The microenvironment and treatment resistance in chronic lymphocytic leukemia
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Dichotomy in NF-κB signaling and chemoresistance in IGHV mutated versus unmutated CLL cells upon CD40/TLR9 triggering

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Abstract
Chronic lymphocytic leukemia (CLL) cells circulating in peripheral blood (PB) differ from the leukemic fraction in lymph nodes (LN) regarding cell division and drug sensitivity. CD40 stimulation of PB CLL in vitro results in chemoresistance and provides a partial model for the LN microenvironment. The TLR9 ligand CpG induces proliferation in IGHV unmutated, but apoptosis in IGHV mutated CLL. In order to juxtapose proliferative with anti-apoptotic signals, we here studied effects of CpG in the context of CD40 ligation in mutated versus unmutated CLL cells. Prolonged CD40 ligation induced classical followed by alternative NF-κB activity in both subgroups, correlating with enhanced Bfl-1 and Bcl-X_L levels, respectively. A dichotomy in NF-κB signaling occurred upon combined CD40/TLR9 triggering. This induced declining p52 and Bcl-X_L levels, and reversed chemoresistance only in mutated cells, whereas unmutated cells proliferated, maintained p52 and Bcl-X_L and remained chemoresistant. The pivotal contribution of Bcl-X_L to chemoresistance was demonstrated by the BH3 mimetic ABT-737 and RNAi. Finally, in ex vivo LN samples p52, p65 and Bcl-X_L levels were highly expressed, corroborating the in vitro findings. Thus, a distinction in NF-κB activation and drug susceptibility in mutated versus unmutated (LN-like) CLL cells was uncovered, which was causally linked to Bcl-X_L levels.
Introduction

The clinical course of chronic lymphocytic leukemia (CLL) is highly variable (1) and prognosis is strongly associated with mutation status of the immunoglobulin variable heavy chain (IGHV) genes (2-4). Patients with unmutated CLL have a rapidly progressive disease compared to patients with mutated CLL. Both mutated and unmutated CLL display a similar gene expression profile (5, 6), but a subset of genes is differentially expressed. Among these genes is the gene encoding the zeta associated protein of 70kDa (ZAP70) (6), which is more frequently expressed in unmutated CLL. In ZAP70+ CLL there is enhanced IgM signaling (7), but how this affects prognosis is still unclear. It has been speculated that enhanced NF-κB signaling in ZAP70+ CLL might play a role in CLL cell survival (8, 9). In addition, ligand-receptor interactions, such as CD40L-CD40 (10), BAFF and APRIL and their various receptors (11) as well as CpG-TLR9 (12), have been studied as inducers of NF-κB activity in CLL. These signaling routes are putative candidates to influence CLL cell survival and thereby the clinical course of the disease.

CpG motifs are present in unmethylated viral and bacterial DNA, but endogenous ligands released during cellular stress are also thought to activate TLR9 receptors (13). A dichotomy was observed in peripheral blood (PB) CLL cells from mutated versus unmutated CLL patients stimulated with CpG in vitro (14). CpG stimulation resulted in proliferation in the majority of unmutated CLL cells, whereas mutated CLL cells underwent apoptosis. Notably, PB CLL cells differ in important aspects from the leukemic fraction in lymph node (LN) CLL cells. While PB CLL cells are prone to apoptosis, sensitive to various drugs and arrested in the G0/G1 phase of the cell cycle, LN CLL cells have an anti-apoptotic profile, are supposedly drug resistant and are proliferating (15-18). CD40L stimulation of both mutated and unmutated PB CLL cells in vitro results in an anti-apoptotic profile with increased expression of Mcl-1, Bcl-XL and Bfl-1 (19), and the development of drugresistance (18). However, CD40L stimulation does not induce significant proliferation (20) and thus provides only a partial model for the LN microenvironment.

Two different NF-κB pathways can be distinguished. In the classical pathway, phosphorylation of IκBα leads to degradation of IκBα in the proteasome. As a result the active NF-κB subunits p65 and p50 translocate to the nucleus and activate their downstream targets (21). Alternative NF-κB activation leads to phosphorylation and degradation of p100, which results in translocation of the subunits p52 and RelB to the nucleus. Hewamana et al showed that in PB CLL cells the classical NF-κB pathway is associated with in vitro cell survival (9). It is currently unknown which NF-κB pathway predominates in LN CLL cells, and which possible downstream NF-κB targets, such as Bcl-XL or Bfl-1 (22) are important in causing drugresistance. Secondly, it is unclear whether mutated and unmutated CLL differ in these respects.

In order to more accurately mimic the in vivo lymph node microenvironment, in the current study, we stimulated mutated and unmutated CLL cells simultaneously with
CD40L and CpG. Earlier studies have linked CD40 triggering and NF-kB activity in CLL (23, 24), but neither the currently recognized clinically important distinction of mutated versus unmutated CLL, nor the alternative NF-κB pathway was included in those studies. TLR9 triggering causes Myd88-dependent activation of NF-κB (25). Other studies have already reported on the proliferation and cytokine production induced by combined TLR9/CD40 triggering (26). Yet, it is not known if and to what extent TLR9 signaling interferes with simultaneous CD40 triggering in mutated or unmutated CLL cells to alter NF-κB signaling pathways and development of drugresistance. We performed time course analyzes of prolonged CD40L stimulation showing sequential activation of the classical and alternative NF-κB pathway. This correlated with early Bfl-1 and late Bcl-X₅ upregulation, respectively. Furthermore, a novel distinction in alternative NF-κB activation and drug susceptibility in mutated versus unmutated CLL was observed; only in mutated CLL combined triggering of CD40 and TLR9 downregulated p52 and Bcl-X₅ levels and abrogated drugresistance. Lastly, in ex vivo CLL LN samples, activation of the classical and alternative NF-κB pathway, as well as Bcl-X₅ expression could be observed, underlining the relevance of the in vitro observations.

Materials and Methods

Patient material
After informed consent patient material was obtained during diagnostic or follow-up procedures at the departments of Hematology and Pathology of the Academic Medical Center Amsterdam. This study was approved by the AMC Ethical Review Board (ERB) and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. LN material, diffusely infiltrated by CLL (unmutated), was freshly frozen in liquid nitrogen directly after surgical removal. Immunohistochemical analysis of LN revealed that greater than 90% of the tissue consisted of tumor cells (18). PB of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, The Netherlands) were frozen and stored as described (27). Expression of CD5 and CD19 (Beckton Dickinson (BD) Biosciences, San Jose, CA, USA) on leukemic cells was assessed by flow cytometry (FACScalibur, BD Biosciences) and analyzed with CellQuest software (BD Biosciences). Patient characteristics are reported in Supplemental table 1.

Reagents
CpG oligonucleotide type B- Human TLR9 ligand (ODN2006) was purchased from Invivogen (San Diego, USA). Bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands). Roscovitine and fludarabine (F-Ara-A) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BAY-11-7082 was obtained from Calbiochem (Amsterdam, The Netherlands). ABT-737 (28) was obtained under MTA from Abbott (Abbott Park, Illinois, USA, courtesy Dr S. Rosenberg).
Cell culture, proliferation assay, detection of apoptosis

PB lymphocytes of CLL patients were stimulated by co-culture with NIH3T3 fibroblasts stably transfected with human CD40L, as previously described (27). Briefly, DMSO-frozen CLL cells were thawed and co-cultured on irradiated (30Gy) control NIH3T3 fibroblasts or NIH3T3 fibroblasts expressing CD40L ± CpG (1,5 μg/ml). The CLL-cell concentration was 1,67x10^6 cells/ml in IMDM containing 10% FCS. After 1, 6, 24 or 72 hrs of culture at 37°C cells were detached and incubated in medium ± cytotoxic drugs for an additional 24-48 hrs. To test the effect of BAY-11-7082 cells were pre-treated for 30 minutes with 1 μM of BAY-11-7082.

To analyze proliferation upon CD40L/CpG stimulation CLL cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands). Cells were resuspended in PBS at 1,0x10^7/mL in 0,5 μM CFSE for 15 min at 37 °C and washed. After stimulation for 72 hours with CD40L/CpG, cells were analyzed by flow cytometry.

For apoptosis induction, cells were treated with 100 μM fludarabine (48 hrs), 30nM bortezomib (24 hrs) or 25 μM roscovitine (24 hrs), and stained with 200 nM MitoTracker Orange (Molecular Probes) for 30 minutes at 37°C followed by flow cytometry (27). Additionally, apoptotic and viable cells were discriminated by Annexin V (IQ Products, Groningen, The Netherlands) and propidium iodine staining (PI; Sigma, St Louis, MO) as described (27).

Western Blot and antibodies

Western blotting was performed as previously described (27). Samples (10-80 μg protein) were separated by 13% SDS-PAGE gel electrophoresis. Blots were probed with polyclonal anti-Bcl-X\_L (catalog#620211, BD Biosciences), antiserum to β-actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal NF-κB2 p100/p52 (catalog#4882 Cell Signaling), polyclonal anti-totalκBα (catalog#9242 Cell Signaling), monoclonal mouse antibody for phospho-IκBα (catalog#9246 Cell Signaling), polyclonal anti-p65 (clone C-20; catalog#sc-372; Santa Cruz), polyclonal anti-Histon H3 (catalog#9715 Cell Signaling), polyclonal antibodies against A1/Bfl-1 were a kind gift of Prof. Dr. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Immunoreactive proteins were visualized using HRP-conjugated Ig (DAKO, Glostrup, Denmark) and enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK). IRDye 680 donkey anti-rabbit IgG, IRDye 800 donkey anti-goat IgG or IRDye 800 donkey anti-mouse IgG (Westburg, Leusden, the Netherlands) were used as secondary antibody when blots were scanned on the Odyssey imager (LI-COR Biosciences, Lincoln, NE). When indicated, Western blots were subjected to densitometry by analysis with the AIDA image analyzer software (AIDA Image Analyzer version 3.52) or the Odyssey software (Odyssey Application software version 3.0).
Enzyme-linked immunosorbent assay (ELISA)
IL-6, IL-8, IL-10 and TNF-α in supernatants were measured using the PeliKine human ELISA kit (Sanquin, Amsterdam, The Netherlands) according to manufacturer’s recommendations. Absorbance was read at 450 nm.

RNAi and nucleofection
CLL cells were transfected using the Amaxa nucleofection technology (Amaxa, Koln, Germany), according to the manufactures’ recommendations and as described (20). Briefly, cells (5,0x10⁶) were resuspended in 100 μL Human B- Cell nucleofector kit solution. RNAi (Bcl-X₇ and Silencer Select Negative Control#1) was obtained from Ambion (ID#1920 and Cat#4390843) and 1,5-3 μg was used. Cell suspensions mixed with RNAi were nucleofected with the Amaxa Nucleofector apparatus using program X-05. Thereafter, cells were immediately transferred into pre-warmed 6-well plate containing NIH3T3-cells expressing CD40L, and co-cultured for 72 hours before protein lysates were obtained and treatment with cytotoxic drugs was initiated.

Statistics
The Shapiro-Wilk normality test was performed to analyze Gaussian distributions. If there was a Gaussian distribution a two-sided t-test was used to analyze differences between the groups. If there was no Gaussian distribution, a two-tailed Mann-Whitney U-test was used to analyze differences between the groups and a Wilcoxon matched paired test to analyze differences between paired samples. Statistically significance was set at P<0.05, with * representing 0.01<P<0.05; ** 0.001<P<0.01; *** P<0.001.

Results
CD40L stimulation of PB CLL cells first activates the classical and then the alternative NF-κB pathway
It is assumed that in vivo CLL cells continuously transit from lymph nodes to peripheral blood. In lymph node proliferation centers CD40-CD40L interaction takes place, which can be mimicked by in vitro co-culture of CLL cells from peripheral blood on feeder cells expressing human CD40L (18, 20, 26, 27, 29). The kinetics of CD40L-induced NF-κB activity in such a system are unknown. In time course experiments (1-72hrs CD40 stimulation), we found sequential activation of the classical and alternative NF-κB pathway in both mutated and unmutated CLL. The classical NF-κB pathway, as detected by phosphorylated IκB, was activated within 1 hour (Figure 1A). Synthesis of p100 and simultaneous appearance of p52 indicating alternative NF-κB pathway activity became apparent from 24-72 hours after CD40 stimulation. Bfl-1 and Bcl-X₇ are members of the anti-apoptotic Bcl-2 family and are both downstream NF-κB targets (22). Increased Bfl-1 protein levels were associated with high levels of pIκBα at 6 hrs of CD40L stimulation,
whereas elevated Bcl-X_L levels were associated with activation of the alternative NF-κB pathway, as indicated by elevated levels of p52 at 24 hrs (Figure 1A).

To investigate whether Bfl-1 was indeed a downstream target of the classical NF-κB pathway, and Bcl-X_L of the alternative pathway, CD40-stimulated CLL cells were treated with an irreversible inhibitor of IκBα phosphorylation (BAY-11-7082). Blocking classical NF-κB activation resulted in a clear downregulation of IκBα phosphorylation and Bfl-1 levels. In comparison, p52 and Bcl-X_L levels were partially maintained after BAY-11-7082 both in mutated and unmutated CLL (Figure 1B). Thus, alternative NF-κB activation was at least partially independent of classical NF-κB activity in CD40-stimulated CLL cells.

Prolonged CD40 stimulation of CLL cells is known to induce resistance to various drugs (20, 24, 27). Co-incubation with BAY-11-7082 did not sensitize mutated and unmutated cells to bortezomib and only partially abrogated the chemoresistance of mutated CD40-stimulated CLL to fludarabine (Figure 1C). For fludarabine, mutated samples appeared more sensitized than unmutated samples, but this did not reach statistical significance (n=7 for both groups) (black bars Figure 1C). To further correlate chemoresistance in conjunction with NF-κB activation, we investigated sensitivity of CLL for fludarabine and bortezomib after 6, 24 and 72 hrs of CD40 stimulation. Significant differences in resistance to bortezomib and fludarabine were observed comparing 6 hrs of CD40 stimulation to prolonged CD40 stimulation (24 and 72hrs) in both patient groups (Figure 1D). In control samples (medium) no significant differences in apoptosis were observed. Thus, we conclude that the development of CD40L-induced drugresistance occurred predominantly in parallel to enhanced activity of the alternative NF-κB pathway and a rise in Bcl- X_L levels.

Combined CD40/TLR9 triggering induces proliferation predominantly in unmutated CLL cells

CD40 ligation alone induced proliferation in CLL cells to a very moderate extent (Figure 2A) and in a small proportion of cells in cultures for up to 7 days (data not shown). Longo and colleagues have demonstrated that the TLR9 ligand CpG induces proliferation mainly in unmutated CLL, whereas mutated CLL undergo apoptosis (14). In order to more accurately mimic the in vivo lymph node microenvironment where CLL cells proliferate, we stimulated CLL cells simultaneously with CD40L and CpG for three and five days. As shown before (14), stimulation with CpG only resulted in proliferation of unmutated, but not mutated CLL (Figure 2A,B). The proliferating fraction upon simultaneous CD40/TLR9 triggering was considerably larger than upon triggering with either of the stimuli alone. Notably, 3 days of CD40/TLR9 triggering of mutated CLL hardly resulted in proliferation. After 5 days of CD40/TLR9 triggering a significant difference in proliferation was observed between mutated and unmutated CLL (Figure 2B). The observed differences were not attributable to differential expression of CD40 or TLR9 in mutated versus unmutated CLL.
**Figure 1. Time course analysis of classical and alternative NF-κB activation and drug resistance in mutated and unmutated CLL cells.**

- **A.** Cells were stimulated with CD40L for the indicated time (0, 1, 6, 24 and 72 hrs). Protein lysates were probed for IκBα, pIκBα, Bfl-1, p100, p52, Bcl-X, levels and β-actin levels as loading control. Bands were visualized using HRP-conjugated Ig and enhanced chemiluminescence. Blots from one representative mutated and one unmutated patient are shown, of a total of four analyzed for each group.

- **B.** Mutated (n=4) and unmutated (n=4) CLL cells were stimulated with 3T3 (control) or CD40L-expressing 3T3 cells for 0, 6 and 72 hours ± classical NF-κB inhibitor BAY-11-7082 (1μM) as indicated. Protein lysates were probed for pIκBα, Bfl-1, p100, p52, Bcl-X, and β-actin as loading control. Western blots were scanned on the Odyssey imager. Blots from a representative mutated and an unmutated patient are shown.

- **C.** Mutated (n=7) and unmutated (n=7) were co-cultured with control 3T3 (control) or CD40L-expressing 3T3 cells for 72 hrs, ± BAY as indicated. After detachment, cells were incubated with bortezomib for 24 hrs or fludarabine for 48 hrs. Apoptosis was measured with MitoTracker staining. Error bars represent standard error of the mean. *0.01 < P < .05; **0.001 < P < .01; *** P < .001.

- **D.** CLL cells were stimulated with 3T3 (control) or CD40L-expressing 3T3 cells for 6, 24 and 72 hrs. After detachment, cells were incubated with the indicated drugs as described in the Materials and Methods and analyzed for apoptosis by MitoTracker staining after 24 hrs (bortezomib 30nM) or 48 hrs (fludarabine 100μM). The number of patient samples analyzed was 5 mutated and 6 unmutated for the 6 hr time point, 9 for both groups for the 24 hr time point, and 8 mutated and 6 unmutated for the 72 hr time point. Error bars represent standard error of the mean. *0.01 < P < .05; **0.001 < P < .01; *** P < .001.
Figure 2. Proliferation of unmutated CLL cells upon combined CD40L/CpG stimulation. A. Mutated and unmutated CLL cells were stimulated with CpG, CD40L and a combination of CpG and CD40L. Cells were labelled with CFSE and monitored after 3 and 5 days by FACS analysis. B. For both patient groups, the fraction of proliferating cells on day 3 and day 5 after the indicated stimuli is plotted. Bars represent the average of four patients ± SE. * .01 < P < .05.
Figure 3. P52 and Bcl-X\(_L\) levels are downregulated in mutated CLL cells but not in unmutated CLL cells stimulated with CD40L and CpG. A. CLL cells were co-cultured with control 3T3 (control) or CD40L-expressing 3T3 cells for 72 hrs, ± CpG as indicated. Protein lysates were probed for p100, p52, Bcl-X\(_L\), pIkB-a, Bfl-1, as indicated and \(\beta\)-actin as loading control. The p52 levels of the unmutated patient depicted were analyzed in two separate Western blots of the same experiment, as indicated by the vertical line. Results are representative of at least four different patients. B. Western blots of p52 and Bcl-X\(_L\) were quantified by use of the AIDA image analyzer software in both mutated and unmutated CLL patients stimulated with CpG, CD40L and a combination of CpG and CD40L. Bars represent the average of seven patients ± SE. P52 and Bcl-X\(_L\) levels in mutated were compared to those in unmutated CLL samples, \(p=0.018\) and \(p=0.007\), respectively). * .01 < \(p\) < .05; ** .001 < \(p\) < .01.
cells at baseline or upon stimulation with CD40L/CpG as measured by flow cytometry and q-PCR (data not shown).

In supernatants of these cultures, cytokine levels were determined by ELISA. In agreement with previous data (26), TNF-α, IL-6 and also IL-10 secretion was enhanced by combined CD40/TLR9 triggering (Supplementary Figure 1). For IL-6, IL-8 and IL-10 no differences were observed between mutated and unmutated CLL stimulated with CD40L/CpG. Regarding TNFα levels, there was a trend towards higher levels in the supernatant of unmutated CLL stimulated with CD40L/CpG as compared to mutated CLL.

Dichotomy in alternative NF-κB activation, Bcl-XL expression, and drug susceptibility in mutated versus unmutated CLL cells upon combined CD40L and CpG stimulation

In view of the above differences we investigated the effect of CpG on CD40L-induced NF-κB activation and concomitant drugresistance. Western blot analysis was performed for p52, Bcl-XL, pIkBα and Bfl-1 after 72 hrs of stimulation. Notably, P52 and Bcl-XL showed a clear decline in mutated CLL cells upon CD40/TLR9 triggering compared to

![Graph A](image1)

![Graph B](image2)

**Figure 4. CpG abrogates CD40L-induced drugresistance in mutated but not in unmutated CLL cells.** A. Mutated and unmutated CLL cells were co-cultured with 3T3 (control) or CD40L-expressing 3T3 cells for 72 hrs, ± CpG as indicated. Sensitivity for bortezomib (30 nM) was tested for 10 mutated and 9 unmutated patients, fludarabine (100 μM) was tested for 8 mutated and 9 unmutated patients. B. Data for the CD40L-CpG stimulation in combination with bortezomib (left panel) or fludarabine (right panel) was compared for the two patient groups. Dots represent individual patients and the horizontal line represents the average. For A and B error bars represent standard error of the mean. * .01 < P < .05; *** P < .001.
CD40 stimulation alone. In contrast, in unmutated cells no such decline in p52 and Bcl-X<sub>L</sub> was observed (Figure 3A upper panel). Densitometric analysis of 7 mutated versus 7 unmutated CLL samples revealed that these differences were statistically significant (Figure 3B). Compared with the 3T3 control, TLR9 triggering showed a modest upregulation of Bcl-X<sub>L</sub> in both groups. A representative sample is showed in Figure 3A. There was a trend that unmutated CLL cells (n=8) stimulated with CD40L/CpG showed higher pκBα levels than mutated CLL cells (n=7) stimulated with CD40L/CpG, but this did not reach statistical significance (Supplemental figure 2). No significant differences in Bfl-1 levels were observed between mutated and unmutated CLL cells stimulated with CD40L/CpG (Figure 3A and Supplemental figure 2). Thus, a dichotomy occurred in alternative NF-κB signaling and Bcl-X<sub>L</sub> levels in mutated versus unmutated CLL cells triggered via CD40 and TLR9.

In line with the study of Longo et al (14), CpG induced apoptosis in mutated CLL, and we observed that this could be completely prevented by simultaneous stimulation with CD40L (Figure 4A). Next, drug sensitivity to bortezomib and fludarabine was measured in mutated and unmutated CLL cells after 72 hours of CD40/TLR9 triggering. In mutated CLL cells, CD40L-induced drugresistance was partially abrogated by adding CpG. In contrast, in unmutated CLL cells, in which CD40L-induced p52 and Bcl-X<sub>L</sub> levels were unaffected by CpG, no effect on drugresistance was observed (Figure 4A). For bortezomib as well as fludarabine these differences between mutated and unmutated CLL were statistically significant (Figure 4B).

**CD40L-induced drugresistance is mediated by Bcl-XL**

The previous experiments indicated that Bcl-X<sub>L</sub> levels are important in the development of drugresistance in CD40-stimulated CLL cells. To functionally address this, Bcl-X<sub>L</sub> levels were lowered in two different ways. Firstly, the Bad like BH3-only mimetic ABT-737 which has high affinity for Bcl-2 and Bcl-X<sub>L</sub> (30) was used. Previously, we and others have shown that CLL cells are highly sensitive for ABT-737 and that this is reduced at least 100-fold upon CD40 triggering (20, 27). When used in a sublethal dose in this setting (0.1μM), ABT-737 significantly increased drug-induced apoptosis in both mutated and unmutated CLL cells stimulated with CD40L/CpG (Figure 5A).

Next, RNAi was used to knock-down Bcl-X<sub>L</sub>. Nucleofection with specific siRNA, but not aspecific control siRNA, resulted in a clear knock-down of Bcl-X<sub>L</sub> (Figure 5B). Quantified data of the Bcl-X<sub>L</sub>/β-actin ratio showed significant differences between CLL cells treated with Bcl-X<sub>L</sub> RNAi and negative control RNAi (Figure 5C). Importantly, knock-down of Bcl-X<sub>L</sub> in CLL cells resulted in enhanced drug susceptibility for bortezomib and roscovitine (Figure 5D). In these experiments, specific apoptosis was calculated and plotted, since background apoptosis was enhanced by the nucleofection procedure. Mean levels of background apoptosis in CD40- stimulated CLL cells were 25,4% ± 7,78% (mean ± SE). For Bcl-X<sub>L</sub> and negative control RNAi treated CLL cells background apoptosis levels were higher, respectively 43,3% ± 10,53% and 42,3% ± 8,88% (mean ± SE). Fludarabine-
induced apoptosis is routinely measured after 48 hours after detachment from 3T40L cells, but at this time point background apoptosis levels in Bcl-XL RNAi treated CLL cells reached approximately 60% and a significant additional effect of fludarabine could not be reliably observed (data not shown). In summary, the combined data from the ABT-737 and RNAi experiments strongly suggested that Bcl-XL levels play an important role in determining drug susceptibility of CLL cells stimulated with CD40L.

Figure 5. Nucleofection of Bcl-XL RNAi and ABT-737 results in enhanced sensitivity to cytotoxic drugs. A. A sublethal dose of ABT-737 (0.1μM) was added after 72 hours of CD40-TLR9 triggering and sensitivity for bortezomib (30 nM) and fludarabine (100 μM) was tested for mutated (n=8) and unmutated (n=6) patients. B. Nucleofection with Bcl-XL or control siRNA was done on CLL cells before co-culturing on CD40L expressing 3T3 cells. Lysates of CLL cells nucleofected with Bcl-XL RNAi or negative control RNAi were probed for expression of Bcl-XL or actin as a loading control. As a control lysates of CLL cells on 3T3 and CD40L expressing 3T3 cells are shown. C. Western blots were quantified with the Odyssey software for 4 CLL patients. D. After nucleofection with Bcl-XL RNAi or negative control RNAi followed by stimulation with CD40L for 72 hrs mutated CLL cells (n=4) were treated with bortezomib (30 nM) and roscovitine (25 μM). For A, B and C error bars represent standard error of the mean * .01 < P < .05; ** .001 < P < .01
PB CLL cells studied in vitro with CD40L and CpG and lymph node proliferation centers in vivo have a similar NF-κB signaling signature

To investigate whether our in vitro model resembled the situation in vivo, lymph node samples obtained from CLL patients (only available from unmutated patients) were compared with those from peripheral blood (mutated and unmutated) (Figure 6). As expected based on previous studies (9, 31), p65 is present in the nuclei of peripheral blood CLL samples, but its levels are significantly higher in lymph node samples. Bfl-1 could be observed in moderate to high amounts in both PB and LN samples, and there seemed to be no major difference among the two compartments. By comparison, in a control sample using our in vitro CD40-stimulation system, higher p65 as well as Bfl-1 levels at 6 and 72 hr time points were observed. Concerning the alternative NF-κB pathway, in the majority of lymph node samples p100 was clearly processed into p52. In peripheral blood samples activity of the alternative NF-κB pathway was lower, which was correlated with a clear difference in Bcl-XL expression in PB versus LN compartment. In most instances, no p100 band could be observed in ex vivo samples for an unknown reason. The levels of p52 and Bcl-XL were comparable between ex vivo samples and CLL cells stimulated in vitro via CD40L. Together, these results strongly suggest that in the LN compartment both classical and alternative NF-κB signaling occur, and at appreciably higher levels than in PB. Secondly, with respect to Bfl-1 and Bcl-XL levels the alternative pathway appears to predominate in LN correlating with our in vitro observations. Thus,

Figure 6. P52, p65 and Bcl-XL are highly expressed in CLL lymph nodes. A. Nuclear or cytoplasmic fraction protein lysates obtained from peripheral blood (PB, n=5) and lymph node (LN, n=7) were probed for p65, Histone H3, Bfl-1 and β-actin as indicated. All lymph node samples were obtained from unmutated CLL patients. The first two PB samples were obtained from mutated CLL patients, the last three samples were obtained from unmutated CLL patients. For comparison, PB CLL cells stimulated in vitro for 6 and 72 hours via CD40 were included. B. Cytoplasmic fractions of the same samples were probed for p100/52, Bcl-XL and β-actin. We have previously described Bcl-XL levels in lymph node samples (27). Western blots were scanned on the Odyssey imager. Vertical lines indicate that images were derived from separate gels.
the in vitro CD40/TLR9 model - which renders CLL cells chemoresistant and endows proliferative capacities - at least partially recapitulates the local NF-κB signaling in lymph node proliferation centers in vivo.

Discussion

The present study demonstrates that prolonged CD40 stimulation of CLL cells induced sequential classical and alternative NF-κB activation which correlated with Bfl-1 and Bcl-XL levels, respectively. Combining CD40L and CpG uncovered a dichotomy in prognostic subgroups with respect to proliferation as well as alternative NF-κB activity, Bcl-XL levels and drugresistance. In addition to the well-known divergent B cell receptor signaling in ZAP70+ versus ZAP70- CLL cells (7), this new distinction in survival signaling upon CD40L/CpG stimulation may have clinical relevance.

The correlation between CD40 stimulation, NF-κB activity, increased survival and drugresistance in CLL cells has been reported earlier (23, 24). In addition, a recent study addressed the basal classical NF-κB activity in unstimulated peripheral blood CLL cells, which was inversely correlated with the response to fludarabine (9). Compared to these studies, our current study provides novel as well as more detailed information. Firstly, we describe sequential classical and alternative NF-κB signaling in time-course experiments, revealing alternative NF-κB activity after prolonged CD40 stimulation. Secondly, expression of the anti-apoptotic Bcl-2 members Bfl-1 and Bcl-XL is shown to be related to classical and alternative NF-κB activation, respectively. Interestingly, induction of Bcl-XL via the alternative NF-κB pathway is related to resistance to various drugs. Thirdly, we separate all our patient data according to the clinically relevant subgroups of mutated (n=15) versus unmutated (n=12) CLL. Furthermore, using the TLR9 ligand CpG, we uncover a hitherto undescribed dichotomy in mutated versus unmutated CLL, both in relation to proliferation and drugresistance. Finally, by investigating ex vivo lymph node samples we demonstrate that the relevant signaling pathways and anti-apoptotic Bcl-2 family members are active players in these proliferation centers. It is known that constitutive CD40 signaling in murine B cells induces alternative NF-κB activation and promotes lymphomagenesis (32). This correlates well with our finding that prolonged CD40 stimulation of CLL cells (24-72 hours) resulted in alternative NF-κB activation and upregulation of Bcl-XL levels. The crucial role of Bcl-XL in the development of drugresistance could be demonstrated by the BH3 mimetic ABT-737 and Bcl-XL RNAi. Simultaneous stimulation with CD40L/CpG has been studied previously in normal and CLL cells to enhance immunogenicity (26). The effect of combined stimulation on drugresistance, or prognostic subgroups and NF-κB signaling were hitherto unknown. We could demonstrate that combined CD40/TLR9 triggering diminished p52 and Bcl-XL levels in mutated CLL cells and partially abrogated drugresistance in mutated, but not unmutated cells. The reason for the dichotomy in CD40L/CpG induced NF-κB
signaling in mutated versus unmutated CLL cells is as yet unknown. It could be related to the well-known difference in ZAP70 expression in mutated and unmutated CLL cells. Indeed, a recent study using unstimulated PB CLL cells reported a relation between ZAP70 status and p65 activity which determined sensitivity to a novel IκB kinase inhibitor (31). In our setting of in vitro CD40/TLR9 stimulation, no changes in ZAP70 expression occurred which could be linked to NF-κB signaling (data not shown). In addition, an earlier report concluded that phospho-IκB correlates with clinical outcome in CLL with lymph node involvement, but is independent from ZAP-70 expression as a prognostic factor of survival (33). We therefore presume that, independently of ZAP70 signaling, the two CLL subgroups might differ intrinsically in their wiring of upstream NF-κB components. It has been shown that both mutated and unmutated CLL cells bear the phenotype of activated B cells (34), but it has been suggested that unmutated CLL cells resemble B cells at an earlier state of activation than mutated CLL cells (35). The degree to which the pre-malignant mutated and unmutated CLL cells have been exposed to TNF members, e.g. CD40L, BAFF or APRIL, which all affect NF-κB signaling, most likely varies depending on the encounter with T helper cells or by location in the LN (36, 37). Thus, it is conceivable that different modes of upstream NF-κB signaling are hard-wired in the two CLL subgroups. Possibly, in mutated CLL cells stimulated with CD40L/CpG, a negative feedback mechanism is activated, which does not occur in unmutated CLL cells. Negative regulation of NF-κB signaling is mediated by a family of deubiquitinating enzymes among which are cylindromatosis (CYLD) and A20 (38). Recent studies have shown inactivation of A20 in various B cell malignancies (39, 40). Furthermore, alternative NF-κB signaling in many cell types relies on NF-κB-inducing kinase (NIK), which is controlled in a complex manner through interaction with TRAF and IAP family members. Important in the context of our studies, regulation of NIK can be under control of CD40 or BAFF signaling (41, 42). If and how CpG/TLR9 signaling interferes with these signaling pathways to affect NF-κB activity, in particular in the context of CLL, is currently unresolved. However, the source for the dichotomy in mutated versus unmutated CLL cells upon CD40/TLR9 triggering might well lie in differential NF-κB signaling mediated through upstream regulators such as cIAP1/2, A20, NIK, and TRAF family members. The complex interplay between these signaling modulators is currently the subject of intense scrutiny (43, 44). In addition, combined CD40/TLR9 triggering induces proliferation in unmutated CLL cells, suggesting that these signaling pathways can also interfere or even replace signals that are presumed to initiate from the B-cell receptor (BCR). In this context, the recent insights into chronic active BCR signaling in diffuse large B-cell lymphoma, which also involves constitutive NF-κB activity for cell survival (45), may also have relevance for CLL.

Based on our current data, Bcl-X<sub>L</sub> appears to be an important downstream target of the alternative NF-κB pathway in determining drugresistance. Treatment with the BH3 mimetic ABT-737 as well as knock-down of Bcl-X<sub>L</sub> resulted in enhanced sensitivity to
cytotoxic drugs. Treatment with ABT-737 showed complete abrogation of drugresistance in both subgroups. At a concentration of 0.1 μM ABT-737 treatment by itself did not induce apoptosis, in line with data from Vogler et al (20). In addition, we observed an association between alternative NF-κB activity and Bcl-X_L levels. Indeed, specifically blocking classical NF-κB activity by BAY-11-7082 strongly abrogated Bfl-1 expression, whereas only a partial effect on Bcl-X_L and p52 expression was observed. Our data indicate that compared to the alternative NF-κB pathway, the classical NF-κB pathway plays a minor role in the NF-κB dichotomy of mutated versus unmutated CLL cells stimulated with CD40L/CpG. To date, there are no specific pharmacological inhibitors available to selectively block the alternative NF-κB pathway. NIK RNAi has been used to target the alternative pathway (46), but CD40 requires NIK for signaling events in both the classical and alternative NF-κB pathway (47). Thus, NIK might not be the appropriate target to selectively block alternative NF-κB activity upon CD40 triggering in CLL.

B cell receptor triggering is proposed to drive CLL proliferation in vivo, especially for the unmutated subtype (8). Various prior studies applied surface IgM crosslinking in vitro to assess [3H] thymidine incorporation (48) or Ki-67 staining (49) in CLL patients, which resulted in minimal proliferation. By combining CD40L and CpG stimulation, we could demonstrate clearly enhanced proliferation in unmutated CLL cells, correlating with the development of drugresistance. By comparison, in mutated CLL cells, CD40L/CpG stimulation resulted in modest proliferation at day 5, and partial abrogation of drugresistance. Although our co-culture system provides an oversimplified model for the complex lymph node microenvironment, various cytokines are produced such as, TNFα, APRIL, IL-6, IL-8 and IL-10 which may be involved in tumor cell survival in vivo (50-52) (Supplemental figure 1 and data not shown). Finally, the in vitro effects of CD40L/CpG stimulation at least partially relate to the in vivo situation as can be deduced from the comparable, high levels of p52, p65 and Bcl-X_L in observed ex vivo CLL lymph node samples. Our findings also underscore that Bcl-X_L is a potential target for therapy, e.g. by BH3 mimetics such as ABT-737, especially in unmutated CLL patients.

Currently, CLL is viewed as a heterogeneous malignancy. In a small subset of CLL patients the disease has a rapid course and requires early treatment. The dynamic cell turnover reported by recent studies (53, 54) may well be linked with cell division rates as well as the period leukemic cells reside in LN proliferation centers. Our findings indicate that modeling of the lymph node characteristics of CLL is feasible and can provide novel information on distinct responses in vitro to cytostatic drugs among prognostic subgroups.

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Conflict of interest
The authors declare no conflict of interest.
Reference List


Table 1. Patient characteristics.

<table>
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1 Percentage of cells positive for CD5 and CD19 surface expression measured by flow cytometry.
2 Mutated IgVH gene (+) denotes >2% mutations compared to germ line sequence.
3 As determined by FISH. Probes for 11q22.3 (ATM), centromere 12 (CEP12), 13q14 (D13S319), 14q32 (IGH) and 17p13 (TP53) were obtained from Abbot-Vysis. Samples with > 10% aberrant signals were considered abnormal.
**Supplemental figure 1.** Cytokine production in mutated and unmutated CLL cells stimulated with CD40L and/or CpG. Mutated and unmutated CLL cells were co-cultured with 3T3 or CD40L-expressing 3T3 cells ± CpG as indicated. After 72 hours supernatants were collected and IL-6, IL-8, IL-10 and TNF-α levels were measured by ELISA. CLL cells from three to six mutated and unmutated patients were used for the experiments. Error bars present standard error of the mean.

**Supplemental figure 2.** Densitometric analysis of classical NF-κB activity and the downstream target Bfl-1 in mutated and unmutated CLL cells stimulated with CD40L and CpG. CLL cells were co-cultured with control 3T3 (control) or CD40L-expressing 3T3 cells for 72 hrs, ± CpG as indicated. Western blots of pIkBα and Bfl-1 were quantified by use of the AIDA image analyzer software in both mutated (n=7) and unmutated (n=8) CLL patients. Protein levels are normalized for β-actin. Error bars present standard error of the mean.