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Characterization of Ly108 in the Thymus: Evidence for Distinct Properties of a Novel Form of Ly108

Mala Dutta* and Pamela L. Schwartzberg†

Ly108 (CD352) is a member of the signaling lymphocyte activation molecule (SLAM) family of receptors that signals through SLAM-associated protein (SAP), an SH2 domain protein that can function by the recruitment of Src family kinases or by competition with phosphatases. Ly108 is expressed on a variety of hematopoietic cells, with especially high levels on developing thymocytes. We find that Ly108 is constitutively tyrosine phosphorylated in murine thymi in a SAP- and Fyn kinase-dependent manner. Phosphorylation of Ly108 is rapidly lost after thymocyte disaggregation, suggesting dynamic contact-mediated regulation of Ly108. Similar to recent reports, we find at least three isoforms of Ly108 mRNA and protein in the thymus, which are differentially expressed in the thymi of C57BL/6 and 129S6 mice that express the lupus-resistant and lupus-prone haplotypes of Ly108, respectively. Notably, the recently described novel isoform Ly108-H1 is not expressed in mice having the lupus-prone haplotype of Ly108, but is expressed in C57BL/6 mice. We further provide evidence for differential phosphorylation of these isoforms; the novel Ly108-H1 does not undergo tyrosine phosphorylation, suggesting that it functions as a decoy isoform that contributes to the reduced overall phosphorylation of Ly108 seen in C57BL/6 mice. Our study suggests that Ly108 is dynamically regulated in the thymus, shedding light on Ly108 isoform expression and phosphorylation.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DP, double positive; IP, immunoprecipitate; ITSM, immunoreceptor tyrosine-based switch motif; PNGase F, peptide N-glycosidase F; RIPA, radioimmunoprecipitation assay buffer; SAP, SLAM-associated protein; SH2, Src homology domain 2; SHIP, SH2 domain containing phosphatase; SLAM, signaling lymphocyte activation molecule; SP, single positive; XLP, X-linked lymphoproliferative disease.

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XLPI and revealed phenotypes not previously appreciated, including defects in germlinal center formation and T cell–B cell interactions (35–37), as well as a lack of NKT cells and other innate-like T cell populations that provide a first-line defense against infection (38, 39). Patients with XLPI exhibit similar defects, including a lack of germinal centers and of NKT cells (38–42). Interestingly, studies in gene-targeted mice have implicated Ly108 in both these phenotypes (37, 43).

The involvement of Ly108 in NKT cell development suggests that Ly108 actively signals during thymic development (43). To examine Ly108 signaling in the thymus, we evaluated Ly108 protein and phosphorylation status in the intact thymus and in isolated thymocytes. We have found that Ly108 is constitutively phosphorylated in the thymus in a SAP and Fyn-dependent manner. Ly108 phosphorylation is rapidly lost upon disaggregation of thymocytes, suggesting that Ly108 is constitutively engaged and dynamically regulated in the thymus. Evaluation of Ly108 message and protein revealed multiple forms that are differentially expressed in lupus-prone and resistant strains of mice. Interestingly, a recently described novel isoform, Ly108-H1, is uniquely expressed in lupus-resistant C57BL/6 mice and is not tyrosine phosphorylated despite the presence of an ITSM motif, suggesting that it acts as a decoy-receptor and alters signaling downstream from Ly108.

**Materials and Methods**

**Abs**

Unconjugated, PE, or biotin-conjugated mouse mAb directed against Ly108 extracellular domain was obtained from eBioscience. Ly108 polyclonal antiserum were generated by Covance Research Products by immunizing rabbits with KLH-conjugated peptides and affinity purified from peptide-conjugated N-hydroxysuccinimide–activated Sepharose columns. Pan-Ly108 rabbit polyclonal Ab was generated using a C terminus–specific peptide CLGESAVLPLKPAKIA. Ly108-2 rabbit polyclonal Ab was generated using a C terminus–specific peptide CKKREETVALTGYNQ-CLGESA VLPLKLPAGKIA. Ly108-2 rabbit polyclonal Ab and incubated at 4˚C with protein A beads for 2 h.

**Mice**

Mice were maintained and used in accordance with the Institutional Animal Care and Use Committee at the National Human Genome Research Institute, National Institutes of Health. SH2Di+/− (SAP-deficient) mice were described previously (44). C57BL/6 mice were obtained from The Jackson Laboratory, and MHC class I- and class II-deficient (B6.129-H-Ab1m1Cru B2m1Hex) (45) mice were obtained from Taconic Laboratories. Ly108-+/− mice were generated from the HGTG-8 C57BL/6 ES cell line and do not express Ly108, as detected by flow cytometry (Z. Kraus, M. Dutta, E.K. Wakeland, and P.L. Schwartzberg, manuscript in preparation).

**Cell lines and cloning**

Ly108-1, Ly108-2, or novel Ly108-H1 isoform were amplified from cDNA made from splenocyte RNA of C57BL/6 mice using the following primers: Ly108 F 5′-AACGCAGTACCATcagttgcagacctgagacgca-3′; Ly108-1 R 5′-TTGCTGTATACAgatgtagctgctggttacga-3′, and Ly108-2 R 5′-TTGCTGTATACAgatgtagctgctggttacga-3′. The Ly108-2 primers also amplified the novel Ly108-H1 isoform, PCR products were cloned in pCTAP (Stratagene) using EcoV/BamHI sites and transfected into the mouse thymoma EL-4 cell line; stable transfectants were selected in G418. Human SH2DiA cloned in a MIGR retroviral vector (46) was used to infect stable cell lines. High Ly108- and SAP-expressing clones were sorted on surface Ly108 and GFP levels, cloned by limiting dilution, and maintained under G418 selection.

**Results**

**Ly108 expression on thymocytes**

To evaluate Ly108 in the thymus, we first examined surface Ly108 expression on thymocytes by flow cytometry. We found that Ly108 is highly expressed on thymocytes from C57BL/6 mice, with greatest expression on double-negative and DP thymocytes, and downregulation at the single-positive (SP) stage (Fig. 1A). Lowest expression was seen on the most mature HSA lo SP thymocytes (Fig. 1B). Similar results were obtained when Ly108 was evaluated on thymocytes by intracellular staining, suggesting that total Ly108 protein was decreased (Supplemental Fig. 1A). The downregulation during development correlated with reduced Ly108 mRNA at SP stages (Supplemental Fig. 1B).
To further examine Ly108 protein, we immunoprecipitated Ly108 using an Ly108 mouse mAb and Western blotting with a pan-Ly108 rabbit polyclonal Ab directed against the extracellular domain of Ly108. Ly108 protein ran as a diffuse band between 60 and 85 kDa that was present in WT and SAP-deficient thymocytes, but absent in the immunoprecipitates of Ly108−/− thymocytes, confirming the specificity of the polyclonal Ab (Fig. 1C). Ly108 has multiple spliced message isoforms that are predicted to generate proteins between 35 and 40 kDa. Analysis of Ly108 using the glycosylation prediction software NetGly 1.0 server (Technical University of Denmark) predicted seven N-linked glycosylation sites (N82, N101, N112, N152, N159, N193, and N218), but no O-linked sites in the extracellular domain, potentially contributing to its diffuse, slow migration pattern. After treatment of immunoprecipitates with PNGase F and immunoblotting with the pan-Ly108 rabbit polyclonal, the diffusely migrating band now collapsed into several distinct bands, some of which migrated in the predicted m.w. range of the Ly108 isoforms (Fig. 1D). Thus, Ly108 is a heavily glycosylated protein that is developmentally regulated in thymocytes.

Contact-mediated Ly108 phosphorylation in intact thymus

Using isolated thymocytes, Zhong and Veillette (15) have shown previously that Ly108 can be tyrosine phosphorylated in response to stimulation by cross-linking with anti-Ly108. Because Ly108 is a self-ligand that is expressed on most thymocytes, we postulated that Ly108 might be constitutively stimulated in the intact thymus because of thymocyte–thymocyte interactions. To evaluate this hypothesis, we directly lysed intact thymus was heavily tyrosine phosphorylated (Fig. 2B). This loss of tyrosine phosphorylation occurred rapidly (within minutes), as soon as thymocytes were dissociated into single-cell preparations. Ly108 phosphorylation could not be re-capitated by merely pelleting the cells together at 37°C (data not shown), but could be reintroduced with pervanadate treatment.

In contrast, TCR-ζ, a protein known to be constitutively tyrosine-phosphorylated in the thymus secondary to TCR engagement with self-MHC (47), was still tyrosine phosphorylated in lysates from single-cell preparations (Fig. 2B, lower panels), which is consistent with previous reports that ζ phosphorylation is lost only after several hours of incubation at 37°C (47, 48). Therefore the regulation of Ly108 phosphorylation appears to be contact mediated and highly dynamic.

To examine whether Ly108 phosphorylation leads to downstream signaling, we examined SAP association. SAP was found to coimmunoprecipitate with Ly108 in both samples from intact thymus as well as pervanadate treated thymocytes, but not from thymocytes in suspension where Ly108 was not phosphorylated (Fig. 2B). These findings differ from those seen with SLAM, where SAP can also be found in association with unphosphorylated ITSMs (49–52).

Ly108 phosphorylation is reduced in the absence of Fyn or SAP

Previous work has demonstrated that SAP and the Src family tyrosine kinase Fyn are required for the induction of Ly108 phosphorylation in response to cross-linking Ab in vitro (15). To evaluate requirements for phosphorylation in vivo, we examined Ly108 phosphorylation in SAP- and Fyn-deficient thymi. Ly108 phosphorylation was completely absent in SAP-deficient thymi and was dramatically reduced in Fyn−/− thymi (Fig. 2C). These data indicate the critical requirement of SAP for Ly108 phos-
phorylation, and implicate Fyn as a key kinase that phosphorylates Ly108 in intact thymi. Nonetheless, the presence of some residual phosphorylation suggests that other tyrosine kinases also contribute to the Ly108 tyrosine phosphorylation. Parallel to the tyrosine phosphorylation of Ly108, coimmunoprecipitation of SAP was drastically reduced in \textit{Fyn} \textsuperscript{−/−} thymi, although some association was still consistently detected (Fig. 2C). SAP was not detected in control IPs from Ly108-deficient thymi, demonstrating the specificity of these coimmunoprecipitations.

Induction of Ly108 phosphorylation is potentiated by concomitant \(\alpha\)-CD3 stimulation

As Ly108 phosphorylation was rapidly lost in single thymocyte suspensions, we evaluated the requirements for induction of Ly108 phosphorylation in cell suspensions using stimulation by crosslinking anti-Ly108 with anti-mouse IgG. We found that induction of tyrosine phosphorylation of Ly108 occurred with relatively slow kinetics, reaching a peak by 15–30 min (Fig. 3A). The peak tyrosine phosphorylation signal appeared to be reduced by 60 min, which also corresponded with reduced Ly108 protein. Induction of Ly108 tyrosine phosphorylation was severely diminished in the absence of SAP and Fyn (Fig. 3B), as reported previously.

To determine whether TCR stimulation, which rapidly activates the Src family kinases Lck and Fyn, contributes to Ly108 phosphorylation, we first examined Ly108 phosphorylation in thymi from mice lacking MHC class I and II, in which the TCR is not engaged. Ly108 phosphorylation was only modestly decreased in thymi from these mice (Fig. 2C). However, as these mice lack mature SP thymocytes, we could not rule out that altered cell populations contributed to any changes in Ly108 phosphorylation. To further examine the effect of TCR stimulation on Ly108 phosphorylation, we cross-linked both Ly108 and the TCR using Abs directed against Ly108 and \(\alpha\)-TCR \(\varepsilon\). We observed that the kinetics of Ly108 stimulation could be potentiated by concomitant TCR stimulation, with the peak Ly108 phosphorylation occurring earlier (7 min) than with Ly108 engagement alone (Fig. 3C, Supplemental Fig. 1D). Thus, TCR engagement helps to potentiate Ly108 phosphorylation.

Ly108 isoforms in C57BL/6 mice

Ly108 encodes multiple isoforms that are generated by alternative splicing affecting the C-terminal cytoplasmic tail (13, 15). Previous data have demonstrated differential expression of Ly108 isoforms in lupus-prone and lupus-resistant strains of mice (13).
To further our understanding of Ly108, we cloned Ly108 cDNA from C57BL/6 mice (Fig. 4A). Similar to initial descriptions of Ly108, we obtained cDNAs encoding Ly108-1 and Ly108-2, which encode proteins that are identical in the N-terminus but vary in the C-termini, resulting in different numbers of ITSMs and non-ITSM tyrosine residues. Ly108-1 (36.4 kDa) has two classical ITSMs (Y295 and Y319) and a non-ITSM tyrosine (AEYS), whereas Ly108-2 (38.6 kDa) has two classical ITSMs (Y295 and Y319) plus one additional nonclassical ITSM (Y335) and another non-ITSM Y (Y349 NYNS). We also identified a novel Ly108-H1 spliced isoform related to the Ly108-2 isoform, which had deleted exon VII encoding one classical ITSM (Y319; Fig. 4A, Supplemental Fig. 2). During the course of our studies, this isoform was also reported by Keszei et al. (16), who designated this species novel Ly108-H1. The lack of this isoform in the lupus-prone haplotype was also confirmed in lupus-prone congenic B6.Sle1b mice that express the lupus-prone SLAM haplotype. Examination of these isoforms by quantitative RT-PCR confirmed that 129S6 mice expressed significantly higher levels of Ly108-1, while expressing similar levels of Ly108-2 isoform as C57BL/6 mice (Fig. 4B). These finding differ slightly from those of Wandstrat et al. (13), who found higher levels of Ly108-2 expressed from the lupus-resistant SLAM haplotype, but the primers used in their study would also amplify Ly108-H1. Notably, using isoform-specific Taqman probes, we found that expression of the novel Ly108-H1 was restricted to C57BL/6 mice that express the lupus-resistant haplotype. Similar results were recently reported by Keszei and colleagues (16).

To start to evaluate the function of the distinct isoforms, we subcloned these isoforms into the pCTAP vector and generated...
stable clones expressing the individual isoforms in the mouse thymoma cell line EL-4 (Fig. 4C). Similar to the endogenous protein, Ly108 expressed in these cell lines appeared as slowly migrating species, which when deglycosylated showed distinct bands of sizes corresponding to the predicted fusion protein products. Western blotting with a pan-Ly108 rabbit polyclonal Ab directed against the N-terminus (extracellular domain) of Ly108 confirmed expression of species having the predicted sizes of the tagged proteins: 44.4, 46.6 and 43.8 kDa, corresponding to Ly108-1, Ly108-2, and the novel Ly108-H1 isoform, respectively (Fig. 4C, top panel). Of note, this Ab was seen to react more strongly with the deglycosylated species of Ly108.

To further evaluate these distinct isoforms, we generated rabbit polyclonal Ab directed against the C-terminus of Ly108. Although we were unable to obtain a Ly108-1–specific Ab, we were able to obtain an Ly108-2 polyclonal Ab directed against a sequence located in the Ly108-2 isoform. This sequence is also represented in the novel Ly108-H1 isoform (Fig. 4A for sequence locations). This Ab recognized the Ly108 species in the cell lines expressing Ly108-2 or novel Ly108-H1, but not the Ly108-1 isoform (Fig. 4C, bottom panel).

Examination of immunoprecipitated Ly108 protein from thymocytes treated with PNGase F from C57BL/6 and 129S6 mice further confirmed that 129S6 mice were largely missing a species that corresponded to the predicted size of the novel Ly108-H1 isoform. Moreover, as predicted, this smaller form reacted with anti–Ly108-2 (Fig. 4D).

Expression and phosphorylation of Ly108 isoforms in C57BL/6 and 129S6 mice

Given the differences in expression of the splice isoforms in 129S6 and C57BL/6 mice, we used these two strains to compare the expression and phosphorylation of Ly108. Surprisingly, DP thymocytes from C57BL/6 mice expressed levels of Ly108 on their surface more than 3-fold higher than 129S6 thymocytes (Fig. 5A). Examination of CD4 and CD8 SP cells revealed that 129S6 mice exhibited a broader range of expression, with some cells having very low levels of Ly108, so that there was an even greater difference in the mean fluorescent intensity of Ly108 expression between C57BL/6 and 129S6. These differences were confirmed with Western blotting. Whereas levels of the downstream signaling molecule Fyn appeared equivalent in thymocytes from these
mouse strains, Ly108 levels were considerably lower in 129S thymocytes (Fig 5A). Lower levels of Ly108 expression were also observed in congenic B6.Sle1b mice (16; M. Dutta, data not shown).

Despite lower expression levels of Ly108 in 129S6 thymi, we found that Ly108 immunoprecipitated from intact 129S6 thymi was more heavily phosphorylated than that from C57BL/6 thymi, when normalized for protein levels (Fig. 5B). To evaluate which isoforms contributed to this increase, we used the Ly108 mouse mAb, which recognizes all the isoforms, as well as specific Ly108-2 polyclonal Ab to immunoprecipitate Ly108 from intact thymi of C57BL/6 and 129S6 mice (Fig. 6). After deglycosylation, tyrosine-phosphorylated species were compared by Western blotting with 4G10 and the pan anti-Ly108 (bottom panel). Loading of IPs was adjusted to normalize levels of Ly108. Relative intensities of pLy108/Ly108 are graphed. Data are representative of three or more experiments.

Ly108 isoforms are differentially phosphorylated

In order to evaluate the phosphorylation potential of each isoform in isolation, we took advantage of the Ly108 isoform-expressing EL-4 cell lines that were cotransfected with SAP. Because we have been unsuccessful in stimulating Ly108 activity by Ab cross linking in these cell lines, the single-isoform—expressing cell lines were treated with pervanadate, followed by Ly108 immunoprecipitation. When adjusted for total amount of protein, the Ly108-1 isoform phosphorylated more efficiently than the Ly108-2 isoform (Fig 6C, top), in agreement with a previous report examining Tacchimeric versions of Ly108 (15). Consistent with these patterns of phosphorylation, SAP association was also increased with Ly108-1 when compared with Ly108-2 isoform (Fig. 6C, bottom). Surprisingly, although the novel Ly108-H1 isoform was expressed, we did not find evidence for its tyrosine phosphorylation, even when overloading the sample to increase its detection (Fig. 6C). Despite the apparent lack of tyrosine phosphorylation, some SAP association could be detected with the novel Ly108-H1 isoform (Fig. 6C, bottom).

Evaluation of the deglycosylated immunoprecipitated proteins from cell lines confirmed the results from intact thymi; the Ly108-1 isoform was more heavily phosphorylated, and moreover the migration of the pLy108-1 resembled that of the pLy108-2 isoform (Fig. 6D). Furthermore, we again did not see evidence for tyrosine phosphorylation of the novel Ly108-H1 isoform, even with overloading of this sample. These data indicate that Ly108-1 isoform is the most highly phosphorylated isoform of Ly108, whereas the novel Ly108-H1 isoform either fails to be or is poorly phosphorylated.

Discussion

Ly108 has been implicated in the development of NKT cells, the regulation of humoral immunity, and T and NK cell cytotoxicity; however, despite its involvement in these important immunologic processes, little is known about Ly108 regulation and signaling. We present evidence that Ly108 is dynamically regulated in the thymus, both at the level of expression and tyrosine phosphorylation.

**FIGURE 5.** Differential expression and phosphorylation of Ly108 in thymocytes from C57BL/6 and 129S6 mice. (A) Thymocytes from C57BL/6 and 129S6 mice were stained for surface expression of CD4, CD8, and Ly108 (left). Ly108 was immunoprecipitated from C57BL/6 and 129S6 thymocytes using anti-Ly108 mouse mAb and was immunoblotted using pan anti-Ly108 (top right). Total Fyn levels in these lysates were compared using aFyn (bottom right). (B) Two examples of Ly108 immunoprecipitated from intact C57BL/6 and 129S6 thymi using Ly108 mAb and immunoblotted for phosphorytrosine, 4G10 (top panel), and pan anti-Ly108 (bottom panel). Loading of IPs was adjusted to normalize levels of Ly108. Relative intensities of pLy108/Ly108 are graphed. Data are representative of three or more experiments.
We further extend data supporting distinct patterns of phosphorylation of different Ly108 isoforms that are differentially regulated in lupus-prone and resistant strains of mice. Importantly, we provide evidence that the novel Ly108-H1, which is associated with protection from lupus, is not tyrosine phosphorylated in the thymus. The implication of Ly108 as one of the SLAM family members involved in NKT cell development suggests that Ly108 is activated during thymic development. Indeed, the widespread expression of Ly108 and its decreased expression in SP thymocytes raises the possibility that it may be involved more broadly in thymocyte differentiation. We found that Ly108 is constitutively phosphorylated in the thymus, suggesting that at least a portion of Ly108 is engaged in the intact organ. We also find evidence of Ly108 phosphorylation in intact lymph nodes, although less pronounced, demonstrating that Ly108 can be found phosphorylated in the periphery as well. Surprisingly, Ly108 phosphorylation appears to be tightly regulated. As soon as thymocyte interactions were disrupted by making a single-cell preparation, Ly108 phosphorylation was lost. This dynamic control of Ly108 phosphorylation was in contrast to phosphorylation of TCR-ζ.
which is relatively stable for several hours after thymic disaggregation.

The rapid loss of Ly108 phosphorylation when cellular contact is lost upon thymic disassociation is a distinctive feature of Ly108 and suggests the rapid action of a phosphatase. Indeed, although SAP has been implicated in the recruitment of the tyrosine kinase Fyn, initial work on SLAM and SAP suggested that SAP could act as a competitor for negative regulatory molecules, such as SHP-1 and SHIP. In this regard, it is of interest that Ly108 has been found to bind and activate SHP-1 in the absence of SAP (22; R. Zhao, J.L. Cannons, M. Dutta, G.M. Griffiths, and P.L. Schwartzberg, submitted for publication). Although we have not been able to detect coimmunoprecipitation with phosphatases in the thymus, the rapid dephosphorylation of Ly108 suggests that a phosphatase is in close proximity—either directly binding to Ly108 or as part of a complex. Whereas phosphorylation of Ly108 is dependent on SAP and partially dependent on Fyn, the net phosphorylation status of Ly108 is likely to be the result of a balance of the actions of kinases such as Fyn and phosphatases such as SHP-1.

Although Fyn appears to be the major kinase responsible for Ly108 phosphorylation, we consistently observed some residual phosphorylation in Fyn-deficient thymi, suggesting that other tyrosine kinases also can participate in Ly108 phosphorylation. Such data are also consistent with data from Snow et al. (22), who found that knockdown of Ly108 prevented restimulation induced cell death in mature T cells, whereas knockdown of Fyn did not. In vitro evidence suggests that Lck can also interact with SAP; therefore, Lck may be one such kinase that can also contribute to Ly108 phosphorylation. Consistent with a role for Lck, we have found that tyrosine phosphorylation of Ly108 is potentiated by CD3 engagement, which leads to rapid and potent Lck activation. Although we do not know whether CD3 coengagement leads to the same patterns of phosphorylation of Ly108 tyrosine motifs, such potentiation of Ly108 signaling by TCR stimulation could also be envisioned if the signaling pathways of these two receptors intersect. Recently, it was reported that 2B4 can use ITAM motif-containing proteins to influence signaling in NK cells (53). It will also be of interest to see whether Ly108 intersects with TCR signaling pathways to cause synergistic downstream effects. Such a potential intersection of signaling would be consistent with a role for Ly108 in affecting T cell development. The kinetics of Ly108 phosphorylation and dephosphorylation also offers some insight into the regulation of Ly108 signaling in the thymus. Although the phosphorylation of Ly108 is slow to peak, its dephosphorylation occurs rapidly. It is possible that such a tight regulation of signaling offers stringency to the thymocyte-thymocyte communication process by allowing only longer duration cell interactions to result in productive signaling. It is possible to speculate that this feature may contribute to the effects of Ly108 on NKT cell development.

During the course of our study, we often observed a reduction in total Ly108 protein as tyrosine phosphorylation was induced either by pervanadate treatment or anti-Ly108 stimulation (Fig. 3A). This pattern of increased phosphorylation accompanied by reduced protein levels was also observed in intact thymi of 129 mice (Fig. 5A, 5B). Whether this reduction of Ly108 upon phosphorylation is the result of induced degradation, a general phenomenon by which many signaling pathways are regulated, is an interesting question.

Ly108 has been implicated as a major contributor to the differential effects of SLAM haplotypes on lupus susceptibility and development of antinuclear Abs. Earlier data implicated the increased ratio of Ly108-1 versus Ly108-2 expression as contributing to the development of antinuclear Abs in lupus prone haplotypes. We also observed relatively increased expression of Ly108-1 in thymocytes from 129S6 (having the lupus-prone haplotype) compared with C57BL/6 mice (having the lupus-resistant haplotype), although we did not observe an accompanying relative decrease in the expression of Ly108-2. However, more recently, expression of a novel spliced Ly108 isoform, Ly108-H1, has been reported and appears to be expressed solely from the lupus-resistant haplotype. Importantly, expression of this isoform conferred resistance to the development of antinuclear Abs, providing evidence for a dominant role for this isoform. We also independently cloned out this isoform and found that it is expressed in C57BL/6 mice but not in 129S6 mice, which carry the haplotype associated with lupus susceptibility. Furthermore, we have found that expression of novel Ly108-H1 is also lacking in congenic mice carrying the lupus-susceptible allele of the SLAM locus introgressed onto C57BL/6 mice. (B6.Sle1b mice; M. Dutta, unpublished data). It is of note that the primers used in the previous study would have also amplified the novel Ly108-H1 isoform, likely accounting for the reported relatively increased expression of Ly108-2 in lupus-resistant strains (13).

Our findings support those of Keszei and colleagues (16), but provide further insight into the signaling capabilities of the different isoforms of Ly108. Consistent with previous studies using Tac chimeras with the cytoplasmic tails of Ly108 (15), we observed that Ly108-1 was also more heavily phosphorylated than Ly108-2 in thymocytes in vivo. We also provide evidence that the novel Ly108-H1 is not phosphorylated in vivo, nor can phosphorylation be induced when expressed in isolation in cell lines. The varying degree of phosphorylation seen in Ly108 isoforms in vivo, which is recapitulated upon pervanadate treatment, suggests that not all forms of tyrosine in the Ly108 cytoplasmic tail are phosphorylated evenly, and that Ly108 phosphorylation might in fact be a sequential process. The development of phospho–site-specific Abs for Ly108 may help to address this issue, as well as the question of whether Ly108 is differentially phosphorylated under different conditions. Our results further suggest that the novel Ly108-H1 isoform could function as a decoy isoform that may be capable of binding Ly108 on other cells and that contributes to the bulk of total Ly108 protein in the C57BL/6 thymus without transmitting phosphorylation dependent downstream signals. In this sense, novel Ly108-H1 could help to confer lupus protection by mitigating total Ly108 signaling. Therefore, we propose that total Ly108 phosphorylation and signaling differences seen in the thymi of lupus-prone and lupus-resistant strains result from the interplay between expression differences of Ly108-1 (lupus promoting) and novel Ly108-H1 (lupus mitigating) isoforms. Preliminary data suggest that Ly108 can potentiate signals downstream from the TCR (M. Dutta, unpublished data). Whether novel Ly108-H1 also differentially affects TCR signaling, or affects other downstream outcomes of Ly108 signaling, remains an important question.

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Disclosures
The authors have no financial conflicts of interest.

References


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Supplemental Figure Legends:

**Figure S1.** Dynamic expression of Ly108 during thymocyte development. (A) WT Thymocytes were stained for intracellular total Ly108 and surface Ly108, CD4 and CD8. Surface Ly108 levels are shown. (B) Real time quantitative RT-PCR for Ly108 was done on sorted DP as well as CD8+ SP thymocytes using a Ly108 specific probe and normalized to 18s RNA. (C). Ly108 is tyrosine phosphorylated in intact lymph nodes. Intact thymus and lymph nodes from 6 week old WT C57Bl/6J mice were lysed in RIPA lysis buffer, immunoprecipitated with αLy108 and probed with phosphotyrosine 4G10 (top) and αLy108(bottom). LN were pooled from 2 mice. (D) Ly108 phosphorylation is potentiated by αCD3 engagement in vitro. Cells were stimulated and immunoprecipitated as in Figure 3C.

**Figure S2.** Sequence of Ly108-H1 compared to Ly108-1 and Ly108-2. Boxes indicate ITSM and non ITSM tyrosines in the C terminus.
### Supplemental S2

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| Ly108-2 | MAVSRAPAPDSACRMRWLFPLVFCLSGSEVSQSSSDPQLMNGVLSAVPLKLPGKIANIIINNYEWEASQVTALV |
| Ly108-H1 | MAVSRAPAPDSACRMRWLFPLVFCLSGSEVSQSSSDPQLMNGVLSAVPLKLPGKIANIIINNYEWEASQVTALV |

| Ly108-1 | INLSNPESPQIMNTDVKKRLNITQSYSLQISNLMTADTGSYTAQITTDKSEIVTFKYILRVFERLGLETNTYTLLENG |
| Ly108-2 | INLSNPESPQIMNTDVKKRLNITQSYSLQISNLMTADTGSYTAQITTDKSEIVTFKYILRVFERLGLETNTYTLLENG |
| Ly108-H1 | INLSNPESPQIMNTDVKKRLNITQSYSLQISNLMTADTGSYTAQITTDKSEIVTFKYILRVFERLGLETNTYTLLENG |

| Ly108-1 | TCQIHLACVLKNQSQTVSVEWQATGNISLGPNVTIFWDPRUNSGDQTYVCRAKNVNSLSVSSTQSLCKGVTNPPWNA |
| Ly108-2 | TCQIHLACVLKNQSQTVSVEWQATGNISLGPNVTIFWDPRUNSGDQTYVCRAKNVNSLSVSSTQSLCKGVTNPPWNA |
| Ly108-H1 | TCQIHLACVLKNQSQTVSVEWQATGNISLGPNVTIFWDPRUNSGDQTYVCRAKNVNSLSVSSTQSLCKGVTNPPWNA |

| Ly108-1 | VWFMTTISIISAVILFVCWSIHVKRSGPLTSQHPESQSTDPGSPGNTVYAQVTRPMQEMKIPKIPKIKNDMTIYS |
| Ly108-2 | VWFMTTISIISAVILFVCWSIHVKRSGPLTSQHPESQSTDPGSPGNTVYAQVTRPMQEMKIPKIKNDMTIYS |
| Ly108-H1 | VWFMTTISIISAVILFVCWSIHVKRSGPLTSQHPESQSTDPGSPGNTVYAQVTRPMQEMKIPKIKNDMTIYS |

| Ly108-1 | IVNHSREETVATGYNPITLKVNTLINYS---- |
| Ly108-2 | IVNHSREETVATGYNPITLKVNTLINYS |
| Ly108-H1 | IVNHSREETVATGYNPITLKVNTLINYS |

| Ly108-1 | IVNHSRE-------------------------AEYS |
| Ly108-2 | IVNHSREETVATGYNPITLKVNTLINYS |
| Ly108-H1 | IVNHSREETVATGYNPITLKVNTLINYS |

| Ly108-1 | ----ETVATGYNPITLKVNTLINYS |
| Ly108-2 | ----ETVATGYNPITLKVNTLINYS |
| Ly108-H1 | ----ETVATGYNPITLKVNTLINYS |