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**The expression and signalling patterns of CD180 toll like receptor
in Chronic Lymphocytic Leukaemia (CLL)**

Sayed, U.

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**The expression and signalling patterns of CD180 toll like receptor
in Chronic Lymphocytic Leukaemia (CLL)**

Uzma Sayed

A thesis submitted in partial fulfilment of the requirements of the University of
Westminster for the degree of Doctor of Philosophy.

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ABSTRACT

Chronic lymphocytic leukaemia (CLL) is characterised by a progressive accumulation of mature CD5+CD20+CD23+ lymphocytes. Despite the remarkable progress in our understanding of the immunobiology of CLL, the aetiology of the disease remains unknown. The consensus is that CLL cells are driven by (auto)antigen(s) through the B cell receptor (BCR) and are regulated by a variety of signals received from the microenvironment, including toll-like receptors (TLR). Our group has previously shown that engagement of the CD180 orphan TLR expressed by approximately 60% of CLL cells, can re-wire the sIgM-mediated signalling from a pro-survival pathway, involving phosphatidylinositol-4,5-bisphosphate3-kinase (PI3K) and protein kinase B (AKT) to the potentially pro-apoptotic pathway through mitogen-activated protein kinase (p38MAPK).

However, little is known about the function of the other BCR - sIgD in CLL and its possible interaction with CD180. Here we studied intracellular signalling and apoptosis of CLL cells following sole or sequential ligation of CD180 and sIgD.

Our data indicated that following sequential ligation of CD180 and sIgD, CLL samples demonstrated enhanced p38MAPK phosphorylation leading to increased apoptosis of CLL cells indicating synergistic relationship between CD180 and sIgD. To better understand the prognostic importance of CD180 expression we sought to determine whether CD180 and other prognostic markers such as CD38 and ZAP70 displayed any correlation with the known cytogenetic aberrations: *TP53* and *DLEU1*. Our results suggested that CLL cells with *DLEU1* deletion are characterised by the negative expression of both, CD180 and CD38, and this might have a significance for CLL prognosis.

To explain this correlation, we hypothesised that interaction of CLL cells with their microenvironment through TLRs leads to the expansion of leukaemic clones, *in vivo*, in lymph nodes. Our results indicated that CD180 is heterogeneously expressed in the paraffin tissue sections of the lymph nodes of CLL patients and its expression positively correlates with the expression of Ki-67.

Our data demonstrated, that although CD180 expression and signaling might have negative prognostic importance in CLL due to the enhanced proliferation of leukaemic cells, its interaction with sIgD would re-direct leukaemic cells towards apoptosis thus opening new opportunities for the disease immunotherapy.

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Finally, I am grateful to God Almighty for giving me the good health and wellbeing that was necessary to complete this PhD.

Acknowledgement of Contribution

I would like to particularly thank all those who contributed in many ways for the completion of this project. My profound appreciation goes to Darellyn Oo for helping with FISH imaging and analysis. I am thankful to Kristina Zaitseva for performing Immunohistochemistry staining of lymph nodes. A special thanks to Nadeeka Rajakaruna for helping me in the early stages of my PhD journey.

Declaration

I, Uzma Sayed, hereby declare that the PhD thesis titled “The expression and signalling patterns of CD180 toll like receptor in Chronic Lymphocytic Leukaemia (CLL)” is a result of research work carried out by me under the supervision of Dr. Nina Porakishvili. Moreover, I confirm that this thesis has not been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:

Date: 22.11.2019

List of Abbreviations

µl	· microlitre
ABT-199	· Venetoclax
ADCC	· Antibody dependent cellular toxicity
ADP	· Adenosine diphosphate
AKT	· Protein Kinase B
APCs	· Antigen Presenting Cells
AP-1	· activator protein 1
APRIL	· A proliferating inducing ligand
ATM	· ataxia telangiectasia mutated
BAFF	· B cell activating factor
BCL2	· B cell lymphoma 2
BCMA	· B cell maturation transmembrane activator
BCR	· B CeLL Receptor
BLNK	· B-cell Linker protein
BM	· Bone Marrow
BRAF	· proto-oncogene B-Raf
BTK	· Bruton's Tyrosine Kinase
CAL-101	· former name of Idealisib
CART	· Chimeric antigen receptor modified T cells
CAP	· Cyclophosphamide doxorubicin prednisone
CD	· Cluster of differentiation
CDA	· Chlorodeoxyadenosine
CLL	· Chronic Lymphocytic Leukaemia
CR	· Complete Remission
CXCL12	· C-X-C motif chemokine ligand 12
DAG	· Diacylglycerol
DD	· Death domain
DAMP	· Damage-associated molecular pattern
DISC	· Death inducing signaling complex
DLBCL	· Diffuse large B Cell Lymphoma
DNA	· Deoxyribonucleic acid
ERK	· Extracellular signal regulated kinase
F(ab)	· Fragment antibody with no Fc portion
FBS	· Foetal Bovine Serum
FC	· Fludarabine cyclophosphamide
FC	· Flow cytometer
FCR	· Fludarabine cyclophosphamide and rituximab
FDC	· Follicular dendritic cells
FISH	· Fluorescence <i>in situ</i> hybridization
FITC	· Fluorescein isothiocyanate
GABA	· Gamma-aminobutyric acid
GCs	· Germinal centres
HBSS	· Hanks Balanced Salts Solution
HPV	· Human papillomavirus
HRP	· Horseradish peroxidase
IFN-β	Interferons beta
IgD	· Immunoglobulin D

IgE	· Immunoglobulin E
IgVH	· Immunoglobulin heavy chain variable region gene
IgM	· Immunoglobulin M
Ig- α	· CD79a
Ig- β	· CD79b
IHC	· Immunohistochemical
IL-4	· Interleukin-4
IMS	· Isopropyl methylated spirit
INF γ	· Immune Interferon γ
IP3	· inositol-1,4,5-triphosphate
ITAM	· immunoreceptor tryocine based activation motif
IWCLL	· International workshop on CLL
JNK	· c-Jun N-terminal kinase
KCL	· Potassium chlorolride
kDa	· Kilo Dalton
LDH	· Lactate dehydrogenase
LDT	· Lymphocyte Doubling Time
LN	· Lymph node
LPS	· Lipopolysaccharide
LRR	· Lucin- rich repeats
Lyn	· LYN proto-Oncogene, Src family Tyrosine kinase
mAb	· Monoclonal antibody
MAP	· Mammalian protein kinase
MAPK	· Mitogen activated protein kinase
MAPKK	· Mitogen-activated protein kinase kinase
MCI	· Mantle Cell Lymphoma
McL-1	· Myeloid cell leukemia 1
M-CLL	· mutataated Chronic Lymphocytic leukaemia
MFI	· Mean fluorescence Intensity
MRD	· Minimal residual disease
MTA	· Material transfer agreement
MyD88	· Myeloid differentiation primary-response protein 88
MZ	· Marginal zone
MZB	· Mantle zone B cells
MZL	· marginal zone lymphoma
NCI	· National Cancer Institute
NF-AT	· Nuclear Factor activated T cells
NF-kB	· Nuclear factor-kB
NK	· Natural killer cells
NLCs	· Nurse like cells
ORR	· Overall Relapse Refractory
OS	· Overall Survival
p53	· protein 53
P13K	· Phosphatidynilisotol 3-kinase
PAMP	· Pathogen-associated molecular pattern
PARP	· poly-ADP-ribose protein
PB	· Peripheral Blood
PBMC	· Peripheral Blood Mononuclear Cells
PBS	· Phosphate Buffered saline

PCs	· proliferation centres
PE-Cy5	· Phycoerytherin cyanine 5
PEST	· peptide sequence that is rich in proline, glutamic acid, serine, and threonine
PFA	· Paraformaldehyde
PFS	· Progression free survival
PH	· pleckstrin homology
PHA	· Phytohaemagglutinin
PI	· Propidium Iodide
PIP3	· Phosphatidylinositol 3 4 5-triphosphate
PK	· Protein kinase
PTEN	· phosphate and tensin homolog deleted on chromosomes ten
PUMA	· P53 upregulated modulator of apoptosis
RAF	· Rapidly accelerated fibrosarcoma
RAS	· Retrovirus-associated DNA sequences
RBS	· Relative Binding sites
RNA	· Ribonucleic acid
RPMI 1640	· Roswell Park Memorial Institute 1640
RP105	· Radio protective 105
RR	· Relapse Refractory
RT	· Room Temperature
SD	· Standard deviation
SF3B1	· Splicing factor 3 subunit 1
SFK	· Src family kinase
SHIM	· somatic hypermutation
SLE	· Systemic lupus erythematosus
SYK	· Spleen Tyrosine kinase
TCR	· T Cell Receptor
T _H Cells	· Helper T cells
TIR	· Toll/Interleukin-1 Receptor
TK1	· Thymidine kinase
TLR	· Toll-like Receptor
TNF	· Tumour necrosis factor
TNF α	· Tumour necrosis factor alpha
UCH	· University College Hospital
UCL	· University College London
U-CLL	· Chronic Lymphocytic Leukemia with unmutated IGVH genes
UoW	· University of Westminster
US	· Unstimulated
VLA-4	· Very late antigen -4
WBC	· White Blood Cells
XLA	· X-Linked agammaglobulinemia
ZAP-70	· Zeta-chain associated Protein-70

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Chapter 1

1.1 Introduction

Chronic lymphocytic leukaemia (CLL) is the most frequently diagnosed adult leukaemia in the Western world. The median age of diagnosis in USA, Europe and Australia is approximately 72 years with about one quarter of patients aged <65 years and approximately 6% less than 50 years at diagnosis. CLL accounts for 1% of all new cancer cases in the UK and 37% of all leukaemia combined. In 2014 there were 3515 new cases of CLL in the UK: 2,204 (63%) in males and 1,311 (37%) in females, giving a male: female ratio of around 17: 10. 2014 statistics demonstrated that there were seven new CLL cases for every 100,000 males in the UK and 4 for every 100,000 females. The gap between males and females in CLL incidence increases with age (Figure 1.1).

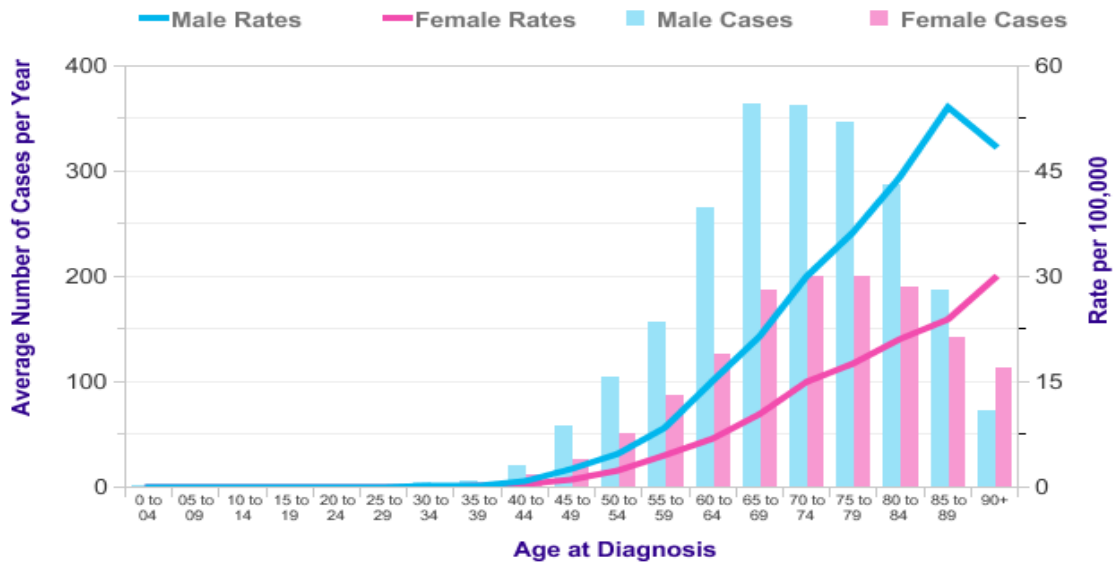


Figure 1.1: Incidence of CLL in the UK

Average number of New Cases per year and Age-specific Incidence rates per 100,000 Population, UK for Chronic Lymphocytic Leukaemia (2012-2014).

<http://www.cancerresearchuk.org/cancer-info/cancerstats/types/leukemia-cll/incidence/>

1.2 Epidemiology

CLL is characterised by populational heterogeneity. Its frequency is higher in Caucasians from North America, Europe and Russia as compared to African males and females. More importantly CLL incidence rate is significantly lower in South Asian countries, particularly in Japan and in China (Nabhan and Rosen, 2014). In Japan age-adjusted incidence for CLL is less than that of mantle cell-Lymphoma (Tamura *et al.*, 2001; Chihara *et al.*, 2014). There is no definite explanation of this predominance, but genetic predisposition is implied since the difference in occurrence is observed in generations after migration. An extensive survey of relatives of CLL patients demonstrated the familial pattern with approximately 10% CLL cases depicting a family history of the disease. Additionally, B cells with typical immunophenotype of CLL cells has been detected in circulating peripheral blood of 13.5% of blood relatives of CLL patients with unknown mode of transmission of the disease (Kawamata *et al.*, 2013; Slager and Zent, 2014).

1.3 Pathophysiology and Diagnosis

Five and 10-year absolute survival from initial diagnosis of CLL patients has been estimated in 2009-2011 as 80.4% and 64.7% respectively in USA and in UK (London Cancer CLL guidelines, 2015). Although with modern therapeutic approaches long-term survival of CLL patients has significantly improved, the underlying causes and aetiology of the disease are still unknown. No significant association with established carcinogens, infection or radiation has been identified. However, some data indicate an increased incidence of CLL in farmers, rubber manufacturers and those working with organic solvents (Brown and Rushton, 2012; Polychronakis *et al.*, 2013) (Pulte *et al.*, 2016).

CLL is clinically heterogeneous disease, characterized by accumulation of long lived morphologically mature, but functionally impaired CD5+ B lymphocytes in bone marrow, blood and lymphoid tissues. The accumulation is due to deficient apoptosis and extended survival of B cell (Wiernic *et al.*, 2003; Billard, 2014). CLL cells proliferation occurs in microanatomical sites called proliferation centres or pseudofollicles (Burger *et al.*, 2009) in bone marrow and in the lymph nodes, from where these cells expand into the circulation (Gradowsky *et al.*, 2012). CLL cells are predominantly monoclonal with respect to both heavy and light Immunoglobulin chains

and gene analysis revealed the association of CLL course with Immunoglobulin gene rearrangement (Munoz *et al.*, 2012; Rosenquist *et al.*, 2017).

The morphology of CLL cells in blood smears is consistent with small mature lymphocytes with a narrow border of cytoplasm, dense nucleus with coarse condensed nuclear chromatin with absence of distinctive nuclei, the typical features being the presence of smudge and smear cells. CLL bone marrow biopsy (taken rarely and not used for a routine diagnosis) show different infiltration patterns: nodular, interstitial, mixed and diffused, whereas in early clinical stages non-diffuse patterns are generally found (Oscier *et al.*, 2016; Gulati *et al.*, 2017)

Initially most CLL cases present without specific symptoms or the patient may present systemic symptoms such as night sweats, tiredness, unintentional weight loss and symptoms of anaemia or infection. Most patients are diagnosed because of routine blood test that show high white blood cell (WBC) count (Mir *et al.*, 2017). Advanced CLL presents itself with lymphadenopathy of cervical, axillary or marginal lymph nodes, hepatomegaly, mild or moderate splenomegaly, and thrombocytopenia thus leading to infection and bleeding and ultimately bone marrow failure. (Rodriguez *et al.*, 2016).

Morbidity and mortality in CLL result from secondary infections with the major cause being abnormal immune function either due to humoral immunodepression inherent to the hematologic disease or through the immunosuppressive effects of the management of CLL. The majority of CLL patients suffer infections with the most common immune defect being hypoglobulinemia rendering patients susceptible to bacterial infections (Nosari, 2012; Rimon *et al.*, 2017).

According to revised guidelines from the international workshop on CLL 2017, the diagnosis of CLL is based on an increase in the number of blood lymphocytes by 50% or more with at least 5000 lymphocytes/ μ l of peripheral blood with a distinctive immunophenotype that remain for more than 3 months (Hallek, 2017). Following clinical and morphological evaluation of patients and peripheral blood, the clonality of these cells are confirmed by flow cytometry. CLL diagnosis with flow cytometry is straightforward and Immunophenotyping not only distinguishes B-cell from T-cell disorder but also identifies CLL cells as CD20+CD5+CD23+ with dim expressions of Immunoglobulin IgM/IgD and CD79b as compared to those found on normal B cells. CD23 expression allows differential diagnosis from Mantle Cell Lymphoma (MCL),

(Nabhan and Rosen, 2014). Other tests are also performed besides flow cytometry to gain an insight into prognostication and to assess the tumour burden. These are mainly disease staging, fluorescence *in situ* hybridisation (FISH), deep sequencing, mutational status of immunoglobulin variable heavy chain domains (*IGHV*), cellular and serum biomarkers and (rarely) bone marrow examination (Mir *et al.*, 2017).

1.4 Rai and Binet staging

At the time of presentation, depending upon various clinical characteristics, CLL patients are categorized into stages according to the Rai (USA) and Binet (Europe) staging system. Rai *et al.*, in 1975 and Binet *et al.* in 1977 developed clinical staging strategy in CLL, these staging systems are still in use today as both classifications are based on clinical features presented at the time of initial examination. Rai and Binet staging systems are considered accurate means of determining the initial prognosis for individual patients and to determine when and whether to start treatment. Rai system (I) identifies five stages starting from 0 to IV, whilst Binet system (II) has three stages A-B-C. Rai staging (USA) revolves around lymphocytosis, lymphadenopathy, hepato/splenomegaly, anaemia and then thrombocytopenia. The Binet staging (Europe) describes symptoms according to the presence and absence of anaemia or thrombocytopenia with lymphadenopathy at single or multiple sites (Table 1). Typical median survival time for patients for Rai stage 0 – 1 and Binet A is 12 years, and these are low risk patients. Median survival time for Rai stage patients IV and Binet C is less than 3 years and they usually fall under the high-risk category. The remaining cases are of intermediate risk (Nabhan and Rosen, 2014; Rai and Stilgebauer, 2018).

Table 1.1: Diagnostic criteria for CLL according to the National Cancer Institute (NCI) and International Workshop on CLL (IWCLL 1996). Both classifications reflect bulk of disease and extent of marrow compromise such as anaemia, thrombocytopenia. Both staging systems have been recognised as simple and reliable predictors of survival. To date the two staging systems are used for assessing the clinical outcome and the survival chances of the patients with CLL.

RAI SYSTEM

Stage	Risk	Clinical features	Median survival (yr)
0	Low	Lymphocytosis in peripheral blood and bone marrow only	> 10
I II	Intermediate	Lymphocytosis with enlarged lymph nodes Lymphocytosis with enlarged spleen/liver	6
III	High	Lymphocytosis and anaemia (Hgb<11g/dL)	2
IV	Very High	Lymphocytosis and thrombocytopenia (platelets <100x10 ⁹ /L)	0

BINET SYSTEM

A	Hgb ≥10g/dL, Plts count ≥100x10 ⁹ /L, and <3 sites involved	> 7
B	Hgb≥10g/dL, Plts count ≥ 100x10 ⁹ /L, and ≥3 sites involved	< 5
C	Hgb <10g/dL, Plts count <100x10 ⁹ /L, regardless of the number of sites involved	< 2

1.5 Prognostic biomarkers

CLL is a heterogeneous disease with many patients showing an indolent clinical course that does not require treatment for many years whilst others will present with an aggressive and symptomatic leukaemia requiring immediate intervention (Parker, 2011; Hallek, 2015). Based on the risk assessment and prognostication CLL cases can be subdivided into categories according to the clinical outcome of the disease and B cell biology.

Following are some of the hallmarks of leukemia that has prognostic significance in CLL which includes Lymphocyte Doubling Time (LDT) Chromosomal aberrations, IGVH mutations, Zap-70 expression, CD38 expression, CD49d, serum markers and novel gene mutations

LDT (Lymphocyte Doubling Time)

The time period needed to double the lymphocyte count in peripheral blood is identified as a simple and valid parameter to assess disease progression particularly at early stages of the disease (Hallek, 2008, 2015, 2017). Comparison between LDT shortly after initial diagnosis compared with the original count of lymphocytes at the time of diagnosis provides distinction between slowly and rapidly progressing disease. LDT is calculated by linear regression and is expressed in months. A stable LDT with no increase or with a slow increase in lymphocyte count for more than 12 months is predicted as a good prognosis while patients with an unstable lymphocyte count for less than 12 months are shown to have an aggressive course of disease with poor survival rate (Scarfo *et al.*, 2016; Molica *et al.*, 1987, 2018). LDT is considered valuable for clinical management of CLL patients as it foretells progression of disease, since clinical prognosis cannot be predicted without proper monitoring of lymphocyte count of patients over a period of time (Strati *et al.*, 2015, 2018)

Chromosomal Aberrations

Fluorescence *in situ* hybridisation (FISH) tests utilising fluorescent DNA probe, have made it possible to carry out analysis of chromosomes with metaphase banding techniques that has prognostic and diagnostic significance in CLL. According to Döhner *et al.*, (2000), 80% of CLL patients demonstrated the presence of genetic abnormality within their malignant clone. These chromosomal defects are found to have an impact on CLL pathology as well as on the clinical characteristics and response to therapy. The most common aberrations stratified using FISH are based on previous studies (Dohner *et al.*, 1999, 1997, 2000; Mayr *et al.*, 2006). With regards to overall patient survival and predicting response to chemotherapy, the most relevant are deletion *13q14*, *trisomy 12*, *deletion 11q23*, *deletion 17p13 (P53 gene)* and complex karyotypes.

13q14 deletion

Deletion of *13q14* is the most frequently identified chromosomal abnormality found in 55% of the CLL patients. It involves structural abnormalities with the loss of the long arm of chromosome 13 with different break points (Parker, 2011). Davids *et al.*, (2015) suggested that *13q14* deletion is due to the loss of two functional genes within this region *DLEU1* and *DLEU2* in CLL which are involved in the regulation of tumour suppressor micro-RNA molecules miR-15a and miR-16-1. Although the expression of micro-RNA molecules is reduced in CLL patients, this does not influence the overall survival time and is associated with indolent course of the disease. (Puiggros, 2014; Rodrigues, 2016).

Trisomy12

Trisomy12 is the second most frequent karyotypic abnormality seen in CLL. 10- 20% of CLL cases exhibit three copies of chromosomes 12. The exact molecular mechanism by which an extra chromosome is acquired, is still unknown. However, this genetic aberration present itself with an atypical morphology and immunophenotype (Baliakas *et al.*, 2016). Initial studies have associated trisomy 12 with aggressive clinical course of the disease (Dohner *et al.*, 2000), but later studies have indicated that poor prognosis of *trisomy 12* correlates with the presence of other genetic abnormalities such as *NOTCH1* mutation, and hence placed it in the intermediate risk group (Puiggros, 2014; Abruzzo *et al.*, 2018).

11q23 deletion

Deletion of the long arm of chromosome *11q* is associated with poor prognosis and results in genomic instability in CLL patients. The deletion is frequently associated with the loss of ataxia telangiectasia mutated (*ATM*) protein which plays a vital role in recruiting tumour suppressor proteins towards the DNA sites and cell cycle regulation (Rose-Zerrili *et al.*, 2014). The deletion often leads to failed apoptosis, therefore damaged cells are not adequately removed. Patients with *11q* deletions express large and multiple lymphadenopathy with poor survival and resistance to conventional chemotherapy (Hernández *et al.*, 2015).

17p13 tumour suppressor gene P53 deletion

17p deletion is commonly seen in approximately 7-8% of newly diagnosed cases of CLL. The median survival of patients with *17p* (*P53*) deletion is 24 months only, irrespective of the size of *P53* deletion (Stilgenbauer *et al.*, 2002). This unfavourable outcome is due to the loss of genetic material encoding for *P53* gene. Monoallelic deletion of various sized portions of the short arm of chromosome 17 results, not only in the deletion of *P53* locus on one allele, but it also causes mutation in the remaining *P53* allele (Yu *et al.*, 2017). Patients harbouring only a single deletion or mutations of *P53* are also reported to have the worst overall median survival. *P53* is required in the cell development where it is responsible for repairing DNA cell damage and arresting cell cycle in G1/S phase. However, if the damage is beyond repair then *P53* promptly induces apoptosis signals leading to the elimination of the cell (Nabhan *et al.*, 2015). Since an intact *P53* pathway is required for DNA repair and for the induction of apoptosis, CLL patients with *P53* deletion demonstrate resistance to the combination of chemotherapeutic regimens: fludarabine-cyclophosphamide (FC) or fludarabine-cyclophosphamide-rituximab (FCR). Thus, patients with *P53* mutations or deletions are included in the high-risk category as they require therapy that impacts treatment choices (Edelmann and Gribben, 2017).

Complex karyotypes

The presence of 3 or more abnormalities in a single genome is considered as a complex karyotype and is seen in almost 20% of CLL cases. CLL patients displaying complex karyotypes more commonly carry *P53* loss, *trisomy 12* and *11q* deletion (*ATM*) (Puiggros, 2014, 2017). Detection of complex karyotypes strongly correlates with the

worst prognosis with shorter overall survival time and progressive disease. The majority of CLL cases displaying complex karyotype are with unmutated *IGVH* status (U-CLL, see below) and positive for CD38 (Malek *et al.*, 2013; Rigolin *et al.*, 2017). CLL patients with complex karyotype have been shown to have inferior outcome after treatment with chemo-immunotherapy (Foà *et al.*, 2014), reduced conditioning with allogeneic stem cell transplant (Grieselhuber *et al.*, 2016) and reduced response to the BTK inhibitor Ibrutinib (Maddocks *et al.*, 2015).

IGVH genes mutations

The mutational status of *IGVH* genes separates CLL patients into two distinct types of the disease grouped according to the shared clinical characteristics. An unmutated status of *IGVH* genes (U-CLL) correlates with the poor prognosis and shorter survival time (11 months) and the patients frequently present with other cytogenetic abnormalities, such as deletion of *11q22*, *13q14*, trisomy 12 and *17p13* deletion or *P53* dysfunction. In contrast, mutated *IGVH* genes (M-CLL) are associated with indolent course of the disease and longer survival time (293 months) and often present with a single *13q14* chromosome abnormality (Dunphy and Cherie, 2010; Pepper *et al.*, 2012; Bulian *et al.*, 2017).

Normal B cells as well as CLL B cells from a large group of patients undergo somatic hypermutation (SHM) in germinal centres (GCs) of lymph nodes. Following antigenic stimulation through the follicular dendritic cells and T cell signals, *IGVH* genes undergo somatic hypermutations aimed at the affinity maturation of the B cell receptor (BCR). Normal B cells with low affinity BCRs for antigens undergo apoptosis, while those B cells with high affinity for antigens preferably differentiate into plasma cells and memory B cell (Chiorazzi and Ferrarini, 2003; Heyman *et al.*, 2016). In CLL, such cells are termed Mutated (M-CLL). However, in the second group of patients CLL B cells go through affinity maturation outside germinal centres without T cell signals. These cells exhibit low affinity maturation as no somatic mutations are introduced in *IGVH* regions therefore these cells are termed Unmutated CLL cells (U-CLL). By various estimates, about 50 -70% of CLL patients have M-CLL clones, and this is a widely accepted prognostic indicator of an indolent disease. A further study suggested that somatic *IGVH* mutations in CLL cells influence the antibody reactivity: U-CLL cells expressed highly polyreactive BCRs, whilst M-CLL cells were characterised by the restricted BCR

reactivity (Herve *et al.*, 2005; Hacken and Burger, 2016). They concluded that whilst both, M-CLL and U-CLL cells are derived from autoreactive B cell precursors, somatic hypermutation can be responsible for the different clinical courses in M-CLL and U-CLL patients.

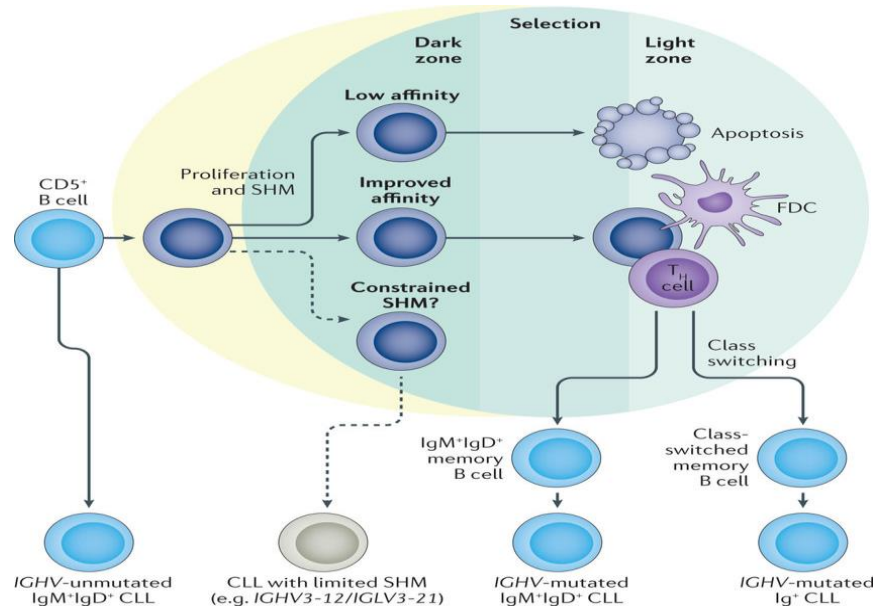


Figure 1.2. Cellular origin of mutated (M) and Unmutated (U) CLL cells via T cell dependent and T cell-independent B cell responses. The M-CLL cells following signals from T cells undergo massive proliferation in germinal centres (GC) dark zones along with somatic hypermutation (SHM). These cells with improved BCR affinity then enter GC light zone and interact with follicular dendritic cells (FDC) and helper T cell (T_H Cells) and undergo class switch recombination of their Ig heavy chain constant region genes. These mutated cells then leave GC and differentiate into either plasma cells or memory B cells. U-CLL cells develop without T cell signals from a marginal zone (MZ). These cells do not experience any SHM and exhibit very low affinity BCRs (Kipps *et al.*, 2016).

ZAP70 expression

Zeta associated protein kinase (70KDa, ZAP70) is a T-cell specific protein tyrosine kinase and its expression in CLL cells is considered to be a negative prognostic indicator. Intracellular ZAP70 expression exhibits a significant correlation with U-CLL, and the patients are characterised by poor overall survival rate (Rossi *et al.*, 2010; Gomes *et al.*, 2017).

Normal mature B cells use Syk protein kinase and not ZAP70 for intracellular BCR signal transduction. However, CLL cells express both, Syk and ZAP70, and the presence of ZAP70 amplifies migration, proliferation and survival of CLL cells through

augmented BCR signalling (Chen *et al.*, 2008; Feng & Wang, 2014). Hence ZAP70 is an attractive target for molecular therapy (Wang *et al.*, 2010).

Table 1.2: Summary of existing biomarkers for CLL and their clinical relevance: The key to risk stratification to standard therapies and/or overall survival.

Biomarkers	Low risk	High risk
Lymphocyte Doubling time (LDT)	>12 months	< 12 months
Chromosomal aberrations		
13q14 del	favorable outcome	
Trisomy 12	intermediate risk	
11q23 del		poor survival
17p13 Tp53		poor survival
Complex Karoyotype		poor survival
IgVH gene mutations		
Mutated(M)	longer survival	
Unmutated (UM)		short survival
Zap70		poor survival
CD38		short survival
CD49d		short survival
Serum markers		
TK1, β_2 -microglobulin and CD23		poor survival
Novel gene mutatiois		
Notch 1, SF3B1, Birc 3		poor survival
MyD88	longer survival	

CD38 expression

Damle *et al.*, (1999) initially highlighted aggressive clinical behaviour of CD38 positive CLL cells indicating overall poor prognosis. CD38 is a surface receptor and acts as a cell surface enzyme (ectoenzyme) expressed on mature B-Lymphocytes and plasma cells (Thompson & Tam, 2014). The functional properties of CD38 are linked with the suppression of B cell lymphopoiesis in the bone marrow and induction of activation, proliferation and cytokine secretions in germinal B cells (Burger *et al.*, 2009; He C *et al.*, 2017). Like Zap-70, CD38 is also linked with BCR signalling: CD38+ CLL cells, *in vitro*, were more responsive towards BCR signaling than CD38- cells that are generally anergic (Morabito *et al.*, 2010 and Benkisser-Petersen, 2015). Initial studies have placed CD38 expression in CLL cells as a useful and reliable prognostic marker

(Bürgler, 2016) based on CD38 expression versus *IGVH* mutation status. However, other studies revealed variability in the expression of CD38 which appears to be unstable on CLL cells throughout the clinical course of the disease (Nabhan, 2015). Moreover, expression of CD38 cannot be correlated with *IGVH* status and hence is considered as an individual prognostic marker, where high expression CD 38 is related to increased proliferation of CLL cells and low survival rate of CLL patients (Cramer and Hallek, 2011).

CD49d

CD49d, also known as very late antigen-4 (VLA-4), is a recently established prognostic marker. It is an $\alpha 4$ adhesion molecule expressed on B and T cells and on CD34+ hematopoietic stem/progenitor cells (Damle, 1999; Bulian, 2014; Baumann, 2016).

Zachetto *et al.*, (2012) confirmed the association between CD49d and CD38 expression by demonstrating that combined high expression of CD38^{high}/CD49d^{high} leads to the poor overall survival and shorter time to first treatment (Zachetto *et al.*, 2012). In 2008 two studies established CD49d as a negative prognostic marker and later studies confirmed it (Gattei *et al.*, 2008; Shanafelt *et al.*, 2008; Giudice 2016). Moreover, Brachtl *et al.*, (2014) maintains that presence of CD38 and CD49d in CLL cells not only signifies an aggressive course of disease but these molecules also play an important role in pathogenesis of CLL. Therefore, these two molecules should be exploited further for developing new novel approaches in combination with currently developed targeted therapies (Brachtl *et al.*, 2014).

Serum markers

There are several serological biomarkers such as serum thymidine kinase (TK1), $\beta 2$ -microglobulin and soluble CD23, which also contribute to the prognosis of CLL at early stages (Hallek *et al.*, 1999; Parker, 2011). Serum TK1 is a cellular enzyme and has a key role in DNA synthesis. Its level is high in dividing neoplastic CLL cells and its expression correlates with tumour mass, therefore can predict disease progression at an early stage (Konoplev *et al.*, 2010). A high concentration of serum $\beta 2$ -microglobulin correlates with a high tumour burden and shorter progression free survival time, and hence it is associated with poor prognosis (Sevov *et al.*, 2012). Serum CD23, a low affinity receptor for IgE is reported to represent an adverse prognostic indicator at the early stages of the disease course. Studies have shown that high levels of CD23 in

CLL cells can lead to diffuse bone marrow infiltration with CLL cells (unfavourable structural feature), decreased lymphocyte doubling time (LDT) and shorter overall survival (Wierda *et al.*, 2005; Nabhan and Rosen, 2014).

Novel gene mutations

Out of the numerous recurrent gene mutations characteristic for CLL, the four most common gene mutations that are extensively researched for better understanding of underlying pathogenesis of CLL are *Notch 1*, *SF3B1*, *BIRC3* and *MYD88*. All of these have demonstrated a direct functional and clinical association with prognostic parameters of CLL, and only MYD88 mutations identified in younger CLL patients has demonstrated favourable outcome (Puente *et al.*, 2011 and Swerdlow *et al.*, 2016).

Notch1

Notch1 mutations are identified in approximately 12% of CLL cases resulting in the decrease in overall survival of CLL patients (Fabbri *et al.*, 2011, 2017). Ninety percent of the activating mutations are detected at the threonine (PEST) domain that generates constitutively active Notch 1 protein with a deficient C-terminal. *Notch1* mutations result in the formation of a variant of plasma cell that support nuclear factor kB (NF-kB) signaling pathway resulting in increased cell survival and resistance to apoptosis in CLL cells (Rossi *et al.*, 2012, 2016). *Notch 1* mutations are reported to be more frequent in patients harbouring trisomy 12 karyotype and unmutated *IGHV* genes. CLL patients with *Notch 1* mutations usually demonstrate a progressive refractory disease with increased risk of Richter syndrome transformation (transformation of CLL to the aggressive diffuse large B cell lymphoma) (Rosati *et al.*, 2018).

SF3B1

Splicing factor, 3b, subunit 1(SF3B1) encodes a protein component that is needed by mRNA prior to protein synthesis. Recurrent mutations result in abnormal splicing. 5-15% of patients displayed *SP3B1* mutations along with the *ATM* mutations and *11q deletion* suggesting an aggressive course of the disease (Wang *et al.*, 2011; Quesada *et al.*, 2012; Nadeu *et al.*, 2016).

BIRC3

Mutations in *BIRC3* results in malfunctioning of MAP3K14 kinase causing an increase in NF- κ B pathway signaling leading to the increase in survival of CLL cells and shorter progression free treatment time (Rossi *et al.*, 2013). *BIRC3* mutations are seen at low frequency in newly diagnosed patients (4%) but at a higher frequency (25%) in relapsed refractory patients (Nadeu *et al.*, 2016). Patients with *BIRC3* mutations have a poor prognosis with overall survival of 3.5 years (Alhourani *et al.*, 2016).

MYD88

Myeloid differentiation primary response 88 (*MYD88*) mutations have been found in only 5% of CLL patients. They occur in Toll like receptors (TLR) signalling pathways coding regions and are most frequent in young patients (Baliakas *et al.*, 2015). Patients with *MYD88* mutations often have low expression levels of CD38 and ZAP70 on CLL cells and belong to M-CLL category (Qin *et al.*, 2017). Overall survival of patients with mutated *TLR/MYD88* is better than without the mutation hence predicting a favourable outcome (Martinez *et al.*, 2016).

1.6 Treatment of CLL

As mentioned earlier, CLL is a heterogeneous disease where patients follow different clinical course depending upon initial diagnosis, age, clinical and prognostic indicators. Depending on the stage at which the disease is diagnosed, a patient may be regularly monitored with a “wait and watch” option. CLL patients with an indolent course of the disease may remain asymptomatic for many years and starting treatment at this early stage will not benefit patients (Gribben, 2010). Treatment is only recommended when the course of the disease gets aggressive with unfavourable prognostic indicators. Currently there are various treatment options available such as chemotherapy with alkylating agents and purine analogues, immunotherapy, molecular therapy, or combination therapy. In more severe cases when CLL spreads to bone marrow or other organs, stem cell transplantation is considered the suitable approach. There is no cure of CLL yet, but modern treatment can lead to long-term remission, although minimal residual disease (MRD) remains a problem (Hallek, 2015)

Chemotherapy

Depending upon fitness of a patient and physical condition at the start of treatment, oral chemotherapy with alkylating agents such as Chlorambucil and Cyclophosphamide had been a first choice of treatment for many years. Both alkylating agents were favoured due to low cost, low toxicity and easy oral route administration but demonstrated low complete remission (CR) and no benefits to overall survival of CLL patients (Eichhorst *et al.*, 2009). Corticosteroids showed promising results initially, but later high incidence of infections was reported with its continued usage. Better responses were observed with combination therapy of chlorambucil and prednisone (CR 60% to 90%) than with Chlorambucil monotherapy, and this combination remains the first choice of treatment when patients are unfit to tolerate stronger available chemotherapy treatments (Rai *et al.*, 2009).

With the discovery of purine analogues Fludarabine monophosphate (Fludara), 2-chlorodeoxyadenosine (2-CDA), physically fit CLL patients had better treatment options than alkylating agents with significant effect on their overall rate (60 - 80% with remission duration 3-5 years). Purine analogues are potent inhibitors of DNA and can induce p53-mediated apoptosis. Multiple studies demonstrated that the overall response rate with Fludarabine monotherapy is significantly higher, 63 to 73%, with complete remission in 7-40% cases, compared to the alkylating CAP regimen (cyclophosphamide, doxorubicin, and prednisone) and Chlorambucil alone (Leporrier *et al.*, 2001; Eichhorst *et al.*, 2006; Catovsky *et al.*, 2007). However, patients with unfavourable prognosis, relapse and need subsequent therapy such as immunotherapy (Robak *et al.*, 2010).

Immunotherapy

Monoclonal antibodies act by employing body's own natural immune responses such as antibody dependent cellular cytotoxicity (ADCC) by Natural Killer (NK) cells, activation of the classical complement pathway and/or apoptosis. Several monoclonal antibodies such as Rituximab and Ofatumumab (directed at CD 20) and Alemtuzumab (directed at CD52) are among the most common frontline therapy for treating CLL (Wierda *et al.*, 2010; Pettitt *et al.*, 2012). Obinutuzumab (GA101) is another novel CD20 mAb that has demonstrated significantly increased ADCC compared with Rituximab.

New mAbs directed at anti-CD37, anti-CD19 and anti-CD40 have shown promising results in treating CLL in clinical trials (Frustaci *et al.*, 2016).

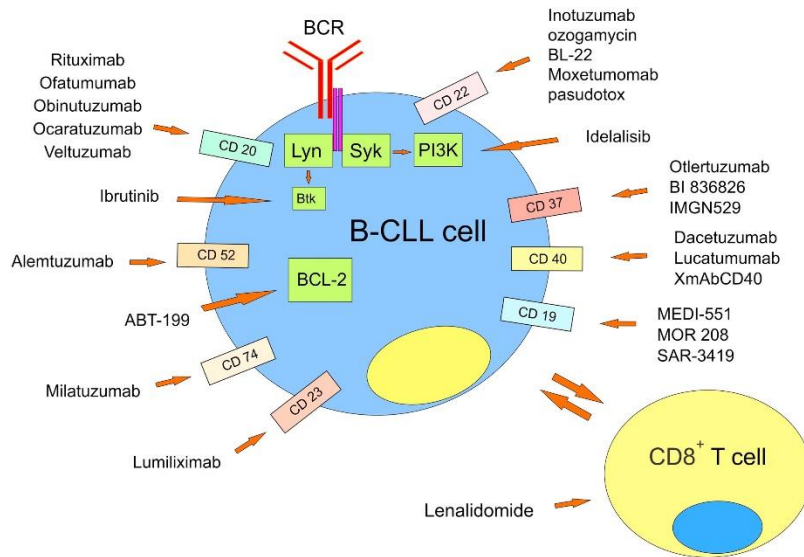


Figure 1.3 Current and Future monoclonal antibodies as frontline treatment of Chronic Lymphocytic leukemia. Monoclonal antibodies (mAbs) act via several mechanisms to recruit immune responses, such as complement activation (CDC), activation of cytotoxic effector cells (ADCC) or phagocytosis (ADP) (Robak *et al.*, 2016).

Combined chemo- and immunotherapy

The introduction of combination chemotherapy known as chemoimmunotherapy in CLL treatment has led to better response and progression free survival. The three main treatment regimen currently being used are FC (fludarabine, cyclophosphamide), FCR (Fludarabine, Cyclophosphamide and Rituximab) and FR (Fludarabine and Rituximab). For young and physically fit patients who have no renal problems and without the 17p deletion and *P53* mutations, the first line of treatment is FC or FCR (Hallek *et al.*, 2010). In relapsed older patients or those with more co-morbidities, FR or combination therapy with Bendamustine and Rituximab showed promising results with response rate approaching 90% (Fischer *et al.*, 2011). Overall survival rate is higher and almost similar in both FC and FCR regimens, but response rate appears higher in case of FCR, whilst the level of toxicity (side effects) is significantly lower in FR regimen. Complete remission (CR) is seen in 47 to 70% patients when treated with FR or FCR regimen respectively (Eichhorst *et al.*, 2014). Ofatumumab is another CD20 mAb that has been combined with FC and FCR to be used as front-line therapy and showed

improved outcome in CLL patients (Rai and Jain, 2016). Alemtuzumab (anti-CD52) when used in combination with FCR in patients with 17p deletions and TP53 mutations showed overall efficacy but with major toxicities therefore was removed from trial (Rai and Jain, 2016.)

Molecular therapy

In the last 4 years, there has been an upsurge in understanding the pathogenesis of CLL and as a result targeted molecular therapies have been developed. This particularly involves targeting BCR signal transduction pathway inhibitors such as inhibitors of Lyn, Syk, Bruton's tyrosine kinase (Btki), phosphatidylinositol 3 kinase (PI3Ki), and BCL2 (BCL2i), have dramatically improved treatment options for patients with high-risk CLL. (Figure 1.3).

Of these the most significant to date are Ibrutinib, Idelalisib and (ABT-199) Venetoclax (Dreger *et al.*, 2018). Pan *et al.*, (2007) and Honigberg *et al.*, (2010) have developed Ibrutinib, an irreversible inhibitor of BTK. It was shown to be more effective than Ofatumumab in the patients with relapsed CLL by inducing higher response rates as well as longer progression free survival (PFS) and overall survival (OS) in the RESONATE trial (Byrd *et al.*, 2013, 2014). A remarkable shrinkage in lymph nodes was observed accompanied by a shift of lymphocytes to peripheral blood. This drug also has a dual mechanism of action on signaling and inhibition of cross-talk in CLL microenvironment (Burger *et al.*, 2015). Idelalisib is a selective and reversible inhibitor of phosphatidylinositol 3-kinase (PI3K) and promotes apoptosis of CLL cells. In patients with relapsed CLL, Idelalisib, in combination with Rituximab or Ofatumumab was shown to induce higher response rates and longer PFS and OS compared to Rituximab monotherapy (Brown *et al.*, 2014; Furman *et al.*, 2014). Both inhibitors, Ibrutinib and Idelalisib are well tolerated in long-term therapy and they could be used in elderly patients with coexistent conditions. Idelalisib was further shown to have remarkable inhibitory effect on lymphocyte trafficking, chemokine networks and T cells in CLL microenvironment (O'Brien *et al.*, 2015). Another recently approved orally bioavailable dual inhibitor of PI3K δ and PI3K γ isoforms is Duvelisib (IPI-145, INK 1197). Duvelisib has shown clinical activity with relapsed or refractory R/R CLL patients with an overall improved median progression-free survival (PFS) by 3.5 months more than Ofatumumab (Lawrence, 2018).

Another successful drug is Venetoclax which is a highly selective antagonist of antiapoptotic protein BCL2. In clinical trials, Venetoclax showed no effects on platelets but induced apoptosis of CLL primary cells containing high expression of BCL2. The overall response rate was high (79%) with 20% demonstrating complete response and 66% showed 15 months progression free survival rate (Roberts *et al.*, 2016). Lenalidomide is another immunomodulatory drug that acts on the CLL microenvironment by inhibiting TNF, altering cytokine secretion and enhancing T-cell immunological synapse formation with CLL B cells (Kater *et al.*, 2014; Byrd *et al.*, 2018). Lenalidomide has shown efficacy in relapsed refractory setting in clinical trials: ORR was 65% and CR was 10% (Facteru *et al.*, 2014; Itchaki and Brown, 2017).

CART-T Cell Therapy

One of the relatively recent advances in CLL therapy is chimeric T cell receptor (TCR) specific for CD19 (CART-19) (Porter *et al.*, 2015; Kalos *et al.*, 2016). Autologous T cells from CLL patients were collected and reintroduced into patients' blood following genetic engineering with the expression of an anti-CD19 chimeric TCR, which is produced by combining an antigen recognition domain of a specific antibody (CD19) with an intracellular domain of the CD3- zeta chain or FcγRIII T cells. After a 4-year follow up of 14 RR-CLL patients, the ORR was 57% and four patients achieved CR. None of the patients in CR have relapsed (Porter *et al.*, 2015, 2016). Recent studies show that CAR-T cell therapy is highly effective in relapsed or refractory CLL patients who failed treatment with Ibrutinib (Turtle *et al.*, 2017).

Despite of these advances in the treatment of CLL, the disease remains incurable. One of the limiting factors is heterogeneity of CLL which means a single therapy is not suitable for all the patients. Therefore there is a need for the development of novel therapeutics approaches that enable patients to receive personalised treatments to achieve a better response.

1.7 B cell receptor signalling in CLL

The role of BCR-mediated signalling in prognostication and stratification of CLL associated with survival and expansion of CLL cells has been under extensive investigation during the last decade. The complexity of BCR signalling permits many distinct outcomes, including survival, tolerance (anergy) or apoptosis, proliferation, and differentiation into antibody-producing cells or memory B cells. BCR activation can

happen in two distinct ways: antigen induced or antigen independent autonomous BCR signaling termed “Tonic signalling” (Figure 1.3). Antigen dependent signaling involves cross-linking of BCR with a specific antigen, ITAMs phosphorylation and consequently activation of a signaling cascade leading to the cell survival and proliferation. In contrast, Tonic signaling involves BCR activation possibly without cross-linking, via auto-antigenor microbial antigens present in the tissue microenvironment (Packam *et al.*, 2014; Koehrer and Burger, 2016).

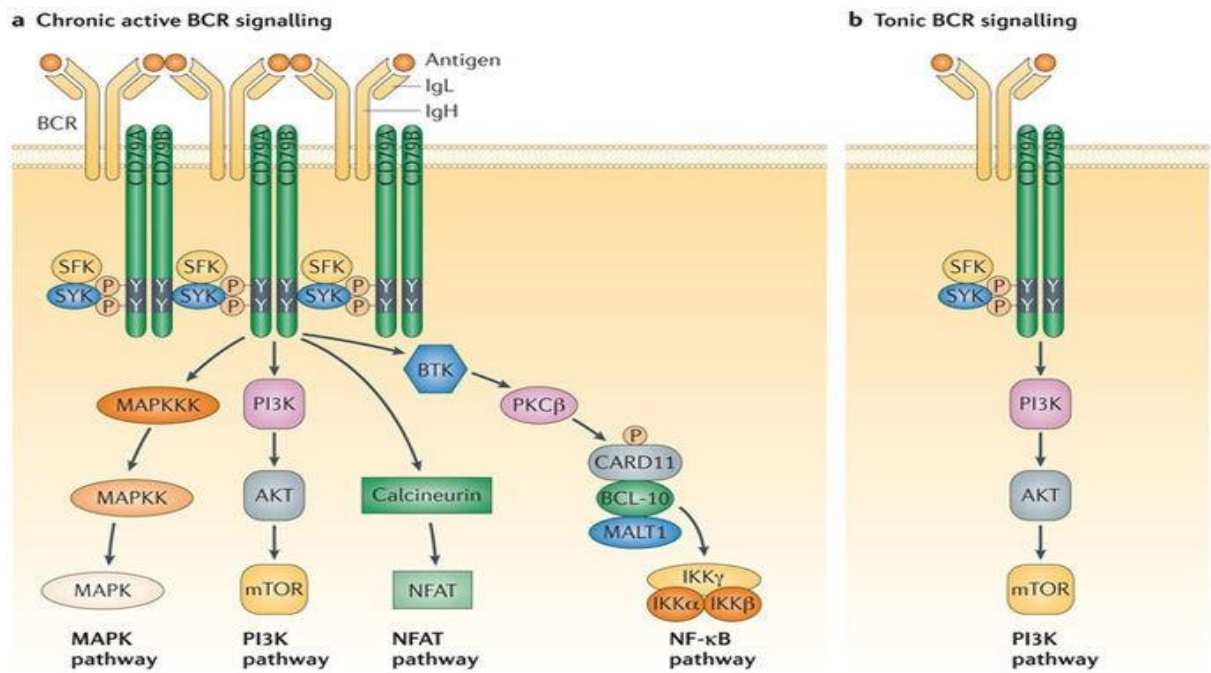


Figure 1.4 Antigen dependent (a) and Antigen independent (Tonic, b) BCR signaling in CLL. CLL cells follow two forms of receptor signaling leading to survival and proliferation. Antigen independent “Tonic signaling” generates within the tissue microenvironment due to the mutations of BCR signaling pathway while antigen dependent signaling results from BCR ligation by specific antigens (Young and Staudt, 2013).

Below I present some of the key components of the BCR-mediated signalling in more details.

Surface Immunoglobulin sIgM and sIgD

All mature B cells co-express IgM and IgD receptor isotypes (Figure 1.5).

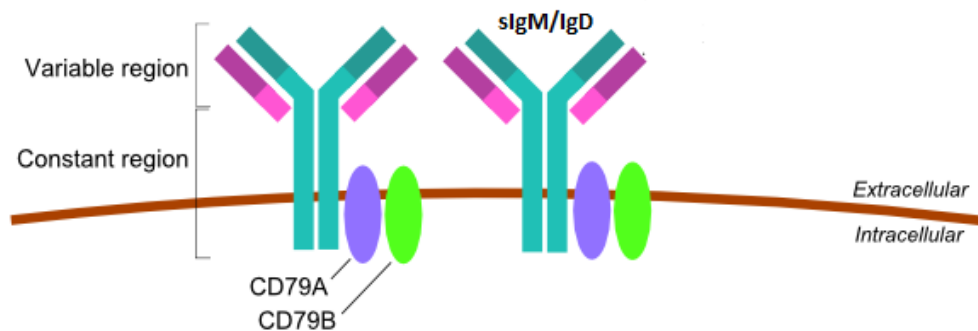


Figure 1.5 B cell receptor (BCR). The BCR complex consists of various components: Ig membrane attached surface IgM/IgD receptors and associated accessory molecules CD79A and CD79B. The B cell receptor extends both outside the plasma membrane (extracellular) and inside the cell (intracellular) (Barnea *et al.*, 2014).

BCR signaling via IgM and IgD is now recognised as the central pathogenic pathway in CLL (Burger and Chiorazzi, 2013). IgM and IgD receptors along with coreceptors CD19 and CD20 help B cell transition from resting state to activated state and thus maintaining the signaling balance and regulation of B cell fate (Mazarello *et al.*, 2017). Due to their structural similarity it was originally thought that their functional responses were similar too following antigenic stimulation. However, studies on knockout mouse models have revealed that their function is interchangeable and IgD can easily substitute for IgM functions in case of low-density expression of IgM or loss of the IgM receptor (Simonetti *et al.*, 2014). CLL cells are characterised by a variable expression of IgM and IgD receptors, and multiple studies on BCR signaling have demonstrated the importance of IgM signaling pathway in CLL cells for survival, cell cycle entry and proliferation. However, studies on the IgD-mediated CLL responses have led to contradicting conclusions, such as cell survival or apoptosis (Stevenson *et al.*, 2014).

Difference in the outcomes following IgM and IgD stimulation is also observed in regard to the *IGVH* mutations. IgM, but not IgD-mediated responses are typically augmented in U-CLL cases (Mockridge *et al.*, 2007).

It has been demonstrated that IgD receptors, following stimulation were rapidly internalized and fail to activate downstream responses (Burger *et al.*, 2009). Haerzschel *et al.*, found that following stimulation there is an amplification of IgD expression and IgD mediated calcium signaling, significantly different from IgM expression and the chemotaxis towards CXCL₁₂ and CCL₂₁ is significantly reduced in IgD but not in IgM (Haerzschel *et al.*, 2016). Although IgM and IgD are similar in structure, differences in their responses probably contribute to the variable clinical behaviour of CLL. Therefore, understanding the details of the BCR signal transduction through the combination of BCR components offers an opportunity for the precise targeting by the PK inhibitory drugs (Apollonio *et al.*, 2013).

CD79a/79b

CD79a (Ig- α) and CD79b (Ig- β) are transmembrane signaling molecules of BCR that form a heterodimer complex on B cells known as BCR complex and have molecular weights of 20 kDa each. Human CD79a is coded by gene *mb-1* which is located on chromosome 19 and the gene coding for CD79b is *B29* located on chromosome 17 (Sims, 2012). CD79a/b play a critical role in B cell maturation, survival and activation. CD79a is expressed at early stages of B cell development persisting from pre-B stage to the last stage of maturation before differentiation into plasma cells (Patterson, 2011). The cytoplasmic tails of the dimer CD79a/b contain Immunoreceptor tyrosine-based activation motifs (ITAMs). Antigen binding to the BCR results in phosphorylation of CD79a and CD79b via ITAMs leading to the recruitment and activation of the kinase Syk. Syk activation then initiates further signal transduction leading to cytoskeletal reorganisation and changes in gene expression for B cell maturation (Hecken and Burger, 2016).

CD79a and CD79b are heterogeneously expressed on CLL cells. Vuillier *et al.* (2005) have reported that glycosylation and folding defects are responsible for the low expression of the CD79a chains in CLL patients. Among other defects that have been recorded are reduced expression of CD79b mRNA (Thompson *et al.*, 1997), somatic mutations of the *B29* gene (Thompson *et al.*, 1997), over-expression of a product of

alternative splicing of CD79b, termed as alternative transcript of CD79b (Δ CD79b) (Alfarano *et al.*, 1999), and abnormal assembly of BCR chains, leading to CD79b accumulation in intracellular compartments (Payelle-Brogard *et al.*, 2002).

PI3K/AKT pathway

AKT (Protein Kinase B) is a serine/threonine-specific protein kinase that is essential for the proper functioning of cellular processes such as glucose metabolism, apoptosis, cell survival and proliferation, transcription and cell cycle progression (Manning and Toker, 2017). AKT is the founding member of PKB family composed of three members AKT1, AKT 2, AKT 3. Activation of these kinases in cells takes place in response to diverse stimuli such as hormones, growth factors, and extracellular matrix components and promotes survival and growth (Fruman *et al.*, 2017). AKT remains in the cytosol in an inactive state, until the cell is stimulated and translocates to the plasma membrane with the help of PI3K. PI3K activates phosphatidylinositol 3, 4, 5, -triphosphate (PIP₃) which recruits phosphatidylinositol-dependent kinase 1 (PDK1). PDK1 partially phosphorylates AKT1 at threonine 308, but full activation of AKT requires additional phosphorylation at serine 473 by other kinases including phosphoinositide-dependent kinase 2 (PDK2), integrin-linked kinase (ILK), mechanistic target of rapamycin complex (mTORC) and DNA-dependent protein kinase (Limon and Fruman, 2014; Manning and Toker, 2017). The cellular levels of PI3K products are controlled by the balance between PI3K activity and the phosphatase activity of PTEN (phosphatase and tensin homolog mapped on chromosome ten). Interestingly, PTEN protein is reduced or not detected in 48% of patients with CLL (Backer *et al.*, 2010, 2016). AKT is dephosphorylated by protein phosphatase 2A (PP2A) and the PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP1/2). In addition, the tumor suppressor phosphatase and tensin homolog (PTEN) inhibits AKT activity by dephosphorylating PIP₃ (Baracho *et al.*, 2011). It has been shown that in CLL cells PI3K pathway is constitutively activated leading to AKT activation with subsequent phosphorylation of other downstream signaling molecules (Figure 1.6). Dysregulation of the PI3K/AKT pathway is implicated in a number of human diseases including cancer, diabetes, cardiovascular disease and neurological diseases (Weistner, 2012; Hue *et al.*, 2016).

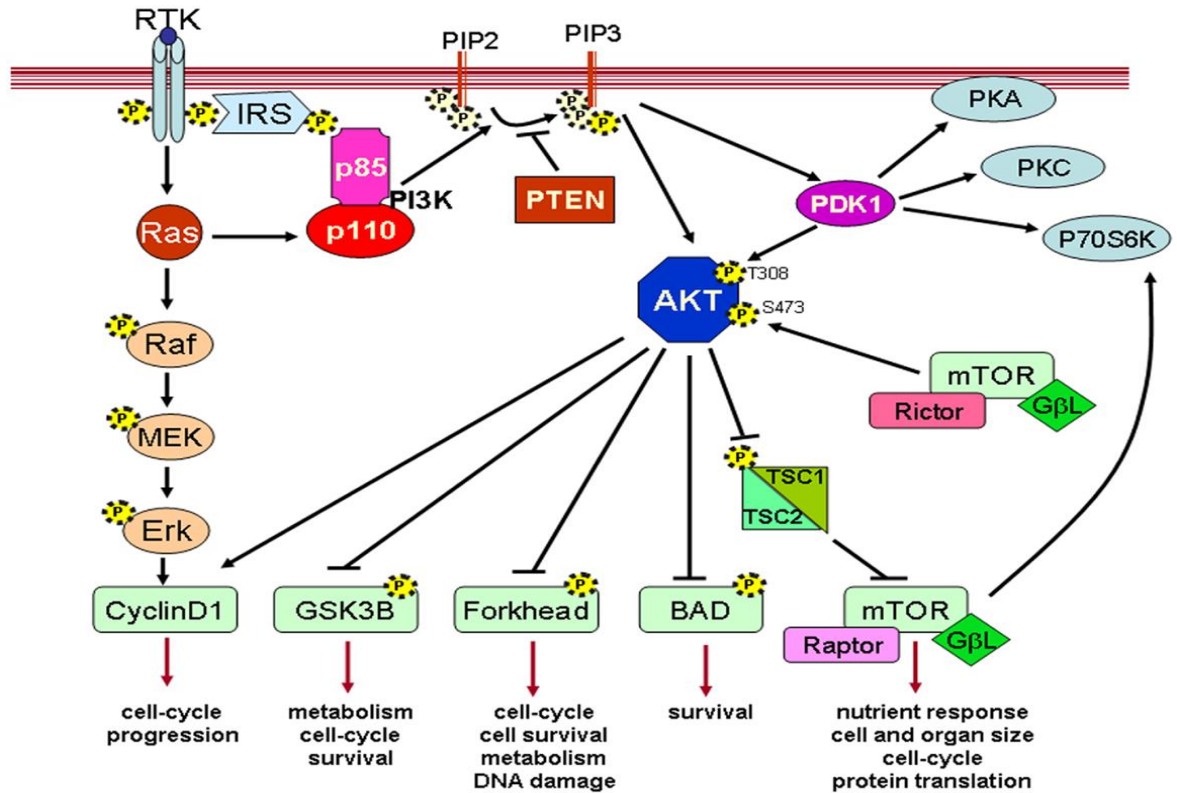


Figure 1.6. AKT/P13K signal transduction pathway. The P13K is required to activate AKT/mTOR pathway and together they regulate various aspects of cellular development such as cell cycle progression, apoptosis and cell survival (Carnero and paramio, 2014).

AKT regulates cell growth through its effects on the TSC1/TSC2 complex and mTORC signalling and contributes to cell proliferation via phosphorylation of the CDK inhibitors p21 and p27. AKT is a major mediator of cell survival through direct inhibition of pro-apoptotic proteins like Bad or inhibition of pro-apoptotic signals generated by transcription factors like FoxO. AKT also regulates NF- κ B signalling by phosphorylating IKK α and Tpl2 (Bozulic and Hemmings, 2009; Hers *et al.*, 2011). It is critically involved in the regulation of metabolism through activation of AS160 and PFKFB2. In addition, AKT has been shown to regulate proteins involved in neuronal function including the GABA receptor, ataxin-1 and huntingtin proteins. AKT contributes to cell migration and invasion via phosphorylation of palladin and vimentin. Barragan *et al.*, (2006) demonstrated that activation of the AKT pathway is possible by another PI3K-independent pathway that takes place via protein kinase C β (PKC β) suggesting the

presence of two different pathways used by CLL cells for AKT Phosphorylation. Due to the critical role of AKT/PKB in regulating diverse cellular functions it is an important therapeutic target for the treatment of human disease.

AKT inhibitors block the anti-apoptotic proteins Mcl-1 which causes an increase in the expression of tumour suppression gene 53 leading to induction of extensive apoptosis of malignant cells (Furman *et al.*, 2010; Fruman *et al.*, 2017). Targeting AKT has been a pharmacological challenge and several promising AKT inhibitors have been developed that either selectively or nonselectively inhibit the three isoforms of AKT by binding to the kinase or PH domains. Two of the most competitive inhibitors A-443654 and AKTi1/2 are reported to inhibit AKT phosphorylation by blocking cell survival and cell cycle progression. A-443654 is a pan AKT-inhibitor which can act against all three isoforms AKT1, AKT2 and AKT3 in equal potency. In contrast AKTi1/2 is a highly selective inhibitor which effects only AKT1 and AKT2 but not AKT3 activity (Luo *et al.*, 2005; Bain *et al.*, 2007). Another known selective inhibitor of isoform PI3K δ is Idealisib (formerly known as CAL-101) that induces apoptosis in CLL cells by inhibiting prosurvival signalling pathways. It also blocks the supportive effect of many micro environmental factors, mainly NLCs activation of the BCR, and of CD40L, BAFF, TNF- α , or fibronectin and promotes CLL cell migration out of lymph node into the blood (Herman *et al.*, 2010, 2014; Ali *et al.*, 2014). Idealisib combined with the anti-CD20 antibody Rituximab significantly improved progression free survival (81%) and overall survival (91%) in relapsed CLL patients (Fruman *et al.*, 2014, 2017). Furthermore, a recent publication showed that combination of Idealisib with Ibrutinib is potentially beneficial for CLL patients (Rooji *et al.*, 2015). Duvelisib (IPI-145) is another potent, dual inhibitor that targets both PI3K δ and PI3K γ isoforms (Winkler *et al.*, 2013). Clinical evaluation of Duvelisib demonstrated that it inhibits BCR mediated signalling and chemotaxis in response to CXCL12 and induces apoptosis in CLL samples (Balakrishnann *et al.*, 2015). Most importantly it has been seen that Duvelisib can overcome Ibrutinib resistance and is potentially beneficial for treating patient's refractory to Ibrutinib (Dong *et al.*, 2014). TGR-1202, copanlisib, GS-9820 and MK2206 are the next-generation PI3K δ inhibitors that have demonstrated activity in patients with advanced haematologic malignancies (Gockeritz *et al.*, 2015; O'Connors *et al.*, 2015).

MAPK/ERK Kinase

The Mitogen activated protein kinase/extra cellular regulated kinase (MAPK/ERK) pathway is another signalling pathway induced by BCR stimulation. This complex pathway plays a key role in many cellular functions including cell proliferation, gene expression, differentiation, cell survival and most importantly inducing apoptosis (Pearson *et al.*, 2001 and Siegfried *et al.*, 2013).

MAPK are serine-threonine protein kinases and so far, 3 classes of mammalian MAP kinases have been identified: the extracellular signal-regulated protein kinases (ERK-1/2), the c-Jun N-terminal kinases (JNK1/2/3) and the p38 stress-activated protein kinases (p38MAPK α , β , δ and γ). ERK, JNK and p38 isoforms are grouped according to their activation motifs and function. ERK predominantly regulates anti-apoptotic and mitogenic gene expression in response to cytokines and growth factors. The other kinases p38 and JNK activation depend on cellular and environmental stress including proinflammatory stimuli and growth factors and mainly control apoptosis and cell cycle progression (De Luca, 2011; McCain, 2013).

Following BCR stimulation, the G proteins recruit retrovirus-associated DNA sequences (Ras) and rapidly accelerated fibrosarcoma (Raf) proteins. Activated Raf phosphorylates and activates mitogen activated ERK protein kinase (MEK). MEKs 1 and 2 phosphorylate ERK, MEKs 3, 4, and 6 phosphorylate p38, whilst MEKs 4 and 7 activate JNK pathways. The transcription factors regulated by MAPK include Elk-1 and cmyc by ERK, c-Jun and ATF-2 by JNK, and ATF-2 and MAX by p38 MAPK (Figure 1.7), and the functional outcome of activation is dependent on the developmental stage of the B cell (Rodriguez and Crespo, 2011; McCain, 2013).

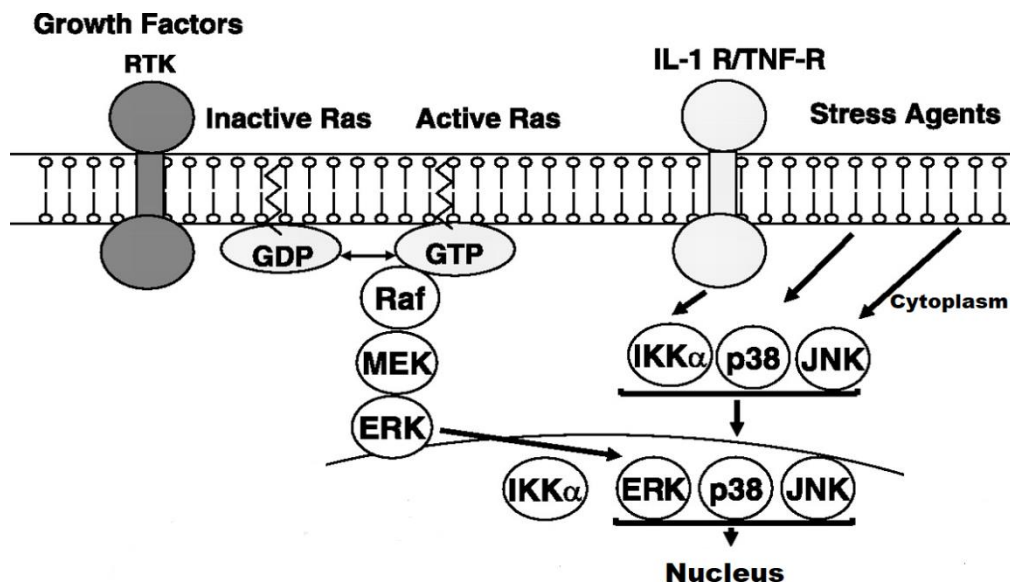


Figure 1.7. Schematic overview of MAPK kinase signalling pathway. The three major factors such as cytokines, cellular stress or inflammatory agents lead to the activation of three major MAPK pathway leading to ERK, p38 or JNK pathway via RAS and RAF signaling and consequently induce its biologic effects on target cells (modified from Hemmaker and Firestein, 2010)

The p38MAPK is known to be associated with proliferation and survival in general but multiple studies have demonstrated its role in inducing apoptosis by cellular stress in CLL. Both, survival and apoptosis response can be mediated by transcriptional and posttranscriptional mechanisms, which directly affect death receptors survival pathway or pro- and anti-apoptotic Bcl-2 family proteins (Shukla *et al.*, 2018). It has been demonstrated that isoform p38α can negatively regulate cell cycle progression at both the G₁/S and G₂/M transitions by a number of mechanisms, including downregulation of cyclins and upregulation of CDK inhibitors (Lake *et al.*, 2016). Additionally, Muzio *et al.*, (2008) suggested that MAPK pathway can become a potential target for pharmacological intervention with therapies as selected cohort of their CLL patients exhibited constitutively phosphorylated MAPK ERK, *in vivo*, following BCR triggering but not, *in vitro*. Their results indicated that constitutive activation pattern of the MAPK kinase pathway in a subset of CLL patients together with increased activity of nuclear factor of activated T cells (NF-AT) in an absence of AKT phosphorylation and unresponsiveness of BCR, may be regarded as molecular characteristics of anergy in human B cells (Muzio *et al.*, 2008; Woyach and Johnson, 2015). The known inhibitors Ibrutinib (BTK), Idealisib (P13K) and Fostamatinib (Syk) have shown to suppress the activity of MAPK kinase signaling in human and murine cells by blocking the activity of chemokine CXCR4 in the proliferation centres (Chen *et al.*, 2016). Two MAPK inhibitors

Vemurafinib and Trametinib target BRAF and MEK1/2 to inhibit MAPK signaling by blocking the proliferation, migration and survival of CLL cells (Crassini *et al.*, 2015; Shukla *et al.*, 2018).

Bruton tyrosine kinase (BTK)

Bruton tyrosine kinase (BTK), a non-receptor cytoplasmic kinase is a critical member of Tec family of kinases due to its key role in B cell development, differentiation, signaling, proliferation and survival. B cells express BTK from early precursor to mature B cells, it is also expressed by myeloid cells but not by plasma cells or T lymphocytes (Satterthwaite, 2000, 2018). Any genetic mutation, loss of function or absence of BTK results in defective B cell development by blocking B cell maturation between the pro and pre-B cell stages causing severe decline in circulating mature B lymphocytes (Buggy, 2012). Genetic deletion of BTK is seen to cause in humans X-linked agammaglobulinemia (Bruton agammaglobulinemia, XLA), which is characterized by virtually the complete absence of B cells and non-production of immunoglobulins to generate humoral responses leading to recurrent bacterial infections (Vetrie *et al.*, 1993). In mice loss of BTK genes causes less severe X-linked immunodeficiency (Xid) (Rawlings *et al.*, 1993).

BTK has 3 functional domains, pleckstrin homology (PH) domain and Src homology SH3 and SH2 domains and each of these domains can potentially interact with proteins critical for activation and regulation of intracellular signaling (Mohammed *et al.*, 2009 and Gusstafson *et al.*, 2017). Following antigenic stimulation of BCR and signal transduction via CD79a and CD79b, BTK translocates to the plasma membrane binding to phosphatidylinositol -3,4,5-triphosphate (3-5) at its PH domain, where it gets partially phosphorylated at Y551 by a Src family kinase (SFK), presumably Lyn. Full activation involves autophosphorylation by Src family kinases at Tyr223 within the SH3 domain. B-cell linker protein (BLNK) at SH2 domain acting along with SH3 domain phosphorylates phospholipase C, gamma 2 (PLC γ 2), which leads to production of the second messenger diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) which results in triggering the downstream signaling. In CLL this triggering causes enhanced activation of P13 kinase, PLC γ 2 and NF- κ B signaling leading to survival and proliferation of malignant cells. BTK is dephosphorylated at SER180 by negative

regulated PKC β , blocking its membrane recruitment and subsequent activation (Hendriks *et al.*, 2014; Smith, 2016).

The role of BTK in the amplification of BCR signaling leading to constitutively active pathway of CLL survival has been very well characterized in recent years. Targeting BTK became a promising strategy and to date Ibrutinib is the most successful orally bioavailable irreversible inhibitor of BTK that has shown excellent results clinically with extended durable remissions in both untreated and relapsed patients (Woyach *et al.*, 2014). Studies have shown that Ibrutinib can interrupt BTK auto phosphorylation following IgM ligation that results in decrease in the expression of intracellular signaling targets via ERK, NF- κ B and AKT leading to survival and proliferation (Herman *et al.*, 2014). In addition, it has been demonstrated that Ibrutinib not only inhibits the proliferation of malignant B lymphocytes *in vivo* but can also inhibit microenvironmental survival proliferation and migration signals by blocking the effect of stromal coculture, *in vitro*, (Burger *et al.*, 2009; Herman *et al.*, 2011; Gayko *et al.*, 2015).

Ibrutinib/PCL-32765 covalently binds to BTK protein through its cysteine residue 481 and inhibits its phosphorylation at TYR223 ATP binding domain of BTK for 24 hours. Although Ibrutinib induces lymphocytosis in CLL patients during their first week of treatment it is resolved within one-month therapy. Therefore, treatment delays or discontinuation of therapy due to side effects of therapy is infrequent (Woyach *et al.*, 2012, 2014). The second-generation BTK inhibitor, Acalabrutinib and two other potential BTK inhibitors CC-292 and ONO-4059 have demonstrated therapeutic efficacy based on their anti-proliferative activity (Burger, 2014; Byrd *et al.*, 2016).

1.8 The role of microenvironment in CLL cell survival

It is widely accepted that CLL cell behaviour is not only regulated by the engagement of BCR by (auto) antigens but CLL cells rely on other stimuli from microenvironment to survive and to proliferate. It has been documented that despite expressing high levels of Bcl-2, CLL cells undergo rapid and spontaneous apoptosis when cultured under, *in vitro*, conditions unless they are cocultured with accessory stromal cells. This demonstrates that prolonged, *in vivo*, lifespan of CLL cells is at least partially due to microenvironmental influences and BCR engagement by antigens (Ghia *et al.*, 2008; Burger *et al.*, 2009; Oppezco and Dighiero, 2013). CLL cells within proliferating centres or “pseudofollicles” modify microenvironment to create optimum surroundings to

facilitate leukemic cell survival, proliferation, homing and tissue retention. Direct contact between CLL cells and accessory cells such as T and NK cells, Nurse like cells (NLCs) and stromal cells supported by secreted cytokines IL-4, TNF α , INF γ and CLCX12 aid in rescuing CLL cells from apoptosis promoting their survival and expansion (Burger, 2011). Messmer *et al.*, (2005) suggested that in the proliferating centres the daily cellular birth rate accounts for approximately 1 to 2% of the entire CLL clone.

Stromal cells are considered critical element of tissue environment of CLL as they may serve to protect CLL cells from spontaneous and drug induced apoptosis. They constitutively secrete chemokine CCL19 and CCL21 which provide an important chemokine gradient for the trafficking and homing of CLL cells from periphery to lymphoid organs (Hacken and Burger, 2014, 2016). NLC secrete CXCL12 (also known as stromal cell-derived-growth-factor 1) and CXCL13 which binds to the receptors CXCR4 and CXCR5 expressed on CLL cells and attracts CLL cells towards the tissue microenvironment. NLCs can also support survival of CLL cells by expressing B cell activating factor (BAFF), and proliferation inducing ligand APRIL which in turn stimulates B cell maturation transmembrane activator (BCMA). Further prosurvival signals are provided by interaction of CD31 expressed on NLCs and CD38 on CLL cells contributing to the failed apoptosis and increased production of CLL cells (Burger, 2013). In addition, in CLL the de-regulated functional capability of T cells and NK cells assists CLL cells to escape immune mediated cytotoxicity (Ramsay *et al.*, 2012; Rainers *et al.*, 2013; Riches *et al.*, 2013).

Taken together (Figure 1.8), the crosstalk between CLL cells and tissue microenvironment deliver signal that favours clonal expansion and drug resistance and disrupting this crosstalk is an attractive therapeutic strategy.

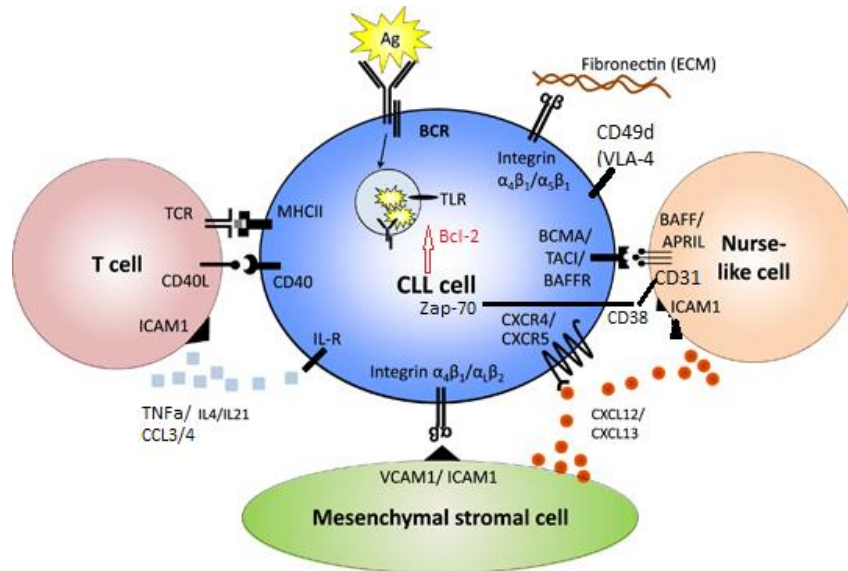


Figure 1.8 CLL cells and lymphoid tissue microenvironment. CLL cells are dependent on pro-survival signals generated from microenvironment elements such as NLCs, MSCs, chemokines, interleukins CD49d and T cells. NLCs and MSCs secrete CXCL12 and CXCL13 and attract CLL cells through CXCR4/CXCR5 receptors. The receptor VLA-4 (CD49d) interacts with MSCs via VCAM1 and Fibronectin and facilitates cell – cell adhesion. The receptor CD31 for CD38 on NLCs induces Zap-70 phosphorylation thus activating BCR mediated signaling pathways. Furthermore, proliferation and survival of CLL cells can be triggered by CD40L and IL4/21 provided by T cells and by the BAFF and APRIL derived from NLCs (Spaargaren *et al.*, 2015)

1.9 Dysregulation of Apoptosis in CLL

Although initially CLL was considered a static disease with uncontrolled accumulation of lymphocytes due to failed apoptosis, the current understanding is that proliferation occurs in secondary lymphoid organs in areas known as proliferation centres of tissue microenvironment leading to expansion and prolong survival of CLL cells *in vivo* (Chiorazzi, 2007; Reed, 2008; Wu *et al.*, 2018)

The major cause of inefficacy of most targeted therapies is acquired resistance towards apoptotic mechanisms, as evading apoptosis is one of the hallmarks of CLL cells. Owing to the fact that apoptosis causes minimum inflammation and damage to the tissue, targeting apoptotic pathways has been a centre of attraction for the development of new improved anti-cancer drugs.

Apoptosis is a cell suicide mechanism that leads to characteristic morphological alterations such as membrane blebbing, reduced cytoplasm, chromatin condensation and fragmentation of genomic DNA eventually causing breakdown of a cell (Parrish *et*

al., 2013; Kalkavan and Green, 2017). It is an irreversible process regulated by a family of cysteine dependent aspartate specific proteases known as caspases. The caspases are divided into initiator caspases and executor caspases. The initiator caspases (8-9-2-10) are responsible for interaction with adaptor proteins in response to apoptotic stimuli. Once activated initiator caspases then cleave effector caspases (3-7-6) thus triggering the activation of caspase cascade. Following initiation, effector caspases cleave a wide range of substrates including poly-ADP-ribose protein (PARP) that results in structural breakdown of cells and finally in apoptosis (Strasser, 2011; Koff *et al.*, 2015).

Apoptosis can be induced by two known pathways (Figure 1.9), either by intrinsic pathway following intracellular activation involving mitochondria (mitochondrial pathway) or by activation of cell surface receptors known as “death receptors”, the extrinsic pathway (Billard, 2014). Activation of the intrinsic pathway is triggered by a wide range of intracellular signals such as radiation, cytotoxic drugs, cellular stress and growth factor withdrawals, and is closely regulated by the Bcl-2 family proteins. Upon stimulation of apoptosis, the balance between pro-apoptotic proteins (Bax, Bak, Bcl-xs and Bcl-G_L) and anti-apoptotic proteins or prosurvival proteins (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and Af/BF1-1) is disturbed resulting in the release of Bax and Bak sequestered by prosurvival member of Bcl-2 family. The anti-apoptotic proteins contain BH1-4 domain and a transmembrane domain, and the proapoptotic proteins have two multi protein domains (Bax, Bak, Bcl-xs and Bcl-GL) and only one BH3 (Bim/Bod, Bad, Bid, Bmf, Bcl-Gs, Blk, Hrk/DP5, Noxa and PUMA/Bbc3). On receiving apoptotic signals BH3 domain proteins bind with prosurvival proteins and antagonise them to release activated Bax and Bak which increases permeabilisation of mitochondrial membrane. This allows the release of cytochrome C which forms an apoptosome containing adaptor protein Apaf-1, inactive initiator procaspase 9 and cytochrome C. Procaspase 9 becomes activated in the apoptosome complex and cleaves caspases -8 and -9 thus triggering the activation of effector caspases. Once the effector caspase cascade is activated, the cell undergoes morphological and biochemical changes regulated by effector caspase 3 and becomes committed to apoptosis (Strasser, 2011; Billard, 2014; Czabotar *et al.*, 2014).

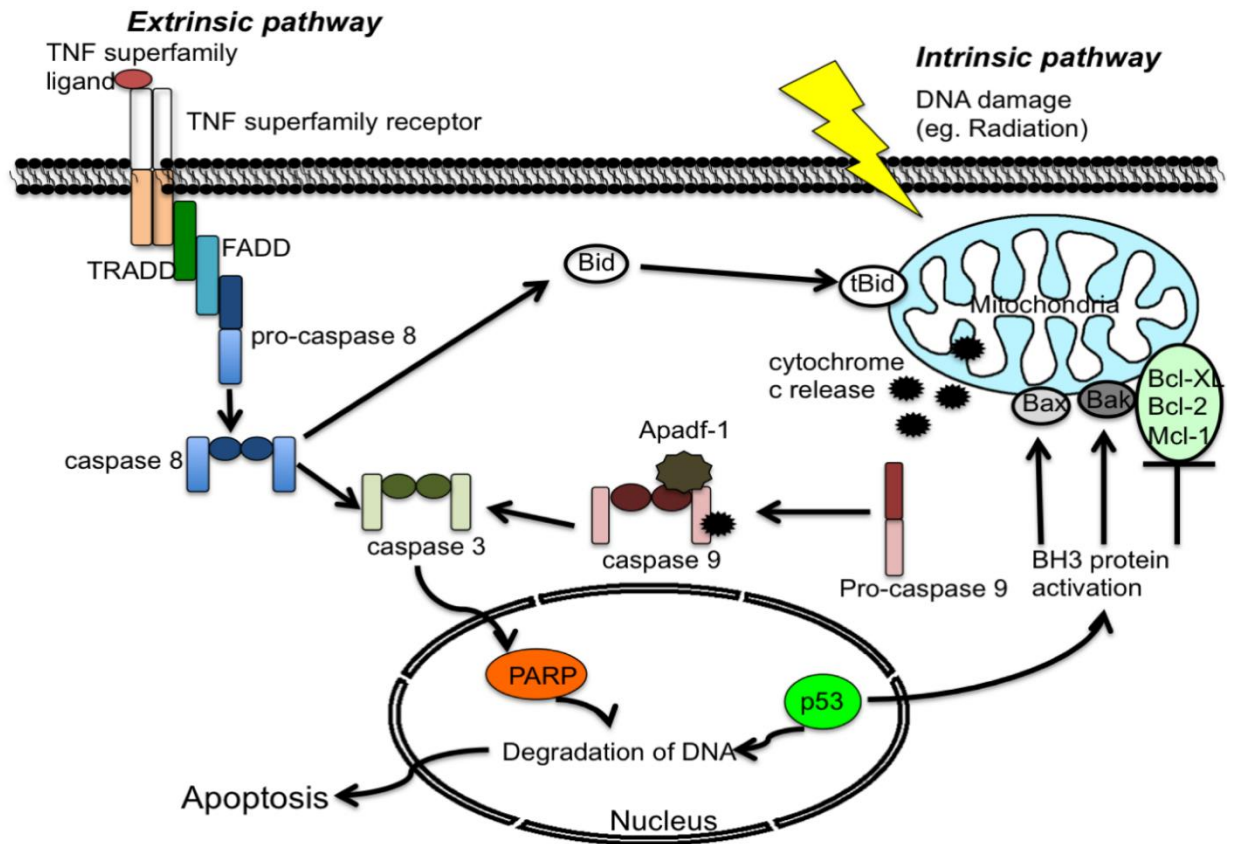


Figure 1.9. Schematic overview of extrinsic (Mitochondria independent) and intrinsic (Mitochondria dependent) apoptosis pathways. The extrinsic pathway activation involves the binding of ligands to the death receptor that results in the formation of death inducing signaling complex (DISC). DISC enables activation of caspase-8, which then cleaves and activates executor caspases 3 and 7 which then lead to demise of the cell. The intrinsic pathway is initiated following cellular stress causing activation of BH3 that leads to the recruitment of pro-apoptotic proteins Bax and Bak, and the release of cytochrome C into the cytosol which forms an apoptosome with procaspase-9 and apoptotic protease activating factor-1 (Apaf-1). Activated caspase-9 then cleaves caspase-3 and 7 allowing apoptosis process to continue to the cell death (Yuan *et al.*, 2012).

The extrinsic pathway also known as mitochondria independent pathway involves the ligation of death receptors which are members of the tumour necrosis factor receptor family such as (TNF-R, FAS, DR4) to their respecting ligands (TNF-L, FAS-L, TRAIL) thus exposing a death domain (DD) on the cytoplasmic site of the receptor. The binding of death receptor to its ligand recruits an adaptor protein and forms a death inducing signal complex (DISC). Within this DISC initiator caspase 8 and effector caspase 3 are cleaved and activated which leads to the demise of the cell.

Apoptosis resistance in CLL is influenced by many factors. CLL cells demonstrate overexpression of Bcl-2 family proteins and the increased Bcl-2 /Bax ratio that induces cell survival instead of apoptosis has led to a significantly poor prognosis in CLL

patients (Packham *et al.*, 2005, 2014). Another anti-apoptotic Bcl-2 family protein Mcl-1 has been observed to promote cell survival in CLL as high expressions of MCL-1 correlated with increased resistance to apoptosis and inability to attain complete remission following cytotoxic therapy (Pepper *et al.*, 2008; Palma *et al.*, 2015).

Another factor that can cause dysregulation of apoptosis is alterations or loss of function of the *P53* gene. Tp53 protein is a transcription factor also known as tumour suppressor protein 53 (TP53). It regulates cellular responses to DNA damages by induction of apoptosis, DNA recombination and cellular senescence. The ataxia telangiectasia-mutated gene (*ATM*) is the activated gene in the *P53* signaling pathway which can block cell cycle progression and stimulate DNA repair. Any defects in *P53/ATM* gene can contribute to a reduced response to apoptosis signals and defective DNA repair mechanism and as a result an increased survival of CLL cells. A variety of factors can damage *P53* gene such as radiation, various chemicals, and viruses such as the Human papillomavirus (HPV) and therefore over 50% of all human cancers have been detected with the mutation of *P53/ATM* genes in cytogenetic analysis (Zenz *et al.*, 2010). In addition to above mentioned factors, changes in the expression of other apoptotic regulators such as the nuclear factor-kappa B (NF- κ B) transcription factor and phosphatidylinositol-3 kinase (P1-3K) signalling pathways can induce cell survival. Many cytokines, including B-cell activation factor (BAFF), a proliferation inducing ligand (APRIL), CD40 ligand and interleukin-4 (IL-4) are activated in CLL cells and promote the survival of CLL cells, and CLL cells appear to be resistant to apoptosis if abnormalities are identified in signalling via cell-surface death receptors. Intracellular signaling following antigenic stimulation via BCR is also been recognised to influence apoptotic pathways in CLL due to presence of overexpression of apoptotic kinases in activated CLL cells (Chen *et al.*, 2010; Hanahan, 2010).

Factors affecting various apoptosis mechanisms are schematically summarised in Figure 1.10.

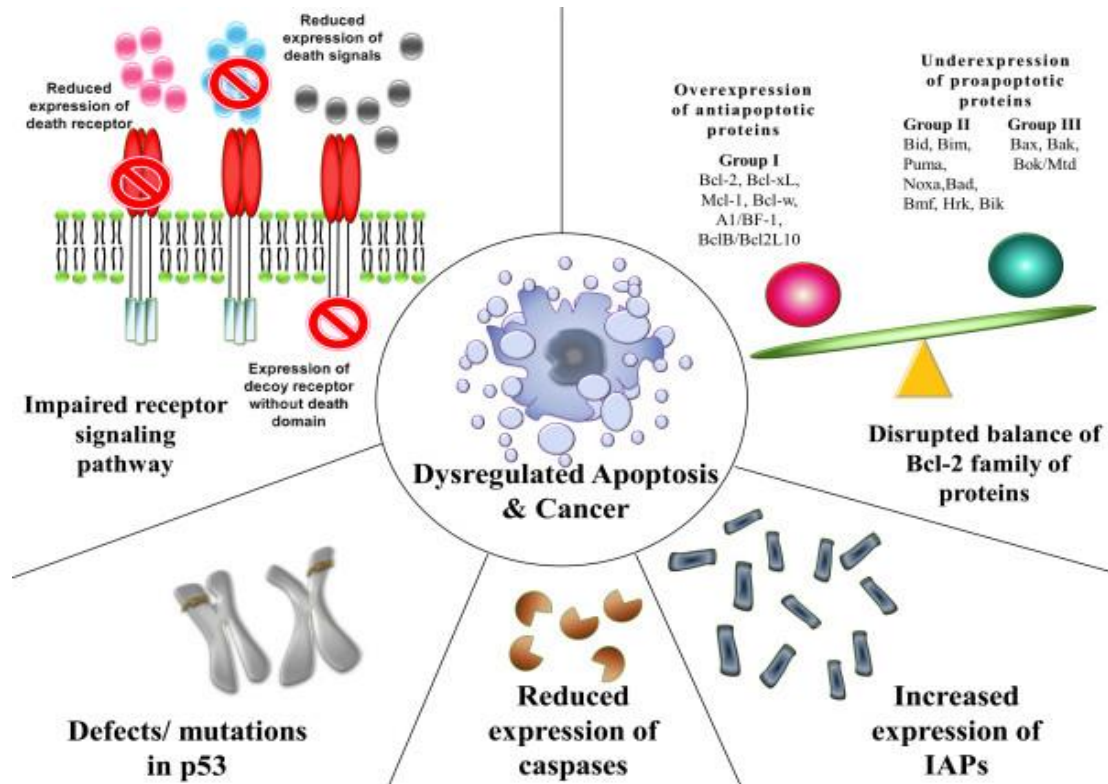


Figure 1.10. Factors involved in the apoptosis resistance. The mechanisms by which the evasion of apoptosis occurs in cancer, including defective genes, reduced caspase function, damaged signaling pathways and overexpression of Bcl-2 proteins (Wong, 2011).

Since dysregulation of apoptosis is a critical factor in the pathogenesis of CLL, strategies that induce apoptosis of malignant cells and bypass Bcl-2 will be therapeutically useful. This would require an improved understanding of a correlation between defective apoptosis in CLL and responses to therapy.

1.10 B cell anergy in CLL

Several studies have demonstrated the presence of anergic cells in CLL (Apollonio *et al*, 2013; Caligaris-Cappio, 2014; Packham *et al*, 2014). Anergy is one of the strategies adapted by immune system to silence autoreactive B lymphocytes on continuous low-affinity recognition of self-antigens (Ag) resulting in cell unresponsiveness when these B lymphocytes were further stimulated through BCR. CLL cells were always been characterized as functionally responsive to BCR triggering but it has been shown that a sizable fraction of CLL patients indeed demonstrate a leukemic clonal expansion of B cells that have anergic features (Cambier *et al.*, 2007; Gururajan, 2014). In mice,

anergized B cells were characterized by low levels of IgM with no effect on IgD expression, elevated basal intracellular calcium concentrations and subsequent constitutive phosphorylation of ERK1/2 and the nuclear translocation of NF-ATc1 in the absence of AKT activation. Such features are also seen in a subset of CLL patients that corresponds with their indolent clinical presentation (Darwiche *et al.*, 2018). However, it has been reported that this biochemical program is not permanent and CLL cell anergy can be reversed experimentally by inhibiting the signaling molecules, *in vivo*, as well, *in vitro*, and BCR responsiveness can be restored (Packham *et al.*, 2014). This feature of anergic cells is more evident in M-CLL/Zap-70 negative cases, and more importantly, the reverted anergic state of B cell is followed by apoptosis induction that may open novel therapeutic avenues (Guarini *et al.*, 2008; Apollonio *et al.*, 2012; Darwiche *et al.*, 2018).

1.11 Toll like receptors as a part of CLL microenvironment

TLRs are a family of transmembrane pattern recognition receptors (PRRs) that play a critical role in early innate immune response by recognizing highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs) expressed by microbial pathogens or endogenous molecules known as danger-associated molecular patterns (DAMPs) released from necrotic or dying cells (Reuven *et al.*, 2014). They are expressed in a variety of leucocytes such as, monocytes, macrophages, natural killer cells, cells of adaptive immunity, T and B lymphocytes and some non-immune cells such as epithelial intestinal cells and fibroblasts and play a significant role in inflammation, immune cell regulation, survival and proliferation (Kawai and Akira, 2011; Huang *et al.*, 2018).

TLRs are homologous to *Drosophila* Toll protein and to date 11 functional TLRs in human (TLR1-TLR11) and 12 in mice has been identified (Figure 1.11), of which TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and 11 are located on the cell surface and primarily respond to bacterial membrane or cell wall associated PAMPs. The remaining TLR3, TLR7, TLR8 and TLR9 are expressed in endosomal/lysosomal compartments and recognise nucleic acid based PAMPs from bacteria and viruses. TLR 10 is a pseudogene (Kawai and Akira, 2010; Rybka *et al.*, 2016).

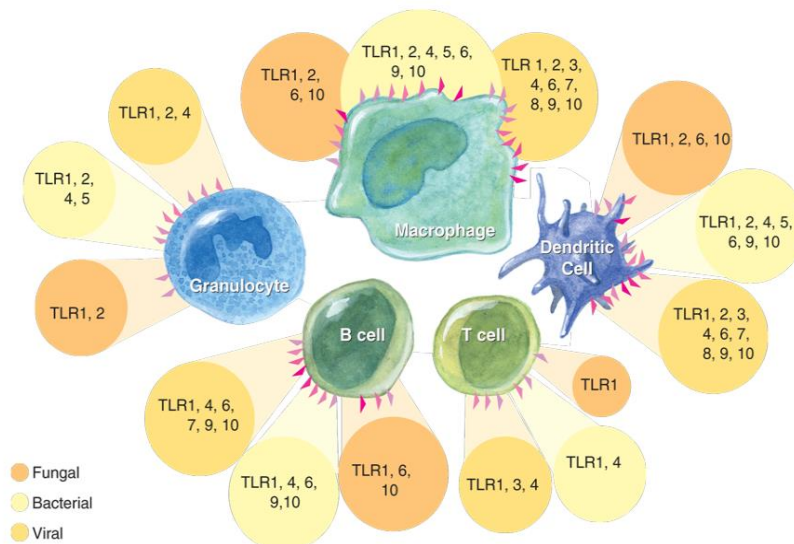


Figure 1.11. Expression of Toll like receptors (TLR) on leukocytes. Each of these TLRs detects different subsets of pathogens (Cognasse *et al.*, 2015).

TLR structure includes variable number of ligands sensing extracellular leucine rich repeats at their N-terminal ends and a cytoplasmic TIR (TLR-IL-1R, interleukin-1-Receptor) homology domain. The TIR domain facilitates signaling between TLRs and adaptor proteins such as MYD88, TRIF, TRAM and TIRAP/MAL recruited for TLR signaling. The activation of TLR signaling can occur in two ways via MYD88 or through a MYD88 independent signaling pathway. MYD88 pathway utilized by most of the TLRs leads to the activation of transcription NF- κ B and the MAPKs, p38 and JNK (c-Jun N-terminal Kinase). MYD88 independent pathway is mostly employed by TLR3 and TLR4, and leads to the activation of both, NF- κ B and IRF3 (interferon Regulatory factor 3) causing activation of other transcription factors including activator protein 1(AP-1) and interferons IFN- β and α (Hua and Hou, 2013; Muzio *et al.*, 2012). Signaling pathways activated downstream of these adaptor molecules promote the expression of pro-inflammatory cytokines, chemokines, and type I and type III interferons (Figure 1.12). However, studies suggest that only TLR4 has shown the ability to employ both pathways sequentially (Barton *et al.*, 2009).

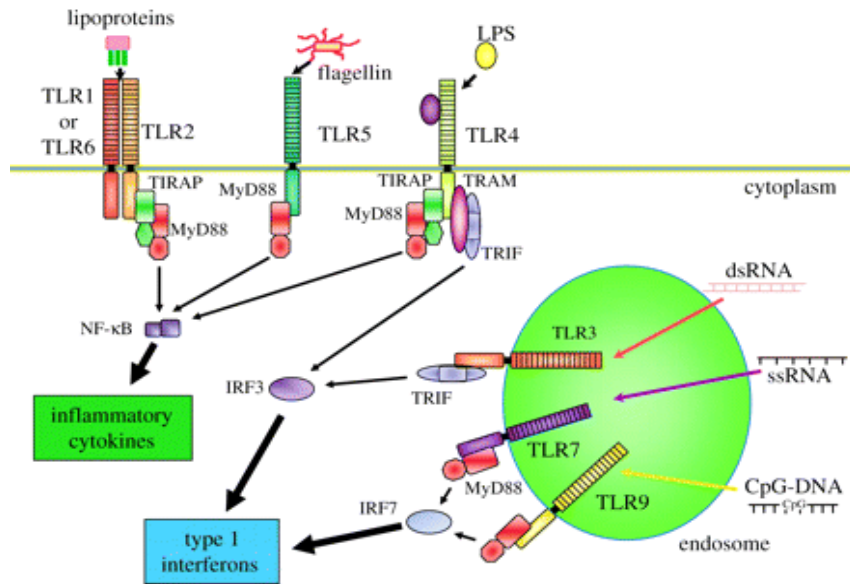


Figure 1.12. Schematic diagram of TLR ligands and signalling pathways. TLR receptors recognize different microbial components: the heterodimer of TLR4 and MD-2 recognizes lipopolysaccharide (LPS); TLR2 recognizes triacyl and diacyl portions of lipoproteins together with TLR1 or TLR6, respectively; TLR5 recognizes flagellin, a major component of flagella; TLR3 recognizes double-stranded RNA; TLR7 recognizes single-stranded RNA and TLR9 recognizes bacterial and viral DNA, the so-called CpG DNA. The signalling pathways of TLRs are mediated by selective usage of adaptor molecules, MyD88, TRIF, TIRAP and TRAM. MyD88 is involved in all TLR signalling except for TLR3. TRIF is involved in TLR3 and TLR4 dependent activation of IRF3 via IKKi/TBK1, resulting in type 1 interferon production. TRAM is responsible for the TLR4-MyD88 dependent pathway involving recruitment of TRIF to the cytoplasmic portion of TLR4. TIRAP is involved in recruiting MyD88 to the cytoplasmic portions of TLR2 and TLR4. TLR7 and TLR9-dependent production of type 1 interferon requires direct interaction of MyD88 and IRF7, which occurs only in plasmacytoid dendritic cells. Nucleic acid-recognizing TLRs (TLR3, TLR7, and TLR9) are present in the endosome (Akira, 2011).

Although TLRs provide protection against a wide variety of pathogens, inappropriate or unregulated activation of TLR signaling can lead to chronic inflammatory and autoimmune disorders. In human naive B cells, TLRs are expressed at low levels, but the expression of some TLRs is induced upon B-cell receptor (BCR) triggering. Consequently, memory B cells express several TLRs at constitutively higher levels than naïve B cells (McGettrik and O’Neil, 2010; Correa, 2014).

It has been suggested that the stimulation of TLRs expressed on CLL cells could potentially contribute to the expansion of leukemic cells. However, several studies suggested that TLR-9 agonists decrease viability of CLL cells and increase susceptibility of CLL cells to apoptosis in cell culture (Rozkova *et al.*, 2010). Ngo *et al.* (2010) reported that MYD88 related oncogenic mutations are highly recurrent in B cell malignancies and are detected in 29% of diffuse large B cell lymphoma (DLBCL) and in 36% of primary central nervous system of lymphoma cases ((Montesinos-Rongen *et al.*, 2011). It was also found that TLRs are involved in transmitting signals via MAPK

and P13K in malignant cells during carcinogenesis (Bhai *et al.*, 2017). A recent study indicated that ibrutinib is an effective inhibitor in blocking TLR and BCR signaling in CLL cells (Herman *et al.*, 2015).

TLLs are heterogeneously expressed in CLL cells. CLL cells showed a variable expression among different CLL samples with high expression of TLR7 and intermediate expression of TLR1, TLR 6 and TLR10 and low expressions of TLR2, TLR4 and TLR 8 and TLR9 (Arviniti *et al.*, 2011)). Barcellini in 2014 reported that CLL patients with low expression of TLR4 displayed higher risk of disease progression and a higher incidence of autoimmune complications (Barcellini *et al.*, 2014).

However, information about various TLR function on CLL cells is scarce, and we are the only group studying the importance of the patterns of CD180 expression and signalling in CLL. Our interest for this receptor was triggered not only by its function as an environmental sensor, but also due to its possible immunomodulatory effect on B cell apoptosis. In a seminal paper published in 1996, Yamashita *et al.*, demonstrated that priming of murine B cells with anti-CD180 (RP105) antibodies sensitised them for anti-BCR (IgM) induced apoptosis. This finding opened opportunities for the development of new therapeutic approaches for CLL using combined stimulation of CD180 and BCR.

1.12 CD180/RP105 toll-like receptor

CD180 and its murine analogue radiation protection 105 (RP105) are type 1 membrane associated orphan receptors which were originally identified as B cell surface molecules mediating activation, proliferation and differentiation and were later recognized as a TLR homologue. Valentine *et al.*, in 1988 identified CD180 by a monoclonal antibody (mAb) designated Bgp95. Later, in 1995 Miyake *et al.*, demonstrated that anti-RP105 mAb in splenic murine B cells promote B cell proliferation as well as resistance against radiation and dexamethasone induced apoptosis.

CD180 shares 74% sequence homology with RP105. CD180 expression is restricted to antigen presenting cells including B cells, monocytes/macrophages and dendritic cells (Miyake *et al.*, 1995). However, on B cells, it is seen mostly on naive B cells in mantle zone with minimum or no expression in the germinal centre cells (Otipoby *et al.*, 2002). The human CD180 gene is located on chromosome 5q12. CD180 has structural

similarities with other TLRs, particularly with TLR4. The structure of CD180 has an extracellular portion that consists of tandem repeats of leucine rich motifs and a cytoplasmic tail domain responsible for cell adhesion or receptor ligand interaction (Ogata *et al.*, 2000). The extracellular leucine rich repeats (LRR) is attached to a secretory molecule MD-1 and together they form a complex CD180/MD-1 (Figure 1.13). CD180/MD-1 working in conjunction with TLR4/MD-2 complex regulates B cell recognition and signaling of lipopolysaccharide (LPS), a membrane constituent of Gram –negative bacteria (Yoon *et al.*, 2011; Karper *et al.*, 2013).

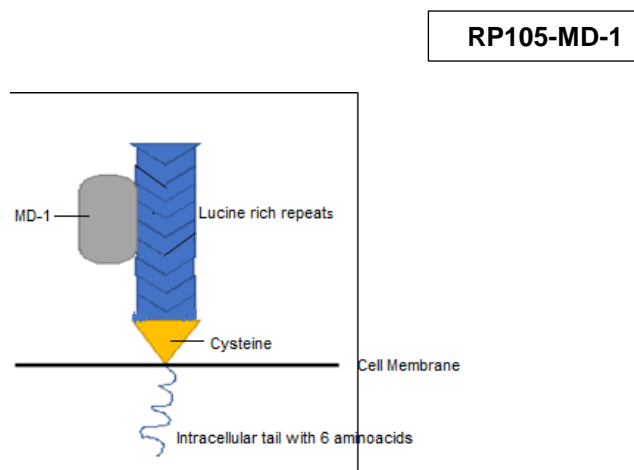


Figure 1.13. Schematic model of CD180 structure. The extracellular part has tandem leucine rich repeats (LRR) with cysteine residues at the end. The cytoplasmic tail consists of 6 amino acids that aid in receptor/ligand interaction. LRR is attached to a satellite molecule MD-1 and forms a complex CD180/MD-1.

CD180 has 61% structure similarities with TLR4 at the extracellular portion with conserved cysteine residues, but differently from TLR4 does not possess intracellular TLR –like domain. MD-1 is required for efficient expression of RP105 as demonstrated by Nagai *et al.* (2002), since MD-1 deficient mice showed impaired LPS induced B cell proliferation, antibody production and CD86 upregulation. To date no natural ligand of CD180 has been identified, and it is assumed that CD180 has to utilize other receptors' pathways to propagate intracellular signals (Chaplin, 2011).

However, TLR4/MD-2 and CD180/MD-1 appear to utilize two distinct signaling pathways. CD180/MD-1 complex following binding with LPS activates Lyn kinases

leading to CD19 phosphorylation whereas TLR4/MD-2 complex binding with LPS triggers both MYD88/IRAK pathway and MYD88 independent (TIR) domain pathway to activate JNK and NFκB (Antoz *et al.*, 2009; Chappel *et al.*, 2014).

Multiple studies have related expression of CD180 with a range of diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (Koarada *et al.*, 2010) and, more recently identifying it as a first immunological marker for marginal zone lymphoma MZL (Rousse *et al.*, 2014). Our group has demonstrated that CD180 is expressed heterogeneously on CLL cell. Whilst the majority of normal B cells expressed high density of CD180, only 60% of CLL samples, preferably M-CLL, expressed CD180 in a clonal manner (Porakishvili *et al.*, 2005). Their follow up study demonstrated that out of these 60% CLL clones that expressed CD180, only half responded with activation and proliferation, when stimulated with anti-CD180 monoclonal antibody, delineating responder and non-responder CLL cells (Porakishvili *et al.*, 2011). Our group has reported that following CD180 stimulation, a strong upregulation is seen in the phosphorylation of ZAP70, p38MAPK, ERK and AKT protein kinases in normal B cells as well as in responder CLL cells which is in line with the pro-survival behaviour of CLL cells (Chiorazzi and Ferrarini, 2005).

1.13 Interaction between CD180 and BCR s (IgM/IgD)

The two BCR receptors, IgM and IgD are present on the surface of most peripheral B cells in mice, humans and variety of other species (Noviski *et al.*, 2018). All immature cells express IgM on their surface but not IgD, but mature B cells express both BCRs. Despite the fact that IgM-mediated signalling has been at the centre of CLL research for many years, the data about the role and the function of IgD on CLL cells are controversial. Likewise, little has been reported on the outcomes of the combined stimulation of B cells through the CD180 and BCR (Figure 1.14). Coligation of CD180 and an autoreactive BCR leads to activation and proliferation of B cells and up-regulation of co-stimulatory molecules, turning them into more efficient APCs. B cells might also secrete cytokines and differentiate into plasma cells.

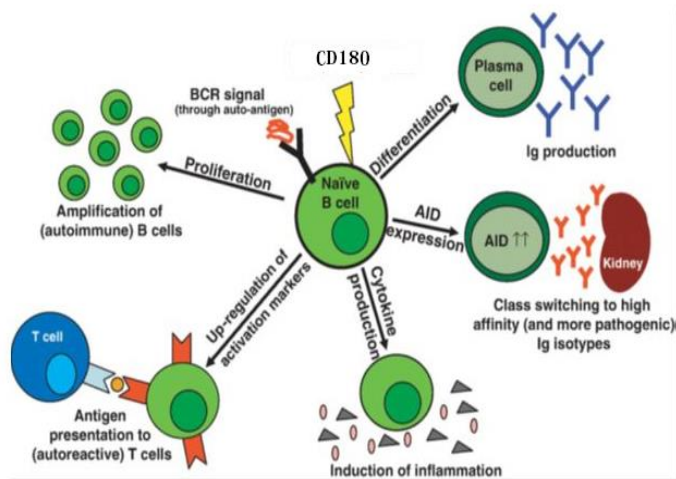


Figure1.14. Functional relationship of CD180 and BCR promote B cell activation and differentiation. Expression of activation induced cytidine deaminase (AID) is directly induced by TLR signals (modified from Claes *et al.*, 2015).

Our group has demonstrated a functional relationship between CD180 and sIgM showing that together they contribute to the activation and survival of CLL cells (Porakishvili *et al.*, 2011). Since CD180 does not have a functional cytoplasmic signaling domain, it might associate with other surface receptors for downstream signalling. Indeed, further investigation of CD180 and sIgM functional relationship through IgM-mediated signaling pathways in CLL indicated that CD180 engagement strongly affects the sIgM signalling pattern and can redirect it from pro-survival to pro-apoptotic pathway (Porakishvili *et al.*, 2015). This involved rewiring sIgM-mediated signalling pathway from PI3K/AKT to p38MAPK in CLL cells, but not in normal B cells (Porakishvili *et al.*, 2015). Since CLL cells have tendency to resist apoptosis, in particular through activation of sIgM-mediated survival, this data opens avenues for the development of new modes of treatment of CLL.

Despite all available information on the structure and high density of expression of IgD as a BCR component on B cells, the exact and unique role of IgD is still unknown. Some studies suggested that IgD functions as an optimized surface receptor improving and regulating B cell recruitment by an antigen. (Roes and Rajewsky, 1993; Hobeika, 2016). It was shown that IgD in mice can substitute for IgM in case of a loss of IgM

(Lutz *et al.*, 1998; Hobeika 2016). Previous studies have suggested that (surface) IgM and sIgD are quantitatively transmitting different downstream signals on normal B cells and on CLL cells (Packham and Stevenson, 2010). IgM and IgD are organized differently on CLL B cells where IgM forms larger and more clusters than IgD as a result the birth rate of CLL cells is more in the proximity of IgM than IgD (Mazarello *et al.*, 2017). IgD expression is absent on immature cells, low on transitional B cells and the highest on mature follicle B cells. However, the relevance of this differential expression of IgM and IgD BCR in CLL is still unclear. On the other hand, the tight regulation of both IgM and IgD expression in B cells and its conservation as seen in mice and humans suggests that IgD along with IgM may perform distinct functions that are specific for the relevant developmental stages in normal B cells as well as in malignant CLL cells (Ubelhart *et al.*, 2015).

Thus far, the pattern of expression and the outcome of IgD interaction with CD180 have not been investigated. Following the suggestion made by Hecken *et al.*, that sIgD might be transmitting a different signal to sIgM signal in CLL cells, it became necessary to study CD180 interaction with sIgD not only to understand its role in CLL but also bearing in mind the possible therapeutic intervention through CD180 and sIgD in CLL (Hacken *et al.*, 2016).

Hypothesis

Interaction between signalling pathways of CD180, and sIgD a B cell receptor, contribute to the regulation of survival and apoptosis of CLL cells.

Aims and Objectives

The aim of the project was to characterise the expression and signalling patterns of CD180 and IgD in CLL cells.

The following objectives were identified to achieve the aims of the project.

1. To identify correlations between CD180 expression patterns and established prognostic indicators in CLL
2. To assess the interaction between CD180 and sIgD-mediated signalling pathways in CLL involving protein kinases BTK, AKT and p38MAPK
3. To assess and effect of CD180 and IgD engagement on survival or apoptosis of CLL cells, following single or combined engagement of sIgD and CD180
4. To determine CD180 expression in CLL cells in stimulated and unstimulated cell cultures in correlation with signalling patterns
5. To analyse expression of CD180 in peripheral blood and in lymph nodes of CLL patients.

Chapter 2

Material & Methods

2.1 Patients

Peripheral blood samples of 60 CLL patients aged 55 to 85 years under the care of Professor Amit Nathwani were collected from University College Hospital after informed consent and ethical approval UCL/UCLH NHS REC 08/H0714/16. The Material transfer agreement (MTA) between the University of Westminster (UoW) and UCH was obtained from the ethical committee of the University of Westminster.

All the patients included in this study were either newly diagnosed or repeat patients who were untreated for 6 months prior to the study. At the time of diagnosis these patients had highly diverse WBC count (from 20 -100 $\times 10^9/L$) and at Binet stages A (40), B (15) and C (5).

Formalin-fixed, paraffin wax embedded (FFPE) CLL lymph node (LN) and normal tonsil sections stored at St Bartholemew's Cancer Institute, Queen Mary University of London, under the care of Dr Sergey Krysov were analysed for CD180 expression on the premises of QMUL as a part of ongoing collaboration.

2.2 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated using Histopaque-1077 (Sigma Aldrich, UK). Blood samples were diluted 1:1 with Hank's Balanced Salts Solution (HBSS, Sigma, UK). Three ml of Histopaque was added to centrifuge tubes and diluted blood was layered on the top. Tubes were centrifuged for 30 minutes, at 400 $\times g$ at room temperature (5810R centrifuge, Eppendorf, UK). PBMC were collected by aspiration of the interphase using a sterile Pasteur pipette and transferred into new centrifuge tubes. The collected cells were washed twice with 5 ml HBSS at 4°C for 10 minutes, by centrifuging at 200 $\times g$. The supernatant was removed, and cell pellet vortexed and re-suspended in 1 ml RPMI-1640 (Roswell Park Memorial Institute 1640, Sigma, UK) supplemented with 10% v/v Foetal bovine serum (FBS, Sigma Aldrich, UK).

The viability of purified cells was checked with Trypan blue which was 95 to 98 %v/v. The number of cells present in a known volume of a sample were estimated using haemocytometer (Millipore, UK). The cell suspension was introduced to a known space of known depth (0.1mm) beneath a cover slip and counted within a grid of area 1mm². This gives the number of cells per 0.1mm³ or 0.1 μ l. Multiplying by 10 gives the number of cells per μ l and multiplying by 10,000 gives the number of cells per ml. Here the

concentration of PBMC was adjusted using RPMI-1640 medium according to the experimental protocol's requirements such as 1×10^6 /ml.

2.3 Immunophenotyping of CLL cells

200 μ l PBMC suspension at a concentration 1×10^6 /ml were placed in the wells of 96 well round-bottom microplates (Corning, Costar, and USA) and centrifuged at 200 xg for 5 minutes at 4°C. The supernatants were discarded, and pellets vortexed to resuspend the cells. 20 μ l human Immunoglobulins (Ig) (ImmunoReagents, USA) at a concentration 2 mg/ml were added into each well to block Fc receptors and the microplate was incubated for 30 min on ice. The microplate was centrifuged for 5 minutes at 200 xg at 4°C and supernatants discarded. After vortexing, 20 μ l of unconjugated primary mouse monoclonal antibodies (mAbs) at a final concentration of 20 μ g/ml were added into each designated well, whilst purified mouse IgG1 was used as isotype control: the antibodies used were anti-CD180, anti-IgM, anti-CD79b, anti-CD38, anti-CD86, anti-CD40 (all from BD Biosciences, UK), and anti-IgD (Sigma Aldrich UK), all of the IgG1 subclass. After incubation on ice for 30 minutes, the cells were washed twice with HBSS for 5 minutes at 200 xg at 4°C. The supernatant was removed and the microplate vortexed. Cells were then treated with 20 μ l of FITC (fluorescein isothiocyanate)-conjugated polyclonal rabbit anti-mouse immunoglobulin F(ab)₂, (Dako, UK) as a secondary antibody diluted at 1:15 in HBSS and incubated on ice in the dark (photosensitive antibody conjugate) for 30 minutes. After 30 minutes the cells were washed twice as above, the supernatant discarded, and the microplate vortexed. To block any unused rabbit anti-mouse F(ab') sites, the cells in each well were treated with 20 μ l of mouse serum at a dilution 1:15 in HBSS (Dako, UK) and kept for on ice for 30 minutes in the dark. The cells were centrifuged for 5 minutes at 4°C at 200 xg , supernatant discarded and microplate vortexed.

In separate experiments, direct staining of freshly isolated PBMCs in RPMI medium was performed using 20 μ l of FITC-conjugated mouse anti-human CD79a (Ancell, USA) at a dilution of 1:25 and 5 μ l of undiluted FITC-conjugated mouse IgG (BD Biosciences, UK) as isotype control were used. Cells were incubated on ice in the dark for 30 minutes, washed twice with HBSS as above, supernatant was discarded and plate vortexed.

All cells were further stained with 5µl of anti-human CD19 PE-Cy5 (phycoerytherin cyanine 5)-conjugated mouse mAb and 5µl of anti-human CD5-PE conjugated mouse mAb (both - BD biosciences, UK), incubated on ice in the dark for 30 minutes, washed twice with HBSS as above, supernatant discarded and microplate vortexed.

For the assessment of intracellular expression of ZAP70 protein kinase PBMCs at a concentration 1×10^6 cells/ml were treated first with 5µl of PE-Cy5 mouse anti human CD19 on ice for 30 minutes in the dark. Cells were washed twice, vortexed and treated with 50µl of Fix and Perm kit medium A (Invitrogen, USA) for 15 minutes in the dark at RT. Fix and Perm kit stops the cells reaction at that stage by fixing them (A) and then Perm reagent (B) permeabilizes the cells for intracellular staining. The cells were then pelleted, the supernatant discarded, the cells washed once as above, and cells were treated with 50µl of medium B for 15 minutes in the dark at RT. The supernatant was removed following centrifugation and cells were resuspended by vortexing. 5ul of FITC conjugated mouse IgG1 isotype control or 5ul of FITC conjugated anti-Zap-70 IgG1 mAb (both BD Biosciences, UK) were added to the designated cells, incubated for 30 minutes on ice in the dark and washed twice as above.

In the case where samples were analysed immediately on the Cyan flow cytometer (Beckman Coulter, UK) 200µl of HBSS were added into each well after two washes and samples were run on flow cytometer. In other cases, 200µl of 2% v/v Paraformaldehyde (PFA, Biolegend, USA) were added to each well and stored at 4°C (no more than for 4 days for surface receptors or 24hr for intracellular receptors) before flow cytometry.

The results of the flow cytometry analysis were expressed as percentages of positive cells vs isotype control as well as antibody relative binding sites/cells (RBS/cell) as previously described (Guyre *et al.*, 1989; Porakishvili *et al.*, 2005). The latter reflects the density of the expression of surface molecules, assessed by indirect immunophenotyping. It was determined by comparison of mean fluorescence intensities of each sample to a standard curve generated with fluorescent microspheres using especially designed Excel template. We consider cells positive for a marker with RBS/cell more than 500 (Porakishvili *et al.*, 2005). Percentages of positive cells were calculated using flow cytometry histograms.

Based on the analysis CLL cells were categorised as CD180+ or CD180-. CD180+ and IgD+ cells were selected for further experiments on signalling and apoptosis. Representative histograms are shown in Figure 2.1a, 2.1b, 2.1c and 2.1d.

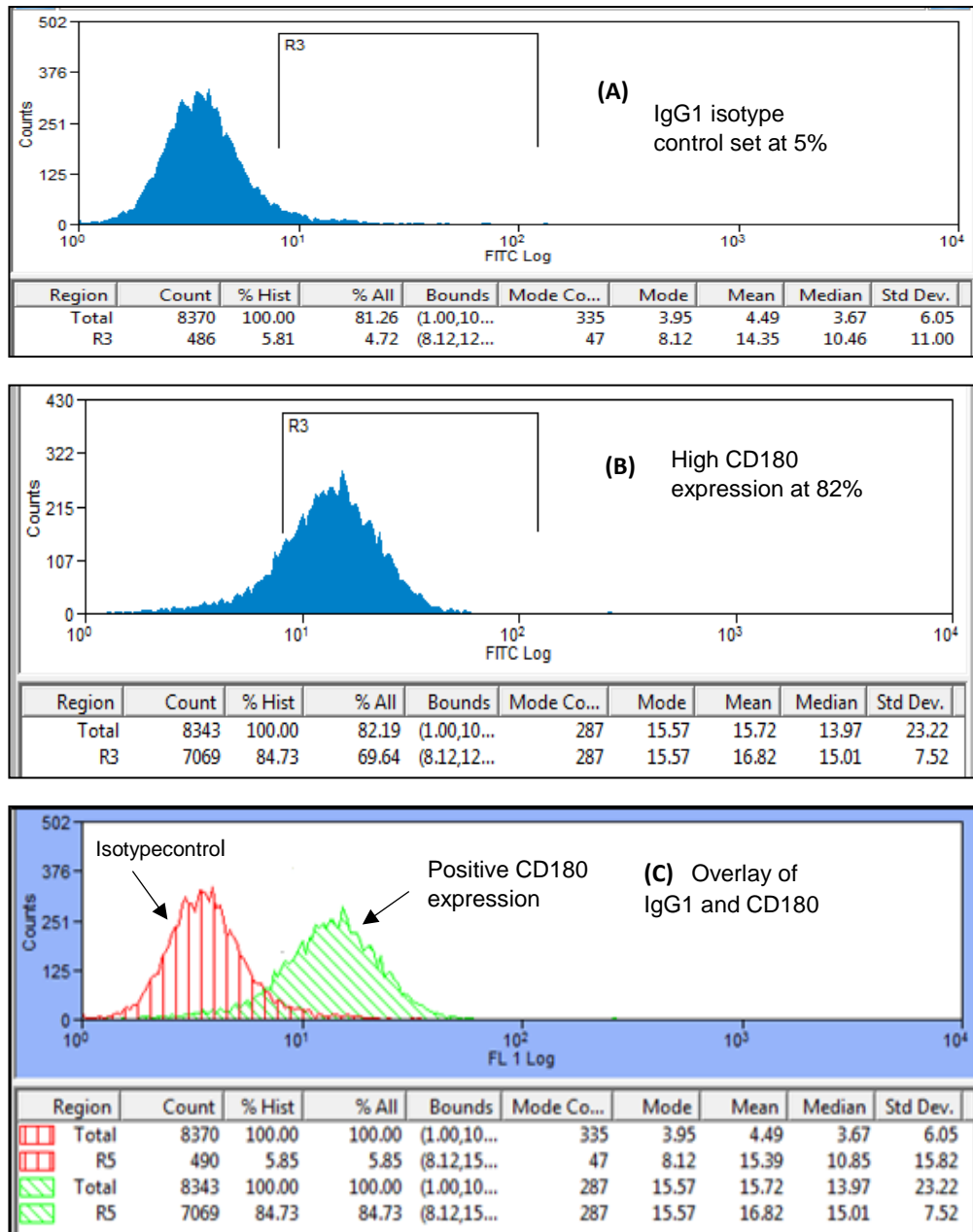


Figure 2.1a: Representative flow cytometer images of the indirect staining using dot plots, histograms and overlays. The purified PBMCs were stained for CD180 and IgG1 isotype control as described above and the flow cytometry data was analysed using Summit 4.3 software CLL cells; **(A)** Histogram of the isotype control IgG1 (5%); **(B)** Histogram of CD180+ CLL cells (84%); **(C)** Overlay plot of A (RED) and B (GREEN).

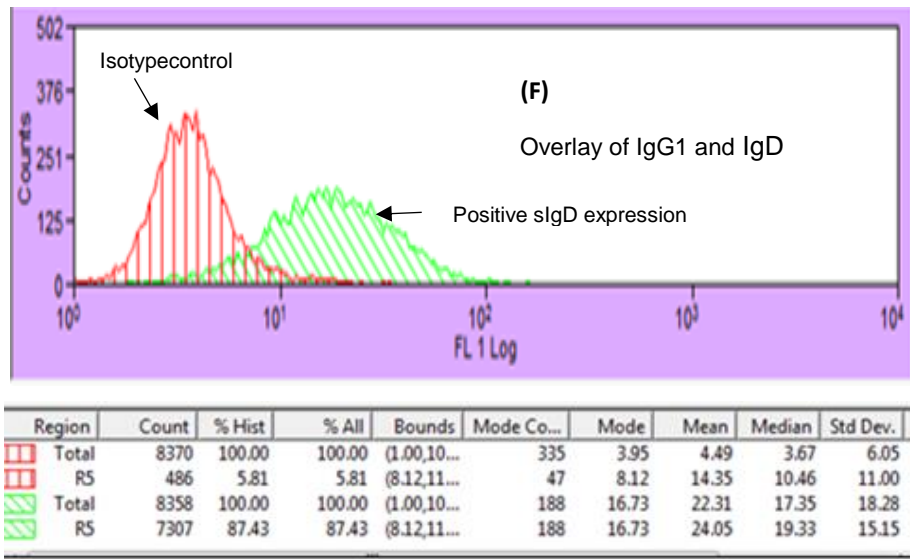
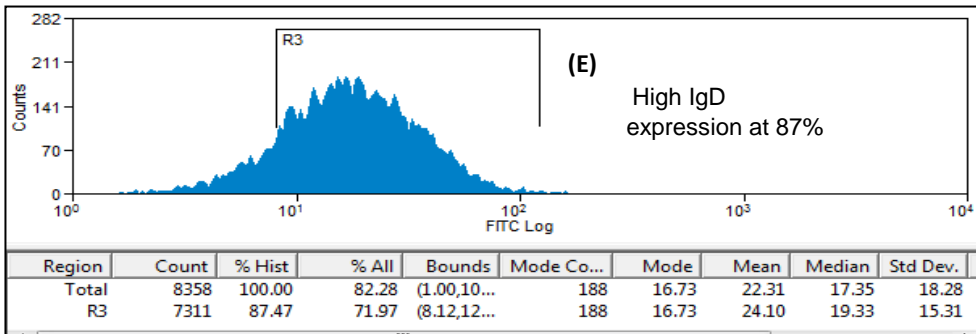
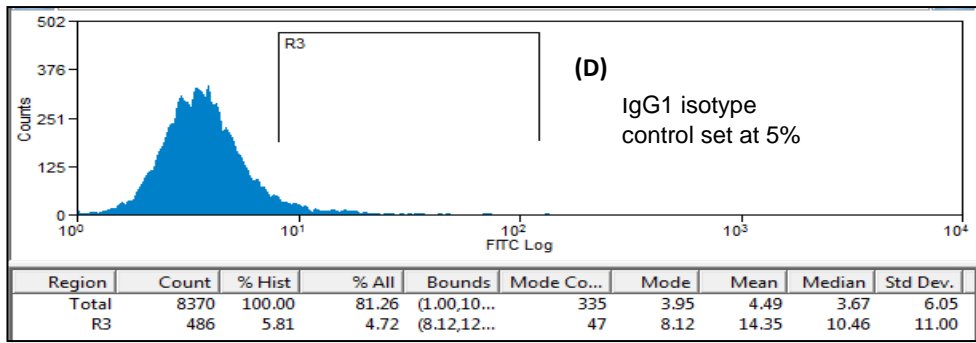


Figure 2.1b: Representative flow cytometer images of the indirect staining using dot plots, histograms and overlays. The purified PBMCs were stained for IgD receptor and isotype control IgG1 as described above and flow cytometry data was analysed using Summit 4.3 software CLL cells; **(D)** Histogram of the isotype control IgG1 (5%); **(E)** Histogram of IgD+ CLL cells at (87%); **(F)** Overlay plot of D (RED) and E (GREEN).

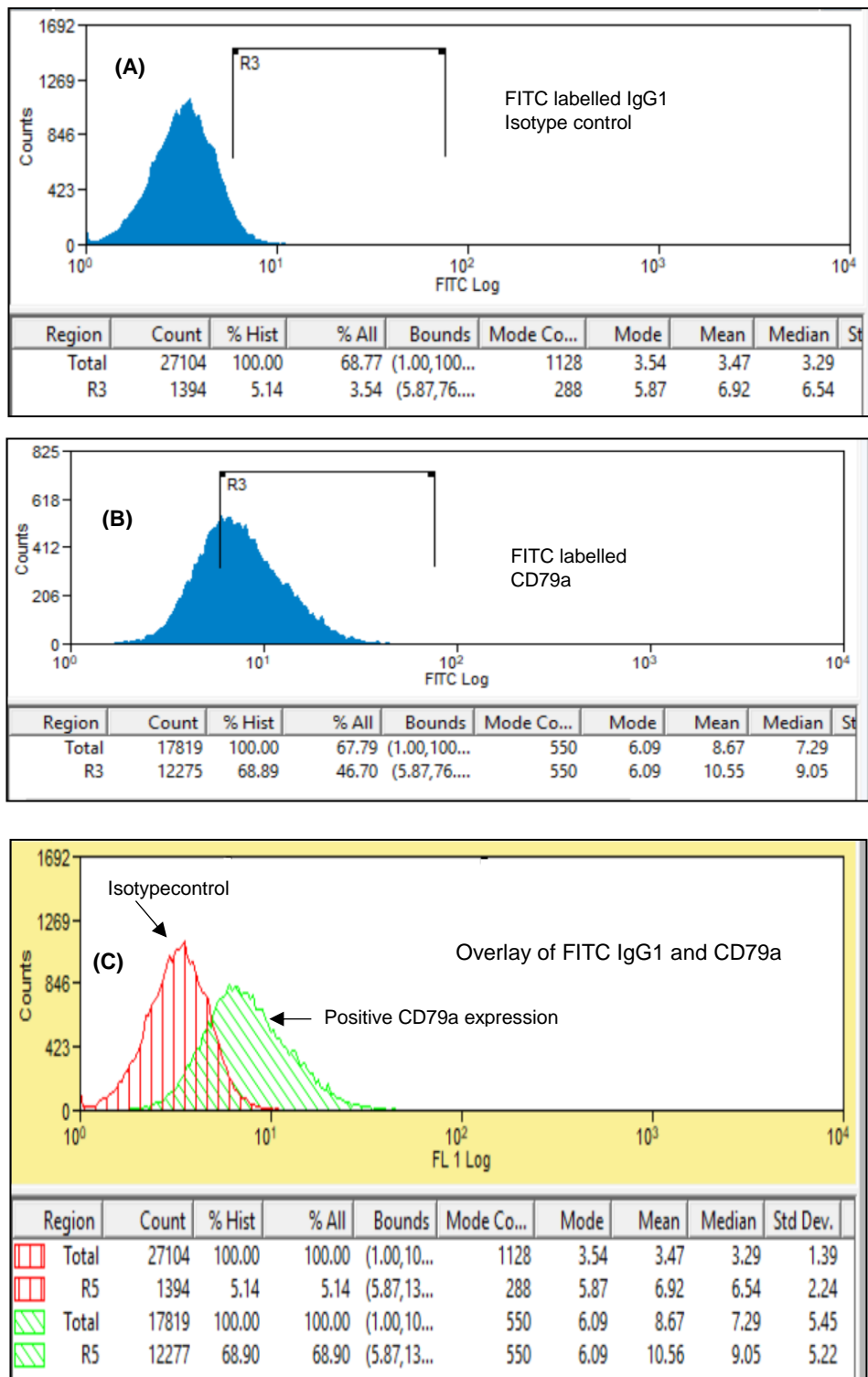


Figure 2.1c: Representative flow cytometer images of the direct staining of CD79a using histograms and an overlay. The purified PBMCs were stained for CD79a and isotype control IgG1 as described above and flow cytometry data was analysed using Summit 4.3 software. **(A)** Histogram of the isotype control IgG1 (5%); **(B)** Histogram of CD79a+ CLL cells (68%); **(C)** Overlay plot of A (RED) and B (GREEN).

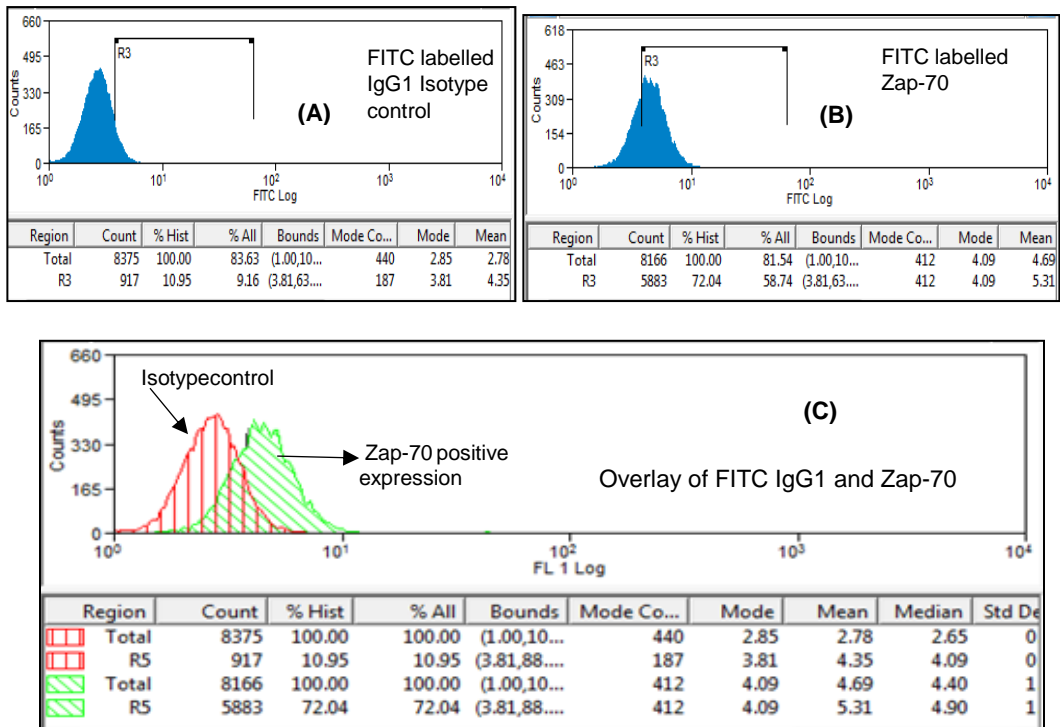


Figure 2.1d. Representative flow cytometer images of the direct staining of Zap-70 using histograms and an overlay. The purified PBMCs were treated with PE Cy5 CD19 and Fix and Perm Medium A & B and stained with FITC conjugated IgG1 or anti- Zap-70 mAb as described above and flow cytometry data was analysed using Summit 4.3 software. (A) Histogram of isotype control IgG1 (10%); (B) Histogram of Zap-70^{pos} CLL cells (72%); (C) Overlay plot of A (RED) and B (GREEN).

2.3 Assessment of the phosphorylation of intracellular protein kinases (PK) and CD79a

200µl CD180+sIgD+ CLL samples at a concentration 10^6 /ml in RPMI-1640 medium supplemented with 10% FBS, were placed in the wells of a 96 well flat bottom microplate (Corning, Costar, USA). The cells were then stimulated with 8µl of sodium azide free anti-CD180 mAb (BD Biosciences, UK) at a final concentration 20 µg/ml for 20 minutes alone or with 8ul of goat anti-human IgD F(ab')₂ (Southern Biotech, Birmingham, USA) at a final concentration 20 µg/ml incubated for 10 minutes at 37°C in 5% CO₂ incubator (Galaxy S, Uk).

In separate experiments for combined stimulation 8µl of anti-CD180 was added first, and cells incubated for 20 minutes and then 8ul of anti-IgD was added and incubated for further 10 minutes. No antibodies were added to the control wells termed as unstimulated.

Following stimulation, the cells were transferred to 96-wells round bottom microplate, pelleted at 200 xg, 4°C, for 5 minutes and supernatant removed. Cells were washed twice with HBSS as before and stained with 5µl of PE-Cy5 conjugated mouse anti-human CD19 mAb. After 30 minutes incubation on ice in the dark, cells were washed twice with HBSS, fixed and permeabilised using Fix/perm kit as described above in section 2.1. Following reaction with Fix/perm kit cells were washed twice at 200 xg for 5 minutes at 4°C and were stained with mAbs raised against human phosphorylated protein kinases: phospho-p38MAPK mouse Alexa Fluor® 488 conjugate (Cell signalling USA, New England Biolabs Ltd, UK), phospho-AKT Rabbit Alexa Fluor® 488 conjugate, (Cell signalling USA, New England Biolabs Ltd, UK), phospho-BTK mouse Alexa Fluor® 647 conjugate (BD Biosciences, UK) and maintained for 30 minutes in the dark at RT. Following incubation cells were washed twice with HBSS.

In separate experiments of CD79a signaling, after fixation and permeabilisation of PBMCs, 20µl of anti-phospho-CD79a unconjugated rabbit antibody (Tyr182) (Cell signaling, USA) were added to stimulated and unstimulated PBMCs and incubated on ice for 30 minutes in the dark. Following twice washing with HBSS as above, and supernatant discarded, stimulated and unstimulated cells were stained with 20µl of goat anti-rabbit Ig secondary antibody conjugated to FITC (BD Biosciences, UK). Following

incubation, cells were again washed twice in HBSS to remove any unwanted reagents and cell debris.

All cells to be analysed immediately were resuspended in HBSS to run on flow cytometer or fixed with 200ul 2% PFA and stored in the dark at 4°C to be analysed later, within 24hr. The results were assessed as percentages of positive cells in stimulated and unstimulated cultures inside gated CD19+CD5+ cell population demonstrated in Figure 2.2 for a representative sample on p38MAPK protein kinase.

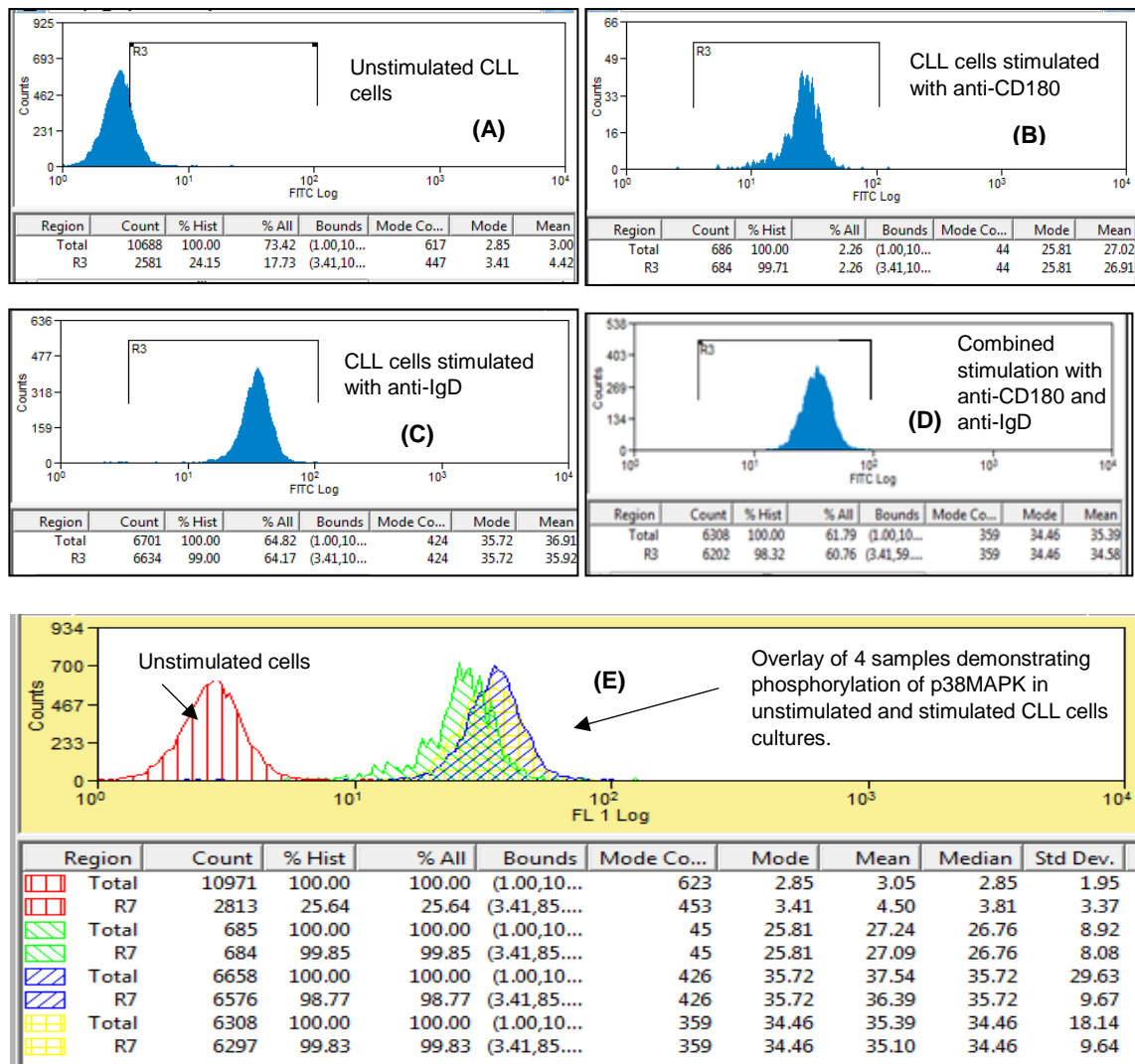


Figure 2.2: Representative flow cytometer images of histogram and overlay of phosphorylation of p38MAPK protein kinase in stimulated and unstimulated CLL cells following stimulation with anti-CD180 and anti-IgD. Purified PBMCs were stimulated with anti-CD180 or anti-IgD or both mAbs sequentially as described above in section 2.3. Control samples were left unstimulated. Cells were washed and stained with anti-CD19 and anti-CD5 mAbs, fixed, permeabilised, and treated with anti-phospho p38MAPK Alexa flour 488 conjugate mAb as described above. The results were analysed by flow cytometry and Summit 4.3 software and expressed as percentages of positive cells inside gated CD19+CD5+ population. **(A)** Histogram representing p38MAPK+CD19+CD5+ unstimulated CLL cells (25%); **(B)** Histogram representing p38MAPK+CD19+CD5+ CLL cells upon anti-CD180 stimulation (99%); **(C)** Histogram representing p38MAPK+CD19+CD5+ CLL cells upon anti-IgD stimulation (98%); **(D)** Histogram representing p38MAPK+CD19+CD5+ CLL cells upon sequential anti-CD180 and anti-IgD stimulation (99%); **(E)** Overlay plot of A (RED), B (GREEN), C (YELLOW) and D (BLUE)

2.4 Assessment of apoptosis of CLL cells

200µl of CD180+sIgD+ CLL samples at a concentration 10^6 /ml in RPMI-1640 medium supplemented with 10% FBS, were placed in the wells of a 96 well flat bottom microplate (Corning, Costar, USA). The cells were then stimulated with 8µl of sodium azide free anti-CD180 mAb (BD Biosciences, UK) at a final concentration 20 µg/ml or with 8ul of goat anti-human IgD F(ab')₂ (Southern Biotech, Birmingham, USA) at a final concentration 20 µg/ml and incubated for 24h at 37°C in 5% CO₂ incubator. In separate experiments for combined stimulation, 8µl of anti-CD180 was added first and incubated for 20 minutes and then 8ul of anti-IgD was added and incubated for 24h. No antibodies were added to the control wells termed as unstimulated.

After 24 hours of incubation, cells were transferred into wells of a round-bottom microplate, washed twice as described before and stained with 5µl of PE-Cy5 conjugated mouse anti-human CD19 mAb. After 30 minutes of incubation on ice in the dark, cells were washed twice with HBSS as before, vortexed and re-suspended in 200µl of binding buffer (diluted in 1:10 in deionized water) supplied in AnnexinV/PI Kit (Sigma Aldrich, UK). 1µl of Propidium Iodide (PI, 100µg/ml in 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaC) and 2 µl of FITC-conjugated Annexin V (~50 µg/ml in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl) were added immediately and incubated at a room temperature for 10 min in the dark. The working concentration for PI and Annexin V-FITC was optimised previously in our group. Following 10 minutes of incubation, samples were analysed by flow cytometry immediately. Viable (Ann-PI-), early apoptotic (Ann+PI-) and late apoptotic (Ann+PI+) cell populations have been identified inside the CD19+ gate and expressed as the percentages as indicated in Figure 2.3 for a representative sample.

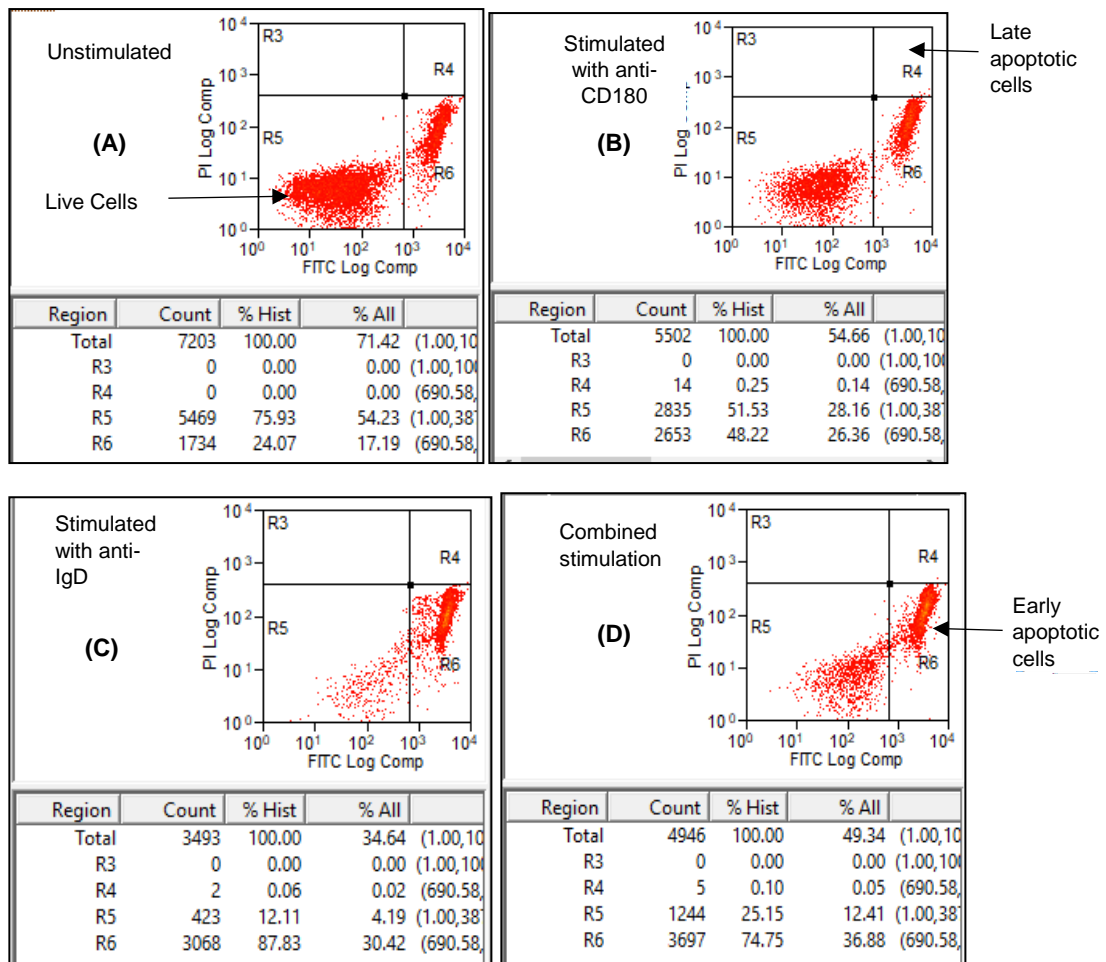


Figure 2.3. Representative flow cytometry profile of cell populations defined by Annexin V/PI staining. PBMCs were stimulated for 24hr with anti-CD180 and/or with anti-IgD mAbs and stained with anti-CD19, Annexin V and PI as described above. **(A)** Unstimulated PBMCs; **(B)** PBMC, stimulated with anti-CD180 mAb; **(C)** PBMCs stimulated with anti-IgD mAb; **(D)** PBMCs, stimulated with both, anti-CD180 and anti-IgD mAbs. R5 – Ann⁻PI⁻ (viable cells); R6 – Ann⁻PI⁻ early apoptotic cells; R4 – Ann⁺PI⁺ late apoptotic cells; R3- cellular debris, dead cells.

2.5 Expression of CD180 in 24, 48, and 72 hours long PBMC cultures

200µl of IgD/IgM positive PBMCs at a concentration 10^6 /ml in RPMI were distributed into wells of 96 flat bottom sterile microplate. Goat anti-human IgD or goat anti-human IgM F(ab')₂ (Southern Biotech, Birmingham, USA) or both were added to some wells in duplicates while some of the wells were left unstimulated. The microplates were kept for incubation for 24 hr, 48hr and 72 hr at 37°C in 5% CO₂ incubator. PBMCs, *ex vivo*, were used as zero-hour controls for the modulation experiments.

Stimulated cells as well as 0-hour control *ex vivo* cells were transferred to 96 round bottom microplates and centrifuged for 5 minutes, 200 xg at 4°C and supernatant discarded. Cells were washed twice in HBSS, vortexed and stained with 5µl PE-Cy5 mouse anti -human CD19 as described above. 5µl of PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180mAb were also added to 0 hours, stimulated or unstimulated wells and left for incubation for 30 minutes in the dark on ice. After incubation, the microplate was centrifuged, the cells washed twice with HBSS as above and supernatants discarded. Cells were vortexed and resuspended in HBSS or PFA and analysed by flow cytometry. The modulation (increase or decrease) of the expression of CD180 on CD19+ cells following 24 hours and 48 hours and 72 hours stimulation, compared with unstimulated cultures and 0 h control, were measured as percentages of CD180+cells as well as Mean fluorescence intensity (MFI) versus isotype control.

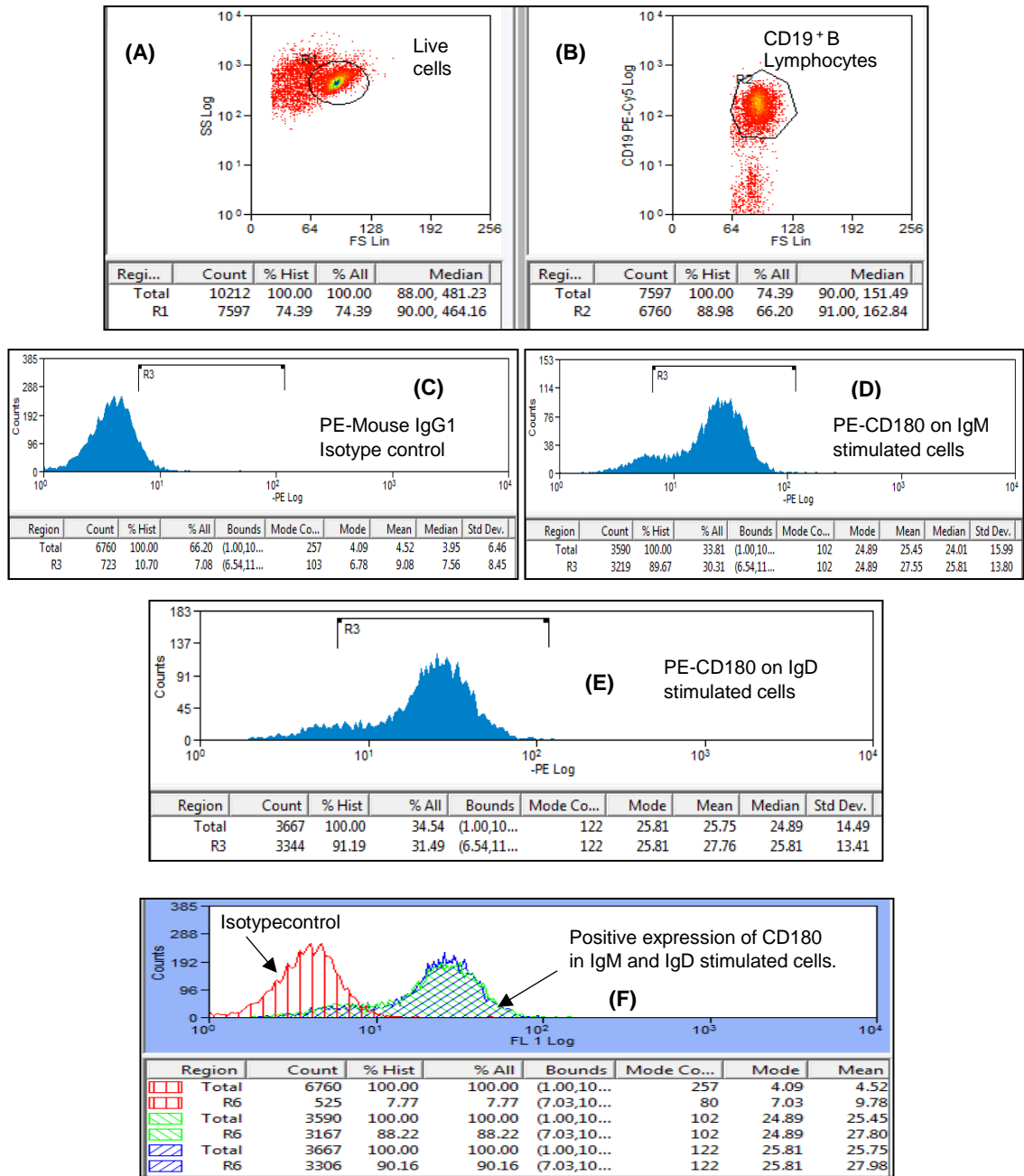


Figure 2.4: Representative flow cytometry images of dot plots, histograms and the overlay of phenotyping by direct staining for CD180 expression on PBMCs from CLL cells. CLL cells were stimulated with anti-IgM and anti-IgD F(ab')₂ and controls were left unstimulated for 24hrs, 48hrs and 72hrs. Cells were then washed and stained with PE-Cy5 conjugated anti-CD19 mAb and PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180mAb. The modulation of the expression of CD180 on CD19+ cells following 24 hours and 48 hours and 72 hours stimulation, compared with unstimulated cultures was analysed by flow cytometry. (A)Dot plot of live cells with gating ;(B) Gated CD19+ B Cells ;(C)Histogram representing negative population of cells (10 %);(D) Histogram representing increased expression of CD180+ cell in IgM stimulated cells(89%);(E)Histogram representing increased expression of CD180+cells in IgD stimulated cells(91%);(F) Overlay plot of CD180+cells compared with negative control cells C(RED) D(GREEN)and E(BLUE).

2.6 Fluorescence in situ hybridization (FISH)

FISH analysis was carried out to determine possible correlations between the expression of CD180 and known chromosomal aberrations in CLL. The CLL samples utilized for FISH experiments were same as used in phenotyping, signaling and apoptosis experiments. All blood samples were obtained from University College Hospital after informed consent as mentioned in section 2.1.

Chromosome and nuclei preparations for FISH analysis were obtained by seeding 0.5 ml of peripheral blood in 10 ml of pre-warmed KREAvital Lymphocyte Karyotyping Medium (KREATECH Diagnostics, Leica Biosystems, UK) in a 25 cm² sterile flask (Thermo Fisher Scientific, Denmark). The blood cultures (at least 2 replicates per patient sample) were incubated at 37°C/ 5% CO₂ for 72 hours. The cell cultures were then pelleted, and supernatant removed. Pre-warmed Hypotonic Solution Potassium Chloride (KCl, 0.075 M) (Life Technologies, UK) was added slowly and cells were left to incubate for 10 minutes at room temperature in sterile conditions. After further centrifugation, the pelleted cells were chemically fixed with cold Carnoy fixative (3:1 Methanol/Acetic Acid). Slides for microscopy observation were prepared by dropping a small amount of the fixed cells with a Pasteur pipette onto the slides and allowed to dry at room temperature. A first assessment of quality of chromosome preparations was performed by Giemsa staining (Invitrogen, Germany) and bright light microscopy analysis.

FISH analysis was undertaken using the following dual-colour (Platinum Bright™550 / Platinum Bright™495) probes: *DLEU1* (KBI-10102), *ATM* (KBI-10103), *TP53* (KBI-10112) (Kreatech Diagnostics, Leica Biosystems, UK), corresponding respectively to the following chromosomal lesions: *del13q14.3*; *del11q22* and *del17q13*. Each of the probes also contain a built-in control to centromeric/ telomeric sequence. Hybridization was carried out as per manufacturer's instructions. Specifically, to visualise and assess copy number of specific DNA sequences on chromosomes and nuclei, slides were denatured in 70% Formamide (Sigma-Aldrich, UK) / 2xSSC (saline-sodium citrate, Sigma-Aldrich, UK) buffer at 72°C for approximately 1 minute 50 seconds (±10 seconds) and then dehydrated in ice cold ethanol series (70%, 90% and 100%) for two minutes in each. The slides were air dry. FISH probes were denatured separately by incubating the probes at 90°C for approximately 9 minutes 50 seconds (±10 seconds).

The denatured probes were then applied to the denatured, air dried chromosomes, followed by application of 22x22 mm glass coverslips sealed with Fixogum (rubber cement) and incubation at 37°C in a humidified chamber. After 24-48 hours, the coverslips were removed, and the slides were washed using 2xSSC, 0.1 Tween-20 and 0.4xSSC 0.1 tween-20 (Sigma Aldrich, UK), for 2 and 1 minutes respectively at room temperature. The slides were then dehydrated in ethanol series (70%, 90% and 100% ethanol) for approximately 1 minute each, allowed to air-dry and finally stained and mounted with Vectashield with DAPI (4',6'-diamidino-2-phenylindole) (Vector Laboratories Vector Shield) The expected fluorescent pattern for normal cells would be two green-two red signals, while cells with deletions of either *DLEU* or *ATM* or *TP53* would present with and 1 red -2 green signals.

The slides were examined on a dedicated FISH workstation housed at UCL Institute for Women's Health, consisting of an Olympus BTX40 microscope powered by Sola light engine coupled with a Digital Scientific Camera (A3472-06) with the Smart Capture 3 software. Image processing and analysis were performed using ImageJ software. ImageJ is image processing programme designed for analysing scientific multidimensional images. Here ImageJ was used to calculate the number of nuclei with or without deletions seen under microscope. FISH data processing cut-off points were determined using the approach previously suggested (Wolff *et al.*, 2007). Values obtained were rounded up, the minimum number of scored nuclei was set at 50 (Wiktor *et al.*, 2006).

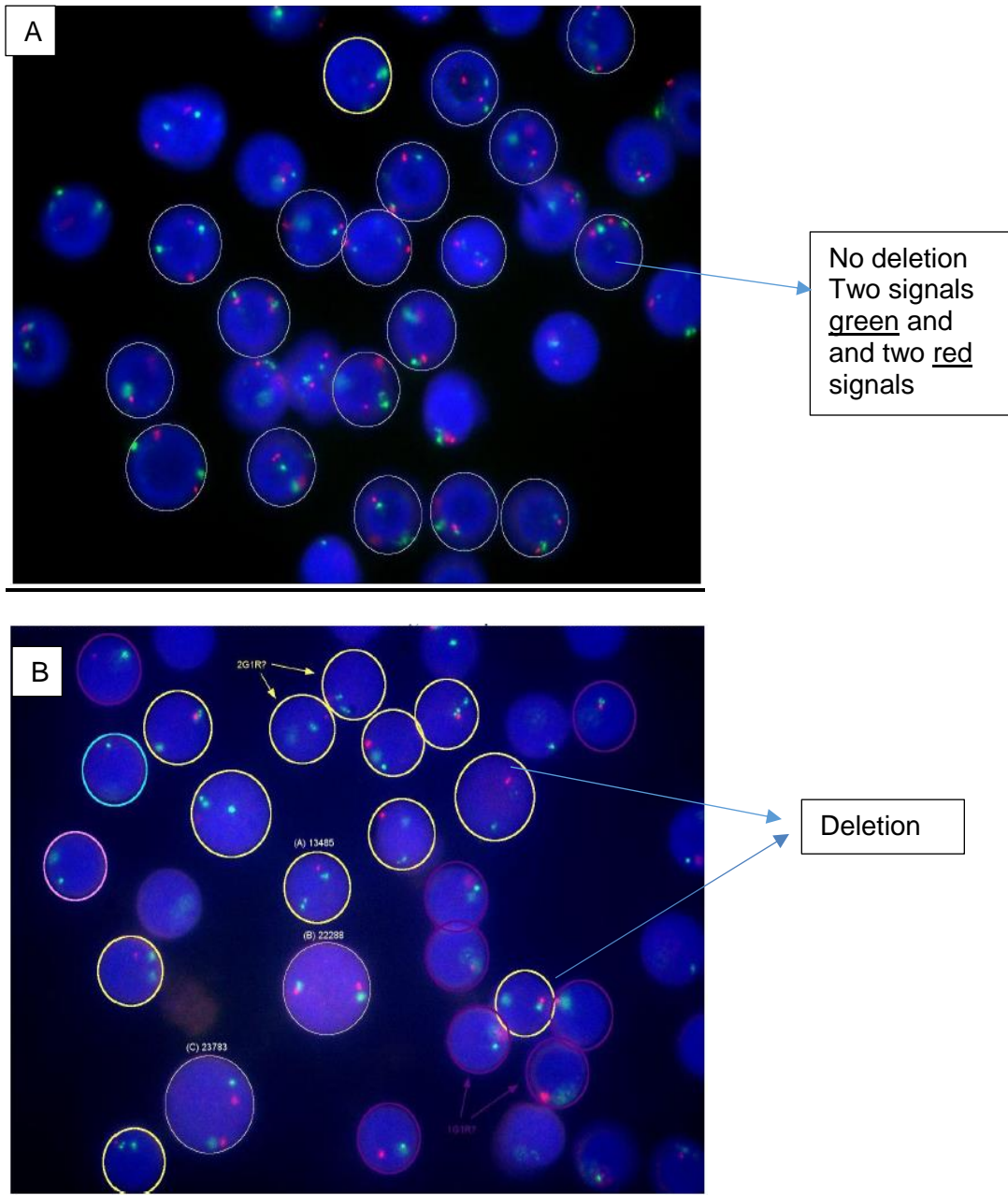


Figure 2.5. Fluorescence *in situ* hybridisation (FISH) representative images for the patient sample

The slides were prepared as above and hybridized with the fluorescent probes *DLEU1* (KBI-10102) designated for the chromosomal lesion del 13q14.3 and labelled with Platinum Bright™550 (red). The control probes for centromeric regions were labelled with Platinum Bright™495 (green). Nuclei were counterstained with DAPI (blue).

(Image **A**) demonstrates that both 11q.22 alleles are intact; whilst (Image **B**) demonstrates presence of the double or single deletion of allele 13q14.3 in the same sample.

The cells selected for the counting are circled

2.7. Immunohistochemistry of lymph nodes from CLL patients

Formalin-fixed, paraffin wax embedded (FFPE) CLL lymph node (LN) and normal tonsil sections stored at St Bartholemew's Cancer Institute, Queen Mary University of London, under the care of Dr Sergey Krysov, cut with microtome approximately 2µm thick, were fully drained and placed in a 60°C oven overnight prior to the experiment. Each tissue section was used in triplicate. Appropriately labelled slides were loaded into the track and dewaxed in xylene twice for five minutes in the fume cupboard to remove the paraffin wax. The slides were then transferred into a denatured alcohol (IMS) solution for at least 2 min followed by IMS solution supplemented with 2% v/v hydrogen peroxide H₂O₂ in order to block any residual endogenous peroxidase activity for 5 minutes twice. The slides were transferred into IMS solution for another 2 minutes and then rinsed in running tap water. The sections were outlined by a hydrophobic pen ImmEdge (Vectors Lab H-4000) and placed into the Tris buffered saline with Tween (Dako wash buffer, Dako, UK) to prevent the sections dehydration.

Primary anti-CD180 polyclonal rabbit antibody (Merck, UK) was used at a dilution of 1:500 in antibody diluent ZUC025-100 (Zytomed, UK) by adding to the sections for 40 minutes, after testing three dilutions – 1:150, 1:500 and 1:1000. The sections were washed in the wash buffer as above and goat anti-rabbit antibodies conjugated to polymerised (poly-) horse reddish peroxidase (HRP, Abcam, UK) were added for 20 minutes. The sections were washed in the wash buffer and ready-made SS-label (BioGenex, UK) was applied for 30 minutes and washed. DAB reagent was prepared according to the manufacturer's instructions (BioGenex, UK) and applied to the sections for further 10 minutes.

The sections were washed in running water, counterstained in hematoxylin for 5 minutes and washed again in running water. The sections were further fixed, mounted and dehydrated using acid alcohol, Scott's solution, IMS and xylene according to the standard procedure and reagents prepared in the lab. After drying in the hood, the sections were assessed microscopically, and images were taken for further assessment. Normal tonsils were the control here.

H-score (or "histo" score) was applied to the triplicate of each tissue section, according to which the membrane staining intensity (0, 1+, 2+, or 3+) was determined for each cell in a fixed field (Hirsh *et al.*, 2003; John *et al.*, 2009). The percentages of cells at

each staining intensity level was calculated, and finally, an H-score was assigned using the following formula: $[1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)]$. The final score ranged from 0 to 300.

The staining was performed in the Bartholemew's Cancer Institute Immunohistochemistry lab by the head technician Mr Andrew Clear and UoW visiting researcher and alumnus Ms Kristina Zaitseva (as a part of our collaboration) and the images (Figure 2.6) and the H-scores were made available to me for further analysis. -

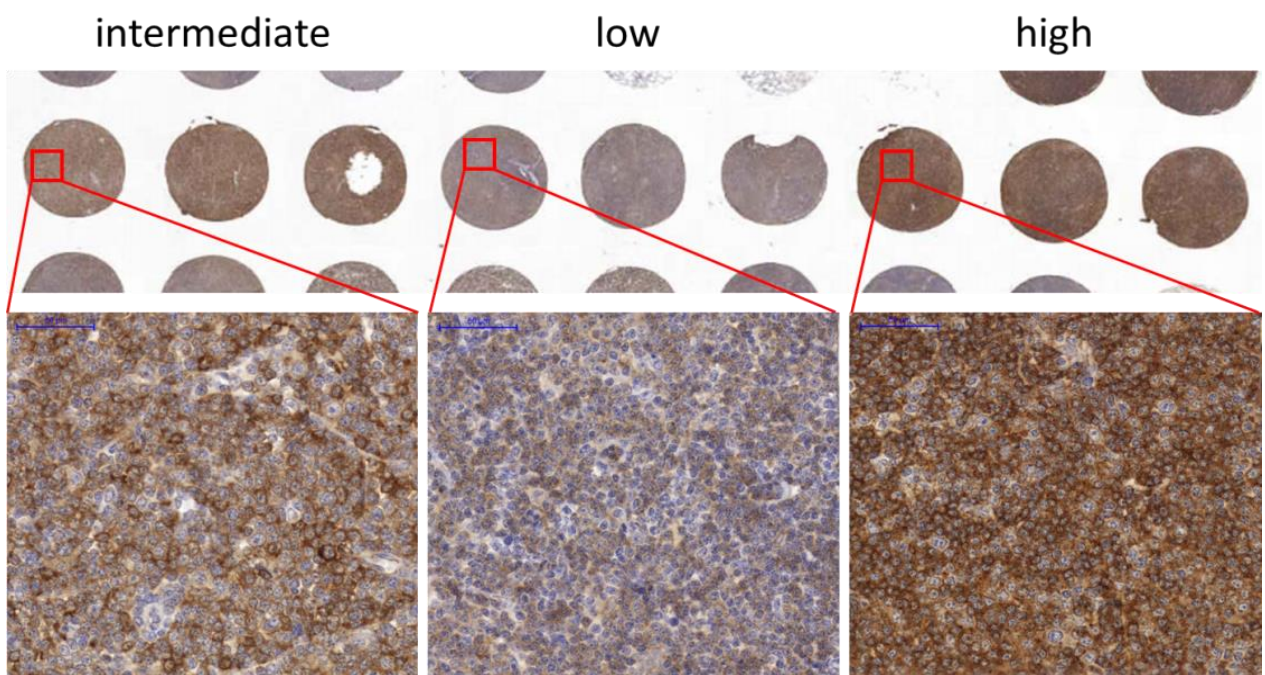


Figure 2.6. CLL LN tissue arrays, immunohistochemistry shows variable patterns of CD180 expression in three samples (triplicates) positive expression of CD180 is shown in color brown.

2.8 Statistical analysis

The results obtained during the course of the project were analysed statistically using appropriate methods. Standard parametrical (Student *t*-test, Pearson's correlation coefficient using SPSS software) or non-parametrical (Mann-Whitney *U*-test, Wilcoxon's signed-rank test using SPSS, Spearman R correlation coefficient) tests were applied. P values <0.05 were considered statistically significant.

Chapter 3

The patterns of CD180 co-expression with other prognostic biomarkers

3.1 Introduction

The clinical course of CLL is highly variable: some patients remain asymptomatic for many years, whereas other patients present with aggressive clinical course and require appropriate treatment soon after diagnosis (Chiorazzi and Ferrarini, 2010). It is essential to be able to predict the clinical course (indolent or the aggressive) of the disease for each patient as well as possible sensitivity to the available treatments in order to apply personalised treatment approach. Some prognostic indicators and biomarkers have been already integrated into medical practice such as mutational status of the immunoglobulin (Ig) heavy (H) chain genes (*IGVH*), lymphocyte doubling time (LDT), expression of ZAP-70 and CD38, chromosomal aberrations and genetic mutations. They are used to help in predicting favourable or unfavourable prognosis of the course of the disease (Ibrahim *et al.*, 2001).

IGVH mutational status has recently been found to correlate with chromosomal aberrations: *del17p* or *del11q* are more likely to be associated with unmutated *IGVH* CLL cells whereas CLL cases with mutated *IGVH* CLL cells are more likely to acquire *del13q* associated with favourable prognosis (Mina *et al.*, 2018). However, determining the *IGVH* mutational status requires high-priced and labour-intensive molecular techniques and these have a limited use in clinical practice (Rodrigues *et al.*, 2016). ZAP-70 protein kinase overexpression in CLL is generally associated with poor prognosis (Moreno and Montserrat, 2008). According to Mina *et al* (2018) in over 90% of the CLL cases, overexpression of ZAP-70 is associated with the expression of U-CLL, and hence unfavourable course of the disease. It has been established that the overexpression of CD38 on CLL cells is distinctly linked with the advanced disease stage, unfavourable prognosis and short-term overall survival. A study by Damle *et al* (1999) showed that the U-CLL cells have elevated expression of CD38 and are more likely require chemotherapy or chemoimmunotherapy.

Chromosomal aberrations with prognostic importance in CLL include: deletion of the long arm of the chromosome number 13 (*del [13q]*) involving *DLEU1* locus, deletion of chromosome 6 (*del[6q]*), chromosome 11 (*del[11q]*) encompassing the ataxia telangiectasia mutated (*ATM*) locus, short arm deletion of the chromosome 17

(del[17p]) which involves the *TP53* locus, chromosome 3 (long arm trisomy- trisomy 3q), trisomy 8q (chromosome 8), trisomy 12q (chromosome 12) and 14q32 translocation. Mina *et al.*, (2018) revealed that different mutations at chromosomal level lead to a different clinical course: CLL cases with del17p associated with the removal of the *TP53* locus demonstrated an unfavourable prognosis along with a short survival period of 32 months, whereas CLL cases with normal karyotype or del13q displayed favourable prognosis with longer survival periods of 10 years or more. The study also stated that the incidence of the del13q was the highest (55%) amongst all the different chromosomal abnormalities such as del11q (18%), a normal karyotype (18%) and trisomy 12 by 16 %.

However, it has been commonly accepted that even within existing prognostic groups, individual heterogeneity of the course of the disease and responses to the available therapies is vast, and the stratification of individual patients in regard to the prognosis and optimal treatment regimen remains a big challenge. Therefore, several prognostic indices and models have been developed to consolidate such prognostic indicators, aiming to enable more definitive prediction of the clinical outcome.

One of the new receptors which is proposed to play a role in the prognostication and stratification of individual CLL cases is CD180 Toll-like receptor. The heterogeneous expression of CD180 receptor on CLL cells with a significant correlation with M-CLL, was reported by Porakishvili *et al.* (2005). Another study by Porakishvili *et al.*, (2011) demonstrated an upregulation in the phosphorylation of the ZAP70 upon CD180 ligation. Here, I investigated if there were any correlations between the expression pattern of CD180 and established CLL biomarkers of prognostic importance.

3.2 Results

The initial analysis of CD180 with a 20% cutoff level categorised blood samples based on CD180 expression into two groups: CD180+ and CD180- . Samples with 20% or more of CD180 expression on CLL cells were considered to be positive (group A, 33 patients) while those with less than 20% of CD180 expression were considered negative for CD180 (group B, 7 patients). Further characterisation of these two groups of samples was performed and the results were highly heterogeneous.

In the 33 CD180+ samples (group A) the median level of CD180 was 58.3±23.9%. The expression of CD40 was characteristically high for these- cells - 93.6±30.0% (Table 3.1). There was no statically significant correlation between the expression of CD180 and other biomarkers. The highest correlation coefficient was seen between the expression of CD180 and CD38 (r =0.3), CD79b (r = 0.4), CD86 (r = 0.3) and IgD (r = 0.4) which reflects the heterogeneity of the disease. The correlation coefficients are shown in Figure 3.1 for each biomarker.

Table 3.1. Percentages of the expression of assessed biomarkers on CD180+ and CD180- CLL cells.

CLL CELLS	N	Biomarkers	MEAN	Std. Deviation
Total patients = 40				
Group A				
CD180+ CLL CELLS	33	CD180	58.28	23.88
		IgM	48.51	27.50
		CD38	30.56	31.03
		CD40	93.63	10.03
		CD79b	36.08	23.58
		CD86	27.70	19.96
		IgD	36.79	29.35
Group B				
CD180- CLL CELLS	7	CD180	6.88	3.94
		IgM	32.69	22.64
		CD38	10.18	9.20
		CD40	74.57	33.02
		CD79b	20.71	17.72
		CD86	22.02	26.30
		IgD	33.60	37.38

Interestingly, seven CD180- samples (group B) demonstrated decreased expression of all the assessed biomarkers (Table 3.1), although these differences were not statistically significant (Figure 3.1). This trend included CD40 and IgD and IgM. Despite

the small number of samples in group B, higher correlations were seen between the expression of CD180 and CD38 and CD86

The expression of all assessed biomarkers between groups A and B were then compared as shown in Figure 3.1a, 3.1b and 3.1c

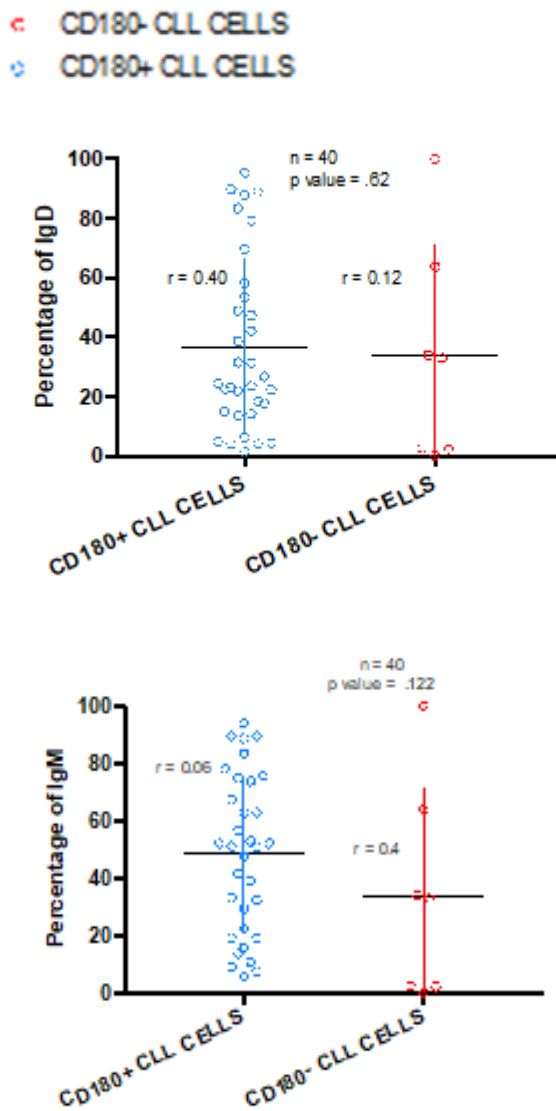


Figure 3.1a Expression of IgD and IgM on CD180+ and CD180- CLL cells.

PBMCs from CLL samples were immunophenotyped as described in Materials and Methods, Chapter 2. CLL samples were subdivided according to the levels of CD180 expression into CD180+ and CD180- groups using 20% cut-off. Centre line shows the mean percentages. 'n' refers to the total number of patient's cells. P (Mann-Whitney) and r (correlation coefficient) values refer to the differences between the expression of IgD and IgM in CD180+ and CD180- CLL cells.

- CD180- CLL CELLS
- CD180+ CLL CELLS

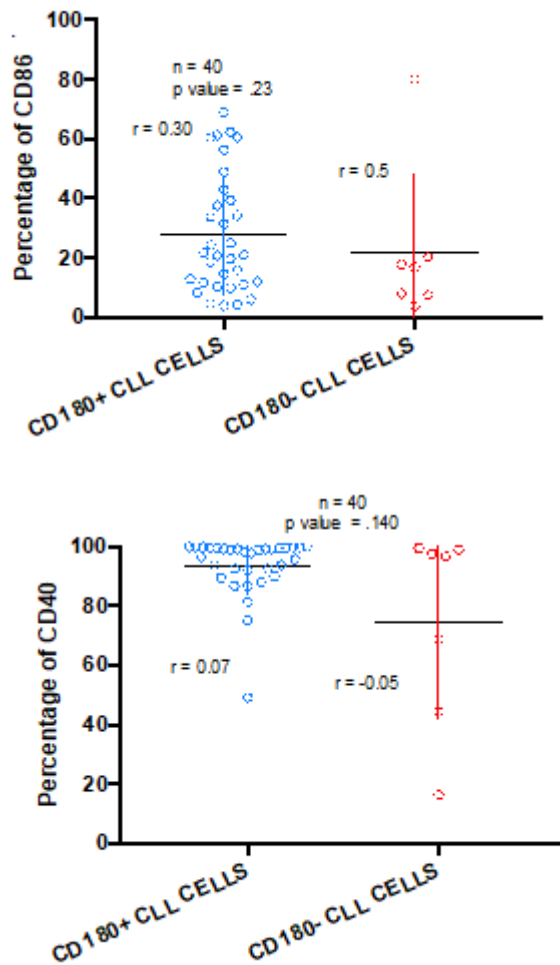


Figure 3.1b Expression of CD86 and CD40 on CD180+ and CD180- CLL cells:

PBMCs from CLL samples were immunophenotyped as described in Materials and Methods Chapter 2. CLL samples were subdivided according to the levels of CD180 expression into CD180+ and CD180- groups using 20% cut-off. Centre line shows mean percentage. 'n' refers to the total number of patients. P (Mann-Whitney) and r (correlation coefficient) values refer to the differences between the expression of CD86 and CD40 in CD180+ and CD180- CLL cells.

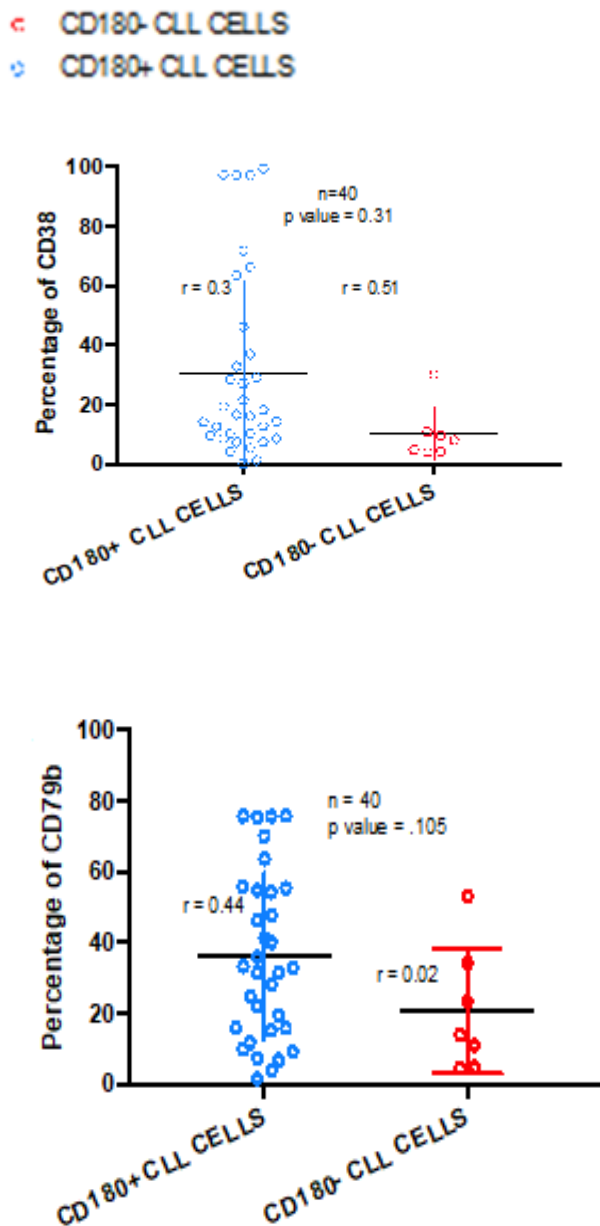


Figure 3.1c Expression of CD38 and CD79b on CD180+ and CD180- CLL cells.

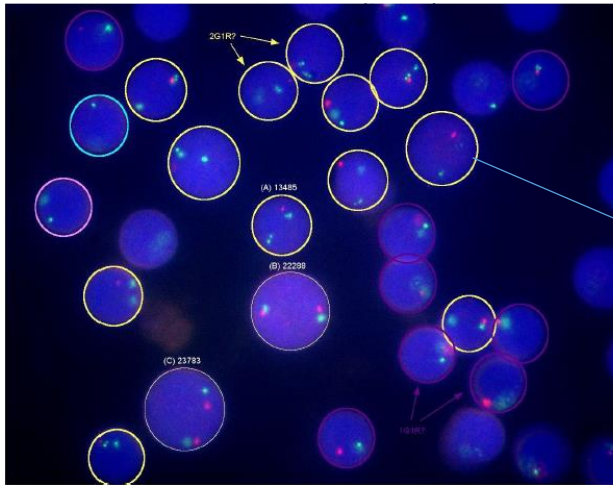
PBMCs from CLL samples were immunophenotyped as described in Materials and Methods Chapter 2. CLL samples were subdivided according to the levels of CD180 expression into CD180+ and CD180- groups using 20% cut-off. Centre line shows the mean percentage. 'n' refers to the total number of patients. P (Mann-Whitney) and r (correlation coefficient) values refer to the differences between the expression of CD38 and CD79b in CD180+ and CD180- CLL cells.

From Figure 3.1a, b, and c, it can be noted that CLL cells from CD180- group (RED) express less CD38- and CD86- compared to the CD180+ group (BLUE). We tested three CD180-CD38- CLL samples (as only three were available due to shortage of samples), for chromosomal aberrations, and all three of them were found to have 13q deletion (*DLEU1*), but no 11q deletion (*ATM*) or 17p deletion (*TP53*), as demonstrated in Figure 3.2 (patients B.02, P.03 and J.04). Cut-off points of each probe was determined to be: *ATM*=17.4%; *DLEU1*=12.3%; *TP53*=18.3%. The percentages of nuclei harboring the *DLEU1* deletion were calculated for each patient as follows: *B.02=87.28%; *J.03=90.48%; *P.04=34.62%.

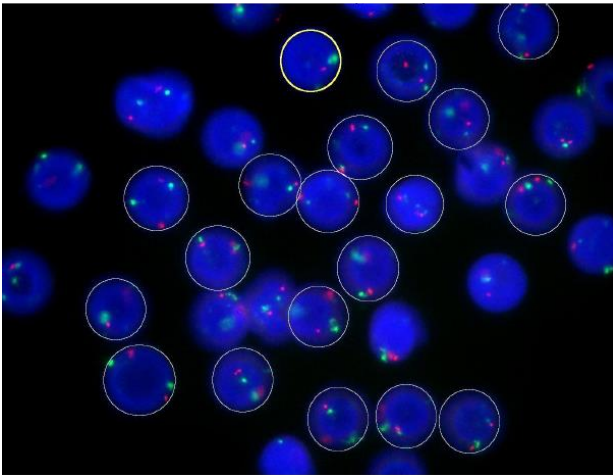
In comparison, the other two patients, for whom the FISH data was analysed were CD180+: one of those (N.01) was CD38+ CD180+, the other (M.05) - CD38-CD180+ (data not shown in this chapter). Due to small sample size statistical analysis was not applied and therefore low number of samples does not allow definite conclusion to be drawn.

	Percentage of Significant BioMarkers				
	ZAP-70	CD38	CD180	IgM	IgD
Sample N.01	0.3	60.61	77.82	57.30	73.67
Sample B.02	2.66	8.40	0.01	62.11	33.80
Sample J.03	72.0	0.8	0.05	23.12	58.15
Sample P.04	36.0	5.58	0.03	31.60	27.61
Sample M.05	7.86	21.65	27.86	33.47	8.22

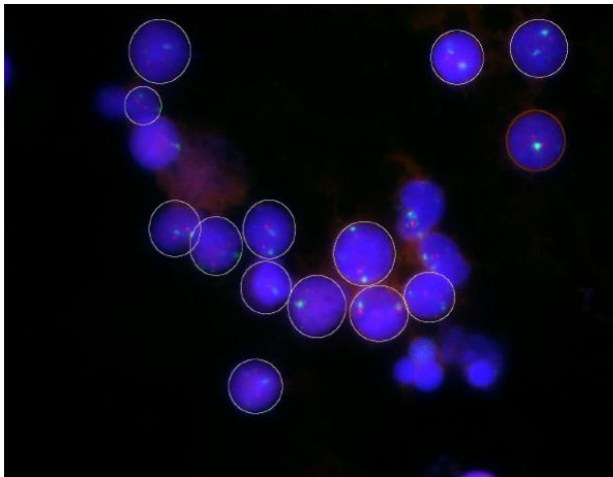
Table 3.2. Comparison of the expression of some of the surface markers and prognostic biomarkers in CLL samples used for FISH analysis. The percentages were obtained by subtracting the % of negative control IgG1 from the total percentage of individual CD markers. Here the IgG1 was set at 5%. CLL cells were purified and loaded with primary antibodies, stained with secondary antibody FITC conjugated and analysed on flow cytometer for surface markers CD38, IgM, IgD and CD180. ZAP70 intracellular phenotyping was carried out using intracellular staining protocol as described in Materials and methods



Sample B.02
Probe *DLEU1*
Chromosome critical region *13q14*
Status: -Deleted

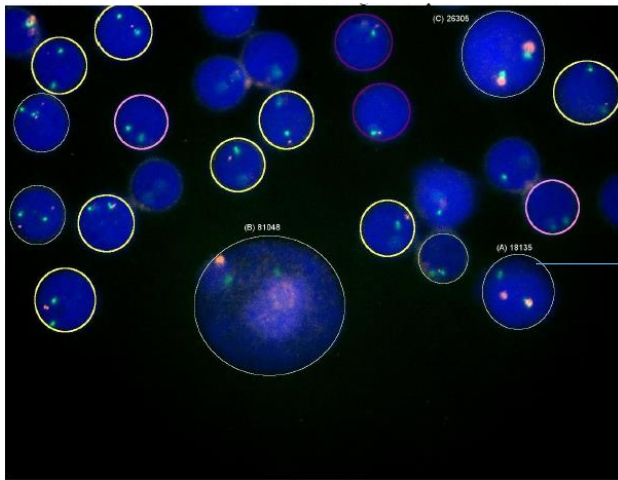


Sample B.02
Probe *ATM*
Chromosome critical region *11q22*
Status: -NO deletion

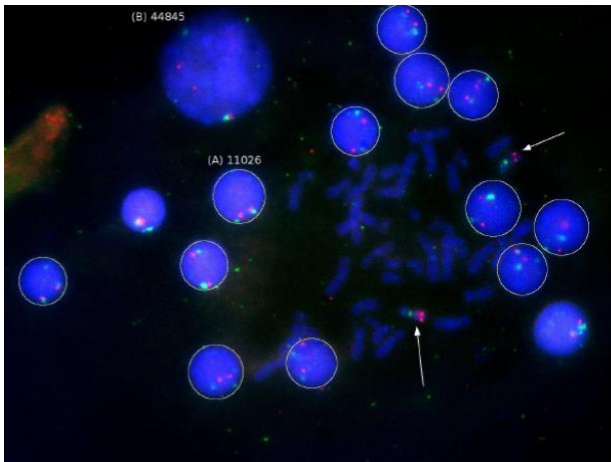


Sample B.02
Probe *TP53*
Chromosome critical region *17p13*
Status: - NO deletion

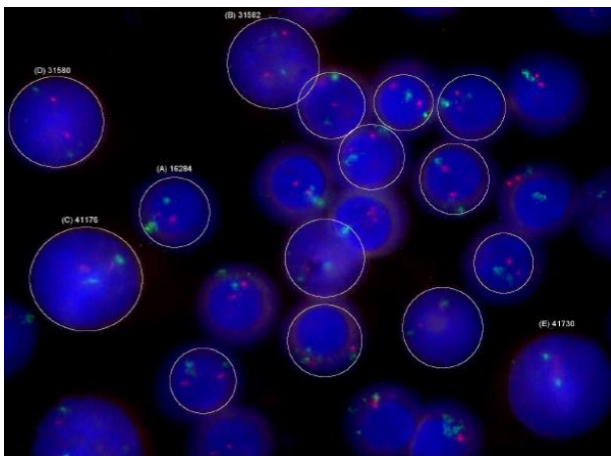
Figure 3.2 a. Annotated fluorescence *in situ* hybridization profiles for CD180/CD38 double-negative CLL sample B.02. PBMCs were stimulated with PHA and processed as described in Materials and methods. Counted cells are shown in outlined circles. Green fluorescent probes delineate control centromere regions; Red fluorescent probes - critical regions. Experiments were repeated when Images obtained were of poor quality. A minimum number of 50 nuclei was scored for each patient per probe (n=50). With an established p-value of $\alpha < 0.05$, the percentages for false-positives was calculated.



Sample P.04
Probe *DLEU1*
Chromosome critical region *13q14*
Status: - Deleted

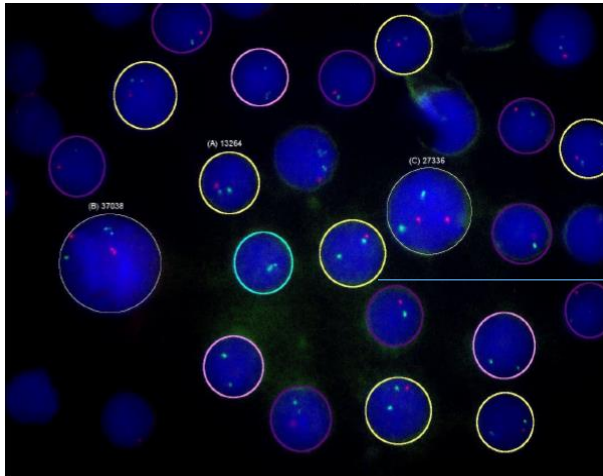


Sample P.04
Probe *ATM*
Chromosome critical region *11q22*
Status: -NO deletion



Sample P.04
Probe *TP53*
Chromosome critical region *17p13*
Status: - NO deletion

Figure 3.2 b. Annotated fluorescence *in situ* hybridization profiles for CD180/CD38 double-negative CLL sample P.04. PBMCs were stimulated with PHA and processed as described in Materials and methods. Counted cells are shown in outlined circles. Green fluorescent probes delineate control centromere regions; Red fluorescent probes - critical regions. Experiments were repeated when Images obtained were of poor quality. A minimum number of 50 nuclei was scored for each patient per probe (n=50). With an established p-value of $\alpha < 0.05$, the percentages for false-positives was calculated

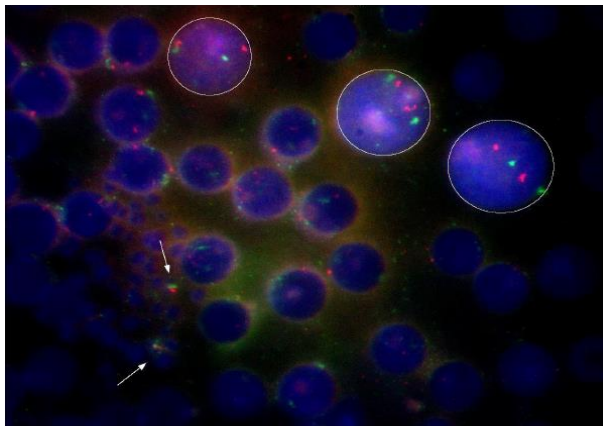


Sample J.03

Probe *DLEU1*

Chromosome critical region *13q14*

Status: -Deleted

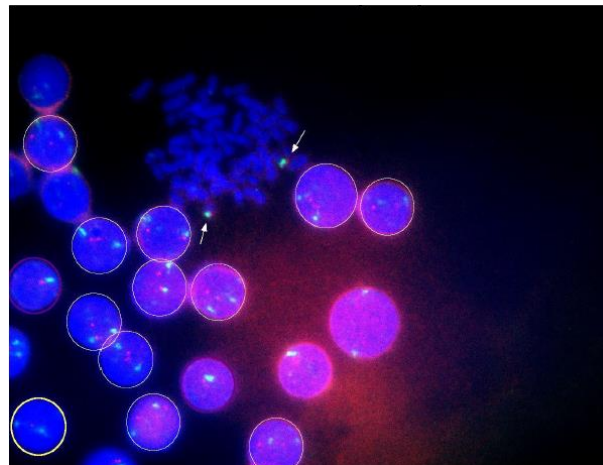


Sample J.03

Probe *ATM*

Chromosome critical region *11q22*

Status: -No deletion



Sample J.03

Probe *TP53*

Chromosome critical region *17p13*

Status: - No deletion

Figure 3.2 c. Annotated fluorescence *in situ* hybridization profiles for CD180/CD38 double-negative CLL sample J.03. PBMCs were stimulated with PHA and processed as described in Materials and methods. Counted cells are shown in outlined circles. Green fluorescent probes delineate control centromere regions; Red fluorescent probes - critical regions. Experiments were repeated when Images obtained were of poor quality. A minimum number of 50 nuclei was scored for each patient per probe (n=50). With an established p-value of $\alpha < 0.05$, the percentages for false-positives was calculated

3.3 Discussion

In this study, blood samples from CLL patients were distributed in two phenotypic groups based on the expression of the CD180 receptor with the cut off for positivity 20% (based on the data available previously in our group). The aim was to assess the correlation of CD180 expression with other prognostically and biologically significant biomarkers, receptors and chromosomal aberrations, associated with favourable or unfavourable course of disease. No statistically significant correlation was established between the expression of CD180 and other surface markers in both CD180+ and CD180- subgroups. The expression of the receptors IgD, IgM and CD79b was heterogenous and varied from patient to patient while expression of CD40 was consistently high (Table 3.1). However, the CD180- group of patients was characterized by lower expression of almost all assessed biomarkers, the phenomenon first established in 2005 by Porakishvili *et al.* However, the CD180- subgroup had high CD38- cells, 85% of all cases (Figure 3.1c) indicating a possible favorable prognostic correlation.

CD38 is an important prognostic biomarker in CLL that helps in determining whether the patient will have favourable or unfavourable disease course. A study by Damle *et al.* (1999) showed that with the cut-off of 30% CD38 can be used to distinguish between the CLL cases based on the prognostic differences such as requirement for chemotherapy. Initially, it was used as a surrogate marker for *IGVH* mutational status as the studies indicated that higher expression of CD38 was associated with unmutated *IGVH* gene status (Zupo *et al.*, 2002; Dal-bo *et al.*, 2009). However, subsequent studies have demonstrated independent prognostic significance of CD38. Although, CD38 expression is extremely heterogeneous amongst CLL cases, it is still valid as an independent prognostic indicator that helps in determining the clinical course, response to treatment and overall survival of CLL patients (Burger and Chiorazzi, 2013). Ibrahim *et al.* (2001) demonstrated that the crucial role of CD38 in lymphocyte proliferation and its anti-apoptotic activities results from the up-regulation of Bcl-2. It has been shown that CD38 ligation led to the suppression of immature B cells in the bone marrow microenvironment (Shanafelt *et al.*, 2003). He *et al.* (2017) described that CLL patients with elevated expression of CD38 were characterized by the 1.92-fold increased risk of a relapse compared to those with lower CD38 expression. A study conducted by

Malavasi *et al.* (2011) suggested that there is an association between the high expression of CD38 with CLL cell proliferation and expansion leading to unfavourable prognosis. According to Porakishvili *et al.* (2005), CD180- samples showed lower expression of CD38 whereas increased CD38 expression was found in CD180+ CLL samples.

The trend of the expression of an important prognostic indicator CD38 on CD180+ CLL cells and its absence on the CD180- CLL cells might suggest that the negative profile for both, CD180 and CD38 receptors, might be associated with favourable prognosis of the disease course whereas co-expression of CD180+ and CD38+ can indicate unfavourable prognosis. The absence of CD38 on CD180 negative CLL cells may lead to increased susceptibility to apoptosis due to the low Bcl-2 expression, and decreased expansion potential, as indicated above. To a certain extent, this suggestion was supported by my data shown in Figure 3.1c that higher proportion (45%) of the CD180+ CLL samples expressed CD86, than in the CD180- subgroup indicating that CD180+CD38+ cells are more likely to be activated, although more samples are required to confirm this hypothesis. CD86 (B7-2) is an established B cell activation marker, a costimulatory molecule generally expressed by antigen presenting cells such as dendritic cells, B cells and macrophages. CD86 is constitutively upregulated on B-cells and is involved in their activation. As a costimulatory molecule, CD86 can promote activation, growth and differentiation of B cells to lower cross-linkage by monoclonal antibody (Suvas *et al.*, 2001). The certain concordance in the expression of CD180 and CD38 on CLL samples might serve as a potential prognostic tool to delineate CLL cases with favourable and unfavourable disease course and hence different approaches for optimal therapies, Bcl-2 antagonists such as Venitoclax in particular (Sargent *et al.*, 2009).

This hypothesis has been further supported by the cytogenetic studies. It has been established that 80% of the CLL samples display at least one cytogenetic abnormality (Döhner *et al.*, 2000; Byrd *et al.*, 2003). In this study CLL samples were hybridised with commercial probes against the following chromosomal aberrations: *13q* deletion (*DLEU1*), *11q* deletion (*ATM*) and *17p* deletion (*TP53*). Metaphase cytogenetic abnormalities were found in three patients with the highest frequency of del(*13q*) [n=3] and rest of the patients lacked any of the genetic aberrations. Del (*13q*) had either mono-allelic or bi-allelic loss in the examined samples. None of the samples displayed the del*17p* (*TP53*) or *11q* deletion (*ATM*). Evidence of other cytogenetic lesions was not found in the CLL samples examined, this could be either because of experimental error or the patient did not have any of the cytogenetic abnormalities. This could have been confirmed by repeating the experiments with substantial chromosomal culture to reach definitive decision but at the time of submitting this thesis it was not possible due to shortage of samples, therefore this analysis was made on a small number of samples.

In this small group of patients all samples with the *DLEU1* deletion were negative for CD180 and CD38. One patient (B.02) was also negative for ZAP70 expression (2.66%). In contrast, the other two -negative for CD180 and CD38 samples were ZAP70+. Deletion del(*13q*) *DLEU1* is the most common chromosomal lesion reported in CLL and has been associated with a favourable prognosis, good clinical outcome, better response to treatment and overall survival (Puiggros *et al.*, 2014; Rahimi *et al.*, 2017). According to Dal-bo *et al* (2009) deletion del (*13q*) is associated with the mutated *IGVH* genes. On the other hand, ZAP-70 expression is known to delineate U-CLL (Table 3.2) (Zap70 cutoff point =15%). Therefore, on this basis it was assumed that patient B.01 was likely to have favourable prognosis as negativity for CD38, ZAP70 and CD180 together with del (*13q*) should be associated with the better clinical outcome along with the possibility of favourable prognosis (Nabhan *et al.*, 2015; Gomes *et al.*, 2017) and better overall survival.

However, the other two cases were more complex. These were patients with concordant negativity for CD180/CD38 and deletion *DLEU1* but were ZAP70 positive. Likewise, one of the patients with normal genotype (N.01) with no chromosomal aberrations were CD180+ and CD38+ but negative for ZAP70 (0.3%). The

prognostication in such a case would remain indecisive since a seemingly “normal” karyotype often harbour genomic mutations associated with unfavourable prognosis and resistance to the therapy (Hallek, 2008; Rodrigues *et al.*, 2016). Conversely, several studies stated that U-CLL samples with complex karyotype (deletion *17p* or *11qdel*) cells are most likely to have unfavourable course of disease and be less responsive to treatments whereas M-CLL cases with “normal karyotype” will have an indolent course of the disease (Rigolin *et al.*, 2017). On one hand, the N.01 sample was positive for CD180 and CD38, presented with no *DLEU1* mutation, which would all suggest favourable prognosis, on the other hand, it was negative for ZAP70 which indicates an M-CLL status and indolent course of disease. Unfortunately, due to small number of samples available, we were not able to draw conclusion, but the concordant pattern of CD180/CD38 expression and *DLEU1* mutation appears as a promising tool for identifying indolent CLL course for personalized therapies.

It would be very interesting to further assess this hypothesis, based on the information presented above: whether a concordance in terms of positive expression of CD180/CD38 and absence of the benign deletion (*del*), all indicating an aggressive CLL course of disease, could outbalance the absence of ZAP70 which is a marker of favourable prognosis. This would evaluate the importance of the triad of biomarkers: co-expression of CD180 and CD38 and absence of the *DLEU1* lesion as a strong indicator stratifying the aggressive course of the disease, independently from ZAP70 expression often linked with *IGHV* mutational status (Dickinson *et al.*, 2005). Likewise, concordant negativity for CD180/CD38 linked to the *DLEU1* deletion, may outbalance the expression of ZAP70, indicating a favourable prognosis. In order to fine-tune the prognostic value of these patterns, the *IGHV* mutational status and other chromosomal aberrations/gene mutations would need to be determined. An approach taken to minimise such errors would be to conduct whole-genome sequencing for each patient (Fabbri *et al.*, 2011; Puente *et al.*, 2011).

3.4 Conclusions

1. Concordant presence or absence of the triad of biomarkers: CD180, CD38 and *DLEU1* deletion may have a stratification significance for the CLL prognosis.
2. Concordant negativity for CD180 and CD38 expression may associate with *13.1q DLEU1* chromosomal aberration and an indolent course of the disease.
3. Concordant positive expression of CD180 and CD38 in the absence of *DLEU1* might indicate unfavourable prognosis for CLL irrespective of ZAP-70 expression.

Chapter 4

Interaction between CD180 and IgD mediated signaling pathways in CLL.

4.1 Introduction

Recent advances in our understanding of the pathobiology of CLL have contributed to the development of new molecular therapies, which, together with immunotherapy, have been gradually replacing conventional chemotherapy in the treatment of this disease (Montserrat *et al.*, 2016; Hallek, 2017). Sequencing of the B-cell receptor (BCR) allows categorization of CLL patients into two clinical subsets according to their immunoglobulin (Ig) VH genes' mutational status, which is now used as a prognostic indicator of the responses to therapies and overall survival. The presence of unmutated *IGVH* genes is usually associated with the expression of zeta-associated protein 70 (ZAP-70), which is not expressed by normal B-cells and is associated with a more aggressive form of CLL (Hamblin *et al.*, 1999; Chen *et al.*, 2005; Zhang and Kipps, 2014). Moreover, the expression of CD38 and CD49d is also associated with an unfavorable prognosis. Both markers have been shown to be involved in CLL cell migration and proliferation (Buggins *et al.*, 2011; Hendy *et al.*, 2016) as discussed in previous chapter.

Furthermore, it has also been established that the tumour microenvironment plays an important role in the pathogenesis of CLL, by providing chemokines and adhesion molecules that contribute to the survival and proliferation of the leukaemic cells (Herishanu *et al.*, 2011; Zhang and Kipps, 2014). In addition, it also promotes the activation of the BCR-mediated signaling through the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in CD79a and CD79b complex-associated proteins, leading to the downstream activation of pro-survival signaling pathways, such as protein kinase B (AKT), extracellular signal-regulated kinase (ERK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathways (Herishanu *et al.*, 2011; Zhang and Kipps, 2014; Porakishvili *et al.*, 2015). Understanding of the regulation of these signaling pathways is paramount for the development of BCR-signaling antagonists, which block the activation of protein kinases leading to the CLL cell survival and expansion (Jones and Byrd, 2014). One of the most prominent examples are Bruton's tyrosine kinase (BTK) inhibitor Ibrutinub and

phosphoinositide 3-kinase (PI3K) inhibitor Idelalisib, currently fully integrated into clinical practice after showing promising results in clinical trials. However, these drugs are associated with several serious side effects (Jones and Byrd, 2014; Rozovski *et al.*, 2014; Hallek, 2017) and hence the search continues for stratification biomarkers that would allow more accurate, if not precise personalized approach to the therapy.

Several studies have demonstrated that toll-like receptors (TLR), with an important role in the regulation of the innate immune system, are also involved in the pathogenesis of CLL (Muzio *et al.*, 2012). Different levels of expression of TLRs were found in CLL cells, and several studies indicated the potential role of TLRs in the modulation of CLL cell signaling response (Muzio *et al.*, 2009; Rozková *et al.*, 2010; Rybka *et al.*, 2016).

CD180 is an orphan receptor that belongs to the TLR family and triggers activation and proliferation of normal B-cells (Valentine *et al.*, 1988; Muzio *et al.*, 2012). It has been shown by our group that CD180 is expressed in approximately 60% of CLL cases and that its expression levels are higher in M-CLL cells (Porakishvili *et al.*, 2005). CD180 ligation on normal B cells and on so called responder (R) CLL cells led to even stronger activation, proliferation and survival of CLL cells compared to those mediated by CD40 or rIL-4. (Porakishvili *et al.*, 2011).

Further studies have shown that CD180 can either promote CLL cell survival and cycling via BTK/PI3K/AKT pathway or induce apoptosis via p38 mitogen-activated protein kinase (p38MAPK) pathway (Porakishvili *et al.*, 2011; Gordienko *et al.*, 2017). These results are consistent with previous studies which demonstrated the ability of CD180 to signal in human and murine B lymphocytes (Valentine *et al.*, 1988, Yazawa *et al.*, 2003, Divanovic *et al.*, 2007) providing the signals for B cell survival and proliferation (Clark *et al.*, 1989; Roshak *et al.*, 1999).

Most importantly, it has been demonstrated that CD180 is able to redirect IgM-mediated signaling from the pro-survival BTK/PI3K/AKT to the pro-apoptotic p38MAPK pathway in CLL cells (Porakishvili *et al.*, 2015) suggesting cross-talk between the signalling pathways operating via IgM and CD180. These findings suggest that CD180 can potentially be used not only as prognostic indicator, but also as a molecular target for the treatment of CLL patients. However, the ligand of this TLR is still unknown, and

further research on CD180 signaling pathways is also required (Porakishvili *et al.*, 2011, 2015).

Importantly no information thus far been provided on the possible interplay between IgD component of BCR and CD180. As reviewed by Hacken *et al.* (2016), most CLL cells express both IgM and IgD components of BCR but signaling through these two isotypes leads to different functional responses in CLL cells, which are not yet fully understood. It has been suggested that signaling through the IgM isoform induces more durable signaling responses in CLL cells, usually via ERK signaling pathway, and that it also stimulates the secretion of C-C motif chemokine ligand 3 (CCL3) and C-C motif chemokine ligand 4 (CCL4), contributing to CLL cell survival and proliferation (Hacken *et al.*, 2016). On the other hand, signaling through the IgD isoform seems to induce the activation of hematopoietic lineage cell-specific protein 1 (HS1) and the polymerisation of F-actin, which results in receptor internalisation and transient downstream signaling responses that fail to promote CLL cell survival (Hacken *et al.*, 2016). In addition, previous studies have suggested that IgM is the main isoform targeted during ibrutinib treatment, however it has been shown that this BTK inhibitor also interferes with IgD-mediated signaling (Hacken *et al.*, 2015; Mazzarello *et al.*, 2017).

The heterogeneity of CD180-mediated signaling pathways and their interaction with BCR isoforms might be affected by the stability and/or modulation of CD180 expression on CLL cells.

As previously reported by Porakishvili *et al.* in 2005, CD180 is heterogeneously expressed on CLL cells, and about 40% of CLL samples are CD180 negative. In contrast, a vast majority (95-98%) of normal B cells, both CD5- and CD5+ express CD180 (Porakishvili *et al.*, 2005).

The variation in the expression of CD180 on B cells has been highlighted before. Koarada *et al.* in 2011 reported that following CD180 stimulation with mAb the expression of CD180 significantly dropped in PBMCs in Systemic Lupus Erythematosus (SLE) patients. Interestingly these stimulated CD180^{neg} cells were the ones spontaneously producing autoantibodies of both, IgG and IgM classes (Koarada and Tada, 2011). Another study reported that in murine B cells signalling through TLR7 and TLR9 also resulted in the reduction of the CD180 expression (You *et al.*, 2015).

CD180 expression levels in individual CLL samples taken from the same patients over a period of 24 months did not change (Porakishvili *et al.*, 2005). However, Tsertsvadze *et al.* (2015) demonstrated that CD180 expression was significantly decreased throughout 0-96 hours in the spontaneous MEC1 cell culture. Hence there appear to be fluctuations in the expression of CD180 in the spontaneous and stimulated long-term cell cultures, whilst the *ex vivo* expression pattern is stable.

Since presence of CD180 was affecting the signaling pattern of CLL cells as seen previously with IgM signaling (Porakishvili *et al.*, 2015), it can be hypothesized that different signalling patterns of CLL cells were partially based on CD180 receptor turnover. Due to the established cross-talk between CD180 and the BCR, this turnover could be affected by the BCR ligation.

Here the aim was to investigate the interaction between IgD and CD180 signalling pathways involving key protein kinases BTK, AKT and P38MAPK as identified previously (Porakishvili *et al.*, 2011; 2015) for IgM-mediated signalling. Additionally, I also attempted to study spontaneous fluctuation of the percentages of CD180+ cells, as well as their modulation by the engagement of sIgD.

4.2 Results

Individual CLL samples exhibited a heterogeneous response to the direct ligation of CD180 or sIgD alone or in combination, assessed by the phosphorylation of AKT and p38MAPK. Our research group has previously established that individual CLL samples can be subdivided into four major categories, by their ability to activate signalling pathways in response to the CD180 ligation. These were AKT-Signalers (AKT-S), p38MAPK-S (p38MAPK-S), non-Signalers (NR), and a very small group of AKT/p38MAPK double signalers (DS) responding as normal B cells belonged to the DS category (Porakishvili *et al.*, 2015). On ligation of sIgM, in our hands, almost all CLL samples demonstrated phosphorylation of AKT, or a lack of response, and only a small proportion of CLL cells (12%) phosphorylated p38MAPK (Porakishvili *et al.*, 2015).

In this study I have categorised CD180+IgD+ CLL samples into AKT-S and p38MAPK-S by their responsiveness to the ligation of CD180 with mAb and studied the pattern of the response of these subtypes of CLL samples to anti-IgD F(ab)₂ stimulatory antibodies. The readout comprised phosphorylation of AKT, p38MAPK and BTK protein

kinases. sIgD was expressed heterogeneously on $61.5 \pm 38.9\%$ CLL cells with an RBS/cell 3579 ± 2579 . The results are shown in Figure 4.1.

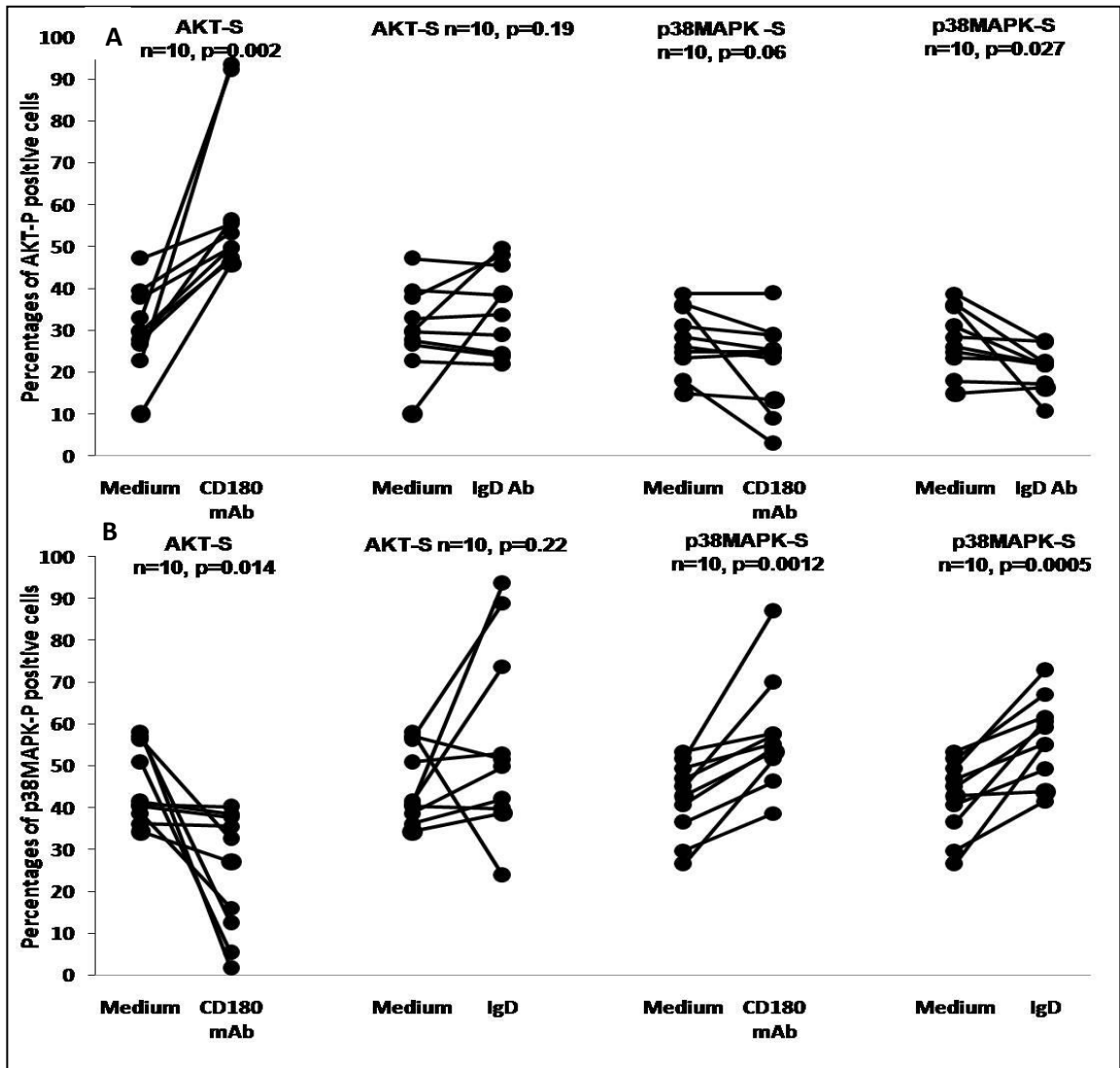


Figure 4.1. Phosphorylation of AKT and p38MAPK protein kinases in AKT-signalers (AKT-S) and p38MAPK signaller (p38MAPK-S) CLL cells following stimulation with anti-CD180mAb or anti-IgD antibodies. Ten CD180+IgD+ CLL samples were stimulated with anti-CD180 mAb or goat anti-human IgD F(ab)₂ for 20 minutes and 10 minutes respectively for sole stimulation. Upon stimulation, cells were treated with anti-CD19 mAb, fixed, permeabilised, and stained with antibodies to AKT-P (Ser473) (A) or p38MAPK-P (B) and assessed by flow cytometry as described in the Materials and methods. Unstimulated samples remain untreated. P values were calculated using the paired *t*-test.

According to my data, individual CLL samples exhibited heterogeneous response to the ligation of CD180 or sIgD alone as assessed by the phosphorylation of AKT and

p38MAPK. However, in contrast to sIgM, in my hands, sIgD-mediated signalling favoured the p38MAPK pathway. Only in 3 out of 10 AKT-S, and in none of the p38MAPK-S CLL samples we detected an increase in AKT phosphorylation following sIgD ligation (Figure 4.1 A). In contrast, all p38MAPK-S and 7 out of 10 AKT-S CLL samples demonstrated enhanced p38MAPK phosphorylation in response to anti-IgD (Figure 4.1 B), leading to an increased apoptosis of CLL cells compared to unstimulated cell cultures (medium), assessed by early apoptotic Annexin+PI- cells from $28.5 \pm 12.9\%$ up to $78.3 \pm 22.4\%$, $n=10$, $p=0.00041$ (Figure 4.2 B).

Porakishvili *et al.* (2015) demonstrated that pre-treatment with anti-CD180 mAb of CLL cells redirected IgM-mediated signalling from AKT to p38MAPK pathway. To study the possible effect of CD180 ligation to sIgD-mediated signalling, I investigated phosphorylation (P) of these protein kinases in response to the sequential ligation of CD180/IgD. Whilst pre-engagement of CD180 did not affect the levels of AKT phosphorylation following the ligation of IgD in AKT-S category of CLL cells (data not shown), it strongly synergised with sIgD-mediated phosphorylation of p38MAPK ($p=0.0032$, Figure 4.2 A, $n=10$).

BTK phosphorylation in response to CD180 or sIgD ligation across the categories of CLL cells was highly heterogeneous (data not shown). We therefore decided to pool the AKT-S and p38MAPK-S data together for both, anti-CD180 and anti-IgD stimulations, and the results are shown in Figure 4.3 ($n=11$). Whilst the response is still very heterogeneous and the differences between stimulated and unstimulated cultures are statistically insignificant, there can be seen a certain trend: in a proportion of CLL samples the ligation of anti-CD180, but not IgD, leads to a strong phosphorylation of BTK, with p values closer to statistically significant ($p=0.094$).

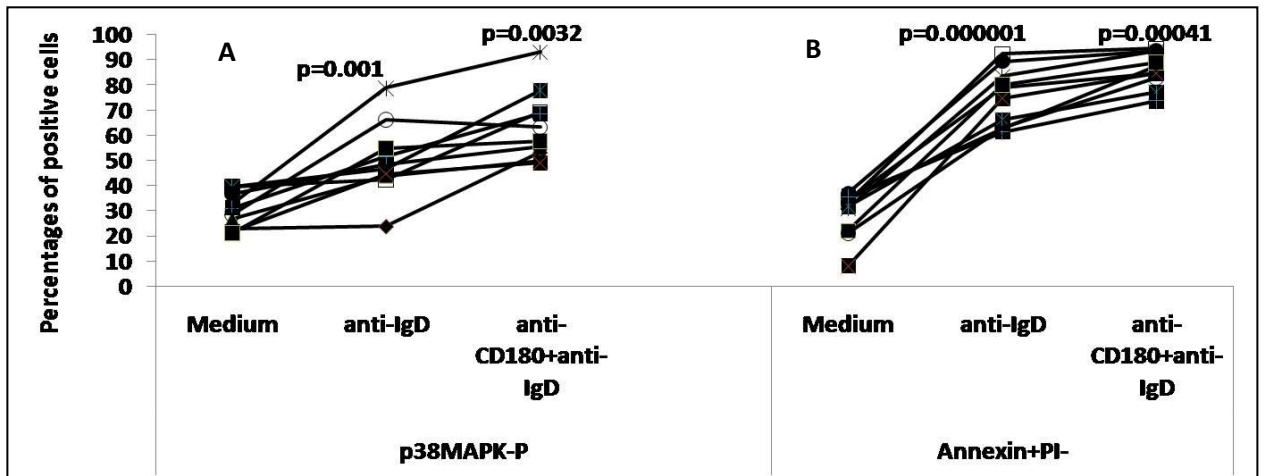


Figure 4.2. Phosphorylation of p38MAPK protein kinases in p38MAPK signaller (p38MAPK-S) CLL cells following sequential stimulation with anti-CD180mAb and anti-IgD antibodies. Ten CD180+IgD+ CLL samples were stimulated with anti-CD180 mAb for 20 minutes and by goat anti-human IgD F(ab)₂ for 10 minutes for single stimulation. For combined stimulation primarily for 20 minutes with anti-CD180mAb and then with anti-IgD for further 10 minutes. Upon stimulation, cells were treated with anti-CD19 mAb, fixed, permeabilised, and stained with antibodies to AKT-P (Ser473) (A) or p38MAPK-P (B) and assessed by flow cytometry as described in the Materials and methods. Unstimulated samples remain untreated. P values were calculated using the paired *t*-test.

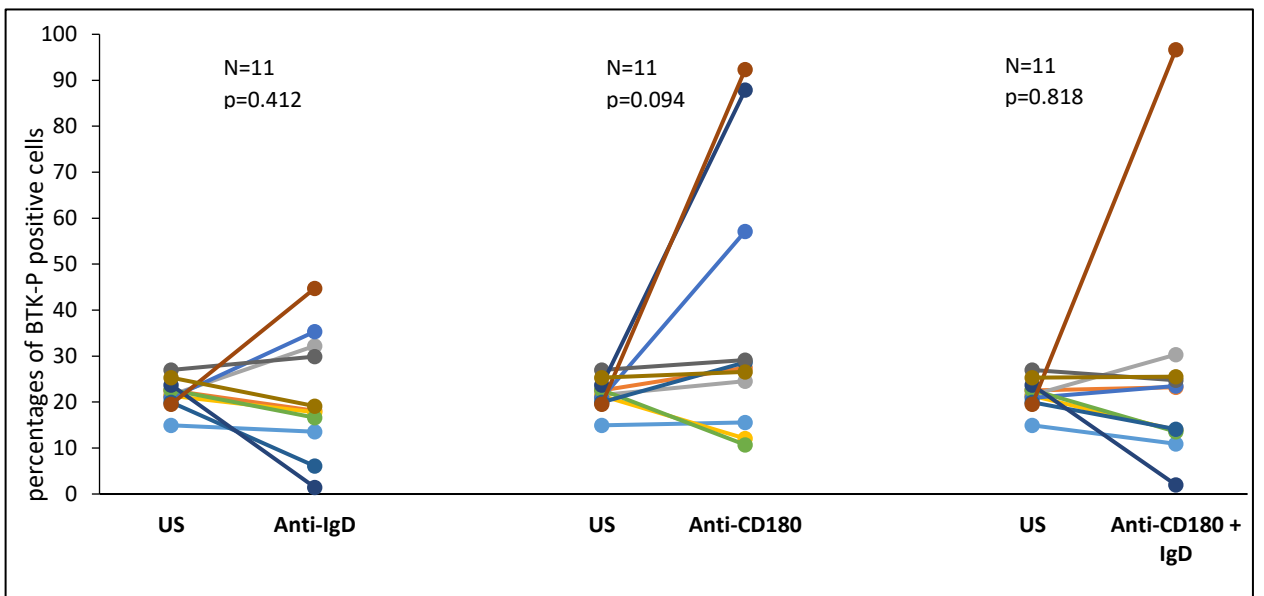


Figure 4.3. Phosphorylation of BTK jointly in AKT-S and p38MAPK-S categories of CLL cells in response to the CD180 and IgD ligation. 11 CD180+IgD+ CLL samples were stimulated with anti-CD180 mAb or goat anti-human IgD F(ab)₂ for 20 minutes and 10 minutes respectively or with a sequential ligation of CD180 and IgD. Upon stimulation, cells were treated with anti-CD19 mAb, fixed, permeabilised, and stained with antibodies to BTK-P and assessed by flow cytometry as described in the Materials and methods. Unstimulated samples remain untreated. P values were calculated using the paired *t*-test.

Importantly, sequential ligation of CD180 and IgD seems to reduce the percentages of BTK-P+ cells (Figure 4.3).

It has been previously demonstrated that CD180-mediated intracellular signalling transmitted via PI3K/AKT leads to the survival of CLL cells, whilst p38MAPK pathway favours apoptosis (Porakishvili *et al.*, 2015). In my experiments I chose to use Annexin V/PI binding which allows to detect membrane-associated and nuclear events and identify early, and late apoptotic cells as discussed in the Material and Methods section.

Table 4.1: Percentages of apoptotic cells in unstimulated and stimulated CLL cells following AnnexinV/Propidium iodide staining: n is the number of samples.

n=16	%		viable cells	early apoptosis	late apoptosis	Total Apop
US	Average %		66.19	27.94	5.87	33.81
	Stdev %		20.96	15.53	10.66	20.96
anti-IgD Stim	Average %		50.28	43.67	6.05	49.72
	Stdev %		18.66	16.75	10.76	18.66
anti-CD 180 Stim	Average %		40.95	48.83	10.22	59.05
	Stdev %		34.10	30.64	17.44	34.10
anti-CD 180+ IgD	Average %		32.84	57.23	9.93	67.16
	Stdev %		28.16	26.23	16.69	28.16

As shown in Table 4.1, the levels of apoptosis were higher in CLL cells stimulated with both anti-CD180 and anti-IgD mAbs (67.16 %; SD \pm 28.16 %) than in unstimulated CLL cells (33.81 %; SD \pm 20.96 %) or in CLL cells stimulated with anti-CD180 (59.05 %; SD \pm 34.10 %) or with anti-IgD (49.72 %; SD \pm 18.66 %) Abs alone. The statistical analysis showed no significant difference ($p \geq 0.05$) in the percentages of viable and early/late-stage apoptotic cells between unstimulated CLL cells and CLL cells stimulated with anti-CD180 mAb. However, there was a significant increase in the percentages of early-stage apoptotic cells in CLL cells stimulated with anti-IgD mAb in comparison to unstimulated CLL cells ($p = 0.039$) (Figure 4.4).

Notably, there was a significant difference in the percentages of viable and early-stage apoptotic cells between unstimulated CLL cells and CLL cells stimulated sequentially with anti-CD180 and anti-IgD mAbs ($p = 0.009$, Figure 4.4). This indicates that the stimulation with anti-IgD following pre-ligation with anti-CD180 mAb favoured the pro-apoptotic pathway in CLL cells. Most importantly there was a concordance of CD180 and IgD-mediated apoptosis, with a combined ligation of the two receptors exceeding that of IgD alone (Figure 4.2B).

Therefore, it could be concluded that sequential ligation of CD180 and IgD leads to the concordant upregulation in the percentages of cells with phosphorylated p38MAPK and those at the early stages of apoptosis.

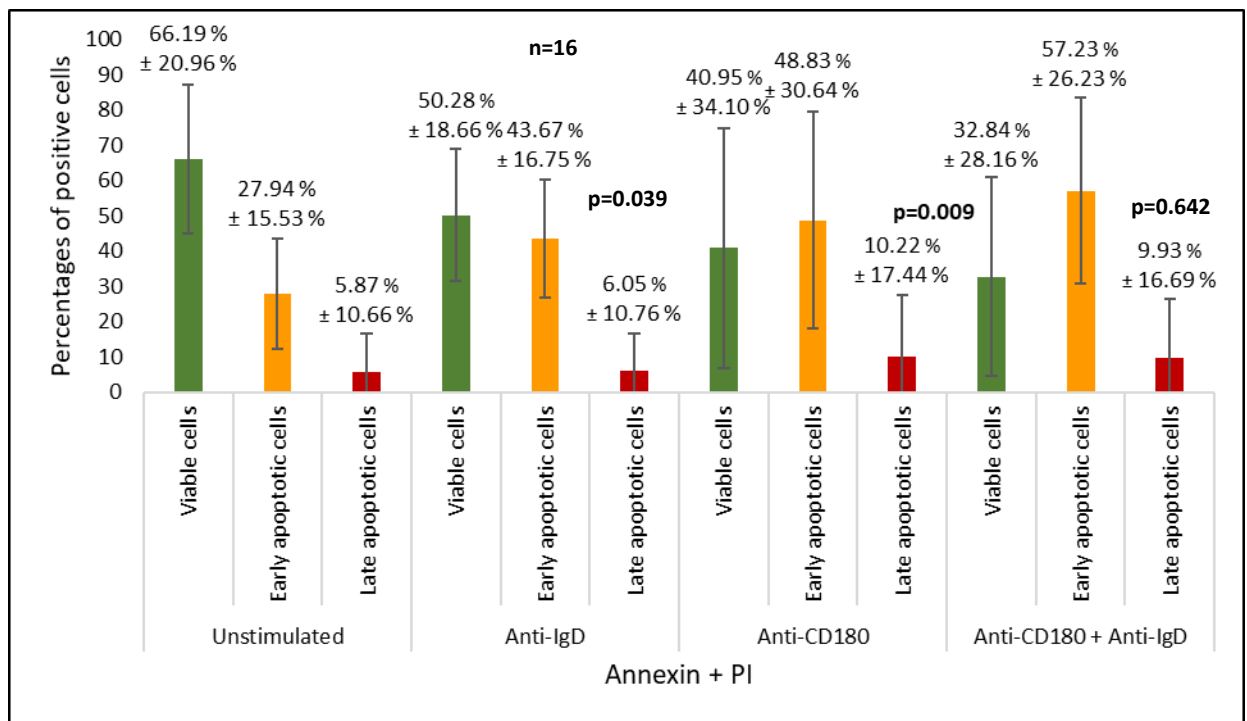


Figure 4.4. The percentages of early and late apoptotic cells in unstimulated CLL cells compared with CLL cells stimulated with anti-IgD Abs, anti-CD180 mAb or both anti-CD180 and anti-IgD Abs. CLL cells were stimulated with anti-CD180 mAb for 20 minutes or goat anti-human IgD F(ab)₂ for 10 minutes, or sequentially by anti-CD180 and anti-IgD as describes previously in Materials and method and incubated for 24 hours at 37°C and 5% CO₂. After incubation, the cells were stained with anti-CD19 mAb, loaded with Annexin V/Propidium iodide (PI) in binding buffer and analysed by flow cytometer. Mann-Whitney non-parametrical *U*-test was used for the statistical analysis.

Further studies were carried out on the stability CD180 expression on CD180+CLL cells in cell cultures for 24, 48 and 72 hours. The surface expression of CD180 receptors on 5 CLL samples was analysed, four of them were CD180+IgM+IgD+and one was

CD180+IgM+IgD-ve. Importantly, all five samples belonged to the AKT-S category, where AKT pathway is associated with proliferation and survival of CLL cells.

The important immediate observation was a significant drop in the levels of the expression of CD180 on unstimulated CLL cells after the first 24h in culture ($p=0.003$), with further decrease for the 48h timepoint ($p=0.009$) in all five CLL samples (Figure 4.5). Four out of five CLL samples demonstrated a low plateau through the incubation time 48-72h apart from one sample which showed the signs of CD180 recovery. Since phenotypically this sample was CD180+IgM+IgD+ as were the other three, hence this result was considered an artefact, albeit more samples were required for conclusive remarks.

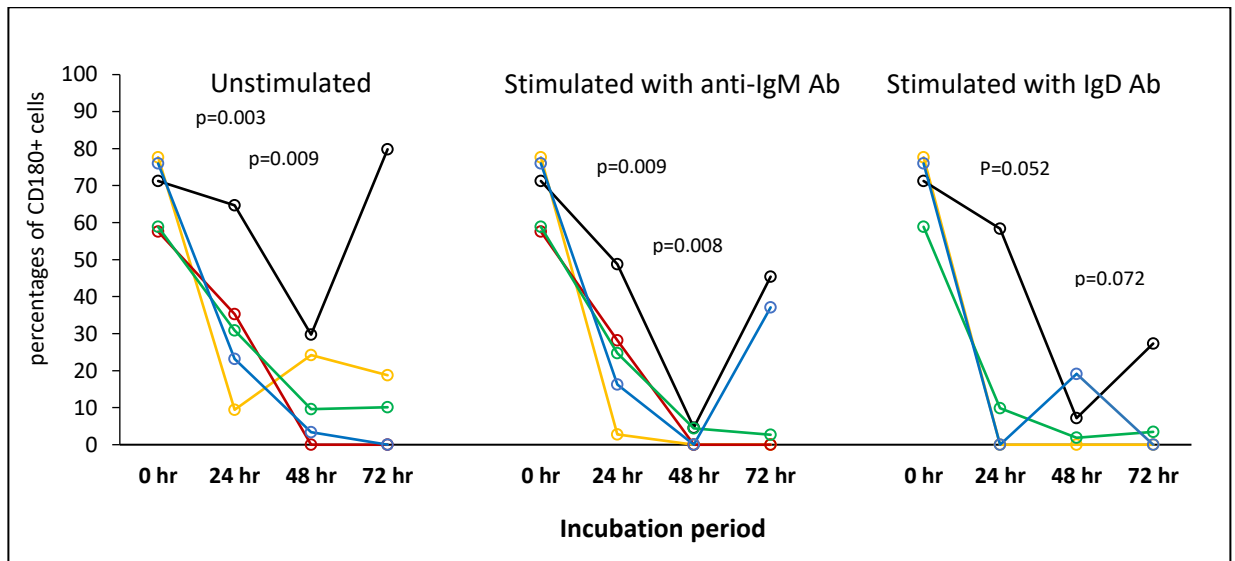


Figure 4.5. Percentages of 180+ CLL cells in 0-72h cell cultures. CD180+CLL samples were incubated for 24,48 and 72 hours unstimulated or stimulated with anti-IgM or anti-IgD F(ab)₂, washed and stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 mAb as described in the Materials and methods. The results were analysed using flow cytometry and Summit 4.3 analytical software and expressed as percentages of CD19+CD180+ positive cells. Isotype control values were subtracted. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control antibody were subtracted. *Ex vivo* (“0” time) CD180 expression was used as a control.

It appears that the application of stimulating anti-IgM or anti-IgD antibodies resulted in the acceleration of the downregulation of CD180, most importantly, both antibodies lead to a very similar drop in the CD180 expression. And this was despite of the fact that anti-IgM treatment resulted in the increased survival, whilst anti-IgD – mostly, in the increased survival or increased apoptosis of these AKT-S CLL samples.

4.3 Discussion

Yamashita *et al.* in 1995 and Yamazaki *et al.* in 2010, observed that pre-treatment of murine B cells with anti-CD180 mAb led to apoptosis following ligation of surface IgM. Porkaishvili *et al.*, (2015) demonstrated that the sequential ligation of CD180 and IgM BCR on CLL cells led to the redirection from pro-survival BTK/P13K/AKT signaling pathway towards pro-apoptotic p38MAPK pathway. Here the aim was to investigate the interplay between signalling pathways of CD180 and sIgD BCR and assess the resulting levels of cell survival/apoptosis.

As previously described by Hacken *et al.* (2016), most CLL cells express both IgM and IgD but the signaling through these two BCR isotypes leads to different functional responses in CLL cells, which are not yet fully understood. In fact, it has been suggested that the IgM isoform induces more durable signaling responses in CLL cells, usually via ERK pathway, and that it also stimulates the secretion of C-C motif chemokine ligand 3 (CCL3) and C-C motif chemokine ligand 4 (CCL4), contributing to CLL cell survival and proliferation (Hacken *et al.*, 2016). On the other hand, signaling through the IgD isoform seems to induce the activation of hematopoietic lineage cell-specific protein 1 (HS1) and the polymerisation of F-actin, which results in receptor internalisation and transient downstream signaling responses that fail to promote CLL cell survival (Hacken *et al.*, 2016). Previous studies have suggested that IgM is the main isoform targeted during Ibrutinib treatment, however it has been shown that this BTK inhibitor also interferes with IgD-mediated signaling (Hacken *et al.*, 2015; Mazzarello *et al.*, 2017). In accordance with Hacken *et al.* (2016), my results showed that all CLL samples analysed were positive for the expression of IgD (84,62 %) and that the levels of expression of this BCR isotype were slightly but not significantly lower from the ones of IgM and CD180 ($p > 0.05$).

It was confirmed here that ligation of CD180 alone led to heterogeneous signalling responses with a well-defined dichotomy to AKT-signallers (AKT-S) and p38MAPK-signallers (p38MAPK-S) (Figures 4.1) as it had been reported previously (Porkaishvili *et al.*, 2015). In addition, my results showed that the stimulation of CLL cells with anti-CD180 mAb led to an increase in both, BTK and p38MAPK phosphorylation levels in

comparison to unstimulated CLL cells, which also indicates that CD180-mediated signaling can either promote survival or induce apoptosis in CLL cells (Figure 4.3).

Furthermore, Porakishvili *et al.* (2015) demonstrated that the stimulation of CD180+ CLL cells with anti-IgM mAb alone led to a significant increase in the activation of the pro-survival BTK/PI3K/AKT signaling pathway, which is involved in CLL cell survival and proliferation. In contrast, my results showed that there was a decrease in BTK phosphorylation levels and an increase in p38MAPK phosphorylation levels in CLL cells stimulated with anti-IgD mAb (Figures 4.1 and 4.3). This was accompanied by a significant increase in the percentages of early-stage apoptotic cells in CLL cells stimulated with anti-IgD mAb in comparison to unstimulated CLL cells (Figure 4.4, $p = 0.039$), suggesting that, as previously proposed by Hacken *et al.* (2016), IgD-mediated signaling induces apoptosis in CLL cells.

It has been well documented that phosphorylation of AKT is essential for the BCR-mediated growth and survival of CLL cells (Downward, 2004; Longo *et al.*, 2008). The drop in AKT-P basal levels in the AKT-S cells following CD180 ligation might indicate that these cells are destined for apoptosis. The role of p38MAPK-mediated signalling in CLL remains unclear. Activation of p38MAPK has been previously associated with proliferation of various cells (Okkenhaug *et al.*, 2007; Herman *et al.*, 2010). In CLL, activation of p38MAPK was reported to be involved in the regulation of cell survival and apoptosis (Barragan *et al.*, 2003; Scuppoli and Pizzola, 2012). In my hands, in CLL activation of p38MAPK-mediated signalling pathways was concordant with the induction of apoptosis.

Bearing in mind possible therapeutic implications, Porakishvili *et al.* (2015) demonstrated that the pre-treatment of CLL cells with anti-CD180 mAb redirected IgM-mediated signaling from the pro-survival BTK/PI3K/AKT signaling pathway to the pro-apoptotic p38MAPK signaling pathway. My results showed that in CLL cells stimulated with both CD180 and anti-IgD mAbs, a decrease in the percentages of AKT-P+ cells and an increase in the p38MAPK phosphorylation levels was observed, in comparison to unstimulated CLL cells (Figures 4.1 and 4.2). Importantly, there was also a significant increase in the percentages of early-stage apoptotic cells in CLL cells stimulated with anti-IgD following the pre-ligation with CD180 mAb in comparison to unstimulated CLL

cells ($p = 0.009$, Figure 4.2 and 4.4). This suggests that CD180 and IgD-mediated signalling could synergistically induce apoptosis in CLL cells via the p38MAPK pathway. Thus, a strong synergism exists between CD180 and IgD mediated pathways in the activation of p38MAPK and the induction of apoptosis of CLL cells.

Dramatic downregulation of the expression of CD180 on CLL cells in spontaneous cultures, as well as those stimulated through IgM and IgD ligation (Figure 4.5), might indicate receptor endocytosis (Stevenson *et al.*, 2011). The process involves internalisation of ligand –receptor complexes and removal of ligands in endosomes. The remaining receptor either degraded or recycled back to the cell surface. The unexpected and intriguing result of my experiments is the spontaneous downregulation of CD180 in cell cultures in all five analysed samples. Internalisation of TLRs upon their ligation has been documented before, such as TLR4 cellular targeting and signaling via the formation of ligand–receptor complex involving the mechanism of endocytosis (Gangloff; 2012). Since CD180 is also homologous to TLR4, it can be reasoned that following CD180 ligation, signaling via CD180 might be recruiting a similar mechanism involving receptor-ligand internalisation. However, no artificial CD180 ligand (mAb) has been used in my experiments, and yet CD180 expression significantly decreased. Before more experiments are carried out on a larger number of samples, I can hypothesise that in these samples CD180 has been ligated, *in vivo*, by a putative ligand, and hence the rapid internalisation occurred, *in vitro*, without exposure to anti CD180 *in vitro*.

That CD180 expression is downregulated on activated B cells in rheumatoid diseases has been documented, although the mechanism of downregulation has not been studied (Kikuchi *et al.*, 2008; Koarada *et al.*, 2014).

In my experiments, since anti-IgM and anti-IgD, both induced acceleration in the downregulation of CD180, this indicates interaction between BCR and CD180 at the very early stages in CLL cell activation, perhaps through the receptor trafficking in lipid rafts. Receptors trafficking and assembly in the lipid rafts has functional importance in intracellular signalling (Villar *et al.*, 2016). Therapeutic antibodies such as anti-CD20 Rituximab and anti-CD52 Alemtuzumab translocated to the lipid's rafts accompanied by the increase in the intracellular Ca^{2+} to facilitate downstream apoptosis signaling

pathways (Janas *et al.*, 2005). There is a possibility that IgM and IgD ligation triggers CD180 receptor co-trafficking towards lipid rafts thus causing instability in the expression profile of CD180 on CLL cells. Of note, all analysed samples belong to the AKT-S category which initiate downstream signalling pathway BTK/PI3K/AKT following CD180 ligation. Interestingly, CD180 downregulation was seen following IgD ligation as well, despite of the activation of p38MAPK route. This indicates that CD180 downregulation in the AKT-S category of cells is an early event of signalling leading to either CLL cell survival, or apoptosis

4.4 Conclusions

1. Individual CLL samples exhibited heterogeneous response to ligation alone of CD180 or sIgD as assessed by the phosphorylation of AKT and p38MAPK.
2. sIgD-mediated signalling favoured the p38MAPK pathway, leading to the increased apoptosis of CLL cells compared to unstimulated cell cultures.
3. Pre-engagement of CD180 synergised with sIgD-mediated p38MAPK-mediated pro-apoptotic signalling in CLL cells.
4. After 24h in culture CD180 is rapidly downregulated from CLL cells, the process accelerated through the ligation of both, IgM or IgD BCR isoforms.

Chapter 5

The patterns of CD180 expression in the lymph nodes of CLL patients

5.1 Introduction

As discussed above, CLL is a lymphoproliferative disease with heterogeneous manifestations. It is characterized by a variable distribution of tumour mass between peripheral blood, bone marrow and lymphoid organs, and this is an important prognostic indicator. Presentation with lymphadenopathy and splenomegaly is used for staging and classification of the disease. Bone marrow and lymph nodes provide lymphoid tissue microenvironment contributing to the pathogenesis of the CLL (Burger *et al.*, 2009). Bone marrow stromal cells when added to CLL cell cultures *in vitro*, aid the survival of the tumour cells which otherwise undergo rapid spontaneous apoptosis (Deaglio *et al.*, 2010). Most of the clonal evolution, survival and proliferation of CLL cells, occurs in specialised structures in the lymph nodes (LNs), and in the bone marrow (BM) called “proliferation centres” (PCs) and found in approximately 90% of CLL cases. The initial studies of lymphoid organs in CLL refer to the PCs as “pseudofollicles” which consist of loosely arranged large cells with prominent nucleoli, in contrast to true B-cell follicles, often surrounded by the small lymphocytic infiltrates (Schmid *et al.*, 1994). As assessed by Ki-67 staining, it appears that CLL cells actively proliferate in “pseudofollicles”, hence the name “proliferation centres” was coined (Burger *et al.*, 2009).

It has been established that the neoplastic transformation of CLL cells occurs in the lymphoid tissues (Burger *et al.*, 2005) supported by the microenvironment in the LNs and BM. However, the histology of lymph nodes or bone marrow in CLL is heterogeneous, and the relationship between different histological patterns and the disease progression has been insufficiently studied, partially, perhaps due to the scarce availability of the resources such as tissue biopsies and BM aspirates which are not necessary for CLL diagnosis or prognosis. Yet better insight into the complex interactions between CLL cells and tissue microenvironment is paramount to our understanding of CLL aetiology, epidemiology and prognostication, as well as for the development of the novel therapeutic approaches.

As stated above, CD180, as a member of the toll-like receptor family, is a microenvironmental sensor, with complex signaling patterns, closely associated with BCR signaling. Our group has previously shown that CD180 is expressed on approximately 60% of CLL samples, although the density of the expression reflected in the antibody binding sites/cell was lesser than that on control B cells (Porakishvili *et al.*, 2005). However, these studies were restricted to CLL cells circulating in the peripheral blood. It was essential to assess expression of CD180 on CLL cells in lymphoid tissues implicating possible role of microenvironment through a putative endogenous ligand. Here analysis of the results of a collaborative study with the St Bartholomew's Cancer Institute, Queen Mary University of London, of CD180 expression on 3 normal tonsillar and 63 paraffin CLL LN tissue sections, under the supervision of Dr Sergey Krysov, performed by Mr Andrew Clear, an expert technician, together with the team of researchers and students from UoW were shown and discussed.

5.2 Results

Since this was the first attempt to visualize CD180 expression in paraffin tissue sections, primarily titration of anti-CD180 rabbit polyclonal antibody (Sigma Aldrich, UK) was performed using three paraffin sections of human tonsils as controls (Figure 5.1).

The titration identified the optimal dilution of the antibody in PBS - that of 1:150, which was further used to stain CLL LN paraffin sections. However, the titration study also revealed high level of expression of CD180 on lymphocytes from the mantle zone of germinal centres and in interfollicular regions. Since T cells do not express CD180, this data is consistent with the intensive expression of CD180 on B cells in normal tonsils. The staining of five normal tonsillar sections produced almost identical results (data not shown).

Further application of optimised concentration of anti-CD180 antibody to the paraffin sections was performed. Unsurprisingly, the results were highly heterogeneous. There was noticeable absence of the germinal centers within these structures. In order to analyse the data, scoring system was applied to the sections stained in triplicates. H-score (or "histo" score) determines the membrane staining intensity (0, 1+, 2+, or 3+) for each cell in a fixed field (Hirsh *et al.*, 2003; John *et al.*, 2009) as it is described in

Material and Methods. The final H- score ranged from 0 to 300 where 0-1+ intensity of staining was considered as low, 2+ as intermediate and 3+ as high (Figure 5.2).

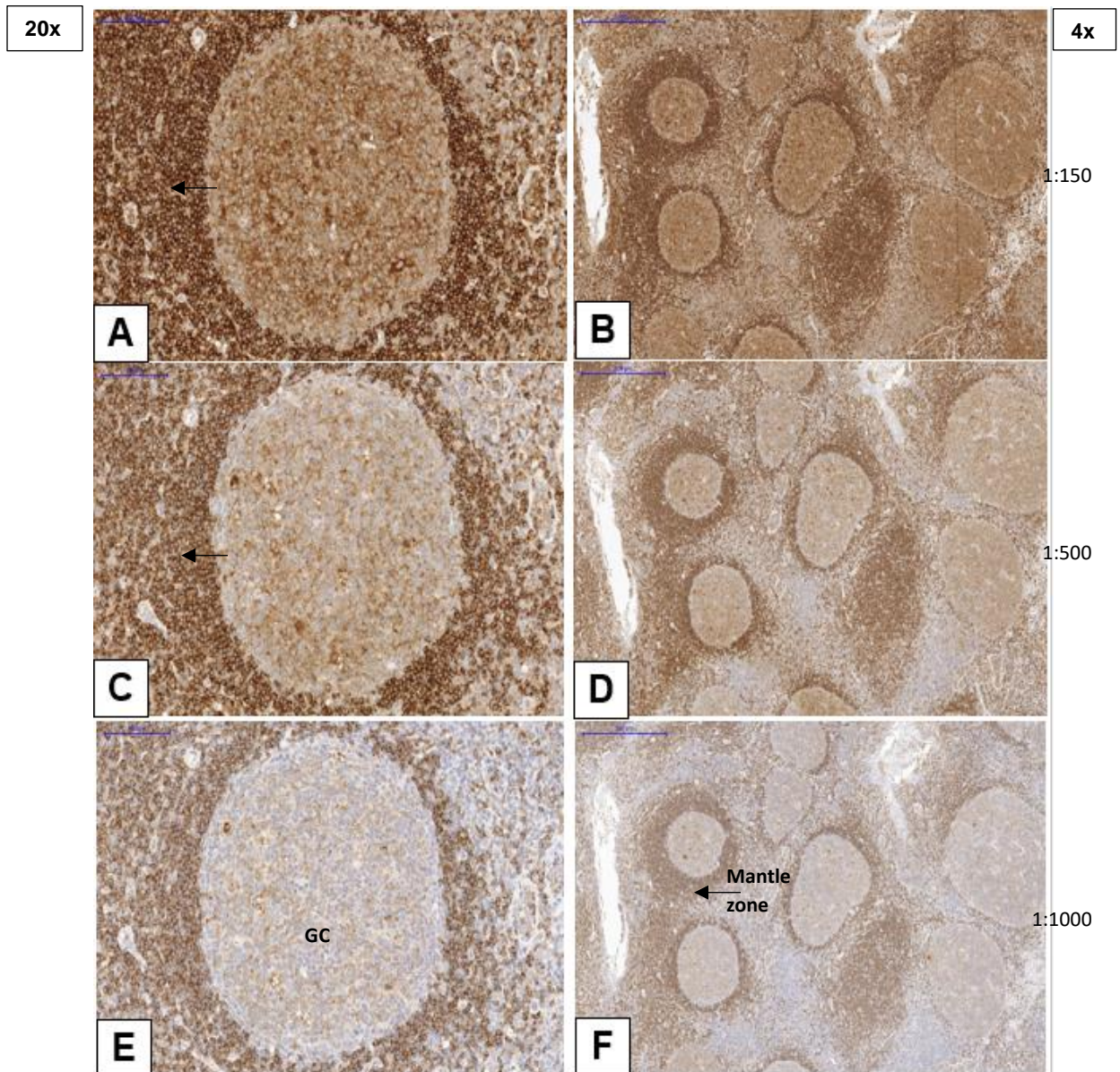


Figure 5.1. Representative profile of immunohistochemical (IHC) staining of paraffin sections of normal human tonsils. 2 μ m thick paraffin embedded sections of the tissues from normal tonsils were stained with different dilutions of rabbit anti-CD180 polyclonal antibodies as described in the Materials and Methods. 20x magnification (A, C and E); 4x magnification (B, D and F). Rabbit anti-CD180 polyclonal antibody dilutions: 1:150 (A and B) 1:500 (C and D) and 1:1000 (E and F). Arrows indicate positive expression of CD180 (brown). The blue line is equal of 50 μ m.

Further attempt was made to find a correlation between the H-scores of the CD180 staining in CLL lymph node sections with clinical and available (pre-performed on the same sections) laboratory data, including prognostic markers and cytogenetic abnormalities, where available. The results indicated two statistically significant correlations between the H-score of the expression of CD180, the stage of the disease (Figure 5.3) and the expression of Ki-67 (Figure 5.4).

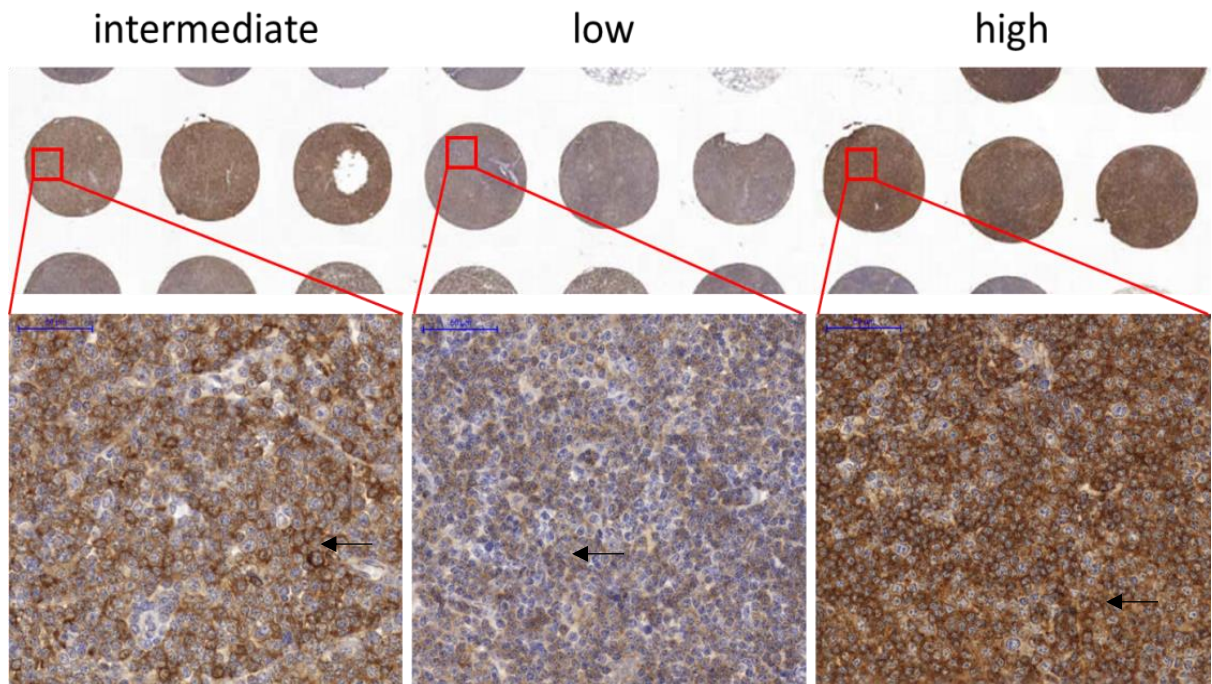


Figure 5.2. Representative patterns of CD180 expression in lymph node tissue sections from CLL patients. (40x magnification). 2 μ m CLL lymph node paraffin embedded sections were stained with the optimal dilution 1:150 of rabbit anti-CD180 polyclonal antibodies as described in the Materials and Methods. The patterns of the expression are based on the scores: 0-1+ (low), 2+ (intermediate) and 3+ (high). Arrows indicate positive expression of CD180 (brown). Poorly organized structures with absence of germinal centres are visible. The blue line is equal of 50mm.

Expression of CD180 in the lymph nodes was significantly lower ($p=0.003$) in CLL patients at Binet stage B, compared to the Binet stage A (Figure 5.3). Unfortunately, the number of the patients at Binet stage C was very low, hence the results regarding this stage were inconclusive, although all four Binet stage C patients which were assessed showed high levels of CD180 expression in the lymph node sections.

Another striking correlation was revealed between the expression of CD180 and Ki-67, a cell cycle-associated protein (Figure 5.4). There was a strong direct correlation between the H-score of CD180 expression and the expression of Ki-67 ($p=0.0003$) indicating that CD180+ cells in the lymph nodes are the ones that preferably enter cell cycle.

No other statistically significant correlations have been found of the expression of CD180 in lymph nodes of CLL patients, and available clinical or laboratory parameters (data not shown).

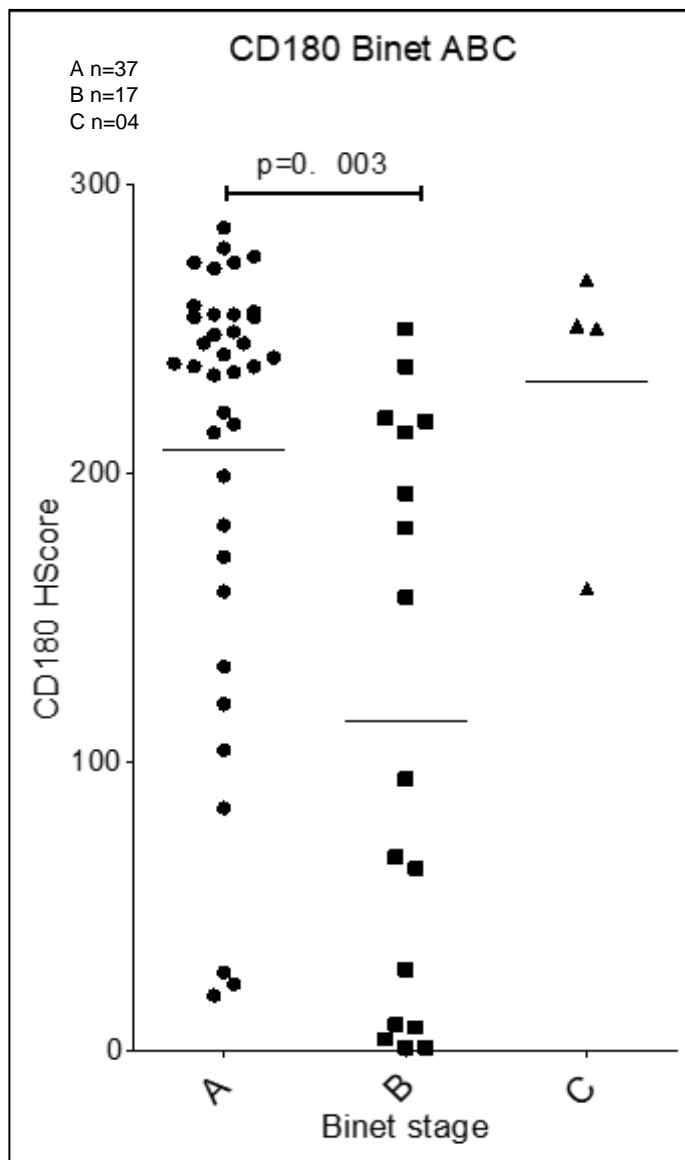


Figure 5.3. CD180 expression in the CLL lymph node paraffin sections at Binet stages A, B and C.

Lymph node paraffin sections from CLL patients at different stages of the disease were stained with the optimal dilution 1:150 of rabbit anti-CD180 polyclonal antibodies as described in the Materials and Methods. The intensity of CD180 expression was assessed using H-score as described in the Materials and methods. Here n is the number of patients used at each CLL stage.

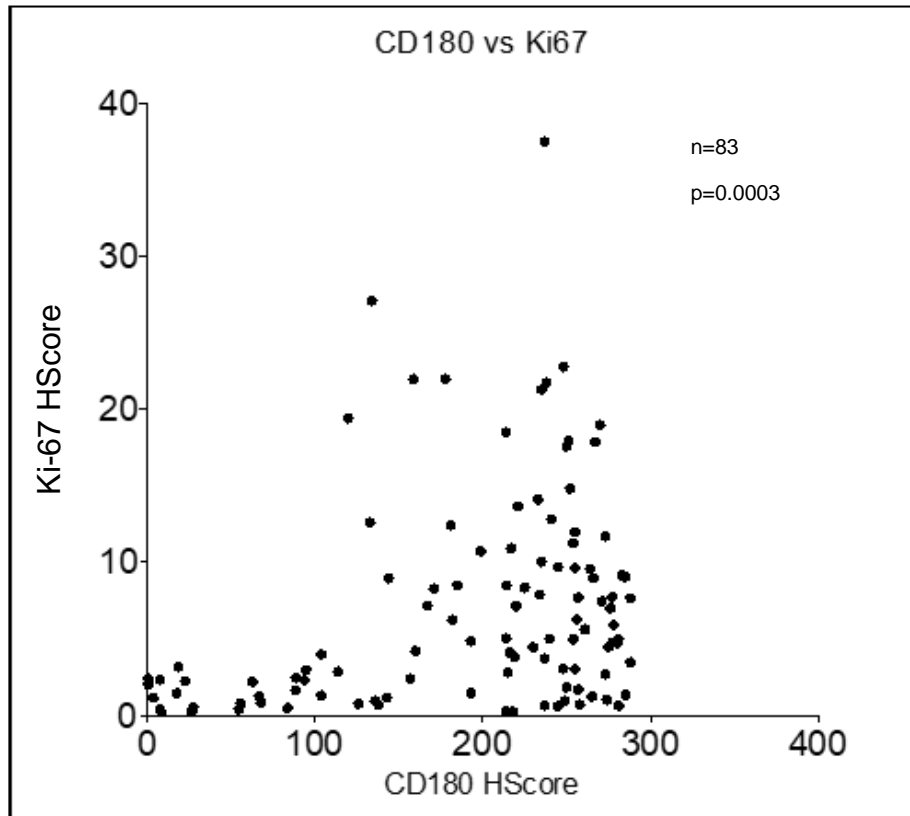


Figure 5.4. Correlation between CD180 and Ki-67 expression in the CLL lymph node sections. Lymph node paraffin sections from CLL patients were stained with the optimal dilution 1:150 of rabbit anti-CD180 polyclonal antibodies as described in the Materials and Methods. The intensity of CD180 expression was assessed using H-score as described in the Materials and methods. Ki-67 analysis was pre-performed on the same sections.

5.3 Discussion

These studies on the CD180 expression in the lymph node sections of CLL patients have further improved our understanding of the dynamics of CD180 expression in lymphoid organs and tissues.

Primarily, the data on three tonsillar samples confirmed previous observations that CD180 is expressed preferably by the mantle zone B cells (MZB) and to the lesser extent by the germinal center (GC) B cells (Miyake *et al.*, 1995; Chaplin *et al.*, 2011; Nagai *et al.*, 2012). Since all these studies were carried out on murine splenic B cells, to the best of our knowledge this is the first report showing the expression of CD180 in human tonsillar MZB cells. Studies had shown that the majority of MZB cells belong to

the activated antigen-experienced cells with mutated *IGVH* genes (Cerruti *et al.*, 2013). The somatic hypermutation and B cells clonal expansion takes place in the germinal centres before B cells migrate to the marginal zone (Tierens *et al.*, 1999). This is in line with previous findings that CD180 is expressed preferentially by the M-CLL cells (Porakishvili *et al.*, 2005), e.g. antigen experienced cells which went through the lymph node germinal centers (Chiorazzi *et al.*, 2005). Therefore, it could be assumed that CD180 is expressed on human B cells after their migration from germinal centres to the marginal zone. Indeed, it had been shown that mice splenic MZB cells undergo activation, proliferation and antibody secretion upon stimulation with anti-CD180 mAb (Chaplin *et al.*, 2011). The data here suggests that CD180 might be considered as a biomarker of antigen experienced normal human B cells migrating from GCs to the marginal zone of lymph nodes.

CLL cell architecture in the LNs was entirely different: it was hard to observe distinct follicles and extra-follicular regions due to infiltrations of neoplastic malignant cells as it had been previously noted (Schmid *et al.*, 1994; Dick *et al.*, 2006).

Very importantly, there was a highly heterogeneous expression of CD180 in individual lymph node sections in CLL, as reported for the peripheral blood by Porakishvili *et al.* (2005). Porakishvili *et al.*, (2005) also showed that individual CLL clones were characterized by a stable pattern of CD180 expression during two years of observations. Together with results analysis presented in this chapter, this suggests that positivity or negativity for CLL expression might represent an intrinsic feature of individual CLL clones. However, to confirm this, PB and LN studies should be carried out on the same CLL patients. Since LN biopsies are not generally performed now in CLL patients, this would be logistically hard to achieve.

Unlike the peripheral blood (Porakishvili *et al.*, 2005) where no correlation was found between the expression of CD180 and the disease stage, the lymph nodes of the patients with more advanced disease (Binet stage B) showed a significantly lower intensity of CD180 expression, compared to patients at disease stage A ($p=0.003$) (Figure 5.3). Importantly, CD180 expression in CLL lymph nodes directly correlated with their entering into the cell cycle as measured by Ki-67 upregulation ($p=0.0003$) (Figure 5.4), indicating that CD180+ could be considered a marker for CLL cells

proliferating in the lymph nodes. It had been shown that CLL cells are often organized into the proliferation centres (PCs) wherein they undergo malignant transformation, survival and expansion as a clone (Schmid *et al.*, 1994; Soma *et al.*, 2006; Ciccone *et al.*, 2011). The PC CLL cells generally express phenotypic markers of activated/proliferating cells evidenced by higher levels of CD38, Ki-67, ZAP70 and IgM, and preferably belong to the U-CLL (Schmid *et al.*, 1994; Soma *et al.*, 2006; Gine *et al.*, 2010). It appears therefore that CD180 expression on the LN CLL cells might be indicative of the cells organized into the proliferation centers.

That CD180+ CLL cells express high levels of Ki-67 is in line with the previous studies of our group showing that the ligation of CD180 on PB CLL cells leads to their proliferation measured by Ki-67 expression (Porakishvili *et al.*, 2011). One can hypothesize that on activation and proliferation of CLL cells in the PCs assisted by the CD180 receptor interaction with a putative ligand, CD180 would be downregulated from CLL cells as it has been shown for B cells in autoimmune diseases (Kikuchi *et al.*, 2008; Koarada *et al.* 2014). My own data presented in Chapter 4, also points towards the process of receptor endocytosis, hence the lower expression of CD180 might be seen in lymph nodes at more advanced Binet stages (Figure 5.3). As for Binet stage C, more data is required in order to make a conclusion regarding possible recovery of CD180 expression in lymph nodes. This study has not been normalized according to the applied treatment of CLL patients at more advanced stages of the disease which represents an additional factor affecting the results.

5.4 Conclusions

1. In normal tonsils CD180+ is expressed preferentially on the mantle zone and interfollicular B cells.
2. CD180 is heterogeneously expressed in the lymph nodes of CLL patients with patterns varying from low to intermediate and high.
3. CD180 expression on the lymph node CLL cells positively correlates with the expression of Ki-67.
4. Intensity of the expression of CD180 on the lymph nodes CLL cells is higher in patients at the Binet stage A, compared to the Binet stage B

Chapter 6

CONCLUDING REMARKS

It has been very well documented that microenvironment plays a major role in the development and progression of chronic lymphocytic leukaemia (CLL). Soluble and cell-bound ligands and receptors in the environment of central and peripheral lymphoid tissues and organs drive proliferation, survival and expansion of CLL cells. Our group has shown previously that non-canonical Toll-like receptor (TLR) CD180 strongly contributes to the regulation of signaling, expansion and survival of CLL cells. Our initial studies have documented that CD180 is expressed heterogeneously on approximately 60% of CLL cells in the peripheral blood (PB), with a significantly higher level of expression on M-CLL, rather than U-CLL samples (Porakishvili *et al.*, 2005). This study also confirms the pattern of highly heterogeneous expression of CD180 in the lymph node (LN) CLL cells with an H-score ranging from 0 to 300 (Figure 5.2). It would have been important to check whether positivity or negativity for CD180 represents an intrinsic feature of a given CLL clone in the LNs and in the PB or whether it can be modulated during CLL cell trafficking in and out of the LNs. Since currently LN biopsies are extremely rare in the process of the CLL patient management, this would be logistically hard to explore. On one hand, previous studies by our group have indicated that individual CLL samples have stably maintained their CD180 expression profile throughout 24 months of observation (Porakishvili *et al.*, 2005). On the other hand, data analysis of this study has shown here that CD180 can be rapidly downregulated, *in vitro*, from the CD180+ CLL cells, the process accelerated by the ligation of BCR (Figure 4.4). However, this drop in the expression of CD180 might be influenced by the removal of CLL cells from their microenvironment or endocytosis of CD180 linked to a putative antigen.

That CD180+ cell samples might be enriched with antigen-experienced CLL cells follows from our observations of its expression on MZB cells and on those in interfollicular spaces (with less frequency) in normal tonsils (Figure 5.1). In addition, some level of correlation between the expression of CD180 and CD38, particularly in U-CLL samples (Porakishvili *et al.*, 2005, and Chapter 3) was also noted. Most importantly the pilot studies performed in this project have indicated that double negative CD180/CD38 CLL cells belong to the prognostically favorable DLEU1

genotype (Chapter 3). All this data would argue that CD180 is associated with CLL cells undergoing survival, proliferation and expansion.

However, not all blood CD180+ CLL samples were found to be responsive to stimulation. Out of CD180+ CLL samples only half responded to the ligation with anti-CD180 monoclonal antibody (mAb) by activation, cycling, and reduced basal apoptosis. CD180-mediated CLL cell activation and survival was comparable or superior to that induced by CD40 ligation or addition of the recombinant IL-4 (Porakishvili *et al.*, 2005). CLL samples which responded to CD180 ligation by increased expression of CD86 (activation) and Ki-67 (cell cycling) were termed responders (R-CLL). In contrast, CD180+ CLL samples that failed to respond to anti-CD180 mAb, despite expressing CD180 receptors, were termed non-responders (NR-CLL).

Our group further demonstrated that CD180 ligation on R-CLL cells and normal B cells, led to a strong upregulation in the phosphorylation of ZAP70/Syk, ERK, p38MAPK, and AKT protein kinases (PK), which was indicative of interaction between or utilization of the same signaling pathways by CD180 and BCR (Porakishvili *et al.*, 2011). Thus, the hypothesis has been put forward that CD180 as a microenvironmental sensor, contributes to or regulates BCR-mediated signaling in response to the (auto) antigen(s). R-CLL cells within the microenvironment of lymphoid tissues in the proliferation centers (PCs) would have an advantage of receiving survival, proliferation and, as a result, expansion signals from both, BCR and CD180. In contrast, CD180-mediated signaling in NR CLL cells did not progress from ZAP70/Syk phosphorylation indicating a block in activation of downstream protein kinases, and possible anergy (Porakishvili *et al.*, 2011).

However, in the same paper it has been noted that although phosphorylation of AKT, ERK, and p38MAPK was significantly increased in R-CLL in response to the CD180 ligation, compared to NR-CLL samples, there was substantial level of heterogeneity in the intensity of responses. More data was required to ascertain whether the range in the intensity of PK activation is associated with a range of CD180 expression on CLL cells in the PB (Porakishvili *et al.*, 2005) and/or lymph nodes (Figure 5.2). In order to clarify the CD180-mediated signaling pathways in CLL downstream from ZAP70/Syk, CLL samples were recategorized into AKT signalers (AKT-S) and p38MAPK signalers

(p38MAPK-S), since these two PKs appear to represent two alternative signaling pathways observed in the majority of CLL samples (Porakishvili *et al.*, 2015). Activation of both, AKT and p38MAPK was seen in a small cohort of CLL samples and they were termed double signalers (DS) whereas the remaining CD180+ CLL samples which did not respond to CD180 ligation by activating either of the two pathways were defined as non-signalers (NS).

There is strong evidence as shown in previous publications by Porakishvili *et al.* to suggest that NS CLL cells are anergic, since they responded poorly to other established CLL stimuli such as anti-CD40 mAb and rIL-4 (Porakishvili *et al.*, 2011). In addition, CD180 expression is higher on M-CLL cells (Porakishvili *et al.*, 2005), and it has been well documented that M-CLL cells respond poorly to various stimuli, including anti-IgM Ab (Chiorazzi *et al.*, 2005; Stevenson *et al.*, 2011), partially because IgM expression on M-CLL cells is low (Porakishvili *et al.*, 2005). However, it is impossible to exclude the possibility, that alternative downstream pathways are used by NS cells rather than being refractive to the ligation of CD180.

It was important to follow CD180-mediated signaling through alternatively AKT or p38MAPK activation downstream to the apoptosis/survival in order to identify physiological consequences of CD180-mediated signaling in CLL. It was found that AKT (and associated PI3K)-mediated pathway leads to the CLL cell survival, whilst p38MAPK – favoured apoptosis (Porakishvili *et al.*, 2015).

Since the same PKs are activated as a result of the signal transduction from sIgM it was paramount to study the interaction between CD180 and sIgM mediated pathways, and how this might have impinged on CLL cell survival. Our group has determined that the ligation of sIgM with the stimulatory polyclonal Ab mostly leads to the activation of BTK/PI3K/AKT signaling pathway and cell survival. Most importantly though, priming of CLL cells with anti-CD180 mAb redirected prosurvival BTK/PI3K/AKT signaling mediated through BCR towards pro-apoptotic p38MAPK pathway. Rewiring of signaling from pro-survival to pro-apoptotic pathway was not observed in control B cells suggesting that the regulatory effect of CD180 ligation on sIgM is unique for CLL cells (Porakishvili *et al.*, 2015).

As a logical follow up of that study, it was important to investigate, how CD180 signaling interacts with the second BCR isoform – sIgD, which was one of the aims of this project. The data obtained demonstrated that IgD ligation on CD180+IgD+ CLL cells with stimulating polyclonal antibody led mostly to the activation of p38MAPK pathway and subsequent apoptosis of CLL cells (Figures 4.1, 4.2 and 4.3). These findings may contradict some previous publications (Haerzschel *et al.*, 2016; Sabouri *et al.*, 2016), however there are also reports confirming our data (Tavolaro *et al.*, 2013; Hacken *et al.*, 2016) In addition our data is skewed towards CD180+IgD+ cells only. This might mean that CD180+IgD+ cell subset is enriched with those CLL cells where IgD ligation leads predominantly to the apoptosis, compared to the CD180-IgD+ cell subset. Most importantly, priming of CLL cells with anti-CD180 mAb led to the concordant activation of p38MAPK and increased apoptosis (Figures 4.2 and 4.3). This means that priming with CD180 will direct signaling by both BCR isoforms – sIgM and sIgD towards activation of p38MAPK and apoptosis of CLL cells (Figure 6.1) thus suggesting new therapeutic approaches for the treatment of CLL.

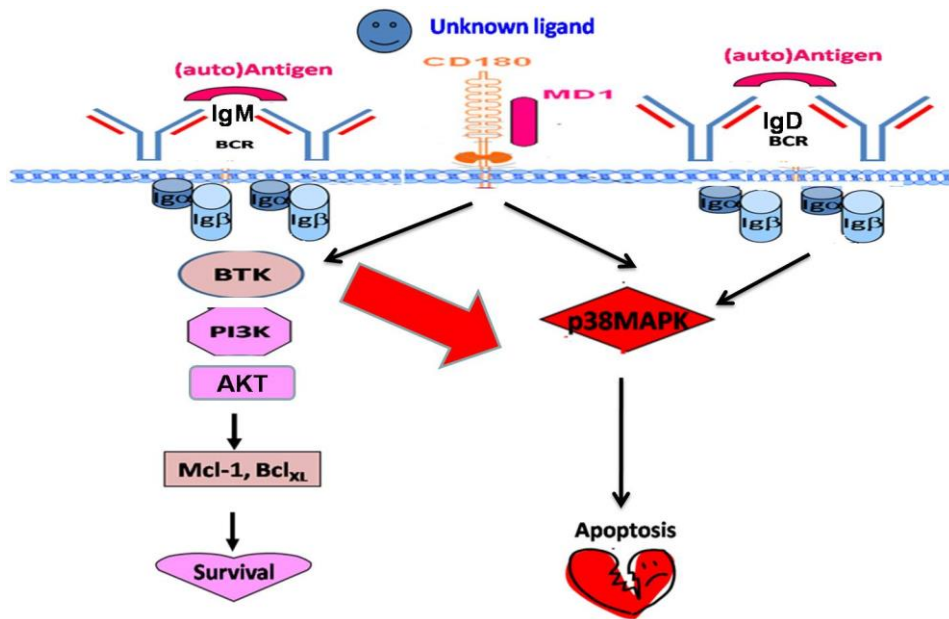


Figure 6.1: Hypothetical scheme of a cross-talk between CD180 and BCR IgM and IgD signaling pathways. CD180-mediated pathway can operate via both, pro-survival BTK/PI3K/AKT or pro-apoptotic p38MAPK pathway, whilst sIgM-mediated signaling mostly operates through BTK/PI3K/AKT, and sIgD – via p38MAPK. Cross-talk between the receptors redirects the signaling pathway from BTK/PI3K/AKT to p38MAPK and subsequent apoptosis.

In the light of molecular therapies with inhibitors of signaling pathways, my data regarding BTK activation was indecisive. I have observed a range of the BTK

phosphorylation responses through CD180 ligation alone, or in combination with the ligation of BCR. In many cases, BTK phosphorylation was seemingly bypassed downstream to the AKT. Ibrutinib resistance in some CLL clones and its toxicity has been reported and the drug itself does not lead to the apoptosis of CLL cells but rather to the accumulation of CLL cells in PB, rather than in the LN and the spleen (Furman *et al.*, 2014; Wiestner, 2015). Therefore prior to the treatment with Ibrutinib, Idealisib or any other inhibitors of signaling pathways, each CLL case should be tested not only for BTK activation upon sIgM engagement, but also a cumulative effect following the ligation of sIgM, sIgD and CD180. The additive effect of the ligation of all three receptors, *in vivo*, might downregulate some of the PKs involved in the pro-survival signaling in CLL cells making inhibition therapy unnecessary and ineffective, and this can be tested, *in vitro*. In addition, Ibrutinib would have no effect on p38MAPK-S CLL cells which do not recruit BTK.

It is becoming obvious that intraclonal diversity plays an important role in CLL progression and outcome. Stratification of these CLL subpopulations is of a paramount importance, in order to identify correct targets for personalized therapy. Our group has established that the pattern of CD180 expression on CLL cells and signaling pathways it utilizes helps to delineate physiological status of these subsets in relation to the precision therapy in conjunction with established prognostic indicators such as CD38 expression and chromosomal aberrations.

Our findings on the modulation of signaling pathways through CD180 and BCR (sIgM and sIgD) and the temporal effects of their ligation is consistent with multiple ligands in the, *in vivo*, microenvironment regulating the survival of CLL cells. Since CD180 can alternate inhibition and promotion of the leukemic growth it may impact clinically relevant tumour host microenvironment interactions. Although it is clear that BCR signals are important for CLL cells survival, my data indicates that involvement of sIgD shifts the balance in favour of apoptotic pathways. CLL cell activation via CD180 does not abrogate pro-apoptotic activity of sIgD but acts synergistically. This opens avenues for the development of new therapeutic approaches for the treatment of CLL.

Future studies

- Confirmation of hypothesised co-localization of IgM and CD180 receptors in the lipid rafts upon stimulation with anti-CD180, anti-IgM and anti-IgD to assess upstream events of the cross-talk of the signaling pathways.
- Identification of the precise chain of intracellular PKs involved in the dichotomy of p38MAPK and AKT-mediated pathways following ligation of CD180.
- Assessment of the stratification value of CD180 in CLL in conjunction with established prognostic indicators.

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