Immunity, Vol. 21, 769–780, December, 2004, Copyright ©2004 by Cell Press

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 *Ly108*, 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 Latour, 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 telomeric 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 CJ7/129Sv-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 *B4galt3*, 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, 48o11, 171k8, 145f9, 77a8, 438k9, and 462j8) (Supplemental Figure S1).

More than 8700 high-quality sequence reads were

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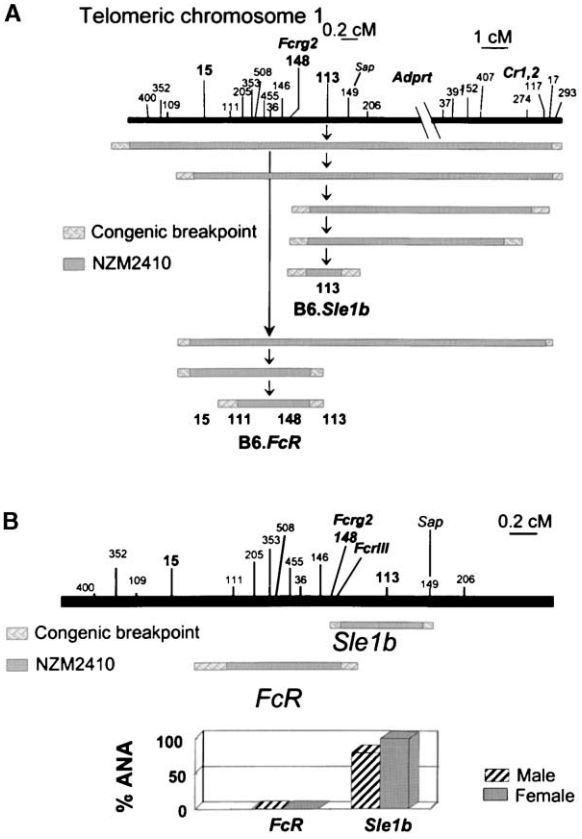

Figure 1. Congenic Derivation of the Minimal *Sle1b* Interval

(A) Genetic map and truncated congenics used to define *Sle1b*. Fine mapping of *Sle1b* was previously described (Morel et al., 2001). A high-resolution map of telomeric chr 1 was produced with 46 microsatellite markers (Dietrich et al., 1996) polymorphic between B6 and NZM2410. Their positions were mapped on 493 (NZM2410 × B6) meioses from two crosses (Morel et al., 1994, 2001), and they were used to genotype (B6.*Sle1* × B6) F1 × B6 progeny for recombinants within the *Sle1* congenic interval.

(B) Progeny carrying selected congenic intervals showing a positive phenotype were intercrossed to homozygosity and expanded. A total of 16 mice/group were aged to 12 months. Sera were screened for antichromatin IgG autoantibodies every other month from 6 to 12 months by ELISA as previously described (Mohan et al., 1998). For interplate comparisons, a serial dilution of an NZM2410 serum was included on each plate to construct a standard curve. The OD value for a 1:100 dilution was assigned a value of 100 units. A sample was deemed positive when it scored greater than 25 units, the mean value + 2 SD of 50 12-month-old B6 controls.

assembled 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

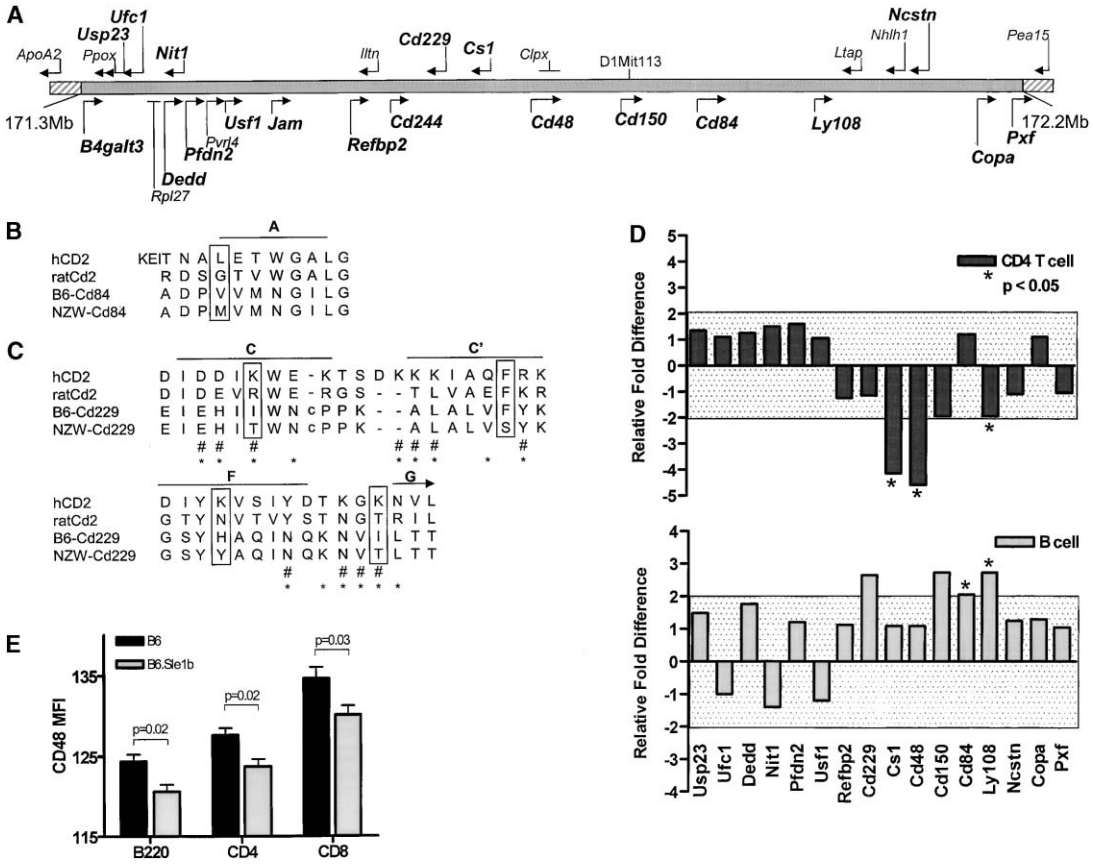

Figure 2. Candidate Gene Analysis of the *Sle1b* Congenic Interval A

(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. β 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, Nit1, 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.ch/prosite]). All of the synonymous and nonsynonymous

Table 1. Analysis of the Productive Sequence Polymorphisms between the B6 and B6.*Sle1b* Alleles

Gene	Function	GenBank Accession Number	Position (Amino Acid)	Amino Acid Change[a] B6	NZW	Other Species[b] Conserved	Not Conserved
Usp23	Intracellular protein breakdown and cell cycle regulation	BAB27431	341	C	R	Human	Rat, *Drosophila*, Yeast
Nit1	Cleaves nitriles and organic amides	NP_036179	22	T	I		Human
Refbp2	RNA and export factor binding protein 2	NP_062357	151	T	A	Human, Rat, Chicken, *Xenopus*	*Drosophila*, *Arabidopsis*
Cd229	Adhesion/costimulatory molecules that regulate the activation threshold in many immune cell lineages	NP_032560	58	I	T	Human	
			70	F	S		Human
			109	H	Y		Human
			118	I	T		Human
			345	K	N		Human
			356	E	K		Human
			571	G	E	Human	
Cs1	See *Cd229*	AAH11154	248	M	T		Human
			253	G	R		Human
Cd48	See *Cd229*	P18181	4	I	R	Human	Rat
			90	N	D	Rat	Human
Cd84	See *Cd229*	NP_038517	27	V	M		Human
Ncstn	Integral membrane protein that interacts with presenilin-1 and -2	AAH19998	21	S	F		Human
			678	T	I	Human	*Drosophila*, *C. elegans*, *Arabidopsis*
			680	V	I	Human, *Drosophila*, *C. elegans*	*Arabidopsis*
Copa	Housekeeping gene	NP_034068	761	S	T	Human, *Drosophila*, *Arabidopsis*	*C. elegans*
			984	N	S	Human	*Drosophila*, *C. elegans*, *Arabidopsis*
			1110	N	S	Human, *Drosophilia*, *C. elegans*	*Arabidopsis*
Pxf	Peroxisomal biogenesis	NP_075528	55	P	S[c]	Human, Rat, Hamster	*Drosophila*[c], *C. elegans*

[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 *Drosophilia*.

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 variations between B6 and B6.*Sle1b* within this region, predominantly in the SLAM/CD2 family cluster. Extensive structural and regulatory polymorphisms distinguished 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, four 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

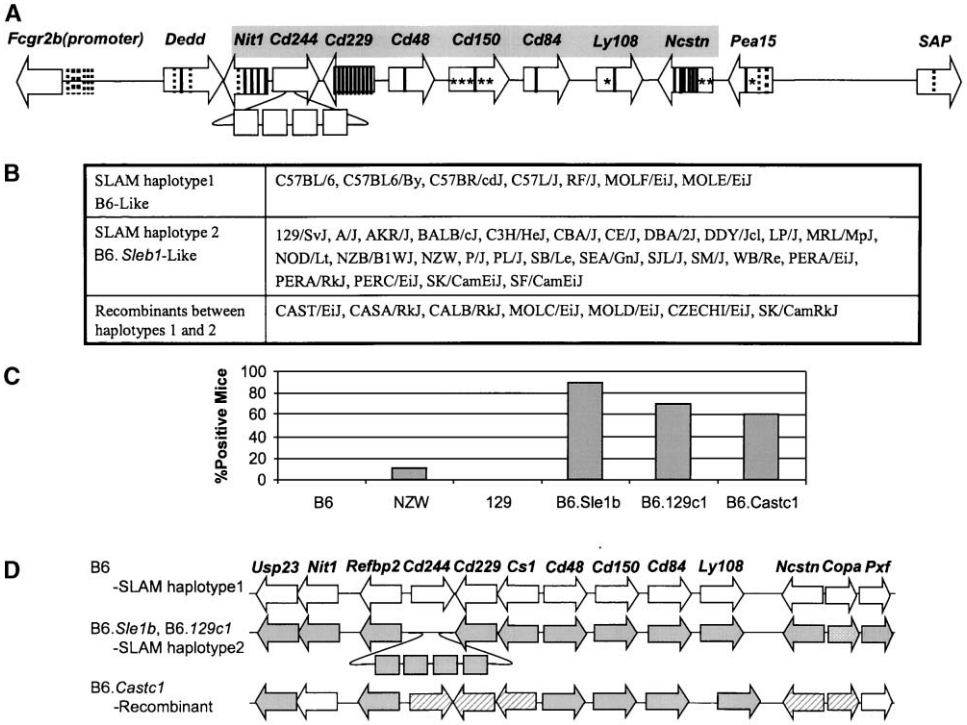

Figure 3. Haplotype Analysis of the *Sle1b* Interval

(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 α 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 *Sle1b* candidate genes. B6 alleles are shown as white arrows. NZM2410 and 129/SvJ share the same alleles, shown as gray arrows. The exception is *Copa*, for which 129/SvJ differs from NZM2410 by a single productive SNP. CAST/Ei alleles not shared with B6 or B6.*Sle1b* are shown as hatched arrows.

across all the inbred laboratory strains assayed. The most conserved segment of the haplotype is centered over the SLAM/CD2 family, and it deteriorates distal to *Dedd* on the centromeric side and proximal to *Pea15* on the telomeric side. A list of all of the SNPs analyzed in the 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/6 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/6/J 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.*Sle1b* mice. While the congenic interval in B6.*Castc1* is similar in size to that of B6.*Sle1* and consequently may contain other genes influencing autoimmunity (although CAST/Ei mice are not reported to be autoimmune-prone), a comparison of the structure of the *Sle1b* region in B6.*Castc1* with B6.*Sle1b* and B6.129c1 provides some useful insights. B6.*Castc1* carries alleles of *Nit1*, *Pxf, Ncstn*, and *Copa* that are similar or identical to B6 (Figure 3D, Supplemental Table S3), arguing against a role for these alleles in autoimmunity caused by *Sle1b*. B6.*Castc1* also excludes the amplification of *Cd244* as a candidate genetic polymorphism for *Sle1b*, in that B6.*Castc1* has a single *Cd244* gene closely related to that of B6. Two SLAM/CD2 family genes, *Cd229* and *Cs1*, are also unique alleles, while the remaining SLAM/CD2 family alleles are shared by B6.*Castc1* and B6.*Sle1b*. These results indicate that B6.*Castc1* carries a SLAM/CD2 family gene cluster that is most identical to B6.*Sle1b* in the telomeric portion of the haplotype.

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 gene family (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 Ca^{2+} 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 Ca^{2+} 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

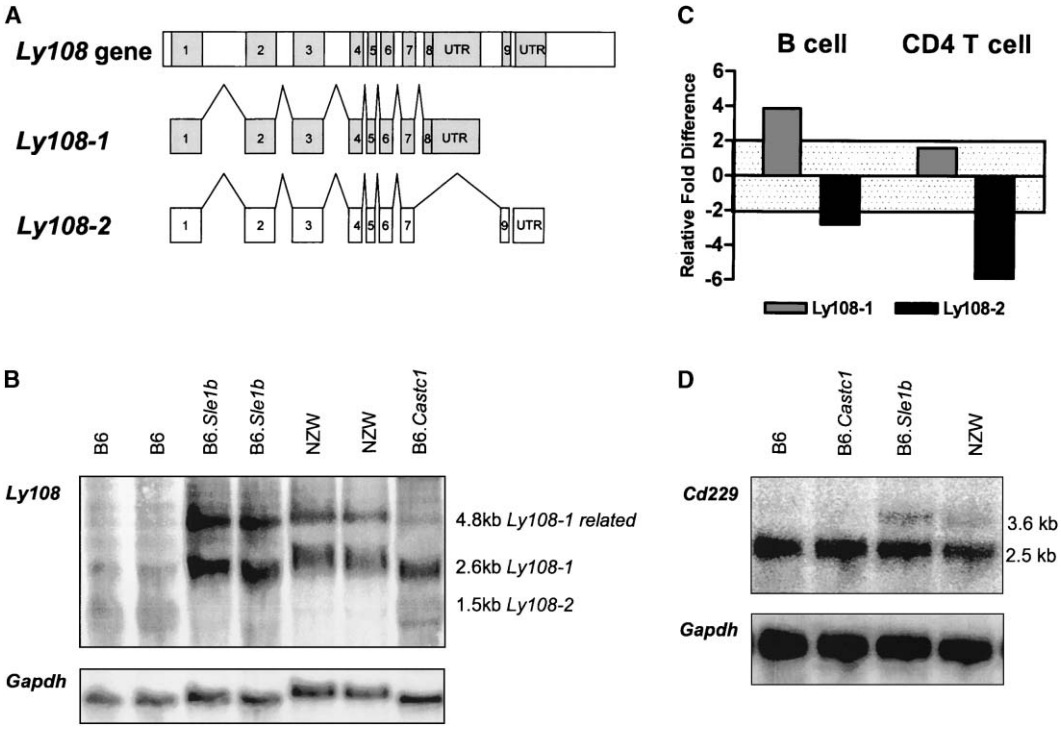

Figure 4. Differential Isoform Expression Associated with the Two SLAM/CD2 Haplotypes

(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/

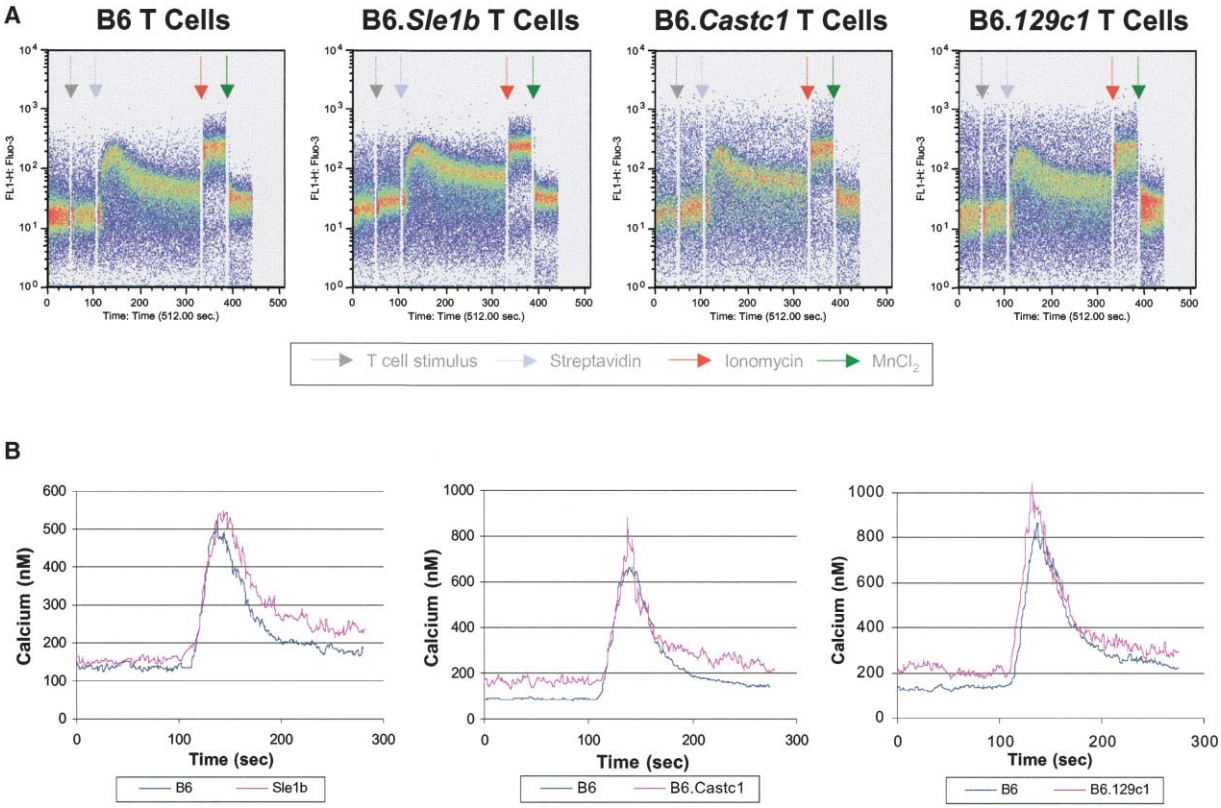

Figure 5. B6.*Sle1b* T Cells Show a Difference in Calcium Flux Compared to B6

(A) T cell-enriched splenocytes from B6, B6.*Sle1b*, B6.*129c1*, and B6.*Castc1* were labeled with Fluo-3 and stimulated with anti-TCRβ, 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

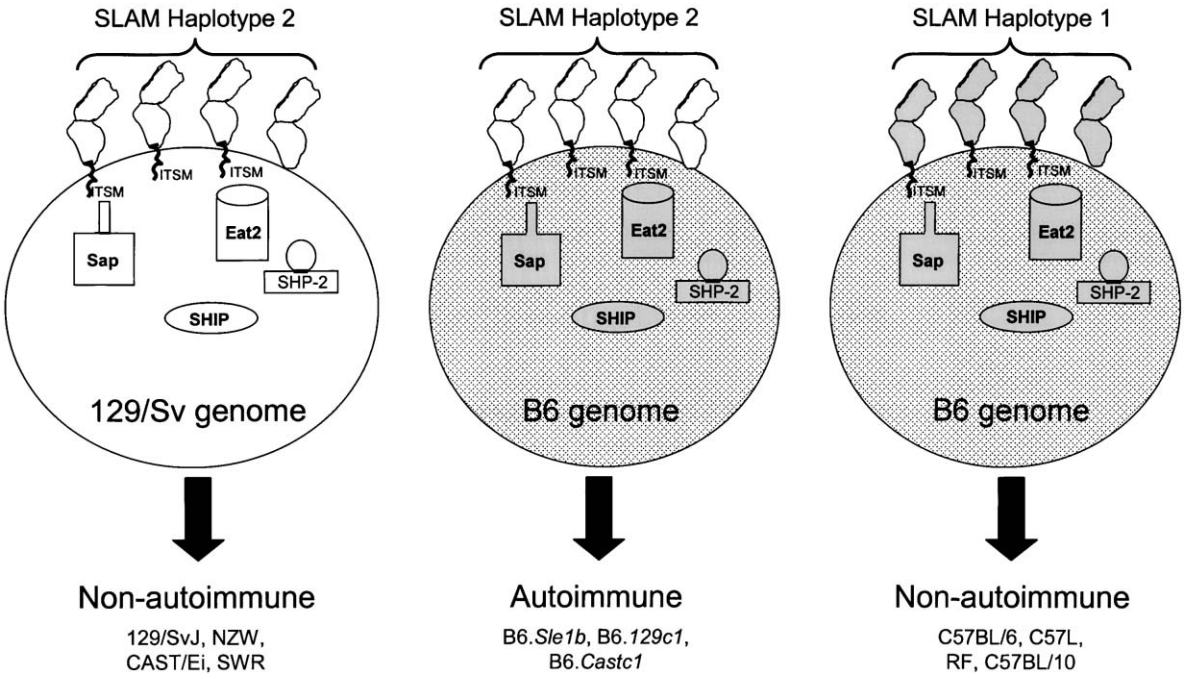

Figure 6. A Model Depicting How SLAM/CD2 Haplotype 2 Mediates Autoimmunity in the Context of the B6 Genome

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.

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 $\times$ 129 hybrid genomes, as we and others have suggested previously (Wakeland et al., 2001; Bygrave et al., 2004) The observed changes in Ca^{2+} 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 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 GenecleanII kit (Qbiogene) and were ligated into the pUC18 vector by using the Rapid Ligation Kit (Roche Boehringer Mannheim). DH5α 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 ($n \geq 5$). 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* 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 Table S5) were labeled with the Rediprime 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 PhosphoImager Storm 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 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 (53-6.7) (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 $MnCl_2$ (Sigma-Aldrich) to achieve minimal response. Analysis was performed on FlowJo version 4.1 (Tree Star). Calcium concentrations were determined as described (Vandenberghe 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 Pure PCR Product Purification Kit (Roche), and sequenced on the Beckman CEQ2000XL. Sequences were analyzed with Sequencher (Gene Codes Corporation).

Flow Cytometric Analysis and Antibodies

Cells were blocked with 2.4G2 (American Type Culture Collection) and stained (1.5×10^6 per antibody cocktail) with FITC, phycoerythrin (PE), PE-Texas Red, Cychrome, PerCPCy5.5, APC, or biotin-conjugated antibodies (Abs) in PBS/2% FCS. The following antibodies from BD Biosciences Pharmingen were used: CD21/35 (7G6), CD23 (B3B4), IgM (R6-60.2), CD48 (HM48-1), CD86 (GL1), CD45R (RA36B2), CD5 (53-7.3), CD11b (M1/70), NK1.1 (PK126), CD25 (PC61), CD69 (H1.2F3), CD4 (H129.19), CD3 (145-2C11), CD19 (1D3), CD62L (MEL-14), CD44 (1M7), CD8 (53-6.7), CD138 (281-2), and Streptavidin-APC. Isotype controls for all antibody combinations were included. Stained cells were analyzed by using a FACsCalibur (BD Biosciences). A total of 40,000 live events within the lymphocyte gate were acquired per sample. Data were analyzed with FlowJo (Treestar).

Total Histone/dsDNA IgG ELISA

Antichromatin ELISAs were performed as described earlier (Mohan et al., 1998), by using the conditions detailed in the figure legends.

Supplemental Data

Supplemental Data including additional information regarding the sequence and expression variations found in the SLAM/CD2 gene family are available at http://www.immunity.com/cgi/content/full/21/6/769/DC1/.

Acknowledgments

We thank Dr. Jose Casco for the maintenance of mice. David Chambliss provided excellent technical assistance in these studies. This work was supported by grants from the National Institutes of Health (NIH), The Alliance for Lupus Research, The Lupus Research Institute, and the Arthritis Foundation (E.K.W.). A.E.W. was supported by an NIH postdoctoral fellowship and by a grant from the Lupus Foundation. N.L. was supported in part by a grant from the National Institute of Allergy and Infectious Disease, T32AI005284.

Received: July 16, 2004
Revised: October 21, 2004
Accepted: October 27, 2004
Published: December 14, 2004

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Accession Numbers

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