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LEUKEMIC CELL/MICROENVIRONMENT INTERACTIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA: ROLE OF JAK/STAT AXIS IN THE SURVIVAL OF NEOPLASTIC CLONE

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
ALL	Acute Lymphocytic Leukemia
alloHSCT	Allogenic Hematopoietic stem cell transplantation
APC	Allophycocyanin
ATM	Ataxia Teleangectasia Mutated
B	Bendamustine
BAD	Bcl-2 Associated Death promoter
Bcl-2	B Cell lymphoma-2
BCR	B Cell Receptor
B-PLL	B cell Prolymphocytic Leukemia
BM	Bone Marrow
BMMC	Bone Marrow Mononuclear Cell
BMSC	Bone Marrow Stromal Cell
BOM	Bone Marrow Biopsy
CDR	Complementarity Determining Region
CD40L	CD40 Ligand
CEB	Cytoplasmic Extraction Buffer
Clb	Chlorambucil
CLL	Chronic Lymphocytic Leukemia
CMV	Cytomegalovirus
GC	Germinal Centre
CR	Complete Remission
EBV	Epstein-Barr virus
ECL	Enhanced ChemiLuminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ERK	Extracellular signal Regulated Kinase
F	Fludarabine
Fab	Antigen binding fragment
FAK	Focal Adhesion Kinase
FBS	Fetal Bovin Serum
Fc	Crystallizable fragment
F/H	Ficoll/Hypaque
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein isothiocyanate
FMO	Fluorescenze Minus One
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulation Factor
GDP	Guanosine diphosphate
GSK3	Glycogen Synthase Kinase 3
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HE	Hematoxylin-Eosin
HIV	Human Immunodeficiecny virus

HLA	Human Leukocyte Antigen
HS1	Hematopoietic lineage cell-Specific protein 1
Hsp90	Heat shock protein of 90kDa
JAK	Janus kinase
JH	Janus homology domain
JNK	c-JUN NH2-Terminale Kinase
hTERT	human Telomerase Reverse Transcriptase
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgV_H	Immunoglobulin heavy chain variable regions
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
mAb	monoclonal Antiboby
MAP	Microtubule-Associated Protein
MAPK	Mitogen Activated Protein Kinase
Mcl-1	Mantle cell lymphoma-1
M-CLL	Mutated-Chronic Lymphocytic Leukemia
MEB	Membrane Extraction Buffer
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIIC	MHC-class-II-peptide-loading-compartment
MMP-9	Matrix metalloproteinase - 9
MSC	Mesenchymal Stromal Cell
MTOC	Microtubule Organizing Centre
mTOR	mammalian Target Of Rapamycin
NEB	Nuclear Extraction Buffer
NF-kB	Nuclear Factor-kappa B
NHL	Non-Hodgkin Lymphoma
NLC	Nurse-like Cell
NK	Natural Killer
OS	Overall Survival
PARP	Poli-ADP-Ribose Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFS	Progression Free Survival
PI	Propidium Iodide
PIAS	Protein Inhibitors of Activated STATs
PI3K	Phosphatidylinositol 3-Kinase
PL	Prolymphocyte
Plcγ2	Phospholipase Cγ2
PMN	Granulocyte
PS	Phosphatidylserine
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatases
R	Rituximab
RBC	Red Blood Cell
RTK	Receptor Tyrosine Kinase

SD	Standard Deviation
SDF-1α	Stromal Derived Factor-1α
SDS-PAGE	Sodium Dodecyl Sulphate/PolyAcrylamide Gel Electrophoresis
Ser	Serine
SFKs	Src Family Kinases
SHM	Somatic Hypermutation
SHP-1	SH2-domain containing Tyrosine Phosphatase
SHIP-1	SH2-domain containing Inositol Phosphatase
sIgM	surface Immunoglobulin M
Syk	Spleen tyrosine kinase
SOCS	Suppressors of Cytokine Signaling
SRBC	Sheep Red Blood Cell
SC	Side Scatter
STAT	Signal Transducers of Activated Transcription
TC	Tri-Color
Th	T helper lymphocyte
Tyr	Tyrosine
TK	Thymidine Kinase
U-CLL	Unmutated-Chronic Lymphocytic Leukemia
VH	Heavy chain Variable region
VL	Light chain Variable region
WB	Western blotting
WBC	White Blood Cell
ZAP-70	Zeta-Associated Protein of 70kDa

Abstract

Chronic Lymphocytic Leukemia (CLL) is characterized by the accumulation of mature clonal CD19+/CD5+/CD23+ B lymphocytes in peripheral blood, bone marrow, and lymphoid tissues. Despite their *in vivo* prolonged lifespan due to intrinsic defects, CLL leukemic cells rapidly undergo spontaneous apoptosis *in vitro*, highlighting the need of extrinsic signals delivered by the microenvironment.

Several molecules, including those released by mesenchymal stromal cells (MSCs), signal through JAK (Janus kinases)-STAT (signal transducers and activators of transcription) pathways. During the PhD course, we particularly focused on JAK2/STAT3 axis since IL-6, one of the most abundant cytokines released in the CLL microenvironment, is the key ligand of the receptor triggering this pathway. The deregulation of JAK2/STAT3 axis may lead to aberrant activation of STAT3 and, as a result, to tumor development in hematopoietic cells.

By western blotting, flow cytometry analysis, subcellular fractionation, and confocal microscopy, we analyzed 76 CLL patients and 23 healthy donors to investigate STAT3 involvement in CLL cell survival.

We demonstrated that STAT3 expression was higher in malignant B cells (MFI: CLL cells 1.28±0.13 *vs* normals 0.61±0.09, Student's *t* test, $p<0.001$) and the protein is significantly phosphorylated at Tyr705 compared with the normal counterpart (B lymphocytes from healthy donors) (MFI: CLL cells 211.3±35.85 *vs* normals 46.50±6.12, Student's *t* test, $p<0.05$), thus showing its constitutive activation in CLL.

Afterwards, we pointed out that the *in vitro* incubation of leukemic B cells with AG490 and Stattic, specific inhibitors of JAK2 and STAT3, respectively, induce a dose-dependent apoptosis of CLL B cells (*e.g.* Cell viability, 24h: CLL cells alone 62.90±4.24% *vs* CLL cells + AG490 50μM 38.30±7.22%, Student's *t* test, $p<0.01$; CLL cells alone 60.96±3.91% *vs* CLL cells + Stattic 10μM 31.78±4.31%, Student's *t* test, $p<0.0001$). Both AG490 and Stattic were able to bypass the microenvironmental protection when

neoplastic B cells were co-cultured with MSCs (*e.g.* Cell viability, 48h: CLL cells 66.80±6.28% *vs* CLL cells + AG490 100μM 22.50±7.50%, Student's *t* test, $p<0.01$; CLL cells 57.51±4.95% *vs* CLL cells + Stattic 10μM 26.25±5.45%, Student's *t* test, $p<0.001$).

In addition to JAK2/STAT3 inhibition, we showed that AG490 treatment on CLL cells can mediate other effects: i) it activates SHP-1, decreasing its phosphorylation at Ser591; ii) it inactivates protein Lyn, reducing the phosphorylation in its active site at Tyr396. Lyn, a Tyr-kinase, and SHP-1, a Tyr-phosphatase, are both involved in the prolonged lifespan of neoplastic CLL cells.

In conclusion, the ability of AG490 and Stattic to induce apoptosis in leukemic B cells, bypassing the pro-survival stimuli provided by the tumor microenvironment, represents a starting point for the development of new therapeutic strategies in CLL, providing at the same time new insights on the cross-talk between JAK/STAT and BCR/Lyn axes in CLL cells.

Riassunto

La Leucemia Linfatica Cronica (LLC) è un disordine linfoproliferativo caratterizzato dall'accumulo di linfociti B maturi, con immunofenotipo CD19+/CD5+/CD23+, nel sangue periferico, in quello midollare e nei tessuti linfoidi. Nonostante le cellule leucemiche *in vivo* non vadano incontro ad apoptosi, se poste *in vitro* muoiono rapidamente; questo evidenzia l'importanza del microambiente nella sopravvivenza del clone neoplastico.

Diverse molecole, incluse quelle rilasciate dalle cellule mesenchimali stromali (MSC) agiscono tramite la via di JAK (*Janus Kinase*)/STAT (*Signal Transducer and Activators of Transcription*).

Nel corso del dottorato, ci siamo concentrati in particolare sull'asse JAK2/STAT3 in quanto IL-6, una delle citochine più abbondanti tra quelle presenti nel microambiente, è il ligando principale del recettore a monte di questa via. La deregolazione dell'asse JAK2/STAT3 può portare all'attivazione incontrollata di STAT3 e, conseguentemente, allo sviluppo di tumori delle cellule ematopoietiche

Tramite *western blotting,* citometria a flusso, frazionamento subcellulare e microscopia confocale, abbiamo analizzato 76 pazienti con LLC e 23 donatori sani al fine di studiare il coinvolgimento di STAT3 nella sopravvivenza della cellula leucemica.

Abbiamo dimostrato come l'espressione di STAT3 sia maggiore nelle cellule neoplastiche (MFI: cellule di LLC 1.28±0.13 *vs* normali 0.61±0.09, Student's *t* test, $p<0.001$) e come sia significativamente fosforilata alla Tyr705 in confronto ai linfociti B di donatori sani (MFI: cellule di LLC 211.3±35.85 *vs* normali 46.50±6.12, Student's *t* test, $p<0.05$), evidenziando così la sua attivazione costitutiva nella CLL.

Dopo di ciò, abbiamo osservato come il trattamento *in vitro* di cellule leucemiche con AG490 e Stattic, rispettivamente inibitori specifici di JAK2 e di STAT3, induca apoptosi dose-dipendente nelle cellule B di LLC (es. Vitalità cellulare, 24h: cellule di LLC 62.90±4.24% *vs* cellule di LLC + AG490 50μM 38.30±7.22%, Student's *t* test, $p<0.01$; cellule di LLC 60.96±3.91% *vs* cellule

di LLC + Stattic 10μM 31.78±4.31%, Student's *t* test, p<0.0001).

Sia AG490 che Stattic si sono dimostrati capaci di superare la protezione fornita dal microambiente quando le cellule neoplastiche sono poste in cultura insieme alle MSC (es. Vitalità cellulare, 48h: cellule di LLC 66.80±6.28% *vs* cellule di LLC + AG490 100μM 22.50±7.50%, Student's *t* test, p<0.01; cellule di LLC 57.51±4.95% *vs* cellule di LLC + Stattic 10μM 26.25±5.45%, Student's *t* test, p<0.001).

Abbiamo dimostrato come AG490 non sia solo in grado di inibire l'asse JAK2/STAT3, ma possa anche mediare altri effetti come: i) l'attivazione di SHP-1 diminuendo la sua fosforilazione in Ser591; ii) l'inibizione della proteina Lyn, riducendo la fosforilazione del sito attivo in Tyr396.
Lyn, una tirosin-chinasi, e SHP-1, una tirosin-fosfatasi, sono coinvolte nella sopravvivenza delle cellule di LLC.

In conclusione, la capacità di AG490 e Stattic di indurre apoptosi nelle cellule B leucemiche superando la protezione fornita dal microambiente, rappresenta un punto di partenza per lo sviluppo di nuove strategie terapeutiche nella LLC, fornendo allo stesso tempo nuove informazioni per la caratterizzazione del *crosstalk* tra l'asse JAK2/STAT3 e il *pathway* BCR/Lyn nelle cellule leucemiche.

INTRODUCTION

1. Chronic Lymphocytic Leukemia (CLL)

1.1 Epidemiology and etiology

B cell Chronic Lymphocytic Leukemia (CLL) is a lymphoproliferative disorder characterized by the accumulation of long-lived monoclonal B cells in peripheral blood, bone marrow and lymphoid tissues. CLL lymphocytes show a CD19+/CD5+/CD23+ membrane phenotype and are blocked in G0/G1 phase of the cell cycle[1]. CLL is the most common adult leukemia in the Western world and is more prevalent in men than in women with a male to female ratio of 1.5-2:1. The incidence rates between men and women are 5.6 (in men) and 4.3 (in women) cases per 100,000 inhabitants per year, respectively. In Europe, these incidence rates are 5.87 and 4.01 cases per 100,000 population per year, respectively. CLL is considered to be mainly a disease of the elderly, with a mean age at diagnosis of 70 years; however, it is not unusual to diagnose it in younger individuals from 30 years of age. The incidence increases rapidly with increasing age[2,3] (Figure 1).

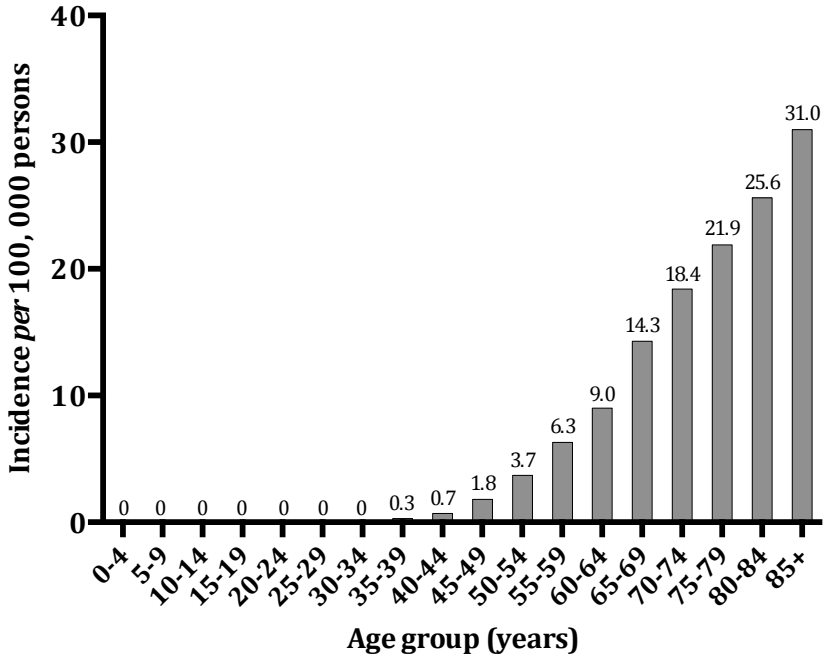

Figure 1. Incidence of CLL Age-specific incidence of CLL (adapted from Redaelli *et al*)[3].

The etiology is still unknown; the exposure to common carcinogens does not seem to be associated with the disease progression. More studies are in progress to assess a potential relation among CLL onset, inflammation, and autoimmune conditions[4,5]. A familiarity of this pathology is well documented in the 8-10% of cases. Moreover, it has been highlighted the phenomenon of the anticipation in which inherited disease is diagnosed at an earlier age and in a more aggressive form in the later generations of a family[6].

1.2 Clinical features

The clinical course of this disorder is highly variable, ranging from a stable to a progressive and severe disease[7]: some patients remain asymptomatic without any treatment, while others present an aggressive outcome that is difficult to control with chemotherapy. About 70% of patients are asymptomatic at diagnosis and it is carried out after detailed examinations followed the finding of lymphocytosis at examination blood count; in other cases, the pathology occurs with asthenia, weight loss, fever, lymphadenopathy, splenomegaly, and hepatomegaly. Some patients could show autoimmune phenomenons, such as hemolytic anemia (11% of cases) or autoimmune thrombocytopenia (2% cases) that are typically present in advanced and multi-treated disease. The typical CLL hypogammaglobulinemia could induce immunodeficiency and high mortality for infections[8,9].

Although the causes of death are often attributed to the underlying disease, in some cases progressing syndromes with a poor prognosis could occur: one of these is the Richter's syndrome in which CLL changes into a fast-growing diffuse large B cell lymphoma. Another evolution could be the B cell prolymphocytic leukemia (B-PLL) that is more aggressive and characterized by malignant B cells larger than average. The occurrence of acute lymphoblastic leukemia is very rare, while acute myeloid leukemia, such as non-hematological disease, could be correlated with CLL immunological deficit and chemotherapy[10].

1.3 Diagnosis

It is crucial to verify that the patient is really suffering from CLL and not by another lymphoproliferative disease that can masquerade as a CLL, such as hairy cell leukemia, or leukemic manifestations of mantle cell lymphoma, marginal zone lymphoma, splenic marginal zone lymphoma with circulating villous lymphocytes, or follicular lymphoma. To achieve this, it is essential to evaluate the blood count, blood smear, and the immune phenotype of the circulating lymphoid cells. The National Cancer Institute diagnostic criteria include[11]:

1. the presence of at least $5x10^9$ B lymphocytes/L (5000/μL) in the peripheral blood. Leukemic cells found in the blood smear are characteristically small (7-10μm diameter), mature lymphocytes with a narrow border of cytoplasm and a dense nucleus, lacking discernible nucleoli, with partially aggregated chromatin. These cells may be found mixed with larger or atypical cells, broken cells, or prolymphocytes, which may comprise up to 55% of the blood lymphocytes. Typically, the "shadows of Gumprecht", that is degenerated cells that are broken in slide preparation, are present in the peripheral blood smear (Figure 2).

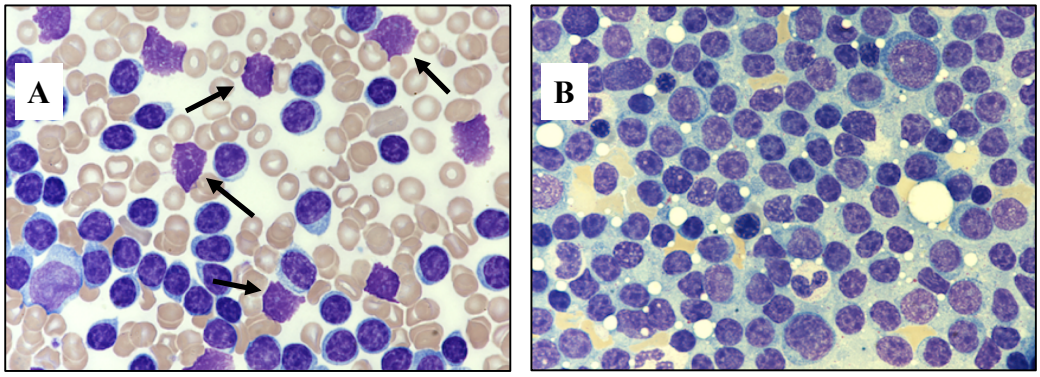

Figure 2. Peripheral (A) and Stromal (B) blood smear of a CLL patient. Arrows indicate Gumprecht shadows typical of CLL.

2. Bone marrow lymphocytic infiltration exceeding 30% of the nucleated cells (Figure 3). Although the type of bone marrow infiltration (diffuse vs not diffuse) reflects the size of the tumor and provides feedback

about the prognosis. Recent studies have shown that the prognostic value of bone marrow biopsy (BOM) can be replaced by new prognostic factors. Bone marrow aspirate and BOM are generally not required for the diagnosis of CLL; however, they can help in the management of the patient to discriminate cytopenias related to bone marrow infiltration than other types;

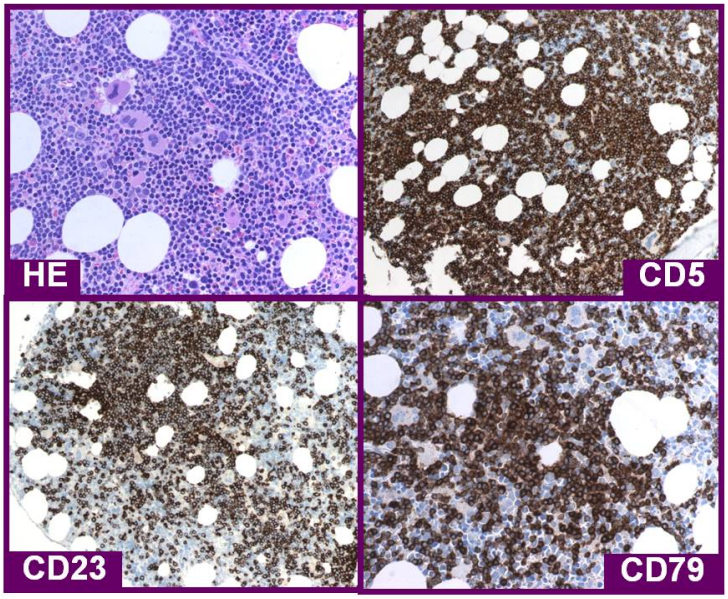

Figure 3. Marrow biopsy of a CLL patient. Preparation stained with hematoxylin-eosin (HE) and three immunohistological pictures that show some diagnostic markers.

3. immunophenotype analysis. CLL phenotype is characterized by three elements:
   a) the expression of a unique type of immunoglobulin light chains (κ or λ);
   b) the co-expression of the T-cell antigen CD5 and the B-cell surface antigens CD19, CD20, and CD23; CD23 is of particular importance in the differential diagnosis with mantle cell lymphoma (CD5+ but CD23-);
   c) low levels of CD79b and surface immunoglobulin (sIg) that in CLL appear to be mainly IgM followed by IgD, IgG, and IgA; it is not unusual to find an IgM and IgD co-expression (Figure 4).

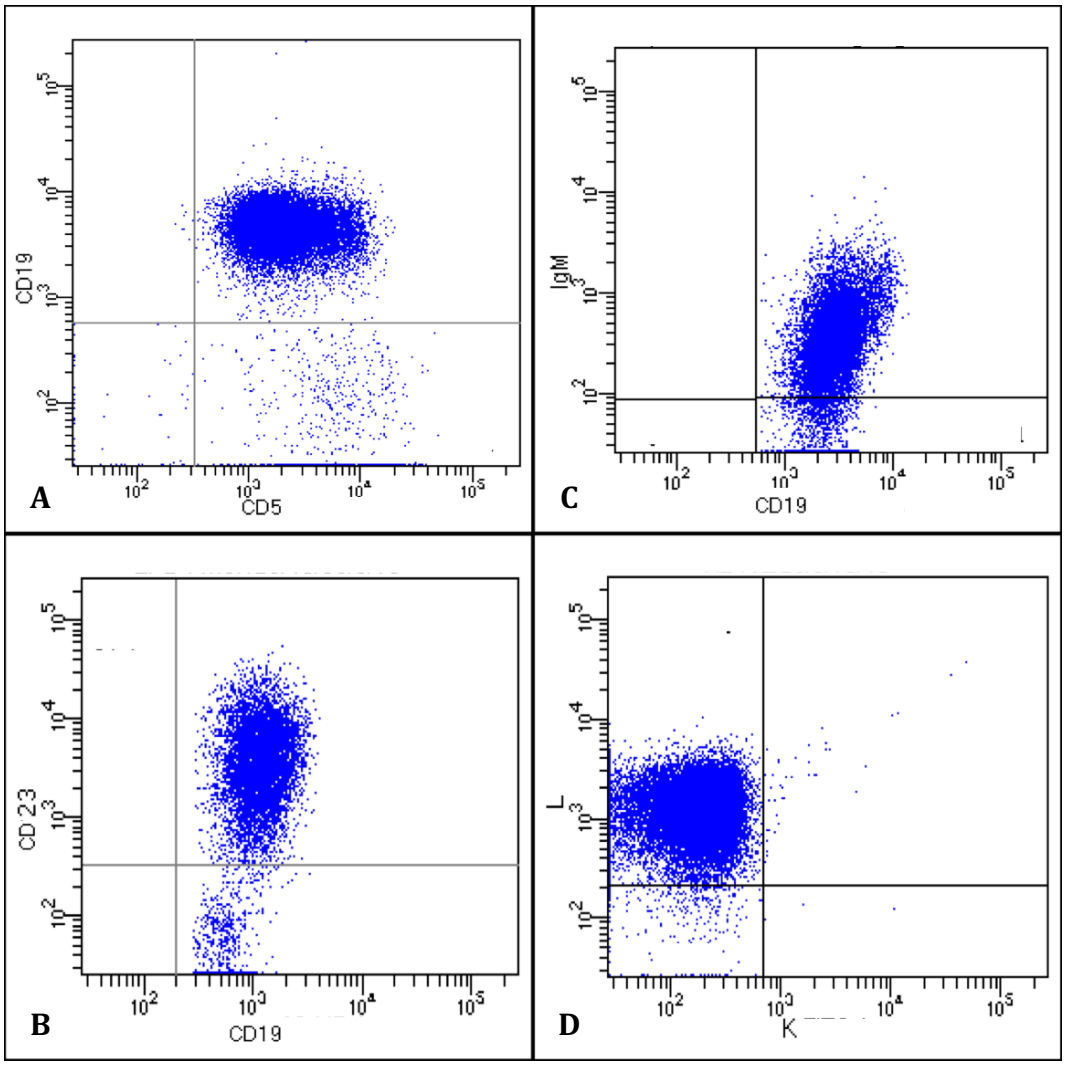

Figure 4. Cytograms of a representative case of CLL. B lymphocytes analyzed (CD19+) are positive to CD5 (**A**) and to CD23 (**B**), surface IgM (sIgM), low density (**C**) express one type of immunoglobuline light chain (λ, **D**).

In CLL, T lymphocytes, in particular CD8+ T cells, are often increased, with a reduced CD4/CD8 ratio. They show activation markers such as CD25 and HLA-DR. Natural-Killer (NK) cells (CD16+/CD56+) are present in high levels. Several analyses are performed to confirm the diagnosis and to prevent complications: serum protein electrophoresis, Ig dosage, Coombs' test, and analysis of renal and liver function. Before starting an immunotherapy, it is important to assess the absence of viral infection, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Human Immunodeficiency virus (HIV)[12].

1.4 Prognosis

Since it is difficult to predict the course of the disease at the time of diagnosis and the value of an early treatment is uncertain, therapy is currently recommended only for patients with a disease progressive, symptomatic, or both[13]. During the years, different parameters or prognostic factors were proposed to define the clinical course of CLL patients.

1.4.1 Clinical prognostic factors

1. **Clinical staging**: There are two widely accepted staging methods in both patient care and clinical trials: the Rai and the Binet system. The original Rai classification was modified to reduce the number of prognostic groups from 5 to 3. Both systems now describe 3 major subgroups with different clinical outcomes. These 2 staging systems are simple, inexpensive, and can be applied by physicians worldwide. Both rely exclusively on physical examination and standard laboratory tests and do not require ultrasound, computed tomography, or magnetic resonance imaging[11].

   The Rai system is so developed:

   - low-risk disease (stage 0): absolute lymphocytosis >15,000/μl and marrow lymphocytosis >40%;
   - intermediate-risk disease (stage I or II): lymphocytosis, enlarged nodes in any site, and splenomegaly and/or hepatomegaly (lymph nodes being palpable or not);
   - high-risk disease (stage III or IV): disease-related anemia (Hb<110g/L) or thrombocytopenia (as defined by a platelet count <100x10^9/L).

   The Binet system is based on the number of involved areas, as defined by the presence of lymph nodes with a diameter greater than 1 cm or organomegaly, and the presence of anemia or thrombocytopenia. It is subdivided into:

- stage A. Hb≥100g/L (10g/dL), platelets ≥100x10^9/L, and up to 2 lymph node areas involved;
- stage B. Hb≥100g/L, platelets ≥100x10^9/L, and lymphoadenopathy greater than that defined for stage A (i.e., 3 or more areas of nodal or organ enlargement);
- stage C. All patients who have Hb <100g/L and/or a platelet count <100x10^9/L, irrespective of lymphoadenopathy.

2. **Lymphocyte doubling time**: it is less than 12 months and it is associated with a worse clinical course.
3. **Bone marrow infiltration**: a diffuse infiltration pattern correlates with a bad prognosis[14].

1.4.2 Biological prognostic factors

The less recent biological prognostic factors are correlated with the expansion of the leukemic clone; they thus become indicative only when the disease is worsening. Their utility is limited because it is not possible to program the therapeutic strategy basing on the progression risk of patients. The biological prognostic factors comprehend:

1. **Prolymphocyte (PL) percentage**: if it is less than or equal to 10% (typical CLL) the probability of PL leukemia evolution is very low; if the percentage is between 11% and 55% there is an intermediate risk of CLL/PL leukemia, and if it is greater than 55% the transformation in PL leukemia may occur[15].
2. **β2 microglobulin**: this parameter is inversely correlated with the survival. It is related with the lymphocyte doubling time so that an increase in β2 microglobulin indicates an high neoplastic cell proliferation[15].
3. **Thymidine kinase (TK) level**: it has been shown that elevated serum thymidine kinase (s-TK) levels predict disease progression in CLL. Patients with s-TK values greater than 7.1U/L have a median

progression free survival (PFS) of 8 months, whereas patients with s-TK values ≤7.1U/L expect a much longer PFS[16].

4. **Soluble CD23 value**: serum CD23 level provides significant additional prognostic information in terms of overall survival (OS) in CLL. Among early stage patients, sCD23 determination, at diagnosis and during the course of the disease, may help to the early identification of patients who will rapidly progress to upper stages. Functions ascribed to sCD23 include prevention of germinal center (GC) B cells from their apoptosis, proliferation of myeloid precursor cells, and, more recently, costimulation of interferon-γ (IFN-γ) production by T cells and triggering of monokine release by monocytes[17].

The progressive discoveries on CLL pathogenesis have identified new prognostic markers that can better determine the clinical course. They describe biological characteristic of leukemic clone that are crucial to evaluate its proliferation and invasion capability. The study of these markers is performed by flow cytometry, cytogenetic and molecular biology techniques. The main markers are:

1. **Somatic Hypermutations** (SHM) of the Ig heavy chain variable region (VH) genes. Based on the numbers of somatic mutations detected in these genes, the cases were divided into 2 categories: "unmutated" (SHM-) or "mutated" (SHM+) (Figure 5-6). Conventionally, patients with <2% differences from the most similar germline gene in both the expressed VH and VL genes were define unmutated; mutated cases were defined as those in which the CLL cells displayed ≥2% differences in either the expressed VH or VL gene[18,19]. In addition, the stereotyped VH3.21 gene is an unfavorable prognostic marker independent of the IgVH mutational status. However, this result has been highlighted especially in patients living in Northern Europe, while it was not confirmed in Mediterranean countries[11].

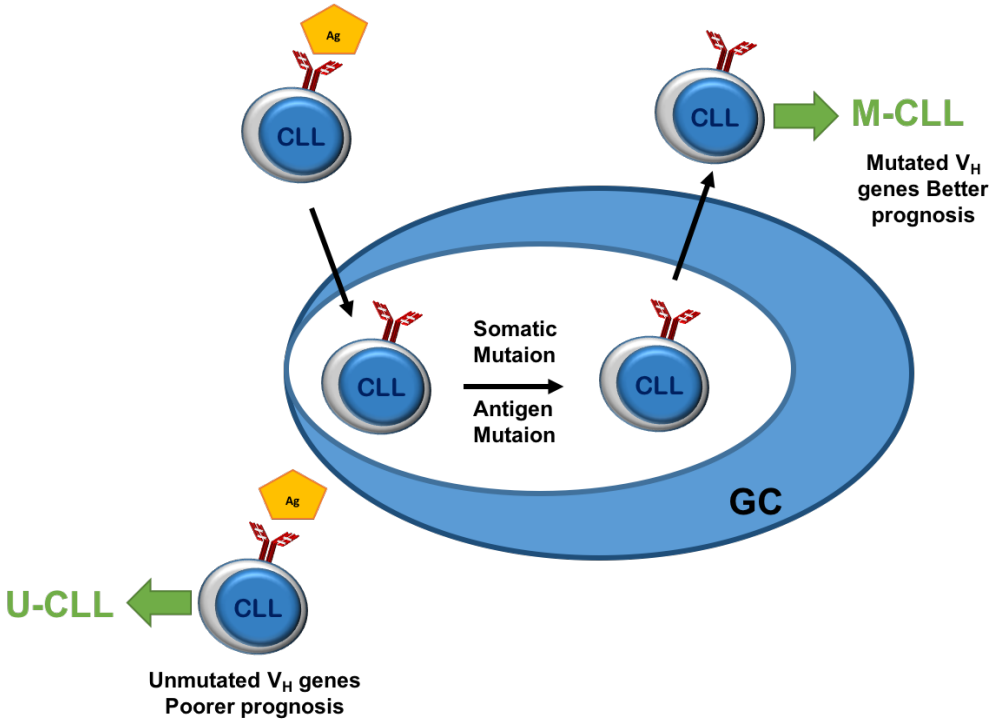

Figure 5. Hypothesis on origins and features of the 2 subsets of chronic lymphocytic leukemia. The development of unmutated CLL (U-CLL) is likely to be from a naive B cell that has encountered antigen but with insufficient stimulus to form a germinal center (GC). This subset has a poorer prognosis. In contrast, mutated CLL (M-CLL) develops from a cell that, following antigen encounter, has undergone somatic mutation and presumably antigen selection in the GC. The final neoplastic event is likely to have occurred after exit from the GC. This subset has a better prognosis. (Adapted from Stevenson et al.[19])

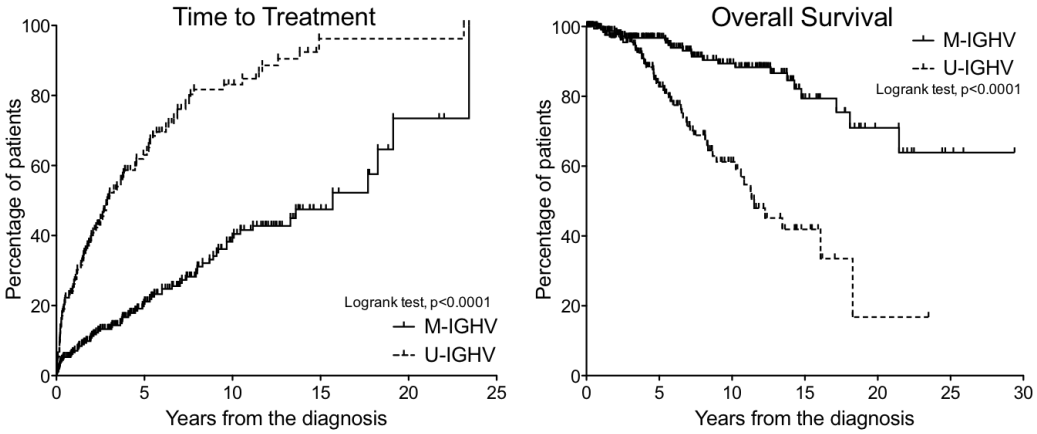

Figure 6. Time to Treatment and Overall Survival in relationship with IGHV prognostic marker. The median time to treatment and Overall Survival for mutated (M-IGHV) and unmutated U-IGHV) CLL patients

2. **CD38 expression**. CD38 is a transmembrane glycoprotein expressed on the surface of cells in a significant percentage of patients with CLL. A previous study suggested that CD38 expression has prognostic value in CLL (Figure 7). Cases with CD38+ B cells >30% show a bad prognosis. Indeed, the cut-off to discriminate CD38+ to CD38- is not

unanimously defined: some authors place it at 20%[20] or to 7%[21] of CD38+ B cells. CD38 has an independent prognostic value. Moreover, variability is the limit of this marker, in particular after treatment: chemotherapy affects mainly CD38- cells, determining an increase of CD38+ cells in a second time[18].

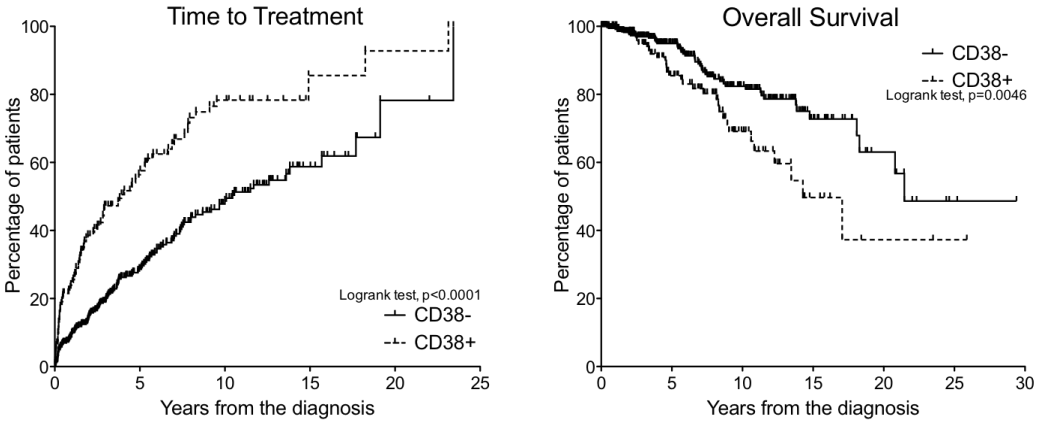

Figure 7. Time to Treatment and Overall Survival in relationship with CD38 prognostic marker. The median time to treatment and Overall Survival for patients with a high (CD38+) or low (CD38-) number of cell expressing this transmembrane glycoprotein.

3. **Intracytoplasmatic expression of protein kinase associated to TCR ζ chain of 70kDa (ZAP-70)**. Zeta-associated protein of 70kDa (ZAP-70) is a cytoplasmic tyrosine kinase which is a key signaling molecule for T lymphocytes and NK cells. ZAP-70 expression may reflect an activation state of the malignant clone associated with progressive disease or may be involved in CLL progression because of its function as a tyrosine kinase that can signal downstream of many surface receptors. The expression of ZAP-70 may change over time in CLL, in particular during clinical progression, suggesting the interest in the evaluation of ZAP-70 during the evolution of the disease (Figure 8)[22]. ZAP-70 expression analysis can be performed with different methods: flow cytometry, immunohistochemistry, western blotting, and Real-Time PCR. Among these, flow cytometry is the most advantageous for its diffusion and easiness of application. Anyhow interlaboratory variation is large and there is neither a consensus nor a regulatory approved methodology[23].

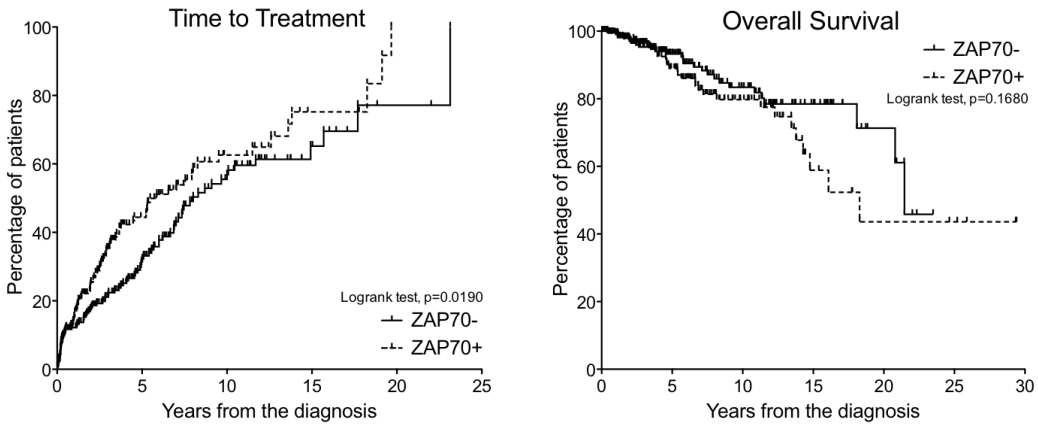

Figure 8. Time to Treatment and Overall Survival in relationship with ZAP70 prognostic marker. The median time to treatment and overall survival for the groups with high (ZAP70+) or low (ZAP70-) expression of this protein.

4. **Chromosomal alterations**. CLL is also characterized by a genomic instability that gives rise to several chromosomal aberrations, 11q, 13q, 17p deletions and 12 trisomy being the most relevant. Clonal genomic aberrations can be identified in approximately 80% of CLL patients by fluorescence in situ hybridization (FISH). While 11q and 17p deletions have been associated with rapid disease progression, the absence of chromosomal abnormalities and the presence of 13q deletion as the sole abnormality are associated with a better prognosis[24]. The presence of chromosome alterations with high risk justifies the use of more aggressive treatment[25]. Chromosome alterations are independent from IgVH mutational status though is evident a more frequency of 11q- and 17p- in unmutated and 13q- in mutated cases. These data show that analysis conducted by cytogenetic could be used as further risk stratification instrument together with the other prognostic factors[26].

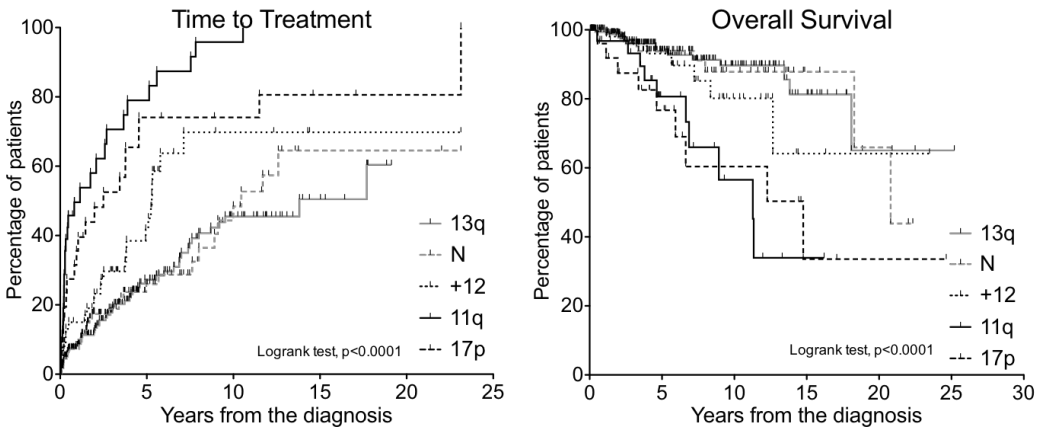

Figure 9. Time to treatment and probability of survival among patients in the most common chromosomal alterations. The median survival times and time to treatment for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as single abnormality were 0.18, 1.75, 3.81, 11.56, and 8.00 years, respectively (adapted from Visentin *et al*)[26].

5. **Telomerase expression and telomere length in CLL**. Activation of telomerase reverse transcriptase (hTERT) is essential for unlimited cell growth and plays a critical role in tumorigenesis[27]. Recently, the levels of telomerase activity (TA) and/or hTERT expression were related to clinical aggressiveness and prognosis in a variety of malignancies, including CLL. CLL cases with high telomerase levels and short telomeres were frequently characterized by an unmutated IgVH status and high-risk chromosomal aberrations. Conversely, CLL cases with low telomerase levels and long telomeres were associated with a mutated IgVH status and low-risk abnormalities (Figure 10). The evaluation of hTERT and telomere length might help the clinician vin the management of CLL patients with mutated IgVH and/or no high-risk chromosomal aberrations since cases with high hTERT/short telomere CLL will progress more rapidly and might require therapy earlier than those with low hTERT/long telomeres[28].

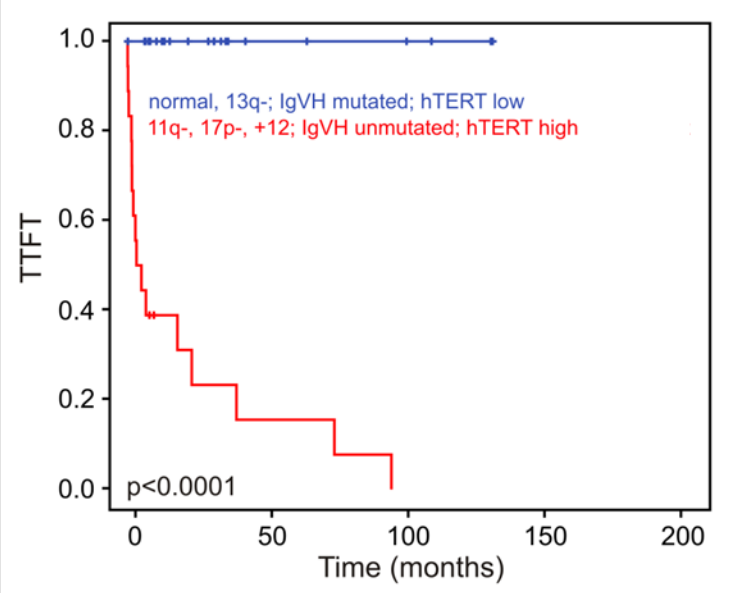

Figure 10. Curves of treatment-free survival. Time from diagnosis to first treatment (TTFT) according to IgVH mutational status, chromosomal categories, and hTERT level/telomere length profile (adapted from Rampazzo *et al*)[28].

1.5 Treatment

Criteria for initiating treatment depend on clinic symptoms, stage and disease activity. Treatment should only be initiated in patients with symptomatic, active disease. Significant B symptoms, cytopaenias not caused by autoimmune phenomena and symptoms or complications from lymphadenopathy, splenomegaly or hepatomegaly, lymphocyte doubling time of <6 months (only in patients with more than $30x10^9$ lymphocytes/l), as well as autoimmune anaemia and/or thrombocytopaenia poorly responsive to conventional therapy define an active disease[29]. In general practice, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should be monitored without therapy unless they have evidence of progression. On the contrary, patients at intermediate (I and II) and high risk stages (III and IV), according to the modified Rai classification or at Binet stage B or C, usually benefit from the start of a treatment; some of these patients (in particular Rai intermediate risk or Binet stage A/B) can be monitored without therapy until they have evidence for progressive or symptomatic disease[11].

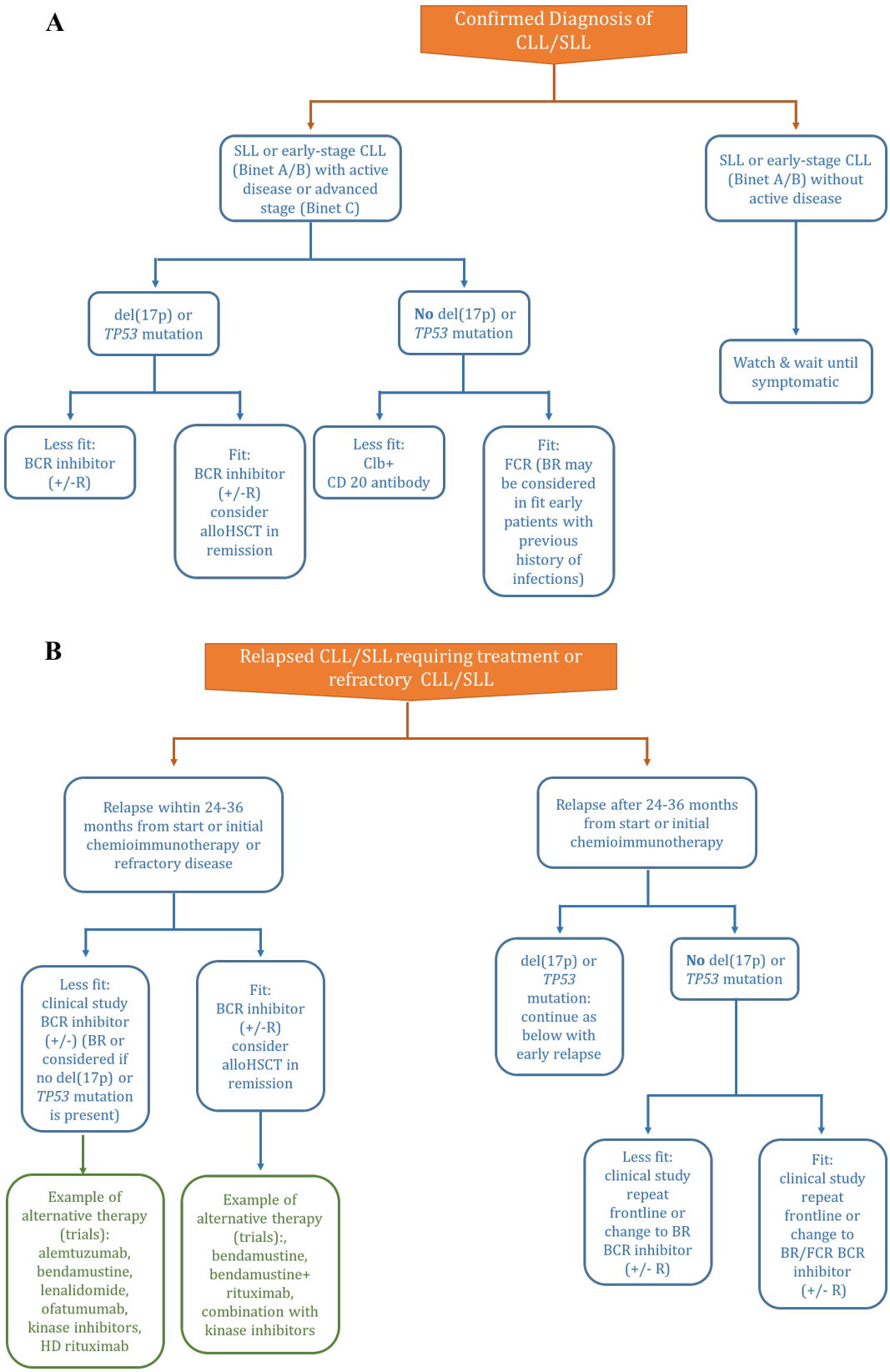

Figure 11. Possible decisional pattern in CLL treatment. Indication for first line treatment (**A**) and for relapse or refractory disease (**B**). R (rituximab), Clb (chlorambucil), F (fludarabine), C (cyclophosphamide), B (bendamustine) and alloHSCT (allogenic hematopoietic stem cell transplantation) (adapted from Eichhorst *et al)*[29].

Therapeutic possibilities comprehend drugs with different mechanisms of action, up to stem cells auto/allotransplantation. Treatment possibilities include both drugs with different mechanisms of action and auto/allogeneic stem cell transplantation. In any case, because of the indolent nature of the disease, the therapy does not aim to complete recovery, that is the elimination of the leukemic clone, but to control the expansion of neoplastic cells.

The choice of the therapy is linked to patient age and general conditions. In physically fit patients (physically active, with no major health problems, normal renal function) without TP53 deletion/mutation, FCR is the standard first-line therapy: improvement of OS has been demonstrated with this first-line chemoimmunotherapy (Figure 11 A)[30].

In patients with relevant co-morbidity, who are usually older, but without TP53 deletion/mutation, the combination of chlorambucil plus an anti-CD20 antibody (rituximab, ofatumumab or obinutuzumab) prolongs progression-free survival (PFS) when compared with monotherapy and is therefore the standard approach[31].

First-line treatment may be repeated if the relapse or progression occurs at least 24-36 months after chemoimmunotherapy and if TP53 deletion/mutation was excluded. If relapse occurs within 24-36 months after chemoimmunotherapy, or if the disease does not respond to any first-line therapy, the therapeutic regimen should be changed (Figure 11 B)[29].

In addition to cytoreductive therapy, CLL treatment includes substitutive and support therapy, such as red cells and platelets transfusions, antibiotic therapy, and intravenous immunoglobulin administrations.

2. Neoplastic B lymphocytes

CLL is a disease characterized by an extremely heterogeneous clinical course, despite a substantial morphologic and immunophenotypic uniformity.

B cells are lymphocytes that participate in humoral immunity by producing antibodies (Abs) in response to antigen (Ag) stimulation. They can

differentiate from "naive" lymphocytes to cells secreting antibodies against specific antigens (plasma cells), or to "memory" long-lived stimulated B lymphocytes that are ready for rapid response to a repeated exposure of the priming antigen.

The B-Cell Receptor (BCR) mediates antigen recognition. BCR is a multimeric complex composed by an sIg homodimer that is linked to plasmatic membranethrough its constant region (crystallizable fragment, Fc)[32,33]; the sIg antigen binding region (Fab) is outward and noncovalently linked to Igα/Igβ (CD79a/CD79b) heterodimer, deputy to intracellular signal transduction[34] (Figure 12). The Fab region comprehends variable regions (V) of sIg light and heavy chains that give BCR specificity for a specific antigen. In turn, V regions are composed by three ipervariable regions, called "complementarity determining regions" (CDR) that allow high affinity binding with the antigen.

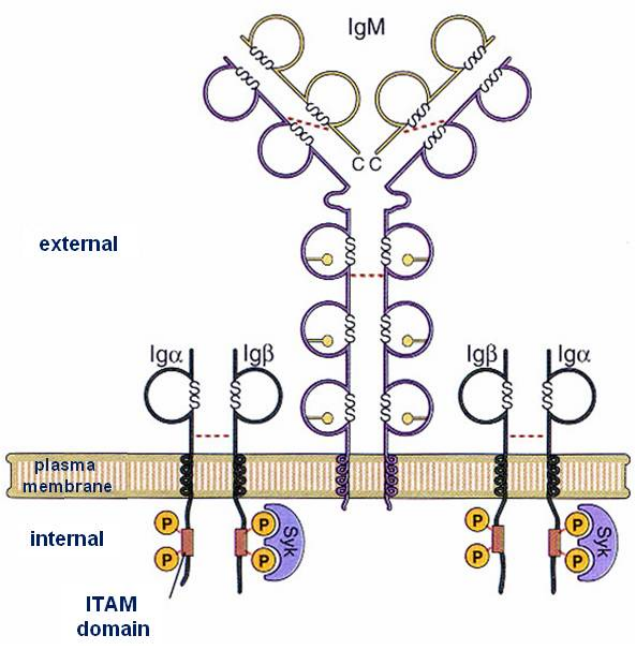

Figure 12. Schematic representation of the BCR. BCR is composed of two immunoglobulin (Ig) heavy and light chains (variable and constant regions), and CD79a and CD79b (Igα/Igβ), which contain an intracellular activation motif that transmits signals to intracellular tyrosine kinases (for example, SYK and Lyn) (adapted form Abbas *et al)*[33].

What differentiates a "naive" B lymphocyte from a "memory" B lymphocyte is the fact that the "naive" one presents an amino acid sequence identical to "germline" sequence, while the "memory" one is characterized by

a different sequence. This is due to somatic hypermutation process that underlies the phenomenon of affinity maturation.

Once recognized a specific antigen, the "naive" B lymphocyte turns on and begins to proliferate inside lymphoid organs. Some of this progeny enters the lymphoid follicles and forms the germinal centre (GC) characterized by an intense proliferation. Here, Ig genes undergo point mutations that lead to the formation of clones with different affinities for the antigen. Clones are selected through contact with follicular dendritic cells expressing antigen: lymphocytes that bind antigen with greater affinity survive, while others undergo apoptosis[35].

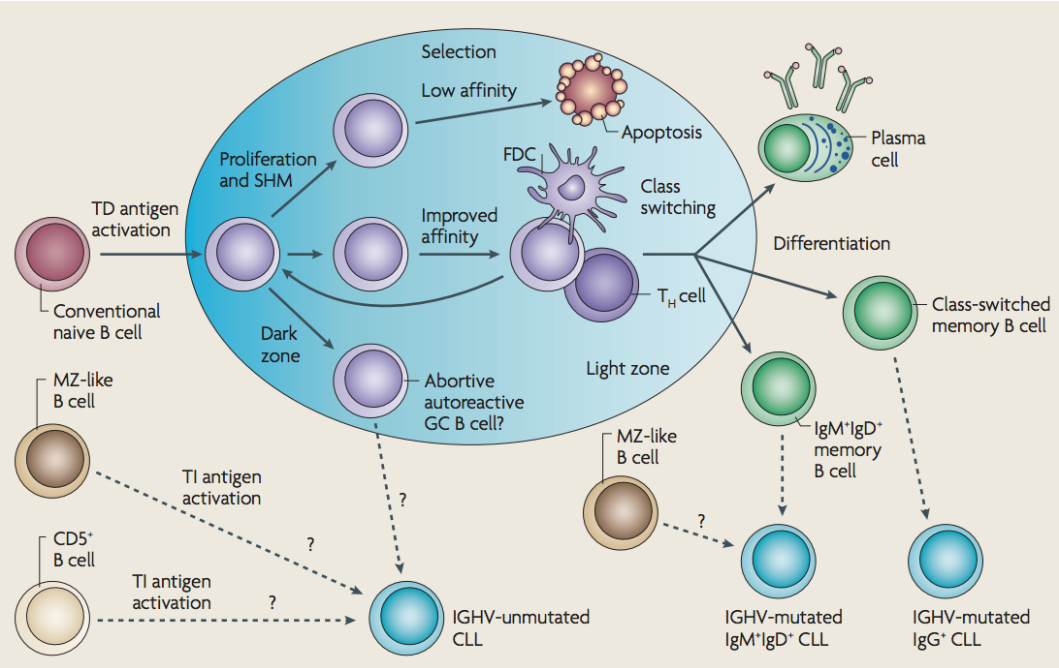

Figure 13. Pathogenesis of CLL. Current thinking is that mutated CLL cells derive from a post-germinal center memory B cell, while not changed one from a B cell, which however has been activated from the antigen; it is unclear if involving the naïve B lymphocyte, the CD5 + B1 or marginal zone B one. Image from Zen *et al*[35].

CLL lymphocytes are small "memory" B cells blocked in G0/G1 and characterized by surface markers recognized by specific monoclonal antibodies; some of these markers, such as CD19 and CD21, are B-related, while others, like CD5, CD23, CD25, and HLA-DR (human Leukocyte Antigen D-related), are not specific for B lymphocytes. In particular, CLL cells express

markers typical of mature B cells localized in the mantle zone of secondary lymphoid follicles.

Recent studies have shown that 50-70% of CLL have undergone IgVH hypermutation, a phenomenon that characterizes normal B cells subjected to a T cell-dependent GC reaction. This finding has led to the hypothesis that CLL cases displaying mutated IgVH may derive from a cell that had transited through the GC, whereas those with germline IgVH may derive from a GC-independent cells. This hypothesis has both biological and clinical relevance since the two subgroups have different prognosis, with M-CLL displaying a better clinical course[36].

The factors involved in CLL pathogenesis comprehend control of apoptosis, signal transduction BCR-mediated, proliferative activity and the microenvironment.

2.1 Intrinsic Factor - Control of apoptosis

The dysregulation of the process of programmed cell death (apoptosis) is now widely recognized as one of the main mechanism in the pathogenesis of many tumors. The accumulation of CLL cells is related to the fact that they do not undergo apoptosis, thus failing the homeostatic mechanism that normally limits the number of circulating cells.

Malignant B cells retain the ability to respond to microenvironmental signals, but have devised a monothematic responsiveness. They have a specific sensitivity to anti-apoptotic signals that favour their survival and become insensitive to pro-apoptotic signals[21].

Apoptosis may be triggered through 2-way (Figure 10)[37]:

1. in the extrinsic way, death extracellular signals (FASL, TNF, TRAIL) are transduced by surface receptors (FAS, TNFR) and the adapter protein FADD activate pro-caspase-8;
2. in the intrinsic way factors such as DNA damage, the presence of reactive oxygen species, pro-apoptotic signals and mitochondrial damage, stimulate the release of cytochrome C into the cytosol where complex with APAF-1 and pro-caspasi 9 forming the apoptosome[38].

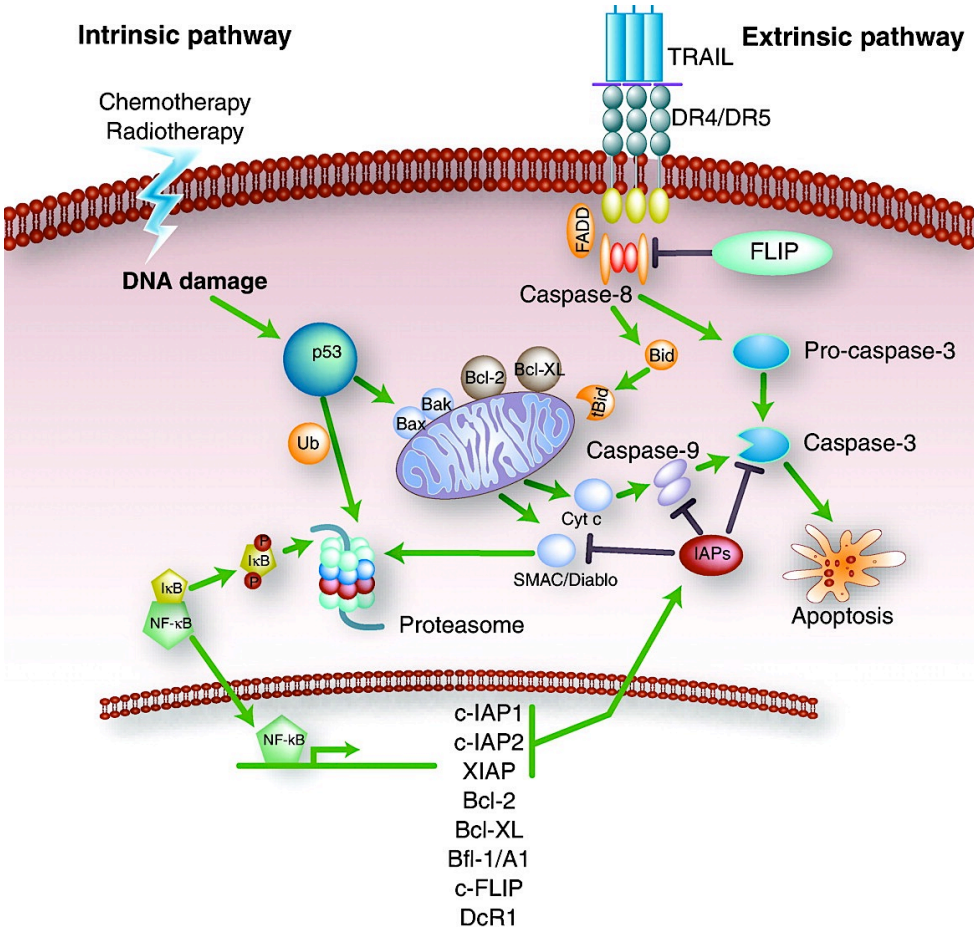

Figure 14. The molecular mechanisms of apoptosis. Apoptosis pathways can be initiated via different stimuli, that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria (intrinsic pathway). Stimulation of death receptors results in receptor aggregation and recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) and caspase-8. Caspase-8 initiates apoptosis by direct cleavage of downstream effector caspases. Mitochondria are engaged via the intrinsic pathway, which can be initiated by a variety of stress stimuli, including ultraviolet (UV) radiation, γ-irradiation, heat, DNA damage, the actions of some oncoproteins and tumour suppressor genes (i.e. p53), viral virulence factors, and most chemotherapeutic agents. CAD, caspase activated DNase; FAS, fibroblast associated antigen. ICAD, inhibitor of CAD; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF related apoptosis inducing ligand[38]. Image from de Vries *et al*[37].

With respect to intrinsic factors, the balance between pro- and anti-apoptotic factors is very important. Among these, the principal apoptosis regulators are proteins of the Bcl-2 family (B-cell lymphoma-2 factors) that play a crucial role in this mechanism by inhibiting (Bcl-2, Bcl-xL, Bcl-w, Bfl-1, and Mcl-1) or promoting (Bax, Bak, Bcl-xS, Bid, Bik, and Hrk) apoptosis. Heterodimerization between pro- and anti-apoptotic members, and their relative levels, may determine the predisposition to respond to a given apoptotic stimulus (Figure 14). Many investigators have reported altered expression of Bcl-2, Bax, and Mcl-1 in CLL[39].

Other intrinsic factors, critical for apoptosis control, are 17p13 and 11q23 deletions containing 2 prominent tumor-suppressor genes mutated at varying proportions: *TP53* (tumor protein 53) and *ATM* (Ataxia telangiectasia mutated), respectively. Mutations of *TP53* and *ATM*, even in the absence of a chromosomal deletion, have been identified to have adverse effects on patient survival. p53 and ATM proteins are central regulators of the DNA-damage-response pathway and their activation leads to cell-cycle arrest and DNA repair, apoptosis, or senescence, depending on the cellular context. Impaired p53 function through mutations and/or deletions is the best-characterized factor associated with chemoresistance in CLL[40].

Moreover, TOSO, also known as Fas-inhibitory molecule 3, was identified as a candidate gene overexpressed in CLL.

TOSO is a transmembrane protein that inhibits Fas-mediated apoptosis by binding Fas-associated death domain (FADD) via its C-terminal intracellular domain. In CLL, high levels of TOSO expression have been correlated with a more aggressive disease[41].

2.2 Intrinsic Factor - BCR-mediated signal transduction

For effective humoral immunity, mature B cells must respond to foreign antigens and generate antigen-specific effector cells; so, it is not surprising that the BCR complex is required for the later stages of B-cell maturation. BCR has two main roles: the first is to transmit signals that regulate B-cell fate decision and the second is to mediate antigen processing leading to the presentation of antigen to T cells, which allows full activation of B cells in the effector phase. The BCR complex consist of immunoglobulin heavy (IgH) and light (IgL) chains associated with Igα and Igβ containing ITAM (Immunoreceptor Tyrosine-based Activation Motif) domains. After BCR ligation by antigen, both the protein tyrosine kinases (PTKs) Syk and Lyn are activated[42]. Then, Lyn phosphorylates ITAMs which, in turn, recruit and facilitate the activation of Syk and Tec-family PTKs. This phosphorylation results in the recruitment of other molecules involved in BCR-mediated signal transduction (Figure 15)[35].

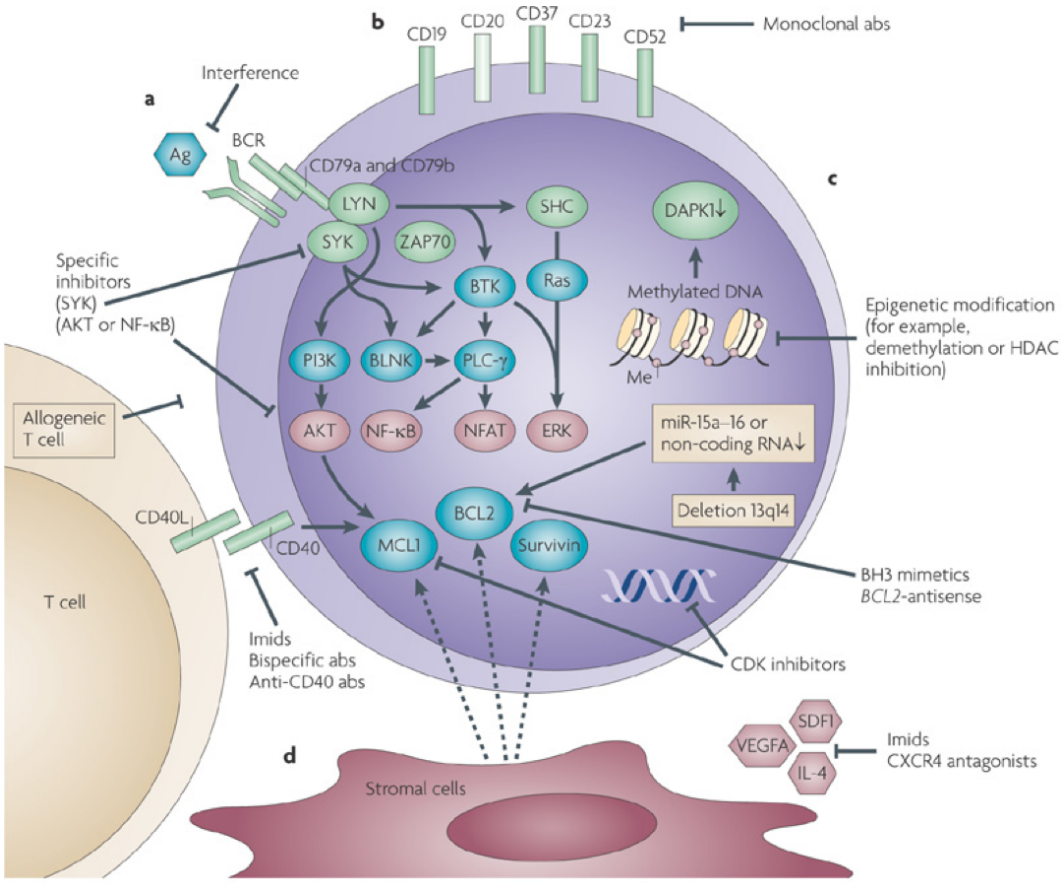

Figure 15. BCR-induced signal transduction pathways. After antigen ligation, tyrosine-kinase Lyn phosphorylates ITAMs of Igα and Igβ, creating binding sites to protein SH2 domain, such as Syk kinase. Follow different biochemical reactions that culminate in the B cell activation, differentiation and/or proliferation. Image from Zen *et al*[35].

BCR signaling can be also regulated by the membrane organization of signaling components. Literature data proposed that in the absence of antigen binding, the BCR is already pre-assembled into oligomeric receptor complexes, which generate a basal level of signaling essential for B-cell maintenance[43]. The low levels of sIgs may explain the reduced ability of CLL cells to capture, present, and respond to antigen. Defects in the BCR of CLL have been attributed to functional deficiency in the CD79 heterodimer, especially CD79b, which is expressed at low levels on these tumor[44].

During B cells activation process, an important function is carried out by plasma membrane microdomains called lipid rafts. These domains are rich in glycosphingolipids and cholesterol which create a liquid-ordered phase within the plasma membrane. Lipid rafts are fluid at physiological temperatures, allowing lateral diffusion of proteins and lipids within the

plane of the membrane. In addition, they are constitutively enriched in certain types of proteins such as glycosphingolipids-linked proteins and lipid chain-modified proteins, including heterotrimeric G proteins, the Src kinases Lyn and Fyn, and other molecules involved in signal transduction, such as Blk, Ras, c-Abl, and actin[45].

Additional proteins, like CD45 and Syk, seem to be excluded from raft and recruited only after BCR translocation into them after the engagement by the Ag. In fact, according to the most recent model proposed to explain BCR functions in the activation of B cells, in resting B cells the BCR is initially excluded from lipid rafts; once having bound the antigen, BCR translocates into the rafts thus starting the signal transduction cascade.

Lyn is a Src-family tyrosine kinase, which, with Blk and Fyn Src-like kinases, is necessary for BCR signaling considering its role in phosphorylation of the ITAM on Igα/Igβ. Tyrosine phosphorylated Igα/Igβ recruits Syk *via* the latter's tandem SH2 domains leading to downstream signaling events. Lyn also provide feedback inhibition of BCR signaling by phosphorylation of cell surface proteins containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These ITIM-containing inhibitory receptors include the inhibitory Fc receptor for IgG, FcγRIIb, and the sialic acid-binding protein expressed on B cells, CD22. The phosphorylation of these ITIMs generates binding sites for the membrane recruitment of phosphatases that inhibit BCR signaling, including the SH2-domain-containing inositol phosphatase (SHIP-1) and the SH2-domain-containing tyrosine phosphatase (SHP-1). The net result of Lyn-deficiency is an exaggerated signaling by the BCR, a phenotype that is moderate in immature B cells and highly pronounced in mature follicular B cells. Lyn also acts inhibiting receptor signaling in myeloid cells and recent studies demonstrated that hyperactivity of myeloid cells contributes, in a relevant manner, to autoimmunity in Lyn-/- mice[46]. In B lymphocytes, Lyn may also be associated to the non-receptor tyrosin-kinase Fak (Focal Adhesion Kinase), involved in different signal transduction cascades. This complex may contribute to cytoskeletal reorganization after antigen binding[47].

Our group demonstrated that in CLL, as compared to normal B cells, protein Lyn is upregulated and shows a different subcellular localization[48]. Moreover, Lyn displays a remarkable constitutive activity, which leads to an increased basal tyrosine protein phosphorylation and a low responsiveness to BCR ligation. Whereas Lyn was concentrated in membrane lipid rafts in normal B cells, the enzyme was present all over the cell surface membrane in CLL cells. Lyn was also detected in the cytosol of the malignant B cells. The release of Lyn into the cytosol following caspase-dependent cleavage of the tyrosine kinase at its N-terminus has been described as a general mechanism in hematopoietic cells during BCR-induced apoptosis[49]. The findings that CLL cells contain a cytosolic Lyn fraction and are defective in programmed cell death suggest that the tyrosine phosphorylation of specific cytosolic targets might account, at least in part, for cell resistance to apoptosis.

The activity of Lyn is critically regulated through its C-terminal Tyr507, which is phosphorylated by the tyrosine kinase Csk and dephosphorylated by the receptor tyrosine phosphatase CD45. In resting B lymphocytes, Lyn is present in its inactive conformation, as result of Csk phosphorylation of Tyr507, which gives rise to an intramolecular association of the phosphorylated residue with Lyn's own SH2 domain[50]. Since Csk, unlike Lyn, was similarly expressed in normal and CLL cells, the constitutive activity of Lyn could be due to the fact that the amount of Csk is insufficient to phosphorylate and down regulate its overexpressed substrate. However, it is likely that other factors are responsible for the presence of the active form of Lyn in CLL cells, first among which might be the dephosphorylation of Lyn at Tyr507 by the tyrosine phosphatase CD45, an abundant membrane protein that, in normal B cells, has access to Lyn only after its migration to lipid rafts induced by BCR engagement[45]. Furthermore, the high concentration of Lyn in CLL cells could promote the kinase intermolecular autophosphorylation at Tyr396, which in turn induces Lyn activation[51].

It is known that, after activation, Src Family Kinase (SFK) level is regulated by the balance of two opposing mechanisms: degradation by ubiquitinylation or rescue by association with Hsp90 (Heat shock protein of 90kDa), a chaperone interacting with the N-terminal lobe of the SFK catalytic

domain[52]. Recently, we demonstrated that, in CLL cells, Lyn is an integral component of an aberrant cytosolic 600kDa complex, where Lyn is associated both with Hsp90 through its catalytic domain, HS1 (Hematopoietic lineage cell Specific protein 1), and SHP-1 through its SH3 domain. Moreover, Hsp90 stabilizes the complex by contributing to converting a network of transient interactions into permanent ones, thus maintaining Lyn in an active conformation and preventing its degradation[53].

HS1, one of the most important Lyn substrate, is an F-actin binding protein involved in the apoptosis of several hematopoietic cell lines. HS1 phosphorylation occurs in a sequential model mediated by Syk and Lyn. It seems that tyrosin-phosphorylation of cortactin, an HS1 homologous protein involved in cell motility, occur by the same mechanism of recruitment of the SFKs.

PI3K and PLCγ2 are both crucial effector enzymes that generate key second messengers in BCR signaling. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdInsP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PtdInsP3), which, in turn, recruits some BCR signaling molecules to the membrane through PH domains. PLCγ2 uses PtdInsP2 to generate inositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG), which are required for the release of intracellular calcium (Ca2+) and activation of protein kinase C (PKC), respectively. Subsequently, Ca2+ flux and PKC activation induce the activation of mitogen-activated protein kinase (MAPK)-family kinases, extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK), p38 MAPK, and transcription factors, including nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFAT). It is probable that the profile of these activated transcription factors then determines B cell fate.

Non-enzymatic adaptor proteins are also important in regulating BCR signaling. Among them, B cell linker (BLNK) efficiently connects Syk and Btk with PLCγ2. Disruption of the BLNK gene leads to impaired activation of PLCγ2 in B cells; BLNK also associates with Vav and Nck, both of which regulate cytoskeletal organization in B cells.

Another B-cell adaptor, B lymphocyte adaptor molecule of 32kDa (BAM32), also binds PLCγ2 and regulates its activation. Since it is recruited to the membrane in a PI3K-dependent manner, BAM32 integrates the PI3K and PLCγ2 pathways. The B cell-specific co-receptor CD19 can work as an adaptor for PI3K also in B cells.

In mature B cells, BCR associates with lipid raft after Ag engagement and the signal transduction induces transcription of genes responsible for B cell activation. Then, the BCR is internalized and can be degraded or sent to an intracellular compartment called MIIC (MHC-class-II-peptide-loading-compartment) where Ag processing and the synthesis of peptide-MHC complexes to Ag presentation occur. These complexes are brought to cell surface, presented to T cells and then recognized by TCR of Th cells which activate B cells through cytokines release.

In immature B cells, BCR is excluded from lipid rafts even after Ag binding and, in this case, cell apoptosis is induced. A behavior similar to that of immature B cells is described also to cells rendered tolerant or anergic by chronic exposure to Ag: BCR is still excluded from rafts even after Ag binding and the result is the lack of a cellular response. Conversely, in B pre-lymphocytes a significant proportion of BCR and signaling molecules, such as PLCγ2 and PI3K, are constitutively associated with rafts and this seems to generate signals of survival and cell differentiation. In these cases, molecule such as PI3K, RAS, RAF, ERK and NF-κB are fundamental in signal transduction in association with BCR. PI3K activates and phosphorylates Akt/PKB which, in turn, phosphorylate cellular targets involved in cell survival including apoptotic factors and glycogen metabolism. One function of Akt is to inhibit the activation of the pro-apoptotic Bcl-2-family member BAD (Bcl-2 antagonist of cell death); moreover, Akt phosphorylates and inhibits glycogen synthase kinase 3 (GSK3) in B cells. In unstimulated cells GSK3 is constitutively active; it phosphorylates and destabilizes Myc and cyclin D, both of which are required for cell-cycle progression. In this way, it seems that Akt functions to promote BCR-induced cell proliferation, as well as survival[42].

2.4 Extrinsic Factor - Microenvironment

Despite their *in vivo* prolonged lifespan due to intrinsic defects, also sustained by the over-expression and constitutive activation of Src kinase Lyn[48], CLL leukemic cells rapidly undergo spontaneous apoptosis *in vitro*, highlighting the need of extrinsic signals delivered by the microenvironment. In fact, at the active sites of disease, such as bone marrow (BM) and secondary lymphoid tissues, CLL cells engage complex cellular and molecular interactions with stromal cells that affect their survival, growth and often confer drug-resistance[54].

Among bone marrow stromal cells, mesenchymal stromal cells (MSCs) show a bidirectional cross-talking with neoplastic B cells. Leukemic cells are supported by stromal cells and, in turn, are also able to activate and induce stromal cell to proliferate and release several mediators (i.e., CXCL12, CXCL13, CCL19 and CCL21) which sustain the ongoing process[55,56].

Although in healthy subjects MSCs represent a small fraction of the stromal cell population, immunohistochemistry studies showed that *in situ* αSMA+ mesenchymal stromal cells, which represent the *in vivo* counterpart of MSCs, are the dominant stromal cell population in CLL microenvironment[57]; thus suggesting a MSC crucial role on favouring malignant cells and disease progression. The interactions between MSCs and neoplastic B cells can occur both through direct cell-cell contact and via soluble factors secreted by the microenvironment[58].

Bone marrow precursors derived from pluripotent stem cells are in contact with stromal cells and generate B cells in an Ag-independent process. BM precursors differentiate to mature virgin B lymphocytes endowed with membrane Ag receptors that migrate to peripheral lymphoid tissues searching an Ag. The encounter with a foreign Ag triggers B cell activation, proliferation, and a second wave of differentiation. The microenvironment for the active social life of a mature B cell is provided by the germinal centres of secondary lymphoid organs. It is within GC that trafficking mature B cells are brought into close contact with specialized T cells and Ag-presenting cells. This dialogue, finely regulated by cytokines, adhesion structures, and

surface molecules, leads to the generation of B memory cells and plasma cell precursors and to the apoptotic elimination of inefficient or potentially dangerous cells. As all normal B cells evolve and operate thanks to microenvironmental cross-talks, it becomes consequent to ask whether the microenvironment may also influence the natural history of B cell malignancies[59].

Isolated CLL cells undergo relatively rapid apoptosis in vitro. This observation has led to the speculation that the microenvironment is necessary and/or plays a pivotal role in maintaining the enhanced survival of CLL cells in vivo. Human bone marrow stromal cells (BMSCs) have been demonstrated to support the survival of CLL cells when both cell types were co-cultured in vitro. Further investigations have suggested that CLL cells need to have intimate contact with BMSC in bone marrow, with T cells in lymph nodes, and with nurse-like cells (NLCs) in lymphatic tissues to maintain survival[60]. Mesenchymal Stromal Cells (MSCs) from both normal healthy donors and CLL patients were able to protect leukemic cells from undergoing spontaneous and drug-induced apoptosis. Our data show that CLL B cells are susceptible to the anti-apoptotic effect of MSCs, favoring neoplastic B cell survival *in vitro* for at least 7 days[61]. Close contact between CLL cells and MSCs is capable to mediate the most effective drug-resistance and it is this latter interaction that could be the most important in providing a niche for residual CLL cells post treatment. We have demonstrated that the analysis of the cleavage pattern of PARP protein detected in CLL patients after 7 day co-culture with MSCs allowed us to distinguish patients into two groups on the basis of their dependence/independence to microenvironmental pro-survival stimuli. The first group is represented by those CLL B cell samples that underwent spontaneous apoptosis in medium alone, but were rescued from apoptosis following exposure to MSCs. On the contrary, the second group identified CLL samples whose viability was high when cultured both in medium alone and in the presence of MSCs, indicating that these CLL clones were able to survive independently from signals coming from the microenvironment[61].

Recent studies demonstrated the significance of CD49d (α4 integrin) in the prognosis of CLL disease[53,54] as well as its biological role of regulating matrix metalloproteinase-9 (MMP-9)[62] Chronic B cell malignancies and bone marrow microenvironment. These observations imply that α4β1 integrin could be a critical mediator of tight interactions between MSCs and CLL cells and MSC-mediated CLL protection.

The signals delivered for normal B-lymphocytes by activated T-cells through CD40L induce B-cell growth, differentiation, and rescue from apoptosis. In contrast to normal B cells, a subset of CLL cells expresses both CD40L and its receptor, enabling an autocrine loop by which CLL cells can promote survival signals on their own. CD40 stimulation of CLL cells had been shown to prevent apoptosis and induce proliferation in vitro. It has been observed a correlation between levels of the anti-apoptotic protein Bcl-2 and survival induced by stromal cell contact, suggesting a mechanism by which stromal cells induce protection of CLL cells against spontaneous apoptosis.

In contrast, it was found that normal CD5+ B lymphocytes were unable to survive in co-culture with stromal cells. Stromal cells can, in addition, induce survival of CLL cells by stromal cell-derived factor-1 (SDF-1/CXCL12), which is a homeostatic chemokine that signals through the CXCR4 chemokine receptor and plays an important role in lymphopoiesis. High-levels of SDF-1 are constitutively produced by stromal cells within the marrow, the primary site of early B cell differentiation. CLL cells express high levels of the CXCR4 surface receptor, and undergo chemotaxis in response to SDF-1. In addition, it has been demonstrated that marrow stromal cells attract CLL cells via the chemokine receptor CXCR4, providing a possible explanation accounting for the infiltration of marrow by CLL cells[55].

All these studies demonstrated the importance of microenvironment: MSCs co-culture represents a reproducible *in vitro* system with functional similarities to *in vivo* bone marrow conditions, pointing out that the heterogeneity of the disease is reflected also in CLL B cell capacity to respond to favorable signals from MSCs[61].

3. Janus family kinase/Signal Transducer and Activator of Transcription pathway

Several signal transduction pathways use a number of mediators and a series of proteins with function of receptor in order to transport the information (transmitted by cytokines, hormones and growth factors) from the microenvironment into the cell. These molecules induce different responses, including: cell proliferation, survival, differentiation and motility. In order to act on cells, these factors must interact with specific receptors able to transduce the signal inside the cell; the most important receptors are:

1. receptors with intrinsic tyrosine kinase activity (Receptor Tyrosine Kinase, RTK);
2. receptors consisting seven transmembrane domains coupled to G proteins (*e.g.* the receptors for chemokines);
3. receptors for cytokines.

Different hormones (*e.g*. erythropoietin, prolactin) and cytokines (*e.g.* IL-6) use receptors for cytokines not presenting intrinsic kinase activity (differently from RTKs), but acquiring it upon binding with the specific ligand; this interaction enables the recruitment, at the cytosolic portion of the receptor, of tyrosine kinases that in turn phosphorylate various substrates, inducing the transduction of different signals (*e.g*. proliferation, survival, etc.)

Among the signaling pathways activated by this class of receptors, one of the most important is represented by tyrosine kinases of Janus family (JAK) and the Signal Transducer and Activator of Transcription (STAT) proteins. Previously four different JAKs (JAK1, JAK2, JAK3 and TYK2) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) have been identified; through different combinations they are able to mediate signal of more than 40 cytokines[63].

The bond with the ligand induces the dimerization of the receptor complex, resulting in a connection of the JAKs and in their cross-phosphorylation.

The activated JAK, in turn, phosphorylates the cytoplasmic domain of the cytokine receptor, thus recruiting and phosphorylating STAT. STAT, once phosphorylated, form activated dimers or tetramers which translocate into the cell nucleus where bind specific DNA sequences called STAT Binding Elements (SBE) and activate the expression of target gene (*e.g.* Mcl-1, Bcl-2, SOCS3, etc) (Figure 16).

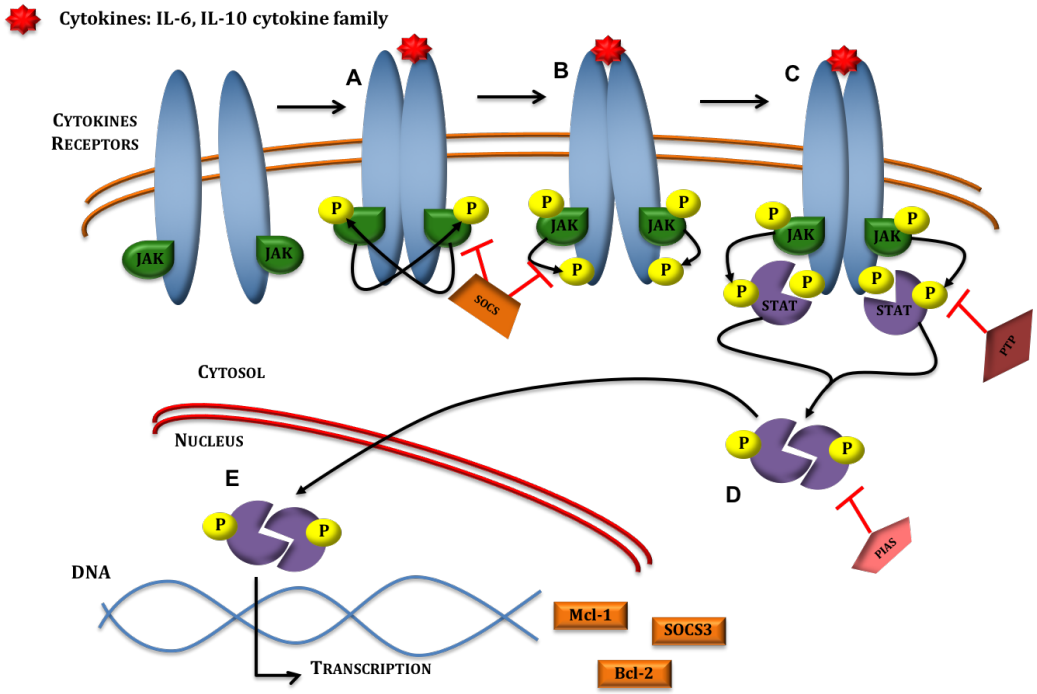

Figure 16. Representation of JAK/STAT molecular pathway. The interaction between receptor and cytokines results in the formation of an activated receptor complex. This complex involves JAK that cross-phosphorylates (A) and phosphorylates the cytokine receptor (B). Subsequently, STAT is recruited and phosphorylated by JAK (C). Activated STAT dimerizes and then translocates to the nucleus (D) where it can induce the transcription of several genes (E), including those for STAT itself, SOCS and others that regulate cell growth.

The target genes of this pathway are genes deputies to the regulation of cell growth, differentiation, proliferation, survival and apoptosis. Deregulation of JAK/STAT signaling pathway may lead to inappropriate activation of STAT and to its subsequent transformation in an oncogene involved in tumor development of hematopoietic cells[64].

Moreover, there are three classes of negative regulators of JAK/STAT pathway: SOCS (Suppressors of Cytokine Signaling), PIAS (Protein Inhibitors of Activated STATs) and PTP (Protein Tyrosine Phosphatases)[65].

SOCS proteins belong to an eight-member family. They present an SH2 domain, a sequence called "SOCS box" in the C-terminal domain and a kinase-

inhibitory region located in the SH2 domain. These proteins create a negative feedback loop in JAK/STAT axes[66]; the activated STAT, in fact, promote the transcription of SOCS genes and, once expressed, these proteins carry out their activities in three different ways:

1. binding to phospho-tyrosine receptors, thus physically blocking the recruitment of STATs;
2. specifically binding JAKs in order to inhibit its kinase activity;
3. interacting with the elongin BC complex, enabling the ubiquitination of JAK and inducing its degradation *via* the proteasome[67].

The second class of negative regulators consists of the PIAS (PIAS1, PIAS3, PIASx e PIASy). These proteins are able to bind STAT activated dimers in order to avoid their interaction with DNA.

Finally, among PTP the best known protein is SHP-1 (Short Heterodimer Partner-1), which contains two SH2 domains and is able to dephosphorylate JAK proteins or phosphorylated receptors to ease their deactivation.

3.1 Janus family kinase (JAK)

Various human malignancies are characterized by an excessive activation of JAK and their associated transcription factors (STATs). There are four JAKs: JAK1, JAK2, JAK3, and TYK2. JAKs molecular weight is about 120-140 kDa and it is composed from seven defined regions of homology called Janus homology domain 1 to 7 (JH1-7) (Figure 17). JH1 is the domain containing the enzymatic activity and with conserved Tyr fundamental for JAK activation (*e.g.* Tyr 1007/1008 in JAK2). Phosphorylation of these tyrosine residues leads to conformational changes in JAK protein thus facilitating the binding of the substrate. JH2 is a "pseudokinase domain", a domain structurally similar to a tyrosine kinase and essential for a normal kinase activity, but lacking enzymatic activity. JH3-JH4 domains show similarity with Src-homology-2 (SH2) domains. JH4-JH7, at the N-terminal

domains, are called FERM domain, that is a protein module involved in localising proteins to the plasma membrane[68].

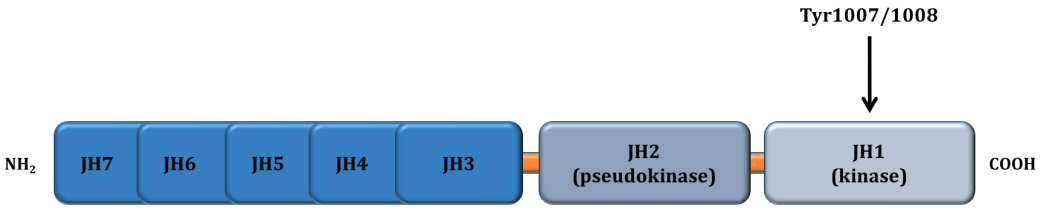

Figure 17. JAK structure. A representation of the domains that form JAK structure: JH1 (kinase), JH2 (pseudokinase) and JH3-7 (FERM domain). The phosphorylation site Tyr1007/1008 characterized JAK2 isoform.

JAK kinases, either alone or in combination with other JAKs, may be preferentially activated depending on the receptor that is going to be activated. The JAK family gets active when two JAKs are brought into close proximity and the trans-phosphorylation is allowed. Once activated, JAKs can phosphorylate additional targets which include both the receptors and their major substrates, the STATs.

Activation of JAK-dependent intracellular signaling is regulated at various levels. Conformational restraint of the JH1 domain through association with JH2 domain maintains the kinase in an inactive form prior to receptor engagement. Meanwhile, the action of tyrosine phosphatases and the expression of specific inhibitors of JAKs limit the duration of JAK-STAT signaling.

Among JAKs, JAK2 has a key role in the activation of JAK/STAT pathway due to its Tyr kinase activity. JAK2 is tied to different cytokine receptors; the binding of the ligands (*e.g.* cytokines such as IL-6, IL-10, etc.) to their receptors triggers the activation of JAK2 that, in turn, phosphorylates Tyr residues on the same receptor thus creating sites for interaction with protein containing p-Tyr-binding SH2 domains, such as STAT3[69].

3.*2 Signal transducer and activator of transcription (STAT)*

Constitutive activation of STATs occurs at very high frequency in different hematopoietic malignancies and solid tumors[70]. Among the

cytokines secreted by microenvironmental cells the most released is interleukin-6 (IL-6)[71].

STAT3 has a pivotal role in promoting cell survival by regulating the expression of anti-apoptotic proteins such as Mcl-1 and Bcl-2, both over-expressed in CLL. For this reason, among all proteins belonging to the STAT family, in this thesis we focused on STAT3, the main mediator of IL-6/JAK/STAT pathway.

This signaling pathway, involved in the pathogenesis of many solid and hematological tumors, starts with the binding of from the structural point of view, it is a glycoprotein of 184 amino acids composed by four alpha helices, with a molecular weight of 22-28 kDa, and encoded by a gene localized in chromosome 7. It was identified in 1986 and named B-cell stimulatory factor 2, because of its ability to stimulate activated B cells to differentiate into plasma cells and to secrete immunoglobulins. Moreover, IL-6 has pleiotropic functions: it is not only an important regulator of inflammatory and immune responses, but also of metabolism, hematopoiesis, angiogenesis and bone homeostasis. IL-6 is produced by different cell types, including monocytes, B and T lymphocytes, fibroblasts, keratinocytes, endothelial cells, adipocytes, and several types of cancer cells.

The interaction between IL-6 and its receptor is not sufficient to activate signal transduction, but requires binding the of the IL-6/IL-6Rα complex to the transducing subunit of the IL-6 cytokines family, known as gp130. The formation of the IL-6/IL-6Rα/gp130 trimeric complex allow the recruitment of the non-receptor tyrosine kinase JAK2 and of STAT3 protein.

STAT3 protein belongs to the STAT protein family, composed of 7 members STAT1, 2, 3, 4, 5a, 5b and 6. STATs are activated by all the members of the IL-6 cytokines family, including: IL-12, IL-13, interferons, IL-10, G-CSF (Granulocyte-Colony Stimulating Factor) and other growth factors[68]. The gene encoding this protein is located on chromosome 17q21.31.

Two functionally distinct STAT3 isoforms are known: STAT3α (complete) and STAT3β (missing of TAD trans-activation domain at the C-terminal). The main isoform, STAT3α, is a protein of 770 amino acids formed by six distinct functional domains (Figure 18):

- a N-terminal domain;
- a leucine zipper coiled-coil domain;
- a DNA-binding domain (DBD) that allow STAT3 to exert its function of transcriptional activator;
- a linker domain;
- a SH2 domain (Src Homology 2) essential for dimerization and recruitment to the receptor; in this domain there is the tyrosine 705 which is phosphorylated by JAK2;
- a C-terminal tail with a TAD trans-activation domain, whose activity is stimulated by serine 727 phosphorylation.

The beta isoform of STAT3, instead, is a protein truncated at amino acid 715; it is produced by alternative splicing in correspondence of the exon 23 and deficits the SH2 domain.

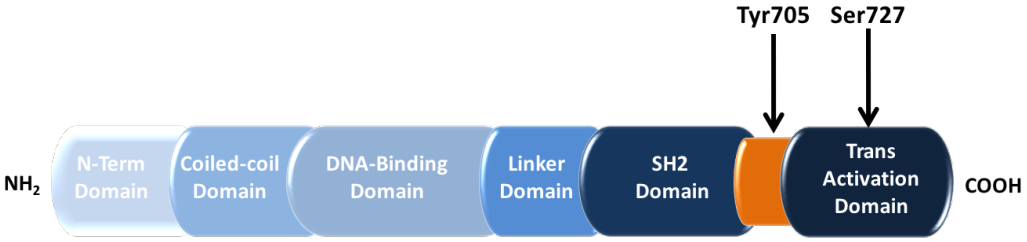

Figure 18. STAT3α structure. It is recognizable the N-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain, the SH2 domain and the trans-activation domain. The two phosphorylation sites of STAT3α, Tyr 705 and Ser727, are shown.

The regulation of STAT3 activity occurs through two different sites of phosphorylation: the tyrosine (Tyr) 705 and serine (Ser) 727. STAT3 phosphorylation at Tyr705 is due to JAK2 kinase and regulates the dimerization through the SH2 domain (which recognize phospho-Tyr). The formation of STAT3 homodimers or STAT3-STAT1 heterodimers are indispensable for the activation of the JAK/STAT pathway; in fact, only in the dimeric form, STAT3 is able to migrate into the nucleus where it binds to the DNA consensus motifs (DBD region) of its target genes.

In addition to tyrosine phosphorylation, which is found to be constitutively present constitutively in various solid and blood tumors, STAT1, 3, 4, 5a and 5b are occasionally phosphorylated at Ser in the C-

terminal transactivation domain. The role of STAT3 Ser727 phosphorylation is still not defined: some studies suggest it has an important role in transcriptional activity, while others demonstrate it plays an inhibitory function. In CLL, STAT3 is constitutively phosphorylated at Ser727 with respect to normal B lymphocytes[72], but the phosphorylation status at Tyr705 is still unclear[73].

3.3 JAK/STAT pathway in oncogenesis

Considering the importance of JAK/STAT axis for neoplastic cell survival, the investigation on this pathway and its regulation/inhibition is crucial. Several studies have shown that the inappropriate activation of JAK/STAT signaling contributes in a direct way to oncogenesis. The aberrant activation of STAT has been described in several cancers and has been well characterized also in leukemias. Several mechanisms involved in constitutive STAT activation have been described in hematological cancer[74]. Among this:

- an increased production of cytokines and their receptors; it can occur either in an autocrine or paracrine way;
- a decreased expression of SOCS proteins due to methylation of their promoter sequence;
- a decrease of tyrosine phosphatases;
- mutations "gain of function" of the receptor. In hematological disorders the most common mutations affect Receptor Tyrosine Kinases (RTKs), whereas they are not frequently found in the cytokine receptors;
- mutations "gain of function" of the kinases that phosphorylate STAT: a point mutation in JAK2 pseudo-kinase domain, responsible for its constitutive activation, is frequently observed in cases of polycythemia vera and in some myeloproliferative disorders. Another example is the t(9:12), found in acute lymphocytic leukemia (ALL), resulting in the fusion between JAK2 and Ets (a

transcription factor), that becomes constitutively active after dimerization;

- oncogene activation by other proteins: the oncogenic fusion protein BCR/ABL (known as Chromosome Philadelphia) derives from t(9:22) and it is found in all cases of chronic myeloid leukemia (CML) and in some cases of ALL. BCR/ABL has the constitutive kinase activity of ABL and activates multiple signaling pathways, including that involving STAT5: BCR/ABL forms a complex with the protein Hck, belonging to the family of Src kinases, resulting in STAT5 activation.

The great number of mechanisms by which the STAT oncogenes are activated and the several hematological malignancies in which they are deregulated, suggest the importance of these proteins in the pathogenesis of various diseases. Furthermore, STATs represent the link between several pathways, that once altered, cause the development of varius neoplasms[75]. STAT3, in fact, connects the pathway triggered by the interaction between the epidermal growth factor (EGF) and its receptor (EGFR) and the phosphoinositol 3-kinase (PI3K) axis. In multiform glioblastoma, EGF/EGFR signaling promotes STAT3 to binding DNA, while PI3K pathway negatively affects it[76].

Moreover, the activity of STAT3 is negatively regulated by RAS/MAP-kinase (Mitogen Activated Protein) axis. The constitutive expression of MEK, one of the activated kinases of the pathway, that in turn activates ERK (extracellular signal-regulated kinase) or the overexpression of ERK in itself, inhibits the activation of STAT3 mediated by IL-6 in CHO and HEK 29322 cell lines.

3.4 Activation of STAT independently of JAK

B cell receptor (BCR) is fundamental for the development of normal B cells and it is also involved in the progression of the most common B cell disorders, such as follicular lymphoma, mantle cell lymphoma and CLL.

In CLL, after the binding with the antigen, BCR triggers several signaling pathways in order to modify gene expression and determine cell destiny. BCR requires the recruitment of the cytosolic protein tyrosine kinase (PTK) to trigger the signaling pathway.

Lyn, a member of SFK, plays a central role in the initiation of the transduction pathway triggering a rapid cascade involving other kinases and second messengers, which are involved in B cell proliferation, differentiation, apoptosis, migration and cell growth arrest. Whereas JAK proteins are not involved in BCR pathway, activation of STAT due to this axis has long been ignored. However, literature data shown that Src kinases can directly phosphorylate the STATs, independently from JAK; this pathway is called no-JAK PTK–STAT axis. In fact, Lyn, stimulated after BCR antigen stimulation, can in turn phosphorylate STAT3 Tyr705 activating it[77]. However, the phosphorylation of STATs mediated by Lyn through BCR is transient. This is probably due to Lyn's dual role in both positive and inhibitory regulation of BCR signaling. For this reason, the functional consequences of Lyn-mediated STATs activation may result in a different set of genes transcribed than more sustained activation by the JAKs[78].

AIM OF THE STUDY

In CLL several molecules released from the microenvironment partners (*e.g.* MSCs), signal through JAK-STAT pathways, thus favoring cell survival[70].

Constitutive activation of JAKs and STATs occurs at very high frequency in different hematopoietic malignancies and solid tumors. Among JAKs, JAK2 has a key role in the activation of JAK/STAT pathway due to its tyrosine (Tyr) kinase activity. JAK2 is tied to different cytokine receptors; the binding of the ligand (*e.g.* IL-6) to its receptors triggers the activation of JAK2 that, in turn, phosphorylates Tyr residues on the same receptor, thus creating sites for interaction with protein containing p-Tyr-binding SH2 domains, such as STAT3[68].

STAT3 has a pivotal role in promoting cell survival by regulating the expression of anti-apoptotic proteins such as Mcl-1 and Bcl-2, both over-expressed in CLL. STAT3 is constitutively phosphorylated at serine (Ser) 727 residue in CLL cells[73], while its Tyr-phosphorylation status is unclear.

During this PhD program we planned to further investigate JAK2/STAT3 axis and to study how this pathway interacts with BCR/Lyn signaling. To this purpose, we were aimed at analysing:

- STAT3 expression and activation in CLL cells;
- inhibition of JAK2/STAT3 pathway on B CLL cells through AG490 and Stattic treatment;
- MSC pro-survival effect on CLL B cells after JAK2/STAT3 inhibition by AG490 and Stattic;
- JAK2/STAT3 pathway after Lyn inhibition by Dasatinb;
- molecules involved in the cross-talk between BCR/Lyn and JAK2/STAT3 pathways.

The aim of this study was to better understand CLL pathogenic JAK2/STAT3 pathway with the ultimate goal of developing new therapeutic strategies for CLL patients.

MATHERIALS AND METHODS

1. Patients

Leukemic B cells will be obtained from CLL patients. We analyzed 76 CLL patients (49 males and 27 females), aged between 49 and 90 years, enrolled by the Hematology Division (chief Prof. G. Semenzato), Padua University School of Medicine.

Clinical characteristics of patients are listed in Table I. In particular, for each patient we reported: the number of white blood cells (WBCs), the percentage of lymphocytes, mutational status of IgVH genes and the expression of ZAP-70. All neoplastic B cells of the 76 patients examined were positive for CD5, CD19 and CD23 markers, typically co-expressed in CLL and those of 16 subjects were also CD38 positive.

B lymphocytes obtained from peripheral blood of 23 healthy subjects were used as normal controls.

Table I. Patients enrolled in the study	
Patients	76
Median age, years (range)	72 (49-90)
Male/Female	49/27
WBCs count, $x10^9$/l (range)	49,789 (4.7-300)
Lymphocytes, % (range)	74 (46-97)
Mutated*	34
ZAP70-positive†	25
CD38-positive‡	16

* Immunoglobulin heavy chain variable region (IgVH) mutational status: patients with <2% differences from the most similar germline gene in both the expressed VH and VL genes were define unmutated; mutated cases were defined as those in which the CLL cells displayed ≥2% differences in either the

† Parameter assessed by flow cytometric analysis (ratiometric method, cut-off> 0.5);

‡ Parameter assessed by flow cytometric analysis (cutoff> 30%);
The cell samples were analyzed by the flow cytometer FACSCanto A (Becton Dickinson; Franklin Lakes, NJ) and data obtained were processed using the software FACSDiva 7. For each analysis 20.000 events were acquired.

2. Isolation of B lymphocytes from peripheral blood

2.*1 Purification of B lymphocytes*

B lymphocytes were isolated from peripheral blood of CLL patients. From a sample of heparinized venous blood, peripheral blood mononuclear cells (PBMCs) cells were obtained proceeding with a layering on Ficoll/Hypaque (F/H) (Amersham Biosciences; San Francisco, CA). This method exploits the difference of density of mononuclear cells (lymphocytes and monocytes) with respect to the other elements of the blood. Mononuclear cells, which have lower density, focus on the layer of F/H while the red blood cells and granulocytes are collected on the bottom of the tube. Considered the high WBC count in CLL patients, peripheral blood was first diluted in 1:6 ratio with 0.9% sodium chloride (saline) at room temperature, gently agitated, and later layered slowly over F/H solution. We proceeded with a centrifugation at 900g for 20 min at 20°C, without brake. The ring of mononuclear cells formed at F/H interface was aspirated and subjected to two successive washes with saline by centrifugation at 400g for 10 minutes at 20°C (Figure 19). The pellet was resuspended in an adequate amount of saline and the cells were counted in a Burker chamber.

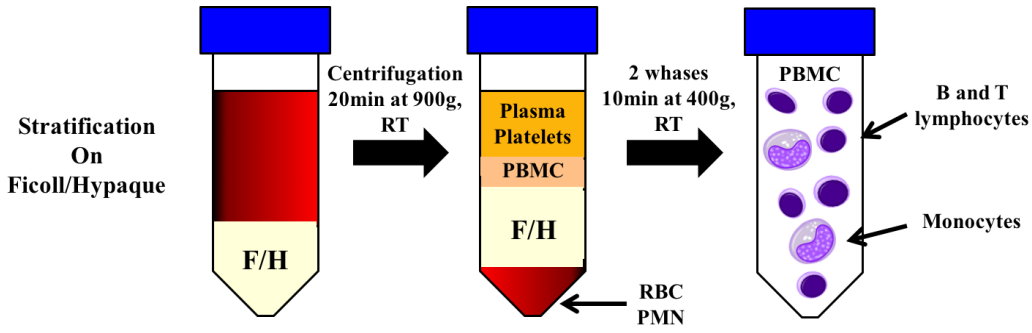

Figure 19. Isolation of mononuclear cells from peripheral blood by stratification on Ficoll/Hipaque. By centrifugation on F/H, mononuclear cells were isolated from peripheral blood. Mononuclear cells and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cells (RBCs) and granulocytes (PMNs) have a higher density than the F/H and collect on the bottom of the tube. RT: room temperature.

2.2 Purification of B lymphocytes with sheep red blood cells (SRBCs)

In most of CLL cases, the percentage of leukemic B cells was greater than 90% of PBMCs (peripheral blood mononuclear cells) isolated.

When the cell population had more than 10% of T lymphocytes, we further performed the purification of B lymphocytes by SRBC method. This purification allows the removal of T cells from the other mononuclear cells, taking advantage from their ability to bind and form complexes, called "rosettes", with SRBCs. The latter, in fact, express on their surface a specific receptor for the T lymphocyte marker CD2; SRBCs treatment with neuraminidase make more accessible the receptor to CD2 binding.

$25x10^6$ aliquots of PBMCs were transferred into a 10ml centrifuge tube and 1ml of SRBC treated with neuraminidase was added. PBMCs and SRBCs were then incubated at 37°C for 25 min, centrifuged at 4°C for 10 min at 200g without brake, and finally subjected to a new incubation at 4°C for 30 min. The supernatant was then aspirated and culture medium RPMI 1640 (Invitrogen; Paisley, UK) was added. The mixture was gently resuspended, layered over F/H solution, and centrifuged at 4°C for 20 min at 900g without brake. T cells, surrounded by SRBCs, accumulated to the bottom of the tube, having a density greater than F/H, while the non-T mononuclear cells (monocytes and B cells) were concentrated above the F/H layer. The interface layer, which contains B cells, was transferred into a 10ml tube. Two washes with saline were performed at 400g at 20°C for 10 min and, in the end, cells were resuspended and counted in a Burker chamber (Figure 20).

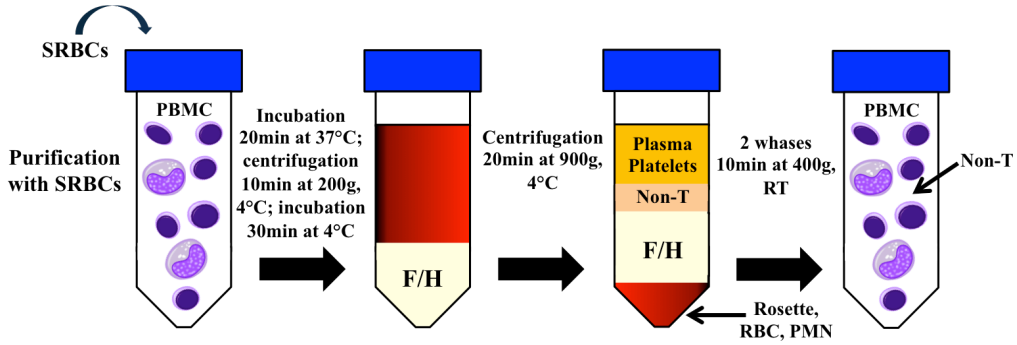

Figure 20. Purification with SRBCs. When the mononuclear cells obtained by separation over F/H contained a percentage of lymphocytes T≥10%, we used SRBCs to remove them. This method exploits the ability of T cells to bind, through their marker CD2, to SRBCs treated with neuraminidase and forming rosettes that are removed by further stratification on F/H.

2.3 Purification of B lymphocytes using RosetteSep kit

We used the RosetteSep kit (StemCell Technologies; Vancouver, CND) to obtain B cells from whole blood of normal healthy donors. The kit consist of a cocktail of antibodies directed against surface antigens expressed by hematopoietic cells (CD2, CD3, CD16, CD36, CD56, CD66b) and glycophorin A expressed by red blood cells. This mixture of antibodies binds "not-B" cells and red blood cells creating immunerosettes. In this way, CD19+ B lymphocytes were isolated from whole blood by negative selection.

Each ml of blood was incubated with 50µl of RosetteSep at room temperature for 20 minutes. The samples were then diluted 1:2 with PBS (Phosphate Buffered Saline)1X + 2% FBS (Fetal Bovine Serum), gently agitated, and then layered over F/H. We proceeded with a centrifugation at 900g for 30 minutes at room temperature, followed by the aspiration of the ring formed at the F/H interface containing B cells. It was resuspended in PBS1X + 2% FBS and centrifuged at 400g for 10 minutes. Finally, the cells were resuspended in PBS1X and counted in a Burker chamber (Figure 20).

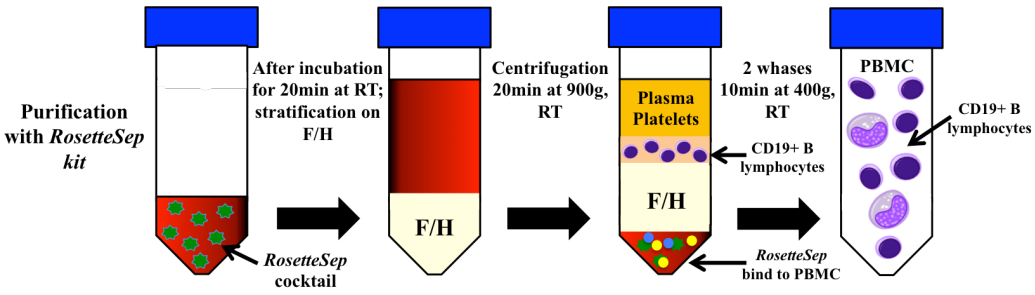

Figure 20. Purification with RosetteSep kit. CD19+ B lymphocytes were isolated from whole blood of healthy donors by negative selection. 10ml of venous whole blood were incubated for 20 min at RT with 500µl of RosetteSep. Afterwards, through stratification on F/H, we get the CD19+ B cells, which are concentrated just above the layer of F/H, while the rest of the cells related to the rosettes were collected on the bottom of the tube.

3. Isolation of MSCs from bone marrow blood

Mesenchymal stromal cells (MSCs) were isolated from the BM blood of CLL patients and healthy subjects. After F/H stratification, BM mononuclear cells (BMMCs) were plated and incubated at 37°C in humidified atmosphere containing 5% CO_2 and allowed to attach for 7 days; the non-adherent

fraction was discarded and adherent cells maintained until confluence and for further expansion. MSC phenotype was characterized through the expression of CD14, CD31, CD34, CD73, CD90, CD45, and CD105 markers. The capability of MSCs to differentiate into mesenchymal lineages (*e.g.* adipocytes and osteocytes) was assessed (Figure 21).

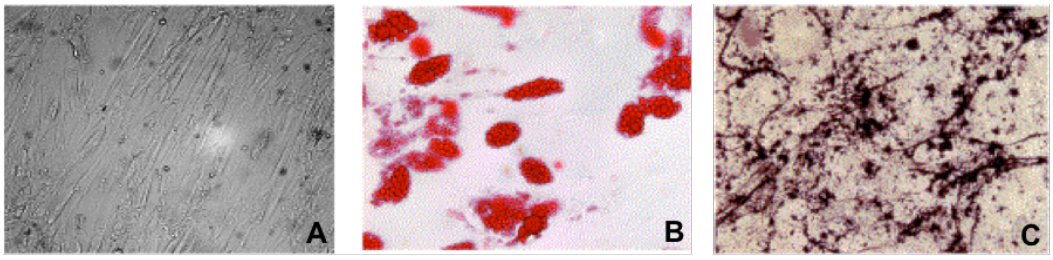

Figure 21. Different lineages from MSCs. A) Undifferentiated MSCs; B) MSCs differentiated into adipocytes - Oil red stained; C) MSCs differentiated into osteocytes - von Kossa stained.

4. Cell cultures condition

Aliquots of B (CD19+/CD5+) and T lymphocytes (CD5+) obtained from patients with CLL, normal B lymphocytes (CD19+) were cultured in RPMI 1640 medium with 10% FBS and antibiotics at a concentration of $2x10^6$/ml in 24- or 48-wells plates. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO2 with or without AG490 (Selleck Chemicals; Munich, Germany) at the concentration of 10, 30, 50 and 100μM and Stattic (Selleck Chemicals) at the concentration of 5, 7.5 and 10μM for 24, 48 and 72 hours. For some experiments we have also used a SHP-1 inhibitor, sodium orthovanadate (Na_3VO_4) at the concentration of 100 μM (Sigma-Aldrich; Milan, Italy), and Dasatinib (Selleck Chemicals) at the dose of 0.1μM.

5. B CLL cells/MSCs co-culture

Some experiments were performed on MSC layer. For co-culture experiments, $2x10^5$/well MSCs from CLL were seeded into 24 well plates and incubated before the experiment at 37˚C in 5% CO_2 up to confluence. Then,

CLL cells were added to MSCs layer at a ratio of 2,5:1. Cells were then treated at the same condition of culture alone to evaluate a possible resistance to drug treatment due to the co-culture with MSCs.

6. Subcellular Fractionation

The subcellular fractionation is a technique that allows the study of proteins in different cellular compartments. The fractions that are obtained are defined "enrichment" of the following components: cytoplasmic/soluble, of the membrane, microsomal, mitochondrial and nuclear.

We used a commercial kit (Thermo Scientific, Rockford, IL, USA) using detergents which could separate cytoplasmic, membrane and nuclear proteins.

10^7 lymphocytes were centrifuged and incubated with different buffers following manufacture's protocol. The buffers are: Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB) with the add of phosphatase and protease inhibitors.

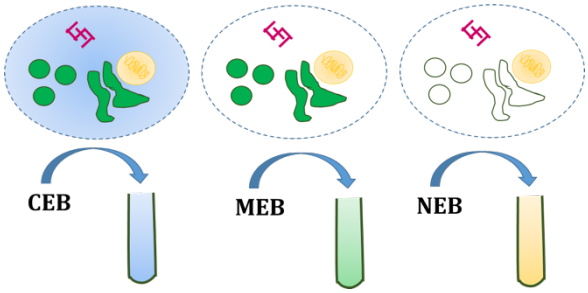

Figure . Representation of cell fractionation protocol. Proteins were collected using several buffers. Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB).

7. Preparation of cell lysates

Cells (5×10^5 for each assay) were prepared by cell lyses with[79]:

- Tris(hydroxymethyl)aminomethane-hydrochloride (TRIS-HCl) pH 6.8 20 mM;

- soidum cloride (NaCl) 150 mM;
- Ethylenediaminetetraacetic acid (EDTA) 2 mM;
- ethylene glycol tetraacetic acid (EGTA) 2 mM;
- Triton X-100 0.5%;
- complete protease inhibitor cocktail (Roche; Mannheim, Germany);
- sodium orthovanadate 1 mM (Calbiochem; Gibbstown, NJ).

To use these lysates for SDS-PAGE (polyacrylamide gel electrophoresis) analysis, the follow substance were added:

- 10% glycerol;
- 2% sodium dodecyl sulphate (SDS);
- 1% β-mercaptoethanol;
- Dye (Pyronin) (Sigma-Aldrich; Milan, IT).

Subsequently, the lysates were vortex, boiled at of 100°C for 5 minutes.

8. Polyacrylamide gel electrophoresis in SDS (SDS-PAGE)

The polyacrylamide gel electrophoresis in SDS is one of the methods used to separate a mixture of proteins on the basis of their molecular weight. SDS is a ionic detergent that binds tightly to proteins causing their denaturation. In the presence of an excess of SDS, approximately 1.4g of detergent will bind to each gram of protein, providing a constant amount of negative charge per unit mass. Therefore, during electrophoresis, all protein-SDS complexes move toward the anode, and thanks to the molecular sieve properties of the gel, their mobility is inversely proportional to their molecular weight. By the migration of standard proteins of known molecular weight simultaneously to samples, it is possible to determine the protein sample weights.

SDS polyacrylamide gel is prepared following Laemmli method. The electrophoretic plate consists of two types of gel:

- *Stacking gel* (Tris-HCl 0.5 M at pH 6.8), which allow concentrates the protein samples so that they are all aligned at the start of electrophoresis.
- *Running gel* (Tris-HCl 1.5 M at pH 8.8), in which the real separation of proteins occurs.

The plate size of 10×8cm is fixed in the Hoefer Mighty Small-If 250 Scientific Instruments machine (Amersham Biosciences). The electrophoresis was run for about 2 hours at 25mA.

9. Western blotting

The western blotting (WB) or immunoblotting is an immunoassay able to detect traces of a specific protein in a heterogeneous mixture, combining the high resolving power of gel electrophoresis with the specificity of the antibodies. The WB is a technique with high sensitivity, able to detect quantities of protein in the order of nanograms.

After SDS-PAGE, proteins are transferred onto a nitrocellulose membrane by the action of an electric field, obtained by applying the appropriate current of 350mA for 2 hours and 30 minutes. The buffer used for the transfer consists of: 25mM Tris, 192mM glycine, 20% methanol and 0.1% SDS with a final pH of 8.0. After the transfer, the membrane is left overnight in the saturation buffer consisting of 50mM Tris-HCl, pH 7.5, 150mM NaCl and 5% bovine serum albumin (BSA), for nonspecific sites saturation. Follows the incubation for 2 hours and 30 min at RT of the primary Abs, diluted in "dilution buffer" (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA).

For our study we used the following antibodies: anti-P-Tyr, which recognizes proteins phosphorylated on tyrosine residues; anti-STAT3, anti-STAT3 Ser727, anti-STAT3 Tyr705, polyclonal anti-Lyn, anti-JAK2, anti-JAK2 Tyr1007/1008 (Cell Signaling Technology, Inc.; Danvers, MA); anti-Lyn Tyr396 (Epitomics Onc.; Burlingame, CA); anti-SHP-1, anti-SHP-1 Ser591 (Millipore; Billerica, MA); anti-α-tubulin and anti-β-actin (Sigma-Aldrich). In

addition, for apoptosis study, we used an anti-PARP (Cell Signaling Technology, Inc.).

Three washes of 10 min, each at RT, were subsequently performed using "washing buffer" (TRIS-HCl 1M, NaCl 3M, Tween20 0.1% at pH 7.5). Membranes were then incubated for 30 minutes with a secondary anti-IgG Ab, obtained against the animal species immunized for the primary Ab. The secondary Ab is conjugated with horseradish peroxidase (Amersham International Biotechnology; Buckingamshire, UK) and diluted in "dilution buffer". After three additional washes with "washing buffer", the membrane was subjected to the detection antibody with the enhanced ChemiLuminescence system (ECL) (Pierce; Rockford, Illinois): the membrane is incubate for 1 min with 1ml of luminol and 1 ml of H_2O_2, which in contact with the peroxidase and, as a result, with the Ag-Ab complex, giving rise to an oxidation reaction with light emission. The membrane was finally revealed into the ImageQuant LAS 500 (Amersham Biosciences) and the bands were scanned and quantified by densitometry, using the program ImageQuantTL (Amersham Biosciences), supplied with the instrument.

10. Flow cytometry

This technique allows a multiparametric evaluation of antigenic characteristic of the single cells by the analysis of visible and fluorescent light they emit when flow through a liquid medium.

The fluorochromes used in this thesis were fluorescein isothiocyanate (FITC), which emits a fluorescence signal at 530nm (green), phycoerythrin (PE) emitting at 585nm, tri-color (TC) that emits at 667nm when hit by a monochromatic laser beam with λ equal to 488nm, and finally, the allophycocyanin (APC) that emits a fluorescence signal at 690nm when excited by a laser beam with λ of 635nm. The cell samples were analyzed by the flow cytometer FACSCanto A (Becton Dickinson; Franklin Lakes, NJ) and data obtained were processed using the FACSDiva 7 software.

10.1 Immunophenotypic analysis

The immunophenotypic analysis on lymphocytes obtained from peripheral blood of CLL patients was performed through flow cytometry. All patients express the typical phenotypic profile according to standard criteria for CLL diagnosis[11].

The immunophenotyping is based on the identification of surface and intracellular Ag using mAb conjugated with fluorochromes. The presence of a certain Ag is revealed and used as an indicator of belonging to a cell line as well as its level of maturation. For apoptosis analysis 20.000 events were acquired.

10.2 Apoptosis analysis

Apoptosis of different cell samples (pathological and normal B lymphocytes and MSCs) was assessed using the Annexin V Apoptosis Detection Kit (Immunostep, Salamanca, Spain).

During the early stages of apoptosis the plasma membrane undergoes profound changes that indicate the status of apoptotic cells to macrophages, which ensure its elimination. Phosphatidylserine (PS), a negatively charged aminophospholipid normally expressed only on the inner side of the plasma membrane, is exposed on the outer surface. The Annexin V is a protein that, in the presence of high concentrations of Ca^{2+}, recognizes and binds selectively the PS, making it useful for the identification of apoptotic cells that expose the phospholipid on their surface. Aliquots of $250x10^3$ cells were harvested, washed, and incubated for 10 min in the dark and at RT with: 100μl of binding buffer, a Ca^{2+}-rich solution that optimizes the binding of Annexin V to the PS, 5μl of Annexin V (FITC), and 5μl of Propidium iodide PI, PE), provided by the kit (1μl/ml final concentration). After the incubation, 100μl of binding buffer were added and cells were analyzed (Figure 22).

For each sample 20.000 events were acquired and the number of apoptotic cells was expressed as percentage of Annexin V positive cells in the total cells analyzed.

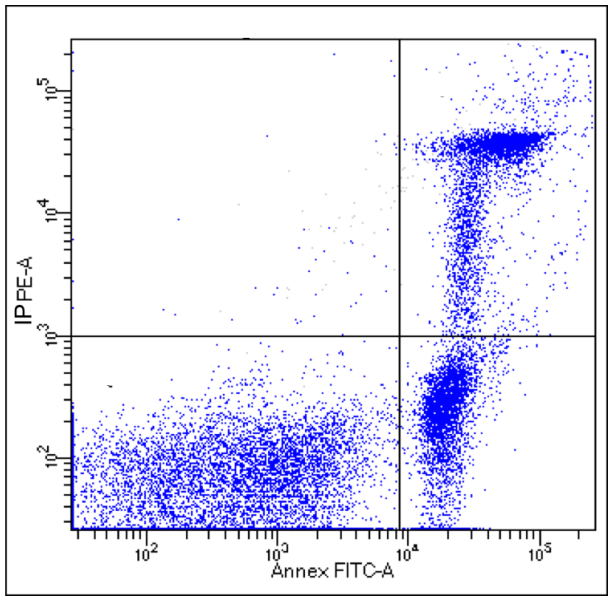

Figure 22. A representative cytogram of Annex V/IP Test.

10.3 Phospho-protein analysis

Cells ($2x10^6$ for each assay) were collected after 15 min and 24h and stained with anti-STAT3 Tyr705 PE (BD Biosciences), anti-STAT3 Ser727 FITC (BD Biosciences), anti-JAK2 Tyr1007/1008 FITC (Abcam, Cambridge, UK) and anti-CD19 APC (BD Biosciences) monoclonal antibodies. Cells were stained with surface antibody anti-CD19 for 10 minutes at room temperature. Therefore, cells were washed, fixed and permeabilized following the manufacture's instructions and incubated with saturating concentrations of the appropriate antibodies (anti-STAT3 Tyr705, anti-STAT3 Ser727 and anti-JAK2 Tyr1007/1008) for 30 minutes at room temperature. After another wash, 30.000 total events were acquired and samples were gated on intact cells by forward scatter (FSC) *vs* side scatter (SSC). For analysis, a second gating step on CD19+ cells was used. To evaluate the quantity of phosphorylated protein, we used the difference between the Mean Fluorescence Intensity (MFI) of fully-stained samples and the Fluorescence Minus One (FMO) controls.

11. Confocal microscopy analysis

Aliquotes of the different cell samples (pathological and normal B lymphocytes from CLL) were collected, washed and plated on poly-L-lysine coated slides for 15 min at room temperature. Cells were then fixed in 4% paraformaldehyde for 10 min, washed twice with PBS 1X and permeabilized with 0,1% Triton X-100 (Sigma-Aldrich) for 4 min. Before staining, non-specific protein binding was blocked by incubating the slides for at least 30 min in 2% bovine serum albumin. Cells were then stained with antibodies against: Lamin B (Santa Cruz), STAT3 and STAT3 Tyr705 (Cell Signaling) diluited 1:250, washed three times with PBS 1X and than incubated with secondary antibody for 30 minutes in the dark. The secondary antibodies used are: anti-mouse-Alexa488 (STAT3 and STAT3 Tyr705) and anti-goat-Alexa594 (Lamin B). Then were performed other three washes in PBS 1X. Slides were mounted with cover slips and fluorescence was detected using the UltraView LCI confocal system (Perkin Elmer; Walthaw, MA, USA) equipped with a fluorescence filter set for excitation at 488 and 594 nm.

12. Statistical analysis

Statistical analysis was performed using Student's *t* test, paired Student's *t* test, Fisher's exact test, Wilcoxon matched pairs test and Pearson's test. Data are reported as mean ± standard deviation (SD) and were considered statistically significant when p values were <0.05 or less.

RESULTS

1. STAT3 is over-expressed in CLL B cells

The aberrant activation of STATs has been observed in solid tumors and leukemias, and different mechanisms involved in STAT constitutive activation have been described, the over-expression of STATs[74] included.

With this as a background, by western blotting analysis, we analyzed the levels of STAT3 protein in freshly isolated neoplastic B cells obtained from 76 untreated CLL patients and in normal B lymphocytes from 23 healthy subjects. A significant difference in STAT3 expression was observed in malignant (1.28±0.13) *versus* normal B lymphocytes (0.61±0.09; Student's *t* test, p<0.001) (Figure 23).

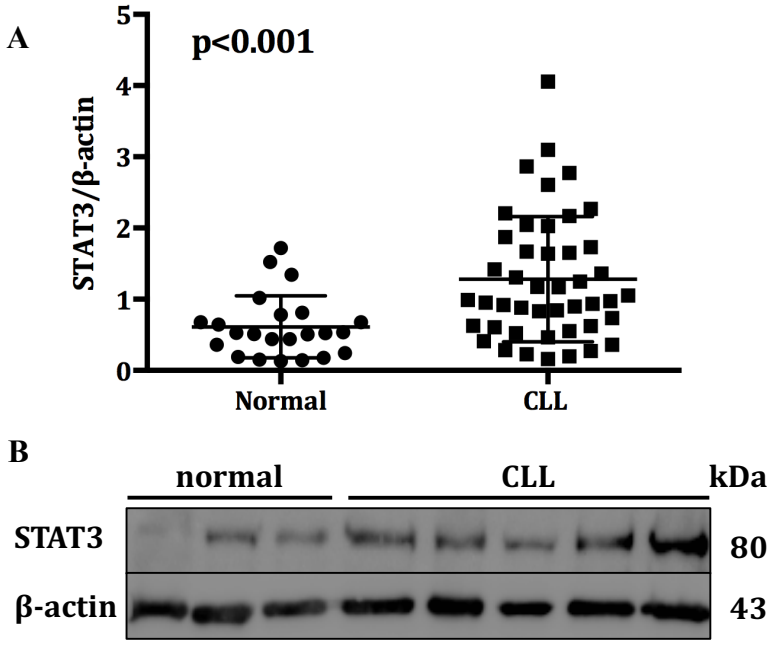

Figure 23. Expression of STAT3 protein in normal and CLL B cells. A. Densitometry of STAT3/β-actin ratio of normal subject (n=23) *vs* CLL patients (n=54), (Student's *t* test, p<0.001)**. B.** The figure shows a representative blot for 3 normal controls and 5 CLL patients. STAT3 expression is higher in CLL samples with respect to donors.

2. STAT3 is constitutively phosphorylated at Ser727 and Tyr705 in CLL B cells

The classical JAK2/STAT3 pathway includes a cytokine/receptor binding with JAK2 recruitment at the receptor and its consequent phosphorylation. Successively, p-JAK2 phosphorylates STAT3 Tyr705, thus prone to be phosphorylated at Ser727 residue by serine kinases, leading to STAT3 dimerization and translocation into the nucleus.

In normal B cells, very low levels of STAT3 phosphorylation are detected at basal conditions. As regards CLL, by flow cytometry and western blot, we firstly confirmed the known constitutive phosphorylation at Ser727[72,80] (Figure 24).

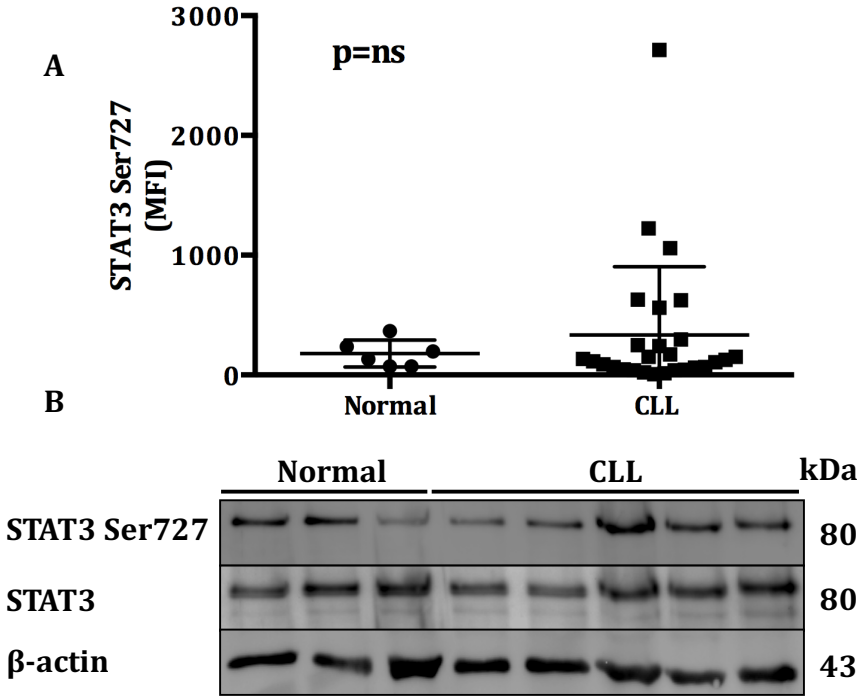

Figure 24. Evaluation of STAT3 phosphorylation at Ser727. A. Flow cytometry analysis of STAT3 phosphorylation at Ser727. Difference between normal (N=6) and CLL (N=27) is not significant (Student's *t* test, p=ns). MFI= Median Fluorescence Intensity. **B.** The figure shows a representative blot from 3 normal controls and 5 CLL patients. STAT3 Ser727/STAT3 ratio is not significantly different between normal and leukemic B cells.

Although STAT3 Ser727 phosphorylation was higher in CLL, we did not observe any significant difference compared with normal B lymphocytes

(MFI: Normal cells, N=6: 178.8±46.2 *vs* CLL cells, N=27: 334.6±109.6; Student's *t* test, p=ns. Figure 24 A).

As far as STAT3 phosphorylation at Tyr705 is concerned, an essential step for STAT3 activation, by flow cytometry and western blot analysis, we demonstrated a constitutive phosphorylation in CLL cells, although in some patients STAT3 Tyr705 phosphorylation is barely detected (MFI: Normal cells, N=6: 46.50±6.12 *vs* CLL cells, N=25: 211.3±35.85; Student's *t* test, p<0.05. Figure 25).

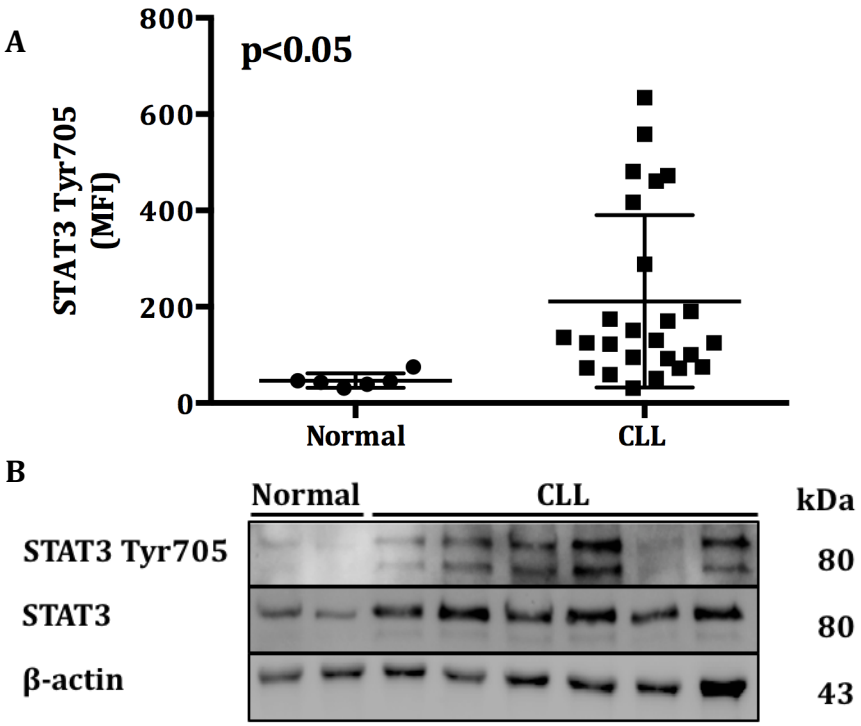

Figure 25. Evaluation of STAT3 phosphorylation at Tyr705. A. Flow cytometry evaluation of STAT3 phosphorylation at Tyr705. Difference between normal (N=6) and CLL (N=25) is significant (Student's *t* test, p<0.05). MFI= Median Fluorescence Intensity. **B.** The figure shows a representative blot from 2 normal controls and 6 CLL patients. Normal B cell have less STAT3 Tyr705 then B CLL cells. Neoplastic cells show a heterogenous expression pattern.

We found a positive correlation between STAT3 Ser727 and STAT3 Tyr705 phosphorylation in 23 patients analysed by flow cytometry (Pearson's test, p<0.0001), accounting for the sequential nature of STAT3 Ser-phosphorylation to STAT3 Tyr-phosphorylation (Figure 26).

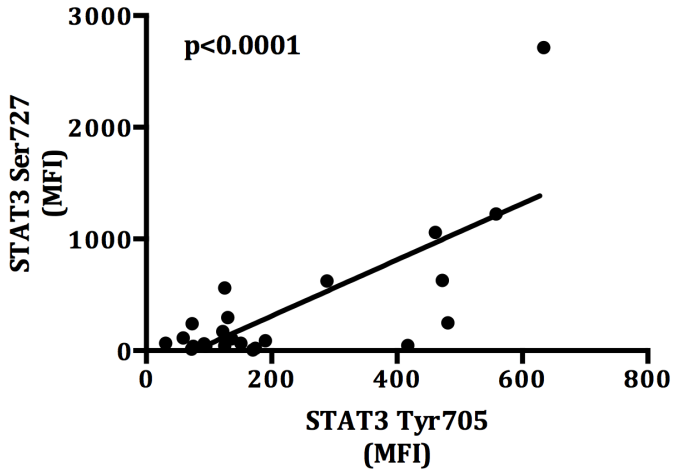

Figure 26. Correlation between STAT3 Tyr705 and STAT3 Ser727. Figure shows the correlation between STAT3 Tyr705 and STAT3 Ser727 in 23 CLL patients (Pearson's test, p<0.0001). MFI: Median Fluorescence Intensity.

3. STAT3 Tyr705 is present in the nucleus of CLL B cells

STAT3 Tyr705 localization was analysed in normal and leukemic B cells. To this purpose, by a subcellular protein fractionation, we separated nuclei from cytosols of cells, detecting STAT3 Tyr705 both in the cytosolic and in the nuclear fractions of CLL B cells (Figure 27A). The purity of the different subcellular fractions was assessed through the presence or absence of PARP and α-Tubulin proteins, localized in nucleus and cytosol, respectively.

These data were supported by confocal microscopy evaluation that highlighted the presence of STAT3 Tyr705 at basal conditions both in the nucleus and in the cytosol of leukemic B cells (Figure 27B). In normal B cells phosphorylated STAT3 is less detectable with respect to neoplastic B cells.

The presence, at basal conditions, of STAT3 phosphorylated at Tyr705 in the nucleus of leukemic, but not of normal, B cells may be a consequence of the constitutive activation of STAT3 pathway in these cells.

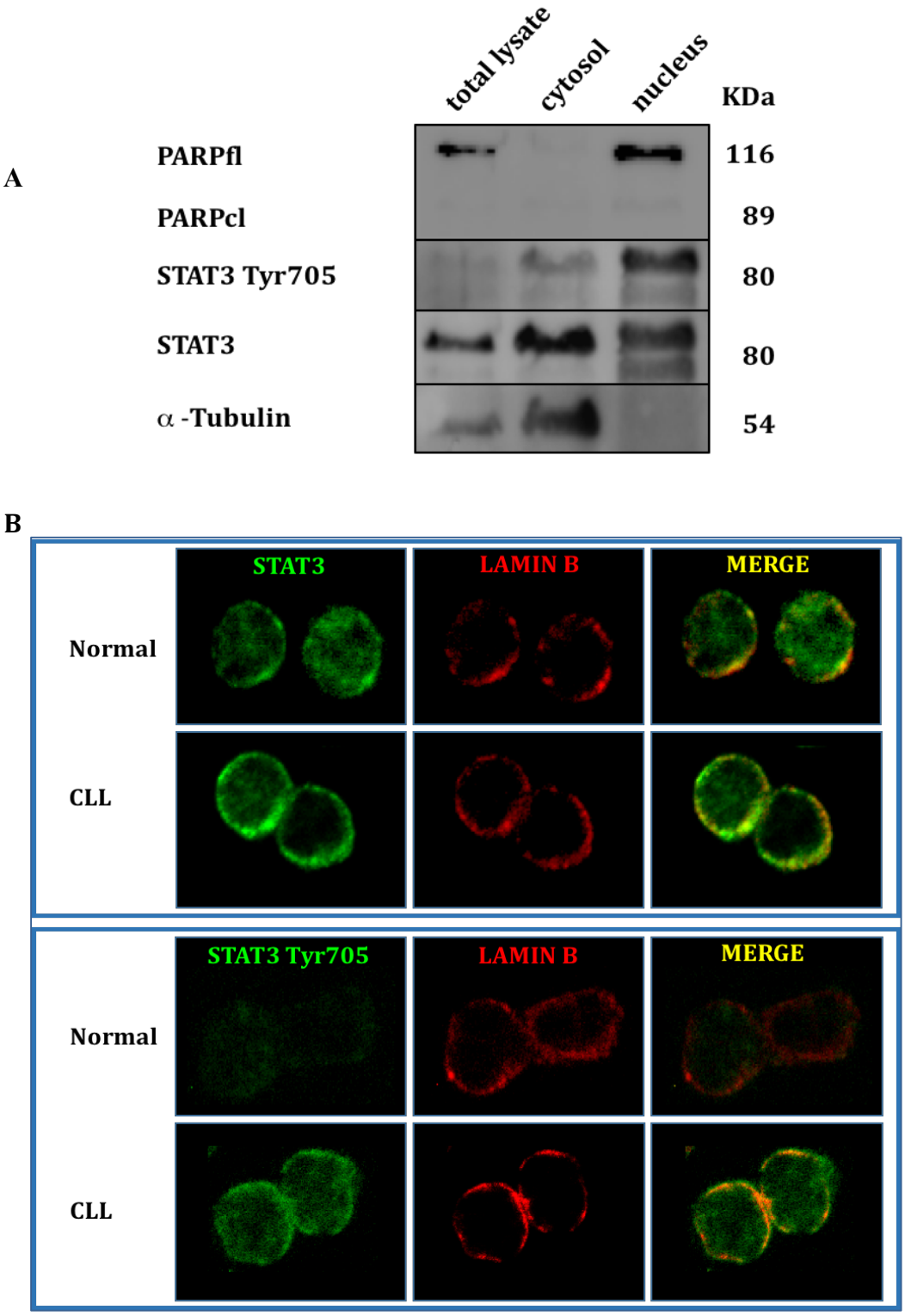

Figure 27. STAT3 subcellular localization. A. The membrane was immunostained with the anti-STAT3 Tyr705, anti-STAT3, anti-PARP and anti-α-Tubulin antibodies. PARP and α-Tubulin proteins were used to assess the purity of nuclear and cytosolic fractions, respectively. STAT3 Tyr705 shows a strong nuclear localization. PARP fl: full length PARP. PARP cl: cleaved PARP. **B.** Confocal microscopy analysis of STAT3 and STAT3 Tyr705 (Alexa 488, green) in normal and CLL B cells obtained from peripheral blood. Lamin B (Alexa 594, red) was used to delimit the nucleus. Both STAT3 and STAT3 Tyr705 are localized in the nucleus.

4. Leukemic microenvironment sustains STAT3 activation

Considering the importance of bone marrow environment in activating JAK/STAT pathways in CLL, by western blotting we evaluated STAT3 phosphorylation at Tyr705 in different conditions:

1. freshly isolated CLL B cells;
2. CLL B cells cultured in medium alone for 24h;
3. CLL B cells cultured for 24h on a layer of mesenchymal stromal cells (MSCs).

We showed that the constitutive phosphorylation of STAT3 at Tyr705 observed in freshly isolated B cells increased when cells were cultivated in medium alone and reached even higher values when CLL B cells were cultured in presence of MSCs (Alone T=0: 0.34±0.06 *vs* Alone T=24h: 0.76±0.17; Wilcoxon matched pairs test, $p<0.05$ and Alone T=0 *vs* +MSCs T=24h: 1.68±0.15; Wilcoxon matched pairs test, $p<0.01$) (Figure 28).

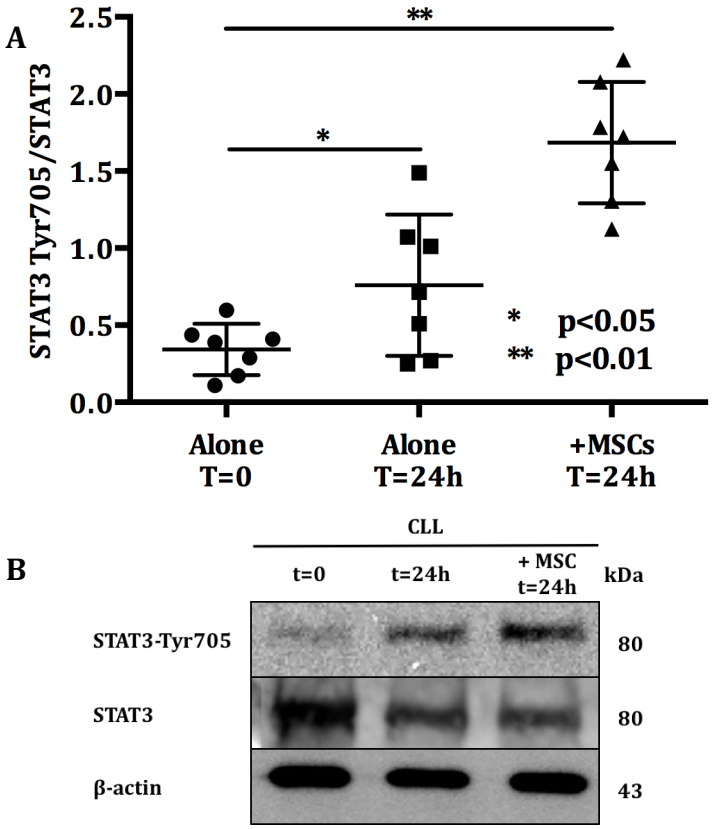

Figure 28. Modulation of STAT3 Tyr705. A. A significant difference in STAT3 phosphorylation at Tyr705 was demonstrated. The graph reports the densitometric analysis of STAT3 Tyr705/STAT3 ratio of 7 samples. (Alone T=0 *vs* Alone T=24h; Wilcoxon matched pairs test, $p<0.05$ and Alone T=0 *vs* +MSCs T=24; Wilcoxon matched pairs test, $p<0.01$). **B.** A representative western blot. MSCs: Mesenchymal Stromal Cells.

Interleukin-6 (IL-6) is the principal activator of JAK2/STAT3 signaling and one of the most abundant cytokine released in the leukemic microenvironment[71]. We analysed JAK2/STAT3 pathway activation in CLL B cells after IL-6 stimulation (Figure 29).

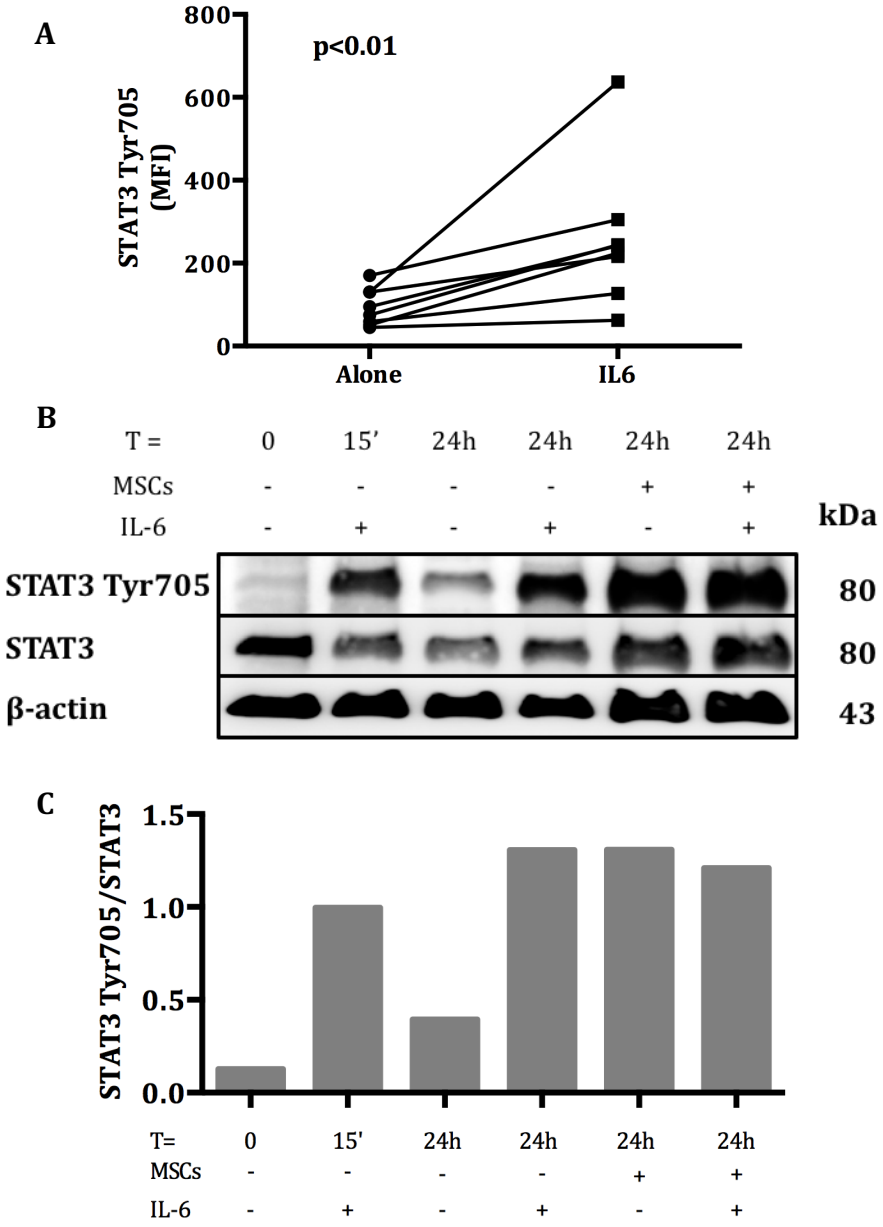

Figure 29. Modulation of STAT3 Tyr705 phosphorylation by IL-6. A. Comparison of STAT3 Tyr705 between B CLL cells alone and treated with IL-6 by flow cytometry (N=8; Wilcoxon test, p<0.01). MFI: Median Fluorescence Intensity. IL-6: Interleukin-6. **B**. STAT3 Tyr705 expression pattern after 15 minutes and 24h with(+) or without(-) MSCs and IL-6. STAT3 Tyr705 level is approximately the same in B cells stimulated with IL-6 or MSCs. MSCs: Mesenchymal Stromal Cells. **C.** Densitometry of western blot of panel B.

As shown in Figure 29 A, by flow cytometry we demonstrated a significant increase of phosphorylation at Tyr705 in response to IL-6 (N=8, Wilcoxon test, $p<0.01$). The data obtained by WB showed that IL-6 up-regulates STAT3 Tyr705 phosphorylation at levels comparable to those obtained when CLL cells are cultured on a stromal layer (Figure 29 B).

5. Inhibition of JAK2/STAT3 induces apoptosis of CLL B cells

We demonstrate the constitutive phosphorylation of STAT3 in CLL B cells. AG490 is a specific inhibitor of JAK2 protein that, in turn, is an established activator of STAT3, through phosphorylation at Tyr705[81]. We treated neoplastic B cells with AG490 to inhibit JAK2/STAT3 axis. We also treated neoplastic B cells with Stattic, a non peptidic small molecule that strongly inhibits STAT3 activation, probably avoiding STAT3 nuclear translocation[82].

B cells from CLL patients were cultured alone or in the presence of different concentration of AG490 (10, 50 and 100μM) or Stattic (5, 7.5, and 10μM), and assessed for cell viability after 24, 48 and 72 hours (Figure 30). The same experiments were performed in presence of MSCs recovered from CLL patients, representing tumor microenvironment and strongly supporting neoplastic B cells survival.

We demonstrated that both AG490 and Stattic were able to induce apoptosis in CLL cells in a dose-dependent manner, as shown by Annexin V/PI tests and the presence of the cleaved form of PARP; in particular, treatment with AG490 (Figure 30 A and B) and Stattic (Figure 30 C and D) were able to bypass environment protection.

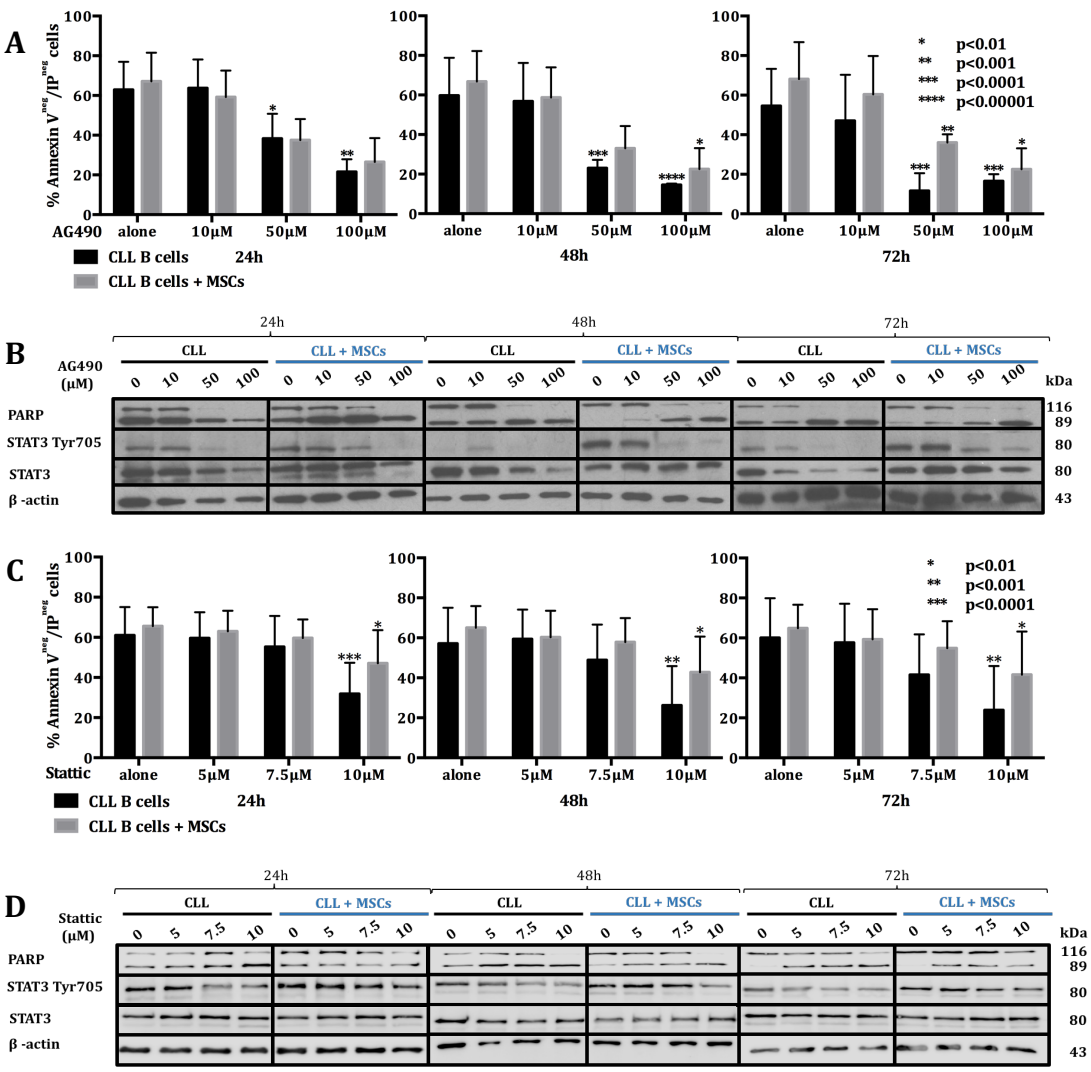

Figure 30. Inhibition of JAK2/STAT3 pathway by AG490 and Stattic. A. Evaluation of CLL B cells viability by flow cytometry, after AG490 (0, 10, 50, 100µM) treatment by Annexin V (FITC)/PI (PE) test with (light grey histograms) or without (black histograms) MSCs at the time points of 24, 48 and 72h. **B.** Evaluation of CLL B cells viability and phosphorylation by western blot, after AG490 (0, 10, 50, 100µM) treatment with/without MSCs at the time points of 24, 48 and 72h. Representative western blots detected sequentially with the anti-STAT3 Tyr705, anti-STAT3, PARP and anti-β-actin antibodies. **C.** Evaluation of CLL B cells viability by flow cytometry, after Stattic (0, 5, 7.5, 10µM) treatment by Annexin V (FITC)/PI (PE) test with (light grey histograms) or without (black histograms) MSCs at the time of 24, 48 and 72h. **D.** Evaluation of CLL B cells viability and phosphorylation by western blot, after Stattic (0, 5, 7.5, 10µM) treatment with/without MSCs at the time of 24, 48 and 72h. Representative western blots detected sequentially with the anti-STAT3 Tyr705, anti-STAT3, PARP and anti-β-actin antibodies. MSCs: Mesenchymal Stromal Cells.

By Student's *t* test statistical analysis, we found a significant difference in cells viability between untreated and treated conditions as follows:

- <u>AG490</u>: 24h, N=8, CLL B cells alone *vs* +AG490 50µM (62.90±4.24% *vs* 38.30±7.22%, p<0.01), CLL B cells alone *vs* +AG490 100µM (62.90±4.24% *vs* 21.50±4.50%, p<0.001); 48h, N=8, CLL B cells alone *vs* +AG490 50µM (59.70±6.03% *vs* 23.00±3.00%, p<0.0001), CLL B cells

alone *vs* +AG490 100μM (59.70±6.03% *vs* 14.50±0.50%, p<0.00001); 72h, N=8, CLL B cells alone *vs* +AG490 50μM (54.50±6.64% *vs* 11.60±5.14%, p<0.001), CLL B cells alone *vs* +AG490 100μM (54.50±6.64% *vs* 16.50±2.50% %, p<0.0001).

These results indicate that AG490 is able to induce apoptosis in a dose-dependent manner.

- AG490+MSCs: 48h, N=4, CLL B cells alone *vs* +AG490 100μM (66.80±6.28% *vs* 22.50±7.50%, p<0.01); 72h, N=4, CLL B cells alone *vs* +AG490 50μM (68.10±7.63% *vs* 11.60±5.14%, p<0.001), CLL B cells alone *vs* +AG490 100μM (68.10±7.63% *vs* 22.50±7.50%, p<0.01).

  These data show that AG490 is able to bypass environment protection.
- Stattic: 24h, N=13, CLL B cells alone *vs* +Stattic 10μM (60.96±3.91% *vs* 31.78±4.31%, p<0.0001), 48h, N=13, CLL B cells alone *vs* +Stattic 10μM (57.15±4.95% *vs* 26.25±5.45%, p<0.001); 72h, N=13, CLL B cells alone *vs* +Stattic 10μM (61.63±5.25% *vs* 25.29±6.64%, p<0.001).

  Stattic affects CLL B cells viability in a dose-dependent manner.
- Stattic+MSCs: 24h, N=9, CLL B cells alone *vs* +Stattic 10μM (65.57±3.14% *vs* 49.20±5.56%, p<0.01); 48h, N=9, CLL B cells alone *vs* +Stattic 10μM (64.70±3.66% *vs* 44.12±5.73%, p<0.01); 72h, N=9 CLL B cells alone *vs* +Stattic 10μM (64.91±4.43% *vs* 44.90±7.85%, p<0.01).

  MSCs are unable to protect neoplastic cells from Stattic-induced apoptosis.

6. Crosstalk between JAK2/STAT3 and BCR/Lyn axes in CLL B cells

6.1 AG490 inhibits both Lyn and SHP-1 in CLL B cells

Lyn is the tyrosine kinase that initiates the phosphorylation cascade triggered by the B Cell Receptor (BCR)-Antigen interaction. Activated Lyn phosphorylates proteins that work as inhibitors of the BCR, such as SHP-1, which acts as phosphatase for several substrates, thus switching off BCR

signaling. Lyn itself is one of SHP-1 target proteins and, once dephosphorylated, becomes inactive.

Recent data on chronic myeloid leukemia (CML) suggest a connection between JAK2 and SHP-1. This relationship is still unclear, but Samanta K *et al.* showed that AG490 inhibits Lyn through SET-PP2A-SHP-1 pathway[83].

Neoplastic B cells from 9 CLL patients were cultured alone or in presence of different concentrations of AG490 (10, 50 and 100μM) and Stattic (5, 7.5, 10μM). By western blotting, the expression and phosphorylation status of SHP-1 and Lyn were assessed.

The treatment with AG490 inhibited the phosphorylation of SHP-1 at Ser591, activating the phosphatase. SHP-1 activation leads to Lyn Tyr396 dephosphorylation/inactivation. Particularly, after treatment with AG490, we observed a decrease in SHP-1 Ser591 and Lyn Tyr396 phosphorylation, while total protein levels remained unaffected.

As expected, the treatment with Stattic did not affect Lyn and SHP-1 phosphorylation since this inhibitor acts downstream, mainly inhibiting STAT3 homodimers binding to DNA (Figure 31).

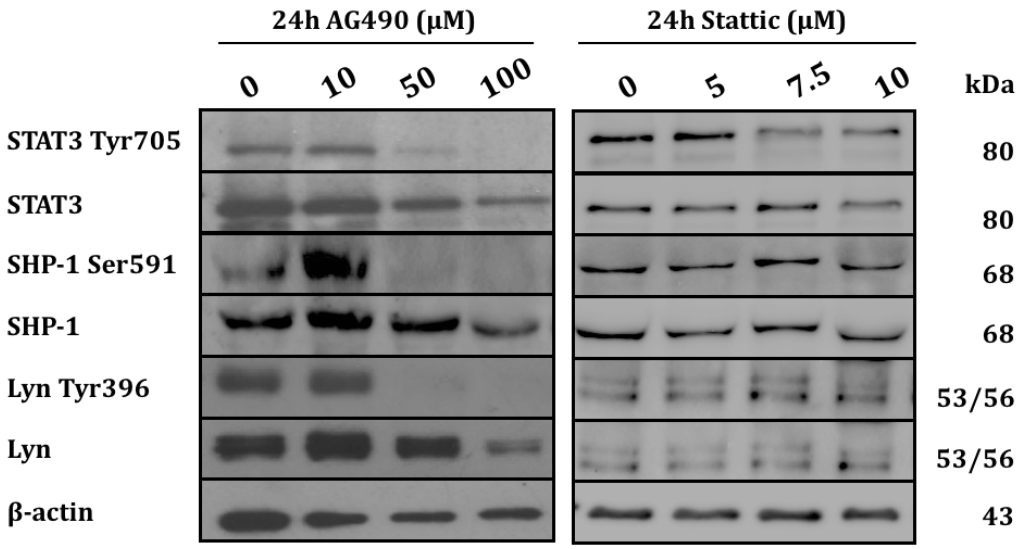

Figure 31. Lyn Tyr396 and SHP-1 Ser591 assessment after inhibition with AG490 and Stattic. CLL B cells were cultivated for 24h with AG490 (0, 10, 50, 100μM) or Stattic (0, 5, 7.5, 10μM) and protein expression pattern evaluated by western blotting. AG490 inhibits STAT3 Tyr705, SHP-1 Ser591 and Lyn Tyr 396 phosphorylation, but Stattic only affect STAT3 Tyr705 activation.

To confirm the link between JAK2 inhibition by AG490 and Lyn dephosphorylation, we added sodium orthovanadate (Na_3VO_4, 100 μM), a phosphatase inhibitor, to cell culture to restore Lyn activation (resulting from the inactivation of SHP-1 phosphatase. As shown in Figure 32, Lyn Tyr396 phosphorylation was restored.

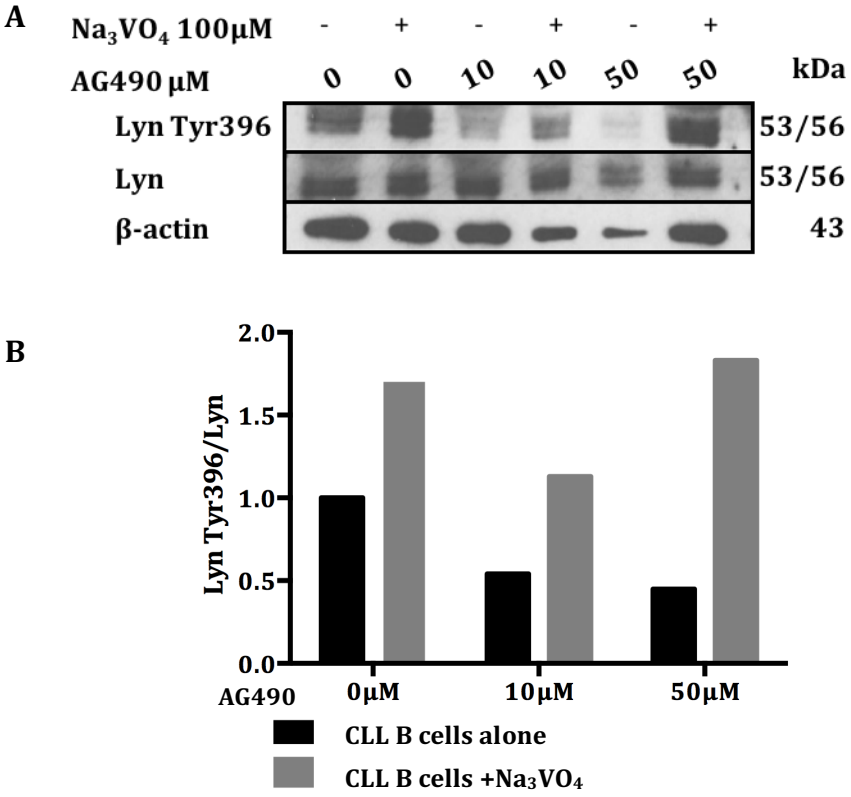

Figure 32. Lyn assessment after SHP-1 inhibition by Na_3VO_4. A. CLL B cells were cultivated with(+)/without(-) Na_3VO_4 (100μM) and with different concentration of AG490 (0, 10, 50μM). Immunostainig with anti-Lyn Tyr396, anti-Lyn and anti-β-actin antibodies highlights the capability of Na_3VO_4 to restore Lyn Tyr396 phosphorylation previously inhibits by AG490 administration. **B.** Histograms represent Lyn Tyr396/Lyn ratio of western blot in panel A. Black bars: CLL B cells alone. Grey bars: B CLL cells + Na_3VO_4. Na_3VO_4: sodium orthovanadate.

6.2 Lyn inhibition by Dasatinib induces STAT3 inactivation

Wang L *et al.*[77] previously demonstrated that a phosphorylation and activation of STAT, independent from JAK, was possible through BCR/Lyn axis. The Src-family kinase inhibitor Dasatinib inhibits Lyn at Tyr396 residue, thus restoring apoptosis of CLL B cells[84].

We treated leukemic B cells with Dasatinib (0.1μM) for 24, 48 and 72h, and evaluated the levels of STAT3 Tyr705 phosphorylation. We

demonstrated that not only Lyn Tyr396, as expected, decreased, but STAT3 Tyr705 phosphorylation was also reduced (Figure 33).

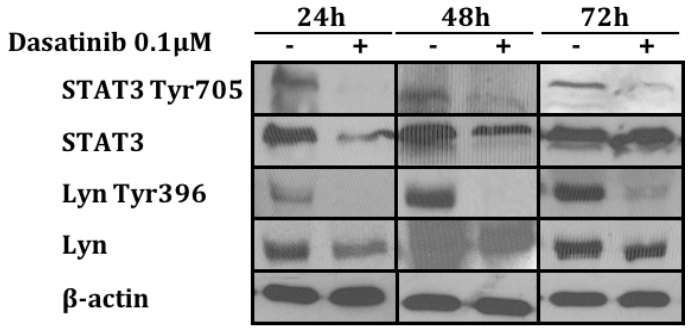

Figure 33. Assessment of STAT3 Tyr705 phosphorylation after Dasatinib treatment. CLL B cells were cultivated for 24, 48, 72h with(+)/without(-) Dasatinib (0.1μM). Dasatinib is able to inhibit both Lyn Tyr396 and STAT3 Tyr 705 phosphorylation.

All these data suggest a link between JAK2/STAT3 and BCR/Lyn axes. In fact, JAK2 may be responsible for Lyn phosphorylation in Tyr396 *via* SHP-1 protein and Lyn could be involved in STAT3 activation.

DISCUSSION

The *in vivo* CLL cell resistance to apoptosis rapidly decreases *in vitro*, marking its dependence on the extrinsic signals coming from the surrounding environment. Herein, by characterizing the activation of JAK2/STAT3 pathway in this disease and investigating its stimulation from tumor microenvironment, we demonstrated the involvement of this axis in CLL cell survival. We showed that STAT3 is constitutively active in CLL cells and the block of JAK2/STAT3 pathway through specific inhibitors affects the viability of the neoplastic clone overcoming the environmental protection. Moreover, the inhibition of Lyn, a Src kinase constitutively activated in CLL, leads to STAT3 dephosphorylation, highlighting a cross-talk between JAK2/STAT3 axis and the signaling initiated by B cell receptor (BCR)/Lyn cascade.

JAK2/STAT3 axis is one of the most described signaling pathway in literature. Its constitutive activation occurs with high frequency in several hematopoietic diseases and solid tumors. STAT3 plays a critical role in pancreatic tumorigenesis[85] and it is an important therapeutic target in head and neck cancer[86]. Moreover, JAK2/STAT3 pathway is involved in the development of large granular lymphocytic leukemia[87] and acute myeloid leukemia[88].

We demonstrated that STAT3 is over-expressed in CLL B cells with respect to normal B lymphocytes. We analysed the two forms of phosphorylated STAT3 (Ser727 and Tyr705), confirming the constitutive phosphorylation at Ser727[72,73]. Noteworthy, we found a constitutive phosphorylation of STAT3 in Tyr705, so far described only after CLL cell stimulation with anti-IgM or IL-6[89]. Actually, the presence of STAT3 Tyr705 phosphorylation in CLL remains controversial, with few studies that show an inducible and transient STAT3 Ty3705 phosphorylation[90], depending on interleukin-6 (IL-6)[73], a STAT3 activator[91]. Indeed, the constitutive activation of STAT3 we observed in CLL cells is probably due to signals coming from the microenvironment, especially to the high levels of IL-6 released from the

neighbouring cells. In fact, our results also showed that STAT3 Tyr705 phosphorylation is further up-regulated when CLL cells were co-cultured with MSCs, thus supporting the key role of the tumor environment in the activation of JAK2/STAT3 pathway. Concerning this, Levidou *et al.* demonstrated that STAT3 is constitutively phosphorylated at Tyr705 in CLL cells residing in the lymph node[92].

We showed the presence of phosphorylated STAT3 Ty3705 into the nucleus of CLL cells, where this protein can exert its function of transcriptional factor through the activation of several pro-survival genes[93]. For instance, STAT3 plays a key role in supporting cell survival by promoting the expression of anti-apoptotic factors such as Mcl-1 and Bcl-2[94–96], both over-expressed in CLL, stressing the role of STAT3 in CLL cell maintenance.

However, the meaning of STAT3 phosphorylations in CLL is still matter of debate, including the hypothesis that STAT3 Tyr705 phosphorylation precedes STAT3 Ser727 phosphorylation[97]. Accordingly, we found an effective positive correlation between STAT3 Ser727 and STAT3 Tyr705 phosphorylation in CLL cells.

STAT3 phosphorylation is mediated by the tyrosine kinase JAK2, whose inhibition leads to STAT3 inactivation.

The kinase inhibitor AG490 is able to selectively block JAK2 activity and to induce apoptosis of myeloma tumor cells inoculated in the immunodeficient murine model SCID[98]. Moreover, AG490 completely blocks the growth of acute lymphoblastic leukemia cells[99]. Our experiments demonstrated that the treatment of CLL B cells with AG490 inhibits JAK2 activation preventing STAT3 phosphorylation and consequently affecting leukemic cells viability.

We also inhibit the pathway downstream JAK2 through Stattic, that selectively inhibits the binding of STAT3 homodimers to DNA and increases the apoptotic rate of STAT3-dependent breast cancer cell lines[82]. Again, we observed a significant decrease in CLL cell viability. The same experiments with AG490 and Stattic were performed on leukemic B cells co-cultured with MSCs. We demonstrated that these inhibitors are able to induce apoptosis in

CLL B cells reverting the resistance to cytotoxic agents induced by the environment, bypassing the provided pro-survival stimuli.

Nowadays, therapeutic approaches aimed at stopping the cross-talk between tumor cells and the microenvironment are often used in the clinical practice. Lenalidomide inhibits the signals triggered by several cytokines (*e.g.* TNFalpha, VEGF, and IL-6) produced by the cells resident in the hematopoietic niche[100]; CXCR4 antagonists inhibit the anti-apoptotic effect of CXCL12 and allow the leukemic cell egress from lymph node, a protective microenvironment, to the circulation[101]; the specific JAK3 inhibitor, PF-956980, bypasses the cytotoxic agent resistance induced by the treatment of CLL cells with IL-4[102]. Our data and these results suggest that JAK2/STAT3 inhibitors deserve further investigation for their use in CLL therapy. The knowledge of the relationships between leukemic cells and the microenvironment will allow the identification of new therapeutic approaches to induce neoplastic cells apoptosis, thereby improving the patient response.

Following BCR engagement, STAT3 is also phosphorylated at Tyr705 *via* Lyn and independently from JAK2[75,77]. With its activity, Lyn can mediated the phosphorylation cascade triggered by BCR engagement and culminating in the expression of several genes involved in cell growth and proliferation. In the past years, we demonstrated a direct correlation between high basal Lyn activity and defects in the induction of apoptosis in CLL B cells[48].

We observed that AG490 treatment of leukemic B cells inhibited the phosphorylation of both Lyn at Tyr396, inactivating Lyn kinase, and SHP-1 at Ser591, activating SHP-1 phosphatase. Lyn and SHP-1 are involved in the prolonged lifespan of CLL B cells[103]. SHP-1 activity is turned on by JAK2 inhibition. Adding sodium orthovanadate (Na_3VO_4, a phosphatase inhibitor) to AG490 pre-treated cells, we restored Lyn phosphorylation at its active site (Tyr396). On the contrary, the treatment of CLL cells with Stattic did not induce any change in SHP-1 status with respect to untreated cells since Stattic works on STAT3, that is a downstream protein compared to JAK2. Since Stattic did not affect SHP-1 activation, it does not impact on Lyn activation/phosphorylation.

The link between JAK2 and SHP-1 is still unclear, but a recent study on chronic myeloid leukemia (CML) cells showed that Lyn inhibition by AG490 is mediated by SET-PP2A-SHP-1 pathway[83]. Moreover, a Lyn activation due to a strengthening SET-mediated inhibition of PP2A sustains oncogenic signaling in CLL[104]. Accordingly, we hypothesize that even in CLL, SET and PP2A may play a key role in connecting the cytokine receptors pathway to BCR/Lyn axis. However, this hypothesis needs to be further investigated.

The Lyn over-expression and constitutive activation in CLL B cells would account for the constitutive phosphorylation of STAT3 at Tyr705 observed even when leukemic cells are not exposed to the microenvironment[77]. As a further support, our data demonstrated that Lyn inhibition by Dasatinib produces STAT3 inactivation.

Summarizing, we hypothesize that the constitutive activation of JAK2/STAT3 pathway in CLL cells leads to SET protein activation. Once activated, SET inhibits PP2A which, in turn, is unable to activate, *via* dephosphorylation of Ser591 residue, SHP-1. An inactive SHP-1 implies a phosphorylated/active Lyn that may sustain STAT3 phosphorylation (Figure 34).

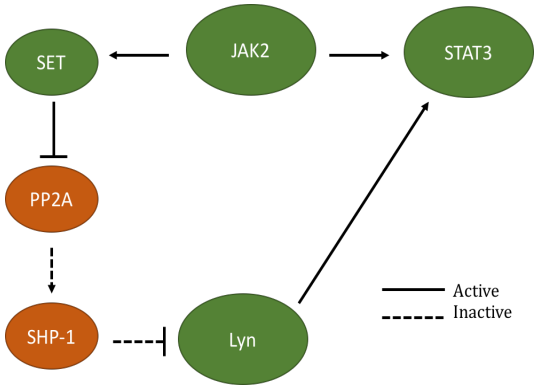

Figure 34. JAK2/STAT3 – BCR/Lyn Crosstalk. A schematic representation of the molecules involved in the crosstalk. Normally in CLL B cells, JAK2 phosphorylation leads to SET activation, therefore PP2A is not activated and SHP-1 is not able to dephosphorylate/deactivate Lyn kinase. Both JAK2 and Lyn can activate STAT3. Green: active; Red:inactive.

Bypassing the pro-survival stimuli provided by the tumor microenvironment, the ability of AG490 and Stattic to induce apoptosis in leukemic B cells offers a starting point for the development of new therapeutic strategies in CLL. This study also provides new insights for the investigation of the pathogenesis of CLL that focus the attention on the cross-talk between JAK/STAT and BCR/Lyn axes.

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