RESEARCH HIGHLIGHT

LCK connects NTB-A and SAP signaling in T cells to restimulation-induced cell death

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Received: August 06, 2014
Published online: October 14, 2014

Signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) is an adaptor protein required for SLAM family receptor signaling. In T cells, signaling from different SLAM receptors (SLAM-Rs) governs differentiation, effector function, and apoptosis specifically through the self-regulatory program of T cell receptor restimulation-induced cell death (RICD). Indeed, SLAM-R signaling and RICD are impaired in X-linked lympho- proliferative disease (XLP) patients that are deficient for SAP, as well as in SAP-deficient mice. Importantly, defective RICD likely contributes to excessive CD8^{+} T cell accumulation and severe immunopathology noted in XLP patients upon infection with Epstein-Barr Virus (EBV). It is well established that SAP signaling through different SLAM-Rs is associated with the recruitment of the Src-family kinase FYN. Surprisingly, we recently discovered that FYN has no role in RICD. Instead, our data suggest that SAP enhances the recruitment and activation of LCK to the SLAM family receptor NK, T, and B cell Antigen (NTB-A), and thus amplifies TCR signaling for optimal RICD. In this research highlight we review the role of SAP in T cells, and describe our recent findings placing LCK as an important player in SAP-mediated NTB-A signaling for T cell apoptosis.

Keywords: SAP; NTB-A; LCK; RICD; XLP

To cite this article: Gil Katz, *et al*. LCK connects NTB-A and SAP signaling in T cells to restimulation-induced cell death. Receptor Clin Invest 2014; 1: e292. doi: 10.14800/rci.292.

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Restimulation-induced cell death (RICD) is an autoregulatory form of apoptosis that is thought to limit T cell expansion during an immune response [1]. Defects in this process may lead to autoimmune and/or lymphoproliferative syndromes associated with damage to self-tissues. One example is X-linked lymphoproliferative disease (XLP), for which patients can develop a fatal fulminant infectious mononucleosis following Epstein-Barr virus (EBV) infection [2, 3]. Severe immunopathology is driven by unbridled expansion of activated CD8^{+} T cells and infiltration into multiple organs, resulting in widespread necrosis. The genetic cause for most XLP cases is a null mutation in signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), a 128 amino

acid adaptor protein composed almost entirely of a single SH2 domain [2]. Since mutations in the SAP gene *SH2D1A* were linked to XLP over 15 years ago, the question of how SAP deficiency translates into a pathological over-accumulation of activated T cells has remained unclear. A systematic analysis of several apoptosis pathways revealed XLP patient T cells are specifically resistant to RICD [4]. Knockdown experiments in normal human T cells confirmed SAP deficiency impairs RICD, particularly for $CD8^+$ T cells [4]. Further analysis showed that T cell receptor (TCR) signaling was compromised in activated SAP-deficient T cells, manifesting in reduced BIM and FAS-ligand (FASL) induction after restimulation. Strikingly, siRNA-mediated silencing of only one SLAM family receptor, NTB-A (SLAMF6), resulted in defective RICD akin to SAP-deficient T cells. Indeed, SAP was recruited to NTB-A receptors after TCR restimulation [4], suggesting the pro-apoptotic function of SAP works through NTB-A to ensure a robust TCR signal that is sufficient to cause RICD in activated effector T cells.

How does SAP, through its association with NTB-A, contribute to stronger TCR signaling necessary for RICD induction? One mechanism involves SAP displacing the SH2 domain–containing protein tyrosine phosphatase (SHP)-1 from NTB-A after TCR restimulation in healthy donor T cells, switching NTB-A from a negative to a positive signaling receptor. SHP-1 remained docked with NTB-A in SAP-deficient XLP patient T cells after restimulation, allowing for modulation of TCR signaling [4]. However, SHP-1-specific siRNA knockdown experiments only partly rescued the RICD defect in XLP patient T cells. These data suggested that apart from preventing a negative effect on TCR signaling, SAP also served to amplify TCR signaling, presumably through the recruitment of a Src-family kinase. Herein we review the current data on SAP expression and function in T cells, signal transduction associated with SAP and SLAM-Rs, and our most recent work on SAP and NTB-A signaling for RICD.

SAP expression and function in T cells

SAP is expressed throughout the T cell lineage; in fact, SAP expression in thymocytes is significantly higher than in resting mature T cells [5]. SAP expression in the thymus is critical for the development of unconventional T cells that are selected by hematopoietic cells, including natural killer T (NKT) cells and other innate-like T cell lineages [6]. In mature T cells, SAP is required for $CD4^+$ Th2 differentiation [7, 8], $CD8^+$ cytotoxicity [9, 10], T follicular helper (T_{FH}) cell differentiation and provision of B cell help for optimal humoral responses [11], and efficient RICD of activated T cells [4]. Defects in these processes tend to be more severe in mice deficient for SAP versus individual SLAM-Rs, implying functional redundancy in the SLAM-R family. For example, at least two SLAM-Rs are known to cooperate in facilitating germinal center responses (CD84 and Ly108, the murine homolog of NTB-A) and cytolysis of target B cells (Ly108 and 2B4) by promoting sustained, SAP-dependent T:B cell interactions [9, 10, 12]. In contrast, SAP appears to constrain the magnitude of a given T cell response by promoting RICD in activated, cycling T cells solely through NTB-A signaling [4]. Although NTB-A deficiency has not been documented in humans, loss of Ly108 in mice rescues certain immune deficits noted in $Sap^{-/-}$ animals [13], underscoring the "switch" like property of SLAM-R signaling dictated by the presence or absence of SAP. Indeed, NTB-A knockdown actually boosts RICD in XLP patient T cells, ostensibly by removing the aforementioned SHP-1-driven modulatory signal [4].

It is now clear that SAP deficiency in mice [7, 14] and humans [2, 3] leads to an acute increase in antigen-specific T cell expansion in response to certain infections, which can result in significant immunopathology. Despite its importance in multiple T cell subsets, relatively little is known about the regulation of SAP expression. Both mouse and human *SH2D1A* (SAP) promoters contain a highly homologous region between –185 to –163 (mouse) and –167 to –134 (human) with an Ets binding site that is crucial to the basal promoter activity [15]. In humans, a single nucleotide polymorphism at position -346 (T->C) was also correlated with *SH2D1A* expression, such that lower SAP mRNA and protein were found in individuals with -346C compared with -346T [16].

Initial TCR stimulation down regulates SAP expression for 24-72 hours through a mechanism that is not well understood [9, 15]. However, both SAP and NTB-A expression increase dramatically as activated T cells expand in the presence of interleukin-2 (IL-2), exceeding expression levels noted in resting T cells [5]. IL-2 also up regulates SAP expression in NK cells with similar kinetics [17]. It is tempting to speculate that increased SAP expression in activated T cells is a major factor in conveying sensitivity to RICD, which is dependent on IL-2-mediated cell cycle progression [1]. In fact, our preliminary studies suggest that while NTB-A expression remains remarkably constant on activated T cells from different individuals, low RICD sensitivity noted in certain human donors correlates with abnormally low SAP expression (A.L. Snow, unpublished data).

Signaling by SLAM-Rs through SAP is classically associated with the Src-family kinase FYN [3]. SAP interacts with the SH3 domain of FYN through arginine 78 (R78) [18, 19], allowing for recruitment to immunoreceptor tyrosine-based switch motifs (ITSMs) in SLAM-Rs. This

interaction results in tyrosine phosphorylation of the receptors themselves and downstream signaling components, such as Dok-1/Dok-2 or Vav-1 when SLAM (CD150/SLAMF1) or 2B4 (CD244/SLAMF4) are phosphorylated, respectively [18, 20]. Beyond recruiting FYN to SLAM-Rs, SAP can directly enhance FYN kinase activity [21]. Phosphorylation of several SLAM-Rs is significantly attenuated in both SAP and FYN-deficient thymocytes and T cells, although some residual phosphorylation is observed in the absence of FYN [19, 21-23], implying that other Src-family kinases may compensate for the loss of FYN in SLAM-R signaling in thymocytes. In accordance with this idea, NKT cells are completely absent in $SAP^{-/-}$ thymi, while a small population of NKT cells persists in $FYN^{-/-}$ thymi [24].

LCK signaling through SAP and RICD

The possibility that a Src-family kinase other than FYN can participate in SAP-dependent SLAM-R signaling was first suggested through overexpression experiments and *in vitro* binding assays. Overexpression of SLAM, SAP, and LCK in 293T cells resulted in robust SLAM phosphorylation, above levels noted when only SAP or LCK were over expressed with SLAM [21]. This study also demonstrated that recombinant SAP could bind *in vitro* translated LCK through its kinase domain, in contrast to the SH3-mediated interaction with FYN. Subsequent work with Sap^{R78A} knock-in mice established that not all SAP-dependent processes require FYN interaction, including T:B cell conjugation and NKT cell effector function [25, 26]. In addition, expression of either WT or R78A SAP restored RICD sensitivity to XLP patient T cells, suggesting FYN was also dispensable for this process [4]. However, no direct association between LCK and a SLAM-R had been detected in primary cells. Furthermore, a clear role for LCK in SAP-mediated SLAM-R signaling had not been demonstrated.

We recently investigated the molecular mechanism by which SAP potentiates TCR signaling through NTB-A for efficient RICD. Our results demonstrated that LCK, but not FYN, associates with NTB-A in activated, cycling human T cells [27]. Consistent with this finding, we also found knockdown or pharmacological inhibition of FYN had no effect on RICD sensitivity. On the other hand, specific inhibition of LCK impaired RICD significantly, akin to SAP deficiency itself. Similar results were obtained by silencing LCK expression using LCK-specific siRNA. We next assessed changes in LCK association with NTB-A both before and after TCR restimulation by immunoprecipitating NTB-A from primary human T cell cultures. We showed that LCK reliably precipitates with NTB-A, and that this association is significantly increased after TCR restimulation. In addition, NTB-A-associated LCK phosphorylation on tyrosine 394 (Y394) and serine 59 (S59), representing the fully active conformation of LCK [28, 29], also increased after TCR restimulation. In contrast, no association of FYN with NTB-A was detectable before or after TCR restimulation. Importantly, the TCR-induced increase in LCK association and phosphorylation at NTB-A receptors was abrogated in both SAP knockdown T cells and XLP patient T cells, consistent with a block in RICD. Given that increased recruitment and phosphorylation of LCK to NTB-A was dependent on SAP, we suspected that NTB-A, SAP, and LCK are found within the same protein complex. We confirmed this hypothesis by showing that immunoprecipitation of SAP reliably pulled down both LCK and NTB-A in primary human T cells, and that these interactions were enhanced after TCR restimulation.

To better characterize the binding of SAP to NTB-A, and its effect on LCK recruitment and phosphorylation, we mutated key tyrosine residues within the cytoplasmic tail of NTB-A to phenylalanines and expressed these mutant receptors in the NTB-A-deficient T cell line PEER [30]. As stated previously, SAP binds to the cytoplasmic tails of SLAM-Rs via phosphorylated tyrosines [3] found within ITSMs. Following stable selection of $NTB\text{-}A^{+}$ clones, we tested both recruitment and Y394 phosphorylation of LCK to NTB-A, as well as SAP binding, before and after TCR restimulation. Our results showed that two ITSM-based tyrosine residues in the NTB-A tail, Y284 and Y308, are critical for SAP binding to NTB-A after TCR restimulation, as well as for the recruitment and Y394 phosphorylation of LCK. In contrast, mutation of Y273 actually enhanced SAP and LCK recruitment, suggesting this tyrosine may play a role in attenuating positive signals conveyed through NTB-A, perhaps by associating with a regulatory molecule like SHP-1.

To confirm that NTB-A-associated LCK kinase activity was enhanced after TCR re-engagement, we also performed *in vitro* kinase assays using NTB-A immunoprecipitates from primary T cells and a well-established substrate for LCK, recombinant GST-CD3ζ [28]. We found that NTB-A-associated LCK kinase activity was significantly increased following TCR restimulation in a SAP-dependent manner. Congruent with these results, we also detected higher levels of endogenous phosphorylated CD3ζ and global tyrosine phosphorylation in normal versus SAP-deficient T cells following restimulation. In summation, these results demonstrate that SAP boosts TCR signaling over the required threshold for RICD induction by enhancing the interaction between active LCK and NTB-A receptors. Increased recruitment and activation of LCK at the immunological synapse, where CD3 and NTB-A are known to colocalize [4], serves to amplify proximal TCR signaling and induce

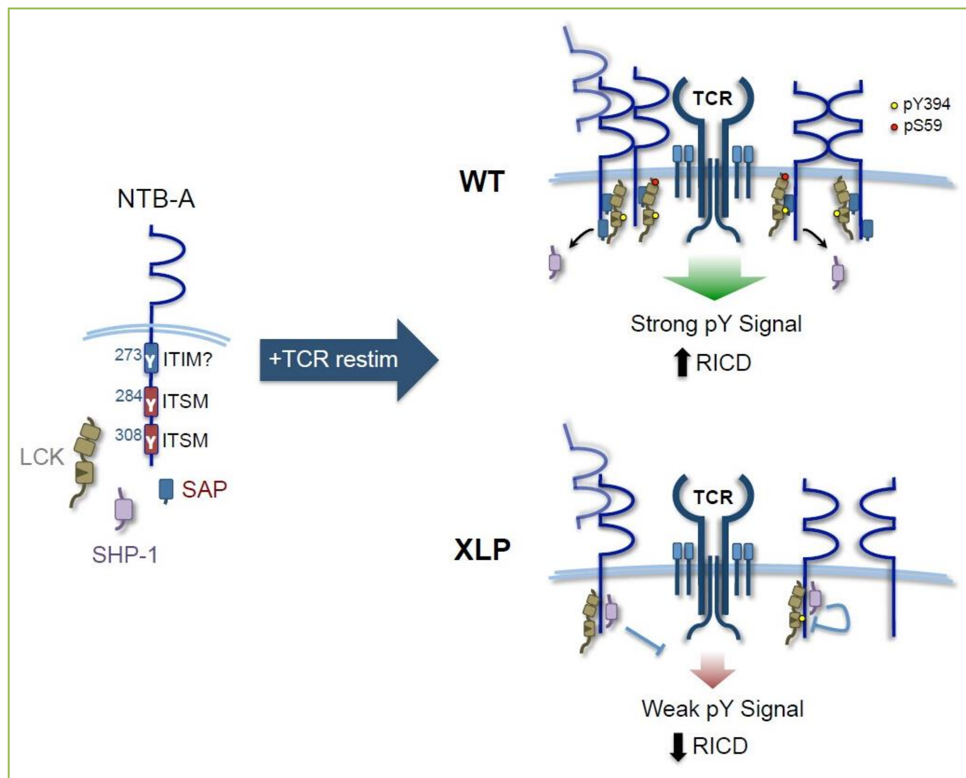

Figure 1. SAP-dependent association of active LCK with NTB-A promotes strong TCR signaling for RICD. *Left panel*: schematic diagram of key signaling molecules involved in determining RICD sensitivity, including the NTB-A receptor containing 2 ITSMs and 1 putative immunoreceptor tyrosine-based inhibitory motif (ITIM) for signaling. *Right panel*: In wild-type (WT) T cells, TCR restimulation induces SAP-dependent recruitment and activation (via Y394 and S59 phosphorylation) of LCK at NTB-A receptors, as well as displacement of SHP-1. Strong colocalization of TCR and NTB-A likely amplifies proximal signaling via LCK to promote RICD. In XLP patient T cells, loss of SAP weakens proximal signaling by leaving more SHP-1 and less LCK associated with NTB-A after TCR restimulation. This manifests as less tyrosine phosphorylation of downstream signaling components, poor induction of pro-apoptotic molecules, and impaired RICD.

downstream pro-apoptotic molecules (e.g. FASL, BIM) that execute the RICD program (Figure 1).

Our data further highlight SAP as a versatile adaptor protein capable of coupling both FYN and LCK to SLAM-R signaling. Although it is not yet clear what conditions favor FYN or LCK binding to SAP and different SLAM-Rs, the formation of these complexes clearly results in different signaling outcomes. While FYN is required for the development and differentiation of several T cell subsets, LCK may be reserved for specific effector functions and RICD. Our results, obtained using primary human T cells under physiologically relevant conditions, lay the groundwork for future studies to determine whether this molecular complex represents a viable therapeutic target for controlling T cell responses by modulating RICD sensitivity.

Conflicting interests

The authors have declared that no competing interests exist.

Acknowledgments

We thank the patients and families for participation, as well as collaborators at Cincinnati Children's Hospital (Dr. Rebecca Marsh) and the National Institutes of Health (Dr. Helen Su and Dr. Michael Lenardo) for facilitating normal donor and patient blood samples. This work was supported by startup grants from the XLP Research Trust (United Kingdom) and Uniformed Services University.

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