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**Mechanistic Elements of BAFF-BCR Crosstalk
in Chronic Lymphocytic Leukemia**

By

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Introduction

Chronic lymphocytic leukemia (CLL) is a cancer characterized by the uncontrolled proliferation and accumulation of B-cell lymphocytes ¹⁸. It is the most commonly diagnosed leukemia in the Western world ³². In 2017, it is estimated that over 20,000 cases will be diagnosed in the United States, comprising of approximately a third of newly diagnosed leukemias ³³. Age-adjusted incidence rates are especially high for the 65 and older population, at 20.6 per 100,000 ³⁴. Such elderly patients are also particularly prone to adverse outcomes driven by comorbidity ³⁵. While CLL prognosis varies, its beginning is often asymptomatic and insidious ^{30,32}. Therefore, CLL detection is contingent on regular checkups and blood tests, and as such, its epidemiological prevalence is likely to be underestimated. While the onset of CLL is gradual, as its name suggests, it nevertheless poses economic strain on both the healthcare system and individual patients while lowering their qualities of life ³¹. For these reasons, CLL is an important public health consideration with significant social and economic costs.

While CLL remains non-curative, progress has been made to better manage the disease. Earliest breakthroughs in treatment include the use of alkylating agents such as chlorambucil, which continues to be used in combination with other therapies ³⁷. Alkylating agents remained the standard of care between the 1950s and 1980s, after which fludarabine, a purine nucleoside analogue, was introduced in CLL therapy ³⁶. Notable studies determined fludarabine's advantage over chlorambucil with respect to response rates and remission duration, and it soon became a mainstay of CLL treatment ^{38,39}. Immunochemotherapy entered serious therapeutic consideration for CLL when rituximab was introduced at the turn of the century. Rituximab targeted CD20 on the surface of B-cells and was incorporated into landmark combination therapies with

fludarabine and cyclophosphamide ³⁶. These combination therapies have since become some of the standard approaches to CLL treatment. More recent findings have established the role of B-cell receptor (BCR) signaling in CLL survival, migration, and proliferation ⁴⁰. There are a number of clinically relevant BCR kinases in CLL. They include phosphatidylinositol-3-kinase (PI3K), Lck/Yes novel tyrosine kinase (LYN), Bruton's tyrosine kinase (BTK), and spleen tyrosine kinase (SYK) ^{41, 42}. The inhibitors of kinases involved in BCR signaling pathways, such as Ibrutinib and Idelalisib, BTK and PI3K inhibitors respectively, have emerged as especially promising therapies ^{43, 44}. At this time, these BCR kinases are the subjects of extensive research toward the development of novel therapies.

The origin of CLL manifestations may be sourced from a variety of gene mutations, but are divided into two main subtypes. These are classified by the degree of mutations in the variable regions of the heavy chain coding segments of the Ig antibody ¹⁹. CLL cells are also identified by uncharacteristic ZAP-70 expression, which are typically found in T-cells, contributing to their activation ²⁰. In malignant B-cells, it is thought that ZAP-70 contributes to cellular activation on a similar axis as antigenic engagement does ²¹. CLL B-cells can also be recognized by the presence of surface markers CD19, CD5, and CD23. There are also reduced levels of surface IgM, IgD, and CD79b on CLL B-cells, a state which resembles that of an activated and mature B-lymphocyte state ²².

While the historical view of CLL is that it is caused by an initial apoptotic defect, more recent findings contradict this view. It is now understood that initial CLL apoptotic evasion may be a result of environmental signals ²². Thus, the disease is exacerbated by survival signals provided by stromal cells in protective niches, such as the lymph nodes and bone marrow ^{2, 3}. Under typical conditions, these survival signals are necessary for healthy B-cell growth and maturation.

On the other hand, in CLL, the environmental signals rescue neoplastic B-cells from apoptosis and allow them to thrive ^{1, 2, 3, 4}. Some of these soluble mediators include tumor necrosis factor receptor (TNFR) superfamily ligands- clusters of differentiation ligand 40 (CD40L), B-cell activating factor (BAFF), and a proliferation-inducing ligand (APRIL). CD40L and BAFF/APRIL are ubiquitously secreted in the stromal niches and promote the survival and activation of neoplastic clones ². BAFF/APRIL ligands and their receptors are indispensable in B-cell survival ^{9, 10, 11, 12}. BAFF/APRIL share homology and can bind to two TNFRs – B-cell maturation antigen (BCMA) and transmembrane activator of the calcium modulator and cyclophilin ligand-interactor (TACI), whereas BAFF alone can bind BAFF receptor (BAFF-R, BR3) ¹³.

Despite progress in understanding the role of BAFF/APRIL signaling in healthy and neoplastic B-cells, the role of BAFF-mediated NF κ B activation in CLL remains understudied.

An important mechanism through which the BAFF and APRIL survival factors work is in the activation of Bcl-2 family proteins ¹³. This system contains pro-apoptotic and anti-apoptotic proteins that are maintained in a balanced fashion. B-cells transducing survival signals from extrinsic factors in their microenvironments encourage the activity of anti-apoptotic proteins of the Bcl-2 family, pushing the balance toward survival ²³. If the pro-apoptotic proteins Bak and Bax are uninhibited to a threshold degree, they oligomerize and permeabilize the mitochondrial membrane ²³. This initiates the release of caspase-activating factors such as cytochrome c into the cytoplasm, which results in the cleavage of cellular proteins and leads to cell death. This response, however, is inhibited by the anti-apoptotic proteins, which indicates that the Bcl-2 family proteins can be harnessed to promote both cell survival and cell death.

There are a number of pathways that mediate survival factors expressed by stromal cells and apoptosis avoidance through the Bcl-2 protein family. CLL cells in the microenvironments of the lymph node, for example, display gene signatures that indicate the activation of the BCR and nuclear factor- κ B (NF κ B) pathways ⁵. The therapeutic inhibition of BCR-associated kinases has significantly impacted CLL outcomes, in part, by causing B-cell migration from niches that provided stromal support ⁶. However, patients who progress on, or are intolerant of BCRi therapy, have poor outcomes ^{7, 8}. Improved understanding of microenvironment signaling through other pathways will foster development of novel therapeutic approaches in CLL. This work focuses on one such pathway, the NF κ B signaling pathway. Like other TNFR ligands, BAFF/APRIL activate NF κ B signaling. This is a major common pathway which mediates anti-apoptotic responses in CLL cells through induction of Bcl-2 family proteins and chemokine networks ^{13, 14, 15, 16, 17}. Both signal through BCMA/TACI to activate the canonical NF κ B in CLL, where the I κ B kinase complex phosphorylates I κ B α , triggering its ubiquitination and leading to nuclear translocation of the NF κ B dimers, predominantly p50/RelA and p50/c-Rel ^{9, 14}. This, in turn, leads to a survival response.

BAFF also works through non-canonical NF κ B signaling and can be initiated via the BAFF receptor, also known as BR3. Its activation mechanism via the BAFF/BR3 interaction will be delineated as follows. When BR3 is stimulated by the BAFF ligand, it frees the NF- κ B inducing kinase (NIK) to phosphorylate the I κ B kinase (IKK α). NIK also directly phosphorylates p100 at two sites: Ser-866 and Ser-870. IKK α activation via NIK phosphorylates p100 at the Ser-822 site ²⁴. Once all three serine sites are phosphorylated, p100 is processed into p52, dimerizes with RelB, enters the nucleus, and initiates the transcription of pro-survival BCL-2 family proteins ²⁴.

Of note, there is a particular mechanism by which NIK is activated to initiate phosphorylation of IKK α and p100. The non-canonical BAFF signaling pathway is constantly repressed²⁵. When no ligand is attached to the BAFF receptor, NIK is constitutively targeted for ubiquitination and then degraded²⁵. Cellular inducer of apoptosis proteins (cIAPs) 1 and 2 are responsible for the ubiquitination of NIK²⁶. However, cIAP does not perform its function autonomously. It first binds to tumor necrosis factor receptor-associated factor 2 (TRAF2), leading to dimerization with TRAF3, which is bound to NIK. The association of TRAF3 and NIK is known to be essential to NIK degradation²⁵. The TRAF2 and TRAF3 complex allows cIAP to ubiquitinate NIK²⁶. This constant process prevents NIK from being active.

Activation of NIK requires that TRAF 3 must be inhibited²⁶. During BAFF receptor activation, the receptor recruits TRAF 3, TRAF 2, and cIAP to its receptor domain²⁷. At this point, cIAP ubiquitinates TRAF 3 and target it for degradation²⁶. Consequentially, without TRAF 3, NIK accumulates until it is energetically favored to phosphorylate IKK α and p100²⁷. Preliminary experiments have suggested that BAFF stimulation has also upregulated the phosphorylation of a protein from a different pathway, which aids in cell survival. BAFF stimulation is not only working through the non-canonical NF κ B pathway, but is likely involved in crosstalk with the BCR pathway through SYK. This investigation focuses on the mechanism of this crosstalk. Since NIK is activated in response to BAFF stimulation, it is possible that NIK is also inducing a BCR response through phosphorylation of SYK. The mechanism of this crosstalk is the subject of investigation.

Methods

Patient samples and cell culture

Peripheral blood was obtained from patients with CLL at the Center for Hematologic Malignancies at the Oregon Health and Science University (Portland, OR) after informed consent. Mononuclear cells were isolated using standard Ficoll-Hypaque techniques (Amersham, Piscataway, NJ), rendering >90% CD5⁺/CD19⁺ cells, as determined by flow cytometry (FACSCanto). CLL cells were cultured in RPMI-1640 supplemented with 15% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 25 mM HEPES, 100 µM non-essential amino acids and 1 mM sodium pyruvate (Life Technologies, Grand Island, NY). For stimulation with soluble factors, CLL cells were seeded at 1x10⁶/mL in the presence of 25 ng/mL soluble human BAFF (sol-BAFF; Cell Signaling Technology, Danvers, MA). For stimulation with stroma, BAFF-expressing Chinese hamster ovary cells (BAFF-CHO) were obtained from Dr. Robert Woodland (University of Massachusetts, Worcester, MA)¹⁸. The cells were maintained in MEM-α supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 µM non-essential amino acids. CHO-K1 cells not expressing BAFF were used as control (American Type Culture Collection [ATCC], Manassas, VA). CLL cells were cultured on BAFF-expressing (or control) cells under the stromal conditions previously described¹⁷. Briefly, stromal cells were seeded to achieve 80-100% confluence; on the following day, CLL cells were plated at a 50:1 ratio and incubated at 37°C in 5% CO₂. Cultures were then treated with drugs as indicated. At harvest, CLL cells were gently washed off the stromal layer. When harvested for protein, CLL cells were transferred to a new plate and incubated for an additional 60 minutes to allow re-attachment of stromal cells, before the CLL

cells were gently washed off and collected. This minimized contamination of CLL cells by the adherent BAFF-expressing or control cells.

Immunoblotting and Immunoprecipitation (IP)

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM Sodium phosphate, 1 mM NaVO₃, 1 mM EDTA, 1 mM EGTA), supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN), phosphatase inhibitor cocktail 2 and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich).

For immunoprecipitation experiments, cell protein lysates were pre-cleared and incubated at 4°C overnight with 2 µg of the indicated primary antibody or with rabbit IgG as isotype-specific control (Santa Cruz Biotechnology, Santa Cruz, CA). Lysates were incubated with 20 µL of 50% protein A agarose beads slurry (Cell Signaling) for 3 hours at 4°C. After washes, samples were heated to 95°C for up to 5 min and analyzed by immunoblotting. 10% of source protein was used as input control.

The following antibodies were used: GAPDH, IKK α , NIK, NF κ B2(p100/52), pSYK^{Y352}, SYK (#2712), SYK (D3Z1E, for IP), TRAF2, TRAF3 (Cell Signaling Technology, Danvers, MA); β -actin (Sigma-Aldrich).

Cell electroporation

Raji cells (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Electroporation of Raji cells was performed using the Amaxa Human B-cell Nucleofection Kit (Lonza, Walkersville, MD). 5x10⁶ cells were mixed with 100 µL of Amaxa Solution V, and 2 µg of DNA was nucleofected using program O-017.

Transfection efficiency, assessed by transfection with 2 μ g pMaxGFP plasmid, was 70-90% with cell viability of >90% at 24 hours. pCMV4-NIK-HA was a gift from Shao-Cong Sun (Addgene plasmid 27554) ²¹.

Results

Figure 1. SYK interacts with NIK/TRAF2/TRAF3 in neoplastic B-cells. (A-B) Raji cells were stimulated with 25 ng/mL sol-BAFF for 30 min. Proteins lysates were subjected to immunoprecipitation experiments using indicated antibodies as described in the methods. A representative blot of three independent experiments is shown. (C) Raji cells were transfected with siNIK vs. control siRNA. 48 h later, cells were stimulated with 25 ng/mL sol-BAFF for 30 min. Whole-cell lysates were subjected to immunoblotting. A representative blot of three independent experiments is shown.

Figure 2. SYK-TRAF2/3 interaction. (A, C) Raji cells were stimulated with 25 ng/mL sol-BAFF for 30 min. Proteins lysates were subjected to immunoprecipitation experiments with TRAF2 or control antibodies. (B, C) CLL cells were cultured on BAFF-expressing stroma for 24 h. Protein lysates were immunoprecipitated with SYK or control antibodies. Representative blots of three independent experiments are shown. (D) Raji cells were transfected with pCMV-NIK or control plasmid. 48 h later, cells were stimulated or not with 25 ng/mL sol-BAFF for 30 min. Whole-cell lysates were subjected to immunoblotting. A representative blot of three independent experiments is shown. (E) CLL cells were co-cultured with BAFF-expressing stroma for 24 h, followed by treatment with IKK inhibitors for 6h. Whole cell protein lysates were subjected to immunoblotting.

Figure 3. BAFF-BCR crosstalk in CLL cells. BAFF-R engagement stabilizes NIK within the NIK/TRAF2/TRAFF2/cIAP1/2 complex, promoting the non-canonical NFκB pathway activity. SYK recruitment to NIK/TRAFF2/TRAFF3 signaling complex assists BAFF-mediated activation of BCR signaling, which contributes to activation of the canonical NFκB. Concurrently, SYK induces STAT3 transcription factor, thereby upregulating Mcl-1, a pro-survival Bcl-2 family member.

BAFF stimulation induces SYK interaction through the NIK/TRAFF2/TRAFF3 signaling complex

Since we found that BAFF promotes CLL cell survival via SYK-mediated upregulation of the canonical NFκB and Mcl-1, we studied how BAFF activates SYK. BAFF-R signals through an intermediary complex, which involves adaptor proteins TRAF2/TRAFF3, NFκB-inducing kinase (NIK), and inhibitor of apoptosis (IAP) family proteins cIAP1/2¹³. While the exact mechanism remains elusive, it is believed that in unstimulated B-cells NIK is constitutively bound to TRAF3 and degraded. When BAFF-R is engaged, the NIK/TRAFF/cIAP complex is recruited to the receptor, followed by TRAF3 repression, thus allowing NIK to persist and activate IKK1. IKK1 catalyzes proteasome-assisted processing of NFκB2 (p100) precursor, thereby inducing the non-canonical NFκB¹³. Since targeting NEDD8 with pevonedistat, which blocks p100 processing¹⁷, did not regulate Mcl-1 in BAFF-stimulated CLL cells, we supposed that NIK and/or IKK1 may be responsible for BAFF-induced SYK activation. To study this, we evaluated whether SYK complexes with NIK/TRAFF2/TRAFF3 in Raji B-cell lymphoma cell line. Immunoprecipitation of cells with SYK monoclonal antibodies showed association of SYK with TRAF3 and TRAF2 in BAFF-stimulated Raji cells (Fig. 1A). Moreover, we were able to detect NIK in our SYK immunoprecipitates. Conversely, TRAF2 co-immunoprecipitated with both SYK and NIK in

Raji cells, while SYK complexed with TRAF2 and TRAF3 in primary CLL cells (Fig. 2A-C). We subsequently confirmed SYK binding in the reverse experiments with NIK monoclonal antibodies (Fig. 1B). By contrast, SYK did not complex with IKK1 in either Raji or CLL cells (Fig. 1A and Fig. 2B-C), strongly suggesting that SYK interacts with submembrane TRAFs in neoplastic B-cells.

As in CLL, BAFF stimulation induced SYK phosphorylation in Raji cells. However, engineered expression of NIK failed to enhance SYK activation either in the absence or in the presence of BAFF (Fig. 2D). A possible explanation for this is the critical requirement for TRAF2/3 scaffold in NIK-SYK interaction: NIK depletion by means of a genetic knockdown did result in reduced SYK activation (Fig. 1C). Meanwhile, pharmacologic targeting of IKKs failed to prevent BAFF-mediated SYK activation (Fig. 2E).

Thus, BAFF-BCR crosstalk in neoplastic B-cells is at least in part mediated by SYK interaction with NIK/TRAF2/TRAF3 complex (Fig. 3).

Discussion

Along with the BCR, many concurrently active pathways ensure survival of the neoplastic B-cells in the protective niche. Others have previously demonstrated that primary CLL cells co-cultured with CD40L-expressing stroma activate the canonical and non-canonical NF κ B pathways, accompanied by upregulation of the pro-survival Bcl-2 family proteins (Bcl-xL), and acquire therapeutic resistance, including to BCRi's^{15, 16, 17}. BAFF was previously shown to co-opt BCR signaling in mouse splenic B-lymphocytes, manifested by phosphorylation of the BCR-

associated CD79A subunit and SYK ²⁸. Here we propose a possible mechanism that facilitates this cross-talk in primary human neoplastic B-cells (Fig. 3).

We observed direct interaction between NIK/TRAF2/TRAF3 complex and SYK in CLL cells, potentially implicating those two kinases in BAFF-BCR cross-talk in neoplastic B-cells (Fig. 3). However, we have not investigated the role of the BCR structures (CD79A/B) or LYN, a BCR-associated kinase constitutively active in CLL cells ²⁹, in this setting. Additional experiments will be required to decipher the exact contributions of the individual kinases and BCR structural components in BAFF-BCR cross-talk in CLL, and will be hampered by the technical challenges of eliminating those individual players in primary B-cells. It is conceivable that multiple conditions need to be fulfilled, where cooperative action involving an intact BCR structure and the SRC family protein kinases is required for NIK-mediated activation of SYK and BCR signal propagation.

Subsequent studies should help elucidate the exact ligand-receptor interactions leading to BCR activation in CLL. BAFF and APRIL bind the two TNFR superfamily members - BCMA and TACI - with high affinity. It would be important to confirm whether APRIL stimulation could replicate some of the BAFF ligand effects. While our data suggest that BAFF-BR3 interaction may be necessary for BCR activation via NIK/TRAF2/TRAF3 complex, isolated and concurrent blockade of the individual signaling receptors ¹⁰ will be necessary to elucidate their importance in regulation of NFκB and BCR signaling pathways, as well as response to BCRi in BAFF-simulated CLL cells.

Thus, this study illuminates the cross-talk between BAFF and BCR signaling pathways in neoplastic B-cells and provides insights into the mechanism of the interaction.

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