

c-Abl regulates Mcl-1 gene expression in chronic lymphocytic leukemia cells

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Chronic lymphocytic leukemia (CLL) is a malignancy characterized by clonal expansion of mature B cells that are resistant to apoptosis. This resistance to apoptosis partly results from Mcl-1 expression because high levels of this protein in CLL cells correlate with poor disease prognosis and resistance to chemotherapy. Thus, understanding the mechanism(s) regulating Mcl-1 expression in CLL cells may be useful in the development of new therapies for this incurable disease. In the present study,

we show a strong relationship between c-Abl and Mcl-1 expression in CLL cells. We show that treatment of CLL cells with Abl-specific siRNA or with imatinib, to inhibit c-Abl activity, results in the down-regulation of Mcl-1 protein and mRNA. A major regulator of Mcl-1 gene expression is STAT3. Our data show that CLL cells expressing high levels of c-Abl also show elevated levels of phospho-STAT3, and that STAT3 phosphorylation in CLL cells is dependent on c-Abl activity. However,

STAT3 phosphorylation by c-Abl requires activation of nuclear factor- κ B, secretion of autocrine interleukin-6, and active protein kinase C. Taken together, our data demonstrate the mechanism involved in c-Abl regulation of Mcl-1 expression in CLL cells, and suggest that c-Abl inhibition has therapeutic application in the treatment of this disease. (*Blood*. 2011; 117(8):2414-2422)

Introduction

Chronic lymphocytic leukemia (CLL) is a malignant disease characterized by the gradual accumulation of mature, nonproliferative B cells expressing a distinctive group of surface markers, including CD19, CD23, and CD5.^{1,2} It is a heterogeneous disease that can follow an indolent or aggressive course, and understanding the nature of this heterogeneity is an area of intense research interest.² CLL cells are resistant to apoptosis, and this is thought to be regulated by survival signals from the microenvironment coupled with intrinsic defects in apoptotic machinery.³⁻⁷ Therefore, understanding in greater detail the molecular basis of such apoptotic resistance may help lead to the discovery of agents effective in CLL treatment.

The more aggressive, poor prognostic course of CLL positively correlates with a series of cellular markers, such as ZAP70, CD38, and unmutated IgV_H.⁸⁻¹⁰ Recently, it has been reported that high expression levels of the antiapoptotic protein Mcl-1 in CLL cells correlate with both poor disease prognosis¹¹ and in vitro and in vivo chemoresistance.¹¹⁻¹⁴ Moreover, down-regulation of Mcl-1 using antisense oligonucleotides results in cell death during in vitro culture,¹⁵ and elevated Mcl-1 protein expression has been shown to prolong the survival of CLL cells exposed to a variety of apoptosis-inducing stimuli.¹⁶ Taken together, this evidence establishes Mcl-1 as a critical mediator of CLL cell survival.

Another signaling protein overexpressed in CLL cells that plays a role in apoptotic resistance is the nonreceptor tyrosine kinase c-Abl.¹⁷ c-Abl appears to be important for normal B-cell development because targeted disruption of the gene encoding this protein in mice results in development of lymphopenia.¹⁸ c-Abl functions in B cells to phosphorylate CD19 after antigen engagement of the B-cell receptor (BCR), highlighting c-Abl as an important regulator

of B-cell signaling.¹⁹ In CLL, the overexpression of c-Abl stimulates activity of the nuclear factor- κ B (NF- κ B) pathway, leading to increased resistance of the malignant cells to spontaneous apoptosis.¹⁷ Thus, a pathophysiologic role for this kinase in CLL is in the regulation of prosurvival signaling.

Active NF- κ B is known to induce the autocrine expression of cytokines, such as interleukin-6 (IL-6).²⁰ IL-6 in CLL is associated with adverse disease features and reduced patient survival.⁴ It is thought that elevated levels of IL-6 can promote disease progression either indirectly through effects on the microenvironment or directly through effects on cell survival and proliferation. An example of the direct effect of IL-6 is its capability of activating the transcription factor STAT3 via the classic IL-6 receptor (IL-6R) pathway.²¹ Such stimulation of STAT3 then promotes up-regulation of antiapoptotic genes, such as Mcl-1.²²⁻²⁴ A role for c-Abl in the activation of STAT3 has been described,²⁵ as well as a connection, in chronic myelogenous leukemia (CML) cells, between c-Abl and Mcl-1.²¹ Therefore, we hypothesize that c-Abl in CLL cells may play a role in the regulation of Mcl-1 expression through a mechanism involving activation of NF- κ B and IL-6-mediated activation of STAT3.

In the present study, we show that c-Abl expression in CLL cells is correlated with both Mcl-1 mRNA levels and STAT3 activation. Furthermore, we also show that treatment of CLL cells with either imatinib (a c-Abl-specific inhibitor) or c-Abl-specific siRNA inhibits STAT3 activation and Mcl-1 transcription. Examination of the mechanism of c-Abl-induced Mcl-1 gene expression in CLL cells shows that NF- κ B, production of autocrine IL-6, and active protein kinase C (PKC) are all involved in this process. Collectively, our findings highlight an

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important antiapoptotic mechanism controlling Mcl-1 expression and CLL cell survival.

Methods

Materials

Anti-Mcl-1, anti-CrkL, anti-I κ B α , anti-Lck, anti-STAT3, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology. Anti-poly (ADP-ribose) polymerase and antihuman IL-6R antibodies were from R&D Systems. Anti-pSTAT3 (Y⁷⁰⁵), anti-pCrkL (Y²⁰⁷), and anti-pI κ B α (S^{32/36}) antibodies were from Cell Signaling Technology (New England Biolabs). Anti-Abl was from BD Biosciences. Imatinib was from Novartis. BAY11-7082, bisindolylmaleimide I, mpV(pic), STAT3 inhibitor VII, SN50 inhibitor, and SN50 control peptides were from Calbiochem (Merck Chemicals). c-Abl and control siRNA were from QIAGEN. Bryostatin 1 and anti- β -actin were from Sigma-Aldrich. Recombinant human IL-6 was from Invitrogen. Mcl-1 and RPL27 primers were from Eurofins MWG Operon. Z-VAD-OMe-FMK was from Bachem.

Patient samples

CLL cells were obtained from the peripheral blood of patients by 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 Leukemia Biobank. When required, cells were thawed, resuspended in culture media, and equilibrated as described previously.¹⁷

Western blotting

CLL cells were lysed, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted as previously described.²⁶ c-Abl protein levels were quantitated in Western blots by comparing the chemiluminescence of c-Abl in cell lysates with that of known amounts of recombinant c-Abl protein.²⁶

Immunoprecipitation

A total of 1×10^7 CLL cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150mM NaCl, 50mM sodium fluoride, 25mM sodium pyrophosphate, 50mM sodium glycerophosphate, 2mM ethylenediaminetetraacetic acid, and 2mM ethyleneglycoltetraacetic acid). Lysates were centrifuged at 16 000g for 30 minutes, and the supernatant was incubated with 2 μ g of anti-Lck (overnight, 4°C), followed by 1 hour with Protein G-agarose (GE Healthcare). Immune complexes were then washed with RIPA buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot.

RT-PCR analysis

RNA extraction, reverse transcription, and real-time amplification were as previously described.²⁶ Mcl-1 was amplified using forward (5'-CTCGTAAGGACAAAACGGGAC-3') and reverse (5'-CTCCTCCATAGCTTCCCAAAC-3') primers. RPL27 was used as an internal control, and was amplified using forward (5'-CAAGTTCATGAAACCTGGGAAG-3') and reverse (5'-GCAGTTTCTGGAAGAACCATT-3') primers. All polymerase chain reactions (PCRs) were performed on a Stratagene Mx3005P quantitative reverse-transcribed (RT)PCR machine as described previously.²⁶

Nucleofection

A total of 5×10^6 CLL cells were transfected using Amaxa Solution V Nucleofector Kit following the manufacturer's instructions (Amaxa). c-Abl or control siRNA (1 μ M) was added to cellular suspensions before using

program U-15 to transfect the cells. After 72 hours, cells were harvested and their protein content was analyzed by Western blot.

IL-6 immunoassay

CLL cells were incubated for 6 hours at 37°C before isolating culture supernatants. IL-6 levels in these supernatants were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems). IL-6 levels were assayed in duplicate with all values expressed as a mean of the 2 determinations. The concentration of IL-6 in the patient samples was determined from a standard curve.

Statistical analysis

Datasets were compared for statistical significance using either Student *t* test or Mann-Whitney U test. The comparisons were performed using Microsoft Excel and SPSS Version 15.0 software, respectively.

Results

c-Abl regulates Mcl-1 expression in CLL cells

Previous work has shown that Mcl-1 and c-Abl expression are important factors contributing to malignant cell resistance to apoptosis and poor disease prognosis in CLL.^{5,11,17,27} A relationship between these 2 factors in CLL cells is suggested by demonstrations of BCR-ABL-mediated regulation of Mcl-1 gene expression in CML cells.^{28,29} To assess the role of c-Abl in Mcl-1 expression in CLL cells, we used imatinib, which is a specific inhibitor of this kinase. To determine the optimal concentration of imatinib needed to inhibit c-Abl in CLL cells, we performed a concentration-response experiment measuring the phosphorylation level of CrkL (v-crk sarcoma virus CT10 oncogene homolog [avian]-like, a protein that is a direct substrate of c-Abl³⁰) in relation to increasing concentrations of imatinib. We found that imatinib had dose-dependent inhibition on CrkL phosphorylation, with the maximal effect achieved at 20 μ M (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

At 20 μ M, imatinib is reported to inhibit c-Kit, platelet-derived growth factor receptor, and Lck. Whereas the former two are not expressed by CLL cells, Lck expression has been observed. To test the effect of imatinib on Lck activity in CLL cells, we used 20 μ M imatinib and 500nM dasatinib (a general src-family kinase inhibitor) and analyzed immunoprecipitated Lck for phosphorylation at its active site using anti-pY⁴¹⁶-Src antibody. We found that the presence of 500nM dasatinib almost completely inhibited Lck phosphorylation at its active site. However, the presence of 20 μ M imatinib only had partial, if any, effect on Lck phosphorylation (supplemental Figure 1E). In whole cell lysates, similar effects of dasatinib and imatinib were observed with respect to active src kinase antibody reactivity (supplemental Figure 1E). Thus, taken together with the other data presented in Figure 1 showing the effect of imatinib on pCrkL levels, these results demonstrate that c-Abl is the kinase that is affected most by imatinib treatment of CLL cells.

We next examined the effect of imatinib on Mcl-1 expression in CLL cells and found a significant reduction in expression of this protein in treated compared with untreated cells (Figure 1A-B). We also observed cleavage of poly (ADP-ribose) polymerase in the treated cells (Figure 1A), indicating that caspases had become active and suggesting that imatinib stimulates CLL cell apoptosis. These results reinforce previous work showing the importance of c-Abl in CLL cell survival¹⁷ and further suggest that a mechanism

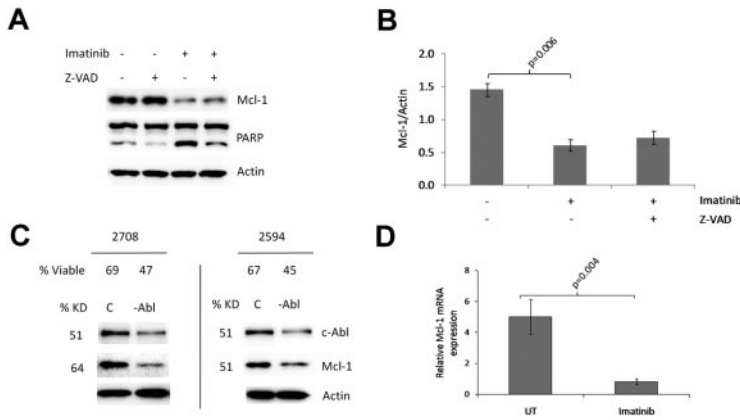


Figure 1. c-Abl inhibition decreases Mcl-1 expression in CLL cells and induces apoptosis. (A) Western blot analysis of Mcl-1 expression in CLL cells treated with imatinib. CLL cells were cultured for 24 hours in the presence or absence of 20 μ M imatinib and/or 50 μ M z-VAD. Lysates were probed by Western blot for the indicated proteins. (B) Graphical representation of the data in panel A. Mcl-1 expression is quantitated relative to β -actin and normalized between patient samples to the levels of Mcl-1 in freshly thawed CLL cells. The data are presented as the mean \pm SEM using the malignant cells from 4 different patient samples. (C) Western blot analysis of Mcl-1 expression of CLL cells transfected with control (c) and c-Abl-specific (-Abl) siRNA. Cell viability (% viable) of the transfected cells, as determined by trypan blue, and percentage knockdown (% KD) of c-Abl and Mcl-1 are indicated. (D) Quantitative RT-PCR analysis of Mcl-1 mRNA levels in CLL cells treated with imatinib. CLL cells were cultured for 24 hours in the presence or absence of 20 μ M imatinib. Mcl-1 mRNA levels were measured relative to those of RPL27 (a housekeeping gene). The results are presented as mean \pm SEM using the malignant cells from 6 different patient samples. Statistical significance in all parts of this figure was determined using a Student *t* test.

of this survival involves c-Abl-mediated control of Mcl-1 expression.

However, Mcl-1 can be cleaved by caspases during the onset of apoptosis.³¹ Thus, the apparent down-regulation of protein expression may be the result of the induction of CLL cell apoptosis and activation of caspases. To test this possibility, we used the pan-caspase inhibitor z-VAD. We found that the presence of 50 μ M z-VAD in cultures of CLL cells that were treated with imatinib failed to restore Mcl-1 protein expression despite inhibiting poly (ADP-ribose) polymerase cleavage in these cells (Figure 1A-B). This result demonstrates that Mcl-1 protein down-regulation by imatinib is independent of caspase activation.

To test whether the effects of imatinib on Mcl-1 expression in CLL cells were the result of inhibition of c-Abl, we used specific siRNA. Figure 1C shows that siRNA-mediated knockdown of c-Abl expression in CLL cells resulted in a concomitant reduction in Mcl-1 expression, whereas the control siRNA had no effect. This demonstrates that imatinib induces Mcl-1 down-regulation through its ability to inhibit c-Abl.

To explore the mechanism of Mcl-1 regulation by c-Abl, we next examined Mcl-1 mRNA levels in CLL cells by quantitative RT-PCR. Figure 1D shows that CLL cells incubated in the presence of imatinib show a significant decrease in Mcl-1 mRNA compared with untreated cells. This suggests that the down-regulation of

Mcl-1 protein after c-Abl inhibition is the result of associated down-regulation of Mcl-1 gene expression.

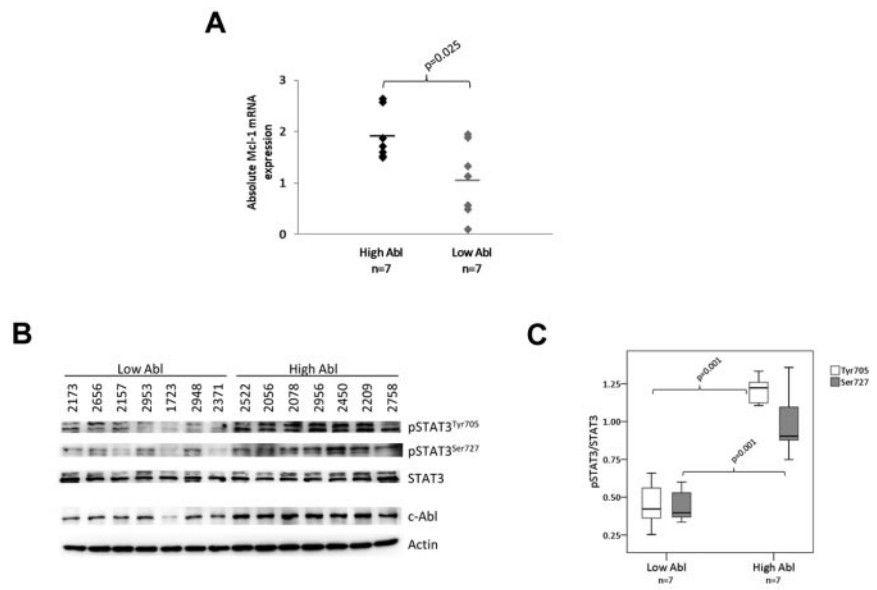
c-Abl levels in CLL cells correlate with Mcl-1 gene expression and STAT3 activation

We further investigated the relationship between c-Abl and Mcl-1 gene expression by comparing the levels of Mcl-1 mRNA in CLL cells containing high levels of c-Abl (cAbl^{high}-CLL cells) with those containing low levels of c-Abl (cAbl^{low}-CLL cells). We found significantly higher Mcl-1 expression in cAbl^{high}-CLL cells than in cAbl^{low}-CLL cells (Figure 2A), suggesting a link between c-Abl protein and Mcl-1 gene expression.

STAT3 is a known regulator of Mcl-1 gene expression,³² and it is known that this protein is constitutively phosphorylated on S⁷²⁷ in CLL cells.^{33,34} Furthermore, it is also known that BCR-ABL stimulates Mcl-1 gene transcription through a mechanism involving activation of STAT proteins.^{28,29} Thus, c-Abl in CLL cells may regulate Mcl-1 by activating STAT3. To investigate this possibility, we used Western blot analysis to determine pS⁷²⁷-STAT3 levels in lysates of cAbl^{high}-CLL and cAbl^{low}-CLL cells. We found that lysates of cAbl^{high}-CLL cells showed significantly higher levels of pS⁷²⁷-STAT3 than did those of cAbl^{low}-CLL cells (Figure 2B-C). This result confirms those of others showing constitutive serine

Figure 2. c-Abl levels in CLL cells correlate with Mcl-1 gene expression and STAT3 activation.

(A) Quantitative RT-PCR analysis of Mcl-1 mRNA expression in cAbl^{high}-CLL and cAbl^{low}-CLL cells (*P* = .025, *n* = 14). Mcl-1 levels are presented relative to those of RPL27. (B) Western blot analysis showing pS⁷²⁷- and pY⁷⁰⁵-STAT3 levels in 7 high and 7 low c-Abl expressing CLL cases. (C) Box plot representation of the data in panel B. Clear box plots represent pY⁷⁰⁵-STAT3 levels; and shaded box plots, pS⁷²⁷-STAT3 levels. Statistical significance was performed using Mann-Whitney U test (*P* = .001, *n* = 14).



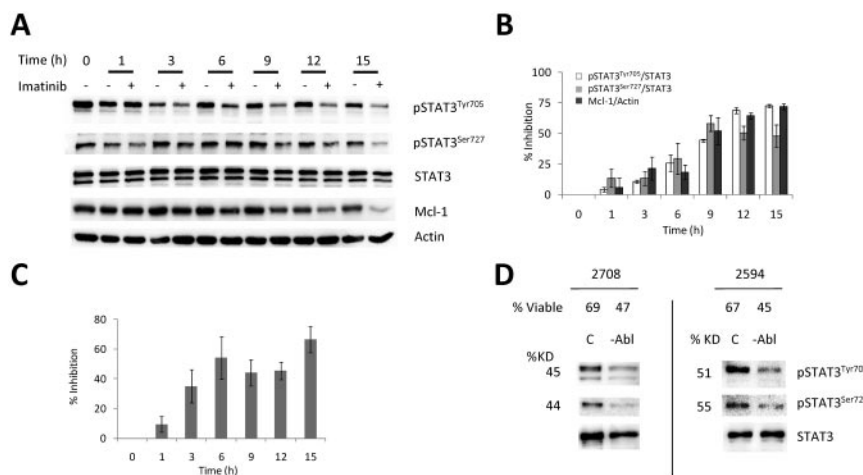


Figure 3. c-AbI regulates STAT3 activation and Mcl-1 transcription in CLL cells. (A) Western blot analysis of the effect of imatinib on pSTAT3 and Mcl-1 protein expression. CLL cells were cultured overnight and then incubated in the presence or absence of imatinib (20 μ M) for the indicated times. (B) Graphical representation of the data in panel A, showing percentage inhibition of pSTAT3 and Mcl-1 expression after imatinib treatment (mean \pm SEM). Results are representative of 3 different CLL patient samples and are expressed as the percentage inhibition relative to the levels of pSTAT3 and Mcl-1 in untreated CLL cells incubated overnight. (C) The effect of imatinib on Mcl-1 gene expression. CLL cells from 3 different patients were incubated overnight before treating with imatinib (20 μ M) for the indicated time points. Mcl-1 mRNA levels were quantified using quantitative RT-PCR. Results are expressed as the percentage inhibition of Mcl-1 mRNA expression relative to the levels in untreated CLL cells incubated overnight (mean \pm SEM). (D) Western blot analysis of STAT3 phosphorylation in CLL cells transfected with control (c) or c-AbI-specific (-AbI) siRNA. Cell viability (% viable) of transfected cells, as measured by trypan blue, and percentage knockdown (% KD) of pS⁷²⁷- and pY⁷⁰⁵-STAT3 are indicated; % KD of c-AbI is presented in Figure 1C.

phosphorylation of STAT3 in CLL cells,^{33,34} and suggests that c-AbI contributes to the level of STAT3 phosphorylation.

We also analyzed STAT3 for pY⁷⁰⁵ despite reports indicating that this residue in STAT3 is not constitutively phosphorylated in CLL cells.^{33,34} In contrast to the results of others, we detected low, but measurable, levels of pY⁷⁰⁵-STAT3 in all CLL cell lysates tested. However, we also observed significantly more pronounced levels of pY⁷⁰⁵-STAT3 associated with cAbI^{high}-CLL than with cAbI^{low}-CLL cells (Figure 2B-C). Thus, these data support the notion that c-AbI may contribute to the level of STAT3 phosphorylation, and, taken together with the data presented in Figure 2A, further suggest that c-AbI may regulate Mcl-1 gene expression through activation of STAT3.

c-AbI regulates STAT3 activation and Mcl-1 transcription

To investigate a possible relationship between c-AbI and STAT3 in the regulation of Mcl-1 expression, we treated CLL cells with imatinib and analyzed STAT3 phosphorylation status and Mcl-1

expression. We found that the presence of imatinib in CLL cell cultures reduced the levels of both pY⁷⁰⁵-STAT3 and pS⁷²⁷-STAT3 and that this corresponded with a concomitant reduction in Mcl-1 mRNA and protein levels (Figure 3A-C; supplemental Figure 2). That c-AbI mediates STAT3 phosphorylation is demonstrated in Figure 3D. CLL cells treated with siRNA targeting c-AbI had reduced levels of pY⁷⁰⁵-STAT3 and pS⁷²⁷-STAT3, whereas those treated with control siRNA did not. The results of this latter experiment agree with those presented in Figure 1C showing that c-AbI-specific siRNA reduces the expression of Mcl-1. Thus, c-AbI regulates Mcl-1 expression in CLL cells probably through a mechanism involving the activation of STAT3.

To address potential controversy between our data and those of others^{33,34} regarding the presence of pY⁷⁰⁵-STAT3 in CLL cells, we analyzed STAT3 phosphorylation over a 24-hour time course (Figure 4A; supplemental Figure 3A). We observed that the levels of phosphorylated STAT3 in CLL cells increased dramatically after 24 hours of culture and that a marked increase in phospho-STAT3

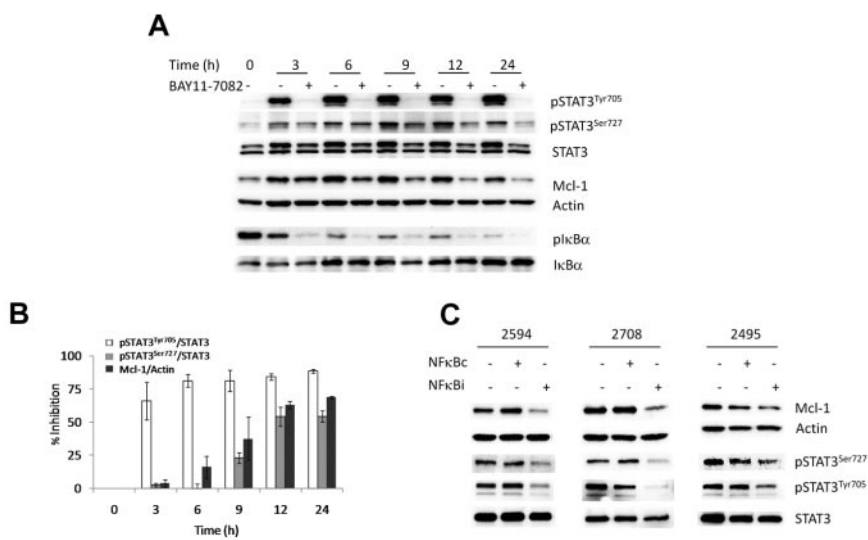


Figure 4. NF- κ B regulates STAT3 activation in CLL cells. (A) Effect of NF- κ B inhibition on pSTAT3 and Mcl-1 expression. CLL cells were incubated for 24 hours in the presence or absence of 2 μ M BAY11-7082, before probing for indicated proteins in CLL cell lysates by Western blot. pI κ B α levels were also measured as a control for the effects of BAY11-7082. (B) Graphical representation of the data shown in panel A, showing the percentage inhibition of pSTAT3 and Mcl-1 after BAY11-7082 treatment. Results are representative of 3 different CLL patient samples and are expressed as the percentage inhibition relative to 0 hours untreated CLL cells (mean \pm SEM). (C) CLL cells were incubated for 12 hours in the presence or absence of a control peptide (c) or the SN50 NF- κ B peptide inhibitor (i), before probing for pSTAT3 and Mcl-1 expression by Western blot.

was apparent, even after 3 hours of culture. Interestingly, the increase in phospho-STAT3 was matched by a similar increase in CrkL phosphorylation (supplemental Figure 3B). Because c-Abl protein levels did not change over the same incubation period, these results suggest that c-Abl activation was being induced. Thus, these data show that STAT3 becomes spontaneously phosphorylated when CLL cells are cultured in vitro. Furthermore, taken together with the other results presented in Figure 3, these data also indicate that spontaneous STAT3 activation in CLL cells is mediated by increased c-Abl activity, resulting in Mcl-1 gene expression and protein up-regulation.

NF- κ B regulates STAT3 activation and Mcl-1 expression in CLL cells

We have previously shown that the inhibition of c-Abl results in suppression of NF- κ B pathway activation in CLL cells.¹⁷ To explore whether this has a role to play in the regulation of c-Abl-mediated Mcl-1 expression in CLL cells, we used BAY11-7082, a compound that specifically inhibits the phosphorylation of the NF- κ B inhibitory protein I κ B α .³⁵ Figure 4A and B shows that treatment of CLL cells with 2 μ M BAY11-7082 inhibited the spontaneous phosphorylation of STAT3 on S⁷²⁷ and Y⁷⁰⁵ and resulted in down-regulation of Mcl-1 expression. The presence of BAY11-7082 also reduced the amount of pI κ B α in CLL cell lysates, indicating it was inhibiting I κ B-kinase activity and suggesting a potential role of the NF- κ B pathway in STAT3 activation. To confirm this potential role, we used the NF- κ B inhibitor peptide SN50. This peptide binds to the nuclear localization sequence of NF- κ B and prevents its translocation into the nucleus.³⁶ Figure 4C shows that the presence of 18 μ M SN50 in CLL cell cultures inhibits both STAT3 phosphorylation and Mcl-1 expression, whereas the control peptide had no effect. Because BAY11-7082 and SN50 inhibit the NF- κ B pathway at different levels, these experiments strongly suggest that activation of the NF- κ B pathway is an important regulator of STAT3 activation and Mcl-1 expression in CLL cells.

NF- κ B pathway activation by c-Abl induces autocrine IL-6 expression and Y⁷⁰⁵ phosphorylation of STAT3 in CLL cells

In other cell types, NF- κ B pathway activation is known to induce autocrine IL-6 production and result in the activation of STAT3.^{20,22,23} We have previously shown that c-Abl can regulate NF- κ B pathway activation in CLL cells,¹⁷ and others have shown that CLL cells are able to secrete autocrine IL-6.³⁷ Taken together with these findings, our observation that the NF- κ B pathway stimulates STAT3 activation suggests a mechanism whereby c-Abl promotes STAT3 phosphorylation and Mcl-1 gene transcription through induction of NF- κ B-mediated autocrine IL-6 production. To investigate this hypothesis, we examined the role of c-Abl in the secretion of IL-6 from CLL cells. Pretreatment of CLL cells with 20 μ M imatinib or 2 μ M BAY11-7082 significantly inhibited IL-6 production as measured by enzyme-linked immunosorbent assay of culture supernatants (Figure 5A). This suggests that c-Abl and NF- κ B are upstream of IL-6 production in CLL cells.

We next treated CLL cells with IL-6R blocking antibodies to investigate the role of autocrine IL-6 in STAT3 activation. Figure 5B shows that blocking the IL-6R on CLL cells completely inhibited spontaneous STAT3 phosphorylation on Y⁷⁰⁵, whereas a control antibody had no effect. However, blocking the IL-6R on CLL cells did not inhibit serine phosphorylation of STAT3, nor did it significantly decrease the expression of Mcl-1 (Figure 5B). When we examined whether BAY11-7082 or imatinib inhibited IL-6R pathway signaling, we found that the presence of these compounds

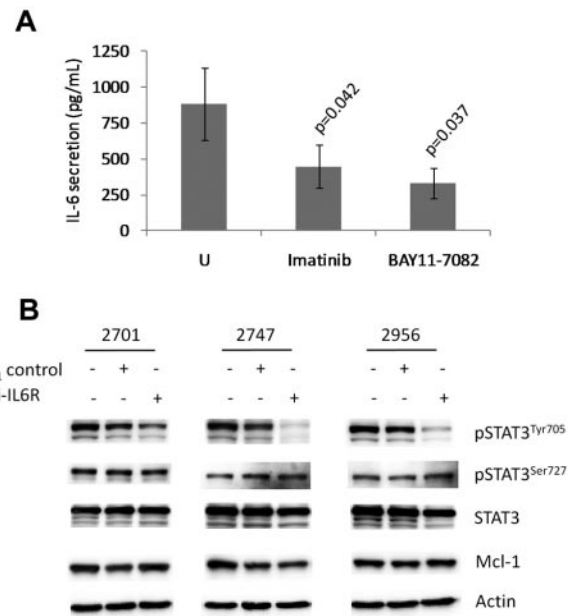


Figure 5. c-Abl-activated NF- κ B in CLL cells causes autocrine IL-6 expression and STAT3 Y⁷⁰⁵ phosphorylation. (A) Enzyme-linked immunosorbent assay measurement of IL-6 secretion from CLL cells cultured for 6 hours in the absence of inhibitor (U), or in the presence of either 20 μ M imatinib or 2 μ M BAY11-7082. The results are representative of 4 different CLL patient samples and are presented as mean \pm SEM. (B) Blocking the IL-6R inhibits IL-6 secretion and Y⁷⁰⁵-STAT3 phosphorylation. CLL cells from 3 different cases were incubated in the presence or absence of 0.5 μ M anti-IL-6R antibody or an IgG₁ isotype control for 12 hours. Lysates of these cells were then probed by Western blot with the indicated antibodies.

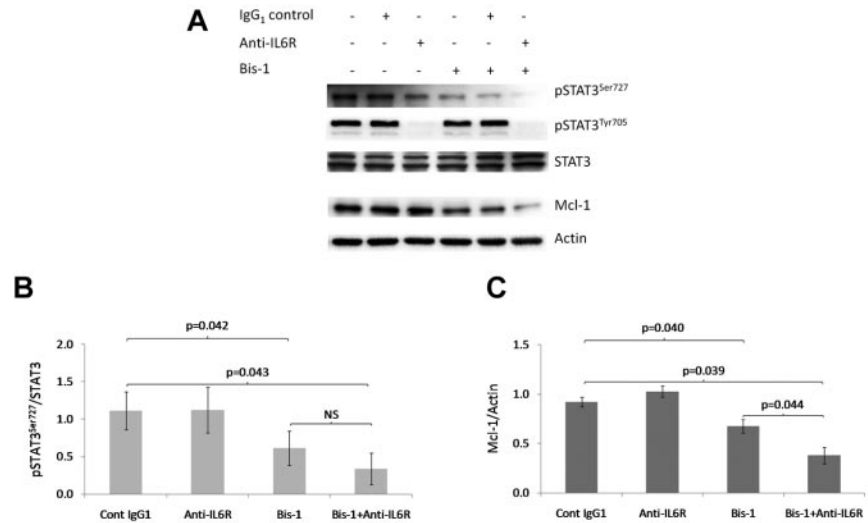
in CLL cell cultures had no effect on the ability of exogenously added recombinant IL-6 to stimulate STAT3 tyrosine phosphorylation (supplemental Figure 4). These data suggest that the induction of Mcl-1 expression by c-Abl-mediated NF- κ B activation requires phosphorylation of S⁷²⁷ in STAT3. Thus, whereas the c-Abl and NF- κ B induction of pY⁷⁰⁵-STAT3 in CLL cells is clearly controlled by autocrine IL-6, the induction of pS⁷²⁷-STAT3 results from a different stimulus.

Induction of pS⁷²⁷-STAT3 in CLL cells by c-Abl and NF- κ B requires activation of PKC

In other cell types, PKC plays an important role in regulating Mcl-1 expression by phosphorylating STAT3.³⁸⁻⁴⁰ To investigate whether PKC is similarly involved in the regulation of Mcl-1 expression in CLL cells, we examined the effect of PKC inhibition on pS⁷²⁷-STAT3 levels and Mcl-1 expression. Figure 6A through C shows that treatment of CLL cells with 1 μ M BisI, a selective PKC inhibitor, significantly reduced both STAT3 S⁷²⁷ phosphorylation and expression of Mcl-1. Interestingly, BisI did not affect pY⁷⁰⁵-STAT3 levels in CLL cells, suggesting that PKC-mediated phosphorylation of STAT3 on S⁷²⁷ is an important part of c-Abl-mediated induction of Mcl-1 expression. To investigate whether pY⁷⁰⁵-STAT3 plays any role in the regulation of Mcl-1 expression, we cocultured BisI-treated CLL cells in the presence of anti-IL-6R antibodies. Figure 6C shows that IL-6R blockade had a small, but significant, additive effect on the inhibition of Mcl-1 expression when used in combination with BisI. Thus, these data suggest that both PKC and the release of autocrine IL-6 are important for STAT3 activation and Mcl-1 expression by c-Abl in CLL cells.

To confirm the role of PKC-mediated STAT3 phosphorylation in regulating Mcl-1 expression in CLL cells, we used the PKC

Figure 6. PKC induces STAT3 S⁷²⁷ phosphorylation and Mcl-1 transcription in CLL cells. (A) Inhibiting PKC and blocking the IL-6R inhibits STAT3 phosphorylation and Mcl-1 expression. CLL cells were incubated for 12 hours in the presence or absence of 1 μ M Bis1 and/or 0.5 μ M anti-IL-6R antibody, or IgG₁ isotype control. Lysates of these cells were then probed by Western blot with the indicated antibodies. (B) Graphical representation of the data shown in panel A. These data highlight the down-regulation of pS⁷²⁷-STAT3 levels that are observed in CLL cells after treatment with Bis1. (C) Graphical representation of the data shown in panel A. These data highlight the down-regulation of Mcl-1 protein levels that are observed in CLL cells after treatment with Bis1. (B-C) Results are representative of 3 different CLL patient samples and are expressed relative to IgG₁ isotype control (mean \pm SEM). Statistical significance was determined using a Student *t* test.



agonist bryostatatin. We found that the treatment of CLL cells with 50nM bryostatatin induced STAT3 phosphorylation on S⁷²⁷ and reversed the reduction in Mcl-1 expression caused by imatinib (supplemental Figure 5A-B). Furthermore, we found that treatment of CLL cells with bryostatatin also reversed the effects of BAY11-7082; however, in this experiment, a complete restoration in Mcl-1 levels was not observed. This is probably because BAY11-7082, by blocking NF- κ B activation, also blocks the positive additive effects of autocrine IL-6 and consequent Y⁷⁰⁵ phosphorylation of STAT3 on Mcl-1 expression in CLL cells (Figure 5A; supplemental Figure 5D). Finally, treatment of CLL cells with the STAT3 inhibitor STAT3i VII blocked bryostatatin-induced Mcl-1 expression (supplemental Figure 5C). This indicates that PKC is involved in Mcl-1 expression upstream of STAT3 activation. Taken together, these results support the notion that PKC-induced phosphorylation of S⁷²⁷ in STAT3 is an important component within the mechanism of Mcl-1 expression by c-Abl.

Discussion

It is widely accepted that Mcl-1 is a critical mediator of malignant cell survival in CLL.¹¹⁻¹⁶ Thus, agents that reduce Mcl-1 expression will have clinical application in this disease. Our laboratory has previously shown that c-Abl is overexpressed in CLL cells where it has a cytoprotective function through its ability to activate the NF- κ B pathway.¹⁷ Here we show that c-Abl-mediated activation of NF- κ B provides CLL cell cytoprotection through up-regulation of Mcl-1. We also show that the pathway downstream of active NF- κ B in CLL cells involves release of autocrine IL-6, which, together with active PKC, acts to induce STAT3 activation and transcription of the Mcl-1 gene. Taken together, these data strongly suggest that c-Abl inhibition may be of potential use for the treatment of CLL.

Previous work by others has demonstrated that c-Abl inhibition, either with imatinib or dasatinib, sensitizes CLL cells to chemotherapeutic agents, such as fludarabine and chlorambucil.⁴¹⁻⁴³ Furthermore, others have reported that reduction of Mcl-1 levels using antisense oligonucleotides or siRNA also sensitizes CLL cells to anticancer drugs.^{12,15} The present work combines these observations and provides a mechanistic explanation for the sensitizing effects of imatinib and highlights the benefits of inhibiting this

kinase in CLL treatment. We demonstrate that c-Abl inhibition, either with imatinib or through down-regulation with siRNA, reduces both Mcl-1 mRNA and protein expression in CLL cells. Such reduction of Mcl-1 protein levels by imatinib treatment of CLL cells would then result in the increased spontaneous apoptosis we have observed in previous work,¹⁷ as well as the sensitization to anticancer drugs others have observed.⁴¹⁻⁴³ Our finding that c-Abl regulates Mcl-1 expression may also provide an explanation for why the malignant cells from some cases of CLL express more Mcl-1 than do those from other cases. An interesting study by Pepper et al¹¹ has demonstrated that high levels of Mcl-1 expression in the malignant cells of CLL correlate with indicators of poor disease prognosis. Our observation that CLL cells containing high levels of c-Abl also contain high levels of Mcl-1 mRNA, taken together with our previous observations that high c-Abl expression correlates with indicators of poor disease prognosis,¹⁷ suggests that c-Abl plays a role in the regulation of Mcl-1 expression in vivo and illustrates an important link between c-Abl and Mcl-1 in the pathobiology of CLL.

In our studies, the concentration of imatinib required to achieve maximum inhibition of c-Abl in CLL cells (20 μ M) was higher than that needed to inhibit the Bcr-Abl oncogene product in CML cells (5 μ M). The reason for this is unclear but could involve expression levels of drug transport proteins because uptake drug transporters, such as OCT1, and multidrug resistance-associated ABC transporters are known to have critical roles in cell influx and efflux of imatinib.⁴⁴ This notion is supported by studies showing that CLL cells express high levels of multidrug resistance-associated ABC transporters,⁴⁵ and by studies showing that OCT1 expression levels correlate with malignant cell sensitivity to imatinib in CML.⁴⁶ Nevertheless, we found that this drug was specific for c-Abl in CLL cells because the effects of imatinib could be replicated using siRNA-mediated knockdown of c-Abl expression. Furthermore, although our data indicate that 20 μ M imatinib can also partially affect Lck activity in CLL cells, we know that specific Lck inhibition affects CLL cell survival through a different mechanism (V. Thompson, F.T., M.Z., and J.R.S., unpublished data). One potential limitation with our work could be that such high concentrations of imatinib may be difficult to achieve during therapy.⁴⁷ Nevertheless, our findings suggest a mechanism of action for other c-Abl inhibitors, such as dasatinib and possibly

also nilotinib, compounds that are effective at much lower concentrations.

The present study expands our previous work showing the role of c-Abl in NF- κ B pathway activation.¹⁷ We used BAY11-7082 and SN50 as inhibitors of NF- κ B pathway activation. These compounds act at different levels to inhibit translocation of NF- κ B into the cell nucleus.^{35,36} Because both BAY11-7082 and SN50 have the same overall effect on Mcl-1 expression, we can exclude any nonspecific effects of these inhibitors in our experiments. Thus, our demonstration that NF- κ B pathway inhibition down-regulates Mcl-1 expression in CLL cells suggests that one of the consequences of c-Abl-mediated activation of NF- κ B is the induction of Mcl-1 gene transcription. That NF- κ B plays a role in the regulation of Mcl-1 expression in CLL cells can be further interpreted in the light of other studies showing the importance of NF- κ B activation in disease prognosis and in malignant cell resistance to chemotherapeutic agents.^{48,49} When taken together with the results of the present work, these studies suggest that the proapoptotic effects of NF- κ B inhibitors on CLL cells will, at least partly, be mediated by a reduction in Mcl-1 expression.

Active NF- κ B is known to induce the autocrine expression of cytokines, such as IL-6,²⁰ and overexpression of IL-6 in CLL is associated with adverse disease features and reduced survival.⁴ Our results show that CLL cells secrete IL-6 in culture and that both imatinib and BAY11-7082 inhibit this secretion. This indicates that c-Abl-induced NF- κ B pathway activation is responsible for IL-6 production by CLL cells. This is important because STAT3 becomes activated during IL-6-IL-6R interaction, and STAT3 is a principal mediator of Mcl-1 gene transcription.^{20,22-24} Indeed, we observe increased phosphorylation of STAT3 in cultured CLL cells that can be inhibited by the presence of either imatinib or BAY11-7082, or by treating CLL cells with siRNA targeting c-Abl. This demonstrates that c-Abl in CLL cells stimulates STAT3 activation through a mechanism involving NF- κ B and IL-6. This observation is supported by a study showing that dependence on exogenous IL-6 in STAT3-mediated survival of B lymphoma cells can be overcome by expression of active c-Abl²⁵ and by studies linking the oncogenic BCR-ABL fusion protein to STAT activation in CML cells.^{28,29}

However, we found that blockade of IL-6-IL-6R signaling with anti-IL-6R antibodies did not significantly reduce Mcl-1 expression in cultured CLL cells despite the inhibitory effect these antibodies had on the induction of STAT3 phosphorylation on Y⁷⁰⁵. We attribute this to the fact that STAT3 is also phosphorylated on S⁷²⁷ in CLL cells. The results of our experiments confirm reports of constitutive phosphorylation of STAT3 on S⁷²⁷ in CLL cells.^{33,34} That pS⁷²⁷-STAT3 is involved in gene transcription has recently been addressed in a study showing that pS⁷²⁷-STAT3 in CLL cells can translocate to the nucleus and bind DNA, irrespective of Y⁷⁰⁵ phosphorylation, to induce transcription of genes coding for antiapoptotic proteins, such as Bcl-XL and Bcl-2.³⁴ That pS⁷²⁷-STAT3 could stimulate Mcl-1 gene transcription in CLL cells is suggested in another report demonstrating the role of S⁷²⁷ phosphorylated STAT3 in Mcl-1-mediated macrophage survival.³² Thus, we show that treatment of CLL cells with BisI inhibits both phosphorylation of STAT3 on S⁷²⁷ and Mcl-1 expression. Treatment of CLL cells with a combination of BisI and IL-6R blocking antibodies induced a further decrease in Mcl-1 expression. Taken together, these results indicate that, although pY⁷⁰⁵-STAT3 induced by IL-6 can mediate Mcl-1 expression in CLL cells, it appears that pS⁷²⁷-STAT3 is probably the major regulator because the effects of IL-6 on Mcl-1 expression in CLL cells are only observed in the

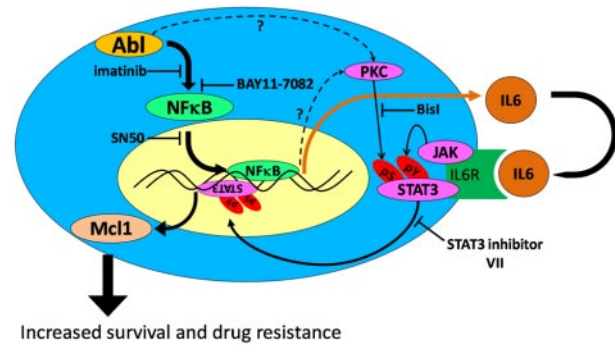


Figure 7. Schematic of the signaling pathway involved in c-Abl-mediated regulation of Mcl-1 expression in CLL cells.

absence of STAT3 phosphorylation on S⁷²⁷. Nevertheless, our demonstration of reduced phosphorylation of STAT3 on both Y⁷⁰⁵ and S⁷²⁷ after treatment of CLL cells with either imatinib or BAY11-7082 indicates that c-Abl and NF- κ B activation are upstream of STAT3 phosphorylation. This role of c-Abl in STAT3 phosphorylation is also supported by our observation that the level of STAT3 phosphorylation correlated with the level of c-Abl expression in CLL cells.

Our observation that the level of STAT3 phosphorylation on Y⁷⁰⁵ increases in cultured CLL cells appears contrary to established reports.^{33,34} The reason for this discrepancy is unclear but could be the result of the sensitivity of the anti-pY⁷⁰⁵-STAT3 antibodies used in the other studies or (possibly more likely) to differing culture conditions where the effects of autocrine IL-6 are diminished.

The kinase responsible for c-Abl-mediated phosphorylation of STAT3 on S⁷²⁷ and activation of Mcl-1 gene transcription is probably PKC. This notion is supported by studies in other cell types showing that novel PKC isoforms, such as PKC δ and PKC ϵ , can target this residue in STAT3.^{38-40,50} Moreover, one study has demonstrated that PKC activation of CLL cells stimulates Mcl-1 expression,⁵¹ and our own work indicates that PKC δ and PKC ϵ are highly expressed in CLL cells.²⁶ In the present study, stimulation of CLL cells with bryostatin induced increased levels of pS⁷²⁷-STAT3 and Mcl-1 protein expression that was dependent on STAT3 activation; treatment of CLL cells with a STAT3 inhibitor before bryostatin stimulation inhibited induction of STAT3 phosphorylation and Mcl-1 expression. That PKC activation is downstream of c-Abl and NF- κ B is indicated by the ability of bryostatin to reconstitute Mcl-1 expression in the presence of imatinib and BAY11-7082. Finally, we used BisI at a concentration of 1 μ M, a concentration that has recently been shown to effectively inhibit PKC activity but not that of p70^{S6K} in studies of whole cells.⁵² Thus, the inhibition of STAT3 phosphorylation on pS⁷²⁷ and partial down-regulation of Mcl-1 expression caused by BisI treatment of CLL cells can be attributed to the effects of this compound on PKC activity and not to the inhibition of other protein kinases, as have been described for this compound.⁵³ Taken together, our results assign therapeutic value to PKC inhibition using compounds, such as BisI, for the treatment of CLL; effective inhibition of PKC would result in decreased STAT3-mediated Mcl-1 expression.

In conclusion, the results of the present study support the signaling model presented in Figure 7. This model indicates that c-Abl, which we have shown is predominantly expressed as a cytosolic isoform in CLL cells,¹⁷ may be a central regulator of different pathways known to have roles in CLL cell cytoprotection. Thus, c-Abl-mediated activation of the NF- κ B pathway results in autocrine IL-6 production and activation of PKC, which together

function to activate STAT3 and result in induction of Mcl-1 gene transcription. The results of the present study more clearly define the pathobiologic role of c-Abl in CLL cells. Future studies investigating the mechanism(s) responsible for the activation of c-Abl, such as a known role for BCR engagement, will now be important because CLL cells expressing high levels of c-Abl and Mcl-1 belong to the poor prognostic group of CLL patients where BCR engagement is an important contributing factor to disease progression.

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Authorship

Contribution: J.C.A. designed the research, performed the experiments, and wrote the paper; F.T. performed experiments; M.Z. designed the research; and K.L. and J.R.S. designed the research and wrote the paper.

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