Microenvironment and anti-CD20 based therapies in CLL
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CD40 stimulation sensitizes CLL cells to lysosomal cell death induction by type II anti-CD20 monoclonal antibody GA101

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Abstract
Sensitivity of CLL cells to anti-CD20 mAbs is low and therefore the efficacy of monotherapy with current anti-CD20 monoclonal antibodies (mAbs) is limited. At present it is not known whether sensitivity of CLL cells to CD20 mAbs is modulated by micro environmental stimuli. Previously we have shown that in vitro CD40 stimulation of peripheral blood derived CLL cells results in resistance to cytotoxic drugs. In this study we show that, in contrast, CD40 stimulation sensitizes CLL cells to the recently described novel type II anti-CD20 mAb GA101. Cell death occurred without crosslinking of GA101 and involved a lysosome-dependent mechanism. Combination of GA101 with various cytotoxic drugs resulted in additive cell death, not only in CD40-stimulated CLL cells, but also in p53 dysfunctional CLL cells. Our findings indicate that GA101 has efficacy against chemoresistant CLL and provide a rationale for combining cytotoxic drugs with anti-CD20 monoclonal antibodies.
Introduction

Although treatment results for Chronic Lymphocytic Leukemia (CLL) have improved considerably over the last decade, a curative drug regimen is still lacking. Similar to other B cell malignancies such as follicular lymphoma and multiple myeloma, in CLL the interaction of the malignant cells with their microenvironment in lymph nodes (LN), spleen and possibly bone marrow, has been shown to play an important role in the biology of the disease. We and others have previously shown that in vitro CD40 stimulation of CLL cells can to a certain extent mimic the LN environment, and results in the induction of resistance of the CLL cells to cytotoxic drugs like fludarabine, chlorambucil, bortezomib and roscovitine. Thus, these microenvironmental niches might be an important localization of minimal residual disease and form the basis for the relapses characterizing this disease. Moreover, upon sequential treatments in up to 50% of the patients selection of p53 dysfunctional clones occurs, also resulting in chemoresistance. Therefore, the urge for new treatments that circumvent microenvironmental chemoresistance and act independently of p53 is high. Such new treatment strategies may include anti-CD20 monoclonal antibody (mAb) containing regimens. However, sensitivity of CLL cells to anti-CD20 monoclonal antibodies (mAbs) in vitro is low and monotherapy with conventional doses of the type I anti-CD20 mAb rituximab (R) has only limited efficacy in CLL. In light of rituximab resistance or unresponsiveness, more potent anti-CD20 mAbs are currently sought. Two types of anti-CD20 mAbs have been described. A prime difference is that in contrast to type I anti-CD20 mAbs, type II mAbs are unable to translocate CD20 into lipid rafts or evoke Ca²⁺ flux. Ofatumumab, a second generation type I anti-CD20 mAb seems promising for the treatment of CLL, although large amounts seem required for administration to patients. GA101 is a novel glycoengineered type II anti-CD20 mAb. When compared to rituximab, GA101 has enhanced direct cell death inducing capacity and improved antibody dependent cellular cytotoxicity (ADCC). GA101 was also more potent than rituximab at equivalent concentration in depleting CLL cells in vitro. Finally, a phase I study with GA101 in heavily pre-treated relapsed/refractory CLL patients showed promising activity when given as single agent. Recent studies showed that type II anti-CD20 mAbs induce homotypic adhesion-related cell death through a lysosome-dependent pathway.

Rather unexpectedly we recently found that, in contrast to its induction of chemoresistance, CD40 stimulation enhanced sensitivity of CLL cells to rituximab via a ROS dependent mechanism. In view of the largely different mechanism of action of type I- vs type II anti-CD20 mAbs, we decided to address the question whether CD40 stimulation also affects sensitivity of CLL cells to GA101 and, if so, by what mechanism.
Methods

Patient samples
Peripheral blood was drawn from CLL patients (diagnosed according to the NCI-WG guidelines). Peripheral blood mononuclear cells (PBMCs) 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 samples contained at least 90% CD5+/CD19+ cells as assessed via flow cytometry. p53 dysfunction of patient samples was assessed with cytogenetics (Del 17p13) in combination with multiplex quantification of p53 target gene induction as described earlier24. The studies were approved by the Ethical Review Board of the Institute and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983.

Antibodies, reagents and western blotting
The anti-CD20 monoclonal antibody (mAb) Rituximab was kindly provided by Roche Nederland BV (Woerden, The Netherlands). The anti-CD20 mAb GA101 was provided by Roche Glycart (Schlieren, Switzerland) and goat anti-human (GAH) Fc-g fragment specific antibody was obtained from Jackson Immunoresearch Europe. The pan-caspase inhibitor Q-VD was purchased by R&D systems (Minneapolis, USA) and Z-VDfmk by Alexis Biochemicals (San Diego, CA, USA). Fludarabine, Chlorambucil, Nutlin, Bendamustine, Concanamycin A, Bafilomycin A, Cytochalasin D, Propidium Iodide (PI) and N-acetylcyesteine were purchased from Sigma Chemical Co (St. Louis, MO, USA). The proteasome inhibitor bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands). MitoTracker orange and Lysotracker Red were purchased from Molecular Probes, Leiden, The Netherlands. FITC-labeled Annexin V was purchased from IQ products BV, Groningen, The Netherlands. Anti-CD2 APC was purchased from Beckton Dickinson (San Jose, CA) (clone SS.2). FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody was from Becton Dickinson, San Jose, CA (catalog #559341). Preparations of cell lysates and western blotting were performed as described previously 25. Blots were probed with rabbit anti-cleaved caspase-3 mAb (catalog # 9664 Cell Signaling), polyclonal rabbit anti-PARP antibody (catalog # 9452 Cell Signaling) or cathepsinB (AF953 R&D systems (Minneapolis, USA) and antiserum to β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) or tubulin (Cell Signaling). Immunoreactive proteins were visualized using IRDye 680 donkey anti-rabbit IgG, IRDye 800 donkey anti-goat IgG or IRDye 800 donkey anti-mouse IgG (Westburg, Leusden, the Netherlands) as secondary antibody. Blots were scanned on the Odyssey imager (LI-COR Biosciences, Lincoln, NE).
MDW933 detection of active glucocerebrosidase (GBA) in CLL cells
GBA from CLL cell extracts were labelled in vitro with green fluorescent MDW933. The lysate (20 µg total protein) was incubated for 30 min at 37°C with the probe dissolved in McIlvaine buffer (150 mM citrate-Na₂HPO₄, pH 5.2, 0.2% (w/v) sodium taurocholate, 0.1% (v/v) Triton X-100). After incubation, samples were resolved by standard conditions on 10% SDS-PAGE. The resulting gels were analysed by fluorescence scanning on a Typhoon Variable Mode Imager (Amersham Biosciences), 600 PMT, medium sensitivity, 200 μm pixel size: MDW933 (488 λ em, 520bp40 λ ex).

In vitro 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. Briefly, 5 x 10⁶ 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 3 days, CLL cells were gently removed from the fibroblast layer and used in further experiments. In analogous experiments CLL cells were co-cultured with autologous T cells activated with 1µg/ml anti-CD3 (clone 1XE, Sanquin, Amsterdam, The Netherlands) and 5 µg/ml anti-CD28 (clone 15E8, Sanquin, Amsterdam, The Netherlands) or soluble anti-CD40 (clone 14G7, Sanquin, Amsterdam, The Netherlands). After 3 days cells were used in further experiments.

Induction and analysis of apoptosis, caspase-3 activation
For apoptosis induction 3T3 or 3T40L stimulated CLL cells (at a concentration of 1.5 x 10⁶/ml) were incubated with the indicated anti-CD20 mAbs (10 µg/ml) for 24h. Crosslinking GAH antibody (indicated as XL) (50 µg/ml) was added 30 minutes after the CD20 mAbs. In combination experiments cells were incubated with GA101 and cytotoxic drugs for 48hrs. Fludarabine was used at a final concentration of 25 and 50 µM, Bortezomib at 15 and 30 nM, Nutlin at 5 and 10 µM, Chlorambucil at 5 and 10 µM and Bendamustine at 50 and 100 µM. The pan-caspase inhibitors Q-VD or Z-VAD-FMK and the ROS scavenger N-acetylcysteine (NAC) were added 30 minutes and cytochalasin D, concanamycin A or Bafilomycin 45 minutes before the indicated 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. In some experiments, data are expressed as specific cell death (due to heterogeneous levels of basal apoptosis), which was defined as: % cell death in stimulated cells - % cell death in medium control.

For analysis of active caspase-3, cells were fixed and permeabilised (eBioscience, San Diego, CA) and subsequently stained with FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (Becton Dickinson, San Jose, CA).
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).

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.

**Light- and confocal microscopy**

HA was assessed with light microscopy. Cells were incubated with the indicated anti-CD20 mAbs in flat bottomed 48 well-plates. 24 hours later, cells were viewed with a Leica DCF 320 inverted microscope (Leica Microsystems CH9435-Heerbrugs) using a x10 or x20 lens. Images were acquired using a Leica DMIL camera type 090-135.002 and were processed with Leica application suite v3.4 software (Leica Microsystems Switzerland, CMS, GmBH).

For confocal microscopy, cells were incubated with 25nM Lysotracker Red for 15 minutes after incubation with GA101 at different time points. Cells were washed with cold PBS and subsequently fixed with 2% paraformaldehyde on ice for 5 minutes. After fixation cells were seeded onto glass object slides for confocal analysis using a Leica TCS SP2 confocal system equipped with an Ar/Kr laser with a 63x objective, and images were taken in glow-over-under mode.

**Lysosomal volume measurement**

To assess lysosomal volume, cells were labeled with 25nM Lysotracker Red at different time points after incubation with GA101. FL-2 fluorescence of Lysotracker labeled cells was measured on a FACS-calibur cytometer (BD biosciences) 1 hour after labeling. Unlabeled cells were used as background control. Number of lysosomes per cell were counted blind by 2 different independent observers.

**Assessment synergistic or additive effects of GA101 with cytotoxic drugs and statistics**

To assess possible synergistic or additive effects, drug interactions were analyzed as described earlier. In short, observed survival corrected for baseline apoptosis of the sample is plotted against expected survival, calculated from the fraction of surviving cells of samples treated with the individual drugs and GA101 (Expected survival = survival drug x survival GA101).

The diagonal line (XY line) represents the situation in which observed survival = predicted survival. Dots beneath this line indicate synergistic interactions (as observed survival <
CD40 stimulation sensitizes CLL cells to GA101-induced lysosomal cell death

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Patient characteristics including sex, age, mutation of IgHV genes, genetic aberrations, p53 function, Rai stage and previous therapy. (F= female, M = male, Mut = mutated IgHV genes, UM = unmutated IgHV genes, Clb = chlorambucil, ClbP = chlorambucil+prednisone, F= fludarabine, FCR= fludarabine, cyclophosphamide, rituximab, Ofa= ofatumumab, P= prednisone, ND = not determined)
expected survival). Dots above the XY line represent additive interactions (observed survival > expected survival, but < survival of most active single drug ($D_{\text{max}}$)). 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 GA101-induced cell death

The induction of cell death by GA101 and GA101 and crosslinker (GXL) was compared with Rituximab plus crosslinker (RXL) in CD40-stimulated (3T40L) CLL cells and unstimulated (3T3) CLL cells of 13 patients. Patient characteristics are listed in Table 1 (ID# 1-13). CD40 stimulation induces resistance to various conventional drugs, such as roscovitine, a cyclin-dependent kinase inhibitor that acts via the Mcl-1/Noxa axis and induces classical caspase dependent apoptosis. In contrast, CD40 stimulation sensitized to anti-CD20-mediated cell death (Figure 1A) both triggered via RXL or GA101, with a maximum at 24 hours at a concentration of 10 µg/ml (data not shown). CLL cells with unmutated and mutated immunoglobulin variable heavy genes (IGHV) had similar sensitivity to anti-CD20 mediated death (Figure 1A, top panels). GA101 induced cell death in the absence of a secondary crosslinking mAb, in contrast to rituximab (Figure 1A, bottom panel). Crosslinking GA101 (GXL) increased cell death.

Figure 1 CD40 stimulation induces drug resistance but sensitizes CLL cells to GA101 induced cell death

A. PBMC of CLL patients were cultured on 3T3 murine fibroblasts or 3T3 expressing human CD40L as described in materials and methods. After 3 days CD40-stimulated (3T40L) and unstimulated (3T3) CLL cells were removed from the fibroblast layer and incubated with the indicated anti-CD20 mAbs (R= Rituximab, G= GA101. Where indicated, crosslinking (XL) GAH antibody (50 µg/ml) was added 30 minutes after the anti-CD20 mAbs (RXL= rituximab crosslinking, GXL= GA101 crosslinking). After 24h apoptosis was analyzed by measuring phosphatidyl serine (PS) and Annexin V exposure by flow cytometry. Top: results from individual patients (mutated: mutated IgHV genes, unmutated: unmutated IgHV genes) Bottom: averaged results from 13 CLL patients (8 mutated and 5 unmutated). Data are presented as percentage specific cell death, whereby background apoptosis in absence of reagents is subtracted from the observed values in each incubation (mean ± SEM). .001<p<.01 **, p<.001 *** Asterisks indicate significant difference from control (3T3).

B. Representative Annexin V/PI FACS plots from unstimulated (3T3) and CD40-stimulated (3T40L) CLL cells incubated 24 h with XL alone, RXL and G respectively.

C. CD40-stimulated CLL cells were incubated 24h with RXL, G and GXL and cell death was analyzed in parallel by Annexin V/PI or mitotracker FACS staining. Stainings gave similar results in percentage of dead cells. Averaged results from 3 independent experiments (n=7 patients) (mean ± SEM). Black bars indicate dead cells as analyzed by Annexin V/PI staining (% dead cells= 100%- AnnV-PI- cells).

D. Percentage subG1 cells in CD40-stimulated CLL cells 24h after incubation with RXL, G and GXL. Averaged results from 5 CLL patients are presented as percentage subG1 cells (mean ± SEM). .01<p<.05 *, .001<p<.01 **, Asterisks indicate significant difference from control (medium).

E. Timecourse of CD40 stimulation (6-24-48 hours) and subsequent incubation with RXL, G, GXL and roscovitine. After 24h apoptosis was analyzed by measuring mitoTracker signal by flow cytometry. Averaged results from 7 CLL patients are presented as percentage specific cell death (mean ± SEM). .01<p<.05 *, .001<p<.01 **.

F. CLL cells stimulated 72 hours with 3T3, 3T40L, CD3/CD28 activated autologous T cells (act T cell) or with soluble anti-CD40 (aCD40) were incubated with RXL, G, GXL and roscovitine. After 24h apoptosis of CLL cells was analyzed by measuring mitoT signal in CD2-minus cells by flow cytometry. Averaged results from 3 CLL patients are presented as percentage specific cell death. .01<p<.05 *, p<.001 ***
Figure 2 GA101 induces non-apoptotic cell death in CD40-stimulated CLL cells

A. Representative FACS plots of intracellular active caspase-3 staining of CD40-stimulated CLL cells incubated with GA101 for 24h. As positive control unstimulated CLL cells incubated with roscovitine are shown. Indicated are low (lo), intermediately (int) and highly (hi) caspase-3 positive cells.

B. Western blot for cleaved PARP and active caspase-3 of cell lysates from CD40-stimulated CLL cells incubated with GA101 for 24 hours. As positive control unstimulated CLL cells treated with roscovitine are shown. Cleaved caspase 3 (CC3) (19 and 17 KD) and caspase-3 induced cleavage fragment of PARP-1 of 89 kDa in the presence or absence of 20 µM of Q-VD are shown. Percentage cell death as assessed with Mitotracker is indicated. Results are representative of 4 experiments.

C. RXL and GA101 induced cell death in CD40-stimulated CLL cells is not blocked by 100 µM of Z-VAD or 20 µM of Q-VD (left). Right: roscovitine induced cell death in unstimulated CLL cells is blocked by Z-VAD or Q-VD (positive control). Data are presented as percentage specific cell death (mean ± SEM from 4 independent experiments). .001<p<.01 **, p<.001 ***. Asterisks indicate significant difference from control (3T3).
death of control 3T3 as well as of CD40-stimulated CLL cells, while XL alone did not induce cell death. Representative Annexin V/PI plots are shown in Figure 1B. Assessment of cell death by evaluation of mitochondrial membrane potential with MitoTracker gave equivalent results as obtained with Annexin V/PI staining (Figure 1C). Furthermore, the induction of a subG1 peak as a result of DNA fragmentation was observed 24 hours after incubation with RXL, G and GXL (Figure 1D). To investigate the kinetics of CD40-induced sensitization to anti-C20 mAbs, cells were stimulated with CD40L for different time points (6-24-48 hours) and subsequently incubated with anti-CD20 mAbs. CD40-induced sensitization to GA101-induced cell death was optimal after 48 hours stimulation with CD40L (Figure 1E). CD40-induced sensitization to GA101 occurred with similar timing as CD40-induced resistance to cytostatic drugs (Figure 1E). Stimulation with CD40L for 72 hours did not further increase sensitivity to CD20 mAbs or drug resistance (data not shown).

A purported source of the CD40L in vivo is T cells residing in the lymph node1,3-5,7,8. Therefore, it was tested whether autologous T cells could provide sufficient CD40 stimulation to enhance GA101-induced cell death. CLL cells were co-cultured with autologous T cells, using the endogenous ratios of CLL versus T cells present in the PBMC fraction. T cells were activated for 3 days via combined anti-CD3/CD28 mAbs which induces expression of CD40L (Supplemental Figure 3), and then the CLL cells were tested for sensitivity to CD20-mediated cell death. Indeed, also activated T cells were able to significantly increase sensitivity to RXL-and GA101-induced cell death (Figure 1F). Of note, the sensitivity towards roscovitine was not affected using activated T cells in this particular set-up, attesting to the fact the types of cell death induced by CD20 stimulation or drugs are distinct. Using only the relative weak stimulus of soluble anti-CD40 (Figure 1F) only showed a slight increase in RXL-induced cell death. Thus, CD40 stimulation of CLL cells via a co-culture system or via endogenous activated T cells sensitized to CD20-mediated cell death. In subsequent experiments GA101 was tested in the absence of a crosslinking mAb, except where indicated.

**GA101 induces cell death in CD40-stimulated CLL cells in a non-apoptotic manner**

After incubation with GA101, CD40-stimulated CLL cells showed processing of the effector caspase-3 as determined by FACS analysis (Figure 2A). Of note, regarding the amount of caspase 3 activation, three categories of cells could be discerned: low, intermediated and highly positive cells (indicated in Figure 2A). Cells treated with GA101 showed increase in the intermediate category, while the positive control for full caspase-3 processing (roscovitine) displayed high staining. PARP is a substrate of caspase-3 and PARP cleavage is a hallmark in classical caspase dependent apoptosis32. On western blot basal levels of cleaved caspase-3 (CC3) and PARP were already observed in CD40-stimulated CLL cells and GA101 induced no further increase (Figure 2B). As positive control, cells were treated with roscovitine. As expected, PARP cleavage and caspase-3 activation by roscovitine were blocked in the presence of 20 µM of the pan-caspase inhibitor Q-VD (Figure 2B). In contrast, no clear correlation
between the amount of caspase-3 activation, PARP cleavage and the percentage of dead cells
was found for GA101. Moreover, although GA101 induced caspase-3 activation (as measured
by intracellular staining), cell death was not blocked in the presence of the pan-caspase
inhibitors Q-VD (20 µM) or Z-VAD (100 µM) (Figure 2C). In addition, using an alternative
measure of viability (MTS assay) for CD40-stimulated CLL cells treated with anti-CD20 mAbs
showed only a partial decrease while control CLL cells treated with roscovitine displayed
a clear reduction in metabolic activity (data not shown). Finally, it should be mentioned
that CD40 stimulation of CLL cells generally does not trigger proliferation either alone33 or
in combination with anti-CD20 mAbs. Altogether, this suggested that cell death by GA101
occurred via a non-classical and/or caspase independent mechanism.

GA101 induces actin polymerization dependent homotypic aggregation and cell death in CD40-stimulated CLL cells

Recent studies show that type II anti-CD20 mAbs induced strong homotypic aggregation
(HA) and subsequent cell death in Raji cells and primary CLL cells21,22. HA requires active
reorganization of the cytoskeleton. It was shown earlier that CD40 stimulation induces
homotypic adhesion in normal B cells34, although for CLL cells this was not confirmed35.
Figure 3 shows mild HA of CLL cells after CD40 stimulation which was strongly increased
by GA101 or GXL. To test whether the process of HA and cell death were functionally linked, cells
were treated with cytochalasin D (cytoD), an agent that inhibits actin polymerization. CytoD
CD40 stimulation sensitizes CLL cells to GA101-induced lysosomal cell death

completely blocked GA101 and RXL induced HA (Figure 3) and cell death in CD40-stimulated CLL cells (Figure 4A), suggesting a clear dependence on actin reorganization.

Figure 4. GA101 induces lysosomal swelling and burst and cell death is blocked with cytochalasin D and concanamycin A
A. Before incubation with RXL, G or GXL, CD40-stimulated (3T40L) CLL cells were pre-treated with 1 µM Cytochalasin D, 100nM Concanamycin or 200nM Bafilomycin. After 24h cell death was analyzed by measuring mitoTracker signal by flow cytometry. Averaged results from 8 CLL patients are presented as percentage cell death (mean ± SEM).
B. Top: Histograms showing Lysotracker signal in CD40-stimulated CLL cells in medium, after 2 and 6 hours incubation with GA101 in the presence (light grey line) and absence (black line) of Concanamycin. Unstained control is shown in shaded grey. In the right upper corner the individual Geomean of lysotracker (LT) of every sample is shown. Bottom: After 1, 2, 4 and 6 hours of incubation with GA101, CD40-stimulated CLL cells were labelled with Lysotracker and Lysotracker signal was analyzed by flow cytometry. .01<p< .05 *, p<.001 ***
CD40 stimulation sensitizes CLL cells to GA101 induced cell death via a lysosome dependent mechanism

Our data suggested non-apoptotic cell death of CD40-stimulated CLL cells by GA101. Lysosomal Membrane Permeabilization (LMP) and subsequent lysosomal cell death resulting from the release of hydrolases into the cytosol is one proposed cell death mechanism induced by type II anti-CD20 mAbs\textsuperscript{21,22}. To test the involvement of lysosomes, CD40-stimulated CLL cells were pre-treated with concanamycin A or Bafilomycin, both agents that inhibit acidification of lysosomes by blocking V-ATPases\textsuperscript{36}. HA was not blocked in the presence of concanamycin A (Figure 3), but GA101-induced cell death was completely blocked in cells pre-treated with concanamycin A or Bafilomycin (Figure 4A). This suggested that the process of HA itself is lysosome-independent, but the subsequent cell death is lysosome dependent. Lysosomal swelling, LMP and burst are characteristics of lysosomal cell death\textsuperscript{37}. Lysosomal volume of cells incubated with GA101 at different time points was assessed using Lysotracker. FACS analysis of CD40-stimulated CLL cells after 2 hours incubation with GA101 showed a peak in Lysotracker signal (Figure 4B), followed by a decrease after 4-6 hours, suggesting an initial increase in volume or numbers of lysosomes after 2 hours followed by LMP and burst after 4-6 hours. This pattern was also reflected by the percentage of dead cells (data not shown) increasing after 4 hours. Furthermore, loss of Lysotracker signal coincided with loss of mitochondrial membrane permeability (data not shown), indicating that the process of lysosomal rupture and cell death are correlated.

Next, Lysotracker-labelled cells incubated with GA101 were visualised by confocal microscopy. As a result of HA, large clusters of cells were observed after incubation with GA101 (Figure 5A, indicated by arrow) and the distribution of lysosomes in individual cells was studied. Strikingly, the distribution and amount of lysosomes was very different between CD40-stimulated and unstimulated CLL cells (Figure 5A+B). CD40-stimulated CLL cells showed increased numbers of bright lysotracker-positive lysosomes, whereas unstimulated CLL cells contained only a small amount of lysosomes in the peri-nuclear region (Figure 5A, medium control). Also, the mean fluorescence intensity of Lysotracker signal in CD40-stimulated cells (3T40L) was 4 times higher than in unstimulated CLL cells (3T3) (Figure 5B). After 2 hours incubation with GA101, CD40-stimulated CLL cells showed both a further increase in Lysotracker signal and in relocalization of lysosomes around the nucleus, that disappeared after 4 hours incubation (Figure 5A), thereby confirming the FACS data (Figure 4B). Importantly, the GA101 induced increase in lysosomal numbers and/or volume and activity was not observed in unstimulated (3T3) CLL cells (Figure 5A). Quantification of the number of lysosomes per cell showed that CD40 stimulation significantly increases the amount of lysosomes per cell (Figure 5B right). GA101 treatment increased the number of lysosomes further (2 hours), whereas a drop in lysosomal number is observed after 6 hours incubation with GA101, suggesting a lysosomal burst. To confirm the observed increase in lysosomal content in CLL cells (patient ID#29-32 Table 1) after CD40 stimulation, levels of the lysosomal hydrolases cathepsin B and active
glucocerebrosidase (GBA), were measured by western blot and by in vitro labeling of active GBA with the recently developed compound MDW93326, respectively. Clearly, an increase in both hydrolases was observed after CD40 stimulation (Figure 5C). These data suggest that by increasing the number and activity of lysosomes in the cell, CD40 stimulation sensitizes CLL cells to lysosomal cell death by GA101. This was confirmed by highest Lysotracker signal after 48 hours of CD40 stimulation (Figure 5D), the time point at which sensitization to anti-CD20 mAbs is at a maximum level (Figure 1E).

Recently, we described that crosslinking of Rituximab (RXL) induces ROS dependent cell death in CD40-stimulated CLL cells. Of note, whereas the ROS scavenger N-acetylcysteine (NAC) significantly inhibited RXL-induced cell death (Figure 5E), no inhibition was observed in GA101-induced cell death in the presence of NAC (Figure 5E), making a role for ROS in GA101-induced cell death less likely. An important question is whether lysosomes are involved in RXL-induced cell death of CD40 stimulated CLL cells. RXL-induced cell death was not blocked in the presence of concanamycin A (Figure 4A). Moreover, Figure 5F shows that in contrast to GA101, RXL induces no increase in Lysotracker signal or relocalization of lysosomes. These data suggest that RXL and GA101 induce cell death in CD40-stimulated CLL cells via a different mechanism.

Finally, the effect of Q-VD, cytochalasin D and concanamycin A on GA101-induced increase in Lysotracker signal in CD40-stimulated CLL cells was tested (Figure 5G). In line with previous results, Q-VD did not block HA or increase in Lysotracker signal (Figure 5G). Interestingly, cytochalasin D blocked HA but did not block the increase in Lysotracker signal. As expected, concanamycin A did not block HA, but blocked the increase in Lysotracker signal (Figure 5G).

Additive cell death induction in drug resistant CLL cells by combination treatment of GA101 and cytotoxic drugs

Next, we tested the effect of a combination treatment of GA101 with cytotoxic drugs in CD40-stimulated CLL cells with mutated (n=7) and unmutated (n=5) IGHV genes and p53 dysfunctional CLL cells (n=3). Patient characteristics are listed in Table 1 (ID # 14-28). The p53-independent proteasome inhibitor Bortezomib (15 and 30 nM) and the following cytotoxic drugs with a p53-dependent mode of action were tested in combination with GA101: Bendamustine (50 and 100 µM), Chlorambucil (5 and 10 µM), Fludarabine (25 and 50 µM) and Nutlin (5 and 10 µM). Bendamustine is a mechlorethamine derivative with alkylating and purine-analog like activity. Chlorambucil is a nitrogen mustard alkylating agent that induces a p53 response after induction of DNA damage. Fludarabine is a purine-analog and triggers apoptosis via a p53 response that induces the expression of Puma. Nutlins are the first highly selective small molecule inhibitors of the p53-MDM2 interaction and are able to induce p53 irrespective of upstream signaling defects. Nutlin-3a has shown promising cytotoxic activity against CLL.

First, sensitivity of unstimulated CLL cells to the different cytotoxic drugs was tested. There was no difference in sensitivity between CLL cells with mutated and unmutated IGHV
Figure 5. CD40 stimulation sensitizes CLL cells to GA101 induced cell death via a lysosome dependent mechanism

A. Confocal microscopy of lysotracker-labelled unstimulated (lower panel) and CD40-stimulated CLL cells (upper panel) after different time points of treatment with GA101. Shown is the distribution of lysosomes in individual cells visualised in glow-over-under (right: glow-over-under color scale; with the highest intensity
genes (Supplemental Figure 1). As expected, p53 dysfunctional CLL cells showed resistance to p53 dependent drugs Chlorambucil, Fludarabine and Nutlin and partial resistance to Bendamustine. However, these cells were sensitive to bortezomib which acts via a p53-independent mechanism (Supplemental Figure 1).

The results from the combination treatment of CD40-stimulated CLL cells with GA101 and cytotoxic drugs are shown in Figure 6A. As expected, CD40-stimulated CLL cells were fully or partially resistant to the cytotoxic drugs alone (left bars in each panel, labeled control). However, cells were sensitive to GA101 and GXL as already shown in Figure 1. When combined with cytotoxic drugs, GA101 and especially GXL induced 80-100% cell death in these drug resistant CLL cells. Importantly, CD40-stimulated p53 dysfunctional CLL cells were
sensitive to GA101 and in combination with cytotoxic drugs apoptosis levels of 80% were observed (Figure 6A lower panel, n=3).
Synergistic or additive effects of GA101 and the cytotoxic drugs (assessed as described in materials and methods) are shown in Table 2 and Supplemental Figure 2. No significant synergistic effects were found.

High concentrations of Chlorambucil and Bendamustine showed statistically significant additive effects in CD40-stimulated CLL cells with mutated and unmutated IGHV genes. At 100 mM, Bendamustine also showed a significant additive effect in CD40-stimulated p53 dysfunctional CLL cells. Nutlin induced significant additive effects in CLL cells with unmutated IGHV genes and in CD40-stimulated p53 dysfunctional CLL cells (Supplemental Figure 2) and for 30 nM Bortezomib additive effects in CD40-stimulated p53 dysfunctional CLL cells were observed. Fludarabine containing regimens have been shown to be effective in CLL11. Furthermore, our recent studies showed synergistic effects of the ROS-inducing drug cisplatin and fludarabine regarding cell death induction of CLL43, and these drugs also enhanced cell death by RXL23. We therefore tested whether low concentrations of cisplatin or fludarabine in combination with GA101 induced cell death in drug resistant CLL cells. Indeed, low concentrations of both cisplatin and fludarabine combined with RXL, but also G and GXL induced strong cell death induction in CD40-stimulated CLL cells (Figure 6B).

### Table 2. Synergistic or additive effects of GA101 and cytotoxic drugs

<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Bortezomib</th>
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Pooled results of 7 mutated (M), 5 unmutated (UM) and 3 p53 dysfunctional (p53d) patient samples treated with GA101 (G) or crosslinked GA101 (GXL) in combination with different cytotoxic drugs. A= additive trend, A*,**,***= statistical significant additive effect. Syn = synergistic trend. 0.1<p<.05 *, .01<p<.05 **, p<.001 ***

Discussion

This study shows for the first time that cell death induced by type II anti-CD20 mAb GA101 is enhanced in primary CD40-stimulated CLL cells. Our main finding is that by increasing lysosomal volume and number, CD40 stimulation sensitizes CLL cells to GA101-induced lysosomal cell death. Hereby we extend recent publications showing type II-induced lysosomal cell death in cell lines21,22, and translate these findings to a physiologically relevant context. In contrast to the type I antibody rituximab, GA101 does not require a secondary crosslinking mAb to induce cell death in vitro. Finally, combination treatment of drug resistant...
CD40-stimulated CLL cells with GA101 and cytotoxic drugs results in additive cell death induction, also in p53 dysfunctional CLL cells. Although the contrasting effects of CD40-induced sensitization to anti-CD20 mAbs and CD40-induced drug resistance occur with similar kinetics, the underlying mechanisms are clearly different. Firstly, CD40-induced sensitization to RXL is based on the formation of ROS whereas CD40-induced sensitization to GA101 is based on increased lysosomal number and volume (this study). Secondly, CD40-induced drug resistance is based on an increase in Bcl-2 family members and decrease in BH3 only proteins resulting in an anti-apoptotic profile. We and others have shown that Bcl-2 members and classical apoptosis involving activated caspase 3 do not play a role in anti-CD20 induced cell death.

To translate the observation that CLL cells cultured on CD40L expressing fibroblasts were more sensitive to anti-CD20 mAbs to a more physiological setting, we also tested CLL cells co-cultured with autologous CD3/CD28 activated T cells. The fraction of T cells in this set-up is far below the CLL cells, and the CD40 stimulation is likely to be less potent than in the co-culture system, but still enhanced sensitivity to anti-CD20 mAbs was detectable. Using only soluble anti-CD40 mAbs did not significantly enhance sensitivity towards anti-CD20 mAbs, probably due to the rather weak CD40 signaling provided. This could also be explained by different epitope binding and the absence of crosslinking in contrast to membrane-bound CD40L in 3T40L cells and activated T cells. Maximal resistance to chemotherapeutic drugs was only observed in CLL cells co-cultured with 3T40L cells. There are several likely explanations for this. First, the mechanism of anti-CD20-induced cell death is clearly different from cell death induced by cytostatic drugs. The latter is classical mitochondrial apoptosis controlled by Bcl-2 members. In addition, induction of full drug resistance in CLL cells requires persistent CD40 and/or NF-κB signaling resulting in strong upregulation of Bcl-2 members Mcl-1, Bcl-XL and Bfl-1. In an experimental setting, such strong signaling can be provided by 3T40L cells that express higher levels of CD40L than activated T cells (Supplemental Figure 3).

As shown previously, homotypic aggregation and cell death induced by GA101 were completely blocked by cytochalasin D. Therefore, the process of HA seems essential for cell death induction, yet the exact mechanism behind GA101-induced HA remains to be determined and is also not clear for other type II mAbs. GA101-induced HA was not dependent on ICAM-1 (data not shown). Increased sensitivity of CD40-stimulated CLL cells to GA101 induced cell death might be linked with increased HA as a result of CD40 stimulation.

Lysosomal cell death has recently been appreciated as actual cell death mechanism, rather than as a downstream event of apoptotic cell death (reviewed in ref. 37). Decreased Lysotracker fluorescence may reflect LMP and/or an increase in lysosomal pH, meaning that this method is not absolutely specific for LMP. However these data, together with complete inhibition of cell death in the presence of concanamycin A or Bafilomycin (Figure 4 A) make lysosomal cell death induced by GA101 in CLL cells likely. Golay et al recently proposed that type II anti-CD20 induced cell death detected by flow cytometry is an artefact of HA. An
argument against this hypothesis is the prominent cell death inhibition by concanamycin A in the presence of HA, as also observed with cathepsin inhibitors in some circumstances. An interesting observation was the increased Lysotracker signal in the absence of HA in CD40-stimulated CLL cells incubated with GA101 plus cytochalasin D. This suggests that the process of actin polymerization, HA and increase in lysosomal number and activity are not functionally linked. We therefore hypothesize that for GA101 induced-LMP and hence cell death, both HA and an increase in lysosomal volume and number are required. Indeed, not for every mAb a direct relationship between HA and cell death is found. For example, the pan-HLA mAb A9-1 induced high levels of HA but relatively low levels of cell death in Raji cells. We found that CD40 stimulation increases the number and activity of lysosomes in CLL cells (as shown by an increase in lysotracker signal, increase in number of lysosomes and increase in lysosomal hydrolases cathepsin B and active GBA), thereby sensitizing them to GA101 induced lysosomal cell death.

In comparison, RXL-induced cell death was not blocked in the presence of concanamycin A (Figure 4A) and induced no increase in lysosomal number and activity (Figure 5F), suggesting a different cell death mechanism. Indeed, we recently showed that RXL induces ROS dependent cell death in CD40 stimulated CLL cells, whereas this was not observed with GA101 (Figure 5E). Thus, the basis for the death-inducing capacity of type I CD20 mAb Rituximab versus GA101 seems to be quite different. Table 3 summarizes the differences between Rituximab and GA101-induced cell death in CD40 stimulated CLL cells. GA101 induces strong HA and lysosomal dependent cell death, whereas RXL induces Ca\(^{2+}\) - and ROS-dependent cell death. Both CD20 mAbs induce caspase activation as bystander phenomenon of a different cell death mechanism. Cell death can be observed with AnnexinV/PI staining as well as with MitoT staining and DNA fragmentation.

In Figure 7 we summarise our findings for GA101-induced lysosomal cell death in CD40 stimulated CLL cells, with inclusion of the indirect caspase activation. Anti-CD20 induced cell death is generally believed to be caspase-independent, however some reports show that caspase activation plays a role. In the present study, GA101 induced incomplete caspase-3 activation as measured by FACS, and by Western blot no full processing of caspase-3 was found. Furthermore, no correlation between the amount of caspase activation and the percentage of dead cells was found. The results suggest at best a partial processing of caspase-3 during anti-CD20 induced cell death. Finally, GA101 induced cell death was not blocked by pan-caspase inhibitors, suggesting participation of caspase-independent, non-classical apoptosis. Cleavage of caspases has also been described in lysosomal cell death. Release of lysosomal proteases into the cytosol are directly involved in the cleavage of pro-apoptotic substrates and/or activation of caspases. This latter process is usually mediated indirectly, through a cascade in which LMP causes the proteolytic activation of Bid (which is cleaved by the two lysosomal cathepsins B and D), which then induces MOMP, resulting in cytochrome c release and apoptosome-dependent caspase activation (reviewed in ref. 45). These sequence of events is likely to occur in CD40-stimulated CLL cells after incubation with GA101 and could
therefore be the result of lysosomal cell death and not reflect classical apoptosis. Of note, the identity of the responsible lysosomal hydrolase(s) is not certain. We applied various inhibitors of cathepsins but were unable to prevent GA101-induced cell death in CLL cells (data not shown). We tested the effect of GA101 in combination with cytotoxic drugs in CD40-stimulated drug resistant CLL cells with mutated and unmutated IGHV genes and in p53 dysfunctional CLL cells. As expected, p53 dysfunctional CLL cells were insensitive to chlorambucil, fludarabine and nutlin which all have a p53-dependent mode of action. However, these cells were partially sensitive to Bendamustine (Supplemental Figure 1). The mechanism of action of Bendamustin has been principally linked to p53 stabilization, however a recent report showed that bendamustine cytotoxicity in CLL cells was p53 independent and mediated by ROS generation and triggering of the intrinsic apoptotic pathway\textsuperscript{53,54} and thus fits well with our data. We observed 80-100\% cell death induction in drug resistant CLL cells by combining GA101 with cytotoxic drugs, also in patients with p53-dysfunctional CLL cells. Especially this latter finding is of importance, since these patients do not respond to conventional therapy and tend to have a rapidly progressive disease\textsuperscript{55}. Moreover, the frequency of p53 dysfunction

**Table 3. Summary of characteristics of RXL and GA101 induced cell death in CD40-stimulated CLL cells**

<table>
<thead>
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</table>

HA=homotypic aggregation, LMP = lysosomal membrane permeabilization, ROS= reactive oxygen species, mitoT = mitotracker, RXL = rituximab + crosslinking. + = present. ++ = clearly present, - = absent

**Figure 7 Model for GA101 induced cell death in CD40 stimulated CLL cells**

GA101 induces homotypic aggregation (HA) which can be blocked by CytoD. CD40 stimulation increases the number of lysosomes, which are susceptible to swelling and burst via GA101 engagement. Both GA101 induced HA and increase in lysosomal number are required to result in Lysosomal Membrane Permeabilisation (LMP) leading to cell death. An increase in lysosomal volume and LMP can be inhibited by concA. Release of cathepsins into the cytosol after LMP leads to caspase activation. Caspase activation, but not cell death is inhibited by Z-VAD and Q-VD.
increases to nearly 50% as the disease progresses and following therapy\textsuperscript{9,10}. We have previously shown that also RXL induces cell death in p53-dysfunctional CLL cells\textsuperscript{23}. Combining RXL, G or GXL with cisplatin or fludarabine results in strong cell death induction already at low concentrations of cytostatic drugs (Figure 7B)\textsuperscript{23}. Altogether, this study provides a rationale for combining cytotoxic drugs with type II anti-CD20 mAbs for the treatment of CLL.

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**Author contributions**

M.Jak designed the research, performed experiments, analyzed the data and wrote the paper, G.G.W van Bochove and W.W. Kallemeijn, J.M. Tromp performed experiments and analyzed data. E. Reits designed and helped with the confocal experiments, C. Klein and P. Umana contributed the new mAb GA101 and designed research, R.A.W. van Lier, M.H.J. van Oers designed research and analyzed data. E. Eldering designed research, analyzed data and wrote the paper.

**Conflict of interest disclosure**

C. Klein and P. Umana has relevant financial relationship(s) to disclose. Name of Organization: Roche. Type of relationship: Employment, Equity Ownership and Patents & Royalties. All other authors have no conflict of interest to disclose.
References


CD40 stimulation sensitizes CLL cells to GA101-induced lysosomal cell death


Supplemental Figure 1 Jak et al.

Supplemental Figure 1
Unstimulated CLL cells from 7 mutated, 5 unmutated and 3 p53 dysfunctional patients were treated with different concentrations bortezomib, nutlin, fludarabine, chlorambucil or bendamustine. After 48 hours cell death was analyzed by measuring mitoTracker signal by flow cytometry. Averaged results are presented as percentage cell death (mean ± SEM). Bor=bortezomib, Nut=nutlin, Fluda=fludarabine, Chlor=chlorambucil, Ben=bendamustine.
Supplemental Figure 2 Jak et al.

Synergistic or additive effects of GA101 and the highest concentration of cytotoxic drugs were assessed as described in materials and methods. The diagonal line (XY line) represents the situation in which observed survival = predicted survival. Dots beneath this line indicate synergistic interactions and dots above the XY line represent additive interactions.  

M=mutated, UM=ummutated, p53 d=p53 dysfunctional
Supplemental Figure 3 Jak et al.

Supplemental Figure 3
Left: CD40L expression on resting (grey) and activated T cells (black) Right: CD40L expression on 3T3 (grey) and 3T40L cells (black)