The microenvironment and treatment resistance in chronic lymphocytic leukemia
Tromp, J.M.

Citation for published version (APA):

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Primary CLL cells are resistant to cellular inhibitor apoptosis protein (cIAP) antagonists

Jacqueline M. Tromp1,2, Judith A. Elias1,2, Anna Malara2, Jan-Jaap Schot1,2, Anja Krippner3, John Silke4, Marinus H.J. van Oers1, Eric Eldering2

Departments of Hematology1 and Experimental Immunology2, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
Institute of Cellular Medicine3, Newcastle University, Newcastle, United Kingdom
The Walter and Eliza Hall Institute4, Melbourne, Australia
Abstract

In the lymph node (LN) microenvironment, CLL cells display increased NF-κB activity as compared to peripheral blood (PB) CLL cells, resulting in chemoresistance. New treatment strategies are mandatory to overcome this chemoresistance. Cellular Inhibitor of Apoptosis Protein (cIAP) antagonists induce apoptosis in various cancer cells in a TNFα-dependent manner via degradation of cIAP1 and cIAP2. Degradation of cIAPs results in TNFR1 mediated apoptosis via formation of a complex of receptor interacting protein kinase (RIP1) with caspase 8. CD40-stimulation of CLL cells in vitro is used as a model to mimic the LN microenvironment and results in NF-κB activation and TNFα production. In this study, we show that cIAP2 is highly expressed in CLL cells and that treatment with cIAP antagonists results in its degradation. However, cIAP2 levels in CLL cells recover during treatment with cIAP antagonists. CD40-stimulation of CLL cells leads to enhanced levels of TNFR1 and TNFR2. Although all components of the so-called ripoptosome complex are present, CD40-stimulated CLL cells are resistant to cIAP antagonists. The exact cause of the inability of TNF(R) signalling to induce CLL cell death remains to be clarified.
Introduction

Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of CD5+ monoclonal B lymphocytes and is a heterogeneous disease with a highly variable clinical course. In peripheral blood (PB) most CLL cells are arrested in G0/G1 of the cell cycle due to a defect in apoptosis. A defect in apoptosis regulation is mainly caused by an impaired balance of pro- and anti-apoptotic proteins (1, 2). In addition, in CLL cells expression levels of inhibitor of apoptosis proteins (IAPs) are found to be higher as compared to non-leukemic lymphocytes (3, 4). Furthermore, high levels of cIAPs are associated with a progressive course of disease in CLL (3).

IAPs are endogenous proteins that can block apoptosis pathways by interfering with the activation of effector caspases (5, 6). The IAP family consist of eight proteins and include X-linked IAP (XIAP) and cellular IAP1 and IAP2 (cIAP1, cIAP2) (7-10). All IAP family members contain at least one baculovirus IAP repeat (BIR) domain. The BIR domain is a 70-80 amino-acid long motif that binds to caspases (11, 12). XIAP is a direct inhibitor of caspases, whereas cIAP1 and cIAP2 also bind to caspases, but are not considered to directly inhibit them (13, 14). cIAP1 and cIAP2 interfere with caspase activity by ubiquitination and thereby proteasomal degradation of caspases (15). Besides the BIR motif, IAP proteins contain other functional domains, such as caspase activating and recruitment domain (CARD) and the really interesting new gene (RING) domain (6). The CARD domain is only present in cIAP1 and cIAP2 and to date its function remains largely unknown. The RING domain is present in XIAP, cIAP1 and cIAP2 and has been shown to exert E3 ubiquitin ligase activity (16-18). E3 ligases are important in ubiquitin linkage. Post-translational modification of proteins by ubiquitination is an important regulatory mechanism which can result in proteasomal degradation (K48 ubiquitin linkage) or signal transduction (K63 ubiquitin linkage) (19). Upon binding of TNFα to the TNFR1, TNF-receptor-associated factor 2 (TRAF2), cIAP1 and cIAP2 are recruited to the intracellular domain of the TNF-receptor. Binding of cIAP1 and 2 to TRAF2 results in receptor-interacting protein 1 (RIP1) modification with K63-linked polyubiquitin chains, resulting in RIP1 proteasomal degradation, leading to NF-κB activation (20, 21). cIAP1 and cIAP2 also act as negative regulators of NF-κB signaling by inducing K48-linked polyubiquitination and proteasomal degradation of NF-κB inducing kinase (NIK), an upstream regulator of the alternative NF-κB signaling pathway (22, 23).

In murine B cells, it has been shown that upon CD40 activation TNF-receptor-associated factor 3 (TRAF3) is degraded in a cIAP1-cIAP2-TRAF2 dependent way (24). Degradation of TRAF3 leads to NIK stabilization via preventing NIK to bind to the cIAP1-cIAP2-TRAF2 ubiquitin ligase complex and this results in activation of the alternative NF-κB pathway.
How the adaptor and signaling proteins TRAF2, cIAP1 and cIAP2 are regulated in unstimulated and CD40-stimulated CLL cells is currently unknown.

Smac/Diablo, an endogenous inhibitor of IAPs released from mitochondria, is an important molecule that binds to the BIR domain and thereby antagonizes IAP mediated caspase inhibition (25, 26). Based on the N-terminus of Smac, which binds and inactivates XIAP, small molecule inhibitors of XIAP function have been developed (27, 28). It has been shown that XIAP inhibitors induce apoptosis in multiple myeloma cells (29). In CLL, XIAP inhibitors sensitize cells to extrinsic cell death inducers like TRAIL and CD95 (30, 31). Compound A is a small molecule which is designed to mimic Smac/Diablo (22). However, in various cancer cell lines treatment with compound A results in cIAP1 and cIAP2 degradation, but has no effects on XIAP degradation (22). Compound A induces cIAP1 and cIAP2 degradation resulting in NF-κB activation and TNFα production. Induction of TNFα-dependent apoptosis via compound A has been described to be effective in various cancer cell lines (22, 23). Inhibition of cIAP1 and cIAP2 enhances the release of RIP1 from TNFR1 and leads to incorporation of RIP1 into a complex with caspase 8 and Fas-associated death domain (FADD) resulting in cell death (32-34). Recently, a death inducing complex containing RIP1, caspase 8 and FADD has been described that assembles independently of death receptor activation, which is called the ripoptosome (35, 36). The fact that cIAP degradation with cIAP antagonists results in spontaneous ripoptosome formation leading to cell death highlights the importance of understanding the molecular mechanisms regulating cIAP expression.

Overexpression of IAP proteins is found in many cancer types and is associated with chemoresistance (5). In CLL, relapses putatively originate from the protective LN microenvironment. New treatment modalities, such as cIAP antagonists, could be important to overcome chemoresistance. The aim of this study was to investigate the various adaptor proteins of the intracellular TNFR signalosome and ripoptosome in unstimulated and CD40-stimulated CLL cells. Furthermore, we explored whether inhibition of cIAP1 and cIAP2, via compound A, induces TNFα-dependent cell death in unstimulated and CD40-stimulated CLL cells either alone or in combination with other death inducing stimuli.

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. Peripheral blood (PB) mononuclear cells of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, The Netherlands) were frozen and stored as described (37). Expression of CD5 and CD19 (both 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 Table 1.

**Reagents**

Compound A was obtained under MTA from Tetralogic (Malvern, PA, USA) dissolved in dimethyl sulfoxide (DMSO) and was used in various concentrations (5, 50, 250 and 500 nM). SuperKiller TRAIL was purchased from Alexis Biochemicals and was used in concentration of 10 ng/ml. Recombinant human TNFα (100 ng/ml) was obtained from Invitrogen (Carlsbad, CA, USA). TNFR1 (FITC) (Lot # LFA 015071), TNFR2 (PE) (Lot # LFB 046021) were obtained from R&D systems (Minneapolis, USA). Anti-human Fas monoclonal antibody (FAS10, 10 μg/ml) was produced at the CLB Sanquin Blood Foundation (CLB, Amsterdam, The Netherlands). CysTNF32W/86T, designated as TNFα-TNFR1 (0.1-1 μg/ml), CysTNF143N/145R, designated as TNFα-TNFR2 (0.1-1 μg/ml) were a kind gift from Anja Krippner, New Castle University, United Kingdom (38, 39).

**Cell culture and detection of apoptosis**

Peripheral blood lymphocytes of CLL patients were stimulated by co-culture with NIH3T3 fibroblasts stably transfected with human CD40L, as described previously (37). Briefly, DMSO-frozen CLL cells were thawed and co cultured on irradiated (30Gy) control NIH3T3 fibroblasts or NIH3T3 fibroblasts expressing CD40L. The final CLL cell concentration was 1.67 x 10⁶ cells/ml in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% FCS. Cells were simultaneously cultured with NIH3T3 fibroblasts ± CD40L and compound A. After 1, 4, 24, 48 or 72 hrs of culture at 37 °C, CLL cells were carefully detached by pipetting and stained with Annexin V APC (IQ Products, Groningen, The Netherlands) and propidium iodine (PI; Sigma, St Louis, MO) as described and analyzed by FACS (37). Additionally, apoptotic and viable cells were discriminated via flow cytometry with MitoTracker Orange (Molecular Probes, Leiden, The Netherlands).

**Western Blot and antibodies**

Western blotting was performed as described previously (37). Samples (60 μg protein) were separated by 7.5-13% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Blots were probed with polyclonal anti-p65 (clone C-20; catalog # sc-372; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-Histon H3 (catalog #9715 Cell Signaling), polyclonal NF-κB2 p100/p52 (catalog #4882 Cell Signaling), monoclonal
mouse antibody for phosphoIκBα (catalog #9246 Cell Signaling), NIK (catalog #4994 Cell signaling) TRAF2 (catalog nr cs-876, Santa Cruz Biotechnology, Santa Cruz, CA) TRAF6 (catalog #4743s Cell signaling), RIP1 (catalog #610458 BD Bioscience) polyclonal antibodies against cIAP1 and cIAP2 (La Trobe University, Melbourne, Australia) and antiserum to β-actin (clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA). IRDye 680 donkey anti-rat IgG, 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. Western blots were scanned on the Odyssey imager (LI-COR Biosciences, Lincoln, NE). When indicated immunoreactive proteins were visualized using HRP-conjugated Ig (Rabbit anti-goat, swine anti-rabbit and goat anti-mouse, DAKO, Glostrup, Denmark) and enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, U.K.).

**Enzyme-linked immunosorbent assay**

Peripheral blood lymphocytes of CLL patients were stimulated with CD40L expressed on NIH3T3 fibroblasts, as described above. The supernatant was collected and used for Enzyme-linked immunosorbent assay (ELISA). TNF-α levels were measured using the PeliKine human ELISA kit (Sanquin, Amsterdam) according to manufacturer’s recommendations. Absorbance was read at 450 nm using an ELISA reader (Biorad, Hercules, Ca).

**RNA isolation and RT-MLPA**

Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St. Louis, MO, USA). Reverse transcription–multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously (40, 41). Only the mRNA levels of NF-κB targets genes are shown. Equal amounts of mRNA were included per reaction and all samples were tested in a single experiment using the same batch of reagents. Relative mRNA expression of the gene of interest is calculated by setting the total signal for each sample at 100% and individual signals of genes of interest were calculated relative to the 100% value.

**Statistical analysis**

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 of the data was set at P < 0.05, with one asterisk (*) representing 0.01 < P < 0.05; two asterisk (**) 0.001 < P < 0.01; three asterisk(***P<0.001.
Results

Primary CLL cells show high cIAP2 levels and CD40 stimulation results in NF-κB activation and increased levels of TNFR1 and TNFR2.

Previously, we have shown that upon prolonged CD40 stimulation the classical and then the alternative NF-κB pathway are activated in CLL cells (42). In accordance, NIK, p52 and plkbα levels were increased in CD40-stimulated CLL cells (Figure 1A). In line with previous studies (3, 4) levels of cIAP2 were found to be much higher than cIAP1.

![Figure 1. Enhanced NF-κB activation and increased levels of TNFR1/TNFR2 in CLL cells induced by CD40 stimulation. A. CLL cells were stimulated with 3T3 or CD40L-expressing 3T3 cells for 48 hrs. Protein lysates were probed for NIK, p100, p52 and plkbα. β-actin was used as loading control. Western blots were scanned on the Odyssey imager. Blots from one representative CLL patient is shown, of a total of nine analyzed. B. After 48 hrs of stimulation ± CD40L, CLL cells were detached from the feeder layer cells. Protein lysates were probed for cIAP1, cIAP2, TRAF2, TRAF6 and RIP1. β-actin as loading control. Western blots were scanned on the Odyssey imager. Blots from one representative CLL patient is shown, of a total of three analyzed. C. CLL cells were co-cultured with 3T3 (n=16) or CD40L-expressing 3T3 cells (n=16). After 72 hours supernatants were collected and TNF-α levels were measured by ELISA. Error bars present standard error of the mean. D. TNFR1 and TNFR2 expression was measured on freshly isolated, 3T3 and 3T40L stimulated CLL cells by flow cytometry (n=8). Error bars present standard error of the mean. * .01 < P < .05; ** .001 < P < .01.](image-url)
in unstimulated peripheral blood (PB) CLL cells (Figure 1B). CD40-stimulation of CLL cells did not influence cIAP1 and cIAP2 levels (Figure 1B). TRAF2 has been described to recruit cIAPs to many of the TNF superfamily receptors, which is important in signal transduction (43, 44). Basal levels of TRAF2 were present in unstimulated CLL cells and CD40 stimulation resulted in increased TRAF2 levels (Figure 1B). Binding of cIAP1 and 2

### Table 1. Patient characteristics.

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1 Percentage of cells positive for CD5/CD19 surface expression measured by flow cytometry. 2 Mutated IgVH gene (+) denotes >2% mutations compared to germline 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. ND = not determined.

4 Clb = chlorambucil, F = fludarabine, FCR = fludarabine, cyclophosphamide, rituximab, FCO = fludarabine, cyclophosphamide, ofatumumab, RCHOP = rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone.

5 P53 functional status was measured via radiation-induced RNA expression of p53 target genes Puma and Bax as described previously F = p53 Functional, D = p53 Dysfunctional.
to TRAF2 results in RIP1 modification which leads to NF-κB activation (20, 21). RIP1 levels also markedly increased upon CD40 stimulation of CLL cells (Figure 1B). CD40-stimulated CLL cells are known to produce TNFα ((42) and Figure 1C). In agreement with a previous report (45), we further confirmed a significant upregulation of TNFR1 and TNFR2 upon CD40 stimulation (Figure 1D). Together, these data demonstrate that CD40 stimulation of CLL cells resulted in NF-κB activation, TNFα production and upregulation of TNFR1 and TNFR2. Moreover, TRAF2, NIK and RIP1 levels increased in CD40-stimulated CLL cells. Thus, all adaptor proteins components which play an important role in the formation of the TNFR1/cIAP signalosome and ripoptosome are present.

Effects of compound A on cIAP2 levels and NF-κB activation

Compound A is a potent inducer of cell death via degradation of cIAP1 and cIAP2 resulting in TNFα production (22, 23). This leads to autocrine TNFR1 signaling resulting in caspase 8 activation. Unstimulated and CD40-stimulated CLL cells were treated with compound A and Western Blot analyses of cIAP1 and cIAP2 were performed. The expression of cIAP1 in CLL was very low, as reported before (3, 4), but upon compound A treatment cIAP1 levels were further decreased (Figure 2A and Figure 2B, upper panel). High levels of cIAP2 were observed in both unstimulated and CD40-stimulated CLL cells and subsequent degradation of cIAP2 was observed upon compound A treatment. Interestingly, after 4 hrs of compound A treatment cIAP2 expression levels partially recovered (Figure 2A and Figure 2B, upper panel). To investigate the effects of compound A treatment on NF-κB activation, protein expression of p65 and p52 from nuclear extracts of unstimulated and CD40-stimulated CLL cells were analysed in the presence or absence of compound A. An obvious increase in p65 levels and a slight increase in p52 levels were observed in unstimulated CLL cells treated with compound A (Figure 2A, lower panel). This was a reproducible phenomenon observed in three different patients. As we have shown previously, levels of p52 and p65 from nuclear extracts of CD40-stimulated CLL cells were high, and only a minor increase in p65 levels was observed after 4 hours of compound A treatment (Figure 2B, lower panel) (42). Despite the obvious increase of p65 levels observed in unstimulated CLL cells only a minor increase in TNFα production upon compound A treatment was observed (Figure 2C). In CD40-stimulated CLL cells no significant change in TNFα was observed upon compound A treatment (Figure 2C). Other downstream targets of NF-κB signaling were investigated such as the anti-apoptotic proteins Bcl-X_L and Bfl-1 in the presence or absence of compound A (48 hours of stimulation)(46). No significant increase in mRNA levels of Bcl-X_L and Bfl-1 were observed upon compound A treatment in unstimulated and CD40-stimulated CLL cells (Figure 2D). Previously, we have been shown that the classical NF-κB pathway induces Bfl-1 levels in CLL cells upon CD40 stimulation (42). In this study, although the classical NF-κB pathway was activated by compound A in unstimulated CLL cells, we did not observe an increase in Bfl-1 levels. In conclusion, we
have shown that cIAP2 levels decrease upon compound A treatment in unstimulated and CD40-stimulated CLL cells. However, cIAP2 levels partially recovered despite treatment. Furthermore, enhanced NF-κB activation was observed in unstimulated CLL cells upon compound A treatment but only a slight increase in TNFα was observed.

Figure 2. The effects of compound A on cIAP levels and NF-κB activation in unstimulated and CD40-stimulated CLL cells. A. CLL cells on 3T3 control feeder layer cells were incubated with compound A (250nM) for 1, 4 and 24 hours. Cytoplasmic protein lysates were probed for cIAP1, cIAP2 and β-actin as loading control (upper panel). Nuclear extracts were probed with p100, p52, p65 and histon H3 as loading control (lower panel). Blots from one representative CLL patient is shown, of a total of three analyzed. B. CLL cells were stimulated with CD40L in the presence or absence of compound A (250nM) for indicated time (0, 1, 4 and 24 hrs) and cytoplasmic protein lysates were probed for cIAP1, cIAP2 and β-actin. Nuclear extracts were probed with p100, p52, p65 and histon H3 as loading control Blots from one representative CLL patient is shown, of a total of four analyzed. C. CLL cells were co-cultured on control or CD40L expressing fibroblasts in the presence or absence of compound A (250nM) for 72 hours. Supernatants were collected and TNF-α levels were measured by ELISA. Error bars present standard error of the mean. D. Bcl-XL and Bfl-1 gene expression profiles were investigated by RT-MLPA as described in materials and methods. CLL cells were cultured for 48 hours in the presence or absence of CD40L ± compound A (250nM) (n=5). Gus was used as a housekeeping gene. Error bars present standard error of the mean.
CLL cells are resistant to compound A

Based on the previous data, we hypothesized that compound A would induce cell death especially in CD40-stimulated CLL cells. However, using varying concentrations of compound A, only a minor induction of apoptosis was observed in CD40-stimulated CLL cells (n=26) and not in unstimulated CLL cells (n=13) (Figure 3A,B). As a control, Kym1 cells (22) were treated with different concentrations of compound A, which resulted in high levels of apoptosis when treated with compound A in low concentrations (0.5 nM) (data not shown). The slight, but significant, increase in apoptosis induced by the highest concentration of compound A (500 nM) in CD40-stimulated CLL cells could

Figure 3. The effects of compound A on apoptosis in unstimulated and CD40-stimulated CLL cells. A. Representative Annexin V FITC/ PI facs plots of unstimulated (left panel) and CD40-stimulated CLL cells (right panel) are shown in the absence (upper panel) or presence of compound A (500nM) (lower panel). B. CLL cells were stimulated with 3T3 (n=13) or CD40L-expressing 3T3 cells (n=26) for 72 hrs in the presence or absence of different concentrations of compound A (5, 50, 250 and 500nM) and analyzed for apoptosis by Annexin V/ PI staining. Bars present standard deviation. C. Exogenous TNFα (5 ug/ml) was added to CD40-stimulated CLL cells ± compound A (n=4). After 72 hrs apoptosis was measured by Annexin V/ PI staining. TNFα was blocked by co-culturing soluble anti-TNFα receptor (ethanercept) with CD40-stimulated CLL cells treated with compound A (n=20) for 72 hrs. Apoptosis was determined by Annexin V/ PI staining. D. CD40-stimulated CLL cells ± compound A were simultaneously treated with FAS and TRAIL for 48 hrs (n=3). Apoptosis was determined by Annexin V/ PI staining. For C, D and E error bars present standard error of the mean. ** .001 < P < .01, *** P < .001.
not be blocked by anti-TNFα, suggesting TNFα independent apoptosis. In addition, no significant increase in apoptosis was observed when exogenous TNFα was combined with compound A treatment compared to compound A treatment only, in CD40-stimulated CLL cells (Figure 3C). To determine whether compound A would act synergistically with extrinsic cell death inducers, CD40-stimulated CLL cells were treated with compound A in the presence or absence of FAS or TRAIL (30, 31). No synergistic effects were observed (Figure 3D). As a positive control for TRAIL- and FAS-induced apoptosis Jurkat cells were used, which readily underwent apoptosis upon TRAIL and FAS triggering (data not shown). In addition, no synergistic effects of compound A with cytotoxic drugs, such as fludarabine, were observed (data not shown). In conclusion, despite compound A-induced cIAP2 degradation, TNFα production and increased expression of TNFR1 in CD40-stimulated CLL cells, compound A, by itself or combined with intrinsic or extrinsic cell death inducers, did not induce specific apoptosis in primary CD40-stimulated CLL cells nor in unstimulated CLL cells.

TNFR1 stimulation combined with compound A treatment does not induce cell death

Distinct functions for the two TNF receptors have been described. TNFR1 carries a cytoplasmic death domain and can activate caspases, leading to apoptosis. In contrast, TNFR2 does not contain a death domain and is known to activate NF-κB leading to enhanced survival (47-50). We investigated whether TNFR1 triggering with a TNFα mutant, which specifically binds to TNFR1 (TNFα-TNFR1), would induce cell death when combined with compound A. As a negative control, a TNFα mutant which specifically binds to TNFR2 (TNFα-TNFR2) was tested in the presence and absence of compound A. Two CLL samples were investigated, one sample with relatively low expression of TNFR1 (pt-1) and another sample with higher expression levels of TNFR1 (pt-2) (Figure 4A). Upon CD40-stimulation levels of TNFR2 increased in pt-1 and levels of TNFR1 increased in pt-2 (Figure 4A). In both samples treatment with compound A combined with specific TNFR1 stimulation did not induce apoptosis in unstimulated nor in CD40-stimulated CLL cells (Figure 4B, C). Anti-TNFα was added to prevent TNFR2 signaling from TNFα produced by CD40-stimulated CLL cells, but also no differences in apoptosis were observed in the presence of anti-TNFα (Figure 4C). As a control for TNFR signaling upon specific TNFR1 and TNFR2 binding with mutant TNFα, expression of CD95 was measured in non-CD40 stimulated CLL cells (51). In pt-2 we observed an increase of CD95 expression upon specific TNFR1 stimulation (Figure 4D). In both patients specific TNFR2 stimulation resulted in an increase of CD95 expression (Figure 4D). In summary, specific TNFR1 stimulation did not result in compound A induced apoptosis in unstimulated and CD40-stimulated CLL cells.
Figure 4. Specific TNFR1 and TNFR2 stimulation in compound A treated CLL cells A. TNFR1 and TNFR2 expression was measured on 3T3 and 3T40L stimulated CLL cells by flow cytometry. B. CLL cells were co-cultured on control fibroblasts. CLL cells were treated with compound A (50-500nM) in the presence or absence of mutant TNFα which specifically binds to TNFR1 (TNFα-TNFR1) and mutant TNFα which has higher affinity to TNFR2 (TNFα-TNFR2). C. CLL cells were stimulated with CD40L and treated with compound A (50-500nM) in the presence or absence of TNFα-TNFR1 and TNFα-TNFR2. Anti-TNFα (ethanercept) was added to CD40L stimulated CLL cells in the presence of TNFα-TNFR1 to block endogenous TNFα activity. D. Percentage of CD95 expression was measured in unstimulated CLL cells in the presence and absence of TNFα-TNFR1 and TNFα-TNFR2. For A, B, C and D pt1: left panel and pt2: right panel.
Discussion

In this study, we show that CD40 stimulation of CLL cells results in increased levels of TNFR1, cIAP2, TRAF2 and RIP1, all of which are important components of the TNFR1/cIAP signalosome. Compound A has been shown to be effective in TNFα-dependent apoptosis via degradation of cIAP1 and cIAP2 in various solid cancer cell lines (22). In this study, we describe that CLL cells express all required components of the TNFR1/cIAP signalosome, yet are resistant to compound A treatment. A potential reason for resistance to compound A is the increase of cIAP levels during compound A treatment which might lead to impaired ripoptosome formation. In addition, other mechanisms possibly inhibiting ripoptosome formation are suppression of the RIP1 deubiquitinating enzyme cylindromatosis (CYLD) or induction of cFLIP. These mechanisms will be discussed in detail below.

In CLL cells expression of cIAP2 levels were markedly higher than cIAP1 levels. It has been shown that cIAP1 degradation occurs at a lower concentration of compound A than cIAP2 (52). We showed that cIAP2 levels are effectively down regulated in CLL cells when treated with compound A, although levels restored during treatment. This phenomenon has been described by Petersen and colleagues in lung carcinoma cell lines (53). Cell lines which were resistant to cIAP antagonists showed initial degradation of cIAP2, but levels restored after 3 hours of treatment. cIAP2 upregulation was suppressed via a PI3K inhibitor (LY294002) and abrogated resistance to cIAP antagonists (53). In our hands, inhibition of the PI3K pathway, via LY294002, did not sensitize unstimulated nor CD40-stimulated CLL cells to compound A (data not shown). Upon compound A treatment in unstimulated CLL cells, we observed activation of the NF-κB pathway. In CLL cells, it has been described that NF-κB regulates cIAP levels (54). Activation of the NF-κB pathway might induce cIAP levels in CLL cells during treatment with compound A. Currently, we are investigating whether this increase in cIAP levels could be inhibited by suppression of the NF-κB pathway and abrogates resistance to cIAP antagonists.

CIAPs play an important role in the regulation of RIP1. In the absence of CIAPs, RIP1 is recruited to form a complex with FADD and caspase-8 resulting in cell death. CIAPs can mediate binding of RIP1 to transforming growth factor β-activated kinase 1 (TAK1) which prevents RIP1 from binding to caspase 8 and FADD (34). Vanlangenakker and collaborators recently showed that lowering TAK1 levels resulted in enhanced binding of RIP1 to FADD, leading to an accumulation of reactive oxygen species and thereby inducing necrosis (56). When cIAP levels restore despite treatment with cIAP antagonists formation of this death inducing complex containing RIP1 might not occur. An important aspect of further studies is to investigate whether a RIP1-FADD-caspase-8 death inducing complex is formed and if not whether this is due to persistent binding of RIP1.
to TAK1 in CLL cells treated with compound A. Besides persistent binding of TAK1 to RIP1, there might be other reasons why RIP1 does not form a death domain, including for example the presence of polyubiquitinated RIP1. The polyubiquitinated form of RIP1 is a component of a protein complex which activates the NF-κB pathway (20). When polyubiquitinated chains are removed from RIP1, RIP1 is able to form a death domain with RIP3 and caspase 8 (57). The deubiquitinating enzyme cylindromatosis (CYLD) is important in deubiquitination of RIP1 (58). Lymphoid enhancer-binding factor 1 (LEF1) has been identified as a transcriptional repressor of CYLD in CLL (58). Thus, another explanation for compound A resistance in CLL cells could be that LEF1 suppresses CYLD which prevents RIP1 from binding to RIP3 and caspase 8. Another protein involved in ripoptosome formation is cellular FLICE inhibitory protein (cFLIP). cFLIP_L represses formation of the ripoptosome (35, 36) and the short form of cFLIP, cFLIP_S promotes ripoptosome formation (35). cFLIP_L can form a heterodimer with caspase 8 and inhibits cell death via cleavage of RIP1 and RIP3 (59). CLL cells express higher levels of cFLIP_L compared to normal B cells (41). In addition, CD40 stimulation of CLL cells results in higher cFLIP_L levels (60). Further studies are needed to investigate whether cFLIP_L plays a role in resistance to compound A treatment in CLL cells.

Combining compound A with other extrinsic cell death inducers such as TRAIL and FAS did not reveal any additive or synergistic effects in CD40-stimulated CLL cells. In contrast, co-treatment of XIAP inhibitors and TRAIL showed a significant induction of apoptosis in primary CLL cells compared to monotherapy with XIAP and TRAIL. Interestingly, almost all unmutated CLL cells were sensitive to this combination (12 unmutated CLL samples out of 14 tested) (31). Upon compound A treatment, we did not observe significant differences in apoptosis between mutated and unmutated CLL cells (data not shown). These results imply that there may be a role for XIAP inhibitors and TRAIL in the treatment of CLL patients with an unfavourable prognosis.

Recently, Weisberg and collaborators showed promising effects of Smac mimetics as a targeted therapy in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) in vitro and in vivo mice models (61). The authors demonstrated inhibition of proliferation when a chemoresistant Ba/F3-derived cell line was treated with Smac mimetics (61). CLL cells stimulated with CD40L hardly show any proliferation, although preliminary experiments show that, after CD40-stimulation in the presence of recombinant IL-21 in the culture medium, CLL cells do proliferate (Tromp et al, chapter 4, this thesis). Currently, we are investigating whether compound A is able to induce inhibition of proliferation and results in enhanced cell death in these cells.

CLL cells which are stimulated with CD40L show an upregulation of anti-apoptotic proteins and become resistant for cytostatic drugs (37, 60). Recently, it has been shown that CD40 ligation resulted in caspase 8 activation in a RIP1 dependent manner in
the absence of TRAF6 (62). When binding of TRAF6 to CD40 was inhibited, activation of caspase 3 and 8 was induced in different carcinoma cell lines. Furthermore, cIAP1 and cIAP2 degradation in those carcinoma cell lines was shown to amplify the CD40-mediated cytotoxic effect (62). These findings could have important implications for CLL cells, because it is thought that CD40-CD40L interaction in lymph nodes of CLL cells play an important role in relapses due to drug resistance. In this study, we show that CD40 stimulation of CLL cells resulted in an upregulation of TRAF6 (Figure 1B). Novel strategies to alter CD40-induced survival signaling in death signaling in CLL cells, for example via inhibition of TRAF-6, could be promising in inducing cell death in the protective microenvironment of CLL.

In conclusion, our data show that CD40-stimulated CLL cells show enhanced NF-κB activity, TNFα production and an upregulation of TNFR1. CLL cells were found to be resistant to cIAP antagonists, putatively due to the inability of death signaling via TNFR1. Compound A treatment resulted in degradation of cIAP2, but those levels restored during treatment, which could be an important aspect in compound A resistance. Further studies are needed to investigate whether a ripoptosome is formed in CLL cells upon compound A treatment. The fact that other TNF receptors besides TNFR1, such as CD40, could also be used to trigger death signaling, rather than survival signaling, may lead to a new field of investigation in targeted therapies for chemoresistant CLL cells.
Reference List


