Resistance to ABT-199 induced by microenvironmental signals in chronic lymphocytic leukemia can be counteracted by CD20 antibodies or kinase inhibitors

A major clinical problem in chronic lymphocytic leukemia (CLL) is development of chemoresistance, which can be caused by genetic lesions but is also strongly influenced by the leukemic microenvironment. Two compartments can be distinguished in CLL; the blood, where quiescent CLL cells accumulate, and the lymphoid microenvironment within the lymph nodes (LN), spleen and bone marrow, where surrounding cells provide external signals that drive CLL proliferation and survival.[1] CLL survival is correlated with NF-κB-mediated upregulation of various protective Bcl-2 family members, notably Bcl-XL, Bfl-1 and Mcl-1 in LN samples compared to peripheral blood (PB).[2,3]

Recently, novel therapeutics that target microenvironmental signals or Bcl-2 family members have entered clinical trials and practice.[1,4] One prominent strategy for CLL and other cancers is to target the apoptosis machinery directly by so-called BH3-mimetics. The Bcl-2-specific compound ABT-199 or Venetoclax is highly cytotoxic for CLL cells and shows improved clinical efficacy and induces no thrombocytopenia as opposed to its predecessor Navitoclax.[4] Peripheral blood lymphocyte counts, lymph node size and bone marrow involvement all diminished early after treatment.[5] A second strategy employs kinase inhibitors that target critical signal transduction pathways controling cell growth, adhesion and survival. Inhibitors of

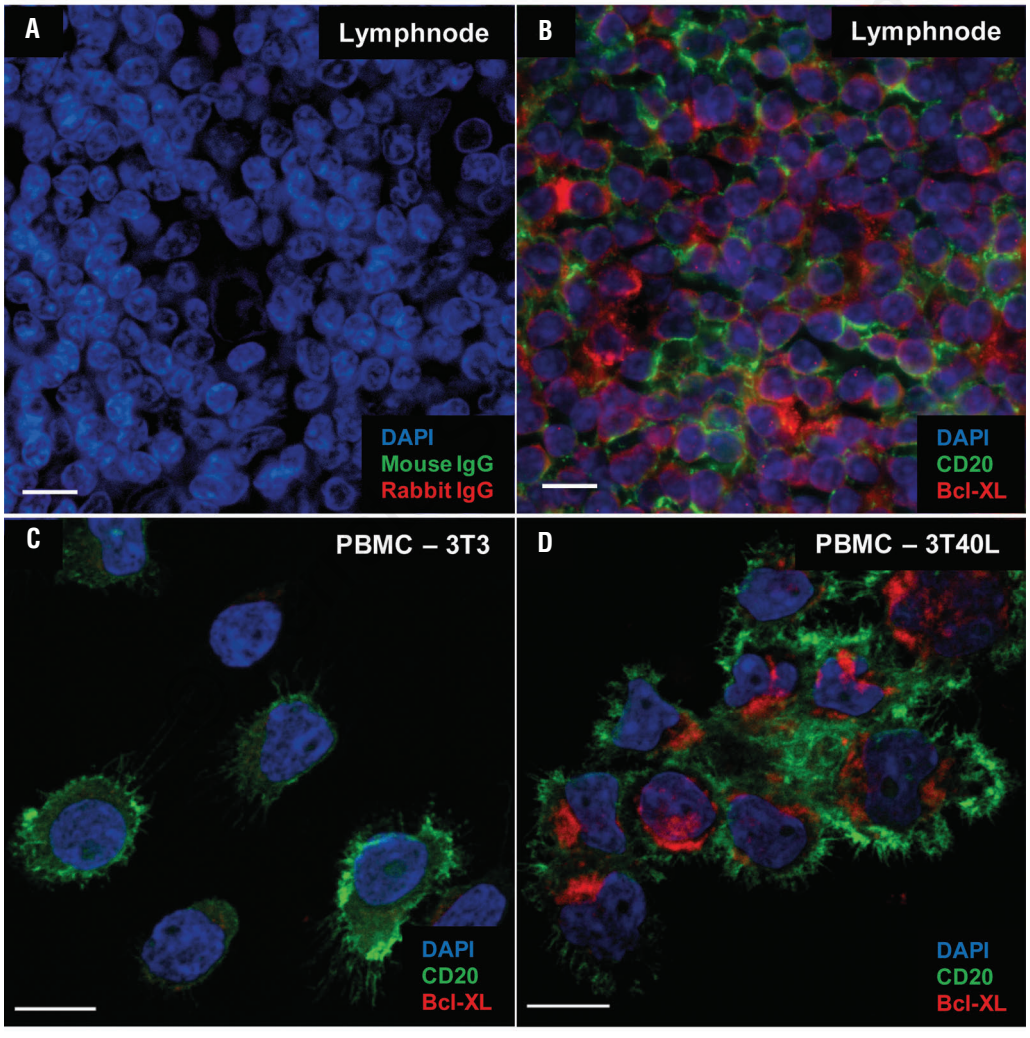

Figure 1. Bcl-XL is expressed in CLL cells in LN tissue. One representative CLL LN sample out of 4 stained is shown and stained for (A) Isotype control staining and nuclear staining with DAPI in blue, scale-bar represent 10 μm. (B) CD20 in green and Bcl-XL in red and nuclear staining with DAPI in blue (C and D). CLL cells (*Online Supplementary Table S1*; patients #23-25) were co-cultured with NIH3T3 fibroblasts transfected with empty vector (3T3) (C) or co-cultured with NIH3T3 fibroblasts transfected with hCD40L (3T40L) (D) for three days. After detachment, cytospins were made and stained for CD20, Bcl-XL and DAPI. Imaging was performed using a Leica TCS SP8-X confocal microscope. One CLL sample is shown of a total of three analyzed, scale-bar represent 10 μm. (A and B) Paraffin-embedded LN samples from CLL patients were incubated with primary antibody anti-CD20 (eBioscience, San Diego, CA, USA) and anti-Bcl-XL (Cell Signaling, Boston, MA, USA) and subsequently incubated with Alexa Fluor 488 labeled goat anti-mouse and Alexa Fluor 594 labeled goat anti-rabbit antibodies (Invitrogen, Camarillo, CA, USA) and counterstained with DAPI. Immunofluorescent imaging (40x) was performed using a Leica DMRA fluorescence microscope.

the B-cell receptor (BCR)-associated kinases Bruton's tyrosine kinase (Btk) and phosphatidylinositol-3-kinase (PI3K) show strong clinical activity and were recently approved for both relapsed/refractory CLL and for CLL harboring 17p deletions.[6,7] A third novel strategy is treatment with next generation anti-CD20 monoclonal antibodies (mAb), for example, with enhanced direct cytotoxicity and antibody-dependent cell mediated cytotoxicity (GA101, obinutuzumab).[8]

As outlined above, pro-survival signals can up-regulate Bcl-2 members, which are not targeted by ABT-199. We therefore analyzed to what extent microenvironmental sig-

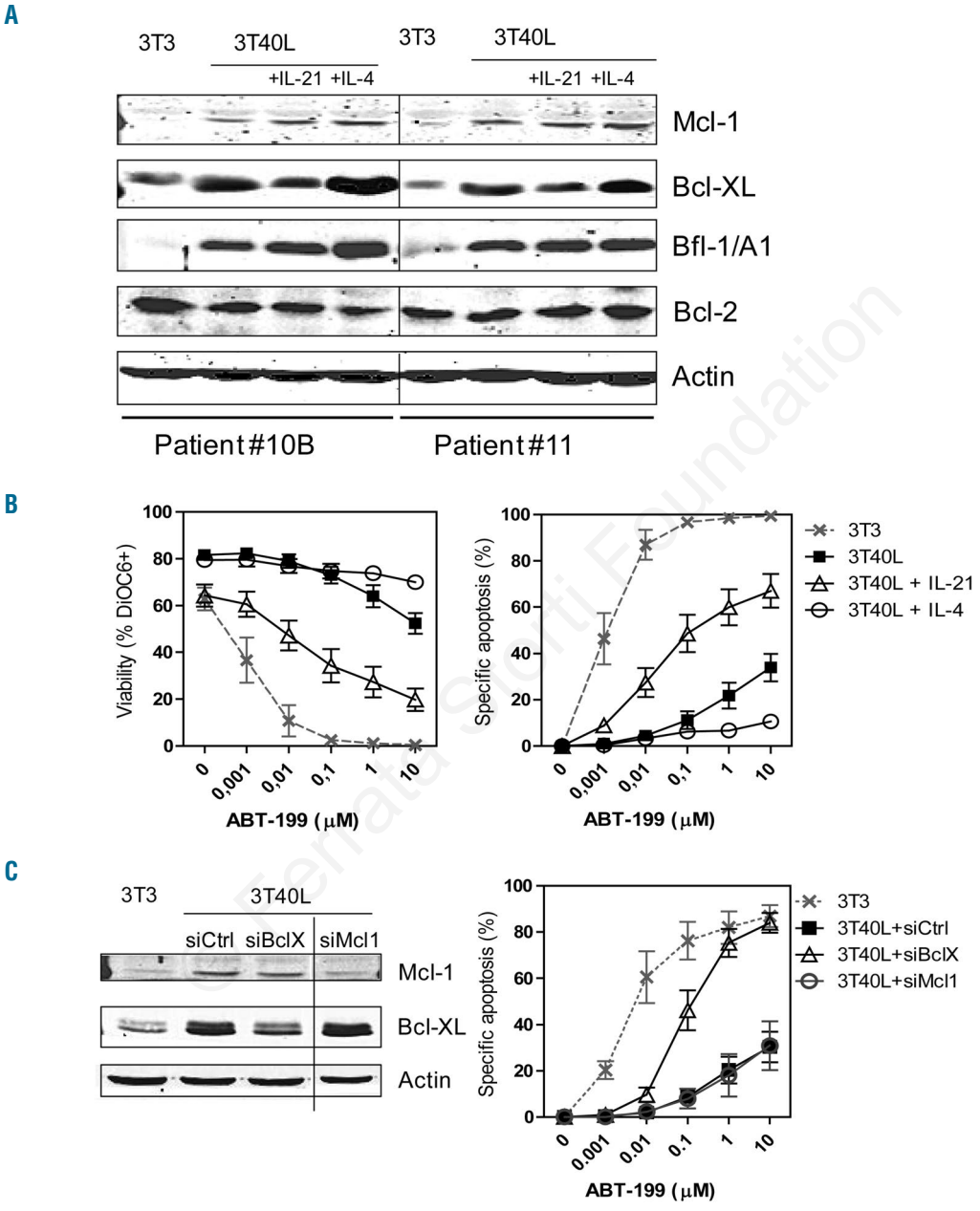

Figure 2. CLL cells become resistant to ABT-199 upon CD40 stimulation. CLL cells were cultured on control 3T3 or 3T40L cells in the presence or absence of 25 ng/ml IL-21 or IL-4 for 72 h. (A) Western blot analysis was performed using standard techniques with anti-Mcl-1, anti-Bcl-2, anti-Bcl-XL, anti-Bfl-1 and anti-β-actin antibodies. Blots from two representative CLL samples are shown of a total of seven analyzed (*Online Supplementary Table S1*; patient #1-3A, 10B, 11). (B) After detachment, cells were incubated with 0.001-10 μM ABT-199 for h 24. Viability was assessed by DiOC6/PI staining. Left panel shows % viable cells, right panel shows specific apoptosis. Results are shown as mean ± SEM, n=8 (3 IgV_H mutated, 3 unmutated, 2 IgV_H status unknown (*Online Supplementary Table S1*; patient #4-11) (C) CLL cells (*Online Supplementary Table S1*; patient #5, 13A, 19) were nucleofected (Amaxa, Koln, Germany) with 3 μg siRNA (Bcl-XL, Mcl-1 and Silencer Select Negative Control) and cultured on 3T40L cells for three days. Left panel: lysates were probed for Bcl-XL, Mcl-1 or actin as a loading control. As a control, lysates of CLL cells on 3T3 are shown. (Right) after nucleofection and culture, cells were incubated with ABT-199 for 24 h, n=3, mean ± SEM. Reagents: anti-Mcl-1 (Cell Signaling), anti-Bcl-2 (Enzo Life Sciences, Raamsdonksveer, The Netherlands), anti-Bcl-XL (BD Biosciences), anti-β-actin (Santa Cruz Biotechnology), polyclonal antibody against Bfl-1 was a kind gift of Jannie Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands), IL-21 and IL-4 (Gibco, Invitrogen, Bleiswijk, The Netherlands), ABT-199 from Abbvie (Abbott Park, IL, USA), siRNA from obtained from Ambion (ID#1920, ID#8583 and Cat#4390843). Specific apoptosis is defined as [% cell death in treated cells] – [% cell death in medium control] / [% viable cells medium control] x 100.

nals can affect sensitivity to ABT-199 in CLL samples, and whether this can be counteracted by combination therapy with other novel therapeutics. This study was approved by the AMC Ethical Review Board and conducted in agreement with the Declaration of Helsinki. Patients' characteristics are available in the *Online Supplementary Table S1*.

We first established by immunofluorescence that Bcl-XL is expressed in LN CLL cells. For comparison, we performed immunostaining on CLL cells stimulated with CD40 ligand *in vitro*, mimicking activated T-cell-mediated signaling.[9] This also showed clear Bcl-XL expression, whereas PB CLL cells were essentially negative (Figure 1 and *Online Supplementary Figure S1*). We next applied *in vitro* CD40 stimulation with IL-21 and IL-4, cytokines produced by activated T cells and follicular helper T cells that can induce proliferation and survival, respectively.[9,10] As reported previously,[10,11] CD40 stimulation of CLL cells increased expression of Bcl-2 members Mcl-1, Bcl-XL and Bfl-1 (Figure 2A). T-cell cytokines had contrasting effects; IL-21 down-regulated Bcl-XL while IL-4 further increased Bcl-XL and Bfl-1 expression. Mcl-1 was not significantly altered by addition of IL-21 or IL-4. Averaged quantification on multiple samples is available in the *Online Supplementary Figure S2*. Unstimulated CLL cells are highly sensitive to ABT-199 (LC50<1nM) (*Online Supplementary Table S2*). Remarkably, CD40 and CD40^{+}IL-4 stimulation resulted in full resistance to 10 μM ABT-199. The effect of IL-21 was less pronounced, probably as a result of down-regulated Bcl-XL in comparison with CD40 stimulation alone[9] (Figure 2A and B). There were differences among patients' samples in the basal sensitivity and extent of resistance induction towards BH3-mimetics, which were reproducible in independent experiments (*Online Supplementary Figure S3*). No differences in response were observed among mutated

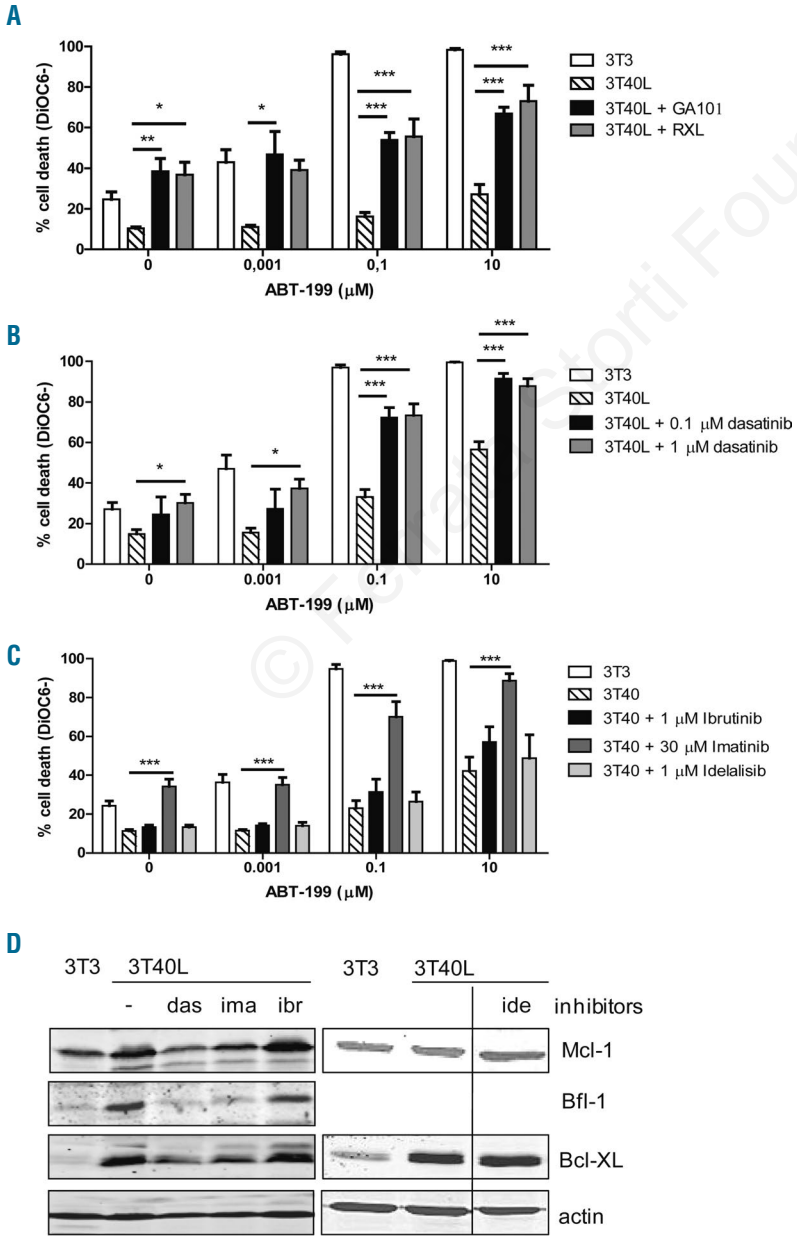

Figure 3. CD40L-induced resistance to ABT-199 can be overcome by combination therapy. (A) CLL cells were co-cultured with 3T3 or 3T40L for three days with 10 μg/mL crosslinked rituximab (RXL) or GA101 as described,[12,13] or in combination with indicated concentrations of ABT-199 and analyzed for apoptosis after 24 h. Averaged results are presented as percentage cell death (mean ± SEM) for 6 CLL samples (*Online Supplementary Table S1*; patient #3B, 5, 7, 10B, 19, 22) for GA101 and 3 CLL samples (*Online Supplementary Table S1*; patient #3B, 7, 10B) for rituximab. (B) CLL cells were co-cultured in the presence of 0.1 or 1 μM dasatinib and after deatchment were incubated with ABT-199. (n=8 *Online Supplementary Table S1*; patient #5, 8, 10B-12, 19-21), (C) CLL cells were co-cultured in the presence of 1 μM ibrutinib, 1 μM idelalisib or 30 μM imatinib. After detachment, cells were incubated with ABT-199. (n=8 *Online Supplementary Table S1*; patient #3B-5, 8, 12, 13B-15). (D) CD40-stimulated CLL cells were co- cultured for 48 h in the presence of dasatinib, imatinib, or ibrutinib as indicated. Lysates were probed for Mcl-1, Bcl-XL and Bfl-1 and actin for loading control. The results are representative of 3 blots. Reagents: GA101 (Roche, Woerden, The Netherlands) , dasatinib (Novartis, Basel, Switzerland), ibrutinib (Pharmacyclics, Sunnyvale, CA, USA), idelalisib (Selleckchem, Houston, TX, USA), imatinib (Novartis, Basel, Switzerland). Statistics by Students *t*-test: **P*<0.05;***P*<0.01; ****P*<0.001.

(n=4) versus unmutated (n=4) IgV$_H$ CLL samples. Nucleofection with Bcl-XL siRNA resulted in restored susceptibility for ABT-199. Unexpectedly, knockdown of Mcl-1 hardly altered the sensitivity to ABT-199 after CD40 stimulation (Figure 2C), although it slightly decreased spontaneous apoptosis (*data not shown*). Unfortunately, no clear knockdown of Bfl-1 could be obtained for unknown reasons. Thus, in the context of CD40 stimulation, especially Bcl-XL plays an important role in shifting the susceptibility of CLL cells for ABT-199. The role of individual Bcl-2 members was separately addressed in immortalized primary B cells as described previously,[11] which established that both Bfl-1 and Mcl-1 can also singly confer resistance to ABT-199 when expressed at sufficient levels (*Online Supplementary Figure S4*).

Next, we investigated combination strategies to revert resistance for ABT-199 induced by CD40 stimulation. We have previously demonstrated that, whereas unstimulated CLL cells are not sensitive to CD20-mediated killing, anti-CD20 monoclonal antibodies induce non-apoptotic cell death in CD40-stimulated CLL cells.[12,13] Combination of GA101 or rituximab with ABT-199 demonstrated that CD40-induced resistance to ABT-199 could be counteracted by anti-CD20 antibodies (Figure 3A). As shown elsewhere, treatment with anti-CD20 Abs does not affect expression of Bcl-2 family members in CD40-stimulated CLL cells.[12]

Secondly, in agreement with previous studies using ABT-737,[11] resistance to ABT-199 in CLL could also be significantly reverted by the Src kinase inhibitor dasatinib at physiologically relevant doses (Figure 3B). Prime targets of dasatinib are Abl and Btk.[14] The Btk inhibitor ibrutinib but also the PI3Kδ inhibitor idelalisib have not been investigated in the context of CD40-mediated drug resistance. We found that ibrutinib and idelalisib had minimal impact on CD40-induced resistance for ABT-199 (Figure 3C). It should be noted in this context that additional BCR stimulation had no effect on CD40-induced resistance for ABT-199 or on induction NF-κB responsive Bcl-2 family members (*Online Supplementary Figure S5*). In contrast, high dose of the c-Abl inhibitor imatinib could reverse the CD40-induced resistance for ABT-199. In agreement with their effects on ABT-199 sensitivity, c-Abl inhibitors dasatinib and imatinib reversed induction of pro-survival Bcl-XL, Mcl-1, and Bfl-1, whereas ibrutinib or idelalisib had no effect (Figure 3D). Of note, the kinase inhibitors by themselves hardly induced apoptosis upon CD40 stimulation of CLL cells (*Online Supplementary Figure S6*). *Online Supplementary Table S2* summarizes the effects of the various stimulations and combination therapies on the LC50 for ABT-199.

Development or selection of resistant clones is a major problem in cancer treatment. Both for conventional chemotherapeutics and novel targeted drugs, there is considerable selection pressure for the cancer cells to escape elimination. Our *in vitro* co-culture model of CLL applies prolonged CD40 stimulation, and represents an extreme system of chemoprotection, where primary CLL cells are converted within three days from highly sensitive to fully resistant to ABT-199. More moderate forms of pro-survival signaling will occur *in vivo* and are also observed by co-culture of CLL cells with autologous activated T cells,[9] and this is also supported by the modulating effect of IL-21 *in vitro*. Still, in CLL lymph node upregulation of Bcl-XL, Bfl-1 and Mcl-1 can be observed,[2,3] and this might be conducive to development of CLL clones or niches with decreased sensitivity for ABT-199. Support for this possibility has been obtained *in vitro* by selection of clones resistant to ABT-199, caused by mutation in Bcl-2 or Bax.[15] To obtain long-term and complete CLL remission *in vivo*, combination strategies of ABT-199 with kinase inhibitors or anti-CD20 antibodies might be attractive. The clinical activities of dasatinib, ibrutinib or idelalisib can be attributed to inhibition of BCR-mediated survival and adhesion, followed by egress of CLL cells from LN into the bloodstream. There, they express almost exclusively Bcl-2 and are, therefore, highly susceptible to ABT-199. The combination of ABT-199 with anti-CD20 monoclonal antibodies is currently being explored in clinical trials. With regard to direct cell death induction, our findings support the effectiveness of such combinations in CLL in relation to drug-resistant niches at lymph-node sites.

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