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Angiopoietine modulieren molekulare und zelluläre Schlüsselprozesse in den chronischen lymphatischen Leukämie Zellen durch Tie2-unabhängige Mikroumgebungs-Signalwege

Angiopoietins modulate key molecular and cellular processes of Chronic Lymphocytic Leukaemia cells through Tie2-independent microenvironmental signalling

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Die vorliegende Arbeit sowie alle diese Arbeit zugrundeliegenden Experiment wurde im Labor für molekulare Hämatologie und Onkologie, Klinik I für Inneres Medizin des Klinikums der Universität zu Köln von März 2012 bis August 2015 in der Arbeitsgruppe von Herrn Professor Dr. med. Karl-Anton Kreuzer von mir under seine Anleitung durchgeführt.

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Die Gedanken sind frei, wer kann sie erraten,
sie fliegen vorbei wie nächtliche Schatten.
Kein Mensch kann sie wissen, kein Jäger erschießen
mit Pulver und Blei: Die Gedanken sind frei!

Fragment from a German Volkslied, ca. 1810-1820

For the great Norberto, who introduced me for the very first time to Gemütlichkeit and Maßkrüge.

INDEX

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

µ	Micro-
7-AAD 7-	Aminoactinomycin D
ABIN-2	A20 binding inhibitor of NF-kappaB-2
ABL	Abelson murine leukaemia viral oncogene
AIHA	Autoimmune haemolytic anaemia
Akt	Protein Kinase B
ALL	Acute lymphoblastic leukaemia
alloHSCT	Allogenic haematopoietic stem cell transplantation
AML	Acute myelogenous leukaemia
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
Ang-Tie2	Angiopoietin-Tie2 signalling receptor system or pathway
BCR	B-cell receptor
bFGF	Basic fibroblast growth factor
bFGFR	Basic fibroblast growth factor receptor
BM	Bone marrow
BMSC	Bone marrow stromal cells
BMX	Cytoplasmic tyrosine-protein kinase BMX
bp	Base pairs
BSA	Bovine serum albumin

CAR	CXCL12-abundant reticular cells
CD	Cluster of differentiation or classification determinant
cDNA	Complementary deoxyribonucleic acid
CLL	Chronic lymphocytic leukaemia
CML	Chronic myelogenous leukaemia
Cq	Quantification cycle
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
DOK-R	Docking protein
DTT	Dithiothreitole
EC	Endothelial Cells
ECD	R-Phycoerythrin-Texas Red® -X
ECL	Endothelial cell Layer
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immnoabsorbent assay
Em	Emission/wavelength nm
eNOS	Endothelial nitric oxide synthase
et al.	et alii (and others)
EtBr	Ethidium bromide
Ex	Excitation/wavelength nm
FACS	Fluorescent activated cell sorter
FAK	Focal Adhesion Kinase
FC	Fludarabine and cyclophosphamide
FCR	Fludarabine, cyclophosphamide, and rituximab
FCS	Foetal calf serum
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate

FL	Filter in flow cytometry
g	Grams
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRB2	Growth factor receptor-bound protein 2
h	Hour
HCL	Hairy cell lymphoma
HEV	High endothelial venules
HRP	Horse radish peroxidase
HSC	Haematopoietic stem cell
HUVEC	Human umbilical vascular endothelial cells
Ig	Immunoglobulin
IgV_H	Immunoglobulin variable region heavy chain
IL	Interleukin
kDa	Kilo Dalton
L/l	Litre
LMHO	Laboratory of Molecular Haematology and Oncology
LN	Lymph node
m	Mili-
M	Mole
MALDI/MS	Matrix Assisted Laser Desorption/Ionization/Mass Spectrometry
MBL	Monoclonal B-cell lymphocytosis or pre-CLL
MCL	Mantel cell lymphoma
ME	Microenvironment
min	Minute
MM	Multiple Myeloma
mM	Milimol
mRNA	Messenger ribonucleic acid

1. INTRODUCTION

1.1 Classification of CLL amongst hematopoietic malignancies

The World Health Organization (WHO) classification of tumours of hematopoietic and lymphoid tissues published in 2001 and updated in 2008 represents the worldwide consensus on the diagnosis of haematopoietic malignancies, which is based on the recognition of distinct diseases using a multidisciplinary approach (1). The WHO classifies chronic lymphocytic leukaemia (CLL) as a mature B-cell neoplasm (2). Earlier, as a norm the CLL diagnosis required a lymphocyte count of ≥10.0x10^9/L to diagnose CLL (3). Currently, CLL diagnosis requires an absolute lower count of >5.0×10^9/L monoclonal B-cells in peripheral blood (1). Lower lymphocytic counts could still be considered to diagnose CLL, provided that the patients present cytopenias and additional symptoms characteristic of a CLL clinical course (2).

Closely resembling CLL, small-lymphocytic lymphoma (SLL) is a neoplasm characterized by small, monomorphic, and round to slightly irregularly shaped B-lymphocytes. However, SLL is a term rather used in non-leukemic cases, regardless of the similar morphology and immunophenotype with CLL.

Healthy individuals may display an aberrant immunophenotype that resembles that of CLL, but characterized by the absence of the gross accumulation of malignant lymphocytes (lymphocytosis). This is denominated as monoclonal B-cell lymphocytes (MBL), a clonal B-cell disorder presenting ≤ 5×10^9 B-lymphocytes/L in peripheral blood, but without the presence of lymphadenopathy or organomegaly (4).

1.2 Epidemiology of CLL

CLL is an incurable and prevalent blood malignancy amongst the elder citizens of western societies (5) and is extremely rare in eastern countries, linking CLL to

genetic pre-dispositions (2). Patients diagnosed with CLL present a heterogeneous collection of chromosomal, genetic, molecular, and cellular characteristics, which allow physicians to accurately diagnose the disease and promptly administer the most optimal treatment; these are denominated prognostic markers.

The female to male ratio of CLL diagnosis is 1:1.5-2. (2) with a disease incidence of approximately 2 patients in 100,00 per year, a number that increases to 12.5 patients in 100,000 starting at 65 years of age and beyond (2). Whilst the average age of patients diagnosed with CLL is between 67 and 72, the available diagnostic tools allow early diagnosis in patients as young as 40 years of age (5). Notably, younger CLL patients display the most severe symptoms attributed to this malignancy (5). As quality of life and life expectancy increase steadily amongst the general world population, it is likely that the morbidity and mortality rates for which CLL is responsible will concomitantly increase in the upcoming years (5).

1.3 Aetiology and pathogenesis of CLL

The underlying course and precise triggers of the overwhelming chronic accumulation of CD5+ and CD19+ B-cells in peripheral blood, bone marrow (BM), and secondary lymphoid nodes (LNs) remain to date a mystery. Healthy B-lymphocytes proliferate upon exposure to antigens and possess a rather short life. In comparison to healthy B-cells, malignant CLL cells fail to undergo timely apoptosis and evade efficient immunological elimination. Nevertheless, reports based on highly sensitive (1 in 10^5 cells) detection of cells positive for CD5, CD19, CD20, and CD79b reveal that up to 3% of healthy individuals show a detectable population of lymphocytes with a malignant profile, that is, a profile identical to that of CLL cells (3). Therefore, CLL is rather a disease of gross accumulation of lymphocytes (Fig. 1.1) failing to undergo timely apoptosis and efficient immunological elimination from the body.

1.4 CLL patients and their clinical picture

The clinical features of CLL are rather heterogonous, with presentation, course, and outcome widely differing amongst patients (2). Besides accumulating in peripheral blood, bone marrow (BM), spleen, and in some instances, malignant CLL cells may infiltrate the liver. CLL cells may also infiltrate tissues lacking lymphoid vessels such

as in the bladder, yet this is described as a very rare instance of CLL cell infiltration (6).

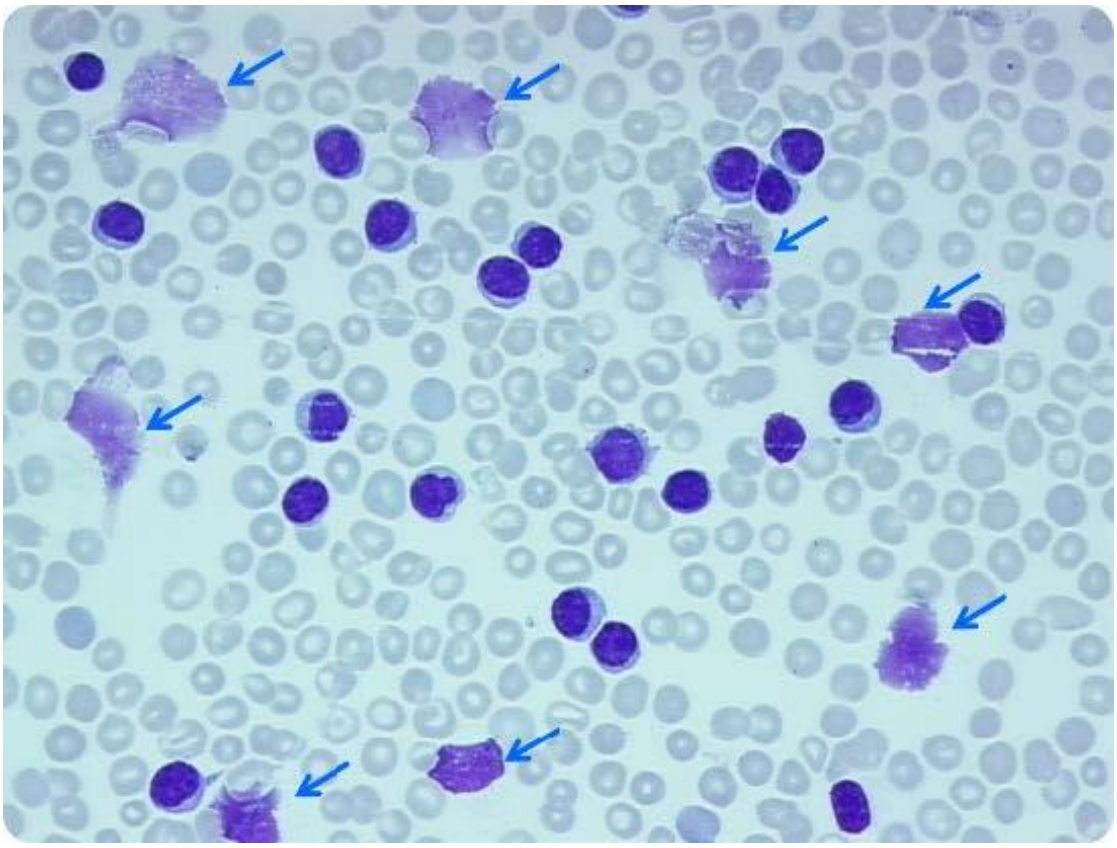

Figure 1.1 Light microscopy shows the numerous and fragile CLL cells. The light micrograph shows the excessive presence of CLL cells in a blood smear. A characteristic of CLL cells is their fragility, which is easily detected as malignant cells burst during the preparation slides. Photograph provided by Prof. med. K-A. Kreuzer from the Laboratory of Molecular Haematology and Oncology (LMHO).

Patients diagnosed with CLL may not require immediate therapy and physicians will “watch and wait” the patient if no other symptoms other than lymphocytosis are detected. However, other CLL patients will progress rapidly into later stages of the disease; between 80-90% of patients will develop severe symptoms after the initial diagnosis (3). Likely symptoms to appear are the enlargement of lymph nodes in the neck, under arms, and groin area, a state denominated as lymphadenopathy. Furthermore, patients may also have enlargement of spleen and liver, symptoms denominated as splenomegaly and hepatomegaly respectively. Two additional symptoms are anaemia, characterised by a low count of red blood cells (haemoglobin <11 g/dL), and thrombocytopenia, characterised by the low counts of platelets

(platelets <100×10^9/L). Other symptoms may include weight loss (≥ 10%) within the preceding 6 months, notorious fatigues, fever for longer than two weeks, or night sweats for longer than 1 month when accompanying infections are not detected (5).

The transformation from asymptomatic or mild CLL to aggressive leukaemia may lead to develop Richter´s syndrome, which occurs at a rate of 0.5% to 1% of patients per year. Richter´s syndrome is characterized by the rapid progression of adenopathy, also known as lymphadenopathy, and an increase in the detection of prognostic markers in serum (7). Further complications in CLL patients are autoimmune haemolytic anaemia (AIHA) and immune-mediated thrombocytopenia (7). Moreover, reactivation of cytomegalovirus, listeria, and pneumocystis infections may also affect the already weakened CLL patient (7). To group and treat patients, physicians utilize two influential scales of stratification to assess disease stage, disease progression, and outcome prediction; these are the Rai and Binet staging systems.

1.5 Clinical stratification of CLL cases: Rai and Binet systems

The CLL disease stratification scales are denominated Rai (1975) and Binet (1977) staging systems. Both systems, summarized on Table 1.1, rely on physical examination and blood counts without the need of expensive equipment, such as ultrasound, computed tomography, or magnetic resonance imaging, and both describe prognostic groups with respective clinical outcomes (5). The Rai and Binet stratifications help physicians to determine extension of disease progression and the possible avenues of individual treatment. In general, under both classifications, CLL patients in early stages present lymphocytosis in peripheral blood and BM. Patients at intermediate risk show lymphocytosis, enlarged LNs, and splenomegaly. High-risk patients show anaemia, extreme weight loss, night sweats, and thrombocytopenia. More specifically, Rai stating is widely employed in the United States of America, whilst Europe prefers the utilization of the Binet system.

Despite the fact that these two clinical staging systems are useful tools to predict overall survival and to determine patient handling, they cannot predict an individual’s risk of disease progression in the early stage (3). Currently, physicians make use of morphology (Fig. 1.1), cytogenetics (Fig 1.2), fluorescence in situ hybridization (FISH,

Fig. 1.3), flow cytometry (Fig. 1.4), and molecular genetics (Fig.1.5) to obtain a more accurate and reliable medical picture of each patient. All together, these are denominated prognostic markers and the following sections briefly discuss them.

Table 1.1 The Rai and Binet staging system with expected median survival.

Rai staging system				
Stage 0	**Stage II**	**Stage II**	**Stage III**	**Stage IV**
Low risk **+ lymphocytosis**	**Intermediate risk** **+ lymphocytosis** **+ lymphadenopathy**	**Intermediate risk** **+ lymphocytosis** **+/- lymphadenopathy** **+/- splenomegaly** **+/- hepatomegaly**	**High risk** **+ lymphocytosis** **+/- lymphadenopathy** **+/- splenomegaly** **+/- hepatomegaly** **+ anaemia**	**High risk** **+ lymphocytosis** **+/- lymphadenopathy** **+/- splenomegaly** **+/- hepatomegaly** **+/- anaemia** **+ thrombocytopenia**
> 10 years	**≈ 6 years**	**≈ 6 years**	**≈ 2 years**	**≈ 2 years**

Binet staging system **		
Stage A **Lymphadenopathy < 3 areas** **- anaemia** **- thrombocytopenia**	**Stage B** **Lymphadenopathy ≥ 3 areas** **- anaemia** **- thrombocytopenia**	**Stage C** **Lymphadenopathy ≥ 3 areas** **+ anaemia** **+ thrombocytopenia**
> 7 years	**< 5 years**	**< 2 years**

**Binet staging system considers the lymph node swollen areas on the neck, under arms, groin area

1.6 Cytogenetics analysis in the CLL context

Cytogenetics allows us to observe the complete chromosomal map of an individual, thus becoming instrumental in describing structural chromosomal defects that may correlate with disease. In the CLL context, classical cytogenetic analyses reveal chromosomal aberrations deletions on the long arm of chromosome 13 involving band 13q14 (del(13q14)), which is the most detected aberration in ca. 55% of patients (5). Additionally, there are deletions of the long arm of chromosome 11 (del(11q)), the band were gene ATM is located, which encodes the proximal DNA damage response kinase ATM. Patients with (del(11q)) display gross lymphadenopathy, rapid disease progression, and shortened survival (5). Trisomy 12 is also common aberrancy in CLL

patients (Fig. 1.2), yet it is rather unclear which genes in this chromosome are involved in CLL pathogenesis.

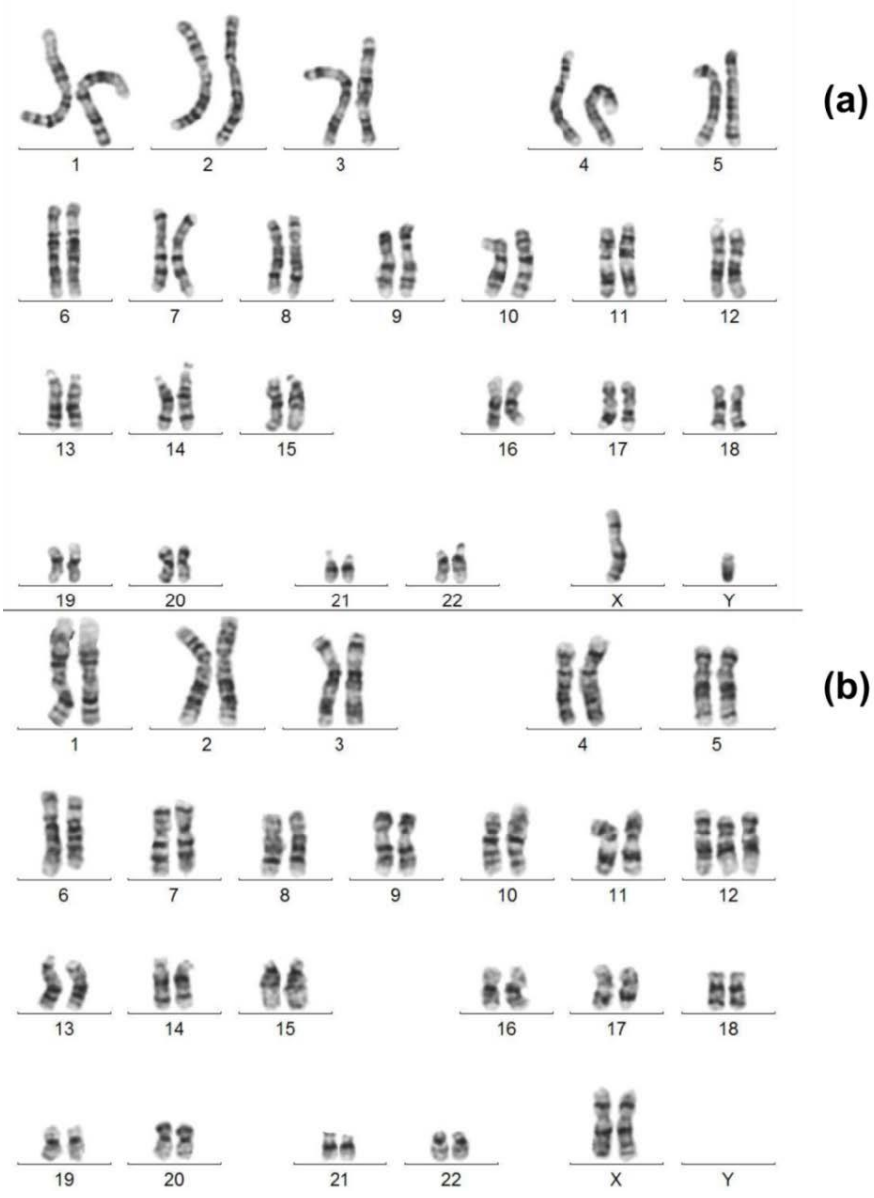

Figure 1.2 Detecting trisomy 12 in a CLL patient.
A healthy patient (a, 46, XY) presenting no aberrations in the chromosomal map. A CLL patient (b, 47, XX) presenting a typical trisomy 12. Diagrams kindly provided by C. Krings-Rocha from the LMHO.

Notoriously influential in CLL pathology are the deletions of the short arm of chromosome 17l(17p)). Specifically, those located at band 17p13. Such a deletion affects the tumour suppressor gene TP53, which encodes p53, a protein that keeps cell cycle in check and is essential in DNA damage repair. Furthermore, when cytogenetics reveal this deletion it becomes a predictor of therapy resistance and also

a strong indicator of bad prognosis (5). Other isolated deletions, such as 13q14, have good prognosis for the patient (3). In some instances, CLL cases carry cytogenetic alterations not characteristic of CLL such as 7q deletions (1).

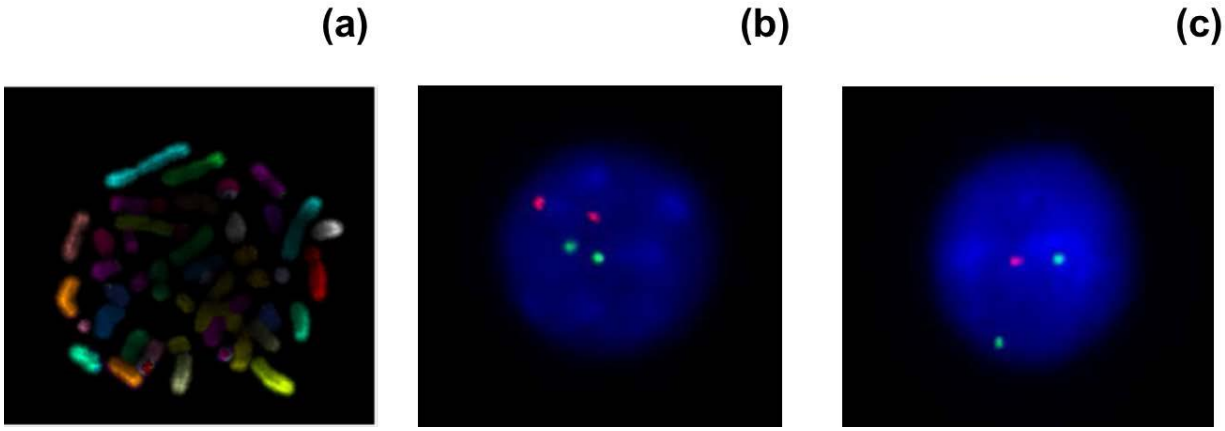

Figure 1.3 FISH in CLL diagnosis.
(a) Example of a multicolour FISH panel. In a healthy cell (ATM/TP53) there is not detection of TP53 deletion, but in this CLL sample (c) FISH (ATM/TP53) detect the TP53 deletion. Diagrams kindly provided by Mr. C. Krings-Rocha from the LMHO.

Due to the large number of CLL patients failing to show genetic aberrations in classical cytogenetics analysis, approaches such as FISH become a great tool to uncover underlying genetic defects (Fig. 1.3). For example, FISH is useful to detect deletions in chromosome band 13q14, deletion of 11q22.3-q23.1, deletion of 6q21-q23, and mutations or deletions of 17p13 (3). Figure 1.3 (c) shows an example of TP53 deletion. Using FISH profiling to predict median survival shows that patients with 17p have the shortest disease duration of circa 32 months and those patients with 13q deletion of circa 133 months (3).

1.7 Prognostic markers in CLL diagnosis

The diagnosis of CLL cells also employs flow cytometry to detect patterns of surface membrane antigens, which are particularly specific of CLL cells and allow for an accurate cellular discrimination and selection. The clusters of differentiation (CDs), commonly targeted on the surface of CLL cells are CD5, CD19, CD20, CD23, CD27, CD40, CD45, and CD45RA. In addition, CLL cells are normally negative for CD10, CD14, CD34, CD122, CD154, and IgG (8). When compared to CLL cells, healthy B-

cells express notably lower levels of surface immunoglobulins (Igs), CD20, and CD179b. Clonal B-cell populations with an atypical CLL phenotype (bright CD20 and Ig, lack of CD23) or even a non-CLL phenotype (CD5 negative) are detectable in healthy individuals (1). This suggests that during the life of a patient these CLL-to-be lymphocytes are exposed to the signals that trigger their proliferation and excessive accumulation, leading to a full CLL clinical development.

Additionally, two important factors utilized in diagnosis are zeta-chain-associated protein kinase-70 (ZAP-70) and cluster of differentiation-38 (CD38). Expression of protein tyrosine kinase ZAP-70 and the type II transmembrane glycoprotein CD38 strongly correlate with poor prognosis and aggressive disease, usually requiring immediate therapy (9).However, unlike all CDs and other surface markers, ZAP-70 is localized in the cell cytoplasm. Figure 1.4 shows four different CLL patients tested for ZAP-70 expression. In order to indicate a positive ZAP-70 diagnosis, there has to be a minimum of 20% CLL cells positive for ZAP-70 in a given sample; only samples (c) and (d) are ZAP-70 positive.

1.8 Genetics analysis of CLL patients

Allegedly, mutation-inducing enzymes produced upon encountering antigens expressed by follicular dendritic cells in the presence of T-cells trigger the somatic mutation in normal B-cells (also known as hypermutations) transforming them into leukemic B-lymphocytes. These mutations putatively occur in “germinal centres” localized to the BM and secondary LNs. Due to their close relation with disease progression, researchers highlight somatic mutations in essential genes such as NOTCH1, MYD88, TP53, ATM, SF3B1, FBXW7, POT1, CHD2, some of which play central function in regulating DNA damage and DNA repair signalling (5).

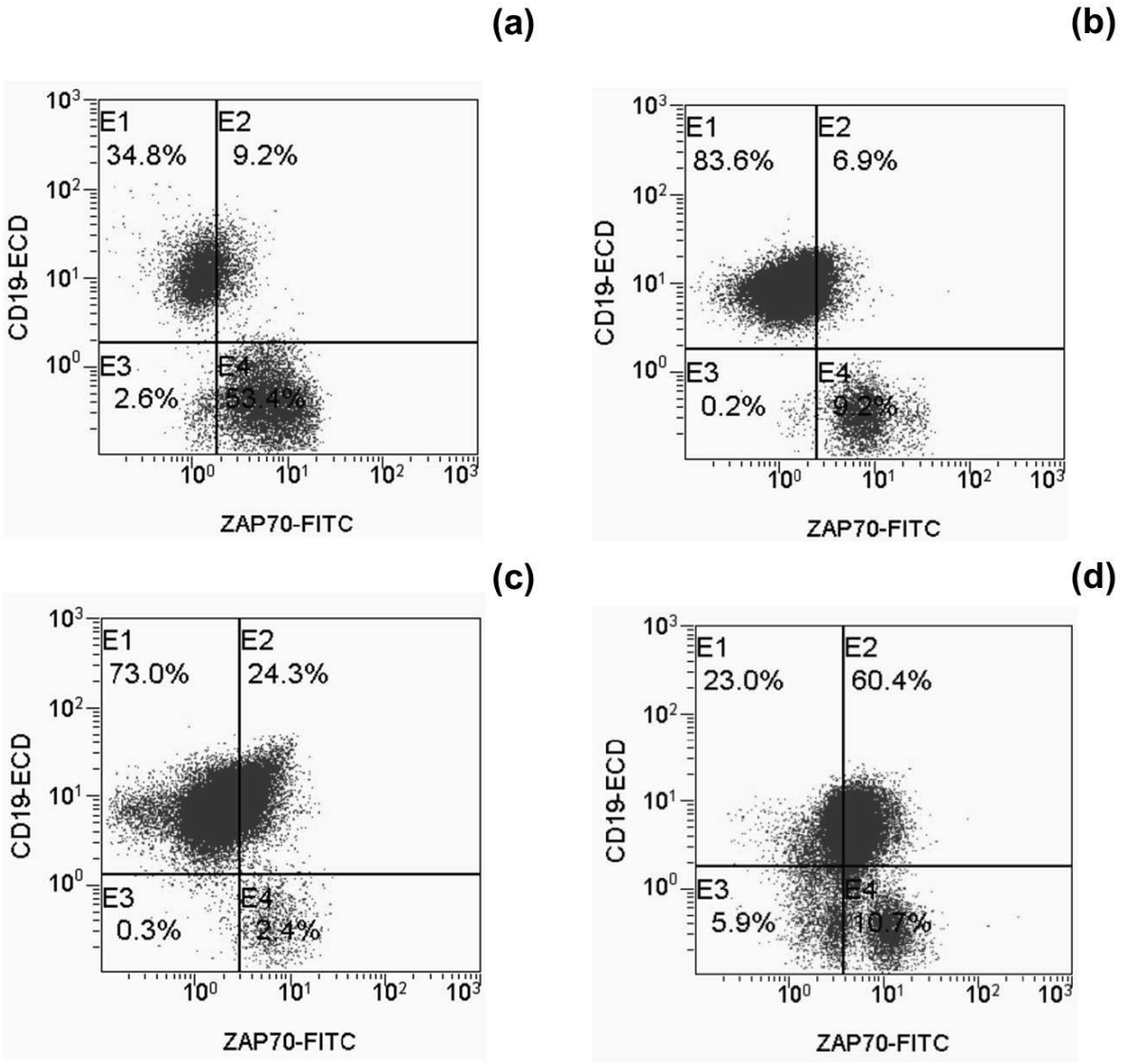

Figure 1.4 Detection of ZAP-70 in CLL patients.
The diagram shows the ZAP-70 expression profile of four CLL patients. Unlike most cluster of differentiation (CDs) commonly screened by diagnostics, ZAP-70 is localized in the CLL cell cytoplasm. In some instances, ZAP-70 expression correlates with bad prognosis and worst survival rates, hence its relevance as a prognostic marker and its use by diagnostic laboratories. Patients (a) and (b) are considered ZAP-70 negative, since diagnostics require a threshold of 20% and above. Patients (c) and (d) are ZAP-70 positive. Images kindly provided by Kristin Kramer and M. Patz from the LMHO.

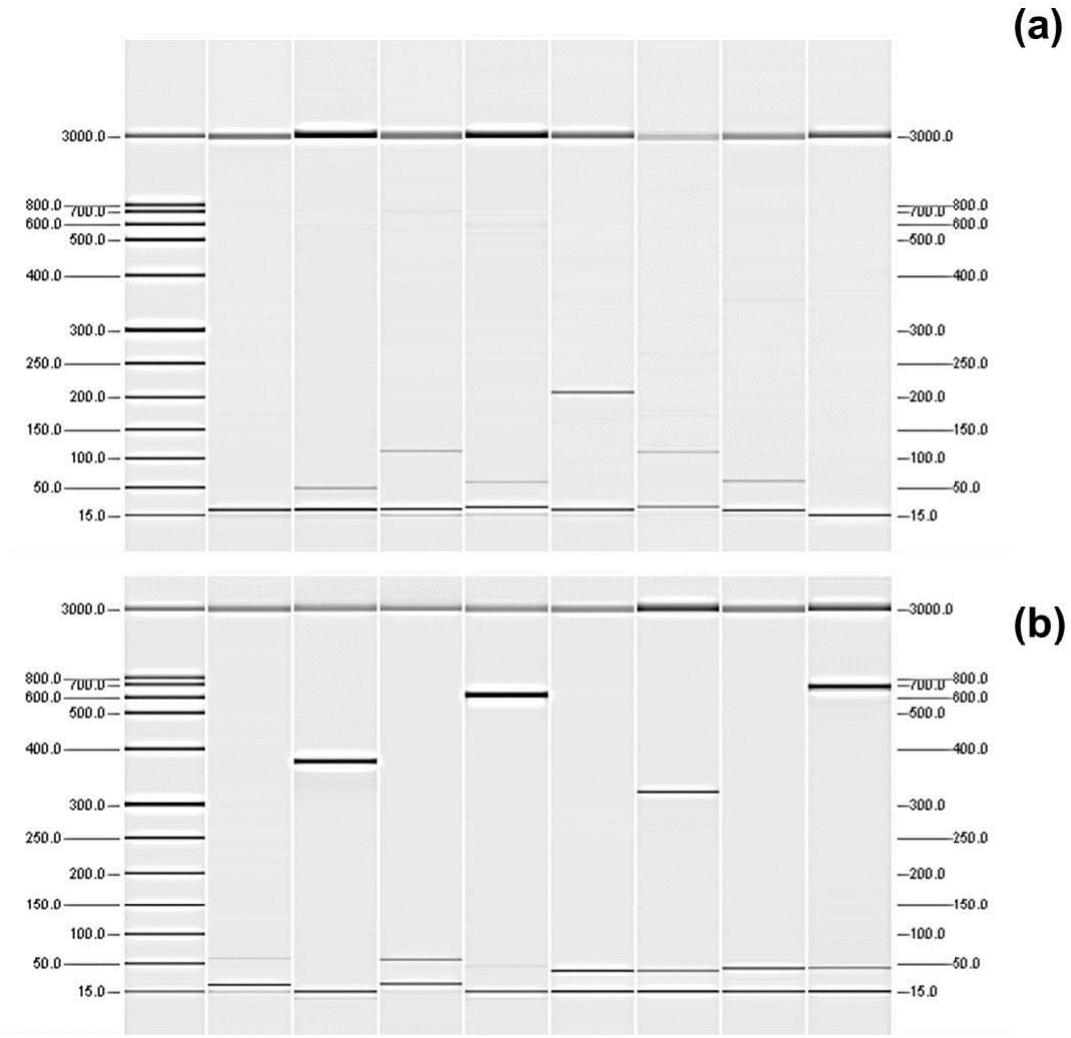

Figure 1.5 Analysis of segments of the IgV_H and TP53 exons. Diagnostic verify the most relevant segment of IgV_H (a) gene and the exons of TP53 (b) to assess their presence and their mutational state. Electrophoresis photographs kindly provided by Elena Krämer from the LMHO.

Mutations of the rearranged variable regions IgV_H are not associated with bad prognosis in CLL. Nonetheless, the IgV_H status has strong repercussion over the expected survival of CLL patients (3). Their sequences and different gene segments are better visualised by capillary electrophoresis (Fig. 1.5, a) and besides IgV_H, other genes, such as TP53 are visualised using this technique and further sequenced to verify mutations in the different exons (Fig. 1.5, b). However, an unmutated IgV_H is strongly associated with poor prognosis (Fig. 1.6). These discrepancies have given rise to two important subsets of CLL cells defined based on the IgV_H mutational status.

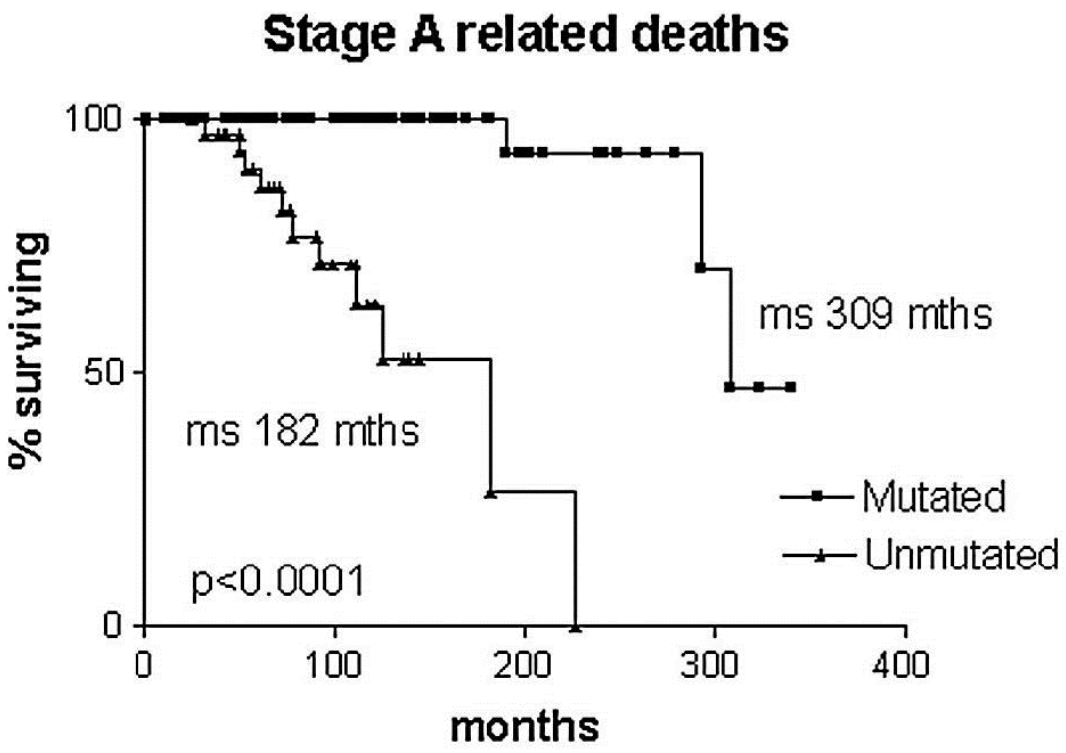

Figure 1.6 Survival of CLL patients and their IgV_H mutational status. The actuarial fitting curve compares patients with mutated and unmutated IgV_H genes in Binet stage A. 114 stage patients were include and non-B-CLL deaths excluded. Retrieved from Kay N E et al. 2002 (3).

1.9 Treatment strategies against CLL

Available therapies against CLL include the B-cell receptor (BCR), multiple chemokine and angiogenic growth factor receptors, adhesion molecules, and members of the tumour necrosis factor (TNF) family such as CD40, BMCA and BAAF-R (10). Currently, to treat CLL physicians include the "gold standard" chlorambucil, a classical cytostatic and alkylating agent of low toxicity, low cost, and oral administration (5). The standard line of treatment is composed of the monoclonal antibody rituximab with the cytostatic drugs fludarabine and cyclophosphamide (known as FCR) or with bendamustine (BR). FCR appears to have more efficacy that BR, nevertheless it presents toxic side effects to the patient (11). In a two-arm prospective trial of FC (fludarabine and cyclophosphamide) versus FCR (FC and rituximab), addition of rituximab showed improved progression-free survival (PFS) and overall survival (OS) in CLL patients (11).

Rituximab is a chimeric type 1 antibody against the B-cell antigen CD20, a protein expressed on the surface of B-cell of non-Hodgkin lymphomas and CLL cells. Obinutuzumab is a monoclonal antibodies which targets receptor CD20 on CLL cells too (5). Additionally, ofatumumab, a CD20 antibody humanized type 1 monoclonal antibody, targets a different epitope of CD20 displaying higher affinity when compared to rituximab (11). Obinutuzumab (GA101), a glycoengineered type 2 antibody also targeting CD20, possesses higher antibody-dependent cytotoxicity and direct non-apoptotic cell death induction (11). Glycoengineering refers to the modification of sugar residues on the antibody surface in order to increase its affinity towards its target protein. The use of obinutzumab correlates with activation of cellular mechanism such as complement-dependent cytotoxicity, opsonisation (increase in phagocytic capabilities) of macrophages that causes antibody-dependent cell-mediated cytotoxicity, and induction of apoptosis.

Together with fludarabine, cladribine (2-CdA-chlorodeoxyadenosine) and pentostatin (DCF, 2′-deoxycoformycin) are two additional purine nucleoside analogues introduced for the treatment of CLL and their use improves PFS in CLL patients (12). The immuno-modulatory agent lenalidomine is a second generation of thalidomine that has been employed with some success in CLL patients (5). Apparently, lenalidomide inhibits the action of important cytokines (TNF α, interleukin-7, and VEGF) stimulating proliferation of T- and natural killer (NK)-cells. Whist lenalidomine possess higher efficacy and fewer side effects, it is a potential inducer of teratogenicity (11).

A focus in current therapeutic CLL research is the BCR signalling pathway, which is essential for CLL cell survival. The Federal Drug Administration (FDA, USA) recently (2014) approved Imvubrica/Ibutrinib, a BTK inhibitor drug that blocks BCR-dependent CLL survival. Ibutrinib binds to cysteine 481 of the enzyme Bruton's tyrosine kinase (BTK), inactivating downstream signally of BCR, resulting in lower migration and proliferation rates of malignant leukocytes possibly by activating their apoptosis (11). This drug is well-tolerated with most of its side effects classified as grade I or II (11). Resistance to ibutrinim is attributed to mutations in cysteine 481 of BTK and mutations in phospholipase Cγ2 (PLCγ2), a downstream enzyme in BCR signalling.

Antagonist of C-X-C chemokine receptor type 4 (CXCR4, also known as fusin or CD184), BCR inhibitors, and PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) inhibitors act by obstructing the direct interaction of CLL cells with other bystander cells

present in the microenvironment, such as stromal, mesenchymal, and endothelial cells (EC) (9). Cellular biologists more accurately define the microenvironment as the extracellular matrix. However, in oncology, the term microenvironment is more common and broadly disseminated, because it better reflects the interaction of multiple tissues, cells, and molecules. The term microenvironment thus will prevail throughout this manuscript. BCR can also be targeted using idelasibib, a phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor. Phosphorylated PI3K associates with activation of nuclear factor-kappa-B (NFκB), and activation and expression of B-cell lymphoma (BCL)-XL and Mcl-1, two factors regulating pro-survival pathways (11). Furthermore, PI3K inhibition has a detrimental effect in CLL cells characterized by the migration of leukemic lymphocytes into microenvironments protecting them from chemo-therapeutic approaches (11).

GS-1101 is an up-to-date PI3Kδ (a PI3K isozyme), specifically a BCR-related inhibitor that promotes CLL apoptosis, migration, and homing (9). GS-1101 not only inhibits CLL cells chemokine-mediated adhesion in response to CXCL12/CXCL13 factors, but also prevents CLL cells from migrating beneath stromal cells. Other drugs regulate leukemic cell adhesion to vascular endothelium and controls migration beneath and underneath bone marrow stromal cells (BMSCs) and examples of these are AMD3100, T140, and ALX40-4C, which target the CXCR4/CXCL12 signalling pathway (9). Furthermore, AMD3100 triggers mobilization of CLL cells from their protective microenvironments to the blood stream, making CLL cells accessible to conventional drugs (9).

In addition to the above mentioned therapies, physicians consider allogenic haematopoietic stem cell transplantation (alloHSCT) appropriate for those CLL patients of young age without significant comorbidities, for those presenting refractory CLL to purine analogues and show relapse within the first two years of purine analogue therapy, or for those displaying a 17p(TP53) mutation (13). The benefits of alloHSCT are visible over time after the transplantation and once the graft-versus-leukaemia state is achieved (13). The allogenic graft-versus-leukaemia effect can overcome resistance toward high-dose therapy and eradicate minimal residual disease. However, donors need to be related (e.g. siblings) which becomes a hurdle to many patients; unrelated donor transplantations are possible, but there is a high-risk of

mortality (12). After alloHSCT, there is PFS of approximately 35-45% in a 5-year monitored period post-transplantation (13).

Whilst some of the above mentioned drugs are safe and well tolerated and lead to high initial response rates, alone or in combination, they may not induce complete remission and some CLL patients show disease relapses. This demonstrates that available therapies are not suitable for all CLL cases and in the case of high risk patients (del(17q) with TP53 mutated), may fail to induce remission and to prevent progression into late stages of disease.

1.10 The cancer microenvironment and CLL pathogenesis

This highlights the importance of molecular cross-talk between CLL cells and those of the microenvironment, which strongly modulate signalling pathways that support not only CLL survival, but also dictate lymphocyte trafficking between BM, LNs, and the blood stream (9). Transmembrane proteins on CLL surface regulate the molecular cross talk between microenvironmental signals that supports survival and proliferation of malignant CLL cells. Targeting the microenvironment interaction is of utmost importance to successfully treat CLL, because CLL cells adhere to stromal cells microenvironment, amongst others, becoming resistant to the cytotoxic effect of common drugs such as corticosteroids and fludarabine (9). The prolonged survival, high proliferative rates, evasion of apoptosis, and resistance to treatment exhibited by CLL cells depends on the tight and efficient molecular intercommunication between the microenvironments and the cytoplasm. It appears that early events in the different cancer microenvironments, namely BM, secondary LNs, and spleen, trigger specific genomic alterations that affect apoptosis in clonal B-cells transforming (By transformation the author refers to mutagenesis) the healthy B-cells into malignant leukemic cells (5).

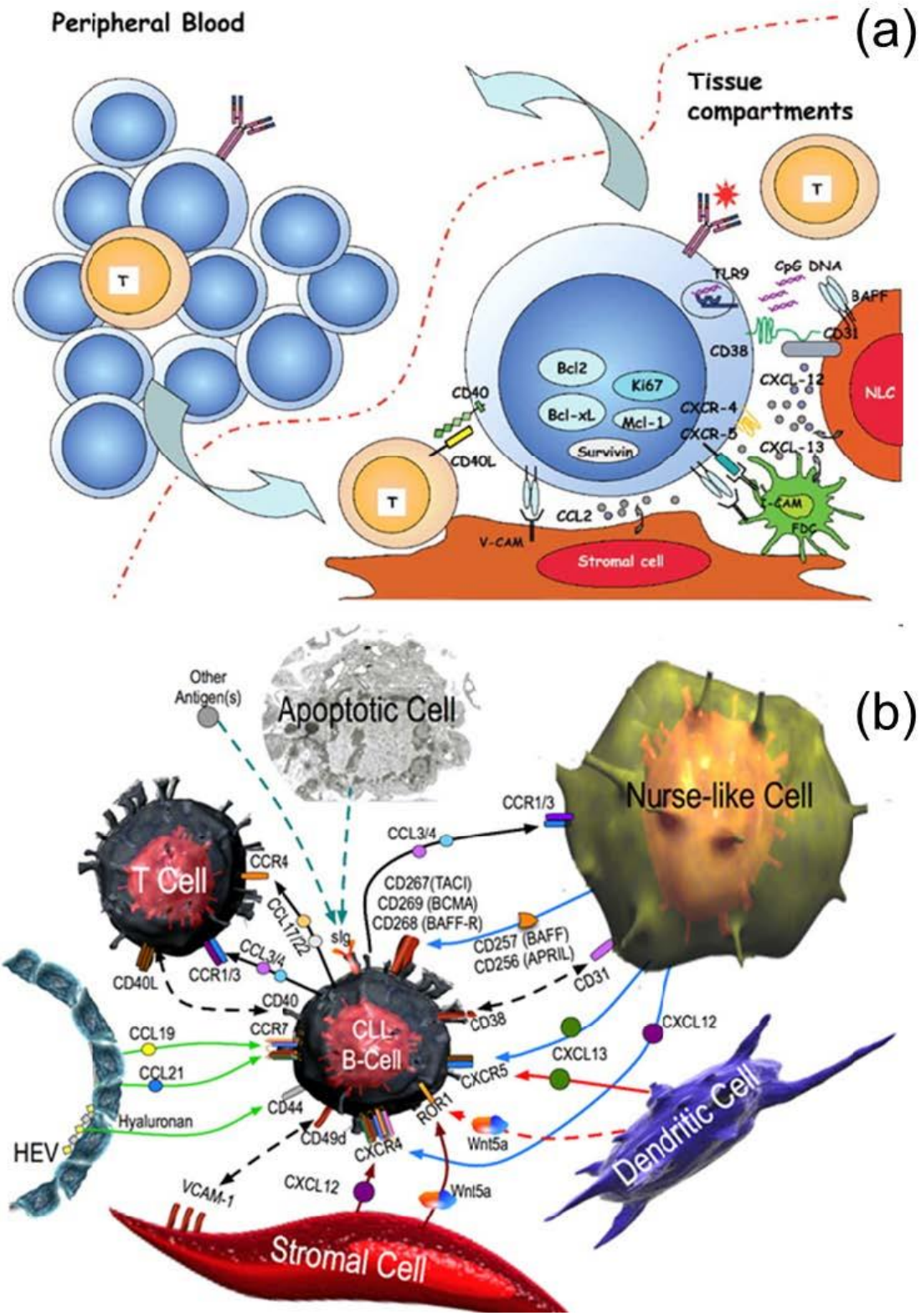

Figure 1.7 Influential models of the CLL microenvironment.
The complex molecular and cellular microenvironment is believed to provide CLL cells with molecules that trigger pro-survival and turn-off pro-apoptotic signalling allowing them to tribe, proliferate, and circulate between the peripheral blood and microenvironmental compartments. Models retrieved from (a) Caligaris-Cappio, 2011 (14) and from (b) Fecteau and Kipps, 2012 (15).

Abnormalities in the immune system correlate with a variety of aberrant defects involving the normal B-cell population, T-cell subsets, and NK-cells that act primarily as defence in malignancy and dendritic cells that regulate prompt cytotoxic T-cell response against tumour cells. (3). It is plausible to infer that functional deficiencies in any of these cell types would render patients more prompt to develop malignancies (3). Figure 1.7 shows the most influential microenvironment in the CLL research field. These proposed models highlight the complex molecular cross talk existing between CLL cells, bystander cells, and tissue in the microenvironment.

The vast assortment of cells comprising the cancer microenvironment reflects the challenges that researchers and physicians face whilst trying to comprehend the CLL niches of proliferation (Fig. 1.8). For example, cellular population of the BM microenvironment includes haematopoietic and non-haematopoietic cell types such as nerve cells, non-melinating Schwann cells, mesenchymal stromal cells, human stem cells (HSC), EC, bone-forming osteoblast, bone-resorbing osteoclast, CXCL12-abundant reticular (CAR) cells, B-lymphocytes, T-lymphocytes, nurse-like cells, adipocytes, fibroblasts (16), macrophages, eosinophils, and basophils, amongst others. Less understood in the CLL context is the LN microenvironment, which establishes immunity maintenance of immunological tolerance (17). Here, stromal and dendritic cells modulate extravasation of lymphocytes from lymphatic vessel to the high endothelial venules (HEV), where T- and B- cell come in close proximity and segregate in cortex and paracortex respectively. However, research of the molecular dynamics of the LN in CLL context is in its infancy. Furthermore, it has been suggested that CLL cells are capable of influencing the cellular composition in the microenvironment, which allows them to avoid immune-mediated destruction (10).

1.11 Angiogenesis and hematopoietic malignancies

All of the aforementioned cells comprising the different microenvironments secrete a plethora of angiogenic inducers, angiogenic inhibitors, cytokines, chemokines, and metabolites. All of the factors must act in combinatorial manner to preserve optimal cellular population, tissue stability, and proper modulation of signalling transduction events that determine cellular fate. It is not surprising that the aberrant and often excessive expression of angiogenic inducers and inhibitors

associates with leaky vessels and accumulation of macromolecules in plasma and tumour tissue (17).

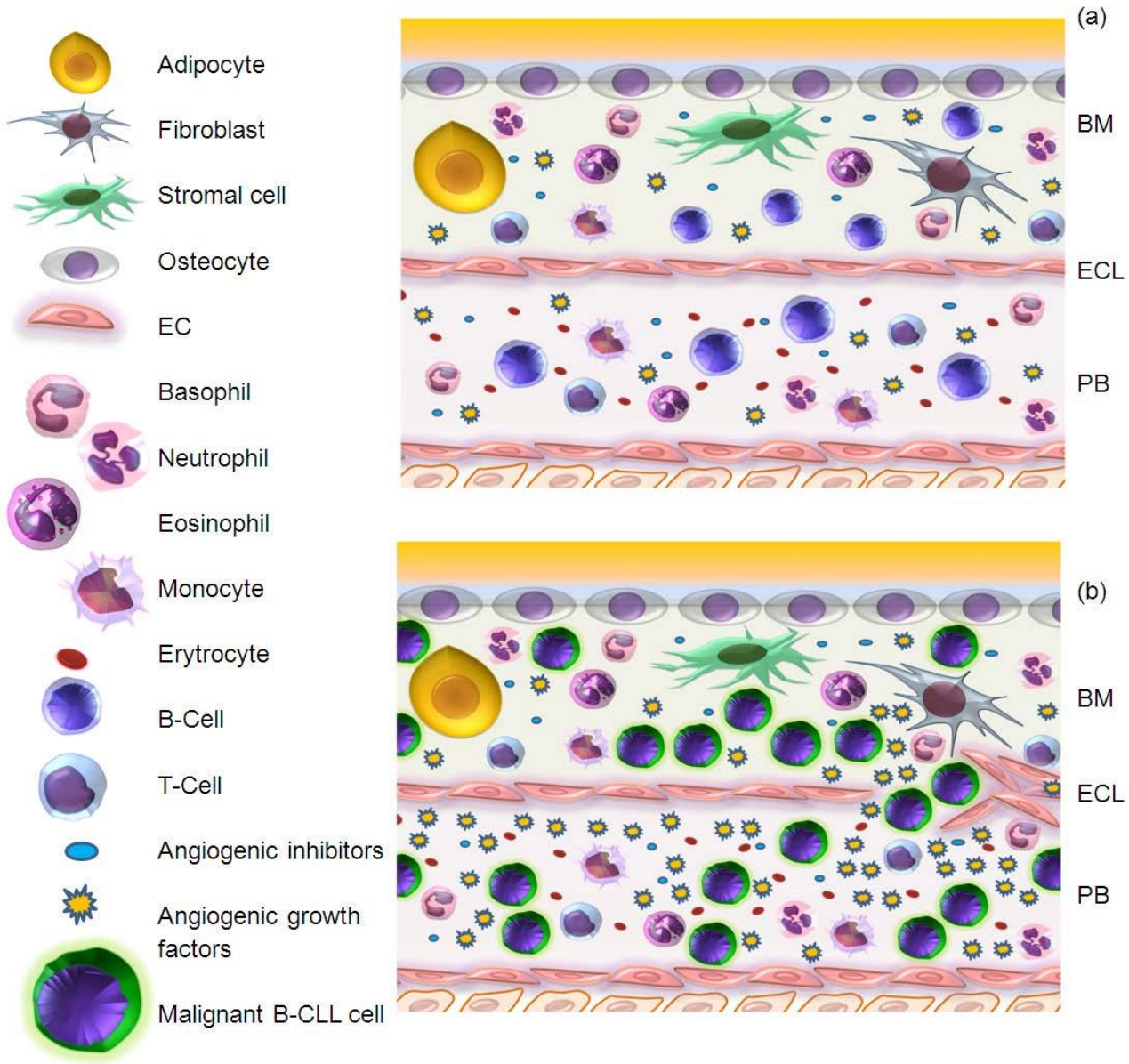

Figure 1.8 The cellular composition of the microenvironment.
The above model depicts the cellular composition normally found in the bone marrow and the blood capillaries in healthy patients (a). In CLL (b), it is believed that the endothelial cell layer (ECL) is disrupted, allowing the fast traffic between peripheral blood and the BM microenvironment and vice versa. Platelets are omitted from the model. Illustration retrieved from Aguirre Palma et al., 2014 (18). BM; bone marrow, ECL; endothelial cell layer, PB; peripheral blood.

Angiogenesis, the development of new blood vessels from pre-existing ones, is highly active during embryogenesis. During adult life, angiogenesis occurs exclusively during wound healing, menstrual cycles (19) and in heart and skeletal muscles due to

extraneous physical activity (20). When J. Folkman proposed angiogenesis as the process accountable for tumour growth in 1971 (21) he challenged the notions suggesting that neovascularization was merely an inflammatory response to necrotic tumour cells and that angiogenesis was a disadvantageous process for the tumour (19).

In healthy organisms, ECs, the main components of blood vessel, capillaries, and HEVs, remain in a quiescence state due to the balance between angiogenic inducers and inhibitors. The main angiogenesis inducers are the basic fibroblast growth factor (bFGF), the platelet derived growth factor (PDGF), and the vascular endothelial growth factor (VEGF), and some of the angiogenic inhibitors, such as angiostatin and endostatin (Fig. 1.9).

Pathological angiogenesis is one of the most dreadful physiological processes tightly linked to the disproportionate development of new blood vessels that support growth and proliferation of solid tumours. Overexpression of angiogenic inducers is the landmark and starting point to pathogenic angiogenesis, which supports the expansion of blood vessel networks that supply solid tumours with nutrients (Fig. 1.9). Expression of angiogenic growth factors supersedes that of angiogenic inhibitors, turning on the “angiogenic switch” that induces uncontrolled vasculogenesis and angiogenesis. The developing network of blood vessel will secure the growth and expansion of tumour cells by providing them with an efficient nutrient exchange.

Since leukaemias are not directly dependent on a network of blood vessels to support their basic physiological requirements it is rather difficult to envision how this process supports circulating malignant cells. Today it is widely accepted that pathological angiogenesis supports blood cancers (3,19,22) and molecular biologists and oncologists focus their effort in understanding the role of angiogenesis in haematological malignancies, including CLL.

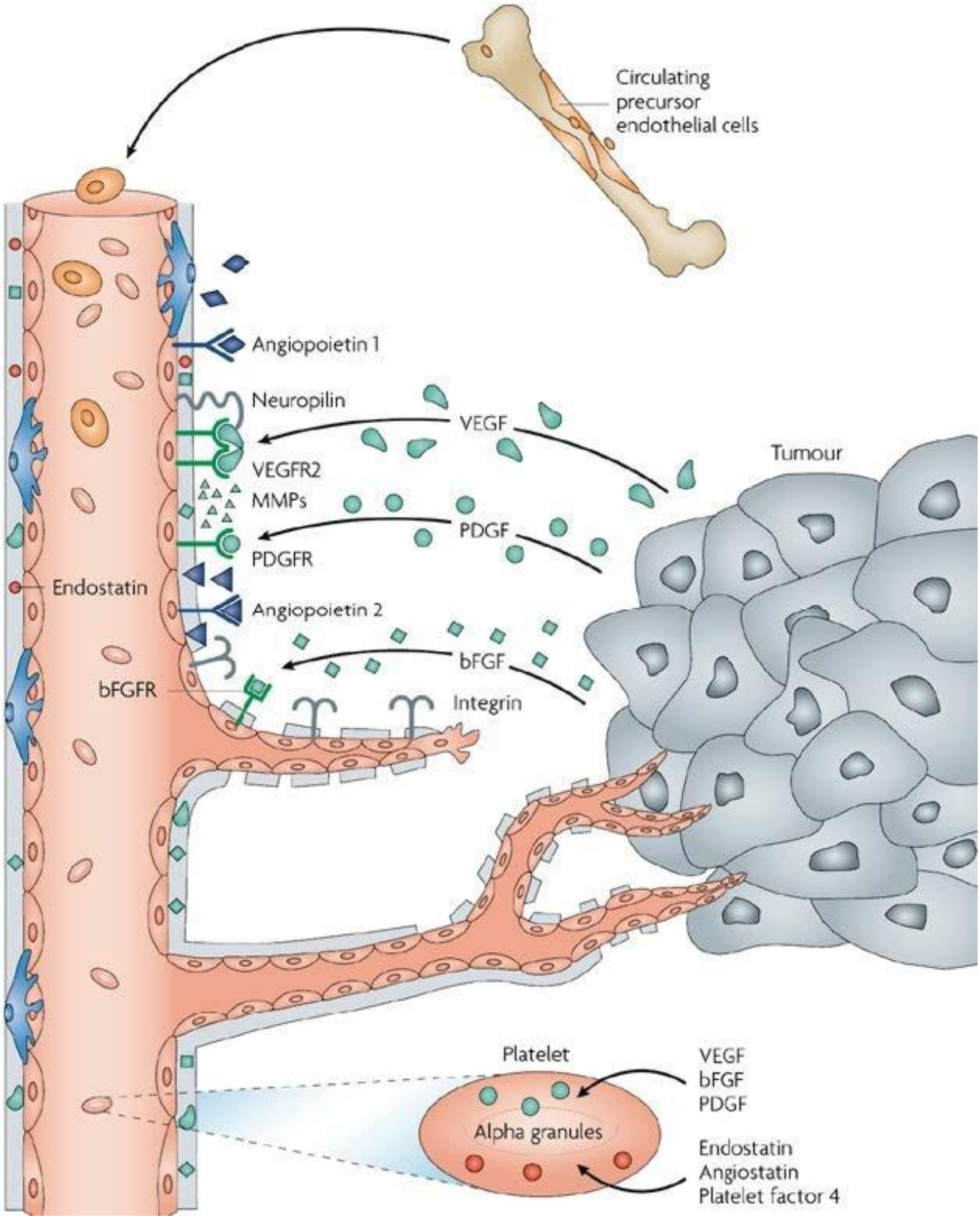

Figure 1.9 Angiogenic factors induces development of blood vessels. Tumour factors such as VEGF, PDGF, Ang2, and bFGF are excessively secreted by tumour cells into the microenvironment, triggering the development of new blood vessels from pre-existing ones. The constant secretion of angiogenic inducers eventually triggers development of a blood vessel network. Please note that Ang1 plays a crucial role in the keeping the endothelial cell layer in a quiescent state. Illustration retrieved from Juda Folkman, 2007 Nature Reviews Drug Discovery (23).

1.12 Classification of angiogenesis inducers and inhibitors

Angiogenic growth factors trigger the activation of angiogenesis-related signalling pathways resulting in the aberrant and pathological development of blood vessels, capillaries, and HEV. There are also identified angiogenic inhibitors, molecules that are involved in regression and growth inhibition of blood vessels. Altogether, they are called angiogenic factors and to avoid confusion they will be referred throughout this work as angiogenic inducers and angiogenic inhibitors. The Angiogenesis Foundation provides a concise list of angiogenic inducers and inhibitors identified to date. This list describes 21 known inducers and 41 known inhibitors. They are in large ligand molecules that recognise receptors on the surface of the cell membrane. Notably, the overexpression of angiogenic inducers tends to correlated with growth, proliferation, metastasis, and invasion of malignant tumours. The most extensively studied angiogenic growth factors are the VEGF, the bFGF, and the PDGF; figure 1.9 emphasises their roles in triggering angiogenesis.

The large number of inducers and inhibitors listed also illustrates the complexity that researchers face whilst studying angiogenesis and their related pathways, not only in CLL, but also in any other given solid or haematopoietic malignancy model. CLL researchers have only investigated a small portion of angiogenesis-related factors. Therefore, the impact of inducers and inhibitors over cellular and molecular mechanisms defining CLL pathogenesis is today not well understood and research remains in its infancy.

1.13 Angiogenesis and CLL pathogenesis

Studies from CLL cases describe abnormal vasculogenesis in the BM and LNs (24) and others illustrate abnormal angiogenesis related to over expression of pro-angiogenic factors (25). The first strong evidence showing that angiogenesis may be involved in CLL pathogenesis came from BM trephine sections derived from CLL patients (Fig. 1.10). Their analysis revealed that abnormal marrow angiogenesis associates positively to increased Rai stage (26). Peterson et al. not only described increased microvessel density in BM biopsies from CLL samples, but also linked this increase to the elevate secretion of bFGF detected in urine of CLL patients (27). Besides secreting high bFGF levels, CLL cells secrete and VEGF and show decreased

levels of trombospondi-1 (TSP-1), endostatin, and interferon-α (IFN- α) (3,25). The notorious high levels of bFGF and VEGF detected in the urine of CLL patients correlate with Rai staging and to increased microvessels density in BM sections of CLL samples (26). Thus, the significant density increase of microvessels and increase in bFGF/VEGF secretion in urine samples of CLL patients became the first central observation to foster the idea that angiogenesis is an active process in CLL.

Table 1.2 Know angiogenic inducers and inhibitors. Retrieved from the Angiogenic Foundation (28)**.**

Angiogenic inducers	Angiogenic inhibitors:
1. Angiogenin 2. Angiopoietin-1 (Ang1) 3. Del-1 4. Fibroblast growth factors: acidic (aFGF) 5. Fibroblast growth factors: basic (bFGF) 6. Follistatin 7. Granulocyte colony-stimulating factor 8. (G-CSF) 9. Hepatocyte growth factor (HGF) /scatter factor (SF) 10. Interleukin-8 (IL-8) 11. Leptin 12. Midkine 13. Placental growth factor (PGF) 14. Platelet-derived endothelial cell growth factor (PD-ECGF) 15. Platelet-derived growth factor-BB (PDGF-BB) 16. Pleiotrophin (PTN) 17. Progranulin (PGN) 18. Proliferin 19. Transforming growth factor-alpha (TGF-alpha) 20. Transforming growth factor-beta (TGF-beta) 21. Tumour necrosis factor-alpha (TNF-alpha) 22. Vascular endothelial growth factor (VEGF) (formerly vascular permeability factor (VPF))	1. Angioarrestin 2. Angiopoietin-2 (Ang2) 3. Angiostatin (plasminogen fragment) 4. Antiangiogenic antithrombin III 5. Arrestin 6. Chondromodulin 7. Canstatin 8. Cartilage-derived inhibitor (CDI) 9. CD59 complement fragment 10. Endostatin (collagen XVIII fragment) 11. Endorepellin 12. Fibronectin fragment 13. Fibronectin fragment (Anastellin) 14. Gro-beta 15. Heparinases 16. Heparin hexasaccharide fragment 17. Human chorionic gonadotropin (hCG) 18. Interferon alphagamma 19. Interferon inducible protein (IP-10) 20. Interleukin-12 21. Kringle 5 (plasminogen fragment) 22. Metalloproteinase inhibitors (TIMPs) 23. 2-Methoxyestradiol 24. PEX 25. Pigment epithelium derived factor (PEDF) 26. Placental ribonuclease inhibitor 27. Plasminogen activator inhibitor 28. Platelet factor-4 (PF4) 29. Prolactin 16kD fragment 30. Proliferin-related protein (PRP) 31. Prothrombin kringle 2 32. Retinoids 33. Soluble Fms-like tyrosine kinase-1 (S-Flt-1) 34. Targeting fibronectin-binding integrins 35. Tetrahydrocortisol-S 36. Thrombospondin-1 (TSP-1) and -2 37. Transforming growth factor-beta (TGF-b) 38. Troponin I 39. Tumstatin 40. Vasculostatin 41. Vasostatin (calreticulin fragment)

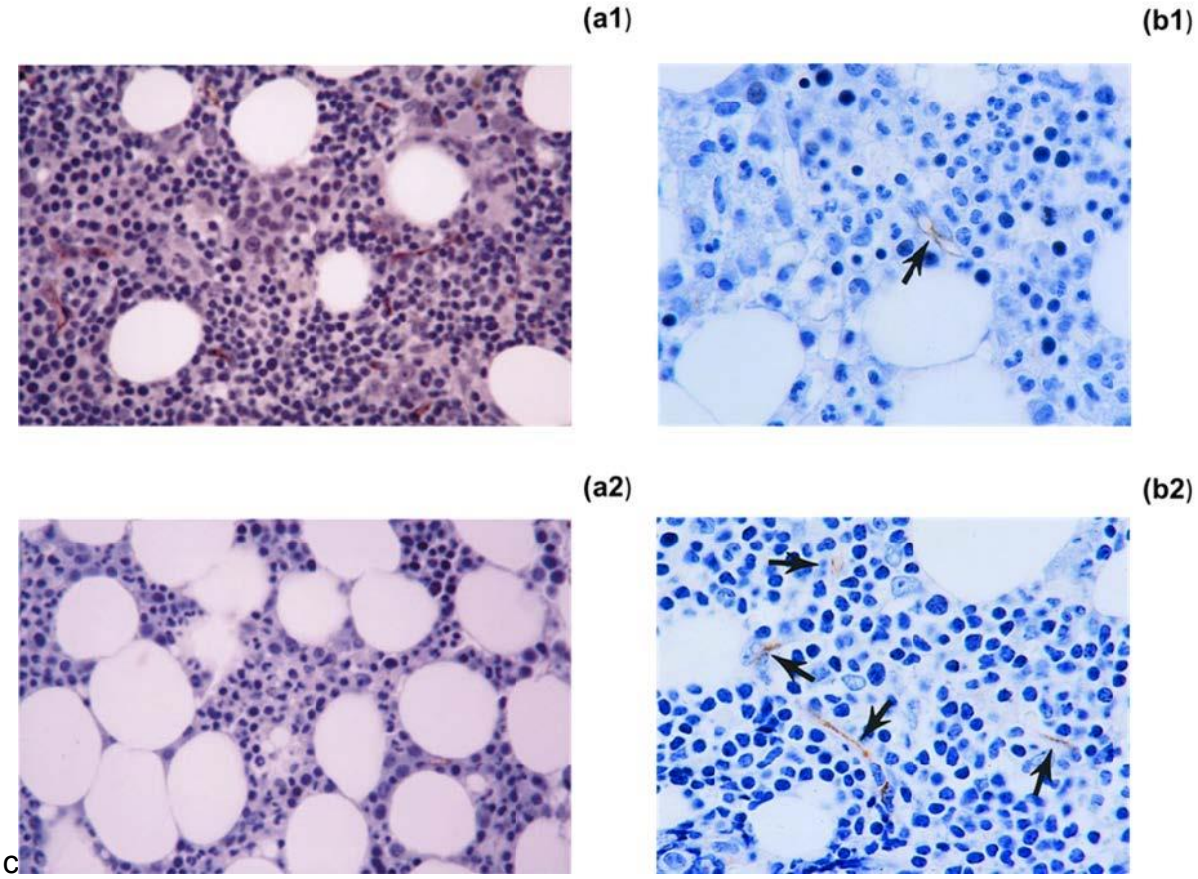

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Figure 1.10 Evidence of angiogenesis in CLL-BM sections
Evidence of angiogenesis involvement in CLL pathogenesis. Inmunohistochemical analysis demonstrated in two different and consecutive publications, 2000 and 2001, that there is an increase of blood vessel development in BM of CLL patients. Photographs a1/b1 and a2/b2 show the staining for microvessel density, which show a clear vascularization. Photographs b1/b2 indicate vessels with black arrows. Both publications target endothelial cell layers on microvessel with antibody that recognizes CD34 on ECs. Photographs a1/a2 retrieved from Kini et al. 2000 (26) and b1/ b2 retrieved from Peterson et al. 2001 (27).

VEGF and angiopoietins are important partners (29) and in association, they recruit inflammatory cells, such neutrophils, macrophages, T- and B-cells, as well as EC (30). Besides the overexpression of pro-angiogenic VEGF and bFGF, in recent years CLL research often highlights the peculiar overexpression of the angiogenic inhibitor angiopoietin-2 (Ang2) in CLL cases. Plasma of CLL patients shows high plasma levels of Ang2 and CLL cells abundantly secrete Ang2 in cell culture, an observation that tightly correlates with severity and disease progression of CLL cases (31,32). Increased Ang2 levels also correlated with shorter time to first treatment (TTFT), increased vascularisation in BM sections of CLL patients, and disease

aggressiveness (33). Furthermore, Ang2 levels negatively impact CLL disease course (34) correlating not only with Binet staging scale, but also with high β-2-microglobulin, unmutated IgV_H, elevated CD38-ZAP-70, and intermediate-high risk cytogenetics factors (32).

Recent epigenetic evidence demonstrated the association between low methylation on the Ang2 promoter and poor prognosis, shorter TTFT, and shorter survival of CLL patients (34). Lower methylation of Ang2 results in higher expression of Ang2 and vice versa, which may translate into higher protein secretion into the microenvironment. Newer reports demonstrate the concomitant expression of high levels of Ang2 and pro-angiogenic VEGF in CLL patients (32). Ang2 overexpression and its correlations to disease suggest that the Ang-Tie2 pathway is potentially relevant in CLL pathophysiology.

1.14 The Ang-Tie2 signalling receptor system

Ang2, together with pro-angiogenic angiopoietin-1 (Ang1), tyrosine kinase with immunoglobulin and endothelial growth factor domain-1 and 2 receptors (Tie1 and Tie2), and vascular endothelial protein tyrosine phosphatase (VE-PTP) comprise the Ang-Tie2 signalling receptor system (Fig. 1.11). This important pathway regulates vessel assembly and maturation during embryogenesis and is instrumental in securing quiescence of the vascular tissue during adult life (35). Also, it is one of the most relevant molecular mechanisms (Others being VEGF-VEGFR, bFGF-FGFR) in charge of controlling angiogenesis (Fig. 1.9). The Ang-Tie2 is relevant in pathology because it tightly associates with inflammation (36) and very strongly to tumour-related angiogenesis (37). This pathway dynamically supports successful proliferation of neoplastic cells, as well as growth of tumour tissue by aiding in the spreading of the new blood vessel network to feed malignant cells and tissue.

The angiopoietins comprise four ligands (Ang1, Ang2, Ang3 and Ang4) of similar structure (38) Ang-1(human) and Ang-4 (mouse) are agonists (inducers) factors, whilst Ang2 and Ang3 are antagonists (inhibitors) (39). An amino-terminal “superclustering” and coiled-coil domain followed by a carboxyl-terminal fibrinogen domain comprise the structure of all four angiopoietins (40). Regardless of their homology, they differ in their biological role (40).

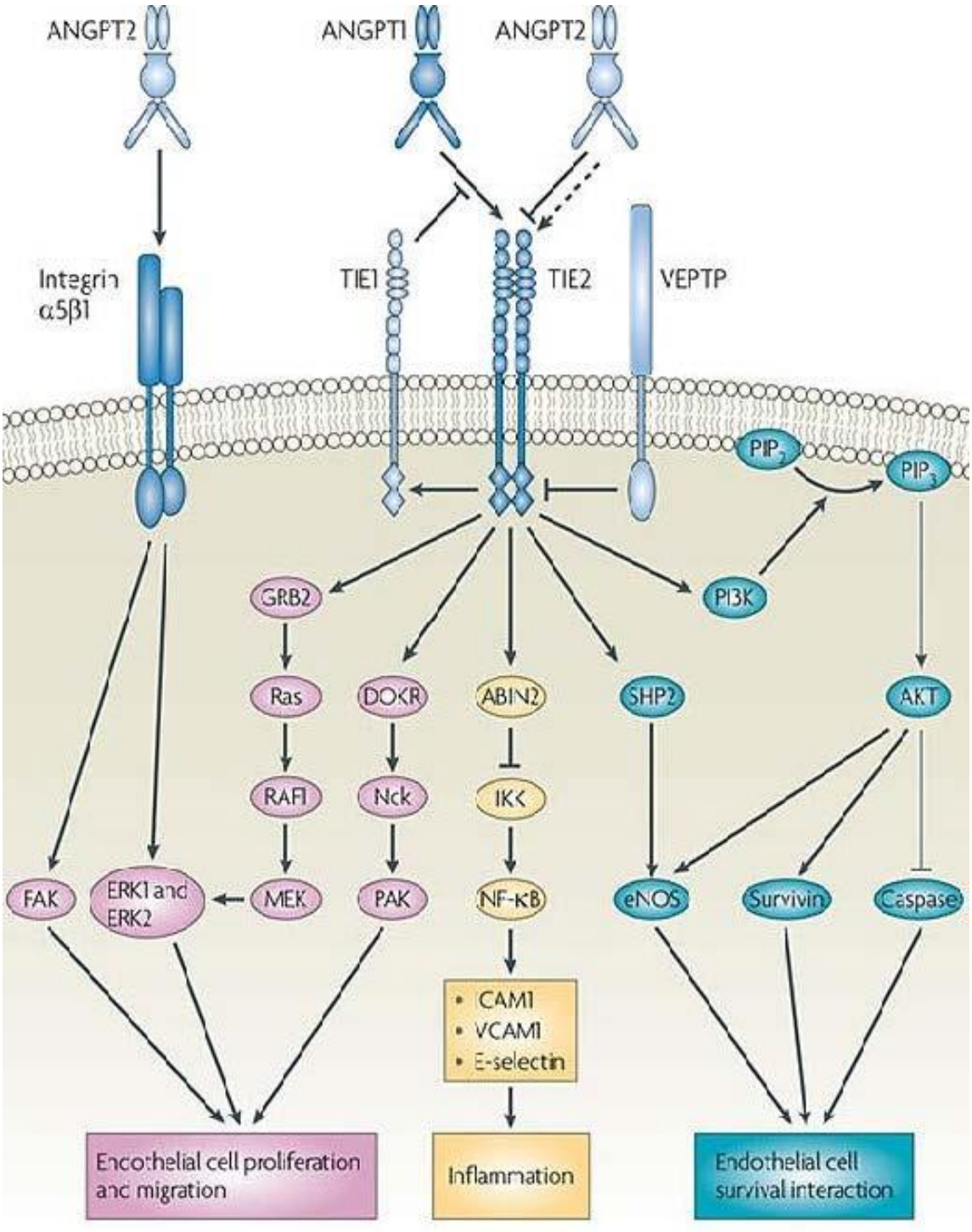


Figure 1.11 The Ang-Tie2 signalling receptor pathway at a glance. This is the most reliable and current representation of the Ang-Tie2 pathway and its molecular dynamics. It presents key the components, known downstream signalling transduction effects they regulate, and their likely effect. Arrow indicates phosphorylation and the blunted lines indicate dephosphorylation. Model retrieved from Huang et al. 2010, Nature Reviews Cancer (36).

Agonist Ang1 promotes EC survival in a dose-dependent manner, supports EC network formation and stabilization, and reduces apoptosis in EC models, an effect increased in the presence of VEGF (41). Its anti-inflammatory role promotes pericyte-dependent vessel integrity and supports a Tie2-constitutively activated state (38). The former is characterized by the maintenance a quiescent ECL of blood vessels (42), decreased vascular permeability (36), and fast repair of damaged and leaky vessels (29). Thus, Ang1 is a key maintainer of the vascular endothelial barrier that will not only prevent leakages but also tissue leukocyte migration (43). Notably, healthy adults present relatively high Ang1 plasma levels (40-50ng/ml) and Ang1 overexpression results in varying degrees of reduced tumour growth in certain models (35).

Homology screening revealed an antagonist to Ang1, a secreted ligand termed Ang2. Ang2 induces aberrant blood vessel formation when overexpressed in mouse embryos (44), a lethal phenotype resembling the lack of Ang1 and Tie2 (45). Ang2 destabilizes and remodels vascular endothelium (38) and its secretion dramatically increases during vasculogenesis and inflammation (43) inducing severe discontinuities in vessel development (44). Ang2 is capable of undergoing cytoplasmic localization inside Weible-Palade bodies. In addition, the half-life of Ang2 of ca. 18 hours and showed that chemical stimulation (phorbol 12-myristate 13-acetate [PMA], thrombin, and histamine) promotes swift Ang2 release from Weible-Palade bodies into the extra cellular space (45). Release of Ang2 into the microenvironment occurs mainly during vasculogenesis and inflammation (43). The high levels of Ang2 associated with severity of disease make Ang2 an attractive prognostic marker (35) in malignancies.

Receptors Tie1 and Tie2 are type 1 transmembrane protein receptor tyrosine kinases (RTKs) of homologous structure involved in proliferation, migration, and survival of endothelial cells which together with VEGF receptors represent exclusive endothelial cell-specific RTKs (39). At the N-terminus Tie1 and Tie2 possess two immunoglobulin-like (Ig), followed by three EGF domains, an additional Ig domain, three fibronectin type III-like repeats, a transmembrane domain and a catalytic tyrosine kinase domain at the C-terminus for transmission and propagation of signal transduction events (46). Tie2-dependent signal transduction is essential during embryogenesis. Experiments based on the lack of Tie2 showed that mice lacking this receptor die in utero due to aberrant blood vessel development (47). The orphan receptor, Tie1, sustains the integrity and survival of vascular ECs in regions undergoing

angiogenesis during mice embryogenesis. Similar to Tie2 deficiency, mice lacking Tie1 also die in utero (48). Whilst the role of Tie2 is well characterized, the molecular and physiological contributions of Tie1 remained for long rather unclear (39).

Receptors Tie1 and Tie2 interact through their ectodomains forming a complex detected even prior and regardless of Ang1 or Ang2 recognition. The kinase domains (endodomain) play no role in neither Tie2 clustering, nor Tie2 localization upon angiopoietin recognition (39). This Tie1-Tie2 complex inhibits Tie2-dependent signal transduction showing that Ang1 and Ang2 are capable of differentially modulating the Tie1-Tie2 complex (39). Remarkably here is the role of Ang1, which binds to Tie2 inducing dissociation of Tie1-Tie2 complex, Tie2 clustering, and Tie2-dependent signal transduction, whilst Ang2 fails to induce a similar effect, yet binds Tie2 preventing further Ang1 binding to the Tie2 acting as competitive antagonist (39).

The molecular mechanics surrounding the Ang-Tie2 signalling pathway were unclear and inaccurate, However, the now available biochemical evidence provide us with a more unified model of Ang-Tie2 signalling, depicting clear roles and interactions of ligands with receptors (37,39,40,49). Both Ang1 and Ang2 bind by complementary surface interaction to the same site on Tie2 (37,49) and recent research describes the specific amino acid residues on the surface of Ang1 and Ang2 that give their definite and contrasting cell-signalling competences (40).

Another member of the Ang-Tie2 signalling pathway is VE-PTP, whose main know function is to regulate Tie2-dependent proliferation during embryonic development and blood vessel remodelling (50). This peculiar regulator of Ang-Tie2 signalling transduction specifically binds and dephosphorylates activated Tie2, attenuating Tie2 activity, and resensitizing Tie2 to new angiopoietin stimuli (51). Ang1 activation of Tie2 shows an evident association of VE-PTP to Tie2 and VE-PTP dissociation enhances Tie2 activation by Ang1 (50–52).

1.15 Missing links between CLL and the Ang-Tie2 pathway

Though long considered inactive on CLL cells due to the absence of Tie2 receptor on CLL cells (25), the molecular mechanics of the Ang-Tie2 signalling system in CLL context become of particular interest for CLL researchers. CLL cells abundantly secrete Ang2 into the microenvironment and CLL patients present high level of Ang2,

which correlates with the severity of the disease (31,32). Intriguingly, CLL cells express the importance of Tie1 receptor and its expression correlates with the disease stage (53). Yet these leukemic B-cells fail to express the Tie2 receptor (25) and no attempts exist in literature to neither describe the role of VE-PTP nor to understand the impact that Ang1 over molecular and cellular behaviour in the CLL context. The implication of Ang-Tie2 signal transduction on CLL pathophysiology and its role in regulating central cellular functions of CLL cells remain to date a mystery.

1.16 The experimental goals and working hypothesis

Since CLL cells lack Tie2 receptor, the Ang-Tie2 pathway has been considered inactive in regulating the fate of CLL cells. The component of the Ang-Tie2 pathway, namely Ang1, Ang2, Tie1, Tie2, and VE-PTP, have been overlooked in CLL research since we only know that Ang2 is overexpressed in CLL cases and that CLL cells secrete Ang1 and Ang2, and express Tie1 on their surface. Therefore, this work aims to clarify to which extent Ang1 and Ang2 modulate essential physiological aspects of CLL cells such as survival and migration. The full contributions of the Ang-Tie2 pathway and its component to CLL pathophysiology at the molecular and cellular levels await elucidation. To assess the impact of angiopoietin on CLL pathophysiology it is essential to:

- Determine the parallel expression pattern of the components of the Ang-Tie2 signalling pathway in CLL cells by qualitative PCR.
- Analyse the circulating levels of ligand Ang1 levels in plasma from CLL patients and Ang1 levels that CLL secrete in cell media.
- Assess the impact of Ang1 and Ang2 on CLL cell survival by mimicking angiopoietin microenvironmental availability utilizing recombinant human Ang1 and Ang2.
- Determine fold changes in relative mRNA levels of Ang1, Ang2, and Tie1 in CLL cells upon exposure to recombinant Ang1 and Ang2.
- Expose CLL cells to angiopoietins and observed if these factor are capable of triggering Tie2 and VE-PTP expression by qualitative PCR.
- Understand the changes in expression patterns of Tie1 receptor on CLL cells upon angiopoietin exposure.
- Assess the ability of angiopoietin to modulate chemotaxis and transendothelial-like migration of CLL cells.
- Monitor the sensitivity of CLL cells to angiopoietins and their role in regulating caspase and ATP relative

2. METHODS AND MATERIALS

2.1 Compliance with ethical standards

Research was performed in accordance with the Declaration of Helsinki, which was drafted by the Word Medical association initially in 1964 as a guideline of ethical principles to be followed by researchers working with humans subjects, human material, and their information (54). Furthermore, the internal revision board of the University Hospital, Cologne, approved research procedures. Prior to drawing peripheral blood, CLL patients were accordingly informed and signed a consent form.

2.2 Confirmation of CLL diagnosis

All CLL patients had a confirmed CLL status prior peripheral blood collection. The CLL status was confirmed by classical haematology (e.g. B-cell lymphocytotis), flow cytometry (e.g. CD3, CD4, CD8, CD5, CD19, CD38, and ZAP-70) molecular genetics (e.g. mutated and unmutated IgV_H status), and cytogenetics (e.g. del(11q), trisome 12,) according to the guidelines of the Laboratory for Molecular Haematology and Oncology (LMHO) of the University Clinic of Cologne. Personal of Department of Internal Medicine I (Haematology and oncology) performed peripheral blood drawing from CLL patients.

2.3 Isolation of CLL cells and PBMCs

Processing of peripheral blood and CLL cell isolation was performed by personal of the Biobank, Centre for Integrated Oncology Cologne/Bonn (CIO), at the University Hospital Cologne. The peripheral blood from CLL and healthy patients was collected in ethylene diamine tetraacetic acid (EDTA)-treated tubes. EDTA is a proton donating acid (polyprotic) composed of four carboxylic acid group and two amines

capable of chelating (sequestrating) Ca++, which results in prevention of blood clothing (55). In addition, EDTA preserves cellular entities and protects cell morphology. The three different formulation currently available for blood preservation are Na_2EDTA, K_2EDTA (UK and USA) and K_3EDTA (Europe and Japan) (55). In a free solution EDTA would yield circa pH 2.5, whilst either of the three used salts (Na_2EDTA, K_2EDTA and K_3EDTA) in a 1% solution would yield a pH of circa pH 7.5 (55).

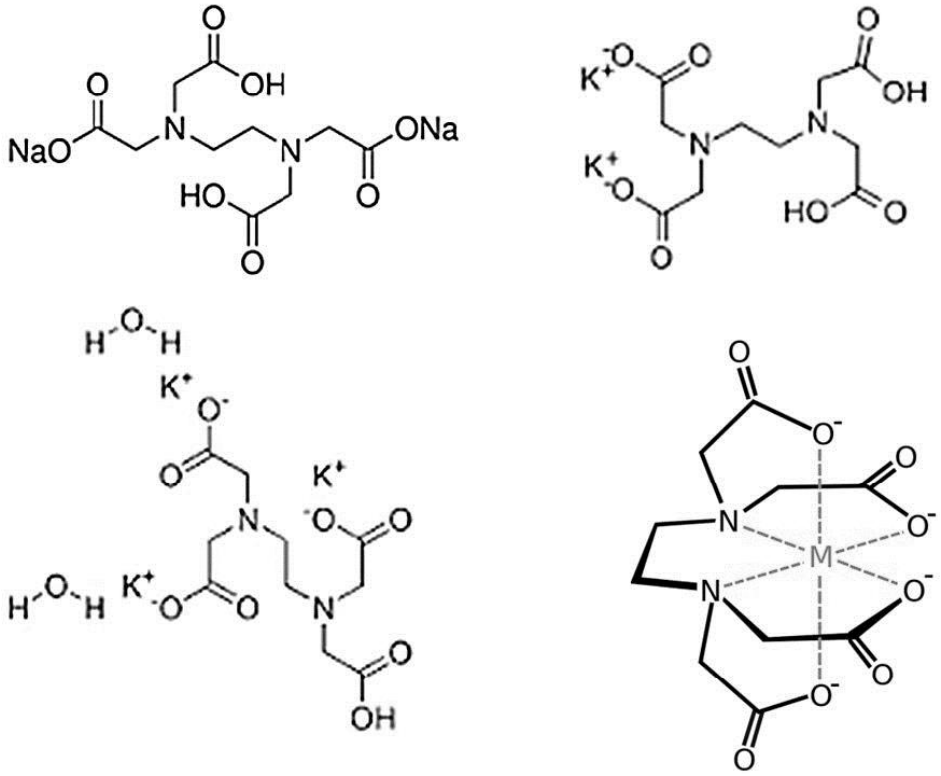


Figure 2.1 EDTA salts and metal sequestration.
There are three widely utilized EDTA salts available to prevent blood coagulation: Na_2EDTA, K_2EDTA and K_3EDTA. Upon encountering metals in blood, such as Ca++, metal sequestration occurs and EDTA undergoes conformational changes.

T-cells (CD4+T-cells, CD8+ T-cells, regulatory CD4+CD25+ T-cells (Tregs), naïve, and memory T-cells), B-cells, plasma cells, dendritic cells, monocytes (macrophages), granulocytes (basophils, eosinophils, neutrophils), NK cells, and platelets, amongst others, are present in blood. In the case of CLL patients, the B-CLL-cell population increases grossly and their isolation is possible by biochemical and mechanical procedures that separates them from all other cells, a technique known as lymphocyte enrichment (Fig. 2.2).

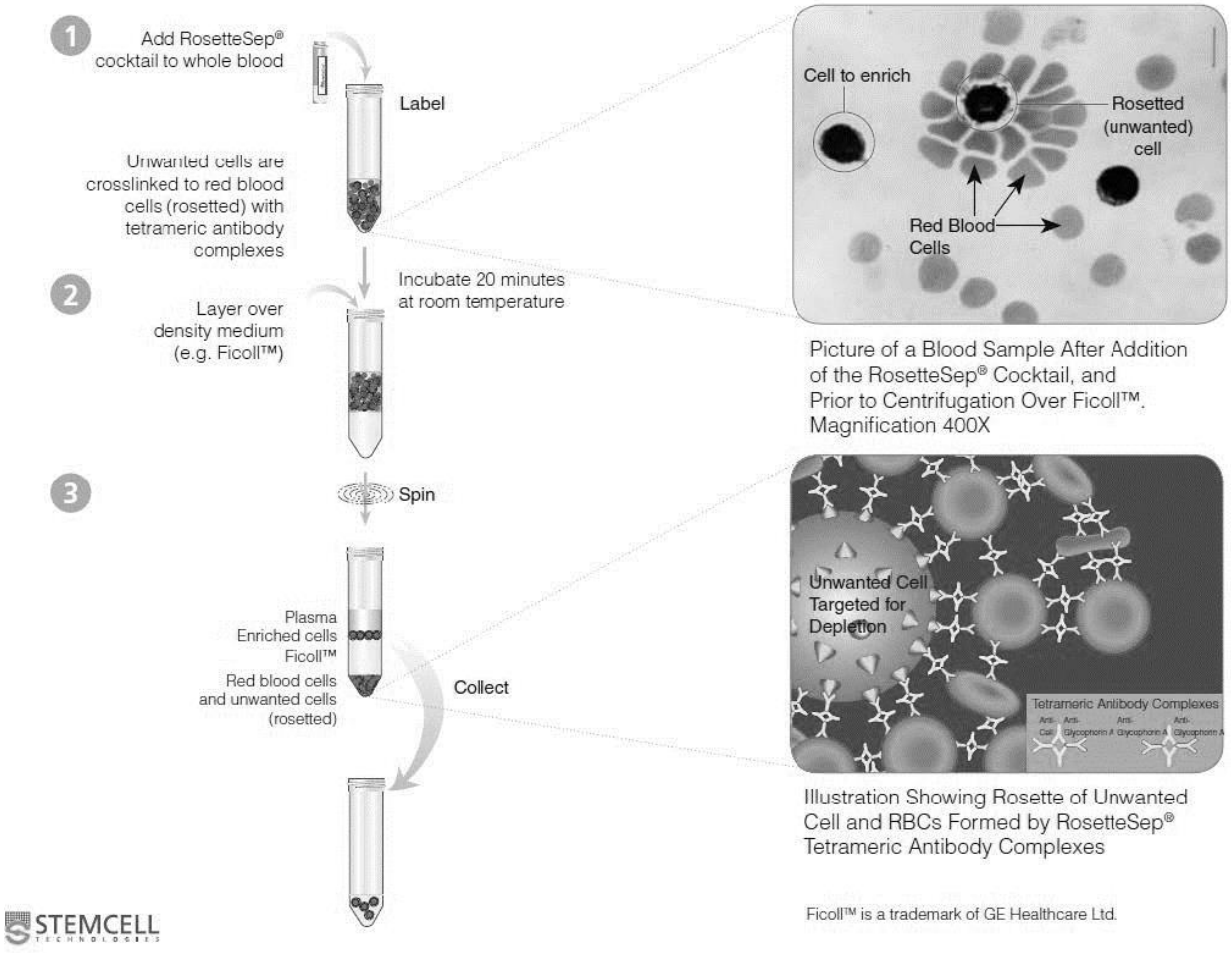


Figure 2.2 Lymphocyte isolation employing enrichment cocktail. The routine of B-CLL cell isolation is achieved by incubation with a cocktail that forms tetrameric antibody complexes between red blood cells to unwanted white blood cells. Light microscopy photograph shows the aggregates to be separated by gradient centrifugation. Because the enriched B-CLL-cell population cannot penetrate the Ficoll gradient, it remains in the interface, giving a richly pure and viable cell population that can processed. Diagrams retrieved from Cell separation products for autoimmunity research brochure from STEMCELL Technologies on July 1st, 2015.

Peripheral blood from CLL patients was incubated with RosetteSep® Human Total Lymphocyte Enrichment Cocktail, which labels every other cell but healthy and malignant lymphocytes to allow their enrichment by negative selection. As illustrated in figure 2.2, the RosetteSep® solution forms a tretrameric antibody complex against transmembrane proteins CD16, CD36, CD66b, and glycophorin a (CD235a). Blood samples were incubated in a rotating platform for 20 min adding 50 µl of RosetteSep ® per millilitre of blood. After incubation, the mixture was poured over a Ficoll bed and

centrifuged for 20 min at 1200 g with the brake off. This centrifugation step resulted in the formation of a gradient that yields a clear separation of the non-B-cell, which form a pellet at the bottom, from lymphocytes. Here the lymphocytes remain enriched over the interface between plasma and the Ficoll gradient. Enriched B-CLL-cells are easily collected from the interface, washed with Dulbecco's PBS, and used in downstream applications. The isolation of peripheral blood mononuclear cells (PBMCs) was similarly performed using a Ficoll gradient, but excluding the incubation step with RosetteSep® in this case we employed peripheral blood from healthy volunteers, which were employed as controls where applicable.

2.4 Assessing purity of isolated CLL cells by flow cytometry

After lymphocyte enrichment procedures, it is necessary to ensure that the CLL cell preparation is pure prior downstream applications and here flow cytometry is instrumental in the assessment of cell purity. Flow cytometry, also known fluorescence-activated cell sorting (FACS), is one of the most revolutionary technical advances in scientific research. Attributed to Wallace Coulter, the Coulter counter, the predecessor to modern flow cytometry, besides counting cells and particles it was the first instrument employed in clinical laboratories that could perform simple cellular discrimination based on cell volume, thus allowing cell counting and differentiation of platelets, erythrocytes, and leukocytes. The first attempt to sort cells was accomplished in Los Alamos National Laboratory, where Mack Fulwyler modified a Coulter counter to sort cells based on their volume. Then Leonard Herzenberg added a mercury arch lamp and light detectors to detect cells stained with fluorochrome-coupled antibodies or ligands, thus allowing identification based on emission besides size; he named the modified Coulter counter as fluorescence-activated cell sorter (FACS) machine (56). Thus, the archaic term FACS refers more precisely to the device commercialized by the company BD Bioscences that was then denominated fluorescence-activated cell sorter or BD FACS in the 1970s (57), but the proper current denomination is flow cytometer.

Besides allowing to identify expression of proteins on the cell membrane (e.g. CDs, VEGFR, Tie1, Tie2), the flow cytometers of today allow to analyse total DNA, specific mRNA expression, calcium concentrations, membrane potential, pH, and

degrees of protein phosphorylation (57). With their sensitive and dynamic range, and speed of information processing, modern flow cytometers detect simultaneously around 10-colours/parameters (e.g. channels/lasers) and process large amount of samples rather rapidly (58)

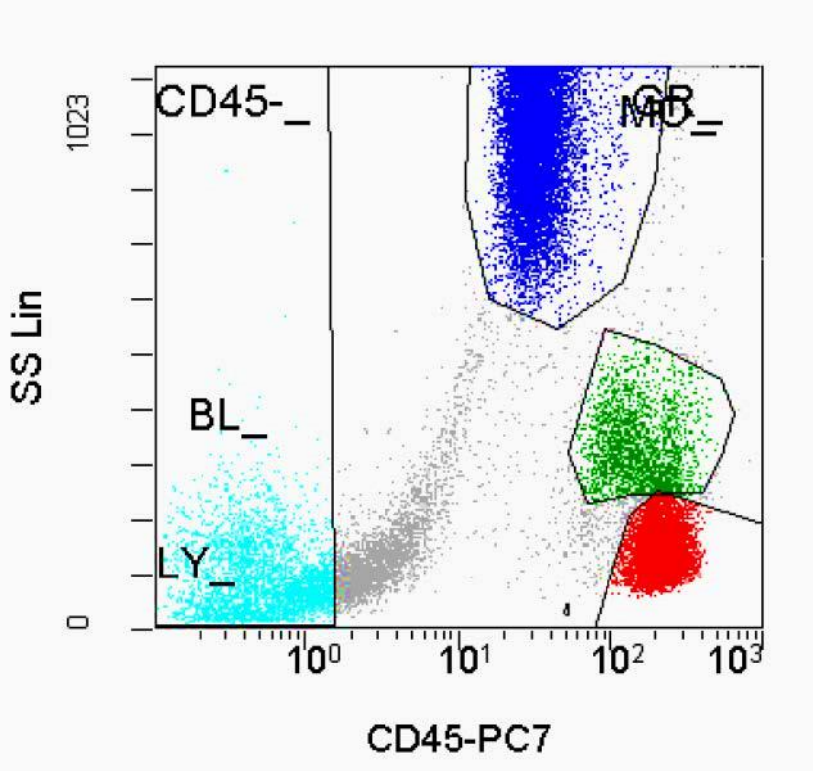


Figure 2.3 Flow cytometry of cell clusters in blood.
The flow cytometer allows us to define the different of cells according to their volume and their granularity. In this panel red blood cells are usually excluded from analysis and left ungated (cyan cluster). The large granulocyte population (blue cluster) stands out at the top of the side scatter, followed by monocytes (green cluster), and the smaller lymphocytes (red cluster).

Flow cytometry measurements reflect the fluorescence intensity emitted (Em-emmision) by cell populations hit by a laser of a given wave length (Ex-excitation). The light sources hit and reflect accordingly to cell area discriminating cells by volume allowing to differentiate between lymphocyte and monocytes; the forward scatter. Also, light can penetrate and be reflected by the internal structure of the cells, indicating the type of granularity, or internal cellular particles (e.g. organelles), allowing discrimination between lymphocytes and granulocytes; the side scatter (57). Figure 2.3 shows how a typical flow cytometry analysis would sort cells found in blood based on granularity and volume. The large population in cyan represents red blood cells, blue population represent the granulocytes, green population monocytes, and red population

represents the lymphocytes. Lymphocytes are the smallest leukocytes and therefore localized at the far bottom in the forward scatter. From this kind of diagram, individual leukocyte population can be gated and individually analysed for their external and internal prognostic markers.

Lymphocytes express specific sets of transmembrane proteins (e.g. surface/receptor), which are coupled to fluorescent antibodies that allow their selection based on per cent of cells expressing the protein of interest. For example, leukocytes have on their cell surface clusters of differentiation (CDs), transmembrane proteins specifically expressed by leukocyte sets. Labelling CDs on B-cells is a standard practice in hematologic diagnosis to identify “molecular markers”, which are taken into consideration in haematological diagnosis.

To analyse purity of the isolated CLL cells, concomitant expression of markers CD5 and CD19 was assessed by flow cytometry. CD5 and CD19 are two characteristic markers employed in molecular diagnostics procedures. Upon isolation of the CLL cells floating over interlayer between Ficoll and plasma CLL cells were washed 3X with sheat fluid (Beckman Coulter) and harvested for 5 min at 400g. Tubes were vortexed to detach cell and incubated for 15 minutes with 10 µl of CD5-FITC (Beckman Coulter) or 5 µl CD19-EDC (Beckman Coulter). After incubation 200 µl of sheat fluid were added to the flow cytometry tubes, vortexed, and analysed using a Gallios flow cytometer (Beckman Coulter).

2.5 Assessing CLL cells viability by flow cytometry

Flow cytometry coupled with two viability selection dyes is the method of choice to discriminate between viable, apoptotic, and necrotic cells in any given cell population. These dyes, namely 7-AAD (7-amino-actinomycin D) and human AnnexinV conjugated (also known as coupling) to fluorescein isothiocyanate (FITC) accurately determine the viability status of cells. 7-AAD is widely utilized in viability assays because it cannot penetrate intact healthy cells, which possess a stable and highly hydrophobilid lipid bilayer, also denominated as the cell membrane. Only apoptotic and necrotic cell have a compromised cell membrane that allows the infiltration and binding of 7-AAD to DNA chains. Phospholipids are the main components of the cell membrane. AnnexinV is a protein with strong and selective affinity against the

phospholipid phosphatidylserine (PS), a phospholipid that remains in inner layer of the cell membrane in healthy cells. When apoptotic signals are activated, PS relocalizes from the inner to the outer cell membrane, making this phospholipid available to AnnexinV. Figure 2.4 shows a test flow cytometric reading to ensure the functionality of viability assay and to discriminate between true viable, apoptotic, and necrotic cells.

To evaluate CLL cell viability by 7-AAD/AnnexinV-FITC double staining $1x10^6$ CLL cells were washed 3x with 2 ml of Dulbecco's PBS, vortexed, harvested for 5 min at 400g. The repeated washes secure the removal of cell media and any attached debris. The pellet was vortexed and washed twice with 100 µl of AnnexinV binding buffer (eBiosciences), cells harvested 5 min at 400 g, detached by vortexing, and resuspended in 20 µl of AnnexinV binding buffer. 1 µl of 7-AAD (Biolengend) and 1 µl AnnexinV-FITC (eBioscience), vortexed, and incubated in the dark for 20 min. After incubation, 200 µl sheat fluid were added, tubes vortexed, and placed in Gallios flow cytometer for analysis. At least 10,000 events were taken into consideration. Flow cytometer analysis processed using Kalusa software. Figure 2.5 show a representative 7-AAD/AnnexinV-FITC double staining of a CLL population exposed to 5% DMSO over night to test the effectivity of assay.

2.6 Automated cell counting and viability assessment by trypan blue

To determine CLL cell concentration we employed a ViCell automated cell counter (Beckman Coulter). This device also allows to determine cell viability by trypan blue staining, thus adding another quality step in assessing CLL cell survival after Ficoll gradient isolation (Fig. 2.5). Trypan blue only penetrates necrotic and apoptotic cells, thus viable cells remain colourless when observed under a light microscope. Please note that trypan blue does not discriminate between apoptotic and necrotic viability status as 7-AAD/AnnexinV-FITC (Fig. 2.4).

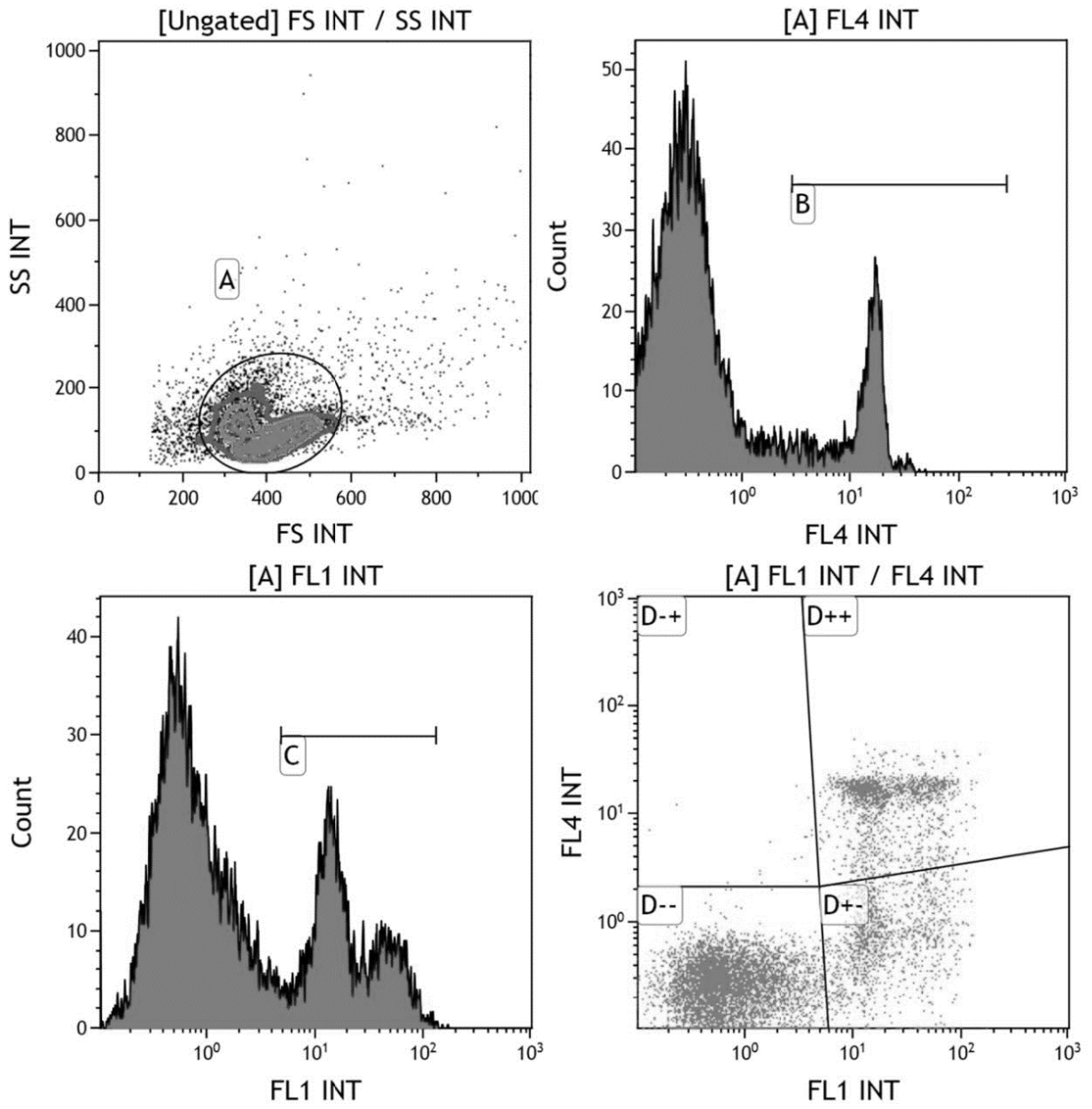


Figure 2.4 CLL cell viability using 7-AAD and AnnexinV-FITC.
The enriched CLL cell population can be easily gated (Gate A) to exclude all the cell debris. There are cells positive for 7-AAD (Gate B) and for AnnexinV-FITC (Gate C) indicating a mixed lymphocytic population of viable and non-viable cells. True viable cells are negative for 7-AAD and for AnnexinV/FITC (Gate D--), since they are not permeable and there is not PS relocalization on their surface. Apoptotic cells are negative for 7-AAD and positive for Annexin V-FITC (Gate D+-), because the cell membrane is not yet permeable but PS has started to relocate to the outer cell membrane indicating the progress of apoptosis. Necrotic cells are positive for both 7-AAD and AnnexinV-FITC (Gate D++), because the permeable cell membrane allows 7-AAD into the cell and PS has relocalized to the outer cell membrane.

2.7 Cell culture of CLL cells and healthy PBMCs

Cell culture is one of the essential assays towards the development of in vivo models, because it allows studying cellular entities in an environment that closely resembles physiological conditions. Conditioned cell culture media contain the most essential supplements to keep cells viable for days. Furthermore, addition of cytokines, chemokines, growth promoters, growth inhibitor, drugs, and many other chemicals, allows studying their specific role over cell systems at the molecular and cellular levels.

During the course of the cell culture procedures, the RPMI medium (Roswell Park Memorial Institute); a media supplemented with vitamins, amino acids and bicarbonate as a buffer system was employed, because this medium supports the culture of normal and neoplastic leukocytes (59). For the culture of human umbilical vascular endothelial cell (HUVEC, batch C-12200, Promocell) a different media was employed, namely Endopan3 (PAN Biotech), a cell media that contains growth factors (e.g. VEGF) required for the expansion and survival of endothelial cells (EC).

All cells, primary cells (PBMCs and CLL cells) and cell lines (HUVEC), were cultured in an incubator under standard conditions at 37 °C with 5% CO_2 supplementation and automatic humidification. For primary CLL cells and PBMCs, RPMI 1640-Glutmax was supplemented with 1%-penicillin/streptomycin (PAA Biotech) and 10% foetal calf serum (PAA Biotech). Antibiotics supplementation prevents bacterial contamination and foetal calf serum provides to the cells in culture essential nutrients for sustained growth and survival, such as growth factors. For HUVEC cells, we employed Endopan3 ready-to-use-media without any type of supplementation. At all times, cell culture procedures and reagents processing were handled in sterile conditions under laminar flow.

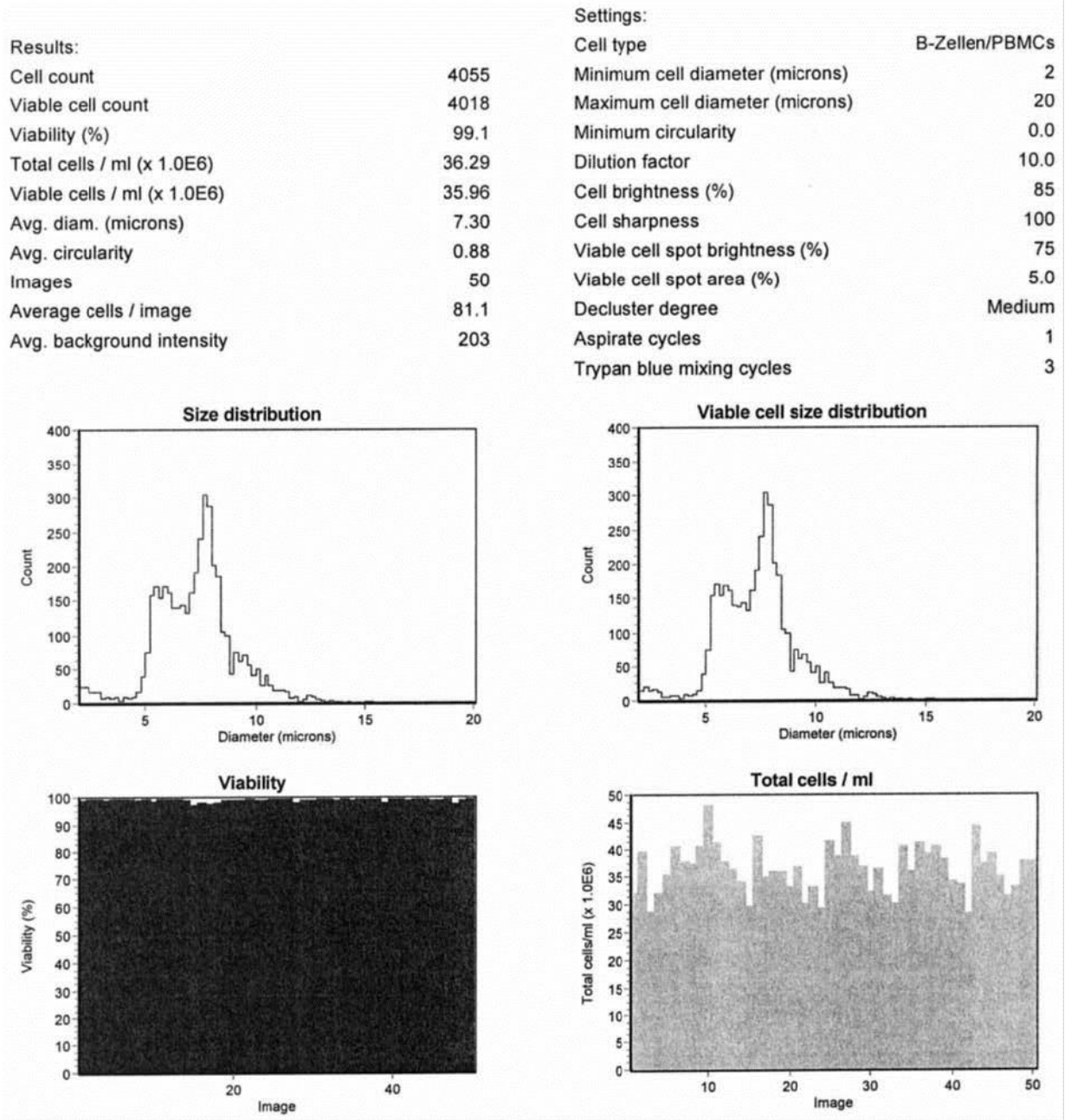


Figure 2.5 CLL cell assessment on an automated cell counter. Counting cells in the automate cell counter to determine cell density also allows to the easy determination of viability by trypan blue. Furthermore, the size distribution of cells is determined by the system. This shows that CLL cells are between 6 and 8 µm in diameter. Increases in size distribution are attributed to cell dimers allowing to further asses the presence of exclusively lymphocytes. Cells around 10 µm are attributed to cell dimers.

2.8 Measuring Ang1 secretion in plasma and cell medium by ELISA

The levels of certain molecules (e.g. VEGF, Ang2, bFGF, disease specific antibodies) in blood, plasma, urine, saliva, breast milk, tears, and supernatant from

cells in culture, give valuable information about the pathophysiological status of a patient. Many available assays measure the concentration of proteins of physiological relevance such as cytokines, chemokines, growth factors, and growth inhibitors. Today enzyme-linked immuno-sorbent assays (ELISA) appear as a qualitative and quantitative technique of choice, since it offers accurate and reliable measurements of protein/antibody concentrations. ELISA assays are carried out using a 96-well plate coated with a monoclonal antibody against the protein of interest to easily perform a sensitive determination of the concentration of a specific protein (e.g. VEGF or Ang1) compared against a control concentration curve (e.g. human recombinant VEGF or Ang1).

To assess the concentration of Ang1 in plasma from healthy and CLL patients, as well as the levels of Ang1 secreted into cell media by healthy PBMCs and CLL cells, we employed a Quantikine ELISA Human Angiopoietin-1 kit (R&D, DANG10). For plasma collection, we depleted cells from peripheral blood of healthy and CLL patients by centrifugation. The plasma was carefully removed from the top of the interface, passed to a new centrifuges tube, centrifuged for 20 min, 800 g and 4°C, and stored at -20°C. At time of processing plasmas was diluted 1:50, using 10 µl plasma and 490 µl of Calibrator Diluent RD5P Concentrate. To measure Ang1 secreted into cell media, healthy PBMCs and CLL cells seeded at a density of $1x10^7$ cells/ml were cultured for 24 h. Cells were depleted from media by centrifuging for 15 min at 1000 g and 4°C, supernatant collected and stored at -20°C. As suggested by the manufacturer, supernatants were not diluted before ELISA-plate processing. The plates were processed together with a Ang1 standard curve. All of the samples and standards were loaded in triplicates. To measure the changes in Ang1 concentration, the ELISA plate was analysed using a µQuant photomether (Bio-TEK instruments) with the suggested OD correction (OD_{real}=OD450–OD565). Raw measurements were processed with Microsoft Excel 2010.

2.9 Assessment of survival after stimulation with Ang1, Ang2, and trebananib

Changes in survival induced upon stimulation with chemokines, cytokines, antibodies, and drugs, give key clues into the implication of these factors in regulating survival, proliferation, apoptosis, and cell homeostasis. We employed recombinant

protein technology to mimic exposure of CLL cells to compartments in the microenvironment where Ang1 and Ang2 concentrations are rather high.

Trebananib, formerly AMG-386 sponsored by AMGEN, is a selective neutralizing peptibody of proven neoplastic activity that selectively and strongly binds ligands Ang1 and Ang2 (60). Since this anti-angiopoietin peptibody is comprised of a peptide that binds Ang1 and Ang2, trebananib prevents interaction of angiopoietins with their receptors (60). Because it is not known whether endogenously secreted Ang1 and Ang2 play any role in autocrine survival advantage in CLL cells, the peptibody was included in stimulation experiments.

Lyophilized recombinant human Ang1 and Ang2 were reconstituted in the same media in which CLL were to be cultured and diluted to a working dilution of 10 µg/ml. Due to the high amounts of proteins present in foetal calf serum and RPMI, recombinant angiopoietins are expected to remain stabilized in these conditions. Trebananib (AMG386), kindly provided by AMGEN, was diluted to a stock solution of 10 µg/ml. CLL cells were seeded to a final concentration of $1x10^7$ and stimulated with either recombinant human Ang1, Ang2 (Prepotech), or trebananib at increasing concentrations of 50, 100, 200, 500 ng/ml. Samples were loaded in triplicates in 96-well cell culture plates and cultured for 1 and 4 days using standard cell culture conditions. Cell viability was assessed as described above by flow cytometry using AnnexinV-FITC/7-AAD viability assay and at least 10,000 events were taken into consideration.

2.10 RNA extraction and purification

The first step to evaluate gene expression is to measure whether genes are being expressed at any given time and if expressed, whether their expression levels are being altered by any given external conditions. Here the extraction and stability of RNA material is the first and most important step in the process of ensuring successful downstream applications. For this purpose, RNA material is extracted from cells and reverse transcribed into cDNA, which allows qualitatively and quantitatively measuring mRNA transcript expression employing PCR and RT-PCR techniques.

Regardless of the downstream application, $3x10^7$ for CLL/B-cells and $1x10^7$ HUVEC were harvested for 10 min at 800 g and 4°C, washed with PBS-3x**,** lysed with 600 µl RLT buffer (Qiagen) supplemented with 0.1% µl β-mercaptoethanol (Sigma), vortexed, snap frozen in liquid nitrogen, and stored at -80°C. Due to their small size, when compared to endothelial cell lines, $3x10^7$ cells instead of $1x10^7$ were employed for RNA extraction. At time of processing, lysates were allowed to thaw on ice and brought to RT with alternate vortexing. RNA material was extracted as suggested by the RNeasy Plus Mini Kit (Qiagen). RNA integrity was verified by electrophoresis on a 1.5 % agarose gel (Fig. 2.6 a) in parallel with a 1000 bp DNA ladder (Thermo Scientific). RNA concentration and purity were assessed photometrically (Fig. 2.6 c) using a NanoPhotometer® P-Class (Implem). Here mRNA samples were considered suitable for further processing if the absorbance ratio of A260nm/A280nm remained between 1.8 and 2.2. More specifically, this is the ratio between the absorbance for the detection of nucleic acids (A260nm) and the absorbance for the detection of aromatic amino acids (proteins/peptides A280nm).

2.11 RNA reverse transcription for cDNA synthesis

RevertAid First Strand cDNA synthesis kit (Thermo Scientific) was employed to synthesise first strand cDNA for PCR and RT-PCR analysis from the extracted RNA. In a DNAase-free PCR tubes 1 µg purified RNA 1 µl prime Oligo$(dT)_{18}$, 1 µl dNTP mix (10 mM each), 4 µl 5X RT buffer, 0.5 µl Thermo Scientific Ribbon RNase inhibitors, 1 µl RevertAid Premium Reverse Transcriptase, and DEPC-treated water were mixed to yield a final reaction volume of 20 µl. The reverse transcription reaction was performed using a Mastercycler apparatus (Eppendorf) for 10 min at 25°C, 50 min at 50°C, 5 min at 80°C, and cooling to 4°C. The product was stored to -20°C until further processing.

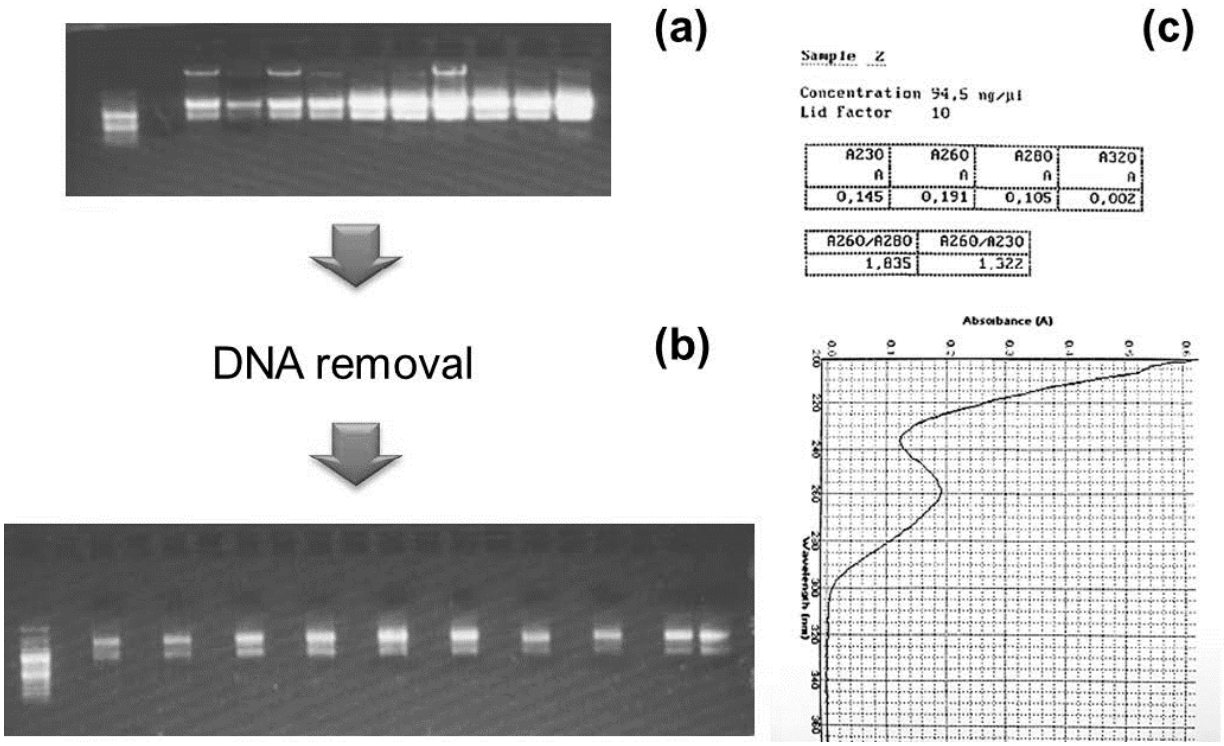


Figure 2.6 Verification of RNA integrity and purity
It is essential to verify the integrity of purified RNA before performing reverse transcription reactions and discard possible genomic DNA contamination. Here, 10 RNA samples extracted from CLL cells were assessed for integrity by running 2 µl from RNA extract on a 1.5% agarose gel supplemented with ethidium-bromide (EtBr) for RNA staining. Integrity was verified under a UV light. Intact RNA shows to bands around 5,000 bp and 2,000 bp). Due to DNA contamination, (a) all samples underwent an additional purification procedure for genomic DNA removal. Samples were again to assess genomic DNA elimination (b). Purify and concentrations were further assessed with a photometer (c). The ratio of A260/A280 remained at all times between 1.800 and 2.200. Once a RNA extract seemed to be integral and possess a proper A260/A280 ratio it was deemed fit for cDNA synthesis.

2.12 Primer design

To test how gene expression is regulated by external factors in the microenvironment, it is essential to test changes in their mRNA expression. The mRNA sequences Ang1, Ang2, Tie1, Tie2, and VE-PTP were obtained from NCBI Reference Sequence and primer pairs picked from the Basic Local Alignment Search Tool (BLAST) query for primer pairs exclusively expanding exon-exon junctions. The primer reference sequences for forward and reverse primer pairs are summarized on table 2.1. A threshold of ≥ 500 bp was set to all possible primer pair so that the same primer pair could be employed for qualitative PCR and semi-quantitative RT-PCR. Identity

and product size were further verified using In-Silico PCR available from the University of California at Santa Cruz (UCSC) Genome Browser. All primers were purchased from TIB MOLBIOL (Berlin, Germany) and solved in DEPC-treated water (Roth, Germany) to a final concentration of 5 µM.

Table 2.1 Primer pairs employed and empirical Tm.

Gene	RefSeq mRNA	Sequence (Forward/Reverse)	Size	Tm
ABL	NM_005157	TGGAGATAACACTCTAAGCATAACTAAAGGT	124	60
		GATGTAGTTGCTTGGGACCCA		
Ang1	NM_001146.3	GAAGGTGTTTTACTAAAGGGAGGA	245	60
		CCAACCTCCCCCATTGACAT		
Ang2	NM_001147.2	CTAAGGACCCCACTGTTGCT	145	62
		TCCATGTCACAGTAGGCCTTG		
Tie1	NM_005424.4	GATGGCCATTGAGTCCCTGA	200	59
		TCAGCTCGTACACTTCATCGT		
Tie2-1	NM_000459.3	GATGGCCATTGAGTCCCTGA	227	61
		TCAGCTCGTACACTTCATCGT		
Tie2-2	NM_000459.3	GGGACAGTGCTCCATCCAAA	444	61
		TGTGAAGCGTCTCACAGGTC		
VE-PTP	NM_001109754.2	AAGCGAAGCATGAACAGTTGC	158	62

Tie2-2 primer pair employed for RT-PCR. The primer pair for Tie2-1 did not perform well under RT-PCR conditions.

Tm refers to the final empirical melting temperature selected from gradient qualitative PCR reactions. Size is in bp and matches the size calculated by BLAST primer search and design, the size of the product predicted by In-Silico PCR and the size seen on agarose gel electrophoresis. RefSeq refers to the number or reference sequence (mRNA) stored in the database of the National Center for Biotechnology Information (NCBI, USA).

Size= The expected size of the amplified PCR product in bp

Tm= The melting temperature used for amplification was calculated empirically

2.13 Qualitative PCR for Tm determination

The theoretical melting temperatures (Tm) provided by In-Silico PCR and TIB MOLBIOL PCR were not taken in consideration to perform PCR reactions. For quality purposes, an empirical Tm was determined to ensure optimal amplification. All primer pairs were subjected to gradient PCR analysis (96 Universal Gradient, peqLAB) using all available cDNAs and non-template controls (NTC) employing at least eight temperatures in the range of 58-64°C. The primer sequence, their expected size in base pairs (bp), and the empirical Tm are summarized on table 2.1.

For the qualitative gradient PCR, reactions of 20 µl were prepared by mixing, 10 µl of 2xFastStart PCR Master Mix (Roche), 1.5 µl forward primer and 1.5 revers primer (5 µM), 6 µl DEPC-treated water, and 1 µl synthesized cDNA.

The standard PCR program was comprised of initial denaturation of 10 min at 95°C, followed by 40 cycles of denaturation of 30 s at 95°C, amplification of 30 s at (Gradient)°C, and elongation of 30 s at 72°C. A final elongation of 2 min at 72°C was added followed by cooling to 4°C. Agarose gel electrophoresis was utilized to verify correct size of all PCR products on 0.8% agarose gel electrophoresis in parallel with a 100bp DNA ladder (ThermoScientific).

2.14 Detecting components of the Ang-Tie2 pathway by PCR

To assess the expression of the main component of the Ang-Tie2 signalling pathways at the mRNA level, we analysed five CLL patients' samples and compared them to healthy PBMCs. Furthermore, we analysed the expression of Ang1, Ang2, Tie1, Tie2, and VE-PTP in parallel, using the same patient's sample for each single gene. The extraction of RNA material and the synthesis of cDNA were performed for these samples as already explained. For qualitative PCR, the same cycling procedures outlined above were employed with the respective Tm°C for each primer pair.

After running PCR reaction, 5 µl of PCR product were loaded in a 0.8% agarose gel together with a DNA 100 bp ladder. The gel was run for 40 min at 100 volts. Additionally, the reference gene ABL was also amplified from samples and observed by gel agarose electrophoresis.

2.15 Semi-quantitative real time-PCR

To determine changes in mRNA expression of Ang1, Ang2, and Tie1 upon exposure of CLL cells to angiopoietins, CLL cells were stimulated with 500 ng/ml of Ang1or Ang2 for 30 min and 6 h.

Prior to analysing changes in gene expression on these samples, the efficiency of primer pairs was validated to ensure compatibility of primer pairs with RT-PCR analysis. All primer pairs were tested at parallel 10-fold serial dilutions over a range of

five magnitudes using all available cDNAs as template and respective non-template controls (Fig. 2.7, a). Efficiency slope was defined at -3.3 (±0.25) (Fig. 2.7, b) as calculated by the LightCycler software (Fig. 2.8 b). Additionally, to discard primer dimers or wrongful product amplification, a melting curve analysis was followed and the melting curve and melting peak verified for single product amplification as show in figure 2.8 (c and d).

CLL cells were lysed and RNA/cDNA processed as described above. We employed 10 µl Maxima SYBR Green qPCR 2xMaster Mix (ThermoScientific), 1 µl cDNA, 1.5 forward primer and 1.5 revers primer (5 µM) to run 20 µl RT-PCR reactions using a Light Cycler System 2.0 (Roche, Mannheim, Germany) and its accompanying software (Fig. 2.7). All RT-PCR reactions were run with a denaturation of 15 min at 95 °C, 40 cycles of denaturation for 30 s at 95°C, amplification for 30 s at Tm°C, and elongation 30 s at 72°C, followed by a final elongation of 1 min at 72°C. Only Tm was adjusted between readings and all other cycling condition remained unchanged during sample measurements.

In order to assess the validity of the RT-PCR reaction and differentiate between a product of primer dimerization and that one of a real RT-PCR product, melting curve analysis was included to confirm identity of RT-PCR products. This is confirmed by the melting curve (Fig. 2.7, c) and the melting peaks, which reveal the variety (if any) of RT-PCR products that have been amplified. Relative changes of gene expressions were calculated by the ΔΔCt method (61) and normalized to the reference gene ABL (Abelson murine leukaemia viral oncogene homolog 1).

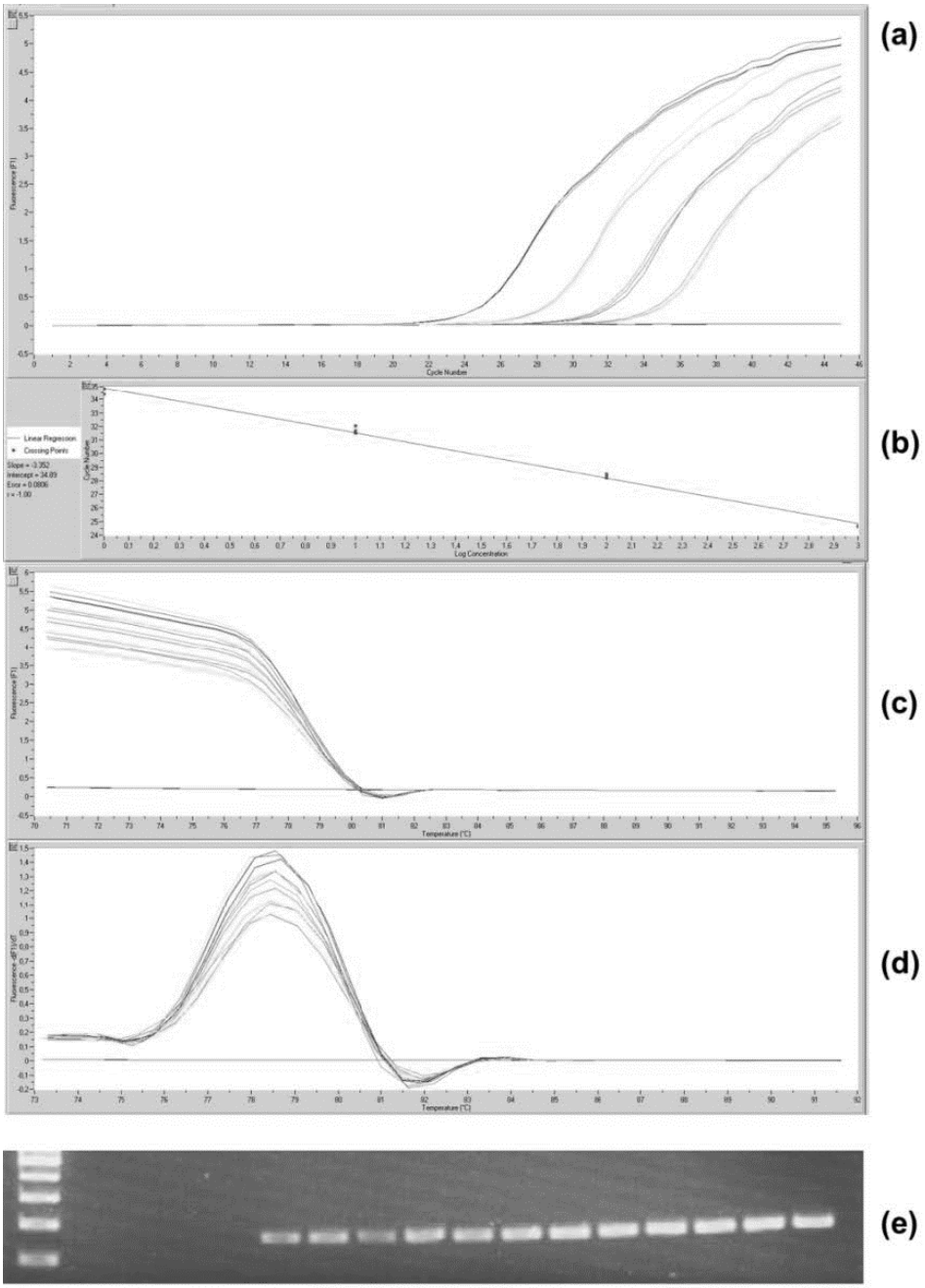


Figure 2.7 Primer pair efficiency and product identity in RT-PCR.
Using 10-fold dilution of five orders of magnitude (a) it is possible to determine the efficiency of a primer to amplify cDNA. If the primer pair is efficient, a slope of efficiency (b) should be close to -3.3. To test the identify DNA product, melting curve (c) and melting peaks (d) analysis are added to each run. Both (c) and (d) show that only a single DNA product has been amplified and that no primer dimers were amplified. Primer pairs have lower melting temperatures and would appear as early signals in both the melting curve and the melting peak. As a further quality check, RT-PCR product is run on an agarose gel (e) to ensure that

the size (bp) matches the size expected by that specific primer (125bp for ABL (e)).

2.16 Detecting transient expression of Tie2 and VE-PTP

CLL cells were seeded to a density of $1x10^7$ cells/ml and stimulated with 500 ng/ml of Ang1 or Ang2 and collected after 30 min and 6 h. Corresponding non-stimulated sample was collected prior to stimulation, as well as at 30 min and 6 h. RNA isolation, cDNA synthesis, and qualitative PCR reactions were processed as described above. As a control of Tie2 and VE-PTP expression, we utilized RNA/cDNA extracted from HUVEC cells. HUVEC is an endothelial cell line of standard use as control in the study of the Ang-Tie2 signalling pathway, because these cells express Tie1, Tie2, VE-PTP, Ang1, and Ang2. Due to suspected low expression of Tie2 and VE-PTP, final PCR products were analysed by capillary gel electrophoresis to confirm expression that could be easily missed by agarose gel electrophoresis. ABL was used as a reference gene and visualized by classical agarose gel electrophoresis.

2.17 Assessing Tie1 and Tie2 expression on Ang1/Ang2 stimulated CLL cells

CLL cells were seeded to a density of $1x10^7$ cells/ml and stimulated with 500 ng/ml of Ang1 or Ang2 for 24 h under standard cell culture conditions. CLL cells were aspirated and transferred into flow cytometry tubes and washed 2X with Isoflow® sheat fluid supplemented with 0.5% BSA. Then $2x10^5$ CLL cells were diluted in 100 µl sheat fluid plus 0.5% BSA and transferred to a new flow cytometry tube. To block the human Fc receptors (FcRs) expressed on the surface of B-cells, we employed Human TruStain FcX (Biolegend Inc.), which blocks FcR-derived signalling that may interfere with antibody specific staining. To the CLL cell suspension, 5 µl of TruStain were added per tube, tubes incubated at RT with mild shaking for 15 min, without washing the FcR blocking after this incubation time.

To determine the qualitative expression density of Tie1 and Tie2 receptors on the surface of CLL cells, cells were stained with either 10 µl of PE-conjugated goat IgG isotype, polyclonal anti-human antibodyTie1-PE-conjugated, or polyclonal anti-human Tie2-PE-conjugated, which were added to the FcR-blocked CLL cell suspension and incubated with mild shaking at 4°C during 1 h. To eliminate any unbound antibody, CLL

cells washed 3X with 3 ml sheat fluid and harvested 5 min at 400 g. After the last wash cycle, cells were resuspended in 100 µl of sheat fluid and analysed with a Gallios flow cytometry (Beckman Coulter) using a 488 nm wavelength

2.18 Assessing chemotaxis and transendothelial-like migration

As shown in figure 2.5, CLL cells have a diameter of approximately 6-8 µm. To test the chemotaxis effect upon stimulation with Ang1 and Ang2 over CLL cells, we employed Transwell Permeable Support (Corning Inc) inserts with a pore size of 5 µm adapted for 24-well plates. To test the transendothelial-like migration effect, we employed ThinCert (Greiner Bio-One) inserts with a pore size of 3 µm adapted for 24-well plates. Wells and inserts were preconditioned with cell culture media for 12 h at standard cell culture conditions. CLL cells were seeded at $1x10^6$ cells/ml and volumes processed as suggested by the manufacturers; 600 µl of cell solution inside the insert (Both manufacturers agreed on this measurement). Media in the bottom chamber was supplemented with either 500 ng/ml of Ang1, Ang2, or Ang1+trebananib or Ang2+trebananib (500 ng/ml of each) to analyse the angiopoietin blockage. Cell culture plates were incubated for 24 h in standard cell culture conditions. After incubation, CLL cells were retrieved from the bottom chamber and counted using a Neunbauer haemocytometer.

2.19 Assessing caspase and ATP activity after angiopoietin stimulation

Caspase activity is a great marker of cellular metabolic fitness since activation of caspases enzymes, such a caspase 3/7, reliably indicates whether external factor activate or inhibit caspase activity. This is relevant to test compounds and protein entities that are believed to regulate cell survival. Caspase activity in CLL samples was assessed using Cell Meter ™ Caspase 3/7 activity (AAT Bioquest, Inc.). The kit quantifies the relative activated caspase 3/7 activity upon exposure to apoptosis inducer or caspase activity inhibitor (62)

Black-wall 96-well plates (Greiner Bio) with transparent bottom were coated with poly-d-lysine (Sigma-Aldrich) at a concentration of 50 µg/ml dissolved in Dulbecco's PBS for 24 h at 4°C with constant shaking. After coating washed with PBS-3x, and allowed to air-dry under aseptic environment with complementary UV-treatment. CLL

cells were seeded at the concentration suggested by the manufacturer with a corresponding caspase content dilution curve of untreated cells (PBMCs and CLL cells), where the undiluted/non-treated cell represent the 100% of caspase activity and is utilized to determine increases or decreases of caspase activity. PBMCs and CLL cells were stimulated with Ang1, Ang2, or trebananib at concentrations ranging from 5 fg/ml to 500 ng/ml for 24 h. After incubation, the cells were processed as suggested by the manufacturer and changes in fluorescence were monitored with a Paradigm Detection Platform connected to Multimode Analysis Software (Beckman Coulter).

Assessing ATP presence in a cellular population is another method to assess cellular metabolic fitness. ATP relative content was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). CLL cells were seeded as suggested with a corresponding ATP content dilution curve of untreated cells, where the undiluted/non-treated control was considered as 100%. Both PBMCs and CLL cells were stimulated with Ang1, Ang2, or trebananib at concentrations ranging from 5 fg/ml to 500 ng/ml in white-wall 96-well plates (Sarsted, Germany) and incubated for 24 h. Plates were analysed with a microplate luminometer (EG&G Berthold).

2.20 Determinating changes in prognostic markers expression

Another way to explore the impact of angiopoietin present in the microenvironment over CLL pathogenesis is measuring changes in the expression of the prognostic (diagnostic) markers. Target prognostic marker and their conjugated fluorophores for flow cytometry detection are summarized on table 2.2. We stimulated cells with 500 µg/ml of Ang1 or Ang2 for 12 h followed by flow cytometry measurements to analyse changes of relevant molecular marker on CLL cells. Samples were analysed and ran in a Gallios flow cytometer (Beckman Coulter) and 10,000 events were taken into consideration. CLL cells from six patients were used for this analysis.

The antibodies used were separated into groups and master mixes prepared for flow cytometry as described on Table 2.2. Creating a master mix for all detection of target CDs would not be feasible, because some antibodies share fluorescent conjugates, which would overlap in flow cytometry analysis. The multicolour flow cytometry employing several conjugates allows for the precise identification of specific cell subtypes and in this context, subtype of different prognostic marker expression in

six single readings per sample (non-stimulated, Ang1-stimulated, and Ang2 stimulated).

Table 2.2 Antibodies mixtures, targets, and their conjugates.

Tube/Conjugate	FITC	PE	ECD	PC 5	PC 7
1	CD8	CD4	CD3	CD56	CD45
2	CD5	CD10	CD19	CD20	CD45
3	FMC7	CD79b	CD19	CD38	CD45
5	Kappa	Lambda	CD19	CD5	CD45
5	CD23	CD43	CD19	CD25	CD45
6	IgM	ROR1	CD19	CD25	CD45

FITC, fluorescein isothiocyanate; PE, phycoerithrine; ECD, R-phycoerythrin-Texas Red® -X; PC 5, R-hycoerythrin-cyanine 5.1; PC 7, phycoerythrin cyanin 7; ROR1, receptor tyrosine kinase-like orphan receptor 1; IgM Immunoglobulin M. Antibody mixes and table adapted from the LMHO protocols.

2.21 Protein isolation: cell lysis and preservation of protein integrity

In order to examine proteins, it is essential to extract them cytoplasm and cell membrane. Lysis procedures allow obtaining protein in solution and eliminating cell membranes and all other non-proteic material. To secure the integrity of proteins, lysis buffer was supplemented with two important reagents; cOmplete, an inhibitory cocktail that prevents protein degradation and PhosSTOP, a phosphatase inhibitory cocktail that preserves the phosphorylated state of proteins.

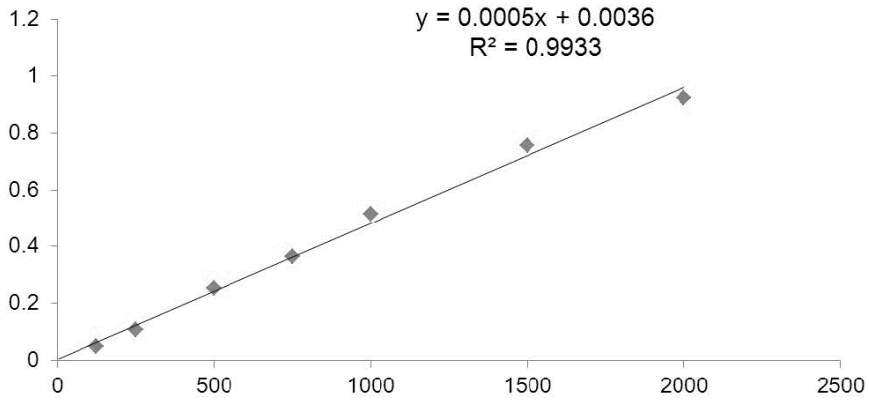
For protein isolation, a total of $1x10^7$ cell were harvested (regardless of the experiment), supernatant discarded, pellet resuspended with 100 µl of ice chilled lysis buffer (0.5% Triton X100, 4 mM EDTA, 20mM Tris, 10 mM NaCl), vortexed, and incubated on ice for 1 h with alternative vortexing every 5 min to ensure maximal protein extraction. The lysis buffer was supplemented with cOmplete ULTRA and PhosSTOP tablets to preserve protein integrity and phosphorylation patterns. After incubation, samples were centrifuges at 4°C for 30 min at 1000 g. Supernatant were transferred to a clean centrifuge tube and centrifuged 20 min at 1000 g at 4°C. Supernatants were then transferred to a new centrifuge tube and stored at -20°C before proceeding to quantify protein concentration.

2.22 Protein concentration: colorimetric assay and photometry

In order to accurately measured protein concentration in CLL cell lysates, it is essential to create a protein dilution curve of known concentrations. To create the dilution curve, a Pre-Diluted Protein Assay Standards Set, which contains seven dilutions of BSA reflecting final concentrations of 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL, was employed (Fig. 2.8). To measure protein, a colorimetric method using Pierce 660 Protein Assay (Thermo Scientific) was utilized. Pierce 660 offers higher sensitivity and requires fewer samples for measurement when compared to Bradford assay. For measurements, 5 µl of each lysate were diluted 1:10 in 0.9% NaCl saline supplemented with 0.05% NaN_3 for protein preservation. Into 96 well-plates 150 µl of Pierce 660 were pipetted per well and 10 µl of standards and samples were pipetted into the reagent. Samples were pipetted in triplicate and corresponding blank samples were simultaneously loaded. Absorbance at 650 nm was measured using a photometer (nanoQuant). A new standard curve was included in each measurement. Lysates were further processed into protein separation by SDS-PAGE gel electrophoresis or phosphorylation patterns recognition by antibody arras assay.

2.23 Protein separation: SDS-PAGE gel electrophoresis

To assess changes in NF-kB phosphorylation upon angiopoietin exposure, $1x10^7$ CLL cells were stimulated with either 500 ng/ml Ang1 or Ang2 for 4 and 12 h. CLL cells were processed as explained above to extract protein. All reagents for SDS-PAGE gel electrophoresis were purchased from Life Technologies. Samples handling, buffers preparation, gel electrophoresis, transfers/blotting, and stains were performed as suggested by manufacture without alterations. We separated proteins used NuPAGE Tris-Acetate precast gels. For loading, each sample was prepared by mixing 10 µg/protein from lysate, 7 µl NuPAGE LD buffer, 3 µl DTT, and adjusted the volume to 20 µl with H_20. Samples were heated to 70°C for 5 min in an Eppendorf thermomixer at 1000 rpm and placed immediately on ice, incubated for 10 min, centrifuged for 5 min at 800 g and supernatants transferred to clean centrifuge tube.



Str µg/ml	A OD 660	B OD 660	C OD 660	OD Average	OD 660 corr	Stdrdev
0	0,142	0,135	0,143	0,140	0,000	0,4%
125	0,181	0,187	0,194	0,187	0,047	0,7%
250	0,228	0,256	0,252	0,245	0,105	1,5%
500	0,381	0,414	0,380	0,392	0,252	1,9%
750	0,510	0,483	0,509	0,501	0,361	1,5%
1000	0,659	0,680	0,622	0,654	0,514	2,9%
1500	0,889	0,892	0,896	0,892	0,752	0,4%
2000	1,033	1,065	1,081	1,060	0,920	2,4%

Figure 2.8 Standard curve to determine protein concentration. The maximum tolerance for the standard deviation was set to 5% in all measurements. Equation was solved for x and employed to determine protein concentration in each given lysate. A new curve was designed each time protein concentration was assessed in new sets of lysates.

We prepared the SDS-PAGE separation chamber and used acetate transfer buffer. The inner reservoir was supplemented with 500 ul NuPAGE Antioxidant mixture. Running buffer was pre-cooled before loading procedure and the assembled loading chamber was placed inside an ice chamber to prevent overheating and ensure smooth lane running across the gel. A coloured protein ladder was loaded together with protein samples. All samples were loaded with a Hamilton pipette. Gels were run for 1 h at 200 volts. After protein separation, gels were processed for transferring on PFVD membrane for western blot analysis or for staining with silver staining.

2.24 Protein detection: Western blot

The PDVF transfer membrane was activated for 10 min in methanol and washed 3X in the respective freshly prepared NuPAGE transfer buffer. The transferring sandwich was prepared as suggested by the manufacturer, covered with ice, and ran for 60 min and 30 volts. The membrane was washed 3X with water and protein transfer

verified with Ponceu dye. After protein transfer, the Ponceu-stained membrane was washed in a solution of PBS-5% milk w/v solution several times until die was detected was no longer observed in the milky washing solution or for at least 2 h.

Once distained, the membrane was washed 3X with PBS. The first antibody was diluted 1 to 2000 in PBS-5% milk w/v solution, added to the membrane and incubated ON for at least 14 h with mild constant shaking at 4°C. The antibody concentration of 1:2000 worked well for all the antibodies. Membrane was washed 3x with PBS-Tween for 15 min. The target antibodies employed were NFkB, phospho-NFkB (Cell Signalling), and β-actin (Sigma, diluted to 1:10,000). After washing steps, second antibody (e.g. horseradish peroxidase coupled anti-rabbit or anti-mouse) was similarly diluted 1:2000 in blocking solution, added to the membrane, incubated for 1 h at RT and washed 3x with PBS-Tween. To detect protein, the PDVF membrane was soaked with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific), excess solution discarded and immediately placed inside an X-ray cassette (Siemens). Inside a photographs dark room, the PDVF membrane was exposed to Lumi-Film Chemiluminescent Detection Film (Roche) and developed using a Curiox60 AFGA developing system.

2.25 Protein identification: Silver staining and Mass Spectrometry

To detect subtle changes of protein expression pattern resulting from separation of protein on SDS-PAGE gel electrophoresis, we employed a silver staining. Silver staining offers a rapid and sensitive method, 50x more sensitive than Coomassie blue staining, being an excellent tool for the detection of low amounts of protein. For this purpose, CLL cells ($1x10^7$ cells/ml) were stimulated with 500 ng/ml of Ang1 or Ang2 for 1 for 24 h, washed with PBS-3x. Cells were lysed and protein concentration measure as explained above. 2 ng of protein/well were loaded using a Hamilton pipette into NuPage 3-8% tris-acetate gel (LifeTechnologies) using accompanying buffers and protein separated for 35 min at 15 volts.

To visualize protein band, the gel was processed for silver staining using SilverQuest Silver Staining Kit (Invitrogen) following the protocol suggested by the manufacturer. By cutting and destining specific band from the gels, it is possible to determine the identity of proteins of by mass spectrometry (MS). Because silver ions

interfere with MS analysis, it is essential to distain silver gels to ensure compatibility with mass spectrometry techniques. From the gel, bands of interest were extracted, distained, and sent to the Proteomics Facilities at CECAD Cologne. Protein identification was carried using Matrix Assisted Laser Desorption/Ionization/Mass Spectrometry (MALDI/MS) which identifies proteins by determining their exact mass, which are compared with the internal database.

2.26 Assay to evaluate changes in phosphorylation of 71 RTKs

Phosphorylation is probably the most important molecular events, due to the fact that phosphorylation and dephosphorylating regulate transduction-signalling pathways that dictate proliferation and activation of survival and apoptosis. For the simultaneous detection of changes in the relative phosphorylation, a RayBio® Human RTK Phosphorylation Antibody Array that evaluates 71 relevant human receptor tyrosine kinases (RTKs) was employed. The relevance of RTKs resides in their role in transmitting signal from the microenvironment into the cell by signal transduction events. Thus, by evaluating 71 RTKs simultaneously, it is possible to understand which signalling pathways are activated or deactivated by angiopoietin present in the microenvironment directly from CLL cell lysate without the need of running individual western blots and fishing for putative targets of phosphorylation.

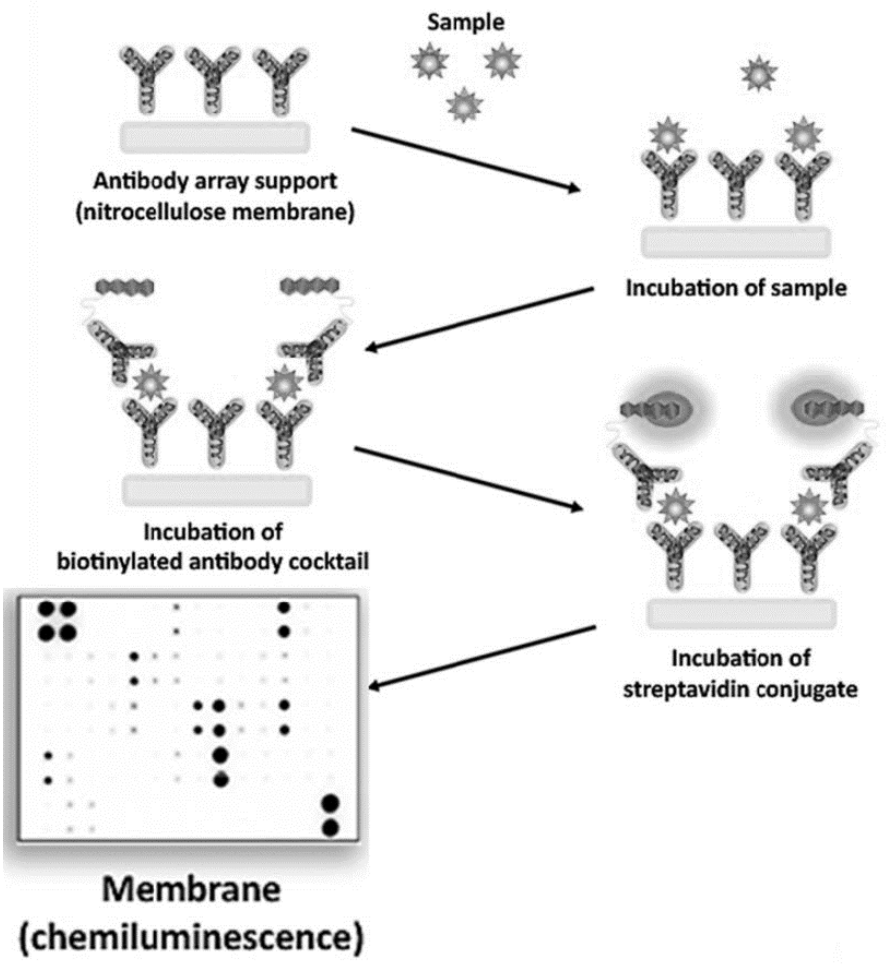


Figure 2.9 The RTK phosphorylation assay.
The 71 relevant RTks are printed as a set of antibodies that will recognize specific phosphorylated protein and changes in phosphorylation intensity in a reverse-western blot fashion. Negative and positive controls are included in the membrane no so additional blank, positive, or negative controls samples are required. Illustration retrieved from the user manual of the Human RTK Phosphorylation Antibody Array 1 (RayBio).

CLL cells were incubated at a density of $1x10^7$ cells/ml in RPMI 1840 media as untreated control or as stimulated sample (500 mg/ml Ang1). We incubated in a cell culture plate in standard conditions for 30 min, 1 h, and 12 h, removed from incubator, and placed immediately in ice. Ice chilled Dulbecco´s PBS was added in excess to the wells and harvested in a pre-cooled centrifuge to 4 °C for 10 min at 800 g. Pellet was immediately resuspended in cell ice-chilled lysis buffer supplemented with protein and protein concentration measured as already described. For the assay, the lysate was processed as suggested by the manufacturer. This assay work as a reverse western blot with antibodies pre-printed on the membranes (Fig. 2.9). The assay includes negative and positive controls thus ensuring a high quality and reliable reading. To

visualize the signal, we exposed the membrane to Lumi-Film Chemiluminescent Detection Film (Roche). Films were developed with AFGA Curix60 developing system.

The name and functional information associated with each single phosphorylated peptide were retrieved from the Universal Protein Resource knowledge base (UniProtKB).

2.27 Statistics and graph design

Results are shown as mean ± bars of standard error of the mean (SEM). Significance was calculated using Windows Microsoft Excel 2010 with a two-tailed student t-test and minimal statistical significance was set at $p \leq 0.05$. Graphs were processed using GraphPad Prism Version 5. For flow cytometry, Kalusa 2.0 software was utilized to interpret data acquisition. For RT-PCR, the LightCycler3 Front Screen software was utilized to visualize data and efficiently calculate slope of amplification.

2.28 Materials

2.28.1 Devices

Autoclave	Variokav Type 400, H+P Labortechnick GmBH
Capillary cooling block	Roche Molecular Diagnostics, Pleasanton, USA
Cell incubator	Heraeus Holding GmbH, Hanau, Germany
Cell separator	MACS, Miltenyi Biotec
Centrifuges Biofuge pico	Heraeus Holding GmbH, Hanau, Deutschland
Centrifuges Labofuge 400R pico	Heraeus Holding GmbH, Hanau, Deutschland
Electrophoresis capillary	Qiaxel, Qiagen, Hilden, Germany
Electrophoresis chamber DNA Sub Cell,	Bio-Rad Laboratories GmbH, Munich, Germany
Flow cytometer Gallos	Beckman Coulter, Inc., Brea, CA, USA
Hemaecytometer Neubauer	Neubauer, Labortechnik
Laminar flow bench-cell lines	HLB 2448, Heraeus Holding GmbH, Hanau, Germany
Luminometer MicroLumatPlus LB 96V	Berthold Tech GmbH & Co., Bad Wildbad, Germany
pH-meter, pH Level1	inoLab, Weiheim, Germany
Photometer µQuant	BioTeck Instruments
Photometer 2	Beckman Coulter, Inc., Brea, CA, USA
Photometer´s software	Beckman Coulter, Inc., Brea, CA, USA
Pipettes, Research Series	Eppendorf, Hamburg, Germany
RT-PCR Light Cycler 2.0	Roche Molecular Diagnostics, Pleasanton, USA
SDS-PAGE system, X-Cell SureLock	Invitrogen, Carlsbad, USA
Shaker MTS4,	Ika, Staufen, Deutschland
Thermocycler-PCR peqSTAR	PeqLab, Erlangen, Germany
Vortex K-550-GE	Bender & Holbein, Bruchsal, Germany
Western bloting Xcell II blot module	Invitrogen, Carlsbad, USA
X-film developer, Curix 60	Agfa-Gevaert N.V, Mortsel, Belgium

2.28.2 Consumables

B-cell isolation columns, MACS	Miltenyi Biotec, Bergisch Gladbach, Germany
Cell culture flasks	Sarstedt, Nümbrech, Germany
Cell cultures multi well plates	Greiner-Bio-One, Frickenhausen, Germany
Centrifuge tubes15, 50 ml	Sarstedt, Nümbrech, Germany
Flow cytometry tubes	Sarstedt, Nümbrech, Germany
Microscope slides	Engelbrecht GmbH, Edermünde, Germany
PCR-strips	Sarstedt, Nümbrech, Germany

Pipettes for cell culture	Sarstedt, Nümbrech, Germany
Reagent tubes	Sarstedt, Nümbrech, Germany
Reagent tubes-PCR grade	Sarstedt, Nümbrech, Germany
RT-PCR capillaries	Roche Molecular Diagnostics, Pleasanton, USA
Serum tubes	Sarsted, Nümbrech, Germany
Filters Sterifix 0.2 µm pore	B. Braun AG, Melsungen, Germany
Syringes	B. Braun AG, Melsungen, Germany
Tips with and w/o filter	Sarstedt, Nümbrech, Germany
Transfer membrane- Filter paper	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Transfer membrane- PVDF	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Transwells inserts- 5 µm	Greiner-Bio-One, Frickenhausen, Germany
Transwells inserts- 3 µm	McCornick, USA

2.28.3 Reagents

Acetic acid	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Agarose	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
BSA- bovine serum albumin	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
DMSO-dimethylsulfoxid	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
β-mercaptoethanol 98%	Sigma-Aldrich GmbH, Seelze, Germany
DTT-dithioteritol	Sigma-Aldrich GmbH, Seelze, Germany
ECL Wester blotting detection	Amersham Bioscience, New York, USA
EDTA-ethilenediaminotetraacetic acid	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Ethidium bromide	Serva GmbH, Heidelberg, Germany
Ethanol	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Formaldehyde	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Hydrochloric acid	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Isopropanol	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Methanol	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Milk powder	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
NaCl-sodium chloride	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
PBS-10x tablets	Life Technologies GmbH, Darmstadt, Germany
Phosphatase inhibitor tablets PhoStop®	Roche, Manheim, Germany
Ponceau S	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Protease inhibitor tablets cOmplete®	Roche, Manheim, Germany
TRIS-tris(hydroxymethyl)aminomethane	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Triton X-100	Carl Roth GmBH + CO. KG, Karlsruhe, Germany

Trypsin-EDTA solution (10x)	PAA, Pasching, Austria
Tween20	Carl Roth GmBH + CO. KG, Karlsruhe, Germany

2.28.4 Cell culture reagents

Lymphocyte Separation Medium 1077	PAA, Pasching, Austria
Penicillin - streptomycin 100x	PAA, Pasching, Austria
RPMI 1640 Medium, GlutaMAX™	Life Technologies GmbH, Darmstadt, Germany
ENDOPAN3 ready-to-use	PAN-Biotech GmbH, Germany
DMSO-Dimethlyssulfoxid	Carl Roth GmBH + CO. KG, Karlsruhe, Germany
Dulbecco´s PBS 1x without Ca & Mg	PAA, Pasching, Austria
Foetal calf serum Gibco®	Life Technologies GmbH, Darmstadt, Germany
EDTA-0.1 M for cell culture	PAA, Pasching, Austria

2.28.5 Special reagents

Quantikine Ang1 ELISA kit	R&D Systems, Minneapolis, USA
Rossette Sep® B-cell enrichment	Stem Cell Technologies, Grenoble, France
RNAeasy RNA isolation kit	Qiagen, Hilden, Germany
Cell titer Glow® Luminiscent	Promega, Madison, USA
Human phospho-RTK array kit	R&D Systems, Minneapolis, USA
cOmplete ULTRA Tablets, mini, EDTA	Roche Molecular Diagnostics, Manheim, Germany
PhosSTOP Phosphatase Inhibitor	Roche Molecular Diagnostics, Manheim, Germany
Protein Assay Standars: BSA Set	Thermo Scientific, Rockford, IL, USA
SuperSignal™ West Femto Maximum	Thermo Scientific, Rockford, IL, USA
Caspase 3/7 Activity Apoptosis Assay	ATT Bioquest ®, Inc. Sunnyvale, CA, USA
IsoFlow™ Sheath Fluid	Beckman Coulter, Inc., Brea, CA, USA
Recombinant Human ANG-1	PrepoTech, Rocky Hill, NJ, USA
Recombinant Human ANG-2	PrepoTech, Rocky Hill, NJ, USA
Trebananib	AMGEN, Thousand Oaks, CA, USA

2.28.6 Ready-to-use solutions

Annexin-V binding buffer	BD, New Jersey, USA
Erytrocyte lysis buffer	Qiagen, Hilden, Germany
NuPage® 10-well 8-12% tris-acetate gel	Life Technologies GmbH, Darmstadt, Germany
NuPage® 15-well acetate gel	Life Technologies GmbH, Darmstadt, Germany
NuPage® acetate running buffer (10x)	Life Technologies GmbH, Darmstadt, Germany
NuPage® Antioxidant	Life Technologies GmbH, Darmstadt, Germany

NuPage® LDS sample buffer (4x)	Life Technologies GmbH, Darmstadt, Germany
NuPage® MES running buffer (10x)	Life Technologies GmbH, Darmstadt, Germany
NuPage® MOPS running buffer (10x)	Life Technologies GmbH, Darmstadt, Germany
NuPage® sample reducing agent (10x)	Life Technologies GmbH, Darmstadt, Germany
NuPage® transfer buffer (20x)	Life Technologies GmbH, Darmstadt, Germany
RNAlater® lysis buffer	Qiagen, Hilden, Germany
NaCl 0.9% solution	B. Braun AG, Melsungen, Germany
Tryptanblau 0.5% solution	Biochrom AG, Berlin, Germany
NuPAGE® Tris-Acetate SDS Buffer	Life Technologies GmbH, Darmstadt, Germany
Calibrator Diluent RD5P (5X)	R&D System, Inc. Minneapolis, MN, USA
SuperSignal™ West Pico Chemiluminescent	Life Technologies GmbH, Darmstadt, Germany
IsoFlow™ Sheath Fluid	Beckman Coulter, Inc., Brea, CA, USA

2.28.7 PCR and RT-PCR reagents

0.1 M DTT	Life Technologies GmbH, Darmstadt, Germany
10 mM dNTP mix	Life Technologies GmbH, Darmstadt, Germany
100 bp DNA ladder	Life Technologies GmbH, Darmstadt, Germany
1000 bp DNA ladder	Life Technologies GmbH, Darmstadt, Germany
5X RT buffer	Life Technologies GmbH, Darmstadt, Germany
6x DNA loading dye	Life Technologies GmbH, Darmstadt, Germany
FastSTART PCR-master mix 2x	Roche Molecular Diagnostics, Manheim, Germany
Oligo(dT)20 Primer	Life Technologies GmbH, Darmstadt, Germany
RNAse H	Invitrogen, Carlsbad, USA
SuperScript® IV Reverse Transcriptase	Life Technologies GmbH, Darmstadt, Germany
SYBR Green RT-PCR master mix2x	Life Technologies GmbH, Darmstadt, Germany
Water, nuclease-free, steam sterilised	Carl Roth GmBH + CO. KG, Karlsruhe, Germany

2.28.8 Flow cytometry antibodies and reagents

Human TrusStain FcX	BioLegend Inc., San Diego, CA, USA
7-AAD	BioLegend Inc., San Diego, CA, USA
AnnexinV-FITC	eBioscience, Viena, Austria
Anti-human Tie1-PE	R&D Systems, Inc., Minneapolis, MN,USA
Anti-human Tie2-PE	R&D Systems, Inc., Minneapolis, MN,USA
PE-conjugated goat IgG isotype	R&D Systems, Inc., Minneapolis, MN,USA
Actin	Sigma Aldrich, Germany
NFkB	Cell signalling Technologies Inc.

phospho-NFkB	Cell signalling Technologies Inc.
CD10-PE	Beckman Coulter, Inc., Brea, CA, USA
CD19-ECD	Beckman Coulter, Inc., Brea, CA, USA
CD20-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD22-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD23-FITC	Beckman Coulter, Inc., Brea, CA, USA
CD25-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD38-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD3-ECD	Beckman Coulter, Inc., Brea, CA, USA
CD43-PE	Beckman Coulter, Inc., Brea, CA, USA
CD45-PC 7	Beckman Coulter, Inc., Brea, CA, USA
CD4-PE	Beckman Coulter, Inc., Brea, CA, USA
CD56-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD5-FITC	Beckman Coulter, Inc., Brea, CA, USA
CD5-PC 5	Beckman Coulter, Inc., Brea, CA, USA
CD79b-PE	Beckman Coulter, Inc., Brea, CA, USA
CD8-FITC	Beckman Coulter, Inc., Brea, CA, USA
IgM	Dako Denmark, Glostrup, Denmark
Kappa-FITC	Dako Denmark, Glostrup, Denmark
Lamda-PE	Dako Denmark, Glostrup, Denmark
ROR1	Miltenyi Biotech, Bergisch Gladbach, Germany

2.28.9 Software

BLAST- Basic Local Alignment Search Tool	National Center for Biotechnology Information, USA
GraphPad Prism 5.0	GrapPad Software, San Diego, USA
Kaluza® Analysis Software	Beckman Coulter GmbH, Krefeld
LightCycler Software Version 3.5	Roche Molecular Diagnostics, Pleasanton, USA
Office 2010	Microsoft Corporation, Redmon, USA
Zotero 4.0.26.4	George Mason University, Fairfax, USA

3. RESULTS

3.1 The enriched malignant CLL-cell fraction is highly pure

Even though lymphocyte enrichment employing the RosetteSep® Human Total Lymphocyte Enrichment Cocktail promises a highly enriched lymphocyte population, a couple of quality checks are required before downstream processing of the CLL cell fractions. First, it is imperative to evaluate the purity of the enriched fraction by antibody labelling, which are then analysed by flow cytometry. Second, because Ficoll exposure could have a cytotoxic effect on CLL cells, it is necessary to assess cell viability to ensure that stimulation experiments start with a batch of healthy leukemic lymphocytes.

For this purpose, flow cytometry offers a quantitative and qualitative analysis of purity. To determine purity of the enriched CLL cells, two CDs characteristic of these leukemic lymphocytes are targeted, namely CD5 and CD19. A pure CLL cell population is expected to have a large number of CD5+/CD19+ cells. Surface markers CD5 and CD19 were targeted with CD5-FITC-conjugated and CD19-ECD-conjugated.

Figure 3.1 shows two representative flow cytometry diagrams comparing the profile of a healthy blood and the enriched CLL sample, as well as their CD5/CD19 profiles. The whole blood of healthy patients presents the four expected populations (red blood cells/cyan, lymphocytes/red, monocytes/green, and granulocytes/blue), whilst an enriched CLL cell population must present a single large lymphocyte/red cluster (Fig. 3.1, a1 and b1). The high purity of the enriched CLL samples is attested by the high percentages of CD5+/CD19+ lymphocytes, which means that most of the isolated lymphocytes are CLL cells. The blood of healthy patients (Fig. 3.1, a2) presents a small population of lymphocytes that are close to 0% of CD5+/CD19+

leukocytes, showing the presence of small clusters of leukocytes with a CLL phenotype in healthy individuals.

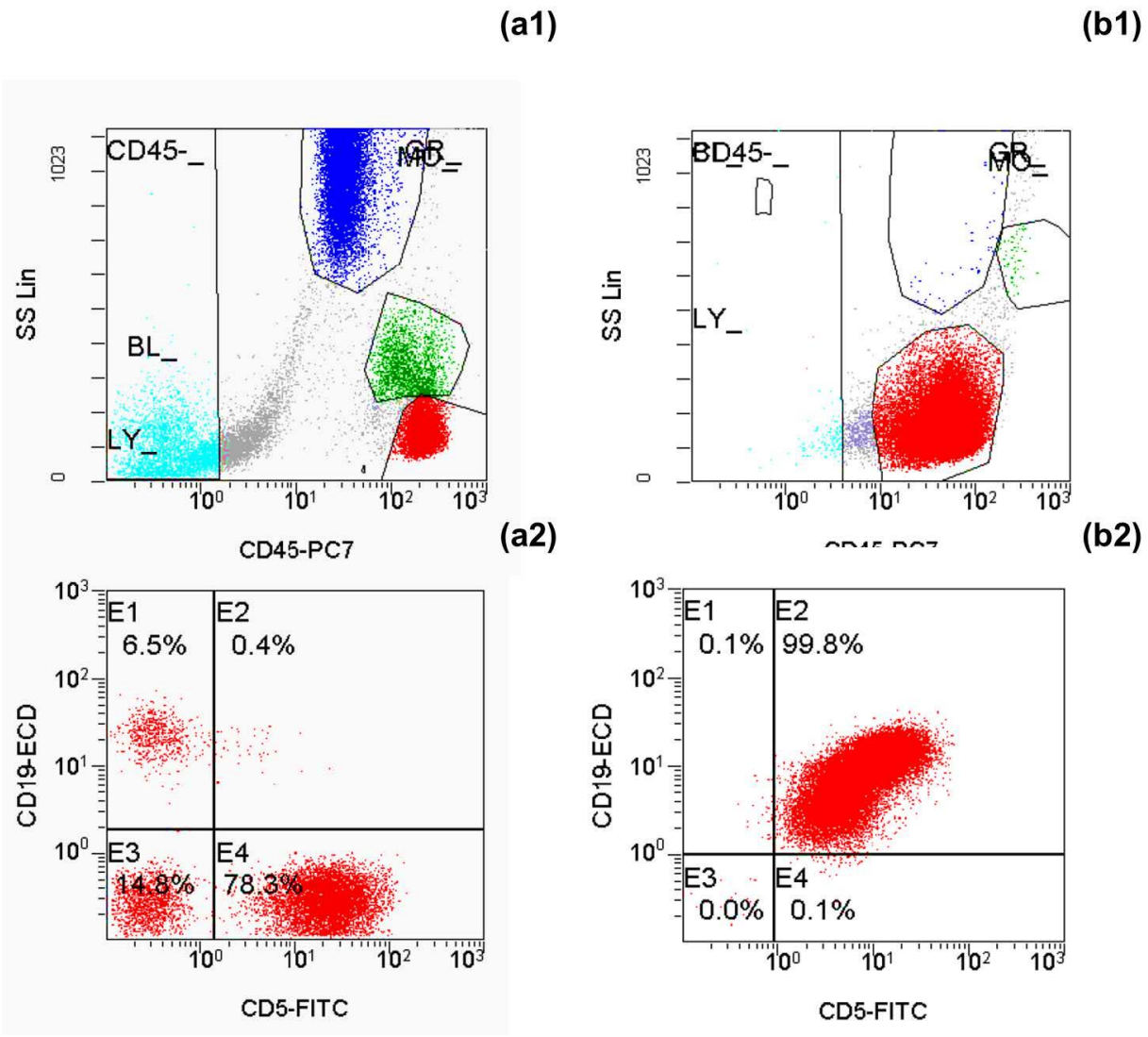


Figure 3.1 Analysing healthy blood and purified CLL cells.
This diagram compares blood (a) from a healthy patient with an enriched CLL population (b). The whole blood population shows the presence of red blood cells (cyan), granulocytes (blue), monocytes (green), and lymphocytes (red) (a1). The gated lymphocyte population (a1 red) shows that in healthy individuals the population of CD5+/CD19+ lymphocytes (a2) is close to 0% (0.4%). The enriched CLL cell population post-Ficoll isolation clusters in a single population (b1 red) of 0% CD3+/CD8+, 0% 57.5% CD39+/CD4+, and should be close to 100% CD5+/CD19+. This indicates a successful isolation procedure composes of a highly pure CLL enriched population.

3.2 Trypan blue shows a high survival rate post-CLL cell isolation

After assessing cell purity, it is necessary to count the cells to seed them at the desired concentration for all downstream experiments. The advantage of using a ViCell automated cell counter is that it also determines cellular viability by staining the cells with trypan blue, becoming the first quality check of cell viability post-isolation. Trypan is a standard and economic way to determine the percentage of viable and necrotic/apoptotic cells. The dye does not penetrate healthy cells with an intact cell membrane, but penetrates equally necrotic and apoptotic cells. However, this technique makes it impossible to discriminate between these two cell populations. The cell automated counter analysis shows that freshly enriched CLL-cells have a viability of ca. 99% (Fig. 2.5). Furthermore, ViCell program also shows the approximate diameter of CLL cells. The size distribution remained between 6 to 8 µm (Fig. 2.5). Since CLL must be counted prior seeding, this procedure was performed for each CLL isolation round giving comparable results in size and viability.

3.3 CLL cells remain highly viable after isolation

Due to its high sensitive, flow cytometry is the method of choice to measure cellular survival rate. An assay employing the double staining 7-AAD and AnnexinV-FITC not only allows to accurately discriminate between viable, apoptotic, or necrotic cells, but also allows to calculate highly accurate percentages of cells in each of these states. Flow cytometry offers two key advantages: high selectivity of dyes and antibody, as well as the analysis of large amount of cells.

Figure 3.2 shows the flow cytometry diagrams typical of a freshly isolated CLL cell population. The pure CLL cell population concentrated in the area in which lymphocytes normally gather, which can be gated allowing the analysis of a semi-homogenous cell population (Fig. 3.2, gate A); gating helps to eliminates debris and unwanted material. It is considered semi-homogenous, because even though it is a single lymphocyte population, different subclonal variants are likely to be found.

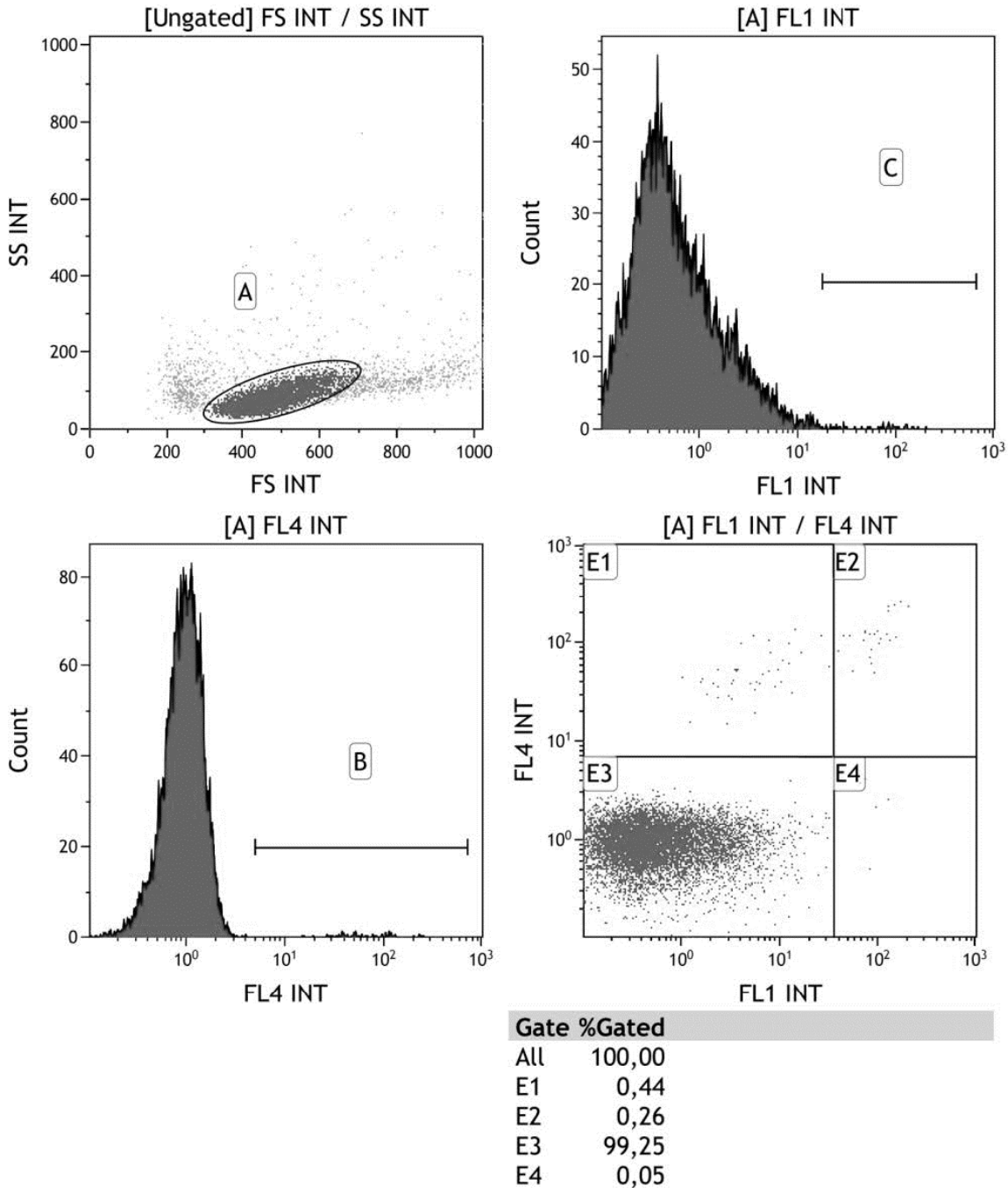
CLL cells were stained with double-viability assay employing 7-AAD detected by FL1 (Fig. 3.2, filter1) and AnnexinV-FITC detected by FL4 (Fig. 3.2, filter 4). A population of highly viable CLL cells shows that there are no CLL cells that can be

bound by AnnexinV-FITC (Fig 3.2, FL1, gate C), since PS remains colocalized to the inner membrane. Similarly, 7-AAD cannot penetrate the membrane of viable CLL cells remaining excluded from CLL cells (Fig. 3.2, FL4, gate B). The FL1 and FL4 histograms are useful, but not sufficient, to calculate the accurate amount of truly viable cells.

The dot plot considers the population detected by FL1 against the cell population of FL4. This shows that the gated lymphocyte population (Fig. 3.2, gate A) is highly viable, because it its negative for both stains (Fig. 3.2, gate E3). The CLL cell population at gate E4 is apoptotic, because PS started to relocalize to the cell membrane where it can be recognized and bound by AnnexinV-FITC. However, this gated population still preserves an intact cell membrane, which makes it 7-AAD-negative (Fig. 3.2, E4). Necrotic cells have a permeable membrane and 7-AAD can penetrate and bind DNA, which makes the population in gate E2 positive for 7-AAD and for AnnexinV-FITC (Fig. 3.2, E2). The double staining 7-AAD and AnnexinV-FITC was employed through this work to assess viability determination. From gate E3 it is possible to determine the percentage of survival advantage attributed to a given substance stimulation.

3.4 CLL plasma and cell media from CLL cells shows Ang1 downregulation

Only the correlation between Ang2 detection in plasma of CLL patients and the secretion of this ligand by CLL cells in vitro has been explored in the CLL context (32,63). However, the ligand Ang1 has been overlooked in CLL research. The amount of Ang1 in plasma and the level of Ang1 that cells secrete in vivo can be assessed employing Quantikine ELISA Human Angiopoietin-1 kit and analysed photometrically to determine the concentration of Ang1.



Gate	%Gated
All	100,00
E1	0,44
E2	0,26
E3	99,25
E4	0,05

Figure 3.2 Exclusion viability test with 7-AAD and Annexin V-FITC.
Flow cytometry analysis indicates that viable cells are negative for 7-AAD and negative for Annexin V/FITC (gate C), since they are not permeable and PS has not relocated to the surface. Necrotic cells are negative for 7-AAD and positive for Annexin V-FITC (gate D), because the cell membrane is not yet permeable but PS has started to relocated to the outer cell membrane indicating the progress of apoptosis. Necrotic cells are positive for both 7-AAD and Annexin V-FITC (gate B), because the permeable cell membrane of the dying cells has allowed 7-AAD penetration into the cell and PS is localized by now on the outer cell membrane. FL1- detection filter for FITC. FL4- detection filter for 7-AAD.

The ELISA reading of plasma samples shows that whilst Ang1 ligands is detected in the plasma of healthy patients, there is a significant (p=0.04) downregulation of Ang1 levels when compared to the plasma of CLL patients. The average level of Ang1 detected in plasma from healthy patients is 4414 ng/ml (Fig. 3.3, a) and from CLL patients is 2336 ng/ml, which represent a relative reduction of roughly 50% in Ang1 levels.

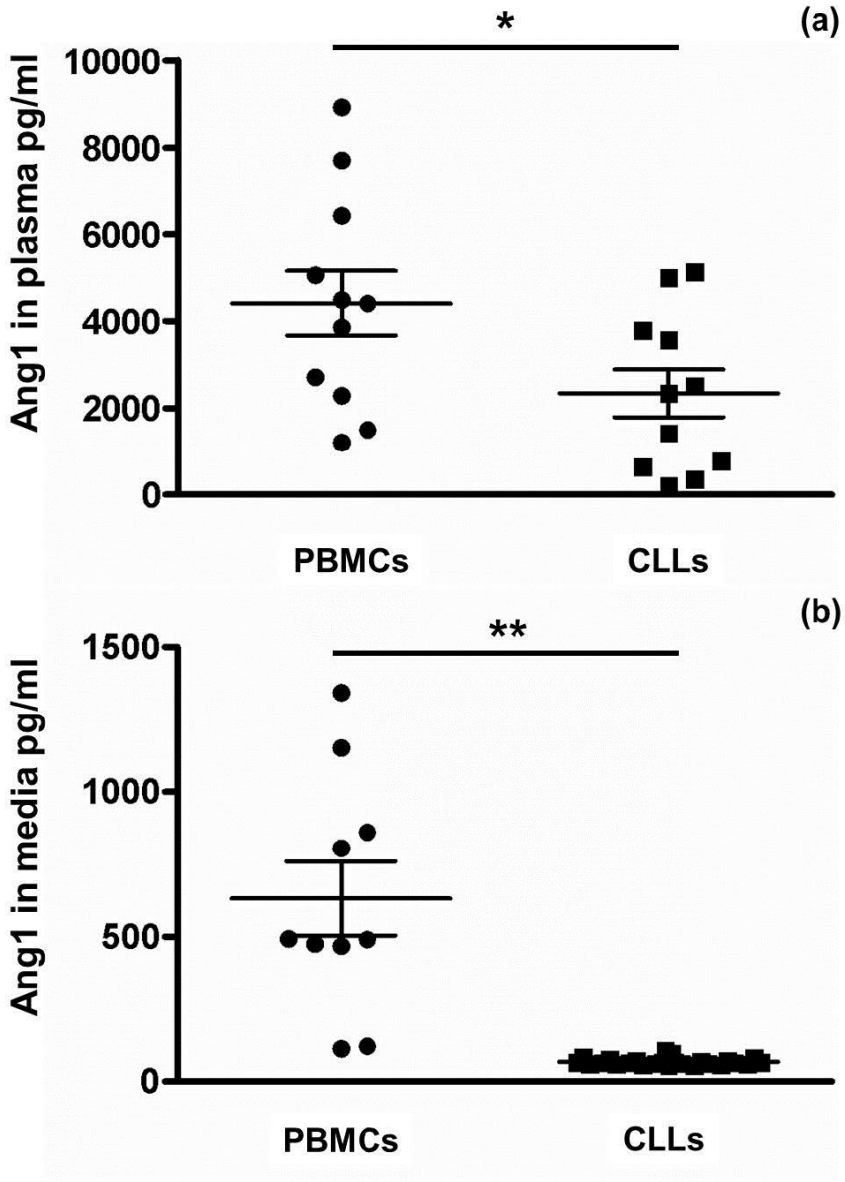


Figure 3.3 Ang1 levels from plasma and cell media.
a) Ang1 levels as detected in plasma from healthy and CLL patients. b) Ang1 levels secreted by PBMCs and CLL cells ($1x10^7$ cells/ml) after 24 h in culture. Plasma samples; healthy n=10, CLL n=10. Cell culture, PBMCs n=10 and CLL samples n=25. Significance set at p≤0.05 (*) and p≤0.005 (**).

After incubating PBMCs and CLL cells, ELISA readings detect a similar discrepancy in the levels of Ang1 (Fig. 3.3, b) secreted by cell in culture. The PBMCs population secreted significantly (p=0.002) higher levels of Ang1 (632 pg/ml) when compared to CLL cells (67 pg/ml), indicating that only a basal level of Ang1 ligand is secreted into the microenvironment by CLL cells.

This downregulation of Ang1 in CLL patients is rather remarkable. Since quiescent Tie2-expressing cells, such as ECs comprising blood vessels and capillaries, require Ang1 to support their quiescent state, downregulation of Ang1 could lead to a leaky state of vessels and capillaries, facilitating perfusion of leukemic lymphocytes between organs, tissues, and the blood stream. Ang1 consequently represents a putative negative prognostic marker, meaning that the lack of a factor, rather than by its overexpression, indicates its association to disease.

3.5 Angiopoietins differently regulate CLL cell survival

Survival is one of the most relevant functional parameter assessing the impact that any given substance in the microenvironment has over the regulation of pro-survival or pro-apoptotic signal transduction events. For this purpose, purified CLL cells were exposed to increasing concentrations of recombinant Ang1 and Ang2. This exposure aimed to mimic microenvironmental availability of angiopoietins that CLL cells may encounter in the different compartments, where different cells and tissues secrete varying concentrations of angiopoietins.

CLL cells were stimulated with increasing concentrations of 50, 100, 200, and 500 ng/ml of either recombinant human Ang1 or Ang2 and cultured for a short period of one day and a longer period of four days. After 1 d post-angiopoietin stimulation, the increasing angiopoietin concentration of Ang1 apparently has no impact on survival advantage (Fig. 3.4, a). Nevertheless, stimulation with Ang2 induces a slight reduction in survival advantage. Whilst this survival advantage remains non-significant throughout increasing concentrations (50 ng/ml not significant (n.s.), 100 ng/ml -4.2% p=0.05, 200 ng/ml -7.1% p=0.05), there is a slight and significant reduction of survival advantage when employing 500 ng/ml (-10.0% p=0.005) suggesting that Ang2 has a pro-apoptotic effect on CLL cells.

Angiopoietin exposure in the long-term showed relevant changes in CLL cell survival advantage when compared to short-term angiopoietin exposure. Ang1 confers significant survival advantage in a dose dependent manner, even at extremely low concentrations (50 ng/ml +4.4% p=0.048, 100 ng/ml +6.7% p=0.01, 200 ng/ml+7.9% p=0.02, 500 ng/ml +11.5% p=0.003,). In addition, stimulation with Ang2 (Fig. 3.4, .b) significantly decreases CLL survival (50 ng/ml n.s., 100 ng/ml n.s., 200ng/ml -8.2% p=0.04, 500 ng/ml -9.0% p=0.04) also in dose dependent manner. The pro-survival effect of Ang1 and the detrimental one of Ang2 in a long-term analysis suggest that the early angiopoietin interaction with CLL cells is sufficient to regulate pro-survival and anti-apoptotic signalling in CLL cell.

Do angiopoietins regulate survival advantage on CLL cell in a paracrine, autocrine, or auto- and paracrine fashion? Since CLL cells constitutively express and secrete Ang1 ligand (Fig, 3.3, b), as well as abundant Ang2 (31) into the microenvironment, it is possible to infer that this low levels could control survival-related signalling. To solve this conundrum, CLL-derived angiopoietins were blocked employing a drug that specifically and strongly binds Ang1 and Ang2 by preventing them from binding to their target receptors and thus induce signal transduction. To understand to which extend basal levels of CLL cell-derived angiopoietins control CLL cells survival, CLL cells were also exposed to increasing concentrations of 50, 100, 200, and 500 ng/ml of trebananib.

Survival measurements after the first day of stimulation with trebananib show no significant changes in survival advantage. However, after stimulating CLL cells with increasing levels of trebananib (Fig. 3.4, c) for four days, there are no significant changes in cell survival at the lowest concentration of trebananib (50 ng/ml n.s., 100 ng/ml n.s., 200ng/ml n.s.). However, there is a significant detriment in survival advantage when employing 500 ng/ml (-12.3%, p=0.01,). Thus, we could infer that endogenous Ang1 and Ang2 derived from CLL cells participate in an autocrine-like manner to regulate survival of CLL cells. Due to the significant effect observed when stimulating CLL cells with 500 ng/ml of Ang1, Ang2, or trebananib, this concentration was employed throughout all further downstream experimental processes.

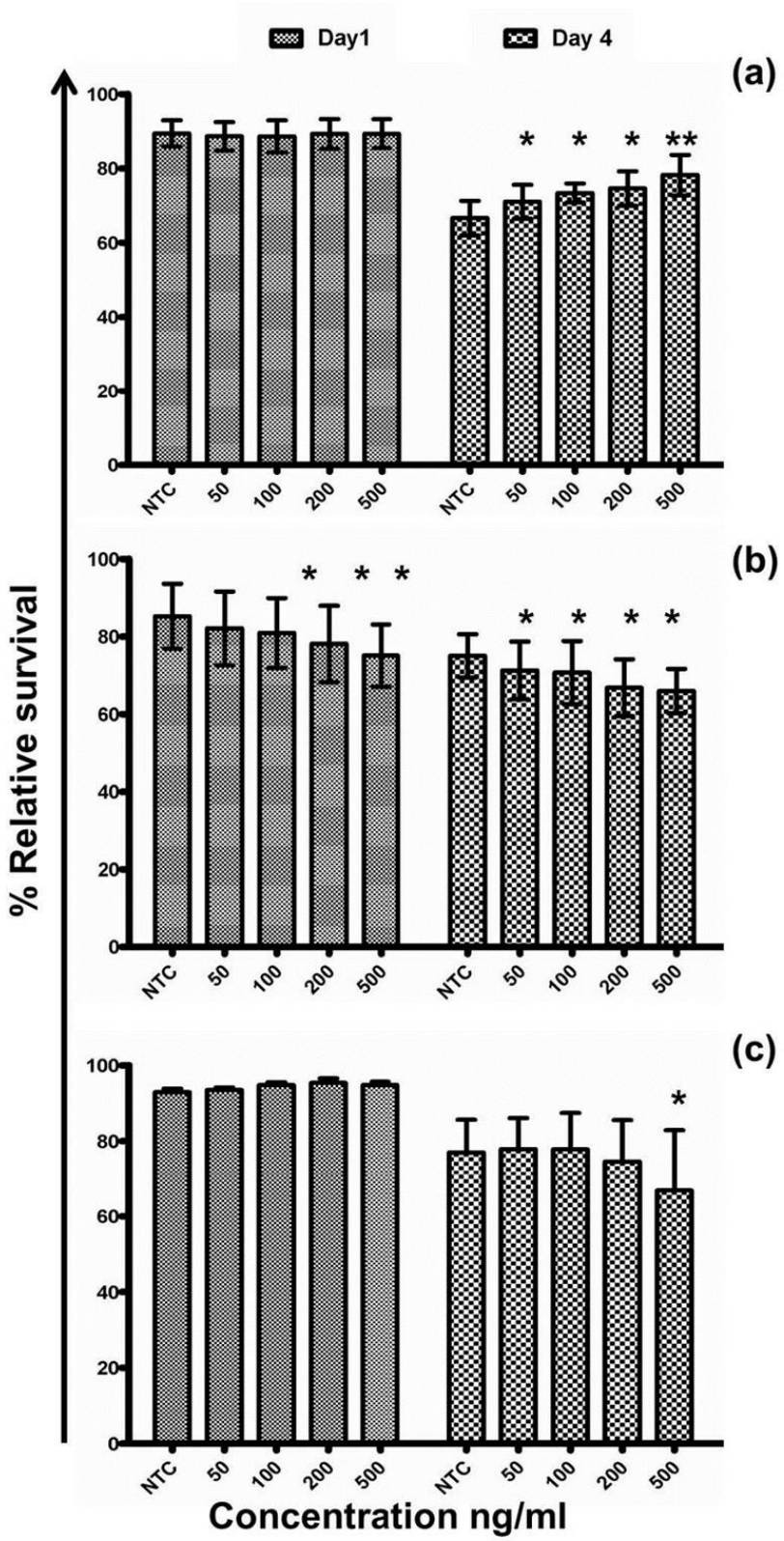


Figure 3.4 Survival assessments of CLL cells.
Changes in survival after 1 and 4 d post-stimulation with (a) Ang1, (b) Ang2, and (c) trebananib. Relative survival expressed in per cent. n=6. Significance set at *p ≤0.05 and **p ≤0.005.

3.6 CLL cells express Ang1, Ang2, Tie1, and lack Tie2 and VE-PTP mRNA

To understand to which extent the Ang-Tie2 signalling pathway is actively regulating the fate of CLL cells, it is imperative to understand the mRNA expression status of key genes involved in the Ang-Tie2 signalling pathway. This is possible through parallel analysis of the expression of Ang1, Ang2, Tie1, Tie2, and VE-PTP at the mRNA level in CLL cells and healthy PBMCs. For this purpose, qualitative PCR was employed for the amplification of synthesized cDNA material from CLL and healthy patients. The results of amplified cDNAs were visualized by classical agarose gel electrophoresis.

The PCR product verification shows that CLL cells express mRNA transcripts for Ang1, Ang2, and Tie1 (Fig. 3.5). However, agarose gel electrophoresis also shows that CLL cells lack mRNA transcripts for receptor Tie2 and Tie-2 regulator VE-PTP. Besides expressing Ang1, Ang2, and Tie1, the healthy PBMC population expresses Tie2. Nevertheless, VE-PTP is not found in the healthy PBMC population.

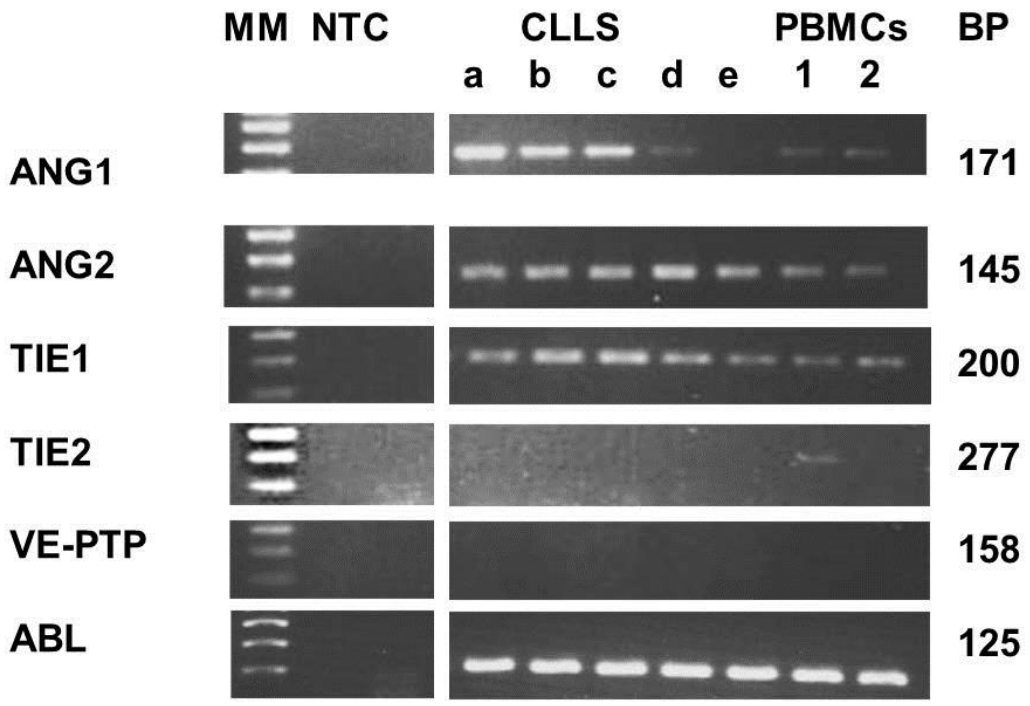


Figure 3.5 Expression of the components of the Ang-Tie2 pathway. Agarose gel electrophoresis showing the detection of Ang1, Ang2, Tie1, Tie2, VE-PTP and reference gene ABL. a-e CLL samples. 1-2 Healthy PBMCs samples. NTC; non-template controls, BP; size in base pairs. MM; molecular marker.

3.7 Ang1 and Ang2 regulate mRNA expression of Ang1, Ang2, and Tie1

The presence of Ang1, Ang2, and Tie1 mRNA in CLL samples is a suggestive indicator of Ang-Tie2 signalling activity in CLL cells. Nevertheless, it is not known whether mRNA expression of Ang1, Ang2, Tie1, and Tie2 (in the case of cells expressing Tie2 receptor) is regulated by angiopoietins present in the extracellular space through a feedback inhibition mechanism.

CLL cell samples were stimulated with 500 ng/ml of either Ang1 or Ang2 to determine whether microenvironmentally available angiopoietins regulate, through a putative feedback loop, expression of Ang1, Ang2, and Tie1 transcripts. When evaluating changes in the relative levels of mRNA expression 1 h post angiopoietin stimulation it appears that both angiopoietins differentially induce changes in mRNA expression of Ang1, Ang2, and Tie1 (Fig. 3.6). With respect to non-stimulated control (1.00), Ang1 stimulation induces a decrease to 0.83 fold (n.s.) of Ang1, a significant increase to 1.14 fold ($p=0.018$) of Ang2, and a significant increase to 1.31 fold ($p=0.029$) of Tie1. Ang2 stimulation induces a significant increase to 1.21 fold ($p=0.18$) of Ang1, a significant increase of 1.55 fold ($p=0.042$) of Ang2, and an increase of 1.31 fold (n.s) of Tie1.

The ability of external angiopoietins to modulate the relative mRNA contents of Ang1, Ang2, and Tie1 on CLL cells illustrate the sensitivity of CLL cells to angiopoietins present in the microenvironment. Thus, it is likely that compartments where CLL cells are localized and their respective angiopoietin concentrations will dictate the expression patterns of Ang1, Ang2, and Tie1 of CLL cells. Since CLL cells lack Tie2 on their surfaces, changes in mRNA expression of Ang1, Ang2, and Tie1 are likely regulated through a Tie2-independent feedback mechanism. Therefore, it is possible to infer that alternate receptors on the surface of CLL cells recognize angiopoietins and in return regulate expression of Ang1, Ang2, and Tie1 mRNA.

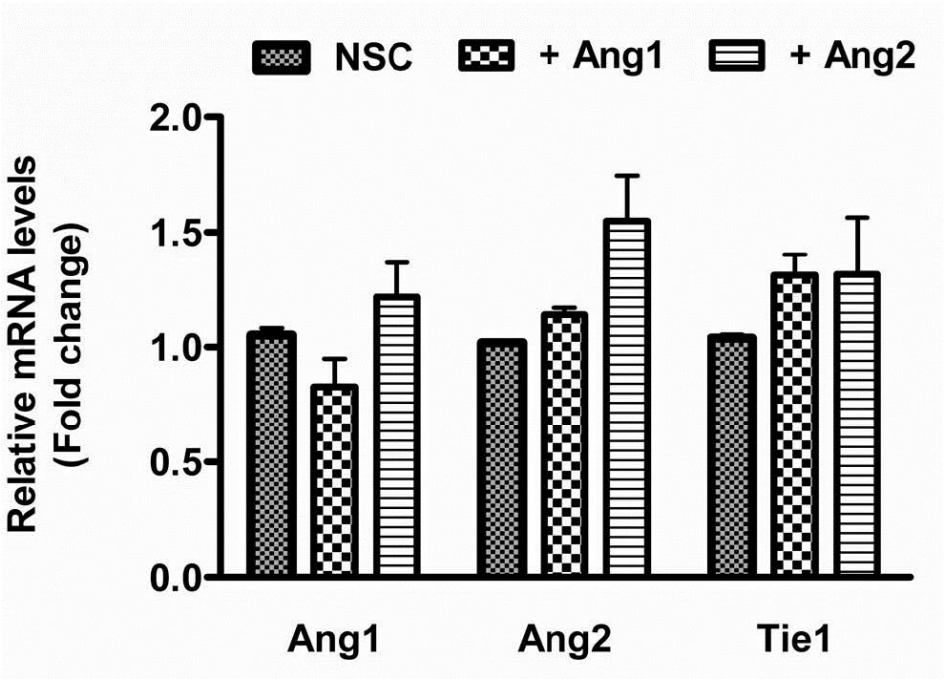


Figure 3.6 Relative changes in Ang1, Ang2, and Tie1 mRNA.
mRNA levels measured upon Ang1 and Ang2 stimulation. Fold changes were assessed by ΔΔCt method and normalized to the reference gene ABL. Relative mRNA expression level of 1.0 refers to basal expression of the non-stimulated control (NSC) n=6

3.8 Ang2 induces transient and concomitant expression of Tie2 and VE-PTP

Qualitative PCR showed that CLL samples express Ang1, Ang2, and Tie1, yet CLL cells from peripheral blood lack Tie2 and VE-PTP mRNA, supporting results of previous works (25,64). However, the case of Tie2 and VE-PTP expression could be transient and their expression may not necessary be present through the entire life span of CLL cells. Transcription and translation could also be brief processes and it was not known if Tie2 or VE-PTP expression could be triggered transiently. After all, only EC constitutively express Tie2 on their surfaces. In addition, there are no reports of VE-PTP expression, the negative regulator of Tie2, in the CLL context.

To assess whether angiopoietins trigger transient expression of either VE-PTP or Tie2 in CLL cells, CLL cell samples were stimulated with 500 ng/ml of Ang1 or Ang2. However, in this occasion CLL cells were stimulated with angiopoietins for a brief period of 30 min and a longer period of 6 h.

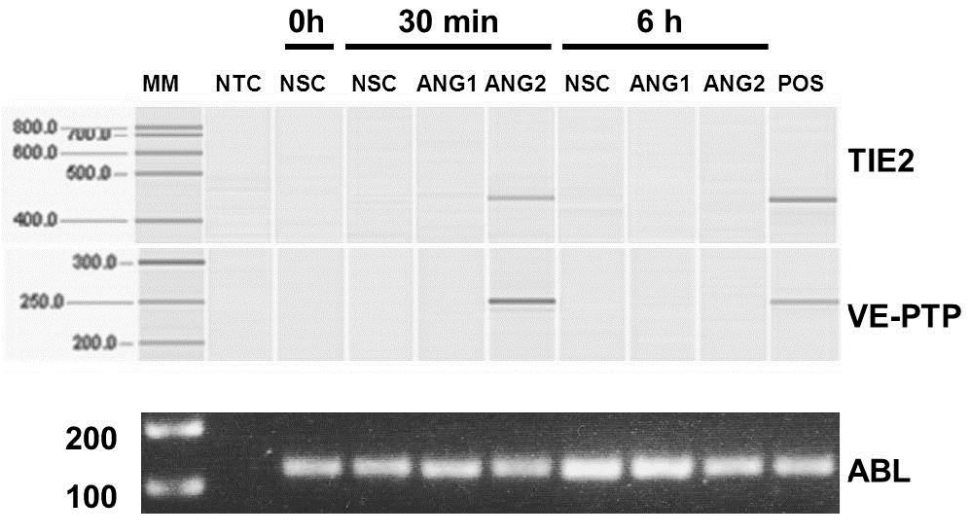


Figure 3.7 Detecting transient expression of Tie2 and VE-PTP transcripts. After 30 min of stimulation only Ang2, but not Ang1, induces expression of Tie2 and VE-PTP. After 6 h, these transcripts are no longer detectable. Tie2 and VE-PTP transcripts were visualised on capillary electrophoresis. HUVEC cells were used as positive control for Tie2 and VE-PTP expression. Reference gene ABL was run on regular gel electrophoresis. BP; base pairs, NTC; non-template control, NSC; non-stimulated control.

The capillary gel electrophoresis showed that there is concomitant expression of both VE-PTP and Tie2 transcripts only after 30 min of angiopoietin stimulation; only Ang2 but not Ang1 induces this expression (Fig. 3.7). In addition, detection of the Tie2 and VE-PTP mRNA transcripts observed upon short-term stimulation with Ang2 is no longer detectable at 6 h post-stimulation. Altogether this indicates that CLL cells react to high amounts of external recombinant Ang2 ligand present in the microenvironment to regulate transient Tie2 and VE-PTP. This also shows the potential of angiopoietins to regulate the components of the Ang-Tie2 pathway at the mRNA level.

3.9 Angiopoietins differentially modulate expression of Tie1 in CLL cells

Tie1 and Tie2 are two receptors easily targeted by antibodies coupled with fluorophores for their detection by flow cytometry. Tie1 receptor is supposedly expressed on the surface of CLL cells, whilst partner Tie2 is absent. As RT-PCR shows, angiopoietins are capable of regulating the expression mRNA level of Ang1, Ang2, and Tie1. Nevertheless, changes in mRNA expression do not necessarily correlate with changes in protein translation. In addition, the lack of Tie2 mRNA does not necessary indicate that Tie2 receptor is not already on the surface of Tie2, since it

is possible to infer that CLL cells transported into the blood stream already possess the Tie2 protein on their surface and CLL cells may no longer manufacture Tie2 mRNA. Thus, flow cytometry was employed to analyse expression of Tie1 and Tie2 on non-treated CLL samples and in CLL samples stimulated with Ang1 and Ang2.

The flow cytometer analysis shows expression of Tie1 (Fig. 3.8, a) on CLL cells and shows that receptor Tie2 is not present on the surface of CLL cells, backing qualitative PCR findings. Stimulation with angiopoietins differentially modulates the expression of Tie1 on surface of CLL cells. After analysing six patients (Fig. 3.8, c), flow cytometry analysis showed that stimulating CLL cells with Ang1 significantly increases the CLL-Tie1 relative expression by 7.7% ($p=0.014$) and stimulation with Ang2 significantly increases the CLL-Tie1 expression to 12.5% ($p=0.006$).

The increase in Tie1 receptor expression upon exposure to Ang1 and Ang2 suggests that angiopoietins present in the microenvironment differentially control Tie1 expression at the mRNA (Fig. 3.6) level and at the protein level. It is evident that receptor Tie1 is already present in CLL cells localized to the blood stream, whilst receptor Tie2 is not present on the outer cellular membrane of CLL cells when transported into the blood stream, nor its expression can be triggered by angiopoietins. Changes in Tie1 expression upon angiopoietin stimulation further support the notion that CLL cells are highly sensitive to microenvironmentally available angiopoietins.

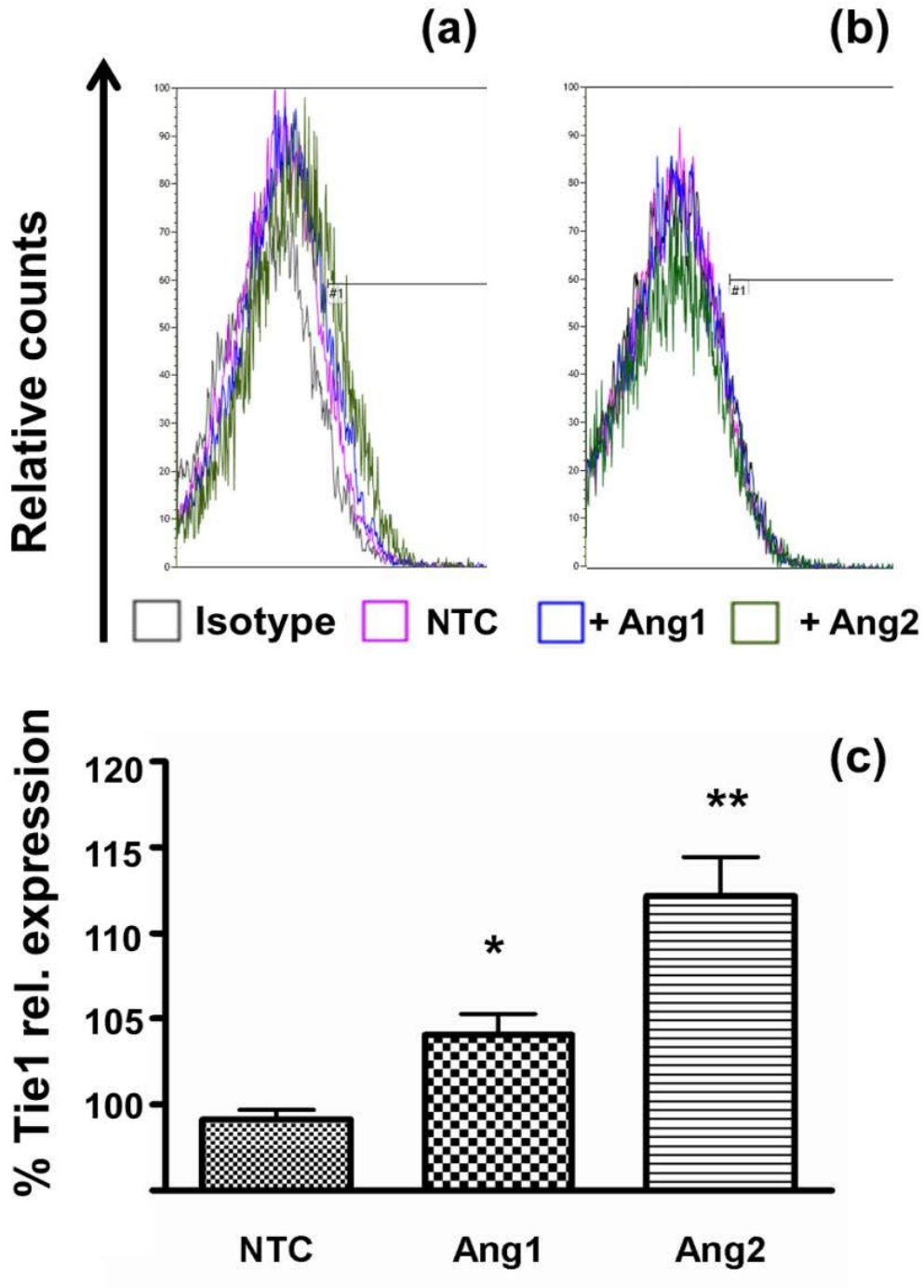


Figure 3.8 Tie1 and Tie2 detection following angiopoietin exposure.
The flow cytometer histogram shows the shift in detecting Tie1-relative fluorescence (a). Histogram shows the lack of Tie2-positive CLL cells (b). Graph shows the relative increase in Tie1-relative expression in non-treated control (Set to 100%), and Ang1 and Ang2 stimulated samples (c). n=6. Significance set at $p \leq 0.05$.

3.10 Ang1 induces chemotaxis and transendothelial-like migration of CLL cells

After regulation of CLL survival, cellular migration is one of the most relevant cellular processes to determine whether any drug or protein regulates key function such as pro-survival or anti-apoptotic signalling. The ability of ligands Ang1 and Ang2 in inducing migration and transport of CLL cells is not understood and therefore a migration assay to test simple chemotaxis and transendothelial-like migration were designed.

CLL cells have a diameter of 6-8 µm and to test the chemotactic effect of angiopoietins on these cells, Transwell Permeable Supports (Corning Inc) inserts with a membrane with pores of 5 µm/diameter were employed to hinder the simple passage of CLL cells into the lower chamber. CLL cells were stimulated with 500 ng/ml of Ang1 or Ang2. Stimulation with Ang1, but not Ang2, induced a robust homing-like effect of CLL cells (Fig. 3.8, a) and the effect is highly significant (Ang1 p=0.0006, Ang2 p= 0.0003). Blocking Ang1 and Ang2 by adding trebananib (Ang1+trebananib and Ang2+trebananib, 500 ng/ml from each compound) to the media significantly abrogates homing-like migration (Ang1+Tre p=0.01, Ang2+Tre p= 0.03).

Transendothelial migration refers to the ability of cells to reshape in order to penetrate a physical barrier. Such a barrier could be the ECL composing capillaries, veins, and HEV. To test whether angiopoietins are capable of inducing transendothelial-like migration of CLL cells ThinCert (Greiner Bio-One) inserts with a membrane with pores of 3 µm/diameter were employed. CLL cells were stimulated with 500 ng/ml of Ang1 or Ang2. This assay showed that Ang1 induces significant transendothelial-like migration (Fig. 3.8 b, Ang1 p=0.0008, Ang2 n.s.). In this instance, trebananib (Ang1+trebananib and Ang2+trebananib, 500 ng/ml from each compound) significantly abrogates CLL movement through the membrane (Ang1+Tre p=0.048, Ang2+Tre p= 0,044).

Apparently, Ang1 not only supports CLL cell survival advantage, but also induces angiopoietin-guided chemotaxis and strikingly transendothelial-like movement, which suggests plasticity of CLL cells upon exposure to high levels of Ang1 ligand.

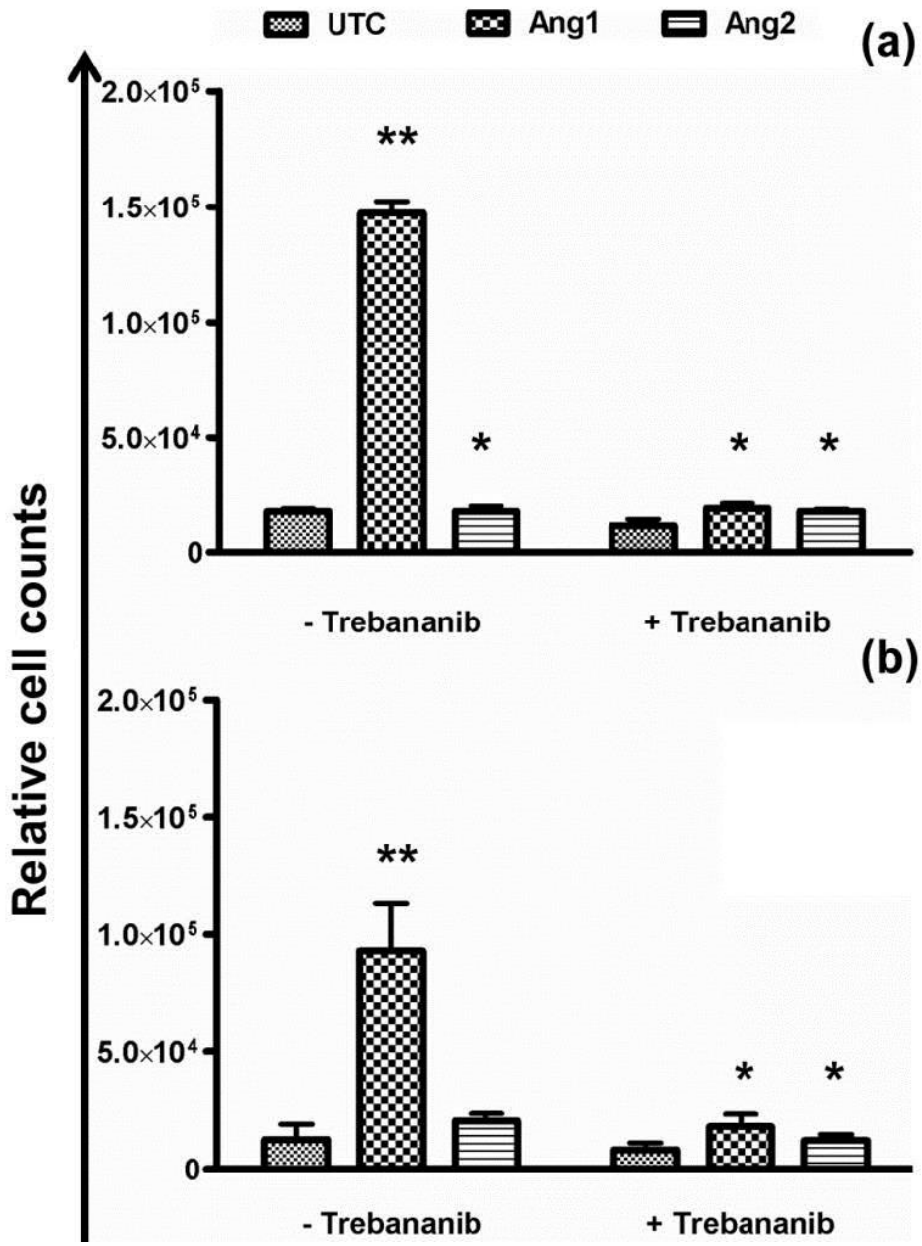


Figure 3.9 Chemotaxis and transendothelial-like migration of CLL cells. (a) Ang1 has a strong effect on homing-like activity on CLL cells and Ang2 display a pattern similar to that of the untreated control (UTC). (b) Ang1, but not Ang2, has a strong effect inducing transendothelial-like migration. Trebananib blocks Ang1 in both (a) and (b), bringing relative values close to a basal level, which are also comparable those of Ang2 stimulation. n=6. Significance set at $p \leq 0.05$.

3.11 Angiopoietin alter caspase and ATP activity in CLL cells

Relative changes in caspase and ATP relative content were assessed to evaluate the impact of angiopoietins on the metabolic fitness of CLL cells. The caspase

activity assay is a great marker of cellular metabolic fitness. This is relevant to test novel compounds and protein entities that are believed to regulate cell survival. To assess changes of caspase activity levels in non-stimulated and stimulated CLL samples, Cell Meter ™ Caspase 3/7 activity (AAT Bioquest, Inc.) assay was employed.

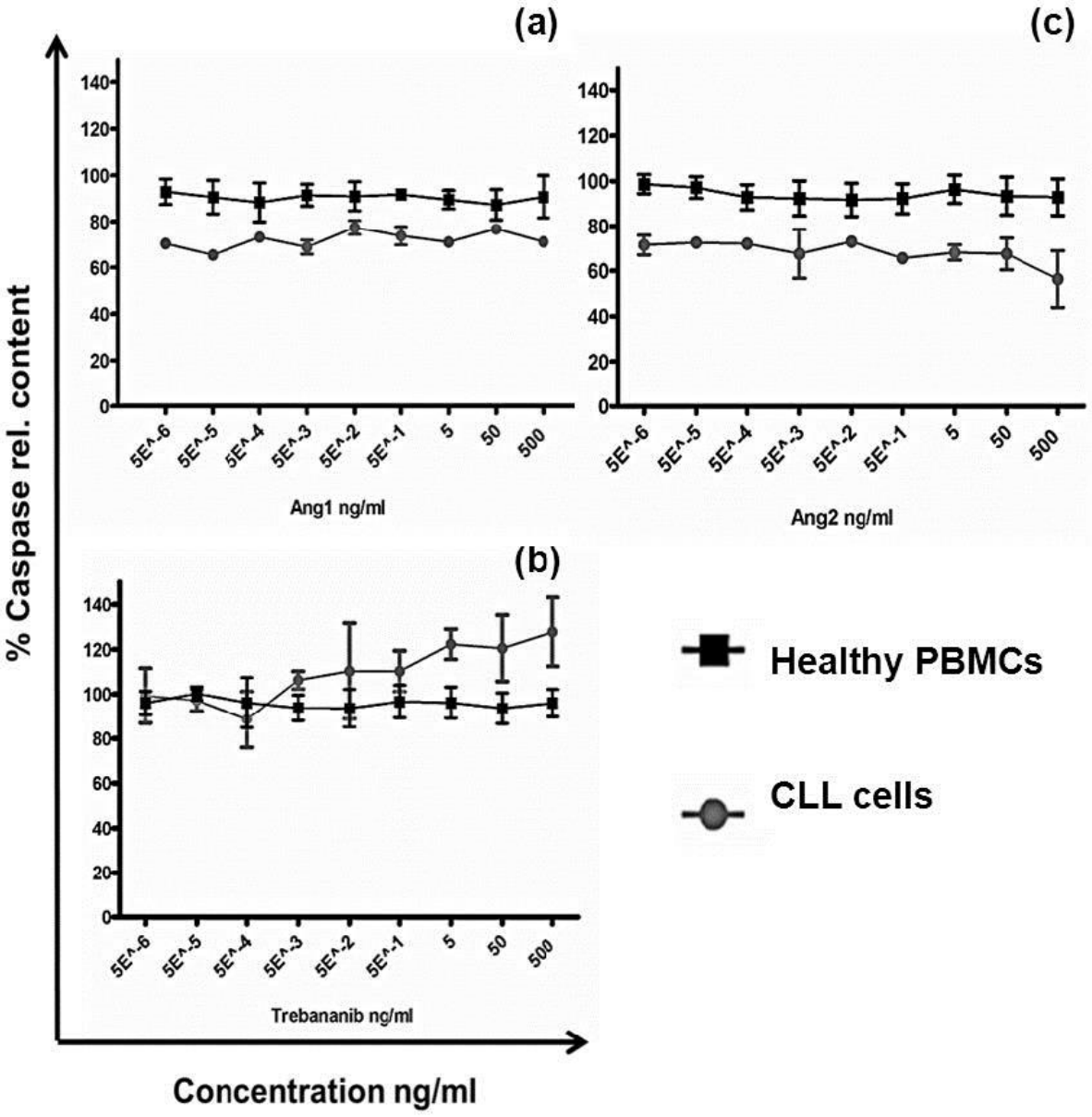


Figure 3.10 Variation of caspase relative contents in CLL cells. Relative changes in fluorescence are expressed as per cent change relative to non-stimulated CLL cells. Cells were stimulated with Ang1, Ang2, or trebananib at increasing concentration of (a) Ang1 (b) Ang2, and (c) trebananib. n=6.

Stimulation with Ang1 and Ang2 after 24 h induces a constant reduction of caspase relative content in CLL cells (Fig 3.9). When comparing to non-stimulated control (Set as 100% reference), stimulation with as low as 5 fg/ml of Ang1 (Fig. 3.9, a) induces a decrease of caspase content, remaining relatively steady as concentration increased (5 fg/ml 70.4%, 50 fg/ml 65.4%, 500 fg/ml 73.2%, 5 pg/ml 68.8%, 50 pg/ml 77.4%, 500 pg/ml 73.8%, 5 ng/ml 71%, 50 ng/ml 76.8%, and 500 ng/ml 71.1%). Stimulation with Ang2 (Fig. 3.9, b) also results in a decrease of caspase content (5 fg/ml 71.5%, 50 fg/ml 72.5%, 500 fg/ml 72%, 5 pg/ml 67.6%, 50 pg/ml 73%, 500 pg/ml 65.6%, 5 ng/ml 68.2%, 50 ng/ml 67.5%, and 500 ng/ml 56.4%). Stimulation with trebananib (Fig. 3.9, c) triggers an increase of caspase activity only on CLL cells (5 fg/ml 99.3%, 50 fg/ml 97.2%, 500 fg/ml 86%, 5 pg/ml 106.3%, 50 pg/ml 110.3%, 500 pg/ml 110.3%, 5 ng/ml 122.3%, 50 ng/ml 120.5%, and 500 ng/ml 127.8%).

In addition, assessing ATP presence in a cellular population is another method to assess cellular metabolic fitness. ATP relative content was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

Slight reduction of ATP relative content remains constant upon Ang1 (Fig. 3.10,a) stimulation (5 fg/ml 67.5%, 50 fg/ml 67.5%, 500 fg/ml-67.4%, 5 pg/ml 66.3%, 50 pg/ml 66.8%, 500 pg/ml 68%, 5 ng/ml 64.1%, 50 ng/ml 71.8%, and 500 ng/ml 72.7%). Ang2 stimulation (Fig. 3.10, b) induces a notorious decrease in ATP content, decreasing as the concentration of Ang2 approaches 500 ng/ml (5 fg/ml 68.6%, 50 fg/ml 67.2%, 500 fg/ml 65.1%, 5 pg/ml 61.8%, 50 pg/ml 63.3%, 500 pg/ml 62.6%, 5 ng/ml 58.1%, 50 ng/ml 55.9%, 500 ng/ml 46.7%). Exposure to trebananib (Fig. 3.10, c) shows deeper decrease of activity than that of Ang1 and Ang2 (5 fg/ml 56.3%, 50 fg/ml 55.3%, 500 fg/ml 56.6%, 5 pg/ml 57.4%, 50 pg/ml 49.5%, 500 pg/ml 49.3%, 5 ng/ml 45.9%, 50 ng/ml 42%, and 500 ng/ml 35.8%). The decrease in ATP detection indicates the sensitivity of CLL cells toward the presence of Ang1 and Ang2 (Fig. 9).

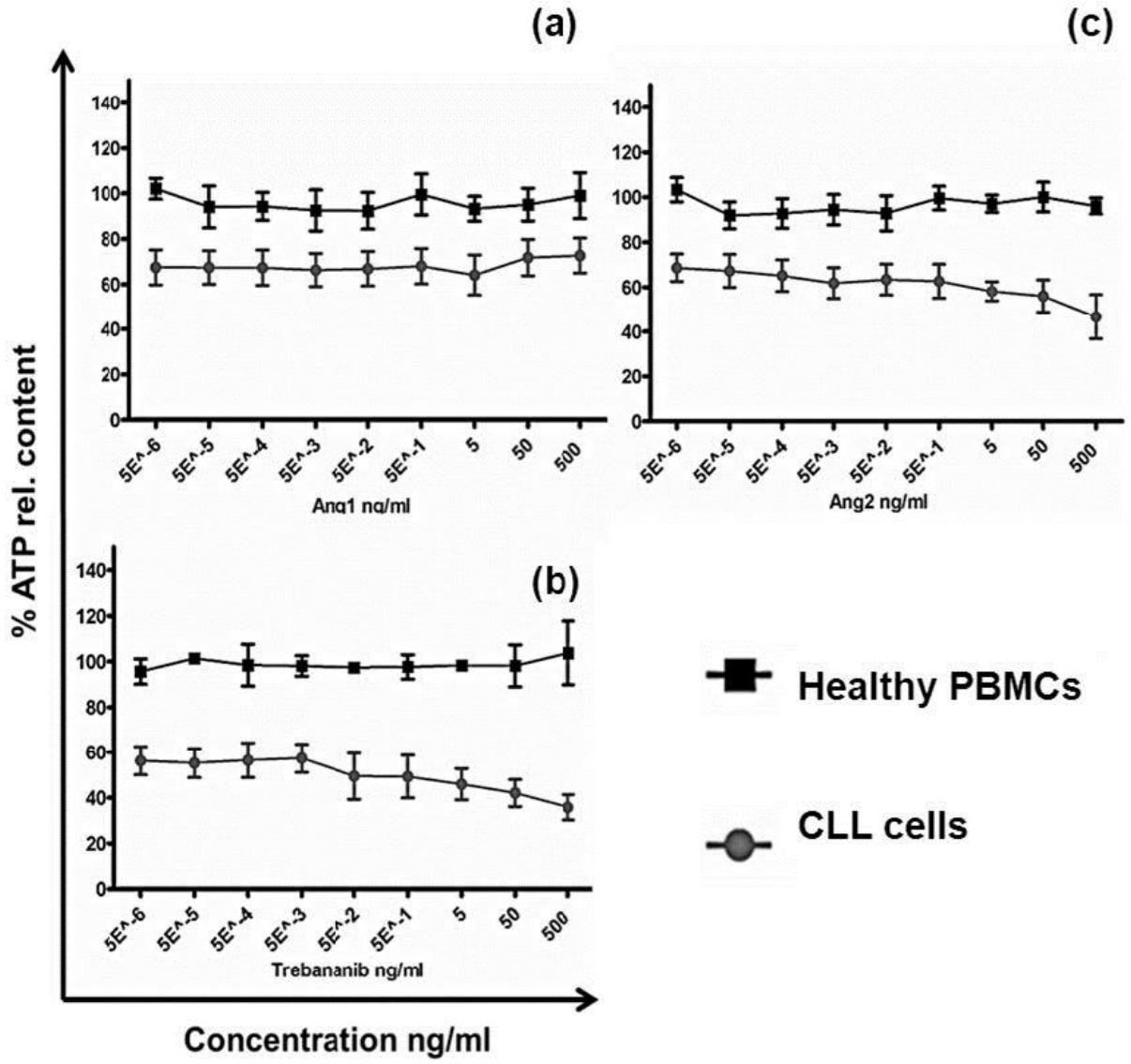


Figure 3.11 Variation of ATP relative contents in CLL cells.
Relative changes in luminosity are expressed as per cent changes relative to non-stimulated CLL cells. Cells were stimulated with increasing concentration of (a) Ang1, (b) Ang2, and (c) trebananib. n=6.

3.12 Ang2 triggers strong dephosphorylating of NF-kB p65 subunit

The NFκB signalling pathway is associated with inflammation responses, carcinogenesis, chemoresistance (65), as well as stress responses, B-cell development, immunity, cell proliferation, and apoptosis. Activation of the pathways is mainly regulated by phosphorylation of NF-kB, which results in ubiquitination of IκB proteins, a post-translational modification that leads in degradation of proteins by the proteasome and translocation of the NF-κB/p65 subunit into the nucleus to regulate gene expression. Due to its essential role in cell homeostasis, the NF-kB status in its normal and phosphorylated state was analysed to understand if angiopoietins have a strong general impact in cellular homeostasis.

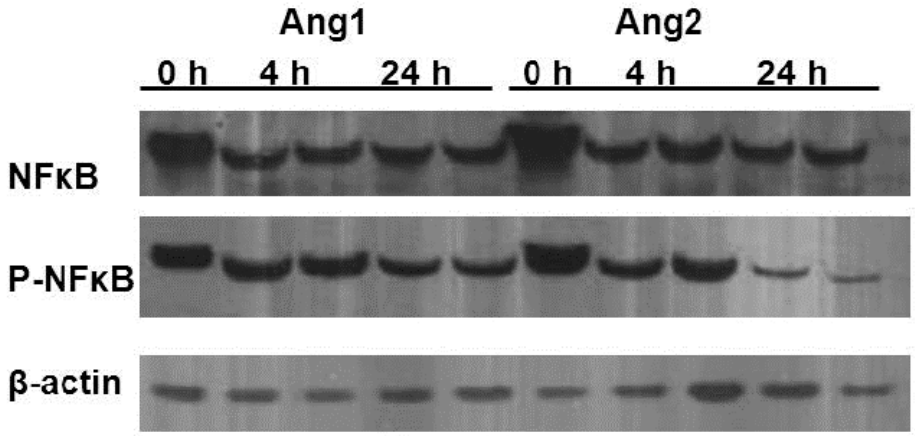


Figure 3.12 Ang2 induces dephosphorylation of NFκB p 65 subunit. After isolation, it is evident that NF-kB is strongly phosphorylated (0h) and this phosphorylation decrease in culture (4h). Ang1 does not trigger changes in NFκB phosphorylation. However, Ang2 induces a strong reduction of phosphorylation after 24 h of stimulation in culture. This gel shows samples loaded in duplicates.

In CLL cells, the NF-kB p65 remains strongly phosphorylated in freshly isolated CLL cells form the blood stream (Fig. 3.11), which shows its high activity in leukemic cells. Stimulation with Ang1 shows no dramatic changes in phosphorylation at either 4 h or 24 h. However, Ang2 induces an evident reduction of NF-kB phosphorylation after 24 h of Ang2 stimulation, suggesting a role of this angiogenic ligand in inducing external stress.

3.13 Ang2 induction of VE-PTP expression is confirmed by mass spectrometry

Protein identification was carried using MALDI/MS, which identifies proteins by determining their exact mass, which are compared with the internal database. Whilst comparing protein patterns from non-stimulated and Ang1/Ang2-stimulated CLL cells (1 h and 12 h), we were able to detect a strong signal around the size of 225 kDa in the Ang2-stimulated sample (Fig. 3.12, arrow). Label-free MS, revealed VE-PTP presence in Ang2-stimulated sample, but not in control bands. Only keratins were identified besides VE-PTP in the Ang2 stimulated sample. Keratins are a common background protein found in most mass spectrometry measurements. The size of the band from the Ang2 stimulated sample is congruent with the previous reports, where the reported size of VE-PTP ranges between 240 and 260 kDa (52). Protein size will vary according to technique of separation and degree of glycosylation, which also and likely varies amongst different cellular systems.

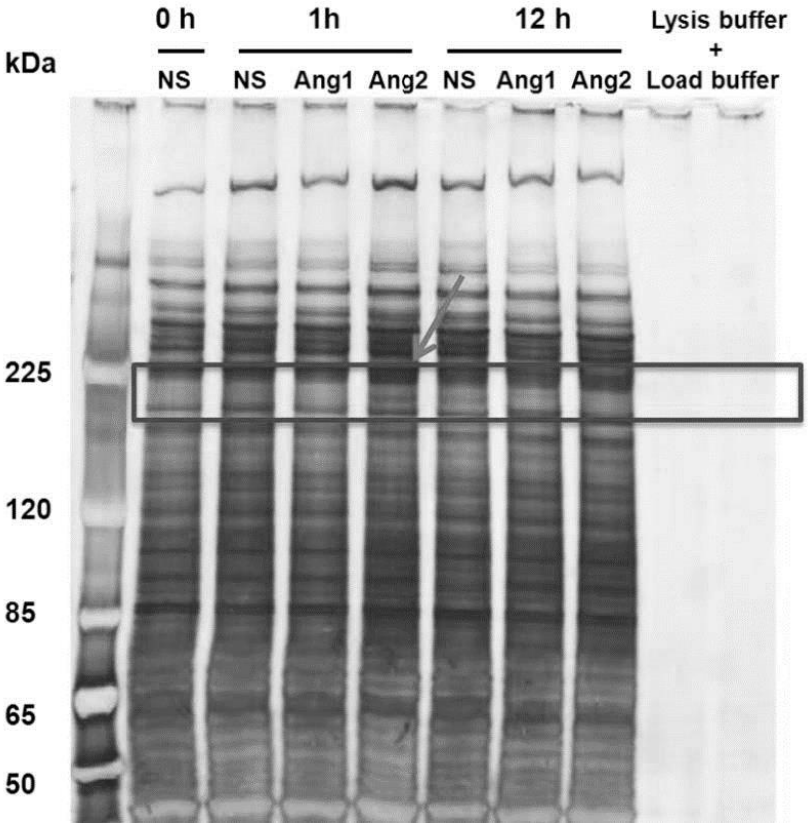


Figure 3.13 Detection of changes in protein patterns by silver staining. The arrow shows the detection site of VE-PTP peptide. This band was only visualised upon Ang2 stimulation for 1 h and the extra band is not seen at 12 h. Selected bands were evaluated by label free MS to identify proteins.

3.14 Ang1 phosphorylates key of tyrosine receptor kinases (RTKs)

The relevance of RTKs resides in their role in transmitting signal from the microenvironment into the cell by signal transduction events. Furthermore, they are ideal therapeutic targets that control the most essential signalling pathway that regulate survival, apoptosis, migration, differentiation, and cell division.

For the simultaneous detection of changes in the relative phosphorylation, a RayBio® Human RTK Phosphorylation Antibody Array that detects 71 relevant human receptor tyrosine kinases (RTKs) was employed. Evaluating 71 RTKs simultaneously offers the convenient advantage of quickly understanding which signalling pathways are putatively activated or deactivated due to Ang1 exposure. This reading is performed directly from CLL cell lysate without the need of running individual western blots and fishing for putative targets of increased/decreased phosphorylation. Figure 3.13 shows the changes in phosphorylation induced by Ang1 stimulation. In freshly isolated cells, there is a basal activation of RTKs (Fig. 3.13, a). Phosphorylation is a brief molecular event. Therefore, CLL cells were stimulated 30 min and immediately collected (Fig. 3.13, b). Collection at this time shows that a number of RTKs becomes strongly phosphorylated. This phosphorylation starts to decrease dramatically at 2 h (Fig. 3.13, c) and by 12 h it has been reduced to levels resembling the non-stimulated sample (Fig. 3.13, d). There is a noticeable phosphorylation of the RTK named BMX (Table 3.1, yellow). Please note that receptors Tie1 (Table 3.1, blue) and Tie2 (Table 3.1, red) remained non-phosphorylated.

Table 3.1 shows the membrane map of the 71 RTKs printed on the membranes. The proteins showing an increase in phosphorylation after 30 min are highlighted in green. The names of these proteins are summarized in table 3.2. Stimulation of Ang1 apparently induces an increase in phosphorylation of 33 out of 71 RTKs as shown by the assay (Fig. 3.13)

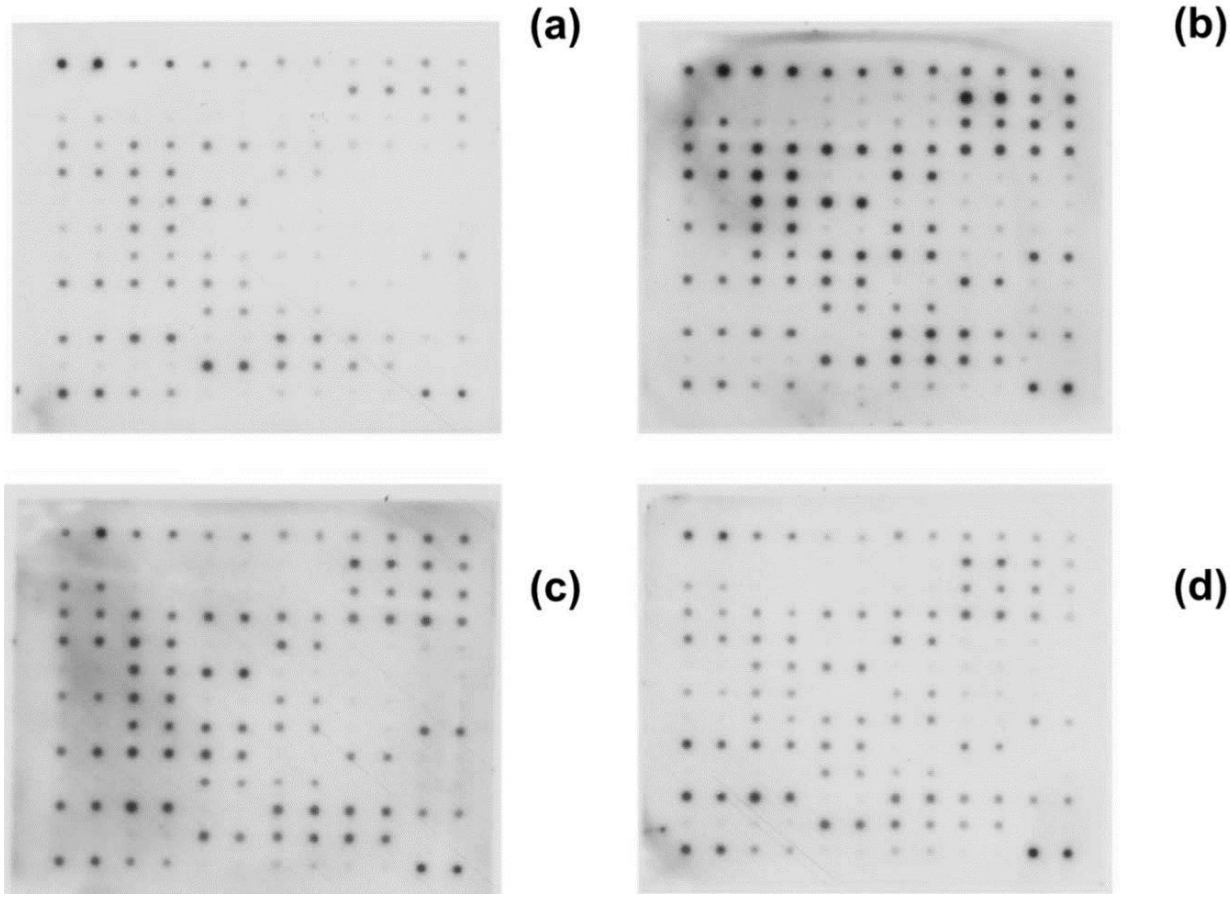


Figure 3.14 Ang1 induces phosphorylation of important RTKs.
CLL cells were stimulated with Ang1 for 30 min (b), 1 h (c), and 12 h (d). The membranes report increases in phosphorylation or phosphorylation de novo. The most striking changes in phosphorylation are observed at 30 min (b) post stimulation. Phosphorylation commences to vanish during the first 2 h (c) and 12 h (d). At 12 h (c) the phosphorylation pattern on the membranes begins to resemble that of the non-stimulated CLL cell sample (a).

Table 3.1 The phosphorylation array map and phosphorylated RTKs.

	A		B		C		D		E		F	
1	POS1	POS1	POS2	POS2	POS3	POS3	ABL1	ABL1	ACK1	ACK1	ALK	ALK
2	NEG	NEG	NEG	NEG	Axl	Axl	Blk	Blk	BMX	BMX	Btk	Btk
3	Csk	Csk	Dtk	Dtk	EGFR	EGFR	EphA1	EphA1	EphA2	EphA2	EphA3	EphA3
4	EphA4	EphA4	EphA5	EphA5	EphA6	EphA6	EphA7	EphA7	EphA8	EphA8	EphB1	EphB1
5	EphB2	EphB2	EphB3	EphB3	EphB4	EphB4	EphB6	EphB6	ErbB2	ErbB2	ErbB3	ErbB3
6	ErbB4	ErbB4	FAK	FAK	FER	FER	FGFR1	FGFR1	FGFR2	FGFR2	FGFR2 (α)	FGFR2 (α)
7	Fgr	Fgr	FRK	FRK	Fyn	Fyn	Hck	Hck	HGFR	HGFR	IGF-IR	IGF-IR
8	Insulin R	Insulin R	Itk	Itk	JAK1	JAK1	JAK2	JAK2	JAK3	JAK3	LCK	LCK
9	LTK	LTK	Lyn	Lyn	MATK	MATK	M-CSFR	M-CSFR	MUSK	MUSK	NGFR	NGFR
10	PDGFR α	PDGFR α	PDGFR ß	PDGFR ß	PYK2	PYK2	RET	RET	ROR1	ROR1	ROR2	ROR2
11	ROS	ROS	RYK	RYK	SCFR	SCFR	SRMS	SRMS	SYK	SYK	Tec	Tec
12	Tie-1	Tie-1	Tie-2	Tie-2	TNK1	TNK1	TRKB	TRKB	TXK	TXK	NEG	NEG
13	Tyk2	Tyk2	TYRO10	TYRO10	VEGFR2	VEGFR2	VEGFR3	VEGFR3	ZAP70	ZAP70	POS4	POS4

POS1, POS2,POS3; possitive controls / NEG; negative controls

Table 3.2 Proteins putatively phosphorylated in CLL cells by Ang1. Names of peptides retrieved from the UniProp database (66) **. Please note that these are not included in the list of abbreviations.**

Protein	Common names	Type
ACK1	Activated CDC42 kinase 1	Non-receptor
ALK	Anaplastic lymphoma kinase or CD246	Receptor
BMX	Cytoplasmic tyrosine-protein kinase BMX	Non receptor
CSK	C-terminal Src Kinase	Non-receptor
BTK	Bruton's tyrosine kinase	Non- receptor
EphA2	Ephrin type-A receptor 2	Receptor
EphA3	Ephrin type-A receptor 3	Receptor
EphA4	Ephrin type-A receptor 4	Receptor
EphA5	Ephrin type-A receptor 5	Receptor
EphA6	Ephrin type-A receptor 6	Receptor
EphA7	Ephrin type-A receptor 7	Receptor
EphA8	Ephrin type-A receptor 8	Receptor
EphB1	Ephrin type-B receptor 4	Receptor
EphB2	Ephrin type-B receptor 5	Receptor
EphB3	Ephrin type-B receptor 6	Receptor
EphB6	Ephrin type-B receptor 7	Receptor
EprB2	Ephrin type-A receptor 8	Receptor
ErbB2	HER2/neu, proto-oncogene Neu, CD340	Receptor
ErbB3	HER3(human epidermal growth factor receptor 3	Receptor
FAK	Focal adhesion kinase 1	Non-receptor
FER	fer (fps/fes related) tyrosine kinase	Non-receptor
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	Non-receptor
FRK	Fyn-related kinase	Non-receptor
HCK	Tyrosine-protein kinase HCK	Non-receptor
Itk	Tyrosine-protein kinase ITK/TSK interleukin-2-inducible T-cell kinase	Non-receptor
JAK1	Janus kinase 1	Non-receptor
JAK2	Janus Kinase2	Non-receptor
LCK	Lymphocyte-specific protein tyrosine kinase	Non-receptor
MATK	Megakaryocyte-associated tyrosine-protein kinase	Non-receptor
MUSK	Muscle, skeletal receptor tyrosine-protein kinase	Receptor
SRMS	src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	Non-receptor
SYK	Spleen tyrosine kinase	Non-receptor
TRKB	Tyrosine receptor kinase B, BDNF/NT-3 growth factors receptor or neurotrophic tyrosine kinase, receptor-2	Receptor
TXK	Tyrosine-protein kinase TXK	Non receptor

Table 3.3 Main known function of the phosphorylated peptides by Ang1. Retrieved from the UniProp database (66).

Protein	Known functions
ACK1	– Cell spreading and migration, cell survival, cell growth and proliferation – Implicated in trafficking and clathrin-mediated endocytosis – Confers metastatic properties on cancer cells and promotes tumour growth by negatively regulating tumour suppressor such as WWOX and positively regulating pro-survival factors such as AKT1 and AR
ALK	– Acts as a receptor for ligands pleiotrophin (PTN) and midkine (MDK), two potent inducers of angiogenesis – Induces anti-apoptotic signalling and regulation of cell proliferation
BMX	– Actin reorganization, cell migration, cell proliferation and survival, cell adhesion, and apoptosis – Adaptive cytoprotection against extracellular stress in different cell systems – May also play a role in the growth and differentiation of hematopoietic cells
BTK	– Regulation of cell apoptosis
CSK	– Regulation of cell growth, differentiation, migration and immune response
EphA2	– Regulates migration, integrin-mediated adhesion, proliferation and differentiation of cells – UV radiation-induced apoptosis – Ligand-independent stimulatory effect on chemotactic cell migration – In angiogenesis, in early hindbrain development and epithelial proliferation and branching morphogenesis during mammary gland development
EphA3	– Regulates cell-cell adhesion, cytoskeletal organization, and cell migration
EphA4	– Modulates cell morphology and integrin-dependent cell adhesion – Plays a role in synaptic plasticity – In angiogenesis playing a role in central nervous system vascular formation
EphA5	– Functions as an axon guidance molecule during development – Plays also a role in synaptic plasticity in adult brain through regulation of synaptogenesis
EphA6	– Not details available
EphA7	– Regulates brain development modulating cell-cell adhesion and repulsion
EphA8	– Regulate integrin-mediated cell adhesion and migration on fibronectin substrate
EphB1	– Axon guidance during development – Angiogenesis, palate development and in inner ear development through regulation of endolymph production

EphB2	– Regulates retinal axon guidance – Regulates chemotaxis, proliferation, and polarity of the hippocampus neural progenitors – Regulate angiogenesis – Cell migration and adhesion
EphB3	– Controls other aspects of development through regulation of cell migration – Includes angiogenesis, palate development, and thymic epithelium development for instance – Regulates migration and adhesion of cells that tubularize the urethra and septate the cloaca
EphB6	– Modulates cell adhesion and migration by exerting both positive and negative effects
ErbB2	– Regulates outgrowth and stabilization of peripheral microtubules (MTs)
ErbB3	– Binds and is activated by neuregulins
FAK	– Regulates cell migration, adhesion, spreading, reorganization of the actin cytoskeleton, cell cycle progression, cell proliferation, and apoptosis – Required for embryonic angiogenesis, normal cardiomyocyte migration and proliferation, and normal heart development – Regulates axon growth and neuronal cell migration, axon branching and synapse formation; required for normal development of the nervous system – Plays a role in osteogenesis and osteoblasts differentiation
FER	– Regulation of the actin cytoskeleton, microtubule assembly, cell adhesion, cell migration, and chemotaxis – Plays a role in leukocyte recruitment and diapedesis in response to bacterial lipopolysaccharide (LPS) – Plays a role in synapse organization, trafficking of synaptic vesicles, the generation of excitatory postsynaptic currents and neuron-neuron synaptic transmission
Fgr	– Regulation of immune responses, including neutrophil, monocyte, macrophage and mast cell functions, cytoskeleton remodelling in response to extracellular stimuli, phagocytosis, cell adhesion, and migration – Regulates actin cytoskeleton reorganization, cell spreading, and adhesion
FRK	– Negatively regulates cell proliferation
HCK	– Found in hematopoietic cells that transmits signals from cell surface receptors and plays an important role in the regulation of innate immune responses, including neutrophil, monocyte, macrophage and mast cell functions, phagocytosis, survival, proliferation, cell adhesion, and migration – During the phagocytic process, mediates mobilization of secretory lysosomes, degranulation, and activation of NADPH oxidase to bring about the respiratory burst – Plays a role in the release of inflammatory molecules – Promotes reorganization of the actin cytoskeleton and actin polymerization
ITK	– Regulation of the adaptive immune response

	– Regulates the development, function, and differentiation of conventional T-cells and nonconventional NKT-cells – Regulates lymphokine production, T-cell proliferation, and differentiation
JAK1	– No function described
JAK2	– Cell growth, development, and differentiation – Mediates essential signalling events in both innate and adaptive immunity
LCK	– Regulates selection and maturation of developing T-cells in the thymus and in the function of mature T-cells – Plays a key role in T-cell antigen receptor (TCR)-linked signal transduction pathways
MATK	– Regulates in the signal transduction of hematopoietic cells – Inhibitory role in the control of T-cell proliferation
Musk	– Modulates the formation and the maintenance of the neuromuscular junction (NMJ), the synapse between the motor neuron and the skeletal muscle – Reorganization of the actin cytoskeleton
Srms	– May be involved in proliferation or differentiation of keratinocytes in the skin
SYK	– Mediates signal transduction downstream of a variety of transmembrane receptors including classical immunoreceptors like the BCR – Regulates innate and adaptive immunity, cell adhesion, osteoclast maturation, platelet activation, and vascular development – Regulates neutrophil degranulation and phagocytosis through activation of the MAPK signalling cascade – Mediates the activation of dendritic cells by cell necrosis stimuli, activation of mast cells, and cell adhesion – Involved in vascular development where it may regulate blood and lymphatic vascular separation – Required for osteoclast development and functioning
TRKB	– Involved in the development and the maturation of the central and the peripheral nervous systems through regulation of neuron survival, proliferation, migration, differentiation, and synapse formation and plasticity – Suppress anoikis, the apoptosis resulting from loss of cell-matrix interactions.
TXLr	– Development, function and differentiation of conventional T-cells and nonconventional NKT-cells

3.15 Angiopoietins differentately modify expression of prognostic markers

Another way to explore the impact of angiopoietins present in the microenvironment over CLL cell pathogenesis is to measure the changes they induce over the expression pattern of prognostic markers. There is no reason whatsoever to believe that angiopoietins have an impact on prognostic marker profile expression. However, since both affect cellular and molecular features of CLL cells, it is plausible to believe that angiopoietins may modulate expression of CDs and additional prognostic marker in CLL cells.

CLL cells were stimulated with 500 µg/ml Ang1 or Ang2 for 12 hours to measure changes in the percentage of relevant CDs on CLL cells. Six patients were taken into consideration. Samples were analysed and ran in a Gallios flow cytometer (Beckman Coulter) and 10,000 events were taken into consideration. The antibodies used were separated into groups and master mixes prepared for flow cytometry as described on Table 2.2. As shown in figure 3.14, in a non-stimulated sample CLL cells can be observed expressing the typical CLL CD-prognostic markers. Similar diagrams were acquired for non-stimulated, Ang1-stimulated, and Ang2-stimulated samples for each patient. The changes in percent of each marker pair is summarized in table. 3.4.

Table 3.4 shows that the changes in CD expression vary tremendously from patient to patient highlighting again the heterogeneity between patients. The instances where a notable change in CD expression is observed have been highlighted in on table 3.2.

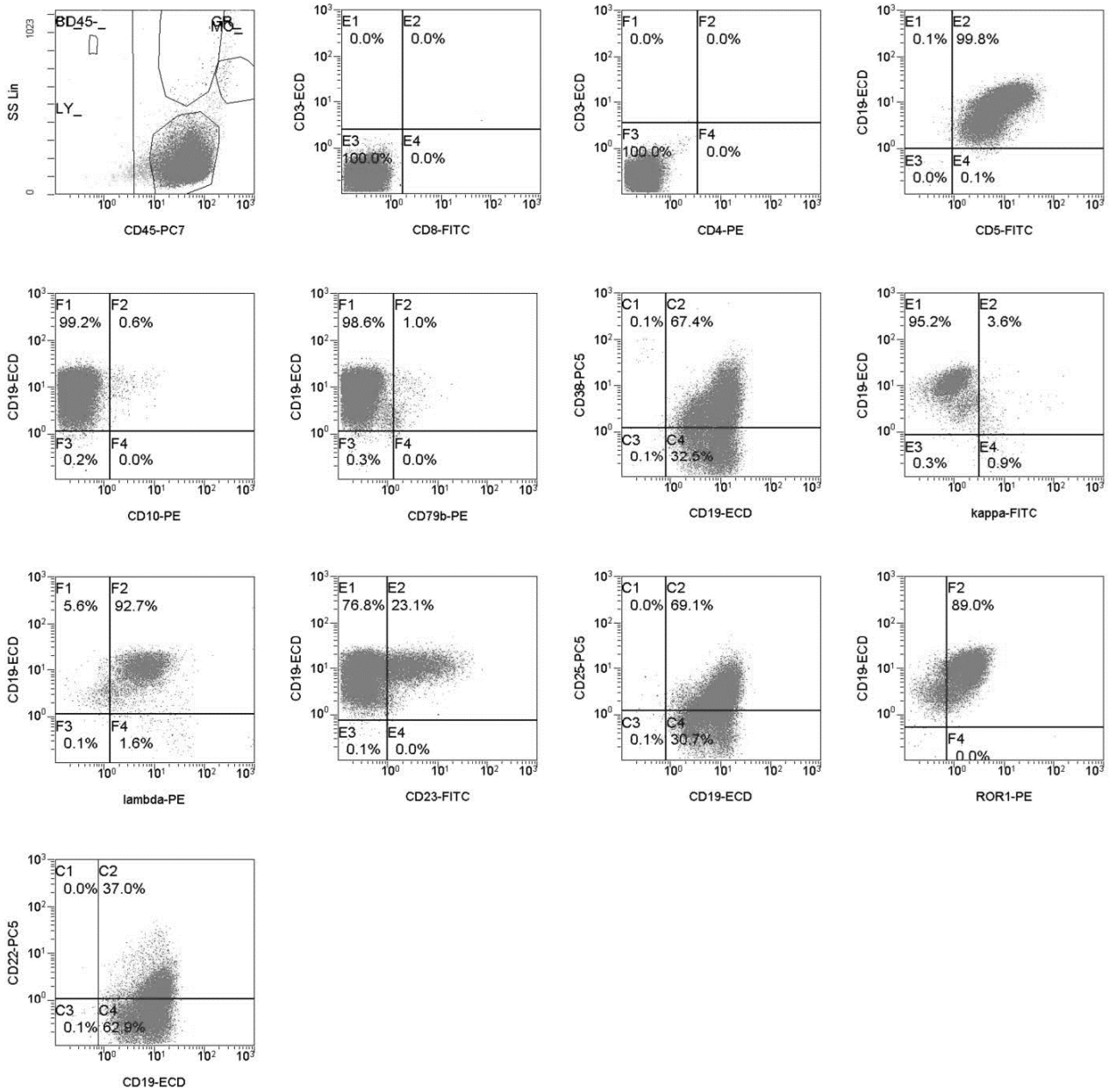


Figure 3.15 Evaluating prognostic markers under angiopoietins exposure. The most relevant surface markers characteristic of CLL cells were analysed by flow cytometry in non-stimulated, Ang1-stimulated, and Ang2-stimulated CLL samples. Cell from six patients were taken into consideration using antibodies mixtures described in table 2.2. Changes surface profiles are summarized for each patient in table 3.4.

Table 3.4 Changes in CLL prognostic markers induced by angiopoietins. Highlighted boxes highlight changes induced by Ang1 and Ang2 compared with untreated (Unt) cells.

	Patient I			
	Markers	**Unt**	**Ang1**	**Ang2**
Quality control	**CD3+CD8+**	**0,0**	**0,0**	**0,0**
	CD3+CD4+	**0,0**	**0,0**	**0,0**
	CD19+CD5+	**99,3**	**99,9**	**99,7**
B-Antigen	**CD19+CD10+**	0,9	1,8	0,4
B-Differentiation	**CD 19+CD79B+**	6,3	7,4	6,5
Activation	**CD19+CD38+**	1,6	2,0	3,2
Immunoglobulins	**CD 19+IgKappa+**	95,6	89,9	87,7
	CD 19+IgLambda+	2,8	2,1	2,4
Activation	**CD19+CD23**	2,0	2,0	1,5
Therapy targets	**CD19+CD25**	59,3	61,6	76,6
	CD19+ROR1	83,0	80,0	88,4
B-Differentiation	**CD19+CD22**	82,6	85,2	92,1

Patient II			
Markers	**Unt**	**Ang1**	**Ang2**
CD3+CD8+	**0,0**	**0,0**	**0,0**
CD3+CD4+	**1,1**	**0,0**	**0,1**
CD19+CD5+	**100,0**	**100,0**	**99,6**
CD19+CD10+	0,5	0,0	0,6
CD 19+CD79B+	3,4	0,5	1,5
CD19+CD38+	78,1	89,1	88,9
CD 19+IgKappa+	85,1	52,9	83,1
CD 19+IgLambda+	2,2	0,5	1,9
CD19+CD23	35,0	18,9	12,4
CD19+CD25	33,5	20,7	36,6
CD19+ROR1	75,4	81,7	90,7
CD19+CD22	82,6	82,0	80,2

	Patient III			
	Markers	**Unt**	**Ang1**	**Ang2**
Quality control	**CD3+CD8+**	**0,0**	**0,0**	**0,0**
	CD3+CD4+	**0,0**	**0,0**	**0,0**
	CD19+CD5+	**97,1**	**98,6**	**98,2**
B-Antigen	**CD19+CD10+**	0,9	0,6	1,3
B-Differentiation	**CD 19+CD79B+**	13,3	11,6	12,5
Activation	**CD19+CD38+**	3,5	4,6	3,6
Immunoglobulins	**CD 19+IgKappa+**	4,9	2,9	3,9
	CD 19+IgLambda+	96,8	96,3	93,7
Activation	**CD19+CD23**	3,7	3,9	4,2
Therapy targets	**CD19+CD25**	25,1	24,0	16,9
	CD19+ROR1	92,0	96,2	93,1
B-Differentiation	**CD19+CD22**	46,0	48,7	39,7

Patient IV			
Markers	**Unt**	**Ang1**	**Ang2**
CD3+CD8+	**0,0**	**0,0**	**0,0**
CD3+CD4+	**0,0**	**0,0**	**0,0**
CD19+CD5+	**98,1**	**97,6**	**97,7**
CD19+CD10+	0,7	0,6	0,6
CD 19+CD79B+	12,3	9,6	10,2
CD19+CD38+	35,0	35,1	36,6
CD 19+IgKappa+	76,9	75,3	75,0
CD 19+IgLambda+	2,2	1,6	1,9
CD19+CD23	19,9	18,3	21,3
CD19+CD25	65,9	64,7	66,9
CD19+ROR1	85,9	94,0	97,1
CD19+CD22	26,2	31,4	32,1

	Patient V			
	Markers	**Unt**	**Ang1**	**Ang2**
Quality control	**CD3+CD8+**	**0,0**	**0,0**	**0,0**
	CD3+CD4+	**0,0**	**0,1**	**0,0**
	CD19+CD5+	**94,2**	**92,5**	**91,3**
B-Antigen	**CD19+CD10+**	0,4	0,5	0,9
B-Differentiation	**CD 19+CD79B+**	28,2	22,9	24,6
Activation	**CD19+CD38+**	3,5	6,2	4,7
Immunoglobulins	**CD 19+IgKappa+**	2,3	1,9	18,5
	CD 19+IgLambda+	96,2	95,6	95,9
Activation	**CD19+CD23**	14,1	15,1	13,6
Therapy targets	**CD19+CD25**	82,1	84,3	79,4
	CD19+ROR1	95,4	94,6	94,3
B-Differentiation	**CD19+CD22**	57,3	55,0	53,9

Patient VI			
Markers	**Unt**	**Ang1**	**Ang2**
CD3+CD8+	**0,0**	**0,0**	**0,0**
CD3+CD4+	**0,0**	**0,0**	**0,0**
CD19+CD5+	**99,8**	**99,6**	**99,0**
CD19+CD10+	0,6	0,6	0,3
CD 19+CD79B+	1,0	1,1	0,7
CD19+CD38+	67,4	67,7	66,1
CD 19+IgKappa+	3,8	3,7	4,4
CD 19+IgLambda+	92,7	95,5	92,2
CD19+CD23	23,1	20,5	17,7
CD19+CD25	69,1	68,8	70,8
CD19+ROR1	89,0	89,3	81,3
CD19+CD22	37,0	38,3	39,7

4. DISCUSSION

4.1 Researching Ang-Tie2 pathway and its components on the CLL context

The detection of high levels of bFGF and VEGF in the urine of CLL patients correlating with Rai staging and the increased microvessel density in BM biopsy trephine sections from CLL patients (26) strongly suggest that CLL pathogenesis is not foreign to angiogenesis. Treating CLL patients with lenalidomine results in the peculiar downregulation of serum levels of both bFGF and VEGF (67). Also, CLL researchers report the concomitant overexpression of Ang2 and pro-angiogenic VEGF in CLL patients (31). Both VEGF and angiopoietins are important angiogenic partners (29) that together induce recruitment of inflammatory cells such as neutrophils, macrophages, T- and B-cells, and ECs (30). By now it is clear that Ang2 overexpression negatively impacts TTFT, disease aggressiveness, and rapid disease progression (33). In addition, the low methylation pattern on the Ang2 promoter also correlate with poor prognosis, shorter TTFT, and shorter survival of CLL patients; lower methylation translates into higher expression of Ang2 (34).

In other malignancies, such as in angiosarcomas, the aberrant expression in components of the Ang-Tie2 pathway also associates with non-vasoformative and aggressive lesions (68). For example, in patients of colorectal cancer, higher levels of Ang2 and soluble Tie2 (sTie2) are found in plasma, and tumour tissues strongly secrete Ang2 and VEGF (Soluble Tie1 and Tie2 in plasma result from cleavage of the ectodomain of both receptors and not by the secretion of the whole proteins into the microenvironment). Additionally, in patients with more advanced disease, Ang2 and VEGF were concomitantly highly expressed (69).

Similarly as in CLL, in AML patients neo-angiogenesis in the BM appears to influence the course of the malignancy (70). Recent reports describe the association

between short survival and elevated Ang2 in AML patients, suggesting the use of Ang2 levels as a prognostic marker (71). AML researchers revealed the relevance of Ang1 is regulating cell cycle progression and showed that Ang2 expression also correlated with low Ang1 mRNA levels (72). Leukaemia cells and primary AML cells with high ecotropic viral integration site-1 (EVI1) expression show a high expression of Ang1 and upon EVL1 silencing there is a concomitant downregulation of Ang1; EVL1 associates with poor prognosis in AML patients (70). Knockdown of Ang1 induces cell cycle progression in EVI1+ leukaemia cells, highlighting the role of the Ang-Tie2 pathway in regulating progression of leukemic cell proliferation by keeping in check the G0/G1 phase (70). Altogether, this suggests a role of the components of the Ang-Tie2 pathway in supporting development of hematopoietic malignancies. However, there is plenty of biochemical evidence to satisfactorily explain the involvement the Ang-Tie2 pathway in determining the fate of CLL cells.

Regardless of its status in CLL, the biochemistry of the Ang-Tie2 pathway was unclear for many years and the putative molecular mechanics were the product of mere speculations. Still by 2010 molecular biology failed to accurately clarify the mechanisms by which Ang1 and Ang2 exercise their radically different biological roles in angiogenesis (36). Even though Ang1 and Ang2 share a high degree of homology, it is evident that they trigger different cellular and molecular events (40). In a series of elegant biochemical experiments Yu et al. unveiled the proteins regions (amino acid residues) responsible for defining the specific biological activity for the agonist Ang1 and for the antagonist Ang2 (40). Furthermore, they demonstrated that the Ang1-Tie2 and Ang2-Tie2 interactions show only a subtle difference in conformation without any radical structural changes or binding affinity. Moreover, only Ang1 and not Ang2 has a role in dissuading the inhibitory Tie1-Tie2 interaction; only binding of Ang1 to Tie2 induces strong Tie2 superclustering translating into phosphorylation of the endodomains activating signal transduction events (40).

Together, the role of ligands properly defined, the description of a Tie1-Tie2 inhibitory interaction, and the identification of VE-PTP as negative regulator of Tie2, is possible to elucidate the function of the Ang-Tie2 pathway in the CLL microenvironment context. This clarification is necessary because it was not understood how angiopoietins regulate CLL cell survival, since CLL cells from the peripheral blood lack Tie2 receptor on their surface. In addition, the potential of

angiopoietins in regulating the expression of Ang1, Ang2, Tie1, Tie2, and VE-PTP at the mRNA level in a feedback manner has not been addressed. Also, the implication of angiopoietins as regulators of migration, metabolic fitness, and activation of kinases in CLL has to date not been clarified. This discussion aims to shed light into the close relationship existing between CLL cells and angiopoietins present in the microenvironment focusing on how angiopoietins modulate the fate of CLL cells.

4.2 Components of the Ang-Tie2 pathway in CLL cells

In order to understand how a molecular pathway functions in any specific cellular model, it is imperative to know which of its components (e.g. ligands, receptors, co-receptors, downstream effectors, and transcription factors) are expressed in a given system, and whether the pathway is activated autocrinally and/or paracrinally. There is a knowledge gap in CLL research regarding the function of the Ang-Tie2 pathway in dictating the fate of CLL cells. Previously Kay et al. analysed the expression of some angiogenic factors and receptors in CLL patients, detecting expression of Tie1 and Ang1 and failing to detect Tie2 transcripts (25). Furthermore, Aguayo et al. described Tie1 expression (73) in CLL samples and correlating its expression levels with CLL disease stage. Whilst extensive work has described Ang2 expression in CLL and its correlation with disease progression (32,34,63), there are not tests to determine the parallel expression of all components of the Ang-Tie2 pathway, namely Ang1, Ang2, Tie1, Tie2, and VE-PTP, in single cell batches from CLL patients. Figure 4.1 represents the current known expression status of the Ang-Tie2 pathway components in CLL cells, as well as additional key secretors of Ang1 and Ang2 ligands.

Figure 3.5 shows the qualitative PCR analysis of five CLL cell samples that express Ang1, Ang2, and Tie1 mRNA; the healthy PBMC population express these factors as well. However, all CLL cell samples fail to express Tie2 and VE-PTP and Tie2 are only found in the healthy PBMCs population. Detection of Tie2 in PBMCs is of particular interest, because it may represent the Tie2 expressed by Tie2-expressing monocytes/macrophages (TEMs), which comprise 2-7% of the total PBMCs population of healthy patients (74). It is still unknown whether the Ang-Tie2 pathway is active in TEMs, however, VE-PTP it is not detected in the PBMCs population. Besides presenting high Ang2 plasma levels, CLL patients also

display increased TEMs counts in peripheral blood, strongly associating with a high-risk cytogenetics status, such as 17p deletion (33).

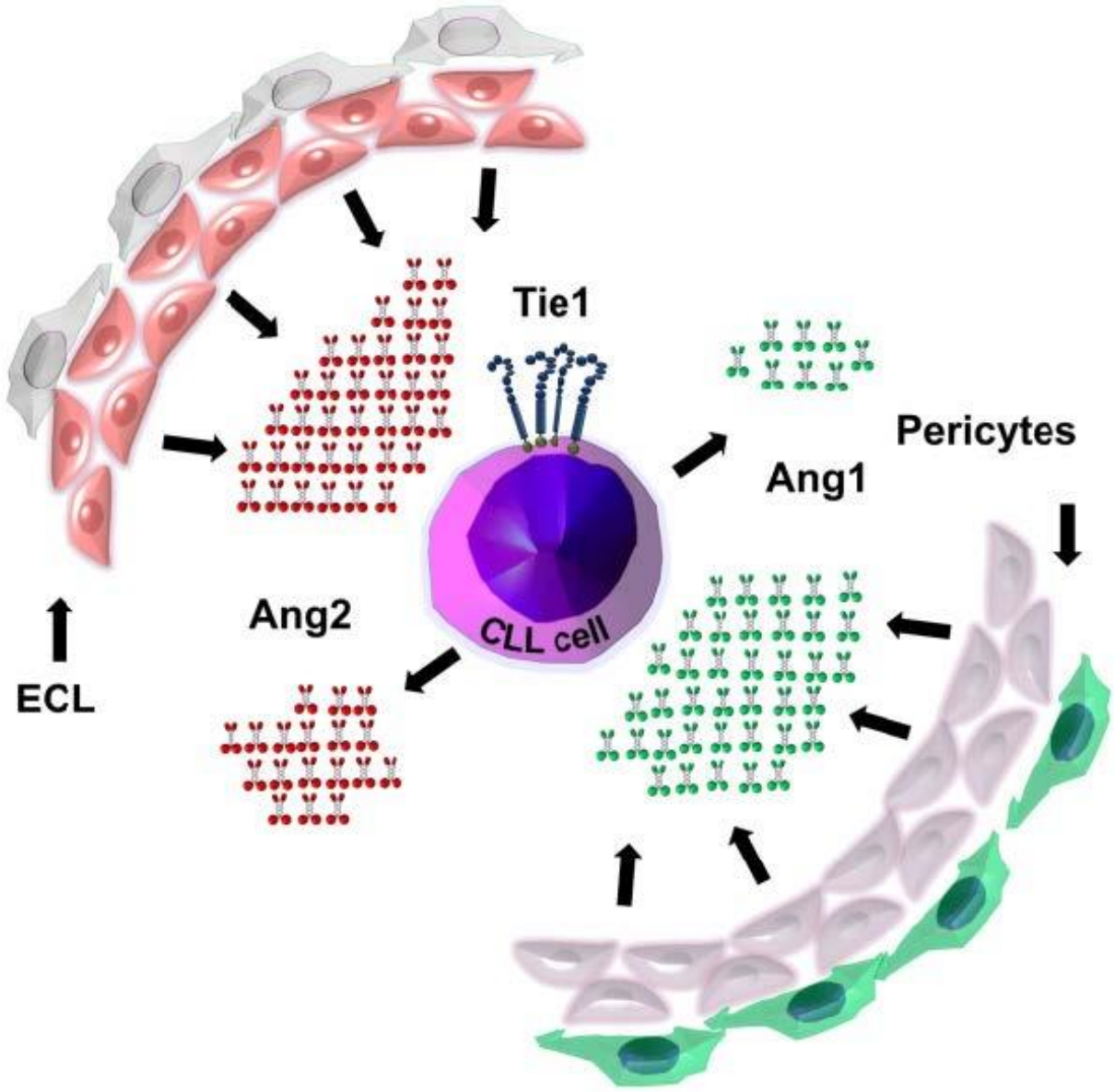


Figure 4.1 CLL cells, the microenvironment, and angiopoietin secretion. CLL cells express Tie1 receptor on their surface, secrete Ang2 abundantly, and secrete Ang1 at basal levels. Most of the angiopoietins that CLL cells will encounter in their microenvironment derive from accessory cells. The pericytes covering the EC layer (ECL) are the main secretors of Ang1 and the ECs are the main secretors of Ang2. Unlike ECs, CLL cells from peripheral blood lack Tie2 and VE-PTP.

Apparently, exposure of circulating TEMs to Ang2 instigates a broader, tumour promoting phenotype of TEMs, an effect supported by a vascularisation increase in tumour models (75). Reduction of TEMs associated with reduced angiogenesis of

tumours probably are due to a reduction of angiogenesis (76). When Ang2 comes in contact with TEMs, these supress T-cell proliferation and support tumour progression by undermining antitumour activity (76). Thus, the importance of TEMs lays on the ability to drive tumour development and tumour-related angiogenesis. Those monocytes lacking Tie2 have a diminished secretion of pro-angiogenic factors, whilst TEMs express higher levels of angiogenic inducers such as VEGF (75). Since qualitative PCR experiments did not detect VE-PTP in the PBMCs population, it suggests that no ECs in population; ECs are positive for both VE-PTP and Tie2.

4.3 Repercussion of the low circulating levels of Ang1 in CLL patients

Whilst qualitative PCR indicates the expression of mRNA transcripts of Ang1, Ang2, and Tie1, this gives no information on the secretion behaviour of the ligands. Are Ang1 and Ang2 secreted from the cells into the microenvironment by CLL cells at all? And if so, is their secretion level up- or downregulated when compared to those from healthy samples? To understand whether angiopoietin levels have any impact in disease, it is necessary to evaluate the expression levels present in plasma from healthy and CLL patients. Culture of healthy PBMCS and CLL cells may also determine to which extend these dominant cell populations contribute to the levels of angiopoietins detected in plasma. Whether there is a remarkable over- or underexpression of angiopoietins in CLL patients, this information will give some insight into the possible source of aberrant angiopoietin expression.

Agonist Ang1 has an anti-inflammatory role, promotes pericyte-dependent vessel integrity, and supports a Tie2-constitutively activated state (38). Regardless, the role of Ang1 in CLL pathophysiology is unclear and has not been associated with Binet/Rai staging, PFS, or TTFT. Nevertheless, in ovarian cancer cell trials, combined values of circulating Ang1 and Tie2 in peripheral blood predict improvement in PFS in bevacizumab-treated patients (77). Antagonist Ang2 has been investigated in the CLL context and is clear that Ang2 upregulation, high plasma levels, and methylation patterns strongly correlate with disease stage and progression; the reported average level of Ang2 is 2459 pg/mL (32,34,63). As shown in figure 3.3, both plasma and supernatants from cell culture show drastic differences in the levels of Ang1 secretion between healthy and CLL samples. First, plasma from CLL patients presents significantly lower levels of Ang1 (2336 pg/ml) when compared to plasma Ang1 levels

(4414 pg/ml) from healthy patients. After culturing CLL cells and PBMCs for 24 h, it is evident that CLL cells secrete far lower levels of Ang1 (67 pg/ml) into the microenvironment when compared to the healthy PBMCs secretion levels (632 pg/ml).

The low levels of Ang1 indicate the inability of CLL cells to secrete optimal levels of Ang1 into the microenvironment. Low secretion of Ang1 could impact the microenvironment, especially those cells that require Ang1 for activation of the Ang-Tie2 pathway. Since ECs rely strongly on Ang-Tie2 to maintain stability, it could be inferred that this reduction of Ang1 has deleterious effects on permeability of those tissues composed mainly of ECs such as vessel, capillaries, and HEVs. Alternatively, it is possible to suggest that the lower levels detected in plasma from CLL patients could derive from pericytes with a compromised secretion of Ang1. Nonetheless, the high levels of Ang2 and the low levels of Ang1 detected in plasma does not say much about how these factors impact survival of CLL cells.

4.4 Angiopoietins as regulators of survival and apoptosis of CLL cells

Whilst any given ligand may induce signal transduction events and a plethora of molecular events, being capable to regulate cell survival will certainly highlight its relevance in research and treatment. CLL cells may encounter changing levels of Ang1 and Ang2 through the different compartments they visit, which are the BM, LNs, spleen, and peripheral blood. Today CLL cells and the Ang-Tie2 pathway appear unrelated, mainly because CLL cells isolated from peripheral blood lack Tie2 receptor. Whilst it seems unlikely that ligands Ang1 and Ang2 regulate survival of CLL cells, such an assumption can be challenged empirically. A strategy to mimic the microenvironmental availability of Ang1 and Ang2 is to expose CLL cells to the recombinant forms of these proteins and perform survival assays to ensure that these ligands confer survival advantage, induce pro-apoptotic activity, or neither to CLL cells in culture.

CLL cells were exposed for one and four days to increasing amounts of Ang1 (Fig. 3.4, a) and Ang2 (Fig. 3.4, b), followed by flow cytometry analysis to reveal any relevant changes in survival patterns. After the first day of stimulation, Ang1 had no effect on survival, whilst Ang2 showed a significant reduction in survival at the highest concentration of 500 ng/ml. On day four, however, the survival assay revealed that Ang1 is capable of conferring significant survival advantage to CLL cells in a dose-

dependent manner. Ang2 on the other hand shows a significant decrease in survival. Because these effects are almost unperceived at day one, possibly the initial and brief contact of CLL cells with angiopoietins is sufficient to activate the downstream events that support a long-term survival effect. It has been reported that Ang2 has a relative half-life of approximately 18 h (45). However, even though two publications claim that the half-life of Ang1 is short (78,79), neither these or the references they cite provide conclusive biochemical evidence that could corroborate the half-life of Ang1. Whilst the half-life of Ang1 remains unknown, it could be inferred that it is close to that of Ang2 due to the high sequence homology existing between these two peptides. If angiopoietins are degraded within hours, CLL cells must then quickly recognize their presence in the microenvironment for the activation of pro-survival (By Ang1) and pro-apoptotic (By Ang2) signal transduction events before Ang1 and Ang2 lose their activation capability by undergoing proteic degradation.

Because CLL cells themselves secrete both Ang1 and Ang2 at basal levels, it is valid to question the ability of CLL-derived angiopoietins to regulate the survival of CLL cells in an autocrine manner. The anti-angiogenic cancer drug trebananib, formerly AMG 386, specifically and strongly binds Ang1 and Ang2 preventing their interaction with target receptors (80). In solid tumour patients, trebananib monotherapy was well tolerated, showed antitumour activity, and impacted tumour vascularity (81,82), most likely by inhibiting and reversing EC proliferation (82). In this work, trebananib was employed as a blocker of CLL-secreted angiopoietins in similar increasing concentrations as those of Ang1 and Ang2 (Fig. 3.4, c). Whilst there are not changes in survival after the first day of stimulation, it is evident that after four days trebananib proves detrimental to the CLL cell survival. This strongly indicates that CLL-derived angiopoietins are capable of regulating CLL cell survival in an autocrine manner, possibly by interacting with target receptors others than Tie2.

4.5 CLL cells and the expression of the components of the Ang-Tie2 pathway

If angiopoietins differentially regulate survival, are they also capable of regulating the expression of mRNA levels of those components from the Ang-Tie2 pathway expressed by CLL cells? To answer this question, CLL cells were stimulated with recombinant Ang1 and Ang2 only for 1 h; transcription can be a short-lived

phenomenon and changes in mRNA expression may change and return to basal levels rather quickly. Collecting samples after 1 h ensures that CLL cells have interacted with angiopoietins whilst angiopoietins are still viable in the microenvironment. As explained above, only the relative 18 h half-life of Ang2 is supported with biochemical data, whilst the half-life of Ang1 is unknown (45,78,79).

When assessing the relative changes of mRNA of Ang1, Ang2, and Tie1 after exposure of CLL cells to recombinant Ang1 and Ang2, it was observed that Ang1 and Ang2 differentially modulate the mRNA expression of Ang1, Ang2, and Tie2 (Fig. 3.6). There is no data suggesting that Ang1 or Ang2 are capable of regulating the components of the Ang-Tie2 system through a feedback inhibition loop. If this is the case, how do CLL cells recognize Ang1 and Ang2 and in turn regulate mRNA transcription? Whilst this work does not offer congruent probe, it is possible to infer that alternative receptors on CLL cells recognize Ang1 and Ang2 either for the purpose of feedback inhibition, or that possibly Tie1 receptor participates in the feedback inhibition mechanism. The role of Tie1 has been defined as that of an inhibitor of Tie2 phosphorylation, but whether it also serves as an expression modulator of Ang1, Ang2, and Tie2 at the mRNA level is to be determined.

Changes in Ang1, Ang2, and Tie1 mRNA expression in CLL cells post-angiopoietin stimulation prompted to ask whether angiopoietins could possible induce a transient mRNA expression of either Tie2 or VE-PTP transcripts. Surprisingly, qualitative PCR showed that upon 30 min of Ang2 stimulation there is transient and concomitant expression of Tie2 and VE-PTP mRNA (Fig. 3.7), which is no longer detected within hours after stimulation. This is the first time that expression of Tie2 and VE-PTP is reported in the CLL context. Observing a transient expression of both Tie2 and VE-PTP upon Ang2 exposure in CLL cells is intriguing. First, this transient expression could be present in only a small subpopulation of CLL cells, inducing a transiently active Ang-Tie2 pathway only in given few as a strategy to promote proliferation and evasion of apoptosis. Nevertheless, agonist Ang1, which promotes survival advantage of CLL cells in the long term, fails to induce the transient expression of Tie2 and VE-PTP. Second, this observation supports the idea that angiopoietins regulate expression of components of the Ang-Tie2 pathway through a feedback inhibition loop. Third, it suggests that CLL cells are capable of having a transient

“complete” Ang-Tie2 system; whether there are additional components to the Ang-Tie2 system is a subject of permanent biochemical research and must be determined.

Moreover, it was possible to detect expression of the VE-PTP peptide employing label-free MS (Fig. 3.12). The peculiarity of this observation resides in that VE-PTP was detected only 1 h post-Ang2 stimulation and by 12 h the band was no longer observed, suggesting a fast degradation of the peptide. MS showed that only background peptides (e.g. keratins) were presents in control, Ang1-stimulated (1 h, 12 h), and Ang2-stimulated (12 h) bands. VE-PTP binding to Tie2 prevents downstream signalling events and the disassociation of VE-PTP from Tie2 negatively affects EC proliferation and compromises vascular remodelling (50). VE-PTP is a novel and relevant component of the Ang-Tie2 pathway and small-molecule inhibitors of VE-PTP catalytic activity activate Tie2, enhancing Ang1-induced Tie2 activation and inducing phosphorylation of signalling molecules downstream of Tie2, including AKT, eNOS, and ERK (83). This emphasises the important function of VE-PTP in balancing Tie2 activity (50) and hence its relevance in tumour development.

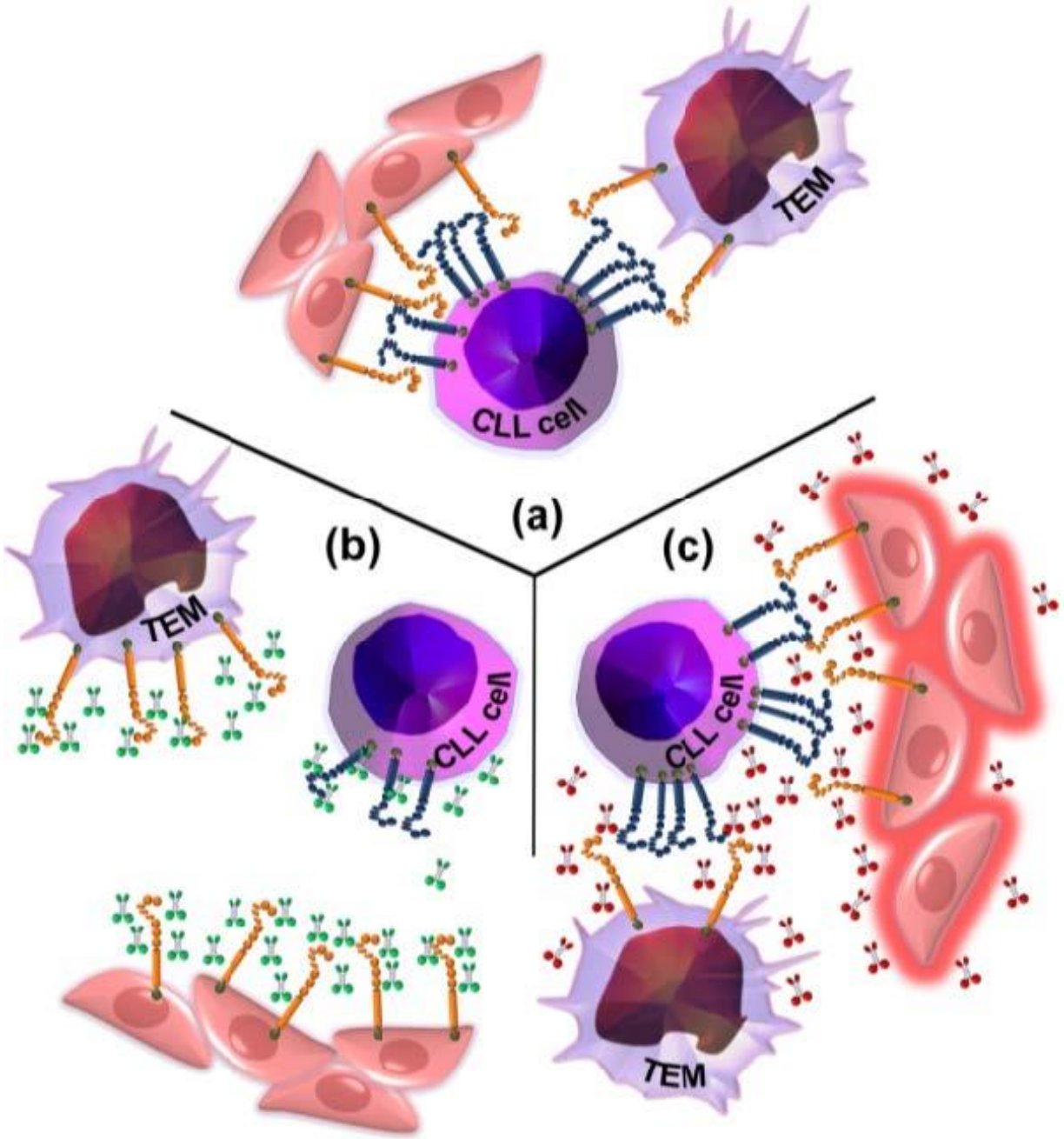


Figure 4.2 Interaction of Tie1-expressing CLL cells with ECs and TEMs. (a) CLL cells adhere to Tie2 positive cells such as endothelial cells (Pink) and Tie2-expressing macrophages/monocytes (TEMs), creating a Tie1-Tie2 inhibitory complex comprised of two different cell entities. (b) When encountering high levels of agonist Ang1 (Green), the inhibitor Tie1-Tie2 complex in dissuaded resulting in activation Ang-Tie2 signal transduction in Tie2+ cells and increased CLL cell survival. (b) However, when high levels of Ang2 (red) are present in the microenvironment the Ang-Tie2 inhibitory complex remains stable, Ang2 binds to the active site of Tie2 preventing Ang1 binding and compromising the constitutively active Ang-Tie2 activity on ECs, which induces instability of the EC layer affecting permeability/perfusion.

4.6 Expression of Tie1 receptor on CLL cells

The Tie1 receptor is essential in angiogenesis processes during embryogenesis and development and is mainly expressed by ECs and haematopoietic lineage cells (84). Expression of Tie1 mRNA by CLL cells becomes a puzzling issue. Why would CLL cells only express the co-receptor of Tie1? Is it possible to infer that Tie1-CLL cells interact with Tie2-expressing cells present in the microenvironment, such as ECs or TEMs, to create the inhibitor Tie1-Tie2 complex? Interaction of Tie1 and Tie2 through the ectodomain is and blocks Tie2-dependent (39). Even though RT-PCR results shows that Ang1 and Ang2 regulate mRNA expression of Tie1, this observation may not necessary correlate with expression of Tie1 receptors on CLL cells. Upon exposure to angiopoietins, flow cytometry analysis shows that there is a clear increase in Tie1 expression on the surface of CLL cells (Fig. 3.8). Whilst Ang1 induces a relative increase of expression of 7.7%, Ang2 induces larger increase of 12.5%. In CLL patients Tie1 expression correlates with Rai stages and becomes a predictor of an aggressive disease course and a marker of shorter survival (73).

Since the Tie1-Tie2 association has an inhibitory character (39), we could infer that Tie1-positive CLL cells interact and associate with Tie2 expressing cells in the microenvironment such as EC cells or TEMs, suggesting a novel paracrinal strategy of Ang-Tie2 pathway regulation involving two cell entities (Fig. 4.2, a). The direct interaction of CLL-Tie1-possitive with Tie2-positive cells in the microenvironment could represent a putative strategy for the regulation of Ang-Tie2 signalling, since interaction of Tie-Tie2 is inhibitory and only Ang1 is capable of inducing its disassociation. Figure 4.2 illustrates the possible binding behaviour of Tie1-expressing CLL cells in the presence of Tie2 positive cells such as TEMs and ECs. It seems that Ang2 induces a homing-like effect on TEMs (74) and Ang1 stimulation triggers aggregation of Tie2 expressing cells by Tie2-dependent trans-association at cell-cell contacts in a bridge-like manner (85). Therefore, the Tie1-Tie2 binding suggested in figure 4.2 (a) could represent trans-association of Tie2 positive cells from the microenvironment with Tie1 positive CLL cells. This interaction is possibly regulated differently by Ang1 (Fig. 4.2, b) and Ang2 (Fig. 4.2, c).la

4.7 Chemotaxis and transendothelial-like migration of CLL cells

Besides regulating growth, cell division, pro-survival, and pro-apoptotic processes, signal transduction events also activate the mechanism of cellular mechanobiology that lead to cell reshaping and movement by undergoing cytoskeleton remodelling in order to deform, reshape, and migrate (86). Changes in mechanical properties trigger biochemical signals activated by molecules and/or proteins (e.g. ligands, cytokines, chemokines) and by physical contact with cells and tissues comprising the microenvironments (86). The putative model of the Ang-Tie2 pathway in figure 1.11 shows that one of the downstream effects of Tie2 activation is the proliferation and migration of ECs, which have a constitutively activated Ang-Tie2 pathway. After determining that angiopoietins regulate gene expression (Ang1, Ang2, Tie1, Tie2, and VE-PTP) and survival of CLL cells, it became of interest to determine whether angiopoietins are capable of inducing chemotaxis and transendothelial-like migration of CLL cells.

The diameter of CLL cells ranges from 6-to-8 μm and therefore a chemotaxis assay is straightforward and employs a membrane with pores of 5 μm/diameter to prevent simple cell passage. As show in figure 3.8, supplementation with ligand Ang1 resulted in a remarkable and significant transport of CLL cells into the lower chamber. Nevertheless, Ang2 did not show a significant effect on migration. Also the concomitant utilization of trebananib and Ang1 abrogated significantly the transport induced by Ang1. However, could this effect be attributed to simple chemotaxis? Or is Ang1 capable of inducing transendothelial-like migration of CLL cells? To tackle this issue, a membrane with pores of 3 μm/diameter was employed; only cells reshaping themselves could be capable of traversing this membrane. Here, ligand Ang1 was capable of inducing the significant transport of CLL cells into to the lower chamber and here trebananib similarly abrogates the migration when employed concomitantly with Ang1 (Fig. 3.8, b). Ang2 on the other hand, showed not significant induction of CLL transport.

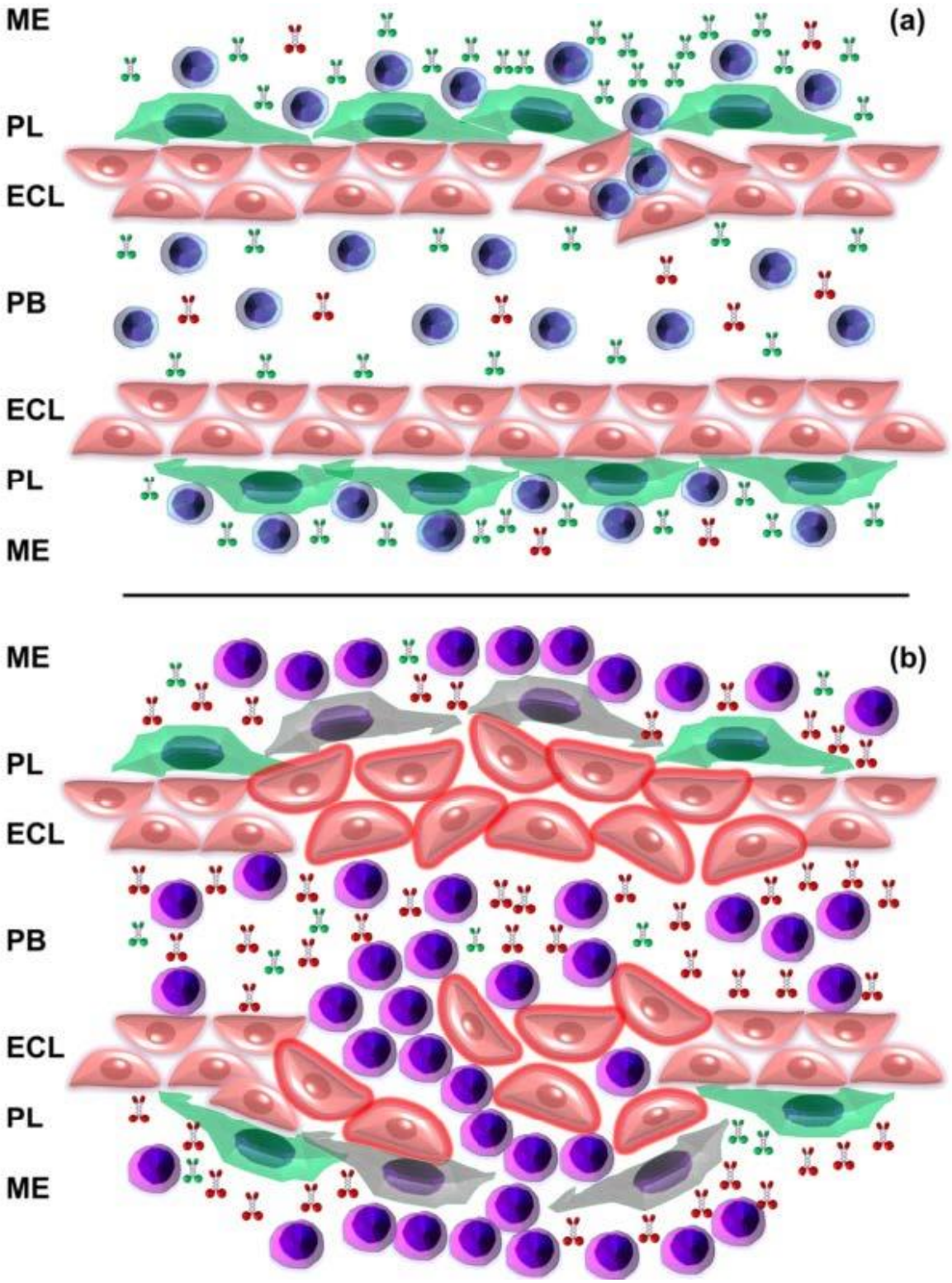


Figure 4.3 Angiopoietins and lymphocyte circulation in CLL.

Healthy B-cells around the pericyte layer (PL) encounter Ang1 (Green) and CLL cells from peripheral blood (PB) and compartments migrate towards the rich Ang1 PL layer. In CLL (b), Ang1 is drastically downregulated, EC layer (ECL) compromised, pericytes fail to maintain a stable ECL, and CLL cells easily recirculate between peripheral blood (PB) and the microenvironment/niche. The high Ang2 (Red) levels will prevent binding of Ang1 to Tie2-posstive cells. CLL will migrate to areas of high Ang1 expression, such as the BM. Whilst passage may be facilitated due to the compromised ECL, CLL cells still must reshape themselves in order to transverse ECL: transendothelial migration. The ECL is permeable, but ECs are not fully disassociated acquiring a "leaky" state.

The strong induction of chemotaxis and transendothelial-like migration by Ang1 indicates that CLL cells are likely to be attracted by cells and towards tissues that express this factor (Fig. 4.3). The effect is also Tie2-independent, since CLL cells lack Tie2 receptor, and it is only Ang2 which induces the transient expression of Tie2 on CLL cells. The synthesis of both Ang1 and Ang2 has been evaluated in different microenvironmental compartments. For example, the synthesis of Ang1 is expressed in different adult tissues and cells such as mural cells, fibroblasts, and non-vascular normal/tumour cells. Ligand Ang2 is synthesized mainly in ECs, which are capable of storing Ang2 in granules denominated Weibel-Palade bodies; cell release Ang2 under stress situation, such as hypoxia (36). Therefore, varying concentrations of angiopoietins in the different microenvironmental compartments will dictate gene expression, survival, and migration patterns of CLL cells. Figure 4.3 shows a model of stability of the ECL in healthy and CLL scenarios that illustrates the migration of healthy B-cell and CLL cells, as well as the instability induced by aberrant angiopoietin secretion.

4.8 Modulation of the CLL microenvironment by angiopoietins

Undoubtedly the CLL microenvironment is essential in CLL pathophysiology, since malignant B-cells fail to undergo timely apoptosis and efficient immunological elimination, resulting in their excessively accumulation in BM, LNs, peripheral blood, and spleen (Fig. 4.3, b). Models of the CLL microenvironment indicate that BM, LN, and spleen are also putative centres of leukemic cell proliferation. CLL cells in these proliferation centres, also denominated psedufollicles or niches, allegedly recirculate between peripheral blood and tissue compartments, dynamically interacting with healthy lymphocytes, accessory cells (87), surrounding tissues, and of course other CLL cells. Whilst the involvement of angiopoietins in lymphangiogenesis is not completely understood, evidence shows that both angiopoietins are essential in formation and development of lymphatic vessels. Angiopoietin overexpression induces lymphatic angiogenesis in skin wound model, in particular Ang1 overexpression, promoting angiogenesis is mouse cornea and skin models (36).

Niches are also local tissue microenvironments supporting haematopoiesis and in BM, HSCs are localized to the niche which has a perivascular localization, adjacent to blood vessels, and located in close proximity to trabecular bone; in response to

haematopoietic stress the niche can shift to extramedullary sites (16). The HSCs are often observed in transit or migrating through vascular barriers (16) supporting the idea that CLL cells may similarly recirculate (Fig. 4.3, b) and transiently reach a perivascular position. This would in return support a fast and efficient exchange of nutrients of the tumour cells with blood capillaries without inducing development of new vessels, a cellular behaviour denominated tumour co-option (88).

The microenvironment also provides mechanical information to the cells, resulting in additional signal transduction that also influences growth, motility, and differentiation (89). Ang1 and Ang2 modulate ECs stability (and leakiness) that could lead to an aberrant expression of angiopoietins resulting in changes of stiffness of different components of the microenvironment. Furthermore, it is possible to infer that accessory cells in the CLL microenvironment, which normally secrete Ang1 and maintain Ang-Tie2 signalling, have lost their Ang1 secretion ability. This situation could alter stiffness in the microenvironment, altering cell function, stem cell differentiation, and tissue homeostasis (89). Changes in the rigidity of the microenvironment possibly contribute to disease development in cancer and fibrosis; stiffness increases close to 10-fold in cancer tissues, also correlating with cancer cell survival and proliferation (89).

The first evidence of Ang1 and Ang2 expression in BM microenvironment showed that up to 75% of all osteoblast and osteoclasts express Ang1, and 25% of these weakly express Ang2 (90). Mural cells, fibroblasts, and non-vascular cells are secretors of Ang1, whilst Ang2 secretion is attributed mainly to ECs (36). Fibroblasts, which lack Tie2, adhere and spread in the presence of Ang1 but not Ang2, further suggesting that migration and adhesion processes induced by Ang1 are independent of Tie2 (91). Pericytes are essential accessory cells and key participants in angiogenesis and vascular integrity; their deficiency in pericyte coverage strongly associates with numerous diseases and uncontrolled angiogenesis (92). Ang2 treatment results in severe disassociation of pericytes and loss of pericyte coverage from tumour vessels, thus inducing vascular permeability (93),(36). As illustrated in figure 4.3, compromised ECLs may allow irregular perfusion and transport of leukemic cells from and to niches.

CLL cells apparently escape treatment and microenvironmental hardships by adhering to stromal, mesenchymal, and ECs present in the cancer microenvironment

to overcome the cytotoxic effects of anti-cancer drugs (9). Drugs such as corticosteroids and fludarabine regulate leukemic cell adhesion to vascular endothelium controlling migration beneath and underneath bone marrow stromal cells (9). Consequently, challenging the physical associations between CLL cells and surrounding tissues and accessory cells comprising the cancer microenvironment becomes a plausible therapeutic strategy (94). However, whether those therapies have any indirect impact on angiogenic pathways such as VEGF-VEGFR, Ang-Tie2, or bFGF-FGFR awaits elucidation.

The chemotactic and transendothelial-like effect that Ang1 has over CLL cells highly suggests that CLL cells will travel to areas were accessory cells express Ang1, such as the BM microenvironment or towards healthy pericytes covering vessels, capillaries, or HEVs. Whilst CLL cells highly secrete Ang2, this ligand fails to induce CLL cell migration and survival assays clearly demonstrate that Ang2 presence becomes detrimental to the survival of leukemic lymphocytes (Fig. 3.3). The low levels of Ang1 in the plasma of CLL patients could induce a pathological state of compromised pericyte coverage, leaky vessels, or higher trafficking rates of leukemic lymphocytes between peripheral blood and the microenvironments, because Ang2 then becomes the dominant angiopoietin (Fig. 4.3 b).

4.9 Angiopoietins modulating molecular event on CLL cells

The molecular mechanics of the Ang-Tie2 pathway outlined in figure 1.11 are based on to those cells expressing Tie2 endogenously, such as ECs comprising vessels, capillaries, and HEVs. Although Ang1-Tie2 signalling promotes maturation and quiescence of blood vessels, mainly regulated by Akt signal transduction via the p85 subunit of PI3K, Tie2 also has proangiogenic activity mediated by MAPK signalling (95). Binding of Ang1 to Tie2 activates the PI3K signalling transduction pathway, promoting EC migration and survival, which results in activation of anti-apoptotic pathways that are controlled by PKB/Akt (96), and downstream-of-kinase-related (Dok-R) docking protein, which mediates cell migration signal transduction, stressing the role of Ang-Tie2 signalling in EC migration (97). The interaction between Ang1 with Tie2 also activates other important factors such as endothelial nitric oxide synthase (eNOS), SH2 domain-containing phosphatase (SHP2), and growth factor receptor-

bound protein 2 (GRB2) (36). Furthermore, Ang-Tie2 signalling triggers anti-apoptotic signals through the A20-binding inhibitor of NFκB-2 (ABIN-2) (98). Thus, activation of Tie2 is key for the cell survival signals and the maintenance of the endothelial barrier and quiescent vasculature (36). Since these are all dependent on Tie2 activation, the molecular activity scenario in CLL cells will likely differ.

If angiopoietins control key cellular processes, even in the absence of Tie2 as observed in CLL cells, this can only be achieved by activation or deactivation of receptors and downstream effectors. Selecting a few key factors could become a lengthy and futile endeavour. Therefore, it is convenient to test the activation and deactivation, meaning phosphorylation and dephosphorylating, of a large number of key RTKs. RTKs are highly relevant to research, because they transmit essential molecular signals that trigger or hinder pro-survival and pro-apoptotic activity. Most drugs target RTKs, since these molecules are most likely to have a relevant detrimental effect to survival and proliferation of malignant cells. Membranes for the detection of phosphorylation of 71 RTKs were employed to detect changes in phosphorylation induced by Ang1 stimulation Analysis of an expanded amount of RTKs aims to give insight into the molecular mechanism through which Ang1 modulates the survival, chemotaxis, and transendothelial-like migration of CLL cells.

Phosphorylation events triggered by any given molecule could be transient and may have a half-life of minutes. Therefore, the phenomenon of phosphorylation must be studied rapidly to ensure that early phosphorylation is recorded, but also at later stages to observe any long-term induction of phosphorylation. As shown in figure 3.13, during the first 30 min of Ang1 stimulation, there is a remarkable phosphorylation of the RTKs listed in table 3.2, which quickly starts to vanish and return to the basal levels of phosphorylation seen before Ang1 stimulation. The roles of these RTKs are summarized in table 3.3.

It was unexpected to see that Ang1 increases phosphorylation of key RTKs, provided that no Tie2 is present in the stimulated cells and that stimulation with Ang1 induces no transient Tie2 expression. Most of the listed phosphorylated RTKs regulate migration, survival, growth, proliferation, adhesion, apoptosis, and immune responses, amongst many other functions (Table 3.3). The phosphorylation of focal adhesion kinase-1 (FAK) is of particular relevance, because in accordance to the model shown

in figure 1.11, Ang2 activates this factor through integrin signalling, resulting in proliferation and migration of EC cells. This non-receptor RTK is required in developmental angiogenesis, osteogenesis, and differentiation of osteoblasts highlighting its role in embryonic stages (66). Here it appears that Ang1 has preponderance to phosphorylate FAK, which could correlate with the advantage survival and migration increase observed in CLL cells. Since FAK is associated with reorganization of the actin cytoskeleton, its phosphorylation may facilitate reshaping of CLL cells and hence their transendothelial-like migration.

In addition, cytoplasmic tyrosine-protein kinase (BMX) appears to be highly phosphorylated by Ang1 during the first 30 min of stimulation (Fig. 3.13, b). BMX is associated with actin skeleton reorganization, cell migration, proliferation, survival, adhesion, and apoptosis (66). More importantly, it is associated with a kind of adaptive cytoprotection against extracellular stress in different cell systems, which may be the key clue that explains the ability of Ang1 to confer CLL cells with survival advantage (66). It appears that factor BMX promotes growth and differentiation of haematopoietic cells.

It is remarkable to observe that seven of the phosphorylated RTKs, namely BMX, EphA2, EphA4, EphB1, EphB2, EphB3, and FAK have been directly associated with the regulation of angiogenesis. Whilst discussing individually RTKs listed in table 3.3 and their possible relation to CLL cell pathophysiology is out of the scope of this work, changes in RTKs phosphorylation profiles strongly suggest a crucial role of Ang1 in regulating essential cellular and molecular functions of CLL cells in a Tie2-independent fashion.

Furthermore, Western blot analysis showed that there is a notable decrease of phosphorylation after 24 h of Ang2 stimulation. As seen in figure 3.11, NFkB is highly phosphorylated in CLL cells freshly isolated from peripheral blood. The reduction of phosphorylation is similar under Ang1 and Ang2 stimulation after 4 h, but after 24 h only Ang2 supports a strong dephosphorylation of NFkB (p65 subunit). This may result into a lack of transcription that leads to cytokines, chemokines, pro-apoptotic and growth factors productions. Ang2, but not Ang1, is detrimental for CLL survival, and this lack of phosphorylation may be linked to the inability of CLL cells to produce key factors. Phosphorylation of NFkB leads to transcription of factor that impact survival,

proliferation, inflammation, immune regulation, lymphagenesis, and B-cell maturation (99).

4.10 Angiopoietins exposure and fluctuating metabolic activity in CLL cells

Cancer cells impair energy homeostasis of healthy tissues and cells in the microenvironment, allowing tumour cells to acquire protective and pro-survival qualities (100). Mitochondrial functions, such ATP production and Ca^{2+} homeostasis, strongly regulate metabolism, apoptosis, necrosis, autophagy, cell migration, gene expression, and cell differentiation (101). Even in the presence of oxygen, most cancers, including CLL, rely on glycolysis as the main energy-generating pathway (i.e. the Warburg effect) and as a source of building blocks for proteins, nucleotides, and lipids (100). Therefore, reduction of ATP activity induced by Ang1 indicates that CLL cells possibly profit from lowering their metabolic activity to preserve cellular energy. This could correlate with increase in survival advantage of CLL cells. In contrast, reduction of ATP activity by Ang2 exposure could correlate with significant reduction in survival observed after four days of Ang2 stimulation (Fig. 3.4).

In models of porcine acute myocardial infarction, the concomitant expression VEGF and Ang1 correlates with neovascularization, reduced caspase-3, and reduced apoptosis (102). In the assays here, both angiopoietins reduce the content of caspase, but only Ang1 offers a clear survival advantage to CLL cells. Exposure to trebananib, a strong blocker of Ang1 and Ang2 activity, increases caspases and whilst reducing ATP activity, showing the high sensitivity of CLL cells towards the concentration of Ang1 and Ang1 present in the microenvironment. Also, the increase in caspase upon trebananib exposure could correlate with the induction of apoptosis observed in CLL cell stimulated with this inhibitor of angiopoietins. Since trebananib blocks CLL-derived angiopoietins, this supports again the notion that CLL-derived angiopoietins play an autocrine role in CLL cells. The unchanged ATP and caspase level in the PBMC population upon Ang1, Ang2, or trebananib exposure indicate that, unlike CLL cells, healthy PBMCs likely possess a stronger mechanism to compensate bioenergetics stresses. It is imperative to acquire additional biochemical data that describes the precise alternative mechanisms of action of angiopoietins over CLL cells independent of Tie2 receptor, which would help to explain these changes in metabolic activity.

4.11 Angiopoietin exposure and prognostic marker expression

Today´s diagnostic services analyse the expression of CDs and other surface markers typically associated with CLL pathogenesis. These surface markers are also considered prognostic markers that may aid therapy selection and monitor therapeutic progress after treatment. Since CDs are transmembrane proteins, their expression patterns are easily detected by flow cytometry. The typical CDs evaluated in CLL are CD3, CD5, CD4, CD8, CD10, CD19, CD22, CD23, CD25, CD38, and CD79b. In addition, Igκ and Igλ chains and receptor tyrosine kinase-like orphan receptor-1 (ROR1) are evaluated in parallel with CDs, since these are involved in B-cell immunological response.

Stimulation with angiopoietins showed heterogeneous changes of CDs and Igs expression patterns on CLL cells stimulated with recombinant Ang1 and Ang2. Flow cytometry detected changes in expression patterns of CD38, CD22 (B-cell activation), Igκ and Igλ, and CD79b (B-cell differentiation), and targets of therapy CD25 and ROR1 between non-stimulated, Ang1, or Ang2 stimulated CLL cells (Table 3.4). However, due to the widely heterogeneous observed in each CLL patient, it is not possible to draw any reliable correlation of marker expression with Ang1 or Ang2. Nevertheless, this suggests that CLL cells react to angiopoietins to modulate expression of surface prognostic markers (heterogeneously), hence angiopoietins are capable of modifying the CLL cell immunological response.

The discovery of subclonal populations is one of the most relevant findings in CLL research of the last years. As seen on table 3.4, the assorted changes in prognostic markers between these six patients highlight the heterogenic response amongst CLL patients. Heterogeneity, however, is also present amongst the cells from a single patient. During a period of seven years, CLL cells were drawn from three CLL patients, throughout which they received alkylator/fludarabine-based chemotherapy or antibody-based treatment (103). Using whole-genome sequencing researchers showed that subclonal population underwent clonal evolution, which is also heterogeneous amongst patients (103). The samples tested showed the presence of five subclonal populations that exhibited two different repopulation behaviours after treatment (Fig. 4.5). It is remarkable to observe that a small sub-population is capable of becoming the dominant subclone after treatment (103). Patients with solid and

haematopoietic malignancies, including AML, MM, and CLL, have different genotypes that explain the mixed patients' subsets, known as intratumoral heterogeneity (103).

Clinically, around 43% of CLL patients commonly display clonal evolution of del(11q) and del(17p) as demonstrated by either FISH or cytogenetics; their frequency increases in unmutated IgV_H patients (104). Therefore genetic evolution is a predictor of increased clinical aggressiveness, which has therapeutic implications (104). Disease aggressiveness is also powered by genetic plasticity that results from mutation over time leading to a positive selection of malignant cells (104). This means that subclonal populations are cable of promoting their own survival by undergoing clonal evolution and mutations that confer resistance against treatment (104). Thus, the progression of a malignancy is the result of continues mutational diversification and clonal selection (104). In this scenario, cancer cells are capable of fooling therapy due to their genetic heterogeneity, because some clones remain stable after therapy, others die, and others dominate after treatment (Fig. 4.4, b). Since Ang1 induces survival advantage and Ang2 induces pro-apoptotic activity, it could be inferred that they, differentially, contribute to promote survival of some of the CLL subclones. Proper definition of subclonal population and their separation by cell sorting, followed by recombinant angiopoietin stimulation, could reveal which subpopulations are sensitive to angiopoietins.

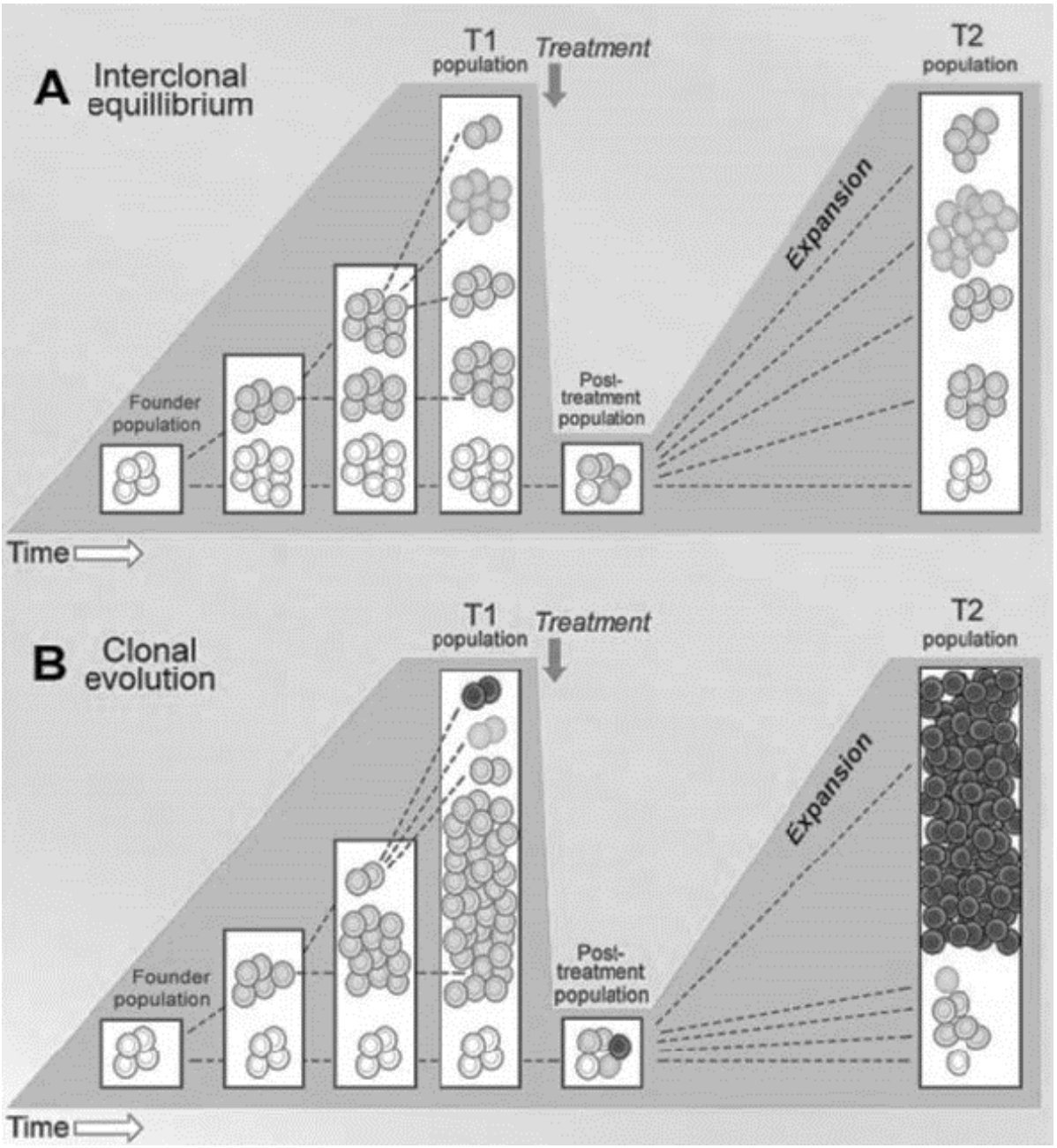


Figure 4.4 Subclonal populations and post-treatment repopulation. The repopulation after treatment follows two patterns. First, the clonal equilibrium (A) is observed through the development of disease, which remains after treatment. Second, there is an emergence of fitter population (B), also known as clonal evolution, in which a clone that represented a minority in the initial population became the dominant after treatment, taking over the dominant clone before treatment. Illustration retrieved from Catherine J. Wu, Blood 2012 (103).

4.12 Perspectives

Intrinsic non-responsiveness and adaptive resistance hinder the efficacy of available anti-CLL therapies. This is mainly due to multiple redundant pathways and the ability of haematopoietic and inflammatory cells to migrate (36), fostering the necessity to search for new avenues in CLL therapy. Even when BCR signalling and CXCR4-CXCL12 axis have been targeted at the therapeutic level in CLL (94), there are to date no therapies approved for the treatment of CLL that target exclusively any of the anti-angiogenesis pathways. Since research linking the Ang-Tie2 pathway with CLL pathophysiology is in its infancy, this pathway requires attention in the CLL context.

CLL cells from peripheral blood lack Tie2 receptors (25,64), yet react to Ang1 and Ang2 exposure, highly suggesting that angiopoietins recognize alternate receptors expressed by CLL cells. Integrins putatively recognize angiopoietins (37) and research indicates that Ang1 binds to integrins inducing cell adhesion (91). Integrins are vital receptors capable of recruiting 150 proteins or more to cell-ECM interaction sites (making them some of the most ubiquitously recognized receptors), which are referred to as the adhesome, shuttling proteins and kinases that influence gene transcription (89). Employing different myocyte cell lines, which lack Tie2, Dallabrida et al. demonstrate that Ang1 and Ang2, together with laminin, fibronectin, vitronectin, and collagen-I, -III, and –IV, induce cell adhesion (105). In addition, Ang1 also induces EC spread, adherence, and migration, effect clearly ablated by Ang2 (91). Additionally, Ang1 confers remarkable survival advantages strongly exceeding the effect of Ang2, laminin, fibronectin, vitronectin, and collagen-I, -III, and –IV (105).

Since CLL cells lack Tie2, these are an opportunity to study the role angiopoietins in regulating survival, migration, and gene expression of malignant cells in a Tie2-independent manner. Whilst it is alluring to accept integrins as the only alternative receptor, biochemistry needs (1) to offer congruent evidence to determine all binding partners of Ang1 and Ang2, (2) to describe phosphorylation patterns induced by angiopoietin (e.g. phosphoproteomics), and (3) to identify feedback inhibition mechanism that regulate the Ang-Tie2 pathway in Tie2-negative cells.

5.ZUSAMMENFASSUNG - ABSTRACT

(Auf deutsche Sprache)

Es ist bereits bekannt, dass ein hohes Ang2 Level im Plasma von CLL Patienten in Zusammenhang mit dem Fortschreiten der Erkrankung steht. Jedoch gibt es bislang keine eindeutigen Nachweise für eine Verknüpfung von Angiopoetinen und dem Ang-Tie2 Signalweg mit der Entwicklung sowie Proliferation von CLL-Patientenzellen. Die vorliegende Arbeit befasst sich mit der Rolle von Angiopoetinen in der Regulation von Migration, Überleben, Metabolismus sowie molekularen Ereignissen in CLL Zellen. Im Gegensatz zum Wachstumsfaktor Ang2, welcher in CLL Patienten bereits von zahlreichen Wissenschaftlern charakterisiert und beschrieben wurde, bleibt die Rolle von Ang1 relativ unbekannt. Ein Vergleich der Ang1 Plasmakonzentration von gesunden Patienten (4414pg/ml) und CLL Patienten (p=0.04/2336 pg/ml) zeigt eine Herunterregulation des Proteins in erkrankten Zellen. Des Weiteren sekretieren gesunde und in Kultur gehaltene mononukleäre Zellen des peripheren Blutes (PBMCs) höhere Mengen von Ang1 als CLL Zellen (p=0.002/67pg/ml). Eine Behandlung von CLL Zellen mit rekombinantem Ang1 resultiert in verbessertem Zellüberleben, während Ang2 langfristig gesehen Apoptose einleitet. Außerdem wirkt sich eine Inhibierung von Angiopoetinen in CLL Zellen mittels Trebananib nachteilig auf das Zellüberleben aus, was einen Tie2-unabhängigen Signalweg vermuten lässt. Zusätzliche Migrationsexperimente demonstrieren, dass Ang1 CLL Zell Chemotaxis (p=0.0006) und transendothel-ähnliche Migration einleitet (Zellen ändern ihre Form um die Zellschicht zu durchdringen (p=0.0008); eine Ergänzung mit Trebananib hebt beide Effekte auf; CLL Zellen exprimieren mRNA von Ang1, Ang2 und Tie1, jedoch nicht von Tie2 und VE-PTP/Tie2-Inhibitor). Diese Beobachtungen zeigten sich nicht bei Ang2 . Stimulationsversuche zeigen unterschiedliche Effekte von Ang1 und Ang2 auf die Expression von Ang1, Ang2 und Tie1 mRNA in CLL Zellen. Vor allem die Ang2 Stimulation, jedoch nicht die von Ang1, steuert eine begleitende und transiente Expression von VE-PTP und Tie2 mRNA. Eine gleichzeitige Behandlung von CLL Zellen mit Ang1 und Ang2 erhöht die Tie1 Expression um 7,7 % (p=0.014), welche auf 12,5 % (p=0.006) steigt wenn die Faktoren einzelnd verabreicht werden. Weiterhin induzieren Angiopoetine Expressionsänderungen der prognostischen Marker CD38/CD22 (B-Zellaktivierung), Igκ/Igλ/CD79b (B-Zelldifferenzierung) und CD25/ROR1 (Therapeutische Ziele). Variationen der relativen Caspase- und ATP Aktivitäten demonstrieren den Einfluss von Angiopoetinen auf die metabolische Fitness von CLL Zellen. Eine experimentelle Evaluierung von 71 RTKs zeigt, dass Ang1 die Phosphorylierung von 33 RTKs erhöht, wovon 7 (BMX, EphA2, EphA4, EphB1, EphB2, EphB3, and FAK) eine direkte Verbindung zur Angiogenese aufweisen. Ein Tie2-Mangel sowie die oben beschriebenen Beobachtungen in CLL Patientenzellen lassen auf eine Tie2-unabhängige Regulation schließen, welche CLL Zellen zu einem geeigneten Modell machen um alternative Angiopoetin Signalwege auf molekularer Ebene zu erforschen. Zusammenfassend beschreibt diese Arbeit, dass Angiopoetine die CLL Zellpathophysiologie dynamisch regulieren und im Zusammenhang mit dem Ang-Tie2 Signalweg neue therapeutische Ansätze zur Behandlung von CLL Patienten bieten.

(In English)

Whilst high Ang2 plasma levels in CLL patients correlate with disease progression, there is not conclusive evidence linking angiopoietins and the Ang-Tie2 pathway to CLL cell development and proliferation. This work addresses the putative involvement of angiopoietins in regulating survival, migration, metabolic fitness, and molecular events of CLL cells. Ang2 in CLL patients is extensively described, yet little is known about Ang1. When compared to healthy patients (4414pg/ml), plasma from CLL patients shows Ang1 downregulation (p=0.04/2336pg/ml). In culture, healthy PBMCs secrete higher Ang1 levels than CLL cells (p=0.002/67pg/ml). Exposing CLL cells to recombinant Ang1 confers survival, whilst Ang2 induces apoptosis in the long-term. However, blocking CLL-derived angiopoietins with trebananib is detrimental to survival, suggesting Tie2-independent autocrine signalling. Migration assays demonstrate that Ang1, but not Ang2, induces CLL cell chemotaxis (p=0.0006) and transendothelial-like migration (cell reshaping to penetrate a cell layer, p=0.0008); trebananib supplementation abrogates both effects. CLL cells express Ang1, Ang2, and Tie1 mRNAs, and fail to express Tie2 and VE-PTP (Tie2-inhibitor). Stimulation assays reveal that Ang1 and Ang2 differentially modulate Ang1, Ang2, and Tie1 mRNA expression in CLL cells. Remarkably, Ang2 stimulation, but not that of Ang1, triggers the concomitant and transient expression of VE-PTP and Tie2 mRNA. Exposure to Ang1 and Ang2 increase CLL-Tie1 expression by 7.7% (p=0.014) and by 12.5% (p=0.006) respectively. Angiopoietins induce expression changes of the prognostic markers CD38/CD22 (B-cell activation), Igκ/Igλ/ CD79b (B-cell differentiation), and CD25/ROR1 (therapeutic targets). Variations in relative caspase and ATP activity demonstrate the ability of angiopoietins to affect metabolic fitness of CLL cells. In an assay evaluating 71 RTKs, Ang1 increases phosphorylation of 33, seven of which (BMX, EphA2, EphA4, EphB1, EphB2, EphB3, and FAK) are directly associated with angiogenesis. Since CLL cells lack Tie2, apparently angiopoietins modulate the aforementioned events in a Tie2-independent fashion, making CLL cells a suitable model to understand alternative mechanisms of angiopoietin signalling. Altogether, this works demonstrates that angiopoietins dynamically regulate CLL cell pathophysiology, highlighting the novel therapeutic potential of angiopoietins and the Ang-Tie2 pathway in the CLL context.

6. REFERENCES

66. * in UniProtKB [Internet]. [cited 2015 Oct 1]. Available from: http://www.uniprot.org/uniprot/
53. Aguayo A, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, et al. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000 Sep 15;96(6):2240–5.
73. Aguayo A, Manshouri T, O'Brien S, Keating M, Beran M, Koller C, et al. Clinical relevance of Flt1 and Tie1 angiogenesis receptors expression in B-cell chronic lymphocytic leukemia (CLL). Leuk Res. 2001 Apr 1;25(4):279–85.
18. Aguirre Palma LM, Gehrke I, Kreuzer K-A. Angiogenic factors in chronic lymphocytic leukaemia (CLL): Where do we stand? Crit Rev Oncol Hematol. 2015 Mar;93(3):225–36.
78. Alfieri A, Watson JJ, Kammerer RA, Tasab M, Progias P, Reeves K, et al. Angiopoietin-1 variant reduces LPS-induced microvascular dysfunction in a murine model of sepsis. Crit Care. 2012 Oct 4;16(5):R182.
28. Angiogenesis | The Angiogenesis Foundation [Internet]. [cited 2015 Mar 4]. Available from: http://www.angio.org/learn/angiogenesis/
35. Augustin HG, Young Koh G, Thurston G, Alitalo K. Control of vascular morphogenesis and homeostasis through the angiopoietin–Tie system. Nat Rev Mol Cell Biol. 2009 Mar;10(3):165–77.
77. Backen A, Renehan AG, Clamp AR, Berzuini C, Zhou C, Oza A, et al. The Combination of Circulating Ang1 and Tie2 Levels Predicts Progression-Free Survival Advantage in Bevacizumab-Treated Patients with Ovarian Cancer. Clin Cancer Res. 2014 Jan 9;20(17):4549–58.
55. Banfi G, Salvagno GL, Lippi G. The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes. Clin Chem Lab Med. 2007 May 1;45(5):565–76.
49. Barton WA, Tzvetkova D, Nikolov DB. Structure of the Angiopoietin-2 Receptor Binding Domain and Identification of Surfaces Involved in Tie2 Recognition. Structure. 2005 Mai;13(5):825–32.
37. Barton WA, Tzvetkova-Robev D, Miranda EP, Kolev MV, Rajashankar KR, Himanen JP, et al. Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2–Tie2 complex. Nat Struct Mol Biol. 2006 Jun;13(6):524–32.
57. Bonetta L. Flow cytometry smaller and better. Nat Methods. 2005 Oct;2(10):785–95.
68. Buehler D, Rush P, Hasenstein JR, Rice SR, Hafez GR, Longley BJ, et al. Expression of angiopoietin-TIE system components in angiosarcoma. Mod Pathol. 2013 Aug;26(8):1032–40.
94. Burger JA, Gribben JG. The microenvironment in chronic lymphocytic leukemia (CLL) and other B cell malignancies: Insight into disease biology and new targeted therapies. Semin Cancer Biol. 2014 Feb;24:71–81.
14. Caligaris-Cappio F. Inflammation, the microenvironment and chronic lymphocytic leukemia. Haematologica. 2011 Mar 1;96(3):353–5.
1. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood. 2011 May 12;117(19):5019–32.
93. Cao Y, Sonveaux P, Liu S, Zhao Y, Mi J, Clary BM, et al. Systemic Overexpression of Angiopoietin-2 Promotes Tumor Microvessel Regression and Inhibits Angiogenesis and Tumor Growth. Cancer Res. 2007 Apr 15;67(8):3835–44.
91. Carlson TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO. Direct Cell Adhesion to the Angiopoietins Mediated by Integrins. J Biol Chem. 2001 Jul 13;276(28):26516–25.

62. Caspase 3/7 Activity Apoptosis Assay Kit *Blue Fluorescence* caspase 3 apoptosis assay caspase activity assay caspase 3 activity assay roche caspase 3 assay [Internet]. [cited 2015 Jul 13]. Available from: http://aatbio.com/gen4prst.pl?Cid=22795&gid=32
76. Coffelt SB, Chen Y-Y, Muthana M, Welford AF, Tal AO, Scholz A, et al. Angiopoietin 2 Stimulates TIE2-Expressing Monocytes To Suppress T Cell Activation and To Promote Regulatory T Cell Expansion. J Immunol. 2011 Jan 4;186(7):4183–90.
75. Coffelt SB, Tal AO, Scholz A, Palma MD, Patel S, Urbich C, et al. Angiopoietin-2 Regulates Gene Expression in TIE2-Expressing Monocytes and Augments Their Inherent Proangiogenic Functions. Cancer Res. 2010 Jan 7;70(13):5270–80.
80. Coxon A, Bready J, Min H, Kaufman S, Leal J, Yu D, et al. Context-dependent role of angiopoietin-1 inhibition in the suppression of angiogenesis and tumor growth: implications for AMG 386, an angiopoietin-1/2-neutralizing peptibody. Mol Cancer Ther. 2010 Oct;9(10):2641–51.
105. Dallabrida SM, Ismail N, Oberle JR, Himes BE, Rupnick MA. Angiopoietin-1 Promotes Cardiac and Skeletal Myocyte Survival Through Integrins. Circ Res. 2005 Apr 3;96(4):e8–24.
20. De Falco S. The discovery of placenta growth factor and its biological activity. Exp Mol Med. 2012 Jan 31;44(1):1–9.
6. Desai V, Isharwal S, Pooli A, Lele S, Feloney M. Chronic lymphocytic leukemia of the bladder: an atypical etiology of gross hematuria. Ther Adv Urol. 2014 Oct;6(5):198–200.
81. Diamond JR, Wu B, Agarwal N, Bowles DW, Lam ET, Werner TL, et al. Pharmacokinetic drug-drug interaction study of the angiopoietin-1/angiopoietin-2-inhibiting peptibody trebananib (AMG 386) and paclitaxel in patients with advanced solid tumors. Invest New Drugs. 2015 Apr 21;33(3):691–9.
13. Dreger P, Montserrat E, (eric) on behalf of the ES for B and MT (EBMT) and the ERI on C. Where Does Allogeneic Stem Cell Transplantation Fit in the Treatment of Chronic Lymphocytic Leukemia? Curr Hematol Malig Rep. 2015 Feb 5;10(1):59–64.
47. Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, et al. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev. 1994 Aug 15;8(16):1897–909.
58. Edwards BS, Sklar LA. Flow Cytometry Impact on Early Drug Discovery. J Biomol Screen. 2015 Jul 1;20(6):689–707.
88. Eikesdal HP, Kalluri R. Drug resistance associated with antiangiogenesis therapy. Semin Cancer Biol. 2009 Oct;19(5):310–7.
51. Fachinger G, Deutsch U, Risau W. Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2. Oncogene. 1999 Oct 21;18(43):5948–53.
46. Fagiani E, Christofori G. Angiopoietins in angiogenesis. Cancer Lett. 2013 Jan 1;328(1):18–26.
15. Fecteau J-F, Kipps TJ. Structure and function of the hematopoietic cancer niche: focus on chronic lymphocytic leukemia. Front Biosci Sch Ed. 2012 Jan 1;4:61–73.
67. Ferrajoli A, Lee B-N, Schlette EJ, O'Brien SM, Gao H, Wen S, et al. Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood. 2008 Jun 1;111(11):5291–7.
45. Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, et al. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood. 2004 Jun 1;103(11):4150–6.
19. Folkman J. Angiogenesis. In: Editors-in-Chief: Sydney Brenner, Jeffrey H. Miller, editors. Encyclopedia of Genetics [Internet]. New York: Academic Press; 2001 [cited 2013 Aug 15]. p. 66–73. Available from: http://www.sciencedirect.com/science/article/pii/B0122270800015457
23. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov. 2007 Apr;6(4):273–86.
22. Folkman J. Angiogenesis-dependent diseases. Semin Oncol. 2001 Dec;28(6):536–42.
21. Folkman J. Tumor Angiogenesis: Therapeutic Implications. N Engl J Med. 1971;285(21):1182–6.
85. Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, et al. Differential function of Tie2 at cell–cell contacts and cell–substratum contacts regulated by angiopoietin-1. Nat Cell Biol. 2008 Mai;10(5):513–26.
10. Hacken E ten, Burger JA. Molecular Pathways: Targeting the Microenvironment in Chronic Lymphocytic Leukemia—Focus on the B-Cell Receptor. Clin Cancer Res. 2014 Jan 2;20(3):548–56.
87. Hacken E ten, Burger JA. Molecular Pathways: Targeting the Microenvironment in Chronic Lymphocytic Leukemia—Focus on the B-Cell Receptor. Clin Cancer Res. 2014 Jan 2;20(3):548–56.
5. Hallek M. Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. Am J Hematol. 2013;88(9):803–16.

9. Han T-T, Fan L, Li J-Y, Xu W. Role of chemokines and their receptors in chronic lymphocytic leukemia: Function in microenvironment and targeted therapy. Cancer Biol Ther. 2013 Oct 22;15(1):3–9.
82. Herbst RS, Hong D, Chap L, Kurzrock R, Jackson E, Silverman JM, et al. Safety, Pharmacokinetics, and Antitumor Activity of AMG 386, a Selective Angiopoietin Inhibitor, in Adult Patients With Advanced Solid Tumors. J Clin Oncol. 2009 Jul 20;27(21):3557–65.
90. Horner A, Bord S, Kelsall AW, Coleman N, Compston JE. Tie2 ligands angiopoietin-1 and angiopoietin-2 are coexpressed with vascular endothelial cell growth factor in growing human bone. Bone. 2001 Jan;28(1):65–71.
65. Hu J, Haseebuddin M, Young M, Colburn NH. Suppression of p65 phosphorylation coincides with inhibition of IκBα polyubiquitination and degradation. Mol Carcinog. 2005 Dec 1;44(4):274–84.
36. Huang H, Bhat A, Woodnutt G, Lappe R. Targeting the ANGPT–TIE2 pathway in malignancy. Nat Rev Cancer. 2010 Aug;10(8):575–85.
70. Ichihara E, Kaneda K, Saito Y, Yamakawa N, Morishita K. Angiopoietin1 contributes to the maintenance of cell quiescence in EVI1high leukemia cells. Biochem Biophys Res Commun. 2011 Dec 16;416(3–4):239–45.
89. Jansen KA, Donato DM, Balcioglu HE, Schmidt T, Danen EHJ, Koenderink GH. A guide to mechanobiology: Where biology and physics meet. Biochim Biophys Acta BBA - Mol Cell Res [Internet]. 2015 [cited 2015 Jun 24]; Available from: http://www.sciencedirect.com/science/article/pii/S0167488915001536
42. Jeansson M, Gawlik A, Anderson G, Li C, Kerjaschki D, Henkelman M, et al. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. J Clin Invest. 2011 Jun 1;121(6):2278–89.
71. Ji C. Prognostic impact of δ-like ligand 4 and Notch1 in acute myeloid leukemia. Oncol Rep [Internet]. 2012 Jul 31 [cited 2013 Sep 13]; Available from: http://www.spandidos-publications.com/or/28/4/1503
97. Jones N, Chen SH, Sturk C, Master Z, Tran J, Kerbel RS, et al. A Unique Autophosphorylation Site on Tie2/Tek Mediates Dok-R Phosphotyrosine Binding Domain Binding and Function. Mol Cell Biol. 2003 Apr;23(8):2658–68.
96. Jones N, Iljin K, Dumont DJ, Alitalo K. Tie receptors: new modulators of angiogenic and lymphangiogenic responses. Nat Rev Mol Cell Biol. 2001 Apr;2(4):257–67.
101. Kasahara A, Scorrano L. Mitochondria: from cell death executioners to regulators of cell differentiation. Trends Cell Biol [Internet]. 2014 [cited 2014 Oct 16]; Available from: http://www.sciencedirect.com/science/article/pii/S0962892414001421
38. Kawaguchi M, Sugaya M, Suga H, Miyagaki T, Ohmatsu H, Fujita H, et al. Serum Levels of Angiopoietin-2, but not Angiopoietin-1, are Elevated in Patients with Erythrodermic Cutaneous T-cell Lymphoma. Acta Derm Venereol. 2013 Jul 1;
25. Kay NE, Bone ND, Tschumper RC, Howell KH, Geyer SM, Dewald GW, et al. B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. Leukemia. 2002 May;16(5):911–9.
3. Kay NE, Hamblin TJ, Jelinek DF, Dewald GW, Byrd JC, Farag S, et al. Chronic Lymphocytic Leukemia. ASH Educ Program Book. 2002 Jan 1;2002(1):193–213.
26. Kini AR, Kay NE, Peterson LC. Increased bone marrow angiogenesis in B cell chronic lymphocytic leukemia. Leukemia. 2000 Aug;14(8):1414–8.
104. Landau DA, Carter SL, Getz G, Wu CJ. Clonal evolution in hematological malignancies and therapeutic implications. Leukemia. 2014 Jan;28(1):34–43.
56. Lanier LL. Just the FACS. J Immunol. 2014 Jan 9;193(5):2043–4.
72. Loges S, Heil G, Bruweleit M, Schoder V, Butzal M, Fischer U, et al. Analysis of Concerted Expression of Angiogenic Growth Factors in Acute Myeloid Leukemia: Expression of Angiopoietin-2 Represents an Independent Prognostic Factor for Overall Survival. J Clin Oncol. 2005 Feb 20;23(6):1109–17.
33. Maffei R, Bulgarelli J, Fiorcari S, Bertoncelli L, Martinelli S, Guarnotta C, et al. The monocytic population in chronic lymphocytic leukemia shows altered composition and deregulation of genes involved in phagocytosis and inflammation. Haematologica. 2013 Jan 7;98(7):1115–23.
31. Maffei R, Martinelli S, Castelli I, Santachiara R, Zucchini P, Fontana M, et al. Increased angiogenesis induced by chronic lymphocytic leukemia B cells is mediated by leukemia-derived Ang2 and VEGF. Leuk Res. 2010 Mar;34(3):312–21.
32. Maffei R, Martinelli S, Santachiara R, Rossi D, Guarnotta C, Sozzi E, et al. Angiopoietin-2 plasma dosage predicts time to first treatment and overall survival in chronic lymphocytic leukemia. Blood. 2010 Jul 29;116(4):584–92.

44. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a Natural Antagonist for Tie2 That Disrupts in vivo Angiogenesis. Science. 1997 Apr 7;277(5322):55–60.
34. Martinelli S, Kanduri M, Maffei R, Fiorcari S, Bulgarelli J, Marasca R, et al. ANGPT2 promoter methylation is strongly associated with gene expression and prognosis in chronic lymphocytic leukemia. Epigenetics [Internet]. 2013 Jul 1 [cited 2013 Sep 13];8(7). Available from: https://www.landesbioscience.com/journals/epigenetics/article/24947/?nocache=426453416
63. Martinelli S, Maffei R, Castelli I, Santachiara R, Zucchini P, Fontana M, et al. Increased expression of angiopoietin-2 characterizes early B-cell chronic lymphocytic leukemia with poor prognosis. Leuk Res. 2008 Apr;32(4):593–7.
16. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014 Jan 16;505(7483):327–34.
43. Moss A. The angiopoietin:Tie 2 interaction: A potential target for future therapies in human vascular disease. Cytokine Growth Factor Rev. 2013 Dec;24(6):579–92.
7. Nabhan C, Rosen ST. Chronic lymphocytic leukemia: a clinical review. JAMA. 2014 Dec 3;312(21):2265–76.
52. Nawroth R, Poell G, Ranft A, Kloep S, Samulowitz U, Fachinger G, et al. VE-PTP and VE-cadherin ectodomains interact to facilitate regulation of phosphorylation and cell contacts. EMBO J. 2002 Sep 16;21(18):4885–95.
60. Neal J, Wakelee H. AMG-386, a selective angiopoietin-1/-2-neutralizing peptibody for the potential treatment of cancer. Curr Opin Mol Ther. 2010 Aug;12(4):487–95.
41. Papapetropoulos A, García-Cardeña G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, Sessa WC. Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. Lab Investig J Tech Methods Pathol. 1999 Feb;79(2):213–23.
4. Parikh SA, Kay NE, Shanafelt TD. Monoclonal B-cell lymphocytosis: update on diagnosis, clinical outcome, and counseling. Clin Adv Hematol Oncol HO. 2013 Nov;11(11):720–9.
27. Peterson L, Kini AR, Kay N. Angiogenesis is increased in B-cell chronic lymphocytic leukemia. Blood. 2001 Apr 15;97(8):2529–30.
61. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 May 1;29(9):e45.
64. Pötzsch B, Gehrke I, Poll-Wolbeck SJ, Flamme H, Kreuzer K-A. Angiopoietin-2/Tie2 Signaling in the Microenvironment of Chronic Lymphocytic Leukemia (CLL). Res Cancer Tumor. 2014;3(1):6–18.
100. Prezma T, Shteinfer A, Admoni L, Raviv Z, Sela I, Levi I, et al. VDAC1-based peptides: novel pro-apoptotic agents and potential therapeutics for B-cell chronic lymphocytic leukemia. Cell Death Dis. 2013 Sep 19;4(9):e809.
48. Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. EMBO J. 1995 Dec 1;14(23):5884–91.
12. Robak T. Recent progress in the management of chronic lymphocytic leukemia. Cancer Treat Rev. 2007 Dec;33(8):710–28.
59. RPMI Media - RPMI-1640 Media [Internet]. Sigma-Aldrich. [cited 2015 Jun 24]. Available from: http://www.sigmaaldrich.com/life-science/cell-culture/classical-media-salts/rpmi-media.html
39. Seegar TCM, Eller B, Tzvetkova-Robev D, Kolev MV, Henderson SC, Nikolov DB, et al. Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. Mol Cell. 2010 Mar 12;37(5):643–55.
83. Shen J, Frye M, Lee BL, Reinardy JL, McClung JM, Ding K, et al. Targeting VE-PTP activates TIE2 and stabilizes the ocular vasculature. J Clin Invest. 2014 Oct 1;124(10):4564–76.
30. Sinnathamby T, Yun TJ, Clavet-Lanthier M-É, Cheong C, Sirois MG. VEGF and angiopoietins promote inflammatory cell recruitment and mature blood vessel formation in murine sponge/Matrigel model. J Cell Biochem. 2014 Aug 1;n/a – n/a.
92. Stapor PC, Sweat RS, Dashti DC, Betancourt AM, Murfee WL. Pericyte Dynamics during Angiogenesis: New Insights from New Identities. J Vasc Res. 2014;51(3):163–74.
17. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nat Rev Cancer. 2012 Mar;12(3):210–9.
2. Swerdlow, Steven, Campo, Elias, Lee Harris, Nancy, Jaffe, Elaine, PIleri, Stefano, Stein, Harald, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. 4th ed. Lyon: Internatioanl Agency for Research on Cancer; 2008. 439 p.
98. Tadros A, Hughes DP, Dunmore BJ, Brindle NPJ. ABIN-2 protects endothelial cells from death and has a role in the antiapoptotic effect of angiopoietin-1. Blood. 2003 Dec 15;102(13):4407–9.

102. Tao Z, Chen B, Tan X, Zhao Y, Wang L, Zhu T, et al. Coexpression of VEGF and angiopoietin-1 promotes angiogenesis and cardiomyocyte proliferation reduces apoptosis in porcine myocardial infarction (MI) heart. Proc Natl Acad Sci. 2011 Jan 2;108(5):2064–9.
11. Tausch E, Mertens D, Stilgenbauer S. Advances in treating chronic lymphocytic leukemia. F1000Prime Rep [Internet]. 2014 Aug 1 [cited 2015 Mar 20];6. Available from: http://f1000.com/prime/reports/m/6/65
24. Van Bockstaele F, Verhasselt B, Philippé J. Prognostic markers in chronic lymphocytic leukemia: A comprehensive review. Blood Rev. 2009 Jan;23(1):25–47.
74. Venneri MA, Palma MD, Ponzoni M, Pucci F, Scielzo C, Zonari E, et al. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood. 2007 Jun 15;109(12):5276–85.
8. Vilpo J, Hulkkonen J, Hurme M, Vilpo L. Surface membrane antigen expression changes induced in vitro by exogenous growth factors in chronic lymphocytic leukemia cells. Leukemia. 2002 Sep;16(9):1691–8.
69. Wang R-H, Xie J-G, Chen H, Ren T-T, Zhang Y-C. [Expression of angiopoietin-2 and vascular endothelial growth factor in human colon cancer]. Nan Fang Yi Ke Da Xue Xue Bao. 2013 Aug 20;33(8):1236–9.
99. Weinberg RA, Ludwig DK. CST Guide: Pahtways and Protocols. First. USA: Cell Signalling Technology, Inc.; 2015. 304 p.
50. Winderlich M, Keller L, Cagna G, Broermann A, Kamenyeva O, Kiefer F, et al. VE-PTP controls blood vessel development by balancing Tie-2 activity. J Cell Biol. 2009 May 18;185(4):657–71.
54. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects [Internet]. 2013 [cited 2015 Apr 16]. Available from: http://www.wma.net/en/30publications/10policies/b3/
84. Woo KV, Baldwin HS. Role of Tie1 in Shear Stress and Atherosclerosis. Trends Cardiovasc Med. 2011 May;21(4):118–23.
103. Wu CJ. CLL clonal heterogeneity: an ecology of competing subpopulations. Blood. 2012 Nov 15;120(20):4117–8.
95. Yamakawa D, Kidoya H, Sakimoto S, Jia W, Naito H, Takakura N. Ligand-independent Tie2 Dimers Mediate Kinase Activity Stimulated by High Dose Angiopoietin-1. J Biol Chem. 2013 Mar 5;288(18):12469–77.
29. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature. 2000 Sep 14;407(6801):242–8.
86. Yang MT. Mechanobiology in harness. Nat Mater. 2014 Jun;13(6):531–531.
40. Yu X, Seegar TCM, Dalton AC, Tzvetkova-Robev D, Goldgur Y, Rajashankar KR, et al. Structural basis for angiopoietin-1–mediated signaling initiation. Proc Natl Acad Sci. 2013 Apr 30;110(18):7205–10.
79. Zhu Y-G, Qu J-M, Zhang J, Jiang H-N, Xu J-F. Novel Interventional Approaches for ALI/ARDS: Cell-Based Gene Therapy. Mediators Inflamm. 2011 Jul 13;2011:e560194.

7. PRELIMINARY PUBLICATIONS

The results and views written on this manuscript have been preliminarily presented in the following forms:

ORIGINAL ARTICLE

Angiopoietins modulate components of Ang-Tie2 pathway, survival, and migration of chronic lymphocytic leukaemia (CLL) cells in vitro.

Aguirre Palma LM, Flamme H, Gehrke I, Kreuzer KA.

Journal of Cancer Microenvironment Sprinter

In press: doi: 10.1007/s12307-016-0180-7

REVIEW ARTICLE

Angiogenic factors in chronic lymphocytic leukaemia (CLL): Where do we stand?

Aguirre Palma LM, Gehrke I, Kreuzer KA

Critical Reviews in Oncology/Hematology, Vol.93, Issue 3, March 2015

In press: doi:10.1016/j.critrevonc.2014.10.007

POSTER PRESENTATION

Angiopoietin 1 and Angiopoietin 2 ligands regulate physiological processes of chronic lymphocytic leukaemia (CLL cells) in a Tie-2 independent manner.

Aguirre Palma LM, Gehrke I, Hallek M, K.A. Kreuzer

Conference: CrossRoad for Biology 13th-14th February, 2014, Cologne, Germany.

8. LEBENSLAUF

Mein Lebenslauf wird aus Gründen des Datenschutzes in der elektronischen Fassung meine Arbeit nich veröffentlicht.