# CD40 triggering enhances fludarabine-induced apoptosis of chronic lymphocytic leukemia B-cells through autocrine release of tumor necrosis factor-α and interferon-γ and tumor necrosis factor receptor-I-II upregulation

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*Background and Objectives*. In chronic lymphocytic leukemia (CLL) B-cells are refractory to activation signals and to apoptosis. CD40 triggering, however, rescues CLL B-cells from their anergic state and upregulates the FAS receptor. We therefore studied whether CD40 triggering enhances CLL B-cell sensitivity to fludarabine, and receptors or cytokines potentially involved in apoptosis.

*Design and Methods*. CD40-activation of CLL B-cells was carried out by co-culture with CD40L-transfected cells. After fludarabine treatment, apoptosis was evaluated by propidium iodide (PI), annexin-V/PI or DiOC6 staining and flow cytometry analysis. Modulation of Bcl-2, of tumor necrosis factor receptor (TNFRI/II) and release of tumor necrosis factor (TNF)α/interferon (IFN)γ was also analyzed. Furthermore, addition of caspase-inhibitors or anti-TNFα/-IFNγ monoclonal antibodies to fludarabine-treated cells allowed us to determine the mediators of apoptosis. Student's t tests or ANOVA variance statistical analysis were performed to evaluate whether any differences observed might be considered significant.

*Results*. CD40 triggering enhanced fludarabine sensitivity of CLL B-cells, downmodulated Bcl-2 and upregulated TNFRI/II. Caspases 1 and 6 were the major caspases involved in fludarabine apoptosis induction in resting B cells, while only anti-TNFα/-IFNγ monoclonal antibodies reduced apoptosis in activated cells. In agreement with this observation, autocrine production of TNFα and IFNγ by CD40-activated CLL B cells was found.

*Interpretation and Conclusions*. B-cells from a considerable proportion of CLL cases studied (11/20) are more prone to fludarabine-induced apoptosis after CD40 triggering; accordingly Bcl-2 expression was lower in activated cells. Moreover, upregulation of TNFRI/II, release of TNFα and IFNγ, and inhibition of apoptosis by anti-TNFα/-IFNγ monoclonal antibodies in CD40-activated cells strongly suggest that these cytokines may play a role in sensitizing B-cells to fludarabine treatment.

Key words: B-CLL, CD40-triggering, fludarabine, apoptosis.

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Chronic lymphocytic leukemia (CLL) is characterized by the abnormal expansion of CD5+ mature B-lymphocytes.[1] In CLL the accumulation of monoclonal mature B-cells results from an extended lifespan of B-cells, their increased capacity to proliferate, and/or defective apoptosis.[2] Apoptosis is a well characterized mode of cell death which occurs throughout normal growth and development, and many factors function in a highly complex controlled process leading to cell death. In CLL, different factors may contribute to enhanced survival and dysregulated apoptosis of leukemic cells, such as Bcl-2 overexpression,[3,4] or increased production of anti-apoptotic cytokines (e.g. interleukin (IL)-4 and interferon (IFN)γ[5,6] by residual T-cells.[7] In addition, the CD95 (FAS) antigen, a 45-kDa membrane protein belonging to the tumor necrosis factor receptor (TNFR) superfamily,[8–10] principally involved in inducing cellular apoptosis, is not expressed on leukemic CLL cells under resting conditions,[11] suggesting that reduced FAS pathway utilization could contribute to the relentless accumulation of malignant cells.

Another member of the TNFR superfamily, the CD40 molecule, expressed on virtually all B-cells, plays a major role in regulating B-cell activation and differentiation.[12,13] Activated CD40L+ T-cells stimulate CD40-bearing B-cells to proliferate, differentiate and express other accessory surface molecules that are important in cognate co-stimulatory cell-cell interactions.[14] Defective CD40L (CD154) expression can result in severe immunodeficiency. In fact, individuals with a genetic defect (or defects) in the X-linked gene encoding CD154 have recurrent bacterial infections secondary to immunodeficiency characterized by an inability to generate antigen-specific secondary antibody responses.[15–18] Similar defects are often also noted in CLL and the reported defective expression of the CD40L[19] and of the CD28 antigen on residual T-cells,[7] as well as the weak expression of CD80/CD86 on B-cells,[20] may account for an impairment in normal co-operative T-B interactions and immunodeficiency in this disease. It has, however, been shown that co-culture between CLL B-cells (CD40+) and CD40L-transfected cells increases surface levels of CD95,[21–23] CD80 and CD86,[24] and that these stimulated CLL cells may activate allogeneic CD4+ and CD8+ cytotoxic T-cells (CTL).[25] Furthermore, CLL B-cells transduced with a replication-defective adenovirus vector (Ad-CD154) to express high titers of CD40L become effective *in vitro* stimulators of autologous T-cells;[22] a phase I study based on this strategy has been designed for CLL patients.[26] CD40-activated CLL B-cells thus become

more susceptible to cytotoxic T-cell mediated immune recognition, but it is still uncertain whether CD40 triggering may impair the B-cells' response to drug therapy. The CD40 molecule, following interaction with its ligand, may behave as a positive or a negative regulator of neoplastic cell growth: it induces apoptosis of non-Hodgkin's lymphoma,[27,28] but it can also promote survival of both normal and neoplastic B-cells.[29-32]

The aim of the present study was to clarify whether CD40-preactivation of CLL B-cells could be helpful in sensitizing these cells to apoptosis induced by fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A). We also investigated how fludarabine induces apoptotic pathways of resting and activated CLL B-cells.

## Design and Methods

### Fludarabine

Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A) was obtained from Schering (Berlin, Germany).

### Patients' specimens

Twenty patients fulfilling the clinical, morphologic and immunophenotypic criteria of CLL were selected at the Hematology Department, University of Genoa and blood samples were obtained after informed consent. Seven of the patients were males and 13 were females, with a mean age of 71 years (range 55-88). According to Rai's staging system,[33] 6 patients were in stage 0, 10 in stage 1, 3 in stage 2 and 1 in stage 3. The median white blood count was $30\times10^9$/L (range: 7-$300\times10^9$/L), with a median lymphocyte count of $21\times10^9$/L (range: 5-$216\times10^9$/L). At the time of the study, 14 patients had never been treated, while the remaining 6 had received only chlorambucil therapy and had been off-treatment for at least three months.

### Enrichment of B-lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation by lymphocyte separation medium (ICN, Costa Mesa, CA, USA), washed twice with RPMI 1640 (Biowhittaker Europe, Verviers, Belgium) and resuspended in the same medium containing 10% fetal calf serum (FCS) (Euroclone, UK), L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel) and penicillin-streptomycin (Mascia-Brunelli, Milano, Italy). B-cells were purified by negative selection with antibody-coated magnetic beads, when residual non-B cells exceeded 10%. Briefly, after 1 hour (h) of incubation at 37°C on plastic to remove monocytes, non-adherent cells were incubated for 30 min at 4°C with magnetic polystyrene anti-CD2 immunobeads (Dynal, Oslo, Norway) at a bead/cell ratio of 4:1 in phosphate-buffered saline (PBS) (Euroclone) with 5% FCS. Rosette-forming $CD2^+$ cells (T- and NK-cells) were removed using a cobalt-samarium magnet and the purity of B-cells obtained was checked by staining with fluorescein isothiocyanate (FITC)-conjugated CD19, CD2 and CD3 monoclonal antibodies (moAbs) (Coulter-Immunotech, Miami, FL, USA) and flow cytofluorimetric analysis (EPICS XL, Coulter).

### Immunofluorescence analyses of cell surface antigen expression

Flow cytometry analyses of total CLL cells and of B-cell enriched fractions were performed by two-color immunofluorescence, as previously described.[34] A panel of moAbs was used to study the phenotype of CLL B-cells both at resting conditions and after co-culture with CD40L-transfected cells: FITC-CD19 (Coulter-Immunotech), -CD20, -CD22 (Becton Dickinson, San José, CA, USA), -CD40, -CD70, -CD95 (Pharmingen, San Diego, CA, USA), moAbs and phycoerythrin (PE)-conjugated -CD19, (Coulter-Immunotech), -CD27 (Pharmingen), -CD5, -CD23, -CD120a, -CD120b (anti-TNFRI and anti-TNFRII receptors) (Caltag, Burlingame, CA, USA) moAbs. FITC and PE goat anti-mouse IgG1, IgG2a, IgG2b (Southern Biotechnology, Birmingham, AL, USA) were also included as negative controls. Briefly, $1\times10^5$ cells were incubated with FITC- or PE-moAbs for 30 min at 4°C. After two washes in PBS with 2% FCS, cells were resuspended in washing solution and analyzed by flow cytometry.

### Cell culture and apoptosis assays

Fresh, purified CLL B-cells were cultured in RPMI 1640 with 10% FCS and antibiotics in 24-well plates at a concentration of $1\times10^6$ cells/mL. Fludarabine (40 μg/mL) was added at the beginning of the cultures. After 72h of culture, cells were recovered, washed, treated with Triton (0.1% in PBS) (Sigma Chemicals, St Louis, MO, USA) and stained with DNA prepstain (containing RNAse and propidium iodide) (Coulter) to analyze cell cycle and to determine the percentage of apoptotic cells. Analysis was performed with dedicated software of the flow cytometer (EPICS XL, Coulter). Early apoptosis induction was also determined by annexin-V-FITC and propidium iodide (PI) (MBL, Nakaku, Nagoya, Japan) double staining, following the manufacturers' instructions. Briefly, $1\times10^6$ cells, fludarabine-treated or untreated, resting or activated, were washed, resuspended in binding buffer 1X and stained with 1 μL of annexin-V-FITC. After 10 min incubation, 1 μL of PI was added and samples were incubated for 5 min. We also evaluated changes in the inner mitochondrial membrane potential ($\Delta\Psi_m$), an event associated with cells undergoing apoptosis, by staining resting and activated B-cells, fludarabine-treated and untreated, with DiOC6 (3,3' dihexylocarbocyanine iodide) (Sig-

**Table 1. Percentage of inhibition of fludarabine-induced apoptosis by caspase inhibitors in CLL B-cells.**

| Case n. | Flu.+ic3 | Flu.+ic8 | Flu.+ic9 | Flu.+ic1 | Flu.+ic6 |
|---|---|---|---|---|---|
| 1 | 20 | 7 | **67** | **87** | **60** |
| 2 | **100** | **100** | 0 | **91** | **41** |
| 3 | 0 | 24 | **100** | **100** | **100** |
| 4 | **100** | **100** | **34** | **100** | **100** |
| 6 | 8 | 16 | **52** | **88** | **72** |
| 7 | 0 | 0 | **81** | **100** | **99** |
| 11 | 0 | **100** | 0 | 0 | **48** |
| 12 | **65** | **40** | 0 | **70** | **75** |
| 13 | **25** | 0 | 0 | **35** | **82** |
| 14 | **35** | **65** | **35** | **57** | **42** |
| 15 | 0 | 0 | 0 | 20 | **46** |
| 16 | 0 | 0 | 23 | **98** | **100** |
| 18 | 0 | 0 | 0 | **58** | **75** |
| 19 | 0 | 0 | 0 | 0 | **100** |

*Apoptosis was evaluated by cytofluorography by staining cells with PI in 14 out of 20 cases studied and values represent the mean of two independent experiments. The mean percentage of fludarabine-induced apoptosis was 55±16 with a control value of 8.1±9.6 (mean±SD). In this table values are expressed as percentage of apoptosis inhibition in comparison with values obtained without inhibitors in fludarabine-treated cells. Values ≥ 25 are considered as statistically significant and indicated in bold. *ic= inhibitor of caspase.*

**Table 2. CD40-activated CLL B-cells.**

| Case n. | Release TNFα/IFNγ | % Apoptosis Inhibition anti-TNFα/anti-IFNγ | %Expression TNFRI /TNFRII |
|---|---|---|---|
| 1 | 13/87 | 66/66 | 36/38 |
| 2 | 32/111 | 51/54 | 44/n.t. |
| 3 | 25/105 | 47/57 | 61/2 |
| 4 | 24/1075 | 50/50 | 28/15 |
| 5 | 35/132 | 42/53 | 16/n.t. |
| 6 | 15/18 | 20/20 | 29/n.t. |
| 7 | 16/0 | 34/34 | 26/40 |
| 8 | 0/202 | 13/12 | 34/76 |
| 9 | n.t./n.t. | 20/25 | 10/36 |
| 10 | 26/50 | 50/48 | n.t./35 |
| 11 | 400/58 | 24/24 | 35/28 |
| 12 | 20/0 | 33/37 | 28/32 |
| 14 | 16/100 | 27/30 | 15/n.t. |
| 16 | n.t./ n.t. | 10/12 | 22/17 |
| 18 | 30/20 | 32/35 | 10/19 |
| 19 | 20/324 | 17/20 | 8/18 |
| 20 | 18/25 | 28/30 | 6/17 |

*Cytokine release was determined by ELISA in supernatants of CLL B-cells (n= 15) cultured with CD40L-transfected cells for 72h; values are expressed in pg/mL. Apoptosis inhibition by anti-TNFα and anti-IFNγ moAbs, expressed as percentage of inhibition in comparison with apoptosis induced by fludarabine without moAbs, was evaluated by cytofluorography after PI staining of CLL B-cells (n=17). Values are representative of two independent experiments. The mean percentage of fludarabine-induced apoptosis was 55±16 with a control value of 8.1±9.6 (mean ± SD). TNFRI and II expression was evaluated in 17 cases and indicated as the percentage of positive cells in immunofluorescence analysis. *nt= not tested.*

ma), a green fluorescent dye. Briefly, 3,3' DiOC6 was added to $1\times10^6$ cells to a final concentration of 40 nmol/L. Cells were then incubated at 37°C for 15 min, washed twice with PBS and immediately analyzed by flow cytometry. Viable cells have a high $\Psi$m and display bright DiOC6 fluorescence, while apoptotic cells display dull DiOC6 fluorescence.

### Co-culture of CLL cells with CD40L-transfected cells

Murine L cells stably transfected with the human CD40L (CD154) molecule[35] (a kind gift from Dr. R. Lemoli, Seragnoli Institute of Hematology and Medical Oncology, University of Bologna, Italy) were cultured in flasks until confluency and used to activate CLL B-cells. The 72h co-cultures of CLL B-cells with CD40L-transfected cells were set up in 24-well plates at a concentration of 1:100 of transfectants and B-cells, respectively. After this incubation time, CLL B-cells were collected, washed, resuspended in culture-medium, counted, checked for viability and stained with specific moAbs to determine the upregulation of antigens such as CD95, CD70, CD120a and CD120b. CD40-activated cells were also re-cultured for a further 72h in medium alone as controls, or with fludarabine (40 μg/mL) in a 24-well plate at a concentration of $1\times10^6$ cells per well. In order to determine apoptosis of preactivated CLL B-cells treated with fludarabine, cells from each well were recovered, washed and stained with PI, annexin-V-FITC/PI or DiOC6, as described above.

### Immunofluorescence analysis of Bcl-2 on resting and on CD40-activated CLL cells

Bcl-2 expression by resting and CD40-activated CLL B-cells was analyzed by flow cytometry, by staining permeabilized cells with FITC-anti-Bcl-2 moAb (Pharmingen). Briefly, resting or activated CLL B-cells were dispensed at a concentration of $5\times10^5$/sample and washed in PBS + 2% FCS. Cells were first stained with PE-CD19, FITC-CD19 or negative controls and incubated at 4°C for 30 min. After two washes with PBS + 2% FCS, cells were fixed and permeabilized with cytofix/cytoperm (Pharmingen) for 20 min at 4°C. Following one wash with Perm/Wash buffer (Pharmingen), cells were stained with FITC-anti-Bcl-2 moAb or a FITC-IgG moAb (Pharmingen) as negative control. After 30 min of incubation, samples were washed with Perm/Wash, resuspended in PBS + 2% FCS and analyzed using a Coulter flow cytometer.

### RNA isolation and first strand cDNA synthesis

Total cellular RNA was isolated from $5\times10^6$ purified B-cells both at resting conditions and after 72h co-culture with CD40L-transfected cells, using

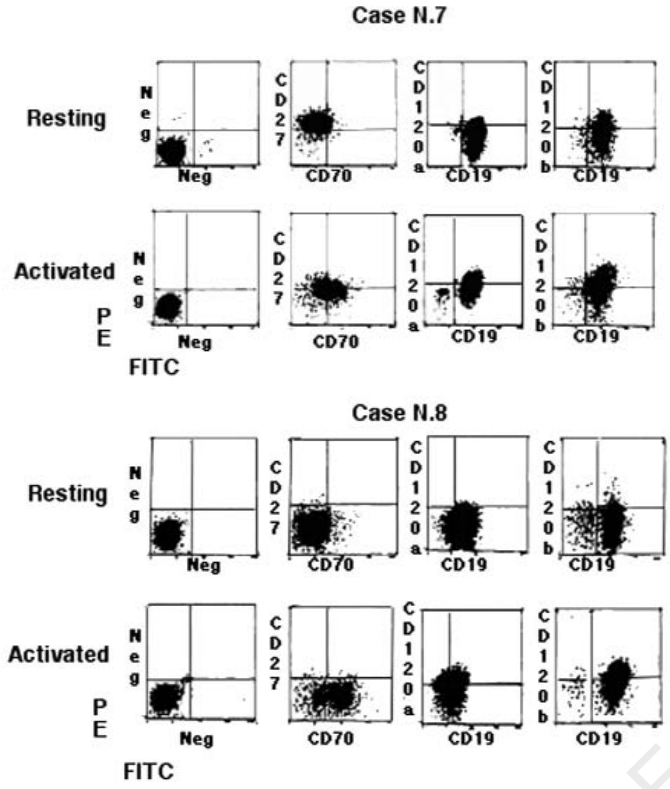


**Figure 1. Flow cytometry analysis of CD120a and CD120b (TNFRI/II) upregulation on purified B-cells from 2 representative CLL cases out of 17 studied following CD40-CD40L interaction. After 72h co-culture between CLL B-cells and CD40L-transfected cells, CD120a and CD120b antigens were strongly upregulated. The CD70 antigen was also highly expressed after CD40 triggering.**

Tripure (Boehringer Mannheim, Germany), according to the manufacturer's instructions. First strand cDNA was synthesized from 2 mg of RNA with the cDNA preamplification system (GIBCO-BRL) using SuperScript II reverse transcriptase (RT) and random hexamers.

### *RT-PCR for TNFα mRNA in CLL before and after CD40 activation*

To evaluate TNFα mRNA expression in CLL before and after CD40 activation, 5μL of cDNAs were amplified using TNF primers (hTNFγ from MBL). PCR reaction samples (50 μL) were obtained according to manufacturer's instructions with 5 μL of first strand cDNA template with the following amplification profile: 96°C 1 min and 59°C for 4 min (2 cycles); 94°C for 1 min and 55°C for 2-5 min (35 cycles); 70°C for 10 min.

In addition, as a positive control we performed GAPDH amplification. GAPDH primer sequences were: 5'-TGAAGGTCGGAGTCAACGGATTTGG-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3'. GAPDH PCR amplification was performed at 95°C for 2 min (1 cycle); 95°C for 30 sec, 72°C for 30 sec and 72°C for 1 min (36 cycles); 72°C for 5 min. PCR products were visualized by gel electrophoresis on a 2.5% agarose gel stained with ethidium bromide.

The gel image was acquired by Gene Snap 4.00.00 and analyzed as described above.

### *Evaluation of caspases mediating fludarabine-induced apoptosis of CLL cells before and after CD40 triggering*

To determine which caspases mediated fludarabine-induced apoptosis of CLL B-cells, $1\times10^5$ cells/well, resting or pre-activated (after 72h of co-culture with CD40L-transfected cells), were cultured with fludarabine (40 μg/mL) or fludarabine and caspase inhibitors-1 (YVAD-FMK), -2 (VDVAD-FMK), -3 (DEVD-FMK), -5 (WEHD-FMK), -6 (VEID-FMK), -8 (IETD-FMK) and -9 (LEHD-FMK) (MBL), in 96-well flat bottom plates for 72h. Fifty micromol of each caspase inhibitor were preincubated with the cells for 2h. In an additional series of experiments, we added 50 μL of anti-TNFα and anti-IFNγ moAb supernatants (a kind gift from Dr. S. Ferrini, National Institute for Cancer Research, Genoa, Italy), in place of the caspase inhibitor. Furthermore in some experiments we added the anti-CD95 blocking moAb ZB4 (MBL) at a concentration of 250 ng/mL to evaluate whether CD95 receptor could be involved in fludarabine-induced apoptosis. After this incubation time, fludarabine or medium alone as control was added in a final volume of 200 μL/well.

### Detection of cytokine production

Supernatants from 72h cultures of purified B-cells and from 72h co-cultures of B and CD40L-transfected cells were tested for TNFα and IFNγ release using an ELISA kit (Endogen Inc. Woburn, MH, USA). Sera of the same patients were also tested in parallel.

### Statistical analysis

Results are reported as the mean of percentage ± standard deviation (SD) of the mean, when not expressly indicated. Two-tailed Student's t tests (paired and unpaired, according to samples) or ANOVA variance analysis were used as appropriate for statistical analysis. Only $p$ values < 0.05 were considered as statistically significant.

## Results

### Upregulation of TNFRI and II (CD120a CD120b) following CD40 triggering of CLL cells

Twenty CLL cases were included in this study and were subjected to a preliminary phenotypic characterization to confirm CD5, CD23 and CD40 expression. In 17/20 cases, we further determined the modulation of TNFRI and II (CD120a and CD120b) on CLL B-cells before and after CD40 triggering. As shown in Figure 1 and Table 2, the two TNF receptors, negative or weakly expressed on resting cells, were strongly upregulated after CD40/CD40L interaction. The mean percentage of TNFRI expression was 7.4±5.71 (mean±SD) at resting conditions, and 25.5±14.79 on CD40-activated cells, while TNFRII expression increased from 9.86±8.49 on resting cells to 28.69±18.12 on CD40-activated cells. In agreement with a previous report,[21] we further confirmed that CD40 triggering strongly upregulated CD70 (Figure 1) and CD95 (FAS) antigens (*data not shown*) on activated cells.

### Effect of CD40 triggering on fludarabine-induced apoptosis in CLL cells

Apoptosis of CLL B-cells exposed *in vitro* to fludarabine was evaluated in 20 patients through PI staining and subsequent flow cytometry analysis. Experiments performed on both resting and activated cells (after CD40 triggering) demonstrated that CD40 preactivation could alter the cells' sensitivity to fludarabine-induced apoptosis. As shown in Figure 2, apoptosis proved enhanced in 11 cases, unchanged in 7, and decreased in 2 (n=20). The mean percentage of apoptosis of CLL B-cells after fludarabine treatment at resting conditions was 36.95±13.39 (vs controls: 10.85±8.14; $p$ <0.0001; n=20), and increased to 57.3±15.55 after CD40 triggering (vs controls: 8.35±9.25; $p$<0.0001; n=20; paired Student's t test). When the two groups of fludarabine-treated cells (activated vs resting) were

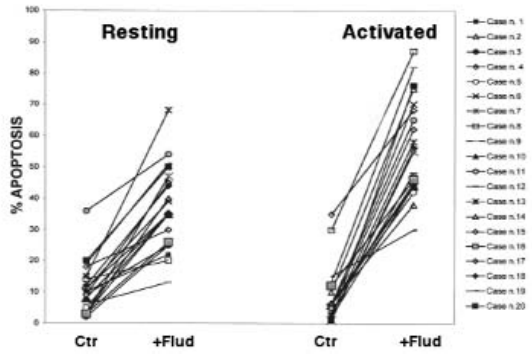


**Figure 2. Fludarabine-induced apoptosis of B-cells from 20 CLL cases before and after CD40 activation. Apoptosis was determined by PI staining and cytofluorimetric analysis. Values indicate the percentage of apoptotic cells at resting and at activated conditions before and after 72h fludarabine treatment and represent mean values of three independent experiments.**

further compared statistically, the overall increase of CLL sensitivity to fludarabine-induced apoptosis, after CD40 triggering, was statistically significant ($p$<0.0007, n=20; paired Student's t test), and even more significant when analyzed in the restricted group of the 11 cases showing clear enhancement of fludarabine-induced apoptosis after CD40 activation (33±13.36 vs 67.64±11.96: resting vs activated fludarabine-treated cells; $p$< 0.0001; n=11 paired Student's t test). In the remaining 7 cases, the mean percentage of fludarabine-induced apoptosis was 40.14±13.72 (vs controls: 6.8±4.7) at resting conditions and 46.86±7.13 (vs controls: 5.7±3.4) in activated cells. Moreover, after CD40 triggering, the apoptosis induced by fludarabine appeared to be more rapid. In fact, as shown in Figure 3 A and D, following 72h of fludarabine treatment and PI staining, we observed that the typical G0/G1 subpeak of apoptotic cells was sharper in activated cells than in resting ones. In agreement with this finding, when apoptosis was determined by annexin-FITC and PI double staining in 10 out of 20 patients, for better discrimination of early and late apoptosis, we demonstrated that, following 72h of fludarabine treatment, the percentage of cells in late apoptosis (annexin+V+/PI+) was higher in CD40-activated cells than in resting ones (Figure 3 B,E). In addition, staining of CLL B-cells with DiOC6, a green cell-permeable fluorochrome that is concentrated in the charged mitochondria of living cells but not in the depolarized mitochondria of apoptotic cells, allowed us to determine that fludarabine induced a drop of membrane mitochondrial potential both in resting and CD40-activated cells (n=10) (Figure 3 C,F). The three different meth-

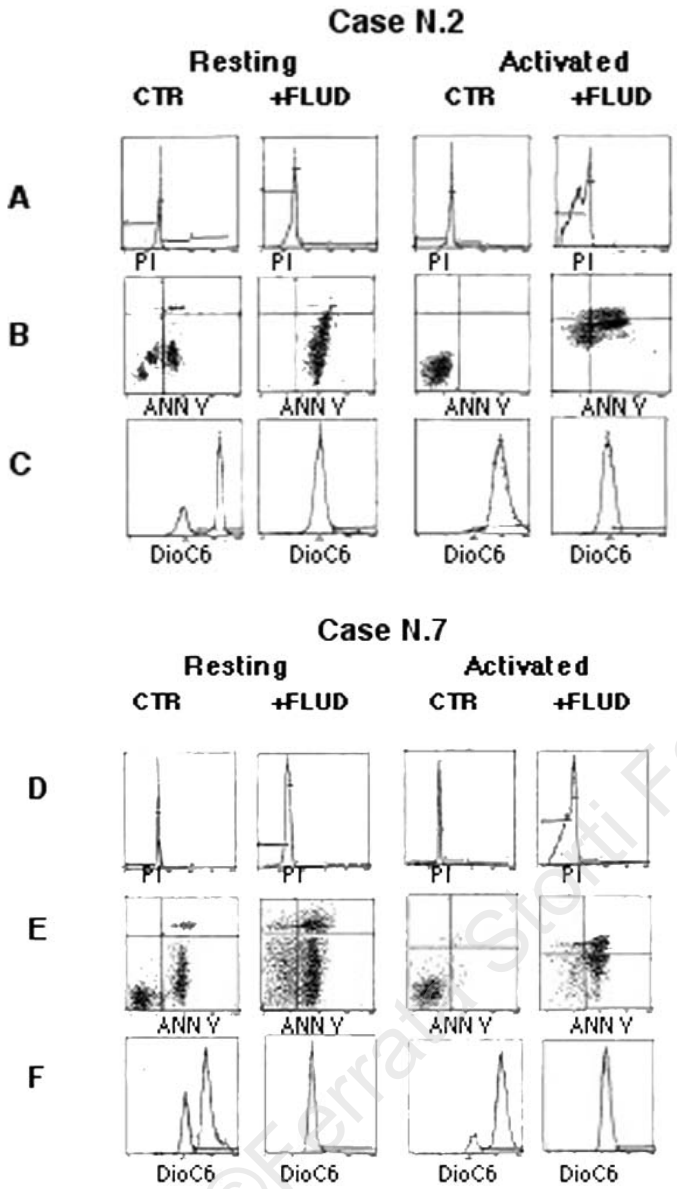


**Figure 3. Fludarabine-induced apoptosis in resting and CD40-activated CLL B-cells evaluated by PI (A,D), annexin-V-FITC/PI (B,E) and DiOC6 (C,F) staining in 2 representative CLL cases (N. 2, 7). Fludarabine-treated cells are indicated as: + Flud and untreated controls as: Ctr in resting and activated B-cells from 2 CLL cases of those studied by three different methods: PI (n=20), annexin-V-FITC/PI (n=10) and DiOC6 (n=10).**



ods employed here to determine apoptosis induction (PI, annexin-V/PI and DiOC6 staining) enabled us to demonstrate that DNA fragmentation, phosphatidyl-serine externalization and mitochondrial depolarization, all typical features of apoptosis, were present in resting, as well as in CD40-activated fludarabine-treated cells.

### *Bcl-2 modulation before and after CD40 triggering of CLL cells*

For many years Bcl-2 has been recognized as one of the major molecules capable of preventing apoptosis in CLL B-cells. By intracytoplasmic staining and subsequent immunofluorescence analysis, we determined Bcl-2 expression on resting and activated CLL B-cells, whether fludarabine-treated or not (n=16). As shown in Figure 4, Bcl-2 expression significantly decreased following CD40-activation and was further downmodulated by fludarabine treatment on both resting and activated cells. Data were statistically analyzed through ANOVA multivariate analysis and the results are summarized in Figure 5. At resting conditions, Bcl-2 was highly expressed, with a mean fluorescence intensity (MFI) value of 9.81±4.23 (mean±SD) that decreased to 6.73±2.31 after CD40-CD40L interaction ($p = 0.0019$). Bcl-2 on resting cells was further downmodulated following fludarabine treatment in resting cells (MFI = 8.1±3.1 versus 4.1±2; $p = 0.0017$) and in activated cells (MFI = 4.8±1.4 versus 2.5±0.5; $p<0.0001$). In addition,

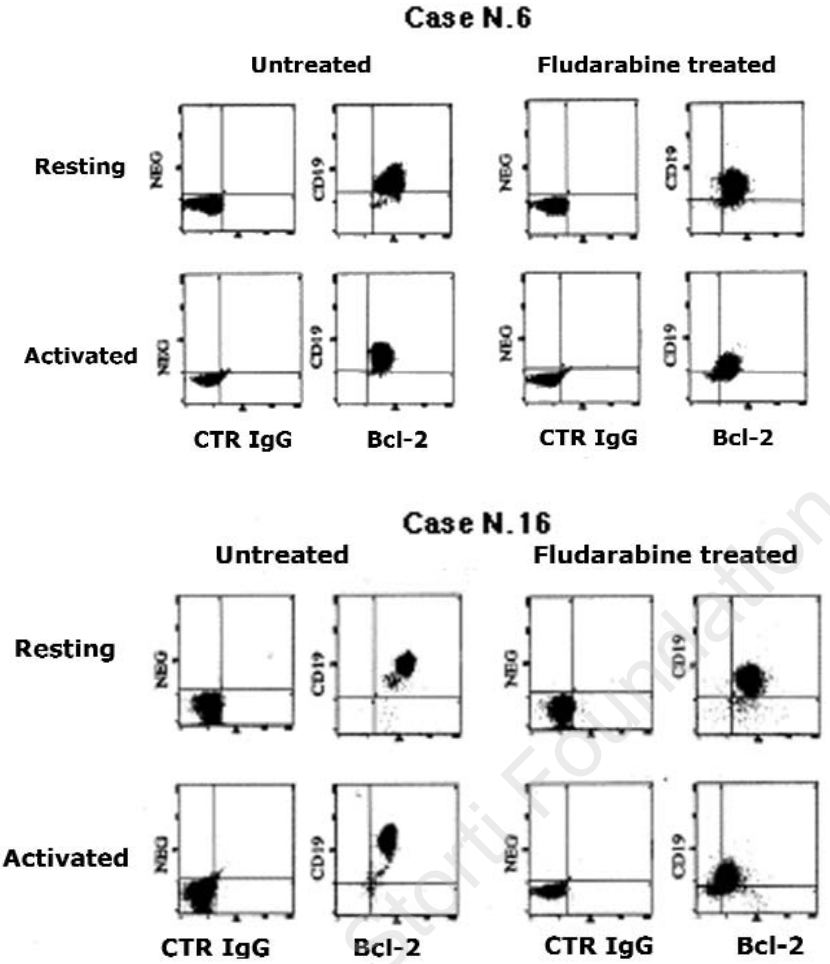


**Figure 4. Bcl-2 downmodulation on CLL B-cells at resting or at activated conditions (following CD40 preactivation) before and after fludarabine treatment. Evaluation of Bcl-2 modulation in 2 representative cases (n. 6 and n. 16) out of the 16 analyzed. Bcl-2 expression was determined by intracytoplasmic staining with anti-Bcl-2 moAb after cell permeabilization. Cells were analyzed after 72h culture in medium alone (untreated) or with fludarabine (treated) either before (resting) and following CD40 triggering (activated).**

Bcl-2 downmodulation proved much more significant in activated cells than in resting ones ($p<0.0001$ and $p=0.0003$ respectively), thus suggesting that a decrease in Bcl-2 expression by CD40-activated cells may parallel their enhanced sensitivity to fludarabine.

### *Caspase inhibitors block fludarabine-induced apoptosis of CLL B-cells at resting conditions but not after CD40 triggering*

The caspases involved in the process of fludarabine-induced apoptosis, before and after CD40 triggering, were investigated by adding inhibitors of caspases 1, 2, 3, 5, 6, 8 and 9 to cells from 14 of the 20 cases studied, treated for 72h *in vitro*. As shown in Table 1 the inhibitor of caspase-6 prevented apoptosis in all the cases tested (n= 14) with a mean percentage inhibition of 74.2±23.4. The inhibitor of caspase-1 blocked apoptosis in 11 out of 14 cases (mean % ± SD= 64.5±37.1). Caspases-9, -3 and -8 appeared to be involved in a smaller number of cases, while inhibitors of caspases -2 and -5 never blocked fludarabine-induced apoptosis (*data not shown*). After CD40 triggering, however, we could no longer prevent fludarabine-induced apoptosis in the presence of the same caspase inhibitors. Fludarabine-induced apoptosis of CD40-stimulated cells was, instead, inhibited by the addition of anti-TNFα and anti-IFNγ moAbs (Table 2). The mean percentages of inhibition were 33.18±15.68 with anti-TNFα moAb and 35.71± 16.39 with the anti-IFNγ moAb (n=17) (Table 2). Moreover, the anti-CD95 blocking moAb ZB4 failed to inhibit fludarabine-induced apoptosis of CD40-activated cells (*data not shown*), suggesting that

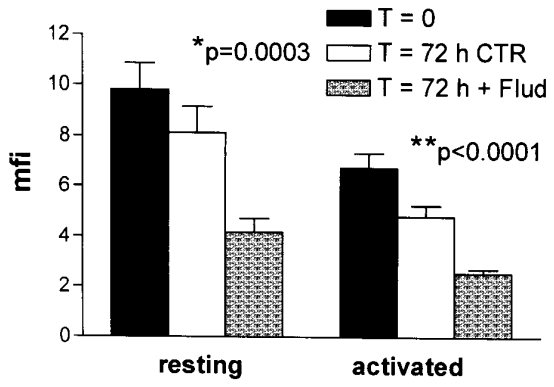


**Figure 5. Bcl-2 downmodulation on CLL B-cells from 16 B-CLL cases following CD40 triggering. The graph represents analyses of Bcl-2 expression as determined by intracytoplasmic staining with anti-Bcl-2 moAb before (resting) and after 72h of co-culture with CD40L-transfected cells (activated). Cells were studied at time 0 (T=0) (fresh cells at resting conditions and immediately after 72h co-culture with CD40L transfectant) and following 72h in culture with medium alone (72h CTR) or with fludarabine (72h + Flud), both for resting cells and after pre-activation. Values are expressed as mean fluorescence intensity (MFI) and error bars indicate the standard deviation about the mean.**

the FAS receptor is not involved in fludarabine-mediated apoptosis.

### *TNFα mRNA expression in CLL cells before and after CD40 triggering*

Based on the evidence that TNFα may contribute to fludarabine-induced apoptosis in CD40-activated CLL cells, we evaluated TNFα mRNA expression in purified B-cells from 5 CLL cases (#4, 6, 8, 9, 16) before and after CD40-activation. As shown in Figure 6, TNFα mRNA (535 bp) was negative or faintly expressed in fresh resting CLL B-cells and became clearly expressed following co-culture with CD40-transfected cells for 72h.

### *TNFα and IFNγ release following CD40 triggering of CLL cells*

To evaluate whether CD40 triggering could modify cytokine production by CLL B-cells we determined the release of TNFα and IFNγ in the supernatants of cultures of purified B-cells from 15 CLL patients activated with CD40L-transfected cells for 72h. As shown in Table 2, in supernatants from 72h cultures of CD40-activated CLL B-cells we detected production of TNFα in 14 (range 13-400 pg/mL) and IFNγ in 13 (range: 18-1,075 pg/mL) of the 15 cases tested. On the other hand, supernatants from fresh purified CLL B-cells cultured for 72h in medium and sera were also tested and found negative in all cases.

## Discussion

The CD40L has been proposed as an immunostimulatory molecule capable of improving host cytolytic T-cell responses to autologous CLL B-cells and a phase I clinical trial of gene therapy utilizing this strategy in CLL patients has given some encouraging preliminary results.[26] In addition, the possibility that CD40-activated cells still could be sensitive, and/or possibly more sensitive to conventional therapy could be relevant.

Taking advantage of an *in vitro* co-culture system between purified B-cells from CLL patients and a CD40L-transfected cell line, we attempted to clarify whether CD40 triggering could sensitize CLL B-cells to fludarabine-induced apoptosis. We here demonstrated that CD40-preactivation of CLL B-cells does not impair but rather enhances CLL sensitivity to fludarabine treatment. The percentage of cells undergoing apoptosis was, in fact, increased in



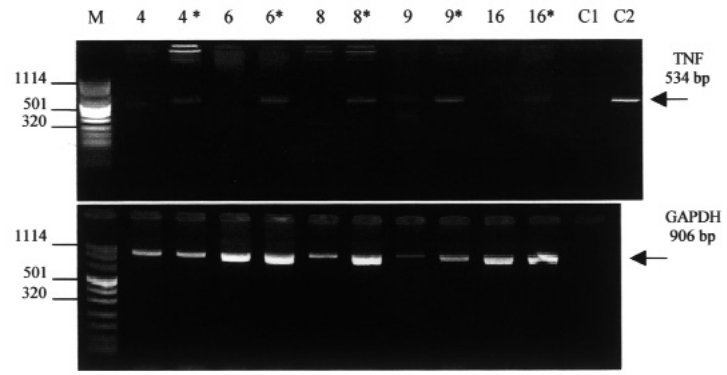


**Figure 6. mRNA expression of TNFα in CLL B-cells before (cases #4, 6, 8, 9, 16) and after CD40 triggering (4*, 6*, 8*, 9*, 16*). mRNA expression was analyzed in purified B-cells from CLL cases before and after 72h co-cultures with CD40L-transfected cells. The TNFα mRNA band (535 bp), faint or negative at resting conditions, was clearly expressed following CD40 triggering. GAPDH mRNA (921 bp) PCR amplification was performed as a positive control. M. DNA molecular weight. C1: blank PCR control. C2: positive control included in the kit.**

11 of the 20 patients studied, while it was unmodified in 7. The different methods employed to analyze cell cycle, early and late apoptosis, and membrane mitochondrial depolarization, all documented a higher sensitivity of CD40-activated cells to fludarabine treatment. Moreover, following CD40 triggering, the kinetics of fludarabine-induced apoptosis appeared to be accelerated, as demonstrated by the sharper subdiploid peak (PI staining) and by the higher percentage of cells already in late apoptosis (annexin-V$^+$/PI$^+$) in fludarabine-treated and activated cells than in cells treated at resting conditions. In addition, the significant Bcl-2 downregulation detected in CLL B-cells following 72h CD40 triggering and, to a further extent, in fludarabine-treated cells both support our findings. In agreement with our observations, Berrebi *et al.*[36] described an increased sensitivity to fludarabine of pokewood mitogen-activated CLL B-cells and Grdisa *et al.*[37] showed that CD40 ligation does not impair fludarabine activity in CLL. However, Romano *et al.*[38] and Kitada *et al.*[21] have reported a decreased CLL fludarabine sensitivity after CD40 activation. These results may appear in contrast with our findings but discrepancies might be related to different experimental conditions employed. In the first work,[38] in fact, CD40 activation of CLL cells was carried out using the agonistic anti-CD40 moAb. We, instead, chose to activate CLL cells through cocultures between CLL cells and CD40L-transfected cells because this method appears to be more efficient in priming CLL cells than that using the antibody alone (anti-CD40) (*personal observations*). In the second study,[21] the authors added fludarabine during the co-culture of CLL cells with CD40L-transfected cells. We, instead, treated previously activated cells with fludarabine in a system free of contaminating CD40L-transfected cells. In our experimental conditions, a preventive CD40 activation reduced spontaneous *in vitro* apoptosis, but also enhanced CLL B-cell fludarabine sensitivity.

We further demonstrated that, at resting conditions, fludarabine apoptosis induction was mediated by caspases-6 and -1. Caspase-6 and caspase-3 are the major pool of caspases activated in various tumor cell lines undergoing apoptosis.[39] The limited involvement of caspases-3 and -8 that we observed in CLL may be related to a possible block of the FAS apoptotic pathway observed by us and others[21] (*and personal observation*). CLL B-cells, unable to activate the FAS-apoptotic pathway, may thus utilize alternative pathways. The finding that, following CD40 triggering, fludarabine-induced apoptosis is no longer blocked by caspases inhibitors, suggests that other and multiple factors may interact to induce death or, on the contrary, survival. Among these factors, cytokines have a prominent role. Interestingly, we found that addition of anti-TNFα or anti-IFNγ moAbs was capable of inhibiting fludarabine-induced apoptosis of CD40-activated cells. In agreement, following CD40 triggering, CLL cells expressed TNFα mRNA and produced TNFα and IFNγ. CD120a and CD120b (TNFRI and TNFRII) were further highly upregulated on CD40-activated CLL B-cells. Trentin *et al.*[40] have previously reported the constitutive expression of p75 (TNFRII) on CLL at resting conditions, suggesting that this receptor has a role in mediating proliferation of malignant cells to autocrine TNFα production. In CLL, residual T-cells may represent an important source of TNFα, and Bojarska-Junes *et al.*[41] recently described that intracytoplasmic TNFα expression is higher in T-cells than in B-cells, and is significantly increased in patients with more advanced disease (stage III-IV). We here observed that leukemic B-cells release TNFα and express high levels of the two TNF receptors (p55/p75) only after CD40 ligation. It has been widely demonstrated that TNFRI is able to mediate apoptosis following association between its cytoplasmic death domain (DD) and the adapter proteins TRADD and FADD.[42] TNFRII, instead, belongs to the subgroup of TNFR molecules, such as CD40 and CD30, that do not contain a death domain and are primarily involved in gene transcription for cell survival, growth and differentiation. However, it now appears clear that these molecules can also induce cell death. A report by Grell *et al.*[43] showed that the cytotoxic effects induced by TNFRII, CD40 and CD30 are mediated by endogenous production of TNFα and autotropic or paratropic activation of TNFRI, and that stimulation of TNFRII and CD40 synergistically enhances TNFRI-induced cytotoxicity. Moreover, Yang *et al.*[44] have recently suggested that both p55 (TNFRI) and p75 (TNFRII) are able to transmit apoptotic signals. It is thus possible that the presence of a complete p55/p75 TNF receptor and significant levels of endogenous TNFα, released following CD40 triggering, may contribute to fludarabine-induced apoptosis in CLL. In contrast to fludarabine, however, in preliminary experiments we found that CD40 triggering increased the resistance of CLL B-cells to dexamethasone, thus suggesting a distinct pharmacological modulation of apoptosis by glucocorticoids (*personal data*). Furthermore, Messmer *et al.*[45] demonstrated that the cytotoxic effect of TNFα on the MCF-7 cell line (breast carcinoma) was due to the downregulation of inhibitor of apoptosis proteins (IAPs) and that this effect was inhibited by dexamethasone treatment. We may thus hypothesize, in agreement with the above reported data, that in our model TNFα released by CD40-activated cells may downregulate IAPs, thus allowing fludarabine- but not dexamethasone-mediated apoptotic effects. In addition, IFNγ may induce susceptibility to TNFα-mediated apoptosis[46] and recently it has been demonstrated that the apoptotic effect of IFNα on hairy cells is mediated by

autocrine TNFα release and downregulation of IAPs.[47] In our system, IFNγ and TNFα were both released by CLL B-cells following CD40 triggering, and may thus have synergistically acted in sensitizing these cells to fludarabine-induced apoptosis.

In conclusion, our data suggest that fludarabine's effect is not impaired in CD40-activated CLL B-cells but indeed may be enhanced through an apoptotic pathway involving TNFα release and related signals. Future investigations will be undertaken to determine whether CD40 engagement could be helpful in overcoming fludarabine resistance in CLL patients and to clarify the role played by cytokines, such as TNFα and IFNγ, and expression of related receptors in sensitizing CLL B-cells to drug-induced apoptosis.

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## Pre-publication Report & Outcomes of Peer Review

*Contributions*

DdT designed the cellular and molecular studies, performed the cellular experiments and flow cytometric analysis and wrote the paper. MPM and MC performed the molecular experiments. PLT contributed to designing the cellular studies and performed the ELISA assays. MC and EB selected the patients, collected the data, performed the statistical analysis and contributed to the interpretation of the results. RF and MG gave substantial contributions to data interpretation and critical review of the draft. All the authors gave final approval for submission of the paper and they are listed according to a criterion of decreasing individual contribution to the work, with exception of the last two authors who had a major role in interpretating the data. We wish to thank Drs T.J. Kipps, W. Wierda and V. Pistoia for their suggestions and critical reading of the manuscript.

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*Disclosures*

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

*Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Dr. Estella Matutes, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Matutes and the Editors. Manuscript received August 22, 2002; accepted December 19, 2002.

In the following paragraphs, Dr. Matutes summarizes the peer-review process and its outcomes.

*What is already known on this topic*

Different factors have been shown to be involved in the dysregulated apoptosis of CLL including BCL-2 overexpression, reduced FAS levels and release of anti-apoptotic molecules by T-cells. CD40 activated CLL cells become more susceptible to cytotoxic T-cells and thus expression of this molecule may be relevant in sensitizing CLL cells to undergo apoptosis.

*What this study adds*

The present study shows that *in vitro* CD40 activation of CLL cells enhances fludarabine-induced apoptosis by downregulating BCL-2, upregulating TNFRI/II and by autocrine production of TFN-α and IFNγ.

*Caveats*

These findings provide further insights into the complexity of factors involved in the impaired apoptosis in CLL cells and suggest that CD40 engagement might potentially be useful to overcome the defective apoptosis of CLL and fludarabine resistance.