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**Functional interaction between CD180 Toll-like receptor (TLR)  
and B cell receptor (BCR) in the biology of Chronic Lymphocytic  
Leukaemia (CLL)**

**Rajakaruna, A.**

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**Functional interaction between CD180  
Toll-like receptor (TLR) and B cell  
receptor (BCR) in the biology of Chronic  
Lymphocytic Leukaemia (CLL)**

**A.V. Nadeeka P. Rajakaruna**

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## Abstract

Chronic Lymphocytic Leukaemia (CLL) is the most common leukaemia in the western world and remains incurable. It is driven by as yet unknown (auto)antigens via the B cell receptor (BCR) and growth, survival and expansion signals it receives from the microenvironment through a range of cytokines and receptors, including Toll-like receptors (TLR). The role of the microenvironment in the development and progression of CLL is currently of major interest. Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) represent exogenous and endogenous microenvironmental factors acting via a range of receptors, including TLR. CD180/RP105 is a membrane-associated orphan receptor of the TLR family which is expressed on various cells of the immune system including macrophages, peripheral blood monocytes, dendritic cells, naive and mature B cells and marginal zone/mantle zone B cells driving normal B-cell activation and proliferation. However, it is not expressed on germinal centre (GC) B cells. CD180 is expressed heterogeneously on CLL cells, and predominantly on CLL with mutated *IGVH* genes (M-CLL). Although approximately 60% of CLL clones expressed CD180, only half responded to ligation with anti-CD180 monoclonal antibody (mAb) by activation, cycling, and reduced basal apoptosis. They were termed responders (R) and CD180<sup>+</sup> CLL samples that failed to respond to anti-CD180 mAb despite expressing a high density of CD180 receptors, were termed non-responders (NR).

Our group has previously demonstrated that CD180 ligation significantly induced phosphorylation of ZAP70/Syk, ERK, p38MAPK, and AKT in R-CLL cells whilst CD180-mediated signalling in NR CLL cells did not progress downstream from ZAP70/Syk phosphorylation indicating a block in activation of downstream protein kinases, and possible energy.

However, the responses were quite heterogeneous and to further understand the CD180-mediated signalling pathways in CLL; downstream signal transduction was studied by defining CLL samples into R and NR through their proximal ability to activate AKT. R-CLL cells could be divided into two categories as AKT signalers (AKT-S) and AKT non-signalers (AKT-NS) based on the ability to increase level of phosphorylation of AKT (Ser 473) compared with the basal levels. AKT-NS showed a significant increase in p38MAPK-P and this group was, therefore, re-categorised as p38MAPK signalers (p38MAPK-S). A small cohort

of patients showed phosphorylation of both AKT and p38MAPK and they were categorised as double signallers (DS) whereas the remaining CLL samples which showed a decrease in percentages of both AKT-P and p38MAPK-P expressing cells and were categorised as non-signallers (NS). Ligation of CD180 with mAb on CLL cells can activate two alternative signalling pathways, one being pro-survival and operating through activation of protein kinases (PK) BTK-AKT (AKT-signallers); and the second - predominantly pro-apoptotic, operating through activation of p38MAPK (p38MAPK-signallers) but not through BTK. This may have implications for CLL therapy where BTK inhibitors are being used.

To assess cross-talk between CD180 and BCR (sIgM) signalling pathways in CLL, the effect of pre-engagement of CD180 on sIgM-mediated signalling was investigated. While sIgM ligation with goat anti-human IgM F(ab)<sub>2</sub> alone led to a significant activation of pro-survival BTK-AKT pathway, pre-treatment with anti-CD180 mAb redirected pro-survival signalling mediated through BCR towards pro-apoptotic p38MAPK pathway. Application of specific inhibitors of AKT and p38MAPK signalling pathways confirmed that, in many of the CLL samples, activation of AKT and p38MAPK pathways is exclusive. This dichotomy appears to be a feature of CLL cells, and not of normal B cells that responded to CD180 ligation as double AKT/p38MAPK signallers.

It is becoming apparent that the clinical outcome of patients with CLL is significantly affected by intraclonal diversity. CLL clones which can respond more vigorously to external stimuli may gain selective advantage for growth and survival and perhaps promote clonal evolution. That CD180 expression modulates in some categories of CD180<sup>+</sup> CLL cells suggests a possible link between dynamic expression of CD180 and CD180-mediated intracellular signalling, survival or apoptosis. Finding on rewiring of signalling pathways from BTK/AKT pro-survival circuit to p38MAPK pro-apoptotic pathway in AKT-S CLL cells may suggest a possible cross-talk between CD180 and BCR in AKT-S is consistent with that CD180<sup>+</sup>sIgM<sup>+</sup> CLL cells may receive simultaneous signals through both receptors *in vivo* and microenvironment plays a major role in the survival of CLL cells. Hence, this study helped define mechanisms leading to the expansion of the leukaemic cells and thus contribute to development of novel therapies of CLL.

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## **Declaration**

I, A. V. Nadeeka P. Rajakaruna, declare that the PhD thesis entitled Functional interaction between CD180 toll-like receptor (TLR) and B cell receptor (BCR) in development and progression of Chronic Lymphocytic Leukaemia (CLL) contains no material that has 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:13.07.2017

## List of abbreviations

µl	- microliter
ADCC	- Antibody-dependent cellular cytotoxicity
AID	- Activation-induced cytidine deaminase
AIF	- Apoptosis inducing factor
AKT-NS	- AKT non-signallers
AKT-S	- AKT signallers
API	- Activating protein I
APC	- Allophycocyanin
APRIL	- A proliferation-inducing ligand
ASCT	- Autologous stem cell transplantation
ATL	- Antitumour lipids
ATM	- Ataxia telangiectasia mutated
B2M	- β2-microglobulin
BAD	- Bcl-2 Associated Death
BAFF	- B-cell activating factor
BAFF-R	- BAFF-receptor
BAG1	- BCL2 Associated Athanogene 1
Bak	- Bcl-2 antagonist/killer
Bax	- Bcl-2-associated X protein
BBRs	- BAFF-binding receptors
BCR	- B cell receptor
B-CLL	- B-cell chronic lymphocytic leukaemia
Bcl2	- B cell lymphoma 2
BCL2A1	- Bcl-2-related protein A1
BCL-xL	- B-cell lymphoma-extra large
BCMA	- B-cell maturation antigen

BH	- Bcl-2 homology
Bid	- BH3 interacting-domain
Bik	- BCL2-interacting killer
BIR	- Baculoviral IAP repeat
BLNK	- B-cell linker protein
BlyS	- B-lymphocyte stimulator
Bmf	- Bcl-2 modifying factor
Bok	- Bcl-2 related ovarian killer
BSA	- Bovine serum albumin
BTK	- Bruton's Tyrosine Kinase
c-FLIP	- cellular FLICE (FADD-like IL-1 $\beta$ -converting enzyme)-inhibitor protein
cADPR	- cyclic Adenosine diphosphate ribose
CAR	- Chimeric antigen receptor
CART	- Chimeric antigen receptor modified T cells
CAT	- Central kinase catalytic
CD	- Cluster of Differentiation
CDDO	- Triteroenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid
CDR3	- Complementarity-determining regions 3
CLL	- Chronic Lymphocytic Leukaemia
CLPD	- Chronic lymphoproliferative disorders
c-MET	- c-mesenchymal-epithelial transition factor
CR	- Complete remission
CSR	- Class switch recombination
CTB	- Cholera Toxin B subunit
CXCL12	- C-X-C motif chemokine ligand 12
CXCR4	- C-X-C motif chemokine receptor 4



DAG	- Diacylglycerol
DAMP	- Damage-associated molecular pattern
DiOC <sub>6</sub>	- 3,3'-dihexyloxacarbocyanine iodide
DISC	- Death-inducing signalling complex
DN	- Dominant negative
DNA	- Deoxyribonucleic acid
DS	- Double signallers
DT	- Doubling Time
DYRK1A	- Dual specificity tyrosine phosphorylated and regulate kinase1A
EBV	- Epstein–Barr virus
ERK	- Extracellular signal-regulated kinase
FADD	- Fas-associated death domain
FasL	- Fas ligand
FBS	- Foetal bovine serum
FDX1	- Ferredoxin
FISH	- Fluorescent <i>in situ</i> hybridization
FITC	- Fluorescein isothiocyanate
FL	- Follicular lymphoma
GC	- Germinal Centre
g/dl	- gramm per decilitre
g/l	- gramm per litre
GFR	- Glomerular filtration rate
GSK-3	- Glycogen synthase kinase-3
Hb	- Haemoglobin
HBBS	- Hank's buffered salt solution
HCDR3	- Heavy chain complementarity-determining region 3

HGF	- Hepatocyte growth factor
HM	- Hydrophobic motif
Hrk	- Harakiri, BCL2 Interacting Protein
HSP27	- Heat shock protein27
IAPs	- Inhibitor of apoptosis proteins
iFISH	- Interphase fluorescence <i>in situ</i> hybridization
Ig	- Immunoglobulin
IgE	- Immunoglobulin E
IKK	- IκB kinase
IL	- Interleukin
IL-R	- Interleukin receptor
IGVH	- Immunoglobulin heavy chain variable region gene
IκBα	- Inhibitor of kappa B
ITAM	- Immunoreceptor tyrosine-based activation motifs
IFN	- Interferon
IP <sub>3</sub>	- Inositol-1,4,5-triphosphate
IPD	- IP3R- derived peptide
IRF	- Interferon-response factor
IRAK	- Interleukin-1 receptor-associated kinase
IWCLL	- International Workshop on CLL
JAK3	- Janus-activated kinase 3
JNK	- Jun amino(N)-terminal kinase
kDa	- kilodalton
l	- Litre
LAM	- Lymphoma-associated macrophages
LDH	- Lactate dehydrogenase
LDT	- Lymphocyte doubling time

LINK	- Linker
LPL	- Lymphoplasmacytic lymphoma
LPS	- Lipopolysaccharide
LRR	- Leucine-rich repeats
LRs	- Lipid rafts
Lyn	- Lck/Yes novel tyrosine kinase
mAb	- monoclonal antibody
MAL	- MyD88-adaptor-like
MAPK	- Mitogen-activated protein kinase
MAPKK	- Mitogen-activated protein kinase kinase
MAPKKK	- Mitogen-activated protein kinase kinase kinase
MBL	- Monoclonal B-cell lymphocytosis
Mcl-1	- Myeloid Cell Leukaemia 1
M-CLL	- chronic lymphocytic leukaemia with mutated <i>IGVH</i> genes
Mda-7	- Melanoma differentiation-associated gene-7
MDM2	- Mouse double minute 2
MDR	- Multidrug resistant
MEK	- Mitogen-activated Protein/Extracellular Signal-regulated Kinase
MHC-I	- Major histocompatibility complex class I
MKK1	- Mitogen-activated protein kinase kinase 1
MKK2	- Mitogen-activated protein kinase kinase 2
mm <sup>3</sup>	- millimetre cubed
mM	- millimolar
MPLA	- Monophosphoryl lipid A
MPN	- Myeloproliferative neoplasm
MRD	- Minimal residual disease
mRNA	- Messenger ribonucleic acid

MSK1	- Mitogen- and stress-activated protein kinase1
MSC	- Mesenchymal stem cells
Mtd	- Matador
mTOR	- Mammalian target of rapamycin
mTORC1	- mTOR complex 1
MyD88	- Myeloid differentiation primary-response protein 88
NAD	- Nicotinamide adenine dinucleotide
NF-AT	- Nuclear factor of activated T cells
NFκB	- Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	- Non-Hodgkin lymphoma
NK	- Natural killer cell
NLC	- Nurse-like cells
NOXA	- NADPH oxidase activator
NS	- Non-signallers
ODNs	- Oligodeoxynucleotides
OS	- Overall survival
oxLDL	- Oxidised low-density lipoprotein
p53	- protein 53
PAMP	- Pathogen-associated molecular pattern
PARP	- Poly (ADP-ribose) polymerase
PBMC	- Peripheral blood mononuclear cells
PC12	- pheochromocytoma12
PCs	- Proliferation centres
PDK1	- Phosphatidylinositol-dependent protein kinase 1
PE	- Phycoerythrin
PE-Cy5	- Phycoerythrin cyanine5

PFA	- Paraformaldehyde
PFS	- Progression-free survival
PH	- Pleckstrin homology
PI3K	- Phosphatidylinositol 3-kinase
PI3,4,5-P3	- Phosphatidylinositol-3,4,5-triphosphate
PI-4,5-P2	- Phosphatidylinositol-4,5-bisphosphate
PKA	- Protein kinase A
PKB	- Protein kinase B
PKC	- Protein kinase C
PLC $\gamma$ 2	- Phospholipase C gamma 2
PLT	- Platelet count
PRK2	- Protein kinase C-related kinase 2
PTEN	- Phosphatase and tensin homolog
PTK	- Protein tyrosine kinase
PUMA	- p53 upregulated modulator of apoptosis
RACPK	- Related to A and C protein kinases
RAF	- Rapidly accelerated fibrosarcoma
Ras	- Retrovirus-associated DNA sequences
RBS	- Relative binding sites
R-CLL	- Responder chronic lymphocytic leukaemic cells
RDX	- Radixin
RP105	- Radio protective 105
RPMI1640	- Roswell Park Memorial Institute 1640
s $\beta$ 2m	- serum $\beta$ 2-microglobulin
sCD23	- soluble cluster of differentiation 23
sCD40L	- soluble cluster of differentiation 40 ligand
SCT	- Stem cell transplantation

SDF-1 $\alpha$	- Stromal-derived factor-1 $\alpha$
SGK	- Serum-and glucocorticoid-induced protein kinase
SH1	- Src homology type 1
slg	- surface/secreted Immunoglobulin
SLE	- Systemic lupus erythematosus
SLL	- Small lymphocytic lymphoma
Smac/DIABLO	- Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
ssRNA	- single-stranded ribonucleic acid
sTK	- serum thymidine kinase
STAT3	- Signal transducer and activator of transcription 3
Syk	- Spleen tyrosine kinase
TACI	- Transmembrane activator and cyclophilin ligand interactor
TAK1	- TGF-P activated protein kinase 1
TCL1	- T-cell leukaemia/lymphoma 1
T-CLL	- T-cell Chronic Lymphocytic Leukaemia
TFS	- Treatment-free survival
TIR	- Toll/IL-1 receptor
TIRAP	- Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TLR	- Toll-like receptor
TNF	- Tumour necrosis factor
TNFR1	- Type 1 TNF receptor
TP53	- Tumour protein 53
TRADD	- TNF receptor-associated death domain
TRAF6	- TNF receptor-associated factor 6
TRAIL	- TNF-related apoptosis-inducing ligand
TRAM	- TRIF-related adaptor molecule

TRIF	-Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing interferon- $\beta$
TTM	- Total tumour mass
U-CLL	- Chronic lymphocytic leukaemia with unmutated <i>IGVH</i> genes
VDJ	- Variable, diversity, joining genes
VCAM-1	- Vascular cell adhesion molecule-1
VLA-4	- Very late antigen 4
WBC	- White Blood cell
WM	- Waldenstrom macroglobulinaemia
Xid	- X-linked immunodeficiency
XIAP	- X-linked inhibitor of apoptosis protein
XLA	- X-linked agammaglobulinaemia
ZAP70	- Zeta-chain associated protein- 70

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**Chapter 1**  
**Literature review**

## 1.1 Epidemiology and aetiology of Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common form of leukaemia in Western countries mainly affecting elderly individuals. It presents with progressive accumulation of leukaemic B cells in the blood, bone marrow and lymphatic tissues (Dighiero, 2005). CLL is most frequently diagnosed among people aged 65-74 with the median age at diagnosis being 71 years. The highest frequency of deaths from CLL is found among people aged 75-84 with a median age at death of 80 ([www.seer.cancer.gov](http://www.seer.cancer.gov)). Between 7% and 20% of patients with CLL in European population are <55 years. These younger patients have adverse prognosis markers with Immunoglobulin Heavy Chain Variable (*IGHV*) unmutated status (U-CLL), expression of ZAP-70 and more likely to have early stage of disease (Parikh *et al.*, 2013). The time to treatment was shorter in patients  $\leq 55$  years than in those older than 55 years (4.0 years *versus* 5.2 years;  $P=0.001$ ). And also, CLL patients who were  $\leq 55$  years had longer survival than patients who were  $> 55$  years (12.5 years *versus* 9.5 years;  $P<0.0001$ ). However, patients  $\leq 55$  years had significantly shorter survival than the age- and sex-matched normal population (12.5 years *versus* not reached;  $P<0.0001$ ) (Parikh *et al.*, 2013). The disease rarely is diagnosed in people <30 years of age (Morrison and Nowakowski, 2016).

The incidence of the disease shows significant geographical differences. CLL is much less common in the Far East than the Western countries. It is possible to suggest that genetic and environmental factors may play a role in geographical differences since the incidence in the Asian population in the USA seems to be lower as well (Morrison and Nowakowski, 2016). When evaluating the average incidence of CLL in Taiwan during the period from 1986-1990 to 2001-2005, there was a drastic increase of CLL incidence in both males and females. However, the incidence remained stable for both sexes for Caucasian Americans, suggesting the involvement of lifestyle and environmental factors in the development of CLL in Taiwanese (Wu, 2010). Eastern CLL patients have been reported to progress earlier, to have a shorter time-to-first-treatment than western patients. They also required treatment twice as often as Caucasian patients in the same hospital suggesting that the outcome difference might be less associated with the availability of medical care or therapeutic medicines but due to the existence of ethnic differences in the disease development (Wu *et al.*, 2013).

Gender is also an important factor as the incidence of the disease is different among males and females. The incidence of CLL in the USA is 6.75 and 3.65 cases per 100,000 population per year in males and females respectively, resulting in a ratio of 1.7:1 between males and females (Morrison and Mayakovski, 2016). Catovsky *et al.*, (1989) were the first to report that women with CLL had a better overall survival (OS) independently of age and the stage of the disease. A statistically significant inferior survival for men compared to women was shown by Kristinsson *et al.*, (2009) in all age groups of CLL patients. Recent clinical trials demonstrated that women had a better overall response to treatment than men although they experienced greater gastro-intestinal toxicity (Catovsky *et al.*, 2014).

CLL cells express mostly CD19<sup>+</sup> B cell lineage phenotype hence the disease is also called B-cell chronic lymphocytic leukaemia (B-CLL). The prediagnosis aetiology of CLL remains unknown. A CLL patient with at least one affected relative is considered familial and approximately 5% of patients have family history of leukaemia (Goldin *et al.*, 2010). One in every ten patients with CLL has either a family history of CLL or another lymphoproliferative disorder and there is a 30-fold increase in the risk of CLL in first-degree relatives of patients (Greer *et al.*, 2009). Several studies have been done to analyse the variations between familial and sporadic CLL. Some studies involving multiplex families have shown familial CLL to have an earlier age at diagnosis compared to sporadic CLL. However opposite results have been demonstrated by some studies based on a large population of CLL patients, by not finding differences in age at diagnosis between two categories. A higher proportion of female cases is shown among familial compared to sporadic CLL (Goldin *et al.*, 2010).

Studies on familial CLL have not discovered genes to clearly relate with the pathophysiology of CLL although the characteristic expression markers of CLL have been found in family members of patients (Goldin *et al.*, 2010). Familial and sporadic CLL cases have shown similar shorter telomeres or CD38 positivity which is a prognostic marker of CLL (Ishibe *et al.*, 2002). The frequency of *IGVH* mutation was significantly higher in familial CLL (in 327 cases) compared to sporadic cases (in 724 cases) (Crowther-Swanepoel *et al.*, 2008). It is also found that B-lymphocyte stimulator levels (BlyS, also known as B-cell activating factor, BAFF) were higher in familial CLL cases than sporadic CLL cases or controls

(Novak *et al.*, 2006). Another small study has shown similar results with higher levels of BAFF in familial CLL than sporadic CLL cases, but the level of expression was significantly lower in the combined group of CLL patients than controls (Molica *et al.*, 2009).

Kristinsson *et al.*, (2008) have observed 3-fold increased risk of non-Hodgkin lymphoma (NHL) and CLL among first-degree relatives of lymphoplasmacytic lymphoma (LPL)/Waldenstrom macroglobulinemia (WM) patients. Although CLL shows substantial geographical variations with a 40-fold difference in rates (the highest among Caucasians in North America and Europe and very low in Asians), environmental risk factors associated with CLL have not been identified (Goldin *et al.*, 2011).

Most chronic lymphoproliferative disorders (CLPDs) are B cell origin. Cases of chronic lymphoid leukaemia of T-cell origin have been reported to account for less than 5% of the total number of CLPDs (Jandi, 1987). A subtle proportion of CLL cases belongs to T lineage, and it is known as T-cell Chronic Lymphocytic Leukaemia (T-CLL). T-CLL accounts for less than 1% of all CLPDs and consists of mature T-lymphocytes with immunophenotype of CD2<sup>+</sup>, CD3<sup>+</sup>, CD7<sup>+</sup>, and CD4<sup>+</sup>/CD8<sup>-ve</sup> (Hoyer *et al.*, 1995). The presence of lymphadenopathy, organomegaly and infiltrating monoclonal B cells with same CLL immunophenotyping, but lack of peripheral blood lymphocytosis is known as small lymphocytic lymphoma (SLL). SLL has been considered as an equivalent form of CLL, but currently, they are regarded as different manifestations of the same entity. The clinical outcome of CLL is extremely heterogeneous as some patients show very indolent disease while others have a more aggressive disease (Santos and O'Brien, 2012).

An increased risk for CLL among individuals with a history of pneumococcal pneumonia has been reported. Those studies have found that pneumonia remained marginally associated with increased risk of CLL for more than a 5-year period after pneumonia episode. The presence of an extended latency period over several years in those individuals could be explained by suggesting that undiagnosed CLL may cause pneumonia (Anderson *et al.*, 2009). Other respiratory tract infections such as sinusitis also increased the risk of CLL. B-cell immunity seems to be important in protection against *Streptococcus pneumoniae*

and *Haemophilus influenza* which involve with respiratory tract infections (Anderson *et al.*, 2009). Some evidence has shown a connection between skin infections like cellulitis and herpes zoster (caused by *Staphylococcus aureus* or *Streptococcus pyogenes* and *varicella zoster virus* respectively) and the increased occurrence of CLL (Anderson *et al.*, 2009). There are few explanations for these interactions. Common community acquired disease such as pneumonia may promote the transition from Monoclonal B-cell lymphocytosis (MBL) to CLL. Since CLL cells have V<sub>H</sub> gene mutations, the importance of antigenic stimulation in CLL can be suggested (Kienle *et al.*, 2006). Since encapsulated bacteria are responsible for causing respiratory tract infections it is also possible to suggest that these bacteria may play a role in the development of CLL. The disruption of immunity in CLL patients before they are diagnosed could be the cause for the frequent presence of community-acquired infections (Anderson *et al.*, 2009).

## **1.2 Diagnosis and clinical staging**

CLL is described as leukaemic, lymphocytic lymphoma by the World Health Organization and it is distinguishable from SLL by its leukaemic appearance. Several diagnostic parameters are used to confirm that the patient has CLL and no other lymphoproliferative disease such as hairy cell leukaemia, or leukaemic manifestations of mantle cell lymphoma, marginal zone lymphoma, splenic marginal zone lymphoma with circulating villous lymphocytes or follicular lymphoma (Hallek *et al.*, 2008).

### **1.2.1 Diagnosis in blood**

The presence of more than or equal to  $5 \times 10^9/L$  B lymphocytes (5000/ $\mu L$ ) in the peripheral blood for the duration of at least three months indicates diagnosis of CLL. The clonality of the circulating B lymphocytes can be confirmed by flow cytometry analysis. The leukaemic cells are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli with partially aggregated chromatin (Hallek *et al.*, 2008).

### **1.2.2 Diagnosis by Immunophenotyping**

CLL cells are characterised by the co-expression of T-cell antigen CD5 and B-cell surface antigens CD19, CD20 and CD23. The levels of expression of CD19 and CD20 are lower compared with normal B cells. Almasri *et al.*, (1992) demonstrated that low CD20 expression is a unique feature of CLL/SLL cells.

Cells showing intense expression of CD20 antigens in a small number of CLL/SLL cases could be due to the presence of biologically or clinically different disorder. The absence of FMC7 and CD22 is also used as a routine diagnostic criterion in patients with CLL (Ibrahim, 2001).

### 1.2.3 Clinical staging

There are two staging systems for CLL depending on clinical characteristics - Rai staging and Binet staging.

**Table 1.1: Rai staging** (Rai *et al.*, 1975).

<i>Clinical stage</i>	<i>Clinical characteristics</i>
Low risk (stage 0)	Lymphocytosis (increase in the proportion of lymphocytes) in blood and/or bone marrow. Absolute lymphocytes count is 15,000/mm <sup>3</sup> or more in blood, with 40% or more lymphocytes in the marrow.
Intermediate risk (stage I or stage II)	Lymphocytosis with enlarged lymph nodes, enlarged spleen (splenomegaly) or liver (hepatomegaly) or both (stage I) and nodes may or may not be enlarged (stage II).
High risk (stage III)	Lymphocytosis with anaemia, haemoglobin (Hb) less than 11 g/100 ml or haematocrit less than 33%. Nodes, spleen or liver may or may not be enlarged.
High risk (stage IV)	Lymphocytosis with thrombocytopenia (platelet count less than 100,000/mm <sup>3</sup> ).

When comparing the survival of patients in different stages according to Rai staging system, Rai *et al.*, (1975) reported that out of 125 patients analysed, all stage IV patients died within 3.5 years from diagnosis and only one patient in stage II survived for five years. The overall survival (OS) of patients in stages II, I and 0 was longer than stage IV and some patients in stage 0 could survive for 23,24 and 32 years after diagnosis.

Binet *et al.*, (1981) proposed a new staging system by analysing two series of patients (a total of 295 patients). They have used a survival method which enabled to identify factors affecting survival. The new staging system has categorised CLL patients into three groups as low-, intermediate- and high-risk patients. The new system was simpler and required only clinical examinations and routing haematological analysis.

**Table 1.2: Binet staging** (Binet *et al.*, 1981).

<i>Clinical stage</i>	<i>Clinical characteristics</i>
Stage A	No anaemia, no thrombopenia, less than three involved areas*. Hb 100 g/L (10 g/dL) or less, platelets 100x10 <sup>9</sup> /L or more and any two of the organomegalies (splenomegaly or hepatomegaly).
Stage B	No anaemia, no thrombopenia, three or more involved areas* Hb 100 g/L (10 g/dL) or less and platelets 100x10 <sup>9</sup> /L or more.
Stage C	Anaemia (Hb less than 10 g/dL) and/or thrombopenia (platelets less than 100,000/mm <sup>3</sup> ), irrespective of organomegaly.

\*The following areas of involvements are considered for staging;

1. Head and neck, including the Waldeyer ring (this counts as one area, even if more than one group of nodes is enlarged).

2. Axillae (involvement of both axillae counts as one area).
3. Groins, including superficial femorals (involvement of both groins counts as one area).
4. Palpable spleen.
5. Palpable liver (clinically enlarged).

The median survival for the patients at Binet stage A or Rai stage 0 (low risk) is over 10 years, 5-7 years for patients in Binet stage B or Rai stage I-II (intermediate risk) and 2-3.5 years for Binet stage C or Rai stage III-IV (high risk) (Cramer and Hallek, 2010).

### **1.3 Prognostic markers**

CLL patients show clinical heterogeneity having some patients die soon after diagnosis despite aggressive treatments whereas the other group can survive for a prolonged period without requiring definitive treatment. Rai and Binet staging systems above mentioned, provide valuable information on survival of patients; however, they are unable to predict which patients will experience an indolent or an aggressive form of the disease especially in early stage or intermediate risks patients. Therefore, the use of several prognostic factors which enable to identify favourable *versus* poor prognoses is necessary to manage patients clinically. These prognostic markers include Immunoglobulin (Ig) heavy (H) chain gene (*IGHV*) mutational status, expression of CD38, lymphocyte doubling time, circulating levels of  $\beta_2$  microglobulin and soluble CD23, serum thymidine kinase levels, bone marrow history, expression of ZAP-70 and cytogenetic abnormalities (Damle ,1999).

#### **1.3.1 Immunoglobulin (Ig) heavy (H) chain gene (*IGHV*) mutational status**

B cell Immunoglobulin genes undergo somatic hypermutations during germinal center reaction to diversify the antibody repertoire resulting in improved affinity of an antibody towards an antigen. Although the early studies suggested that CLL cells did not show somatic mutations in complementarity-determining regions of the antibody genes, Cai *et al.*, (1992) were the first to show that the  $V_H$  and  $V_C$  genes rearranged and/or expressed in CLL cells have few and random mutations. They described unmutated CLL cases being “preantigen” and the mutated CLL being “postantigen”. Hamblin *et al.*, (2002) demonstrated that out of total number



of 38 patients 45.2% showed  $\geq 98\%$  sequence homology with the nearest germline  $V_H$  genes (considered “unmutated, U-CLL”) whereas 54.8% showed 2% somatic mutations (considered “mutated, M-CLL”) arguing that U-CLL cells arise from pre-germinal centre naïve B cells whilst M-CLL cells arise from post-germinal centre memory B cells. Patients with U-CLL showed significantly worse survival irrespective of the stage as well they showed the progressive disease, advanced stage, atypical morphology and trisomy 12. CLL patients with somatic mutations showed abnormalities at 13q14 and it has been previously shown that patients with trisomy 12 had poor clinical outcomes than patients having 13q14 abnormalities (Juliusson, 1990).

The observation that females have a more favorable clinical outcomes than males was further confirmed by Damle *et al.*, (2004) who demonstrated that about 10% of women showed unmutated *IGVH* genes and  $\geq 30\%$  CD38 expression and poor outcomes and about 50% had mutated status and  $<30\%$  CD38 expression with good outcomes. They also demonstrated that those CLL cells expressing  $\geq 30\%$  CD38 and with unmutated status display surface markers which are characteristic of B cells that have not entered a germinal centre (GC).

There are approximately 51  $V_H$  genes in the repertoire available for recombination to generate functional  $VH-DH-JH$  transcriptional units. Normal B cells are stimulated by antigens and enter the lymphoid follicle after genetic recombination. Point mutations in the variable region genes can cause somatic hypermutations arising the differentiation process. Selection of  $V$  gene sequences in the presence of limited antigens to increase affinity can lead to an increase in the replacement amino acids in complementary-determining regions (CDRs). Fais *et al.*, (1998) indicated that the  $V_H$  genes of approximately 50% of the IgM+ B-CLL cells and approximately 75% of the non-IgM+ B-CLL cells can exhibit somatic mutations. Somatic mutations CLL cases may have arisen from B cells that have undergone mutational events but without losing expression of IgD or not having isotype-switch before entering to the blood (Oscier, 1997).

Unmutated  $V_H$  genes were significantly associated with *V1-69* and *D3-3* usage, with atypical morphology; isolated trisomy 12, advanced stage and progressive disease. However, the reason for this biased association is not known (Hamblin, 1999). Similar results were published earlier by Fais *et al.*, (1998) showing that

the use of  $V_H$  gene among CLL patients is not random. The expression of  $V_H$  and  $V_L$  genes among  $CD5^+$  normal B cells and CLL cells was different. They found that  $V_H$  4-34, 3-07 and 1-69 were the most commonly expressed  $V_H$  genes in CLL cells. They also found that mutation frequencies vary depending on the  $V_H$  family expressed by the CLL cells. The presence of somatic mutations in approximately 50% of  $IgM^+$  CLL cells and approximately 75% of non- $IgM^+$  cells suggests that these cells have been exposed to antigens previously and they are “experienced” or “memory”  $CD5^+$  B cells. CLL biased differential expression of immunoglobulin genes among M-CLL and U-CLL cells was also reported by Dal-Bo in 2011. He observed that certain immunoglobulin genes such as *IGHV1-69*, *IGKV1-33/ID-33*, and *IGLV3-21* are more commonly found in nonmutated rearrangements whereas other genes such as *IGHV4-34*, *IGKV2-30* and *IGLV2-8* are more frequent in the mutated rearrangement.

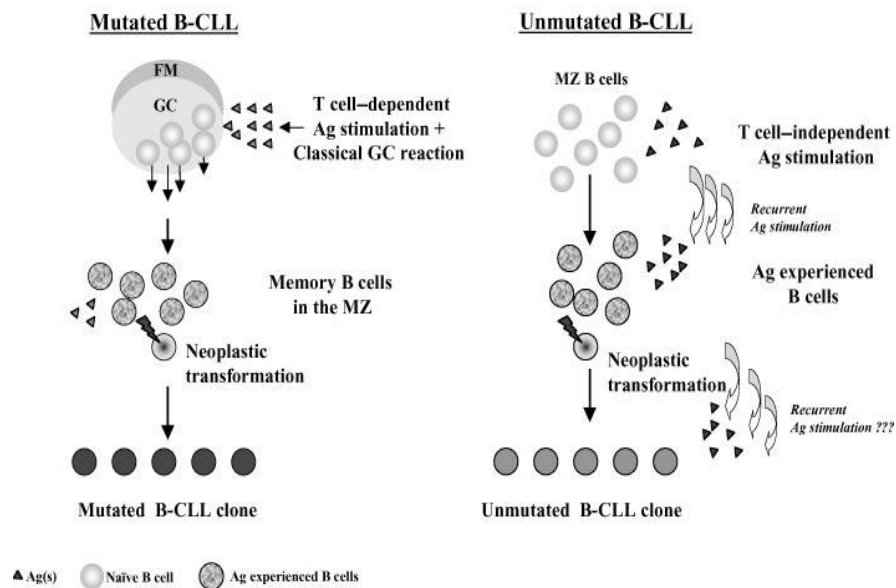
The presence of homologous (“stereotyped”) complementarity-determining regions 3 (CDR3) sequences in both mutated and unmutated cases of CLL patients has been reported by several groups. Stamatopoulos *et al.*, (2007) described 48 different subsets of *IGHV*-D-J sequences with homologous heavy-chain CDR3 (HCDR3) among 916 CLL patients of Mediterranean origin and they were named ‘subset #1’ to ‘subset #48’. By analysing the association between stereotyped BCR and clinical/phenotypic features or outcome for selected subsets of CLL patients it was suggested that specific antigen binding sites might be important in determining clinical presentation and prognosis. Oxidised low-density lipoproteins (oxLDL) may be formed by the conjugation of metabolites of lipid peroxidation and can be found on apoptotic cells and microbial surfaces. It was recently reported that mAbs from CLL cases of subset # 1 and subset # 32 could recognise malondialdehyde (MDA)-modified oxLDL epitopes. Non-stereotypical CLL mAbs from *IGHV3-21UM*, *IGHV1-2UM* and *IGHV3-30M* could recognise these epitopes suggesting most CLL mAbs have the capacity to bind different epitopes, all of which may be derived in oxidation processes (Myhrinder,2008; Rosen,2010).

Most U-CLL cells express low-affinity BCRs that are polyreactive to self- and exo-antigens such as DNA, lipopolysaccharide (LPS), insulin, apoptotic cells, oxLDL and the cytoskeletal antigens myosin and vimentin whereas M-CLL BCRs are

generally not polyreactive (Herve,2005; Catera,2008; Chu,2008; Binder,2010). Beta-(1,6)-glucan is a polysaccharide found in the cell wall of certain yeast, filamentous fungi, Basidiomycetes and bacteria. It was identified that a subset defined as V3-7Sh (a subset of patients expressing mutated *IGHV3-7* and semi-identical *IGKV2-24* genes) could recognise  $\beta$ -(1,6)-glucan. Mutated V3-7Sh accounted for 0.3% of all CLL cases and responded to the natural antigenic determinant shared by some pathogens such as commensal yeast species of *Candida*, *Trichosporon*, *Malassezia* and *Saccharomyces*, filamentous fungus *Aspergillus*, spores and conidia of *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Fonsecaea pedrosoi*, and *Rhizopus oryzae* (Hoogeboom,2013).

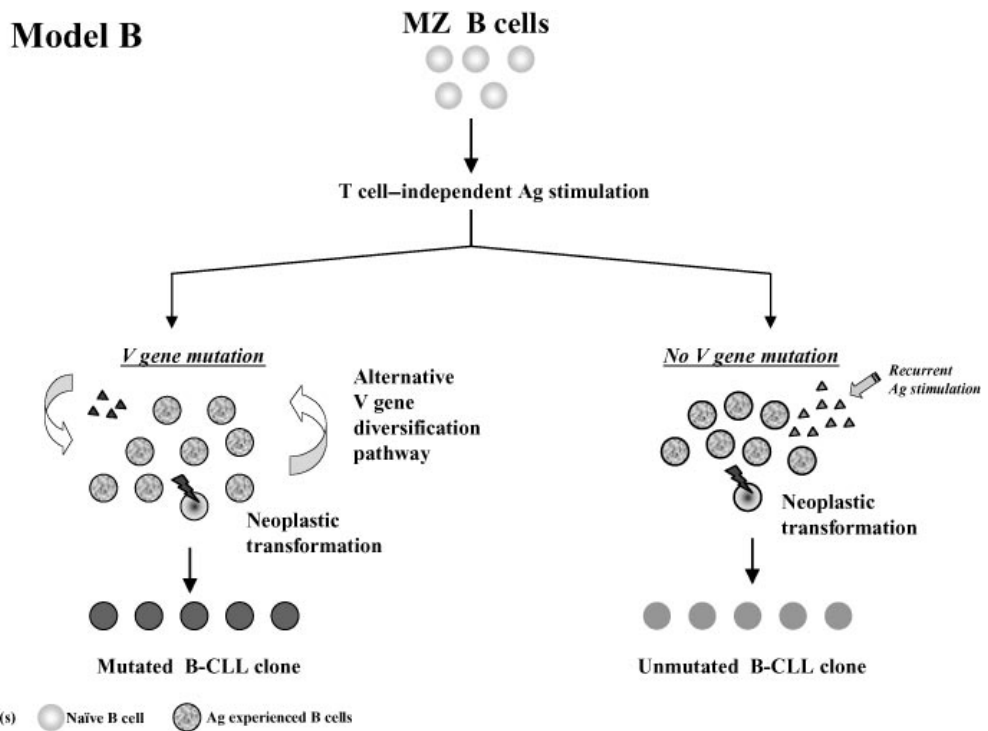
Monoclonal antibodies found in CLL patients were found to be similar to natural IgM Abs with low affinity to microbes, lacking somatic hypermutation and with autoreactivity/polyreactivity. Auto- and polyreactivity is conversely associated with the mutational load of immunoglobulin genes in BCR. It was observed that by reversing the amino acid structure in monoreactive M-CLL Ab into germline genes, the monoreactivity can be converted into polyreactivity. This suggests that most CLL B cells derive from normal B lymphocytes with poly- or autoreactive BCRs (Herve, 2005, Hoehn, 2010). sIgs of CLL showed reactivity against certain Gram-positive and Gram-negative bacteria such as *Streptococcus pyogenes*, *Enterococcus faecium*, *Enterococcus faecalis* and *Enterobacter cloacae*. *IGHV1-69* UM mAbs particularly had higher binding capability for the antigens from those bacteria. Certain viral infections were also associated with CLL and associations were observed between subset # *IGHV-34* with persistence infections with Epstein-Barr virus (EBV) and cytomegalovirus (CMV) and some CLL cases with hepatitis C virus (HCV) (Gunnarsson,2011; Kostareli,2012). Moreover, respiratory tract infections (Anderson, 2009), sinusitis, pneumonia (Landgren, 2007), skin infections such as cellulitis and herpes zoster (Landgren, 2007; Anderson, 2009) have shown that there is a possibility of having an association of these infections with CLL, suggesting the importance of antigenic stimulation for  $V_H$  gene mutation.

## Model A



**Figure 1.1: Model to explain the derivation of CLL cells via T cell-dependent and T cell-independent manner.** The mutated CLL cell derives from a B cell stimulated by T-cell-dependent antigens which activate the cell to undergo classical germinal centre (GC) reactions. The U-CLL cell derives from a marginal zone (MZ) B cell by a T cell-independent process (Chiorazzi *et al.*, 2003).

The phenotypic differences observed between the B-CLL cells and their putative progenitors is a significant factor to explain the model shown in figure 1.1. If these progenitors are the IgM<sup>+</sup>, IgD<sub>low</sub> MZ B cells, the process of activation to which the B-CLL cells are subjected, either by self antigen stimulation and/or leukemic transformation, may induce the appearance of new surface markers. Activation of resting MZ B cells induces the expression of a number of activation markers including CD5, CD23, and CD38. Considering this, the phenotypic profile of the two subgroups suggests that the mutated and unmutated B-CLL cases are frozen at different degrees of activation (Damle *et al.*, 2002). It becomes more challenging to explain the phenotypic differences if one assumes that mutated and unmutated B-CLL cases are generated by different progenitors and that the mutated cases derive from memory B cells, especially considering that memory B cells homing to the MZ are activated cells.



**Figure 1.2: Model to explain the derivation of CLL via T cell-independent pathway.** IgM<sup>+</sup>IgD<sup>low</sup> subset of MZ B cells is stimulated by autoantigens in T cell-independent manner. Some of these cells undergo somatic hypermutations via V gene differentiation pathway and/or isotype class switching and others do not develop mutations to become U-CLL clones (Chiorazzi *et al.*, 2003).

However, these two models are not mutually exclusive, and therefore the mutated B-CLL cells could follow either of the two differentiation pathways explained. Indeed, the segregation of B-CLL cases into two subgroups defined by the presence or absence of V gene mutations may be too simplistic, and additional subgroups within these two larger groups may exist. Because the MZ contains three B cell subsets defined by the surface expression of IgD (memory B cells and the two subsets of MZ B cells), further distinction at the gene expression level may reveal more subsets (Chirazzi *et al.*, 2003)

It was demonstrated that Binet stage A CLL patients more frequently had V<sub>H</sub> mutated status (91 of 112 at the less than 97% and 106 of 132 at the less than 98% V<sub>H</sub> homology cut off) with good clinical outcome whereas the patients diagnosed at the same Binet stage A, but with unmutated V<sub>H</sub> status had inferior outcome (Krober, 2002).

### 1.3.2 Expression of surface CD38

CD38 (T10 antigen) was initially characterised as a T cell differentiation antigen expressed on T cells and in different haematopoietic and non-haematopoietic tissues such as CD34<sup>+</sup> progenitor cells, germinal centre B cells, activated mature lymphocytes, plasma cells and myeloid precursors (Ibrahim, 2001). It is a type 2 trans-membrane glycoprotein type II membrane which is similar to the type I class in that they span the membrane only once, but they have their amino terminus on the cytoplasmic side of the cell and the carboxy terminus on the exterior and acts as a complex ecto-enzyme with adenosine diphosphate-ribosyl cyclase activity to catalyse the conversion of nicotinamide adenine dinucleotide (NAD) into cyclic Adenosine diphosphate ribose (cADPR). This causes an inositol 1,4,5-trisphosphate (IP<sub>3</sub>) independent mobilisation of intracellular Ca<sup>2+</sup>. Exposure of murine B cells to anti-CD38 mAb caused IP<sub>3</sub>-independent Ca<sup>2+</sup> reflux further confirming the involvement of CD38 in lymphocyte activation and proliferation (Zupo, 1994). CD38 also has cyclic adenosine diphosphate-ribose hydrolase activities (Howard, 1993).

CD38 is expressed at different stages of B cell development especially when cell-to-cell interactions are significant (Hamblin *et al.*, 2002). Immature B cells, germinal centre (GC) B cells and plasma cells in humans have a high level of expression of CD38 whereas mature resting B cells show negativity for CD38 expression or very weak level of positivity. Since they are highly expressed in GC B cells, it can be suggested that CD38 may be involved with the selection process of GC B cells (Zupo, 1994). It has been demonstrated that anti-CD38 in GC B cells could prevent apoptosis and increase the expression of B cell lymphoma 2 (*bcl-2*) proto-oncogene expression. Deaglio *et al.*, (2010) studied the signalling mechanism mediated by CD38 in human B cells and found that it was driven via three stages. Firstly, CD38 interacts with CD31, a non-substrate ligand surrogated by ligation with agonistic monoclonal antibodies, then localisation of the receptor-ligand complex into membrane lipid rafts and finally the generation of functional associations with other proteins CD19/CD81 complex.

CD38 is expressed heterogeneously among CLL patients. It is highly expressed in lymph nodes compared with bone marrow or peripheral blood. Ozren (2004) demonstrated that there was no significant difference in CD38 expression between peripheral blood and bone marrow. Another study by Khoudoleeva

(2011) showed that expression of CD38 on CLL cells varied from almost negligible to 100%. Depending on the level of CD38 expression, they observed different subgroups of CLL patients as follows: patients with 30% or more B cells with CD38 are positive, 30%-80% intermediate positive, more than 80% high positive and those with less than 30% CD38 expressed B cells are negative.

Damle *et al.*, (2007) demonstrated that U-CLL cells were characterised by high levels of CD38 expression (30% or more cells in a clone), and this was associated with the more aggressive disease with a worse prognosis. CD38 expression on M-CLL cells was low and this was associated with indolent disease and with significantly longer survival times with good prognosis.

There is an elevated level of ZAP-70 expression in CD38<sup>+</sup> CLL clones compared to CD38<sup>neg</sup> CLL clones and this may contribute to the increased ability of BCR mediated signal transduction which is associated with ZAP-70 (Damle, 2007). They also have shown a correlation between CD38 expression and Ki-67, a cellular marker of proliferation in CLL cells suggesting CD38<sup>+</sup> CLL clones enter the cell cycle more frequently than CD38<sup>neg</sup> CLL clones. Khoudoleeva (2011) has demonstrated that although there is a significant correlation between CD38 expression and Ki-67 expression in the bone marrow, the relationship between the expression of CD38 and Ki-67 in lymph nodes and the spleen was more complicated. Combined analysis of CD38 and ZAP-70 enabled categorisation of CLL patients into three subgroups: CD38<sup>neg</sup>/ZAP-70<sup>neg</sup> with good prognosis, patients with discordant CD38/ZAP-70 (CD38<sup>+</sup>/ZAP-70<sup>neg</sup> or CD38<sup>neg</sup>/ZAP-70<sup>+</sup>) results in an intermediate prognosis and CD38<sup>+</sup>/ZAP-70<sup>+</sup> results in poor prognosis. When concordant CD38<sup>+</sup>/ZAP-70<sup>+</sup> and discordant patients were compared with concordant CD38<sup>neg</sup>/ZAP-70<sup>neg</sup> patients, the presence of other negative prognostic markers such as 17p13 deletions, 11q22-23 deletions and trisomy 12 was significantly higher (Schroers, 2005). Genetic analysis by Damle *et al.*, (2004) showed that CD38/CD31 interaction could lead to upregulation of genes controlling leukocyte transendothelial migration, actin cytoskeleton and focal adhesion and downregulation of genes controlling interactions with extracellular matrix and coding for cell adhesion molecules. This suggests that the long-term interaction of CD38/CD31 modulate CLL cells competence to grow and progress by responding to microenvironmental signals and conditions.

Stromal derived factor-1 $\alpha$  (SDF-1 $\alpha$ )/CXCL12, which is mainly produced by stromal and nurse-like cells (NLCs), is effective in recruiting circulating CLL lymphocytes toward secondary lymphoid organs via the specific CXCR4 receptor. CD38<sup>+</sup>/ZAP-70<sup>+</sup> patients are characterized by enhanced migration toward SDF-1 $\alpha$ . Suggesting the association between CD38 and ZAP-70. In ZAP-70<sup>+</sup>/CD38<sup>+</sup> CLL patients, activation of CD38 results in the phosphorylation of ZAP-70 suggesting CD38 mediated signalling depends on the presence of ZAP-70. This was further confirmed showing CD38<sup>+</sup>/ZAP-70<sup>neg</sup> CLL patients were consistently unable to signal via CD38 (Deaglio, 2007). Correlation between the expression of CD38 and the concentration of  $\beta_2$ -microglobulin or sCD23 in a distinct subset of B-CLL has been reported by Poeta (2001). There are some controversial data on CD38 expression and apoptosis showing CD38<sup>+</sup> CLL cells undergo apoptosis quickly after treatments with anti-immunoglobulin antibodies whilst CD38<sup>neg</sup> cells are resistant to these antibodies suggesting receptor stimulus can lead to either apoptosis or survival (Zupo, 1996). Previous studies also have demonstrated similar results showing that expression of CD38, enabled to identify different CLL clones that show either increased or decreased chance of survival by transducing signals via CD38 (Zupo, 1994).

CLL cells of some patients are homogeneously negative or positive for CD38 whereas, in a proportion of patients, leukaemia cells show concomitant presence of CD38 leading to bimodal distribution (Scielzo *et al.*, 2007). In CLL, expression of CD38 above the threshold level, expression of protein kinase ZAP-70 and absence of mutations in the *IGVH* gene are considered as reliable negative prognostic markers (Deaglio, 2010). Several studies have shown a correlation between CD38 expression and poor clinical outcomes of CLL patients. One study demonstrated that CD38<sup>+</sup> patients required more frequent treatments than CD38<sup>neg</sup> patients (Domingo-Domenech, 2003). A higher percentage of CD38 correlated with lack of complete responses to six courses of fludarabine monophosphate as first line approach (Del Poeta, 2001). However, as a surrogate marker, the association between CD38 and *IGVH* mutational status is not considered as absolute (Cramer and Hallek, 2010). Oscier *et al.*, (2002) suggested that CD38 expression can be used to give additional information in patients with an established mutational status but cannot be used as an independent marker.



### 1.3.3 Expression of intracellular ZAP-70

ZAP-70 (zeta-associated protein-70) is a receptor-associated protein tyrosine kinase expressed by T lymphocytes, natural killer (NK) cells, activated tonsillar and splenic B cells and U-CLL cells. The role of ZAP-70 in CLL cells is related to the enhancement of BCR-mediated signalling, promoting survival and/or proliferation. Expression of ZAP-70 is a negative prognostic marker for CLL (Catherwood *et al.*, 2006) as ZAP-70 is an essential signalling molecule in T lymphocytes and natural killer cells (Chen, 2002;2005). CD38 ligation resulted in phosphorylation of ZAP-70 in Jurkat T cells showing the importance of ZAP-70 in CD38 mediated signalling pathway (Zubiaur, 1997). ZAP-70 is a member of the Syk-ZAP-70 protein kinase family and its function in normal T cells and natural killer cells is to play a critical role in development and differentiation. ZAP-70 is not expressed in normal B lymphocytes (Hus,2005). It was reported that the expression of ZAP-70 is superior in the prediction of the time to first treatment when compared with the expression of CD38 or *IGVH* mutational status. Also, ZAP70 expression was 5.54-fold higher in U-CLL cells compared to M-CLL (Cramer and Hallek, 2010). There was a strong correlation between the presence of *IGVH* mutations and the percentage of leukaemic cells expressing ZAP-70. Moreover, there was 100 percent probability (95 percent confidence interval, 89 to 100) of the absence of somatic hypermutations in the presence of more than 20 percent ZAP-70 positive CLL cells and 87.5 percent probability of *IGVH* mutations in the presence of a low percentage of ZAP-70 positive cells. The reason for this correlation is unknown. The level of expression of ZAP-70 did not change over time and the presence of ZAP-70 was associated with rapid progression and poor survival. Therefore, analysis of expression of ZAP-70 could be used as a simple and reliable surrogate marker to identify *IGVH* mutations (Crespo, 2003; Schroers, 2005). Studies using DNA microarrays have shown a way to distinguish U-CLL from M-CLL by the differential expression of a small number of genes including the gene which encodes the ZAP-70 protein (Rosenwald, 2001; Wiestner, 2003).

Additionally, Sabbatini (2007) assessed the role of ZAP-70 on bone marrow biopsy specimens of CLL patients using immunohistochemistry and confirmed the potential for ZAP-70 as a surrogate marker for mutational status. This study

also demonstrated a small subset of discordant cases with unmutated status and low expression of ZAP-70 had more favourable clinical outcomes. Hus (2006) demonstrated that a significant difference between ZAP-70 positive and ZAP-70 negative patients in terms of CLL prognostic parameters such as stage of the disease, WBC count, lymphocytosis, platelet count (PLT), haemoglobin level and lactate dehydrogenate (LDH) serum level compared with CD38 positive and negative patients (WBC count, lymphocytosis and LDH activity). Another study performed by Krober (2006) found a correlation between the presence of prognostic genetic features and discordant ZAP-70 and VH mutational status, moreover, 25% of cases studied had discordance ZAP-70 expression and VH mutation status. Subgroups with V3-21 usage and 17p or 11q deletion had a higher proportion of discordant cases (39%). ZAP-70 positive/VH mutated cases had a higher usage of V3-21 (89%). The association between ZAP-70 expression and VH mutational status was stronger in cases without V3-21 usage, 17p deletion, or 11q deletion (84%) and the distribution of ZAP-70 positive/VH mutated vs. ZAP-70 negative/VH unmutated cases were balanced (55%, vs. 45%). It is found that the percentage of ZAP-70 positive B cells in the peripheral blood of CLL patients ranged from 0% to 85.4% and the level of expression was significantly higher in B lymphocytes taken from lymph nodes and spleen of CLL patients (Khoudoleeva, 2011).

#### **1.3.4 CLL cell kinetics**

The time taken to double the absolute lymphocyte count is known as lymphocyte doubling time (LDT) and it reflects the disease activity in CLL. An LDT of less than 12 months is associated with an aggressive course, decreased progression-free survival (PFS) and OS. If the lymphocyte count increases more than 50% in 2 months, or the LDT is less than six months then the patient is considered to have a status of active disease and treatments are needed as per the guidelines published by International Workshop on CLL (IWCLL) (Cramer and Hallek, 2010).

Some studies have shown that there is a correlation between *in vivo* LDT and telomere length and telomerase activity. Patients with shorter LDTs (less than 12 months or more) had significantly higher telomerase activity than patients who had longer LDTs (Damle, 2002). CLL kinetics has been measured using deuterated water to see the incorporation of <sup>2</sup>H into the deoxyribose moiety of

DNA of newly divided CLL cells. This method has demonstrated that the leukaemic cells of each patient had definable and substantial lymphocyte birth rates varying from 0.1% to greater than 1.0% of the entire clone per day. The patients who had lymphocyte birth rates higher than 0.35% per day showed more active disease and developed progressive disease than those who had lower lymphocyte birth rates (Messmer,2005). The doubling time (DT) of total tumour mass (TTM) which is measured using the square root of the number of peripheral blood lymphocytes per nl, the diameter of the largest palpable lymph node in centimetres, and the enlargement of the spleen below left costal margin in centimetres (Jaksik and Vitake, 1981) in untreated patients was found to be associated with the type of response to treatment and prognosis (Jaksic, 1981). Molica and Alberti (1987) demonstrated that the trends in LDT could be used as a proper parameter to predict the progression of the disease. Their findings suggested that higher the LDT, longer the survival. Also, many of patients who had higher LDT did not have treatments indicating slower progression of the disease.

### **1.3.5 Serum markers**

Serum  $\beta_2$ -microglobulin (B2M), serum thymidine kinases (sTKs) and soluble CD23 (sCD23) are the most important serum prognostic markers in CLL. s $\beta_2$ m is an extracellular protein which is non-covalently bound to the  $\beta$ -chain of the class I MHC (major histocompatibility complex). Increased level of s $\beta_2$ m is linked with adverse prognostic features such as the expression of CD38 and ZAP-70 (Schroers *et al.*, 2005). sTK involves with salvage pathway for DNA synthesis and is found only in dividing cells and its elevated levels are associated with rapid disease progression.

#### **sCD23 (Soluble CD23)**

CD23 is expressed by mature B cells, antigen presenting cells and platelets. It is a 45KDa transmembrane protein and a low-affinity IgE receptor. CD23 is proteolysed into soluble CD23 (sCD23) molecules of variable molecular weight. Elevated levels of its soluble form are characteristic of CLL exclusively and are associated with reduced survival (Cramer and Hallek, 2010). Several biological activities of CD23 and its soluble factor have been reported. This includes

regulation of normal B and T cell proliferation (Swendeman,1987; Armitage,1989), control of synthesis of human IgE (Sarfati,1988), proliferation of myeloid precursors (Mossalayi,1990) and the rescue of germinal centre B cells from apoptosis (Liu,1991). Synergistic effect of CD23 and IL-1 had been found on the maturation of prothymocytes (Mossalayi, 1990). CD21 which is the receptor for the EBV is the ligand for CD23 and CD21/CD23 interaction involves in cell adhesion of human B-lymphocytes (Aubry, 1992). The expression patterns of CD23 in CLL have been studied using immunohistochemistry and found that CD23 is more strongly expressed in the proliferating cells from the splenic white pulp and lymph nodes than in the small CLL cells outside the proliferating centres (Lampert, 1999). Lopez-Matas *et al.*, (2000) demonstrated that compared to normal B lymphocytes, CLL cells have low expression of CD21 and increased levels of CD23 facilitates normal B-cells entering the cell cycle explained by the higher expression of CD23 in CLL (Fournier,1994). Moreover, CD23/CD21 interaction plays a major role in B cell activation and proto-oncogenes expression such as *c-fos* leading to cell proliferation and tumour mass formation (Luxembourg,1994).

Reinisch (1994) demonstrated that there is a significant reciprocal relationship between serum sCD23 and LDT in CLL. Serum deoxythymidine kinase activity and TTM score but not absolute lymphocyte count were positively correlated with sCD23. The cross-linking of the CD23 molecule delivered a negative growth signal to the leukaemic B cells and the CD23 gene was abnormally regulated in CLL (Fournier,1992). The doubling time of sCD23 level has been to be prognostically significant for OS of the entire group of patients studied and for disease progression among stage A patients. The doubling of sCD23 level increased the risk of death of the whole population of CLL patients analysed by 3.2 times (Sarfati, 1996).

### ***Serum thymidine kinase (sTK)***

Thymidine kinase is an enzyme involve with pyrimidine metabolic pathway and has a significant role in the complementary alternative salvage pathway of DNA synthesis and conversion of deoxythymidine into deoxythymidine monophosphate is catalysed by this enzyme (Hallek, 1992; Hannigan, 1993). There are two different isoenzymes of TK in mammalian cells which are cytosolic

TK (TK1) accounting for 95% of serum TK activity and mitochondrial TK (TK2). TK1 levels vary throughout the cell cycle and the highest activity is seen at the G1/S boundary (Wintersberger, 1992; Johansson, 1997). Elevated TK activity was found in patients with unmutated IGHV status, poor prognostic chromosomal abnormalities such as trisomy 12, del11q23, del17p13,  $\geq 15\%$  CD38 expression and adverse clinical outcomes with aggressive and progressive disease. The level of TK was elevated in ZAP-70 positive patients compared to ZAP-70 negative patients and significantly higher levels of TK were found in different  $V_H$  gene usage subgroups of patients with  $V_{H4-39}$ ,  $V_{H1-69}$  and  $V_{H3-2}$  than other gene usage subgroups (Matthews, 2006). Furthermore, Levy *et al.*, (1999) showed that both p27<sup>kip1</sup>, the cyclin-dependent kinase inhibitor and s-TK levels were strongly associated with either LDT or disease progression. Magnac *et al.*, (2003) demonstrated that s-TK indicates the proliferative potential of the malignant clone and a predictor of mutational status. sTK levels correlated with the proliferative activity of CLL cells and related with number of dividing tumour cells (Hallek, 1999).

### ***Serum $\beta_2$ -microglobulin***

$\beta_2$ -microglobulin (B2M) is a polypeptide with 11.6 kDa which stabilises the tertiary structure of the MHC-I molecules present on cell surface of nucleated cells (Bjorkman and Burmeister, 1994). The use of B2M as a prognostic marker is very useful over other prognostic markers due to its cheapness, easiness to implement and availability in many non-specialised laboratories. B2M levels are influenced by patient's renal function (Filler *et al.*, 2002). As the B2M level approximately correlates with tumour mass, it can be suggested that the levels could vary with the progression of the disease and relatively low level in the early stage when the tumour mass is low. Gentile *et al.*, (2009) demonstrated that B2M levels and CD38 expression were correlated in 128/219 CLL cases studied by having both B2M positivity and  $CD38 \geq 30\%$ . Furthermore, B2M level was correlated with *IGHV* mutational status in 125/195 CLL cases (64.1%) by having B2M<sup>+</sup>/*IGHV* unmutated status and 29/62 (46.8%) B2M<sup>-</sup>/*IGHV* mutated cases. B2M negative cases had a significantly higher treatment-free survival (TFS) compared to B2M positive cases.

There are possible explanations for the mechanism of increased level of B2M in CLL cases. Lymphocytes continuously shed B2M and hence with the progressive expansion of leukaemic clone, the level of B2M also increases showing a close correlation between CLL stage and B2M level. Contrast to that; there was a substantial population of CLL patients in Binet stage A with high levels of B2M suggesting those cells are more activated *in vivo* and shed more B2M (Gentile *et al.*, 2009). Molica *et al.*, (1999) studied the combining effect of sCD23 and B2M to verify whether these two markers have an additive effect. They integrated the levels of B2M and sCD23 into Binet staging system and stratified patients belonging to the Binet low and intermediate risk categories as *low risk group*, stage A (low B2M <3mg/dl, low sCD23 <2300 U/ml or one increased), *intermediate risk group*, stage A (high B2M >3mg/dl/high sCD23 >2300U/ml) stage B (B2M/sCD23 both low or one increased), high risk group, stage B (B2M/sCD23 both increased) and Stage C. However B2M levels also rise with worsening renal dysfunction, leading some investigators to suggest a measure of B2M adjusted for glomerular filtration rate (GFR-adjusted B2M) (Delgado *et al.*, 2009).

### 1.3.6 Chromosomal aberrations

Interphase fluorescence *in situ* hybridization (iFISH) analysis shows that CLL cells from 80% of patients have at least one cytogenetic abnormality. These abnormalities include deletion of the long arms of chromosome 13 (del[13q]), chromosome 11 (del[11q]) and chromosome 6 (del[6q]), deletion of the short arm of chromosome 17 (del[17p]), trisomy of the long arm of chromosome 3 (trisomy 3q), chromosome 8 (trisomy 8q), chromosome 12 (trisomy 12q) and translocation 14q32. The worst prognosis is associated with del(17p) followed by del(11q) which is related to removal of *TP53* locus and Ataxia telangiectasia mutated (ATM) gene respectively (Cramer and Hallek, 2010).

Stilgenbauer *et al.*, (1999) demonstrated that among 285 CLL patients, 21 cases (7%) had del[6q]. Those patients had adverse clinical parameters such as higher white blood cell counts and more extensive lymphadenopathy. However, there was no significant difference in the OS and the treatment-free intervals between CLL patients who had del[6q] compared to the normal chromosome. Krober *et al.*, (2006) analysed chromosome aberrations among CLL patients and detected

72% of patients (104 of 144) had chromosome aberrations with most common 13q deletion (52%) followed by 11q deletion (21%), 17p deletion (12%), trisomy 12 (8%), 14q involving aberrations in 4% and least of 1% 6q deletion of cases. They observed that the association between ZAP-70 expression and VH mutational status differed significantly depending on presence or absence of additional genetic high-risk features. The subgroups with V3-21 usage and 17p or 11q deletion had a large proportion of discordant cases and the cases with V3-21 usage were almost ZAP-70 positive/VH mutated. The cases without V3-21 usage, 17p deletion or 11q deletion had a stronger association between ZAP-70 expression and VH mutation status. They observed that the CLL cases using V3-21 with ZAP-70 expression predicted poor clinical outcomes regardless of *IGVH* mutational status but the majority who had unmutated *IGVH* with 11q or 17p deletions did not predict unfavourable clinical outcomes. Therefore, it was proposed that high-risk genomic aberrations are independent prognostic factors in addition to mutational status and ZAP-70 expression.

Byrd *et al.*, (2006) demonstrated that CLL patients with high-risk interphase cytogenetics such as del(11q22.3) or del(17p13.1) representing one-fifth of the patients studied showed significantly shorter PFS and OS. Compared with other cytogenetic abnormalities, all patients with del(17p13.1) responded to rituximab and fludarabine therapy partially with a shorter duration and had a short remission. Following autologous transplantation, patients with del11q23 is usually associated with bulky lymphadenopathy and high incidence of residual disease (Dohner *et al.*, 1997). Patients with normal karyotype or deletion of 13q14 had a better prognosis than those with a complex karyotype or deletion of 11q23 or 17p13 (Stilgenbauer *et al.*, 2000; Shanafelt *et al.*, 2006). They identified a commonly deleted segment in band 11q22.3-923.1 which contains the genes encoding for ATM, radixin (RDX) and ferredoxin (FDX1). CLL patients with 17p13 deletion resist to purine analogues due to loss or mutation of the *p53* gene (El *et al.*, 1993; Dohner *et al.*, 1995). CLL patients with 17p deletion affecting *TP53* had failure after treatment with alkylating agents, purine analogues and rituximab (Dohner *et al.*, 1995). Mutations of *TP53* have been found in 4% to 37% of patients with CLL (Catovsky *et al.*, 2007; Grever *et al.*, 2007). Moreover, Zenz *et al.*, (2008) showed the equal poor survival of CLL patients with 17p deletion plus *TP53* mutation, *TP53* mutation only and 17p deletion only.

10% to 15% of CLL patients have shown structural alterations and point mutations of *p53* tumour-suppressor gene (Rouby *et al.*, 1992). It was reported that wild-type *p53* protein represses the activity of the human multidrug resistant (*MDR1*) gene promoter *in vitro* whereas it is stimulated by mutant *p53* protein (Chin *et al.*, 1992). Deletion of the *p53* locus is associated with poor prognosis and *p53* mutant CLL cells could cause a more aggressive disease (Rosenwald *et al.*, 2004). Sandoval *et al.*, (1996) and Genini *et al.*, (2000) demonstrated that *p53* and *p53*-dependent target genes can be activated by incorporating triphosphate of fludarabine into the DNA of CLL cells during DNA repair which could lead to killing of CLL cells. However, the significance of intact *p53* is controversial as *p53*-independent killing in some CLL cases has been reported (Gartenhaus *et al.*, 1996; Johnston *et al.*, 1997). Rosenwald *et al.*, (2001) found that fludarabine could activate a *p53* response in all CLL patients studied suggesting the potential to select for outgrowth of *p53* mutant subclones that are cross-resistant to many of other chemotherapeutic agents. Rouby *et al.*, (1993) demonstrated an association between *p53* gene mutations and an aggressive form of CLL characterised by advanced Rai stage, rapid LDT, increased drug resistance as measured by a poor response to chemotherapy and shortened survival duration.

The most common chromosomal abnormality is trisomy 12. However, the structural aberrations involving long arm of chromosome 13 also frequent among CLL patients. Juliusson *et al.*, (1990) analysed abnormal karyotypes in CLL patients using data from five collaborating European centres and found that almost half the cases had trisomy 12 and aberrations of chromosome 13 as single aberrations suggesting their involvement in the pathogenesis of the disease. Others have reported that important genes are localised on bands q13 to q22. The retinoblastoma gene may be involved on chromosome 13 as most of the aberrations were deletions of q14 and adjacent bands or translocations involving 13q14. They also confirmed the position of trisomy 12 as a prognosis marker and association with survival was observed only in patients with single aberrations. The presence of trisomy 12 in complex karyotypes had not shown a clinical significant. Several studies have demonstrated that trisomy of chromosome 12 derives from the duplication of one chromosome rather deletion and triplication of the other chromosome (Einhorn *et al.*, 1989). Patients with other adverse



prognostic markers such as ZAP-70 expression or unmutated *IGHV* genes have higher chromosomal instability and show a greater risk of acquiring chromosomal aberrations leading to an aggressive form of the disease during the course of the disease (Shanafelt *et al.*, 2006).

As mentioned earlier although the reasons for the better outcome of CLL in women remain unclear, better prognostic factors have been identified in women. These include significantly higher incidence of mutated *IGHV* genes, lower expression of CD38 and ZAP-70, lower beta-2 macroglobulin levels and fewer cytogenetic abnormalities such as *TP53* deletion/mutation and/or 11q deletion (Catovsky *et al.*, 2014). Genotypic male: female ratios have been studied in 4698 patients and found significantly higher male: female ratio (2.5) in patients with 11q del, alone or other abnormalities than in patients with trisomy 12, 13q del or 17p del (1.5). The exact reasons for different prognostic factors between men and women not known (Cantu *et al.*, 2013). Even though those data supported the association of prognostic factors and sex, the prevalence of good prognostic factors in women does not solely account for better outcomes in women, underpinned by the findings that even women with unmated *IGHV* genes had longer survival than their male counterpart (Catovsky *et al.*, 2014).

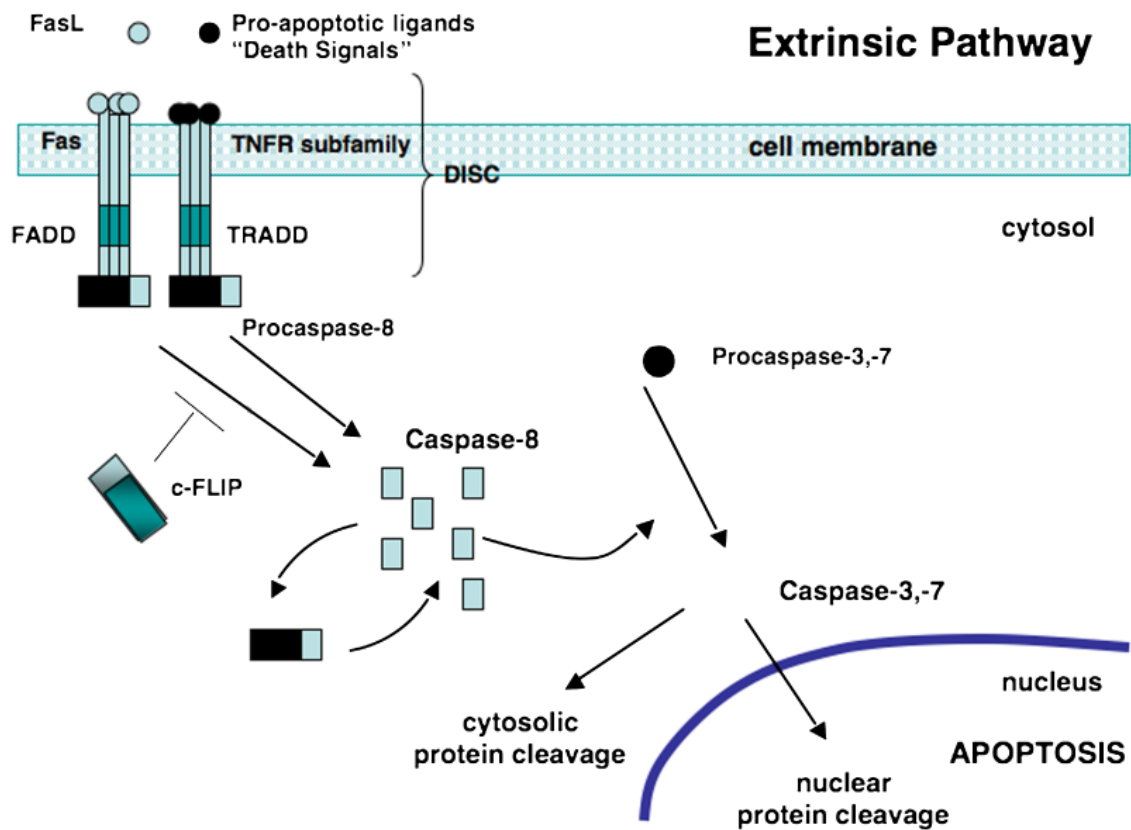
#### **1.4 Resistance to apoptosis**

It has been documented that CLL cells expressed high levels of Bcl-2 protein and associated with resistance to apoptosis. CLL cells are characterised by the increased Bcl-2/Bax ratio which favours cell survival (Pepper *et al.*, 1999). CLL cells are generally resistant to apoptosis induced by tumour necrosis factor (TNF)-family death ligands, such as TNF- $\alpha$ , Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) (Pedersen *et al.*, 2002). MDM2 (Mouse double minute 2) is a negative regulator of an important apoptosis regulator protein p53, and it is over expressed in many human cancers including CLL (Vassilev *et al.*, 2004). Also, some cytokines such as IL-1, IL-6, IL-8, and interferon- $\gamma$  are known to be produced by CLL cells and apparently could act in an autocrine fashion enhancing survival from apoptosis (Aguilar-Santelises *et al.*, 1999).

Peripheral blood CLL cells are mostly non-proliferating and arrested in G0/G1 phase of the cell cycle suggesting CLL is a malignancy of failed apoptosis whereas cell division occurs in proliferation centres in some patients causing an

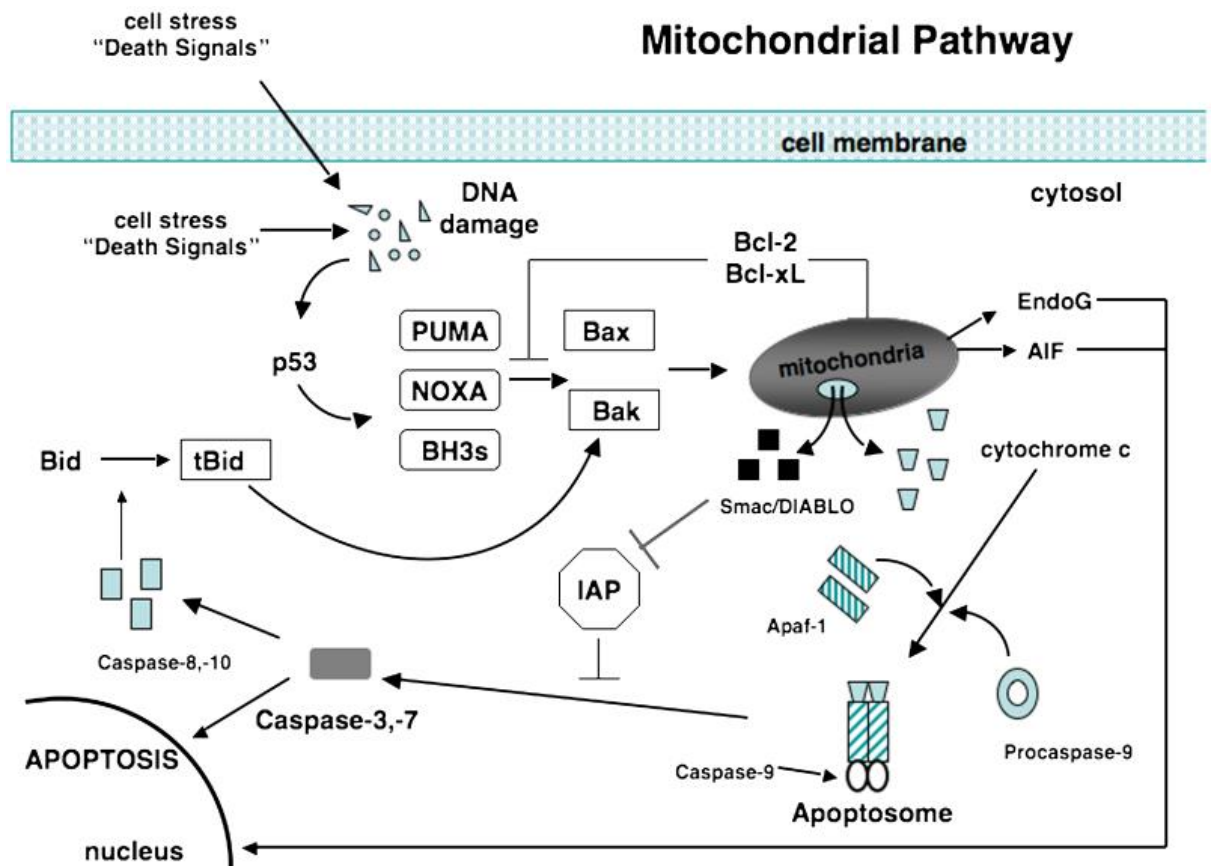
increase in the tumour load (Burger *et al.*, 2002; Hallek *et al.*, 2010). The mechanism of apoptosis can be explained by using three pathways. The commonly described pathways are the intrinsic (or mitochondrial) and extrinsic (or death receptor), and the less well-known initiation pathway is the endoplasmic reticulum pathway. Caspases act as both initiators and executioners in apoptosis pathways (Wong, 2011).

In extrinsic pathway (Figure 1.3), ligands such as TNF and FasL bind with their respective death receptors, type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95). Intracellular death domain associated with these death receptors recruit adaptor proteins such as TNF receptor-associated death domain (TRADD), and Fas-associated death domain (FADD). This ligand-receptor-adaptor protein complex is known as the death-inducing signalling complex (DISC) (Wang, 2011). FasL is expressed mainly by activated T cells and natural killer cells (Watanabe *et al.*, 2002) whereas TNF- $\alpha$  is produced predominantly by activated monocytes/macrophages and lymphocytes (Klener *et al.*, 2006). This DISC complex recruits and assembles initiator caspases 8, a cysteine protease which can release active caspases in turn (O'Brien, 2008) (Figure 1.3).



**Figure 1.3: The extrinsic or death receptor (DR) pathway of apoptosis.** Binding of pro-apoptotic ligands, death signals and Fas ligand (FasL) to Fas or TNFRs, groups the death domain (DDs) of DRs and form a binding site for an adaptor molecule. Death-inducing signalling complex (DISC) is formed by the ligand-receptor-adaptor protein complex and the initiator caspase-8 is recruited and assembled to release active caspase enzyme molecules into the cytosol. The activated effector caspases-3 and -7 causes nuclear protein cleavage and the initiation of apoptosis (O'Brien and Kirby, 2008).

Initiation of the intrinsic mitochondrial pathway of apoptosis happens within the cell in response to cellular stresses such as DNA damage, radical oxygen species, radiation, hormone or growth-factor deprivation, chemotherapeutic agents, cytokines and glucocorticoids (O'Brien and Kirby, 2008). This initiation can eventually release pro-apoptotic proteins from the mitochondria to activate caspase enzymes and trigger apoptosis (Figure 1.4). The intrinsic pathway depends on the balance of activity between pro-apoptotic and anti-apoptotic members of the Bcl-2 superfamily of proteins (Coultas *et al.*, 2003; Letai *et al.*, 2005).



**Figure 1.4: The mitochondrial or intrinsic pathway.** When p53 is induced by DNA damage, the pro-apoptotic proteins Bcl-2-associated X protein (Bax) and BCL2 antagonist/killer (Bak) are activated. This activation is done through the conversion of Bcl-2 homology 3 (BH3) interacting-domain (Bid) to tBid by caspase-8 or -10 and through activation of p53 upregulated modulator of apoptosis (PUMA), Noxa or other BH3 initiator proteins. Oligomerisation of activated Bax and Bak at the mitochondrial membrane causes the mitochondria to release mitochondrial factors including cytochrome c. It combines with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 to form apoptosome. Caspase 3 and 7 are activated by activated caspase 9 allowing apoptosis to proceed. Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) proteins inactivate inhibitor of apoptosis protein (IAP) and EndoG and apoptosis inducing factor (AIF) stimulate apoptosis independent of caspase (O'Brien and Kirby, 2008).

Kerr *et al.*, (1972) suggested the importance of apoptosis for eliminating potentially malignant cells, hyperplasia and tumour progression showing the vital role of reduced apoptosis or its resistance in carcinogenesis. The Bcl-2 family of proteins contain both pro-apoptotic and anti-apoptotic proteins that regulate apoptosis (Gross *et al.*, 1999). Bcl-2 is the leading member of this protein family which is coded by the *BCL2* gene. Members of the Bcl-2 family can be further

divided into three groups based on their functions and Bcl-2 homology (BH) domains. The first group consists of anti-apoptotic proteins such as Bcl-2, Bcl-xL, myeloid cell leukaemia 1 (Mcl-1), Bcl-w, Bcl-2-related protein A1 (BCL2A1/Bfl-1) and Bcl-B/Bcl2L10. The second group consists of BH-3 only proteins which act as pro-apoptotic proteins such as BH3 interacting-domain (Bid), Bim, PUMA, Noxa, Bcl-2 associated death (Bad), Bcl-2 modifying factor (Bmf), Harakiri, BCL2 Interacting Protein (Hrk) and BCL2-interacting killer (Bik). The third group also consists of pro-apoptotic proteins but with all four BH domains. Some examples are Bcl-2-associated X protein (Bax), Bcl-2 antagonist/killer (Bak) and Bcl-2 related ovarian killer/ Matador (Bok/Mtd). Dysregulation of apoptosis can happen due to disruption in the balance of anti-apoptotic and pro-apoptotic members of Bcl-2 family. This can be due to overexpression of one or more anti-apoptotic proteins or under expression of one or more pro-apoptotic proteins or both (Dewson and Kuc, 2010). Another mechanism in apoptosis which leads to carcinogenesis is the low levels of caspases or impairment in caspase function. It was also suggested that abnormalities in death receptor pathway also leads to evasion of the extrinsic apoptosis pathway and hence leads to carcinogenesis (Wong, 2011).

The p53 protein is also known as tumour protein53 (TP53) is a tumour suppressor protein. The tumour suppressor gene *TP53* located on the short arm of chromosome 17 (17p13.1) encodes for p53. The p53 protein plays a role in the induction of apoptosis, cell cycle regulation, development, differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence (Oren and Rotter, 1999). More than 50% of human cancers are associated with defects in the *p53* tumour suppressor gene (Bai and Zhu, 2006). Apoptosis, cytokinesis and signal transduction can be regulated by a group of structurally and functionally similar proteins called the inhibitor of apoptosis proteins (IAPs) and eight IAPs have been identified. IAPs inhibit caspase activity by binding to their conserved baculoviral IAP repeat (BIR) domains to the active site of caspases which leads to promoting degradation of caspases and prevent the binding of substrates to caspase active sites (Wei *et al.*, 2008). Abnormalities in death signalling pathways have been identified which lead to evasion of apoptosis intrinsic pathway. Also, Friesen *et al.*, (1997) demonstrated that CD95-resistant and doxorubicin-resistant leukaemia and neuroblastoma cells displayed

resistance for induction of apoptosis *in vivo* and it was associated with reduced expression of CD95. Evasion of the death signalling pathway in various cancers is also related to reduced membrane expression of death receptors and abnormal expression of decoy receptors (Fulda, 2010).

In CLL, cells express high levels of anti-apoptotic proteins of Bcl-2 and low levels of pro-apoptotic proteins such as Bax *in vivo*. Activation of the Bcl-2 pathway in CLL cells was confirmed by the examination of caspase-8 and caspase-9 levels. Inhibition of caspase-9 by LEHD, a cell permeable irreversible inhibitor of caspase 9, could inhibit the activation of caspase-3 in the apoptotic cells. Nevertheless, there was a preferential activation of caspase-9 over caspase-8 in CLL cells further confirming that the Bcl-2 pathway is functional in apoptosis of CLL cells (Goolsby *et al.*, 2005). It was demonstrated that CD40L ligation in CLL cells upregulates the expression of the anti-apoptotic proteins, Bcl-X<sub>L</sub> and Mcl-1 (Kitada *et al.*, 1999). Robertson *et al.*, (1996) analysed the survival of 33 untreated CLL patients and revealed significantly poorer survival in patients with higher expression levels of Bcl-2. The relationship between drug-induced apoptosis to *p53* gene mutation and Bcl-2/Bax proteins was analysed in CLL patients after inducing apoptosis with a camptothecin analogue, 9-amino-20(s)-camptothecin, or a purine analogue, fludarabine. They found that drug-induced apoptosis was *p53* independent whereas the cells with none to low Bcl-2/Bax ratios were sensitive to the drug compared to drug-resistant CLL cells with intermediate to high ratios (Thomas *et al.*, 1996).

It was thought that combination of Mcl-1 and Bcl-2 has an additive effect on CLL cells to protect against apoptosis induced by chemotherapeutic drugs *in vivo*. *In vivo* dynamics of CLL cells may regulate *Mcl-1* and *Bcl-2* genes to have either Bcl-2 or Mcl-1 but not both anti-apoptotic proteins, so the CLL cells which express both genes have an additional advantage to confront with anticancer drugs. Higher levels of Mcl-1 were significantly associated with failure to attain complete remission (CR) (Kitada *et al.*, 1998). Bcl-2 binding protein, BCL2 Associated Athanogene 1 (BAG-1) expression was found to associate with failure to attain CR and poorer *in vivo* response to chemotherapy suggesting BAG-1 can interact with Bcl-2 to give an additive effect to enhance the resistance to apoptosis. Circulating CLL cells expressed Bcl-2, Mcl-1, BAG-1, Bax, Bak and Caspase-3 but not B-cell lymphoma-extra large (Bcl-X<sub>L</sub>) and BAD proteins (Kitada *et al.*, 1998).

The effect of antitumour triterpenoids, CDDO (triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid) has been observed in CLL cells *in vitro* and found to induce apoptosis through different mechanisms than those induced by cytotoxic chemotherapeutic agents. Triggering of CLL cells by CDDO activated caspase-8 followed by activation of caspase-3 suggesting CDDO triggers the same pathway of apoptosis as TNF/Fas-family cytokine receptors (Pedersen *et al.*, 2002).

MicroRNAs (miRNA) are small non-coding RNAs involved in several cellular processes and expressed in a tissue-specific manner. They regulate gene expression, and their deregulation can alter expression levels of genes involved in development/progression of tumors. miRNA can function as oncogenes or tumor suppressors in CLL and can also serve as markers for CLL onset/progression. Another study has shown that downmodulation of miRNA, *miR-15a* and *miR-16* which act as negative regulators of anti-apoptotic proteins, has been correlated with expression of anti-apoptotic proteins such as MCL1 in CLL (Calin and Carlo, 2009). Bcl2 expression in CLL was found inversely correlated with the expression of *miR-15a* and *miR-16* as they negatively regulated Bcl2 at a posttranscriptional level (Cimmino *et al.*, 2005). It was demonstrated that Bcl-2-IP<sub>3</sub>R interaction could be disrupted by an IP<sub>3</sub>R- derived peptide (IPD) and high amplitude Ca<sup>2+</sup> elevation and thereby apoptosis was induced in primary CLL cells *in vitro* (Zhong *et al.*, 2011). Miyake *et al.*, (1994) demonstrated that novel mAb RP/14 could protect B cells from irradiation-dependent apoptosis by delivering signals to block apoptosis in mice. Several mechanisms which lead to evasion of apoptosis and carcinogenesis are summarised in figure 1.5 below.

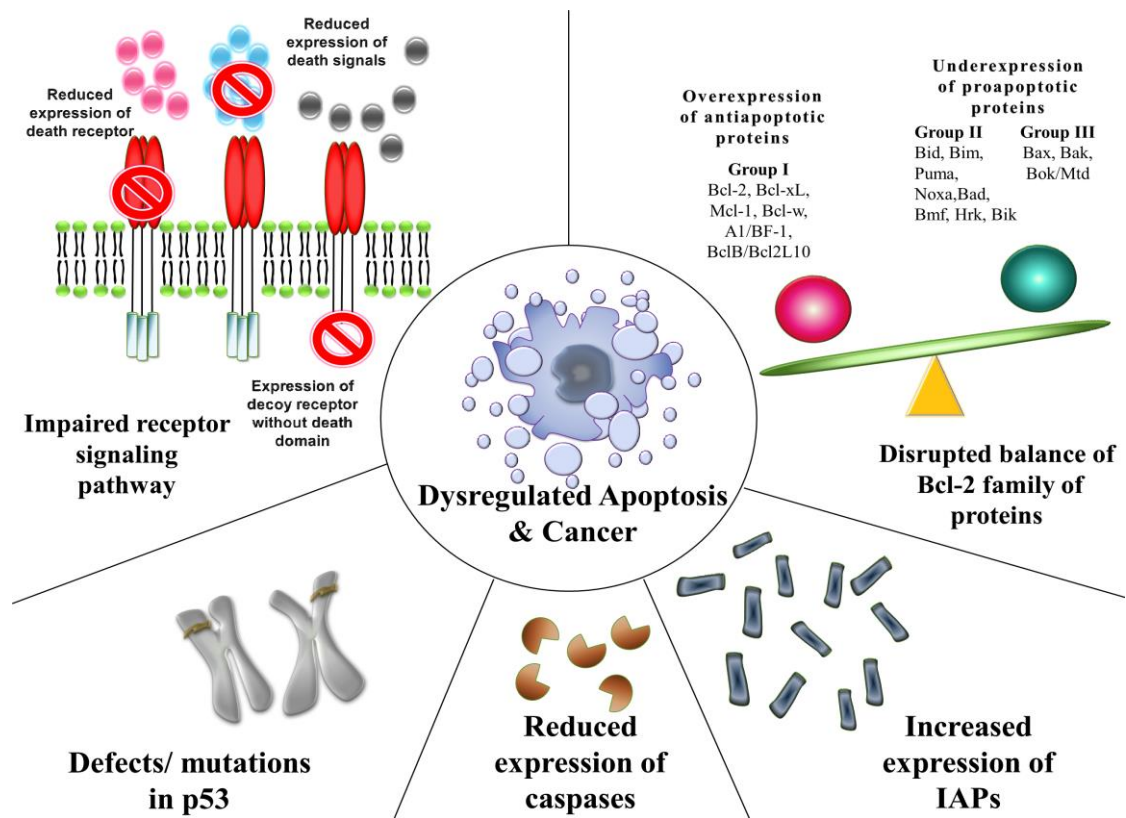


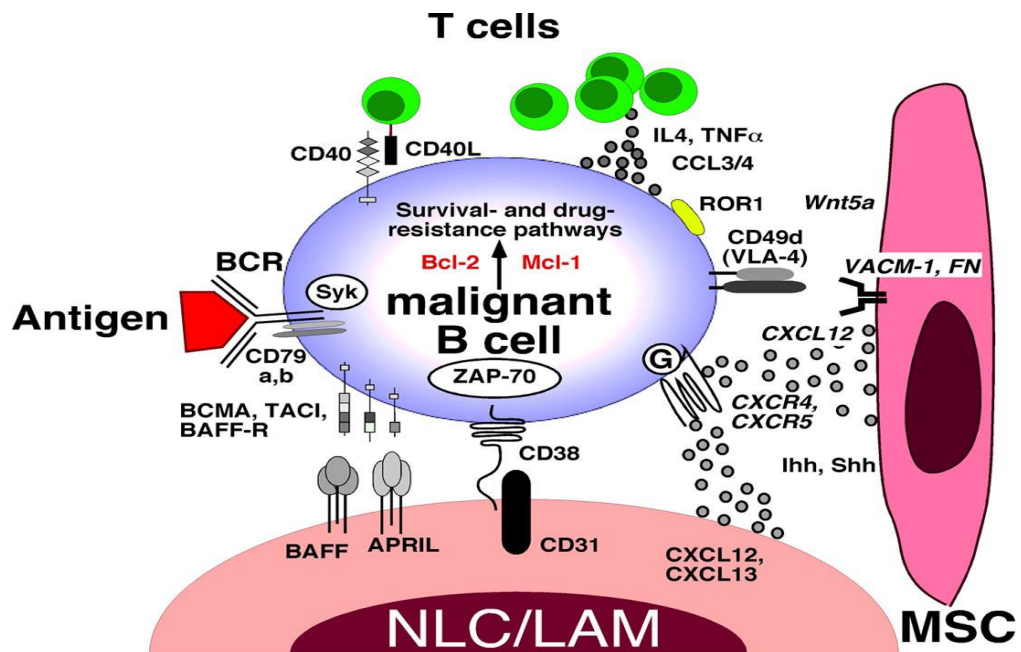
Figure 1.5: Summary of the mechanisms that contribute to evasion of apoptosis and carcinogenesis (Wang, 2011).

### 1.5 Microenvironmental signals

Leukaemic B lymphocytes which show prolonged survival and persistence *in vivo*, usually undergo apoptosis when cultured *in vitro* suggesting the importance of other factors probably originating from the microenvironment for *in vivo* accumulation. Stromal cells/“nurse-like cells” and T-lymphocytes are two potential cellular components in their microenvironment. It was shown that *in vitro* spontaneous apoptosis of CLL cells can be prevented by the co-culture with nurse-like cells or stromal cells or activated autologous T cells (Gionnoni *et al.*, 2014). The action of T cells can be replaced by the presence of T cell-derived cytokines (IL-4) and soluble CD40 ligand (sCD40L). CD40 stimulation also induced CLL proliferation and activation reflected in upregulation of CD80, CD86 and CD95 (Porakishvili *et al.*, 2011). CLL cells recruit accessory cells to create a microenvironment which supports their survival. Increased number of CD3<sup>+</sup> T cells with CD40L<sup>+</sup> and CD4<sup>+</sup> could stimulate CLL cells by interaction between CD40 and CD40L and it could be synergised with BCR signalling (Scieizo *et al.*, 2011). This ligation could lead to activation of NF- $\kappa$ B/Rel transcription factors and Janus-activated kinase 3 (JAK3), signal transducer and activator of transcription



3 to induce high levels of the anti-apoptotic proteins BCL-XL and MCL-1 (Totero *et al.*, 2006). Stimulation of CD40 in CLL cells could increase their proliferation. CLL cells produce chemokines such as CCL3, CCL4, CCL22 and interleukin-8 to interact with accessory cells such as T cells and monocytes to ensure the presence of signals for malignant growth and survival (Oppezzo and Dighiero, 2013). Activation-induced cytidine deaminase (AID) expression results mainly from signals received through CD40-CD40L pathway. It was demonstrated that AID expression was restricted to a subpopulation of CLL cells which undergoes class switch recombination and aggressive course of the disease suggesting B lymphocytes are being constitutively activated in specific tumour microenvironment (Oppezo *et al.*, 2003). CLL progression was favoured by stimulation of CLL cells through BCR which is considered as another possible mechanism of the microenvironmental impact (Ghia *et al.*, 2008). This was confirmed by the evidence that CLL cells possess phenotyping profiles of activated B cells via antigen (Ag) interactions (Damle *et al.*, 2002) and expression of antigen-experienced B lymphocytes gene profiles (Klein *et al.*, 2001).



**Figure 1.6: Molecular crosstalk between CLL B cells and the microenvironment.** Interactions between CLL cells and nurse-like cells/ lymphoma-associated macrophages (NLCs/LAMs) or Mesenchymal stem cells (MSCs) is established and maintained by chemokine receptors and adhesion molecules. CLL cells are attracted by NLCs expressing chemokines CXCL12, CXCL13 and MSCs expressing CXCL12 via G protein-coupled chemokine receptor CXCR4 and CXCR5 expressed on CLL. They also make cell-to-cell adhesion with stromal cells using integrins such as very

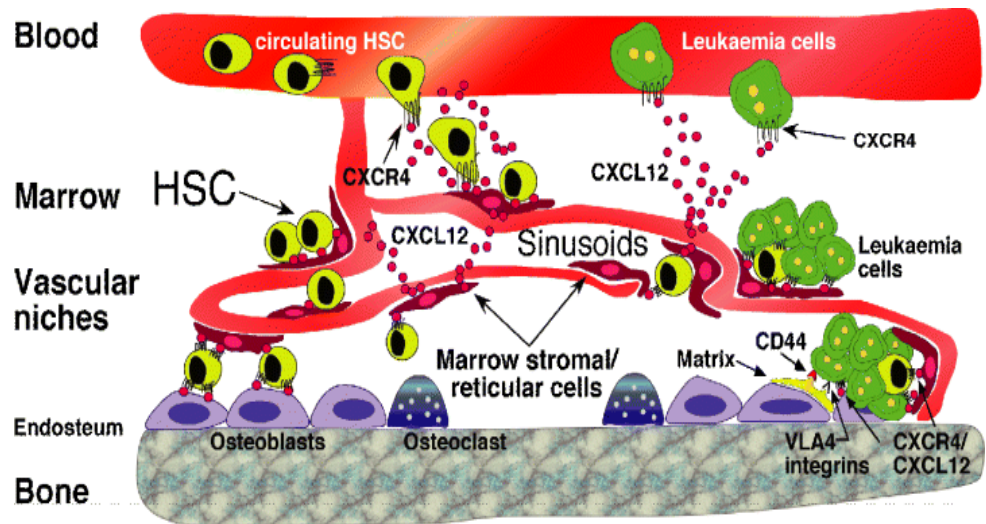
late antigen 4 (VLA-4) integrins (CD49d) present on CLL cells and respective ligands such as vascular cell adhesion molecule 1 (VCAM-1) and fibronectin/FN present on stromal cells. Interactions between TNF family members BAFF and proliferation-inducing ligand on NLCs and corresponding receptors BCMA, TACI and BAFF-R provide survival signals for CLL. CLL cells also interact with NLCs and stromal cells via CD38 ligand and its ligand CD31 expressed on those cells which activates ZAP-70 and downstream signalling pathway. Stimulation of BCR and CD79a, b complex induces downstream signalling by activation spleen tyrosine kinase (Syk) and ZAP-70 enhancing the higher secretion of CCL3 and CCL4 to attract T cells thereby CD40L<sup>+</sup> T cells found in proliferation centres (pseudofollicles) interact with CLL cells via CD40. Cytokines produced by T cells and CLL cells also act as an important survival regulator in CLL cells (Burger *et al.*, 2009).

CLL cells mainly reside in the spleen, lymph nodes and bone marrow where they receive microenvironmental support for expansion. Antigenic stimulation upregulated CD40L on T cells and initiated antibody production. B cell migration to the germinal centre in B cells was stimulated by CD40/CD40L interactions which lead to somatic hypermutations and class switch recombination (CSR) (Schatner *et al.*, 2000; Grdisa *et al.*, 2003). Stimulation of CLL cells led to up-regulation of apoptosis protein, survivin confirming the dependence of CLL cells on CD40/CD40L microenvironment (Granziero *et al.*, 2001). Kostareli *et al.*, (2012) found that mutated and unmutated cases of CLL are different by having distinct Toll like receptors (TLRs). They also demonstrated that TLRs functional in CLL cells in a heterogeneous manner in stereotyped subsets. As an example, CLL subset # 1 is aggressive and subset #4 is indolent as well as they have different gene expression profiles in TLR signalling pathway and responsiveness to TLR ligands indicating CLL cells receive both BCR dependent and independent pro-survival signals from its microenvironment.

Giannoni *et al.*, (2011) observed that hepatocyte growth factor (HGF) and C-X-C motif chemokine 12 (CXCL12) were highly produced by stromal cells which can give survival signals to CLL via ligation to HGF receptor, c-mesenchymal-epithelial transition factor (c-MET). Levels of HGF in sera of CLL patients were higher than normal controls. Giannoni *et al.*, (2011) also showed that HGF/c-MET interaction could activate signal transducer and activator of transcription 3 (STAT3) phosphorylation in nurse-like cells together with activation of Indoleamine-2,3-dioxygenase enzyme in monocytes which modulates T-cell

proliferation and IL-10. Both nurse-like cells and monocytes in CLL patients significantly inhibited T-cell proliferation suggesting the dual pathophysiological function of HGF either directly through enhancement of survival of the leukaemic clone and indirectly by favouring T-cell immunosuppression.

Normal activated CD3<sup>+</sup>, CD4<sup>+</sup> and CD40L<sup>+</sup> T cells are mainly gathered into proliferation centres (PC) in CLL, which is a characteristic structure of CLL suggesting T cell-mediated activation in CLL *in vivo*. CLL cells produce selected chemokines such as CCL22/MDC and CCL-17/TARC which can recruit activated T lymphocytes to provide favourable growth and survival signals (Ghia *et al.*, 2008). C-X-C motif chemokine receptor 4 (CXCR4) expressed highly on CLL cells may be used to move away from T cells towards stromal cell-derived factor-1 (SDF-1) which is also known as C-X-C motif chemokine ligand 12 (CXCL12) produced by stromal cells to avoid T-mediated limit of expansion. It was also observed that co-culturing of CLL cells with marrow stromal cells secreting CXCL12 induced CLL cell migration beneath the stromal cells. CLL cells are attracted by NLC via CXCR4 and protect them from spontaneous or drug-induced apoptosis in a contact-dependent manner. This suggests the role of CXCR4 in CLL trafficking and recirculation between blood and the bone marrow or lymphoid tissues (Figure 1.7) (Bleul *et al.*, 1996; Burger *et al.*, 1999). Therefore, CXCR4-CXCL12 axis acts as an important therapeutic target in CLL and Burger *et al.*, (2004) demonstrated that small peptide antagonists of CXCR4 could block CXCL12 induced activation, migration and signalling of CLL cells. An activated fibroblast cell type, mesenchymal  $\alpha$ -smooth muscle actin<sup>+</sup> ( $\alpha$ -SMA<sup>+</sup>) stromal cells in stromal compartment secrete CXCL12 to promote tumour growth and endothelial progenitor recruitment (Orimo *et al.*, 2005; Ruan *et al.*, 2006). It was demonstrated that Phosphatidylinositol 3-kinase (PI3K) inhibitors can effectively inhibit the signals derived from CXCL12 and CLL-MSK interactions for migration, survival and drug-resistance showing the importance of microenvironment (Niedermeier *et al.*, 2009).



**Figure 1.7: Role of the CXCR4 chemokine receptor in the homing of haematopoietic stem cells and leukaemia cells to the marrow microenvironment.** Both, haematopoietic stem cells and leukaemia cells expressing CXCR4, which directs chemotaxis of haematopoietic progenitor cells (HPC) and leukaemia cells from the circulation to the marrow along the gradients of CXCL12 (SDF-1). There is a vascular network called 'sinusoids' locating reticular stromal cells which HPC migrate in to. Leukaemia cells can adopt this mechanism to access niches that generally are restricted to HSC and to get favourable conditions for their survival. Another mechanism to explain the cross-talk of HSC and leukaemia cell in the marrow microenvironment is that providing a niche for HSC is by mesenchymal-derived osteoblasts which are specialised fibroblasts secreting CXCL12 (Burger et al., 2007).

## 1.6 Treatment of CLL

The stage of the disease and prognosis determine the necessity of treatment in CLL. There are several treatment options as follows.

### 1.6.1 Chemotherapy

The common nitrogen mustard alkylating agents used for CLL treatment are chlorambucil and cyclophosphamide to induce cell death through p53 independent pathway. Induction of apoptosis by chlorambucil was related to increasing p53 and MDM-2 proteins whereas patients who had p53 mutations, increased cell apoptosis via p53 independent manner (Begleiter *et al.*, 1996). The combination of chlorambucil and prednisolone produced initial response rates between 60%-90% and complete remission in 60% of patients analysed (Keating

*et al.*, 1998). Particularly patients who are resistant to alkylating agents are treated with fludarabine, which is a purine analogue and potent inhibitor of DNA repair (Dillman *et al.*, 1989). Moreover, it was reported that combination of fludarabine and cyclophosphamide (FC) give a higher response rate than fludarabine alone (O'Brien, 1998). Rai *et al.*, (2000) reported that fludarabine was superior to chlorambucil in previously untreated CLL patients in terms of rate of complete remission, overall rate of response, duration of response and progression-free survival. Another group has demonstrated that 2-chlorodeoxyadenosine (2-CDA)+prednisolone is an effective treatment for CLL patients who resist to chlorambucil+prednisolone and for patients who had early relapse after treating with alkylating agents (Robak *et al.*, 2000). Fludarabine had a superior activity compared to CAP regimen (cyclophosphamide, doxorubicin and prednisone) and chlorambucil in terms of complete remission rate and remission duration (Dilman *et al.*, 1989; Rai *et al.*, 2000).

Chemotherapy treatment of CLL was reported to relate with *p53* mutations and selective drug resistance mainly associated with alkylating agents (Sturm *et al.*, 2003). They suggested that *p53* mutations may be related to DAN-damaging therapies which result in *p53* inactivation. *In vitro* or *in vivo* drug resistance can be caused by several biological factors including abnormal *p53* function, overexpression of *bcl-2* and incubation of tumour cells with interleukin-4 (IL-4) (Dohner *et al.*, 1995; Thomas *et al.*, 1996). It was reported that chlorambucil could delay the disease progression whereas no effect on survival. However, analysis of the percentages of chlorambucil treated and untreated CLL patients progressed into stage B suggested that early exposure to the drug may select for resistant clones which may be related to poor prognosis of patients who have no response to early therapy (Dighiero *et al.*, 1998).

### **1.6.2 Immunotherapy**

Receptors on CLL are targeted via antibody-dependent cellular cytotoxicity (ADCC), causing apoptosis of CD20<sup>+</sup> B cells and clearance of immune complexes (Shaw *et al.*, 2003). Rituximab is the first monoclonal antibody approved for CLL treatment directed to CD20 phosphoprotein. Due to low density of CD20 on CLL cells, Rituximab treatment has a weak response in CLL patients (O'Brien *et al.*, 2001). CD52, a ubiquitous antigen in CLL with heterogeneous density is targeted by Campath-1H (alemtuzumab). Campath-1H has a significant effect on CLL cells

in the blood and bone marrow, but its effectiveness on targeting cells in secondary lymphoid tissues is lower confirming their protection by microenvironmental interactions with NLC and other stromal cells (Bentz *et al.*, 1995). CD52 is expressed by both B and T lymphocytes and monocytes. It is reported that Campath-1H is concomitant with a significant incidence of opportunistic infections associated with T-cell immunodeficiency. Monoclonal antibodies are effective in the management of minimal residual disease (MRD) and the administration of moderate amounts of Campath-1H can clear residual tumour (Montillo *et al.*, 2002; Ferrajoli *et al.*, 2003;). Furthermore, when CD20 monoclonal antibody, rituximab is used as a single agent, it can induce only partial responses. The response is mainly limited to the lymph nodes and of short duration (Itala *et al.*, 2002; Hainsworth *et al.*, 2003). Therapeutic outcome of rituximab can be improved when it is used with other drugs such as fludarabine mono-phosphate (fludara) or fludara and cyclophosphamide (Ferrajoli *et al.*, 2003).

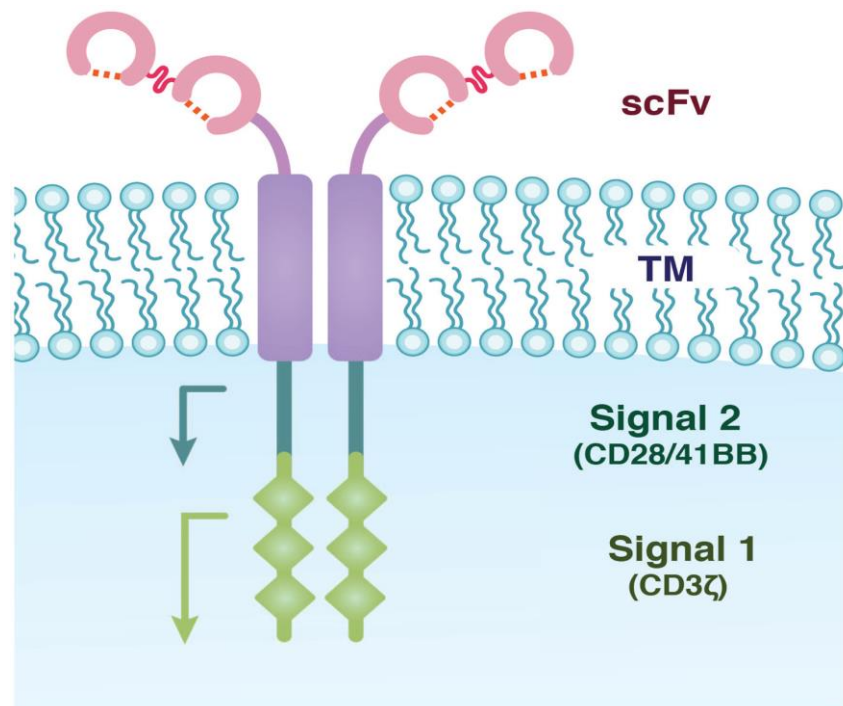
### **1.6.3 Haematopoietic stem cell transplantation**

Allogenic and Autologous stem cell transplantation (SCT) are increasingly considered for treatment of patients with CLL. Autologous stem cell transplantation (ASCT) may increase the response rate and prolong the time to progression, but in comparison with chemotherapy, it does not show a longer survival (Sutton *et al.*, 2011). Since they have done their trial before monoclonal antibodies are started to use, it is important to analyse whether incorporation of rituximab into front line therapy can improve the results or including rituximab as an additional treatment after ASCT. The efficacy of ASCT depends on the administered cytotoxic therapy and it can improve patients' outcome with defined poor-risk features (Dreger and Montserrat, 2002). Allogenic stem cell transplantation from related and well-matched or partially matched unrelated donors could increase long-term MRD-negative survival in 50% of poor-risk CLL patients studied independently of the underlying genomic risk profile (Dreger *et al.*, 2010).

### **1.6.4 Novel therapies**

Recent studies have shown that autologous CD19 redirected T cells expressing genetically modified chimeric antigen receptor (CART19) (Figure 1.8) are very effective in targeting CD19 on CLL cells through transduction of chimeric antigen receptor linked to potent signalling domains (Porter *et al.*, 2011). A patient's T

cells are genetically modified and transferred back to the patient's body mediating killing of malignant and normal B cells (Davila and Brentjens, 2013). It was reported that CLL patients with *TP53* deletions have short remissions after standard therapies (Dohner *et al.*, 1995) and for CLL patients who have advanced stages of the disease, the only approach is allogenic bone marrow transplantation to induce long-term remission (Gribben *et al.*, 2011). However, allogenic bone marrow transplantation is associated with potent graft-versus-tumour effect contributing to morbidity especially in older patients (Sorrer *et al.*, 2008; Gribben *et al.*, 2011). Genetically modified autologous T cells may avoid this limitation and unlike antibody-mediated therapy, chimeric antigen receptor modified T cells have the potential to replicate *in vivo*. This can lead to long-term persistence and sustained tumour control (Porter *et al.*, 2011).



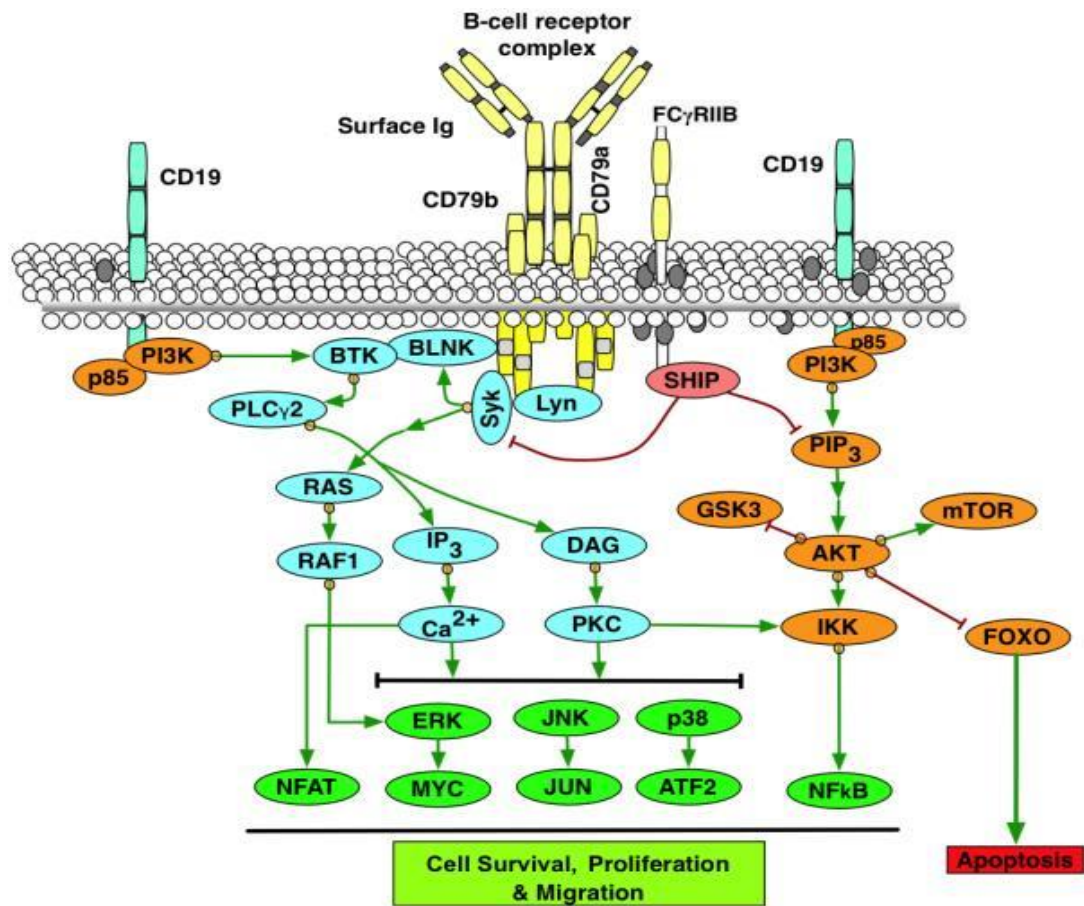
**Figure 1.8: The chimeric antigen receptor (CAR).** Most CARs consist of three parts, the antigen binding domain of a scFv, transmembrane region (TM) of a protein such as CD8 and a signal transduction domain associated with a T cell receptor. Antigen binds with scFv and the two signal transduction domains mediate T cell activation (Davila and Brentjens, 2013).

### 1.7 BCR-mediated signalling in normal B cells and CLL cells

BCR consists of membrane immunoglobulin IgM, IgD homodimers and non-covalently bound heterodimers Igα/Igβ (CD79a/CD79b) which are essential for receptor expression and signal transduction. When IgM is ligated with a soluble

or membrane bound antigen, the activated BCR recruits Src family kinases Lck/Yes novel tyrosine kinase (Lyn) and spleen tyrosine kinase (Syk). Activated BCR phosphorylates the tyrosine residues in the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) portion of CD79a and CD79b (Choi *et al.*, 2012). This phosphorylation activates Bruton's tyrosine kinase (BTK) and PI3K which in turn induces the mobilization and activation of other downstream kinases such as protein kinase C- $\beta$  (PKC- $\beta$ ), mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) to recruit the signalosome. Dual phosphorylation of CD79a/CD79b performed by Syk and Lyn provides continued amplification of the BCR by recruiting protein kinases and forming complex with CD19 and other co-stimulatory molecules. Antigen ligation results in BCR aggregation and microcluster formation in lipid rafts. This cascade activation promotes survival and proliferation of B cells by upregulating transcription factors such as nuclear factor kappa light-chain enhancer of activated B cells (NF- $\kappa$ B). Negative regulators of BCR signalosome such as CD22, CD5, CD72 and Fc $\gamma$ RIIB control the duration and intensity of BCR signalling (Stevenson *et al.*, 2011). BCR is critical for normal B lymphocyte survival and is retained in most B-cell malignancies. Low expression of BCR compared to normal B cells is a characteristic feature of CLL cells (Oppezzo and Dighiero, 2013). U-CLL cells are characterised by significantly higher levels of the expression of sIgM compared to M-CLL cells (Porakishvili *et al.*, 2005) and better BCR-mediated responses (Guarini *et al.*, 2008). Guarini *et al.*, (2008) showed that increased expression of genes involved with ERK and JNK (Jun amino-terminal kinases) MAPK pathways, downmodulation of genes expression in p38MAPK pathway, upregulation of genes expression for proteins involved in cell proliferation and genes related to anti-apoptotic pathway in U-CLL cells upon BCR ligation. The BCR of different CLL patients are often structurally very similar either in antigen binding site contributed by VH, DH, JH genes or the entire antigen binding site coded by both H and L chains suggesting their binding with similar and relevant antigens for the pathogenesis of the disease (Chiorazzi *et al.*, 2005). BCR response might be heterogeneous among CLL patients due to the variable levels of sIgM (Porakishvili *et al.*, 2005; Stevenson, 2011).





**Figure 1.9: B-cell receptor (BCR) mediated signalling.** The BCR complex consists of surface Ig and accessory molecules CD79a and CD79b, which are phosphorylated in response to antigen binding, and recruit signalosome molecules to promote cell survival and proliferation by various downstream pathways (Choi *et al.*, 2012).

Figure 1.9 shows the structure of B-cell receptor complex and major protein kinases involve with BCR signalling. They are further described below.

**1.7.1 Bruton tyrosine kinase (BTK) –** BTK is a non-receptor tyrosine kinase belongs to Tec kinase family which is primarily expressed in hematopoietic cells particularly B cells. It is also expressed on mast and myeloid cells but not expressed on T cells and plasma cells (Geneviev *et al.*, 1994). Mutations in the gene encoding BTK result in B cell deficiency disease X-linked agammaglobulinemia (XLA) in human and X-linked immunodeficiency (xid) in mice demonstrating the importance of BTK in B cell development. XLA is associated with absence of peripheral B cells, decreased levels of serum immunoglobulin and increased susceptibility to infections whereas xid is associated with reduced mature B cell numbers in murine counterpart (Rawlings *et al.*, 1993; Tsukada *et al.*, 1993; Ventrie *et al.*, 1993). Tyrosine kinases, such as

Lyn and Syk activate BTK, upon BCR activation in turns activate transcription factors needed for B cell proliferation and differentiation (Petro *et al.*, 2000). BTK involves in other receptor-mediated signalling such as chemokine receptors, CXCR4 and CXCR5 which are needed for B cell migration and adhesion (Spaargaren *et al.*,2006; Ortolanno *et al.*,2010). Syk plays a major role in activation of BTK and it was reported that BTK activity in Syk negative cells is significantly reduced (Kawakami *et al.*, 2000). Y551 (site 1) within the Src homology type 1 (SH1) domain is phosphorylated by Src-kinases Lyn and/or Syk in BTK, significantly increasing its BTK kinase activity. SH3 domain contains a second phosphorylation site at Y223 which is autophosphorylated by BTK. BTK is activated not only by BCR but also a wide array of receptors can activate BTK in B cells and other cell types including activated G-protein coupled receptors, IgE receptors on mast cells, cytokines and their cytokine receptors and CD40 stimulation (Muller *et al.*, 2004).

It was demonstrated that BTK plays a significant role in BCR/NF- $\kappa$  signalling axis using a BTK-deficient B cell line and primary B lymphocytes isolated from BTK-deficient mice. Moreover, BCR could induce degradation of inhibitor of kappa B (I $\kappa$ B $\alpha$ ). Nevertheless, NF- $\kappa$ B required BTK and the activity of I $\kappa$ B kinase (IKK) which frequently targets the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  for degradation was stimulated by BCR induction in BTK<sup>+</sup> cells whereas it was not observed in BTK-deficient B cells in mice (Petro *et al.*, 2000). The importance of BTK in chemokine-mediated homing and adhesion of B cells was also reported by Spaargaren *et al.*, (2003).

In BCR signalling, BTK is recruited to the membrane-bound signalosome in the early stage of B-cell activation and following phosphorylation by Syk and Lyn, phospholipase C gamma 2 (PLC $\gamma$ 2) is phosphorylated which leads to production of the second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP<sub>3</sub>). In CLL this pathway is amplified and leads to pro-survival signals through its effects on PI3K, PLC $\gamma$ 2, and NF- $\kappa$ B. Inhibition of BTK by ibrutinib interrupts BTK autophosphorylation after IgM ligation hence reduces the expression of downstream targets of BCR activation including ERK, NF- $\kappa$ B, and v-AKT murine thymoma viral oncogene homolog (AKT) (Ringshausen *et al.*, 2002; Herman *et al.*, 2011). Several studies demonstrate that BTK involves with MAP kinase activation. Hence it plays a role in the activation of ERK2, JNK and p38 (Kurosaki, 1999; Kawakami *et al.*, 1997). Involvement of BTK in several constitutively active

pathways of CLL survival was reported by Petro *et al.*, (2000; 2001). PCI-32765 is a potent inhibitor of BTK which binds specifically and irreversibly with cysteine residue in the BTK protein and inhibits its phosphorylation on Tyr<sup>223</sup>, therefore, the enzymatic activity (Honigberg *et al.*, 2010). PCI-32765 can increase favourable clinical outcomes of CLL patients in terms of rapid resolution of lymphadenopathy and/or organomegaly (Advani *et al.*, 2010; Burger *et al.*, 2010).

**1.7.2 AKT** – One of the targets of PI3K is the serine-threonine kinase c-AKT. It is also known as PKB (protein kinase B) and RACPK (related to A and C protein kinases) (Bellacosa *et al.*, 1990; Jones *et al.*, 1991). AKT kinases belong to the AGC kinase family, related to AMP/GMP kinases and protein kinase C. The three conserved domains they consist of are an N-terminal pleckstrin homology (PH) domain, a central kinase catalytic (CAT) domain, and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM). The PH domains are ~80% identical among AKT isomers and ~30% identical to PH domains in pleckstrin and other proteins. The PH domain is connected to the CAT domain by the linker (LINK) region which is poorly conserved among the AKT isoforms (17–46% identical) and has no significant homology to any other human protein (Kumar and Madison, 2005). The consensus CAT domains are ~90% identical among the AKT isoforms and are closely related to the PKC, protein kinase A (PKA), serum- and glucocorticoid-induced protein kinase (SGK), and S6 sub families of the AGC kinase family whilst the C-terminal EXT is ~70% identical among the AKT isoforms and is most closely related to the PKC family (Kumar and Madison, 2005). AKT is regulated by both phosphorylation and the direct binding of PI3K lipid products to its PH domain. However, PI3K independent mechanisms to activate AKT have been identified as well (Burgering and Cohen, 1995; Franke *et al.*, 1995).

Phosphorylation of phosphatidylinositol-4,5-bisphosphate (PI3,4,5-P<sub>2</sub>) by PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5-P<sub>3</sub>) which in turn binds with PH domain of AKT protein and phosphatidylinositol-dependent protein kinase (PDK1) recruiting them to the membrane. Akt activation involves the phosphorylation of two residues, Thr308 and Ser 473. Thr308 is phosphorylated by PDK1 in the activation loop and Ser473 is phosphorylated by PDK2 localising into c-terminal HM (Franke, 2008). mTOR forms part of two different complexes which are mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)

needed for regulating different proteins. Upstream mTORC2 is essential to phosphorylate AKT whereas mTORC1 acts downstream of AKT (Zoncu *et al.*, 2011). AKT inhibits its substrates including the members of the Forkhead family of transcription factors and glycogen synthase kinase-3 (GSK-3). Inhibition of GSK-3 induces the up-regulation of the anti-apoptotic protein MCL-1 (Maurer *et al.*, 2006). The phosphorylation of the Forkhead family member FoxO3a and GSK-3 is induced and MCL-1 protein is increased by the activation of PI3K/AKT pathway in CLL. Inhibition of PI3K induces loss of cell viability, dephosphorylation of FoxO3a and GSK-3, and decrease in the level of MCL-1 protein (Ringshausen *et al.*, 2002; Petlickovski *et al.*, 2005). Higher capacity for cell cycle progression is associated with a stronger activation of AKT pathway in CLL cells from patients with progressive disease (Longo *et al.*, 2007). This group has demonstrated the importance of activation of AKT in cell cycle progression and proliferation of CLL cells. When specific pharmacological inhibitors are used for PI3K/AKT/mTOR, ERK and JNK pathways, the complete inhibition of the proliferative response was observed indicating the importance of these signalling pathways in cell survival of CLL. Nevertheless, constitutively activated AKT kinases can increase leukaemic cell viability and expression of the anti-apoptotic proteins Mcl-1, Bcl-xL and X-linked inhibitor of apoptosis protein (XIAP). Longo *et al.*, (2007) also demonstrated that the strength of AKT, ERK and/or JNK activation determines the different proliferative responses in various CLL subsets. Survival of CLL cells is also promoted by AKT upon activation by several pro-survival signals such as IL-4, phorbol ester, human albumin and immobilised anti-IgM (Barragan *et al.*, 2002; Jones *et al.*, 2003). AKT phosphorylation was inhibited by PI3K inhibitor, LY294002 and PKC inhibitor suggesting the existence of two different pathways for AKT phosphorylation in CLL cells (Barragan *et al.*, 2006).

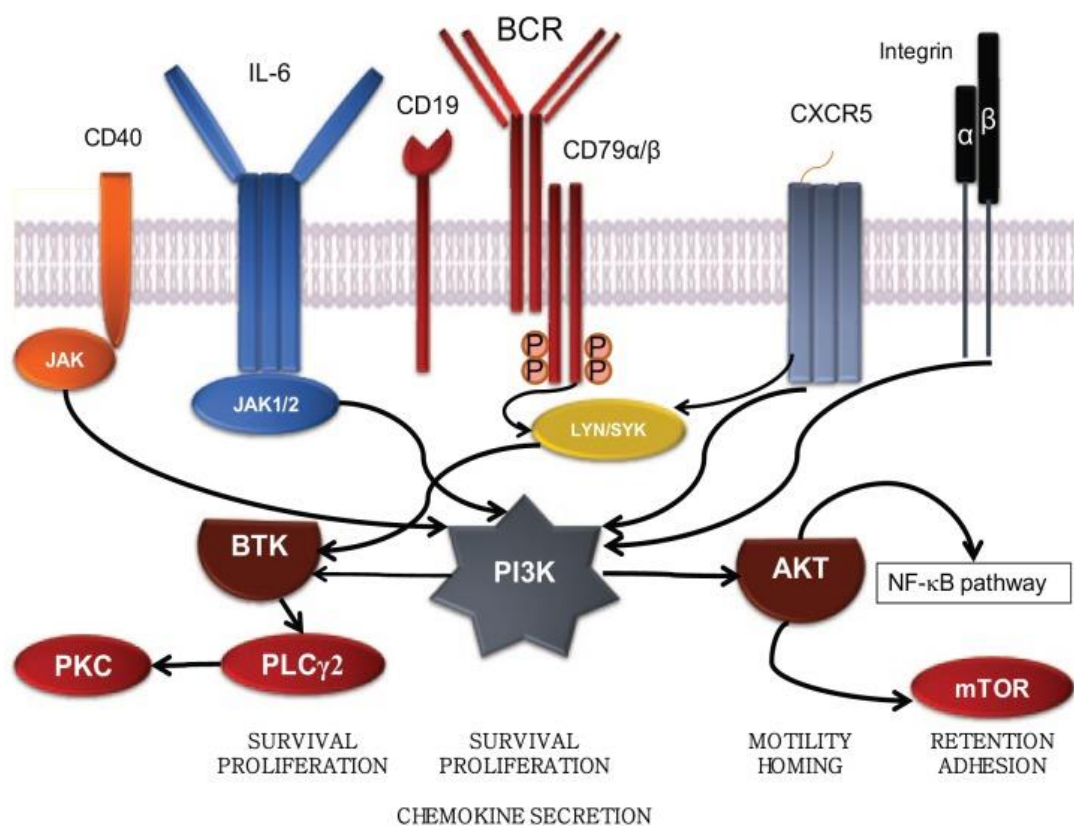
AKT is expressed on macrophages regulate their survival pathway. AKT-1 is constitutively activated in human macrophages and the addition of PI3K inhibitor, LY294002 suppressed the activation of AKT-1 and induced cell death confirming constitutive activation of AKT was regulated by PI3K serine/threonine kinase. It was also reported that inhibition of PI3K or use of a dominant negative (DN) AKT-1 to suppress AKT-1 resulted in loss of mitochondrial membrane potential and thereby activation of caspase-9 and -3 and DNA fragmentation in mice (Hongtao *et al.*, 2001). PI3K/AKT signalling pathway is also present in the lipid rafts of mantle cell lymphoma (MCL) and survival of MCL cells depends on raft-mediated

AKT activation. The synthetic antitumour lipids (ATLs), are a family of compounds which can induce apoptosis in tumour cells and they could inhibit PI3K/AKT survival signals by dephosphorylating AKT and displacing AKT and key enzyme regulators from lipid rafts (Reis-Sobreiro *et al.*, 2013). AKT inhibitors are used to inhibit the phosphorylation of AKT thereby to inhibit cell survival and cell cycle progression. A-443654 is a potent, ATP-competitive and reversible inhibitor of AKT catalysed phosphorylation activity. It is a pan-AKT inhibitor which effect on AKT1, AKT2 or AKT3 (Luo *et al.*, 2005; Shi *et al.*, 2005). It was reported that A-443654 inhibits other protein kinases as well including PKA, protein kinase C-related kinase 2 (PRK2), mitogen- and stress-activated protein kinase1 (MSK1) and dual-specificity tyrosine-phosphorylated and -regulated kinase1A (DYRK1A) showing less selectivity. An ATP non-competitive AKT inhibitor, Akti-1/2 is a highly selective AKT inhibitor, which blocks AKT1 and AKT2 but not AKT3 activity. Akti-1/2 requires the PH domain for its activity (Barnett *et al.*, 2005; Logie *et al.*, 2007). Novel specific inhibitor AiX can antagonise AKT activation by direct blockade and induces apoptosis in U-CLL cells (Hofbauer *et al.*, 2010).

**1.7.3 Phosphatidylinositol-3-kinases (PI3Ks)** - PI3Ks consist of a lipid kinase family characterised by its ability to phosphorylate inositol ring 3'-OH group in inositol phospholipids. There are three primary groups of PI3Ks depending on their structure and substrate specificity and the most important group involve with B cell response is class I PI3Ks. These are heterodimers composed of a catalytic (CAT) subunit of 110-120 kDa (i.e., p110) and associated an adaptor/regulatory subunit (i.e., p85). There are three catalytic subunits in mammalian PI3Ks sharing 42-58% amino acid sequence identity called p110 $\alpha$ , p110 $\beta$  and p110 $\delta$  in subclass IA which are activated by receptors with protein tyrosine kinase activity. Their regulatory subunits are p85 $\alpha$ , p85 $\beta$  and p55 $\delta$ . The subclass IB (PI3K) is activated by receptors coupled with G proteins (Fruman *et al.*, 1998).

Upon activation of growth factor receptor protein tyrosine kinases, autophosphorylation on tyrosine residues takes place to recruit PI3K to the membrane by directly binding to phosphotyrosine consensus residues of growth factor receptors or adaptors using one of the two SH2 domains in the adaptor subunit. This activates CAT subunit and activation of PI3K leads to the production of the second messenger PI3,4,5-P3 from the substrate PI-4,5-P2. PI3,4,5-P3 then recruits a subset of signalling proteins with PH domains to the membrane,

including PDK1 and AKT (Fruman *et al.*, 1998; Fresno-Vara *et al.*, 2004). AKT activates a positive regulator of the survival factor NF $\kappa$ B which is IKK (Hussain *et al.*, 2012). It was reported that inhibition of PI3K by wortmannin and LY294002 could block induction of cyclin D2 and impaired phosphorylation of activation residues in BCR-mediated mitogen-activated protein/extracellular signal-related kinase kinase (MEK)1/2 and p42/44ERK in splenic B cells (Piatelli *et al.*, 2004). Sirinivasan *et al.*, (2009) analysed the role of PI3K in 'tonic' BCR signalling, which is likely to be antigen-independent. They have performed *in vivo* BCR ablation which leads to rapid cell death of mature B cells but it was prevented by constitutive PI3K activation.



**Figure 1.10: The role of PI3K in the biology of B cells.** The PI3K/AKT/mTOR pathway controls most hallmarks of cancer, including cell cycle, survival, metabolism, motility and genomic instability (Ortiz-Maldonado *et al.*, 2015)

The PI3K-AKT-mTOR pathway controls most hallmarks of cancer (Figure 1.10). (Hanahan and Weinberg, 2000; Fruman and Rommel, 2014). PI3K receives signals through CD40, IL-6, BCR, CXCR5 and integrin. Only Class I isoforms of PI3K have an important role in cancer. PI3K $\alpha$  is a frequent genetic driver, while PI3K $\beta$  is usually found in tumours with loss of phosphatase and tensin homolog (PTEN) (Jia *et al.*, 2008). PTEN is an enzyme with opposite action of PI3K which

dephosphorylates PIP3. PI3K $\delta$  is more specific for leukocytes and has a major role in the survival of normal B cells. It acts as a second messenger of many cell receptors such as BCR, CD40, IL-6 and CXCR5 for cell survival, proliferation, chemokine secretion, motility and adhesion to endothelial and stromal cells (Marshall *et al.*, 2000; Davies *et al.*, 2004). PI3K $\gamma$  is found in leukocytes, but it is mostly involved in inflammation (Baracho *et al.*, 2011). Moreover, PI3K $\gamma$  is also important in supporting the growth and survival of lymphoid malignancies, particularly in response to chemokines (Liu *et al.*, 2007; Konrad *et al.*, 2008). Idelalisib is one of the most advanced clinically developed PI3K $\delta$  inhibitor, formerly known as GS-1101 or CAL-101. It is a selective inhibitor of the  $\delta$  isoform that promotes apoptosis in primary CLL cells *ex vivo* despite adverse prognostic factors such as 17p or 11q deletions. The effectiveness of Idelalisib in M-CLL cells mostly dependent on tonic BCR signalling and in U-CLL cells, it relies primarily on chronic active BCR signalling pathways (Herman *et al.*, 2010).

**1.7.4 Mitogen-activated protein kinases (MAPKs)** – MAPKs are a family of conserved protein kinases that phosphorylate specific serine and threonine residues of target protein substrates and regulate many cellular activities such as gene expression, mitosis, cell movement, metabolism, cell survival and apoptosis. MAPK activity is regulated through three-tiered cascades composed of a MAPK, Mitogen-activated protein kinase kinase (MAPKK, MKK, or MEK) and a MAPK kinase kinase or MAPK/ERK kinase (MAPKKK or MEKK) (Chang and Karin, 2001). In mammals, there are at least four distinct groups of MAPKs are expressed which are extracellular signal-related kinases (ERK)-1/2, Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38 $\alpha/\beta/\gamma/\delta$ ) and ERK5. ERK1/2 are phosphorylated by specific MAPKKs, Mitogen-activated protein kinase kinase 1 (MKK1) and Mitogen-activated protein kinase kinase 2 (MKK2). Upstream MAPKKKs such as rapidly accelerated fibrosarcoma (RAF) proteins activate MKK1/2 via retrovirus-associated DNA sequences (Ras)/ Rapidly accelerated fibrosarcoma (Raf)/MEK pathway and those Ras protein functions are regulated by many growth factors and the proto-oncogene *RAS*. Three JUN-amino-terminal kinases (JNK1/2/3) and four p38 protein kinases (p38 $\alpha/\beta/\gamma/\delta$ ) are phosphorylated by MKK4/7 and MKK3/6, respectively in response to stress stimuli. Several MEKKs for JNKs and p38 protein kinases have been identified and some activate both JNK and p38 cascades. A fourth MAPK, ERK5, is exclusively activated by MEK5, which can be phosphorylated by two MAPKKKs, MEKK2 and MEKK3

(Chang and Karin, 2001). Several authors have reported that constitutive activation of p38 MAPK in B-cell tumours can contribute to B-cell tumour growth. p38 has shown to have a role in cytokine-dependent cell growth and cell survival. Inhibition of p38 activity has demonstrated inhibition of proliferation in erythropoietin (EPO)-dependent erythroid cell line HCD57 cells but did not protect cells from apoptosis induced by EPO withdrawal (Jacobs-Helber *et al.*, 2000). Korus *et al.*, (2002) demonstrated that NF- $\kappa$ B activation by Bcr, the oncogenic fusion protein was not mediated by nuclear translocation, but rather by p38MAPK dependent modification of the RelA/p65 transactivation domain.

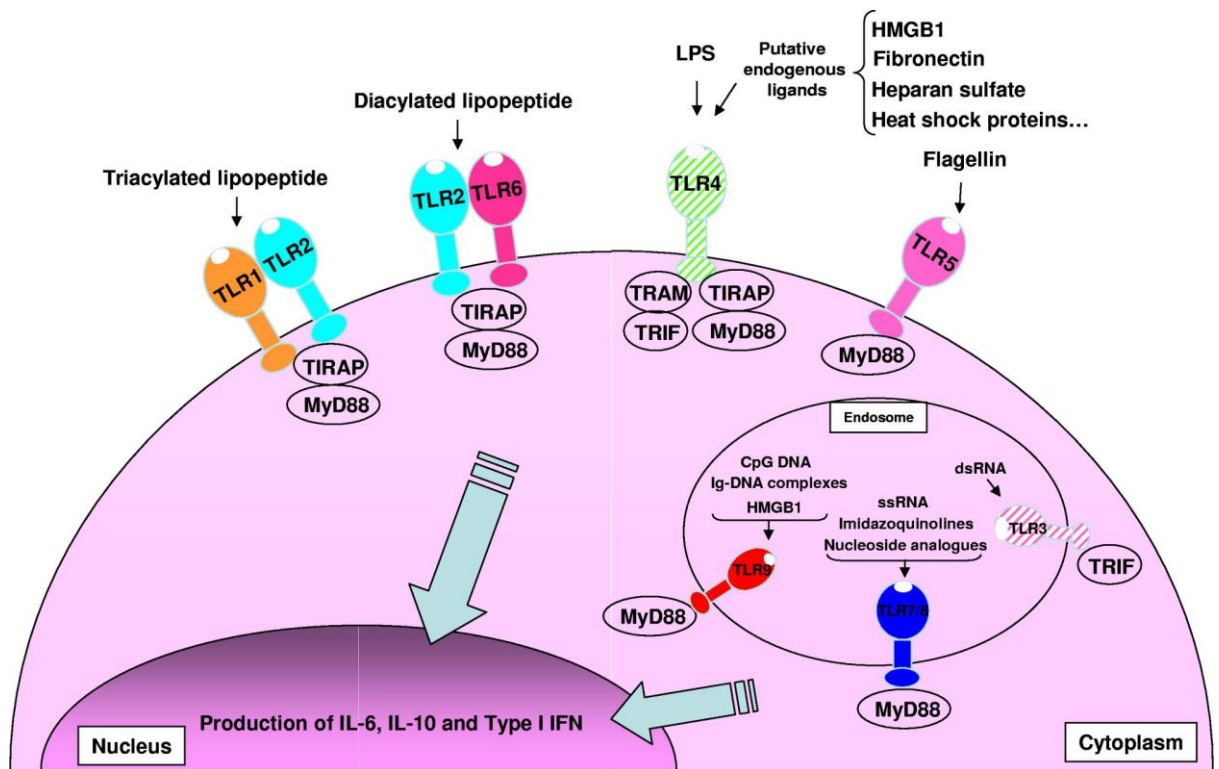
It was reported that the Noxa/Mcl-1 ratio strongly correlates with sensitivity to ABT-737, a BH3 mimetic inhibitor of Bcl-xL, Bcl-2 and Bcl-w in CD40-stimulated CLL cells and both the NF- $\kappa$ B and p38 MAPK signalling pathways involve in regulating Noxa and Mcl-1 levels in CD40-stimulated CLL cells and can, therefore, modulate sensitivity for ABT-737. This was confirmed by the observation that both Noxa and Mcl-1 levels declined in the presence of the p38 inhibitor SB203580 (Tromp *et al.*, 2011). Various treatments in cancer cell lines, including bortezomib, UVB irradiation, and cisplatin, have shown activation of the p38 MAPK signalling to induce Noxa. Muzio *et al.*, (2008) demonstrated that MAPK ERK is constitutively phosphorylated in a proportion of CLL patients who did not show respond to BCR triggering *in vitro*. They suggested that MAPK pathway may be regarded as a potential target for new therapeutics in a selected cohort of patients. Their findings indicated that constitutive activation of MAPK signalling pathway together with nuclear factor of activated T cells (NF-AT) transactivation in the absence of AKT activation represent the molecular characteristics of anergic human B lymphocytes. CLL cells consistently express high levels of tumour suppressor Mda-7/IL-24 protein, melanoma differentiation-associated gene-7 (*mda-7*) and IL-24 mRNA. p38 MAPK is shown to be activated by endogenous Mda-7/IL-24 protein or by exogenous IL-24 in CLL (Sainz-Perez *et al.*, 2006). Pro-apoptotic activity of p38 MAPK was reported in some cell lines. Inhibition of p38 MAPK by its specific inhibitor SB203580 abrogated the induction of apoptosis by mda-7 (IL24) antibody in several model systems and human melanoma cells (Sarkar *et al.*, 2002). However, an upstream activator of p38 MAPK, MKK6b overexpression could lead to induction of apoptosis in Jurkat T cells. Furthermore, MKK6b activity was found to be required for Fas-induced apoptosis (Huang *et al.*, 1997) suggesting the type of stimuli and cell type,



activation of the SAPK/p38 MAPK pathways may be sufficient to induce cell death. Sainz-Perez *et al.*, (2006) reported that p38 MAPK does not have pro-apoptotic activity in CLL cells since fresh cells express p38 MAPK. Use of the p38MAPK inhibitor SB203580 and silencing of Mda-7/IL-24 induced apoptosis in CLL cells showing its role in cell survival. Graves *et al.*, (1998) demonstrated the functional role of p38 MAPK to regulate transcriptional or translational events needed for BCR induced apoptosis by showing SB203580 inhibited BCR-induced apoptosis but not apoptosis induced by cross-linking Fas in two human B cell lines B104 and BJAB. p38MAPK also activates NF- $\kappa$ B which resulted in oncogenic transformation by the oncogenic fusion protein p210 Bcr-Abl (Korus *et al.*, 2002).

### **1.8 Toll-like Receptors (TLRs)**

TLRs are expressed by different types of leukocytes and in some non-immune cells present in sites of barrier function such as intestinal and airway epithelial cells. Their key roles are regulating innate immunity by recognition of exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) (Muzio *et al.*, 2012). TLR4 was first identified as a mammalian homologue of *Drosophila* Toll receptor which is needed for induction of genes involved in inflammatory response and later several proteins which are structurally similar to TLR4 have been identified and named TLRs. The TLR family consists of 10 members designated TLR1-TLR10 in human and 12 in mice. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are present on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are localised into intracellular vesicles such as endosomes (Takeda and Akira, 2004; Muzio *et al.*, 2012). The cytoplasmic domain of the TLR, Toll/IL-1 receptor (TIR) domain) has similarity with IL-1 receptor family. TLRs have leucine-rich repeats (LRRs) in the extracellular domain differ to Ig-like extracellular portion of IL-1 receptors. Each TLR can recognise specific components of pathogens playing a significant role in mammalian immune system (Takeda and Akira, 2004) (Figure 1.11).



**Figure 1.11: Different types of TLRs, TLR specific ligands and their signalling complexes in normal and malignant human B cells (Chiron *et al.*, 2008).** TLR3, 7, 8 and 9 recognise highly conserved viral structures and TLR1,2,4,5,6,7,8,9 recognise bacterial structures. Heterodimerization of TLR2 with TLR1 or TLR6 is triggered by bacterial lipopeptides, whereas TLR3 is activated by double-stranded RNA. TLR4 is activated by LPS, TLR5 is activated by flagellin, TLR7 and TLR8 are activated by single-stranded RNA (ssRNA), and TLR9 is activated by unmethylated CpG DNA motifs. TLRs are also activated by endogenous ligands released during cellular stress or matrix degradation such as heat-shock proteins, fibronectin, heparin sulphates (Rifkin *et al.*, 2005). The ligand for TLR10 is not identified yet and it may functionally have a link to specific naïve and memory B cell actions rather function to recognise pathogens (Domer *et al.*, 2009). Several studies have demonstrated that peripheral blood-derived naïve and memory B cells express distinct levels of TLR6, 7, and 9. Circulating naïve B cells do not express TLR6, 7, 9, and 10 (Bernasconi *et al.*, 2003) whereas memory B cells display a higher sensitivity to TLR activation with a concomitant higher capacity for differentiation into plasma cells. Therefore, the sensitivity of naïve B cells to circulating PAMP is very low and to mature them to plasma cells they require additional signals. High expression levels of TLR are found in naïve, germinal centre, and memory B cells in the tonsils suggesting human B cell localisation influences expression of TLRs (Mansson *et al.*, 2006). It was also reported that TLR4 is also expressed in CD4<sup>+</sup> T cells and involve with CD4<sup>+</sup> T-cell proliferation and survival *in vitro*.

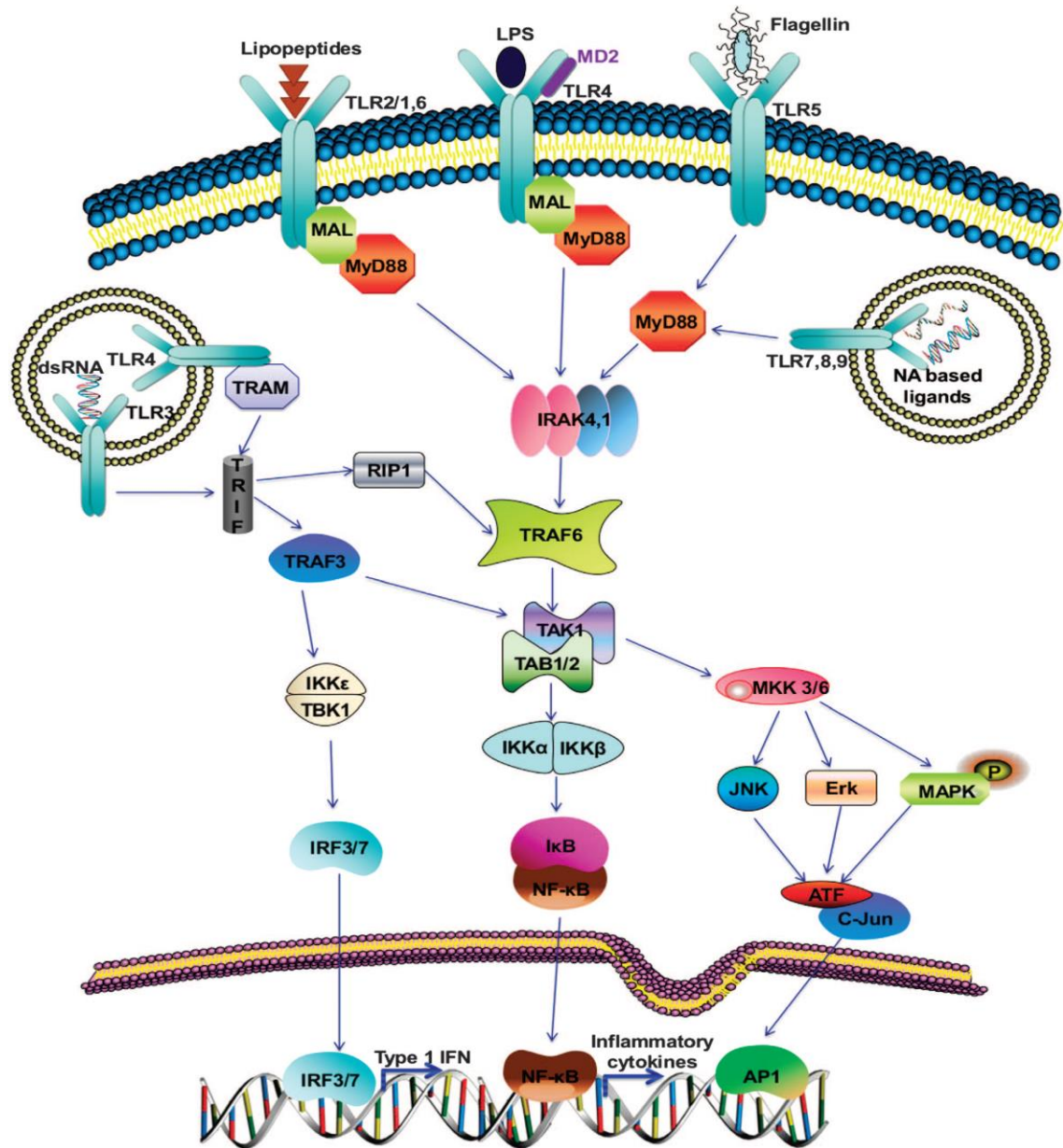
These CD4<sup>+</sup> T cells and  $\delta\gamma$  T cells are activated by TLR4 which contributes to autoimmune inflammation (Reynolds *et al.*, 2012).

A fraction of circulating B cells which are IgG<sup>-</sup> and CD27<sup>-</sup> with intermediate expression levels of CD19 and CD69 and most tonsillar B cells express TLR2 (Mansson *et al.*, 2006; Ganley-Leal *et al.*, 2006). Increased TLR expression in tonsillar B cells may be caused by local infections. Expression of TLR7, 9 and 10 in resting B cells is increased by the activation via the BCR and/or CD40L, or by CpG, oligodeoxynucleotides (ODNs) (Bernasconi *et al.*, 2003, Bourke *et al.*, 2003). Furthermore, TLR7 and MyD88 expression in naive peripheral blood B cells are activated by type I interferon (IFN) produced by pDCs during infections (Bekeredjian *et al.*, 2005). CD180 (Radio protective 105, RP105) structurally resembles TLRs but lacks a TIR domain and promotes LPS-driven B-cell responses in mice whereas it acts as a negative regulator of TLR4 signalling in monocytes and DCs (Divanovic *et al.*, 2007). Expression of TLRs in CLL is heterogeneous, but most of CLL cases express TLR1, TLR2, TLR6, TLR10 on the cell surface, and TLR7, TLR8, TLR9 within endosomes resembling expression patterns in normal mature B cells (Muzio *et al.*, 2009). The level of expression of TLR7 and CD180 is high, TLR1, TLR6, TLR10 is intermediate and TLR2, TLR4, TLR8 and TLR9 is low in CLL cells at cohort levels. TLR7 and TLR8 levels are varied among different samples and there is an up-regulation of TLR8 and down-regulation of TLR4 mRNA in the unmutated subgroup (Arvaniti *et al.*, 2011). Oncogenic mutations were identified in MyD88 in different B cell malignancies. Single point mutations were found in 29% of ABC type diffuse large B cell lymphoma cases (Ngo *et al.*, 2010), 13% of splenic marginal zone lymphoma cases (Yan *et al.*, 2012), 36% of primary central nervous system lymphoma cases (Montesinos-Rongen *et al.*, 2011) and 3-10% of CLL cases (Puente *et al.*, 2011; Wang *et al.*, 2011). Since these mutations may affect specific signalling pathways in leukaemic cells, they are considered as novel therapeutic targets. Stimulation of TLR7 increases the expression of costimulatory molecules such as CD25, CD80, CD86 on leukaemic cells and produces inflammatory cytokines such as TNF $\alpha$  and IL-6 (Spaner *et al.*, 2005). TLR9 ligand CpG induces the production of TNF $\alpha$ , IL-10, IL-6 and CD25 expression (Decker *et al.*, 2000; Rozkova *et al.*, 2010). Therefore, agonists of both TLR7 and TLR9 have been studied for immunotherapy approaches in preclinical models of CLL *in vitro* (Hoogendoorn *et al.*, 2004; Spaner *et al.*, 2006).

Primary B cells are activated by antigens and LPS to upregulate CD69 expression and co-engagement of these two receptors induces a synergistic B cell response suggesting a cross talk between TLR4 and BCR (Minguet *et al.*, 2008).

Triggering of BCR of naive B-cells can lead to a rapid up-regulation of TLR9 expression suggesting the synergism of two receptors (Bernasconi *et al.*, 2003). However, to induce full activation, proliferation and differentiation of naive B-cells, co-stimulation with three different signals from BCR, CD40 and TLR (Muzio *et al.*, 2012) and cytokines (Porakishvili *et al.*, 2011) are required. Comparison of M-CLL and U-CLL showed up-regulation of TLR8 mRNA and down regulation of TLR4 in U-CLL (Muzio *et al.*, 2012). Some studies have reported that TLR9 induction via CpG-B oligonucleotides may induce apoptosis in M-CLL and proliferation in U-CLL samples (Muzio *et al.*, 2012) as well as upregulation of the expression of CD40 suggesting the synergy with IL-2 and/or CD40L (Chiron *et al.*, 2008). To summarise, there is an evidence of the role of TLRs as microenvironmental sensors (Miyake *et al.*, 1994, 1995; Chan *et al.*, 1998; Miura *et al.*, 1998; Roshak *et al.*, 1999; Yazawa *et al.*, 2003). Since there is increased frequency and severity of infections and autoimmune complications in CLL patients, it can be suggested that inflammation or autoimmunity mediated by distinct TLR may also involve with regulating the development, progression and/or accumulation of CLL (Muzio *et al.*, 2012).

## 1.9 TLR-mediated signalling



**Figure 1.12: Schematic representation of TLR mediated signalling pathway.**

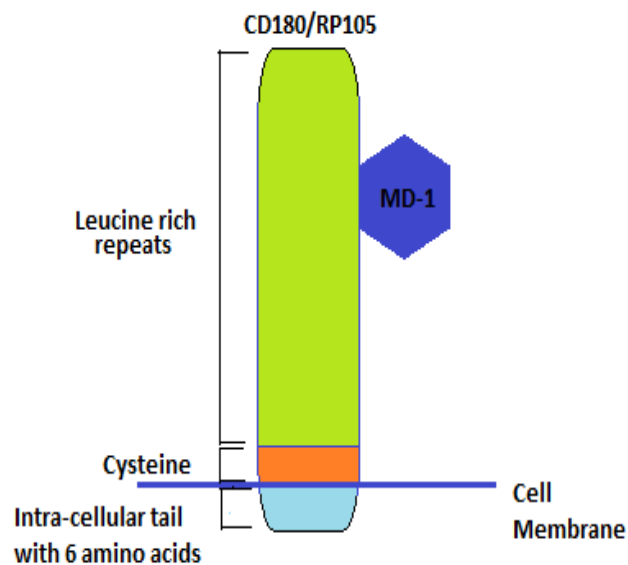
When TLRs are stimulated by their respective ligands, they dimerize and recruit downstream adaptor molecules, such as myeloid differentiation primary-response protein 88 (MyD88), MyD88-adaptor-like (MAL), Toll/interleukin (IL)-1 receptor, TIR-domain-containing adaptor-inducing interferon- $\beta$  (TRIF), TRIF-related adaptor molecule (TRAM), which activate other downstream molecules leading to the activation of signalling cascades that converge at the nuclear factor- $\kappa$ B (NF- $\kappa$ B), interferon (IFN) response factors (IRFs) and mitogen-activated protein (MAP) kinases. Transcription of several pro-inflammatory molecules, such as interleukin (IL)-6, IL-8, IL-12, and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) are induced by those molecules. The secretion of these molecules is responsible for attacking microbes and helps activate other immune components. (Anwar *et al.*, 2013).

TLR signalling pathway is activated from the cytoplasmic TIR domain which acts as a binding site for downstream adaptor molecules to mediate signals from other molecules. TIR domain recruits two main adaptor proteins, one is MyD88, which is recruited to the TLR-TIR domain together with Mal (MyD88 adaptor-like) which is also known as TIRAP (TIR-domain-containing adaptor protein). The second adaptor is TRIF (Toll-receptor-associated activator of interferon) (Akira, 2006). The induction of specific gene expression in MyD88 pathway is mediated by the NF- $\kappa$ B transcription factor, AP-1 (activating protein 1) or IRF1, 5 and 7 (interferon-response factor). TRIF associated pathway is regulated by IRF3 and NF- $\kappa$ B (Honda *et al.*, 2006). Except for TLR3, all other TLRs trigger signalling through MyD88 whereas TLR4 can trigger downstream signals through both pathways. The death domain of MyD88 recruits Interleukin-1 receptor-associated kinase (IRAK) family members to the TLR signalling complex, activate them and transmit the signal to TNF receptor-associated factor 6 (TRAF6) which allows the phosphorylation of IKK. The pathway continues with the activation of NF- $\kappa$ B and the recruitment of TGF- $\beta$  activated protein kinase 1 (TAK1) that induces the MAPK pathways. These signalling cascades eventually induce the transcription of inflammatory cytokines such as IL-6, IL-8, IL-12 and TNF- $\alpha$ , type I or II interferons and chemokines (Akira, 2006).

### **1.10 CD180 receptor**

CD180 is the human analogue of the murine surface receptor RP105 sharing 74% sequence homology. CD180 receptor was originally discovered on murine B cells and identified on human B cells by Bgp95 mAb (Valentine *et al.*, 1988). It is a membrane-associated orphan receptor with a molecular weight of 105kD. The extracellular portion of CD180 consists of tandem repeats of leucine-rich motif separated from the single transmembrane domain by a carboxy-flanking region thought to be involved in processes such as cell adhesion or receptor-ligand interaction (Figure 1.13). The presence of conserved cysteine residues in the carboxy-flanking region in CD180 is a similarity with TLRs (Chan *et al.*, 1998). CD180 is expressed on antigen presenting cells such as human B lymphocytes, monocytes (Zarembek and Godowski, 2002) and dendritic cells (Kadowaki *et al.*, 2001). Histological studies have shown RP105 is mainly expressed on mature B cells in mantle zones whereas its expression in germinal centre cells is either very low or negative (Miuro *et al.*, 1998). RP105 forms a complex with MD-1 to elicit responses to bacterial LPS which is structurally related to TLR4/MD-2 complex.

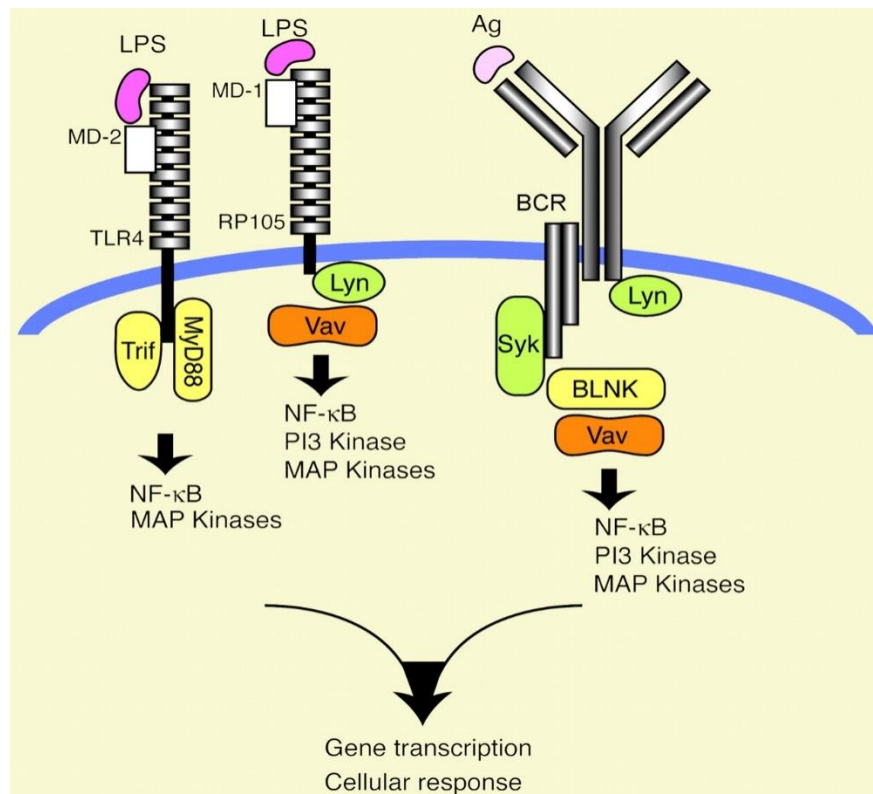
The importance of MD1 for RP105 activity has been demonstrated by showing that MD-1 null mice had impairment in LPS induced B-cell proliferation, antibody production and B7.2/CD86 upregulation (Nagai *et al.*, 2002).



**Figure 1.13: Schematic diagram to show the structure of CD180 receptor on cell surface.** The extra-cellular domain consists of tandem repeats of leucine-rich motif and conserved cysteine residues and is associated with MD-1. The intra-cellular domain consists of 6 amino acids and is not associated with adaptor molecules.

CD180 is homologous to TLR4 but lacks its intracellular TLR-like domain with 61% sequence similarity in the extracellular domain (Chappell *et al.*, 2013). These two receptors use two distinct signalling pathways. LPS binding to CD180/MD1 induces Lyn activation and CD19 phosphorylation whereas LPS binding to TLR4/MD-2 activates MyD88/IRAK and MyD88-independent Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP) pathways to activate JNK and NF $\kappa$ B (Antoz *et al.*, 2009).





**Figure 1.14: Schematic diagram to show expression of TLR4/MD2, RP105/MD1 and BCR receptor complexes on cell membrane.** RP105 signalling is unclear, but the involvement of Lyn and Vav, was envisaged by the activation of Lyn by RP105 stimulation and downstream defects in RP105 signalling between Lyn<sup>-/-</sup> and Vav1<sup>-/-</sup>Vav2<sup>-/-</sup> B cells in mice (Kurosaki, 2005).

Although CD180 regulates B cell sensitivity to LPS in mice, its natural ligand is yet unknown since CD180 molecule lacks LPS-binding pockets, unlike TLR4. Stimulation of RP105 with an antibody protects B cells from irradiation or dexamethasone-induced apoptosis in murine B cells and activates proliferation (Miyake *et al.*, 1994). Chaplin *et al.*, (2011) reported that CD180 signals could induce B cells for strong polyclonal B cell proliferation and Ig production. Our group demonstrated that CD180 involves in B cell activation, proliferation, and survival from apoptosis (Porakishvili *et al.*, 2005, 2011). CD180 was shown to increase in cell size, expression of the costimulatory molecule CD80 and DNA synthesis upon stimulation (Miuro *et al.*, 1998). Roshak *et al.*, (1999) demonstrated for the first time that incubation of human peripheral blood leukocytes with anti-RP105 sera induced proliferation. CD180 expression in mice B cells was analysed by Nagai *et al.*, (2002) and showed it is 3-fold higher on marginal zone B cells than follicular and B1 cells and was down regulated on germinal centre cells. Valentine *et al.*, (1998) showed that ligation of CD180 with



mAb could trigger B cell activation and proliferation. Another study has reported that anti-CD180 increased both transitional and mature B cells, marginal zone B cell proliferation and CD86, but not CD80 expression. Anti-CD180 had no effect on cytokine production from B cells, but it increased IL-6, IL-10, and TNF- $\alpha$  production in combination with LPS or CpG in mice (Chaplin *et al.*, 2011).

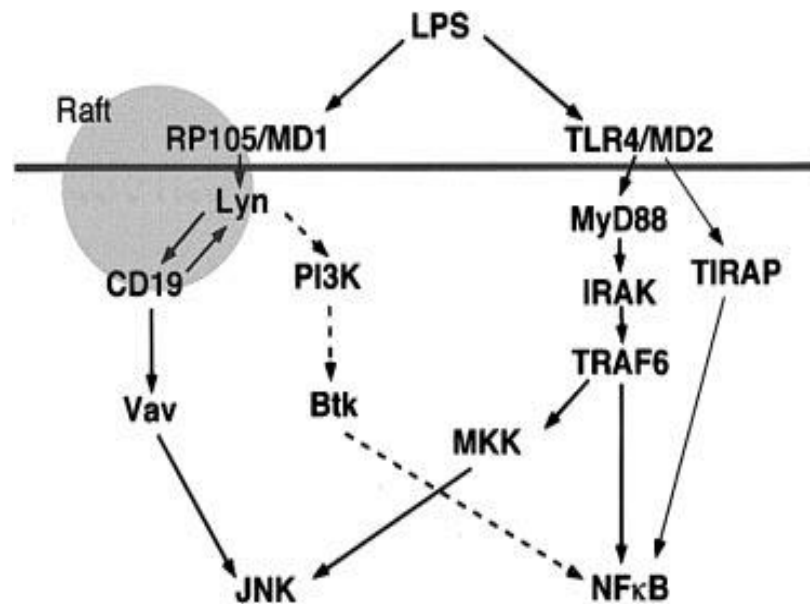
It was also reported that after 24 hours LPS stimulation, CD180 expression was significantly decreased in normal and leukaemic CD19<sup>+</sup> cells (Antosz *et al.*, 2009)., LPS stimulation has shown to 100-fold down regulate CD180 expression in monocytic leukaemia cell line THP-1 (Zarembek and Godowski, 2002). Nevertheless, reduction of CD180 expression in unstimulated peripheral blood CD19<sup>+</sup> CLL cells was four-times lower than that of normal CD19<sup>+</sup> lymphocytes suggesting constant stimulation of CD180 receptor in CLL by unknown antigen or autoantigen. It can also be suggested that unknown antigen resembles the structure of LPS of gram-negative bacteria (Antosz *et al.*, 2009). This observation may provide evidence to suggest that repeated polyclonal activation of leukaemic B cells by microbial molecule during natural infection or inflammation is responsible for initiating oncogenic process of CLL (Chiron *et al.*, 2008).

CD180 is expressed heterogeneously on CLL cells, and predominantly on M-CLL (Porakishvili *et al.*, 2005). Although approximately 60% of CLL clones expressed CD180, only half responded to ligation with anti-CD180 mAb by activation, cycling, and reduced basal apoptosis. This was comparable or superior to that induced by anti-CD40 mAb or IL-4. These CLL clones were termed responders (R-CLL). In contrast, CD180<sup>+</sup> CLL samples that failed to respond to anti-CD180 mAb were termed non-responders (Porakishvili *et al.*, 2005). Furthermore, CD180 ligation led to a strong upregulation in phosphorylation of ZAP70, p38MAPK, ERK and particularly AKT protein kinases in normal B cells and R-CLL cells (Porakishvili *et al.*, 2011), which is an indication of its pro-survival function in responder CLL (Chiorazzi and Ferrarini, 2003). B-cell activation decreases expression of CD180 *in*as germinal centre (GC) B cells, and most B-cell lines do not express CD180 (Otipoby *et al.*, 2002) and many activated B cells from patients with rheumatoid diseases are CD180 negative (Koarada *et al.*, 1999).

### 1.11 CD180 mediated signalling in normal B cells

Several studies have supported the importance of CD180 in B-cell survival, activation, proliferation and/or differentiation. However, the mechanisms or pathways of signalling are not yet fully understood. Although TLRs have an intracellular TIR domain and an adaptor molecule Myd88 which activates the various downstream PTKs, CD180 does not have a functional cytoplasmic signalling domain, and it cannot independently propagate an intracellular signal. It must, therefore, recruit or converge with other receptor pathways. RP105 mediated signalling pathway was independent of MyD88 expression, and its functioning is regulated by CD19. In mice, RP105 ligation recruits CD19 into lipid rafts and induces the phosphorylation of CD19 which in turns amplifies Src kinase Lyn activity (Chan *et al.*, 1998). Phosphorylated CD19 recruits Vav, which is important to act upstream of JNK pathway. Therefore, CD19 may mediate the interaction between Lyn and Vav in RP105 mediated signalling. This was confirmed by the fact that CD19 deficient human B cells showed diminished phosphorylation of Vav following RP105 ligation. In fact, this study has demonstrated that PI3K and NF- $\kappa$ B activation by CD180 ligation were shown to be independent of CD19 (Figure 1.15) (Yazawa *et al.*, 2003).

It was also demonstrated that Vav1/2-mutant B cells failed to proliferate *in vitro* in response to LPS or CD180 in mice whereas deficiency of Vav1 alone prevented CD180- driven proliferation. Nevertheless, activation of ERK in response to CD180 and induction of CD86 and CD25 by anti-CD180 also required both Vav1 and Vav2 (Hebeis *et al.*, 2005). Involvement of Lyn, BTK, PKC $\beta$ I/II and MAP kinases in both sIgM- and CD180 mediated activation suggest a common signalling pathway(s) for both receptors. Activation of Lyn kinase by anti-IgM stimulation is relatively stronger than by anti-CD180 causing more efficient recruitment of Lyn to BCR complex. This results in blocking of CD180 induced MAP kinase activation during simultaneous ligation (Chan *et al.*, 1998).



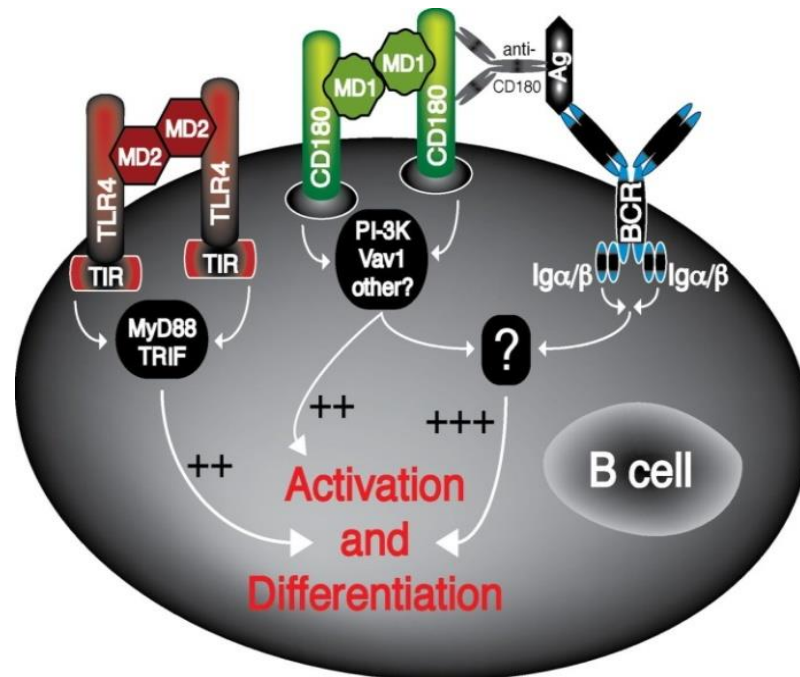
**Figure 1.15: Model to represent the mediation of LPS signalling in B lymphocytes.** B cells are activated by LPS through B-cell specific RP105 and universally expressed TLR4. Binding of LPS to RP105 induces Lyn activation and CD19 phosphorylation. Phosphorylated CD19 augments Lyn kinase activity and mediates the Lyn and Vav interaction, which is needed for JNK activation. PI3-kinase and BTK are activated independent of CD19 and regulate  $[Ca^{2+}]$  responses. LPS binding to TLR4 activates MyD88/IRAK and MyD88-independent TIRAP pathways to activate JNK and NF- $\beta$  (Yazawa *et al.*, 2003).

Src-family protein tyrosine kinase Lyn, Protein kinase C  $\beta$  I/II (PKC $\beta$ I/II) and ERK2-specific mitogen-activated protein (MAP) kinase (MEK) are essential for CD180 mediated signalling cascade (Chan *et al.*, 1998). Involvement of these molecules in both sIgM and CD180 mediated activation suggest the existence of common signalling pathway(s) for both receptors since BTK and PKC  $\beta$ I/II contribute to anti-CD180 and IgM-mediated B cell activation.

### 1.12 Cross-talk between CD180 and B cell receptor (BCR)

Previous reports suggested that sequential engagement of the BCR and intracellularly expressed TLR9 (Leadbetter *et al.*, 2002) and TLR7 (Lau *et al.*, 2005) can augment B-cell activation. CD69 expression in primary B cells is upregulated by antigen and LPS whereas co-engagement of these receptors induces a synergistic B-cell response (Minguet *et al.*, 2008). It was shown that specific Ag must be linked to anti-CD180 to induce IgG Ab responses suggesting

the requirement of both BCR and CD180 ligation on the same cell, to produce specific Ab (Chappell *et al.*, 2014).



**Figure 1.16: Role of CD180 and TLR4 in promoting B cell activation and differentiation together with BCR.** The extracellular domains of CD180 and TLR4 have 61% sequence similarity and 29% sequence identity and interact with 25 kDa molecules MD-1 and MD-2, respectively. CD180 can activate PI-3K and Vav-1, and synergise with BCR signalling via an unidentified pathway to induce B-cell activation and differentiation (Chappell *et al.*, 2014).

Interaction of R-CLL cells with the microenvironment through CD180 may contribute to expansion of leukaemic clones *in vivo*, in lymph nodes and bone marrow within "proliferation centres" (PCs). The ligand(s) for CD180, soluble or cell bound, in the PCs, could act as co-stimulatory molecules to BCR signalling together with CD40/CD40L and cytokines to provide signals for CLL cell expansion and survival. The classical BCR pathway is regulated by the signalosome proteins (PI3K, BTK, BLNK, PLCγ and PKC) and leads to the elevation of phosphorylated ERK (Mizuno and Rothstein, 2005). Therefore, other pro-survival signals such as those from CD180 may rewire the BCR signalling in CLL cells. Roshak *et al.*, (1999) observed B lymphocyte proliferation in humans after inducing it with anti-human RP105 sera. Exposure to anti-CD180 sensitised murine B cells to anti-IgM-mediated apoptosis and growth arrest (Yamashita *et al.*, 1996). Porakishvili *et al.*, (2009) demonstrated for the first time that CD180 modulates BCR-signalling. It is important therefore to study the effect of CD180

ligation on regulation of apoptosis of CLL cells and how it impinges on BCR mediated signalling.

Since the microenvironment plays a significant role in development and progression of CLL, this study was focussed on to understand the role of local microenvironment of CLL through interaction of surface molecules such as BCR and CD180. CD180 plays a major role in CLL cells in interacting with their microenvironment. Thus, the interaction of R-CLL cells with the microenvironment through CD180 may contribute to expansion of leukaemic clones, *in vivo*, in lymph nodes and bone marrow within "proliferation centres" (PCs). CD180 ligation with putative endogenous or exogenous ligand would, therefore, contribute to the pro-survival intracellular signalling in CLL cells. Hence, it was a major interest to study the effect of CD180 ligation on protecting CLL cells from apoptosis and how it impinges on BCR mediated signalling. This project would, therefore, help to define mechanisms leading to the expansion of the leukaemic cells and progression of the disease through interaction of CLL cells with its microenvironment and thus to contribute to developing new therapies of CLL.

### **The major aims of the research**

#### ***Aim 1. To study CD180-mediated intracellular signalling pathways in CLL***

To define transduction pathways of CD180-mediated signal downstream of ZAP70/Syk, the phosphorylation of intracellular protein kinases BTK, AKT and p38MAPK was assessed upon stimulation with anti-CD180. This will be discussed in chapter 3.

#### ***Aim 2. To evaluate the modulatory effect of CD180 ligation on sIgM-mediated signalling pathways***

To assess whether CD180 ligation can modulate the intensity of BCR signalling and whether the two pathways are functionally linked, CLL cells were pre-treated with anti-CD180 mAb followed by the sIgM ligation and *vice versa* and phosphorylation of intracellular protein kinases was assessed as described in Aim 1. This will be discussed in chapter 3.

#### ***Aim 3. To assess regulation of apoptosis of CLL cells following CD180 ligation***

Specific inhibitors of the CD180-mediated signalling pathways as in Aim 1 (BTK, AKT and p38MAPK), were used to block specific intracellular pathway alone and

in combination to study their involvement in CD180-mediated regulation of apoptosis. This will be discussed in chapter 4.

***Aim 4. To assess the spontaneous modulation of CD180 expression and modulation of CD180 expression upon anti-IgM stimulation***

To evaluate modulation of expression patterns of CD180 on unstimulated CLL cells and when stimulated with anti-IgM, cells were immunophenotyped at baseline hour and following 24, 48 and 72 hours anti-IgM stimulation. At the same time, CLL cells were left unstimulated in medium to immunophenotype for CD180 at each time point. This will be discussed in chapter 5.

**Chapter 2**  
**Materials and methods**

## 2.1 Patients and CLL samples

90 patients with CLL, aged between 47-89 years (median age 67.9 years) were included in the study. Patients were mainly Binet stage A and Rai stage- Low risk. The stages and white blood cell count of patients are shown in the table 1. From this cohort, 44 patients have been identified as IGHV M-CLL and 37 patients as IGHV unmutated U-CLL. The genotype of the remaining 9 patients was unknown. Patients were considered as untreated if they had received no treatment during the 6 months prior to the study. Twenty-four patients during the course of the study underwent treatment with a variety of therapeutic agents including chlorambucil (n=8), fludarabine (n=7), Ofatumumab + CAL101 (n=2), rituximab (4), Campath (2) and Ibrutinib (n=1). Ten age-matched (50-78 years, median age 63.5 years) healthy volunteers served as controls.

Ethical approval was obtained from the ethical committee at University College London Hospital (UCLH, 08/H0714/6), NHS trust. Prior to the study, the informed consent was obtained from the patients under the care of Professor Amit Nathwani, UCL CLL clinic. The University of Westminster ethical committee approval was obtained regarding MTA Material Transfer Agreement (MTA) with UCL.

**Table 2.1: CLL stages of the patient cases studied and the corresponding WBC counts**

<b>Binet stage</b>	<b>Number of patients</b>	<b>WBC count <math>\times 10^9/L</math> Range</b>
Rai-Low Risk Binet stage A	42 11	8.5–100.1
Rai-Intermediate Risk	20	6.6–196.0
Rai-High Risk	13	1.1-200.4

## 2.2 Isolation of peripheral blood mononuclear cells (PBMC)

Six to ten ml of whole blood were taken from CLL patients into heparinised test tubes by specially qualified staff at UCLH CLL clinic. Hanks' buffered salt solution (HBSS) and Histopaque 1077 (Sigma, UK) density medium were allowed to equilibrate to room temperature before the experiment. After dilution of the samples in an equal volume of HBSS, 6-8 ml of blood sample was carefully

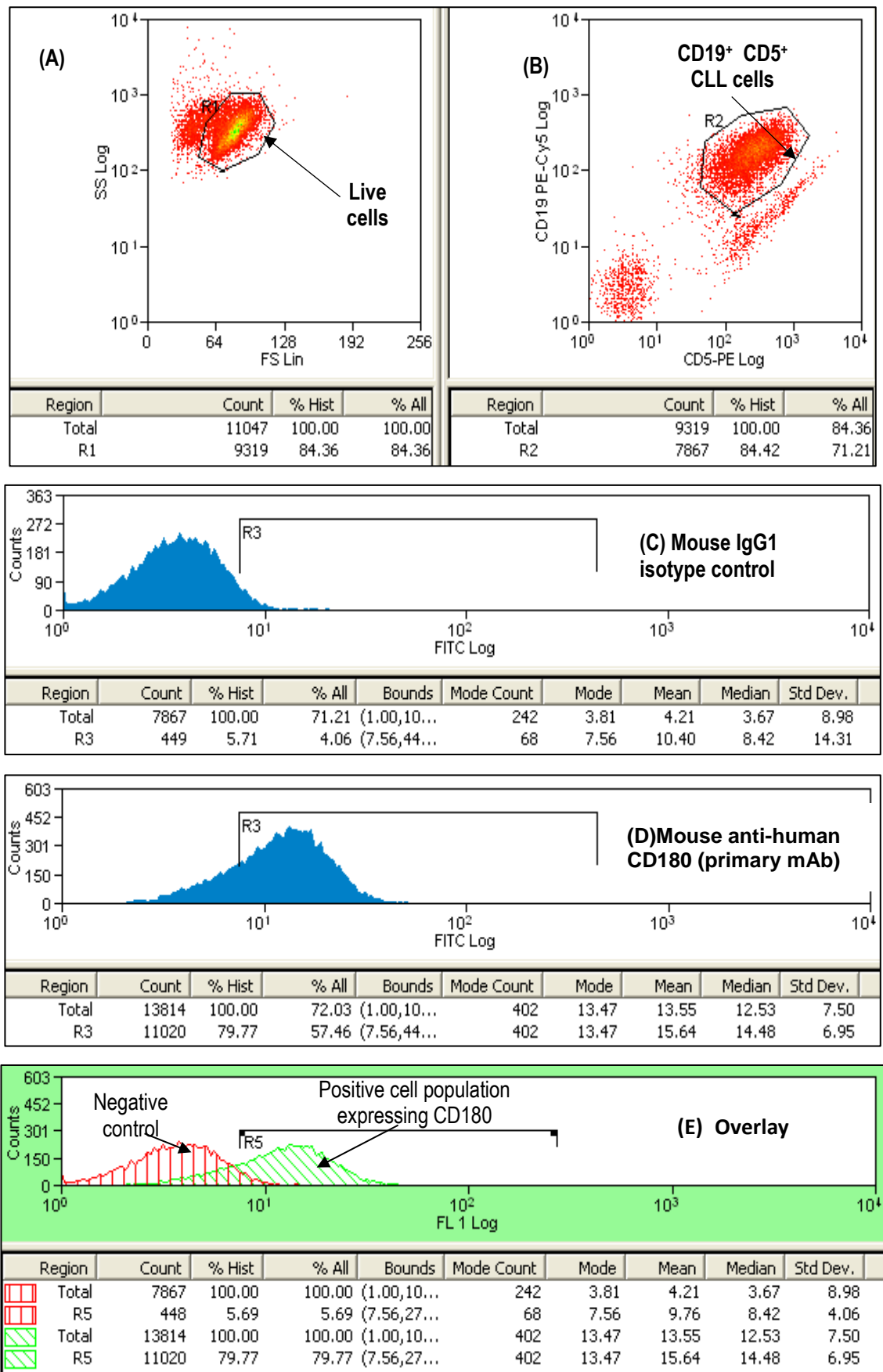


layered onto 3 ml density medium. Upon centrifugation (5810 R centrifuge Eppendorf, UK) at 400 g for 30 min at 22 °C, PBMCs were collected by aspiration of the interphase. The cells were washed twice with 5 ml of HBSS, by centrifuging for 10 min, 400 g at 4 °C. The cells were resuspended in 1ml of Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 10% w/v foetal bovine serum (FBS) (both from Sigma-Aldrich, UK). The lymphocytes were counted using a haemocytometer (Millipore, UK) and cell concentration was adjusted to  $10^6$  cells/ml by resuspending in RPMI-1640.

### **2.3 Immunophenotyping**

200µl of PBMC suspension was added into each well of a 96 well round bottom microplate (Corning, VWR UK) at a concentration of  $10^6$ /ml. The plate was centrifuged for 5 min, 400 g at 4 °C. The supernatant was discarded and the pellet was vortexed (Rotamixer, UK). In order to prevent non-specific binding of the specific monoclonal antibodies (mAb) via Fc receptors, 20 µl of 2 mg/ml human immunoglobulin (Ig) (ImmunoReagents, UK) was added into each well and the plate was incubated for 30min on ice. The plate was centrifuged for 5 min, 400 g at 4 °C, supernatant was removed and vortexed. 20 µl of the following mouse anti-human mAbs at a final concentration 20 µg/ml was added into designated wells: IgG1 isotype control, anti-CD180 (IgG1 clone G28-8), anti-IgM, anti-CD79b, anti-CD38, anti-CD86 and anti-CD40 (all - BD Pharmingen, UK), and anti-IgD (Sigma, UK). Cells were incubated for 30 min on ice and washed twice with 200 µl HBSS for 5 min, 400 g at 4 °C. As the secondary antibody, 20 µl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig (Dako, UK), at an optimal dilution 1:15 in Phosphate Buffered Saline solution (PBS) (Sigma, UK) was added into each well. The plate was incubated in the dark on ice for 30 min, centrifuged at 400 g and cells were washed twice as before. The cells were further incubated with 20 µl of mouse serum (Dako, UK) for 30 minutes on ice in the dark (1:15 diluted in PBS) to block any free rabbit anti-mouse F(ab) sites and centrifuged for 5 min, 400 g at 4 °C. The supernatant was removed, vortexed and 5 µl of phycoerythrin cyanine 5 conjugated (PE-Cy5) mouse anti-human CD19 monoclonal antibody (mAb) (BD Pharmingen, UK) and 5 µl of phycoerythrin cyanine 5 conjugated (PE-Cy5) mouse anti-human CD5 monoclonal antibody (mAb) (BD Pharmingen, UK) were added and incubated for 30 min on ice in the dark. Cells were washed twice as before and fixed with 200 µl of 2% w/v paraformaldehyde (PFA) (Sigma, UK) in PBS and stored (no more than 4 days)

at 4 °C until analysis by flow cytometry using Cyan (Beckman Coulter, UK) flow cytometer and Summit software v4.3. The results were expressed as percentages of positive cells as well as antibody relative binding sites (RBS)/cell as previously described (Guyre *et al.*, 1989; Porakishvili *et al.*, 2005). This is a method of evaluating the level of a cell surface molecule, reflecting its density on the cell membrane. Briefly, the number of secondary antibody-binding sites (RBS) per cell versus isotype control was determined by comparison with the mean fluorescence intensities to a standard curve generated with fluorescent microspheres (Dako Cytomation, UK) containing beads with five different levels of fluorochrome molecules per bead as described earlier (Porakishvili *et al.*, 2005). In some cases, the relative mean fluorescence intensity (RMFI) was used as a measure of positivity (RMFI = MFI of sample/MFI of isotype control). CLL clones were then categorised as CD180+ and CD180- for future studies, or double positive for CD180 and surface IgM (sIgM).



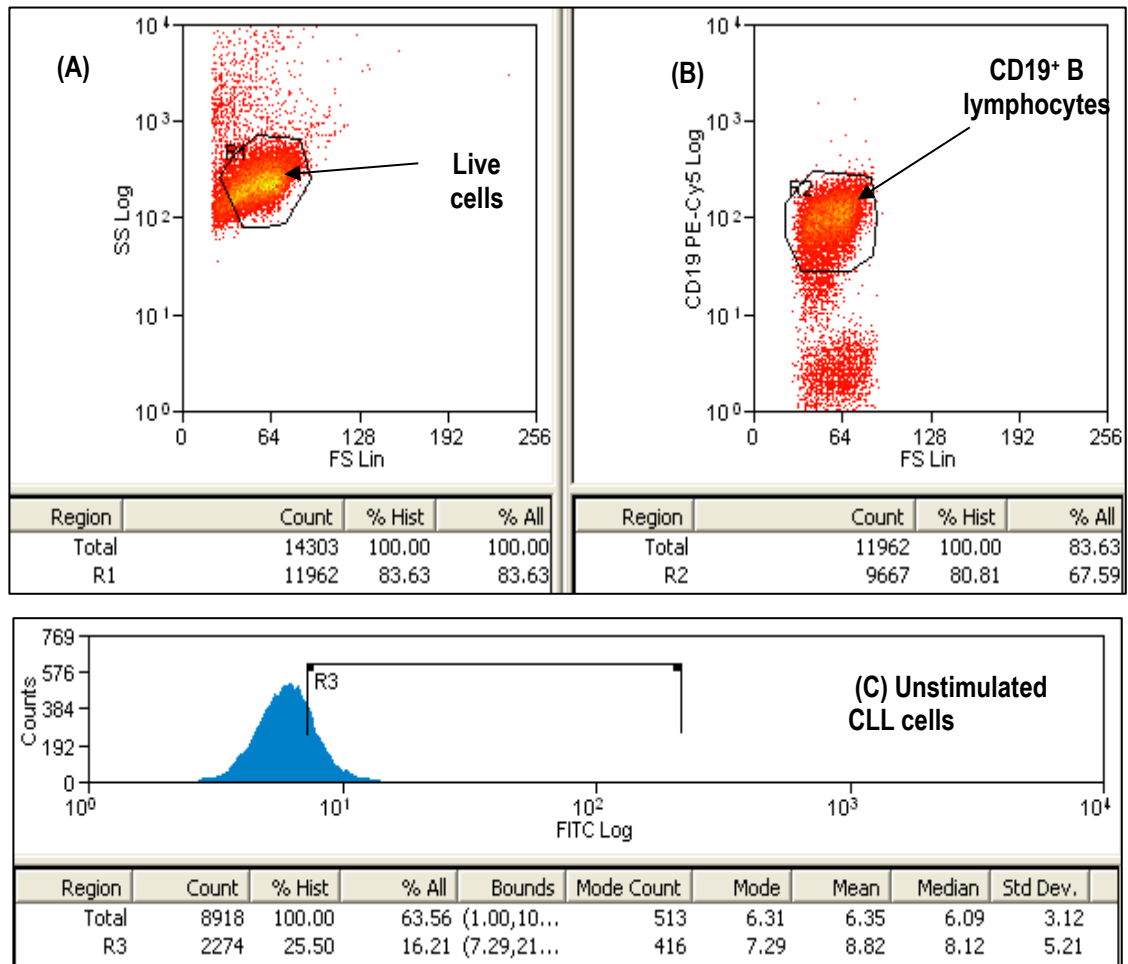
**Figure 2.1: Representative flow cytometry image of dot plots, histograms and the overlay of phenotyping of PBMCs from a CLL patient. The CLL sample was incubated with human immunoglobulin (Ig), stained with mouse IgG1k isotype**

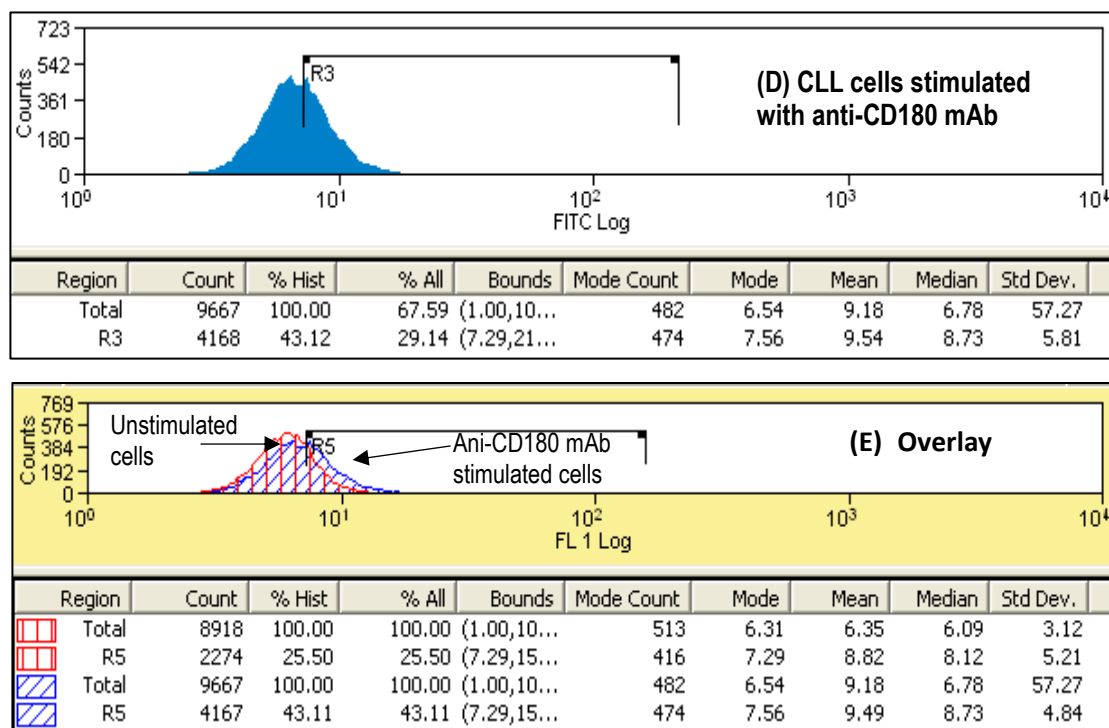
control or mouse anti-human CD180 (primary mAb). Following the incubation with mouse serum, the sample was stained with FITC-conjugated rabbit anti-mouse Ig (secondary mAb), PE-Cy5-conjugated mouse anti-human CD19 and PE-conjugated mouse anti-human CD5. The sample was analysed by CYAN flow cytometer and Summit (4.3) analytical software. The gating of the first dot plot represents live cells (Panel A) and the second gating represents CD19 and CD5 positive CLL cells (Panel B). The first histogram shows the negative population of cells (Panel C) and the second histogram shows CD180<sup>+</sup> cell population (Panel D). The overlay plot shows the CD180 positive CLL cells compared with the negative control cells inside CD19<sup>+</sup> gated population (Panel E).

## **2.4 Phosphorylation of intracellular protein kinases**

200 µl of isolated PBMCs from CD180+sIgM<sup>+</sup> CLL samples and PBMCs from healthy volunteers (concentration of 1x10<sup>6</sup> cells/ml in RPMI+10% w/v FBS) were added to 96 well flat bottom microplates (Corning, VWR UK) and incubated with 8µl of sodium azide free anti-CD180 mAb (BD Pharmingen, UK) or goat anti-human IgM F(ab)<sub>2</sub> (Southern Biotech, Birmingham, USA) under sterile conditions at a final concentration of 20 µg/ml and incubated at 37 °C, 5% CO<sub>2</sub> for 30 min (short-term cultures) or 24 hours (long-term cultures). In separate experiments, cells were sequentially incubated with anti-CD180 for 20 min followed by anti-IgM F(ab)<sub>2</sub> for 10 min or *vice versa*. The optimal stimulation time for short-term cultures has been identified in previous experiments of our research group (Porakishvili *et al.*, 2011). Unstimulated cells were used as negative controls. Upon stimulation, cells were transferred into 96 well round bottom plates, centrifuged at 400 g, 5 min at 4 °C and the supernatant was removed. Cells were washed twice with HBSS as before, the supernatant was discarded, vortexed and cells were stained with 5 µl of PE-Cy5-conjugated anti-CD19 mAb and incubated for 30 min on ice in dark. After two washes, as before, cells were fixed with 50 µl of solution A of Fix/Perm kit (ADG, Kaumberg, Austria) by incubating in the dark at room temperature for 15 min. The cells were washed once with HBSS as before and permeabilised with 50 µl solution B of the Fix/Perm kit. Cells were incubated for another 15 min at room temperature in the dark and centrifuged at 400 g, 5 min at 4 °C. The supernatant was discarded, vortexed and cells were further stained with either of the following anti-human antibodies to phosphorylated (P) intracellular protein kinases: anti-AKT-P Alexa Fluor-488 (FITC conjugated) rabbit mAb, anti-p38MAPK-P Alexa Fluor-488 (FITC conjugated) mouse mAb (Cell Signaling, Danvers, MA, USA), anti-BTK-P Alexa

Fluor-647 (PE-conjugated) mouse mAb (BD Pharmingen, UK) and incubated at room temperature for 30 min in the dark. After incubation, the cells were washed once with HBSS as above, fixed with 200µl 2% v/v paraformaldehyde (PFA) and stored in the dark (for ≤ 24h) at 4 °C for flow cytometry. The results of the experiment were expressed as percentages of positive cells inside gated CD19<sup>+</sup> cell population.





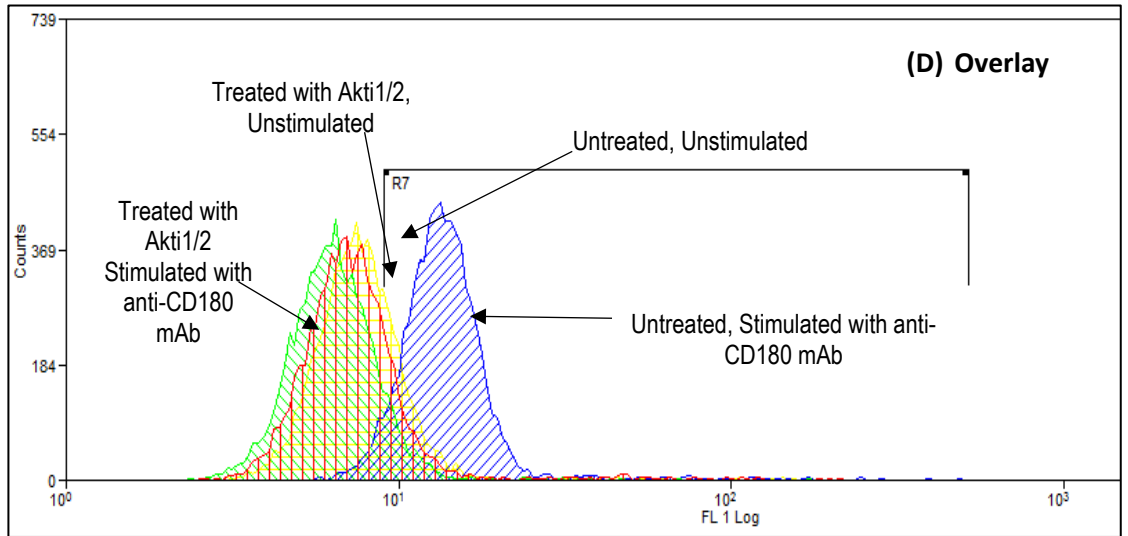
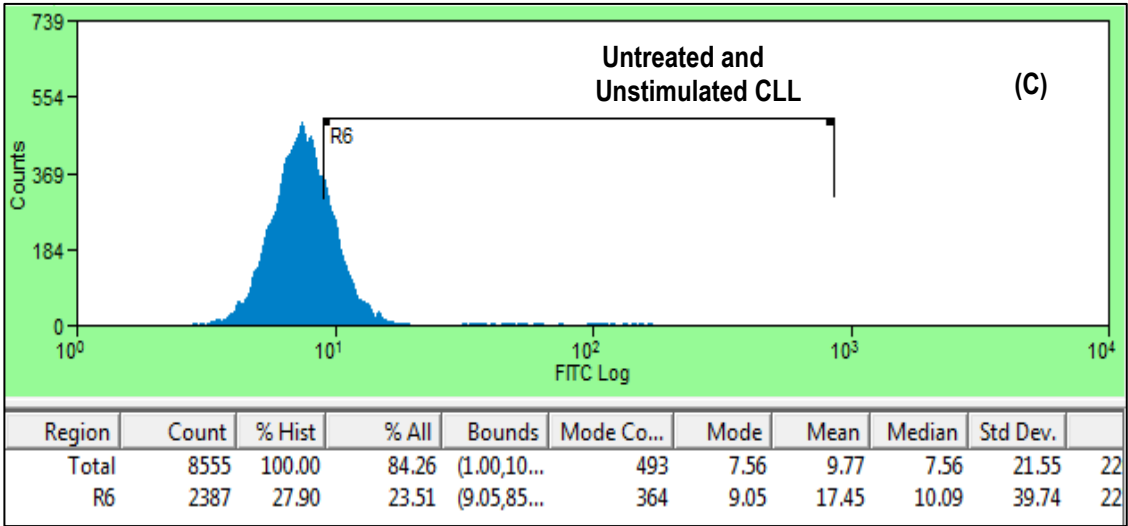
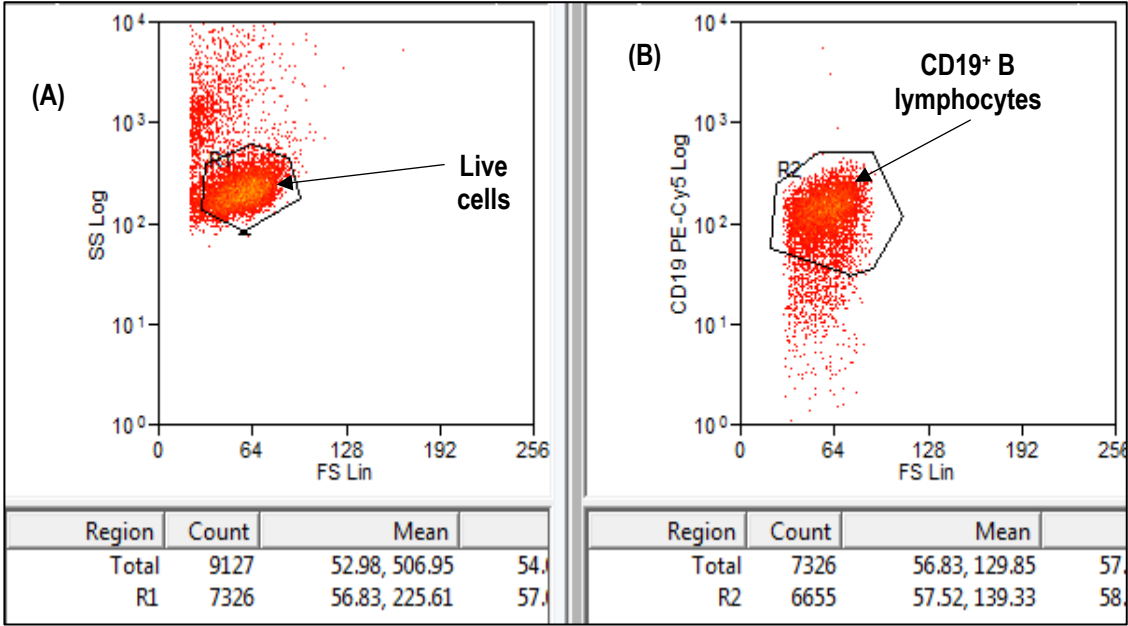
**Figure 2.2: Representative flow cytometry image of dot plots, histograms and the overlay of Phosphorylation of AKT protein kinase of PBMCs from a AKT-S CLL patient.** CLL cells were left unstimulated in medium or incubated with anti-CD180 mAb for 20 min (CD180 mAb), washed, stained with anti-CD19 mAb, fixed, permeabilised, stained with anti-AKT(Ser473)-P as described in 2.4 of Materials and methods, analysed by CYAN flow cytometer and Summit 4.3 analytical software and expressed as percentages of positive cells inside gated CD19<sup>+</sup> population. The gating of the first dot plot represents live cells (Panel A) and the second gating represents CD19 positive B lymphocytes (Panel B). The first histogram shows CLL cell population with phosphorylated AKT (p-AKT) when unstimulated (Panel C) and the second histogram shows CLL cell population with p-AKT when stimulated with anti-CD180 mAb (Panel D). The overlay plot shows CLL cell population with p-AKT when stimulated with anti-CD180 mAb compared with unstimulated cells (Panel E).

## 2.5 Analysing the effect of protein kinase inhibitors on phosphorylation of corresponding protein kinases by flow cytometry

200  $\mu$ l of isolated PBMCs (concentration of  $1 \times 10^6$  cells/ml in RPMI+10% FBS) were added to 96 well flat bottom microplates. In order to assess the optimal concentrations of protein kinase inhibitors, pilot experiments were carried out with range of concentrations. Final concentration of 5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M was tested for SB203580, 2  $\mu$ M, 5  $\mu$ M and 10  $\mu$ M was tested for Akti1/2 and 2  $\mu$ M, 5  $\mu$ M and

10  $\mu$ M was tested for Ibrutinib inhibitor (data not shown). CD180+ CLL cells from CD180+ patients were treated with protein kinase inhibitors as follows; 4  $\mu$ l of p38 MAPK inhibitor, SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) (Sigma, UK) was added at a final concentration of 20  $\mu$ M (Wilk-Blaszczal *et al.*, 1998) and incubated at 37°C, 5% CO<sub>2</sub> for 2 hours (Young *et al.*, 1997).

2  $\mu$ l of AKT 1/2 kinase inhibitor (1,3-Dihydro-1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl) phenyl) methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate, Akt Inhibitor VIII trifluoroacetate salt hydrate, Akti-1/2 trifluoroacetate salt hydrate) (Sigma, UK) was added at a final concentration of 5  $\mu$ M and incubated at 37°C, 5% CO<sub>2</sub> for 2 hours (Frias *et al.*, 2009). 2  $\mu$ l of BTK kinase inhibitor, Ibrutinib (PCI-32765), (Selleckchem, UK) was added at a final concentration of 10  $\mu$ M and incubated at 37°C, 5% CO<sub>2</sub> for 2 hours (Herman *et al.*, 2011). Cells were transferred into 96 well round bottom plates centrifuged at 400 g, for 5 min at 4 °C, the supernatant was removed and vortexed. Cells were washed twice with HBSS as before, the supernatant was discarded and vortexed. The pellet was resuspended in 200  $\mu$ l of RPMI and transferred into a 96 well flat bottom microplate. Non-treated CLL cells were used as negative controls. Both treated and untreated CLL cells were incubated with 8  $\mu$ l of sodium azide free anti-CD180 mAb under sterile conditions at a final concentration of 20  $\mu$ g/ml and incubated at 37°C, 5% CO<sub>2</sub> for 30 min. Unstimulated CLL cells were used as negative controls. After the stimulation, the rest of the experiment was carried out as explained above in section 2.4.



**Figure 2.3: Representative flow cytometry image of dot plots, histogram and the overlay of Phosphorylation of AKT protein kinase of PBMCs from a AKT-S CLL patient showing the effect of Akti1/2 treatment. CLL cells were**



incubated with Akti1/2 inhibitor for 2 hrs, washed and left unstimulated in medium or incubated with anti-CD180 mAb for 20 min (CD180 mAb). The sample was washed, stained with anti-CD19 mAb, fixed, permeabilised, stained with anti-AKT(Ser473)-P as described in 2.4 of materials and methodology, analysed by CYAN flow cytometer and Summit 4.3 analytical software and expressed as percentages of positive cells inside gated CD19<sup>+</sup> population. The gating of the first dot plot represents live cells (Panel A) and the second gating represents CD19<sup>+</sup> B lymphocytes (Panel B). The histogram shows the CLL cell population with phosphorylated AKT (p-AKT) when untreated with Akti1/2 and unstimulated (Panel C). The overlay plot shows the comparison of CLL cell population with p-AKT, stimulated with anti-CD180 mAb when untreated/treated with Akti1/2 and CLL cell population with p-AKT when unstimulated and untreated/treated with Akti1/2 (Panel D).

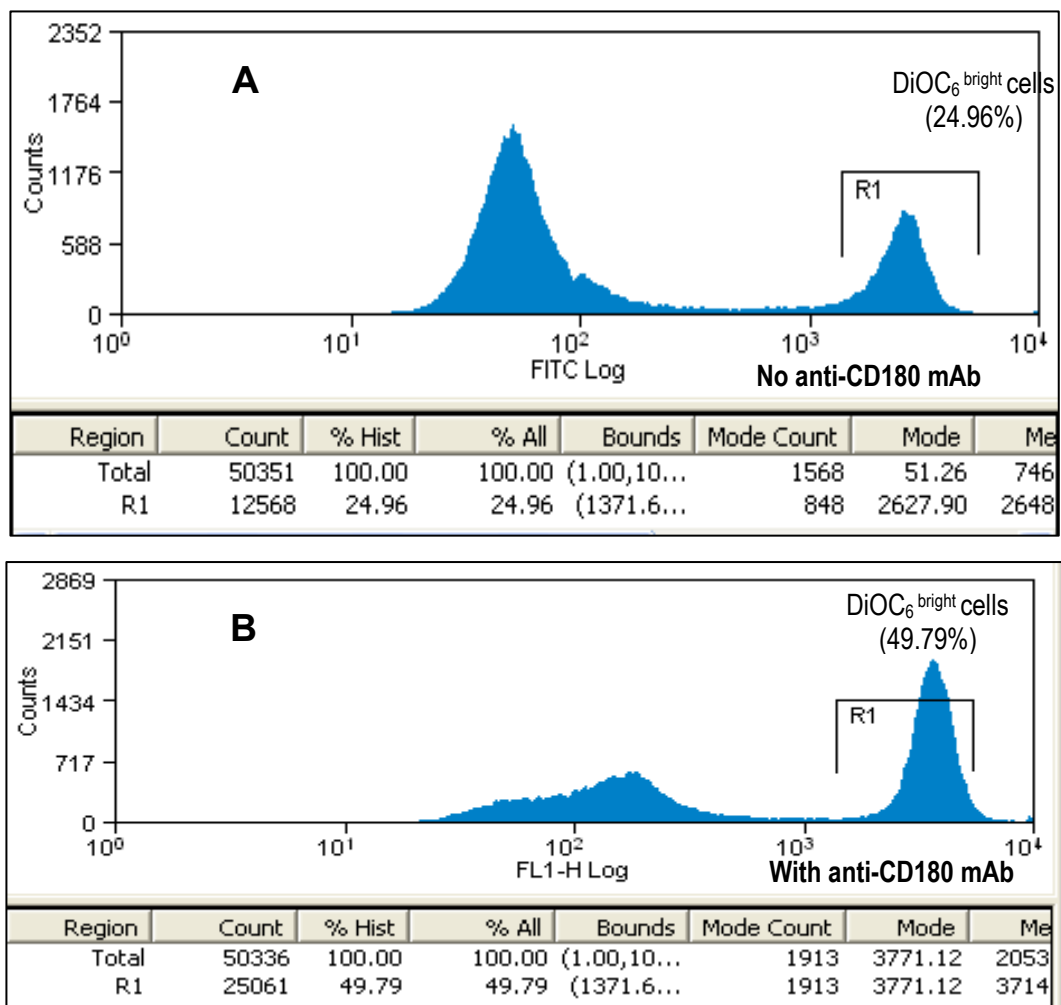
## **2.6 Apoptosis assay**

### **2.6.1 Assessment of apoptosis by the changes in mitochondrial membrane potential**

3,3'-dihexyloxocarbocyanine iodide (DiOC<sub>6</sub>) is a fluorescent dye that discriminates between bright fluorescent viable and dim fluorescent apoptotic cells upon exposure to blue light (482nm) which excites DiOC<sub>6</sub> to fluoresce green (504nm) (Koning et al., 1993). Mitochondrial membrane potential ( $\Delta\psi_m$ ) is an important parameter of mitochondrial function and an indicator of cell health. Depletion of  $\Delta\psi_m$  suggests the loss of mitochondrial membrane integrity and thus the initiation of the proapoptotic signal. A cell-permeant, lipophilic dye, DiOC<sub>6</sub> accumulates in mitochondria due to their large negative membrane potential and can be applied to monitor the mitochondrial membrane potential using flow cytometric detection (Chang *et al.*, 2013). The optimum concentration of DiOC<sub>6</sub> was previously determined by our research group (Porakishvili et al., 2005). DiOC<sub>6</sub> is reported to be a useful probe for labelling endoplasmic reticulum and our research group has confirmed the validity of assessing mitochondrial membrane potential of CLL cells using DiOC<sub>6</sub> compared to annexin V-FITC (data not shown).

200  $\mu$ l of PBMCs at a concentration 10<sup>6</sup> cells/ml in RPMI+10% FBS were stimulated with 8  $\mu$ l of sodium azide free anti-CD180 mAb as above for 24 h in 96 well flat bottom microplates. Non-stimulated cells were used as negative controls. Upon stimulation, cells were transferred into 96 well round bottom microplates, centrifuged, washed once as described above, stained with 5  $\mu$ l of

PE-Cy5 conjugated anti-CD19 mAb and incubated for 30 min on ice in the dark. Cells were washed twice with HBSS as before, supernatant was removed and vortexed. Cells were resuspended in 200  $\mu$ l of HBSS and transferred into 96 well flat bottom microplates and probed with 20  $\mu$ l (0.2  $\mu$ mol/l) of DiOC<sub>6</sub> (3,3'-dihexyloxacabocyanine iodide; Molecular Probes, Eugene, USA). Cells were incubated for 20 min at 37°C, washed twice with HBSS, resuspended in HBSS and analysed by flow cytometry immediately. The level of apoptosis was determined by the percentages of DiOC<sub>6</sub><sup>dim</sup> cells gated on the CD19<sup>+</sup> population versus unstimulated cultures as previously described (Porakishvili *et al.*, 2009, 2011).

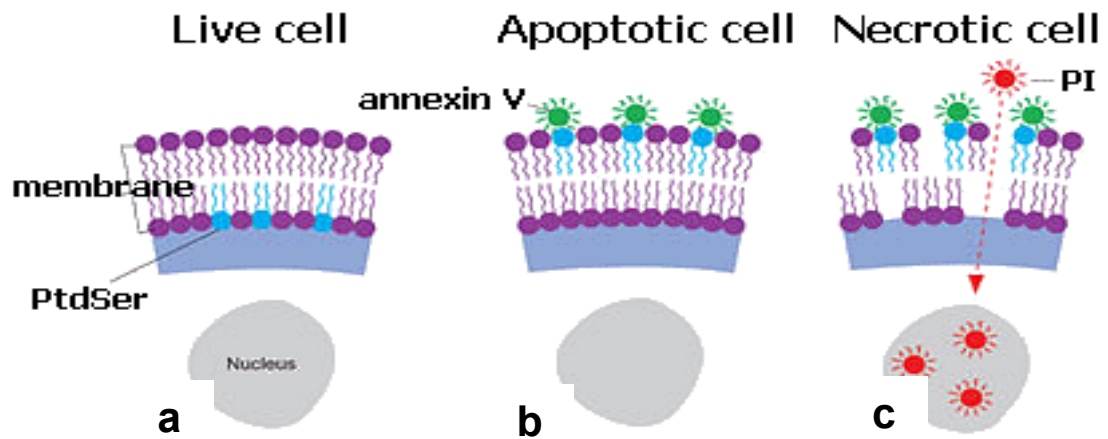


**Figure 2.4: Representative flow cytometric profiles of 24 h CLL cell cultures without CD180 antibody (A) and after incubating with anti-CD180 mAb (B) showing the percentages of DiOC<sub>6</sub><sup>bright</sup> cells gated on CD19<sup>+</sup> lymphocytes.** PBMC from CD180<sup>+</sup> CLL patients were cultured in the absence and presence of 10  $\mu$ g/mL anti-CD180 mAb for 24h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and loaded with 0.4  $\mu$ M of DiOC<sub>6</sub> Dye for 20 min at 37°C. Flow

cytometric analysis was performed immediately. DiOC<sub>6</sub><sup>bright</sup> cells were counted gating on CD19<sup>+</sup> population and identified as viable cells (Porakishvili *et al.*, 2011).

### **2.6.2 Assessment of apoptosis by the loss of phospholipid asymmetry in cell membranes**

The annexins are a group of proteins which bind to phospholipids in the presence of calcium (Pigault *et al.*, 1994; Trotter *et al.*, 1995). Annexin V-FITC is a fluorescent probe which binds to phosphatidylserine in the presence of calcium. The Mg-ATP-dependent enzyme, aminophospholipid translocase transports phosphatidylserine to the inside of the lipid bilayer in living cells (Trotter *et al.*, 1995) (Figure 2.5a). At the onset of apoptosis, phosphatidylserine, which is normally found on the internal part of the plasma membrane, becomes translocated to the external portion of the membrane and becomes available to bind to the annexin V-FITC conjugate in the presence of calcium. Therefore, the binding of annexin V-FITC to phosphatidylserine in the membrane of cells is an indication of the beginning of the apoptotic process (Figure 2.5b) and the binding of PI to the cellular DNA in cells indicates that the cell membrane has been totally compromised (Figure 2.5c).

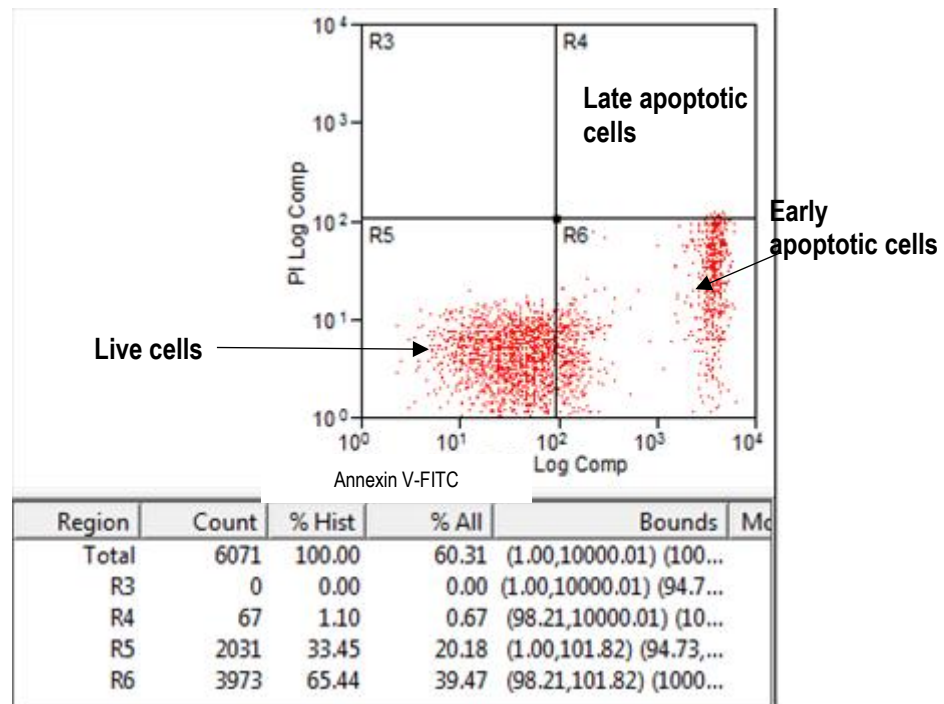


**Figure 2.5: Binding of Annexin V-FITC and Propidium Iodide (PI) at different stages of apoptosis.**

In apoptotic cells, the membrane phospholipid phosphatidylserine (PtdSer) is translocated from the inner to the outer leaflet of the plasma membrane thereby exposing PtdSer to the external cellular environment. Annexin V binds to cells with exposed PtdSer in the presence of  $\text{Ca}^{2+}$  thus acting as a sensitive probe for flow cytometry analysis of cells that are in the earliest stage of apoptosis. Viable and early apoptotic cells exclude PI, whereas the membranes of dead and damaged cells are permeable to PI hence acting as a probe to analyse cells that are in the end stage of apoptosis or necrosis (Givan, 2000).

200  $\mu\text{l}$  of PBMCs at a concentration  $10^6$  cells/ml in RPMI+10% FBS were stimulated with 8  $\mu\text{l}$  of sodium azide free anti-CD180 mAb as above for 24h in 96 well flat bottom microplates. Unstimulated cells were used as negative controls. Upon stimulation, cells were transferred into 96 well round bottom microplate, centrifuged, washed twice as described above, stained with 5  $\mu\text{l}$  of PE-Cy5 conjugated anti-CD19 mAb and incubated for 30 min on ice in the dark. Cells were washed twice with HBSS as before, the supernatant was removed and vortexed. Cells were resuspended in 200  $\mu\text{l}$  of binding buffer (diluted 1:10 in deionized water) and transferred into 96 well flat bottom microplates. Each well was incubated with 1  $\mu\text{l}$  of propidium iodide (PI, 100 $\mu\text{g}/\text{ml}$  in 10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) and 2  $\mu\text{l}$  of annexin V-FITC conjugate ( $\sim 50$   $\mu\text{g}/\text{ml}$  in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl). The optimum concentration of PI and Annexin V-FITC was previously determined in our laboratory (data not shown). Cells were incubated for exactly 10 min at room temperature in the dark and analysed by flow cytometry immediately. The level of

apoptosis was determined by the percentages of PI and Annexin V-FITC positive cells gated on the CD19<sup>+</sup> population versus unstimulated cultures.



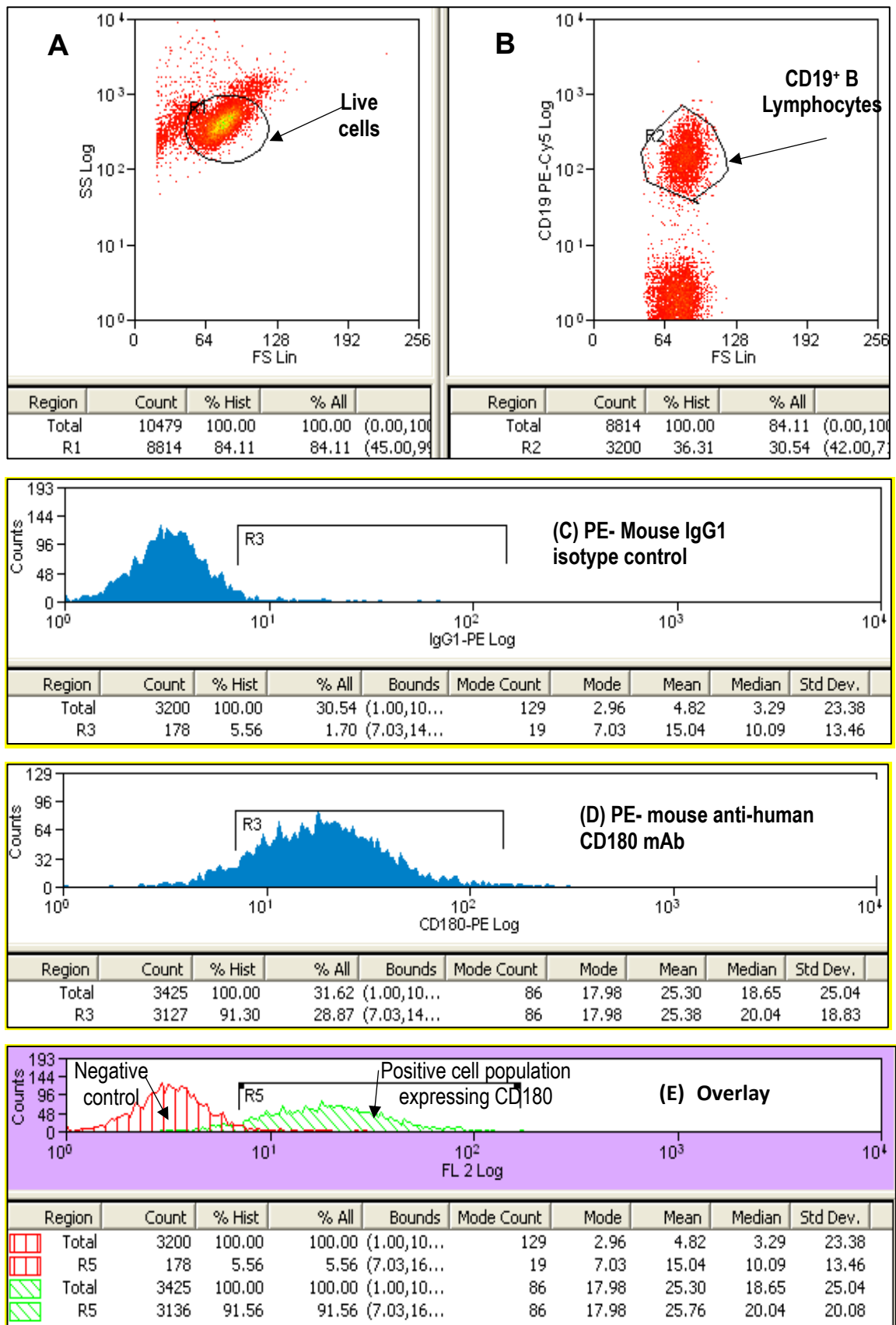
**Figure 2.6: Representative flow cytometry image of a dot plot of CLL cells at different stages of apoptosis.** CLL samples were stained with 5µl of PE-Cy5 conjugated anti-CD19 mAb, 1 µl of propidium iodide (PI) and 2 µl of annexin V-FITC conjugate upon stimulation with anti-CD180 mAb and incubated at 37°C, 5% CO<sub>2</sub> for 30 min. The sample was analysed by CYAN flow cytometer and Summit (4.3) analytical software. Viable cells are negative to both Annexin V-FITC and PI, early apoptotic cells are positive to Annexin V-FITC while negative to PI and cells that are in late apoptosis or already dead are positive to both Annexin V-FITC and PI.

## 2.7 Analysing the spontaneous and IgM ligated modulation of CD180 level of expression in CD180<sup>+</sup> and CD180<sup>neg</sup> CLL cells

In order to assess the level of CD180 expression *ex vivo*, 200 µl of isolated PBMCs from CD180<sup>+</sup> CLL samples (concentration of 1x10<sup>6</sup> cells/ml in RPMI+10% FBS) were added to 4 wells of 96 well flat bottom microplates. The plate was centrifuged for 5 min, 400 g at 4 °C. The supernatant was discarded and the pellet was vortexed. In order to prevent non-specific binding via Fc receptors, 20 µl of 2 mg/ml human immunoglobulin (Ig) was added into each well and incubated for 30 min on ice. The plate was centrifuged for 5 min, 400g at 4 °C, supernatant was removed and vortexed. 5 µl of PE-conjugated mouse IgG1 isotype control and 5µl of PE-conjugated mouse anti-human CD180 mAb (both from BD Pharmingen,

UK) was added (n=2). 5 µl of PE-Cy5 conjugated anti-CD19 mAb was added into all 4 wells. Cells were incubated for 30 min on ice in the dark and washed twice with 200 µl HBSS for 5 min, 400g at 4 °C. Cells were fixed with 200 µl of 2% PFA in PBS and stored (no more than 4 days) at 4 °C until analysis by flow cytometry using Cyan flow cytometer and summit software v4.3. The results of the experiment were expressed as percentages of CD180 positive cells gated on the CD19<sup>+</sup> population. In some experiments cells were incubated for 24, 48 and 72 hours at 37 °C, 5% CO<sub>2</sub> in 96 well flat bottom microplates, transferred into 96 well round bottom microplates, centrifuged at 400 g, 5 min at 4 °C and the supernatant was removed. Cells were washed twice with HBSS as before, the supernatant was discarded and vortexed. The same staining and analysis procedure was carried out as in the *ex vivo* experiment to analyse the percentages of CD180 positive cells gated on the CD19<sup>+</sup> population. Same procedure was followed again for CD180- CLL samples in order to compare the level of expression of CD180 *ex vivo* and after 24, 48 and 72 hr incubation.

In order to analyse the effect of IgM ligation on expression of CD180 on the CLL cells, 200 µl of isolated PBMCs from IgM positive CLL samples (concentration of 1x10<sup>6</sup> cells/ml in RPMI+10% w/v FBS) were added to 96 well flat bottom microplates. Cells were stimulated with goat anti-human IgM F(ab)<sub>2</sub> under sterile conditions at a final concentration of 20 µg/ml and incubated at 37 °C, 5% CO<sub>2</sub> for 24, 48 and 72 hours in 96 well flat bottom microplates, transferred into 96 well round bottom microplates, centrifuged at 400 g, 5 min at 4 °C and the supernatant was removed. Cells were washed twice with HBSS as before, the supernatant was discarded and vortexed. The same staining and analysis procedure was carried out as before.



**Figure 2.7: Representative flow cytometry image of dot plots, histograms and the overlay of phenotyping by direct staining for CD180 expressed on PBMCs from a CLL patient. CLL sample was incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse**

anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19. The sample was analysed by CYAN flow cytometer and Summit (4.3) analytical software. The gating of the first dot plot represents live cells (Panel A) and the second gating represents CD19 positive B lymphocytes (Panel B). The first histogram shows the negative population of cells (Panel C) and the second histogram shows CD180<sup>+</sup> cell population (Panel D). The overlay plot shows the CD180 positive CLL cells compared with the negative control cells inside CD19<sup>+</sup> gated population (Panel E).

## **2.8 Statistical analysis**

The results obtained during the course of the project were analysed statistically using appropriate methods. The following statistical methods were applied where required;

Standard parametrical tests:

Pearson's correlation coefficient using SPSS software (provided by the University of Westminster, IT systems, UK)

Student *t*-test (MS excel)

Non-parametrical tests:

Man-Whitney *U*-test using MINITAB software (provided by the University of Westminster, IT systems, UK)

Wilcoxon's signed-rank test using SPSS software

P values <0.05 were considered to be statistically significant.



## **Chapter 3**

# **BCR and CD180 mediated signalling in CLL cells and crosstalk between them**

### 3.1 Introduction

Our group has previously shown that CD180 is expressed heterogeneously in CLL cells whilst its expression in the majority of CD19<sup>+</sup> control B cells is high (5548±2271 RBS/cell, range 2392-13870 RBS/cell) compared to CD180<sup>+</sup> CLL cells (1500±932 RBS/cell, range 499-4287 RBS/cell). Classification of CLL cells as either CD180 positive or negative was based on the levels of anti-CD180 antibody RBS on a small CD180 negative population seen in the control CD19<sup>+</sup>B cells: 316 ± 88 RBS/cell, range 201–470 RBS/cell. The upper limit for the negative population was determined as mean + 2SD (Porakishvili *et al.*, 2005). The percentages of CD180-negative B cells in control B cell population was recorded as [mean ±SD (range)] 2.6 ±1.5% (0.8-7.3%) (Koarada *et al.*, 2001). The ratio between CD180<sup>+</sup> versus CD180<sup>neg</sup> CLL cell samples obtained from individual patients was approximately 2:1.

In the initial study, no difference between CD180 expression was detected between untreated patients, patients who have been treated 6 months prior to the research or those undergoing various treatments (Porakishvili *et al.*, 2005). It was also reported that the expression of CD180, sIgM, CD79b and CD40 on both, M-CLL and U-CLL cells was significantly lower than in control B cells. Whilst there were no significant differences in the expression of CD79b, CD38, CD86 or CD40 on U-CLL and M-CLL cells it was interesting to note that CD38<sup>+</sup> U-CLL samples expressed a higher density of CD180 compared with CD38<sup>neg</sup> U-CLL samples, but this was not observed in M-CLL samples (Porakishvili *et al.*, 2005). Most importantly in this publication, it was demonstrated that U-CLL samples were characterised by the significantly higher expression of sIgM and significantly lower expression of CD180, compared to M-CLL cells.

Porakishvili *et al.*, (2005) further subdivided CD180<sup>+</sup> CLL samples into two groups depending on their responsiveness to CD180 ligation reflected in their activation and proliferation. Those CD180<sup>+</sup> CLL clones which responded to CD180 ligation by a significant increase in the expression of CD86 and Ki-67 were termed as responders, (R) whereas CD180<sup>+</sup> CLL cells which failed to respond to anti-CD180 mAb by elevation of CD86 and Ki-67 were termed non-responders, (NR).

These results on activation and cycling of CLL cells upon stimulation with anti-CD180 mAb were consistent with previous studies which demonstrated the ability of anti-CD180 mAb to activate normal human B lymphocytes and murine B cells

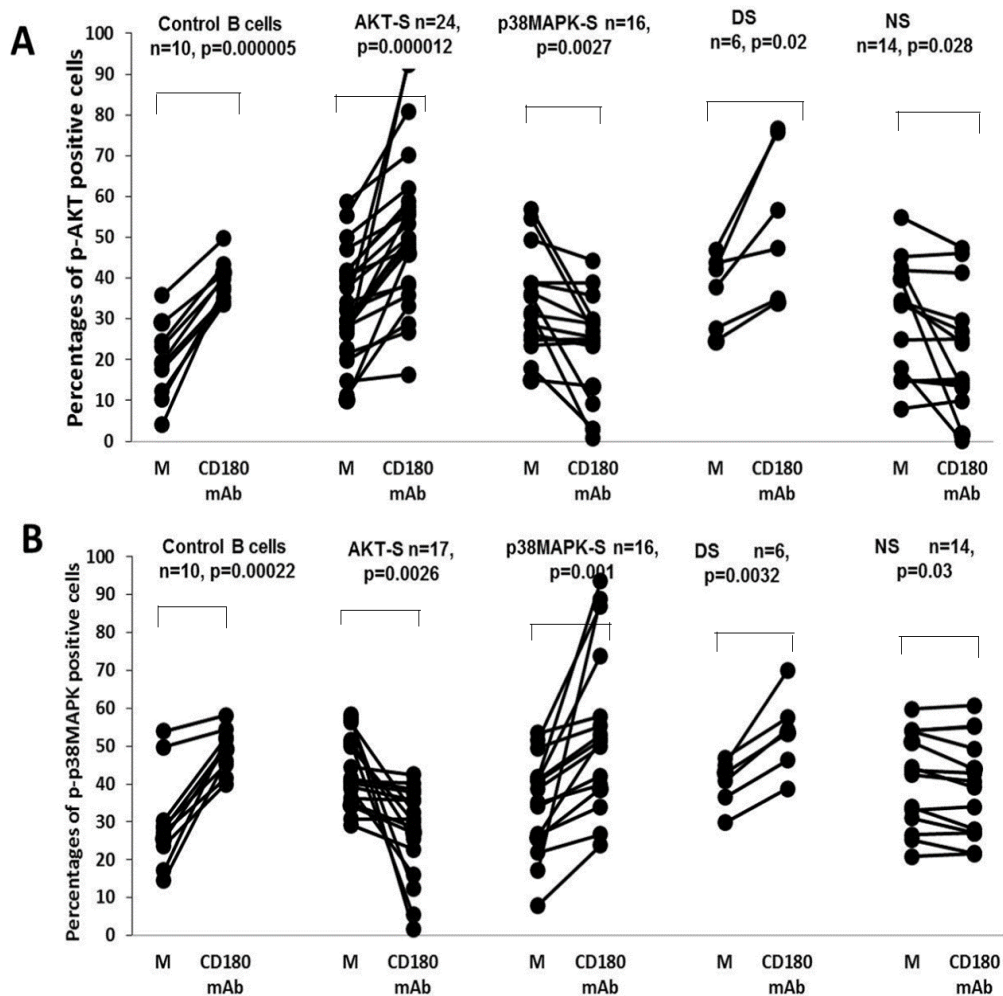
(Valentine *et al.*, 1988; Chan *et al.*, 1998). CD180 ligation led to comparable or even greater levels of activation and proliferation of normal B cells and R-CLL cells than optimal concentrations of anti-CD40 mAb or recombinant IL-4 (rIL-4) (Porakishvili *et al.*, 2005). The role of CD40 ligation in CLL has been reported in previous studies. CD40 ligation has been shown to both stimulate proliferation of CLL cells and protect against apoptosis by inducing cMyc (Clark *et al.*, 1989; Mentz *et al.*, 1996). CD40 ligation also led to upregulation of anti-apoptotic proteins of the B-cell lymphoma (Bcl) family and cellular FADD-like IL-1 $\beta$ -converting enzyme (FLICE)-inhibitory protein c-FLIP (Liu *et al.*, 1989; Tuscano *et al.*, 1996). IL-4 protects B cells and CLL cells from spontaneous-, cytokine-, drug-induced (Dancescu *et al.*, 1992; Mentz *et al.*, 1996; Mainou-Fowler *et al.*, 2001) and Fas-mediated (Foote *et al.*, 1996) apoptosis.

Since CD180 lacks the TIR intracellular signalling domain and has only 11 intracellular amino acids (Miyake *et al.*, 1995; Miura *et al.*, 1996; Fugier-Vivier *et al.*, 1997; Roshak *et al.*, 1999), it may either recruit an unidentified B cell specific protein for upstream signalling (Yazawa *et al.*, 2003) or cooperate/converge with other signalling pathways. In order to identify the signalling pathway(s) utilized by CD180 in CLL and the possible defect in NR-CLL clones, Porakishvili *et al.*, (2011) determined the phosphorylation of several important enzymes involved in signalling and they were the first to demonstrate that normal B cells and R-CLL cells, but not NR-CLL cells responded to CD180 ligation by significant increase in the phosphorylation of protein kinases ZAP70/Syk, p38MAPK, ERK and AKT as well as the relative fluorescence intensity (RFI) of antibody binding which indicating the level of phosphorylation/cell. However, the responses were quite heterogeneous and here, using a larger cohort of patients, R-CLL cells could be divided into two categories as AKT signallers (AKT-S) and AKT non-signallers (AKT-NS) based on increased levels of phosphorylation of AKT (Ser 473) compared with the basal levels. It was important to determine the characteristics of the two categories of cells in terms of other signalling molecules, and this has been one of the major aims of my project.

## 3.2 Results

### 3.2.1 CD180 ligation leading to alternative phosphorylation of either AKT or p38MAPK

Stimulation with anti-CD180 mAb of 24 CLL samples which are CD180+ led to a significant increase in the percentages of cells expressing phosphorylated AKT, and these cells were termed, as previously, AKT-signallers (AKT-S) ( $p=0.000012$ , Figure 3.1A). Importantly, simultaneously CD180 ligation in the same cells induced significant downregulation of p38MAPK-P basal levels ( $p=0.0026$ , Figure 3.1B). Whereas 16 CLL samples previously identified as AKT-NS showed a significant increase in p38MAPK-P ( $p=0.001$ , Figure 3.1B) accompanied by the drop in AKT-P below basal levels ( $p=0.0027$ , Figures 3.1A). This group of CLL samples was therefore re-categorised by us as p38MAPK signallers (p38MAPK-S). These data support the hypothesis that CD180 ligation leads to two possible alternative pathways as indicated by the elevation of AKT-P or p38MAPK-P. Only six out of 60 CLL samples responded to CD180 ligation by increasing levels of both AKT-P and p38MAPK-P as did all control B cells ( $p=0.02$  and  $p=0.0032$  respectively). They were marked as double-signallers (DS) (Figures 3.1A and B). The remaining 14 CLL samples showed a significant decrease in percentages of both AKT-P and p38MAPK-P expressing cells and were categorised as non-signallers (NS) ( $p=0.028$  and  $p=0.03$  respectively, Figures 3.1A and B). Therefore, four patterns of CD180-mediated signalling in CLL cells were identified: AKT-signallers (AKT-S), p38MAPK-signallers (p38MAPK-S), non-signallers (NS) and a minor subset of double AKT/p38MAPK signallers (DS). The dichotomy of CD180-induced signalling by elevation of AKT-P or p38MAPK-P could be related to the surface density of CD180. However, no significant differences in CD180, CD38 or CD79b expression were observed between AKT-S, p38MAPK-S or NS cells, although DS CLL cells surprisingly expressed the lowest levels of CD180. Porakishvili *et al.* (2011) previously reported that CD180 ligation of control B cells resulted in simultaneous activation of protein kinases ZAP70/Syk, ERK, p38MAPK and AKT. This was further confirmed by the present study showing phosphorylation of both, AKT and p38MAPK protein kinases in control B cells ( $p=0.000005$  and  $p=0.00022$  respectively in Figures 3.1A and 3.1B). Therefore, the dichotomy of AKT *versus* p38MAPK activation appears to be a feature of CLL cells.



**Figure 3.1: Phosphorylation of (A) AKT protein kinase (B) p38MAPK protein kinase in control B cells, AKT-signaller (AKT-S), p38MAPK signaller (p38MAPK-S), double signaller (DS) and non-signaller (NS) cells following stimulation with anti-CD180 mAb.** CLL cells and control B cells were incubated with anti-CD180 mAb for 20 min (CD180 mAb) or left unstimulated in medium (M), washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-AKT(Ser473)-P (A) or with anti-p38MAPK-P (B) mAbs as described in the Materials and methods, analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

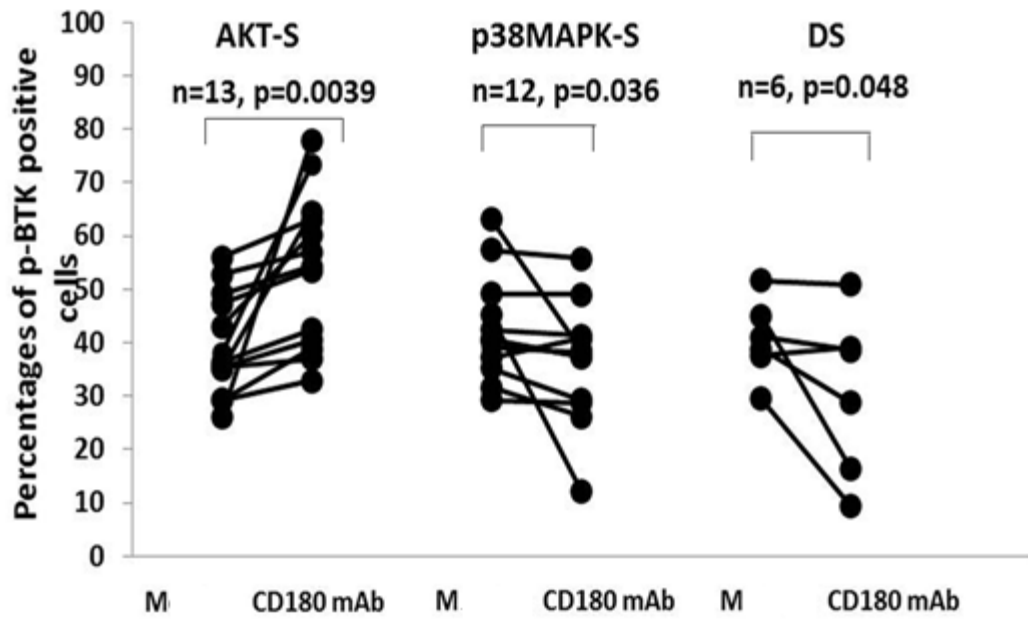
### **3.2.2 CD180-induced AKT-mediated signalling in CLL cells involves BTK and leads to the survival of CLL cells, while the p38MAPK pathway favours apoptosis**

Zhuang *et al.*, (2010) demonstrated that CLL clones consistently express activated AKT which plays a pivotal role in maintaining cell survival. This was in accord with previous studies showing the significance of AKT in cell survival upon stimulation via soluble anti-IgM (sol-IgM) (Petlickovski *et al.*, 2005) and anti-CD40 (Cuni *et al.*, 2004). Herman *et al.*, (2011) demonstrated that BTK protein and its mRNA are significantly overexpressed in CLL cells compared with normal B cells suggesting the importance of BTK in CLL cell survival mediated through the BCR. Therefore, it was important to determine whether CD180-mediated survival of CLL cells involved BTK as well as AKT pathway.

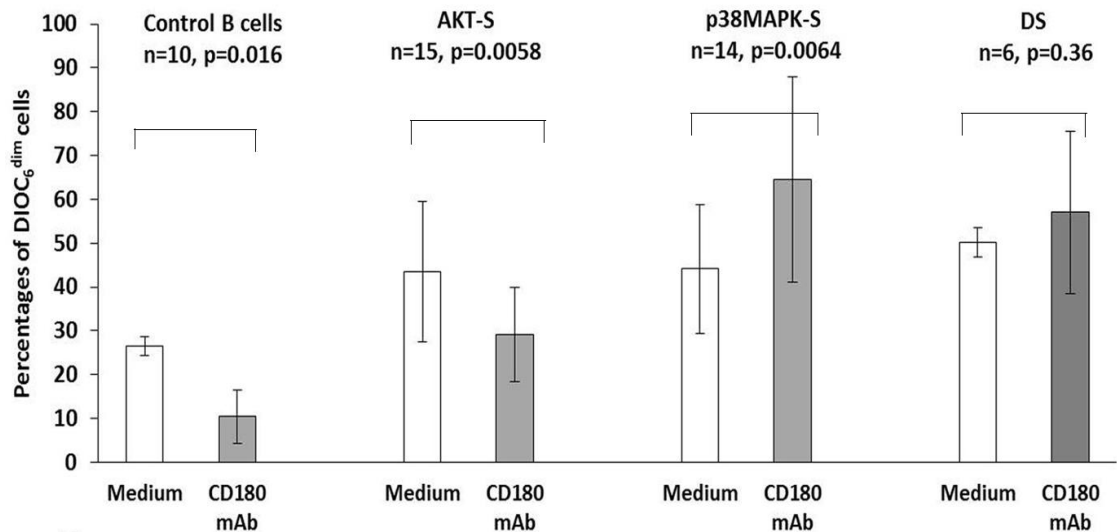
CD180 ligation of all 13 AKT-S CLL samples induced upregulation of BTK-P ( $p=0.0039$ , Figure 3.2). This was accompanied by a substantial survival from apoptosis shown by a significant decrease in DiOC<sub>6</sub><sup>dim</sup> apoptotic cells in 15 AKT-S CLL samples ( $p=0.0058$ , Figure 3.3). Control B cells also exhibited increased survival following CD180 ligation which was shown by a significant decrease in DiOC<sub>6</sub><sup>dim</sup> apoptotic cells ( $p=0.016$ , Figure 3.3 and [Porakishvili *et al.*, 2011]).

In contrast, there was a significant decrease in the percentages of BTK-P<sup>+</sup> cells below the basal level in 11 out of 12 p38MAPK-S CLL samples, following the treatment with anti-CD180 mAb ( $p=0.036$ , Figure 3.2). Most importantly, a significant (if heterogeneous) increase in DiOC<sub>6</sub><sup>dim</sup> apoptotic p38MAPK-S cells after CD180 ligation in 14 p38MAPK-S CLL samples analysed was observed ( $p=0.0064$ , Figure 3.3).

BTK phosphorylation and associated apoptosis in a small cohort of 6 DS CLL samples was analysed and there was a decrease in BTK-P in DS ( $p=0.048$ , Figure 3.2) whilst there was no CD180-mediated change in the cell survival as analysed by percentages of DiOC<sub>6</sub><sup>dim</sup> apoptotic cells ( $p=0.36$ , Figure 3.3).



**Figure 3.2: Percentages of BTK-P<sup>+</sup> cells in AKT-S, p38MAPK-S and DS categories of CLL cells.** Cells were incubated with anti-CD180 mAb for 20 min (CD180 mAb) or left unstimulated (Medium), washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-BTK-P mAb as described in the Materials and methods and analysed by flow cytometry. P values were calculated using the paired *t*-test.



**Figure 3.3: The percentages of DiOC<sub>6</sub><sup>dim</sup> (apoptotic) cells in control B cells, AKT-S, p38MAPK-S and DS CLL cells, compared to unstimulated cultures (Medium).** Cells were stimulated with anti-CD180 mAb for 24h, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means  $\pm$  SD, p values were

calculated using a non-parametrical *U*-test. White colour bars represent % of dead cells in unstimulated cultures (Medium) and grey colour bars represent % of dead cells in stimulated cultures.

### **3.2.3 sIgM-induced activation favours the pro-survival signalling pathway in AKT-S CLL cells, but not a pro-apoptotic pathway in p38MAPK-S CLL cells**

Several studies reported the significance of AKT in CLL survival, by playing a vital role in BCR-mediated pro-survival signalling (Packham *et al.*, 2010; Wickremasinghe *et al.*, 2011; Scuppoli *et al.*, 2012). Bichi *et al.*, (2002) demonstrated that activation of sIgM is associated with higher expression levels of T-cell leukaemia/lymphoma 1 (TCL1) protein which is an AKT activator and increased phosphorylation of AKT. Overexpression of TCL1 causes expansion of CD5<sup>+</sup> B cells which develop into CLL-like disease in transgenic mice showing the importance of AKT phosphorylation in development of CLL upon sIgM activation. Hence, it was important to study and compare sIgM induced signalling pathways with CD180 mediated signalling patterns which were identified in the major categories of CLL cells defined above. Double positive CD180<sup>+</sup>sIgM<sup>+</sup> CLL samples were selected for this study.

In the AKT-S category of cells, striking similarities between CD180 and sIgM-mediated signalling could be observed. There was a significant increase in phosphorylation of AKT ( $p=0.00063$ , Figure 3.4A) and BTK ( $p=0.0006$ , Figure 3.4C) above the basal level in AKT-S CLL samples upon treatment with anti-IgM whereas downregulation of the basal levels of p38MAPK-P ( $p=0.019$ , Figure 3.4B). However, anti-CD180 induced activation of BTK/AKT was observed in all AKT-S CLL samples studied (Figures 3.1 and 3.2), anti-IgM effect on this BTK/AKT circuit was more heterogeneous. 4/20 (20%) AKT-S CLL samples responded to anti-IgM by downregulation of AKT-P (Figure 3.4A) and 2/15 (13%) AKT-S samples responded by phosphorylation of p38MAPK (Figure 3.4B). However, overall 70% of AKT-S CLL samples responded to anti-CD180 and anti-IgM similar pattern and led to activation of BTK/AKT whilst downregulation of p38MAPK-P.

The anti-IgM effect on survival of AKT-S CLL cells was studied by analysing DiOC<sub>6</sub><sup>dim</sup> apoptotic cells upon stimulation. Interestingly, anti-IgM protected AKT-S CLL cells from apoptosis in 9 CLL samples as shown by a significant reduction in

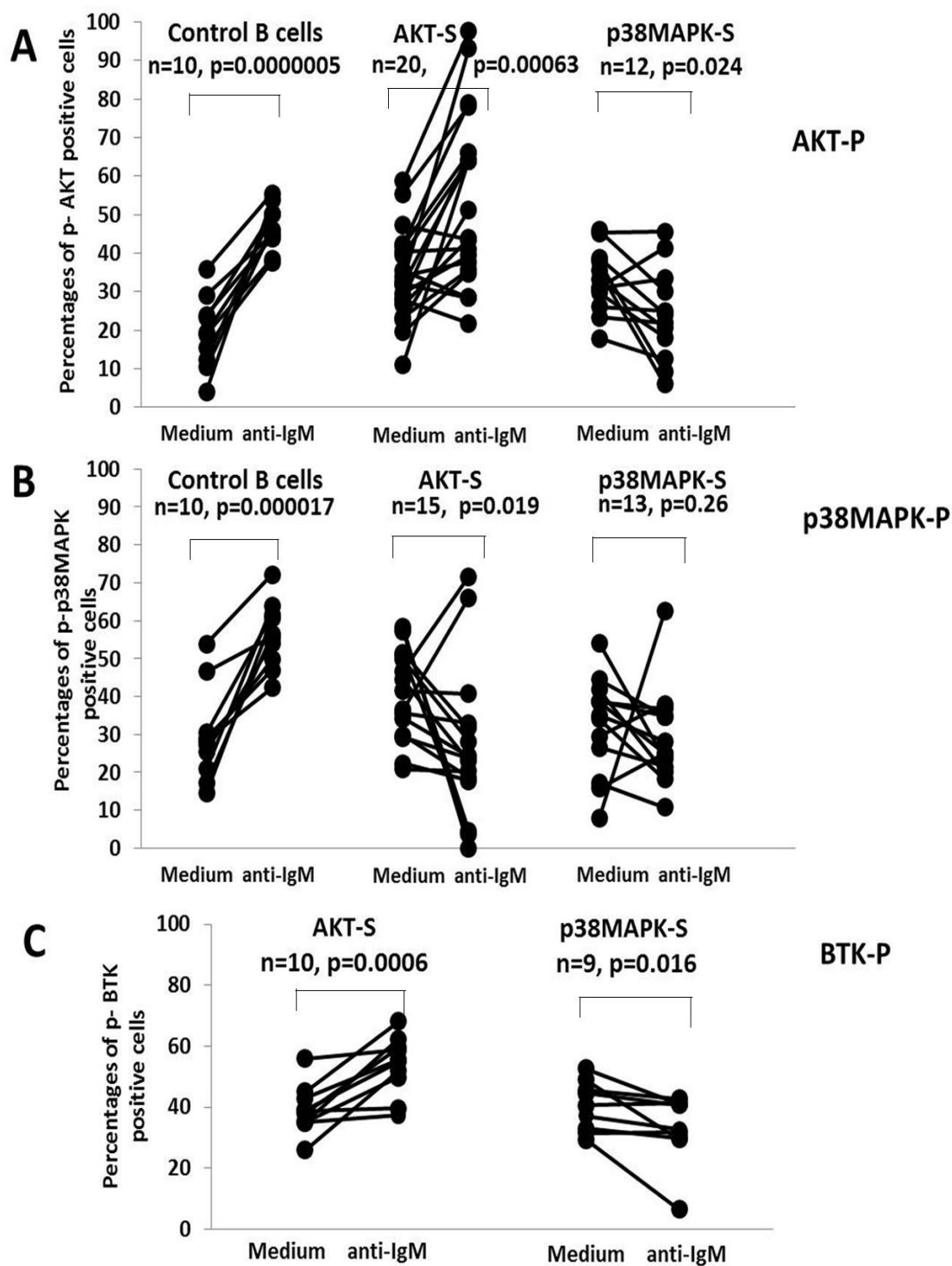


DiOC<sub>6</sub><sup>dim</sup> apoptotic AKT-S cells ( $p=0.020$ , Figure 3.5) similar to the result shown by CD180 ligation in this category of cells.

By contrast, sIgM ligation resulted in an increase in phosphorylation of p38MAPK in only 3/13 p38MAPK-S samples (23%) ( $p=0.26$ , Figure 3.4B) suggesting phosphorylation of p38MAPK upon stimulation with anti-IgM is a less frequent event compared to CD180 ligation. However, there was a significant decrease in expression of BTK-P in all 9 p38MAPK-S CLL samples studied ( $p=0.016$ , Figure 3.4C).

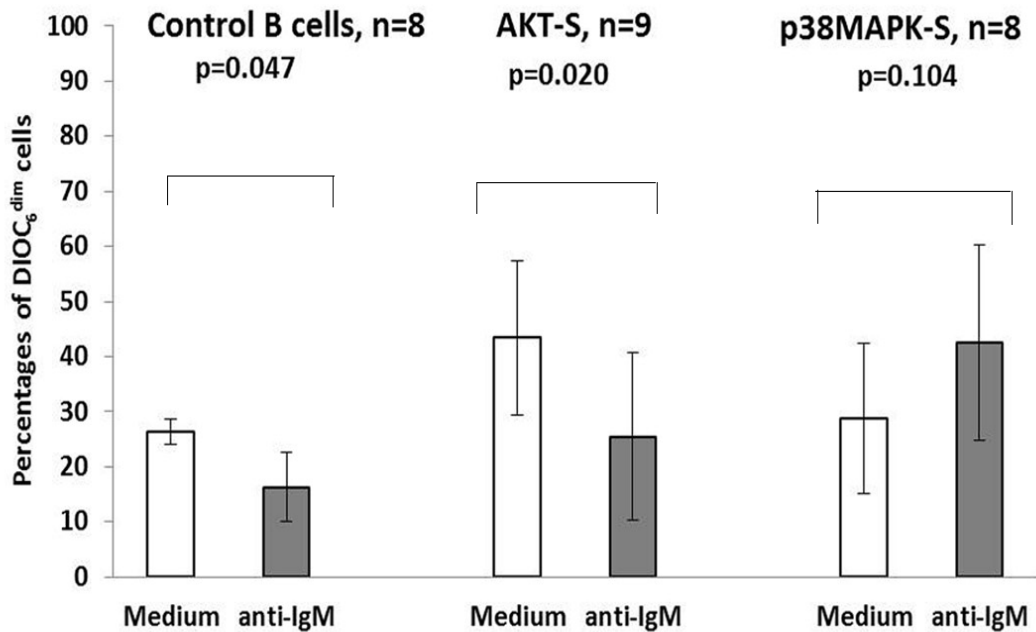
Effect of sIgM ligation on the survival of p38MAPK-S cells was assessed by analysing the changes in mitochondrial membrane potential and there was no significant difference in DiOC<sub>6</sub><sup>dim</sup> apoptotic p38MAPK-S cells upon stimulation with anti-IgM ( $p=0.104$ , Figure 3.5). Therefore, new data indicate that phosphorylation of p38MAPK is vital for the induction of apoptosis in p38MAPK-S category of cells that has not been observed after sIgM ligation compared to CD180 ligation. As similar to CD180 ligation, sIgM ligation also resulted in phosphorylation of both AKT and p38MAPK protein kinases in control B cells ( $p=0.0000005$  and  $p=0.000017$  respectively, Figures 3.4A and B).

Hence, these data suggest that a pro-survival signalling pathway operating via BTK/AKT in CLL cells can be activated by both sIgM and CD180 ligation whereas only anti-CD180 mAb can activate an alternative pathway mediated via p38MAPK which leads to apoptosis in p38MAPK-S cells. This p38MAPK activation is a rare event caused by anti-sIgM alone.



**Figure 3.4: Anti-IgM mediated phosphorylation of AKT, p38MAPK and BTK.**

(A, B and C) Control B cells or CLL cells from the AKT-S and p38MAPK-S groups were incubated with anti-IgM F(ab)<sub>2</sub> for 10 minutes (anti-IgM) or left unstimulated in medium (Medium), washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-AKT-P, anti-p38MAPK-P and anti-BTK-P mAbs as described in the Materials and methods. The results were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.



**Figure 3.5: The percentages of DiOC<sub>6</sub><sup>dim</sup> apoptotic cells in control B cells, AKT-S, p38MAPK-S and DS upon stimulation with anti-IgM F(ab)<sub>2</sub>.** Control B cells and CLL cells were stimulated with anti-IgM F(ab)<sub>2</sub> or left unstimulated (Medium) for 24 h, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values represent mean  $\pm$  SD, p values were calculated using the non-parametrical *U*-test. White colour bars represent % of dead cells in unstimulated cultures (Medium) and grey colour bars represent % of dead cells in stimulated cultures.

### 3.2.4 Rewiring of sIgM-mediated intracellular signalling from pro-survival BTK/PI3K/AKT to a pro-apoptotic p38MAPK Pathway

Yamashita *et al.*, (1996) showed that pretreatment of murine B cells with anti-CD180/RP105 led to anti-IgM-induced apoptosis. Hence, it was interesting to study whether pre-engagement of CD180 on CLL would affect BCR mediated signalling in CLL. CLL samples which are CD180<sup>+</sup>sIgM<sup>+</sup> were selected for this study. In all 15 AKT-S CLL samples, pre-treatment with anti-CD180 mAb, followed by anti-IgM F(ab)<sub>2</sub>, led to a significant decrease in the percentages of AKT-P<sup>+</sup> cells (Figure 3.6A, p=0.000013) albeit not to the baseline level, compared with anti-IgM alone. Simultaneously, a decrease in BTK-P<sup>+</sup> cells from 44.3 $\pm$ 11.4% (anti-IgM alone) to 32.6 $\pm$ 13.5% (anti-CD180+anti-IgM, n=11, p=0.02) with baseline levels of 38.3 $\pm$ 6.5% was observed. Most interestingly, in 14 out of 15 of these AKT-S samples, reduction in BTK/AKT phosphorylation was accompanied

by a significant increase in the phosphorylation of p38MAPK following sequential ligation of CD180 and IgM ( $p=0.0057$  Figure 3.6A).

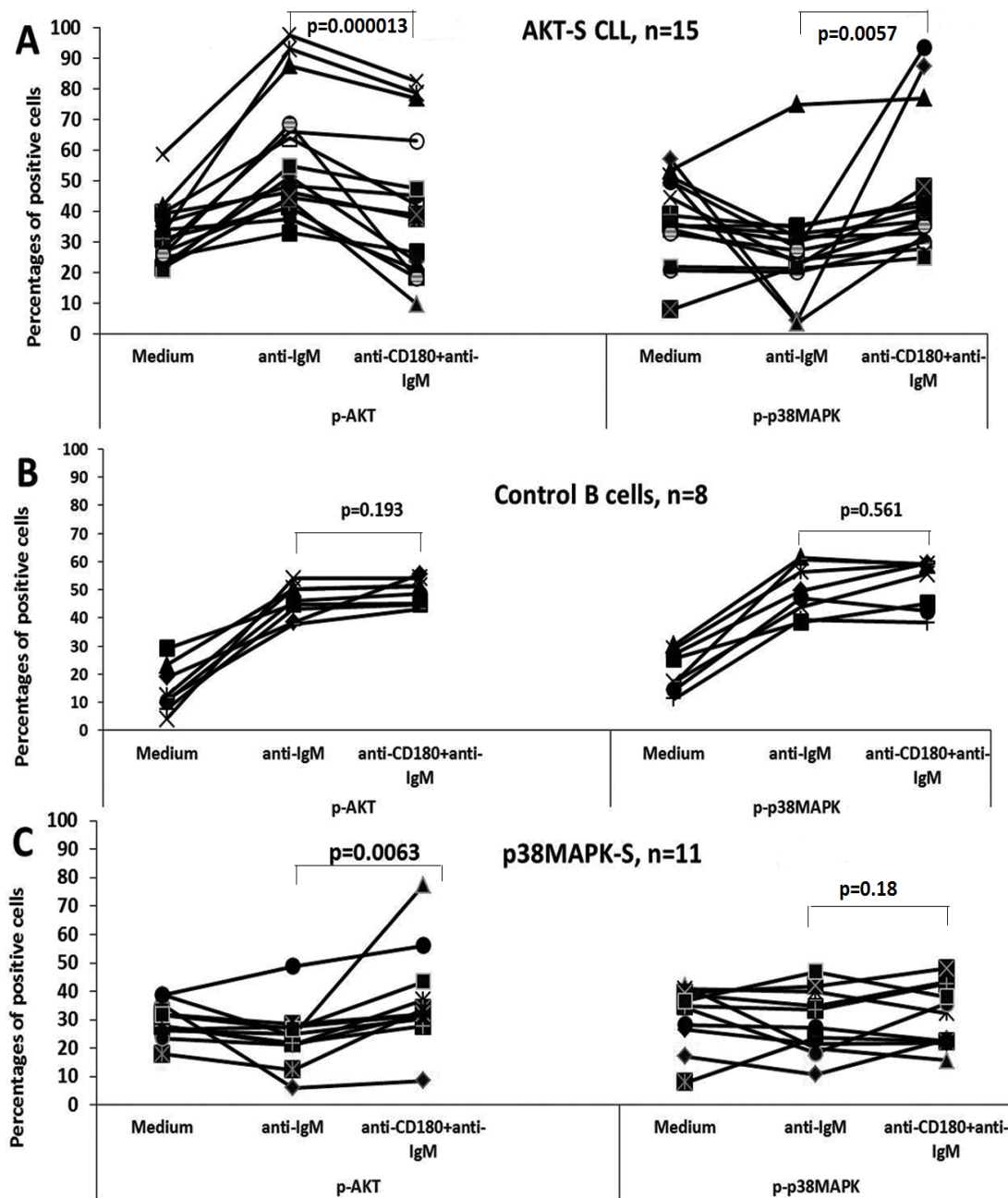
The effect of pre-treatment of AKT-S CLL cells with anti-CD180 mAb on their survival was also studied. Importantly, there was a significant increase in apoptotic cells compared with the engagement of IgM alone ( $p = 0.025$ ) and above baseline apoptosis ( $p = 0.026$ , Figure 3.7). Therefore, these data show that sensitization of AKT-S CLL cells with anti-CD180 leads to rewiring of anti-IgM mediated pro-survival signalling via BTK/AKT to the pro-apoptotic p38MAPK pathway. However, a similar result was not observed in control B cells as there was no significant difference in the percentages of AKT-P<sup>+</sup> cells ( $p=0.193$ , Figure 3.6B) and p38MAPK-P<sup>+</sup> ( $p=0.561$ , Figure 3.6B) following sequential ligation of CD180 and IgM. Hence, the modulation appears to be a characteristic of CLL cells. Differences in the optimal stimulation time for anti-CD180 and anti-IgM antibodies did not affect the redirection phenomenon described in this study. The percentages of cells expressing AKT-P following stimulation with anti-IgM for 30 min were somewhat lower than those after 10 min ( $49.3 \pm 8.9\%$  versus  $59.4 \pm 11.0\%$ ,  $p = 0.09$ ). However, this did not affect an outcome of pre-stimulation with anti-CD180 where the numbers of AKT-P cells further dropped down to  $26.4 \pm 13.5\%$ ,  $p = 0.028$ . The percentages of p38MAPK-P expressing cells did not differ in the range of 10 to 30 min stimulation with anti-IgM (data not shown).

It was important to study the effect of sensitization with anti-IgM antibodies in AKT-S CLL samples. Striking similarities between the modulation caused by the sensitization with anti-CD180 mAb and anti-IgM antibodies could be observed. In all 12 AKT-S CLL samples, there was a significant downregulation of CD180-mediated expression of AKT-P to basal levels ( $p=0.0039$ , Figure 3.8A) accompanied by a significant increase in the expression of p38MAPK-P compared to CD180 ligation alone ( $p=0.016$ , Figure 3.8A). In addition, pre-treatment with anti-IgM led to downregulation of percentages of cells expressing BTK-P from  $45.0 \pm 15.8\%$  (anti-CD180) down to  $35.8.4 \pm 11.3\%$  (anti-IgM + anti-CD180,  $n = 11$ ,  $p = 0.019$ ) with the baseline levels of  $38.6 \pm 7.1\%$ .

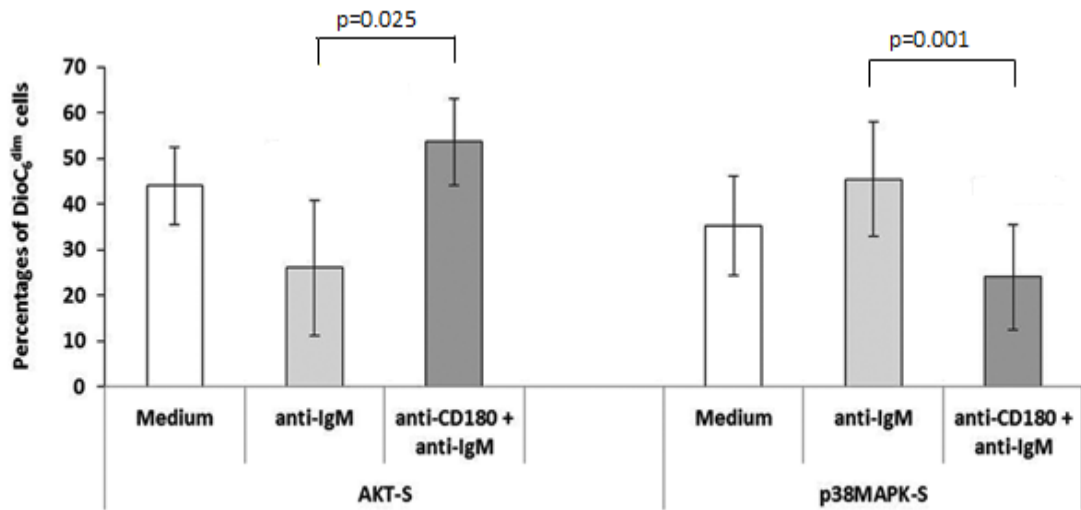
Although pretreatment of control B cells with anti-CD180 did not make a significant difference in percentages of cells with AKT-P and p38MAPK-P, pretreatment of control B cells with anti-IgM had an additive effect with anti-CD180 which led to a significant increase in expression of AKT-P in all 8 control samples ( $p=0.00006$ , Figure 3.8B). However, there were no changes in the levels

of p38MAPK-P in control B cells ( $p=0.278$ , Figure 3.8B). These data support the conclusion that there is cross-talk between the BCR and CD180 in the AKT-S CLL samples, leading to rewiring of BTK/AKT pro-survival signalling pathway to p38MAPK pro-apoptotic pathway.

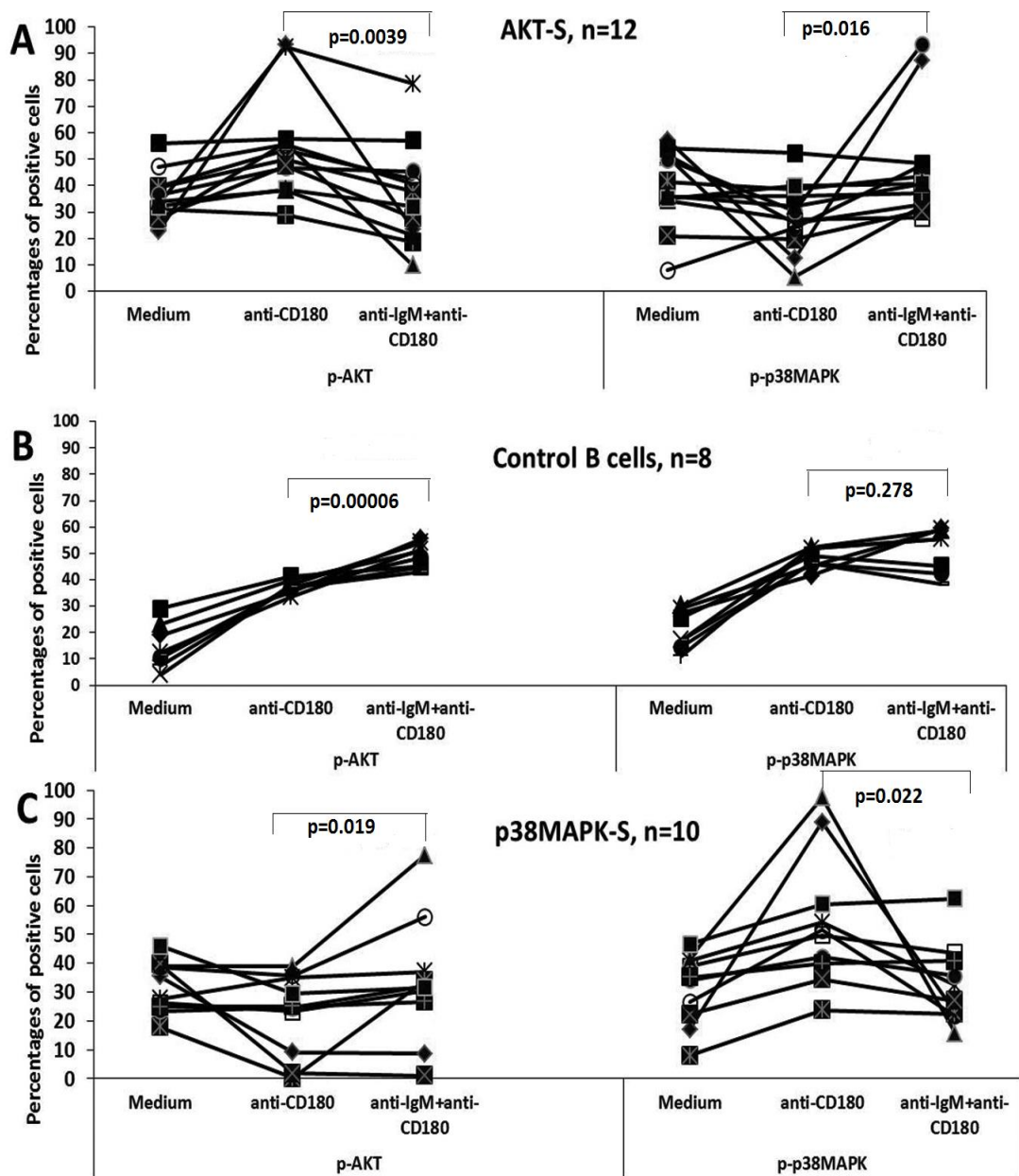
It was decided to study the effect of pretreatment of p38MAPK-S CLL samples with anti-CD180 and anti-IgM antibodies next. Interestingly, in all eleven p38MAPK-S samples, pretreatment with anti-CD180 had an additive effect with anti-IgM mediated levels of AKT-P ( $p=0.0063$ , Figure 3.6C) whilst there was no significant difference in p38MAPK-P upon sequential ligation of anti-CD180 mAb and anti-IgM antibodies ( $p=0.18$ , Figure 3.6C). This was accompanied by increased survival of p38MAPK-S CLL cells as shown by a significant decrease in percentages of DiOC<sub>6</sub><sup>dim</sup> apoptotic cells compared to sIgM ligation alone ( $p=0.001$ , Figure 3.7). Similarly, pretreatment of p38MAPK-S CLL cells with anti-IgM amplified the CD180-mediated percentages of AKT-P expressing cells in 8/10 samples ( $p=0.019$ , Figure 3.8C) but decreased the expression of p38MAPK-P compared to CD180 ligation alone ( $p=0.022$ , Figure 3.8C). Hence these data show that in contrast to AKT-S cells, pretreatment of p38MAPK-S cells with either anti-CD180 or anti-IgM often leads to additive phosphorylation of AKT. Therefore, it can be concluded that in AKT-S CLL cells, ligation of either sIgM or CD180 lead to a substantial activation of the pro-survival BTK/AKT pathway and presensitization of the other receptor redirected intracellular signalling towards the potentially pro-apoptotic p38MAPK pathway, whereas in p38MAPK-S CLL samples similar preengagement favours the BTK/AKT pathway.



**Figure 3.6: Modulation of sIgM-mediated signalling by sensitisation with anti-CD180 mAb.** (A-C) Control B cells or CD180<sup>+</sup>sIgM<sup>+</sup> CLL samples were treated with anti-CD180 mAb or anti-IgM F(ab)<sub>2</sub> alone or first with anti-CD180 for 20 minutes followed with anti-IgM F(ab)<sub>2</sub> for another 10 minutes as described in the Materials and methods. Unstimulated cultures (Medium) were used as controls. The cells were then stained with anti-CD19~Cy5-PE mAb, fixed, permeabilised and stained with antibodies to AKT-P and p38MAPK-P. p values were calculated using the paired *t*-test.



**Figure 3.7:** The percentages of DiOC<sub>6</sub><sup>dim</sup> (apoptotic) cells in unstimulated CLL cells (Medium) or stimulated with anti-IgM F(ab)<sub>2</sub> alone or first with anti-CD180 followed by anti-IgM F(ab)<sub>2</sub> for 24 h, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> and analysed by flow cytometry. The values represent mean ± SD, p values calculated using the nonparametrical *U* test.



**Figure 3.8: Modulation of CD180-mediated signalling by sensitisation with anti-IgM F(ab)<sub>2</sub>.** (A-C) Control B cells or CD180<sup>+</sup>sIgM<sup>+</sup> CLL samples were treated with anti-CD180 mAb or anti-IgM F(ab)<sub>2</sub> alone or first with anti-IgM F(ab)<sub>2</sub> for 10 minutes followed by anti-CD180 for 20 minutes as described in the Materials and methods. Unstimulated cultures (Medium) were used as controls. The cells were then stained with anti-CD19~Cy5-PE mAb, fixed, permeabilised and stained with antibodies to AKT-P and p38MAPK-P as described in above. p values were calculated using the paired *t*-test.

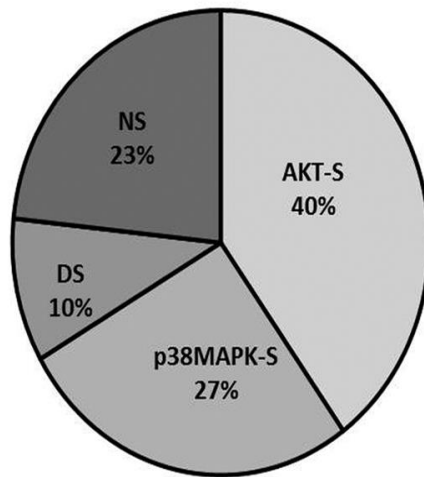


### 3.3 Discussion

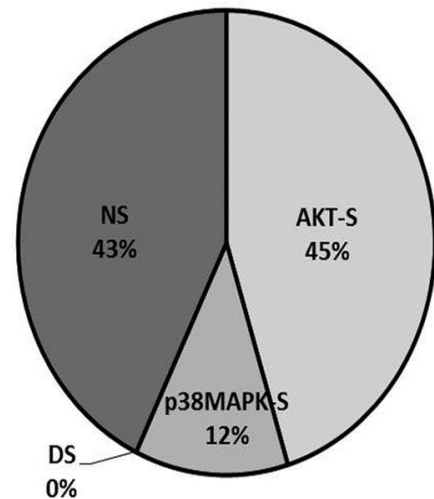
#### 3.3.1 CD180 ligation on B-CLL cells leads to alternative phosphorylation of either AKT or p38MAPK

Four patterns of CD180-mediated signalling in CLL cells have been identified: AKT-signallers (AKT-S); p38MAPK signallers (p38MAPK-S); nonsignallers (NS); and a minor subset of double AKT/p38MAPK signallers (DS). 24 out of the 60 CD180+ CLL samples, responded to CD180 ligation by a significant upregulation of AKT-P (AKT-S) (Figures 3.1A). It was reported that activation of AKT is essential for the BCR-mediated growth and survival of B lymphocytes (Woodland *et al.*, 2008) as well as the survival of CLL cells (Longo *et al.*, 2008; Packham *et al.*, 2010; Downward, 2014). CD180 ligation resulted in increased phosphorylation of p38MAPK in 16 out of 36 remaining samples (p38MAPK-S) (Figures 3.1B) and only in a small cohort of 6 CLL samples showed activation of both AKT-P and p38MAPK-P (DS) upon stimulation with anti-CD180 mAb (Figures 3.1A and B). The use of specific inhibitors of AKT and p38MAPK signalling pathways to confirm that, activation of AKT and p38MAPK pathways is exclusive will be discussed in chapter 4. Activation of alternative pathways appears to be a feature of CLL cells, and not of normal B cells as they responded to CD180 ligation by increasing both AKT-P and p38MAPK-P (Figures 3.1A, B). CD180 ligation led to significant drop in p38MAPK-P basal levels in the AKT-S cells (Figures 3B) and AKT-P basal levels in p38MAPK-S cells (Figures 3A). Petlickovski *et al.*, (2005) reported that BCR stimulation causes a decrease in signal intensity of p38MAPK, rather than an increase, but not linked to AKT activation. This data suggests the regulatory influence of one pathway on another. 14 CD180+ CLL samples did not respond to CD180 ligation by activating either of the two pathways (NS, Figures 3.1 A and B). It is possible to suggest that those NS CLL cells may use an alternative pathway or that they are totally refractive to ligation of CD180. Interestingly, those CD180 NS CLL samples remained unresponsive to the ligation of sIgM suggesting their anergic status (Packham *et al.*, 2014). The distribution of CD180-mediated signalling categories of CLL cells which was identified is shown in Figure 3.8 below.

Ligation of CD180 on CD180+ CLL cells



Ligation of IgM on CD180+slgM+ CLL cells



**Figure 3.9: Proportion of CLL samples exhibiting four different patterns of signalling.** Treatment with anti-CD180 mAb of CD180+ CLL samples or treatment with anti-IgM F(ab)<sub>2</sub> of CD180+slgM+ CLL samples. AKT-S, AKT-signallers; p38MAPK-S, p38MAPK signallers; DS, double AKT/p38MAPK signallers; NS, nonsignallers (Porakishvili *et al.*, 2015)

Porakishvili *et al.*, (2005) have demonstrated that more U-CLL samples (71%) are CD180+slgM+ than CD180+slgM<sup>neg/low</sup> attributing U-CLL samples mostly to the AKT-S and NS signalling categories. However, in this study M-CLL samples were found to be evenly distributed between AKT-S, p38MAPK-S and NS categories. Interestingly, all six DS CLL samples belonged to M-CLL group, which is associated with increased anergy (Packham *et al.*, 2014). There were no differences in surface expression of the CD180, slgM, CD79b or CD38 between the four signalling groups (data not shown). However, p38MAPK-S cells expressed significantly higher *ex vivo* levels of slgD compared to AKT-S cells: 83.5±15.1% versus 48.5±33.0%, p=0.0055, n=10. Although the relevance of this is currently unclear, it is possible to suggest that slgD could be involved in the p38MAPK-pathway mediated through CD180 which our research group is currently investigating.

### **3.3.2 CD180-mediated AKT-signalling pathway in CLL involves activation of BTK and is pro-survival, while p38MAPK activation favours apoptosis**

There are several studies showing that BTK plays an important role in BCR signalling pathway (Burger *et al.*, 2010). Moreover, its role in CLL cell survival has also been reported (Ponader *et al.*, 2012). As per this data, CD180 ligation in AKT-S CLL samples activated pro-survival BTK kinase while suppressing p38MAPK pathway. Activation of BTK led to the reduction in apoptosis in this category of CLL samples (Figure 3.2). However, recruitment of BTK in p38MAPK-S samples was not observed, resulting in the increased apoptosis (Figure 3.2). The role of p38MAPK-mediated signalling in CLL is so far unclear. Activation of p38MAPK has been associated with proliferation of various cells in response to CpG-ODN. Takeshita *et al.*, (2004) reported that CpG DNA/TLR9-mediated cellular signalling involves in activation of p38MAPK in macrophages. CPG-DNA driven activation of p38MAPK in murine macrophage cell line, RAW264.7 has been reported by Ahmad-Nejad *et al.*, (2002). Moreover, in mice dendritic cells, CpG DNA induced survival signals via activation of PI3K and p38MAPK (Park *et al.*, 2002). Peng (2005) showed the importance of activation of p38MAPK in TLR4 signalling in B cells for transcription of inflammatory genes and in TLR9 signalling for activation, proliferation and Ig secretion. CpG DNA augmented BCR-mediated signals for the activation of p38MAPK which lead to synergistic production of cytokines and induction of splenic mature B cells proliferation (Kyung-Ae *et al.*, 2003). On the contrary, Ntoufa *et al.*, (2012) demonstrated that p38MAPK activation led to tolerant status in CLL cells.

Moreover, it has been documented that p38MAPK is associated with regulation of cell survival in CLL (Grumont *et al.*, 1998; Furman *et al.*, 2000; Piatelli *et al.*, 2004). p38MAPK has been shown to induce apoptosis in murine splenocytes and human lymphoma B-cell lines (Graves *et al.*, 1998; Yan *et al.*, 2008). Later Negro *et al.*, (2012) demonstrated the significance of p38MAPK in apoptosis of CLL cells. Current data strongly confirm that CD180-mediated activation of p38MAPK is associated with apoptosis of CLL cells. This might suggest the potential use of p38MAPK-signalling as a new profiling tool for those patients who are unresponsive to BTK inhibitors.

To summarise, current data show that CD180 ligation activates BTK/AKT signalling pathway in AKT-S cells leading to their survival but not in p38MAPK-S or NS CLL samples (Figures 3.1, 3.2 and 3.3). Therefore, considering that CLL

cells receive various stimuli from their microenvironment *in vivo*, including via CD180, these findings suggest that BTK inhibitors might be limited to AKT-S CLL cells.

### **3.3.3 sIgM ligation favours pro-survival BTK/AKT signalling pathways in AKT-S CLL cells**

Since CD180 may play a significant role in interacting with microenvironmental ligands in CLL, it was important to compare the sIgM-mediated signalling patterns with CD180-mediated signalling in the major categories of CLL cells as defined above. Current data show a striking similarity of CD180- and sIgM-mediated signalling in AKT-S CLL samples by activation of pro-survival BTK/AKT pathway following the ligation of either receptor (Figures 3.1 and 3.4A) leading to increased survival of CLL cells (Figures 3.3 and 3.5). In contrast to CD180 activation, BCR engagement did not show an activation of p38MAPK in p38MAPK-S (Figure 3.4B) or DS CLL samples. This could be explained by a significant drop of BTK-P<sup>+</sup> cells below the basal levels following stimulation of sIgM in p38MAPK-S cells since it has been previously shown that BCR signalling was abrogated in the absence of BTK activation (Petro *et al.*, 2000). Moreover, Efremov *et al.*, (2007) also failed to detect p38MAPK activation in CLL cells following BCR ligation. This suggests that the lack of BTK phosphorylation abrogates both “arms” of BCR signalling (via AKT and via p38MAPK), whilst leaving CD180-mediated p38MAPK activation intact.

Recent data indicate that CD180 is able to bypass BTK and carry signals downstream to p38MAPK favouring apoptosis over survival. Therefore, it suggests that although there is a substantial overlap of CD180 and sIgM mediated activation of BTK/AKT pro-survival signalling circuit, only ligation of CD180 can lead to activation of an alternative pro-apoptotic pathway mediated via p38MAPK. Hence, it was important to study how sequential ligation of CD180 and BCR would impact signal transduction through the AKT and p38MAPK pathways.

### **3.3.4 Pretreatment of CLL cells with Anti-CD180 antibodies rewires the sIgM signalling pathway from pro-survival to pro-apoptotic**

Yamashita *et al.*, (1996) demonstrated that anti-IgM induced murine B cells can be sensitised for apoptosis by treatment with anti-CD180 mAb, whereas combining signalling of CD180 and BCR induced rapid proliferation and antigen-

specific antibody responses in mice *in vivo* (Chaplin *et al.*, 2013). Recent data clearly indicate that pretreatment of AKT-S CLL cells with anti-CD180 mAb significantly reduced the pro-survival pathway operated via BTK/AKT circuit induced via subsequent ligation of sIgM and redirected it towards p38MAPK pathway which leads to apoptosis (Figure 3.6 and 3.7). Similar results were observed when AKT-S CLL cells were stimulated with by ligating two receptors in the reverse order (Figure 3.8).

There was no rewiring of signalling from pro-survival to pro-apoptotic pathway in control B cells (Figure 3.6B and 3.8B), although the reasons for absence of a cross-talk between CD180 and BCR in normal cells is unclear, recent data show that modulation is unique to CLL.

Since CLL cells are believed to express BCR in proliferation centres (Ferrarini *et al.*, 2014) and pilot experiments using immunohistochemical staining of our research group has shown that CD180 is expressed in bone marrow and lymph nodes (data not shown), it is possible to suggest that CD180<sup>+</sup>sIgM<sup>+</sup> CLL cells may receive simultaneous signals through both receptors *in vivo*. Continuous BCR mediated signalling in CLL has been reported (Burger *et al.*, 2013) and it is possible to hypothesise that CD180-mediated activation of CLL cells by an unknown microenvironmental ligand such as generated soluble ligand may mimic this continuous BCR-mediated signalling. Rewiring of intracellular signals from BTK/AKT pro-survival circuit to p38MAPK pro-apoptotic pathway in AKT-S CLL cells may suggest a possible cross-talk between CD180 and BCR in AKT-S. Such modulation of signalling could influence blood cell counts, long-term disease progression and could provide a basis for therapy. In order to understand the significance of these pathways for prognosis and clinical criteria, future experiments will be carried out.

In conclusion, current data help to hypothesise that interaction between CD180 and sIgM leads to convergence of certain key signalling pathways. Whereas the pro-survival pathway appears to be operating through the BTK/AKT circuit, the pro-apoptotic pathway is activated via p38MAPK perhaps leading to inhibition of the BTK/AKT. The elements of the pro-apoptotic pathway upstream to p38MAPK have yet to be identified. ~~This New~~ data suggest that in a substantial number of CLL samples preengaging of CD180 could prevent further pro-survival signalling

mediated via sIgM and, instead, induce CLL cell apoptosis, which opens the door to the new strategies for the treatment of a large cohort of CLL patients.

## **Chapter 4**

# **Modulation of CD180-mediated signalling and survival of CLL cells through specific kinase inhibitors**

## 4.1 Introduction

### 4.1.1 AKT Inhibitors

The Phosphatidylinositol-3-kinase (PI3K) pathway was described in chapter one as the most critical pathway in the survival of CLL cells. AKT (serine-threonine kinase) also known as protein kinase B (PKB) is one of the important targets of PI3K products. Low-activity conformation of AKT resides in the cytosol and it is activated by phosphorylation at Thr<sup>308</sup> and Ser<sup>473</sup> through recruitment to cell membranes. When activated, AKT is capable of promoting cell survival via phosphorylation and inactivation of components of apoptotic cascade. Substrates of AKT include the members of the forkhead family of transcription factors and glycogen synthase kinase-3 (GSK-3) (Maurer *et al.*, 2006). Inhibition of GSK-3 induces the up-regulation of the anti-apoptotic protein MCL-1. Moreover, inhibition of PI3K induces a loss of cell viability, dephosphorylation of FoxO3a and GSK-3, and a subsequent decrease in the level of MCL-1 protein. CLL patients with progressive disease have shown a higher capacity for cell cycle progression in CLL cells upon stronger activation of AKT (Longo *et al.*, 2007). The AKT inhibitors reduce phosphorylation of downstream targets such as GSK3 $\alpha/\beta$ , FOXO3, TSC2 and mTOR in a dose-dependent manner. Phosphorylation of further downstream signalling molecules in the AKT pathway such as S6 protein are also reduced by AKT inhibitors (Luo *et al.*, 2005).

The three AKT isoforms: AKT1, AKT2 and AKT3 are closely related and consist of a conserved amino-terminal (N-terminal) pleckstrin homology (PH) domain which involves with intracellular signalling, a central kinase catalytic domain and a carboxy-terminal (C-terminal) regulatory hydrophobic motif (HM) (Hanada *et al.*, 2004). The three AKT isoforms are highly homologous. However, it is unclear whether they are functionally redundant (Datta *et al.*, 1999).

Studies on tissue expression patterns of AKT isoforms revealed that AKT1 and AKT2 are ubiquitously expressed, whereas AKT3 expression is mainly limited to brain, testis, lung, heart, kidney, mammary gland and fat (Brodbeck *et al.*, 1999; Masure *et al.*, 1999; Yang *et al.*, 2003). Knockout of AKT isoforms in mice revealed distinct phenotypes: deficiency of AKT1 resulted in a smaller animal with significant growth defects (Cho *et al.*, 2001). AKT2 deficient mice were unable to maintain glucose homeostasis (Cho *et al.*, 2001) and AKT3 knockout mice had a



smaller brain, but were otherwise normal (Yang *et al.*, 2004). Overexpression of AKT2, but not AKT1 and AKT3, increased the phosphatidylinositide 3-kinases (PI3K)- dependent effect on adhesion, invasion and metastasis of breast and ovarian cancer cells (Arboleda *et al.*, 2003). AKT has been proposed as a potential therapeutic target for cancer (Bellacosa *et al.*, 2005). Downregulation of AKT1 by antisense oligonucleotides inhibited anchorage-independent cell growth and induced apoptosis in cancer cells (Liu *et al.*, 2001). It was shown that the targeted decrease of AKT3 levels with siRNA or by expressing active phosphatase and tensin homolog (PTEN) protein stimulated apoptosis and inhibited melanoma tumour development (Stahl *et al.*, 2004). Jetzt *et al.*, (2003) demonstrated that adenovirus-mediated expression of an AKT kinase-dead mutant of AKT induces apoptosis selectively in tumour cells and suppresses tumour growth in mice. It is a commonly held perception that tumour cells are dependent upon activated AKT for survival and sensitive to inhibition of AKT, particularly those cells with PTEN mutations and/or a high basal level of activated AKT (Bellacosa *et al.*, 2005).

### **Akti-1/2**

It is an ATP non-competitive AKT inhibitor blocking AKT1 and AKT2, but not AKT3 activity. Akti-1/2 also prevents phosphorylation at Ser<sup>308</sup> by phosphoinositide-dependent protein kinase (PDK)1 *in vitro* and at Ser<sup>308</sup> and Thr<sup>473</sup> in cells without inhibiting PDK1 activity (Barnet *et al.*, 2005). It was also reported that Akti-1/2 could sensitise tumour cells to induce apoptosis when combined with some chemotherapeutic agents and biological agents, such as TNF-related apoptosis-inducing ligand (TRAIL) (Lindsley *et al.*, 2005). The chemical structure of Akti1/1 is shown in Figure 4.1.

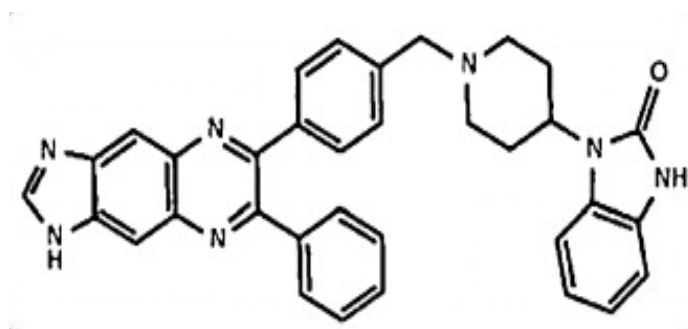


Figure 4.1: Chemical structure of AKTi-1/2 (Gilot *et al.*, 2010)

Akti-1/2 inhibits  $\text{Ca}^{2+}$ /CaM-dependent protein kinase (CaMK)  $\alpha$  activity. The efficiency of inhibition of recombinant CaMK $\alpha$  activity is the same as the CaMK inhibitor KN-93. Moreover, Akti-1/2 prevents the nuclear translocation of aryl hydrocarbon receptor (AhR) in MCF-7 cells in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure, which has been demonstrated to require CaMKI activity. This shows that Akti-1/2 has off-target effects at concentrations equipotent with AKT inhibition which should be considered in therapeutic applications (Gilot *et al.*, 2010). Akti-1/2 could induce dose-dependent apoptosis in CLL cells, and the Akti1/2 mediated apoptosis was also confirmed by induction of Poly (ADP-ribose) polymerase (PARP) cleavage demonstrating that activated AKT in CLL cells play a role in the survival of the malignant cells. It was also reported that Akti-1/2 at the concentration inducing substantial apoptosis in CLL cells did not induce apoptosis in normal cells suggesting normal PBMC cells are less dependent on AKT for survival than CLL cells. AKT inhibitor-induced apoptosis was associated with rapid loss of induced myeloid leukaemia cell differentiation protein (MCL1) through proteasome degradation and increased expression of p53 (Zhuang *et al.*, 2010).

Induction of apoptosis in CLL cells by akt1/2 is dose-dependent and it is irrespective of TP53 mutational status. Furthermore, B cells from patients with CLL were more sensitive to AKT inhibitors than T cells from leukemic patients, and B or T cells from healthy donors. Apoptosis induced by AKT inhibitors was not blocked by CLL survival factors such as IL-4 and stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ). Interestingly, inhibition by Akti-1/2 did not lead to any change in the mRNA expression profile of genes involved in apoptosis. However, it induced p53 upregulated modulator of apoptosis (PUMA) and NADPH oxidase activator (NOXA) proapoptotic protein levels, and decreased MCL-1 anti-apoptotic protein level (Frias *et al.*, 2009).

Zhuang *et al.*, (2010) also demonstrated that activated AKT in CLL cells can be inhibited by selective inhibitors can induce apoptosis in CLL cells. Furthermore, some AKT inhibitors such as AiX induce apoptosis preferentially in U-CLL cells (Hofbauer *et al.*, 2009). Hence AKT inhibitors have been tested as a treatment for CLL. Presently, the allosteric AKT inhibitor MK-2206 (an analog of Akti-1/2) is under investigation at the phase II level in advanced ovarian, fallopian tube and peritoneal carcinomas (NCT01283035) (Correa *et al.*, 2014).

#### 4.1.2 p38MAPK inhibitors

##### **SB203580**

P38MAPK is a Ser/Thr protein kinase activated by various inflammatory cytokines and a variety of stress stimuli. It is activated by dual phosphorylation of Thr<sup>180</sup> and Tyr<sup>182</sup> in a Thr-Gly-Tyr activation loop by a dual specificity MAP kinase kinase 6 (MKK6) (Kumar *et al.*, 1999). SB203580 (4-(4-fluorophenyl)2,2-(4-methylsulfinylphenyl)25-(4-pyridyl) Imidazole) is a pyridinyl imidazole inhibits p38 kinase catalytic activity by binding to the ATP site. It has the ability to bind to both the inactive and activated forms of p38 kinase and it is a highly selective inhibitor of the p38 family of MAPKs (Wilson *et al.*, 1997; Young *et al.*, 1997). SB203580 was originally discovered as an inhibitor of LPS-induced cytokine synthesis in the human leukaemia monocytic cell line, THP-1 (Laydon *et al.*, 1994). Kumar *et al.*, (1999) demonstrated that SB203580 inhibits only the activity of p38 but not its activation by MKK6 using THP-1 monocytes because the binding of SB203580 in the ATP binding pocket of p38 MAPK does not interfere with the ability of MKK6 to phosphorylate and activate p38 (Kumar *et al.*, 1999).

MAP kinase-activated protein (MAPKAP) kinase-2 (MAPKAP kinases-2) which is activated by p42 or p44 isoforms of MAP kinase, promotes phosphorylation of heat shock protein27 (HSP27). It is suggested that HPS27 may be involved with cell survival and SB203580 has shown inhibitory effect on MAPKAP kinase-2 and hence on HPS27 on pheochromocytoma (PC12) cells (Cuenda *et al.*, 1995). Due to the high selectivity, the inhibitor did not show inhibition of Jun N-terminal kinase, ERK or other kinases (Cuenda *et al.*, 1995, 1997; Kumar *et al.*, 1997). It was demonstrated that SB203580 do not act distally by blocking calcium channels or altering their voltage dependency (Wilk-Blaszczak *et al.*, 1998).

The melanoma differentiation associated gene-7 (mda-7) is a gene induced during terminal differentiation in human melanoma cells and classified as IL-24. Administration of mda-7/IL-24 by means of a replication-incompetent adenovirus (Ad.mda-7) induced apoptosis selectively in diverse human cancer cells without affecting normal fibroblast or epithelial cells. It was demonstrated that treatment of melanoma cells with SB203580 inhibited Ad.mda-7-induced apoptosis as well as Ad.mda-7-mediated induction of the GADD family of genes in a time and dose-dependent manner, and Ad.mda-7-mediated down-regulation of the anti-

apoptotic protein BCL-2 (Sarkar *et al.*, 2002). The chemical structure of SB203580 is shown in Figure 4.2.

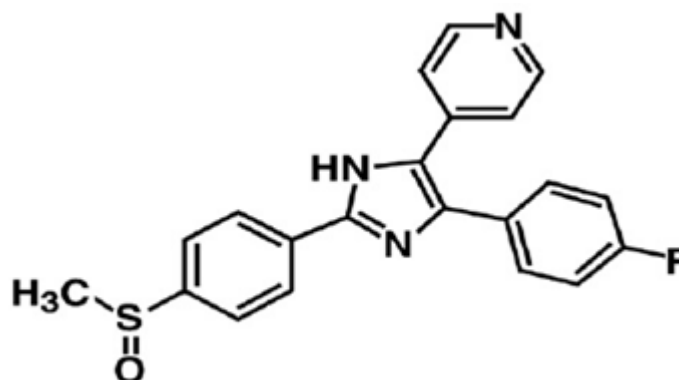


Figure 4.2: Chemical structure of SB203580 (Young *et al.*, 1997)

Erythroleukaemic SKT6 cells respond to glycoprotein hormone erythropoietin (Epo) and induce haemoglobinization and Epo-dependent cell growth. All MAP kinases ERK1, ERK2, p38, JNK1 and JNK2 are activated by Epo stimulation in SK6 cells and SB203580 inhibited Epo-dependent differentiation and haemoglobinization. In Epo-dependent FD-EPO cells, inhibition of p38 suppressed cell growth (Nagata *et al.*, 1998).

The activation of p38 in Epo-dependent HCD57 cells in response to EPO suggests a role for p38 in EPO-induced proliferation or survival. SB203580 suppressed proliferation of Epo-induced HCD57 cells. However, the treatment with this inhibitor neither caused apoptosis in the presence of EPO nor protected these cells from apoptosis when EPO was withdrawn (Jacobs-Helber *et al.*, 2000). In contrast, Shan *et al.*, (1999) reported that inhibition of p38 protected HCD57 cells from apoptosis following EPO withdrawal for 4 days. In CD40 stimulated CLL cells, treatment with SB203580 diminished the levels of NOXA and MCL-1. Therefore, SB203580 had not shown significant alteration in Noxa/MCL-1 ratio (Tromp *et al.*, 2011). The ability of r-IL-24 and LPS, MAPK activators to reverse SB203580-mediated apoptosis in CLL cells was reported by Sainz-Perez *et al.*, (2006).

Korus *et al.*, (2002) demonstrated elevated levels of p38MAPK in the breakpoint cluster region (BCR)-transfected COS-7 cells, which is a gene associated with the Philadelphia chromosome and pathogenesis of CML and ALL. Elevated levels of p38 MAPK were not observed in control cells, or in the presence of the

inhibitor suggesting that BCR can activate endogenous p38 MAPK and that the inhibitor can block this activation. SB203580 affects NF- $\kappa$ B activation since, in the presence of the inhibitor, activation of both the NF- $\kappa$ B-luc and Gal4-p65 by BCR was substantially reduced (Korus *et al.*, 2002)

### 4.1.3 BTK inhibitors

#### ***Ibrutinib***

BTK is a key signalling molecule that plays a significant role in the BCR mediated signalling pathway (Burger *et al.*, 2010) and in the survival and proliferation of malignant B cells (Maas *et al.*, 2012). Moreover, its significance in CLL cell survival has also been reported (Ponader *et al.*, 2012). BTK is overexpressed in CLL cells compared with normal B cells suggesting its involvement in CLL cell survival mediated through the BCR (Herman *et al.*, 2011).

Ibrutinib which was originally named as PCI-32765 is a BTK inhibitor that binds specifically and irreversibly to cysteine residue in the BTK kinase domain and hence blocks its enzymatic activity. Ibrutinib is the first BTK-targeting agent to secure approval by the US Food and Drug Administration (FDA) and other health authorities globally (Gaykoet *et al.*, 2014). The chemical structure of Ibrutinib is shown in Figure 4.3.

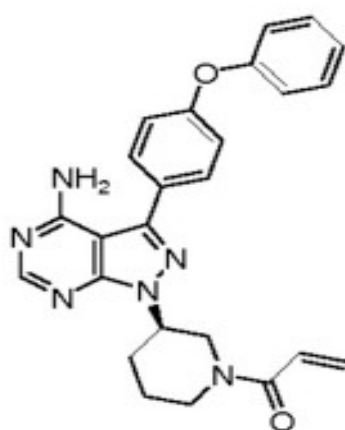
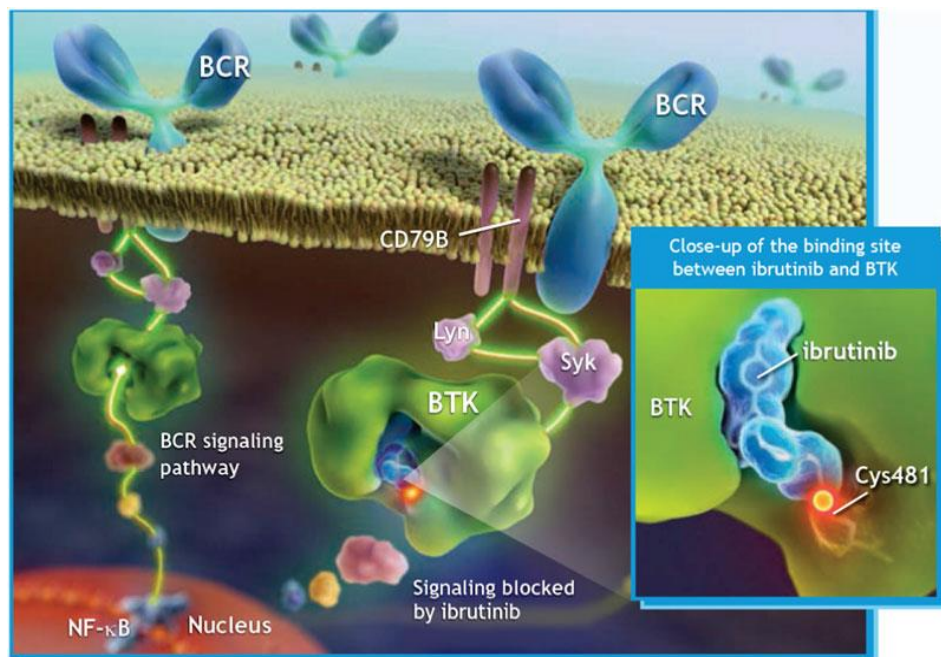


Figure 4.3: Chemical structure of Ibrutinib (Woyach *et al.*, 2012)

Ibrutinib binds covalently to the amino acid cysteine at position 481 (Cys-481) in the BTK active site and prevents adenosine triphosphate from binding to BTK

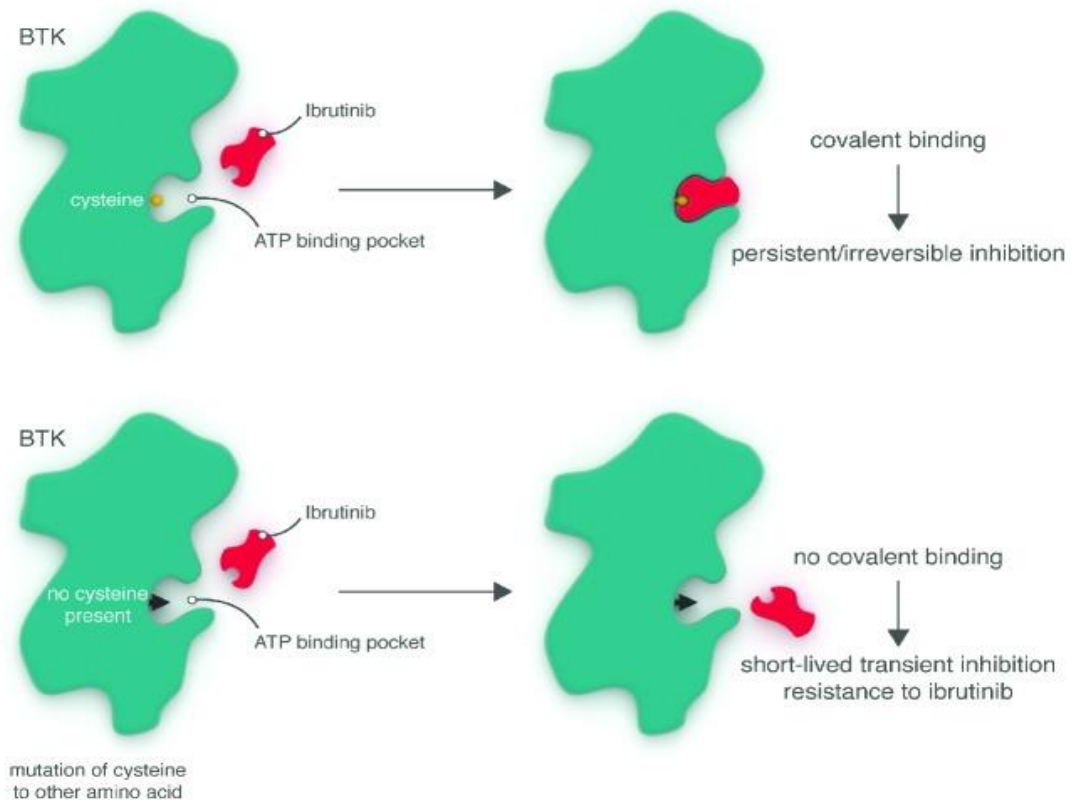
active site and thus its activation (Figure 4.4). It has a short half-life, and due to the formation of covalent bonds, provides a high degree of pharmacologic selectivity (Gaykoet *et al.*, 2014)



**Figure 4.4: Mechanism of action of Ibrutinib via B cell receptor pathway.**

When the B cell receptor binds to its specific antigen, BTK is phosphorylated (activated) by Syk/Lyn. Ibrutinib blocks the downstream signalling by forming a covalent bond with Cys481 at the active site of BTK (Gayko *et al.*, 2014).

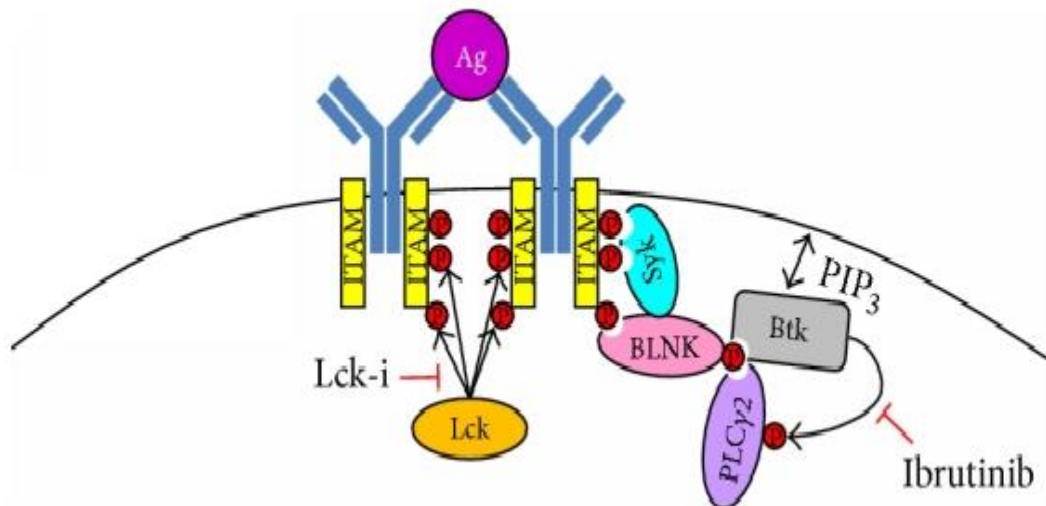
Some CLL patients show Ibrutinib resistance, and it was suggested that the C481S mutation disrupts the formation of covalent bonds between Ibrutinib and BTK leading to loss of inhibition of BTK enzymatic activity and finally Ibrutinib resistance (Furman *et al.*, 2014; Wiestner, 2015, Figure 4.5).



**Figure 4.5: Mechanism of BTK inhibition by Ibrutinib is compared with the effect of resistant mutations.** Ibrutinib covalently binds to a cysteine at position 481 (Cys481) in the ATP binding pocket of BTK which leads to irreversible inhibition of the kinase molecule. In patients with Ibrutinib resistance, mutations that result in replacement of Cys481 by another amino acid have been identified. In the absence of Cys481, Ibrutinib cannot form a covalent bond and inhibition of the kinase is reversible and short-lived, consistent with the rapid clearance of Ibrutinib (Wiestner, 2015).

Inhibitory action of Ibrutinib was recorded in several preclinical studies on numerous processes including ERK signalling, NF- $\kappa$ B DNA binding, cytosine-phosphate-guanine (CpG)-mediated CLL cell proliferation and tumour cell migration. Byrd *et al.*, (2013) demonstrated that the response in patients with CLL as evaluated according to the criteria of the International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) (Hallek *et al.*, 2008) is greater in patients with U-CLL cells than in those with M-CLL cells (77% partial or complete response and 13% partial response with lymphocytosis *versus* 33% partial or complete response and 42% partial response with lymphocytosis). Although lymph node shrinkage and clinical benefit occur in patients with both CLL cells subsets, lymphocytosis tends to persist in patients with M-CLL (Woyach *et al.*, 2014). Ibrutinib expressed several mechanisms of action in CLL cells *in vitro*: induction

of apoptosis, blocking signalling and activation in response to BCR and CD40 pathway stimulation, as well as disrupting the protective effect of stromal cell co-cubation (Herman *et al.*, 2011). Point of inhibition of Ibrutinib in BCR signalling pathway is shown in Figure 4.6.



**Figure 4.6: Point of inhibition for Ibrutinib.** Ibrutinib inhibits the ability of BTK to phosphorylate and activate PLCγ2 (Slupsky, 2014).

Ibrutinib is also effective in blocking integrin  $\alpha(4)\beta(1)$ -mediated adhesion to fibronectin and VCAM-1 in CLL cells, as well as signalling and homing in response to CXCL12 and CXCL13 (De Rooji *et al.*, 2012; Ponader *et al.*, 2012). It also could reduce secretion of the BCR-dependent chemokines such as CCL3 and CCL4 (Ponader *et al.*, 2012).

In this experiment, specific inhibitors of the CD180-mediate signalling pathways were used to block specific intracellular pathways alone and in combination to confirm their involvement in CD180-mediated signalling.

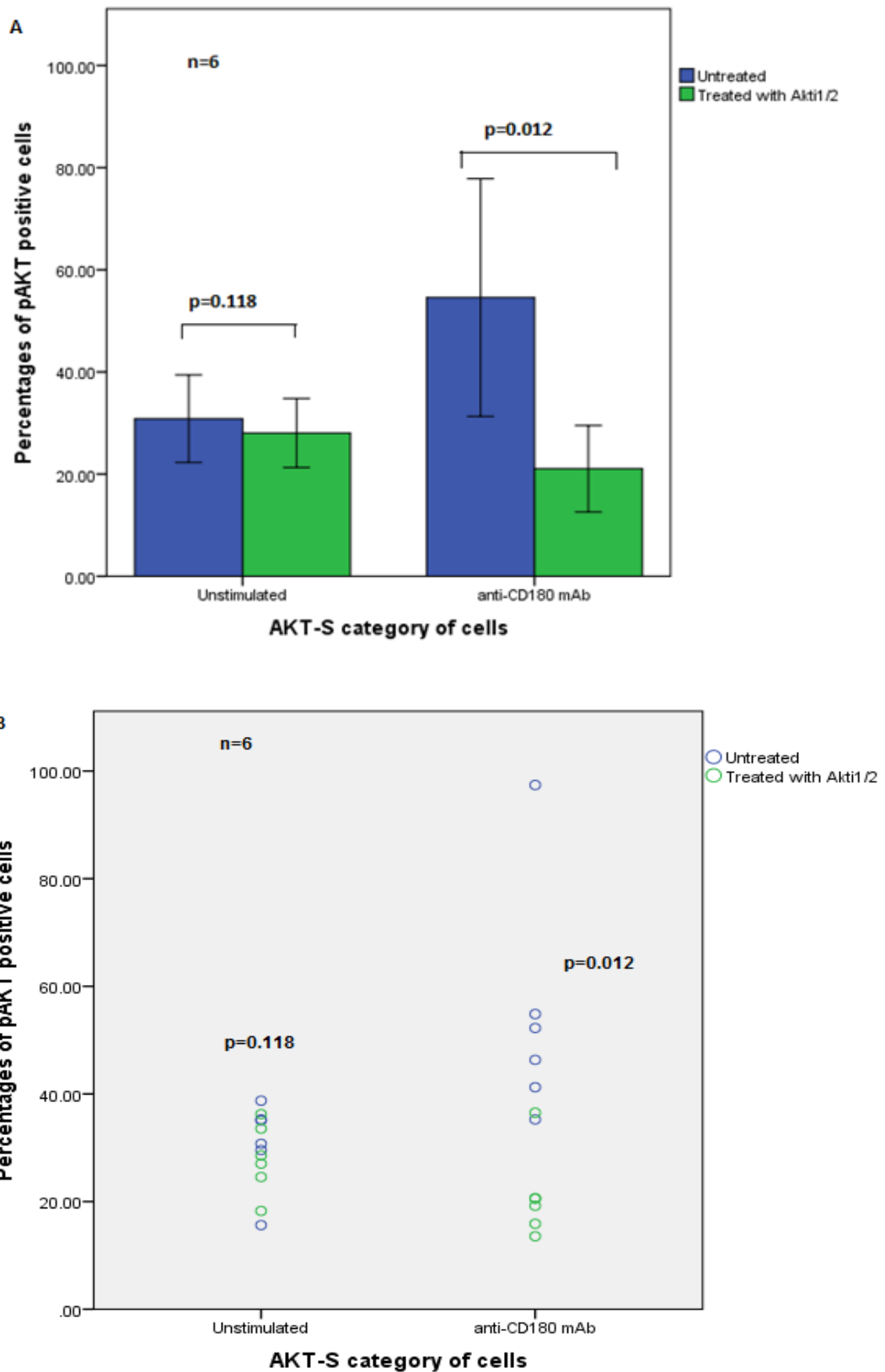
## 4.2 Results

CD180<sup>+</sup> CLL samples were categorised into two groups as AKT-S and p38MAPK-S according to alternative phosphorylation of AKT or p38MAPK protein kinases following CD180 ligation with mAb as described in Chapter 3 and Porakishvili *et al.*, (2015). In this study, AKT-S and p38MAPK-S CLL samples were pre-treated with specific protein kinase inhibitors as explained in Materials and methods.



#### **4.2.1 Effect of Akti1/2 on AKT phosphorylation and cell survival in AKT-S and p38MAPK-S CLL cells**

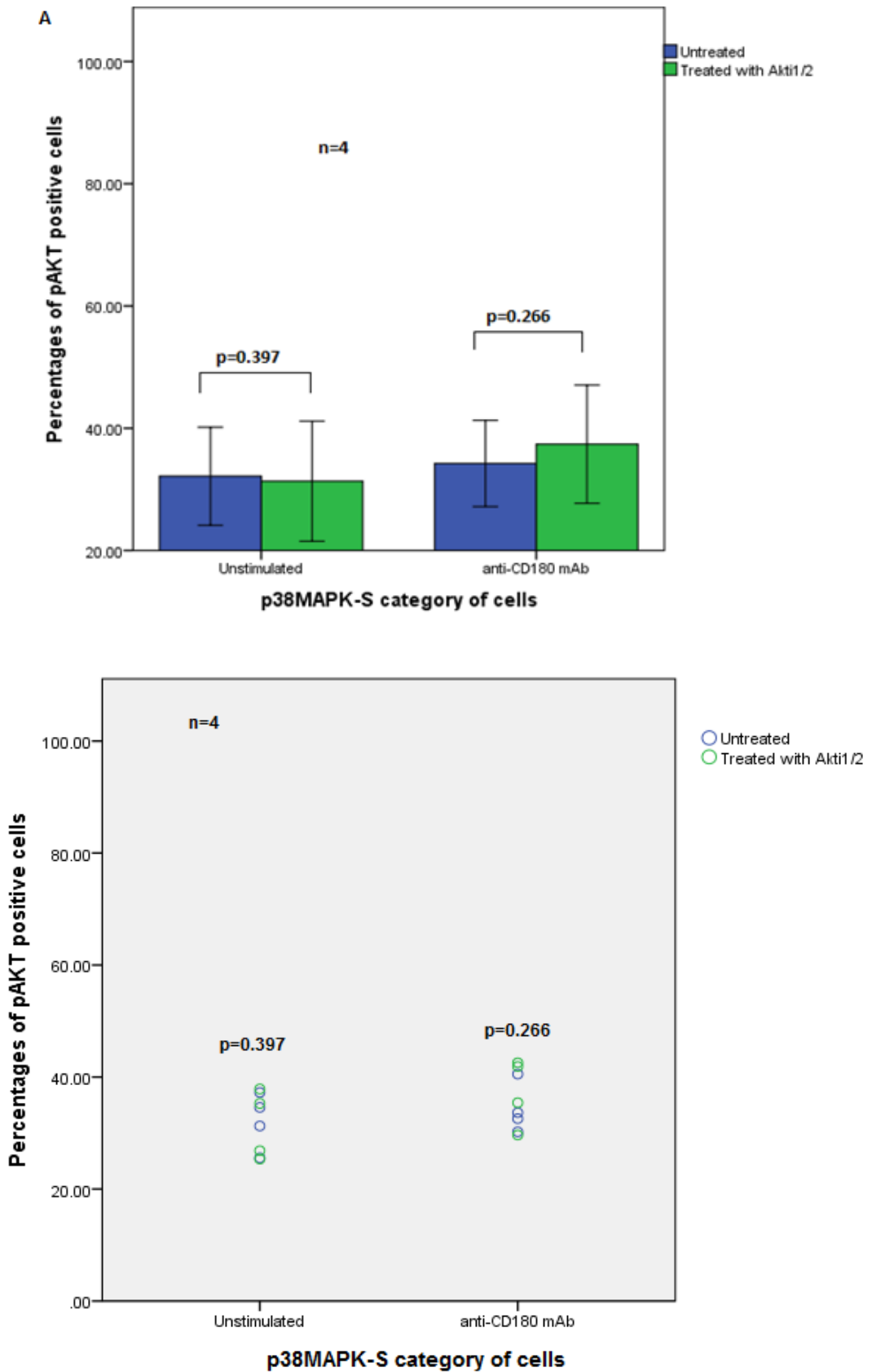
To assess the effect of Akti-1/2 on AKT activation in CLL samples, the phosphorylation status of AKT (after treating with inhibitor followed by mAbs) was studied. In all six AKT-S CLL samples, the percentage of cells with pAKT were significantly increased following CD180 ligation compared with basal levels ( $p=0.019$ , Figure 4.7). Interestingly, pre-treatment of unstimulated AKT-S cells with Akti1/2 did not exert an apparent effect on spontaneous phosphorylation of AKT, when untreated and treated ( $p=0.118$ , Figure 4.7). As expected, in all six AKT-S CLL samples stimulated with anti-CD180 mAb, Ser473 phosphorylation was significantly reduced when pre-treated with Akti1/2 compared to untreated samples, down to the basal levels ( $p=0.012$ , Figure 4.7).



**Figure 4.7: Percentages of cells expressing p-AKT in unstimulated and anti-CD180 mAb stimulated AKT-S CLL samples untreated or treated with Akti1/2 inhibitor: Bar chart (A) and scatter plot (B).** CLL cells were incubated with AKTi1/2 inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for

20 min (CD180 mAb) or left unstimulated in medium, then washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-AKT(Ser473)-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

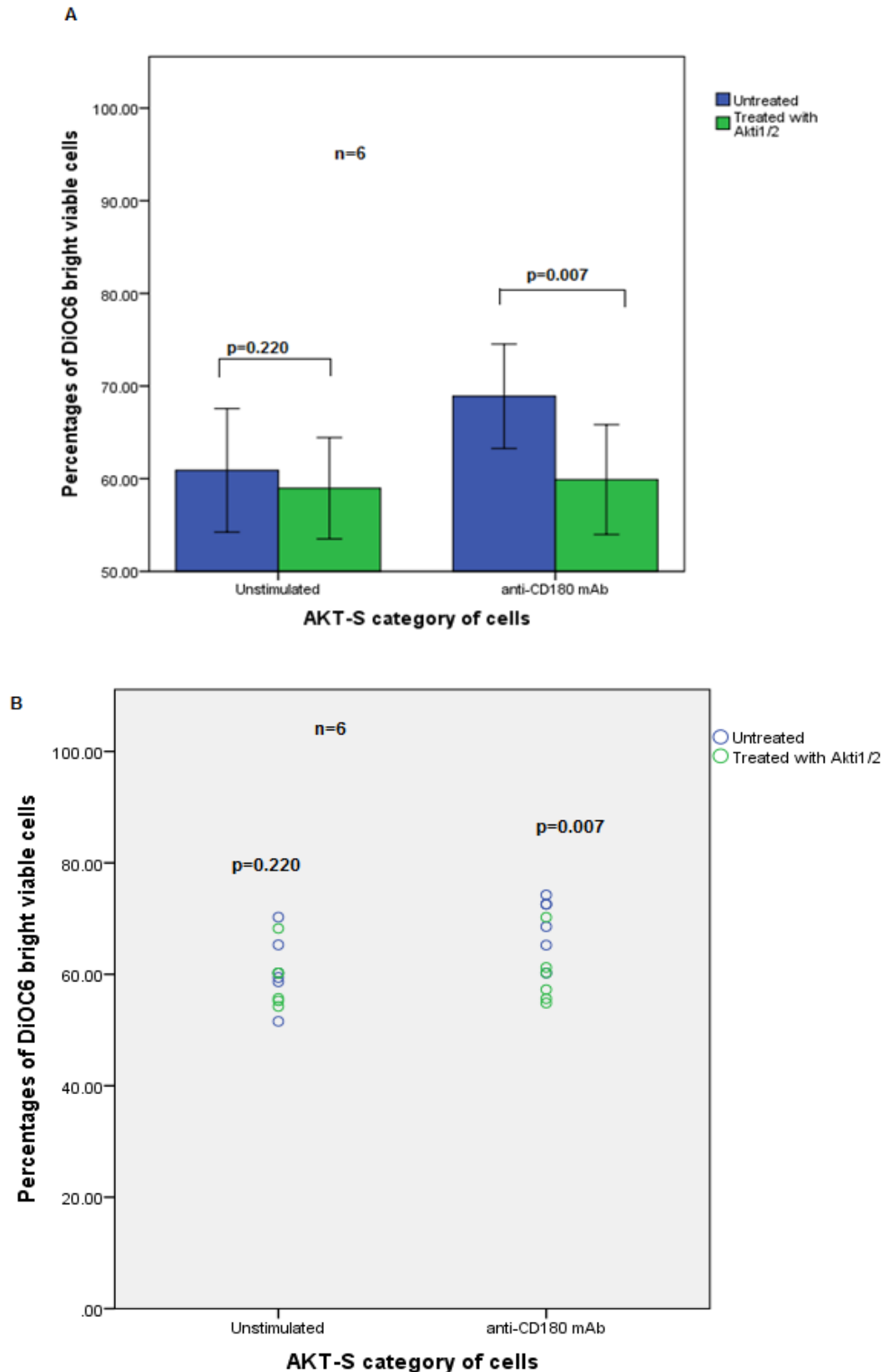
Importantly, pre-treatment of p38MAPK-S CLL cells with AKT inhibitor Akti1/2 had no effect on phosphorylation of AKT as there was no reduction in basal ( $p=0.397$ , Figure 4.8), or anti-CD180-stimulated percentages of pAKT cells ( $p=0.266$ , Figure 4.8) following pre-treatment with Akti1/2.



**Figure 4.8: Percentages of cells expressing p-AKT in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL samples untreated or treated with AKTi1/2 inhibitor: Bar chart (A) and scatter plot (B).** P38MAPK-S CLL cells were incubated with AKTi1/2 inhibitor or left untreated, washed and stimulated with anti-

CD180 mAb for 20 min (CD180 mAb) or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-AKT(Ser473)-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

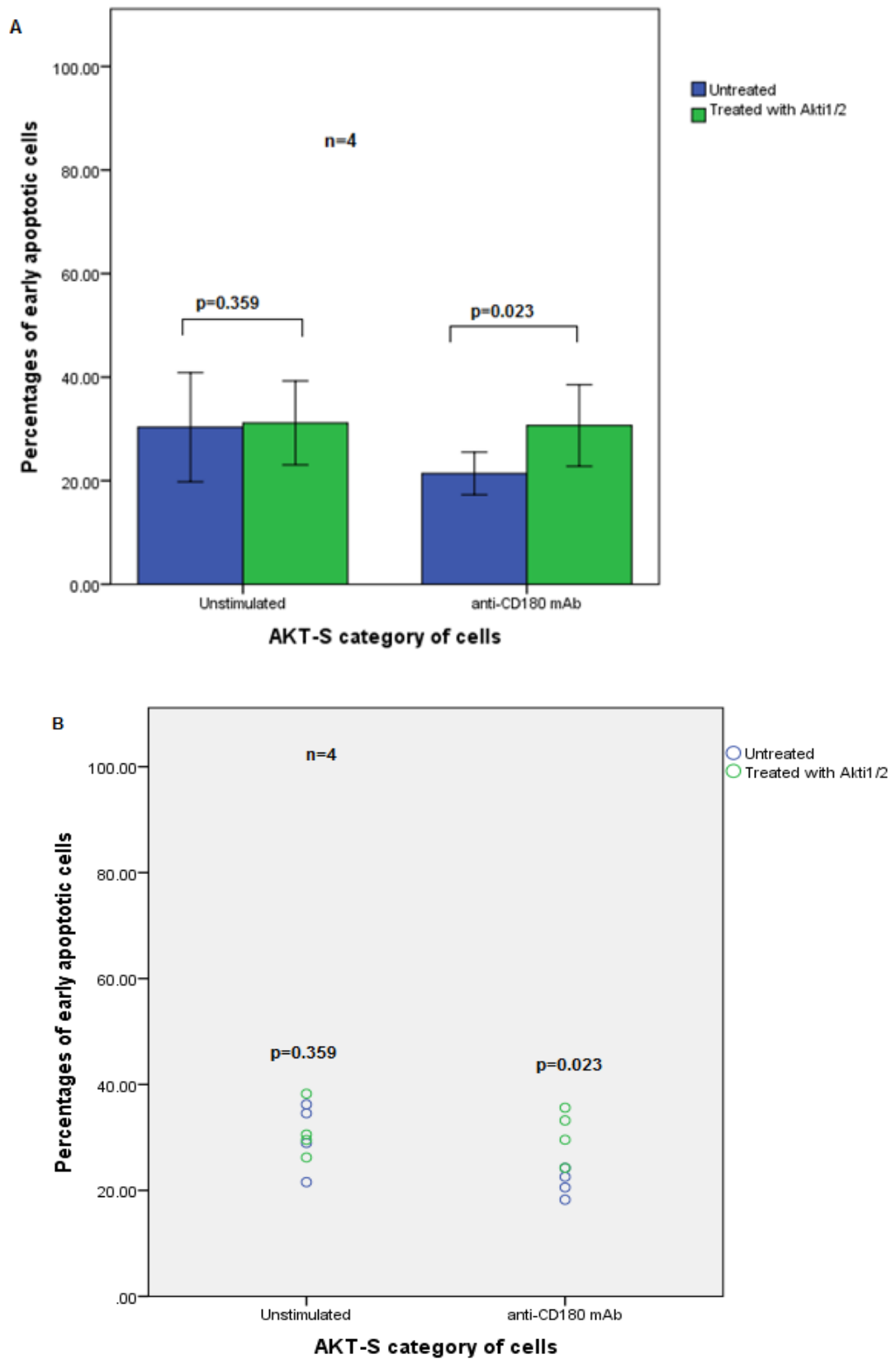
Since it has been documented that AKT activation is essential for the survival of CLL cells (Ringshausen *et al.*, 2002; Petlickovski *et al.*, 2005; Longo *et al.*, 2007) evaluating possible changes in the survival of CLL cells following CD180 ligation with and without AKT inhibitors was important. The percentages of viable cells were measured by assessing changes in mitochondrial membrane potential using DiOC<sub>6</sub> dye. These recent data confirmed previous observations that stimulation of CLL cells with anti-CD180 mAb enhanced their survival (Porakishvili *et al.*, 2005). Indeed, there was a significant increase in the percentages of viable cells ( $p=0.045$ ) following CD180 ligation compared with basal levels confirming AKT-mediated anti-apoptotic activity in CD180-induced AKT-S cells. Interestingly, pre-treatment of unstimulated AKT-S cells with Akti1/2 did not exert any effect on apoptosis ( $p=0.220$ , Figure 4.9). As expected, in all six AKT-S CLL samples stimulated with anti-CD180 mAb, percentages of viable cells were significantly reduced when pre-treated with Akti1/2 compared to untreated samples ( $p=0.007$ , Figure 4.9), down to the basal levels confirming the inhibitory action of Akti1/2 on AKT-mediated pro-survival pathway in AKT-S CLL cells.



**Figure 4.9: Percentages of DiOC<sub>6</sub><sup>bright</sup> viable cells in unstimulated and anti-CD180 mAb stimulated AKT-S CLL cells untreated or treated with Akti1/2 inhibitor: Bar chart (A) and scatter plot (B).** Cells were incubated with Akti1/2, washed and stimulated with anti-CD180 mAb for 24h, washed, stained with anti-CD19

mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

In some AKT-S CLL samples, changes in the survival of CLL cells following CD180 ligation with and without AKT inhibitors were assessed using the annexin/PI method which detects the loss of phospholipid asymmetry in cell membranes during apoptosis (Terasaki, 1989; Koning *et al.*, 1993). Indeed, there was a significant decrease in the percentages of annexin<sup>pos</sup>PI<sup>neg</sup> cells ( $p=0.011$ ) following CD180 ligation compared with basal levels confirming AKT-mediated anti-apoptotic activity in CD180-induced AKT-S cells. Similar to the results seen with DiOC<sub>6</sub>, pre-treatment of unstimulated AKT-S cells with Akti1/2 did not exert any effect on the level of apoptosis ( $p=0.359$ , Figure 4.10). As expected, in all four AKT-S CLL samples stimulated with anti-CD180 mAb, percentages of Annexin<sup>pos</sup>PI<sup>neg</sup> cells were significantly increased when pre-treated with Akti1/2 compared to untreated samples ( $p=0.023$ , Figure 4.10), further confirming the inhibitory action of Akti1/2 on AKT-mediated pro-survival pathway in AKT-S CLL cells.



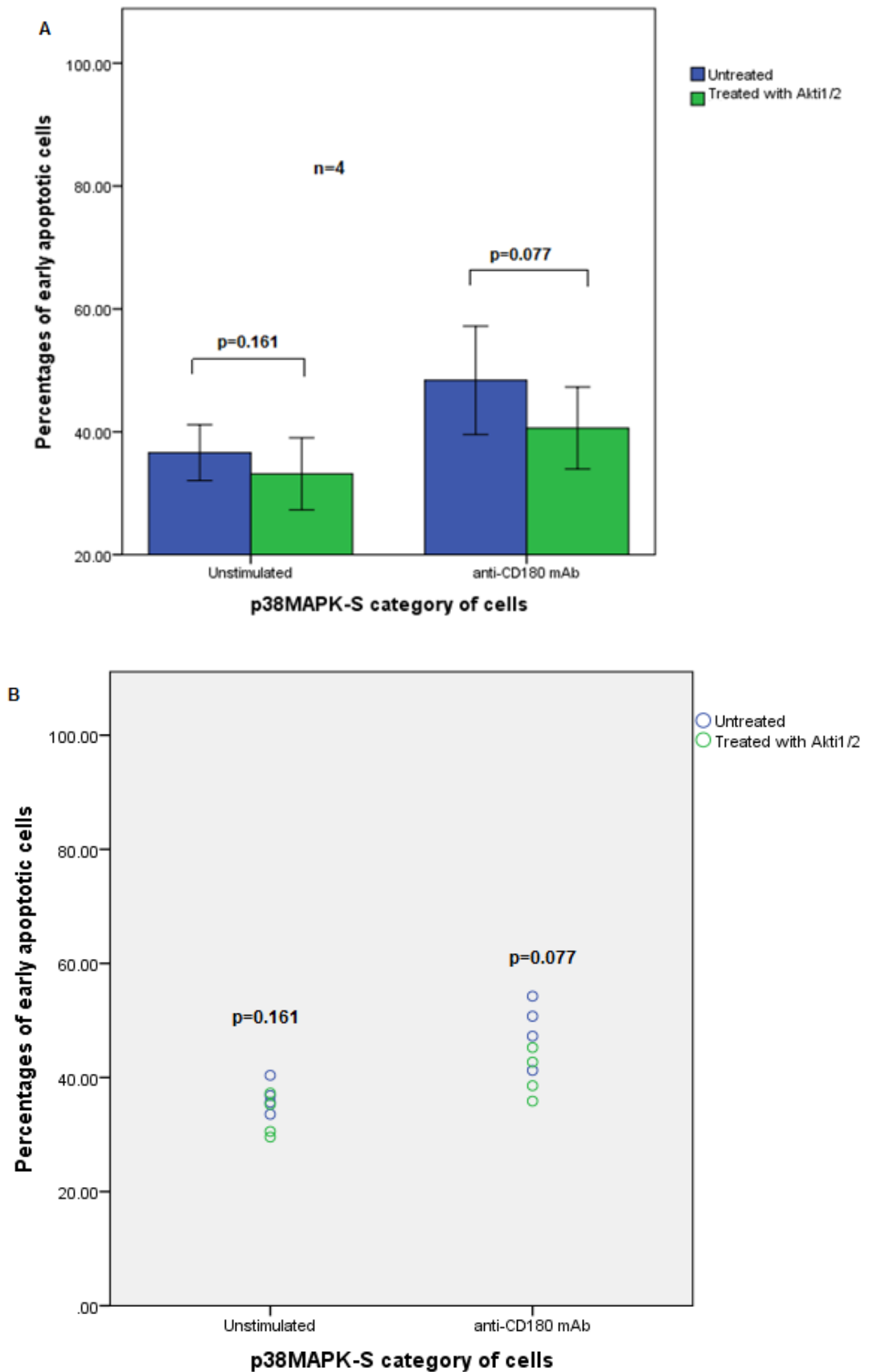
**Figure 4.10: Percentages of early apoptotic cells in unstimulated and anti-CD180 mAb stimulated AKT-S CLL cells untreated or treated with Akti1/2 inhibitor: Bar chart (A) and scatter plot (B).** CLL cells were incubated with Akti1/2 inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for 24h or left



unstimulated, washed, stained with anti-CD19 mAb, washed, resuspended in binding buffer, loaded with PI and Annexin V-FITC for 10 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

Interestingly, AKT inhibitor elevated percentages of apoptotic cells to the level characteristic for unstimulated CLL cells suggesting that a fraction of CLL cells was susceptible to plasticity of a survival vs. apoptosis.

The effect of Akti1/2 on the survival of p38MAPK-S CLL cells was assessed by using Annexin-PI assay. In line with the previous data in all four p38MAPK-S CLL samples, the percentages of early apoptotic cells were significantly increased following CD180 ligation compared with basal levels ( $p=0.011$ , Figure 4.11) confirming p38MAPK- mediated pro-apoptotic effect of CD180 ligation in p38MAPK-S CLL cells. As expected pre-treatment of p38MAPK-S CLL cells with AKT inhibitor Akti1/2 had no effect on survival in all 4 p38MAPK-S CLL samples as there were no significant changes in basal ( $p=0.161$ , Figure 4.11), or anti-CD180-stimulated percentages of early apoptotic cells ( $p=0.077$ , Figure 4.11) following pre-treatment with Akti1/2.



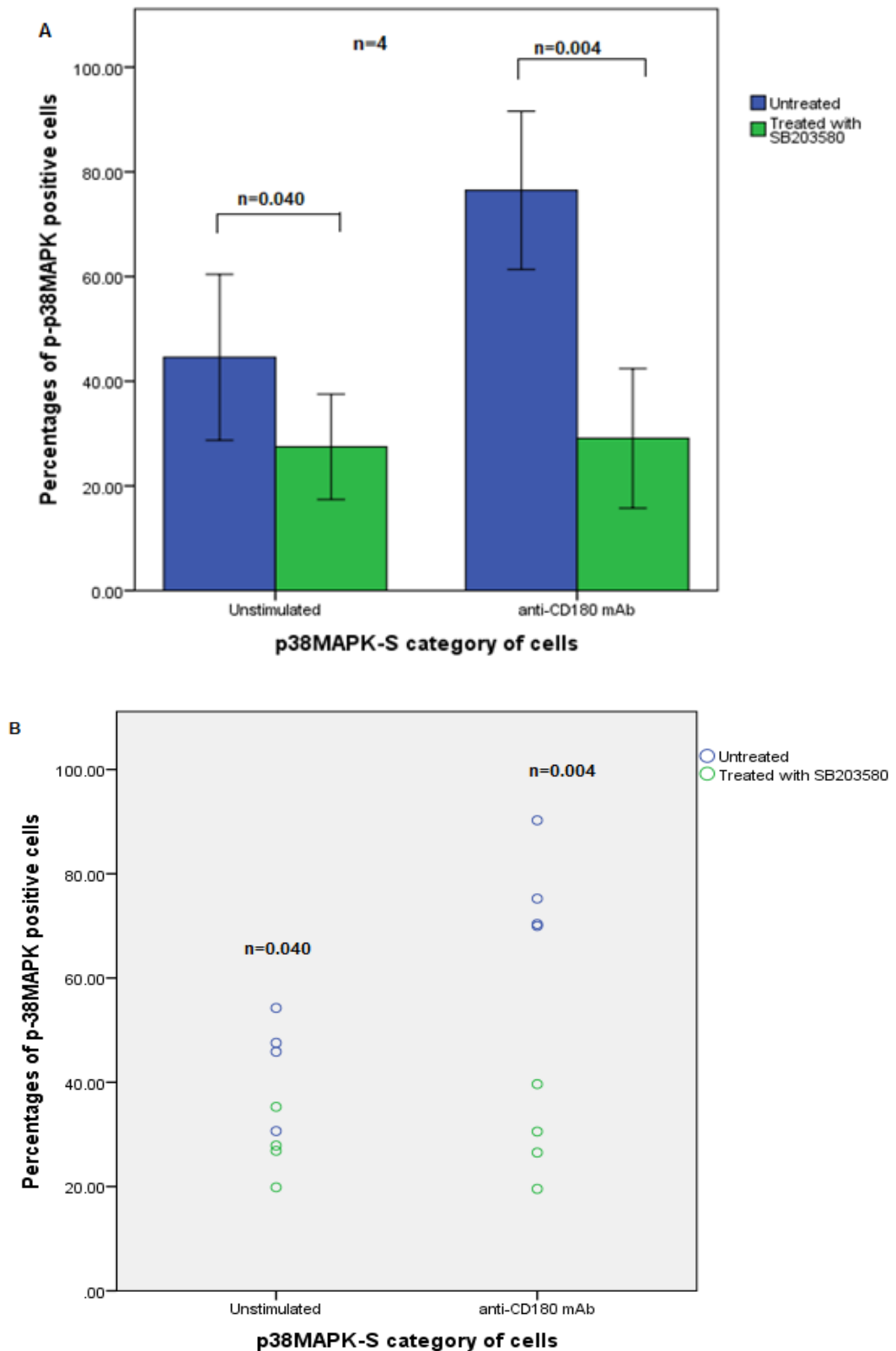
**Figure 4.11: Percentages of early apoptotic cells in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL cells untreated or treated with Akti1/2 inhibitor: Bar chart (A) and scatter plot (B). CLL cells were incubated**

with Akt1/2 inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for 24h or left unstimulated, washed, stained with anti-CD19 mAb, washed, resuspended in binding buffer, loaded with PI and Annexin V-FITC for 10 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

#### **4.2.2 Effect of SB203580 on p38MAPK phosphorylation and cell survival in p38MAPK-S and AKT-S CLL cells**

In line with the previous publications of our research group (Porakishvili *et al.*, 2011) in all four p38MAPK-S CLL samples, a significant increase in the percentages of cells with p38MAPK-P was observed, compared with basal levels following stimulation with anti-CD180 mAb ( $p=0.022$ , Figure 4.12). To assess the effect of SB203580 on p38MAPK activity in CLL cells, the phosphorylation status of p38MAPK after pre-treating cells with the p38 inhibitor, SB203580 was studied.

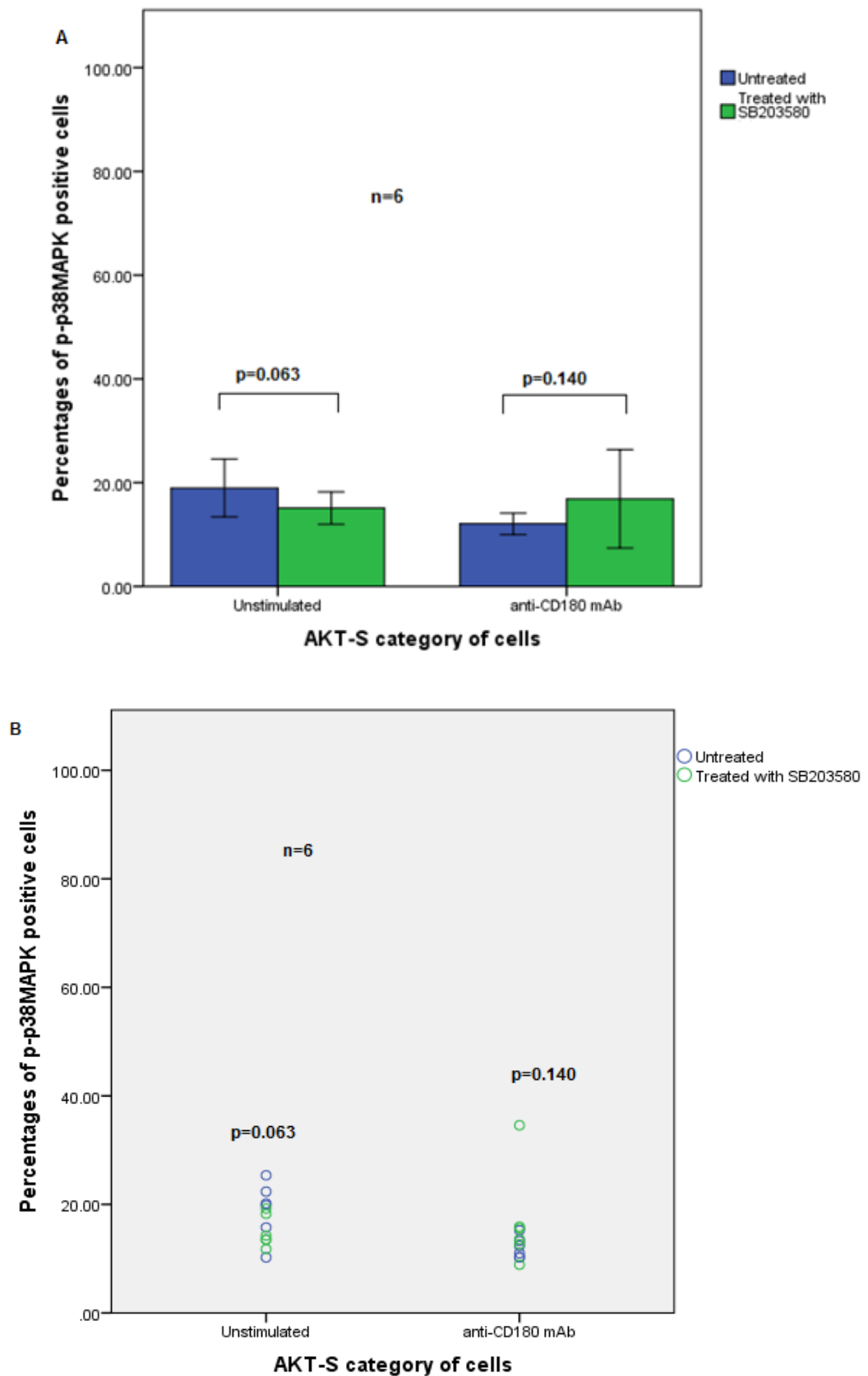
In contrast to the effect shown by Akt1/2 inhibitor, pre-treatment of unstimulated p38MAPK-S cells with SB203580 significantly reduced spontaneous phosphorylation of p38MAPK compared to untreated samples ( $p=0.040$ , Figure 4.12). As expected, in all four p38MAPK-S CLL samples stimulated with anti-CD180 mAb, phosphorylation of p38MAPK was significantly reduced when pre-treated with SB203580 compared to untreated samples, down to the basal levels ( $p=0.004$ , Figure 4.12).



**Figure 4.12: Percentages of cells expressing p38MAPK-P in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL samples untreated or treated with SB203580 inhibitor: Bar chart (A) and scatter plot (B). CLL cells were incubated with SB203580 inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for 20 min (CD180 mAb) or left unstimulated in medium, washed,**

stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-p38MAPK-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

Assessing the effect of SB203580 on AKT-S CLL cells has shown that pretreatment with SB203580 had no effect on phosphorylation of p38MAPK protein kinase in all 6 AKT-S CLL cells as there was no substantial reduction in basal ( $p=0.063$ , Figure 4.13) or anti-CD180-stimulated percentages of p38MAPK-P cells ( $p=0.140$ , Figure 4.13).

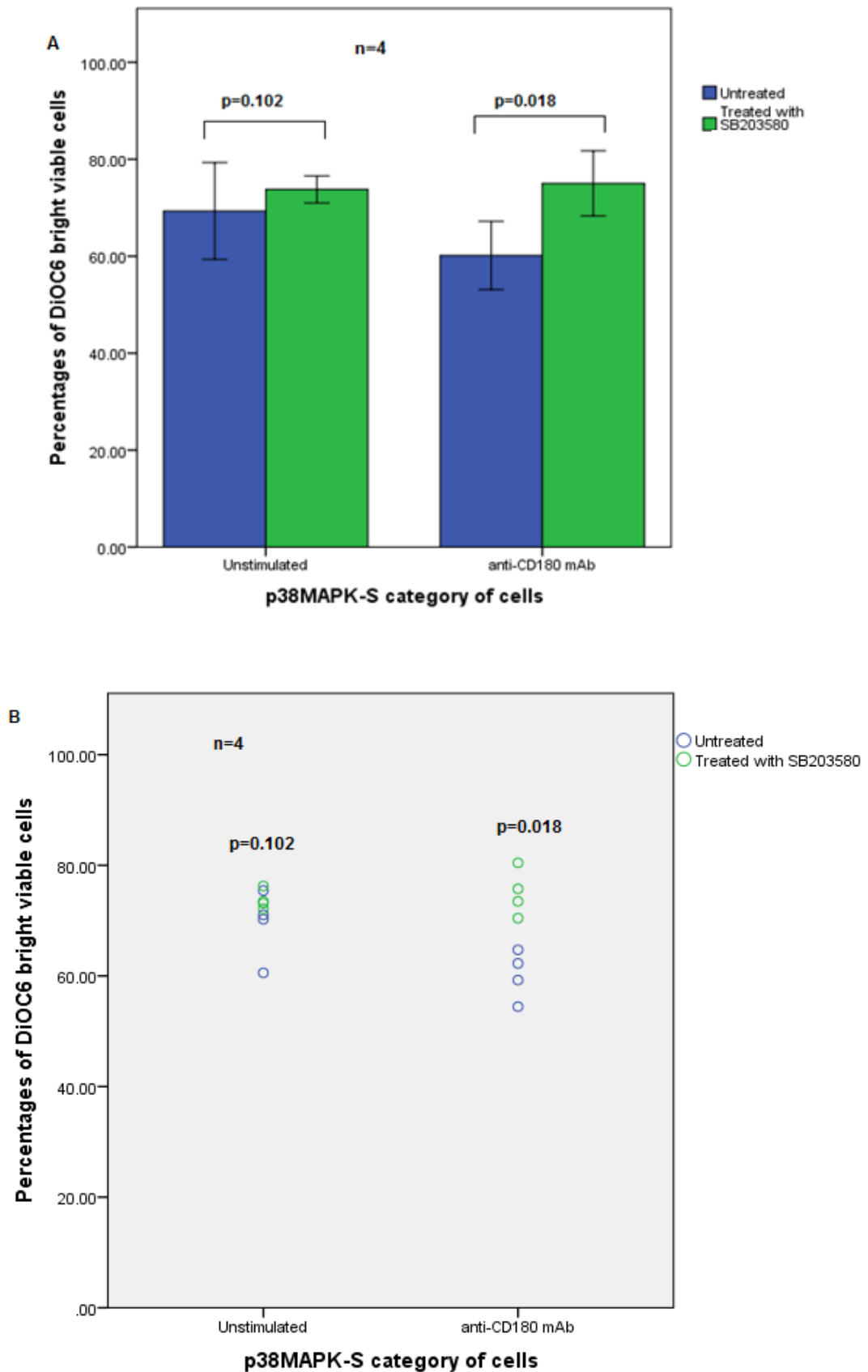


**Figure 4.13: Percentages of cells expressing p38MAPK-P in unstimulated and anti-CD180 mAb stimulated AKT-S CLL samples untreated or treated with SB203580 inhibitor: Bar chart (A) and scatter plot (B).** CLL cells were incubated with SB203580 inhibitor or left untreated, washed and stimulated with anti-

CD180 mAb for 20 min (CD180 mAb) or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-AKT(Ser473)-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

Although the role of p38MAPK-mediated signalling in CLL is yet unclear, it has been documented that p38MAPK activation is associated with proliferation of various cells in response to CpG-ODN (Ahmed-Nejad *et al.*, 2002; Park *et al.*, 2002; Takeshita *et al.*, 2004; Peng *et al.*, 2005). In CLL it seems that p38MAPK is involved in the regulation of cell survival (Grumont *et al.*, 1998; Piatelli *et al.*, 2004), establishment of cell tolerance status (Ntoufa *et al.*, 2012) and apoptosis (Negro *et al.*, 2012). Therefore, it was essential to assess possible changes in the survival of CLL cells following CD180 ligation with and without SB203580 inhibitors. As described in chapter 3 and the previous publication (Porakishvili *et al.*, 2015), current data confirmed that CD180-mediated activation of p38MAPK could induce apoptosis in CLL cells.

The percentages of viable cells were measured using DiOC<sub>6</sub> dye and this data yet again confirmed that stimulation with anti-CD180 mAb led to apoptosis of p38MAPK-S CLL cells. Indeed, there was a significant decrease in the percentages of DiOC<sub>6</sub><sup>bright</sup>, viable cells ( $p=0.001$ , Figure 4.14) following CD180 ligation compared with basal levels confirming p38MAPK mediated pro-apoptotic activity. However, pre-treatment of unstimulated p38MAPK-S cells with SB203580 did not exert a significant effect on survival of p38MAPK-S CLL cells ( $p=0.102$ , Figure 4.14). As expected, in all four p38MAPK-S CLL samples stimulated with anti-CD180 mAb, percentages of viable cells were significantly increased when pre-treated with SB203580 compared to untreated samples ( $p=0.018$ , Figure 4.14), confirming the inhibitory action of SB203580 on p38MAPK-mediated pro-apoptotic pathway in p38MAPK-S CLL cells.

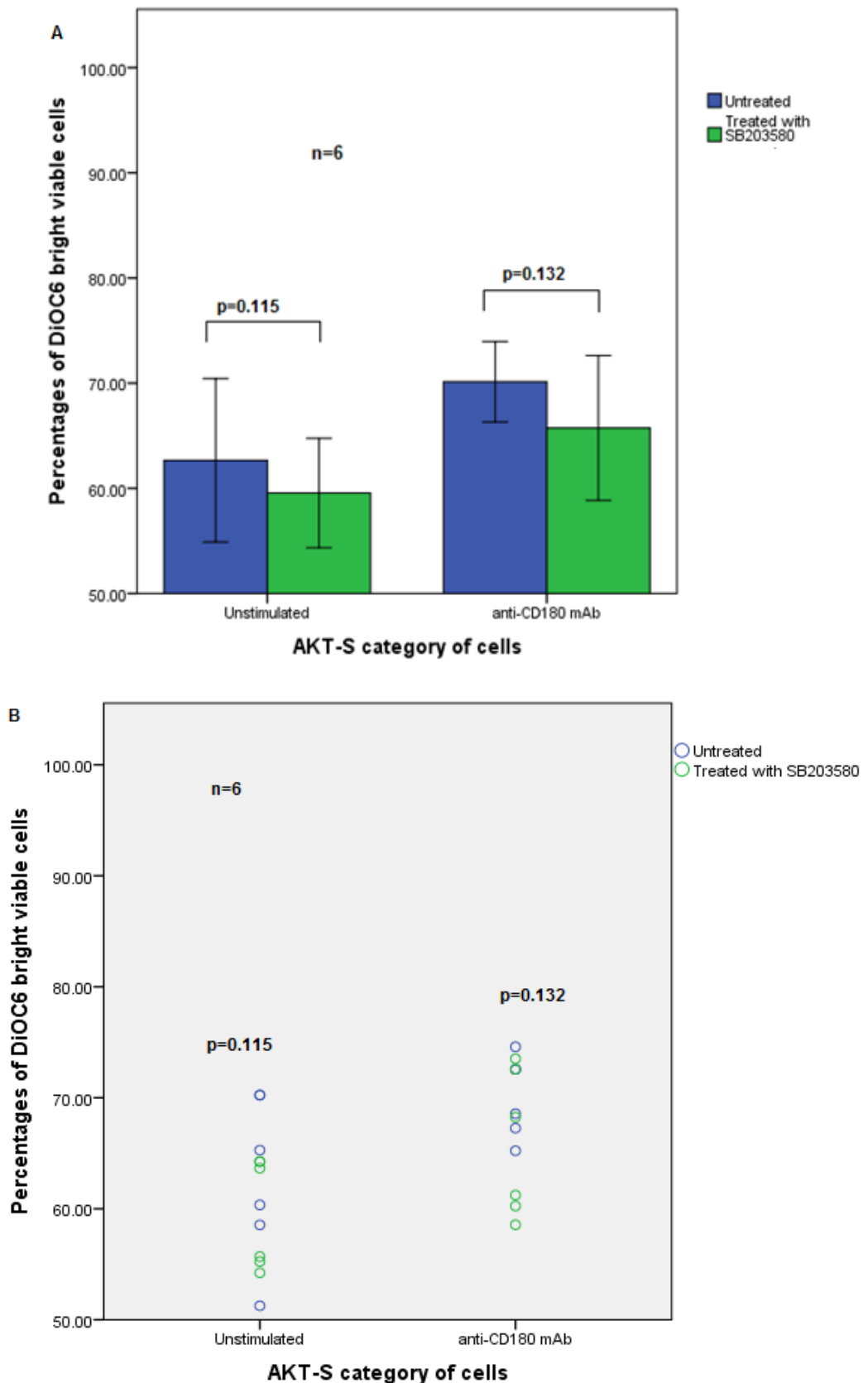


**Figure 4.14: Percentage of viable cells in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL cells treated or untreated with SB203580 inhibitor: Bar chart (A) and scatter plot (B). Cells were pretreated with SB203580**



or left untreated, washed and then stimulated with anti-CD180 mAb for 24h, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

Moreover, the effect of SB203580 on the survival of AKT-S CLL cells was assessed. Importantly, pre-treatment of AKT-S CLL cells with p38MAPK inhibitor SB203580 had no appreciable effect on survival in all 6 AKT-S CLL cells as there was no significant change in basal ( $p=0.115$ , Figure 4.15), or anti-CD180-stimulated percentages of viable cells ( $p=0.132$ , Figure 4.15) following pre-treatment with SB203580.



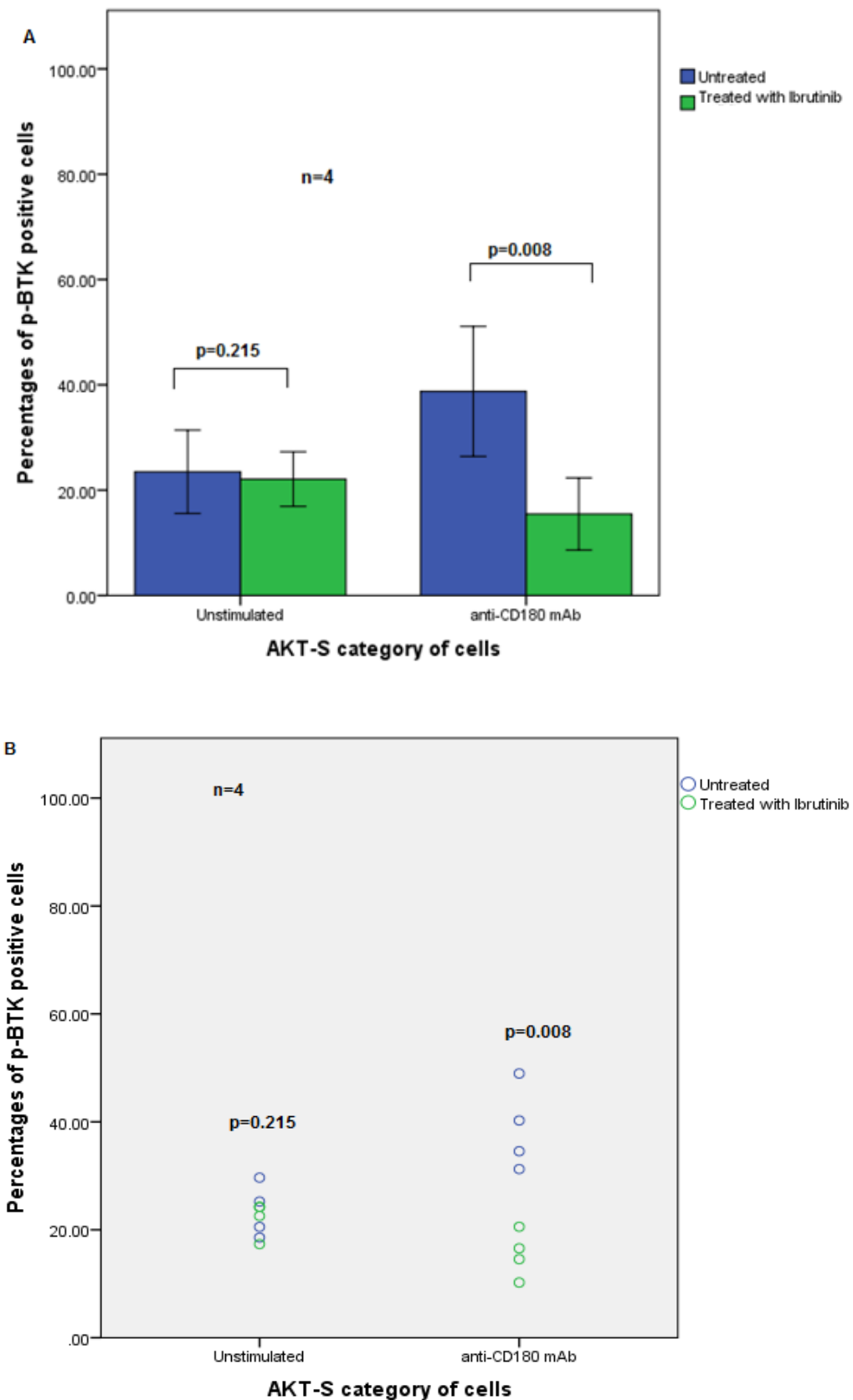
**Figure 4.15: Percentage of viable cells in unstimulated and anti-CD180 mAb stimulated AKT-S CLL cells untreated or treated with SB203580 inhibitor: Bar chart (A) and scatter plot (B).** Cells were pretreated with SB203580, washed and then stimulated with anti-CD180 mAb for 24h, washed, stained with anti-CD19 mAb,

loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

#### **4.2.3. Effect of Ibrutinib on BTK Phosphorylation and cell survival in p38MAPK-S and AKT-S CLL cells**

The effect of Ibrutinib on BTK activation in both AKT-S and p38MAPK-S categories of CLL cells were assessed by the phosphorylation status of BTK after treatment with this specific BTK inhibitor. In all five AKT-S CLL samples analysed, the percentages of cells with phosphorylated BTK were significantly increased compared to basal levels after ligating with anti-CD180 mAb ( $p=0.005$ ).

Interestingly, in four out of five samples, pretreatment with Ibrutinib significantly reduced CD180 mediated phosphorylation of BTK down to the basal levels ( $p=0.008$ , Figure 4.16). Similar to the results obtained for Akt1/2, pre-treatment of unstimulated AKT-S cells in those four samples did not show any significant change in spontaneous phosphorylation of BTK, when untreated and treated ( $p=0.215$ , Figure 4.16).



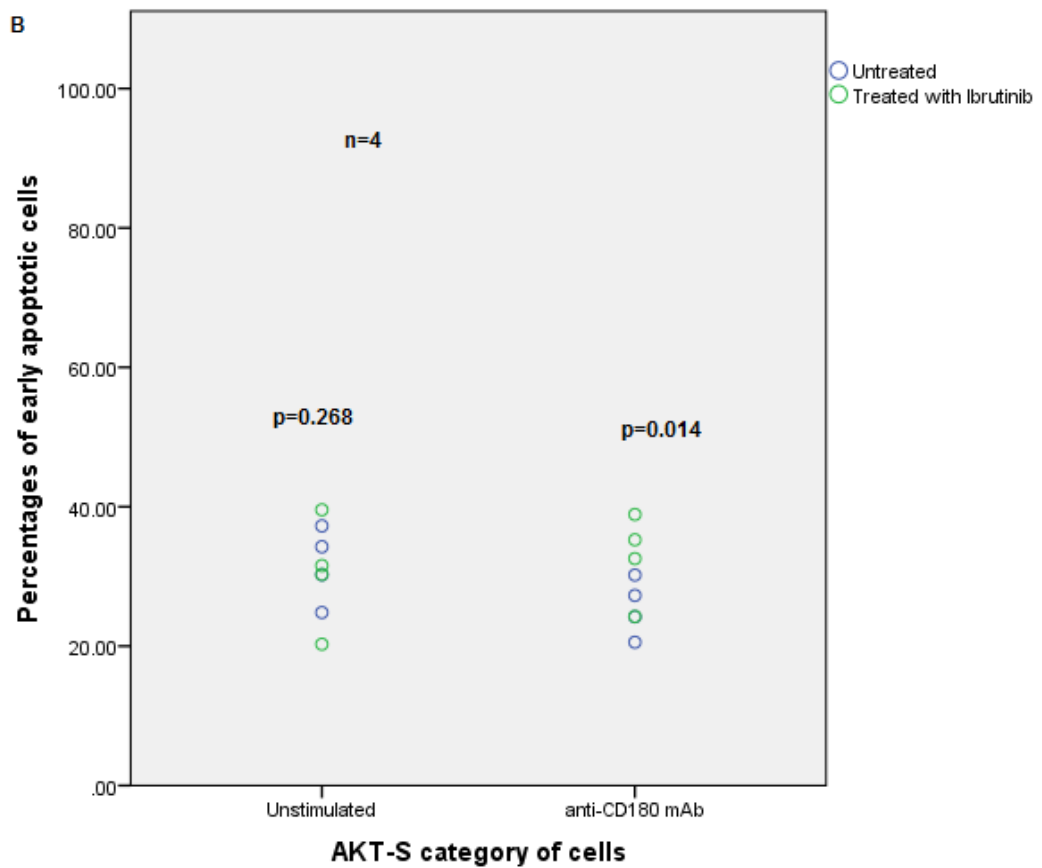
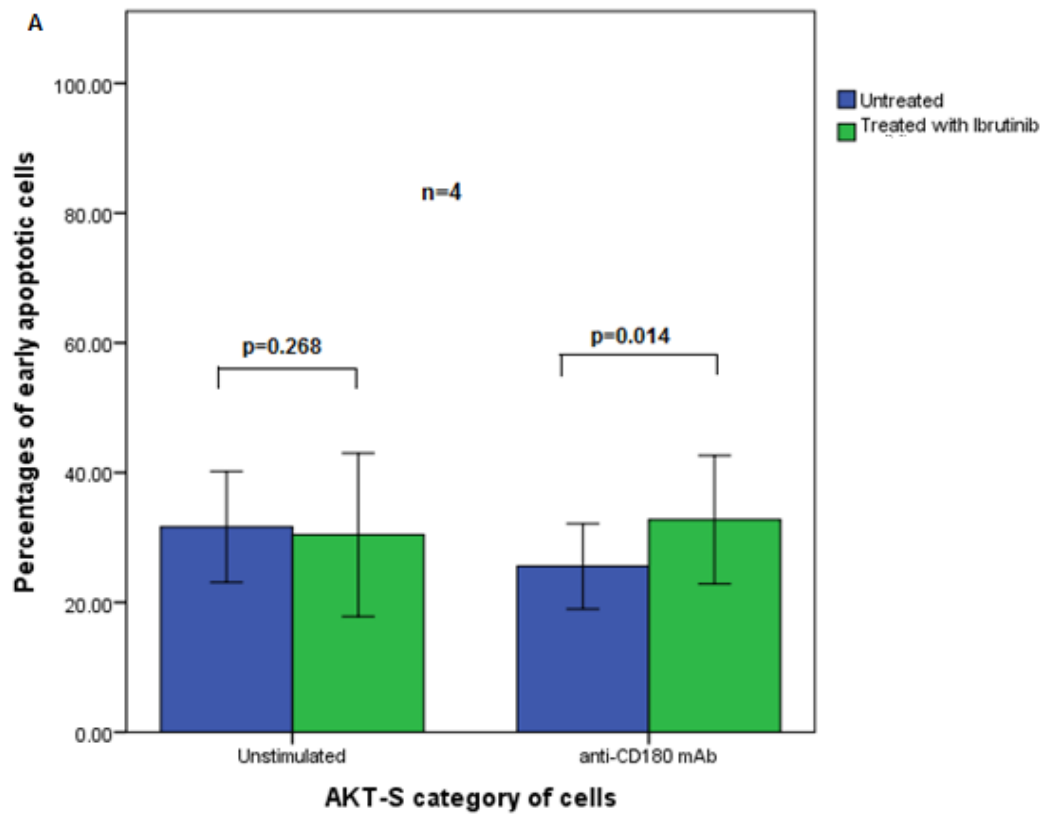
**Figure 4.16: Percentages of cells expressing BTK-P in unstimulated and anti-CD180 mAb stimulated AKT-S CLL samples when untreated or treated with Ibrutinib: Bar chart (A) and scatter plot (B).** CLL cells were incubated with Ibrutinib inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for 20

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min (CD180 mAb) or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-BTK-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

Since it has been documented that BTK activation is an essential contributor to the survival of CLL cells (Petro *et al.*, 2000, 2001; Honigberg *et al.*, 2010) the possible changes in the survival of CLL cells caused by Ibrutinib treatment were studied and compared, followed by CD180 ligation. Percentages of early apoptotic cells were measured by analysing the percentages of Annexin<sup>pos</sup>PI<sup>neg</sup> cells. This data yet again confirmed that stimulation of AKT-S CLL cells with anti-CD180 mAb involves phosphorylation of BTK which leads to the CLL cell survival.

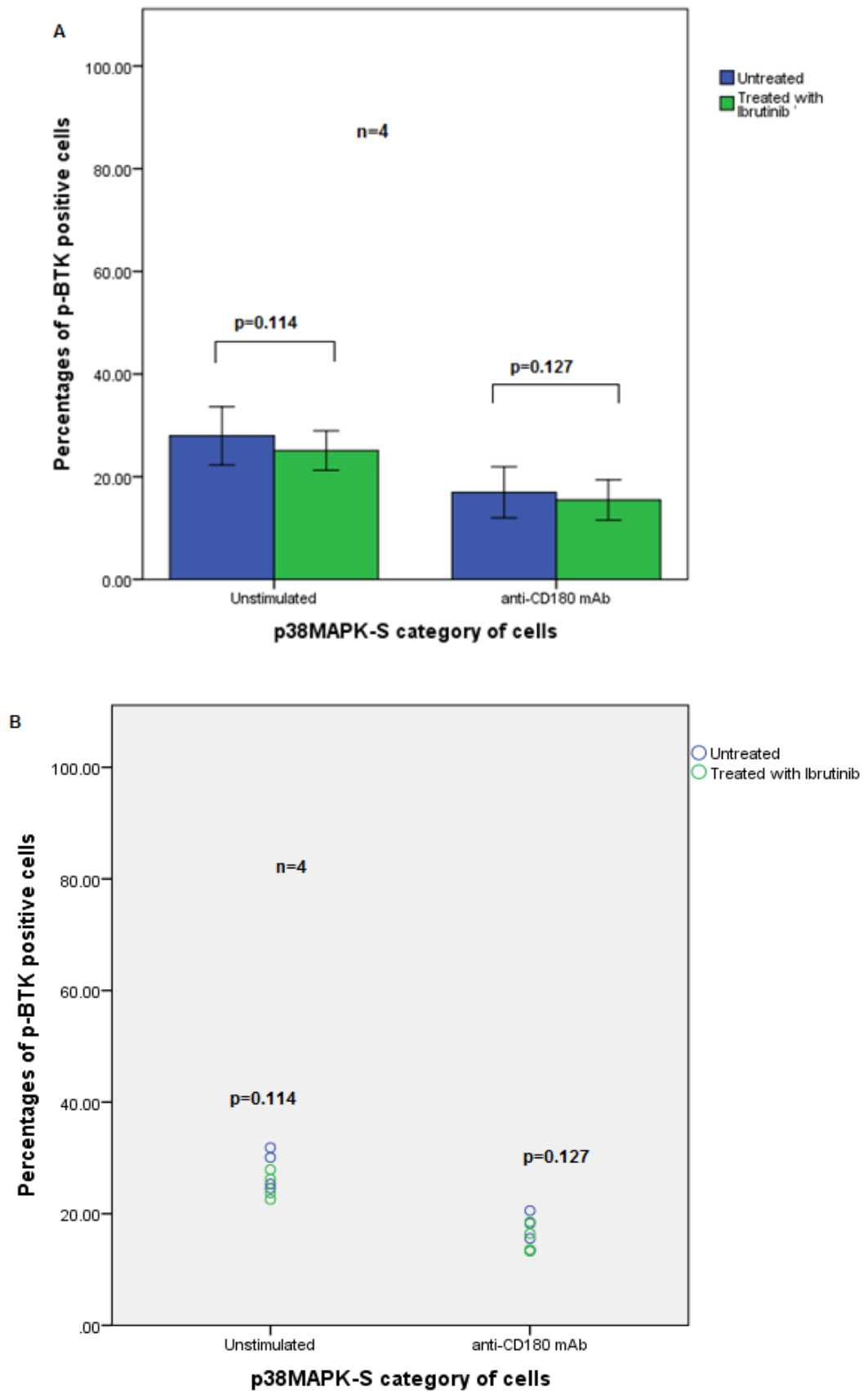
Indeed, there was a significant decrease in the percentages of early apoptotic cells in all five samples analysed ( $p=0.001$ ) following CD180 ligation compared with basal levels confirming BTK mediated pro-survival activity in CD180-induced AKT-S cells. Interestingly, pre-treatment of unstimulated CLL cells with Ibrutinib did not exert a noticeable effect on apoptosis, ( $p=0.268$  Figure 4.17). In four out of five AKT-S CLL samples, pretreatment with Ibrutinib significantly increased anti-CD180 stimulated percentages of early apoptotic cells ( $p=0.013$ , Figure 4.17), confirming that BTK involves in CD180-mediated survival in AKT-S cells.



**Figure 4.17: Percentages of early apoptotic cells in unstimulated and anti-CD180 mAb stimulated AKT-S CLL cells untreated or treated with Ibrutinib: Bar chart (A) and scatter plot (B).** CLL cells were incubated with Ibrutinib or left

untreated, washed and stimulated with anti-CD180 mAb for 24h or left unstimulated, washed, stained with anti-CD19 mAb, washed, resuspended in binding buffer, loaded with PI and Annexin V-FITC for 10 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

To assess the effect of Ibrutinib on BTK activation in p38MAPK-S CLL samples, the phosphorylation status of BTK was examined after treating with inhibitor followed by anti-CD180 mAb. In all four p38MAPK-S CLL samples, the percentages of cells with pBTK were significantly decreased following CD180 ligation compared with basal levels ( $p=0.0002$ ) confirming the previous results in Chapter 3 and the previous publication (Porakishvili *et al.*, 2015), which is the reduction in phosphorylation of BTK in p38MAPK-S when stimulated with CD180 mAb. Importantly, pre-treatment of p38MAPK-S CLL cells with BTK inhibitor Ibrutinib had no effect on phosphorylation of BTK protein kinase in all 4 p38MAPK-S CLL cells as there was no substantial reduction in basal ( $p=0.114$ , Figure 4.18) or anti-CD180-stimulated percentages of BTK-P cells ( $p=0.127$ , Figure 4.18) following pre-treatment with Ibrutinib.

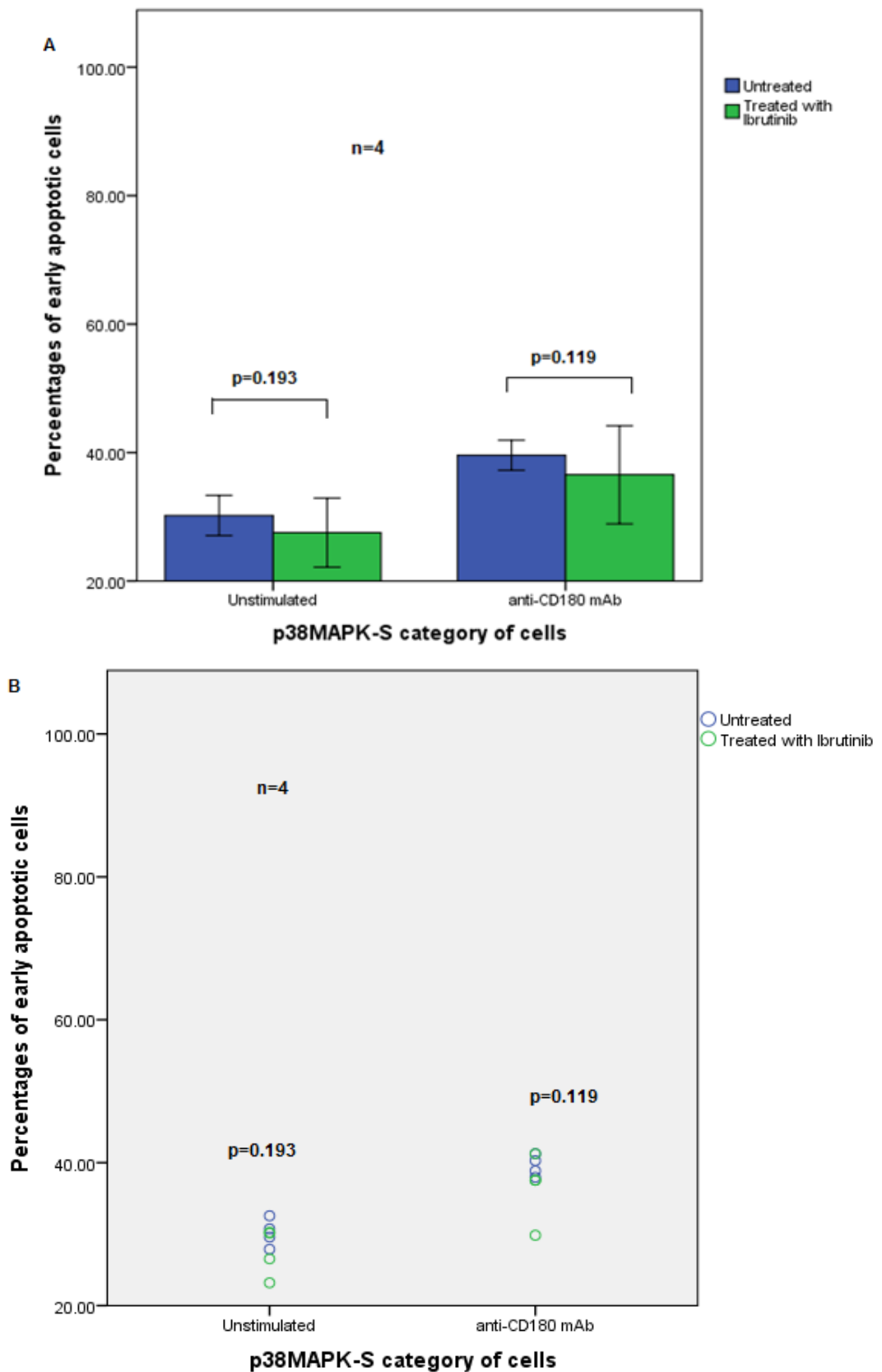


**Figure 4.18: Percentages of cells expressing BTK-P in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL samples when untreated or treated with Ibrutinib: Bar chart (A) and scatter plot (B). CLL cells were**



incubated with Ibrutinib inhibitor or left untreated, washed and stimulated with anti-CD180 mAb for 20 min (CD180 mAb) or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-BTK-P as described in the Materials and methods. Samples were analysed by flow cytometry and expressed as percentages of positive cells. P values were calculated using the paired *t*-test.

It was necessary to assess the effect of Ibrutinib on the survival of p38MAPK-S CLL cells. Indeed, there was a significant increase in the percentages of early apoptotic cells in all four samples analysed ( $p=0.003$ ) following CD180 ligation compared with basal levels confirming CD180-mediated signalling in p38MAPK-S can lead to pro-apoptotic pathway although there is no recruitment of BTK kinase in signalling of this category of cells. Interestingly, pre-treatment of Ibrutinib had no appreciable effect on survival in all 4 p38MAPK-S CLL samples as there was no significant change in basal ( $p=0.193$ , Figure 4.19), or anti-CD180-stimulated percentages of early apoptotic cells ( $p=0.119$ , Figure 4.19) following pre-treatment with Ibrutinib.



**Figure 4.19: Percentages of early apoptotic cells in unstimulated and anti-CD180 mAb stimulated p38MAPK-S CLL cells untreated or treated with Ibrutinib: Bar chart (A) and scatter plot (B).** CLL cells were incubated with Ibrutinib or left untreated, washed and stimulated with anti-CD180 mAb for 24h or left

unstimulated, washed, stained with anti-CD19 mAb, washed, resuspended in binding buffer, loaded with PI and Annexin V-FITC for 10 min and analysed by flow cytometry as described in the Materials and methods. The p values were calculated using a non-parametrical *U*-test.

### 4.3 Discussion

#### 4.3.1 AKTi1/2 inhibited CD180-mediated phosphorylation of AKT protein kinase and AKT-S CLL cell survival

In all CLL samples, a significant increase in the percentages of cells with phosphorylated AKT was observed after stimulating with anti-CD180 mAb compared to unstimulated cells. CD180 mediated phosphorylation of AKT in CLL was previously reported by our group (Porakishvili *et al.*, 2011, 2015). Although it has been shown by some authors that CLL cells constitutively express phosphorylated AKT (Cuni *et al.*, 2004; Petickovski *et al.*, 2005; Ticchioni *et al.*, 2007; Zhuang *et al.*, 2010), a significant change in the constitutive phosphorylation of AKT following the treatment with AKTi1/2 inhibitor was not observed in this study (Figure 4.7). Current data indicate that AKT protein kinase is not activated (at least in AKT-S category of CLL cells) without receiving a specific stimulation, the opinion also shared by others (Bernal *et al.*, 2001; Barragan *et al.*, 2002; Ringshausen *et al.*, 2002; Muzio *et al.*, 2008). In all six AKT-S CLL samples stimulated with anti-CD180 mAb Ser473 phosphorylation of AKT was significantly reduced when pre-treated with Akti1/2, down to the basal levels (Figure 4.7). This confirms that CD180 mediated signalling recruits AKT protein kinase in its signalling pathway. Inhibition of AKT by AKTi1/2 in a dose-dependent manner in CLL has been reported by several other studies (Frias *et al.*, 2009; Zhuang *et al.*, 2010) linking it with the induction of PUMA and NOXA proteins the well-known p53-inducible pro-apoptotic members of the Bcl-2 family and a decrease in anti-apoptotic MCL-1 protein.

As expected there was no effect on phosphorylation of AKT seen in p38MAPK-S CLL cells in both unstimulated and anti-CD180 mAb stimulated cells when comparing untreated and AKTi1/2 pre-treated cells (Figure 4.8). This confirms our previous findings (Porakishvili *et al.*, 2015) regarding the dichotomy of CD180-mediated signalling pathways recruiting either AKT or p38MAPK. Although the direct substrates affected by AKT inhibition were not assessed, the

effect of AKTi1/2 on apoptosis of CLL cells was examined. Using both, DiOC<sub>6</sub> and Annexin/PI staining assays, a significant increase in the percentages of viable CLL cells following CD180-ligation was observed compared to basal levels confirming anti-apoptotic activity of CD180 mediated signalling in AKT-S CLL cells (Figure 4.9). That the pre-treatment of AKT-S CLL samples with AKTi1/2 inhibitor significantly reduced the percentages of viable cells upon CD180-ligation and inhibited the CD180 mediated anti-apoptotic activity confirms the hypothesis that CD180-mediated CLL cell survival is operating via AKT (Figures 4.9 and 4.10). Several earlier studies have shown that inhibition of AKT induces apoptosis in CLL cells and inhibit the expression of anti-apoptotic proteins (Frias *et al.*, 2009; Zhuang *et al.*, 2010).

Interestingly, pre-treatment of unstimulated AKT-S cells with Akti1/2 did not exert any effect on apoptosis, indicating as above a lack of spontaneous recruitment of AKT in CLL cells. Importantly, pre-treatment of p38MAPK-S CLL cells with AKT inhibitor Akti1/2 had no effect on the survival of p38MAPK-S CLL cells unstimulated or stimulated with anti-CD180 mAb as there was no reduction in basal or anti-CD180-stimulated percentages of early apoptotic cells (Figure 4.11) confirming that CD180 ligation in AKT-S CLL cells does not recruit p38MAPK protein kinase. Up to my knowledge, our research group was the first to report on a dichotomy of CD180-mediated signalling pathways in CLL cells.

#### **4.3.2 SB203580 inhibited CD180 mediated phosphorylation of p38MAPK protein kinase and p38MAPK-S cell apoptosis**

In all four p38MAPK-S CLL samples analysed, a significant increase in the percentages of cells with phosphorylated p38MAPK was observed after stimulating with anti-CD180 mAb, compared to the basal levels (Figure 4.12). CD180 mediated phosphorylation of p38MAPK in CLL cells was previously reported by our group (Porakishvili *et al.*, 2011; 2015).

Contrary to the data shown for AKT above, pre-treatment of unstimulated p38MAPK-S cells with SB203580 significantly reduced spontaneous phosphorylation of p38MAPK compared to untreated samples. This suggests the presence of constitutively phosphorylated p38MAPK in unstimulated CLL cells. Similar results regarding the presence of phosphorylated p38MAPK in all fresh CLL cells but not in normal B cells were reported others (Ringshausen *et al.*,

2004; Sainz-Perez *et al.*, 2006; Blix *et al.*, 2012). It has been proposed that constitutive activation of the p38MAPK, observed in the majority of CLL cases was critical for matrix metalloproteinase-9 (MMP-9) production, which plays an important role in tumour-angiogenesis and tumour homing (Molica *et al.*, 2003). As expected, in all four p38MAPK-S CLL samples stimulated with anti-CD180 mAb, phosphorylation of p38MAPK was significantly reduced when pre-treated with SB203580 compared to untreated samples, down to the basal levels confirming that CD180-mediated signalling recruits p38MAPK in p38MAPK-S cells (Figure 4.12). It was suggested that SB203580 has no effect on activation of p38MAPK by mitogen-activated protein kinase 6 (MKK6), which is an essential component of the MAP kinase signal transduction pathway, but the continuous presence of SB203580 blocks the ability of p38MAPK to phosphorylate its downstream substrates (Kumar *et al.*, 1999), the question we might address in our future studies. That SB203580 had no effect on phosphorylation of p38MAPK in AKT-S CLL cells (Figure 4.13) further confirmed the hypothesis that CD180 ligation operates via two alternative signalling pathways in AKT-S and p38MAPK-S categories of CLL cells.

p38MAPK-mediated apoptosis only in p38MAPK-S category of CLL cells is a phenomenon first detected by us. Conflicting data has been published previously regarding the role of p38MAPK in apoptosis. Earlier on it was demonstrated that p38MAPK inhibitor, SB203580 could significantly inhibit BCR-induced apoptosis in B104 cells, human B lymphoma line (Graves *et al.*, 1998). It was further reported that Rituximab activates a CD20-mediated signalling pathway to induce apoptosis of the CLL cells in p38MAPK activation-dependent manner (Pederson *et al.*, 2002). p38MAPK was shown to be a key mediator of glucocorticoid-induced apoptosis in all lymphoid cells (Miller *et al.*, 2005). However, Sainz-Perez *et al.*, (2006) have demonstrated a paradoxical effect of p38MAPK which acts as an important intermediate for the function of Mda-7/IL-24, tumour suppressor proteins by inducing apoptosis in diverse cancer cells, whereas promoting the survival in CLL through p38MAPK activation. Also, Sarkar *et al.*, (2002) demonstrated SB203580 mediated inhibition of mda-7 induced apoptosis in human melanoma cells. In the light of these contradictory data, it was important to study the effect of SB203580 on the survival of CLL cells. The current data strongly indicate that p38MAPK selective inhibitor abrogates CD180-mediated

pro-apoptotic action of this protein kinase (Figure 4.14) in p38MAPK-S CLL cells, as suggested earlier (Porakishvili *et al.*, 2011). Interestingly, any significant changes in the percentages of viable cells in unstimulated p38MAPK-S CLL cells were not observed when pre-treated with SB203580 (Figure 4.14), although pre-treatment of unstimulated p38MAPK-S cells with SB203580 significantly reduced spontaneous phosphorylation of p38MAPK compared to untreated samples (Figure 4.12). This might indicate that although SB203580 inhibits phosphorylation of constitutively activated p38MAPK, the latter is not a sole contributor to the induction of apoptosis of p38MAPK-S CLL cells, and some complementary upstream or downstream events are required which can only be initiated upon stimulation with anti-CD180. As I mentioned above, these signalling molecules are still to be identified.

That, similar to the results seen with AKTi1/2 inhibitor, pre-treatment of AKT-S CLL cells with p38MAPK inhibitor SB203580 had no effect on survival of AKT-S CLL cells (Figure 4.15) once more confirming the existence of two alternative signalling pathways upon ligation of CD180.

#### **4.3.3 Ibrutinib inhibited CD180 mediated phosphorylation of BTK protein kinase and cell survival in AKT-S CLL cells while not affecting CD180-mediated BTK phosphorylation or cell apoptosis in p38MAPK-S CLL cells**

In line with our previous publications (Porakishvili *et al.*, 2011; 2015) in all five AKT-S CLL samples analysed, there was a statistically significant increase in the percentages of cells with phosphorylated BTK, compared with basal levels following stimulation with anti-CD180 mAb (Figure 4.16). This validates the previous findings that BTK is involved in CD180 mediated signalling in AKT-S CLL cells (Porakishvili *et al.*, 2015). The significance of BTK activation for BCR-mediated signalling has been previously demonstrated. Upon BCR activation, BTK becomes activated by other tyrosine kinases, such as Lyn and SYK, resulting in activation of transcription factors needed for B-cell proliferation and differentiation (Petro *et al.*, 2000). BTK is involved in signalling pathways of other receptors related to B cell migration and adhesion, such as chemokine receptors (CXCR4 and CXCR5) and adhesion molecules. It seems that BTK is critical for the development and function of normal B lymphocytes, and its expression appears to be required for CLL development (Kil *et al.*, 2013). However, the

precise role of the kinase function of BTK in the initial development of CLL, as well as the disease expansion phase, remains unclear (Woyach *et al.*, 2014).

There were no significant changes in spontaneous phosphorylation of BTK, confirmed by other studies with Ibrutinib (Figure 4.16). It appears that BTK is preferentially activated after receiving signals via BCR or CD40 (Herman *et al.*, 2011). In this study, the stimulation for the activation of BTK is driven by anti-CD180 mAb. Interestingly, in four out of five CLL samples stimulated with anti-CD180 mAb, phosphorylation of BTK was significantly reduced when pre-treated with Ibrutinib compared to untreated samples, down to the basal levels confirming that Ibrutinib inhibits CD180 mediated signalling via BTK in AKT-S cells (Figure 4.16). Current data also indicate that some of the key signalling PKs contributing to BCR-mediated signalling pathways are shared by CD180, as it has been suggested in Chapter 3.

Importantly, although four out of five AKT-S samples demonstrated a significant reduction in the levels of BTK-P induced by Ibrutinib, one sample showed resistance towards Ibrutinib. It has been recently reported that 5.3% of CLL patients have disease progression following the treatment with Ibrutinib, suggesting resistance of those CLL cells to the inhibitor. The mechanism of resistance to Ibrutinib is thought to be associated with C481S mutation of BTK active site which prevents covalent binding between Ibrutinib and BTK (Furman *et al.*, 2014; Wiestner, 2015).

It is well documented that BTK is involved in pro-survival signalling in both, normal B lymphocytes and CLL cells (Woyach *et al.*, 2014) as well as CLL-cell trafficking (Spaargaren *et al.*, 2003; de Gorter *et al.*, 2007; Byrd *et al.*, 2013;2014). Herman *et al.* (2011) reported that Ibrutinib induces apoptosis in CLL cells stimulated by pro-survival factors such as CD40L, BAFF, IL-6, IL-4, TNF- $\alpha$ , fibronectin, stromal cell contact. Burger *et al.*, (2010) demonstrated the inhibitory action of Ibrutinib on CLL cell survival, proliferation and leukaemic cell migration towards the tissue homing chemokines such as CXCL12 and CXCL13. They have also reported that Ibrutinib downregulated secretion of BCR-dependent chemokines such as CCL3 and CCL4 *in vitro* and in CLL patients receiving Ibrutinib suggesting Ibrutinib may work in part by modulating the CLL cell interaction with the microenvironment rather than by direct cytotoxicity. Recently, Guo *et al.*, (2016) showed that U-CLL cells display higher proliferative capacity correlated with higher BTK-P levels

compared to M-CLL. However, they failed to detect any differences in the Ibrutinib mediated apoptosis between U-CLL and M-CLL samples.

Since our group has previously shown that BTK phosphorylation upon CD180 ligation led to the survival of CLL cells (Porakishvili *et al.*, 2011; 2015), analysing the effect of Ibrutinib on CD180-mediated survival of CLL cells was important. Current data have shown that in four AKT-S samples stimulated with anti-CD180 mAb, Ibrutinib treatment has significantly increased the percentages of early apoptotic cells (Figure 4.17). This confirms that CD180-induced pro-survival role of BTK is effectively inhibited by Ibrutinib in AKT-S. However, no significant change in the number of apoptotic cells observed in the sample which was resistant to Ibrutinib treatment.

Moreover, the effect of Ibrutinib on phosphorylation of BTK in p38MAPK-S CLL cells was studied and interestingly, in all four samples there was no significant change in basal or anti-CD180 mAb stimulated percentages of BTK-P cells (Figure 4.18). This confirms that BTK is not recruited in p38MAPK-S category of cells for CD180-mediated apoptosis (Figure 4.19) as proposed in Chapter 3 and the previous publication Porakishvili *et al.*, (2015). That Ibrutinib had no effect on phosphorylation of BTK in p38MAPK-S CLL cells (Figure 4.18) further confirmed the hypothesis that CD180 ligation operates via two alternative signalling pathways. Similarly, Ibrutinib had no effect on survival of p38MAPK-S CLL cells as there was no reduction in basal or anti-CD180-stimulated percentages of early apoptotic cells (Figure 4.19) confirming the hypothesis that CD180 ligation in p38MAPK-S CLL cells does not recruit BTK protein kinase. Nevertheless, this suggests that therapeutic application of the BTK inhibitors might be limited to the AKT-S CLL cells

In conclusion, specific inhibitors of p-AKT, p-p38MAPK and p-BTK were used to confirm the involvement of these protein kinases in CD180-mediated signalling. As expected, pretreatment of AKT-S CLL cells with AKT inhibitor Akti1/2 resulted in a significant decrease in the number of p-AKT cells, while no effect was seen for p38MAPK-S cells. Likewise, p38MAPK inhibitor SB2035804 suppressed CD180-stimulated levels of p-p38MAPK in p38MAPK-S, but not in AKT-S cells. Moreover, pBTK inhibitor, ibrutinib could suppress phosphorylation of BTK and survival only in AKT-S CLL cells. Thus, the current data suggest that CD180-mediated intracellular signalling in CD180<sup>+</sup> CLL cells can engage two major



pathways, either via BTK/AKT favouring survival or via p38MAPK favouring apoptosis. Application of specific inhibitors confirmed that, in many of the CLL samples, activation of AKT and p38MAPK pathways is exclusive. This appears to be a feature of CLL cells, and not of normal B cells that responded to CD180 ligation as double AKT/p38MAPK signallers.

## **Chapter 5**

# **Modulation of CD180 expression on CLL cells**

## 5.1 Introduction

CD180, previously called RP105, contains a leucine-rich extracellular domain but also possesses an 11-amino acid cytoplasmic domain with no homology to Toll-like receptor 4 (TLR4) or other known proteins (Miyake *et al.*, 1995). Nagai *et al.*, (2002) demonstrated that CD180 is associated with a small molecule called MD1 which is needed for recognition of lipopolysaccharide (LPS) which are found in outer membrane of gram-negative bacteria, surface expression and intracellular distribution of CD180. MD-1-null mice showed impairment in LPS-induced B cell proliferation, antibody production and B7.2/CD86 up-regulation (Nagai *et al.*, 2002). As previously described, the CD180 mediated signalling mechanism is less studied, however it seems that cross-linking of CD180 by antibody leads to activation of extracellular signal-regulated kinase (ERK), Jun amino(N)-terminal kinase (JNK) and p38-Mitogen activated protein kinase (p38MAPK) (Miyake *et al.*, 1994; Chan *et al.*, 1998; Porakishvili *et al.*, 2011). Current evidence suggests that MyD88 does not participate in CD180 mediated signalling as B cells deficient in MyD88 have shown proliferation following CD180 crosslinking. However, cells deficient in protein kinase C  $\beta$  (PKC $\beta$ ) or from X-linked immunodeficient (*xid*) mice lacking normal Bruton tyrosine kinase (BTK) function, responded poorly to CD180 ligation suggesting the importance of these proteins in CD180 mediated signalling (Chan *et al.*, 1998). It was also reported that optimal proliferative signals after CD180 cross-linking require that the B cell co-receptor CD19 forms a complex with the Lck/Yes novel tyrosine kinase (Lyn) and Vav following CD180 ligation (Yazawa *et al.*, 2003).

Stimulation of cells with LPS could significantly affect expression of CD180 on CD19<sup>+</sup> cells and there was a significant decrease in expression of CD180 after 24hr LPS stimulation in normal and leukaemia CD19<sup>+</sup> cells suggesting that chemical structure of unknown antigen for CD180 resembles the lipopolysaccharide of gram-negative bacteria (Antosz *et al.*, 2009) and it was correlated with reduced expression of CD180 mRNA (Zarembek *et al.*, 2002). This provided confirmation of previous studies which showed 100-fold down-regulation of CD180 expression in the monocytic leukaemia cell line, THP-1 after LPS stimulation (Zarembek and Godowski., 2002). Nagai *et al.* (2005) demonstrated that in CD180 knockout (KO) mice, B cell responses to LPS are impaired, and the level of constitutive serum IgG3 is reduced. This supported a

model of CD180 as a required coreceptor for B cell responses to bacterial cell wall components, to bind with LPS and form heterodimers with TLR4 to enhance signalling. Porakishvili *et al.*, (2005) demonstrated that control B cells express higher levels of CD180 than do CLL cells. Similarly, Antosz *et al.*, (2009) studied the expression patterns of CD180 receptor on the CD19<sup>+</sup> subpopulation of normal and CLL lymphocytes and demonstrated that CD180 expression in unstimulated normal B cells is four times higher than in unstimulated CLL cells suggesting constant stimulation of CD180 receptor in CLL by unknown antigen or auto-antigen. Ogata *et al.*, (2000) demonstrated the functional interaction between CD284 and CD180 in LPS-mediated nuclear factor  $\kappa$ B (NF $\kappa$ B) activation and confirmed that CD180 protein plays a significant role in B cell development acting both as LPS sensor and regulator of B cell proliferation. However, the natural ligand of CD180 is yet unknown. In contrast, Divanovic *et al.*, (2005) reported that the antigen for CD180 is not LPS as CD180 does not bind with this antigen and CD180 complex does not contain the required LPS-binding pocket (Tsuneyoshi *et al.*, 2005). They suggested that although CD180 regulates B cell sensitivity to LPS, the mechanism of CD180 to support TLR4 signalling remains unknown. Two opposing functions of CD180 have been reported; an essential co-receptor for LPS/stimulator of B cells (Nagai *et al.*, 2005) and a specific TLR4 inhibitor in dendritic cells (DCs) with no physiological effect on B cells (Divanovic *et al.*, 2007).

As previously described, the human myeloid differentiation factor 1 (MD-1) molecule has 66% and 38% amino acid sequence similarity to mouse and chicken MD-1 respectively and interacts with CD180 to play a significant role in cell surface expression (Miura *et al.*, 1998). MD-1 and MD-2 are both members of group I of the MD-2- related lipid-recognition (ML) family that is characterized by a lipid binding function (12). MD-1 shares ~20% sequence identity with MD-2 which is an adaptor molecule needed by TLR4 for the recognition of bacterial lipopolysaccharides on the cell surface. MD-1 is associated with and functions with radioprotective 105 (RP105). It was reported that without MD-1, the majority of human CD180 was held in the cell and CD180 was not able to undergo intracellular maturation or traffic to cell surfaces (Miura *et al.*, 1998). Nagai *et al.*, (2005) demonstrated that CD180/MD-1 that may not directly recognise lipoproteins and LPS, is displayed at high levels on marginal zone (MZ) B cells

and is essential for both polyclonal and specific IgG3 Ab responses to T-independent (TI) type 1 vaccines.

Arvaniti *et al.*, (2011) also reported the high level of expression of CD180 in a large cohort of 192 CLL patients by gene expression profiles, suggesting CD180 may promote the activation of both CLL and normal B cells. Good *et al.*, (2009) demonstrated that CD180 is highly expressed on naïve and memory B cells however, memory cells show 2-5-fold higher expression compared to naïve cells. Moreover, signalling through CD180 in combination with CD40L decreased the time taken to enter division for naive and memory B cells compared with CD40L alone, and it had a more potent effect on naive B cells than on memory B cells (Good *et al.*, 2009).

Roshak *et al.*, (1999) reported that anti-human CD180 sera could induce proliferation of lymphocytes in humans in a concentration-dependent manner and this proliferative response was equal to the response given by anti-CD40 induction. Moreover, anti-CD180 induction in murine cells leads to proliferation of murine splenocytes and it was reported that anti-CD180 activates >85% both human and mouse B cells *in vitro* causing them to proliferate (Miyake *et al.*, 1994). Kulikova *et al.*, (2013) studied CD180 expression patterns in the CLL cell line, MEC1 and reported that there was a decrease in CD180<sup>+</sup> cells number during the activation and a negative correlation with the number of cells expressing CD180 and CD86 intensity.

It was reported that the number of CD180-negative B cells is significantly increased in PBMCs from systemic lupus erythematosus (SLE) patients and is positively associated with the disease activity (Koarada *et al.*, 2011). These CD180<sup>neg</sup> B cells from SLE patients could produce IgG and IgM *in vivo* spontaneously, suggesting that only CD180<sup>neg</sup> B cells can produce auto-antibodies. Interestingly in SLE, ligation of CD180 inhibited the tyrosine phosphorylation of signal transducer and activator of transcription 2 (STAT-2) induced by IFN- $\alpha$  via a Lyn-PI3K-BTK-dependent pathway. Moreover, TLR7 and TLR9 signalling could downregulate CD180 expression and attenuate the inhibitory effect of anti-CD180 on the activation of IFN-  $\alpha$  signalling in murine B cells (You *et al.*, 2015). In SLE, the percentages of CD180<sup>+</sup> B cells decreased as the disease turned inactive and disappeared in the peripheral blood from inactive patients treated with corticosteroids. Moreover, CD180<sup>neg</sup> B cells underwent

spontaneous and dexamethasone-induced apoptosis in contrast to CD180<sup>+</sup> B cells. They have also shown that CD180<sup>neg</sup> B cells in SLE can produce IgG and IgM antibodies *in vitro* suggesting at least partially the CD180<sup>neg</sup> B cells may include pathogenic autoreactive B cells subset(s) in SLE (Koarada and Tada, 2011).

As previously described elsewhere, histological studies have shown that CD180 is expressed mainly on mature B cells in mantle zones whereas germinal centre cells are either dull or negative (Miuro *et al.*, 1998). Mestrallet *et al.*, (2016) reported a high expression of CD180 by neoplastic follicular lymphoma (FL) cells when they reside in lymph nodes (LNs). As the expression of the CD180 may support interaction between lymphoma cells and their microenvironment, their findings suggest that CD180 could potentially be playing a role in FL pathogenesis. They also demonstrated that the expression of CD19 is conserved by neoplastic FL B cells, whereas the expression of the negative BCR regulator, CD22 is down-regulated suggesting the importance of BCR signalling in FL pathogenesis and significant overlap between the TLR and BCR pathways. Similar to the role of CD180 in CLL, the TLR/CD180 pathway and its interaction with CD19 signalling were reported to be involved in the enhanced BCR activity via the tyrosine kinase Syk in FL (Leseux *et al.*, 2006).

Chaplin *et al.*, (2011) demonstrated that anti-CD180 mAb *in vivo* induces rapid polyclonal B cell expansion and immunoglobulin production, especially IgG1 and IgG3 subclasses and it was inhibited by co-administration of diverse TLR ligands. However, anti-CD180 synergizes with ligands for all MyD88-dependent TLRs to increase B cell proliferation. Combining activity of anti-CD180 with TLR signals increased cytokine production from purified B cells; however, it does not, by itself, induce cytokine production. Yamashita *et al.*, (1996) demonstrated that exposure to anti-RP105 sensitised murine B cells to anti-IgM-mediated apoptosis and growth arrest.

Previous studies by our group on surface expression of CD180 on CLL cells have shown that CD180 is heterogeneously expressed on CLL cells. However, the majority of control CD19<sup>+</sup> B cells express a high density of CD180 (5548±2271 RBS/cell) compared to CD180<sup>+</sup> CLL cells (1500±932 RBS/cell) (Porakishvili *et al.*, 2005). This could be related to activation, as CD180<sup>neg</sup> B cells increased in patients with SLE and showed higher levels of activation markers than CD180<sup>+</sup> B

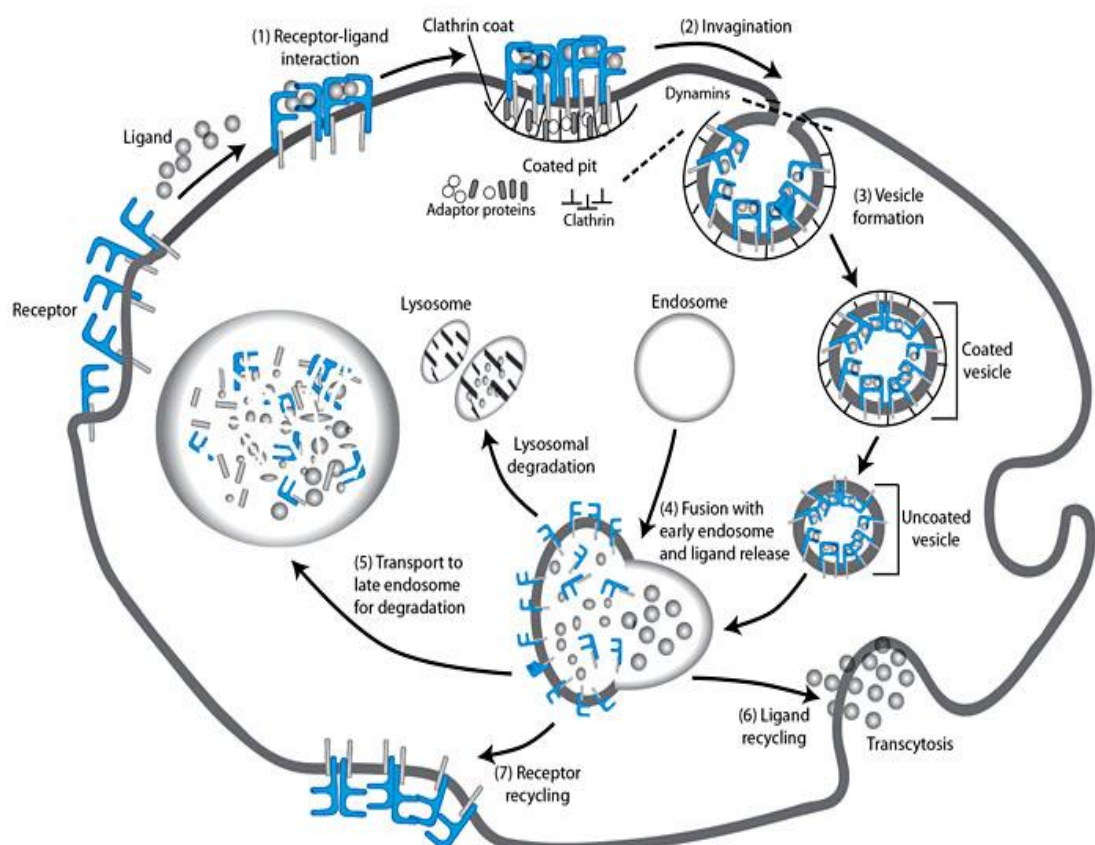
cells (Kadowaki *et al.*, 2001). CLL cells were classified as CD180<sup>neg</sup> based on a small CD180<sup>neg</sup> population seen in the control B cells (316±88 RBS/cell). It was reported that the ratio of the CD180<sup>+</sup> versus CD180<sup>neg</sup> CLL patients was about 2:1 (50 and 28 respectively). However, there was no difference in CD180 expression between the untreated patients, those treated six months prior to the investigation or those who were receiving treatment during the research as well as there was no relationship with disease stage or WBC counts (Porakishvili *et al.*, 2005). Interestingly, the level of CD180 expression in those CLL cells with high expression of CD180 remained high and those with low density stayed low for up to 24 months suggesting that CLL using UM-*IGVH* genes might be continuously maintained in a more heightened state of BCR mediated activation, consistent with lower levels of CD180 (Porakishvili *et al.*, 2005). Our previous and current data have also shown that there is no relationship between CD180 and sIgM, CD79b or CD38 expression in CLL patients. However, the expression of sIgM and the BCR-associated molecule, surface CD79b is variable from patient to patient, but significantly lower on CLL cells compared with control B cells (Zupo *et al.*, 1996; Thompson *et al.*, 1997; Damle *et al.*, 1999). CD180 expression level is higher in M-CLL group than UM-CLL group in contrast to sIgM levels. Although most of the UM-CLL cells were negative for CD180, positive cells have shown an increased expression of CD38 (Porakishvili *et al.*, 2005).

Interactions between CD180- and BCR mediated signalling in B cells have been reported in several studies. Chan *et al.*, (1998) demonstrated that both CD180 and BCR mediated signalling in mice use Lyn protein and BCR can recruit Lyn more efficiently to the BCR complex compared to the putative CD180 signalling complex suggesting the simultaneous ligation of BCR and CD180 will reduce the amount of Lyn that could be used by CD180 signalling complex and block the CD180-induced MAP kinase activation.

Tsertsvadze *et al.*, (2015) studied the correlation of the expression of CD32 and CD180 on CLL cells and the MEC1 cell line, which are derived from a CLL patient with M-CLL, and found that expression of CD32 is significantly increased on CLL cells throughout 0-96 hours compared to control B cells as well as in long-term MEC1 cell culture. In contrast, CD180 expression on MEC1 cells significantly decreased throughout 0-96 hours of MEC1 cell culture. The negative correlation

between CD180 and CD32 expression on cycling MEC1 cells could be limited to M-CLL.

Instability of CD180 expression could be partially explained by its endocytosis. Although dynamics of the ligation-induced CD180 endocytosis in CLL or B cells has not been documented, termination of immune responses by receptor endocytosis has been reported for other types of receptors such as G-protein-coupled receptors, which are important in various biological processes. Binding of a ligand to their receptor complex induces phosphorylation and conformational changes of the different subunits of the receptor activating signals. This is followed by internalisation of ligand-receptor complexes and their direction into endosomes where the ligand is removed and degraded whereas the receptor is either degraded or is recycled back to the cell surface (Kane *et al.*, 2014). Key steps in internalisation of receptors are summarised in figure 5.1.



**Figure 5.1: Termination of signal transduction by receptor endocytosis.**

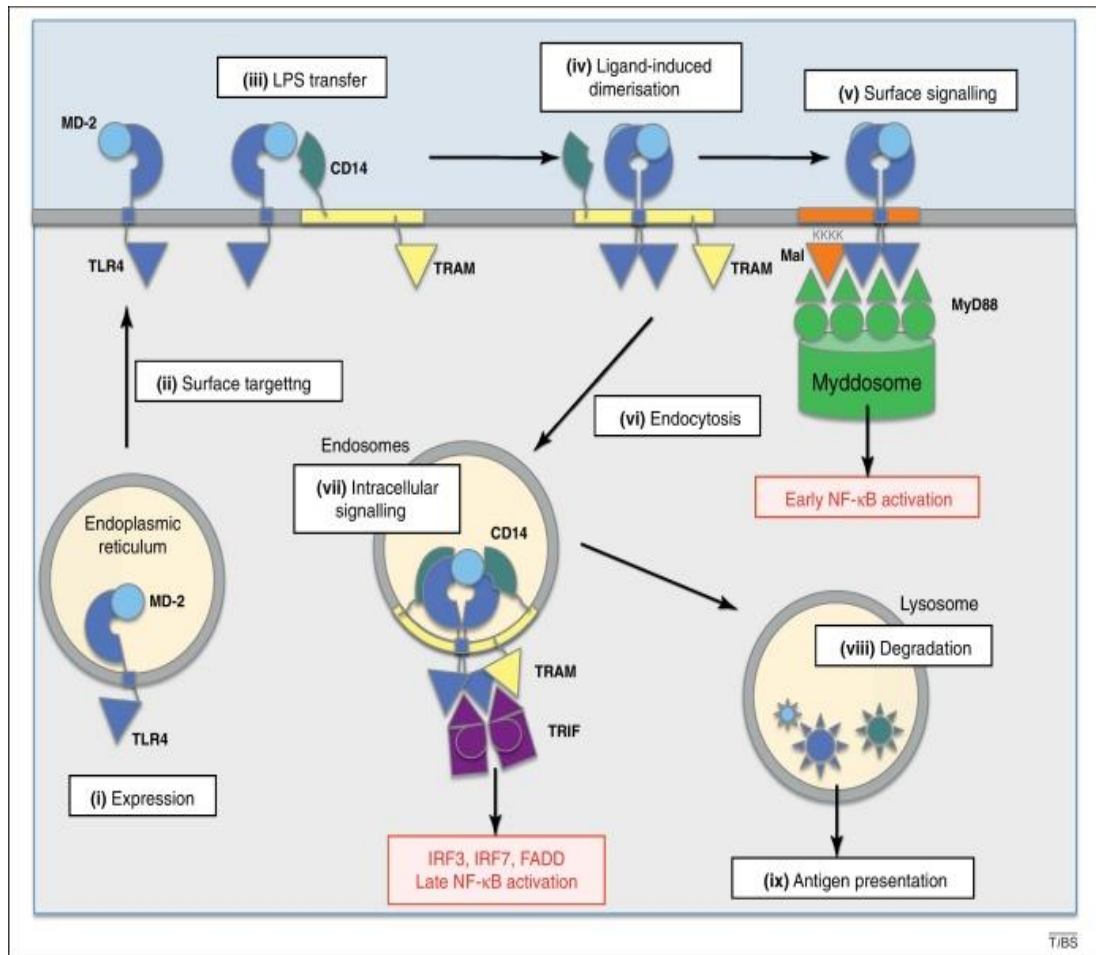
Ligand binding to surface receptors transduces activating signals (1) and simultaneously initiates signals that cause lateral diffusion of receptor-ligand complexes to clathrin-coated pits, membrane invagination (2) and generation of coated vesicles (3). The vesicles then undergo a process of uncoating (a precondition for vesicles to join to other



membranes), fuse with early endosomes and release ligands (4). The ligand undergoes proteosomal and/or lysosomal degradation after being transported either to late endosome and/or lysosome (5) or is recycled and secreted by transcytosis (6). Similarly, receptors may undergo proteosomal degradation or recycle to the cell surface (7) (Kane *et al.*, 2014).

As shown in Figure 5, binding of a ligand to complex receptors induces phosphorylation and conformational changes of the different subunits of the receptor leading to transduction of activation signals. This is followed by internalisation of ligandreceptor complexes and their transport into the endosomes. In the endosomes, the ligand is removed and undergoes rapid degradation, while the receptor is either degraded or is recycled back to the cell surface (Kane *et al.*, 2014).

Gangloff (2012) proposed a mechanism for TLR4 cellular targeting and signalling which involves the formation of ligand-receptor complex leading to endocytosis as shown in figure 5.2. Since CD180 is a TLR4 homologue lacking the intracellular TLR4 signalling domain, it may be plausible to suggest that CD180 mediated signalling recruits a similar mechanism which leads to endocytosis of the receptor-ligand complex.



**Figure 5.2: Proposed mechanism for TLR4 cellular targeting and signalling.**

(i) TLR4 is expressed in the endoplasmic reticulum. (ii) It relies on MD-2 among other protein partners for surface targeting. (iii) LPS is transferred from CD14 to TLR4-MD-2. (iv) Receptor dimerization after ligand binding (v) Mal has a phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) binding motif (depicted as KKKK) that targets the receptor complex to a membrane microdomain. PIP<sub>2</sub> is exclusively located at the plasma membrane. Surface signalling involves the myddosome and leads to early NF-κB activation. (vi) CD14 and TRAM-related adaptor molecule (TRAM) have lipid-raft localization signals and are engulfed along the TLR4-MD-2-LPS complex. Mal is not translocated out of the membrane. (vii) Endosomal signalling results in the recruitment of TRAM and Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β (TRIF) in the case of LPS and monophosphoryl lipid A (MPLA). This leads to the activation of Interferon-response factor3 (IRF3), IRF7, NF-κB and Fas-associated death domain (FADD), respectively. (viii) In the lysosome, all endocytosed complexes are targeted for degradation (ix) antigen presentation (Gangloff, 2012).

Lipid rafts (LRs) are specialised microdomains located in the outer plasma membrane of cells that play important roles in various cellular functions, including cell signalling, secretory and endocytic pathways. Many surface antigens

expressed on CLL cells are localised or translocated into LRs and carry out different functions. LRs act as critical binding sites for therapeutic antibodies such as anti-CD20 rituximab and anti-CD52 alemtuzumab used for CLL patients. It has been reported that rituximab binds to CD20 on CLL cells and translocates it into LRs to increase intracellular  $Ca^{2+}$  level to trigger downstream apoptosis signalling pathways (Janas *et al.*, 2005). Alomari *et al.*, (2014) have identified 643 proteins in LRs of CLL including 30 proteins with no previous known association to the LRs.

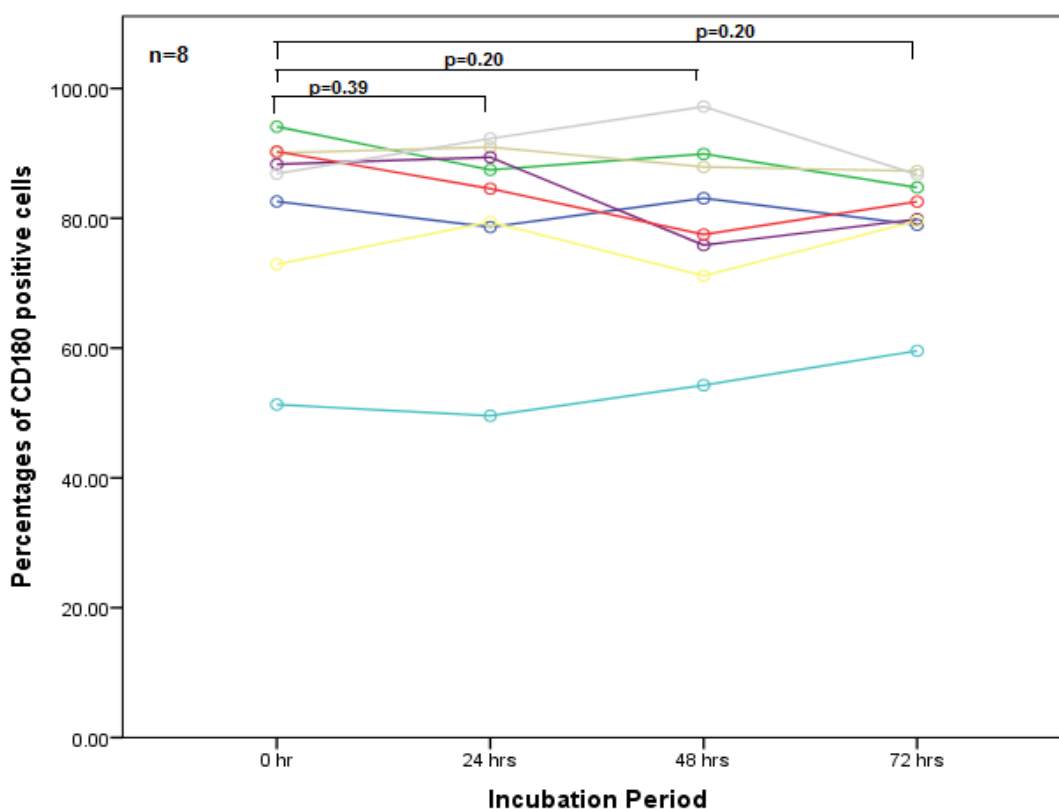
Here, the stability of CD180 expression on CD180<sup>+</sup> CLL cells in cell cultures up to 72 hours was studied. This was important since it would help to understand the dynamics of CD180-mediated intracellular signalling, survival or apoptosis, discussed in the previous chapters better. Both spontaneous change and change of expression of CD180 upon anti-IgM stimulation have been analysed. And also, the CD180 expression in CD180<sup>neg</sup> cells in cell cultures up to 72 hours was studied to assess the stability of CD180 expression profile on CLL cells. This experiment was carried out to evaluate modulation of expression patterns of CD180 on unstimulated CLL cells and when stimulated with anti-IgM which helped to suggest a possible link between dynamic expression of CD180 and CD180 mediated intracellular signalling.

## **5.2 Results**

The surface expression of CD180 receptor on 17 CLL CD180<sup>+</sup> CLL samples was analysed and three patterns in the dynamics of CD180 expression during 0-72h in culture were observed.

### **5.2.1 Stable spontaneous expression of CD180 on a fraction of CLL samples (Pattern I)**

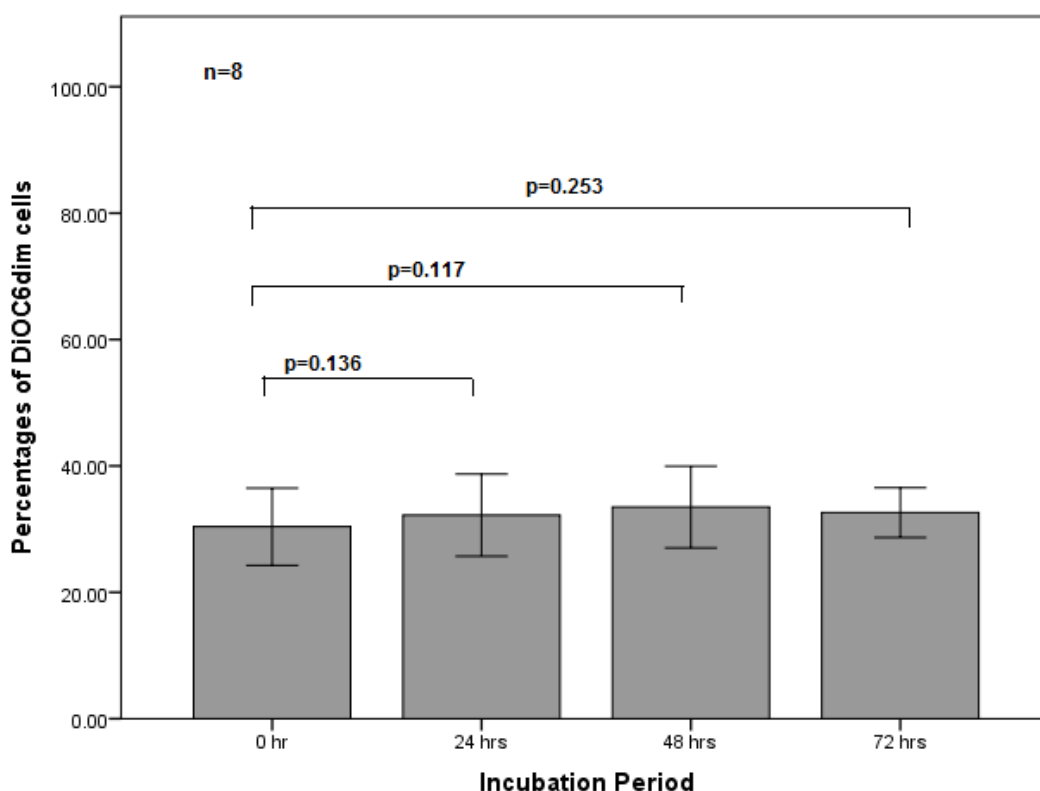
In 8 out of 17 CLL samples, there were no significant differences in the percentages of CD180<sup>+</sup> CLL cells after 24, 48 or 72 hours of incubation ( $p=0.39$ ;  $p=0.20$  and  $p=0.20$  respectively, Figure 5.3). Importantly, all of these 8 CLL samples belonged to the category of non-signallers (NS) as categorised in Chapter 3.



**Figure 5.3: Percentages of CD180<sup>+</sup> cells in a fraction of CLL samples with a stable spontaneous expression of CD180 up to 72 hours (Pattern I).** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 as described in Materials and methods, analysed by CYAN flow cytometer and Summit (4.3) analytical software and expressed as percentages of positive cells. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control was subtracted from the percentages of cells binding to anti-CD180 mAb.

### 5.2.2 Cell viability of a fraction of CLL samples with a stable spontaneous expression of CD180

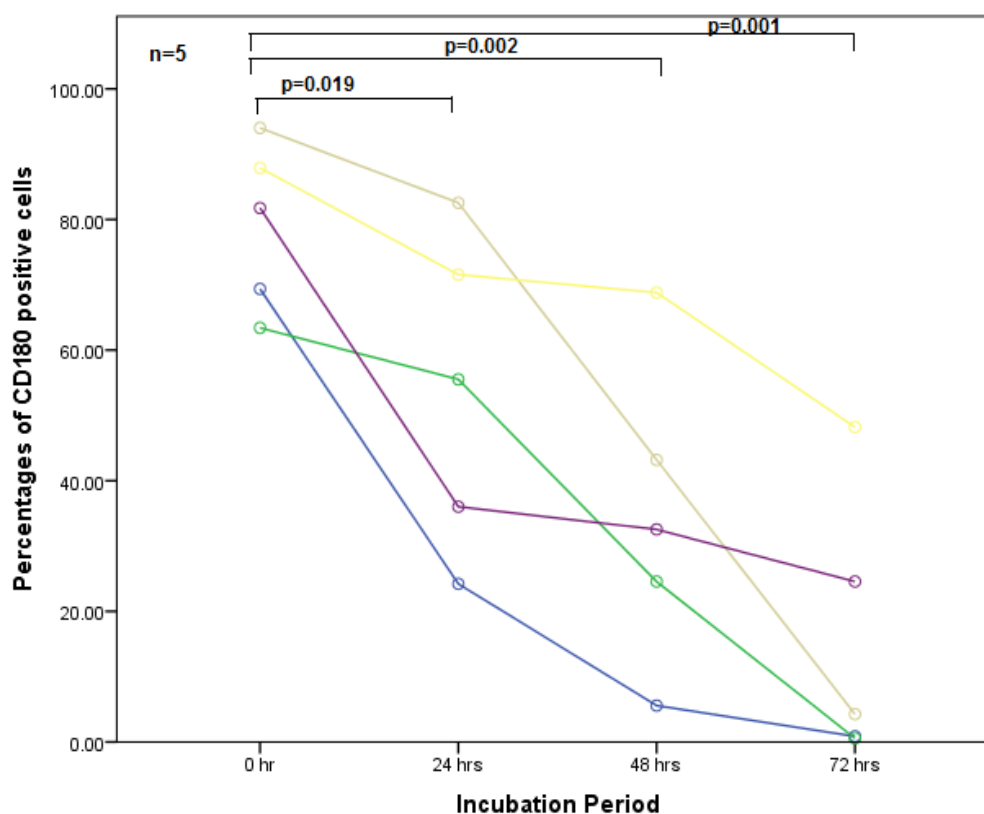
Since the surface expression of CD180 and cell viability may have a functional relationship, it was important to measure the percentages of viable cells by assessing changes in mitochondrial membrane potential as previously described in Materials and methods. There were no significant differences in the percentages of viable cells after 24, 48 or 72 hours of incubation ( $p=0.136$ ;  $p=0.117$  and  $p=0.253$  respectively, Figure 5.4).



**Figure 5.4: Percentages of DiOC6<sup>dim</sup> (apoptotic) cells in a fraction of CLL samples with a stable spontaneous expression of CD180 up to 72 hours (Pattern I).** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means  $\pm$  SD, p values were calculated using the paired *t*-test.

### **5.2.3 Decreased spontaneous expression of CD180 on a fraction of CLL samples (Pattern II)**

Interestingly, in 5 out of 17 CLL samples, the percentages of CD180<sup>+</sup> CLL cells significantly reduced after 24, 48 or 72 hours of incubation ( $p=0.019$ ;  $p=0.002$ , and  $p=0.001$  respectively, Figure 5.5). Moreover, four out of five CD180<sup>+</sup> CLL samples became completely negative for CD180 expression after 72 hours of incubation; one sample remained as CD180<sup>+</sup> but with a reduced expression compared to 0 hour. Moreover, decrease in CD180 expression was significant from 24-48 hours ( $p=0.029$ ) and 48-72 hours ( $p=0.017$ ). It was interesting to identify that all 5 CLL samples belonged to the category of p38MAPK-signallers (p38MAPK-S).



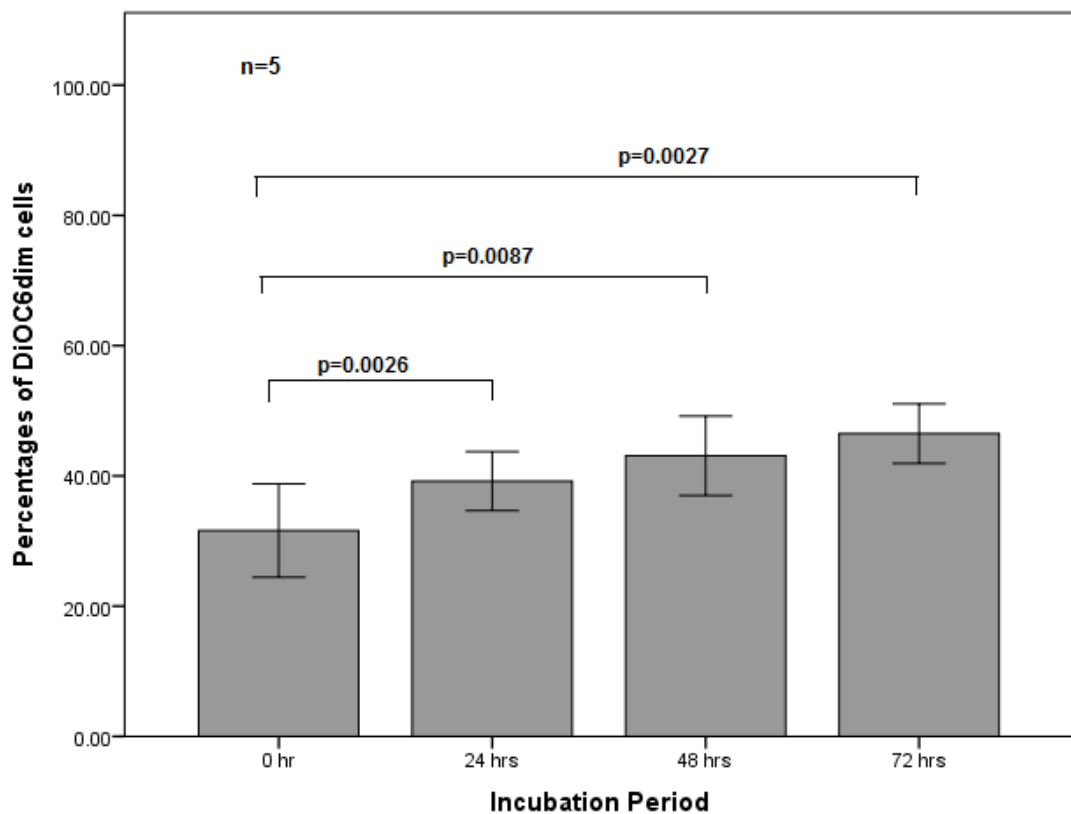
**Figure 5.5: Percentages of CD180<sup>+</sup> cells in a fraction of CLL samples with a decreased spontaneous expression of CD180 up to 72 hours (Pattern II).**

CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 as described in Materials and methods, analysed by CYAN flow cytometer and Summit (4.3) analytical software and expressed as percentages of positive cells. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control was subtracted from the percentages of cells binding to anti-CD180 mAb.

#### **5.2.4 Cell viability of a fraction of CLL samples with a decreased spontaneous expression of CD180**

Since the reduction of expression of CD180 may be associated with survival of cells, it was essential to assess possible changes in the survival of CLL cells in cultures up to 72 hours. The viability of cells was measured by using DiOC<sub>6</sub> dye as previously mentioned. The percentages of DiOC<sub>6</sub><sup>dim</sup> apoptotic cells were significantly increased after 24, 48 or 72 hours of incubation ( $p=0.0026$ ;  $p=0.0087$  and  $p=0.0027$ , Figure 5.6) showing a positive correlation between decrease of CD180 expression and apoptosis of those CD180<sup>+</sup> CLL cells. Increased

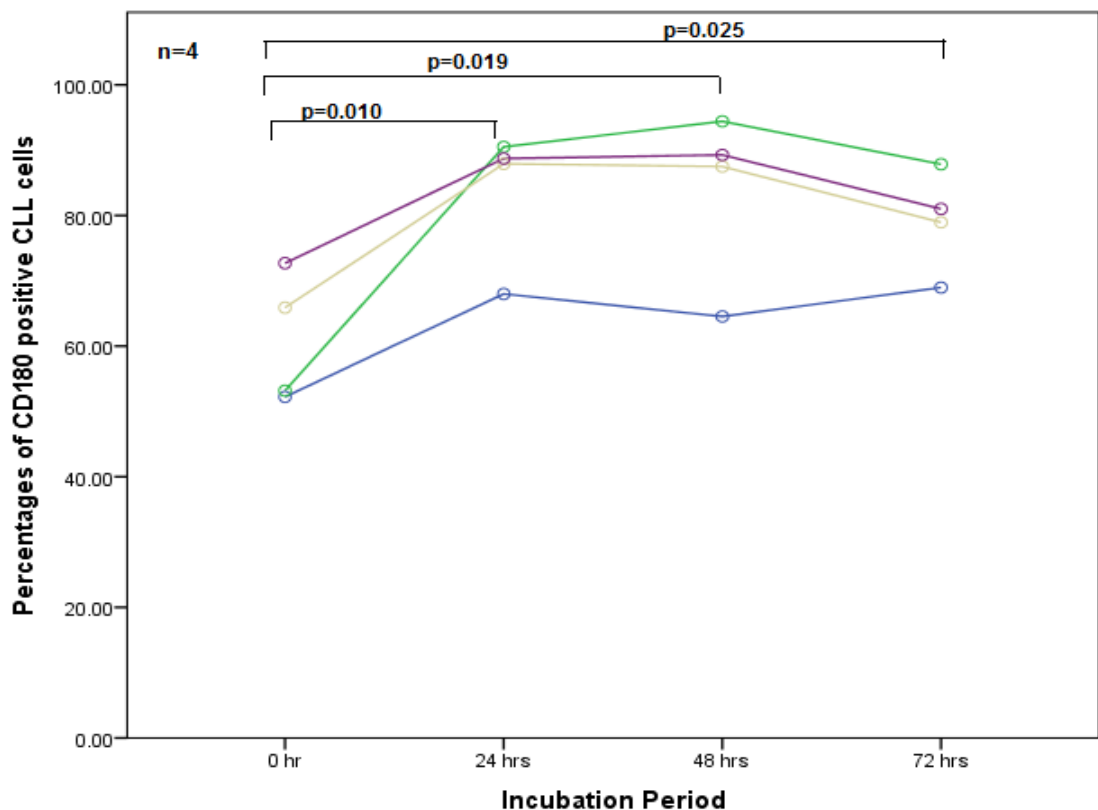
apoptosis was successive from 24-48 hours ( $p=0.049$ ) and 48-72 hours ( $p=0.006$ ).



**Figure 5.6: Percentages of DiOC6<sup>dim</sup> (apoptotic) cells in a fraction of CLL samples with a decreased spontaneous expression of CD180 up to 72 hours (Pattern II).** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means  $\pm$  SD,  $p$  values were calculated using the paired  $t$ -test.

### 5.2.5 Increased spontaneous expression of CD180 on a fraction of CLL samples (Pattern III)

In 4 out of 17 CLL samples, the percentages of CD180<sup>+</sup> CLL cells significantly increased after 24, 48 or 72 hours of incubation ( $p=0.010$ ;  $p=0.019$ , and  $p=0.025$  respectively, Figure 5.7). However, there was no significant difference of percentages of CD180<sup>+</sup> CLL cells between 24 and 48hr cultures ( $p=0.463$ ) or 48 and 72hr cultures ( $p=0.111$ ) confirming although there is a substantial increase of CD180 expression up to 24 hours, after 24 hours the expression remains stable. Importantly, all 5 CLL samples belonged to the category of AKT-signallers (AKT-S).



**Figure 5.7: Percentages of CD180<sup>+</sup> cells in a fraction of CLL samples with an increased spontaneous expression of CD180 up to 72 hours (Pattern III).**

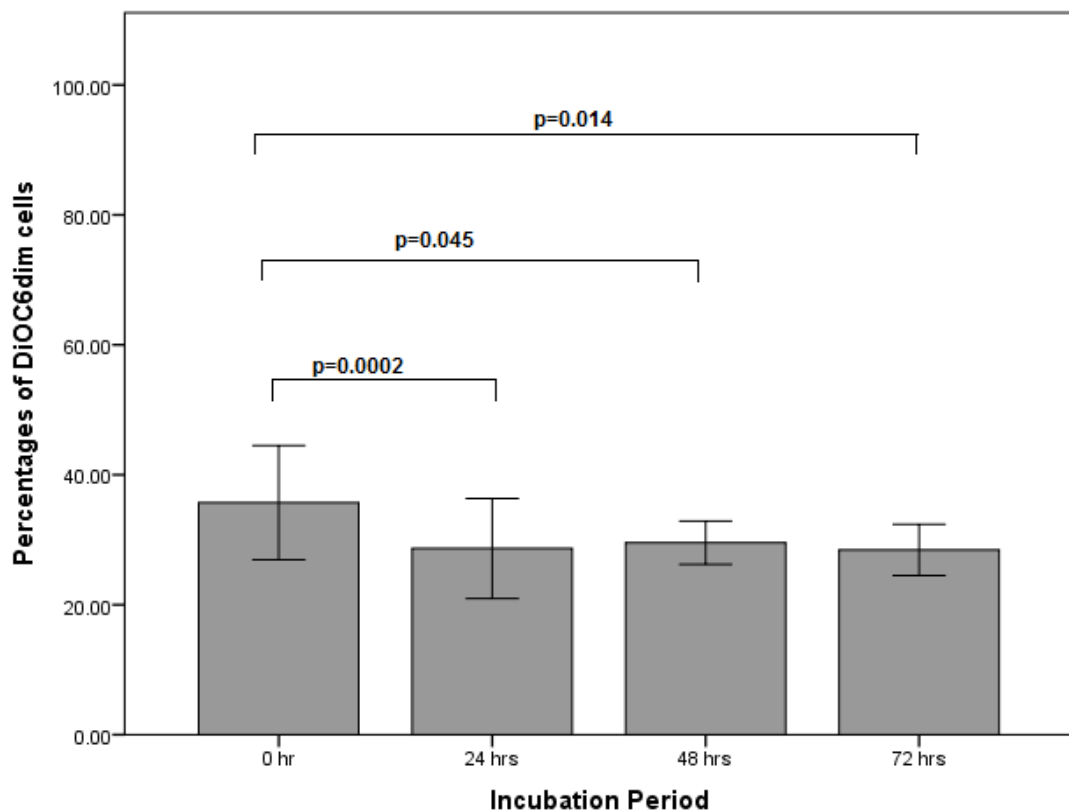
CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 as described in Materials and methods, analysed by CYAN flow cytometer and Summit (4.3) analytical software and expressed as percentages of positive cells. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control was subtracted from the percentages of cells binding to anti-CD180 mAb.

### **5.2.6 Viability of CD180 positive CLL samples which had an increased expression of CD180 up to 72 hours when unstimulated**

It was important to assess whether the increase in CD180 expression up to 24 hours in those CD180<sup>+</sup> CLL cells was associated with cell viability in cultures. Interestingly, the percentages of DiOC6<sup>dim</sup> apoptotic cells were significantly decreased after 24, 48 or 72 hours of incubation ( $p=0.0002$ ;  $p=0.045$  and  $p=0.014$ , Figure 5.8) showing a correlation between increased CD180 expression and survival of cells. However, there was no significant difference of percentages of



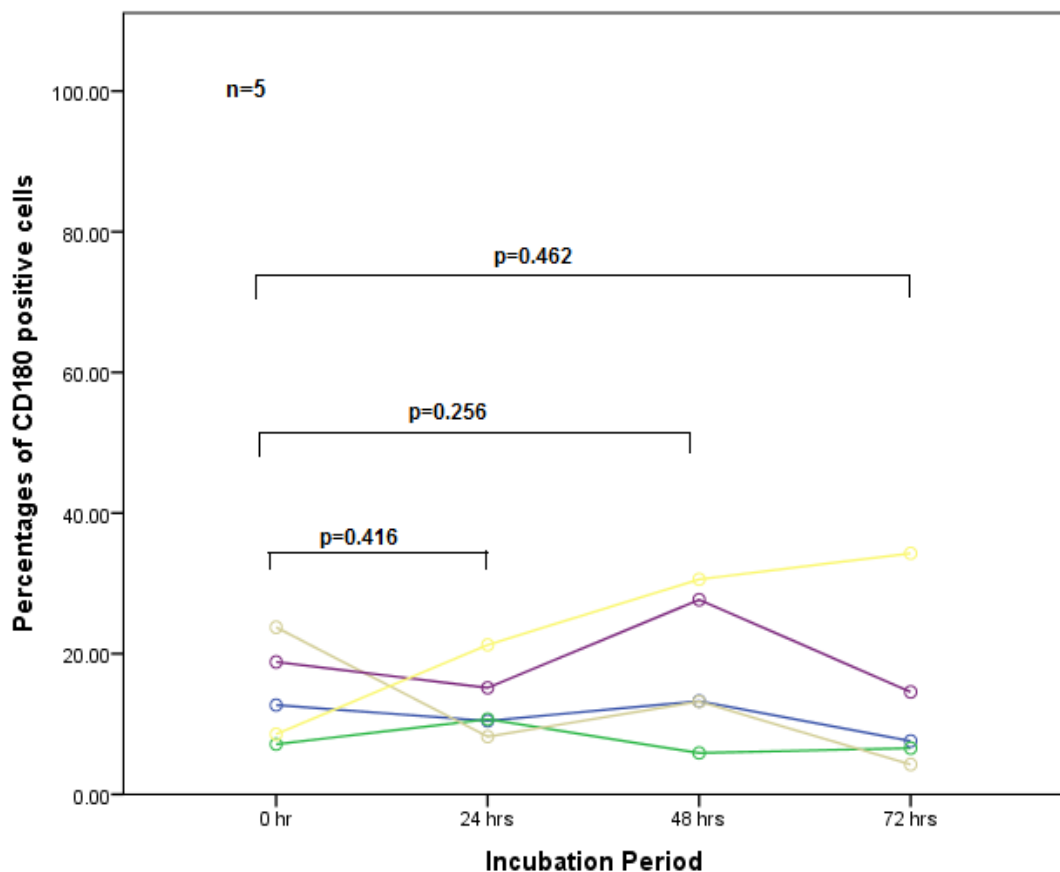
viable cells between 24 and 48hr cell cultures ( $p=0.356$ ) or 48 and 72hr cell cultures ( $p=0.179$ ).



**Figure 5.8: Percentages of DiOC6<sup>dim</sup> (apoptotic) cells in a fraction of CLL samples with an increased spontaneous expression of CD180 up to 72 hours (Pattern III).** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means  $\pm$  SD, p values were calculated using the paired *t*-test.

### 5.2.7 Stable spontaneous expression of CD180 on CD180<sup>neg</sup> CLL samples

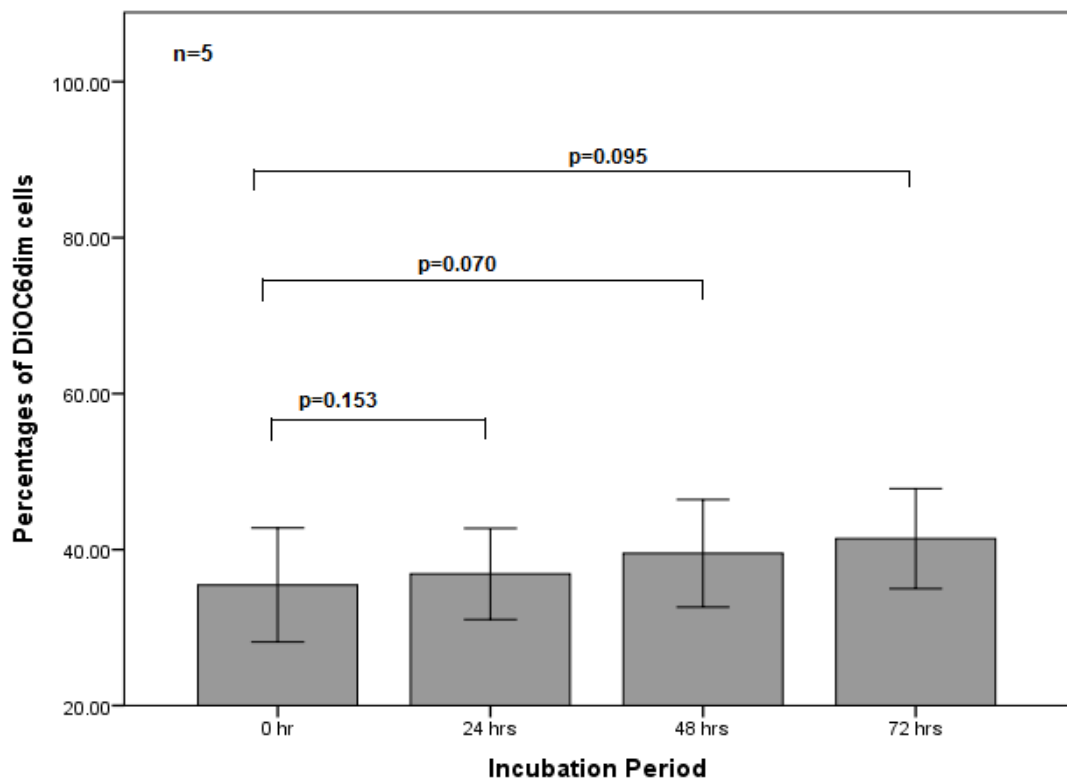
CD180 expression on 5 CD180<sup>neg</sup> CLL samples was studied to assess the stability of CD180 expression profile in CD180<sup>neg</sup> CLL cells. As expected there were no significant differences in the percentages of CD180<sup>+</sup> CLL cells after 24, 48 or 72 hours of incubation ( $p=0.416$ ;  $p=0.256$  and  $p=0.462$  respectively, Figure 5.9).



**Figure 5.9: Percentages of CD180<sup>+</sup> cells in CD180<sup>neg</sup> CLL samples up to 72 hours.** CD180<sup>neg</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 as described in Materials and methods, analysed by CYAN flow cytometer and Summit (4.3) analytical software and expressed as percentages of positive cells. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control was subtracted from the percentages of cells binding to anti-CD180 mAb.

### 5.2.8 Viability of CD180 negative cells up to 72 hours of incubation

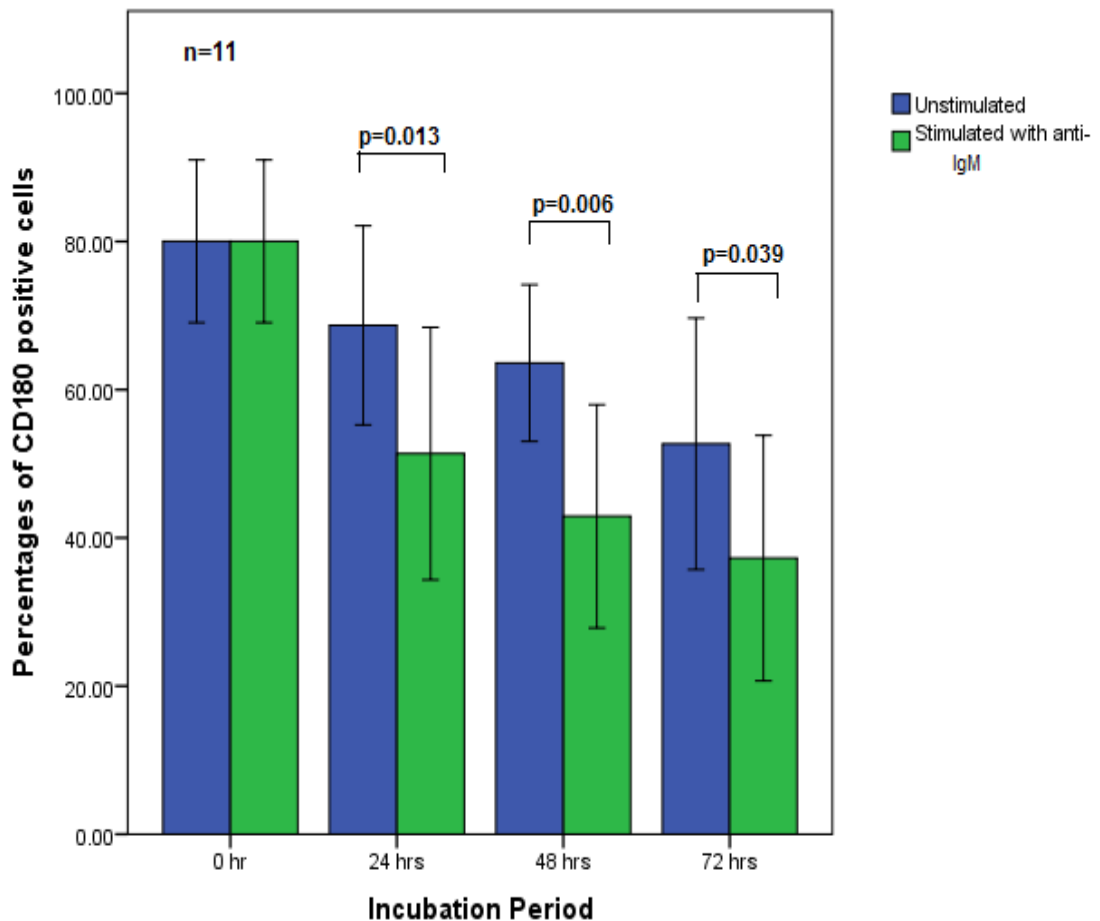
Analysis of viability of those CD180<sup>neg</sup> CLL samples showed strikingly similar results shown to those CD180<sup>+</sup> samples with a stable spontaneous expression of CD180. Indeed, there were no significant differences in the percentages of viable cells after 24, 48 or 72 hours of incubation ( $p=0.153$ ;  $p=0.070$  and  $p=0.095$  respectively, Figure 5.10).



**Figure 5.10: Percentages of DiOC6<sup>dim</sup> (apoptotic) cells CD180<sup>neg</sup> CLL samples up to 72 hours.** CD180<sup>neg</sup> CLL cells were taken at 0 hour or incubated for 24, 48 and 72 hours, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means ± SD, p values were calculated using the paired *t*-test.

### 5.2.9 Modulation of CD180 expression on CD180<sup>+</sup> CLL samples upon anti-IgM stimulation

Since several studies have demonstrated that activation could significantly affect surface expression of CD180 in different types of cells (Antosz *et al.*, 2009; Chankotadze, 2013; You *et al.*, 2015), it was necessary to assess the effect of IgM ligation on surface expression of CD180 in CLL cells. CLL samples with CD180<sup>+</sup>IgM<sup>+</sup> CLL cells were used for this study and interestingly, in all 11 samples, the percentages of CD180<sup>+</sup> cells significantly decreased following anti-IgM ligation compared with basal levels after 24, 48 and 72 hours of incubation ( $p=0.013$ ;  $p=0.006$  and  $p=0.039$ , Figure 5.11). Moreover, 7 out of 11 CLL samples belonged to the category of p38MAPK-signallers and the rest was AKT-signallers. Furthermore, the percentages of CD180<sup>+</sup> cells significantly decreased after 24, 48 or 72 hours of incubation in unstimulated cultures ( $p=0.021$ ;  $p=0.014$  and  $p=0.002$ ).

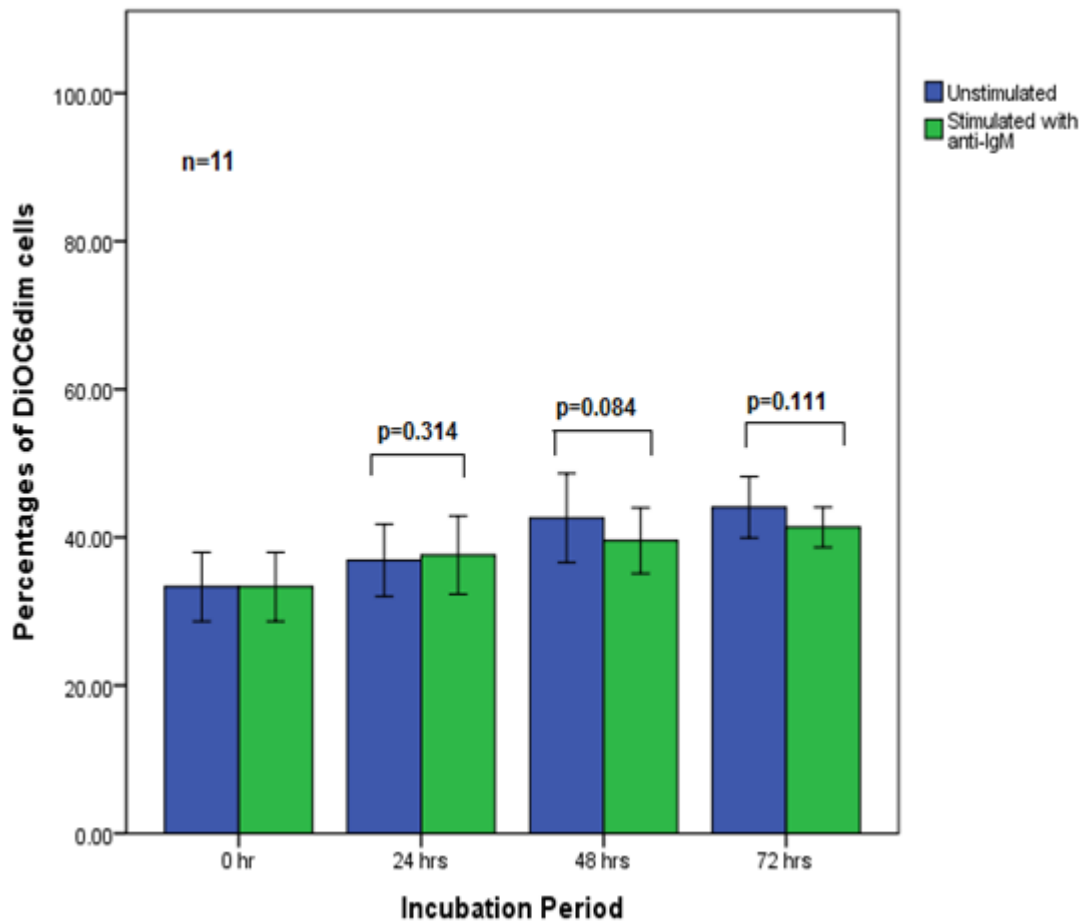


**Figure 5.11: Modulation of CD180 expression in CD180<sup>+</sup> CLL samples upon anti-IgM F(ab)<sub>2</sub> stimulation.** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated with anti-IgM mAb for 24, 48 and 72 hours, washed, incubated with human immunoglobulin (Ig), stained with PE-conjugated mouse IgG1 isotype control or PE-conjugated mouse anti-human CD180 mAb and PE-Cy5-conjugated mouse anti-human CD19 as described in Materials and methods, analysed by CYAN flow cytometer and Summit (4.3) analytical software and expressed as percentages of positive cells. p values were calculated using the paired *t*-test. Percentages of cells binding to the isotype control was subtracted from the percentages of cells binding to anti-CD180 mAb.

#### 5.2.10 Effect of anti-IgM stimulation on viability of CD180 positive CLL cells

Since the decrease of expression of CD180 upon anti-IgM stimulation may be associated with survival of cells, it was essential to assess possible changes in the survival of CLL cells in anti-IgM stimulated cultures up to 72 hours. The viability of cells was measured by determining changes in mitochondrial membrane potential as previously mentioned. There were no significant differences in the percentages of viable cells following anti-IgM ligation compared with basal levels after 24, 48 and 72 hours of incubation ( $p=0.314$ ;  $p=0.084$  and

$p=0.111$  respectively, Figure 5.12) in all 11 CLL samples. However, the percentages of DiOC<sub>6</sub><sup>dim</sup> apoptotic cells were significantly increased after 24, 48 or 72 hours of incubation in unstimulated cultures ( $p=0.047$ ;  $p=0.00016$  and  $p=0.00007$ ).



**Figure 5.12: The percentages of DiOC<sub>6</sub><sup>dim</sup> (apoptotic) cells in anti-IgM F(ab)<sub>2</sub> stimulated CD180<sup>+</sup> CLL cells compared to unstimulated cultures.** CD180<sup>+</sup> CLL cells were taken at 0 hour or incubated with anti-IgM for 24, 48 and 72 hours, washed, stained with anti-CD19 mAb, loaded with DiOC<sub>6</sub> for 20 min and analysed by flow cytometry as described in the Materials and methods. The values are means  $\pm$  SD, p values were calculated using the paired *t*-test.

## **5.3 Discussion**

### **5.3.1 Stable spontaneous expression of CD180 on a fraction of CLL samples with no change in cell survival**

Current data has shown that on a fraction of CD180<sup>+</sup> CLL samples (8 out of 17), the surface expression of CD180 did not significantly change from 0 to 72 hours (Figure 5.3). Analysis of a possible correlation between modulation of CD180 expression and survival of cells has confirmed that CLL cells with a stable spontaneous CD180 expression had a stable cell viability throughout 0- 72 hours in cultures (Figure 5.4). As previously described in Chapter 3 and the previous publication Porakishvili *et al.*, (2015), there are four patterns of CD180-mediated signalling in CLL cells and here, all 8 CLL samples belonged to the non signalers (NS) category. It has been previously demonstrated that NS CLL samples are unresponsive to CD180 ligation suggesting that NS may use an alternative pathway or they are totally refractive to ligation of CD180. Moreover, NS samples were unresponsive to the ligation of sIgM suggesting their anergic state (Packhan *et al.*, 2014). Two explanations for instability of CD180 expression in cultures might be either internalisation of ligand-receptor complex (endocytosis) as shown by TLR4 and other biologically important receptors or change of the percentages of CD180<sup>+</sup> cells due to activation of either pro-apoptotic or anti-apoptotic pathways upon stimulation of cells by a specific ligand. Interestingly, all 8 samples which showed a stable expression of CD180 were NS and this suggests that either a natural ligand was not present in the body or those NS CLL cells did not respond to the intrinsic ligand which was present in the body at the time of the samples taken due to their anergic state. Therefore no triggering of endocytosis or activation of either pro-survival or pro-apoptotic signalling pathway has been taken place.

### **5.3.2 The level of CD180 surface expression decreased on a fraction of CLL samples with a significant increase in cell viability**

A fraction of CD180<sup>+</sup> CLL samples showed a significant decrease of CD180 surface expression from 0 to 72 hours (Figure 5.5) while the percentages of apoptotic cells in cultures significantly increased (Figure 5.6). Similar results were reported by Tsertsvadze *et al.*, (2015) showing a decrease in CD180 expression in MEC1 cells throughout 0-96 hours. Current data on downmodulation of CD180 expression suggest that CD180 was already stimulated by an intrinsic ligand

causing further endocytosis of the receptors in cultures. Although dynamics of the ligation-induced CD180 endocytosis in CLL or B cells has not been reported yet, lipid raft formation of many of the surface antigens aberrantly expressed on CLL (Janas *et al.*, 2005; Alomari *et al.*, 2014) and ligation-induced endocytosis of other receptors have been reported by others (Ferguson, 2001; Spat *et al.*, 2004; Takayama *et al.*, 2005; Nakayama *et al.*, 2013 and Kane *et al.*, 2014) including endocytosis of TLR4 (Gangloff, 2012). Interestingly, all five CLL samples with decreased CD180 expression were p38MAPK-signallers. Although the role of p38MAPK-mediated signalling in CLL is unclear, previous data in Chapter 3 confirm that CD180-mediated activation of p38MAPK is associated with apoptosis in CLL cells. In line with previous findings, there was an increase in the percentages of apoptosis cells throughout 0-72 hours further supporting the hypothesis that those cells were already stimulated by an intrinsic ligand, and therefore, p38MAPK-mediated pro-apoptotic pathway is activated. This may have led to increasing the percentages of CD180<sup>neg</sup> cells in cultures. As previously described in Chapter 3 and the previous publication Porakishvili *et al.*, (2015), CD180 ligation can redirect sIgM-mediated signalling from the pro-survival to the pro-apoptotic pathway. Hence the reduction in the expression of CD180 on cycling CLL cells suggests that this may lead to a weakening of redirection effect and enhance further survival and expansion of CLL cells in proliferative centres of lymphoid tissues.

### **5.3.3 The level of CD180 surface expression increased on a fraction of CLL samples with a significant increase in cell viability**

Moreover, a fraction of CD180<sup>+</sup> CLL samples showed a significant increase of CD180 surface expression from 0 to 24 hours spontaneously followed by a stable expression from 24 to 72 hours in cultures (Figure 5.7). Interestingly, all 4 CLL samples were AKT-signallers. Previous signalling data have demonstrated that CD180 ligation on AKT-S CLL cells activates the pro-survival pathway. Hence it is credible to suggest that these AKT-S CLL cells were already stimulated by a putative CD180 antigen which was present in its microenvironment and activated pro-survival pathway thereby increasing the number of CD180<sup>+</sup> CLL cells in cultures. Confirming this, the viability of cells of all four samples significantly decreased from 0 to 24 hours. However, the signal generated by receptor ligation might not be robust enough to continue its pro-survival effect in long-term cultures

as the viability did not change significantly after 48 or 72 hours compared with 24 hours culture (Figure 5.8).

#### **5.3.4 Stable spontaneous expression of CD180 on CD180<sup>neg</sup> CLL samples while no change in cell survival**

There was no significant change in CD180 surface expression and apoptosis of CD180<sup>neg</sup> CLL cells from 0 to 72 hours of incubation as expected (Figure 5.9). Since CD180<sup>neg</sup> CLL cells possess relatively less CD180 RBS/cell ( $\leq 316 \pm 88$  RBS/cell) to ligate with antigen and initiate a response, either the level of CD180 expression or the percentages of apoptotic cells were not affected by any ligand-receptor interaction (Figure 5.10). Consequently, these CD180<sup>neg</sup> CLL cells remained as a negative population of cells for CD180 expression up to 72 hours.

#### **5.3.5 The anti-IgM treatment significantly reduced the expression of CD180 while not affecting survival of cells**

There was a statistically significant decrease in the percentages of CD180<sup>+</sup> CLL cells when stimulated with anti-IgM for 24, 48 and 72 hours compared to basal levels (Figure 5.11) suggesting a functional relationship between CD180- and BCR mediated signalling. Preliminary data of our research group indicated that CD180 is endocytosed upon ligation with anti-CD180 mAb (data not shown). Hence it is possible to suggest that in these samples, CD180 was already stimulated by an unknown antigen present *in vivo* initiating the endocytosis process and anti-IgM treatment triggered further endocytosis in cultures. In that case, the putative ligand of CD180 and anti-IgM should recognise different epitopes on CD180. Hypothesised co-localization of IgM and CD180 receptors in the lipid rafts will be assessed in our future studies to understand the interaction between the two receptors better. Chappell *et al.*, (2014) suggested that the combination of signals via CD180 and antigen-engaged BCRs appears to be responsible for effective immunisation in mice. Significantly higher expression of CD180 in CD19<sup>+</sup> subpopulation of unstimulated normal B cells than in unstimulated CLL cells has been previously reported (Porakishvili *et al.*, 2005; Antosz *et al.*, 2009) suggesting constant stimulation of CD180 receptor in CLL by an unknown antigen or auto-antigen. Furthermore, 7 out of 11 CLL samples were p38MAPK-S and 4 out of 11 CLL samples were AKT-S. However, there was a significant decrease of CD180 expression and percentages of viable cells in unstimulated cultures throughout 0-72 hours (Figure 5.12). This data further



confirmed that at least for p38MAPK-S, the intrinsic ligand has already stimulated pro-apoptotic pathway.

In conclusion, current data suggest that at least for p38MAPK-S and AKT-S CLL cells, the surface expression of CD180 is dynamic in cultures and this dynamic expression might be related to the interaction of CD180 with an unknown ligand present in the microenvironment. In my opinion, either the intrinsic ligand of CD180 was present in the body at the time of the samples taken, or it could be a generated soluble molecule such as a heat shock protein (HSP). Although the dynamics of ligation-induced CD180 endocytosis in CLL cells has not been documented so far, it is plausible to suggest that upon receiving signals via binding of ligands to surface receptors, activating signals are transduced, and endocytosis of receptor-ligand complex is initiated. This provides a probable elucidation for dynamics of CD180 expression observed in p38MAPK-S and AKT-S CLL cells. Furthermore, current data suggest that the internalisation is further triggered by the activation signals received via BCR ligation. Moreover, a stable spontaneous expression of CD180 shown by nonsignallers confirms that the dynamics of CD180 expression in cultures is mediated by the signals received from their microenvironment. Hence, the anergic state of NS CLL cells for CD180- and BCR mediated signalling prevented modulation of CD180 expression in this category of cells. In conclusion, current data show that modulation of CD180 expression in cultures is a characteristic of some categories of CLL cells suggesting a possible link between dynamics of the receptor expression and CD180-mediated intracellular signalling, survival or apoptosis.

## **Chapter 6**

### **Summary and conclusion**

The role of microenvironment in the development and progression of chronic lymphocytic leukaemia (CLL) is currently of major interest. Pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) represent exogenous and endogenous microenvironmental factors. These factors act via a range of receptors, including Toll-like receptors (TLR). CD180/RP105 is a membrane-associated orphan receptor that belongs to the TLR family, is expressed by professional antigen-presenting cells, and drives normal B-cell activation and proliferation. It is expressed heterogeneously on CLL cells, and predominantly on CLL with mutated *IGVH* genes (M-CLL). Our group has previously shown that approximately 60% of CLL samples expressed surface CD180 (Porakishvili *et al.*, 2005), but only half responded to ligation with anti-CD180 monoclonal antibody (mAb) resulting in activation, cycling, and reduced basal apoptosis. This was comparable or superior to that induced by anti-CD40 mAb or IL-4 (Porakishvili *et al.*, 2011). These CLL samples which upregulated CD86 and Ki-67 upon stimulation with anti-CD180 mAb were termed responders (R). In contrast, CD180<sup>+</sup> CLL samples that failed to respond to anti-CD180 mAb, despite expressing a high density of CD180 receptors, were termed non-responders (NR). Porakishvili *et al.*, (2011) further demonstrated that in R-CLL cells and normal B cells, CD180 ligation led to a strong upregulation in phosphorylation of ZAP70/Syk, Erk, p38MAPK, and AKT protein kinase (PK) in a Ca<sup>2+</sup> independent manner, compared to untreated cells. Thus, the interaction of R-CLL cells with the microenvironment through CD180 may contribute to the expansion of leukaemia cell clones, *in vivo*, in lymph nodes and bone marrow within proliferation centres (PCs). In contrast, CD180-mediated signalling in NR CLL cells did not progress downstream from ZAP70/Syk phosphorylation indicating a block in activation of downstream protein kinases, and possible energy.

However, our group has previously noted that although the levels of phosphorylated AKT, ERK, and p38MAPK were significantly increased in R-CLL compared to NR-CLL samples), there was substantial heterogeneity within both anti-CD180 R and NR subsets of CLL. Therefore, to clarify the CD180-mediated signalling pathways in CLL, downstream signal transduction was studied and samples were recategorized into R and NR through their proximal ability to activate AKT. This was more appropriate compared to distal event of CD86

upregulation, which may depend on other factors such as T cell interactions and/or cytokines. Although the responses were quite heterogeneous, using a larger cohort of patients (n=60), R-CLL cells could be divided into two categories; AKT signallers (AKT-S) and AKT non-signallers (AKT-NS) based on the ability to increase the level of phosphorylation of AKT (Ser 473) compared to basal levels. It was important to determine the characteristics of the two categories of cells in terms of other signalling molecules which was one of the major aims of my project. Hence the major signalling protein kinases; BTK, AKT and p38MAPK which were associated with the BCR signalling pathway were studied during this project.

## **Summary**

The results of this study helped to establish the following:

- Activation of alternative pathways appears to be a feature of CLL cells and there is a dichotomy in the CD180 mediated signalling pathways in CLL cells as BTK/AKT or p38MAPK.
- This dichotomy in CD180-mediated signalling has opposing effects – BTK/AKT signalling pathway in AKT-S cells leads to survival whereas CD180-mediated activation of p38MAPK is associated with apoptosis.
- Based on the differential pathways activated following CD180 ligation, four patterns of CD180-mediated signalling in CLL cells were identified: AKT-signallers (AKT-S); p38MAPK signallers (p38MAPK-S); nonsignallers (NS); and a minor subset of double AKT/p38MAPK signallers (DS).
- Pretreatment of CLL cells with anti-CD180 antibodies rewires the sIgM signalling pathway from pro-survival to pro-apoptotic in AKT-S CLL cells whereas pretreatment of p38MAPK-S cells with either anti-CD180 or anti-IgM often leads to additive phosphorylation of AKT.
- Application of specific inhibitors of AKT and p38MAPK signalling pathways confirmed that, in many of the CLL samples, activation of AKT and p38MAPK pathways is exclusive.
- Modulation of CD180 expression studies showed that spontaneous expression of CD180 on p38MAPK-S and AKT-S CLL cells is dynamic in cell cultures which may be associated with dynamics of CD180-mediated

intracellular signalling, whereas spontaneous CD180 expression is stable on NS and CD180<sup>neg</sup> categories of CLL cells.

- Activation of BCR mediated signalling by anti-IgM ligation, significantly reduce the expression of CD180 in CD180<sup>+</sup>IgM<sup>+</sup> CLL cells.

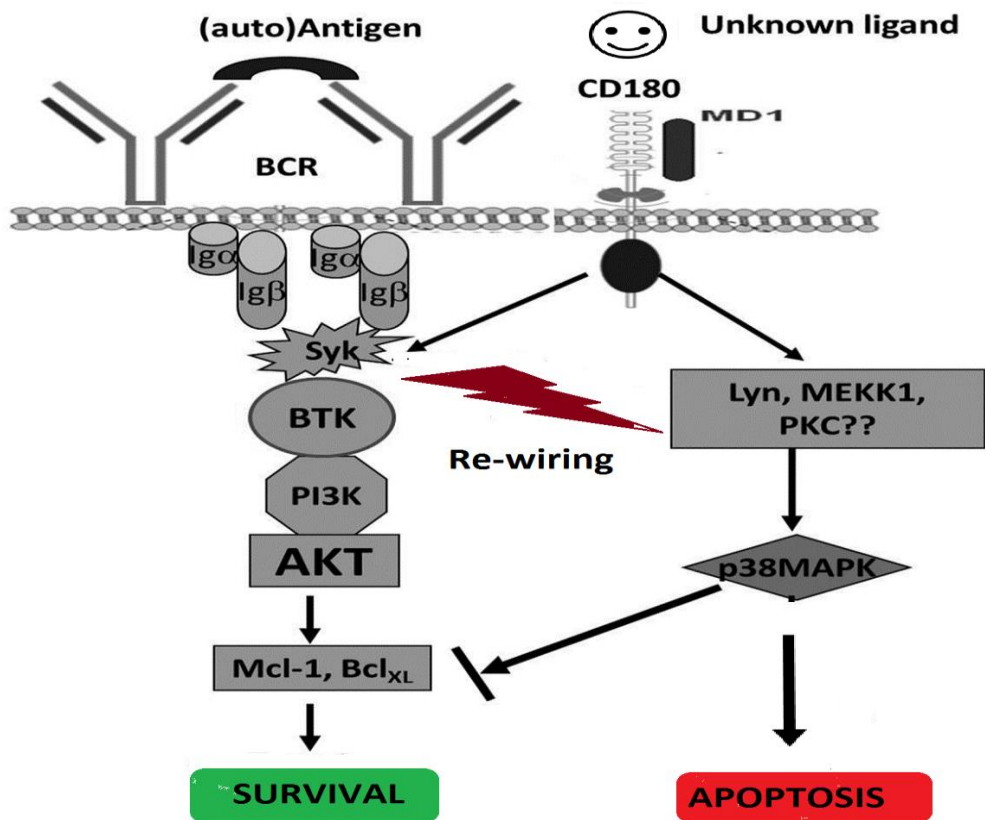
Signalling experiments included 60 CLL patients and 10 control samples. CD180<sup>+</sup> CLL samples which responded to CD180 ligation by a significant upregulation of AKT-P were termed AKT-signallers (AKT-S, ~40%) and the samples which upregulated p38MAPK-P were termed p38MAPK-signallers (p38MAPK-S, ~27%). Activation of both AKT and p38MAPK was seen in a small cohort of CLL samples and they were termed double signallers (DS, ~10%) whereas the remaining CD180<sup>+</sup> CLL samples which did not respond to CD180 ligation by activating either of the two pathways were defined as non-signallers (NS, ~23%). This suggests that NS may use an alternative pathway or they are totally refractive to ligation of CD180. Therefore, as described elsewhere, four patterns of CD180-mediated signalling in CLL cells were identified: AKT-S, p38MAPK-S, NS and a minor subset of double AKT/p38MAPK signallers.

It was important to compare CD180-mediated changes in survival of AKT-S and p38MAPK-S categories of CLL cells. CD180 ligation in AKT-S cells, activated protein kinase, BTK leading to a significant increase in pro-survival while suppressing p38MAPK pathway. A similar result was observed with control B cells. However, CD180 ligation in p38MAPK-S did not recruit BTK and importantly, CD180 ligation stimulated pro-apoptotic pathway in this category of cells. In conclusion, ligation of CD180 with anti-CD180 mAb on CLL cells can activate two alternative signalling pathways; pro-survival: operate through activation of protein kinases BTK/AKT (AKT-S) predominantly and pro-apoptotic: operate through activation of p38MAPK (p38MAPK-S).

sIgM induced signalling pathways were studied and compared with CD180 mediated signalling patterns which were identified in the major categories of CLL cells. Striking similarities were observed between two signalling pathways in AKT-S CLL cells. Anti-IgM treatment increased the phosphorylation of AKT whereas downregulated the phosphorylation of p38MAPK in AKT-S category of cells. However, CD180 ligation induced activation of BTK/AKT in all AKT-S CLL samples, anti-IgM effect on this BTK/AKT circuit was more heterogeneous. Similar to anti-CD180 ligation, anti-IgM ligation also protected AKT-S CLL cells

from apoptosis. Phosphorylation of p38MAPK was a less frequent event with anti-IgM stimulation compared to CD180 ligation as only 23% of samples showed activation of p38MAPK. There was no significant difference in apoptosis in this category of cells suggesting that phosphorylation of p38MAPK is vital for the induction of apoptosis in p38MAPK-S cells. There was a significant decrease in expression of BTK-P in all p38MAPK-S CLL samples upon stimulation with anti-IgM. Similarly, BCR engagement did not show an activation of p38MAPK in DS CLL samples. This could be due the lack of BTK phosphorylation abrogates both “arms” of BCR signalling (via AKT and p38MAPK), whilst leaving CD180-mediated p38MAPK activation intact. Interestingly, the ability of CD180 to bypass BTK and carry signals downstream to p38MAPK, favours apoptosis over survival. This suggests, although there is a substantial overlap of CD180 and sIgM mediated activation of BTK/AKT pro-survival signalling circuit, only ligation of CD180 can lead to activation of an alternative proapoptotic pathway mediated via p38MAPK.

Since the previous data indicate that CD180 and IgM operate through similar signalling pathways, particularly via BTK/AKT, leading to increased survival of CLL cells, it was interesting to investigate the effect of pre-engagement of CD180 on signalling through sIgM (the BCR) investigated. Whilst sIgM ligation with goat anti-human IgM F(ab)<sub>2</sub> alone led to a significant activation of pro-survival BTK-AKT pathway in AKT-S cells, pre-treatment with anti-CD180 mAb redirected pro-survival signalling mediated through BCR towards pro-apoptotic p38MAPK pathway. Rewiring of signalling from pro-survival to pro-apoptotic pathway was not observed in control B cells suggesting that modulation is unique for CLL cells. Hence it is possible to hypothesise that CD180-mediated activation of CLL cells by an unknown microenvironmental ligand may mimic this continuous BCR-mediated signalling. Therefore, rewiring of intracellular signals from BTK/AKT pro-survival circuit to p38MAPK pro-apoptotic pathway in AKT-S CLL cells may suggest a possible cross-talk between CD180 and BCR. A hypothetical scheme for cross-talk between CD180 and the BCR is shown below.



**Figure 6.1: Hypothetical scheme of a cross-talk between CD180 and BCR signalling pathways.** CD180-mediated pathway can operate via both pro-survival BTK/AKT or pro-apoptotic p38MAPK pathway, while sIgM-mediated signalling mostly operates through BTK/AKT. Cross-talk between the two receptors redirects signalling pathway from BTK/AKT to p38MAPK. Hypothetical precursors of p38MAPK activation are suggested (Adapted from Porakishvili *et al.*, 2015).

However, in contrast to AKT-S cells, pretreatment of p38MAPK-S cells with either anti-CD180 or anti-IgM often leads to additive phosphorylation of AKT favouring the BTK/AKT antiapoptotic pathway. Although this is a rare event, it emphasises the importance of individual tailor-made immunotherapeutic approaches to the treatment of CLL.

Specific inhibitors of AKT, BTK and p38MAPK have been used to confirm the exclusivity of activation of AKT and p38MAPK pathways. In all AKT-S CLL samples pretreatment with AKT inhibitor, Akti1/2 significantly reduced the CD180 mediated activation of AKT but did not affect the phosphorylation of AKT in p38MAPK-S CLL cells. Moreover, CD180 mediated pro-apoptotic activity in AKT-S CLL cells was inhibited by Akti1/2 as measured by DiOC<sub>6</sub> and Annexin/PI staining assays. Pretreatment with inhibitor did not affect the phosphorylation of AKT or survival of cells in unstimulated samples, indicating AKT protein kinase is

not activated (at least in AKT-S category of CLL cells) without a specific stimulation. Interestingly, Akti1/2 had no effect on CD180-mediated survival of p38MAPK-S CLL cells. In contrast, pretreatment of unstimulated p38MAPK-S cells with p38MAPK inhibitor, SB203580 significantly reduced spontaneous phosphorylation of p38MAPK compared to untreated samples suggesting the presence of constitutively phosphorylated p38MAPK in unstimulated CLL cells. As expected, SB203580 treatment inhibited CD180 mediated phosphorylation of p38MAPK in all p38MAPK-S CLL but did not affect p38MAPK phosphorylation in AKT-S CLL cells.

Recent data strongly indicate that p38MAPK selective inhibitor abrogates CD180-mediated pro-apoptotic action in p38MAPK-S CLL cells as the pretreatment significantly increased the percentages of viable cells compared to unstimulated cells. This suggests that although SB203580 inhibits phosphorylation of constitutively activated p38MAPK, phosphorylation on its own is not a sole contributor to the induction of apoptosis of p38MAPK-S CLL cells, and some complementary upstream or downstream events are required which can only be initiated upon stimulation with anti-CD180. As expected pre-treatment of AKT-S CLL cells with p38MAPK inhibitor had no effect on survival of AKT-S CLL. Hence the use of both Akti1/2 and SB203580 confirmed that, in many of the CLL samples, activation of AKT and p38MAPK pathways is exclusive.

CD180 mediated phosphorylation of BTK was inhibited in a majority of AKT-S CLL samples when treated with Ibrutinib, a BTK specific inhibitor, suggesting sharing of essential protein kinases such as BTK by both CD180 and BCR mediated signalling. Only one sample was resistant to Ibrutinib treatment in duplicates indicating Ibrutinib resistance in some CLL clones, as reported by others (Furman *et al.*, 2014; Wiestner, 2015). That pretreatment with Ibrutinib significantly reduced survival of AKT-S CLL cells when stimulated with anti-CD180 mAb further confirmed that CD180-mediated AKT/BTK pro-survival pathway in this category of cells. Confirming previous data in Chapter 3, Ibrutinib had no effect on phosphorylation of BTK or survival of p38MAPK-S CLL cells suggesting CD180 ligation does not recruit BTK in p38MAPK-S cells.

Recent data on modulation of surface expression of CD180 in 26 CLL samples confirmed the dynamic expression of CD180 in p38MAPK-S and AKT-S categories of CLL cells. However, there was a stable spontaneous expression of



CD180 on NS and CD180<sup>neg</sup> CLL cells. Downmodulation of CD180 expression in p38MAPK-S suggests that possible endocytosis of CD180 upon ligation of putative ligand present in its microenvironment and/or activation of the pro-apoptotic pathway. Interestingly, the spontaneous increase of CD180 expression in AKT-S CLL cells was associated with activation of pro-survival pathway suggesting a plausible ligand-receptor interaction *in vivo* triggering pro-survival. NS and CD180<sup>neg</sup> CLL samples did not modulate surface expression of CD180 in cultures indicating the significance of ligand-receptor interaction for the dynamics of CD180 expression. Moreover, stimulation with anti-IgM significantly reduced the surface expression of CD180 in CD180<sup>+</sup> CLL samples throughout 0-72 hours suggesting possible colocalization of both CD180 and BCR into membrane lipid rafts and further triggering of CD180 endocytosis by anti-IgM ligation.

There are some restrictions that limit further understanding of the role of microenvironmental influences in CLL. For instance, due to the invasiveness of the procedure, availability of bone marrow and lymph nodes biopsies is limited and *in vitro* studies classically carried out using peripheral blood-derived tumour cells. Moreover, mimicking the microenvironment *in vitro* requires further understanding of the microenvironment *in vivo*. Therefore, the majority of studies based on the peripheral blood cells only provide a partial image of the real interactions in the solid tissues. Thus, the contribution of host microenvironment to the proliferation and survival of CLL cells *in vivo* remains insufficiently defined. Issues related to getting enough samples from CLL patients may also limit the sturdiness of results and further studies.

Through the findings of this research project, it was established that CD180 expression on CLL cells helps to identify different subsets and to delineate their physiological status. Since intraclonal diversity plays an important role in the clinical outcome of patients with CLL, identification of subsets which respond more vigorously to external stimuli is of primary importance. Recent findings on modulation of signalling pathways through CD180 and sIgM and the temporal effects of their ligation is consistent with multiple ligands in the, *in vivo*, microenvironment playing an important role in the survival of CLL cells. In conclusion, sensitisation of AKT-S CLL cells with anti-CD180 leads to re-wiring of anti-IgM signalling from pro-survival BTK/AKT to pro-apoptotic p38MAPK

pathway, opening the door to new strategies for the treatment of a substantial cohort of CLL patients. I anticipate that this study helps to define in detail mechanisms leading to the expansion of the leukaemia cells *in vivo*, the progression of the disease and thus leads to the development of novel therapies of CLL.

### **Future studies**

- Hypothesised co-localization of IgM and CD180 receptors in the lipid rafts upon stimulation with anti-CD180 will be assessed to explore the interaction between the two receptors. Control and R-CLL cells will be stimulated with APC-conjugated anti-CD180 mAb and its co-localisation in FITC-conjugated probe for the lipid rafts (FITC-CTB) and sIgM stained with PE-conjugated antibody will be studied using confocal analysis.
- Inhibitors of protein kinases, which are upstream and downstream to p38MAPK including for Lyn will be used to understand whether complementary upstream or downstream events are required which can only be initiated upon stimulation with anti-CD180 and the inhibitors of NF- $\kappa$ B to determine whether this is the key protein involved in CD180 induced survival.
- Western blotting will be used to confirm the flow cytometry data and to assess activation of protein kinases where flow cytometry antibodies are not yet commercially available including the effect of PI3K inhibitor CAL101 on CD180 mediated signalling.

## References

- Advani R., Sharman J.P., Smith S.M., et al., (2010) Effect of Btk inhibitor PCI-32765 monotherapy on responses in patients with relapsed aggressive NHL: Evidence of antitumour activity from a phase I study. *J Clin Oncol.* 28(15s):8012.
- Aguilar Santelises, M., Rottenberg, M.E., Lewin, N., Mellstedt, H. and Jondal, M. (1996) Bcl-2, Bax and p53 expression in B-CLL in relation to in vitro survival and clinical progression. *Int J Cancer.* 69:114–119.
- Ahmad-Nejad P., et al. (2002) Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* 32:1958–68.
- Akira S. (2006) TLR signaling. *Curr Top Microbiol Immunol.* 311:1-16.
- Alomari M., Mactier S., Kaufman K.L., Best O.G., Mulligan S.P. and Christopherson R.I. (2014) Profiling the Lipid Raft Proteome from Human MEC1 Chronic Lymphocytic Leukaemia Cells. *Proteomics Bioinform.* 87.
- Antoz H., Sajewicz J., Marzec-Kotarska B., Kocki J. and Dmoszynska A (2009) Different expression of CD180, CD284 and CD14 receptors on the CD19+ subpopulation of normal and B-CLL lymphocytes. *Folia Histochemica.* 47:593-598.
- Armitage R. J., Goff L.K. and Beverley P.C.L. (1989) Expression and functional role of CD23 on T cells. *Eur. J. Immunol.* 19:31-35.
- Arvaniti E., Ntoufa S., Papakonstantinou N., Touloumenidou T., Laoutaris N., Anagnostopoulos A., Lamnissou K., Caligaris-Cappio F., Stamatopoulos K., Ghia P., Muzio M., Belessi C. (2011) Toll-like receptor signaling pathway in chronic lymphocytic leukaemia: distinct gene expression profiles of potential pathogenetic significance in specific subsets of patients. *Haematologica.* 96(11):1644-52.
- Aubry J.P., Pochon S., Graber P., Jansen K.U., Bonnefoy J. (1992) CD21 is a ligand for CD23 and regulates IgE production. *Nature.* 358:505-7.
- Bai L. and Zhu W.G. (2006) p53: structure, function and therapeutic applications. *J Cancer Mol.* 2(4):141-153.
- Baracho G., Miletic A., Omori S., Cato M. and Rickert R. (2011) Emergence of the PI3-kinase pathway as a central modulator of normal and aberrant B cell differentiation. *Curr Opin Immunol.* 23:178–183.
- Barnett S.F., Defeo-Jones D., Fu S., Hancock P.J., Haskell K.M., Jones R.E., et al., (2005) Identification and characterization of pleckstrin-homologydomain- dependent and isoenzymespecific Akt inhibitors. *Biochem J.* 385:399-408.
- Barragan M., Bellosillo B., Campas C., Colomer D., Pons G. and Gil J. (2002) Involvement of protein kinase C and phosphatidylinositol 3–kinase pathways in the survival of B-cell chronic lymphocytic leukaemia cells. *Blood.* 99:2969-2976.
- Barragan M., Frias M D., Iglesias-Serret D., Campas C., Castano E., Santidrian A.F., Coll-Mulet L., Cosialls A.M., Domingo A., Pons G. and Gil J. (2006) Regulation of Akt/PKB by phosphatidylinositol 3-kinasedependent and independent pathways in B-cell chronic lymphocytic leukaemia cells: role of protein kinase C $\beta$ . *Journal of Leukocyte Biology.* 80:1473-1479.
- Bekeredjian-Ding I.B., Wagner M., Hornung V., et al., (2005) Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J Immunol.* 174:4043-4050.

- Bellacosa A., Testa J.R., Staal S.P., and Tschlis P.N. (1991) A retroviral oncogene, akt, encoding a serine threonine kinase containing an SH2-like region. *Science*, 254, 274–277.
- Bernasconi N.L., Onai N., Lanzavecchia A. (2003) A role for Toll-like receptors in acquired immunity: upregulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood*. 101:4500-4504.
- Bernal A., Pastore R.D., Asgary Z., Keller S.A., Cesarman E., Liou H.C., *et al.*, (2001) Survival of leukaemic B cells promoted by engagement of the antigen receptor. *Blood*. 98(10):3050-7.
- Bichi R., Shinton S.A., Martin E.S., Koval A., Calin G.A., Cesari R. *et al.*, (2002) Human chronic lymphocytic leukaemia model in mouse by targeted TCL1 expression. *Proc Natl Acad Sci USA*. 99:6955–60.
- Binder M., Lechenne B., Ummanni R., Scharf C., Balabanov S., Trusch M., Schluter H., Braren I., Spillner E. and Trepel M. (2010) Stereotypical chronic lymphocytic leukaemia B-cell receptors recognize survival promoting antigens on stromal cells. *PLoS ONE*. 5(12).
- Bjorkman P.J. and Burmeister W.P. (1994) Structures of two classes of MHC molecules elucidated: crucial differences and similarities. *Current Opinion in Structural Biology*. 4:852–856.
- Bleul C.C., Fuhlbrigge R.C., Casasnovas J.M., Aiuti A., Springer T.A. (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med*. 184:1101–9.
- Blix E.S., Irish J.M., Husebekk A., Delabie J., Forfang L., Tierens A.M., Myklebust J.H. and Kolstad A. (2012) Phospho-specific flow cytometry identifies aberrant signaling in indolent B-cell lymphoma. *BMC Cancer*.12:478.
- Bourke E., Bosisio D., Golay J., Polentarutti N., Mantovani A. (2003) The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. *Blood*. 102:956-963.
- Burger J.A. and Burkle A. (2007) The CXCR4 chemokine receptor in acute chronic leukaemia: a marrow homing receptor and potential therapeutic target. *British Journal of Haematology*. 137:288-296.
- Burger J.A. and Kipps T.J. (2002) Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukaemia B cells. *Leukaemia & lymphoma*. 43(3):461–466.
- Burger J.A. and Kipps T.J. (2006) CXCR4: a key receptor in the crosstalk between tumour cells and their microenvironment. *Blood*. 107(5).
- Burger J.A., Burger M., Kipps T.J. (1999) Chronic lymphocytic leukaemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood*. 94:3658–67.
- Burger J.A., O'Brien S., Fowler N., *et al.*, (2010) The Bruton's tyrosine kinase inhibitor, PCI-32765, is well tolerated and demonstrates promising clinical activity in chronic lymphocytic leukaemia (CLL) and small lymphocytic lymphoma (SLL): an update on ongoing phase 1 studies [abstract]. *Blood*. 116(21):32a.

- Burgering B.M., and Coffey P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature*. 376:599–602.
- Byrd J.C., Gribben J.G., Peterson B.L., Grever M.R., Lozanski G., Lucas D.M., Lampson B., Larson R.A., Caligiuri M.A. and Heerema N.A. (2006) Select High-Risk genetic features predict earlier progression following chemo immunotherapy with fludarabine and rituximab in chronic lymphocytic leukaemia: Justification for risk-Adapted Therapy. *J Clin Oncol*. 24:437-443.
- Byrd J.C., Furman R.R., Coutre S.E., Flinn I.W., Burger J.A., Blum K.A., Grant B., Sharman J.P., Coleman M., Wierda W.G., Jones J.A., Zhao W., Heerema N.A., Johnson A.J., Sukbuntherng J., Chang B.Y., Clow F., Hedrick E., Buggy J.J., James D.F. and O'Brien S. (2013) Targeting BTK with Ibrutinib in Relapsed Chronic Lymphocytic Leukaemia. *The new England Journal of Medicine*. 369(1):32-42.
- Byrd J.C., Brown J.R., O'Brien S., Barrientos J.C., Kay N.E., Reddy N.M. *et al.*, (2014) Ibrutinib versus Ofatumumab in Previously Treated Chronic Lymphoid Leukaemia. *N Engl J Med*. 371: 213–223.
- Cai, J., Humphries, C., Richardson, A. and Tucker, W. P. (1992) Extensive and selective mutations of a rearranged VH5 genes in human B Cell Chronic Lymphocytic Leukaemia. *J. Exp. Med*. 176:1073-1081.
- Calin G.A. and Croce C. (2009) Chronic lymphocytic leukaemia: interplay between noncoding RNAs and protein-coding genes. *Blood*. 114:4761–4770.
- Cantu E.S., McGill J.R., Stephenson C.F., Hoffmann H.M., Tang L., Yan J. *et al.*, (2013) Male to female sex ratios of abnormalities detected by fluorescence in situ hybridization in a population of chronic lymphocytic leukaemia patients. *Hematol Rep*. 5(1):13-7.
- Cappio, F. (2001) Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukaemia. *Blood*. 97:2777-2783.
- Catera, R., Silverman G.J., Hatzi K., Seiler T., Didier S., Zhang L., Herve M., Meffre E., Oscier D.G., Vlassara H., *et al.* (2008) Chronic lymphocytic leukaemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol. Med*. 14:665–674.
- Catovsky D., Richards S., Matutes E., *et al.*, (2007) Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. *Lancet*. 370:230-239.
- Catovsky D., Wade R. and Else M. (2014) The clinical significance of patients' sex in chronic lymphocytic leukaemia. *Haematologica*, 99(6):1088-1094.
- Catovsky, D., Fooks, J. & Richards, S. (1989) Prognostic Factors In Chronic Lymphocytic Leukaemia: The Importance Of Age, Sex And Response To Treatment In Survival. A Report From The Mrc Cll 1 Trial. Mrc Working Party On Leukaemia In Adults. *Br J Haematol*. 72:141-9.
- Chan, V. W. F., Mecklenbräuker, I., Su, I., Texido, G., Leitges, M., Carsetti, R., Lowell, C.A., Rajewsky, K., Miyake, K., and Tarakhovskiy, A. (1998) The Molecular Mechanism of B Cell Activation by *tol*-like Receptor Protein RP-105. *J.Exp.Med*. 188(1):93-101.
- Chang H., Huang H., Huang T., Yang P., Wang Y. and Juan H. (2013) Flow cytometry detection of mitochondrial membrane potential. *BioProtocol*, 3(8).
- Chang L. and Karin M. (2001) Mammalian MAP kinase signalling cascades. *Nature*. 410: 37–40.

- Chankotadze R. (2013) A study of specific stimulation-induced response in B-chronic lymphocytic leukaemia cell line - MEC1 cells.
- Chaplin J.W., Chappell C. P. and Clark E. A. (2013) Targeting antigens to CD180 rapidly induces antigen-specific IgG, affinity maturation, and immunological memory. *Journal of experimental medicine*. 2013:2135-2146.
- Chaplin J.W., Kasahara S., Clark E.A. and Ledbetter J.A. (2011) Anti-CD180 (RP105) Activates B Cells To Rapidly Produce Polyclonal Ig via a T Cell and MyD88-Independent Pathway. *Journal of Immunology*. 187:4199-4209.
- Chappell, C.P., Giltiay, N.V., Dresch, C. and Clark, E.A. (2014) Controlling immunity responses by targeting antigens to dendritic cell subsets and B cells. *International Immunology*. 26(1):3-11.
- Chen L., Apgar J., Huynh L., Dicker F., Giago-McGahan T., Rassenti L. *et al.*, (2005) ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukaemia. *Blood*. 105:2036–2041.
- Chen L., Widhopf G., Huynh L., Rassenti L., Rai K.R., Weiss A. *et al.*, (2002) Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukaemia. *Blood*. 100:4609–4614.
- Chin K.V., Ueda K., Pastan I., Gottesman M.M. (1992) Modulation of activity of the promoter of the human MDRI gene by ras and p53. *Science*. 255:459.
- Chiorazzi, N. and Ferrarini, M. (2003) B cell chronic lymphocytic leukaemia Lessons learned from studies of the B cell Antigen Receptor. *Annu Rev. Immunol*. 21:841-94.
- Chiorazzi, N., Rai, K.R. and Ferrarini, M. (2005) Mechanisms of disease Chronic Lymphocytic Leukaemia. *The new journal of medicine*. 352(8):804-815.
- Chiron D., Bekeredjian-Ding I., Pellat-Deceunynck C., Bataille R., Jego G. (2008) Toll-like receptors: lessons to learn from normal and malignant human B cells. *Blood*. 112:2205-2213.
- Chu C.C., Catera R., Hatzi K., Yan X.J., Zhang L., Wang X.B., Fales H.M., Allen S.L., Kolitz J.E., Rai K.R., and Chiorazzi N. (2008) Chronic lymphocytic leukaemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood*. 112:5122–5129.
- Cimmino A., Calin G. A., Fabbri M., Iorio M.V., Ferracin M., Shimizu M., Wojcik S.E., Aqeilan R.I., Zupo S., Dono M., Rassenti L., Alder H., Volinia S., Liu C., Kipps T.J., Negrini M., Croce C., (2005) miR-15 and miR-16 induce apoptosis by targeting BCL2, *Proc. Natl. Acad. Sci. U.S.A.* 102:13944–13949.
- Coultas L. and Stasser A. (2003) The role of the Bcl-2 protein family in cancer. *Semin Cancer Biol*. 13:115–123.
- Cramer, P. and Hallek, M. (2011) Prognostic factors in chronic lymphocytic leukaemia-what do we need to know?. *Nat.Rev.Clin.Oncol*. 8:38-47.
- Crespo M., Bosch F., Villamor N., Bellosillo B., Colomer D., Rozman M., Marce S., Lopez-Guillermo A., Campo E. and Montserrat E. (2003) ZAP-70 expression as a surrogate for Immunoglobulin-Variable-Region Mutations in chronic lymphocytic leukaemia. *The new England journal of medicine*. 348:18.

Crowther-Swanepoel D., Wild R., Sellick G., *et al.*, (2008) Insight into the pathogenesis of chronic lymphocytic leukaemia (CLL) through analysis of *IgVH* gene usage and mutation status in familial CLL. *Blood*. 111:5691–5693.

Cuenda A., Cohen P., Bue´e-Scherrer V. and Goedert M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6): comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO Journal*. 16:295–305.

Cuenda A., Rouse J., Doza Y.N., Meier R., Cohen P., Gallagher T.F., Young P.R. and Lee J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin. *FEBS Letters*. 364(1):229–233.

Cunì S., Pérez-Aciego P., Pérez-Chacòn G., Vargas J.A., Sánchez A., Martín-Saavedra F.M., *et al.*, (2004) A sustained activation of PI3K/NFkappaB pathway is critical for the survival of chronic lymphocytic leukaemia B cells. *Leukaemia*. 18(8):1391-400.

Dal-Bo M., Del Giudice I., Bomben R., Capello D., Bertoni F., Forconi F., Laurenti L., Rossi D., Zucchetto A., Pozzato G., Marasca R., Efremov D.G., Guarini A., Del Poeta G., Foa R., Gaidano G., Gattei V. (2011) B-cell receptor, clinical course and prognosis in chronic lymphocytic leukaemia: the growing saga of the IGHV3 subgroup gene usage. *Br. J. Haematol*. 153:3-14

Damle R. N., Wasil T., Fais F., Ghiotto F., Valetto A., Allen S. L., Buchbinder A., Budman D., Dittmar K., Kolitz J., Lichtman S. M., Schulman P., Vinciguerra V. P., Rai K. R., Ferrarini M. and Chiorazzi N. (1999) Ig V Gene Mutation Status And Cd38 Expression As Novel Prognostic Indicators In Chronic Lymphocytic Leukaemia. *Blood*. 94:840-7.

Damle R., Batliwala F., Allen S.L., Kolitz J.E., Vinciguerra V.P., *et al.* (2004) Telomere length and telomerase activity in chronic lymphocytic leukaemia B cells. *Blood*. 103:375-382.

Damle, R.N., Ghiotto, F., Valetto, A., Albesiano, E., Fais, F., Yan, X. J., Sison, C.P., Allen, S.L., Kolitz, J., Schulman, P., Vinciguerra, V.P., Budde, P., Frey, J., Rai, K.R., Ferrarini, M. and Chiorazzi, N. (2002) B-cell chronic lymphocytic leukaemia cells express a surface membrane phenotype of activated, antigen experienced B lymphocytes. *Blood*. 99:4087–4093.

Darce J.R., Arendt B.K., Wu X., Jelinek D.F. (2007) Regulated expression of BAFF-binding receptors during human B cell differentiation. *J Immunol*. 179:7276–86.

Davies C., Mason J., Wakelam M., Young L. and Eliopoulos A. (2004) Inhibition of phosphatidylinositol 3-kinase- and ERK MAPK regulated protein synthesis reveals the pro-apoptotic properties of CD40 ligation in carcinoma cells. *J Biol Chem*, 279: 1010–1019.

Davila M.L. and Brentjens R. (2013) CAR Therapy for CLL: What are the challenges?. *Hematol Oncol Clin North Am*. 27(2):341-353.

de Toterò D., Meazza R., Zupo S., Cutrona G., Matis S., Colombo M. *et al.*, (2006) Interleukin-21 receptor (IL-21R) is up-regulated by CD40 triggering and mediates proapoptotic signals in chronic lymphocytic leukaemia B cells. *Blood*. 107:3708–3715.

de Gorter D.J., Beuling E.A., Kersseboom R., Middendorp S., van Gils J.M., Hendriks R.W. *et al.*, (2007) Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine controlled B cell migration and homing. *Immunity*. 26: 93–104.

De Rooij M.F., Kuil A. and Geest C.R. (2012) The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine controlled adhesion and migration in chronic lymphocytic leukaemia. *Blood*. 119(11):2590–4.

Deaglio S., Vaisitti T., Aydin S., Bergui L., D'Arena G., Bonello L., Omede P., Scatolini M., Jaksic O., Chiorino G., Efremov D., and Malavasi F. (2007) CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. *Blood*. 110:4012-4021.

Deaglio S., Vaisitti T., Billington R., Bergui L., Omede P., Genazzani A.A. and Malavasi F. (2010) CD38/CD19: a lipid raft-dependent signalling complex in human B cells. *Blood*. 109:5390-5398.

Deaglio, S., Aydin, S., Grand, M. M., Vaisitti, T., Bergui, L., D'arena, G., Chiorino, G. & Malavasi, F. (2010) Cd38/Cd31 Interactions Activate Genetic Pathways Leading To Proliferation And Migration In Chronic Lymphocytic Leukaemia Cells. *Mol Med*. 16:87-91.

Decker T., Schneller F., Sparwasser T., Tretter T., Lipford G.B., Wagner H., Peschel C. (2000) Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukaemia B cells. *Blood*. 95(3):999-1006.

Del Poeta G., Maurillo L., Venditti A. *et al.*, (2001) Clinical significance of CD38 expression in chronic lymphocytic leukaemia. *Blood*. 98:2633–2639.

Delgado J., Pratt G., Phillips N., Briones J., Fegan C., Nomdedeu J., Pepper C., Aventin A., Ayats R., Brunet S., Martino R., Valcarcel D., Milligan D. and Sierra J. (2009) Beta<sub>2</sub>-microglobulin is a better predictor of treatment-free survival in patients with chronic lymphocytic leukaemia if adjusted according to glomerular filtration rate. *British Journal of Haematology*. 145:801-805.

Dewson G. and Kluc R.M. (2010) Bcl-2 family-regulated apoptosis in health and disease. *Cell Health and Cytoskeleton*. 2:9-22.

Dighiero G., Maloum K., Desablens B., Cazin B., Navaffo M., Lebay R., Leporrier M., Jaubert J., Lepeu G., Dreyfus B., Binet J., Travade P. (1998) Chlorambucil in indolent chronic lymphocytic leukaemia. *The new England journal of medicine*. 338:1506-1514.

Dighiero, G. (2005) Chronic Lymphocytic Leukaemia, CLL Biology and Prognosis. *American Society of Hematology*. 1:278-284.

Dillman R.O., Mick R., McIntyre O.R. (1989) Pentostatin in chronic lymphocytic leukaemia: a phase II trial of cancer and leukaemia group B. *J Clin Oncol*. 7:433.

Divanovic S., Trompette A., Atabani S., Madan R., Golenbock D. T., Visintin A., Finberg R.W., Tarakhovskiy A., Vogel S.N., Belkaid Y, *et al.* (2005) Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat. Immunol*. 6: 571–578.

Divanovic S., Trompette A., Petiniot L.K., Allen J.L., Flick L.M., Belkaid Y., Madan R., Haky J.J. and C. Karp C. L. (2007) Regulation of TLR4 signaling and the host interface with pathogens and danger: the role of RP105. *J. Leukoc. Biol*. 82: 265–271.

Dohner H., Fischer K., Bentz M., *et al.*, (1995) p53 gene deletion predicts for poor survival and nonresponse to therapy with purine analogs in chronic B-cell leukaemias. *Blood*. 85:1580-1589.

Dohner H., Fischer K., Bentz M., Hansen K., Benner A., Cabot G., Diehl D., Schlenk R., Coy J., Stilgenhauer S., Volkmann M., Galle P.R., Poustka A., Hunstein W., Lichter P.



- (1995) P53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell lymphocytic leukaemia. *Blood*. 85:1580.
- Dohner H., Stilgenbauer S., James M.R., *et al.*, (1997) 11q deletions identify a new subset of B-cell chronic lymphocytic leukaemia characterized by extensive nodal involvement and inferior prognosis. *Blood*. 89:2516-2522.
- Domer M., Brandt S., Tinguely M., Zucol F., Bourquin J., Zauner L., Berger C., Bernasconi M., Speck R.F. and Nadal D. (2009) Plasma cell toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production. *Immunology*. 128:573-579.
- Downward, J. (2004) PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol*. 15:177–82.
- Dreger P. and Montserrat E. (2002) Autologous and allogenic stem cell transplantation for chronic lymphocytic leukaemia. *Leukaemia*. 16:985-992.
- Dreger P., Dohner H., Ritgen M., Bottcher S., Busch R., Dietrich S., Bunjes D., Cohen S., Schubert J., Hegenbart U., Beelen D., Zeis M., Stadler M., Hasenkamp J., Uharek L., Scheid C., Humpe A., Zenz T., Winkler D., Hallek M., Kneba M., Schmitz N., Stilgenbauer S. and German C. L. L. S. G. (2010) Allogeneic Stem Cell Transplantation Provides Durable Disease Control In Poor-Risk Chronic Lymphocytic Leukaemia: Long-Term Clinical And Mrd Results Of The German Cll Study Group Cll3x Trial. *Blood*. 116:2438-47.
- Efremov, D.G., Gobessi, S. and Longo, P.G. (2007) Signaling pathways activated by antigen-receptor engagement in chronic lymphocytic leukaemia B-cells, *Autoimmunity Reviews*. 7(2):102-8.
- El Rouby S., Bayona W., Pisharody S.M., Newcomb E.W. (1992) p53 mutations in B-cell chronic lymphocytic leukaemia. *Cum Top Immunol Microbiol*. 182:313.
- El Rouby S., Thomas A., Costin D., *et al.*, (1993) p53 gene mutation in B-cell chronic lymphocytic leukaemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood*. 82:3452-3459.
- Engelman J.A. (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nature Reviews Cancer*. 9(8):550-562.
- Fais F., Ghiotto F., Hashimoto S., Sellars B., Valetto A., Allen S. L., Schulman P., Vinciguerra V. P., Rai K., Rassenti L. Z., Kipps T. J., Dighiero G., Schroeder H. W., Ferrarini M. and Chiorazzi N. (1998) Chronic Lymphocytic Leukaemia B Cells Express Restricted Sets Of Mutated And Unmutated Antigen Receptors. *J Clin Invest*. 102:1515-25.
- Ferguson S.S.G (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signalling. *Pharmacol Rev*. 53: 1–24.
- Ferrajoli A., Thomas D.A., Albitar M., *et al.*, (2003) Alemtuzumab for minimal residual disease in CLL. *Blood*. 22:569.
- Ferrarini, M., Toterod, Colombo, M. and Cutrona, G. (2014) Facts and Speculations on the Onset and Clonal Expansion of Chronic Lymphocytic Leukaemia Cells. *Int Trends Immunity*. 2 (1):1-10.
- Filler G., Priem F., Lepage N., Sinha P., Vollmer I., Clark H., Keely E., Matzinger M., Akbari A., Althaus H. and Jung K. (2002) Betatrace protein, cystatin C, beta2-

microglobulin, and creatinine compared for detecting impaired glomerular filtration rates in children. *Clinical Chemistry*. 48:729–736.

Franke T.F. (2008) PI3K/Akt: getting it right matters. *Oncogene*. 27:6473–6488.

Franke T.F., Yang S.I., Chan T.O., Datta K., Kazlauskas A., Morrison D.K., Kaplan D.R., and Tschlis P.N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 81:727–736.

Fresno-Vara J.A., Casado E., DeCastro J., Cejas P., Belda-Iniesta C., Gonzales Baron M. (2003) PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev*. 30:193–204.

Friedman D.R., Lanasa M.C. and Davis P.H. (2013) Perifosine treatment in chronic lymphocytic leukaemia: results of a phase II clinical trial and in vitro studies. *Leukaemia Lymphoma*. 55(5):1067-75.

Friesen C., Fulda S. and Debatin K.M. (1997) Deficient activation of the CD95 (APO-1/ Fas) system in drug resistant cells. *Leukaemia*. 11(11):1833-1841.

Fruman D.A., Snapper S.B., Yballe C.M., *et al.*, (1999) Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85a. *Science*. 283:393-397.

Fulda S. (2010) Evasion of apoptosis as a cellular stress response in cancer. *International Journal of Cell Biology*. 2010.

Furman R.R., Asgary Z., Mascarenhas J.O., Liou H.C., Schattner E.J. (2000) Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukaemia B cells. *J. Immunol*. 164:2200–6.

Furman, R.R. (2010). Prognostic markers and stratification of chronic lymphocytic leukaemia. *Haematology*. 77- 81.

Furman R.R., Cheng S., Lu P., Setty M., Perez A.R., Guo A., Racchumi J., Xu G., Wu H., Ma J., Steggerda A.M., Coleman M., Leslie C. and Wang Y.L. (2014) Ibrutinib Resistance in Chronic Lymphocytic Leukaemia. *New England Journal of medicine*. 370:24.

Gahrton G., Robert K.H., Friberg K., Juliusson G., Biberfeld P., Zech L. (1982) Cytogenetic mapping of the duplicated segment of chromosome 12 in lymphoproliferative disorders. *Nature*. 297:513–14.

Gangloff M. (2012) Different dimerisation mode for TLR4 upon endosomal acidification?. *Trends in Biochemical Sciences*. 37 (3).

Ganley-Leal L.M., Liu X., Wetzler L.M. (2006) Toll-like receptor 2-mediated human B cell differentiation. *Clin Immunol*. 120:272-284.

Gartenhaus R.B., Wang P., Hoffman M., Janson D., Rai K.R. (1996) The induction of p53 and WAF1/CIP1 in chronic lymphocytic leukaemia cells treated with 2-chlorodeoxyadenosine. *J Mol Med*. 74:143-147.

Gayko U., Fung M., Clow F., Sun S., Faust E., Price S., James D., Doyle M., Bari S. and Zhuang S.H. (2014) Development of the Bruton's tyrosine kinase inhibitor ibrutinib for B cell malignancies. *Ann.N.Y.Acad.Sci*. 1358:82-94.

Genevier H.C., *et al.*, (1994) Expression of Bruton's tyrosine kinase protein within the B cell lineage. *European journal of immunology*. 24:3100–3105.

Genini D., Adachi S., Chao Q., *et al.*, (2000) Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukaemia cells by damaging the DNA and by directly affecting the mitochondria. *Blood*. 96:3537-3543.

Ghia, P., Chiorazzi, N. and Stamatopoulos, K. (2008) Microenvironmental influences in chronic lymphocytic leukaemia: the role of antigen stimulation. *J Intern Med*. 264:549-562.

Giannini, P., Pietra, G., Travaini, G., Quarto, R., Shyti, G., Benelli, R., Ottaggio, L., Mingari, M.C., Zupo, S., Cutrona, G., Pierri, I., Balleari, E., Pattarozzi, A., Calvaruso, M., Tripodo, C., Ferrarini, M. and Toterò, D. (2014) Chronic lymphocytic leukaemia nurse-like cells express hepatocyte growth factor receptor (c-MET) and indoleamine 2,3-dioxygenase and display features of immunosuppressive type 2 skewed macrophages. *Haematologica*. 99 (6):1078-1087.

Giannoni P., Scaglione S., Quarto R., Narcist R., Parodi M., Balleart E. *et al.*, (2011) An interaction between hepatocyte growth factor and its receptor (c-MET) prolongs the survival of chronic lymphocytic leukaemic cells through STAT3 phosphorylation: a potential role of mesenchymal cells in the disease. *Haematologica*. 96(7):1015-23.

Gilot D., Giudicelli F., Lagadic-Gossman D. and Fardel O. (2010) AKTi-1/2, an allosteric inhibitor of AKT 1 and 2, efficiently inhibits CaMKI $\alpha$  activity and aryl hydrocarbon receptor pathway. *Chemico-Biological Interactions*. 188:3546-552.

Goldin, L. R. S., Slager, S.L. and Caporaso, N. E. (2010). Familial Chronic Lymphocytic Leukaemia. *Current Opinion in Hematology*. 17(4):350-355.

Good K.L., Avery D.T. and Tangye S.G. (2009) Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naïve B cells. *The Journal of Immunology*. 182:890-901.

Granziero L., Ghia P., Circosta P. *et al.*, (2001) Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukaemia. *Blood*. 97:2777-83.

Graves J.D., Draves K.E., Craxton A., Krebs E.G. and Clark E.A. (1998) A comparison of signalling requirements for apoptosis of human B lymphocytes induced by the B cell receptor and CD95/Fas. *The journal of immunology*. 161:168-174.

Grdisa M. (2003) Influence of CD40 ligation on survival and apoptosis of B-CLL cells in vitro. *Leuk Res*. 27:951-956.

Grever M.R., Lucas D.M., Dewald G.W., *et al.*, (2007) Comprehensive assessment of genetic and molecular features predicting outcome in patients with chronic lymphocytic leukaemia: results from the US Intergroup Phase III Trial E2997. *J Clin Oncol*. 25:799-804.

Gribben J.G., Hosing C., Maloney D.G. (2011) Stem cell transplantation for indolent lymphoma and chronic lymphocytic leukaemia. *Biol Blood Marrow Transplant*. 17:S63-S70.

Gross A., McDonnell J.M., Korsmeyer S.J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*. 13:1899-1911.

Grumont R.J., *et al.* (1998) B lymphocytes differentially use the Rel and nuclear factor kappa B1 (NF-kappa B1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. *J. Exp. Med*. 187:663-74.

Guarini, A., Chiaretti, S., Tavoraro, S., Maggio, R., Paragine, N., Citarella, F., Ricciardi, M. R., Santangelo, S., Marinelli, M., De propriis, M. S., Messina, M., Mauro, F.R., Giudice,

I D., and Foa, R. (2008) BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgVH unmutated chronic lymphocytic leukaemia (CLL) cells. *Blood*. 112 (3):782-792.

Gunnarsson R. and Rosenquist R. (2011) New insights into the pathobiology of chronic lymphocytic leukaemia. *J. Hematopathol.* 4:149-163.

Guo A., Lu P., Galanina N., Nabhan C., Smith S.M., Coleman M., Wang Y.L. (2016) Heightened BTK-dependent cell proliferation in unmutated chronic lymphocytic leukaemia confers increased sensitivity to ibrutinib. *Oncotarget*, 7(4).

Hainsworth J.D., Litchy S., Barton J.H., *et al.*, (2003) Single-agent Rituximab as first-line and maintenance treatment for patients with chronic lymphocytic leukaemia or small lymphocytic lymphoma: a phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol.* 21:1746-1751.

Hahne M., Kataoka T., Schroter M. *et al.* (1998) APRIL, a new ligand of the tumor necrosis factor family, stimulates tumour cell growth. *J Exp Med.* 188:1185–90.

Hallek M. *et al.*, (2010) Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *The Lancet.* 376(9747):1164–1174.

Hallek M., Langenmayer I., Nerl C., Knauf W., Dietzfelbinger H., Adorf D., Ostwald M., Busch R., Kuhn-Kallek I., Thiel E. and Emmerich B. (1999) Elevated serum thymidine kinase levels identify a subgroup at high risk of disease progression in early nonmoulding chronic lymphocytic leukaemia. *Blood.* 93(5):1732-1737.

Hallek M., Wanders L., Strohmeyer S., Emmerich B. (1992) Thymidine kinase: a tumour marker with prognostic value for non-Hodgkin's lymphoma and a broad range of potential clinical applications. *Ann Hematol.* 65:1–5.

Hanada M, Feng J, Hemmings B. A. (2004) Structure, regulation and function of PKB/AKT-a major therapeutic target. *Biochim Biophys Acta.* 1697:3-16.

Hannigan B.M., Barnett Y.A., Armstrong D.B., McKelvey- Martin V.J., McKenna P.G. (1993) Thymidine kinases: the enzymes and their clinical usefulness. *Cancer Biother.*, 8:189–197.

Hassan M., Alaoui A., Feyen O., Mirmohammadsadegh A., Essmann F., Tannapfel A., *et al.*, (2008) The BH3-only member Noxa causes apoptosis in melanoma cells by multiple pathways. *Oncogene.* 27:4557–68.

Hebeis B., Vigorito E., Kovesdi D. and Turner M. (2005) Vav proteins are required for B-lymphocyte responses to LPS. *Blood.* 106:635-640.

Herman S.E., Gordon, A.L., Wagner, A.J., Heerema, N.A., Zhao, W., Flynn, J.M., Jones, J., Andritsos, L., Puri, K.D., Lannutti, B.J., Giese, N.A., Zhang, X., Wei, L., Byrd, J.C. and Johnson, A.J. (2010) Phosphatidylinositol 3-kinase-d inhibitor CAL- 101 shows promising preclinical activity in chronic lymphocytic leukaemia by antagonizing intrinsic and extrinsic cellular survival signals. *Blood.* 116:2078–2088.

Herman S.E., Gordon A.L., Hertlein E., Ramanunni A., Zhang X., Jaqlowski S., Flynn J., Jones J., Blum, K.A., Biggy J.J., Hamdy, A., Johnson A.J. and Byrd J.C. (2011) Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukaemia and is effectively targeted by PCI-32765. *Blood.* 117(23):6287–6296.

- Hervé M., Xu K., Ng Y.S., Wardemann H., Albesiano E., Messmer B.T., Chiorazzi N., Meffre E. (2005) Unmutated and mutated chronic lymphocytic leukaemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J. Clin. Invest.* 115:1636-1643.
- Hoehn D., Medeiros L.J., Konoplev S. (2010) Molecular pathology of chronic lymphocytic leukaemia. In: *Hematopathology: genomic mechanisms of neoplastic diseases*, eds.: D. Crisan. Humana Press, New York. 255-291.
- Hoellenriegel J., Meadows S.A., Sivina M., Wierda W.G., Kantarjian H., Keating M.J., Giese N., O'Brien S., Yu A., Miller L.L., Lannutti B.J., Burger J.A. (2011) The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukaemia. *Blood.* 118(13):3603-12.
- Hofbauer S. W., Pinon J. D., Brachtl G. et al., (2010) Modifying Akt signalling in B-Cell chronic lymphocytic leukaemia cells. *Cancer Res.* 70:7336-7344.
- Honda K. and Taniguchi T. (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol.* 6(9):644-658.
- Honigberg L.A., Smith A.M., Sirisawad M., et al., (2010) The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. *Proc Natl Acad Sci U S A.* 107(29):13075-13080.
- Hoogeboom R., van Kessel K.P., Hochstenbach F., Wormhoudt T.A., Reinten R.J., Wagner K., Kater A.P., Guikema J.E., Bende R.J., van Noesel C.J. (2013) A mutated B cell chronic lymphocytic leukaemia subset that recognizes and responds to fungi. *J. Exp. Med.* 210:59-70.
- Hoogendoorn M., Wolbers J.O., Smit W.M., Schaafsma M.R., Barge R.M., Willemze R., Falkenburg J.H. (2004) Generation of B-cell chronic lymphocytic leukaemia (B-CLL)-reactive T-cell lines and clones from HLA class I-matched donors using modified B-CLL cells as stimulators: implications for adoptive immunotherapy. *Leukaemia.* 18(7):1278-1287.
- Howard M., Grimaldi J.C., Bazan J.F., et al., (1993) Formation and hydrolysis of cyclic ADP-ribose catalysed by lymphocyte antigen CD38. *Science.* 262:1056-1059.
- Hoyer J. D., Ross C. W., Li C. Y., Witzig T. E., Gascoyne R. D., Dewald G. W. & Hanson C. A. (1995) True T-Cell Chronic Lymphocytic Leukaemia: A Morphologic And Immunophenotypic Study Of 25 Cases. *Blood.* 86:1163-9.
- Huang P., Feng L., Oldham E.A., Keating M.J., Plunkett W. (2000) Superoxide dismutase as a target for the selective killing of cancer cells. *Nature.* 407 (6802):390-5.
- Huang S., Y. Jiang Z. J. Li E. Nishida P. Mathias S. C. Lin R. J. Ulevitch G. R. Nemerow and Han J. H. (1997) Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity.* 6:739.
- Hus I., Podhorecka M., Bojarska-Junak A., Rolinski J., Schmitt M., Sieklucka M., Wasik-Szczepanek E. and Dmoszynska A. (2006) The clinical significance of ZAP-70 and CD38 expression in B-cell chronic lymphocytic leukaemia. *Annals of Oncology.* 17:68-690.
- Hussain A.R., Ahmed S.O., Ahmed M., Khan O.S., AlAbdulmohsen S., Plataniias L.C., et al., (2012) Cross-talk between NFkB and the PI3 kinase/Akt pathway can be targeted

in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. *PLoSOne*. 7(6):e39945.

Ikeda H., Hideshima T. and Fulciniti M. (2010) PI3K/p110 $\delta$  is a novel therapeutic target in multiple myeloma. *Blood*. 116(9):1460-1468.

Ishibe N., Prieto D., Hosack D.A., *et al.*, (2002) Telomere length and heavy-chain mutation status in familial chronic lymphocytic leukaemia. *Leuk Res*. 26:791–794.

Itala M., Geisler C.H., Kimby E., *et al.*, (2002) Standard-dose anti-CD20 antibody Rituximab has efficacy in chronic lymphocytic leukaemia: results from a Nordic multicentre study. *Eur J Haematol*. 69:129-134.

Jacobs-Helber S.M., Ryan J.J. and Sawyer S.T. (2000) JNK and p38 are activated by erythropoietin (EPO) but are not induced in apoptosis following EPO withdrawal in EPO-dependent HCD57 cells. *Blood*. 96:933-940.

Jager U., Fridrik M., Zeitlinger M., Heintel D., Hopfinger G., Burgstaller S., *et al.*, (2012) Arbeitsgemeinnnschaft Medikamentose Tumourtherapie (AGMT) Investigators. Rituximab serum concentrations during immune-chemotherapy of follicular lymphoma correlate with patient gender, bone marrow infiltration and clinical response. *Hematologica*. 97(9):1431-8.

Jaksic B. and Vitale B. (1981) Total tumour mass score (TTM): A new parameter in chronic lymphocytic leukaemia. *Br J Haematol*. 49:405-414.

Janas E., Priest R., Wilde J.I., White J.H. and Malhotra R (2005) Rituxan (anti-CD20 antibody) induced translocation of CD20 into lipid rafts is crucial for calcium influx and apoptosis. *Clin Exp Immunol*. 139: 439-446.

Jandl J.H. *et al.*, (1987) Chronic lymphocytic leukaemia, in *Blood: A Textbook of Hematology*. Boston, MA, Little, Brown. 64.

Jia S., Liu Z., Zhang S., Liu P., Zhang L., Lee S. *et al.*, (2008) Essential roles of PI(3)K-p110 $\beta$  in cell growth, metabolism and tumorigenesis. *Nature*. 454:776–779.

Johansson M., Brismar S., Karlsson A. (1997) Human deoxycytidine kinase is located in the cell nucleus. *Proc Natl Acad Sci USA*, 94:11941–11945.

Jones D.T., Ganeshaguru K., Anderson R.J., Jackson T.R., Bruckdorfer K.R., Low S.Y. *et al.*, (2003) Albumin activates the AKT signaling pathway and protects B-chronic lymphocytic leukaemia cells from chlorambucil- and radiation-induced apoptosis. *Blood*. 101:3174–3180.

Jones P.F., Jakubowicz T., Pitossi F.J., Maurer F., and Hemmings B.A. (1991). Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger family. *Proc. Natl. Acad. Sci. USA*. 88:4171–4175.

Juliusson G., Oscier D. G., Fitchett M., Ross F.M., Stockdill G., Mackie M.J., Parke A.C., Castoldi G.L., Guneo A., Knuutila S., Elonen E. and Gahrton G. (1990) Prognostic subgroups in B-CLL chronic lymphocytic leukaemia defined by specific chromosomal abnormalities. *N Engl J Med*. 323:720-4.

Kadowaki, N., Ho, S., Antonenko, S., de Waal Malefyt, R., Kastelein, R.A., Bazan, F. and Yong-Jun, L. (2001) Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine*. 194:863–870.

Kane B.A., Bryant K.J., McNeil H.P. and Tedla N.T. (2014) Termination of Immune Activation: An Essential Component of Healthy Host Immune Responses. *Journal of Innate Immunity*. 6:727-738.

Kawakami Y., Miura T., Bissonnette R., Hata D., Khan W. N., Kitamura T., Maeda-Yamamoto M., Hartman S. E., Yao L., Alt F. W. and Kawakami, T. (1997) Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. *Proc Natl Acad Sci U S A*. 94: 3938-3942.

Kawakami, Y., Kitaura, J., Hartman, S. E., Lowell, C. A., Siraganian, R. P. and Kawakami, T. (2000) Regulation of protein kinase C $\beta$  by two protein-tyrosine kinases, Btk and Syk. *Proc Natl Acad Sci U S A*. 97:7423-7428.

Kerr J.F.R., Wyllie A.H., Currie A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 26:239-257.

Kienle D., Benner A., Krober A., Winkler D., Mertens D., Buhler A., Seiler T., Jager U., Lichter P., Dohner H., Stilgenbauer S. (2006) Distinct gene expression patterns in chronic lymphocytic leukaemia defined by usage of specific VH genes. *Blood*. 107:2090–2093.

Kil L.P., de Bruijn M.J., van Hulst J.A., Langerak A.W., Yuvaraj S., Hendriks R.W. (2013) Bruton's tyrosine kinase mediated signaling enhances leukaemogenesis in a mouse model for chronic lymphocytic leukaemia. *American Journal of Blood Research*. 3(1):71-83.

Kishi H., Nakagawa K., Matsumoto M., Suga M., Ando M., Taya Y. *et al.*, (2001) shock induces G1 arrest through p53 phosphorylation at ser33 by activating p38MAPK without phosphorylation at ser15 and ser20. *J Biol Chem*. 276:39115–39122.

Kitada S., Andersen J., Akar S., *et al.*, (1998) Expression of apoptosis-regulating proteins in chronic lymphocytic leukaemia: Correlations with in vitro and in vivo chemo responses. *Blood*. 91:3379–3389.

Kitada S., Zapata J.M., Andreeff M., Reed J.C. (1999) Bryostatins and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. *Br J Haematol*. 106:995–1004.

Klein U., Tu Y., Stolovitzky G.A. *et al.*, (2011) Gene expression profiling of B cell chronic lymphocytic leukaemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med*. 194:1625–38.

Klener P., Andera L., Klener P. *et al.*, (2006) Cell death signalling pathways in the pathogenesis and therapy of haematologic malignancies: overview of apoptotic pathways. *Folia Biol (Praha)*. 52:34–44.

Koarada, S., Yoshifumi, T., Ushiyama, O., Morito, F., Suzuke, N., Ohta, A., Miyake, K., Kimoto, M. and Nagasawa, K. (1999) B cells lacking rp105, a novel B cell antigen, in systemic lupus erythematosus. *Arthritis & Rheumatism*. 42:2593-2600.

Koarada S., Tada Y., Sohma Y., Haruta Y., Suematsu R., Mitamura M., Inoue H., Ehara H., Tokoro Y., Ohta A. and Nagasawa K. (2010) Autoantibody-producing RP105<sup>neg</sup> B cells, from patients with systemic lupus erythematosus, showed more preferential expression of BCMA compared with BAFF-R than normal subjects. *Rheumatology*. 49:662-670.

Koning, A.J., Lum, P.Y., Williams, J.M. and Wright, R. (1993) DiOC6 staining reveals organelle structure and dynamics in 177 living yeast cells. *Cell motility and the cytoskeleton*. 25(2):111–128.

Korus M., Mahon G.M., Ceng L. and Whitehead I. P. (2002) p38 MAPK-mediated activation of NF- $\kappa$ B by the RhoGEF domain of Bcr. *Oncogene*. 21:4601-4612.

Kostareli E., Gounari M., Agathangelidis A. and Stamatopoulos (2012) Immunoglobulin gene repertoire in chronic lymphocytic leukaemia: Insight into antigen selection and microenvironmental interactions. *Mediterr J Hematol Infect Dis*. 2035-3006.

Kristinsson S.Y., Bjorkholm M., Goldin L.R., McMaster M.L., Turesson I., Landgren O. (2008) Risk of lymphoproliferative disorders among first-degree relatives of lymphoplasmacytic lymphoma/ Waldenstrom macroglobulinemia patients: a population-based study in Sweden. *Blood*. 112:3052–3056.

Kristinsson S.Y., Dickman P.W., Wilson W.H., Caporaso N., Bjorkholm M., Landgren O. (2009) Improved survival in chronic lymphocytic leukaemia in the past decade: a population based study including 11,179 patients diagnosed between 1973-2003 in Sweden. *Haematologica*. 94(9):1259-65.

Krober A., Bloehdorn J., Hafner S., Buhler A., Seiler T., Kienle D., Winkler D., Bangerter M., Schlenk R.F., Benner A., Lichter P., Dohner H. and Stilgenbauer (2006) Additional genetic high-risk features such as 11q deletion, 17p deletion and V3-21 usage characterize discordance of ZAP-70 and V<sub>H</sub> mutational status in chronic lymphocytic leukaemia. *J Clin Oncol*. 24:969-975.

Krober A., Seiler T., Benner A., Bullinger L., Bruckle E., Lichter P., Dohner H. and Stilgenbauer S. (2002) V<sub>H</sub> mutation status, CD38 expression level, genomic aberrations and survival in chronic lymphocytic leukaemia. *Blood*. 100:1410-1416.

Kumar S., McDonnell PC., Gum RJ., Hand AT., Lee JC. and Young PR. (1997) Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochemical Biophysical Research Communication*. 235:533–538.

Kumar C. C., Madison V. (2005) Akt crystal structure and Akt-specific inhibitors. *Oncogene*. 24:7493–501.

Kurosaki T. (1999) Genetic analysis of B cell antigen receptor signaling. *Annu Rev Immunol*. 17:555-592.

Kurosaki T. (2005) Vav: a newcomer in innate receptor signalling. *Blood*. 106:635.

Lamba V. Lamba J. Yasuda K., Strom S., Davila J., Hancock M.L. *et al.*, (2003) Hepatic CYP2B6 expression: gender and ethnic differences and relationship to CYP2B6 genotype and CAR (constitutive androstane receptor) expression. *J Pharmacol Exp Ther*. 307(3):906-22.

Lampert I.A., Wotherspoon A., Van Noorden S., Hasserjian R.P. (1999) High expression of CD23 in the proliferation centers of chronic lymphocytic leukaemia in lymph nodes and spleen. *Hum Pathol*. 30:648.

Landgren O., Gridley G., Check D., Caporaso N.E., Morris B.L. (2007) Acquired immune-related and inflammatory conditions and subsequent chronic lymphocytic leukaemia. *British Journal of Haematology*. 139:791–798.



Landgren O., Rapkin J.S., Caporaso N.E., Mellekjær L., Gridley G., Goldin L.R., Engels E.A. (2007) Respiratory tract infections and subsequent risk of chronic lymphocytic leukaemia. *Blood*. 109:2198–2201.

Lannutti B.J., Meadows S.A., Herman S.E.M., Kashishian A., Steiner B., Johnson A.J., Byrd J.C., Tyner J.W., Loriaux M.M., Deininger M., Druker B.J., Puri K.D., Ulrich R.G. and Giese N.A. (2011) CAL-101, a p110 $\delta$  selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signalling and cellular viability. *Blood*. 117:591-594.

Lau C. M., Broughton C., Tabor A. S., Akira S., Flavell R. A., Mamula M. J., Christensen S. R. *et al.*, (2005) RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J. Exp. Med.* 202:1171–1177.

Leadbetter E. A., Rifkin I. R., Hohlbaum A. M., Beaudette B. C., Shlomchik M. J. and Marshak-Rothstein A. (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature*. 416:603–607.

Leseux L., Hamdi S.M., Al Saati T., Capilla F., Recher C., Laurent G., Bezombes C. (2006) Syk-dependent mTOR activation in follicular lymphoma cells. *Blood*. 108:4156–4162.

Letai A. (2005) Pharmacological manipulation of Bcl-2 family members to control cell death. *J Clin Invest*. 115:2648–2655.

Levy V., Nataf J., Zompi S., Thomas F., Brouzes C., Casadevall N., Hallek M., Delmer, A., Ajchenbaum-Cymbalista F. (1999) Prognostic significance of serum thymidine kinase levels and p27KIP1 expression in chronic lymphocytic leukaemia. *Blood*. 94:321a.

Lindsley C.W., Zhao Z., Leister W.H., Robinson R.G., Barnett S.F., Defeo-Jones D., Jones R.E., Hartman G.D., Huff J.R., Huber H.E. and Duggan M.E. (2005) Allosteric AKT (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. *Bioorganic & Medicinal chemistry letters*. 15:761-764.

Lioni M., Noma K., Snyder A., Klein-Szanto A., Diehl J.A., Rustgi A.K., *et al.*, (2008) Bortezomib induces apoptosis in esophageal squamous cell carcinoma cells through activation of the p38 mitogen-activated protein kinase pathway. *Mol Cancer Ther*. 7:2866–75.

Liu H., Perlman H., Pagliari L.J. and Pope R.M. (2001) Constitutively activated Akt-1 is vital for the survival of human monocyte-differentiated macrophages: Role of Mcl-1, Independent of Nuclear factor (NF)-Kb, Bad, or caspase activation. *J.Exp. Med.* 113-125.

Liu Y. J., Cairns J.A., Holder M.J., Abbot S.D., Jansen K.U., Bonnefoy J.Y., Gordon J., and MacLennan I. C. M. (1991) Recombinant 25-kDa CD23 and interleukin 1 $\alpha$  promote the survival of germinal center B cells: evidence for bifurcation in the development of centrocytes rescued from apoptosis. *Eur. J. Immunol*. 21:1107-1114.

Logie L., Ruiz-Alcaraz A.J., Keane M., Woods Y.L., Bain J., Marquez R., *et al.*, (2007) Characterization of a protein kinase B inhibitor in vitro and in insulin treated liver cells. *Diabetes*. 56:2218-27.

Longo P.G., Laurenti L., Gobessi S., Petlickovski A., Pelosi M., Chiusolo P., *et al.*, (2007) The Akt signaling pathway determines the different proliferative capacity of chronic lymphocytic leukaemia B-cells from patients with progressive and stable disease. *Leukaemia*. 21:110-20.

Longo P.G., Laurenti L., Gobessi S., Sica S., Giuseppe L. and Efremov D. G. (2008) The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukaemia B cells. *Blood*. 111: 846-855.

- Lopez-Matas M., Rodriguez-Justo M., Morilla R., Catovsky D. and Matutes E. (2000) Quantitative expression of CD23 and its ligand CD21 in chronic lymphocytic leukaemia. *Haematologica*. 85:1140-1145.
- Luo Y., Shoemaker A.R., Liu X., Woods K.W., Thomas S.A., de Jong R., *et al.*, (2005) Potent and selective inhibitors of Akt kinases slow the progress of tumours *in vivo*. *Mol Cancer Ther*. 4:977-86.
- Luxembourg A.T., Cooper N.R. (1994) Modulation of signalling via the B cell antigen receptor by CD21, the receptor for C3 $\delta$  and EBV. *J Immunol*. 153:4448-57.
- Maas A. & R.W. Hendriks. (2001) Role of Bruton's tyrosine kinase in B cell development. *Development Immunology*. 8:171–181.
- Macias-Perez. I.M. and I. W. Flinn. (2013) GS-1101: a delta-specific PI3K inhibitor in chronic lymphocytic leukaemia. *Current Hematologic Malignancy Reports*, 8(1):22–27.
- Mackay F., Schneider P., Rennert P., Browning J. (2003) BAFF and APRIL: a tutorial on B cell survival. *Annu Rev Immunol*. 21:231–64.
- MacLennan I., Vinuesa C. (2002) Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. *Immunity*. 17:235–8.
- Maganac C., Porcher R., Davi F., Nataf J., Payelle-Brogard B., Tang R.P., Oppezzo P., Levy V., Dighiero G. and Ajchenbaum-Cymbalista F. (2003) Predictive value of serum thymidine kinase level for Ig-V mutational status in B-CLL. *Leukaemia*. 17:133-137.
- Mansson A., Adner M., Hockerfelt U., Cardell L.O. (2006) A distinct Toll-like receptor repertoire in human tonsillar B cells, directly activated by PamCSK, R-837 and CpG-2006 stimulation. *Immunology*. 118:539-548.
- Marshall A., Niiro H., Yun T. and Clark E. (2000) Regulation of B-cell activation and differentiation by the phosphatidylinositol 3-kinase and phospholipase C gamma pathway. *Immunol Rev*. 176:30–46.
- Matthew C., Catherwood M.A., Morris T.C.M., Kettle P.J., Drake M.B., Gilmore W.S., Alexander H.D. (2006) Serum TK levels in CLL identify Binet stage A patients within biologically defined prognostic subgroups most likely to undergo disease progression. *Eur J Haematol*. 77:309-317.
- Matthews C., Catherwood M.A., Morris T.C.M., Kettle P.J. Levy V, Nataf J, Zompi S, Thomas F, Brouzes C, Casadevall N, Hallek M, Delmer, A, Ajchenbaum-Cymbalista F. (1999) Prognostic significance of serum thymidine kinase levels and p27KIP1 expression in chronic lymphocytic leukaemia. *Blood*. 94:321a.
- Maurer U., Charvet C., Wagman A.S., Dejardin E., Green D.R. (2006) Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell*. 21:749-60.
- Messmer B.T., Messmer D., Allen S.L., Kolitz J.E., Kudalkar P., Cesar D., Murphy E.J., Koduru P., Ferrarini M., Zupo S., Cutrona G., Damle R.N., Wasil T., Rai K.R., Hellerstein M.K. and Chiorazzi N. (2005) In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukaemia B cells. *The journal of clinical investigation*. 115(3):755-764.
- Mestrallet F., Sujobert P., Sarkozy C., Traverse-Glehen A., Callet-Bauchu E., Magaud J., Salles G. and Baseggio L. (2016) CD180 Overexpression in Follicular Lymphoma Is Restricted to the Lymph Node Compartment. *Cytometry Part B (Clinical Cytometry)*. 433-439.

Miguet S., Dopfer E.P., Pollmer C., Freudenberg M.A., Galanos C., Reth M., Huber M. and Schamel W.W. (2008) Enhanced B-cell activation mediated by TLR4 and BCR crosstalk. *European Journal of Immunology*. 38:2475-2487.

Miguet L., Lennon S., Baseggio L., Traverse-Glehen A., Berger F., Perrusson N., Chenard M.P., Galois A.C., Eischen A., Mayeur-Rousse C. *et al.*, (2013) Cell-surface expression of the TLR homolog CD180 in circulating cells from splenic and nodal marginal zone lymphomas. *Leukaemia*. 27:1748–1750.

Miura Y., Shimazu R., Miyake K., Akashi S., Ogata H., Yamashita Y., Narisawa Y. and Kimoto M. (1998) RP105 is associated with MD-1 and transmits an activation signals in Human B cells. *Blood*. 92:2815-2822.

Miyake, K., Yamashita, Y., Hitoshi, Y., Takatsu, K. and Kimoto, M. (1994) Murine B cell proliferation and protection from apoptosis with an antibody against a 105-kD Molecule: unresponsiveness of X-linked Immunodeficient B Cells. *J.Exp.Med*, 180:1217-1224.

Mizuno, T. and Rothstein, T.L. (2005) B Cell Receptor (BCR) Cross-Talk: CD40 engagement creates an alternate pathway for BCR signaling that activates I $\kappa$ B Kinase/I $\kappa$ Ba/NF- $\kappa$ B without the need for PI3K and phospholipase C $\gamma$ . *J Immunol*. 147:6062-6070.

Molica S, Digiesi G, Mauro F, *et al.*, (2009) Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukaemia. *Leuk Res*. 33:162–165.

Molica S. and Alberti A. (1987) Prognostic value of the lymphocyte doubling time in chronic lymphocytic leukaemia. *Cancer*. 60:2712-2716.

Montesinos-Rongen M., Godlewska E., Brunn A., Wiestler O.D., Siebert R. and Deckert M. (2011) Activating L265P mutations of the MYD88 gene are common in primary central nervous system lymphoma. *Acta Neuropathol*. 122:791–792.

Montillo M., Cafro A.M., Tedeschi A., *et al.*, (2002) Safety and efficacy of subcutaneous Campath-1H for treating residual disease in patients with chronic lymphocytic leukaemia responding to fludarabine. *Haematologica*. 87:695-700.

Moore P.A., Belvedere O., Orr A. *et al.* (1999) BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science*. 285:260–3.

Mossalayi M. D., Lecron J.C., Dalloul A.H., Sarfati M., Bertho J.M., Hofstetter H., Delespesse G. and Debr P.W. (1990) Soluble CD23 (FcERII) and interleukin 1 synergistically induce early human thymocyte maturation. *J. Exp. Med*. 171:959-964.

Mossayali M. D., Arock M., Bertho J. M., Blanc C., Dalloul A.H., Hofstetter H., Sarfati M., Delespesse G. and Debr P. (1990) Proliferation of early human myeloid precursors induced by interleukin-1 and recombinant soluble CD23. *Blood*. 75:1924-1927.

Muller B., Wirth T., and Brunner C. (2004) Role of Bruton's tyrosine kinase in innate and adaptive immunity. *Recent Res. Devel. Experimental Med*. 121-5.

Muller C., Murawski N., Wiesen M.H., Held G., Poeschel V., Zeynalova S. *et al.*, (2012) The role of sex and weight on rituximab clearance and serum elimination half-life in elderly patients with DLBCL. *Blood*. 119(14):3276-84.

Muzio M., Scielzo C., Bertilaccio M.T., Frenquelli M., Ghia P., Caligaris-Cappio F. (2009) Expression and function of toll like receptors in chronic lymphocytic leukaemia cells. *Br J Haematol*. 144(4):507-516.

Muzio M., Apollonio B., Scielzo C., Frenquell M., Vandoni I., Boussiotis V., Caligaris-Cappio F. and Ghia P. (2008) Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood*. 112(1):188-95.

Muzio M., Fonte E. and Caligaris-Cappio F. (2012) Toll-like Receptors in Chronic Lymphocytic Leukaemia. *Mediterr J Hematol Infect Dis*. 4(1).

Myhrinder A.L., Hellqvist E., Sidorova E., Söderberg A., Baxendale H., Dahle C., Willander K., Tobin G., Bäckman E., Söderberg O., Rosenquist R., Hörkkö S., Rosén A.(2008) A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukaemia antibodies. *Blood*. 111:3838-3848.

Nagai Y., Kobayashi T., Motoi Y., Ishiguro K., Akashi S., Saitoh S., Kusumoto Y., Kusumoto Y., Akira S., Matsumoto M., Takatsu K. and Miyake K. (2005) The Radioprotective 105/MD-1 Complex Links TLR2 and TLR4/MD-2 in Antibody Response to Microbial Membranes. *The Journal of Immunology*. 174:7043-7049.

Nagai Y., Shimazu R., Ogata H., Akashi S., Sudo K., Yamasaki H., Hayashi S., Iwakura Y., Kimoto M. and Miyake K. (2002) Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood*. 99:1699-1705.

Nagata Y., Takahashi N., Davis R.J. and Todokoro K. (1998) Activation of p38 MAP Kinase and JNK But Not ERK Is Required for Erythropoietin-Induced Erythroid Differentiation. *Blood*. 92(6): 1859-1869.

Nakayama M, Nakayama A, van Lessen M, Yamamoto H, Hoffmann S, Drexler HCA, et al: Spatial regulation of VEGF receptor endocytosis in angiogenesis (2013) *Nat Cell Biol*. 15: 249–260.

Negro R., et al. (2012) Overexpression of the autoimmunity-associated phosphatase PTPN22 promotes survival of antigen-stimulated CLL cells by selectively activating AKT. *Blood*. 119:6278–87.

Ngo V.N., Young R.M., Schmitz R., Jhavar S., Xiao W., Lim K.H., Kohlhammer H., Xu W., Yang Y., Zhao H., Shaffer A.L., Romesser P., Wright G., Powell J., Rosenwald A., Muller-Hermelink H.K., Ott G., Gascoyne R.D., Connors J.M., Rimsza L.M., Campo E., Jaffe E.S., Delabie J., Smeland E.B., Fisher R.I., Braziel R.M., Tubbs R.R., Cook J.R., Weisenburger D.D., Chan W.C., Staudt L.M. (2010) Oncogenically active MYD88 mutations in human lymphoma. *Nature*. 470(7332):115-119.

Niedermeier M., Hennessy B.T., Knight A.A., Henneberg M., Hu J., Kurtova A.V., Wierda W.G., Keating M.J., Shokat K.M. and Burger J.A. (2009) Isoform-selective phosphoinositide 3'-kinase inhibit CXCR4 signaling and overcome stromal cell-mediated drug resistance in chronic lymphocytic leukaemia: a novel therapeutic approach. *Blood*. 113:5549-5557.

Novak A.J., Grote D.M., Ziesmer S.C., et al., (2006) Elevated serum B-lymphocyte stimulator levels in patients with familial lymphoproliferative disorders. *J Clin Oncol*. 24:983–987.

Ntoufa, S., Vardi, A., Papakonstantinou, N., Anagnostopoulos A., Aleporou-Marinou, V., Belessi, C., Ghia, P., Caligaris-Cappio, F., Muzio, M. and Stamatopoulos, K. (2012) Distinct Innate Immunity Pathways to Activation and Tolerance in Subgroups of Chronic Lymphocytic Leukaemia with Distinct Immunoglobulin Receptors. *Mol Medicine*. 18:1281-1291.

- O'Brien M.A. and Kirby R. (2008) Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and desregulation in disease. *J Vet Emerg Crit Care*. 18(6):572-585.
- O'Brien S. (2012) Small lymphocytic lymphoma and chronic lymphocytic leukaemia: are they the same disease? *Cancer J*. 18(5):396-403.
- O'Connor B.P., Raman V.S., Erickson L.D. *et al.* (2004) BCMA is essential for the survival of long-lived bone marrow plasma cells. *J Exp Med*. 199:91–8.
- Ogata H., Su I., Miyake K. *et al.* (2000) The Toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *J Exp Med*.192:23-29.
- Oppezzo P., Vuillier F., Vasconcelos Y., Dumas G., Magnac C., Payelle-Brogard B. *et al.*, (2003) Chronic lymphocytic leukaemia B cells expressing AID display a dissociation between class switch recombination and somatic hypermutation. *Blood*. 9:9.
- Oppezzo, P. and Dighiero, G. (2013) Role of the B-cell receptor and the microenvironment in chronic lymphocytic leukaemia. *Blood cancer journal*. 3:149.
- Oren M. and Rotter V. (1999) Introduction: p53—the first twenty years. *Cell Mol Life Sci*. 55:9-11.
- Orimo A., Gupta P.B., Sgroi D.C., Arenzana-Seisdedos F., Delaunay T., Naeem R., Carey V.J., Richardson A.L. and Weinberg R.A. (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumour growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 121:335–348.
- Oscier D.G., Gardiner A.C., Mould S.J. *et al.*, (2002) Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of p53 gene are independent prognostic factors. *Blood*. 100:1177–1184.
- Oscier D.G., Thompsett A., Zhu D. and Stevenson F.K. (1997) Differential rates of somatic hypermutation in VH genes among subsets of chronic lymphocytic leukaemia defined by chromosomal abnormalities. *Blood*. 89(11):4153-4160.
- Otipoby, K.L., Nagai, Y., Shu, G.L., Miyake, K. and Clark E.A. (2002) CD180 (RP105/Bgp95) workshop report. In: Leukocyte Typing VII. White Cell Differentiation Antigens (ed. by D.Y. Mason), pp. 120–123. Oxford University Press, Oxford.
- Ozren J., Mirjana M. K. P., Ika K. S., Rajko K., Vlatko P., and Branimir J., (2004) CD38 on B-cell chronic lymphocytic leukaemia cells has higher expression in lymph nodes than in peripheral blood or bone marrow. *Blood*. 103(5):1968-1969.
- Parikh S. A., Rabe K.G., Kay N. E., Call T.G., Ding W., Schwager S.M., Bowen D. A., Conte M., Jelinek D.F., Slager S.L. and Shanafelt T.D. (2014) Chronic lymphocytic leukaemia in young ( $\leq 55$  years) patients: a comprehensive analysis of prognostic factors and outcomes. *Haematologica*. 99(1):140-147.
- Park Y., Lee S.W., Sung Y.C. (2002) Cutting edge: CpG DNA inhibits dendritic cell apoptosis by up regulating cellular inhibitor of apoptosis proteins through the phosphatidylinositide-3-OH kinase pathway. *J. Immunol*. 168:5–8.
- Patten P.E., Buggins A.G., Richards J. *et al.*, (2008) CD38 expression in chronic lymphocytic leukaemia is regulated by the tumour microenvironment. *Blood*. 111:5173–81.
- Pedersen, I.M., Kitada, S., Schimmer, A., Kim, Y., Zapata, J.M., Charboneau, L., Rassenti, L., Andreeff, M., Bennett, F., Sporn, M.B., Liotta, L.D., Kipps, T.J. and Reed,

- J.C. (2002) The triterpenoid CDDO induces apoptosis in refractory CLL B cells. *Blood*. 100 (8):2965-2972.
- Peng, S.L. (2005) Signaling in B cells via Toll-like receptors. *Curr Opin Immunol*. 17(3): 230–236.
- Petlickovski, A., Laurenti, L., Li, X. Chiusolo, P., Sica, S., Leone, G. and, Efremov, D.G. (2005) Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukaemia B cells. *Blood*. 105:4820–7.
- Petro J.B., Khan W.N. (2001) Phospholipase C-gamma 2 couples Bruton's tyrosine kinase to the NF-kappa B signaling pathway in B lymphocytes. *J Biol Chem*. 276:1715-9.
- Petro J.B., Rahman S.M.J., Ballard D.W. and Khan W. N. (2000) Bruton's Tyrosine Kinase Is Required for Activation of Ikb Kinase and Nuclear Factor kB in Response to B Cell Receptor Engagement. *J.Exp.Med*. 174:1745-1753.
- Piatelli M.J., Wardle C., Blois J., Doughty C., Schram B.R., Rothstein T.L. and Chiles T.C. (2004) Phosphatidylinositol 3-Kinase-Dependent Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase 1/2 and NF-B Signaling Pathways Are Required for B Cell Antigen Receptor-Mediated Cyclin D2 Induction in Mature B Cells. *The Journal of Immunology*. 172:2753-2762.
- Poeta G.D., Maurillo L., Venditti A., Buccisano F., Epiceno A.M., Capelli G., Tamburini A., Suppo G., Battaglia A., Principe M.I.D., Moro B.D., Masi M., and Amadori S. (2001) Clinical significance of CD38 expression in chronic lymphocytic leukaemia. *Blood*. 98: 2633-2639.
- Ponader, S., Chen, S.S., Buggy, J.J. Balakrishnan, K., Gandhi, V., Wierda, W.G., Keating, M.J., O'Brien, S., Chiorazzi, N. and Burger, J.A. (2012) The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukaemia cell survival and tissue homing *in vitro* and *in vivo*. *Blood*. 119(5):1182–1189.
- Porakishvili N., Kulikova N., Jewell A. P., Youinou P.Y., Yong K., Nathwani A., Heelan B., Duke V., Hamblin T.J., Wallace P., Ely P., Clark E.A. and Lydyard P.M. (2005) Differential expression of CD180 and IgM by B-cell chronic lymphocytic leukaemia cells using mutated and unmutated immunoglobulin VH genes. *British journal of Haematology*. 131:313-319.
- Porakishvili N., Memon A., Atik C., Clark E., Kulikova N., Natwani A., Kaufman M.S., Rai K.R., Damle R.N., Chiorazzi N. and Lydyard P.M. (2009) IWCLL meeting, Barcelona, *Hematologica*. 94(s3):S37.
- Porakishvili N., Memon A., Vispute K., Clark E.A., Rai K.R., Nathwani A., Damle R.N., Chiorazzi N. and Lydyard P. (2011) CD180 functions in activation, survival and cycling of B chronic lymphocytic leukaemia cells. *British journal of Haematology*. 153:486-498.
- Porakishvili N., Vispute K., Steele A.J., Rajakaruna N., Kulikova N., Tsertsvadze T., Nathwani A., Dample R.N., Clark E.A., Rai K.R., Ciorazzi N., and Lydyard P.M. (2015) Rewiring of sIgM-Mediated Intracellular Signaling through the CD180 Toll-like Receptor. *Molecular Medicine*. 21(1):46-57
- Porter D. L., Levine B. L., Kalos M., Bagg A. and June C. H. (2011) Chimeric Antigen Receptor-Modified T Cells In Chronic Lymphoid Leukaemia. *N Engl J Med*. 365:725-33.
- Puente X.S., Pinyol M., Quesada V., Conde L., Ordonez G.R., *et al.*, (2011) Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 7;475(7354):101-105.

Rai K.R., Peterson B.L., Appelbaum F.R., *et al.*, (2000) Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukaemia. *N Engl J Med.* 343:1750-1757.

Rai K.R., Peterson B.L., Appelbaum F.R., Kolitz J., Elias L., Shepherd L., Hines J., Threatte G.A., Larson R.A., Cheson B.D., Schiffer C.A. (2000) Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukaemia. *The New England Journal of medicine.* 343:1750-1757.

Rai K.R., Sawitsky A., Cronkite E.P., Chanana A.D., Levy R.N., Pasternack B.S. (1975) Clinical staging of chronic lymphocytic leukaemia. *Blood.* 46(2):219-34.

Rai, K. R. (1997) Chronic lymphocytic leukaemia in the elderly population. *Clin Geriatr Med.* 13:245.

Rawlings D.J., *et al.*, (1993) Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science.* 261:358–361.

Reinisch W, Willheim M, Hilgarth M, *et al.*, (1994) Soluble CD23 reliably reflects disease activity in B-cell chronic lymphocytic leukaemia. *J Clin Oncol.* 12:2146-2149.

Reis-Sobreiro M., Roue G., Moros A., Gajate C., de la Iglesia-Vincente J., Colomer D. and Mollinedo F. (2013) Lipid raft-mediated Akt signalling as a therapeutic target in mantle cell lymphoma. *Blood Cancer Journal.* 3(5).

Reynolds J.M., Martinez G.J., Chung Y. and Dong C. (2012) Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *PNAS.* 109:13064-13069.

Ringshausen I., Schneller F., Bogner C., Hipp S., Duyster J., Peschel C., Decker T. (2002) Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood.* 100(10):3741-3748.

Robak T., Blonski J.Z., Kasznicki M., Blasinska-Morawiec M., Krykowski E., Dmoszynska A., Mrugala-Spiewak H., Skotnicki A.B., Nowak W., (2000) Cladribine with prednisone versus chlorambucil with prednisone as first-line therapy in chronic lymphocytic leukaemia: report of a prospective, randomized, multicentre trial. *Blood.* 96:2723-2729.

Robertson L.E., Plunkett W., McConnell K., Keating M.J., McDonnell T.J. (1996) Bcl-2 expression in chronic lymphocytic leukaemia and its correlation with the induction of apoptosis and clinical outcome. *Leukaemia.* 10:456.

Rosén A., Murray F., Evaldsson C., Rosenquist R. (2010) Antigens in chronic lymphocytic leukaemia – implications for cell origin and leukaemogenesis. *Semin. Cancer Biol.* 20:400-409.

Rosenwald A., Alizadeh A.A., Widhopf G., Simon R., Davis R.E., Yu X. *et al.*, (2001) Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukaemia. *J Exp Med.* 194:639–1647.

Roshak, A.K., Anderson, A.M., Holmes, S.D., Jonak, Z., Bolognese, B., Terrett, J., and Marshall, L.A. (1999) anti-human RP105 sera induces lymphocyte proliferation. *Journal of Leukocyte Biology.* 65:43-49.

Rouby S.E., Thomas A., Costin D., Rosenberg C.R., Potmesil M., Silbe R. and Newcomb E.W. (1993) p53 gene mutation in B-cell chronic lymphocytic leukaemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood.* 82(11):3452-3459.

Rozkova D., Novotna L., Pytlik R., Hochova I., Kozak T., Bartunkova J., Spisek R. (2010) Toll-like receptors on B-CLL cells: expression and Functional consequences of their stimulation. *Int J Cancer*. 126(5):1132-1143.

Ruan J., Hyjek E., Kermani P., Christos P.J., Hooper A.T., Coleman M., Hempstead B., Leonard J.P., Chadburn A. and Rafii S. (2006) Magnitude of stromal hemangiogenesis correlates with histologic subtype of non-Hodgkin's lymphoma. *Clin Cancer Res*. 12: 5622– 5631.

Sabattini E., Orduz r., Campidelli C., Zinzani P.L., Callea V., Zupo S., Cutrona G., Morabito F., Ferrarini M. and Pileri S. (2007) B cell chronic lymphocytic leukaemia/small lymphocytic lymphoma: role of ZAP-70 determination on bone marrow biopsy specimens. *J Clin Pathol*. 60:627-632.

Sainz-Perez a., Gary-Gouy H., Portier A., Davi F., Merle-Beral H., Galanaud P. and Dalloul A. (2006) High Mda-7 expression promotes malignant cell survival and p38 MAP kinase activation in chronic lymphocytic leukaemia, *Leukemia*. 20:498-504.

Sandoval A., Consoli U. and Plunkett W. (1996) Fludarabinemediated inhibition of nucleotide excision repair induces apoptosis in quiescent human lymphocytes. *Clin Cancer Res*. 2:1731-1741.

Santos F.P., O'Brien S., Almasri, N.M., Duque, R.E., Iturraspe, J., Everett, E. & Braylan, R.C. (1992) Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukaemia. *American Journal of Hematology*. 40:259–263.

Sarfari M., Chevret S., Chastang C., Biron G., Stryckmans P., Delespesse G., Binet J., Merie-Beral H. and Bron D. (1996) Prognostic importance of serum soluble CD23 levels in chronic lymphocytic leukaemia. *Blood*. 88:4259-4264.

Sarfati M., and Delespesse G. (1988) Possible role of FcERII in the regulation of human IgE synthesis. *J. Immunol*. 41:2195-2199.

Sarkar D., Su Z., Lebedeva I.V., Sauane M., Gopalkrishnan R.V., Valerie K., Dent P. and Fisher P.B. (2002) mda-7 (IL-24) mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *PNAS*. 99:10054-10059.

Sarkar D., Su Zao-zhong., Lebedeva I.V., Sauane M., Gopalkrishnan R. V., Dent P. and Fisher P.B. (2002) *mda-7* (IL-24): Signaling and Functional Roles. *Bio Techniques*. 33:S39-S39.

Saxena, N.K., Titus, M.A., Ding, X., Floyd, J., Srinivasan, S., Sitaraman, S.V. and Anania, F.A. (2004) Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. *The FASEB journal*. 18(13):1612–1614.

Schattner E. J. (2000) CD40 ligand in CLL pathogenesis and therapy. *Leuk Lymphoma*. 37:461-472.

Schneider P, MacKay F, Steiner V et al. (1999) BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*. 189:1747–56.

Schroers R., Griesinger F., Trumper L., Haase D., Kulle B., Klein-Hitpass L., Sellmann L., Duhrsen U., and Dung J. (2005) Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukaemia. *Leukaemia*. 19:750-758.



Scielzo C., Apollonio B., Scarfo L., Janus A., Muzio M., Ten Hacken E. *et al.*, (2011) The functional *in vitro* response to CD40 ligation reflects a different clinical outcome in patients with chronic lymphocytic leukaemia. *Leukaemia*. 25:1760–1767.

Scuppoli, M.T. and Pizzolo, G. (2012) Signaling pathways activated by the B cell receptor in chronic lymphocytic leukaemia. *Expert Rev. Hematol.* 5(3):341–348.

Shanafelt T.D., Witzig T.E., Fink S.R., Jenkins R.B., Paternoster S.F., Smoley S.A., Stockero K.J., Nast D.M., Flynn H.C., Tschumper R.C., Geyer S., Zent C.S., Call T.G., Jelinek D.F., Kay N.E. and Dewald G.W. (2006) Prospective evaluation of clonal evaluation during long term follow-up of patients with untreated early-stage chronic lymphocytic leukaemia. *J Clin Oncol.* 24:4634-4641.

Shi Y., Liu X., Han E.K., Guan R., Shoemaker A.R., Oleksijew A., *et al.*, (2005) Optimal classes of chemotherapeutic agents sensitized by specific small-molecule inhibitors of akt *in vitro* and *in vivo*. *Neoplasia*. 7:992-1000.

Siebert R., Deckert M. (2011) Activating L265P mutations of the MYD88 gene are common in primary central nervous system lymphoma. *Acta Neuropathol.* 122(6):791-792.

Slupsky J.R. (2014) Does B Cell Receptor Signalling in Chronic Lymphocytic Leukaemia Cells Differ from That in Other B Cell Types? *Blood*. 118:4313-4320.

Sorrer M.L., Storer B.E., Maloney D.G., Sandmaier B.M., Martin P.J., Storb R. (2008) Outcomes after allogeneic hematopoietic cell transplantation with nonmyeloablative or myeloablative conditioning regimens for treatment of lymphoma and chronic lymphocytic leukaemia. *Blood*. 111:446-52.

Spaargaren M., Beuling E.A., Rurup M.L., Meijer H.P., Klok M.D., Middendorp S. *et al.*, (2003) The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J Exp Med.* 198: 1539–1550.

Spaner D.E., Shi Y., White D., Mena J., Hammond C., Tomic J., He L., Tomai M.A., Miller R.L., Booth J., Radvanyi L. (2006) Immunomodulatory effects of Toll-like receptor-7 activation on chronic lymphocytic leukaemia cells. *Leukaemia*. 20(2):286-295.

Spaner D.E., Shi Y., White D., Mena J., Hammond C., Tomic J., He L., Tomai M.A., Miller R.L., Booth J., Radvanyi L. (2005) Immunomodulatory effects of Toll-like receptor-7 activation on chronic lymphocytic leukaemia cells. *Leukaemia*. 20(2):286-295.

Spat A, Hunyady L: Control of aldosterone secretion: a model for convergence in cellular signaling pathways (2004) *Physiol Rev.* 84: 489–539.

Srinivasan L., Sasaki Y., Calado D., Zhang B., Paik J., DePinho R. *et al.*, (2009) PI3 kinase signals BCR-dependent mature B cell survival. *Cell*. 139:573–586.

Stamatopoulos K., Belessi C., Moreno C., Boudjograh M., Guida G., Smilevska T., Belhoul, L., Stella S., Stavroyianni N., Crespo M., Hadzidimitriou A., Sutton L., Bosch F., Laoutaris N., Anagnostopoulos A., Montserrat E., Fassas A., Dighiero G., Caligaris-Cappio F., Merle-Beral H., Ghia P. and Davi, F. (2007) Over 20% Of Patients with Chronic Lymphocytic Leukaemia Carry Stereotyped Receptors: Pathogenetic Implications And Clinical Correlations. *Blood*. 109:259-70.

Stevenson, F.K., Krysov, S., Davies, A.J., Steele, A.J. and Packham, G. (2011) B-cell receptor signaling in chronic lymphocytic leukaemia. *Blood*. 118:4313-4320.

Stilgenbauer S., Bullinger L., Benner A., Wildenberger K., Bentz M., Dohner K., HoA.D., Lichter P. and Dohner H. (1999) Incidence and clinical significance of 6q deletions in B cell chronic lymphocytic leukaemia. *Leukaemia*. 13(9):1331-4.

Sturm I., Bosanquet A.G., Hermann S., Guner D, Dorken B. and Daniel P.T. (2003) Mutation of 53 and consecutive selective drug resistance in B-CLL occurs as a consequence of prior DNA-damaging chemotherapy. *Cell death and differentiation*. 1:477-484.

Sutton, L. *et al.*, (2011) Autologous stem cell transplantation as a first-line treatment 186 strategy for chronic lymphocytic leukaemia: a multicenter, randomized, controlled trial from the SFGM-TC and GFLLC. *Blood*. 117(23):6109–6119.

Suzuki H., Terauchi Y., Fujiwara M., *et al.*, (1999) Xid-like immunodeficiency in mice with disruption of the p85a subunit of phosphoinositide 3-kinase. *Science*. 283:390-392.

Swendeman S., and Thorley-Lawson D.A. (1987) The activation antigen BLAST-2, when shed, is an autocrine BCGF, for normal and transformed B cells. *Eur. Mol. Biol. Organ J*. 6:1637-1642.

Takayama Y, May P, Anderson RGW, Herz J: Low density lipoprotein receptor-related protein 1 (LRP1) controls endocytosis and c- CBL-mediated ubiquitination of the platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ) (2005). *J Biol Chem*. 280: 18504–18510.

Takeda, K. and Akira, S. (2004) TLR signaling pathways. *Seminars in Immunology*. 16:3-9.

Takeshita F., *et al.* (2004) Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. *Semin. Immunol*. 16:17–22.

Thomas A., El Rouby S., Reed J.C., *et al.*, (1996) Drug-induced apoptosis in B-cell chronic lymphocytic leukaemia: relationship between p53 gene mutation and bcl-2/bax proteins in drug resistance. *Oncogene*. 12:1055–1062.

Thomas A., El Rouby S., Reed J.C., Krajewski S., Silber R., Potmesil M., Newcomb E.W. (1996) Drug-induced apoptosis in B-cell chronic lymphocytic leukaemia: Relationship between p53 mutation and bcl-2/ bax proteins in drug resistance. *Oncogene*. 12:1055.

Thompson J.S., Schneider P., Kalled S.L. *et al.* (2000) BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population. *J Exp Med*.192:129–35.

Ticchioni M., Essafi M., Jeandel P.Y., Davi F., Cassuto J.P., Deckert M. *et al.*, (2007) Homeostatic chemokines increase survival of B-chronic lymphocytic leukaemia cells through inactivation of transcription factor FOXO3a. *Oncogene*. 26(50):7081-91.

Tromp J. M., Geest C. R., Breij E. C. W. *et al.*, (2011) Tipping the Noxa/Mcl-1 balance overcomes ABT-737 resistance in chronic lymphocytic leukaemia. *Clin Cancer Res*. 18:487-498.

Tsukada S. *et al.*, (1993) Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. *Cell*. 72:279–290.

Tsuneyoshi N., Fukudome K., Kohara J., Tomimasu R., Gauchat J.F., Nakatake H., and Kimoto M. (2005) The functional and structural properties of MD-2 required for lipopolysaccharide binding are absent in MD1. *J. Immunol*. 174: 340–344.

Valentine M.A., Clarke E.A., Shu G.L., Norris N.A., Ledbetter J.A. (1988) Antibody to a novel 95-kda surface glycoprotein on human B cells induces calcium mobilization and B cell activation. *J Immunol.* 140:4071-4078.

Vassilev L.T., Vu B.T., Graves B., Carvajal D., Podlaski F., Filipovic Z., Kong N., Kammlott U., Lukacs C., Klein C., Fotouhi N. and Liu E.A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* 303(5659):844–848.

Vetrie D. *et al.*, (1993) The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature.* 361:226–233.

Wang L., Lawrence M.S., Wan Y., Stojanov P., Sougnez C., Stevenson K., Werner L., Sivachenko A., DeLuca D.S., Zhang L., Zhang W., Vartanov A.R., Fernandes S.M., Goldstein N.R., Folco E.G., Cibulskis K., Tesar B., Sievers Q.L., Shefler E., Gabriel S., Hacohen N., Reed R., Meyerson M., Golub T.R., Lander E.S., Neuberger D., Brown J.R., Getz G., Wu C.J. (2011) SF3B1 and other novel cancer genes in chronic lymphocytic leukaemia. *N Engl J Med.* 365(26):2497- 2506.

Watanabe N., Ikuta K., Nisitani S., *et al.*, (2002) Activation and differentiation of autoreactive b-1 cells by interleukin 10 induce autoimmune hemolytic anemia in Fas-deficient antierythrocyte immunoglobulin transgenic mice. *J Exp Med.* 196(1):141–146.

Wei Y., Fan T., Yu M. (2008) Inhibitor of apoptosis proteins and apoptosis. *Acta Biochim Biophys Sin.* 40(4):278-288.

Wiestner A., Rosenwald A., Barry T.S., Wright G., Davis R.E., Henrickson S.E. *et al.*, (2003) ZAP-70 expression identifies a chronic lymphocytic leukaemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood.* 101:4944–4951.

Wiestner A. (2015) The role of B-cell receptor inhibitors in the treatment of patients with chronic lymphocytic leukaemia. *Haematologica.* 100(12): 1495-1507.

Wilk-Blaszczak M.A., Stein B., Xu S., Barbosa M.S., Cobb M.H. and Belardetti F. (1998) The Mitogen-Activated Protein Kinase p38-2 Is Necessary for the Inhibition of N-Type Calcium Current by Bradykinin. *The journal of Neurosciences.* 18(1):112-118.

Wilson K.P., McCaffrey P.G., Hsiao K., Pazhanisamy S., Galullo V., Bemis G.W., Fitzgibbon M.J., Caron P.R., Murcko M.A. and Su M.S. (1997) The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. *The journal of Biological Chemistry.* 4:423– 431.

Wintersberger E., Rothebader H., Seiser C. (1992) Regulation of thymidine kinase during growth, cell cycle and differentiation. *Adv Enzyme Regul.* 241–254.

Wong R.S.Y. (2011) Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Research.* 30:87.

Woodland R.T., Fox C.J., Schmidt M.R., Hammerman P.S., Opferman J.T., Korsmeyer S.J., Hilbert D.M. and Thompson C.B. (2008) Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood.* 111(2): 750–760.

Woyach J.A., Bojnik E., Ruppert A.S., Stefanovski M.R., Goettl V.M. and Smucker K.A. (2014) Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukaemia (CLL). *Blood.* 123(8):1207-13.

Woyach J.A., Jonson A.J. and Byrd J.C. (2012) The B-cell receptor signalling pathway as a therapeutic target in CLL. *Blood.* 120:1175-1184.

Wu S.J., Chiang C.J., Lin C.T., Tien H.F. and Lai M.S. (2013) Improving but Inferior Survival in Patients with Chronic Lymphocytic Leukaemia in Taiwan: A Population-Based Study, 1990–2004. *PLOS ONE*. 8(4):2930.

Wu S.J., Huang S.Y., Lin C.T., Lin Y.J., Chang C.J., *et al.*, (2010) The incidence of chronic lymphocytic leukaemia in Taiwan, 1986–2005: a distinct increasing trend with birth-cohort effect. *Blood*. 116:4430–4435.

Yamashita, Y., Mlyake, K., Miura, Y., Kaneko, Y., Yagita, H., Suda, T., Nagata, S., Nomura, T., Sakaguchi, N. and Kimoto, M. (1996) Activation Mediated by RP105 but Not CD40 Makes Normal B Cells Susceptible to Anti-IgM-induced Apoptosis: A Role for Fc Receptor Coligation. *Exp. Med.* 184:113-120.

Yan Y., Huang Y., Watkins A.J., Kocalkowski S., Zeng N., Hamoudi R.A., Isaacson P.G., de Leval L., Wotherspoon A., Du M.Q. (2012) BCR and TLR signalling pathways are recurrently targeted by genetic changes in splenic marginal zone lymphomas. *Haematologica*. 97(4):595-8.

Yazawa N., Fujimoto M., Sato S., Miyake K., Asano N., Nagai Y., Takeuchi O., Takeda K., Okochi H. and Akira S. (2003) Cd19 Regulates Innate Immunity By The Toll-Like Receptor Rp105 Signaling In B Lymphocytes. *Blood*. 102:1374-1380.

Yeretssian G., Doiron K., Shao W., Leavitt B.R., Hayden M.R., Nicholson D.W. *et al.*, (2009) Gender differences in expression of the human caspase-12 long variant determines susceptibility of *Listeria monocytogenes* infection. *Proc Natl Acad Sci USA*. 106(22):9016-20.

Yi A.K., Yoon J.G., Krieg A.M. (2003) Convergence of CpG DNA- and BCR mediated signals at the c-Jun N terminal kinase and NF-kappaB activation pathways: regulation by mitogen-activated protein kinases. *Int. Immunol.* 15:577–91.

You M., Dong G., Li F., Ma F., Ren J., Xu Y., Yue H., Tang R., Ren D. and Hou Y. (2015) Ligation of CD180 inhibits IFN- $\alpha$  signaling in a Lyn-PI3K-BTK-dependent manner in B cells. *Cellular & Molecular Immunology*. 1-11.

Young, J.W. *et al.*, (1992) The B7/BB1 antigen provides one of several costimulatory signals for the activation of CD4+ T lymphocytes by human blood dendritic cells in vitro. *Journal of Clinical Investigation*. 90(1):229.

Young P.R., McLaughlin M.M., Kumar S., Kassis S., Doyle M.L., McNulty D., Gallagher T.F., Fisher S., McDonnell P.C., Carr S.A., Huddleston M.J., Seibel G., Porter T.G., Livi G.P., Addams J.L. and Lee J.C. (1997) Pyridinyl Imidazole Inhibitors of p38 Mitogen-activated Protein Kinase Bind in the ATP Site. *The journal of Biological Chemistry*. 272(18):12116-12121.

Zarembek, K.A. and Godowski, P.J. (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocyte responses to microbes, their products and cytokines. *Journal of Immunology*. 168:554–561.

Zenz T., Krober A., Scherer K., Habe S., Buhler A., Benner A., Denzel T., Winkler D., Edelmann J., Schwanen C., Dohner H. and Stilgenbauer S. (2008) Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukaemia: results from a detailed genetic characterization with long-term follow-up. *Blood*. 112:3322-3329.

Zhong F., Harr M.W., Bultynck G., Monaco G., Parys J.B., DeSmedt H., Rong Y.P., Molitoris J.K., Lam M., Ryder C., Matsuyama S., Distelhorst C.W. (2011) Induction of Ca<sup>2+</sup>-driven apoptosis in chronic lymphocytic leukaemia cells by peptide-mediated disruption of Bcl-2–IP3 receptor interaction. *Blood*. 117:2924–2934.

Zhuang, J., Hawkins, S.F., Glenn, M.A., Lin, K., Johnson, G.G., Carter, A., Cawley, J.C., and Pettitt, A.R. (2010) Akt is activated in chronic lymphocytic leukaemia cells and delivers a pro-survival signal: the therapeutic potential of Akt inhibition. *Haematologica*. 95 (1):110–118.

Zoncu R., Efeyan A., Sabatini D.M. (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol*. 12:21–35.

Zubiaur M., Izquierdo M., Terhorst C., Malavasi F., Sancho J. (1997) CD38 ligation results in activation of the Raf-1/mitogen-activated protein kinase and the CD3-zeta/zeta-associated protein-70 signaling pathways in Jurkat T lymphocytes. *J Immunol*. 159 (1):193–205.

Zupo S., Isnardi L., Megna M., *et al.*, (1996) CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukaemias with different responses to anti-IgM antibodies and propensity to apoptosis. *Blood*. 88:1365-1374.

Zupo S., Rugari E., Dono M., Tamborelli G., Malavasi F., Ferrarini M. (1994) CD38 signaling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. *Eur J Immunol*. 24:1218-1222.

Zupo S., Rugari E., Dono M., Tamborelli G., Malavasi F., Ferrarini M., CD38 signaling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. (1994) *Eur J Immunol*. 24:1218-1222.