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The Receptor Ly108 Functions as a SAP Adaptor-Dependent On-Off Switch for T Cell Help to B Cells and NKT Cell Development

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SUMMARY

Humans and mice deficient in the adaptor protein SAP (*Sh2d1a*) have a major defect in humoral immunity, resulting from a lack of T cell help for B cells. The role of SAP in this process is incompletely understood. We found that deletion of receptor Ly108 (*Slamf6*) in CD4$^+$ T cells reversed the *Sh2d1a*$^{-/-}$ phenotype, eliminating the SAP requirement for germinal centers. This potent negative signaling by Ly108 required immunotyrosine switch motifs (ITSMs) and SHP-1 recruitment, resulting in high amounts of SHP-1 at the T cell:B cell synapse, limiting T cell:B cell adhesion. Ly108-negative signaling was important not only in CD4$^+$ T cells; we found that NKT cell differentiation was substantially restored in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice. The ability of SAP to regulate both positive and negative signals in T cells can explain the severity of SAP deficiency and highlights the importance of SAP and SHP-1 competition for Ly108 ITSM binding as a rheostat for the magnitude of T cell help to B cells.

INTRODUCTION

The formation of germinal centers is critical for the development of most humoral immunity. This includes affinity maturation, long-lived plasma cell generation, and an overall effective neutralizing antibody response (Allen et al., 2007; Tarlinton, 2008). The development of germinal centers is controlled by follicular helper CD4$^+$ T (Tfh) cells, which are the specialized T cells for B cell help (Crotty, 2011). Therefore, understanding Tfh cells and germinal centers is important for rational approaches to vaccine design and new therapeutic approaches for autoimmune diseases involving B cells. Tfh cell differentiation is dependent on the transcription factor Bcl6 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), and Tfh cells express proteins that facilitate colocalization with B cells (high CXCR5 and CXCR4, concomitant with low CCR7 and S1P receptor expression or function) and molecules important for T cell help to B cells, such as IL-21, IL-4, and CD40L (Crotty, 2011). Tfh cells within germinal centers (GC Tfh cells) have notably high expression of signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) (Ma et al., 2009; Yusuf et al., 2010).

Sh2d1a, the gene encoding SAP, was originally cloned as the causal locus of the frequently lethal human immunodeficiency X-linked lymphoproliferative disease (XLP), characterized by difficulty controlling a variety of pathogens (Cannons et al., 2011). It was then determined that SAP plays a central role in the development of B cell immunity, because SAP is required in CD4$^+$ T cells for germinal center B cell development and development of long-term humoral immunity (Crotty et al., 2003). In the absence of SAP, virtually no memory B cells, long-lived plasma cells, or sustained antibody responses are present after acute viral infections (Crotty et al., 2003; Kamperschroer et al., 2006; McCausland et al., 2007; Moyron-Quiroz et al., 2009), chronic viral infections (Chen et al., 2005; Crotty et al., 2006; Harker et al., 2011), parasite exposure (Cannons et al., 2006), immunizations with a variety of simple or complex protein antigens (Cannons et al., 2006, 2011; Veillette et al., 2008), or when ablated in autoantibody-prone mice (Jennings et al., 2008; Linterman et al., 2009). XLP patients were later confirmed to have severe loss of germinal centers and memory B cells (Ma et al., 2005, 2006; Malbran et al., 2004). SAP has also been shown to be critically important for the development of NKT cells (Chung et al., 2005; Griewank et al., 2007; Nichols et al., 2005; Pasquier et al., 2005).

These findings have spurred extensive interest in delineating the molecular functions of SAP and the SLAM family receptors. Structurally, SAP consists primarily of an SH2 domain (Poy et al., 1999). SAP is expressed in the cytoplasm and binds tyrosines found in the intracellular domains of SLAM family receptors such as SLAM (CD150, *Slamf1*), CD84 (*Cd84*), Ly9 (SLAMF3, *Slamf3*), 2B4 (CD244, SLAMF4, *Cd244*), and Ly108 (*Slamf6*, NTB-A in humans). Multiple SLAM family receptors

are expressed on both T and B cells and have been shown to bind SAP through an uncommon tyrosine motif, termed an immunotyrosine switch motif (ITSMs) (Sayos et al., 1998; Shlapatska et al., 2001). All SAP-binding SLAM family members contain two or more ITSMs in their cytoplasmic tail. SLAM family receptors all possess a similar ectodomain structure and most exhibit homotypic adhesion (e.g., SLAM-SLAM, Ly108-Ly108) (Cao et al., 2006; Mavaddat et al., 2000; Yan et al., 2007). The receptor tails serve as docking sites for multiple signaling molecules upon homotypic receptor binding (Cannons et al., 2011; Latour et al., 2001; Schwartzberg et al., 2009; Veillette et al., 2009). SLAM family receptors are known to mediate IL-4 production by $CD4^+$ T cells (Davidson et al., 2004; Wang et al., 2004; Yusuf et al., 2010), cytotoxicity by NK cells (Nakajima et al., 2000; Pasquier et al., 2005; Tangye et al., 2000), thymic selection of NKT cells (Griewank et al., 2007), and bactericidal activity of neutrophils (Howie et al., 2005) and macrophages (Berger et al., 2010), among other functions. Although findings on inhibitory aspects of SLAM family receptor signaling are present in the literature (Cannons et al., 2011; Veillette et al., 2009), some of this was controversial (Latour et al., 2001; Nichols et al., 2001; Sayos et al., 1998), and most recent analysis has focused on the positive SAP-dependent signaling activities of SLAM family receptors (Cannons et al., 2010b; Chan et al., 2003; Latour et al., 2003; Yusuf et al., 2010; Zhong and Veillette, 2008). Most notably, SAP plays a central role in $CD4^+$ T cell adhesion to B cells (Qi et al., 2008). *Sh2d1a*$^{-/-}$ $CD4^+$ T cells were unable to form long-term conjugates with cognate B cells in vivo and in vitro (Cannons et al., 2010a; Qi et al., 2008). SLAM family members CD84 and Ly108 were shown to collaborate in this process in vitro in a SAP-dependent manner, and CD84 was shown to be important for germinal center development after some protein immunizations, though it did not fully phenocopy SAP deficiency (Cannons et al., 2010a). Here we show that single gene ablation of *Slamf6* or *Cd84* does not result in significant germinal center or antibody defects after an acute viral infection, unlike the severe germinal center defect seen in *Sh2d1a*$^{-/-}$ mice. This left an inability to explain the severity of the humoral immunity defects globally observed in the absence of SAP. To better understand the role of Ly108 signaling in T cell help to B cells, we developed a *Sh2d1a*$^{-/-}$*Slamf6*$^{-/-}$ double-deficient mouse. Surprisingly, the absence of Ly108 eliminated the requirement for SAP in $CD4^+$ T cells for B cell help. This observation led us to identify a potent Ly108-negative signaling pathway, active in both $CD4^+$ T cells and NKT cells, which may act as a general regulator of lymphocyte:lymphocyte adhesion.

RESULTS

Ly108 Expression and Function in an Acute Viral Infection

We have been examining the role of individual SAP-binding SLAM family receptors in the process of T cell help to B cells in the germinal center. With the exception of 2B4 (CD244), which binds to CD48 and is not expressed by $CD4^+$ T cells, the remaining SLAM family members are homophilic receptors. Murine CD319 (CRACC, *Slamf7*) does not bind SAP (Bouchon et al., 2001). Mice deficient in individual SLAM family members have provided evidence that Ly9 (*Slamf3*) is not required for germinal centers (Graham et al., 2006). SLAM (*Slamf1*) is not required for Tfh cell differentiation or adhesion (Cannons et al., 2006; McCausland et al., 2007) but is required for GC Tfh cell IL-4 production (Cannons et al., 2010b; Yusuf et al., 2010). Interestingly, Ly108 (NTB-A in humans, *Slamf6*) is known to bind SAP (Bottino et al., 2001; Zhong and Veillette, 2008) and Ly108 isoforms are linked to the development of autoantibody-mediated autoimmune diseases (Keszei et al., 2011; Wandstrat et al., 2004). Therefore, we evaluated whether Ly108 expression is modulated on Tfh cells and B cells in the context of an acute viral infection, lymphocytic choriomeningitis virus (LCMV). Activated $CD4^+$ T cells express elevated levels of Ly108 compared to naive $CD4^+$ T cells (Figure 1A). Tfh cells express more Ly108 than other virus-specific $CD4^+$ T cells (predominately Th1 cells), consistent with previous observations (Cannons et al., 2010b). The Tfh cells present in germinal centers (GC Tfh cells) are known to have elevated levels of SAP and BCL6 (Kroenke et al., 2012; Ma et al., 2009; Yusuf et al., 2010) but had equivalent Ly108 to other Tfh cells (Figure 1A). Given that Ly108 is a homophilic receptor, we measured Ly108 expression on activated B cells during an LCMV infection. We observed that both germinal center B cells and plasma cells have elevated Ly108 expression compared to naive B cells (Figure 1B). $CD4^+$ T cells and B cells from *Slamf6*$^{-/-}$ mice are shown for comparison, demonstrating constitutive expression of Ly108 by both $CD4^+$ T cells and B cells (Figures 1C and 1D).

To investigate the effects of loss of Ly108, we scrutinized antiviral B cell and T cell responses by wild-type (WT), *Slamf6*$^{+/-}$, and *Slamf6*$^{-/-}$ mice. Eight days after an acute LCMV infection, germinal center B cell numbers were unaffected by absence of Ly108 (Figure 1E). Additionally, antibody responses 30 days after infection were normal in *Slamf6*$^{-/-}$ mice (Figure 1G). In contrast, *Sh2d1a*$^{-/-}$ mice display a striking germinal center defect 8 days after LCMV infection and have severely reduced LCMV antibody titers 30 days postinfection (Figures 1F and 1H).

Previous work demonstrated that CD84 has a partial role in germinal center development after protein immunizations (Cannons et al., 2010a), but we observed no defect in *Cd84*$^{-/-}$ mice in the context of an acute LCMV infection (Figure S1 available online). We therefore examined a second infection model, vaccinia virus (VACV), and again no defect in germinal centers or Tfh cells was observed in *Cd84*$^{-/-}$ mice (Figure S1 and data not shown). These observations implicated a robust Tfh cell functional redundancy between SLAM family receptors, which has also been observed for SLAM family receptor participation in NKT cell development (Griewank et al., 2007).

Ly108 Function in the Absence of SAP

To strategically delineate the requirements of Ly108 in the SAP-deficient phenotype, we generated *Sh2d1a*$^{-/-}$*Slamf6*$^{-/-}$ double-deficient mice. Surprisingly, the removal of Ly108 expression from SAP-deficient mice eliminated the SAP requirement for germinal center formation in response to an LCMV infection ($p < 0.001$; Figure 2A). Plasma cell development was also recovered in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice, in stark contrast to *Sh2d1a*$^{-/-}$ mice ($p < 0.001$; Figure 2B). Tfh cell percentages were unaffected (Figure 2C). *Sh2d1a*$^{-/-}$ mice have a severe defect in GC Tfh cell formation (Yusuf et al., 2010). In contrast to the absence of GC Tfh cells in *Sh2d1a*$^{-/-}$ mice, GC Tfh cell

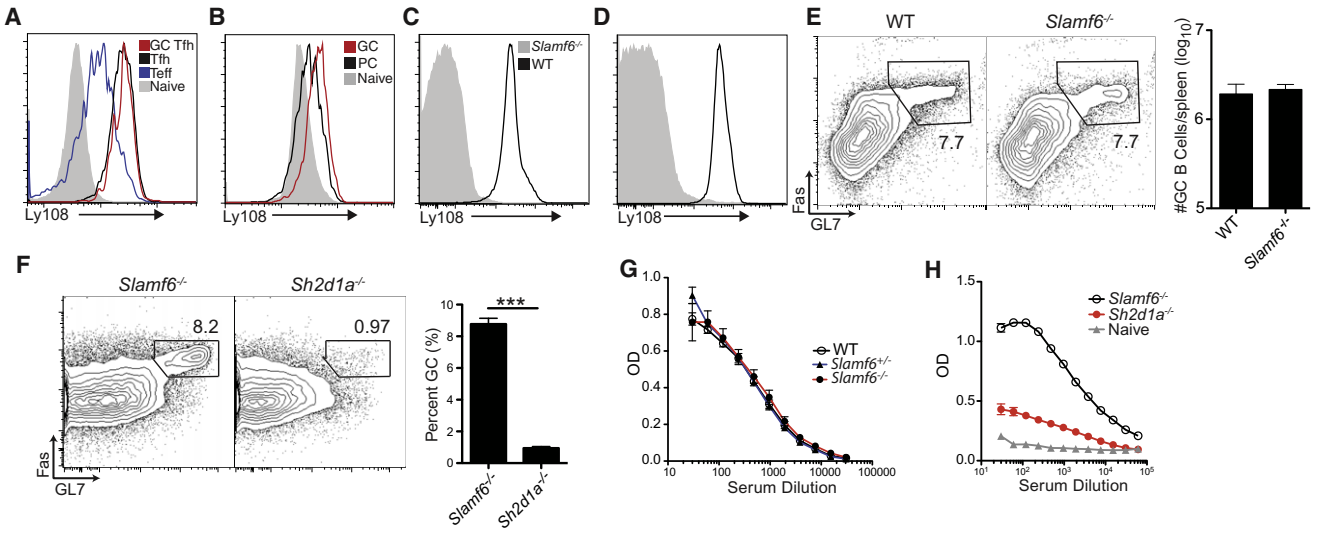

Figure 1. Ly108 Expression and Function in an Acute Viral Infection

(A and B) Ly108 expression on splenic (A) effector (Th1) (CXCR5^{lo}), Tfh (CXCR5^{hi}), and GC Tfh (CXCR5^{hi}PD1^{hi}) CD44^{hi}CD4^{+} cells 8 days after LCMV infection, and (B) naive B cells, plasma cells (CD19^{+}CD138^{hi}IgD^{lo}), and germinal center B cells (CD19^{+}GL7^{hi}Fas^{hi}) 8 days after LCMV infection.

(C and D) CD4^{+} T cells (C) and B cells (D) from uninfected wild-type (WT) and *Slamf6*$^{-/-}$ mice.

(E and F) *Slamf6*$^{+/+}$ and *Slamf6*$^{-/-}$ mice (E) or *Slamf6*$^{-/-}$ and *Sh2d1a*$^{-/-}$ mice (F) were infected with LCMV and splenocytes were analyzed for GC B cells 8 days later, gated on CD19^{+} cells.

(G) WT, *Slamf6*$^{+/-}$, and *Slamf6*$^{-/-}$ mice were infected with LCMV- and virus-specific serum IgG was measured day 30 after infection.

(H) *Slamf6*$^{-/-}$ and *Sh2d1a*$^{-/-}$ mice were infected with LCMV, and virus-specific serum IgG was measured day 8 postinfection.

Data are representative of two or more independent experiments; n = 4 or more per group. ***p < 0001. Error bars are SEM. See also Figure S1.

numbers in the combined absence of SAP and Ly108 (*Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$) were 80% of WT GC numbers ($p < 0.001$; Figure 2D). Antibody responses to an acute viral infection were recovered in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice, in contrast to *Sh2d1a*$^{-/-}$ mice ($p < 0.01$; Figure 2E). In contrast, neither *Slamf1*$^{-/-}$*Sh2d1a*$^{-/-}$ mice (Yusuf et al., 2010) nor *Cd84*$^{-/-}$*Sh2d1a*$^{-/-}$ mice (Figure S2) showed evidence of amelioration of the negative signaling occurring in the absence of SAP, with germinal center defects equal in severity to *Sh2d1a*$^{-/-}$ mice. These data suggested that Ly108 transmits a potent negative signal in the absence of SAP, potentially explaining the gross defect of humoral immunity in SAP-deficient mice and humans.

CD4^{+} T Cell-Intrinsic Effects of Ly108 Signaling

SAP has been shown to be required in CD4^{+} T cells for humoral immunity and, with the exception of one study, not required in B cells or APCs for the development of germinal centers (Cannons et al., 2011; Veillette et al., 2008). To determine whether Ly108 inhibitory signaling in the absence of SAP was CD4^{+} T cell intrinsic, we transferred naive WT, *Sh2d1a*$^{-/-}$, or *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ purified SMARTA TCR transgenic CD4^{+} T cells ("SM," LCMV gp66-77 I-A^{b}-specific) into *Sh2d1a*$^{-/-}$ recipients. Given that *Sh2d1a*$^{-/-}$ mice are unable to mount an endogenous germinal center response to LCMV, any germinal center B cells that develop are a direct result of the transferred virus-specific SM CD4^{+} T cells. Eight days after LCMV, mice receiving *Sh2d1a*$^{-/-}$ SM CD4^{+} T cells failed to mount a germinal center response, whereas *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4^{+} T cells rescued germinal center formation (58% of WT *Sh2d1a*$^{-/-}$ versus *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$, $p < 0.001$; Figure 3A). *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM cells also supported robust plasma cell development in response to the acute infection (90% of WT), in contrast to *Sh2d1a*$^{-/-}$ SM cells ($p < 0.001$; Figure 3B). Whereas *Sh2d1a*$^{-/-}$ SM cells had a defect in Tfh cell frequency ($p < 0.05$; Figure 3C; Yusuf et al., 2010), this defect was reversed in the absence of Ly108 (*Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ versus *Sh2d1a*$^{-/-}$ SM, $p < 0.01$; Figure 3C). Notably, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4^{+} T cells were able to differentiate into GC Tfh cells ($p < 0.001$; Figure 3D), consistent with the strong B cell response observed in the recipient mice. These data show that the germinal center phenotype seen in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice is due to a CD4^{+} T cell-intrinsic function, revealing a potent Ly108 inhibitory pathway that requires counteraction by SAP.

One challenge to studying SLAM family receptors is that the genes are clustered together on chromosome 1. This impacts the interpretation of certain experiments because of SLAM family receptor allelic differences between mouse strains (Keszei et al., 2011; Veillette et al., 2006; Wandstrat et al., 2004). Therefore, we designed an experiment to confirm that *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ reversal of the severe *Sh2d1a*$^{-/-}$ humoral immunity defect phenotype was specifically due to the absence of Ly108. Ly108 was reintroduced by retroviral vector (RV) transduction of *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4^{+} T cells (Figure 3E). The two widely expressed Ly108 isoforms, Ly108-1 and Ly108-2, both of which contain two canonical ITSMs, were investigated for activity. Introduction of either isoform of Ly108 into *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM cells "reversed" the phenotype of these cells and suppressed germinal center development ($p < 0.05$ and $p < 0.01$; Figure 3E). Plasma cell responses to the viral infection

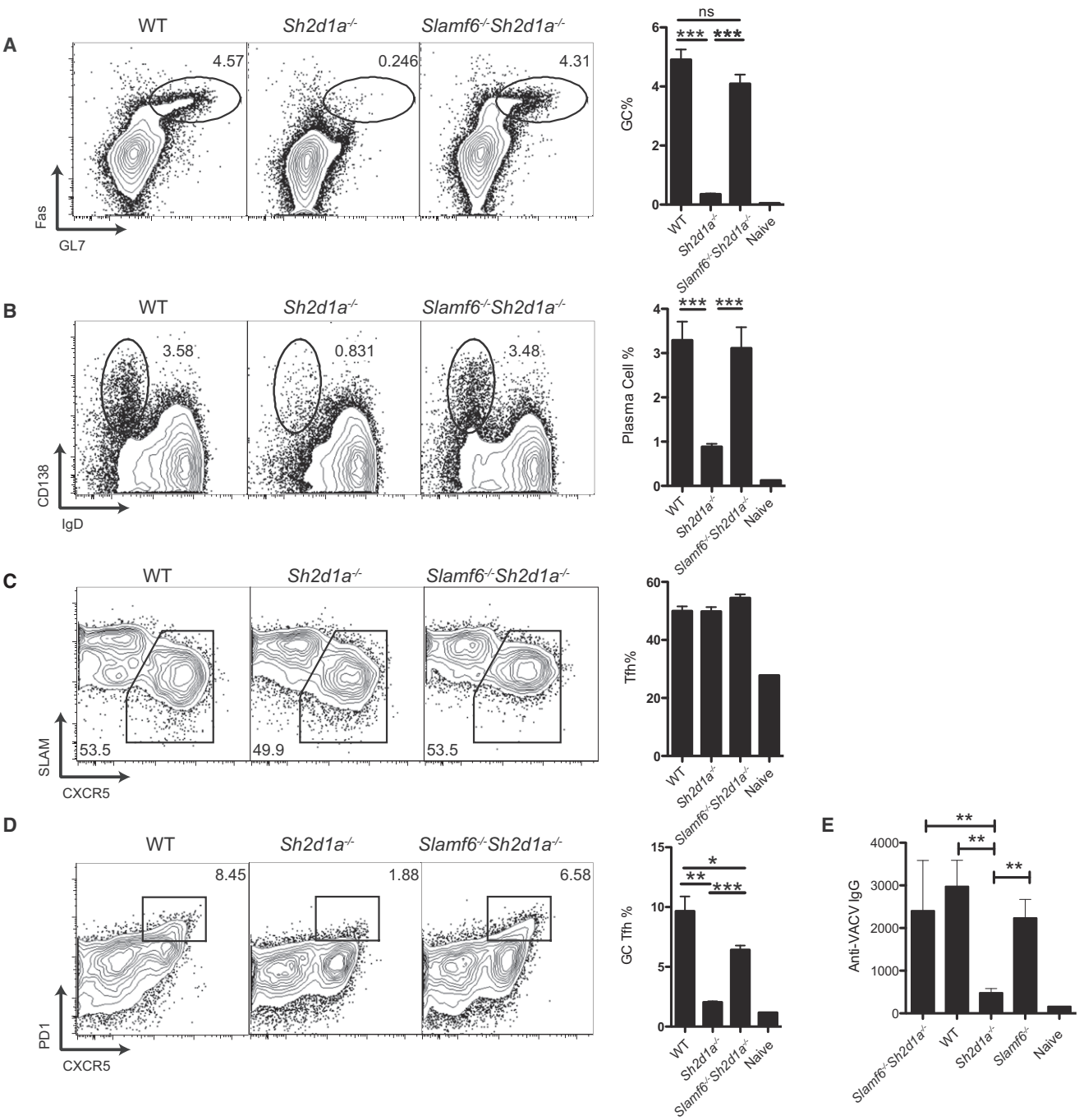

Figure 2. Loss of Ly108 Eliminates the SAP Expression Requirement for Germinal Center Development

(A–D) Wild-type, *Sh2d1a*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice were infected with LCMV. Splenocytes were analyzed day 8 after infection.

(A and B) Germinal center B cells (Fas^{hi}GL7^{hi}) (A) and plasma cells (IgD^{lo}CD138^{hi}) (B) are shown as a percent of total B cells (CD19^{+}CD4^{-}).

(C and D) Tfh cells (CXCR5^{+}SLAM^{lo}) (C) and GC Tfh cells (CXCR5^{+}PD1^{hi}) (D) are shown as a percent of activated CD4^{+} T cells (CD44^{hi}CD4^{+}).

Data are representative of three (A–C) or two (D) independent experiments; n = 5 per group.

(E) Wild-type, *Sh2d1a*$^{-/-}$, *Slamf6*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice were infected with vaccinia virus. Serum antibody titers were analyzed day 8 after infection.

*p < 0.05, **p < 0.005, ***p < 0001. n.s., no statistically significant difference (p > 0.05). Error bars are SEM. See also Figure S2.

were also inhibited by Ly108 expression ($p < 0.012$ and $p < 0.04$; Figure 3F). Inhibition of germinal center and plasma cell development by Ly108 expression in the absence of SAP was incomplete, probably because of moderate expression of Ly108 by the RV expression vector (Figure 3G). This experimental setting allowed for the demonstration of negative signaling involving Ly108, but it also allowed for complementary experiments demonstrating that overexpression of SAP in

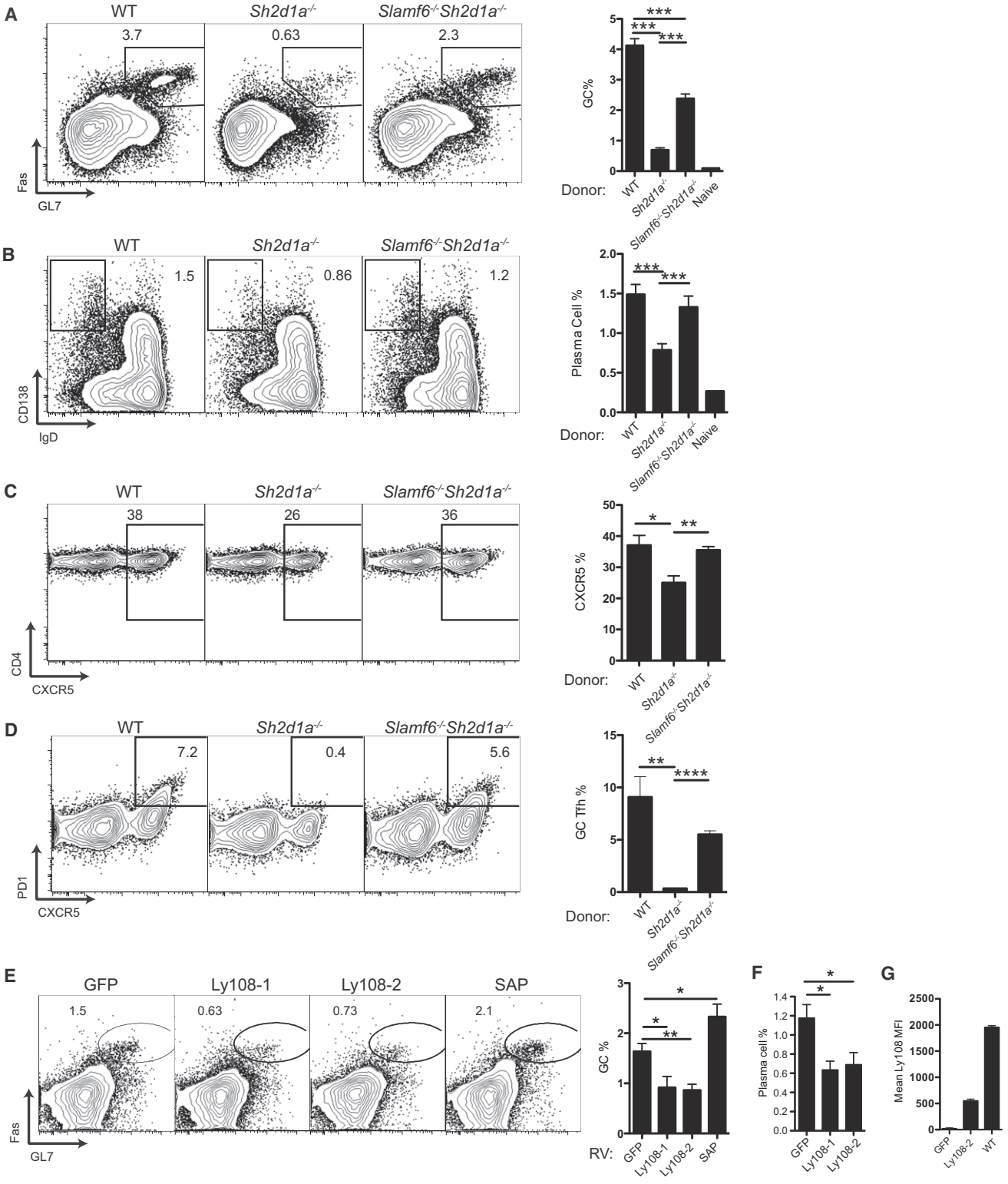

Figure 3. Loss of Ly108 Reverses the SAP Requirement in CD4+ T Cells for T Cell Help to B Cells

(A–D) WT, *Sh2d1a*$^{-/-}$, or *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD45.1^{+} SM TCR transgenic CD4^{+} T cells were transferred into *Sh2d1a*$^{-/-}$ recipient mice subsequently infected with LCMV. Splenocytes were analyzed day 8 after infection.

(A and B) Germinal center B cells (Fas^{hi}GL7^{hi}) (A) and plasma cells (CD138^{+}IgD^{lo}) (B) shown as percent of total B cells (CD19^{+}CD4^{-}).

(C and D) Tfh cells (CXCR5^{+}) (C) and GC Tfh cells (CXCR5^{+}PD1^{hi}) (D) shown as a percent of SM (CD45.1^{+}CD4^{+}B220^{-}).

Slamf6$^{-/-}$ *Sh2d1a*$^{-/-}$ CD4$^+$ T cells led to enhanced germinal center formation ($p < 0.05$; Figure 3E). This is consistent with SAP both blocking a negative signal through Ly108 and providing a positive signal for CD4$^+$ T cell help to B cells. Taken together, these results provide strong evidence for a CD4$^+$ T cell-intrinsic effect of Ly108 inhibition of humoral immunity that is modulated by SAP expression.

The Role of Ly108 in Formation of T Cell:B Cell Conjugates

To evaluate how Ly108 functions in the absence of SAP, we asked what aspect of T cell help to B cells is inhibited by Ly108. Previous studies have shown that SAP plays an important role in CD4$^+$ T cell:B cell conjugate contact time in germinal centers. SAP deficiency leads to decreased contact time. This defect can be recapitulated in vitro by a flow cytometry-based T cell:B cell conjugation assay (Cannons et al., 2010a; Qi et al., 2008) wherein absence of SAP expression in CD4$^+$ T cells results in a severe defect in adhesion to B cells in the presence of cognate peptide (Figures 4A and 4B). We therefore examined whether T cell:B cell conjugates were impacted by the absence of Ly108 in *Sh2d1a*$^{-/-}$ CD4$^+$ T cells. Combined loss of SAP and Ly108 again reversed the *Sh2d1a*$^{-/-}$ CD4$^+$ T cell phenotype, back to T cell:B cell conjugate percentages seen for WT CD4$^+$ T cells (Figures 4A and 4B). These data demonstrate that Ly108 signaling can actively inhibit T cell:B cell adhesion and indicate that this is the likely cause of the in vivo Ly108-dependent block to germinal center development.

ITSM Phosphotyrosine Motifs Are Required for Ly108 Inhibitory Signals

Based on the previous experiments, we hypothesized that Ly108 transmits a potent negative signal to CD4$^+$ T cells in conditions of low or absent SAP protein. SAP protein levels are low in naive CD4$^+$ T cells and many activated CD4$^+$ T cells, but SAP protein expression is substantially upregulated in Tfh cells, both in mice (Yusuf et al., 2010) and humans (Kroenke et al., 2012; Ma et al., 2009). We therefore addressed how Ly108 transmits this negative signal. Ly108 isoforms contain multiple tyrosines, which may be potential docking sites for inhibitory signaling molecules. In addition to two ITSMs, Ly108-1 contains one additional unique tyrosine, whereas Ly108-2 contains two additional unique tyrosines (Cannons et al., 2011). Because Ly108-1 and Ly108-2 both are able to inhibit germinal center formation in the absence of SAP (Figures 3E–3G), we focused on Ly108-2 and generated a Ly108-2 ("Ly108") mutant expression construct in which all cytoplasmic tyrosines were mutated to phenylalanines (Ly108-AllF) (Figure S3). We also evaluated a construct that contained only the single conserved noncanonical ITSM (Ly108-Y3) to test the importance of the ITSMs for Ly108-negative signaling. *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells were transduced with Ly108, Ly108-AllF, Ly108-Y3, or a control construct (RV-GFP) and transferred into *Sh2d1a*$^{-/-}$ recipients. RV-GFP-transduced *Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells transferred into *Sh2d1a*$^{-/-}$ recipients were used as a negative control. After cell transfer, mice were infected with LCMV and germinal center formation evaluated at day 8. As anticipated, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM + RV-GFP drove significantly more germinal centers than did *Sh2d1a*$^{-/-}$SM + RV-GFP ($p < 0.05$; Figures 4C and 4D). Additionally, reintroduction of Ly108 in *Slamf6*$^{-/-}$ *Sh2d1a*$^{-/-}$ SM cells inhibited germinal center formation ($p < 0.05$; Figures 4C and 4D). However, Ly108-Y3- ($p < 0.01$) or Ly108-AllF- ($p < 0.05$) expressing *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM cells were unable to inhibit germinal center formation in comparison to *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM cells expressing intact Ly108 (Figures 4C and 4D). In follow-up experiments with Ly108-AllF, the inhibition of plasma cell development by Ly108 in the absence of SAP was lost when CD4$^+$ T cells expressed the Ly108-AllF mutant ($p < 0.013$; Figure 4E). Thus, ITSM phosphotyrosines are important for the transduction of inhibitory signals through Ly108 that restrict humoral immune responses.

To examine the roles of individual ITSMs in Ly108-negative signaling, we created two additional Ly108 mutants containing only the first or second ITSM tyrosine (Ly108-Y1 and Ly108-Y2). *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells were transduced with RV-Ly108, Ly108-Y1, or Ly108-Y2 and transferred into *Sh2d1a*$^{-/-}$ recipients that were then infected with LCMV. Ly108 with only a single ITSM, either the first ITSM ($p < 0.05$) or second ITSM ($p < 0.05$), provided strong inhibitory signaling in the absence of SAP protein (Figures 4F and 4G). Because the ITSMs are the SAP binding motifs, these results indicate that the ITSM phosphotyrosines can serve as an on-off switch for CD4$^+$ T cell help to B cells via competition for phosphotyrosine binding between SAP (positive signaling) and an unknown negative signaling protein.

Increased SHP-1 Association with Ly108 in the Absence of SAP and Disruption of the T Cell:B Cell Synapse

Previous literature suggested that in addition to binding SAP, different SLAM family members could recruit negative signaling molecules including the protein phosphatases SHP-2, SHP-1, Cbl, or Csk (Chen et al., 2006; Eissmann et al., 2005; Kim et al., 2010; Sayos et al., 1998; Snow et al., 2009; Zhong and Veillette, 2008), as well as the lipid phosphatase SHIP-1 (Latour et al., 2001; Shlapatska et al., 2001). Limited evidence was also available for recruitment of negative signaling molecules to Ly108 (or NTB-A) (Bottino et al., 2001; Snow et al., 2009; Valdez et al., 2004; Zhong and Veillette, 2008). To understand the mechanisms by which Ly108 transduces an inhibitory signal, we immunoprecipitated Ly108 from WT and *Sh2d1a*$^{-/-}$ CD4$^+$

(E–G) *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells (CD45.1$^+$) were transduced with Ly108-1, Ly108-2, SAP, or GFP vector in vitro and transferred into *Sh2d1a*$^{-/-}$ recipient mice, and mice were infected with LCMV.
(E) Germinal center B cells (Fas^{hi}GL7^{hi}) are shown 8 days after infection, as a percent of total B cells.
(F) Plasma cell (CD19$^+$ IgD^{lo}CD138$^+$) frequencies, as a percent of total B cells.
(G) MFI of Ly108 expression on *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells transduced with empty vector (GFP) or Ly108 vector (Ly108-2), in comparison to endogenous Ly108 expression on WT SM cells.
Data are representative of four (A–C, E) or two (D, F, G) independent experiments; n = 4 or more mice per group. *$p < 0.05$, **$p < 0.005$, ***$p < 0001$. Error bars are SEM.

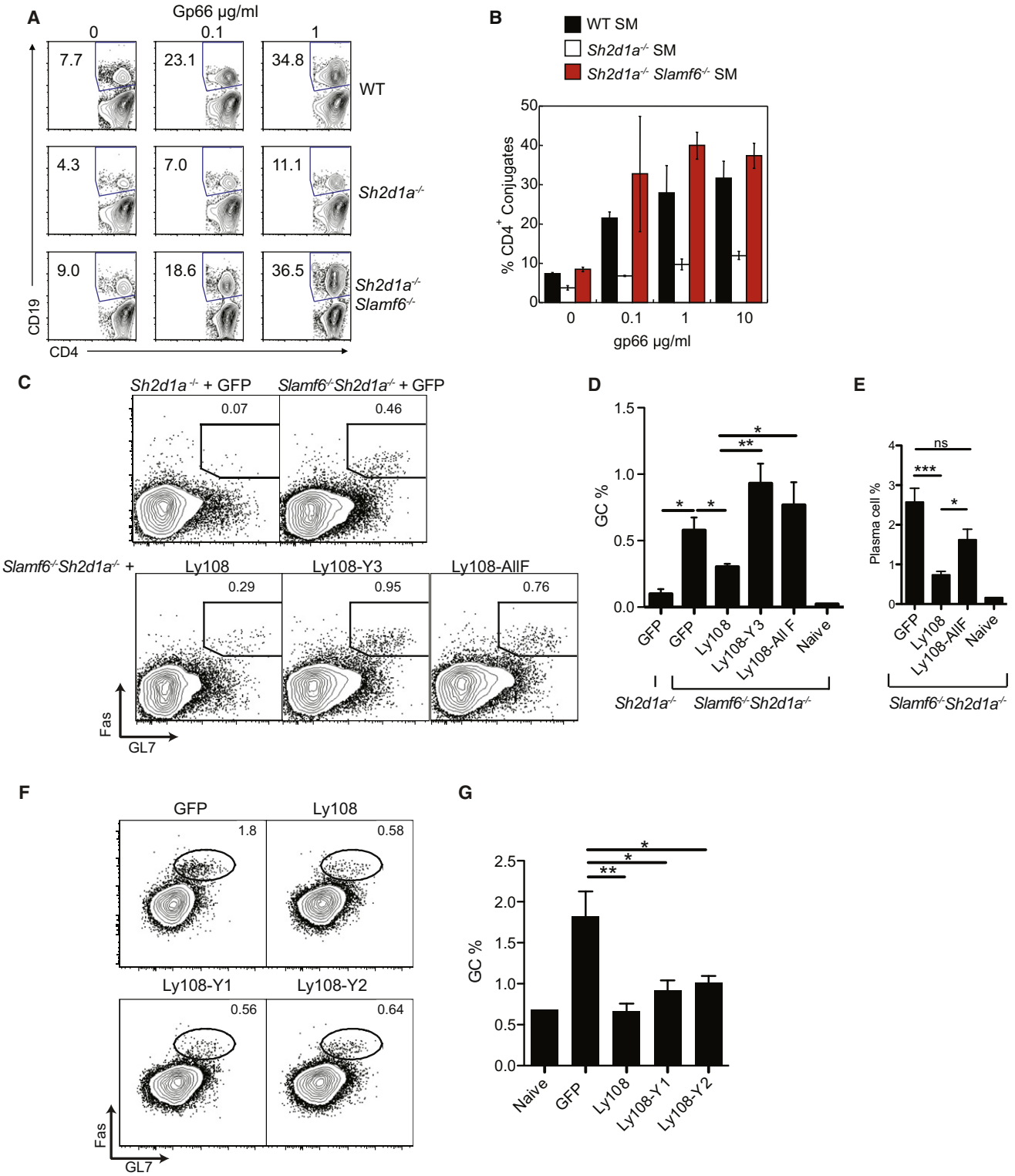

Figure 4. *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T Cells Form Stable Conjugates with B Cells, and ITSM Phosphotyrosine Motifs Are Required for Inhibitory Signals Transmitted by Ly108 In Vivo

(A and B) Conjugation efficiency of WT, *Sh2d1a*$^{-/-}$, and *Slamf6*$^{-/-}$ *Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells with B cells pulsed with cognate peptide (LCMV gp66-77).

(A) Representative flow cytometry plots, gated on CD4$^+$ T cells.

(B) Mean frequency of CD4$^+$CD19$^+$ conjugates in total CD4$^+$ events. n = 2.

(C–G) Roles of Ly108 tyrosines in vivo.

T cells after stimulation with peptide-pulsed B cells. Stimulation with B cells led to a specific increase in SHP-1 association with Ly108 in the *Sh2d1a*$^{-/-}$ CD4$^+$ T cells (Figure 5A), with less SHP-1 association in WT CD4$^+$ T cells. Thus, in the absence of SAP, CD4$^+$ T cells show increased association of the phosphatase SHP-1 with Ly108.

We subsequently used confocal immunofluorescence microscopy to examine SHP-1 localization in T cell:B cell conjugates. SHP-1 accumulated at the synapse of WT, *Sh2d1a*$^{-/-}$, and *Sh2d1a*$^{-/-}$*Slamf6*$^{-/-}$ CD4$^+$ T cell conjugates with WT B cells pulsed with cognate peptide (Figure 5B, top). Rotation of the images and a more detailed en face evaluation of the T cell:B cell synapse revealed marked differences in SHP-1 localization (Figure 5B, bottom). WT CD4$^+$ T cells exhibited a clearance of SHP-1 from the center of the B cell contact site, resulting in an O-shaped SHP-1 pattern around the perimeter of the synapse (Figures 5B–5D). In contrast, *Sh2d1a*$^{-/-}$ CD4$^+$ T cells failed to restrict SHP-1 localization and SHP-1 was diffusely spread throughout the entire T cell:B cell synapse (Figures 5B–5D). Strikingly, SHP-1 was cleared from the central synapse in a large fraction of *Sh2d1a*$^{-/-}$*Slamf6*$^{-/-}$ T cell:B cell conjugates (Figures 5B–5D). Together, these data suggest that the impaired clearance of SHP-1 from the immune synapse has a pronounced negative impact on the adhesion of *Sh2d1a*$^{-/-}$ CD4$^+$ T cells with antigen-presenting B cells.

To validate the requirements of the Ly108 tyrosine residues and SHP-1 recruitment, *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ SM T cells were retrovirally reconstituted with either WT Ly108 or Ly108-AllF. After stimulation with peptide-pulsed B cells, SHP-1 selectively associated with WT Ly108 in the *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cells (Figure 5E), consistent with the increased SHP-1 associated with Ly108 observed in *Sh2d1a*$^{-/-}$ cells (Figure 5A). Additionally, to visualize Ly108 and SHP-1 localization, Ly108 constructs were designed as fusion proteins with GFP and introduced into *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ SM T cells. Ly108 is present both in central and peripheral regions of the synapse, as seen in *Slamf6*$^{-/-}$ cells reconstituted with Ly108-GFP or Ly108-AllF-GFP (Figures 5G, 5H, and S4). SHP-1 is predominantly restricted to an outer ring at the synapse in *Slamf6*$^{-/-}$ + Ly108-GFP cells (Figures 5F and 5G), comparable to WT and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cells (Figures 5B–5D). *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cells reconstituted with Ly108 show increased SHP-1 recruitment in the center of the synapse (Figures 5F and 5G), consistent with enhanced SHP-1 recruitment by Ly108 in the absence of SAP. However, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cells reconstituted with the Ly108-AllF mutant show SHP-1 localization in an outer ring, comparable to WT CD4$^+$ T cells (Figures 5F and 5H). These experiments support a model whereby Ly108 recruits SHP-1 to the central area of the synapse in the absence of SAP and confirms the requirements for the tyrosine residues for such recruitment. Thus, both in vitro and in vivo experiments suggest that in the absence of SAP, Ly108 mediates a potent negative signal primarily via SHP-1 phosphatase recruitment to ITSM motifs.

Sh2d1a$^{-/-}$ T Cell:B Cell Adhesion Can Be Restored by Inhibition of SHP-1 or Reduced Ly108 Expression

If the negative signaling through Ly108 depends on SHP-1 recruitment, disruption of SHP-1 recruitment or function is predicted to prevent Ly108-negative signaling. Furthermore, disruption of SHP-1 recruitment or function would be predicted to reverse the severe cell:cell adhesion defect observed for SAP-deficient CD4$^+$ T cells. To test this hypothesis, we preincubated SM CD4$^+$ T cells with sodium stibogluconate (SSG), a specific SHP-1 inhibitor (Iype et al., 2010), and evaluated T cell:B cell conjugate formation. *Sh2d1a*$^{-/-}$ CD4$^+$ T cell adhesion to B cells in the presence of cognate peptide is severely defective (Figures 6A and 6B). Strikingly, treatment of *Sh2d1a*$^{-/-}$ CD4$^+$ T cells with SSG restores T cell:B cell adhesion to the same level achieved by wild-type CD4$^+$ T cells (Figures 6A–6C). Thus, excessive Ly108-mediated SHP-1 recruitment is the primary functional cause of the profound *Sh2d1a*$^{-/-}$ CD4$^+$ T cell adhesion to B cell defect.

Ly108 is a self-ligand. This implies a requirement for Ly108 on both the CD4$^+$ T cell and B cell for Ly108- and SHP-1-dependent inhibitory signaling to occur in CD4$^+$ T cells. Inhibition of T cell:B cell adhesion does require Ly108 expression on both the CD4$^+$ T cells and the B cells, as shown by the fact that *Sh2d1a*$^{-/-}$ CD4$^+$ T cells exhibit adhesion to *Slamf6*$^{-/-}$ B cells that is comparable to WT CD4$^+$ T cell adhesion to WT B cells in the presence of high-dose cognate peptide (Figure 6D). In addition, at 100-fold lower peptide concentrations, *Sh2d1a*$^{-/-}$ CD4$^+$ T cells exhibit reduced adhesion to *Slamf6*$^{-/-}$ B cells compared to WT CD4$^+$ T cell adhesion to *Slamf6*$^{-/-}$ B cells (Figures 6B and 6D). We infer from these results that positive SAP-dependent signaling through SLAM family receptors is most important under limiting concentrations of antigen (Figure 6D), whereas negative signaling through Ly108 can be potent at all antigen concentrations (Figure 6B). In summary, ligation of Ly108 on CD4$^+$ T cells by Ly108 on B cells triggers SHP-1 recruitment in the absence of SAP, resulting in truncated synapse formation and abortive T cell help (Figure S5). This potent inhibitory mechanism downstream of Ly108 can be reversed by selective inhibition of SHP-1.

(C–E) *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4$^+$ T cells were transduced with GFP, Ly108-2 ("Ly108"), Ly108-Y3 mutant, or Ly108-AllF mutant RV and transferred into *Sh2d1a*$^{-/-}$ recipient mice. An additional group received *Sh2d1a*$^{-/-}$ SM cells transduced with RV-GFP. Mice were infected with LCMV and B cell responses in spleen were analyzed 8 days after infection.
(C) Representative germinal center B cell FACS plots are shown, gated on total B cells (CD19$^+$CD4$^-$).
(D) Quantitation of GC B cells as gated in (C).
(E) Quantitation of the plasma cell response.
(F and G) *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM -Ly108-Y1 mutant or -Ly108-Y2 mutant CD4$^+$ T cells were transduced with RV-GFP, -Ly108-2 ("Ly108") and transferred into *Sh2d1a*$^{-/-}$ recipient mice subsequently infected with LCMV.
(F) Representative germinal center B cell FACS plots are shown, gated on total B cells (CD19$^+$CD4$^-$), analyzed at day 8 after infection.
(G) Quantitation of GC B cells as gated in (F).
Data are representative of two independent experiments; n = 4 or more per group. *$p < 0.05$, **$p < 0.005$; error bars are SEM. See also Figure S3.

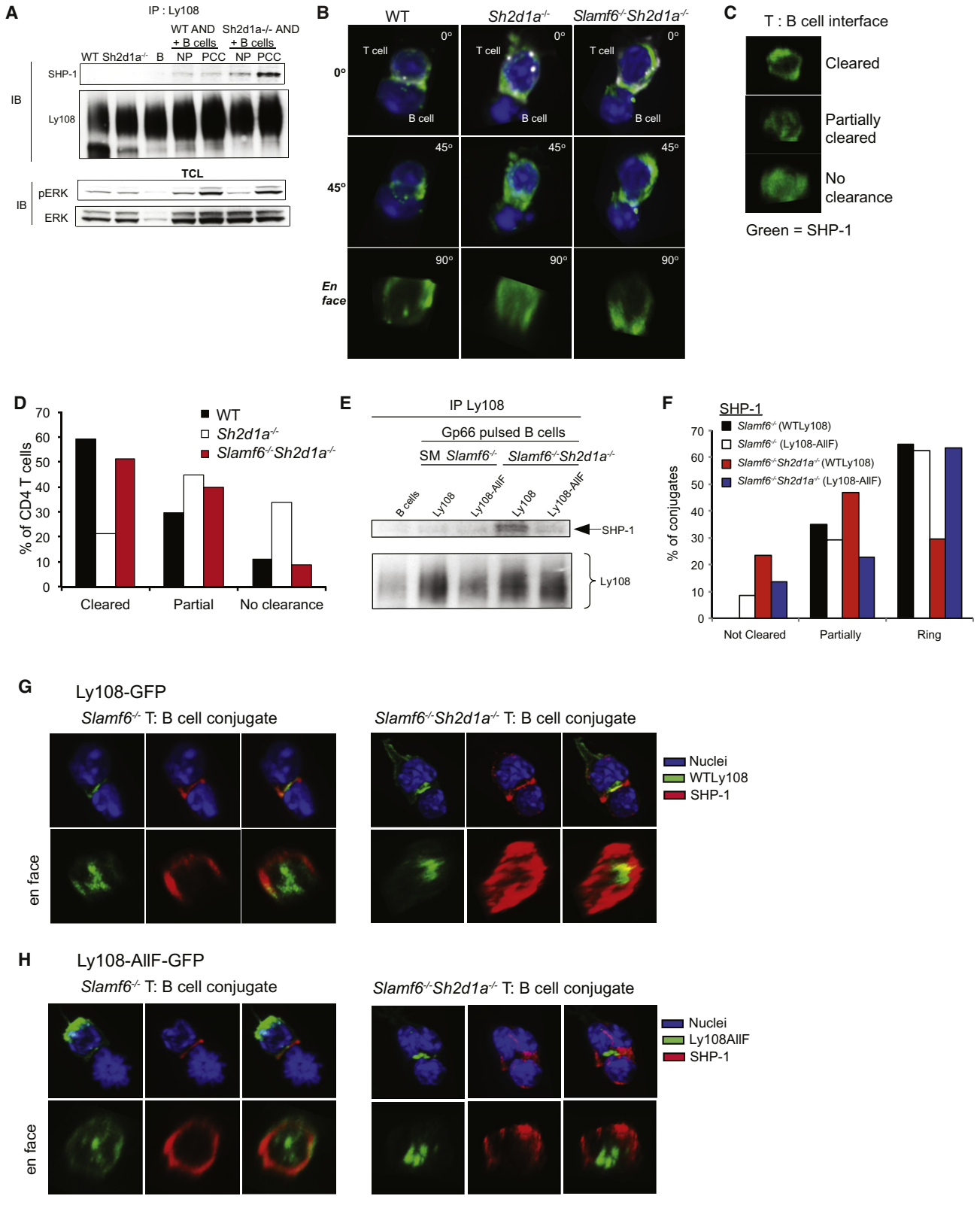

Figure 5. Ly108 Recruits SHP-1 to the T Cell:B Cell Immunological Synapse

(A) Activated WT and *Sh2d1a*$^{-/-}$ AND TCR transgenic (PCC-specific) CD4^{+} T cells were lysed without stimulation or postincubation with LPS-activated B cells that were untreated or pulsed with PCC peptide. Lysates were immunoprecipated for Ly108 and blotted for SHP-1 and Ly108. Total cell lysates (TCL) were examined for pERK activation and total ERK protein levels. NP, no peptide.

NKT Cell Development Is Rescued in *Sh2d1a*$^{-/-}$ Mice by the Elimination of Ly108

In addition to the dramatic humoral immunity defect, a severe defect in NKT cell development is a second prominent phenotype in both SAP-deficient mice and humans (Griewank et al., 2007; Ma et al., 2005; Nichols et al., 2005; Pasquier et al., 2005). SLAM family receptors Ly108 and SLAM have been shown to contribute to the development of NKT cells in the thymus (Griewank et al., 2007). We therefore examined whether the absence of NKT cells in *Sh2d1a*$^{-/-}$ animals was related to negative signaling by Ly108. WT, *Sh2d1a*$^{-/-}$, *Slamf6*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice were analyzed for the presence of splenic NKT cells. Consistent with previous studies, NKT cells were absent in *Sh2d1a*$^{-/-}$ mice (99.9% loss, $p < 0.0001$), whereas *Slamf6*$^{-/-}$ animals had 61% reduced frequencies compared to WT ($p < 0.0001$; Figures 7A and 7C). Quantifying absolute cell numbers gave the same results ($p < 0.001$, $p < 0.002$; Figure 7B). Surprisingly, NKT cell frequencies were substantially rescued by deletion of *Slamf6* from *Sh2d1a*$^{-/-}$ mice (252-fold NKT cell increase in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ versus *Sh2d1a*$^{-/-}$ mice; $p < 0.0001$; Figures 7A and 7C). The *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice did, however, have fewer splenic NKT cells than did WT ($p < 0.0001$). Notably, the *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice also had fewer splenic NKT cells than *Slamf6*$^{-/-}$ mice ($p < 0.0034$; Figures 7A and 7C).

NKT cell development depends on thymocyte-thymocyte interactions, where CD1d on one thymocyte is engaged by the TCR of another thymocyte (Bendelac et al., 2007). NKT cell development in *Sh2d1a*$^{-/-}$ mice is blocked at an extremely early stage, referred to as stage 0. NKT cell thymic development is rescued in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice (*Sh2d1a*$^{-/-}$ versus *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$, 0.008% and 2.62%, respectively; Figures 7D and 7E). However, the rescue does not completely restore developing thymic NKT cell numbers to wild-type numbers (Figures 7D and 7E). Liver is a major site of NKT cells in the periphery. Whereas *Sh2d1a*$^{-/-}$ mice have a complete absence of liver NKT cells, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice have substantial liver NKT cells (0.007% versus 3.1%; Figures 7G and 7H). Nevertheless, the liver NKT cell frequency in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice is still reduced in comparison to wild-type mice (Figures 7G and 7H), comparable to the NKT cells in spleen. *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ NKT cells are fully differentiated, with normal proportions of stage 2 (NK1.1^{-}) and stage 3 (NK1.1^{+}) NKT cells (Figures 7F and 7I). Overall, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice have a significant loss of NKT cells, in the rank order: wild-type > *Slamf6*$^{-/-}$ > *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$. This shows the positive signaling role of Ly108 in NKT cell development also. In conclusion, inhibitory signaling by Ly108 is potent in both CD4^{+} T cell function and NKT cell development.

DISCUSSION

A central role for SAP has been shown in Tfh cell differentiation and function and the generation of B cell immunity, both in mice and humans; however, how SAP facilitates these feats has been incompletely understood. We have found that Ly108 can transmit positive and negative signals to CD4^{+} T cells and NKT cells, such that absence of SAP results in both the loss of a positive signal and the exacerbation of a negative signal. Our findings help resolve the conundrum of why SAP deficiency results in such a severe humoral immunity defect encompassing virtually all T cell-dependent immune responses. Equally importantly, these findings reveal a mechanism whereby Ly108 appears to serve as a rheostat for T cell:B cell interactions and other lymphocyte:lymphocyte interactions (e.g., NKT cell thymic selection), via modulation of the ratio of positive and negative signals transmitted by SAP and SHP-1 competing for occupancy of the same ITSMs of Ly108. The broader role of Ly108 is confirmed by similar findings in CD8^{+} T cells (Zhao et al., 2012, this issue of *Immunity*).

Negative signaling by SLAM family receptors was previously observed, but this was primarily in the context of 2B4 in SAP-deficient human NK cells (Moretta et al., 2001; Nakajima et al., 2000; Parolini et al., 2000), and it was generally considered to be an unusual feature of NK cell receptor inhibitory signaling biology (Moretta et al., 2001; Raulet et al., 2001; Veillette et al., 2009) that was extended to include both 2B4 and NTB-A (*Slamf6*) (Bottino et al., 2001). Our CD4^{+} T cell and NKT cell work presented here, and new studies on CD8^{+} T cells (Zhao et al., 2012, this issue; Palendira et al., 2011), indicate that a potent negative signaling role of Ly108 is broadly active in lymphocytes. This is not a generalized property of SLAM family receptors. *Slamf1*$^{-/-}$*Sh2d1a*$^{-/-}$ mice phenocopy the severe germinal center defect of *Sh2d1a*$^{-/-}$ mice, indicating that SLAM has only positive signaling functions (Yusuf et al., 2010). Disruption of *Cd84* also failed to rescue the negative signaling observed

(B–D) WT, *Sh2d1a*$^{-/-}$, and *Slamf6*$^{-/-}$ *Sh2d1a*$^{-/-}$ SM CD4^{+} T cell conjugates with LPS-activated B cells pulsed with cognate peptide (LCMV gp66-77) were stained with Hoechst (blue) and antibodies to CD4 (white) and SHP-1 (green).

(B) Cells were examined from the side as a confocal projection in the x-y plane (top row), at 45° (middle row), and 90° (en face, bottom row) rotations in the y-z plane.

(C) Representative immunofluorescence images of SHP-1 localization at the immune synapse.

(D) Quantification of SHP-1 localization at the immune synapse. Data represent two independent experiments with more than 40 conjugates scored/genotype for each experiment.

(E) Activated *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4^{+} T cells expressing Ly108-GFP or Ly108-AllF-GFP (Ly108-AllF) constructs were incubated with activated B cells pulsed with cognate peptide (LCMV gp66-77). Lysates were immunoprecipated for Ly108 and blotted for SHP-1 and Ly108.

(F–H) *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4^{+} T cells were transfected with either Ly108-GFP (WTLy108) or Ly108-AllF-GFP and conjugated to WT B cell targets pulsed with cognate peptide (LCMV gp66-77). Cells were stained with Hoechst (blue) and antibodies against SHP-1 (red). Green is Ly108-GFP fluorescence.

(F) Quantitation of SHP-1 localization at the synapse.

(G) Representative immunofluorescence images of cells expressing Ly108-GFP.

(H) Representative immunofluorescence images of cells expressing Ly108-AllF-GFP.

Further examples are shown in Figure S4. Data for each experiment depicted are representative of two or more experiments.

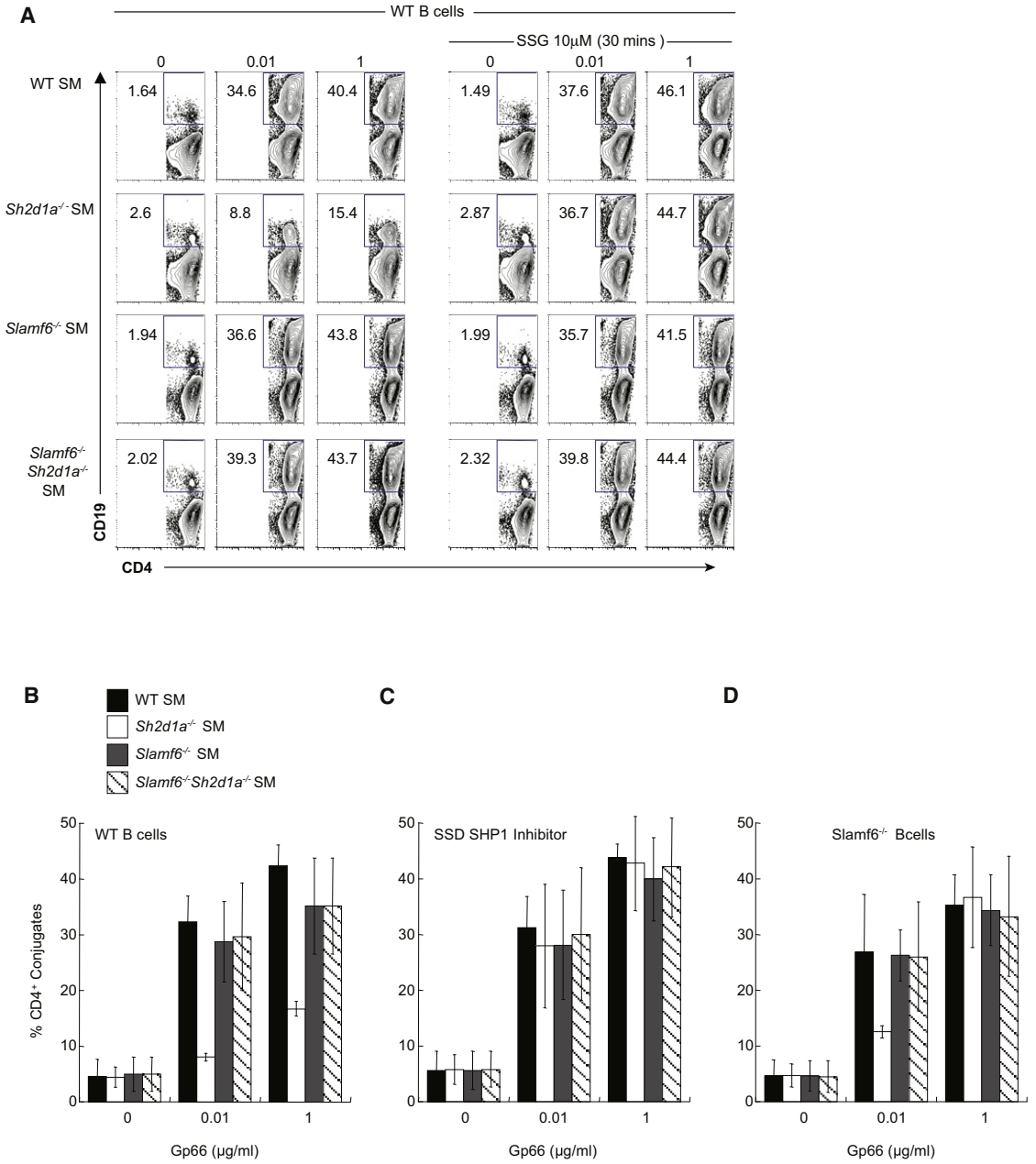

Figure 6. Inhibition of SHP-1 Reverses the Adhesion Defect of SAP-Deficient CD4+ T Cells

(A–C) Activated WT, *Sh2d1a*$^{-/-}$, *Slamf6*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ SM CD4+ T cells were incubated with activated WT B cells pulsed with cognate peptide (LCMV gp66-77. 0, 0.01, or 1.0 μg/ml), in the presence or absence of SSG, followed by flow cytometry.

(A) Representative flow cytometry plots, gated on CD4+ T cells.

(B and C) Mean frequency of T cell:B cell conjugates in total CD4+ events, in the absence (B) or presence (C) of SSG.

(D) CD4+ T cells incubated with activated *Slamf6*$^{-/-}$ B cells pulsed with cognate peptide (LCMV gp66-77. 0, 0.01, or 1.0 μg/ml). Data are shown from one of two experiments with equivalent results. See also Figure S5.

in *Sh2d1a*$^{-/-}$ mice. Therefore, the capacity for both negative and positive signaling appears to be restricted to Ly108 and 2B4 SLAM family receptors, of which only Ly108 is expressed on CD4+ T cells and developing NKT cells.

The potent negative signaling by Ly108 is primarily mediated by SHP-1. *Ptpn6*$^{-/-}$ (*motheaten*, SHP-1-deficient) mice have a phenotype consistent with the importance of SHP-1 in Ly108-negative signaling and balancing signaling through Ly108, as shown by the fact that *Ptpn6*$^{-/-}$ mice have rapid hyperglobulinemia (Green and Shultz, 1975; Shultz and Green, 1976). SHP-1 is a key protein phosphatase, functioning in a variety of signaling pathways, and the importance of SHP-1

is reinforced by the severe phenotype of *Ptpn6*$^{-/-}$ mice, which become severely ill and die at approximately 3 weeks of age as a result of autoimmune pneumonitis and other autoimmune sequelae. Further examination of the kinetics of SHP-1 regulation of T cell:B cell interactions and other Ly108-dependent functions of lymphocytes is an important area for future investigation, which will require refined experimental approaches.

Positive signaling mediated by SAP has been shown previously for multiple SLAM family receptors, including SLAM, CD84, and Ly108. Positive signaling through SLAM has been studied in the most molecular detail and has served as a template for understanding SLAM family receptor signaling (Veillette, 2006). SAP binds phosphotyrosines of the SLAM ITSM motifs and recruits Fyn kinase and PKC-θ, and together this signaling complex mediates induction of IL-4 expression (Cannons et al., 2011; Crotty, 2011). The biological role of this pathway in CD4$^+$ T cells has recently become clearer, as GC Tfh cells produce IL-4 (Crotty, 2011; Harada et al., 2012; Vijayanand et al., 2012) and SLAM receptor engagement induction of SAP signaling is required for expression of IL-4 by GC Tfh cells (Yusuf et al., 2010). A positive signaling role for SAP in CD4$^+$ T cells is also seen for CD84 adhesion and Ly108 adhesion (Cannons et al., 2010a). A positive signaling role for Ly108 is clearest from the study of NKT cell development. A consistent ~50% reduction in NKT cell numbers was observed in *Slamf6*$^{-/-}$ mice (Griewank et al., 2007) and confirmed here. Notably, *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ mice have fewer NKT cells than do *Slamf6*$^{-/-}$ mice. This indicates that not only does Ly108 provide a negative signal in the absence of SAP and a positive signal in the presence of SAP, additional SLAM family receptors also provide positive SAP-dependent signals that facilitate NKT cell development. The role of SLAM itself in NKT cell development was primarily revealed only in the combined absence of Ly108 and SLAM, highlighting the redundancy between SLAM family receptors for positive signaling via SAP.

The positive signaling contribution of SAP in CD4$^+$ T cells is best revealed in the studies of *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cell-intrinsic defects. Both germinal center B cell and GC Tfh cell frequencies were ~50% lower compared to WT CD4$^+$ T cell recipients. This shows a genetic requirement for positive signals through SAP and/or Ly108, paralleling the NKT cell biology. Furthermore, SAP overexpression in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CD4$^+$ T cells enhanced germinal center B cell numbers, again showing a positive role for SAP signaling. A positive signaling role for Ly108 in CD8 T cells is seen in reduced signaling and killing by *Slamf6*$^{-/-}$ CD8$^+$ T cells (Zhao et al., 2012, this issue).

The observation that Ly108 can transmit both positive and negative signals led us to examine the molecular mechanism of this process. We found that the negative signal is ITSM dependent and requires SHP-1 recruitment to Ly108 and the immunological synapse. This intimate linking of both positive and negative signaling to a single Ly108 binding site, the ITSM, forces a direct competition between SAP and SHP-1 for occupancy. Therefore, the simplest interpretation of these data is that the magnitude of negative or positive signaling transmitted by engaged Ly108 during T cell:B cell interaction is determined by the ratio of available SAP versus SHP-1 in the local subcellular microenvironment. This suggests that the ratio of SAP and SHP-1 occupancy of Ly108 acts as a rheostat for the magnitude of T cell help to B cells. Ly108 expression is dynamically regulated on many hematopoietic cells. SAP expression is dynamically regulated in T cells. SHP-1 recruitment to membrane is dynamically regulated. We suggest that this Ly108 rheostat concept may apply to a variety of cell:cell interactions.

SAP's impact on the duration of cell:cell interaction is particularly important for Tfh cell function. Tfh cells are specialized for B cell help, which is primarily provided via cell:cell interactions (Crotty, 2011). In addition, Tfh cell differentiation itself is strongly dependent on T cell:B cell interactions. Tfh cell differentiation is a multistage process (Crotty, 2011), such that in the absence of SAP there is still sufficient T cell:B cell interaction for early Tfh cell differentiation (Bcl6$^+$CXCR5$^+$) but not full polarization to GC Tfh cells (Bcl6^{hi}PD1^{hi}) (Choi et al., 2011; Crotty, 2011; Deenick et al., 2010; Qi et al., 2008; Yusuf et al., 2010). This is also important for the cognate B cells, because in the absence of extended T cell:B cell interactions, germinal center B cells fail to develop (Qi et al., 2008). Furthermore, in the absence of sufficient survival signals from CD4$^+$ T cells, germinal center B cells apoptose within hours (Liu et al., 1989). GC Tfh cells regulate maintenance of germinal center B cells (Eto et al., 2011; Linterman et al., 2010; Victora et al., 2010), and altering the duration of T cell:B cell contact controls the quantity of information transfer between the two cells, thereby controlling germinal center B cell survival and further differentiation (Crotty, 2012). Altering the duration of T cell:B cell contact also probably alters the quality of the information transferred, because some information transfer probably takes the form of an initial contact-dependent B cell→T cell signal (i.e., MHCII-TCR engagement) and then "help" from the T cell back to the B cell after a lag phase of additional signal integration and protein translation. The duration of the contact is critical for such information transfer. As such, Ly108 modulation of the overall time of adhesion appears to serve as a powerful rheostat for T cell→B cell help, indirectly influencing a range of receptor:ligand interactions. This is consistent with the observation that GC Tfh cells have the highest SAP protein expression among CD4$^+$ T cells. NKT cell development is also consistent with this, given that thymocyte interactions are potentially of insufficient duration to facilitate early NKT cell development in the thymus in the absence of SAP. In support of this model, recently it has been shown that thymic NKT cell development depends on strong sustained TCR signaling (Moran et al., 2011). Further understanding the stages of Ly108 and SLAM family-mediated cell:cell communications is important for unraveling germinal center biology.

The characterization of the ITAM and ITIM motifs has greatly informed our understanding of how lymphocytes interpret interactions with other cells. The ITSM motif has proven to be challenging to understand. The data herein highlight the bimodal positive-negative signaling that can occur through this motif. The fact that SAP or SHP-1 need only one pY for binding, whereas Ly108 and other SLAM family receptors have two ITSMs, adds complexity to the signaling competition possibilities. The presence of non-ITSM tyrosine motifs adds a further level of complexity yet to be examined. A third isoform of Ly108 with only a single ITSM was recently identified, and this isoform ameliorates autoimmunity (Keszei et al., 2011). It is also worth noting that PD-1, a potent inhibitory receptor

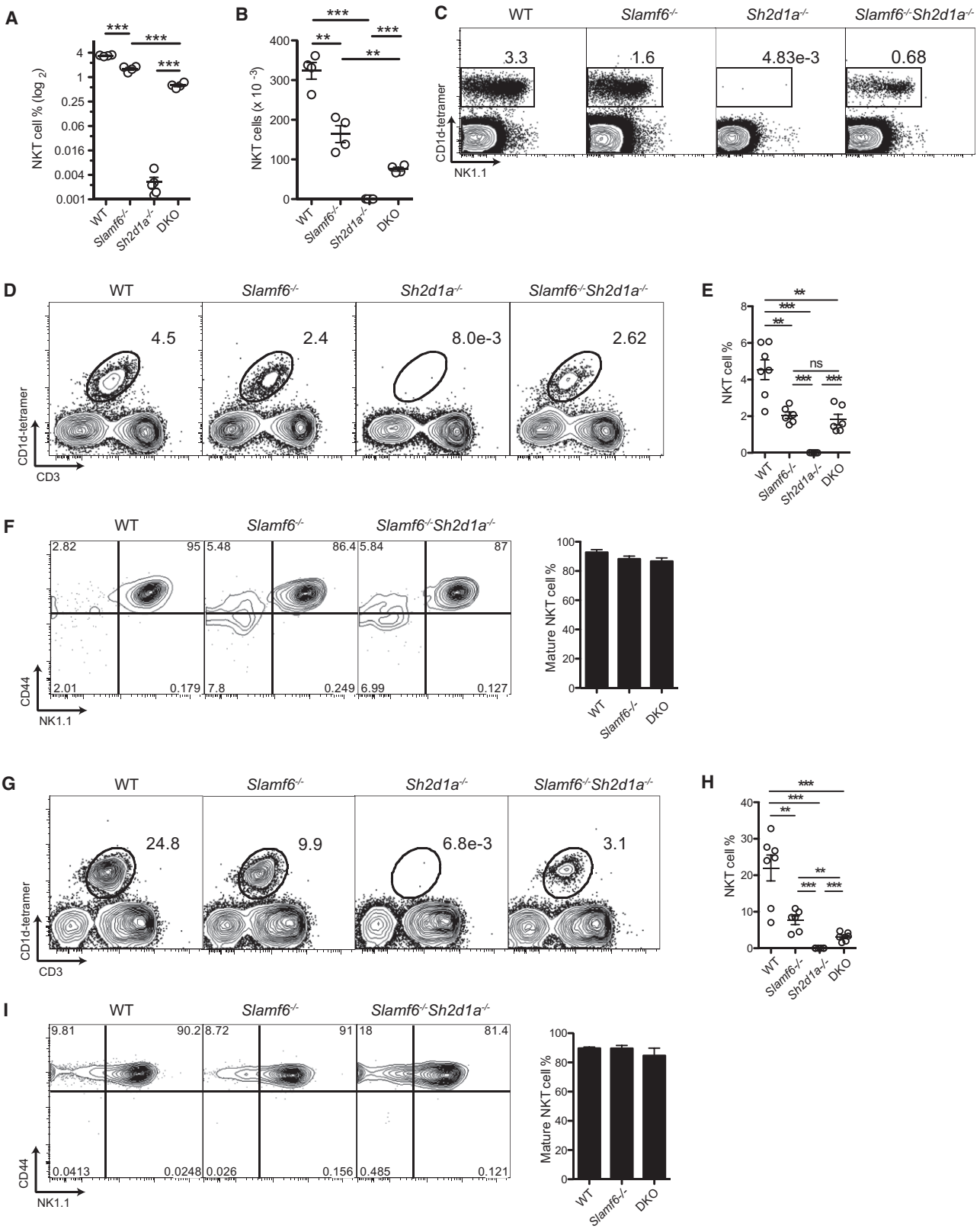

Figure 7. Ly108 Provides Both Positive and Negative Signals for NKT Cell Development

WT, *Slamf6*$^{-/-}$, *Sh2d1a*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ (DKO) mice were analyzed for NKT cells.

(A) Frequencies of splenic NKT cells, gated as shown in (C).

expressed on T cells with great interest as an immunotherapeutic target for treating chronic viral infections and tumors (Barber et al., 2006), is unusual in that it possesses a single ITSM (Sidorenko and Clark, 2003) and has not been reported to bind SAP. In conclusion, these surprising results illuminate several interesting aspects of lymphocyte biology centered on the elucidation that the severe humoral immunity and NKT cell development defects observed in SAP deficiency stem from the duality of Ly108 functions.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6J (B6) mice were purchased from the Jackson Laboratory. *Sh2d1a*$^{-}$, *Slamf6*$^{-/-}$, *Slamf6*$^{-/-}$*Sh2d1a*$^{-}$, SMARTA TCR transgenic (SM, LCMV gp66-77 I-A^{b} specific) CD45.1^{+}, *Sh2d1a*$^{-}$ SM, *Slamf6*$^{-/-}$ SM, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-}$ SM mice were all on a fully B6 background and bred at LIAI. AND TCR transgenic mice were purchased from Jackson. *Cd84*$^{-/-}$ mice were generated as previously described (Cannons et al., 2010a). *Slamf6*$^{-/-}$ mice were generated by Lexicon Genetics on the 129 background via homologous recombination targeting exon 1 of *Slamf6*. The neomycin resistance gene cassette remains. Ly108 protein expression is completely absent (Dutta and Schwartzberg, 2012). *Slamf6*$^{-/-}$ mice have no gross B cell, CD4^{+} T cell, or CD8^{+} T cell defects. Mice were obtained through the NIH KOMP program and then backcrossed ten generations to the B6 background at LIAI. Whole-genome microsatellite analysis through the University of California, Los Angeles, Southern California Genotyping Consortium verified that the *Slamf6*$^{-/-}$ mice were 99% B6. The remaining 1% was of the Sv129 background around the SLAM locus, incorporating the region between the SNP markers mCv22849619 and rs13476259. Expression of SLAM and CD84 on *Slamf6*$^{-/-}$ lymphocytes is normal (data not shown). *Sh2d1a*$^{-}$ mice were greater than 99% B6 by SNP analysis, with a small region of the X chromosome remaining Sv129. All animal experiments were conducted in accordance with approved animal protocols.

Adoptive Transfers, Retroviral Transductions, and Transfections

Sh2d1a (SAP) expressing retroviral vector (pMIG-SAP) was reported previously (McCausland et al., 2007). Ly108-1 and Ly108-2 sequences were cloned into the pMIG vector. Site-directed mutagenesis of Ly108-2 was done to create single tyrosine to phenylalanine mutants. Viral particles containing expression constructs of interest (RV) were produced from the Plat-E cell line as previously described (Johnston et al., 2009). SM CD4^{+} T cells were purified from spleen by negative selection with magnetic beads (Miltenyi). Cell transfers were done with either 5 × 10^{3} for naive cells or 2.5 × 10^{4} for retrovirally transduced cells by intravenous injection via the retro-orbital sinus. For biochemical analysis, retrovirally transduced cells were sorted based on GFP expression and restimulated with peptide-pulsed B cells. Ly108 and Ly108-AllF were cloned into a GFP fusion expression construct (Zhao et al., 2012, this issue). Constructs were introduced into cells via Amaxa nucleofactor as previously described (Qi et al., 2008).

Viruses

LCMV Armstrong stocks were prepared and quantified as previously described (McCausland et al., 2007). All infections were done by bilateral intraperitoneal injection of 2 × 10^{5} plaque-forming units of LCMV Armstrong per mouse. Vaccinia virus Western Reserve strain (VACV-WR) stocks (mature virion) were prepared and quantified as previously described (Benhnia et al., 2009). Mice were infected with VACV by bilateral intraperitoneal injection of 2 × 10^{5} plaque-forming units per mouse.

Flow Cytometry

Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption. Monoclonal antibodies against surface markers were used with FACS buffer (PBS + 0.5% BSA): GL7, Fas, and CD138-biotin (281-2) came from BD PharMingen; CD4 (RM4-5 and GK1.5), CD8a (Ly-2), B220 (RA3-6B2), PD1 (J43), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), Ly108 (13G3-19D), and CD44 (IM7) came from eBioscience; and SLAM (TC15-12F12.2) came from Biolegend. FITC-labeled peanut agglutinin (PNA) was from Vector Laboratories. CXCR5 staining was done with purified anti-CXCR5 (2G8, BD PharMingen), followed by biotinylated goat anti-rat IgG (Jackson Immunoresearch), and then PE- or APC-labeled streptavidin (Caltag Laboratories) with each staining step done in PBS + 0.5% BSA + 2% FCS + 2% Normal Mouse Serum on ice; samples were acquired without fixation. CD1d tetramers were provided by M. Kronenberg (Sidobre and Kronenberg, 2002). All FACS samples were washed twice with FACS buffer, acquired with an LSRII or Canto (BD Biosciences), and then analyzed with FlowJo (Tree Star).

ELISA

Serum from mice 30 days after LCMV infection was used. Anti-LCMV IgG was quantified by ELISA with LCMV-infected cell lysate as the capture antigen. 96-well Polysorp microtiter plates (Nunc) were coated overnight with LCMV-infected cell lysate in PBS. After incubation of sample serum, HRPO-conjugated goat anti-mouse IgGγ (Invitrogen) was used for detection. VACV ELISAs used a similar procedure, with VACV antigen (Moyron-Quiroz et al., 2009).

Immunoprecipitation and Immunoblotting

LPS-activated B cells were untreated or pulsed with PCC peptide for 1 hr at 37°C, washed, and mixed with either WT or *Sh2d1a*$^{-/-}$ antigen-specific CD4^{+} T cells for 20 min at 37°C. Note that pervanadate treatment of T cells did not result in Ly108 SHP-1 binding, indicating that physiological Ly108 ligation is required (data not shown). Stimulated cells were lysed with cold HNGT buffer (pH 7.4) containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, and protease inhibitors. Lysates were incubated overnight with 2.5 μg/ml anti-mouse Ly108 (eBio13G3-191) at 4°C, followed by 2 hr incubation with protein-A (Santa Cruz). Immunocomplexes were washed and boiled in nonreducing SDS sample buffer for 5 min at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with TBS containing 5% BSA, 0.1% Tween-20. For immunoblotting, the following reagents were used: rabbit anti-SHP-1, rabbit anti-pERK, rabbit anti-ERK (Cell Signaling Technologies), mouse anti-Ly108 (eBioscience), and rabbit anti-Ly108 (Dutta and Schwartzberg, 2012). HRP-conjugated secondary reagents were from Jackson ImmunoResearch Laboratories.

Conjugate Adhesion Assay

SMARTA CD4^{+} T cells (5 × 10^{5}/well) were incubated for 30 min (37°C) in 96-well U-bottom plates with LPS-activated B cells (2 × 10^{6}/well) pulsed with gp66 peptide (LCMV gp66-77, or gp61-80 in some assays). Conjugate frequencies were enumerated by flow cytometry after the cell mixture was stained at 4°C for CD4 and CD19 as previously described (Qi et al., 2008). Sodium stibogluconate (SSG, Calbiochem) was resuspended in H_2O at 100 mg/ml.

(B) Absolute numbers of splenic NKT cells.
(C) Representative flow cytometry plots of splenic NKT cells, gated on total CD4^{+} cells. Data are representative of two independent experiments.
(D) Representative flow cytometry plots of thymic NKT cells, gated on total live CD8^{-} cells.
(E) Quantitation of (D), combining two independent experiments.
(F) Developmental profiles of thymic NKT cells, gated as described in (D), from one of two representative experiments.
(G) Representative flow cytometry plots of liver NKT cells, gated on total live CD19^{-} mononuclear cells.
(H) Quantitation of (G), combining two independent experiments.
(I) Developmental profiles of liver NKT cells, gated as described in (G), from one of two representative experiments.
**$p < 0.005$, ***$p < 0.0005$. Error bars are SEM.

Immunofluorescence Microscopy

LPS-activated B cells pulsed with gp66 LCMV peptide for 1 hr at 37°C were washed and mixed with either activated, WT SMARTA, *Sh2d1a*$^{-/-}$ SMARTA, *Slamf6*$^{-/-}$ SMARTA, or *Sh2d1a*$^{-/-}$*Slamf6*$^{-/-}$ SMARTA CD4^{+} T cells at a 1:1 ratio in serum-free media for 5 min at 37°C to allow conjugates to form. Conjugates were plated on glass multiwell slides for 15 min at 37°C. Samples were fixed and permeablized with −20°C methanol, washed several times in PBS, and blocked with PBS containing 0.1% BSA for 20 min at room temperature. Samples were incubated with primary antibody in PBS containing 0.1% BSA for 1 hr at room temp, washed five times with PBS followed by staining with secondary reagents for 40 min at room temp. For immunofluorescence the following reagents were used: rabbit anti-SHP-1 (AbCam) and rat anti-CD4 (BD PharMingen). Secondary reagents conjugated with Alexa Fluor dyes (excited at 488, 568, 633) were purchased from Invitrogen. Hoechst staining was completed in PBS for 5 min at room temp. Conjugates were examined by immunofluorescence with a Zeiss LSM 510 confocal microscope with a 63× oil immersion objective. Three-dimensional reconstruction of z stacks were made with the Imaris Scientific 3D/4D image processing and analysis software (Bitplane Scientific Software). 40 conjugates were examined per genotype per experiment.

Statistical Analysis

Statistical tests were performed with Prism 5.0 (GraphPad). p values were calculated by two-tailed unpaired Student's t tests with a 95% confidence interval. Error bars depict the standard error of the mean (SEM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at doi:10.1016/j.immuni.2012.05.016.

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