Control of T Lymphocyte Signaling by Ly108, a Signaling Lymphocytic Activation Molecule Family Receptor Implicated in Autoimmunity† §

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The signaling lymphocytic activation molecule family of receptors has been implicated in the pathophysiology of autoimmunity in humans and mice. One member of the family, Ly108, was strongly linked to lupus susceptibility in mice. High expression of a Ly108 isoform, Ly108-1, was observed in lymphocytes of lupus-prone mice. Herein, we examined the molecular basis for the influence of Ly108 on lupus susceptibility by studying Ly108 signal transduction in T cells. We observed that Ly108 was able to mediate a tyrosine phosphorylation signal implicating Ly108, Vav-1, and c-Cbl in a manner strictly dependent on engagement of the extracellular domain of Ly108 and co-expression of the Src homology 2 (SH2) domain-containing adaptor signaling lymphocytic activation molecule (SLAM)-associated protein (SAP). Evaluation of T cells from mice carrying mutations in the SAP-FynT pathway indicated that Ly108-triggered protein tyrosine phosphorylation was due to the capacity of SAP to recruit FynT. Importantly, Ly108-1 was more apt at triggering tyrosine phosphorylation signals in T cells when compared with the predominant Ly108 isoform found in non-lupus-prone mice, Ly108-2. This difference was due in part to the presence in Ly108-1 of a unique intra-cytoplasmic tyrosine-based motif that promoted Ly108 signal transduction. Together these data provided a molecular explanation for the involvement of Ly108 in lupus susceptibility in mice.

Susceptibility to autoimmune diseases such as systemic lupus erythematosus and diabetes is greatly influenced by genetic factors. In studies of lupus-susceptible mouse strains like NZM2410, several gene loci cooperate toward disease predisposition (1). One locus, Sle1, causes a loss of immune tolerance, leading to production of anti-nuclear antibodies. Sle1 is composed of four sub-loci, termed Sle1a–d, that all contribute to disease susceptibility. Of these, the best studied and most influential locus is Sle1b, which corresponds to the slam family locus on mouse chromosome 1 (2).

The slam family of genes encodes six immune cell-specific receptors named signaling lymphocytic activation molecule (SLAM) (CD150), 2B4 (CD244), Ly-9 (CD229), CD84, Ly108 (also named natural killer, T- and B-cell antigen (NTB-A) in humans) and CD2-like receptor activating cytotoxic cells (CRACC, also referred to as CD319) (3, 4). SLAM-related receptors (SRRs) possess immunoglobulin (lg)-like domains in their extracellular region, a single transmembrane domain and a cytoplasmic region bearing multiple tyrosine-based motifs. With the exception of 2B4, which binds CD48, all SRRs are self-ligands.

Through tyrosine phosphorylation sites in their cytoplasmic domain, SRRs associate with the SLAM-associated protein (SAP) family of adaptors that includes SAP, EAT-2, and ERT (3, 4). These adaptors are composed primarily of a Src homology 2 (SH2) domain and link SRRs to intracellular signals. In the case of SAP, a second binding surface in the SH2 domain enables SAP to couple SRRs to the Src-related protein-tyrosine kinase FynT, thereby triggering protein tyrosine phosphorylation signals (5–8). EAT-2 and ERT possess one or two carboxyl-terminal tyrosines that undergo phosphorylation and link SRRs to alternative signals (9). Interestingly, SAP is mutated in X-linked lymphoproliferative disease, a human immunodeficiency characterized by a faulty immune response to Epstein-Barr virus.

Ly108/NTB-A (hereafter named Ly108) is a self-ligating member of the SLAM family expressed on T cells, B cells and, at least in humans, natural killer (NK) cells (10, 11). Early studies showed that engagement of Ly108 was able to promote SAP-dependent natural cytotoxicity by human NK cells (11–14). Subsequently, it was shown that stimulation of human CD4+ T cells by an anti-Ly108 monoclonal antibody (mAb) enhanced T helper 1 (Th1) cytokine release (15). Furthermore, injection of C57BL/6 mice with a Ly108-Fc fusion protein (presumed to block Ly108-Ly108 homotypic interactions) delayed the onset of disease.

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The abbreviations used are: SLAM, signaling lymphocytic activation molecule; SRR, SLAM-related receptor; NK, natural killer; SAP, SLAM-associated protein; NTB-A, natural killer, T- and B-cell antigen; SH2, Src homology 2; Th1, T helper 1; DC, dendritic cell; mAb, monoclonal antibody; IL, interleukin.
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of T<sub>4</sub>-1-dependent autoimmune disease experimental autoimmune encephalomyelitis. These observations suggested that Ly108 may be implicated in the pathophysiology of autoimmune diseases by regulating T<sub>4</sub>-1 cytokine production by CD4<sup>+</sup> T cells. Paradoxically, analyses of T cells from mice engineered to lack most of the extracellular domain of Ly108 (ly108<sup>HE2 +</sup>) suggested that Ly108 played a role in T helper 2 (T<sub>h</sub>2) cytokine production (16). Although the basis for these contradictory results remains to be established, these data nonetheless offered a compelling indication that Ly108 participates in normal immunity and autoimmune pathologies.

Further support for the idea that Ly108 is involved in autoimmune diseases was obtained with the characterization of the Sle1b locus in the lupus-susceptible mouse strain NZM2410 (2). This study identified several polymorphisms in slam family genes between susceptible and non-susceptible mice. The most statistically significant alteration was a polymorphism in ly108. This polymorphism resulted in up-regulated expression of an alternatively spliced isoform of ly108, ly108-1, as well as down-regulated expression of another ly108 isoform, ly108-2, in T cells and B cells of disease-prone animals. In mature T cells these alterations correlated with an enhancement of antigen receptor-triggered responses in vitro.

Herein we examined the molecular basis for the greater responsiveness of T cells expressing high levels of the lupus-associated Ly108 isoform, Ly108-1. We found that all Ly108 isoforms were able to mediate tyrosine phosphorylation signals in T cells that involve Ly108 itself, Vav-1, and to a lesser extent, c-Cbl. These signals were dependent on self-engagement of the extracellular domain of Ly108 and co-expression of SAP. In addition, they required the aptitude of SAP to recruit the Src-related protein-tyrosine kinase FynT. Importantly, Ly108-1 was more apt than Ly108-2 at triggering this protein tyrosine phosphorylation signal. This difference was due in part to the presence of an additional tyrosine-based motif in the cytoplasmic domain of Ly108-1.

**EXPERIMENTAL PROCEDURES**

**Cells**—BI-141 is a mouse T-cell line (17). Derivatives stably expressing various forms of Ly108 or Tac-Ly108 with or without SAP were generated by transfection (6). Cells expressing Tac-SLAM or Tac-2B4 in combination with SAP were described (6, 18). Thymocytes, splenocytes, splenic T cells, splenic B cells, splenic dendritic cells (DCs), peritoneal macrophages, splenic NK cells, and liver NK-T cells were obtained from C57BL/6 mice (Harlan, Chicago, IL) or the indicated mouse strain according to standard protocols. Whenever cells were purified, purity was confirmed to be >90% (data not shown). In some cases mice were injected intraperitoneally with the polyclonal NK cell activator poly(I:C) (150 μg in 150 μl of phosphate-buffered saline (PBS); Sigma-Aldrich) or PBS alone 24 h before cell isolation (19). Interleukin-2 (IL-2)-activated NK cells were generated by propagating purified splenic NK cells in medium supplemented with IL-2 (1000 units/ml). Fibroblasts expressing Ly108 were generated by transfection of DCEK cells (L929 fibroblasts stably expressing I-E<sup>+</sup>) with a cDNA encoding a cytoplasmic domain-deleted version of Ly108. Cells expressing the puromycin resistance marker alone were used as control.

**Mice**—SAP-deficient (sap<sup>−/−</sup>) mice, Fyn-deficient (fyn<sup>−/−</sup>) mice, and mice expressing the SAP arginine 78-to-alanine 78 mutant (sap<sup>R78A</sup>) were reported previously (8, 20, 21).

**cDNAs, Mutagenesis, and Constructs**—A cDNA encoding Ly108 (clone number 8397616) was obtained from American Type Culture Collection. cDNAs encoding Ly108-1 and Ly108-2 were generated by PCR using primers designed from sequences available from the Ensembl data base and the original ly108 cDNA as template. Variants in which the sequences encoding the extracellular and transmembrane regions of Ly108 were replaced by those of Tac were created by PCR. For expression in BI-141 T cells or DCEK, cDNAs were cloned in the expression plasmid pSrα- puro, which confers resistance to puromycin. The expression vector encoding SAP (pNT-neo-SAP) was reported elsewhere (6). The cDNA coding for the Ly108-Fc fusion was produced by PCR using mouse ly108 cDNA and a human IgG<sub>1</sub> cDNA (obtained from Dr. Nicole Beauchemin, McGill University) as templates. Point mutations or truncation of the cytoplasmic segment (leaving intact the eight most membrane proximal residues of the cytoplasmic domain) were introduced in ly108 cDNAs using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) or PCR, respectively. All cDNAs were fully sequenced to make certain that they carried no undesired mutations (data not shown).

**Antibodies**—Polyclonal rabbit antisera against Ly108 were generated by immunizing rabbits with a TrpE fusion protein encompassing the cytoplasmic domain of Ly108-1. Rabbit antibodies directed against SAP, Fyn, Vav-1, c-Cbl, and phosphotyrosine were described (6, 18, 22, 23). A rabbit anti-Tac serum against the extracellular domain of Tac was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. A mAb recognizing mouse Ly108 (mAb 3E11) was generated in rats using the Ly108-Fc fusion protein as immunogen. This antibody recognizes Ly108 but not the other SRRs (supplemental Fig. 1). mAb 3E11 is an IgG<sub>2a</sub>/κ (data not shown). An isotype control was purchased from ebiosciences (San Diego, CA). For flow cytometry both antibodies were coupled to Alexa 647 according to the protocol outlined by the manufacturer (Invitrogen). mAbs against CD4, CD8, CD28, B220, NKR-P1c (NK1.1), CD11c, CD11b, F4/80, and CD49b (mAb DX-5) were obtained from ebioscience or BD Biosciences. Anti-Tac mouse mAb 7G7 and anti-SLAM mAb 12F12 were purified from culture supernatant (24, 25). For some experiments, biotinylated mAb 3E11 or mAb 7G7 was produced.

**Cell Stimulation**—Stimulation of Tac chimeras on BI-141 was performed as described (6). For antibody-mediated ligation of Ly108 on thymocytes, cells (20 × 10<sup>6</sup> cells) were triggered for the indicated times at 37 °C with rat anti-Ly108 mAb 3E11, biotinylated or not, followed by avidin or rabbit anti-rat IgG (6). For self-ligation of Ly108 on thymocytes, thymocytes (10 × 10<sup>6</sup>) from wild-type mice were incubated for 40 min at 37 °C with adherent DCEK fibroblasts expressing or not a cytoplasmic domain-truncated version of Ly108, previously plated in tissue culture dishes. Stimulation was stopped by harvesting non-adherent cells from the plates. After stimulation, cells were
lysed in TNE buffer (1 × TNE is 50 mM Tris, pH 8.0, 1% Nonidet P-40, and 2 mM EDTA) supplemented with protease and phosphatase inhibitors.

**Immunoprecipitations and Immunoblots—**Immunoprecipitations and immunoblots were performed as described in an earlier report (26). Immuneactive products were detected using either 125I-labeled protein A, horseradish peroxidase (HRP)-coupled protein A, 125I-labeled rabbit anti-mouse IgG, or HRP-sheep anti-mouse IgG. All secondary reagents were purchased from GE Healthcare. Radioactive signals were quantitated using a Storm 860 PhosphorImager (GE Healthcare).

**RESULTS**

**Mouse Ly108 Is Expressed on T Cells and B Cells but Not on Most Mature NK Cells—**Newly generated anti-Ly108 mAb 3E11 was used to study the expression of Ly108 on mouse immune cells (Fig. 1). In thymus, Ly108 was expressed on CD4+CD8−, CD4−CD8+, CD4+CD8+, and CD4−CD8− cells (Fig. 1A). Generally, all cells in these subpopulations expressed Ly108, except CD4−CD8− cells, which contained a small (<10%) Ly108-negative subset. Whether these Ly108-negative cells were of lymphoid origin remains to be established. In spleen, all CD4+ T cells, CD8+ T cells, and B cells also possessed Ly108 (Fig. 1B). This expression was further augmented when T cells or B cells were activated in vitro (Figs. 1, E and F). In contrast, only a small subset (~10–20%) of splenic NK cells expressed Ly108 (Fig. 1C). This was true for cells from untreated mice and mice injected with the NK cell activator poly(I:C). These Ly108-positive NK cells were lost when cells were propagated and activated in vitro with IL-2. The absence of Ly108 on most mouse NK cells was surprising given that human NK cells uniformly express high levels of NTB-A, the human Ly108 equivalent (11–14). Ly108 was present on all liver NK-T cells, in keeping with a recent report (27) (Fig. 1D). Last, Ly108 was expressed on all splenic DCs but not on macrophages. Thus, in the mouse, Ly108 is expressed on T cells, B cells, NK-T cells, and DCs but not on most NK cells.

**Ly108 Mediates a SAP-dependent Tyrosine Phosphorylation Signal in T Cells—**To elucidate the signals triggered by Ly108, full-length Ly108 (Ly108-1) was expressed in the absence or in the presence of SAP in the mouse T cell line Bl-141. This cell line was chosen because it normally lacks both Ly108 and SAP (data not shown). Moreover, it was previously used to define the pathways linked to other SRRs (5, 6, 18). The impact of Ly108 on protein tyrosine phosphorylation was studied by immunoblotting of cell lysates with anti-phosphotyrosine antibodies (Fig. 2A, first panel). Without SAP (lane 3), cells containing Ly108 exhibited no increase in protein tyrosine phosphorylation compared with cells lacking Ly108 (lane 1). However, with SAP (lane 4), they showed a striking increase in tyrosine phosphorylation of polypeptides of 65 (p65), 95 (p95), and 120 (p120) kDa. An increase in tyrosine phosphorylation of p120 and, to a lesser extent, p95 was also seen in cells containing SAP alone (lane 2). Although the basis of this observation is not known, this signal may be due to SAP-dependent signaling via Ly-9, a SRR expressed in small amounts in parental Bl-141 cells (data not shown).
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The 65-kDa substrate was consistent with being Ly108 itself. To examine this possibility lysates were immunoprecipitated with anti-Ly108 antibodies and probed by anti-phosphotyrosine immunoblotting (Fig. 2B, first panel). The tyrosine-phosphorylated p65 seen in cells expressing Ly108 and SAP (lane 4 – 6) was efficiently immunoprecipitated with anti-Ly108 (lane 4) but not anti-SLAM (lane 5) or anti-CD4 (lane 6) antibodies. No tyrosine-phosphorylated substrate was noted in cells lacking SAP (lanes 1 – 3). We also ascertained whether SAP was physically associated with Ly108 by probing Ly108 immunoprecipitates with anti-SAP antibodies (second panel). SAP was present in immunoprecipitates obtained with anti-Ly108 (lane 4) but not irrelevant (lanes 5 and 6) antibodies.

Like human NTB-A, mouse Ly108 is a self-ligand (supplemental Fig. 2). Hence, the data of Fig. 2B did not address whether engagement of Ly108 was required to trigger SAP binding and SAP-dependent tyrosine phosphorylation. To ascertain this, we created a chimeric receptor in which the extracellular and transmembrane regions of Ly108 were replaced by those of Tac (human IL-2 receptor α chain). Stimulation with antibodies against Tac induced a tyrosine phosphorylation signal involving polypeptides of 65 (probably Tac-Ly108), 95, and 120 kDa in cells containing Tac-Ly108 (TLy108) and SAP (Fig. 2C, first panel, lane 4) but not Tac-Ly108 alone (lane 2). Importantly, no increase in protein tyrosine phosphorylation was detected in the absence of anti-Tac stimulation (lane 3), indicating that Ly108 engagement was essential to induce the SAP-dependent signals.

Similar results were obtained when Tac-Ly108 was immunoprecipitated (second panel). To address if this reflected the ligand-dependent association of Ly108 with SAP, Tac immunoprecipitates were probed by immunoblotting with anti-SAP antibodies (third panel). In the absence of engagement (lane 3), Tac-Ly108 was minimally associated with SAP. A much more extensive association was observed after Tac stimulation (lane 4). The ligand-inducible nature of the Ly108-SAP interaction was in contrast to the ligand-independent association between SLAM and SAP (6). Therefore, Ly108 engagement was required to induce full Ly108-SAP association and SAP-dependent protein tyrosine phosphorylation in T cells.

Ly108 Signaling Is Caused by the Src-related Protein Tyrosine Kinase FynT—Previous studies indicated that the ability of SLAM and 2B4 to mediate tyrosine phosphorylation signals in the presence of SAP was mediated in large part by the capacity of SAP to bind the Src-related protein-tyrosine kinase FynT (5, 6, 8, 28). This function required a second binding surface in the SAP SH2 domain that is centered on Arg-78 of SAP and directly interacts with the SH3 domain of FynT. However, a more recent report suggested that alternative mechanisms can also enable SRRs to mediate tyrosine phosphorylation signals (29).

To appraise whether SAP-dependent Ly108 signaling was mediated by FynT, Ly108-triggered protein tyrosine phosphorylation was examined in T cells from mice carrying mutations in the SAP-FynT pathway, including sap<sup>−/−</sup>, sap<sup>R78A</sup> (in which the SAP-FynT interaction was disrupted because of a germ-line mutation of Arg-78 to alanine), and fyn<sup>−/−</sup> mice (Fig. 3). Thymocytes from these animals were stimulated or not with anti-Ly108 antibodies, and Ly108 tyrosine phosphorylation was
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We wanted to ensure that Ly108 tyrosine in thymocytes could also be induced by the physiological ligand of Ly108; that is, Ly108 itself. To this end, thymocytes from wild-type mice were incubated at 37 °C in the presence of fibroblasts expressing or not a cytoplasmic domain-truncated version of Ly108 (Fig. 3D).

After cell lysis, Ly108 tyrosine phosphorylation was monitored as detailed for Figs. 3, A–C (Fig. 3E). This analysis revealed that stimulation of thymocytes with fibroblasts expressing Ly108 (lane 2) caused a greater extent of tyrosine phosphorylation of Ly108 in thymocytes in comparison to fibroblasts lacking Ly108 (lane 1). Hence, self-ligand-induced phosphorylation of Ly108 occurred on normal T cells in the absence of overexpression.

Ly108 Signaling Is Distinct from SLAM Signaling and Involves Vav-1 and c-Cbl—In addition to Ly108, CD4+ T cells express other SRRs including SLAM (30). To address whether Ly108 and SLAM might mediate redundant signals and, presumably, functions in these cells, the tyrosine phosphorylation signals induced by the two receptors were compared (Fig. 4). To prevent effects due to the distinct extracellular domains of the receptors, chimeric Tac receptors were used in these experiments. Whereas Tac-Ly108 stimulation (Fig. 4A, first panel, lane 4) provoked the tyrosine phosphorylation of polypeptides of 65 (Tac-Ly108), 95, and 120 kDa, engagement of Tac-SLAM (lane 6) triggered an increase in the phosphorytousine content of proteins of 54, 56, 62, 70, and to a lesser extent, 150 kDa. The latter set of substrates represents the adaptor molecules Shc, Dok-2 and Dok-1, Tac-SLAM, and SH2 domain-containing 5′-inositol phosphatase (SHIP)-1, respectively (6).

We also compared the signal induced by Ly108 with that provoked by 2B4, a SRR found on NK cells and some CD8+ T cells (30) (Fig. 4A, first panel, lanes 7 and 8). Engagement of the Tac-2B4 chimera stimulated the tyrosine phosphorylation of...
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substrates of 65, 95, and 120 kDa (lane 8), previously identified as Tac-2B4, guanine nucleotide exchange factor Vav-1, and ubiquitin ligase c-Cbl, respectively (18). This pattern of tyrosine phosphorylation was similar to that triggered by Tac-Ly108 (lane 4).

The latter finding suggested that the Ly108-triggered substrates might be Vav-1 and c-Cbl. To address this possibility, lysates were immunoprecipitated with antibodies against Vav-1 or c-Cbl, and the phosphorylation content was analyzed by anti-phosphotyrosine immunoblotting (Fig. 4B, first and third panels). Tyrosine phosphorylation of Vav-1 (first panel) was markedly enhanced upon stimulation of cells expressing Tac-Ly108 and SAP (lane 4). A much weaker induction of Vav-1 tyrosine phosphorylation was noted in cells expressing Tac-SLAM (lane 6), although a strong response was observed in cells bearing Tac-2B4 (lane 8). Similar results were obtained when tyrosine phosphorylation of c-Cbl was examined (third panel). Hence, in T cells, Ly108 induced a tyrosine phosphorylation signal involving Vav-1 and c-Cbl. This signal was clearly distinct from that evoked by SLAM but was analogous to the one provoked by 2B4.

Ly108-1, the Isoform Associated with Heightened Lupus Susceptibility in Mice, Mediates a More Robust Tyrosine Phosphorylation Signal Than Ly108-2 in T Cells—Mouse T cells express two major Ly108 isoforms, Ly108-1 and Ly108-2 (2, 10). These proteins are generated by alternative splicing of exons coding protein tyrosine phosphorylation, a time-course of stimulation was also conducted (Fig. 5C). Once again, stimulation of Tac-Ly108-1 (first panel, lanes 1–6) resulted in more robust protein tyrosine phosphorylation than ligation of Tac-Ly108-2 (lanes 7–12). A quantitation is depicted in Fig. 5D.

To explain these differences, we compared the association of the two Ly108 isoforms with SAP (Fig. 6). This analysis was performed utilizing cells expressing full-length Ly108 isoforms in lieu of the Tac-Ly108 chimeras, as the former cells exhibited more easily detectable binding of Ly108 to SAP. With several independent clones expressing similar levels of Ly108-1 or Ly108-2 (Fig. 6A and the fourth panel of B), SAP was more extensively associated (on average 1.5-fold) with Ly108-1 than Ly108-2 (Fig. 6B, first panel). However, this disparity was much less marked than the difference in tyrosine phosphorylation of Ly108-1 and Ly108-2 (third panel), suggesting that the differences in SAP binding did not fully explain the differences in Ly108 tyrosine phosphorylation. A quantitation is shown in Fig. 6C.

Structural Basis of Signaling Differences between Ly108-1 and Ly108-2—Ly108-1 contains one unique tyrosine-based motif in its cytoplasmic domain (supplemental Fig. 3). Because hyperactive T cells from lupus-susceptible mice bearing the Sle1b locus express high levels of Ly108-1 in comparison to Ly108-2 (2), we studied the possibility that Ly108-1 and Ly108-2 differed in their signaling properties (Fig. 5). For this purpose Tac chimeric receptors containing the cytoplasmic domain of Ly108-1 or Ly108-2 were expressed in B1-141 T cells in the presence of SAP. Two individual clones expressing equivalent amounts of Tac were selected for further experiments (Fig. 5A).

Ligation of the Ly108-1 chimera (Fig. 5B, first panel, lanes 2 and 4) resulted in a stronger tyrosine phosphorylation signal, in comparison to triggering of the Ly108-2 chimera (lanes 6 and 8). This was true for phosphorylation of the chimeric receptor (Tac-Ly108) and, to a lesser albeit appreciable extent, p95 (presumably Vav-1) and p120 (presumably c-Cbl). To ensure that these differences did not simply reflect variations in the kinetics of

![Figure 4](image-url) Comparison of the signals transduced by Ly108, SLAM, and 2B4. A, overall protein tyrosine phosphorylation. BI-141 derivatives expressing the indicated Tac chimeras and SAP were stimulated for 7 min with biotinylated anti-Tac mAb 7G7 and avidin. Protein tyrosine phosphorylation was examined by immunoblotting of total cell lysates with anti-phosphotyrosine (P.tyr) antibodies (first panel). The abundance of the chimeras and SAP was verified by immunoblotting lysates with anti-Tac (second panel) and anti-SAP (third panel) antibodies, respectively. The positions of the major tyrosine-phosphorylated substrates, the Tac chimeras, and SAP are shown by arrowheads on the left, whereas those of prestained molecular mass markers are indicated on the right. B, tyrosine phosphorylation of Vav-1 and c-Cbl. The experiment was as outlined for A, except that tyrosine phosphorylation of Vav-1 was directly assessed by probing anti-Vav-1 immunoprecipitates (IP) with anti-phosphotyrosine antibodies (first panel). The abundance of Vav-1 was verified by reprobing the membrane with anti-Vav-1 antibodies (second panel). The extent of tyrosine phosphorylation of c-Cbl was also determined by probing anti-c-Cbl immunoprecipitates with anti-phosphotyrosine antibodies (third panel). The abundance of c-Cbl was verified by reprobing the membrane with anti-c-Cbl (fourth panel). The migrations of Vav-1 and c-Cbl are indicated by arrowheads on the left. TLY108, Tac-Ly108; TSLAM, Tac-SLAM; 2B4, Tac-2B4.

for the cytoplasmic domain of Ly108 and are distinguishable solely by their divergent carboxyl-terminal segments (supplemental Fig. 3). Because hyperactive T cells from lupus-susceptible mice bearing the Sle1b locus express high levels of Ly108-1 in comparison to Ly108-2 (2), we studied the possibility that Ly108-1 and Ly108-2 differed in their signaling properties (Fig. 5). For this purpose Tac chimeric receptors containing the cytoplasmic domain of Ly108-1 or Ly108-2 were expressed in B1-141 T cells in the presence of SAP. Two individual clones expressing equivalent amounts of Tac were selected for further experiments (Fig. 5A).

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Ly108-2 (Fig. 7D, first panel, compare lanes 7–10 with lanes 3–6; see quantitation in Fig. 7E) had no effect. Therefore, the disparity in the signaling capabilities of Ly108-1 and Ly108-2 was dictated in part by the unique tyrosine found in Ly108-1. However, because the effect of mutating this tyrosine was not complete, it is likely that additional factors were also involved (see “Discussion”).

**DISCUSSION**

Studies of lupus-susceptible NZM2410 mice provided compelling indication that SRRs, in particular Ly108, are implicated in the pathophysiology of autoimmune diseases (2). It was observed that lymphocytes from lupus-prone mice expressed greater amounts of Ly108-1 relative to Ly108-2 when compared with lymphocytes from non-susceptible animals. This difference correlated with greater T cell antigen receptor-mediated calcium fluxes *in vitro*. To determine the mechanism by which differential utilization of these isoforms influenced T cell responsiveness and lupus susceptibility, we characterized their signaling capabilities. As a first step, we ascertained whether, like other SLAM family members, Ly108 was coupled to protein tyrosine phosphorylation signals in T cells. Using variants of a T cell line expressing Ly108 with or without SAP, we found that full-length Ly108 was apt at stimulating intracellular protein tyrosine phosphorylation, in a manner that was strictly reliant on SAP. Moreover, using a Tac-Ly108 chimeric receptor in which the self-ligating extracellular domain of Ly108 was replaced by phenylalanines, and the ability of the Tac-Ly108 chimeras to trigger tyrosine phosphorylation signals was determined (Figs. 7, B–E). Mutation of the unique tyrosine of Ly108-1 (Fig. 7B, first panel, compare lanes 5–8 with lanes 1–4; see quantitation in Fig. 7C) reduced the ability of Ly108-1 to transduce tyrosine phosphorylation signals. Nevertheless, this effect was not complete, as the mutant triggered signals that were still superior to those of Ly108-2 (lanes 9–12). By opposition, mutation of the two unique tyrosines found in the ability of Ly108 to associate with SAP as well as to mediate tyrosine phosphorylation signals was strongly dependent on engagement of Ly108. This was in contrast to SLAM, which interacts with SAP in a ligand-independent manner (6).

Next, we examined the role of the interaction between SAP and the Src family kinase FynT in Ly108 signal transduction. Using T cells from mice carrying various mutations in this cascade, it was observed that Ly108-induced protein tyrosine phosphorylation was eliminated in thymocytes lacking SAP, in
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FIGURE 6. Differential association of Ly108-1 and Ly108-2 with SAP. A, cell surface expression of Ly108-1 and Ly108-2. BI-141 T cells were stably transfected with cDNAs encoding full-length Ly108-1 or Ly108-2 in the presence of SAP. Cell surface expression of Ly108 on three independent clones of each type was assessed by flow cytometry with anti-Ly108 antibodies (first panel). The interaction of Ly108 with SAP (first panel) was probed by immunoblotting of anti-Ly108 immunoprecipitates (IP) with anti-SAP antibodies. The presence of Ly108 in the immunoprecipitates was revealed by reprobing the membrane with anti-Ly108 antibodies (second panel). Ly108 tyrosine phosphorylation was monitored by probing lysates with anti-phosphotyrosine (P-tyr) antibodies (third panel), whereas the abundance of Ly108 and SAP was verified by immunoblotting of lysates with anti-Ly108 mAb 3E11 (fourth panel) and anti-SAP (fifth panel) antibodies, respectively. C, quantitation. The extent of association of Ly108-1 and Ly108-2 with SAP as well as their extent of tyrosine phosphorylation in the experiment depicted in B was quantitated using a PhosphorImager.

agreement with the results obtained with the T cell line. More significantly, this signal was also severely reduced in thymocytes expressing SAP R78A, a SAP mutant unable to bind FynT. Likewise, it was abrogated in thymocytes from FynT-deficient mice. Thus, these data provided firm evidence that Ly108-triggered protein tyrosine phosphorylation was largely mediated through coupling of SAP to FynT by way of the Arg-78-based motif of SAP.

Because T cells express other members of the SLAM family in addition to Ly108, it was important to determine whether the signals provided by these receptors and Ly108 were redundant. To address this, we focused on SLAM, the best characterized SLAM family receptor expressed in T cells. Whereas engagement of Ly108 induced tyrosine phosphorylation of Vav-1 and, to a lesser extent, c-Cbl, triggering of SLAM provoked the tyrosine phosphorylation of SHIP-1, Dok-1, Dok-2, and Shc. Only a small degree of Vav-1 tyrosine phosphorylation was evoked by SLAM engagement. Considering these differences, it seems probable that Ly108 and SLAM do not have redundant functions in CD4+ T cells. It is noteworthy, however, that Ly108 evoked signals very similar to those triggered by 2B4, another SLAM family receptor. Although 2B4 is not found in CD4+ T cells, it is broadly expressed in NK cells. The resemblance in the SAP-dependent signals triggered by Ly108 and 2B4 may explain why both can efficiently promote cytoxicity in human NK cells in a SAP-dependent manner (11–14).

Having defined the tyrosine phosphorylation signal mediated by Ly108 in T cells, we tested whether the two Ly108 isoforms, Ly108-1 and Ly108-2, differed in their capacity to trigger this signal. Interestingly, we observed that Ly108-1, the isoform expressed in greater amounts in lupus-prone mice, was more apt than Ly108-2 at triggering SAP-dependent tyrosine phosphorylation signals. This effect was especially obvious for tyrosine phosphorylation of Ly108 itself (~10-fold). However, it was also clearly observed for Vav-1 and c-Cbl, although the differences seen with these substrates were smaller (~2-fold). At first glance, the smaller magnitude of the latter differences may raise questions about their relevance. Nonetheless, it should be pointed out that differences of this scale are typical of “modifiers” of autoimmunity. Disease modifiers usually operate in the context of other modifiers, with which they cooperate to cause full-blown pathology. Thus, the differential ability of Ly108-1 and Ly108-2 to trigger tyrosine phosphorylation signals may very well explain their influence on lupus susceptibility.

Does the type of tyrosine phosphorylation signal triggered by Ly108 elucidate the functions previously ascribed to Ly108 in T cells as well as the differential impact of Ly108-1 and Ly108-2 on mature T cell reactivity? We believe that this is likely to be the case. Our results imply that self-engagement of Ly108, in response to interactions between a T cell and a Ly108-positive antigen-presenting cell (such as a DC or a B cell) or two T cells as suggested elsewhere (30), would trigger signals that can intersect with those emanating from the T cell antigen receptor. The major Ly108-regulated substrate, Vav-1, is a guanine nucleotide exchange factor for Rac-1 and cdc42 that promotes cytoskeletal reorganization during T cell activation (31, 32). It is activated by tyrosine phosphorylation and is required for T cell antigen receptor-induced cytoskeletal reorganization, proliferation, and effector functions. These activities can easily explain the stimulatory impact of anti-Ly108 antibodies on antigen receptor-induced T cell cytokine secretion in normal CD4+ T cells and the defect in T1(2) cytokine secretion observed in Ly108-deficient CD4+ T cells (15, 16). They probably also underlie the greater TCR-triggered calcium signals seen in T cells preferentially expressing Ly108-1 over Ly108-2 (2).

At first glance the role of c-Cbl tyrosine phosphorylation in Ly108-dependent biological effects is less clear. c-Cbl is an E3 ubiquitin ligase that usually plays an inhibitory role during T cell activation (33). However, c-Cbl was also postulated to function as an adaptor molecule that, upon tyrosine phosphorylation, may catalyze the formation of signaling complexes and facilitate cell activation. It is of note that the effect of Ly108 engagement on c-Cbl was not as marked as that on Vav-1. Thus, c-Cbl may not play as central a role as Vav-1 in Ly108 signaling. It is also possible that c-Cbl participates in a negative feedback mechanism aimed at restricting the stimulatory impact of Ly108 engagement in CD4+ T cells. In support of this, c-Cbl tyrosine phosphorylation in response to Ly108 engagement tended to occur with slower kinetics than Vav-1 tyrosine phosphorylation (see Figs. 5 and 7 for examples).

What is the structural basis for the signaling differences seen between Ly108-1 and Ly108-2? Because the two isoforms...
exclusively differ within the carboxyl-terminal portions of their cytoplasmic domain, we tested the possibility that unique tyrosine-based motifs in these segments were involved. Our studies showed that mutation of the tyrosine in the unique motif of Ly108-1 caused an appreciable reduction of Ly108-1-triggered protein tyrosine phosphorylation. In contrast, replacement of the tyrosines in the two motifs uniquely found in Ly108-2 had no effect. These observations implied that part of the differences in the signals triggered by Ly108-1 and Ly108-2 were due to the unique tyrosine-based motif in Ly108-1. It is possible that this motif undergoes tyrosine phosphorylation, which could facilitate the recruitment of effectors such as SAP and Vav-1. Alternatively, it may regulate the cellular distribution or conformation of Ly108, perhaps independently of tyrosine phosphorylation, thereby promoting the coupling of Ly108 to its effectors.

Because mutation of the unique tyrosine of Ly108-1 did not reduce the Ly108-triggered signal to the same level as that seen with Ly108-2, it is probable that additional structural features also participate in their signaling differences. Along these lines, we examined the signaling capabilities of another, albeit rare, Ly108 isoform named Ly108-3 (supplemental Fig. 4). This isoform possesses a third type of carboxyl-terminal sequence distinct from those of Ly108-1 and Ly108-2 (supplemental Fig. 3). Our analyses showed that Ly108-3 caused a tyrosine phosphorylation signal that was intermediate between those of Ly108-1 and Ly108-2 (supplemental Fig. 4B). Combined with Fig. 7, this observation suggested that sequences in the unique carboxyl-terminal segment of Ly108-2, distinct from the unique tyrosines, might also participate in the distinct signaling potentials of the two isoforms. Hence, the mechanism by which Ly108-1 signals more efficiently than Ly108-2 in T cells is probably multifactorial.

Greater amounts of Ly108-1 over Ly108-2 are present not only in T cells, but also in B cells, of mice bearing the lupus susceptibility locus Sle1b (2). Intriguingly, unlike T cells, B cells containing higher amounts of Ly108-1 were found to exhibit diminished, rather than augmented antigen receptor-triggered responses (1). It was proposed that such a decrease would compromise tolerance induction against self-antigens in immature B cells, thereby favoring the accumulation of self-reactive B cells. At this time, the mechanism by which a preponderance of Ly108-1 over Ly108-2 would have opposite functional consequences in

**FIGURE 7. Role of the unique tyrosines in Ly108-1 and Ly108-2.** A, amino acid sequences of the cytoplasmic domain of Ly108-1 and Ly108-2. The amino acid sequences, common and specific, of the cytoplasmic domain of Ly108-1 and Ly108-2 are shown. Intra-cytoplasmic tyrosines are underlined, whereas putative SAP binding motifs are boxed. B-E, these experiments were performed as detailed in the legend of Fig. 5, except that pools of two different clones expressing mutant forms of Tac-Ly108-1 (B and C) or Tac-Ly108-2 (D and E) in which the unique tyrosine(s) was mutated to phenylalanine (TLY108-1 Y3F and TLY108-2 Y3F,Y4F mutants, respectively) were analyzed. Protein tyrosine phosphorylation was examined by immunoblotting of total cell lysates with anti-phosphotyrosine (P.tyr) antibodies (Figs. 7, B and D, first panel). The abundance of the chimeras and SAP was verified by immunoblotting of cell lysates with anti-Tac (second panel) and anti-SAP (third panel) antibodies, respectively. The positions of the major tyrosine-phosphorylated substrates, the Tac chimeras, and SAP are shown by arrowheads on the left, whereas those of prestained molecular mass markers are indicated on the right. The extent of tyrosine phosphorylation of Tac-Ly108 (TLY108), p95 (presumably Vav-1), and p120 (presumably c-Cbl) was quantitated using a PhosphorImager (C and E). Data are represented as absolute numbers of radioactivity.
immature B cells and mature T cells is not known. A probable explanation is that, contrary to CD4+ T cells, most B cells do not express SAP. Hence, Ly108 is probably coupled to other effectors in B cells. One candidate is EAT-2, a SAP-related adaptor that does not recruit FynT and couples SRRs to distinct signals (9).

In summary, recent data firmly indicated that ly108 plays an important role in defining lupus susceptibility in mice (2). A relative increase in the expression of Ly108-1 over Ly108-2 was observed in T cells and B cells from lupus-prone mice. Herein, we found that Ly108-1 was more efficient than Ly108-2 at transducing SAP-dependent signals involving Vav-1 and, to a lesser extent, c-Cbl in T cells. This observation provides a molecular explanation for the enhanced T cell responsiveness conferred by higher expression of Ly108-1 in mice. Interestingly, a lupus susceptibility locus in humans also maps to, or close to the SLAM gene family (34–36). Considering this, it will be interesting to determine whether altered expression and/or splicing of NTB-A (the human ly108 equivalent), leading to changes in NTB-A signaling in lymphocytes, also contribute to defining autoimmune disease susceptibility in humans.

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