Targeting B cell anergy in Chronic Lymphocytic Leukemia.

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Running Title: MAPK and NF-AT inhibitors in CLL
KEY POINTS

1) A sizable fraction of CLL patients is characterized by the expansion of clonal B cells with anergic features.

2) The constitutive biochemical signature of B cell anergy can be efficiently targeted in CLL for therapeutic purposes

Keywords: Chronic Lymphocytic Leukemia (CLL), B cell anergy, B Cell Receptor (BCR) signalling, inhibitors
Abstract

B-Cell Receptor (BCR) triggering and responsiveness have a crucial role in the survival and expansion of Chronic Lymphocytic Leukemia (CLL) clones. Analysis of in vitro response of CLL cells to BCR triggering allowed the definition of two main subsets of patients and lack of signalling capacity was associated with constitutive activation of ERK1/2 and NF-ATc1, consistent with the idea that at least one group of CLL patients derives from the abnormal expansion of anergic B cells. In the present paper we have further investigated the anergic subset of CLL (defined as the one with constitutive ERK1/2 phosphorylation) and found that it is characterized by low levels of surface IgM and impairment of calcium mobilization after BCR engagement in vitro. Chronic BCR triggering promoted CLL cell survival selectively in pERK(+) samples and the use of MAPK and NF-AT signalling inhibitors specifically induced apoptosis in this group of patients. Apoptosis induction was preceded by an initial phase of anergy reversal consisting in the loss of ERK phosphorylation and NF-AT nuclear translocation, and by the restoration of BCR responsiveness, reinforcing the idea that the anergic program favours the survival of leukemic lymphocytes.
Introduction

Chronic Lymphocytic Leukemia (CLL), a chronic lymphoid malignancy characterized by the accumulation of monoclonal, CD5⁺ B lymphocytes in peripheral blood (PB), bone marrow (BM) and secondary lymphoid organs, is clinically heterogeneous. Patients may present with an indolent disease and a life expectancy similar to normal individuals or with an aggressive tumour, shorter survival and early treatment requirements. Biological predictors of clinical outcome, such as the mutational status of the immunoglobulin (IG) heavy chain variable genes (IGHV) and the expression of signalling molecule ZAP70, suggest a key role for the CLL cell membrane IG in the pathogenesis of the disease. In addition, molecular and functional analyses of the B-Cell Receptor (BCR) strongly support the hypothesis that a stimulation through the BCR is involved in the selection and expansion of the malignant CLL clone.

In line with this, several groups (reviewed by Stevenson et al.) have found that a number of molecules along the signalling pathways originating from the BCR are constitutively activated in all CLL patients, providing the basis for effective therapeutic targeting, currently tested in prospective clinical trials (reviewed by Woyach et al.).

We previously reported that in approximately half of CLL cases leukemic cells constitutively express phosphorylated Extracellular Regulated Kinases 1/2 (ERK1/2) and MAP/ERK Kinases 1/2 (MEK1/2) together with activated Nuclear Factor of Activated T cells c1 (NF-ATc1) transcription factor. Such cases are also characterized by cellular unresponsiveness to sIgM ligation in terms of phosphotyrosine induction. In addition, Mockridge and colleagues demonstrated that cells from CLL patients that express low levels of surface IgM fail to mobilize intracellular calcium upon BCR triggering. Taken together, these features recapitulate the biochemical program observed in mouse models of B cell anergy. Anergy is one of the mechanisms that the immune system
adopts to silence autoreactive B lymphocytes upon low affinity recognition of self antigens (Ag)\textsuperscript{13}. The state of BCR desensitization induced by chronic Ag binding \textit{in vivo} results in cell unresponsiveness when B lymphocytes are further stimulated \textit{in vitro} by an Ag-like activation\textsuperscript{14}. In mouse models, anergized B cells share a common biochemical signature that includes low levels of surface IgM (due to constant BCR internalization and recycling\textsuperscript{15}), elevated basal intracellular calcium concentrations and subsequent constitutive activation of ERK1/2 and NF-ATc1\textsuperscript{11,16}. This biochemical program is not permanent, but it is reversible and lasts as long as B lymphocytes are exposed to the Ag\textsuperscript{14}.

All together these observations led us to propose that constitutive activation of MAP kinase signalling pathway along with NFATc1 transactivation represents the molecular signature also of human anergic B lymphocytes and thus that CLL cases presenting this signature may be taken as a human model of leukemic expansions of B cells with anergic properties\textsuperscript{9}.

In this manuscript, we investigated the molecular features of the CLL anergic subset (defined as the one that presents constitutive pERK) and we also took advantage of the use of small chemical compounds that specifically block the activation of both ERK1/2 and NF-ATc1. We conclude that anergy induction and maintenance favours the survival of leukemic lymphocytes and may be effectively targeted for therapeutic purposes.
Material and Methods

Tissue samples and cell purification

Leukemic lymphocytes were obtained from peripheral blood of CLL patients, diagnosed according to the International Workshop on CLL (iwCLL)/National Cancer Institute Working Group (NCIWG) guidelines. All patients were either untreated or off therapy for at least 6 months before the beginning of the study. The following parameters were analyzed for each patient: age, gender, disease stage at diagnosis according to Binet or modified Rai criteria, CD38 expression, IGHV gene mutational status, ZAP70 expression, history of treatment, characterization of disease as progressive or stable as defined by the iwCLL/NCIWG and survival time.

Leukemic cells were purified immediately after blood withdrawal, by negative depletion, using a B-lymphocyte enrichment kit (RosetteSep; StemCell Technologies). Purity of all preparations was always more than 99%, and the cells coexpressed CD19 and CD5 on their cell surfaces as checked by flow cytometry (FC500; Beckman Coulter); preparations were virtually devoid of natural killer (NK) cells, T lymphocytes, and monocytes.

All tissue samples were obtained with approval by the institutional review board of San Raffaele University Hospital (Milan, Italy). Informed consent was obtained in accordance with the Declaration of Helsinki.

MEC1 CLL cell line was purchased from DSMZ.

Antibodies and reagents.

Antibodies

For flow cytometry, anti CD19 and anti CD5 were purchased from Beckman Coulter and IgM from Southern Biotechnology. Alexafluo488-pERK1/2 antibody was from BD Biosciences Pharmingen. Cells were analyzed with a FC500 flow cytometer (Beckman-Coulter).
For Western blotting, anti phospho-ERK1/2 (Y202/Y204), anti phospho-RSK1/2 (T359/S363) were purchased from Cell Signaling Technology; anti ERK1/2 and anti RSK1 were from Santa Cruz.
For Western blot analysis, specific secondary antibodies horseradish peroxidase (HRP)-conjugated, (goat anti-rabbit Ig and goat anti mouse Ig) were purchased from Upstate Biotechnology.
For BCR triggering, goat F(ab)2 anti–human IgM antibodies were purchased from CALTAG Laboratories.

**Inhibition and Stimulation**
U0126 was purchased from Calbiochem; CI1040 and AZD6244 were from Selleck Chemicals. For in vitro studies 11R-VIVIT peptide was purchased from Calbiochem, for in vivo experiments the peptide was chemically synthesized by Genescript (purity>98%) and resuspended in water at the concentration of 5mg/ml. NMS6E was provided by Nerviano Medical Sciences.
As positive control for phospho-flow staining, processed murine splenocytes were stimulated with PMA (100 ng/ml, Sigma).

**Cell culture and stimuli**
Purified lukemic cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 15 µg/ml gentamicin (complete RPMI; Invitrogen). Lymphocytes were cultured at the concentration of 3x10^6 cells/ml in the presence or the absence of different stimuli or inhibitors as indicated.

**Western blot analysis**
Cells were lysed with ice cold Lysis Buffer (NaCl 0.15M, 1% v/v NP40, 1mM EDTA pH=8, 50mM Tris-HCl pH=7) with fresh protease and phosphatase inhibitors cocktail (Roche).
Whole protein extracts (30 µg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDSPAGE), and proteins from gel were electron-transferred onto nitrocellulose membranes and incubated with the indicated antibodies. Antibodies were diluted in a solution of PBS+0,1% Tween (PBST) containing 5% of non fat dry milk or 5% of BSA (Bovin Serum Albumin) according to manufacturers’ instructions. Immunoreactivity was revealed by incubation with specific secondary antibodies conjugated with HRP, and was followed by enhanced chemiluminescence (ECL) reaction (Pierce) and film exposures. Densitometric analysis of ERK-specific bands was performed using ImageQuant Software (GE Healthcare). The values of individual patients were calculated as percentage of the positive control (MEC1 cell line) and determined as the ratio of the OD of phospho-ERK and OD of total ERK.

**NF-ATc1 activation assay**

*Nuclear extraction*

Nuclear and cytoplasmic extracts were obtained using Nuclear Extract Kit from Active Motif. Briefly, 8.8x10^6 cells (left untreated or treated with the indicated stimuli/inhibitors) were collected in ice cold PBS in the presence of phosphatase inhibitors (provided with the kit). The cells were then resuspended in Hypotonic Buffer (20mM Hepes, pH=7.5; 5mM NaF; 10µM Na2MoO4; 0,1mM EDTA) and incubated on ice for 15 minutes. Addition of Detergent caused leakage of the cytoplasmic proteins in the supernatant. After collection of the cytoplasmic fraction, the nuclei were lysed and the nuclear proteins were solubilized in Lysis Buffer in the presence of the Protease inhibitor cocktail (all solutions were provided with the Kit). Nuclear extracts were quantified (by using BCA Assay) and used for the evaluation of NF-ATc1 activation.
**NF-ATc1 ELISA based assay**

The level of NF-ATc1 activation was determined by using TransAM transcription factor kit from Active Motif. Nuclear extracts, purified as described in the previous section, were incubated with oligonucleotides containing the NF-AT specific consensus sequence immobilized on a 96-well plate. Active NF-AT contained in nuclear extracts bound specifically to this oligonucleotide and was detected with a specific antibody against NF-AT. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provided colorimetric readout that was quantified by measuring absorbance at 450 nm. To confirm specificity, we also performed competition experiments by adding 2μl of free oligonucleotide to the wells before nuclear extract (data not shown).

**Analysis of cell viability and apoptosis**

After each different treatment at indicated doses and time points, the percentage of viable cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay kit, according to the manufacturer's instructions (Promega). All measurements were performed in duplicate and the viability of treated cells was normalized to the relative untreated control.

Induction of apoptosis was evaluated by double staining with FITC (fluorescein isothiocyanate)-conjugated Annexin V and PI (propidium iodide), using the Annexin V-FITC Apoptosis Detection Kit (eBioscience), according to the manufacturer’s instructions. Samples were analyzed on a FC500 flow cytometer (Beckman Coulter).

**Intracellular calcium flux**

Intracellular calcium flux was measured by using the fluorogenic probe Fluo3AM (Invitrogen) as previously described\(^9\).
Briefly, purified leukemic cells (at concentration of $10^7$ cells/ml in complete RPMI) were incubated with 4μM of Fluo3-AM and 0.02% (vol/vol) of Pluronic F-127 (Sigma) for 30 minutes at 37°C. Cells were then washed and resuspended at 5x10^6 cells/mL in complete RPMI at room temperature. Cells (250 μL) were warmed to 37°C for 5 minutes prior to acquisition of background fluorescence (i.e., of unstimulated cells), followed by addition of 20 μg/ml goat F(ab)2 anti-human IgM or IgD (Southern Biotechnology) and data acquisition for a further 5 minutes.

Data were acquired on a BD FACScalibur and analyzed by using the Flowjo software (Tree Star).

In order to calculate the percentage of cells responding to BCR triggering, we first established a baseline fluorescence threshold (T) for each sample (by acquiring samples for 40 seconds without any stimulus) at the fluorescence intensity of the 85th percentile of unstimulated cells. We then calculated the peak percentage of cells that exhibited an increase in fluorescence intensity above the threshold following treatment with anti-IgM/IgD. As previously described10, we classified patient as signallers when the percentage of cell responding to anti-IgM stimulation was higher than 5%.

**In vivo experiments**

*Rag2−/−γc−/−* mice were kindly provided by CIEA and Taconic. All mice were housed and bred in a specific pathogen free animal facility, treated in accordance with the European Union guidelines and approval of the San Raffaele Scientific Institute Institutional Ethical Committee.

Eight-week-old Rag2−/−γc−/− female mice were challenged subcutaneously in the left flank with 10×10^6 MEC1 cells in 0.1 ml of saline through a 27-gauge needle, as previously described22. 10 days later, mice bearing subcutaneous tumour were intraperitoneally injected with a specific schedule (5 days on-2
days off), with 11R-VIVIT (10 mg/kg, dissolved in PBS) or with phosphate buffer solution (PBS-saline) as control.

Animals were monitored twice a week for weight and tumor growth (measuring 3 perpendicular diameters) and sacrificed when the mean tumor volume reached 1000 mm$^3$ or when animals experienced clinical signs and symptoms.

Peripheral blood and/or tissue (spleen, lymph nodes and femoral bone marrow) single cell suspensions were depleted of red blood cells by incubation in an ammonium chloride solution (ACK) lysis buffer (NH$_4$Cl 0.15 M, KHCO$_3$ 10 mM, Na$_2$EDTA 0.1 mM, pH 7.2–7.4). After blocking fragment crystallizable receptors with Fc block (BD Biosciences Pharmingen) for 10 minutes at room temperature to avoid nonspecific binding of antibodies, cells from peripheral blood, bone marrow, peritoneal exudates, lymph nodes (when present) and spleen were stained with anti human CD19 antibody, to investigate the presence of MEC1 cells in the different compartments, and analyzed with a FC500 flow cytometer (Beckman-Coulter).

**Phospho-Flow Cytometry**

ERK1/2 phosphorylation status was analyzed by flow cytometry on processed murine spleens. Briefly, single cell suspensions were fixed in Lyse/Fix Solution (BD Biosciences Pharmingen) for 10 minutes at 37°C. Cells were then washed and permeabilized by using a Perm Buffer (0.5% saponin, 5% FCS, 10mM Hepes) for 20 minutes at room temperature. Cells were washed and incubated with anti human CD19 and anti human pERK1/2 for 30 minutes at 4°C and analyzed with a FC500 flow cytometer. PMA-stimulated cells for 10 minutes at 37°C were used as positive control (data not shown).
**Statistical analysis**

Data were compared using either the paired or unpaired Student’s t-tests or the nonparametric Mann Whitney test. Comparison of survival curves was performed with the use of the log-rank test. Analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA). A *P*-value <0.05 was considered as statistically significant.
Results

Identification of the molecular features of anergy in CLL

Based on our previous observation of the existence of anergic features (i.e. constitutive ERK1/2 phosphorylation and NF-ATc1 activation) in a subset of CLL patients, we started by characterizing an unselected cohort of 52 CLL patients. CLL cases with constitutive phosphorylated ERK1/2 (pERK1/2+) were 23 out of 52, they preferentially showed absence of CD38 expression (p=0.01), low ZAP70 expression (p=0.02) and a more stable clinical course (p=0.007). They also showed an association trend with mutated status of IGHV genes even if this correlation did not reach statistical significance (p>0.05) (Table 1 and Supplementary Figure 1). As expected, at biochemical level constitutive ERK1/2 phosphorylation correlated with higher NF-ATc1 nuclear translocation (Figure 1A) as leukemic cells from the pERK(+) subset showed a mean NF-ATc1 activation of 80.7 ± 8.2 (N=23) while those from the pERK(-) group had a mean NF-ATc1 activation of 48.4 ± 5.9 (N=29) (p=0.004).

We next aimed at evaluating the ability of CLL cells from the anergic subset to mobilize calcium after stimulation with 20 μg/mL of soluble Goat F(ab)2 anti-human IgM, as anergic B cells are characterized by BCR desensitization with consequent unresponsiveness to Ag triggering.

As previously reported and as described in Methods Section, patients were considered as able to signal (so called “signallers”) through the BCR when more than 5% of cells were able to increase the calcium-associated fluorescence. In detail, 13/16 pERK(+) samples were unable to increase intracellular Ca²⁺ upon BCR stimulation and were considered “non signallers” (Figure 1B). In contrast, 12/16 pERK(-) samples fully responded by mobilizing calcium (p=0.0005). Interestingly, ERK1/2 phosphorylation was associated with a lower expression of sIgM (Figure 1C, p=0.03), recalling normal B cells chronically triggered by Ag.
We next aimed at evaluating whether the above-detected features of CLL cells might translate into a higher resistance to apoptosis. To this end, we cultured *in vitro* leukemic cells from 10 pERK(+) and 9 pERK(-) samples and analyzed spontaneous apoptosis after 24 and 48 hours. As shown in Figure 1D, pERK(+) B cells are more protected from spontaneous apoptosis than pERK(-) cells both at 24 (p=0.001) and at 48 hours (p=0.004). This might possibly indicate that persistent *in vivo* BCR engagement may be responsible for maintaining survival in pERK(+) cells.

Functional anergy, as shown by BCR desensitization, is a reversible process that can be reverted by simply culturing cells *in vitro* long enough to allow the removal of BCR occupancy. Thus we next aimed at evaluating whether the biochemical features associated with anergy might also be reversible and might correlate with the changes in the response through the BCR. To this end, we cultured CLL cells from 6 pERK(+) cases and from 4 pERK(-) cases for 48 hours and we started measuring ERK1/2 phosphorylation, NF-ATc1 and sIgM changes over time. Both ERK (Figure 2A) and NF-ATc1 (Figure 2B) activation were lost upon culture and this effect was accompanied by the re-expression of sIgM (Figure 2C). As expected, cultured leukemic cells which had lost the biochemical features of anergy regained the ability to fully respond to BCR triggering (Figure 2A, left panel). On the other hand, no changes were observed in the case of pERK(-) samples (Supplementary Figure 2).

**Targeting anergic BCR signalling pathways: how to reprogram functional response**

Based on these evidences (Figure 2), we then asked whether the direct inhibition of the signalling molecules active in the biochemical program of anergy could also have an effect on the reversal of BCR responsiveness.
To this end, we used different small chemical inhibitors including: a) U0126 and CI1040 that specifically target the activation of MEK1/2, the direct upstream kinase of ERK; b) NMS6E, a new generation ERK1/2 inhibitor; c) a cell permeable version of VIVIT, an inhibitory peptide that blocks NF-AT nuclear translocation. As shown in Supplementary Figures 3A, B, C, treatment with the different compounds is able to block the activation of each specific target after short-term incubation (maximal inhibition observed after 1 hour).

In addition, time course experiments revealed that exposure to VIVIT not only inhibited NF-AT nuclear translocation, but also blocked ERK1/2 phosphorylation (maximal inhibition observed after 4 hours of treatment, Figure 3A). Conversely, treatment with U0126 induced inhibition of NF-AT activation (maximal inhibition at 6 hours, Figure 3B) besides pERK blockade.

To verify whether the direct blockade of ERK and NF-AT might restore the ability of anergic cells to fully respond to antigen-like stimulation, we pre-treated pERK(+) cells with 10µM of U0126 (1 hour, 7 patients samples) and with 10µM of VIVIT (4 hours, 4 patients samples), washed out the inhibitors at the indicated time point, left cell untreated or stimulated with soluble anti-IgM for additional 5 minutes, and measured the activation levels of ERK1/2. Both U0126 (Figure 4A) and VIVIT (Figure 4B) decreased the levels of pERK1/2 and restored the cell ability to respond to anti-IgM stimulation. The same evidence was observed with NF-ATc1 (Figure 4C) as both U0126 and VIVIT inhibited its activation and restored the responsiveness to BCR triggering in the 6 patients tested. As no modification of sIgM was observed over the inhibition time period (data not shown), these results suggest that CLL cells restore their signalling capacity independent of the levels of sIgM expression.
Prolonged blockade of anergic signalling pathways induces apoptosis

To verify whether prolonged blockade of anergic signalling might affect leukemic cell survival especially of the pERK(+) subset, we treated freshly purified leukemic cells for 48 hours with increasing concentrations of MEK1/2 inhibitors (U0126, CI1040), ERK1/2 inhibitor (NMS6E) or NF-AT inhibitor (VIVIT), evaluated cell viability by using a luminescence-based assay and performed dose-response curves (Supplementary Figure 4, two representative samples). In order to analyze the response to treatment and to verify potential differences among patients based on ERK1/2 phosphorylation status, we analyzed the level of cell viability after 48 hours of treatment with 10 μM of each compound.

The use of MEK inhibitors induced a significant decrease of cell viability in pERK(+) patients (Figure 5A: U0126 and Figure 5B: CI1040). In details, pERK(+) samples treated with U0126 had a mean percentage of survival of 36.6% ± 5.2 compared to 60.6% ± 6.6 survival of the pERK(-) subset (p=0.01; n=22). Similarly, pERK(+) samples treated with CI1040 had a mean percentage of survival of 36.6% ± 4.1 compared to 59.2% ± 6.3 survival of the pERK(-) subset (p=0.01; n=17). A smaller cohort of patients (5 pERK(+) and 4 pERK(-)) was treated with an additional MEK1/2 inhibitor, AZD6244 (Supplementary Figure 5), that was shown to be very effective in pERK inhibition in clinical trials for solid tumours24. This inhibitor induced cell death in pERK(+) samples (60.0% ± 4.9 of cell viability in pERK(+) vs 81.5% ± 3.5 in pERK(-), p=0.03).

Likewise also the direct inhibition of ERK1/2 with 10 μM of NMS6E induced a strong decrease of the viability (29.4% ± 8.1 mean survival) compared to pERK(-) samples (68.1% ± 8.8) (Figure 5C; p=0.01; n=16).

In parallel, NF-AT inhibition with VIVIT reduced cell viability in the cells from 14 pERK1/2(+) patients (21.3% ± 4.2 mean survival) as compared to those from the pERK(-) group (20 cases, 42.9 ± 5.5 mean survival, p=0.01)
The effect correlated also with NF-AT activation status (29.5% ± 7.5 a mean survival for NF-AT(+) group of and 53.6% ± 6.1 mean survival for the NF-AT(-), p=0.02; n=20) (Figure 5E).

Taken together, our results demonstrate that the presence of anergic features is associated with the maintenance of cell survival in CLL and that the specific inhibition of the signalling pathways involved in this biochemical program may selectively induce cell death among pERK(+) patients.

As our results (Figure 3A-B) suggested that MAPK and NF-AT are interconnected, we tested whether the concomitant inhibition of both pathways could modify the pro-apoptotic effect on CLL cells. To this end, we treated pERK(+)/NF-AT(+) cells (n=6) with 10 µM of each inhibitor (NMS6E, U0126, VIVIT) alone or in combination and we analyzed cell viability after 48 hours. The simultaneous inhibition of both MAPK and NF-AT signalling did not enhance apoptosis (Figure 5F), thus confirming that both molecules lay on the same signalling pathway of anergy, that is effectively blocked by single inhibitors.

NF-ATc1 inhibition slows leukemia progression in vivo.

To strengthen our in vitro data, we evaluated the effect of NF-AT inhibition in vivo, following VIVIT administration in the xenograft Rag2⁻/⁻γc⁻/⁻ mouse model subcutaneously transplanted with the CLL cell line MEC122, that shows specific features of anergy (constitutive pERK1/2, constitutive NF-ATc1 nuclear translocation). Mice were subcutaneously injected with 10x10⁶ cells and then challenged with VIVIT (10mg/kg) or with vehicle alone starting from day 10 after tumour injection. The effect of the inhibitor was monitored by tumour volume growth. Mice were sacrificed when the tumour volume reached 1000 mm³ or when the animals showed clinical signs and symptoms.
VIVIT administration delayed tumour growth (Figure 6A, day 33, day 38, day 40 p<0.05) and improved mice survival (Figure 6B) the median survival of VIVIT treated group being 52.5 days as compared to the untreated group (46 days; p=0.006).

At the time of sacrifice, organs and tissues (spleen, bone marrow, peritoneal exudate, lymph nodes when enlarged and peripheral blood) were collected, processed and stained for human CD19 to identify human leukemic cells.

To further explore *in vivo* anergy reversal, cells processed from spleen were also stained for pERK1/2 (Figure 6C). VIVIT treatment significantly decreased ERK1/2 phosphorylation in CD19+ cells, indicating that the biochemical anergic program can be reverted also *in vivo*, in a mouse model of CLL, and anergy reversal is associated with a survival benefit for the animals.
Discussion

For many years CLL has been considered a leukemia of functionally inactive B lymphocytes. In apparent contrast, more recently it has been shown that a portion of cases is actually responsive to external stimuli including the activation though the BCR. That notwithstanding, a sizable fraction of CLL patients are indeed characterized by the expansion of clonal B cells that have anergic features. Anergy is one of strategies adopted by the immune system to silence autoreactive B cells, the others being clonal deletion and tolerance. Based mainly on studies in transgenic mouse models it is believed that anergy may be the result of the continuous binding of low affinity self Ag to surface IG that induces the activation of a biochemical program resulting in cell unresponsiveness when B lymphocytes are further triggered via the BCR. Phosphorylation of ERK, together with the nuclear translocation of NF-AT in the absence of AKT activation are the hallmarks of mouse B cell anergy. We have previously shown that such features are present in a subset of CLL patients who have an indolent clinical presentation.

In this paper, we demonstrate that in CLL cells this biochemical signature associates with cellular and functional features of anergy, including in vitro inability to mobilize intracellular calcium upon anti-IgM stimulation and the reduced expression of surface IgM. The latter features are reminiscent of B cells facing chronic Ag-stimulation that favours continuous recycling of B cell receptor.

A recent work demonstrated that NF-ATc1 is constitutively activated in all CLL samples tested. We here show that this activation occurs at variable levels in the different patients. Interestingly, constitutive phosphorylation of ERK1/2 kinases correlates with higher NF-ATc1 activation, suggesting that the different basal levels of NF-ATc1 activation, being higher in the pERK(+) subset (Figure 1A), might induce a different downstream transcriptional program that
culminates in the induction of B cell anergy, due to a “dosage-dependent” activation of the transcription factor and/or to different NF-AT-interacting partners. All these issues could be investigated in the future by the direct analysis of the NF-ATc1 interacting proteins.

In vivo the activation of the biochemical program that culminates in the induction of B-cell anergy is known to be transient as it requires chronic BCR triggering. Accordingly we show here that Ag removal, obtained by culturing CLL cells in vitro, associates with the loss of the biochemical anergic signature (reduction of pERK and nuclear NF-ATc1 levels) along with the re-expression of surface IgM and restores the ability of CLL cells to fully respond to BCR triggering, as previously shown in a mouse model of B cell anergy as well as in CLL. This transient capacity to be anergized after a chronic stimulation through the BCR is independent of and likely successive to the supposedly autonomous signalling occurring in all cases of CLL as recently suggested. The actual relationship between these two signalling activities deserves further investigation.

We also show that by inhibiting the signalling molecules involved in anergy we can revert anergy both in vitro and in vivo, as revealed by the restoration of BCR responsiveness following the loss of ERK phosphorylation and NF-AT nuclear translocation. Reversal of anergy could also be obtained by simply culturing cells for 48 hours after blood withdrawal and this paralleled the delayed onset of the apoptosis among anergic leukemic lymphocytes in vitro. This is in line with the hypothesis that chronic antigen engagement may be responsible also for a prolonged cell survival in vivo. It came then with no surprise that by using of a number of small chemical inhibitors that specifically target, at different levels, the anergy-related pathways we were able to induce apoptosis of the anergic CLL subset, opening a new possibility of therapeutic intervention. This occurred both by blocking MAPK with two different classes
of molecules that target both MEK1/2 and ERK1/2 and by inhibiting NF-ATc1 nuclear translocation with a cell permeable version of VIVIT peptide. Apoptosis occurred at later time points and the use of NF-AT and ERK1/2 inhibitors induced an initial phase of anergy reversal as revealed by the restoration of BCR responsiveness that mirrored the loss of ERK phosphorylation and NF-AT nuclear translocation at biochemical level.

We additionally tested the effect of NF-AT inhibition by administering VIVIT in xenograft model of CLL with Rag2−/−γc−/− mice subcutaneously injected with MEC1 cell line22. MEC1 cells have anergic features as they display constitutive ERK1/2 and NF-ATc1 activation, high basal level of calcium and general unresponsiveness to BCR triggering together with low levels of surface IgM. The reason why features typical of a reversible biochemical program (anergy) are maintained in a cell line without any apparent ongoing antigen stimulation is unclear. A plausible explanation is that yet unknown gene abnormalities acquired during the immortalization process may maintain the anergic program in an antigen-independent fashion. Alternatively it might be speculated that self antigens able to stimulate the MEC1 BCR are released by apoptotic cells in culture,

Mice treated with VIVIT had a delay in tumour growth and an increased survival compared to the untreated group. Of interest, leukemic cells from treated mice showed a decreased expression of ERK1/2 phosphorylation, thereby paralleling and confirming our in vitro findings. These data further support the idea that blocking anergic pathways may be highly effective not only in vitro but also in vivo with an obvious potential clinical implications at least for the subset of patients whose cells are characterized by anegic features. The use of small molecules inhibitors is now becoming extremely promising (see BTK and PI3K inhibitors) due to the specific targeting and the limited side effects. For this reason, anergy reversal could be envisaged of potential use in
the sizable fraction of patients who have a rather indolent still progressive disease. It may be asked whether the risk exists that the reversal of B cell anergy in vivo might lead to a more aggressive disease, considering that absence of ERK1/2 phosphorylation is associated with a worse prognosis. However, according to our findings it is reasonable to believe that blocking B cell anergy in vivo would trigger an even higher apoptosis rate than that observed in vitro. This effect could be due to the combination of two different mechanisms: on one side the inhibition of a survival program (anergy) and on the other side the restoration of the signalling capacity, the latter being involved in the induction of apoptosis in CLL, as previously reported.

In conclusion, our results demonstrate that a subset of CLL patients, identified by the constitutive ERK1/2 phosphorylation, is characterized by the abnormal expansion of B cells with anergic properties. In these cells the constitutive activation of ERK and NF-ATc1, leading to functional unresponsiveness, can be efficiently targeted and cells regain their ability to respond to BCR stimulation. More importantly, B-cell anergy reversal is followed by apoptosis induction possibly opening new perspectives in the clinical management of these patients.
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Autorship contribution
B.A. designed the study, performed in vitro and in vivo experiments, analyzed the data and wrote the manuscript; C.S. performed in vitro experiments; M.T.S.B. performed in vivo experiments; E.T.N. performed in vitro experiments; L.S. provided patients samples and clinical data; P.R. performed in vitro experiments; F.S. and G.P. assisted in writing the manuscript; P.G., M.M. and F.C.C. designed the study, analyzed the data and wrote the manuscript.

Conflict-of-interest disclosure
The authors declare no competing financial interests.

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REFERENCES


Table 1

<table>
<thead>
<tr>
<th></th>
<th>pERK1/2(+)</th>
<th>pERK1/2(-)</th>
<th>p value</th>
</tr>
</thead>
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<tr>
<td>CD38 positive (≥30%)</td>
<td>10.5% (2/19)</td>
<td>44.4% (12/27)</td>
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<tr>
<td>Unmutated IGHV genes</td>
<td>42.6% (8/19)</td>
<td>57.7% (15/26)</td>
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<td>ZAP70 positive (≥20%)</td>
<td>35.3% (6/17)</td>
<td>66.7% (18/27)</td>
<td>0.02</td>
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<td>Disease progression</td>
<td>26.7% (4/15)</td>
<td>76.5% (13/17)</td>
<td>0.007</td>
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Figure legends

Figure 1. *Molecular determinants of B cell anergy.* A) *ERK1/2 constitutive phosphorylation significantly correlated with NF-ATc1 nuclear translocation* (*p*=0.004). Constitutive ERK1/2 and NF-ATc1 activation (each normalized to specific positive controls) were evaluated in a cohort of 52 freshly purified samples. Samples were grouped based on the ERK1/2 phosphorylation status (23 pERK(+), with relative % pERK>10%, and 29 pERK(-), with relative % p-ERK<10%) and the percentage of NF-ATc1 activation was reported on the y axis.

B) *ERK1/2 activation and calcium flux.* Leukemic cells from 32 patients were labeled with the calcium-sensitive dye Fluo-3–AM and analyzed by flow cytometry before and after addition of F(ab’)2 anti-IgM. Samples were grouped based on the ERK1/2 activation status. Graph shows individual data point and means (horizontal line). Data were analyzed by using Mann-Whitney t test (*p* value is indicated).

C) *ERK1/2 activation and IgM expression.* Leukemic cells from 24 patients were analyzed for surface IgM expression (MFI) and grouped based on pERK1/2 status. Graph shows individual data point and means (horizontal line). Data were analyzed by using Mann-Whitney t test (*p* value is indicated).

D) *Anergic cells display an increasing survival in vitro.* Anergic signature correlates with increased in vitro survival of CLL cells. Freshly purified leukemic cells (10 pERK(+) and 9 pERK(-) samples) were cultured for 24 or 48 hours and cell viability was analyzed by Annexin/PI staining. Samples were grouped based on the ERK1/2 activation status and data were analyzed by using Mann-Whitney test (*p* value is indicated).

Figure 2. *Biochemical signature of B cell anergy is a reversibile event.* Cells from 6 different anergic samples were cultured for 48 hours. ERK1/2
phosphorylation (Figure 2A), NF-ATc1 nuclear translocation (Figure 2B) and IgM expression levels (Figure 2C) were analyzed both immediately after purification and after 48 hours of cell culture. Each Figure shows one representative case (left panel) and the dot plot analysis of all the analyzed samples (right panel).

Figure 3. Small chemical compounds: analysis of target inhibition.

A-B) CLL cells were treated with 10uM of U0126 or VIVIT for increasing time points (as indicated). ERK1/2 activation was measured by western blot (Figure 3A) and NF-ATc1 nuclear translocation was quantified by specific ELISA assay (Figure 3B). NF-ATc1 values are normalized to the untreated control.

Figure 4. Signalling inhibitors: how to reprogram antigen response.

Inhibition of both ERK1/2 and NF-ATc1 by treatment with U0126 and VIVIT respectively, reverse the anergic phenotype and restore the ability of CLL cells to respond to anti-IgM stimulation in vitro. A-B) CLL cells were treated with U0126 (1 hour, Figure 4A) or with VIVIT (4 hours, Figure 4B), washed and left untreated or stimulated for additional 5 minutes with anti IgM antibodies. The phosphorylation status of ERK1/2 was analyzed by western blotting and band intensity was quantified by densitometry. pERK1/2 values are normalized to the control-untreated sample. C) CLL cells were treated with U0126 (6 hours) or with VIVIT (2 hours), washed and stimulated for additional 20 minutes with anti IgM antibodies. NF-ATc1 activation was measured by specific ELISA assay. NF-ATc1 values are normalized to the untreated control.

Figure 5. Longer blockade of anergic signalling induces apoptosis in the pERK(+) subset. A) Leukemic cells were left untreated or treated for 48 hours with 10uM of U0126 and cell viability was measured by using a luminescent-
based cell viability assay. Each value from treated samples was normalized to the untreated control, and samples were grouped based on the ERK1/2 activation status. Data were analyzed by using Mann-Whitney t test (p value is indicated).

**B)** Leukemic cells were left untreated or treated for 48 hours with 10uM of CI1040 and cell viability was measured by using a luminescent-based cell viability assay. Each value from treated samples was normalized to the untreated control, and samples were grouped based on the ERK1/2 activation status. Data were analyzed by using Mann-Whitney t test (p value is indicated).

**C)** Leukemic cells were left untreated or treated for 48 hours with 10uM of NMS6E and cell viability was measured by using a luminescent-based cell viability assay. Each value from treated samples was normalized to the untreated control, and samples were grouped based on the ERK1/2 activation status. Data were analyzed by using Mann-Whitney t test (p value is indicated).

**D-E)** Leukemic cells were left untreated or treated for 48 hours with 10uM of 11R-VIVIT and cell viability was measured by using a luminescent-based cell viability assay. Each value from treated samples was normalized to the untreated control, and samples were grouped based on the ERK1/2 (Figure 5D) or NF-ATc1 (Figure 5E) activation status. Data were analyzed by using Mann-Whitney t test (p value is indicated).

**F)** ERK1/2 and NF-ATc1 inhibitors do not exert any synergistic effect. Samples from 9 anergic CLL patients were treated with 10 uM of each inhibitor (U0126, NMS6E, VIVIT) or with a MAPK inhibitor in combination with VIVIT for 48 hours and cell viability was measured. Viability is expressed as percentage of the untreated control.

**Figure 6. 11R-VIVIT slows leukemia progression in CLL xenograft model.**
MEC1 CLL cell line was injected subcutaneously into Rag2^−/−γc^−/− mice. Mice
were challenged with 11R-VIVIT (daily intraperitoneal administration of 10mg/Kg) or with vehicle alone starting from day 10 after tumour injection. 

**A) In vivo VIVIT administration reduces tumour growth.** Tumour growth curves obtained in Rag2−/−γc−/− mice that received a transplant subcutaneously in the left flank of MEC1 cells (10×10^6 cells). Ten days later, mice bearing MEC1 tumour were randomly assigned to one of the following daily intraperitoneal treatments: saline solution (PBS, n=7) or 10mg/Kg VIVIT (n=8). Tumour size was evaluated by measuring perpendicular diameters by a caliper. Animals were sacrificed when the tumor volume reached 1000 mm^3 or when animals showed clinical signs and symptoms. Measurements were stopped when 75% of originally treated mice were still surviving. Statistical analysis was performed using the Student’s T test (day 33, day 38 and day 40 PBS vs VIVIT, p<0.05).

**B) In vivo VIVIT administration improves mice survival.** Kaplan-Meier survival plot for Rag2−/−γc−/− mice challenged subcutaneously in the left flank with MEC1 cells (10×10^6 cells). Ten days later, mice bearing MEC1 tumor were randomly assigned to one of the following daily intraperitoneal treatments: saline solution (PBS, n=15) or 10mg/Kg VIVIT (n=14). Animals were sacrificed when the tumor volume reached 1000 mm^3 or when animals showed clinical signs and symptoms (median survival of untreated group 46 days, median survival of VIVIT treated group 52,5 days, p=0.006). Data is from three independent experiments.

**C) In vivo anergy reversal: pERK1/2.** pERK1/2 expression on MEC1 cells present in the spleen of animals injected with the cell line. At time of sacrifice, spleens were collected, processed and stained for CD19 and phosphorylated ERK1/2, as described in Material and Methods Section. Statistical analysis was performed using Student’s T test.
FIGURE 2

A

B

C
FIGURE 4

A

B

C
FIGURE 5

A  B  C

D  E

F
Targeting B cell anergy in chronic lymphocytic leukemia

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