binding of this molecule (Romero et al., 2004).

The SLAM/CD2 alleles of B6.Sle1b also differed from those of B6 in gene copy number. Genomic analysis of Cd244, a SLAM/CD2 family gene expressed in NK cells and CD8 T cells (but not B cells), determined that this gene is expanded from a single locus in B6 to a 4-locus cluster (Supplemental Figure S2). Transcripts from three of these Cd244 genes were detected in splenic cDNA preparations, and all three encode binding domains that are highly divergent from that of the single Cd244 molecule expressed in B6. Sequence analysis of B6.Sle1b splenic cDNAs suggests that one of these three Cd244 genes is expressed preferentially, although transcripts from all three genes are detectable in splenocytes. The functional consequences of these extensive variations in Cd244 structure on NK cell function remain to be determined.

Six non-SLAM/CD2 family genes in the Sle1b interval have one or more nonsynonymous mutations (Table 1). Usp23, Nitt1, Refbp2, Ncstn, Copa, and Pxf are ubiquitously expressed genes that have functions in metabolic and cellular processes, with no obvious immunological roles (Sacksteder et al., 2000; Smith and Southan, 2000; Pekarsky et al., 1998; Yu et al., 2000; Quek and Chow, 1997). Nicastrin (Ncstn) is a component of the γ-secretase complex, which functions as a protease to transduce signals of cell surface receptors. Although this complex has been shown to process type I integral membrane proteins, including Cd44 and the Notch ligands Delta and Jagged, the functional implications remain unclear (Kimberly and Wolfe, 2003). Furthermore, the mutations in these genes are not located in highly conserved positions (Table 1) or in motifs impacting protein phosphorylation, acetylation, and/or glycosylation (based on analysis with PROSITE [www.expasy.org/prosite]). All of the synonymous and nonsynonymous
Table 1. Analysis of the Productive Sequence Polymorphisms between the B6 and B6.Sle1b Alleles

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>GenBank Accession Number</th>
<th>Position (Amino Acid)</th>
<th>Amino Acid Changea</th>
<th>Other Speciesb</th>
<th>Other Speciesc</th>
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<tr>
<td>Usp23</td>
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<td>T&gt;I</td>
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<td></td>
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<tr>
<td>Refbp2</td>
<td>RNA and export factor binding protein 2</td>
<td>NP_062357</td>
<td>151</td>
<td>T&gt;A</td>
<td>Human, Rat, Chicken, Drosophila, Arabidopsis</td>
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<td>Cd229</td>
<td>Adhesion/costimulatory molecules that regulate the activation threshold in many immune cell lineages</td>
<td>NP_032560</td>
<td>58</td>
<td>I&gt;T</td>
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<td></td>
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<tr>
<td>Cs1</td>
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<td>M&gt;T</td>
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<td></td>
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<tr>
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<td>P18181</td>
<td>4</td>
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<td>Rat</td>
</tr>
<tr>
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<td>27</td>
<td>V&gt;M</td>
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<tr>
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<td>S&gt;F</td>
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<td>Human, Rat, Hamster</td>
<td>Drosophila, C. elegans</td>
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</tbody>
</table>

a The amino acids that are underlined represent the conserved version.
b The sequence was not available for every species.
c S is conserved in Drosophila.

polymorphisms found in the SLAM/CD2 family and non-SLAM/CD2 family genes are presented in Supplemental Table S1.

Four members of the SLAM/CD2 family, Cd48, Ly108, Cd84, and Cs1, vary in their expression in splenic B and/or T cells between B6 and B6.Sle1b (Figure 2D). Cd84 and Ly108 are upregulated in B cells from B6.Sle1b mice, while Cs1, Cd48, and Ly108 are downregulated in B6.Sle1b T cells. This differential expression was confirmed in B and T cell subsets at the cell surface level for Cd48, the only member for which a commercially available monoclonal antibody that recognizes both the B6 and B6.Sle1b alleles exists (Figure 2E). These variations in expression were detected in young female mice (<12 weeks) well prior to the development of ANA or any other autoimmune phenotypes. In contrast, no variations in expression were found among the non-SLAM/CD2 family positional candidates (Figure 2D).

In summary, genomic analyses detected extensive structural and regulatory polymorphisms distinguishing six of the seven genes in the SLAM/CD2 cluster. Many of these structural mutations impact molecular domains essential for the functions of these molecules. In addition, many of these genes vary between B6 and B6.Sle1b in their lymphocyte expression levels. In contrast, non-SLAM/CD2 family members exhibit much less structural variability and have no detectable changes in expression between B6 and B6.Sle1b.

Polymorphic SLAM/CD2 Family Haplotypes and the Development of Autoimmunity

The Sle1b genomic region was characterized in a panel of inbred laboratory strains to determine whether the highly divergent SLAM/CD2 family gene cluster in B6.Sle1b is a unique feature of the genome of the NZM2410 strain. Sequence analysis of the extracellular Ig regions of the SLAM/CD2 family revealed that, surprisingly, most of these strains carry alleles throughout the Sle1b interval that are indistinguishable from those of B6.Sle1b. The analysis was extended to a set of SNPs in genes flanking the SLAM/CD2 family. As shown in Figure 3A, the SNPs within this genomic segment form two stable haplotypes.
Figure 3. Haplotype Analysis of the Sle1b Interval

(A) The Ig domains of the SLAM/CD2 genes, and a subset of SNPs from flanking genes, were sequenced in 34 inbred laboratory strains. Two stable, divergent haplotypes (Haplotypes 1 and 2) were observed. SNPs that distinguish between the two haplotypes are shown as solid black lines, and the genes falling into the haplotype block are highlighted in gray. **Cd244** shows gene expansion into a four-gene locus in Haplotype 2. SNPs that lie outside the haplotype region and do not follow strain-specific patterns are shown as dotted gray lines. Asterisks represent additional SNPs found in a single strain.

(B) SNPs in the SLAM/CD2 family region define two separate haplotypes among all inbred laboratory strains. This table shows strains that share the B6-like version (SLAM/CD2 Haplotype 1) and the B6.Sle1b-like version (SLAM/CD2 Haplotype 2). Analysis of 14 wild-derived inbred strains identified additional strains carrying either haplotype, or some recombinants with part of Haplotype 1 and part of Haplotype 2.

(C) Penetrance of ANA production in congenics at the Sle1b locus, derived from NZM2410, 129/SvJ, and CAST/Ei. Sera from nine or more 9-month-old female mice were assayed for total histone/dsDNA IgG ANA by ELISA. Sera were assayed at a dilution of 1:800. For interplate comparisons, serial dilutions of a chromatin-specific hybridoma supernatant were included to construct a standard curve. Sera were considered positive if they scored higher than the mean value + 4 SD of 11- to 12-month-old B6 females.

(D) Allelic comparisons between the autoimmune-prone B6.Sle1b, B6.129c1, and B6.CASTc1 congenic mice and B6 were performed for all the productive polymorphisms previously found in the 129-derived SLAM/CD2 haplotype 2 on the B6 background have highly penetrant ANA production region and the subset that define the SLAM/CD2 family haplotype, along with the inbred laboratory strains used, can be found in Supplemental Table S2. The autoimmune-associated haplotype of B6.Sle1b, termed SLAM/CD2 haplotype 2, is the most common version in laboratory strains, being detected in every autoimmune-prone strain analyzed as well as many nonautoimmune strains such as 129/SvJ (Figure 3B). The prevalence of SLAM/CD2 haplotype 2 among nonautoimmune strains indicated that either this haplotype interacts with an additional gene(s) from the C57BL/6J genome to breach tolerance, or that an unidentified mutation unique to the SLAM/CD2 haplotype 2 in B6.Sle1b is responsible for the development of autoimmunity. To address this issue, we utilized marker-assisted selection to introgress a minimal congenic interval containing the SLAM/CD2 haplotype 2 from the nonautoimmune 129/SvJ strain onto the C57BL/6J background (Wakeland et al., 1997). As shown in Figure 3C, although age-matched 129/SvJ mice are ANA negative, B6.129c1 mice homozygous for the 129-derived SLAM/CD2 haplotype 2 on the B6 background have highly penetrant ANA production and are phenotypically indistinguishable from B6.Sle1b. Interestingly, Bygrave et al. recently reported a similar ANA phenotype in a B6.129c1 congenic strain encompassing the Sle1b interval (Bygrave et al., 2004). These findings suggest that the SLAM/CD2 haplotype 2 of 129/SvJ and B6.Sle1b are equivalent in their capacity to mediate autoimmunity and that one or more additional allelic variations in the B6 genome interact with this haplotype to cause autoimmunity. This result illustrates the importance of epistatic interactions with the B6 genome in the autoimmune phenotypes associated with Sle1 (Morel et al., 1999; Wakeland et al., 2001). Although the Sle1b interval occurs as two stable haplotypes among the standard inbred strains, a detailed analysis of wild mice revealed extensive additional di-
versity and recombination throughout the entire region (N.L., K. Belobrajdic, A.Y.C., A.E.W., F. Bonhomme, S. Edwards, and E.K.W., unpublished data). Fourteen fully inbred strains, derived from wild mouse stocks of different Mus subspecies, were also analyzed for the SLAM/CD2 family haplotype. Several of these strains carry recombinant versions of this region that share some alleles with B6, some with B6.Sle1b, and some unique alleles (Figure 3B, Supplemental Figure S3). These strains may prove to be invaluable in further mapping the gene(s) mediating autoimmunity in this region. Most notably, we find that the CAST/Ei strain, which is derived from wild Mus m. castaneus stocks, carries one such recombinant version of the region. As shown in Figure 3C, B6.Castc1 congenic mice, which carry a CAST/Ei-derived Sle1b interval introgressed onto the C57Bl/6 background (Wakeland et al., 1997), also develop highly penetrant ANA and are phenotypically indistinguishable from B6.

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Genetic Variations in Expression of SLAM/CD2 Family Members between Nonautoimmune B6 and Autoimmune-Prone Congenic Strains

Previous studies have reported several splice-generated isoforms of members of the SLAM/CD2 family gene (Wang et al., 2001; Peck and Ruley, 2000; Palou et al., 2000; Stepp et al., 1999; de la Fuente et al., 1999). Polymorphisms in splice isoform expression are likely to have functional consequences and have been implicated as candidates for other autoimmune susceptibility loci (Blomhoff et al., 2004; Ueda et al., 2003; Muschen et al., 1999). Comparisons of SLAM/CD2 family isoform levels in B6 and B6.Sle1b revealed significant differences in isoform usage for both Cd229 and Ly108. As illustrated in Figure 4A, Ly108 produces two splice isoforms (here termed Ly108-1 and Ly108-2) that differ by expressing alternative exons that encode divergent cytoplasmic signaling domains (Peck and Ruley, 2000).

Interestingly, Ly108-1 contains two TxYxxV/I/A ITSM signaling motifs, while Ly108-2 contains three. As shown by Northern analysis in Figure 4B, B6 splenocytes preferentially express isoform Ly108-2, while B6.Sle1b splenocytes preferentially express isoform Ly108-1. An additional high-molecular weight band was also observed in B6.Sle1b, which is thought to be a partially spliced variant (Peck and Ruley, 2000). Hybridizations with isoform-specific probes suggest that it is related to Ly108-1, and not Ly108-2 (data not shown). Real-time quantitative RT-PCR of the Ly108-1 and Ly108-2 isoforms in B and CD4 T cells from B6 and B6.Sle1b confirms that these isoforms are divergently expressed between B6 and B6.Sle1b (Figure 4C, seven mice/group). This variation in isoform expression results in a ∼5-fold increase in the ratio of Ly108-1 to Ly108-2 in B6.Sle1b B and T cells. Analyses of RNA from B6.Castc1, NZW, and a panel of inbred strains confirm that preferential expression of the Ly108-1 isoform is characteristic of SLAM/CD2 haplotype 2 (Figure 4B and Supplemental Figure S4). In addition to differential isoform expression of Ly108, an additional splice variation in Cd229 was also observed in B6.Sle1b and NZW splenocytes, although this isoform was absent from both B6 and B6.Castc1 (Figure 4D).

Autoimmune-Associated SLAM/CD2 Family Alleles Modify CD4 T Cell Activation

The role of SLAM/CD2 family genes in modulating signaling during cellular interactions suggests that these polymorphisms might mediate autoimmunity via modulating cellular responses to activation. Consequently, we assessed the properties of T, B, and monocyte populations in young B6.Sle1b mice extensively, with the intention of identifying intrinsic functional changes correlated with a predisposition to autoimmunity. At 4–6 weeks of age, B6.Sle1b mice do not have detectable autoimmunity, and their lymphocytic cellular profiles are indistinguishable from normal B6 (Mohan et al., 1998) (Supplemental Table S4); although, as described above, they do vary from B6 in the transcriptional expression of specific SLAM/CD2 family genes. As shown in Figure 5, comparisons of B6 versus B6.Sle1b CD4 T cells reveal a significant change in both the peak value and persistence of Ca2+ flux levels produced by stimulation through the TCR complex with a cocktail of monoclonal Abs. These results are consistent with an intrinsic increase in the activation of B6.Sle1b-derived CD4 T cells by TCR stimulation. Similar results were obtained with both B6.Castc1-derived and B6.129c1-derived CD4 T cells, indicating that this activation phenotype correlates with both predisposition to autoimmunity and the presence of the autoimmune-prone SLAM/CD2 family haplotype. A similar analysis of B cells from these strains failed to reveal any measurable differences in Ca2+ flux levels in young mice (data not shown).

Discussion

Genomic analysis of the critical interval for Sle1b has identified members of the SLAM/CD2 family gene cluster as the strongest candidate genes for the potent autoimmune phenotypes associated with Sle1b. The gene cluster is highly polymorphic, and autoimmunity is elicited in three independently derived congenic strains carrying identical alleles for the telomeric portion of the SLAM/CD2 gene cluster. This suggests that the SLAM/CD2 haplotype 2-derived alleles of Cd48, Cd150, Cd84, and Ly108 are the strongest susceptibility gene candidates. Although Ly108 exhibits an intriguing preferential isoform expression, making it the strongest single gene
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Figure 4. Differential Isoform Expression Associated with the Two SLAM/CD2 Haplotypes

(A) Ly108 has two alternative splice forms that differ in the 3' end (Peck and Ruley, 2000). Ly108-1 is composed of exons 1–8, whereas Ly108-2 shares exons 1–7 and splices to exon 9.

(B) Northern blotting of splenic total RNA reveals differences in isoform expression between the two SLAM/CD2 haplotypes. Ly108-1 is upregulated, while Ly108-2 is downregulated in B6.Sle1b, NZW, and B6.Castc1, compared to B6.

(C) Isoform expression of Ly108 in B6 and B6.Sle1b (n = 7) was confirmed by real-time RT-PCR in B and CD4 T cells by using isoform-specific primers.

(D) Cd229 exhibits a difference in expression between B6 and B6.Sle1b via Northern blotting. B6.Sle1b and NZW show an additional possible isoform, while the autoimmune B6.Castc1 resembles B6.

candidate, it is equally feasible that more than one of these alleles participates in susceptibility to autoimmunity. In such a scenario, autoimmunity would be caused by the combined impact of two or more divergent alleles in the SLAM/CD2 family, possibly by influencing the functional properties of multiple immune cell lineages. The association of autoimmunity with a polymorphic haplotype, rather than a single allele, is reminiscent of the long-established association of polymorphisms in the major histocompatibility complex with autoimmunity (Vyse and Todd, 1996), as well as the recently reported association of Crohn’s disease with polymorphisms in the human cytokine gene cluster (Rioux et al., 2001). SLAM/CD2 family molecules have been shown to transmit both stimulatory and inhibitory signals during cell-cell interactions between T cell, B cell, monocyte, and NK cell lineages (Tangye et al., 2000; Sidorenko and Clark, 2003; Engel et al., 2003). Each SLAM/CD2 family molecule is expressed in a specific set of immune cell lineages, and their expression is upregulated by a variety of stimuli, including activation by antigen receptor systems, Toll receptors, and cytokines. Their costimulatory functions are activated via their adhesion during cognate antigen recognition between effector cells and APCs or targets. Phosphorylation of the ITSM motifs in their cytoplasmic domains activates their capacity to modulate a variety of immune functions, depending on the availability of downstream molecules in their signaling pathways (for reviews, see Engel et al., 2003; Veillette and Latour, 2003; Veillette, 2004). Due to the complexities of these signaling processes, the functional consequences of stimulation through these molecules are dependent upon a variety of factors, including their expression levels, the functional avidity of their interactions, and the milieu of downstream signal transduction molecules expressed within the interacting cells. A variety of studies have demonstrated that their activation can modulate numerous immune functions, including T cell activation, cytokine secretion, and cytotoxicity (Howie et al., 2002; Shlapatska et al., 2001).

Our analysis of Sle1b has revealed that the SLAM/CD2 gene cluster is extremely diversified in wild mice and that two haplotypes are prevalent among standard laboratory mouse strains. SLAM/CD2 haplotypes 1 and 2 encode molecules that differ by multiple structural polymorphisms in regions critical to ligand binding, in expression levels on B and T lymphocytes, and in their preferential expression of specific isoforms. Such changes would be predicted to modulate their signaling properties during cellular interactions in the immune system and significantly impact immune activation. The preferential expression of a specific isoform of Ly108 in SLAM/
Figure 5. B6.Sle1b T Cells Show a Difference in Calcium Flux Compared to B6

(A) T cell-enriched splenocytes from B6, B6.Sle1b, B6.129c1, and B6.Castc1 were labeled with Fluo-3 and stimulated with anti-TCR, anti-CD4, and anti-CD8. The representative raw dot plots depict Fluo-3 intensity as a function of time, and the arrows indicate when each reagent was added.

(B) Calcium concentrations were plotted as a function of time. B6.Sle1b, B6.129c1, and B6.Castc1 (four or more mice/strain) exhibit an exaggerated response after the initial calcium spike. A paired Student’s t test reveals a p value of 0.014 between B6 and B6.Sle1b (n = 4).

CD2 haplotype 2 is especially intriguing in this regard, since splice variants of Cd244 have been shown to differentially mediate the molecule’s inhibitory and stimulatory functions (Schatzle et al., 1999). Thus, the variation in Ly108 splice variants that distinguish SLAM/CD2 haplotypes 1 and 2 may reflect a significant change in the function of this molecule. Similar splice variations have been implicated in allelic variations associated with other autoimmune susceptibility genes (Blomhoff et al., 2004; Ueda et al., 2003; Muschen et al., 1999).

The extensive diversity detected in immunoregulatory gene clusters such as the SLAM/CD2 gene cluster may be a consequence of pathogen-driven selection, which is anticipated to favor the maintenance of polymorphisms that functionally diversify immune responses against microbial pathogens (Slev and Potts, 2002). Several studies associate polymorphisms in the SLAM/CD2 family with resistance to viral diseases, including a prominent role in the regulation of EBV infections (reviewed in Sidorenko and Clark, 2003). This connection is intriguing with respect to the candidacy of SLAM/CD2 family polymorphisms as susceptibility alleles in human SLE. The human SLAM/CD2 family cluster is located in 1q23, a region in the human genome that has been associated with SLE susceptibility in multiple human linkage studies (Tsao et al., 1997; Shai et al., 1999; Moser et al., 1998). Interestingly, EBV infection has been implicated as an environmental trigger for SLE, raising the possibility that SLAM/CD2 cluster-mediated variations in the immune response to EBV infection may potentiate the development of autoimmunity (James et al., 2001; Moon et al., 2004; Kang et al., 2004). The link between autoimmunity and the SLAM/CD2 gene cluster is strengthened by evidence that mice deficient in the adaptor molecule SH2D1A or SLAM-associated protein (SAP), which directly binds to various members of this family of receptors, are protected from the experimental pristane-induced model of lupus (Hron et al., 2004). Given our association of variations in the SLAM/CD2 cluster with lupus susceptibility in mice, further work on the relationship of polymorphisms in the SLAM/CD2 cluster with susceptibility to SLE in humans is clearly warranted.

Our analyses demonstrate that SLAM/CD2 haplotype 2 alleles cause autoimmunity when they are expressed in the B6 strain, but not in several other laboratory strains, including 129/SvJ and NZW. As illustrated in Figure 6, these results suggest that the combination of the signaling properties of SLAM/CD2 haplotype 2 alleles with the downstream signal transduction pathways expressed in the B6 genome results in spontaneous autoimmunity. However, a variety of studies have demonstrated that
We propose that the autoimmunity mediated by Sle1b is a consequence of the SLAM/CD2 family alleles, in combination with genetic variations in downstream signaling molecules. In the context of the 129/SvJ or BALB/c genomes, SLAM/CD2 haplotype 2 does not cause elevated ANA production. On the other hand, the imbalance between the SLAM/CD2 haplotype 2 alleles and signaling molecules found in the B6 genome (shown in the center panel) results in spontaneous autoimmunity, as seen in the B6.Sle1b, B6.129c1, and B6.Castc1 congenic lines.

Figure 6. A Model Depicting How SLAM/CD2 Haplotype 2 Mediates Autoimmunity in the Context of the B6 Genome

the functional consequences of the signals transmitted by SLAM/CD2 molecules are dependent upon their interactions with several signal transduction pathways (reviewed in Veillette and Latour, 2003; Veillette, 2004; Engel et al., 2003), and genetic variations affecting the functions of these pathways would be predicted to influence the consequences of SLAM/CD2 family signaling on the immune system. Thus, the signaling characteristics of SLAM/CD2 haplotype 2 are balanced by the downstream signal transduction properties expressed in the immune systems of inbred strains such as 129/SvJ, C3H, or BALB/c, and as a result, these mice are nonautoimmune. However, the combination of SLAM/CD2 haplotype 2 and the signal transduction milieu expressed in B6 mice results in a poorly regulated immune system that is prone to autoimmunity. Finally, the observed autoimmunity of B6.129c1 mice suggests caution in the interpretation of autoimmune phenotypes expressed in mice with gene ablations that have B6/SvJ, C3H, or BALB/c, and as a result, these mice are nonautoimmune. However, the combination of SLAM/CD2 haplotype 2 and the signal transduction milieu expressed in B6 mice results in a poorly regulated immune system that is prone to autoimmunity. Finally, the observed autoimmunity of B6.129c1 mice suggests caution in the interpretation of autoimmune phenotypes expressed in mice with gene ablations that have B6 × 129 hybrid genomes, as we and others have suggested previously (Wakeland et al., 2001; Bygrave et al., 2004).

The observed changes in Ca²⁺ flux properties of CD4 T cells as a consequence of moving SLAM/CD2 haplotype 2 onto B6 are consistent with modification in the activation status of the immune system.

These results provide a clear example of the manner in which epistatic interactions between polymorphic genetic systems can lead to autoimmune susceptibility. In this model, SLAM/CD2 haplotype 2 alleles in combination with one or more polymorphic genes in the B6 genome lead to an imbalanced immune system. The importance of contributions from the B6 genome to the autoimmune phenotypes of Sle1 was previously revealed by our analysis of interactions between NZW and B6.Sle1; in these analyses, we identified four loci in NZW, which we designated Sles1–Sles4 (for SLE suppressor [Morel et al., 1999]), that suppress the autoimmunity elicited by B6.Sle1. It is tempting to speculate that some of these suppressive modifiers may impact the signal transduction pathways that interface with SLAM/CD2 family molecules.

Experimental Procedures

Mice
Mice were kept under specific pathogen-free (SPF) conditions at the University of Texas Southwestern Medical Center. The introgression of SLAM/CD2 from 129/SvJ onto C57BL/6 was performed by using marker-assisted selection protocols (Wakeland et al., 1997) and required five backcross generations to C57BL/6. The B6.129c1 congenic strain carries a 129-derived congenic interval from D1MIT148 to D1MIT115 (roughly 4 Mb) and includes Sle1b, but excludes Sle1a, Sle1d, and Sle1c. The B6.Castc1 congenic strain has a congenic interval similar in size to that of B6.Sle1.

BAC DNA Extraction and Insert Sequencing
BAC DNA was prepared by following the Wellcome Trust Centre for Human Genetics and Institute of Molecular Medicine protocol (http://www.molbiol.ox.ac.uk/jmejia/more_protocols.html). For sequencing libraries, BAC DNA was sheared in a nebulizer (Glas-Col, Inc.) at 150 psi nitrogen for 2.5 min. Fragment ends were mended by incubation with 0.2 mM dNTPs, 40 U T4 DNA polymerase for 15 min at RT and the addition of 25 U Klenow for 1 hr at RT. DNA was purified by phenol–chloroform extraction and ethanol precipitation and was run on a 1% agarose gel. 1–3 kb fragments were extracted
by using the GeneClean kit (Qbiogene) and were ligated into the pUC18 vector by using the Rapid Ligation Kit (Roche Applied Science). DHEs TM-competent cells (Invitrogen) were transformed and plated onto LB plates with 100 μg/ml ampicillin, 50 μg/ml X-gal, and 1 mM IPTG. Plasmid DNA was extracted by using an AutoGen 740 (AutoGen) and was sequenced by using M13 primers on the Beckman CEQ 2000XL (Beckman Coulter). Sequence was assembled with Phred, Phrap, and Consed (see http://www.phrap.org).

Quantitative RT-PCR
Splenic B cells were obtained by using Dynabeads mouse pan B (B220) magnetic beads (Dynal), and total RNA was isolated by using RNeasy Mini kit (Qiagen). cDNA was synthesized with the TaqMan RT kit (Applied Biosystems). Quantitative real-time PCR was performed on 10 ng cDNA on the GeneAmp 5700 with SYBR green (Applied Biosystems); primer sequences are shown in Supplemental Table S5. For each primer set, standard curves were generated by using serial dilutions of cDNA. Gapdh was used to normalize for loading. Data are presented as mean fold differences of B6.Sle1b relative to B6 (n = 5). A Student’s t test was used to determine significance.

Northern RNA Analysis
Total splenic RNA was isolated from 3-month-old or younger B6, B6.Sle1b, NZW, and B6.CastC1 t mice by using Trizol (Invitrogen). 10 μg was run out on a 1% denaturing formaldehyde gel and transferred to a Hybond-N nylon membrane (Amersham Biosciences) with the Turboblotter system (Schleicher & Schuell Biosciences). Ly108, Cd229, and Gapdh probes (primer sequences are listed in Supplemental Data) were labeled with the Rediprim II kit (Amersham) and were purified with Quick Spin G-50 Sephadex Columns (Roche). ULTRAhyb solution (Ambion) was used for hybridization according to manufacturer’s directions. Membranes were washed with 2× SSC/0.1% SDS, followed by 0.1× SSC/0.1% SDS, and were exposed with the Phosphor Screen and PhosphorStorm System (Amersham).

Calcium Flux Analysis
Splenic B and T cells from 1- to 2-month-old B6 and B6.Sle1b mice were enriched by negative selection by using Dynabeads mouse pan T (Thy1.2) and B (B220) magnetic beads (Dynal), respectively. A total of 10^6 cells were labeled with 4 μM CFSE (Molecular Probes) and 200 μg/ml Flucon Pluronic F-127 (Molecular Probes) at 37°C for 30 min. Experimental runs were performed on the FACSScan (Becton Dickinson). 1.7 μg/ml biotin TCR β chain H57-597, 1.7 μg/ml biotin CD4 (GK1.5), and 1.7 μg/ml biotin CD8a (S3-6.7) (BD Pharmingen) were used, followed by crosslinking with 20 μg/ml streptavidin (Roche). 2 μM ionomycin (Calbiochem) was added to achieve maximal response, followed by 2 mM MnCl2 (Sigma-Aldrich) to achieve minimal response. Analysis was performed on FlowJo version 4.1 (Tree Star). Calcium concentrations were determined as described (Vandenberge and Ceuppens, 1990). Mean calcium concentrations were determined at the baseline, at peak response, and postresponse. Paired Student’s t tests were used to determine significance.

SNP Analysis
Genomic DNA was obtained from Jackson Laboratories. Regions containing SNPs that were initially found between B6 and B6.Sle1b alleles of candidate genes were amplified, purified with the High Nature Genomic DNA was obtained from Jackson Laboratories. Regions D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A., O’Connor, T.J., Dietrich, W.F., Miller, J., Steen, R., Merchant, M.A., Damron-Boles, A.L., and Harley, J.B. (2001). Systemic lupus erythematosus in adults

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The C57B16/J sequence has been deposited under GenBank accession number AY819658.