Grade's final project

# *Ex vivo* study of the effect of an anti-proliferative drug in Chronic Lymphocytic Leukemia cells mimicking proliferative niches microenvironment

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# 1. INTRODUCTION

### 1.1 Definition and prognostic markers

Chronic lymphocytic leukemia (CLL), the most common sort of adult leukemia in western countries, is a neoplastic disease characterized by the accumulation and clonal proliferation of immunoincompetent, mature-appearing B lymphocytes in peripheral blood (PB), bone marrow (BM) and secondary lymphoid tissues such as lymph nodes (LN) and spleen.[1] The monoclonal population of CLL cells immunophenotype is unique and clearly characterized by the expression of low levels of surface immunoglobulins IgM/IgD, surface markers CD19 and CD20 (particular B cell linage biomarkers) and the concomitant expression of CD5, and CD23 (crucial for not confusing CLL with other B cell chronic lymphoproliferative disorder).[2] Nevertheless, they do not express CD79b (or it is only expressed by less than 5% of the CLL cases).[3,4,5]

The course of the disease is variable, some patients diagnosed with CLL can hold a normal life span whereas others develop early symptoms of BM failure, repeated infections, and transformation into more aggressive forms of the disease. This last group tend to die within five years after diagnosis.[6] Identification of clinical and laboratory features associated with disease progression and the likelihood for a favourable response to therapy could allow for risk-adapted management of patients with CLL that ultimately could improve outcome.

The mutational status of immunoglobulin heavy variable (IGHV) genes became the first biological marker with clinical prognosis value at the moment of diagnosis. The expression of mutated IGHV genes is correlated with a longer time to clinical progression, longer time to treatment and a prolonged median survival.[7,8] The explanation why CLL cells carrying V-gene mutations have more favourable prognostic markers than unmutated CLL cells rest, on one hand, on the fact that mutated CLL cells own an inability to bind antigens due to changes in the conformation of B-cell receptor (BCR) caused by IGHV mutations (antigen does not fit BCR, driving to a "clonal ignorance"). On the other hand, these mutations are also able to damage the BCR signalling despite an adequate fit of the antigen, fact which will also entail a CLL cell less capable of survive, proliferate and prevent apoptosis.

CLL cells expressing unmutated IGHV genes can be differentiated from those that use mutated IGHV genes through the discordant expression of a small subset of genes.[9] One of these genes encodes the cytoplasmatic tyrosine kinase Zeta-chain-

associated protein of 70 kDa (ZAP-70). ZAP 70 is a major component of the signalling cascade downstream of the T cell receptor (TCR), and the Fc receptor (FcR) in[10] NK cells. Nonetheless, subsequent investigations  found that unmutated CLL cells regularly expressed levels of ZAP-70 protein that were comparable to those present in normal blood T cells.[10,11] In contrast, mutated CLL cells generally do not express detectable levels of ZAP-70 protein. Therefore, ZAP-70 expression found in  leukemia cells can be used as a surrogate marker for IGHV mutational status[12] which can segregate patients that have significantly different tendencies for disease progression.

Although ZAP-70 expression in CLL has an adverse prognostic influence, its role in the biology of the tumoral B cell is not fully defined. In this regard, the expression of ZAP-70 protein in CLL cells has been related to an enhanced BCR signaling.[13,14]

### 1.2 BCR and signal transduction

Signal transduction in cells of the immune system by BCRs, TCRs and FcRs is accomplished by a conceptually similar mechanism. Currently, it is postulated that, in an appropriate microenvironment, the BCRs of CLL  lymphocytes become engaged by foreign microbial or autoantigens[15,16] which, in association with other co-stimulatory signals, trigger specific signalling cascades to the nucleus of the cells supporting CLL clone expansion.  Interestingly, there are evidences submitting that a certain type or a similar group of antigens are relevant to CLL pathogenesis, since BCR's IGHV gene sequences against concrete antigens have been found in a percentage of the patients much higher than that which could be randomly expected in subsets of patients.

BCR is constituted by an antigen-specific membrane immunoglobulin and the non- covalently bound, disulfide-linked heterodimers Ig-α/Ig-β (CD79α/CD79β). Upon antigen recognition, BCR induces phosphorylation of the immunoreceptor tyrosine activating motifs (ITAMs), present in the cytoplasmatic tails of CD79α/CD79β, through Src family kinases such as Lyn.[17] Consecutively, spleen tyrosine kinase (Syk) is recruited and bound to the ITAMs via ITAMs' phosphotyrosine and Syk's Src homology 2 (SH2) domain, where is phosphorylated (necessary step since neither the TCR nor the BCR complex have intrinsic protein tyrosine kinase (PTK) activity). After activation, Syk phosphorylates multiple signal intermediates including Bruton's tyrosine kinase (Btk, member of the Tec Kinases family) in association with the B cell linker protein (BLNK), which is responsible for downstream signalling molecules  activation such as Raf, mitogen-activated protein kinase (MEK), extracellular signal-regulated kinase (ERK) and nuclear factor-kB (Figure 1). Phosphoinositide 3-kinase (PI3K) is activated

by the coordinated action of Syk and Btk, stimulating its downstream pathway, which includes AKT kinase phosphorylation[18,19], activation that allows NFAT functionality.
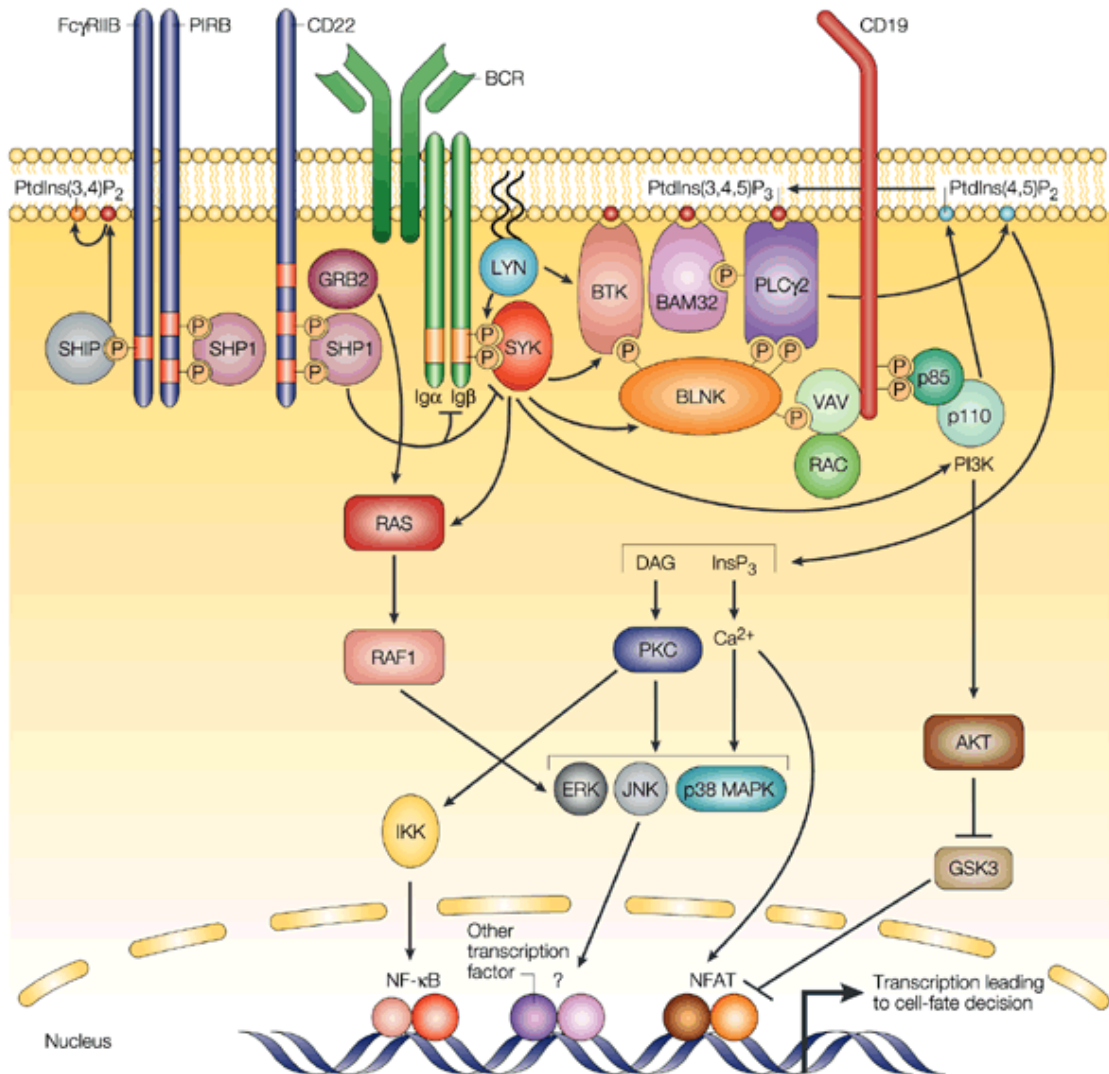


***Figure 1. Schematic representation of B lymphocyte signalling pathways involved in cell survival, development and proliferation.*** *Syk is an essential trigger for B lymphocyte downstream signalling implicated in several downstream kinases activation such as BTK, BLNK, PI3K and ERK. These kinases contribute to avoid cell death through apoptotic proteins inhibition and to cell proliferation stimulating certain transcription factors like NF-κB.*

The crucial role of ZAP-70 in proximal signalling after TCR engagement and its high homology with Syk, prompted the idea that ZAP-70 may augment signalling through BCR, thus, providing a biological explanation for the more aggressive clinical

outcome of the ZAP-70 positive subgroup of patients. As mentioned above, the specific molecular mechanisms underlying the role of ZAP-70 in BCR signalling remain largely unknown. In several reports it was shown that the requirement for ZAP-70 expression was not absolute in BCR activation pathway, as some CLL cases not expressing ZAP-70 were able to respond to BCR stimulation equally well as ZAP-70 positive ones.[20,11].

## 1.3 Changing overview, CLL as an active proliferative leukemia

CLL has historically been attributed to a process of deficient apoptosis rather than a B lymphocyte increased proliferation, since CLL cells show high levels of expression of anti-apoptotic proteins such as Bcl-2,[21] Bcl-x$_L$, Mcl-1 and Bag-1 and low levels of the pro-apoptotic proteins Bax or Bcl-x$_S$.[22] However, recent clinical and biological observations are questioning this concept. Clinically, the different prognostic between patients[23] (a third of the CLL patients suffer an aggressive disease and present a fatal course) and the divergences in lymphocyte counts between patients (in some of them shorter lymphocyte duplication time is exhibited), among others, suggest the existence of cellular proliferation phenomena, which could be crucial in the CLL activity and clinical progression. From the biological standpoint, several studies have shown the importance of proliferation; Messmer and cols. performed the first study of in vivo CLL cell kinetics [Messmer BT, J Clin Invest, 2005] suggesting that not only leukemic cells are dividing, but also that they do it in a higher rate than normal B lymphocytes. Recent studies proved that the size of the proliferative compartment is associated with several adverse prognostic factors, both clinical and biological (ZAP-70, CD38 and CD49d expression). This had an important impact on the time to require treatment and overall survival [Giné E, Haematologica, 2010]. Besides, by histological studies, a group of patients with a higher active proliferation centers in LN (expanded pseudofollicles with a higher number of mitosis or Ki-67 expression) was identified; the presence of these expanded and/or highly active proliferation centers was associated with an aggressive clinical behaviour. Finally, recent data about gene expression profiling has evidenced a higher expression of genes related to proliferation in LN and BM than in PB [Herishanu Y, ASH annual meeting abstracts, 2009], which corroborates the important role of microenvironment in proliferation and disease maintenance.

## 1.4 CLL cells microenvironment

The relevance of microenvironment signals is shown by the incapacity of CLL cells to avoid spontaneous apoptosis *in vitro* in the absence of certain stimuli (eg. CD40 ligand or IL-4) despite CLL cells are able to have a long half-life *in vivo*,[24,25] which suggests that such co-stimulatory signals and contact with non-leukemic cells (CD4+ T cells, stromal cells (BMSCs), follicular dendritic cells (FDC) or "nurse-like" cells (NLC)) play a role in CLL cells survival in vivo and even in the response to treatment (Figure 2).[26] Mentioned stimuli are mediated by soluble cytokines which interact either with BCR or other receptors such as CD40 or Toll-like receptors (TLRs). TLRs seem to play an important role in CLL disease, since they are capable of recognizing different molecular patterns from antigens and, therefore, promote B lymphocytes maturation and proliferation. CLL cells express larger amount of TLR9 in particular, which can be stimulated by oligodeoxinucleotides-CpG inducing cellular proliferation, cytokine production and an immunogenic phenotype in a variable range.[27] Signals triggered by CD40L have been shown to be critical for the function of B lymphocytes at multiple steps of the T cell–dependent immune response.
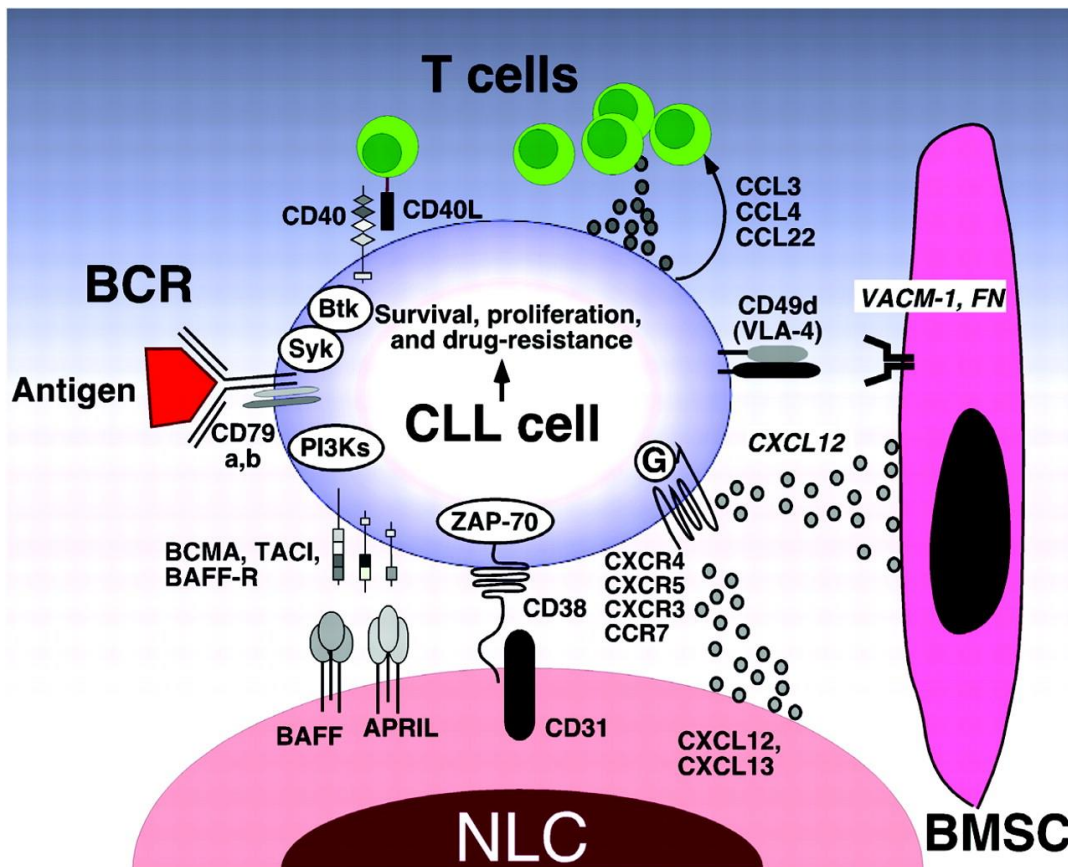


***Figure 2. Molecular interactions between CLL cells and microenvironment.***
*Molecular interactions between CLL and BMSCs cells in the BM and lymphoid tissue*

*microenvironments that are considered important for CLL-cell survival and proliferation, CLL-cell homing, and tissue retention.*

There is growing in vitro [Burger JA, Blood, 1999; Burkle A, Blood, 2007] and in vivo [Sipkins DA, Nature, 2005; calissano C, Blood, 2008] evidence suggesting that chemokine receptors and adhesion molecules expressed on CLL cells are responsible for their trafficking between the blood and the tissues, and their homing and/or retention within these tissue microenvironments, guided by accessory cells that establish gradients of the respective ligands. Stromal cell-derived factor 1 (SDF-1, a CXC chemokine also called CXCL12) becomes a potent chemokine able to bind the plasma membrane and form a concentration gradient embracing BMSCs, which secrete high levels of this factor. Having settled that CLL cells express functional receptors for CXCL12 (CXCR4), among other chemokine receptors as CXCR3 or CXCR5, some investigations have proved the existence of CLL cells mobilization *in vitro* linked to CXCL12 exposure, which triggers endocytosis of CXCR4, mobilization of calcium, actin polymerization and chemotaxis under physiological flow conditions. Furthermore, there are other important chemoretractants such as Chemokine (C-C motif) ligand 21 (CCL21) and (CCL19) which also play an important role in B and T cell migration to secondary lymphoid organs by binding to its chemokine receptor CCR7[28] and C-X-C motif chemokine 13 (CXCL13) also known as B lymphocyte chemoattractant (BLC), secreted by NLCs and stromal cells, regulates lymphocytes homing and positioning within lymph follicles interacting with its receptor CXCR5.[29]

### 1.5 Conventional treatment and new approaches

What has been said above, and unlike the ancient hypothesis, highlights the importance of cellular proliferation in CLL, fact that propose that new assays testing drugs that inhibit the proliferative component of CLL cells should be performed. The majority of tested drugs, until recent years, have been investigated using CLL cells cultured in suspension or using apoptosis-protecting models. On the other hand, xenograft models lack of interactions with T and NK lymphocytes, since they are immunodepresive animals.[30] Moreover, transgenic animals ( transgenic of TCL-1 or deleted of Leu2/mir15a/16-1)[31] do not reflect the molecular pathogenesis of the disease.

At present, there is no curative therapy for CLL. Due to the extremely variable clinical course of CLL patients, finding a cure for this type of leukemia is a remarkable

challenge. Nowadays, the major operative therapeutic regimens in use for CLL treatment consist of conventional chemotherapy combinations with rituximab, the monoclonal antibody against CD20. Nonetheless, these treatments are compromised in a subset of patients with certain genetic aberrations.[32] Furthermore, these regimens have been registered as provoking significant toxicity.

Alternatives to conventional treatments are purine analogs such as Fludarabine (Fludara) (Sigma), which are defined as the most efficient active group of drugs against CLL until present. These drugs own a complex mechanism of action including induction of apoptosis.

Notwithstanding the molecular mechanisms responsible for BCR signalling regulatation are not entirely defined. Several inhibitors which have their target in enzymes involved in BCR signaling have been developed in recent years and most of them are in clinical trials today. Some examples are GS-1101 (formerly CAL-101 (Calistoga Pharmaceuticals )) (small molecule that selectively can inhibit the delta isoform PI3K),[33] Ibrutinib ( also called PCI-32765 (FiercePharma )[34,35] and AVL292 (Avila Therapeutics)[36] (Btk inhibitors) and Sorafenib (Bayer) (acts blocking ERK pathway).[37,38,39]

Novel studies using Syk deficient mice highlight the central role of Syk for B cell malignancies survival and development,[40,41,42] proposing that the Syk kinase could be a potential rational target for therapeutic intervention. Selective Syk inhibitors already exists. The most popular is Fostamatinib (also called R406, FosD or R788 in its oral formulation) (Rigel Pharmaceuticals)[43,44] which is in clinical trials at present. FosD is launching objective responses rates of 55% for CLL patients.[45,46]

In this study, a possible alternative syk-inhibitor drug (TAK659) is tested in preclinical trials.  TAK659 mechanism of action consist of blocking Syk kinase activity by coupling it's active ingredient to Syk's tyrosine kinase domain, expecting, hence, a decrease in CLL B cells proliferation and survival, as well as less chemotaxis toward tissue homing chemokines.

# 2. OBJECTIVES

## 2.1 General objective

Evaluation of the action of an anti-proliferative drug against CLL cells in an *ex vivo* system which reproduces the cellular microenvironment in proliferative niches.

## 2.2 Specific objectives:

1. *Ex vivo* reproduction of antiapoptotic and proliferative stimuli coming from CLL cells microenvironment.
2. Proliferative and apoptotic rates analysis of drug treated CLL cells.
3. Correlation of the response grade with the observed effect of the anti proliferative drugs in their therapeutic targets.

# 3. MATHERIALS AND METHODS

## 3.1. CLL samples

### 3.1.1. Isolation and culture of primary CLL cells.

27 patients diagnosed with CLL were included in this study, which was approved by the ethic committee for clinical experimentation (CEIC) from the institution where the project was executed (VHIR). A written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Paque Plus (GE Healthcare) density gradient, washed twice with PBS, resuspended in FBS with 20% DMSO and stored in liquid nitrogen until analysis. Only samples with >85% of CLL cells (CD19+/CD5+ cells, as assessed by flow cytometry (FC)) were incorporated in the study.

### 3.1.2.Clinical and biological data gathering from patients.

Clinical and biological data from all patients diagnosed in CLL have been collected from standard diagnostic checking. Registered parameters are the following:

sex, age, symptoms, ganglionar damaged areas, lymphocytary duplication time, morphology and immunophenotype, marrow infiltration grade, Rai/Binet state, LDH, ß2microglobuline, sCD23, sTK, ZAP-70, CD38, conventional cytogenetics and chromosomical alterations studies(del13q, +12, del11q y del17p).

### 3.1.3.Cell lines.

UE6E7T-2 cell line, derived from mesenchimal human cells (BMSC) from bone marrow was obtained through RIKEN cell bank. Cells were cultured at 37º in 5% $CO_2$ atmosphere in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 2mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/mL penicillin/streptomycin. The T-cell acute lymphoblastic leukemia cell line Jurkat and Burkitt's lymphoma cell line Ramos were obtained from ATCC and cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/mL penicillin/streptomycin. Both cell lines were used as positive controls.

### 3.1.4. Co-culture conditions.

BMSCs were seeded at a concentration of $1,5x10^4$ cells/mL in 24-well plates and incubated for 24hours to allow cells adherence. CLL cells were cultured at a ratio of 100:1 ($1,5x10^6$ cells/mL) on confluent layers of BMSCs in supplemented RPMI with 1μg/mL CD40-L (P eprotech) and 1,5 μg/mL CpG ODN (ODN2006; invitrogen). 10μg/mL of anti-Human IgM (Gt F (ab') 2 fraction)(invitrogen) was also added in determined wells. At the indicated time points, CLL cells were harvested by gently washing off, leaving the adherent stromal cell layer intact.

### 3.2. Reagents.

TAK659 (kindly provided by Millenium), Fludarabine (Sigma) and R406((Fostamatinib o R788 y R406, Selleckchem) were dissolved in DMSO (Dimethyl sulfoxide) and stored at -80ºC.

### 3.3 Experimental design

#### 3.3.1. Western blotting assays.

Whole cell protein extracts were prepared from $3x10^6$ cells using 50μL lysis buffer containing 20 mM Tris (hydroxymethyl) aminomethane (Tris) pH 7.4, 1 mM EDTA, 140 mM NaCl, 1% NP-40 supplemented with 2mM sodium vanadate and

protease inhibitor cocktail (Sigma-Aldrich) for 1 hour at 4ºC. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad). 25 µg of denatured protein were resolved by 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Membranes were blocked for 1 hour at room temperature (RT) in 5% non-fat milk (BD) dissolved in TBS-T( containing 1M Tris pH 7.5, 5M NaCl and 10% Tween 20) or 5% phosphoblocker (Cell Biolabs)/TBS-T. Membranes where incubated overnight at 4ºC with primary antibodies against phosphorylated-Syk/phosphorylated-Zap (Cell Signaling Technology), GAPDH and alpha-actin (Abcam), total Syk and Zap proteins (Upstate and Zymed respectively), phosphorylated ERK and total ERK protein (Cell Signaling Technology), phosphorylated AKT  and total AKT protein (Cell Signaling Technology). Immunodetection was done with the corresponding IgG HRP-linked secondary antibodies (Dako North America), and the detection systems ECL chemiluminescence detection system (GE Healthcare) (for total specific proteins detection), and SuperSignal West Pico Chemiluminescent Substrate detection system (for phosphorylated proteins) (Thermoscientific, Pierce). Chemiluminescent images were acquired with the LAS-4000 system (GE).

### 3.3.2.  Assessment of apoptosis.

Apoptosis was assessed analyzing the binding of annexin V-FITC (fluorescein isothiocyanate ) and the incorporation of Propidium iodide (PI) by FC. Staining was performed according to the manufacturer's instructions using the annexin V-FITC apoptosis detection kit (Bender Medsystems). Results were analyzed using the FCS Express 4 software (De Novo Software).

### 3.3.3.  Proliferation assays.

The rate of proliferation was analyzed by determining the expression of Ki-67 by FC. Intracellular staining was performed using a FITC-labelled antibody against Ki-67 (Becton Dickinson) after fixation and permeabilization using the BD Intrasure kit (Becton Dickinson) following the manufacturer's instructions. Results were analyzed using the FCS Express 4 software (De Novo Software).

### 3.3.4.  Chemotaxis assays.

Migration to the chemokine CXCL12 and to CCL19 was determined in primary CLL cells from 5 patients by using a transwell migration assay across bare polycarbonate membranes (Corning, New York, NY, USA). A total of 100 µL of RPMI-10% FBS containing $1 \times 10^6$ cells was added to the top chamber of a 24-mm-diameter transwell culture insert with a pore size of 5 µm. For migration toward CXCL12, 600 µL

of RPMI-10% FBS alone or with 200 ng/mL of CXCL12 were added to the lower chamber. For migration to CCL19, 600 µL of RPMI-10% FBS alone or with 0.1 µg/mL of CCL19 were added to the lower chamber.  Chambers were incubated for 6 hours at 37ºC in 5% $CO_2$ and cells in the lower chamber were counted with a Navios cytometer under a defined flow rate for 5 minutes. The migration index was calculated as the number of cells transmigrating with chemokine divided by the number of transmigrating cells with control medium only.

### 3.4. Statistical analysis

Results are expressed as the mean +/- standard error of the mean (SEM) of at least three independent experiments. The statistically significant difference between groups was analyzed using the Mann-Whitney test or one or two-way ANOVA (t test), and pvalue<0.05 was considered significant. Lethal dose 50 ($LD_{50}$) values were calculated with GraphPad Prism software version 5.0(San Diego, CA, USA). Analyses were performed using the biostatistics software package SPSS version 17 (IBM, Chicago,IL, USA). Results were graphed with GraphPad Prism software.

# 4. RESULTS

**The co-culture of primary CLL cells with BMSCs, CD40-L, CpG ODN and anti-IgM induces active proliferation and survival of CLL cells**

CLL cells placed in BM and secondary lymphoid tissues can receive diverse survival and proliferative signals from the microenvironment, thanks to which, they become more resistant to external apoptotic signals. With the aim of partially *ex vivo* mimic the microenvironment found in the proliferative niches, primary CLL cells were co-cultured with the mesenchimal cell line UE6E7T-2 and stimulated them with soluble CD40-L, CpG ODN and anti-IgM. Proliferative responses assessed by Ki-67 expression were already observed after 24 hours and further notably increased after 48 hours of co-culture (Figure 3A). A decreased percentage of Ki-67 positive cells was observed when adding anti-IgM in the co-culture system. A possible biological explanation is that BCR stimulation triggers cell survival rather than cell renewal. This hypothesis is supported by the apoptosis assay, CLL cell survival is increased when anti-IgM is added when compared with CLL cells in co-culture without BCR stimulation (Figure 3B).
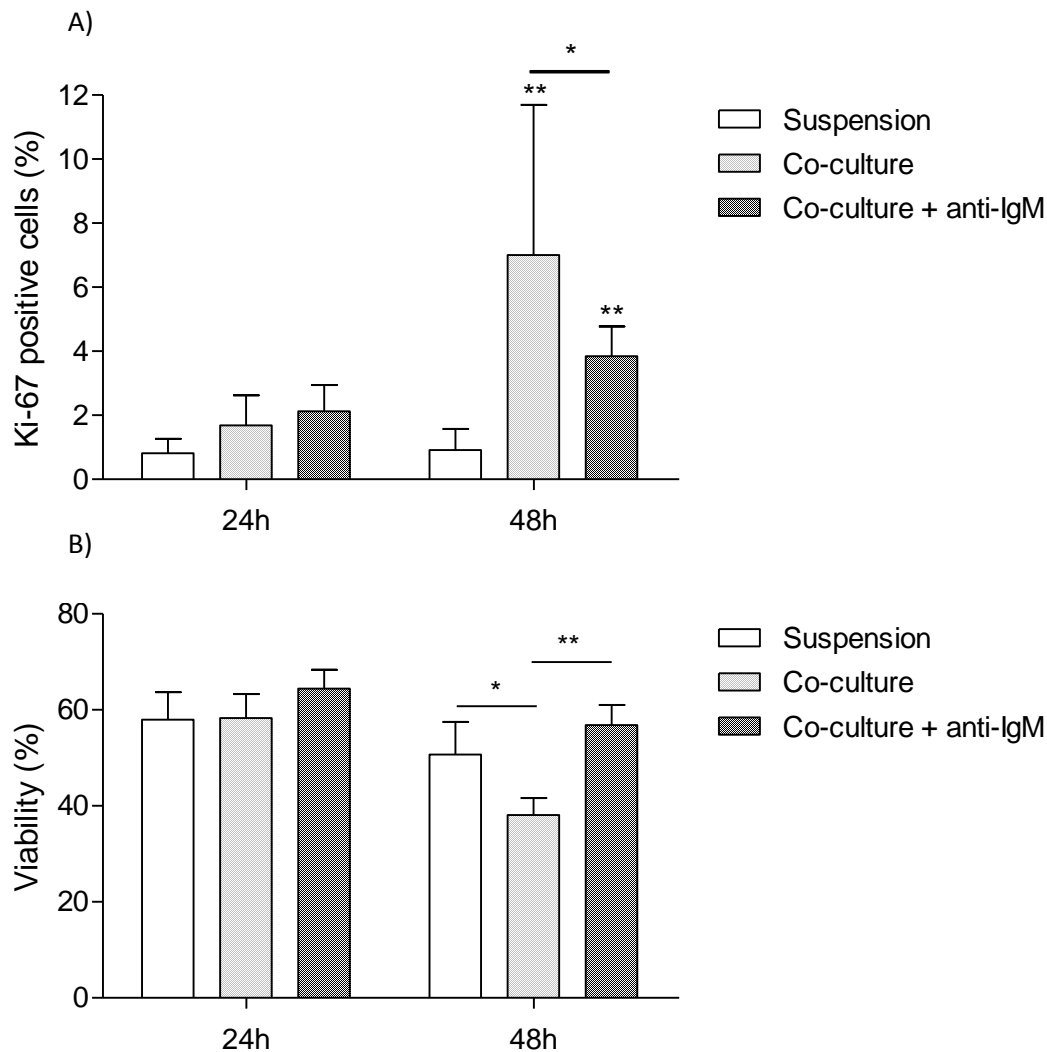
***Figure 3. The co-culture of primary CLL cells with BMSCs, CD40L and CpG ODN induces active proliferation and survival of CLL cells****. CLL cells from 10 patients were cultured in suspension or in co-culture with or without anti-IgM for 24 and 48 hours. Fig.3A) Analysis of Ki-67 expression by FC. Fig.3) Analysis of apoptosis in 10 patients assessed by annexin V-FITC binding and the incorporation of propidium iodide by FC. Annexin V/PI double negative cells were considered viable cells. Graphs show mean ± SEM (\*P<0.05, \*\*P<0.01, two-way ANOVA, Bonferroni post-test).*

## SYK inhibition with TAK659 abrogates BCR-signaling in primary CLL cells

To determine the efficacy of TAK659 in blocking BCR downstream signaling, was analyzed phosphorylation of Syk and other downstream kinases of the BCR pathway (ERK1/2 and Akt) by western blotting after stimulate co-cultured CLL cells with anti-IgM prior one hour of incubation with increasing doses of TAK659.

As depicted in figure 2, CLL cells did not display baseline activation of Syk, Akt and ERK1/2. The co-culture of CLL cells with the addition of anti-IgM induced Syk and

downstream kinases phosphorylation, supporting the microenvironment relevance for CLL survival and proliferation. Interestingly, short treatment of CLL cells with increasing concentrations of TAK659 resulted in a dose-dependent reduction in Akt and ERK1/2 phosphorylation. In contrast, an increase in the amount of Syk phosphorylated at Y352 was observed in these experiments at higher TAK659 concentrations. Since this site in Syk is phosphorylated by Lyn, the most likely explanation for this finding is that Syk is involved in a negative regulatory circuit that inactivates Lyn after BCR engagement.

On the other hand, ZAP-70 expression in both patients was analyzed and a differential expression of this protein between these patients was observed. Patient number 09 displayed an evident higher expression of ZAP-70 protein than patient number 30, in agreement with previous unreported FC analysis. As mentioned above, ZAP-70 has been related with an enhancement of BCR signaling which may explain the need for higher TAK659 doses for decrease Akt phosphorylation or even the incapability of decrease ERK1/2 phosphorylation in patient number 09.
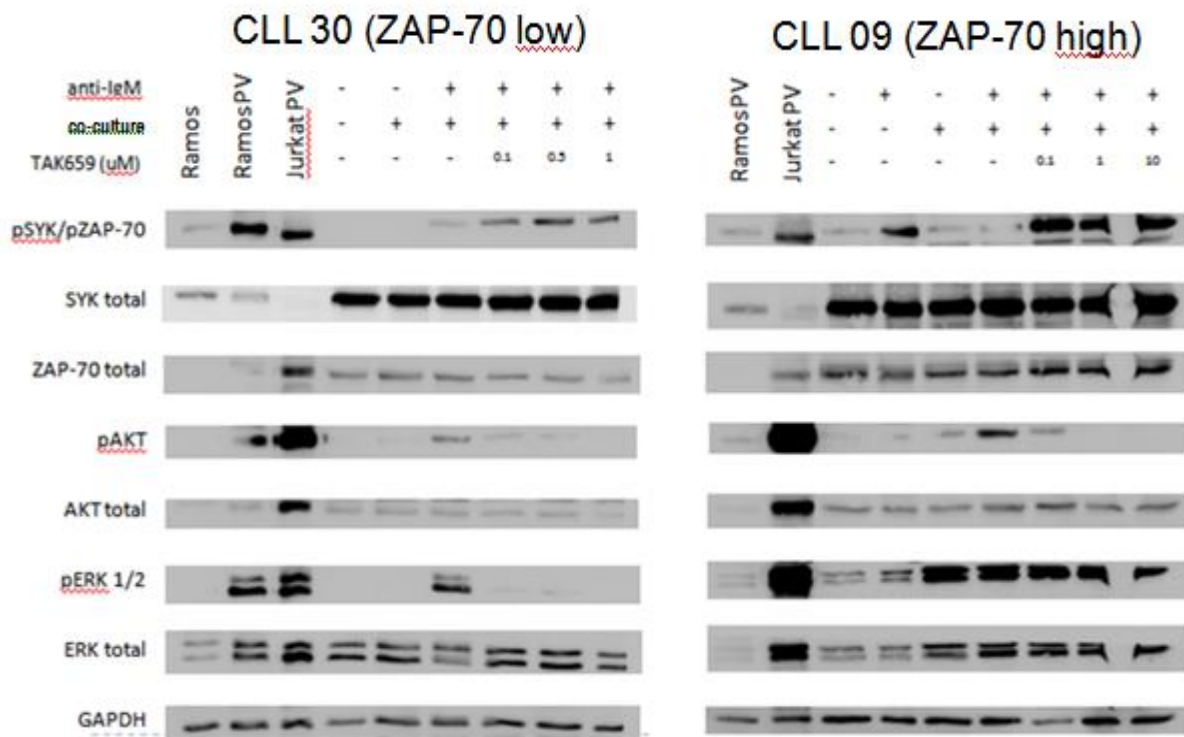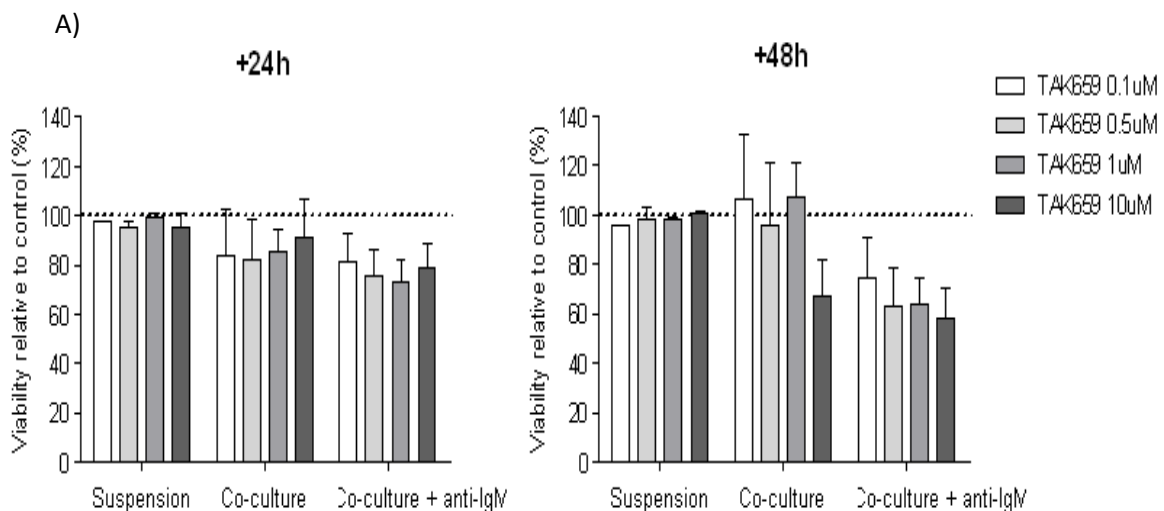


*Figure 4. TAK659 inhibits BCR signaling in primary CLL cells. Stimulation of primary co-cultured CLL cells (n=10) for 15 minutes with anti-IgM prior to 1 hour of incubation with increasing doses of TAK659. Displayed are immunoblots from primary CLL cells from 2 representative patients. Ramos and Jurkat pervanadate (PV) cells lines were used as positive controls for phosphorylated kinases. "P" indicates immunoblotting for the active, phosphorylated form.*

**TAK659 induces apoptosis in primary CLL cells in a dose dependent manner.**

In order to asses the role of Syk in the cytotoxicity induced by TAK659 treatment, primary CLL cells cultured for 24 and 48 hours in suspension or in co-culture ± stimulation with anti-IgM were treated with different concentrations of TAK659 (Figure 5A). The analysis of annexin V-PI cells showed that TAK659 induced apoptosis in a dose dependent manner in co-culture conditions already after 24 hours, especially with previous BCR-stimulation, though it was more evident after 48 hours. Letal dose 50 (LD50) was not achieved. No statistical significant differences in TAK659 sensitivity among patients were found according to ZAP-70 expression (Figure 5B), suggesting that TAK659 induces apoptosis in primary CLL cells regardless ZAP-70 expression.

A wide variability is noticed among patients, as is represented in figure 5C. This event is attributable to a kind of compensation between sensitive and resistant patients to TAK659 treatment. Resistant cases should be studied individually, such patient number 33, whose viability was partially enhanced after TAK659 treatment. A possible explanation could be acquired polymorphisms that led CLL cells to this resistant behavior.
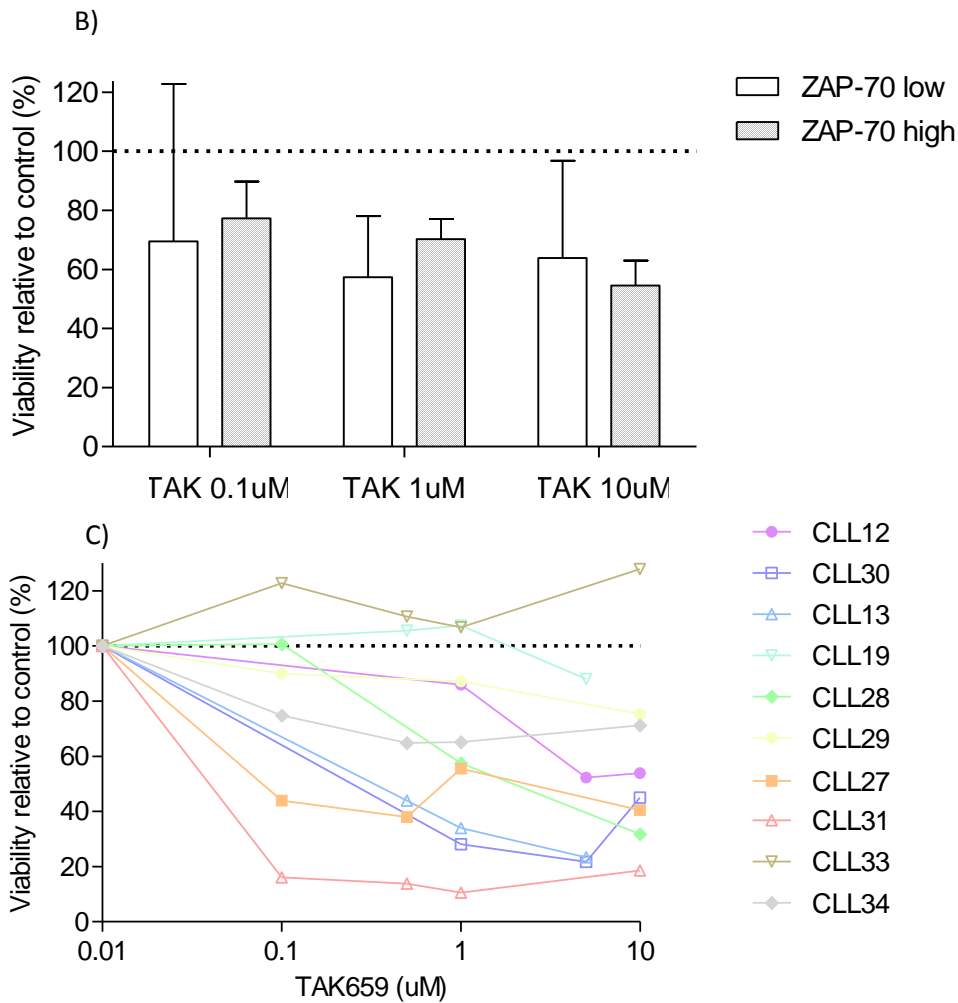
A)

***Figure 5. The Syk inhibitor TAK659 induces CLL cell apoptosis.*** *Viability was determined 24 and 48 hours after incubation of primary CLL cells from 10 patients in suspension or in co-culture supplemented with CD40L and TLR9L with or without 10ug/ml of anti-IgM prior treatment with increasing doses of TAK659 for one hour. A, B and C, viability related to controls is displayed. Annexin V/PI double negative cells were considered viable cells. Graphs show mean ± SEM.*

## Active proliferation of co-cultured CLL cells is partially abrogated by TAK659 treatment

The ability of TAK659 to decrease proliferation in active proliferative co-cultured CLL cells was determined by measuring Ki-67 expression. No differences were observed between drug concentrations in suspension condition, where proliferation is already very low in conventional conditions. However, TAK659 significally decreased proliferation cells' rate from dosis 0.5 µM in a dose-dependent manner, arriving to a rate of proliferation less than 50 per cent compared to control with TAK659 10 µM (Figure 6).
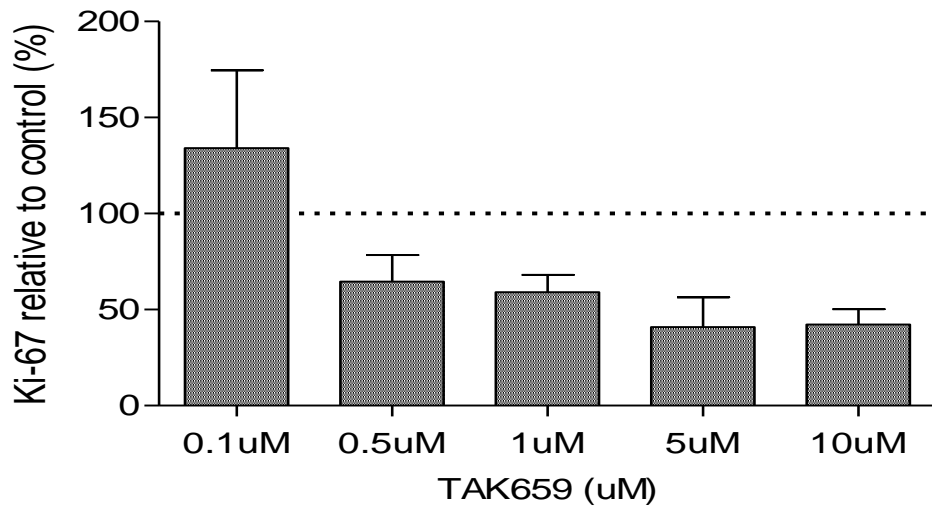
***Figure 6. Analysis of Ki-67 expression after TAK659 treatment.*** *Primary CLL cells from 6 patients were treated with increasing doses of TAK659 for one hour. Subsequently, CLL cells were cultured in suspension or in co-culture for 48 hours and harvested for FC analysis of Ki-67 expression. The graph represents the mean relative Ki-67 expression of CLL cells± SEM.*

## Syk inhibition with TAK659 abrogates CXCL12-induced chemotaxis of primary CLL cells

CLL cells are known to migrate to BM and LN toward the chemokine CXCL12 after the activation of its receptor CXCR4. Since Syk seems to be involved in migration of normal and tumoral cells, cytotoxicity of TAK659 was evaluated in a chemotaxis assay. Migration to CXCL12 was significally lower after TAK659 treatment, not only in each case analyzed, but also when comparing all patients together, despite the wide variable responses among patients (Figure 7).
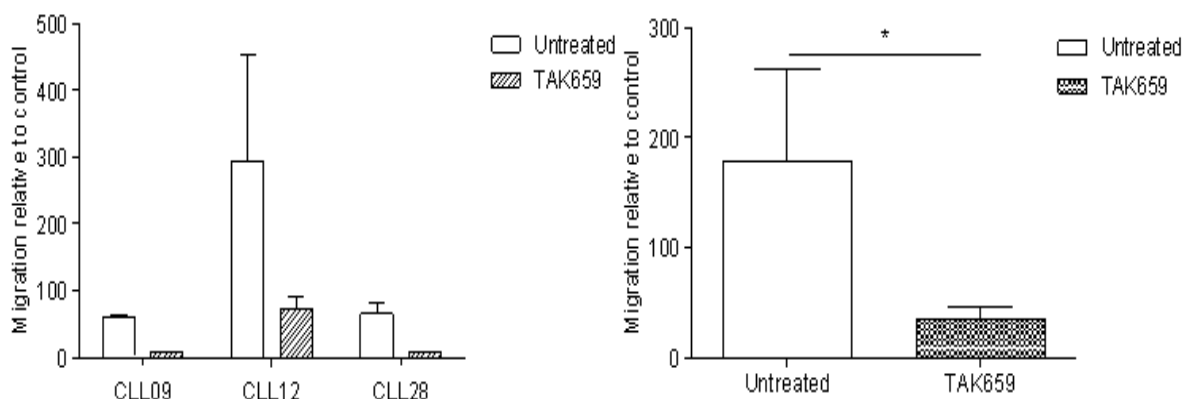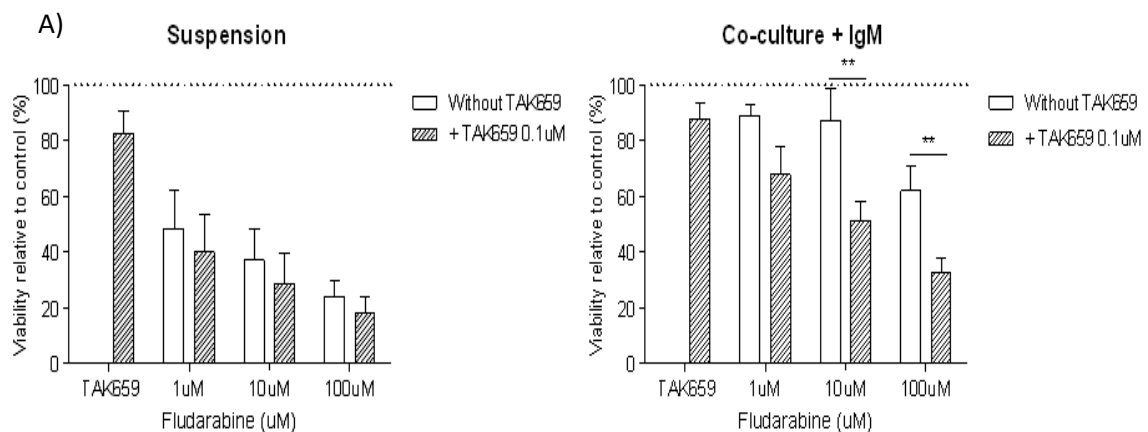


***Figure 7. Syk inhibition with TAK659 decreases chemotaxis of CLL cells toward CXCL12.***
*Primary CLL cells from 3 patients were cultured in suspension and with or without TAK659 0.1 µM for one hour. Afterwards, primary CLL cells were allowed to migrate to a medium containing 200 ng/mL of*

*CXCL12 for 6 hours. Simultaneously, primary CLL cells were placed in a non-containing CXCL12 medium as control. Displayed are the mean ± SEM supernatant concentrations of triplicates from 3 patients. (\*P<0.05, two-way ANOVA, Bonferroni post-test).*

## Enhanced cytotoxic effect of fludarabine regims when combined with TAK659 in primary CLL cells.

Fludarabine is a highly effective chemotherapeutic drug that produces higher response rates than conventional treatments. Toxicity rate produced by the adding of fludarabine to our tested drug TAK659 in primary CLL cells was evaluated (Figure 8). Despite a notably dose dependent decrease of cell survival in suspended CLL cells, no divergences between fludarabine alone and fludarabine in combination with TAK659 were appreciated in suspended CLL cells. However, looking at co-culture with anti-IgM condition CLL cells apoptosis was significaly magnified when using the combination regim of fludarabine and TAK659 compared to fludarabine treatment alone from the 10 µM dose (Figure 8A). The cytotoxic augmented effect of fludarabine in combination with TAK659 was evident when seeing LD50. LD50 of fludarabine alone in CLL cells co-cultured was 332 µM (95% CI 37.35-2944), while LD50 for those treated with combined regims of TAK659 and fludarabine was 11.031 µM (95% CI 4.05-30.03)(Figure 8B).

B)



| | Without TAK659 | | TAK659 0.1uM | | |
|---|---|---|---|---|---|
| | LD50 | 95%CI | LD50 | 95%CI | P value |
| Suspension | 2.28 | 0.56-8.73 | 0.98 | 0.27-3.62 | 0.36 |
| Co-culture + IgM | 332 | 37.35-2944 | 11.03 | 4.05-30.03 | 0.0008 |

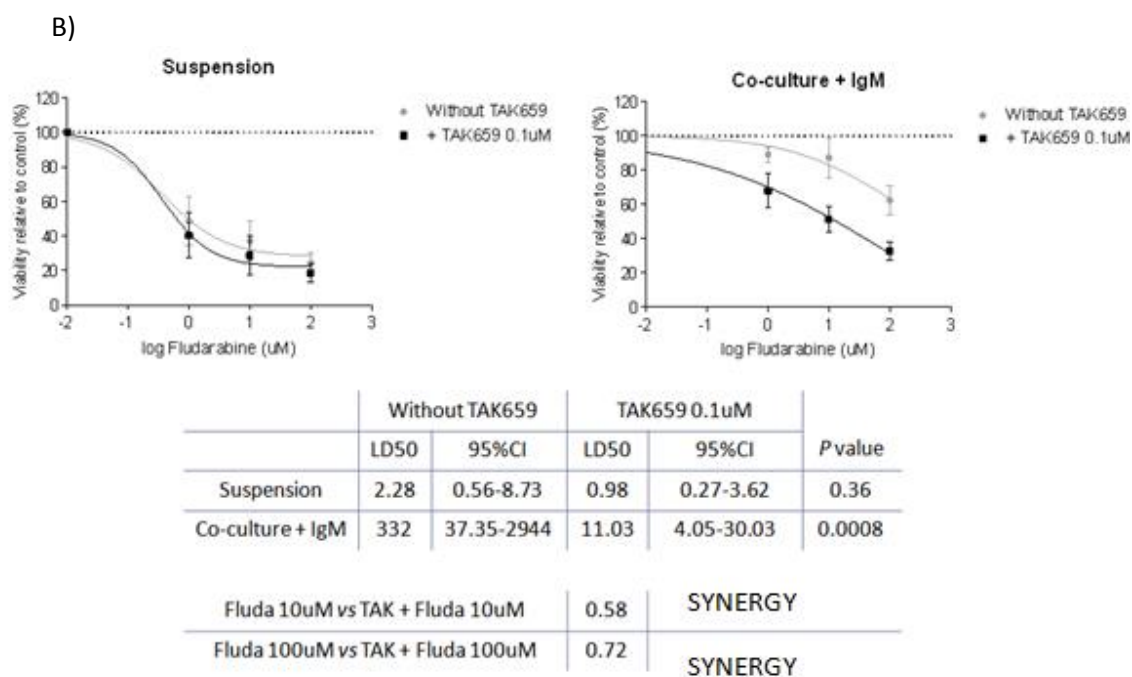| | | |
|---|---|---|
| Fluda 10uM vs TAK + Fluda 10uM | 0.58 | SYNERGY |
| Fluda 100uM vs TAK + Fluda 100uM | 0.72 | SYNERGY |

*Figure 8. TAK659 combined with fludarabine produces a synergic cytotoxical effect on primary CLL cells. Primary CLL cells from 6 patients were treated with increasing doses of fludarabine alone or fludarabine in combination with TAK659 (0.1 µM) for one hour. Afterwards, CLL cells were cultured in suspension or in co-culture with anti-IgM for 48 hours and harvested for FC analysis of Annexin V/PI. LD50 of fludarabine alone and combined with TAK659 is compared in Figure 8B both for suspension and co-culture conditions. Synergy rate is calculated in order to asses whether the effect of the combination of the drugs is attributed to the addition of the individual drug actions or a synergic phenomenon. Graphs represents the mean ± SEM.*

# 5. DISCUSSION

Growing evidences reinforces the crucial role that microenvironment found in BM and secondary lymphoid tissues plays in the maintenance and expansion of CLL cells.[47,48,49] Leukemic cells are supported by accessory cells in these niches, which supply them with anti-apoptotic and proliferative signals. Importantly, these interactions also confer resistance against drugs[50,51] making CLL cells located in these proliferative centers difficult to completely eradicate. For this reason, there is an increased emphasis placed on therapeutically targeting CLL cells from these proliferative niches.

The hypothesis which supports the project is that *ex vivo* mimicking of the microenvironment found in proliferative centers would partially reproduce *in vivo* CLL

situation and, therefore, premature CLL cells death could be avoided when cultured *in vitro*. Moreover, these proliferative and anti-apoptotic conditions would allow testing anti-proliferative drugs *ex vivo* in a model close to *in vivo* conditions. The co-culture of primary CLL cells and BMSCs along with soluble CD40L, CpG ODN and antibodies against IgM induced  marked proliferative and survival responses.

Advances in the knowledge of the pathogenesis of CLL and recent preclinical[52,53,54] and first clinical trials[44,55] sustain the necessity of locate BCR signaling involved proteins as the next generation  targets for treatment of patients with CLL and other non-hodgkin malignancies. Over the past few years, more competent therapies involving target-specific drugs have emerged. BCR-associated kinases Syk[44], Btk[55] and Pi3K[33] inhibitors have been the most preferently studied, obtaining promising results.

The tyrosine kinase Syk was as an essential trigger for BCR signaling due to its involvement in various signal transduction pathways, leading to cell survival by anti-apoptotic signals inhibition, cell proliferation and mobilization to proliferative centers.[56,57,58]

Several studies have already evidenced modulatory effects of Syk activity: R406 (the active metabolite of the prodrug fostamatinib (R788; Rigel/Astra Zeneca),  was shown to effectively antagonize CLL cell survival after BCR stimulation and inhibit chemokine secretion from BMSCs in co-culture conditions. R406 is nowadays in clinical trials. However a complicating issue for R406 is the fact that is that has rather limited specificity towards SYK due to its activity of ATP-competitive kinase inhibition. Indeed, R406 has been shown to inhibit several other kinases and non-kinase targets.[46]

In this project, toxicity of TAK659, another anti-proliferative drug which has its target in Syk, is studied and analyzed in preclinical trials under conditions which recreate the proliferative niches, expecting better results than reached with the actual anti-Syk inhibitors, since TAK659 is theoretically characterized by an special specificity to Syk. Remarkably, TAK659 exhibited significant *ex vivo* anti-tumoral activity in CLL cases irrespective of prognosis markers such as ZAP-70. Therefore, these results demonstrates the ability of TAK659 to promote apoptosis under condition mimicking tumor microenvironment.

The proliferative compartment in CLL represents only a small percentage of the analyzed malignant cells, however, increasing  evidence suggest that such actively proliferating cells are responsible for disease persistence after treatment and the eventual relapse observed almost in all patients. Targeting this small but crucial

compartment with TAK659 successfully reduced Ki-67+ CLL cells, building promising efficacy against relapse and disease expansion.

One of the most relevant effects of Syk inhibitors and other inhibitors of BCR-associated downstream kinases is their capacity to cause lymph nodes size reduction, which is usually accompanied by lymphocytosis.[44] These clinical indications points that kinase inhibitors motive CLL cells mobilization from tissues to PB and moderates chemotaxis to BM and LN. If CLL cells remain in the blood, are more susceptible to apoptotic signals because of the absence of microenvironment support. The finding that TAK659 effectively avoided chemotaxis of primary CLL cells toward CXCL12 confirm earlier findings with R406 and reiteratively indicate that these effects are attributed to Syk inhibition.

## 6. CONCLUSIONS

These results demonstrate that the co-culture system herein employed promoted proliferation, induces Syk expression and protects primary CLL cells from apoptosis induced by cytotoxic agents. In this setting, targeting protein kinase Syk by treatment with TAK659 is able to efficiently overcome microenvironment-mediated cell protection, proliferation and migration and has specific effect on actively proliferating CLL cells. Therefore, TAK659 might be a potent therapeutic drug complementing our growing armamentarium against CLL.

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