Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia

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In B-cell chronic lymphocytic leukemia (B-CLL), defective apoptosis causes the accumulation of mature CD5+ B cells in lymphoid organs, bone marrow (BM), and peripheral blood (PB). These cells are the progeny of a proliferating pool that feeds the accumulating compartment. The authors sought to determine which molecular mechanisms govern the proliferating pool, how they relate to apoptosis, and what the role is of the microenvironment. To begin to resolve these problems, the expression and modulation of the family of inhibitor of apoptosis proteins (IAPs) were investigated, with consideration given to the possibility that physiological stimuli, such as CD40 ligand (CD40L), available to B cells in the microenvironment, might modulate IAP expression. The in vivo data on mononuclear cells from PB or BM of 30 patients demonstrate that B-CLL cells on CD40 stimulation express Survivin and that Survivin is the only IAP whose expression is induced by CD40L. Through immunohistochemistry, in vivo Survivin expression in lymph node (LN) and BM biopsies was evaluated. In reactive LN, Survivin was detected only in highly proliferating germinal center cells. In LN from patients with B-CLL, Survivin was detected only in pseudofollicles. Pseudofollicle Survivin+ cells were actively proliferating and, in contrast to Survivin− B cells found in normal GC, were Bcl-2+. In B-CLL BM biopsies, CD5+, Survivin+ cells were observed in clusters interspersed with T cells. These findings establish that Survivin controls the B-CLL proliferative pool interfacing apoptosis and that its expression may be modulated by microenvironmental stimuli. (Blood. 2001;97:2777-2783)

Introduction

B-cell chronic lymphocytic leukemia (B-CLL), the most common leukemia in the Western world, is characterized by the relentless accumulation of monoclonal mature CD5+ B cells in lymphoid organs, bone marrow (BM), and peripheral blood (PB).1 Virtually all circulating B-CLL lymphocytes are long-lived elements arrested in the G0/early G1 phase of the cell cycle,2 but focal aggregates of proliferating cells form the so-called pseudofollicles in lymph nodes (LN)3 and are scattered in the BM, which is a preferred site of relapse.4

The progressive rise of lymphocytes, despite the very low proportion proliferating cells, has led to the notion that B-CLL is primarily related to defective apoptosis.1 Genetic defects of the neoplastic cell and external stimuli may both influence the cell’s abnormal behavior.5 Investigations on the intrinsic ability of B-CLL cells to avoid apoptosis have been mainly centered on the Bcl-2 gene family.6 Even if the t(14;18) translocation is exceedingly rare, the Bcl-2 gene product, which is the prototype antidote to apoptosis, is consistently overexpressed in B-CLL cells7,8 together with high levels of Mcl-1.5,10 The microenvironment likely plays a prominent role because the malignant cells progressively accumulate in vivo, whereas they rapidly undergo apoptosis when cultured in vitro. BM stromal cells11,12 and follicular dendritic cells13 may be involved in the natural history of the disease, but the most attractive candidates are T lymphocytes. Their numbers are increased in B-CLL patients, and activated CD4+ T cells strikingly predominate in BM and LN.14,16

All these considerations lead to the central issue of the disease. CLL cells accumulate because of defective apoptosis that causes extended survival. These cells are the progeny of a pool (albeit small) of proliferating elements that seed the downstream accumulating compartment. The questions are (1) which molecular mechanisms govern the proliferating pool, (2) how are patients interfaced with apoptosis, and (3) what, if any, is the role of the microenvironment. We have approached these problems by investigating the expression and modulation of the family of inhibitor of apoptosis proteins (IAPs)17 and by considering the possibility that physiological stimuli available to B cells in the microenvironment might modulate IAP expression.

IAPs were originally identified in baculoviruses18 and are highly conserved across species. Similar to their viral counterparts, expression of human IAP genes can inhibit apoptosis induced by a variety of stimuli. Six human IAPs have been described so far: NAIP,19 cIAP1, cIAP2,20 XIAP,21 Survivin,22 and Apollon.23 They all appear to suppress apoptosis by caspase and procaspase inhibition,24 therefore representing the last chance for a cell to escape its apoptotic fate. Recent evidence suggests that in vivo caspases are inactive in B-CLL cells, though they are functional
when the cells are cultured in vitro. Therefore, it is conceivable to postulate a role for IAPs in maintaining the disease. Among IAPs, Survivin not only controls apoptosis but also participates in cell-cycle progression, thereby integrating apoptosis and cell division.

As for microenvironmental stimuli, the presence of CD4+ T cells in involved BM and LN led us to focus on the interactions between the B-cell–associated molecule CD40 and its natural ligand (CD40L, CD154). CD40 belongs to the tumor necrosis factor (TNF) receptor superfamily, and its stimulation appears to rescue B-CLL cells from apoptosis and to induce proliferation. CD40L is a member of the TNF family that is expressed on activated T cells.

Our experiments indicated that Survivin plays a role in the pathophysiology of B-CLL for the following reasons: (1) B-CLL cells, on CD40 stimulation, express Survivin; (2) Survivin is the only IAP whose expression is induced in B-CLL cells by CD40L; (3) in vivo Survivin+ cells are proliferating elements that co-express Bcl-2 and are confined within the LN pseudofollicles and in clusters that gather in the BM, interspersed with T cells. These data provide a link between microenvironmental factors, here represented by CD4+ T cells, and the proliferation/apoptosis dilemma of B-CLL cells.

Patients, materials, and methods

Patients

We analyzed PB or BM from 28 patients with B-CLL and from 2 patients with B-prolymphocytic leukemia (B-PLL). Diagnosis was based on standard clinical and laboratory criteria, including cell morphology. All patients were studied either before or at least 3 months after chemotherapy. Patients were staged according to Rai. Table 1 summarizes the main clinical data of the patients.

We used tonsils, obtained as discarded tissues, from children undergoing tonsillectomy. Retrieval of all tissue samples followed the institutional guidelines at Ospedale Mauriziano Umberto I, Torino, Italy.

Cells

Mononuclear cells from PB, BM, and manually disaggregated tonsils were isolated by centrifugation through a Ficoll-Hypaque density gradient. Cells were washed with phosphate-buffered saline solution (PBS) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies, San Giuliano Milanese, Milan, Italy), 2 mM L-glutamine, and 15 µg/mL gentamicin (complete RPMI, cRPMI). Cells were cultured in 6-well plates at a concentration of $1 \times 10^6$ cells/mL with or without 0.5 µg/mL trimeric human CD40L protein (Immunex, Seattle, WA). A fusion protein of CD40L and CD8 was also used, kindly provided by Dr. P. Lane (Oxford, United Kingdom). Mec1 and Mec2 cell lines were maintained in culture in cRPMI.

Flow cytometric analysis and cell sorting

Surface staining of cells was performed using the following monoclonal antibodies (mAbs): fluorescein isothiocyanate (FITC)–labeled anti-CD19 (Becton Dickinson Immunocytometry Systems, San Diego, CA); phycoerythrin (PE)–labeled anti-CD23 and anti-CD38 (Becton Dickinson Immunocytometry Systems, San Diego, CA); tricolor (FITC–PE–peridinin chlorophyll protein–biotin)–labeled anti-CD5, anti-CD19, anti-CD20, and anti-CD38 (Becton Dickinson Immunocytometry Systems, San Diego, CA); and PE-conjugated rabbit antihuman IgM, IgG, IgA, and IgD, antihuman IgG, IgA, and IgD, antihuman kappa light chain, and polyclonal PE-conjugated rabbit antihuman lambda light chain, and polyclonal PE-conjugated rabbit antihuman kappa light chain, and polyclonal PE-conjugated rabbit antihuman lambda light chain, and polyclonal PE-conjugated rabbit antihuman kappa light chain, and polyclonal PE-conjugated rabbit antihuman lambda light chain, and polyclonal PE-conjugated rabbit antihuman lambda light chain.

Intracellular staining was performed after fixation and permeabilization

Table 1. Clinical data of the patients studied

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*B-PLL
R, CD40L-responder; NR, CD40L-nonresponder; ND, not done.
using the Fix & Perm kit (Caltag Laboratories) following the manufac-
turer’s instructions. A mouse monoclonal antihuman Survivin (8E2 clone) was used (D. Altieri, unpublished data, January 2000). FITC-labeled goat antimouse immunoglobulin (SBA) was used as a secondary reagent.

Flow cytometric analysis and cell sorting were performed on a Facsccalibur equipped with the sorter module using Cellquest research software (Becton Dickinson). Gernalimal center B cells were sorted from tonsils as CD19+ CD38- IgD- elements, whereas CLL B cells were identified and sorted as CD19+ CD5- cells.

RNA preparation and RT-PCR

Total RNA was extracted from 1 to 3×10⁶ FACS sorted and unsorted cells and during the 7 days of culture using guanidinium thiocyanate method (RNAzol; Biotecx Laboratories, Houston, TX). The obtained RNA was reverse-transcribed into cDNA using 200 U Superscript II, RNase H reverse transcription (Life Technologies Italia S.tl, San Giuliano Milanese, Milan) and amplified by polymerase chain reaction (PCR). The PCR reaction was performed in 20 μL with 0.2 mM dNTPs, 1× GeneAmp buffer (PerkinElmer Italia, Monza, Italy), 0.5 U AmpliTaq DNA polymerase (PerkinElmer), and 10 pmol each specific primer for: cIAP1, forward 5'-GAC ATC TCT TCA TCG AGG 3'; reverse 5'-CCA CAG GTG TAT TCA TCA TGA C 3'; cIAP2, forward 5'-TCC TAG CTG CAG A TT CGT TC 3'; reverse 5'-GGT AAC TGG CTT GAA CTT GAC 3'; NAIP, forward 5'-AGG AAG TIC ATG GCC ACC CAG CAA 3'; reverse 5'-CTC CTC CAG CAG TAA TTG AGA AAC TGG ACC 3'; XIAP, forward 5'-GGG GAT TAA TCA TGA CTT TTA ACA GTT TTG AAG GA T 3'; reverse 5'-CTC TCG AGC ATG CCT ACT AGA GAG TTA GA 3'. Survivin, forward 5'-GCACCTACATCAGAACGATTG-GCC3'; reverse 5'-TGCCCTTCTTCTGCAG3'.

Cycling conditions were as follows: 1 cycle at 94°C for 2 minutes, 35 cycles at 94°C for 1 minute, annealing temperature for 45 seconds (54°C for cIAP1, 54°C for cIAP2, and Survivin and 52°C for NAIP and XIAP), 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Amplified products were resolved by electrophoresis on a 1.5% agarose gel.

Quantitative assessment of apoptosis

Binding of Annexin V-FITC was used to follow phosphatidylserine exposition on early apoptotic cells. Staining was performed according to the manufacturer’s instructions using the Annexin V-based apoptosis detection kit (R&D Systems, Flanders, NJ). Briefly, 10×10⁶ cells were incubated with saturating concentrations of Annexin V-FITC for 15 to 30 minutes at room temperature and were immediately analyzed on a Facsccalibur (Becton Dickinson).

Cell-cycle analysis

Determination of the percentage of B-CLL cells at each stage of the cell cycle was made by an assessment of DNA content after staining with propidium iodide using DNA con3 kit (Consul T.S., Rivalta, Italy) and amplified by polymerase chain reaction (PCR). The PCR reaction was performed in 20 μL with 0.2 mM dNTPs, 1× GeneAmp buffer (PerkinElmer Italia, Monza, Italy), 0.5 U AmpliTaq DNA polymerase (PerkinElmer), and 10 pmol each specific primer for: cIAP1, forward 5'-GAC ATC TCT TCA TCG AGG 3'; reverse 5'-CCA CAG GTG TAT TCA TCA TGA C 3'; cIAP2, forward 5'-TCC TAG CTG CAG A TT CGT TC 3'; reverse 5'-GGT AAC TGG CTT GAA CTT GAC 3'; NAIP, forward 5'-AGG AAG TIC ATG GCC ACC CAG CAA 3'; reverse 5'-CTC CTC CAG CAG TAA TTG AGA AAC TGG ACC 3'; XIAP, forward 5'-GGG GAT TAA TCA TGA CTT TTA ACA GTT TTG AAG GA T 3'; reverse 5'-CTC TCG AGC ATG CCT ACT AGA GAG TTA GA 3'. Survivin, forward 5'-GCACCTACATCAGAACGATTG- GCC3'; reverse 5'-TGCCCTTCTTCTGCAG3'.

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Results

IAP expression in freshly isolated B-CLL cells

We analyzed mononuclear cells from PB or BM of 30 patients, 28 with B-CLL and 2 with B-PLL (Table 1). B-CLL cells coexpressed CD19, CD5, and CD23 and had weak surface immunoglobulin expression. B-PLL cells had a CD19+, CD5−, CD23+ phenotype and expressed bright surface immunoglobulin. In agreement with published studies,8,10 Western blot analysis revealed that all the patients had high levels of Bcl-2 and Mcl-1 and low levels of Bcl-XL (data not shown).

The percentage of leukemic B cells ranged between 90% and 95% of mononuclear cells. In 5 patients with CLL, CD19+, CD5+ cells were also FACS-separated to a purity level greater than 97%. The expression of cIAP1, cIAP2, NAIP, XIAP, and Survivin was assessed by RT-PCR on both sorted and unsorted samples, and the results were identical.

cIAP1, cIAP2, NAIP, and XIAP were consistently positive in all samples. On the contrary, Survivin expression was undetectable in most patients (25 of 30, 83%). In the remaining 5 patients (patients 5, 8, 9, 18, 25; Table 1) and in 2 CLL cell lines (MEC1 and MEC215), Survivin mRNA was readily amplifiable. This evidence was confirmed at the protein level by cytofluorometric and Western blot studies (Figures 1A, 2). Cell-cycle analysis of some patients (Figure 3A) revealed that the proportion of cycling cells was more pronounced than in Survivin+ patients. Although the number of patients is too small to allow clinical correlation, it is worth mentioning that all 5 Survivin+ patients had active disease, as mentioned previously. One of them (patient 9; Table 1) had B-PLL. In all the other patients a proportion of larger, atypical cells, ranging between...
20% and 40%, was observed in PB smears. On the contrary, only 4 of the remaining 25 patients who did not constitutively express Survivin had active disease requiring treatment.

Survivin expression in CLL cells is modulated by CD40 engagement

We next investigated the in vitro behavior of CLL cells. As expected, these cells started undergoing spontaneous apoptosis after 24 hours of culture in conventional medium. To assess the effect of CD40 stimulation, we set up time-course experiments with cells cultured in the presence or absence of human soluble CD40L. Viability and proliferation were tested every 24 hours up to 7 days.

Two different populations of patients were observed: CD40L responders and CD40L nonresponders. In 15 of 22 (68%) studied patients (Table 1; patients 1-22), CD40L stimulation reduced the entity of spontaneous apoptosis and improved cell viability by 20% to 38% after 48 to 96 hours of culture. These patients were considered CD40L responders. The entity of response was identical irrespective of whether the cells were exposed to monomeric or trimeric soluble CD40L. The increased viability (Figure 4) resulted in a higher number of cells in stimulated versus control cultures. The increase in the proportion of proliferating cells, evaluated on the basis of DNA content assay after 3 days of CD40 stimulation, ranged between 0.5% and 5.3% (Figure 3B) compared to unstimulated controls, indicating that a number, albeit small, of B-CLL cells entered the cell cycle in response to CD40 ligation. The remaining patients (7 of 22, 31%) were considered CD40L nonresponders because both viability and proliferative activity of cultured cells were unmodified after stimulation.

Even if the number of patients studied is too small to draw any statistically significant clinical correlation, we evaluated the possibility that the response to CD40L might be related to a number of known clinical parameters. The response to CD40L did not correlate with stage or duration of disease, nor did it correlate with previous treatments. Also unrelated were disease activity, evaluated according to NCI guidelines,36 the number of atypical cells in PB smears, and the immunoglobulin isotype of the malignant clone.
Given that it has been shown that B-CLL patients can be divided into 2 groups with different prognoses on the basis of CD38 expression, we also evaluated the possibility of a correlation between the expression of CD38 on the cell surface and the response to CD40L. As shown in Table 1, no correlation was observed. No modification in the expression of Bcl-2, Mcl-1, and Bcl-xL proteins were observed in either CD40L responders or nonresponders (data not shown).

We then evaluated the possibility that IAP expression in B-CLL might be modulated by CD40L stimulation. The expression of cIAP1, cIAP2, NAIP, and XIAP remained unmodified in both CD40L responders and CD40L nonresponders. On the contrary, Survivin expression was up-regulated after 48 to 96 hours of CD40 engagement in all CD40L responders. Up-regulation was first observed by RT-PCR (Figure 5) and then confirmed at protein level by cytofluorometric analysis (Figure 1B) and Western blot (data not shown). Cells of patients who were already Survivin+ at day 0 maintained Survivin positivity throughout the culture period if they were exposed to CD40L (Figure 1B). In the tested patients, Survivin expression was irreversibly lost shortly after the beginning of in vitro culture if the cells were cultured in medium alone. No modulation of Survivin expression was observed in the cells of the 7 CD40L nonresponders cultured for up to 7 days in the presence or absence of CD40L.

Survivin expression in CLL lymph nodes and bone marrow

As in vitro data were ascribing a role to Survivin, we evaluated its in vivo expression in LN and BM biopsies by immunohistochemical analysis. In reactive LN, Survivin was detected only in highly proliferating germinal center (GC) cells (Figure 6A-B). Immunohistochemical data were confirmed by RT-PCR analysis on FACS-purified GC cells (data not shown).

Lymph nodes from 4 patients with B-CLL were examined, and the findings were consistent and reproducible. In B-CLL LN, Survivin was detected only in pseudofollicles (Figure 6C-D). Pseudofollicle Survivin+ cells were actively proliferating, as documented by the Ki67-positivity (Figure 7A). At the same time they were uniformly and intensely Bcl-2+ (Figure 7B), indicating a remarkable resistance to undergo apoptosis. Only few elements in pseudofollicles expressed the cyclin kinase inhibitor p27kip1 (Figure 7C). The overwhelming majority of malignant cells invading LN outside pseudofollicles were p27kip1-, Bcl2+ but Survivin+ Ki67+ (Figures 6, 7).

![Figure 5. Survivin expression is induced by CD40 stimulation.](image)

![Figure 6. Survivin expression in reactive and B-CLL lymph nodes (immunoperoxidase staining).](image)

![Figure 7. Serial section analysis of pseudofollicles in B-CLL lymph node.](image)
Discussion

The salient feature of B-CLL natural history is the in vivo accumulation of monoclonal resting B cells. Several defects in the induction of apoptosis, including the overexpression of Bcl-2 and Mcl-1,2,39 concur to create an accumulation compartment that progressively enlarges. More generally, the overall pattern of the expression of the Bcl-2 gene family favors the extended survival of B-CLL cells. On these grounds, B-CLL is considered a malignancy whose destiny is decisively influenced by absent or defective apoptosis.

This interpretation has to be consistent with the obvious need for a proliferative compartment that supplies the accumulation compartment and with the contention that the B-CLL cell genetic abnormalities do not entirely account for all aspects of cell accumulation. The microenvironment presumably exerts a strong influence in vivo as malignant cells very rapidly undergo apoptosis once cultured in vitro. In this work, we demonstrate that Survivin expression in resting B-CLL cells is induced in vitro by CD40 stimulation, that Survivin is the only IAP whose expression is modulated by CD40L, and that in vivo Survivin+ cells are localized in LN pseudofollicles and in rare BM clusters interspersed with T cells. Survivin+ cells proliferate and, at the same time, express Bcl-2. These findings establish 2 major points. First, they indicate that Survivin has an important role in controlling the B-CLL proliferative pool and its ability to nourish the accumulation compartment. Second, they demonstrate that Survivin expression may be modulated by microenvironmental stimuli. A further ancillary point is that the constitutive expression of the anti-apoptotic proteins NAIP, cIAP1, cIAP2, and XIAP by B-CLL cells circulating in the PB may contribute to their accumulation.

Survivin is a protein that prevents apoptosis by blocking caspase activity.38 Furthermore, it is expressed in the G2/M phase of the cell cycle, where it associates with the mitotic spindle microtubules.28 It has been suggested that caspase activity occurs during each cell cycle and that Survivin functions to block this activity.39 In fact, Survivin forms a supramolecular assembly together with caspase-3 and the cyclin-dependent kinase inhibitor p21waf1 within centrosomes,40 thereby preventing apoptosis and preserving p21waf1 integrity during normal mitotic progression.40 In this way, Survivin interfaces cell survival and cell proliferation. By immunostaining reactive LN, we demonstrate that in normal lymphoid tissues, the expression of Survivin is concentrated in GC that represent the B-cell proliferation compartment of lymphoid follicles41 (Figure 6). This observation fits very well with the intense Survivin positivity observed in highly aggressive lymphomas of GC origin.22 In B-CLL, the proliferating pool is located in LN pseudofollicles, where proliferating B-CLL cells coexpress both Survivin and Bcl-2 (Figures 6, 7). This is in striking contrast with normal GC cells that are Bcl-2+.42 Thus, pseudofollicle-proliferating B-CLL cells differ from normal GC cells because they have lost the possibility to undergo apoptosis and are bound to accumulate. The overwhelming majority of LN B-CLL cells are Bcl-2+, p27kip1+ but Survivin−, Ki-67+. Although it remains to be established which mechanisms control the switch-off of Survivin expression in the accumulating compartment, we have acquired information about the mechanisms that control the re-expression of Survivin in cells hidden within the Survivin−accumulation compartment. In vitro we mimicked the stimulus provided in vivo by activated T cells by using soluble CD40L. Our data demonstrate that the stimulation of CD40 is able to induce Survivin expression in B-CLL cells obtained from PB or BM (Figure 5). Therefore, the observation that clusters of Survivin+ cells are detected in the BM (Figure 8), which is a privileged site of disease localization and relapse, and are interspersed with T cells stands as an interesting link between in vitro and in vivo data. This finding is clearly consistent with the notion that many activated CD4+ T cells are present in tissues involved by B-CLL.15

It may be asked why not all patients with B-CLL respond to CD40L stimulation. There are several possible answers. First, our in vitro stimulus may not be strong enough to detect all responsive cells. The number of circulating malignant cells inducible by CD40L may be variable, so that some occurrences are easily disclosed while others remain unnoticed. Second, CD40L might be one of a number of favoring microenvironmental stimuli. Other stimuli, perhaps provided by BM stromal cells12 or follicular dendritic cells,13 may be involved. Third, the term B-CLL probably does not indicate a single homogeneous disease but may cover a wider spectrum of disorders with different stimuli requirements. It has been shown that the presence of somatic mutations of IgV genes and of high percentages of CD38+ cells defines 2 groups of patients with B-CLL with significantly different prognoses.35,36 In this small series, the ability to respond to CD40L does not correlate with the expression of CD38 (Table 1) nor (P. Ghia et al, unpublished results, March 2000) with the presence or absence of immunoglobulin somatic mutations, and it remains to be established whether the responsiveness or unresponsiveness to CD40L may define more subclasses of CLL. Forthcoming clinical studies on a larger series of patients will address this issue and the issue of whether the constitutive expression of Survivin has a clinical correlate, and they may identify a subset of patients with a more aggressive form of the disease as recently shown in diffuse large B-cell lymphomas.44

Taken together, the data obtained lead to a model of B-CLL growth and expansion in which a small proliferating pool within LN pseudofollicles is governed by the expression of Survivin. These cells are bound to accumulate because they also express Bcl-2. They leave the pseudofollicle microenvironment, down-regulate Survivin expression, and stop proliferating. During their long lifespans, they circulate and may easily reach the BM, a privileged site of disease relapse and a sanctuary for CD4+ T cells. In the BM, microenvironmental stimuli such as the CD40 stimulation provided by activated CD4+ T cells may recruit Survivin− B
cells hidden in the accumulating compartment, persuade them to re-express Survivin, and perpetuate a vicious circle. According to this model, microenvironmental stimuli, namely those provided by T cells, help to propagate the disease and give ample justification to treatment approaches with purine nucleoside analogues or monoclonal antibodies such as Campath 1H, which, because they are able to damage both B and T cells, interrupt the interactions between malignant B cells and microenvironmental bystander nontumoral cells. Understanding the mechanisms that allow the progeny of LN pseudolymphocytes to switch off Survivin expression will give therapeutically useful information; the presence of circulating cells that retain Survivin positivity is associated with a higher proliferative activity (Figure 3) and might lead to a more aggressive clinical behavior. Identifying molecules that disrupt Survivin-caspase interactions can provide a new way of eradicating B-CLL by promoting the elimination of the proliferating pool.

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References


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