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Dissecting the crosstalk between Chronic Lymphocytic Leukemia cells and microenvironment: role of GS-1101 a specific PI3K delta inhibitor

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INTRODUCTION

I. BIOLOGY OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

Chronic Lymphocytic Leukemia (CLL), the most frequent form of leukemia in Western countries, is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in the blood, bone marrow, lymph nodes and spleen (1). The monoclonal population of B cells in CLL expresses CD19, CD5, and CD23 and has reduced levels of membrane IgM, IgD, and CD79b, a phenotype of mature, activated B lymphocytes (2). Historically, CLL was viewed as a tumor caused by the accumulation of long-lived but mainly resting lymphocytes with low proliferation rate (3). This model has been challenged in fact CLL contains a small fraction of actively proliferating cells, with approximately 2% of cells newly generated each day. Lymphocytes in the peripheral blood (PB) are predominantly resting, but specific structures known as proliferation centers, localized in the lymph nodes and in the bone marrow, replenish CLL cell population (4). Clinically, CLL is characterized by a marked degree of heterogeneity, ranging from patients that harbor highly stable disease with a nearly normal life expectancy to patients with rapidly progressive disease who are destined to succumb in a short time. The variable course of CLL is driven, at least in part, by heterogeneity in the disease biology (5).

1. The origins and properties of CLL cells

Although the precise cell of origin of CLL is still under investigation, immunogenetic studies and gene expression profiling (GEP) analyses have provided important information regarding the putative CLL progenitor (6). The B cell receptor is a multimeric complex formed by immunoglobulins noncovalently associated Igα/Igβ (CD79a/CD79b) (7). CD79a and CD79b have an extracellular part and an intracellular part that contains sequences termed immunoreceptor tyrosine-based activation motifs (ITAM) that can be phosphorylated by intracellular tyrosine kinases and bind other kinases (7). The heterodimer CD79a/CD70b is the signal transduction unit of the BCR whose activation triggers a series of downstream reactions. Depending on the B-cell developmental stage and Ag structure, these reactions may lead to events as different as activation, anergy and apoptosis. Molecular

investigations of the B cell receptor (BCR) indicate that 60%-65% of CLL cells carry immunoglobulin heavy-chain variable (IGHV) genes with evidence of somatic hypermutation in their variable regions, a process that occurs in the germinal center (GC) and may modify BCR affinity for antigens (8). Conversely, 35%-40% of CLLs are devoid of IGHV somatic mutations. The association with IGHV gene mutations suggests that a fraction of cases (CLL with mutated IGHV genes also called M-CLL) derived from germinal center experienced B cells and the other cases (CLL with unmutated IGHV genes also called UM-CLL) derive from B cells that have undergone differentiation in germinal center independent fashion (6). The B-cell receptors of CLL cells from various patients are often structurally very similar, suggesting that the antigens these receptors bind are similar and relevant to the pathogenesis of CLL (9). In some cases, there are common features in the portion of the antigen-binding site contributed by the H chain (V_H, D, and J_H genes). In these cases, each V_H gene exibits special patterns of mutations and preferential combinations with particular D or J_H segments, which generate distinct features in the antigen-binding pocket. These V_HDJ_H rearrangements and characteristic of antigen-binding pockets differ from the diversity found in B cells from normal persons (10). In other groups of cases, the structural similarity of the receptors involves the entire antigen-binding site, coded by both the H and L chains (VH, D, JH, and VL and JL genes). In these instances, the receptors from various patients are very similar or virtually identical. These findings are very striking since, given the number of possible combinations of V-gene segments that can encode antigen-binding domains, one would not expect to find 2 cases of CLL with such structurally similar B-cell receptors in more than 1 million cases (11). These cases suggest that a limited set of promoting antigens induces division of the leukemic cells, but are unknown. It is possible that latent virus or commensal bacteria activate particular B-cell clones through BCR or environmental antigens or autoantigens could induce clonal expansion. CLL cells frequently have polyreactive receptors, which bind multiple antigens, including autoantigens (12), allowing stimulation by both autoantigens and microbial antigens. For antigenic stimulation to underlie clonal expansion, the BCR must propagate a signal to the cell nucleus. Cross-linking BCR with antibodies to IgM in vitro mimics

the engagement of antigens with B-cell receptors and transmits signals to the cell nucleus in 50 percent of cases of CLL in particular with unmutated CLL (13). Once signal transduction is initiated by the BCR, B lymphocytes progress into the cell cycle or die, cross-linking of surface IgM in CLL cells can transduce a signal that can cause or prevent apoptosis (14) (Figure 1). The distinction between M-CLL ans UM-CLL is clinically relevant because germline IGHV genes predict poor outcome. The worse prognosis predicted by unmutated IGHV genes is probably due to the enrichment of some genetic lesions that confer higher aggressiveness among UM-CLL and to the predisposition of theses cases to undergo clonal evolution (15).

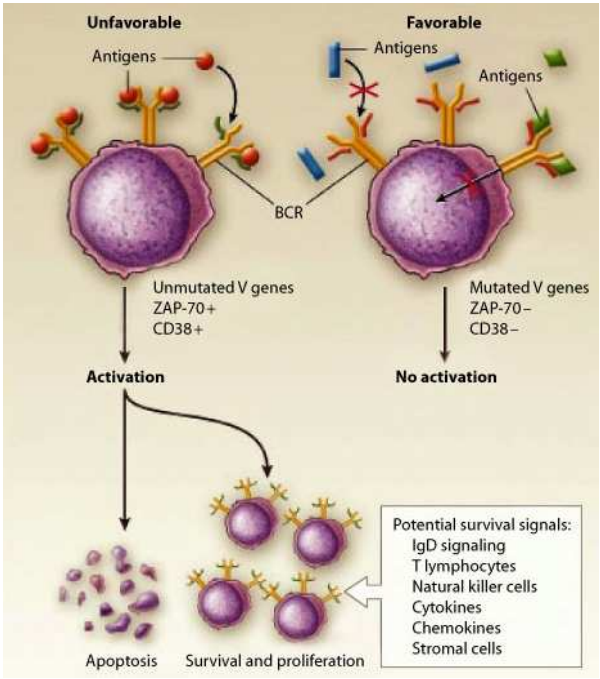


Figure 1. The promoting role of antigen stimulation and accessory signals in CLL. B cells in patients with CLL who have unfavourable prognostic markers (left side of figure) are stimulated by the binding of self-antigens to the B-cell receptor. The dynamic balance of negative and positive signals derived by the B-cell receptor and the survival signals determine whether the leukemic cell proliferates or dies by apoptosis. B cells from patients with CLL who have favourable prognostic markers (right side of figure) are less capable to triggering apoptosis, survival or proliferation owing to inability to bind antigen. This lack of receptor stimulation may be a factor associated with a less aggressive for of disease (2).

2. Genetic lesions in CLL

CLL genome is largely devoid of the chromosomal translocation and aberrant somatic hypermutations. These observations are consistent with a post-GC or GC-independent derivation of CLL (16). Karyotipic investigations revealed the association of CLL with chromosomal deletions and amplifications, the most frequent ones being trisomy of chromosome 12 (16%) and deletions of chromosomal regions 11q (18%), 17p (7%) and 13q14 (55%) (17).

- Trisomy 12 alters the gene dosage of a candidate proto-oncogene. In particular deletions of chromosomal region 11q22-q23 comprise the ataxia telangiectasia (ATM) gene (5).The deficiency of ATM gene causes genomic instability given the inability to correctly signal and repair DNA-damage is thought to contribute to CLL pathogenesis by allowing the accumulation of genetic mutations during cellular proliferation (18).
- Deletion of 13q14 is the most frequent alteration in CLL and probably represent an early event in the disease. Based on the analysis of a large number of CLL cases with monoallelic 13q14 deletion, a minimal deleted region (MDR) has been defined that contains: 1) the long non-coding RNA deleted in leukemia (DLEU)-2, which bears no similarity with any other non-coding RNA, but is conserved among vertebrates, and 2) the first exon of DLEU1 gene, another sterile transcript (19). Two microRNAs, miR-15a and miR16-1 that are expressed as a cluster, were identified within intron 4 of DLEU2. Any genetic alteration that affects the normal mRNA expression of DLEU2 also affects miR-15a/16-1 expression. Downregulation of DLEU2 and miR15a/miR16-1 expression compared to normal B cells has been described also for CLL without 13q14 deletion, suggesting an epigenetic mechanism suppressing the DLEU2/mir-15a/16-1 cluster lacking 13q14 deletion (16). In vitro assays in which the DLEU2 mRNA was introduced into a 13q14-homozygous deleted cell line failed to demonstrate any effects on cell death or proliferation (20). MiR-15a/16-1 have been the subject of intensive investigations aimed at identifying their target mRNAs encoding gene products involved in regulating proliferation and

apoptosis (21). The corresponding genes include cyclins (CCND1 and CCND3) and cyclin–dependent kinases (CHK6) as well as genes involved in apoptosis (BCL2).

- In approximately 5%-10% of untreated CLL patients, del17p13 disrupts the TP53 tumor suppressor gene. Many cases of CLL with del17p13 display inactivation of the second TP53 allele by point mutation. Altered TP53 gene mutation is an important predictor of chemorefractoriness and is associated with reduced survival (22).

Recently, whole genome sequencing studies have revealed recurrent genetic lesions that affect genes implicated in different biological pathways of potential pathogenetic relevance for CLL: NOTCH1, BIRC3, splicing factor 3b subunit 1 (SF3B1) and myeloid differentiation primary response gene 88 (MYD88).

- NOTCH1 encodes a ligand-activated transcription factor that regulates several downstream pathways important for cell growth control. In CLL the frequency of NOTCH1 mutations at the time of diagnosis is approximately 10%, occur among UM-CLL and cluster with trisomy 12 (23). NOTCH1 mutation identify a group of high risk CLL with poor survival comparable to that associated with TP53 abnormalities (24).
- SFB3B1 is a critical component of both major and minor spliceosomes (25), regulating the alternative splicing program of genes involved in the control of cell cycle progression and apoptosis points to a potential contribution of SF3B1 mutations in modulating tumor cell proliferation and survival (26). At diagnosis SFB3B1 is mutated in 5%-10% of CLLs and predicts reduced survival independent of other clinical and biological risk factors (27).
- BIRC3 negatively regulates MAP3K14, an activator of the non-canonical pathway of NF-KB signaling (28), so CLLs harboring BIRC3 disruption display constitutive NF-KB activation. At CLL diagnosis, BIRC3 disruption associated with unfavorable clinical and genetic features and predicts poor outcome independent of other risk factors (29) Figure 2.

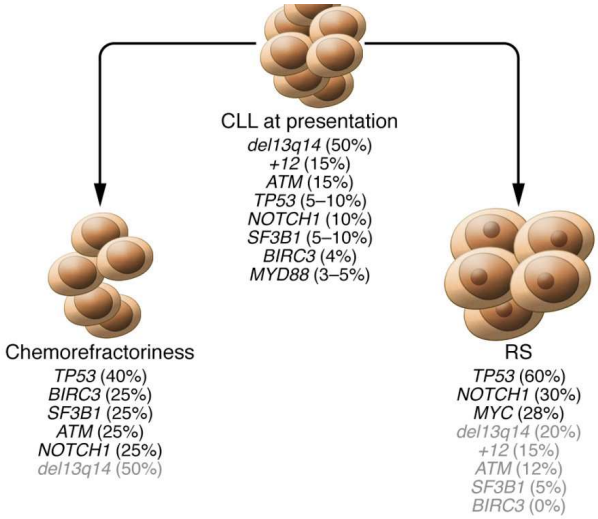


Figure 2. Genetic lesions of CLL at different phases of the disease. The frequency (in parentheses) of genetic lesions is shown for CLL at presentation and for two different types of CLL progression: chemorefractoriness without evidence of histologic transformation, and histologic transformation to RS. The two types of CLL progression follow distinct molecular pathways in terms of type and frequency of genetic lesions (30).

3. Expression of specific proteins in or on CLL cells

Expression of CD38 and CD49d in and ZAP-70 in CLL cells predict outcome in CLL (31, 32).

- CD38 has the characteristics of a coreceptor and its physical and functional association with the BCR suggests that may act as a fine modulator of the threshold of B-cell activation. CD38 has a central role in initiating and modulating a series input signals from the microenvironment. The percentage of cells within CLL clone that display CD38 is an indicator of the potential and degree of cellular activation of the clone: those with higher percentage are more responsive to activation signals or are activated and are therefore more often more aggressive (33). In particular CD38+ fractions of CLL clones are enriched in cells expressing Ki-67 and ZAP-70 and CD38+ cells proliferated more than CD38- cells (34). Expression of CD38 is a measure of cell division and a reflection of growth in vivo and this feature of CD38+ cells appears to be correlated to their ability to migrate and take advantage of interactions with the microenvironment.

CD38 expression is significantly higher in lymph nodes than in peripheral blood or bone marrow neoplastic B cells obtained from the same CLL patient, suggesting that this subset may constitute a reservoir of CLL cells that continuously contributes to the tumor burden (35).

- Also the percentage of CD49d+ is an independent indicator of prognosis in CLL, in particular higher levels (more than 30%) are correlated with shorter survival times. CD49d is an α-integrin subunit ($\alpha 4$) that can pair with CD29 (the $\beta 1$ subunit) to form a complete integrin that binds VCAM-1 and fibronectin on the accessory cells. For this reason this integrin is involved in leucocyte trafficking, activation and survival and also facilitates interactions between leucocytes and stromal cells (33). CD49d and CD38 are often expressed on CLL cells and a large molecular complex comprising CD49d, CD38, CD44v, and MMP-9 has been identified on UM-CLL clones, bringing these prognostic marker in a biological network (36).
- Intracellular expression of ZAP-70 protein is an important indicator of time-to treatment and survival in CLL (cut-off of 20%) and is an independent marker of clinical outcome (32). ZAP-70+ CLL cells are more likely to express adhesion molecules such as CD49d chemokine receptors, in particular CCR7,that promote migration toward a series of integrin and inhibit apoptosis. An important component of ZAP-70 expression is trafficking to solid tissue niches where signaling through chemokine receptors and BCRs might promote survival and proliferation (37, 38) (Figure 3).

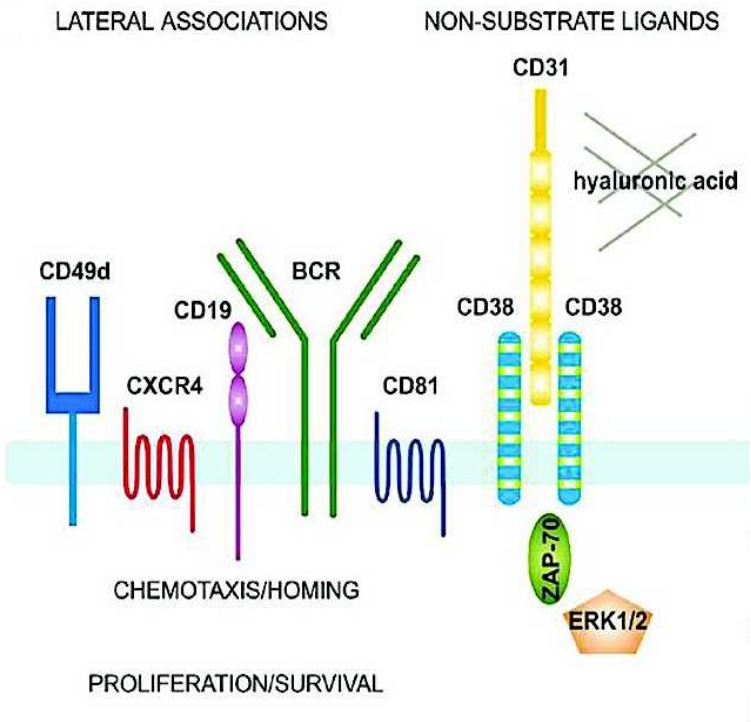


Figure 3. **Structural and functional characteristics of the human CD38 molecule.** CD38 is expressed as an integral surface membrane molecule on B lymphocytes, CD38 interacts with nonsubstrate ligands, including CD31 and hyaluronic acid, which regulate cell-cell and cell-matrix contact. CD38 is preferentially localized in membrane lipid microdomains in close contact with the BCR complex (CD19/CD81) and with molecules regulating homing (CXCR4 and CD49d). CD38 engagement by natural (CD31) or surrogate (agonistic mAb) ligands triggers activation of intracellular signaling pathways that include ZAP-70 and ERK1/2 as major players. These signals increase chemotaxis and proliferation of neoplastic B cells (33).

4. Defective apoptosis and signals from microenvironment

The progression of CLL is influenced by defective or absent apoptosis. The process of apotosis is tighly regulated by the expression of several proteins that exert a positive or a negative regulation of cell death and control the cell's fate by their interaction and relative balance (39). The ability of CLL cells to escape apotosis is mainly due to Bcl-2 expression. Bcl-2 gene family is an extended family of genes with a role in the control of apoptosis: Bax codes for a protein partner of Bcl-2 protein that blocks its repression of apoptosis. Bcl-XL cooperates with Bcl-2. Bcl-2 is overexpressed in CLL cells that also express Bcl-XL and Bax. The relative balance of all these apoptotic proteins favors the suppression of apoptosis in CLL cells (40).

CLL B cells accumulate in vivo, but undergo spontaneous apoptosis *in vitro*, unless they are co-cultured with supportive stromal cells. This suggests that in vivo CLL cells interact with accessory cells in tissue microenvironments which provide growth- and survival-signals (41). Previous studies demonstrated that co-culture with different types of stromal cells, such as monocyte-derived nurselike cells (NLC)(42), bone marrow stromal cells (BMSC) (43, 44) and endothelial cells (EC) (45, 46) promotes CLL cell survival and protects from spontaneous or drug-induced apoptosis. It is also well recognized that CLL cell growth occurs in characteristic lymphatic tissue areas called proliferation centers or pseudofollicles (47), where leukemia cell proliferation accounts for a daily turnover of up to 1 to 2% of the entire CLL cell clone (48). Hence, based on *in vitro* and in vivo studies it is now recognized that crosstalk between CLL cells and the tissue microenvironment plays a critical role in regard to the expansion of the CLL clone (49) (Figure 4).

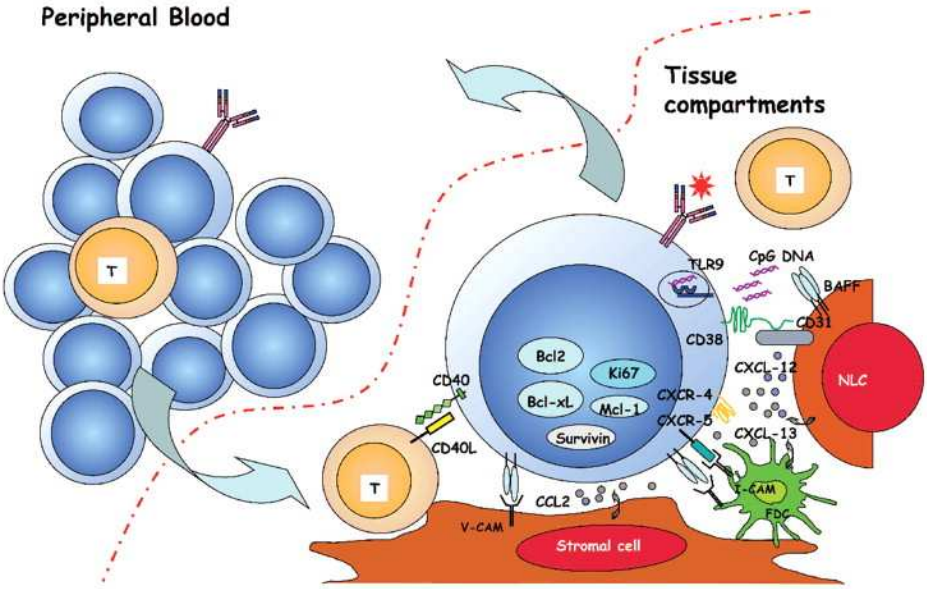


Figure 4. A proposed model of CLL clonal expansion from a CLL cell/microenvironmental perspective that includes the main actors. NLC: nurse-like cells. FDC: follicular dendritic cells (50).

II. THE TUMOR MICROENVIRONMENT IN CLL

The microenvironment is the compilation of accessory cells that within individual organs work as a team through cell-cell contacts and active molecular crosstalk to provide functional scaffolding to parenchymal cells. In solid tumor, a microenvironmental instrumental to the survival and propagation of malignant cells is built up by the concurrence of inflammatory cells that produce growth factors, new vessel formation and provides nutrient and immune tolerance that avoid immune-mediated elimination (51). Blood cancers develop in specialized tissue microenvironments, bone marrow and secondary lymphatic tissues these microenvironments are characterized by different populations of accessory cells and T cells that interact with malignant cells and promote tumor growth and drug resistance (52). The microenvironments of CLL are maintained trough a dynamic coevolution of tumor and normal cells with the presence of stromal, immune and endothelial cells populations, and have features deriving from the mature B-cell nature of CLL cells, including the mechanism that regulate B-cell homing and the antigen stimulation (53).

1. Bone marrow and secondary lymphatic tissues

In CLL disease, the proliferating compartment is represented by focal aggregates of proliferating prolymphocytes and para-immunoblasts that give rise to the called pseudofollicles or proliferation centers. Pseudo-follicles are the histological CLL hallmark in lymph nodes, splenic white pulp and bone marrow where they appear as nodular areas never surrounded by a mantle zone. These areas are usually infiltrated with an important number of CLL cells that after interaction with T cells and /or stromal/follicular dendritic cells, are able to express the proliferation marker Ki-67 and the progression disease molecules such as CD38 and CD49d (54).

Bone marrow and secondary lymphoid organs present different microenvironment, each finely tuned to support several aspects of lymphocyte maturation and differentiation. The bone marrow harbors hematopoietic stem cells (HSCs) and hosts the development of mature B

cells from committed progenitors. The development of mature B cells is primarily concerned with the events that lead to the production of cells with a functional antigen receptor (B cell receptor). Moreover in bone marrow, reticular cells secrete high levels of the chemokine SDF-1 (CXCL12) and form vascular niches. Secretion of CXCL12 can attract circulating neoplastic B cells via CXCR4 receptors expressed on CLL cells and also other malignant B cells and favor the homing to the marrow where contact with reticular cells provides growth and survival signals. These cellular interactions also confer drug resistance to leukemia cells. Mature B cells can also migrate to secondary lymphatic tissue where they are exposed to antigen within germinal centers of secondary lymphoid follicles. In this microenvironment B cells interact with CD4+ T cells for the necessary help on antigen recognition and with follicular dendritic cells for the quality control and affinity maturation. The antigen encounter triggers the proliferation, maturation and final differentiation into effector plasma cells and memory cells. Interactions between CLL and accessory cells within proliferation centers are critical for providing growth and survival signals to CLL B cells, including their proliferation and resembling interactions between normal, antigen-stimulated B cells and accessory cells during germinal centers reaction. CLL cells outside the proliferation centers are resting and considered the nonproliferative compartment (41). In lymphoid organs, but also in minor degree in bone marrow, CLL form aggregates of larger cells (prolymphocytes and paraimmunoblasts) with other cells and supporting stroma called “pseudofollicles”. These pseudofollicles are the presumed sites of proliferation in CLL and constituted the proliferation compartment (55) where leukemia cell proliferation accounts for a daily turnover of up to 1 to 2% of the entire CLL cell clone (56) (Figure 5).

The pattern of tissue infiltration by CLL cells may be variable. More frequently, malignant cells are seen only or predominantly in the peripheral blood and in the bone marrow. In some instances lymph nodes involvement is observed together with modest peripheral blood involvement. These clinical observations point to the existence of mechanism that selectively control the trafficking and homing of malignant lymphocytes to distinct microenvironments. One mechanism might be accounted by chemokines and chemokine receptors. CLL cells may express specific sets of

chemokine receptors and/or respond to specific chemokines produced by microenvironmental elements that selectively attract individual cells to explicit anatomical sites (57).

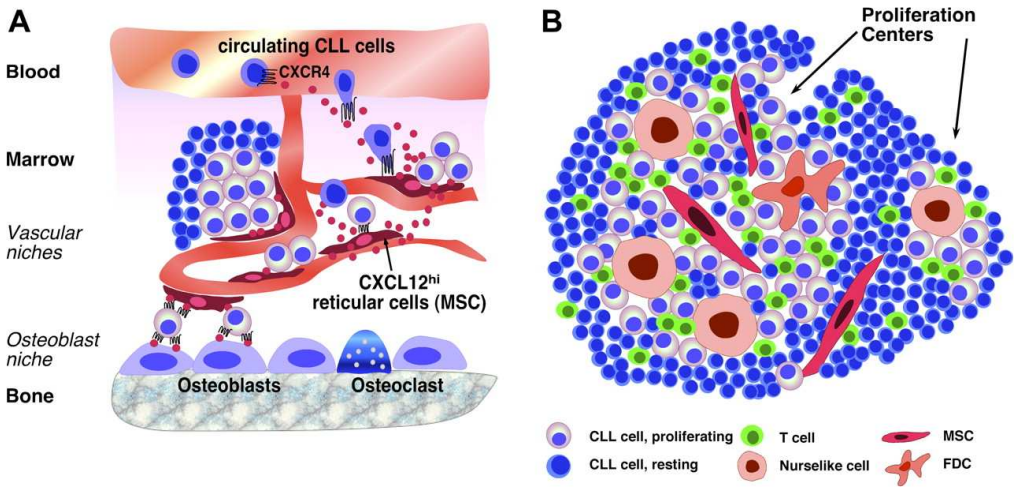


Figure 5. Cellular Interactions. (A) Marrow and (B) lymphatic tissue microenvironments in mature B-cell tumors(41) .

2. CLL cell trafficking, chemokine receptors and adhesion molecules

Lymphocytes recirculate continuously from blood to tissue and back to the bloodstream again. This recirculation, also called “homing”, is organized by tissue-specific expression of chemokines and ligand/activation-regulated expression of chemokine receptors on lymphocytes, cooperating with adhesion molecules and their ligands. This interaction is mediated by a multistep process that involves 1) lymphocytes rolling, 2) rapid activation of lymphocytes integrin, 3) adhesion to endothelial ligands through activated integrins, and 4) diapedesis. Multiple protein families, with peculiar functions, are involved in the traffic signals. Selectins mediate the attachment or tethering of flowing lymphocytes to the vessel wall and permit lymphocytes to enroll in the direction of flow, β1 and β3 integrin families that mediate interaction with extracellular matrix; matrix metalloproteinases, cadherins and associated molecules that mediate stable intercellular adhesions.

Lymphocytes exit from the bloodstream interacting with vascular endothelium in specialized postcapillary venules, termed high endothelial venules (HEV) in lymph nodes and Peyer patches. First of all there is a loose tethering engagement leading to a rolling movement of the lymphocyte over the vascular endothelium. Not only selectin family can allow the effective interaction with their sialomucin ligands but under certain conditions, the integrins α4β1 and α4β7 and CD44 can mediate rolling. Lymphocyte rolling is transient and reversible, unless followed by a chemokine signal leading to integrin activation. In fact chemokines displayed on the luminal surface of the endothelium activate chemokine receptors on rolling lymphocytes, which in turn triggers integrin activation. This causes arrest, firm adhesion, and transendothelial migration into the tissues, where stromal cells organize the localization and retention of the lymphocytes via chemokine gradients. Heparin sulfate proteoglycans (HSPGs) expressed on endothelium or extracellular matrix contribute to integrin activation and promotes diapedesis by concentrating and presenting chemokines to their receptors. This causes activation of several signaling molecules such as PI3K, Tec/Btk-family kinases, phospholipase C

and Ras-family GTPases. Chemokines including CXCL12 (SDF-1), CXCL13, CCL21, CXCL10, and CCL17 trigger affinity and avidity of lymphocyte integrins, resulting in rapid lymphocyte arrest under flow conditions. These integrins, for example LFA-1 (lymphocyte-associated antigen 1), $\alpha 4\beta 1$ and $\alpha 4\beta$, mediate stable adhesion and promote migration of lymphocytes across the vessel wall. In the tissues, lymphocytes engage chemoattractant gradients directing the trafficking to the correct microenvironment. Adhesion molecules and chemokine receptors on its cell surface determine the final fate of lymphocytes in the microenvironment allowing tissue-specific homing (58).

Several evidences confirm that many of the physiologic mechanism of tissue-specific territoriality are also functional in neoplastic lymphocytes, such as CLL cell.

For example, CXCR4 chemokine receptors are expressed on blood CLL cells, and it's able to mediate CLL cell chemotaxis, migration across vascular endothelium, actin polymerization, and migration beneath and underneath bone marrow stromal cells (BMSC) in response to CXCL12 gradients. CXCL12 downregulates CXCR4 on CLL cells via receptor endocytosis, this can be used to distinguish tissue-derived from blood CLL cells, $CXCR4^{dim}$ CLL cells from CXCL12-abundant tissues (bone marrow, lymph nodes) from $CXCR4^{high}$ CLL cells from blood. In vivo deuterium labeling of CLL cells revealed that patients with higher CXCR4 expression on blood CLL cells had delayed appearance of newly produced, $CD38^{+}$ cells in the blood, an increased risk for lymphoid organ infiltration and poor outcome. Higher CXCR4 expression on CLL cells favors prolonged tissue retention and proliferation. Moreover there is an intraclonal heterogeneity of CXCR4, with an enrichment of CLL cells expressing lower CXCR4 surface levels in the $CD38^{+}/CD5^{bright}$ fraction, along with increased deuterium incorporation. These evidences indicate that lower blood CXCR4 surface levels label a fraction of CLL cells that has recently exited the tissues into the blood (59).

3. The cellular microenvironment in CLL

- **Bone marrow stromal cells**

Bone marrow stroma cells (BMSCs) are of mesenchymal origin and are similar to actin (αSMA^+)-positive mesenchymal stromal cells (MSCs) in other tissues such as secondary lymphatic tissues. A dense infiltration of αSMA^+ stromal cells in lymphatic tissues from CLL patients suggests that interactions between CLL cells and this type of stromal cells plays a role also outside the bone marrow. BMSCs were the first stromal cells characterized to support CLL cells. In CLL, BMSCs create niches within the bone marrow in which CLL cells lodge and are nourish and protected from different cytotoxic agents. In particular BMSC-derived survival and drug-resistance signals are largely adhesion-dependent, in fact CLL coculture with BMSCs results in the adhesion and rapid, spontaneous migration of a fraction of CLL cells beneath and underneath the BMSCs (pseudoemperipolesis) and this seems to be dependent from CXCR4 and VLA-4 expression by leukemia cells. Both murine and human BMSC are very similar in the ability to protect CLL cells from apoptosis, in particular a murine in vivo model of CLL showed that the murine BM microenvironment was able to support CLL cell growth, suggesting that key cellular and molecular pathways in the bone marrow are conserved between mice and humans (60). The cross-talk between CLL and BMSCs is bidirectional causing activation of both CLL cells and BMSCs in particular activation of both Erk and Akt can occur in BMSCs by direct contact with CLL B cells and their soluble factors (61, 62).

Interaction between CLL cells and primary marrow stromal elements derived from CLL bone biopsy affects levels of anti-apoptotic proteins and induces dramatic alterations in the levels of secreted pro-and anti-angiogenic cytokines. In particular this interaction increases the production of VEGF and platelet-derived growth factor (PDGF), along with a diminution of thrombospondin-1. PDGF can bind to the PDGFR on MSCs to trigger downstream PI3K-Akt activation. PDGFR-PI3K-Akt activation further triggers downstream programs that result in increased VEGF production, which subsequently leads to increased survival/drug resistance in CLL

cells, as well as promoting neovascularization, which is related to disease progression (62).

- **Nurse-like cells (NLCs)**

Nurse cells were first recognized in situ in the thymus, where they form characteristic complexes with immature T lymphocytes and play an important role in thymocyte maturation and differentiation. This cellular interaction is characterized by the active invasion into thymic nurse cells by thymocytes. When cultured, the PBMC from patients with CLL developed abundant numbers of NLC that became the predominant population of adherent cells. In contrast, PBMC of healthy donors rarely generated such NLC when cultured under identical conditions. This may be because of a difference between the blood of patients with CLL and that of healthy donors in the relative proportion of cells that can give rise to such NLC. Patients with CLL may have greater numbers of circulating NLC progenitor cells, possibly secondary to the infiltration of the marrow by leukemia B cells. Phenotypic characterization of CLL NLC suggests they are related to marrow stromal cells. These cells lack expression of B-cell or T-cell differentiation antigens and NLC do not share cytogenetic abnormalities with the CLL B-cell clone. NLC are noted to express stromal cell markers, such as vimentin and STRO-1 and express mRNA for SDF-1.

In vitro, NLCs differentiate from blood monocytes into large, round, adherent cells cocultured with CLL cells that attract and protect them from spontaneous apoptosis or drug-induced cell death in a contact dependent fashion. In vivo, NLCs can be found in the spleen and lymphoid tissues of CLL patients (63). NLC represent a model for the microenvironment in secondary lymphatic tissues in fact gene expression profiles of CLL cells cocultured with NLC are very similar to CLL cells isolated from secondary lymphatic tissues, which show signs of BCR and NF-KB activation and upregulation of BCR target genes such as CCL3 and CCL4. CLL-NLC cross-talk is characterized by important pathways and showed interesting therapeutic targets of CLL-microenvironment cross-talk. In particular NLCs attract CLL cells secreting CXCL12 and CXCL13 and protect CLL cells from spontaneous or drug-induced apoptosis through CXCL12, B cell-

activating factor of the tumor necrosis factor family (BAFF), CD31 and plexin-B1 (63).

- **T lymphocytes**

In untreated CLL, the peripheral number of T cells is increased in both CD4 and CD8 compartments. It's still unknown whether this increased number of T cells is due to interaction with the CLL clone, with microbial antigens or for other reasons. T cells interact with CLL cells and stimulate CLL growth and by adhesion molecules and production/ secretion of several cytokines, for example interleukin-4 or 2 (IL-4/IL-2) or tumor necrosis factor α (TNF-α) or interferon γ (IFNγ). These cytokines permit to rescue CLL cell from apoptosis by upregulating Bcl-2 protein (64).

CD4+ T cells express CD40L (CD154), a member of the TNF superfamily that may bind to $CD40^+$ CLL cells, which can support the growth of CLL cells by CD40 ligation. CD40 crosslinking on CLL cells induces up-regulation of CD80 and CD54 and turns non immunogenic CLL cells into T-cell stimulators. This proliferative CLL B cells activated through CD40 express also survivin, a member of the family protein of inhibitor of apoptosis. In fact surviving positive cells have an extended survival, an increased proliferative rate and retain Bcl-2 positivity. Induced expression of CD154 on CLL cells by adenoviral gene transfer can crosslink CD40 on CLL cells and induce activation and immune recognition. This concept can be used into the clinic as a novel form of immunotherapy, in particular patients infused with their cells transduced in vitro with adenovirus encoding CD40L were shown to make antibodies to ROR1 an oncofetal antigen restricted to CLL cells. CD40 activation of CLL cells can result in activation of prosurvival and proliferation pathways or immune recognition and induction of a specific response. In general ligation of CD40 on CLL cells induces phenotypic and biochemical changes that facilitate CLL cell-T cell interactions and enhance the sensitivity of CLL cells to clearance by adaptive and innate immune-effector mechanisms (65). Proliferation centers contain activated CD4+ T cells adjacent to leukemic cells, likely indicating adhesion and bidirectional signals. CLL cells secrete CCL22, CCL3 and CCL4, which are involved in T cell recruitment to the lymph-

nodes, so leukemic cells themselves play an active role in the accumulation of T lymphocytes. Moreover migration in response to CXCL12, CCL21 and CCL19 of T cells from CLL patients is defective compared to T cells from healthy adults. T cells in proliferation centers help CLL cells survival and proliferation, and the low migratory response towards CXCL12 in T cells from ZAP70- CLL patients is believed to favor the indolent clinical course of the disease in these patients (42, 63, 66).

T cells from CLL patients are dysfunctional and are not able to form an effective immune synapse. This molecular defect is due, in part, to interactions with the malignant cells and is reversed by using an immunomodulatory drug, Lenalidomide. This agent induce stimulation of T cells through CD28 and enhancement of the expression of cytokines (including IL-2 and IFN-γ), regression of regulatory T cells with concomitant induction of Th17, and increase of NK- and of antibody-dependent cytotoxicities (67).

Moreover reprogramming of autologous T cells to target specific tumor antigens involves the use of an antibody-derived antigen-binding moiety fused with an internal signaling domain such as CD3ς to forma a chimeric antigen receptor (CAR). In this way it's possible to use the patient's own cells, they can be created quickly, and the same chimeric antigen receptor can be used for multiple patients. Preliminary results show that low doses of autologous T cells infected with a CD19-targeted CAR infused into a patient induce tumor lysis syndrome followed by clinical response (68).

- **Endothelial cells**

CLL cells may interact with endothelial cells (EC) through different mechanisms that allow cell migration and angiogenesis. Endothelial cells cover the inner surface of blood vessels and interact with CLL cells through different molecules allowing CLL-EC crosstalk for example CD38, expressed on CLL cells, interact with CD31 on endothelial cells; VCAM-1 expressed by activated endothelium binds CD49d on CLL surface (69, 70). This cellular interaction permits adhesion and transmigration of neoplastic B cells for tissue colonization. In CLL patients with clinically evident lymphadenopathy, it has been shown an autocrine loop that involves

vascular endothelial growth factor and vascular endothelial growth factor receptor. Intimate contact between leukemia cells and ECs in marrow niches and the increased microvessel density in marrows infiltrated by CLL cells support the concept of crosstalk between CLL and ECs via the VEGF and the Ang-2/Tie2 axes, and indicate that such interactions are an integral part of the marrow microenvironment. In bone marrow and lymph nodes of patients with CLL, microvascular density is significantly increased. This is probably due to the ability of CLL cells to secrete pro-angiogenic factors in the surrounding microenvironment. Leukemic cells are able to progressively disrupt the normal architecture of tissue microenvironments to generate a favourable soil for their survival and growth. As part of this process, the CLL capacity to modify the vascular structures of infiltrated tissues, subverting the stability of pre-existing vessels and thus determining abnormal vessel formations, may be crucial to provide oxygen and metabolities to allow dissemination though-out the body and to establish positive cellular interactions. Notably increased serum or plasmatic levels of pro-angiogenic factors such as bFGF, VEGF and Ang2 were reported in CLL patients. In particular CLL cells spontaneously express both VEGF and Ang2 and secrete these pro-angiogenic factors in the surrounding microenvironment (70). Secretion of Ang2 and VEGF by CLL cells is able to stimulate the morphogenetic changes resembling capillary-like structure tube formation in a in vitro angiogenesis test. The role of endothelial cells in mediating CLL cells survival is still under investigation. Some evidences indicated that:

1. Soluble factors released from endothelial cells in enriched of dimeric interleukin 6 with a peculiar antiapoptotic activity on CLL cells (71, 72);
2. Cell-cell interactions between CLL cells and endothelial hybrid cells prevented the expression of apoptosis;
3. CLL cells show elevated levels of the anti-apoptotic proteins Bcl-2, Mcl-1 and Bcl-XL, increased expression of CD38 and CD49d and NF-KB activation were reported in CLL cells co-cultured with endothelial cells (73);

4. CLL cells in adhesion to EC layer were protected from undergoing spontaneous apoptosis through cell-cell contact (69).

- **Selected molecular pathways in the CLL microenvironment**

The CXCR4-CXCL12 axis*:* the CXCR4 (CD184) receptor is highly expressed on CLL cell surface and mediates leukemia cell chemotaxis, migration across vascular endothelium, actin polymerization and migration beneath and underneath bone marrow stromal cells in response to CXCL12 gradients. The stromal chemokine, that binds CXCR4 receptor, is CXCL12 (formerly called stroma-derived factor-1 SDF-1), originally characterized as a pre-B cell growth factor (74). CXCL12 causes not only migration towards stromal cells, but provides also survival signals to CLL cell, for example in NLC co-cultures. Both of these effects are mediated through CXCR4 receptor, which is downregulated via receptor endocytosis one activated by CXCL12. In particular this characteristic can be used to distinguish circulating CLL cells in the peripheral blood from resident tissue CLL cells (lymphatic and bone marrow), in fact circulating CLL cells typically express high levels of surface CXCR4 and CLL resident in bone marrow or lymph nodes show lower levels of surface CXCR4. Upon stimulation by CXCL12, signaling through the CXCR4 receptor has pleiotropic effects on CLL cells, including activation of PI3 kinases, serine phosphorylation of signal transducer and activator of transcription 3 (STAT3) and p44/42 MAP kinases as well as effects on calcium mobilization (75). Proliferating CLL cells from bone marrow and lymphatic tissues display significantly lower levels of CXCR4 than nonproliferating CLL cells. Moreover in vivo deuterium labeling CLL cells revealed an enrichment of CLL cells expressing lower CXCR4 surface levels in the $CD38^{+}/CD5^{bright}$ fraction along with increased deuterium incorporation, these data indicate that CLL subclones with lower blood CXCR4 surface levels are a fraction of cells that has recently exited the tissues into the blood (76). BCR signaling results in the down-modulation of CXCR4, along with enhanced chemotaxis toward CXCL12 and CXCL13 (77, 78). This may explain why $ZAP\text{-}70^{+}$ CLL cells display increased chemotaxis and survival in response to CXCL12 compared with $ZAP\text{-}70^{-}$ CLL cells, given that ZAP-70 expression is

associated with higher responsiveness to BCR stimulation (78). Additionally, interactions between CXCR4 and CD38 may play an important role because CD38+ CLL cells appear to have enhanced chemotaxis and CD38 activation increases chemotaxis toward CXCL12, whereas a blocking anti-CD38 mAb inhibits this process (79). CXCR4 can be specifically blocked by CXCR4 antagonists, inhibiting CLL cell activation by CXCL12 and reverse stromal cell-mediated drug resistance. On these basis clinical trial in which CLL patients are treated with a combination of rituximab and plerixafor, a small molecule CXCR4 antagonist. In these clinical trials the aim is to mobilize leukemia cells from the tissues using a CXCR4 antagonist and to target these cells outside of the protective niches (80).

the CXCR5-CXCL13 axis: CXCR5 (CD185) regulates lymphocyte homing and positioning within follicles of secondary lymphoid tissues. CXCR5 is expressed by mature recirculating B cells, a small subset of CD4+ and CD8+ T cells, and skin-derived migratory dendritic cells (81).

CXCR5 gene-deleted mice display defective formation of primary follicles and germinal centers in the spleen and Payer patches and lack inguinal lymph nodes. The ligand for CXCR5 is B cell-attracting chemokine 1 (BCA-1) also known as CXCL13. CXCL13 is costitutively secreted by stromal cells in B cell areas in secondary lymphoid tissues (follicles), where B cells encounter antigen and differentiate (82).

CXCL13 gradients induce recruitment of circulating naive B cell to follicles, and are involved in the microanatomic positioning of B cells within the germinal center. In addition to regulate lymphocytes migration and microarchitecture in secondary lymphoid tissues, CXCL13-CXCR5 axis has a role in trafficking of B1 B cells, a possible normal counterparts of CLL cells (83) . CLL cells express high levels of CXCR5, and stimulation with CXCL13 induces activation via Gi proteins, PI3Ks, and p44/42 MAPK, resulting in actin polymerization, CXCR5 endocytosis, and chemotaxis. CXCL12 seems to be involved in establishment and maintenance of the micro-architecture of lymphoid tissues infiltrated by CLL cells, characterized by proliferation centers (pseudofollicles). CXCL13 mRNA and protein are expressed by NLC in vitro and in vivo (84).

Moreover CXCR5 plays a role in CLL-cell positioning and cognate interactions between CLL and CXCL13-secreting NLCs in lymphoid tissues.

***CCL3, CCL4, and CCL22*:** CCL3 and CCL4, called also as Macrophage inflammatory proteins-1 alpha and beta (MIP-1α, β) are chemokines of the CC subfamily and inducible in different hematopoietic cells involved in adaptive immune response such as dendritic cells, macrophages and B/T lymphocytes. CCL3 signals through the chemokine receptor CCR1 and CCR5, whereas CCL4 signals only through CCR5, both are chemoattractans for monocytes and lymphocytes (85). Several studies demonstrated CCL3 and CCL4 overexpression and secretion by activated CLL cells, moreover CCL3 expression in B cells is induced in response of BCR stimulation and CD40 ligand and repressed by Bcl-6 (86).

CLL patients display elevated plasma levels of CCL3 and CCL4, and plasma levels of CCL3 were strongly associated with established prognostic markers and time to treatment. A multivariable analysis revealed that CCL3, advanced clinical stage, poor-risk cytogenetics, and CD38 expression were independent prognostic markers in a cohort of 351 CLL patients (87). CLL cells upregulate and secrete CCL3 and CCL4 in response to BCR stimulation and in co-culture with nurselike cells.

Conceivably, CLL cell-derived CCL3/CCL4 may induce trafficking and homing of accessory cells and in particular of T cells to activated, CD38+/Ki67+ CLL cells for cognate T-CLL cell interaction that foster CLL-cell proliferation (88).

Conceptually, by attracting T cells and other immune cells, CLL-cell-derived chemokines foster the coevolution of CLL cells and their supportive microenvironment, thereby actively creating a favorable microenvironment. Several studies indicated that CCL3 and CCL4 display a specific function in lymphatic tissues, demonstrated that B cell activation within lymphoid tissues results in CCL3/4 secretion leading to the recruitment of CCR5+ regulatory T cells for cognate interaction and antigen presenting cells. CLL cells in the proliferative compartments are interspersed with T cells and NLC. By attracting T cells and other immune cells for cognate interactions with the leukemic cells, CLL cell-derived CCL3/4 foster the co-evolution of

CLL cells and their microenvironment where CLL cells interact with T cells and other accessory cells that deliver survival- and proliferation- signals.

***Adhesion molecules*:** Integrins are heterodimeric glycoproteins consisting of various α and β subunits, the function of which is to mediate cell-cell and cell-matrix adhesion. Integrins are a family of cell adhesion molecules that regulate cell growth and function in the stromal microenvironment. **β1** integrins are very late activation antigens (VLAs) that have the same **β1** subunit but various α chains (**α1-α6).** The **α4β1** integrin VLA-4 (CD49d) is a receptor for fibronectin and vascular cell adhesion molecule-1 (VCAM-1/CD106).

VLA-4 plays a particularly important role in interactions between normal and malignant hematopoietic cells and the bone marrow microenvironment. This integrin is important in CLL playing a role in the homing and retention of CLL cells in the microenvironments. In particular VLA-4 integrin cooperates with CXCR4 in CLL-cell adhesion to BMSCs. VLA-4 expression on CLL cells has prognostic impact, high VLA-4 expression correlates with poorer prognosis indicating the relevance of these interactions in vivo (89). Patients with high VLA-4 expression have significantly increased CLL infiltration in bone marrow, in fact blocking VLA-4 induces inhibition of in vivo homing of CLL cells to the bone marrow. In addition the association of VLA-4 with CXCR4 expression in CLL cells suggesting a coordinated role in CLL cells trafficking to lymphoid tissues (90).

These studies indicate that VLA-4 integrin plays a key role in the adhesion of CLL and other leukemia cells to stromal cells and the extracellular matrix.

Other important adhesion molecules in CLL include LFA-1, L-selectin (CD62L) and CD44. LFA-1, integrin lymphocyte function associated antigen-1, has an aberrant behavior in CLL cells compared to normal cells. Reduced levels of LFA-1 on CLL cells is correlated with an impairment of the migratory pattern in CLL cells (91).

Selectins are carbohydrate-binding molecules that permit and facilitate adhesion of malignant cells to tissues inducing activation of integrins and release of chemokines. Another important molecule in CLL cell trafficking is VCAM-1 (vascular cell adhesion molecule-1) expressed on the surface of

endothelial and stromal cells. VCAM-1 provides direct survival signals to CLL cells that adhere to it. Moreover increased levels of soluble VCAM-1 are associated with CLL tumor burden (92, 93).

***CD40-CD154 interactions*:** Several studies showed that CD154 induces pro-survival signals and drug resistance to CLL cells. Within proliferation centers, a significant number of T cells display CD40 ligand (CD154) that can bind to CD40 on CLL cells, rescuing them from apoptosis.
Conversely, CD40 cross-linking also induces up-regulation of CD80 and CD54 and turns nonimmunogenic CLL cells into effective T-cell stimulators (93).
CLL cells engineered to express CD154 by adenoviral gene transfer can cross-link CD40 on bystander CLL cells and induce the same sequence of activation and immune recognition.
CLL patients infused with CLL cells transduced in vitro with an adenovirus encoding CD40L (Ad-CD154) can make antibodies to ROR1, an oncofetal antigen restricted to CLL cells, indicating that this approach can overcome immune tolerance.
These data suggest that CD40 activation of CLL cells can result in different outcomes that are not mutually contradictory: activation of prosurvival and proliferation pathways if triggered by CD154+ T-cells in the context of proliferation centers, or immune recognition and induction of a specific immune response if triggered in the context of Ad-CD154-transduced CLL cells (94).

***BAFF and APRIL*:** BAFF and APRIL are related TNF family ligands that bind to members of the TNF receptor family such as B-cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI). BAFF also binds another TNF receptor, the BAFF receptor (BAFF-R). Binding to heparin sulfate proteoglycans allows for tissue retention of APRIL.
Via BCMA, TACI and BAFF-R expressed on the CLL cells, NLC-derived BAFF and APRIL induce activation of the canonical NF-kB pathway and protect CLL cells from apoptosis (95).

Male v-Myc myelocytomatosis viral oncogene homolog (c-Myc) transgenic mice develop a CD5 B-cel leukemia resembling CLL after cross-breeding with Baff-transgenic mice, with Baff-induced enhancement of c-Myc, suggesting an important relationship between BAFF and c-MYC in CLL (96).

Aging April-transgenic mice develop an expansion of CD5+ B1 B cells with organ infiltration, which can be reversed with anti-April mAbs.

BCR and BCR-associated Kinases (Syk, Btk, and PI3K delta): In CLL microenvironment, the BCRs of CLL cells become engaged by microbial or auto-antigens, which, along with other costimulatory signals, promote the expansion of the CLL clone playing a role in the pathogenesis of CLL (97).

Engagement of BCRs by antigen induces phosphorylation of immunoreceptor tyrosine-based activation motifs in the cytoplasmic tails of Ig-α and Ig-β, with subsequent recruitment of Syk to BCR microclusters, followed by downstream activation of Btk and PI3Ks. Upon phosphorylation, Sky, Btk, and PI3Ks propagate BCR-derived signals by activating downstream signaling pathways, including calcium mobilization and activation of AKT kinase, ERK1/2, and myeloid cell leukemia-1 (MCL-1).

The notion that ZAP-70 and unmutated CLL cells are more responsive to BCR stimulation and other microenvironmental signals suggests that patients with high-risk disease features may be particularly well suited for alternative treatments that target the microenvironment (98, 99).

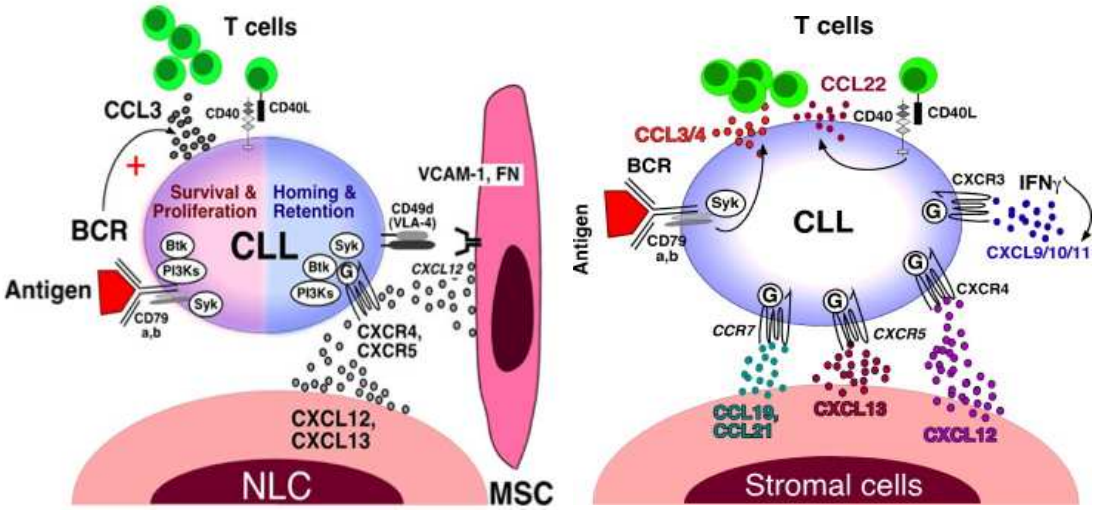


Figure 6. CLL Microenvironment. Accessory cells in the tissue microenvironments, collectively referred to as “stromal cells” constitutively secrete the chemokines CXCL12, CXCL13, CCL19, and CCL21. Through corresponding chemokine receptors expressed on the surface of the CLL cells, the leukemia cells migrate and home to stromal cell niches in the tissue compartments (57, 100)

III. Role of kinase targeted strategies in CLL

In the last years there have been recent improvements in prolonging survival of CLL patients with combination of different chemoimmunotherapy regimens, but chronic lymphocytic leukemia remains an incurable disease with conventional therapies. Although these therapies are often effective at killing circulant leukemic cells in the peripheral blood, the problem is that residual disease remains inside the protective niches in the bone marrow and lymph nodes. In these sites CLL cells are rescued from apoptosis and received protection from a variety of treatments through pro-survival signals fostered by the microenvironments. The existence of growth-favorable niches appears to be critical to the survival of CLL cells. Once resident in the stroma, CLL cells are protected from treatments, in this scenario strategies designed to mobilize CLL cells out of these protective niches (chemosensitization) as well as strategies to prevent CLL cells from migrating into the stroma hold great promises (101).

- **Spleen tyrosine kinase (SYK), and its inhibitor, fostamatinib (R788)**

SYK is a member of the SYK/ZAP70 family of nonreceptor kinases and activates pathways downstream of BCR signaling molecule.
SYK-deficient mice have several defective B lymphopoiesis in particular B cell development is blocked at the stage of pro-B to pre-B cell transition correlating Syk with a role in pre-B-cell receptor signaling.
In vivo studies demonstrate that SYK also regulates survival and maintenance of mature normal and malignant B cells and modulates leukocyte adhesion and chemotaxis of normal B cells. These data suggest that Syk participates in tissue homing and retention of activated B cells.
Fostamatinib (FosD, also called R788), the clinically used oral formulation is a pro-drug converted in vivo into the bioactive form R406 and is the only Syk inhibitor in clinical use. R406 is not a highly selective SYK inhibitor and displays lesser activity against Flt3, Jak and Lck. In vivo, R406 plasma levels are dose dependent with a variable concentration between 1ug/ml and > 5ug/ml after a single dose of R406.

After encouraging results in a Phase I/II study in patients with relapsed B cell lymphomas in particular patients with CLL, FosD was primarily developed in rheumatoid arthritis (RA) and now is in phase 3 trial.
In the lymphoma trial, highest response rates were seen in CLL patients, where the objective response rate was 55%.
The investigators reported a transient lymphocytosis and concurrent resolution of lymphadenopathy, but details about the degree and duration of lymphocytosis were not specified.
One CLL patient actually discontinued therapy because of lymphocytosis before the redistribution phenomenon was recognized.
More specific SYK inhibitors are active in preclinical models of CLL and diffuse large B-cell lymphoma and it's expected that SKY inhibitors will re-appear on the clinical stage in selected B cell malignancies in the near future (77, 102, 103).

- **Bruton's tyrosine kinase (BTK) and its inhibitor, Ibrutinib**

BTK is a non-receptor tyrosine kinase of the Tec kinase family and plays a central role in BCR signaling. BTK is primarily expressed in hematopoietic cells, particularly in B cells but not in T cells and plasma cells.
BTK deficiency is the genetic basis for X-linked agammaglobulinemia (XLA), a primary immunodeficiency characterized by low serum immunoglobulin levels and lack of peripheral B cells.
After BCR triggering, BTK is activated by Lyn and SYK, causing the activation of transcription factors necessary for B-cell proliferation and differentiation.
Besides its role in BCR signaling, BTK is also involved in signaling of receptors related to B cell migration and adhesion, such as the CXCR4 and CXCR5 chemokine receptors and adhesion molecules (integrins).
Ibrutinib, also called PCI-32765, is a BTK inhibitor which binds specifically and irreversibly to a cysteine residue in the BTK kinase domain and thereby blocks BTK phosphorylation and enzymatic activity. Ibrutinib displays encouraging clinical activity in patients with B-cell malignancies, particularly in CLL and mantle cell lymphoma patients.

Moreover this drug can antagonize CLL cell survival after stimulation with various factors (CD40L, BAFF, IL-6, IL-4, TNF-α, fibronectin, stromal cell contact), as well as CpG-induced CLL cell ploriferation. Ibrutinib is able to inhibit CLL cell survival and proliferation derived from BCR and nurse-like cells (NLC).

Interestingly, the secretion of the BCR activation-dependent chemokines CCL3 and CCL4 by CLL cells was downregulated both in vitro and in vivo in plasma from CLL patients receiving therapy with ibrutinib on these chemokines, indicating that CCL3 and CCL4 plasma levels are robust biomarkers for kinase inhibitors targeting the BCR signaling axis.

In an adoptive transfer TCL1 mouse model of CLL, we demonstrated inhibition of CLL progression and redistribution of CLL cells into the blood. Ibrutinib effectively inhibits CLL cell survival and migration explaining some of the characteristic clinical activity of CLL cell redistribution. Lastly Ibrutinib interfere with CLL chemotaxis and integrin mediated CLL cell adhesion, in fact it's able to inhibit BCR-controlled signaling and integrin α(4)β(1)-mediated adhesion to fibronectin and VCAM-1 in CLL cells.

They also confirmed that ibrutinib inhibits CLL cell migration towards CXCL12, CXCL13 and CCL19. These data explain the ability of this drug to induce redistribution of CLL cells from tissues into the peripheral blood.

Clinically, the ibrutinib-induced lymphocytosis is variable among patients and directly related to the presence of the drug: when ibrutinib was given in an intermittent fashion with a monthly 7-days-off-drug period, a saw-toothed pattern of absolute lymphocyte counts (ALC) was noticed, where ALC rapidly dropped during the off-drug period and then increased again, once ibrutinib was restarted (103-106).

- **The PI3Kδ inhibitor GS-1101.**

PI3Ks integrate and transmit signals from different surface molecules, such as the BCR, chemokine receptors and adhesion molecules, thereby regulating important cellular functions, including cell growth, survival and migration.

PI3Ks are divided in 3 classes. The class I kinases contain 4 isoforms designated PI3Kα, β, γ and δ. PI3Kα and β isoforms are ubiquitously

expressed, PI3Kγ isoform has a particular role in T-cell activation, and PI3Kδ expression is largely restricted to hematopoietic cells. PI3Kδ plays a critical role in B-cell homeostasis and function, based on studies with gene deleted mice which harbor reduced numbers of B1 and marginal zone B cells, display reduced immunoglobulin levels and poor responses to immunization, as well as defective BCR and CD40 signaling and can develop inflammatory bowel disease.

In CLL cells, PI3K are constitutively activated, presumably by growth and survival signals from the microenvironment, such as adhesion to BMSC, CXCR4 activation, and B cell receptor (BCR) activation. Unmutated, high risk CLL patients show overexpression of PI3K.

GS-1101 (previously called CAL-101) is a potent and highly selective PI3Kδ inhibitor, and the first PI3Kδ inhibitor in clinical use.

GS-1101 induces apoptosis in B-cell lines and primary cells from patients with different B-cell malignancies, such as CLL, mantle cell lymphoma and multiple myeloma. Moreover, it also inhibits constitutive and CD40, TNF-alpha, fibronectin and BCR derived PI3K signaling leading to suppression of Akt activation.

GS-1101 interferes with CLL cells chemotaxis towards CXCL12 and CXCL13 and migration beneath stromal cells (pseudoemperipolesis) and induces disruption of survival signals could be a critical mechanism for the clinical activity of GS.1101. These in vitro results are corroborated by clinical data showing marked reductions in circulating CCL3, CCL4 and CXCL13 levels, and rising lymphocytosis during GS-1101 treatment.

GS-1101 treatment induce in CLL patients an initial leukemic cell re-destribution from tissues into the blood along with a rapid lymph node size reduction and a transient lymphocytosis during the first weeks of treatment.

In general, GS-1101 displays a dual mechanism of action, directly decreasing cell survival while reducing interactions that retain CLL cells in the tissue microenvironments (103, 107-109) Figure 7.

The similarities in clinical responses of CLL patients to treatment with a SYK-, BTK-, or PI3Kδ inhibitor suggest overlapping functions of these kinases in BCR signaling, CLL cell migration, and homing.

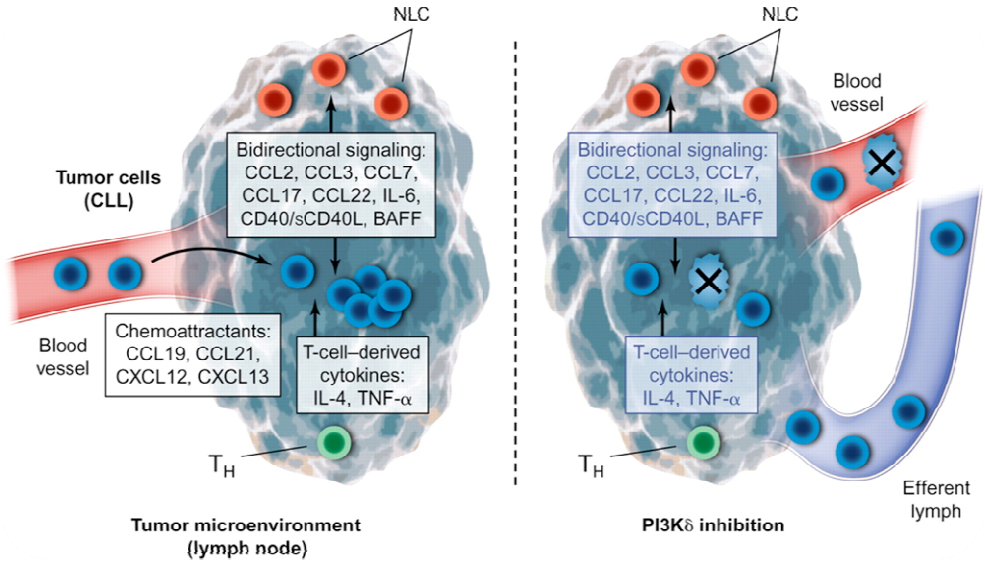


Figure 7. Mechanism of action of GS-1101. In CLL patients, GS-1101 causes early lymphocytosis and rapid lymph node shrinkage, suggesting that this plays a role for CLL cell migration, tissue homing and survival.

AIM and RATIONALE

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in adults. Clinically CLL is characterized by a marked degree of heterogeneity driven at least in part by differences in the disease biology (30).
For this reason, CLL remains an incurable disease with conventional therapies. In fact although therapies are often effective at killing CLL cells in the peripheral blood, residual disease remains in the bone marrow and lymph nodes.
In the tissues, bidirectional interactions between CLL cells and non-transformed cells of stromal and immune compartments extend CLL-cell survival and protect from the effects of chemotherapeutics (101).

In this study we investigated the interaction between CLL and endothelial or bone marrow stromal cells in an in vitro co-culture system. Although an in vitro model is never going to be able to recapitulate the in vivo microenvironment completely, it may be useful for dissecting specific cellular signals ascribed to different elements directly interacting with leukemic cells in vivo. This project was designed to examine and highlight cellular pathways and molecular networks involved in the crosstalk between CLL cells and endothelial or bone marrow stromal cells, with particular interest on integrin and adhesion pathways.
Given the critical role of integrin in CLL trafficking between blood and secondary lymphoid tissues, we analyzed CLL cell adhesion to endothelial and bone marrow stromal cells under static and shear flow conditions.
We therefore, examined the possibility to interfere with CLL survival and integrin function and signaling using a PI3K delta inhibitor, GS-1101.

MATERIAL AND METHODS

CLL cell purification, reagents

After obtaining inform consent in accordance with the Declaration of Helsinki on protocols approved by the Institutional Review Board, peripheral blood samples were collected from 35 untreated CLL patients fulfilling clinical and immunophenotypic criteria for CLL at Hematology Division of Modena Hospital and at MD Anderson Cancer Center. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Pacque (GE Healthcare, Waukesha, Wi, USA) and were used fresh or cryopreserved into fetal bovine serum (FBS) (BD Biosciences, San Diego, CA, USA) plus 10% dimethylsulfoxide (Sigma-Aldrich, St Louis, MO, USA). In genome wide expression profiling (GEP) analysis, PBMCs were incubated with CD19Microbeads (Miltenyi Biotech, Auburn, CA, USA), obtaining a purity >99% as assessed by flow cytometry using PE-conjugated CD19 (Miltenyi Biotech). GS-1101 was provided by Gilead Seattle and was dissolved in DMSO at 10mM and stored at -20°C until use.

Cell cultures

The murine stromal cell lines KUSA-H1 and 9-15c, both derived from bone marrow of a C3H/He mouse, were purchased from the RIKEN Cell Bank (Ibaraki, Japan) and maintained in RPMI 1640 medium supplemented with 2.05 mM L-glutamine (Hyclone, Logan, UT), 10% FBS (SACF Biosciences) and penicillin-streptomycin (Cellgro). The human mesenchymal cell line StromaNKtert, derived from bone marrow and immortalized by human telomerase reverse transcriptase (hTERT)(110) containing also exogene MFG-tst-IRES-neo was purchased from RIKEN and maintained in minimum essential Medium Eagle with Earl salts and L-glutamine (α-MEM; HyClone) supplemented with 12.5% FBS (SACF Biosciences, Lenexa, KS), 12.5% human serum (Cellgro), 1μM hydrocortisone (Sigma-Aldrich, St. Louis, MO) and 100μM 2-mercaptoethanol (Sigma-Aldrich). Primary human MSCs, isolated from bone marrow of CLL patients, were isolated and expanded as previously described(111). Human umbilical vein endothelial cells (HUVEC, Cascade Biologics, Portland OR) pooled from multiple donors, were

cultured in M200 PRF medium supplemented with Low Serum Growth Supplement kit (Cascade Biologics) with a final concentration of 2% FBS, 1μg/ml hydrocortisone, 10ng/ml human epidermal growth factor, 3 ng/mL basic fibroblast growth factor and 10 μg/mL heparin. The human microvascular endothelial cell line HMEC-1 was purchased from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and was maintained in MCDB131 medium (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (SACF Biosciences), 10 mM/L L-glutamine (Hyclone), 10ng/mL Epidermal Growth Factor (Becton-Dickinson) and 1μg/mL hydrocortisone (Sigma-Aldrich). The murine vascular endothelial cell line UV-2, transformed by ultraviolet, was purchased from RIKEN and maintained in DMEM medium supplemented with 10% FBS (SACF Biosciences), 2.05 mM L-glutamine (Hyclone) and penicillin-streptomycin (Cellgro). RAMOS cells (ATCC, LGC Standards, UK) were cultured in RPMI 1640 supplemented with 2.05 mM L- glutamine (Hycloe, Logan, UT), 10% FBS (SACF Biosciences) and penicillin-streptomycin (Cellgro).

CLL viability in EC and BMSC co-cultures

For co-culture experiments two different EC (HUVEC, UV-2) and 2 different BMSC (NKtert, KUSA-H1) were seeded the day before the experiment onto a 24-well plate at a concentration of $5x10^4$ cells/well and incubated at 37°C in 5% CO_2 in the corresponding medium. After confirming the confluence of the cell layer by phase contrast microscopy, CLL cells were added onto the cell layer at a final concentration of $2x10^6$/ml. As control, CLL cells were cultured in medium suspension at a concentration of $2x10^6$/ml.

For inhibition studies, cells were incubated for 1 h with the following blocking antibodies at 10 mg/mL: anti-CD11a (clone 25.3; Immunotech, Marseille, France), anti-CD18 (clone L130; BD Biosciences), anti-CD49d (clone HP2/1; Immunotech) and anti-CD29 (clone LIa1/2; Immunotech) for CLL and anti-CD106 (clone 51-10C9; BD Biosciences) and anti-CD54 (clone BBIG-I1; R&D Systems, Minneapolis, MN, USA) for HUVEC before co-culture. An isotypic antibody (IgG, clone 11711, R&D Systems) was added as an irrelevant control. To evaluate the effect of GS-1101 on CLL cells viability, co-cultures were treated with 5μM GS-1101. At the indicated

time-points, CLL cells were collected and tested for cell viability. Determination of CLL cell viability in the presence or absence of GS-1101 was assessed with the analysis of mitochondrial transmembrane potential by 3,3' dihexyloxacarbocyanine iodide (DiOC6; Molecular Probes, Invitrogen) and cell membrane permeability to Propidium Iodide (PI; Sigma-Aldrich)(42). CLL cells viability was determined at baseline and after 24, 48 and 72 hours.

Genome-wide expression profiling

Purified CD19+ CLL cells were suspended at a final concentration of $1X10^6$/ml in RPMI 1640+10%FBS medium (Invitrogen, Carlsbad, CA, USA) and then plated in 24-well plates alone (CLL only) or onto endothelial layers (CLL HC) formed by HUVEC. For genome-wide expression profiling (GEP) analysis, all samples collected from co-culture conditions were investigated by flow cytometry using PE-conjugated CD19 (Miltenyi Biotech) to exclude HUVEC contamination. GEP analysis was performed on total RNA extracted from purified CD19+ cells (RNeasy Midi kit Plus, QIAGEN, Valencia, CA, USA) isolated from 9 individual CLL patients which were separated in 3 experimental subsets: (i) freshly isolated cells (CLL baseline), (ii) CLL cells cultured in medium alone for 48 hours (CLL only) and (iii) CLL cells co-cultured 48h on HUVEC layer (CLL HC). High quality RNAs were amplified and Cy3-labeled using Low Input Quick Amp Labeling kit (Agilent Technologies, Palo alto, CA, USA). Agilent RNA One-Color Spike-In was added in each sample to provide positive controls for monitoring the microarray workflow from sample amplification and labelling to microarray processing. It contains 10 *in vitro* synthesized, polyadenylated transcripts derived from the Adenovirus E1A transcriptome that are premixed at various ratios. All cRNA products were purified using RNeasy columns (QIAGEN). Samples had to contain at least 6 pmole of cyanine dye/μg of cRNA to be considered suitable for subsequent hybridization. Cy3-labeled cRNA (1.65 μg) were fragmented to an average size of 50-100 nt by incubation at 60℃ for 30 min using *in situ* Hybridization kit-plus (Agilent). Samples were hybridized for 17 hours at 65℃ on 4×44K Whole Human Genome Microarray (Agilent) and then

scanned using laser scanner (Agilent Technologies). Fluorescence data were analyzed with Feature Extraction Software v.10.5 (Agilent Technologies) an QC Chart tool v.1.3. Agglomerative two-dimensional clustering analysis and supervised analyses based on t-test were performed using Gene Spring GX (Agilent) software. Genes were defined as differentially expressed between groups at a significant level of $p<0.05$ and with a fold change cut off $\pm$ 2 in all the pair wise comparisons. Gene Ontology Tool (http://www.geneontology.org/) and PANTHER Classification System (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships, http://www.pantherdb.org/) were used to unravel biological function and pathway represented in gene lists.

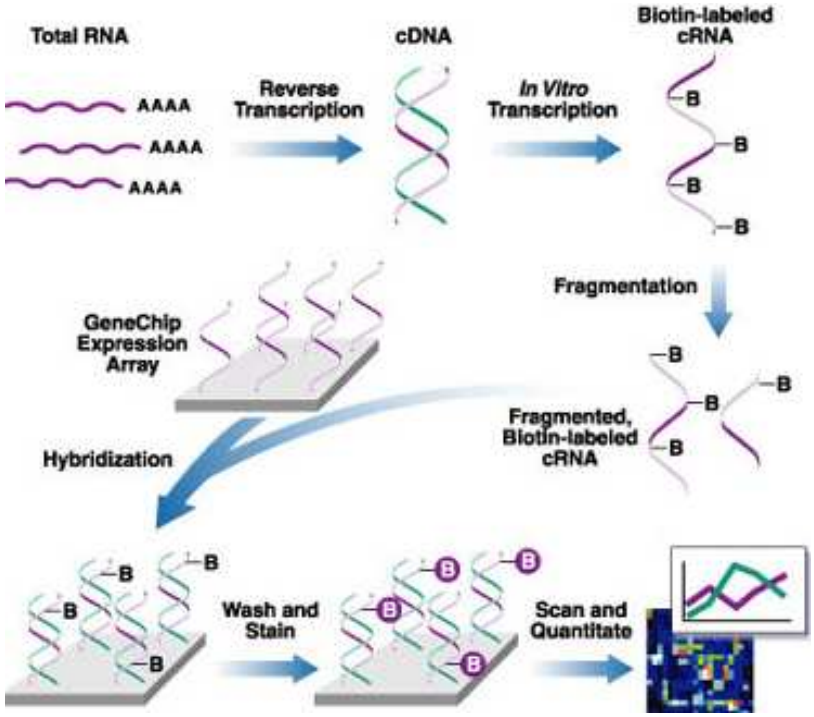


Figure 8. Genome wide expression profiling procedure.

Confocal microscopy

For confocal microscopy studies, CLL HC layers were fixed with paraformaldehyde (4% in phosphate-buffered saline), per- meabilized using saponin (0.1% in phosphate-buffered saline) and saturated using non-immune goat serum. HUVEC were stained with a mouse anti-human CD31 Alexa 647-conjugated (AbD Serotec, Oxford, UK). Nuclei were counter-stained with DAPI (Invitrogen, Milan, Italy). Slides were analyzed using a Leica TCSSP5 confocal micro- scope at 63x magnification. Images were acquired using LAS AF (Leica, Milan, Italy) and processed using Photoshop (San Jose, CA, USA) software.

VCAM-1 and VLA-4 flow-cytometry staining

HUVEC and HMEC-1 were grown in the appropriate cell culture medium until confluence and then stimulated with TNFα (R&D Systems) at a concentration of 10ng/ml for 24 hours and then detached with 5mmol/L EDTA/EGTA in Dublecco's PBS (HyClone), pelleted and washed with RPMI 1640 + 0.5% bovine serum albumin (BSA). Then cells were stained with PE-conjugated VCAM-1 antibodies (BD Biosciences) or isotype-matched control antibodies (mouse IgG1-PE; BD Biosciences) and incubated for 20 minutes at 4°C. VLA-4 expression in CLL cells was detected in 10 CLL samples using anti-CD19 APC and anti VLA-4 PE (BD Biosciences) or isotype control. Samples were washed with FACS Buffer and resuspended in 400 μl for assessment on a FACS Calibur (BD Biosciences).

Static cell adhesion assay

Two different EC (HUVEC and HMEC-1) and two different BMSC (9-15c and CLL-MSC) were seeded for 24 hours onto 24-well plate at a concentration of $0.8x10^5$ cells/ml in the appropriate cell culture medium and then stimulated for 24 hours with 10ng/mL TNFα (R&D Systems, Minneapolis, MN)(112). After 1 hour incubation at 37°C and 5% CO_2 in complete medium, CLL cells were added onto the confluent endothelial and stromal cell layers to a final concentration of $5x10^6$/well in presence or

absence of 5 μM GS-1101, and the plates were incubated at 37°C for 4 hours. After incubation the cells that were not adhered to the EC and BMSC cell layers were removed by washing the wells with RPMI 1640 medium. The complete removal of non-adherent cells and the integrity of the cell layer was assessed by phase contrast microscopy and documented photographically. The EC and BMSC layers containing the adherent CLL cells then were detached by incubation for 1 minute with trypsin/EDTA solution pre-warmed at 37°C (Invitrogen), followed by neutralization of the trypsin with 1 ml of RPMI/10% FBS. Cells then were washed and suspended in a final volume of 0.6 ml medium for counting by flow cytometry (FACS Calibur,BD Biosciences) for 20 seconds at 60μl/min in triplicates. A lymphocyte gate was set using the different relative size and granularity (forward scatter and side scatter) to exclude endothelial and stromal cells from the count, as described before(113). The number of adherent cells under each condition was expressed as percentage of the control for each experiment.

Parallel plate flow detachment assay

Slides were coated with HUVEC in culture media for 24 hours and then stimulated with TNFα (10ng/ml) (R&D systems) in fresh medium for 24 hours. After 1 hour incubation in complete medium at 37°C in 5% CO_2, $5x10^6$ CLL cells, treated with 5μM GS-1101 for 1 hour at 37°C and untreated controls, were injected into the flow chamber and allowed to settle on the slides for 20 minutes. For cell adhesion to VCAM-1 integrin (R&D Biosystems), slides were coated with 10 μg/ml VCAM-1 or ovalbumine (Sigma-Aldrich) overnight at 4℃. The slides were washed with PBS and blocked with 2% ovalbumine before addition of Ramos. $5x10^6$ Ramos cells were either treated or not treated with 5 μM GS-1101 for 1 hour at 37℃ and allowed to adhere to VCAM-1 slides for 20 minutes. Using a computer controlled syringe pump (Harvard Apparatus Holliston, MA), an increasing linear gradient of shear flow was applied over the adherent cells for 300 seconds and the number of cells remaining adhered was recorded by digital microscopy. Shear stress calculations were determined every 50 seconds where the shear stress in dynes/cm^2 was

defined as $6\mu Q/wh^2$ where μ is the viscosity of the medium (0.007), Q is the flow rate in cm^3/s, w is the width of the chamber (0.3175 cm) and h is the height of the chamber (0.0254 cm). All the samples were assayed in triplicates.

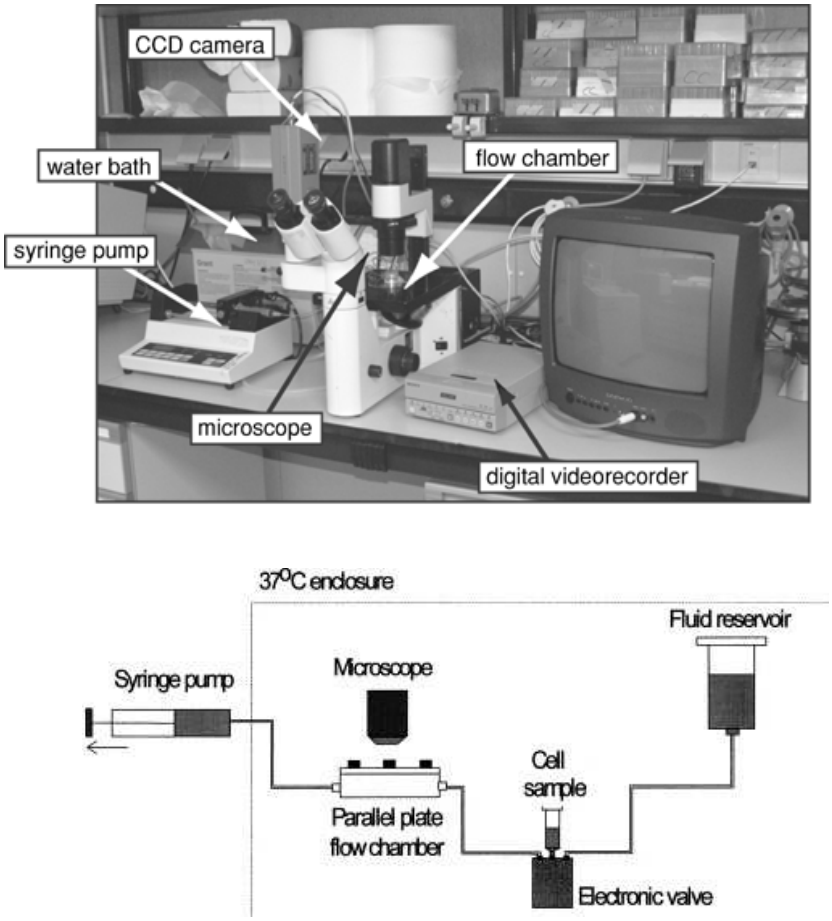


Figure 9. A parallel-plate fluid flow chamber is a benchtop (in vitro) model that simulates fluid shear stresses on various cell types exposed to dynamic fluid flow in their natural, physiological environment

Detection of phospho-proteins by flow cytometry

Phospho-protein expression in CLL cells was detected after 1 hour and 24 hours of co-culture with HMEC-1 and after 1 hour of stimulation with a monoclonal antibody for $\alpha_4\beta_1$ integrin (19H8)(114) with antibody crosslinking using F(ab') 2 fragments of goat anti-mouse IgG (Invitrogen) in presence or absence of 5μM GS-1101. The following antibodies were used for detection of activated phospho-proteins: anti-pAkt T308 AlexaFluor 488 (Cell Signaling Tecnology) and anti-CD19 PE-cy5 (BD Biosciences) or an isotype-matched control antibody (rabbit IgG-AlexaFluor 488, Cell Signaling Tecnology). $1x10^6$ CLL cells were supended in PBS containing 4% paraformaldehyde to block stimulation reaction. After 10 minutes incubation, cells were washed once in cold PBS and stored overnight. Then cells were washed and resuspended in PBS containing 1% bovine serum albumin and incubated with antibodies for 1 hour at room temperature. Samples were washed with PBS + 1% BSA and resuspended in 350 μl PBS + 1% BSA for assessment on a FACS Calibur (BD Biosciences).

Immunoblotting

CLL cells were starved in RPMI + 0.5% BSA for 2 hours at 37°C then cultured in suspension or co-cultured with HMEC-1 for 1 hour and 24 hours at 37°C. Cells were washed once with cold PBS and lysed ice for 30 minutes with lysis buffer containing 25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl2, 0.5% sodium deoxycholate, 20 mM glycerophosohate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitor. Cells were pelleted with centrifugation at 14.000 rpm for 15 minutes at 4°C and supernatant was stored at -80°C. Protein content was determined using the detergent compatible (DC) protein assay kit, according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Aliquots (50 ug) of total cell protein were boiled with Laemmli sample buffer and loaded onto 4% to 12% SDS-polyacrylamide gradient gels and transferred to nitrocellulose membranes (GE, Osmonics Labstore, Minnetonka, MN). Membranes were blocked for 1 hour in PBS-Tween containing 5% nonfat dried milk and incubated with

primary antibodies either overnight or for 3 hours followed by species-specific horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10000) for 1 hour. The blots were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL) and normalized to the actin levels in each extract. Membranes were probed at 4°C with the following primary antibodies: anti-total AKT, phospho-AKT (T308), (Cell Signaling, Danvers, MA), and β-Actin (Sigma- Aldrich). Immunoreactive bands were visualized using peroxidase-conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence detection system (Pierce Biotechnology).

Data analysis and statistics

Results are shown as mean ± standard error mean (SEM) of at least 3 experiments. Analyses were performed using GraphPad Prism 4 Software for Macintosh (GraphPad software Inc., La Jolla, CA, USA). Student paired or unpaired T tests were used for statistical comparison. The Spearman test was used for estimating correlations between quantitative parameter. A P value ≤ 0.05 was considered statistically significant. Flow cytometry data were analyzed using FlowJo version 8.8.7 software (Treestar).

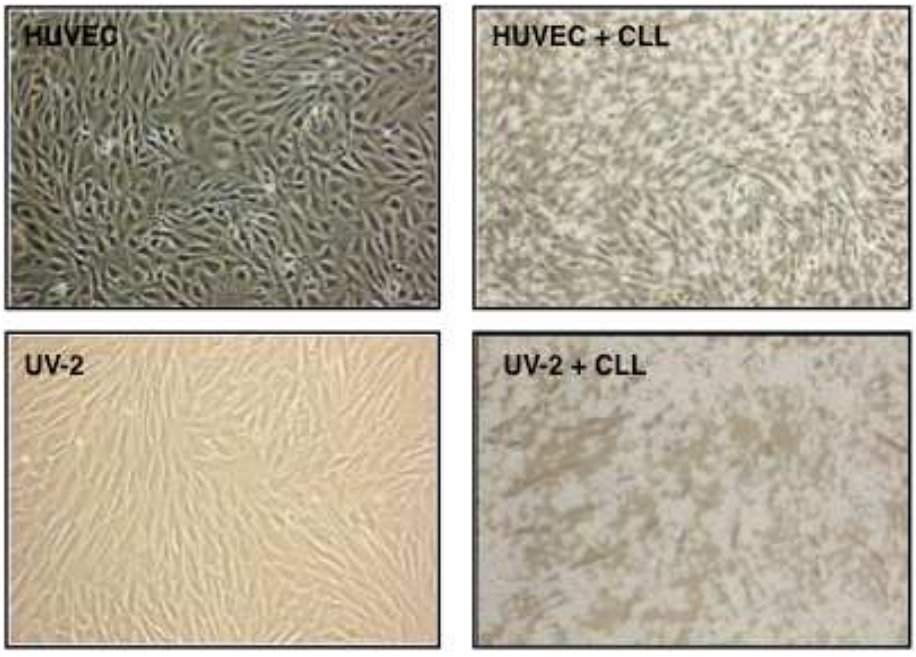
RESULTS

1. EC and BMSC rescue CLL cells from apoptosis

Microenvironmental accessory cells are well recognized for their capacity to protect CLL cells from apoptosis. Here we tested in a direct comparison assay the ability of BMSC and EC to protect CLL cells from spontaneous apoptosis. CLL cells were cultured in direct contact with endothelial cells (HUVEC, UV-2) or stromal cells (KUSA-H1, Nk-Tert) or in medium alone for 72h (Figure 10). CLL viability was determined at 24h, 48h and 72h using DiOC6-PI staining by flow cytometry.

ENDOTHELIAL CELLS

A)



STROMAL CELLS

B)

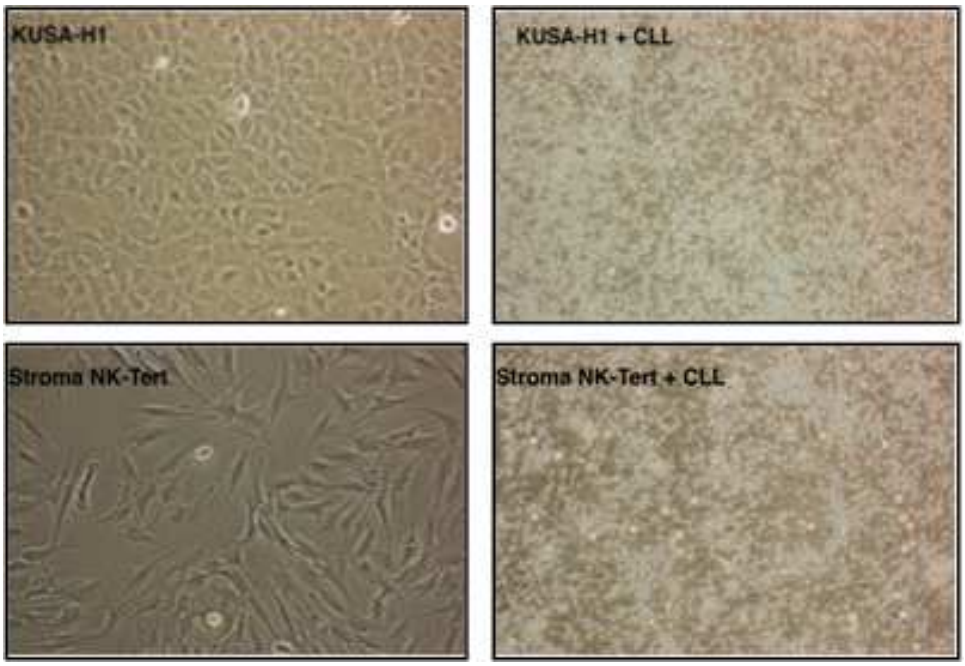


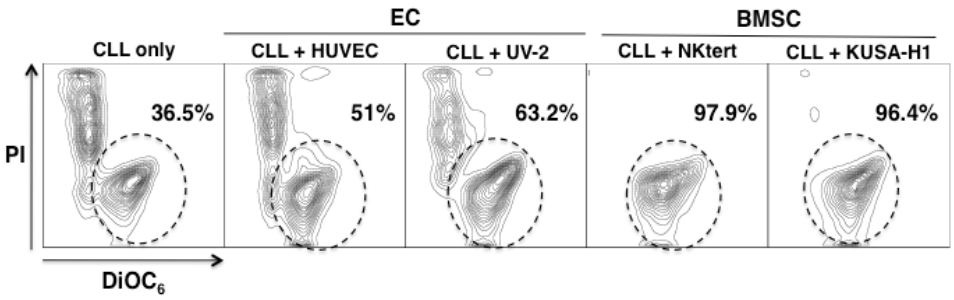
Figure 10. Phase-contrast photomicrographs show four different cell lines used to test CLL viability in co-culture: A) Endothelial cells (EC) and B) Stromal cells (BMSC).

Both EC and BMSC significantly protected CLL cells from apoptosis at different time-points. The mean viability of CLL cells cultured in medium alone was 44.1% (±4.1%) after 24 hours, 37.5% (±4.1%) after 48 hours and 38.2% (±4.25%) after 72 hours (n=7). In presence of human (HUVEC) or murine (UV-2) endothelial cells, the mean viabilities of CLL cells were significantly higher at 56.8% (±3.2%, $p<0.05$, n=7) or 63.7% (±5.9%, $p<0.05$, n=7) after 24 hours, 53.2% (±4.3%, $p<0.05$, n=7) or 61.9% (±5.3%, $p<0.01$, n=7) after 48 hours and 50.7% (±4.7%, ns) or 58.6% (±4.6%, $p<0.01$, n=7) after 72 hours. Human (NKtert) and murine (KUSA- H1) BMSC were even more effective in preserving CLL cell viability, the mean viability of CLL

cells were 72.0% (±2.1%, p<0.01, n=7) or 78.0% (±4.1%, p<0.01, n=7) after 24 hours, 96.7% (±0.6%, p<0.01, n=7) or 93.7% (±1.0% p<0.01, n=7) after 48h and 98.5% (±0.2%, p<0.01, n=7) or 95% (±0.9%, p<0.01, n=7) after 72 hours (Figure 11).

Our data demonstrate that endothelial and marrow stromal cells both support the viability and protect CLL cells from apoptosis. While both, EC and BMSC provided CLL cells with protection we noted comparably less protection from spontaneous apoptosis when compared directly with BMSC.

A)



B)

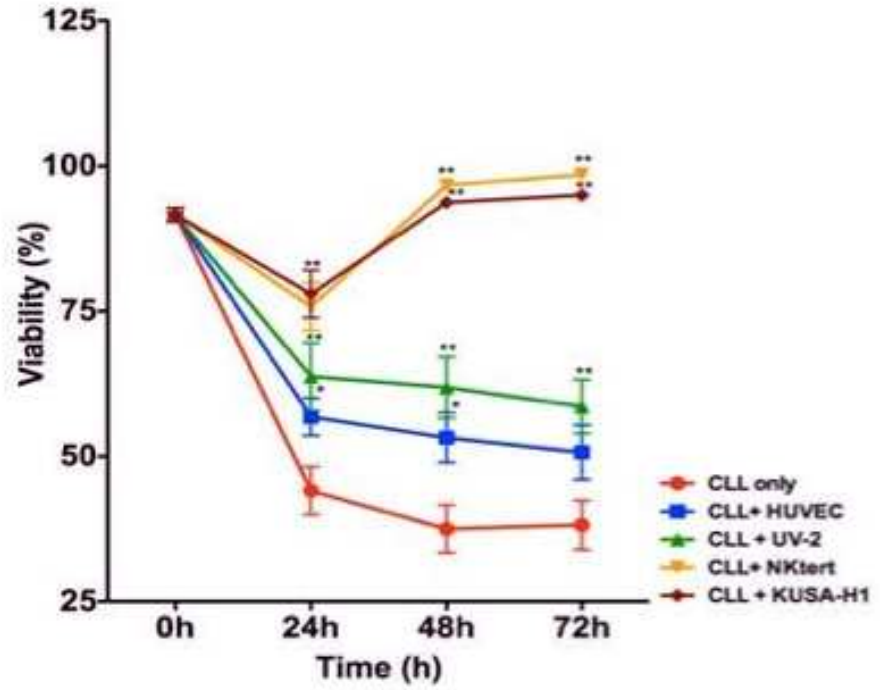


FIGURE 11. (A) CLL cells are cultured in medium alone, in co-culture with two EC (HUVEC and UV-2) and in co-culture with two BMSC (NKtert and KUSA-H1) for three different time-points: 24, 48, 72 hours. Contour plots display a representative CLL sample viability after 48 hours. The gates represent the viable cells positive to $DiOC_6$ and negative to PI. (B) The line graph display mean±SEM CLL viabilities from 7 different patients after 24, 48, 72 hours of culture. Both EC and BMSC significantly support CLL viability at different time-points (*$p<0.05$; **$p<0.01$) compared to CLL culture in medium alone.

2. EC and BMSC induce a peculiar gene expression profile in CLL cells

Given the ability of endothelial cell to protect CLL cells from apoptosis, we investigated the mechanisms underlying the EC-CLL cell crosstalk by comparative gene expression profile of CLL cultured in medium alone or in contact with endothelial cell (HUVEC). From each patient, three set of samples were collected: (i) CLL cells from peripheral blood (CLL at baseline), (ii) CLL cells cultured 48h in medium alone (CLL only) and (iii) CLL cultured 48h in contact with endothelial cell layer (CLL HC). The whole-genome gene expression profile of all CLL samples were inspected by microarrays. We compared gene expression profiles between CLL cultured in contact with EC layer with CLL at baseline to unravel the transcriptional modifications induced by EC cells. Overall 1944 genes were found to be modulated by treatment ($FC \geq 2$, $p<0.05$): 1217 genes were up-regulated in HC condition and 727 genes were down-regulated. Differentially expressed genes were analyzed with the GeneSpring Gene Ontology browser tool to identify the Gene Ontology categories most represented in gene-list (Table 1). CLL cells in co-culture with EC up-regulated a series of genes involved in angiogenesis (GO: angiogenesis) and regulation of endothelial cell function (GO: blood vessel development, vasculature development, blood vessel morphogenesis and patterning of blood vessels). Moreover, genes involved in cell motion and migration as well as in reorganization of actin cytoskeleton were significantly modulated (GO: regulation of cell motion, regulation of cell migration, actin cytoskeleton reorganization and biogenesis). Finally, we found an enrichment of genes involved in response to external stimuli, to stress condition, and to hypoxia (GO: response to external stimuli, response to stress and response to hypoxia). The most represented cellular pathways included the TGFβ and Wnt signalling pathways, inflammation

mediated by chemokine and cytokine signaling pathways and the integrin signaling pathways as well as a set of genes coding for BTB-Kelch proteins (Figure 12).

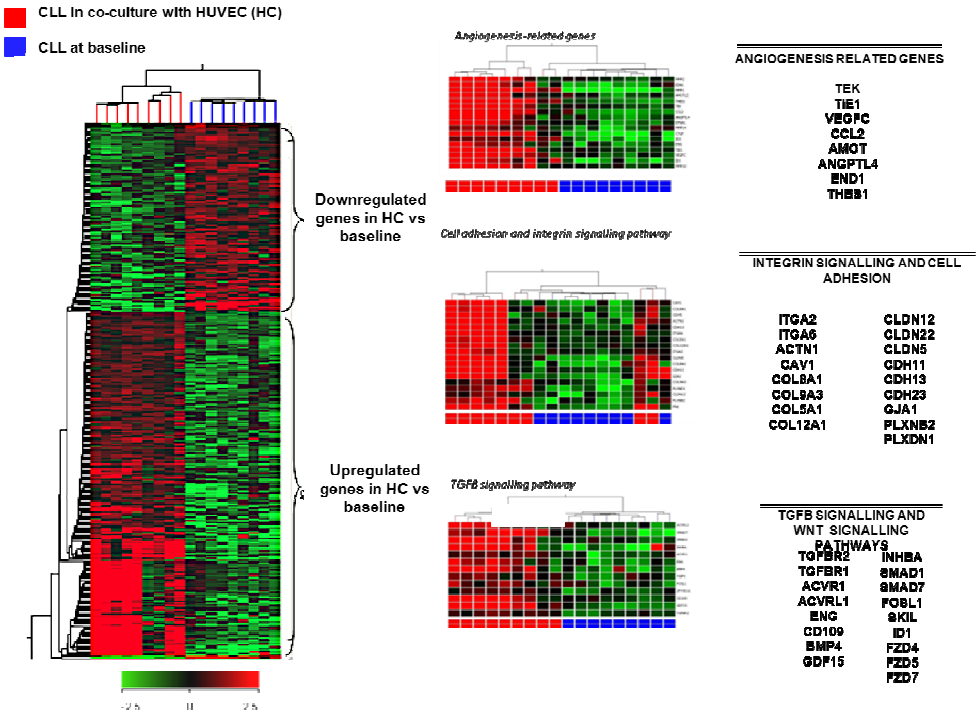


Figure 12. Heat map depicting differentially expressed genes between CLL cells at baseline and CLL cells in co-culture for 48 h. Sets of differentially expressed genes involved in angiogenesis, cell adhesion and the TGFb signaling pathway are indicated. The data are represented in a grid format in which each column represents a case and each row a single gene. Normalized intensity signals are depicted in the pseudo color scale as indicated.

Table 1 GO categories of modulated genes in CLL by co-culture with endothelial cell layer

GO ACCESSION	GO Term	p-value
GO:0001568	blood vessel development	1.0E-06
GO:0048514	blood vessel morphogenesis	1.0E-06
GO:0001944	vasculature development	1.0E-06
GO:0001525	angiogenesis	9.3E-06
GO:0048646	anatomical structure formation	1.5E-05
GO:0051270	regulation of cell motion	4.3E-04
GO:0030334	regulation of cell migration	4.9E-04
GO:0009887	organ morphogenesis	4.9E-04
GO:0042060	wound healing	5.8E-04
GO:0001569	patterning of blood vessels	6.1E-03
GO:0050793	regulation of developmental process	6.1E-03
GO:0009968	negative regulation of signal transduction	1.2E-02
GO:0046872	metal ion binding	1.7E-02
GO:0005925	focal adhesion	1.7E-02
GO:0006937	regulation of muscle contraction	2.2E-02
GO:0008360	regulation of cell shape	2.2E-02
GO:0050878	regulation of body fluid levels	2.3E-02
GO:0005924	cell-substrate adherens junction	2.5E-02
GO:0043167	ion binding	2.5E-02
GO:0065007	biological regulation	2.5E-02
GO:0007596	blood coagulation	2.8E-02
GO:0050817	coagulation	2.8E-02
GO:0043169	cation binding	2.8E-02
GO:0009966	regulation of signal transduction	4.6E-02
GO:0030055	cell-substrate junction	4.6E-02
GO:0006950	response to stress	5.0E-02
GO:0046914	transition metal ion binding	5.2E-02
GO:0007599	hemostasis	5.6E-02
GO:0022604	regulation of cell morphogenesis	6.2E-02
GO:0009605	response to external stimulus	7.4E-02
GO:0009653	anatomical structure morphogenesis	7.5E-02
GO:0001666	response to hypoxia	8.6E-02
GO:0048513	organ development	8.6E-02
GO:0050789	regulation of biological process	8.9E-02
GO:0030036	actin cytoskeleton organization and	9.2E-02

	biogenesis	
GO:0032502	developmental process	9.8E-02
GO:0048661	positive regulation of smooth muscle cell proliferation	9.8E-02

We next compared our gene expression profiling to those previously found in other co-culture of CLL cells with nurse-like cells and the bone marrow-derived stromal cell line. We found that different microenvironmental elements are able to induce distinct gene expression signatures in CLL cells with only few genes being modulated in a similar way by different cell types (Figure 13).

1) Contact with HS-5 stromal cells influences several genes involved in important cellular networks such as MYC, p38 MAPK, NFKB, INFλ as well as AKT and ERK1/2. Moreover microarray data show an upregulation of a variety of chemokines and cytokines in CLL samples under survival-inducing culture conditions in particular CCL4, IL6, CXCL1, CXCL5 (42).

2) The most highly up-regulated genes by nurse-like co-culture are: the CC chemokine CCL4, the BCMA (TNFRSF17), early growth response protein 2 gene (EGR2) and the Fc receptor-like 5 gene (FCRL5)(115).

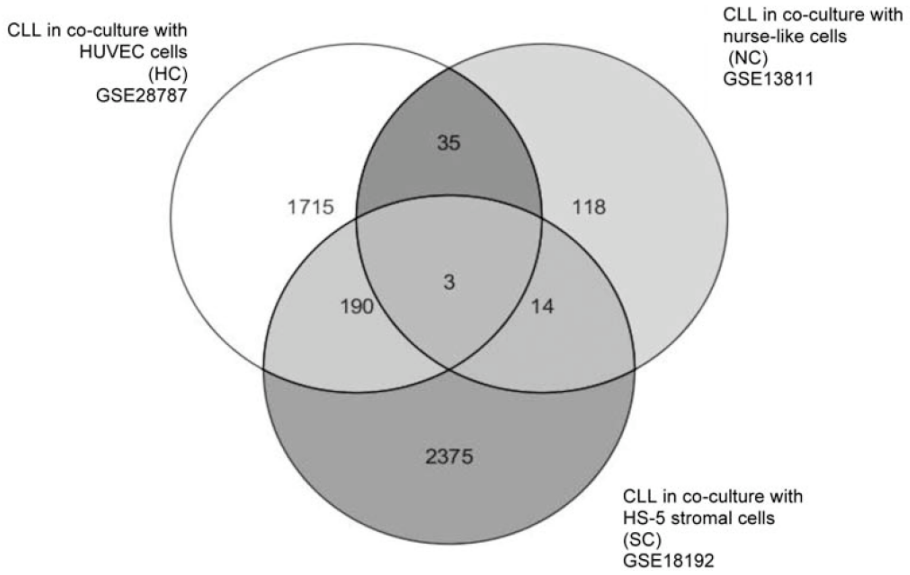


Figure 13. Venn diagram comparing three sets of differentially expressed genes (all FC=2, P<0.05) in CLL co-cultured on HUVEC (HC), on nurse-like cells (NC) and on HS-5 stromal cells (SC). Few genes were shared by HC, NC and SC culture conditions.

3. Contact with endothelial cells increases the pro-angiogenic and anti-apoptotic profile of chronic lymphocytic leukemia cells

Deeper analyses of GEP data were performed focusing on two categories of differentially expressed genes: (i) those coding for soluble factors involved in angiogenesis and/or regulating microenvironment components, and (ii) those coding for proteins involved in apoptosis regulation (*Table 2*). We found that CLL cells in culture with EC showed a 22.6-fold increase of the C-C motif chemokine CCL2, capable to inducing pro-angiogenic stimuli on EC, recruiting tumor-associated monocytes (TAM) and suppressing cytotoxic lymphocytes (*P*=0.0032). In addition, a 6.5-fold increase of PDGFC expression, a chemoattractant for mesenchymal cells, was induced in CLL cells by EC co-culture (*P*=0.0051). Other soluble factors up-regulated by EC/CLL contact compared to baseline were VEGFC (FC=9.4, *P*=0.0061), ANGTL4 (FC=8.6, *P*=0.015), ENG (FC=4.0, *P*=0.025), EDN1 (FC=9.2, *P*=0.0061), AMOTL2 (FC=4.3, *P*=0.019) and THBS1 (FC=45.1, *P*=0.0004) as well as the metalloproteases MMP2 (FC=8.3, *P*=0.02) and MMP14 (FC=3.0, *P*=0.039). All these transcriptional modifications were not detected in the GEP of CLL cells cultured alone. In order to confirm the modulation of vascular cytokines in CLL cells cultured in the presence of the EC layer, we measured the secreted levels in conditioned media collected after 48 h-culture (Figure 14). We found that HUVEC cultured in medium alone secreted VEGFC (643.3±71.7 pg/mL), Ang2 (1054.9±570.0 pg/mL), ENG (910.0±10.0 pg/mL), THBS-1 (1602.0±310.0 ng/mL) and CCL2 (772.5±60.0 pg/mL) but small levels of Ang1 (24.5±3.1 pg/mL). When cultured alone, CLL cells secreted low levels of VEGFC (33.4±27.9 pg/mL), Ang2 (31.4±8.6 pg/mL), Ang1 (24.5±3.5 pg/mL) and CCL2 (11.1±1.7 pg/mL) and no detectable levels of THBS-1 or ENG. The CLL/EC co-culture condition increased the secretion of VEGFC (1043.2±57.7 pg/mL), Ang2 (4935.7±1325.1 pg/mL), ENG (1603.1±273.7 pg/mL), THBS-1 (2774.8±357.4 ng/mL) and CCL2 (3162.1±721.2 pg/mL) whereas it maintained low Ang1 secretion (32.7±3.7 pg/mL). In addition, CLL cells (n=3) were cultured for 48 h on the HUVEC layer and then collected and cultured for a further 48 h in fresh medium. We found that media conditioned by CLL cells previously stimulated by EC contact compared to

media conditioned by unstimulated CLL cells were enriched in THBS-1 (from 0 to 5 ng/mL), Ang2 (from 27 to 59 pg/mL), ENG (from 0 to 370 pg/mL), VEGFC (from 89 to 897 pg/mL) and CCL2 (from 11 to 3188 pg/mL) but not Ang1 (from 27 to 21 pg/mL).

Table 2. Differentially expressed genes in CLL cells co-cultured on endothelial layer

Cellular Pathway	Up/ down	Gene symbol	Gene name	Fold change	p-value
TGFβ signalling	UP	TGFBR2	TGF-beta receptor type-2	2.3	0.00276
	UP	TGFBR1	TGF-beta receptor type-1	2.6	0.01437
	UP	ACVR1	Activin receptor type-1	2.8	0.01160
	UP	ACVRL1	Serine/threonine-protein kinase receptor R3	30.7	0.00580
	UP	ENG	Endoglin	4.0	0.02540
	UP	CD109	CD109 antigen	11.7	0.01829
	UP	BMP4	Bone morphogenetic protein 4	8.3	0.02216
	UP	GDF15	Growth/differentiation factor 15	18.6	0.00073
	UP	INHBA	Inhibin beta A chain	11.4	0.03279
	UP	ZFYVE16	Zinc finger FYVE domain-containing protein 16	2.4	0.00496
	UP	SMAD7	Mothers against decapentaplegic homolog 7	14.0	0.00140
	UP	SMAD1	Mothers against decapentaplegic homolog 1	6.6	0.02538
	UP	FOSL1	Fos-related antigen 1	2.5	0.04631
	UP	SKIL	Ski-like protein	4.0	0.00014
	UP	ID1	DNA-binding protein inhibitor ID-1	14.7	0.00098

	DOWN	CITED1	Cbp/p300-interacting transactivator 1	-2.6	0.00821
Wnt signaling	UP	FZD4	Frizzled-4	16.1	0.0026
	UP	FZD5	Frizzled-5	5.3	0.0044
	UP	FZD7	Frizzled-7	4.3	0.0060
Angiogenesis	UP	TIE2	Angiopoietin-1 receptor	10.7	0.0170
	UP	TIE1	Tyrosine-protein kinase receptor Tie-1	18.9	0.0088
	UP	VEGFC	Vascular endothelial growth factor C	9.4	0.0061
	UP	CCL2	C-C motif chemokine 2	22.6	0.0032
	UP	AMOTL2	Angiomotin-like protein 2	4.3	0.0191
	UP	ANGPTL4	Angiopoietin-related protein 4	8.6	0.0154
	UP	EDN1	Big endothelin-1	9.2	0.0061
	UP	THBS-1	Thrombospondin-1	45.1	0.0004
	DOWN	FLT4	Vascular endothelial growth factor receptor 3	-2.1	0.0048
Response to hypoxia	UP	HIF1A	Hypoxia-inducible factor 1 alpha	3.9	0.0111
	UP	HIF2A/EPAS1	Endothelial PAS domain-containing protein 1	12.9	0.0067
	UP	HIF3A	Hypoxia-inducible factor 3 alpha	2.2	0.0330
Integrin signalling	UP	ITGA2	Integrin alpha-2	5.2	0.0219
	UP	ITGA6	Integrin alpha-6 light	6.7	0.0213

			chain		
	UP	ACTN1	Alpha-actinin-1	11.9	0.0067
	UP	CAV1	Caveolin-1	18.9	0.0163
	UP	COL8A1	Collagen alpha-1(VIII) chain	15.8	0.0025
	UP	COL9A3	Collagen alpha-3(IX) chain	2.9	0.0413
	UP	COL5A1	Collagen alpha-1(V) chain	4.0	0.0173
	UP	COL12A1	Collagen alpha-1(XII) chain	5.4	0.0371
	DOWN	ITGB7	Integrin beta-7	-2.4	0.0047
Cell adhesion	UP	CLDN12	Claudin-12	4.1	0.0100
	UP	CLDN22	Claudin-22	4.2	0.0383
	UP	CLDN5	Claudin-5	14.5	0.0041
	DOWN	CLDN11	Claudin-11	-2.2	0.0177
	UP	CDH11	Cadherin-11	9.7	0.0293
	UP	CDH13	Cadherin-13	7.1	0.0297
	UP	CDH5	Cadherin-5	5.1	0.0304
	DOWN	CDH23	Cadherin-23	-2.1	0.0038
	UP	GJA1	Gap junction alpha-1 protein	18.4	0.0136
	UP	PLXNB2	Plexin-B2	5.7	0.0311
	UP	PLXND1	Plexin-D1	6.9	0.0025
Others	UP	CCR6	C-C chemokine receptor type 6	7.3	0.0050

	UP	ABCB1	Multidrug resistance protein 1	6.3	0.0445
	UP	PDGFC	Platelet-derived growth factor C, receptor-binding form	6.5	0.0051

** Up- or down-regulated genes in CLL cells co-cultured for 48h on endothelial layer compared to CLL at baseline*

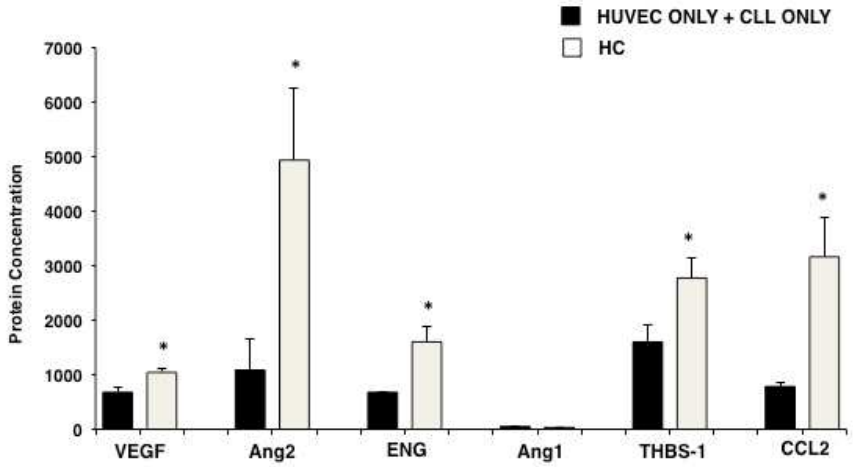


Figure 14. Purified CLL cells were co-cultured on a HUVEC layer near confluence for 48h at which time the conditioned medium (CM) was harvested for ELISA determination of VEGF, Ang2, ENG, Ang1, THBS-1 and CCL2. Histograms represent mean protein concentration ± SEM for eight separate experiments. HUVEC only + CLL only columns represent the sum of protein levels obtained from CM of HUVEC and CLL cultured in medium alone for 48h, whereas HC columns represent protein levels in CM obtained from CLL co-culture on a HUVEC layer. Note the increase in secretion of several angiogenesis-related proteins when CLL cells were co-cultured on HUVEC compared to secretion obtained from both separated cellular elements. Values presented are in pg/ml for VEGFC, Ang2, ENG, Ang1 and CCL2 and in ng/ml for THBS-1. *P<0.05 for HC compared to HUVEC and CLL cells separately.

4. Physical contact with endothelial cells through β_1- and β_2- integrins is essential for protection from spontaneous apoptosis

Next, we investigated whether the intimate contact between leukemic cells and supporting endothelial cells was necessary to protect CLL cells from spontaneous apoptosis.

We collected media from CLL co-cultured on HUVEC for 48 h (HC CM) and then added them to CLL cultures of five patients. A significant reduction of dead cells was measured in CLL cells cultured with the addition of HC CM (41.2±3.4%) compared to cells in unconditioned medium or in medium obtained from CLL cells cultured alone in suspension (52.7±2.3%) (P<0.05). In addition, CLL cells were co-cultured in close contact with the HUVEC layer for 48 h and then leukemic cells were collected and cultured alone in the same HC medium for another 2 days. We found that CLL cells, always maintained in medium alone for 96 h, had a level of 15.4±13.7% of live cells, whereas CLL cells stimulated by contact with HUVEC and then cultured for 48 h alone showed 57.8±12.2% of live cells, comparable to the survival of CLL cells in the permanent co-culture condition (58.2±14.7%) (Figure 15).

These observations suggest that soluble factors present in the co-culture conditioned medium partially contribute to the inhibition of apoptosis in CLL cells.

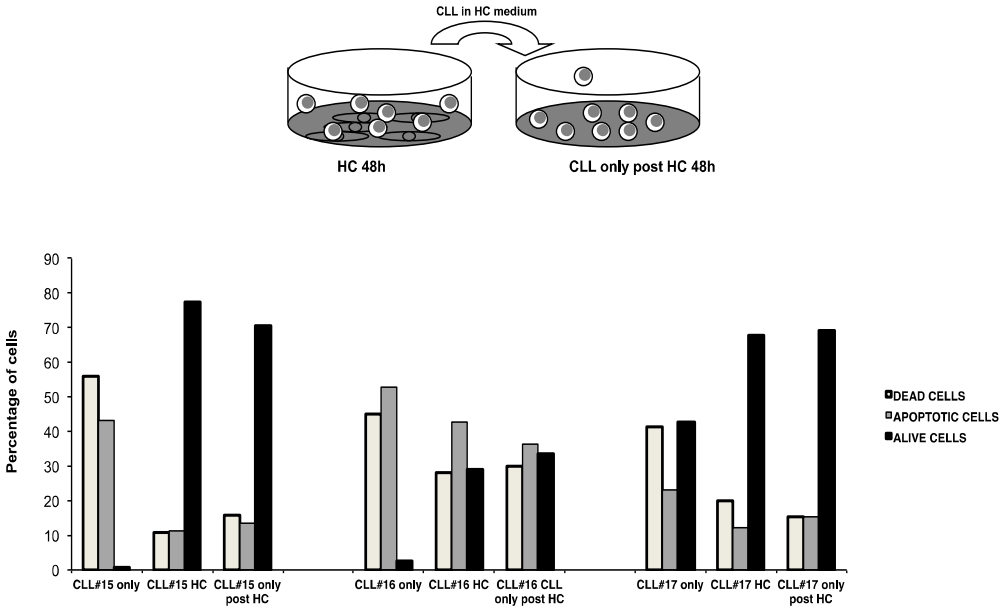


Figure 15. CLL cells were cultured in medium alone (CLL only) or on HUVEC layer (HC) for 96 hours. In addition, as depicted in the above schematic cartoon, CLL were cultured on HUVEC layer for 48 hours and then CLL cells were separated from EC and cultured alone for further 2 days in the same HC medium (CLL only post HC). Data are presented as percentage of dead, apoptotic and alive cells for 3 patients at 96 hours. Note that HC-stimulated CLL maintained increased viability even if separated by EC layer.

CLL cells were cultured in the presence of conditioned medium collected from HUVEC cultured alone near confluence (70–80%) (HUVEC CM) and apoptosis was evaluated at 48h by flow cytometry. HUVEC CM did not exert a protective effect on CLL cells (apoptotic cells 50.2±9.1% in CLL only *versus* 56.1±5.5% CLL with HUVEC CM) (Figure 16). These data indicate that a direct cellular interaction is necessary to mediate CLL survival. To investigate the mechanism of adhesion between CLL cells and HUVEC, neutralizing monoclonal antibodies were added to co-cultures to antagonize β_1- and β_2- integrin pathways.

Blocking CD106 on endothelial cells or CD18 on CLL cells led to almost complete abrogation of apoptosis protection (>70% inhibition of viability compared to the HC condition). Anti-CD49d, anti-CD29 and anti-CD11a induced moderate inhibition of survival (nearly 50% inhibition). There was a slight alteration of the HC system with blocking of CD54 (22% inhibition). With simultaneous blocking of CD49d, CD29 and CD106 or CD11a, CD18 and CD54, 76% and 85% inhibition was observed (Figure 17). Adherent CLL cells to HUVEC or underneath the monolayer were documented by confocal immunofluorescence analysis for CD31 (white) and DAPI (blu) and by May-Grunwald Giemsa staining (Figure 18).

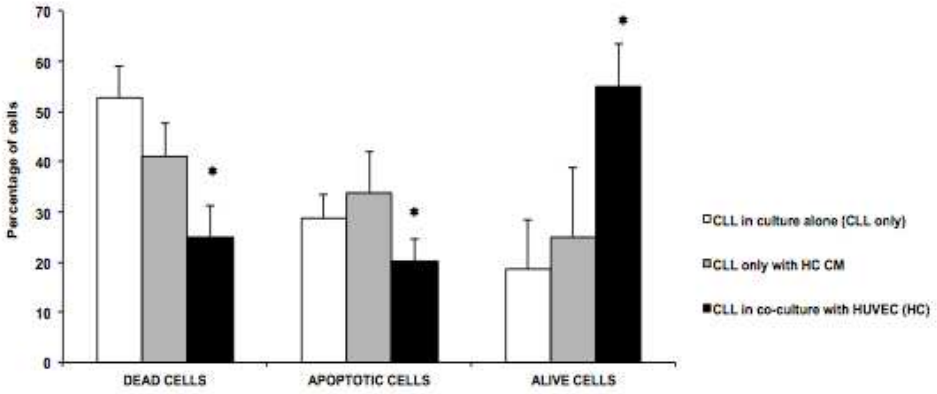


Figure 16. Purified CLL cells (n=5) were cultured in the presence of conditioned medium collected from HUVEC near confluence (HUVEC CM). Following 2 days of culture, leukemic cells were harvested and analyzed for cell apoptosis. * P<0.05 in HC compared to CLL only.

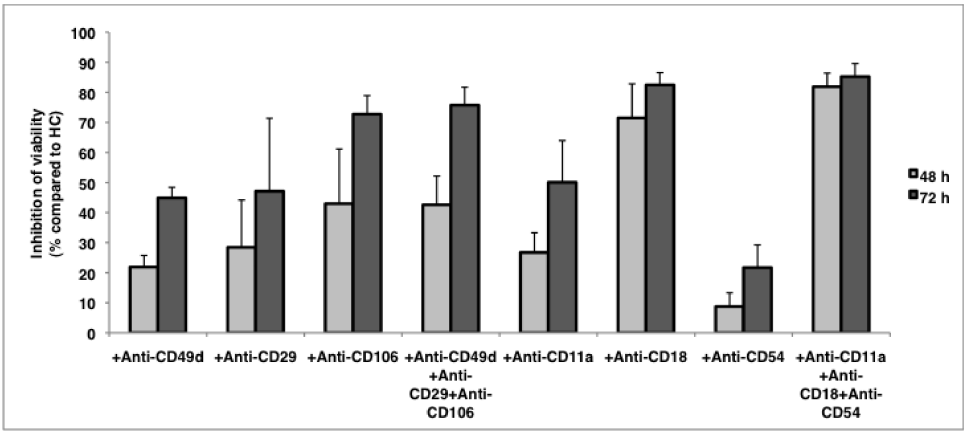


Figure 17. CLL or HUVEC were pre-incubated for 1h with blocking antibodies before starting the co-culture condition. Then, leukemic cells (n=6) were cultured for 48-72h either in medium alone or on a HUVEC layer in the presence or absence of 10 μg/ml of the indicated monoclonal antibodies. Data are presented as mean ± SEM. histograms represent the percentage of viability inhibition determined by blocking antibodies relative to the viability observed in the HC condition with irrelevant isotypic control antibody, added.

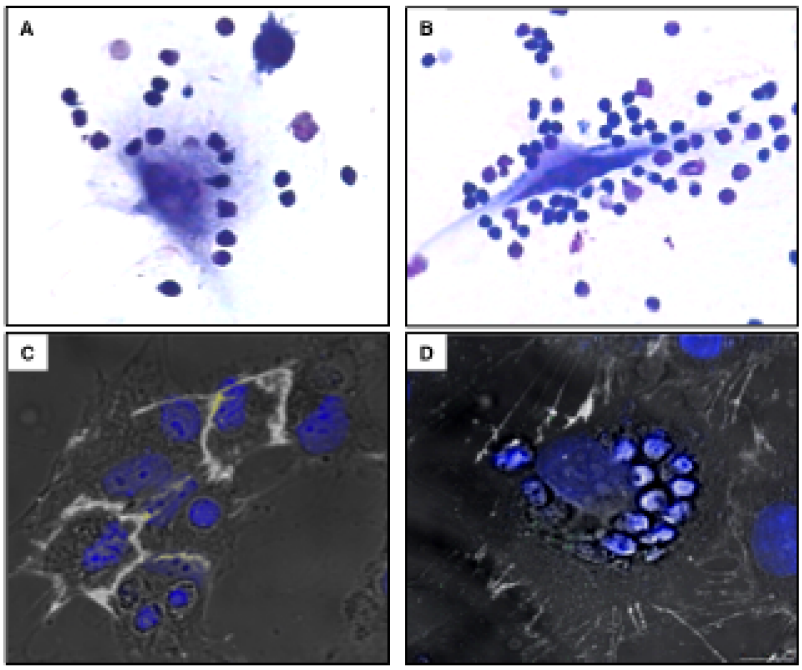


Figure 18. CLL cells from representative patients were co-cultured for 48h on HUVEC, and non-adherent cells were carefully removed before May-Grunwald Giemsa staining (A-B). Specimens were photographed at 400X magnification. Confocal immunofluorescence analysis are shown in panels (C and D) HUVEC were stained using anti-CD31 monoclonal antibody (white), while nuclei were visualized using DAPI (blue). Slides were analyzed using a TCSSP5 Leica confocal microscope at 63x magnification. Confocal planes are shown after merging.

5. *Treatment with TNFα increasesVCAM-1 expression on EC and BMSC and CLL cell adhesion*

Next we investigated the ability of CLL cells to adhere to VCAM-1 on EC and BMSC. Both EC and BMSC were stimulated for 24h with TNFα (10ng/ml) and analyzed for VCAM-1 expression. We compared VCAM-1 expression before and after TNFα treatment on EC and BMSC and we found that TNFα treatment strongly upregulated VCAM-1 expression on the surface of HUVEC and HMEC-1 (n=3), as shown in Figure 19.

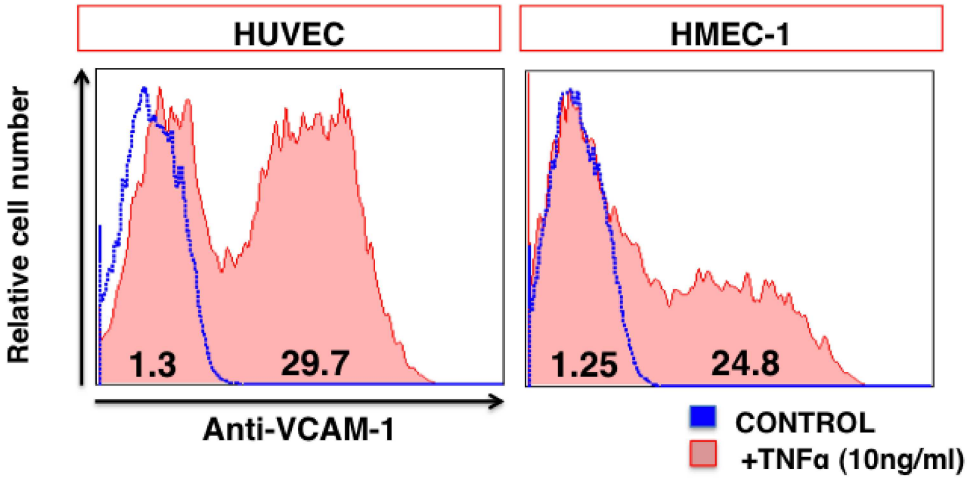


Figure 19. Displayed are fluorescence histograms depicting the relative fluorescence intensity of HUVEC and HMEC-1 stained with anti-VCAM-1 mAb either treated or not treated (CONTROL) with TNFα (10ng/ml) for 24 hours. Mean fluorescence intensity ratio was calculated by dividing the mean fluorescence intensity for VCAM-1 by the mean fluorescence of the isotype control in both conditions.

Primary CLL cells were cultured onto the confluent endothelial and stromal cell layers for 4 hours and after incubation the cells that had not adhered to the EC and BMSC cell layers were removed. TNFα significantly increased adhesion to both EC and BMSC stimulated with TNFα when compared to unstimulated controls, as documented in phase-contrast photomicrographs (Figure 20).

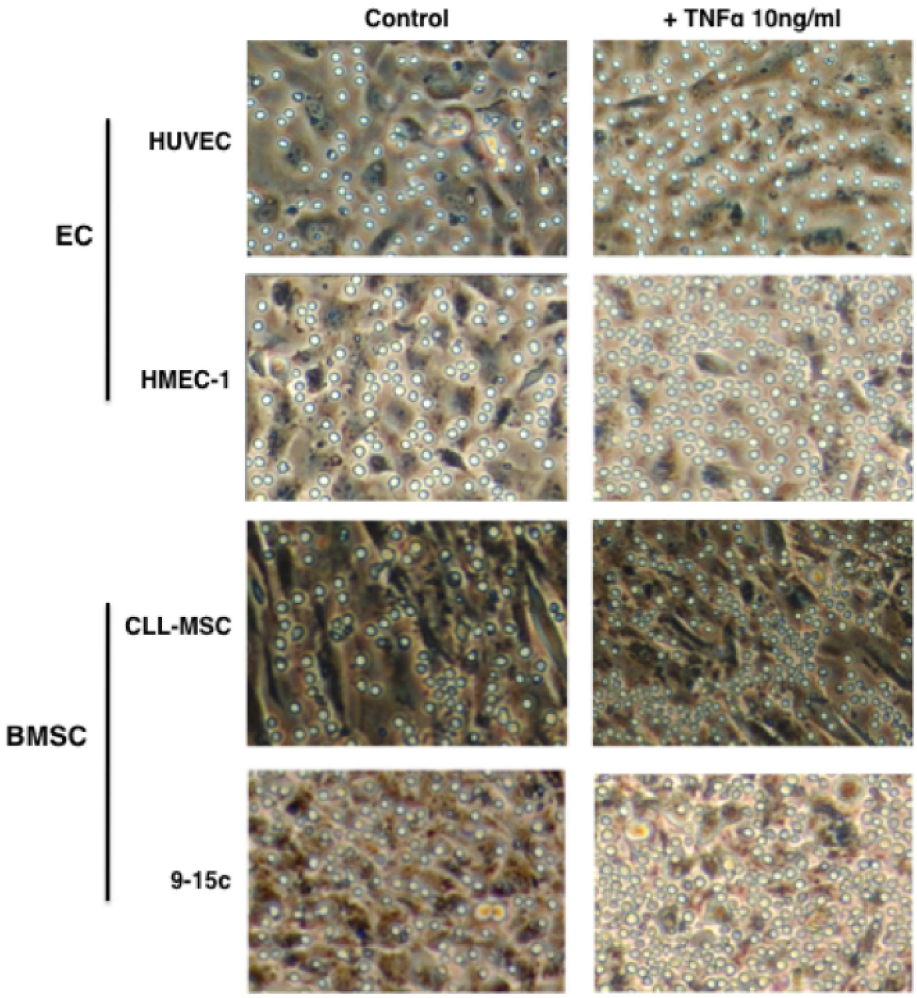


Figure 20. Phase contrast photomicrographs demonstrating CLL cell adhesion to EC and BMSC either treated or untreated (CONTROL) with TNFα.

Next, we addressed the effect of increased adhesion mediated by TNFα to more adhesive interactions between VLA-4 on CLL cell and VCAM-1 expressed on EC and BMSC. At this purpose we analyzed VLA-4 expression on CLL cells showing variable expression of VLA-4 integrin ranging from 1% to 78%, as shown in 3 positive representative samples (cut-off of 30%) in Figure 21. Given the high expression of VCAM-1 on EC/BMSC and the variable VLA-4 expression on CLL cells, we compared the relative adhesion of CLL cell to activate endothelium with their individual VLA-4 expression. We observed a significant positive correlation between VLA-4 expression and adhesion to TNFα-treated HUVEC (Figure 22); r=0.414, p=0.004). These data suggest that interaction between VLA-4 and VCAM-1 increased the ability of CLL cells to adhere to EC and BMSC.

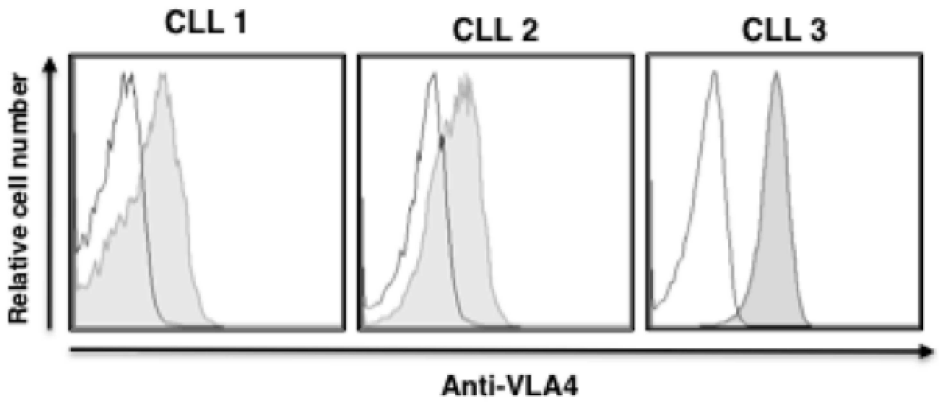


Figure 21. Representative fluorescence histograms of CLL cells stained with anti-VLA-4 (grey histograms) or the corresponding isotype control (white histograms).

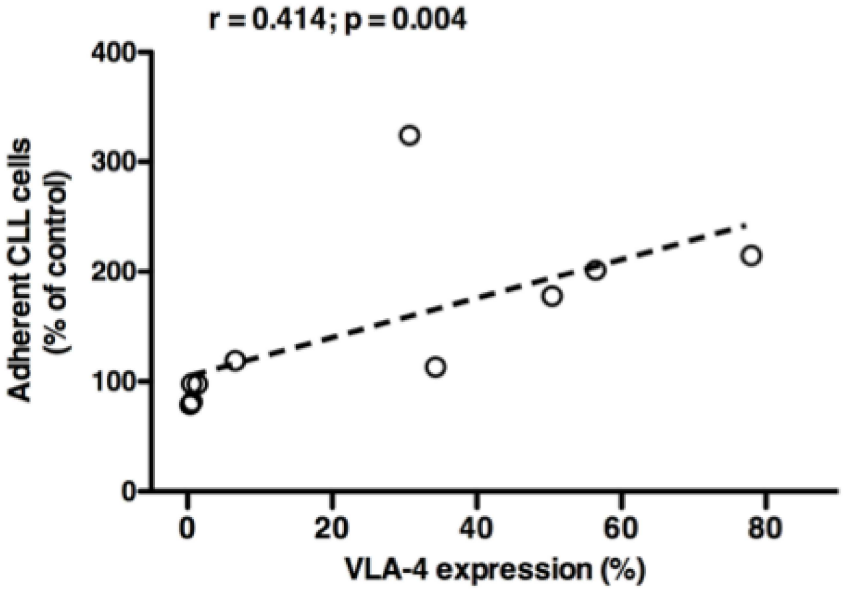


Figure 22. A positive correlation is displayed between VLA-4 expression and the relative adhesion of CLL cells to HUVEC stimulated with TNFα (n=10).

6. GS-1101 inhibits CLL cell adhesion to EC and BMSC

Next, we explored the effects of GS-1101 to interfere with CLL adhesion. As previously described, primary CLL cells displayed a significant increased adhesion to both EC and BMSC stimulated with TNFα when compared to unstimulated controls. For example the mean relative number (±SEM) of CLL cells adherent to TNFα-treated HMEC-1 increased to 144.3% ± 8.3% ($p<0.01$, n=8) and to 143.9% ± 16.9% on TNFα-treated CLL-MSC ($p<0.05$, n=8) when compared to respective untreated controls (Figure 23). Next, we found that CLL cell adhesion to TNFα-stimulated EC and BMSC was inhibited by GS-1101. The mean relative adhesion (±SEM) of CLL cells to EC was significantly reduced from 162.8% (±27.7%) to 95.7% (±12.1%) using HUVEC, and from 144.3% (±8.3%) to 99.4% (±7%) using HMEC-1 cells. CLL cell adhesion to TNFα-stimulated BMSC was significantly reduced from 128.9% (±7.3%) to 94.1% (±7.9%) using 9-15c cells, and from 143.9% (±16.9%) to 100.7% (±10.1%) using CLL-MSC (Figure 23). GS-1101 also reduced CLL cell adhesion to unstimulated EC and BMSC, but these differences did not reach statistical significance. For example, CLL cell adhesion to unstimulated EC or BMSC was reduced by GS-1101 to 91.2% (±6.8%, ns, n=8) on HMEC-1, to 87.5% (±11%, ns, n=8) on HUVEC, to 73.9% (±6.2%, $p<0.01$, n=8) on 9-15c, and to 93.9% (±6.9, ns, n=8) using CLL-MSC. Phase-contrast photomicrographs document increased numbers of adherent CLL cells after TNFα-treatment and reduced numbers of adherent CLL cells after treatment with GS-1101 (Figure 24).

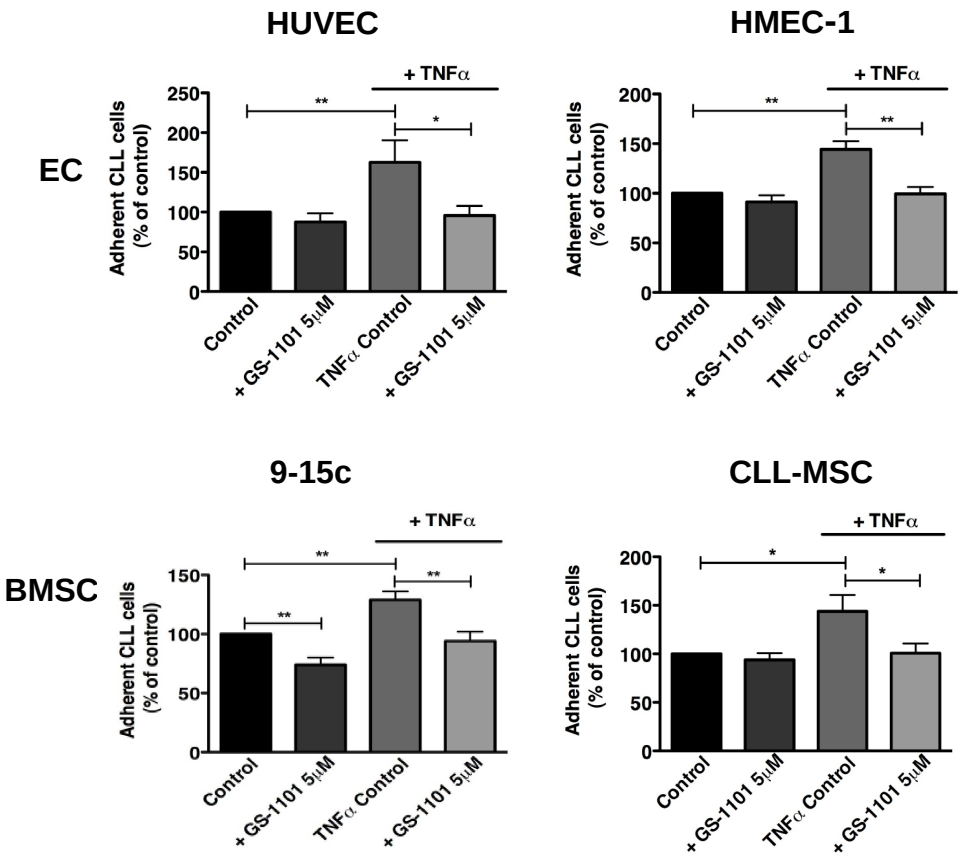


Figure 23. Bar diagrams represent the mean relative adhesion (±SEM; n=8) of CLL cells in the presence or absence of GS-1101 to EC and BMSC compared with the control. TNFα treatment increase the adhesion of CLL cells to EC and BMSC and in presence of GS-1101 is significantly inhibited (*p<0.05; **p<0.01).

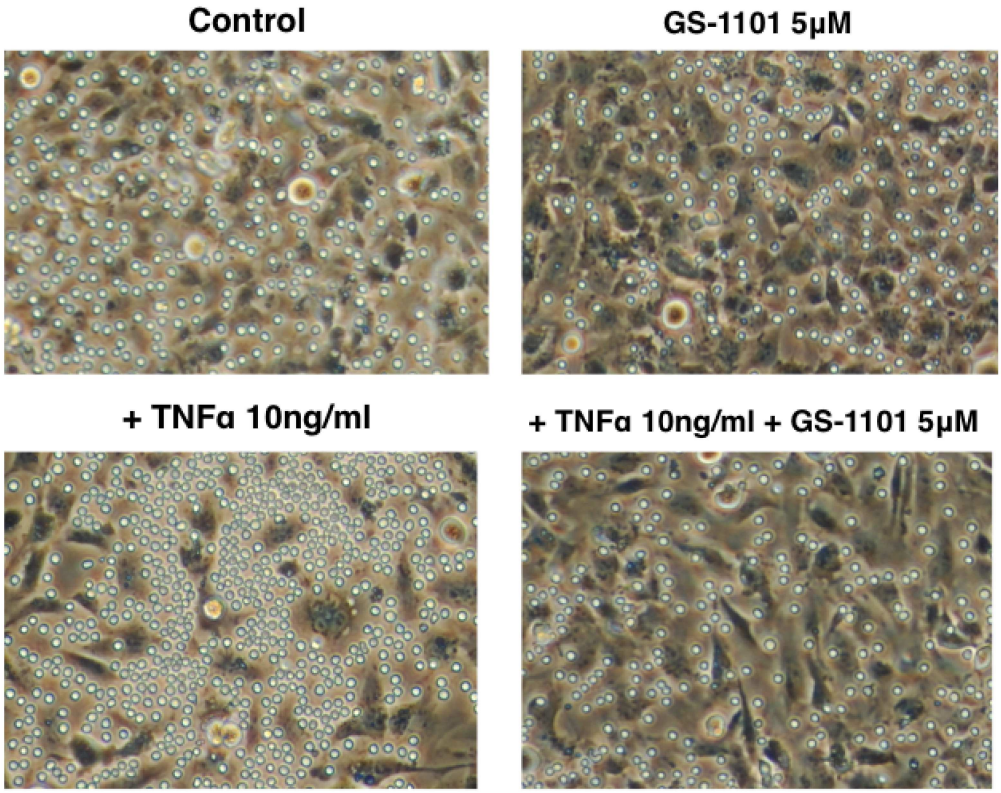


Figure 24. Representative phase contrast photomicrographs demonstrating CLL cell adhesion to HMEC-1 either treated or untreated (CONTROL) with TNFα. Treatment with TNFα increased the number of adherent CLL cells to HMEC-1 in comparison to the control (on the bottom left); in presence of GS-1101 the number is reduced both in presence or absence of TNF treatment.

We than investigated the ability of GS-1101 to interfere with CLL adhesion in VLA-4 high and VLA-4 low groups. The mean relative adhesion (±SEM) of CLL cells on TNFα-treated HUVEC layer significantly decreased from 206% ±34.2± to 118.5%±20.5% (n=5, $p<0.05$) in VLA-4 high expression group, and from 115.6%±18.3 to 98.8%±14.8% in VLA-4 low expression group (n=5, p=ns). Using GS-1101 to interfere with VLA-4-VCAM-1 interaction, we found a strong inhibition of CLL adhesion to TNFα-treated endothelium in VLA-4 high expression group (40.6% inhibition of adhesion) compared with VLA-4 low expression group (12.1% of inhibition of adhesion) (Figure 25, n=10, $p<0.05$). Collectively, our data suggest that

GS-1101 significantly inhibits CLL adhesion to EC and BMSC interfering with VLA-4 and VCAM-1 interaction.

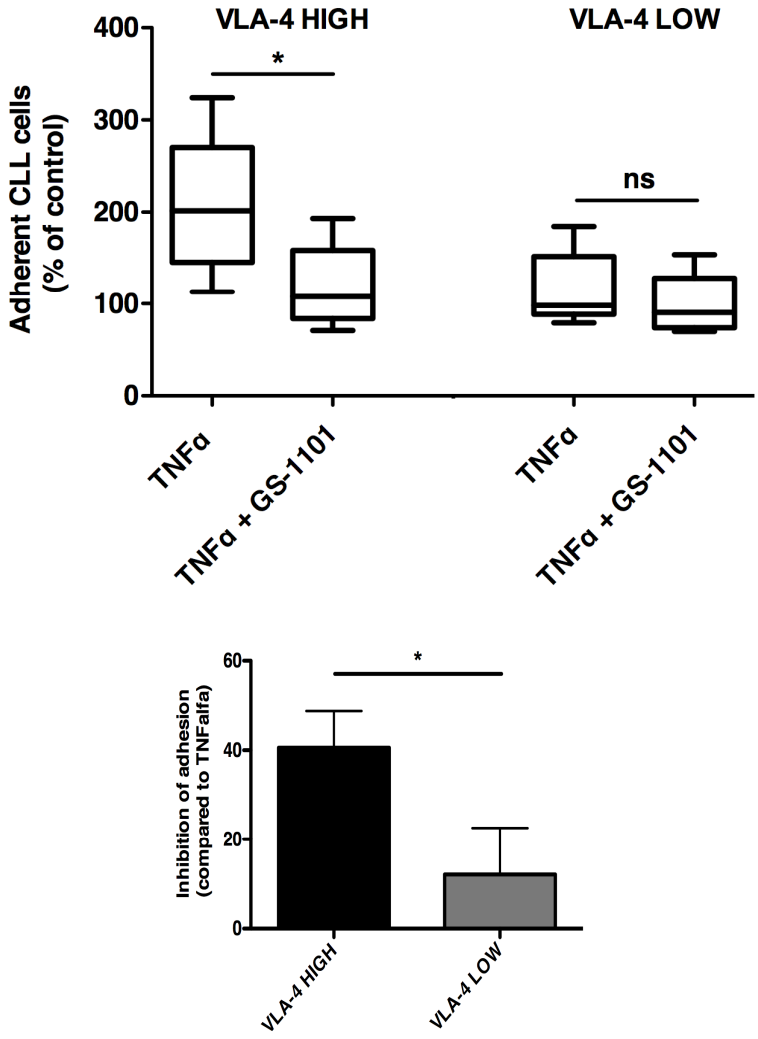


Figure 25. The box plot display a comparison of CLL cell adhesion to HUVEC TNFα-stimulated in presence or absence of GS-1101 in VLA-4 high (n=5) and VLA-4 low (n=5) expression groups. Bar diagram represents the percentage of inhibition in CLL adhesion to HUVEC stimulated with TNFα induced by GS-1101 in VLA-4 high and VLA-4 low CLL samples relative to adhesion observed without GS-1101. Data are shown as mean ±SEM (*p<0.05).

7. ***GS-1101 interferes with cell adhesion to activated endothelium and VCAM-1 under shear flow conditions.***

To investigate effects of GS-1101 on CLL cell adhesion to EC under conditions that mimic physiologic flow conditions within blood vessels, we used parallel plate flow assays. CLL cells treated with 5μM GS-1101 or unstimulated control cells were allowed to adhere to TNFα-stimulated HUVEC. As shown in Figure 26A, TNFα treatment increased CLL cell adhesion to HUVEC when compared with unstimulated controls. GS-1101 effectively inhibited CLL cell adhesion to EC under shear flow conditions at low (15 dynes/cm^2) and higher shear forces (30 dynes/cm^2). As illustrated in Figure 26B, the mean (±SEM) relative number of adherent CLL cells on HUVEC increased from 10.8 % (±2%) to 23.3 % (±2.6%) after TNFα stimulation ($p<0.01$, n=5) at 15 dynes/cm^2. GS-1101 decreased CLL cell adhesion to TNFα-activated HUVEC from 23.3% (±2.6%) to 13.9% (±2.4%) at low shear force of 15 dynes/cm^2 ($p<0.05$, n=5). At high shear force 30 dynes/cm^2 the mean (±SEM) number of cells adherent to HUVEC increased from 4.1% (±0.7%) to 12.9 (±1.8%) in presence of TNFα stimulation ($p<0.01$, n=5) that decreased to 7.8% (±1.4%) in presence of 5μM GS-1101 ($p<0.05$, n=5).

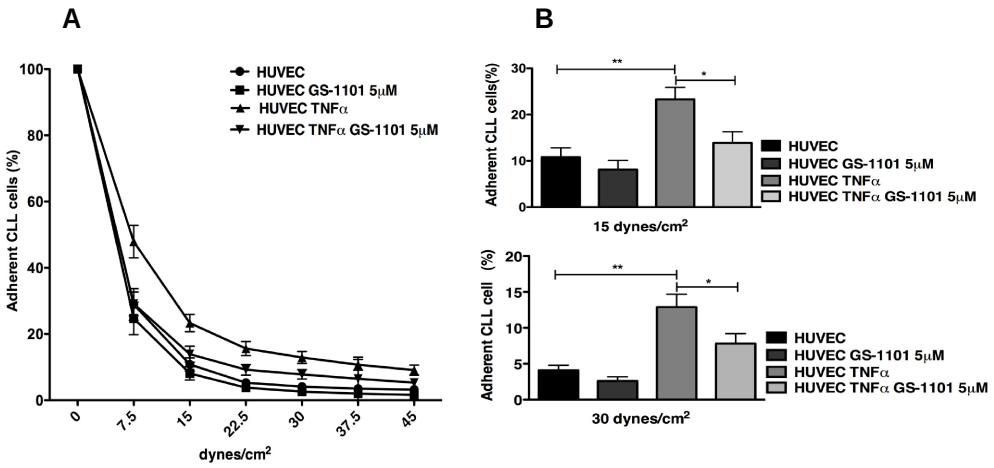


Figure 26. (A) A linear gradient of shear flow increasing from 0-45 dynes/cm^2 is applied over the adhered cells and the number of adherent cells is determined by the percentage of cells remaining every 50 seconds. (A) CLL cells were either treated or not treated with GS-1101 and allowed to adhere to HUVEC stimulated or not stimulated (CONTROL) with TNFα (n=5). The data displayed the mean relative adhesion (±SEM) at different shear forces compared with the baseline adhesion for each condition. **(B)** The bar diagrams display the percentage of cells adherent to HUVEC at two representative shear forces (15 and 30 dynes/cm^2).

We further investigated specifically if adhesion mediated by VCAM-1 was affected by GS-1101. Ramos cells were treated and not treated with GS-1101 and allowed to adhere to slides coated with VCAM-1. As shown in Figure 27, GS-1101 significantly inhibited Ramos cell adhesion to VCAM-1 compared to ovalbumin control in the untreated sample. At a shear force of 22.5 dynes/cm2 the mean (±SEM) number of cells adherent to VCAM-1 decreased from 41.6%±5.8% to 17.7%±6.1% in presence of GS-1101 (n=3; $p<0.05$). Collectively, these data confirm the earlier results in the static adhesion assays, corroborating that GS-1101 inhibits CLL cell adhesion to activated endothelium and in particular confirm an interference with the interaction between VLA-4 and VCAM-1.

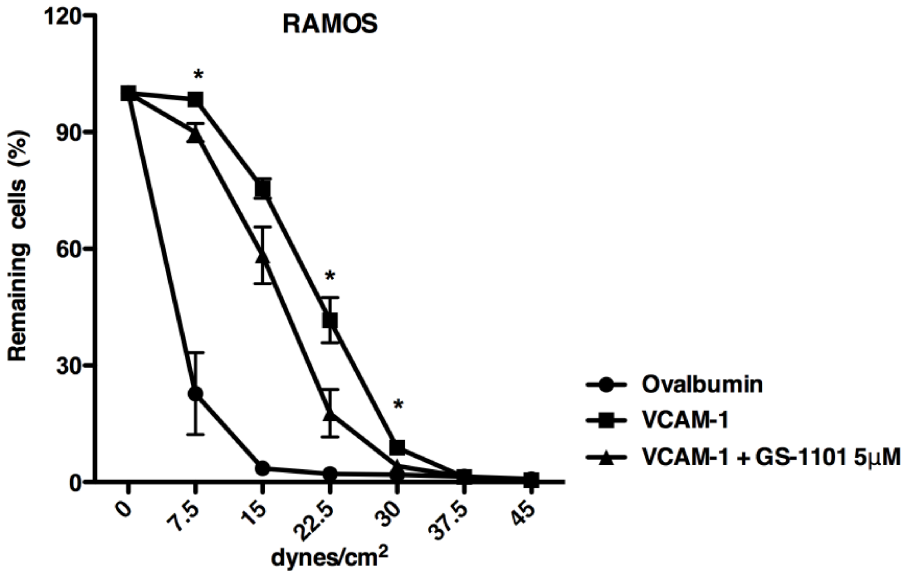


Figure 27. Ramos cells were treated or not treated with GS-1101 and allowed to adhere to slides coated with VCAM-1 or ovalbumin (CONTROL) (n=3, *$p<0.05$).

8. *GS-1101 inhibits CLL cell survival in EC and BMSC co-cultures*

To test the effect of GS-1101 on EC- and BMSC- mediated CLL cell protection, CLL cells were cultured on EC (HUVEC and UV-2) or BMSC (NKtert and KUSA-H1) in presence or absence of 5μM GS-1101 and CLL cell viabilities were assessed at 24, 48, 72 hours. In a representative case (Figure 28A), GS-1101 reduced CLL cell viability from 51.7% to 41.9% with HUVEC, 47.9% to 41% with UV-2, 97.5% to 64.9% with NK-tert and 92.9% to 85.1% with KUSA-H1 after 48 hours. We also found a significant reduction in the viability of CLL cells co-cultured with EC and BMSC in presence of GS-1101 compared to the control at all time-points. At 24 hours, the mean relative viability of CLL cells in presence of GS-1101 was 73.9% (±2.2%, $p<0.01$, $n=7$) with HUVEC, 81.7% (±2.5% $p<0.01$, $n=7$) with UV-2, 63.0% (±2.4% $p<0.01$, $n=7$) with NKtert and 71.1% (±4.1% $p<0.01$, $n=7$) with KUSA-H1 (Figure 28B). These data demonstrate that GS-1101 can overcome EC- and BMSC- mediated CLL cell protection. In addition the impact of GS1101 is roughly equivalent in its ability to overcome CLL B cell protection for both cell types.

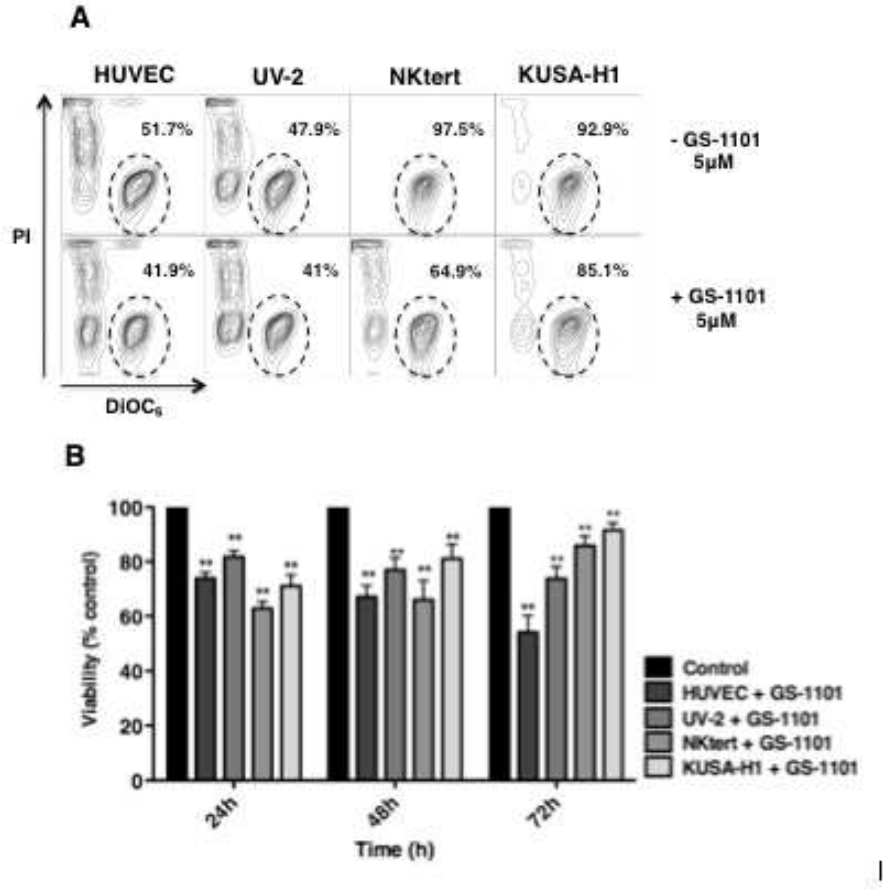


Figure 28. (A) The gates in the contour plots exemplify viable CLL cells in one representative sample. (B) The bar diagrams represent the mean relative viabilities of CLL cells co-cultured in normal conditions (CONTROL) or in presence of 5µM GS-1101 with EC and BMSC. Viabilities in GS-1101-treated samples were normalized to the corresponding viabilities of control samples at the respective time-point (100%) to account for differences in spontaneous apoptosis. Displayed are mean±SEM from 7 different samples (*p<0.05; **p<0.01).

9. GS-1101 inhibits EC- and VLA-4-induced signaling in CLL cells

To determine effects of GS-1101 on EC-induced signaling, we analyzed AKT phosphorylation in presence or absence of GS-1101 after co-culture with HMEC-1 by flow cytometry. Overlay histograms depict relative pAKT expression in a representative CLL sample co-cultured with HMEC-1 for 24 hours in presence or absence of GS-1101 (Figure 29A). In this and other CLL cases, we consistently found that GS-1101 decreases EC-induced AKT phosphorylation. Figure 29B shows mean relative levels of pAKT, measured after 1 and 24 hours of EC co-culture. While differences after 1 hour of co-culture were small, AKT phosphorylation was increased after 24 hours in co-culture with HMEC-1 from 100.9% (±6.8%) to 117.2% (±7.6%) ($p<0.01$, $n=4$). GS-1101 significantly decreased EC-induced pAKT levels to 76.7% (±8.8%) after 24 hours ($p < 0.05$). These findings were confirmed by immunoblot analyses, which demonstrated decreased EC-induced AKT phosphorylation (T308) in two representative CLL samples after GS-1101 treatment (Figure 29C).

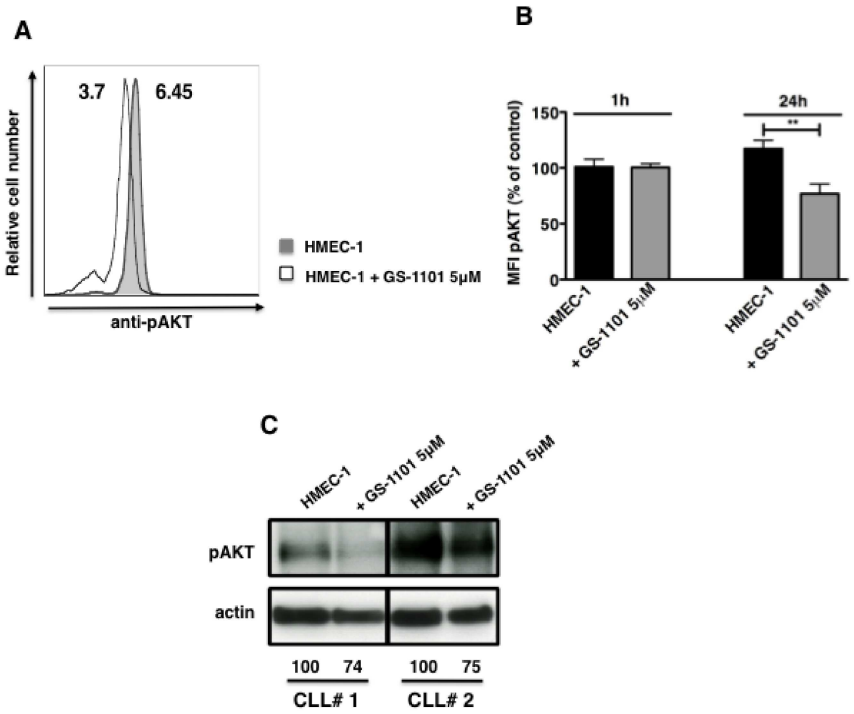


Figure 29. A) Overlay histograms depict the mean AKT phosphorylation (pAKT) fluorescence intensity of CD19+ CLL cells in a representative case. The solid gray histogram depicts pAKT staining of CLL cells in co-culture with HMEC-1 without GS-1101 and the black line histogram depicts of CLL cells in co-culture with HMEC-1 in presence of 5 µM GS-1101. AKT phosphorylation was analyzed in CLL cells after 1 hour and 24 hours of co-culture with HMEC-1 either in presence or absence of GS-1101. (B) The bar diagrams represent the mean fluorescence intensity ratio (MFIR) for AKT phosphorylation in CLL cells co-cultured with HMEC-1 normalized for the MFIR of CLL cultured alone (CONTROL). (C) Displayed are immunoblots from 2 representative CLL samples of 4 patients co-cultured with HMEC-1 in presence or absence of GS-1101 for 24 hours. Lysates were probed with antibodies to pAKT (Tyr 308) and actin.

To investigate if VLA-4 activation can also induce AKT activation, we incubated CLL cells with activating anti-CD49d (19H8) mAbs for 1 hour, followed by crosslinking in the presence or absence of GS-1101. As shown in Figure 30, AKT phosphorylation induced by CD49d stimulation was significantly inhibited by GS-1101 ($p<0.05$). The crosslinking secondary antibody alone did not stimulate AKT phosphorylation (data not shown). This result was confirmed also by immunoblot with 3 different CLL samples (Figure 30). These data demonstrate that GS-1101 is able to inhibit AKT phosphorylation induced by EC and CD49d-triggering, indicating that GS-1101 interferes with CLL cell adhesion by inhibition of VLA-4 integrin function and signaling.

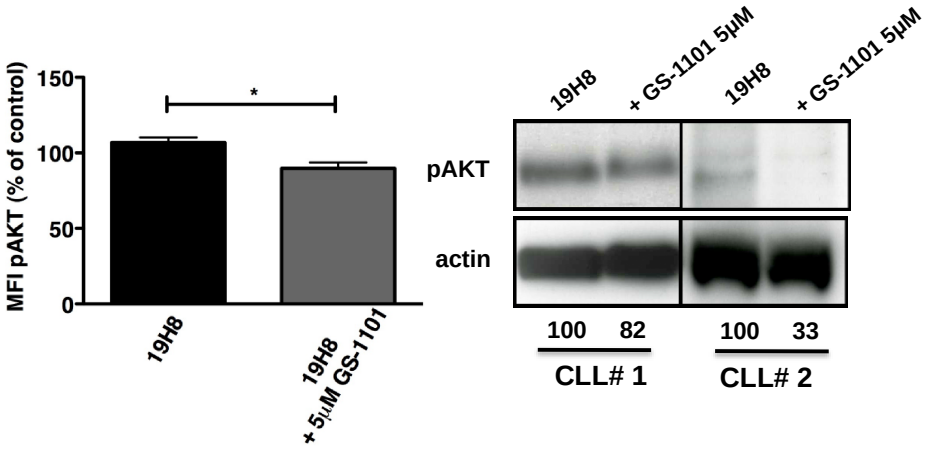


Figure 30. The bar diagrams represent the mean relative fluorescence intensity ratio of CLL cells stimulated with 19H8 mAb (CD49d) either in presence or absence of GS-1101. Mean fluorescence intensity ratio were normalized for the corresponding MFIR at baseline. Displayed are the means (±SEM) from 3 different patients (*$p<0.05$; **$p<0.01$, n=3). The immunoblot on the right hand side depicts AKT activation in two representative CLL samples stimulated with 19H8 anti-VLA4 mAbs in presence or absence of GS-1101.

DISCUSSION

Several evidences have suggested that the biology of chronic lymphocytic leukemia cells in vivo depends on their anatomic localization in fact in the blood CLL cells display characteristics of resting cells whereas in the lymph node and bone marrow demonstrate increased proliferation. These observations indicate that CLL is a tumor “addicted to the host” (101).

This study was devised in light of several observations. First, although CLL B cells exhibit characteristics consistent with prolonged cell survival in vivo, when cultured in vitro these cells often undergo spontaneous apoptosis (116). It suggests that extrinsic signals from microenvironmental elements surrounding leukemic cells in vivo are essential to support prolonged CLL B-cell survival. Second, lymphocytes interact transiently and reversibly with different accessory cells through selectins and integrins resulting in cell arrest, firm adhesion and transendothelial migration into tissues. This suggests that CLL cell-survival is a consequence of adhesion to accessory cells. Third a potent and selective inhibitor of PI3K delta, GS-1101 is able to interfere with the crosstalk between CLL cells and their microenvironment affecting CLL migration beneath bone marrow stromal cell, chemotaxis towards CXCL12 and CXCL13 and disrupts BCR signalling.

Moreover increased vascularisation in bone marrow (BM) and lymph nodes (LN) may provide infiltrated tissues with oxygen and metabolite supply, as well as with signals and conditions involved in regulating leukemic cell homing and recirculation. We hypothesize that the presence of activated endothelial cells (EC) and stromal cells (BMSC) in BM and LN tissues as well as the direct interaction with EC and BMSC during recirculation from PB to tissues may also contribute to CLL survival and may be involved in drug resistance, thus facilitating disease progression and relapse after conventional therapy.

Thus, we co-cultured CLL cells on endothelial and stromal layers allowing the establishment of functional interactions throughout intimate contact between cellular elements. We found that BMSC were consistently more effective in maintaining CLL cell viability when compared with EC. The exact mechanism for this is unclear but seems not too surprising given that BMSC are critical and more numerous components of the supporting cellular infrastructure for hematopoietic cells in the marrow

microenvironment. This microenvironmental protection is mediated by integrins and soluble factors. In fact, conditioned medium collected from HUVEC near confluence lacked to recapitulate the survival improvement observed in direct co-culture system. These results indicate that the intimate contact between leukemic cells and supporting endothelial elements is necessary to protect CLL cells from spontaneous apoptosis. Blocking mAbs against α4β1/VCAM-1 and αLβ2/ICAM axes demonstrated that both signals were involved in interaction between CLL and endothelium. Disrupting the integrin-mediated contact significantly decreased the survival advantage in co-culture system. Approximately 70% to 80% inhibition of survival could be obtained with anti-CD106 or anti-CD18.

However, we surprisingly observed that, when CLL cells were removed from EC layer, leukemic cells maintained a survival advantage compared to CLL cultured in suspension. In addition, conditioned medium collected from two-days CLL/EC co-culture significantly inhibited spontaneous apoptosis of CLL cells. Overall, our data demonstrated that physical contact between CLL cells and endothelium is essential to trigger the cellular secretion of several soluble factors mediating additional survival hits on leukemic cells.

To explore the mechanisms underlying the enhanced survival consequent to co-culture with EC cells, we performed a genome-wide profiling of CLL transcriptome changes that occur as a consequence of CLL-EC interaction. We found that CLL cells in direct contact with endothelium underwent functional modifications that induced dramatic alterations in the secreted levels of several angiogenesis-related factors. This finding is consistent with the reported ability of stromal cells to activate CLL cells to secrete VEGF and basic FGF in co-culture assay (69,72). This imbalance in the production of angiogenic regulators may determine in CLL cells a microenvironmental-driven angiogenic switch. In particular, we found that intimate contact with endothelium determined on CLL cells the up-regulation of endothelin-1 (EDN1), endoglin (ENG) , angiomotin-like protein 2 (AMOTL2), angiopoietin-related protein 4 (ANGPTL4), angiopoietin-2 (Ang2), vascular endothelial growth factor C (VEGFC), and thrombospondin 1 (THBS-1). EDN1 modulates various stages of neovascularisation, including endothelial-cell proliferation, migration,

invasion, protease production and tube formation as well as stimulates the production of VEGF by increasing hypoxia-inducible factor 1α and matrix metalloproteases. We found that CLL cells increased the secretion of ENG involved in activation and survival of endothelial cells. ENG (CD105) is a disulfite-linked homodimeric transmembrane glycoprotein, component of the receptor complex of TGF-β. ENG is mainly expressed by activated vascular endothelial cells but also by several neoplastic cells, including solid tumors such as meningiomas, ovary and breast carcinomas and hematological malignancies such as refractory anemia with excess of blasts, blast crisis of chronic myelogenous leukemia, the most immature subtypes of acute leukemias and also CLL (117,118). Other soluble factors up-regulated by CLL cells in contact with endothelium were AMOTL2 involved in EC migration and tube formation and two angiopoietins (Ang2 and ANPTL4) able to induce destabilization of pre-existing vessels and neovascularisation as well as migration and immunosuppressive phenotype of monocytes.(4) Another angiogenesis-related factors secreted by CLL in EC contact was a member of VEGF family, VEGFC. CLL B cells were reported to secrete VEGF, to constitutively express VEGFR-1 and VEGFR-2 (KDR) and to respond to exogenous VEGF by increasing apoptotic resistance through STAT3 activation and up-regulation of XIAP and Mcl-1 expression (119,120). Based on these observations, a VEGF autocrine pathway was postulated to be involved in CLL survival. VEGFC has been characterized as a lymphangiogenic and angiogenic growth factor and shown to signal through the receptors VEGFR-3 and VEGFR-2. Aside from its effect on angiogenesis, VEGFC signalling was reported to induce proliferation, promote cell survival and protect leukemic cells from chemotherapy-induced apoptosis by induction of Bcl2 in a subset of acute leukemias (120). In contrast with the reported diminution of thrombospondin-1 (THBS-1) in CLL cells adhering to stromal cells, we found that CLL cells when co-cultured on endothelial layer up-regulated THBS-1. Classically considered an anti-angiogenic factor,(121) THBS-1 can also be considered a stimulator of tumor progression and angiogenesis by inducing proteolytic degradation of the extracellular matrix, cell migration and invasion throughout the regulation of plasminogen/plasmin system, the up-regulation of MMP9 and the activation of TGF-β1.(122) Of interest, CLL

was reported to secrete THBS-1 and to express the thrombospondin receptor CD36 (123). Overall, our finding that the interaction between CLL cells and endothelium lead to an increase in several pro-angiogenic cytokines involved in survival, proliferation and migration of endothelial cells, and vessel sprouting indicate that a kind of circularly sustained angiogenic milieu may be present in CLL-infiltrated tissues in order to maintain increased microvessel density and to improve growth condition for the CLL clone.

Another important result from the microarray experiments was the observation that endothelial cells induce expression changes of factors able to orchestrate the cellular composition and function of several microenvironmental elements including stromal fibroblasts and macrophages such as Ang2, PDGFC and CCL2. In particular, the C-C motif chemokine CCL2 is one of the key chemokines regulating migration and infiltration of monocytes/macrophages via activation of CCR2 receptor. In addition, CCL2 can directly mediate tumor angiogenesis and augments the Th2 response.(124) Of interest, about half of CLL clones was reported to express CCR2 receptor. Very recently, CCL2 protein was detected in supernatants obtained from CLL co-culture with bone marrow-derived stromal cell line HS-5, implying a chemotactic activity on monocytes (125). We have also demonstrated that interaction between endothelium and CLL cells affect the levels of several anti-apoptotic factors including Bcl-2 and BIRC5/Survivin. When circulating CLL cells were cultured in medium alone, they experienced a rapid decrease of Bcl-2 expression. Endothelial cell contact was able to maintain elevated levels of Bcl-2 comparable to CLL in vivo expression and to increase significantly expression of Survivin. It suggest that physical contact with endothelium may trigger signals able to preserve the anti-apoptotic phenotype of CLL cells and to deliver additional survival hits.

Currently, several inhibitors of kinases downstream of B cell receptor are developed in clinical trials in CLL, and are emerging as promising alternative targeted therapies for CLL patients. These new agents include the spleen tyrosine kinase (Syk) inhibitor fostamatinib (102), Bruton's tyrosine kinase (BTK) inhibitor ibrutinib (formerly PCI32765)(103), and the PI3Kδ inhibitor GS-1101 (formerly CAL-101)(109), and all have shown

similar characteristic clinical activity. Typically, these agents cause rapid resolution of lymphadenopathy and/or organomegaly with redistribution of CLL from tissues into the blood, resulting in a surge in lymphocytosis during the first weeks of therapy, which later oftentimes slowly resolves. This characteristic activity suggests that these agents interfere with the CLL cell adhesion and other tissue retention signals.

Previous works on GS-1101 (109), ibrutinib (104), and R406 (77) suggests that disruption of chemokine receptor signalling and function by these inhibitors are involved in the CLL cell redistribution phenomenon. Mechanistically, this concept, which is largely based on chemotaxis assays and signalling studies, likely does not fully explain the clinical findings given the plethora of means by which CLL B cells can adhere to stromal cells. To better characterize and understand the nature of the GS-1101-induced CLL cell redistribution, we explored the effects of GS-1101 on CLL cell adhesion to different stromal cell types present in the CLL microenvironment (BMSC, EC). We found that GS-1101 significantly inhibited CLL cell adhesion to both, BMSC and EC under static and shear flow conditions. Furthermore, we noticed higher levels of CLL cell adhesion to TNFα-treated EC and BMSC, indicating that this increased adhesion was mediated by upregulation of VCAM-1 on the respective stromal cells. These results were also confirmed using a cell line, Ramos, to better explain the role of GS-1101 interfering with VLA-4 and VCAM-1 integrins. In this system, GS-1101 is able to affect the adhesion of RAMOS mediated by VCAM-1 supporting our findings with CLL cells on activated endothelium. Moreover, in our group of CLL patients, differences on VLA-4 expression play a critical role in the adhesion process defining the response to GS-1101. All these data together indicate that GS-1101 reduces CLL cell adhesion to stromal cells by interference with VLA4 (CD49d)-mediated adhesion of the leukemia cells to VCAM-1 (CD106) on the surface of the stromal cells.

Our data corroborate the concept that GS-1101 inhibits VLA-4 integrin-mediated adhesion of primary CLL cells to VCAM-1 and proposed this activity to explain the CLL cell redestribution. This is of relevance to the *in vivo* situation in that these cells will more closely resemble the *in vivo* microenvironment. In this scenario, CLL cells enter and accumulate into lymph nodes where adhere to accessory cells using different integrins and

in particular VLA-4. This induces an increase in lymph nodes size supported by different growth and survival signals. GS-1101 is able to interfere with integrin mediated adhesion, in particular with VLA-4 on CLL cells and VCAM-1 on EC/BMSC, causing a lymph node shrinkage with a redestribution of CLL into the blood.

Besides tissue homing and retention, increased CLL cell survival is an expected consequence of adhesion to stromal cells, but effects of GS-1101 on EC- and BMSC-mediated CLL cell survival have not previously been studied in detail. GS-1101 significantly interferes with these pro-survival effects implies that the adhesive interaction, and not exclusively BCR activation and signaling, contribute to CLL cell activation and survival in these tissue sites. Indeed, we find increased AKT activation in stromal co-cultures that are downmodulated by GS-1101. The finding that anti-VLA4 mAbs similarly activate AKT in a GS-1101-sensitive fashion further support the concept of VLA4-VCAM-1 mediated activation of CLL cells.

In conclusion, these results demonstrate that treatment with GS-1101 inhibits CLL cell adhesion to EC and BMSC and Akt related survival pathways. This provides new explanations for the tissue to blood distribution of CLL cells commonly seen in CLL patients given GS-1101.

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