

File ID 116945
Filename Chapter 5: Persistent Mcl-1/Bim protein signature after CD40 ligation in chronic lymphocytic leukemia is associated with specific drug sensitivity

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type Dissertation
Title 'From the cradle to the grave' : novel therapeutic approaches to attack the microenvironment in chronic lymphocytic leukemia
Author D. Hallaert
Faculty Faculty of Medicine
Year 2008
Pages 176

FULL BIBLIOGRAPHIC DETAILS:

<http://dare.uva.nl/record/284714>

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chapter 5

Persistent Mcl-1/Bim protein signature after CD40 ligation in Chronic Lymphocytic Leukemia is associated with specific drug sensitivity

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Manuscript in preparation

ABSTRACT

Relapse in CLL might originate from a niche where CLL cells proliferate and are protected from chemotherapeutic drugs. It is therefore important to understand how CLL cells from lymph nodes (LN) differ from the cells which have moved into the circulation. To mimic this *in vivo* LN setting we used CLL cells stimulated via CD40. The aim of the present study was to monitor expression of apoptosis regulatory genes, in relation to sensitivity for various types of drugs (fludarabine, bortezomib, GSI-1 and roscovitine) during and following a CD40-ligand stimulus. CD40 ligation resulted in enhanced NF- κ B activity and increased expression of target genes Bcl-X_L and Bfl-1. Furthermore, Mcl-1 and Bim_{EL} protein, but not RNA levels, were increased and decreased respectively. Four days after cessation of CD40L stimulation there was a dichotomy: NF- κ B activity, Bcl-X_L and Bfl-1 gene expression gradually declined, but Mcl-1 and Bim_{EL} protein changes persisted. This was accompanied by reversal of resistance to drugs, except for roscovitine, a cyclin dependent kinase (CDK) inhibitor, where the apoptosis-inducing capacity strongly depends on Mcl-1 and Bim. These data illustrate the long-lasting, non-transcriptional effects of CD40 signals on Mcl-1 and Bim in CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) remains an incurable disease and although multi-agent treatment can result in peripheral lymphocyte depletion, the CLL cells in the protected microenvironmental niches (lymph node (LN), bone marrow (BM) and spleen) are less effectively targeted. Therefore, it is important to assess the efficacy of therapeutics directed towards CLL cells residing in those niches¹.

CLL cells in the peripheral blood (PB) are arrested in the G₀/G₁ phase of the cell cycle, and replication occurs in proliferation centers (pseudofollicles). Recently it was shown that the disease process is more dynamic than previously considered and is characterized by proliferating as well as dying cells². Selected microenvironmental signals delivered by accessory cells, such as follicular dendritic cells (FDCs), BM stromal cells, IL-6-producing endothelial cells, SDF-producing nurse-like cells, or CD40 ligand (CD40L/CD154) expressing CD4⁺ T cells, have been shown to increase the apoptotic threshold *in vitro*³⁻⁷. CD40L is expressed on activated T cells and plays an important role in B cell activation, proliferation, isotype switching, normal germinal centre formation^{8,9} and normal specific antibody responses^{10,11}. In B cells, the response to CD40 is complex and it is well established that CD40 can support cell survival through upregulation of the expression of genes encoding antiapoptotic proteins such as Bcl-X_L and Bfl-1¹²⁻¹⁵. The roles of the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and nuclear factor-kappa B (NF-κB) pathways in mediating CD40 stimulation are well described¹⁶. NF-κB signaling is divided into two pathways: the canonical pathway and the alternative pathway. Activation of the canonical pathway proceeds through the degradation of phosphorylated IκBα and subsequent nuclear translocation of active heterodimers (composed of p50, p65, and/or c-Rel). Activation of the alternative NF-κB pathway results in nuclear translocation of p52 along with RelB. Translocation of active heterodimers to the nucleus influences the expression of various genes^{17,18}. Studies suggest that CLL cells may have constitutively activated canonical NF-κB activity, which enhances cell survival^{19,20}.

The Bcl-2 family of proteins plays an important role in the response to chemotherapeutic drugs and includes anti-apoptotic and pro-apoptotic members. The Bcl-2 family members Bax and Bak directly trigger cytochrome *c* release from the mitochondria. In turn, this is under the control of the anti-apoptotic Bcl-2 family members (Bcl-2, Bfl-1, Mcl-1 and Bcl-X_L). This balance can be tipped by the pro-apoptotic BH3-only subgroup of the family^{21,22}.

In a recent comparative *ex vivo* study of apoptosis regulatory genes and proteins in neoplastic B cells derived from CLL LN proliferation centers and from PB, we observed

specific changes in mRNA expression: Bcl-X_L and Bfl-1 were upregulated and Noxa was downregulated. Furthermore, although Mcl-1 mRNA levels were unaltered, protein expression was upregulated in LN samples¹⁴. Studies on Bcl-2 family members in CLL cells stimulated *in vitro* with CD40L showed upregulation in mRNA expression of Bcl-X_L, Bfl-1, Bid, and survivin and downregulation of Noxa¹²⁻¹⁵. Again, Mcl-1 was clearly increased upon CD40 triggering^{13;14}, on the protein level, but not at the RNA level. Thus, *in vitro* CD40L stimulated CLL and *ex vivo* LN CLL cells displayed a highly analogous anti-apoptotic phenotype compared to PB CLL. Therefore, *in vitro* CD40L stimulation of CLL cells seems to be a useful model to mimic the *in vivo* LN setting. Mapping this transit from LN to PB at the molecular level in relation to drug sensitivity could provide new insights into the efficacy of chemotherapeutic regimens. We observed a distinctive trend where reversible transcriptional changes are contrasted with long-lasting post-transcriptional modulation of Mcl-1 and Bim proteins. Together, these changes dictate short- and long-term effects with respect to drug sensitivity.

MATERIALS AND METHODS

CLL cells

PB from CLL patients was obtained in the setting of routine diagnostic or follow-up procedures at the department of Hematology of the Academic Medical Center Amsterdam. Patients had to give informed consent and the study was approved by the AMC Ethical Review Board. This study was conducted in accordance with the ethical standards in our institute and in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral blood (PB) mononuclear cells (PBMCs) obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, The Netherlands) were frozen in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with L-Glutamine, 25 mM HEPES (Biowhittaker, Europe) containing, 2mM L-Glutamine (Invitrogen), 50 mg Gentamycine (Invitrogen) 3.57 x 10⁻⁴% (v/v) β-mercapto ethanol (Merck, Darmstadt, Germany) and, 10 % dimethyl sulphoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and 15% fetal calf serum, (FCS; ICN Biomedicals GmbH, Meckenheim, Germany), and stored in liquid nitrogen. Expression of CD5 and CD19 (both Becton Dickinson (BD) Biosciences, San Jose, CA, USA) on leukemic cells (>90% purity) was assessed by flow cytometry (FACScalibur, BD Biosciences) and analysed with CellQuest software (BD Biosciences).

RNA isolation and RT-MLPA

Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich). Reverse transcription–multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously^{23;24}.

Reagents

The proteasome inhibitor bortezomib was obtained from Janssen-Cilag (Tilburg the Netherlands). GSI-1²⁵ (gamma-secretase inhibitor-1; Z-LLNle-CHO – Cat.nr. 565750) and cycloheximide were obtained from Calbiochem (Amsterdam, the Netherlands). Roscovitine and fludarabine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Analysis of apoptosis, Western blot and antibodies

For apoptosis induction, 5×10^6 CLL/ml cells were incubated with 100 μ M fludarabine (48 hrs), 30nM bortezomib, 25 μ M roscovitine or 5 μ M GSI-1 (24 hrs), and stained with 200 nM MitoTracker Orange (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C and analysed by FACS. Western blotting was performed as described previously²³. Cells were lysed in Laemmli Sample Buffer, and samples (10-30 μ g protein) were separated by 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels for Erk). Blots were probed with the following antibodies: polyclonal anti-Mcl-1 (catalog no. 554103; Pharmingen, BD Biosciences), monoclonal anti-Noxa (clone 114C307.1; Imgenex, San Diego, CA), monoclonal anti-Bim (clone 14A8; Chemicon, Temecula, CA), antiserum to β -actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-Bcl-XL (catalog no. 620211, BD Biosciences), polyclonal anti-Bcl-2 (catalog no. 210-701-C100, Kordia, Leiden the Netherlands) polyclonal anti-phospho-Erk (catalog no. #9102 Cell Signaling), polyclonal anti-Erk (catalog no. #9101 Cell Signaling), monoclonal anti-phospho-I κ B α (clone 5A5, Cell Signaling), polyclonal anti-I κ B α (catalog no. #9242, Cell Signaling), polyclonal anti-p100/p52 (catalog no. #4882, Cell Signaling), polyclonal anti-phospho-GSK-3 β (sc-11757, Santa Cruz Biotechnology) and polyclonal anti-GSK-3 β (clone 1H8, Santa Cruz Biotechnology).

In vitro CD40 stimulation

CLL cells were stimulated by co-culture with fibroblasts (NIH3T3) that had been stably transfected with a plasmid encoding human CD40L (3T40L). Fibroblasts were irradiated (30Gy) and plated in culture-treated 6-wells plates (6×10^5 cells/well). CLL cells were thawed and 5×10^6 cells per well were added (day-3) to the adhered

fibroblasts in 3 ml IMDM containing 10% FCS and incubated at 37°C. After 3 days (day 0) CLL cells were gently removed and transferred to new 6-well plates and incubated for 4 days (day 1-4) in 3 ml IMDM containing 10% FCS. There were no residual fibroblasts in the transferred CLL cell culture.

Proteasome activity assay

Cytoplasmic extracts (Assay buffer: 250 mM HEPES (pH7.5), 5 mM EDTA, 0.5% NP-40 and 0.01% SDS) from freshly isolated PBMCs from CLL patients (CD40L stimulation and time point of lyses is indicated in legend) were used to measure proteasome activity using a 20S proteasome activity assay kit (Chemicon, part of Millipore; Billerica, USA) following the manufacturer's instructions. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LVVY-AMC. The free AMC fluorescence was quantified using a 380/460-nm filter set in a VICTOR² D fluorometer (Wallac-PerkinElmer, Massachusetts, USA). Proteasomal activity was calculated from the changes in fluorescence over time and expressed per µg of protein in the extract.

Statistical analysis

The Mann Whitney *U* test was used to analyze weather differences in proteasomal activity between control 3T3 and 3T40L stimulated CLL cells at day 0 and day 4 (4 days after cessation of CD40 stimulation) were statistically significant. *P* values below 0.05 were considered statistically significant.

RESULTS

The anti-apoptotic expression profile resets after termination of CD40 stimulation

CLL cells were co-cultured with non transfected 3T3 cells (control), or with 3T3 cells stably transfected with a huCD40L-encoding plasmid (3T40L). We evaluated mRNA expression profiles of different apoptosis genes by means of RT-MLPA^{14;24} from cells of CLL patients during, and at different time points after, CD40 triggering. Cells were stimulated for three days (day -3 to day 0), followed by four days culture in the absence of CD40 signals (day 1-4). As reported previously, CD40L stimulation resulted in increased mRNA expression of Bcl-X_L, Bfl-1, Bid and Survivin and decreased mRNA expression of Noxa (Fig. 1; day 0)^{12-14;26}. Expression of Bcl-X_L, Bfl-1 and Bid mRNA declined gradually to pre-stimulation levels after cessation of CD40 stimulation. Noxa downregulation, however, was not only observed in CLL cells cultured on 3T40L cells

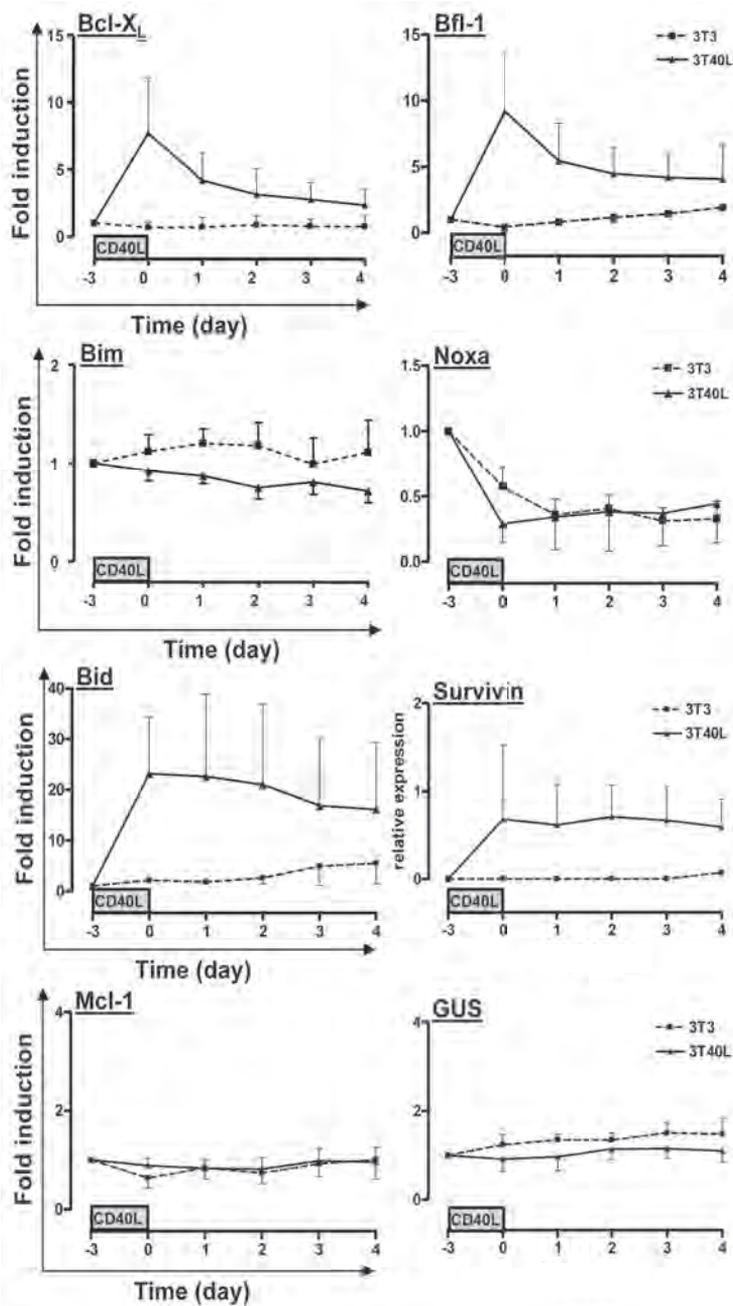


Figure 1. Apoptosis gene expression profile of CLL cells after termination of CD40 stimulation.

Changes in the mRNA expression of Bcl-X_L, Bfl-1, Mcl-1, Noxa, Bim, Bid, survivin and GUS were investigated by RT-MLPA. CLL cells were co-cultured for 3 days on control 3T3 cells (dashed lines) or CD40L-transfected 3T3 cells (solid lines). After detachment and washing, cells were incubated in medium for 4 days without stimulation. mRNA expression is shown at day -3 (prior to stimulation), 0 (3 days co-culture), 1, 2, 3 and 4 (days after termination of stimulation). Data represent mean \pm SD from 5 independent experiments.

but also on 3T3 cells and the mRNA levels remained low under both conditions. Mcl-1 and Bim mRNA expression was not altered upon CD40, similar to the housekeeping gene GUS which showed stable expression throughout the experimental period. In conclusion, the altered gene expression by *in vitro* CD40 engagement of CLL cells was in agreement with previous results^{12-14;26}. Cessation of CD40 stimulation resulted in a gradual decline to baseline expression.

CLL cells selectively regain chemosensitivity after termination of CD40 stimulation

Next, sensitivity to different chemotherapeutic drugs was tested, in relation to

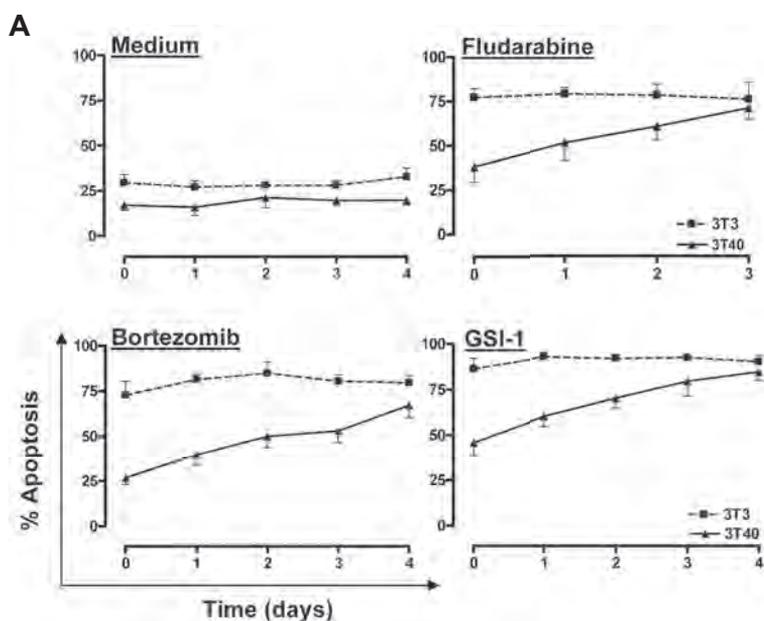
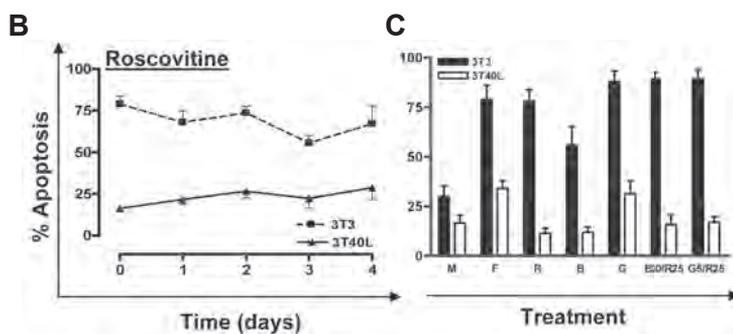


Figure 2. Apoptosis response after termination of CD40 stimulation in CLL.

(A) CLL cells were co-cultured for 3 days on 3T3 cells (dashed lines) or CD40L-transfected 3T3 cells (solid lines). After detachment and washing, cells were directly or after 1-4 days of additional CD40L free culture, incubated with bortezomib (30 nM) or GSI-1 (5 μ M) for 24 hours and with fludarabine (100 μ M) for 48 hours. Apoptosis response was analyzed by MitoTracker staining. Data represent mean \pm SD from 6 independent experiments.

(B) Apoptosis response upon 24 hours incubation with roscovitine (25 μ M) of CLL cells cultured as described in A. Data represent mean \pm SD from 4 independent experiments.

(C) CLL cells were co-cultured for 3 days on 3T3 cells (black bars) or CD40L-transfected 3T3 cells (white bars). After detachment and washing, cells were incubated with fludarabine (F) (100 μ M) for 48 hours or roscovitine (R) (25 μ M), bortezomib (B) (30 nM) and GSI-1 (G) (5 μ M) for 24 hours. Also the following combinations of drugs were used: bortezomib (30 nM) + roscovitine (25 μ M) (B30/R25) or GSI-1 (5 μ M) + roscovitine (25 μ M) (G5/R25). M=medium. Apoptosis response was analyzed by MitoTracker staining. % Apoptosis is shown at day 0 (+/- 3 days CD40 stimulation). Data represent mean \pm SD from 6 independent experiments.



the culture regimen described above. Figure 2 shows that at day 0 CLL cells had become resistant to all drugs tested: the purine analog fludarabine, the proteasome inhibitors bortezomib and GSI-1 (gamma-secretase inhibitor-1), and the CDK inhibitor roscovitine. Also the combination of roscovitine with bortezomib, GSI-1 (Fig. 2C) or fludarabine (data not shown), did not induce apoptosis at day 0. After cessation of CD40 stimulation CLL cells became gradually sensitive again to fludarabine, bortezomib and GSI-1, but not to roscovitine (Fig. 2A&B; day0-4). This surprising dichotomy was further investigated.

Prolonged changes in Mcl-1 and Bim^{EL} protein expression after termination of CD40 triggering in CLL

Previous research showed that roscovitine induced apoptosis via the Mcl-1/Noxa axis. Rapid (4 hours) Mcl-1 degradation is the initial event in roscovitine-induced apoptosis^{27;28} and the BH3-only proteins Noxa and Bim are involved as specific mediators in this process²⁷.

Changes in Mcl-1, Bcl-X_L and Noxa were determined in CLL cells treated for 24 hours with roscovitine or bortezomib at two different time points; after three days of CD40L stimulation (day 0) and four days after termination of CD40L stimulation (day 4). In accord with previous data^{12-14;27;28}, Mcl-1 and Bcl-X_L protein expression was upregulated after CD40L stimulation at day 0 compared to unstimulated (3T3) CLL cells (Fig. 3A). At day 4, the levels of Bcl-X_L were declined compared to day 0, whereas Mcl-1 protein expression was still upregulated. Also, Noxa protein expression was higher after CD40L stimulation at day 0 and 4, in contrast with mRNA levels observed in figure 1. Finally, CD40 stimulation increases proteasomal degradation of Bim^{EL}. Figure 3B illustrated the decrease in Bim^{EL} protein, which persisted up to day 4.

Concerning drug responses in this setup, roscovitine was functional, as measured by Mcl-1 degradation, at day 4 but not at day 0. Nevertheless, at both time points

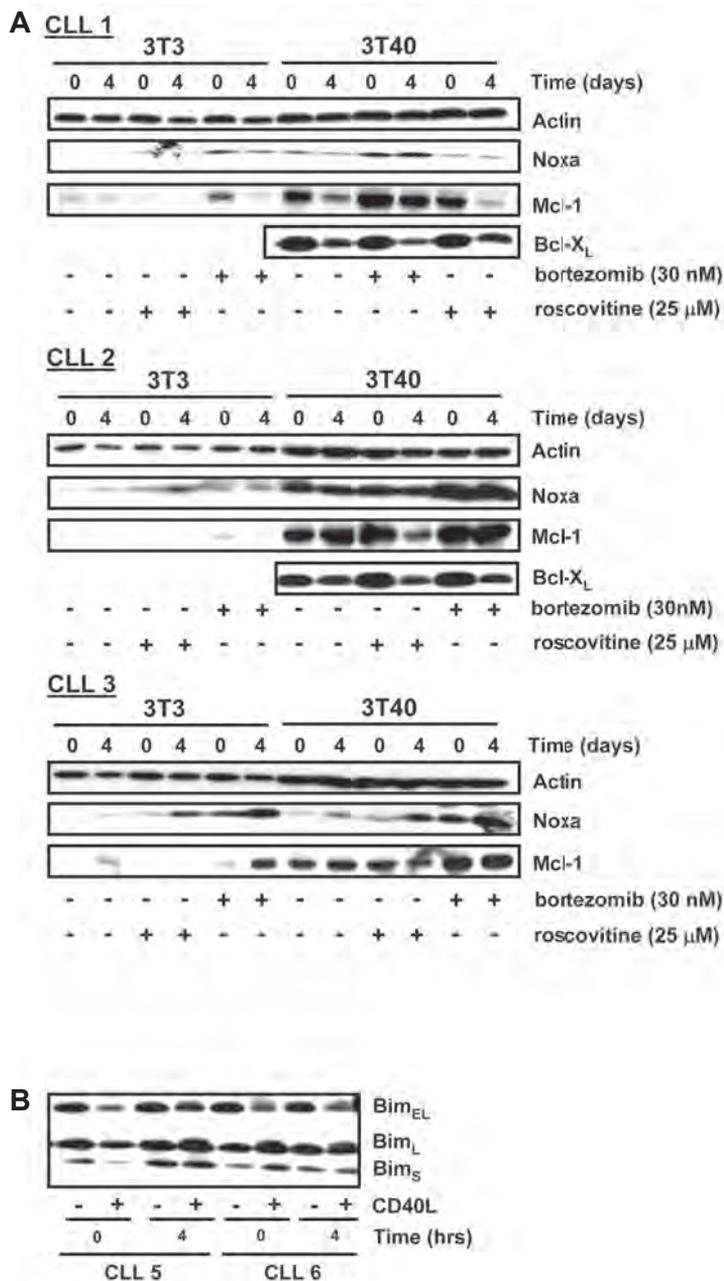


Figure 3. Protein expression of Mcl-1, Bcl-X_L, Noxa and Bim after termination of CD40 stimulation in CLL.

(A) CLL cells were co-cultured with control 3T3 or CD40L-transfected 3T3 cells for 3 days (day 0). After detachment and washing, cells were directly (day 0) or after 4 days (day 4) of CD40L free culture, incubated with the indicated drugs (24 hours). Lysates were probed for Mcl-1, Noxa and Bcl-X_L as indicated and actin as loading control.

(B) CLL cells were co-cultured as in A. Lysates were probed for Bim as indicated and actin as loading control.

roscovitine did not induce apoptosis. Furthermore, as expected bortezomib induced accumulation of Noxa and Mcl-1^{14;29}, at both time points (Fig 3A).

In conclusion, Mcl-1 and Bcl-X_L protein expression were increased after CD40 triggering. After termination of CD40 triggering, Bcl-X_L expression gradually decreased, however, Mcl-1 and Noxa remained high. Also the Erk-mediated Bim_{EL} degradation continued after cessation of the CD40 signal.

CD40 signaling results in NF- κ B, in GSK-3 β and long-lasting ERK activation

Various signaling pathways are known to affect levels of Bcl-2 family members. First, we examined the canonical and alternative NF- κ B activity in three CLL samples stimulated with CD40L. Figure 4A illustrates the phosphorylation of I κ B α and the processing of p100 to p52 at day 0, demonstrating activation of the canonical and alternative NF- κ B pathway upon CD40 triggering. At day 4, activation of the canonical pathway was decreased to baseline expression in CLL 4&5 and reduced approximately 50% in CLL 6. The alternative pathway was decreased in CLL 4&6, but still increased in CLL 5.

Next, it is known that Erk signaling mediates the proteasomal degradation of Bim_{EL} in model systems^{30;31}. Figure 4B shows that Erk is phosphorylated upon CD40 stimulation and this was maintained and even increased at day 4.

Third, Mcl-1 is a known target for phosphorylation and subsequent proteasomal degradation. Cytokine withdrawal in murine cell lines causes decreased PI3K-Akt/PKB signaling to activate GSK-3 β which in turn phosphorylates Mcl-1, thus marking it for proteasomal degradation³². In CLL, CD40 ligation induced both total and phosphorylated (inactive) forms of GSK-3 β (Fig. 4C).

Taken together, these data demonstrated activation of various CD40L-induced survival pathways in CLL. After cessation of the CD40 signal, NF- κ B in general returns to baseline, as also indicated by mRNA data (indicated by Bcl-X_L and Bfl-1 expression). Erk remained significantly activated, while GSK-3 β appeared persistently blocked.

Proteasomal activity does not account for dysregulated protein expression after CD40 stimulation in CLL

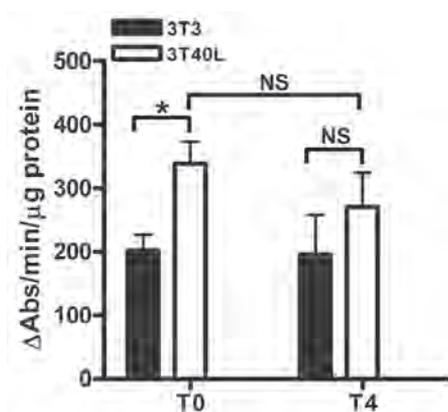
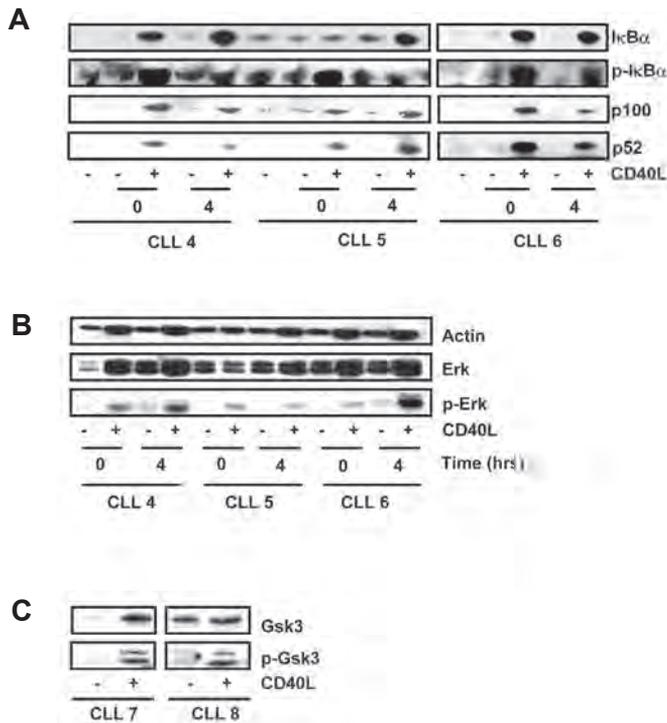
Next, we tested whether the altered protein profile (of Mcl-1 and Bim_{EL}) observed after CD40 stimulation was a consequence of altered proteasomal activity. In figure 5 proteasomal activity was measured in CLL cell lysates obtained after 3 days incubation of the CLL cells with CD40L (day 0; n=13) and four days after cessation of CD40L stimulation (day 4; n=4). Proteasomal activity was significantly increased after CD40 stimulation at day 0. This persisted four days after termination of CD40 stimulation.

Figure 4. Activity of NF- κ B, Erk and GSK-3 β upon CD40 engagement in CLL.

(A) CLL cells were co-cultured with control 3T3 or CD40L-transfected 3T3 cells for 3 days (day0). After detachment and washing (day 0), cells were incubated for 4 days in medium (day 4). Lysates were probed for I κ B α , p-I κ B α and p100/52 as indicated. Shown are representative examples of 3 CLL patients.

(B) CLL cells were co-cultured with control 3T3 or CD40L-transfected 3T3 cells for 3 days (day0). Lysates were probed for Erk and p-Erk as indicated and actin as loading control. Shown are representative examples of 3 CLL patients.

(C) CLL cells were co-cultured with control 3T3 or CD40L-transfected 3T3 cells for 3 days (day 0). Lysates were probed for GSK-3 β and p-GSK-3 β as indicated. Shown are representative examples of 2 CLL patients.

**Figure 5. Proteasomal activity in CLL cells upon CD40 stimulation.**

CLL cells were co-cultured with control 3T3 or 3T40L cells for 3 days. After detachment and washing (day 0). CLL cells were incubated for 4 days in medium (day 4). At day 0 (n=13) and 4 (n=4), CLL cells were lysed, the fluorogenic proteasome substrate LVVY-AMC was added and the enzymatic activity of the 20S proteasome was measured as the amount of AMC fluorescence per minute (counts/min). Results are expressed as change in AMC fluorescence per minute per μ g protein.

$P < 0.01$ with the Mann Whitney U test are indicated by an asterisk (*).

NS = not significant.

CD40 ligation does not influence Mcl-1 and Bim protein turnover in CLL

Finally, we examined whether the half-life of Mcl-1 changed upon CD40 ligation. Therefore, the stability of Mcl-1 was assessed following inhibition of protein synthesis by treatment with cycloheximide. Previous reports in cell lines showed that Mcl-1 protein was rapidly lost with a half-life of around 30 minutes^{33;34}. In CLL, after CD40 stimulation, the half-life of Mcl-1 was similar (Fig. 6). These results suggest that CD40 ligation does not considerably influence the half-life of Mcl-1 in CLL cells.

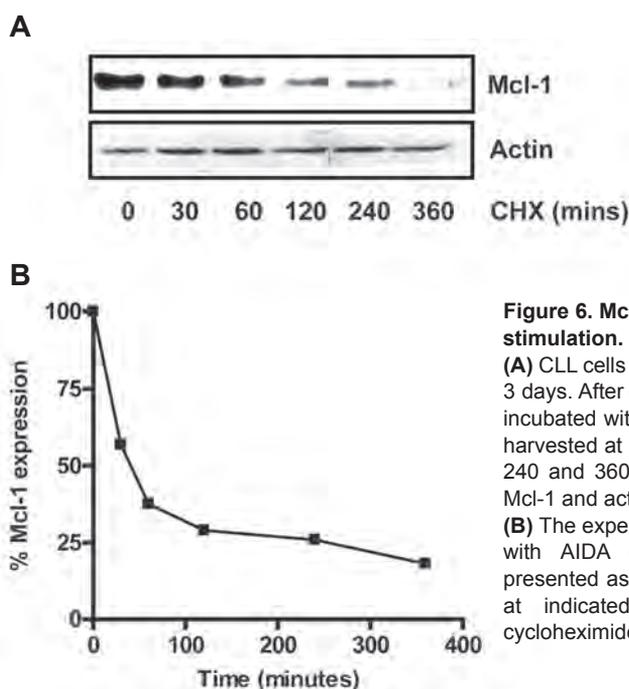


Figure 6. Mcl-1 turnover upon CD40 stimulation.

(A) CLL cells were co-cultured with 3T40L cells for 3 days. After detachment and washing, cells were incubated with 5 μ g/ml cycloheximide. Cells were harvested at indicated time points (0, 30, 60, 120, 240 and 360 minutes). Lysates were probed for Mcl-1 and actin as loading control.

(B) The experiment described in A, was quantified with AIDA evaluation software. Results are presented as the percentage of Mcl-1 expression at indicated time points after addition of cycloheximide (5 μ g/ml).

DISCUSSION

The aim of this study was to mimic the transition of CLL cells as they egress from LN to PB, and relate this to drug sensitivity. The major finding of the present work was that CD40 stimulation has long-lasting effects on protein expression of Mcl-1 and Bim, and this was paralleled by prolonged resistance to the CDK inhibitor roscovitine.

In CLL, CD40 ligation affects various signaling pathways with subsequent transcriptional consequences^{16;20;35}. We observed that both the canonical and alternative NF- κ B pathways were stimulated, and as a result, the NF- κ B target genes Bcl-X_L and Bfl-

1, were upregulated. This expression signature was accompanied by broad drug resistance. Termination of CD40 signals resulted in diminished NF- κ B activity, subsequent Bcl-X_L and Bfl-1 downregulation and pre-sensitization to proteasome inhibitors and fludarabine. Importantly, the resistance to the CDK inhibitor roscovitine persisted. Recent studies by us, and others, have shown that Noxa, Bim and Mcl-1 are crucial mediators in roscovitine-induced apoptosis²⁷. Therefore, the persistent increased Mcl-1/Bim protein ratio observed after cessation of CD40 triggering might mediate the resistance to roscovitine. Remarkably, although roscovitine did initiate Mcl-1 degradation at day 4, this obviously did not trigger apoptosis. We speculate that the Mcl-1 reservoir still dominates and thus continuously neutralizes pro-apoptotic proteins, such as Bim and Noxa. In previous studies we demonstrated the role of Mcl-1, Bim and Noxa in roscovitine-induced cell death²⁷. It would be interesting; however, to test the specific role of these proteins in the resistance to roscovitine through gene knockdown or overexpression experiments in CD40 stimulated CLL cells. Additionally, there might be unknown mechanisms of action of roscovitine, other than the described fast effect on Mcl-1²⁷. In this context, it would be interesting to test the effects of the BH3-mimetic ABT-737^{36;37} or similar compounds, in combination with roscovitine, as it has been shown that roscovitine increased ABT-737 toxicity in human leukemia cell lines³⁸. Mcl-1¹⁴ and Bim protein levels in CD40 triggered CLL are under posttranscriptional control. Both proteins are known targets for phosphorylation and proteasomal degradation, but via different upstream routes. Activated Erk can mediate Bim_{EL} degradation^{30;39;40}, whereas termination of PKB signaling has been linked with GSK-3 β -mediated Mcl-1 phosphorylation and subsequent degradation³². Since both phosphorylated Erk and reduced Bim_{EL} protein levels persist after termination of CD40 signaling (Fig. 3B), it appears likely that this pathway is indeed continuously active in the CLL cells. The control of Mcl-1 protein levels seems less clear. In the two CLL samples tested, CD40 stimulation induced phosphorylation of GSK-3 β , thereby inactivating it. In growth factor withdrawal-mediated apoptosis, this depends on decreased PKB activity and lead to decreased Mcl-1 turnover³². PKB activity was however unaffected upon CD40 stimulation in CLL and the PI3 kinase inhibitor LY294002 did not induce apoptosis (Hallaert, unpublished observation). More surprisingly, although protein levels were persistently increased, the half-life of Mcl-1 was still quite short (<45 minutes). This value is close to the reported basal half-life of approximately 30 minutes in other cells^{33;34}. It would seem therefore, that CD40 triggering does not result in a drastically increased half-life of Mcl-1, although direct comparison with un-triggered CLL cells was not feasible due to very low Mcl-1 levels in those cells. In view of these observations, another possible control mechanism may be involved. Other studies suggested translational regulation of Mcl-1 through

the initiation factor eIF^{41;42}. It is known that phosphorylation eIF2 α can repress translation of Mcl-1 resulting in downregulation of Mcl-1 protein⁴¹. Whether Mcl-1 protein expression after CD40 triggering in CLL is also regulated via eIF2 α or other posttranscriptional mechanisms remains to be determined.

Our earlier work has shown that Noxa is highly expressed in circulating CLL levels²³, but is low in LN^{14;23}. This can be mimicked to a certain extent by *in vitro* CD40 signals, but some inconsistencies concerning Noxa protein levels have come to light in the present studies. First, prolonged *in vitro* culture of CLL on 3T40L cells but surprisingly also 3T3 cells caused persistent lowering of Noxa mRNA as detected by MLPA. In various CLL samples however, a rise of Noxa protein was observed upon prolonged CD40 stimulation. Since the regulation of Noxa at transcriptional level and especially protein level is uncertain⁴³⁻⁴⁶, we can only speculate about the underlying mechanism. First, the rapid suppressive effect of CD40 triggering as well as the slower effect of prolonged co-culture with 3T3 cells on Noxa mRNA indicates that *in vivo* circulating CLL cells lack signals that might suppress this high basal transcriptional rate. In support of this, the basal Noxa mRNA levels are low in LN niches¹⁴. This difference is unlikely to be due to CD40 signals, since we observed that prolonged *in vitro* CD40 triggering can in fact cause a gradual increase in Noxa protein. The underlying mechanism of this puzzling discrepancy between Noxa mRNA and protein remains unexplained. It could on one hand be caused by unknown modes of post-transcriptional regulation of Noxa, or also result from the various forms of regulation of its binding partner Mcl-1. If the latter were true, the observed changes in Noxa are not an intrinsic property, but would result merely from passive accumulation as Mcl-1 protein levels change. More research is required to discriminate among these different options.

Taken together, prolonged CD40 triggering induced not only reversible changes in transcription patterns, but also seems to have long-lasting effects on post-transcriptional regulation of Mcl-1 and Bim. Using this model system to mimic the *in vivo* LN setting could help to predict responses to new drugs.

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