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Importance of microenvironment and antigen in the regulation of growth and survival of CLL cells

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"*Science never solves a problem without creating ten more"*

George Bernard Shaw

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells rapidly die when put in culture implying that microenvironmental signals delivered by accessory cells confer CLL cells with a growth advantage. Recent findings show that CLL cells are antigen experienced and antigen binding play a critical role in the pathogenesis of the disease. The overall aim of this thesis was to study the influence of the microenvironment and antigen binding in CLL.

In paper I, we studied the influence of the small redox-regulatory molecule thioredoxin (Trx) on CLL cell survival and proliferation. We found Trx to be highly expressed in CLL lymph nodes (LNs), secreted from stromal cells surrounding proliferating CLL cells in proliferation centers, indicating growth promoting properties. Secreted Trx was also shown to protect CLL cells from apoptosis.

In paper II, oxidized LDL was added to subset #1 CLL cells. However, in contrast to our hypothesis, we could not observe activation and proliferation of CLL cells. Instead subset #1 CLL cells were unresponsive/anergic through the B cell receptor (BcR). This anergic state could however be overcome by "wash out" of bound antigen or addition of toll-like receptor 9 stimulation in some patients.

Gene expression profiles differ between groups of CLL patients and in peripheral blood (PB) and LN compartment, due to different microenvironments. However, it is not known whether these differences also apply for DNA methylation. In paper III, we identified various genes that were alternatively methylated between IGHV mutated (M) and unmutated (UM) groups. For example prognostic genes, *CLLU1* and *LPL,* genes involved in B cell signaling, *IBTK,* as well as numerous TGF-β and NF-κB/TNF pathway genes.

The intensity and duration of BcR signals are fine-tuned by enhancing or inhibitory co-receptors. SHP-1 inhibits BcR-signals by dephosphorylation. In paper IV, we compared the expression and activity of SHP-1 in CLL cells from LN with matched PB samples. However, in contrast to our hypothesis, SHP-1 activity/phosphorylation status in PB and LN, did not differ significantly.

This thesis, add another piece to the puzzle, on how the microenvironment and antigens influence CLL pathogenesis. Since great variations among individuals are seen, further studies in different groups of patients are necessary to elucidate the importance of antigen for the development of CLL.

HUR PÅVERKAR MIKROMILJÖN OCH ANTIGEN I LYMFKNUTOR UTVECKLING AV KLL?

Kronisk lymfatisk leukemi (KLL) är den vanligaste formen av leukemi hos vuxna i västvärlden. Varje år diagnostiseras ca 500 patienter med KLL i Sverige och medianåldern för diagnos är runt 70 år. Det är en heterogen sjukdom, där en del patienter överlever många år utan behandling, medan andra avlider inom bara några få år. Det finns för nuvarande ingen botande behandling för dessa patienter, men utvecklingen av nya läkemedel går snabbt framåt och forskning pågår hela tiden för att utveckla nya effektiva läkemedel. KLL är en sjukdom där en viss typ av immunceller s.k. B-lymfocyter delar sig ohämmat, exakt vilken typ av B-cell som är ursprungscellen eller vad som orsakar och driver sjukdomen är fortfarande inte helt känt och studeras med högsta prioritet av forskare världen över. Flera rapporter visar att KLL-cellerna är beroende av omgivande celler i sin mikromiljö och att de även visar tecken på att ha aktiverats av antigen. Detta har man kommit fram till då man observerat att KLL-cellerna som cirkulerar i blodet är mer vilande jämfört med de leukemiceller som befinner sig i vävnad t.ex. lymfknuta. Där har KLL-cellerna andra celler tätt inpå sig, är mer aktiva och delar sig. Dessutom har man sett att KLL-celler i lymfknutor skiljer sig åt när man tittar på vilka gener som uttrycks. Vår grupp och andra forskargrupper har visat att KLL-antikroppar kan binda till kroppsegna strukturer s.k. autoantigen, men även till främmande strukturer som finns på t.ex. bakterier och virus. En frisk B-cell som befinner sig i lymfknuta ska normalt inte aktiveras av autoantigen (sk. tolerans eller anergi). Vi vet nu att KLL B-celler uppträder annorlunda vid konfrontation med autoantigen dvs. bindning till B-cellsreceptorn, jämfört med friska B-celler. Syftet med min avhandling har varit att få bättre förståelse för varför sjukdomen uppstår, vad som driver utvecklingen av den och mer specifikt att ta reda på hur mikromiljön och antigen är involverat och på vilket sätt det påverkar och aktiverar leukemicellerna. Denna kunskap utgör grunden till hur celldelningen hos leukemicellerna kan stoppas.

I det första delarbetet fann vi att ett litet protein med reduktions-oxidations (redox)-egenskaper kallat thioredoxin, frisätts från s.k. stromaceller, som finns runtomkring aktiva, delande leukemiceller i lymfknutan. Vi kunde även se att thioredoxin förbättrade överlevnaden hos leukemicellerna.

I det andra delarbetet såg vi, tvärtom mot vad vi hade trott, att stimulering av KLL-celler från en viss grupp av KLL patienter, med sitt specifika autoantigen (oxiderat LDL), trots sitt aggressiva sjukdomsförlopp, varken gav cellaktivering eller celldelning. Denna brist på cellsvar berodde troligtvis på kronisk tillgång och stimulans av antigen till cellernas receptorer i kroppen. Detta tillstånd kunde dock brytas om antigenet, som redan satt bundet till receptorerna tvättades bort eller om cellerna dessutom stimulerades via andra s.k. toll-liknande receptorer.

Eftersom man tidigare sett att olika undergrupper av patienter med olika sjukdomsstadier, samt celler från blod och lymfknuta, uttrycker olika gener, ville vi i delarbete tre titta på skillnader i DNA-metylering hos dessa grupper. DNA-metylering är ett sätt för celler att reglera geners uttryck, som en ”PÅ/AV” knapp. Vi fann många gener som var olika metylerade när vi jämförde celler från patienter med mindre aggressivt sjukdomsförlopp med de som hade ett mer aggressivt förlopp, många av dessa var involverade i cellsignalering Däremot var metyleringsmönstret i princip oförändrat vid jämförelse över tid eller mellan blod och lymfknuta i samma patient.

Eftersom man tidigare sett att B-cellsaktivering via B-cellsreceptorn är viktig för utveckling av KLL samt att dessa signaler skiljer sig mellan blod och lymfknuta, ville vi i delarbete fyra studera ett protein, SHP-1, som reglerar B-cellsreceptorsignalering och jämföra uttryck och aktivitet av detta protein i celler från blod och lymfknuta. Vi kunde dock inte se någon skillnad i uttryck eller aktivitet av proteinet.

Sammanfattningsvis bidrar forskningsprojekten till att öka förståelsen för hur mikromiljön och antigen kan påverka KLL celler. Eftersom sjukdomen är mycket heterogen och ter sig olika hos olika individer krävs mer forskning på olika grupper av patienter för att ta reda på vilken roll antigen spelar för utveckling av sjukdomen.

LIST OF PAPERS

This thesis is based on the following Papers, referred to in the text by their Roman numerals (I-IV):

Eva Bäckman, **Ann-Charlotte Bergh**, Irena Lagerdahl, Björn Rydberg, Christer Sundström, Gerard Tobin, Richard Rosenquist, Mats Linderholm, Anders Rosén. Thioredoxin, produced by stromal cells retrieved from the lymph node microenvironment, rescues chronic lymphocytic leukemia cells from apoptosis in vitro. *Haematologica 2007;92: 1495-1504.*

Ann-Charlotte Bergh, Chamilly Evaldsson, Lone Bredo Pedersen, Christian Geisler, Kostas Stamatopoulos, Richard Rosenquist, and Anders Rosén. Silenced B-cell receptor response to autoantigen in a poor-prognostic subset of chronic lymphocytic leukemia. *Haematologica 2014;99: 1722-30*

Nicola Cahill, **Ann-Charlotte Bergh**, Meena Kanduri, Hanna Göransson Kultima, Larry Mansouri, Anders Isaksson, Fergus Ryan, Karin Ekström Smedby, Gunnar Juliusson, Christer Sundström, Anders Rosén and Richard Rosenquist. 450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments*. Leukemia 2012;27: 150-158.*

Ann-Charlotte Bergh, Zahra El-Schich, Payam Delfani, Ohlsson Lars, Anders Rosén and Anette Gjörloff Wingren. B cell receptor signaling suppressor SHP-1 is active in CLL lymph node and peripheral blood. *Manuscript*

CONTENTS

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APRIL	A proliferation-induced ligand
BAFF	B cell activating factor
BcR	B cell receptor
BM	Bone marrow
BTK	Bruton´s tyrosine kinase
C	Constant
CDR	Complementarity determining region
CLL	Chronic lymphocytic leukemia
CSR	Class switch recombination
D	Diversity
DNMT	DNA methyltransferase
FDC	Follicular dendritic cell
FISH	Fluorescence *in situ* hybridization
FR	Framework region
FRC	Follicular reticular cell
GSH	Glutathione
HC	Heavy chain
IGHV	Immunoglobulin heavy chain variable
IGLV	Immunoglobulin lambda variable
IL	Interleukin
ITAM	Immunoreceptor tyrosine based activation motif
J	Joining
LC	Light chain
LN	Lymph node
mAb	Monoclonal antibody
MAP	Mitogen activated protein
M	Mutated
MDA-LDL	Malondialdehyde modified low density lipoprotein
MSC	Mesenchymal stromal cell
MZ	Marginal zone
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κB

NLC	Nurse-like cell
oxLDL	Oxidized low density lipoprotein
PBMC	Peripheral blood mononuclear cell
PC	Proliferation center
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
PRR	Pattern recognition receptor
Pre-B cell	Precursor B cell
Pro-B cell	Progenitor B cell
PTP	Protein tyrosine phosphatase
ROS	Reactive oxygen species
SHM	Somatic hypermutation
SYK	Spleen tyrosine kinase
TLR	Toll-like receptor
Trx	Thioredoxin
UM	Unmutated
V	Variable
ZAP70	70 kDa zeta chain associated protein kinase

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of long-lived, monoclonal B-lymphocytes infiltrating bone marrow (BM), peripheral blood (PB) and lymphoid tissue. There is currently no curative treatment.
Over the last decade, immunogenetic analysis of B cell receptor immunoglobulins (BcR IGs), which resulted in the observations that groups of CLL patients share remarkable similar IG gene rearrangements, have revolutionized the field of CLL. These studies highlights the BcR as a key-player in CLL, and the hypothesis that antigens (Ags) play a critical role in the pathogenesis was put forward (1-3).

In this thesis I will focus on the interplay between the malignant CLL B cells and tumor microenvironment as well as the role for Ags in the leukemogenesis. For you to understand these parts, I will first guide you through the basics of the immune system, BcR structure, normal B cell development and interaction with Ag.

The immune system

The immune system is composed of two subdivisions; the innate immune system and the adaptive immune system.

The innate immunity

The innate, non-adaptive defenses form the earliest barriers to infection. Most microorganisms that we come into contact with are destroyed by innate defense mechanisms. Innate immune responses are not specific to a particular pathogen (as the adaptive immune responses are) but depend on proteins and phagocytes that recognize conserved structures on pathogens. The first barriers are physical such as skin and mucosal surfaces or chemical such as secretion of antimicrobial peptides. If the pathogen should pass these barriers inflammation occurs and the complement system is activated. The complement system helps to clear the pathogens by recruiting phagocytes, mediating uptake through complement receptors or by causing direct lysis of the pathogen.

Recruited phagocytes bear pattern recognition receptors (PRRs), such as scavenger receptors (SC) and toll-like receptors (TLRs), that recognize repetitive structures on bacteria, and pathogen-associated molecular patterns (PAMPs) such as bacterial DNA which contains unmethylated repeats of dinucleotide CpG and viral double-stranded RNA (4, 5). Recent studies have shown that besides pathogen-derived ligands, PRRs can also recognize self-derived ligands such as oxidation-specific epitopes formed during apoptosis and oxidation (6). These epitopes can also be recognized by natural antibodies (Abs) produced by innate B cells.

The adaptive immunity

The adaptive immune system is triggered when a pathogen evades the innate immune system and generates a threshold level of Ags. The adaptive immune responses are initiated in the local lymphoid tissue in response to Ags presented by dendritic cells. Ag-specific T cells and Ab secreting B cells are generated by clonal expansion and differentiation.

The B cell receptor immunoglobulin

B cells originate and mature in the BM, they account for 10-15% of circulating lymphocytes and are the main component of the adaptive immune system with its chief functions being Ag presentation and Ab production in order to eliminate foreign pathogens (7). The human body makes millions of B lymphocytes each day that circulates in the blood and in the lymphatic system as naïve B cells that play a role of surveillance in the immune system. Each B cell has a unique BcR on its surface that recognizes Ags on foreign pathogens. Upon encounter and BcR-binding to the specific Ag, the naïve B cell undergoes Ab affinity maturation and differentiates either into a long-lived memory B cell or an Ab secreting plasma cell. The BcR is also an active and dynamic signal transmitter that forwards signals from the surrounding microenvironment, which can induce the cell to proliferate, become anergic, edit its BcR or undergo apoptosis. The outcome of Ag stimulation depends on multiple factors such as the cells in the surrounding microenvironment, co-receptor interaction, and the type and affinity of the Ag.

The immunoglobulin structure

The IG molecule is composed of four polypeptide chains, two identical light chains (LC) and two identical heavy chains (HC) (Figure 1). Each chain has both a variable (V) and a constant (C) region. The four chains are joined in the final IG molecule to form a flexible Y-shape. At the tip of each arm of the Y-shaped molecule is an area called the Ab-combining site, which is formed by a portion of the HC and LC. The V domain of IG HC is encoded by 3 types of genes called variable (IGHV), diversity (IGHD) and joining (IGHJ), while the V domain of LCs is encoded by IGKV/IGLV and IGKJ/IGLJ genes only. Each V domain can be split into four regions of relatively constant sequences termed the framework regions (FRs) and three regions with high sequence variability, termed the complementarity determining regions (CDR1, CDR2 and CDR3). The CDR3 is at the junction of the IGHV-IGHD-IGHJ genes and has the highest variability. Constant regions have essentially the same amino acid sequence in all Ab molecules of the same class (IgG, IgM, IgD, IgA, or IgE) and specifies the isotype of the Ig and exert various effector functions (8-10).

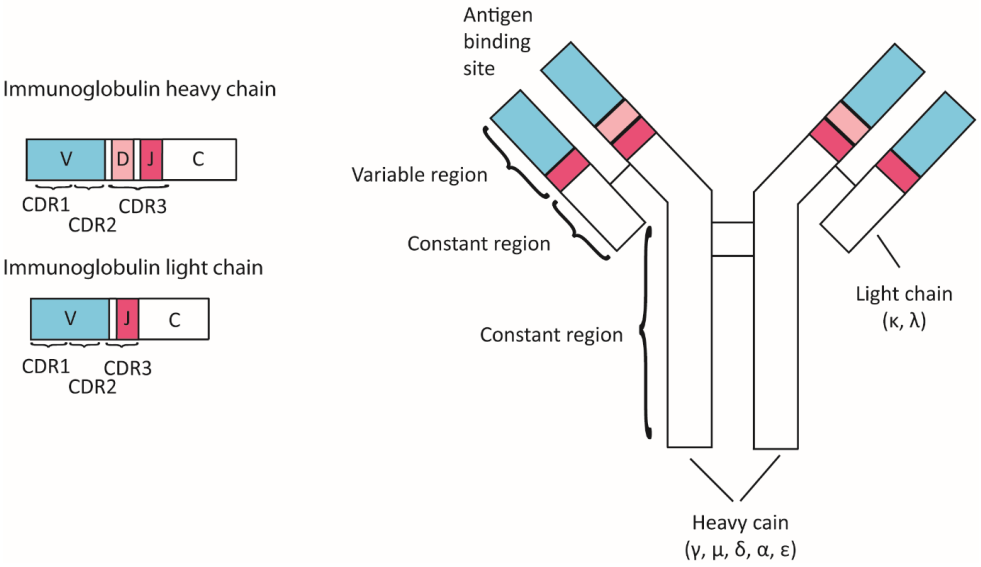

Figure 1. *Immunoglobulin structure*

B cell development and generation of immunoglobulin diversity

Antigen-independent rearrangement

Early B cell development occurs stepwise in the bone marrow and is dependent on interaction with the surrounding stromal cells for their differentiation and proliferation. The earliest B-lineage cells are called progenitor B cells (pro-B cells) and derive from lymphoid stem cells. Pro-B cells express the surface markers CD19 and CD43. It is in this developmental stage the IGHV gene rearrangement starts with the joining of an IGHD gene to an IGHJ gene, followed by the joining of an IGHV gene to the rearranged IGHD-IGHJ genes in the late pro-B cell. Productive IGHV-IGHD-IGHJ rearrangement leads to expression of a precursor (pre)-BcR which in combination with a surrogate light chain is found both in the cytoplasm and on the surface of the large pre-B cell. Signaling through this receptor induces cell division, giving rise to small pre-B cells in which the rearrangement of the IGH locus halts and the light-chain rearrangement starts. The small-pre-B cell now loses the surface expression of CD43 but retains the expression of CD19. Once a productive light chain, κ or λ, is produced, a complete IgM-molecule is expressed on the cell surface and together with signaling chains, Igα and Igβ a functional BcR complex is formed (7, 11, 12). The cell is now defined as an immature B cell. The BcR is now tested for central tolerance to self-Ags. If the B cell turns out to be strongly self-reactive, depending on the nature of the Ag, the cells can have different fates; either they die through apoptosis or clonal deletion, produce a new receptor through receptor editing, or become anergic. Immature B cells that do not bind strongly to self-Ags are allowed to mature, leave the BM and migrate to the spleen as a mature naïve B cell (7). The primary repertoire of IGs that takes place without Ag involvement has been formed.

B cell interaction with antigen

B cell activation requires two distinct signals, and results in B cell differentiation into memory B cells or plasma cells. The first activation signal occurs upon Ag binding to the BcR. Upon binding to the BcR, the Ag is internalized by receptor-mediated endocytosis, digested, and complexed with MHC II molecules on the B cell surface (13). The second activation signal occurs either via a T-dependent or a T-independent (TI) mechanism. Most B cell responses to Ag require the interaction of B cells with T helper cells. Activated

T cells provide a second activation signal to the B cell, which can occur through a variety of proteins. However, there are a few types of TI Ags that can directly provide the second B cell activation signal, i.e. type 1 (TI-1) and type 2 (TI-2) (13). While secondary signals in a TI-1 response can be given by TLRs, TI-2 Ags usually contains highly repetitious molecules such as bacterial or viral polysaccharides, which can extensively cross-link the BcR and thus activate the B cell (14).

The germinal center reaction

Germinal centers (GCs) are important sites of B cell clonal expansion, somatic hypermutation (SHM), and affinity based selection, which in the end results in in the production of high affinity Abs (Figure 2) (15). Upon encounter with Ag in the circulation or in the primary follicle the naïve B cell migrate to the T cell zone of the lymphoid tissue. In the T cell zone the B cell becomes fully activated after interaction with $CD4^+$ T cells and Ag-presenting cells (15, 16). The Ag selected B cells are induced to differentiate into short-lived Ab producing cells or enter GCs. Ag activated cells differentiate into centroblasts which undergoes clonal expansion and forms the dark zone of the GC. During proliferation the centroblasts undergo the second phase of IG diversification which is achieved by SHM, and class switch recombination (CSR). SHM introduces single nucleotide exchanges into the rearranged IGV region, giving rise to mutated BcRs on the surface of the B cell with altered Ab affinity for Ag (17, 18). SHM is associated with DNA strand breaks and requires the activity of activation-induced cytidine deaminase (AID) (18). Eventually some centroblasts reduce their rate of division, differentiate into centocytes and move to the light zone. In the light zone these smaller non-dividing centocytes are situated in a mesh of follicular dentritic cells (FDCs), T cells and macrophages. Clones with the highest affinity for Ag are favored for survival and develops into Ab-secreting cells. In CSR the original C region is replaced by an alternative C region, in this way the functional diversity of the IG repertoire is increased. The first Ag receptors expressed on a B cell are of IgM and IgD isotypes. Later in an immune response the same assembled V region can be expressed in IgG, IgA or IgE Abs. Ag selected centrocytes eventually differentiate into memory or plasma cells (15, 19, 20).

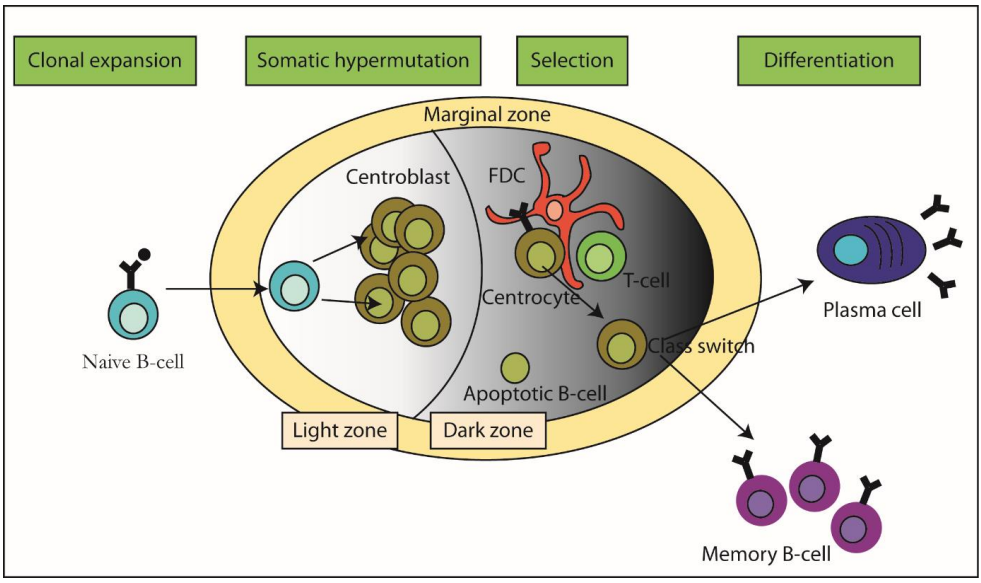

***Figure 2**. Germinal center reaction*

Tolerance mechanisms

About 50-75% of newly generated B cells are autoreactive and must be silenced by tolerance mechanisms to prevent autoimmune diseases (21, 22). This silencing is known to occur by three different mechanisms; receptor editing, clonal deletion or anergy, while anergy seem to be the most common mechanism used (23, 24). Receptor editing occurs when the primary IG gene rearrangement is modified so that it gains different specificity, by undergoing a secondary LC rearrangement at the IGK or IGL locus. If the cells fail to eliminate high affinity for self, the cells undergo clonal deletion, which is mediated by apoptosis of the autoreactive cells in the BM (25, 26). The decision of which silencing mechanism to use is dependent on receptor affinity, and autoantigen avidity. Higher avidity favors deletion and editing. Low avidity favors anergy. The immune system is constantly challenged to discriminate between self vs non-self and mediate the right response. Ag binding to cell surface Ag-receptors in combination with secondary signals from i.e. CD40L, cytokines, chemokines or TLR agonists leads to transduction of downstream signaling pathways. Chronic Ag-receptor stimulation in the absence of secondary signals however leads to anergy (27). Chronic BcR stimulation induce biased mono-phosphorylation of immune receptor tyrosine based activation motifs (ITAMs) which in turn lead to biased activation of Lyn. Lyn activate inhibitory signaling pathways

involving SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1) and SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP). Anergized B cells are characterized by low sIgM, as a result of constant BcR internalization and recycling, elevated basal intracellular Ca^{2+} concentration, and subsequent constitutive activation of extracellular signal-regulated kinase (ERK)1/2 (28).

B cell subsets

In humans, different B cell subpopulations can be distinguished in peripheral blood and other tissues on the basis of differential expression of various surface markers. These different subsets correspond to different stages of maturation, activation and differentiation and play different roles in the immune system. B1 cells or marginal zone (MZ) B cells have been discussed as potential origin for CLL. These subsets are described below.

B1 cells

B1 cells have been best described in mouse and the identity and existence of a human counterpart to murine B1 cells have been debated. B1 cells play critical roles in immediate defense against microbial invasion and in housekeeping scavenging of cellular debris (29-32). They are the main producers of natural IgM Abs, which are germline encoded, highly polyspecific, spontaneous secreted in the absence of infection and bind to both self-Ags and microbial Ags (33-35). B1 cells are the main B cell population in the peritoneal and pleural cavities. They develop earlier than follicular (B2) cells hence the name B1. In addition B1 cells differ functionally from B2 cells in efficiently presenting Ag to T cells, furthermore they show some similar characteristics to anergic B cells, such as tonic signaling with high cytoplasmic free Ca^{2+} concentration in the absence of specific stimulation. They are non-responsive to BcR engagement and cannot mobilize Ca^{2+} or proliferate in response to BcR-cross-linking. Instead they have an enhanced ability to respond to innate immune signals such as TLR agonists (36-39). Two subpopulations have been found in mice; B1a and B1b. Both subsets share phenotypic characteristics and are $CD19^{hi}CD45^{lo}CD43^{+}CD23^{-}IgM^{hi}IgD^{lo}$. B1a cells express CD5, while B1b cells do not (36). B1b cells are regulated separately from B1a and also have distinct functions. IGHV gene usage is much more restricted in B1 cells than adult B2 cells. B1 cells secrete Abs that are specific for self-Ags such as oxidized lipids and Ags on apoptotic cells. In addition

B1 cell derived Abs have specificity for pathogen expressed molecules such as phosphorylcholine and LPS. Recently, Griffin et al (40) found a small subset of B cells in human PB and cord blood that express CD20, CD27 and CD43 that show several functional characteristics similar to murine B1 cells. The main characteristics of this cell population were spontaneous IgM secretion, efficient T cell stimulation, and tonic intracellular signaling. An intense debate is still going on however if these "human B1 cells" really are true B1 or if they are pre-plasmablasts (41-44).

Marginal zone B cells

Marginal zone (MZ) B cells reside foremost in the MZ of the spleen but in humans they can also be found circulating in blood. MZ-like regions exist also in tonsil subepithelial areas, dome regions of Peyer patches, and subcapsular regions of LNs. They are characterized by a $sIgM^{high}$, $sIgD^{low}$, $CD27^{+}$, $CD23^{-}$, $CD21^{+}$, $CD1^{+}$, $CD5^{-}$ phenotype. Pathogens reaching the blood-stream are efficiently trapped in the MZ by macrophages, MZ B cells then respond rapidly to the pathogens, in a T cell independent way, by developing into plasmablasts which secrete IgM. They can express either mutated or unmutated IGVs however, about 90% of all MZ B cells bear SHM (45).

Chronic lymphocytic leukemia

Epidemiology

CLL accounts for about 30% of mature B cell malignancies (46) and is the most common form of adult leukemia in the Western countries with approximately 500 cases diagnosed annually in Sweden. The incidence rate is twice as high in men compared to women with a median age at diagnosis of 72 years (47-49). Few, if any, environmental factors have been linked to CLL, rather there seem to be a genetic and familial predisposition for the pathogenesis of the disease. Epidemiological studies show that there is a familial susceptibility in approximately 5-10% of cases (50). Furthermore the disease has been shown to appear more frequently in certain geographic areas, Western Europe and North America and is very rare in for example Asia (51, 52).

CLL is characterized by the accumulation of high numbers of long-lived, monoclonal B lymphocytes infiltrating BM, PB, and lymphoid tissue. The characteristic immunophenotype of lymphoid cells in CLL is weak surface IgM, with or without IgD, positive for surface CD5, CD19, CD20 (weak), CD22 (weak), CD79a, CD23, CD43 (53, 54). Immunophenotyping is used as a diagnostic criteria as well as a lymphocytosis of > 5 x 10^9 B lymphocytes/L in PB (55). Clinical staging can be performed according to Binet or Rai staging systems (Tables 1 and 2 respectively) (56, 57).

Table 1. Clinical staging according to Binet (56)

Stage	Number of involved lymph node areas	Hemoglobin g/L	Platelets x10^9/L
A	< 3	> 100	> 100
B	> 2	> 100	> 100
C	any	< 100	< 100

Table 2. Clinical staging according to Rai (57)

Stage	Blood and bone marrow involvement	Lymphadenopathy	Splenomegaly or hepatomegaly	Hemoglobin g/L	Platelets x10^9/L
0	Yes	No	No	> 109	> 99
I	Yes	Yes	No	> 109	> 99
II	Yes	Yes or No	Yes	> 109	> 99
III	Yes	Yes or No	Yes or No	< 110	> 99
IV	Yes	Yes or No	Yes or No	any	< 100

Prognostic markers

Prognostic factors traditionally used in the clinic are Rai and Binet and the lymphocyte doubling time (LDT: the time required for doubling of the absolute lymphocyte count). Although these factors are helpful in defining the extent of the disease and of disease progression, they cannot predict the long-term clinical prognosis of patients with low-stage disease, which today concerns the majority. The variability in the clinical course of CLL has driven researchers to look for better prognostic markers that can be used for CLL prognostication and can lead to a more individualized treatment strategy. Several prognostic markers based on genetic, phenotypic, and molecular characteristics of CLL B cells have emerged in the past decade.

IGHV gene analysis

The fact that half of the CLL patients have somatically mutated IGHV genes was already reported in 1994 (58). In 1999 the prognostic importance of this characteristic was demonstrated by two independent groups (59, 60). They showed that CLL patients with mutated (M) IGHV genes had a median overall survival significantly longer compared with patients with unmutated (UM) IGHV genes. A cut-off of more than or equal to 98% identity to the corresponding germline IGHV gene is classified as UM. IGHV mutational status is a reliable marker and does not change over time, it is an established and commonly used prognostic marker in CLL.

Expression of biomarkers

CD38 is a transmembrane glycoprotein expressed on the surface of B cells and other immune cells. It is involved in interaction with the microenvironment, cell-signaling, adhesion and Ca^{2+}-flux (61). CD38 has been of much interest as a prognostic marker in CLL. Patients with a high percentage within the CLL clone of CD38 positive cells are more prone to activation, are more often IGHV UM and have a more aggressive disease. In contrast those with CD38 negative cells have M IGHV genes, a more indolent disease course, requires no or minimal treatment and have a longer survival (59, 62). Further studies by others have revealed however, that this correlation with the IGHV mutational status is not absolute and that CD38 expression may change over time (63). Furthermore, there are also discussions on which is the best cut-off value for determining positivity. The initial cut-off value of 30% was later lowered to 20% and then to 7% (64, 65). Nonetheless, despite these controversies, CD38 expression has proven to be a valuable prognostic marker in CLL.

ZAP70 (70-kDa zeta-chain-associated protein kinase) is an intracellular protein important for cell signaling. It was first described in T cells and natural killer cells, but was later also found in normal B cells, particularly when activated (66-68). ZAP70 was identified to be expressed in CLL B cells and also to be the most differentially expressed gene when CLL patients with M and UM IGHV genes were compared, high expression level of ZAP70 have been shown to correlate to a more aggressive disease (69, 70). Although it is a valuable independent prognostic marker, there has been problems with the standardization of the protocol and techniques used for ZAP70 analysis.

Genomic aberrations

Chromosomal aberrations have been found by fluorescence in-situ hybridization (FISH) technique in 82% of CLL patients, however no common single genetic aberration have been found in all CLL cases (71).

Deletion at 13q14 is the most frequently occurring chromosomal aberration, occurring in approximately 55-60% of all cases (71). It is, as sole aberration, associated with a favorable prognosis and indolent disease course. It was recently discovered that deletion of this region is associated with the loss of microRNAs (miRNAs), miR15a and miR16-1 (72). miR15a/16-1 negatively regulates the expression of the anti-apoptotic protein BCL2 and the deletion of these miRNAs correlates with overexpression of the *BCL2* gene (73).

Deletion at 11q is the second most common alteration in CLL and occurs in about 12-18% of cases, most often in patients with UM IGHV, and is associated with poor clinical outcome (71). The Ataxia telangiectasia mutated (ATM) gene is identified as the most likely candidate gene in del(11q) CLL. ATM is crucial for DNA repair and known to play a role in activating p53 (74).

Trisomy 12 is the third most common alteration and occurs in about 11-16% of cases (71). It is associated with an intermediate survival and shorter time to treatment. Trisomy 12 has recently been shown to be associated with mutations in the *NOTCH1* gene (75, 76).

Deletion of 17p occurs in about 7% of CLL cases (71) and patients with this aberration always suffer from a very aggressive clinical course and chemotherapy resistance, because of loss of *TP53*. Most patients with del(17p) carry mutations within the remaining *TP53* allele (77, 78). The p53 tumor suppressor protein plays a key role in inducing apoptosis and cell cycle arrest after DNA damage (79).

Novel mutations

In recent years, with new generation sequencing (NGS) technique available, researchers have been able to find several new mutated genes with prognostic value. Some of these genes include *NOTCH1*, *MYD88, SF3B1* and *BIRC3* (75, 80-83). *NOTCH1* and SF3B1 will be described below.

NOTCH1 mutations, a ligand activated transcription factor gene, occur in about 8-12% of patients at diagnosis and have been associated with a shorter time to first treatment and shorter overall survival (75, 76). A high frequency of *NOTCH1* mutations have been found in patients with trisomy 12 (84, 85).

Mutations of the ***SF3B1*** gene were documented in 5-17% of patients and were associated with advanced stage disease and with short time to first treatment and overall survival (80).

Treatment

CLL is the most frequently diagnosed leukemia in the Western world, yet it remains essentially incurable. Prognostic markers have evolved that help to predict the clinical course and to find high risk patients. Therapy of CLL has therefore significantly improved the past decade, resulting in prolonged survival.

In general newly diagnosed asymptomatic patients with early stage disease should be monitored without treatment and the standard approach for these patients is a watch-and-wait strategy (86). Treatment of asymptomatic early stage disease patients with alkylating agents does not give any survival advantage (55, 87). When the disease start to progress the standard and most effective treatment is combination immunotherapy with fludarabine, cyclophosphamide and rituximab (FCR). This combination of purine analogue, alkylating agent and monoclonal CD20 Ab gives an overall response rate of about 90% (88, 89). This treatment regimen can, however, be associated with high toxicity, which of course is a significant problem because most CLL patients are older. Other treatment options are therefore needed for the elderly. Patients with aggressive disease with poor prognostic markers such as del(17p) are resistant to fludarabine. Allogeneic hematopoietic stem cell transplant remains the only potentially curative treatment for patients with CLL, but is associated with high rates of treatment related mortality. This treatment should only be considered for young patients with poor prognosis (55).

Recent studies on treatment options have been focused on BcR signaling pathway inhibitors. The understanding of the importance of BcR signaling for CLL pathogenesis has opened up for the development of small molecule inhibitors of BcR-associated

kinases, including spleen tyrosine kinase (SYK), bruton´s tyrosine kinase (BTK) and phosphoinositide 3 kinase (PI3K). Inhibition of these kinases leads to redistribution of lymphoma cells from LN to PB and promote apoptosis of CLL cells *in vitro* (90, 91) Ibrutinib is a BTK-inhibitor, which blocks BcR signaling *in vitro* and *in vivo*. It impairs microenvironmental induced survival, proliferation migration and adhesion of CLL cells (90, 92-94). Ibrutinib is now approved by the FDA for use in CLL patients. Idelalisib is a PI3K-inhibitor, which as in analogy with Ibrutinib inhibits microenvironmental and BcR signals and reduce CLL cell survival and migration (95-97). Idelalisib was recently approved by the FDA for use in CLL.

The CLL microenvironment and B cell signaling

Cellular microenvironment

Several studies indicate that the microenvironment has a critical role in CLL cell survival and accumulation (98, 99). CLL cells circulating in the PB are most often arrested in the G0/G1 stage of the cell cycle but a small proportion of cells accumulate in structures termed pseudofollicles or proliferation centers (PC) in BM and LN, a hallmark finding of CLL histopathology (100). The microenvironment in PC constitute a tight 'cellular architecture' similar to that found in normal GCs of Ag-driven proliferation (Figure 3). These consist of mesenchymal stromal cells (MSCs) in the BM, monocyte derived nurse-like cells (NLCs) and T cells in the LNs (101, 102). Microenvironmental interactions have been shown to be essential for inducing CLL cell survival and proliferation. In *in vitro* conditions CLL cells often undergo spontaneous apoptosis implying that *in vitro* cultured CLL cells lack essential survival signals that are present in the *in vivo* microenvironment. Unless CLL cells are cultured in the presence of other cell types or soluble cytokines and chemokines or other molecules that engage important cell surface receptors such as the BcR, CD38, and CD40, they undergo apoptosis more rapidly than normal B lymphocytes (101-104). Historically, CLL was viewed as an accumulative disease of cells with a defect in apoptosis. Although the CLL cells in the blood are regarded as non-dividing, a small fraction of CLL cells replicate in PCs in BM and LNs. Recent studies using deuterated water labelling showed that the birth rate of the CLL cells varied from 0.1% to greater than 1% per clone and day and therefore was more pronounced than previously thought (105).

The number of T cells is increased in CLL compared to healthy individuals (106). In PCs CLL cells interact with CD40-ligand (CD40L) expressing $CD4^+$ T cells that bind to CD40 on CLL cells, rescuing them from apoptosis (107). CD40-cross-linking also up-regulates CD80 and CD54 on CLL cells which makes them effective T cell stimulators (108). The cross-talk between CLL cells and the accessory cells is bidirectional, causing interaction of both cell types. In the PCs CLL cells also interact with MSCs or NLCs. NLCs differentiate from blood monocytes, and can attract CLL cells and protect them from undergoing spontaneous or drug-induced apoptosis. NLCs can be found in the spleen and in the secondary lymphoid tissue of CLL patients. Gene expression patterns from CLL cells co-cultured with NLCs and from CLL cells isolated directly from LN tissue are very similar with signs of BcR and nuclear factor (NF)-κB activation and up-regulation of BcR-target genes such as CCL3 and CCL4 (109, 110). In addition, CLL cells also secrete these chemokines following BcR-stimulation or after co-culture with NLCs (109). NLCs secrete CXCL12 (stromal cell derived factor-1 [SDF-1]; ligand for CXCR4), CXCL13, B cell activating factor (BAFF), a proliferation inducing ligand (APRIL)(111) and express CD31 (ligand for CD38), plexin B1, and vimentin (112), which all are important molecules for NLC-CLL cross-talk supporting CLL cell survival, and are important therapeutic targets. Also FDCs are important for the support of CLL cells, partly dependent on ligation of CD44 on CLL cells and up-regulation of the anti-apoptotic protein myeloid cell leukemia 1 (MCL1) (113) as well as CD100/plexinB1 cross-talk (114).

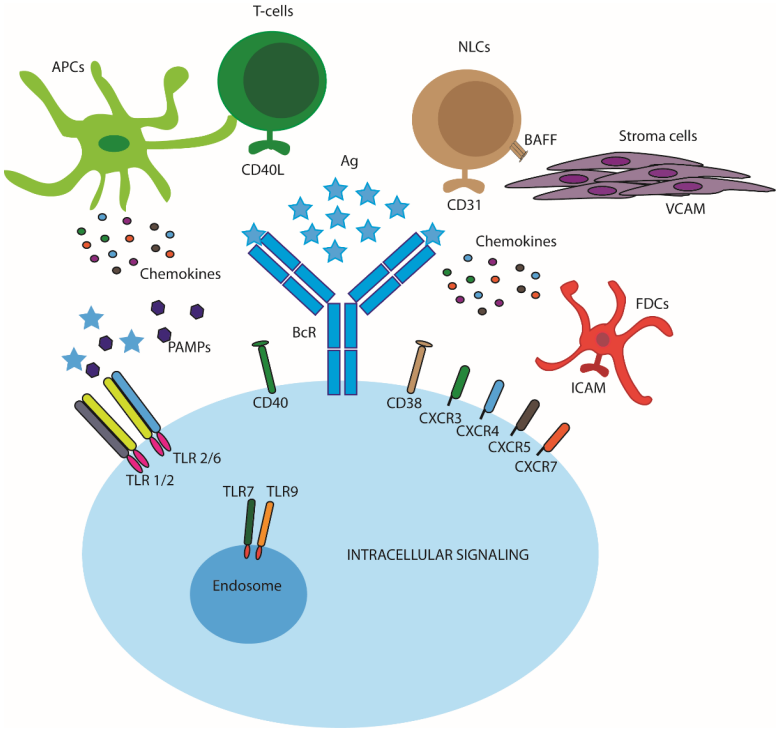

Figure 3. *Interactions of CLL cells with their microenvironment*

The redox system

ROS are products of normal metabolism and depending on concentration can be beneficial or harmful to cells and tissues. At physiological low levels ROS functions as signaling molecules in intracellular signaling and regulation, however excess ROS can induce oxidative modifications, inhibit protein function and promote cell death. Various pathologies, including cancer, can result from high oxidative stress (115). Therefore it is important that the cells have functioning reducing systems to maintain the redox balance. CLL cells suffer from a high level of ROS and oxidative stress, which renders them more dependent on the redox regulatory systems to maintain the redox balance (116, 117).

The three most important redox systems commonly found within the cells are: nicotinammide adenine dinucleotide phosphate ($NADPH/NADP^+$), thioredoxin

(*TRX_{red}/TRX_{ox}*) and glutathione (*GSH/GSSG*). In paper I in this thesis I have studied effects of Trx, therefore I will focus on the Trx system below.

Trxs are a family of proteins that have a conserved catalytic site (-Cys-Gly-Pro-Cys-) that undergoes reversible oxidation to the cystine disulfide (Trx-S2) through the transfer of electrons from the catalytic site cysteine residues (Cys) to a disulfide substrate (X-S2). The oxidized Trx is then reduced back to the Cys form [Trx-(SH)2] by the NADPH-dependent flavoprotein Trx reductase (Figure 4) (118). Human Trx-1 is a small 12 kDa protein. The central role of the Trx system is to provide a reducing environment and to protect the cell from effects of oxidative stress that ultimately leads to apoptosis. Trxs have been implicated in a number of mammalian cell functions and activity has been found outside the cell (cell growth stimulation and chemotaxis), in the cytoplasm (as an antioxidant and a reductant cofactor), in the nucleus (regulation of transcription factor activity), and in the mitochondria (119).

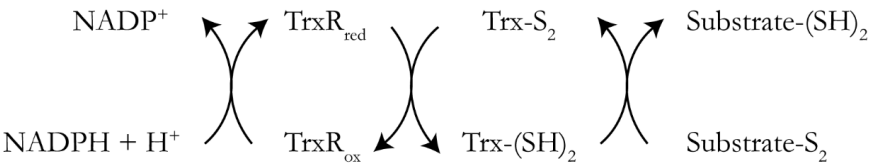

Figure 4. Scheme of reactions catalyzed by the thioredoxin-dependent system

Emerging evidence has shown that PTPs are highly susceptible to oxidation. The Cys residue in PTPs act as a redox sensor in response to proximal ROS. Oxidation of catalytic Cys residue results in enzymatically inactivated PTPs. The oxidized PTPs lose their enzymatic activity and are therefore incapable of dephosphorylating their substrates. In response to extracellular stimuli, which induce the production of active oxidants, PTPs are transiently inactivated allowing full activation of the protein tyrosine phosphorylation signaling cascade. Soon after the ROS burst is terminated, oxidized PTPs are converted back to the active state via GSH and other endogenous reductants, down-regulating tyrosine phosphorylation signals. If the cellular redox potential is altered and the redox-balance is destroyed, it might lead to pathological consequences including carcinogenesis. In cancer cells that generate large amounts of ROS the active site Cys of

PTPs might be constitutively oxidized, leading to uncontrolled tyrosine phosphorylation level.

A recent study reveal that BM stromal cells increase the ability of CLL cells to maintain the redox balance and promote cell survival and drug resistance by converting cystine to cysteine. Cysteine is then released into the microenvironment and CLL cells can in contrast to cystine take up cysteine and promote GSH synthesis which leads to increased CLL cell survival and drug resistance (120).

BcR signaling

The LN microenvironment is also an important site for BcR activation. BcR activation is most often induced by Ag, but can be ligand-independent (tonic), and triggers a down-stream signaling cascade that cause B cell selection, proliferation, differentiation and Ab production (7). In CLL, BcR signaling plays a critical role in pathogenesis, however the mechanism of BcR stimulation are heterogenous and not yet fully understood. Gene expression studies demonstrate that BcR-signaling is the key regulatory pathway activated in CLL cells in LNs (110). Studies have revealed that cases with high levels of CD38, ZAP70, and carrying UM IGHV are more responsive to BcR-triggering than are cases with M IGHV genes (121). M CLL cells usually show a more anergic profile with constitutive phosphorylation of signaling proteins, including ERK kinase and reduced levels of responsiveness to BcR stimulation (122, 123). Recent clinical trials with agents that target the BcR signaling pathway, such as inhibitors of SYK, BTK, and PI3Kδ, are showing considerable activity in patients with CLL, further underscoring that the leukemic cells rely on BcR signals for growth and survival (90, 92, 95).

Engagement of the BcR by Ag induces aggregation of BcR components that lead to phosphorylation of ITAMs in the cytoplasmic tails of Ig-α (CD79A) and Ig-β (CD79B) by LYN kinase which in turn activates SYK, BTK and PI3Kδ kinases and downstream pathways, including calcium mobilization, activation of phospholipase C (PLC)γ, protein kinase C (PKC)β, NF-κB-signaling, mitogen activated protein (MAP) kinases, and nuclear transcription (Figure 5). Collectively, these signaling events promote B cell survival and proliferation. The intensity and duration of the BcR signal are controlled, modulated and fine-tuned by various positive (CD19) and negative regulatory co-receptors and phosphatases. Phosphorylation of the inhibitory receptors CD22, FcβRIIb, CD5 and CD72

brings the phosphatases SHP-1 and SHIP-1 in the vicinity of the Ag-stimulated BcR and inhibit the signal by dephosphorylating activated components of the BcR-signaling pathway (124, 125). Importantly, some of these negative regulators are also activated by LYN, which functions as both a positive and negative regulator of BcR-signaling. Several molecules involved in BcR signal transduction, such as LYN, SYK, PIK3, and PKC are constitutively active in CLL (126-129).

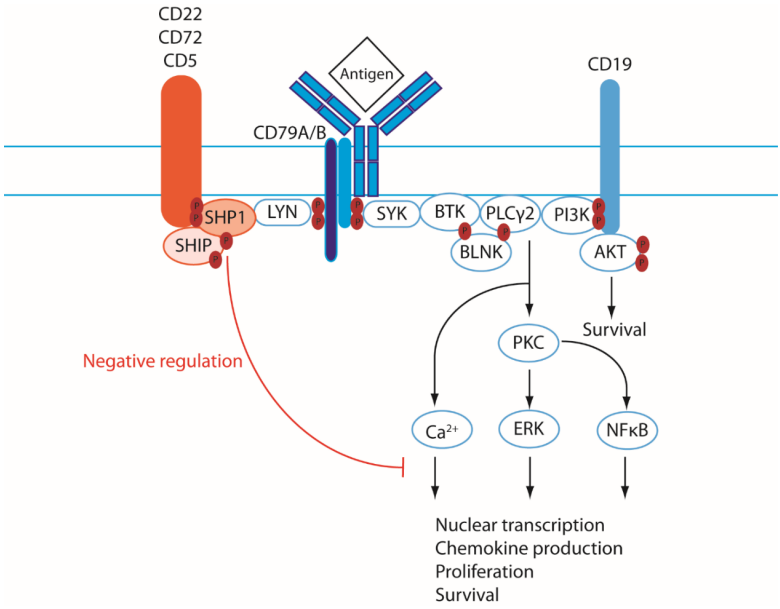

Figure 5. *B cell receptor signaling*

TLRs

Other key molecules such as TLRs have been shown to act concomitantly with the BcR and to have a role in CLL development and progression (130). TLRs are part of the innate immune system and trigger an immediate immune response upon engagement. TLRs can act to bridge innate and adaptive immunity by acting as co-stimulatory signals for B cells and induce maturation, proliferation and Ab secretion (131). TLR7 and TLR9 can together with the BcR participate in responses to autoantigens. Expression of TLRs in CLL B cells are similar to that of B cells from tonsil and memory B cells and express functional TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10 (132-134). Subgroups of CLL cases, especially

those belonging to subsets with stereotyped BcRs, show differential responses to immune stimulation through the TLRs, and these responses may extend to cell proliferation control, apoptosis, B-cell anergy, or TLR tolerance (130).

Stereotyped B cell receptors in CLL

CLL was previously thought of as a homogenous disease derived from naïve, Ag-inexperienced B cells. This view has over the past decade changed dramatically. Findings from immunogenetic studies that CLL patients can be subdivided into two groups with different prognosis depending on the mutational status of the rearranged IGHV genes (59, 60) started an interest among researchers for IG genes and the involvement of Ag in the disease pathogenesis. Today, it is well known and accepted that IGHV and IGLV gene usage in CLL B cells is highly biased and non-random showing preference for certain IGHV genes such as IGHV1-69, IGHV3-7, IGHV3-21 and IGHV4-34 (135, 136). Perhaps the strongest evidence for Ag involvement is the existence of different subsets of cases with highly restricted or "stereotyped" VH CDR3 sequences, characterized by shared amino acid motifs. This suggests that clones with stereotyped BcRs, recognize similar antigenic epitopes. There are today more than 200 subsets of patients that express such stereotyped BcRs (1, 135, 137, 138). More recent studies based on large amount of sequences collected from different countries, and with the development of new bioinformatics tools, reveal that stereotopy is more common amongst UM CLL than M CLL. Phylogenetically related IGHV genes can be members of the same group, and groups of patients share clinical and biological features such as for example genomic aberrations, epigenetic modifications and cell-signaling via innate and/or adaptive signaling pathways (2, 3, 139, 140). For example, subsets #1 (IGHV1/5/7/IGKV1-39), #2 (IGHV3-21/IGLV3-21) and #4 (IGHV4-34/IGKV2-30) have different clinical outcomes, with indolent disease in subset #4 and aggressive disease in subset #1 and subset #2 (141).

Antigens in CLL

The observations, as mentioned above, that a large number of CLL patients share stereotypic BcRs/mAbs suggests recognition of similar antigenic epitopes and a role for Ags in the disease onset and/or development. Early studies have shown that CLL Abs are polyreactive and recognize autoantigens such as single stranded DNA (ssDNA), double

stranded DNA (dsDNA), histones, cardiolipin or cytoskeletal components (142, 143). Along with the findings of stereotyped receptors in CLL and the development of new techniques to produce recombinant Abs the interest to search for Ag specificity took off again. The results that emerged from these studies revealed that only the UM CLL Abs had an auto- and polyreactive nature whereas the M CLL Abs had lost this ability due to SHM. Studies also showed that M CLL patient IGs regained their polyractivity when reverted back to its germline configuration, indicating that both UM and M CLL B cells derive from self-reactive precursors (144). Interestingly, the combined message from several studies regarding CLL IG specificity is that CLL Abs bind to molecular epitopes on apoptotic cells and that CLL Abs assigned to the same subset exhibit similar Ag-reactivity profiles. More specifically, CLL mAbs have been found to bind to vimentin, filamin B, cofilin-1, proline-rich acidic protein 1 (PRAP-1), phosphorylcholine, cardiolipin, non-muscle myosin heavy chain IIA (MYHIIA), and metabolites of lipid peroxidation all of which are exposed on the cell surface upon apoptosis (145-147). Not only self but also microbial, viral and fungal Ags have been recognized as targets of CLL BcRs (148). The same oxidized epitopes found on apoptotic cells are also exposed on some bacteria ie *Streptococcus pnemoniae* and on oxidized low density lipoprotein (oxLDL) (145). Viral infections have also been suggested to drive the CLL clone. For instance infections by Epstein-Barr virus or Cytomegalovirus have been associated with subset #4 (149). Autonomous signaling have also been described in CLL, where CLL derived Igs induce Ag-independent cell-autonomous signaling due to self-recognition of an intrinsic IGHV motif (150). However, the relevance of these interactions still remains unclear. A combination of signals generated by cell autonomous BcR interactions and interactions with external low-affinity autoantigens could be important to modulate the disease and to provide important co-stimulatory signals (151).

Origin of CLL

The transformation events that select normal B cells to become CLL B cells remain at present unknown. The cellular origin of CLL cells has been an area of considerable debate. It has been proposed that CLL cells derive from B1-like cells, MZ B cells or transitional B cells, based on cell surface phenotype and molecular and functional characteristics.

For about two decades ago CLL was thought off as an accumulative disease with cells originating from naïve non-proliferating immune incompetent cells. Later studies showed that CLL could be divided into subgroups with CLL clones using either M or UM IGHV genes which showed different clinical outcomes (59, 60). These observations gave rise to the hypothesis that the different subgroups originated from distinct cell types. UM CLL cells were to derive from naïve $CD5^+$ pre-GC B cells and the M CLL cells from a $CD5^+$ memory cell. This model have since been modified, since both subsets have been shown to express surface markers and gene expression profiles of activated, Ag-experienced cells. Furthermore, gene expression studies showed similarities to memory B cells and also revealed very few differences between the two subsets, suggesting a single precursor (152). However, the definition "Ag-experienced" do not need to be restricted to mature B cells reacting with foreign Ags but can apply to any cell regardless of maturation status, after the expression of a rearranged IGH.

Today, we know that CLL cells produce Abs with binding specificities resembling natural Abs. CLL BcRs are often skewed toward polyreactivity with binding to autoantigens as well as exoantigens. There are at present three different cell subsets discussed to be the CLL cell counterpart, the MZ B cell, the B1 cell and the transitional B cell. The MZ B cell shares many CLL cell features. MZ B cells can display an activated phenotype after Ag encounter. They express BcRs which is coded by UM and M IGV genes, which are polyreactive and can bind autoantigens as well as microbes. However, the MZ B cell has a different surface phenotype than CLL cells, in that they are $CD5^-CD23^-CD22^+$ (45). This difference in phenotype however could reflect activation status. The B1 cells produce germline encoded, polyreactive, autoreactive natural Abs, which serve as a part of the innate immune system to protect against microbes and to eliminate catabolic debris (153). Furthermore, it has been shown that B1 cells can give rise to a CLL like disease in aging mice (154). What contradicts B1 cells as CLL origin is the lack of a truly identified human counterpart and that they almost exclusively express UM *IGHV* genes (153). Transitional B cells express CD5, can be autoreactive and express both M and UM IGVs (155, 156). Findings against transitional B cells as CLL origin are expression of CD10 not present on CLL cells, absence of CD27 expressed on CLL cells and lack of responsiveness to BAFF which effectively supports CLL cell survival (156).

With the evidence for stereotyped receptors in some but not all CLL clones, the concept of a single cell origin of CLL is being challenged. Leukemogenesis in CLL may follow a long stepwise process, where transforming events can occur at any time during B cell maturation. Thus, it is possible that CLL can be derived from multiple CLL precursors (157).

DNA methylation

The current definition of epigenetics is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence". Epigenetic modifications include methylation of cytosine bases in DNA, post-translational modifications of histone proteins as well as the positioning of nucleosomes along the DNA. Not all genes are active at all times. DNA methylation is one of several epigenetic mechanisms that cells use to control gene expression. Methylation of DNA is a common epigenetic signaling-tool that cells use to lock genes in an off-position. Proper DNA methylation is essential for cell differentiation and embryonic development. DNA methylation in mammals primarily occurs by the covalent modification of cytosine residues in CpG dinucleotides of DNA which are converted to 5-methylcytosine by DNA methyltransferase (DNMT) enzymes. The altered cytosine residues are usually immediately adjacent to a guanine nucleotide, resulting in two methylated cytosine residues sitting diagonally to each other on opposing DNA strands. In mammals CpG dinucleotides are concentrated in GC rich regions where high CpG contents are found, these regions are called CpG-islands. CpG islands are positioned in the 5´ end of many human genes and occupy ~60% of human gene promoters. The majority of CpG islands remain unmethylated during development and differentiation allowing gene expression to occur. In contrast most of the CpG sites scattered in the genome are methylated (158-160). Given the critical role of DNA methylation in gene expression and cell differentiation, failure of the proper maintenance of epigenetic marks can result in inappropriate activation or inhibition of various signaling pathways and lead to disease states such as cancer.

DNA methylation in tumorigenesis

Normal cells have genes that promote cell proliferation (oncogenes) and those that suppress cell proliferation (tumor suppressor genes). Mutations of oncogenes can result

in constitutive activation of these genes. In contrast mutations and/or chromosomal deletions of suppressor genes result in their inactivation. Oncogene activation and tumor-suppressor gene inactivation can lead to uncontrolled cell proliferation and development of cancer. Recent research have shown that not only mutations and/or deletions of certain genes but also aberrant DNA methylation contribute to cancer pathogenesis. 3-6% of all cytosines are methylated in normal human DNA. Methylable CpG dinucleotides are not randomly distributed in the human genome but instead CpG-rich regions known as CpG islands, which span the 5´ end region of many genes, are usually unmethylated in normal cells. In cancer cells, the CpG-island promoter of some tumor-suppressor genes are hypermethylated and therefore silenced (Figure 6). DNA methylation is regarded as a relatively stable modification and is now considered one of the hallmark mechanisms of aberrant gene silencing in cancer.

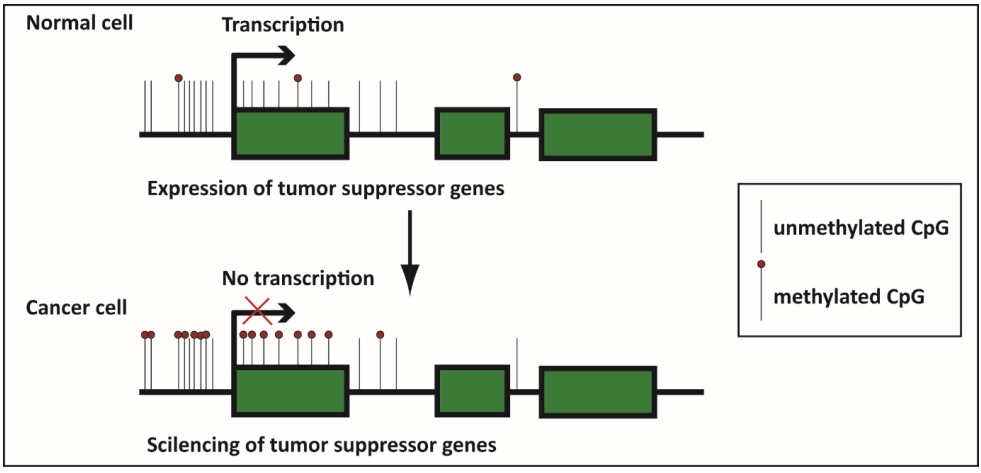

Figure 6. DNA methylation in normal and cancer cells

Role of DNA methylation in CLL

Aberrant DNA-methylation has over the past two decades evolved as a key player in the pathogenesis of CLL. Early studies of DNA methylation revealed the importance of hypermethylation as a key event promoting genomic instability and oncogene activation in CLL and other cancers. Later studies revealed DNA from CLL patients to be globally hypomethylated compared to PB cells from normal healthy individuals (161) which is true also for other cancers. In the early days of epigenetics, methylation patterns were determined in single genes. In this way, several gene promoters have been shown to be

hypermethylated and some of these can also be coupled to disease progression and survival. DNA methylation of *TWIST2,* a transcription factor and known silencer of p53, has shown to be more frequent within IGHV M CLL patients, (162) similarly silencing of *HOXA-4* (163), transcription factor important for cell development, and *ZAP70* (164) through DNA methylation also correlated to IGHV mutational status. With the development of new more global, high-resolution microarray and sequencing technologies, it became possible to determine DNA methylation on a global level. Kanduri M *et al* demonstrated a differential methylation pattern distinguishing poor prognostic IGHV UM from favorable prognostic IGHV M CLL patients. Several tumor suppressor genes were shown to be methylated in UM cases. Furthermore, genes involved in cell signaling such as MAPK and NF-κB were shown to be unmethylated in UM cases (165). Comparison of methylation profiles of three distinct stereotyped subsets with different prognosis; subset #1 and #2 (poor-prognosis) and subset #4 (favorable prognosis), revealed significantly different methylation profiles. Interestingly the majority of the differentially methylated genes were involved in B cell activation. Furthermore, these genes were found to be more methylated in subset#1 than in subset#2 and #4 (166). Recently it was shown that M CLL and UM CLL maintain an epigenetic signature of memory B cells and naïve B cells, respectively (167). Later the same group showed that this epigenetic classification was a strong predictor of time to first treatment along with Binet stage (168). In recent years dysregulation of miRNAs have been demonstrated (166) in CLL, which has an effect on the expression of tumor suppressor and oncogenes (169-171). The mechanisms underlying altered miRNA expression are poorly known but could be due to epigenetic manipulation. Bear et al. have by integrating DNA methylation and miRNA promoter data identified 128 recurrent miRNA targets for aberrant promoter DNA methylation in CLL (172).

Epigenetic based treatment

Since epigenetic modifications do not involve changes in the DNA sequence, the change is reversible. Methylated genes can be re-expressed after treatment with DNA methyltransferase inhibitors. The only approved DNA methylation inhibitor present is 5´-Azacytidine and is used for patients with myeloplastic syndrome only (173).

AIMS OF THE THESIS

The overall aim of this thesis was to study the influence of the microenvironment on the B cells of both peripheral blood cells and cells from lymph nodes. The specific aims were as follows:

Paper I

- To study the production of thioredoixn from the microenvironmental cells in the CLL LN compartment.
- To access the influence of thioredoxin on CLL cell viablility.

Paper II

- To study the binding of CLL subset #1 Abs to native antigen malondialdehyde modified low density lipoprotein (MDA-LDL)
- To evaluate the effect of antigen binding to CLL cell BcR on cell activation and/or proliferation.

Paper III

- To study DNA methylation profiling of different prognostic subgroups.
- To evaluate changes in the global DNA methylation profiles of CLL patients over time and in patient-matched LN and PB samples.

Paper IV

- To study SHP-1 expression and activity in CLL patients using matched LN and PB samples.

MATERIALS AND METHODS

Since detailed description of methods used is written in the material and methods section in each paper, I will only present the patient material below.

Patient samples and patient characteristics

Primary CLL cells from patient PB and/or LNs were used in Paper I-IV.

In Paper I, LNs from 13 CLL patients and PB from five CLL patients were collected from consecutively attending patients at the Hematology Clinic at Linköping University Hospital. In addition, LN sections from 12 CLL patients were collected at the Department of Pathology at Uppsala University Hospital.

In Paper II, PB samples from 12 CLL patients belonging to the stereotyped subset #1 (Clan I IGHV/IGKV1(D)-39) were collected, six patients from Papanicolaou Hospital, Thessaloniki, Greece, four patients from Rigshospitalet, Copenhagen, Denmark and two patients from Uppsala University Hospital.

In Paper III, a total of 36 PB samples were obtained from the Swedish part of the SCALE study. For DNA-methylation studies sorted PB samples from 9 IGHV mutated and 9 IGHV unmutated cases were analyzed. PB samples from these same patients were also collected at later time-points for DNA-methylation analysis over time. In addition, 10 patient-matched PB and LN samples were collected from Linköping University and Uppsala University hospitals. PB from three age-matched normal individuals and one whole-genome amplified DNA derived from PB were included as controls.

In Paper IV, LNs and matched PB samples were collected from 6 CLL patients. Five patients from Linköping University Hospital and one patient from Uppsala University Hospital. In addition PB was collected from 14 CLL patients from Linköping university Hospital.

All samples were diagnosed according to the WHO classification and the international Workshop on Chronic Lymphocytic Leukemia criteria (IWCLL) (55). In Paper I all samples were analyzed unsorted. In Paper II some samples were negatively selected for $CD19^+/CD5^+$ cells before experimentally used and some were used unsorted as detailed in the paper. In Paper III and IV all samples were $CD19^+/CD5^+$ sorted. Patient characteristics are outlined in each paper. Informed consent was obtained according to the Declaration of Helsinki.

Ig gene sequencing

In Papers I-IV IG gene sequencing was performed by PCR amplification of DNA using IGHV gene specific primers. The PCR products were sequenced and aligned against germline sequences in the IMGT® database using the IMGT/V-QUEST online tool (http://www.imgt.org) (174). Samples with less than 98% homology to the germline IGHV sequences were considered as mutated. Patients were assigned to different stereotypic subsets following recently established criteria

Cell purification

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-gradient centrifugation (GE Healthcare) and were negatively selected using magnetic beads specifically developed for CLL cell purification (MACS microbeads, Miltenyi Biotec) when indicated. The $CD5^+/CD19^+$ cells were >97% as assessed by flow cytometry.

Stromal cells including FRC and FDC were purified from CLL lymph nodes and tonsil tissue. Single cell suspensions were retrieved after mincing the lymph nodes and tonsils in a stainless steel mesh. The cells were seeded in tissue flasks and adherent cells were fed every fourth day by replacing 50% of the medium until confluence. Phenotype of the adherent cells was analyzed by flow cytometry.

RESULTS AND DISCUSSION

Thioredoxin produced by stromal cells influence CLL cell survival (Paper I)

The CLL BM and LN microenvironment has been shown to be essential in several ways for CLL cell survival and proliferation. CLL cells undergo spontaneous apoptosis when cultured *in vitro* which implies that they lack essential growth factors present in the *in vivo* microenvironment. In this paper, we wanted to further elucidate important growth-promoting interactions and focused on the Trx system. Trx is a small redox-active and multifunctional molecule expressed at low amount in all cells in the body, containing an active site motif with amino acid Cys-X-X-Cys (118) that undergoes reversible NADPH-dependent reduction by selenocysteine containing flavoprotein Trx reductases. It is a potent antioxidant and protein disulfide oxidoreductase. Intracellular Trx has anti-apoptotic as well as growth-promoting effects and additionally, some types of cells have the capacity to release Trx where the extracellular form of Trx has cytokine and chemokine activities (175). Interestingly, exogenous Trx was previously shown to protect malignant CLL cells from apoptosis *in vitro* (176). In paper I, we analyzed the expression of Trx *in vivo* in frozen sections from CLL LNs, and *in vitro* by culturing the Trx expressing cells. Furthermore, we analyzed the effect of secreted Trx on CLL B cells.

In this study, we found Trx in CLL LNs to be highly expressed in cells with macrophage- and/or fibroblast/dendritic-like morphology (Figure 7), the tumor cells themselves however expressed very low levels. The Trx expressing cells were identified as FDCs and follicular reticular cells (FRCs). Expression of Trx correlated with the presence of stromal cells and proliferating Ki-67$^+$ leukemic cells and the Trx expressing cells were mainly localized to and surrounded by Ki-67$^+$ leukemia cells, indicating that Trx is a potential tumor survival factor. Furthermore, these stromal cells were able to spontaneously secrete Trx *ex vivo*. Co-culturing of stromal cells with CLL B cells extended B cell survival, which could be blocked by a neutralizing Trx Ab (Figure 8), indicating that stromal derived Trx is a pro-survival factor for CLL B cells.

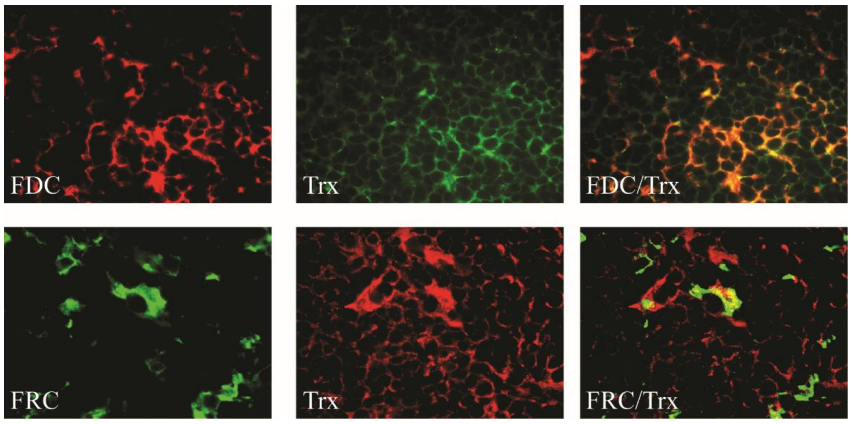

Figure 7. In vivo expression of thioredoxin

Stromal cells (FDCs; Follicular dendritic cells and FRCs; Follicular reticular cells) overexpress thioredoxin. Frozen lymph node sections were stained with antibodies for FDC or FRC together with anti-Trx (2G11).

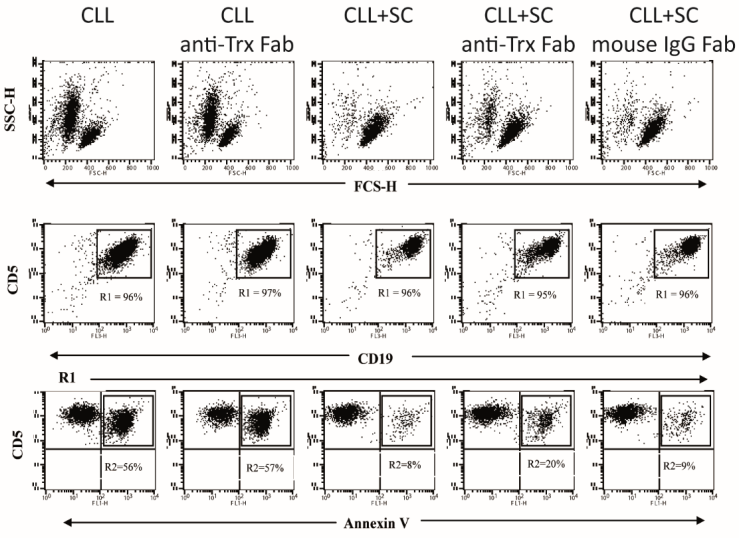

Figure 8. Thioredoxin-mediated improvement of leukemic cell survival after co-culturing with stromal cells from CLL lymph nodes.

Leukemia cells from the blood of CLL patients were cultured with and without stromal cells purified from tonsils or CLL lymph nodes in the presence or absence of anti-thioredoxin Fab fragments

The detailed molecular mechanism behind the improved Trx-mediated CLL survival remains to be elucidated. However, it is known that the multifunctional Trx protein exerts several redox-regulatory functions and physically associates with a large number of target proteins, modulating their three-dimensional structure (and functions) by catalyzing thiol-disulfide exchange reactions. Trx, through its redox activity, regulates the activity of enzymes such as apoptosis signal-regulating kinase 1 (ASK-1) (177) and PKC α, γ ,ε, and δ (178) and either directly or indirectly modulate the DNA binding activity of transcription factors, including NF-κB (179), the glucocorticoid receptor (180), p53 (181) redox protein redox factor-1 (Ref-1) (182) and activator protein-1 (AP-1) (183), thus affecting various aspects of cellular responses including redox homeostasis, cell growth, and survival. Trx is a key protein in inducing synthesis of several cytokines, including IL-4, interferon-γ, and TNF-α, which are known to exert survival effects on CLL cells (184). Recently it was demonstrated that Trx catalytically interacts with a single principal target protein on the surface of lymphocytes. This target protein was identified as the TNF receptor superfamily member 8 (TNFRSF8) also known as CD30 (185). CD30 is a receptor of activated lymphocytes involved in the regulation of inflammation (186). Trx catalyzes disulfide exchange dynamically, activating or inactivating the CD30 pathway in response to the redox environment, thus affecting lymphocyte effector functions (185). Furthermore, we have shown (see paper III) that *TNFRSF8* is differentially methylated in IGHV M and IGHV UM CLL cases.

Trx expression is increased in several human primary cancers, including lung, colon, cervix, liver, pancreatic, colorectal, and squamous cell cancer (118). In contrast to other cancers where Trx has been shown to be overexpressed in the tumor cells *per se,* we found Trx to be minimal expressed in CLL cells but overexpressed in the accessory cells surrounding the leukemic cells. However despite the source, Trx seem to be linked to aggressive tumor growth and inhibition of apoptosis (187). Recently, in line with our results, Li et al found Trx to be highly expressed in diffuse large B cell lymphoma (DLBCL), not only in the tumor cells but also in stromal cells in the surrounding microenvironment (188). A recent study show that transfection of human MCF-7 breast cancer, human HT-29 colon cancer and mouse WEHI7.2 lymphoma cell lines with Trx-1 have increased levels of HIF-1α protein during both normoxic and hypoxic conditions, which promote angiogenesis (189). This may provide a mechanism to explain the aggressive tumor

growth observed in tumors overexpressing Trx. Inhibitors targeting Trx pathway provide a promising therapeutic strategy for cancer prevention and intervention (190-192), however no such studies have been performed in CLL.

Chronic autoantigen-BcR engagement induce un-responsiveness/anergy in subset #1 CLL (Paper II)

Several lines of evidence suggest that the development and evolution of CLL is dependent upon the interplay between genetic defects and stimuli originating from the microenvironment, including important cellular components and soluble factors as well as antigenic stimulation through the BcR. Molecular and functional studies support the concept that CLL cells are Ag-experienced, where the strongest evidence for Ag involvement is the existence of different subsets of cases with highly similar or "stereotyped" IGs in their BcRs, indicating selection by Ag in CLL ontogeny (193, 194). Within stereotyped subsets, similarities between different cases extend from BcR IG sequences to biological and prognostic features, clinical presentation and even outcome (2, 193-195).

Studies by our group, confirmed by others, showed that CLL Abs may recognise oxidation-specific neo-epitopes on lipoproteins and apoptotic cells. Interestingly, these structures may also share molecular identity with epitopes on infectious pathogens so called molecular mimicry (145, 147, 196, 197).

Stereotyped subset #1 is the largest subset among the IGHV UM CLL with frequencies about 2.5%-3% (2, 3, 140, 198). We recently found that IgM from subset #1 CLL cells bind to oxidized phospholipids (145), more specifically to malondialdehyde (MDA). MDA is a major degradation product of unsaturated lipids reacting with reactive oxygen species. Although there is no doubt that Ag-BcR interaction is of great importance in the pathogenesis of CLL this interaction has not been studied with cognate Ag. The discovery of subset #1 cognate Ag enabled us to study the contribution of antigen in triggering proliferation and/or differentiation of CLL cells.

In paper II, we hypothesized that the cognate multivalent Ag oxLDL could induce a full proliferative response on its own in this poor-prognostic subset. However, in contrast to

our expectation, although subset #1 Abs were shown to bind specifically to oxLDL (Figure 9), by analyzing BcR-signaling events we found that oxLDL alone was not sufficient for induction of Ca^{2+}-flux, proliferation, or IgM secretion, indicating a state of BcR unresponsiveness/molecular anergy including constitutive ERK1/2-phosphorylation. Interestingly, this unresponsiveness could be relaxed after 48 h in culture (Figure 10).

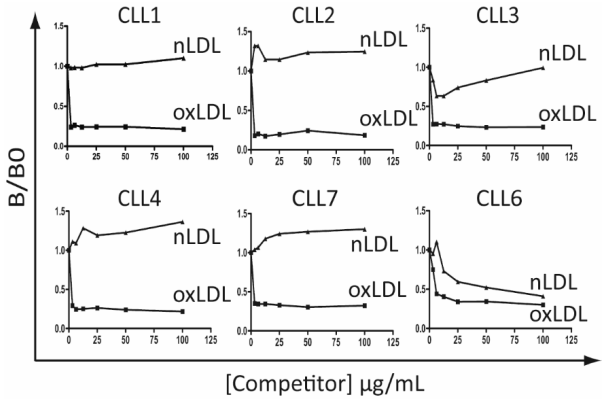

Figure 9. Subset #1 CLL IgM antibodies bind specifically to oxLDL

Specificity of secreted subset #1 CLL IgM Abs tested in competition ELISA. B/B_0 indicates ratio of IgM bound in the presence of competitor (oxLDL or nLDL) divided by IgM bound without competitor.

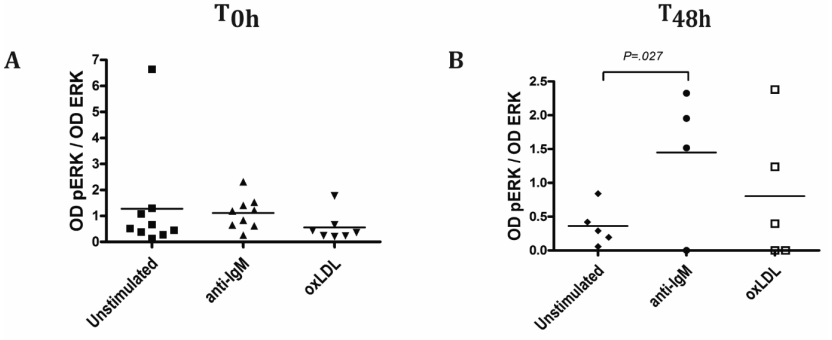

Figure 10. BcR responsiveness at start (0 h) and after 48 h of in vitro culture.

(A). *Subset #1 CLL cells were analyzed for phosphorylation of ERK1/2 by Western blot analysis at basal level (T0h), and* ***(B)*** *after 48-h in vitro culture (T48h) in the presence or absence of the indicated stimuli for 5 min*

It has been assumed for many years that CLL cells are anergic since they have very low surface expression of IgM and do not respond properly to BcR stimulation. BcR unresponsiveness in a context of B cell anergy is brought about by a condition in which self-reactive B cells are silenced upon chronic exposure to low-affinity autoantigens *in vivo*. Anergized B cells are characterized by low sIgM, as a result of constant BcR internalization and recycling, elevated basal intracellular Ca^{2+} concentration, and subsequent constitutive activation of ERK1/2 (28). Previous studies have shown that CLL cells are heterogeneous in their ability to respond to stimulation via the BcR (122). UM CLL cases preferentially respond to cross-linking with anti-IgM, exhibiting prominent Ca^{2+} mobilization and phosphorylation of intracellular kinases, whereas M CLL cases usually are more unresponsive and therefore regarded as being more anergized. Anergy appears to be isotype specific since cases that fail to signal via surface IgM are capable of signaling via surface IgD. Observations that expression of surface IgM and the ability to signal upon its engagement, could be recovered by incubation *in vitro*, strongly suggests that Ag engagement occurs *in vivo* and that the cells partly recover when they circulate in the blood (122, 123, 199).

Not only the BcR but also several other molecules/receptors are important for the B cells to sense their microenvironment, including CD40, TLRs, BAFF and APRIL. Evidence is increasing that membrane associated as well as endosomal TLRs have a role in CLL development and progression (130, 200). TLR-stimulation provides a signal that synergizes with BcR-triggering and T cell help to amplify human B cell responses to Ag (201). Hence, stimulation with TLR-agonists increases the expression of co-stimulatory molecules, which in turn raise the surface expression of activation markers such as CD25 and CD86 (123, 133, 202). Furthermore, functional interactions between the BcR and TLR signaling pathways are implicated in the control of B cell anergy. As recently shown, subgroups of CLL cases, especially those belonging to subsets with stereotyped BcRs, exhibit differential responses to immune stimulation through the TLRs, and these responses may extend to cell proliferation control, apoptosis, B cell anergy, or TLR tolerance (130). The state of paralysis may be recovered by exogenous or endogenous factors breaching the unresponsiveness and this is relevant for understanding CLL clonal dynamics. Indeed, co-signals from exogenous microbes or, alternatively, aberrant signals via endogenous innate receptors such as NOTCH1 may circumvent normal controls. TLR-

signaling has been reported to breach B cell anergy (28) as was also the case in roughly half of the CLL cases of the present study. Most likely, therefore, a combination of several signals (including environmental/microbial TLR-ligands) are required to surpass a critical threshold for allowing S-phase entry in these CLL cells.

DNA methylation patterns: a comparison between resting and proliferative compartment as well as over time (Paper III)

Recently, using 27K DNA methylation microarrays, Kanduri et al recognized differential methylation pattern for IGHV UM and IGHV M CLL patients (165). They found a number of proliferation related genes to be unmethylated in the IGHV UM subgroup of patients. In paper III, we wanted to study DNA methylation pattern in CLL on a larger scale, utilizing 450K global arrays. More specifically, we sought to determine DNA methylation changes over time and to search for changes attributed to alternative microenvironments, i.e. resting and proliferative compartments. A total of 2239 CpG sites were found to be differentially methylated between IGHV M and U CLL, with the majority of sites outside of known annotated CpG islands and within gene bodies, in line with the recent findings by Kulis et al (167).

Ontology analysis of 1826 differentially methylated genes identified a number of enriched physiological and molecular functionalities, most of which has not been implicated in CLL, however many of the identified genes were members of recognized signaling pathways and also genes shown to be involved in other cancers.

A more focused search for genes implicated in CLL revealed differential methylation of known CLL prognostication genes (summarized in Table 3), such as *ZAP70, LPL* and *CLLU1.* We found *ZAP70* to be preferentially methylated in IGHV M cases compared to IGHV UM cases, this is in line with results from Corcoran *et al*. (164). Recent studies have shown that methylation of *ZAP70* correlates well to *ZAP70* expression (164). For the first time, the CLL prognostic genes *LPL* and *CLLU1*, were found to be epigenetically regulated. Gene expression of *LPL* and *CLLU1* has been shown to be higher in IGHV UM patients compared to IGHV M cases Accordingly, higher DNA methylation of the *CLLU1* gene was

present in IGHV M CLL compared with IGHV UM cases. Also higher DNA methylation of the *LPL* gene was found in IGHV M compared to IGHV UM cases (203-205). Interestingly, we found *NOTCH1* to be higher methylated in IGHV M than IGHV UM subsets. *NOTCH1* has been identified as mutated gene in CLL and a predictor of poor survival (76). Furthermore, we found differentially methylated genes related to apoptosis (*LEF-1* (206) and *TCF3* (207)), proliferation (*CD80* (208), *CD86* (209)) and BcR signaling pathways (*IBTK* (210)). Other genes found were related to epigenetic regulation (*HDAC, HDAC9, DNMT3B*).

Table 3. Functionalities and genomic positions of differentially methylated CpG sites

Gene	Function/ implication in cancer	Normal B cell controls	MUTATED CLL	UNMUTATED CLL	Region in relation to CpG Island ǂ	CpG site(s) location within gene structure ǂ
BCR related signaling genes						
IBTK	B cell signaling inhibitor (210)	**Hypermeth**	**Unmeth**	**Hypermeth**	Shore	TSS1500
Survival related genes						
LEF1	Lymphoid enhancing binding factor Involved in WNT signaling. Pro-survival factor in CLL (206)	**Hypermeth**	**Meth**	**Unmeth**	NA	Gene body
TCF3	Transcriptional regulator. Regulation of B cell proliferation and VDJ recombination. Suspected regulator CD38 expression (211)Transcriptional regulator. Regulation of B cell proliferation and VDJ recombination. Suspected regulator CD38 expression (211)	**Hypermeth**	**Unmeth**	**Hypermeth**	Shore	Gene body
Prognostic CLL genes						
ZAP70	T and B cell signaling regulator Upregulated in unmutated CLL (69)	**Meth**	**Hypermeth**	**Unmeth**	NA	TS1500
CLLU1	CLL upregulated gene Upregulated in unmutated CLL (204)	**Hypermeth**	**Hypermeth**	**Unmeth**	NA	Gene body
LPL	Lipoprotein lipase Upregulated in unmutated CLL (212)Upregulated in unmutated CLL (212)	**Hypermeth/ Meth**	**Hypermeth/ Meth**	**Unmeth**	Shore	Gene Body TSS1500
NOTCH1	Transmembrane (76) protein receptor Mutated in CLL	**Low meth**	**Meth**	**Unmeth**	Shore	Gene Body
Epigenetic regulating genes						
DNMT3B	DNA methyl- transferase (213) Suspected Aberrant de novo DNA	**Unmeth**	**Meth**	**Unmeth**	Shore	5'UTR

HDAC4	Histone deacetylase, Deactylases histones (214)	**Hypermeth**	**Unmeth**	**Hypermeth**	Island/ Shore/Shelf	Gene body
HDAC9	Histone deacetylase Deactylases histones (214)Histone deacetylase Deactylases histones (214)	**Hypermeth**	**Unmeth**	**Meth**	NA	Gene Body
Tumor Suppressors						
ABI3ˇ	Tumor suppressor (215) Tumor suppressor (215) Implicated in lung metastasis	**Meth**	**Unmeth**	**Meth**	NA	Gene body/TSS200
WISP3ˇ	Tumor suppressor (216) Expression loss in breast cancer. WNT signaling	**Hypermeth**	**Hypermeth**	**Low meth /Meth**	NA	TSS200/ 1st Exon/ 5'UTR
Proliferation/survival related genes						
CD86ˇ	B-cell co-stimulatory molecule (208)	**Hypermeth**	**Meth**	**Hypermeth**	NA	5'UTR/body
CD80	B-cell co-stimulatory molecule (208)	**Hypermeth**	**Unmeth**	**Hypermeth/Meth**	NA	TSS1500
BCL2L1	Apoptosis regulator (217) BCL2L1 underexpressed in CLL	**Hypermeth/ Meth**	**Unmeth**	**Hypermeth**	Shore	Gene body
SPNˇ	B cell development Cell trafficking and lymphocyte activation.(218)B cell development Cell trafficking and lymphocyte activation.(218)	**Hypermeth**	**Hypermeth**	**Meth**	Shelf	TS1500
FAS	Member of TNF receptor family Regulation of programmed cell death (219)Regulation of programmed cell death (219)	**Hypermeth**	**Low Meth**	**Hypermeth**	Shore	Gene body
MYB	Proto-oncogene Regulates miR 155 in CLL (220)	**Meth**	**Hypermeth**	**Low meth**	Shelf NA	Gene Body 3'UTR

Unmeth; ≤0.2 in average beta value, Low Meth; 0.3-0.4 in average beta value, Meth >0.4-0.6 in beta value, Hypermeth; >0.7 in average beta value. ˢ Note the methylation status provided maybe indicative of the methylation status at more than one site for the listed gene. In some cases the CpG site may be represented in different transcripts and therefore in different gene regions.

One of the enriched canonical pathways highlighted through ingenuity pathway analysis (IPA) was the "molecular mechanisms of cancer pathway". Within this pathway we could identify established genes/pathways implicated in CLL, such as genes belonging to the TGF-β and the NF-κB/TNF pathways. The NF-κB/TNF pathway is highly interesting since TNF, TNFRSF8 (CD30), and two TNF-receptor genes, TNFSF1A and TNFRSF1B, recently shown to be redox controlled by protein-disulfide isomerase (PDI) in complex with Trx (221), were found to be differentially methylated in M and UM CLL.

Currently, the extent to which DNA methylation levels change with respect to time and treatment remains largely unknown in cancer. In the second part of paper III, we sought to determine changes of for the global DNA methylation pattern potentially occurring over time. By comparing paired diagnostic and follow-up (5-8 years) samples, we could for the first time show that the global methylation pattern is relatively stable over time. No recurrent differentially methylated sites were noted over time within the IGHV M subgroup and very few changes in the IGHV UM cases. When analyzing the intra-individual changes however, a larger difference in the number of non-recurrent differentially methylated sites were noted over time between IGHV M and IGHV UM cases. More specifically, analysis revealed IGHV UM patients to have a higher number of differentially methylated sites compared with IGHV M patients when measured longitudinally. These non-recurrent changes in IGHV UM cases are however minor (overall global changes <1%), taking into account the 485,000 sites available on the array.

The activity status and proliferative activity are known to differ in CLL PB and LN compartments. Recently, differential gene expression profiles have been found for patient-matched samples originating from the PB and LN in CLL. Genes involved in proliferation and active BcR signaling were shown to be highly expressed in the LN compartment compared with the PB (110). In the third part of paper III, we hypothesized that DNA methylation may partake in the mechanism of differential gene expression seen within the different compartments of CLL. In contrast to our hypothesis, while comparing patient-matched CLL cells derived from PB and LN, we found that global DNA methylation is rather similar in the two compartments. In summary, it seems like, unlike gene expression, DNA methylation of CLL cells from resting and proliferative compartments is less influenced by microenvironmental factors.

SHP-1 expression and activity: comparison of resting and active compartments in CLL (Paper IV)

SHP-1 is a protein phosphotyrosine phosphatase (PTP) which modulates intracellular signaling and thus regulates cell activation, proliferation, differentiation, and migration. It has also been shown to contribute to maintenance of tolerance to self-Ags (124). SHP-1 is a negative regulator of signal transduction induced by a number of cell receptors including the BcR. SHP-1 is often downregulated in lymphoid malignancies and it has been shown that lymphoma/leukemia cells with highly aggressive profile lack SHP-1 expression *in vitro,* suggesting that loss of SHP-1 expression might be associated with both malignant transformation and tumor cell aggressiveness (222, 223). As also described above, there are now several lines of evidence highlighting the importance of chronic Ag stimulation and BcR signaling in CLL pathogenesis (2, 3, 140, 145, 224). Considering that SHP-1 is a key phosphatase for the control of BcR signaling, we hypothesized in paper IV that, SHP-1 expression and/or activity should be lower in the LN proliferative compartment compared with the resting PB compartment. However, in contrast to our hypothesis, SHP-1 expression and activity/phosphorylation status did not differ significantly in PB CLL cells as, compared to LN derived CLL cells from the same donor.

SHP-1 protein was previously found to be equally expressed in CLL PB cells and normal B cells from healthy donors (225). In CLL, SHP-1 was expressed both in cytosolic and membrane fractions in contrast to normal B cells where it was only expressed in the cytosol. In CLL, CD5 is phosphorylated by LYN, and by this phosphorylation becomes a docking site for SHP-1. Once at the plasma membrane, SHP-1 is phosphorylated by LYN only at tyrosine residues and hence becomes activated and thus can de-phosphorylate downstream signaling molecules. SHP-1 in the cytosol is phosphorylated at serine S591 and its activity is thus inhibited (226). While phosphorylation of tyrosine Y536 and Y564 have been shown to activate SHP-1 and to increase its PTP activity, phosphorylation of serine S591 have been shown to be inhibitory when targeted by PKC (227, 228). We studied SHP-1 mRNA, protein expression and phosphorylation of whole cell lysates from CLL PB and LN. Although we see differences in SHP-1 mRNA levels with higher levels of SHP-1 expressed in PB than in LN, we could not show any significant differences in SHP-1 protein levels (Figure 11).

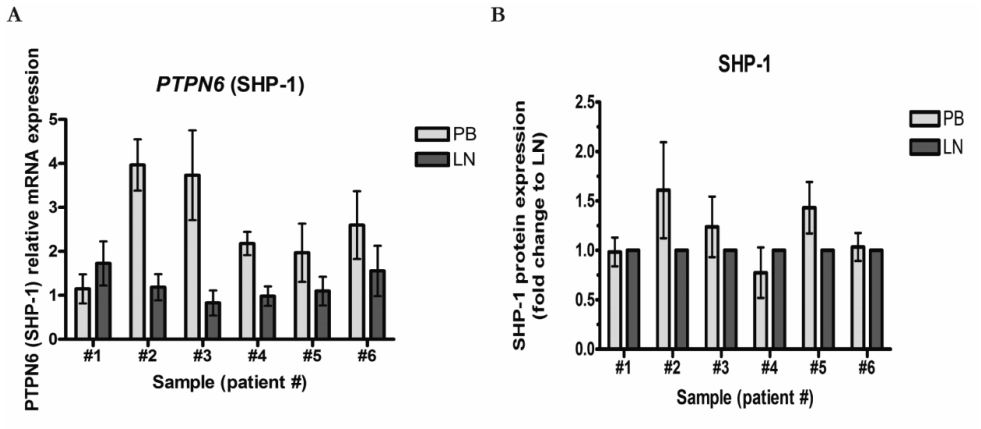

Figure 11. PTPN6 and SHP-1 expression in PB and LN samples

$CD5^+CD19^+$ cells from 6 matched PB and LN patient samples were purified by negative selection. ***A****. PTPN6 (SHP-1) gene expression levels were determined by Q-PCR.* ***B****. Total cell extracts were prepared and the amounts of SHP-1 and β-actin were determined by Western blot analysis. Densiometric analysis were performed and relative expression was calculated as optical density (OD) ratio of SHP-1/OD β-actin.*

We could detect SHP-1 phosphorylation on both tyrosine and serine residues in all patients, however no significant differences were found in the two compartments (Figure 12). Phosphorylation of SHP-1 was however, down-regulated in BcR/ anti-IgM stimulated vs unstimulated PB cells (Figure 13).

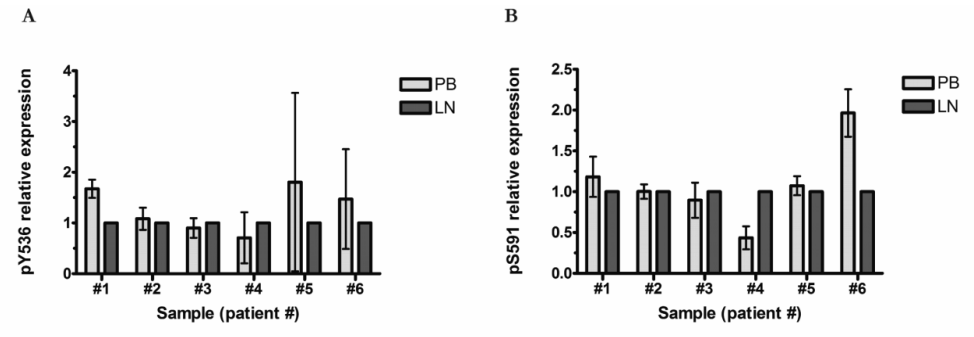

Figure 12. SHP-1 phosphorylation in matched PB and LN samples

$CD5^+CD19^+$ cells from 6 matched PB and LN patient samples were purified by negative selection. Whole-cell lysates were analyzed by immunoblotting with anti-pY536-SHP-1, anti-pS591-SHP-1, anti-SH-PTP1 or anti-β-actin. Densiometric analysis were performed and relative expression was calculated as optical density (OD) ratio of pY536/OD of total SHP-1 (A) or OD ratio of pS591/OD of total SHP-1 (B) and normalized to β-actin.

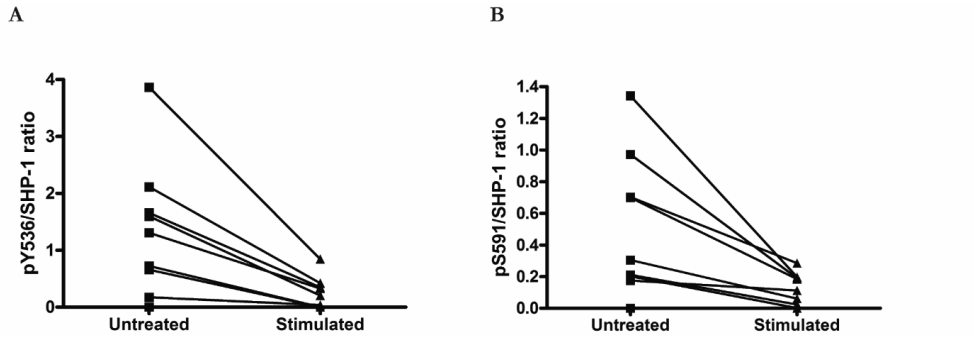

Figure 13. Effect of anti-IgM in PB cells on SHP-1 phosphorylation

*Purified $CD5^+CD19^+$ cells from 10 matched PB and LN patient samples were cultured in the absence or presence of 16 μg/ml anti-IgM for 48 h. Whole-cell lysates were analyzed by immunoblotting with anti-pY536-SHP-1, anti-pS591-SHP-1, anti-SH-PTP1 or anti-β-actin. Densitometric analyses of untreated and anti-IgM treated (stimulated) samples are shown and data are presented as optical density (OD) ratio of pY536/OD of total SHP-1 (**A**) or OD ratio of pS591/OD of total SHP-1 (**B**) and normalized to β-actin.*

In conclusion, we see de-phosphorylation/inactivation of SHP-1 *in vitro*, however *in vivo* SHP-1 is still activated and phosphorylated in activated LN cells. B cells with an anergic/unresponsive profile is maintained *in vivo*. This is in line with findings in B1 cells in which CD5-SHP-1 association persists upon BcR engagement. This limits B cell response and retain B1 cells in its anergic state (229). This observation is noteworthy, since CLL B cells share several properties with B1 B cells, including $CD5^+$ expression and the anergic condition (224, 230). Studies have shown that the active site cysteines of PTPs can be oxidized, thus inhibiting its enzymatic activity. This could also be the case in our study. Further studies are necessary to evaluate activity of SHP-1 in cells from PB and LN.

CONCLUSIONS

In this thesis we have investigated influence of the cellular microenvironment as well as antigen on the pathogenesis of chronic lymphocytic leukemia and we found that:

Paper I

- The small redox molecule Trx was secreted from FRCs and FDCs positioned in the proliferation centers in close contact with proliferating CLL cells. Inhibition of Trx in culture promoted increased cell death, implicating an important role for Trx in CLL cell survival. Trx is probably one soluble molecule among several others important for survival and cell growth of CLL cells.

Paper II

- Cognate antigen for the aggressive CLL subset #1, oxLDL, could not activate or induce proliferation in subset #1 CLL cells. This inability to respond to antigen via BcR was due to un-responsivity/anergy as could be shown by high basal level of pERK as well as regained responsiveness after 48h antigen wash-out. Furthermore, TLR9 engagement was able to breach the anergic state in some patients. These results indicates that clonal expansion in CLL subset #1 are brought about by several other factors and or that sub-clones with different responsivity exists.

Paper III

- On a larger scale than previously reported we revealed a large number of differentially methylated CpG sites between IGHV M and IGHV UM cases, with the majority of sites positioned outside annotated CpG islands. For example the known CLL prognostic genes *CLLU1*, *LPL*, *ZAP70* and *NOTCH1*, the inhibitor *IBTK* gene and numerous TGF-β and NF-κB/TNF pathway genes were alternatively methylated between subgroups. These data support the notion that aberrant DNA methylation contributes to the differential outcome that can be seen in different prognostic CLL subgroups.

- When searching for differences in DNA methylation over time we could conclude that the global methylation pattern was rather stable with few recurrent changes noted within subgroups, indicating that aberrant DNA methylation events are early events in CLL pathogenesis. Also between patient-matched CLL cells derived from different compartments, PB (resting) and LN (proliferating), the global DNA methylation was rather similar. Overall, it appears that unlike gene expression, DNA methylation of CLL cells from different compartments is less influenced by microenvironmental factors.

Paper IV

- SHP-1 protein expression and activity/phosphorylation status in PB CLL cells, compared to LN derived CLL cells from the same donor, did not differ significantly. Absence of SHP-1 protein and *PTPN6* gene downregulation and/or activation in proliferative centers/LNs *in vivo* may be part of a strategy for anergy maintenance in CLL, or alternatively it may indicate loss of function by oxidation or mutation of the catalytic site amino acids.

Taken together, this thesis, add another piece to the puzzle, on how the microenvironment and antigens influence CLL pathogenesis. Since great variations among individuals are seen, further studies in different groups of patients are necessary to elucidate the importance of antigen for the development of CLL.

In the long run, studies on microenvironmental influence, cell signaling, genomic and epigenetic modifications, comparing different subsets of patients, could lead to a more individualized treatment.

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