ARTICLE

SLP76 integrates into the B-cell receptor signaling cascade in chronic lymphocytic leukemia cells and is associated with an aggressive disease course

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Haematologica 2016
Volume 101(12):1553-1562

ABSTRACT

In the last decade, the B-cell receptor has emerged as a pivotal stimulus in the pathogenesis of chronic lymphocytic leukemia, and a very feasible therapeutic target in this disease. B-cell receptor responsiveness in chronic lymphocytic leukemia cells is heterogeneous among patients and correlates with aggressiveness of the disease. Here we show, for the first time, that SLP76, a key scaffold protein in T-cell receptor signaling, is ectopically expressed in chronic lymphocytic leukemia cells, with variable levels among patients, and correlates positively with unmutated immunoglobulin heavy chain variable gene status and ZAP-70 expression. We found that SLP76 was functionally active in chronic lymphocytic leukemia cells. A SYK-dependent basal level of phosphorylated SLP76 exists in the cells, and upon B-cell receptor engagement, SLP76 tyrosine phosphorylation is significantly enhanced concomitantly with increased physical association with BTK. B-cell receptor-induced SLP76 phosphorylation is mediated by upstream signaling events involving LCK and SYK. Knockdown of SLP76 in the cells resulted in decreased induction of BTK, PLCγ2 and IκB phosphorylation, as well as cell viability after B-cell receptor activation with anti-IgM. Consistent with our biochemical findings, high total SLP76 expression in chronic lymphocytic leukemia cells correlated with a more aggressive disease course. In conclusion: SLP76 is ectopically expressed in chronic lymphocytic leukemia cells where it plays a role in B-cell receptor signaling.

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Received: November 9, 2015.

Accepted: July 12, 2016.

Pre-published: July 21, 2016.

doi:10.3324/haematol.2015.139154

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/101/12/1553

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal, $CD5^+$ B cells in the peripheral blood, bone marrow and secondary lymphoid organs.[1] Despite the fact that CLL is currently incurable by standard chemo-immunotherapy, impressive clinical responses can be obtained which prolong overall survival.[2]

B-cell receptor (BCR) signaling is a crucial component of normal B-cell development, and plays an important role in the differentiation, survival, proliferation and antibody secretion of these cells.[3] In mature B cells, antigen engagement of the BCR induces coordinated downstream signaling cascades. These initial events include the recruitment and activation of Lyn to phosphorylate the immunoreceptor tyrosine-based activation motifs of the Igα/Igβ components of the BCR. These events are followed by further recruitment and activation of additional kinases and adaptor molecules such as SYK, Bruton tyrosine kinase (BTK), phosphatidylinositol 3-kinases (PI3K), B-cell linker (BLNK or SLP65) and PLCγ2 which form a micro-signalosome that enables the amplification and propagation of the signal through a

number of downstream cascades.[3]

BCR signaling also plays a critical role in the pathogenesis of CLL, and antigen engagement is presumed to be a key regulator of CLL cell survival and proliferation *in vivo*.[4,5] There are two main subgroups of CLL based on immunoglobulin heavy chain variable (*IGHV*) gene mutational status.[6-8] CLL with mutated *IGHV* is characterized by stable or slowly progressive disease, while the unmutated *IGHV* CLL subtype has a more aggressive clinical course.[6,7] *In vitro* studies have shown that activation of the BCR protects CLL cells from apoptosis[9,10] and promotes entry into the cell cycle.[11,12] However, responsiveness of CLL cells to BCR activation is heterogeneous.[13] CLL cells with unmutated *IGHV* are usually BCR-signaling competent, while those with mutated *IGHV* generally respond weakly to BCR activation.[8] The zeta chain-associated protein kinase of 70 kD (ZAP-70), which is normally expressed in T cells, is involved in T-cell receptor (TCR) signaling. ZAP-70 is ectopically expressed in most cases of CLL with unmutated *IGHV* CLL and less often with mutated *IGHV*.[14-16] Expression of ZAP-70 in CLL cells is associated with an augmented response to BCR activation[17] and correlates with a more aggressive clinical course.[16,18-20] Given the essential role of BCR signaling in the pathogenesis of CLL, this pathway has now become a target for anti-CLL therapy and small-molecules directed against kinases such as SYK, BTK, or PI3K have impressive clinical activity.[21,22]

The SH2 domain-containing leukocyte protein of 76 kDa (SLP76, also known as LCP2) is a hematopoietic adaptor protein known to be important in multiple biochemical signaling pathways and is expressed in all hematopoietic lineages except for mature B cells.[23,24] In T cells, SLP76 functions as a critical signal transducer downstream of the TCR.[25] Engagement of the TCR leads to consecutive activation of kinases including LCK, which phosphorylates the immunoreceptor tyrosine-based activation motifs of this receptor.[26] These phosphorylated motifs recruit and activate ZAP-70 which then phosphorylates SLP76 and the transmembrane adaptor LAT.[27] Activated LAT recruits SLP76 from the cytosol to the cell membrane to form a multimolecular complex comprising a number of signaling molecules,[28] including PLCγ1, VAV and NCK (which bind to SLP76 tyrosine residues Y112 and Y128), ITK (which binds SLP76 Y145),[29] ADAP, LCK and HPK1.[30] Formation of the SLP76-LAT multimolecular complex allows proximity between the signaling molecules and facilitates an efficient propagation of various TCR signaling processes.[30]

Since CLL cells express ZAP-70 and LCK,[15,16,31,32] we examined whether they also express additional TCR-associated molecules. Here we report, for the first time, the aberrant expression of SLP76 in CLL cells, which plays a role in the BCR signaling pathway and correlates with an aggressive clinical course.

Methods

Patients and samples

After signing an informed consent form approved by the Institutional Review Board according to the Helsinki declarations, blood samples were collected from patients fulfilling the standard criteria for CLL[33] and also from healthy controls. The patients' characteristics are shown in *Online Supplement S1*. The sample handling protocol is available in *Online Supplement S2*.

Antibodies and reagents

The antibodies and reagents used in this study are detailed in *Online Supplement S2*.

CD19 enrichment

Peripheral blood mononuclear cells were magnetically labeled using CD19 microbeads (Miltenyi Biotec, Inc., Auburn, CA, USA), and separated (more than 95% purity) on a magnetic cell separation LS column (Miltenyi Biotec, Inc.) according to the manufacturer's instructions.

Western blotting and co-immunoprecipitation

Western blot and co-immunoprecipitation protocols are described in *Online Supplement S2*.

mRNA extraction and cDNA synthesis

RNA was extracted using an RNeasy kit (Qiagen, CA, USA), and reverse transcription was performed using a Verso cDNA kit (Thermo Fisher Scientific/ABgene, Epsom, UK), according to the manufacturers' instructions.

Quantitative reverse transcriptase polymerase chain reaction

Gene transcripts were quantified by quantitative polymerase chain reaction using the Absolute Blue QPCR SYBR Green ROX mix (Thermo Fisher Scientific/ABgene) and a Rotor-Gene RG6000 apparatus (Corbett Research, Mortlake, Australia). The primers used are presented in *Online Supplement S2*.

Immunoglobulin heavy chain variant gene and ZAP-70 analysis

The *IGHV* gene was amplified as described elseswhere.[7] The protocol is available in *Online Supplement S2*. ZAP-70 expression was assessed by western blotting of $CD19^+$ purified CLL cells.

Flow cytometry

Cells were stained using Cytofix fixation buffer and Phosflow Perm/Wash buffer I according to the manufacturer's instructions. Samples were acquired by a FACSCalibur and analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA).

In vitro B-cell receptor stimulation

CLL cells ($1x10^7$/mL) were stimulated with goat $F(ab')_2$ anti–human IgM (10 μg/mL) at 37°C for the indicated times. For inhibition assays, cells were incubated prior to IgM stimulation in the absence or presence of the following: 10 μM PP2 for 15 min, 10 μM SYK inhibitor II for 15 min, 0.5 μM ibrutinib for 1 h, 20 μM cytochalasin B for 30 min, 10 mM MβCD for 30 min, 40, 200, and 1000 nM LCK inhibitor for 2 h, and 0.2, 1, and 5 μM R406 for 30 min. These concentrations were chosen on the basis of previous publications,[32,34-37] and in this study were titrated to obtain a maximal effect without killing the cells. Inhibitors were dissolved in dimethylsulfoxide, while controls were treated accordingly with dimethylsulfoxide.

Short interfering RNA transfection

Cells were transfected with siRNA using the 4D-Nucleofectordevice (Lonza Group Ltd, Basel, Switzerland) according to the manufacturer's instructions. The protocol is described in *Online Supplement S2*.

Cell apoptosis assay

Cell apoptosis was detected using a MEBCYTO Apoptosis Kit (MBL Co., Ltd. Nagoya, Japan) according to the manufacturer's instructions. The protocol is available in *Online Supplement S2*.

Statistical analysis

We compared continuous variables between groups using the Student t-test. A P value <0.05 was considered statistically significant. Survival curves were created using the method of Kaplan and Meier, and the log-rank test was used to assess differences between the subgroups. A P value <0.05 was considered statistically significant. All statistical analyses were performed using Graphpad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

Results

SLP76 is aberrantly expressed in chronic lymphocytic leukemia cells

Western blotting of CD19$^+$ purified CLL cells revealed that the scaffold protein SLP76 is aberrantly expressed in CLL cells, and not in the control, mature B-cell lines, Raji (Figure 1A) and Daudi (*data not shown*). The levels of SLP76 expression in CLL cells varied among patients (Figure 1A), and correlated with SLP76 mRNA levels (*Online Supplement S3*). To exclude any possibility that SLP76 expression originated from contaminating T cells in the CD19$^+$ selected B-cell samples, the blots were also analyzed for CD3ε, a T-cell specific molecule. As shown in Figure 1A, CD3ε was undetectable in the purified CLL samples, but readily found in the Jurkat T-cell line. BLNK, the counterpart adaptor molecule of SLP76 which is normally expressed in B cells, was detected in all CLL cell samples at comparable levels (Figure 1A). To further verify these findings, SLP76 expression was also measured using intracellular flow cytometry. As shown in Figure 1B,C,

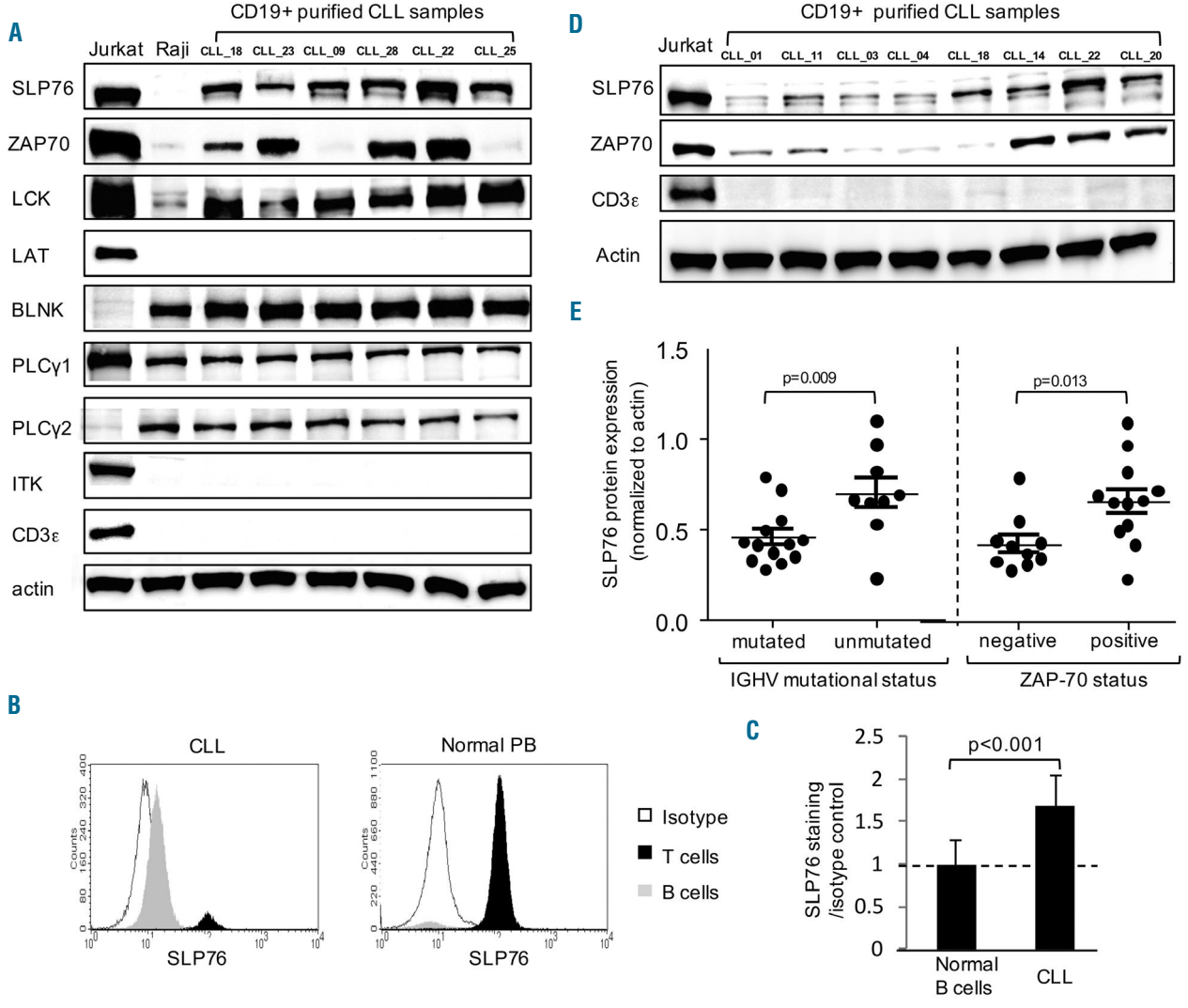

Figure 1. SLP76 is ectopically expressed in CLL cells and is associated with unfavorable prognostic markers. (A) Detection of total SLP76 as well as ZAP-70, LCK, LAT, ITK, PLCγ1, PLCγ2, BLNK (SLP65) and CD3ε levels by western blot in primary CD19$^+$ purified CLL cells and in cell lines including Jurkat (positive control for T-cell-related proteins) and Raji (negative control for T-cell-related proteins). Actin was used to verify equal loading (n=22). A representative image is shown. (B-C) Flow cytometry analysis of SLP76 levels in T cells and B-cells in peripheral blood samples of healthy individuals and CLL patients. (B) Representative cases showing SLP76 expression in normal and CLL blood samples. (C) A summary of SLP76 mean fluorescence intensity (average ± SD) normalized to isotype control in gated B cells of healthy subjects (n=6) and CLL patients (n=6). (D) Detection of ZAP70 levels in four samples of primary CD19$^+$ purified CLL cells with low SLP76 levels and four with high SLP76 levels by western blot. The Jurkat cell line was used as a positive control. CD3ε was used to verify that there was no T-cell contamination. Actin was used to verify equal loading. (E) Correlation between SLP76 protein levels, in CD19$^+$ purified CLL cells (detected by western blot then quantified and normalized to actin), and *IGHV* mutational status and ZAP-70 expression (detected by western blot, n=22).

SLP76 was detected in both $CD19^+$ and $CD3^+$ gated populations in CLL samples, but levels were lower in CLL cells than in T cells present in the same sample. In contrast to CLL cells, in peripheral blood lymphocytes of healthy individuals SLP76 was only detected in T cells, and was not present in B cells (Figure 1B).

Since SLP76 is a downstream signaling molecule of the TCR pathway, we examined whether other components of this pathway are also expressed in CLL cells. As previously reported, the proximal signaling kinases LCK and ZAP-70 were detected in CLL cells at varying levels (Figure 1A). However the more distal downstream TCR molecules (LAT and ITK) were not expressed in CLL samples, while both PLCγ2 and PLCγ1 were detected at near comparable levels (Figure 1A). In most cases examined, patients' samples containing high levels of SLP76 proteins also had high levels of ZAP-70 expression (Figure 1D). Quantitative analysis of SLP76 expression at the protein level showed that CLL cells with unmutated *IGHV* or which were positive for ZAP-70 expressed higher levels of SLP76 than CLL cells which had mutated *IGHV* or were negative for ZAP-70 (Figure 1E)

B-cell receptor engagement phosphorylates SLP76 in chronic lymphocytic leukemia cells independently of ZAP-70

In order to examine whether aberrant SLP76 expression in CLL cells plays a role in BCR signaling, we activated CLL cells by BCR cross-linking. Upon TCR engagement, tyrosine residues Y113, Y128, and Y145 on the SH2 domain binding motifs in the N-terminal of SLP76 are phosphorylated.[23] In contrast, SLP76 phosphorylation on serine 376 generates a delayed negative signal that regulates T-cell activation.[38] As shown in Figure 2A,B, after surface IgM engagement, SLP76 undergoes tyrosine phosphorylation (Y128) in a time-dependent manner, with this process reaching maximal intensity at 15 min, decreasing after 45 min, and returning to near basal levels by 120 min (Figure 2A). In contrast, serine 376 (S376) phosphorylation had increased by 15 min, peaked and remained elevated at 45 and 120 min, respectively (Figure 2A), concomitant with the decrease in Y128 phosphorylation. Activation of ERK and AKT followed SLP76 Y128 residue phosphorylation, and diminished concomitantly with the phosphorylation of SLP76 S376 residue (Figure 2A). Overall, Y128 phosphorylation following IgM engagement was variable among patients, with an average increase of over 30% (Figure 2C). Despite the correlation between total SLP76 and ZAP-70 expression, there was no correlation between ZAP-70 expression and the degree of SLP76 phosphorylation after BCR activation (Figure 2D,E). In fact, significant SLP76 phosphorylation also occurred in some ZAP-70-negative CLL cells (Figure 2E), indicating that ZAP-70 is not essential for SLP76 activation in CLL cells. In a similar manner phosphorylation of the S376 residue did not correlate with ZAP-70 expression (*Online Supplement S4*).

B-cell receptor-triggered phosphorylation of SLP76 in chronic lymphocytic leukemia cells is mediated through LCK and SYK

Since SRC family kinases, in particular, LYN, play a pivotal role in the proximal signaling cascade triggered by BCR engagement,[36] we examined whether SLP76 phosphorylation was dependent on SRC family kinases activity. As shown in Figure 3A, pre-incubation of CLL cells with PP2, an inhibitor of SRC family kinases, blocked SLP76 phosphorylation almost completely. In a similar manner, the SYK inhibitor-SYKII abrogated the phosphorylation of SLP76, and even depleted the basal level. PP2 and SYKII also partially inhibited the phosphorylation of SLP76 S376 residue (Figure 3B). Cytochalasin B (a cytoskeleton inhibitor) and methyl-β-cyclodextrin (MβCD, extracts membrane-associated cholesterol) had no effect on SLP76 tyrosine phosphorylation, indicating that the integrity of the cytoskeleton or the cholesterol-rich membrane domains is not necessary to initiate activation of SLP76 in response to BCR engagement (Figure 3A). As expected, PP2 and SYKII abrogated AKT phosphorylation, which was used to confirm the activity of each one of these inhibitors (Figure 3). Due to the critical role of LCK in TCR upstream signaling, and its ectopic expression in CLL cells, we also determined its role in the activation of SLP76 after BCR triggering. LCKi, a highly selective inhibitor of LCK that does not affect LYN activity,[32] reduced SLP76 phosphorylation in response to BCR engagement to baseline level (Figure 3C). The clinically-used SYK inhibitor R406 completely abolished SLP76 phosphorylation (Figure 3D), in a manner similar to the activity of SYKII (Figure 3A).

SLP76 binds BTK, an association enhanced by B-cell receptor engagement

It is established that in CLL cells, BTK associates with the adaptor protein BLNK.[39] As shown in Figure 4A,B, immunoprecipitation of SLP76 in unstimulated CLL cells resulted in co-precipitation of BTK. Moreover, engagement of the BCR led to a significant increase in the association of SLP76/BTK. Although, SLP76 and BTK are physically associated in CLL cells, BCR-mediated induction of SLP76 phosphorylation was only slightly affected by the potent BTK inhibitor ibrutinib (Figure 3A).

SLP76 downregulation partially abrogates B-cell receptor signaling

In an attempt to examine the direct role of SLP76 in BCR signaling, SLP76 expression was down-regulated using siRNA. Transfection of CLL cells with siRNA against SLP76 resulted in a decrease of this gene after 24 h of culture, as measured by reverse transcriptase polymerase chain reaction (*data not shown*), while a maximum decline in protein level was evident after 36 h (approximately 50%, Figure 4C). Modulations in BCR signaling were also examined using the knockdowns and control CLL cells after anti-IgM cross-linking. As shown in Figure 4C,E, down-regulation of SLP76 significantly decreased BTK phosphorylation in BCR-activated CLL cells. In addition, the BCR-mediated activation of PLCγ2, a downstream kinase in the BCR cascade, was also significantly inhibited by the down regulation of SLP76 (Figure 4C,F). We further investigated the role of SLP76 in the regulation of distal signaling events following BCR cross-linking. Knockdown of SLP76 significantly reduced IκB activation in BCR-stimulated CLL cells, while phosphorylation of ERK and AKT was left intact (Figure 5A,B). In addition, knockdown of BLNK, the counterpart adaptor molecule of SLP76 in B cells, resulted in a similar effect of reduction in BTK (P=0.04) and IκB activation in anti-IgM-stimulated CLL cells (Figure 4D and Figure 5A,B), without a significant impact on ERK and AKT activation. Thus, our data indicate that SLP76 as well as BLNK are specifically required

for BCR-induced activation of NF-κB. In light of the above, the viability of control CLL cells cultured for 48 h in the presence of anti-IgM antibody was improved compared to that of unstimluated cells, but this protective effect was partially abrogated by SLP76 knockdown (Figure 5C).

SLP76 expression correlates with progression of chronic lymphocytic leukemia

Based on the observation that SLP76 expression is heterogeneous among patients with CLL, and considering that SLP76 is phosphorylated after BCR activation, we also evaluated the possible clinical significance of SLP76 expression in CLL. In this analysis, the median value of SLP76 protein levels was used to separate patients into subgroups with low and high expression. As shown in Figure 5D, patients with CLL who had higher SLP76 levels had a shorter time to disease progression or first treatment compared to patients with lower SLP76 levels.

Discussion

In this study we demonstrate that the scaffold protein SLP76, which plays a critical role in the TCR signaling pathway,[23] is aberrantly expressed in CLL cells. The level of SLP76 varies among patients and correlates with unmutated *IGHV* gene status, positive ZAP-70 expression, and a shorter time to disease progression. Similar to SLP76, the

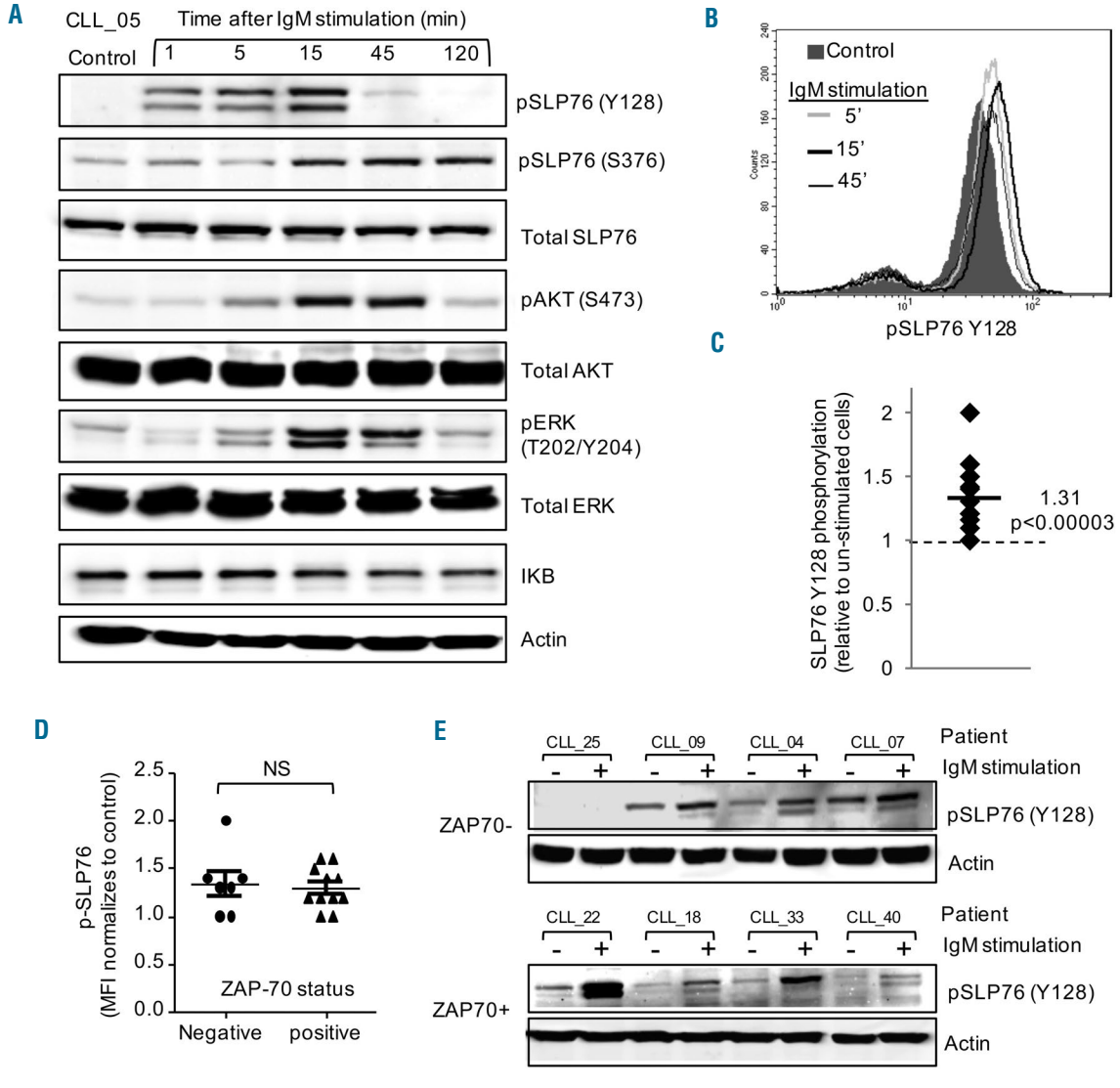

Figure 2. SLP76 is phosphorylated upon BCR activation in CLL cells. Peripheral blood CLL cells were incubated with goat F(ab')$_2$ anti-human IgM (10 μg/mL) for different times. (A) A representative western blot analysis showing SLP76 tyrosine (Y128) and serine (S376) phosphorylation, as well as AKT (S473), ERK (T202/Y204) activation and IκB expression (n=4). Actin was used to verify equal loading. (B) A representative analysis of SLP76 tyrosine phosphorylation (Y128) determined by flow cytometry in CD19$^+$ gated CLL cells after BCR stimulation with goat F(ab')$_2$ anti-human IgM (10 μg/mL) for 5, 15, and 45 min compared to unstimulated cells. (C) A summary of the phosphorylation levels of SLP76 (Y128) after IgM stimulation with goat F(ab')$_2$ anti-human IgM (10 μg/mL) for 15 min, determined by flow cytometry in CD19$^+$ gated CLL cells compared to unstimulated cells (n=20). (D) SLP76 tyrosine phosphorylation (Y128) determined by flow cytometry, in ZAP-70 negative (n=7) and ZAP-70 positive (n=11) patients. (E) Western blot analysis of SLP76 tyrosine phosphorylation (Y128) following 15 min of IgM stimulation, in ZAP-70 negative (n=4) and ZAP-70 positive (n=4) patients.

proximal TCR signaling molecules LCK and ZAP-70 are ectopically expressed in CLL cells, with varying levels among patients, while being mostly undetectable in peripheral blood B cells from healthy individuals.[31,40] Like SLP76, ZAP-70 expression is also associated with more aggressive disease and correlates with unmutated *IGHV* status. LCK, a SRC family protein kinase that targets ZAP-70 as one of its principal substrates, as well as ZAP-70 itself, both appear to mediate BCR signaling in CLL cells.[17,32,41,42] ZAP-70 has also been shown conclusively to enhance BCR response in CLL cells.[17,41,42]

In normal T cells, phosphorylation of SLP76 at multiple tyrosine residues (Y112, Y128, and Y145) is required for a functional TCR,[23] and here we demonstrate that SLP76 is not only ectopically expressed in CLL cells, but is also functional. Engagement of the BCR in CLL cells induces immediate transient tyrosine phosphorylation of SLP76 followed by phosphorylation of an inhibitory serine residue, in a similar manner to that occurring on TCR activation in T cells. In agreement with previously published data regarding BCR signaling, BCR-mediated activation of SLP76 was independent of cholesterol-rich lipid rafts.[43] We

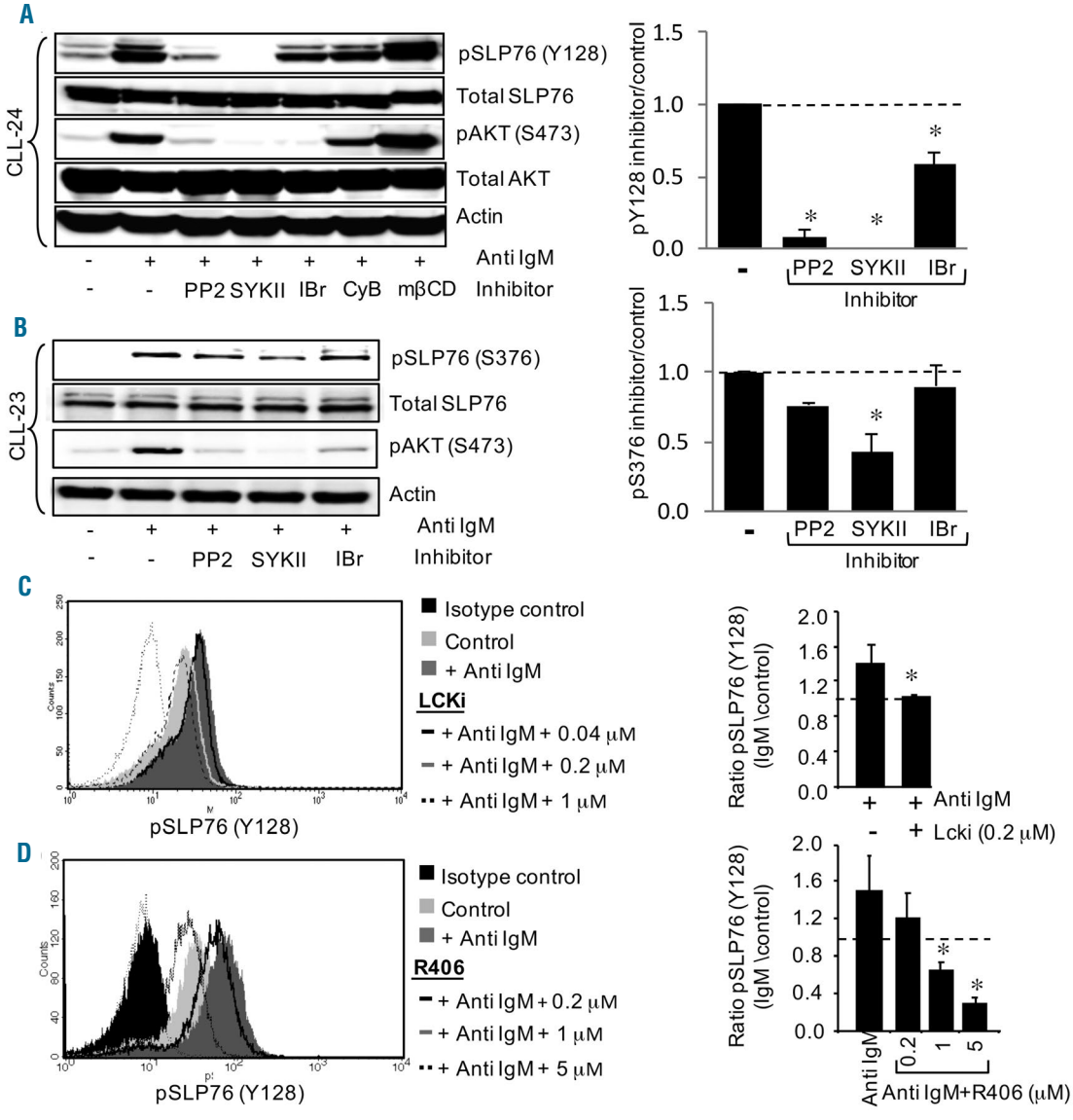

Figure 3. BCR-induced SLP76 phosphorylation is mediated by LCK and SYK. (A-B) Peripheral blood CLL cells were pre-incubated with the specified inhibitors (PP2: 10 μM for 15 minutes, SYKII: 10 μM SYK inhibitor II for 15 minutes, Ibr: 0.5 μM Ibrutinib for 1 hour CyB: 20 μM Cytochalasin B for 30 minutes, mβCD, 10mM methyl β cyclodextrin for 30 minutes), followed by activation with goat F(ab')$_2$ anti-human IgM (10 μg/mL). Protein was extracted and analyzed by western blot. Representative analyses showing in A-Left: induction of SLP76 tyrosine phophorylation (Y128), 15 min after BCR activation and in B-left: SLP76 serine phophorylation (S376), 45 min after anti-IgM engagement, in the presence or absence of each one of the inhibitors. AKT phosphorylation was used to confirm the inhibitory activity of each inhibitor. A-right and B-right: A summary of three experiments showing quantification of SLP76 tyrosine and serine phophorylation in BCR-activated CLL cells pre-incubated with the indicated inhibitors and normalized to control (BCR-activated CLL cells without inhibitors). (C-D) Peripheral blood CLL cells were incubated with: (C) 0.04, 0.2, or 1 μM of LCKi inhibitor for 2 h. DMSO treated cells served as controls. (D) 0.2, 1, or 5 μM of the SYK inhibitor R406 for 30 min. DMSO treated cells served as controls. Both control and inhibitor-treated cells were then activated with goat F(ab')$_2$ anti-human IgM (10 μg/mL) for 15 min. SLP76 tyrosine phosphorylation (Y128), was then determined by flow cytometry in CD19$^+$ gated cells. C-left and D-left: One representative analysis. C-right and D-right: A summary of SLP76 phosphorylation levels (C) n=4, (D) n=3.

also showed that phosphorylation of SLP76 after BCR engagement occurs regardless of ZAP-70 expression, as it is also induced in ZAP-70-negative CLL cells. However, both LCK and SYK participate in the upstream signaling of SLP76 in CLL cells, as evident from the inhibition of SLP76 tyrosine phosphorylation using inhibitors directed to LCK or Syk.[30,32] In addition, SYK but not LCK, appears to maintain a basal level of SLP76 phosphorylation independently of BCR engagement. Our findings are compatible with previous reports which show that SLP76 is phosphorylated in a SYK-dependent manner in several non-T-cell subtypes.[29,44] Furthermore, LCK has been reported to mediate phosphorylation of SYK but not ZAP-70 in BCR-stimulated CLL cells,[32] whereas tyrosine residues required for ZAP-70 kinase activation are not phosphorylated in CLL cells following BCR stimulation.[41,42]

Another key signaling adaptor protein of the TCR pathway is LAT, which is constitutively localized to the membrane glycolipid-enriched microdomains.[23] Upon TCR stimulation, phosphorylation of LAT recruits SLP76 through GADS to the cell membrane, and the two adaptor proteins serve as a scaffold for the formation of a multimolecular signaling complex.[23] In contrast to T cells, we show that LAT is not expressed in CLL. In T cells, LAT is essential for activation of PLCγ1 and the Ras pathway upon TCR ligation,[45] while in other cell types signaling involving SLP76 may propagate independently of LAT. In this respect, Mizuno *et al.* showed that murine B-cell lines express SLP76 and that knockdown of SLP76 attenuated BCR signaling, despite the fact that these cells do not express LAT.[46] Additionally, there seems to be a differential requirement of LAT in platelets, and LAT-deficient mice show no bleeding diathesis unlike those animals that are SYK$^{-/-}$ or SLP76$^{-/-}$.[47,48]

Here we also show that ITK is not expressed in CLL cells, while BTK is constitutively physically linked to SLP76, an association which increases significantly in response to BCR engagement. In a similar manner, BTK has also been shown to be constitutively associated with SLP76 in mast cells, which increased association after FcεRI ligation.[49] It seems that SLP76 operates as an adaptor protein that mediates SYK-dependent BTK activation in CLL cells. Accordingly, the BTK inhibitor ibrutinib had minimal effect on BCR-mediated SLP76 phosphorylation, while knockdown of SLP76 resulted in down-regulation of BTK phosphorylation after BCR stimulation. SLP76 knockdown also significantly inhibited the BCR-induced

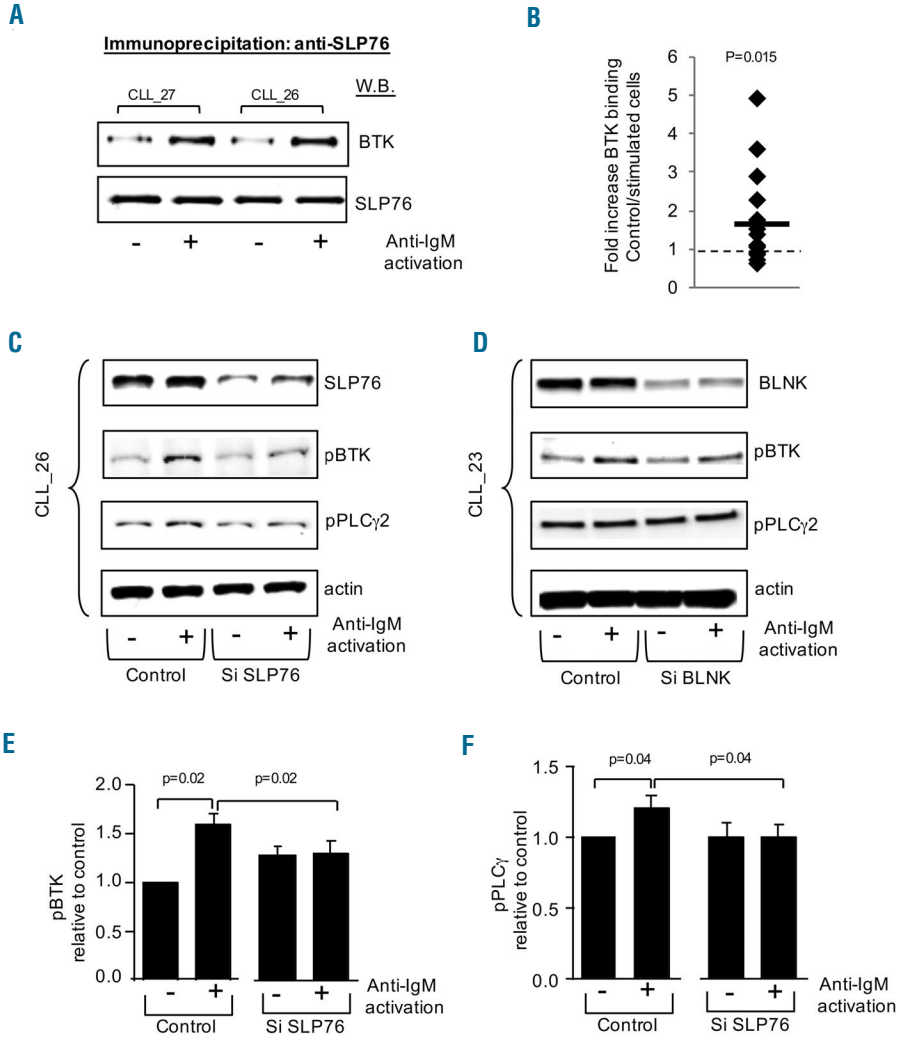

Figure 4. SLP76 associates with BTK and modulates BCR signaling. (A, B) IgM activated (for 15 min) and control CLL cell extracts were immunoprecipitated with anti-SLP76 antibody and probed with anti-SLP76 and anti-BTK antibodies. (A) Representative western blot analysis of two patients' samples. (B) Summary of immunoprecipitation results. BTK levels are indicated as percentage of control (n=17). (C-F) Peripheral blood CLL cells were transfected with either control or SLP76-specific siRNA or with BLNK-specific siRNA. After 36 h, cells were stimulated with 10 μg/mL goat F(ab')$_2$ anti-human IgM for 15 min. Following stimulation, cells were lysed and SLP76 levels, BLNK levels, BTK phosphorylation (Y223) levels and PLCγ2 phosphorylation (Y759) levels were detected by western blot. (C, D) Representative western blot analysis showing control and SLP76-knockdown cells (C) or control and BLNK-knockdown cells (D) either stimulated or unstimulated with goat F(ab')$_2$ anti-human IgM (10 μg/mL). (E-F) The average + SE of the phosphorylation responses of BTK (E) n=12 and PLCγ2 (F) n=10 in control and SLP76-knockdown cells either stimulated or unstimulated with goat F(ab')$_2$ anti-human IgM (10 μg/mL).

phosphorylation of PLCγ2, a more distal signaling molecule of the BCR cascade. Similarly, SLP76 has also been shown to mediate PLCγ2 activation in different cell types.[50] Examination of the various distal BCR-activated signaling pathways in SLP76 knockdown CLL cells revealed intact activation of ERK and AKT activation, but impaired activation of the canonical NF-κB pathway, which plays a critical role in CLL pathogenesis.[5,51] Our finding, that BLNK knockdown resulted in a similar inhibitory pattern on BTK and IκB, further suggests that SLP76 and BLNK have overlapping or complementary activity, namely orchestrating the formation of the BTK-PLCγ2 axis that regulates NF-κB activation in BCR-stimulated CLL cells. Similarly, Tan *et al.* previously reported that in BCR-stimulated mouse BLNK$^{-/-}$ B cells, activation of NF-kB is impaired, while induction of AKT and mitogen-activated protein kinases are intact.[52] Given that the NF-κB pathway has been implicated in regulation of genes essential for B-cell survival, it is not surprising that knockdown of SLP76 partially blocked the pro-survival protection induced by BCR activation in CLL cells.

In this study we also demonstrate that higher SLP76 protein levels correlate with a shorter time from diagnosis to disease progression or the initiation of first treatment, which provides support for the biological role of SLP76 in the pathogenesis of CLL. The strong association between SLP76 and other BCR pathway components, such as ZAP-70 and *IGHV* mutational status, suggests that the clinical correlation between SLP76 levels and disease progression is not solely related to SLP76, but is part of a variety of factors that interact to enhance BCR signaling. Some of these components and in particular those indicative of "T-cell origin", may share a common transcriptional regulation involving ZAP-70, LCK, and SLP76.

Experimental evidence accumulated over recent years indicates that BCR-mediated responses in CLL cells are probably very different from those occurring in normal mature B cells. Although key components of the TCR signaling complex are apparently expressed by CLL cells, which are B cells, their biochemical function in CLL cells

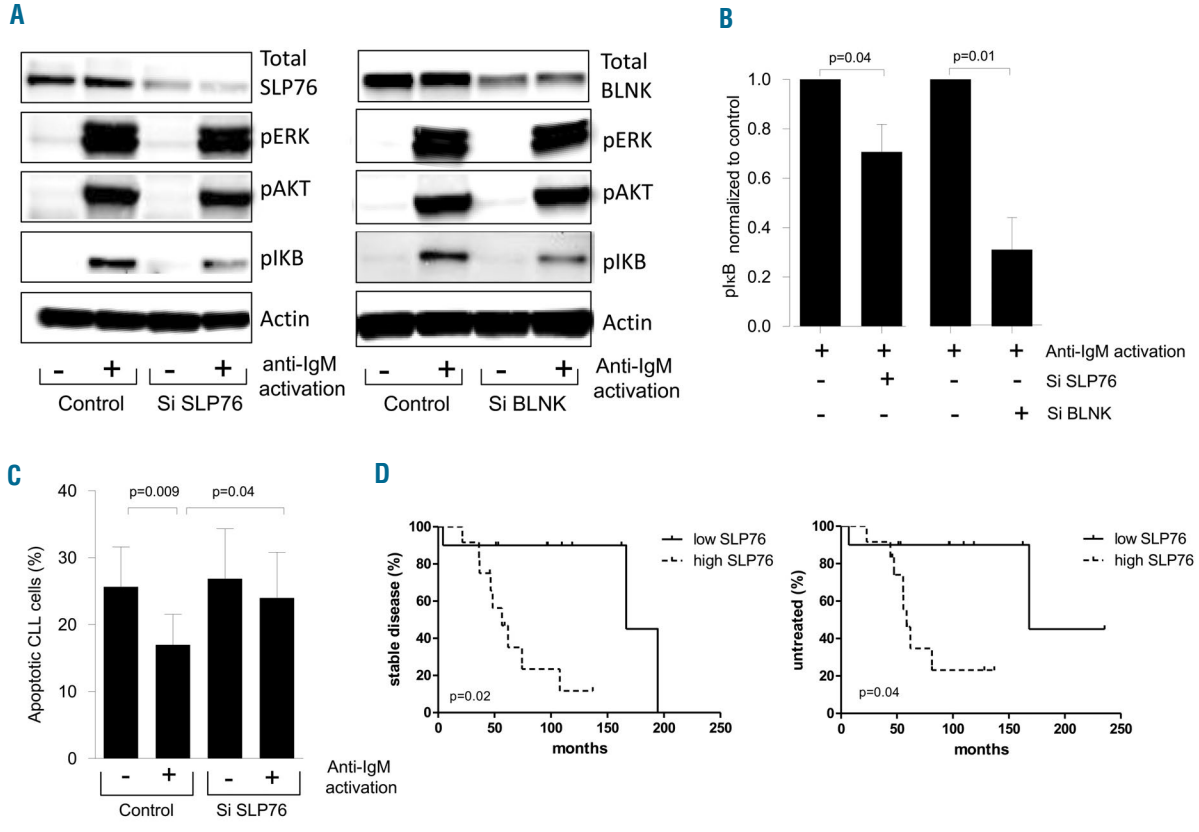

Figure 5. SLP76 expression correlates with disease progression and cell survival. (A, B) Peripheral blood CLL cells were transfected with either control or SLP76-specific siRNA or with BLNK-specific siRNA. After 36 h, cells were stimulated with 10 μg/mL goat F(ab')$_2$ anti-human IgM for 15 min. Following stimulation, cells were lysed and SLP76, BLNK, ERK (T202/Y204) phosphorylation, AKT (S473) phosphorylation and IκB (S32) phosphorylation levels were detected by western blot. Actin was used to verify equal loading (A) Representative western blot analysis showing control and SLP76-knockdown cells (left) and control and BLNK-knockdown cells (right) either stimulated or unstimulated with goat F(ab')$_2$ anti-human IgM (10 μg/mL). (B) The average + SE of the phosphorylation responses following IgM stimulation of IkB in SLP76-knockdown (n=6) and BLNK-knockdown (n=4) cells. (C) Peripheral blood CLL cells were transfected with control or SLP76-specific siRNA. After 36 h, cells were stimulated with 10 μg/mL goat F(ab')$_2$ anti-human IgM for 48 h. Following stimulation, cells were analyzed for apoptosis by flow cytometry. To determine the percentage of apoptotic cells, annexin V-positive cells were gated out of CD23$^+$ CD5$^+$ cells (n=8). (D) Kaplan-Meier analysis of time to first disease progression (n=22) (left) and time to first treatment (right) dichotomized according to whether patients had low or high SLP76 protein expression. SLP76 expression was determined in purified CLL cells using western blot, quantified and normalized to actin. Separation into low and high subgroups was based on the median SLP76 level. Details of the patients' characteristics are available in *Online Supplement S1*; the low SLP76 subgroup includes patients CLL_01 through CLL_11 and the high SLP76 subgroups consists of patients CLL_12 through CLL_22.

is fundamentally different from that evident in the context of the TCR cascade. Our study shows that, unlike in T cells, the scaffold protein SLP76 in CLL cells operates independently of LAT, ZAP-70 or ITK, and also promotes BTK, PLCγ2 and IκB activation upon BCR engagement. Thus, BCR activation, which is essential in CLL pathophysiology, as well as the subsequent signaling cascade it induces are enhanced in CLL cells *via* this novel modality.

Acknowledgments

This research was performed in partial fulfillment of the requirements for a Ph. D. degree of Nili Dezorella, at the Sackler Faculty of Medicine, Tel-Aviv University, Israel.

Funding

This work was supported by the Varda and Boaz Dotan Foundation (YH, BK) and the Israeli Science Foundation (YH).

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