A novel isoform of the Ly108 gene ameliorates murine lupus

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Abbreviations used: ANA, antinuclear antibody; BAC, bacterial artificial chromosome; dsDNA, double-stranded DNA; ES, embryonic stem; ITSM, immuno-tyrosine switch motif; mRNA, messenger RNA; PNGaseF, peptide:N-glycosidase F; SAP, SLAM-associated protein; SLAM, signaling lymphocytic activation molecule; SLAMF, SLAM family; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; ssDNA, single-stranded DNA.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies against a wide spectrum of self-antigens, especially from the cell nucleus. Genes in multiple pathways participate in mediating disease pathogenesis, and epistatic interactions among these genes influence the severity of disease. SLE-related genes include, for instance, those involved in physiological pathways of waste disposal mechanisms, regulating the removal of circulating immune complexes and apoptotic cells by the mononuclear phagocyte system, or those involved in T cell functions. Several of the signaling lymphocytic activation molecule (SLAM) family (SLAMF) genes, which encode cell surface receptors and which affect both antigen-presenting cell and T cell functions, may also regulate thresholds for tolerance and activation of T and B lymphocytes as well as reactivation-induced cell death (Wandstrat et al., 2004; Kumar et al., 2006; Carlucci et al., 2007; Calpe et al., 2008; Cunninghame Graham et al., 2008; Snow et al., 2009; Detre et al., 2010; Kim et al., 2010; Morel, 2010; You et al., 2010).

In mice, the SLAMF cell surface receptors encode nine genes (Slamf1–9; Calpe et al., 2008) that vary between two sets of mouse strains: Slamf1–9-haplotype 1, e.g., C57BL/6 (B6), and Slamf1–9-haplotype 2, e.g., NZW or 129 (Wandstrat et al., 2004; Detre et al., 2010). In the lupus-prone congenic mouse strains, B6.Sle1b mice, DNA segments derived from NZW or 129, respectively, are embedded in the B6 genome. These NZW or 129 segments contain several genes, including Slamf1–7.

Studies of human systemic lupus erythematosus patients and of murine congenic mouse strains associate genes in a DNA segment on chromosome 1 with a genetic predisposition for this disease. The systematic analysis of lupus–prone congenic mouse strains suggests a role for two isoforms of the Ly108 receptor in the pathogenesis of the disease. In this study, we demonstrate that Ly108 is involved in the pathogenesis of lupus–related autoimmunity in mice. More importantly, we identified a third protein isoform, Ly108–H1, which is absent in two lupus–prone congenic animals. Introduction of an Ly108–H1–expressing transgene markedly diminishes T cell–dependent autoimmunity in congenic B6.Sle1b mice. Thus, an immune response–suppressing isoform of Ly108 can regulate the pathogenesis of lupus.

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Autoantibodies develop because of an epistatic interplay between one or more Slamf-haplotype 2 genes with B6 genes (Bygrave et al., 2004; Wandstrat et al., 2004; Carlucci et al., 2007). Based on the studies with the Sle1b mouse (Wandstrat et al., 2004; Kumar et al., 2006), Ly108 (Slamf6) has been suggested as a candidate susceptibility gene for SLE. An initial study using BALB/c splenocytes identified two Ly108-coding cDNAs (Peck and Ruley, 2000), later termed Ly108-1 (GenBank/EMBL/DDBJ accession no. AF248635.1) and Ly108-2 (GenBank accession no. AF248636.1). These two splice variants of Ly108 are thought to encode protein isoforms with identical extracellular domains but differing cytoplasmic tails. The ratio of transcripts encoding Ly108-1 and Ly108-2 differs in Sle1b and B6 lymphocytes, which is thought to influence early B cell development (Kumar et al., 2006), resulting in increased autoantibody production. The B cell experiments led to the conclusion that “the normal Ly108-2 allele, but not the lupus-associated Ly108-1 allele, was found to sensitize immature B cells to deletion and RAG re-expression” (Kumar et al., 2006).

In this study, we use gene-targeted mutant and congenic mice in combination with bacterial artificial chromosome (BAC)-based transgenic mice to provide evidence that Ly108 is involved in the regulation of SLE in a CD4+ T cell–dependent manner. In addition to Ly108-1 and Ly108-2, we identify a novel protein isoform, Ly108-H1, which is present only in Slamf-haplotype 1 mice and not in the lupus-prone Sle1b or B6.129chr1b strains. Introduction of one copy of an Ly108-H1 BAC-based transgene into the Sle1b dramatically reduces lupus-related autoimmunity. Thus, although the Slamf-haplotype 2 segment in the congenic Sle1b mouse contains circa 20 genes, the Ly108-H1 isoform plays a dominant role in suppressing the pathogenesis of SLE.

RESULTS

The congenic Ly108−/− [129 × B6] mouse does not develop SLE, in contrast to the B6.129chr1b congenic mouse, which contains the same 129-derived segment

To examine whether disruption of the Ly108 gene would affect the development of murine lupus, we backcrossed our congenic Ly108−/− [129 × B6] mouse, which was generated with 129-derived embryonic stem (ES) cells (Howie et al., 2005), with the WT B6 mouse. In addition, we generated a new B6.Ly108−/− mouse using B6 ES cells (Fig. S1, A–D). We hypothesized that the congenic Ly108−/− [129 × B6] mouse would not develop lupus if Ly108 played a dominant role in the pathogenesis of the disease. In contrast, Ly108−/− [129 × B6] would develop disease if genes other than Ly108 that are present in the selected 129-derived segment were of equal or greater importance.

We found that in aged Ly108−/− [129 × B6] females, anti-nucleosome autoantibody levels were similar to those in B6 mice, whereas these antibodies were detected in the serum of the lupus-prone B6.129chr1b [129 × B6] and Sle1b [NZW × B6] mice. Furthermore, the B6.Ly108−/− mice did not develop lupus (Fig. 1 A). As expected in the case of a T cell–dependent autoimmune disease (Crispin et al., 2010b), the percentage of effector/memory CD4+ T cells was increased in aged B6.129chr1b and Sle1b mice (Fig. 1 B). In contrast, the percentage of effector/memory CD4+ T cells...
in the aged Ly108−/− [129 × B6] mice was equivalent to that in WT B6 mice (Fig. 1 B). Low-resolution microsatellite marker analysis of the boundaries of the 129-derived segment in Ly108−/− [129 × B6] and B6.129chr1b mice showed that they were similar (Fig. 1 C and Fig. S1 F). Support for the presence of the 129-derived segment (e.g., Slamf-haplotype 2) in both mouse strains comes from staining with haplotype 2–specific α-Hap2 antibody (Fig. S1 E). To fine-map the exact breakpoint between the B6 genetic background and the 129 congenic insert in Ly108−/− [129 × B6] and B6.129chr1b mice, we used a single nucleotide polymorphism (SNP) genotyping microarray. Notably, on the centromeric side of the congenic insert, the first 129 SNP allele was rs32120942 (National Center for Biotechnology Information [NCBI] SNP database accession no.) for both the Ly108−/− [129 × B6] and B6.129chr1b analysis (Fig. 1 C and Table S2). However, the exact breakpoint upstream of SNP rs32120942, which spans 4.8 Mb, could not be determined as no sequence differences among the 1,406 B6 and 129 SNP alleles were included on the array. This observation argues that the centromeric side recombinant region belongs to an identical large haplotype block in 129 and B6 mice; thus, there is no reason to attempt to find the exact breakpoint. On the telomeric side, the 129-derived segment is 2 Mb longer in Ly108−/− [129 × B6] than in B6.129chr1b and contains 18 RefSeq genes (Fig. S1 G). None of the possible differences between Ly108−/− [129 × B6] and B6.129chr1b genomic sequence overlap with any of the described SLE suppressor regions in 129 or NZW (Morel et al., 1999; Subramanian et al., 2005). Thus, the disruption of the Ly108 gene in a congenic mouse anehorizes disease found in a very similar congenic mouse strain that harbors the intact 129-derived Ly108 gene.

Because the homophilic receptor Ly108 is expressed on both T and B cells, we examined whether specific T cell–dependent antibody responses would be affected by the Ly108null mutation. As shown in Fig. 1 D and Fig. S1 H, T cell–dependent low- and high-affinity antibody responses to haptenated proteins by the congenic Ly108−/− [129 × B6] mice are identical to those by WT B6 mice. We conclude that the absence of the lupus-susceptibility gene Ly108 significantly reduces antinucleosome antibody responses and CD4+ T cell expansion in a B6 mouse, which contains a short 129-derived segment.

**Protein isoform analysis detects Ly108–H1, a novel protein isoform, which is coexpressed with Ly108–1 and Ly108–2 in Slamf-haplotype 1 mice**

Because the difference in expression levels of the two Ly108 alleles in the Sle1b and B6 mouse was based on quantitative PCR (Wandstrat et al., 2004), we set out to examine whether the Ly108-1/2 isoform variability was reflected at the protein level of Slamf-haplotype 1 and 2 mice. First, we assessed the expression pattern of Ly108 on the surface of hematopoietic cells derived from known Slamf-haplotype 1 and 2 mice using our anti-Ly108 monoclonal antibody, 13G3, which is specific for the extracellular region of the receptor (Fig. 2 A). Interestingly, CD4+ 8 (double negative) and CD4+ 8 (double positive) thymocyte subsets derived from the Slamf-haplotype 1 mouse, B6 and MOLF/EiJ, expressed considerably more Ly108 than thymocyte subsets isolated from Slamf-haplotype 2 mice, e.g., BALB/c, 129, and Sle1b (Fig. 2 A).

The difference in Ly108 cell surface expression in Slamf-haplotype 1 mice appeared to be a reflection of increased transcription of the Slamf6 gene, as judged by quantitative PCR (Fig. S2). We therefore evaluated whether the difference in cell surface expression among strains of the two Slamf-haplotypes could be attributed to an increase in Ly108 protein expression, particularly among the two isoforms. To this end, immunoprecipitates made with monoclonal α-Ly108 (13G3) were subjected to SDS-PAGE, and the protein isoforms Ly108–1 and Ly108–2 were identified by Western blotting with two polyclonal antibodies. One antibody (R4) was directed...
against a unique amino acid sequence in the C terminus of the Ly108-2 cytoplasmic tail and the second antibody (R1) against a cytoplasmic tail segment shared by the two isoforms (Fig. 3 D). The glycosylated Ly108-1 and Ly108-2 proteins, when precipitated from thymocytes and CD4+ T cells of B6 or BALB/c mice, ran as indistinguishable bands upon Western blotting with R1. However, when the immunoprecipitates were blotted with the R4 antibody, samples from B6 mice consistently showed higher Ly108-2 expression as compared with the same cell subsets of the BALB/c animals (Fig. 2 B). Upon deglycosylation with the enzyme PNGaseF (peptide: N-glycosidase F), Ly108-1 and Ly108-2 protein isoforms migrated according to their predicted molecular masses, 34 kD and 36 kD, respectively (Fig. 2 C, top). Surprisingly, when the membranes were reprobed with the R4 antibody (Fig. 2 C, bottom), two proteins were detected in B6 thymocytes. In addition to the 36-kD Ly108-2 protein, a smaller 30-kD protein, designated Ly108-H1, was present only in the B6 thymocytes. The Ly108-H1 protein was also present in B lymphocytes of B6 mice but not in 129, BALB/c, or NOD B lymphocytes (Fig. 2 D).

Thus, the presence of a previously unidentified protein isoform, Ly108-H1, expressed exclusively in Slamf-haplotype 1 mouse strains, could, at least in part, have accounted for the elevated Ly108 expression in B6 thymocytes. Because quantification by Western blotting of immunoprecipitates is inherently difficult, a difference in protein expression of Ly108-1 and Ly108-2 could not be excluded.

The Ly108-H1 protein is encoded by a splice variant of Ly108 that lacks both exons 7 and 8.

The detection of Ly108-H1 by R4 but not by R1, which was raised against a peptide sequence encoded by exon 7, suggested that the R1 peptide sequence, containing the second immuno-tyrosine switch motif (ITSM), was absent from Ly108-H1 (Fig. 3 D). Based on the protein analyses, we tested the possibility that lymphocytes from Slamf-haplotype 1 mice expressed an Ly108 splice variant lacking sequences for exons 7 and 8. Using exon 1– and exon 9–specific primer pairs, two PCR products, presumably Ly108-2 and Ly108-H1, were amplified from B6 thymocytes (Fig. 3 A). Subsequent cloning and sequence analysis of the cDNAs confirmed that B6 thymocytes indeed express a transcript that lacks both exons 7 and 8 (GenBank/EMBL/DDBJ accession no. ACF05482).

To determine whether this novel transcript encodes Ly108-H1, three cDNAs were transiently transfected into the Ly108-negative T cell line BI-141 (Fig. 3 B). Immunoprecipitation followed by deglycosylation with PNGaseF, SDS-PAGE, and immunoblotting verified that Ly108-exon 7/8 cDNA encoded the Ly108-H1 protein, as judged by its

Figure 3. The Ly108–H1 protein is encoded by a splice variant of Ly108 that lacks both exons 7 and 8. (A, left) Diagram of the nine exons of the Ly108 gene. Both exons 8 and 9 contain a 3′ untranslated region. Location of the exon 1–9 primers is indicated with red arrowheads. (right) RT-PCR using mRNA from B6, 129, BALB/c, and NOD thymocytes. (B) Ly108 cell surface expression on CD4+ T hybridoma cells that were transiently transfected with cDNAs encoding Ly108-1, Ly108-2, or Ly108-H1. Surface expression was evaluated by flow cytometry 24 h after transfection. (C) Lysates of the Ly108-1, Ly108-2, or Ly108-H1 BI-141 transfected cell lines were immunoprecipitated with α-Ly108, treated with PNGaseF, subjected to SDS-PAGE, and evaluated by Western blotting (WB) with the R4 polyclonal antibody. IP, immunoprecipitate. (D) Schematic illustration of the three Ly108 protein isoforms. Antibody R1 binds a segment shared by the isoforms Ly108-1 and Ly108-2. Antibody R4 is directed against the C-terminal amino acid sequence of isoforms Ly108-2 and Ly108-H1. (E) RT-PCR analyses of isoform mRNAs isolated from thymocytes of Slamf-haplotype 1 and 2 WT and congenic mouse strains. Primers specific for exons 5 and 8 recognize Ly108-1, and primers specific for exons 5 and 9 recognize Ly108-2 and Ly108-H1. Plasmids containing the cDNA sequences of individual isoforms (pLy108-1, pLy108-2, and pLy108-H1) were used as positive controls. (F) RT-PCR analyses of isoform mRNAs isolated from B cell lines. B6-206 and B6-208 are cloned B cell lymphoma lines derived from B6 mice. WEHI-231 and A20 are derived from BALB/c mice (Slamf-haplotype 2). All T cell experiments were performed two times; the B cell analysis was performed once using two different cell lines from each of the haplotypes.
molecular weight and reactivity with R4 (Fig. 3 C). Collectively, these data clearly demonstrate that T and B lineage cells from haplotype 1 but not haplotype 2 mice express Ly108-H1, in addition to the Ly108-1 and Ly108-2 proteins (Fig. 3 D).

Next, expression of Ly108-H1 in T and B cells from the lupus-prone congenic strains Sle1b and B6.129chr1b and from various Slamf-haplotype 1 and 2 mice was examined by RT-PCR. To this end, oligonucleotide primers that either spanned exons 5–8 (detecting Ly108-1) or exons 5–9 (common to Ly108-2 and Ly108-H1) were used. Ly108-H1 was detectable in Slamf-haplotype 1 but not in any haplotype 2 thymocytes (Fig. 3 E). More importantly, Ly108-H1 is absent in the congenic mouse strains Sle1b and B6.129chr1b, suggesting that the presence of this novel protein isoform might affect the development of lupus.

To evaluate whether single cells express all three isoforms, we used B cell lymphoma cell lines from different origins. These experiments showed that the B6-derived lines B6-206 and B6-208 coexpressed transscripts for all three isoforms, whereas the BALB/c-derived A20 and (BALB/c × NZB)F1-derived WEHI-231 cells coexpressed only Ly108-1 and Ly108-2 (Fig. 3 F).

The data with Sle1b and B6.129chr1b lymphocytes suggest that the alternate splice form, Ly108-H1, might have been generated by regulatory elements within the short 129-derived segment in these congenic mouse strains and perhaps by elements that are part of, or in proximity to, the Ly108 gene. To evaluate this, a B6-derived and a 129-derived BAC clone were each transfected into Jurkat T cells. Both transfectant cells expressed Ly108-1 and Ly108-2, but only in transfectants generated with the B6-derived BAC clone could Ly108-H1 be detected (Fig. S3, A and B). Our sequence analyses indicated that the splice donor and acceptor sequences in the introns surrounding exons 6–8 are identical between B6 and the congenic mouse strains Sle1b and B6.129chr1b (Fig. S3 C). However, two pyrimidine-rich tandem repeat sequences were only present in intron 6 of the B6 mouse (Fig. S3 C), which could be the cause for the alternate splicing events that generate the protein isoform Ly108-H1 (Wagner and Garcia-Blanco, 2001; Black, 2003).

Collectively, these data clearly demonstrate that T and B lymphocytes from Slamf-haplotype 1 but not haplotype 2 mice coexpress the Ly108-1, Ly108-2, and Ly108-H1 protein isoforms. We conclude that Ly108-H1 is generated by elements within the B6-derived BAC clone, most likely by sequences in intron 6, which could negatively regulate splicing and which are absent in Slamf-haplotype 2 mice.

Faithful expression of an Ly108-H1 BAC-based transgene BACLy108-H1 in Sle1b mice

Because Ly108-H1 is not expressed in the congenic Sle1b and B6.129chr1b mice, we hypothesized that this isoform could have a protective role in murine lupus. To this end, we introduced the Ly108-H1 isoform into the Sle1b mouse. However, introduction of an Ly108-H1–specific transgene might be complicated by regulatory elements in and in proximity to the Ly108 gene and by the possibility of overexpressing the transgene in a tissue-biased manner. We therefore introduced into the Sle1b mouse a B6-derived BAC clone–based transgene, which only expressed Ly108-H1. This required several alterations of the BAC clone RP23-77A8 (Osoegawa et al., 2000). First, through recombineering in Escherichia coli, we removed a DNA fragment containing Ly108 exons 7 and 8, thus preventing potential expression of Ly108-1 and Ly108-2 by the transgene. Next, the genomic sequences encoding SLAM (Slamf1) and CD84 (Slamf5) were removed by two subsequent recombineering steps. Because the Ly108 amino acid sequence does not differ between haplotype 1 and haplotype 2 strains, the transgene simply reconstructed a missing splicing event in the transcriptome of Sle1b mice. The resulting ∼100-kb genomic BACLy108-H1 vector should only contain the genomic cis-sequences that are requisite for faithful transcription of only the Ly108-H1 isoform (Fig. 4 A).

Transgenesis of the BACLy108-H1–H1 mouse into Sle1b mice resulted in the Sle1b.BACLy108-H1 mouse with the transgene located in one area of the genome, as judged by fluorescent in situ hybridization (Fig. S4 A). Semiquantitative RT-PCR indicated that Ly108-H1 was expressed in thymocytes derived from hemizygous transgenic Sle1b.BACLy108-H1 mouse, while absent in Sle1b thymocytes (Fig. 4 B). To further evaluate the faithful transcription of all three of the Ly108 isoforms, we took advantage of the fact that Ly108 transcripts originating from B6 and Sle1b differ from each other in several synonymous SNPs (NCBI mouse SNP database). Indeed, the Sle1b.BACLy108-H1 mice express exons 2–4 both of Slamf-haplotype 1 (encoded by BACLy108-H1) and of Slamf-haplotype 2 (encoded by Sle1b; Fig. 4 C). Examination of the RFLPs of thymocyte-derived Ly108 cDNA confirmed that Ly108-1/2 expression in Sle1b.BACLy108-H1 thymocytes was controlled by the Slamf-haplotype 2 segment of the Sle1b mouse (Fig. 4 D, left). The BAC transgene did not express SLAM and CD84, as judged by the use of two different SNP-based RFLPs (Fig. 4 D, middle and right).

Cyttofluorimetric analyses of Ly108 expression on the surface of T lineage cells isolated from Sle1b, Sle1b.BACLy108-H1, or B6 mice (Fig. 4 E) supported the notion that Ly108 surface expression on T cells was slightly higher in hemizygous Sle1b.BACLy108-H1 mice than their Sle1b transgene-negative littermates. And, as expected for mice that are hemizygous for the Ly108-H1 transgene and homozygous for the Slamf-haplotype 2 forms of Ly108, surface expression was not as high as on the surface of Slamf-haplotype 1, e.g., B6 thymocytes (Figs. 2 and 4 E). The overall T and B lymphocyte development was normal in young Sle1b.BACLy108-H1 mice (Fig. S4 B). Collectively, these results indicate that, at most, one copy of Ly108-H1 was expressed in Sle1b.BACLy108-H1–derived T lineage cells and that cell surface expression and balance between the three isoforms was similar to that found in the B6 mouse.

The presence of the Ly108-H1 isoform in Sle1b mice ameliorates the development of lupus-related autoimmunity

To assess whether Ly108-H1 would affect the spontaneous development of SLE in Sle1b mice, we analyzed a cohort
of aged female hemizygous Sle1b.BACLy108-H1 mice and transgene-negative Sle1b littermate controls along with B6 females. Whereas the Sle1b mice (6–8 mo old) had high titers of antinuclear antibodies (ANAs), as judged in a HEp-2 cell–based fluorescence quantitative assay, spontaneous development of these autoantibodies was significantly lower in their transgenic littermates (Fig. 3A). First, antinucleosome IgG and antichromatin IgG titers were dramatically lower in aged Sle1b.BACLy108-H1 mice than in B6 mice. Thymic and splenic T cells were incubated with the monoclonal α-Ly108 antibody (13G3) conjugated with DyLight 649 and then washed and analyzed by flow cytometry. The mean fluorescent intensity (MFI) of gated cell populations in the DyLight 649 channel is shown (three to four individual mice per group). PCR experiments were performed a minimum of two times. DN, double negative; DP, double positive.

We conclude that the presence of Ly108-H1 partially suppresses the key humoral autoimmune features of Sle1b mice, i.e., autoantibody production, spontaneous activation of peripheral T cells, and expansion of germinal center B cells. This is remarkable because the DNA segment derived from NZW, i.e., Slamf-haplotype 2, contains 7 Slamf genes and >12 other genes, which might somehow contribute to Sle1b–based lupus (Wandstrat et al., 2004; Calpe et al., 2008).

Figure 4. Expression of an Ly108–H1 BAC-based transgene in Sle1b mice. (A) The BACLy108-H1 transgene vector encoding Ly108-H1 was generated by deleting exons 7 and 8 of Ly108 and the SLAM and CD84 genes from the B6 BAC clone RP23–7778A. Alteration of the 200-kb B6 BAC clone by Red/ET recombineering is described in Materials and methods. The resulting DNA fragment BACLy108-H1 is ~100 kb. S1; S2, S3, and S4 are homology arms for Red/ET recombination. KANA is a kanamycin resistance gene cassette. (B) RT-PCR prepared with thymic mRNA using Ly108-H1–specific primers. (C) Haplotype-specific RFLP fragments were obtained by BsrI digestion of PCR-amplified thymic Ly108 cDNA. B6 and Sle1b mice exclusively express haplotype 1– and haplotype 2–specific fragments, respectively. Because Sle1b.BACLy108-H1 mice express both fragments, the transgene expresses the B6 based fragment. PCR primer pairs specific for Ly108 exons 2–4 (common for Ly108-1, Ly108-2, and Ly108-H1) were used, as described in Materials and methods. (D, left) RT-PCR primers specific for Ly108 exons 2–7 (common for Ly108-1 and Ly108-2 only) were used to isolate a cDNA from the thymus. Haplotype 1– and haplotype 2–specific RFLP used BsrI digestion, as in C. (middle) RT-PCR primers specific for SLAM exons 1–5 were used to isolate a cDNA from the thymus. Haplotype 1– and haplotype 2–specific RFLP fragments were obtained by TaqI digestion. (right) RT-PCR primers specific for CD84 exons 1–2 were used to isolate cDNA. Haplotype-specific RFLP fragments were obtained by MspI digestion. (E) Cell surface expression of Ly108 using T lineage cells isolated from Sle1b, Sle1b.BACLy108-H1, or B6 mice. Thymic and splenic T cells were incubated with the monoclonal α-Ly108 antibody (13G3) conjugated with DyLight 649 and then washed and analyzed by flow cytometry. The mean fluorescent intensity (MFI) of gated cell populations in the DyLight 649 channel is shown (three to four individual mice per group). PCR experiments were performed a minimum of two times. DN, double negative; DP, double positive.

Ly108-H1 in CD4+ T cells ameliorates autoimmunity in a transfer model of SLE

Whereas previous studies indicate that a defect in early B cell development is a major contributor to lupus in the Sle1b mouse (Kumar et al., 2006; Chang et al., 2009), a role for peripheral T cells cannot be excluded in this congenic mouse. We therefore directly tested the possibility that peripheral T cells could induce autoantibody production in an established transfer model of SLE (Morris et al., 1990). As shown in Fig. 6A, the transfer of Sle1b splenocytes into bm12 recipients induces much higher anti–double-stranded DNA (dsDNA) IgG titers than the transfer of splenocytes derived from WT B6 mice, particularly at 4 wk after transfer. Second, both purified Sle1b CD4+ T cells or CD62L+ naive CD4+ T cells (Fig. 6B and Fig. S5, A–E) consistently induced stronger
autoantibody responses when transferred into bm12 mice than the transfer of the same cells isolated from WT B6 mice. This result strongly supported a role for peripheral CD4+ T cells in the spontaneous Sle1b disease.

A comparison of autoantibody responses to the transfer of cells derived from Sle1b or Sle1b.BACLy108-H1 mice into bm12 recipients showed that Ly108-H1-expressing splenocytes or CD4+ T cells ameliorate autoreactive responses (Fig. 6, C and D; and Fig. S5, D and E). Furthermore, transferring Sle1b.BACLy108-H1 splenocytes or CD4+ T cells into bm12 recipients induces less CD4+ T cell activation (Fig. 6 E) than transfer of Sle1b-derived cells. Similarly, less B cell activation is found upon transfer of Sle1b.BACLy108-H1 cells compared with Sle1b cells (Fig. S5 F).

An in vitro experiment confirmed the notion that Ly108-H1 affects T cell proliferation when naive CD4+ T cells isolated from Sle1b, Sle1b.BACLy108-H1, and B6 mice were stimulated with limiting amounts of α-CD3. Under the conditions used, Sle1b CD4+ T cells responded with a robust proliferation (Fig. 6 F), suggesting that they are indeed intrinsically prone to undesirable immune activation. However, as predicted by our in vivo observations (Fig. 6 D and Fig. S5 E), this phenotype of naive CD4+ Sle1b T cells was dramatically reduced in naive Sle1b.BACLy108-H1 CD4+ T cells (Fig. 6 F). Similar results were obtained with measuring proliferation by CFSE dilution (Fig. S5 G).

Collectively, the outcomes of this set of experiments demonstrate that, in contrast to previous suggestions that Ly108 controls T and B cell development (Kumar et al., 2006; Kanta and Mohan, 2009), peripheral Sle1b CD4+ T cells play a major role in the pathogenesis of SLE. More importantly, the transfer of Ly108-H1—expressing Sle1b T cells causes significantly less disease than the transfer of the same number of Sle1b T cells. We conclude that a balanced expression of the Ly108-H1 isofrom in CD4+ T cells ameliorates SLE in Sle1b mice and may dampen in vivo and in vitro T cell activations.

**DISCUSSION**

In mice, genome-wide linkage studies have implicated the syntenic region to human 1q23 in three different models of spontaneous lupus: the (NZB × NZW)F2 intercross, the NZM/Aeg2410 New Zealand mice, and the BXSB mice (Kono et al., 1994; Rozzo et al., 1996; Hogarth et al., 1998). The phenotype of these mice is very similar to that in SLE patients, with the production of autoantibodies, as well as multiorgan involvement, including severe nephritis. In congenic mice derived from the NZM2410 mouse strain and B6, the locus on chromosome 1, i.e., Sle1, by itself was sufficient to generate a strong, spontaneous, humoral ANA response. Sle1 also led to an expanded pool of histone-reactive T cells. Thus, Sle1 may lead to the presentation of chromatin in an immunogenic fashion or directly impact tolerance of chromatin-specific B cells. Consequently, Sle1 is thought to be a major player in orchestrating selective loss of B cell and T cell tolerability to chromatin. Fine mapping of the Sle1 locus determined that three loci within this congenic interval, termed Sle1a, Sle1b, and Sle1c (Morel et al., 2001), could independently cause a loss of tolerance to chromatin, which is a necessary step for full disease induction. The Sle1b region, an ~0.9-Mb Slamf-haplotype 2-derived, i.e., NZW-derived, DNA segment which includes the Slamf locus, was implicated as a major contributor to the role of Sle1b in tolerance (Wandstrat et al., 2004).

Similar to the Sle1b mouse, B6.129chr1b mice also develop lupus (Bygrave et al., 2004; Carlucci et al., 2007). In these mice, the Slamf genes are derived from the 129
Figure 6. The presence of Ly108-H1 in Sle1b CD4+ T cells ameliorates SLE in a transfer model of SLE. (A) 5 × 10^6 splenocytes isolated from individual B6 or Sle1b mice were transferred into bm12 recipients by i.p. injection. After 4 or 10 wk, anti-dsDNA antibodies in the serum were determined by ELISA and were expressed as ELISA units. (B) 7 × 10^6 CD4+ T cells isolated from B6 or Sle1b mice were transferred into bm12 recipients by i.p. injection. After 4 or 10 wk, anti-ssDNA antibodies in the serum were determined by ELISA and are expressed as ELISA units (EU). (C and D) Statistical analyses were performed as in Fig. 1. Median values are determined SLE in a transfer model of SLE.

Several recent studies support a model in which T cells isolated from SLE patients surprisingly express protein isoforms that are infrequently found in cells from healthy individuals. For instance, unusual CD3-ζ and CD44 isoform mRNAs have been found in lupus patients, which lead to mutant CD3-ζ proteins that alter TCR/CD3 signaling (Nambiar et al., 2001; Tuszaka et al., 2003; Crispin et al., 2010). The experiments in this paper set out to demonstrate that elimination in genetically associated with lupus (genome-wide association study SNP), are highly expressed in patients but not in healthy individuals (Graham et al., 2006). Furthermore, only the short isoform of CD244 (SLAMF4), which is an alternative splicing product, was associated with lupus (Kim et al., 2010). How the alternate splice forms of mouse Ly108-1 and Ly108-2 are generated is not known in detail, but the presence of two 3′ untranslated region sequences in all healthy mice suggests that the decision process might be stochastic (Fig. S3 B). Although the presence or absence of Ly108-H1 protein marks the major difference between Ly108 in Sle1b and B6 mice, we cannot exclude the idea that subtle differences in expression of Ly108-1 and Ly108-2 contribute to cooperation between the three isoforms in a given cell. In addition to the two well-characterized isoforms, Zhong and Veillette (2008) recently

samples (10^6 cells/well) generated from the pool of two animals. The result is representative of two independent experiments (statistical analysis by two-tailed unpaired t test).
reported a rare transcript termed Ly108-3. This isoform, when ligated, mediates a tyrosine phosphorylation signal that is intermediate between Ly108-1 and Ly108-2. This protein isoform could not be detected in our experiments.

Transgenesis of the BACLy108-H1 vector encoding Ly108-H1 into Sle1b mice had a dramatic effect on the spontaneous development of lupus. Remarkably, expression of Ly108-H1 significantly decreased autoantibody titers, percentages of spontaneously activated T and B cells in the spleen, and expansion of germinal center B cells in aged Sle1b.BACLy108-H1 mice compared with their Sle1h littermate controls. Despite not completely reversing the autoimmune phenotype normally observed in Sle1b mice, our results indicate that Ly108-H1 is a major player in regulating Ly108-mediated autoimmunity. This lack of a full phenotype reversal can be explained in part by our semi-quantitative PCR data, which suggest that Ly108-H1 is expressed at lower levels in the hemizygous transgenic animals than in the B6 mouse (Fig. 4 B). Moreover, in Sle1b mice, the putative pathogenic isoform, Ly108-1, has an elevated expression level compared with the B6 mouse (Wandstrat et al., 2004), which might be difficult to overcome by inhibitory signals.

Although activation of both T and B cells was affected by the presence of Ly108-H1, we focused on the role of peripheral CD4+ T cells in transfer experiments. Although T cells in the Sle1 congenic mice show a broad range of autoimmune phenotypes (e.g., spontaneous CD4+ T cell activation, decreased number of T regulatory cells, presence of histone-specific T cells, and increased proliferation and cytokine production; Morel et al., 2001; Chen et al., 2005), the Sle1b subcongenic mice carry only a fraction of these defects (i.e., increased percentage of activated T cells and elevated calcium influx after receptor cross-linking [Wandstrat et al., 2004; Chen et al., 2005]). In this study, we show that the transfer of peripheral CD4+ cells derived from Sle1b mice into bm12 recipients induces T and B cell activation and autoantibody responses that are much more robust than after the transfer of B6 cells, work that directly links, for the first time, T cell–intrinsic phenotypes of Sle1b with lupus development. The transfer of Sle1b cells expressing Ly108-H1 results in a much lesser autoantibody and T cell responses compared with the responses to Sle1b cells. This ameliorating effect of Ly108-H1 is caused by a mechanism that results in dampening in vivo and in vitro T cell proliferation and not by reduced activation-induced cell death.

Recently, experiments with Sle1b-derived B cells led to the conclusion that Ly108-2, in contrast to Ly108-1, is able to sensitize immature B cells to deletion (Kumar et al., 2006). Preliminary transfections in WEHI-231 cells (unpublished data) suggest that in contrast to Ly108-2, Ly108-H1 does not affect apoptosis. In the model in Fig. 7, we therefore hypothesize that Ly108-1 is a pathogenic allele that operates in immature B cells and peripheral T cells. Ly108-2 and the novel isoform Ly108-H1 are both associated with disease protection: whereas Ly108-2 contributes to sensitizing T and B cells to apoptosis, Ly108-H1 is an effective suppressor of pathogenic T cell proliferation in Sle1b. Based on these functional differences between the Ly108 isoforms, it is likely that Ly108-H1 mediates distinct inhibitory signaling events rather than passively interfering with signals initiated by Ly108-1 and Ly108-2. This hypothesis is supported by the observation that the BACLy108-H1 transgene only causes a small increase in total Ly108 surface expression on peripheral CD4+ T cells, making it improbable that Ly108-H1 is affecting clustering of the other Ly108 molecules in the immunosynapse. Additionally, expression of the Ly108-H1 transgene dampens proliferation in CD4+ T cells, whereas Ly108-/- mice maintain normal levels of proliferation (Howie et al., 2005; unpublished data generated with B6.Ly108-/-), further supporting the notion that Ly108-H1 is an active signaling molecule. As yeast two-hybrid screenings have identified that both ITSMs, associated with the Y295 and Y319 of Ly108, are capable of binding SAP (Fraser et al., 2002), it is likely that Ly108-H1, which also contains one of these motifs, is also capable of recruiting the adapter protein SAP. Additionally, as Ly108 was recently shown to participate in stabilizing T cell–B cell conjugate formation in a SAP-binding dependent manner (Cannons et al., 2010), dissecting the role of Ly108 isoforms in cell–cell networking processes that govern autoantibody production could be an exciting area for further investigations. As an interest in gene isoform–dependent mechanisms is rapidly increasing and because isoform expression appears to be altered in lupus patients, the outcomes of our experiments relate to a larger concept that an interplay between isoforms provides for a plethora of regulatory possibilities in developmental biology, as well as in pathogenesis of diseases.

MATERIALS AND METHODS

Mice. B6, NOD/LtJ, MOLF/EfJ, BALB/c, and B6.C-H-2bm12/KhEg (bm12) mice were obtained from the Jackson Laboratory. 129/SvEvTac (129) mice were obtained from Taconic. B6.Sle1b (Sle1b; Morel et al., 2001) mice were provided by M. Botto (Imperial College London, London, England, UK). B6.Sle1b (Sle1b; Morel et al., 2001) mice were provided by L. Morel (University of Florida, Gainesville, FL). Ly108-/- [129 × B6] (Howie et al., 2005) was backcrossed six times with B6, and breeders were selected for the smallest congenic interval. Animal experiments were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

![Figure 7. Schematic outline of the proposed role of the Ly108 protein isoforms in the pathogenesis of disease in Sle1b mice.](image-url)
As described by Morris et al. (1990), Ly108 was precipitated with BS-BSA–precoated Immunolon (Dynatech) plates were incubated with purified CD62L+ naïve T cells, or purified CD62L− CD4+ naïve T cells (magnetic bead cell separation; Miltenyi Biotec) from age- and sex-matched donors.

**Generating the SleI.BACly108-H1 mice.** RP23-77A8 BAC clone, containing full-length B6 Ly108, CD84, and SLAM, was obtained from the BACPAC Resources Center at Children's Hospital Oakland Research Institute. Exons 7–8 and the surrounding intronic region of the Ly108 gene were removed by Red/ET recombination (Muyrers et al., 1999) using a commercial kit (Gene Bridges GmbH) according to the manufacturer's protocol. In brief, S1 (5'-TTAACGACCATGCTCATAGTGAAGCAG-3') and S2 (5'-AATACACAGATGAAGCACTTAGTTGTTACATTTTG-3') homology arms were used to target a 2.8-kb fragment containing Ly108 exons 7 and 8 and replacing it with a single Pmel site. To remove a 130-bp genomic region containing SLAM and CD84, we performed a second recombination step using S3 (5'-AGGTCTCATCTATCTAACTCTTCCGATGAGTCA-3') and S4 (5'-GTTATGGACATGCTGATCTCATGTGCATTGGAATGATTC-3') homology arms. Vector was injected into SleI oocytes by the Beth Israel Deaconess Transgenic Core Facility. Screening of founders was performed by PCR.

**Murine transfer model of lupus.** As described by Morris et al. (1990), 7–9-wk-old naïve female b6.12 mice were injected i.p. with spleenocytes, purified CD4+ T cells, or purified CD62L+ CD4+ naïve T cells (magnetic bead cell separation; Miltenyi Biotec) from age- and sex-matched donors.

**RT-PCR.** RNA was isolated from cells using the RNaseasy kit (Qiagen) or TRIzol (Invitrogen). RT was performed with the ProtoScript cDNA kit (New England Biolabs, Inc.). A full-length RT-PCR was performed on cDNA templates with the following primers with introduction of restriction sites: 5'-XhoI-Ly108-1/2-H1 start, 5'-GGCTTCAGATGGCTGGT-CATCTACAGGTCTG3'-3' Ly108-1 end XbaI, 5'-GGTCTCAGATTTAGATGGGATTCAATTGGAATGTTG3'-3' and 3'-Ly108-2/H1 end XbaI, 5'-GGCTTCTAGATCGAGTTATAGGTGTTAATTGGAATGTTG3'-3'. A shorter PCR was performed for better separation of the isoforms with the following primers: 5'- (in exon 5) 5'-TTTGCAGCATCCACTCC-3'; 3'- Ly108-1, 5'-TTAACGACCATGCTCATAGTGAAGCAG-3'; and 3'- Ly108-2/H1, 5'-TGCAGGATTTATGGGATGATG-3'. RT-PCR for selective amplification of Ly108-H1 was performed with the primers Ly108-H1 forward, 5'-CCTACCTCTCTCTCTCTCTCTCTCT-3', and Ly108-H1 reverse, 5'-CCTGTTAAGGACCTACTTCTCTCTCT-3'. GAPDH-specific primers were obtained from New England Biolabs, Inc.

**Expression vectors.** Ly108 isoforms were amplified by PCR with primers introducing XhoI and XbaI restriction sites and cloned into pCR2.1-TOPO (Invitrogen) before subcloning into the mammalian expression vector PCDM. (Promega). Ly108-1 and Ly108-2 cDNAs were provided by E. Ruley (Vanderbilt University School of Medicine, Nashville, TN; Peck and Ruley, 2000). Ly108-H1 was amplified from B6 thymus cDNA.

**Cell transfection.** Ly108 isoforms were transfected into 1–2 × 10^7 cells by electroporation (250 V and 960 μF) with 10 μg plasmid DNA in 400 μl OptiMEM (Invitrogen) using a cuvette with a 4-mm electrode gap (Bio-Rad Laboratories) and analyzed 24 h later.

**Flow cytometry.** Single cell suspensions of spleens and thyromes were stained with the following antibodies after blocking nonspecific binding with CD16/32 (93) and 20% rabbit serum or 10% rabbit serum, respectively: α-CD3 (17A2), α-CD4 (L3T4), α-CD8-α (53–67), α-CD19 (1D3), α-CD21 (eBio4E3), α-CD23 (B3B4), α-CD44 (IM7), α-CD62L (Mel-14), α-CD69 (H1.2F3), α-CD86 (GL-1), α-CD138 (281–2), α-B220 (RA3-6B2), α-Fas (Jo2), α-GL7 (GL-7), α–TCR-β (H57–597) purchased from eBioscience, BD, or BioLegend. PB857-loaded CD14 tetramer was provided by the National Institutes of Health tetramer facility. When surface Ly108 staining was performed, we used cytS- or DyLight 660–conjugated anti-Ly108 (13G3) or IgG2a isotype control. Data were acquired with a cytometer (LSRII; BD) and analyzed using FlowJo software (Tree Star). Dead cells were excluded upon DAPI uptake. For intracellular staining, we used IFN-γ (XMG1.2) and IL-2 (JES6-514) antibodies (from BioLegend and BD, respectively) after 5-h PMA (50 ng/ml) and ionomycin (1 μg/ml) activation and cell permeabilization (BioLegend kit). When aged mice from a cohort were analyzed on different days (Fig. 1 B), each flow cytometry assay included at least one age-matched WT control.

**ELISA.** Titer of antinucleosome (antihistone–DNA complex) antibodies in mouse sera were determined by ELISA as described previously (Mohan et al., 1998). In brief, met-BSA–precoated Immunomul (Dynatech) plates were selected based on reactivity with Ly108-Fc, as judged by ELISA.

**Polyclonal antibodies.** Antiseras R1 and R4 directed at the cytoplasmic tails of Ly108 isoforms were generated by immunizing female New Zealand white rabbits with peptides conjugated via the N-terminal cysteine to KLH (Thermo Fisher Scientific). Antiserum R1, which recognizes both Ly108-1 and Ly108-2, was generated against the peptide Cys-KNDSMTIY-SIVNHKRE. Antiserum R4, recognizing Ly108-2 and Ly108-H1, was directed against the peptide Cys-ALTGYQNPIKLKVNLNINGYNs.

**Immunoprecipitation and Western blotting.** Ly108 was precipitated from cell lysates with α-Ly108 (13G3) and protein G agarose (Invitrogen). Purified proteins were resolubilized before deglycosylation at 37°C for 2 h with immobilized carbohydrate binding domain–PNGaseF fusion protein (CBM-PNGaseF), donated by A. Warren (University of British Columbia, Vancouver, British Columbia, Canada). Isoforms were separated on a 12% continuous SDS–PAGE gel with MOPS (morpholino propane sulfonic acid) running buffer (Invitrogen). After transfer to polyvinylidene fluoride membrane, Western blotting was performed with the indicated rabbit primary and anti-rabbit, light chain–specific secondary horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch Laboratories, Inc.). Reactivity was detected by chemiluminescence with SuperSignal (Thermo Fisher Scientific).

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**Genotypic analysis.** Genomic DNA of Ly108−/− [129 × B6]) and B6.129/chrlb mice was analyzed with polymorphic genetic markers. PCR was performed using 10 markers on chromosome 1 and at least 1 marker per other chromosomes. Primer sequences were obtained from the Mouse Genome Informatics database. Fine mapping of congeneric boundaries was performed by the Jackson Laboratory (JAX Mouse Diversity Genotyping Array Service). In brief, mouse genomic DNA was labeled and hybridized on a custom Affymetrix array. The mean resolution of the analysis was one SNP every 4.3 kb.
coated overnight with dsDNA and then with total histone solutions (Sigma-Aldrich). Samples were incubated on plates in various dilutions between 1:600 and 1:1,200, and then plates were washed, and autoantibodies were detected with anti-mouse IgG-HRP (GE Healthcare).

Autoantibody titer was expressed as ELISA units, comparing OD values of samples with a standard curve prepared with serial dilutions of ANA-positive NZM2410 serum pool. Antichromatin and anti-dsDNA titers were determined as for the antinucleosome levels, except for the preparation of ELISA plates. UV-irradiated Immunolun plates were incubated overnight with 3 μg/ml chicken chromatin (Cohen and Maldonado, 2003) or mung bean nuclease (New England Biolabs, Inc.)–treated dsDNA (Sigma-Aldrich). Anti–single-stranded DNA (ssDNA) was determined as described previously (Walter et al., 2010).

**ANA.** Specificity of autoantibodies was determined by indirect immunofluorescence using permeabilized HEP–2 cells (Antibodies Inc.). After incubation with various dilutions of mouse sera, HEP–2 slides were developed with anti-mouse IgG F(ab′)2 secondary antibodies. Quantitative analysis was performed by acquiring fluorescent images (Axioimager M1; Carl Zeiss) and determining main fluorescent intensity of HEP–2 nuclei (Volocity; PerkinElmer).

**T cell proliferation assay.** Splenic naive CD62L+ CD4+ T cells were purified using magnetic cell purification (Miltenyi Biotec) and activated by 0.3 μg/ml plate-bound anti-CD3 (145–2C11) and 1 μg/ml anti-CD28 (37.51) for 3 d on 96-well plates. Proliferation was assessed by incorporation of [3H]thymidine (1 µCi/well), which was added for the last 16 h of each culture, or by CFSE (Invitrogen) dilution by loading cells according to the manufacturer’s protocol.

**Metaphase fluorescent in situ hybridization.** Metaphase chromosome preparations were derived from 10 μg/ml LPS (Sigma-Aldrich)–activated splenocytes. RP23-77A8 (SLAM, CD84, and Ly108) BAC clone was labeled with the biotin–nick translation method (Roche) and hybridized overnight with the metaphase preparations. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinconjugated streptavidin followed by DAPI counterstaining.

**Online supplemental material.** Fig. S1 shows the generation and description of Ly108−/− (B6) mice and the boundaries of the 129 segments in congenic Ly108−/− (B6 × 129) and B6.129chr1b mice. Fig. S2 supports protein expression experiments in Fig. 2 A. Fig. S3 describes the Sle1b BAC clone and determining functional expression experiments in Fig. 2 A. Fig. S4 describes the genetic difference between haplotype 1 and haplotype 2 mouse strains, which leads to the selective expression of Ly108-H1. Fig. S5 supports autoantibody and in vitro proliferative data of Fig. 6. Table S1 describes cellular changes in aged B6, Sle1b, and Sle1b:BACLy108-H1 mice. Table S2 provides information about the congenic breakpoint SNP markers. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101653/DC1.

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