CD180/RP105 Toll-like Receptor (TLR) mediated signalling in Chronic Lymphocytic Leukaemia

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Abstract

The role of the 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, respectively) represent exogenous and endogenous microenvironmental factors acting 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.

We have previously shown that approximately 60% of CLL samples expressed surface CD180 but only half responded to ligation with anti-CD180 monoclonal antibody (mAb) resulting in activation, cycling, and reduced basal apoptosis and were termed responders (R). In contrast, CD180+CLL samples that failed to respond to anti-CD180 mAb, despite expressing a high density of CD180 receptors, were termed non-responders (NR). We further demonstrated that in R-CLL cells, CD180 ligation significantly induced phosphorylation of ZAP70/Syk, ERK, p38MAPK, and AKT. 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 anergy.

To further clarify the CD180-mediated signalling pathways in CLL, here we studied signal transduction downstream from ZAP70/Syk by delineating CLL samples into R and NR through their proximal ability to activate AKT. We have studied major signalling protein kinases associated with the BCR signalling pathway: PI3K, Btk, ERK, p38MAPK and AKT.

Segregation of CLL samples responding to CD180 ligation by signalling via pAKT, rather than by CD86 upregulation, revealed that CD180 ligation on CLL cells can activate two alternative signalling pathways: pro-survival that operates via PI3K-Btk-AKT protein kinases, or mostly pro-apoptotic, that operates via p38MAPK but not through Btk. This may have implications for CLL therapy where Btk inhibitors are being used.

Here we demonstrate that albeit ligation of sIgM alone also activates pro-survival PI3K-Btk-AKT pathway pre-engagement of CD180 redirected BCR-mediated signalling towards the potentially pro-apoptotic p38MAPK pathway that opens new horizons for immunotherapy.

Since the tissue microenvironment plays a crucial role in generation and survival of the CLL clones, studies pertaining to CD180 expression in the lymphoid tissues were undertaken. Our pilot data suggests that in normal tonsils CD180 is expressed by the mantle zone (MZ) B cells and not the germinal centre (GC) B cells. However in CLL lymph nodes complete obliteration of the normal tissue architecture and a weak expression of CD180 has been detected, whilst expression of CD180 on bone marrow CLL cells was heterogeneous. Since CLL cells migrate to and from the solid tissues into the peripheral circulation in any CLL clone, there is always an intra-clonal kinetic heterogeneity through a suggested continuum between the 'proliferative' or $CXCR4^{dim}CD5^{bright}$, and 'resting' $CXCR4^{bright}CD5^{dim}$ fractions. Here we report that the 'resting' compartment was enriched for CD180+ cells compared to the 'proliferating' subset. In contrast, sIgM+ cells were more frequent in the proliferating fraction. Since the “resting” subset of CLL cells is also considered as the one “returning” to the solid tissues supported by the increased expression of CXCR4, our data might suggest possible attraction of the CD180+ cells towards the putative ligand in the lymphoid tissues.

It is becoming apparent that intraclonal diversity plays an important role in the clinical outcome of patients with CLL. Subsets of the CLL clone that respond more robustly to external stimuli may well gain a growth and survival advantage and possibly promote clonal evolution. Identification of these CLL subpopulations was therefore of prime importance, as these cells may be preferred targets for future therapeutics. We have established that CD180 expression on CLL cells helps identifying different subsets and delineating their physiological status. Our 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. Since TLR can shuttle between inhibition and promotion of leukemic growth they may play a

key role in immune evasion impacting on clinically relevant tumour-host microenvironment interactions. The identification of distinct CD180-mediated signalling pathways that promote tumour cell proliferation and survival will allow specific targeting of key players in the pathways with immunotherapy and chemotherapy.

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List of abbreviations

µl- microlitre

ATM- Ataxia-teleangiectasia mutated gene

BCR- B cell receptor

Btk- Bruton's Tyrosine Kinase

Bcl2- B cell lymphoma 2

CLL- Chronic Lymphocytic Leukaemia

CDR3- Complementarity determining region 3

CMV- Cytomegalovirus

CXCR4- CXC chemokine receptor 4

CXCL12- CXC motif chemokine ligand 12

DAMP- Damage associated molecular pattern

EBV- Epstein Barr Virus

ERK- Extracellular signal regulated kinase

FCγIIB- Fc gamma receptor II B

FCR- Fludarabine, cyclophosphamide, rituximab

FISH- Fluorescent in situ hybridization

GC- Germinal Centre

g/l- gram per litre

Hb- Haemoglobin

Hsp- Heat-shock proteins

HMGB1- High mobility group box protein

Ig- Immunoglobulin

IL-R- Interleukin receptor

IGVH- immunoglobulin heavy chain variable gene

ITAM- immunoreceptor tyrosine-based activation motifs

IWCLL- International Workshop for Chronic Lymphocytic Leukaemia

IFN- interferon

JNK- c-Jun N-terminal kinase

kD- kilo dalton

LPS- lipopolysaccharide

LRR- Leucine Rich repeats

LDH- Lactate dehydrogenase

mAb- monoclonal antibody

MAPK- Mitogen activated protein kinase

MZ- Marginal zone

MZB- Marginal zone B cells

MyD88- myeloid differentiation primary response gene 88

Mcl-1- myeloid cell leukemia-1

mTor- mammalian target of rapamycin

NCI- National Cancer Institute

NFκB- Nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT- Nuclear factor of activated T cells

NK- Natural killer cells

P53- protein 53

PAMP- Pathogen associated molecular pattern

PI3K- phosphatidylinositol 3-kinase

PLCy2-phospholipase C gamma 2

PKC- protein kinase C

PTK- protein tyrosine kinase

RP105- radio protective 105

SDF1- stromal derived factor-I

sIg- surface immunoglobulin

SLE- systemic lupus erythematosus

Syk- spleen tyrosine kinase

TIR- Toll/IL-1 receptor

TLR- Toll like receptor

TNF- tumour necrosis factor

VDJ- variable, diversity, joining

WBC- white blood cell

ZAP70 - zeta-chain associated protein- 70

Table of Contents

Table of Figures

Chapter 1 Literature Review

Immunobiology of CLL

1.1 Chronic Lymphocytic Leukaemia and its occurrence

Chronic Lymphocytic Leukaemia (CLL) is the most common leukaemia in the western world and forms approximately 30% of all adult leukaemias. According to the statistics by the Leukaemia and Lymphoma society, an estimated 105,119 people are living with (or in remission from) CLL in 2011 in the USA alone (http://www.lls.org). Based on the rates of diagnosis from 2007-2009, 1 in 202 men and women will be diagnosed with CLL during their lifetime (http://seer.cancer.gov/statfacts/html/clyl.html). CLL is more common in people of Russian and European descent, and rare in people from China, Japan, or Southeast Asian countries. Some recent studies have however, shown increasing occurrence of CLL in Taiwan (Wu *et al.,*2010). The reason(s) for this geographic difference is not known (www.cancer.net).

The incidence of CLL increases significantly among people aged 50 years and older. The median age at diagnosis is 72 years. The median survival period is approximately 5 years. About 10% of CLL patients are reported to be younger than 55 years (http://www.lls.org). The disease can be aggressive with patients dying relatively soon after diagnosis, or indolent with patients surviving for many years. Older patients may die from other causes, or they may succumb to the consequences of the disease or its many complications (Young *et al.,* 2006). There is also evidence of CLL patients being prone to development of secondary malignancies (Tsimberidou *et al.,* 2009). Approximately 29% of deaths are unrelated to CLL, mainly other cancers (12%), haemolytic anaemia (10%) and cardiovascular complications (16%) (Catovsky *et al.,* 1989). Another common complication affecting CLL patients is called Richter's transformation or Richter's syndrome and affects 5% patients at some point during the course of the disease. Richter's syndrome (RS) is characterized by the development of high-grade non-Hodgkins lymphoma (NHL) in a patient with (CLL). The large cells of RS may arise through transformation of the original CLL clone or represent a new neoplasm. RS

may be triggered by viral infections, such as Epstein-Barr virus (EBV). Trisomy 12 and chromosome 11 abnormalities, as well as multiple genetic defects, have been described in patients with RS. These abnormalities may cause CLL cells to proliferate and, by facilitating the acquisition of new genetic abnormalities, to transform into RS cells (Tsimberidou *et al.,* 2006)

The number of Male (M) patients diagnosed with CLL is almost twice that of Female (F) with an M: F ratio of 1·8: 1. The M: F ratio is lower, 1·5:1, in patients aged 70 or over. Women are more likely to have early-stage disease and, regardless of stage and age, they have a better prognosis than men, but the mechanism(s) responsible for the improved survival in women is unknown. (Catovsky *et al.,*1989; Greer *et al.,* 2009). In the Medical Research Council (MRC), UK CLL clinical trial 1 in a cohort of 660 patients, results reported were: better prognosis CLL (or stage A CLL - detailed further) was the most common, among women of all ages, in contrast to men for whom increased chances of survival with the disease only predominated in the older age group. The majority of deaths in patients presenting with the bad prognosis (Stage C and Stage B-detailed later) were CLL-related. Women always fared better than men and this was independent of stage and age. This and other features documented in the trial suggest a major biological difference between the sexes which has not been widely recognized (Catovsky *et al.,*2008). The cause of CLL is unknown. There is no definitive link to radiation, cancer-causing chemicals, or viruses. Familial CLL cases have been detected, but the pattern of inheritance is yet completely unknown (Greer *et al.,* 2009). A CLL patient with at least one affected relative is considered "familial". It is estimated in some population that 5-10% of the cases may be associated with the familial form of CLL. One in every 10 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). The pattern of immunoglobulin gene usage and the frequency of somatic mutation are similar in familial CLL (Sakai *et al.,* 2000). Importantly monoclonal $CD5^{+}B$ lymphocytes with an immunophenotype very close to that observed in CLL have been found in otherwise healthy adults. Similar phenomenon has been identified in family members of patients. Studies involving CLL cases from multiplex families have

generally shown familial CLL to have an earlier age at diagnosis compared to sporadic CLL. However, larger population-based studies have not found differences in age at diagnosis between sporadic and familial cases (Goldin *et al.,*2010). The genetic aetiology of CLL is unknown and early work on familial CLL has not yet uncovered any obvious gene or group of genes that can be clearly related to the pathophysiology of CLL (Goldin *et al.,*2010). In a small patient cohort based study, increased serum BAFF (B-cell activating factor of the TNF family) level was shown to be associated with familial CLL (Stefano *et al.,*2009).

CLL cells express mostly CD19+ B-lineage phenotype hence the disease is also commonly called B-cell Chronic Lymphocytic leukaemia (CLL) with a very small proportion of CLL cases belonging to T lineage. T-cell CLL is also called 'T-cell Prolymphocytic Leukaemia' and consists of mature T-lymphocytes with immunophenotype of CD2+, CD3+, CD7+ and CD4+/CD8-ve (Hoyer *et al.,*1995). Small lymphocytic leukaemia (SLL) is a form of B-cell lymphoma structurally related to CLL but with a slightly different prognostic aspect. SLL is also be referred to 'CLL in the nodal regions' (Pangalis *et al.,*1999). In a broader perspective, CLL is a heterogeneous disease with a variable clinical course. The treatment varies according to the stage of the disease, age of the patient and the other secondary clinical conditions arising due to the immune dysregulation.

1.2 CLL diagnosis and staging

The World Health Organization classification of hematopoietic neoplasias describes CLL as leukaemic, lymphocytic lymphoma, being only distinguishable from SLL by its leukaemic appearance. The guidelines for the diagnosis and treatment of CLL were revised by the International Workshop on CLL in 2008 (IWCLL). Criteria for CLL are as follows: the presence in the peripheral blood of 5 x 10^9/L monoclonal B lymphocytes for at least 3 months. The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry. Typical immunophenotype of CLL lymphocyte is $CD5^+$, $CD23^+$, $CD43^{+/-}$, $CD10^-$, $CD19^+$. Dim expression of CD20 and surface immunoglobulin is highly characteristic of CLL and this can be useful in distinguishing from mantle cell lymphoma, especially in those rare cases that lack expression of CD23. Bone marrow examination is not required

for diagnosis and a CT scan not required for staging, but flow cytometry is crucial for correct diagnosis (Hallek *et al.,*2008). In most patients in the earlier stages, there are no distinct symptoms and the diagnosis is more or less 'by chance' sometimes following a routine blood test resulting in abnormally high white blood cell count. Depending on various clinical characteristics, the CLL diagnosis is further categorized according to either of the staging criteria (discussed further). However, as leukemia cells replace the bone marrow, the number of red blood cells, normal white blood cells, and platelets in the blood decreases. That is why several seemingly unrelated symptoms and signs occur in lymphocytic leukemia (Hallek *et al.,*2008):

Painless lumps in the neck, armpit, or groin

- Fatigue and shortness of breath due to anemia
- Fever and repeated infections
- Pain in bones, ribs, or abdomen
- Easy bruising and bleeding due to low numbers of platelets
- Loss of appetite

Depending on various clinical characteristics, the CLL diagnosis is further categorized according to either of the following staging criteria:

Rai staging system categorizes CLL as a 'low-risk' disease for patients who have lymphocytosis (increase in the proportion of lymphocytes) with leukemic cells (cells with leukemic phenotype) in blood and/or bone marrow (lymphoid cells approximately 30%; earlier considered Rai stage 0). Those with lymphocytosis, enlarged lymph nodes, splenomegaly (enlargement of spleen) and/or hepatomegaly (enlargement of the liver) (lymph nodes being palpable or not) are defined as 'intermediate-risk' disease (formerly considered Rai stage I or stage II). 'High-risk' disease includes patients with disease-related anaemia (as defined by a haemoglobin [Hb] level approx.110 g/L [11 g/dL]; formerly stage III) or

thrombocytopenia (as defined by a platelet count approx. $100x10^9$/L; formerly stage IV) (Rai *et al.,*1975; Hallek et .al. 2008)

Binet staging CLL is classified as: Stage A - Hb 100 g/L (10 g/dL) or less and platelets $100x10^9$/L or more and any two of the organomegalies (splenomegaly or hepatomegaly). Stage B - Hb 100 g/L (10 g/dL) or less and platelets $100x10^9$/L or more plus organomegaly greater than that defined for stage A (i.e. three or more areas of nodal or organ enlargement). Stage C -All patients who have Hb less than 100 g/L (10 g/dL)and/or a platelet count less than 100 10^9/L, irrespective of organomegaly. The organomegaly described by this system includes:

- Head and neck, including the Waldeyer ring (this counts as one area, even if more than one group of nodes is enlarged)
- Axillae (involvement of both axillae counts as one area)
- Groins, including superficial femorals (involvement of both groins counts as one area);
- Palpable spleen;
- Palpable liver (clinically enlarged) (Binet *et al.,*1981; Hallek *et al.,*2008).

The staging system used in practice is usually subjective to the clinician. The latest CLL staging system consists of a combination of the both the systems above depending on the symptoms of the patient (www.cancer.org). However, the heterogeneous nature of the disease entails further fine tuning of the staging systems.

1.3 CLL Prognosis

While both the Rai and Binet staging systems continue to provide the most useful tools for assessing prognosis in CLL, however both often cannot identify subsets of patients that may, or may not, benefit from therapy as well as the disease progression patient survival or resistance to chemotherapy. Therefore majority of CLL research is focussed on identifying biological markers of the disease that dictate the course/fate of the disease in a patient. Currently the mutation status of

IGVH genes of CLL cells has been officially implemented as a prognostic criterion in developed countries whilst the validity of other markers is in the process of further evaluation (Bazargan *et al.,*2012). It must be stressed that although quite important, these criteria are not necessarily applicable for each individual case and sometimes the use of a combination of prognostic markers may be recommended. These markers of prognosis help in assessment and further study or aggressiveness and progression of the disease. Some of the most widely clinically accepted prognostic markers are detailed below.

Lymphocyte doubling time (LDT)

LDT is defined as the period of time needed for lymphocytes to double in number the amount found at diagnosis. The prognostic value of LDT is expressed in months and obtained by linear regression (Molica *et al.,*1987). The LDT of more than 12 months (>12 months) usually manifests as a less aggressive disease and a better life expectancy and a median survival time of higher than 5 years. The opposite is true for LDT less than 12 months (<12 months) where the median survival time is approximately 36 months. The study of LDT as prognostic marker is independent of age, sex, lymphocyte count, anaemia, and thrombocytopenia. Initially it was thought that CLL is an accumulative disease arising due to failure of the cell apoptosis. A study based on the oral administration of heavy water (^2H_2O) to CLL patients demonstrated that CLL cells have an *in vivo* birth rate/proliferation rate of 0.1% to >1% of the total leukemic clone per day (Messmer .*et al.,* 2005). Therefore the study of LDT is validated as a 'proliferation' marker. It is concluded that since LDT appears to predict the progression of the disease, it is useful in the clinical management of CLL. However, it is also a retrospective marker and needs monitoring of he patient blood count for a few months before making any clinical predictions (Damle *et al.,*2010; García-Muñoz *et al.,*2012).

<u>Immunoglobulin Variable Heavy chain (IGVH) gene mutational status</u>

In normal B cell development, B cells that are stimulated by antigen enter lymphoid follicles in the secondary lymphoid organs. A germinal centre (GC) is

formed, and under the influence of T cells and in the presence of antigen presented by follicular dendritic cells, affinity maturation takes place. The process is based on the generation of random somatic mutations in the variable region of the immunoglobulin gene, resulting in random changes of the antibody's affinity for the antigen. Only B cells with a high antigen binding affinity survive and differentiate further. Although there is some evidence that somatic hypermutation is not restricted to the GC reaction, it is generally accepted that B cells with mutated IGVH genes are post-GC, antigen-experienced cells (Klein *et al.,*2001; Chiorazzi *et al.,* 2003; Jumaa *et al.,* 2005).

Study of somatic hypermutations in the Immunoglobulin Variable Heavy chain (IGVH) genes for CLL cells showed that approximately 50% of the patients displayed mutations of their IGVH genes. CLL cases carrying IGVH genes with less than 98% homology to the closest germline gene (considered "mutated, M") generally follow a more indolent course than those with 98% or more homology (considered "unmutated, U").The patients with the U genotypes exhibit aggressive proliferation of lymphocytes and a poorer clinical prognosis (Damle *et al.,* 1999, Hamblin *et al.,*1999). Certain immunoglobulin genes (eg, IgHV1-69, IgKV1-33/1D-33, IgLV3-21) are preferentially used in U rearrangements, whereas others (eg, IgHV4-34, Ig_KV2-30, IGLV2-8) are more frequent in M rearrangements. This feature is "CLL-biased," because it does not appear in the normal B-cell IGVH repertoire.

Also, when the VH and VL gene expressions were compared between the normal CD5+ B cells and CLL cells, it was observed that the VH gene usage was discordant between the two cell types. The CLL cells showed higher reactivity toward a possible antigen experience via VH genes. The discordance between the biased use of certain V genes in the H and L chains of CLL cells in relation to normal B cell repertoires implies that reactivity with the antigens that determined selection of these B cells depends more on the structure of the VH (Fais *et al.,*1998; Chiorazzi *et al.,* 2003)

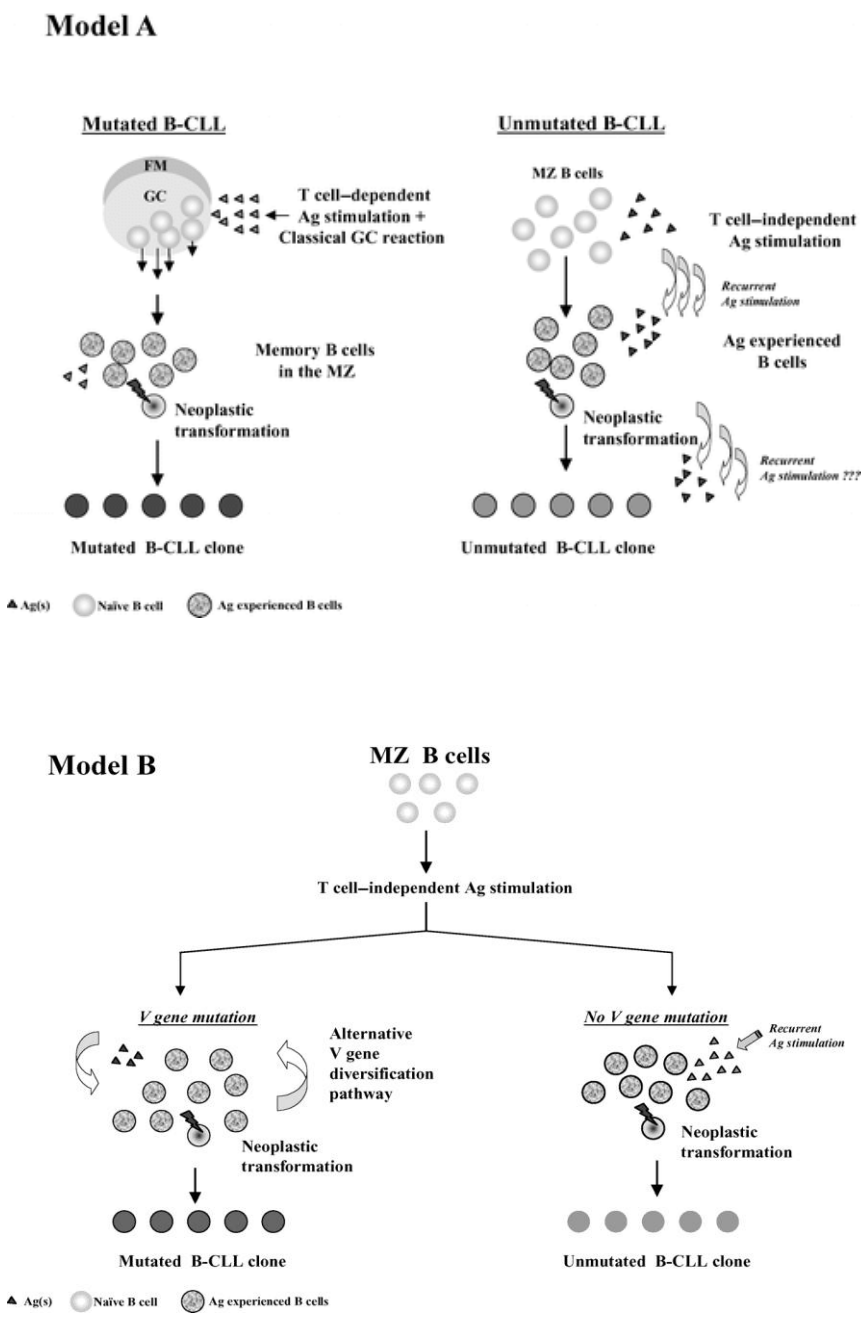


Figure 1. 1 Putative models to explain the derivation of a CLL cell from different cell types and/or distinct differentiation pathways

Model A suggests derivation of the mutated CLL cell from a B cell stimulated by a T cell–dependent antigen that drives the cell through a classical GC reaction. In this model, the U CLL cell derives from an MZ B cell driven by a T cell–independent process that does not elicit T cell help or somatic mutations. Model B suggests derivation of both the M and U CLL cells from the $IgM^{+}IgD^{low}$ subset of MZ B cells that are triggered independent of T cells and either do not or do develop somatic mutations. B cells that develop somatic mutations do so via a T cell–independent alternative V gene differentiation pathway. The available data do not exclude a hybrid model in which CLL cells derive from cells and differentiation pathways of both models (adapted from Chiorazzi N *et al.,* 2003).

CLL cases with remarkable similarity of the BCR (V regions of the H and L chains) have been identified (Tobin *et al.,*2003; Ghiotto *et al.,*2004). Various groups have reported subsets of CLL cases carrying closely homologous or "stereotyped" complementarity-determining region 3 (CDR3) sequences among both M and U cases. Stereotyped BCRs are strikingly similar BCRs, often arising from the use of common H and L chain V region gene segments that share CDR3 structural features (length, amino acid composition, and unique amino acid residues at recombination junctions)(Bühler *et al.,* 2010). Stamatopoulos *et al.,* (2007) studied 927 Ig sequences from a number of different centers in the Mediterranean and found that over 20% of patients carried a stereotypical BCR belonging to one of 48 stereotyped subsets (simply named 'subset #1' to 'subset #48). This analysis was performed using adapted criteria so that unlike previous studies, sequences did not necessarily have to use the same IGHV gene, the essential criteria being >60% HCDR3 amino acid similarity .This study also revealed intriguing connections between certain subsets and clinical outcome. Another similar study describes five sets of patients, mostly with U or minimally mutated IGVH genes, with strikingly similar B cell antigen receptors (BCRs) arising from the use of common H and L chain V region gene segments that share CDR3 structural features such as length, amino acid composition, and unique amino acid residues at recombination junctions. The data imply that either a significant fraction of CLL cells was selected by a limited set of antigenic epitopes at some point in their development and/or that they derive from a distinct B cell subpopulation with limited Ig V region diversity.. The remarkable B cell receptor (BCR) similarity in unrelated and geographically distant cases implies the recognition of individual, discrete antigens or classes of structurally similar epitopes, likely selecting the leukemic clones

(Ghia *et al.,* 2005; Stamatopoulos *et al.,* 2005). For example, some molecular evidence exists for EBV and CMV persistence in a subset of patients with CLL expressing the particular BCR stereotype of IGHV4-34 (Kostareli *et al.,*2009).

Additionally, it has also been documented that CLL cells, with U IGVH genes, also have polyreactive receptors able to bind to autoantigens and multiple microbial antigens (Broker *et al.,* 1988; Sthoeger *et al.,* 1989; Diaw et al., 1997; Dighiero *et al.,*1999). Some studies have suggested the role of antigens like molecular motifs on oxidized LDL and apoptotic cells as targets of antibodies produced by CLL cells (Dahle *et al.,*2008). Indicated by anepidemiological report was an increased risk for CLL among individuals with a history of pneumococcal pneumonia (Landgren *et al.,*2007). Additionally, some evidence exists for the connection between infections like cellulitis and herpes zoster (caused by *Staphylococcus aureus* and *varicella zoster virus* respectively*)* and the increased occurence of CLL (Anderson *et al.,*2009). Most recently, a study with a subset of M-CLL, expressing stereotypic BCRs highly specific for β-(1,6)-glucan, which is a major antigenic determinant of yeasts and filamentous fungi, has been published. This study showed the specificty of the the particular BCR stereotype to the antigen β-(1,6)-glucan. Also CLL cells expressing these stereotypic receptors proliferated in response to β-(1,6)-glucan (Hoogeboom *et al.,*2013).

All or most of these antigens are recognised and/or are ligands for Pattern recognition receptors (PRRs) of the innate immunity like Toll-like receptors (TLR), NOD-like receptors (NLRs) and scavenger receptors, which are expressed by CLL cells (Damle *et al.,*2002)(discussed later).

Expression of ZAP70

Even though the study of mutational status of the BCR is a powerful prognostic marker, IGVH sequencing is difficult to perform in a routine diagnostic laboratory and is expensive. Thus this assay is currently unavailable to most of the CLL patients. This reason dictates the need for a surrogate prognostic marker which is cheaper and easier to assess in a routine diagnostic setting.

The protein tyrosine kinase ZAP70 is normally expressed along with the T-cell receptor in T-cells and also in Natural Killer (NK) cells. ZAP70 also shows characteristic expression in CLL cells. Studies have shown that expression of ZAP70 is associated with increased/enhanced B-cell receptor downstream signalling in chronic lymphocytic leukemia (Kong *et al.,* 1995; Chen *et al.,*2002). In addition to the BCR, ZAP70 also facilitates downstream signalling from the receptors expressed by CLL cells like CD38 and CXCR4 (Richardson *et al.,* 2006). ZAP70 appears to be involved in CLL cell trafficking as well through the receptor CXCR4 and its ligand SDF1 (CXCL12), which govern CLL cell migrations to and from the lymphoid tissues, also the primary sites of CLL cell birth and proliferation (Burger *et al.,*1999; 2007). Another study shows that increased expression of ZAP70 is associated with TLR-9 ligation response in IgM+ve B cells which causes these cells to acquire CLL like phenotype, further indicating the role of this protein in CLL evolution (Bekeredjian-Ding *et al.,* 2008). Further, ZAP70 gene was found to be more highly expressed in U CLL than in M CLL and its expression could distinguish these 2 subsets with high statistical significance (Rosenwald *et al.,*2001). Another similar study expanded this analysis by profiling gene expressions in purified CLL samples from 107 patients and showed that ZAP70 expression is a preferential discriminator of M and U CLL. Further shown in this study was that ZAP70 expression identifies patients with a more aggressive clinical course and, therefore, has the potential to be a clinically useful molecular marker of prognosis in CLL (Weistner *et al.,*2003; Rassenti *et al.,*2004). CLL cells are considered to be positive for ZAP-70 when at least 20% of the CD 19+ cell population express this antigen in flow cytometry profiles (Luz *et al.,* 2006; Del Principe *et al.,* 2006).

However, contradicting the earlier data was a protein expression analysis for ZAP70 which showed 23% discordance when compared according to the M and U CLL cases. In this study, 23% of the patients with M CLL were positive for expression of ZAP70 as against the U CLL cases which lacked this expression. Further monitoring and co-relating with the clinical factors, (with an arbitrary threshold of 20% or higher cells expressing ZAP-70 considered positive for this factor) the expression of ZAP-70 showed a better co-relation to the median

survival times than the IGVH mutational status. Also, increased expression of ZAP70 by CLL cells proved a stronger predictor of the need for treatment than the presence of an unmutated IGVH gene. Since flow cytometry can be used reliably to assess blood samples for ZAP70, it is more amenable for application in clinical laboratories than nucleic acid–sequence analyses of the rearranged IGVH gene. Moreover, because the expression of ZAP-70 appears to be constant over time, in spite of the minor shortcomings, it is still a reliable prognostic marker which can be used at the time of disease diagnosis to predict the treatment options and disease progression in CLL (Rassenti *et al.,*2004).

Expression of CD38

CD38 is a BCR co-receptor, constitutively expressed by mature B-lymphocytes and plasma cells. It plays a dynamic role in the B-cell compartment by drastically modifying the functional properties of B-cells whereas it blocks B cell lymphopoiesis in the bone marrow and rescues germinal centre B cells from apoptosis (Zupo *et al.,*1994; Burger *et al.,*2007; Damle *et al.,*2010). An explanation for this apparently contradictory behaviour is likely to be found through investigation of the role of the micro-environment in providing soluble or cell-bound ligand(s) for CD38. CD31/platelet-endothelial cell adhesion molecule 1 (PECAM-1) is thus far the only reported cell surface–bound ligand for CD38 and it has been shown that CD31/CD38 interactions control an active signalling pathway in circulating and residential lymphocytes (Deaglio *et al.,*2000). *In vitro* analysis showed that ligation of CD38 on B-lymphocytes with monoclonal antibody increased cellular proliferation and induces intracellular Ca2+ fluxes leading to increase in anti-apoptopic protein Bcl-2 (Deaglio *et al.,*2003).

In CLL cells as well, CD38 expression can augment BCR signalling (Lund *et al.,*1996) and regulate both IgM and IgD induced apoptosis (Zupo *et al.,* 2000). Further, it has been shown that CD38 induces proliferation of CLL cells and increases their survival (Deaglio *et al.,* 2010) as been shown that CD38 induces proliferation of CLL cells and increases their survival (Deaglio *et al.,* 2010). The increased proliferation may be the result of interaction between CD38 and CD31, which upregulates CD100 (a survival receptor from the semaphorin family)

involved in sustaining CLL growth and survival (Elhabazi *et al.,* 2003; Kumanogoh *et al.,* 2003). CD38 expression is noted to be higher within the bone marrow and the lymph nodes, where CLL cells are to proliferate in special zones called proliferation centres (Jaksic *et al.,*2004; Soma *et al.,*2006). Moreover, within each CLL clone, cells expressing CD38 are enriched in Ki-67, suggesting that CD38+ cells are a cycling subset (Damle *et al.,*2007). The micro-environment in CLL has been suggested to play a primary role in the pathogenesis of CLL, rescuing the cells from apoptosis and CD38 has been shown to be an important mediator of this interaction (Deaglio *et al.,*2006; Chiorazzi *et al.,* 2005, 2011) . In support of this concept, it was demonstrated that CD38 expression delineates populations of CLL cells that are activated and express proliferation markers (Damle *et al.,* 2007). A study showed that approximately 1% of the CLL clone proliferates everyday and depending on the clonal kinetics, the cells express differentially receptors -CXCR4 and CD5. The fraction expressing lower levels of surface CXCR4 was enriched in CD38+ cells comprising largely of the cell population that have recently exited a solid tissue, after undergoing proliferation (Messmer *et al.,*2005; Deaglio *et al.,*2010; Vaisitti *et al.,*2010; Calissano *et al.,*2011; Thomson *et al.,*2013).

Presence of a distinct CD38+ cell population within the leukemic clone, with a numerical cut off definition at 30% or more as positive, correlates with IGVH gene mutational status and identifies CLL patients with a poorer prognosis or aggressive form of the disease (Silvia *et al.,* 2006). In fact, it has been well documented that patients expressing CD38 on more than 20% on their malignant cells have a disadvantage in survival compared to patients with percentage of CD38+cells (Damle *et al.,* 1999; Ke *et al.,* 2002)

However, as mentioned above, expression of CD38 varies between the cells in the lymphatic tissues and bone marrow compared to that in the peripheral blood (Patten *et al.,*2008). Also, it has been reported that cell surface expression of CD38 is not constant during the course of disease (Chang and Cleveland, 2002). Furthermore, in as many as 1 in 4 patients, there may be a significant variability of CD38 expression during the course of the disease (Hamblin *et al.,*2002), although there are CLL cases where CLL cells never express CD38 over the course of time

(Ghia *et al.,*2003). Thus, whilst it would be convenient if CD38 positivity were a reliable surrogate marker for IGVH mutational status (a technically much more demanding laboratory test), in many cases the two variables are discordant and both retain independent prognostic significance (Hamblin *et al.,*2002; Thomson .*et al.,*2013).

Serum Markers

β_2-microglobulin : The level of soluble β_2-microglobulin has been observed to correlate with the stage of disease in CLL patients. High serum levels of β_2-microglobulin were found in CLL patients with a rapidly progressing disease (Giovanni *et al.,*1988). In addition, serum β_2-microglobulin levels correlated with the treatment free survival time (TFS). TFS is the time measured in months between two courses of treatment (Molica *et al.,*2009).

Serum CD23 (sCD23): Serum CD23 antigen (low affinity receptor for IgE) is a membrane glycoprotein which is cleaved into soluble fragments having pleiotropic effect. Previous studies showed that CD23 protein and gene expression are abnormally regulated in CLL (Fournier *et al.,*,1992). The level of CD23 in the serum of CLL patients can vary between 3 to 500 fold more as compared to control subjects or other lymphoid malignancies and correlates with the tumour burden/stage of the disease (Sarfati *et al.,*,1996).

Serum Lactate Dehydrogenase (LDH): Lactate Dehydrogenase (LDH) is an isozyme which exists in many different cell systems and subsequent to tissue or cell damage, serum LDH levels may be elevated. The level of the LDH in the blood also correlates with anaerobic metabolism and increased glycolysis in the cytoplasm of malignant cells accompanied by a high turnover rate. A relationship between various neoplasias and increased LDH levels has thus been reported by many groups (Lundh *et al.,*1967; Ferrara *et al.,*1996). In CLL, high level of this isozyme is commonly observed and correlates with the stage of the disease (Shen *et al.,*2007). The haematology unit at UCH, where we obtained CLL samples from, LDH is routinely assayed as a prognostic marker for CLL.

Even though comparatively convenient to assay, the shortcomings of the use of these serum markers, is that the tumour load cannot be accurately predicted in early stages of CLL given the low expression of these markers. Hence the use of serum markers for prognosis of the course of the disease is controversial (Molica *et al.,*1999).

Chromosomal aberrations

Recurrent losses or gains of chromosomal material as well as mutations of key tumour suppressors (*ATM* and *TP53*) have been identified in CLL (Zenz *et al.,*2010.). These aberrations are believed to be important "drivers" of the disease as well as its clinical characteristics. Fluorescence *in situ* hybridization (FISH) analysis demonstrated that chromosomal abnormalities can be found in up to 80% of CLL cases. The most frequent aberrations are deletions of chromosomes 13q (55% of cases), 11q (12%), 17p (8%) and trisomy of chromosome 12 (15%). Genetic studies on the affected loci have allowed the identification of specific genes that may play a relevant role in the pathogenesis of CLL (e.g. microRNA genes on chromosome 13, *ATM* (Ataxia Telangiectasia Mutated) gene on chromosome 11 and *TP53* on chromosome 17). Deletions/mutations of the *TP53* (tumour suppressor gene encoding for the protein p53 on chromosome 17) gene have been shown to be associated with resistance to treatment and were considered as independent markers for poor survival (Juliusson *et al.,*1990).

13q14 deletion: this is the most common type of genetic aberration and can be either homozygous or heterozygous. This abnormality also occurs in other lymphomas, apart from CLL. Patients with this deletion have better prognosis than the other chromosomal deletions in CLL. The median survival time for patients with this genotype is approximately 133 months (Dohner *et al.,*2000).

Trisomy 12: 10-20% of CLL patients manifest trisomy at chromosome 12. More often this genotype is associated with CLL of the 'atypical' cellular morphology (Matutes *et al.,*1998). In addition, it is associated with an early disease progression and shorter 'treatment free' survival as compared to the 13q14 deletion (Dohner *et al.,*2000). The median survival time for patients with trisomy 12 is better than

those with 17p deletion and lesser than the patients with 13q14 deletion (Zenz *et al.,*2011).

11q23 deletion: About one fifth of patients with treatment indications will exhibit 11q deletions. Patients with 11q deletion have a more rapid progression of disease, shorter survival and extensive lymphadenopathy (Dohner *et al.,*1997). The minimal consensus region in bands 11q22.3–q23.1 harbours the *ATM* gene in almost all cases. But only a subset show biallelic inactivation of *ATM* by simultaneous mutations. Mutations of the *ATM* gene have been shown to occur in 12% of all patients with CLL, and in 30% of patients with 11q deletion and are associated with poorer outcome (Austen *.et al.,*2005). The role of other genes in 11q22.–q23.1 remains unresolved . There is a very strong association between the presence of the deletion 11q and an U IGHV mutation status. The biological basis for this association is currently unclear (Zenz *.et al.,*2011).

17p13 deletion and mutation of the key tumour suppressor gene *TP53*: the deletion of 17p13 occurs in 3-5% of cases with CLL. It is more common in patients with refractory or relapsed CLL post-treatment and rarely detected at diagnosis (Zenz *et al.,*2010). This chromosomal deletion covers most of the short arm of chromosome 17 which also contains the TP53 locus. Very few cases with 17p deletion will show functional p53 tumour suppressor pathway (Zenz *et al.,*2010). Mutation of *TP53* is not entirely dependent on the 17p deletion. Though the genetic complexity for *TP53* mutation is not yet completely defined, recent study demonstrated that poorer prognosis was observed in patients with p53 mutation in absence of 17p chromosomal deletion (Zenz *et al.,*2008).

1.4 CLL cells and apoptosis

CLL is commonly considered as a paradigm for a malignancy of failed apoptosis, as CLL cells circulating in the blood are largely non-proliferating and arrested in the G0/G1 phase of the cell cycle. Cell division occurs mainly in the 'proliferation centres' in tissue microenvironments, accounting for the increase in the tumour load in some patients (Burger *et. al.,* 2002; Hallek *et. al.,* 2010; Chiorazzi *et. al.,*

2011). However, lack of apoptosis is still considered a major component of the dysregulation of normal B-cell homeostasis in all subsets of this malignancy.

Apoptosis is an important physiological process that helps to regulate the normal levels of all cells. Apotosis can be defined as a form of cell death, also known as 'programmed cell death', in which a 'suicide' program is activated within a cell, leading to fragmentation of the DNA, shrinkage of the cytoplasm, membrane changes and cell death without lysis or damage to neighboring cells. When the cellular DNA is damaged or the cell is under stress due to any number of stimuli, cell division and differentiation are balanced by apoptosis, through a number of intra cellular biochemical reactions manifesting distinct morphological features, including a decrease in cell volume, chromatin condensation and the formation of membrane-bound apoptotic bodies (Alberts *et. al.,* 2002). Alteration in susceptibility to apoptosis is an important feature of many human cancers.

The intracellular machinery responsible for apoptosis depends on a family of proteases called 'caspases' (cysteine proteases with aspartate specificity). Caspases are synthesized in the cell as inactive precursors, or *procaspases,* and usually activated by cleavage at aspartic acids by other caspases. Activation of the caspases results further in an amplifying proteolytic signalling cascade. The end result of this cascade being irreversible breakdown of the nuclear lamina, cleavage of the cellular DNA and ultimately cellular apoptosis (Alberts *et. al.,* 2002; Lamkanfi *et. al.,* 2007).

The caspases cascade can be activated by two main pathways of apoptosis. The 'intrinsic' cell death pathway- which is activated by a very wide range of stimuli, including radiation, cytotoxic drugs, cellular stress and growth factor withdrawal. This cascade involves the release of proteins, including cytochrome *c*, from the mitochondrial intermembrane space. Cytoplasmic cytochrome *c* combines with an adaptor molecule- Apaf-1 (Apoptotic protease activating factor 1) and an inactive 'initiator' caspase, procaspase 9, within a multiprotein complex called the apoptosome. This leads to the activation of caspase 9 which then triggers a cascade of reactions including the cleavage of the caspase from its substrate PARP (poly(ADP-ribose) polymerase), resulting in the morphological and biochemical

changes leading to apoptosis i.e. intracellular signalling mediated by the 'effector' caspase i.e. caspase 3 (Cory *et. al.,* 2003; Green *et. al.,* 2004; Packham *et. al.,* 2005). The second cell-death pathway called the 'extrinsic' cell-death pathway, mainly functions independently of mitochondria. This pathway involves engagement of particular 'death' receptors that belong to the tumor necrosis factor receptor (TNF-R) family and, through the formation of the death-inducing-signalling-complex (DISC) (Ashkenazi *et. al.,* 1998), leads to a cascade of activation of caspases, including caspase-8 and caspase-3, which in turn induce apoptosis (Danial *et. al.,* 2004).

Successful treatment of CLL aims at effectively targeting the malignant clone to induce apoptosis with minimal adverse effects. Chlorambucil and prednisolone which are commonly used chemotherapeutic agents for CLL, induce cytotoxic activity through the activation of the 'effector' caspases caspase-3 and caspase-7 (King *et. al.,* 2001). The immunotherapeutic agent Rituximab (monoclonal antibody to CD20) which has demonstrated high effectiveness in treatment of CLL, has shown to induce apoptosis involving the activation of caspase 9, caspase 3 and PARP cleavage via modulation of Mcl-1 (Byrd *et. al.,* 2002).

Bcl-2 family proteins play a key role in controlling mitochondrial function associated with the 'intrinsic' cell-death pathway, either by preventing or promoting the release of cytochrome *c.* Some members of this family, like Bcl-2 itself or Bcl-X_L, inhibit apoptosis at least partly by blocking the release of cytochrome *c* from mitochondria (anti-apoptopic). Other members of the Bcl-2 family, contrastingly, promote procaspase activation and cell death (pro-apoptopic). Out of these pro-apoptopic molecules, e.g. *Bad*, function by binding to and inactivating the death-inhibiting members of the family, whereas others, like *Bax* and *Bak,* stimulate the release of cytochrome *c* from mitochondria. If the genes encoding *Bax* and *Bak* are both inactivated, cells are remarkably resistant to most apoptosis-inducing stimuli, indicating the crucial importance of these proteins in apoptosis induction. The relative expression (or activity) of various anti-apoptotic and pro-apoptotic Bcl-2 family proteins is a critical determinant of

apoptosis sensitivity in cells (Cory *et. al.,* 2003; Kirkin *et. al.,* 2004; Packham *et. al.,* 2005).

Many studies have consistently validated that Bcl-2 is over expressed in CLL cells (Moore *et. al.,* 2007; Letai 2011). The exact genetic alteration responsible for this over expression has not yet been clearly established. The expression of Bcl-2 and Bax and, perhaps more significantly, the relative expression of these functional antagonists, is an important variable in CLL. CLL cells have increased Bcl-2/Bax ratios (favouring cell survival) compared to normal controls (Pepper *et. al.,* 1997; Saxena *et. al.,* 2004). Individual variation in the expression of Bcl-2/Bax correlates with apoptosis and clinical outcome including resistance to therapy. For example, decreased Bcl-2/Bax ratios are associated with increased sensitivity to cytotoxic drugs *in vitro* and improved responses to chemotherapy in patients (Thomas *et. al.,* 1996; Pepper *et. al.,* 1997; Moore *et. al.,* 2007).

Other protein of the Bcl-2 family which contributes to apoptosis control and over-expressed in CLL cells, includes the anti-apoptopic molecule Mcl-1 (myeloid cell leukemia sequence 1). During apoptosis reaction, Mcl-1 forms a very efficient substrate for caspases (Clohessy *et. al.,* 2009). Caspase cleavage of Mcl-1 simultaneously inactivates the survival function of this protein and converts Mcl-1 into a cell death-promoting molecule, activating a positive feedback loop that results in increased caspase activation (Michels *et. al.,* 2005). Therefore, Mcl-1 acts as a molecular switch during apoptosis, converted from a molecular bodyguard to assassin, by proteolytic cleavage (Packham *et. al.,* 2005). In CLL, Mcl-1 expression has *in vitro* and *in vivo* significance and its expression co-relates with the expression of other bad prognostic markers of the disease. Additionally, Mcl-1 is also a key controller of CLL drug resistance and is an important regulator of disease progression and outcome in CLL (Saxena *et. al.,* 2004; Pepper 2008).

Further regulating the apoptosis pathways are Bcl-2 family members e.g. Puma and Noxa which induce apoptosis by binding to and neutralizing the ability of antiapoptotic proteins, including Bcl-2 and Bcl-XL (Jeffers *et. al.,* 2003) . The important apoptosis regulator protein, p53 up-regulates transcription of these proteins in response to cellular stress/DNA damage and involves the activation of

caspase 9 signalling cascade (Jin *et. al.,* 2001; Harris *et. al.,* 2005; Vazquez *et. al.,* 2008). p53 is a transcription factor that induces senescence, cell cycle arrest (at G1 and/or G2 phase) or apoptosis in response to DNA damage, oncogene activation, hypoxia, cellular stress and the loss of normal cell contacts thus preventing aberrant cell growth (Giaccia *et. al.,* 1998; Lohrum *et. al.,* 1999). Tumour supressor gene encoding the protein p53 or Tp53 is dysfunctional or mutated in cancers and regulated by the oncogene MDM2 (Mouse double minute 2). MDM2 is a negative regulator of p53 and binds to it with high affinity thus negatively modulating its transcriptional activity and stability. Overexpression of MDM2, found in many human cancers including CLL, effectively impairs p53 function and therefore through uncontrolled cell division and failure to apoptosis induction leads to tumourogenesis (Haupt *et. al.,* 1997; Burns *et. al.,* 1999; Vassilev *et. al.,* 2004). Studies have identified alternative non-transcriptional mechanism of p53 by binding to anti-apoptotic Bcl-2 family proteins at the mitochondrial surface, resulting in Bax activation and apoptosis (Mihara *et. al.,* 2003). This direct interaction of p53 with mitochondrial antiapoptotic proteins including Bcl-2 is the major route for apoptosis induction in CLL cells. p53's transcriptional targets in CLL cells include proteins that impede this non transcriptional pathway (Grever etal 2007). Treatment-induced p53 activity is predominantly found in the mitochondrial fraction of CLL cell extracts and is associated with the antiapoptotic protein Bcl-2. Therefore, strategies that block up-regulation of p53-mediated transcription may be of value in enhancing apoptosis induction of CLL cells by p53-elevating drugs (Steele *et. al.,* 2008).

Additionally, to the defective intra cellular apoptopic pathways, the extrinsic cellular microenvironment plays an important role in CLL cell survival as evidenced by the spontaneous *in vitro* cell death (Burger *et. al.,* 2002; Chiorazzi *et. al.,* 2005). A number of factors, including co-incubation with cytokines, can rescue CLL cells from cell death, and the role of these mechanisms is important in the understanding of the behaviour of the disease *in vivo*. Some of these cytokines (e.g. IL-1, IL-6, IL-8, and interferon-γ) are known to be produced by CLL cells, and presumably can act in an autocrine fashion (Aguilar-Santelises *et. al.,* 1999). T-cells

in the CLL patients also produce supporting cytokines e.g. TNFα, IL4 and IL10 which aid their survival (Jewell *et. al.,* 1994; Mainou-Fowler *et. al.,* 2001)

In addition to being responsive to cytokine-mediated signals, CLL cells are also responsive to cell-mediated signals which help them evade apoptosis. CLL cells express a range of adhesion molecules that determine the tissue distribution of the cells and regulate the ability to recirculate through different immunological compartments. As cells migrate through tissues, they interact with other cell types. Some examples of such interactions are those with bone marrow stromal cells, direct cellular interaction with CD4-positive T cells (Tretter *et. al.,* 1998), follicular dendritic cells and other cells of the lymph nodes(Herishanu *et. al.,* 2011; Burger *et. al.,* 2013). The intra-cellular modulation triggered by the stimulation of the BCR is another major feature of the CLL cells. Overexpression of the various anti-apoptopic protein kinases activated through the BCR ligation is a characteristic of the CLL cells (Stevenson *et. al.,* 2011) (discussed further in detail).

Therefore, in brief, these data suggests it is likely that the accumulation of the malignant clone in CLL is at least partly dependent on the dysregulation of apoptosis pathways leading to prolonged cell survival. This, however, is not an intrinsic function of CLL cells and depends upon continued stimulation by cytokines and cell interactions. Strategies to disrupt autocrine and paracrine survival pathways may lead to improved clinical management of the disease.

1.5 Treatment

The necessity of therapy in CLL is dictated by the stage of the disease and prognosis. For example expression of ZAP-70 and U IGVH genes indicate poor prognosis and these patients require treatment almost immediately at diagnosis. On the other hand in case of patients with a good prognosis e.g. M IGVH and low ZAP-70 expression, the 'wait and watch' regime is followed by close monitoring of the clinical symptoms.

Chemotherapy: Chlorambucil and cyclophosphamide are the common alkylating agents used for CLL treatment. These agents induce cell death through p53 independent pathway (Begleiter *et al.,* 1996). Combinations of chlorambucil and prednisone were considered the benchmark for CLL therapy (Keating *et al.,* 1998). Chlorambucil used alone or with prednisone produced initial response rates between 60% and 90%, and a complete remission in 60% of patients. Its efficiency depends on the dose administered and response criteria (Rai *et al.,* 2000; Robak *et al.,* 2000). Chlorambucil usually reduces the WBC count, decreases lymphadenopathy and splenomegaly, but rarely returns the bone marrow to normal. Randomized, controlled trials begun in the early 1990s demonstrated superior response rates, progression-free survival, and quality of life for patients treated with fludarabine-based therapy rather than chlorambucil or other alkylating-agent regimens (Shanafelt *et al.,*2012). Fludarabine is a purine analogue [monophosphate (Fludara), 2-chlorodeoxyadenosine (2-CDA)], and potent inhibitor of DNA repair (Dillman *et al.,* 1989; Keating *et al.,* 1998) and gives higher remission rates particularly in patients resistant to the alkylating agents. Fludarabine combination therapies, in particular the combination of fludarabine and cyclophosphamide (FC), seem to have the potential to yield higher response rates than fludarabine alone (O'Brien, 1998). However, all patients eventually relapse and the overall prognosis of advanced CLL (Binet stage C, Rai stage III–IV) has remained poor, with a median survival of 2–3 years (Byrd *et al.,* 1998). This warrants the necessity of new improved therapies. The use of multiple courses of purine analogue therapy in patients can cause bone marrow suppression leading to anaemia, neutropenia, thrombocytopenia, which ultimately, has limited the use of this type of therapy in CLL (Robak *et al.,* 2000; Rai *et al.,* 2000). In addition, conventional therapy with purine analogues also leads to drug resistance and the disease remains incurable.

Immunotherapy: is mainly the targeting of receptors on CLL cells via antibody dependent cellular cytotoxicity (ADCC), causing direct apoptosis of CD20+ B cells and/or clearance of the formed immune complexes (Shaw *et al.,* 2003). The first monoclonal antibody (mAb) which was approved for the clinical treatment of CLL was Rituximab, directed to the CD20 phosphoprotein. However, clinical trials have

observed a low responsiveness in CLL patients to Rituximab treatment, possibly as a result of the low density of CD20 on CLL cells. Further studies on the dose-dependent response of Rituximab showed that tripling the 'once a week dose schedule' enhanced the response up to 40% (O'Brien *et al.,* 2001). Another study has documented that the patients receiving Fludarabine in addition to Rituximab had a significantly better progression-free survival and overall survival (Byrd *et al.,*2005).

Haematopoietic Stem Cell transplantation: Considering the side effects of the use of drugs and the response to treatment in patients, other forms of treatment also need to be opted for. Sutton *et al.,* (2011) reported the results of a randomized clinical trial exploring the role of autologous stem cell transplantation (ASCT) in patients with chronic lymphocytic leukemia (CLL), showing that ASCT may increase the response rate and prolong the time to progression but it does not result in a longer survival, in comparison with chemotherapy treatment (Sutton *et al.,*2011). Allogenic stem cell transplantation has been studied in clinical trials for patients with poor-risk CLL and showed long term minimum residual disease (MRD) free survival in 50% of the patient cohort, independent of the genomic profiles (Dreger *et al.,*2010).

Novel therapies: Therapies to circumvent the complication arising from those listed above, are in the process of clinical trials. One such therapy is the use of autologous CD19 redirected T cells expression a genetically modified chimeric antigen receptor (CART19). A recent study has shown that the use of these CART19 cells is highly potent in targeting CD19 (and other targets) through transduction of chimeric antigen receptor linked to potent signalling domains. Unlike antibody-mediated therapy, these modified T cells have the potential to replicate *in vivo*, and long-term persistence could lead to sustained tumor control (Porter *et al.,*2011).

Further potential immunotherapeutic targets include proliferating cells such as CD38+ B cells as well as BCR-mediated and ZAP-70-mediated signal transducers. Targeting the various kinases in the BCR pathways has also received a lot of importance and few clinical trials have efficiently shown success in the drugs used

for such targeted therapy.

B-cell receptor (BCR) and its role in pathogenesis of CLL

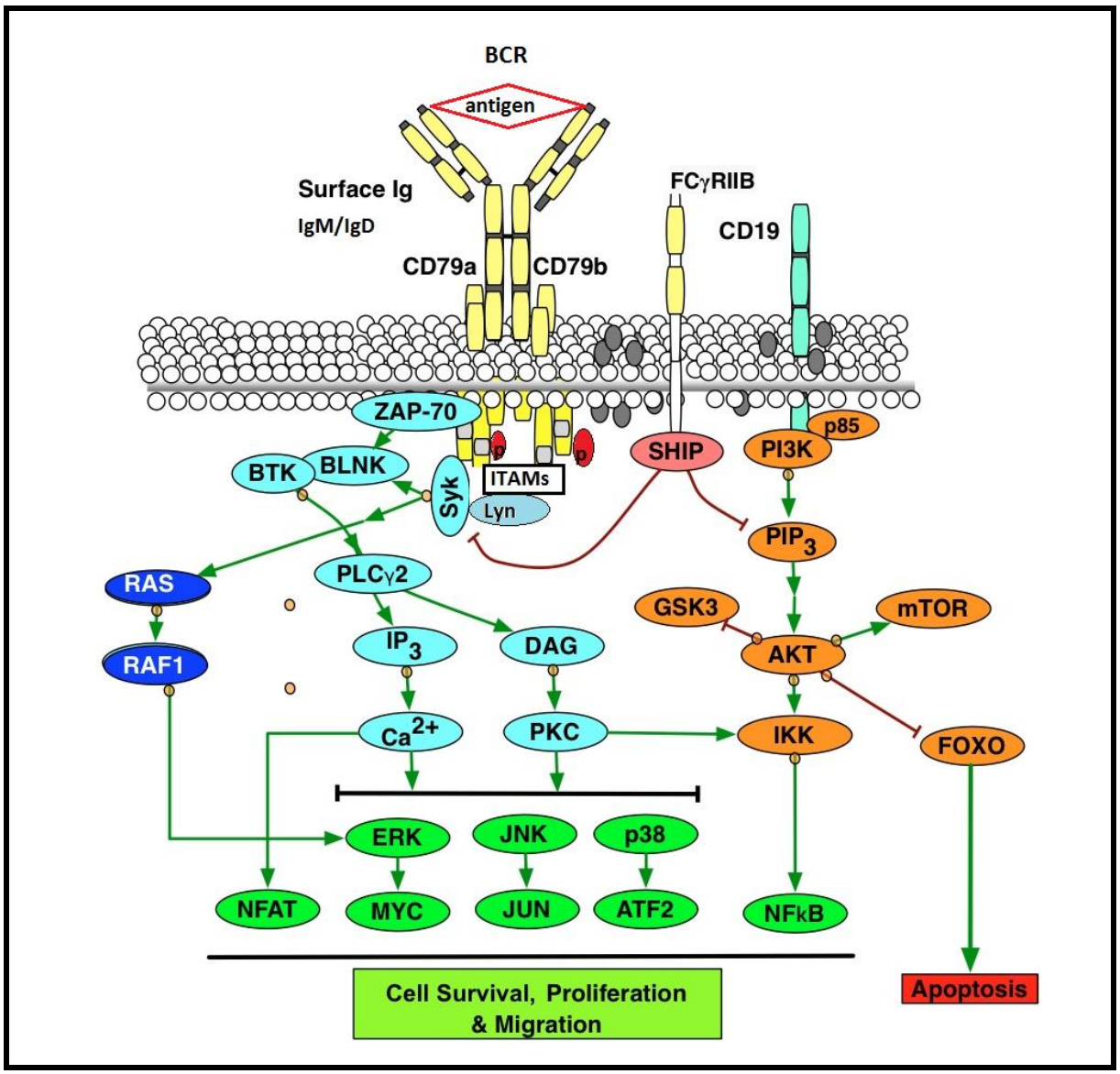


Figure 1. 2: B cell receptor (BCR) complex and the major signalling pathways

The BCR complex is comprised of the antigen receptors IgM and IgD, associated with two polypeptides, Igα and Igß, also known as CD79a and CD79b respectively. Surface immunoglobulins are transmembrane molecules, (with the intra-cytoplasmic part only a few amino acids long), thus using CD79a and CD79b molecules for signalling through (immunoreceptor tyrosine-based activation motifs) ITAMs. (adapted from Choi and Kipps 2012).

As shown in the Figure 1.2, the BCR complex consists of the surface immunoglubulins (IgM or IgD), the associated with polypeptides CD79a and CD79b and the intra-cytoplasmic ITAMs within their cytoplasmic tail that initiates signal transduction following BCR aggregation (Flaswinkel and Reth, 1994). All mature naïve splenic B cells are positive for surface IgM (sIgM) and surface IgD (sIgD), while immature B cells express sIgM combined with variable surface expression of lgD. This differential expression suggests that sIgM and slgD are quantitatively transmitting different signals (Norvell *et al.,* 1996; Packham *et al.,*2010).

Initially the src-family protein tyrosine kinases, mainly Lyn and Syk (protein tyrosine kinases) PTKs are in proximity with the BCR through the phosphorylated ITAMs of resting BCR (Clark *et al.,* 1992; Pleiman *et al.,* 1994). Upon BCR stimulation with an antigen followed by receptor aggregation, these kinases along with the ITAMs partition into glycosphingolipid-rich microdomains of the plasma membrane or 'lipid rafts'(Guo *et al.,*2001; Cheng *et al.,* 2001). The formation of the lipid rafts and receptor aggregation followed by phophorylation of the kinases Syk and Lyn, leads to recruitment of downstream PTKs. This activity results in a progressive amplification of ITAM phosphorylation and promotes the subsequent recruitment and activation of additional effector molecules (Johnson *et al.,* 1995). The important effector enzymes include (phosphatidyl 3-kinase) PI3K and (phospholipase Cy2) PLCy2. The second messenger (phosphatidylinositol-3,4,5-triphosphate) PIP3 is generated by PI3K which then recruits other BCR molecules as well as to activate the kinase AKT furthering the signal to activation of transcription factor (nuclear factor κB) NFκB. The activation of this factor largely transmits a survival/proliferation signal for the cell (Brazil *et al.,*2001). In parallel is the PLC-γ2 activation which leads to the intracellular release of calcium (Ca2+) through (inisitol phosphate 3) IP3 and activation of (protein kinase C) PKC through (diacylglycerol) DAG, considered to be crucial for the activation of (mitogen-activated protein kinases) MAPKs. These MAPKs include extracellular signal regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38 MAPK. In addition to these two direct pathways, there are a number of other complex

signalling pathways activated after antigenic stimulation of the BCR. The final fate of the B-cell i.e. survival, proliferation, migration, apoptosis and/or anergy is dictated by the result of PTK modulations of the downstream regulators including transcription factors. (Dal porto *et al.,*2004; Kurosaki *et al.,*2011; Choi and Kipps 2012; Woyach *et al.,*2013).

1.6 Signalling through the B-Cell Receptor in CLL

There is strong evidence that signalling via the BCR plays a major role in the development of CLL and determines the variable clinical behaviour. Chronic active BCR signalling due to point mutations in CD79b has recently been identified as a key pathogenic mechanism resulting in constitutive NF-κB activation and cell survival/proliferation (Davies et al., 2010). In contrast, CLL cells also have been shown to manifest gene expression characteristics of resting B cells and cells from the M-CLL subtype have been described as anergic and unresponsive to BCR activation (Guarini et al., 2008). The BCR of many CLL cells share characteristics with natural antibody-producing B cells that recognize microbial antigens and self-antigens, leading to the hypothesis that antigen selection plays a role in the ontogeny of CLL (Ghia et al., 2008). A number of studies have elucidated the role of antigenic stimulation in the pathogenesis of CLL. As mentioned earlier, stereotypes of BCR observed in CLL cells, the evidence of bacterial and viral infections affecting CLL patients (before or after diagnosis) as well as the phenotypic characteristics of the CLL cells defining them to be memory cells, all add to the evidence that antigenic stimulation dictates the pathogenecity of the disease (Hulkkonen et al., 2002; Chiorazziet al., 2005; Efremov et al., 2007; Stamatopoulos et al., 2010; Stevenson et al., 2011; Scupoli et al., 2012). Further validating the significance of the BCR pathways in CLL are the numerous kinase inhibitors that have shown success in inducing apoptosis of the CLL cells both *in vitro* and *in vivo* through clinical trials.

The major protein kinases, which play an important role in BCR mediated signalling and extensively studied as treatment targets in CLL are discussed below.

BTK- (Bruton's Tyrosine Kinase) BTK is a non-receptor tyrosine kinase and member of the Tec family of kinases (Schaeffer et al., 2002). It is an intermediate signalling molecule in the B-cell receptor signalling pathway that mediates the survival and expansion of both normal and malignant B cells through various signalling mechanisms. The importance of BTK in the B-cell receptor signalling pathway has been established by direct evidence in humans and in mouse models (Buggy et al., 2012). In humans, mutations in the kinase domain of BTK result in a primary immunodeficiency known as X-linked agammaglobulinemia (XLA), a disease that occurs only in young boys because the gene coding for the BTK protein is located on the X chromosome (Vihinen et al., 2000). There is experimental evidence that BTK plays a critical role in the function and survival of B cells. Mice with a mutation in this gene have severe immune defects that mostly affect the B-cell compartment. BTK is activated upon BCR cross-linking by a two-step mechanism involving PI3K and the Src family PTK Lyn (Mohamed et al., 1999). Further to activation of BTK in normal B cells, striking enhancement of extracellular calcium influx is also observed which is in turn responsible for activating other downstream kinases (Fluckiger et al., 1998). Evidence exists for BTK linking the BCR stimulation to the transcription factor NfκB activation (Bajpai et al., 2000). BTK is also required for BCR-mediated activation of ERK and c-JNK-1(Jiang et al., 1998) which beling to the family of MAPKs. Most importantly, with regards to CLL cell survival, the anti-apoptopic molecule AKT, is also dependant on BTK for its activation and survival effect (Craxton et al., 1999). Owing to its role in activation of survival kinases, within the last several years, BTK has become the focus of targeted therapies designed to disrupt the activity of the B-cell receptor signal transduction pathway in various B-cell malignancies. Ibrutinib, a small molecule that interferes with BTK activity, has been shown to disrupt B-cell survival in vitro and has demonstrated efficacy in phase I and II clinical trials, with particularly encouraging responses and duration of response reported in patients with CLL and mantle cell lymphoma (Burger et al., 2013). Ibrutinib, is in phase III clinical trials in patients with CLL after showing dramatic therapeutic activity in the phase I and phase II clinical trials (Burger et al., 2013).

PI3K - The (phosphatidylinositide 3-kinases) PI3K-induced pathway plays a pivotal role in CLL cell survival and growth. PI3Ks generate phosphoinositide lipids in response to extracellular stimuli, regulating survival, proliferation, differentiation and migration (Manning & Cantley, 2007). Out of the three classes of PI3K isoforms, PI3K class I are heterodimers that consist of a catalytic subunit which are the 110 kDa (p110α, p110β and p110δ) proteins and a regulatory subunit which is the p85. The p110δ along with p85 subunit is highly expressed in cells of hematopoietic origin, being predominantly detected in leukocytes (Chantry et al., 1997). Genetic and pharmacologic approaches that specifically inactivate the p110δ isoform have demonstrated its important role in B-cell signalling. Antigenic binding to the BCR sets in motion the signalling events in which the regulatory subunit p85 provides a binding site for the protein Ras, an event indispensible for further signal transduction (Domin et al., 1997; Ringhausen et al., 2006). PI3K is involved in several signal transduction pathways in B cells such as those initiated through CD40 signalling, BCR signalling, Toll-like receptor signalling and signalling through a variety of cytokines (Aagard et al., 1996; Andjelic et al., 2000). It has been shown that PI3K activates the serine/threonine kinase AKT/protein kinase B (PKB). AKT binds to the products of PI3K, PI 3,4-P_2, and PI 3,4,5-P_3 and becomes itself activated by phosphorylation. In addition to AKT activation, another important effect of PI3K signalling is activation of PKC isoforms (Toker et al., 1994). PKC is a family of iso-enzymes classified by their dependence on cofactors and may be regulated by several independent mechanisms. Both AKT and PKC play a role in cell cycle regulation, proliferation and cell survival (Kandell et al., 1999; Miyamoto et al., 2002).

It has been shown that PI3K is constitutively active in CLL. Additionally, a sustained activation of PI3K/NFκB pathway is critical for the survival of CLL B cells (Cuni et al., 2004). Also, blocking of activation of PI3K leads to apoptosis of the CLL cells, independent of AKT phosphorylation, implicating its important role in the CLL cell survival (Ringhausen et al., 2002).

Inhibitors to PI3K have shown some success at killing CLL cells. Wortmannin a broad range PI3K inhibitor targets CLL cells but has many negative side effects due

to phosphorylation inhibition of essential PI3K isoforms (Wymann et al., 1996). On the other hand, specific inhibitor to the isoform PI3K-δ, which is expressed exclusively by haematopoetic cells, CAL-101, has recently shown success as a therapy in numerous clinical trials (Hoellenriegel et al., 2011; Castillo et al., 2012).

AKT- The serine/threonine kinase AKT, also known as protein kinase B (PKB), is a central node in cell signalling downstream of growth factors, cytokines, and other cellular stimuli especially implicated in its anti-apoptopic role in malignant cells. As mentioned above AKT is a key mediator of the BCR induced signalling pathway through PI3K. CLL clones consistently contain activated AKT which plays a pivotal role in maintaining cell survival. Sustained activation of AKT is required to drive cell-cycle progression of CLL B cells stimulated with CpG oligonucleotides (Longo et al., 2007). The PI3K/AKT pathway controls the expression and function of many proteins that are essential for cell survival. These include members of the Bcl-2 family of proteins, such as proapoptotic Bcl-2 antagonist of cell death BAD or antiapoptotic Mcl-1, which can be inhibited or up-regulated by AKT, respectively (Datta et al., 1997; Liu et al., 2001). In addition, AKT can increase the expression of NF-κB target genes, such as Bcl-xL and A1, by activating the (IκB kinase) IKK to induce the degradation of IκB (Suzuki et al., 2009). AKT can also prevent apoptosis by directly phosphorylating and inactivating caspase-9 or by inducing the expression of distal negative regulators of apoptosis, such as the X-linked inhibitor of apoptosis protein (XIAP)(Cardone et al., 1998; Dan et al., 2004). In addition to being activated by phosphorylation through the PI3K dependent pathway, AKT can also be activated in CLL cells through another PI3K-independent pathway mediated through the protein kinase Cβ (PKCβ) (Barragan et al., 2006). Specific inhibition of AKT induces extensive apoptosis of CLL cells, which is associated with both a rapid loss of Mcl-1 through proteasomal degradation and increased expression of the tumour suppression gene p53. Various AKT inhibitors (e.g. MK-2206) are currently undergoing clinical trials for CLL and have a high therapeutic effect even for various other malignancies.

MAPK- Cells recognize and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade that leads to activation of the (mitogen-activated protein kinases) MAPKs. All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate diverse cellular activities running the gamut from gene expression, mitosis, and metabolism to motility, survival and apoptosis, and differentiation. To date, five distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, γ, andδ , ERKs 3 and 4, and ERK5 (Chen et al., 2011). Although each MAPK has unique characteristics, a number of features are shared by the MAPK pathways studied to date. ERK are distributed throughout quiescent cells, but upon stimulation, a significant population of ERK accumulates in the nucleus. ERK signalling has been implicated as a key regulator of cell proliferation, and for this reason, inhibitors of the ERK pathway are entering clinical trials as potential anticancer agents (Kohno et al., 2003). BCR-proximal Lyn and Syk kinases induce PLCγ2 phosphorylation and Ras activation. Ras binds to and activates Raf kinase that subsequently activates MEK1 and MEK2 that lay immediately upstream of ERK (Muzio et al., 2008). Additionally ERK is constitutively phosphorylated in more than half of CLL cases. CLL cases presenting this signature may be taken as a human model of anergic B cells aberrantly expanded (Gauld et al., 2006; Muzio et al., 2008).

Further in the MAPK pathways is the p38MAP kinase which has been found to be important in many cellular apoptosis systems. A dependence of p38MAPK activity in B-cell–mediated apoptosis has, for example, been demonstrated in B-cell receptor (BCR)–mediated apoptosis in the B104 B-cell line (Graves et al., 1998). These data suggests that the p38 MAPK pathway plays a complex role in different apoptosis pathways in B cells. Rituximab (anti-CD20 antibody used as a immunotherapeutic agent for CLL) activates a CD20-mediated signalling pathway that results in apoptosis of the CLL cells. This action has been found to be dependent on p38MAPK activation (Pederson et al., 2002). *In vitro*, CLL cells are usually hyporesponsive to proliferative signals that activate normal B-lymphocytes (Fluckiger et al., 1994). However, CLL cells can be induced to proliferate by certain stimuli, such as triggering of the CD40 receptor or stimulation with CpG-

oligodeoxynucleotides (CpG ODN) (Decker et al., 2000;2002). The latter mimic CpG motifs present in unmethylated bacterial DNA, which are recognized by (Toll-like receptor 9) TLR9. This interaction activates several downstream signalling pathways, including NF-κB, phosphatidylinositol 3-kinase (PI3K)/AKT and the MAPKs. p38MAPK is also a key mediator in glucocorticoid-induced apoptosis in all lymphoid cells (Miller et al., 2005). The role of p38MAPK in CLL cell survival and apoptosis is paradoxical, whereby, in Mda-7 and IL-24, inducers of apoptosis in diverse cancer cells, promote the survival of CLL B-cells through p38MAPK activation (Sainz-Perez et al., 2006), which is in contrast to the apoptosis inducing mechanism mentioned before.

Additionally, constitutive activation of the p38MAPK is also observed in majority of CLL cases and is critical for matrix metalloproteinase-9 (MMP-9) production, which plays a critical role in tumor-angiogenesis and tumor homing (Molica et al., 2003). Elevated serum levels of MMP-9 which thus correspond to the activation of p38MAPK, might predict an early disease progression in CLL and survival of CLL cells on bone marrow stromal cells (Ringshausen et al., 2004).

Thus, the studies on the BCR signalling pathways and the protein kinases therin have brought an important contribution to the understanding the patho-physiology of CLL and contribution to the identification of effective therapies.

Further discussed in the results section are my own studies elucidating the role and activation of these enzymes in response to CD180 and BCR ligation in CLL cells and normal B-cells.

Role of Micro-environment in CLL

When CLL cells are removed from their natural micro-environment they spontaneously undergo apoptosis *in vitro*, suggesting that certain external stimuli support growth and survival of CLL cells *in vivo*, and that these are essential for the expansion of the leukemic cells(Ghia *et al*., 2005),. The *in vitro* culturing of CLL

cells with nurse-like cells, stromal cells and endothelial cells rescue them from apoptosis (Ghia *et al.,* 2008). There is increasing interest in the role of pro-survival signals provided by micro-environment in CLL. It has been observed that the host micro-environment and the resulting interplay between the genetic background and environmental influences play a crucial role in disease progression, as well as in resistance to treatment and resistance to apoptosis. Examples of interaction between CLL cells and lymph nodes (LN) micro-environment are shown in the Figure 1.3 below.

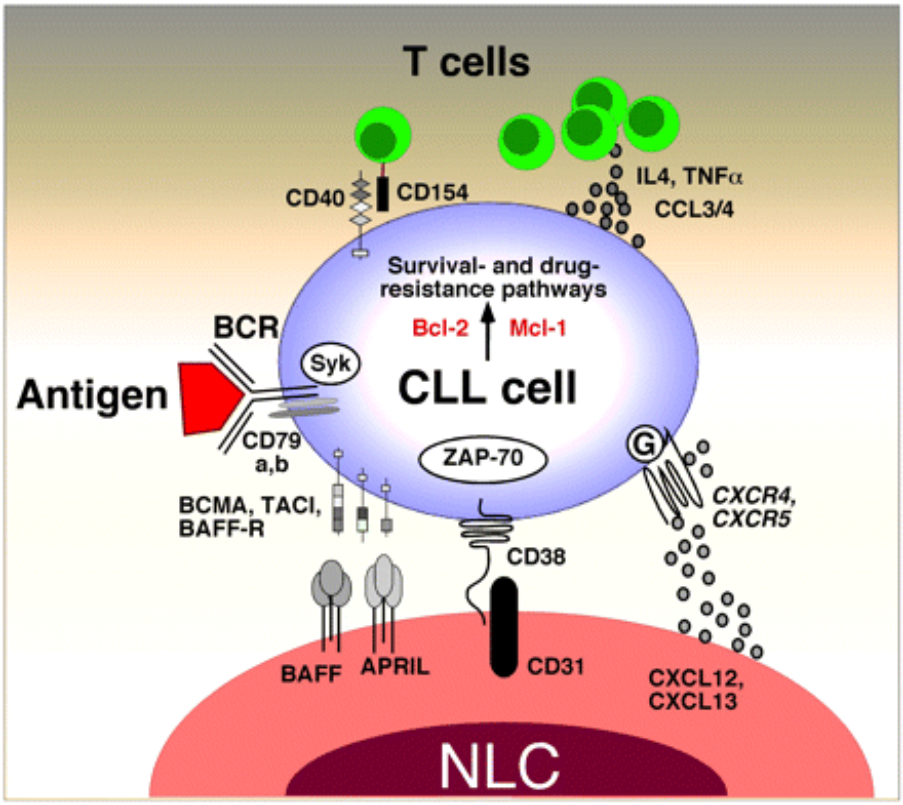


Figure 1. 3 Model for cross-talk between CLL cells and the lymph node micro-environment

Molecules involved in cross-talk between CLL cells and accessory cells in the lymphoid tissue micro-environments are shown in the figure. Contact between CLL cells and nurse like cells (NLCs) is established and maintained by chemokine receptors and adhesion molecules. NLCs express the chemokines CXCL12 and CXCL13. NLCs attract CLL cells via the G protein–coupled chemokine receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells. NLCs also express the tumour necrosis factor family members BAFF and APRIL, providing survival signals to CLL cells via corresponding receptors (BCMA, TACI, BAFF-R). CD38 expression allows CLL cells to interact with CD31, the ligand for CD38, expressed by stromal and nurse like cells. Ligation of CD38 activates ZAP-70 and downstream survival pathways. Self and/or environmental antigens (Ags) are considered a key factor in stimulation and expansion of the CLL clone. Stimulation of the B-cell antigen receptor (BCR) complex (BCR and CD79a,b) induces downstream signalling by recruitment and activation of Syk and ZAP-70. BCR stimulation and co-culture with NLC also induces CLL cells to secrete high levels of the chemokines CCL3 and CCL4, which are potent T cell–attracting chemokines. Through this mechanism, CLL cells can actively recruit T cells for cognate T-cell interactions with CLL cells. $CD154^+$ T cells are preferentially found in CLL pseudofollicles and can interact with CLL cells via CD40. Cytokines secreted by T cells or CLL cells, such as IL-4 or tumour necrosis factor α, are considered important regulators of CLL cell survival. Collectively, this cross-talk between CLL cells and accessory cells results in activation of survival and drug resistance pathways, such as those provided by Bcl-2 and Mcl-1 (Burger *et al.*,2009)

Stromal cells, that are found in the tissues admixed with small leukaemic lymphocytes (Burger *et al.,*2006) together with a number of other accessory cells, show the capacity to sustain prolonged viability of the leukaemic clone when this is placed *in vitro* for culture (Ghia *et al.,*2005). Similarly, adherent nurse-like cells, though not present in lymphoid tissue but obtained from PB of patients with CLL, are also able to protect leukaemic cells from spontaneous apoptosis (Burger *et al.,*2005). The same occurs when activated autologous T cells are co-cultured with CLL cells; though this action can be somehow replaced by the presence of T-cell derived cytokines (i.e. IL-4) and the exposure to T-cell-related molecules (i.e. sCD40L) (Ranheim *et al.,*1993). Besides rescuing leukemic cells from apoptosis, CD40 stimulation can also induce their proliferation and activation as witnessed by the up-regulation of several molecules on the cell surface (e.g. CD80, CD95), as well as the induction of chemokine production (e.g. CCL-22/MDC, CCL-17/TARC) and apoptosis regulators like Survivin (Granziero *et al.,*2001). Experimental findings suggest a scenario where CLL cells infiltrating lymphoid tissues interact with activated T cells that influence leukaemic B cell proliferation and provide a short-term anti-apoptotic support, whilst stromal cells (and other accessory cells, e.g. nurse-like cells) provide a long-term support that favours the extended survival and relentless accumulation of leukaemic cells (Ghia *et al.,*2005). These and other pieces of experimental evidence indicate that different cellular micro-environmental components deliver fundamental and specific signals for the maintenance and expansion of leukaemic B cells at different time points in the natural history of CLL. At the same time, CLL cells are active players in shaping the micro-environment according to their needs, seen by the production of selected chemokines (i.e. CCL22/MDC and CCL-17/TARC) which recruit activated T lymphocytes that will ensure provision of survival signals (e.g. IL-4 and CD40 ligation).

Historically, CLL has been viewed as an accumulative disease of cells with a defect in apoptosis. Consistent with this view, the majority of peripheral blood CLL cells are arrested in G0/G1 and show a gene expression profile of resting cells. However, recent studies using *in vivo* deuterated water labelling of CLL cells indicate a more important role of tumour proliferation in the progression of CLL

than was previously unappreciated (Messmer *et al.,*2005). LN has been identified a site of CLL cell activation and tumour proliferation by comparing the gene expression of CLL cells located to different anatomic compartments. Less pronounced changes were observed in the BM, suggesting distinct effects of the LN and BM micro-environment on the activation of signalling pathways and CLL tumour biology (Burger *et al.,*2006). Also, observed in the same study was that the expression of the anti-apoptopic transcription factor NF-kB was more pronounced in the LNs, through activation of the BCR signalling pathways, than in the PB cells in CLL (Herishanu *et al.,*2011). Immunohistochemistry for the cell-cycle marker Ki67 suggests that CLL proliferation occurs in the BM and secondary lymphoid organs, in spots or clusters of cells referred to as 'proliferation centres' (PCs). This characteristic of PCs in CLL also reinforces the concept of a sustained/persistent immune stimulation in CLL. Additionally, the CD38 has been shown to highly expressed by the cells in the PCs along with a higher expression of IgM indicating the role of these two receptors in proliferation of CLL cells (Soma *et al.,*2006; Deaglio *et al.,*2010). The signals and interaction of the cells in these PCs govern tumour proliferation and cellular migration, but have not been fully elucidated because most *in vitro* systems are not able to support similar environmental dynamics completely (Herishanu *et al.,*2010).

Chemokine receptors play an important role in coordinating the trafficking and organization of haematopoietic and non-haematopoietic cells within various tissue compartments and are constitutively expressed in distinct tissue micro-environments. CLL cells express high levels of the chemokine receptor CXCR4. Co-culture of CLL cells with marrow stromal cells that secrete CXCL12 induces the neoplastic B cells to migrate to and then underneath the stromal cells in a CXCR4-dependent fashion (Burger *et al.,*1999). Similar to marrow stroma, nurse-like cells attract CLL cells via CXCR4 and protect CLL cells from spontaneous or drug-induced apoptosis in a contact-dependent fashion (Burger *et al.,*2009). These observations support a model proposing that expression of CXCR4 by CLL cells allows for their recirculation between the blood and the marrow or lymphoid tissues, where they receive protective survival signals. Because CXCL12 not only attracts CLL cells to supportive micro-environments but also directly stimulates

CLL cell survival (Burger *et al.,*2000), the CXCR4-CXCL12 axis constitutes an important therapeutic target in CLL (Domanska *et al.,*2012; Burger *et al.,*2012). Also, it was observed by that stromal cell-mediated protection from spontaneous or Fludarabine-induced apoptosis of CLL cells was partially blocked by the use of CXCR4 antagonists suggesting a potential role of CXCR4 antagonists in combination with a B-cell targeted therapy in the treatment of CLL (Burger *et al.,*2005; 2010; 2012). Figure 1.4 demonstrates the role of the CXCR4 receptor and its interaction with CXCL12 in naïve and Pre-B and Pro-B cell developments particularly in the hypoxic environment which is common to all malignancies.

However, a number of technical restrictions limit further understanding the role of micro-environmental influences in CLL. For instance, *in vitro* studies typically analyze PB-derived tumour cells because BM and LN biopsies are often not available. In most of the patients with early stage CLL, the tissue biopsies are not performed because of the invasiveness of the procedure. Plus, mimicking the micro-environment *in vitro* requires further understanding of the micro-environment *in vivo*. The majority of studies are based on the peripheral blood cells which is in fact a "snapshot" of the real interactions in the solid tissues. Thus, the contribution of the host micro-environment to the proliferation and survival of CLL cells *in vivo* remains insufficiently defined.

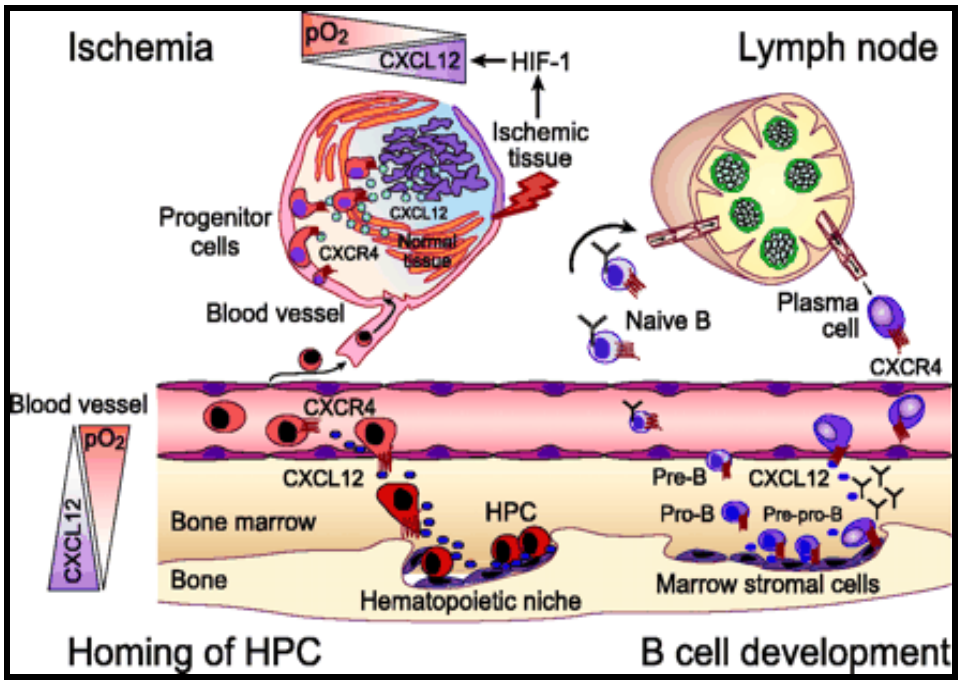


Figure 1. 4 Model for the CXCR4 chemokine receptor in homing and migration of haematopoietic progenitors, B-lymphocyte development, and progenitor recruitment to sites of ischemic tissue damage in various haematopoietic malignancies

(adapted from Burger *et al.*,2006).

Toll-like receptors (TLR) as micro environmental sensors in CLL

1.7 Toll-like receptors expressed by normal B cells

Receptors for pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) representing exogenous and endogenous micro-environmental factors respectively alongside BCR, are candidates for driving CLL cells. These pattern recognition receptors include Toll-like receptors (TLR) and there is increasing evidence for a role of both membrane-associated and endosomal TLRs in CLL. The 10 identified human TLRs and their respective ligands are shown in Figure 1.5. TLRs belong to the Interleukin-1 Receptor/Toll-Like Receptor superfamily and represent type 1 integral membrane glycoproteins, with molecular weights ranging 90-115kD. The presence of an extracellular domain containing leucine rich repeats (LRR) and a cytoplasmic toll/IL-1 receptor (TIR) domain, similar to that of IL-1, is characteristic of the TLR family (Shizuo *et al.,*2003). TLRs recognize molecules that are broadly shared by pathogens but distinguishable from host molecules (PAMPs), whilst certain TLRs, most commonly TLR2 and TLR4, also recognize DAMPs, which are endogenous ligands such as Heat-shock proteins (Hsp), High mobility group box protein (HMGB1) or viral endogenous glycoproteins (Gp96) (Park .*et al.,*2004; Asea.*et al.,*2001). CD14 has been identified as a cell surface adaptor molecule which assists the TLR signalling.

TLRs, apart from LRRs share conserved characteristic cysteine residues (Kumar *et al.,*2009). The last discovered TLR10 is an orphan receptor (unknown ligand); however, sequence analysis as well as chimeric receptor experiments suggested that human TLR10 and TLR1 share common mechanisms of innate immune sensing but not signalling (Guan *et al.,*2010). The TLR expression pattern is quite specific and unique for each cell type and species; in normal human B-cells TLR1, TLR2, TLR6, TLR7, TLR8, TLR9 and TLR10 are prevalently expressed.

Another receptor that belongs to the TLR superfamily, - CD180/RP105 was first identified in splenic murine B cells. Miyake *et al.,* (1995) named this antigen as Radio Protective 105 (RP105) as it protected B cells from radiation-dependent apoptosis. This is the main focus of my study, and it will be discussed further.

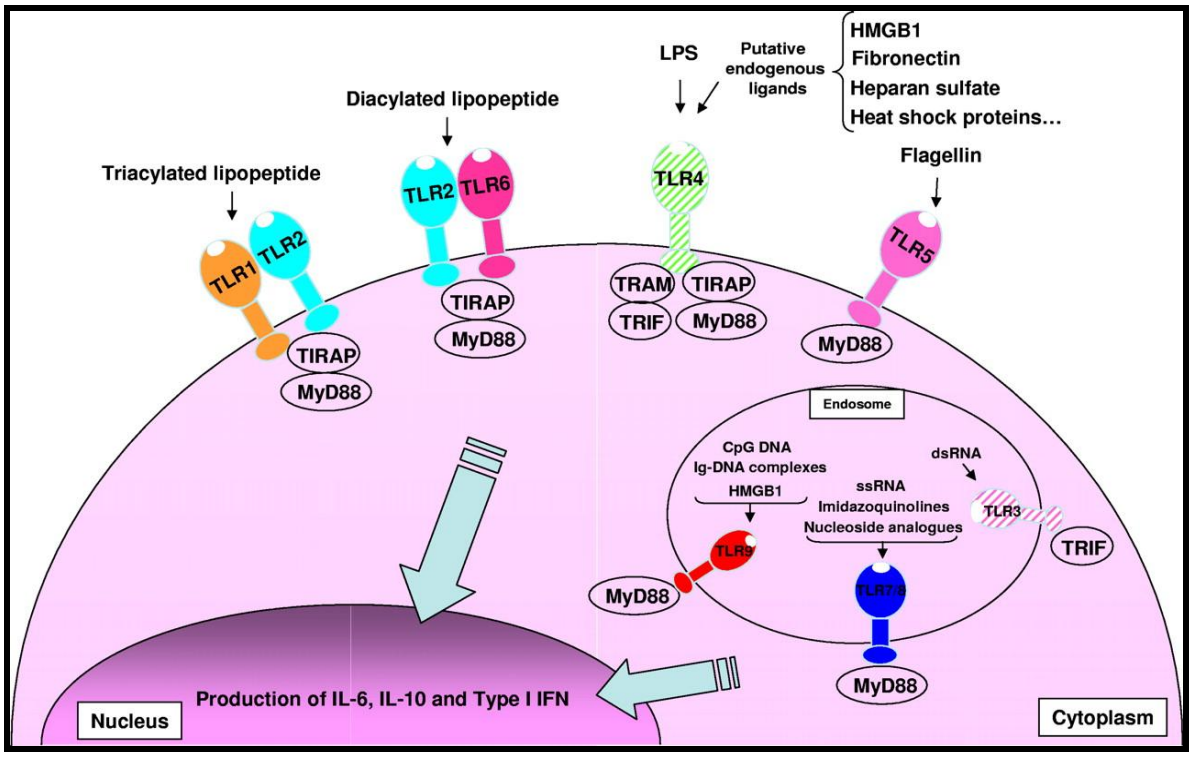


Figure 1. 5 Overview of TLRs-TLR ligands and their signalling complexes in normal and malignant human B cells

TLR2 associates with TLR6 or TLR1 to form receptors that recognize diacylated and triacylated lipopeptides, respectively. TLR3 recognizes virally derived dsRNA. TLR4 recognizes LPS from Gram-negative bacteria, and several putative endogenous ligands. TLR5 recognizes bacterial flagellin. TLR7 and TLR8 recognize ssRNA from viruses, imidazoquinolines, and nucleoside analogs. TLR9 recognizes CpG DNA from bacteria and viruses, immunoglobulin-DNA complexes, and HMGB1. Unlike TLR1, 2, 5, 6, 7/8, and 9, which are expressed by normal and malignant human B cells, TLR3 and TLR4 are expressed solely by malignant B cells and are shown hatched. A total of 4 signalling adaptors are involved in TLR signalling: MyD88, TRIF, TRAM, and TIRAP. TLR signalling pathways result in the production of IL-6, IL-10, and type I IFN (Chiron *et al.,*2008).

1.8 TLR-mediated signalling

The activation of TLR signalling pathways originates from the cytoplasmic TIR domains. In the signalling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, plays a crucial role in downstream signalling. TLR signalling pathways, besides a MyD88-dependent pathway (Figure 1.6.a.) that is common to all TLRs, include a MyD88-independent pathway that is specific to the TLR3- and TLR4 (FIgure 1.6.b.) (Akira *et al.,*2001; Takeda *et al.,*2003).

It has been suggested that the B cell stimulation and activation in response to LPS is brought about by the cooperative functioning of TLR-4 and RP105/CD180, the two molecules that share structural homology (Divanovic *et al.,*2005).

The two different mechanisms of activation of the signalling pathways, through TLR4, are elucidated in Figures 1.6.a and 1.6.b.

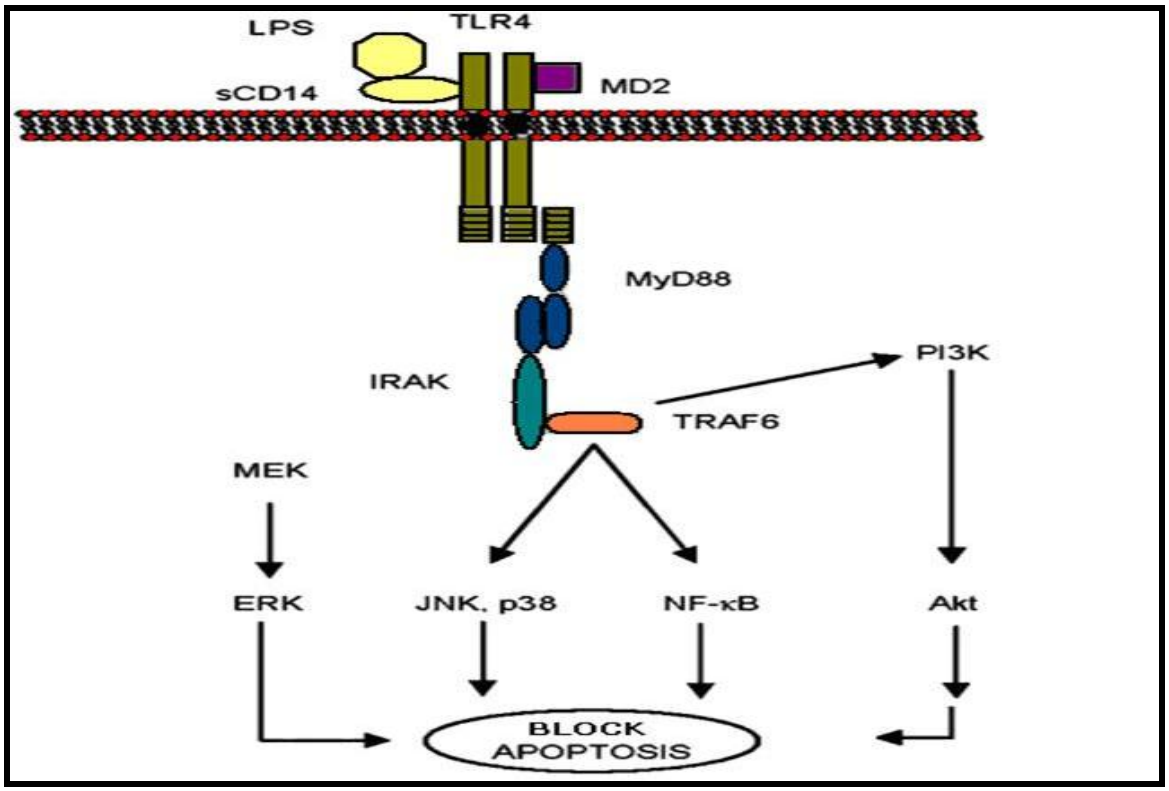


Figure 1. 6 a MyD88 dependent mechanism of TLR-4/MD-2 signalling

The TLR-4/MD-2 forms a complex with soluble CD14. This complex binds to LPS and recruits the myeloid differentiation protein (MyD88) molecule, TIRAP (TIR-containing adaptor protein), TRIF (TIR-containing adaptor protein inducing interferon ß) and TOLLIP (Toll-interacting protein).This leads to the attachment of IRAK4 to the receptor-protein complex. This binding of IRAK4 promotes the transphosphorylation of IRAK1. The phosphorylation results in the attachment of IRAK1 to TNF receptor-associated factor-6 (TRAF6). The IRAK1- TRAF6 complex is functional and signals the activation of various molecules which eventually lead to the release of NF-κB. This NF- κB enters the nucleus and initiates transcription. The IRAK1-TRAF6 also cause the activation of ERK, JNK, and p38 MAPKs kinases. The binding of LPS to the TLR-4/MD-2 also activates PI3 kinase. These molecules bring about phosphorylation which activates various second messengers which in turn activate AKT. This AKT is important for NF- κB activity in the nucleus. The NF- κB along with ERK, JNK, p38 and AKT inhibit the apoptotic signal in the B cells (Medzhitov *et al.,*2001; Takeda *et al.,*2003; Singh *et al.,*2003).

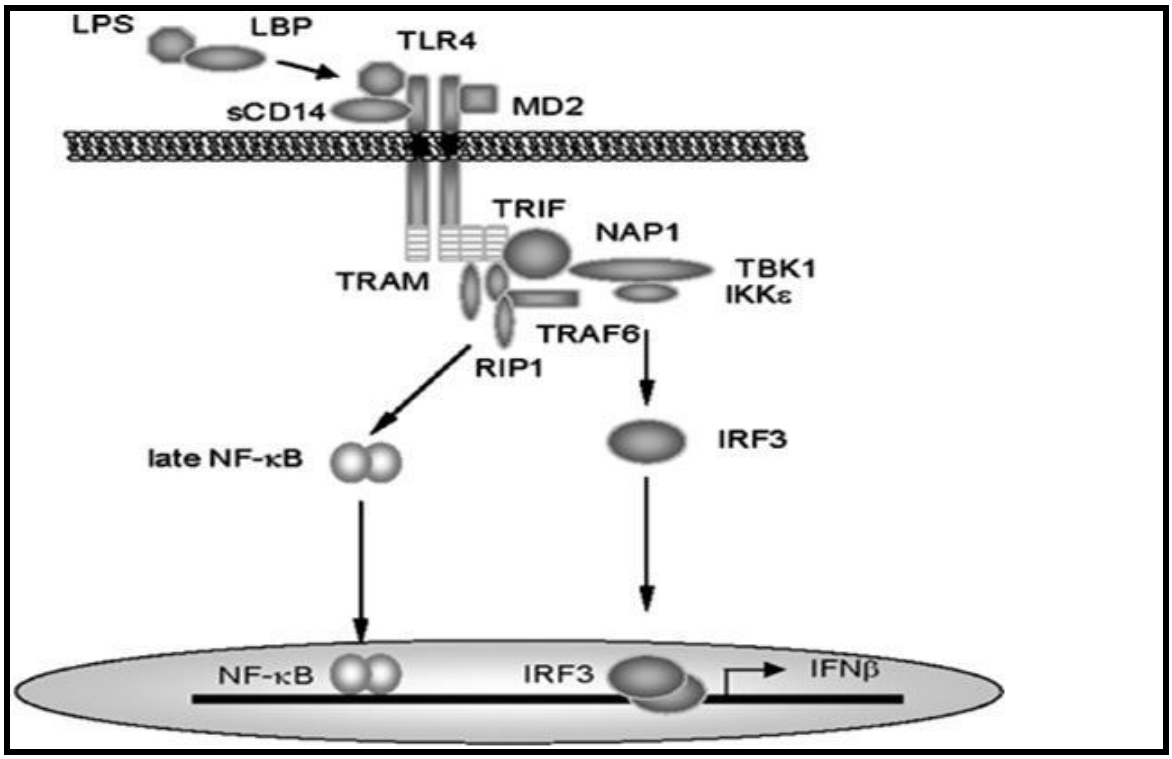


Figure 1.6.b MyD88 independent mechanism of TLR-4/MD-2 signalling

The TLR4 also functions in the absence of MyD88. The TRIF-related adaptor molecule (TRAM) binds to the cytoplasmic domain of TLR4. A molecule called TRIF then binds to this TLR4-TRAM complex formed. TRIF further activates IFN regulatory factor 3 (IRF3) and NF- κB through a series of activation steps. The TLR4-TRAM-TRIF complex binds to the TRAF6 and RIP1 which activate NF- κB and translocate it into the nucleus. In a parallel pathway, the TRAF family member-associated NF-κB activator (TANK) and IκB kinase (IKK) bind to the complex which phosphorylates IRF3. This activated IRF3 translocates into the nucleus. In the nucleus, NF-κB and IRF3 promote the activity of IFNß. This ultimately inhibits apoptosis of the B cells (Medzhitov *et al.,*2001; Takeda *et al.,*2003).

1.9 Toll-like receptors in CLL

We are only at the beginning of a large-scale investigation of possible roles of TLRs as environmental sensors in CLL and information is scarce. Some reports indicated that CLL cells display the same pattern of TLR expression as normal B-cells, yet with overexpression of TLR9. Furthermore, TLR7 and TLR9 appear to be functional and liable to respond to ligands, respectively imidazoquinolines and CpG-ODN, thus potentially opening new therapeutic approaches (Grandjennete *et al.*,,2007).

TLR expression repertoire of freshly isolated CLL cells appeared to be similar to the pattern described in B-cells from tonsils and in memory B-cells and is unrelated to disease stage, mutational status of *IGHV* genes, expression of CD38 or ZAP70 (Bernasconi *et al.*,, 2003; Bourke *et al.*,, 2003).

Since a number of stimuli from the micro-environment play a role in the survival and proliferation of CLL cells, the idea that TLR ligands play a similar role in CLL is intriguing. TLR9 ligands have been previously shown to induce an immunogenic phenotype in CLL cells as defined by the expression of costimulatory molecules and specific cytokines (Spaner *et al.,* 2007). Muzio *et al.,*(2008) demonstrated that TLR ligands for TLR1/2, TLR6/2 and NOD2 are capable of inducing activation of CLL cells determined as an increase in CD25 and/or CD86 expression.

TLR7 ligands were shown to enhance CLL cell sensitivity to chemotherapy and immunotherapy (Shi *et al.*,, 2007; Spaner & Masellis, 2007), and to sensitize them to *in vitro* apoptosis (Spaner *et al.,*2006; Grandjenette *et al.,* 2007). On the other hand, a heterogeneous response was observed when CLL cells were exposed to TLR9 ligands (CpG-ODN) and analysed for apoptosis and/or cell proliferation. In some cases an apoptotic effect was observed while in others an initial triggering of proliferation was detected (Decker *et al.,*2000,2002; Castro *et al.*,, 2006; Longo *et al.,*2007) suggesting that TLR9 ligation may exert both a "pro-tumour" and/or "anti-tumour effect".

A model has been proposed, in which the co stimulation of three different signals derived from BCR, CD40 and TLR is required to induce full activation, proliferation

and differentiation of naive B-cells (Ruprecht *et al.,*2006). It was shown that a specific culture system using CpGs, together with sequential steps for T-cell-independent activation of naive human B cells, can induce plasma-cell differentiation (Huggins *et al.,*2007)

CLL is often associated with an increased frequency and severity of infections. It was suggested that common infections may play a role in CLL aetiology which could be due to underlying immune disturbance in CLL patients, and/or to a direct effect of microbial antigens on the leukaemic clone. Given all this, one could hypothesize that inflammation or autoimmunity mediated by distinct TLRs may also play a role in regulating the development, progression and/or accumulation of CLL. Indeed, in mouse models of CLL the lack of the inhibitory receptor TIR8, which allows TLR-mediated stimulation, triggers leukaemia progression *in vivo* (Bertilaccio *et al.,*2011). Since TLRs can improve immune response but may also be involved in modulating tumour cell proliferation or apoptosis, the possibility that TLR activity may shuttle between defence from and promotion of leukaemic growth has to be taken into account. All the data available also supports the hypothesis that in addition to endogenous micro environmental factors, foreign microbial components may have a role in sustaining the malignant clone also *in vivo*. However, it is yet unclear whether TLRs contribute and to what extent to early or late phases of the natural history of CLL.

CD180/RP105 in B-cells and CLL cells

CD180/RP105, has been reported to contribute significantly to specific phenotypical and functional characteristics of B cells and CLL cells (Porakishvili *et al.,*2005; 2011). The molecule CD180 was originally identified on human B cells by a monoclonal antibody (mAb) designated Bgp95 (Valentine *et al.,* 1988). Subsequently, CD180 was found on naive B cells but not on germinal center (GC) B cells (Otipoby *et al.,* 2002). As I mentioned above, anti-RP105 cross-linking promoted murine B-cell proliferation as well as resistance against radiation- and dexamethasone-induced apoptosis (Miyake *et al.,* 1995). Kobe and Deisenhofer,

(1994) suggested the involvement of RP105 in protein-protein interactions, such as cell adhesion or receptor-ligand binding and piqued interests in its function in B cells.

1.10 CD180/RP105 structure and function

CD180 is the human analogue of the murine surface receptor RP105 sharing 74% sequence homology. It is included in the TLR family since it shares structural similarity with the TLRs. It is homologous to TLR4 but lacks the intracellular TIR-like domain (Miyake *et al.,*1995; Divanovic *et al.,*2007). CD180 is a type I transmembrane protein of 661 amino acids (105 kDa) comprising of 22 tandemly repeated extracellular leucine-rich repeats (LRR) and a short cytoplasmic domain of 6 to 11 amino acids. Conserved cysteine residues are also present which are essential for signal transduction through RP105/CD180 (Figure 1.7) (Miyake *et al.,* 1995; Muira *et al.,*1998; Divanovic *et al.,* 2007). Fluorescence in situ hybridization (FISH) on the sequence revealed the location of murine and human RP105 gene to be chromosome 5q12 (Madzhitov *et al.,*2001).

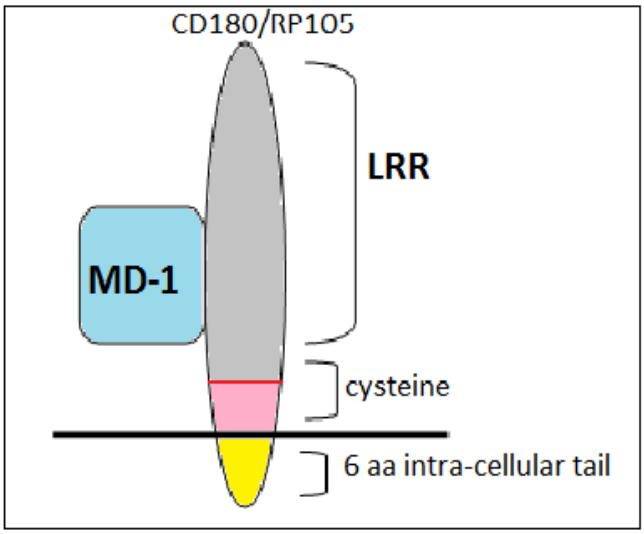


Figure 1. 7 structure of CD180 on cell surface.

CD180 is a 105-kDa type1 membrane protein of the TLR family. The extra-cellular motif contains 22 LRRs and conserved cysteine residues. It is physically associated with MD-1. The intra-cellular tail is very short with only 6 amino acids and absence of adaptor signalling molecule.

CD180 is an orphan receptor (unknown natural ligand), expressed on various cells of the immune system including macrophages, peripheral blood monocytes and dendritic cells, naive and mature B cells and marginal zone/mantle zone B cells but not on germinal center (GC) B cells (Otipoby *et al.,*2002; Divanovic et.al,2005; Nagai *et al.,*2012). CD180 is physically associated with molecule MD-1 which is indispensable for its cell-surface expression (Figure 1.7) and analogous to TLR4/MD2 complex. MD-1 plays an important role in cell surface expression of CD180 such that without MD-1, the majority of human CD180 is held in cytoplasm without being expressed on the surface (Miura *et al.,*1998). It was shown that MD-1 down-regulation with the antisense oligodeoxynucleotides led to impairment in LPS-induced CD80/CD86 up-regulation on bone marrow-derived dendritic cells indicating also its importance in the function of the CD180/MD-1 complex (Gorczynski *et al.,* 2000). Murine B-cell responses to LPS binding and activation in absence of MD-1 were impaired (Miura *et al.,*1998; Ogata *et al.,*2000). Murine B cells require the signal through CD180/MD-1 and also through TLR4/MD-2 for CD86 (B7.2) upregulation, proliferation, and antibody production (Nagai *et al.,*2012).

In antigen presenting cells, LPS binds directly to MD-2 to form a LPS/MD-2 complex which then associates with TLR4 to initiate signalling. However, the co-expression of CD180/MD-1 inhibits LPS-TLR-MD-2 complex formation thus providing a direct evidence of CD180/MD-1 physiological negative regulator of TLR4 responses (Divanovic *et al.,*2007).

On the other hand, in murine splenic marginal zone (MZ) B cells, Lipid A moeity of LPS (TLR4 ligand) plus CD180 stimulation induces massive proliferation and expression of Bcl-xL and c-Myc which in turn contribute to TLR4-mediated anti-apoptotic responses in MZ B cells.

Importantly from the point of CLL studies it has been shown that B cell lymphoblasts stimulated with anti-CD180 mAb underwent apoptosis after cross-linking of surface IgM. In B cell lymphoblasts activated through IgM, co-ligation of Fc gamma receptor IIB (FcγIIB) aborted the downstream signalling. Contrastingly, in CD180-stimulated blasts, co-ligation of FcγIIB with sIgM augmented, rather than

aborted, signalling. This response was specific to CD180 blasts and not comparable to those activated through anti-CD40 (Yamashita *et al.,*1996). Anti-CD180 mAb induced strong polyclonal activation, proliferation and Ig production (mainly IgG1 and IgG3) in mature/marginal/transitional B cells (Chaplin *et al.,*2011). More recently, it has been shown that CD180/MD-1 complex is indispensable for TLR4/MD-2-dependent proliferation and IgM-secreting plasma cell differentiation of MZ B cells (Nagai *et al.,*2012).

1.11 CD180-mediated signalling in normal B cells

As previously discussed, a number of studies demonstrated that CD180 has a role in B-cell survival, activation (assessed by up-regulating CD86), proliferation and/or differentiation. However, the mechanisms or pathways through which the receptor signals are not yet sufficiently understood.

Since CD180 does not have a functional cytoplasmic signalling domain, it cannot independently propagate an intracellular signal. It has to therefore recruit or converge with other receptor pathways. As mentioned in section 2.1 (Figures 1.6.a, b) all TLRs have an intracellular TIR domain and an adaptor molecule Myd88 which activates the various downstream PTKs. The functioning of CD180 is, however, is not regulated by MyD88 but by CD19. This is consistent with the fact that CD19 deficient human B cells show diminished proliferation following CD180 ligation. In mice the binding of LPS to the CD180/MD-1 complex results in the phosphorylation of CD19. This leads to the translocation of CD19 into the lipid rafts where the Src kinase Lyn is located. The translocated CD19 in turn activates the signalling molecules Lyn and Vav. CD19 forms a bridge between the interaction of Lyn and Vav. Lyn and Vav interaction is considered crucial for JNK activation. Importantly, in view of this study, PI3K and NF-κB activation by CD180 binding was shown to be independent of CD19 (Figure 1.8). (Yazawa *et al.,* 2003).

The LPS binding to RP105/MD-1 also phosphorylates PI3-kinase which recruits Btk (Bruton tyrosine kinase) to the cell membrane which causes the Ca2+ mobilization to the extracellular region. The Ca2+ mobilization is important for the phosphorylation and translocation of NK-κB to the nucleus although Btk can be

activated by Lyn. Moreover, CD19 is not required for the CD180 induced Ca2+ mobilization. The proteins JNK, NK-κB, p38MAPK and ERK together induce a signal to prevent apoptosis, thus explaining partly the role of CD180 in cell survival (Yazawa *et al.,*2003; Hebeis *et al.,*2005; Divanovic *et al.,*2007). Another CD180 mediated pathway study demonstrated that in addition to Lyn, protein kinase C β I/II (PKCβI/II), and Erk2-specific mitogen-activated protein (MAP) kinase (MEK) are essential and probably functionally connected elements of the CD180 mediated signalling cascade in B cells (Chan *et al.,*1998).

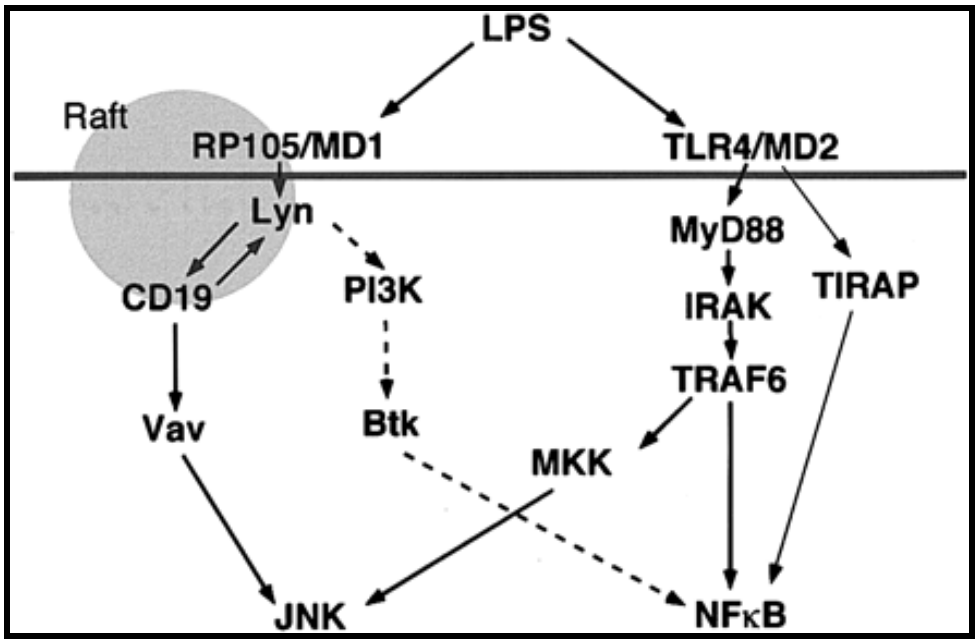


Figure 1. 8 Signalling pathway for CD180/RP105

RP105/CD180 on binding to its ligand, induces Lyn activation and CD19 phosphorylation, which leads to augmentation of Lyn activity and mediates interaction of Lyn with Vav, leading to activation. (adapted from Yazawa *et al.,* 2003).

Various studies have focused on the expression of CD180 on B cells in different diseases. The expression of CD180 on B cells of systemic lupus erythematous (SLE) patients was lesser than its expression on B cells of rheumatoid arthritis patients There was no significant difference in the expression of CD180 on the B cells of rheumatoid arthritis and normal healthy patients (Koarada *et al.,*2001). There was a significant loss of CD180 expression on B cells in dermatomyositis, whereas polymyositis patients have similar levels of CD180 to those of the normal subjects (Kikuchi *et al.,*2001). More recently, Miguet.*et al.,* (2012) showed that circulating cells from marginal zone lymphoma (MZL) exhibited strong surface expression of CD180, significantly higher than that by normal B-cells. This study identified CD180 as the first positive immunological marker for MZL, able to distinguish MZL from other B cell malignancies (Miguet *et al.,*2012).

1.12 Expression patterns and function of CD180 on CLL cells

Our group at the University of Westminster and UCL was the first to demonstrate that CD180 was heterogeneously expressed on approximately 2/3 of CLL samples (Porakishvili *et al.,* 2005).

Significantly higher expression of CD180 was observed on M-CLL cells. In contrast, the expression of sIgM was significantly higher on U-CLL cells. The research group further demonstrated that approximately half of CD180+ samples responded to ligation with anti-CD180 mAb by activation (upregulation of CD86), cycling (upregulation of Ki-67), and reduced basal apoptosis (assessed by changes in mitochondrial membrane potential) (*Porakishvili et al.,2011*). These CLL clones were termed responders (R). In contrast, CD180+CLL samples that failed to respond to anti-CD180 mAb by activation and cycling were termed non-responders (NR). CD180 ligation delivered a comparable, or superior, to CD40 and IL-4 activation, survival, and cell cycling signal for both normal B cells and CLL cells. We further showed that the defect in activation of the unresponsive $CD180^{+}$ CLL clones following ligation of CD180 is downstream of a ZAP70/Syk-dependent signalling pathway (Porakishvili *et al.,*2011).

Aims and hypothesis

The working hypothesis of the study undertaken was that since CD180 plays an important role in interaction of CLL cells with their micro-environment it may contribute to activation and expansion of leukaemic clones, *in vivo,* in lymph nodes and bone marrow within "proliferation centers" (PCs). CD180 ligation with putative endogenous or exogenous ligand would therefore contribute to the pro-survival intracellular signalling in CLL cells. To this end CD180 could act as co-stimulatory molecule together with CD40/CD40L and cytokines to provide signals for CLL cell expansion and survival.

Since CLL cells receive the major pro-survival stimuli from BCR, interaction between CD180 and BCR-mediated signalling pathways were studied with an aim to investigate the regulatory effects of CD180-signalling on BCR.

The major aims of this study were:

1. To assess the activation of intracellular protein kinases in CLL cells following CD180 ligation;
2. To establish the modulatory effect of CD180 engagement on the survival of CLL cells;
3. To establish putative interactions between the CD180 mediated and the BCR induced signalling pathways in CLL;
4. To study the expression of CD180 in control and CLL lymphoid tissues.

Chapter 2 Materials and methods

2.1 Patients

Eighty-five CLL patients, aged between 39-90 years, were included in the study. Patients were mainly Binet stages A and Rai Stages – Low Risk. The stages and white blood cell counts of patients are shown in Table 2.1. Forty two patients were M-CLL and thirty-six were identified as U-CLL. The genotype of the remaining seven patients was unknown. The most common chromosomal aberration detected was 13q deletion (n=14) and 17p deletion (n=14). Three patients were detected with trisomy 12 chromosomal aberration. No chormosomal abberations were detected for the remaining fifty four patients. Patients were considered to be untreated if they had received no treatment during the 6 months prior to the study. Fourty eight patients during the course of the study underwent treatment with a variety of therapeutic agents including chlorambucil (n=7), fludarabine (n=6), alemtuzumab (n=33) and rituximab (n=2).

The control B cells were age matched and collected from prior frozen samples at the laboratories in Feinstein Institute for Medical Research, USA (courtsey of Dr. Nicholas Chiorazzi).

The sample collection from and immunohistochemical staining (for the lymph nodes, bone marrow and tonsils) was performed at the laboratory of Dr. Teresa Marafioti at UCL department of Pathology. This work was undertaken as a collaborative project and under complete guidance of Mrs. Jennifer Paterson.

Ethical approval was obtained (both for the blood and solid tissue samples) according to the ethical committee at University College London Hospital (UCLH) NHS trust and informed consent from the patients themselves who were under the care of Dr. Amit Nathwani. Additionally, the University of Westminster ethical committee approval was obtained according to the MTA (Material Transfer Agreement) with UCL. In the USA, Institutional Review Board of the North Shore–Long Island Jewish Health System and the Feinstein Institute for Medical Research ethically approved the studies undertaken.

Table 2.1 CLL stages of the patient cases studied and the co-responding WBC counts

Rai Stage and Binet Stage	Number of Patients	WBC count $x10^9/L$ Range
Rai- Low Risk Binet stage A	33 8	4.21-100.6
Rai- Intermediate Risk	21	6.6 – 196.0
Rai- High Risk	12	7.1 – 200.0

2.2. Isolation of peripheral blood mononuclear cells (PBMCs) in the density gradient

Ten millilitres of whole blood was taken from CLL patients into heparinised test-tubes by specially qualified staff. Hanks' buffered salt solution (HBSS) and Histopaque 1077 (Sigma, U.K.) density medium were allowed to equilibrate to room temperature before the experiment. After dilution of the samples in equal volume of HBSS, 6-8 ml of sample was carefully layered onto 3 ml density medium. By centrifugation (5810 R centrifuge Eppendorf, U.K.) at 400g (30 min, room temperature), the samples were separated and (peripheral blood mononuclear cells) PBMCs were collected by aspiration of the interphase. The cells were washed in 10 ml of HBSS, by centrifuging for 15min, 400g at 4°C. After discarding the supernatant the cells were washed again with 5 ml Roswell Park Memorial Institute 1640 (RPMI) (Gibco, U.K.) supplemented with 10% Foetal bovine serum (FBS) (Sigma, U.K.) by centrifuging for 10min, 400g, 4°C. The supernatant was discarded, the cells were re-suspended in 1ml of RPMI-1640 (supplemented with

10%FBS), counted in a haemocytometer and the concentration of cells adjusted to the required for each experiment.

2.3 Isolation of B-CLL cells by positive selection

After counting, the cells were centrifuged at 400g for 5 min at 4°C, resuspended in 5mL iMAC buffer (Miltenyi Biotec, USA) composed of phosphate buffered solution (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2μM EDTA (Sigma, USA) and washed by centrifugation for 10 min, 4°C at 450g. 80μL of iMAC buffer and 20μL of CD19 multisort microbeads (Miltenyi Biotec, USA) were added for every 10^7 cells and incubated at 4°C for 15 min. Following incubation, 10mL of iMAC buffer was added to the mixture and the cells were centrifuged at 450g for 7 min, 4°C. Excess fluid was removed and 0.5mL of iMAC buffer was added to enable the re-suspension of the cells by gentle tapping of the tube. The iMAC column (Miltenyi Biotec, USA) was washed with 0.5mL of iMAC buffer and the cell suspension was carefully pushed through followed by three washes using each time 0.5mL of MAC buffer. Cells were centrifuged at 450g for 5 min at 8°C, re-suspended in 1mL of RPMI-1640 and counted with haemocytometer. The purity of the B-CLL cells, ascertained by staining with PE-Cy5-conjugated mouse anti-human CD19 mAb (BD biosciences, USA) and analysed by flow cytometry with BD FACS*Verse*™ using BD FACSuite™ software (Becton Dickinson Immunocytometry Systems, USA). This was found to be 95% or greater for each selection.

2.4 Phenotyping of CLL cells (peripheral blood and tissues) and normal B-cells

Into each well of a 96 well round bottomed microplate (Nunc, Fisher Scientific, U.K.) 200μl of PBMC suspension was distributed at a concentration $1x10^6$/ml. Plate was centrifuged at 400g for 5min at 4°C to concentrate the cells and supernatant discarded. Into each well, 20μl of 2mg/ml human immunoglobulins (Ig) (Sigma Aldrich, U.K.) was added in order to block any non-specific binding via Fc-receptors. The microplate was incubated on ice for 20min and cells washed with 200 μl HBSS by pipetting and centrifuging for 5 minutes at 400g (repeated twice). 20μl of 20μl/ml IgG1 isotype control (BD Pharmingen, U.K.) was added in the first

well followed with 20µl of primary (unconjugated) mouse antibodies in the next 7 wells to the following receptors: anti-CD180 (BD Pharmingen, U.K.), anti-IgM (BD Pharmingen, U.K.), anti-CD79b (Fitzgerald, U.S.A.), anti-CD38 (Fitzgerald, U.S.A.), anti-CD86 (Clone Bu63, a gift from Prof. B. Chain, UCL, U.K.), anti-IgD (Sigma, U.K.) and anti-CD40 (a gift from Prof. E. Clarke, University of Washington, Seattle, U.S.A.) for a final concentration of 20µl/ml. The plate was incubated on ice and in dark for 30min followed by washing the cells twice with HBSS (same as above). As the secondary antibody, 20µl of FITC-conjugated rabbit anti-mouse Ig (Dako, U.K.), was optimally diluted 1:15 in Phosphate Buffered Saline solution (PBS) (Sigma, U.K.) and was added into each well. The plate was incubated in the dark on ice for 30min, centrifuged at 400g and cells were washed twice as before. To block any free rabbit anti-mouse F(ab') sites, the cells were further incubated with 20µl of mouse serum (Dako, UK) for 30 minutes, (1:15 diluted in PBS), centrifuged, supernatant discarded, and stained with 15µl of PE-Cy5-conjugated mouse anti human CD19 mAb (BD Pharmingen, U.K.).

Cells in each well were fixed with 200µl of 2% Paraformaldehyde (PFA)(Sigma, U.K.) in PBS and stored (not more than 4 days) at 4°C until analysis by flow cytometry using Cyan (Beckman Coulter, UK) flow cytometer and Summit software v4.3. Alternatively, if the flow cytometry analysis was performed instantaneously, 200µl of HBSS was added in PBS into each well prior to the analysis. The results were expressed as percentages of positive cells as well as Relative binding sites RBS/cell as previously described (Guyre *et al.,* 1989; Porakishvili *et al.,* 2005). This is a method of evaluation of the level of the expression 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 (Sphereotech, USA or 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).

2.5 Assessment of the expression of CD86 and Ki-67

200µl of cell suspension at a concentration $1x10^6$ cells/ml in RPMI+10%FBS were distributed into a 96-well flat-bottom microplate (Nunc, Fisher Scientific, U.K.). Under sterile conditions 8µl of anti-CD180 monoclonal antibody (mAb), sodium azide free stimulatory (clone G28.8, a gift from Professor Edward Clark, University of Washington, Seattle, U.S.A.) was added to wells to stimulate CLL cells, whilst the control wells were left without additives, followed by 72h incubation in 5% CO_2 at 37°C. Upon incubation the cells were then transferred to 96 well round-bottom microplate (Nunc, Fisher Scientific, U.K.) while slowly acclimatizing them to room temperature and washed twice in HBSS at 400g, 5min, 4°C, and the supernatant discarded. The cells were stained with 10µl of PE-Cy5 conjugated anti-CD19 mAb (BD Pharmingen, U.K.) and 10µl of PE conjugated anti-CD86 mAb (eBiosciences, U.K.) and incubated on ice in the dark for 30min. The cells were washed twice in HBSS by centrifuging at 400g, 5min, 4°C.

In separate experiments following 72h long incubation and washing cells were permeabilized and fixed using Cytofix/Cytoperm reagent (BD Biosciences,U.S.A.). 8µL of FITC conjugated IgGI isotype control or 8µL of FITC conjugated anti-Ki-67 mAb (BD Pharmingen, U.S.A.) were added to the designated wells and the plate incubated for 30 min on ice in the dark. Delayed washing for intracellular staining was carried out by adding 200µL of PBS wash buffer and leaving the plate on ice for 15 min prior to centrifugation.

Finally the cells were re-suspended in 200µl of 2% paraformaldehyde (PFA) and stored in the dark at 4°C until Flow Cytometry analysis was performed. The percentages of CD86+ or Ki-67+ cells were calculated, by gating on CD19+ cells.

2.6. Detection of intracellular phosphorylated protein kinases:

2.6.1 by flow cytometery

200µl of isolated PBMCs from each patient at a concentration of $1x10^6$ cells/ml in RPMI+10%FBS were added to 4x4 wells of a 96 well flat bottom microplate. Under sterile conditions 8µl of sodium azide free stimulatory anti-CD180 mAb (clone

G28.8, a gift from Professor Edward Clark, University of Washington, Seattle, U.S.A.) was added to 2x4 wells with the cells. The plate was incubated at 37°C, 5% CO_2 for 30 min (short-term cultures) or for 24h (long-term cultures). In some experiments, the cells were incubated with anti-CD180 mAb for 15 min followed by the addition of anti-IgM antibodies (goat $F(ab')_2$ anti-human IgM; Southern Biotech, U.S.A) and incubated for another 15 minutes or 24h. Following incubation cells were washed twice in 200µl HBSS, centrifuging at 400g, 5min, 4°C, discarding the supernatant and vortexing the plate. The cells were then transferred to 96 well round-bottom microplate. 10µl of Cy5-conjugated CD19 mAb were added to each well and incubated in the dark, on ice for 30minutes followed by two washing steps performed using 200µl HBSS. For fixation, 70µl of solution A of the Fix/Perm Kit (Caltag Laboratories, U.S.A.) were added to each well and incubated in the dark at room temperature for 15min. The cells were washed once with HBSS as before. 70µl of permeabilisation solution B of the Fix/Perm kit was applied and incubated in the dark at room temperature for 15min. The cells were centrifuged at 400g, 5min, 4°C, the supernatant discarded, and the plate vortexed. The following antibodies were applied: 10µl of Alexa Fluor 647 conjugated anti-ZAP70/Syk (p) (Cell Signalling, U.S.A.), FITC conjugated anti-ERK(p) (BD Pharmingen, U.K.), FITC conjugated anti-p38MAPK(p) (Cell Signalling, U.S.A.), FITC conjugated anti-AKT(p) (Cell Signalling, U.S.A.), Phospho-Btk (Tyr223)PE conjugated (Cell signalling, U.S.A) and incubated in the dark, on ice for 30min. After incubation the cells were washed as above. Finally the cells were fixed with 200µl 2% PFA and stored in the dark at 4°C for analysis by Flow cytometry, for no longer than 24h.

2.6.2 by Immunoblotting

Upon stimulation of CLL cells as described in 2.6.1 but at a higher concentration of 10^7 cells/ml, cultures were centrifuged in 1.5ml tubes (450g, 5min) and the supernatant discarded. Cell pellet was then washed with 1ml of HBSS as above and re-suspended in approximately 80 µl of whole cell lysis solution including 1µl of PMSF (phenylmethanesulfonylfluoride, Sigma, U.K.) diluted in Dimethyl sulfoxide (DMSO, Sigma, U.K.) and 1 µl Protease Inhibitor cocktail (PI) (Sigma, U.K.). The cells were centrifuged for 10 min at 17000g. Carefully supernatant was pipetted taking

care that the DNA pellet does not contaminate the protein sample. The protein solutions were then transferred to separate 1.5ml centrifuge tubes and stored at -80°C until further procedures.

20µl of the protein samples extracted from CLL cells as above, were added to a 10µl of Loading dye mix containing 7.5 µl loading dye, (Invitrogen,U.K.) and 2.5 µl 1M Dithiothreitol (DTT) (Sigma, U.K.). For assaying molecular weights of the proteins, we used the High-Range Rainbow Molecular Weight Markers HMW (RPN756) (Invitrogen, U.K.) with the loading dye and 10µl water as above. The samples were heated at 70°C for 10 mins in heat blocks and were ready to be loaded on the pre-casted 1% gel (4-12% tris glycine gels EC6035BOX) (Invitrogen, U.K.). The samples were loaded and the gel was run at 125V, 40mA, 10 w for 1 hour in the tank filled with MOPS running buffer (Invitrogen, U.K. filled). 500 µl of anti-oxidant (Invitrogen, U.K.) was also added to the buffer.

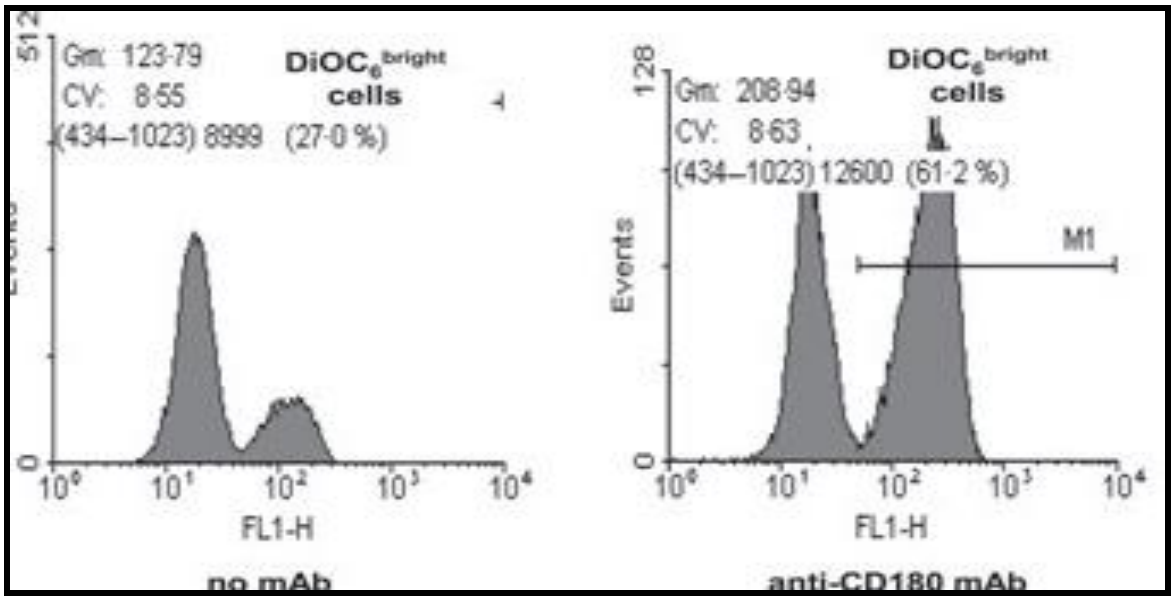
For protein transfer 200 ml of transfer buffer was used per gel cassette (400ml methanol, 150ml distilled water and 200 µl anti-oxidant). A 3mm filter paper (Whatman, GE Healthcare, U.K.) soaked in the transfer buffer was placed on the isolated gel followed by a piece of wetted HYBOND (Amersham, U.K.) membrane. The gel was placed in the 'Invitrogen wet transfer system', the cassette was covered with the transfer buffer and the tank attached to the power pack to allow the transfer of protein for 1 hour at 25V, 125mA, 15W.

After the transfer, the HYBOND membrane was removed and stained with Ponceau S (Sigma, U.K.) for 30 seconds, followed by washing the membrane with wash buffer and blocking with FCS and Polyvinylpyrrolidone (Sigma, U.K.) diluted 1:10 in 10X TBS for 40 mins. The following primary unconjugated antibodies were then added to blocking buffer at the optimal concentrations along with 0.1% Sodium Azide and incubated overnight: The next day, the blot was washed with the washing buffer and incubated with the secondary horse-radish peroxidase (HRP) conjugated antibodies to pAKT, pp38MAPK, pErk, Mcl-1, pP13K, Bcl-2, Bcl-xL (all from Cell Signalling, USA) for 2 hours and washed. The visualization of the protein bands was performed with Enhanced

chemiluminescence reagent(ECL)(Amersham Biosciences, U.K.) and the Fuji XRay film.

2.7. Assesment of apoptosis by the changes in mitochondrial membrane potential

CLL cells were stimulated as described in 2.6.1 and incubated for 24h. Upon stimulation the cells were washed as described above and treated with 10μL of PE-Cy5 conjugated anti-CD19 mAb for 30min on ice in the dark. Following two washes with PBS-AB buffer, the cells were re-suspended in 200μL of HBSS and 0.2μM of $DiOC_6$ was added to each well. The optimum concentration of $DiOC_6$ was previously determined in our laboratory (data not shown). $DiOC_6$ [(3) - (3,3'-dihexyloxacarbocyanine iodide] is a fluorescent dye that discriminates between bright fluorescent viable and dim fluorescent apoptotic cells upon exposure to blue light which excites $DiOC_6$ to fluoresce green (Terasaki, 1989; Koning *et al.,* 1993). The plate was incubated for 20 min at 37°C and 5% $C0_2$ and cells analysed by flow cytometry immediately. The level of viability was assessed by the percentages of $DiOC_6^{bright}$ cells gated on the CD19+ cell population.



(A) **(B)**

Figure 2. 1 representative flow cytometric profile of 72 h cell cultures: without CD180 antibody (a) and after incubation with anti-CD180 mab (b)

PBMC from normal controls and B-CLL patients were cultured in the absence and presence of 10μg/mL anti-CD180 mAb. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and 0.4μM of DiOC6. Flow cytometric analysis was performed immediately, DiOC6^{bright} cells were identified as viable cells (Porakishvili *et al.,* 2011)

2.8 Immunohistochemical staining of bone marrow aspirates and lymph node biopsies

The affinity isolated rabbit anti-CD180 antibody (Sigma, UK) was chosen for use in this study due to its reported specificity for CD180 and suitability for use with formalin-fixed paraffin-embedded (FFPE) tissues. 2μm-thick sections of reactive tonsil were used for antibody optimization on the Bond-III automated staining platform (Leica Biosystems, UK). Sections underwent automated dewaxing and endogenous peroxidase was blocked using 3-4% (v/v) hydrogen peroxide. The antibody was tested with a range of heat induced epitope retrieval methods using citrate-based (pH 6.0) and EDTA-based (pH 9.0) epitope retrieval conditions. Dilution curves for anti-CD180 Ab were carried out with 15 minute incubation at ambient temperature, and signal visualized using Bond Polymer Refine Detection kit (DS9800) and haematoxylin counterstain. The slides were reviewed and optimal conditions chosen based upon the criterion of background-free selective cellular labeling and taken forward for use on cases of CLL.

2.9 CD180 expression in subsets of CLL cells categorized according to the surface expression of CD5 and CXCR4

CLL clones can be divided into distinct fractions on the basis of inverse surface expression of CXCR4 and CD5 also defining intra-clonal kinetic differences (Callisano *.et al.,*2011) (Figure 2.2). In order to assess CD180 expression on 'proliferative'($CXCR4^{dim}CD5^{bright}$) and 'resting' ($CXCR4^{bright}CD5^{dim}$) fractions of CLL cells, 2×10^5 isolated PBMCs were re-suspended in FACS buffer (PBS + 10% fetal bovine serum + 1% sodium azide) and incubated with murine anti–human monoclonal antibodies (mAbs) to: CD5~FITC, CXCR4~PerCP, CD19~APC and CD180~PE or IgG1~PE isotype control (all from BD Biosciences, USA) at 4°C for thirty minutes. Cells were washed and analysed with BD FACS*verse*™ flow cytometer (Becton Dickinson Immunocytometry systems, USA) using FlowJo v7.2.4 version software.

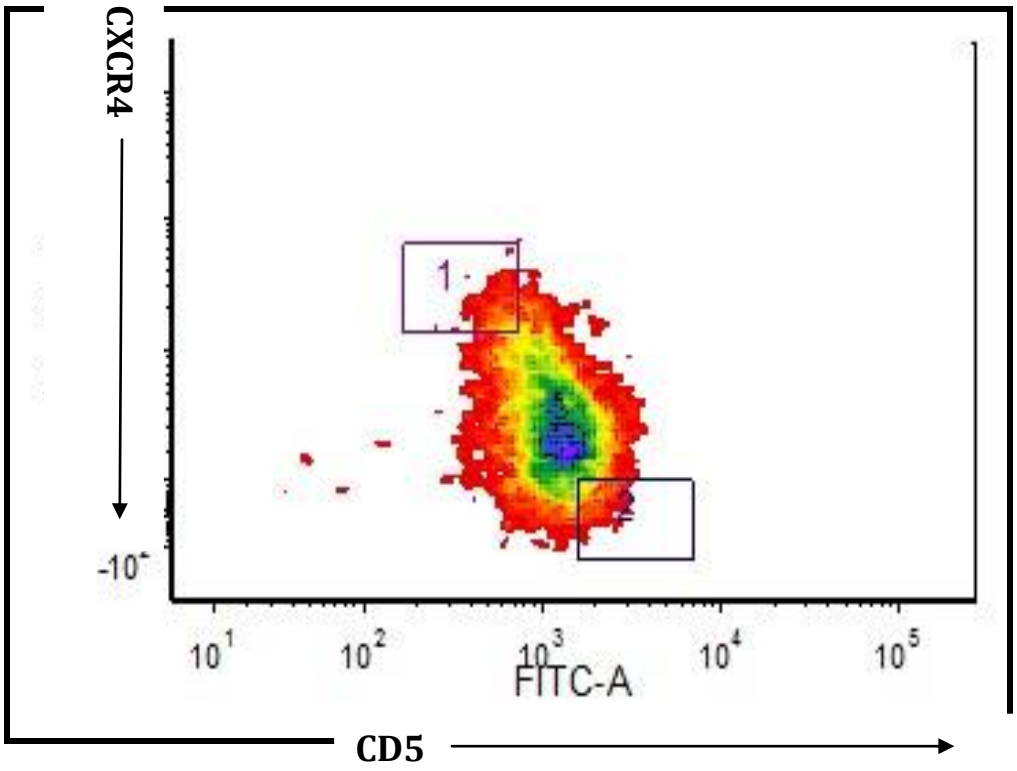


Figure 2. 2 contour plot of CD19+ve CLL cells plotted on the basis of CXCR4 and CD5

The CD180 expression in the 'proliferative'($CXCR4^{dim}CD5^{bright}$) (1) and 'resting' ($CXCR4^{bright}CD5^{dim}$) (2) fractions was determined. The expression of CD180 was expressed was percentage of positive cells in each subset, compared to the isotype control

2.10 Statistical Analysis

The following statistical methods were applied where required:

- Wilcoxon's non-parametric paired test using SPSS (software package used for statistical analysis)(provided by the University of Westminster, IT systems, UK)
- Mann-Whitney non-parametric U-test using MINITAB software (provided by the University of Westminster, IT systems, UK);
- Pearson's correlation coefficient using SPSS software;
- Student *t*-test (MS excel);
- Paired *t*-test

P-values <0.05 were considered significant in each case.

Chapter 3 CLL cells response to CD180 ligation

Results

Previous studies by our group have shown that CD180 is heterogeneously expressed by CLL cells in a clonal fashion (Porakishvili *et al.,* 2005). The level of the expression of this surface molecule was measured by the number of relative binding sites per cell (RBS/cell). The results, demonstrated that most normal (control) CD19+ B cells expressed a high density of surface CD180 (5548 ± 2271 RBS/cell), although a small population of CD180- negative cells [mean±S.D. (range)] 2.6±1.5% (0.8–7.3%) were evident (Koarada *et al.,*2001). Based on the data with normal B cells, in CLL cells, the negative CD180 clones were therefore defined by the level of CD180 RBS/cell on this small CD180- population (316 ± 88 RBS/cell, range 201-470 RBS/cell). The limit for the negative population was determined as the mean ± 2SD, as described previously (Porakishvili *et al.,* 2005). Also demonstrated by our group previously is that though the expression of CD180 in CLL clones is heterogeneous, this expression is significantly higher in cases with M IGVH genes compared to the U IGVH CLL cases (Porakishvili *et al.,* 2005). In addition also shown earlier was that CD180 ligation delivers a comparable or superior to CD40 and IL-4 activation, survival, and cell cycling signal for both normal B cells and CLL cells.

In the studies outlined below, the intra-cellular effects of CD180 ligation with mAb, in CLL and normal B-cells, have been outlined.

3.1a CD180 ligation leads to the activation of CLL cells in the 'Responder' group of patients as compared to the 'Non-Responder' group

Further to the studies with the surface expression of CD180, also previously published by our research group was that half of the CD180+ clones respond via activation and cycling and termed Responders: R while the other half which fail to respond were termed Non-Responders: NR CLL (Porakishvili *et al.,*2005). In my small study of 13 CD180+ CLL clones, 6 responded to CD180 ligation by activation (R CLL) measured by upregulation of CD86, while the other 7 CD180+ CLL clones failed to respond (NR CLL)(Figure 3.1.a) thus confirming the previous studies. The reason for only studying 13 patients was because this study was undertaken to confirm the same previous previous study also a part of the publication (Porakishvili *et.al.,2011)*

We also studied the expression of MD-1 in the CD180+ R CLL and NR CLL cells, since it an important molecule required for the cell surface expression of CD180 (Miura *et al.,* 1998; Nagai *et al.,* 2002). The lack of responsiveness by NR CLL cells was also not related to the level of the expression of MD-1 as shown for normal human B cells. We observed there was no statistical difference in the expression of MD-1 by R CLL (RFI: 1.86 ± 0.09) and by NR CLL (RFI: 2.75 ± 1.21, p= 0.15) clones (Porakishvili *et al.,*2011).

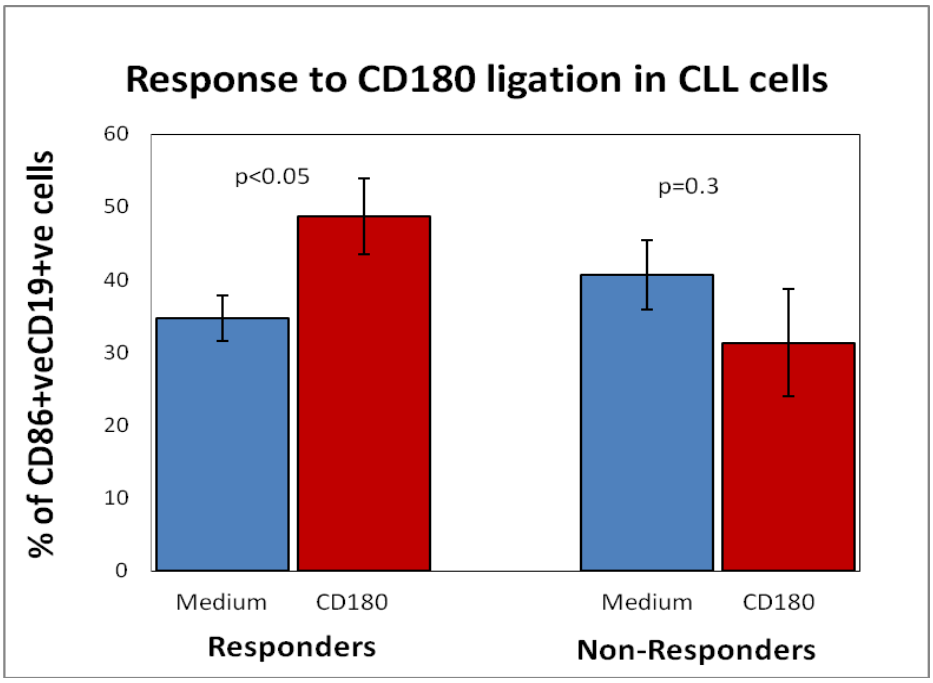


Figure 3. 1 a) Expression of CD86(B7.2) after anti-CD180mAb stimulation for 72 hours in CD180+ R CLL and NR CLL

PBMCs from 6 R CLL and 7 NR CLL were stimulated with 10µg/ml of anti-CD180 mAb for 72h and stained with PE-Cy5-conjugated anti-CD19 mAb and PE-conjugated anti-CD86 mAb. The cells were analyzed by flow cytometry. Data were analysed using the Mann–Whitney U-test, and represent mean ± standard deviation. P values were calculated using student t-test.

3.2a Ligation of CD180 on normal B cells, and R and NR CLL cells leads to upstream signalling events, with no or low effector enzyme phosphorylation in NR CLL cells

In order to localize the defect in activation of the NR CLL cells, it was important to identify the signalling pathways activated through CD180 ligation in CLL cells. Our working hypothesis for this study was that in the NR CLL cells, there could a block in the pathways which are active in the R CLL cells. To this effect, we measured phosphorylation of various intracellular signalling molecules- ZAP70/Syk, ERK, p38MAPK and AKT, in normal B cells, R and NR CLL. These intra-cellular molecules form an intrinsic part of the BCR mediated signalling pathways and we wanted to compare the activation of these with CD180 stimulated pathways,

Normal B cells demonstrated a significant increase in the percentages of cells with these phosphorylated signalling kinases (Figure 3.2.a). Similarly ligation of CD180 on R CLL cells resulted in the phosphorylation of all protein kinases tested as measured by the increase in the percentages of positive cells (Figure 3.2.b) although the responses were more heterogeneous. The degree of phosphorylation of R CLL cells following CD180 ligation was comparable to that measured in normal B cells with exception of pERK which was lower ($p=0.028$). We detected significantly higher levels of constitutive expression of pZAP70/Syk in CLL cells compared to normal controls ($55.5\pm25.8\%$ vs $29.4\pm7.6\%$, $p=0.0057$), which resulted in higher phosphorylation of ZAP70/Syk in CLL cells following CD180 engagement ($p=0.00001$, Figures 3.2.a and 3.2.b).

Results were totally different for NR CLL cells (Figure 3.2.c). Although there was increase in phosphorylation of the ZAP70/Syk in NR CLL cells following CD180 ligation ($p=0.049$), and to the lesser extent, of ERK (not significant), almost no responses were detected for phosphorylated p38MAPK, and, particularly pAKT ($p=0.008$ as compared to R CLL). Western blotting analysis confirmed the high phosphorylation of AKT in R CLL cells and the lack of pAKT in NR CLL cells following CD180 ligation (Figure 3.2.d).

We therefore concluded that the protein kinases ZAP70/Syk, ERK, p38MAPK and AKT were involved in signalling through CD180 ligation in normal B cells and R CLL cells. Also, in case of the NR CLL cells, the cellular activation was blocked downstream to ZAP70/Syk since this kinase was activated in response to CD180 ligation in this category of cells.

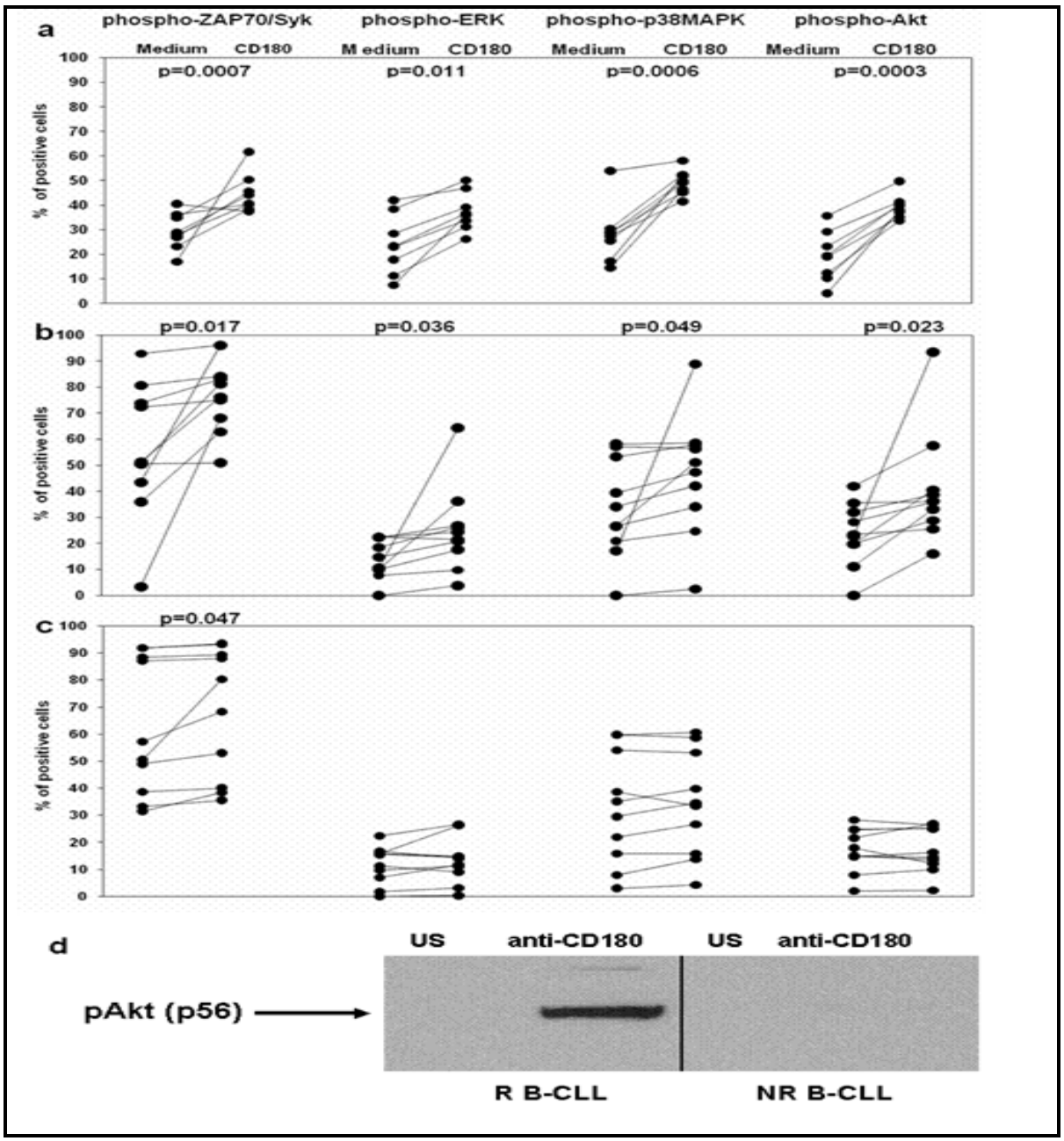


Figure 3. 2 a,b and c: Intracellular signalling following stimulation with anti-CD180 mAb.

Phosphorylation of protein kinases ZAP70/Syk, ERK, p38MAPK and AKT in (a) Control normal B cells, (b) Responder CLL cells and (c) Non-responder CLL cells. (d) A representative western blot showing the level of pAKT in R and NR CLL cells.

(a-c) Purified normal B cells, R and NR CLL cells were stimulated with 10 μg/ml of anti-CD180 mAb for 20 minutes, fixed, permeabilised and treated with antibodies to phosphorylated protein kinases- pZAP70/Syk, pERK, pp38MAPK and pAKT. The results are shown as percentages of positive cells in unstimulated and stimulated cultures. p values were calculated using paired t-test. (d) Cells from R and NR CLL patients were incubated unstimulated (US) or with 10 μg/ml of anti-CD180 mAb (anti-CD180) as above and analysed by Western blotting as described in materials and methods. The phospho-AKT antibody detected a major band of 56kDa after stimulation with anti-CD180 in cells from Responder but not Non Responder CLL cells.

3.3a Responder CLL clones can be subdivided into Early Responders (ER) and Late Responders (LR) by their activation of AKT protein kinase

As mentioned above, CD180 ligation response in CLL cells divided them into R CLL or NR CLL and that treatment of R CLL cells with anti-CD180 mAb for 30 min led to the increase in phosphorylation, specifically of AKT. In contrast, NR CLL cells failed to respond to the CD180 ligation by the activation of AKT (Porakishvili *et al.,*2011). However, since the increase in the percentages of pAKT+ R CLL clones following CD180 ligation was highly heterogeneous, we investigated this variability in more detail.

We therefore studied the pAKT positive cells at different time points after CD180 ligation in culture. It appeared that some of the CLL samples previously categorised as NR CLL demonstrated substantial increase in phosphorylation of AKT when the time of exposure to anti-CD180 mAb was increased to 24h as analysed by flow cytometry and immunoblotting. No appreciable induction of AKT activation has been detected after 6h or 18h in culture. This allowed us to re-categorise R-CLL samples into Early AKT Responders (ER-AKT) and Late AKT Responders (LR-AKT) based on a significant increase in the percentages of pAKT+ cells above the basal level (Figure 3.3.a). Upregulation of the levels of pAKT in both ER-AKT and LR-AKT was confirmed by immunoblotting (Figure 3.3.b). The magnitude of the pAKT response in the two subgroups did not differ significantly. Interestingly 5 out of 19 ER-AKT samples also showed durable AKT phosphorylation within 30min-24h time-scale. CLL samples that failed to phosphorylate AKT protein kinase following stimulation with anti-CD180 mAb irrespective of the incubation period were categorised as NR-CLL (Figure 3.3.a and 3.3.b). Lack of the increase in pAKT was not caused by an aberrant deficiency of the non-phosphorylated enzyme since total AKT levels were comparable in ER-AKT, LR-AKT and NR-CLL cells (Figure 3.3.b).

To assess if the difference in the density of CD180 could play a role in the pace of the AKT phosphorylation, we determined the levels of CD180 expression in the two subgroups. ER-AKT (n=11) and LR-AKT (n=12) did not differ significantly in

the expression of CD180 (RBS/cell: 2342±1725 vs 1425±1127, p=0.15; % of positive cells: 60.1±27.0 vs 66.2±25.5%, p=0.45; respectively). However, interestingly, ER-AKT cells, compared to LR-AKT cells, expressed significantly higher levels of sIgM (RBS/cell: 3254±2327 vs 865±845, p=0.02; % of positive cells: 67.2±18.4 vs 35.1±29.3% p=0.003, respectively) and CD79b (RBS/cell: 1859±1538 vs 384±396, p=0.019; % of positive cells: 48.8±28.3 vs 27.7±22.1%, p=0.017, respectively).

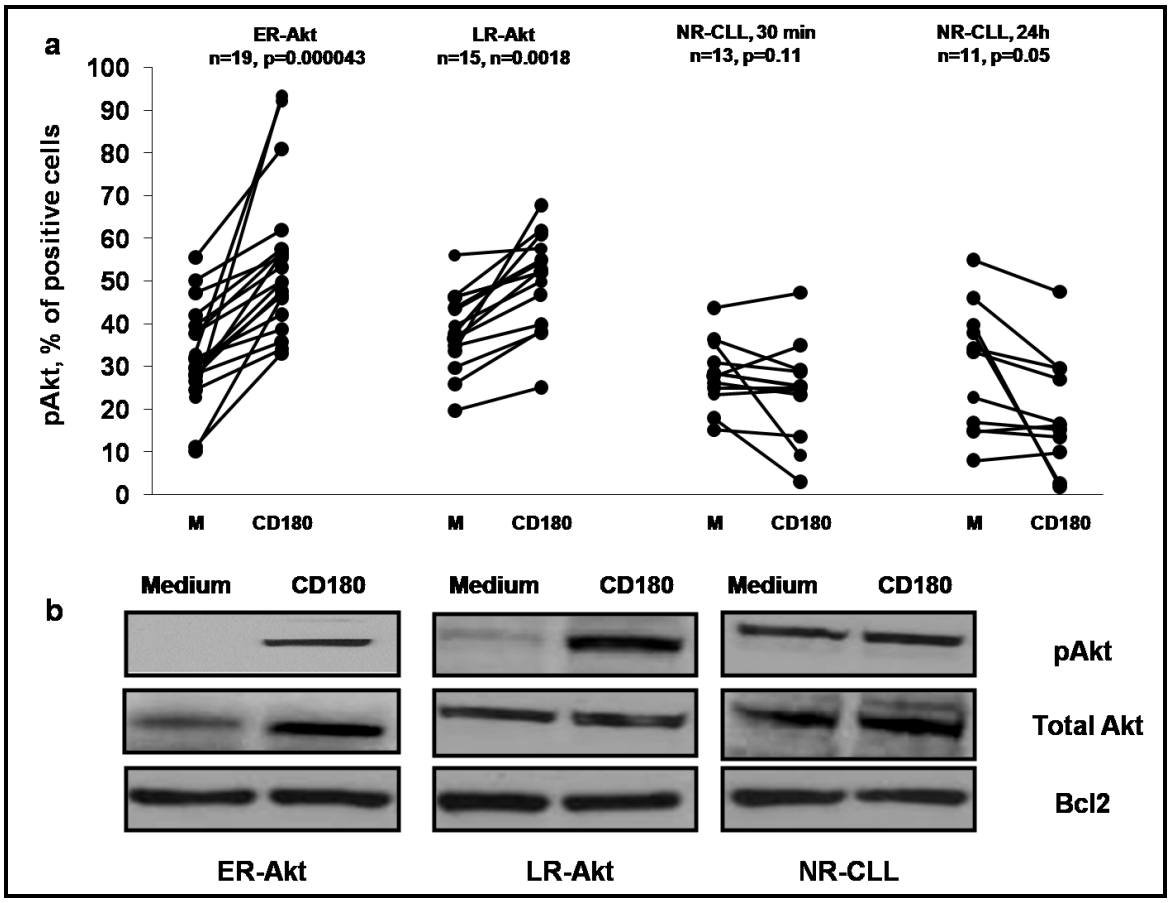


Figure 3. 3 a,b: Phosphorylation of AKT protein kinase in Early AKT Responder (ER-AKT), Late AKT Responders (LR-AKT) and Non-Responder (NR-CLL) cells following stimulation with anti-CD180 mAb for 30 min or 24h.

(a) CLL cells were incubated with anti-CD180 mAb for 30 min or 24h or left unstimulated in medium (M), washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-pAKT(Ser473) mAb 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. **(b)** Representative immunoblots are shown indicating the levels of total AKT and pAKT in ER-AKT, LR-AKT and NR CLL samples following stimulation with anti-CD180 mAb (CD180) as described in the Materials and Methods. Unstimulated CLL cultures in medium (Medium) were used as controls. The bands represent total AKT and phospho-AKT visualised by anti-AKT or anti-pAKT(Ser473) Abs; Bcl-2 was used as a loading control.

3.4a CD180 ligation on CLL cells leads to either activation of AKT or p38MAPK protein kinases

Since some NR-CLL were re-categorised as AKT-Responders following prolonged stimulation with anti-CD180 mAb we re-grouped CLL samples based on their ability to increase phosphorylation of AKT. We further examined if ER-AKT, LR-AKT and NR CLL samples responded differently to CD180 ligation by activation of other protein kinases involved in CD180-mediated signalling as mentioned above-ZAP70/Syk, p38MAPK and pERK (Porakishvili *et al.,*2011). Unexpectedly, stimulation of ER-AKT CLL clones with anti-CD180 mAb lead to a significant downregulation of the basal levels of phosphorylated p38MAPK (Figure 3.4.c). In contrast, short-term stimulation (30 minutes) of a substantial number of AKT-non-responsive CLL samples led to a significant upregulation of phospho-p38MAPK (Figure 3.4.c) confirmed by immunoblotting (Figure 3.4.c). We did not detect any appreciable differences in phosphorylation of ERK between the defined subgroups (data not shown). These data are consistent with a hypothesis that elevation of activated AKT or p38MAPK represents two possible alternative pathways downstream from Syk following CD180 ligation. However, out of the pooled 34 ER-AKT and LR-AKT samples 5 also responded to CD180 ligation by elevated levels of both – pAKT and pp38MAPK as did all tested control B cells (Figures 3.4.b and 3.4.c).

We have therefore identified four major patterns of CD180-mediated signalling in CLL cells: ER-AKT, LR-AKT, ER-p38MAPK , a minor subset of double AKT/p38MAPK signallers and NR. The differences between the subgroups appear downstream from ZAP70/Syk via different signalling routes (AKT or p38MAPK), or no signalling (NR), as phophorylation of ZAP70/Syk was observed in all cells (Figure 3.4.a). Importantly, in control B cells, CD180 ligation resulted in simultaneous activation of all protein kinases tested, including ERK (Figure 3.4.a, 3.4.b and 3.2.a) so the detected dichotomy (AKT vs p38MAPK) appears to be a feature of CLL cells.

No significant differences were detected in the expression of CD180 (density or percentages of positive cell) between AKT-signallers and p38MAPK-signallers. However, ER-p38MAPK cells appeared to be "better equipped" with surface receptors and expressed significantly higher *ex vivo* levels of sIgM, IgD, CD79b and CD38 expressed as RBS/cell and percentages of positive cells, compared with the pooled AKT-signalling cells (ER-AKT and LR-AKT). Of note, AKT-signallers were largely negative for CD38 expression (Table 3.4). However all double AKT/MAPK signallers expressed low levels of CD180 as compared with pooled AKT-signallers and p38MAPK-signallers (426±212 RBS/cells, p=0.014 and p=0.015 respectively; 42.4±22.4%, p=0.043 and p=0.016 respectively) and were negative for CD79b expression (125±122 RBS/cell, p= 0.006, p=0.045 respectively, and 12.6±10.0%, p=0.14 and p=0.002 respectively).

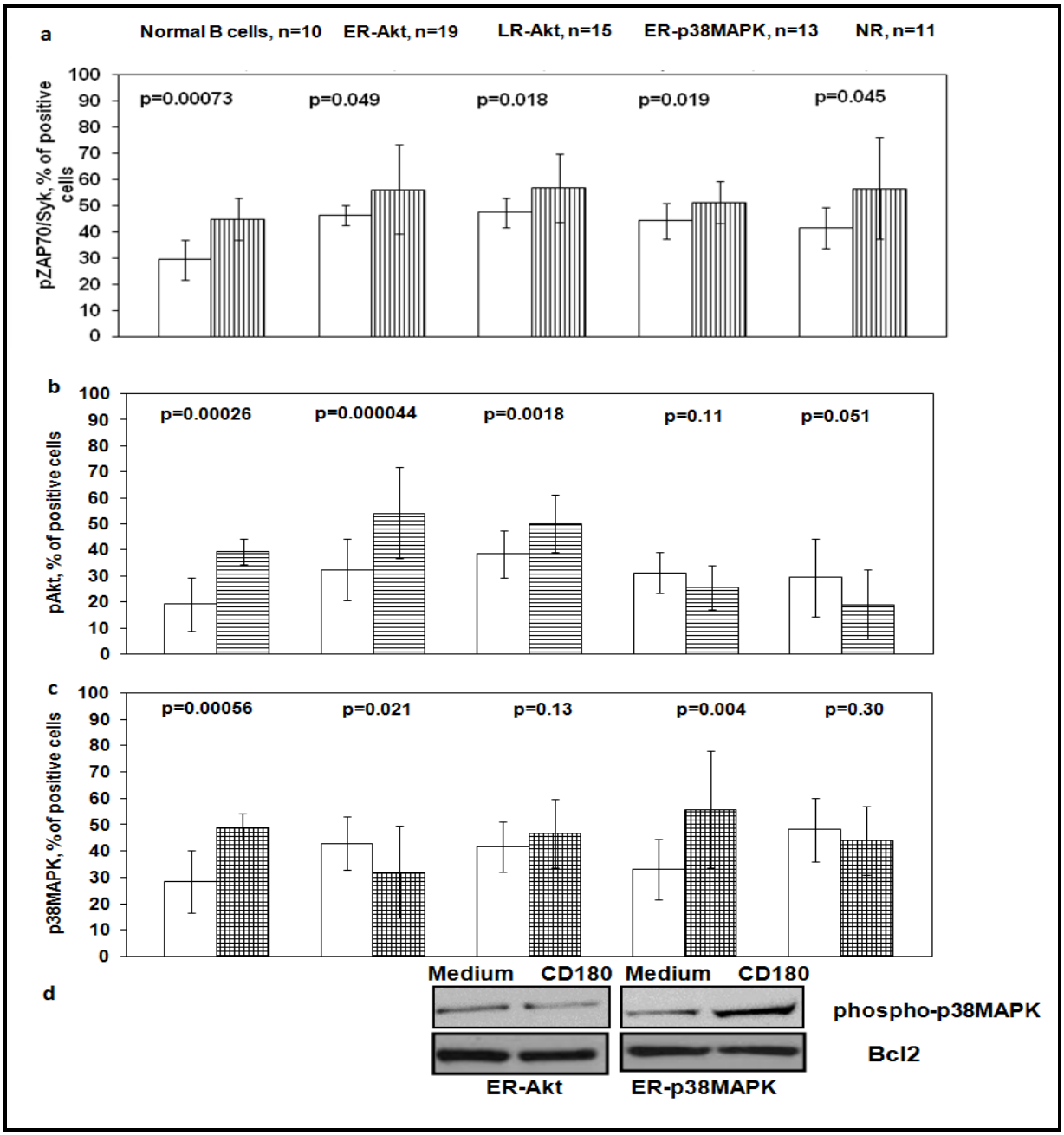


Figure 3. 4 .a,b,c,d: Anti-CD180 mediated phosphorylation of AKT and p38MAPK in control B cells and in different categories of CLL cells

ER-AKT, LR-AKT, ER-p38MAPK. (a,b and c) Control B cells and CLL cells were incubated with anti-CD180 mAb for 30 minutes (ER) or 24h (LR) or left unstimulated in medium (M), washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-pZap70/Syk anti-pAKT(Ser473) and anti-phospho-p38MAPK(Thr180/Tyr182) 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. (d) Representative immunoblots showing the levels of phospho-p38MAPK without (Medium) and after stimulation with anti-CD180 mAb (CD180) in ER-AKT and ER-p38MAPK CLL cells visualized by anti-phospho-p38MAPK(Thr180/Tyr182) mAb as described in the Material and Methods. Bcl2 was used as a loading control.

	Pooled ER-AKT and LR-AKT, n=20		ER-p38MAPK, n=10			
Receptor	**RBS/cell**	**%**	**RBS/cell**	**%**	**$P_{RBS/cell}$**	**$P_{\%}$**
CD180	**1954 ± 1721**	**62.3±26.7**	**1752 ± 1223**	**68.5±18.4**	**0.372**	**0.236**
sIgM	**1663 ± 1601**	**50.2±29.4**	**11300±6800**	**61.8±26.8**	**0.044**	**0.107**
sIgD	**2186 ± 1358**	**47.9±25.8**	**6775 ± 3537**	**69.0±24.6**	**0.048**	**0.049**
CD79b	**976 ± 914**	**37.9±22.5**	**5168 ± 3383**	**61.6±26.2**	**0.047**	**0.046**
CD38	**544 ± 470**	**21.8±24.0**	**1742 ± 1533**	**39.9±18.2**	**0.048**	**0.049**

Table 3.4. Phenotypic characteristics of CLL cells that signal via AKT or p38MAPK protein kinases following stimulation with anti-CD180 mAb:

CLL cells were blocked with human immunoglobulins, treated with unconjugated mAbs to CD180, IgM, IgD, CD79b and CD38, washed, stained with rabbit-anti-mouse FITC-conjugated Ab, blocked with mouse serum and stained with PE-Cy5-conjugated anti-CD19 mAb as described in the Material and Methods. The results were analysed by flow cytometry and expressed as antibody Relative binding sites per cell (RBS/cell) and percentages of positive cells. P values were calculated using the Mann-Whitney non-parametric U-test.

3.5a CD180-induced AKT-mediated signalling in CLL cells involves Btk and PI3-K

Since AKT (Zhuang *et al.,*2010) and associated PI3-K (de Frias *et al.,*2009; Barragan *et al.,*2006; Nedellec *et al.,*2005) mediated pathways have been shown to be important in survival of CLL cells, we next determined whether these pathways were involved in the survival of CLL cells mediated by CD180 as shown previously by our group (Porakishvili *et al.,*2005 and 2011).

Our data demonstrate that in those CLL cells that responded to CD180 ligation with phosphorylation of AKT, the levels of pPI3-K, measured by immunoblotting were also upregulated. This was seen in AKT-responder CLL cells but not in ER-p38MAPK CLL cells (Figure 3.5.a), despite a substantial level of non-phosphorylated enzyme in the latter group as defined by total PI3-K (Figure 3.5.a). It has been recently reported that BCR-mediated survival of CLL cells operates via early activation of Bruton agammaglobulinemia tyrosine kinase (Btk) (Woodland *et al.,*2008; Packham *et al.,*2010). We therefore tested whether CD180-mediated signalling in AKT-responder CLL cells also involves Btk. As expected, in ER-PI3-K/AKT-responder CLL cells, CD180 ligation induced significant upregulation of pBtk. In contrast, the levels of pBtk were significantly decreased in the ER-p38MAPK category of cells (Figure 3.5.b).

Taken together our data indicate that ligation of CD180 on CD180+ CLL cells, either leads to the activation of a signalling pathway Btk/PI3-K/AKT, or a signal is diverted through p38MAPK. The consequences of the differential signalling were therefore studied next.

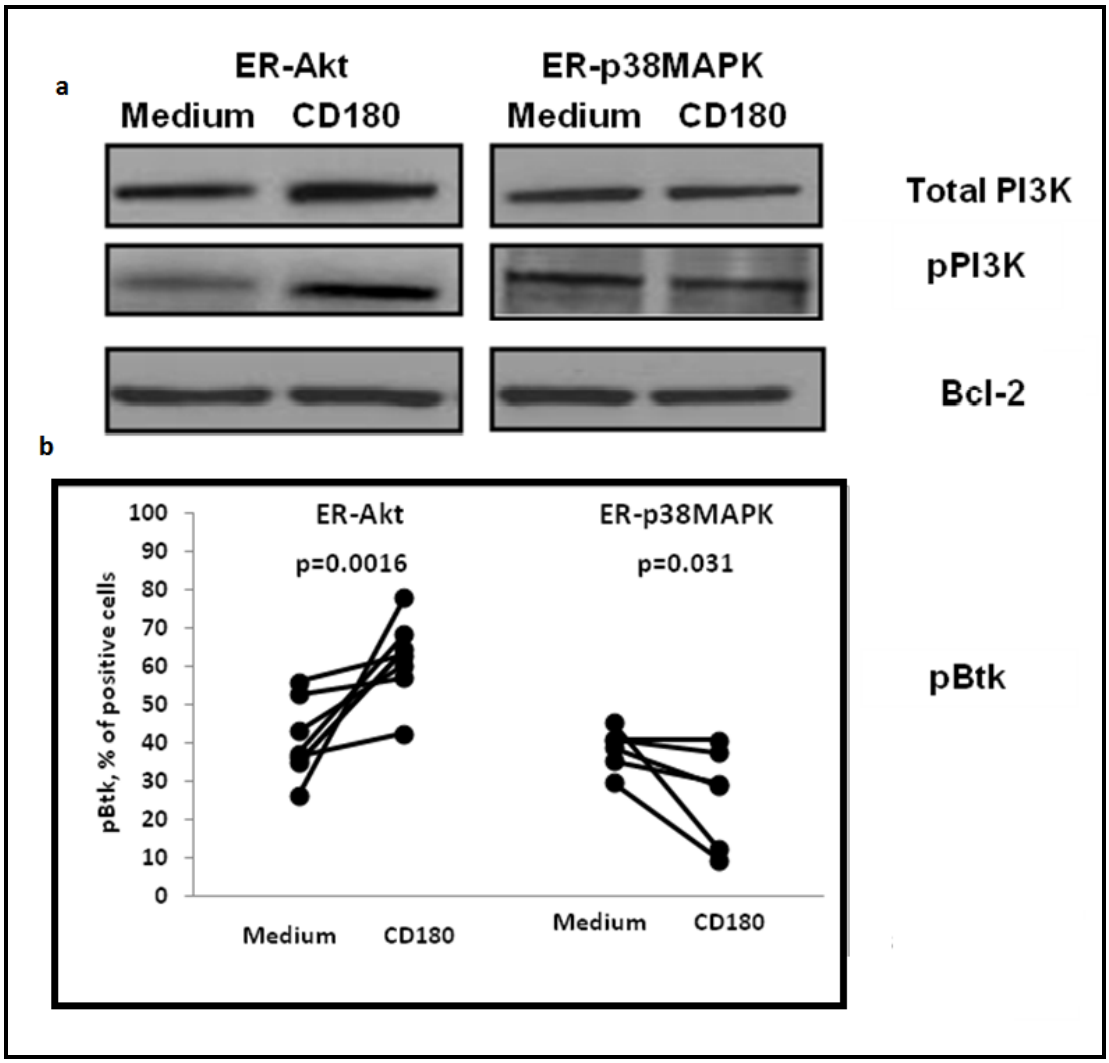


Figure 3. 5 a,b: Anti-CD180 mediated phosphorylation of PI3-K, Btk in ER-AKT and ER-p38MAPK CLL cells.

(a) Representative immunoblots show the levels of total PI3-K, phospho-PI3-K and Mcl-1 in ER-AKT and ER-p38MAPK CLL cells following stimulation with anti-CD180 mAb (CD180) as described in the Materials and Methods. Unstimulated CLL cultures in medium (Medium) were used as controls. The bands represent total PI3-K and phospho-PI3-K visualized by anti-PI3-K(p85) or anti-pPI3-K(tyr458)/p55(tyr199) mAbs respectively or anti-Mcl-1 mAb. Bcl2 was used as loading control. (b) Percentages of pBtk+ cells in ER-AKT and ER-p38MAPK categories of CLL cells following stimulation with anti-CD180 mAb measured by flow cytometry. CLL cells were incubated with anti-CD180 mAb for 30 minutes or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-pBtk mAb as described in the Materials and Methods. P values were calculated using the paired *t*-test.

3.6a CD180-induced Btk/PI3-K/AKT signalling pathway leads to pro-survival while p38MAPK pathway leads to pro-apoptosis in CLL cells

Having established that the CD180 mediated signalling in CLL cells leads to a dichotomy in signalling, either through pAKT ot p38MAPK activation, we next examined the effect of this dichotomy on survival of CLL cells. The important anti-apoptopic protein kinase Mcl-1, belonging to the Bcl2 anti-apoptopic family, has been shown play a role as a downstream target of the PI3-K-AKT pathways via IgM engagement in CLL (Gandhi *et al.,*2008). Therefore we hypothesized that this kinase is active in the CD180 mediated AKT signalling. Most certainly our data demonstrated that there was an appreciable increase in the levels of Mcl-1 following CD180 ligation in ER-AKT cells (Figure 3.6.a), but not in ER-p38MAPK CLL cells. Further, changes in the mitochondrial membrane potential, measured as level of apoptosis, (Petit *et al.,*1995; Porakishvili *et al.,* 2011) showed there was an increase in the percentages of $DiOC_6^{dim}$ apoptotic cells in ER-p38MAPK cells (Figure 3.6.b).

Thus, to sum up, in our hands CD180-ligation induced significant suppression of basal (spontaneous) apoptosis after 24hr in culture in both control B cells and Btk/PI3-K/AKT-responder CLL cells (pooled ER-AKT and LR-AKT cells). In contrast, the percentage of apoptopic cells was increased in the ER-p38MAPK responder cells.

Thus, our data suggest that CD180-mediated intracellular signalling in CD180+ CLL cells can engage two major pathways: pro-survival that operates via Btk/PI3-K/AKT protein kinases, or pro-apoptotic that operates via p38MAPK. Importantly, simultaneous activation of both pathways observed in normal B cells only resulted in their survival (Gauld *et al.,*2002), but not apoptosis, hence endorsing this result as a CLL specific characteristic.

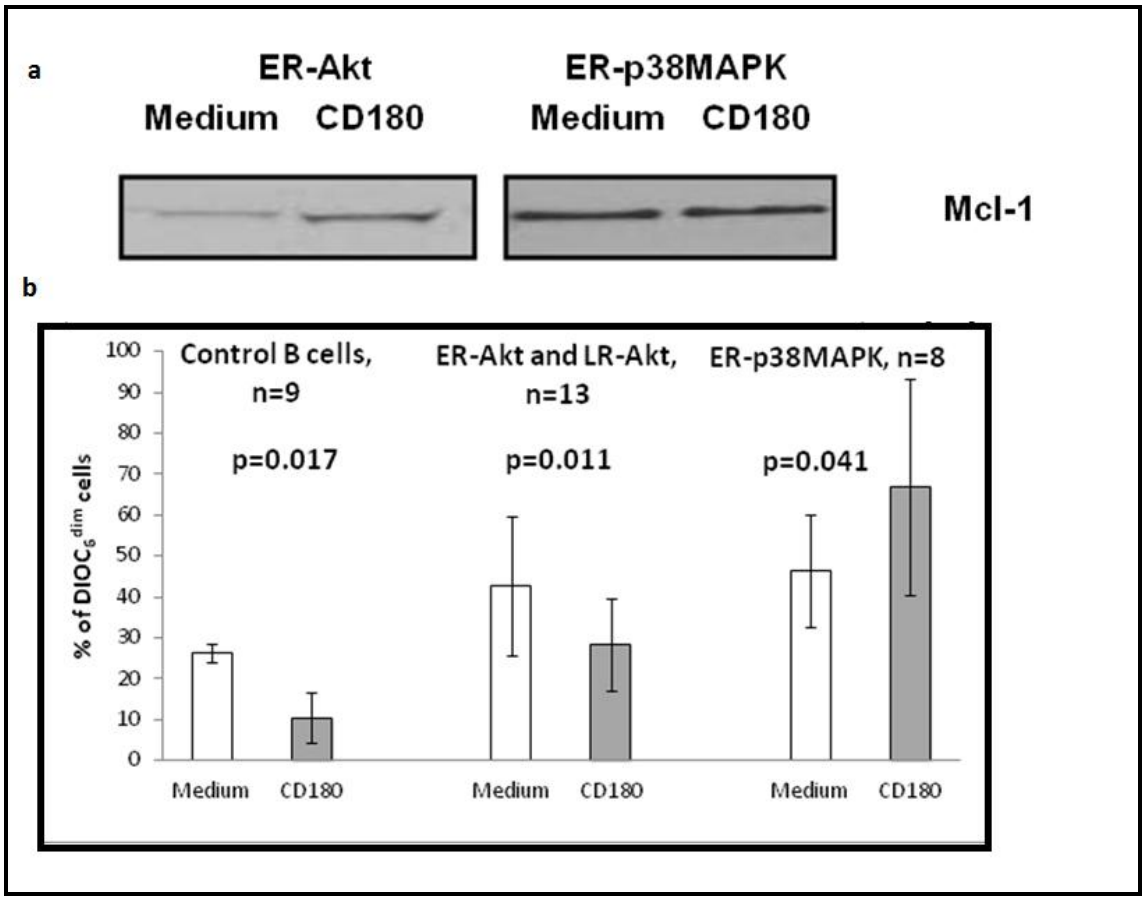


Figure 3. 6.a, b: (a)Representative immunoblots show the levels of Mcl-1 in ER-AKT and ER-p38MAPK CLL cells following stimulation with anti-CD180 mAb (CD180) (b) The percentages of $DiOC_6^{dim}$ apoptotic cells in control B cells, pooled ER-AKT and LR-AKT, and ER-p38MAPK CLL cells following CD180 stimulation

(a) as described in the Materials and Methods, cells were stimulated with CD180 mAb and unstimulated CLL cultures in medium (Medium) were used as controls. The bands represent total Mcl-1 as visualized by anti-Mcl-1 mAb.(b)percentages of ER-AKT and LR-AKT cells following stimulation with anti-CD180 mAb as described in the Material and Methods. Control B cells and CLL cells were stimulated with anti-CD180 mAb for 24h, washed, stained with anti-CD19 mAb, loaded with $DiOC_6$ for 20 min and analysed by flow cytometry. P values were calculated using the paired *t*-test.

Discussion

3.1.b CD180 ligation activates the 'Responder' group of CLL cells while the 'Non-responder' group remains unresponsive

Our research group has previously shown that normal human CD19+B cells express CD180, and its ligation resulted in a strong upregulation of the lymphocyte activation marker CD86. However only 2/3 of CLL clones constitutively expressed CD180 and approximately half of the CD180+ clones respond to ligation (Porakishvili *et al.,* 2005, 2011). In my study, this observation was confirmed (Porakishvili *et al.,* 2011). Out of 13 CLL cell samples 6 CD180+ clones responded by upregulation of CD86 upon CD180 ligation in culture. These clones were termed Responders: R, while the other unresponsive CD180+ CLL clones were termed Non-responders: NR (Fig. 3.1). Increased CD180-mediated survival of murine B cells was associated with extensive proliferation (Chaplin *et al., 2011*). In another model system, CD180 engagement protected murine B lymphocytes from radiation and dexamethasone-induced apoptosis (Miyake *et al.,* 1994, 1995). Together with this data, our findings suggest that engagement of CD180 by various environmental signals leads to the activation of R CLL clones. Also, the surface density of CD180 and the outcome of its ligation were consistently reproducible over 48 months (Porakishvili *et al.,* 2005, 2011), indicating that the function of CD180 was a constitutive characteristic of leukaemic clones. Additionally, it is important to note that in this study we assay the expression of CD86 after 72 hours in culture. Hence there is a possibility that many other factors that are produced in the cell culture over this period e.g. various number of cytokines could support the cellular activation and expression of CD86. Studies to this end aimed at assaying micro-environmental influences in the culture, playing a role in CD180 ligation response would be need to be undertaken in the future.

3.2b Phosphorylation of intracellular signalling enzymes post CD180 ligation

Unlike most TLRs 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). Therefore CD180 may either recruit an unidentified B-cell specific protein for upstream signalling (Yazawa *et al.,* 2003) or cooperate/converge with other signalling pathways. As an initial step towards identifying the signalling pathway(s) utilized by CD180 in CLL and the possible defect in NR CLL clones, we determined the phosphorylation of several important enzymes involved in signalling.

Normal B cells responded to CD180 ligation by a robust increase in the phosphorylation of all protein kinases studied (Figure 3.2.a). Importantly R CLL cells exhibited comparable levels of kinase phosphorylation (Figure 3.2.b), particularly that of ZAP70 and AKT. RP105-mediated signalling in murine B cells uses the Src-family protein tyrosine kinase Lyn, protein kinase CbI/II (PKCbI/II), and Erk2-specific MAP kinase (MEK) (Miura *et al.,* 1998). Furthermore, murine RP105 engagement in B cells may exploit two divergent signalling pathways (a) via Lyn, CD19 and Vav downstream to JNK, and (b) via Lyn, PI3-K and Btk downstream to NF-κB (Yazawa *et al.,*, 2003). To our knowledge, this is the first report showing recruitment of phosphorylated AKT following CD180 ligation in human CLL cells and B cells (Figures 3.2.a, 3.2.b and 3.2.d).

In contrast, in NR CLL cells, we found a block of phosphorylation downstream from ZAP70/Syk protein kinases, particularly that of AKT in response to CD180 ligation (Figure 3.2.c, $p=0.003$ compared to R CLL and Figure 3.2.d). This lack of AKT activation may be a reason for apparent global unresponsiveness of this subset of CLL cases.

Therefore, our data can be also considered in a wider context, possibly indicating anergy of NR CLL clones. In CLL cells with unresponsiveness to BCR engagement, the inability to activate AKT was associated with high constitutive phosphorylation of ERK (Muzio *et al.,* 2008). Lack of AKT phosphorylation coupled with constitutive

activation of the p38MAPK signalling pathway is found in murine B cells rendered anergic by BCR-signalling (Merrell *et al.*,, 2006). In our hands there were high levels of constitutive pZAP70/Syk and p38MAPK in both R and NR CLL clones, but only in NR CLL cells was this associated with a lack of AKT phosphorylation. These data are indicative of a possible cross-talk between BCR and CD180 signalling pathways.

Our data therefore indicate a significant defect in distal signalling events in NR CLL cells whilst proximal phosphorylation of ZAP70/Syk appears to be functional.

3.3.b CD180 ligation response in CLL is better validated by the AKT phosphorylation and can be either an early (30 minutes) or late (24hours) event

Activation of AKT has been reported to be essential for the general growth and survival of B lymphocytes (Woodland *et al.,*2008) as well as survival of CLL cells following BCR ligation (Packham *et al.,*2010; Downward *et al.,*2004). It also appears to be paramount for TLR-mediated cell expansion and survival. Ligation of RP105/CD180 on naïve control B cells lead to the survival from apoptosis operating through phosphorylation of AKT (Yamazak *et al.,*2010) whilst stimulation of proliferation of CLL cells through the engagement of TLR9 by CpG-ODNs was also transmitted through AKT and ERK (Longo *et al.,*2007)

As discussed above, activation of AKT was a central event in CD180-mediated signalling and differentiated the NR CLL from R CLL. However, the levels of pAKT varied through individual clones even in R CLL group. Here with more data at hand (studying CD180 ligation responses at different time points) we re-categorized the responsiveness of CLL clones to CD180 ligation based on their ability to phosphorylate AKT, rather than expression of CD86 as before (as discussed in 3.1.b; Porakishvili *et al.,*2005,2011).We analyzed the level of phosphorylation of protein kinases following long term stimulation (24h), in addition to the short-term (30 min) stimulation with soluble anti-CD180. This extension in receptor stimulation time was considered since it has been shown before that prolonged phosphorylation of AKT might be required for pro-survival signalling in CLL cells

stimulated with soluble anti-IgM (Petlickovski *et al.,*2005; Packham *et al.,*2010) and for proliferation after the treatment with CpG-ODN (Longo *et al.,*2007).

Here we demonstrate that out of 55 CLL samples tested CD180 ligation with mAb lead to the speedy phosphorylation of AKT above the basal levels in 19 CLL samples, whilst in further 15 samples appreciable increase in pAKT was detected after 24h in culture by flow cytometry (Figure 3.3.a) and Immunoblotting (Figure 3.3.b). We therefore defined these CLL cells as Early AKT responders (ER-AKT) and Late AKT Responders (LR-AKT). It must be noted that five out of 19 ER-AKT samples also showed durable AKT phosphorylation within 30min-24h time-scale. The remaining 24 CLL samples did not respond to the CD180 ligation by exceeding the basal levels of pAKT in the time-scale of 30min-24h (Non-responders, NR, Figure 3.3.a and 3.3.b).

We further studied whether differences in the speed of AKT phosphorylation in response to CD180 ligation were dependant on the level of the expression of CD180 receptor assessed by the percentages of positive cells as well as antibody relative binding sites/cell (RBS/cell) indicative of the density of the receptor expression (Porakishvili *et al.,*2005; 2011). Interestingly, ER-AKT and LR-AKT did not differ in the levels of the expression of CD180. However ER-AKT cells, compared to LR-AKT cells, expressed significantly higher levels of sIgM and CD79b. This, in our opinion, indicated a possible cross-talk between BCR and CD180 signalling pathways (Porakishvili *et al.,*2011; Efremov *et al.,*2007; Packham *et al.,*2010).

Further co-relation with the available clinical data showed that the CLL clones from patients with progressive disease and poorer prognosis (U IgVH) responded to CD180 ligation by robustly activating AKT immediately at 30 minutes (categorized as ER-AKT CLL clones). On the other hand the CLL cells from patients with an indolent disease stage and better prognosis required a minimum of 24 hours stimulation through CD180 to activate AKT significantly (categorized as LR-AKT). These observations are synchronous with the study by Longo *et al.,*(2006) showing that the amplitude and duration of AKT activation, in response to TLR ligation, could determine the cell proliferative capacity and corresponded with the

clinical status. They observed the cells from cases with bad prognosis were highly proliferative and responsive to TLR stimulation only upto one hour in culture, by robust AKT phosphorylation (similar to our findings with CD180 ligation in ER-AKT) (Longo *et al.,*2006). In this study we did not investigate the rate of cell proliferation in the two subsets- ER-AKT and LR-AKT post CD180 ligation but our group has shown earlier that the cells responsive to CD180 ligation show increased capacity for proliferation evidenced by upregulation of cell cycle marker Ki67 (Porakishvili *et al.,*2005; 2011). However in the future, assay of (possible) differential rate of proliferation mediated through CD180, in the ER-AKT and LR-AKT subsets, could be of prognostic value.

3.4b CD180 ligation on CLL cells leads to the alternative activation of AKT or p38MAPK signalling pathways

As the ER-AKT cells and LR-AKT cells expressed differential level of sIgM and CD79b, in our opinion, this indicated a possible cross-talk between BCR and CD180 signalling pathways and prompted us to further re-assess the levels of activation of other key protein kinases associated with the IgM signalling pathways such as ERK and p38MAPK (Porakishvili *et al.,*2011; Efremov *et al.,*2007; Packham *et al.,*2010).

We did not find significant differences in the patterns of phosphorylation of ERK between ER-AKT, LR-AKT or NR-AKT categories of cells.

However, the most intriguing data was obtained for p38MAPK. We detected a significant drop (p=0.021) in phospho-p38MAPK basal levels in ER-AKT category of CLL cells following CD180 ligation, assessed by flow cytometry and confirmed by Immunoblotting (Figure 3.4.b and c). Some decrease in the signal intensity of p38MAPK upon stimulation of BCR has been reported before (Petlickovski *et al.,*2005), but never linked with the AKT activation. In contrast, thirteen CLL samples formerly defined as NR-AKT, responded to the ligation of CD180 by a strong phosphorylation of p38MAPK after 30 min (Figure 3.5.c, p=0.004), confirmed by Immunoblotting (Figure 3.5.d), but not 24h in culture (Figure 3.5.c). The remaining 11 CLL samples were unresponsive by activation of either AKT or p38MAPK during the time points used (Figure 3.5.b and c).The phosphorylation of upstream ZAP70/Syk was significantly observed in all subsets irrespective of the downstream enzyme activity (Figure 3.5.a).

Thus we have indentified two alternative signalling pathways following CD180 ligation, downstream of ZAP70/Syk, operating via AKT or p38MAPK. This exclusivity of pro-AKT and pro-p38MAPK pathways appears to be a feature of CLL cells and does not apply to control B cells where CD180 ligation induced simultaneous activation of AKT and p38MAPK (Figure 3.5.a,b and c; Porakishvili *et al.,*2011). Interestingly, out of pooled ER-AKT and LR-AKT 34 samples 5 responded to CD180 ligation in a manner similar to that of normal control B cells: by activation of both – AKT and p38MAPK. This minor subset of CLL cells (those

which respond to CD180 ligation by uoregulation of both p38MAPK and AKT) remains enigmatic since it is characterised by extremely low levels of expression of CD180 – opposite to normal B cells, where expression of CD180 significantly exceeds that of CLL cells (Porakishvili *et al.,*2005).

Therefore, based on our current data, CLL cells in relation to CD180 can be further re-categorised into three major categories: AKT-Responders, p38MAPK-Responders and Non-responders, and a minor subset of double AKT/p38MAPK Responders.

The role of p38MAPK-mediated signalling in CLL remains unclear. Activation of p38MAPK has been previously associated with proliferation of various cells in response to CpG-ODN (Peng *et al.,*2005; Efremov *et al.,*2007). In CLL, activation of p38MAPK was reported to be involved in the regulation of cell survival and apoptosis (Yi *et al.,*2003; Piatelli *et al.,*2004).

It was therefore important to further study how differential signal transduction in response to CD180 translates into the regulation of survival and apoptosis of CLL cells.

3.5b CD180-mediated AKT-signalling pathway in CLL cells involves activation of PI3-K and Btk protein kinases and is pro-survival, whilst p38MAPK activation favors apoptosis

AKT protein kinase is one of the most important targets of the products of phosphatidylinositol-3-kinase (PI3-K) δ isoform which is upstream to AKT (Efremov *et al.,*2007; Manning *et al.,*2007) . PI3-K is expressed exclusively by hematopoietic cells and plays a key role in BCR signalling (Okkenhaug *et al.,*2007). Constitutive expression of PI3-K is elevated almost three-fold in CLL cells compared to normal B cells (Herman *et al.,* 2010). PI3-K pathway appears to be critical for the survival of CLL cells activated through BCR as shown previously by various groups including us (Bernal *et al.,*2001; Barragan *et al.,*2002;Ringshausen *et al.,*2002; Cuni *et al.,*2004; Nedellec *et al.,* 2005)

Inhibition of PI3-K completely abrogated expression of Mcl-1 following BCR ligation (Petlickovski *et al.,*2005) indicating that PI3-K/AKT activation is paramount for the BCR-mediated resistance to apoptosis. Importantly, engagement of RP105 on murine B cells was shown previously to lead to the activation of PI3-K (Chan *et al.,*1998), followed by the recruitment of AKT (Hebeis *et al.,*2005).

More recently it has been demonstrated that activation of Bruton's tyrosine kinase (Btk), a signalling element of the BCR pathway (Pone *et al.,*2012) is important for the survival of CLL cells. Specific Btk inhibitor PCI-32765 suppressed CLL cell proliferation, migration and survival *in vitro* and *in vivo* mouse models (Burger *et al.,*2010; Ponader *et al.,*2012). Activation of Btk is initiated through its recruitment to the plasma membrane facilitated by interaction with phosphatidylinositol-3,4,5-trisphosphate (PIP3), a product of PI3-K activity (Salim *et al.,*1996; Thien *et al.,*2001).

Since Btk-PI3-K-AKT represents an important signalling pro-survival circuit we next studied activation of PI3-K and Btk following CD180 ligation on CLL cells in various categories of CLL cells (Figure 3.5.a,b). As expected, the levels of pPI3-K were elevated in ER-AKT, but not in ER-p38MAPK cells (Figure 3.5.a) as assessed by immunoblotting. This was not due to the PI3-K deficiency since ER-p38MAPK category of CLL cells contained appreciable levels of total PI3-K (Figure 3.5.a). Likewise, treatment with anti-CD180 mAbs led to a significant upregulation of pBtk in pooled ER-AKT and LR-AKT cells ($p=0.0016$), but not in ER-p38MAPK (Figure 3.5.b).

It has been suggested previously that Btk can be activated in cells with functionally deficient PI3-K through another mode of Btk recruitment (Fukuda *et al.,* 1996). However, in case of ER-p38MAPK category we see the lack of the recruitment of all three pro-survival PKs: Btk, PI3-K and AKT.

Therefore our data demonstrates that signal transduction in approximately half of CLL samples following CD180 ligation operates via Btk-PI3-K-AKT protein kinase circuit, whist a fourth of the samples activate p38MAPK instead.

To ascertain whether activation of these PKs has an effect on CLL cell survival we simultaneously measured the levels of Mcl-1 in all categories of cells since Mcl-1 induction has been linked to activation of the PI3-K/AKT protein kinases (Longo *et al.,*2008). Inhibition of AKT activation resulted in apoptosis preceded by a decline in Mcl-1 (Zhuang *et al.,*2001). Indeed, in our hands CD180-mediated induction of Mcl-1 was seen only in ER-AKT CLL cells (Figure 3.6.a).

It has been shown previously that pharmacological inhibition of PI3-K activity prevented induction of Mcl-1, and the deficiency of the latter promoted apoptosis (Petlickovski *et al.,*2005). Indeed, measurement of the levels of apoptosis in various categories of CLL cells in 24h long cell cultures revealed that in ER-p38MAPK cells ligation of CD180 led to a significant induction of apoptosis ($p=0.041$). In contrast both control B cells and pooled AKT-responder CLL cells demonstrated significant drop in the percentages of apoptotic cells ($p=0.017$ and $p=0.011$ resepctively) as shown in Figure 3.6.b.

Importance of the speed in AKT phosphorylation (Early vs Late AKT Responders) for pro-survival effect of CD180-ligation is unclear. It has been shown that magnitude and duration of CpG-ODN-induced AKT signalling response affects the proliferation capacity of CLL clones (Longo *et al.,*2007). However we could not find significant differences in the magnitude of AKT activation (Figure 3.3.a and 3.3.b) between ER-AKT and LR-AKT CLL cells. Nor have we detected differences in the basal levels of pAKT between these categories of cells (Figure 3.3.a).

Our data strongly suggests that CD180 ligation has pleiotropic effect on apoptosis of CLL cells. In approximately half of CLL samples CD180 engagement activates pro-survival signalling nodule Btk-PI3-K-AKT accompanied by a suppression of p38MAPK signalling. In a fourth of CLL samples pro-survival PKs are not recruited, and instead recruitment of p38MAPK leads to the induction of apoptosis.

There are few reports on the role of p38MAPK-mediated signalling in CLL cells. Recruitment of p38MAPK appeared to have little effect on CpG-ODN induced CLL cell proliferation (Longo *et al.,*2007) and was linked with the tolerant status of CLL cells (Ntoufa *et al.,*2012). Activation of Syk and Lyn, but not Btk alone was shown

to be essential for the phosphorylation of p38MAPKinase (Malavasi *et al.,*2011). Since in our hands ZAP70/Syk but not Btk activation (Figure 3.4.a,b,c) was associated with downstream engagement of p38MAPK, Lyn as well as PKC and Vav (Piatelli *et al.,*2004) appear as candidates for an upstream PKs involved in this pathway. These studies are currently underway.

It is also important to follow up a significant difference in CD38 expression detected by us between AKT and p38MAPK responders (Table 1), indicating that the latter population represents an expanding subset of CLL cells, since CD38 was shown to drive CLL cell proliferation and chemotaxis (Dal Porto *et al.,*2004). Although in this study ZAP70 and ERK were pinpointed as major players downstream to CD38, our data indicates possible involvement of p38MAPK.

It is unclear whether in the remaining one forth of "genuine" NR CLL cells downstream from ZAP70/Syk signal is blocked or there is another pathway, operated via different PKs from those studied (Figure 3.2.a and 3.4.a) (Porakishvili *et al.,*2011).

The current view is that CLL cells sense microenvironment via BCR, assisted by TLRs and cytokines receptors. It was important therefore to assess sIgM-mediated signalling in the categories of CLL cells defined through their responses to CD180(studies outlines in the next chapter).

Chapter 4 CD180 and IgM interactions

Introduction

Studies with CD180 have shown that ligation on B cells with specific mAbs leads to activation and proliferation of murine B cells (Yamashita *et al.,* 1996, Miyake *et al.,* 1998). However, no specific intracellular pathway has been identified as CD180 lacks the toll/interleukin receptor (TIR) domain, which is mandatory for signalling. Activated RP105 in mice has been reported to signal through pathways similar to the BCR complex (Yazawa *et al.,* 2003). In the previous chapter we have shown that this is indeed the case as the protein kinases Zap70/Syk, Btk, PI3K, p38MAPK, AKT and Mcl-1 which play a role in signalling through the BCR, were also activated when CD180 was stimulated with mAb. Our group has previously expression of CD180 and sIgM is differential in CLL cells (Porakishvili *et al.,*2005). We further hypothesized that the expression of these two receptors on the surface of CLL cells was possibly a determining factor in the course/fate of the CLL cell due to antigenic stimulation. Also, the dichotomy established in the signalling pathways, with the ligation of CD180, further fabricated the idea of comparing the effects of CD180 ligation and sIgM ligation on the CLL cells. Considering that AKT activation was a more rational marker for assaying the CD180 response, we grouped the previously studied R and NR CLL clones (based on effect of CD86 upregulation by CD180 ligation) into ER-AKT, LR-AKT, ER-p38MAPK and Non-responders and a minor subset of double responders. In this chapter, further assayed are the effects of sIgM stimulation in these subsets of cells.

Results

4.1a ER-AKT and LR-AKT CLL cells, but not ER-p38MAPK or NR CLL cells activate pro-survival signalling pathway through the engagement of sIgM

Since AKT plays central role in pro-survival signalling in CLL cells through the engagement of the BCR (Packham *et al.,*2010; Wickremasinghe *et al.,*2011) and the anti-apoptotic effect of CD180 in our hands also appears to be operating via AKT, it was important to compare signalling patterns of sIgM and CD180 in the major categories of CLL cells defined by us above.

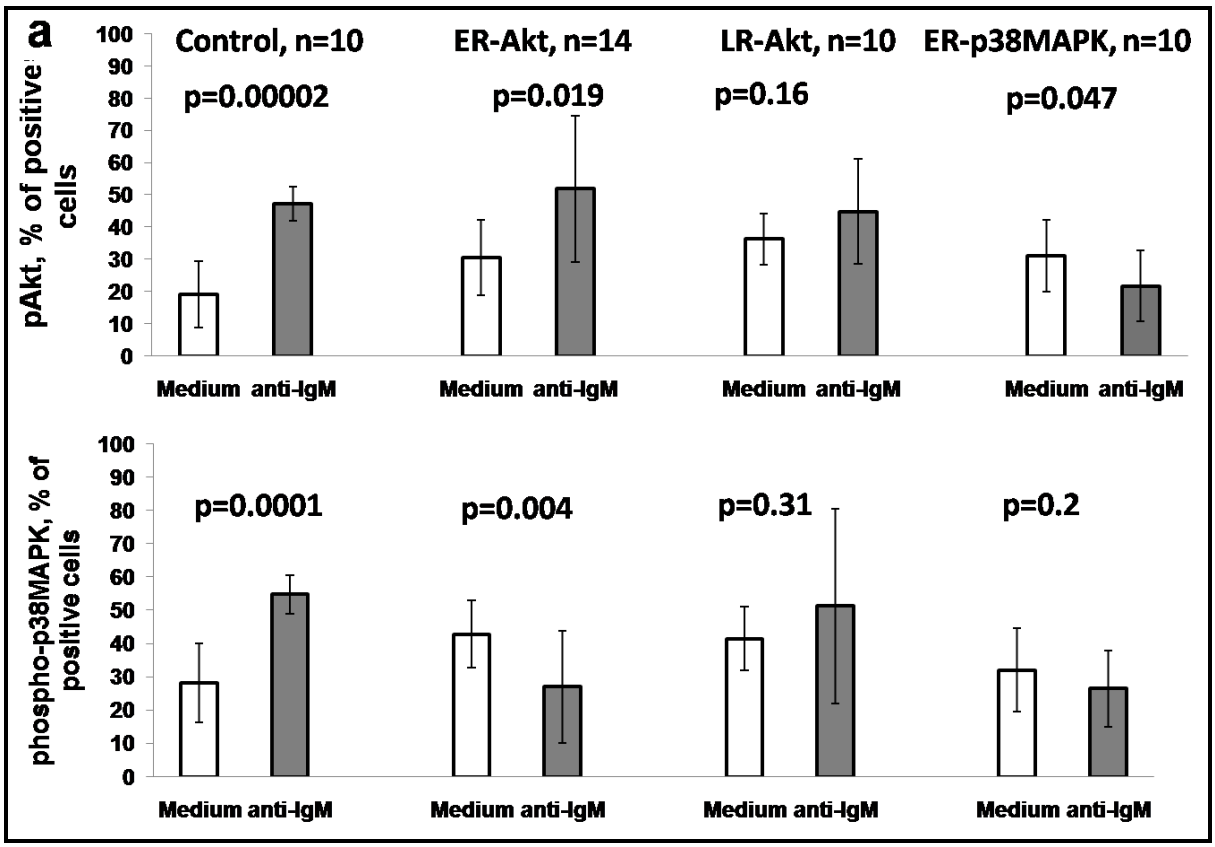
Our data (Figure 4.1.a, b and c) demonstrated striking similarities between CD180 and sIgM-mediated signalling pathways in the ER-AKT category of cells regarding activation of Btk, PI3-K and AKT. Anti-IgM Ab induced significant phosphorylation of these protein kinases in ER-AKT CLL clones, but not in LR-AKT clones. A possible explanation could be lower density of sIgM and CD79b on LR-AKT cells compared to the ER-AKT cells, (discussed in chapter 3) indicating the importance of a certain threshold of the BCR density for initiation of signalling.

Similar to CD180 ligation, there was a significant downregulation of the basal levels of phospho-p38MAPK in ER-AKT cells. Ligation of sIgM on ER-AKT cells also led to upregulation of NF-κB (Figure 4.1.b), and increased protection from apoptosis (Figure 4.1.d). The pro-survival effect of sIgM ligation was only detectable in control B cells and ER-AKT cells, but not in LR-AKT or ER- p38MAPK cells. As in case of CD180, we could not find activation of the enzymes studied following sIgM ligation in NR-CLL group.

However, in contrast to the CD180 mediated signalling we did not detect appreciable activation of p38MAPK through the ligation of sIgM in any of the CLL categories tested (Figure 4.1.a and 4.1.b), including ER-p38MAPK group. Instead, in ER-p38MAPK cells we observed a drop in the basal levels of both pBtk and pAKT (Figures 4.1.a and c) and lack of protection from apoptosis (Figure 4.1.d). This

indicates that the pro-survival effect of sIgM-mediated signalling was blocked in this category of cells, but through a pathway different from that through p38MAPK.

Thus, our data suggest that both CD180 and sIgM ligation on CLL cells results in activation of a pro-survival signalling pathway operating via AKT. However, whereas anti-CD180 mAb can activate a pro-apoptotic pathway mediated via p38MAPK, ligation of sIgM alone does not lead to the activation of this protein kinase.



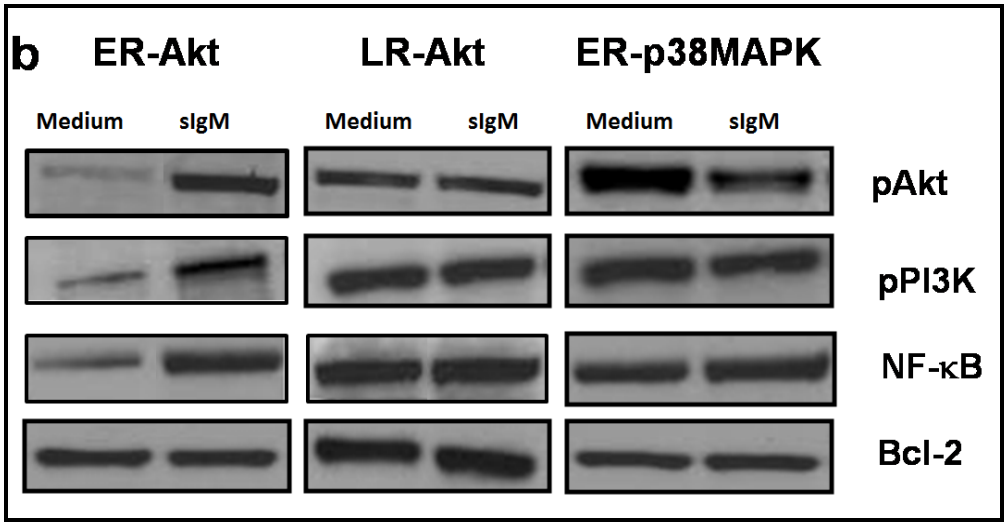
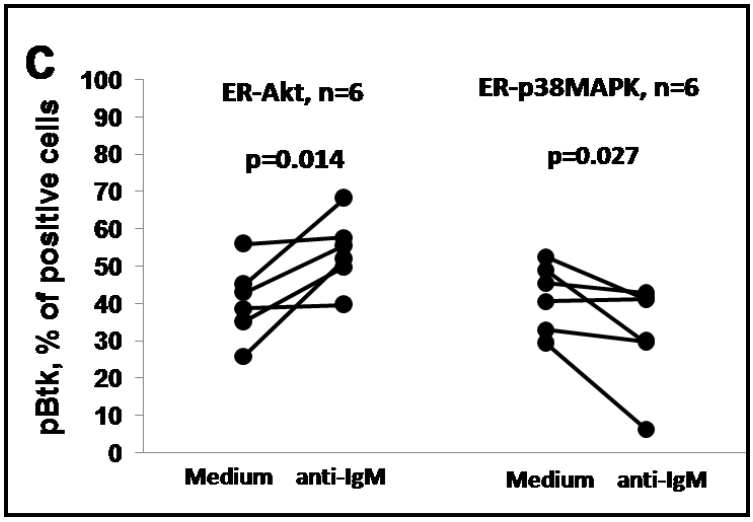


Figure 4. 1 a,b: Anti-IgM mediated phosphorylation of AKT, PI3-K, NF-κB and p38MAPK in different categories of CLL cells described above.



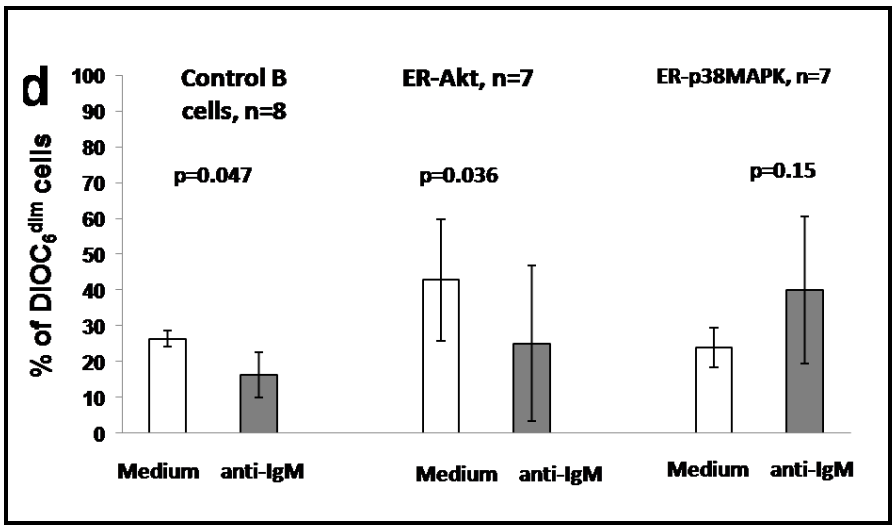


Figure 4.1.c,d: Anti-IgM mediated phosphorylation of Btk and survival of control B cells and different categories of CLL cells

(a and c) Control B cells and CLL cells were incubated with anti-IgM Ab for 30 minutes (ER) or 24h (LR) or left unstimulated in medium, washed, stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-pAKT(Ser473), anti-phospho-p38MAPK(Thr180/Tyr182) and anti-pBtk 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. **(b)** Representative immunoblots showing the levels of pAKT, pPI3-K and NF-κB in ER-AKT, LR-AKT and ER-p38MAPK CLL cells following stimulation with anti-IgM Ab as described in the Materials and Methods. Unstimulated CLL cultures in (Medium) were used as controls. The bands were visualized respectively by anti-pAKT(ser473), anti-pPI3-K(tyr458)/p55(tyr199) or anti- NF-κBp65(D14E12) mAbs. Bcl2 was used as a loading control. **(d)** Percentages of $DiOC_6^{dim}$ cells in control B cells, ER-AKT and ER-p38MAPK CLL cells following stimulation with anti-IgM Ab as described in the Material and Methods. Control B cells and CLL cells were stimulated with anti-IgM Ab for 24h, washed, stained with anti-CD19 mAb, loaded

with $DiOC_6$ for 20 min and analysed by flow cytometry. P values were calculated using Mann-Whitney *U*-test.

4.2.a Pre-treatment of CLL cells with anti-CD180 mAb re-wires sIgM-mediated intracellular signalling from PI3-K/AKT to p38MAPK pathway

Our data indicates that CD180 and IgM operate through similar signalling pathways, particularly that of Btk/PI3-K/AKT, leading to the increased survival of CLL cells. We next tested whether pre-engagement of CD180 would affect signalling through sIgM (the BCR). This was important to determine since, Yamashita *et al.,*1996, demonstrated that Ab-mediated cross-linking of RP105 on mouse B cells led to sensitization to apoptosis in response to BCR ligation.

In 11 of the 16 CLL samples tested, pre-treatment with anti-CD180 mAb, followed by the stimulatory anti-IgM Ab, lead to a significant decrease in the levels of phosphorylation of AKT (Figure 4.2, a and b) and PI3-K (Figure 4.2.b) compared with anti-IgM alone. Simultaneously, we observed a decline in the percentages of pBtk+ cells from 41.9 ± 18.7% down to 34.7 ± 19.1% (n= 9, p=0,029). The levels of the anti-apoptotic proteins Mcl-1 and Bcl_{XL} were also appreciably decreased (Figure 4.2.b) indicating a significant reduction of pro-survival signalling. Interestingly a drop in the levels of pERK was also detected, in half of these CLL samples (Figure 4.2.c). However, the most important observation was that reduction in Btk/AKT/PI3-K signalling in these 11 samples was accompanied by a significant upregulation of phosphorylated p38MAPK (Figure 4.2.a). In contrast, pre-treatment of the remaining 5 out of 16 CLL samples with anti-CD180 mAb followed by engagement of sIgM, led to a significant increase in the levels of pAKT and pPI3-K, but no changes were detected in the levels of activated p38MAPK or anti-apoptotic proteins (Figures 4.2.d and 4.2.e).

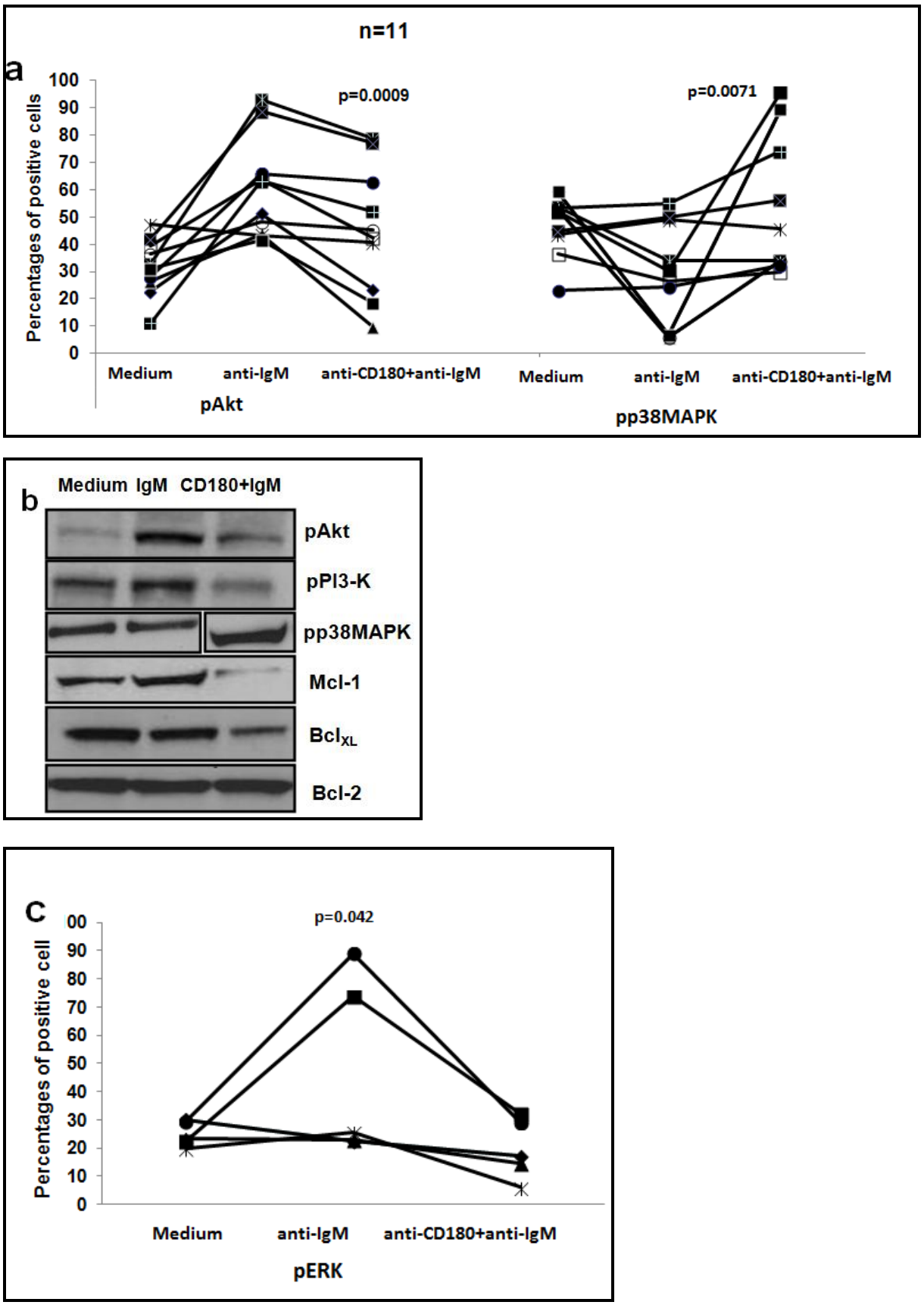
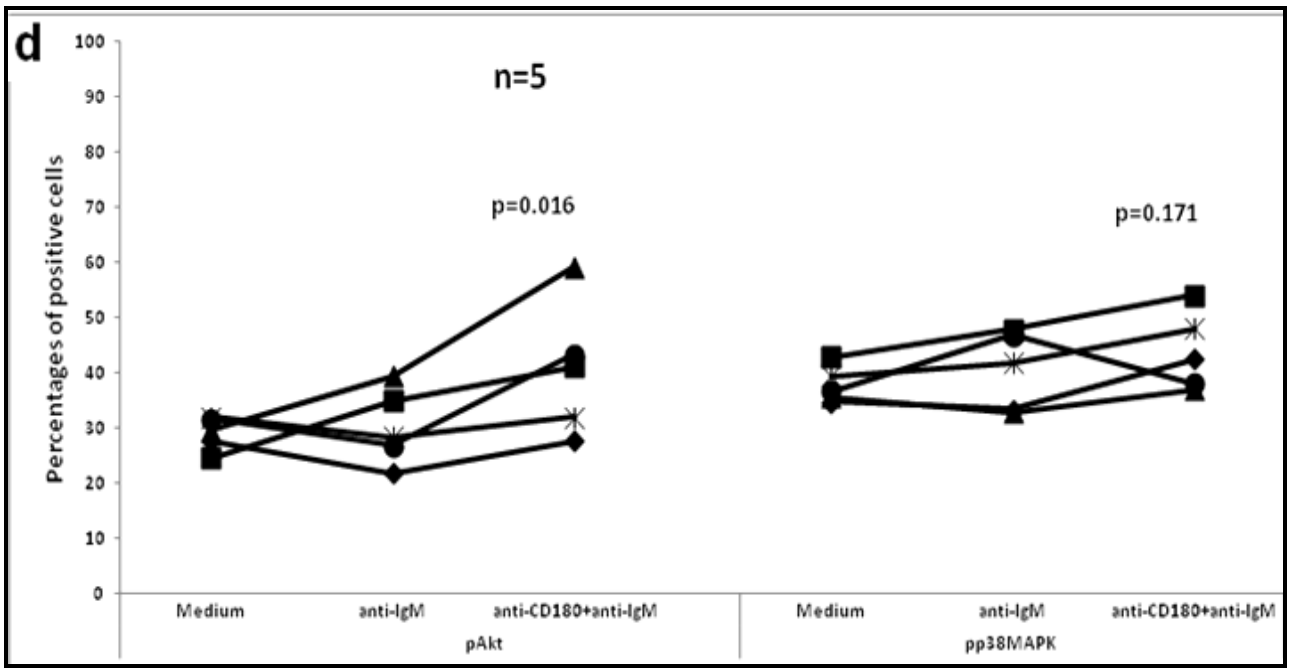


Figure 4. 2.a,b,c. Activation of intracellular protein kinases and anti-apoptotic proteins following sequential ligation of CD180 and sIgM.



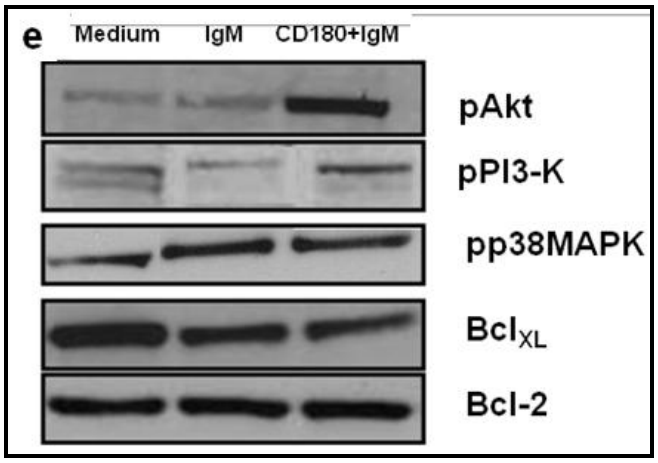


Figure 4.2.d,e:. Activation of intracellular protein kinases and anti-apoptotic proteins following sequential ligation of CD180 and sIgM.

(a, c and d) CLL cells were treated with anti-CD180 mAb for 15 minutes and with anti-IgM Ab for another 15 minutes as described in the Material and Methods. The cells were stained with anti-CD19 mAb, fixed, permeabilised and stained with anti-pAKT(Ser473), anti-phospho-p38MAPK(Thr180/Tyr182) and anti-pERK(44/42) 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. **(b and e)** Representative immunoblots of pAKT, pPI3-K, pp38MAPK, Mcl-1 and $Bcl_{XL,}$ following sequential ligation of CD180 followed by IgM. Bcl-2 was used as a loading control. The bands were assessed and visualised as described by the Materials and Methods.

Interestingly, the two groups of CLL clones (with and without modulation of sIgM-mediated signalling) differed in the levels of AKT phosphorylated in response to the ligation of sIgM alone: it was twice as high in the first group compared with the minor group that showed no changes – 60.3±18.4% vs 30.3±6.9%, p=0.0003.

Also notable was that the first group was characterised by the higher levels of expression of CD38: 1105±676 vs 374±192 RBS/cell, p=0.024; 42.3 ± 20.9% vs 19.5±12.3%, p=0.045 (n=11 and n=5 respectively).

In contrast to CD40 and IL4 mediated activation (measured by the expression of CD86) and cycling (assessed through Ki-67) of CLL cells demonstrated previously by our group (Porakishvili *et al.,*2011) there was no additive effect between CD180 and sIgM mediated CLL cell activation or cycling (studied later and personal communication with Miss Nadeeka Rajakurna, continuing PhD student in our research group at University of Westminster) which is consistent with convergence of CD180 and sIgM signalling pathways. Furthermore it indicates that re-wiring of the sIgM-induced AKT-mediated signalling pathways by CD180 does not affect cell activation and cycling, but only their survival and apoptosis.

We therefore conclude that in those CLL cells where ligation of sIgM led to a substantial activation of the pro-survival Btk/PI3-K/AKT pathway, pre-engagement of CD180 redirected BCR-mediated signalling towards the potentially pro-apoptotic p38MAPK pathway.

4.3a CD180 negative CLL clones demonstrate impaired responses to sIgM stimulation

Since CD180 engagement had such a profound effect on rewiring of the sIgM-mediated pro-survival signalling pathway, we next wanted to determine if in the absence of CD180, sIgM-signalling would be intact. Surprisingly, ligation of sIgM on CD180neg CLL cells did not result in an appreciable activation above basal level of all protein kinases tested, apart from Btk where a substantial increase has been detected (Figure 4.3a). Furthermore, activation of CD180neg CLL cells measured by the level of expression of CD86 (in 24h - 72h cultures) was also decreased when

compared to R-CLL clones (Figure 4.3b, p value between CD180+ and CD180neg CLL cells = 0.013).

Interestingly, CD180neg CLL cells (n=17) were characterised by a significantly lower expression of CD38, as compared to CD180+ CLL cells (n=33): 828 ± 762 vs 387 ± 365 RBS/cell, p=0.026; and 33.0 ± 23.4 vs 18.2 ± 16.5%, p=0.045.

Taken together with our group's previous observation that activation and cycling of CD180 negative CLL cells in response to anti-CD40 mAb or the addition of rIL-4 was low (Porakishvili *et al.,*2011), our data are consistent with the hypothesis that CD180 negative CLL cells respond poorly to microenvironmental signals.

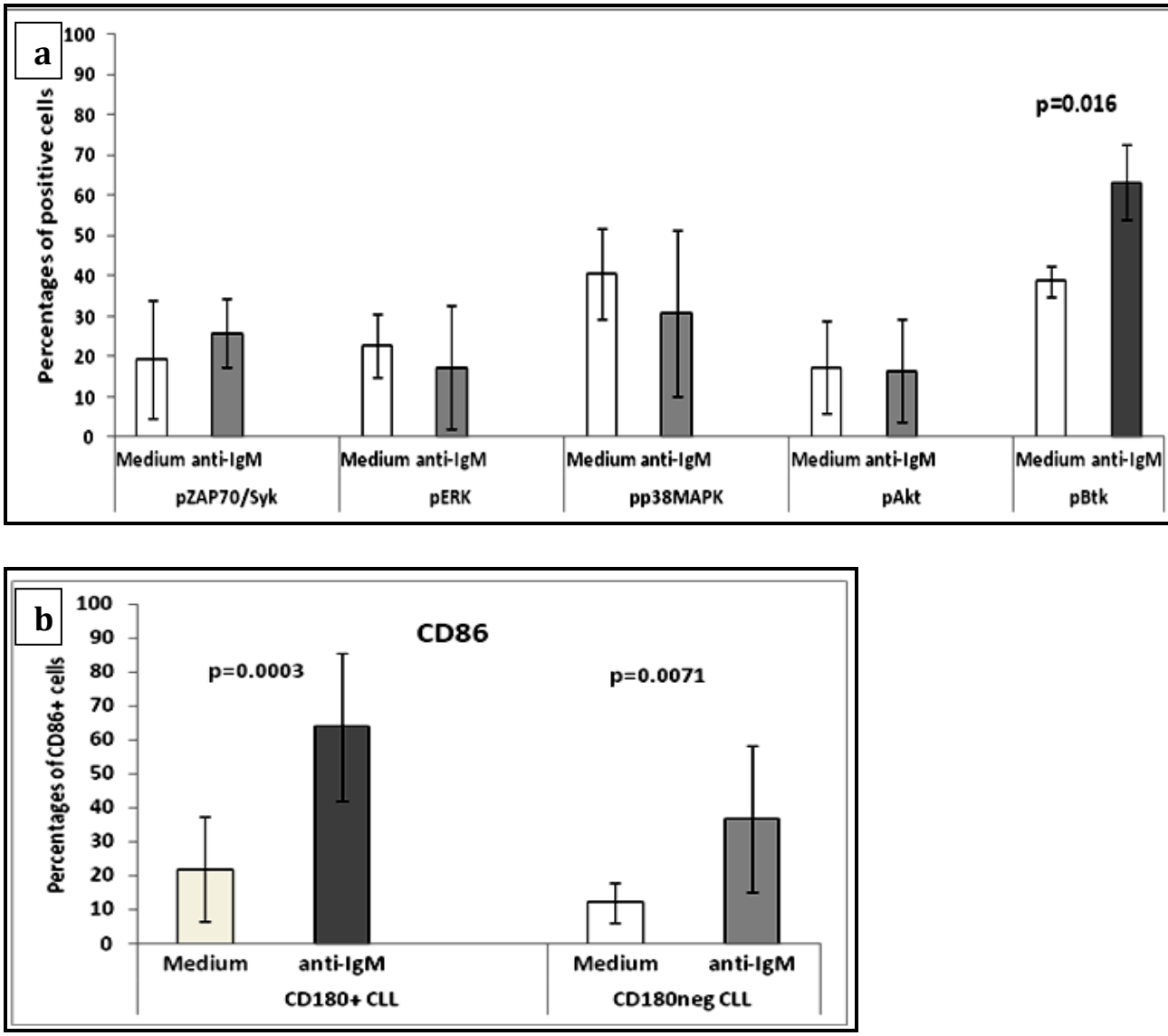


Figure 4. 3 a,b sIgM-induced signalling, activation of CD180-IgM+ CLL cells.

(a) Ten CD180neg CLL clones were treated with anti-IgM Ab for 30 minutes and the levels of activated protein kinases assessed as described in Materials and methods **(b)** Ten CD180+IgM+ and ten CD180-IgM+ CLL clones were treated with anti-IgM mAb for 48-72h as described in the Material and Methods, washed and counterstained with anti-CD19 and anti-CD86 mAbs using unstimulated cultures (Medium) as controls. The results were analysed by flow cytometry and expressed as percentages of CD86+ cells. p values were calculated using the Mann-Whitney non-parametrical U-test.

Discussion

4.1.b Pro-survival Btk-PI3K-AKT pathway, but not pro-apoptotic p38MAPK signalling pathway, is activated via CD180 and sIgM in ER-AKT cells

It has been shown for B cells that PI3K-signalling is more important for transducing BCR-signalling, than TLR-signalling (Pone *et al.,*2012). However, our data strongly suggests that ER-AKT CLL cells activate pro-survival Btk-PI3K-AKT pathway following the ligation of either receptor – CD180 (Figure 3.6.a,b) or sIgM (Figure 4.1.a,b,c,d). In our hands there was a striking resemblance between PK activation profiles in this category of cells: substantial activation of Btk, AKT and PI3K following sIgM ligation (Figure 4.1a, b and c) leading to significant survival of CLL cells from apoptosis (p=0.036), comparable with that of control B cells (p=0.047, Figure 4.1.d). This was accompanied by a substantial increase in the levels of NF-κB (Figure 4.1.b), which is often activated downstream to Btk and AKT and PI3K (Ringshausen *et al.,*2002; Herman *et al.,*2010). However, differently from CD180, we were unable to detect activation of PKs in the LR-AKT category of cells following stimulation via sIgM (Figure 4.1a and 4.1 b). This could be explained by a low expression of sIgM and CD79b on ER-AKT category of cells discussed above. It appears that the level of expression, in particular, the density of sIgM and CD79b reflected in antibody RBS/cell (Porakishvili *et al.,*2005; 2011) on these cells is below threshold for BCR engagement.

Importantly, like in case of CD180, we have observed significant reduction in the basal levels of activated p38MAPK in ER-AKT cells following engagement of sIgM (Figure 4.1a, p=0.004).

However, unlike CD180, we failed to detect any activation of p38MAPK in ER-p38MAPK responder cells. It was predictable, since in this category of CLL cells we also established significant drop of pBtk+ cells below the basal levels following stimulation of sIgM (Figure 4.1c), and it has been previously shown that BCR signalling completely failed in Btk-deficient B cells, assessed by the absence of proliferation and the lack of NF-κB activation produced by BCR engagement (Khan

*et al.,*1995; Petro *et al.,*2000), in line with our observations (Figure 4.1b). Others also failed to detect p38MAPK activation in CLL cells following BCR ligation (Efremov *et al.,*2007). What it means though is that incapability to activate Btk abrogates both "arms" of BCR signalling (via AKT and p38MAPK), but leaves CD180-mediated p38MAPK activation intact (Figure 3.5.b). In other words, our data indicates, that differently from BCR, CD180 can signal downstream to p38MAPK bypassing Btk. This may have implications for CLL therapy where Btk inhibitors are being used since CLL cells might receive Btk-independent stimuli from microenvironment via CD180.

Interestingly, out of the double-responder AKT/p38MAPK subset of cells only one/seven was characterized by activation of both PKs following ligation of sIgM, whilst the rest only demonstrated phosphorylation of AKT. In contrast, our pilot experiments suggest that some CD180 NR-CLL cells are responding to sIgM ligation by activation of Btk (data not shown). Overall, we found that activation of p38MAPK following sIgM engagement is a rare event, compared to the ligation of CD180, a view shared by Efremov et al (2007).

This means that although there is a substantial overlap in CD180 and sIgM intracellular signalling pathways, they could be quite different. However in one third of tested CLL samples – ER-AKT, CD180 and sIgM signal transduction converged towards pro-survival Btk-PI3K-AKT pathway. This prompted us to study how signal transduction would proceed if we "clash" the pathways through sequential ligation of CD180 and BCR, moreover that the likelihood of CLL cells receiving both microenvironmental stimuli *in vivo* should be quite high.

4.2b Pre-treatment of CLL cells with anti-CD180 antibodies re-wires the sIgM signalling pathway from pro-survival to pro-apoptotic

Interactions between CD180/RP105 and BCR signalling pathways in CLL have not been reported before. In a paper published in 1996, Yamashita *et al.,* reported that treatment with anti-RP105 mAb of murine B cells sensitized them towards anti-IgM induced apoptosis. To our knowledge, there were no other reports on the

subject, involving human or mouse B cells or CLL cells. Likewise there is little data on interaction between CD180/RP105-mediated and other signalling pathways in B cells. RP105 was reported to enhance CpG DNA-induced proliferation and survival by naïve B cells through upregulation of the expression of TLR9 (Yamazaki *et al.,*2010), as well as TLR4-dependent survival, proliferation and plasma cell generation of mantle zone B cells (Nagai *et al.,*2012).

Our pilot histochemical analysis identified substantial expression of CD180 on CLL cells in the bone marrow aspirates and on CLL cells and normal B cells in lymph node biopsies (discussed in chapter 5). Hence it was intriguing to explore using stimulatory antibodies the "fate" of CD180+sIgM+ CLL cells when they receive both stimuli mimicking *in vivo* interactions with microenvironmental putative CLL ligand and sIgM (auto)antigen.

Our data proves that 15 minute long pre-treatment of CLL cells with anti-CD180 mAbs substantially diminished pro-survival Btk-PI3K-AKT signalling pathway mediated by subsequent ligation of sIgM and re-directed it towards pro-apoptotic p38MAPK pathway in two thirds of CLL samples tested (Figure 4.2a,b,c). Not only levels of activated pBtk, pAKT and pPI3K dropped significantly (Figures 4.2.a,b,c,d,e), but this was accompanied by a decrease in the anti-apoptotic proteins Mcl-1 and Bcl_{XL} (Figure 4.2.b,e). In CLL, McL-1 is associated with chemoresistance, resistance to apoptosis and poor prognosis (Pepper *et al.,*2008; Scupoli *et al.,*2012). Our data strongly suggests that the mechanism of the suppression of pro-survival signals during sequential ligation of CD180 and sIgM involves favoring of pro-apoptotic p38MAPK pathway. It is interesting that in half of the samples were re-wiring towards apoptosis was observed, we found a parallel significant decrease in the levels of activated Erk. Since Erk was reported as a key element of CLL cell expansion *in vivo* driven by CD38 (Malavasi *et al.,*2011) pre-ligation of CD180 might also lead to decreased potency of CLL expansion *in vivo*. This rewiring of sIgM-induced anti-apoptotic signals towards pro-apoptotic by the ligation of CD180 confirms earlier observations (Yamashita *et al.,*1996) and could have important implications in future strategies for therapy of CLL.

In a one third of CLL samples where sIgM ligation alone resulted in a poor activation of AKT-PI3K pathway, pre-engagement of CD180 lead to the opposite effect: potentiation of the pro-survival signal. Intriguingly, all of these CLL samples belonged to the category of AKT/p38MAPK double responders (also discussed in chapter 3). We have previously demonstrated that, in contrast to CD40 and IL4 mediated cell activation and cycling, there was no additive effect between CD180 and sIgM ligation in the expression of CD86 or Ki-67 (Porakishvili *et al.,*2011). This might indicate that increased survival of this category of CLL cells induced by co-ligation of CD180 and sIgM is not accompanied by their proliferation/expansion. Indeed we found that all these CLL samples were CD38 negative.

We therefore conclude that in those CLL cells where ligation of sIgM led to a substantial activation of the pro-survival Btk-Pi3K-AKT pathway, pre-engagement of CD180 redirected BCR-mediated signalling towards the potentially pro-apoptotic p38MAPK pathway. However additive pro-survival effect follows co-ligation of CD180 and sIgM in case when either receptor alone provides sub-optimal stimuli. Although the second scenario seems to be rare, it emphasises once more the importance of individual tailor-made immunotherapeutical approaches to the treatment of CLL.

In the light of these findings it was interesting to establish signalling pattern of CD180negIgM+ CLL. We found that in the absence of CD180 sIgM-induced CLL cell signalling is impaired (Figure 4.3.a,b). Only Btk has been significantly activated in this category of CLL cells with no appreciable increase in the levels of pZAP70/Syk, pERK, pAKT or pp38MAPK (Figure 6), and cell activation was also diminished (Figure 4.3b). We have previously shown that CD180neg cells were also poorly responding to the ligation of CD40 and addition of recombinant IL-4 (Porakishvili *et al.,*2011). Thus it appears that CD180 represents an essential component of CLL signalling machinery through its interaction with and modulation of sIgM-mediated responses.

It is intriguing that sequential transduction of two pro-survival signals via CD180 and sIgM results in its abrogation. Likewise we have also shown previously that pre-treatment of CLL cells with anti-sIgM leads to the abrogation of CD180-

mediated PI3-K/AKT signalling (Vispute *et al.,*2011). We hypothesize that interaction between CD180 is due to the convergence of certain key signalling pathways (Figure 4.4). Whereas pro-survival pathway appears to be operating through Btk-PI3K-AKT circuit, the upstream to p38MAPK elements of pro-apoptotic pathway have yet to be identified.

Our data strongly suggests that in a substantial number of CLL samples by pre-engaging CD180 we could prevent further pro-survival signalling mediated via sIgM and, instead, induce CLL cell apoptosis, which opens doors to new strategies for the treatment of CLL.

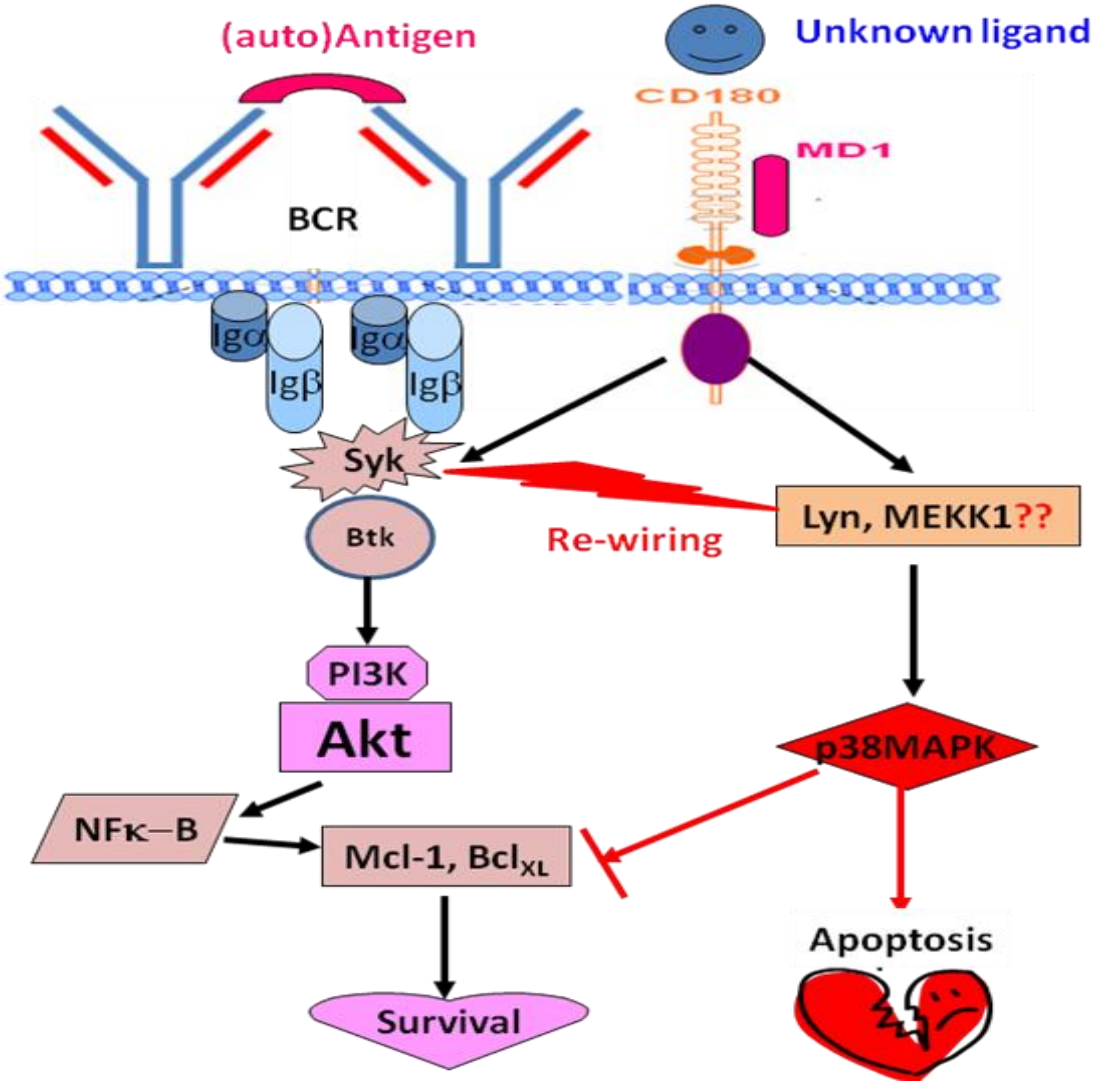


Figure 4. 4: Hypothetical model for interactions between CD180 and sIgM pathways

Chapter 5 Expression of CD180 in lymphoid tissues

Introduction

CLL is a lymphoproliferative disease, with heterogeneous manifestations and variable prognosis. Patients with CLL often have lymphadenopathy and splenomegaly. CLL is characterized by highly variable distribution of tumour mass between peripheral blood, bone marrow and lymphoid organs which is important for staging, classification and prognosis. There is an increasing evidence of the role of lymphoid tissue microenvironment providing pro-survival stimuli for the leukaemic cells and hence contributing to the pathogenesis of the diseases (Burger *et al.,*2009). CLL cells when cultured *in vitro* undergo apoptosis indicating that the *in vivo* microenvironment supports their growth/proliferation and survival. Bone marrow stromal cells when added to CLL cell cultures *in vitro*, aid the survival of the malignant cells (Deaglio *et al.,*2010). Recently it has been shown that the majority of the clonal evolution of the CLL cells, and proliferation, occurs in specialized structures in the tissues (bone marrow 'BM' and/or lymph nodes 'LN') referred to as 'pseudofollicles' or 'proliferation centres'. These structures were found in approximately 90% of CLL cases. They consist of loosely arranged larger cells that often contain prominent nucleoli, in contrast to true B-cell follicles, which may be found entrapped within the small lymphocytic infiltrates in sections of lymphoid organs in CLL (Schmid *et al.,*1994). Assessment of the cell-cycle marker Ki-67 suggests that CLL proliferation occurs in these proliferation centres in BM or LN (Burger *et al.,*2009). Better understanding complex interactions between CLL cells and tissue microenvironment, would add to the knowledge of CLL epidemiology and novel therapeutic approaches.

Our previous studies have shown CD180 to be expressed by 2/3rd of the CLL samples and a majority of control B cells. However, these studies account for the trafficking cells in the peripheral blood. It was essential to assess expression of CD180 on CLL cells in lymphoid tissues implicating their possible interaction with microenvironment through a putative endogenous ligand. In this pilot study, we attempted to determine CD180 expression on CLL cells in BM and LN, comparing it

with normal reactive tonsils. In three cases we were able to assess concordance of this CD180 expression in lymphoid tissue with the peripheral blood.

Results

5.1 CD180 expression in lymphoid tissues is heterogeneous

We studied the normal reactive tonsil tissue and LN and BM samples from CLL patients using histochemical staining with a specific CD180 antibody as described in Materials and methods. In brief, 2μm-thick paraffin embedded tissue sections (Tonsils, LN or BM) were used for staining with anti-CD180 mAb, blocked using 3-4% (v/v) hydrogen peroxide and counterstained with haematoxylin. The slides were reviewed with light microscopy and optimal conditions chosen based upon the criterion of background-free selective cellular labelling and images obtained.

The normal control reactive tonsil sample showed strong expression of CD180 (brown) by the cells in the mantle zone region of the secondary follicles (indicated with an arrow)(Figures 5.1 and 5.2). In contrast, the germinal centre (GC) cells showed extremely weak CD180 expression except of a few morphologically evident macrophages (indicated with arrows Figures 5.1 and 5.2).

In case of the 2 CLL LN samples studies, we observed very weak positive surface staining of CD180 (Images 5.3 and 5.4). The CLL cells were conspicuous by their monomorphic aggregates (as indicated in the darker stained sections).

In case of the bone marrow samples studied from 6 CLL cases, we saw a variable expression of CD180. Images 5.6,5.7,5.8,5.9 and 5.10 show areas in BM with CLL cell infiltrates (possibly 'proliferation centres'; indicated with arrows) with cells showing variable surface expression of CD180. Images 5.9 and 5.10 show relatively weaker CD180 expression compared to the CLL cases in images 5.5,5.6,5.7 and 5.8

We thus, with this pilot study, show that though strongly expressed by marginal zone cells in normal tonsil, CD180 expression pattern in the CLL tissues (BM and LN) is heterogeneous. Further studies with larger number of samples are necessary to validate our preliminary findings.

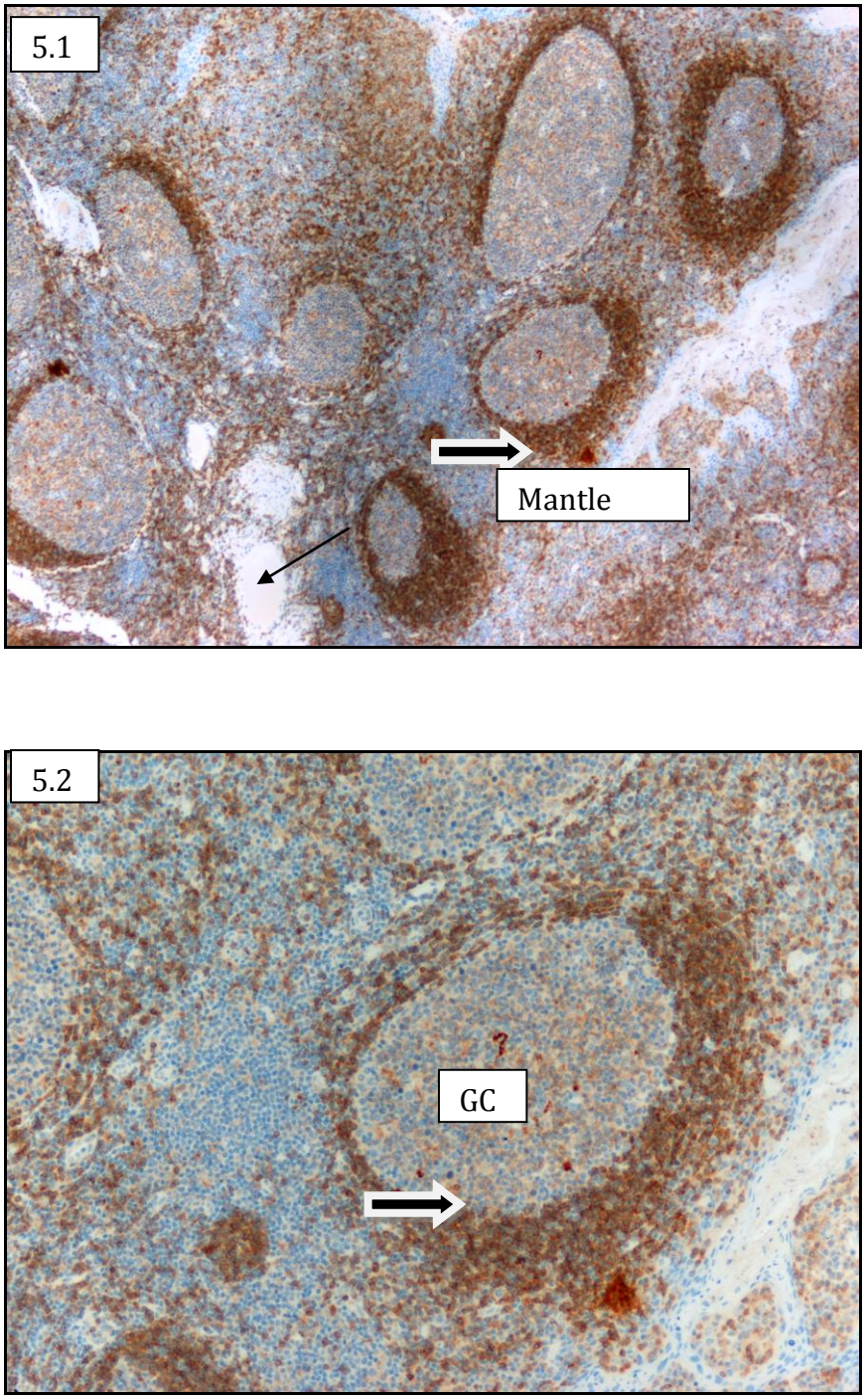
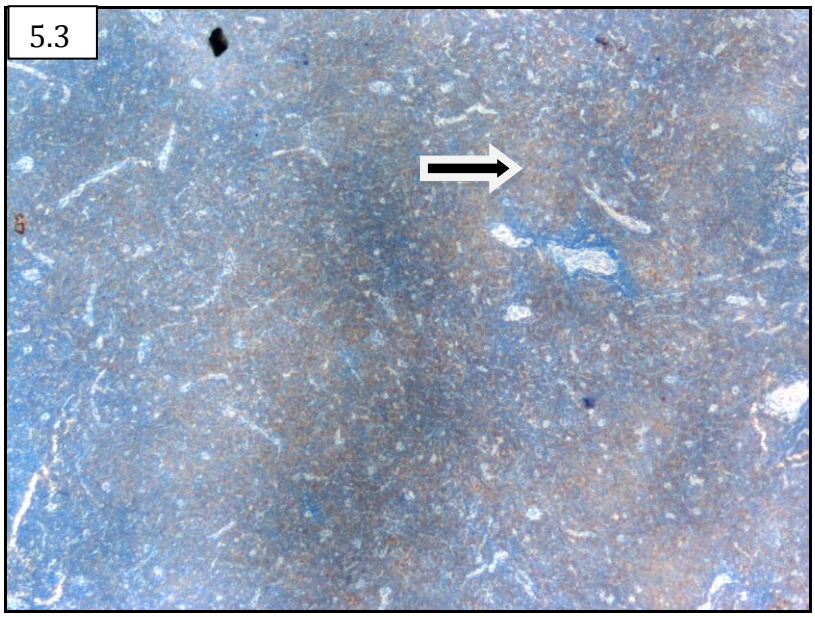


Figure 5. 1, 2: Histochemical staining for the expression of CD180 in normal reactive tonsil sample

(1) Normal reactive tonsil 10x magnification **(2)** 40x magnification of marked secondary follicle; 2µm thick paraffin embedded sections of the tissues from normal or CLL cases were stained with haematoxylin and anti-CD180 mAb, fixed and analysed with light microscopy as described in Materials and Methods. Arrows indicate the positive expression of CD180 observed (brown).



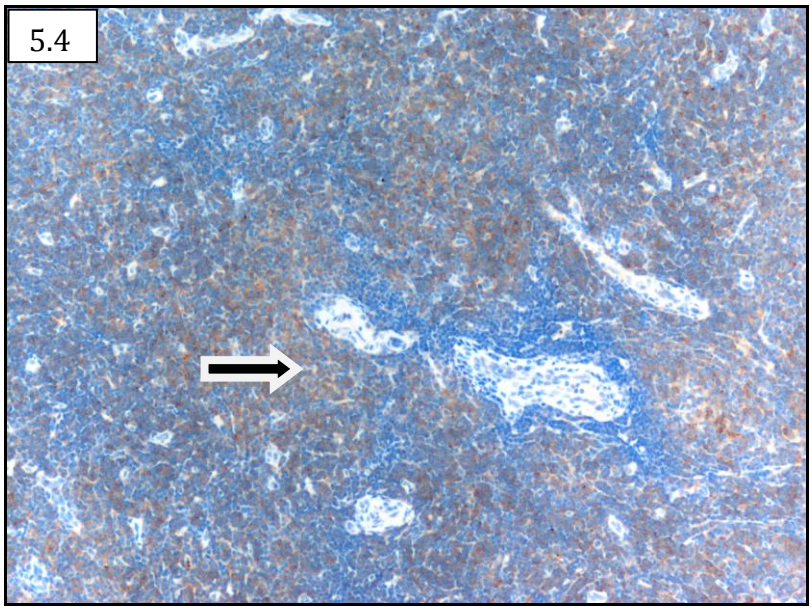
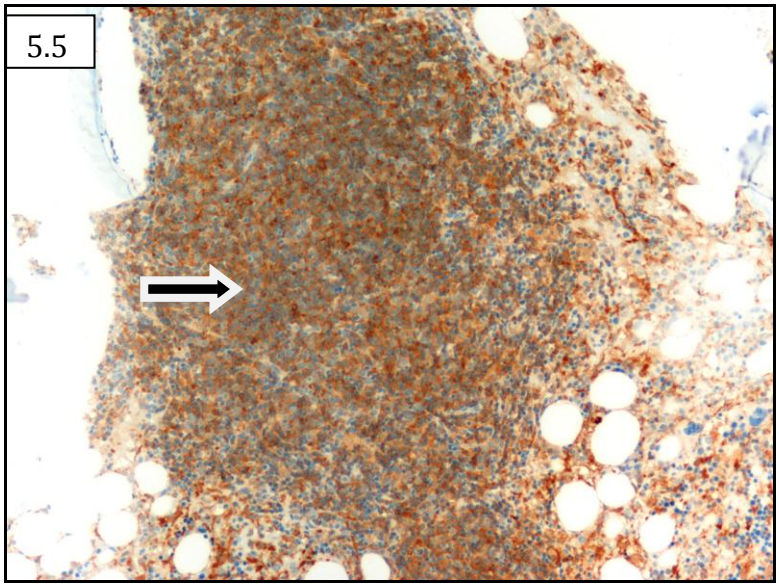
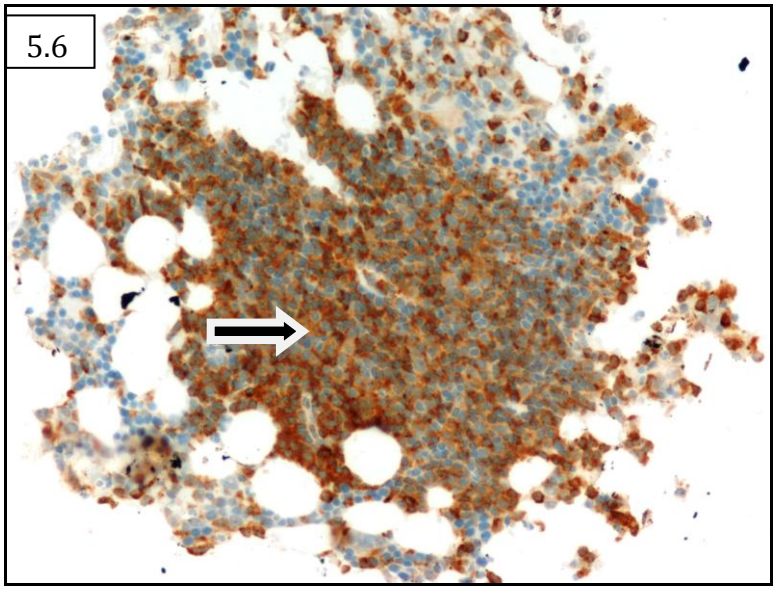


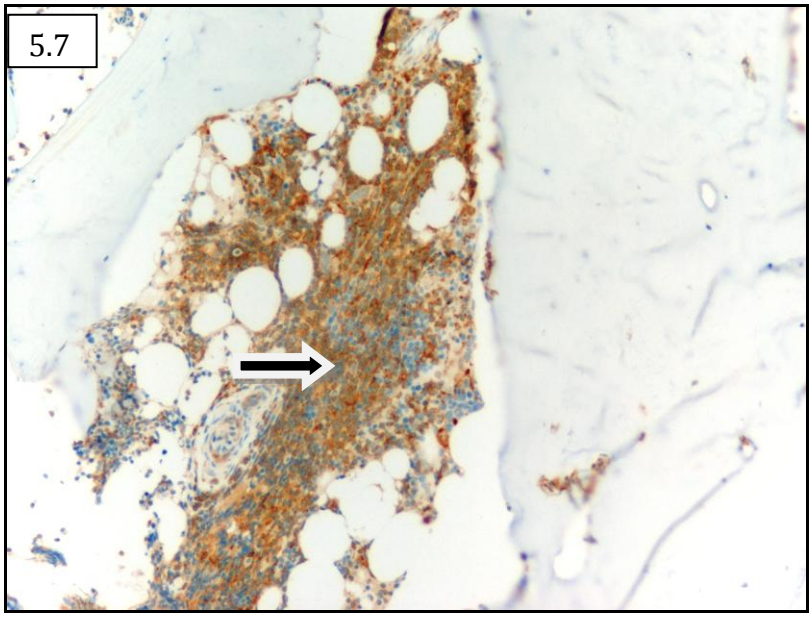
Figure 5. 3 , 4: Immunohistochemical staining for CD180 expression in CLL lymph nodes

Weak expression of CD180 is observed in the areas referred to as the 'proliferation centres'. Arrows indicate weak surface expression of CD180. 2µm thick paraffin embedded sections of the tissues from CLL cases were stained with haematoxylin and anti-CD180 mAb, fixed and analysed with light microscopy as described in

Materials and Methods. Arrows indicate the positive expression of CD180 observed (brown).



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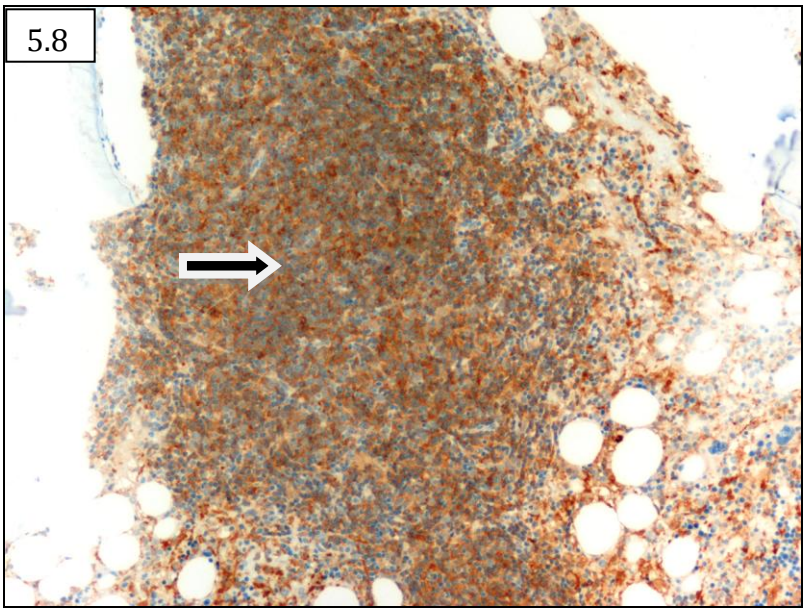
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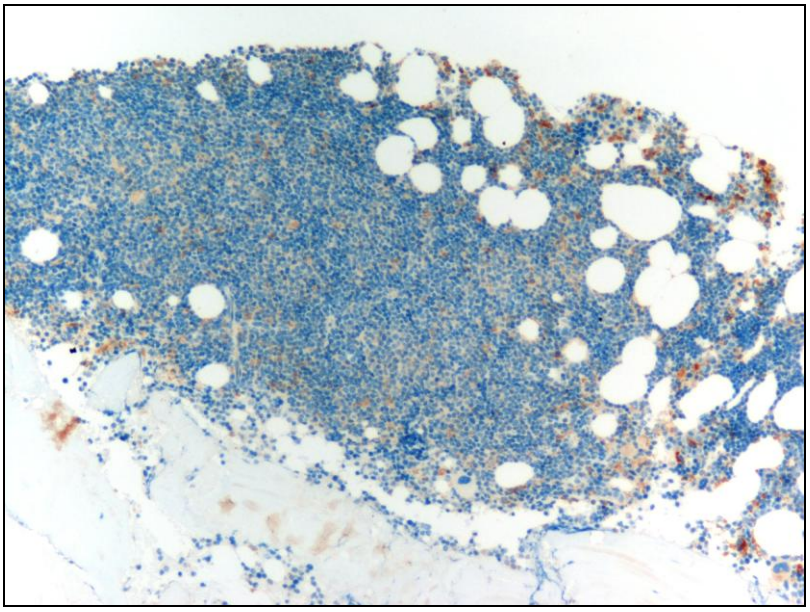


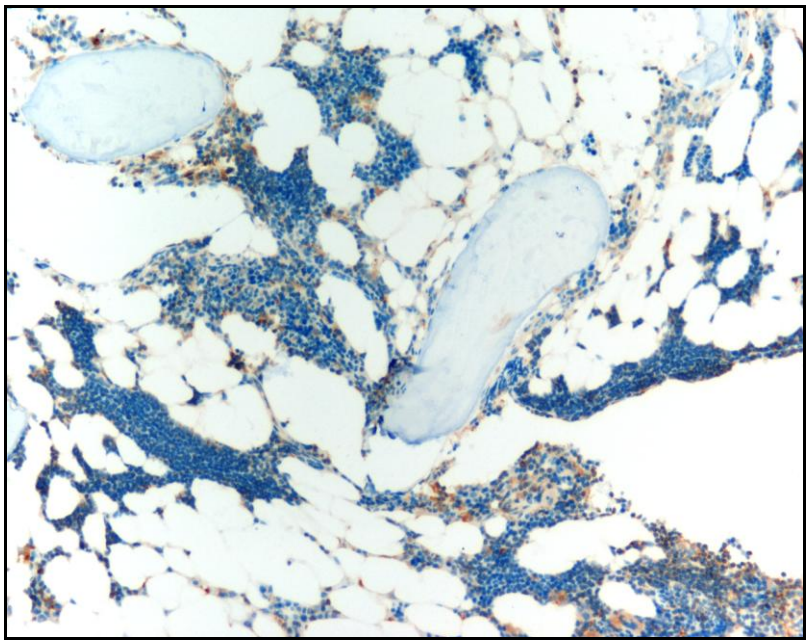


Figure 5. 5, 5. 6, 5. 7, 5. 8, 5. 9, 5. 10 : Histochemical staining of the expression of CD180 in CLL bone marrow samples

Figure 5.5 - 5.8 show distinct strong expression of CD180 as indicated. 5.9 and 5.10 show weak or even negative expression of CD180 in the CLL bone marrow samples. 2µm thick paraffin embedded sections of the tissues from CLL cases were stained with haematoxylin and anti-CD180 mAb, fixed and analysed with light microscopy as described in Materials and Methods. Arrows indicate the positive expression of CD180 observed (brown).

5.2 The level of the expression of CD180 is higher in Peripheral Blood (PB) than Bone Marrow (BM) of CLL patients

In three CLL cases we were able to analyse possible concordance of the CLL expression in the peripheral blood and in bone marrow. PBMCs were isolated from tissues (BM aspirates and PB) and suspended in medium as described in Materials and methods. The cells were stained using fluorochrome tagged antibodies to CD180 and CD19. Cells were analysed with flow cytometery by gating on CD19+ cells and calculating the relative MFI of CD180 expression with that of the isotype control. We observed that in all three cases studied, the surface expression of CD180 was significantly higher in the PB than in the BM ($p=0.03$) (Figure 5.11).

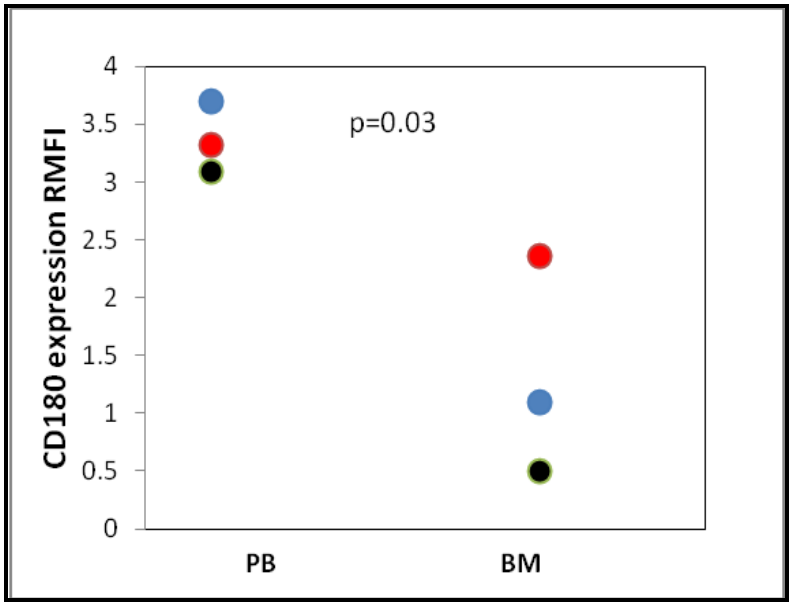


Figure 5. 11 CD180 expression pattern in peripheral blood and bone marrow cells from 3 CLL cases

CLL cells from peripheral blood and bone marrow aspirates from 3 patients were isolated and stained with fluorochrome conjugated anti-CD180 mAb as described in Materials and methods. The expression was assayed with flow cytometry and expressed as relative-MFI compared to the isotype controls. P values were obtained using paired *t*-test.

Discussion

It has been established that the neoplastic transformation of the CLL clone occurs in the lymphoid tissues (Burger *et al.,*2005). However, the histology of lymph nodes or bone marrow in CLL is heterogeneous and the relationship between different histological patterns and clinical outcome of the disease has been insufficiently studied. The main reason is the shortcoming in the availability of tissue samples for analysis owing to the fact that tissue biopsy, being highly invasive, is not a standard procedure for diagnosis.

We studied the expressions of CD180 in various lymphoid tissues for better understanding of its role. Our pilot data, firstly confirms the observations by many others that CD180 is expressed by the MZB cells and much less likely by the GC cells. Also, the expression of CD180 on MZB cells was 3 times higher than that seen with the B cells in the peripheral blood(Miyake *et al.,*1995; Chaplin *et al.,*2011; Nagai *et al.,*2012). Since all the other studies have demonstrated these in murine splenic B cells, to the best of our knowledge this was the first report showing the expression of CD180 in human tonsillar MZB cells. The MZB cells are largely consisting of B cells with a pre-activated phenotype and express somatically mutated IGVH genes indicating possibly previous antigenic experience (Cerruti *et al.,*2013). The somatic hypermutations could be acquired in germinal centres before migration of the cells to the marginal zone and the occurrence of clonal expansion (Tierens *et al.,*1999). We have shown previously that CD180 is expressed preferentially by the M IGVH CLL cells too (Porakishvili *et al.,*2005). Hence, presumably, CD180 is expressed on the cell surface after the migration of cells from germinal centres to the marginal zone. Another study with mice splenic MZB cells has indicated anti-CD180 mAb stimulation showed robust increase in marginal zone B cell proliferation, and CD86 upregulation, along with antibody secretions in the same subset (Chaplin *et al.,*2011). Our data indicates CD180 expression in the tonsil MZ is indicative of an antigenic experience for the cells. Therefore, to sum up, our hypothetical model suggests - the naive B cells in the GC undergo antigenic stimulation, mature into memory or a pre-activated B cell

subset, migrate to MZ where upon the CD180 gets expressed on the surface. Here, engagement of CD180 through the unknown ligand(s) drives the cells into proliferation, activation (CD86 upregulation) and antibody secretion, further to which the receptor is probably internalized before entering the peripheral blood. Our data fits in with these previous observations that B cells lose CD180 upon activation (Kikuchi *et al.,*2008).

Further we ventured into assessing the expressions of CD180 in CLL solid tissues. The data observed was different from that seen with the normal reactive tonsil since the lymphoid tissues in CLL show complete obliteration of the normal tissue architecture (Dick *et al.,*2006). No distinct follicles and extra-follicular regions are observed in the CLL lymph nodes due to massive infiltrations with the neoplastic malignant cell (Schmid *et al.,*1994). In these tissues, as defined by many, are present specialized regions called proliferations centres (PCs) wherein the major transformation, developement and survival of the neoplastic clone takes place (Schmid *et al.,*1994; Soma *et al.,*2006; Ciccone *et al.,*2011). Our data demonstrated that CD180 was very weakly expressed in both the LN samples studied, which were mainly indicative of areas with the PCs. The PC cells generally express phenotypic markers of activated/proliferating cells evidenced by higher levels of molecule Ki67, CD38, higher expression of ZAP70 and IgM, in most cases with U IGVH (Schmid *et al.,*1994; Soma *et al.,*2006; Gine *et al.,*2010). We have discussed above that CD180 expression is downregulated in activated cells, preferential in M IGVH and differential with sIgM. Therefore, the observation of diminished levels of CD180 in the PC cells was be-fitting. However, to account for the extremely low positivity observed in two samples, we reason that since CD180 is also expressed by dendritic cells, it is possible that the weakly stained regions observed were dendritic cells. This is based on the knowledge that proliferation centres and surrounding tissues also contain a delicate follicular dendritic cell network (Schmid *et al.,*1994)

In case of our studies with the BM from 6 CLL cases, 4 cases showed strong expression of CD180 (Figures 5.5, 5.6,5.7,5.8) whilst and 2 cases were extremely weak or even negative for CD180 on BM lymphocytes (Figures 5.9 and 5.10)

indicating, possibly, that similar to our observation with the PB where we have shown that in about 1/3 of CLL cases CLL cells are negative for CD180 expression in peripheral blood.

Thus, CLL BM can be either CD180+ve or CD180-ve, i.e. the expression of CD180 in the BM of CLL patients is heterogeneous.

It is known that BM architecture varies with two different infiltration patterns commonly observed in CLL - diffused and interstitial. Some studies have stated that the diffused patterns are indicative of a poor disease prognosis and lower median survival time, whilst the interstitial infiltration is associated with a better prognosis (Carbone *et al.,*1978; Rozman *et al.,*1981; Pangalis *et al.,*1984). However we were unable to establish correlation between the BM architecture and the pattern of expression of CD180.

We are yet uncertain of the physiological role of this heterogeneity and further studies comparing the simultaneous expressions in PB and BM of the same CLL cases is crucial to be undertaken in the future.

In three CLL cases, we studied with FACS analysis, the comparative expressions of CD180+ in PB and corresponding BM cells. Interestingly in all three cases, PB CD19+ cells expressed significantly higher levels of surface CD180 compared to BM CD19+ cells (Figure 5.11). That the trafficking cells expressed higher levels of CD180 is intriguing and might suggest the involvement of CD180/ligand interaction with the expansion and/or homing of CLL cells *in vivo.* This suggestion, to certain extent is supported by our data on the expression of CD180 on proliferating and homing subsets of CLL cells presented in the next chapter.

Chapter 6 CD180 expression by functionally distinct CLL subsets

Introduction

Traditionally CLL has been considered to result from the accumulation of relatively immature, and possibly incompetent B lymphocytes arising due to defect in the apoptosis, rather than being a proliferating cell malignancy (Dameshek *et al.,*1967; Chiorazzi *et al.,*2003). However, further studies revealed that although the majority of circulating CLL cells are not proliferating, a small proliferative compartment does exist (Chiorazzi *et al.,*2005). CLL cells arise from the bone marrow and infiltrate lymphoid organs such as lymph nodes and the spleen considered to be sites of leukemic cell proliferation as very few actively dividing cells are observed in the blood (Ghia *et al.,*2000; Dighiero *et al.,*1991). Clinically in some patients, the disease remains indolent without necessitating therapy, in others, it progresses with unknown causes into a more aggressive disease. The progressive disease is often associated with genomic changes, and is suggestive of clonal evolution (Shanafelt *et al.,*2009).

Studies of the CLL clonal kinetics in lymphoid organs and in peripheral blood have been intriguing, but the most informative studies have been carried out relatively recently. Messmer *et al.,*(2005) used deuterium (a nonradioactive isotope detectable by mass spectrometry) that was administered in the form of deuterated "heavy" water (^2H_2O) to CLL patients. The idea was to examine *in vivo,* newly synthesized DNA of dividing cells by labelling with the nonradioactive isotope. The data obtained demonstrated that CLL cells proliferate at definable and, in some cases, substantial rates (about 0.1% to greater than 1.0% of the clone per day). In most instances, patients whose clonal birth rates exceeded 0.35% per day had symptoms or signs of active or progressive CLL. Thus, these studies highlighted the dynamic nature of CLL clones and defined sizeable rates of birth and death that were previously unappreciated. Further studies by Calissano *et al.,*(2009; 2011), aimed at better understanding the phenotype of these cells, in correlation to the cell cycle or kinetics, within each CLL clone. Using the same "heavy" water approach as before, they observed phenotypic intra-clonal heterogeneity related to the expressions of CD38, CXCR4 and CD5. Delineating the CLL clones on the basis of reciprocal densities of chemokine receptor 4 (CXCR4) and CD5 revealed that the

$CXCR4^{dim}CD5^{bright}$ (proliferative) fraction contained more ^{2}H-labeled DNA and hence divided cells than the $CXCR4^{bright}CD5^{dim}$ (resting) fraction. This enrichment was confirmed by the higher expression of cell cycle–associated molecule Ki-67 in the same fractions, and gene expression profiling (Calissano *et al.,*2011).

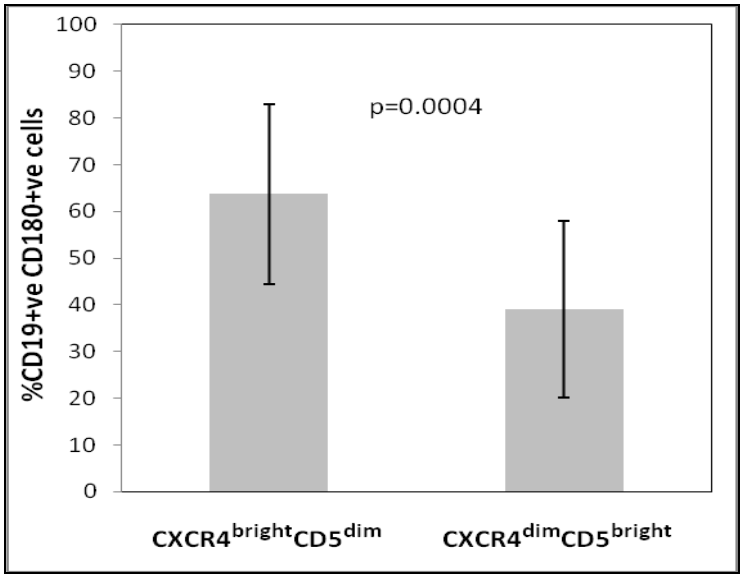
In the previous chapter we have outlined our pilot studies on the expression of CD180 on CLL cells in lymphoid tissues. In our hands CD180+CLL cells were found in the marginal zones of the lymph nodes, but not in the germinal centres that suggests some association between CLL cells kinetics and the expression of CD180. We therefore used the approached developed by Calissano et al to assess expression of CD180 on "proliferative" and "resting" CLL cell subsets. Since we have previously shown that CD180 and IgM are differentially expressed by CLL cells in peripheral blood (Porakishvili *et al.,* 2005), and our recent data implies strong interaction between CD180 and sIgM signalling pathways, we have studied the expression of both receptors on these CLL populations.

Results

6.1. Differential expression of CD180 and sIgM by the 'proliferating' ($CXCR4^{dim}CD5^{bright}$) and the 'resting' ($CXCR4^{bright}CD5^{dim}$) CLL fractions

CLL cells from 16 cases were isolated and stained with compatible fluorochrome-labeled mAbs against CD180 or sIgM, CD19, CXCR4 and CD5 and data analysed by flow cytometry (as defined in the Materials and methods). Percentages of CD180+ or sIgM+ CLL cells on $CXCR4^{dim}CD5^{bright}$ or $CXCR4^{bright}CD5^{dim}$ subsets were determined by gating on CD19+ cells in comparison with the corresponding isotype controls.

Our data indicates that significantly more $CXCR4^{bright}CD5^{dim}$ cells expressed CD180 compared to the $CXCR4^{dim}CD5^{bright}$ counterparts (Figure 6.1.A). In contrast, the latter cell subset was enriched by sIgM+ cells (Figure 6.1.B). This would suggest that CD180 is preferably expressed by the "resting" population of CLL cells whilst the sIgM+ cells mostly belong to the "proliferating" category. This observation is concordant with our previous report indicating an often alternative expression of the two receptors on CLL cells (Porakishvili *et al.,* 2005).



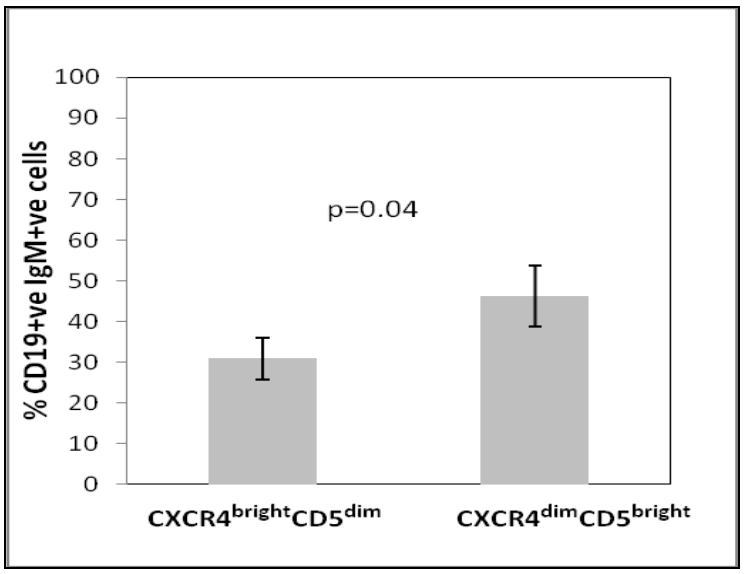


Figure 6. 1 .A, B: CD180 and sIgM expressions on CXCR4^{bright}tCD5^{dim} 'resting' and CXCR4^{dim}CD5^{bright} 'proliferative' CLL subsets

PBMCs from 16 CLL cases were incubated with fluorochrome-labeled mAbs reactive with CD19, CD5, CXCR4 and CD180 or sIgM. Cells were first gated for CD19 and then gated for corresponding CXCR4^{bright}CD5^{dim} and CXCR4^{dim}CD5^{bright} fractions which accounted for ~ 5% of total cell populations in each case. CD180 or sIgM expression was assayed in these fractions as defined in Materials and methods. Statistical significance of the data analysed was assayed by student *t*-test and p-value <0.05 was considered significant. The bars indicate standard deviation.

Discussion

The phenotypes ascertaining the differential clonal kinetics of CLL cells were determined by Calassino *et al.,* (2009 and 2011). CD5 is associated with B cell activation whereas CXCR4, is an important chemokine receptor, involved in maintaining B-cell contact with stromal elements of solid lymphoid tissues and migration of the cells to the solid tissues (Burger *et al.,*2001). It was established that the pattern of expression of these two markers correlated with the proliferative capacity of CLL cells. A hypothetical lifecycle for individual CLL cells was conjured, representing a continuum between the $CXCR4^{dim}CD5^{bright}$, $CXCR4^{int}CD5^{int}$ and $CXCR4^{bright}CD5^{dim}$ fractions (Figure 6.2). At one extreme is the proliferative fraction ($CXCR4^{dim}CD5^{bright}$), highly enriched in young, vital cells that recently left a solid lymphoid tissue where activation and proliferation occurred. At the other end is the resting compartment, containing older, less robust cells that may have been circulating in the periphery longer and are attempting through high CXCR4 levels to migrate into a solid tissue niche to avoid death ($CXCR4^{bright}CD5^{dim}$). In this model, the intermediate fraction, which is the bulk of the clone, links the extremes and is the fraction from which most of our current knowledge on circulating CLL cells is derived (Calissano *et al.,*2011).

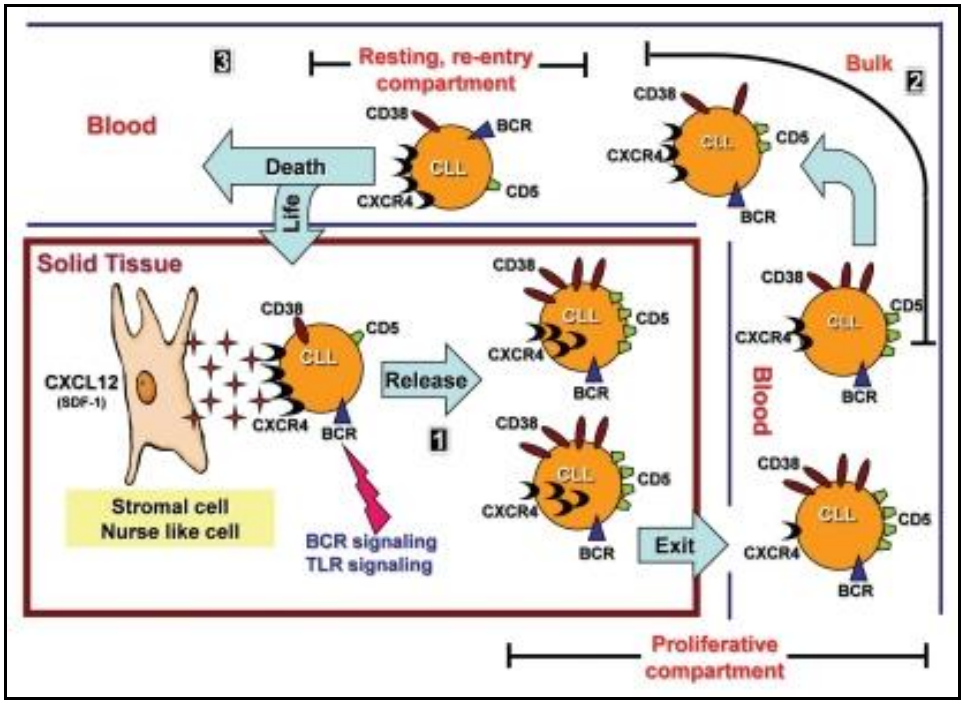


Figure 6. 2 Hypothetical model of the lifecycle of a CLL B cell.

Part 1: CLL cells rest on the stroma supported by CXCR4-CXCL12 interactions. When stimulated, cells are activated and divide, upregulating CD5, internalizing CXCR4 and detaching from stroma. The process could be ligand-induced (for example, BCR or toll-like [TLR] or other pathways) or spontaneous. Low CXCR4 levels increase the chances of recently divided CLL cells ($CXCR4^{dim}CD5^{bright}$ phenotype) exiting solid tissue and reaching peripheral blood. Part 2: Recently born/divided CLL cells reach peripheral blood as members of the $CXCR4^{dim}CD5^{bright}$ fraction. Over time, possibly because of a lack of trophic input from the solid tissue microenvironment, cells begin to reexpress CXCR4 to trek back to nutrient-rich niches. This leads to expression of a $CXCR4^{int}CD5^{int}$ and then $CXCR4^{bright}CD5^{dim}$ membrane phenotype. The model considers the three fractions to be linked as a continuum. Part 3: $CXCR4^{bright}CD5^{dim}$ CLL cells have the greatest chance of detecting and following a CXCL12/SDF1 gradient, thereby reentering lymphoid solid tissue and receiving prosurvival stimuli. Those that do not reenter die by exhaustion (Calissano *et al.*,2011**).**

In our studies we observed that the cells from the 'resting' compartment characterized by the phenotype $CXCR4^{bright}CD5^{dim}$ were enriched for CD180+ cells compared to the 'proliferating' $CXCR4^{dim}CD5^{bright}$ subset. In contrast sIgM+ cells were more frequent in the proliferating fraction (Figures 6.1 A and B). This supports our previous data demonstrating that CLL cells differentially express these two receptors (Porakishvili *et al.,*2005).

It has been shown earlier that BCR stimulation leads to downregulation of CXCR4 expression (Quiroga *et al.,*2010). According to Callisano *et al.,* (2011) this subset of the CLL cells represent actively proliferative and expanding compartment of the clone. Cell proliferation is often preceded by activation, and it has been demonstrated previously that CD180 expression is considerably downregulated on activated B cells (Kikuchi *et al.,*2001).

As for the "resting" CLL clonal fraction, enriched for CD180+ cells, it is considered to represent "ageing" antigen experienced cells. We have tested CD180 expression on CD19+CD27+ and CD19+CD27neg CLL cells (unpublished data not shown) but it has been inconclusive. To this end we are uncertain whether CD180 is predominantly expressed by antigen experienced cells.

Since the "resting" subset of CLL cells is also considered as the one "returning" to the solid tissues supported by the increased expression of CRCX4, our data might suggest possible involvement of CD180 through its interaction with putative endogenous ligand in the homing of the CLL cells as a part of a circulation of the clone *in vivo*. This is further supported by our data presented in Chapter 5 which indicates preferable expression of CD180 on the marginal zone (MZ) CLL cells and normal B cells.

Bone marrow stromal cells also attract CLL cells via the chemokine receptor CXCR4 that leads to infiltration of BM by the CLL cells and provides strong survival signals (Burger *et al.,*2001; Barretina *et al.,*2003).

As MZ B cells can be actively recruited back into circulation, we can speculate that ligation of CD180 on CLL cells could possibly transform them from the 'resting' state to the 'proliferative' state. The proliferative fraction of a CLL clone is of major interest for several reasons. First, the 'proliferative compartment' may contain cells that developed new structural DNA abnormalities which lead to a more aggressive disease (Damle *et al.,*2007). Furthermore, the most recently born fraction may be a progeny of putative leukaemic stem cells. Finally, targeting these cells with therapy will therefore abort clonal evolution (Calissano *et al.,*2011; Chiorazzi *et al.,*2005). Hypothesizing that the CD180 ligation drives the cells into cycling or proliferation which corresponds to increasing the expression of CD5 and downregulating the expression of CXCR4 and CD180 (Figure 6.2), could therefore prevent the cells from re-entering solid tissue. This process would in turn thwart the cells from receiving survival signals via CXCR4 and keep them in circulation easier to target with therapy.

Most of the therapeutic options available at the moment are focussed on eliminating the entire neoplastic clone, which show a number of unfavourable repercussions (Lukenbill *et al.,*2013; Stephens *et al.,*2013). Delineating the CLL clone according to the proliferative and resting compartments and then targeting individual compartments with a specific phenotype, rather than eliminating the entire clone which might also contain healthy cells, is a putative approach to therapy currently in consideration by many groups (Chiorazzi *et al.,*2011). CD180 could therefore serve as a potential marker for the resting fraction of CLL cells and ligating these cells through mAbs to CD180 could drive them into cycling and proliferation, hence providing putative target for therapy and disrupting the migration of the cells towards their pro- survival niches in the solid lymphoid tissues.

Chapter 7 Conclusions

Part I

The role of the 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, respectively) represent exogenous and endogenous microenvironmental factors acting 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.

We have previously shown that approximately 60% of CLL samples expressed surface CD180 (Porakishvili *et al.,* 2005), but only half of these samples 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 upregulated CD86 and Ki-67 upon stimulation with anti-CD180 mAb and were termed responders (R). In contrast, CD180+CLL samples that failed to respond to anti-CD180 mAb, despite expressing a high density of CD180 receptors, were termed non-responders (NR).

We further demonstrated that in R-CLL cells, treatment with anti-CD180 mAb significantly induced phosphorylation of ZAP70/Syk, Erk, p38MAPK, and AKT in a Ca^{2+} independent manner, compared to untreated cells. 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 anergy (Porakishvili *et al.,* 2011).

However, we have noted that although the levels of phosphorylated AKT, ERK, and p38MAPK were significantly increased in R-CLL (in contrast to NR-CLL samples), there was substantial heterogeneity within both anti-CD180 R and NR subsets of CLL. To further clarify the CD180-mediated signalling pathways in CLL, we studied signal transduction downstream from ZAP70/Syk by delineating CLL samples into

R and NR through their proximal ability to activate AKT rather than the distal event of CD86 upregulation, which could be dependent on other factors such as T-cell interactions and/or cytokines. We have studied major signalling protein kinases associated with the BCR signalling pathway: PI3K, Btk, ERK, p38MAPK and AKT.

Out of 55 CLL samples tested, ligation of CD180 with mAb lead to the speedy phosphorylation of AKT above the basal levels in 19 CLL samples, whilst in a further 15 samples an appreciable increase in pAKT was only detected after 24h in culture. We therefore defined these CLL cells as Early AKT responders (ER-AKT) and Late AKT Responders (LR-AKT).

Segregation of CLL samples responding to CD180 ligation by signalling via pAKT, rather than by CD86 upregulation, revealed that some of those CLL samples that fail to signal along the ZAP70/Syk-AKT pathway early after CD180-stimulation, switch to the ZAP70/Syk-p38MAPK pathway. "Real" NR-CLL samples did not follow either of these pathways. In approximately half of CLL samples, CD180 signalled primarily through the AKT pathway (categorised as AKT-Responders, AKT-R), whilst in approximately 25% of CLL samples, ligation of CD180 led to the alternative phosphorylation of p38MAPK (categorised as p38MAPK-Responders, p38MAPK-R). The remaining 25% of CLL cells failed to respond to CD180 ligation through either of the pathways (non-responders, NR).

This dichotomy of the signalling pathways has profound effect on the survival of CLL cells. CD180 ligation of the AKT-R, but not on p38MAPK-R CLL cells led to appreciable upregulation of the levels of pPI3K and Mcl-1 and significant activation of Btk. In contrast, the levels of pBtk were significantly decreased in the p38MAPK-R signaller CLL cells. It was crucial to establish whether survival of PI3K/Btk/AKT-R and p38MAPK-R CLL cells also differed. In our hands CD180-ligation induced significant suppression of basal (spontaneous) apoptosis (measure by the percentages of $DiOC_6^{dim}$ cells) after 24hr in culture in both control B cells and PI3K/Btk/AKT signaller CLL cells. In contrast, there was an increase in the percentages of $DiOC_6^{dim}$ apoptotic cells in the p38MAPK signallers

Our data indicate that CD180 ligation on CLL cells can activate two alternative signalling pathways: pro-survival that operates via PI3K-Btk-AKT protein kinases, or mostly pro-apoptotic, that operates via p38MAPK, but not through Btk. This may have implications for CLL therapy where Btk inhibitors are being used.

Since AKT plays a central role in pro-survival signalling in CLL cells through the engagement of the BCR, and the anti-apoptotic effect of CD180 in our hands also appears to be operating via AKT, it was important to compare the signalling patterns of sIgM with those mediated by CD180 in the major categories of CLL cells as defined above. Our data suggest that both CD180 and sIgM ligation on CLL cells results in activation of a pro-survival signalling pathway operating via PI3K-Btk-AKT. However, whereas anti-CD180 mAb can activate a pro-apoptotic pathway mediated via p38MAPK, ligation of sIgM alone does not lead to the activation of this protein kinase.

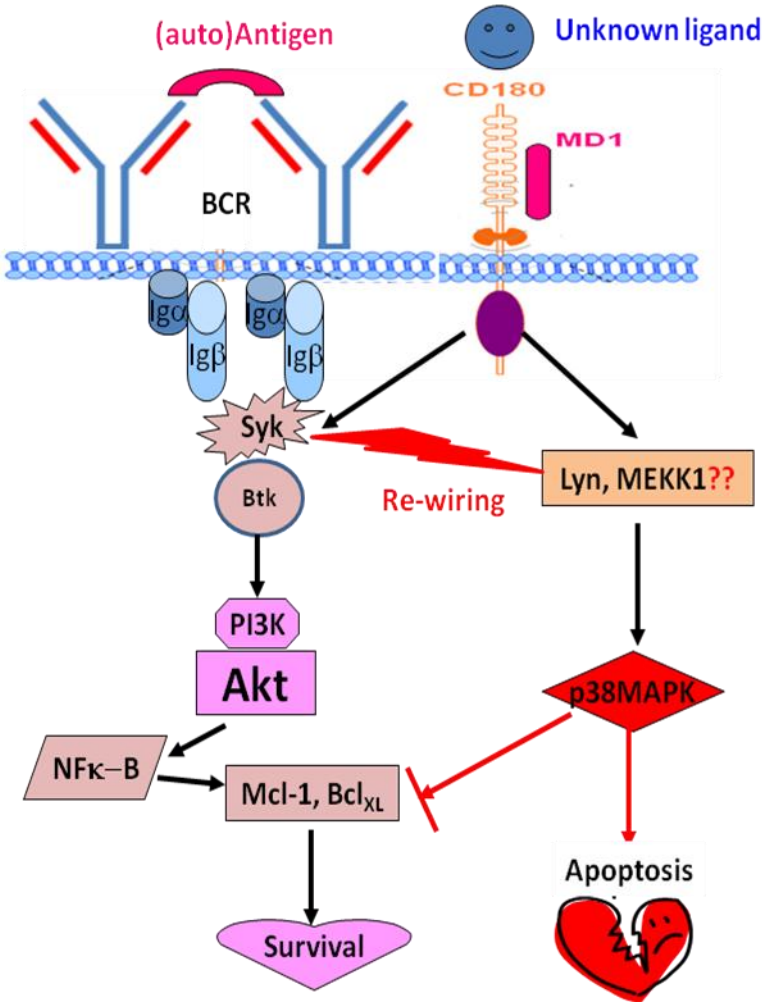


Figure 7. 1: Hypothetical model of the interaction between CD180 and BCR-sgnalling pathways based on our data.

Since our data indicate that CD180 and IgM operate through similar signalling pathways, particularly via PI3K/Btk/AKT, leading to increased survival of CLL cells, we next tested whether pre-engagement of CD180 would affect signalling through sIgM (the BCR). Here we demonstrate that in those CLL cells where ligation of sIgM led to a substantial activation of the pro-survival PI3K/Btk/AKT pathway, pre-engagement of CD180 redirected BCR-mediated signalling towards the potentially pro-apoptotic p38MAPK pathway (Figure 7.1). However, an additive pro-survival effect follows co-ligation of CD180 and sIgM in case when either receptor alone provides sub-optimal stimuli. Although the second scenario seems to be rare, it emphasises once more the importance of individual tailor-made immunotherapeutical approaches to the treatment of CLL.

Part II

Since the tissue microenvironment plays a crucial role in generation and survival of the CLL clones, studies pertaining to CD180 expression in the lymphoid tissues were undertaken. Our pilot data suggests that in normal tonsils CD180 is expressed by the mantle zone (MZ) B cells and not the germinal centre (GC) B cells. This, to the best of our knowledge is the first report on the expression of CD180 in human lymphoid tissues. The MZB cells are largely consisting of B cells with a pre-activated phenotype and express somatically mutated *IGVH* genes indicating possibly previous antigenic experience. This is in line with our previous observations that CD180 is predominantly expressed by M-CLL. The somatic hypermutations could be acquired in germinal centres before migration of the cells to the marginal zone and the occurrence of clonal expansion. Hence, presumably, CD180 is expressed on the cell surface after the migration of cells from germinal centres to the marginal zone, and CD180 expression in the tonsil MZB is indicative of an antigenic experience for the cells. Therefore, to sum up, the naive B cells in the GC undergo antigenic stimulation, mature into memory or a pre-activated B cell subset, migrate to MZ where upon the CD180 gets expressed on the surface. Here, engagement of CD180 through the unknown ligand(s) drives the cells into proliferation, activation (CD86 upregulation) and antibody secretion.

Differently from normal lymphoid tissues, LN biopsies in CLL showed complete obliteration of the normal tissue architecture and a weak expression of CD180. In line with our previous observations that individual CLL samples in peripheral blood differ in the level of expression of CD180 and one third of individual samples were negative for CD180 expression, we demonstrate here heterogeneous expression of this receptor on BM CLL cells. These are the pilot studies, we were the first to report expression of CD180 in lymphoid tissues in CLL, and further studies comparing the simultaneous expressions in PB and BM of the same CLL cases is crucial to be undertaken in the future.

CLL cells migrate to and from the solid tissues into the peripheral circulation and it is in the solid tissues that they receive the crucial survival benefit. Therefore in any CLL clone, there is always an intra-clonal kinetic heterogeneity which can be determined by the differential phenotypes. These have been suggested as a continuum between the 'proliferative' or $CXCR4^{dim}CD5^{bright}$, and 'resting' $CXCR4^{bright}CD5^{dim}$ fractions. Here we report that the 'resting' compartment was enriched for CD180+ cells compared to the 'proliferating' subset. In contrast, sIgM+ cells were more frequent in the proliferating fraction. This supports our previous data demonstrating that CLL cells differentially, and sometimes alternatively, express these two receptors. Since the "resting" subset of CLL cells is also considered as the one "returning" to the solid tissues supported by the increased expression of CXCR4, our data might suggest possible attraction of the CD180+ cells towards the ligand possibly in the lymphoid tissues as CXCR4 gravitates towards the SDF-1 ligand expressed by stromal cells.

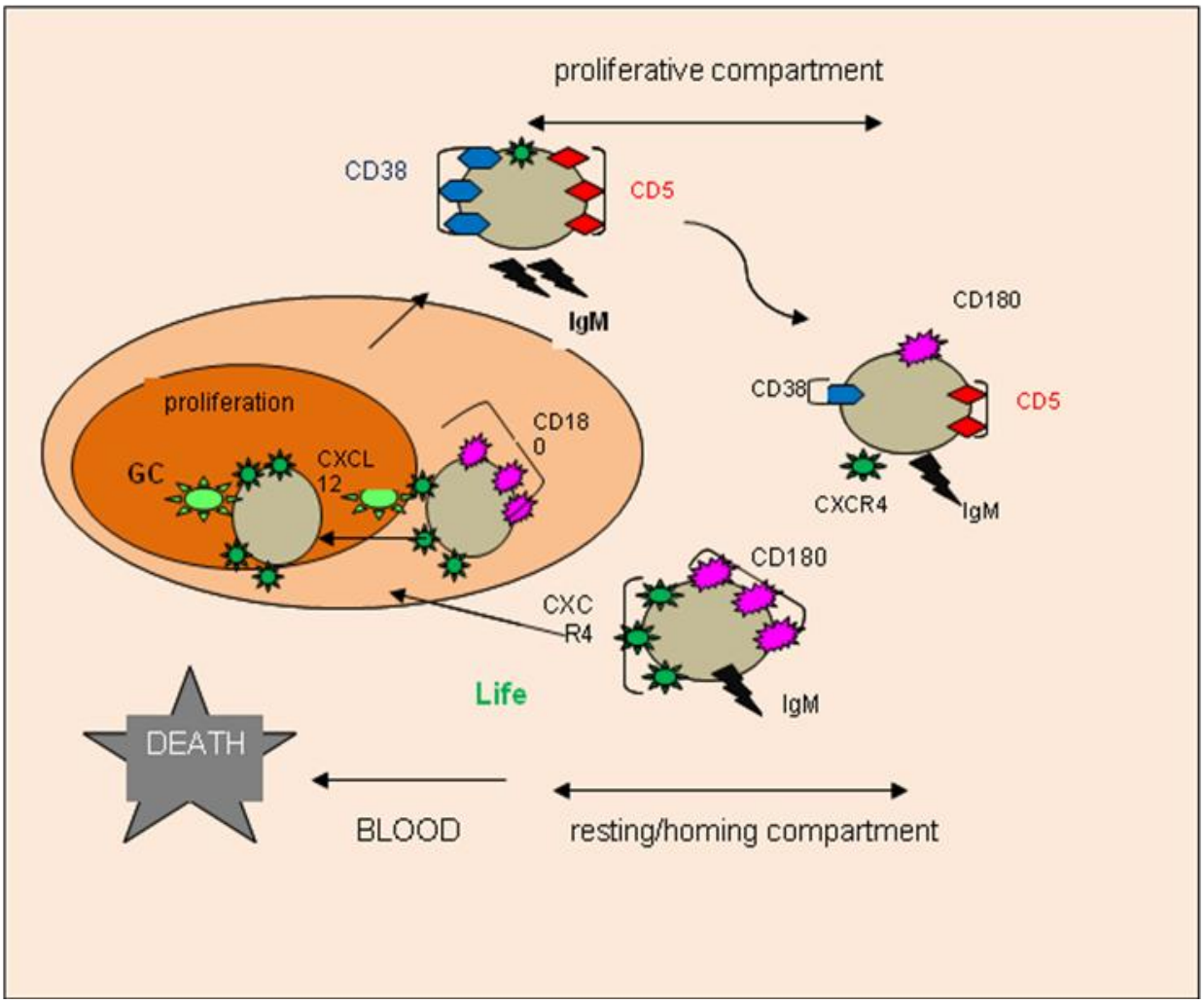


Figure 7. 2: Hypothetical model for the expression and migration of CD180+ CLL cells in the lymphoid tissues and in peripheral blood

As indicated, the 'proliferative' compartment of cells expresses higher level of CD5, CD38 and IgM. These receptors are internalized are the cells circulate through the peripheral blood. At the same time, the receptors CXCR4 and CD180 are increasingly expressed. At this stage the cells have reached a 'quiescence' stage and have two options- undergo apoptosis by further circulation or migrate back to the solid tissues. The CXCR4 expression on the cells attracts these cells towards the CXCL12 (ligand for CXCR4) in the solid tissue. From our previous study, we showed CD180 expressed by the marginal zone B cells, also indicated in this model. However, the physiological function of CD180 in these MZB cells is yet unclear. Once the cells receive the necessary activation and survival stimuli, the robust and newly divided 'young' cells exit the tissue back to the periphery.

As MZ B cells can be actively recruited back into circulation, we can speculate that ligation of CD180 on CLL cells could possibly transform them from the 'resting' state to the 'proliferative' state and thus contribute to the turnover of CLL cells *in vivo,* the hypothetical model presented in Figure 7.2

It is becoming apparent that intraclonal diversity plays an important role in the clinical outcome of patients with CLL. Subsets of the CLL clone that respond more robustly to external stimuli may well gain a growth and survival advantage and possibly promote clonal evolution. Identification of these CLL subpopulations was therefore of prime importance, as these cells may be preferred targets for future therapeutics. Through our work we have established that CD180 expression on CLL cells helps identifying different subsets and delineating their physiological status. Our 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. Since TLR can shuttle between inhibition and promotion of leukemic growth they may play a key role in immune evasion impacting on clinically relevant tumour-host microenvironment interactions. The identification of distinct CD180-mediated signalling pathways that promote tumour cell proliferation and survival will allow specific targeting of key players in the pathways with immunotherapy and chemotherapy.

Summary

The results of my study thus helped establish the following:

- There is a dichotomy in the CD180 mediated signalling pathways in CLL cells - ZAP70/SYk-BTK-PI3K-AKT-Mcl-1 or ZAP70/Syk-p38MAPK.
- This dichotomy in CD180-mediated signalling has antithetical effects - AKT mediated pathway leads to survival of the cells while p38MAPK mediated pathway leads to apoptosis.
- Based on the differential pathways activated following CD180 ligation, we identified 4 major subgroups of CLL clones - AKT responders, p38MAPK responders, Non-Responders and a very minor subset of double responders i.e. AKT and p38MAPK both activated.
- Activation of CD180-mediated signalling can re-wire the BCR pro-survival pathway operating via AKT – to p38MAPK-mediated pro-apoptotic signalling;
- The expression of CD180 in lymphoid tissues is heterogeneous with predominant expression on mantle zone B cells. There are CD180+ and CD180neg CLL clones, both in peripheral blood and in bone marrow.
- CD180 is preferentially expressed on the $CXCR4^{bright}CD5^{dim}$ 'resting/homing' CLL clonal compartment, rather than on 'proliferating' $CXCR4^{dim}CD5^{bright}$ subset.

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