CD40 stimulation sensitizes CLL cells to rituximab-induced cell death

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Abstract

In vitro CD40-stimulated Chronic Lymphocytic Leukemia (CLL) cells are resistant to cytotoxic drugs. In sharp contrast we here show that CD40 stimulation sensitizes CLL cells to rituximab-mediated cell death. This increased sensitivity is specific for anti-CD20 treatment. Rituximab-mediated death in CD40-stimulated CLL cells shows rapid kinetics (within hours) and is caspase- and p53-independent, but depends on extracellular Ca\(^2\) and ROS production. By increasing basal ROS production CD40 stimulation sensitizes CLL cells to rituximab-mediated death. Our findings provide a rationale for combination treatment of CLL with cytotoxic drugs and anti-CD20 monoclonal antibodies.
**Introduction**

CLL is characterized by progressive accumulation of CD5⁺CD19⁺CD23⁺ malignant B cells in blood, bone marrow and lymphoid organs. Although treatment results have improved considerably over the last decade, a curative treatment is still not available, with the possible exception of allogeneic stem cell transplantation. The incurability of CLL might be due to the interaction of CLL cells with their micro-environment, notably in proliferation centers in the lymph nodes. In these niches CLL cells not only receive signals driving proliferation but also multiple signals resulting in resistance to cytotoxic drugs. Indeed, there is histopathologic evidence that in proliferation centres in lymph nodes CLL cells are exposed to CD40L⁺ T cells. We have previously shown that in vitro CD40 stimulation of peripheral blood derived CLL cells can to a certain extent mimic the lymph node microenvironment and result in resistance to cytotoxic drugs.

Recently it has been shown in a large randomized phase III trial that addition of the chimeric anti-CD20 monoclonal antibody (mAb) rituximab (R) to FC (Fludarabine, Cyclophosphamide) improves both progression free survival (PFS) and overall survival (OS) in p53 functional, previously untreated CLL patients. This is remarkable in view of the relatively low efficacy of rituximab when used as monotherapy at dosages used in the treatment of lymphoma. This poor efficacy has been explained by low CD20 expression levels of CLL cells, the presence of complement inactivating molecules (CD55,CD59) on CLL cells, the presence of soluble CD20, shaving and downmodulation of CD20 after rituximab treatment, resistance to ADCC by NK cells in vitro and -last but not least- the intrinsic anti-apoptotic profile of CLL cells.

In the present study we show that, in sharp contrast to the induction of resistance to cytotoxic drugs, CD40 stimulation of CLL cells considerably increases sensitivity to anti-CD20 mediated cell death. Furthermore we demonstrate that rituximab-induced cell death of CD40-stimulated CLL cells is caspase- and p53-independent, and mediated by calcium-dependent ROS production.

**Materials & Methods**

**Patient samples**

Peripheral blood was drawn from CLL patients (diagnosed according to the NCI-WG guidelines). Peripheral blood mononuclear cells (PBMC’s) were isolated by Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands) and either used immediately or stored in liquid nitrogen. During all in vitro experiments, cells were maintained in culture medium: Iscove’s modified Dulbecco medium (IMDM: Gibco Life technology, Paisley, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100µg/ml gentamycin and 0.00036% b-mercaptoethanol. All PBMC samples from CLL patients contained at least 90% CD5⁺/CD19⁺ cells as assessed via flow cytometry.
studies were approved by the Ethical Review Board of the Institute and from all participants written informed consent was obtained.

**Antibodies and reagents**

The anti-CD20 monoclonal antibody (mAb) Rituximab was kindly provided by Roche Nederland BV (Woerden, The Netherlands). The anti-CD20 mAb Ofatumumab was kindly provided by Genmab (Utrecht, The Netherlands). The anti-CD52 antibody alemtuzumab (Ilex pharmaceuticals San Antonio, TX, USA), goat-anti human (GAH) Fc-γ fragment specific antibody (Jackson Immunoresearch Europe), mouse anti-human IgM mAb (MH15, Sanquin, The Netherlands), CD20-FITC clone B9E9 (Beckman Coulter, France), FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (Becton Dickinson, San Jose, CA) were all obtained commercially. 

The pan-caspase inhibitor Q-VD was purchased by R&D systems (Minneapolis, MN, USA) and Z-VADfmk by Alexis Biochemicals (San Diego, CA, USA). Roscovitine, Fludarabine, Cisplatin, Indo1-AM, Propidium Iodide (PI), N-acetylcytseine (NAC), hydroxychloroquine (HCQ), Triton-X 100 and EGTA were purchased from Sigma Chemical Co (St. Louis, MO, USA). The proteasome inhibitor bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands). BAPTA-AM was purchased by Calbiochem. RNAse (DNAse free) was obtained by Roche. 2,7-dichlorodihydrofluorescein diacetate DCFH-DA (DCF-1), MitoTracker orange and Ionomycin were purchased by Molecular Probes, Leiden, The Netherlands. FITC-labeled Annexin V was purchased by IQ products BV, Groningen, The Netherlands.

**Flow Cytometry**

To determine CD20 expression levels on CLL cells, PBMC were stained using FITC-labeled antibodies against CD20 and mean fluorescence intensity (MFI) was determined.

For intracellular staining, cells were fixed and permeabilised (eBioscience, San Diego, CA) and subsequently stained with FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (mAb). Samples were analyzed on the FACSCalibur flow cytometer (BD biosciences) and CellQuest software (Beckton Dickinson) was used for data acquisition. Data were analyzed with FlowJo software (TreeStar, San Carlos, CA, USA).

**In vitro CD40 ligand stimulation of CLL cells**

PBMC from CLL patients (> 90% CD5+ CD19+ cells) were stimulated with CD40 ligand (CD40L) transfected NIH3T3 (3T40L) cells as described previously 6. Briefly, 5.10^6 CLL cells/well were added to 6-well plates coated with irradiated (30 Gy) CD40L transfected NIH3T3 cells. Non-transfected 3T3 cells were used as negative controls. After 2 days, the CLL cells were gently removed from the fibroblast layer and used in further experiments.

**Induction and analysis of apoptosis**

For apoptosis induction 3T3 or 3T40L stimulated CLL cells were incubated with the indicated mAbs (10 µg/ml) for 24h as described previously 17. Crosslinking GAH antibody (indicated
CD40 stimulation sensitizes CLL cells to rituximab-induced cell death

as XL) (50 µg/ml) was added 30 minutes after the indicated mAbs. As a positive control for apoptosis induction cells were treated with different cytotoxic drugs: fludarabine 50 µM, 25 µM roscovitine, a cyclin dependent kinase inhibitor and potent inducer of apoptosis in CLL or bortezomib 30 nM. The pan-caspase inhibitors 20mM Q-VD or 200 mM Z-VADfmk were added 30 minutes before the indicated mAbs or roscovitine. To chelate extracellular calcium, EGTA was added at a concentration of 5mM and added 30 minutes prior to addition of mAbs. As intracellular calcium chelator BAPTA-AM was used in a concentration of 5 µM and added 30 minutes prior to addition of mAbs. The Reactive Oxygen Species (ROS) scavenger NAC was used in a concentration of 50 mM and added 30 minutes prior to respective mAbs.

Apoptosis was analyzed by evaluation of mitochondrial membrane potential with MitoTracker orange (Molecular probes, Leiden, The Netherlands) according to the manufacturer’s recommendations or by Annexin V PI staining as described previously. The percentage apoptotic cells was calculated as follows: 100% - annV-/PI- (viable) cells. Due to heterogeneous levels of basal apoptosis, data are expressed as relative cell death, which was defined as: % cell death in stimulated cells - % cell death in medium control.

**FACS analysis for sub-G1 DNA fragmentation**

DNA fragmentation was analysed using flow cytometry after PI labeling of apoptotic nuclei as described previously. In brief, after apoptosis induction (as described above), cells were put on ice and subsequently centrifuged. While vortexing, cells were resuspended in 200 µl hypotonic solution containing 25 µg/ml PI, 100 µg/ml RNAse and 0.1% Triton-X100 in 0.1% Na-citrate solution. Cells were incubated on ice for 1 h in the dark and analyzed by flow cytometry. The subG1 peak represents apoptotic cells.

**Electron microscopy**

5.10^6 stimulated cells were washed twice in PBS (to remove all FCS) and centrifuged. Pellets were fixed in Karnovsky’s fixative (0.1 M cacodylate buffer pH 7.4 containing 4% w/v formaldehyde and 2.5% w/v glutaraldehyde) and postfixed in 1% osmium tetroxide plus 0.5% uranyl acetate and embedded in Epon Resin (Hexion Specialty Chemicals, Hoogland, The Netherlands). Ultra-thin sections were stained with uranyl acetate and lead citrate and subsequently examined in a Philips CM10 electron microscope; images were obtained and measured with iTEM software (Soft Imaging System GmbH).

**Determination of intracellular ROS production**

ROS production was detected on FACSscan using 2,7-dichlorodihydrofluorescein diacetate, DCFH-DA as described previously. Briefly, 0.2 10^6 cells were incubated with rituximab (10 µg/ml) and crosslinking GAH antibody (50 µg/ml) for 1, 4 or 8 h in culture medium. In some experiments, cells were treated with RXL in the presence or absence of 50mM NAC or 5mM EGTA. After incubation with antibodies, cells were washed in warm (37°C) phenolred free DMEM medium. The pellet was resuspended in 100 µl DMEM medium with 1µM DCFH-DA.
and incubated for 30 min at 37 °C to allow cellular incorporation. ROS-dependent DCF-1 fluorescence in cells was analysed by flow cytometry (FACSCalibur flow cytometer, BD biosciences).

**Measurement of Ca^{2+} mobilization**

Ca^{2+} mobilization was measured as described previously 23. Briefly, after stimulation on 3T3 or 3T40L fibroblasts, cells were gently removed from the fibroblast layer and resuspended in loading medium (Hank’s buffered salt solution (HBSS) containing 1mM Ca^{2+} and Mg^{2+} and 1% FCS) at a concentration of 1-10.10^6 cells/ml and loaded with 2 µM Indo-1 AM for 30 minutes at 37°C while gently shaking. Then cells were washed and resuspended in loading medium at 1.10^6 cells/ml. Calcium responses were measured on a Beckton Dickinson FACS Vantage SE with UV excitation. Data were collected and displayed as the relative ratio of intensities of Indo fluorescence (Ca-bound Indo violet emission 405nm/free Indo blue emission 485 nm) for each cell over time and analyzed with FlowJo software (TreeStar, San Carlos, CA, USA). To analyze CD20 induced Ca^{2+} mobilization, cells were pre-incubated with rituximab (10µg/ml) for 10 minutes and excess antibody was washed away prior to the start of the experiment. Samples (5.10^5 cells per 10 minute assay) were analyzed 1-2 minutes to obtain a baseline, followed by the addition of 50 µg/ml GAH antibody (CD20 induced Ca^{2+} mobilization) or 10 µg/ml mouse anti-human IgM mAb (MH15) to measure B-cell receptor (BCR) induced Ca^{2+} mobilization. As a control GAH antibody alone (50 µg/ml) was added (on non rituximab pre-incubated cells). Ionomycin was used to control for intracellular loading of Indo-1 AM.

**Reverse transcription–multiplex ligation-dependent probe amplification assay**

Reverse transcription–multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously 24. Data were normalized by setting the sum of all signals at 100% and expressing individual peaks relative to the 100% value.

**Statistics**

The Shapiro-Wilk normality test was performed to test Gaussian distribution. When there was Gaussian distribution a two-sided paired t-test was used to analyze differences between the groups. The two-tailed Mann-Whitney U test was used to analyze differences between 2 groups and a Wilcoxon matched paired test was used to analyze differences between paired samples when there was no Gaussian distribution. P-values < 0.05 were considered to be statistically significant.
Results

CD40 stimulation sensitizes CLL cells to anti-CD20 mediated death

Previous studies have shown that CD40-stimulated CLL cells are resistant to various cytotoxic drugs and CD95L \( ^5;^6;^25 \). But the sensitivity of CD40-stimulated CLL cells to CD20 antibodies is not known. Therefore rituximab-mediated cell death of CD40-stimulated CLL cells from 17 CLL patients was tested. Patient characteristics are listed in Table 1. In initial experiments anti-CD52 mAbs were used for comparison. Figure 1A shows typical Annexin V PI FACS plots of rituximab-induced cell death \( ^2^6 \). Maximal levels of cell death were induced at a rituximab concentration of 10 µg/ml (not shown). Assessment of cell death by evaluation of mitochondrial membrane potential with MitoTracker and by Annexin V/PI staining gave similar results (data not shown).

In line with previous studies \( ^1^7;^2^7 \) rituximab- and anti-CD52 mAb only induced apoptosis after crosslinking (XL) (Figure 1A+B). XL alone did not induce apoptosis (Figure 1B). Surprisingly, CD40-stimulated (3T40L) CLL cells were more sensitive to rituximab-mediated death than unstimulated (3T3) CLL cells (Figure 1 p= 0.0064). This was in contrast to cell death caused by CD52XL (Figure 1A+B) which was clearly inhibited by CD40 stimulation (p= 0.0023), indicating

| Patient characteristics including sex, age, mutation of IgHV genes, genetic aberrations, p53 function, Rai stage and previous therapy. (F= female, M = male, M = mutated IgHV genes, UM = unmutated IgHV genes, Clb = chlorambucil, ClbP = chlorambucil+prednisone, F= fludarabine, ND = not determined

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Figure 1 CD40 stimulation sensitizes CLL cells to anti-CD20 mediated death in vitro

PBMC of CLL patients were cultured on 3T40L or 3T3 mouse fibroblasts as described in materials and methods. After 2 days CD40-stimulated (3T40L) and unstimulated (3T3) CLL cells were incubated with the indicated mAbs (10 µg/ml) for 24h (B) or cytotoxic drugs (C). Where indicated, crosslinking (XL) GAH antibody (50 µg/ml) were added 30 minutes after the indicated mAbs. After 24h apoptosis was analyzed by Annexin V PI staining.

A. Upper panel: FSC-SSC FACS plots showing blast formation after CD40 stimulation. Lower panel: representative Annexin V PI FACS plots of 3T3 (left) or 3T40L (right) stimulated CLL cells incubated with Rituximab (R), crosslinked Rituximab (RXL), CD52 or crosslinked CD52 (CD52XL) respectively.

B. Averaged results from 17 CLL patients (5 patients with unmutated and 12 with mutated IGHV genes). Relative cell death was calculated as described in materials and methods. Data are presented as percentage relative cell death (mean ± SEM).

C. Averaged results from 8 CLL patients (3 patients with unmutated and 5 with mutated IGHV genes). Data are presented as percentage relative cell death (mean ± SEM. RXL: rituximab+ XL, OfXL: ofatumumab + XL)

D. Averaged results from 7 CLL patients (with mutated IGHV genes) treated with fludarabine 50 µM, roscovitine 25 µM or bortezomib 30 nM. Data are presented as percentage apoptosis (mean ± SEM).

.01<p< .05 *, .001<p<.01 **, p<.001 ***
that CD40-induced enhancement of rituximab-induced cell death is specific. To exclude that the CD40 mediated sensitization is rituximab specific we also tested Ofatumumab, another so-called type I anti-CD20 antibody (for review see28). Ofatumumab also induced increased cell death in CD40-stimulated CLL cells as compared with unstimulated CLL cells (Figure 1C). Like rituximab, Ofatumumab only induced cell death in the presence of a crosslinking antibody (data not shown).

In agreement with our previous studies5,6, CD40-stimulated CLL cells were resistant to fludarabine, roscovitine and bortezomib (Figure 1D). CD40-stimulated CLL cells with unmutated and mutated immunoglobulin variable heavy-chain (IGHV) genes had similar sensitivity to rituximab-mediated death (data not shown) and no differences in rituximab-induced cell death was observed between never treated and previously treated patients. Importantly, p53 dysfunctional CD40-stimulated CLL cells were also sensitive to rituximab-mediated death in vitro (Supplemental Figure 1).

It has been shown that CD20 levels determine the in vitro susceptibility to rituximab11,29. Thus, a rather trivial explanation for the increased sensitivity of CD40-stimulated CLL cells to rituximab-mediated cell death might be CD40-induced upregulation of CD20 expression levels. However, CD20 expression levels of unstimulated (3T3) and CD40-stimulated (3T40L) CLL cells did not differ (Supplemental Figure 2).

Rituximab-mediated death in CLL is caspase-independent

In line with previous studies using B cell lines17, inhibition of caspases with Q-VD or Z-VAD did not block rituximab-induced cell death of unstimulated (3T3) CLL cells (data not shown) or CD40-stimulated CLL cells (Figure 2A). In contrast, roscovitine-induced apoptosis19 was completely blocked in the presence of Q-VD or Z-VAD (Figure 2A).

Despite the lack of a blocking effect of pan-caspase inhibitors, rituximab-induced cell death displayed 3 hallmarks of apoptosis. The first classical hallmark was phosphatidyl serine (PS) exposure as measured with Annexin V (Figure 2A). Second, rituximab induced partial activation of caspase 3 (as detected by intracellular FACS analysis), which was significantly higher in CD40-stimulated CLL cells (Figure 2B, left panel). However, after roscovitine treatment the increase in active caspase 3+ cells was significantly higher in unstimulated CLL cells than in CD40-stimulated cells5 (Figure 2B, right panel). Interestingly, rituximab-induced activation of caspase 3 could not be blocked by ZVAD in contrast to roscovitine-induced caspase 3 activation (Figure 2C). This is in agreement with our finding that Z-VAD blocked roscovitine-induced cell death but not rituximab-induced cell death. Figure 2C shows different caspase 3 histograms of unstimulated CLL cells incubated with roscovitine or CD40 stimulated CLL cells treated with RXL. Roscovitine induced a distinct peak of active caspase 3+ cells whereas this was not observed with RXL. Clearly, Z-VAD completely blocked roscovitine induced caspase 3 activation, whereas caspase 3 activation by RXL was not blocked by Z-VAD. These data suggest that the FACS assay for caspase-3 cleavage can detect alternatively or partially
processed caspase-3, as has been described in BCR induced ongoing caspase 3 processing in the presence of Z-VAD 30.

The third classical hallmark of apoptosis was the induction of a subG1 peak as a result of DNA fragmentation. A subG1 peak was seen both in CD40-stimulated (Figure 2D, upper panel) and unstimulated CLL cells (data not shown). Similar to its lack of effect on caspase 3 activation, Q-VD pre-treatment did not inhibit rituximab-induced DNA fragmentation, whereas roscovitine-induced DNA fragmentation was inhibited in the presence of Q-VD (Figure 2D).

Finally, rituximab-induced cell death displayed remarkable fast kinetics. The decrease in Annexin V/PI- cells and caspase 3 activation occurred within 1 hour after addition of rituximab and XL (data not shown).

In summary, our data show that rituximab-induced cell death in CLL cells occurs within hours and is caspase-independent.

Rituximab crosslinking causes strong homotypic aggregation (HA) in CD40-stimulated CLL cells

Recently Ivanov et al 26 described a homotypic adhesion (HA) -related cell death by type II anti-CD20 mAbs in cell lines. It has been shown that HA occurs after CD40 stimulation of normal B cells 31. In primary CLL cells we also observed HA upon CD40 stimulation (Figure 3). As expected from a type I antibody 32, rituximab alone did not induce HA, neither in unstimulated nor in CD40-stimulated CLL cells (Figure 3). Crosslinked rituximab strongly

CD40 stimulation sensitizes CLL cells to rituximab-induced cell death
increased HA in CD40-stimulated CLL cells (Figure 3). In contrast, crosslinking of anti-CD52 induced only weak HA in CD40-stimulated CLL cells (Figure 3). In control experiments XL alone did not induce HA (Figure 3). Blocking LFA-1 antibodies showed no effect on HA or cell death (data not shown), suggesting a $\beta_2$ integrin-independent mechanism of HA.

**Figure 3** Rituximab crosslinking causes strong homotypic aggregation (HA) in CD40-stimulated CLL cells

CD40-stimulated (3T40L) and unstimulated (3T3) CLL cells were incubated with 10 µg/ml rituximab or CD52 mAb with or without XL antibody or with XL antibody alone for 24h, at which point HA was assessed by light microscopy. Original magnification: x20. Data are representative for 10 experiments.

**Figure 4** No morphological signs of classical apoptosis in rituximab-treated CD40-stimulated CLL cells

Electron microscopy (EM) of CD40-stimulated CLL cells treated with RXL showed massive vacuolization of cytoplasm with relatively intact nuclei (lower panel, left). Unstimulated cells treated with roscovitine showed classical signs of apoptosis like apoptotic bodies (arrows) and chromatin condensation (lower panel, right). Scale bars in the lower right corner of figures indicate magnification.
No morphological signs of classical apoptosis in rituximab-treated CD40-stimulated CLL cells

To test whether RXL induced classical apoptosis in CD40-stimulated CLL cells, we performed electron microscopy (EM). RXL induced cytoplasmic vacuolization in the presence of relatively intact nuclei in CD40-stimulated CLL cells (Figure 4). The latter has been described as the pattern characteristic for cell death induced by anti-CD20 \(^{17,26}\). In contrast to roscovitine-treated cells (Figure 4), rituximab-treated CD40-stimulated CLL cells displayed no morphological signs of classical apoptosis, like chromatin condensation, pyknotic nuclei, cell shrinkage, disintegration of cytoplasmic organelles and apoptotic bodies.

Rituximab-mediated death of CD40-stimulated CLL cells is Ca\(^{2+}\) and ROS- dependent

It has been shown that CD20 can function as Ca\(^{2+}\) channel \(^{23,33,34}\) and that anti-CD20 mediated cell death is Ca\(^{2+}\)-dependent \(^{32,35}\). In agreement with previous studies \(^{23,34}\), we found that in the presence of a crosslinking antibody rituximab induced a Ca\(^{2+}\) flux in

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**Figure 5** Rituximab-mediated cell death of CD40-stimulated CLL cells is dependent on extracellular calcium

CD40-stimulated CLL cells were pre-incubated with 5 mM EGTA or 5 \(\mu\)M BAPTA-AM 30 minutes prior to addition of rituximab and XL antibody.

A. representative histograms of MitoTracker (MitoT) staining. Left: untreated cells with or without EGTA or BAPTA-AM. Right: RXL treated cells in the presence or absence of EGTA or BAPTA. Markers indicate percentage mitoT cells.

B. Averaged results from 8 independent experiments. Data are presented as percentage relative cell death (mean ± SEM). \(.01<p<.05\) *, \(.001<p<.01\) **, \(p<.001\) ***
CLL cells (Supplemental Figure 3). The pattern was different from the Ca\(^{2+}\) flux after BCR stimulation with anti-IgM mAb (MH15) (Supplemental Figure 3). When used alone, the XL antibody did not induce a Ca\(^{2+}\) flux (data not shown).

To test whether rituximab-induced cell death was Ca\(^{2+}\) dependent, CLL cells were incubated with EGTA (to chelate extracellular calcium) or BAPTA-AM (a chelator of intracellular calcium) prior to the addition of rituximab and XL. In CD40-stimulated CLL cells EGTA significantly
Figure 6 Involvement of ROS in rituximab-mediated cell death of CD40-stimulated CLL cells.

A. Representative DCF-1 FACS plots of unstimulated (3T3 med) and CD40-stimulated (3T40Lmed) CLL cells treated with RXL for 1 hour. Left: FSC SSC plots with ungated debris. Right: DCF-1 FACS plots with gated DCF-1 positive cells. Histogram shows Geometric Mean (MFI) of DCF-1 positive cells (thin grey line 3T3 medium, bold grey line 3T3 RXL, thin black line 3T40 medium, bold black line 3T40 RXL).

B. Left panel: ROS production after 1, 4 and 8 hours of RXL in unstimulated (3T3) and CD40-stimulated (3T40L) CLL cells. Data are presented as Geometric Mean (MFI) of DCF-1 positive cells gated as described above (mean ± SEM from 5 independent experiments). Right panel: percentage cell death (MitoT- cells) in unstimulated (3T3) and CD40-stimulated CLL cells after 1, 4 and 8 hours of RXL. This was paralleled by the percentage of dead cells (Figure 6B right panel). Figure 6C shows the fold increase in ROS production in 5 individual patients after 1 hour RXL. As shown in Figure 6D this increase is blocked by EGTA indicating dependence on extracellular calcium.

To test whether ROS play a role in rituximab-mediated cell death, CD40-stimulated CLL cells were incubated for 30 minutes with the ROS scavenger N-acetylcysteine (NAC) prior to the addition of rituximab and XL. NAC was found to inhibit both the rituximab-induced increase in ROS production (Figure 6D) and rituximab-induced cell death (Figure 6E, p=0.03). With the ROS scavenger Tiron similar results were obtained (data not shown). Importantly NAC did not inhibit roscovitine or anti-CD52- induced cell death (data not shown), indicating that effects of NAC are specific for rituximab-induced cell death. In summary our data show that CD40 stimulation of CLL cells increases basal ROS production, which is further increased by addition of rituximab and XL. Moreover, rituximab-induced ROS generation and

blocked rituximab-mediated cell death (p=0.0043) but BAPTA-AM did not (Figure 5B). Representative MitoTracker histograms are shown in Figure 5A. Analysis of the role of Ca\(^{2+}\) in rituximab-mediated cell death of unstimulated CLL cells was not possible because these cells died in the presence of EGTA or BAPTA-AM (data not shown). These data suggest that rituximab-mediated cell death of CD40-stimulated CLL cells is dependent on extracellular Ca\(^{2+}\). It is known that a rise in intracellular Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_i\) results in a higher mitochondrial Ca\(^{2+}\) concentration [Ca\(^{2+}\)]\(_m\) which induces the production of reactive oxygen species (ROS)\(^{36}\). ROS are associated with different types of cell death mechanisms (classical apoptosis, necrosis and autophagy)\(^{38-43}\). CD40 stimulation induced an almost 2-fold increase in basal ROS production when compared to unstimulated CLL cells. (Figure 6A, Figure 6B left panel, 3T40 medium versus 3T3 medium). RXL induced a significant further increase of ROS production in CD40-stimulated CLL cells but not in unstimulated CLL cells. This was already apparent after 1 hour of RXL, and maintained after 4 and 8 hours (Figure 6B left panel). This was paralleled by the percentage of dead cells (Figure 6B right panel). Figure 6C shows the fold increase in ROS production in 5 individual patients after 1 hour RXL. As shown in Figure 6D this increase is blocked by EGTA indicating dependence on extracellular calcium.

CD40 stimulation sensitizes CLL cells to rituximab-induced cell death

Figure 6 Involvement of ROS in rituximab-mediated cell death of CD40-stimulated CLL cells.

A. Representative DCF-1 FACS plots of unstimulated (3T3 med) and CD40-stimulated (3T40Lmed) CLL cells treated with RXL for 1 hour. Left: FSC SSC plots with ungated debris. Right: DCF-1 FACS plots with gated DCF-1 positive cells. Histogram shows Geometric Mean (MFI) of DCF-1 positive cells (thin grey line 3T3 medium, bold grey line 3T3 RXL, thin black line 3T40 medium, bold black line 3T40 RXL).

B. Left panel: ROS production after 1, 4 and 8 hours of RXL in unstimulated (3T3) and CD40-stimulated (3T40L) CLL cells. Data are presented as Geometric Mean (MFI) of DCF-1 positive cells gated as described above (mean ± SEM from 5 independent experiments). Right panel: percentage cell death (MitoT- cells) in unstimulated (3T3) and CD40-stimulated (3T40L) CLL cells after 1, 4 and 8 hours of RXL.

C. ROS production in CD40-stimulated CLL cells incubated with rituximab and XL antibody for 1 hour. Data are presented as fold increase of DCF-1 where the DCF-1 signal in medium is set as 1 (mean ± SEM from 5 independent experiments. Mean 1.23 ± 0.152 p=0.0277).

D. ROS production in CD40-stimulated (3T40L) CLL cells in the presence or absence of NAC, EGTA and RXL. Shown is one representative experiment. Data are presented as mean fluorescent intensity of DCF-1.

E. Averaged results from 8 independent experiments. N-acetylcysteine (NAC) significantly blocked rituximab-induced cell death of CD40-stimulated CLL cells. (p=0.03). Data are presented as percentage relative cell death (mean ± SEM).

.01<p=.05 *, .001<p<.01 **, p<.001 ***
resulting cell death are dependent on extracellular calcium. Recent studies from our group show synergistic effects of the ROS inducing drug cisplatin and fludarabine as to cell death induction of CLL cells. In line with this RXL also sensitized to fludarabine induced cell death in CD40 stimulated CLL cells (Figure 7). Moreover, in contrast to fludarabin, cisplatin was able to induce cell death in CD40 stimulated cells (Figure 7) and combination treatment of RXL and cisplatin induced strong cell death (Figure 7).

Discussion
The major findings of our study can be summarized as follows: 1) whereas CD40 stimulation renders CLL cells resistant to various cytotoxic drugs, it sensitizes CLL cells to anti-CD20 mediated cell death, also in p53 dysfunctional CLL cells. 2) This increased sensitivity can not be explained by a change in CD20 expression levels and appears not to be a feature of all monoclonal antibody-mediated cell death since it is not observed with anti-CD52 treatment. 3) Rituximab-mediated death in CD40-stimulated CLL cells is caspase-independent, shows rapid kinetics (within hours) and is dependent on extracellular Ca^{2+} and the production of reactive oxygen species (ROS). 4) In CD40-stimulated CLL cells, RXL induces strong homotypic aggregation.

There is controversy concerning the possible mechanisms of anti-CD20-induced cell death. Although some reports indicated that cell death induced by CD20 crosslinking has features of classical apoptosis, others were unable to confirm these findings. A number of findings in our study strongly suggest that rituximab-induced cell death is a form of non-classical apoptosis. First, although 3 classical hallmarks of apoptosis were observed (Annexin V exposure, caspase 3 activation and DNA fragmentation), broad spectrum caspase inhibitors did not block these features or cell death. Theoretically, this lack of inhibition by
pan-caspase inhibitors might be due to inactivation of Z-VAD or Q-VD by the ROS induced by rituximab. However, incubation of pan-caspase inhibitors with oxygen radicals had no effect on the activity of these compounds (data not shown). Also, the observed caspase 3 cleavage which could not be blocked by caspase inhibitors might be explained as a bystander phenomenon, rather than an as initiator of cell death. This has recently also been described in the caspase independent cell death of glioblastoma cell lines incubated with taurolidine. Moreover, ongoing processing of caspase 3 in the presence of Z-VAD has been described before in B cells after BCR triggering and interestingly also after treatment with the ROS inducing agent CCCP.

Secondly, the rapid kinetics of rituximab-induced cell death in CLL cells (within hours) are certainly not characteristic for classical apoptosis. These rapid kinetics make it unlikely that rituximab-mediated death in CLL cells is at the level of transcriptional or translational regulation. This notion is supported by multiplex mRNA analysis: In contrast to CLL cells treated with fludarabine that showed a significant upregulation of p53 induced apoptosis-regulating genes p21 and Puma, no evidence for transcriptional regulation of apoptosis-regulating genes by rituximab was observed (Supplemental Figure 4). Furthermore, CD20 binding in CLL cells did not have an effect on Raf-1 kinase inhibitor protein (RKIP) levels (data not shown), previously reported to be upregulated in NHL B-cell lines after anti-CD20 treatment.

Thirdly, our EM data in rituximab-treated CD40-stimulated CLL cells did not reveal classical signs of apoptosis but – in agreement with a recent report – rather gross vacuolization of cytoplasm with relatively intact nuclei. In contrast to recent studies describing the involvement of autophagy in anti-CD20 mediated death of Burkitt lymphoma cell lines, we found no evidence for RXL induced autophagy in CD40-stimulated CLL cells (data not shown).

CD20 has been described as Ca\textsuperscript{2+}-channel. Type I anti-CD20 antibodies are thought to induce direct cell death by inducing a Ca\textsuperscript{2+} flux (reviewed in). Indeed, upon RXL CLL cells showed a rapid Ca\textsuperscript{2+} flux preceding cell death. Rituximab-induced cell death could be blocked by EGTA but not by BAPTA-AM, in agreement with recent data showing that extracellular calcium plays a pivotal role in anti-CD20-induced cell death. At present it is unclear whether it is the CD20 molecule itself that allows Ca\textsuperscript{2+} entry or another anion channel that opens upon CD20 binding.

Intracellular Ca\textsuperscript{2+} can directly enter mitochondria via the Ca\textsuperscript{2+} uniporter. High [Ca\textsuperscript{2+}]\textsubscript{m} results in the formation of ROS. We therefore hypothesized that a rise in [Ca\textsuperscript{2+}]\textsubscript{i} would induce production of ROS in CLL cells after ligation of CD20. Indeed, ROS were increased in CD40-stimulated CLL cells after RXL, whereas this was not observed in unstimulated CLL cells. Moreover, rituximab-induced ROS production and cell death were inhibited in the presence of the ROS scavenger NAC. Recently, the involvement of ROS was also described in cell death induced by anti-HLA-DR mAbs. Rituximab-induced ROS production was impaired in the presence of EGTA, supporting our hypothesis that Ca\textsuperscript{2+} is important for rituximab-induced ROS. Zhou et al showed that CLL cells with higher basal ROS are more sensitive to 2-Methoxyestradiol (2-ME), a compound that induces apoptosis via a free radical.
mediated mechanism. Based on our data we propose CD40 stimulation sensitizes CLL cells to rituximab-mediated death by increasing basal ROS production, which is further increased by rituximab. ROS production after CD40 stimulation has been described before in B cells and CD40-induced ROS play critical roles in CD40-mediated B cell regulation e.g. NF-kB signaling. Our data indicate that constitutive CD40 signaling results in activation of CLL cells and increased basal ROS production.

The increased sensitivity of CD40 stimulated CLL cells to the ROS inducing agent cisplatin is in line with CD40 induced ROS dependent sensitization to rituximab. Furthermore, combination treatment of RXL and fludarabine showed synergistic effects in CD40 stimulated CLL cells (Figure 7). We hypothesize that RXL induces ROS and thereby sensitizes to fludarabine induced cell death, correlating with earlier findings that fludarabine shows synergistic effects with cisplatin as ROS inducing agent. Importantly, this increase in ROS production appears to be rituximab specific because cytotoxic drugs like fludarabine and roscovitine did not induce ROS in CD40-stimulated CLL cells (unpublished data). Finally, we showed that CD40 stimulation of CLL cells not only induced sensitization to rituximab- but also to ofatumumab- (another type I anti-CD20 mAb) induced cell death. Whether CD40 stimulation also sensitizes to type II anti-CD20 mAbs is subject of our ongoing research.

In conclusion, this study shows that CD40 stimulation sensitizes CLL cells to rituximab-induced cell death via a Ca²⁺ dependent increase in ROS production. Since CD40 stimulation is considered to be part of the micro-environmental stimuli rendering CLL cells less sensitive to cytostatic drugs, our data provide a strong rationale for adding rituximab to chemotherapy regimens, like for example Fludarabine and Cyclophosphamide (FC). By bypassing the anti-apoptotic machinery, RXL is able to induce caspase-independent cell death in CD40-stimulated CLL cells that are resistant to various drugs as result of upregulation of Bcl-2 family members. Moreover, our findings suggest that it might be very relevant to combine rituximab with other ROS inducing drugs like arsenic trioxide or cisplatin.

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Reference List


84


Supplemental Figure 1 CD40 stimulation sensitizes p53 dysfunctional CLL cells to rituximab-mediated death
CD40-stimulated (3T40L) p53 dysfunctional CLL cells of 2 CLL patients were incubated with rituximab (10 µg/ml) and XL antibody (50 µg/ml) for 24h. After 24h apoptosis was analyzed by MitoTracker staining by flow cytometry. Data are presented as percentage cell death (MitoT- cells).

Supplemental Figure 2 Increased sensitivity to anti-CD20 mediated cell death of CD40-stimulated CLL cells is not caused by an increase in CD20 expression
CD20 expression (Geometric Mean) analyzed after 48 hours stimulation on 3T3 or 3T40L fibroblasts. PBMC of 13 CLL patients were stained with FITC-labeled anti-CD20 and analyzed by flow cytometry. Data are presented as mean fluorescence intensity (MFI) ± SEM.

Supplemental Figure 3 RXL induces a Ca²⁺ flux in CLL cells
Ca²⁺ mobilization was measured as described in materials and methods. Data are displayed as the relative ratio of intensities of Indo fluorescence (Ca-bound Indo violet emission 405nm (FL4)/free Indo blue emission 485 nm (FL5). The upper graph shows a Ca²⁺ flux in CD40-stimulated CLL cells after RXL. Lower graph shows a Ca²⁺ flux in CD40-stimulated CLL cells after stimulation of the BCR.
Supplemental Figure 4 MLPA analysis

Relative expression levels of apoptosis-regulating genes, measured via RT-MLPA (n=4; see methods). Results of 39 individual apoptosis-regulating genes are shown as expression relative to the total signal in the sample. Bars graph represents the mean ± SEM. No differentially expressed genes were observed in CLL cells treated with rituximab (R) or crosslinked rituximab (RXL) after 24 hours. As control, CLL cells were treated with 50 µM fludarabine for 24 hours. Here, a significant upregulation of p53 dependent genes (p21, Puma, Bax) was observed (59). Shown are the results from 4 mutated (upper graph) and 4 unmutated (lower graph) CLL patients. .01<p< .05 *, .001<p<.01 **, p<.001 ***

CD40 stimulation sensitizes CLL cells to rituximab-induced cell death