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INCREASED SURVIVAL OF CLL B CELLS IN THE PRESENCE OF MARROW MESENCHYMAL STROMAL CELLS: A NOVEL MODEL TO DEFINE NEW TARGETS FOR THERAPY

Direttore della Scuola: Ch.mo Prof. PAOLA ZANOVELLO

Supervisore: Ch.mo Prof. GIANPIETRO SEMENZATO

Correlatore: Ch.mo Prof. LIVIO TRENTIN

Dottorando: MONICA CASTELLI

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ABBREVIATIONS

Abs	Antibodies
Ag	Antigen
APC	Allophycocyanin
APRIL	A Proliferation-Inducing Ligand
ATM	Ataxia Teleangiectasia Mutated
BAFF	B cell Activating Factor
Bcl-2	B cell lymphoma 2
BCR	B Cell Receptor
BIRC3	Baculoviral IAP Repeat Containing 3
BM	Bone Marrow
BR	Bendamustine, Rituximab
BSA	Bovine Serum Albumin
Btk	Bruton's tyrosine kinase
CAP	Cyclophosphamide, Doxorubicine, Prednisone
Cbfa1	Core-binding factor alpha 1
CCL3	Chemokines C-C motif Ligand 3
CD40L	CD40 Ligand
CDR	Complementarity Determining Region
CHOP	Cyclophosphamide, Doxorubicine, Vincristine, Prednisone
CIRS	Cumulative Illness Rating Scale
CLL	Chronic Lymphocytic Leukemia
CM	Conditionated Medium
CpG	Cytosine-phosphate-Guanine
CR	Complete Remission
CT	Computed Tomography
CTLA-4	Cytotoxic T-Lymphocyte-Associated protein 4
CXCL12	Chemokines C-X-C motif Ligand 12
CXCR4	C-X-C motif Receptor 4
DMEM	Dulbecco's Modified Eagle Medium
ERK	Extracellular signal-Regulated Kinase
ET-1	Endothelin-1
F/H	Ficoll/Hypaque
Fab	Antigen binding fragment
FBS	Fetal Bovine Serum
FC	Crystallizable Fragment
FCR	Fludarabine, Cyclophosphamide, Rituximab
FDCs	Follicular Dendritic Cells
FISH	Interphase Fluorescent *in situ* Hybridization
FITC	Fluorescein Isothiocyanate
FL	Follicular Lymphoma
FLU/Cy	Fludarabine/Cyclophosphamide
GC	Germinal Centre
GCLLSG	German Chronic Lymphocytic Leukemia Study Group
GEP	Gene Expression Profile
Hb	Hemoglobin
HLA-DR	Human Leukocyte Antigen D-related
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
IGVH	Immunoglobulin Heavy chain Variable Region
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
LDT	Lymphocyte Doubling Time

MBL	Monoclonal B-Lymphocytosis
MCL	Mantle Cell Lymphoma
MFI	Mean Fluorescence Intensity
MSC-CM	Mesenchymal Stromal Cell-Conditionated Medium
MSCs	Mesenchymal Stromal Cells
MYD88	Myeloid Differentiation primary response 88
NF-κB	Nuclear Factor-κB
NKGD2	Natural Killer Group 2 member D
NKp30	Natural Killer cell p30-related protein
NLCs	Nurse-Like cells
NOTCH1	Notch Homolog 1, Translocation-associated
ORR	Overall Response Rate
OS	Overall Survival
PARP	Poli-ADP-Ribose Polymerase
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed cell Death protein 1
PD-L1	PD-1 Ligand
PE	Phycoerythrin
PFS	Progression Free Survival
PKCβ	Protein Kinase C β
PLL	Pro-Lymphocytic Leukemia
Plts	Platelets
PMN	Polymorphonuclear Neutrophil
Pparγ2	Proliferator Activated Receptor γ2
PS	Phosphatidylserine
RBC	Red Blood Cell
SD	Standard Deviation
SF3B1	Splicing Factor 3B subunit 1
SH2	Src Homology 2
SHIP 1/2	SH2 domain containing Inositol 5-Phosphatases 1/2
SHM	Somatic Hypermutations
SHP1	SH2 domain containing protein tyrosine Phosphatase-1
sIg	Surface Immunoglobulin
SLL	Small Lymphocytic Lymphoma
STAT-3	Signal Transducer and Activator of Transcription 3
Syk	Spleen tyrosine kinase
TC	Tri-Color
TF	Transcription Factor
TGFβ	Transforming Growth Factor β
TTT	Time To Progression
VH	Heavy chain Variable region
WB	Western Blotting
WHO	World Health Organization
ZAP-70	Zeta-Associated Protein of 70kDa
α-MEM	Modified Eagle Medium
αSMA+	α-Smooth Muscle Actin positive

ABSTRACT

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the Western World, accounting for about 30% of adult leukemia, and it is characterized by the clonal expansion and accumulation of mature CD19+/CD5+/CD23+ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs. Despite their apparent longevity in patients, *in vitro* CLL leukemic B cells rapidly undergo spontaneous apoptosis. The selective survival advantage is due both to intrinsic defects on apoptosis mechanism and to signals delivered by accessory cells at the active site of the disease. Previous studies demonstrated that mesenchymal stromal cells (MSCs), derived from bone marrow, and $CD68^+$ nurse-like cells, derived from peripheral blood, are involved in CLL clone longevity and migration, suggesting a crucial role of MSCs on favouring disease progression.

Therefore, in this thesis we evaluated the effect of MSCs, the main stromal population in the bone marrow of CLL patients, on the survival of leukemic B cells and their role in drug resistance.

MSCs were isolated from the bone marrow of 46 CLL patients; their immunophenotypic characterization was based on the expression of CD105, CD73 and CD90 and the negativity of CD14, CD34, CD45 and CD31. Co-culturing MSCs and CLL B cells, we confirmed that MSCs are able to support malignant B cell survival, providing an *in vitro* culture system that closely approximates CLL microenvironment *in vivo*. We observed that different leukemic clones demonstrated a large variety in the pro-survival effect. Evaluating the cleavage pattern of PARP, we revealed two subsets of CLL clones with different sensitivity to MSCs pro-survival signals. Our results indicate that both cell-cell contact and soluble molecules are actors in the relationship between malignant B cells and the MSCs, promoting CLL B cell survival and migration.

Later, we evaluated the role of the MSCs on CLL B cells during the most common cytotoxic therapy used in clinical practice. Our data demonstrate that MSCs are able to protect leukemic B cells from apoptosis during Fludarabine and Cyclophosphamide treatment, both *in vitro* and *in vivo*. We tested MSCs protective role also during CLL B cells treatment with Ibrutinib, a novel inhibitor of Btk involved in the BCR signaling pathway, and we found that the treatment counteracts the MSC pro-survival effect. To better understand the effect of Ibrutinib on the cross-talk between CLL B cells and MSCs, we evaluated its role on leukemic B cell migration, also analyzing the expression levels of

CCR7 and CXCR4, two chemokine receptors that are central in the homing of the neoplastic clone. We demonstrated that malignant B cell migration is not significantly affected by the Btk inhibitor; since cell-cell contact with MSC is crucial for CLL B cell survival, we analyzed the adhesion of leukemic B cells to MSCs after treatment with Ibrutinib. We found a significant reduction in leukemic B cells and MSCs interactions mediated by the CD49d integrin.

In this thesis, we demonstrate that MSCs enhance the survival of leukemic B cells through the release of soluble factors and cell-cell direct contact and that each CLL clone reveals a peculiar response to the anti-apoptotic signals delivered by MSCs. These observations could be relevant to identify patients more responsive to druggable targets on marrow microenvironment and also to find putative new strategies for CLL therapy. A better understanding on the complexity of the cross-talk between CLL cells and their microenvironment during CLL therapy could also help to define mechanisms of drug resistance and treatment failure, as well to plan randomized clinical trials comparing new compounds and their combinations with standard chemo-immunotherapy.

RIASSUNTO

La Leucemia Linfatica Cronica (LLC) è considerate la più comune leucemia del mondo occidentale, rappresentando circa il 30% delle leucemie dell'adulto, ed è caratterizzata dalla proliferazione clonale e dall'accumulo nel sangue periferico, nel midollo osseo e negli organi linfatici secondari di linfociti B maturi CD19+/CD5+/CD23+. Nonostante i linfociti B leucemici mostrino un'aumentata sopravvivenza nei pazienti affetti da LLC, *in vitro* vanno rapidamente incontro ad apoptosi. Il vantaggio sulla sopravvivenza è legato sia a difetti intrinseci del meccanismo di apoptosi sia a segnali forniti da cellule accessorie, presenti nel sito attivo della malattia. Precedenti studi hanno dimostrato che le cellule mesenchimali stromali (MSC) e le cellule accessorie ("nurse-like") derivate rispettivamente dal midollo osseo e dal sangue periferico, sono coinvolte nell'aumentata longevità e mobilità del clone leucemico, suggerendo un ruolo cruciale delle MSC nel favorire la progressione della malattia.

In questa tesi abbiamo valutato l'effetto delle MSC, la principale popolazione stromale nel midollo osseo dei pazienti affetti da LLC, sulla sopravvivenza dei linfociti B neoplastici e il loro ruolo sulla resistenza ai farmaci.

Le MSC sono state isolate da campioni di sangue midollare provenienti da 46 pazienti affetti da LLC; la loro caratterizzazione immunofenotipica è stata effettuata sulla base dell'espressione di CD105, CD73 e CD90 e sulla negatività di CD14, CD34, CD45 e CD31. Allestendo co-colture di MSC e linfociti B leucemici, abbiamo confermato la capacità delle MSC di incrementare la sopravvivenza delle cellule B neoplastiche, fornendo un sistema di coltura *in vitro* che mima profondamente il microambiente della LLC *in vivo*. Abbiamo osservato una grande varietà sulla vitalità dimostrata dai diversi cloni leucemici e, mediante la valutazione del frammento clivato della proteina PARP, abbiamo identificato due differenti gruppi di cloni di LLC, con una diversa sensibilità ai segnali di stimolo provenienti dalle MSC. I nostri risultati indicano che sia il diretto contatto cellula-cellula che la presenza di molecole solubili sono coinvolte nell'interazione tra le cellule B leucemiche e le MSC, promuovendo la sopravvivenza e la migrazione della cellula B leucemica.

Successivamente, abbiamo valutato l'effetto delle MSC sui linfociti B neoplastici durante un trattamento chemioterapico di uso comune nella pratica clinica. I nostri dati hanno dimostrato che le MSC sono in grado di proteggere le cellule B leucemiche

dall'apoptosi durante il trattamento con Fludarabina e Ciclofosfamide, sia *in vitro* che *in vivo*. Abbiamo esaminato il ruolo protettivo delle MSC anche durante il trattamento dei linfociti neoplastici con Ibrutinib, un nuovo inibitore della Btk, una chinasi coinvolta nella cascata del segnale del BCR, e abbiamo dimostrato che il trattamento delle cellule B con Ibrutinib è in grado di contrastare l'effetto anti-apoptotico delle MSC. Per meglio definire l'azione di Ibrutinib nell'interazione tra le cellule B di LLC e le MSC, abbiamo valutato il suo ruolo sulla mobilità dei linfociti B leucemici, analizzando inoltre i livelli di espressione di CCR7 e CXCR4, due recettori chemiochinici fondamentali nella migrazione del clone neoplastico. Abbiamo dimostrato che la migrazione delle cellule B neoplastiche non è significativamente influenzata dall'inibitore del Btk; inoltre, considerando che il diretto contatto cellula-cellula con le MSC è di notevole importanza per la sopravvivenza dei linfociti B leucemici, abbiamo analizzato l'adesione delle cellule B alle MSC dopo il trattamento con Ibrutinib, evidenziando che la loro adesione era significativamente ridotta.

In questa tesi abbiamo dimostrato che le MSC incrementano la sopravvivenza delle cellule B neoplastiche attraverso il rilascio di fattori solubili e mediante il diretto contatto cellula-cellula, e che ogni clone leucemico rivela una peculiare risposta ai segnali anti-apoptotici rilasciati dalle MSC. Queste osservazioni potrebbero essere determinanti al fine di identificare i pazienti più sensibili a trattamenti mirati a colpire il microambiente midollare ed a trovare potenziali nuove strategie terapeutiche per la LLC. Una migliore comprensione della complessità delle interazioni tra i linfociti leucemici e il loro microambiente nel corso del trattamento potrà inoltre aiutare a chiarire i meccanismi di chemioresistenza e refrattarietà, così come a pianificare studi clinici randomizzati che confrontino nuovi farmaci e la loro combinazione con i trattamenti chemio-immunoterapici già in uso.

INTRODUCTION

1. Chronic Lymphocytic Leukemia

1.1 Incidence and epidemiology

Chronic Lymphocytic Leukemia (CLL) is a lymphoproliferative disorder characterized by the clonal proliferation and accumulation of mature, typically CD5+ B cells within the blood, bone marrow (BM), lymph node and spleen (1). Recently, it has been reported that in CLL the capacity to generate clonal B cells might be acquired at the hematopoietic stem cell (HSC) stage, suggesting that the primary leukemogenic event might involve multipotent, self-renewing HSCs (2). CLL is the most prevalent leukemia in the Western world with an incidence of about 6-9% (3). The median age diagnosis lies between 67 and 72 years with an incidence increasing rapidly with age; more male than female are affected by this disease and only about 10% of CLL patients are reported to be younger than 55 years (4). The proportion of younger patients with early stage CLL and minimal symptoms seems to increase due to more frequent blood testing. The etiology is still unknown; moreover, there is an inherited genetic susceptibility, with an increased risk for family members of CLL patients (5).

1.2 Diagnosis

The World Health Organization (WHO) classification of hematopoietic neoplasias describes CLL as a leukemic lymphocytic lymphoma, distinguishable from small lymphocytic lymphoma (SLL) only by its leukemic appearance. The diagnosis of SLL in fact requires a number of B lymphocytes in the peripheral blood not exceeding 5,000μL. Other lymphoma entities to be separated from CLL are marginal zone lymphoma, lymphoplasmacytic lymphoma and mantle cell lymphoma (MCL). CLL is always a disease of neoplastic B cells, because the entity beforehand described as T cells Chronic Lymphocytic Leukemia (T-CLL) is now called T-cell pro-lymphocytic leukemia (T-PLL) (6).

The diagnosis of CLL requires the following criteria (7):

- The presence of ≥ 5,000μL monoclonal B lymphocytes in the peripheral blood for the duration of at least 3 months. The clonality of the circulating leukemic B cells is confirmed by flow cytometry observing the restriction to expression of either kappa or lambda immunoglobulin light chains; CLL B cells co-express the T-cell antigen CD5 and B cell surface antigens CD19, CD20 and CD23 (Fig. 1). Typically, the expression levels of surface immunoglobulin, CD20 and CD79b are lower than normal B cells (8).

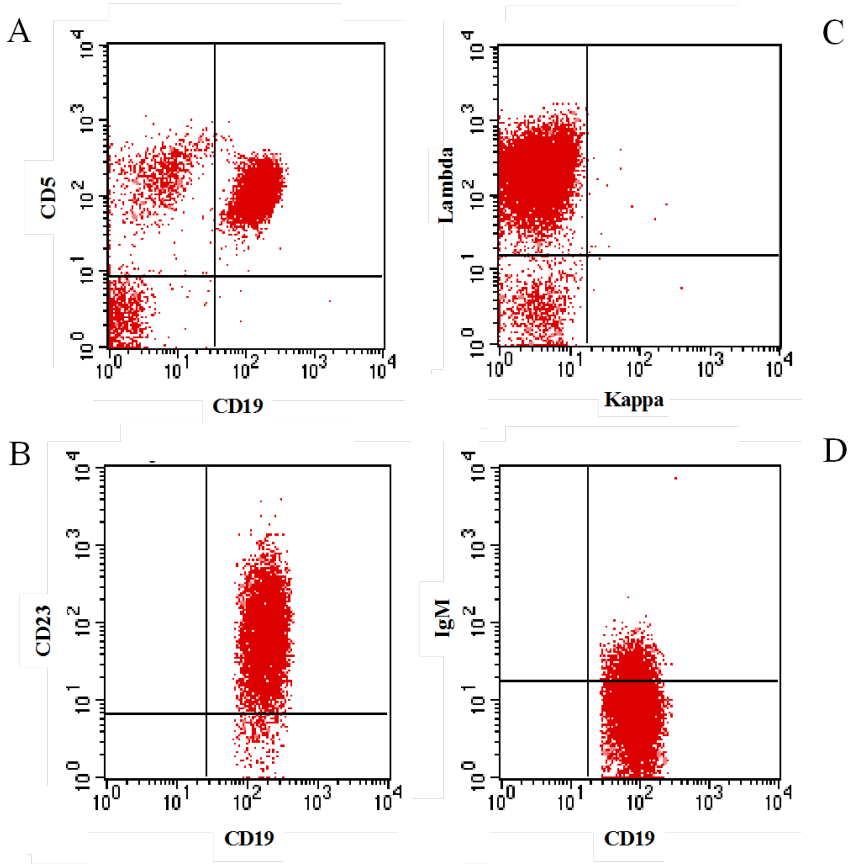


Figure 1. Cytograms of a representative case of CLL. B lymphocytes analyzed (CD19+) are positive to CD5 (panel A) and to CD23 (panel B), express one type of immunoglobuline light chain (λ, panel C), and surface IgM (sIgM), low density (panel D).

- The leukemic B lymphocytes in the blood smear are small and mature, with a narrow border of cytoplasm, a dense nucleus lacking discernible nucleoli and a partially aggregated chromatin (Fig. 2). Gumprecht nuclear shadows, or smudge cells, found as cells debris, are other characteristic morphologic features found in CLL. Larger atypical lymphocytes (pro-lymphocytes) may be seen in the blood smear but they must not exceed 55%; the evidence of pro-lymphocytes in excess of 55% would indicate a diagnosis of pro-lymphocytic leukemia (PLL).

In absence of lymphoadenopathy, organomegaly, cytopaenia and clinical symptoms, the presence of fewer than 5,000/μL monoclonal B lymhocytes is defined monoclonal B-lymphocytosis (MBL), which can be detected in 5% of subjects with normal blood count. Progression to CLL occurs in 1-2% of MBL cases per year (9).

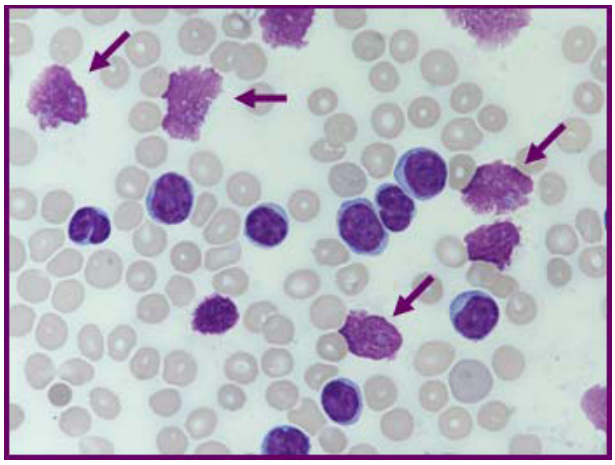


Figure 2. Peripheral blood smear of a CLL patient. Arrows indicate Gumprecht shadows typical of CLL.

1.3 Clinical features

Currently, 70-80% of CLL patients present with a low tumor burden and are frequently diagnosed from a blood count performed for an incidental reason, while other patients may present an aggressive disease (10). The patients could be asymptomatic or present B symptoms (asthenia, weight loss, fever, night sweats) and lymphadenopathy, splenomegaly and hepatomegaly. In advanced disease, we could observe hemolytic anemia (11% of cases) or autoimmune thrombocytopenia (2%). Frequently, CLL patients show hypogammaglobulinemia that could induce acquired immunodeficiency and high risk for infections. The evolution of the disease to Richter's syndrome (a diffuse large B cell lymphoma) or to PLL could be observed in some CLL cases with a poor prognosis.

1.4 Clinical staging

In everyday clinical practice, two widely accepted staging methods co-exist, the Rai and the Binet systems. The original Rai classification was modified to reduce from five to three the number of prognostic groups. Both Rai and Binet staging classify patients into three major prognostic clusters; these classifications are simple and cheap and require only physical examination and routine laboratory tests. The Binet staging system is established

on the number of involved areas, defined by the presence of enlarged lymph nodes (>1cm) or organomegaly and on the evaluation of anemia or thrombocytopenia (Tab. 1) (11). The areas of involvement considered are head and neck, the Waldeyer ring, axillae, groins, palpable spleen and liver.

Table 1. Binet staging system. Hb: hemoglobin; plts: platelets

STAGE	DEFINITION	MEDIAN SURVIVAL
BINET A	Hb ≥10g/dL, plts ≥100x10^9/L, <3 lymph node regions	≥10 years
BINET B	Hb ≥10.0 g/dL, plts ≥100x10^9/L, ≥3 lymph node regions	>8 years
BINET C	Hb <10.0g/dL, plts <100x10^9/L	6.5 years

The modified Rai staging system defines as low-risk disease patients with lymphocytosis (RAI 0); patients with lymphocytosis, enlarged nodes in any site or/and organomegaly are classified in an intermediate risk disease (RAI I, II). High risk stage includes patients with anemia or thrombocytopenia disease-related (Tab. 2, RAI III, IV) (12).

Table 2: Rai staging system. Hb: hemoglobin; plts: platelets.

STAGE	DEFINITION	MEDIAN SURVIVAL
RAI 0	Lymphocytosis >15x10^9/L	>10 years
RAI I	Lymphocytosis + lymphadenopathy	8 years
RAI II	Lymphocytosis + hepatomegaly/splenomegaly ± lymphadenopathy	
RAI III	Lymphocytosis + Hb <11g/dL ± lymphadenopathy/organomegaly	6.5 years
RAI IV	Lymphocytosis + plts <100 x10^9/L ± lymphadenopathy/organomegaly	

1.5 Biological prognostic factors

Additional biological markers are available to predict the prognosis of CLL patients, in particular at early stage. 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. Conventionally, patients with <2% somatic mutations from the most similar germline gene in both the expressed VH and VL genes were define unmutated (SHM-); mutated cases (SHM+) were defined as those in which the CLL cells displayed ≥2% differences in either the expressed VH or VL gene. About 50% of CLL patients present an unmutated immunoglobulin heavy chain variable region (IGVH) status (13). CLL cells have a higher genetic instability with a higher risk of unfavourable genetic mutations. Overall survival (OS) and time to treatment (TTT) are shorter in this CLL patients group (Fig. 3A). The stereotyped VH3.21 gene is an unfavorable prognostic marker independent of the IGVH mutational status.

2) CD38 expression. CD38 is a transmembrane protein that supports B cell interaction and differentiation through the binding of CD31, a cell-adhesion molecule expressed by cells of the CLL microenvironment. Patients with higher CD38 expression have a faster progression and a shorter life expectancy (14). A previous study suggested that CD38 expression has an independent prognostic value in CLL (Fig. 3B).

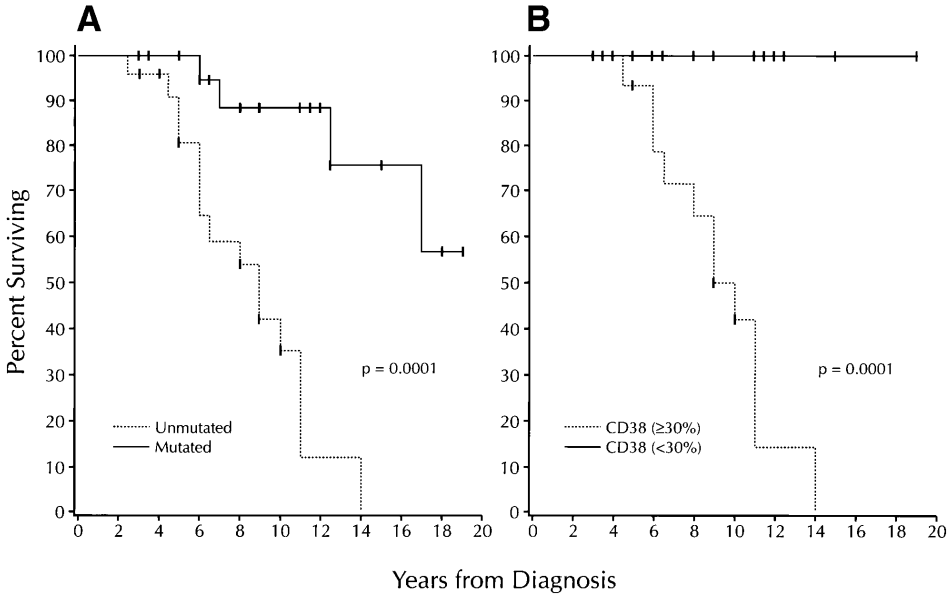


Figure 3. CLL patients survival based on V gene mutation (A) and CD38 expression (B) (13).

3) CD49d. The expression percentage of CD49d+ cells is an independent indicator of prognosis in CLL, with higher levels (≥30%) being correlated with a shorter survival. CD49d is an α subunit (α4) that together with CD29 (the β1 subunit) forms the integrin α4β1. α4β1 binds fibronectin and VCAM-1 and is involved in anchoring cells to tissues via extracellular matrix, which can result in cell survival and can also be a first step in cell

migration. Cell migration function is very important for CLL cells survival and explains the prognostic relevance of CD49d expression (15).

4) 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 working as a key signaling molecule for T lymphocytes and NK cells. ZAP-70 expression reflects an activation state of the malignant clone and may be associated with a CLL progression (16). ZAP-70 value could change over time in CLL; its analysis can be performed with flow cytometry, immunohistochemistry, western blotting and Real-Time PCR. Flow cytometry is the most useful for its diffusion and easiness of application.

5) Molecular Cytogenetics. Using Interphase Fluorescent *in Situ* Hybridization (FISH), cytogenetic lesions can be identified in more than 80% of all CLL cases (17). Deletions on the long arm of chromosome 13, involving band 13q14 *[del(13q14)]* represent the single most frequently observed cytogenetic aberration, occurring in about 55% of all cases and it is characterized by a benign CLL course. Additional frequent chromosomal aberrations include deletions in the long arm of chromosome 11 *[del(11q)].* These deletions usually encompass band 11q23 harboring the gene Ataxia teleangiectasia mutated (ATM), which encodes for the proximal DNA damage response kinase ATM. Patients with a *del(11q)* clone show a rapid CLL progression, bulky lymphadenopathy and reduced overal survival. Trisomy 12 is observed in 10-20% of CLL patients that demonstrated a shorter survival in respect to those with a normal FISH. There is an association between trisomy 12 and the presence of mutations in the Notch homolog 1, translocation-associated (NOTCH1) gene leading to a less favorable course of the disease. Deletions of the short arm of chromosome 17 are found in 5-8% of never treated patients. These deletions usually consist in band 17p13, including the main tumor suppressor gene TP53. *Del(17p)* patients show marked chemo-immunotherapy resistance (18). Mutations of TP53 are found in 4-37% of CLL patients and are associated with a very poor prognosis (19). Whole genome sequencing analyses identified additional recurrent mutations (20) (>5% cases at diagnosis) affecting NOTCH1 (21), splicing factor 3B subunit 1 (SF3B1) (22), baculoviral IAP repeat containing 3 (BIRC3) (23) and myeloid differentiation primary response (MYD88) genes. These mutations usually coexist with some of the genetic abnormalities analyzed by the FISH: SF3B1 mutations with del(11q), NOTCH1 with trisomy 12 and MYD88 with del(13q); generally, these associations resulted in a poor patient outcome. There was a clear relationship between NOTCH1 mutation and a shorter time to therapy with resistance to

treatment and Richter's transformation.

6) Serum markers. According to different studies, also CD23, thymidine kinase and β_2–microglobulin may predict survival and progression-free survival. Nevertheless, their relative value in the management of CLL patients is not validated (24), (25), (26).

1.6 Other test performed at diagnosis

In order to complete the risk assessment, the following examinations are also recommended (27): patient performance status, medical hystory, serum chemistry (including lactate dehydrogenase, bilirubin, serum immunoglobulins), direct antiglobulin test, the status of relevant infections (hepatitis B and C, cytomegalovirus, human immunodeficiency virus). Although a bone marrow biopsy is not required for diagnosis, it is recommended for the diagnostic evaluation of unclear cytopaenias, or FISH and molecular genetics if peripheral blood cell lymphocytosis does not allow adequate flow cytometry analysis. Imaging studies by computed tomography (CT) scans could help to asses the tumor load before starting treatment or to clarify unclear symptoms, but they should not generally be performed in asymptomatic patients for clinical staging.

1.7 Indications to treatment

Patients at intermediate and high risk stages, according to the modified Rai classification, or stage B and C, according to the Binet classification, usually benefit from treatment but some of these patients can be only monitored until they have evidence for progressive or symptomatic disease. The absolute lymphocyte count should not be used as the only indication for starting therapy. Active disease should meet at least one of the following criteria (28): 1. evidence of progressive marrow failure (development or worsening of anemia and/or thrombocytopenia; 2. massive or progressive and symptomatic splenomegaly; 3. massive node or progressive and symptomatic lymph nodes; 4. progressive lymphocytosis increasing more than 50% in months or lymphocyte doubling time (LDT) of less than 6 months (excluding factors contributing to lymphocytosis or lymphadenopathy other than CLL); 5. autoimmune anemia and/or thrombocytopenia poorly responding to other standard therapy; 6. constitutional symptoms as unintentional

weight loss of 10% or more in the last 6 months, significant fatigue, fever higher than 38°C for more than 2 weeks and night sweats for more than 1 month without infections.

2. Neoplastic B lymphocytes

B lymphocyte participates in humoral immunity producing antibodies (Abs) in response to antigen (Ag) stimulation. B cell can differentiate from "naive" lymphocyte to cell secreting antibodies against specific antigens (plasma cells), or to "memory" long-lived stimulated B lymphocyte, which is ready for rapid response to a repeated exposure of the priming antigen. The B cell receptor (BCR) mediates B lymphocyte antigen recognition; BCR is a multimeric complex composed by the antigen-specific surface immunoglobulin (sIg) homodimer, linked to the plasmatic membrane through its constant region (crystallizable fragment, Fc). The sIg antigen binding fragment (Fab) is outward and noncovalently linked to Igα/Igβ (CD79a/CD79b) heterodimer, responsible of intracellular signal transduction (29) (Fig. 4). 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 hypervariable regions, called "complementarity determining regions" (CDR) that allow high affinity binding with the antigen. "Naive" B lymphocyte presents an amino acid sequence identical to "germline" sequence, while the "memory" B cell is characterized by a somatic hypermutation process that underlies the phenomenon of affinity maturation. The "naive" B lymphocyte, after specific antigen identification, turns on and proliferate inside lymphoid organs. Some of this progeny enters the lymphoid follicles to create the germinal centre (GC). In the GC, Ig genes undergo somatic point mutations leading 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. CLL B cells are small "memory" B cells blocked in G0/G1 and characterized by specific surface markers: CD19 and CD21 are B-related, while CD5, CD23, CD25 and HLA-DR (Human Leukocyte Antigen D-related) are not specific for B lymphocytes. In particular, malignant B lymphocytes express markers typical of mature B cells localized in the mantle zone of secondary lymphoid follicles. CLL pathogenesis mechanisms comprehend a defective control of apoptosis, alterations in BCR-mediated signaling transduction and proliferative activity and the microenvironment.

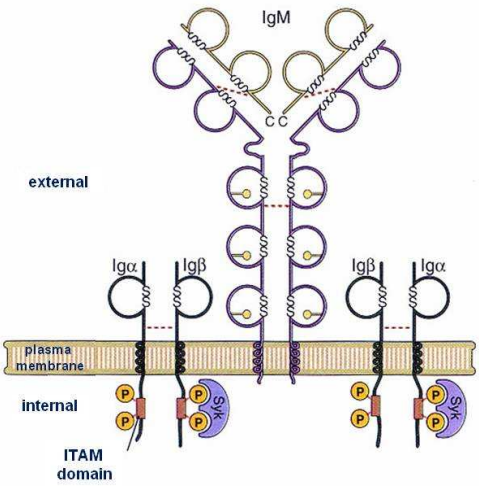


Figure 4. Schematic representation of the BCR. The complex is composed by a sIg, and Igα and Igβ that mediate signal transduction after antigen binding.

2.1 Control of apoptosis

The dysregulation of the programmed cell death (apoptosis) is one of the main mechanisms in CLL pathogenesis, leading to the accumulation of CLL B cells. When CLL B cells were cultured *in vitro*, a substantial proportion of them spontaneously died by apoptosis (30). This evidence suggested that the CLL B cell defective apoptosis has to be ascribed not only to intrinsic defects of the neoplastic cells, but also to extrinsic factors. Malignant B cells retain the ability to respond to microenvironmental signals, but show a specific sensitivity to anti-apoptotic signals that favour their survival and become insensitive to pro-apoptotic signals (31). The balance between pro- and anti-apoptotic factors is very important. 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. Other intrinsic factors, involved in apoptosis control, are *del(17p)* and *del(11q)*, because of the mutation of two relevant tumor-suppressor genes, TP53 and Ataxia Teleangectasia Mutated (ATM) (32).

2.2 BCR-mediated signal transduction

BCR is responsible to transmit signals that regulate B-cell fate decision and to mediate antigen processing leading to the presentation of antigen to T cells, which allows full activation of B cells in the effector phase (33). Antigen binding to the sIg induces activation of upstream kinases, including spleen tyrosine kinase (Syk) and the Src kinase Lyn, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmatic tails of CD79a and CD79b. This activates the hematopoietic cell-specific Lyn substrate (HS1) protein (34) and the related F-actin polymerization ad other upstream kinases, including Syk, Bruton's tyrosine kinase (Btk) and phosphoinositide 3-δ (PI3kδ) kinases and downstream pathways, including calcium mobilization, activation of phospholipase Cγ2, protein kinase C β (PKCβ), nuclear factor κB (NF-κB) signaling, mitogen-activated protein kinases and nuclear transcription. Activation of phosphatases, including Src homology 2 (SH2) domain containing protein tyrosine phosphatase-1 (SHP1), SH2 domain containing inositol 5-phosphatases 1/2 (SHIP1/2) and negative co-receptors (CD22, CD5) contributes to negative regulation of the BCR signaling response (Fig. 5). The precise mechanism triggering BCR activation (antigen-dependent or independent) is still controversial, but several line of evidence support the relevant role of BCR in CLL pathogenesis. The prognostic importance of mutational status of immunoglobulin heavy chain variable regions (IGVH) genes indicates that CLL BCR encounters antigens, which promote a degree of somatic hypermutations, which influence the clinical prognosis of the disease. Gene expression profile (GEP) studies demonstrated that BCR signaling is the key regulatory pathway activated in CLL cells in lymph nodes (35).

Naive B cells are characterized by the presence of a functional surface immunoglobulin of the M isotype (sIgM); in secondary lymphoid organs naive B cells undergo further maturation, including expression of immunoglobulins of the D isotype (sIgD). Most of CLL B cells express both sIgM and sIgD isotypes. sIgM signaling has a dominant role and previous studies demonstrated a different responsiveness to IgM stimulation for CLL carrying unmutated IGVH genes (U-CLL) vs mutated IGVH (M-CLL). U-CLL are more responsive to BCR triggering whereas cells from patients with M-CLL are generally less responsive to BCR cross-linking.

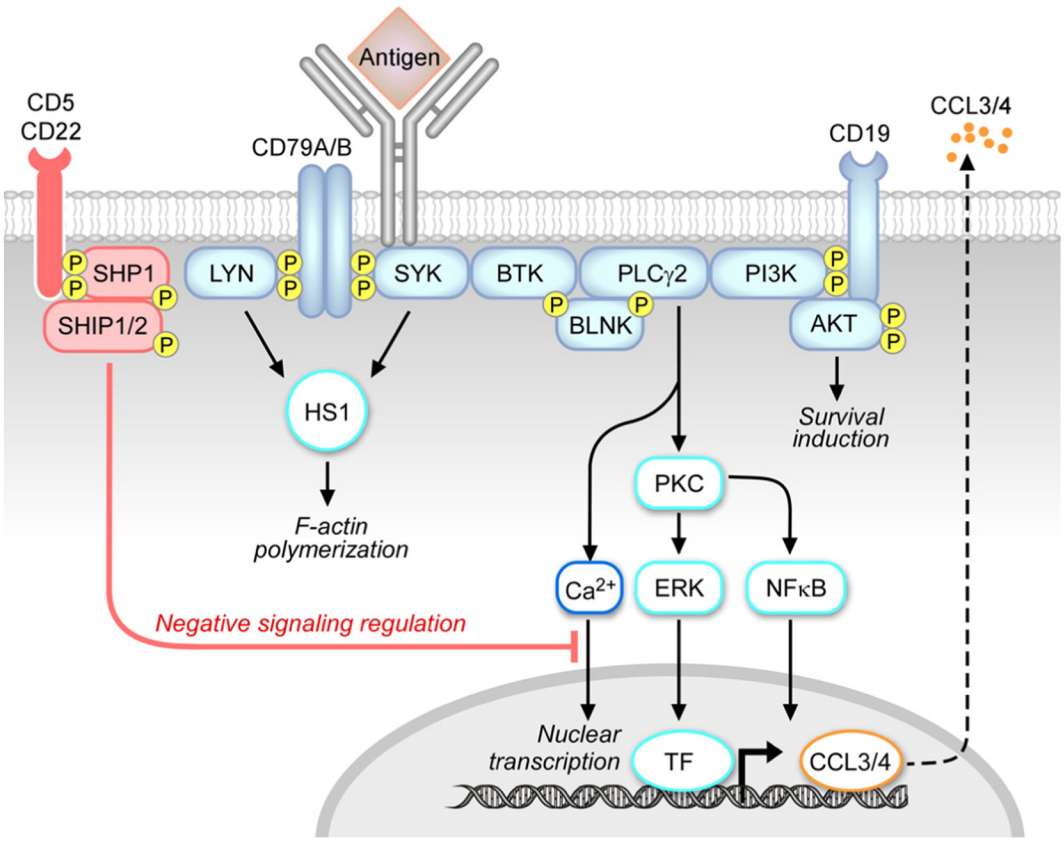


Figure 5. The BCR signaling pathway. BCR triggering by an antigen induces activation of early kinases (Lyn and Syk), which transduce the signal to cytoskeletal activators, including HS1 protein, and to other early effectors of the signaling response, including Btk kinase. Through the BLNK adaptor, Btk activates PLCγ2, and subsequent downstream responses, including calcium signaling (Ca2+), PKC, NFκB and ERK kinase, and nuclear transcription factor (TF). The positive co-receptor CD19 contributes to the activation of the PI3K–Akt pathway and to survival induction. The signaling response ultimately promotes activation of nuclear transcription, including CCL3 and CCL4 chemokine genes, which are then produced and secreted. The signaling response is tightly modulated by negative coreceptors (CD22, CD5) and phosphatases, including SHP1 and SHIP1/2 (41).

Prolonged extracellular signal-regulated kinase (ERK) activation after sIgM triggering supports expression of the proto-oncogene Myc, promoting cell-cycle entry and CLL B cell growth (36). The role of sIgD signaling is less defined but anti-IgD responsiveness was described to impact prognosis (37). Both IgM and IgD BCRs have the same antigen specificity and both sIgM and sIgD derived signals govern overall BCR pathway activation. The BCR signaling patway is central to CLL activation and likely to be triggered by antigens expressed in the tissue microenvironment. Inhibitors targeting BCR-associated kinases, including ibrutinib and idelalisib, have changed the landscape of treatment for CLL patients.

3. Cellular microenvironment in CLL

Bone marrow (BM) precursors originate from pluripotent stem cells and are in close contact with stromal cells. BM precursors are able to differentiate into mature virgin B lymphocytes that migrate to peripheral lymphoid tissues searching for a foreign antigen that will trigger B cell activation, proliferation and a second wave of differentiation. The germinal center in secondary lymphoid organs provides the microenvironment for mature B cells in which they can keep close contact with specialized T cells and antigen-presenting cells. This cross-talk is regulated by chemokines, cytokines and adhesion structures and generate B memory cells, plasma cell precursors and to the apoptosis of dangerous or inefficient cells. As in normal B cells, the microenvironment plays an essential role also in the natural hystory of B cell malignancies. In the BM and secondary lymphatic tissues, CLL B cells engage complex cellular and molecular interactions with stromal cells and matrix, that are called as "the microenvironment" (38). Several studies are gradually defining the critical pathways for leukemic B cells and the microenvironment that could affect cell survival and response to therapy, which now provide a rationale for targeting the CLL microenvironment (Fig. 6). The main cellular actors are MSCs, monocyte-derived nurse-like cells (NLCs), endothelial cells and follicular dendritic cells, T and NK cells (39).

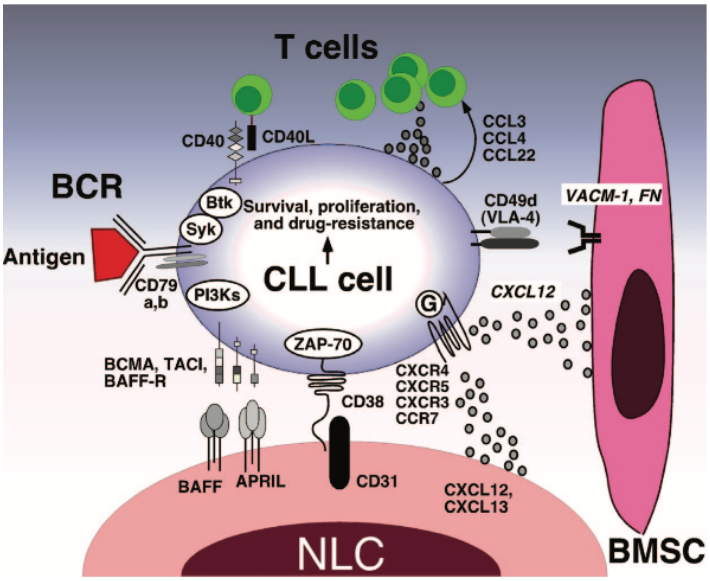


Figure 6. Molecular interactions in the CLL microenvironment. Molecular interactions between CLL B cells and stromal cells in the BM and lymphoid tissue considered relevant for CLL B cells survival and proliferation, homing and tissue retention. BMSC: bone marrow mesenchymal stromal cell, NLC: nurse-like cell. (39).

3.1 Nurse-like cells

NLCs share features similar to thymic nurse cells that nurture developing thymocytes, so they were designated as “nurse-like cells”. NCLs differentiate from monocytes into large, round and adherent cells attracting and protecting CLL B cells from apoptosis in a contact dependent manner. In CLL patients, NLCs can be detected in secondary lymphoid tissues and in the spleen (40). GEPs of CLL B cells after co-culture with NLCs, revealed an activation of BCR and NF-κB signaling pathways with similar gene signatures in leukemic B cells isolated from neoplastic lymph nodes. NLCs are involved in the chemotaxis and survival of CLL cells through the production of chemokines C-X-C motif ligand 12 (CXCL12), CXCL13, expression of TNF family members, B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL). NLCs also express vimentin and calreticulin that are able to activate BCR on CLL B cells and CD31, which is the ligand for CD38 (41). These findings suggest that NLCs could be a relevant model system for studying the lymphatic tissue microenvironment in B cell malignancies.

3.2 Endothelial cells and follicular dendritic cells

Endothelial cells and follicular dendritic cells (FDCs) are additional cellular elements with a crucial role for tissue homing and CLL B cell retention. CLL B cells bind on the surface of microvascular endothelial cells to β1 and β2 integrins and to BAFF and APRIL. The interaction between endothelin 1 (ET-1), exposed on B CLL cells, and the endothelin subtype A receptor (ETAR) on endothelial cells promotes cell survival and drug resistance (42). *In vitro* cultures with FDCs rescues CLL cells from apoptosis by direct cell contact, based on ligation of CD44 on leukemic B cells. The cross-talk between CLL B cells and FDCs dependent on CXCR5-CXCL13 and the lymphotoxin beta receptor/lymphotoxin alpha beta signaling pathways seems to be relevant for CLL cells retaining in lymphoid follicles and for the disease progression (43).

3.3 T and NK cells

The interaction between CD40, expressed on B cells, and CD40 ligand (CD40L) on

activated CD4+ T cells, is critical for the antigen presentation and the induction of normal B cell responses. CD40 ligation is also able to activate CLL B cells promoting their survival. In CLL the increased number of effector memory CD4+ and terminally differentiated CD8+ lymphocytes is associated with a more advanced disease stage. CD4+ and CD8+ cells display higher expression of exhaustion markers, including programmed cell death protein 1 (PD-1), while CLL B cells express high levels of PD-1 ligand (PD-L1); blocking antibodies, interfering with PD-1/PD-L1 axis, are able to prevent CLL progression and restore immune effector function (44). In CLL patients, T cells show an increased expression of the inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and increased proliferation when CTLA-4 is blocked.

In CLL patients a defective NK-cell function is also demonstrated. The reduction of NK cell cytotoxicity has been associated to low expression levels of the activating receptors natural killer cell p30-related protein (NKp30) and natural killer group 2 member D (NKGD2) (45). NK cells can produce also BAFF, interfering with NK-cell mediated lysis after rituximab therapy.

3.4 Bone marrow MSCs

In healthy subjects MSCs represent a small fraction of the stromal cell population, about 0.01-0.0001% of mononuclear cells, and decrease with age ($1/10^4$ in the newborn, $0.5/10^6$ in the older age) but MSCs are the dominant stromal cell population in CLL microenvironment. MSCs, after isolation from bone marrow and *in vitro* expansion in culture systems, remain in an undifferentiated state. When exposed to specific stimuli, MSCs are able to differentiate into the different mesodermal lineages, such as adipogenic, osteogenic, chondrogenic and myogenic, property attesting the nature of these stem cells (46).

MSCs provide an attachment site and growth factors for normal haematopoiesis and, both in CLL, are thought to create in the BM a niche to support and protect CLL B cells (47). MSCs are of mesenchymal origin and are similar to α-smooth muscle actin (αSMA+)-positive mesenchymal stromal cells in other tissues, such as the secondary lymphoid tissues. The observation of a diffuse increase in αSMA+ cell incorporation throughout the stromal compartment of indolent subtype of CLL/SLL and follicular lymphoma (FL), rather than other aggressive B-cell lymphoma subtypes, invest MSCs with a crucial role on favouring malignant cells and disease progression (48). CLL B cells have

a high affinity for MSCs; in co-culture CLL cells have shown a rapid adhesion and migration toward MSCs (49). The protective effect of MSCs is largely dependent on close proximity between stromal cells and leukemic B cells. A murine *in vivo* model of CLL demonstrated that the murine BM microenvironment consists in similar capacity to protect CLL B cell from apoptosis and was also adequate to sustain the disease progression (50).

MSCs constitutively secrete chemokines that organize CLL B cell migration and tissue homing and provide additional signals supporting leukemic B cells survival and promoting drug resistance. MSCs induce up-regulation in CLL B cells of ZAP70, CD38 and the down-regulation of C-X-C motif receptor 4 (CXCR4) (51). MSCs have also been shown to down-modulate the expression of CD20 from the surface of malignant B cells, with possible implications for resistance to anti-CD20 antibody therapy (52). Moreover, MSCs promote in CLL B cells glutathione synthesis and induce glycolysis through NOTCH-mediated c-Myc activation, promoting cell survival and drug resistance (53). Several studies support the relevance of a bidirectional cross-talk between leukemic B cells and MSCs; CLL B cells release microvesicles enriched in activated signaling proteins and are able to activate the Akt pathway in MSCs. MSCs activation by malignant B cells results also in the induction of PKCβII expression and NF-κB pathway activation (54). Over the last years, several soluble factors, cytokines and chemokines released from MSCs have been described, involved in CLL B cell homing, survival and proliferation, which now provide a rationale for targeting the microenvironment.

3.4.1 Immunophenotypic characterization

Without a single distinctive marker, phenotypic characterization of human MSCs is based on their positivity for some antigens, not exclusive of the MSCs, and the absence of some antigens, typically expressed by cells of hematopoietic origin. MSCs express the following markers: CD44 (receptor for hyaluronic acid), CD90 and CD29 (adhesion molecules present on stromal cells) (55), CD105 [endoglin receptor type III of Transforming Growth Factor β (TGF-β)], CD73 (5'-ectonucleotidasi membrane, present in cultures derived from bone marrow stromal cells), STRO-1 (antigen present in non-hematopoietic precursors of the bone marrow) and CD54. MSCs are negatives for CD34 and CD45 (markers of hematopoietic precursors and of hematopoietic cells) and CD31 (endothelial marker).

3.4.2 Expansion and functional characterization

MSCs are isolated from cellular suspensions derived from *ex vivo* bone marrow and resuspended in liquid medium. Mononuclear cells are seeded in plates with a 10,000 cells/cm^2 density in modified Eagle Medium (α-MEM) or Dulbecco's Modified Eagle Medium (DMEM), added of fetal bovine serum (FBS) and antibiotics. After 24 hours some round cells are already adherent to the plate, while the others remain in suspension and are removed after 7 days, with the first change of the medium culture. In the following days appear the first foci of proliferation, constituted by aggregates of highly proliferating cells that tend to mutual confluence, condition that leads to the stop of proliferation and to the spontaneous differentiation of the cells into pre-adipocytes. When the monolayer reaches the semi- confluence (70-80% of surface covered by the cells), the cells are detached with trypsin and seeded in other plates to expand the culture. After 5-7 weeks, it is possible to obtain a homogeneous population of adherent cells with fibroblastic appearance, which continues to proliferate up to 40 generations without spontaneously differentiating (56).

In the presence of appropriate conditioned media, MSCs are able to differentiate into the different lines of mesodermal origin, such as the adipogenic, osteogenic, chondrogenic and myogenic, properties attesting the nature of these stem cells. The adipogenic differentiation is induced with medium containing dexamethasone (1μM), insulin and 3-isobutyl-1-methylxanthine, factors that activate the pathways of lipid synthesis. MSCs progressively accumulate lipidic drops in the cytoplasm, revealed by specific colors as Oil Red-O or Sudan Black (Fig. 7).

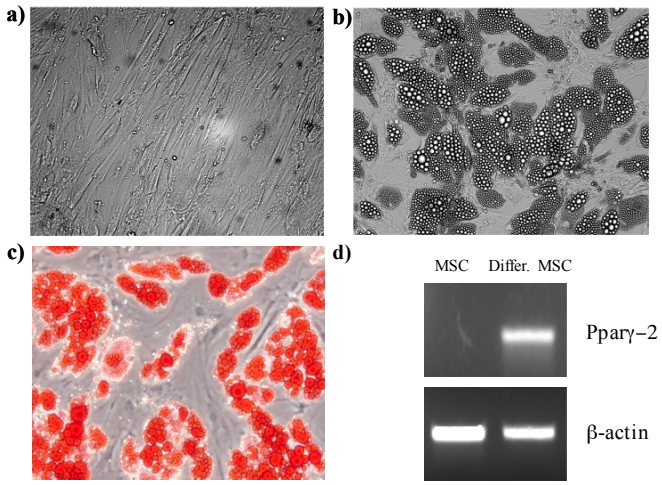


Figure 7. MSCs cultures of adipogenic differentiation. a) Undifferentiated control for adipocytes; b) adipocytes; c) Oil Red-O colouration for adipocytes; d) electrophoresis of gene Proliferator Activated Receptor γ2 (Pparγ2), activated during adipogenic differentiation, and of gene house-keeping β-actin, obtained after reverse transcription of mRNA extracted from differentiated MSC and from control MSC. MSC: mesenchymal stromal cell; Differ. MSC: differentiated mesenchymal stromal cell.

The osteoblastic differentiation is induced with a culture medium containing dexamethasone in smaller quantities than that used in the adipogenic differentiation (0.1μM), ascorbic acid and β-glycerophosphate. The cells assume a polygonal shape and collect in the extracellular space a mineralized matrix refracting light at optical microscope. The mineralized matrix is revealed by intense colors, like von Kossa or alkaline phosphatase reactions (Fig. 8).

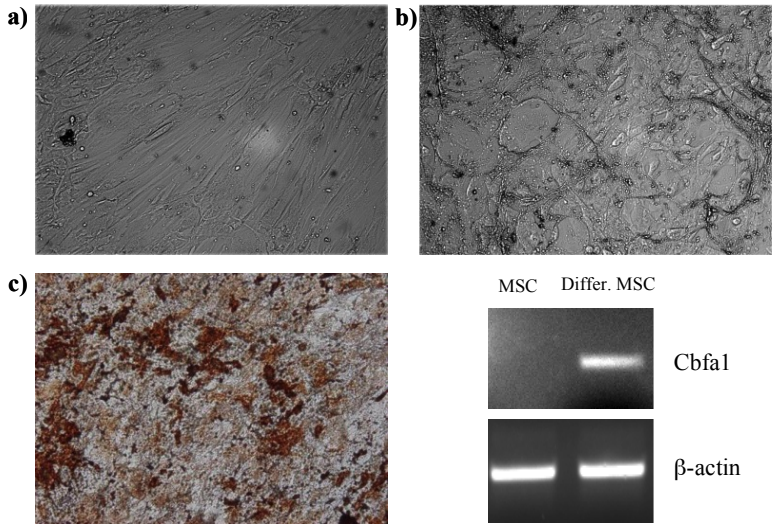


Figure 8. MSCs cultures of osteogenic differentiation. a) Undifferentiated control for osteocytes; b) osteocytes; c) von Kossa colouration for osteocytes; d) electrophoresis of gene Core-binding factor alpha 1 (Cbfa1), activated during osteogenic differentiation, and of gene house-keeping β-actin, obtained after reverse transcription of mRNA extracted from differentiated MSC and from control MSC. MSC: mesenchymal stromal cell; Differ. MSC: differentiated mesenchymal stromal cell.

3.5 Role of chemokines in CLL microenvironment

CLL B cell chemotaxis and homing to bone marrow and lymph nodes is finely regulated by the activation of chemokine receptors and adhesion molecules on the CLL cells. Chemokines, as a family of about 50 peptides, were first proposed as "chemotactic cytokines" in 1992, with a role in regulating homing of immune cells, leukocyte trafficking and maturation (57). CLL B cell migration towards stromal cells is promoted by the chemokine CXCL12 (previously called stromal cell derived factor 1 or SDF-1) (58), secreted both by MSCs and NLCs (Fig. 9). The CXCR4 chemokine receptor (CD184) is expressed on the surface of peripheral CLL B cells in response to CXCL12 gradients; CXCR4 is regulated by receptor endocytosis after CXCL12 binding leading to low CXCR4 surface levels in lymph nodes and bone marrow were CXCL12 levels are high (59). CXCR4 is close in proximity to CD38 on the surface of leukemic B cells and CD38 synergizes with

CXCR4 signaling to promote homing and chemotaxis to CXCL12 (60). CXCR4 stimulation contributes to prolong CLL B cell survival *in vitro* and lead to the activation of ERK and to the 3 activation of signal transducer and activator of transcription 3 (STAT-3) signaling (61). On the other side, activated CLL B cells secrete high levels of the chemokines C-C motif ligand 3 (CCL3) and CLL4 following BCR stimulation or in co-culture with NLCs (62). CCL3 and CCL4 presumably recruit T cells and monocyte or macrophages to tissue sites for interactions with CLL cells. High plasma levels of CCL3 and CCL4 seems to be associated with an inferior clinical outcome in CLL patients. CCL21 (also known as secondary lymphoid tissue chemokine) and CCL19, produced by the stromal cells of extrafollicular zones of lymph nodes, are also potent B-cell chemoattractant binding the receptor CCR7, expressed on lymphocytes. CCR7 expression is higher in patients with lymphoadenopathy (63).

4. Treatment

The right choice of the treatment for a CLL patient is based on the evaluation of the clinical stage of the disease, on the fitness of the patient, on the genetic risk of the leukemia and on the treatment line (first line *vs* second line and response *vs* non response of the last treatment) (64).

4.1 First line treatment

In patients with active symptomatic disease or advanced stage, treatment should be started. Patients in good physical condition ("go go") as defined by a normal creatinine clearance and a low score at the "cumulative illness rating scale" (CIRS) (65) should receive combination therapies such as Fludarabine and Cyclophosphamide (FLU/Cy) or FCR (Fludarabine, Cyclophosphamide and Rituximab). Fludarabine is a purine analogue exstensively studied in CLL; Fludarabine monotherapy produces superior overall response rates (ORR) compared with other treatment regimens containing alkylating agents or corticosteroids (66). Fludarabine induced more remissions than other conventional therapies like CHOP (Cyclophosphamide, Doxorubicine, Vincristine, Prednisone), CAP (Cyclophosphamide, Doxorubicine, Prednisone) or Chlorambucil, but did not improve overall survival when used as single agent (67), (68). A major advance was achieved using the combination of different treatment modalities and, particularly, the most studied

association chemotherapy in CLL is Fludarabine plus Cyclophosphamide. Different randomized trials showed that FLU/Cy combination improves the complete response (CR), OR and progression free survival (PFS) as compared to Fludarabine monotherapy. The FLU/Cy treatment resulted in a higher frequency of neutropenias but the rate of severe infections was not significantly increased (69), (70). In an open-label randomized trial by the German Chronic Lymphocytic Leukemia Study Group (GCLLSG), the activity and safety of FLU/Cy regimen (409 patients) was compared to that of FLU/Cy plus Rituximab, an anti-CD20 antibody (71). FCR was more effective than FLU/Cy in CR rate (44% *vs* 22%), PFS (at 3 years 65% *vs* 45%, fig. 9) and OS (at 3 years 87% *vs* 83%). During FCR treatment, it was observed a higher rate of grade 3-4 neutropenia but not a significant increase in the infection rate and no differences in the health related quality of life. The presence of *del(17p)* was the strongest unfavourable prognostic variable for PFS and OS. Patients not eligible for FCR regimen, should be treated with a less toxic therapy, in order to control CLL and to prolong OS, mantaining a good quality of life. The association of Bendamustine and Rituximab (BR) should be considered as front-line therapy in fit but elderly patients. The BR regimen was investigated as first line therapy in 117 patients and resulted in an overall response rate of 88% with a CR rate of 23.1% and a partial response rate of 64.9% and less neutropenias than FCR regimen (72).

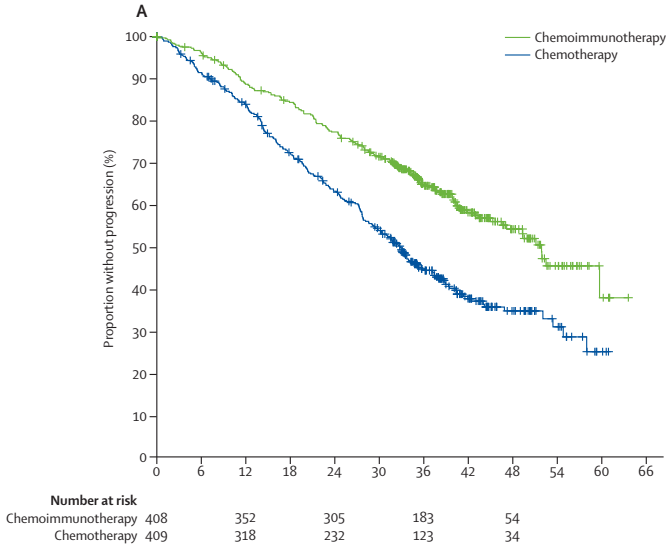


Figure 9. Progression-free survival in all patients treated with chemoimmunotherapy (Fludarabine, Cyclophosphamide and Rituximab) and chemotherapy (Fludarabine and Cyclophosphamide) (18).

Patients with impaired physical condition ("slow go") may treated either with Chlorambucil alone or in combination with an anti-CD20 antibody (Rituximab,

Ofatumumab or Obinutuzumab), or with a dose-reduced Fludarabine containing regimen with a CD20 antibody. In these patients the main goal of the treatment is to control symptoms; nevertheless, the combination of Chlorambucil plus an anti-CD20 antibody prolongs the PFS when compared with monotherapy (73). Patients with active disease and the presence of adverse biological prognostic factors, like *del(17p)* or *TP53* mutations, as first line treatment, should receive FCR or an alemtuzumab-containing therapy. The response is generally poor and short-lived; all yield response rates above 50%. In these patients, if possible, it should be considered the treatment with novel inhibitors (Ibrutinib, Idelalisib), the enrollment in clinical trials with new drugs or an allogeneic stem cell transplantation (HSCT) (6) (74). Manteinance therapy in CLL cannot be generally recommended, except for clinical trials.

4.2 Second-line treatment

As for the first-line treatment, therapy in relapsed patients should be starting only in the presence of active and symptomatic disease. First-line treatment is repeated if the relapsed or the progression occurs more than 24-36 months after the first therapy. If relapse occurs within 24-36 months after first-line therapy, or the disease is refractory to any previous treatment, the choice of therapy should be changed with other chemoimmunotherapy combinations. Whenever possible, refractory patients should be treated with newly approved drugs, like kinase inhibitors (Idelalisib or Ibrutinib), Lenalidomide or enrolled in clinical trials with other new compounds (75), (76). In fit patient with early relapse from chemoimmunotherapy and/or *del(17p)* or *TP53* mutation should be considered also an HSCT (77). Less fit patients could be treated with BCR inhibitors or, if it is not present *del(17p)* or TP53 mutation, BR and FCR-Lite regimens (Fig. 6).

4.3 New drugs for CLL treatment

In recent years, the CLL treatment has undergone a major innovation due to the increasing number of very hopeful new drugs. The two main classes of novel agents are the BCR signaling inhibitors (78) and the Bcl-2 antagonist (79) (Fig. 10); these drugs are orally

bioavailable and demonstrated a good efficacy and tolerability compared with conventional chemoimmunotherapy. Furthermore, these drugs showed activity also in CLL patients with *del(17p)* or TP53 mutation.

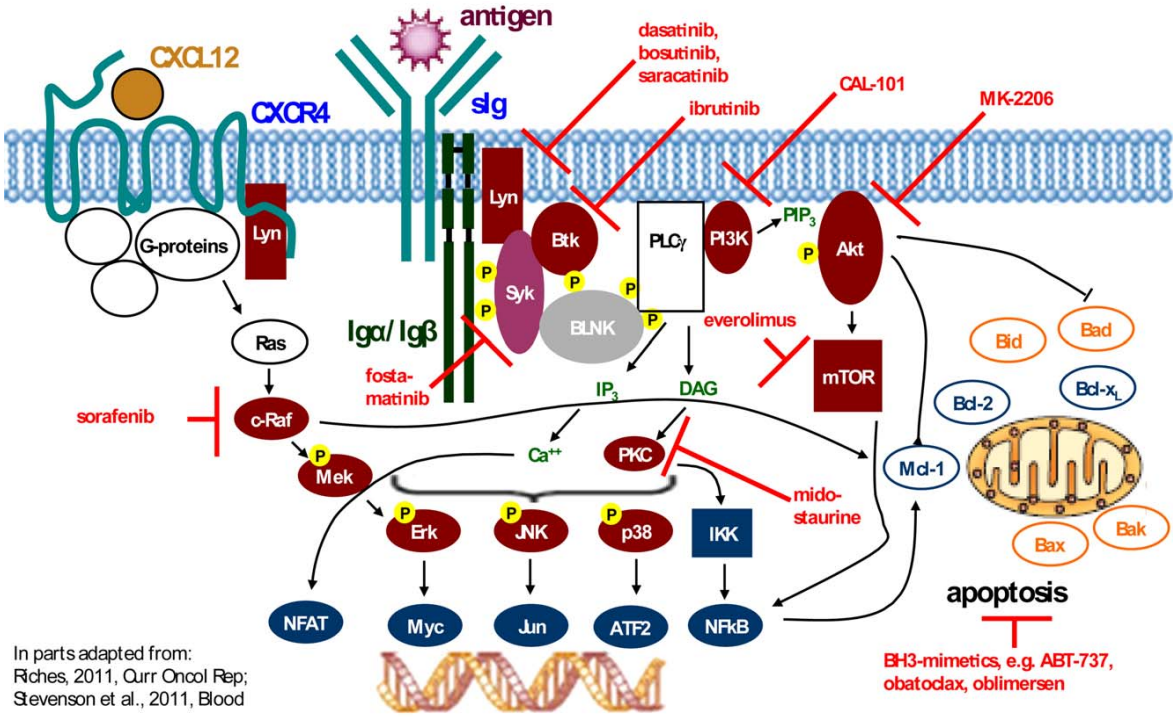


Figure 10. Survival signaling in CLL. Targeting of the BCR as a therapeutic strategy in CLL. Red symbols and letters indicate new drugs (6).

4.3.1 BCR signaling inhibitors

BCR signaling plays an important role in the development, survival, proliferation, functional differentiation and migration of B cells. PI3Kδ, Syk and Btk are essential for BCR signal transduction and their knockout in mouse models leads to impaired antigen-driven maturation and expansion of B cells. In the last decade, an increasing number of B cell malignancies (lymphomas and CLL) were ascribed on BCR signaling for proliferation and survival (80). PI3Ks are divided into three classes and class I is composed by four different isoforms (α, β, γ and δ). PI3Ks regulate several cell functions, including survival, migration, chemokine receptor and integrin signaling activation. The predominant form expressed by hematopoietic cell is PI3Kδ, harvesting a critical role in B cell homeostasis and function. Syk activates signaling pathways downstream of the BCR, chemokine and integrin receptor, suggesting the involvment in tissue homing and retention of activated B cells (81). Btk is a non-receptor tyrosine kinase of the Tec family, rapidly activated by Lyn

and Syk kinases, resulting in the activation of NF-κB signaling, B cell proliferation and differentiation. It is essential for activation of several constitutively active pathways of CLL cell survival, including Akt and the ERK (82). Btk is also involved in regulation of migration and adhesion via CXCR4/CXCR5 and integrin signaling (83). Given the importance of BCR receptor signaling in CLL, an attractive strategy is to target inhibition of this kinase.

Idelalisib: Idelalisib (CAL-101) is an oral PI3Kδ selective inhibitor promoting CLL cells apoptosis in a time and dose-dependent mode without inducing apoptosis in normal T cells or natural killer cells. Idelalisib reduces survival signals derived from the BCR, inhibits CLL cell chemotaxis and migration and also down-regulates secretion of chemokines in stromal co-cultures and after BCR triggering (84). Idelalisib was approved by the FDA in 2014 for the treatment of relapsed/refractory CLL patients in combination with rituximab. Idelalisib has been tested as single agent or in combination with other conventional drugs and demonstrated excellent efficacy and tolerability. Idelalisib pivotal phase III study was conducted in heavily pretreated CLL patients; 220 patients were randomly assigned to receive rituximab/placebo or rituximab/idelalisib. The study resulted in an 85% reduction of the risk progression with a 12-months PFS of 66% in Idelalisib/Rituximab arm, compared with 13% for the placebo/Rituximab arm. PFS and response rates were not affected by adverse prognostic factors, including del *del(17p)/*TP53mut, ZAP70 expression or IGVH mutational status (85).

Fostamatinib: Fostamatinib disodium is the first clinically available oral Syk-inhibitor; it induces apoptosis disrupting B cell receptor signaling. Fostamatinib induced partial responses in replapsed CLL patients in phase I/II study (86).

Ibrutinib: Ibrutinib, previously called PCI-32765, is the first BCR inhibitor approved for treatment in CLL; it is a small orally active molecule that inhibits Btk, that plays a role in the signal transduction of the BCR, inducing apoptosis in CLL cells. Ibrutinib covalent binds to the cysteine-481 amino acid of the Btk enzyme; preclinical studies showed that it inhibits numerous processes, including ERK signaling, NF-κB DNA binding, cytosine-phosphate-guanine (CpG)-mediated CLL-cell proliferation and tumor cell migration. Differently from most regimens used for CLL, Ibrutinib does not have toxic effects on normal T cells (87), (88). Previous studies fixed a dose of 420mg/day; patients with relapsed/refractory disease experienced a 90% ORR, with durable responses and an

estimated PFS of 69% at 30 months. The response was independent of clinical stage, previous therapies and adverse genomic risk factors, including 17p deletion (Fig. 11) (89). In the study of previously untreated patients over 65 years, the ORR was 84%, with an estimated PFS of 96% at 30 months. Treatment with Ibrutinib was generally well tolerated, with the most common adverse events being transient diarrhea (58%), fatigue (28%), infections (32%) and bleeding (61%). Ibrutinib usually causes a transient increase in blood lymphocyte levels, concurrent with a riduction in lymph node and speen size. This effect has been seen also with other agents targeting BCR and it is not consider a sign of progressive disease; continuing Ibrutinib therapy, this asymptomatic lymphocytosis is led to resolution (90). The optimal duration of therapy has not been yet determined, it could be resonable to continue treatment but we do not know yet the long-term effects and the resistance mechanisms need to be better clarified.

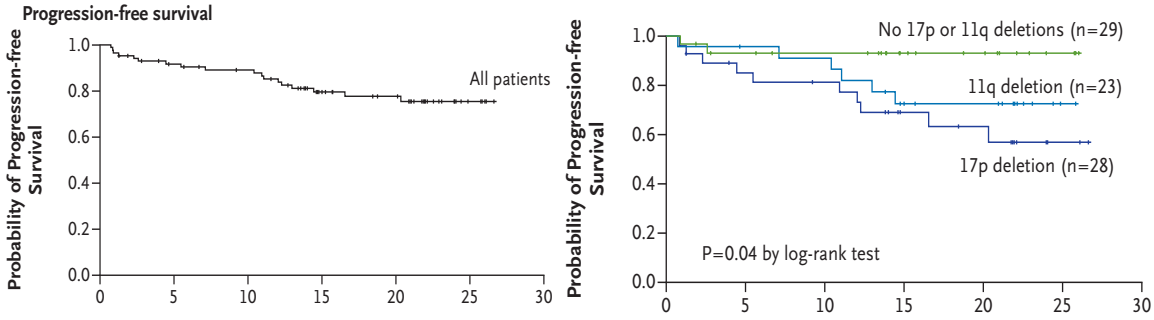


Figure 11. Kaplan-Meier curves for PFS for 85 relapsed/refractory CLL patients, treated with Ibrutinib (left panel) and PFS according to cytogenetic status, with respect to the *del(17p)* and *del(11q)* deletions (right panel) (89).

Dasatinib: Dasatinib is a Src- and Abl- kinase inhibitor that induces apoptosis in CLL cells. Dasatinib seems also to increase the apoptotic effects of Fludarabine, Chlorambucil, dexamethasone and other agents (91). In previous studies, Dasatinib showed efficacy in reduction of nodular tumor masses but it is less effective on peripheral blood lymphocytes.

4.3.2 Bcl-2 inhibitors

Proteins in the B cell lymphoma 2 (Bcl-2) family work as key regulators of the cell apoptosis. Bcl-2 family consists in pro-apoptotic and pro-survival proteins; cancer cells are able to evade apoptosis shifting the balance of Bcl-2 proteins toward the prosurvival effect (92).

ABT199: ABT199 is a highly potent, orally bioavailable and Bcl-2 selective inhibitor that blocks the growth of Bcl-2 dependent tumors *in vivo*. ABT199 showed a promising effect for the treatment of Bcl-2 dependent hematological cancers, including CLL (93).

AIM OF THE STUDY

CLL is a malignancy characterized by the accumulation of monoclonal mature B lymphocytes which are dependent on interactions with the tissue microenvironment for their survival and proliferation. Consequently, exploring and targeting the CLL microenvironment is of progressive increasing interest, also for the development of novel therapeutic strategies and for a better understanding of drug-resistance mechanisms. We focused our attention on MSCs derived from the bone marrow, which provide attachment site and growth factors for normal hematopoiesis and represent a critical component of the CLL microenvironment.

The aim of this PhD project was to further investigate the role of MSCs in CLL pathogenesis and in the treatment of the disease. In particular, we planned to establish an *in vitro* culture system based on MSCs, shaping *in vivo* conditions for determining CLL niche interactions involved in neoplastic cell survival. Employing an *in vitro* system that closely approximate the *in vivo* bone marrow conditions, we tried to better explain the connection between CLL B cells and the microenvironment with the ultimate goal to identify patients who may benefit from compounds targeting CLL microenvironment. Finally, we studied the role of MSCs in the apoptosis of CLL cells during a conventional therapy in general practice, such the combination of Fludarabine and Cyclophosphamide, performing both *in vivo* and *in vitro* experiments. Furthermore, we analyzed the complexity of the cross-talk between malignant B cells and MSCs in the presence of a Btk inhibitor, Ibrutinib, actually considered a really promising and efficient drug in the treatment of CLL. In particular, we planned to examine CLL cells survival and ability of migration and adhesion after the Ibrutinib pre-treatment, in the presence of MSCs.

MATHERIAL AND METHODS

1. Patients

MSCs were isolated from 46 CLL patients (31 males and 15 females, median age 60 years) referred to the Hematology and Clinical Immunology division of Padua University Hospital. The main disease characteristics of the patients are shown in table III, which reports the RAI classification, the percentage of bone marrow infiltration and the quote of CD19/5+ lymphocytes. In particular, 19 patients were classified in stage RAI 0, 15 in stage 1, 10 in stage 2 and 2 patients in stage 3. None of the patients were in stage 4. The mean bone marrow infiltration was 47.4±22.6 and the amount of the CLL B cells in peripheral blood ranged from 1 to 98%.

Malignant B lymphocytes utilized in this study were obtained from 45 CLL patients (27 males, 18 females, median age 65 years) which clinical characteristics are listed in table IV. According to RAI system, 4 patients were classified in stage 4 and 7 in stage 2. Overall 31 patients (69%) were in stage RAI 0 and 1; staging of 3 patients was not determined (nd). Cytogenetic most common lesions were identified using interphase FISH: deletion in the long arm of chromosome 13 (13q-), trisomy of chromosome 12 (12+), deletion in the long arm of chromosome 11 (11q-) and in the short arm of chromosome 17 (17p-) or a normal karyotype (N). Based on the number of SHM of the Ig VH genes, the cases were divided into two categories: 21 unmutated (UM) and 20 mutated (M). Conventionally, mutated status was defined as having a frequency of mutations greater than 2% from germline VH. In 4 patients SHM status was not available (na) or not determined. Using cytofluorimetric analysis, we detected also ZAP70 and CD38 expression. The cut-off used to determine ZAP70 was 20% and 30% for CD38.

Table III. Clinical characteristics of CLL patients studied for MSCs isolation.

MSCs #	RAI stage	Age	Sex	BM infiltration (% Ly)	CD19/5(%)
MSC#1	0	75	M	34	71
MSC#2	1	65	M	70	92
MSC#3	1	78	M	87	92
MSC#4	0	66	F	70	83
MSC#5	1	70	M	90	98
MSC#6	3	75	M	40	68
MSC#7	0	50	F	18	1
MSC#8	0	49	M	56	72
MSC#9	2	60	F	5	2
MSC#10	2	72	M	75	46
MSC#11	1	68	M	13	11
MSC#12	2	61	M	26	54
MSC#13	2	55	M	6	8
MSC#14	0	51	M	54	86
MSC#15	2	47	M	78	86
MSC#16	2	59	F	19	34
MSC#17	0	66	M	40	75
MSC#18	0	54	M	42	68
MSC#19	0	51	M	27	40
MSC#20	1	48	M	47	83
MSC#21	1	55	M	52	70
MSC#22	1	55	M	18	60
MSC#23	0	49	M	62	81
MSC#24	1	69	M	56	50
MSC#25	0	51	M	37	54
MSC#26	1	66	M	64	70
MSC#27	1	60	F	43	56
MSC#28	0	60	M	37	54
MSC#29	1	70	F	70	50
MSC#30	1	63	M	43	70
MSC#31	0	63	M	55	67
MSC#32	1	60	M	40	43
MSC#33	0	56	F	52	45
MSC#34	0	71	F	25	22
MSC#35	0	49	F	62	69
MSC#36	0	60	M	75	85
MSC#37	0	58	F	60	43
MSC#38	3	59	F	50	77
MSC#39	2	72	F	75	85
MSC#40	2	55	M	17	35
MSC#41	2	63	M	90	93
MSC#42	0	56	F	32	50
MSC#43	1	57	M	34	91
MSC#44	0	52	M	21	44
MSC#45	2	67	F	73	81
MSC#46	1	56	F	40	48

Table IV. Clinical characteristics of CLL patients

CLL#	Age	Sex	RAI stage	Cytogenetic	IgVH mutational status (M/UM)	ZAP70	CD38
#01	64	M	0	na	M	pos	neg
#02	74	M	1	na	M	neg	neg
#03	56	F	2	13q-	M	nd	nd
#04	71	M	4	13q-	UM	pos	pos
#05	54	F	1	na	M	nd	pos
#06	68	F	1	11q- 12+	UM	pos	nd
#07	79	F	1	13q-	UM	neg	pos
#08	89	M	2	na	M	nd	nd
#09	67	F	2	17p- 13q-	nd	nd	nd
#10	77	M	0	13q-	M	neg	neg
#11	69	M	0	N	UM	pos	neg
#12	59	M	0	13q-	UM	neg	neg
#13	45	M	1	13q-	M	pos	neg
#14	52	F	2	na	UM	pos	pos
#15	63	F	0	13q-	M	pos	neg
#16	81	M	1	12+	UM	pos	pos
#17	92	F	1	17p-	UM	neg	nd
#18	45	M	4	17p-	UM	neg	pos
#19	63	F	1	13q-	M	nd	nd
#20	51	M	0	17p- 13q-	M	neg	neg
#21	79	M	1	13q-	M	neg	neg
#22	49	M	1	11q- 13q- 12+	UM	pos	pos
#23	55	F	1	na	M	na	na
#24	61	M	1	11q- 12q-	UM	pos	neg
#25	59	M	0	N	UM	pos	neg
#26	66	M	1	N	M	pos	neg
#27	57	F	0	13q-	UM	pos	neg
#28	71	F	1	13q-	M	neg	pos
#29	50	M	2	13q-	UM	pos	pos
#30	67	M	0	13q-	M	pos	pos
#31	81	M	4	na	M	pos	neg
#32	53	M	4	11q-	UM	pos	neg
#33	85	M	0	N	UM	pos	neg
#34	62	M	0	N	M	pos	nd
#35	54	M	1	13q-	M	pos	neg
#36	61	F	0	11q- 13q- 12+	UM	pos	pos
#37	54	F	1	11q- 12+	nd	pos	pos
#38	67	F	0	nd	UM	nd	nd
#39	80	M	1	13q-	M	pos	neg
#40	69	F	2	11q- 13q-	UM	neg	pos
#41	66	F	1	13q-	M	neg	neg
#42	78	F	nd	13q-	M	neg	neg
#43	71	M	nd	12+	na	neg	neg
#44	53	M	2	11q-	UM	neg	pos
#45	49	M	nd	na	na	na	pos

2. Isolation of MSCs from CLL bone marrow

Bone marrow blood samples were obtained from 46 CLL patients, after written informed consent. We collected 1-2 ml of blood from each bone marrow aspirate; mononuclear cells, including MSCs, were obtained proceeding with a layering on Ficoll/Hypaque (F/H, GE Healthcare; Fairfield, Connecticut). This technique is based on the difference of density of mononuclear cells (lymphocytes and monocytes) with respect to the other blood elements. Mononuclear cells, with lower density, focus on the layer of F/H while the red blood cells and granulocytes are collected on the bottom of the tube. We proceeded with a centrifugation at 900g for 20 min at 20°C, without brake. The ring of mononuclear cells at F/H interface was aspirated and washed twice with saline by centrifugation at 400g for 10 minutes at 20°C (Fig. 12). The pellet was resuspended in an appropriate amount of saline and the cells were counted in a Burker chamber.

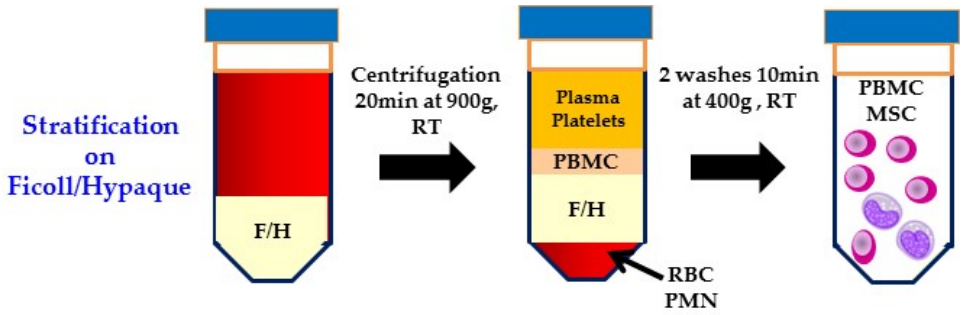


Figure 12. Isolation of MSCs from bone marrow by stratification on Ficoll/Hipaque. By centrifugation on F/H, MSCs were isolated from bone marrow of CLL patients. Mononuclear cells (PBMC) and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cell (RBC) and polymorphonuclear neutrophil (PMN) have a higher density than the F/H and collect on the bottom of the tube. RT: room temperature.

3. *Ex vivo* expansion of MSCs

Mononuclear cells were cultured in 7 ml of Dulbecco's Modified Eagle Medium (DMEM, Invitrogen; Paisely, UK) with 10% of FBS, 1% of PenStrep (Penicilline 5000U/ml, Streptomicine 5,000µg/ml, Invitrogen) and 10µg/ml of Ciprofloxacine (Ciproxin, Bayer; Leverkusen, Germany) at 37°C in humidified atmosphere containing 5% CO_2. Nonadherent cells were removed carefully after 7 days and fresh medium was replaced. When primary cultures became almost confluent, the culture was washed with 2 ml of Phosphate Buffer Saline (PBS)1X and treated with 1 ml of 0.25% trypsin (Invitrogen)

in order to enlarge the culture. Detached cells were transfered in a medium with 10% of FBS and resuspended in DMEM to be seeded in plates with a 10.000 cells/cm^2 density.

4. Immunophenotyping of MSCs

The immunophenotypic analysis was performed through flow cytometry, a technique allowing a multiparametric evaluation of antigenic characteristic of the single cells by the analysis of visible and fluorescent light emission. The immunophenotyping helps to identify surface and intracellular Ag using mAb conjugated with fluorochromes. The presence of a certain Ag is used as an indicator of cell lineage and maturation level. We used flow cytometer FACS Calibur (Becton Dickinson; Milan, Italy) and data obtained were processed using the program Cell Quest. For each analysis 20.000 events were acquired. The fluorochromes used 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 allophycocyanin (APC) that emits a fluorescence signal at 690nm when excited by a laser beam with λ of 635nm. MSCs were removed from plates using 500μl of Accutase solution (Sigma; Saint Louis, USA) and resuspended in 100μl of PBS1X. MSCs were stained with 5μl of mAb for surface expression of CD14 (FITC, Becton Dickinson; Franklin Lakes, USA), CD31 (FITC, Becton Dickinson), CD34 (PE, Becton Dickinson), CD90 (Abd Serotech, Oxford, UK), CD73 (PE, Becton Dickinson) and with 2.5μl of mAb for expression of CD45 (FITC, Caltag-Invitrogen, Paisely, UK) and CD105 (PE, Caltag-Invitrogen).

5. Isolation of CLL B cells from peripheral blood samples

B lymphocytes were isolated from peripheral blood of CLL patients. From a sample of heparinized venous blood, mononuclear cells were obtained proceeding with a layering on F/H. In patients with a quote of B lymphocytes less than 90% of peripheral blood mononuclear cells (PBMCs) isolated we used the RosetteSep kit (StemCell Technologies; Vancouver, Canada). The kit consists 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 immunorosette. CD19+ B lymphocytes are 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 PBS1X + 2% FBS, gently agitated, and then layered over F/H. We proceeded with a centrifugation at 900g for 30 minutes at RT, 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 (Fig. 13). Finally, cells resuspended in PBS1X were counted in a Burker chamber. We used the RosetteSep kit also to isolate B lymphocytes from *buffy coat*, used as normal controls.

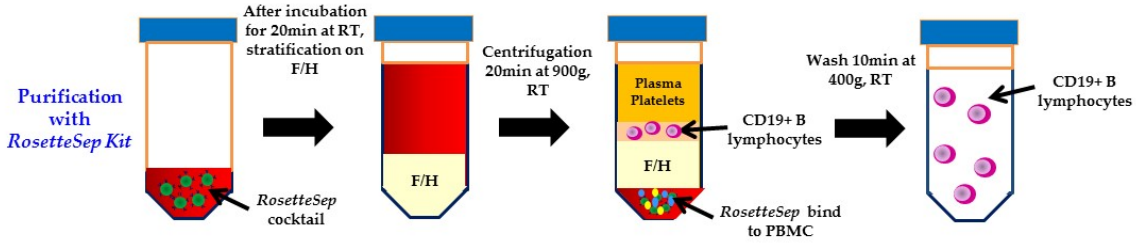


Figure 13. Purification with RosetteSep kit. CD19+ B lymphocytes were isolated from whole blood of healthy donors by negative selection. 10ml of venous whole blood were incubated for 20 min at RT with 500µl of RosetteSep. Afterwards, through stratification on F/H, we get the CD19+ B cells, which are 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.

6. Culture conditions

For co-culture experiments, purified leukemic and normal B cells ($2x10^6$/ml) and MSCs ($1x10^5$/well seeded into 12 well plates) were cultured in complete RPMI-1640 (Sigma-Aldrich; Milan, Italy) at 37°C in a humidified atmosphere containing 5% CO_2. B cells were added to MSCs layer at 20:1 ratio in complete RPMI-1640 medium. For spontaneous apoptosis assays, leukemic cells from the suspension were collected at 3, 5 and 7 days. Therefore, we performed co-cultures of malignant B lymphocytes in direct contact with MSCs and in presence of MSCs separated by a 0.4 µm porous polycarbonate filter (transwell) that allows the exchange of soluble factors.

For analysis of *in vitro* drug-induced cell death, CLL B cells were treated with 20µM Fludarabine (FLU, TEVA; Milan, Italy) and 5mM Cyclophosphamide (Cy, Baxter; Rome, Italy) for 3 and 12 hours; cells were then washed and plated alone and with MSCs for 3, 5 and 7 days. In other experiments, FLU and Cy were added directly in CLL B cells

and CLL B cells-MSCs co-cultures. At the same time, we cultured MSCs with leukemic B cells collected from 10 CLL patients undergoing the first cycle of FLU/Cy chemotherapy *in vivo*, before starting drug infusion and at the third day of treatment. Viability status was examined by staining with Annexin V at 3, 5 and 7 days. At least, we tested the effect of 5μM Ibrutinib, a Btk inhibitor, on CLL B cells isolated from 12 patients cultured alone and with MSCs for 3, 5 and 7 days.

7. Analysis by flow cytometry

Apoptosis of different cell samples (CLL B cells and normal B lymphocytes) was assessed using the Annexin V Apoptosis Detection Kit (Immunostep; Salamanca, Spain). During the early stages of apoptosis, the plasma membrane undergoes profound changes indicating the status of apoptotic cell to macrophages, which ensure its elimination. Phosphatidylserine (PS), a negatively charged aminofosfolipide expressed normally only on the inner side of the plasma membrane, become exposed on the outer surface. Annexin V is a protein that, in the presence of high concentrations of Ca^{2+}, recognizes and selectively binds PS, helping to identify apoptotic cells by phospholipid exposure on their surface.

Aliquots of $5x10^5$ cells were harvested, washed, and incubated for 10 min in the dark and at RT with anti-CD19 APC (Invitrogen). Then cells were washed and 100μl of binding buffer, a Ca^{2+}-rich solution that optimizes the binding of Annexin V to the PS, plus 5μl of Annexin V-FITC were added for further 10 minutes in the dark and at RT. After the incubation, 100μl of binding buffer were added and cells were analyzed by flow cytometer FACS Canto (Becton Dickinson). For each sample 20.000 events were collected using FACS Diva software.

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 1.4 grams 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 was prepared following Laemmli method96. The electrophoretic plate consisted of two types of gel:
- Stacking gel at pH 6.8, which concentrates the protein samples so that they are all aligned at the start of electrophoresis;
- Running gel 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 high sensitive technique, able to detect very small quantities of proteins. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane through an electric field, obtained 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 (buffer A). After the transfer, the membrane was left overnight in the saturation buffer consisting of 50mM Tris-HCl, pH 7.5, 150mM NaCl and 5% bovine serum albumin (BSA) (buffer B), for non specific sites saturation. Follows the incubation for 2 hours and 30 minutes at room temperature of the primary Abs, diluted in: 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA (buffer C).

For our study we used the following antibodies: anti- Poli-ADP-Ribose Polymerase (PARP), anti-Btk Tyr223, anti-Btk (Cell Signaling Technology Inc; Danvers, Massachusetts, USA) and anti-β-actin (Sigma-Aldrich).

Three washes of 10 min, each at RT were subsequently performed, using buffer C supplemented with 0.1% Tween. 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 was conjugated with horseradish peroxidase (Amersham International

Biotechnology; Buckingamshire, UK) and diluted in buffer C. After three additional washes, the membrane was subjected to the detection antibody with the enhanced ChemiLuminescence system (ECL) (Pierce; Rockford, Illinois): the membrane was incubated for 1 minute with 1ml of luminol and 1 ml of H_2O_2, which in contact with the peroxidase and the Ag-Ab complex, give rise to an oxidation reaction with light emission. The membrane was relevated into ImageQuant LAS 500 (Amersham). The densitometric analysis was performed using the Image J program.

10. Chemotaxis assay

MSCs were cultured for 48h in complete RPMI-1640 medium (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2 to obtain the MSC-conditioned medium (MSC-CM). The migration of pre-treated CLL B cells in response to MSC-CM was evaluated using 12-well Corning chemotaxis chamber (Corning Life Sciences; Acton, MA). $2.5x10^6$ cells were incubated in 0.5ml RPMI medium with and without Ibrutinib for 1h at 37°C. Then, cells were transferred into the top chambers of transwell culture insert with a pore size of 3µm. Filters were then placed onto wells containing complete RPMI medium or MSC-CM, and CLL B cells were allowed to migrate for 3h at 37°C. Migrated cells in the lower chamber were then collected and counted on a FACSCanto for 60 seconds in triplicates.

11. Evaluation of CLL B cell adhesion to MSC layer

CLL B cells were suspended to a concentration of $2x10^6$ cells/ml with or without 5µM Ibrutinib and incubated for 1h at 37°C in 5% CO_2 in complete RPMI medium. After incubation, CLL cells were added to the MSC layers and the plates were incubated at 37°C in 5% CO_2 overnight. Cells that had not adhered into the stromal cell layer were removed by vigorously washing 3 times with RPMI 1640 medium. The complete removal and the integrity of the stromal cell layer containing adherent B cells were assessed by phase-contrast microscopy Olympus IX-81 and documented photographically. The layer of cells was detached by incubation with Accutase (Sigma-Aldrich); cells were stained with anti-CD19 APC to exclude MSCs and counted by flow cytometry.

12. Flow cytometry analysis

CLL B cells were cultured with and without MSCs and treated with 5μM Ibrutinib. $5x10^5$ cells for each assay were collected after 48h, leaving intact the adherent layer, and stained with anti-CD49d PE (BD Biosciences), anti-CCR7 FITC (R&D Systems Inc., Minneapolis, MN, USA), anti-CXCR4 PE (R&D Systems Inc.), and anti-CD19 APC (BD Biosciences) monoclonal antibodies. Cells were washed with PBS1X and incubated with saturating concentrations of the appropriate antibodies for 15 minutes at room temperature. 20,000 total events were acquired using FACSCanto (Becton Dickinson) and the data were analysed by FACSDiva 7 software. Samples were gated on intact cells by forward light scatter (FSC) *vs* right-angle light scatter (SSC). For analysis, it was used a second gating step on CD19+ cells. Here, we used a difference between the Mean Fluorescence Intensity (MFI) of fully-stained samples and the Fluorescence Minus One controls.

13. Statistical analysis

Statistical analysis of apoptosis in patients analyzed was performed using Student's *t* test, paired Student's *t* test. Data were expressed as mean±standard deviation (SD) and were considered statistically significant when p values were <0.05.

RESULTS

1. MSCs isolation from bone marrow of CLL patients

MSCs were isolated from the bone marrow of 46 CLL patients afferent to the Hematology and Clinical Immunology division of Padua University Hospital. For each bone marrow sample, the mononuclear cells were seeded in plates with a 10,000 cells/cm^2 density. After 48 hours, we were able to observe the adhesion of some round cells in the plate; after 7 days, with the first change of the medium culture, the cells were extended, in a similar fibroblastic morphology. After 14 days, the cells created highly proliferating aggregates (Fig. 14A), with the achievement of the mutual confluence in 30/40 days (Fig. 14B). All the cultures maintained a homogeneous morphology and an undifferentiated status in the different steps.

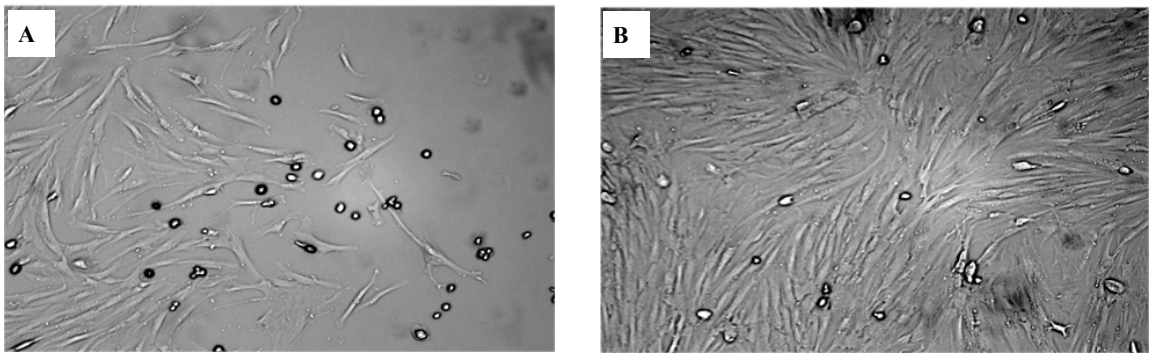


Figure 14. MSCs cultures after 14 (A) and 30 (B) days. Mononuclear cells obtained from bone marrow samples were seeded in plates with DMEM. After 7 days, some cells with a similar fibroblastic morphology were adherent to the plate; the suspended cell were removed with the first change of medium. The first proliferation aggregates were observed after 14 days and the culture obtained the confluence in 30-40 days (10X enlargement with Olympus BX60 microscope).

2. MSCs immunophenotypic characterization

The characterization of the expanded cell population was defined through flow cytometry analysis between third and fourth subculture; the immunophenotypic characterization was based on the expression of CD105, CD73 and CD90 and the negativity of CD14, CD34, CD45 and CD31. We were able to identify in our cell cultures the peculiar phenotype of MSCs, confirming that our cell population was homogeneous and not of hematopoietic derivation (Fig. 15).

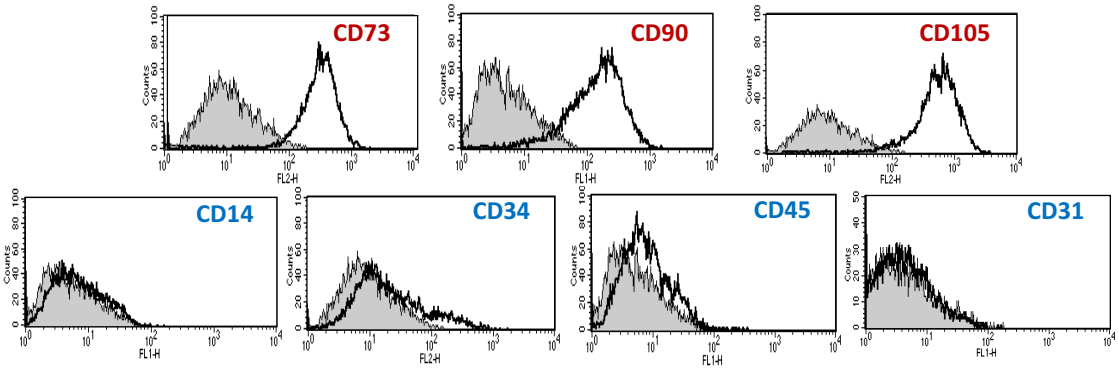


Figure 15. Immunophenotypic characterization of MSCs. Representative case of MSC flow cytometry evaluation. The grey area represents the negative control; the white area indicates the positive expression of the marker.

3. MSCs from CLL patients support *in vitro* neoplastic B cells survival

We tested the effect of MSCs on the survival of leukemic B cells isolated from 30 CLL patients, and normal B cells obtained from 11 healthy controls; B lymphocytes were incubated in direct contact with a confluent layer of MSCs (20:1 ratio). By Annexin V staining, we assessed B cell viability at 3, 5 and 7 days. We found that leukemic B cells underwent apoptosis when cultured in medium alone, but their survival was rescued when cultured with MSCs; CLL B cell viability after 7 days was 13.3%±13.2% in medium alone *vs* 59.2%±17.1% in co-culture with MSCs ($p<0.0001$) (Fig. 16A). Normal B cell viability after 7 days was 6.7%±4.3% in medium alone *vs* 34.9%±15.7% when co-cultured with MSCs ($p<0.001$) (Fig. 16B), indicating that MSCs display a major protective effect on neoplastic B cells. Therefore, we confirmed that MSCs are able to support CLL B cell survival providing an *in vitro* culture system that closely approximate CLL microenvironment *in vivo*.

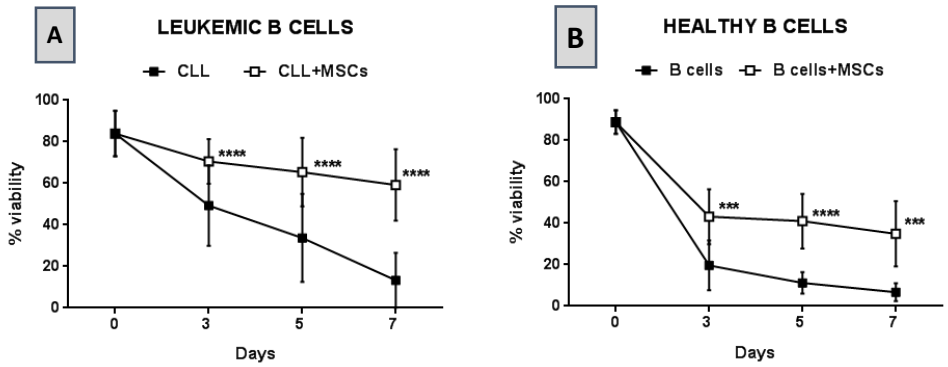


Figure 16. CLL and normal B cell viability cultured alone and with MSCs. Cell apoptosis was assessed by Annexin V test. The graph shows the percentage of cell viability after 3, 5 and 7 days, data are expressed as mean ±SD; ***$p<0.001$, ****$p<0.0001$, Student's *t*-Test.

4. Detection of PARP 89kDa fragment reveals two subsets of CLL clones

On the basis of the high variability observed in CLL B cell viability co-cultured with MSCs (Fig. 17), we evaluated the cleavage pattern of PARP protein after 7 days of culture in a cohort of 27 untreated CLL patients.

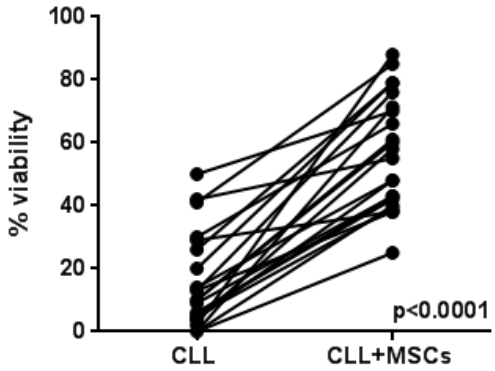


Figure 17. Percentage of CLL B cells viability before and after co-culture with MSCs. CLL B cells apoptosis was assessed by Annexin V test. The graph shows the percentage of cell viability of CLL B cells from different patients at 7 days; p<0.0001, Student's *t*-Test.

We identified two different patterns of CLL clones with different sensitivity to MSCs pro-survival signals: 1) one observed in a group of 15 patients in which CLL clones displayed the cleaved PARP, but the full length protein in presence of MSCs (classified as "dependent" from microenvironmental stimuli) (Fig. 18, left panel); 2) a second pattern observed in a group of 12 CLL patients in which CLL clones displayed the full length PARP with and without the presence of MSCs (classified as "independent" from microenvironmental stimuli) (Fig. 18, right panel).

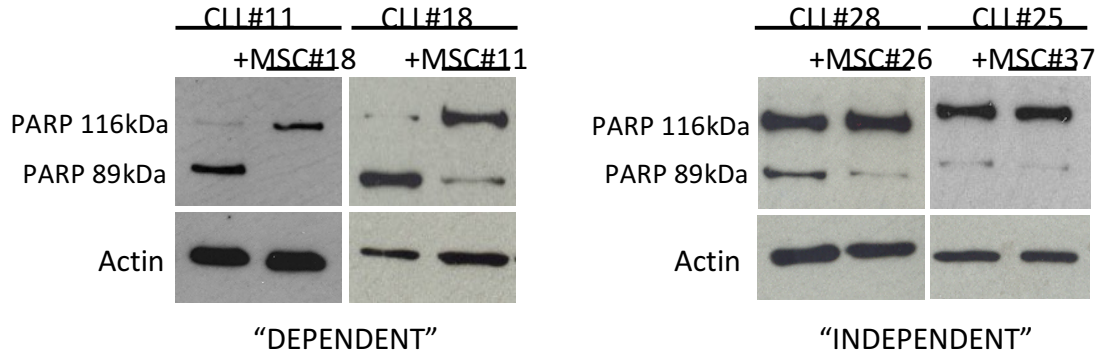
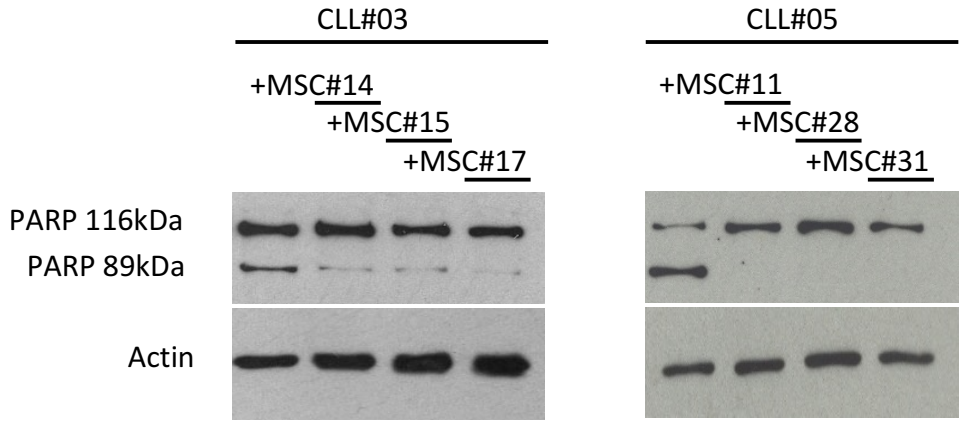


Figure 18. Analysis of PARP protein expression in CLL B cells cultured with and without MSCs. CLL B cells were cultured alone and in the presence of MSCs for 7 days. The total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected sequentially with anti-PARP Ab, to highlight the apoptosis, and anti-β-actin Ab. The figures show two representative cases of CLL B cells "dependent" from microenvironmental stimuli (left panel) and two cases "independent" from MSCs signals (right panel).

We also analyzed the detection of PARP 89kDa fragment from co-cultures of the same CLL clone with different MSCs. Interestingly, the results indicated that CLL clone response to microenvironmental signals did not change in co-culture with MSC obtained from different patients (Fig. 19A). Conversely, co-culturing the same MSC line with different CLL clones, we demonstrated different patterns in the cleavage pattern of PARP (Fig. 19B), indicating that the heterogeneity of leukemic clones is likely to be related to intrinsic features of neoplastic B cells rather than to external stimuli.

A)



B)

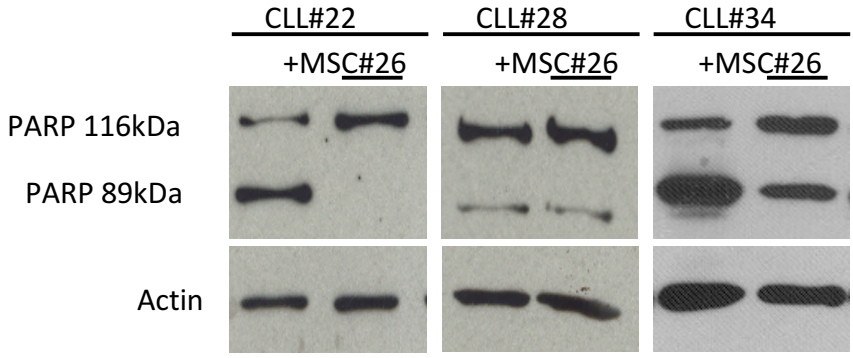


Figure 19. Analysis of PARP pattern in CLL B cells cultured in absence and in presence of MSCs for 7 days. The total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected sequentially with anti-PARP Ab, to highlight the apoptosis, and anti-β-actin Ab. **A)** The figures show two representative cases of a single neoplastic clone (CLL#) cultured with MSCs (MSC#) obtained from different CLL patients; in all the conditions, leukemic B cells displayed a similar response also in the presence of diverse MSCs types. **B)** Representative cases of CLL B cells (CLL#) from distinct patients exposed to the effect of the same MSC line (MSC#); in this condition, CLL clones displayed different responses in the presence of the same MSCs.

5. Cell-cell contact and soluble factors are involved in the cross-talk between MSCs and CLL B cells

We cultured malignant B lymphocytes from 12 CLL patients alone, in direct contact to MSCs and in presence of MSCs separated by a 0.4µm porous polycarbonate filter (transwell) that allows the exchange of soluble factors. We demonstrated that CLL cell survival was increased in presence of MSCs in both conditions; in fact, after 7 days, CLL B cell viability was 13.8%±13.4% when cultured alone *vs* 61.5%± 18.3% in direct contact with MSCs ($p<0.0001$) and 47.5%±7.8% in presence of MSCs separated by transwell ($p<0.0001$). These results indicated that both cell-cell contact and soluble molecules are actors in the relationship between malignant B cells and the MSCs (Fig. 20).

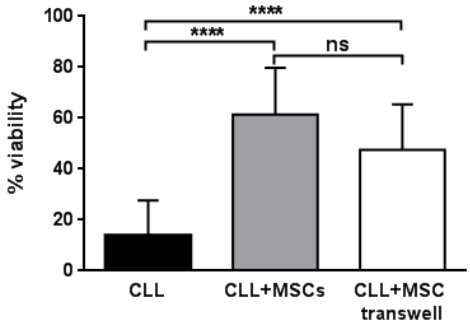


Figure 20. Histograms of cell viability percentage of CLL B cells cultured alone, with MSCs and co-cultured with MSCs in the presence of transwell. CLL B cells apoptosis was assessed by Annexin V test. Histograms show the mean±SD of the percentage; ****$p<0.0001$, Student's *t*-Test.

Considering that MSCs release a high amount of chemoattractants, we also tested the ability of MSCs to influence cell movement. We evaluated the migration of CLL B cells obtained from 21 patients in response to MSC-conditioned medium (MSC-CM) using a 12-well Corning chemotaxis chamber. We found that the number of moved cells was 2.809±1.318 in presence of MSC-CM *vs* 651±543 in medium alone ($p<0.0001$) (Fig. 21).

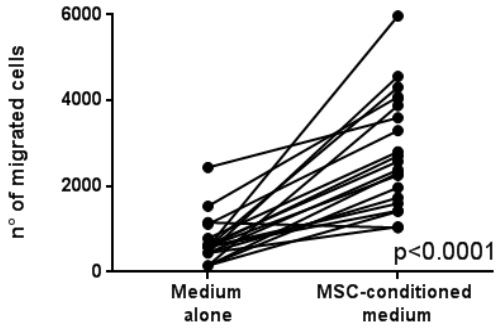


Figure 21. CLL B cell migration toward MSC-CM. Migration tests were performed using a 12-well Corning chemotaxis chamber; migrated cells in the lower chamber were collected and counted on a FACSCanto; $p<0.0001$, Student's *t*-Test.

6. MSCs protect CLL B cells during Fludarabine and Cyclophosphamide treatment, *in vitro* and *in vivo*

We cultured MSCs with leukemic B cells collected from 10 CLL patients undergoing the first cycle of chemotherapy, before starting drug infusion and at the third day of treatment. Patients were treated according FLU/Cy regimen (3 days of therapy for each cycle). Using Annexin V staining, we tested the effect of MSCs on the survival of CLL B cells after 3, 5 and 7 days of co-cultures. After 7 days, the viability of CLL cells isolated before the chemotherapy was 55.2%±18.2% in presence of MSCs *vs* 9.4%±13.6% in absence of MSCs ($p<0.0001$) (Fig. 22A, left panel). After 7 days, CLL cells isolated from the same patients at the end of the first cycle of FLU/Cy, showed a viability of 34.2%±21.6% with MSCs *vs* 13.1%±16.7% without MSCs ($p<0.0001$) (Fig. 22A, right panel). We observed that MSCs were able to enhance the survival of leukemic B cells and to maintain a significant pro-survival effect also during an *in vivo* cytotoxic therapy.

At the same time, we performed an *in vitro* parallel experiment assessing the MSCs protective role on CLL B cells exposed to Fludarabine and Cyclophosphamide. In particular, CLL B cells isolated from 8 patients were pre-treated with FLU/Cy for 3 and 12 hours and then cultured alone or in presence of MSCs for 3, 5 and 7 days. The cell viability at 7 days, after a 3 hours pre-treatment, was 27%±20% in culture with MSCs *vs* 3.6%±3.4% in alone culture ($p<0.05$) (Fig. 22B, left panel); the cell viability at 7 days, after a 12 hours pre-treatment, was 25.1%±20.3% in presence of MSCs *vs* 0.86%±0.69% in absence of MSCs ($p<0.05$); (Fig. 22B, right panel). CLL cell apoptosis levels were increased after a longer drug exposition, but MSCs were still able to maintain a significant pro-survival effect.

In other *in vitro* experiments, FLU and Cy were added directly, at the same doses in CLL B cells and CLL B cells co-cultured with MSCs. The cell viability at 7 days resulted in 9.8%±4.5% in presence of MSCs *vs* 1.8%±1.3% in alone cultures ($p<0.01$), (Fig. 22C). Therefore, MSCs were able to protect CLL B cells from apoptosis during FLU/Cy treatment, both *in vitro* and *in vivo*.

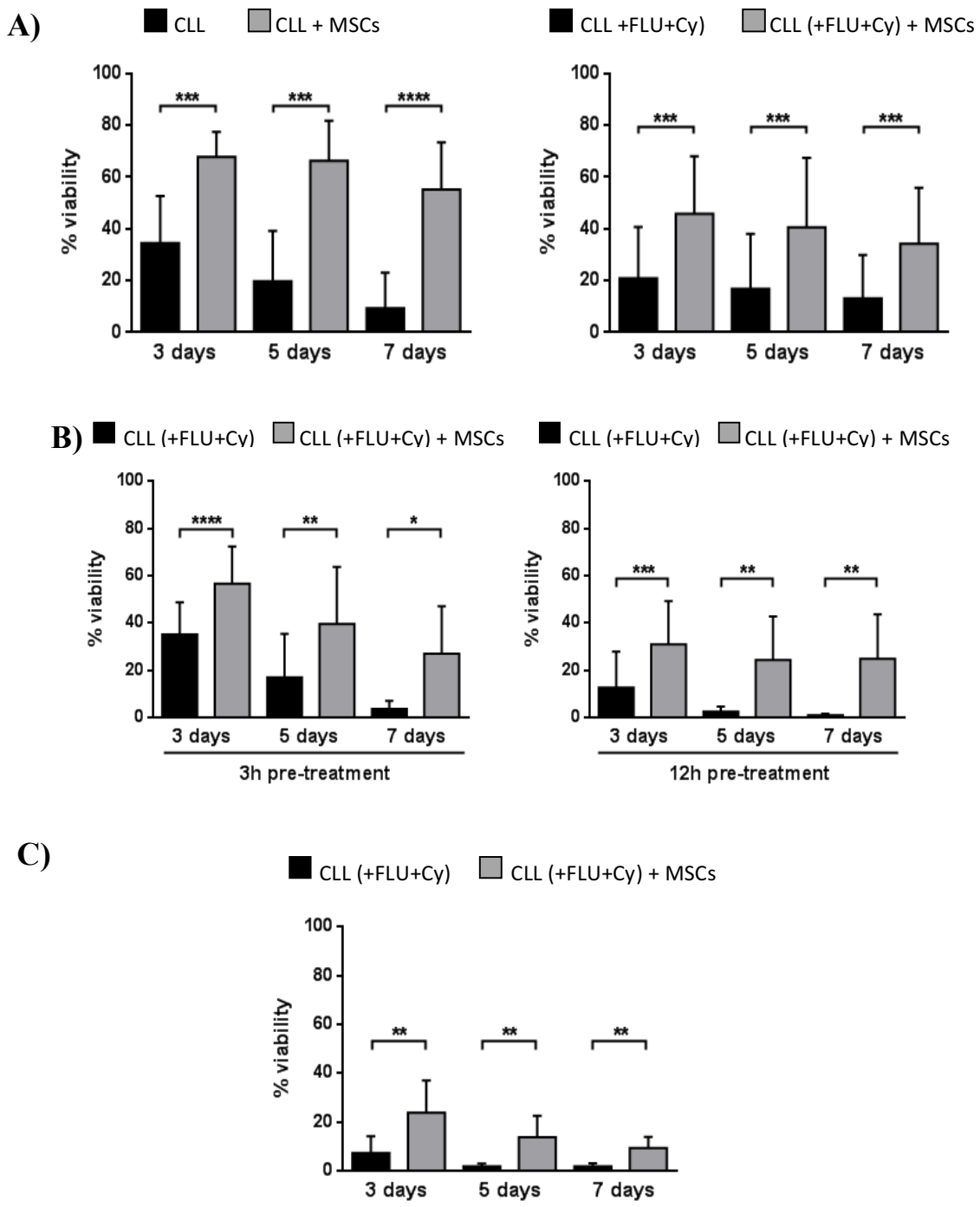


Figure 22. MSCs protect CLL B cells from drug induced apoptosis. A) Viability percentage of CLL B cells cultured alone and with MSCs. CLL B cells were collected from 10 CLL patients before starting chemotherapy (left panel) and at the end of the first cycle of FLU/Cy treatment (right panel). **B) Viability percentage of CLL B cells cultured alone and with MSCs after a 3 and a 12 hours *in vitro* pretreatment with FLU/Cy.** CLL B cells were collected from 8 CLL patients and treated with 20μM fludarabine and 5mM cyclophosphamide for 3 hours (left panel) and 12 hours (right panel); cells were then washed and plated alone and with MSCs for 3, 5 and 7 days. **C) Viability percentage of CLL B cells treated *in vitro* with FLU/Cy and cultured alone and with MSCs.** CLL B cells were collected from 8 CLL patients, directly treated with 20μM Fludarabine and 5mM Cyclophosphamide and plated alone and with MSCs for 3, 5 and 7 days. Viability was examined by staining with Annexin V. Histograms show the mean±SD of the percentage; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 Student's *t*-Test.

7. MSCs are not able to support B leukemic cell survival after treatment with Ibrutinib

Based on MSCs ability to protect CLL B cells from apoptosis induced by conventional therapy, we tested their protective role also during CLL B cell treatment with Ibrutinib, an inhibitor of Btk kinase involved in BCR signaling pathway. By Annexin V test we evaluated neoplastic B cell survival after 3, 5 and 7 days of co-culture with MSCs, finding that, after 7 days, malignant B cell viability was 85.7%±4.1% in absence of the kinase inhibitor *vs* 37.7%±14.7% with Ibrutinib ($p<0.0001$) (Fig. 23).

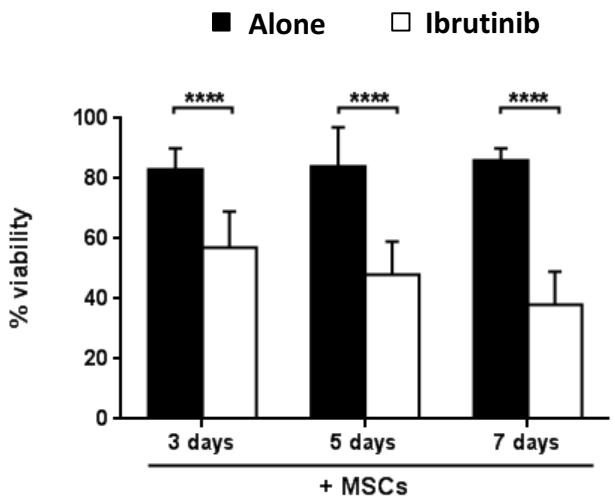


Figure 23. Viability percentage of CLL B cells cultured with MSCs after treatment with ibrutinib. CLL B cells were collected from 12 CLL patients and plated with MSCs alone and with 5μM Ibrutinib for 3, 5 and 7 days. Viability status was examined by staining with Annexin V. Histograms show the mean±SD of the percentage; ****$p<0.0001$, Student's *t*-Test.

Our experiment showed that MSCs were not able to protect CLL cells from apoptosis after treatment with the Btk inhibitor. We confirmed these data by western blotting analysis of PARP protein finding that Ibrutinib was able to induce a cleavage of PARP despite the presence of MSCs (Fig. 24). We also verified that ibrutinib treatment resulted in the inhibition of Btk phosphorylation in Tyr223 both in CLL cells alone and in leukemic B cells co-cultured with MSCs.

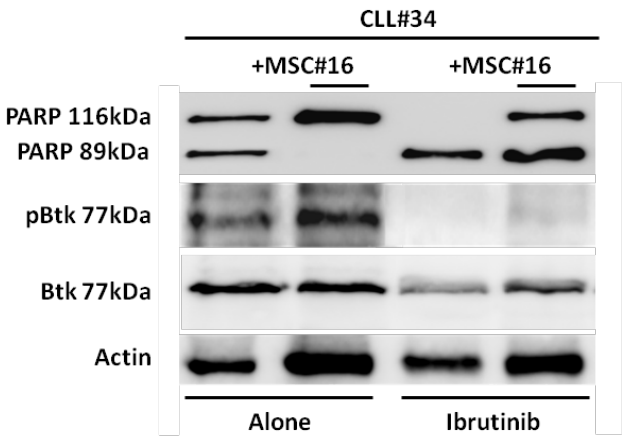


Figure 24. Ibrutinib treatment counteracts the MSC pro-survival effect. CLL B cells were cultured alone and with MSCs and treated with 5μM Ibrutinib. The total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected sequentially with anti-PARP Ab, anti-Btk Tyr223, anti-Btk and anti-β-actin Ab. The figure shows a representative cases of CLL B cell.

8. Ibrutinib treatment does not reduce CLL B cells migration to BM stroma

To better understand the effect of Ibrutinib on the cross-talk between CLL B cells and MSCs, we evaluated its role on B leukemic cell migration toward MSC-CM. CLL B cells from 10 patients were incubated with and without Ibrutinib for 1h at 37°C and then transferred into transwell systems, containing MSC-CM. We counted the cells migrated in the lower chamber after 3 hours and we found that the treatment with the Btk inhibitor did not reduce CLL B cells movement. Migrated leukemic B cells were 8,858±7,920 toward medium alone *vs* 20,391±6,184 toward MSC-CM and 18,772±10,094 toward MSC-CM, after the pre-treatment with Ibrutinib; $p<0.01$ (Fig. 25).

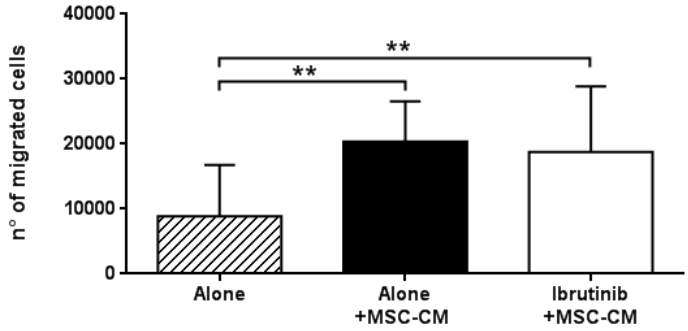
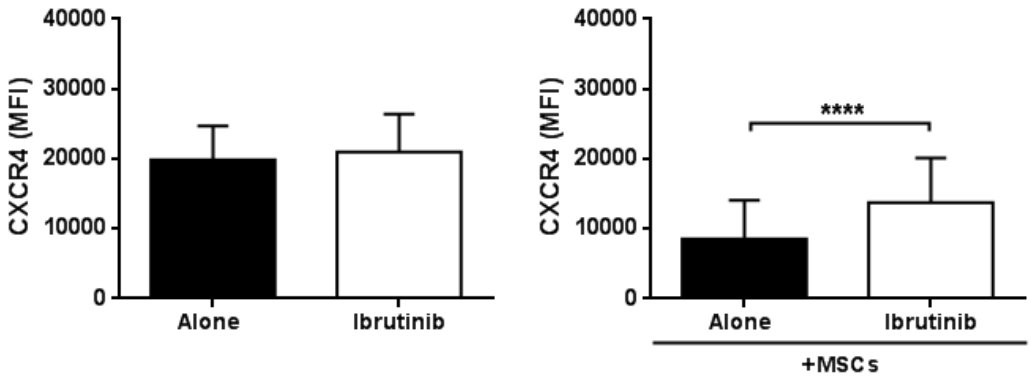


Figure 25. MSC-conditioned medium preserves CLL B cell migration after Ibrutinib treatment. Chemotaxis assay was performed using a 12-well Corning chamber; CLL B cells from 10 patients were incubated for 1h at 37°C with and without 5μM Ibrutinib and then transferred into the top chambers of the transwell system (pore size of 3μm). Cells were allowed to migrate for 3h at 37°C, then collected from the lower chamber and counted on a FACSCanto; histograms show the mean±SD, **p<0.01, Student's *t*-Test.

Considering that cell migration is mediated by the interactions between chemokines with their receptors, by flow cytometry we evaluated the expression levels of two main receptors, CXCR4 and CCR7, on the surface of CLL B cells treated with Ibrutinib and cultured alone and with MSCs. In alone cultures, CXCR4 levels were 19,880±4,858 without the Btk inhibitor treatment *vs* 24,442±6,105 with Ibrutinib exposure, and the difference was not statistically significative. CCR7 expression levels were 5,085±1,308 without the treatment *vs* 4,498±890 with Ibrutinib ($p<0.05$). In co-culture with MSCs, CXCR4 levels were 8,561±5,513 without Ibrutinib *vs* 13,776±6,374 in presence of the drug ($p<0.0001$) (Fig. 26A); CCR7 levels were 5,229±1,237 in cultures alone *vs* 4.904±1.04 with the Btk inhibitor treatment, without statistically significative difference (Fig. 26B). Data are expressed as MFI.

A)



B)

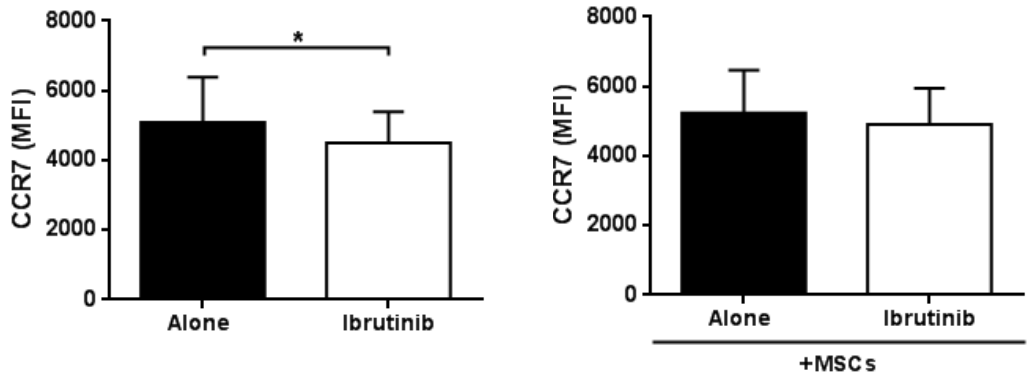


Figure 26. CLL B cells obtained from 12 patients were treated with 5μM Ibrutinib and cultured with and without MSCs. After 48h, the expression levels of CXCR4 (panel A) and CCR7 (panel B) were evaluated by flow cytometry using FACSCanto; data are expressed as Mean Fluorescence Intensity (MFI), mean±SD; *$p<0.05$, ****$p<0.0001$, Student's *t*-Test.

9. Ibrutinib treatment affects CLL B cell adhesion

Having observed that the treatment with Ibrutinib did not affect CLL B cell movement in response to MSC stimuli, we also analyzed the adhesion of leukemic B cells to MSCs after treatment with the Btk inhibitor since cell-cell contact with MSC is crucial for CLL B cell survival. We found that the percentage of leukemic B cells adherent to MSCs was significantly reduced by Ibrutinib (7.7%±3.8% alone *vs* 3.3%±2.4, $p<0.05$); (Fig. 27).

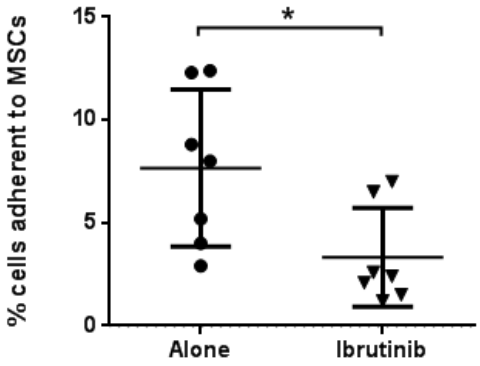


Figure 27. Percentage of cell adherent to MSCs with and without Ibrutinib. CLL B cells from 7 patients were treated with and without 5μM Ibrutinib and incubated for 1h at 37°C in 5% CO_2 in complete RPMI medium. Cells not adherent were removed by washing 3 times with RPMI medium. The layer of cells was detached by incubation with Accutase; cells were than stained with anti-CD19 APC and counted by flow cytometry; mean±SD *$p<0.05$, Student's *t*-Test.

Using phase-contrast microscopy Olympus IX-81 we documented photographically that CLL cells adhesion to MSCs was reduced after Ibrutinib treatment (Fig. 28)

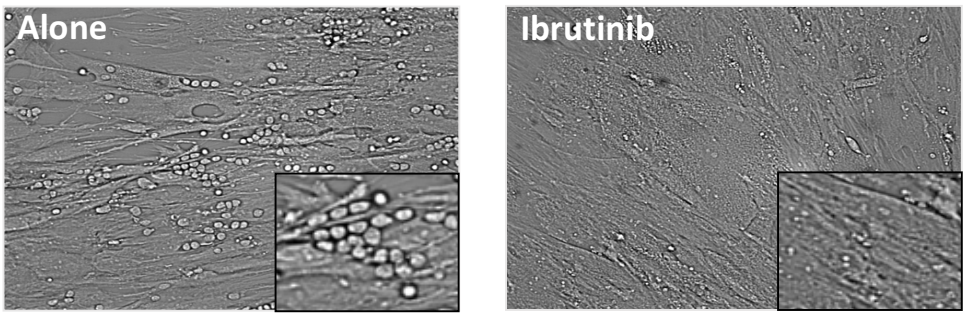


Figure 28. Microscopy analysis of CLL B cell adhesion to MSCs with and without Ibrutinib. CLL cells from 7 patients were treated with or without 5μM Ibrutinib and incubated for 1h at 37°C in 5% CO_2 in complete RPMI medium. CLL B cell adhesion to MSCs was documented photographically using phase-contrast microscopy Olympus IX-81.

Considering these results, we also examined the expression of CD49d, the α4 subunit of the α4β1 integrin heterodime involved in CLL migration and retention in lymph node and bone marrow microenvironment. The levels of CD49d on the surface of CLL B cells cultured with MSCs resulted lower after Ibrutinib treatment (MFI ratio 0.89±0.05, $p<0.01$); (Fig. 29).

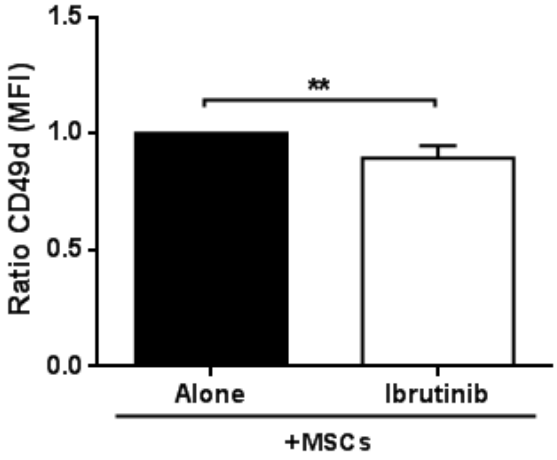


Figure 29. CD49d expression in CLL B cells. Leukemic B cells from 7 patients were co-cultured with MSCs for 24h alone and with 5μM Ibrutinib. CD49d expression was evaluated using FACSCanto. Data are expressed as MFI±SD; **p<0.01, Student's *t*-Test.

DISCUSSION

Chronic Lymphocytic Leukemia is the most common leukemia in adults and is characterized by the accumulation of clonal CD19+/CD5+/CD23+ B lymphocytes, due to uncontrolled growth and resistance to apoptosis. Intrinsic factors, such as genetic lesions, anti-apoptotic proteins, and aberrant signaling networks within leukemia cells have long been the main focus of CLL research. However, over the past years, it became increasingly clear that external signals from the microenvironment make pivotal contributions to CLL progression. In healthy subjects MSCs represent a small fraction of the stromal cell population, about 0.01-0.0001% of mononuclear cells, but are the dominant stromal cell population in CLL microenvironment. MSCs provide an attachment site and growth factors for normal haematopoiesis and, both in CLL, are thought to function in a similar fashion, creating a niche within the BM in which CLL B cells lodge and are nourished and protected from cytotoxic agents (38).

In this project, MSCs from BM of 46 CLL patients were co-cultured with leukemic B cells in order to mimic the neoplastic *in vivo* microenvironment. Our results demonstrated that malignant B cells are susceptible to the antiapoptotic effect of MSCs, favouring neoplastic B cell survival *in vitro* for at least 7 days. This evidence is relevant considering that CLL B cells spontaneously undergo apoptosis once they are removed from the *in vivo* microenvironment and placed in suspension cultures without the supportive stromal cells. This effect was less relevant in normal B lymphocytes, suggesting the presence of a peculiar receptor structure which allows the neoplastic B cell to respond to pro-survival stimuli produced from the elements of the microenvironment in which it is localized. In fact, leukemic B cell expresses specific chemokine and cytokine receptors and responds selectively to soluble factors produced by the compounds of the microenvironment into the active sites of disease (39). Despite CLL B cells are characterized by a typical phenotype and cytogenetic abnormalities, the disease displays heterogeneous clinical courses, suggesting that each malignant clone could present intrinsic features that affect the interactions with the microenvironment. CLL natural history, including response to treatment and drug resistance, is determined both by causal and influential genes and by the relationships that malignant B cells entertain with their supportive microenvironments

(38). Therefore, studying the role of microenvironment we may provide essential strategies to treat and eradicate the disease.

We observed a high variability in the viability of CLL B cells cultured with MSCs and, to better understand these data, we analysed the cleavage pattern of the PARP protein, indicator of caspase activity. The detection of PARP 89kDa fragment in CLL patients, after 7 days of co-culture with MSCs, revealed two subsets of CLL clones with different sensitivity to MSCs pro-survival stimuli. The first group was classified as "dependent" from microenvironmental signals, when CLL B cells underwent spontaneous apoptosis in medium alone, but were rescued by the presence of MSCs. The second group, classified as "independent" from microenvironmental stimuli, identified CLL clones whose viability was high both when cultured in medium alone and in the presence of MSCs, indicating that these leukemic cells were able to survive independently from pro-survival signals coming from stromal cells. Further experiments allowed us to establish that the different behaviour displayed from neoplastic clones is likely to be related to intrinsic features of neoplastic B cells rather than to the variety of the microenvironment. These observations could be relevant to identify patients more responsive to druggable targets on marrow microenvironment and also to find putative new strategies for CLL therapy. In fact, it is likely that a clone "dependent" from microenvironmental stimuli will be more easily affected by a treatment that aims to interactions with it; on the other hand, a clone "independent" from the signals of the microenvironment probably will not show a particular sensitivity to therapies which target the CLL-microenvironment cross-talk.

The increase of malignant B cell viability in the presence of MSCs might be mediated by soluble factors and/or cell-cell contact. Our data indicated that both cell-cell contact and soluble factors are relevant for the survival of malignant clone. We observed an increased migration of neoplastic B cells in the presence of the conditioned medium of MSCs, demonstrating that MSCs from CLL patients are able to produce factors that promote the recruitment of CLL B cell toward a favourable niche for the maintenance of the leukemic clone. Considering that MSCs constitutively secrete a high amount of chemokines, which organize CLL cell trafficking and homing (41), the identification of humoral and cellular factors responsible for the pro-survival effect *in vivo* could be useful also to find new therapeutical targets for CLL and possible promising approaches to manage the disease.

CLL cell interactions with MSCs play a critical role in the disease pathogenesis, in particular in CLL survival, homing, proliferation but also in treatment failure. The cross-

talk between MSCs and the leukemic B cell seems to play a key role in inhibiting apoptosis induced by drugs. We demonstrated that the presence of MSCs protects leukemic B cells from apoptosis after the *in vivo* exposure to Fludarabine and Cyclophosphamide, a chemotherapy regimen considered as the gold standard for first-line treatment in CLL patients (5). Furthermore, our results confirm the protective role of MSCs also performing *in vivo* experiments, with leukemic B cells collected from patients after the first cycle of FLU/Cy therapy. These data could partially explain the reason why in some CLL patients, maybe with a neoplastic clone more "dependent" on microenvironment stimuli, it is more difficult to obtain a complete or long-lasting response. These results, in fact, point out the protective role of microenvironment not only toward malignant B cells *in vitro* but also on apoptosis induced by the *in vivo* administration of chemotherapy, maybe causing therapy refractoriness or an unsatisfactory response to treatment.

The management of CLL is undergoing profound changes; several new drugs have been approved for CLL treatment and many others are in advanced clinical development in the pipeline to be approved for this disease (6). The CLL microenvironment has gained extensive attention during the last few years, thanks to the progressive understanding of the mechanisms involved in CLL B cell proliferation and survival and to the introduction of several novel small molecule inhibitors, which target the CLL-microenvironment cross-talk (81). Different drugs, used in CLL treatment, have been demonstrated to induce *in vitro* apoptosis of CLL B cells, whereas their effect is reduced when administered *in vivo*, probably due to the presence of pro-survival signals coming from the surrounding environment. Therefore, it is suitable to test the effect of new therapeutic agents in the presence of microenvironmental compounds (such as MSCs) which might interfere with the biological effects of the drugs, allowing the identification of subgroups of patients who may benefit from treatments targeting the cross-talk with supportive cells at the sites of the disease.

Considering the relevance of the BCR in the support of the neoplastic B cell, we evaluated the role of MSCs during the treatment with Ibrutinib, a novel Btk inhibitor who targets proteins essential in signaling transduction mediated by the BCR. We tested the effect of the Btk inhibitor, known to reduce CLL B cell migration and to induce *in vitro* apoptosis (94), in our co-culture system. We found that Ibrutinib is able to induce leukemic cell apoptosis independently from MSCs presence, confirming its potential high efficacy in the treatment of CLL. By contrast, the Btk inhibitor did not affect B cell migration toward

a MSC-conditioned medium, rich in cytokines and chemokines, suggesting that malignant B cells do not lose their ability to move toward a protective niche in presence of Ibrutinib.

To better understand these data, we studied some of the receptors involved in cell migration. CLL B cells express on their surface high levels of chemokine receptors; in particular CXCR4 and CCR7 are involved in BM and lymphoid tissues homing of the neoplastic clone (95). We observed that malignant B cells, co-cultured with MSCs and treated with Ibrutinib, displayed an increase of CXCR4 expression levels. The CXCR4 chemokine receptor is expressed at high levels on the surface of CLL B cells and mediated chemotaxis, migration across vascular endothelium and actin polymerization in response to CXCL12 gradients, produced by MSCs (49). CXCL12 also induces a direct pro-survival effect on leukemic B cells. CXCR4 surface expression is regulated by its ligand CXCL12 via receptor endocytosis. In the presence of MSCs, in CLL B cells not treated with Ibrutinib, CXCR4 is internalized into the cytoplasm, indicating the successful receptor-ligand interaction. Although the exact mechanism of CXCR4 up-regulation is still not completely clear, our results indicated that the use of kinase inhibitor could induce modifications in chemokine receptors, in particular interfering with CXCR4-CXCL12 axis. Instead the expression of CCR7, the receptor for both CCL21 and CCL19, correlates with clinical lymphadenopathy, a clinical feature of more advanced disease (96) (97). Although little is known about the mechanisms determining lymph node enlargement in CLL, some studies suggested that CCR7 engagement by CCL21 and/or CCL19 stimulates CLL B cell entry into lymph nodes (96). Considering that Ibrutinib *in vivo* treatment rapidly decrease lymph node size, we evaluated CCR7 expression levels after the *in vitro* treatment with the Btk inhibitor. Our results demonstrated lower CCR7 levels after Ibrutinib exposure, suggesting a possible interaction with this receptor. Nevertheless, in co-culture with MSCs CCR7 was not affected by the Btk inhibitor treatment, suggesting that, probably, the *in vivo* effect of Ibrutinib on CCR7 is more complex and need further studies to be clarified.

Since the direct cell-cell contact with stromal cells is crucial for CLL B cell survival, we tested the effect of Ibrutinib on neoplastic B cell adhesion to a MSCs layer. We found a significant reduction of CLL B cells adhesion through a down-modulation of CD49d expression, an integrin involved in anchoring cells to tissues via extracellular matrix. Our results suggest that Ibrutinib is not only able to reduce CLL B cell viability, but that it could also interfere with cell-cell contact, fundamental in cell survival but also as a first step in cell migration. Therefore, we could suppose that, in presence of Ibrutinib, MSCs are not able to further protect CLL B cells that could remain on peripheral blood and potentially

more exposed to pro-apoptotic stimuli. Ibrutinib is a potent drug and highly selective with great results in CLL therapy; however, it does not completely eliminate the malignant clone in CLL and results mainly in partial responses. A better understanding on the complexity of the cross-talk between CLL cells and their microenvironment could help to define also mechanisms of resistance to Ibrutinib and treatment failure, as well to plan randomized clinical trials comparing new inhibitors of BCR signaling and their combinations with standard chemo-immunotherapy.

In conclusion, this project demonstrated that 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 favourable signals from MSCs (98). Our findings on the role of MSCs and their effect on neoplastic B lymphocytes during chemotherapy exposure open a new scenario to better identify the most effective drugs or drug combinations. Furthermore, we showed that Ibrutinib treatment, inactivating enzymes in the BCR signaling pathway, which are aberrantly activated in CLL, is able not only to inhibit CLL B cell proliferation and survival but also to interact with the cross-talk with the stromal cells, a necessary condition for the eradication of the disease. Additional *in vivo* and *in vitro* studies will accelerate the development of these new concepts and will help to define the best drug combinations.

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