'From the cradle to the grave': novel therapeutic approaches to attack the microenvironment in chronic lymphocytic leukemia

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Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity

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The gradual accumulation of chronic lymphocytic leukemia (B-CLL) cells is presumed to derive from proliferation centers in lymph nodes and bone marrow. To what extent these cells possess the purported anti-apoptotic phenotype of peripheral B-CLL cells is unknown. Recently, we have described that in B-CLL samples from peripheral blood, aberrant apoptosis gene expression was not limited to protective changes but also included increased levels of pro-apoptotic BH3-only member Noxa. Here, we compare apoptosis gene profiles from peripheral blood B-CLL (n=15) with lymph node B-CLL (>90% CD5+/CD19+/CD23+ lymphocytes with Ki67+ centers; n=9). Apart from expected differences in Survivin and Bcl-XL, a prominent distinction with peripheral B-CLL cells was the decreased averaged level of Noxa in lymph nodes. Mcl-1 protein expression showed a reverse trend. Noxa expression could also be reduced in vitro by CD40 stimulation of peripheral blood B-CLL. Direct manipulation of Noxa protein levels was achieved by proteasome inhibition in B-CLL and via RNAi in model cell lines. In each instance, cell viability was directly linked with Noxa levels. These data indicate that suppression of Noxa in the lymph node environment contributes to the persistence of B-CLL at these sites and suggest that therapeutic targeting of Noxa might be beneficial.
INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by a progressive accumulation of monoclonal CD5+ CD23+ mature B cells in the secondary lymphoid tissues, bone marrow, and blood. Previously, it was assumed that B-CLL is associated with a defective regulation of programmed cell death (apoptosis), rather than uncontrolled cell proliferation. Indeed, high expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 has been associated with rapid disease progression and a poor response to chemotherapy. Paradoxically, investigation of virtually all direct apoptosis regulators known at present revealed that, in addition to these anti-apoptotic alterations, the pro-apoptotic proteins Noxa and Bmf are also abundantly expressed in B-CLL. How the elevated expression of these pro-apoptotic proteins is associated with the reputed increased life span of the B-CLL cells is currently unknown.

The vast majority of the circulating B-CLL cells is arrested in G0/G1 phase of the cell cycle, which has contributed to the view that B-CLL is an indolent disease. However, isotopic labeling of leukemic cells in vivo revealed that a substantial fraction of the B-CLL cells does proliferate. It seems logical to assume that the generation of new cells takes place in so called proliferation centers frequently found in lymph nodes and bone marrow of B-CLL patients. This is supported by the numerous Ki67+ and Survivin+ cells present in these structures. The microenvironment not only plays an essential role in the induction of proliferation but presumably also in the suppression of apoptosis. In vitro experiments revealed that various cell types can support the survival of B-CLL cells. Apart from follicular dendritic cells (FDC), bone marrow stromal cells, IL-6 producing endothelial cells, VCAM-1 and SDF-producing nurse-like cells, CD4+ T cells can also aid in providing a microenvironment where B-CLL cells can survive and proliferate. The importance of the microenvironment for the survival of B-CLL cells is also shown by the finding that despite the relentless accumulation of the B-CLL cells in vivo, culturing the leukemic cells in vitro results in spontaneous apoptosis. In vitro culture of B-CLL cells in the presence of CD40L rescues the cells from spontaneous and drug-induced apoptosis, suggesting that such co-stimulatory signals play a role in the survival of B-CLL cells in vivo and even in the response to treatment.

To date, B-CLL is an incurable disease. Although multi-agent treatment can result in a profound peripheral lymphocyte depletion, the B-CLL cells in the bone marrow and/or lymph nodes are less effectively targeted. Persistence of B-CLL cells in the bone marrow is associated with an increased risk of relapse. Therefore, more molecular data about the B-CLL cells in the lymphoid tissues and bone marrow are
necessary, preferably coupled with assessment of efficacy of therapeutics towards B-CLL residing in those niches. We here initiated such an effort by comparing a large panel of apoptosis regulators in circulating B-CLL cells and B-CLL cells residing in lymph nodes. Although the expression of most apoptosis regulators was remarkably comparable, a prominent difference was the expression of the BH3-only protein Noxa. Furthermore, we demonstrate that CD40 engagement of peripheral B-CLL cells can largely reproduce the altered apoptosis profile found in lymph node B-CLL cells. Finally, we show that in vitro manipulation of Noxa expression has a significant and direct effect on B-CLL cell survival. Together, these data provide a new link between the anti-apoptotic microenvironment in the lymph nodes and suppression of Noxa, which suggests that drugs that increase Noxa levels, such as proteasome inhibitors, may be of therapeutic benefit in B-CLL.

MATERIAL EN METHODS

Patient material and cell lines
Patient material was obtained after routine diagnostic or follow-up procedures at the departments of Hematology and Pathology of the Academic Medical Center Amsterdam. All patients were diagnosed according to the WHO classification system. Lymph node (LN) material diffusely infiltrated by B-CLL cells was freshly frozen in liquid nitrogen directly after surgical removal. Immuno-histochemical analysis (see below) of these lymph nodes revealed that more than 90% of the tissue consisted of tumor cells. Peripheral blood (PB) mononuclear cells (PBMC) of B-CLL patients were obtained after Ficoll density centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands). PBMCs from B-CLL patients contained >75% CD5+, CD19+ cells as assessed by flow cytometry and were stored in liquid nitrogen as cell suspensions in 10% DMSO (Merck, Darmstadt, Germany) in heat-inactivated FCS (Invitrogen, Breda, The Netherlands). Clone FSA of the Burkitt's lymphoma cell line Ramos with enhanced response to CD95 has been described previously. Cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen), supplemented with 10% (v/v) heat-inactivated FCS (ICN Biomedicals GmbH, Meckenheim, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM L-glutamine (Invitrogen). This study was conducted in accordance with the ethical standards in our institutional medical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, revised in 1983.
RNA isolation and reverse transcription-multiplex ligation-dependent probe amplification assay (RT-MLPA)

Total RNA was isolated using the Nucleospin RNA isolation kit (Macherey-nagel, Düren, Germany). RT-MLPA procedure was performed as described previously. Briefly, 100 ng total RNA was reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probes. Annealed oligonucleotides were covalently linked by Ligase-65 at 54°C (MRC, Amsterdam, The Netherlands). Ligation products were amplified by polymerase chain reaction (PCR; 33 cycles, 30 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C) using one unlabelled and one 6-carboxy-fluorescein (FAM)-labeled primer (10 pM). PCR products were run on an ABI 3100 capillary sequencer in the presence of 1pM ROX 500 size standard (Applied biosystems, Warrington, UK). Results were analyzed using the programs Genescan analysis and Genotyper (Applied Biosystems). Category tables containing the area for each assigned peak (scored in arbitrary units) were compiled in Genotyper and exported for further analysis with Microsoft Excel spreadsheet software. Data were normalised by setting the sum of all signals at 100%, and expressing individual peaks relative to the 100% value.

Immunohistochemistry

Monoclonal antibodies specific for CD5 (clone 4C7 Lab vision, Neomarkers, Fremont, CA), CD3 (clone SP7), CD23 (clone 1B12), Bcl-6 (clone PG-B67), were used on formalin-fixed paraffin-embedded lymph node specimens. When necessary, antigen retrieval was achieved using a TRIS-EDTA buffer pH 9.2. Antibody detection was performed with the Powervision+ system (ImmunoVision Technologies, Daly City, CA) which was succeeded, for the single antibody staining, by peroxidase visualization with 3,3’-diaminobenzidine (DAB) (Sigma), 0.03% H₂O₂ in Tris-HCl pH 7.6. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted in perbx. For the CD3/Ki67 double stainings the Ki67 MIB-1 clone (Dako, Glostrup, Denmark) was used, for the CD20/Ki67 double staining the Ki67 SP6 clone (Neomarkers), and the L26 clone from Dako. After antibody detection with the Powervision+ system (ImmunoVision) the Liquid permanent red kit (Dako) was used, followed by peroxidase visualization with DAB (Sigma). Finally the slides were counterstained with haematoxylin and mounted in Vectamount.

Flow cytometry

Purified B-CLL cells were incubated FITC- or PE-conjugated mAbs directed against CD5 (Sanquin), CD19 (Sanquin) and CD3 (Becton and Dickinson, San Jose, CA) and
analyzed by flow cytometry with the CellQuest program on a FACS Calibur (Becton and Dickinson).

**In vitro CD40 stimulation**

B-CLL samples were enriched to >95% purity from PBMCs via negative depletion as described previously\(^{26}\). In brief, T cells, monocytes and granulocytes were depleted using anti-CD3, anti-CD14 and anti-CD16 immunomagnetic beads on a magnetic particle concentrator (Dynal A.S. Oslo, Norway). The B-CLL cells were stimulated for three days in culture-treated 24-wells plates (Costar, Corning NY, USA). Each well contained 5 x 10^6 B-CLL cells and 1.5 x 10^5 irradiated (30 Gy) CD40L-transfected or untransfected fibroblast (NIH3T3).

**Retroviral constructs and transduction**

To knock-down Noxa, pRetro-super was used, which contains the polymerase III H1-RNA promoter (pol3) for transcription of the siRNA probe and the phosphoglycerin kinase (pgk)1 promoter driving GFP expression\(^{27}\). The siRNA sequences were: N7 5’GAAGGTGCATTCATGGTG3’ and N85’GTAATTATTGACACACATTTCC3’. The retroviral plasmids were transfected into the helper virus amphotropic producer cell line Phoenix with Fugen-6 (Roche Diagnostics, Almere, The Netherlands). For transduction, Ramos-FSA cells were exposed overnight to viral supernatant (containing vector GFP-only or one of the two Noxa RNAi-targeting sequences) on retronectin-coated (Takara Shuzo, Otso, Japan) 24-well plates. GFP-positive cells were sorted using a FACS-Aria (BD Biosciences) cell sorter to >90% purity for further experiments.

**Analysis of apoptosis**

PB B-CLL cells were stimulated at a concentration of 5x10^6 cells/ml with 20 nM bortezomib (Janssen-Cilag, Tilburg, The Netherlands) for four hours. The cells were washed twice with IMDM (when indicated) and incubated at given time points with FITC-labeled Annexin-V (IQ products, Groningen, The Netherlands) for 20 minutes. Prior to analyses, PI was added (final concentration 5 μg/ml). Viable cells were defined by Annexin V/PI staining. Ramos.FSA clones expressing either control-GFP or Noxa-RNAi (N7 or N8) were stimulated at a concentration of 5x10^5 cells/ml with 30 nM bortezomib for 24 hours, harvested and incubated with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C, washed and double-stained with APC-labeled Annexin-V (IQ products). Fludarabine, staurosporine and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-human Fas10 (agonistic antibody to the CD95 receptor) were a kind gift from Prof. Dr. L. Aarden (Sanquin, Amsterdam, The Netherlands).
Western blotting was done as described previously. Protein samples were separated by 13% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. Blots were probed with the following antisera: polyclonal Mcl-1 (cat. no 554103, Pharmingen, BD Biosciences), monoclonal anti-Noxa (clone 114C307.1, Imgenex, San Diego, CA, USA), monoclonal anti-Bim (clone 14A8, Chemicon, Temecula, CA, USA) and antiserum to β-actin (clone I-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein bands were quantified using high resolution (1200 dpi) scanned images of exposed films and AIDA image analyzer software v3.5 (Raytest Gmbh; Straubenhardt, Germany). Exposed films were only considered when software indicated that bands were not overexposed. In each sample, background corrected intensity of Mcl-1 or Noxa bands were normalized for actin.

Statistical analyses
The Mann Whitney U test was used to analyze if differences in gene expression between the PB and LN B-CLL were statistically significant. P-values < 0.01 were considered statistically significant. Densitometric scans of western blots and MLPA analyses of CD40-triggered CLL cells were analyzed with Student’s T-test. P-values <0.05 were considered statistically significant.

RESULTS
Patients characteristics and immunohistochemistry
Lymph nodes from 9 B-CLL patients and peripheral blood samples from 15 B-CLL patients were included in the study. From 2 patients (B-CLL25 and B-CLL31) both lymph node (LN) tissue and a peripheral blood (PB) sample was available (Table 1). All B-CLL expressed CD5, CD23 and CD19/CD20. The B-CLL cells of the patients with LN involvement expressed unmutated immunoglobulin heavy chain (IgVH) genes. Of the peripheral blood B-CLL, 10 expressed mutated IgVH genes and 8 unmutated IgVH genes (Table 1). In the peripheral blood samples, at least 75% of the leucocytes were lymphocytes. Due to low levels of CD5 expression, the standard FACS gating yielded low percentage of CD5/CD19+ cells in some patients; Patient 30 had in addition low numbers of circulating cells and was first diagnosed as small lymphocytic leukemia (SLL). Immunohistochemistry demonstrated that >90% of the LNs consisted of leukemic lymphocytes. Ki67+ cells were present in all LNs, either diffusely or in proliferation centers. These cells were of B cell origin, as demonstrated by double staining which showed that all Ki67+ cells were also CD20+. In contrast, the
scattered CD3+ T cells were generally negative for Ki67. Absence of clusters of Bcl-6+ or CD21+ (data not shown) cells excluded the presence of germinal center remnants in these LNs (Fig. 1).

Figure 1: Histology of lymph node infiltrated by B-CLL cells. See color figures.
Ubiquitously present B-CLL cells were positive for CD23 and CD5. Scattered CD3+ T cells were present throughout the LN. The absence of clusters of Bcl-6+ cells excluded the presence of germinal center remnants in the LNs. Ki67/CD20 and Ki67/CD3 double staining indicate that all cycling Ki67+ cells (pink) were of CD20+ (brown) origin - see also inset -, while the CD3+ T cells were predominantly Ki67 negative. Magnification 40x.

Profiling of apoptosis genes in peripheral blood and LN samples of B-CLL
The relative expression 34 known apoptosis regulators was investigated by RT-MLPA in PB samples of 13 B-CLL patients, LN samples of 7 B-CLL patients (Fig. 2A) and paired PB and LN samples of 2 B-CLL patients (Fig. 2B and data not shown). The relative expression of the majority of the investigated genes was remarkably comparable between the PB and LN samples. We have described previously that, compared to normal tonsillar B cell fractions, in PB B-CLL several anti- and pro-apoptosis genes (e.g. Flip, Bcl-2, Noxa and Bmf) are aberrantly expressed, and this was also found in LN samples of B-CLL. Interestingly, 3 genes were differentially expressed in PB B-CLL cells as compared to LN samples (Fig. 2C). In agreement with previous reports, the IAP family member Survivin was not expressed in any of the PB B-CLL samples whereas it was clearly expressed in LN B-CLL (P=0.0005). Also, the anti-apoptotic Bcl-2-family member Bcl-XL was more abundantly expressed in the LN samples (P=0.0003). The most striking difference in expression was observed for the BH3-only member Noxa. As found previously, this apoptogenic gene is abundantly expressed in PB B-CLL cells, but its expression was clearly lower in LN
B-CLL cells (averaged relative expression of 15.6 ± 9.8 in PB B-CLL versus 3.0±1.1 in LN B-CLL; P<0.0001 Fig. 2C). Of note, a difference in Noxa expression was also observed between the paired PB- and LN samples of an individual patient (relative expression 9.6 in the PB sample versus 3.4 in the LN sample) (Fig. 2B). Western blot analyses confirmed that the differences in Bcl-X\textsubscript{L} and Noxa mRNA expression were also present at the protein level. The B-CLL LN samples generally expressed lower levels of Noxa than the PB B-CLL samples, and the reverse was observed for

\begin{figure}
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\caption{Apoptosis gene expression profile of B-CLL cells in peripheral blood and lymph nodes. (A) Relative expression of 34 apoptosis regulators was investigated in 15 PB B-CLL (black bars) and 9 LN B-CLL (grey bars). Results of individual apoptosis regulatory genes are shown as expression relative to the total signal in the sample, with standard deviation. Non-apoptosis genes included as housekeeping genes are β2-microglobulin (B2M), Ferritin Light chain (FLT), β-glucoronidase (GUS), and poly(A)-specific ribonuclease (PARN). (B) RT-MLPA data from PB and LN sample of B-CLL-25. (C) The expression of Noxa, Survivin, Bcl-X\textsubscript{L} and Mcl-1 in individual patients are depicted as dots. Asterix (*) indicates statistical significance (P<0.001) of differences in gene expression between PB and LN B-CLL.}
\end{figure}
Bcl-X\textsubscript{L} (Fig. 3). Comparison of the RT-MLPA data with the Western blot data revealed a clear correlation between the levels of Noxa mRNA and Noxa protein. As reported previously, no differences were observed in expression of these apoptosis genes among IgV\textsubscript{H}-mutated versus unmutated cases\textsuperscript{5}.

Since Noxa can selectively interact with the anti-apoptotic protein Mcl-1 and this may influence the degradation of Mcl-1\textsuperscript{29}, we investigated the expression of this Bcl-2 family member in PB B-CLL and LN B-CLL. Although RT-MLPA showed no difference in mRNA expression (Fig. 2C), Western blot analyses revealed that in most LN B-CLL, where Noxa levels were low, Mcl-1 was slightly elevated. Furthermore, the PB samples that expressed higher levels of Noxa showed a decreased expression of Mcl-1 (Fig. 3). This is further illustrated by a paired PB/LN protein sample, where in fact Noxa expression was almost equal, but in this case Mcl-1 protein levels were clearly higher in the LN compartment. Densitometric scanning of Noxa and Mcl-1 protein levels also showed divergence between LN and PB, of which the differences in Noxa levels were statistically significant (Fig. 3B). In summary, the majority of the apoptotic regulators are expressed equally in PB - and LN B-CLL, but a novel and prominent distinction in Noxa level was found.

![Western blot images and densitometric scanning plots](image_url)

**Figure 3: Comparison of Noxa, Mcl-1 and Bcl-X\textsubscript{L} protein in PB vs. LN B-CLL.**

Protein lysates of 7 PB samples and 6 LN samples were subjected to Western blot analyses. 

(A) Blots were stained with antibodies directed against Noxa, Mcl-1 or Bcl-X\textsubscript{L}, and reprobed with an antibody against \(\beta\)-actin as a loading control. In case of Bcl-X\textsubscript{L}, aspecific staining at the upper cutting edge of the blot is visible and precluded analysis of the rightmost two samples.

(B) Densitometric scanning was performed, and averaged Noxa/actin and Mcl-1/actin ratios are plotted in PB and LN samples. Unpaired T-test showed that Noxa ratios were statistically significant (\(P=0.0026\)), and Mcl-1 ratios showed a non-significant trend.
Noxa expression is modulated by CD40 engagement in B-CLL cells

In the LNs the CD40+ B-CLL cells are in close contact with T cells that may express CD40L (Fig. 1). To investigate the effect of this interaction on the expression of the apoptotic regulators, PB B-CLL samples (n = 11) were co-cultured for 1-5 days with CD40L-transfected or untransfected 3T3 fibroblasts (Fig. 4). As reported previously, CD40 stimulation resulted in increased expression of Bcl-XL, A1/Bfl-1, Bid and Survivin. Interestingly, in accordance with our findings in the LN B-CLL cells, CD40L-stimulated PB B-CLL cells also showed a diminished expression of Noxa (Fig. 4A). The effects were observed after one day of CD40 stimulation and RT-MLPA performed at day three and day five showed that the expression of the apoptosis regulators did not alter significantly after that (Fig. 4B). It should be noted that the levels of Noxa mRNA as compared to t=0 also decreased after culture on the control 3T3 cells (p=0.019). The reason for this is not known, however a stronger decline in Noxa levels was consistently observed after CD40 ligation (p=0.004), and the difference between control and CD40-treated cells was statistically significant (p=0.016). These differences were further investigated at the protein level for three patients (see Fig. 4C). Concordant with RT-MLPA analyses, Noxa levels decreased after 3 days culture in presence of CD40L-expressing cells, and Bcl-XL levels increased. Mcl-1 protein levels also clearly increased upon CD40-triggering, although this was not observed via RT-MLPA. So, similar to findings in LN samples (Fig. 3), Mcl-1 levels were apparently under post-transcriptional control.

A prominent distinction between CD40-stimulated B-CLL and LN B-CLL was found for expression of the apoptogenic BH3-only protein Bid. In contrast to LN B-CLL, CD40L-stimulated B-CLL showed a strong and continuous induction of Bid (Fig. 4B). Thus, the altered gene expression in LNs can be mimicked largely but not entirely by in vitro CD40 engagement of B-CLL cells.
Figure 4: CD40 stimulation of peripheral blood B-CLL results in an apoptosis gene expression profile similar to lymph node B-CLL.

(A) Apoptosis gene expression profile was investigated by RT-MLPA in PB samples of 11 freshly isolated B-CLL patients without culturing (black bars) and after three days of culturing on either 3T3 cells (grey bars) or CD40L-transfected 3T3 cells (white bars). Data plus standard deviation are presented as in figure 2.

(B) The expression of Bcl-XL, Bfl-1/A1, Bid and Noxa are shown at day 1, 3 and 5 of culturing on 3T3 cells (white triangles) or CD40L-transfected 3T3 cells (black dots). Statistical analysis of day 0 vs. day 1 samples showed that in all cases the CD40L treated values were significantly different (P<0.01). In case of Noxa, there was also a small but significant decrease for the 3T3 control cells (P=0.019), and a more pronounced effect for CD40L treated cells (P=0.004; difference between 3T3 and CD40L-treated cells P=0.0159, indicated by **).

(C) Western blot of t=0 samples in comparison of CD40L treated cells at day 3 for Noxa, Mcl-1 and Bcl-XL showed that Noxa protein levels decrease while Mcl-1 and Bcl-XL increase. For B-CLL sample 226 the Mcl-1 levels at t=0 were in fact undetectable (see also Fig. 3).
Bortezomib-induced Noxa upregulation causes apoptosis of PB B-CLL cells

To establish a functional relationship between Noxa expression levels and apoptosis sensitivity of B-CLL cells, we made use of recent findings that proteasome inhibitors rapidly and specifically upregulate Noxa. To reduce a widespread impact of proteasome inhibition on protein levels and transcription dependent processes, PB B-CLL cells were transiently exposed to bortezomib for 4 hours. The reversible proteasome inhibitor was then either washed away or incubation was continued. As expected, a pulse of bortezomib treatment already caused a rise in Noxa protein, and this was sufficient to impair survival of B-CLL cells (Fig. 5). Continuous exposure to bortezomib resulted in a massive increase in Noxa levels that was accompanied by almost 100% cell death at 48 hrs. Over the course of this experiment, Mcl-1 protein levels first increased (4 hr timepoint), most likely due to proteasome inhibition, and then declined when cells went into apoptosis. This decline could be prevented by blocking caspase activity with z-VAD (data not shown), and is thus in accord with reports that Mcl-1 is a caspase substrate. Next, we investigated whether the level of Bim, another pro-apoptotic binding partner of Mcl-1, was also subject to change upon bortezomib treatment, and might thereby trigger apoptosis. However, Bim levels were unaffected, both as detected by RT-MLPA (data not shown), and by Western blotting (Fig. 5A). Thus, pharmacological manipulation of the levels of Noxa protein in B-CLL cells appeared to be directly related to viability in an in vitro setting.

Figure 5: Noxa upregulation via transient treatment with bortezomib impacts CLL survival.
Freshly isolated peripheral blood B-CLL cells were treated for 4 hrs with 20 nM of the proteasome inhibitor bortezomib. Cells were then washed and cultured in fresh medium, or incubation was continued.
(A) At the indicated timepoints, cell lysates were prepared and probed for expression of Noxa, Mcl-1, Bim and Actin protein by Western blot. Indicated below the lanes: untreated (M), bortezomib washed away after 4 hrs (B+), and bortezomib without washing (B-). The decrease in Mcl-1 levels in bortezomib treated cells at 24 and 48 hrs could be inhibited by the pan-caspase inhibitor z-VAD (data not shown). Due to massive cell death after 48 hrs in the presence of bortezomib, these lysates did not yield sufficient protein for analysis.
(B) Apoptosis of cells was determined via AnnexinV staining. Spontaneous apoptosis in medium was approximately 50%, which was increased by bortezomib treatment. Results are representative for 3 separate experiments.
Noxa-deficient cells exhibit resistance to bortezomib-induced cell death

Apoptosis regulatory genes as detected via RT-MLPA were not affected during the short-term bortezomib treatment in the previous experiments (data not shown). Yet, it can not be excluded that other genes and proteins besides Noxa that might impact survival were affected by bortezomib. Therefore, to investigate a direct role for Noxa in bortezomib-induced apoptosis, we employed a model system. Ramos B cells (clone FSA)\textsuperscript{24} were transduced with distinct retroviral constructs encoding Noxa siRNAs (N7 or N8) or control-GFP. GFP-positive cells were sorted and Western blot analysis revealed a suppression of Noxa-levels to approximately 50-75% compared to the control-GFP (Fig. 6A). Both Ramos FSA cell lines expressing Noxa RNAi exhibited a significant resistance to bortezomib-induced apoptosis compared to the mock-transduced cells (Fig. 6B). The partial resistance to proteasome inhibitor-mediated apoptosis matched the partial knock-down of Noxa protein. Of note, also in Noxa RNAi cells, bortezomib treatment caused a rapid increase in Noxa protein (data not shown), thus explaining that apoptosis still occurred at higher concentration of the drug. These data are in good agreement with effects of Noxa knock-down in other celltypes (melanoma, mantle cell lymphoma and T cell leukemia)\textsuperscript{22,23,30}. In addition, we obtained similar findings with another protease inhibitor (MG132; data not shown). In contrast, no effect of Noxa protein reduction was observed on apoptosis triggered via other pathways such as fludarabine or staurosporin treatment, or triggering of the CD95 receptor (Fig. 6C). In conclusion, these data demonstrate that decreased expression of Noxa has a direct and specific impact on the susceptibility to apoptosis induced by proteasome inhibitors. Conversely, the death-inducing effect of proteasome inhibition observed in B-CLL cells may therefore rely predominantly on shifts in Noxa expression.
DISCUSSION

There is increasing awareness that the B-CLL population in lymphoid proliferation centers differs fundamentally from the well studied fraction in PB and that this distinction may have clinical relevance\(^8,35\). Here, we present a first direct comparison of these two populations, focusing on the expression of 34 apoptosis regulatory genes. Apart from expected differences in proliferation-related genes (Survivin and Ki67) and anti-apoptotic Bcl-XL, we observed a prominent divergence in the expression of pro-apoptotic Noxa. Previously we described that, compared to non-malignant tonsil or peripheral B-cell fractions, B-CLL cells in the periphery display

Figure 6: Noxa reduction via RNAi specifically prevents apoptosis induction by proteasome inhibitors.

Ramos Burkitt lymphoma cells were retrovirally transduced with two RNAi constructs targeting Noxa (N7 or N8), or GFP control.

(A) Western blot demonstrating reduced Noxa expression in Ramos-N7 and -N8. Equal protein loading is shown by reprobing for β-Actin.

(B) Mock, N7 and N8 transduced Ramos FSA cells were cultured 24 hours in the presence of indicated concentration of bortezomib. Viability was assessed by AnnexinV/mitotracker staining and FACS analysis. Data represent mean ± SD from three independent experiments.

(C) Cells were incubated for 24 hrs in medium containing 100 μM fludarabine (fluda), 0.25 μM staurosporine (stauro), or 5 μg/ml α-CD95, and analysed as in B.
significantly increased levels of this BH3-only member of the Bcl-2 family, in a p53-independent manner. The high levels of Noxa and another BH3-only member Bmf, contrasted with the purported anti-apoptotic phenotype of B-CLL cells but remained functionally unexplained. Our new findings show that the Noxa level is considerably lower in LN CLL and that this is linked with survival capacity. Therefore, targeting Noxa expression or function could be of clinical benefit, also in p53 deficient cases. In vitro CD40 stimulation of PB B-CLL cells resulted in a clear reduction of Noxa expression. Within the LN microenvironment, CD40 stimulation is most likely delivered by CD40L+ T cells. Several groups have investigated the effects of in vitro CD40 engagement in B-CLL cells but an effect on Noxa expression was not yet reported. It is well known that CD40-stimulated B-CLL cells are more resistant to spontaneous or drug-induced apoptosis. This is most probably due to the induction the transcription factor nuclear factor κB (NFκB) and as a consequence, the expression of various anti-apoptotic genes, such as Bcl-XL, cIAP2, A20 and Flip. Previously, Noxa was proposed to be a p53-response gene, but in B-CLL cells, Noxa is apparently not under control of p53, as illustrated by the clearly divergent expression of Puma and Noxa upon p53 stimuli. Later, various transcription factors were proposed to regulate Noxa such as E2F1, p73 and hypoxia inducible factor HIF-1α. Therefore at present it is difficult to definitely assign a specific signaling route that mediates Noxa expression. Very recently though, it was reported that HIF-1α is overexpressed in peripheral B-CLL cells, which may constitute a potential link to the increased Noxa levels in B-CLL.

Although CD40 stimulation of PB B-CLL cells resulted in a similar apoptosis gene expression profile to LN B-CLL, several genes deviated from this profile, most prominently Bid, as reported previously, but also A1/Bfl-1. This indicates that in the LN, B-CLL cells also receive other stimuli than CD40. Indeed, apart from CD4+ T cells expressing CD40L, other cell types can support survival of B-CLL cells. In vitro culture with an FDC cell line or dendritic cells protects B-CLL cells from spontaneous apoptosis. FDC-mediated survival was reported to depend on the expression of the Bcl-2 family member Mcl-1 and in vitro experiments revealed that Mcl-1 levels decline in B-CLL cells undergoing apoptosis. Interestingly, recent data indicate that Mcl-1 is a preferred binding partner of Noxa, and we have indeed observed association of Mcl-1 with Noxa in primary B-CLL samples (D. Hallaert; manuscript in preparation). Furthermore, in 293T cells, Noxa has been described to mediate the degradation of Mcl-1. If this mechanism also holds true for B-CLL cells, it may explain the increase in Mcl-1 protein we observed in LN B-CLL, which was not accompanied by an increase in Mcl-1 mRNA. Accordingly, augmented Mcl-1 protein levels are a consequence of the downregulation of Noxa in the LNs, rather than a
environmental effect on Mcl-1 RNA expression. In addition, in vitro triggering of CD40 on B-CLL cells also influenced Mcl-1 levels in a post-transcriptional fashion (Fig. 4). Thus, the differences in protein levels observed by us for Noxa, Mcl-1 and Bcl-XL levels in the B-CLL LN environment, correspond with current models based on the differential interaction potential of these Bcl-2 family members\textsuperscript{29,34}, and support the anti-apoptosis phenotype of B-CLL cells at this location compared to PB. In addition, spontaneous apoptosis in vitro of B-CLL cells may be connected with the high levels of Noxa which eventually saturate the short-lived Mcl-1 protein\textsuperscript{29,48}.

Two separate experimental approaches supported a direct role for Noxa in survival capacity of B-CLL cells. First, we used the recently discovered rapid and direct effect of bortezomib on Noxa levels\textsuperscript{22,23,30} to demonstrate that short term bortezomib exposure also quickly induced Noxa protein in B-CLL cells, with a corresponding decrease in viability (Fig. 5). Although the levels of Bim did not change upon bortezomib treatment, a role for Bim during the actual triggering phase of apoptosis cannot be excluded. In model systems, Bim is capable of actively triggering Bax activation, while Noxa functions in as ‘sensitiser’\textsuperscript{49,50}. Secondly, a complementary experiment was performed in a model system where only Noxa levels were modified via RNAi. Here, a clear inhibitory effect of Noxa reduction towards apoptosis mediated by proteasome inhibition was observed, while other apoptosis pathways were unaffected (Fig. 6).

Taken together, our data support a model where the viability of the malignant B-CLL clone within the LNs and possibly also the bone marrow corresponds with low levels of Noxa and an upregulation of Bcl-XL and Mcl-1. In addition to these anti-apoptotic gene expression alterations, the B-CLL cells also receive proliferative stimuli as indicated by the Ki-67+ and Survivin+ cells. When the B-CLL cells enter the circulation these stimuli are lost, Noxa is upregulated, and Bcl-XL, Mcl-1 and Survivin are downregulated. As a result, the B-CLL cells may become prone to apoptosis, which can however still be prevented by the continuous high expression of Bcl-2. To what extent circulating B-CLL are actually undergoing apoptosis is difficult to detect directly. Freshly isolated CLL cells are mostly non-apoptotic but undergo rapid ‘spontaneous’ apoptosis in vitro, and recent calculations point to appreciable in vivo death rates\textsuperscript{8}. Together, this suggests that apoptotic B-CLL cells are rapidly cleared from circulation in vivo. It is generally assumed that in the LNs and bone marrow the B-CLL cells are relatively protected against therapeutic drugs\textsuperscript{29}. The circulating B-CLL cells that are already prone to apoptosis are more easily targeted, but the residual B-CLL cells in the LN/bone marrow will eventually lead to a relapse. Currently, there is much interest in application of novel, p53-independent drugs to treat B-CLL\textsuperscript{51-53}. Our data provide new insight into the regulation of the apoptotic behavior of B-CLL cells, and also afford new clues for therapeutic intervention by targeting Noxa expression.
ACKNOWLEDGEMENT

We are grateful to the patients for donating samples and the clinicians involved for their collaboration. This study was initiated after suggestions from Professor Steven Pals (Dept of Pathology of the AMC) that investigation into survival and apoptosis of B-CLL cells should include lymph nodes. The authors would like to thank JBG Mulder and AR Musler for immunohistochemical stainings.
Table 1. Patient and B-CLL sample characteristics.

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LN indicates lymph node; PB, peripheral blood; ND, not done.
*Lymphocytes were investigated in the lymph node samples by immunohistochemistry and in the peripheral blood samples by FACS analysis.
†IgVH mutations were positive if 2% of the IgVH gene was mutated.
‡These samples were used only for Western blot analyses.
§These samples displayed low CD5 staining; therefore, the combined CD5/CD19 gate yielded low values.
REFERENCES


