Stimulation of Toll-Like Receptor-7 Enhances BAFF and APRIL Pathways of Survival in Chronic Lymphocytic Leukemia Cells

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Abstract: CLL cells are resistant *in vivo* to apoptosis, in part, through the autocrine action of the survival factors BAFF and APRIL. We show here, by flow cytometry, that stimulation of Toll-like receptor-7 (TLR-7) with imidazoquinolines induces increases in the expression by CLL cells of BAFF, APRIL and their receptors, results confirmed by Western blot. Moreover, sBAFF and sAPRIL released by leukemia cells are increased in supernatants from Resiquimod-treated cells. These events correlated with an increased resistance against apoptosis, both APRIL and BAFF contributing to this protection. Ligation of TLR-7 activated the canonical and alternative pathways of NF-κB activation. An inhibitor of IκBα phosphorylation largely prevented these effects. Addition of Resiquimod to CLL cells also elicited the activation of several members of the AP-1 family, notably phosphorylation of c-Jun, and the protection against apoptosis was mostly reverted with a specific inhibitor of JNK.

TLR-7 signaling therefore stimulates apoptosis resistance through activation of the BAFF/APRIL pathways. TLR-7 may be activated *in vivo* by PAMPs (pathogen-associated molecular patterns) of microorganisms and/or antigens expressed by cells undergoing apoptosis, as already reported for stimulation of the BCR in CLL cells. The engagement of TLRs could be involved in the aetiology of CLL.

Keywords: CLL, TLR, BAFF, APRIL, BAFF-R, NF-κB.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in Western countries and still remains an incurable disease, despite recent progress [1]. It affects mostly people over 60 years and consists in the accumulation in the blood and lymphoid organs of a mono- or oligoclonal population of $CD5^+$ B lymphocytes. About 80% of the patients exhibit various cytogenetic alterations, yet CLL cells do not express a typical translocation. A small pool of highly proliferating cells has been identified in the bone marrow and lymph nodes that feed the blood compartment [2]. The latter pool consists in anergic and non-dividing small B-lymphocytes, 95-98% of them being arrested in the G_0/G_1 phase of the cell cycle. Although these cells are highly resistant to apoptosis *in vivo*, they become sensitive when cultured *ex vivo* and die rather rapidly, unless they are incubated in the presence of stromal cells that can rescue them from programmed cell death. This suggests that the microenvironment protect them from apoptosis *in vivo* and therefore CLL is a disease of both proliferation and accumulation. A hallmark of CLL cells resides in their dramatic resistance to both spontaneous and drug-induced apoptosis. Several mechanisms that prevent leukemia cells or rescue them from entering the process of programmed cell death have been identified, yet the early signal conferring apoptosis resistance to the tumor cells are still poorly understood.

However, there is now a growing consensus to incriminate an interaction between CLL precursor cells with self-antigens and/or microbial antigens in the aetiology of this leukemia. Indeed, cells from a significant fraction of CLL patients display strikingly similar BCR, indicating that these BCRs, resembling those of mAb's reactive with carbohydrates of bacterial capsules or viral coats and with certain auto antigens, could bind the same antigenic epitopes. These findings suggested that the B-lymphocytes that gave rise to these leukemia cells were selected for this unique BCR structure [3]. The relatively high frequency in CLL patients (about 20%) of these so-called stereotyped receptors imply that either a significant fraction of CLL cells was selected by a restricted number of antigenic epitopes during their development and/or that they derive from a distinct B cell subpopulation with limited Ig V region diversity [4]. CLL cells very frequently express BCR with a monoclonal immunoglobulin displaying characteristic features of antibodies against self-antigens or against microbial antigens [5].

Recently, several antigens binding to monoclonal Ig from various CLL patients and cell lines have been identified and included molecular motifs exposed on apoptotic cells/blebs and bacteria [6]. Their results showed limited target structure recognition and indicated that CLL B cells derive from a cell compartment that produces "natural antibodies," aimed at the elimination and scavenging of apoptotic cells and pathogenic

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bacteria. Clonal expansion in CLL may thus be stimulated by auto antigens occurring during apoptosis, some of the latter epitopes being similar to those on bacteria and microbes [7]. These data suggest that CLL may derive from normal B cells whose function is to remove cellular debris, and also to provide a first line of defence against pathogens. Moreover, several lines of evidence suggest that the encounter of the leukemia cells (or their precursors) with such antigens might be involved in the oncogenic process and is a driving force for the proliferation and differentiation of the tumor cells [4, 8].

The nature of the antigen(s) binding the BCR of CLL cells therefore strongly suggests that the structures bearing these antigenic motifs may be also recognized by the receptors in charge of innate immunity, i.e. the Toll-like receptors or TLR [9]. Several functional TLR are present at the membrane or in the cytosol of CLL cells [10, 11]. TLR recognize PAMPs (pathogen-associated molecular patterns) and their engagement lead to NF-κB activation [9, 12, 13]. We thus made the hypothesis that the stimulation of TLR by PAMPs/autoantigens displayed by autologous dying cells and/or foreign micro-organisms might contribute, in addition to BCR engagement, to the survival of the leukemic clone. Indeed, we showed recently that addition of Resiquimod, a specific TLR-7 ligand, to CLL cells resulted in a NF-κB dependent up-regulation of the inducible nitric oxide synthase (iNOS) and of NO production [14]. Several years ago, we demonstrated that NO released by an endogenous iNOS contributed to the resistance of the leukemia cells to apoptosis [15], mainly through the inhibitory effect of NO on the active site of caspases [16].

We reported previously that BAFF and APRIL contribute, through paracrine and autocrine pathways, to the enhanced resistance of CLL cells to apoptosis [17]. In the present work, we made the hypothesis that the stimulation of TLR-7 by specific agonists could stimulate the expression of BAFF and APRIL, therefore leading to an enhanced survival of CLL cells resulting from an elevated resistance to apoptosis. Indeed, our experiments indicate that ligation of TLR-7 results in an enhanced expression of BAFF and APRIL and of the specific BAFF receptor, BAFF-R, that parallels an increased survival of the leukemia cells. These data reinforce the importance of the stimulation of TLR by auto antigens and/or micro-organisms-derived antigens in the induction of apoptosis resistance in CLL cells

MATERIALS AND METHODOLOGY

Patients, Cells and Cells Cultures

CLL patients' blood samples were obtained from the Hematology Department of Hôtel-Dieu hospital (Paris, France) after informed consent of the patients, in agreement with the revised Helsinki protocol rules. Diagnosis was established according to standard clinical criteria and to the international workshop on CLL (IWCLL), including lymphocyte morphology and co-expression of CD5, CD20 and CD23 antigens. A total of 19 patients (12 men and 7 women) with a mean age of 67 ± 9 years (range 50-85 years) were tested. The time since diagnosis varied between 0 (newly diagnosed patients) and 9 years. All patients were Binet stage A and were previously untreated. The leukemia B cells were isolated as previously described [15] with purity greater than 96%.

All cell cultures were carried out in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% FCS (PAA Laboratories, Les Mureaux, France) at 37°C in an humidified atmosphere containing 5 % CO_2. Freshly isolated CLL cells were seeded at $2x10^6$/ml. The MEC-1 cell line, established from the peripheral blood of a 61-year-old man with CLL in prolymphocytic transformation [18], was purchased from DSMZ (Braunschweig, Germany). These cells display a similar pattern of TLR expression as leukemia cells freshly isolated from CLL patients [11].

Reagents

The TLR-7 agonists Imiquimod/R-837 [1-(2-Methyl-propyl)-1H-imidazo[4,5-c]quinolin-4-amine] [19] and Resiquimod/R-848 [4-Amino-2-(ethoxymethyl)-a,a-dimethyl-1*H*-imidazo(4,5-c)quinoline-1-ethanol] [20] were obtained from Axxora (San Diego, CA, USA). These reagents were dissolved in DMSO as 1 mM stock solutions and kept at -20°C until use. Wedelolactone (7-Methoxy-5,11,12-trihydroxy-coumestan), a furanocoumarin isolated from *Eclipta prostrata* and a powerful specific inhibitor of the I-κB kinase (IKK) complex [21] was purchased from Biomol (Enzo Life Sciences, Plymouth Meeting, PA, USA). Human BCMA-Fc was a kind gift from Dr A. Tsapis (Créteil). Recombinant human BAFF-R-Fc chimera was purchased from R & D (Abingdon, UK). The potent cell-permeable selective and reversible inhibitor of c-Jun N-terminal kinase, JNK Inhibitor II or Anthra(1,9-cd)pyrazol-6(2H)-one], that blocks the phosphorylation of c-jun [22] was purchased from Calbiochem (Merck Chemicals, Nottingham, UK).

Flow Cytometry Analysis

The expression of BAFF, APRIL and their receptors at the membrane of CLL cells was analyzed by indirect immunofluorescence and flow cytometry. The following goat polyclonal IgG were used: TALL-1(BAFF) (Y-15 sc-5743) and TALL-1 (C-16 sc-5744) ; APRIL (R-15 sc-5739) ; BCMA (N-16 sc-11743) ; TACI (C-20 sc7332) and TACI (N-19 sc-7333), all from Santa Cruz Biotechnology (Tebu-Bio, Le Perray en Yvelines, France) and anti-hBAFF-R from R&D (AF 1162). Purified rabbit IgG (Sigma Aldrich, Saint Quentin Fallavier, France) was used as isotype control and FITC-conjugated affinipure $F(ab')_2$ fragment donkey anti-goat IgG(H+L) (Jackson Immunoresearch, Interchim, Montlucon, France) was used as second antibody. In addition, membrane BAFF and BAFF-R were also detected by direct fluorescence with fluorescein conjugated mouse IgG1 mAb anti-hBAFF (R&D IC1241F) and anti-hBAFF-R (Abcam mAb38977) respectively, using FITC mouse IgG1 as isotype control (Becton Dickinson BD555748, BD, Le Pont de Claix, France). For the detection of TLR-7, cells were permeabilized with the CytoFix/CytoPerm kit (Becton-Dickinson) and incubated with a mouse monoclonal anti TLR-7 antibody (IgG1κ; clone 4F4) (Axxara) or mouse IgG_1 (Jackson Immunoresearch) as control; binding was revealed with a fluorescein-conjugated, affinity-purified goat $F(ab')_2$ fragment anti-mouse IgG (H+L) (Rockland, Gilbertsville, PA). Cells were analysed by flow cytometry (EPICS Altra or Coulter Epics XL, Beckman Coulter France, Roissy, France) using the CellQuest program. The estimation of the D-values

(roughly equivalent to the percentage of positive cells) resulting from the subtraction of the histogramme of the isotypic control from the histogramme of the test antibody, was performed according to the technique of Kolmogorov-Smirnov. In some instances, the Δ MFI (differences between the mean fluorescence intensity of test and control antibody) were also presented.

NF-κB Activation

Activation of the different NF-κB members was measured with the TransAM NF-κB family ELISA kit (Active Motif, Carlsbad, CA), according to the specifications of the manufacturer. Briefly this test is based on the specific binding of activated NF-κB components contained in nuclear extracts to a κB consensus site (5´-GGGACTTTCC-3´) that has been immobilized in the wells of a microtitration plate. After treatment with Resiquimod or Imiquimod for 6-8 h, B-CLL cells were lysed using the "nuclear extract kit" from Active Motif according to the manufacturer and protein content was quantified using the Bradford assay. The binding was monitored with antibodies recognizing epitopes on p50, p52, p65, c-Rel or RelB subunits that are accessible only when NF-κB is activated and bound to its target DNA, followed by an HRP-conjugated secondary antibody and colorimetric readout at 405 nm with a WallacVictor 2 (Perkin Elmer, Courtaboeuf, France) microplate spectrophotometer.

AP-1 Activation

The quantification in nuclear lysates of the various members of the AP-1 family of transcription factors was performed in a similar way as for NF-κB with the TransAM AP-1 Family Kit (Active Motif), according to the specifications of the manufacturer.

DNA Fragmentation Assay

Apoptosis was also quantified by DNA fragmentation as evaluated by detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) in cell lysates from aliquots of 20,000 cells using an ELISA with anti-histone and anti-DNA fragment mAbs (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics, Indianapolis, IN, USA) as previously described [14].

Caspase 8 Activity

It was measured as described previously [14]. Briefly, aliquots of cell lysates (20 μg) were incubated with the fluorogenic caspase-8 substrate IETD-aminocoumarin, IETD-AMC (caspase-8 cellular assay, Biomol). The release of AMC was recorded every 10 min during 80 min in a Victor-2 microplate reader thermostated at 37°C (excitation wavelength: 380 nm; emission wavelength: 460 nm). The specificity of the reaction was assessed by the addition of unlabeled Ac-IETD-CHO caspase-8 inhibitor (Biomol).

Western Blot Analysis

The expression of BAFF and BAFF-R was studied by Western blotting, as detailed previously [23]. Briefly, equal amounts of the cell lysates (generally 30-50 μg of proteins in SDS-reducing buffer) were electrophoresed by SDS-PAGE (8%). The goat polyclonal IgG against BAFF/TALL-1 (C-16 sc-5744, Santa Cruz Biotechnology, CA, USA) and anti-hBAFF-R (AF 1162, R&D) were used for the detection and the immunoblotted proteins were revealed with a polyclonal HRP-coated rabbit anti-goat antibody (DakoCymation, Glostrup, Denmark) and a system of chemiluminescence (Western lightning chemiluminescence reagent plus; Perkin Elmer, Boston, MA, USA). Finally, the membranes were also hybridized with a mouse anti-β actin mAb (clone C4; ICN, Costa Mesa, CA, USA) for protein content monitoring and standardization. Films were analyzed with a digital imager (Vilber Lourmat, Marne-la-Vallée, France) system and the NIH Image 1.44b11 software was used for the quantification of the intensity of the bands.

Quantification of Soluble BAFF and APRIL (sBAFF and sAPRIL) in Cell Supernatants

CLL cells were adjusted at 4 x 10^6/ml in RPMI-1640 medium and allowed to incubate for 48 h in the presence or not of varying concentrations of Resiquimod. After centrifugation of the cells, the supernatants were collected and the levels of sBAFF estimated by a specific ELISA (Quantikine human BAFF/BlyS, R&D, Abingdon, UK) as previously described [24]. In parallel, sAPRIL was measured with the human APRIL ELISA kit (AbCys, Paris, France) according to the specifications of the manufacturer. Recombinant soluble BAFF (amino acids 134-285, 17 kDa) was from BioVision Research Products (Mountain View, CA).

Statistical Analysis

Experiments were performed with duplicate or triplicate cell cultures. Statview software was used for statistical analysis. Test and control groups were compared using the unpaired two tail Student's t test.

RESULTS

Enhanced Survival of CLL Cells Following the Ligation of TLR-7

We showed previously that incubation of CLL cells with two specific ligands of TLR-7, Resiquimod and Imiquimod, improved their viability after three days of culture *ex vivo* [14]. This pro-survival effect of the engagement of TLR-7 results from a decreased spontaneous apoptosis, as attested by a marked reduction in cytoplasmic nucleosomes, a surrogate for internucleosomal DNA cleavage (Fig. **1**). Treatment of the CLL cells of three patients for 48 h with 1 μM Resiquimod was found to result in a significant decrease (range 40-60%) in the enrichment of cytoplasmic nucleosomes. Similar results were obtained with Imiquimod (not shown). This was accompanied by a marked reduction in the activity of caspase 8, as shown in Fig. (**2**), where incubation of CLL cells with Resiquimod resulted in a dose-dependent decrease of the cleavage of the caspase 8 specific substrate IETD-AMC. Altogether, these data confirm that the engagement of TLR-7 leads to an early enhanced viability and resistance of the leukemia cells to spontaneous apoptosis.

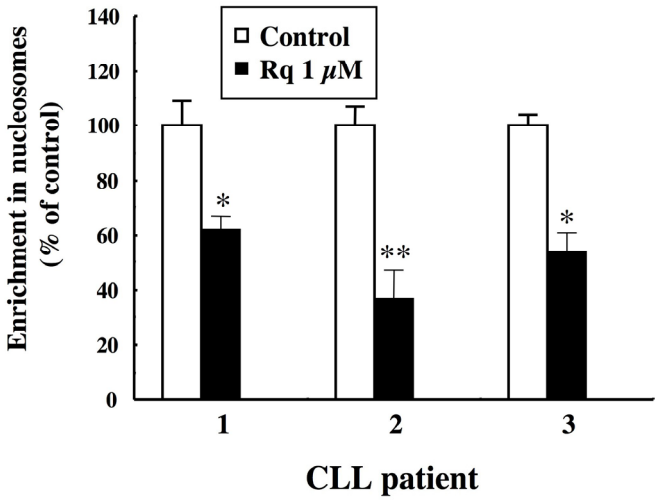

Fig. (1). Stimulation of TLR-7 leads to protection against spontaneous apoptosis. CLL cells (2 x 10^6 / ml) from three different patients were incubated for 48 h in the presence or not of Resiquimod 1 μM. At the end of the incubation period, aliquots were collected and the amount of nucleosomes in the cytoplasm was quantified by the "Cell Death detection kit " (Roche) as described in Materials and Methods. Results from triplicate samples are expressed as % of enrichment in cytoplasmic nucleosomes, in comparison with control unstimulated cells taken as 100%. The differences between control and Resiquimod-treated cells were analysed with the Student's t test. * $p < 0.05$; ** $p < 0.01$

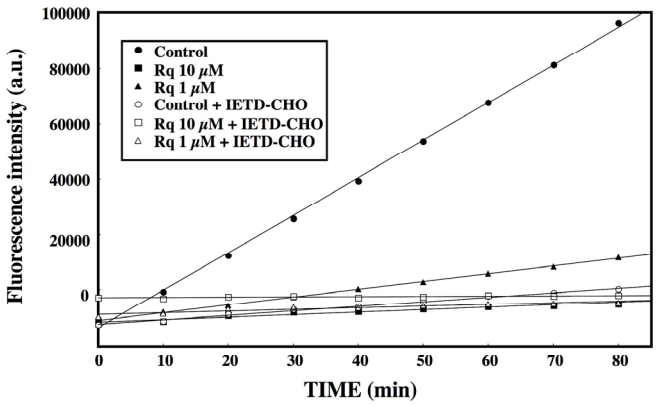

Fig. (2). The TLR-7 ligand Resiquimod inhibits caspase 8 activity in CLL cells. CLL cells were incubated for 20 h in the presence of medium alone or with 1 μM or 10 μM of Resiquimod. Caspase 8 activity in cell lysates was estimated by cleavage of the fluorescent substrate IETD-AMC over an 80 minutes period as described in Materials and Methods. The specificity of the reaction was assessed by the abolition of the activity observed in the presence of the specific caspase 8 inhibitor IETD-CHO.

Ligation of TLR-7 Stimulates the Expression of BAFF, APRIL by CLL Cells

We hypothesized that the enhanced viability of the CLL cells resulting from TLR-7 engagement could be due, in part, to the induction of a response of these cells to pro-survival factors, such as BAFF and APRIL. We thus tested the expression of BAFF and APRIL at the surface of the leukemia cells. As shown in Fig. (**3**) for one representative CLL patient, BAFF and its the closely related APRIL were detected at low but significant levels at the membrane of these cells, in agreement with our previous report [17]. Incubation of the cells with Resiquimod 1μM was found to result in an enhanced expression of BAFF and APRIL as shown in Fig. (**3**). These increases were highly significant as estimated by Kolmogorov-Smirnov. Comparable results were observed with the MEC-1 cell line derived from a B-CLL patient, as shown in Fig. (**4**). Although the spontaneous expression of BAFF and APRIL by MEC-1 cells was lower than that displayed by primary CLL cells, it was still significantly increased in the presence of Resiquimod.

Ligation of TLR-7 Stimulates BAFF-R Expression and is Dependent on NF-κB Activation

The specific receptor for BAFF, BAFF-R, was also found to increase following stimulation of TLR-7 with Resiquimod, as detected by FACS analysis. In comparison with BAFF and APRIL, the expression of BAFF-R, already high in unstimulated leukemia cells (as previously described [24]), is further increased significantly after incubation with Resiquimod (Fig. **5**). Resiquimod-driven increase in BAFF-R expression was almost maximum at 1μM, a plateau of stimulation being observed at higher concentrations (Table **1**). Similar data were obtained with Imiquimod (not shown). The augmentation in membrane BAFF-R was largely reverted in the presence of wedelolactone, an inhibitor of the I-κB kinase (IKK) complex and of NF-κB activation (Fig. **5**). The latter treatment was found to result in a loss of the protective effect against apoptosis afforded by Resiquimod alone (not shown).

Western Blot Study of BAFF and BAFF-R in CLL Cells Following Engagement of TLR-7

The elevated expression of BAFF in CLL cells following Resiquimod treatment was further confirmed by Western blotting. Dose-dependent effects of Resiquimod and of another TLR-7 ligand, Isiquimod, on BAFF expression are depicted in Fig. (**6A**, **B**). In parallel, the presence of BAFF-R on CLL cells is increased following incubation with both TLR-7 ligands (Fig. **6C**). Interestingly, BAFF and BAFF-R expressions also appear to be up regulated by BAFF itself (Fig. **6B**, **C**).

TLR-7 Stimulation Favors the Release of sBAFF and sAPRIL by CLL Cells

The enhanced expression of BAFF and APRIL was associated with an increased release of their soluble form, sBAFF and sAPRIL, in the supernatants of Resiquimod-treated cells (Fig. **7**). In contrast, the expression of the two other BAFF receptors, TACI and BCMA, that are also detected albeit to lower levels than BAFF-R on CLL cells [24], was roughly unaffected by Resiquimod (not shown).

TLR-7-Driven BAFF and APRIL Increases Contribute Both to the Protective Effect on Apoptosis

The contribution of the augmented expression and release of BAFF and APRIL to the protection against apoptosis afforded by TLR-7 stimulation was investigated by using neutralizing soluble forms of their receptors. As seen in

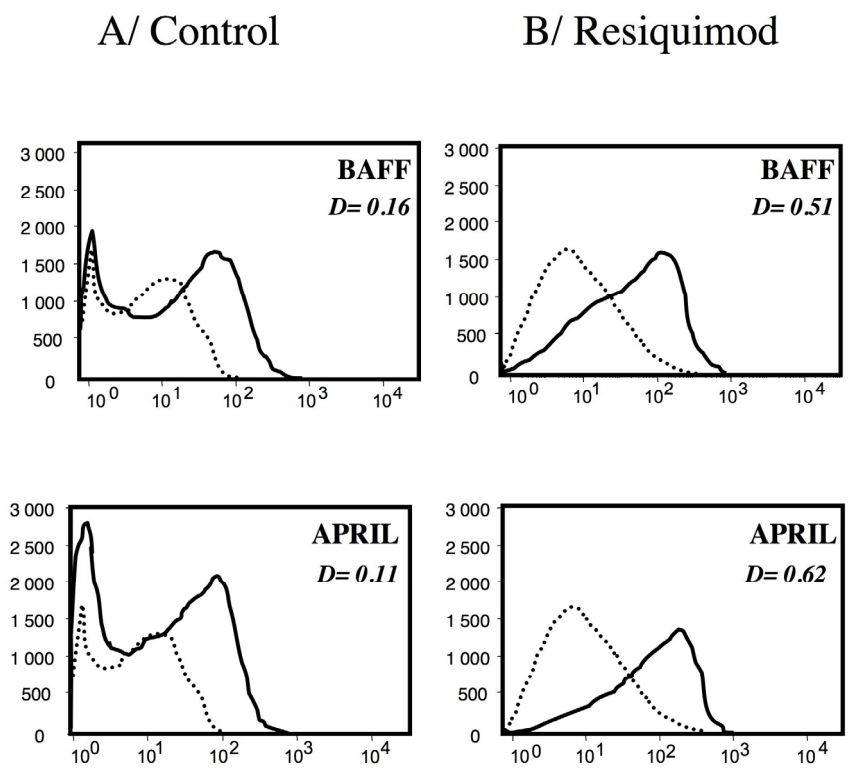

Fig. (3). Stimulation by Resiquimod of BAFF and APRIL expression in CLL cells. Leukemia cells from a CLL patient were incubated overnight in the presence of medium alone (Control, left part) or with Resiquimod 1 µM (right part). After washings, cells were labeled either with goat IgG (isotypic control, dotted lines) or polyclonal goat antibodies against BAFF and APRIL respectively (full lines), revealed with a FITC-conjugated affinipure $F(ab')_2$ fragment donkey anti-goat IgG(H+L) and analyzed by flow cytometry (Epics Altra). The histogrammes are presented and submitted to statistical analysis according to Kolmogorov-Smirnov. The resulting D values that roughly represent the percentages of positive cells are indicated. The differences in the D values between control and Resiquimod-treated cells are highly significant for the two markers ($p < 0.00001$). Results are from one patient representative of four that gave comparable results.

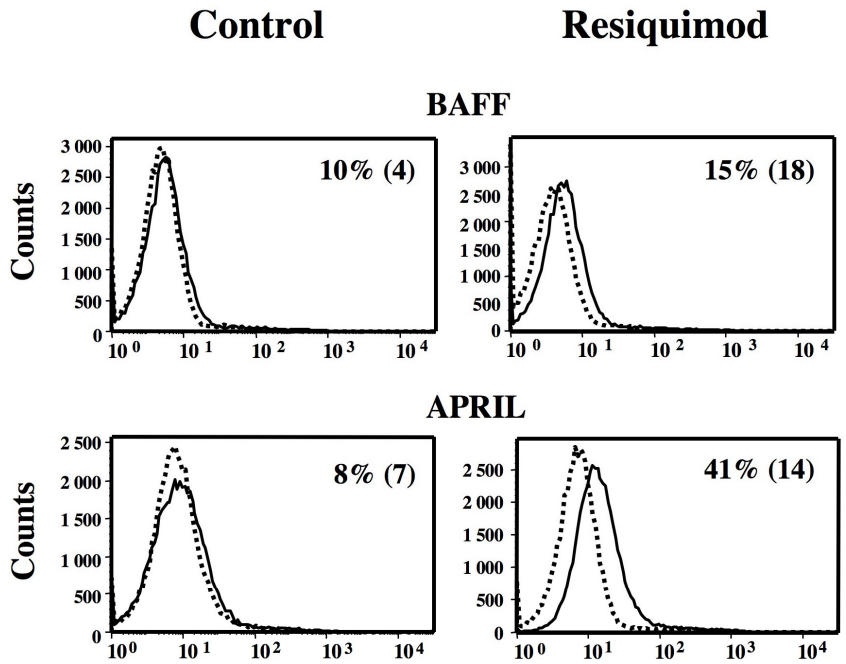

Fig. (4). Effect of Resiquimod on BAFF and APRIL expression by MEC-1 cells. MEC-1 cells were incubated overnight in the presence of medium alone (Control, left part) or with Resiquimod 1 µM (right part) and the membrane expression of BAFF and APRIL was estimated as before by flow cytometry. The percentages of positive cells were estimated by Kolmogorov-Smirnov analysis. The Δ MFI are indicated in brackets and the differences between control and Resiquimod-treated cells are significant for the two markers. Results are from one experiment out of two that gave comparable results.

Table 1. Stimulation by Resiquimod of BAFF-R Expression in CLL Cells

Resiquimod (μM)	D-Value (K-S Analysis) CLL Patient Case			
	1	2	3	4
0	0.53	0.51	0.48	0.55
1	0.67	0.87	0.82	0,79
5	0.70	0.91	0.83	0.85
10	0.71	0.92	0.84	0.89

Leukemia cells from four CLL patients (1, 2, 3, 4) were incubated in the presence of increasing concentrations of Resiquimod. After 48 h incubation, the expression of BAFF-R was determined by flow cytometry. D-values were estimated by Kolmogorov-Smirnov analysis and their increase in the presence of the various concentrations of Resiquimod were highly significant (at least $p < 0.01$).

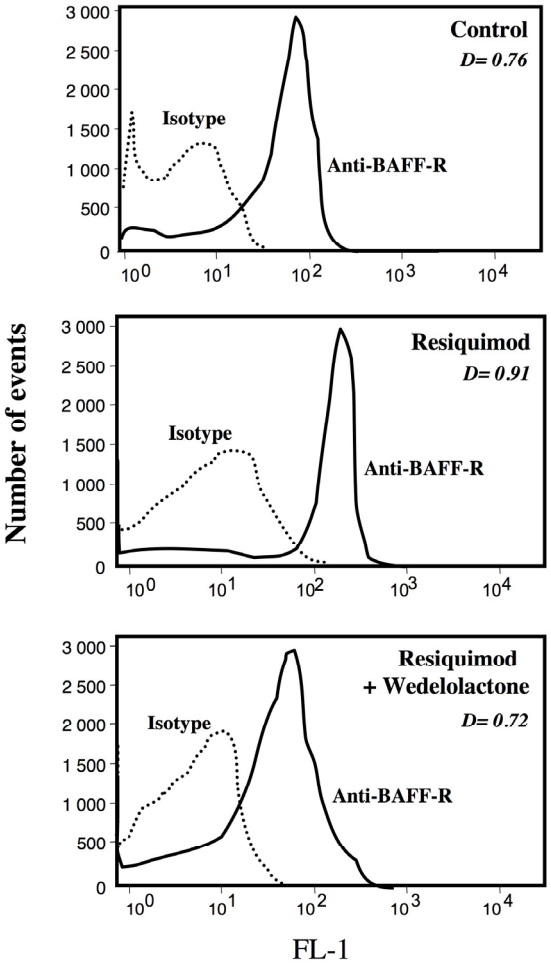

Fig. (5). Resiquimod stimulates BAFF-R expression in CLL cells through NF-κB activation. CLL cells were cultured at 2 x 10^6/ml in the presence of medium (control), Resiquimod 1 μM or Resiquimod 1 μM + Wedelolactone 10 μM. After 48 h of incubation, cells were collected, washed and labeled with either goat IgG (isotypic control, dotted lines) or polyclonal goat antibody against BAFF-R (full lines), revealed with a FITC-conjugated affinipure F(ab')$_2$ fragment donkey anti-goat IgG(H+L), followed by FACS analysis as previously described. Dotted line: isotypic control (goat IgG) ; full line : anti-BAFF-R antibody. D-values were estimated by K-S analysis. Results are from one representative CLL patient out of four that gave comparable results.

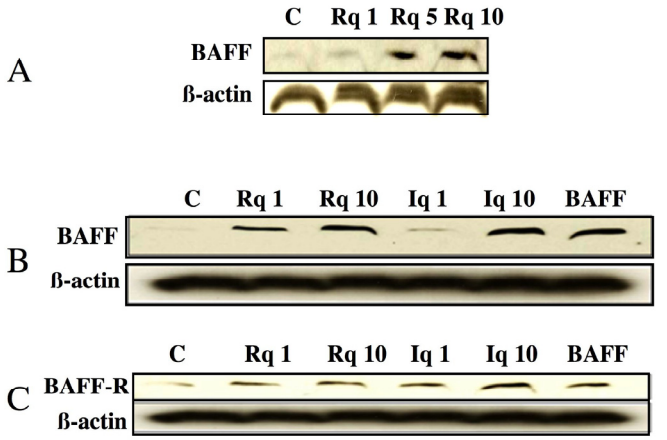

Fig. (6). Resiquimod and Isiquimod triggers the expression of BAFF and BAFF-R in CLL cells. A/ Leukemia cells from one CLL patient were incubated for 20 h in the presence of medium alone (C; control) or with varying concentrations of Resiquimod (Rq) 1, 5 or 10 μM. At the end of the incubation time, cells were lysed and the content in BAFF was analysed by Western blot as described in Materials and Methods, using ß-actin as internal control of equal protein loading. **B** and **C**/ Same as previously, but cells were incubated with Resiquimod (Rq) 1 μM or 10 μM, Imiquimod (Iq) 1μM or 10 μM, or BAFF 250 ng/ml. Cell lysates were analyzed as before for the expression of BAFF (**B**) and BAFF-R (**C**).

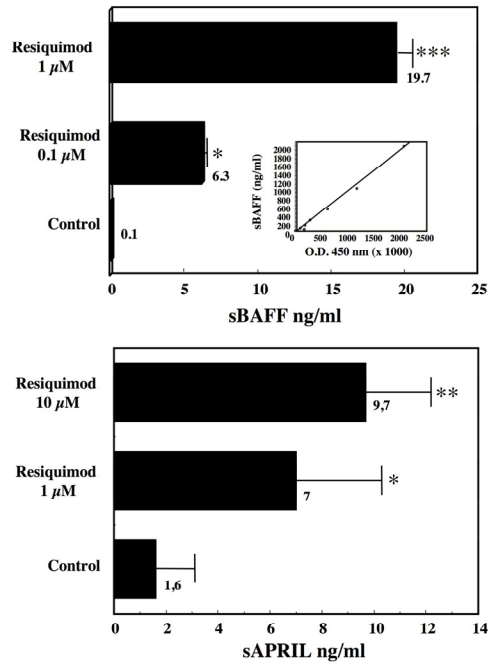

Fig. (7). Resiquimod stimulates sBAFF and sAPRIL release by CLL cells. Leukemia cells were adjusted at 4 x 10^6/ml in complete RPMI-1640 medium and allowed to incubate for 48 h in the presence or not of Resiquimod 0.1 μM or 1 μM. The concentration of soluble BAFF, sBAFF, released in the supernatant was determined from triplicate samples with the sBAFF kit (R &D) using purified sBAFF for the calibration. The concentration of soluble APRIL, sAPRIL, was determined with the human APRIL ELISA kit (AbCys). The Student's t test was used to compare the values between control and Resiquimod-treated cells: * $p < 0.05$; **$p < 0.01$; *** $p < 0.001$. Results are from one representative CLL patient out of three that gave comparable results.

Table **2**, the decrease in spontaneous apoptosis induced by Resiquimod was largely, but not totally, abrogated in the presence of an excess of BCMA-Fc that neutralizes both BAFF and APRIL. In contrast, the protection was only partially alleviated in the presence of BAFF-R-Fc that neutralizes BAFF but not APRIL. These results indicate that the protective effects of the TLR-7 ligand are dependent on the increases in both APRIL and BAFF and some additional factors might also be involved.

Table 2. Comparative Inhibitory Effects of BAFF-R-Fc and BCMA-Fc on Resiquimod-Driven Resistance to Apoptosis of CLL Cells

Treatment	% of Enrichment in Cytoplasmic Nucleosomes	p Value/Control
Control	100 ± 5	-
Rq	59 ± 3	< 0.001
BAFF-R-Fc	98 ± 2	NS
BCMA-Fc	101 ± 6	NS
Rq + BAFF-R-Fc	73 ± 3	< 0.02
Rq + BCMA-Fc	92 ± 4	0.05

Cells from one CLL patient were cultured at 2 x 10^6/ml in the presence or not of Resiquimod 1 µM, together or not with BAFF-R-Fc (50 µg/ml) or BCMA-Fc (50 µg/ml). After 48 hours of incubation, 20 000 cells were collected and the percentage of enrichment in cytoplasmic nucleosomes was estimated from triplicate samples by a specific ELISA, as described in Materials and Methods. Statistical significances in comparison with control (unstimulated) cells, taken as 100%, were estimated with the modified Student's t test for a small number of samples. Data are from one patient out of three that gave comparable results.

TLR-7 Stimulates NF-κB Activation in CLL Cells

NF-κB activation has been described as a major component of TLR signalling. Accordingly, we observed that the ligation of TLR-7 with Resiquimod resulted in a stimulation of several members of the NF-κB family of transcription factors in CLL cells, as shown in Fig. (**8**) for two patients. Both the classical and non-canonical pathways of activation appear to be triggered, inasmuch as significant increases in the nuclear expression of p65/RelA and of p52 could be detected. We previously reported that Resiquimod elicited a dose-dependent phosphorylation of the inhibitor IκBα, leading to its subsequent ubiquitination and degradation by the proteasome [14].

Activation of AP-1 Family Members by the Engagement of TLR-7

The addition of Resiquimod to CLL cells also stimulates the activation of several members of the AP-1 family of transcription factors. As seen in Fig. (**9**), the TLR-7 ligand induced a marked increase in the nuclear expression of Jun-B and in the phosphorylated form of c-Jun. A small augmentation in the nuclear expression of Jun-D already spontaneously elevated was also detected, whereas the other members, such as c-Fos, FosB, Fra-1 and Fra-2 were roughly unaffected. Addition of JNK II inhibitor, an inhibitor of c-Jun phosphorylation, largely reversed the protection toward apoptosis afforded by Resiquimod, as shown by return to basal levels of the enrichment in cytoplasmic nucleosomes

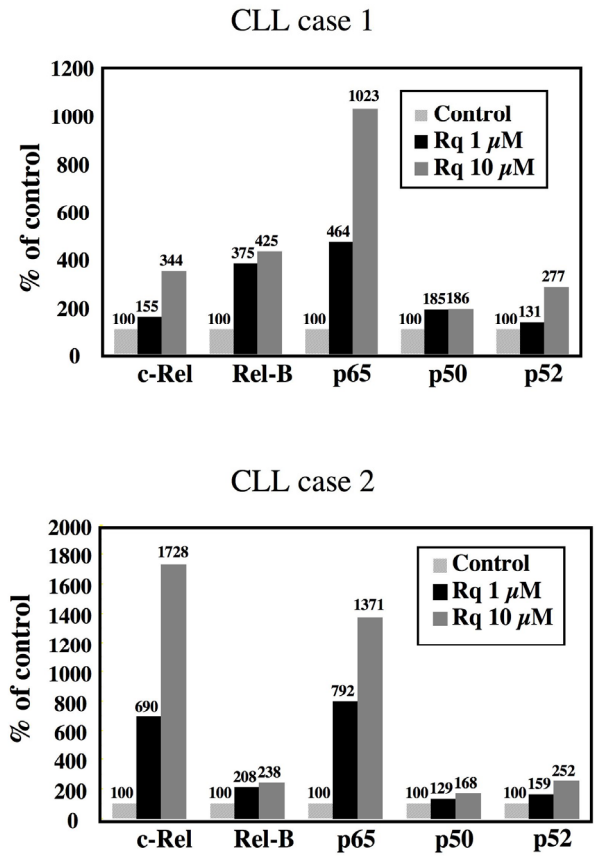

Fig. (8). Resiquimod stimulates NF-κB activation in CLL cells. Leukemia cells from two representative CLL patients were suspended at 4 x 10^6/ml and cultured in the presence of medium alone or with Resiquimod 1 µM or 10 µM. After 6 hours of incubation, cells were collected, washed and nuclear extracts were prepared. The activation of the different NF-κB members was estimated by measuring their binding to a consensus NF-κB sequence with the Transam NF-κB family ELISA kit, as described in Materials and Methods. Results are expressed as the percentage of binding in comparison with control unstimulated cells taken as 100%.

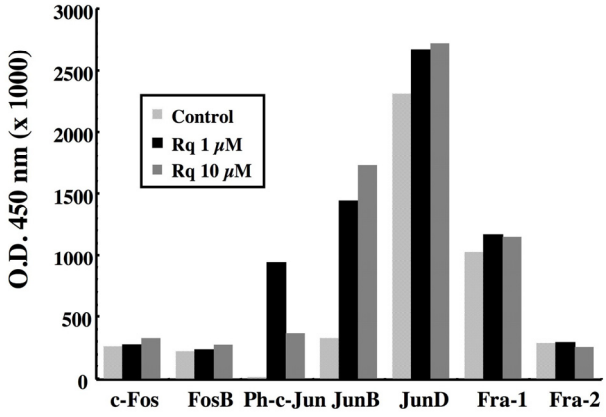

Fig. (9). Resiquimod stimulates the activation of members of the AP-1 family. Leukemia cells from one representative CLL patient, out of three that gave comparable results, were treated as for NF-κB determination and the presence of various members of the AP-1 family of transcription factors in nuclear extracts was tested with the Transam AP-1 family ELISA kit (Active Motif) as described in Materials and Methods.

(Fig. **10**). The Resiquimod-driven increase in the expression of BAFF and APRIL was also largely counteracted in the presence of this inhibitor (Table **3**), emphasizing the link between the activation of AP-1 and the expression of these survival factors.

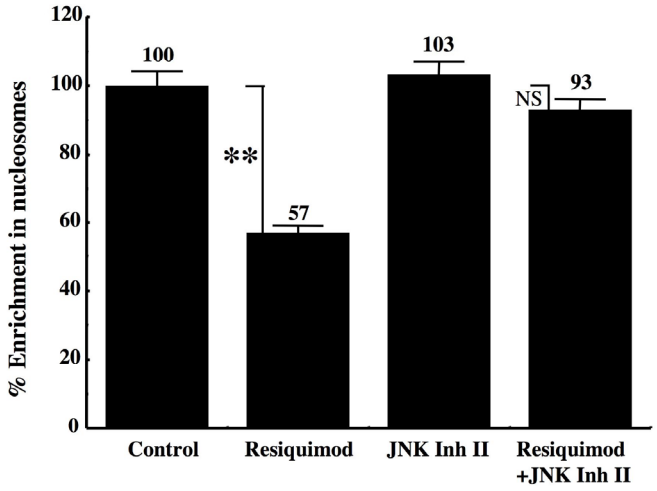

Fig. (10). Resiquimod-induced protection toward apoptosis is reverted by the JNK II inhibitor. Cells from one CLL patient were cultured at 2 x 10^6/ml in the presence or not of Resiquimod 1 µM, together or not with JNK Inh II (50 µg/ml). After 48 h of incubation, 20000 cells were collected and the percentages of enrichment in cytoplasmic nucleosomes were estimated from triplicate samples by a specific ELISA, as described previously. Statistical significance in comparison with control (unstimulated) cells, taken as 100%, was estimated with the modified Student's t test for small numbers of samples. ** $p < 0.02$; NS: not significant. Data are from one patient out of three that gave comparable results.

Table 3. The JNK II Inhibitor Reverts the Resiquimod-Driven Increase in BAFF and APRIL Expression by CLL Cells

		D-Value (K-S Analysis)		
	Patient	**Control**	**Rq**	**Rq + JNK Inh II**
BAFF	1	22	53	29
	2	18	49	23
APRIL	1	17	57	22
	2	15	54	19

Leukemia cells from two CLL patient were incubated or not in the presence of Resiquimod (Rq) 1 µM, together or not with JNK Inh II (50 µg/ml). After 48 h incubation, the expression of BAFF and APRIL was determined by flow cytometry as described previously in the legend of Fig. (**3**). D values were estimated by Kolmogorov-Smirnov analysis as before.

DISCUSSION

The engagement of TLR-7 with a specific ligand such as Resiquimod was found to enhance the viability of the leukemia cells and to protect them from spontaneous apoptosis. This was accompanied by a decreased activity of caspases 8 and, as already reported [14], of the effector caspase 3. In this previous work, we showed that the protection afforded by TLR-7 engagement was due, in part, to the up-regulation of the iNOS in these cells and was reduced in the presence of specific iNOS inhibitors [14]. Here we show, in addition, that stimulation of TLR-7 leads to an up-regulation of two major CLL survival factors, BAFF and APRIL. Both the total amount and the membrane expression of these factors were increased. Moreover, the expression of the specific receptor for BAFF, BAFF-R was also increased by TLR-7 stimulation. This suggested that the enhanced resistance to apoptosis conferred to CLL cells by TLR-7 stimulation could be due to an increased efficiency of the BAFF and APRIL autocrine survival pathways. This hypothesis was supported by the observation that the neutralizing BCMA-Fc and BAFF-R-Fc significantly reversed the protective effect resulting from TLR-7 ligation.

TLR-7 has been reported to signal mainly through the activation of the MyD88/NF-κB pathway [19, 25, 26]. In agreement, we found that TLR-7 engagement resulted in the activation of several NF-κB components, notably p65/RelA and p52, suggesting that both the canonical and alternative pathways were stimulated. Inasmuch as the BAFF gene exhibits several κB responsive elements in its promoter region [27] and is transcriptionally regulated by NF-κB dependent mechanism [28, 29], it is therefore likely that TLR engagement leads to BAFF transcriptional activation. The transcription factors regulating APRIL expression are less well known, but could also involve NF-κB. As regards BAFF-R, its enhanced expression following the engagement of TLR-7 in CLL cells was also dependent on the activation of NF-κB, since it was markedly impaired in the presence of wedelolactone, an inhibitor of IκBα phosphorylation. In addition, the stimulation of BAFF and APRIL expression elicited by Resiquimod appears to involve the activation of the AP-1 transcription factor and the phosphorylation of c-Jun. Furthermore, both the Resiquimod-driven protection toward apoptosis and the increased BAFF and APRIL expression were markedly inhibited in the presence of JNK Inhibitor II, a specific inhibitor of the phosphorylation of c-Jun. In agreement, it was previously reported that, in a human T cell line, the expression of BAFF is regulated through a transduction pathway involving the c-jun NH2-terminal kinase [30].

The up-regulation of BAFF, APRIL and BAFF-R resulting from TLR-7 engagement favors autocrine loops of survival. In turn, BAFF and APRIL can amplify the activation of NF-κB [17] and eventually contribute to stimulate iNOS expression and NO production. Indeed, we observed that addition of exogenous BAFF or APRIL to *ex vivo* cultures of CLL cells triggers an increase in the production of NO by the leukemia cells, in about 25 % of the cases, notably those displaying a low spontaneous expression of iNOS (unpublished observations). In addition to their own pro-survival effects, BAFF and APRIL could amplify the biosynthesis of the anti-apoptotic agent NO when the production of the latter is not optimum.

The physiological ligand of TLR-7 has been identified as single strand RNA (ssRNA) [31, 32]. It is thereof likely that CLL cells or their precursors could be triggered through TLR-7 by ssRNA present in their microenvironment, either as microbial components (PAMPs, pathogen-associated molecular patterns) or as degradation products of self origin. This would provide to these cells, through the stimulation of

the BAFF/APRIL pathways, an enhanced resistance toward spontaneous apoptosis favoring their accumulation.

It could also be suggested that a simultaneous co-ordinated stimulation of the BCR *via* these auto-antigens or microbial antigens would reinforce this resistance and contribute to the aetiology of this leukemia. This hypothesis is supported by the sequences of the monoclonal immunoglobulins of the BCR of CLL cells that, for many of them, present a bias suggestive of an encounter with type 2 T-independent antigens such as some auto-antigens and antigens of microbial origin. The role of TLR-7 is therefore compatible with the hypothesis of a chronic stimulation of B-lymphocytes or their precursors with a peculiar set of antigens (auto antigens or microbial-derived antigens) and PAMPs (pathogen-associated molecular patterns) in the onset of CLL.

BAFF is required for the development of mature B cell. Self-reactive B cells have reduced responsiveness to BAFF and die due to the limiting levels of BAFF available *in vivo*. Elevated BAFF expression provided by TLR-7 ligation rescue self-reactive B cells normally deleted late during maturation [33]. This could explain the large proportion of sIg from both mutated and unmutated CLL patients displaying antibody activity against self components [6].

BAFF-R is highly expressed on murine $CD5^+$ B1 lymphocytes and the engagement of TLR-9 augments the surface expression of BAFF receptors and renders them responsive to BAFF co stimulation, mostly through BAFF-R up-regulation [34]. In turn, BAFF stimulates CLL survival through activation of the canonical pathway of NF-κB activation [35].

Attempts have been made to increase the immuno-genicity of CLL cells through stimulation of TLR-7 and/or TLR-9 in order to favor their elimination by the immune system [reviewed in 36]. In contrast to TLR-9-activated cells, however, TLR-7-activated CLL cells are weak stimulators of T cells proliferation and additional signals are required. The response of CLL cells to TLR agonists may also vary depending on their state of activation and the microenvironment: quiescent cells in the blood compartment versus proliferating cells in the bone marrow and the lymph nodes [37]. Moreover, CLL cells activated by TLR-7 agonists become more sensitive to cycle-active cytotoxic drugs [38] and are also killed more easily by CTLs *in vitro*, although the mechanisms involved are not quite elucidated [39].

In conclusion, our results demonstrate that the engagement of TLR-7 leads to increased resistance of CLL toward apoptosis due to a stimulation of the BAFF and APRIL survival pathways. They support the hypothesis of a chronic stimulation of TLR-7 by ssRNA of self or microorganism origine at the onset of the disease. They also indicate that the therapeutic attempts to increase the immunogenicity of CLL cells (or their sensitivity to cell cycle acting drugs) through stimulation with TLR agonists must take into consideration the risk of simultaneously stimulating their resistance to apoptosis. In the near future, we will try, in association with classical chemotherapy, to inhibit the BAFF and APRIL survival pathways, notably by preventing their stimulation through TLR.

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