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SAP Facilitates Recruitment and Activation of LCK at NTB-A Receptors during Restimulation-Induced Cell Death

Gil Katz,* Scott M. Krummey,† Sasha E. Larsen,* Jeffrey R. Stinson,* and Andrew L. Snow*

Upon TCR restimulation, activated, cycling T cells can undergo a self-regulatory form of apoptosis known as restimulation-induced cell death (RICD). We previously demonstrated that RICD is impaired in T cells from patients with X-linked lymphoproliferative disease, which lack SLAM-associated protein (SAP) expression. Both SAP and the specific SLAM receptor NK, T, and B cell Ag (NTB-A) are required for RICD, but the mechanism by which these molecules promote a strong, proapoptotic signal through the TCR remains unclear. In this article, we show that the Src-family kinase LCK, but not FYN, associates with NTB-A in activated human T cells. This association increased after TCR restimulation in a SAP-dependent manner, requiring both immunoreceptor tyrosine-based switch motifs in the NTB-A cytoplasmic tail. Both NTB-A–associated LCK phosphorylation and kinase activity were enhanced in restimulated T cells, amplifying proximal TCR signaling. In contrast, TCR-induced LCK association with NTB-A, as well as phosphorylation and kinase activity, was reduced in T cells from patients with X-linked lymphoproliferative disease or normal T cells transfected with SAP-specific small interfering RNA, consistent with RICD resistance. Collectively, our data reveal how SAP nucleates a previously unknown signaling complex involving NTB-A and LCK to potentiate RICD of activated human T cells. The Journal of Immunology, 2014, 192: 4202–4209.

During an immune response, Ag-specific T cells undergo extensive clonal expansion that gives rise to effector and memory cells (1). This proliferative phase must be tightly regulated to prevent excessive T cell accumulation and immunopathological damage to self-tissues (2). One mechanism that maintains T cell homeostasis in this context is restimulation-induced cell death (RICD). RICD is an autoregulatory form of apoptosis that constrains the size of the effector T cell pool at the peak of the immune response (3). Following TCR re-engagement in the presence of IL-2, activated cycling T cells upregulate certain proapoptotic molecules, such as FAS ligand (FASL) and BIM, which participate in apoptosis induction. However, the biochemical mechanism that converts TCR signaling into a proapoptotic signal for activated T cells remains poorly understood.

Recently, the clinical significance of this process was underscored in patients with X-linked lymphoproliferative disease (XLP) (4–6). Most cases of XLP are linked to mutations or deletions in SH2D1A, which encodes the signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) (7). XLP is characterized by reduced Ab responses, dysregulated cytokine production, and impaired T cell and NK cell cytotoxicity (7). Despite these deficiencies, XLP patients often present with lymphadenopathy and transitory bouts of excess T cell expansion, concomitant with viral infections. In particular, infection with EBV can lead to a serious, life-threatening form of fulminant infectious mononucleosis driven by massive CD8⁺ T cell expansion and organ infiltration (7, 8). We previously found that RICD was specifically impaired in the T cells of XLP patients, suggesting this apoptosis defect contributes to excessive accumulation of activated T cells. Both SAP and the SLAM family receptor NK, T, and B cell Ag (NTB-A) are required for optimal RICD of normal human T cells (4).

RICD requires a quantitatively strong TCR signal to commit T cells to apoptosis (3). Indeed, the inability of SAP-deficient T cells to undergo RICD is linked to weaker TCR signaling upon restimulation, resulting in impaired upregulation of FASL and BIM. SAP is a small SH2 domain-containing adapter protein used by SLAM receptors (SLAM-Rs) for signal transduction in hematopoietic cells (8). Most SLAM-Rs, including NTB-A, interact homophilically, triggering phosphorylation of immunoreceptor tyrosine-based switch motifs (ITSMs) in their cytoplasmic tails that serve as docking sites for SAP. SAP can facilitate SLAM-R signaling both by displacing modulatory phosphatases like SH2 domain–containing protein tyrosine phosphatase (SHP)-1, and by recruitment and activation of Src-family kinases that convey a positive signal, most notably FYN (9). In activated human T cells, our previous work suggested that SAP dislodges the phosphatase SHP-1 from NTB-A shortly after restimulation, removing a negative regulatory checkpoint for TCR signaling. However, reducing SHP-1 expression in the T cells of XLP patients did not fully rescue RICD, suggesting SAP also transmits a positive signal through NTB-A that potentiates RICD (4).

In this study, we investigated the biochemical link between NTB-A, SAP, and strong TCR signaling during RICD by characterizing endogenous NTB-A–associated protein complexes before and after TCR restimulation in activated, primary human T cells. Contrary to the established paradigm of SLAM-R signaling, we...

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Abbreviations used in this article: FASL, FAS ligand; ITSM, immunoreceptor tyrosine-based switch motif; KD, knockdown; LCK, LCK inhibitor I; NP-40, Nonidet P-40; NS, nonspecific; NS KD, (T cell) transfected with nonspecific small interfering RNA; NTB-A, T cell transfected with SAP-specific small interfering RNA; SH2D1A, small interfering RNA; SLAM, signaling lymphocyte activation molecule; SLAM-R, SLAM receptor; WCL, whole-cell lysate; WT, wild-type; XLP, X-linked lymphoproliferative disease.
confirmed that FYN is not associated with NTB-A in activated T cells, and does not influence RICD sensitivity. Instead, we found that the Src-family kinase LCK associates with NTB-A. Upon TCR restimulation, both LCK association with NTB-A and its phosphorylation were increased in a SAP-dependent manner. Moreover, we show that SAP enhanced NTB-A–associated LCK kinase activity and CD3ζ phosphorylation upon TCR restimulation, suggesting a link between this novel NTB-A-SAP-LCK signaling complex and strong proximal TCR signaling required for RICD.

**Materials and Methods**

**Cell culture**

Patients were enrolled and blood samples were obtained with informed consent under protocols approved by the Institutional Review Boards of Cincinnati Children’s Hospital and the National Institutes of Health. Anonymous normal donor blood was kindly provided by Dr. Michael Lenardo and the National Institutes of Health Blood Bank. Activated T cells were derived and cultured as previously described (4). All subsequent assays were conducted with activated T cells cultured in RPMI 1640 + 10% FCS + 1% Pen/strep + 100 U/ml IL-2 (PeproTech) for ≥ 7 d. The NTB-A–negative T cell line PEER (10) was a generous gift from Dr. Arthur Weiss at University of California, San Francisco.

**Apoptosis assays and flow cytometry**

To test RICD sensitivity, activated human T cells were transfected in triplicate with anti-CD3ε mAb OKT3 (1–500 ng/ml; Ortho Biotech), as previously described (4). For some assays, cells were pretreated for 30 min with 0.2 μM LCK inhibitor I or SU6656 (EMD Millipore) versus DMSO solvent control. At 24 h after TCR restimulation, cells were stained with propidium iodide (Sigma-Aldrich) and collected on constant time (30 s per sample) on an Accuri C6 flow cytometer (BD Biosciences). Cell death was quantified as percentage cell loss = (1 – [number of viable cells (treated) / number of viable cells (untreated)]) × 100. For surface staining, cells were stained with PE-conjugated Abs against NTB-A (R&D), CD3ε, or an isotype control Ab (BD Biosciences).

**RNAi**

Activated, IL-2–cultured human T cells were transfected with 200 pmol small interfering RNA (siRNA) oligonucleotides per 1 × 10⁷ cells, using the Amaxa 4D Nucleofector (Lonza). siRNAs specific for each target protein or a nonspecific negative control were purchased from Life Technologies. All subsequent assays were performed 4 d after transfection.

**NTB-A mutagenesis and transfection**

The human NTB-A open reading frame was PCR amplified using primers NTBA-F (including EcoRI site and Kozak sequence), 5′-ATTTCGCGAATTTCGGCCCGCAGAATTCATCCATCTGCTCTTACG-3′, and NTBA-R (including BamHI site), 5′-TTCTACCCATCTTCACCGACGTGCAAGGGCG-3′, and cloned into the p3XFLAG-CMV-14 expression vector (Lonza). siRNAs specific for each target protein or a nonspecific negative control were purchased from Life Technologies. All subsequent assays were performed 4 d after transfection.

**In vitro kinase assay**

NTB-A bead immunoprecipitates were incubated in protein kinase buffer (25 mM HEPES [pH 7.2], 3 mM MgCl₂, 0.1% NP-40, 20 μM ATP; Ref. 11) containing 100 ng recombinant GST-tagged CD3ζ (Novus Biologicals) for 30 min at 30°C. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose, blocked in 5% BSA in TBS/0.1% Tween, and probed with the following Abs: anti-SAP (rabbit), anti-FYN, anti-φospho-Src-Family (Tyρ356) (Cell Signaling Technology); anti-SAP (mouse) (eBioscience); anti-LCK, anti-CD3ζ (Santa Cruz Biotechnology); anti–NTB-A (Novus); anti-phosphotyrosine (4G10; EMD Millipore); anti-FASL (Ab3; EMD Millipore); anti-BIM (Enzo); and anti-β-actin (Sigma-Aldrich). Bound Abs were detected using HRP-conjugated secondary Abs (Southern Biotech, eBioscience) and ECL (Thermo Scientific). Spot densitometry analysis was performed using ImageJ software.

**ELISA**

Detection of soluble FASL was performed on cell supernatants from restimulated T cells using the Quantikine Human Fas Ligand/TNFsf6 Immunoassay Kit (R&D Systems) according to product protocol. ELISA plates were read using a Synergy H1 Hybrid Reader (BioTek), and concentrations of soluble FASL were calculated in picograms per milliliter, using Gen5 data analysis software (BioTek).

**Results**

**RICD of human T cells is not dependent on FYN**

SAP-deficient T cells from XLP patients are significantly less sensitive to RICD, but not to other apoptotic pathways (4). Our previous work suggested that SAP, via NTB-A, boosts TCR signal strength past the threshold required to trigger RICD in activated T cells. However, the mechanism by which NTB-A and SAP enhance TCR signaling during restimulation is still unclear. Two Src-family kinases are primarily involved in initiating TCR signaling: LCK and FYN (12). FYN is the major Src-family kinase shown to mediate signal transduction of SLAM-Rs (including NTB-A) in thymocytes and NK cells (13–20). To examine the role of both FYN and LCK in RICD, we first used highly selective inhibitors for FYN (SU6656) (21) or LCK [LCK inhibitor I (LCKi)] (22). Surprisingly, inhibition of FYN did not affect RICD (Fig. 1A). However, LCK inhibition significantly reduced RICD (Fig. 1A). This reduction in RICD was consistent with decreased upregulation of BIM and FASL after TCR restimulation (Supplemental Fig. 1). To further confirm these results, we performed siRNA-mediated silencing [knockdown (KD)] of FYN or LCK in activated human T cells derived from healthy normal donors, prior to assaying RICD. SAP-specific siRNA was used as a positive control to reduce RICD sensitivity (4). As shown in Fig. 1B, siRNA KD of FYN expression did not reduce RICD, whereas siRNA KD of LCK or SAP blocked RICD. Because maximal RICD was achieved at a dose of 100 ng/ml OKT3, all subsequent RICD experiments were performed using 100 ng/ml of OKT3 as the saturating dose.

We next performed NTB-A immunoprecipitations in activated T cells from normal donors to directly test whether FYN and/or LCK interacts with NTB-A before and during TCR restimulation. As expected, we detected association of SAP with NTB-A in activated human T cells that increased during restimulation (Supplemental Fig. 2) (4). Surprisingly, no association of FYN
with NTB-A could be detected before or during TCR restimulation (Fig. 1C). In contrast, LCK was associated with NTB-A, and this association was further increased during restimulation (Fig. 1C). Moreover, we also detected a TCR-triggered increase in phosphorylation of NTB-A–associated LCK on tyrosine 394 (Y394) (Fig. 1C), which is indicative of an active conformation (12). In fact, an additional higher m.w. band for phospho-LCK was detected in NTB-A immunoprecipitates from several donors, which also increased with TCR restimulation (Fig. 1C, control 2). This band likely represents the fully active form of LCK phosphorylated at serine 59 (S59). Immunoblots of WCL input controls are shown below for comparison; β-Actin served as a loading control. Data in (A) and (B) are each representative of three independent experiments. RICD values (mean ± SD) for LCKi were significantly lower than those for SU6656 or DMSO (p ≤ 0.02) (A), and RICD values (mean ± SD) for LCK siRNA or SAP siRNA were significantly lower than those for FYN siRNA or NS siRNA (p ≤ 0.03) (B) for all OKT3 doses tested. (C) Immunoprecipitation (IP) of NTB-A from activated T cells restimulated for 0, 10, and 30 min with OKT3. NTB-A IPs were immunoblotted as above for FYN, LCK, phospho-LCK (Y394), and NTB-A. Asterisk denotes putative band for fully active LCK phosphorylated at serine 59 (S59). Immunoblots of WCL input controls are shown below for comparison; β-actin served as a loading control. Two representative control donors, Control 1 and Control 2, are shown, out of ≥14 separate control donors. (D) SAP IPs from restimulated T cells (0, 10, 30 min) were separated by SDS-PAGE and immunoblotted for LCK, FYN, NTB-A, and SAP. Asterisk denotes putative band for fully active LCK phosphorylated at S59. Input WCLs were immunoblotted for comparison; β-actin served as a loading control. Data are representative of two independent experiments using different donors.

**FIGURE 1.** RICD of human T cells is not dependent on FYN. (A) Activated human T cells were tested for RICD sensitivity using anti-CD3 mAb (OKT3), following 30 min of pretreatment with specific inhibitors for FYN (SU6656), LCK (LCKi), or DMSO as a solvent control. Cell death was measured by propidium iodide staining and flow cytometry 24 h post restimulation (B) Left panel, Activated T cells were transfected with siRNA specific for FYN, SAP, LCK, or a NS control and cultured for 4 d in IL-2. Cells were restimulated with increasing doses of OKT3, as above. Right panel, WCLs prepared from siRNA-transfected cells were separated by SDS-PAGE and immunoblotted for proteins listed at right to check KD efficiency. β-Actin served as a loading control. Data in (A) and (B) are each representative of three independent experiments. RICD values (mean ± SD) for LCKi were significantly lower than those for SU6656 or DMSO (p ≤ 0.02) (A), and RICD values (mean ± SD) for LCK siRNA or SAP siRNA were significantly lower than those for FYN siRNA or NS siRNA (p ≤ 0.03) (B) for all OKT3 doses tested. (C) Immunoprecipitation (IP) of NTB-A from activated T cells restimulated for 0, 10, and 30 min with OKT3. NTB-A IPs were immunoblotted as above for FYN, LCK, phospho-LCK (Y394), and NTB-A. Asterisk denotes putative band for fully active LCK phosphorylated at serine 59 (S59). Immunoblots of WCL input controls are shown below for comparison; β-actin served as a loading control. Two representative control donors, Control 1 and Control 2, are shown, out of ≥14 separate control donors. (D) SAP IPs from restimulated T cells (0, 10, 30 min) were separated by SDS-PAGE and immunoblotted for LCK, FYN, NTB-A, and SAP. Asterisk denotes putative band for fully active LCK phosphorylated at S59. Input WCLs were immunoblotted for comparison; β-actin served as a loading control. Data are representative of two independent experiments using different donors.

Increased recruitment and Y394 phosphorylation of NTB-A–associated LCK during restimulation is SAP dependent

Because RICD is reduced in the absence of SAP (Fig. 1B and Ref. 4), we speculated that increased recruitment and phosphorylation of LCK at NTB-A receptors during restimulation is SAP dependent. We therefore compared the association and phosphorylation of LCK in NTB-A immunoprecipitates from activated T cells transfected with nonspecific (NS) siRNA (NS KD) or SAP-specific siRNA (SAP KD). In contrast to NS KD T cells, substantially less recruitment and Y394 phosphorylation of NTB-A–associated LCK were observed in SAP KD T cells during restimulation (Fig. 2A). The putative higher m.w. form of phospho-LCK (Y394 and S59) was also reduced in SAP KD T cells. Importantly, these results correlated directly with reduced RICD in SAP KD T cells (Fig. 2B). Equal expression of NTB-A and CD3ε on both NS and SAP KD T cells suggested that impaired recruitment of LCK to NTB-A and phosphorylation could not be explained by changes in receptor expression (Supplemental Fig. 3, top panel).

We next asked whether association and Y394 phosphorylation of LCK with NTB-A receptors are also compromised in SAP-deficient T cells of patients with XLP during restimulation. Indeed, we observed no increase in LCK association with NTB-A in XLP patient T cells compared with normal control T cells during restimulation (Fig. 3A). Moreover, Y394 phosphorylation of NTB-A–bound LCK, including the putative S59 form, was also reduced in XLP patient T cells (Fig. 3A). We observed a consistent trend of reduced recruitment of LCK to NTB-A and phosphorylation could not be explained by changes in receptor expression (Supplemental Fig. 3, bottom panel).
and phosphorylation of LCK at NTB-A receptors in restimulated T cells are dependent on SAP, and correlate with RICD sensitivity.

Y284 and Y308 in the cytoplasmic tail of NTB-A are critical for LCK recruitment and Y394 phosphorylation after TCR restimulation

SAP is a small 14-kDa protein largely composed of a single SH2 domain. The cytoplasmic tail of NTB-A contains three tyrosine-based motifs that regulate signal transduction: Y273 is part of a putative ITIM, whereas Y284 and Y308 are each part of an ITSM (8). Because SAP binding to NTB-A is reported to be dependent on phosphorylation of one or more of these tyrosines (19, 20), we next asked which tyrosines in the NTB-A cytoplasmic tail are important for increased SAP-dependent LCK recruitment and phosphorylation during restimulation. To address this question, we stably expressed wild-type (WT) NTB-A or single tyrosine-to-phenylalanine (Y→F) NTB-A cytoplasmic tail mutants (Y273F, Y284F, or Y308F) in the NTB-A–negative T cell line PEER (10). We also stably expressed a truncated form of NTB-A, lacking all three tyrosine-based motifs (ΔCT) (Fig. 4A). Similar expression levels of each NTB-A variant were detected in each stably selected PEER cell line (Fig. 4B). When WT NTB-A was immunoprecipitated from PEER cells before and after TCR restimulation, both

FIGURE 2. Increased recruitment and Y394 phosphorylation of NTB-A–associated LCK during TCR restimulation is SAP dependent. (A) Left panel, NTB-A IPs from restimulated T cells (0, 10, 30 min) transfected with either NS- or SAP-specific siRNA. All NTB-A IPs were loaded on the same gel and immunoblotted for LCK, p-LCK (Y394), and NTB-A. IgH, H chain of immunoprecipitation (IP) Ab. Asterisk denotes putative band for fully active LCK phosphorylated at serine 59 (S59). Immunoblots of input WCL are shown for each transfection; β-actin served as a loading control. Right panel, Fold increase in p-LCK (Y394) or LCK in restimulated T cells (10, 30 min) relative to unstimulated (0 min) was quantified by spot densitometry. Values (mean ± SEM) are normalized for total NTB-A in each IP. (B) Cells prepared in (A) were assayed for RICD sensitivity, as described in Fig. 1B (NS KD, ●; SAP KD, □). Data in (A) and (B) are representative of five and three independent experiments, respectively, from separate donors. RICD values (mean ± SD) for SAP KD (B) were significantly lower than those for NS KD (p < 0.03), with the exception of the 1 ng/ml dose of OKT3.

FIGURE 3. Decreased recruitment and phosphorylation of NTB-A–associated LCK upon restimulation of XLP patient T cells. (A) Left panel, NTB-A immunoprecipitation (IP) from restimulated T cells (0, 10, 30 min) derived from a normal control donor (Control) or an XLP patient. All NTB-A IPs were loaded on the same gel and immunoblotted for proteins listed at right. IgH, IgH of IP Ab. Asterisk denotes putative band for fully active LCK phosphorylated at serine 59 (S59). Immunoblots of input WCL are shown for each donor; β-actin served as a loading control. Right panel, Fold increase in p-LCK (Y394) or LCK in restimulated T cells (10, 30 min) relative to unstimulated (0 min) was quantified by spot densitometry. Values (mean ± SEM) are normalized for total NTB-A in each IP. (B) Cells prepared in (A) were assayed for RICD sensitivity, as described in Fig 1B (Control, ●; XLP, □). Data in (A) and (B) are representative of three independent experiments from separate normal donors and XLP patients. RICD values (mean ± SD) for XLP (B) were significantly lower than those for the normal control (p < 0.01), with the exception of the 1 ng/ml dose of OKT3.
recruitment and Y394 phosphorylation of LCK were detected, compared with unstimulated cells (Fig. 4C, WT). Of interest, the Y273F mutation resulted in significantly enhanced LCK recruitment and phosphorylation in PEER cells before and after TCR restimulation, well above levels observed for WT NTB-A (Fig. 4C, Y273F). In contrast, both Y284F and Y308F mutations independently abolished recruitment and Y394 phosphorylation of LCK to NTB-A receptors in restimulated PEER cells (Fig. 4C, Y284F and Y308F). As expected, no recruitment of LCK was detected in restimulated NTB-A ΔCT-expressing PEER cells (Fig. 4C, ΔCT). Importantly, the recruitment and phosphorylation of LCK at NTB-A receptors in restimulated PEER cells closely paralleled the binding of SAP. Although SAP association with Y273F was markedly elevated relative to WT NTB-A, SAP binding to NTB-A was substantially reduced for Y284F and Y308F, and completely abolished for ΔCT. We noted a higher m.w. form of NTB-A, likely corresponding to phosphorylated NTB-A, in immunoprecipitates from restimulated WT and Y273F NTB-A–expressing PEER cells ± TCR restimulation (with a more distinct higher m.w. band in restimulated cells; Fig. 4C). In contrast, this band was not observed for the Y284F, Y308F, and ΔCT mutants. The lower band of NTB-A noted in several lines likely represents a hypoglycosylated form of NTB-A, approximating the m.w. of α-galactoside F–treated NTB-A (∼40 kDa) (23). Taken together, our results show that both Y284 and Y308 are essential in LCK recruitment to NTB-A and Y394 phosphorylation during TCR restimulation, congruent with the amount of SAP binding to NTB-A. Our data also suggest that Y273 may modulate SAP and LCK recruitment to NTB-A, consistent with ITIM-directed signaling.

**NTB-A–associated LCK kinase activity is enhanced during restimulation and is SAP-dependent**

To directly test whether NTB-A–associated LCK kinase activity is enhanced during TCR restimulation, consistent with increased Y394 phosphorylation, we performed an in vitro kinase assay using NTB-A immunoprecipitates isolated from activated human T cells ± TCR restimulation, using recombinant CD3ξ (as a GST-fusion protein) as a known LCK substrate (12). Recombinant GST-CD3ξ was tyrosine phosphorylated in vitro when exposed to NTB-A immunoprecipitates purified shortly after TCR restimulation, reflecting enhanced kinase activity of NTB-A–bound LCK (Fig. 5A). Increased, restimulation-dependent GST-CD3ξ phosphorylation was observed in NTB-A immunoprecipitates isolated from normal control donors, including T cells transfected with NS siRNA (Fig. 5B, 5C). In contrast, little or no increase in GST-CD3ξ phosphorylation was observed upon restimulation in NTB-A immunoprecipitates from either SAP siRNA–transfected T cells (Fig. 5B) or T cells from XLP patients (Fig. 5C). These results demonstrate that NTB-A–associated LCK kinase activity is enhanced during restimulation, and this enhancement is dependent on SAP. Importantly, we also observed substantially decreased phosphorylation of CD3ξ in vivo in SAP-deficient T cells (SAP KD or XLP patient) compared with SAP-sufficient T cells (NS KD or normal control) after TCR restimulation (Fig. 6A, 6B). This in vivo reduction of CD3ξ phosphorylation in SAP-deficient T cells reflects reduced TCR signaling overall, as evidenced by decreased global tyrosine phosphorylation after TCR restimulation (Supplemental Fig. 4). In summation, these data suggest that enhanced NTB-A–associated LCK kinase activity helps to amplify proximal TCR signaling in restimulated T cells. Moreover, our results imply that the substantial decrease in both TCR signal strength and RICD sensitivity noted in XLP patient T cells is due in part to the loss of SAP-dependent association and activation of LCK at NTB-A receptors.

**Discussion**

In this article, we show that the Src-family kinase LCK, but not FYN, associates with NTB-A receptors in primary, activated human T cells, and that this association is increased in a SAP-dependent manner upon TCR restimulation. Our data also demonstrate that a SAP-dependent increase in phosphorylation of NTB-A–associated LCK (Y394 and possibly S59) occurs following TCR restimulation, consistent with the enzymatically active form of LCK. Indeed, NTB-A–associated LCK kinase ac-

**FIGURE 4.** Both Y284 and Y308 in the NTB-A cytoplasmic tail are critical for recruitment and phosphorylation of NTB-A–associated LCK after TCR restimulation. (A) Schematic representation of NTB-A cytoplasmic tail (aa 248–331) with indicated positions of mutated tyrosine (Y) residues to phenylalanine (F) residues within ITIM (Y273F) or ITSM (Y284F, Y308F) motifs. ΔCT, truncated NTB-A cytoplasmic tail lacking ITIM and ITSM motifs (aa 248–271, white line). (B) Flow cytometry profiles of WT, Y273F, Y284F, Y308F, or truncated NTB-A (ΔCT) constructs stably expressed in the NTB-A–negative T cell line PEER. PEER T cells transfected with empty vector (no NTB-A cassette) are shown for comparison. Thick line, anti–NTB-A; thin line, isotype control Ab. (C) NTB-A IPs from restimulated PEER cell lines (0 versus 30 min), expressing WT, Y273F, Y284F, Y308F, and ΔCT NTB-A. NTB-A IPs were immunoblotted for LCK, phospho-LCK (Y394), SAP, and NTB-A. IgH, IgH of IP Ab. For comparison, input WCLs from each restimulated PEER cell line were immunoblotted for SAP, LCK, and β-actin (loading control). Data in (B) and (C) are representative of three independent experiments.
activity is enhanced in restimulated T cells when SAP is present. Taken together, our findings expose a novel signaling complex incorporating NTB-A, SAP, and LCK that potentially enhances proximal TCR signaling strength to drive RICD in activated human T cells. In XLP patient T cells lacking SAP, our data suggest TCR signal strength is likely compromised without reinforcement from activated, NTB-A–associated LCK.

The fact that FYN did not play a role in RICD, and was not associated with NTB-A in restimulated human T cells, was surprising. FYN is considered the key Src-family kinase required for SLAM-R signaling, based on its SAP-dependent association with several other SLAM-Rs (13–20). Although LCK was previously detected in phosphorylation of both SLAM and CD84 (17, 24), no direct association between LCK and SLAM-R was detected to date. To our knowledge, our report represents the first association between LCK and a SLAM-R, which suggests that SAP would only serve to physically recruit more LCK to NTB-A with TCR complexes on the surface of restimulated T cells. In the latter case, it is possible that cell type–based differences in the expression of FYN, LCK, SAP, or the SLAM family receptor examined could account for differences in the associations detected. Alternatively, other cell-specific adapter proteins, such as EAT-2, could potentially regulate the interaction of a SLAM-R with either FYN or LCK via SAP in non–T cells, especially considering SAP binds to different domains of FYN versus LCK (SH3 domain versus kinase domain, respectively) (17, 25). In the latter case, it is possible that cell type–based differences in the expression of FYN, LCK, SAP, or the SLAM family receptor examined could account for differences in the associations detected. Alternatively, other cell-specific adapter proteins, such as EAT-2, could potentially regulate the interaction of a SLAM-R with either FYN or LCK via SAP in non–T cells, especially considering SAP binds to different domains of FYN versus LCK (SH3 domain versus kinase domain, respectively) (17, 25). However, EAT-2 expression is negligible in mature T cells and thus unlikely to play a role in RICD (26).

The SAP-dependent increase in NTB-A–associated LCK kinase activity during restimulation could be explained by at least three different mechanisms. First, an overall increase in recruitment of LCK to NTB-A, including a roughly equivalent proportion of active phospho-LCK, would likely lead to an increase in kinase activity as we observed in bulk NTB-A immunoprecipitates following restimulation. Second, LCK is also known to be activated by aggregation (12). We showed previously that NTB-A colocalizes with CD3 clusters on activated T cells after restimulation (4). Therefore, the aggregation of NTB-A receptors and clustering with TCR complexes on the surface of restimulated T cells may drive proximity-induced activation of LCK. In both scenarios, SAP would only serve to physically recruit more LCK to NTB-A receptors regardless of its activation state. In contrast, the third possibility posits that SAP can directly enhance LCK activity, enhancing NTB-A–associated LCK kinase activity following TCR restimulation is SAP dependent. (A) NTB-A immunoprecipitations (IPs) from restimulated T cells (0, 10, 30 min) were incubated for 30 min with recombiant GST-CD3ζ fusion protein as a substrate for LCK. Proteins were separated on a single SDS-PAGE gel, and in vitro tyrosine phosphorylation of GST-CD3ζ (p-GST-CD3ζ) was detected by immunoblotting, compared with total GST-CD3ζ and NTB-A. Input WCL derived from the same restimulated T cells were immunoblotted for NTB-A and β-actin. Data are representative of at least three independent experiments. (B) Left panel, NTB-A IPs from restimulated T cells (0, 10, 30 min) transfected with either NS- or SAP-specific siRNA were tested for in vitro LCK kinase activity, as described in (A). Samples were separated on a single SDS-PAGE gel, and in vitro tyrosine phosphorylation of the GST-CD3ζ substrate (p-GST-CD3ζ) was detected by immunoblotting, compared with total GST-CD3ζ and NTB-A. Input WCLs derived from the same restimulated T cells were immunoblotted for NTB-A, SAP, and β-actin. Right panel, Spot densitometry quantification (mean ± SD) of p-GST-CD3ζ in restimulated T cells (10, 30 min) relative to unstimulated (0 min) for NS (●) versus SAP (○) siRNA transfectants, normalized for total NTB-A in each IP. (C) Left panel, NTB-A IPs from restimulated normal donor (Control) versus XLP patient T cells were assayed for LCK kinase activity in vitro and immunoblotted as in (B). Right panel, Spot densitometry quantification (mean ± SD) of p-GST-CD3ζ (10, 30 min) relative to unstimulated (0 min) for normal control (●) versus XLP patient (○) T cells, normalized to total NTB-A for each IP. Data in (B) and (C) are representative of two independent experiments using separate donor.
two independent experiments using separate donors. CD3 and lysed as above. Lysates were immunoblotted for phospho-CD3 T cells from a normal donor (Control) or an XLP patient were restimulated min) and lysed. Lysates were loaded on a single SDS-PAGE gel and with NS or SAP-specific siRNA were restimulated with OKT3 (0, 10, 30

z

ylation of the CD3 chain. Hence NTB-A signaling for RICD in

A

B

FIGURE 6. Restimulation-induced tyrosine phosphorylation of CD3ζ is reduced in vivo in SAP-deficient T cells. (A) Activated T cells transfected with NS or SAP-specific siRNA were restimulated with OKT3 (0, 10, 30 min) and lysed. Lysates were loaded on a single SDS-PAGE gel and immunoblotted for phospho-CD3ζ, CD3ζ, SAP, and β-actin. (B) Activated T cells from a normal donor (Control) or an XLP patient were restimulated and lysed as above. Lysates were immunoblotted for phospho-CD3ζ, CD3ζ, SAP, and β-actin. Data in (A) and (B) are representative of at least two independent experiments using separate donors.

much like binding of SAP to FYN has been shown to enhance FYN kinase activity in vitro (17). SAP may aid in inducing or stabilizing the active conformation of LCK upon association with NTB-A, which may have a greater affinity for the NTB-A/SAP docking interface. This model is consistent with our detection of some NTB-A–associated LCK with minimal enzymatic activity in SAP-deficient T cells. For each possibility, SAP-dependent LCK recruitment and activation may be further heightened by the concomitant loss or inactivation of modulatory phosphatases such as SHP-1 that can also dock with NTB-A.

Enhanced NTB-A–associated LCK kinase activity during T cell restimulation provides a plausible link between a SAP/NTB-A-dependent augmentation of TCR signaling strength and RICD sensitivity. Because NTB-A colocalizes with the TCR in restimulated T cells (4), a SAP-dependent increase in NTB-A–associated LCK kinase activity likely amplifies proximal TCR signaling. This NTB-A–SAP–LCK association (8). Our data suggest that both ITSMs assist in the SAP-dependent recruitment and/or activation of LCK at NTB-A because little or no binding of SAP to Y284F or Y308F was observed in restimulated PEER T cells. Our results agree with previous work that showed NTB-A–mediated cytotoxicity was significantly impaired in an NK cell line expressing Y284F or Y308F NTB-A, compared with WT or Y273F NTB-A (25). Of note, we also observed increased recruitment and phosphorylation of LCK with Y273F NTB-A, before and more prominently after TCR restimulation, consistent with increased SAP binding. Y273 in NTB-A is found within a classic ITIM motif, which is typically associated with recruitment of proteins that downmodulate lymphocyte signaling, such as SHP-1, SHP-2, or SHIP (28). Enhanced SAP and phospho-LCK recruitment to this mutant may therefore depend on excluding such negative regulators from NTB-A that either sterically interfere with SAP binding or directly dephosphorylate LCK or NTB-A ITSM tyrosine motifs required for SAP docking. This mechanism remains unclear, given that SHP-1 is very difficult to detect in NTB-A immunoprecipitates. Our previous work suggested that SHP-1 contributes in part to RICD resistance in XLP patient T cells, perhaps through a more sustained interaction with NTB-A (4). Similarly, enhanced SHP-1 recruitment and decreased Src-family kinase activity is observed in Ly108-containing cytotoxic immune synapses formed between target B cells and SAP-deficient murine T cells, resulting in poor adhesion and target cell killing (29). Collectively, these data agree with current models placing SAP as a molecular “switch” for governing both activating and inhibitory signals through SLAM receptors (9). Thus, in addition to augmenting TCR signaling for RICD by enhancing recruitment and/or activation of LCK at NTB-A receptors (positive signaling), SAP interferes with the association and/or activity of phosphatases like SHP-1 at NTB-A receptors following TCR restimulation (preventing negative signaling).

In conclusion, our data demonstrate that SAP nucleates a previously unknown endogenous signaling complex involving NTB-A and LCK to potentiate RICD of activated human T cells by amplifying proximal TCR signaling. This NTB-A–SAP–LCK axis for TCR signal amplification is significant because reduced NTB-A–associated LCK activity correlates with reduced proximal TCR signaling and impaired RICD in the T cells of XLP patients. Devising pharmacological strategies for specifically targeting this signaling complex in activated T cells may prove clinically useful for regulating T cell expansion by manipulating RICD sensitivity.

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Disclosures
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References


