Adhesion of ZAP-70+ chronic lymphocytic leukemia cells to stromal cells is enhanced by cytokines and blocked by inhibitors of the PI3-kinase pathway

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CLL cell survival and proliferation is enhanced through direct contact with supporting cells present in lymphoid tissues. PI3Ks are critical signal transduction enzymes controlling B cell survival and activation. PI3K inhibitors have entered clinical trials and show promising therapeutic activity; however, it is unclear whether PI3K inhibitor drugs differentially affect ZAP-70 positive versus negative CLL cells or target specific microenvironmental interactions. Here we provide evidence that CD40L + IL-4, IL-8 or IL-6 enhance adhesion to stromal cells, with IL-6 showing a selective effect on ZAP-70 positive cells. Stimulatory effects of IL-8 or IL-6 are fully reversed by PI3K inhibition, while the effects of CD40L + IL-4 are partially reversed. While CD40L + IL-4 is the only stimulation increasing CLL cell survival for all patient groups, IL-6 protects ZAP-70 positive cells from cell death induced by PI3K inhibition. Altogether, our results indicate that targeting the PI3K pathway can reverse protective CLL–microenvironment interactions in both ZAP-70 positive and negative CLL despite their differences in cytokine responsiveness.

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent form of adult leukemia in Western countries and remains incurable. CLL is characterized by the progressive accumulation of mature, monoclonal CD5+ CD19+ CD23+ B lymphocytes in the peripheral blood, lymph nodes, spleen and bone marrow [1]. CLL cells can present an absence of mutations in the immunoglobulin variable region genes (IgVH) and can over-express zeta-chain TCR-associated protein kinase 70 kDa (ZAP-70) which both are correlated with the aggressiveness of the disease [2]. Nowadays, they are defined as CLL biomarkers.

Lymphoid tissue microenvironments play a major role in CLL by providing extrinsic survival signals for CLL cells. Spontaneous CLL cell apoptosis observed in vitro can be prevented with T-cell-derived cytokines (such as CD40L and IL-4) [3,4] or by co-culture with mesenchymal stromal cells derived from bone marrow [5,6]. Stromal cells promote early B cell development and mature B cell survival through direct cell contact and soluble factors [7,8]. Additional microenvironmental factors may influence CLL, including inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8), which are elevated in plasma from CLL patients with worse clinical course [9–11].

The current treatments for CLL (Chlorambucil, Fludarabine, anti-CD20 Rituximab, etc.) induce apoptosis in CLL cells but lead to significant immune-suppression and patients often develop drug resistance [12]. There is currently no approved treatment for CLL that specifically block signaling pathways promoting CLL cell survival; however several kinase inhibitors are currently being evaluated, including inhibitors of Syk, Btk and phosphoinositide 3-kinase (PI3K). Recent evidence has strongly implicated PI3Ks as critical signaling enzymes impacting CLL survival and response to microenvironmental stimuli [13,14]. Critical receptors on CLL such as antigen receptor (BCR) and IL-4 receptor activate the PI3K enzymes (p110α, β, γ, δ), which then phosphorylate membrane phosphoinositides on the D3 position to generate second messengers such as phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 recruits and activates Akt and a number of other signaling proteins involved in differentiation, growth, proliferation and survival of B...
cells [15]. The PI3K signaling pathway is constitutively active in CLL cells and inhibiting this pathway reduces CLL survival in vitro [16]. Recent work indicates that pan-PI3K inhibitors untie CLL cells from stromal cells and induce CLL cell apoptosis [13]. CAL-101/ GS-1101, a PI3K p110δ inhibitor promoted apoptosis in primary CLL cells ex vivo and this toxicity was not diminished by co-culture on stromal cells [17,18]. Moreover, clinical trials using CAL-101 showed promising preliminary results in the ability to release CLL cells from lymph nodes into the blood, where cells are less protected and more sensitive to treatments [18]. It is currently not clear whether CLL cells from the ZAP-70 positive and negative prognostic groups differ in their sensitivity to PI3K inhibitors or whether the response to these new therapeutics are affected by inflammatory cytokines present in some patients.

In this study, we investigated whether or not CLL cells from ZAP-70 positive and negative patients differ in their capacity to adhere to stromal cells in response to cytokine stimulation and their sensitivity to PI3K pathway inhibition. We discovered that ZAP-70 positive CLL cells show higher adhesion capacity in response to some inflammatory cytokines. PI3K inhibitors are effective in reducing stromal cell adhesion of both ZAP-70 positive and negative CLL cells and can reverse the enhanced adhesion induced by inflammatory cytokines. These results suggest that ZAP-70 positive and negative CLL interact differently with the stromal microenvironment, and this can potentially be influenced by inflammatory cytokines which may impact their respective retention and survival in lymphoid tissues. PI3K inhibition may be an effective strategy for blocking stromal cell interaction with therapeutic benefit for both ZAP-70 positive and negative CLL patients.

2. Methods

2.1. CLL patients and prognostic markers

Peripheral blood from CLL patients was obtained from the Manitoba CLL Clinic and Tumor Bank following informed consent, with the approval of the Research Ethics Board at the University of Manitoba. CLL cells were stained with anti-CD19-V450, anti-CD5-APC, anti-CD3-PE-Cy7, anti-CD38-PE (all from BD Biosciences) for 15 min at room temperature (RT). After washing, cells were fixed and permeabilized using IntraPrep Reagent 162 (Beckman Coulter) and further stained with anti-ZAP-70-FITC (Beckman Coulter). 7-AAD (BD Biosciences) was also added as a control of the fixation/permeabilization process. After washing, cells were analyzed by flow cytometry using a FACS Canto II or LSR-II instrument (BD Biosciences). The cutoff for CD38 and ZAP-70 positivity was 7% and 25%, respectively as these cutoffs were recently shown to be more clinically relevant [19,20]. IgVλ mutation status was determined by PCR amplification and DNA sequencing as previously described [21].

2.2. CLL cell isolation

Mononuclear cells were isolated using a Ficoll-Paque density gradient as previously described [21]. Contaminating cells were removed using B Cell Enrichment Rosettes (StemCell Technologies) according to the manufacture’s protocol. Residual red cells were removed using ACK lysis buffer (Sigma). CLL cells were cultured in complete RPMI-1640.

2.3. CLL cell co-culture with stromal cells

S17 stromal cells were generously provided by Dr. Kenneth Dorshkind, UCLA [22]. 48-well plates were seeded with S17 cells at 5 x 10^3/well in complete Opti-MEM and cultured over-night. Culture medium was then aspirated from wells and CLL cells at 5 x 10^5/mL in complete RPMI-1640 were added. Recombinant human CD40L, IL-4, IL-6 or IL-8 (all from R&D Systems) were reconstituted in sterile PBS solution and used at 50 ng/mL (final concentration). PBS was used as a control. The PI3K inhibitor PI-103 [23] (Cayman Chemical Company), CAL-101 and GDC-0941 (both from Selleck) were dissolved in DMSO (Sigma) and used at a final concentration of 1.43 μM, 5 μM and 5 μM, respectively. An equal final concentration of DMSO was added to control wells.

2.4. Co-culture adhesion assay

We used an assay adapted from Kurtova et al. [5]. After 24 h co-culture, CLL cells not adhered to the stromal cells were harvested by pipetting three times. Stromal cell-bound cells were trypsinized for 5 min in the presence of 0.25% EDTA (Sigma).

Trypsinized cells were harvested and wells were washed once with complete RPMI-1640. Both stromal cell-bound and unbound cell fractions were stained for 15 min at RT with anti-CD19-V450, anti-CD5-APC, anti-CD3-APC-Cy7, 7-AAD and AnnexinV-FITC (all from BD Biosciences). Of note, trypsinization did not affect the expression of cell markers (data not shown). The absolute numbers of live B cells in the stromal cell-bound and unbound fractions were determined by flow cytometry analysis on a FACs Canto-II instrument (BD Biosciences) by acquiring in 30’s after gating on CD19+CD5+CD3−7-AAD−AnnexinV− cells. The percentage of adhesion was calculated by dividing the number of stromal cell-bound CLL cells by the number of total cells (bound + unbound fractions).

2.5. Co-culture survival assay

After 24 h or 48 h co-culture, CLL cells were harvested and the percentage of live (7-AAD−Annexin−), apoptotic (7-AAD−Annexin+) and dead (7-AAD+Annexin+) cells were determined by flow cytometry.

3. Results

3.1. Inhibition of the PI3K pathway blocks binding of CLL cells to stromal cells

CLL cells were co-cultured with stromal cells for 24 h resulting in binding of CLL cells. The bound and unbound fractions were separated by repeated washing of the stromal layer and each cellular fraction was analyzed by flow cytometry (Fig. 1A). The PI3K signaling enzymes are proposed to play important roles in CLL cell interaction with microenvironments and are thus being actively pursued as new therapeutic targets [13,17,24]. We examined the impact of PI3K pathway inhibition on stromal cell binding, and asked whether ZAP-70 positive and negative subgroups may differ in their dependence on this pathway. We treated cells with PI-103, a potent PI3K inhibitor and precursor to the drug GDC-0941 that has recently entered clinical trials [23]. PI-103 treatment was found to decrease stromal cell adhesion by 35% on average (Fig. 1B), consistent with recent studies [13]. The PI3K inhibitors GDC-0941 or CAL-101 reduced CLL cell binding to stromal cells to a similar extent as PI-103 (Fig. 1B). Next, we assessed the effect of PI3K inhibitors on Akt activation and observed that all three inhibitors decreased Akt phosphorylation (Figure S1). Of 56 CLL patients examined (Table S1), 7 patients did not show any difference in CLL cell adhesion to stromal cells after PI-103 treatment. Interestingly, most of these non-responders were either ZAP-70 negative patients, discordants (ZAP-70 negative and unmutated IgVλ or ZAP-70 positive and mutated IgVλ) or previously treated (Table S1). We further compared the impact of PI3K inhibition on adhesion capacity of ZAP-70 positive and negative patients. When all samples were normalized to their own control (DMSO treatment), we observed significant decreases in both groups: 22% decrease in the ZAP-70 negative group and 39% decrease in the ZAP-70 positive group (Fig. 1C). These results suggest that PI3K pathway inhibition can partially block CLL cell adhesion to stromal cells and tends to be more effective in reversing the adhesion capability of ZAP-70 positive CLL cells.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.08.001.

3.2. Inhibition of the PI3K pathway impairs survival of CLL cell in stromal cell co-culture

Stromal cell binding and other microenvironmental interactions are known to influence CLL cell survival [25,26]. We also examined the effect of PI3K inhibition on CLL cell survival in stromal cell co-cultures using 7-AAD and AnnexinV [Fig. 2A]. Inhibition of the PI3K pathway using different inhibitors resulted in a significant decrease in CLL cell survival (Fig. 2B). After 24 h co-culture, we observed a significant decrease in survival with PI-103 treatment.
Fig. 1. PI3K inhibition impairs CLL adhesion to S17 stromal cells. (A) Representative dot plots showing scatter profiles and CD19/CD3 staining of non-adherent and adherent CLL cells harvested from stromal cell co-cultures. The relative numbers of live B cells in the bound and unbound fractions were determined by flow cytometry by acquiring for exactly 30 s after gating on live CD19+ CD3− cells. (B and C) Pan-PI3K inhibitor PI-103 (500ng/mL), PI3Kα-selective inhibitor CAL-101 (5 μM), PI3K inhibitor GDC-0941 (5 μM) or vehicle control DMSO were added to CLL-stromal cell co-cultures and percent adhesion was determined. (B) Normalized percentage of CLL cells bound to S17 stromal cells with different PI3K inhibitor treatment. To normalize, the reference used for each sample was the percent adhesion with DMSO control treatment. Results shown represent median normalized numbers from 13 different patients. Error bars represent the interquartile range. Statistical significance (*p < 0.05) between treatments was analyzed by Friedman test with Dunn’s multiple comparison post test. (C) Normalized percentages of ZAP-70 positive and negative CLL adhesion to stromal cells. The data is from 56 different patients. Statistical significance between DMSO and PI-103 treatments was analyzed by Wilcoxon paired test. Statistics reported were calculated with absolute percentages.

Fig. 2. PI3K inhibition induces CLL apoptosis. CLL cells were co-cultured with stromal cells in the presence or absence of PI3K inhibitors. After one or two day co-culture, CLL cells were harvested and percentage of live (7-AAD− and AnnexinV− cells), apoptotic (7-AAD− and AnnexinV+ cells) and dead (7-AAD+ and AnnexinV+ cells) cells were determined by flow cytometry. (A) Representative dot plots showing AnnexinV/7-AAD staining profiles with or without PI-103. (B) Percentage of live CLL cells with DMSO, PI-103 (500 ng/mL), CAL-101 (5 μM) or GDC-0941 (5 μM) treatment. Results are shown in box plot as median numbers of live cells, first and third quartiles, maximum and minimum values from 13 different patients. Statistical significance (*p < 0.05) between DMSO and PI3K inhibitor treatments was analyzed by Friedman test with Dunn’s multiple comparison post test. (C and D) Percentage of live CLL cells with DMSO or PI-103 (500 ng/mL) treatment after one (C) or two (D) day co-culture in ZAP-70 positive and negative patient groups. The data is from 56 different patients. Statistical significance (*p < 0.05) between DMSO and PI-103 treatments was analyzed by Wilcoxon paired test.
in both ZAP-70 positive and negative patient groups (Fig. 2C). Similar results were seen after 48 h co-culture (Fig. 2D). However, 5 patients showed no change in cell survival in response to PI-103 treatment. Interestingly, 4 of these patients were ZAP-70 positive and have an unmutated IgH using VH1 (U VH1 – Table S1). These data demonstrate that PI3K inhibition effectively impairs CLL cell survival in the presence of stromal cells for the majority of ZAP-70 positive and negative CLL patients.

3.3. Impact of PI3K inhibition on CLL survival in the context of stromal cells, CD40L and cytokines

We further examined the significance of PI3K signaling in CLL survival and adhesion in the context of pro-inflammatory stimuli present in CLL patients. T cell-derived factors CD40L and IL-4 are thought to be present in tissue-associated proliferation centers and support CLL survival [3,4]. Although their role in CLL is not well defined, IL-6 and IL-8 are elevated in plasma from CLL patients with worse clinical course [9–11] and are associated with poor prognosis in elderly CLL patients [27]. We performed CLL-stromal cell co-cultures in the presence of IL-6, IL-8 or CD40L + IL-4 and determined their impact on CLL survival in co-culture. After 24 h or 48 h co-culture, the only stimuli giving rise to a significant increase of CLL cell survival was CD40L + IL-4 stimulation for both ZAP-70 positive and negative groups (Fig. 3A and B). PI3K inhibition by PI-103 results in a significant decrease of CLL cell adhesion in all conditions (Fig. 3 – significance † not shown). Only CD40L + IL-4 stimulation significantly protected both ZAP-70 positive and negative CLL cells from PI-103 induced death (Fig. 3). IL-8 did not have a significant effect on CLL cell survival in co-culture and did not protect from PI-103 induced cell death whereas IL-6 did partially protect cells from PI-103 in the ZAP-70 positive group only (Fig. 3B). These results suggest that PI3K inhibition can significantly impact survival of both ZAP-70 positive and negative CLL in the presence of a variety of microenvironmental factors. However, CD40L + IL-4 stimulation in combination with stromal cells may partially protect death from stimulated by PI3K inhibition. This finding can be extended to IL-6 stimulation as well but only for CLL cells expressing higher level of ZAP-70.

3.4. Inflammatory cytokine-induced increases in CLL cell adhesion to stromal cells are correlated with ZAP-70 expression and blocked by PI3K inhibitor

We examined the impact of inflammatory and T cell associated stimuli on interactions between CLL and stromal cells in co-culture. CD40L + IL-4 stimulation resulted in ~2-fold increase in CLL cell adhesion in both ZAP-70 positive and negative patients (Fig. 4A). Interestingly, IL-6 stimulation resulted in significantly increased stromal cell adhesion only among ZAP-70 positive patients (Fig. 4A). IL-8-induced responses were highly variable, with a subset of patients showing large increases in adhesion (up to 4.5-fold) and others showing no enhancement. Notably, IL-6 and CD40L + IL-4 (but not IL-8)-induced adhesion was correlated with ZAP-70 expression (Fig. 4B). For both groups of patients, PI-103 treatment significantly inhibited cytokine-induced adhesion (significance † not shown in Fig. 4) and no significant difference was observed between patient groups. However, within the ZAP-70 positive group, CD40L + IL-4 stimulation rendered cells partially resistant to PI-103 inhibition of adhesion (Fig. 4A). These results suggest that inflammatory stimuli may differentially impact stromal cell adhesion capability of ZAP-70 positive and negative CLL cells, but the PI3K pathway is universally required.

Fig. 3. Influence of T cell derived or inflammatory cytokines and ZAP-70 status on PI3K inhibitor-induced CLL apoptosis. PBS, IL-6, IL-8 or CD40L + IL-4 (all at 50 ng/mL) were added to CLL-stromal cell co-cultures, with or without PI-103. After one (A) or two (B) day co-culture, the percentage of live (7-AAD– and AnnexinV– cells), apoptotic (7-AAD– and AnnexinV+ cells) and dead (7-AAD+ and AnnexinV+ cells) cells were determined by flow cytometry. Results are shown in box plot as median numbers of live cells, first and third quartiles, maximum and minimum values from 23 different patients. Statistical significance (⁎ p < 0.05) between DMSO and PI-103 treatments was analyzed by Wilcoxon paired test. Since these statistics reflect significant differences for all conditions, they are not shown in the figure. Statistical significance (⁎⁎ p < 0.05) between cytokines and PBS stimulations was analyzed by Friedman test with Dunn’s multiple comparison post test.

4. Discussion

Interaction of CLL cells with stromal cells within lymphoid tissues can provide protective niches where leukemic cells receive survival signals and are thus difficult to eradicate through conventional therapies [28,29]. Interventions that disrupt CLL cell–stromal cell interactions are an attractive therapeutic concept and several such interventions are under study, including blocking individual chemokines and adhesion molecules involved in this process [28,30]. The intracellular signaling processes driving CLL adhesion to stromal cells are poorly understood, but will likely present additional targets for therapy. Here we evaluate the significance of the PI3K lipid kinases in CLL stromal cell adhesion and survival in
co-culture. PI3Ks represent attractive drug targets and are currently the subject of numerous clinical trials for various cancers including leukemia and lymphomas [17,18,23]. Since ZAP-70 is expressed in a subset of CLL cells, we used ZAP-70 to categorize patients groups and examined its potential significance as a factor affecting response to PI3K inhibitor treatment. To take into account the presence of additional factors present in lymphoid tissue microenvironments of some patients, we have further investigated the interplay of stromal cells, IL-6, IL-8 and CD40L+IL-4, and the potential significance of these factors for PI3K inhibitor treatment.

We found substantial evidence that PI3Ks influence CLL–stromal cell interaction and our results support the potential of PI3K inhibitor therapy to act at the level of the leukemia microenvironment to disrupt supportive niches, even in the presence of factors that promote CLL adhesion such as pro-inflammatory cytokines.

Our results are consistent with recently published work showing that PI3K inhibitors can disrupt CLL adhesion and survival in stromal cell co-culture [13,17,18]. We further compared the response of ZAP-70 positive and negative patients and demonstrate that PI3K inhibition may be particularly effective in blocking

**Fig. 4.** PI3K inhibition impairs cytokine-induced adhesion to S17 stromal cells. CLL–stromal cell co-cultures were stimulated with IL-6, IL-8, CD40L+IL-4 (all at 50 ng/mL) or PBS control. Percent adhesion after one day was assessed as previously. (A) In some conditions, DMSO or PI-103 was added to the co-culture system. To normalize, the reference used for each sample was the percent adhesion with PBS control treatment. Results shown represent median normalized numbers. Error bars represent the interquartile range. Statistical significance (\(p < 0.05\)) between DMSO and PI-103 treatments was analyzed by Wilcoxon paired test. Since these statistics reflect significant differences for all conditions, they are not shown in the figure. Statistical significance (\(p < 0.05\)) between cytokines and PBS stimulations was analyzed by Friedman test with Dunn’s multiple comparison post test. Statistical significance (\(p < 0.05\)) between ZAP-70 positive and negative groups was analyzed by Kruskal–Wallis test with Dunn’s multiple comparison post test. Statistics reported were calculated with absolute percentages. (B) Correlation between ZAP-70 expression and percentage of adhesion after IL-6, IL-8 or CD40L+IL-4 treatment. Correlation significance was analyzed by Spearman test. Results from all panels are from 23 patients.
stromal cell adhesion capabilities of ZAP-70 positive CLL cells. This is important, as we and others find that the CLL cells from this patient group frequently display enhanced adhesion capacity and may have distinctive interactions with the tissue microenvironment [31].

Furthermore, it is likely that cytokines and other immunological signals influence interaction of CLL with stromal cells within patient lymphoid tissues. Since a significant number of CLL patients show elevated levels of immunological mediators such as IL-6 and IL-8 [9–11], we tested whether the presence of these stimuli influence stromal cell adhesion. We have recently found that levels of IL-6 and IL-8 correlate with poor prognosis, and that these cytokines can enhance CLL binding to stromal cells in vitro [27]. Here we find that only the ZAP-70 positive group showed significant enhancement in adhesion capacity in the presence of IL-6. Given that IL-6 signals via the STAT3 pathway [32], it is attractive to imagine that ZAP-70 could amplify STAT3 signaling, resulting in more CLL adhesion to the supportive microenvironment. Importantly, PI3K inhibition was able to inhibit CLL binding to stromal cells and CLL survival in the presence of the inflammatory cytokines tested.

Our experiments showed that T cell-associated stimuli CD40L+IL-4 had a distinct effect on CLL cell survival and adhesion to stromal cells. Notably, CD40L+IL-4 induced increases in stromal cell binding and survival that were only partially reduced by PI3K inhibition. This suggests that CD40L+IL-4 may enhance CLL stromal cell binding and survival capacity through PI3K-independent mechanisms. This conclusion is consistent with findings that most CD40L-induced responses in murine B cells, such as proliferation and isotype switching, are not blocked by PI3K inhibitors or genetic deficiency in PI3K [33,34]. In contrast, most murine B cell responses to IL-4 were shown to depend highly on PI3K [35]. Thus, combined targeting of PI3K and CD40 may be necessary to thoroughly disrupt the interaction of CLL cells within their supportive niches in lymphoid tissues. It would be of interest to determine whether combining a PI3K inhibitor such as CAL-101/NS-1101 with an inhibitor targeting the CD40/CD40L system such as dacetuzumab (SGN-40, a humanized anti-CD40 monoclonal antibody) [17,36] is more effective for ZAP-70 positive CLL patients. This will be part of future investigations.

Stromal cells can promote CLL survival via both cell contact-dependent and soluble factors. It seems likely that the effect of PI-103 on CLL survival is at least partly due to disruption of cell adhesion and thus disruption of contact-dependent survival signals. In addition to impacting CLL cell signaling, PI3K inhibition may also affect stromal cell biology by modulating the expression of cell surface or secreted molecules. However, under the conditions of our experiments we did not observe significant effects of PI-103 on stromal cell survival, adhesion or morphology (data not shown). Moreover, a few CLL patients were identified (12.5%) who were apparently completely resistant to the inhibitory effects of PI-103 on adhesion or cell survival in coculture, suggesting the stromal cells remain relatively intact and functional in the presence of the drug. Interestingly, these PI-103 resistant patients often show discordant ZAP-70 and IgVH mutation status and may define a subgroup of CLL patients who are less dependent on the PI3K pathway. It is possible that specific genetic abnormalities may be associated with PI3K inhibitor resistance and it will be interesting to examine the cytogenetic status of these patients. Thus while our results suggest that these drugs may provide therapeutic benefit in both ZAP-70 positive and negative CLL cells by disrupting protective niches; they also provide clinical and biomarker data regarding a small subset of patients whose CLL cells may be less dependent on this pathway.

In summary, our study identifies heterogeneity in CLL cell binding to stromal cells that correlates with ZAP-70 expression and can be influenced by the inflammatory milieu. PI3K inhibition appears to be a relatively robust strategy to disrupt CLL cell-stromal cell interaction regardless of ZAP-70 status or presence of inflammatory cytokines, with the possible exception that increased CLL adhesion and survival induced by CD40L may be less sensitive to PI3K inhibitors. Our results indicate that treatment of CLL patients with PI3K inhibitors may be effective in disrupting protective interactions with lymphoid tissue microenvironments and sensitizing to other therapeutics.

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Conflict of interest

The authors declare no competing financial interests.

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Contributions: STL performed experiments. STL and AJM analyzed results and made the figures. STL, JBM, SBG and AJM designed the research. STL and AJM wrote the paper.

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