Published in final edited form as:
Immunity. 2012 June 29; 36(6): 1003–1016. doi:10.1016/j.immuni.2012.05.017.

Positive and Negative Signaling through SLAM receptors regulate Synapse Organization and Thresholds of Cytolysis

Fang Zhao[1,2], **Jennifer L. Cannons**[1], **Mala Dutta**[1], **Gillian M. Griffiths**[2,*], and **Pamela L. Schwartzberg**[1,*]

[1]Genetic Disease Research Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

[2]Department of Medicine, Cambridge Institute for Medical Research, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0XY, UK

SUMMARY

X-linked lymphoproliferative syndrome, characterized by fatal responses to Epstein-Barr virus infection, is caused by mutations affecting the adaptor SAP, which links SLAM family receptors to downstream signaling. Although cytotoxic defects in SAP-deficient T cells are documented, the mechanism remains unclear. We show that SAP-deficient murine $CD8^+$ T cells exhibited normal cytotoxicity against fibrosarcoma targets, yet had impaired adhesion to and killing of B cell and low avidity T cell targets. SAP-deficient cytotoxic lymphocytes showed specific defects in immunological synapse organization with these targets, resulting in inefficient actin clearance. In the absence of SAP, signaling through the SLAM family members Ly108 and 2B4 resulted in increased recruitment of the SHP-1 phosphatase, associated with altered SHP-1 localization and decreased activation of Src kinases at the synapse. Hence, SAP and SLAM receptors regulate positive and negative signals required for organizing the T:B cell synapse and setting thresholds for cytotoxicity against distinct cellular targets.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are capable of killing virally infected and tumorigenic cells rapidly and with great specificity through the regulated secretion of specialized secretory lysosomes, or lytic granules. This process requires constant scanning by the T cell receptor (TCR) which, upon recognition of cognate peptide-MHC Class I on target cells, leads to the formation of a distinct topological structure at the CTL-target cell interface known as the immunological synapse (IS) or supramolecular activation complex (SMAC). Initial receptor activation triggers early signaling microclusters at the IS (Beal et al., 2009; Campi et al., 2005) and an accumulation of actin across the interface (Kupfer et al., 1994; Ryser et al., 1982). Actin is then largely cleared so that it is enriched in an outer ring (Stinchcombe et al., 2001; Stinchcombe et al., 2006), supporting a dramatically reorganized structure of receptors: a centrally localized cluster of TCRs and signaling proteins that coalesce to form the central (c)SMAC, surrounded by a ring of adhesion molecules, including integrins and the associated adaptor talin, that form the peripheral (p)SMAC

*Co-corresponding authors: GMG: Cambridge Institute for Medical Research, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0XY, UK; PLS: National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892, USA, pams@mail.nih.gov.

(Monks et al., 1998). TCR signaling also initiates a concurrent polarization of the centrosome, which moves up to and docks at the cSMAC (Stinchcombe et al., 2006), causing the reorganization of the microtubule cytoskeleton towards the target cell. This process is coordinated by actin reorganization and allows the targeted movement of granules along microtubules toward the docked centrosome, where they fuse at a distinct secretory domain on the plasma membrane, releasing their cytolytic components into the target cell.

Mutations in the *SH2D1A* gene, which encodes the signaling lymphocytic activation molecule (SLAM)-associated membrane protein (SAP), cause massive immune dysregulation in patients with X-linked lymphoproliferative syndrome Type 1 (XLP1) (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). Through its Src-homology (SH2) domain, SAP binds to phosphorylated tyrosine-based motifs in the cytoplasmic tails of the SLAM family surface receptors (including SLAM, 2B4, Ly108, Ly9 and CD84), and links these receptors to downstream signal transduction networks by recruiting the Src family kinase FynT (Chan et al., 2003; Feldmann et al., 2003; Latour et al., 2003; Li et al., 2003). XLP1 is characterized by fatal infection with Epstein-Barr virus (EBV), development of lymphomas, hypogammaglobulinemia, as well as hemophagocytic syndrome; abnormalities in natural killer (NK) cell and T cell function have been documented in both XLP1 patients and SAP-deficient (*Sh2d1a*$^{-/-}$) mice (Booth et al., 2011; Cannons et al., 2011). Studies of human cells have shown defects in CD8^{+} T cell cytolytic activity against EBV-infected B cell targets (Dupre et al., 2005; Hislop et al., 2010; Palendira et al., 2011; Sharifi et al., 2004) and impaired restimulation-induced cell death (Snow et al., 2009). Nevertheless, the molecular mechanisms behind defective CTL activity against EBV-infected B cells are unknown.

SAP-deficient (*Sh2d1a*$^{-/-}$) mice exhibit alterations in both CD8^{+} and CD4^{+} T cell function, including altered expression of cytokines and defective T follicular helper cell function, associated with impaired germinal center formation and long-term humoral immunity (Cannons et al., 2011). It was further observed that *Sh2d1a*$^{-/-}$ CD4^{+} T cells have shorter-lived interactions with B cells, but not with dendritic cells (Qi et al., 2008). These observations raise the possibility that SLAM-SAP signaling specifically affects T:B cell interactions and that these defects contribute to the phenotypes of XLP1 (Schwartzberg et al., 2009).

Here, we have examined the role of SAP in CTL activity and show that, through its involvement in conjugate formation and assembly of the IS, SAP, in conjunction with SLAM family members, was specifically required for optimal CTL interactions with and killing of B cell targets. SAP-deficient CTLs exhibited specific defects in actin organization and centrosome docking at the T:B cell IS. We further present evidence that SAP-deficiency led to a negative signal resulting from increased association of Src homology region 2 domain-containing phosphatase-1 (SHP-1) with the SLAM receptors, Ly108 (CD352, Slamf6) and 2B4 (CD244, Slamf4), and reduced SHP-1 clearance at the synapse. This was accompanied by decreased activation of Src family kinases and tyrosine phosphorylation, some of the earliest events in IS formation. Conversely, when SAP was expressed, Ly108 engagement augmented TCR-mediated signals and enhanced lytic function. Together, these data support an indispensable role for SAP and SLAM family members in positive and negative signals that modulate the threshold of T cell signaling required for establishing organization of the T: B cell synapse and effector CTL function.

RESULTS

SAP-deficient CTLs Exhibit Differential Killing of Various Target Cells

Studies of $CD8^+$ T cells from XLP1 patients revealed a defect in the ability of SAP-deficient cells to kill autologous B cells (Dupre et al., 2005; Sharifi et al., 2004). However, recent data suggest that the absence of SAP does not affect cytolysis of fibroblast targets (Hislop et al., 2010; Palendira et al., 2011). To dissect the requirements for SAP in $CD8^+$ T cell cytolytic activity under defined conditions, we utilized the OT-I TCR transgenic mouse model, which expresses a clonal TCR recognizing the specific ovalbumin peptide, SIINFEKL ($OVA_{257–264}$), in the context of H-$2K^b$ (Hogquist et al., 1994). This system permitted the comparison of cytolysis of different targets using the same well-defined antigen. At optimal peptide doses (1μM), both WT and SAP-deficient (*Sh2d1a*$^{-/-}$) OT-I CTLs displayed equivalent levels of cytotoxicity against $OVA_{257–264}$ peptide-loaded EL4 thymoma or MC57 fibrosarcoma target cell lines (Figure 1A). However, *Sh2d1a*$^{-/-}$ CTLs exhibited impaired cytotoxicity of LPS-activated WT B cells, even at saturating levels of peptide (Figure 1A). Similar results were seen *in vivo*, using transferred OT-I cells and peptide pulsed B cell targets (Figure S1A and S1B). In addition, *Sh2d1a*$^{-/-}$ CTLs showed reduced IFN-γ production upon stimulation with B cells (Figures 1B and S1C). Thus, CTLs show a discriminatory ability to kill different targets in the absence of SAP.

The avidity of T cell-target interactions affects subsequent effector functions through modulation of T cell signaling (Smith et al., 2012). In the OT-I system, avidity can be reduced by either decreasing peptide concentration or using an altered peptide ligand (APL), SIIGFEKL (G4), that binds to the OT-I TCR with lower affinity and is unable to sustain early TCR signaling (Daniels et al., 2006; Jameson et al., 1993; Rosette et al., 2001). Both WT and *Sh2d1a*$^{-/-}$ CTLs exhibited similar cytolysis of MC57 fibrosarcoma targets across a wide range of $OVA_{257–264}$ peptide doses and in response to the G4 APL (Figures 1C and 1D). However, modulating TCR interactions with EL4 targets, by either reducing the $OVA_{257–264}$ peptide dose or using G4 (Figures 1C and 1D), uncovered a killing defect by *Sh2d1a*$^{-/-}$ CTLs. Examination of peptide dissociation revealed similar levels by WT and *Sh2d1a*$^{-/-}$ CTLs for either the $OVA_{257–264}$ or G4 peptides, suggesting there were no differences in TCR affinity for antigen between the genotypes (Figure S1D). Moreover, the activation status of WT and *Sh2d1a*$^{-/-}$ CTLs appeared similar, as assessed by CD44, 2B4 and SLAM expression (Figure S3A). Together, these data suggest a defect in the ability of *Sh2d1a*$^{-/-}$ CTLs to modulate thresholds of signaling against distinct cellular targets.

SAP-deficient CTLs Have Weaker Interactions with B cell and Low Avidity EL4 Targets

Previous work demonstrated that SAP is necessary for efficient adhesion of $CD4^+$ T cells to B cells (Qi et al., 2008). To determine whether there is a similar requirement in $CD8^+$ T cells, we used a flow-based conjugate assay. *Sh2d1a*$^{-/-}$ CTLs displayed decreased percentages of conjugates with B cell targets across a range of TCR avidities (Figure 1E), as well as with EL4 cells loaded with low $OVA_{257–264}$ peptide doses or the G4 APL (Figure 1F), consistent with the observed defects in cytolysis. However, if shear stress was reduced by fixing T:B cell conjugates with paraformaldehyde, the magnitude of the defect was reduced in *Sh2d1a*$^{-/-}$ CTLs (Figure S1E), suggesting that initial adhesion was normal. Moreover interactions of *Sh2d1a*$^{-/-}$ CTLs with high avidity EL4 targets were similar to WT. These data suggest that SAP-deficiency impairs the stability of CTL interactions with B cell and low-avidity EL4 targets.

Sh2d1a$^{-/-}$ CTLs Exhibit Reduced Actin Clearance in Conjugates with B cell and Low Avidity EL4 Targets

Interactions of T cells with their cognate targets lead to initial receptor triggering and conjugate formation, resulting in the reorganization of surface receptors to form the IS (Freiberg et al., 2002; Monks et al., 1998). An early event in synapse organization is the accumulation of actin at the site of contact, which is subsequently largely cleared and enriched in a peripheral actin ring, allowing the delivery of cytotoxic granules in the cytolytic synapse (Stinchcombe et al., 2006). To determine whether these processes were intact in SAP-deficiency, we examined actin organization in CTLs directed against different cellular targets by immunofluorescence confocal microscopy. A similar proportion of WT and *Sh2d1a*$^{-/-}$ CTLs exhibited actin accumulation at the synapse with B cell targets (Figure S2A). However, a more detailed *en face* evaluation of the T:B cell IS revealed striking differences between WT and *Sh2d1a*$^{-/-}$ CTLs (Figure 2A and S2B). While WT CTLs formed a characteristic actin ring, actin failed to be centrally cleared in a significant fraction of *Sh2d1a*$^{-/-}$ CTL conjugates (Figure 2A, 2B and S2B). This defect was observed as early as 5 min after cell interaction (data not shown). A similar defect was observed in *Sh2d1a*$^{-/-}$ CTLs conjugated with low-avidity EL4 targets (Figure 2C). Nonetheless, actin organization in *Sh2d1a*$^{-/-}$ CTL conjugates with high avidity EL4 targets remained indistinguishable from that observed in WT (Figure 2D). Talin, a marker of the pSMAC that links the actin cytoskeleton with integrins, also exhibited a similar defect in synaptic clearance in *Sh2d1a*$^{-/-}$ CTLs conjugated with B cell, but not EL4, targets at high avidities (Figures 2A, S2C and S2D). Thus, SAP appears to be required for establishing the fundamental organization of the T:B cell synapse.

Impaired Centrosome Polarization in *Sh2d1a*$^{-/-}$ CTLs towards B cell and Low Avidity Targets

Polarization and docking of the centrosome at the cSMAC is essential for directing lytic granules to the IS and CTL-mediated cytotoxicity (Stinchcombe et al., 2006). Prior studies provided a link between actin clearance and centrosome movement, suggesting that the forces generated by actin movement serve to pull the centrosome forward to dock at the synapse. Using antibody staining against Lck as a synapse marker and γ-tubulin as a centrosome marker, we observed that *Sh2d1a*$^{-/-}$ CTLs polarized and docked their centrosomes at the IS with high avidity EL4 targets (Figures 2E–F and S2E), yet exhibited impaired centrosome docking with B cell targets (Figures 2G and S2E). Nevertheless, staining for both actin and γ-tubulin revealed a strong correlation between actin clearance and centrosome docking, with both WT and *Sh2d1a*$^{-/-}$ CTLs showing similar patterns of centrosome positioning for each stage of actin organization (Figures 2H–J). These data suggest that impaired centrosome docking in *Sh2d1a*$^{-/-}$ CTLs is intimately connected with the actin defect and does not result from an independent deficiency in the CTL polarization machinery.

SHP-1 Recruitment and Maintenance at the Immunological Synapse of *Sh2d1a*$^{-/-}$ CTLs

SAP is the major transducer of signaling through the SLAM family receptors, which interact primarily through homotypic interactions to affect lymphocyte function (Cannons et al., 2011; Schwartzberg et al., 2009). Although SAP has been shown to promote signaling through recruitment of Fyn, other data suggest that SLAM family members can recruit distinct signaling molecules in the absence of SAP (Bottino et al., 2001; Cannons et al., 2011; Eissmann et al., 2005; Parolini et al., 2000; Sayos et al., 1998; Snow et al., 2009), including the tyrosine phosphatase SHP-1, which is known to affect TCR signaling (Chiang and Sefton, 2001; Ren et al., 2011; Stefanova et al., 2003). To determine potential effects of SAP-deficiency on the synaptic recruitment of other proteins, we examined the localization of SHP-1. In WT CTLs conjugated with either B cell or EL4 targets, we found SHP-1 in

microclusters near the peripheral actin ring (Figures 3A–C), resembling recently described inhibitory microclusters associated with the IgA Fc receptor (Pfirsch-Maisonnas et al., 2011). However, in *Sh2d1a*$^{-/-}$ T: B cell conjugates, SHP-1 was diffusely spread throughout the synapse (Figures 3A and 3C). Indeed, the difference in SHP-1 localization at the synapse of *Sh2d1a*$^{-/-}$ CTLs with B cell targets was even more profound than the defect in actin clearance (Figures 3C and 2B). These data raise the possibility that altered SHP-1 localization contributes to the primary defect in SAP-deficient CTL.

Enhanced SHP-1 Recruitment by Ly108 and 2B4 in *Sh2d1a*$^{-/-}$ CTLs

The altered recruitment of SHP-1 to the cytolytic synapse in the absence of SAP suggested the involvement of SLAM family members. Evaluation of WT and *Sh2d1a*$^{-/-}$ CTLs revealed similar levels of 2B4, SLAM, Ly108, CD84, Ly9 and CD48 (Figure S3A). In accord with published data (Cannons et al., 2010; De Salort et al., 2011), LPS-activated B cells showed high expression of multiple SLAM family receptors and ligands, including CD48, the ligand for 2B4, and Ly108 (Figure 3D and S3B), two receptors implicated in cytolysis (Bottino et al., 2001; Parolini et al., 2000). In comparison, EL4 cells exhibited slightly reduced surface CD48 and SLAM, as well as very low Ly108 expression. Notably, MC57 cells displayed the lowest overall expression of SLAM family members, expressing only CD48 (Figure 3D).

To determine whether SLAM family members directly contribute to altered SHP-1 recruitment, we immunoprecipitated Ly108 and 2B4 from CTLs stimulated with B cells and looked for the presence of SHP-1. Upon stimulation of WT CTLs, there was minimal association of SHP-1 with Ly108. However, in *Sh2d1a*$^{-/-}$ CTLs, Ly108 showed increased interactions with SHP-1 (Figures 4A). In contrast, recruitment of C-src tyrosine kinase (Csk), Casitas B-lineage Lymphoma (Cbl) family members, and SH2 domain-containing Inositol Phosphatase (SHIP), other inhibitory proteins implicated as potential binding partners for Ly108 and 2B4, was similar or only variably increased in *Sh2d1a*$^{-/-}$ CTLs (Figure S4A). To exclude the possibility that the SHP-1 association with Ly108 came from B cells, we exploited self-presentation to activate Ly108. Notably, we detected increased SHP-1 recruitment in *Sh2d1a*$^{-/-}$ CTLs compared to WT cells upon stimulation with the G4 peptide APL (Figure S4B). Additionally, increased SHP-1 co-immunoprecipitated with 2B4 in *Sh2d1a*$^{-/-}$, but not WT, CTLs upon stimulation with B cells or via self-presentation with low $OVA_{257–264}$ peptide doses (Figures 4B, S4C and S4D). Thus, in SAP-deficient CTLs, Ly108 and 2B4 show increased recruitment of SHP-1 in response to stimulation with B cells targets. Interestingly, we did not observe SHP-1 association with Ly108 in CTLs stimulated with pervanadate (Figure S4E), suggesting that ligand engagement may be essential for SHP-1 recruitment.

To further examine the association of Ly108 with SHP-1, we assessed their localization concurrently following expression of either WTLy108-GFP or a mutant of Ly108 in which the four cytoplasmic tyrosines were mutated to phenylalanine (Ly108-AllF-GFP). Reconstitution into Ly108-deficient (*Slamf6*$^{-/-}$) and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs permitted evaluation of these receptors in the absence of endogenous Ly108. Both WTLy108 and Ly108-AllF were primarily recruited centrally to the IS in either *Slamf6*$^{-/-}$ or *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs conjugated with WT (Figures 4C–D), but not *Slamf6*$^{-/-}$, B cells. Thus, synaptic recruitment of Ly108 was dependent on receptor engagement, but not on SAP or the cytoplasmic tyrosines of Ly108 in the CTL. In *Slamf6*$^{-/-}$ CTLs reconstituted with either WTLy108 or Ly108-AllF, SHP-1 was peripherally localized (Figures 4C, 4D left and 4E). However, when WTLy108 was re-expressed in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs, SHP-1 was more diffusely localized throughout the synapse, consistent with our observations in *Sh2d1a*$^{-/-}$ CTLs, and partially overlapped with the pattern of Ly108 expression (Figures 4C right and 4E). Nevertheless, SHP-1 failed to be centrally recruited in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$

CTLs reconstituted with Ly108-AllF, localizing again in a peripheral ring (Figures 4D right and 4E). Thus, Ly108 is centrally recruited to the T:B cell IS and the Ly108 cytoplasmic tyrosine residues are required for the abnormal synaptic recruitment of SHP-1 in the absence of SAP.

To confirm the requirement of the cytoplasmic tyrosines for Ly108 interactions with SHP-1, *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ OT-I cells were retrovirally reconstituted with WTLy108 or Ly108-AllF and cells were sorted to obtain transduced cells (Figure S4F). Upon stimulation with B cells, SHP-1 associated with WTLy108, but not Ly108-AllF, in reconstituted *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs (Figure 4F). Together, these data demonstrate that the Ly108 cytoplasmic tyrosine residues are essential for SHP-1 association.

Reduced phosphorylated Src Kinases at the Immunological Synapse of *Sh2d1a*$^{-/-}$ CTLs

To understand the consequences of altered SHP-1 recruitment, we examined potential targets of this phosphatase. One of the major targets of SHP-1 is Lck, the primary Src family kinase expressed in T cells and the first kinase activated by and required for TCR signaling (Stefanova et al., 2003). Of note, a recent study has shown that Lck is essential for actin clearance and centrosome docking at the synapse (Tsun et al., 2011). To determine whether SAP deficiency affects Lck activation and localization, we used an antibody that detects tyrosine phosphorylation on the activation loop of the kinase domain of Src kinases (p-Src) for immunofluorescence microscopy. Upon engagement of a target cell, phosphorylated Src kinases, including Lck, are recruited into signaling microclusters at the contact site (Campi et al., 2005; Freiberg et al., 2002). These microclusters continue to form in the periphery of the mature IS before the coalescence of Lck into the cSMAC. In accord with previous reports on CD4^{+} T cells (Beal et al., 2009; Campi et al., 2005; Varma et al., 2006), a large proportion of mature WT CTL conjugates displayed a ring of p-Src microclusters at the periphery (Figure 5A–C and S5). Similar patterns of p-Src localization were observed in *Sh2d1a*$^{-/-}$ CTLs conjugated with high avidity EL4 targets (Figure 5B and S5). Interestingly, peripheral p-Src localization was reduced in *Sh2d1a*$^{-/-}$ CTLs conjugated with B cell targets, and p-Src microclusters were not well detected in a substantial percentage of cells (Figures 5C and S5). Consistent with reduced p-Src microclusters, *Sh2d1a*$^{-/-}$ CTLs also displayed lower overall tyrosine phosphorylation at the T:B cell IS (Figure 5D).

One mechanism for the blocking of cytotoxicity by inhibitory receptors on NK cells is the early SHP-1-mediated dephosphorylation of the guanine nucleotide exchange factor Vav1, an important regulator of actin-dependent activation signals (Stebbins et al., 2003). Vav1 phosphorylation is enhanced following 2B4 costimulation of NK cells (Riteau et al., 2003) and Ly108 ligation in thymocytes (Zhong and Veillette, 2008). Interestingly, we found that *Sh2d1a*$^{-/-}$ CTLs, restimulated with B cells or via self-presentation, displayed reduced Vav1 phosphorylation relative to WT CTLs (Figure 5E and data not shown). Thus, multiple SHP-1 targets required for actin organization and cytolytic activity are affected in SAP-deficient CTL:B cell conjugates.

Inhibition of SHP-1/2 Rescues Cytotoxicity and Adhesion in *Sh2d1a*$^{-/-}$ CTLs

To definitively link SHP-1 to impaired cytolysis by *Sh2d1a*$^{-/-}$ CTLs, we examined whether cytolytic function could be rescued *in vitro* with a SHP-1 inhibitor. Pre-incubation of *Sh2d1a*$^{-/-}$ CTLs with the SHP-1/2 inhibitor NSC-87877 restored killing of B cell and low avidity EL4 targets to WT levels (Figures 6A and 6B). Additionally, *Sh2d1a*$^{-/-}$ CTLs pre-incubated with the SHP-1 inhibitor SSG showed improved adhesion to B cells (Figure S6). Together, these results argue that defective cytolysis by *Sh2d1a*$^{-/-}$ CTLs results directly from SHP-1-mediated inhibitory signals induced by SLAM family members, implicating these pathways in fine-tuning CTL responses.

Ly108 expression on target cells modulates WT and *Sh2d1a*$^{-/-}$ CTL function

The demonstration of increased SHP-1 recruitment to Ly108 and 2B4 in the absence of SAP suggests that the presence of SLAM family members is required for phenotypes associated with SAP deficiency. To investigate this hypothesis, we evaluated killing of *Slamf6*$^{-/-}$ B cell targets, which should fail to engage Ly108 on CTLs. Upon LPS-activation, these B cells expressed similar levels of CD48 and SLAM as WT B cells, but no Ly108, resembling the surface phenotype of EL4s with respect to SLAM family member expression (Figure S3B). Cytolysis of *Slamf6*$^{-/-}$ B cells by *Sh2d1a*$^{-/-}$ CTLs appeared normal, with the loss of Ly108 interactions actually rescuing the cytolytic defect caused by SAP deficiency (Figure 7A, left). Actin clearance at the IS was similarly improved (Figure 7A, right). To further test the contribution of Ly108, EL4 cells were transduced with constructs expressing Ly108 (Figure S7A). Expression of high levels of Ly108 on EL4 cells resulted in their impaired killing by *Sh2d1a*$^{-/-}$ CTLs, even at high peptide doses, similar to B cell targets (Figure 7B, left). Furthermore, *Sh2d1a*$^{-/-}$ CTLs exhibited reduced actin clearance against Ly108-expressing EL4 cells (Figure 7B, right), but not against conventional EL4 cells transfected with an empty vector.

To further evaluate the effects of Ly108 for phenotypes associated with SAP-deficiency, we examined CTL interactions with WT and *Slamf6*$^{-/-}$ B cells. Loss of Ly108 on B cells improved *Sh2d1a*$^{-/-}$ CTL adhesion at high peptide doses (Figure 7C). However, an even larger increase in adhesion was observed with *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ compared to *Sh2d1a*$^{-/-}$ CTLs, suggesting that Ly108 expression on the T cells may have a greater influence on T:B cell interactions (Figure 7C, 4th panel). IFN-γ production was also improved in *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$, compared to *Sh2d1a*$^{-/-}$, CTLs (Figure S1C). Thus, the presence of SLAM family members is required for and plays an active role in the defective CTL responses against B cells associated with of SAP-deficiency. Together, these experiments support a model in which negative signals mediated by Ly108 in the absence of SAP contribute to the defects in B cell adhesion and cytolysis by SAP-deficient CTLs.

Finally, to establish whether Ly108 engagement plays a functional role in WT cells, CTLs were evaluated for lysis of EL4 targets expressing low or high levels of Ly108, as well as WT or *Slamf6*$^{-/-}$ B cell targets. Although the differences were subtle, WT CTLs exhibited decreased lysis of *Slamf6*$^{-/-}$ B cell targets (Figure 7D). Conversely, WT, but not *Slamf6*$^{-/-}$, CTLs showed increased killing of EL4 cells expressing high levels of Ly108 (Figure 7E). Moreover, Ly108 costimulation of WT T cells resulted in elevated and prolonged ERK activation compared to CD3 stimulation alone (Figure 7F). Thus, Ly108 engagement in the presence of SAP provides a positive signal, augmenting TCR-mediated signaling pathways and increasing lytic function. Together, these results suggest that, depending on the expression of SAP, SLAM family members mediate positive and negative signals that modulate TCR signaling required for immune synapse organization and CTL function.

DISCUSSION

In this study, we provide compelling evidence that SAP, in conjunction with the SLAM family receptors Ly108 and 2B4, differentially regulate CTL-mediated cytolytic activity against distinct cellular targets. Our studies reveal a novel role for SAP in the fundamental organization of the CD8^{+} T:B cell IS and provide mechanistic insight into how SAP and SLAM family members help fine-tune the strength of TCR signaling by modulating the recruitment and organization of phosphatases at the T:B cell synapse. Our results further demonstrate that SLAM family members are critical for the phenotypes of SAP-deficiency and that these phenotypes do not result solely from a lack of signaling from SLAM family receptors. Together, our observations help account for the profound defects seen in the absence of SAP.

SAP-deficiency, in the disease XLP1, is associated with a diverse array of seemingly disparate phenotypes, including impaired EBV clearance leading to hemophagocytosis, impaired humoral immunity associated with defective germinal center formation, the development of lymphomas and other lymphoproliferative syndromes, an absence of natural killer T (NKT) cells, and impaired T cell restimulation-induced cell death (Booth et al., 2011; Cannons et al., 2011). However, whether common mechanisms underlie these diverse phenotypes was largely unknown. We previously found that SAP-deficiency in mice specifically affects $CD4^+$ T:B cell interactions (but not T cell interactions with DCs), raising the possibility that phenotypes of XLP1 are linked by a common mechanism involving altered T and NK cell interactions with B cells, which express high levels of SLAM family members (Cannons et al., 2010; Qi et al., 2008; Schwartzberg et al., 2009). By using a defined antigen-specific system, we demonstrated that SAP-deficiency specifically impairs CTL-mediated cytolysis of activated B cells and hematopoietic targets that express SLAM family members, without affecting the killing of other cellular targets. This concept of B cell-driven phenotypes is supported by recent findings that $CD8^+$ T cells from XLP1 patients show impaired effector function against autologous EBV-infected B cells, but less severe defects against other cellular targets pulsed with EBV-peptides (Hislop et al., 2010; Palendira et al., 2011). Such observations provide important insight into why XLP1 patients are uniquely sensitive to EBV but not other infectious agents. Whether defective lymphocyte interactions contribute to other manifestations of XLP1, such as impaired cell death, is an intriguing question.

Although phenotypes of SAP-deficiency have been linked to the role of SAP downstream of the SLAM family members, loss of individual SLAM receptors only partially phenocopy SAP-deficiency. This observation has been attributed to functional redundancy between SLAM family members, as has been shown for NKT cell development and T:B cell adhesion (Cannons et al., 2010; Griewank et al., 2007). However, other data support a role for SAP in competing and preventing the recruitment of negative signaling molecules, including SHP-1, SHP-2, SHIP, Csk and Cbl, to SLAM family members (Bottino et al., 2001; Cannons et al., 2011; Eissmann et al., 2005; Li et al., 2003; Parolini et al., 2000; Sayos et al., 1998; Shlapatska et al., 2001; Snow et al., 2009). Our results provide physiologic evidence that SAP-deficiency causes phenotypes as a direct result of negative signals initiated by SLAM family members, in addition to those resulting from a lack of SLAM-SAP-Fyn signaling, thereby fundamentally changing the earliest events in TCR signaling. While we have not yet found evidence for inhibitory functions of SLAM family members in WT T cells, our results suggest that, in certain contexts, depending on the level of SAP and SLAM family receptors, SLAM-SAP signaling may provide an important modulatory pathway for TCR signaling that alters T cell interactions with other hematopoietic cells. This scenario closely resembles signaling from activating and inhibitory receptors in NK cells (Davis et al., 1999; Eissmann et al., 2005; Long, 2008; Vyas et al., 2002) and argues that CTLs are also regulated by inhibitory receptor signaling from SLAM family members, particularly Ly108 and 2B4. These data also help provide a conceptual framework for why other immunodeficiencies that affect TCR signaling can lead to fatal responses to EBV.

Notably, our studies highlight the differential nature of cytolytic targets and their role in dictating characteristics of the cytolytic synapse and CTL function, allowing finer distinctions in the requirements for killing hematopoietic targets expressing SLAM family members. The concept of CTL regulation by cellular targets is supported by data showing differential induction of IFN-γ by cellular targets, depending on their expression of B7 molecules (Markiewicz et al., 2005). Our results further suggest a key role for SLAM family members in determining nuances in the nature of lymphocyte interactions required for cross-regulation in the immune system, through control of the earliest stages of T cell activation

and organization of the IS. Indeed, we observe parallel defects in SAP-deficient $CD4^+$ T cells that are also dependent on Ly108 (Kageyama et al., 2012).

Although the role of the IS in T cell activation remains controversial, the IS has been implicated both in terminating TCR signals and in potentiating TCR signals from weak ligands (Cemerski et al., 2008; Varma et al., 2006). The TCR, adhesion proteins, and intracellular signaling molecules are all involved in modulating the functional state of the IS. While we cannot rule out that IS abnormalities in SAP-deficient T cells are secondary to their defects in adhesion, our results argue that SAP influences early events of CTL:B cell synapse formation by affecting proximal signaling, which in turn affects stable formation of the IS and adhesion. It is intriguing that the localization of p-Src at the *Sh2d1a*$^{-/-}$ CTL contact with B cells resembles that seen with low affinity targets (Beal et al., 2009). It is also notable that the IS that forms between T cells and B cells differs in structure from that formed with DCs and other cells (Brossard et al., 2005; Stinchcombe et al., 2001), suggesting that this special structure may be critical for the stability of adhesion of highly motile T:B cell conjugates.

In NK cells, SAP has been found co-localized with 2B4 at the IS (Roda-Navarro JI 2004); similarly, both SAP and SLAM localize to the T cell-APC contact site (Cannons et al., 2004; Howie et al., 2002) and NTB-A co-localizes with TCR upon capping (Snow et al., 2009). Our data supports the idea that SLAM family members are normally recruited to the IS by association with their ligands. SAP recruitment can then link SLAM receptor engagement to positive effector signaling pathways through the SH3-mediated binding of Fyn (Chan et al., 2003; Latour et al., 2003; Li et al., 2003). However, in the absence of SAP, SHP-1 is instead recruited to the SLAM family receptors, Ly108 and 2B4, and maintained throughout the T:B cell IS. This leads to a down-regulation of positive signaling and impaired synapse organization, which may in turn affect cellular adhesion, one of the defects we see in SAP-deficient T cells. While we cannot rule out negative feedback from other signaling molecules including Cbl family members and SHIP, especially in other cell populations that express SLAM family receptors, the high degree of rescue by the SHP-1/2 inhibitor strongly supports a major role for SHP family phosphatases in this pathway in CTLs. This model is strengthened by previously reported findings of SAP and SHP-1 association with 2B4 (Eissmann et al., 2005; Parolini et al., 2000) and NTB-A (Bottino et al., 2001; Snow et al., 2009), and the finding that inhibitory effects of triggering these receptors on primary NK and $CD8^+$ T cells from XLP1 patients can be reversed by the mAb masking of both receptors (Bottino et al., 2001; Hislop et al., 2010; Palendira et al., 2011). Whether negative signaling through SHP-1 is a specific characteristic of Ly108 and 2B4 awaits further evaluation. SHP-1 has been found to inhibit activation of the guanine nucleotide exchange factor Vav1 (Stebbins et al., 2003) upon the triggering of inhibitory receptors on NK cells, thereby blocking early actin-dependent activation signals. Our finding of decreased Vav1 phosphorylation in SAP-deficient CTLs also supports a fundamental role for these pathways in regulating CTL function.

Our results therefore suggest that, by affecting the recruitment of Src family kinases and phosphatases, positive and negative signaling through SLAM family members play a critical role in modulating IS formation and structure, which is required for productive interactions with B cell and other hematopoietic targets. Together, this system provides a critical feature of immunoregulation required for normal lymphocyte interactions and function. The importance of this regulation is highlighted by the profound phenotypes of XLP1.

EXPERIMENTAL PROCEDURES

Mice and Reagents

Sh2d1a$^{-/-}$ (Czar et al., 2001), OT-I TCR transgenic (Hogquist et al., 1994) and C57Bl/6J mice (Jackson Laboratories) were maintained in accordance with guidelines of the NHGRI Animal Care and Use Committee at the National Institutes of Health. *Slamf6*$^{-/-}$ mice were generated from the HGTC-8 C57BL/6J ES cell line and do not express Ly108, as detected by flow cytometry and immunoblotting (Figures S3B, S7B and manuscript submitted). Antibodies and reagents are listed in supplemental material.

Cell Culture and Generation of Mouse CTLs

Splenocytes from 8–12 week old WT and *Sh2d1a*$^{-/-}$ OT-I mice were stimulated *in vitro* with 10 nM $OVA_{257–264}$ peptide in complete medium (RPMI 1640 medium with 10% FCS, L-glutamine, sodium pyruvate, 50uM β-mercaptoethanol, and 50 U/mL penicillin and streptomycin). After 3 days, cells were washed and cultured in complete medium plus 10 U/mL recombinant human IL-2. Experiments were performed with CTLs between 6–8 days after primary stimulation. B cells were purified from C57BL/6J splenocytes by negative selection (Miltenyi Biotec) and activated with 10 μg/mL LPS (Sigma) in complete medium for 3 days. H-2K^{b} EL4 (C57Bl/6 thymoma, ATCC TIB-39) and MC57 (C57Bl/6 fibrosarcoma, ATCC CRL-2295) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS, L-glutamine, and 50 U/mL penicillin and streptomycin. Generation of Ly108 expression constructs and Ly108 expressing cells are described in supplemental methods.

Flow Cytometry, Intracellular Cytokine Staining and Adhesion Conjugate Assay

Staining and flow cytometry are in supplemental methods. For analyses of conjugates, CFSE-labeled target cells were pulsed with peptide for 1 hr at 37°C and washed 3 times in medium. CTLs labeled with the membrane dye PKH26 were mixed with target cells at a ratio of 1:1, spun down gently, and incubated for 15–30 min at 37°C. Samples were resuspended and analyzed by flow cytometry, with the CFSE^{+} PKH26^{+} double positive population representing CTL-target cell conjugates. Alternatively, conjugates were assessed as described (Qi et al., 2008).

In Vitro Cytotoxicity Assay

Antigen-specific cytolytic activity of OT-I CTLs against targets, pulsed with peptide for 1 hr at 37°C, was assayed *in vitro* after a 4 hr incubation at 37°C by measuring LDH-release using the Cytotox96 cytotoxicity assay (Promega), following manufacturer's instructions.

Immunofluorescence Microscopy

Target cells, pulsed with peptide for 1 hr at 37°C, were washed and mixed with OT-I CTLs at a 1:1 ratio in serum-free RPMI for 5 min at 37°C to allow conjugate formation. Conjugates were plated on glass multi-well slides and incubated for 15 min at 37°C, then fixed and permeabilized with −20°C methanol, washed several times in PBS, and blocked in PBS plus 1% BSA for 1 hr at room temperature (RT). Samples were incubated with primary antibody in PBS plus 0.1% BSA for either 1 hr at RT or overnight at 4°C, washed 4–5 times, followed by staining with secondary antibodies for 40 min at RT. Cells were washed an additional 4–5 times, and Hoechst stained in PBS for 5 min at RT. Laser scanning confocal microscopy was performed using the revolution spinning disk confocal system or the Zeiss LSM 510 confocal microscope with a 63X or 100X (Plan-Apochromat, NA 1.40) oil immersion objective. Three-dimensional reconstructions of z-stacks were made using the Imaris Scientific 3D/4D Image Processing & Analysis Software (Bitplane Scientific

Software). Between 35 and 100 conjugates were examined per condition for each experiment.

Immunoprecipitation and Immunoblotting

CTLs were stimulated with $OVA_{257–264}$ pulsed B cells or via self-presentation for 20 min at 37°C, and lysed in ice-cold HNTG buffer (pH 7.4) containing 50mM HEPES, 150mM NaCl, 1mM EDTA, 1mM $MgCl_2$, 10% glycerol, 1% Triton-X-100, and 1mM protease inhibitors. Lysates were incubated overnight at 4°C with antibodies, followed by a 2 hr incubation at 4°C with Protein A or G beads (Santa Cruz), washed, and resuspended in non-reducing SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% w/v SDS, 30% glycerol, and 0.03% w/v bromophenol blue) for 5 min at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Membranes, blocked with TBS plus 5% BSA and 0.1% Tween-20, were incubated with primary antibodies overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies for 30–60 min at RT (Jackson ImmunoResearch Laboratories). Signals were detected using enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech).

Statistical Analyses

Statistical significance between analyzed groups was determined using GraphPad Prism by the Student's t test or Paired Student's t test, as appropriate. Values of $p < 0.05$ were considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank J. Reilley, R. Handon, S. Wincovitch, S. Anderson and J. Fekecs for assistance. This work was funded in part by the intramural program of the National Human Genome Research Institute. FZ was a Scholar in the NIH-Oxford-Cambridge Scholars in Biomedical Research program.

REFERENCES

Beal AM, Anikeeva N, Varma R, Cameron TO, Vasiliver-Shamis G, Norris PJ, Dustin ML, Sykulev Y. Kinetics of early T cell receptor signaling regulate the pathway of lytic granule delivery to the secretory domain. Immunity. 2009; 31:632–642. [PubMed: 19833088]

Booth C, Gilmour KC, Veys P, Gennery AR, Slatter MA, Chapel H, Heath PT, Steward CG, Smith O, O'Meara A, et al. X-linked lymphoproliferative disease due to SAP/SH2D1A deficiency: a multicenter study on the manifestations, management and outcome of the disease. Blood. 2011; 117:53–62. [PubMed: 20926771]

Bottino C, Falco M, Parolini S, Marcenaro E, Augugliaro R, Sivori S, Landi E, Biassoni R, Notarangelo LD, Moretta L, Moretta A. NTB-A, a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. J Exp Med. 2001; 194:235–246. [PubMed: 11489943]

Brossard C, Feuillet V, Schmitt A, Randriamampita C, Romao M, Raposo G, Trautmann A. Multifocal structure of the T cell - dendritic cell synapse. Europ J Immunol. 2005; 35:1741–1753.

Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. J Exp Med. 2005; 202:1031–1036. [PubMed: 16216891]

Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, Wakeland EK, Germain RN, Schwartzberg PL. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity. 2010; 32:253–265. [PubMed: 20153220]

Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol. 2011; 29:665–705. [PubMed: 21219180]

Cannons JL, Yu LJ, Hill B, Mijares LA, Dombroski D, Nichols KE, Antonellis A, Koretzky GA, Gardner K, Schwartzberg PL. SAP regulates T_H2 differentiation and PKC-θ-mediated activation of NF-κB1. Immunity. 2004; 21:693–706. [PubMed: 15539155]

Cemerski S, Das J, Giurisato E, Markiewicz MA, Allen PM, Chakraborty AK, Shaw AS. The balance between T cell receptor signaling and degradation at the center of the immunological synapse is determined by antigen quality. Immunity. 2008; 29:414–422. [PubMed: 18760640]

Chan B, Lanyi A, Song HK, Griesbach J, Simarro-Grande M, Poy F, Howie D, Sumegi J, Terhorst C, Eck MJ. SAP couples Fyn to SLAM immune receptors. Nat Cell Biol. 2003; 5:155–160. [PubMed: 12545174]

Chiang GG, Sefton BM. Specific dephosphorylation of the Lck tyrosine protein kinase at Tyr-394 by the SHP-1 protein-tyrosine phosphatase. J Biol Chem. 2001; 276:23173–23178. [PubMed: 11294838]

Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, Cahn AP, Durham J, Heath P, Wray P, et al. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet. 1998; 20:129–135. [PubMed: 9771704]

Czar MJ, Kersh EN, Mijares LA, Lanier G, Lewis J, Yap G, Chen A, Sher A, Duckett CS, Ahmed R, Schwartzberg PL. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene *SH2D1A/DSHP/SAP*. Proc Natl Acad Sci USA. 2001; 98:7449–7454. [PubMed: 11404475]

Daniels MA, Teixeiro E, Gill J, Hausmann B, Roubaty D, Holmberg K, Werlen G, Hollander GA, Gascoigne NR, Palmer E. Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature. 2006; 444:724–729. [PubMed: 17086201]

Davis DM, Chiu I, Fassett M, Cohen GB, Mandelboim O, Strominger JL. The human natural killer cell immune synapse. Proc Natl Acad Sci USA. 1999; 96:15062–15067. [PubMed: 10611338]

De Salort J, Sintes J, Llinas L, Matesanz-Isabel J, Engel P. Expression of SLAM (CD150) cell-surface receptors on human B-cell subsets: from pro-B to plasma cells. Immunol Letters. 2011; 134:129–136.

Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, Arico M, Aiuti A, Roncarolo M-G. SAP controls the cytolytic activity of $CD8^+$ T cells against EBV-infected cells. Blood. 2005; 105:4383–4389. [PubMed: 15677558]

Eissmann P, Beauchamp L, Wooters J, Tilton JC, Long EO, Watzl C. Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244). Blood. 2005; 105:4722–4729. [PubMed: 15713798]

Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachee-Chardin M, Chedeville G, Tamary H, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003; 115:461–473. [PubMed: 14622600]

Freiberg BA, Kupfer H, Maslanik W, Delli J, Kappler J, Zaller DM, Kupfer A. Staging and resetting T cell activation in SMACs. Nat Immunol. 2002; 3:911–917. [PubMed: 12244310]

Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, Mamchak AA, Terhorst C, Bendelac A. Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. Immunity. 2007; 27:751–762. [PubMed: 18031695]

Hislop AD, Palendira U, Leese AM, Arkwright PD, Rohrlich PS, Tangye SG, Gaspar HB, Lankester AC, Moretta A, Rickinson AB. Impaired Epstein-Barr virus-specific $CD8^+$ T cell function in X-linked lymphoproliferative disease is restricted to SLAM family positive B cell targets. Blood. 2010; 116:3249–3257. [PubMed: 20644117]

Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. Cell. 1994; 76:17–27. [PubMed: 8287475]

Howie D, Simarro M, Sayos J, Guirado M, Sancho J, Terhorst C. Molecular dissection of the signaling and costimulatory functions of CD150 (SLAM): CD150/SAP binding and CD150-mediated costimulation. Blood. 2002; 99:957–965. [PubMed: 11806999]

Jameson SC, Carbone FR, Bevan MJ. Clone-specific T cell receptor antagonists of major histocompatibility complex class I-restricted cytotoxic T cells. J Exp Med. 1993; 177:1541–1550. [PubMed: 8496675]

Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, Schwartzberg PL, Crotty S. Ly108 functions as a SAP-dependent on-off switch for T cell help to B cells and NKT cell development. Immunity. 2012

Kupfer H, Monks CR, Kupfer A. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. J Exp Med. 1994; 179:1507–1515. [PubMed: 7513010]

Latour S, Roncagalli R, Chen R, Bakinowski M, Shi X, Schwartzberg PL, Davidson D, Veillette A. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. Nat Cell Biol. 2003; 5:149–154. [PubMed: 12545173]

Li C, Iosef C, Jia CY, Han VK, Li SS. Dual functional roles for the X-linked lymphoproliferative syndrome gene product SAP/SH2D1A in signaling through the signaling lymphocyte activation molecule (SLAM) family of immune receptors. J Biol Chem. 2003; 278:3852–3859. [PubMed: 12458214]

Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. Immunol Reviews. 2008; 224:70–84.

Markiewicz MA, Carayannopoulos LN, Naidenko OV, Matsui K, Burack WR, Wise EL, Fremont DH, Allen PM, Yokoyama WM, Colonna M, Shaw AS. Costimulation through NKG2D enhances murine $CD8^+$ CTL function: similarities and differences between NKG2D and CD28 costimulation. J Immunol. 2005; 175:2825–2833. [PubMed: 16116168]

Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. Nature. 1998; 395:82–86. [PubMed: 9738502]

Nichols KE, Harkin DP, Levitz S, Krainer M, Kolquist KA, Genovese C, Bernard A, Ferguson M, Zuo L, Snyder E, et al. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. Proc Natl Acad Sci USA. 1998; 95:13765–13770. [PubMed: 9811875]

Palendira U, Low C, Chan A, Hislop AD, Ho E, Phan TG, Deenick E, Cook MC, Riminton DS, Choo S, et al. Molecular pathogenesis of EBV susceptibility in XLP as revealed by analysis of female carriers with heterozygous expression of SAP. PLoS Biol. 2011; 9:e1001187. [PubMed: 22069374]

Parolini S, Bottino C, Falco M, Augugliaro R, Giliani S, Franceschini R, Ochs HD, Wolf H, Bonnefoy JY, Biassoni R, et al. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. J Exp Med. 2000; 192:337–346. [PubMed: 10934222]

Pfirsch-Maisonnas S, Aloulou M, Xu T, Claver J, Kanamaru Y, Tiwari M, Launay P, Monteiro RC, Blank U. Inhibitory ITAM signaling traps activating receptors with the phosphatase SHP-1 to form polarized "inhibisome" clusters. Sci Signal. 2011; 4 ra24.

Qi H, Cannons JL, Klauschen F, Schwartzberg PL, Germain RN. SAP-controlled T-B cell interactions underlies germinal centre formation. Nature. 2008; 455:764–769. [PubMed: 18843362]

Ren L, Chen X, Luechapanichkul R, Selner NG, Meyer TM, Wavreille AS, Chan R, Iorio C, Zhou X, Neel BG, Pei D. Substrate specificity of protein tyrosine phosphatases 1B, RPTPalpha, SHP-1, and SHP-2. Biochemistry. 2011; 50:2339–2356. [PubMed: 21291263]

Riteau B, Barber DF, Long EO. Vav1 phosphorylation is induced by beta2 integrin engagement on natural killer cells upstream of actin cytoskeleton and lipid raft reorganization. J Exp Med. 2003; 198:469–474. [PubMed: 12885870]

Rosette C, Werlen G, Daniels MA, Holman PO, Alam SM, Travers PJ, Gascoigne NR, Palmer E, Jameson SC. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. Immunity. 2001; 15:59–70. [PubMed: 11485738]

Ryser JE, Rungger-Brandle E, Chaponnier C, Gabbiani G, Vassalli P. The area of attachment of cytotoxic T lymphocytes to their target cells shows high motility and polarization of actin, but not myosin. J Immunol. 1982; 128:1159–1162. [PubMed: 7035558]

Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, van Schaik S, Notarangelo L, Geha R, Roncarolo MG, et al. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature. 1998; 395:462–469. [PubMed: 9774102]

Schwartzberg PL, Mueller KL, Qi H, Cannons JL. SLAM receptors and SAP influence lymphocyte interactions, development and function. Nat Rev Immunol. 2009; 9:39–46. [PubMed: 19079134]

Sharifi R, Sinclair JC, Gilmour KC, Arkwright PD, Kinnon C, Thrasher AJ, Gaspar HB. SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease. Blood. 2004; 103:3821–3827. [PubMed: 14726378]

Shlapatska LM, Mikhalap SV, Berdova AG, Zelensky OM, Yun TJ, Nichols KE, Clark EA, Sidorenko SP. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J Immunol. 2001; 166:5480–5487. [PubMed: 11313386]

Smith C, Miles JJ, Khanna R. Advances in direct T-cell alloreactivity: function, avidity, biophysics and structure. Am J Transplant. 2012; 12:15–26. [PubMed: 22152064]

Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, Hoff Jv, Dhar D, Nichols KE, Filipovich AH, et al. SAP augments proximal T cell receptor signal strength necessary for restimulation-induced apoptosis of activated T lymphocytes. J Clin Invest. 2009; 119:2976–2989. [PubMed: 19759517]

Stebbins CC, Watzl C, Billadeau DD, Leibson PJ, Burshtyn DN, Long EO. Vav1 dephosphorylation by the tyrosine phosphatase SHP-1 as a mechanism for inhibition of cellular cytotoxicity. Mol Cell Biol. 2003; 23:6291–6299. [PubMed: 12917349]

Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. Nat Immunol. 2003; 4:248–254. [PubMed: 12577055]

Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. Immunity. 2001; 15:751–761. [PubMed: 11728337]

Stinchcombe JC, Majorovits E, Bossi G, Fuller S, Griffiths GM. Centrosome polarization delivers secretory granules to the immunological synapse. Nature. 2006; 443:462–465. [PubMed: 17006514]

Tsun A, Qureshi I, Stinchcombe JC, Jenkins MR, de la Roche M, Kleczkowska J, Zamoyska R, Griffiths GM. Centrosome docking at the immunological synapse is controlled by Lck signaling. J Cell Biol. 2011; 192:663–674. [PubMed: 21339332]

Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. Immunity. 2006; 25:117–127. [PubMed: 16860761]

Vyas YM, Maniar H, Dupont B. Cutting edge: differential segregation of the SRC homology 2-containing protein tyrosine phosphatase-1 within the early NK cell immune synapse distinguishes noncytolytic from cytolytic interactions. J Immunol. 2002; 168:3150–3154. [PubMed: 11907066]

Zhong MC, Veillette A. Control of T lymphocyte signaling by Ly108, a signaling lymphocytic activation molecule family receptor implicated in autoimmunity. J Biol Chem. 2008; 283:19255–19264. [PubMed: 18482989]

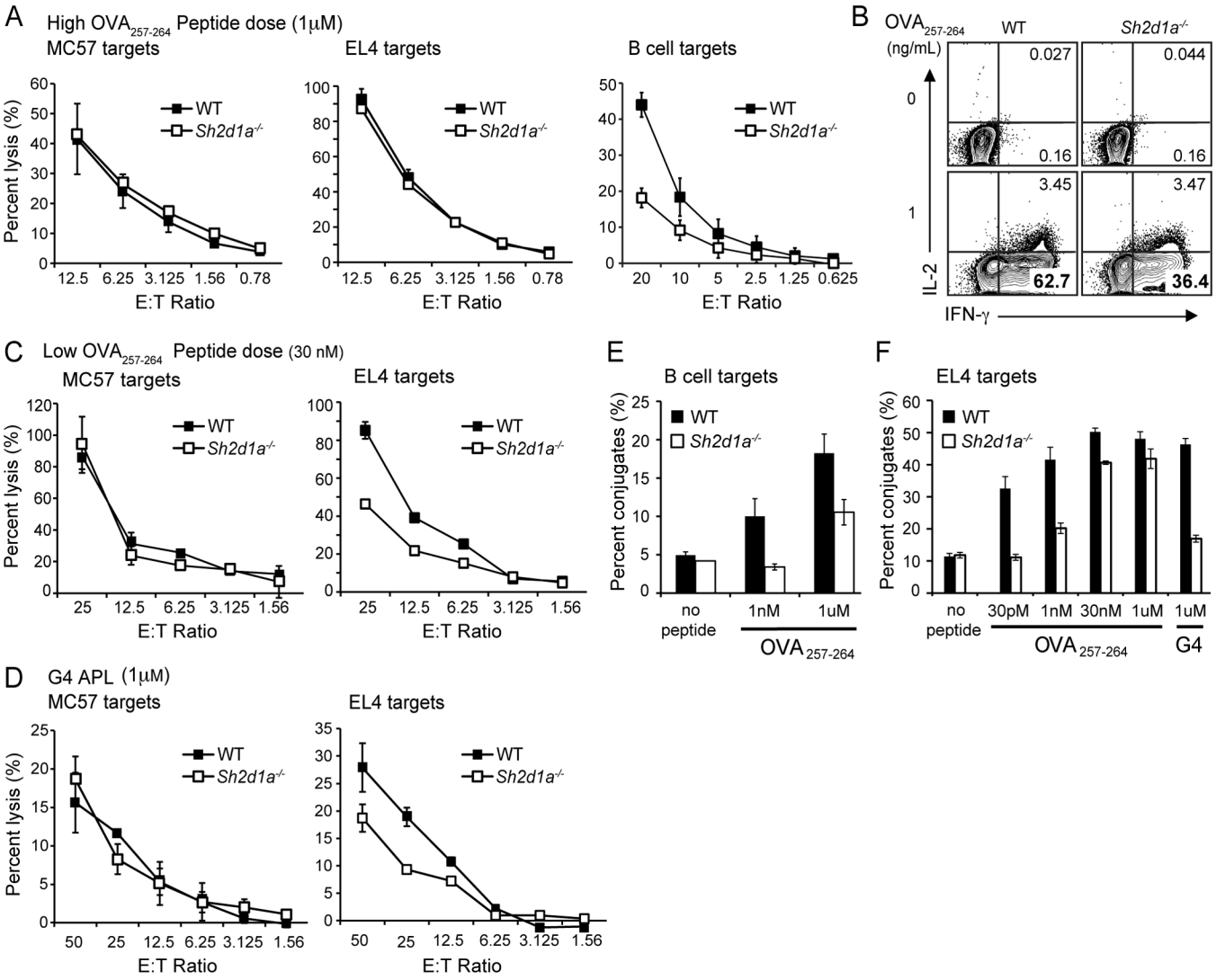

Figure 1. SAP-deficient CTLs have a selective defect in cytolysis of and adhesion to B cell and low avidity T cell targets

(A) Cytolysis of MC57, EL4 and LPS-activated WT B cell targets, pulsed with 1 μM $OVA_{257–264}$, by WT and *Sh2d1a*$^{-/-}$ CTLs. (B) Intracellular IFN-γ and IL-2 production by WT and *Sh2d1a*$^{-/-}$ CTLs after stimulation with $OVA_{257–264}$ pulsed B cells. Representative of 3 independent experiments. (C–D) Cytolysis of MC57 and EL4 targets pulsed with 30 nM $OVA_{257–264}$ (C) or 1 μM G4 APL (D) by WT and *Sh2d1a*$^{-/-}$ CTLs. Graphs show the average cytotoxicity from triplicate wells ±SD for varying effector to target (E:T) ratios, representative of 4 or more experiments. (E–F) Adhesion of WT and *Sh2d1a*$^{-/-}$ CTLs to WT B cells (E) or EL4 cells (F), pulsed with the indicated concentrations of $OVA_{257–264}$ or the G4 APL. Graphs show the average of triplicates ±SD, representative of 3 independent experiments.

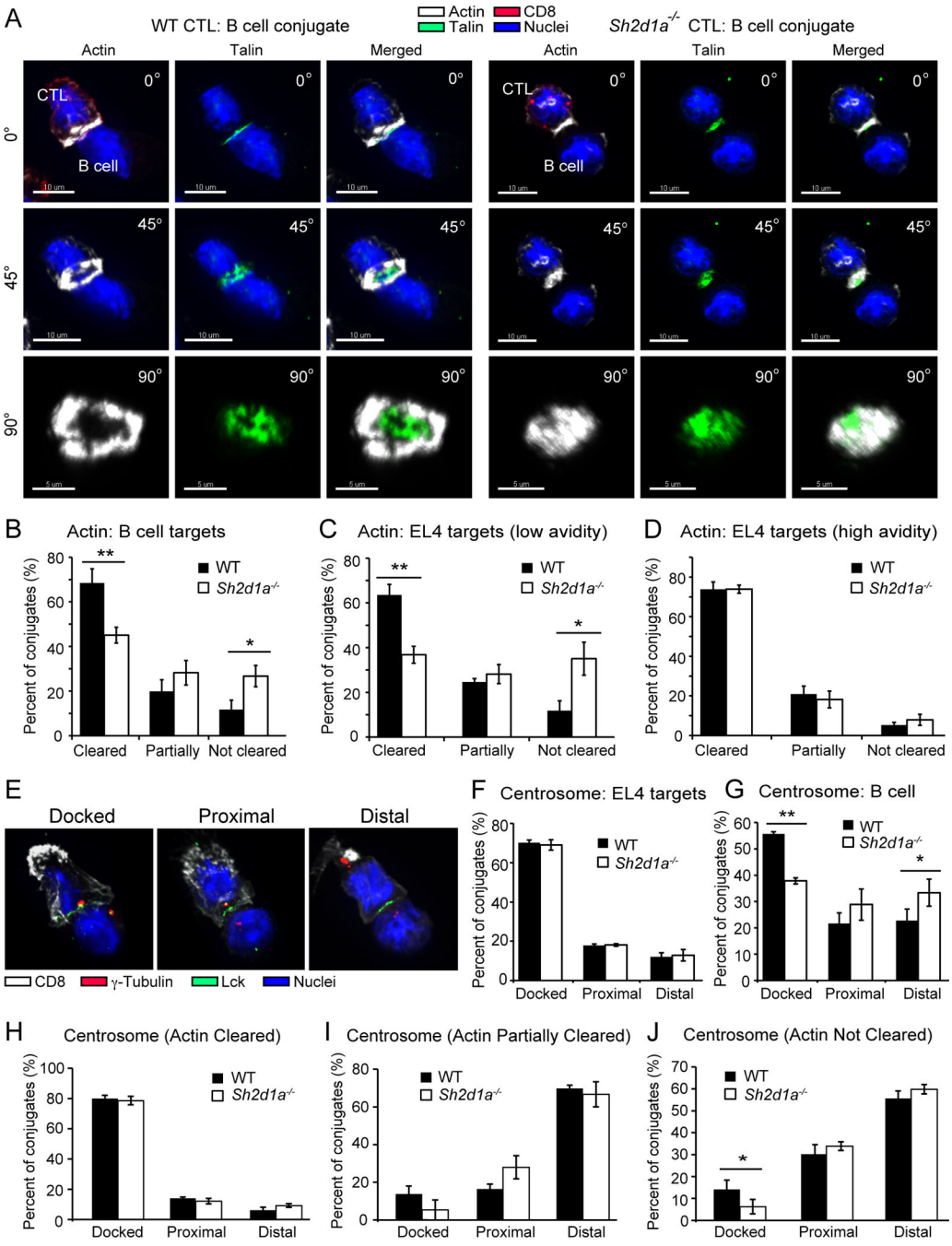

Figure 2. SAP-deficient CTLs exhibit abnormal actin organization and reduced centrosome docking at the T:B cell IS

(A) LPS-activated WT B cell targets, pulsed with 1 μM $OVA_{257–264}$, conjugated with WT or *Sh2d1a*$^{-/-}$ CTLs, examined from the side, as confocal projections in the x–y plane (top row), or at 45° (middle row) and 90° (*en face*, bottom row) rotations in the y–z plane. Scale bars represent either 10 μm (top and middle row) or 5 μm (bottom row). Nuclei (blue), CD8 (red), talin (green), and actin (white). (B–D) Quantification of actin localization at the IS with WT B cell targets pulsed with 1 μM $OVA_{257–264}$ peptide (B), EL4 targets pulsed with 30 nM $OVA_{257–264}$ peptide (C), or EL4 targets pulsed with 1 μM $OVA_{257–264}$ peptide (D). (E) Centrosome localization, shown as confocal projections in the x–y plane. Nuclei (blue),

CD8 (white), Lck (green), and γ-tubulin (red). (F–G) Quantification of centrosome positioning in WT and *Sh2d1a*$^{-/-}$ CTL conjugates with EL4 (F) or WT B cell (G) targets pulsed with 1 μM OVA$^{257–264}$ peptide. (H–J) Quantification of centrosome localization in WT and *Sh2d1a*$^{-/-}$ CTLs exhibiting the phenotypes of actin cleared (H), partially cleared (I), or not cleared (J) at the IS with WT B cell targets pulsed with 1 μM $OVA_{257–264}$ peptide. Data shown are averages of 3 or more experiments ±SEM, with >45 conjugates scored per genotype for each experiment. *$p < 0.05$, **$p < 0.005$, as determined by Paired Student's T-tests.

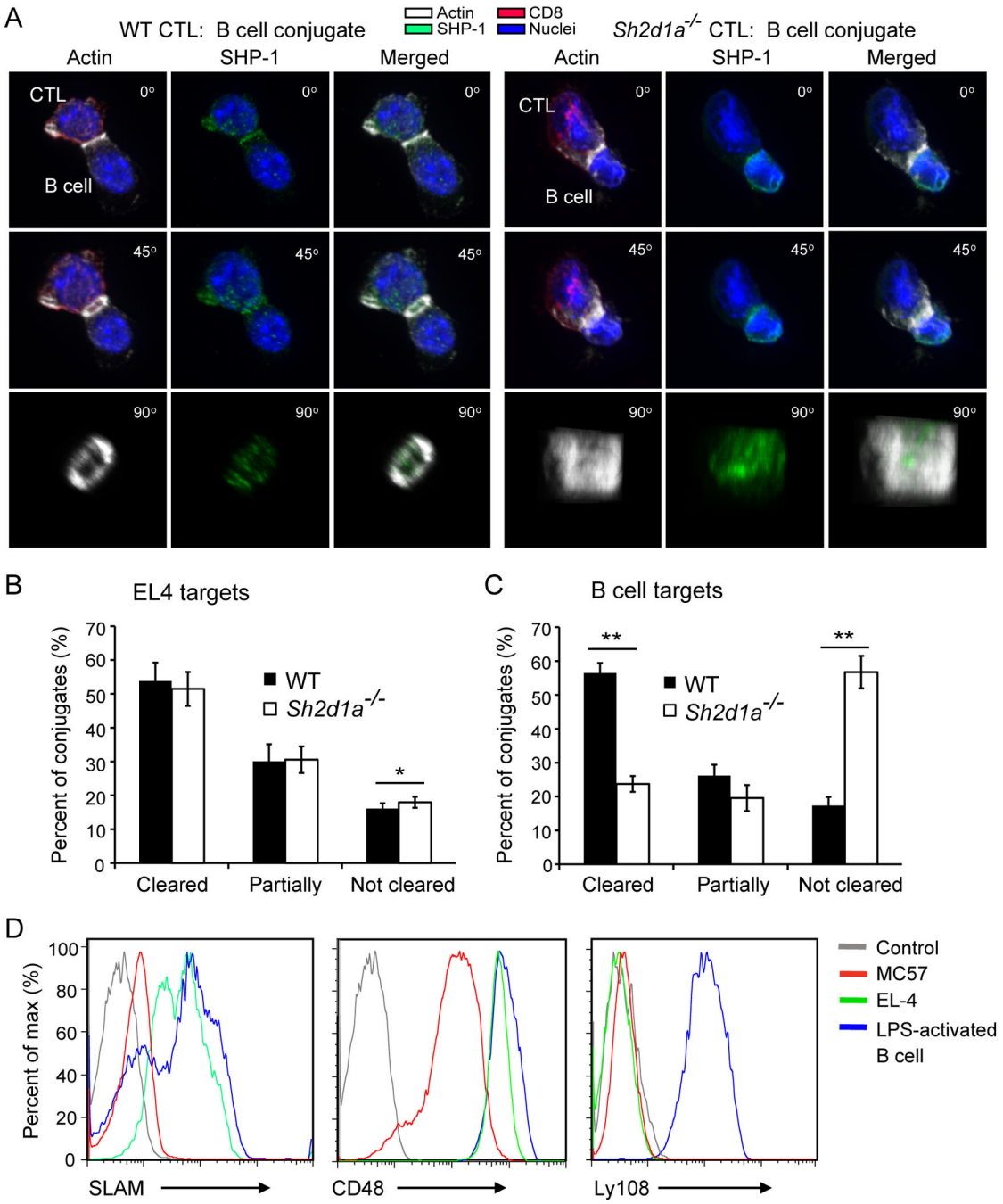

Figure 3. SAP-deficient CTLs exhibit abnormal SHP-1 accumulation at the T:B cell IS

(A) WT and *Sh2d1a*$^{-/-}$ CTL conjugates with LPS-activated WT B cells, examined as in Figure 2A. Nuclei (blue), CD8 (red), SHP-1 (green), and actin (white). (B–C) Quantification of SHP-1 localization at the IS of WT and *Sh2d1a*$^{-/-}$ CTL conjugates with EL4 (B) or LPS-activated WT B cell (C) targets pulsed with 1 μM $OVA_{257–264}$ peptide. Data depicted are the average of 3 or more experiments ±SEM, with 35–60 conjugates scored per genotype for each experiment. **p < 0.005, as determined by Paired Student's T-tests. (D) Expression of SLAM, CD48 and Ly108 on MC57, EL4 and LPS-activated WT B cell targets.

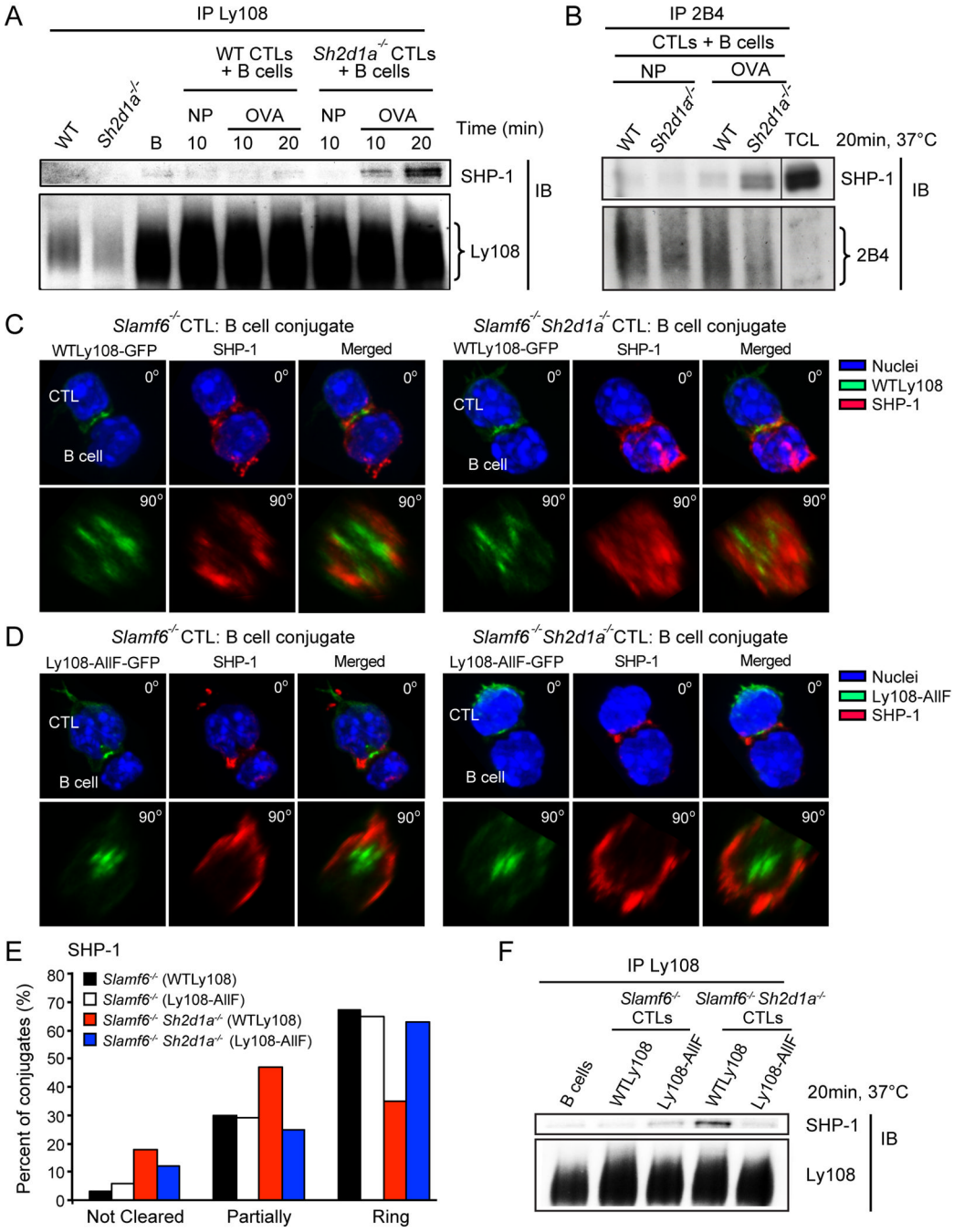

Figure 4. Ly108 and 2B4 show increased association with SHP-1 in the absence of SAP: requirement for Ly108 intracellular tyrosines

(A–B) Lysates from WT and *Sh2d1a*$^{-/-}$ CTLs, stimulated with WT B cells (NP, no peptide, or 1 μg/mL $OVA_{257–264}$ pulsed), were immunoprecipitated for Ly108 (A) or 2B4 (B) and immunoblotted for SHP-1 and Ly108 or 2B4, respectively. Representative immunoblots shown, from 3 or more independent experiments. TCL, total cell lysate. (C–E) *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs, transfected with either WTLy108-GFP (C) or Ly108-AllF-GFP (D), were conjugated with WT B cell targets and shown as confocal projections in the x-y plane (top row) or *en face* (lower row). Nuclei (blue) and SHP-1 (red). (E) Quantification of SHP-1 localization at the T:B cell IS of *Slamf6*$^{-/-}$ and

Slamf6$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs transfected with WTLy108-GFP or Ly108-AllF-GFP. Data shown are representative of two experiments. (F) *Slamf6*$^{-/-}$ and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs, transduced with retroviral vectors expressing either Ly108 or Ly108-AllF, were stimulated with 1 μg/mL $OVA_{257–264}$ pulsed WT B cells. Lysates were immunoprecipitated for Ly108 and immunoblotted for SHP-1 and Ly108. Representative immunoblot shown from 2 independent experiments.

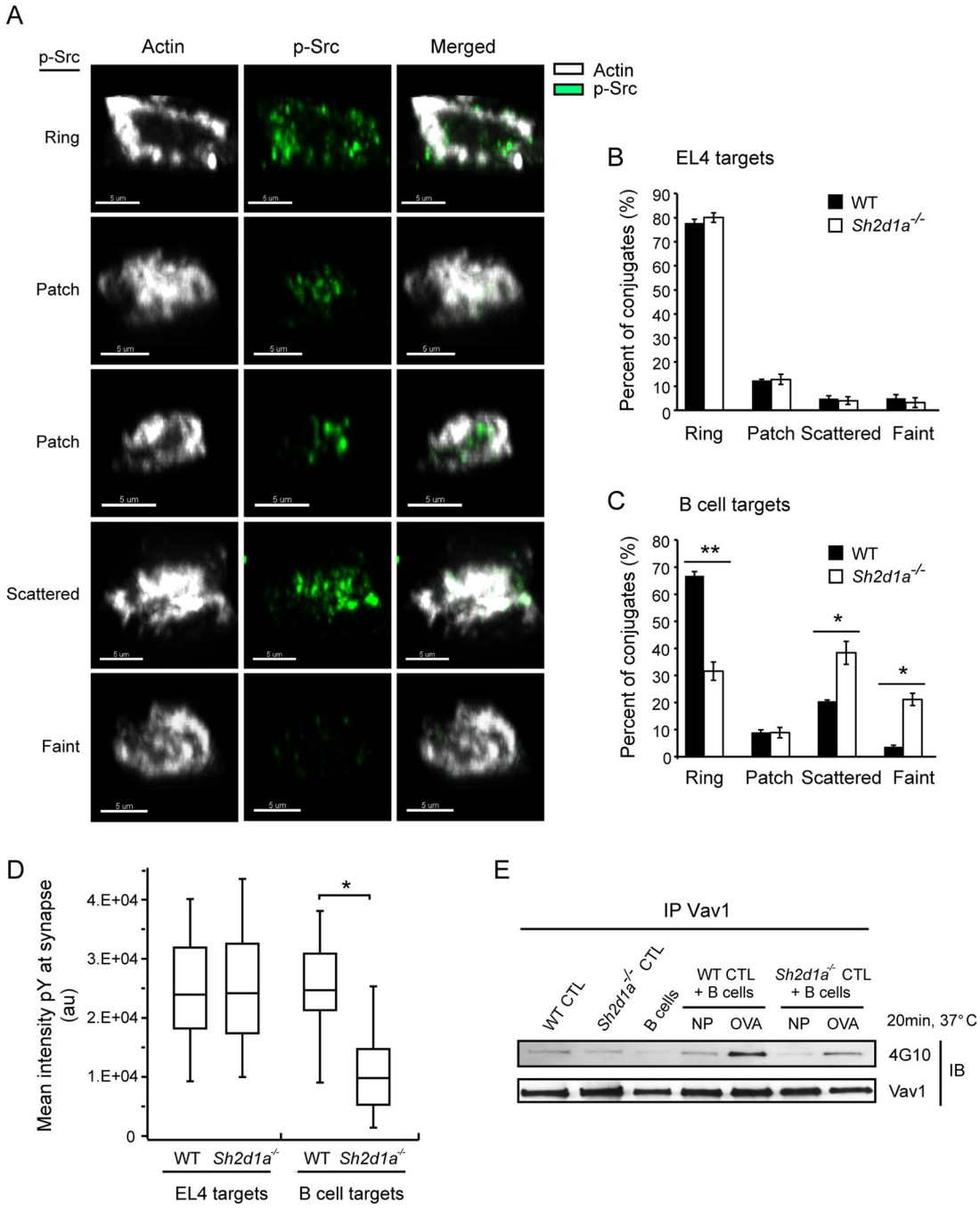

Figure 5. SAP-deficient CTLs exhibit abnormal p-Src localization and decreased tyrosine phosphorylation at the T:B cell IS and reduced Vav1 phosphorylation

(A) Representative immunofluorescence images of p-Src (pY416) localization, shown as *en face* reconstructions: p-Src (green) and actin (white). Scale bars represent 5 μm. (B–C) Quantification of p-Src (pY416) localization at the IS of WT and *Sh2d1a*$^{-/-}$ CTL conjugates with EL4 (B) or WT B cell (C) targets pulsed with 1 μM $OVA_{257–264}$ peptide. Data are the average of 3 experiments ±SEM, with over 40 conjugates scored per genotype for each experiment. *$p < 0.05$, **$p < 0.005$, as determined by Paired Student's T-tests. (D) Mean intensity of pTyr staining at the IS of WT and *Sh2d1a*$^{-/-}$ CTL conjugates with EL4 or WT B cell targets pulsed with 1 μM $OVA_{257–264}$ peptide. Data shown is representative of 3

independent experiments. *$p < 0.0001$, as determined by Student's T-tests. (E) Lysates from WT and *Sh2d1a*$^{-/-}$ CTLs, stimulated with 1 μg/ml $OVA_{257–264}$ pulsed WT B cell targets, were immunoprecipitated for Vav1 and blotted for phosphotyrosine (4G10) and Vav1. Representative immunoblot shown from 3 independent experiments.

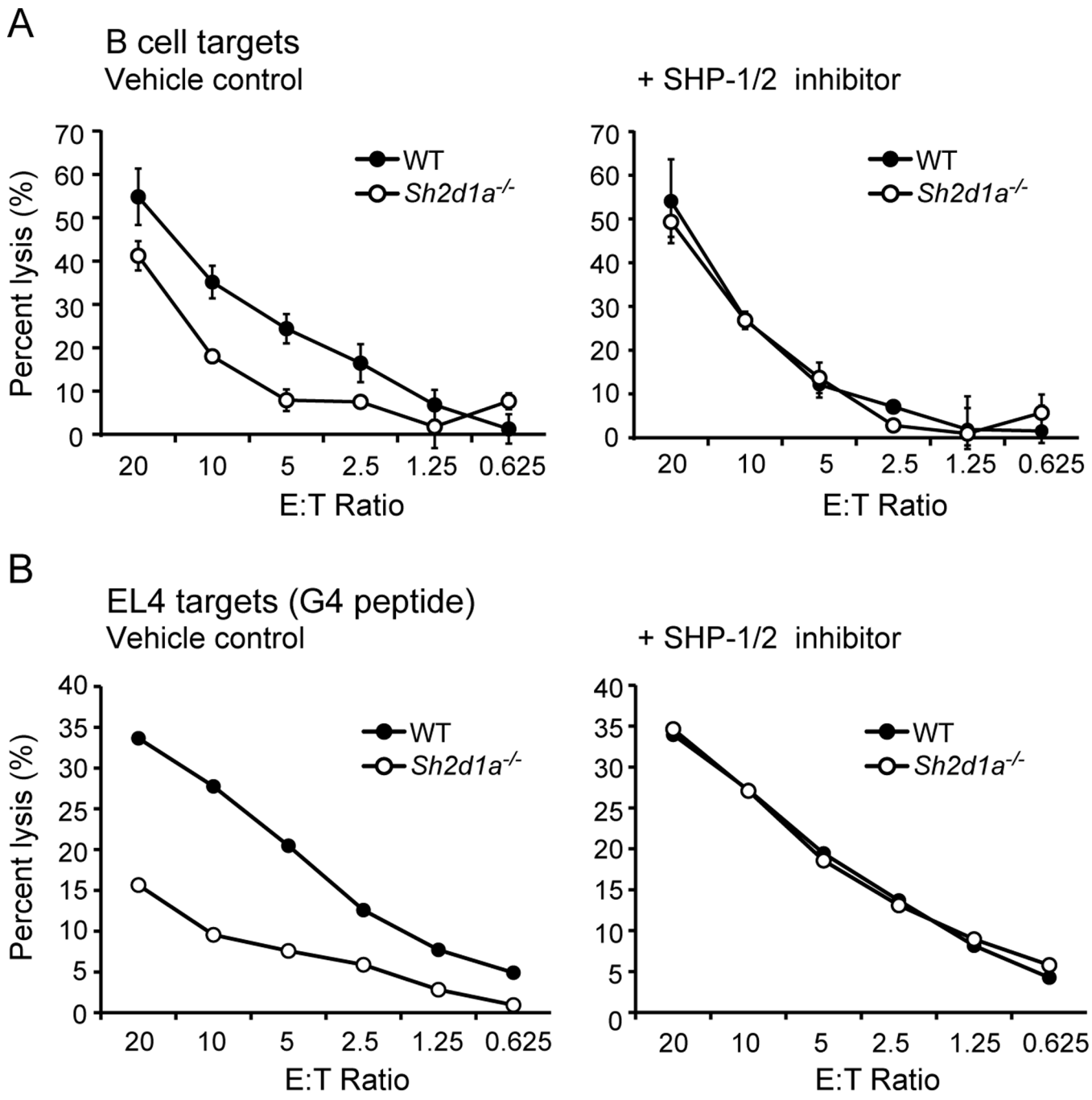

Figure 6. Negative Signaling through the SLAM family receptors is mediated through SHP-1

(A–B) WT and *Sh2d1a*$^{-/-}$ CTLs were serum-starved and pre-incubated with the SHP-1/2 inhibitor NSC-87877 for 2 hr at 37°C, before examination of cytotoxicity against WT B cell targets pulsed with 1 μM $OVA_{257–264}$ peptide (A) or EL4 targets pulsed with 1 μM G4 APL (B). Data shown are the average of triplicate wells ±SD for a range of effector to target (E:T) ratios. Representative of 3 independent experiments.

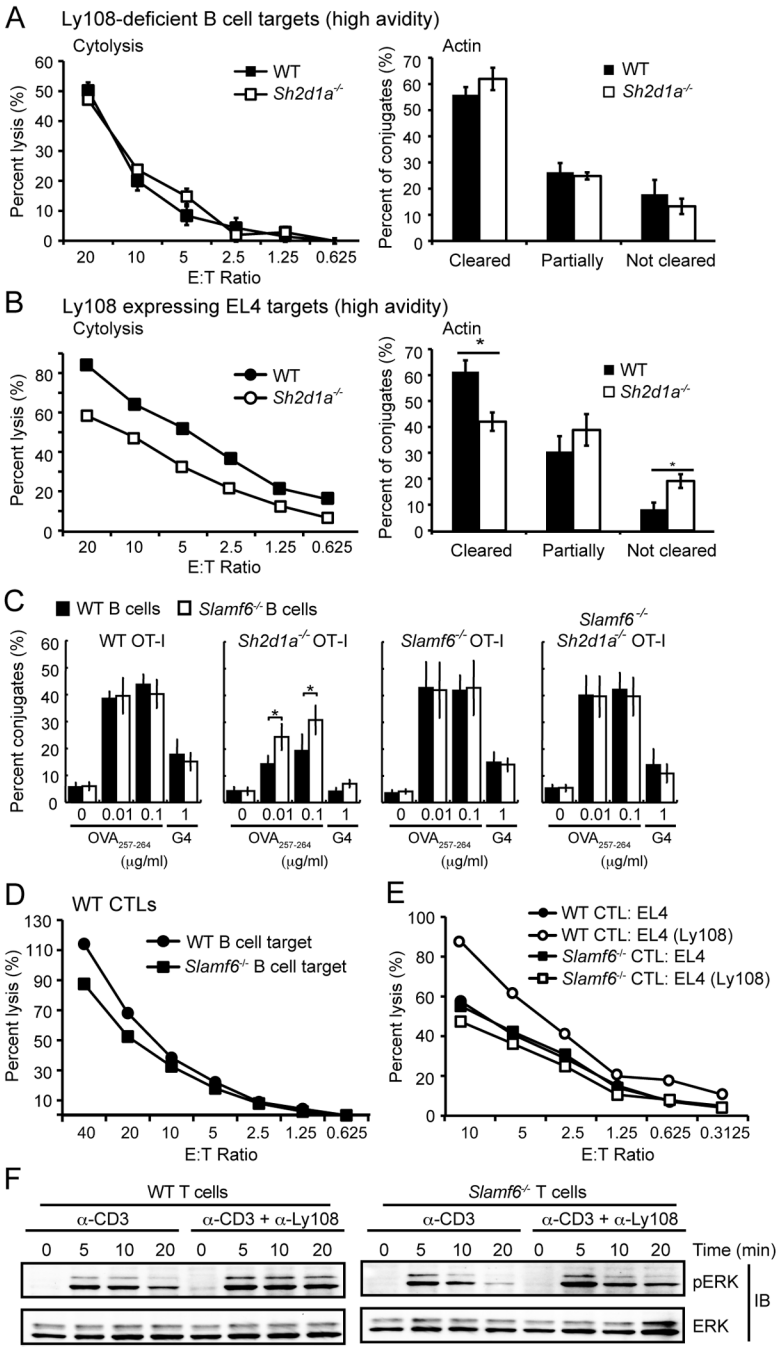

Figure 7. SLAM family receptor engagement is required for the phenotype of SAP-deficient CTLs

(A–B) Cytotoxicity (left) and actin organization at the IS (right) of WT and *Sh2d1a*$^{-/-}$ CTLs against *Slamf6*$^{-/-}$ B cells (A) or Ly108-expressing EL4 targets (B) pulsed with 1 μM $OVA_{257–264}$ peptide. Quantifications of actin organization depict the average of 3 experiments ±SEM and represent more than 40 conjugates per genotype for each experiment. *$p < 0.05$ as determined by Paired Student's T-tests. (C) Adhesion of WT, *Sh2d1a*$^{-/-}$, *Slamf6*$^{-/-}$, and *Slamf6*$^{-/-}$*Sh2d1a*$^{-/-}$ CTLs to WT or *Slamf6*$^{-/-}$ B cells, pulsed with the indicated concentrations of $OVA_{257–264}$ or the G4 APL. Graphs show the average of 3 independent experiments. *$p < 0.05$ as determined by Paired Student's T-tests. (D–E)

Cytotoxicity of WT CTLs against WT or *Slamf6*$^{-/-}$ B cells (D), or of WT and *Slamf6*$^{-/-}$ CTLs against control or Ly108-expressing EL4 targets (E). Cytolysis shown is an average of triplicate wells ±SD for a range of effector to target (E:T) ratios, representative of 3 or more experiments. (F) Lysates from WT and *Slamf6*$^{-/-}$ T cells, stimulated by cross-linking antibodies against anti-CD3 alone or anti-CD3 and anti-Ly108, were immunoblotted for ERK phosphorylation. Representative immunoblot shown from 3 independent experiments.