LCK Is an Important Mediator of B-Cell Receptor Signaling in Chronic Lymphocytic Leukemia Cells

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Abstract

B-cell receptor (BCR) signals promote survival of chronic lymphocytic leukemia (CLL) cells, and it is believed that overexpressed and constitutively active Lyn mediates this signaling. Here, we show that CLL cells express lymphocyte-specific protein tyrosine kinase (LCK) and that inhibition of this Src family tyrosine kinase with the specific inhibitor [4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane (Lck-i)], or reduction of its expression with siRNA, blocks the induction of CD79a, Syk, inhibitor of IκB kinase (IKK), Akt, and extracellular signal-regulated kinase (ERK) phosphorylation by BCR cross-linking in these cells. Furthermore, we show that CLL cells with high levels of LCK expression have higher levels of BCR-mediated Akt, and extracellular signal-regulated kinase (ERK) phosphorylation as well as cell survival than CLL cells with low levels of LCK expression. We also show that treatment of CLL cells with Lck-i inhibits BCR cross-linking–induced cell survival. Taken together, these data show a major role for LCK in proximal and distal BCR-mediated signaling in CLL cells and suggest that LCK expression is important in the pathogenesis of this disease. On a clinical level, these studies advocate the use of specific LCK inhibitors in the treatment of progressive CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is a malignancy of mature B cells that accounts for more than 30% of all leukemias within the United Kingdom and other Western countries (1). This incurable disease is associated with considerable morbidity and mortality due to the suppression of immune function and hematopoiesis that results from expansion of CLL cells within lymphoid organs and bone marrow (2). It is widely accepted that signals generated by engagement of the B-cell receptor (BCR) play an important role in the pathogenesis of CLL (3). This is because the structure of the BCR in CLL cells from different patients can show a very high degree of similarity, indicating that antigens of a similar nature drive development of the disease (4, 5). Such similarity of BCR structure may be particularly important for CLL cases with progressive disease where the leukemic cells bear unmethylated immunoglobulin heavy chain variable (IgHV) genes [unmutated-CLL (UM-CLL)]. In these cases, the expressed BCR is poly-reactive and binds to a variety of self and foreign antigens (6). Work by Krysov and colleagues (7) has shown that the BCR expressed on CLL cells, particularly from patients with UM-CLL, shows features that are associated with continuous in vivo exposure to antigen, whereas others have shown that such continuous BCR-stimulation is reflected in the pattern of gene expression observed in freshly isolated cells (8). Taken together, these observations suggest that targeting this continuous BCR signaling may show therapeutic benefit in patients with CLL with progressive disease, and new drugs that inhibit particular elements within the BCR signaling pathway such as phosphoinositide 3-kinase 6 (PI3Kδ), spleen tyrosine kinase (Syk), and Bruton’s tyrosine kinase (BTK) seem to show promise (9–11). However, the concentrations of these compounds needed to observe an effect in treated cells are disproportionate to those needed to inhibit the specific kinases they target. Moreover, kinases such as BTK and Syk have other signaling roles in B cells, so it remains unclear whether the therapeutic benefit of these new drugs is due to inhibition of BCR signaling. Therefore, a clearer understanding of the mechanism of BCR signal transduction in CLL cells is needed to gain insight into how these reagents are working.

Induction of BCR signaling in CLL cells is thought to follow a model where the Src-family kinase (SFK) Lyn functions to phosphorylate the immunomodulatory tyrosine activation motifs (ITAM) within CD79a and b following receptor interaction with antigen. This event results in the formation of a “signalosome” consisting of Syk, B cell linker protein (BLNK), BTK, phospholipase C γ2 (PLCγ2), CD19, and PI3K. Activation of this signalosome then leads to induction of calcium signaling, as well as of Akt, NF-κB, and mitogen-activated protein kinase (MAPK) pathway...
activation (12). Lyn and Syk are thought to be key mediators of BCR signaling in CLL cells because both these kinases are overexpressed, and both have been shown to have cytoprotective roles (13, 14) in studies using SFK inhibitors such as dasatinib (15) as well as Syk inhibitors such as fostamatinib disodium (16). However, the mechanism of BCR signaling in CLL cells is incompletely understood because additional factors not normally involved in BCR signaling are also present.

For example, CLL cells can express proteins typically involved in TCR signaling, and, in this respect, ZAP70 is expressed. Moreover, considering the importance of BCR signaling in CLL cells because both these kinases are overexpressed, and both have been shown to have cytoprotective roles (13, 14) in studies using SFK inhibitors such as dasatinib (15) as well as Syk inhibitors such as fostamatinib disodium (16). However, the mechanism of BCR signaling in CLL cells is incompletely understood because additional factors not normally involved in BCR signaling are also present.

For example, CLL cells can express proteins typically involved in TCR signaling, and, in this respect, ZAP70 expression is correlated with unmutated IgH genes and bad patient prognosis (16–18). Working investigating the role of ZAP70 in CLL cells has shown that BCR signaling is enhanced in cells expressing high levels of this kinase (19, 20), partially explaining why ZAP70 expression is associated with poor prognosis. Another TCR signaling protein that may have a role in CLL cells is the SFK LCK. This protein is a primary mediator of TCR signaling (21), and targets ZAP70 as one of its principal substrates. However, LCK is reported also to be present in a subset of B cells known as B-1 cells (22–24) where evidence for its function is limited and controversial; one study has reported that the presence of LCK potentiates BCR signaling (24), whereas another has indicated that LCK suppresses BCR signaling (23). In CLL cells, LCK expression was first reported in a study by Majolini and colleagues (22), and subsequent work confirming this observation has further shown that the levels of this SFK vary between individual patients (25, 26). Does LCK play a role in CLL cells? There is no clear answer to this question; one study has suggested that the presence of LCK in CLL cells imparts resistance to glucocorticoids (27), whereas another suggests that LCK has no cytoprotective effect at all (25).

The present study addresses the role of LCK in CLL cells. We hypothesized that LCK in CLL cells has an important function in mediating BCR signaling based on its role in antigen receptor signaling in T cells and, possibly, in B-1 cells. Our experiments confirm this hypothesis and show that during BCR engagement on CLL cells LCK functions to participate in the induction of CD79a phosphorylation, and to induce activation of the Akt, NF-kB, and MAPK pathways resulting in enhanced survival of stimulated CLL cell clones. This work, therefore, supports a role for LCK in potentiating BCR signaling in the subset(s) of B cells where it is expressed. Moreover, considering the importance of BCR signals to CLL pathogenesis, our observations also strongly suggest that LCK is a key contributor to this process and that it might be a therapeutic target for the treatment of this disease.

Materials and Methods

**Materials**

Anti-inhibitor of IκB kinase (IKK)β, anti-LCK, anti-Lyn, anti-p122, and anti-p130–ERK (active extracellular signal–regulated kinase (ERK)), anti-ERK, mouse anti-Syk, rabbit anti-ZAP70, mouse anti-pY193-ZAP70 (active ZAP70), and horseradish peroxidase–conjugated goat anti-mouse and goat anti-rabbit antibodies were from Santa Cruz Biotechnology. Anti-pS77–Akt (active Akt), anti-Akt, anti-pY185–CD79A, anti-CD79a, rabbit anti-pZAP70 (tyrosine 319)/Syk (tyrosine 352), anti-pSrc (tyrosine 416), and anti-pS192–IKKα/β antibodies were purchased from Cell Signaling Technology (New England Biolabs). Monoper-oxo(picolinate)oxovanadate (mpVpic), 4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane (Lck-i), 3,3′-dihexyloxacarbocyanine iodide (DiOC6) and U0126 were from Calbiochem (Merck Chemicals). LCK and control siRNA were from Dharmacon and from Life Technologies (Invitrogen). Propidium iodide (PI) and anti–β-actin were from Sigma-Aldrich. Anti-human immunoglobulin M (IgM) was from Jackson ImmunoResearch. Mouse anti-ZAP70 was from Millipore. MEC1 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

**Patient samples**

CLL cells were obtained from the peripheral blood taken from patients with informed consent and with the approval of the Liverpool Research Ethics Committee. All CLL cell samples used for this study had been cryopreserved and stored within the Liverpool Leukaemia Biobank. When required, cells were thawed, resuspended in culture media, and equilibrated as described previously (28). All cases used in this study were chosen at random and had a minimum viability of 80%. CLL samples with less than 95% CLL cells were purifled by negative selection to greater than 95% using a MACS system (Miltenyi Biotec).

**Cell culture and BCR cross-linking**

Unless specified, suspensions of 1 × 10⁷ cells/mL were cultured under 5% CO₂ at 37°C. The culture medium used was RPMI-1640 [supplemented with 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.5% bovine serum albumin (BSA)]. When required, these cell suspensions were treated with Lck-i (1 µmol/L), U0126 (10 µmol/L), or with dasatinib (150 nmol/L) for 2 hours. BCR stimulation was conducted using 20 µg/mL of F(ab′)₂ goat anti-human IgM for 15 minutes. Cell suspensions were then harvested, washed with ice-cold PBS, and then lysed in SDS lysis buffer.

**Western blotting**

The procedures used here are described in Abrams and colleagues (29). Briefly, whole-cell lysates were prepared by lysing CLL cell pellets with SDS-PAGE sample buffer, sonicating the lysate to shear DNA followed by incubation at 95°C for 5 minutes. Proteins within whole-cell lysates or within immunoprecipitates were separated by SDS-PAGE and then electroblotted on to Immobilon membranes. These membranes were then incubated with primary and secondary antibodies followed by visualization using enhanced chemiluminescence as we have previously described (29). Lck expression in CLL cells was quantitated using a modified...
Immunoprecipitation and in vitro kinase assays

A total of $1 \times 10^7$ CLL cells were lysed in either lysis buffer A or lysis buffer B (50 mmol/L Tris, pH 7.4, 10% glycerol, 150 mmol/L NaCl, 50 mmol/L sodium fluoride, 25 mmol/L sodium pyrophosphate, 50 mmol/L sodium glycerophosphate, 2 mmol/L EDTA, and 2 mmol/L EGTA) with the addition of 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (buffer A), or 0.5% Igepal (buffer B). Lysates were centrifuged at 16,000 $\times$ g for 30 minutes, and the supernatant was incubated with 2 $\mu$g of rabbit anti-p85c(y9146), mouse anti-Syk, or rabbit anti-ZAP70 antibodies. Antibody-lysate mixtures were incubated overnight at 4°C, to be followed by 1 hour with Protein G-agarose (GE Healthcare) at 4°C. Immunocomplexes were then washed with lysis buffer A ready for SDS-PAGE and Western blotting, or with lysis buffer B for in vitro kinase assays. Kinase assays were conducted according to the method described in ref. (30) and used 2 $\mu$g of recombinant Csk-binding protein (Enzo Life Sciences UK Ltd.) as a substrate for immunoprecipitated Lyn and Lck.

Nucleofection

Nucleofections were carried out according to established protocol (31). Briefly, $1 \times 10^7$ CLL cells were transfected using the Amaxa solution V transfection kit according to the manufacturer’s instructions (Lonza Biologics plc). CLL cell suspensions were briefly incubated with no siRNA, or with 2 $\mu$mol/L of either control siRNA or siRNA-targeting LCK before using and program U13 to initiate transfection. The CLL cells were then cultured on poly-2-hydroxyethyl methacrylate (polyHEMA)–coated tissue culture plates for 48 hours. After this time, transfected CLL cells were harvested and divided into unstimulated and BCR stimulated cells. BCR stimulation was achieved by incubating CLL cells with 20 $\mu$g/mL F(ab')$_2$ anti-IgM for 15 minutes. Nucleofection of MEC1 cells (2 $\times$ 10$^6$) was achieved using Amaxa solution V and program X-01.

CLL cell viability assay

CLL cells, at $5 \times 10^6$ cells/mL, were cultured for 3 days with RPMI-1640 plus 0.5% BSA supplemented with $\gamma$-glutamine and streptomycin in 24-well culture plates (Falcon, BD Biosciences) coated with polyHEMA. Cell viability was assessed by flow cytometry using DiOC6 to measure mitochondrial integrity and PI incorporation (as a measure of dead cells) according to established protocol (29). Viable cell count was determined by taking an aliquot of cell suspension and measuring the number of DiOC6$^+$/PI$^-$ events (live cells) counted during a fixed time setting of 30 seconds where the flow cytometer (FACSCalibur) was set to medium flow. In some experiments, CLL cells were incubated with 1 $\mu$mol/L Lck-i for 2 hours before cross-linking the BCR with 20 $\mu$g/mL F(ab')$_2$, fragments of goat anti-human IgM antibody to promote CLL cell survival.

Statistical analysis

Data were sorted with Microsoft Excel and compared for statistical significance using paired Student $t$ test. Mann–Whitney $U$ tests were conducted with the assistance of PASW Statistics version 17 software.

Results

Lck expression levels in CLL cells govern BCR signal strength

CLL cells from different patients express varying levels of LCK (22, 25, 26), however, the functional significance of this expression has not been fully clarified. In T cells, LCK functions to mediate antigen receptor signaling (32). Although LCK is expressed in B cells, a potential role in BCR signaling has also been proposed (23, 24). Thus, it is probable that LCK in CLL cells has an important function in mediating BCR signaling. If this is the case, it may be that BCR-induced signaling may vary according to the level of endogenous LCK expression in CLL cells from different patients. To investigate this possibility, we compared BCR-induced phosphorylation/activation of IKK, ERK, and Akt in CLL cells containing high and low levels of LCK.

Figure 1A confirms the data of previous studies and shows using purified CLL cells that Lyn is expressed at relatively equal levels in the malignant cells from different patients, whereas LCK expression can vary quite considerably. We quantitated the levels of LCK expression in 39 cases of CLL and show a range of expression levels from 19.5 to 235 $pg$ LCK/$\mu g$ total cellular protein (Table 1). When we examined the effects of BCR cross-linking on CLL cells expressing very low ($<75 pg$ Lck/$\mu g$ total cellular protein) or very high (>150 pg Lck/$\mu g$ total cellular protein) levels of LCK, we found that stronger induction of ERK, Akt, and IKK phosphorylation was associated with the cells having high levels of LCK (Fig. 1B–E). Moreover, we also examined a functional relationship between LCK expression and BCR-induced CLL-cell survival. We found that the prosurvival effect of BCR cross-linking was stronger in CLL cells bearing high levels of LCK than in cells expressing lower levels of this SFK (Fig. 1F). Importantly, the cases used for comparison in Fig. 1F express similar levels of ZAP70 (Fig. 1G). Finally, we quantitated ZAP70 levels in some CLL cases using Western blot analysis of whole-cell lysates and determining the band density ratio of ZAP70 and $\beta$-actin (Table 1). Although this method showed that high ZAP70 expression was associated with
levels of LCK. Cells were incubated for 1 day following addition of 20 nM Lck-i and determined that BCR-induced IKK, ERK, and Akt phosphorylation in CLL cells was maximally inhibited by a concentration of 1 µmol/L of this compound (Fig. 2A–C). Interestingly, we also found that IKK phosphorylation stimulated by BCR engagement on CLL cells seemed more sensitive to the effects of Lck-i inhibition than were the induction of ERK and Akt.

Within the paradigm of BCR signaling, Lyn has a proximal role in catalyzing the phosphorylation of the ITAMs within CD79a and b and initiating the signaling cascade (3). Therefore, it is possible that Lck-i mediates its effects by inhibiting this SFK during BCR cross-linking. To investigate this possibility, we examined Lyn activity in CLL cells using a Western blot analysis approach where whole-cell lysates or immunoprecipitated Lyn was probed using an anti-pY512-Src antibody, which cross reacts with the pY396 of active Lyn (36), or by using an in vitro kinase assay to directly measure immunoprecipitated Lyn activity. Figure 2D and E
shows, as expected (13), that Lyn is constitutively active in CLL cells when observed either by Western blot analysis or by kinase assay. Although treatment of CLL cells with Lck-i did not affect Lyn activity, treatment with dasatinib, a pan-SFK inhibitor, resulted in a marked reduction in Lyn activity in both our assay systems. We next examined the effect of Lck-i on LCK activation itself. Here, we used the anti-pY^{416}-Src antibody to immunoprecipitate active SFKs from CLL cell lysates and then probed Western blot analyses of these immunoprecipitates for the presence of LCK (Fig. 2F). This approach was used in preference to directly immunoprecipitating LCK and examining it for activity (as we did for Lyn).
for technical reasons; immunoprecipitation of LCK from CLL cells lysed with mild detergent buffers to lead to coimmunoprecipitation of other SFKs that interfered with assays measuring kinase activity (data not shown), and the use of strong detergent buffers to lyse CLL cells either inhibited our ability to immunoprecipitate LCK or interfered with assays measuring kinase activity. Because anti-pY416-Src antibody also cross reacts with pY394, the phosphorylation of which depends on LCK activity (32), immunoprecipitated LCK will be in an active conformation. Figure 2F shows that detectable levels of active Lck were present in resting CLL cells, and that incubation of CLL cells with the tyrosine phosphatase inhibitor mpVpic markedly increased these levels. However, although BCR cross-linking of CLL cells resulted in a change in molecular weight of LCK consistent with ERK-mediated serine phosphorylation, the effect was not achieved following BCR cross-linking and is represented by the graph. Each point represents mean ± SE of n = 3 experiments using different cases of CLL. D and E, effect of Lck-i on Lyn activity in CLL cells. CLL cells were incubated for 2 hours in the presence of 1 μmol/L Lck-i or 150 nmol/L dasatinib before harvest. D, Lyn immunoprecipitated with anti-pY416-Src and Lyn antibodies by Western blot analysis. E, in vitro kinase assays were conducted on immunoprecipitated Lyn using recombinant Cbp as a substrate (left). The extent of Lyn activation is represented by graphical representation of n = 3 cases (mean ± SE; right). F, effect of Lck-i on LCK activity in CLL cells. CLL cells were stimulated by incubating with 100 μmol/L, mpVpic for 30 minutes or by BCR cross-linking. LCK activity was inhibited in stimulated CLL cells by pretreating with 1 μmol/L Lck-i for 2 hours. Analysis of LCK activity was conducted by immunoprecipitating CLL cell lysates with anti-pY416-Src antibody and then analyzing for the presence of LCK in Western blot analyses. The presented experiment is representative of n = 3 using cells from different donors. G, active ERK mediates the appearance of the slower migrating LCK band. Western blot analysis of whole CLL cell lysates for LCK and β-actin (top blots) and phospho-ERK and ERK (bottom blots). CLL cells were treated with 1 μmol/L Lck-i or 10 μmol/L U0126 for 2 hours before BCR cross-linking. Lysate control, whole-cell lysate precipitated only with protein G sepharose; antibody (Ab) control, anti-pY416-Src antibody precipitated from lysis buffer; BCR-XL/CLL, BCR stimulation; UT, untreated cells.

Figure 2. Lck-i affects LCK but not Lyn activity in CLL cells. A to C, concentration-dependent inhibitory effect of Lck-i on BCR-induced IKK, ERK, and Akt activation in CLL cells. CLL cells were incubated with the indicated amounts of Lck-i for 2 hours before BCR cross-linking with 20 μg/mL F(ab’2) goat anti-human IgM for 15 minutes. CLL cell lysates were analyzed for the presence of active IKK (A), ERK (B), and Akt (C) by Western blot analysis (see inset figures) using pS505/516-IKKα/β, pT202/pY204-ERK, and pS473/Akt antibodies, respectively. Induction of phosphorylation was quantitated relative to the maximal phosphorylation observed following BCR cross-linking and is represented by the graph. Each point represents mean ± SE of n = 3 experiments using different cases of CLL. D, immunoprecipitated Lyn and whole CLL cell lysates were probed with anti-pY416-Src and Lyn antibodies by Western blot analysis. E, in vitro kinase assays were conducted on immunoprecipitated Lyn using recombinant Cbp as a substrate (left). The extent of Lyn activation is represented by graphical representation of n = 3 cases (mean ± SE; right). Treatment of CLL cells with dasatinib significantly (P < 0.05) reduced the level of Lyn activation in CLL cells. F, effect of Lck-i on LCK activity in CLL cells. CLL cells were stimulated by incubating with 100 μmol/L, mpVpic for 30 minutes or by BCR cross-linking. LCK activity was inhibited in stimulated CLL cells by pretreating with 1 μmol/L Lck-i for 2 hours. Analysis of LCK activity was conducted by immunoprecipitating CLL cell lysates with anti-pY416-Src antibody and then analyzing for the presence of LCK in Western blot analyses. The presented experiment is representative of n = 3 using cells from different donors. G, active ERK mediates the appearance of the slower migrating LCK band. Western blot analysis of whole CLL cell lysates for LCK and β-actin (top blots) and phospho-ERK and ERK (bottom blots). CLL cells were treated with 1 μmol/L Lck-i or 10 μmol/L U0126 for 2 hours before BCR cross-linking. Lysate control, whole-cell lysate precipitated only with protein G sepharose; antibody (Ab) control, anti-pY416-Src antibody precipitated from lysis buffer; BCR-XL/CLL, BCR stimulation; UT, untreated cells.
phosphorylation of S49 (refs. 37, 38; Fig. 2G), it did not induce any apparent increase in LCK activity. Finally, Fig. 2F also shows that the presence of 1 μmol/L Lck-i in resting, BCR-stimulated, and mpVpic-treated CLL cells effectively blocked our ability to detect active LCK in lysates of these cells. Taken together, these results show that Lck-i, when used at a concentration of 1 μmol/L, effectively inhibits Lck but not Lyn activation in CLL cells.

Examination of the effect of 1 μmol/L Lck-i on BCR-induced phosphorylation of ERK, Akt, and IKK in CLL cells showed that the presence of this compound significantly reduced BCR signaling to these pathways (Fig. 3A–C).
also examined the effect of Lck-i on the viability of unstimulated and BCR-stimulated CLL cells. Figure 3D shows that CLL cell viability decreases in cultures over time and that BCR engagement reduces this decrease. We found that while the presence of Lck-i had only a small effect on the viability of unstimulated CLL cells, its presence completely inhibited the prosurvival effects of BCR cross-linking. Thus, inhibition of LCK function blocks the ability of BCR to provide prosurvival signals to CLL cells.

siRNA-mediated reduction of LCK expression in CLL cells reduces BCR-induced IKK, ERK, and Akt signals

To confirm the specificity of Lck-i and show that LCK is an important mediator of BCR signaling in CLL cells, we next used specific siRNAs to reduce the expression of this SFK. Figure 4A shows that the level of LCK expression in CLL cells was reduced to approximately 60% (P = 0.039; n = 3) of endogenous levels following treatment with specific siRNA. This reduction in LCK expression resulted in a decline of the levels of BCR-induced ERK and IKK phosphorylation to 58.52% ± 8.03% (P = 0.029; n = 3) and 31.78% ± 6.56% (P = 0.042; n = 3), respectively, of the levels induced in control siRNA-transfected cells (Fig. 4B and C). We also observed a 14.85% ± 3.87% decrease in BCR-induced Akt phosphorylation, which, although small, was nevertheless significant (P = 0.026; n = 4; Fig. 4D). To ensure that the reduction in BCR-induced IKK and ERK phosphorylation was not due to off-target effects of the siRNA (from Dharmacon), we repeated these experiments with siRNA from Invitrogen and obtained similar results (data not shown). Moreover, we also examined the effect of the siRNA we used to target LCK on the level of Lyn expression. Here, we used MEC1 cells (a CLL cell line derived from a patient undergoing prolymphocytoid transformation; ref. 39) and show that Lck siRNA reduces the expression of LCK but has no effect on the expression of Lyn in these cells (Fig. 4E). Thus, the effects we observe in CLL cells treated with siRNA-targeting LCK are due to the specific reduction of LCK. These data, taken together with our studies using Lck-i and comparing BCR signaling between CLL cases expressing high and low levels of LCK, strongly suggest that LCK plays an important role in mediating BCR-induced signals in CLL cells.

LCK mediates proximal BCR signaling events in CLL cells

We next wished to determine whether LCK had a role in BCR-induced phosphorylation of CD79a. Figure 5A and B shows that resting CLL cells have low, but detectable levels of tyrosine phosphorylated CD79a. BCR cross-linking of CLL cells increased the level of phospho-CD79a approximately 2-fold, and prior treatment of CLL cells with 1 µmol/L Lck-i blocked this BCR-induced increase. siRNA-mediated knockdown of LCK expression also resulted in inhibition of BCR-induced CD79a phosphorylation in CLL cells, validating our results using Lck-i and confirming a role for LCK in this process (Fig. 5C and D). However, LCK is not solely responsible for CD79a phosphorylation in CLL cells. This is because BCR-induced CD79a phosphorylation was reduced to below base line levels in CLL cells that were pretreated with 150 nmol/L dasatinib (Fig. 5B and C) indicating a role for an additional SFK, possibly Lyn.

LCK mediates phosphorylation of Syk but not ZAP70 in BCR-stimulated CLL cells

In T cells one of the principal targets of active LCK following antigen receptor engagement is Y319 in ZAP70 (40). CLL cells from a subset of patients also express ZAP70, and a high level of expression of this tyrosine kinase is associated with poor disease prognosis (2). We next investigated whether ZAP70 in CLL cells is a target of LCK. The anti-pY319-ZAP70 antibody we used to explore this phenomenon has cross-reactivity with an analogous site (pY352) in Syk, so it was important to determine the actual target of active LCK in CLL cells. We immunoprecipitated Syk and ZAP70 from resting and BCR-stimulated CLL cells and found that the anti-pY319-ZAP70 antibody was only reactive with Syk immunoprecipitated from BCR-stimulated CLL cells and not with immunoprecipitated ZAP70 (Fig. 6A). Thus, when we reduced LCK expression in CLL cells with siRNA, the resulting decrease in pY319-ZAP70 antibody reactivity in the Western blot analyses of BCR-stimulated CLL cells can only be attributed to a reduction in pY352-Syk levels (Fig. 6B and C). Taken together with the additional data in Fig. 6A showing that the presence of Lck-i blocked the induction of Syk phosphorylation in BCR-stimulated CLL cells, these results show that LCK preferentially mediates the phosphorylation/activation of Syk in CLL cells responding to BCR cross-linking.

Discussion

Our knowledge of BCR signaling in CLL cells is based on current understanding of how BCR signals are initiated and propagated in normal B cells. The most recent review on BCR signaling in CLL cells supports a paradigm, whereby Lyn is responsible for mediating proximal and distal signaling events following BCR engagement on CLL cells (3). The results we present in the current study provide further insight into this paradigm. We show that LCK in BCR-stimulated CLL cells catalyzes the proximal phosphorylation of CD79a as well as the induction of distal signaling events involving phosphorylation of Syk, activation of MAPK, NF-κB, and PI3K/Akt signaling pathways and enhanced cell survival. Because BCR-generated signals are key contributors to CLL cell survival and disease pathogenesis, we propose that LCK may be a good therapeutic target for the treatment of this disease.

We use two approaches to show that LCK plays a role in mediating BCR signaling in CLL cells; siRNA and a specific inhibitor Lck-i. In the first instance, we found that siRNA treatment of CLL cells partially reduced LCK expression, and that this resulted in inhibited BCR-induced activation of IKK (59% inhibition), ERK (32% inhibition), and Akt (15% inhibition). This observation is complimented by experiments showing that the levels of induced pIKK, pERK,
**Figure 4.** siRNA knockdown of LCK expression inhibits BCR-mediated signaling in CLL cells. LCK expression in CLL cells was reduced by treating cells with specific siRNA according to the method described. Whole-cell lysates were then analyzed by Western blot analysis for the indicated antibodies. A, top, Western blot analysis of whole-cell lysates for LCK expression. Bottom, graphical representation of $n = 3$ experiments showing that LCK was reproducibly reduced by 42.81% ± 5.08% in LCK-siRNA–treated cells compared with nonspecific siRNA–treated control cells. B, analysis of BCR-induced ERK activation in CLL cells by Western blot analysis of cell lysates using pT202/pY204-ERK antibodies. BCR induction of ERK activation was reduced by 31.78% ± 6.56% ($n = 3$) in LCK-siRNA–treated cells. C, analysis of BCR-induced IKK activation in CLL cells by Western blot analysis of cell lysates using pS180/181-IKKα/β antibodies. BCR induction of IKK activation was reduced by 58.52% ± 8.03% ($n = 3$) in LCK-siRNA–treated cells. D, analysis of BCR-induced Akt activation in CLL cells by Western blot analysis of cell lysates using pS473-Akt antibodies. BCR induction of Akt activation was reduced by 14.85% ± 3.87% ($n = 4$) in LCK-siRNA–treated cells. E, Western blot analysis showing that LCK-siRNA reduces LCK but does not affect Lyn expression in transfected MEC1 cells. Graphical representations (right) show mean ± SD of LCK and Lyn expression levels in $n = 3$ experiments. For all parts of this figure, UT indicates mock-transfected CLL cells, C indicates nonspecific siRNA–treated CLL cells, and LCK indicates LCK-siRNA–treated CLL cells. X-L indicates CLL cells that have been stimulated by BCR cross-linking. Statistical analysis was conducted using a Student t test for paired data. For parts A–D repeat experiments were carried out using CLL cells from different patients with CLL.
and pAkt are higher in BCR-stimulated CLL cells expressing high levels of LCK than in BCR-stimulated CLL cells expressing low levels of LCK. Together, these observations suggest a direct relationship between the level of LCK expression and the intensity of the BCR signaling response in CLL cells. We then further investigated the role of LCK in CLL cells using Lck-i. When used at 1 μmol/L, we found that this compound effectively inhibits LCK without affecting Lyn. This finding concurs with those of other studies reporting the specificity of Lck-i (33–35), and is important because Lyn is constitutively active in CLL cells (13). Thus, treatment of CLL cells with 1 μmol/L Lck-i significantly reduces BCR-induced activation of the NF-κB, ERK, and PI3K/Akt signaling pathways. Combined with our results using LCK-siRNA, these data provide compelling evidence to support a role for LCK in BCR signaling in CLL cells. Interestingly, the selective effect of LCK knockdown by siRNA on these signaling pathways could be reproduced in concentration-response experiments using Lck-i to inhibit BCR-induced signaling in CLL cells. These observations suggest that some signaling pathways in BCR-stimulated CLL cells are more dependent on LCK than others. In particular, BCR-induced activation of the NF-κB pathway is known to involve activation of PLCγ2 and protein kinase CB (41, 42). A key mediator of PLCγ2 activation during BCR engagement is Syk (43), and our data show that the presence of either LCK-siRNA or Lck-i inhibits Syk activation in CLL cells. These data suggest that LCK may play a key role in the induction of Syk activity in CLL cells. Additional other possible roles of LCK in distal signaling events have also been described (44).

A role for LCK in antigen receptor signaling in normal B cells is controversial, and published evidence suggests that LCK can promote (24) or inhibit (23) BCR signaling in B-1a cells. Our data support a role for LCK in the promotion of BCR signals, and is the first study to show this in CLL cells. Previous reports have suggested that LCK expression may be functionally coupled with CD5 (22) and that it protects CLL cells against glucocorticoids (27). One report suggested that LCK has no direct cytoprotective role in CLL cells by showing that its knockdown does not induce apoptosis (25). Our data showing that Lck-i does not largely affect unstimulated CLL cell viability supports this latter observation. Our data show that Lck mediates BCR-induced activation of the NF-κB, ERK, and PI3K/Akt pathways, signaling pathways that are responsible for CLL cell survival following BCR cross-linking (3, 45, 46). We suggest that it is within this context that LCK has a cytoprotective role when we show that the presence of Lck-i blocks the prosurvival effects of BCR-cross-linking on CLL cells.

The current model of BCR-induced CD79 phosphorylation in CLL cells suggests that Lyn encounters and

Figure 5. BCR cross-linking on CLL cells induces LCK activation and phosphorylation of CD79. A, CLL cells were treated with Lck-i (1 μmol/L) or dasatinib (150 nmol/L) for 2 hours before BCR cross-linking, and phosphorylation of CD79a was assessed in CLL cell lysates by Western blot analysis using pY419-CD79a. B, graphical representation of the mean ± SD of pCD79 induction using the data from A with n = 6 different cases of CLL. C, CLL cells were mock transfected (UT) or treated either with control (C) or LCK-specific siRNA (Lck) for 48 hours. CLL cells were then stimulated by BCR cross-linking, and expression of LCK and induction of CD79 phosphorylation were then assessed in lysates of these cells by Western blot analysis. D, graphical representation of showing the effect of reducing LCK expression by LCK-siRNA on BCR-induced CD79a phosphorylation. LCK levels in control and LCK-siRNA-treated samples were normalized to the levels observed in mock-transfected CLL cells. The data represent mean ± SD of n = 3 experiments using different CLL cells from different patient samples. Statistical analysis for this figure was conducted using a Student t test for paired data.
phosphorylates CD79 once it enters lipid rafts following BCR cross-linking (47). However, the results of the current study show that the presence of Lck-i blocks induction of CD79 phosphorylation by BCR cross-linking without inhibiting the activity of Lyn. Because siRNA knockdown of LCK expression also inhibits BCR-induced CD79 phosphorylation, we conclude that LCK mediates this proximal signaling event in CLL cells. However, these experiments do not rule out a role for Lyn in CD79 phosphorylation. Use of the pan-SFK inhibitor dasatinib shows that the presence of this compound in CLL cell cultures reduces CD79 phosphorylation to below the baseline levels we observe in resting CLL cells. Thus, while LCK may be responsible for the induction of CD79 phosphorylation following BCR cross-linking, Lyn, or another SFK, may be responsible for basal phosphorylation of this protein. Such SFK-mediated basal phosphorylation of CD79 may be pathophysiologically important in CLL because pan-SFK inhibitors are cytotoxic to the malignant cells of this disease (13). Constitutively, active Lyn may also generate signals, which downregulate BCR signaling. Lyn is able to phosphorylate immunomodulatory tyrosine inhibitory motifs (ITIM) in proteins such as CD5 (48), and thereby attract phosphatases such as SHP1, SHP1, and PTPN22 to the cell membrane where they downregulate Lyn-mediated positive signaling events (48–51). Moreover, monophosphorylation of the ITAM within

![Figure 6](image_url)

**Figure 6.** LCK mediates Syk phosphorylation in BCR-stimulated CLL cells. A, Lck-i inhibits BCR-induced Syk phosphorylation. Lysates of resting and BCR-stimulated (X-L) CLL cells were immunoprecipitated with anti-Syk antibodies. The immunoprecipitates were then analyzed by Western blot analysis for the presence of pY352-Syk/pY319-ZAP70 using an antibody that is cross-reactive to both epitopes (anti-pY319-ZAP70 antibody from Cell Signaling Inc.). Right, a graphical representation of induced phosphorylation of immunoprecipitated Syk. The graph illustrates mean ± SD of n = 3 experiments using cells from different donors. B, lysates of resting and BCR-stimulated CLL cells were immunoprecipitated with anti-ZAP70 antibodies. The immunoprecipitates were then analyzed by Western blot analysis for the presence of pY352-Syk/pY319-ZAP70. Right, a graphical representation of induced phosphorylation of immunoprecipitated ZAP70. The graph illustrates mean ± SD of n = 3 experiments using cells from different donors. C, siRNA-knockdown of LCK expression inhibits BCR-induced Syk phosphorylation. CLL cells were treated with LCK-specific (Lck) and nonspecific (C) siRNA, or were mock-transfected (UT). CLL cells were then stimulated by BCR cross-linking (X-L). Induction of pY352-Syk in CLL cell lysates was then assessed by Western blot analysis using the anti-pY352-Syk/pY319-ZAP70 antibody. Right, a graphical representation of induced phosphorylation, and illustrates mean ± SD of n = 3 experiments using cells from different donors. pZAP70 levels in control and LCK-siRNA-treated samples were normalized to the levels observed in mock-transfected CLL cells. Statistical analysis for this figure was conducted using a Student t test for paired data. Lysate control (LC), whole-cell lysate precipitated only with protein G sepharose; antibody (Ab) control (AC), anti-Syk or anti-ZAP70 antibody precipitated from lysis buffer.

![Diagram](image_url)
CD79a, possibly mediated by Lyn, has recently been proposed to play a role in the maintenance of B-cell anergy through activation of an inhibitory signaling circuit consisting of SHIP-1 and Dok-1 (52). The net effect is a balance between positive and negative signaling. The results presented in the current study suggest that LCK acts to tip this balance in favor of positive signals when CLL cells are stimulated through the BCR.

Does LCK in CLL cells become activated in response to BCR cross-linking? Our data indicate that LCK maintains a low constitutive activity in resting CLL cells, and that this activity does not significantly change when CLL cells are subjected to BCR cross-linking. In this way, the function of LCK in CLL and T cells may be similar; a recent report has shown that approximately 40% LCK in T cells is in a constitutively active state and does not change during antigen receptor engagement (53). The role of active LCK in this context may be to determine potency and rapidity of antigen receptor signaling (53), and this may also apply to CLL cells that express high levels of LCK. Interestingly, in lysates of BCR-stimulated CLL cells we saw the appearance of a second, slower migrating band in Western blot analyses probed with LCK antibody. The appearance of this band has been observed by others (37, 38) and correlates with ERK-catalyzed phosphorylation of LCK at S592. Our experiments using the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor U0126 suggest that this event occurs in BCR-stimulated CLL cells, and may be indicative of the proportion of LCK that is in an active state because most of the active LCK we immunoprecipitate from CLL cell lysates with the anti-pY146-Src antibody seems to shift to an increased molecular weight in BCR-stimulated cells. To show that LCK is active in CLL cells, we found that we had to first immunoprecipitate active SFKs from CLL cell lysates with anti-pY416-Src antibody, and then examine these SFKs for the presence of LCK by Western blot analysis. Optimally, we would have chosen to analyze directly immunoprecipitated LCK for evidence of kinase activity, however, in our experiments we were unable to efficiently immunoprecipitate LCK from CLL cell lysates in a state that allowed for further analysis. Nevertheless, because phosphorylation of Y592 in LCK requires activity of this SFK (32), our experiments showing that immunoprecipitation of LCK with anti-pY146-Src is effectively blocked if CLL cells are first cultured in the presence of Lck-i indicates that this is a valid approach. Particularly because we can show that Lck-i does not inhibit the activity of Lyn.

One important target of active LCK in T cells following antigen receptor engagement is Y319 in ZAP70 (40). Phosphorylation at this residue acts to enhance the catalytic function of ZAP70, as well as play a scaffolding role within ZAP70 by providing a binding site for LCK and other proteins such as P3K, Grb2, and CrkII (54, 55). The malignant cells from a subset of patients with CLL also express ZAP70 (17) where it functions to enhance BCR signaling (19, 20). Thus, ZAP70 may be a target of LCK in CLL cells that express this protein. However, using a phospho-ZAP70 antibody (anti-pY319-ZAP70) that also cross reacts with phospho-Syk at an analogous site (pY352), we found that although LCK is involved in the induction of Syk phosphorylation following BCR cross-linking of CLL cells, ZAP70 remains unphosphorylated. This result agrees with a published report showing that BCR cross-linking induces Syk but not ZAP70 phosphorylation in CLL cells (19). That LCK is directly able to phosphorylate Syk on Y352 in BCR-stimulated CLL cells is not shown by our data. Although there is potential for LCK to target Syk based on the high homology of amino acid sequences flanking Y352 and Y319 in Syk and ZAP70, other SFKs could also be involved. It has been suggested that LCK is able to act as a downstream amplifier of Syk in antigen receptor-stimulated T cells by binding pY525/526 residues via its SH2 domain (56, 57), and, in this way, holds Syk in a confirmation that allows Lyn or another SFK to catalyze the phosphorylation of Y322 (57). Such a mechanism may occur in CLL cells because recent work has shown that phosphorylation of pY525/526 in Syk enhances downstream signaling (58). Alternatively, inhibition of LCK-mediated CD79 phosphorylation in BCR-stimulated CLL cells may inhibit Syk activation and Y352 phosphorylation, thereby accounting for the reduction we observe. Whatever the mechanism of LCK-mediated Syk activation may be, our results suggest that the role of ZAP70 in enhancing BCR signaling in CLL cells is independent of the functional relationship it plays with LCK in T cells. This independence is supported both by our data and those of others (25) showing that there is no correlation between LCK and ZAP70 expression in CLL cells.

In the present article, we show that LCK expression varies in the malignant cells from different patients, an observation that others have also made (22, 25–27). These results are in contrast to the study of Contri and colleagues (13) who show no LCK expression in CLL cells from 8 cases studied. On the basis of the number of cases we studied, combined with those of others, we believe that it is a general feature of CLL cells to express LCK. Therefore, any discrepancy with the results of Contri and colleagues may be due to their selection of patients where LCK expression in CLL cells is very low, and/or to technical problems because there is no positive control to show LCK antibody reactivity in what are uniformly negative Western blot analyses. We show that varying LCK levels may be pathophysiologically important because not only does LCK inhibition block the prosurvival effects of BCR cross-linking in CLL cells, but the expression levels of this SFK contribute to the strength of prosurvival signals generated by this stimulus. This finding implies that LCK expression may be prognostically important and high LCK expression may be an indicator of aggressive disease given the established role of BCR signaling to disease pathogenesis in CLL (3). However, we find that the levels LCK expression in UM-CLL compared with mutated-CLL (M-CLL) cells is similar, and that there is no relationship between the expression of LCK and ZAP70. Interestingly, in the malignant cells from the cohort of patient samples used, we found that higher ZAP70 expression was associated with UM-CLL cell cases. This observation is important because ZAP70 expression is reported higher in UM-CLL cases (16, 18).
where it has a clear but undefined role in enhancing BCR signals (19, 20). Thus, the lack of correlation between LCK expression and ZAP70/IGHV status despite our shown role for this SFK in BCR signaling suggests the involvement of additional factors that are not yet apparent within the current study. As an indicator of disease prognosis, LCK expression is likely to be independent of IGHV status, but whether this is true will need more cases than were analyzed to make strong conclusions and is outside the scope of the present study.

In conclusion, this study shows that LCK plays an important role in mediating BCR signaling. This SFK participates in proximal phosphorylation of ITAM motifs in CD79 and induces distal activation of Syk, ERK, NF-κB, and Akt signaling as well as increased CLL cell survival. This places LCK at the heart of CLL pathogenesis and changes the paradigm on the importance of Lyn in this process. As a therapeutic approach, LCK inhibition may be an attractive option because of the importance of BCR signaling to the pathophysiology of CLL cells, and because small-molecule compounds that target LCK are being developed to inhibit antigen receptor–mediated T-cell activation as a means to control organ rejection as well as autoimmune diseases (59). Future in vivo experiments will require models where there is clear evidence of on-going BCR stimulation to assess the value of Lck-i in the treatment of CLL.

References


LCK Is an Important Mediator of B-Cell Receptor Signaling in Chronic Lymphocytic Leukemia Cells

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