The interplay between microenvironmental signaling and novel targeted drugs in CLL

Thijssen, R.

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IL-21 AND IL-4 DISTINCTLY AFFECT NF-κB BINDING ON THE BCL-XL PROMOTER IN CLL CELLS


ABSTRACT

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in western countries, remains an incurable disease. A major clinical problem is the acquired drug resistance in response to therapy, which is strongly influenced by the lymphoid 'microenvironment' within the lymph nodes (LN) and spleen. In the microenvironment, cross-talk between supporting cells, such as CD40L-expressing T cells, and CLL cells deliver critical survival signals. CD40 stimulation drives drug resistance predominantly by upregulation of the anti-apoptotic regulator Bcl-XL in CLL cells.

Here we examined how Bcl-XL is regulated in CLL in the context of CD40 stimulation and follicular T cell cytokines IL-21 and IL-4. p65 (RelA) and p52 (NF-κB2), operating in the canonical or non-canonical NF-κB pathway, respectively, both bind to the Bcl-XL promoter and activate Bcl-XL upon CD40 stimulation. Inhibition of both NF-κB pathways decreases Bcl-XL expression and partially reverts CD40-induced drug resistance. Furthermore, IL-4 and IL-21 exert opposing effects on CD40-mediated Bcl-XL expression. IL-21/STAT3 signalling reduces Bcl-XL transcription whereas IL-4/STAT6 signalling augments BCL-XL transcription. Our data indicate that STAT3 and -6 affect NF-kB activity directly at the Bcl-XL promoter. Further elucidation of the IL-21/STAT3 pathway to suppress Bcl-XL may lead to novel therapeutic targets.

Rachel Thijssen1,2, Arnon P. Kater2,3, Eric Eldering1,3

Departments of Experimental Immunology1 and Hematology2, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. 3Lymphoma and Myeloma Center Amsterdam, LYMMCARE, The Netherlands.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in western countries, remains an incurable disease. The main problem of CLL is the acquired drug resistance in response to treatment and despite the discovery of novel kinase inhibitors, relapses are common.

One of the hallmarks of CLL is apoptosis dysregulation by overexpression of the anti-apoptotic protein Bcl-2. This led to the development of the BH3-mimetic drug, the BCL-2 inhibitor, ABT-199/venetoclax. ABT-199 has proven highly successful in clinical trials in CLL and was recently approved for use in CLL with 17p chromosomal deletion by the FDA. However, a low percentage of complete remission was reached in CLL patients and this may be related to microenvironment-induced localized resistance.

The microenvironment within the lymph nodes (LN) and spleen plays an important role in the acquired drug resistance of CLL cells. In the microenvironment, CLL cells interact with follicular T cells and other surrounding cells that provide stimuli that further increase apoptosis resistance. In the microenvironment CD4+ T cells interact with CLL cells via CD40L-CD40 interaction. We demonstrated that CD40L stimulation of CLL cells in vitro increased their expression of the anti-apoptotic proteins Mcl-1, Bcl-XL and Bfl-1. In addition, the expression of Mcl-1, Bcl-XL and Bfl-1 can be found in LN samples of CLL patients. Bcl-XL plays a particularly important role in shifting the sensitivity versus resistance of CLL cells to ABT-199 in the context of CD40L stimulation. However, the mechanism(s) that control Bcl-XL expression in CD40L-stimulated CLL are unknown. Regulators of Bcl-XL expression might provide valuable therapeutic targets for treatment of CLL.

The NF-κB pathway is activated after CD40L stimulation in healthy B cells and CLL cells and this correlates with the expression of Bcl-XL and Bfl-1. Upon CD40 activation, the canonical NF-κB pathway is activated by the recruitment of TRAF1, 2 and 6 and activation the transforming growth factor-β-activated kinase 1 (TAK1) which leads to subsequent IkB kinase (IKK) phosphorylation. Activated IKK phosphorylates IkB which is part of a complex with NF-κB1 p50 and RelA/p65. Upon phosphorylation, IkB is degraded leading to the translocation of p50 and p65 to the nucleus where transcriptional activation of target genes occurs.

NIK is degraded through the TRAF2/TRAF3/cIAP1/2 complex. Upon CD40L stimulation, the non-canonical NF-κB pathway is activated by recruitment of TRAF2, TRAF3 and cIAP1/2 to the receptor and stabilization of NIK. Stabilized NIK induces phosphorylation of the IKKα homodimers and subsequent NF-κB2/p100 phosphorylation. Upon phosphorylation, p100 is transformed in p52 and together with RelB translocate to the nucleus where activation of target genes occurs.

The exact mechanism by which NF-κB regulates Bcl-XL in CLL cells remains unresolved. In this study, we show that RelA/p65 and NF-κB2/p52, respectively, both bind to the Bcl-XL promoter. Furthermore, endogenous signalling pathways can affect Bcl-XL expression and drug resistance. Accordingly, IL-4 augmented whereas IL-21 reduced CD40-induced resistance to fludarabine and ABT-199 in CLL cells. We demonstrated that IL-21/STAT3 signalling reduces Bcl-XL transcription whereas IL-4/STAT6 signalling augments Bcl-XL transcription.
MATERIAL & METHOD

Patient material
After informed consent, patient blood 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 and conducted in agreement with the Declaration of Helsinki. Blood mononuclear cells of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, The Netherlands) were cryopreserved and stored as previously described. Expression of CD5 and CD19 (both Beckton Dickinson (BD) Biosciences, San Jose, CA, USA) on leukemic cells was assessed by flow cytometry (FACScanto; BD Biosciences). CLL samples included in this study contained 85-99% CD5+/CD19+ cells.

Reagents
ABT-199 was purchased from Active Biochem (Bonn, Germany). Bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands) and fludarabine (F-Ara-A) from Sigma Chemical Co. (St. Louis, MO, USA). BAY-11-7082 was obtained from Calbiochem (Amsterdam, The Netherlands).

Cell culture and detection of apoptosis
Lymphocytes of CLL patients were co-cultured with NIH3T3 fibroblasts stably transfected with human CD40L or negative control as described before. After 24 hours, CLL cells were detached and incubated with or without drugs for an additional 24 hours. CLL cell viability was measured as before. Specific apoptosis is defined as [% cell death in treated cells] – [% cell death in medium control] / [% viable cells medium control] x 100.

Knock down of Bcl-XL and NIK in primary CLL cells
CLL cells were transfected using the Amaxa nucleofection technology (Amaxa, Koln, Germany) as previously described with 3 µg siRNA targeting Bcl-XL and non-targeting siRNA (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) or 3 µg siRNA targeting NIK and non-targeting siRNA (Dharmacon, Lafayette, CO, USA). After nucleofection, cells were cultured on 3T40L for 24 hours before drugs sensitivity assay was performed or protein lysates were obtained.

Western blot analysis
Western blot analysis was performed using standard techniques. Membranes were probed with the following antibodies: anti-Bcl-XL, p-p65, p100/p52, STAT3, p-STAT3, STAT6, pSTAT3 (Cell Signaling, Boston, MA, USA), actin (Santa Cruz Biotechnology, Dallas, TX, USA), and anti-A1/Bfl-1 was a kind gift of Prof. Dr. J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Odyssey Imager (Li-Cor Biosciences) was used as a detection method according to the manufacturer’s protocol.
Luciferase reporter gene experiments

The basic pGL3 luciferase reporter vector (Promega, Madison, WI, USA) was used to construct reporter plasmids with various lengths of the Bcl-XL (BCL2L1) and NFκB2 promoter. The primers used for the BCL2L1 promoter were: 5’-CAGACAAAGTGCTTAAACCACAAAG-3’ and 5’-TTTATAATAGGGATGGGCTCAACC-3’. The primers used for the NFκB2 promoter were: 5’-AGAGGTTGCAGTGAGCCAAAATCC-3’ and 5’-GTTGTGGTGTCAGCGTACC-3’.

Additional Bcl-XL promoter plasmid with STAT3 site-specific mutations was constructed (pGL3_Bcl-XL_STAT3m). The primers used for site-specific mutagenesis for the first STAT3 binding site were: 5’-ACCCCCGTCCTATCGACATGCCTT-3’ and 5’-AAGGCATGTCATAAGGACGCGGGGTAGAAA-3’ and for the second STAT3 binding site were: 5’-CTGAAGGGAGAAAGAGCATCAGAATGAAAAAATAATT-3’ and 5’-GGCATGATTAATATTTTTTTGATGCTCTTTTCTC-3’.

The plasmids pCMV4_NIK-HA, CMV4_p100 and pCMV_STAT6 were obtained from Addgene (Cambridge, MA, USA). HEK293T cells were transfected with 1 µg of luciferase reporter plasmid with or without 0.1 µg NIK, p100 or STAT6 plasmid and 0.3 µg pcDNA3.1/zeo-eGFP as internal control. GENIUS (Westburg, the Netherlands) was used for transfection and cells were stimulated with or without IL-4 or IL-6 (10ng/ml, Gibco, Invitrogen, Life Technologies). After 48 hours, luciferase activity was determined by using BioTek Synergy-HT (Winooski, VT, USA).

Reverse transcription-multiplex ligation-dependent probe amplification assay

RNA was isolated using the GenElute Mammalian Total RNA Miniprep kit (Sigma-aldrich). Reverse transcription-multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure (MRC, Amsterdam, the Netherlands) was performed as described previously by Eldering et al.26. Results were analyzed using the programs Genescan analysis and Genotypes (Applied Biosystems, Life technologies). Data was further analyzed with Microsoft Excel Spreadsheet software. Normalization was performed by setting the sum of all data per sample at 100% and individual peaks were calculated relative to the 100% value.

p65 and p52 DNA binding

Nuclear extracts of CLL cells were prepared using NucBuster protein extraction kit (Novagen, Merck Millipore, Darmstadt, Germany). p65 and p52 DNA binding was determined using TransAM NF-κB kit (Active Motif, Carlsbad, CA, USA).

Statistics

The student T-test was used to analyze paired observations. The one-way ANOVA was used to analyze differences between groups. * p <0.05; ** p<0.01; *** p<0.001.
RESULTS

Bcl-XL plays an important role in CLL drug resistance

CLL cells become resistant to a broad range of chemotherapeutic drugs after CD40L stimulation and recent studies demonstrated that Bcl-XL plays an important role in resistance to ABT-737 and ABT-199. To determine whether Bcl-XL plays an important role in resistance to other therapeutics in CLL cells, we silenced Bcl-XL after CD40L stimulation and performed drug sensitivity assays (Figure 1A, B). Sensitivity to the Bcl-2 inhibitor ABT-199, the chemotherapeutic fludarabine and the proteasome inhibitor bortezomib was restored in CD40L-stimulated cells after Bcl-XL silencing (Figure 1B). These results indicate that Bcl-XL is a major regulator of drug resistance in CLL cells.

Next, we further investigate which NF-κB regulators control the upregulation of Bcl-XL in response to CD40L stimulation. CD40L stimulation induced Bcl-XL mRNA expression as early after 1 h with a peak at 4 h post stimulation (Figure 1C). Both the canonical and non-canonical NF-κB pathway are upregulated early after CD40 activation and this correlates with the induction of Bcl-XL expression. Potential NF-κB binding sites in the human Bcl-XL promoter consisted of three NF-κB1/p65 and four NF-κB2/p52 motifs, identified within the ~2000-bp region preceding the transcriptional start site (Figure 1D). To assess the role of NF-κB in Bcl-XL expression, studies with truncated Bcl-XL promoter reporters were performed in HEK293T cells. Stepwise deletion of both types of NF-κB binding sites gradually reduced the Bcl-XL promoter activity (Figure 1D). These data indicate that NF-κB1/p65 and NF-κB2/p52 can both activate transcription at the Bcl-XL promoter.

Crosstalk between the canonical and non-canonical NF-κB pathway activates Bcl-XL

The activation of the canonical NF-κB pathway has previously been reported to correlate with Bfl-1 expression. Indeed, BAY-11-7082 [BAY] treatment to block the canonical pathway, showed dose-dependent repression of p65 phosphorylation and completely blocked Bfl-1 expression, while BAY only partially inhibited Bcl-XL expression (Figure 2A-B). Furthermore, BAY treatment reduced p100/p52 expression after CD40 activation. Crosstalk between the two NF-κB pathway has been previously mentioned and the p100/NF-κB2 promoter contains consensus binding sites for p65. To determine the effect of canonical pathway activation on p100/NF-κB2 promoter activation, we made a p100/NF-κB2 promoter construct and gradually deleted the p65 binding sites. Deletion of the first p65 binding site strongly reduced p100/NF-κB2 promoter activation (Figure 2C). Furthermore, CD40L stimulation induced p100/NF-κB2 transcription with a peak at 4 hours post stimulation (Figure 2D). These results show that there is crosstalk between the two NF-κB pathways, also in the context of CLL.

Non-canonical NF-κB activation correlates with Bcl-XL expression

Correlation between the conversion of p100 into p52 and the induction of Bcl-XL expression has been observed in CLL cells. To elucidate the role of p100/p52 in Bcl-XL expression, we performed Bcl-XL reporter luciferase activity studies. Transfection of p100 and NIK into HEK293T cells induced the transformation of p100 into p52 (Figure 3A). Bcl-XL promoter
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Figure 1. Bcl-XL level controls sensitivity to drugs. A-B) CLL cells were nucleofected with Bcl-XL or control siRNA and cultured on fibroblasts (3T3) transfected with the human CD40L (3T40L) for 24 hours. A) Protein lysates were probed for Bcl-XL and actin for loading control. B) After detachment, cells were incubated with 1μM ABT-199 or 25nM bortezomib for 24 hours or 25μM fludarabine for 48 hours. Averaged data of 6 CLL samples are shown for ABT-199 and averaged data of 3 CLL samples are shown for bortezomib and fludarabine. Bars represent the mean ± SEM, *p<0.05, ***p<0.001 (one-way ANOVA). C) Bcl-XL mRNA expression by CLL cells over time after stimulation with CD40L measured by real-time PCR, duplicates were normalized to HPRT. D) HEK293T cells were transfected with luciferase reporter gene constructs carrying Bcl-XL promoter fragments of different lengths. NF-κB binding sites were predicted using JASPAR database48. NF-κB binding sites were removed in 5'-deletion mutants of the Bcl-XL promoter and promoter activity was measured. Results are shown as mean ± SEM of the luciferase activation normalized to the vector pGL3-basic (n=2).

activity was further increased upon NIK plus p100 transfection (Figure 3B), indicating that the NIK/p100/p52 pathway activates Bcl-XL expression. As there are no specific inhibitors of the non-canonical pathway, we determined the effect of NIK knock down on Bcl-XL transcription and protein expression in CLL cells. NIK silencing reduced Bcl-XL transcription in CD40L-stimulated CLL cells (Figure 3C-D). In contrast, Bfl-1 transcription was unaffected.
**Figure 2.** Canonical NF-κB inhibition partially downregulates p100 and Bcl-XL. A-B) CLL cells were cultured on 3T3 or 3T40L with or without the indicated concentration of BAY for 24 hours. Protein lysates were probed for p100/p52, p-p65, Bcl-XL, Bfl-1 and actin as loading control. A) Blots from two representative CLL samples are shown of four analyzed. B) Densitometric analysis of p-p65, p100, p52, Bcl-XL and Bfl-1 are shown. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA). C) HEK293T were transfected with luciferase reporter gene constructs carrying different lengths of the NF-κB2/p100 promoter. NF-κB1/p65 binding sites were predicted using JASPAR database. NF-κB binding sites were removed in 5′-deletion mutants of the NF-κB2/p100 promoter and promoter activity was measured. Results are shown as mean ± SEM of the luciferase activation normalized to the vector pGL3-basic. D) NF-κB2/p100 mRNA expression by CLL cells over time after stimulation with CD40L measured by real-time PCR, triplets were normalized to unstimulated CLL cells.
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Figure 3. Non-canonical NF-κB downregulation inhibits Bcl-XL expression. A) HEK293T cells were transfected with an empty vector (mock) or HA-tagged NIK with or without p100. Protein lysates were probed for p100/p52, p-p65, HA and actin as loading control. B) HEK293T cells were transfected with Bcl-XL promoter luciferase construct with HA-tagged NIK and with or without p100. Promoter activity was measured and results are shown as mean ± SEM of the luciferase activation normalized to the vector pGL3-basic (n=2). C-I) CLL cells were nucleofected with NIK or control siRNA and cultured on fibroblasts (3T3) transfected with the human CD40L (3T40L) for 24 hours. NIK (C), Bcl-XL (D) and Bfl-1 (E) mRNA expression by CLL cells after stimulation with CD40L or CD40L and NIK silencing was measured by real-time PCR, normalized to HPRT. F-H) Protein lysates were probed for p100/p52, p-p65, Bcl-XL and actin as loading control. F) Blots from two representative CLL samples are shown. G-H) Densitometric analysis of p-p65, p100, p52 (G) and Bcl-XL (H) are shown. *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA). I) After detachment, cells were incubated with 0.001-10μM ABT-199 for 24 hours. Averaged data of 3 CLL samples are shown and bars represent the mean ± SEM.
upon NIK knock down (Figure 3E). Moreover, NIK silencing partially blocked p100 to p52 transformation, while phosphorylation of p65 was not affected (Figure 3F-G). In addition, Bcl-XL protein was reduced by NIK silencing (Figure 3F, H). Since NIK silencing reduces Bcl-XL expression and levels of Bcl-XL dictate the threshold for sensitivity of CLL cells to ABT-199\(^{14}\), we investigated the effect of NIK silencing on ABT-199 sensitivity. NIK silencing partially enhanced ABT-199 sensitivity (Figure 3I). These results support that CD40L-induced non-canonical NF-κB pathway activation promotes Bcl-XL expression.

**Bcl-XL expression is negatively regulated by IL-21/STAT3 and positively regulated by IL-4/STAT6**

In a previous study, we focussed on stimuli from the microenvironment that affect drug sensitivity of CLL cells. IL-4 and IL-21 produced by follicular T helper cells in the LN had opposing effects on drug sensitivity. IL-4 augmented and IL-21 reversed CD40L-induced resistance to fludarabine and ABT-199\(^{14}\). Correspondingly, IL-4 and IL-21 were shown to exert opposing effects on CD40L-mediated Bcl-XL expression\(^{14}\). We sought to determine the mechanisms by which IL-4R or IL-21R stimulation affect CD40L-driven Bcl-XL expression. IL-4R or IL-21R stimulation alone did not induce Bcl-XL expression (Figure 4A). CD40L-induced Bcl-XL expression was further increased by IL-4 and downregulated by IL-21. IL-4R and IL-21R stimulation leads to differential STAT phosphorylation; IL-4 induced STAT6 phosphorylation and IL-21 induced p-STAT3 (Figure 4A). We next addressed whether inhibition of STAT phosphorylation affects Bcl-XL expression. Blocking the JAK/STAT pathways with a JAK inhibitor [INCB] abrogated the effects of IL-4 and IL-21 on CD40L-mediated Bcl-XL expression (Figure 4B). Since Bcl-XL expression is controlled by CD40L-induced NF-κB activation in CLL cells, we investigated the effects of the STATs on NF-κB signaling. STAT3 was suggested to bind to NF-κB regulators in the cytoplasm\(^{31,32}\). We considered that STAT3 can bind to p100 and thereby negatively affect p100 to p52 conversion. CD40L-induced p100 to p52 transformation or p65 phosphorylation was not affected by IL-21R stimulation, while Bcl-XL expression was reduced (Figure 4C). These data suggest that STAT3 does not interfere with the NF-κB factors during processing in the cytoplasm.

**IL-21/STAT3 reduces Bcl-XL transcription whereas IL-4/STAT6 augments Bcl-XL transcription**

To elucidate the effects of IL-4 and IL-21 on Bcl-XL transcription, we performed MLPA assays. Among a wide panel of anti- or pro- apoptotic regulators (data not shown), IL-4 and IL-21 primarily had opposing effects on CD40L-mediated Bcl-XL transcription (Figure 4A). Bfl-1 transcription was largely unaffected by IL-4R and IL-21R stimulation (Figure 4A).

To investigate if the opposing effects of IL-4 and IL-21 on Bcl-XL transcription is a consequence of effects on NF-κB nuclear translocation, we assessed the DNA-binding of the NF-κB regulators. CD40L stimulation induced nuclear translocation of p65 and p52 and subsequently DNA-binding (Figure 4B). We determined that nuclear translocation and DNA binding activity of p52 and p65 was unaffected by IL-4 and IL-21 (Figure 4B).
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Finally, we investigated whether STAT6 and STAT3 directly affect Bcl-XL transcription. Two STAT6 and STAT3 motifs were identified within the Bcl-XL promoter region (Figure 5C). To assess the role of STAT6 and STAT3 in Bcl-XL expression, signaling studies with the Bcl-XL promoter reporter were performed. HEK293T were transfected with STAT6 and treated with IL-4 to induce pSTAT6 (Figure 5D). STAT6 phosphorylation by IL-4 signaling significantly increased luciferase activity driven by the Bcl-XL reporter (Figure 5E). STAT3 is endogenously present in HEK293T and its phosphorylation by IL-6 downregulated Bcl-XL promoter activity (Figure 5F). Mutations of the STAT3 binding sites in the Bcl-XL promoter abrogated the IL-6 downregulation of the luciferase activity of the Bcl-XL construct (Figure 5F). We conclude from these data that STAT6 positively and STAT3 negatively regulates Bcl-XL expression by binding to its promoter.
Figure 5. STAT6 positively and STAT3 negatively regulate Bcl-XL transcription. A) Bcl-XL and Bfl-1 mRNA expression by CLL cells 24 hours after stimulation with CD40L with or without IL-21 or IL-4 were measured by RT-MLPA, normalized to the sum of all data. Bars represent the mean ± SEM (n=3), **p<0.01, ***p<0.001 (one-way ANOVA). B) NF-κB subunit activation in nuclear extracts of CLL cells 24 hours after stimulation with CD40L and/or IL-21 or IL-4, measured by DNA binding ELISA. Bars represent the mean ± SEM (n=6), *p<0.05, ***p<0.001 (one-way ANOVA). C) STAT6 and STAT3 binding sites on the Bcl-XL promoter were predicted using JASPAR database48. D) HEK293T cells were stimulated with IL-6 or transfected with STAT6 plasmid and stimulated with IL-4. Protein lysates were probed for pSTAT3, STAT6, pSTAT6 and actin for loading control. E) HEK293T cells were transfected with Bcl-XL promoter luciferase construct with STAT6 and stimulated with IL-4. Promoter activity was measured and results are shown as mean ± SEM of the luciferase activation normalized to the vector pGL3-basic (mock). *p<0.05 (Student T-test). F) HEK293T cells were transfected with Bcl-XL promoter luciferase construct or Bcl-XL promoter luciferase construct containing mutations in the two STAT3 binding sites (Bcl-XL STAT3m) and stimulated with IL-6. Promoter activity was measured and results are shown as mean ± SEM of the luciferase activation normalized to the vector pGL3-basic (mock). ***p<0.001 (Student T-test).

DISCUSSION

Acquired resistance in response to treatment is a major problem for CLL and Bcl-XL is a key regulator of drug resistance14,33,34. Novel drugs that directly target Bcl-XL were in clinical trials in CLL35,36. ABT-737 was the first drug that binds and antagonizes Bcl-2 and Bcl-XL35. However, platelets are dependent on Bcl-XL and ABT-737 causes dose-
dependent thrombocytopenia\textsuperscript{37,38}. The recently introduced BH3 mimetic ABT-199 only targets Bcl-2 in CLL. However, Bcl-XL upregulation renders these cells insensitive to ABT-199\textsuperscript{14,39}. Therefore, understanding the processes that control upregulation of Bcl-XL in response to microenvironmental stimuli may identify novel targets for therapy. Bcl-XL expression correlates with the activation of the non-canonical NF-\kappa B pathway\textsuperscript{15}. However, the canonical p65 and non-canonical p52 can bind to the Bcl-XL promoter and they both most likely activate Bcl-XL transcription. Conversely, Bfl-1 expression is thought to correlate with the activation of the canonical NF-\kappa B in CLL cells\textsuperscript{15}. However, in thymocytes the non-canonical p52/RelB heterodimer can also bind to the Bfl-1 promoter. Direct binding of p52 and p65 to the Bcl-XL promoter after CD40L stimulation in CLL cells will have to be verified by ChIP assay.

Inhibition of the canonical NF-\kappa B pathway by BAY partially downregulates Bcl-XL. However, p100 and p52 are also inhibited by BAY and this can also negatively affect Bcl-XL expression. Since BAY downregulates Bcl-XL, we need to further investigate if BAY treatment can revert CD40L-induced ABT-199 resistance. Mentioned in literature\textsuperscript{21}, but not well studied is the mechanism that the canonical pathway can induce the expression of NF-\kappa B2/p100. We found p65 binding sites in the NF-\kappa B2/p100 promoter and promoter activity is lost after truncation of these binding sites. NF-\kappa B2/p100 promoter activity was reduced after deletion of the first p65 binding site and we have to further validated the requirement of this p65 binding site using site-specific mutagenesis. Further studies have to determine direct binding of p65 to the NF-\kappa B2/p100 promoter using ChIP assay. Upon CD40 activation, the canonical NF-\kappa B pathway is first activated and later the non-canonical. Our data are compatible with the following: CD40L-induced p65 activates target gene NF-\kappa B2/p100, this induces p100 protein expression which is phosphorylated by CD40-stabilized NIK. Following this, p100 is transformed in p52, which subsequently translocates to the nucleus to activate Bcl-XL expression. Crosstalk between the two NF-\kappa B pathways has already been mentioned before\textsuperscript{23,28}, NIK activates p65 in in mouse embryonic fibroblasts\textsuperscript{28}. Indeed, we also demonstrate that NIK transfection leads to phosphorylation of p65, showing there is positive crosstalk between the two pathways.

Bcl-XL is upregulated after CD40, BCR or LPS triggering (data not shown), while CLL cells have high basal levels of the nuclear canonical NF-\kappa B transcription factors\textsuperscript{18}. Furthermore, CLL patients with a NFKBI/E deletion showed increased p65 phosphorylation and translocation, while the Bcl-XL gene was not significantly differently expressed\textsuperscript{40}. This suggest that non-canonical pathway activation is needed to upregulate Bcl-XL. Partially silencing NIK downregulates Bcl-XL expression and partially reverts ABT-199 sensitivity in CLL cells. Future studies will determine whether completely blocking the non-canonical NF-\kappa B by siRNA or inhibitors will fully downregulate Bcl-XL expression and reverts drug resistance in CLL cells.

We demonstrate that IL-21/STAT3 signaling downregulates Bcl-XL transcription. Several studies demonstrated a positive correlation with STAT3 expression and NF-\kappa B signaling for survival of cells\textsuperscript{41-43}. In contrast, we demonstrate that STAT3 binds to the Bcl-XL promoter and negatively affects Bcl-XL transcription. This suggests that STAT3 interferes with the p52 or p65 binding to the Bcl-XL promoter, since STAT3 has no effect on NF-\kappa B
activation and translocation. Direct binding of STAT3 to the Bcl-XL promoter in CLL cells will have to be verified using ChIP assays.

IL-21 signaling in mantle cell lymphoma has previously been reported to induce apoptosis by downregulation of Bcl-XL via pSTAT1\(^{44}\). IL-21 signaling in CLL cells results in low levels of STAT1 phosphorylation and did not correlate with Bcl-XL downregulation (data not shown). IL-21 signaling in CLL has been reported to induce apoptosis by upregulation of BIM\(^{45}\), however we did not observe an upregulation of BIM transcription in CD40L-stimulated CLL cells by using MLPA assay (data not shown). Furthermore, we previously demonstrated that the levels of BIM did not dictate the threshold for sensitivity of CLL cells to several drugs, including fludarabine and ABT-199\(^{14,25}\). Our data suggest that recombinant IL-21 may be useful as a combination therapy with ABT-199 in CLL. Recombinant IL-21 has been used in an clinical trial in CLL in addition to an anti-CD20 antibody and this combination showed increased cytotoxicity of CLL cells\(^{46}\). However, IL-21 plus CD40L also promotes proliferation of CLL cells\(^{47}\).

Taken together, we have identified Bcl-XL as the key regulator of CLL cell sensitivity to drugs and targeting Bcl-XL is warranted to avoid the development of drug resistance. IL-21 signaling downregulates Bcl-XL transcription, which involves STAT3 binding sites in the Bcl-XL promoter. The IL-21/JAK/STAT3 pathway may thus provide potential targets for downregulation of Bcl-XL, which could be applied in combination therapy.
REFERENCES


