Characterising CD31/CD38 signalling in primary Chronic Lymphocytic Leukaemia cells

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This thesis is submitted in requirement of the University of Cardiff for the Degree of

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CD38/CD31 signalling in primary CLL cells. Poster presentation: Postgraduate research Day. Cardiff University. November 2011.

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Oral presentations

The role of CD31/CD38 signalling in the Pathogenesis of CLL. Internal presentation for the CLL research group. University Hospital of Wales; multiple July 2010-June-2013.

Characterising CD31/CD38 signalling in CLL. Internal presentation for the Infection, Immunity and Biochemistry department. Cardiff University June 2012.

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Abbreviations

ADP	Adenosine diphosphate
ADPRc	ADP-ribosyl cyclase
AID	Activation induced cytidine deaminase
APRIL	A proliferation inducing ligand
ATM	Ataxia Telangiectasia mutated
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BCL-2	B cell lymphoma-2
BCR	B-cell receptor
BM	Bone marrow
BMSC	Bone marrow stromal cell
BrdU	Bromodeoxyuridine
BTK	Bruton tyrosine kinase
cADP	cyclic-ADP
cADPR	cyclic-ADP-ribose
CD	Cluster of differentiation
CIRS	Cumulative illness rating scale
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CT	Computed Tomography
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbecco’s modified eagles medium
DMSO	Dimethyl sulphoxide
EBV	Epstein-Barr virus

ECOG	Eastern Cooperative Oncology group
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FCR	Fludarabine, cyclophosphomide and Rituximab
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box-O
GEP	Gene expression profiling
GPI	glycosylphosphatidylinositol
GSK-3	Glycogen Synthase kinase-3
HSCT	Haematopoietic stem cell transplantation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HTLV	Human T-lymphotropic virus
Ig	Immunoglobulin
IGHV	Ig heavy chain gene variable region
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
IWCLL	International workshop for CLL
JAK	Janus Kinase
KDa	Kilo-Dalton
LN	Lymph node
LDT	Lymphocyte doubling time
LPS	Lipopolysaccharide
MDM2	Murine double minute 2
MESNA	2-mercaptoethansulphonate

MFI	Mean fluorescent intensity
miR	Micro-RNA
MMP-9	Matrix metalloproteinase-9
mRNA	Messenger RNA
M	Mutated
MW	Molecular weight
MZ	Marginal Zone
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCIWG	National Cancer institute working group
NF-κB	Nuclear factor-κ
NK	Natural killer
NLC	Nurse like cell
NLS	Nuclear Localization signal
NTL	Non-transfected mouse fibroblast L-cells
OS	Overall survival
PB	Peripheral blood
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule
PET	Position emission topography
PI	Propidium iodide
PI3-K	Phosphatidylinositol 3-Kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C

PIP	Phosphatidylinositol-phosphate
PL	Pro-lymphocytes
PLC-γ 1	Phospholipase C-γ1
PVDF	Polyvinylidene diflouride
RPE	R-phycoerythrin
RT	Reverse transcriptase
S6	S6-ribosomal protein
SDF-1	Stromal derived factor-1
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SH-2	Src homology-2
SLL	Small lymphocytic lymphoma
SNP	Single nucleotide polymorphism
sIg	Surface Ig
STAT	Signal tranducer and activator of transcription
STELA	Single telomere length analysis
Syk	Spleen tyrosine kinase
TCF-1	T-cell transcription factor-1α
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
TFR	Tumour flare reaction
TK	Thymidine Kinase
TLR	Toll like receptor
TLS	Tumour lysis syndrome
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TTFT	Time to first treatment

U	Unmutated
VCAM	Vascular cell adhesion molecule
VDJ	Variable, diverse and joining regions
VEGF	Vascular endothelial growth factor
VEGFR-2	VEGF receptor-2
VH	Variable Heavy chain gene
WHO	World health organisation
XIAP	X-linked inhibitor of apoptosis protein
Zap-70	Zeta-associatedprotein-70

Abstract

In this study a CD31-expressing co-culture system was used to establish whether differential CD31/CD38 signalling may contribute to the poor prognosis associated with CD38 expression in CLL. Using western blot analysis, a PKB phospho-substrate antibody was used in combination with phospho-specific antibodies to identify ribosomal protein S6 and GSK3β as key signalling molecules that were augmented following short-term CD31-expressing co-culture. CD31-expressing co-culture did not alter the phosphorylation of STAT6. However, the addition of IL-4 to the cultures was a potent mediator of this signalling pathway. This highlights the specificity of signalling molecules to different external stimuli. Both CD31-expressing co-culture and NTL co-culture induced changes in the phosphorylation of target proteins therefore it was not possible to reach absolute conclusions about the role of CD31 as opposed to co-culture with non-transfected fibroblasts in the parameters measured.

Multi-colour flow cytometry was employed to quantify the expression of cell surface activation markers as well as intracellular phospho-proteins. The CD31-expressing co-culture led to a significant up regulation of the activation markers CD38, CD49d and CD69. Selective pharmacological inhibition of the phosphorylation of S6, STAT6 and ERK resulted in the down regulation of activation markers. Furthermore, the inhibition of p-STAT6 and p-ERK resulted in increased levels of apoptosis, which indicates that these signalling pathways are directly involved in CLL cell survival.

Multi-colour flow cytometry was also used to quantitate the levels of phospho proteins, p-S6 and p-ERK. Similar to the results observed by antibody detection following western blotting, basal and inducible levels of p-S6 and p-ERK were elevated in primary CLL cells expressing high levels of CD38.

Taken together, the work carried out in this project highlights the importance of using co-culture systems to stimulate CLL cells *in vitro* in order to mimic some of the key stimuli encountered *in vivo*. The dissection of the signalling pathways activated as a result of CD31/CD38 interactions provides a rational for the poor prognosis associated with elevated CD38 expression in this disease and identified candidate therapeutic targets that might particularly benefit this group of patients.

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1 Introduction

1.1 CLL

Chronic lymphocytic leukaemia (CLL) is a malignant disorder of mature B-lymphocytes, characterized by the monoclonal expansion of B-cells in the peripheral blood (PB), bone marrow (BM) and lymphoid organs, a large proportion of these malignant cells are arrested in the G0/G1 phase of the cell cycle (Deaglio and Malavasi 2009). CLL has previously been described as a disease characterised by the failure of CLL cells to undergo apoptosis, and was not considered a proliferative disease. However, there is now a growing body of evidence to suggest that malignant CLL cells have undergone substantial cell division, most likely within proliferative compartments in lymphoid tissues to produce an expanding clone, with as much as 1% of the entire clone dividing each day (Deaglio and Malavasi 2009; Messmer et al. 2005). The proliferation of these B-lymphocytes can lead to a compromised immune response, anaemia and thrombocytopenia (Keating et al. 2003).

CLL is the most commonly diagnosed leukaemia in the western world and makes up between 30-40% of all leukaemia cases diagnosed globally (Foon et al. 1990). The median age of diagnosis of CLL is 70 years of age (Oscier et al. 2004). CLL is generally considered a disease of the elderly, however up to 30% of CLL patients are diagnosed below the age of 55 (Oscier et al. 2004). CLL occurs predominantly in males (2:1 ratio) for reasons that are still uncertain (Finch and Linet 1992). CLL is a very heterogeneous disease with a highly variable clinical course and whilst this disease can be treated with chemotherapy to reduce the tumour burden, CLL is still largely considered to be incurable. Through better understanding of the pathological mechanisms involved in this highly variable disease, it is hoped that treatment options for CLL patients will be developed leading to improved response rates and enhanced survival (Abbott 2005; Wu et al. 2013).

The latest world health organisation (WHO) classification scheme defines CLL as a mature B-cell neoplasm and does not distinguish the disease from small lymphocytic leukaemia (SLL) that has identical cell phenotype but which is confined to the lymph nodes (Jaffe 2009).

1.1.1 A brief History: The origin of CLL cells

Over several decades different cell types have been hypothesised as being those which give rise to CLL cells. Some of these suggestions have been disproven with the use of emerging technologies available. CLL cells resemble activated B-lymphocytes and for this reason the cellular origin of CLL cells cannot be deduced by phenotypic analysis alone.

CLL is a disease that shows a high level of heterogeneity, key prognostic markers such as the mutational status of *IGHV* genes can define distinct patient subgroups. CLL patients with unmutated *IGHV* genes (U-CLL) represent a cohort of patients who have not undergone somatic hypermutation in response to antigen and this is associated with poor clinical outcome, whereas patients with mutated *IGHV* genes (M-CLL) are antigen experienced and associated with a better clinical outcome (Damle et al. 1999; Hamblin et al. 1999). These two types of CLL may originate from two distinct cell types (Rosenwald et al. 2001). However, this theory has been called into question since microarray analysis has revealed a small number of differences between U-CLL and M-CLL subgroups but much bigger differences between normal B-cells and CLL cells, irrespective of the *IGHV* mutational status. Furthermore, genetic profiling revealed that U-CLL and M-CLL subgroups shared similarities with memory B-cells, which would indicate that all CLL cells originate from antigen experienced B-cells (Klein et al. 2001). These findings suggest that there may be a single originating cell and other non-genetic factors may be responsible for the differences observed between U-CLL and M-CLL in terms of clinical outcome. In contrast, experiments carried out by Seifert *et al* in 2012 compared global gene expression of M-CLL and U-CLL patients to human mature B-cell subsets and showed that $CD5^+$ normal B-cells had the most similarities to both M-CLL and U-CLL cohorts (Seifert et al. 2012).

Another theory is that marginal zone B-cells are the precursors of both M-CLL and U-CLL. The marginal zone (MZ) is where normal adult B-cells, which produce antibodies against viral or bacterial carbohydrates, reside (Chiorazzi et al. 2005). In humans 70-80% of total MZ B-cells located in the spleen are *IGHV* mutated (Chiorazzi et al. 2005). MZ B-cells, like CLL cells, produce surface IgM and IgD, but unlike CLL cells the surface phenotype of MZ B-cells is $CD5^-CD23^-CD22^+$. However, these phenotypic differences could be attributed to the activation of CLL cells leading to up-regulation of both CD5 and CD23 (Chiorazzi et al. 2005).

1.1.2 Aetiology and epidemiology

Advanced age, Caucasian race and a familial history of other haematological malignancies have all been shown to increase the risk of developing CLL, however the aetiology of CLL remains uncertain (Chiorazzi et al. 2005). The incidence of CLL cases has increased since the 1950's, but this is more likely to be due to more sophisticated detection methods rather than an actual increase in occurrence. Global CLL rates are between 1 to 5 people diagnosed per 100,000 of the population. However, Asian populations have a significantly lower incidence of CLL diagnoses than Caucasian, Americans and European populations; interestingly these lower frequencies are maintained in Asian populations who have migrated to USA (Pan et al. 2002).

CLL is generally not associated with any environmental or external factors; however studies have linked the development of CLL with exposure to occupational chemicals including benzene, radioisotopes and pesticides (Schnatter et al. 2005). Viruses such as HTLV, EBV and CMV have also been proposed as risk factors for the development of CLL (Crowther-Swanepoel et al. 2010).There are also rheumatologic conditions, which have been associated with a risk of developing lympho-proliferative disorders, such as CLL (Mellemkjaer et al. 1996).

Approximately 5% of individuals diagnosed with CLL report a family history of this disease or another lympho-proliferative disorder, thus genetic predisposition is the best-understood risk factor in CLL. Genome-wide association analysis has been used to identify several genetic loci, which together gave an accumulated risk of the development of CLL (Crowther-Swanepoel et al. 2010).

1.1.3 Diagnosis of CLL

Patients with CLL can be asymptomatic and are diagnosed during a routine blood test which returns with a higher than normal white blood cell count (Hoeller et al. 2013; Shanafelt and Kay 2007). However, more often than not patients are diagnosed with this disease after suffering from persistent infections and increased lethargy or malaise due to anaemia (Keating et al. 2003). As CLL progresses symptoms can include swollen lymph nodes, spleen and liver as well as anaemia and thrombocytopenia. A diagnosis of CLL is made when there is a B-lymphocyte count of $5x10^9$/L in the peripheral blood present for at least 3 months; clonality of these cells needs to be established with flow cytometry.

1.1.4 Clinical features

CLL is an extremely heterogeneous disease with high variability in clinical course that is often extremely difficult to predict. The disease can remain stable for many years, and clinicians often implement a 'watch and wait' approach before determining which treatment course would be the most beneficial and appropriate for individual patients. In some patients CLL disease progression is very rapid and despite treatment ends in a fatal outcome. However, survival of patients with CLL is between 8 and 10 years (Hallek et al. 2008). A wide range of laboratory tests can now be used to more accurately predict disease progression; there are also universal clinical parameters used as a standard guide for clinicians, these are outlined below.

1.1.5 CLL cell morphology

The World Health Organization (WHO) describes CLL as a leukaemic lymphocytic lymphoma, which can be distinguished from small lymphocytic leukaemic lymphoma (SLL) only by its morphological appearance and locality. To avoid misdiagnosis, a full blood count and blood smear should be routinely performed on all patients. Figure 1.1 represents a blood smear for a case of CLL. Lymphocytes are small (average 7.33μm in diameter (Kuse et al. 1985)) and appear mature. The nucleus is dense and inhabits most of the cell; the nucleus is contained within a very narrow cytoplasm. The nucleus lacks any observable nucleoli and dark staining within this region represents aggregated chromatin (Hallek et al. 2010). Smudge cells are a common characteristic of CLL cells; these cells are likely to have been smeared during the slide preparation process and demonstrate the increased fragility of the CLL cell membrane.

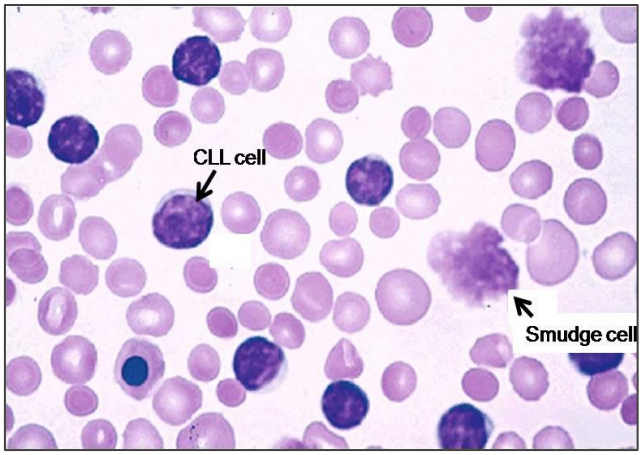


Figure 1.1 Blood film illustrating typical CLL cells and smudge cells (Adapted from Brandon Guthery, M.D., and Nasir Bakshi, M.D. Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City)

1.1.6 Classification systems: Rai and Binet

The Rai and Binet classification systems were developed over 30 years ago but remain a valuable diagnostic tool for clinicians when managing treatment decisions in CLL. Both staging systems are based on clinical features and are thought to help to accurately predict the prognostic outcome of CLL patients. These staging systems are both simple and inexpensive and hence are used as a standard by physicians' worldwide (Binet et al. 1981; Rai et al. 1975).

Table 1.1 shows the Rai classification system, which ranges from stage 0 to stage IV. This system uses the presence of persistent lymphocytosis with or without lymphadenopathy, hepato/splenomegaly, anaemia and thrombocytopenia as markers of disease progression. Stages III and IV represent more advanced disease and a less favourable outcome. Table 1.2 represents the Binet classification system. This system is based on the presence or absence of anaemia or thrombocytopenia with lymphadenopathy at single or multiple sites.

Table 1.1Rai staging System (Adapted from Rai *et al.,* 1975)

Low	
0	Lymphocytosis only
Intermediate	
I	Lymphocytosis + lymphadenopathy
II	Lymphocytosis + splenomegaly with/without lymphadenopathy or hepatomegaly
High	
III	Lymphocytosis + anaemia, with or without organomegaly
IV	Lymphocytosis + thrombocytopenia +/- organomegaly

Table 1.2 Binet staging system (Adapted from Binet *et al.,* 1981)

Stage **A**	Patients have fewer than three areas of enlarged lymphoid tissue. Enlarged lymph nodes of the neck, underarms, and groin, as well as the spleen, are each considered "one group" whether unilateral (one sided) or bilateral (on both sides).
Stage **B**	Patients have more than three areas of enlarged lymphoid tissue. No anaemia or thrombocytopenia
Stage **C**	Patients have anaemia and/or thrombocytopenia

1.1.7 Immunophenotype

CLL cells co-express normal B-cell antigens CD19, CD20 and CD23 but also express CD5, which is characteristically a T-cell marker. Lymphocytes in CLL also show the weak expression of either kappa (κ) or lambda (λ) light chains on the cell surface (Matutes et al. 1994). CD79b and CD22 are either very weakly expressed or not present on the CLL cell surface; both of these molecules are associated with cell signalling. Markers FMC7 and TdT are also weakly expressed or absent on CLL cells, these molecules are associated with B-cell maturity. The levels of expression of these two markers may demonstrate the point of B-cell transformation and thus could be indicative of disease severity (Craig 2007). Flow cytometric analysis of CLL cells is an extremely important tool in aiding diagnosis, classification and prognosis of this disease as well as deciding how best to manage the treatment of this disease.

Table 1.3 Immunophenotypic scoring system for CLL (Adapted from Moreau *et al.*, 1997)

Marker	Expression	Score	Expression	Score
Surface Ig	Weak	1	Mod/strong	0
CD5	Positive	1	Negative	0
CD23	Positive	1	Negative	0
FMC7	Negative	1	Positive	0
CD79b	Weak	1	Strong	0

1.1.8 Other Diagnostic Tests

The following tests/markers are not essential for the diagnosis of CLL but may provide a better indication of disease progression for sufferers of this highly heterogeneous disease.

1.1.8.1 Lymphocyte Doubling Time

The Lymphocyte doubling time (LDT) has been used as an indicator of clinical course since the mid 1980's (Montserrat et al. 1986). The LDT correlates with other prognostic indicators, including CD38 and ZAP70 expression, it has been shown that used alone the LDT is an accurate method of measuring CLL progression (Montserrat et al. 1986). In 1986 Montserrat stated that a LDT of greater than 12 months represents a cohort of patients with a more favourable clinical outcome, a LDT of less than or equal to 12 months is associated with much poorer survival rates. Patients who have a short LDT in the early stages of the disease are likely to have a more rapidly developing disease (Montserrat et al. 1986). Pepper *et al* (2012) showed that LDT was the most prognostic parameter for predicting time to first treatment (TTFT) in a large-scale study of 1152 CLL patients, all in Binet Stage A with a median follow up period of 8 years (Pepper et al. 2012).

1.1.8.2 Molecular cytogenetics

Around 80% of CLL patients will have identifiable chromosomal abnormalities in their malignant clone (Dohner et al. 2000). Genomic aberrations can be identified using fluorescence *in situ* hybridisation (FISH). The most common cytogenetic abnormalities include a 13q deletion, found in approximately 55% of CLL patients (Parker et al. 2011). Other prevalent aberrations are 11q and 17p deletions and trisomy 12 (Dohner et al. 2000). Both the 17p and 11q deletions are mostly found in patients who have unmutated *IGHV* genes and these deletions are associated with a poor prognosis (Stilgenbauer et al. 2002). Patients with a deletion in 17p display resistance to chemotherapy using alkylating drugs and purine analogues (Bentz et al. 1995). A retrospective study which analysed 620 CLL patients showed that 17p deletion and *IGHV* mutational status held the most prognostic power out of various biological and clinical markers analysed with 17p deletion being an important predictor of TTFT (Bulian et al. 2012).

The identification of patients with chromosomal abnormalities is important since these genomic aberrations can identify patients with lower response rates to chemotherapeutic therapies. For example Hewamana and Dearden looked at a cohort of Binet stage C relapsed or refractory patients and discovered that the inactivation of the tumour-suppressor gene TP53 occurred in between 35-50% of these patients, this cohort of patients showed poor response rates to chemotherapeutics including alkylating agents and purine analogues (Hewamana and Dearden 2011). A number of other studies have also revealed correlations between cytogenetic abnormalities and clinical outcome in CLL (Garcia-Marco et al. 1994; Oscier 2005). Therefore, early detection of such chromosomal abnormalities may be able to provide vital information when deciding what treatment course is the most appropriate.

1.1.8.3 Serum Markers

Elevated levels of β_2-microglobulin, thymidine kinase (TK), and soluble CD23 have all been proposed as independent prognostic markers in CLL (Delgado et al. 2009; Hallek et al. 1999; Meuleman et al. 2008). Low levels of β_2-microglobulin have been associated with longer overall survival (OS) and higher remission rates following fludarabine-based chemotherapy (Hallek et al. 1996). High levels of TK are produced by dividing cells and elevated levels of this kinase in the blood serum is indicative of increased CLL proliferation (Hallek et al. 1999). Soluble CD23 is produced by CLL cells so high levels of this molecule detected in serum is a direct measure of tumour burden, and increased levels of soluble CD23 have been linked to shorter TTFT and OS (Meuleman et al. 2008). Serum markers are considered to be reliable independent markers of disease progression, however the routine testing of serum is not conducted, since there is not yet a clearly defined standardised threshold for the levels of these markers in CLL patients (Parker and Strout 2011).

1.1.8.4 CLL Transformation Richter's syndrome

Richter's syndrome was first described by Maurice Richter in 1928 and is characterised by the transformation of CLL cells to a diffuse large cell non-Hodgkin lymphoma (Tsimberidou and Keating 2005). Richter's syndrome is associated with a poor clinical outcome; however a large-scale study has shown that survival of patients who develop this malignancy can vary between a few weeks and up to 15 years (Tsimberidou et al. 2006). Richter's syndrome occurs in between 1-10% of all CLL cases and is more prevalent in patients under 55 years of age (Ghofrani et al. 2007). The risk of

developing Richter's syndrome is independent of disease stage, disease duration or prior treatments. The clinical features of Richter's transformation are non-specific and laboratory tests such as β_2-microglobulin cannot distinguish between CLL and Richter's syndrome. Genetic abnormalities including c-MYC mutations and TP53 inactivation have been shown to be the two most prevalent genetic disruptions in patients with Richter's syndrome (Rossi et al. 2011).PET/CT scanning is a method available which may help to detect Richter's transformation of CLL cells (Bruzzi et al. 2006).

1.2 Treatment of CLL

There are currently no total curative therapies available for the treatment of CLL. Early intervention with chemotherapy versus just observing disease progression has not been shown to increase overall length of survival (Dighiero and Hamblin 2008). The appropriate timing of treatment can delay the natural course of the disease; at present treatment is initiated when a patient displays progressive and/or symptomatic disease. Since there is no curative therapy for CLL and drugs used have a range of unpleasant side effects, the decision to introduce therapy has to be managed very carefully. As the understanding of the biology of this disease has improved, so has the range of treatment options available to patients. The National Cancer Institute working group (NCIWG) has developed a set of criteria to assist clinicians in determining when to begin treatment, these include the development of stage "B" symptoms (weight loss, fevers which last longer than 2 weeks, extreme fatigue and night sweats), as well as increased anaemia and or thrombocytopenia, autoimmune cytopenias, progressive splenomegaly, progressive lymphadenopathy and a LDT of less than 6 months (Hallek et al. 2008).

1.2.1 Assessing patient fitness to determine suitability for treatment

Formal and Informal assessment methods are implemented when deciding whether patients are fit enough to receive treatment for CLL. A formal system commonly used is the Eastern Cooperative Oncology Group Performance Status (ECOG PS), this system uses a grading system of 0-5 to convey how CLL affects day-to-day living, a score of 0 represents physical function equal to pre disease status and a low score would deem a patient fit enough to receive conventional treatments (Oken et al. 1982). Other systems are also used including the Cumulative Illness Rating Scale (CIRS); this system looks at co-morbidities and works by assigning points to separate conditions in other organs of the body. The number of points across all organs in the body is calculated and a low score is associated with higher patient fitness levels (Hudon et al. 2007). However, clinicians may often use personal judgement and previous experience when deciding whether a patient is fit for treatment.

In fit patients Fludarabine, Cyclophosphamide and Rituximab (FCR) treatment (discussed below) has been shown to be the most effective treatment in terms of overall response rates. However, this treatment regime may not be suitable for older or 'less fit' patients due to the toxicities associated with this treatment including cytopenias and infections. An agent that is quite often used in less fit or older patients is the alkylating

agent Chlorambucil, this drug has been established in the treatment of CLL for over 50 years and is administered orally, with fewer adverse side-effects (Van der Jagt et al. 2012).

1.2.2 Single agent therapies

Patients can either receive single agent therapies or several different agents in combination, which are known to act synergistically. Discussed below are three classes of single agent therapies for the treatment of CLL.

1.2.2.1 Alkylating agents

Chlorambucil (phenylbutyric acid nitrogen mustard) was synthesised over 50 years ago and started being used as a therapy for CLL shortly thereafter (Robak and Kasznicki 2002). The exact mechanism of this alkylating agent remains largely undetermined, however it is believed that Chlorambucil acts by inducing DNA crosslinking in CLL cells, Chlorambucil has also been shown to directly induce apoptosis in CLL cells (Robak and Kasznicki 2002). Chlorambucil has response rates in between 47% and 71% of patients and can be used in combination with other alkylating agents, however no differences have been observed in progression-free survival (PFS) or OS when Chlorambucil is used alone or in combination in clinical trials (Hansen et al. 1988; Montserrat et al. 1986).

1.2.2.2 Purine nucleoside analogues

Nucleoside analogues such as Fludarabine, Cladribine and Pentostatin represent a group of cytotoxic agents, which are also very effective in the treatment of CLL (Robak and Kasznicki 2002). The most extensively studied of these three purine analogues is Fludarabine. Fludarabine acts in part by decreasing DNA synthesis, however several *in vitro* studies have confirmed that Fludarabine treatment triggers cell apoptosis (Robak and Kasznicki 2002). As a single agent Fludarabine has been shown to provide higher response rates in terms of PFS compared with treatment of Chlorambucil as a monotherapy (Ricci et al. 2009). The downside of using nucleoside analogues is that these agents can lead to adverse side effects including myelosuppression, significant lymphosuppression and the development of secondary acute myeloid leukaemia (Abbott 2005).

1.2.2.3 Monoclonal antibodies

The emergence of monoclonal antibody therapies represents a more targeted treatment for CLL. The first monoclonal antibody developed for the treatment of CLL was Alemtuzumab (anti-CD52); which targets both mature B- and T-cells within the CLL patient (Mavromatis and Cheson 2003). Treatment with this antibody typically gives response rates of around 30% in patients with advanced disease. However, infusion reactions and immunosuppression pose serious limitations with this agent and, therefore, it is not recommended for use in patients who are immunosuppressed and susceptible to infection (Mavromatis and Cheson 2003; Peleg et al. 2007).

Another monoclonal antibody in clinical use is Rituximab, an anti-CD20 antibody. Rituximab targets CLL B-cells and is used as both a single agent and in combination with other chemotherapeutic agents (Jaglowski and Byrd 2010). This antibody treatment has proven to be highly effective in treating CLL, however studies to determine the specific mechanism of action of Rituximab in CLL cells are still required (Jaglowski and Byrd 2010).

GA-101 is a next generation anti-CD20 antibody; it is the first glyco-engineered anti-CD20 monoclonal antibody developed. The German CLL11 trial is a phase III multicentre study designed to look at the safety and efficacy of GA-101 as well as comparing the efficacy of this monoclonal antibody treatment to other monoclonal antibody treatments. The first stage of this study has been completed and has shown an improvement in PFS in patients treated with GA-101 plus Chlorambucil compared to patients treated with Chlorambucil alone. Another next generation anti-CD20 monoclonal antibody for the treatment of CLL is Ofatumumab. This antibody has shown enhanced killing in Rituximab resistant cell lines (Teeling et al. 2006) and has also shown to be effective in high-risk patient cohorts (Nabhan and Kay 2011).

1.2.3 Combination treatment-Chemoimmunotherapy

The use of combination chemotherapy has been shown to increase levels of complete remission of CLL (Desai and Pinilla-Ibarz 2012). The FCR combination is considered to be the ‘gold standard’ treatment option for CLL by many clinicians (Hamblin 2009). The mechanism by which Fludarabine inhibits excision repair of DNA lends itself to the combined use with Cyclophosphamide, which induces DNA breaks, and these drugs have been shown to synergise in laboratory experiments (Alas and Bonavida 2001).

Additionally, it has also been discovered that Fludarabine and Rituximab could work in synergy since Rituximab acts by sensitizing leukaemic cells to apoptosis by down-regulating the anti-apoptotic protein BCL2 (Alas et al. 2001). A study conducted by Hallek *et al* looked at a large cohort of physically fit CLL patients aged between 30 and 81 years. One group of 408 patients were treated with chemoimmunotherapy that comprised of Fludarabine, Cyclophosphamide and Rituximab, whilst the second cohort of 409 patients was treated with chemotherapeutic agents Fludarabine and Cyclophosphamide only, patients received six doses of treatment over a 28-day period. After 3 years 65% of patients in the chemoimmunotherapy group displayed PFS compared to 45% of patients treated with chemotherapy agents only (Hallek et al. 2010). Although FCR is currently used as a standard treatment choice in CLL patients, new combinations of therapeutic agents are currently being developed which will begin to emerge into clinical use within the next decade. A major benefit of using combinations of therapeutic agents is that individual drugs can be administered at a lower dosage, which can reduce the severity of dose-dependent side effects whilst maintaining efficacy.

1.2.4 Kinase-targeted therapy

Aberrant signalling through the B-cell receptor (BCR) provides growth signals to CLL cells which are central in driving the pathogenesis and progression of this disease (Robak and Robak 2013). Following antigen engagement of the BCR, BCR-associated kinases are recruited and activated these include spleen tyrosine kinases (Syk), Brutons tyrosine kinase (BTK) as well as phosphatidylinositol 3-kinases (PI3K). Several kinases in the BCR signalling pathway are suitable targets for potential therapies in CLL these kinases include LYN, SYK and PI3K(Robak and Robak 2013).

SYK initiates and activates the BCR signalling pathway (Friedberg et al. 2010). *In vitro* treatment of primary CLL cells with the SYK inhibitor Fostamatinib (R788) induces apoptosis at an increased level and antagonises the protective effect of stromal cells, Fostamatinib is an ATP-competitive kinase inhibitor and was originally developed to treat inflammatory diseases but studies have shown that SYK is an interesting target to pursue in CLL (Friedberg et al. 2010; Wiestner 2012b). *In vivo* treatment with Fostamatinib has been conducted using the Eμ-TCL1 transgenic mouse model of CLL. In this context, Fostamatinib demonstrated the ability to inhibit survival and

proliferation of malignant B-cells; and interestingly this SYK inhibitor did not affect the normal B-cell population (Suljagic et al. 2010).

Ibrutinib (PCI-32765) is a potent inhibitor of BTK and has shown very promising results in CLL patients (Burger and Buggy 2013), even in patients not suitable for conventional treatments due to co-morbidities and genetic aberrations such as del 17p, which generally fail to respond to treatment (Barrientos and Rai 2013; Burger and Buggy 2013). Treatment with Ibrutinib results in migration of CLL cells from the lymph nodes into the blood, where they are more treatable (Robak and Robak 2013). Treatment with this inhibitor results in the rapid shrinkage of lymph nodes and prolonged treatment with this agent results in the lymphocyte count returning to normal and remission in a large proportion of patients (Barrientos and Rai 2013).

Dasatinib is an inhibitor that can target both SRC and ABL kinases and is taken orally. *In vitro* studies have shown variable levels of phosphorylation of these kinases in CLL patients; pro-apoptotic effects can be observed following treatment with Dasatinib but this effect is seen to be antagonised when CLL cells have stromal cell contact or CD40 stimulation (Wiestner 2012b).

The PI3K pathway is central in linking many signalling pathways and is responsible, in part for cellular growth, proliferation and cell survival (Liu et al. 2009). The PI3K pathway acts by amplifying the BCR signal and mediating functional effects of antigen-dependent BCR activation. GS-1101 (CAL-101) is a selective inhibitor specific to PI3Kδ isoform and acts by inhibiting PKB and ERK, inhibiting the secretion of cytokines and chemokines both *in vitro* and *in vivo* (Macias-Perez and Flinn 2013; Wiestner 2012b). A phase I study was carried out in 2010 in 37 CLL patients, with resulting reductions in lymphadenopathy in all 37 patients and shrinkage in lymph node legions (of up to 50%) observed in 91% of this patient cohort (Furman 2010). Furthermore, other studies have used GS-1101 in combination with Rituximab or Bendamustine in pre-treated CLL patients and also observed significant reductions in lymphadenopathy (Lu and Wang 2012). GS-1101 induces apoptosis of CLL cells *in vitro* regardless of culture conditions and has shown to be effective with CD40L activation as well as when added to culture with nurse-like stromal cells (Wiestner 2012b).

1.2.5 Immunomodulatory drugs

Lenalidomide is an immunomodulatory drug, which has been used in the treatment of CLL. The exact mechanism of action of Lenalidomide on CLL cells is not fully understood however it may work through enhancement of helper and cytotoxic T cells or inhibition of pro-survival signalling from stromal cells. Furthermore, Lenalidomide has been shown to effectively reduce the proliferation of regulatory T-cells known to be associated with markers of poor prognosis in CLL (Cortelezzi et al. 2012; Schulz et al. 2013). Chanan-Khan *et al* first demonstrated the use of Lenalidomide in the treatment of CLL, patients were treated for 21 days out of a 28-day cycle and 45 patients took part in the study (Chanan-Khan et al. 2006). The overall response rate was 47%, however tumour lysis syndrome (TLS) and tumour flare reaction (TFR) posed major limitations in this treatment (Chanan-Khan et al. 2006) and future studies have used a much lower dose or an escalation dose system whereby they start patients on a dose of 5mg and work up to a dose of 25mg to avoid such reactions (Cortelezzi et al. 2012). Lenalidomide has also been shown to reduce the migratory potential of CLL cells which limits the homing of CLL cells to lymph nodes (Schulz et al. 2013).

1.2.6 Stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is not considered a suitable treatment option for the majority of CLL patients, due to high treatment-related morbidity and mortality (Gribben 2009). However, the use of allogeneic transplants may be considered in younger CLL patients or patients with short remission times (<1-2 years). Patients who may be considered for HSCT are those who have p53 mutations or have experienced an early relapse after purine analogue combination therapy (Ferrajoli 2010).

1.3 Biological and molecular markers

The variability in clinical outcome in CLL is strongly related to several factors including CD38 expression, ZAP70 expression and the mutational status of the *IGHV* genes (Deaglio 2001a). The heterogeneity of this disease means it is essential to identify prognostic markers, to indicate whether individual patients have an indolent or progressive form of the disease.

1.3.1 ZAP70

Zeta-chain associated protein kinase (ZAP70) has a molecular weight of 70kDa and is situated close to the cytoplasmic membranes of both T-cells and natural killer (NK) cells (Chan et al. 1992). ZAP70 is involved in T-cell signalling, but is also needed for pre B-cell development; however the expression of this protein is lost in normal mature B-cells (Chan et al. 1992). The increased expression of ZAP70 in CLL has been associated with a poor prognosis in CLL (Parker and Strout 2011). The precise role of ZAP70 in CLL is unclear, but this protein kinase has been shown to improve the effectiveness of B-cell signalling in CLL cells, particularly in CLL patients expressing unmutated *IGHV* genes (Chen et al. 2008).

1.3.2 *IGHV* mutational status

In the late 1990's, two groups independently reported that CLL could be divided into two subgroups based on the amount of mutations in the immunoglobulin heavy-chain variable region genes (*IGHV*) (Damle et al. 1999; Hamblin et al. 1999). Patients with more than 98% sequence homology of *IGHV* genes to germline are deemed to have an unmutated phenotype and those patients with fewer than 98% homology within this region have a mutated phenotype. A higher-risk cohort of patients are those who display few mutations within the DNA in the *IGHV* antibody gene region, whereas the lower risk cohort of patients show substantial mutations of the DNA in the *IGHV* gene region indicating more antigen experienced CLL cells. It has been shown that patients diagnosed with stage A disease with mutated *IGHV* genes have a median survival three times longer than patients diagnosed with stage A with unmutated *IGHV* genes (Oscier et al. 1997).

1.3.3 Telomere Dynamics

Telomere length analysis can be carried out using a high-resolution single molecule PCR strategy (STELA); telomere length was examined using this highly sensitive approach in a large cohort of CLL patients (Lin et al. 2010b). It was discovered that telomere length was significantly shorter in more advanced Binet stages of CLL, thus short telomere length could be used as an indicator of poor prognosis (Lin et al. 2010a). Furthermore, another key finding of this work is that some early stage patients also had very short telomeres, which were predictive of an unfavourable clinical course prior to clinical disease progression (Lin et al. 2010a). A major hallmark of telomere dysfunction is telomere-telomere fusion events. Consistent with this, samples derived from patients with the most eroded telomeres showed the highest frequency of fusion events and demonstrated genomic instability that was focussed at the telomeric ends of chromosomes (Lin et al. 2010a).

1.4 CD38

The CD38 protein was discovered using monoclonal antibody typing of lymphocytes and was initially thought to be a lymphocyte-specific antigen (Reinherz et al. 1980). Subsequent studies have shown that that CD38 is expressed on many cell types including thymocytes, activated T-lymphocytes, B-cell precursors, plasma cells, NK cells, monocytes as well as dendritic cells (Deaglio 2007). CD38 has also been found on neurons in the human brain (Mizuguchi et al. 1995). CD38 is a glycoprotein, with dual functionality with enzymatic and a receptor capabilities (Deaglio 2003a). Pathways involved in CD38 signalling have been investigated in murine and human B-lymphocyte cell lines. The ligation of CD38 in an immature human B-lymphocyte cell line resulted in the rapid tyrosine phosphorylation of many proteins including the p85 subunit of Phosphatidylinositol 3-kinases (PI3K), as well as phospholipaseCγ (PLCγ) amongst others (Shubinsky and Schlesinger 1997).

The enzymatic activity of CD38 was first described in 1993 in a murine model and established in the human model shortly thereafter (Howard et al. 1993). The enzymatic capabilities of CD38 include the conversion of nicotinamide adenine dinucleotide (NAD) into cyclic adenosine diphosphate ribose (cADP ribose) and hydrolysis of cADP ribose to ADP ribose (Lee 2006). In murine lymphocytes CD38 ligation is followed by a rapid flux in the concentration of intracellular Ca^{2+} (Howard et al. 1993). It has also been shown that the overexpression of human CD38 leads to an increase in intracellular levels of cADP, initiating the mobilisation of $Ca^{2+}$from intracellular stores, resulting in the activation of lymphocytes (Lee 2006).

1.4.1 Phylogeny of CD38

The development of sophisticated phylogenetic analysis techniques has revealed that CD38 is derived from the ancient sea mollusc *Aplysia Californica,* and shares a strikingly similar amino acid sequence with an enzyme found in this sea creature called *Aplysia* ADP-ribosyl cyclase, a soluble protein 256 residues in length located at elevated levels in the ovotestis of this mollusc (Deaglio 2008a). A sequence comparison revealed that 86 of the 256 residues of ADP-ribosyl cyclase from the *Aplysia* mollusc are identical to the human CD38 protein.

A paralogue of CD38 is the GPI-anchored antigen CD157 (Deaglio et al. 2008). *Aplysia,* CD38 and CD157 share around 25-30% sequence identity and there is a nine-

residue sequence, located in the central region of the CD38 protein, which is a highly conserved region amongst the three proteins. CD38, CD157 and ADP-ribosyl cyclase can cyclise NAD to cADPR as well as being able to produce NAADP using NADP as a substrate (Malavasi et al. 2008). A study conducted by Goodrich *et al* has suggested that there may be a fourth member of this cyclase family which is a GPI-anchored protein found in the human parasite *Schistosoma mansoni* which shares 21% homology with CD38 which also is an enzyme producing NAADP from NADP as well as aiding in the hydrolysis of NAD to ADP-ribose (Goodrich et al. 2005).

CD38 and CD157 appear to have evolved in parallel and both function as GPI membrane-anchored proteins. Over the course of evolutionary history the CD38 and CD157 proteins have not only maintained their enzymatic abilities but have also acquired new properties which include membrane anchorage allowing for the cell surface receptor capabilities. The dual functionality of these paralogues as both ecto-enzymes and cell surface receptors are likely to have arisen as a result of evolutionary pressure for the once soluble enzyme to become a more complex and multi-faceted molecule (Deaglio et al. 2006).

1.4.2 CD38 Genetics

Both CD38 and CD157 genes are located on the short arm of chromosome 4 (Quarona et al. 2013). CD38 and CD157 genes are arranged as follows; telomere → CD157 → CD38 → centromere which suggests that CD157 and CD38 have arisen through gene duplication (Malavasi et al. 2006). CD38 is a comparatively large gene and consists of 8 exons that make up 98% of the 80kb fragment. The promoter region of CD38 lacks a TATA box, but contains a CpG island, which indicates epigenetic regulation (Ferrero et al. 1999). The methylation of the promoter region of CD38 negatively correlates with the surface expression of CD38 (Ferrero et al. 1999). A study conducted on 168 CLL patient samples, with the CD38 cut-off point for positivity set at 7%, revealed that 96% of the CD38 negative samples had CD38 gene methylation whereas in the CD38 positive cohort just 25% of patients displayed CD38 gene methylation (Del Poeta et al. 2001). Methylation was not observed in healthy controls, which indicates that this event is CLL specific. A single nucleotide polymorphism is located at the 5' end of intron 1 of CD38; the frequency of the G allele is significantly higher in CLL patients who have clinical and molecular markers of poor prognosis. Moreover the C to G mutation is an

independent risk factor for the development of Richter's syndrome (Del Poeta et al. 2001).

1.4.3 Structure of CD38

The CD38 protein is a type II 45kDa trans-membrane glycoprotein. CD38 has a small 21 amino acid cytoplasmic region, a 23 amino acid single chain trans-membrane domain and a much larger 256 amino acid extracellular domain, which can roughly be divided, into two main regions. The extracellular amino domain is composed of 156 amino acids made up of 5 α-helices which are adjacent to a carboxyl domain made up of 4 β-sheets, these sheets are flanked by another four α-helices, two of which are short and the other two much longer. These two regions are linked at multiple points along the amino acid sequence; the linking of amino acids between the two domains acts as a hinge mechanism and allows for the conformational changes of the CD38 protein upon binding with other molecules (Malavasi et al. 2008).

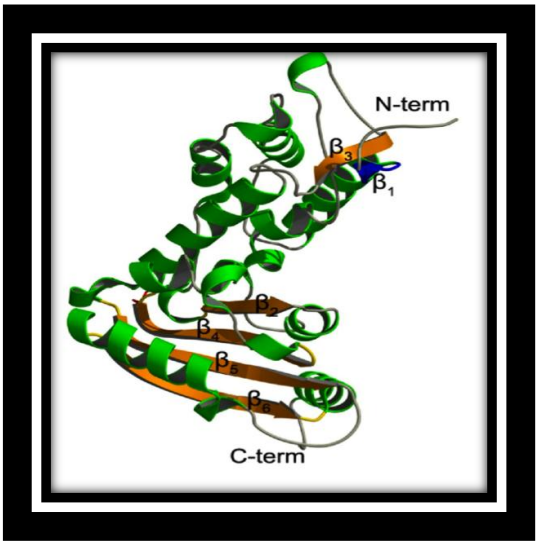


Figure 1.1Crystal structure of CD38(Adapted from Liu *et al*,. 2005)

1.4.4 CD38 in normal lymphocyte development

The expression of CD38 during B-cell ontogeny is very tightly regulated; CD38 appears on bone marrow precursor cells and terminally differentiated plasma cells but is lost on resting mature B-lymphocytes (Campana et al. 2000; Hamblin et al. 2002). CD38 mediates the adhesion of B-lymphocytes to stromal cells found in the bone marrow and lymphoid organs (Zupo et al. 1994). It appears that CD38 expression may be increased

at times during B-cell development when cell-to-cell interactions are required, with CD38 expression stimulated upon naïve B-cell activation, increasing when B-cells enter the germinal centre and rapidly decreasing upon B-cell differentiation (Liu et al. 2008). CD38 expression is absent in the memory B-cell subset. There have been relatively few studies that focus on the role of CD38 in human mature B-cell development.

1.4.5 CD38 in CLL

CD38 is thought to be involved in a number of human diseases including HIV infection, diabetes, acute pro-myelocytic leukaemia, obesity and CLL (Malavasi et al. 2011; Malavasi et al. 2008; Savarino et al. 2000; Stevenson 2006). CD38 is a well-established prognostic marker in CLL; high surface CD38 expression signifies a poor prognosis with shorter overall survival (Damle et al. 1999; Deaglio 2011; Ibrahim et al. 2001). The prognostic relevance of this molecule was first recognised by Damle *et al* in 1999 when analysing a cohort of 47 patients who had been phenotyped for CD38 expression. Damle *et al* discovered that the high expression of CD38 (on greater than 30% of CLL cells) correlated with inferior survival and it was this study that initially established CD38 expression as a means of predicting outcome of newly diagnosed CLL patients (Damle et al. 1999). To reaffirm the prognostic significance of CD38, Ibrahim *et al* (2001) looked at CD38 expression in a larger cohort of patients (Ibrahim et al. 2001). The CD38 expression of 218 patients was correlated with clinical characteristics. CD38 was expressed in 20% or more leukaemic cells in 43% of CLL patients. Patients deemed to have high CD38 expression (greater than 20% of the CLL clone) were shown to have significantly shorter survival times. Furthermore, high CD38 expression identified a sub-group of patients with progressive disease but who were considered to have early stage disease according to the Rai staging classification protocol (Ibrahim et al. 2001).

An accumulating body of evidence indicates that CD38 is not just a marker of activated clonal B-cells but rather it plays a role in the pathology of CLL. A study conducted by Pittner *et al* in 2005 looked at a cohort of 90 patients, of which 43 were uniformly CD38 positive (greater than 20%) and 47 were uniformly CD38 negative (less than 7%) and discovered that cell surface markers CD49d, CD18 and CD20 were more highly expressed in the CD38 positive cohort of patients. Pittner *et al* also looked at the cell cycle related protein APC/C5 and discovered that higher levels could be detected in CD38 positive patients. This is indicative that cells from the CD38 positive cohort of patients have more recently exited the cell cycle and are also more primed to re-enter

the cell cycle (Pittner et al. 2005). In 2007, Pepper and colleagues conducted experiments whereby gene expression analysis was carried out in CD38 positive and CD38 negative CLL cells isolated from 30 bi-modal patients (patients with two distinct populations of CD38+ and CD38- cells) to try and uncover the biological rationale behind the prognostic relevance of CD38. It was shown that there were 62 differentially expressed genes between the $CD38^{+}$ and $CD38^{-}$ populations, 35 genes were over expressed in the $CD38^{+}$ CLL cells and 27 were under expressed in the $CD38^{+}$ CLL cells. VEGF and the anti-apoptotic gene MCL1 showed augmented expression in the $CD38^{+}$ population of CLL cells. The differential gene expression discovered between the two populations of cells from the same patient in some part explains the prognostic significance of the CD38 protein (Pepper 2007).

1.5 Microenvironment

CLL cells *in vivo* exhibit increased survival and levels of apoptosis remain low when compared to normal B-lymphocytes. In stark contrast, when CLL cells are removed from the body they die spontaneously and can be very difficult to keep alive (Ghamlouch et al. 2013). This knowledge highlights the importance of external signals and essential growth factors from accessory cells, which provide supportive interactions to the CLL cell that appear to be critical for the maintenance and progression of CLL (Caligaris-Cappio 2011). CLL cells accumulate in the bone marrow, neoplastic growth follicles and lymphatic tissues where they receive survival and/or growth signals from surrounding cells. CLL cells possess multiple surface adhesion molecules, which include integrins, selectins and immunoglobulins, which facilitate interactions between CLL cells and the extracellular matrix as well as accessory cells, aiding in the migration, localisation and survival of CLL cells (Burger and Montserrat 2013; Caligaris-Cappio 2011; Friedberg 2011). These interactions may be responsible for the emergence of resistance to conventional clinical therapeutics and may account for minimal residual disease (MRD) and relapses following treatment (Audrito et al. 2013). Furthermore, as well as promoting the survival and proliferation of CLL cells signals within the microenvironment are likely to promote the accumulation of new genetic mutations and aid in the expansion of pre-existing mutated sub-clones which may further drive disease progression. Components of the *in vivo* microenvironment thought to promote CLL cell growth and proliferation are discussed below.

1.5.1.1 Bone Marrow stromal cells

Bone marrow stromal cells (BMSCs) were the first stromal cells identified to support CLL cell survival *ex vivo* (Burger et al. 1999). BMSCs are known to be very important in the process of normal haematopoiesis providing growth factors and docking sites to which haematopoietic precursors are able to bind (Burger 2011). It is thought that for CLL cells BMSCs can provide a niche environment within the bone marrow where CLL cells can lodge and be shielded from cytotoxic agents, accounting for MRD (Burger 2011). Co-culture systems with BMSCs have shown that following the rapid adhesion of CLL cells to the stromal layer, a sub population of the CLL clone is able to migrate beneath the BMSCs, this phenomenon is known as pseudo-emperipolesis and is reliant upon the expression of the chemokine receptor CXCR4 as well as expression of the adhesion molecule CD49d by CLL cells (Burger et al. 1999). CXCL12 also known as

stromal cell-derived factor 1 (SDF-1) is a CXC chemokine expressed by stromal cells which signals through CXCR4, a G protein coupled chemokine receptor known to play a role in the trafficking and homing of CLL cells to the bone marrow. The adherence of CLL cells to stromal cells depends upon interactions between CD49d on the CLL cell surface and vascular cell adhesion molecule-1 (VCAM-1) expressed by stromal cells (Buchner et al. 2010; Burger et al. 1999).

1.5.1.2 Nurse-like cells

Nurse-like cells (NLCs) are a subset of blood cells derived from monocytes, which have the ability to differentiate to form large adherent cells, which attract CLL cells and protect them from apoptosis as well as drug-induced cell death *in vitro* through contact-dependent mechanisms(Burger et al. 2000). FISH analysis has proven that NLCs do not arise from the CLL clone; phenotypic characterisation of NLCs has revealed that these cells closely resemble marrow stromal cells (Burger et al. 2000) but also express markers CD45, CD14 and CD68, which are found on monocytic precursor cells (Tsukada et al. 2002). NLCs are found in the spleen and secondary lymphoid tissues of CLL patients and also express CXCL12, CXCL13, CD31, plexin B1, BAFF and APRIL, which promote CLL cell survival (Burger 2011). Furthermore, gene expression profiling (GEP) studies have revealed that CLL cells co-cultured with NLCs have similar gene expression profiles to CLL cells isolated from secondary lymphatic tissues, suggesting that co-culture with NLCs may provide a relevant system to study the CLL cell microenvironment (Burger 2011).

1.5.1.3 T-lymphocytes

Untreated CLL patients show elevated numbers of both $CD4^+$/$CD8^+$ circulating T-cells (Mellstedt and Choudhury 2006), it is unclear whether this expansion is due to interactions with the CLL clone or due to microbial agents which have a greater effect on CLL patients (Burger 2011; Burger et al. 1999). T-lymphocyte numbers are further elevated in CLL patients with a poor clinical outcome (D'Arena et al. 2011). Cytokines such as IL-4, derived from T-cells are able to inhibit apoptosis of CLL cells (Rossmann et al. 2002). Patten *et al* demonstrated that close interactions between CLL cells and T-cells within pseudo follicles *in vivo* result in an activated subset of CLL cells which have elevated CD38 surface expression (Patten et al. 2008).

1.5.2 *In vitro* systems used in CLL research

Recent emphasis in CLL research has focused on targeting the leukaemic cell microenvironment. Previous studies have shown that co-culturing CLL cells with different adherent cell types, collectively known as stromal cells, are able to maintain CLL survival. Some of these co-culture systems are discussed below.

1.5.2.1 CD40 signalling in CLL

CD40 is a 45kDa protein expressed on both normal and malignant B-cells and is a member of the TNF receptor superfamily. A key role of this molecule on B-lymphocytes is to enhance antigen presentation to T-lymphocytes (Bishop and Hostager 2003). The CD40 ligand (CD40L/CD154) is a molecule expressed on the surface of activated T-lymphocytes, which plays a central role in providing helper signals required for the activation, proliferation, differentiation, and prevention of apoptosis in haematopoietic cells (Bishop and Hostager 2003). The activation of $CD4^+$ T-cells leads to an up regulation of CD40L on the T-cell surface. This ligand delivers signals to the CLL cell by binding to CD40 present on the CLL cell surface leading to an up-regulation of pro-survival signalling pathways including PI3K and PLCγ. Furman *et al* demonstrated that *in vitro* CD40 ligation could inhibit apoptosis in CLL cells by inducing the transcription factor NF-κB (Furman et al. 2000). As well as enhancing CLL survival, it has been shown that CD40 signalling promotes proliferation of CLL cells, with augmented expression of cyclin-dependent kinases CDK4 and CDK6 and down regulation of cell cycle inhibitory kinase $p27^{kip1}$(Harnett 2004).

1.5.2.2 Interleukin 4 (IL-4)

IL-4 is a pluripotent gamma chain cytokine, first identified by its proliferative effect on B-lymphocytes (Kay and Pittner 2003). IL-4 can induce the expression of surface molecules involved in immune activation and immune recognition. IL-4 is predominantly secreted by $CD4^+$T-cells and can protect CLL cells from apoptosis *in vitro*(Kay et al. 2001). Circulating $CD8^+$ T-cells from CLL patients also express elevated levels of IL-4 when compared to normal healthy donors (Mu et al. 1997). Cytoplasmic IL-4 can also be detected in clonal B-cells (Douglas et al. 1997). There are two types of IL-4 receptors, one found on haemopoietic cells and the other on every other cell type. CLL cells possess IL-4 receptors, and IL-4 produced by both B-cells and T-cells may impact upon CLL survival via autocrine and paracrine mechanisms (Kay et

al. 2001). The binding of IL-4 to the IL-4 receptor activates Jak1 and Jak3, two members of the Janus family of tyrosine kinases, which results in the phosphorylation and activation of the transcription factor STAT6 (Kay and Pittner 2003).

Experiments conducted by Dancescu *et al* have demonstrated that the addition of IL-4 to culture medium leads to an increase in the expression of anti-apoptotic protein BCL2, when compared to cells which have not been stimulated by IL-4. This implies that the mechanism by which IL-4 rescues CLL cells from apoptosis is, at least to some extent, BCL2-dependent (Dancescu et al. 1992). IL-4 can partially counteract the effect of chemotherapeutics including treatment with Chlorambucil and Fludarabine; increased CLL cell viability is observed when IL-4 is present in drug-treated cultures (Mentz et al. 1996). Studies such as those conducted by Mentz *et al* and Dancescu *et al* highlight the importance of targeting elements of the CLL microenvironment to counteract drug resistance mechanisms.

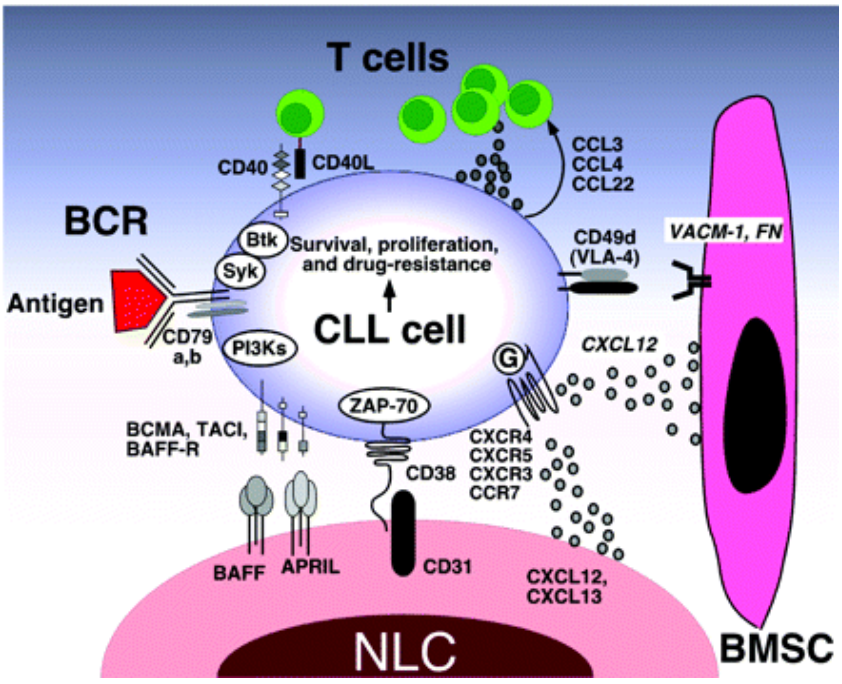


Figure 1.2 CLL cell microenvironment (copied from (Burger et al. 2009))

1.5.3 CD31

CD31 (Cluster of differentiation 31, also known as platelet endothelial cell adhesion molecule (*PECAM-1*)), is encoded by the *PECAM-1* gene located on chromosome 17 (Deaglio 1996). CD31 is a 130kDa member of the Immunoglobulin (Ig) superfamily and is expressed on endothelial cells, platelets, neutrophils, monocytes and naïve B-lymphocytes (Deaglio 2003b; Ibrahim et al. 2003). The extracellular domain of CD31 is composed of 574 amino acids made up of 6 homologous sections linked to a single 118 amino acid intracellular domain via a transmembrane channel (Ibrahim et al. 2003). The expression of CD31 on the surface of B-lymphocytes changes throughout the B-cell maturation process and CD31 plays a fundamental role in determining key adhesion-mediated biological events (Ibrahim et al. 2003). In 1998 Deaglio *et al* identified CD31 as the ligand for CD38 (Deaglio 1998).

Studies have been conducted in an attempt to determine whether the surface expression of CD31 on B-lymphocytes has any prognostic value in CLL. Maniou-Fowler *et al* analysed the surface density of CD31 expressed on the CLL cells in a cohort of 120 patients. CD31 expression was significantly lower in patients with advanced or progressive CLL (Binet stages B and C), and higher in patients with stage A disease. An inverse correlation was established between CD31 and CD38, and all CLL-related deaths that occurred within the duration of this study were patients who had low CLL cell surface expression of CD31. It was shown that low CD31 expression was associated with poor clinical outcome, irrespective of patient age (Mainou-Fowler et al. 2008). In contrast, Ibrahim *et al* looked at a cohort of 120 patients and showed that patients who had low surface expression of CD31 and CD38 had the most favourable outcome compared to all other combinations of expression of these two proteins. They also showed that patients with high CD31 expression but low CD38 expression had a poor clinical outcome, which was analogous to that of the CD38 positive cohort suggesting that increased expression of CD31 is of prognostic significance (Ibrahim et al. 2003). Furthermore, Poggi *et al* showed that members of the BCL2 family associated with cell survival were up-regulated in CLL cells with high surface density of CD31. After studying the effects of CD31 ligation, they observed significant up-regulation of the PI3K/PKB signalling pathway as well as subunits p65 and p52 of NF-κB (Poggi et al. 2010).

Independent immuno-histochemical studies on lymph node sections revealed a direct association between the number of endothelial cells ($CD31^+$) and the level of CD38 expression by CLL cells (Deaglio et al. 2010). The lymph nodes are proposed to be where CLL cells proliferate and $CD38^+$ CLL cells are characterised by a specific genetic profile showing up-regulation of proliferation and survival pathways (Deaglio 2010; Pepper 2007). A method has been developed whereby $CD38^+$ CLL cells can bind to murine fibroblasts transfected with the CD31 ligand with resulting increased growth and survival. *In vivo* $CD31^+$ cells can be found in lymphoid organs often in close contact with $CD38^+$ CLL cells (Patten et al. 2008). Experiments carried out by Deaglio *et al* have revealed that CD31/CD38 interactions drive activation and proliferation of distinct lymphocyte populations (Deaglio et al. 2010).

1.6 Signalling pathways in CLL

1.6.1 B-cell receptor (BCR)

1.6.1.1 Structure of the B-cell receptor (BCR)

Mature B-cells have two types of B-cell receptor, which are IgM and IgD. The BCR is made up of membrane immunoglobulin (mIg) made up from a heavy chain, which is composed of 4 domains in the IgD isotype and 5 domains in the IgM isotype. This heavy chain is linked to a very short intracellular domain, via a transmembrane connective domain, three amino acids in length with the sequence lysine, valine, lysine (KVK) (Matsuuchi and Gold 2001). The mIg is linked to a CD79a Igα/ CD79b Igβ heterodimer which has immunoreceptor tyrosine-based activation motifs (ITAM), a highly conserved sequence which is made up of only 4 amino acids and includes a tyrosine separated from either leucine or isoleucine by any 2 amino acids (Treanor 2012).

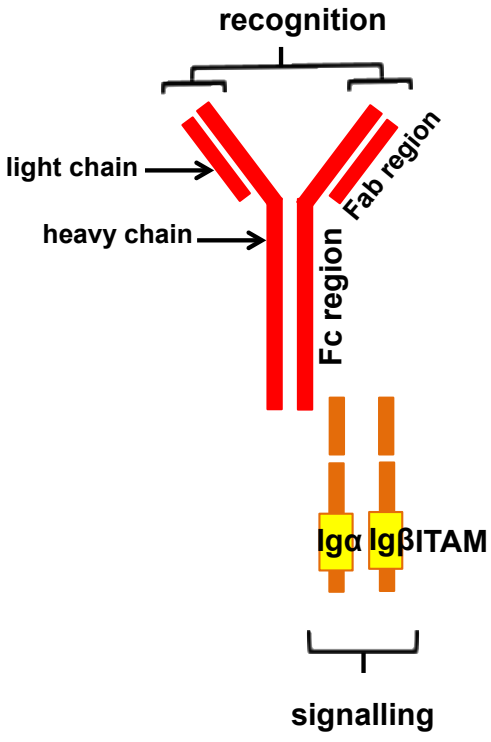


Figure 1.3 B-cell receptor

1.6.1.2 Normal BCR signalling

The ligation of an antigen to the BCR results in phosphorylation of tyrosine residues within the ITAM region of the Igα/ Igβ heterodimer by the SRC family kinase LYN as

well as Spleen Tyrosine kinase (SYK) (Matsuuchi and Gold 2001)(Yamamoto et al. 1993). Phosphorylation of the ITAM region leads to the recruitment of the signalosome, which includes other kinases and adaptor proteins including SYK, BTK and LYN and VAV proteins and adaptor proteins GRB2 and B cell linker (BLNK) (Rolli et al. 2002; Wickremasinghe et al. 2011). BCR signalling occurs through many different pathways, including phospholipase C gamma 2 (PLCγ2), PI3K as well as BTK. Following the recruitment of SYK and LYN to the ITAM region after phosphorylation, BLNK binds via SRC homology 2 (SH2) domain on the non-ITAM Igα portion of the BCR (Engels et al. 2001). Upon binding BLNK it is rapidly phosphorylated and can act as a scaffold protein to bring into contact SYK, BTK, BLNK and PLCγ2. The dual phosphorylation of PLCγ2 via SYK and BTK produces secondary messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate from the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2). DAG can activate Protein kinase C (PKC) that in turn leads to the downstream amplification of BCR signalling (Rolli et al. 2002). IP3 production leads to enzyme activation and an influx of Ca^{2+} from the endoplasmic reticulum and extracellular space. This Ca^{2+} influx activates transcription factors including NF-κB (Woyach et al. 2012).

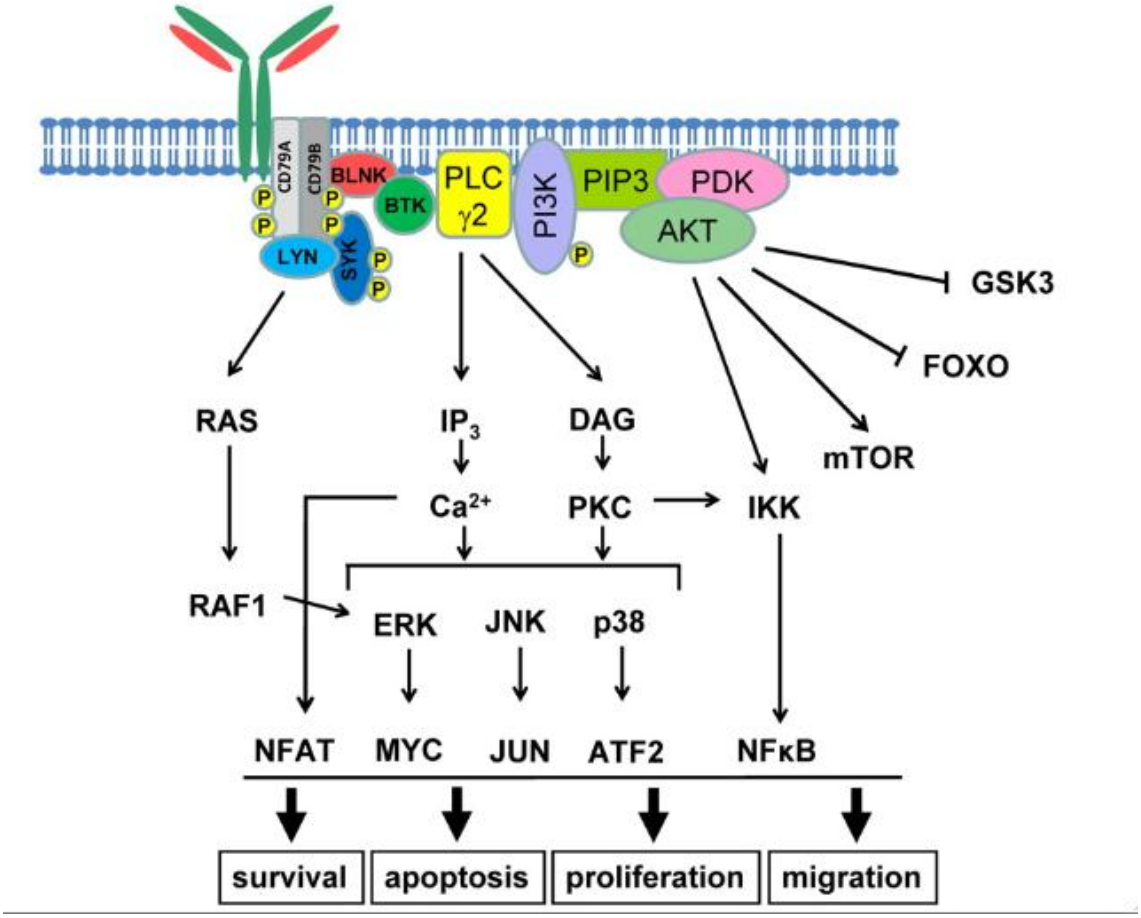


Figure 1.4 Schematic to show the BCR signalling pathway taken from (Stevenson et al. 2011)

The tight regulation of the BCR is required since the constitutive activation of this pathway can result in malignancies and autoimmune diseases (Woyach et al. 2012). This pathway is kept under control via inhibitory regulators FcγRIIb, SH2 domain-containing inositol 5'-phosphatase (SHIP), SH2 domain containing phosphatase (SHP-1) and LYN kinase amongst others (Engels et al. 2001; Matsuuchi and Gold 2001).

An important role of the BCR in normal B-cells is to mediate signals which facilitate entry to the lymph node, where the B-cell can undergo genetic diversification to form a mature cell which is specific to the encountered antigen (Agenes et al. 2000). The cell then differentiates and is either retained as a mature memory B-cell or forms an antibody-secreting plasmacytoid cell. Whilst the B-cell is undergoing the differentiation process, the BCR continues to respond to external stimuli to promote the development of the cell or induce programmed cell death (Agenes et al. 2000).

1.6.1.3 BCR signalling in CLL

The maintenance and progression of CLL is thought to be partly due to aberrant BCR signalling (Stevenson et al. 2011). A characteristic trait of CLL cells is the low surface expression of IgM/IgD, compared to normal B-cells; therefore, it may be assumed that the ability of the CLL cell to signal through the BCR complex is limited. However, Gene expression profiling (GEP) has revealed that CLL cells share many features with antigen activated mature normal B-cells and this suggests a role for BCR signalling in the pathogenesis of CLL.

As previously discussed, the absence of somatic mutations in the immunoglobulin variable heavy chain region (*IGHV*) genes in the sIg is indicative of a poor prognosis. After discovering the prognostic relevance of the *IGHV* genes mutational status, it was established that responsiveness of CLL cells following BCR ligation significantly correlated with *IGHV* mutational status; 80% of unmutated cases responded to ligation compared to only 20% of mutated CLL cases (Lanham et al. 2003). Furthermore, a study by Rosenwald *et al* using GEP revealed that CLL patients share a common gene expression "signature" regardless of *IGHV* mutational status, this would suggest that CLL is in fact not 2 distinct diseases based on mutational status. However, in the unmutated *IGHV* group it was found that there was an up-regulation of genes expressed during mitogenic BCR signalling, the expression of these up regulated genes were used to help in the clinical staging of this highly heterogeneous disease (Rosenwald et al. 2001).

Unmutated *IGHV* genes are strongly associated with the increased expression of ZAP70, which is also a marker of poor prognosis in CLL (Chen et al. 2008; Chen et al. 2002). High ZAP70 protein expression on CLL cells has been shown to lead to increased BCR signalling in the peripheral blood which aids in the migration of CLL cells to microenvironments towards chemokines (Richardson et al. 2006). Despite the fact that ZAP70 expression shows a strong correlation with *IGHV* mutational status CLL patients with unmutated *IGHV* genes and increased ZAP70 expression show augmented SYK phosphorylation following BCR stimulation. Furthermore, microarray analysis of bone marrow and lymph node tissues of CLL patients revealed an increase in BCR signalling in these tissues compared to peripheral blood regardless of the *IGHV* mutational status or ZAP70 expression (Herishanu et al. 2011).

As discussed previously the tumour microenvironment in which CLL cells reside promotes CLL cell survival and proliferation. This may be partly due to increased activation of the BCR signalling pathway. GEP from CLL cells taken from the peripheral blood compared to the lymph node revealed significant increases in genes associated with the BCR signalling pathway in CLL cells derived from the lymph nodes (Jaksic et al. 2004) . Tight regulation of BCR signalling in normal B-cells ensures there is no aberrant signalling, however in CLL deregulation of BCR signalling is observed. This can be due to the constitutive activation of particular kinases.

1.6.1.4 Tyrosine Kinases

1.6.1.4.1 Tyrosine Kinases in normal B-lymphocytes

The SRC kinase LYN initiates BCR signalling and is the mediator of activation and termination of BCR signalling (Wiestner 2012b). In normal resting B-lymphocytes, a significant increase in LYN activity occurs following BCR engagement. LYN plays a central role in mediating both survival and apoptosis following BCR activation in B-lymphocytes (Contri et al. 2005). Interestingly, LYN knockout mice display hyper responsiveness developing lethal autoimmune glomerulo-nephritis, this demonstrates that LYN has a critical role in the regulation of the BCR (Woyach et al. 2012).

LYN phosphorylates SYK with a resulting amplification of BCR signalling which leads to the activation of downstream signalling targets. The activation of SYK is crucial in BCR signalling since this event initiates the formation of the signalosome, which connects key signalling molecules together to coordinate cellular events including

survival, proliferation or differentiation. It is the balance of these signalling molecules that determines the B-lymphocyte fate (Scupoli and Pizzolo 2012).

The ERK signalling pathway is also activated as a result of BCR ligation. ERK regulates transcription factors and, in early stages of B-cell maturation, this event can lead to proliferation and survival whereas at later stages of B-cell development, phosphorylation of ERK can result in apoptosis (Woyach et al. 2012). Downstream proteins involved in cell survival include pro-apoptotic proteins BAD and BIM which are inhibited as a result of ERK activation (Scupoli and Pizzolo 2012).

1.6.1.5 Tyrosine Kinases in CLL cells

The SRC kinase LYN displays increased protein expression in CLL cells compared to normal B-lymphocytes. The distribution of LYN protein expression varies between normal B-lymphocytes and CLL cells. In normal B-lymphocytes LYN protein expression is sporadic across the plasma membrane, whereas in CLL cells dense LYN protein expression could be observed in the whole plasma membrane (Contri et al. 2005). LYN mRNA levels were found to be similar in normal B-lymphocytes and CLL cells, so the differences in protein expression of this kinase are likely to be due to de-regulation in protein turnover. *In vitro* kinase assays have revealed that LYN is constitutively activated in CLL cells, and this activation is independent of BCR engagement (Contri et al. 2005). Following BCR engagement no flux in LYN protein expression was observed, but this may be due to basal levels of this kinase already being elevated in CLL patients. Increased LYN protein activity is linked to an increase in survival pathways and a decrease in cell apoptosis, both features of CLL.

In many CLL patients SYK is overexpressed at both mRNA and protein level. The basal levels of phospho-SYK are also much higher in CLL cells compared to B-cells from normal age-matched individuals (Buchner et al. 2009). However, Gobessi *et al* found no link between the levels of increased SYK activity and CLL disease progression (Gobessi et al. 2009). Increased levels of activated SYK have been discovered in CLL cells located in the lymph node. Both SYK and ZAP-70 are cytoplasmic tyrosine kinases and have been shown to work synergistically, the increased expression of ZAP-70 leads to an enhanced BCR response that displays prolonged activation of SYK (Wiestner 2012a). ZAP70 expression increases the response of CLL cells to BCR activation; this finding provides a rationale for why ZAP70 expression is associated with poor prognosis and a rapidly progressing disease (Gobessi et al. 2007).

Constitutive ERK phosphorylation has been shown in almost half of CLL patients analysed (Muzio et al. 2008), the phosphorylation of ERK has been associated with a lack of BCR responses. Increased ERK phosphorylation as a result of anti-IgM stimulation has been shown to correlate with markers of poor prognosis in CLL and increased phosphorylation of this tyrosine kinase is indicative of a more rapidly progressing disease (Scupoli and Pizzolo 2012). The ability for ERK phosphorylation to influence increased cell survival in CLL has not been fully elucidated, however, the

phosphorylation of ERK may influence the survival potential of the cell since phosphorylated ERK promotes the phosphorylation and degradation of the pro-apoptotic protein BIM in the proteasome (Ley et al. 2005)

1.6.1.6 Serine/threonine kinases

1.6.1.6.1 PKA signalling in normal cells

Protein Kinase A (PKA) is a tetramer composed of two homo or heterodimer subunits, each bound to a catalytic subunit. PKA is a serine/threonine kinase, which is cAMP dependent (adenosine 3′5′ cyclic monophosphate); activation of PKA only occurs when cAMP is present (Kleppe et al. 2011). PKA can alter the function of enzymes through direct phosphorylation or activation of transcription factors CREB, CREM (a cAMP response element modulator) as well as ATF-1 (activating transcription factor). PKA has the ability to coordinate different cellular processes within the same cell, this is due to temporal and spatial regulation of cAMP within different cellular components (Kleppe et al. 2011).

1.6.1.6.2 PKA signalling in CLL cells

Aberrations within the PKA signalling pathway have been implicated in human diseases. A link has been established between the activation of PKA and hyperproliferation and tumourigenesis. Mutations in PKA have been implicated in GH-secreting pituitary tumours as well as advanced thyroid cancers (Kirschner et al. 2009). Increased PKA signalling has also been associated with increased apoptosis in CLL cells. The murine T-lymphoma cell line S49 has been previously used to demonstrate the pro-apoptotic effect of cAMP via PKA signalling (Murray and Insel 2013). Treatment with agents that elevate the intracellular levels of cAMP show elevated levels of apoptosis of CLL cells. Agents such as these may prove to be effective in clinical use to enhance this pro-apoptotic signalling pathway (Lerner et al. 2000).

1.6.1.7 PKC signalling in normal cells

Protein kinase C (PKC) represents a family of closely related Serine/Threonine protein kinases. PKCs are controlled by a variety of extracellular stimuli and facilitate physiological processes through the phosphorylation of PKC and downstream targets of this kinase. PKC was first identified in 1977 as a calcium-activated enzyme (Takai et al. 1977), as well as a major target of lipid metabolite diacylglycerol (DAG). Shortly after

the discovery of PKC it was established that multiple isoforms of PKC exist and interestingly multiple isoforms of PKC may be present in a single cell or tissue (Teicher 2006). PKCs are divided up into 3 main sub-families: the first is the classical Ca^{2+} isoforms (α, βI/ II and γ), the second subfamily is the novel Ca^{2+}-dependent isoforms and the third subfamily is the atypical isoforms (Abrams et al. 2007; Koivunen et al. 2006). PKCs contain both a regulatory and catalytic domain, which are joined by a hinge type region (Steinberg 2008). In normal B-cells PKCξ, PKCβ, PKCδ and PKCε represent important mediators of BCR signalling (Abrams et al. 2007). Upon activation, PKC is recruited to the plasma membrane and PKCs are known to be involved in many processes including regulation of cell growth, as well as regulating immune responses and transcription (Steinberg 2008).

1.6.1.8 PKC signalling in CLL

Constitutive activation of PKC in CLL cells has been identified in multiple studies (Barragan et al. 2002; Gschwendt 1999; Nishikawa and Shirakawa 1992). Work conducted by Alkan *et al* in 2005 looked at the expression of different PKC isoforms in CLL patients and showed that CLL patients consistently displayed expression of PKC isoforms β and γ as well as δ and ζ. However, the expression of other PKC isoforms was more varied. Following pharmacological inhibition of PKC isoforms with Safingol induction of apoptosis was observed regardless of whether patients had received previous treatment or not (Alkan et al. 2005). Holler *et al* used PKCβ deleted TCL1 transgenic mice to assess the effect of this PKC isoform on the initiation and development of CLL disease in the mouse model. It was shown that PKCβ deficient mice did not develop a malignant clone; moreover the targeting of CLL cells *in vitro* with PKCβ specific inhibitor resulted in elevated levels of apoptosis (Holler et al. 2009).

Abrams *et al* 2007 showed that levels of protein kinase CβII (PKCβII) in CLL cells were 7-fold greater than levels of this PKC isoform in normal B cells (Abrams et al. 2007). PKCβII has been shown to negatively regulate Ca^{2+} flux following BCR engagement and it has been proposed that in CLL the overexpression of this kinase maintains BCR signalling at a level that is insufficient to trigger apoptosis following antigen encounters (Abrams et al. 2007).

High PKCβII expression in CLL patients correlated with increased white blood cell count and disease stage. This is indicative that the regulation of BCR signalling by PKCβII is functionally relevant *in vivo* (Barragan et al. 2006).Work conducted by

Lutzny *et al* this year has shown that CLL cells can induce the expression of PKCβII in BMSCs, which could also mediate stromal cell survival (Lutzny et al. 2013). Therefore this particular isoform of PKC may have dual functionality in CLL, expression of this kinase in CLL cells may lead to the evasion of pro-apoptotic signalling through activation of PI3K/PKB signalling, and expression of PKCβII on stromal cells may mediate microenvironment survival.

1.6.2 Phosphatidylinositol 3-Kinase (PI3K)/ Protein kinase B (PKB) signalling

1.6.2.1 Phosphatidylinositol 3-Kinase (PI3K)/ Protein kinase B (PKB) signalling in normal cells

Phosphatidylinositol 3-kinases (PI3K) are an evolutionarily conserved family of intracellular lipid kinases which have been identified in species ranging from yeast to humans. To-date PI3K family members have been identified in every eukaryotic organism examined (Engelman et al. 2006). The regulation of this pathway is under tight control via a complex multi-stage activation process. A schematic of this signalling cascade is depicted in Figure 1.5. Activation of this pathway is triggered through the binding of growth factors to various receptors on the CLL cell surface, which in turn phosphorylates PI3K, initiating the conversion of phosphatidylinositol (3,4)-bis-phosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-tris-phosphate (PIP_3). PKB the target of PI3K then binds to PIP_3at the plasma membrane exposing T^{308} within the "activation loop" to be phosphorylated by PDK1. Phosphorylation of T^{308} results in partial PKB activation, to attain full PKB activation phosphorylation of S^{473} in the carboxy terminal hydrophobic motif must occur (Hemmings and Restuccia 2012).

Protein Kinase B (PKB) also known, as AKT is a well-characterised target of PI3K. PKB belongs to a sub-family of protein kinases termed the AGC (cAMP-dependent protein kinase A/ Protein kinase G/ Protein kinase C) protein kinases, which also include PKA and PKC. PKB is a highly conserved serine/threonine kinase; in fact PKB was identified through the high degree of homology to PKA and PKC and therefore named.

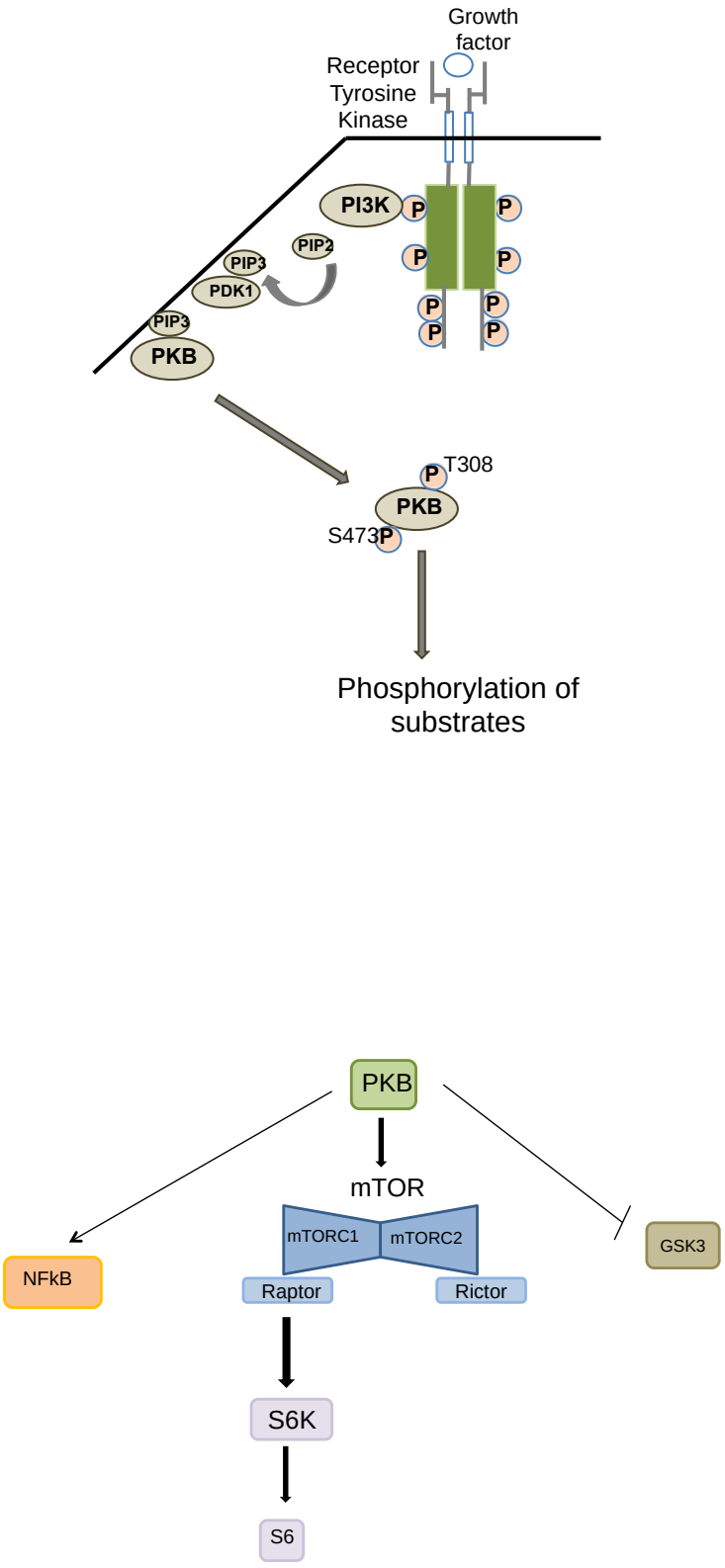


Figure 1.5 Schematic of the PI3K/PKB signalling cascade

1.6.2.2 Phosphatidylinositol 3-Kinase (PI3K)/ Protein kinase B (PKB) signalling in CLL cells

The PI3K signalling pathway has an important role in the biology of human cancers (Barragan et al. 2006); components within this signalling pathway have been found to be deregulated in a wide range of solid tumours as well as haematological malignancies (Engelman et al. 2006). In the context of CLL, PI3K has been described as having a crucial role in cell survival; a huge wealth of knowledge has been gathered on this signalling cascade regarding both CLL and other malignancies (Engelman et al. 2006; Lawlor and Alessi 2001).

A study conducted by Bernal *et al* in 2001 stimulated CLL cells *in vitro* with F (ab') 2 antibody fragments to human IgM. This stimulation resulted in a significant decrease in spontaneous apoptosis and an inhibition in caspase activity as well as augmented expression of anti-apoptotic proteins BCL2 and MCL1. These pro-survival effects observed upon antigen-receptor binding were counteracted by the treatment of CLL cells with PI3K inhibitor LY294002; this implicates PI3K as being a critical mediator of survival signalling in CLL (Bernal et al. 2001).

Evidence from transgenic mice show that when PKB is constitutively activated the mice rapidly develop aggressive B-cell lymphomas with leukaemic involvement (Zhuang et al. 2009). Activated PKB phosphorylates and de-activates many proteins involved in apoptosis and consequently increases cell survival. Although PKB has been identified as a key survival factor in CLL, the precise targets of this kinase remain poorly characterised (Barragan et al. 2006).

1.6.2.2.1 Downstream targets of PI3K/PKB signalling

Following the full activation of PKB, which occurs very rapidly following cell stimulation, PKB is released from the cell membrane and enters the cell nucleus. PKB phosphorylates various substrates at serine and threonine residues within RXRXXS/T consensus motifs in both the cytoplasm and the nucleus, where X represents any amino acid (Kane et al. 2002; Lawlor and Alessi 2001). PKB phosphorylation arbitrates a number of cellular processes including survival, growth, proliferation as well as transcription. PKB substrates reside at different sub-cellular compartments including the cytoplasm, nucleus and mitochondrial membrane and usually become inhibited by the phosphorylation event (Kane et al. 2002).

PKB can alter cell survival; two PKB substrates that are directly involved in apoptosis are BAD and the pro-death caspase-9. BAD, a BCL2 family member promotes apoptosis by forming a heterodimer with the survival factor BCLXL, when BAD is phosphorylated by PKB this interaction is inhibited, which increases the survival potential of the cell (Lawlor and Alessi 2001). Pro-death caspase-9 is also inhibited following phosphorylation by PKB, which results in the phosphorylation and activation of MDM2 (murine double minute-2), promoting the destabilization of p53 and preventing cell death (Feng et al. 2004).

There are other mediators of the PKB signalling pathway, which alter the survival potential of the CLL cell indirectly through transcriptional regulation. Examples of this are the forkhead box O (FOXO) family of transcription factors, so named because they have a very distinct forkhead shaped DNA binding domain (Myatt and Lam 2007). Upon phosphorylation by PKB, FOXO transcription factors are inhibited and translocated out of the cell nucleus preventing any transcriptional activity. The FOXO transcription factors direct the transcription of genes involved in cell cycle arrest and apoptosis (Seoane et al. 2004).

The mammalian target of Rapamycin (mTOR) protein is another important mediator of the PI3K/PKB signalling cascade. The mTOR protein is a kinase, one of this kinase's functions is to regulate cell proliferation, this is achieved through the phosphorylation of the well characterised downstream effector of mTOR; S6 kinase (S6K), which in turn leads to the phosphorylation of the 32kDa S6 ribosomal protein. Ultraviolet crosslinking studies have shown that S6 interacts with tRNA, initiation factors and mRNA and is involved in the regulation of translation initiation (Nygard and Nilsoon 1990). The precise role S6 has in translation is not known but it is thought that following the phosphorylation of this molecule the rate of protein synthesis is increased, it has also been suggested that S6 regulates cell size. The deletion of S6 in mice resulted in a block in ribosome biogenesis and prevented cell cycle progression (Volarevic et al. 2000)

GSK3β is a 46kDa serine/threonine kinase that has enzymatic activity regulated by many signalling pathways.GSK3β has 2 phosphorylation sites that influence the catalytic activity of the protein, Serine 9 is the phosphorylation site for PKB, and phosphorylation of this residue inactivates the protein. In contrast, phosphorylation of Tyrosine 216, located on the activation loop increases catalytic activity(Plate 2004). Phosphorylation of serine 9 prevents phosphorylation of β-catenin, a cytoplasmic

signalling molecule which, when phosphorylated, is degraded however when unphosphorylated β-catenin is translocated to the nucleus. In the nucleus β-catenin interacts with the transcription factors TCF and LEF-1 to initiate the expression of many genes, including cyclin D1, which permits cell cycle progression (Piao et al. 2008).

1.6.3 CD38 signalling

Almost two decades after CD38 was first described as being expressed on the surface of CLL cells it was demonstrated that, as well as being an ecto-enzyme involved in the mobilisation of intracellular Ca^{2+}, CD38 also has a signalling-receptor capacity in lymphocytes (Deaglio 2003a). In the late 1990's, Malavasi and colleagues used a non-substrate agonistic antibody for CD38 to show that CD38 ligation could stimulate intracellular signalling pathways. The molecules activated, as a result of this stimulation were similar to those induced through engagement of the T-cell receptor (TCR).

The earliest experimental work on CD38 signalling was carried out by Zubiaur *et al* on T-cells showing that CD38 ligation could induce tyrosine phosphorylation of the CD3-ζ and CD3-ε components of the TCR (Zubiaur et al. 1999). Downstream targets of CD38 signalling were identified as ERK and PKC signalling cascades amongst others. An experiment using a mutant CD3-ζ TCR resulted in the defective binding to CD38, although tyrosine phosphorylation of CD3-ζ was not achieved, downstream effectors of CD38 signalling were stimulated, indicating that CD3-ε is sufficient to the phosphorylation and activation of some downstream effectors. It is likely that CD3-ζ and CD3-ε work in synergy to attain maximal CD38 signalling, since CD3-ζ has been shown to have the ability to recruit ZAP-70 to the TCR (Zubiaur et al. 1999).

1.6.3.1 CD38 signalling in normal B-cell development

In normal B-lymphocytes CD38 is expressed on the cell surface at very specific stages during B-cell maturation. CD38 is expressed on the surface of progenitor B-cells with a role in aiding lymphopoiesis in the bone marrow. When monoclonal antibodies were used to block CD38 ligation in CLL cells followed by stimulation by cytokines and co-culture with supportive stromal cells, B-cell lymphopoiesis was inhibited. This demonstrates that CD38 is an essential signalling molecule in B-cell maturation process (Kumagai et al. 1995). Investigations by Kitanaka *et al* in 1996 and 1997 revealed that CD19 is an important mediator of CD38 signalling. CD19 is able to facilitate in the recruitment of kinases to the inner membrane that, in turn, allows CD38 signalling via

the PI3K signalling pathway amongst others, this occurs in normal B-cell progenitors. Normal mature B-lymphocytes do not express surface CD38, but re-expression of CD38 marks B-cells that are differentiating or have been stimulated by antigens (Kitanaka et al. 1997; Kitanaka et al. 1996).

1.6.3.2 CD38 signalling in CLL

CD38 is well established as a marker of poor clinical outcome in CLL. In the last decade multiple research groups have been investigating the signalling capabilities of CD38 specifically involving proliferation and survival mechanisms in CLL.

In 2002 Durig *et al* compared the gene expression profiles of a cohort of CD38 positive and CD38 negative patients and revealed differences in the expression of multiple genes including genes that are central in survival signalling (Durig et al. 2002). In 2007 Pepper used GEP to analyse CLL cells attained from CD38 bi-modal patients and showed that the CD38 positive sub-clones had a distinct gene expression profile compared to the CD38 negative sub-clones derived from the same patient (Pepper 2007).

In 2003 Deaglio *et al* used an agonistic CD38 antibody to stimulate surface CD38 in CLL patients (Deaglio 2003a). Two patients out of a cohort of eight patients showed a slight Ca^{2+} flux following cross-linking. The cytokine IL-2 has been shown to up-regulate the expression CD38 when CD38 is already present on the cell surface. The addition of IL-2 to the agonistic CD38 antibody resulted in a marked increase in Ca^{2+} in CLL samples which were previously unresponsive to CD38 ligation alone (Deaglio 2003a). Further work carried out by Deaglio *et al* in 2006 indicate that there is a minimum threshold that is based on the cell surface density of CD38, which is required for CD38 signalling to occur (Deaglio 2006). The capacity of IL-2 to facilitate CD38 signalling in previously unresponsive CLL patients may be due to the ability of this cytokine to modify the arrangement of CD38 with other accessory molecules to allow for signalling to occur (Deaglio 2006). Furthermore, it has been shown that both CD38 and CD19 were recruited alongside CD38 to form lipid rafts (Deaglio 2007), illustrating that to induce CD38 signalling the recruitment of accessory molecules may be required. Since it has been shown that the lymph nodes, microvasculature and peripheral blood all contain accessory molecules this supports the notion that the combination of signals within complex microenvironments *in vivo* are essential for the activation, proliferation and survival of CLL cells.

It has been reported that there may be synergy between the BCR and CD38 protein signalling, which may enhance the survival and proliferation of CLL cells. A report by Lanham *et al* has shown that increased CD38 expression is associated with an increase in BCR signalling (Lanham et al. 2003). Lund *et al* demonstrated that increased CD38 expression lowered the threshold for BCR signalling in murine B-cells, which are responsive to BCR stimulation (Lund et al. 1996). The evidence revealing disruption of BCR signalling in CLL makes the prospect of pharmacological inhibition of this pathway promising therapeutically.

1.6.4 Other signalling pathways implicated in CLL pathogenesis

Aberrant JAK/STAT signalling has been reported in both solid and haematological malignancies. In acute leukaemia' the constitutive activation of STAT transcription factors are commonly observed (Lin et al. 2000). CLL cells possess constitutively high levels of the IL-4 receptor (Dietrich et al. 2012). IL-4 signalling occurs mainly through JAK1 and JAK3, and IL-4 mediated JAK phosphorylation results in the phosphorylation and activation of STAT6 (Dietrich et al. 2012).

The expression of pattern recognition receptors known as toll-like receptors (TLRs) in CLL patients has been reported as being very heterogeneous, however most patients were found to have gene expression profiles which closely resemble normal mature B-lymphocytes with TLRs 1,2,6 and 10 found on the cell surface and TLRs 7,8 and 9 within endosomes (Muzio et al. 2008). When comparing different sub groups of CLL patients it was discovered that patients with mutated *IGHV* genes had very few differences in TLR signalling molecules however TLR8 was found to be up regulated in patients with unmutated *IGHV* genes (Muzio et al. 2012). Gene expression profiling of the TLR signalling pathway was carried out on 192 CLL patients in a study conducted by Arvaniti *et al*. It was established that TLR7 had the highest expression of all the TLR receptors in this cohort of CLL patients (Arvaniti et al. 2011). Furthermore, in more recent experiments Efremov *et al* looked at the capacity of CLL cells to respond to TLR9 signalling. This study showed that patients with a more aggressive form of CLL responded more efficiently to TLR9 stimulation compared to patients who have a less aggressive form of CLL. Thus the capability of CLL cells to respond to TLR9 signalling may be of prognostic value (Efremov et al. 2013)

Constitutively activated NF-κB has been identified in several haematological malignancies including acute myeloid leukaemia, chronic myeloid leukaemia as well as

CLL (Cuni et al. 2004; Furman et al. 2000; Guzman et al. 2001). Three members of the NF-κB family are more abundant in CLL; these are p50, RelA and c-Rel (Cuni et al. 2004; Furman et al. 2000; Hewamana et al. 2008). In CLL, other microenvironmental stimuli have been shown to increase NF-κB activation which in turn leads to increased CLL cell survival; these include AKT activation, CD40 ligation as well as exposure to IL-4 and BAFF (Yamagishi et al. 1997). The downstream targets of NF-κB signalling are highly complex but are known to include members of the BCL2 family as well as inhibitor of apoptosis proteins (IAPs) (Pepper et al. 2009).

1.7 Project Aims:

The precise biological role of CD38 in the pathology of CLL remains elusive. CD38 has been shown as a marker of a more progressive disease type and in around 30% of CLL cases CD38 is highly expressed on the CLL cell surface. Evidence has shown that CD38 is not only a marker of poor prognosis but also has distinct signalling capabilities. CD31 is the non-substrate ligand for CD38 and interactions between CD38/CD31 have been shown to increase CLL cell survival.

The dissection of the key survival signalling pathways in operation in CLL cells, using *in vitro* co-culture systems, should help to provide information about the critical signals that help to protect CLL cells from chemotherapeutic agents in the tissue microenvironments. The central hypothesis of this project is that CD38 ligation leads to changes in key survival signalling pathways resulting in lower rates of CLL cell apoptosis. In order to test this hypothesis, four main objectives were formulated:

1. Assess the ability of CD38 ligation to induce changes in the phosphorylation of protein kinases known to be involved in cell survival, and furthermore to uncover whether CD38 positivity increases the phosphorylation of protein kinase substrates.

2. To assess the ability of other co-culture systems, including CD40L and the Th2 cytokine IL-4, to modulate the phosphorylation of the substrates identified in (1.) and also to compare the effects of these co-culture systems on CLL cell phenotype and viability.

3. To observe changes in CLL cell surface phenotypic markers following CD31 co-culture and to establish whether treatment with pharmacological inhibitors can block the phosphorylation of key signalling pathways.

4. To compare and contrast the effect of different co-culture systems on CLL cell extracellular and intracellular markers of activation.

2 Materials and Methods

2.1 List of general Materials and laboratory equipment

Material	Source	Code
Phosphate buffered saline tablets	Oxoid	BR0014G
15ml tubes	Greiner	188271
50ml tubes	Corning	430291
Haz Tabs	Guest Medical	H8801
Histopaque	Sigma Aldrich	1077
T175 tissue culture flask	Nunc. Thermo Sci	178883
T75 tissue culture flask	Greiner	658175
6-well plates	Nunc. Thermo Sci	140675
12-well plates	Greiner	665180
24-well plates	Nunc. Thermo Sci	150687
5ml Pipettes	Corning	4487
10ml Pipettes	Corning	4101
25ml Pipettes	Corning	4251
Test tubes (flow cytometry)	BD falcon	352054
*Paraformaldehyde	Sigma	P6148

*Paraformaldehyde

The preparation of paraformaldehyde was carried out in a fume hood for health and safety purposes. To make a 1% paraformaldehyde solution, 1 gram of paraformaldehyde (Sigma) was dissolved in 100ml PBS, the solution was heated to 70°C to ensure all paraformaldehyde was dissolved, this solution was cooled and aliquoted into 20 ml universal containers and stored at 4°C in darkness to prevent de-polymerisation and the release of formaldehyde.

2.2 Primary Cell isolation

2.2.1 Patient samples and ethical approval

Professor Chris Fegan and Dr Guy Pratt provided CLL patient samples, which were taken in outpatient clinics at Llandough Hospital, the University Hospital Wales (UHW) and Birmingham Heartlands Hospital. All patients' informed consent was gained in accordance with the ethical approval granted by the South East Wales Research Ethics committee in accordance with the declaration of Helsinki. Patients were considered eligible for this study following a definitive diagnosis of CLL and were subsequently selected for use in this project according to the surface expression of CD19 and CD38.

2.2.2 Density centrifugation of peripheral blood (PB) to obtain CLL cells from patient's samples

CLL cells were isolated from peripheral blood samples by density gradient centrifugation using histopaque reagent (Sigma Aldrich, catalogue no: 1077). Upon arrival in the laboratory, blood sample tubes were inverted several times to ensure they were well mixed. The histopaque reagent was added to labelled tubes corresponding to patient samples. For a 4ml blood sample, 3ml of histopaque was used. Using a pastette, blood samples were layered gently on top of the histopaque layer in a dropwise fashion, excess blood in the collection tubes were rinsed out with phosphate buffered saline (PBS) to make the total volume up to 14ml. The samples were then centrifuged for 20 minutes at 2000 x g with the centrifuge brake switched off to avoid any disruption to the mononuclear (interface) layer. The removal of the brake means that this centrifugation process took approximately 40 minutes. Following centrifugation a monolayer of low-density white blood cells (WBC), which were mainly CLL cells were apparent as an interface layer underneath the blood plasma and on top of the clear histopaque phase. This was removed with a pastette and transferred to a 15ml labelled tube. These cells were washed in 10ml of sterile PBS, and centrifuged for 5 minutes at 1200 x g. To lyse any contaminating red blood cells, 2ml of sterile H_2O was added to the pellet and briefly mixed with a pastette before being topped up to 12ml with sterile PBS to restore the isotonic solution. The cells were centrifuged for 5 minutes at 1200 x g and if any red blood cells were still visible in the pellet the lysis step was repeated. Once the cells were washed the pellet was resuspended in 1-10ml of PBS depending on the amount of cells isolated from the patients' blood sample.

2.2.3 Counting CLL cells

The Beckman Coulter Vi-cell analyser was used to count the number of viable CLL cells. In order to do this, 50µl of the CLL cell sample was added to 450µl of PBS in a Vi-cell counting cup and placed in the cell counting carousel at the appropriate position (position 1-12). This gives a 1 in 10 dilution factor and this information, as well as the cell type and cell size, was entered into the Vi-cell software and set to run. The Vi-cell analyser uses a Trypan Blue exclusion assay to ascertain cell viability and takes 25 images of the sample before giving an average viable cell count per/ml of cells. Data were subsequently printed and used for experimental purposes.

2.2.4 Testing CD19/CD38 status

Following CLL cell isolation and counting, cells were stained for CD19 and CD38. $3x10^5$of each sample was added to a FACS tube (2 tubes per sample; one no antibody control and the other containing all of the relevant antibodies). Using this number of cells ensured that the antibodies were present in excess and that all cells were uniformly stained. The volume in the tubes was then made up to 100µl with sterile PBS. The two antibodies purchased from Invitrogen were added to isolated CLL cells, allophycocyanin-labelled CD19 (catalogue number:MHCD1905) and phycoerythrin-labelled CD38 (catalogue number: MHCD3804), 4µl of each were added to the test sample tube and briefly mixed before being placed in the dark for 10 minutes. After this time 3ml of PBS was added to each of the FACS tubes and tubes were inverted to wash the cells followed by centrifugation for 5 minutes at 1200 x g to pellet the cells. The supernatant was tipped off and the stained (or unstained controls) cells were re-suspended in 1% paraformaldehyde (200µl). Cells were fixed for at least 10 minutes at 5°C followed by analysis on an Accuri C6 flow cytometer using Cflow software. A template folder was used for all samples, which gated out debris as well as any non-viable cells. This approach ensured that all operators analysed the cells in a standard fashion.

2.3 Tissue Culture

2.3.1 Eukaryotic cell culture

Material	Source	Code	Storage
Dulbecco's Modified Eagles Media (DMEM)	Gibco	41965	4°C
Foetal Calf Serum (FCS)	Gibco	31870	-20°C
Penicillin (5000U/ml)/Streptomycin (5000U/ml)	Gibco	12319018	-20°C
Sodium Pyruvate	Gibco	11360070	4°C
0.5% Trypsin EDTA	Gibco	25300	-20°C
L-glutamine x 100 (200μM)	Gibco	25030	-20°C
Interleukin 4	R & D Systems	204-IL-010	-20°C

2.3.2 Co-culture Cell lines

Cell Line	Source
CD31/NTL	Professor Silvia Deaglio
CD40L/NTL	Dr Aneela Majid

2.3.3 Culture media for adherent cells

Cell lines were cultured in Dulbecco's Modified Eagle's Media (DMEM)growth medium supplemented with 10% foetal bovine serum (FBS), Penicillin plus Streptomycin (100U of penicillin/ml and 100μg streptomycin/ml) and Sodium Pyruvate (5X). The media was inverted to mix well before being stored at 4˚C.

2.3.4 Cell culture storage

The co-culture cell lines used in this project were stored in liquid nitrogen in vials containing ~8 x10^6cells in a 500µl solution containing 50% FCS, 40% DMEM (plus the additives described above) and 10% DMSO. Precautionary measures were taken when obtaining cells from liquid nitrogen as well as general liquid nitrogen safety training (lab coat, gloves and face mask). Cells were thawed quickly by placing into a 37°C water bath and added drop-wise to a 15ml tube containing 9.5ml of pre-warmed culture medium to remove the toxic dimethyl sulphoxide (DMSO) as quickly as possible. The mix was then centrifuged at 1200g for 5mins to pellet the cells, the supernatant was removed and the cells were re-suspended in 8ml of fresh warmed growth medium before being transferred into a T25 culture flask and placed in a humidified incubator maintained at 37°C, 5% CO_2. Once the cells were confluent in the T25 flask they were transferred to a larger T75 flask and once confluent in this flask cells were transferred into a T175 flask.

2.3.5 CD31 and CD40 co-culture

The basis of this project was to attempt to characterise signalling events occurring *in vivo* in CLL patients within specific microenvironments in the body that promote CLL cell growth. In order to achieve this *in vitro*, genetically modified adherent mouse fibroblast cell lines were used which had been genetically modified to express either human CD31 or human CD40L, ligands to CD38 and CD40 respectively.

2.3.6 Irradiation of Murine fibroblast cell lines

To inhibit fibroblast growth in co-culture experiments the adherent CD31-expressing or non-transfected cells were removed from culture in a large T175 flask, and re-suspended in 25ml of supplemented DMEM growth medium, 1-5 ml of the cells were placed into a fresh T175 culture flask depending on experimental requirements for continual growth. The rest of the cells were irradiated at 75 Gray (29 minutes in the presence of Caesium-137, γ-emission).

2.3.7 Co-culture conditions

Following irradiation cells were counted using the Vi-Cell Coulter counter, 1 x $10^6$of the fibroblast cells were transferred into each well of a 6-well plate, the final volume of media was then made up to 3ml. The irradiated fibroblast cell lines were left overnight to allow the cells to adhere to the tissue culture plate and were between 90-95% confluent. The media was removed and any fibroblasts which had not adhered were removed by a PBS wash. CLL cells were then added to the co-culture well for a time period according to experimental design in 2-3ml of fresh media. Most of the co-culture experiments in this project were between 1 and 24 hours although longer time points were occasionally used.

2.3.8 Inhibitors

When inhibitors were used in this project they were added to CLL cells for a pre-co-culture step. Appropriate volumes of the inhibitors were added to CLL cells and incubated at 37°C for 30 minutes before being transferred for a further time period (between 1 and 48 hours) in their specific culture conditions. The cells were transferred with the inhibitors in media either to supplemented media only (LQ) or added to culture withCD31-expressing fibroblast or NTL. After the appropriate time point CLL cells were removed from culture and lysed for western blotting or fixed for flow cytometry analysis.

2.4 Cell Lysis and Sample Preparation

2.4.1 Cell lysis and sample preparation reagents

2 x Lysis Buffer (made in 100ml volume and stored at 4°C)

HEPES (1M stock)	10ml (100mM final conc)
NaF (MW 42)	0.042g (10mM final conc)
Iodoacetamide (MW 185)	0.185g (10mM final conc)
NaCl (MW 58.4)	0.8756g (150mM final conc)

*Make up to 100ml total volume with deionized H_2O

NP-40 (10% solution stored at room temperature)

1ml NP-40 added to 9ml H_2O

Protease Inhibitors

Purchased from Sigma (Cat no P-8340) as a 100 x stock in DMSO-stored at -20°C

Phosphatase Inhibitor Cocktail 3

Purchased from Sigma (cat no-2850) as a 100x stock in DMSO-stored at 4°C (Cocktail frozen at 10°C) has to be removed from fridge several hours prior to use to ensure it has properly thawed.

Phosphatase Inhibitor Cocktail 2

Purchased from Sigma (Cat no P-5726) as a 100x aqueous stock (stored at 4°C).

PMSF (MW 100mM) (100 x stock, store at 4°C)-wear mask as harmful if inhaled.

Add 87mg of PMSF to 5ml ethanol and allow to dissolve.

Sodium Orthovanadate (100 x stock; store at -20°C)

Add 0.2 Na_3VO_4 (mw 183.9) to 5ml H_20 and allow to dissolve, aliquot into 500µl tubes and store at -20°C.

Lysis buffer should be made fresh before use:

For 10ml Lysis Buffer:

2 x Lysis Buffer…….5ml

10 x NP40……..1ml

Protease Inhibitors…….100µl

Phosphatase Inhibitors 1…….....100µl

Phosphatase Inhibitors 2……100µl

PMSF…….100µl

Na_3VO_4……..100µl

dH_20……...3.5ml

Reducing Sample Buffer (1ml)

NuPAGE SDS sample buffer 4 x…….....250µl

NuPAGE Reducing agent 10 x…….....100µl

Deionised Water……650µl

2.4.2 Lysis method

Following the appropriate time period CLL cells were removed from co-culture ensuring enough force was used to make sure CLL cells had not adhered to the bottom of the well but not so the fibroblasts were also lifted from the bottom of the plate. The samples were removed from co-culture into a labelled 4ml FACS tubes and centrifuged at 300g for 5 mins at 4°C. The supernatant was removed and cells were washed in 3ml of PBS followed by another 5-minute centrifugation. Lysis buffer was made fresh before use (see appendix), and 500µl of lysis buffer was added to the cell pellet and pipetted up and down several times to re-suspend cells. The tubes were then placed on ice for 20 mins and vortexed at regular intervals (or put at -20°C for a maximum of 2 weeks).

2.4.3 Sample preparation

After the CLL cells were lysed, the samples were vortexed well and the 500µl lysate was transferred to 1.5ml eppendorfs and centrifuged at 13,000-x g for 20 mins to pellet any cell debris. The supernatant was then carefully removed and added to 0.7 volume of acetone (for 500µl lysate 350µl of acetone used). Samples were then stored at -20°C for at least 1 hour. Samples were centrifuged at 13,000 x g for a further 20 mins at 4°C to produce a protein pellet.

2.4.4 Lysis/ reducing buffer heating etc.

In order to resolve proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) the proteins had to be denatured. This was achieved by using reducing sample buffer mix. The SDS sample buffer acts by denaturing proteins by wrapping around the polypeptide backbone. SDS binds to proteins in a mass ratio of 1.4:1, and confers a negative charge to the polypeptide. Following the addition of the reducing sample buffer, the mixture was boiled for 5-10 minutes. The denaturation step enabled the SDS-PAGE separation of proteins based on their molecular weight and not the intrinsic electrical charge of each polypeptide. After the samples were boiled they were removed from the heat block and briefly centrifuged to ensure that the all sample was at the bottom of the tube and no condensation was collected in the tube lid.

2.5 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting

2.5.1 Reagents

Pre Cast Gels: Purchased from Invitrogen

NuPAGE 4-12% Bis-Tris 1.00mm-10 wells; Cat no: NP0321

NuPAGE 4-12% Bis-Tris 1.00mm-12 wells; Cat no: NP0322

Pre-stained molecular weight protein marker (Invitrogen): SeeBlue Plus 2 pre-stained standard (500µl LC5925)

Electrophoresis running buffer: (Invitrogen) 50ml of readymade running buffer solution was diluted in 950ml-deionized water to make a 1x solution, to be stored at room temperature.

Non-reduced Transfer Buffer: (Invitrogen) 50ml of transfer buffer (20x) was diluted with 100ml of methanol(200ml if more than 1 gel to transfer in blot cassette) and 850ml of deionised water (for 2 gels 750ml) to make a 1x working solution which was stored at room temperature.

1 X PBS (Phosphate buffered saline): 10 PBS tablets were dissolved in 1 L distilled water and stored at room temperature.

PBS-Tween:contained 0.1%vTween-20 detergent (V/V) in 1 x PBS

IBT-Tween (Blocking buffer): PBS-Tween was prepared with 1ml (0.05%) of tween detergent added and then heated to 80°C in a water bath; 2g of I-block was added (0.2% v/v) to the solution and dissolved in the heated PBS on a magnetic stirrer. Once the solution had cooled to room temperature 4g of sodium azide was added (0.02%) and the solution can then be stored for use at 4°C for up to 1 month.

Alkaline phosphatase assay (APA) buffer (Tropix Inc.)

CDP-Star development reagent (Tropix Inc.) ready to use solution, to be stored at 4°C

MESNA stripping buffer: prepared as a 1 x working solution, made up from 6.25mM Tris-HCl pH 6.8, 2% w/v SDS and 50mM 2-mercaptoethansulfonate (MESNA; Sigma). This buffer was only kept for a 2-week period and stored at 4°C.

Western Blot antibodies- all purchased from cell signalling technology.

Phospho- (Ser/Thr) PKA Substrate Antibody; Cat number: 9621

Phospho- (Ser/Thr) PKB Substrate Antibody; Cat number: 9611

Phospho- (Ser) PKC Substrate Antibody; Cat number: 2261

Phospho-S6 Ribosomal Protein (Ser235/236) Antibody; Cat number: 4856

Phospho- GSKβ (Ser9) Antibody; Cat number: 9336

Phospho- Stat6 (Tyr641) Antibody; Cat number: 9361

2.5.2 SDS-PAGE

Pre-cast gels purchased from Invitrogen (Cat no: NP0321 and NP0322) were used for all SDS-PAGE experiments in the study. Gel cassettes were first removed from a plastic pouch and rinsed with deionised water. A length of white tape covering an opening at the back of the cassette was removed and in one smooth motion the comb can be removed from the top of the gel to expose the loading wells. The wells were rinsed carefully with 1 x electrophoresis running buffer and the gel was placed into a mini cell such that the notched 'well-side' of the gel faces inwards toward the buffer core. The gels were seated on the bottom of the mini-cell and locked into place with a gel tension wedge. If only one gel was run a buffer dam was used in place of the second gel.

The gel tank was filled with running buffer which was poured into the central chamber and overfilled so that the tank was around a third full in the external buffer section.

A pre stained molecular weight protein marker was added to the first well to ascertain the molecular weight of the resolved proteins detected with western blotting. Appropriate volumes of samples were then loaded into the subsequent wells using gel-loading tips. Gels used were either 10 or 12 wells and up to 30μl of sample per well was loaded. The lid was then placed on the gel tank and whilst the power was off, the electrodes were connected to the power supply. Gels were run for 50-55 minutes at 200V (120mA, 25W) and stopped when the dye front reached the open ridge at the bottom of the gel.

Once the run was complete the lid was removed from the gel tank followed by the unlocking the gel tension wedge and removal of the gel cassette/s from the tank. After rinsing off any running buffer on the cassettes with deionised water the cassettes were laid flat on the bench and the gel was gently removed from the cassette prior to transferring the gel onto a polyvinylidene difluoride PVDF membrane for western blotting.

2.5.3 Transfer of resolved proteins onto a polyvinylidene difluoride (PVDF) membrane

Once proteins had been resolved by SDS-PAGE they were transferred onto a PVDF membrane. The PVDF membrane was first soaked in methanol to prevent unspecific binding to the membrane and then equilibrated by soaking in transfer buffer. The

polyacrylamide gels were placed on top of PVDF membrane and sandwiched between 2 pieces of Whatman 3MM filter paper ensuring no trapped air bubbles were present between the layers. The gel was then placed between 5 pre-soaked sponges in a blotting cassette. Holding the blot module together, the unit was slid into the guide rails in the lower buffer chamber and secured with the gel tension wedge. The sealed blot chamber was then filled with transfer buffer until the gel/ membrane assembly was completely submerged. Any residual air bubbles were removed at this stage by tapping the unit gently on the bench. The outer buffer chamber was filled with deionised water to dissipate heat during the transfer process. Protein transfer was carried out at 30V for 1 hour and thirty minutes. After transfer the membranes were removed from the blot module and washed three times in PBS tween for five minute time periods consecutively.

2.5.4 Immunostaining of western blotted membranes

The membrane was then blocked in 30ml I-Block Tween (IBT-Tween) in a large weighing boat for at least 1 hour at room temperature (or overnight at 4°C) before being transferred to a 50ml tube and incubated with a primary antibody at the correct dilution in IBT-Tween overnight at 4°C on a roller. The primary antibody was poured out of the tube and the blot was washed 3 times for 10 mins with PBS-Tween. This ensured that any excess or unbound antibody was removed thereby decreasing any background staining. The membrane was probed for 1 hour with an appropriate secondary antibody at room temperature followed by a further three 10-minute washes with PBS-Tween to reduce background staining. The membrane was then washed with a 1 x alkaline phosphatase (AP) buffer for 5 mins; excess AP buffer was removed from the membrane by blotting on a paper towel and was then placed on a plastic sheet. 600µl substrate (Tropix, CDP-star ready to use) was dropped onto the membrane to enable detection of protein bands probed for. The detection reagent was removed from the plastic sheet and the films are placed into a cassette case ready to be exposed to photographic film in the developing room. The membranes were initially exposed to the photographic film for 10 minutes and subsequent incubation times were determined for further exposures if required.

2.5.5 Stripping blots for repeated staining of western blotted membranes

If repeated staining was required it was possible to strip antibodies from western blots using Sodium 2-mercaptoetanesulfonate (MESNA) stripping buffer; 30ml of MESNA stripping buffer was added to the blot within a sealed polyethene bag and placed in a water bath for 30 mins at 50°C. Regular shaking of the bag was required to ensure an even spread of the buffer. After this time-point the blots were washed in 10 ml of SDS wash buffer followed by 30 minutes washing in PBST ensuring the wash was changed every 10 mins. The blots were then covered with 30ml of blocking buffer in a sealed polyethene bag and put on a rocker overnight at 4°C. After this time point blots were ready for repeated immunostaining.

2.6 Flow Cytometry

Flow cytometry was used in this project to assess CLL cell viability and the expression and phosphorylation of both intracellular and extracellular molecules of interest.

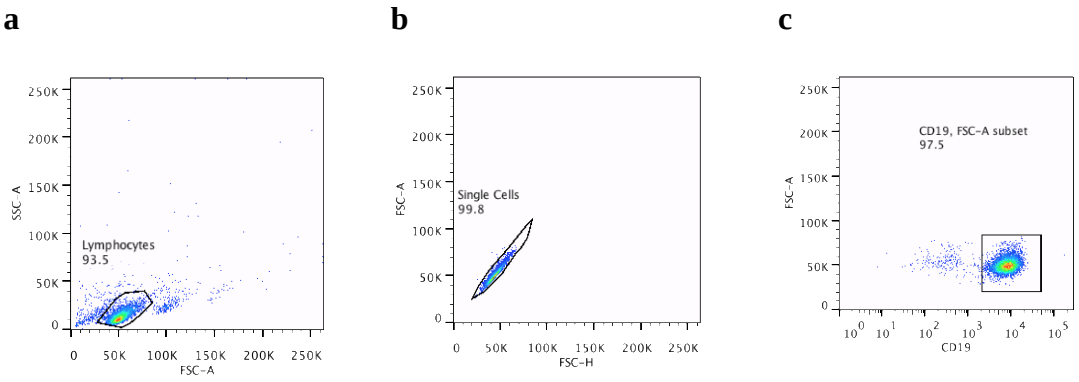


Figure 2.1 Gating strategy for the analysis of CD19$^+$ CLL lymphocytes

(a) Lymphocytes were primarily gated from forward and side scatter profiles. Using an accuri C6 flow cytometer (b) Single cells were gated from forward scatter height and area profiles (c) Within the single cell gate CD19$^+$ lymphocytes were gated to identify the CLL cell population.

2.6.1 Preparation of primary CLL cells for Flow Cytometry

Up to 750µl of CLL cell culture suspension was removed and pelleted by centrifugation for 5 mins at 1200 x g. The cell pellet was resuspended in 100µl of PBS and relevant

antibody/antibodies were added (see below for list of all antibodies used and concentrations). In all antibody panels CD19 was added to identify the B-cell population. CLL cells were incubated with appropriate antibodies for 15 mins in the dark, after this time period CLL cells were washed to remove excess antibody with 2ml of PBS, pelleted and fixed in 250µl of 1% paraformaldehyde.

2.6.2 Fixing and Permeabilising CLL cells for Intracellular staining

In order to quantitatively measure intracellular CLL proteins, flow cytometry was used. Intracellular staining of phospho-S6 (BD Phosflow; cat no: 560434), phospho-STAT6 (561203) and phospho-ERK1/2 (560115) was conducted using a fixing and permeabilisation method. To do this, cells were removed from culture and extracellular staining was carried out for CD38 and CD69 as well as CD19 to identify the B-cell population. After the appropriate staining time for the extracellular proteins the CLL cells were washed in 2ml of PBS and centrifuged at 1500 x g for 5 mins. 100µl of PBS was added as well as 50µl of fixing reagent A (caltag). Cells were put in the dark for a 10 min period and after this time cells were washed in PBS and re-suspended in 1ml of PBS as well as 50µl of Phosflow permeabilisation reagent with the appropriate intracellular antibodies. Following further 15-minute incubation in the dark samples were washed in 2 ml of PBS and resuspended in 500µl of 1% paraformaldehyde. Samples were left to fix for at least 15 minutes in the fridge before being run on the flow cytometer.

Antibody	Fluorochrome	Company	Code
CD19	APC	Invitrogen	MHCD1905
CD38	RPE	Invitrogen	MHCD3804
CD5	PerCP/Cy5.5	Biolegend	300620
CD49d	FITC	AbD Serotec	MCA2503F
CD69	PE/Cy7	Biolegend	310912
CD25	APC-H7	BD Pharmagen	560225
Phospho-S6	Alexa Fluor 488	BD Phosflow, BD	560434

		Biosciences	
Phospho-Stat6	V450	BD Phosflow, BD Biosciences	561203
Phospho-ERK1/2	PerCP/Cy5.5	BD Phosflow, BD Biosciences	560115

2.7 Viability Analysis using Propium Iodide and Annexin V

Annexin V is a calcium-dependent phospholipid binding protein. When cells undergo apoptosis phosphatidyl serine residues flip from the inside to the outside of the plasma membrane. Annexin V has a high affinity for phosphatidyl serine and can be used to assess apoptosis by flow cytometry once it is conjugated to a fluorescent marker.

2.8 Statistical Analysis

Both paired and unpaired *t*-test was used as a means of determining differences between paired samples, which were subjected to different conditions. When correlating protein expression in a cohort of patients, the Spearman's rank test was employed.GraphPad Prism 5.0 (Graphpad Software Inc.) was used to carry out all statistical analysis.

When multiple comparisons were being made the repeated measures ANOVA test was used.

3 Characterising Protein Kinase B signalling in primary CLL cells following CD31 co-culture

3.1. Introduction

CLL has previously been defined as a disease characterised by the resistance of malignant B-lymphocytes to undergo apoptosis (Sanhes et al. 2003). Whilst CLL cells *in vivo* display increased survival when compared to normal B-lymphocytes, when CLL cells are removed from the body they often undergo spontaneous apoptosis (Deaglio 2010). Co-culture systems have been shown to rescue CLL cells from spontaneous and drug-induced apoptosis *in vitro*(Burger et al. 2000; Deaglio et al. 2005; Panayiotidis et al. 1996),by providing signals which maintain CLL cell survival and, under some conditions, result in growth and proliferation (Friedberg 2011). By investigating the signalling pathways that are activated in CLL cells as a consequence of CD31 co-culture it may be possible to identify some of the signals provided by the *in vivo* microenvironment that contribute to the maintenance of CLL cells.

The expression of CD38 on the surface of primary CLL cells is associated with a progressive disease type and is an independent marker of poor clinical outcome in this disease(Ibrahim et al. 2001; Parker and Strout 2011; Pepper 2007). CD38 expression in CLL is modulated by microenvironmental factors and CD38 is not only a prognostic marker in CLL but also a functional molecule, possessing cell surface receptor as well as adhesion capabilities (Deaglio 2010). A role of CD38, in CLL, is to aid in the delivery of proliferative and migratory signals to CLL cells (Deaglio 2011). The receptor capacity of CD38 is modulated, in part, through the binding to its non-substrate ligand CD31 (Deaglio 2010) which is expressed by both CLL cells and endothelial tissues *in vivo* (Willimott et al. 2007). Interactions between CD31 and CD38 have been shown to be able to drive the activation and proliferation of distinct lymphocyte populations (Poggi et al. 2010). Independent immunohistochemical studies on lymph node sections have revealed a link between the numbers of endothelial cells which express CD31 and the level of CD38 expressed on the surface of CLL cells (Deaglio et al. 2010; Patten et al. 2008). The lymph nodes are proposed to be where the proliferative core of the diseases resides and GEP has shown that CLL cells isolated from the lymph nodes displayed activation of signalling pathways, which have the

ability to sustain CLL cell survival and proliferation (Herishanu et al. 2011). CD31/CD38 static interactions are likely to take place in lymph nodes where CLL cells have sustained contact with $CD31^+$ residential stromal cells (Deaglio 2010; Patten et al. 2008).

The deregulation of growth mechanisms in cancer cells is largely due to changes in signalling pathways, some of which are involved in cell proliferation, differentiation, growth and apoptosis (Barragan et al. 2006). Protein kinases are an essential group of enzymes, which regulate the protein activity involved in most cellular processes(Cheng et al. 2011). A valuable approach adopted when attempting to uncover specific components of signalling pathways mediated by protein kinases is the use of phospho-specific antibodies (Alessi et al. 1996b; Obata et al. 2000; Zhang et al. 2002). These antibodies allow for the detection of phosphorylated substrates of a particular kinase and in this chapter such antibodies have helped to identify targets of protein kinase B in primary CLL cells.

In order to simulate the *in vivo* growth permissive lymph node microenvironment, a murine fibroblast cell line that exogenously expresses human CD31 ligand was utilised. In experiments conducted by Deaglio *et al* in 2010, a gene expression analysis was performed following 5 days of primary CLL cell co-culture withCD31-expressing fibroblasts. It was shown that 1645 genes were modulated as a result of CD38/CD31 ligation, some of which are involved in apoptosis regulation, migration and proliferation (Deaglio 2010). This work supports the hypothesis that the maintenance and progression of CLL is modulated through accessory signals provided by the microenvironment supporting the localisation of CLL cells to growth permissive sites such as primary lymph nodes (Deaglio 2010). In contrast to these findings, work conducted by Tonino *et al*(2008) used GEP to show that no altered expression of any known regulators of apoptosis were detected following CD31/CD38 ligation (Tonino S et al. 2008). These contradictory findings call in to question the functional significance of CD38 on the surface of CLL cells.

By studying signals that CD31/CD38 interactions are capable of generating, it may be possible to predict which signalling pathways are activated *in vivo*. In this study phospho-substrate antibodies directed against the substrates of the kinases PKA, PKB and PKC were used. Individual phospho-specific antibodies and pharmacological

inhibitors were then used to study phosphorylated proteins that may contribute to cell survival in CLL.

3.2. Results

3.2.1. CD31 expression is similar in all CLL patients

CD31 is the only known ligand of CD38 but it also has the ability to bind other CD31 molecules a homotypic fashion. This has been shown to result in downstream signalling effects and cell:cell adhesion in primary CLL cells (Poggi et al. 2010).

The expression of CD31 was analysed in CLL patients to investigate whether homotypic interactions should be considered when conducting the experiments in this thesis. Primary cells from 30 CLL patients with differing levels of basal CD38 expression were analysed with anti-CD19 to identify the B-cell population as well as anti-CD38 and anti-CD31 to measure the expression of these molecules. The CD38 MFI values were plotted against the CD31 MFI values in each CLL patient.

Figure 3.1a shows that there was little variation in the CD31 MFI values in the cohort of CLL patients analysed in this experiment and hence no correlation between CD31 and CD38 expression. Figure 3.1b shows a box and whisker plot, which represents the $CD38^{lo}$ and $CD38^{hi}$ cohort of patients. The box and whisker blot shows the median CD31 MFI value for the $CD38^{hi}$ and $CD38^{lo}$ cohorts of patients, the box represents the upper and lower quartiles of the data and the upper and lower limits are represented by the outer bars (whiskers). There was no significant difference detected between the MFI of CD31 in the $CD38^{hi}$ cohort of patients or the $CD38^{lo}$ cohort of patients.

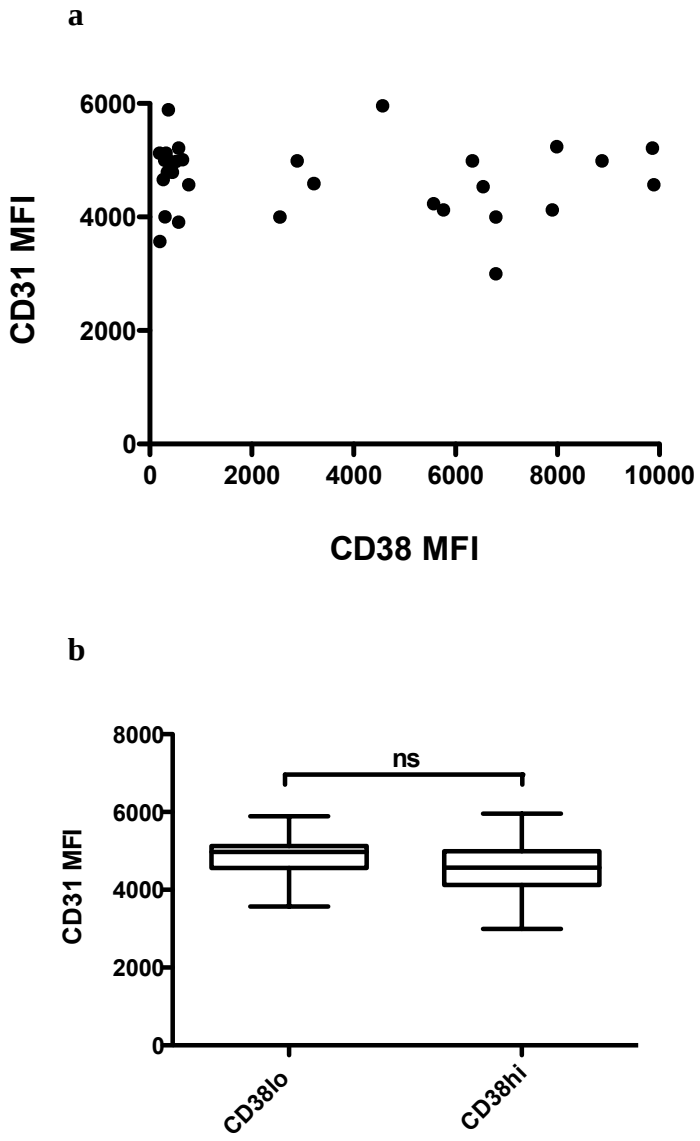


Figure 3.1 There is no significant difference in CD31 expression in CD38^{hi} and CD38^{lo} CLL patients

5 x 10^5 CLL cells were taken from freshly isolated whole blood samples. The surface expression of CD31 and CD38 was measured on CD19$^+$ CLL lymphocytes within the lymphocyte gate identified from forward and side scatter plot. The cut off for CD38^{lo} is <5% CD38 positive cells, CD38^{int} is between 20-40% and CD38^{hi} is >50%. a) The surface expression of CD38 was plotted against the surface expression of CD31, b) A paired *t*-test was used to compare CD31 expression between the CD38^{lo} and CD38^{hi} cohort of patients.

3.2.2. CD38 expression is increased on CLL cells following co-culture with CD31-expressing fibroblasts

Studies to analyse CD38 expression on the surface of CLL cells located within the lymph node and bone marrow microenvironments are limited due to the difficulties associated with obtaining CLL cells from these tissues. However in a study conducted by Jaksic *et al* in 2004 it was shown that CLL cells isolated from the lymph nodes had increased CD38 expression when compared to both the peripheral blood and bone marrow (Jaksic et al. 2004). There is now strong circumstantial evidence to suggest that the expression of CD38 is transient (Calissano et al. 2009; Damle et al. 2007),with CD38 expression being up regulated on the surface of CLL cells located within the lymph node microenvironment and subsequently lost upon re-entry into the peripheral blood. The increase in expression of CD38 on the cell surface when CLL cells enter these growth permissive microenvironments may enhance disease progression and cell survival through CD38 receptor capabilities. For the purpose of these experiments patients are deemed to be $CD38^{hi}$ if over 50% of the CLL cell population express CD38, whereas $CD38^{lo}$ patients have less than 5% of the CLL population expressing CD38.

To establish the stimulating ability of the CD31-expressing co-culture system, primary CLL cells from 20 patient samples with differing levels of basal CD38 expression were co-cultured with fibroblasts transfected with the CD31 ligand (31) as well as non-transfected fibroblasts (Non-transfected mouse L-cells: NTL) for two and five days respectively. Two fluorophore-conjugated antibodies were used, anti-CD19 to identify the B-cell population and anti-CD38 to quantify the expression of this molecule. The CD38 MFI values presented in this chapter relate to the $CD19^{+}$ lymphocyte-gated population in each sample.

Figure 3.2a shows that augmented CD38 MFI was detected following incubation of CLL cells for 2 and 5 days in CD31-expressing co-culture (P=0.0009 and P=0.0002 respectively). A significant increase in CD38MFI was observed following 5 days on CD31-expressing co-culture compared to just 2 days (P=0.0002) suggesting that there is a time-dependent component to the regulation of CD38 under these conditions (Figure 3.2b). In contrast, there was no significant increase in CD38 MFI in cells cultured with non-transfected co-culture for 2 and 5 days (P=0.47 and P=0.39 respectively).

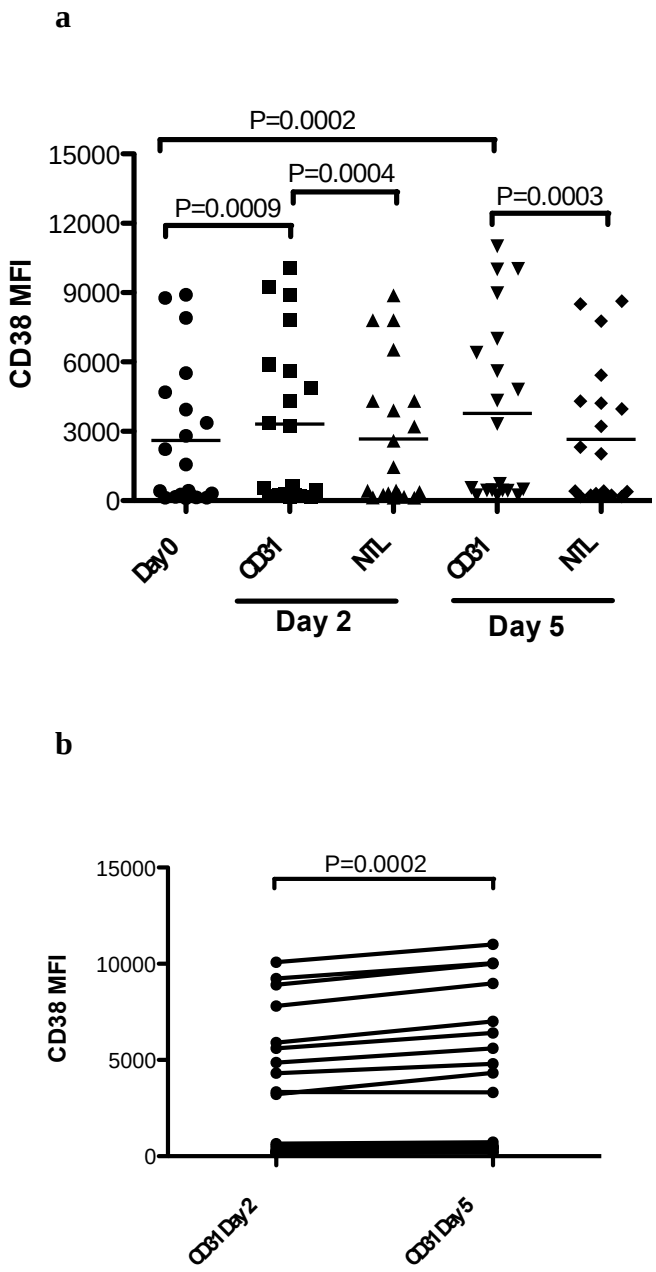


Figure 3.2 A significant increase was observed in CD38 expression following incubation with CD31-expressing fibroblasts at 2 and 5 days

1 x 10^6 CLL cells were placed into co-culture with 1.5 x10^6 NTL or CD31-expressing fibroblasts (90-95% confluent). (a) The surface expression of CD38 was measured on day 0, day 2 and day 5 and the MFI values were plotted for each patient (n=20). CD38 expression was measured on $CD19^+$ CLL lymphocytes within the lymphocyte gate identified from forward and side scatter plot. A paired *t*-test was used to compare CD38 expression. (b) The line graph shows CD38 MFI at 2 and 5 days in CD31 co-culture.

3.2.3. Protein phosphorylation changes were detected using substrate antibodies against protein kinase A, protein kinase B and protein kinase C

Having established that incubation of primary CLL cells with CD31-expressing co-culture augmented the expression of CD38 on the CLL cell surface, the next step of this project involved investigating short-term changes in protein phosphorylation induced by CD31-expressing co-culture.

A schematic of the workflow used for this experiment is shown in Figure 3.3. The first experiment was carried out using two CLL patient samples chosen to represent the extremes of CD38 expression in this disease. The first patient was strongly CD38 positive ($CD38^{hi}$) with CD38 expression on 95% of the CLL cells and the second patient was CD38 negative ($CD38^{lo}$) with only 1% of CLL cells expressing CD38. Three culture conditions were used for each patient: liquid culture (LQ), co-culture with CD31-expressing fibroblasts (31) or co-culture with a non-transfected fibroblast cell line (NTL). Three protein kinase substrate antibodies were utilised, which allowed for the detection of phosphorylated substrates of PKA, PKB and PKC, all of which have been implicated in the pathogenesis of CLL (Abrams et al. 2007; Nakagawa et al. 2006).

Figure 3.4showsthree western blots loaded with protein extracts derived from two CLL patients, one $CD38^{hi}$ and one $CD38^{lo}$. Distinctive patterns of protein phosphorylation were detected with the three protein kinase substrate antibodies. A higher number of phosphorylated substrate bands were detected following CD31-expressing co-culture compared to LQ or NTL co-culture. Given the wide range of changes seen with the different antibodies, it was decided to focus on the changes detected with just one of the antibodies. The PKB substrate antibody is probably the most widely used of the three antibodies (Alnagar et al. 2010; Kane et al. 2002; Manning et al. 2002; Obata et al. 2000)and for this reason it was decided that the PI3K/PKB signalling pathway would be the focus of this chapter.

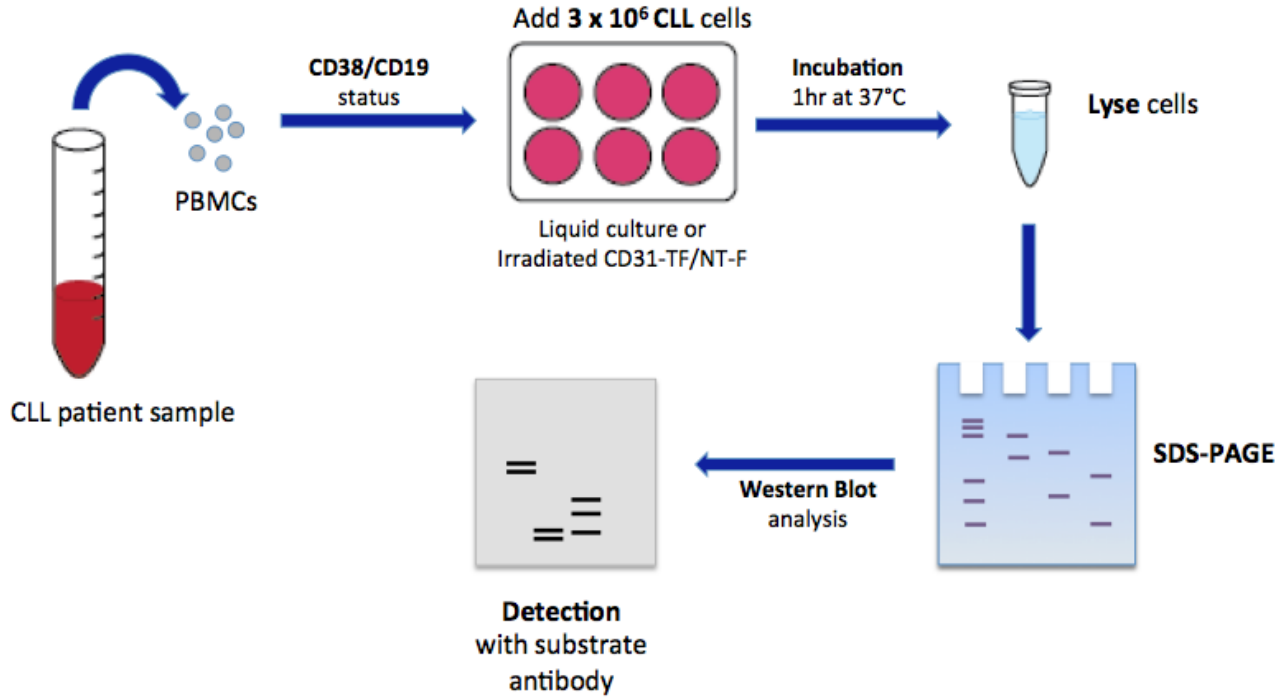


Figure 3.3 - Schematic to demonstrate workflow

PBMCs were isolated from whole blood samples, cells were counted and stained for CD19 to identify B-cell population and the CD38 status was determined within the $CD19^{+}$ population. CLL cells were incubated with CD31-expressing fibroblasts for 1 hour. Following this time CLL cells were removed from culture, lysed and proteins were resolved by SDS-PAGE followed by western blot analysis and detection with a protein kinase substrate antibody.

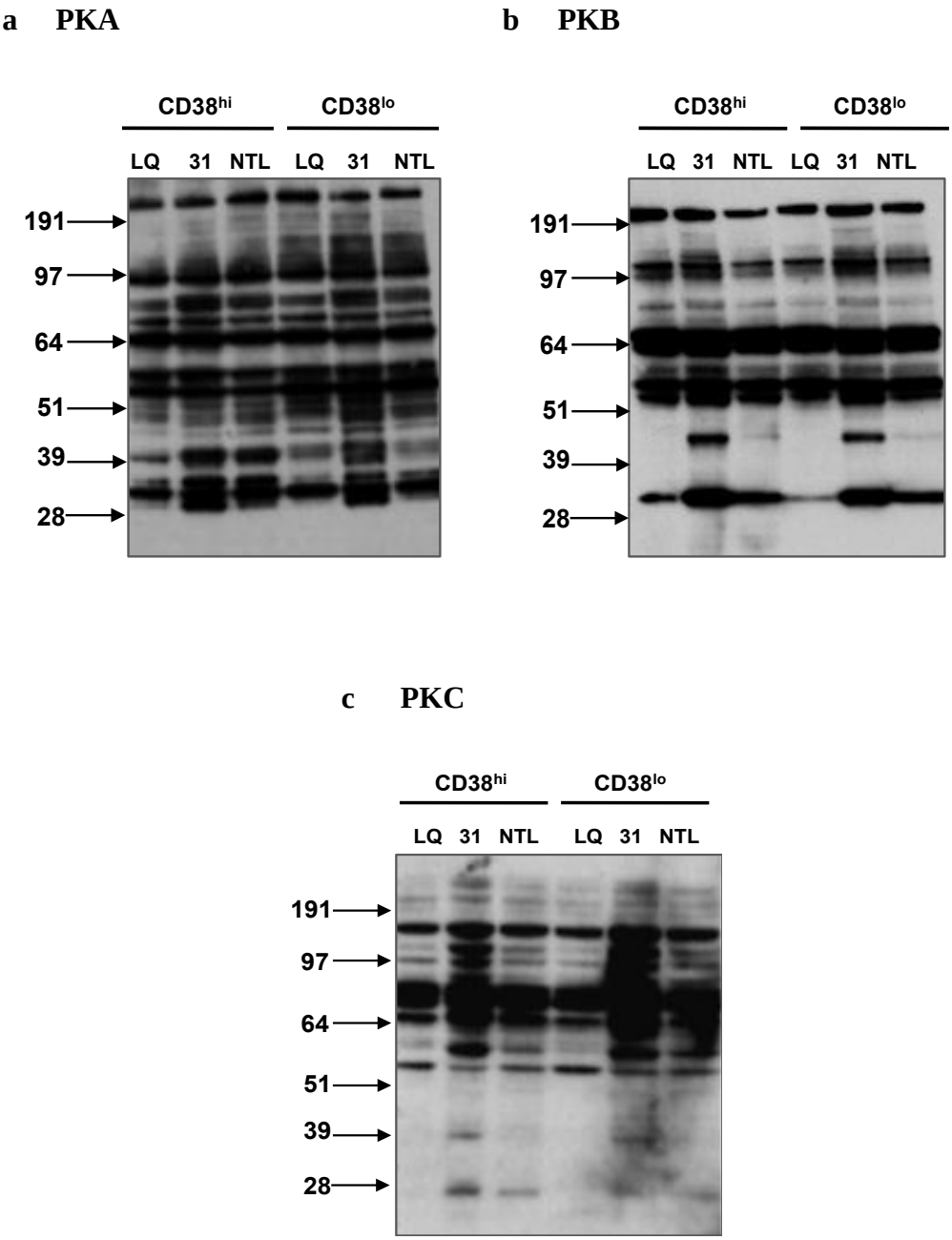


Figure 3.4 Analysis of the same 2 CLL samples with 3 different protein kinase substrate antibodies.

Primary CLL cells were separated from whole blood samples of 2 patients the first a $CD38^{hi}$ patient (CD38 expressed on 95% of CLL cells) and the second a $CD38^{lo}$ patient (CD38 expressed on 1% of CLL cells). CLL cells were incubated with CD31-expressing co-culture cells (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting. Three identical PVDF membranes were probed with different protein kinase substrate antibodies, (a) Protein kinase A, (b) Protein kinase B and (c) Protein kinase C. All antibodies were purchased from Cell Signalling Technologies (1:1000 dilution was used for all antibodies in this chapter).

3.2.4. The protein kinase B substrate antibody detects protein phosphorylation in primary CLL cells

The PKB signalling pathway has been linked to CLL cell proliferation and survival (Deaglio 2001b). The peptide motif for PKB-mediated phosphorylation has been established as Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd (Xaa represents any amino acid, Yaa and Zaa represent small residues other than glycine and Hyd signifies a large hydrophobic residue such as phenylalanine or leucine)(Alessi et al. 1996a). This motif is present in over 400 different proteins (Nicholson and Anderson 2002). Approaches have searched orientated peptide libraries and a motif-profile scoring algorithm has identified more than 14,000 targets phosphorylated by PKB in 9,500 vertebrate protein sequences (Lawlor and Alessi 2001; Obata et al. 2000; Yaffe et al. 2001). CLL cells have altered expression of regulatory molecules that modulate the PKB signalling pathway, and activated PKB regulates the function of numerous substrates involved in apoptosis including Bad, glycogen synthase kinase (GSK) and the forkhead family of transcription factors (Barragan et al. 2006). The PKB signalling pathway is essential for cell proliferation and survival (Zhou et al. 2008) and CLL clones have been shown to contain constitutively activated PKB.

To assess the phosphorylation of PKB substrates in CLL patients' further, $1x10^6$ primary CLL cells were incubated in the conditions previously described. Following incubation, CLL cells were harvested and lysed to generate protein extracts, resolved using SDS-PAGE, transferred onto a PVDF membrane by western blotting and subsequently probed with a PKB substrate antibody. The cut off positivity's for CD38 expression in this project are CD38 low <5%, CD38 intermediate is between 20-40% and CD38 high is >50%.

Figure 3.5shows three CLL patients, the first a $CD38^{lo}$ patient (14% CD38), the second a $CD38^{int}$ patient (37% CD38) and finally a $CD38^{hi}$ patient (84% CD38). Following the incubation of CLL cells with CD31-expressing co-culture, six phospho-proteins appeared to be consistently increased over a range of molecular weights (pp): pp240 pp97, pp65, pp55, pp47 and pp32 (marked with arrows in Figure 3.5). Interestingly, the intensity of some bands representing phosphorylated substrates of PKB remained unchanged following CD31-expressing co-culture, such as the pp65 band.

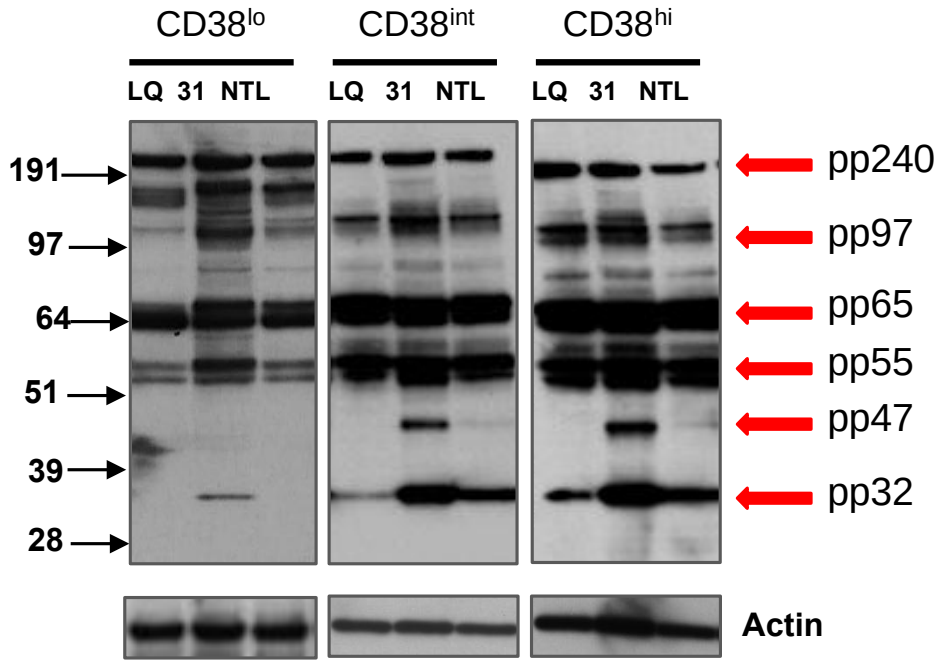


Figure 3.5 – Three representative western blot derived from CLL samples probed with the PKB substrate antibody

Primary CLL cells were separated from three 3 patient samples ($CD38^{lo}$ (14% positive), $CD38^{int}$ (37% positive), $CD38^{hi}$ (84% positive). $1x10^6$ cells were incubated with CD31-expressing co-culture cells (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour in a 6-well plate. Following co-culture the primary CLL cells were removed from culture and cell lysates generated. Protein was extracted, resolved by SDS-PAGE, followed by Western Blotting and detection with an antibody that detects phospho-motifs generated by the kinase PKB. These blots are representative of a further six blots.

3.2.5. Ribosomal protein S6 and glycogen synthase kinase 3 beta are phosphorylated in CLL cells following CD31-expressing co-culture

The next aim was to try and establish the identity of some of the phosphorylated proteins identified with the PKB substrate antibody consistently increased under CD31 co-culture conditions, highlighted by the red arrows in Figure 3.5 (pp240 pp97, pp55, pp47 and pp35). Barragan *et al* have previously used the same PKB substrate antibody to analyse PKB target proteins and found it a useful method to screen for targets of this signalling pathway in CLL (Barragan et al. 2006). In the first instance, the 35kDa protein (pp35) and the 47kDa protein (pp47) detected with the PKB substrate antibody were analysed.

Freshly isolated CLL cells were incubated under three conditions: LQ, 31 and NTL. After 1 hour, CLL cells were removed from the culture conditions and identical samples were resolved by SDS-PAGE on parallel gels. After western blotting, one of the PVDF membranes was probed with a PKB substrate antibody whilst the other was probed with antibodies specific to phospho-S6 and phospho-GSK3β. Phospho-S6 and phospho-GSK3β were considered as potential candidates for the pp35 and pp47 bands identified with the PKB substrate antibody due to comparable molecular weights (Kane et al. 2002). This method allowed for direct comparison of both the size of the band detected and the phosphorylation state of the protein.

Figure 3.6 shows the band detected with the phospho-specific S6 antibody was a comparable size and followed a similar phosphorylation pattern to the pp35 band detected using the PKB substrate antibody. Furthermore the band detected by the phospho-GSK3β antibody was a comparable size and followed a similar phosphorylation pattern to the pp47 band detected by the PKB substrate antibody, thus providing circumstantial evidence for the identity of these phospho protein bands.

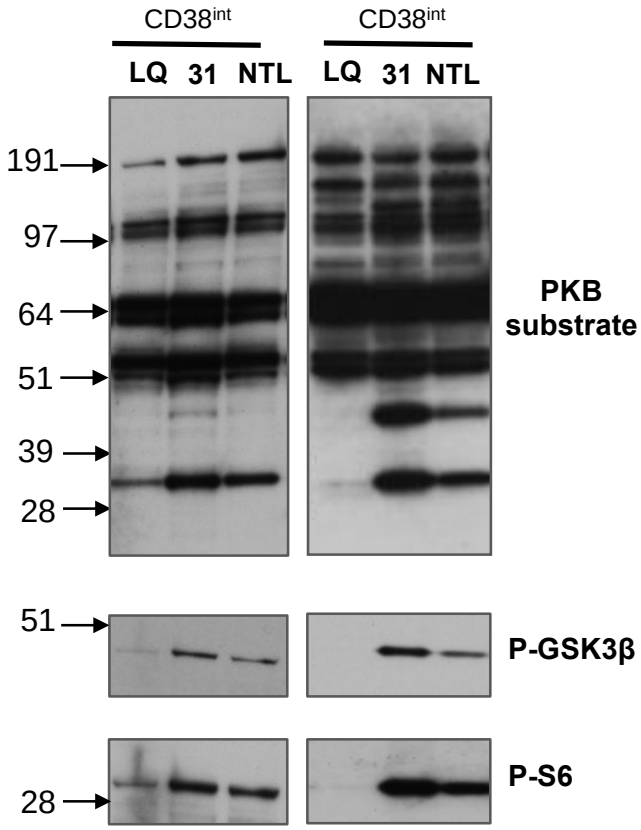


Figure 3.6 Analysis of a CLL sample with a PKB substrate antibody a phospho-S6 antibody and a phospho-GSK3β

Primary CLL cells were separated from a patient sample. CLL cells were incubated with CD31 expressing co-culture cells (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detection with 2 antibodies, the first a PKB substrate antibody that detects phospho motifs generated by the kinase PKB. The second an antibody specific to phospho-S6 ordered from Cell Signalling Technologies.

3.2.6. CD38 positive patients display increased basal S6 phosphorylation

The phosphorylation of ribosomal S6 is associated with protein translation and cell growth (Ibrahim et al. 2003). In a study conducted by Blix *et al* in 2012, different CLL cell stimuli, including CD40 ligation and BCR engagement, were used to assess the inducible and basal phosphorylation of S6 in CLL patients (Blix et al. 2012). Blix *et al* were able to identify variable levels of p-S6 in both unstimulated and CD40L activated CLL cells (Blix et al. 2012), however they did not study whether augmented S6 phosphorylation correlated with CD38 expression.

Therefore, the next step of this study was to investigate whether short-term CD31-expressing co-culture augmented the phosphorylation of ribosomal S6in multiple CLL patients; an additional aim was to determine whether CD38 expression on the surface of CLL cells had an effect on the basal and inducible levels of p-S6. To test the hypothesis that CD38 expression correlates with increased phosphorylation of ribosomal S6, p-S6 levels were measured in $CD38^{hi}$ and $CD38^{lo}$ CLL samples. Primary CLL cells from 6 patient samples (3 $CD38^{hi}$ and 3 $CD38^{lo}$) were incubated for 1 hour in LQ, 31 or NTL. Cell extracts were generated and one $CD38^{hi}$the other $CD38^{lo}$ per gel were resolved by SDS-PAGE followed by western blotting and detection with a phospho-S6 antibody.

Figure 3.7 show that basal p-S6 detected in LQ conditions was elevated in $CD38^{hi}$patients (Patient 1, Patient 3 and Patient 5) compared to $CD38^{lo}$ patients. Furthermore, p-S6 was elevated following 1 hour incubation with CD31-expressing co-culture (31) in all six patients, irrespective of the CD38 status, compared to basal levels of p-S6. Patients 1, 2, 3, 4 and 5 showed comparable levels of p-S6 following CD31-expressing co-culture. However patient 6 show that much lower levels of basal and inducible p-S6 were detected. Interestingly, p-S6 was also augmented following co-culture with non-transfected cells (NTL) cells compared to LQ. In four of the six samples (patients 3.4,5 and 6), cells expressing CD31 caused a higher increase in p-S6 compared to NTL.

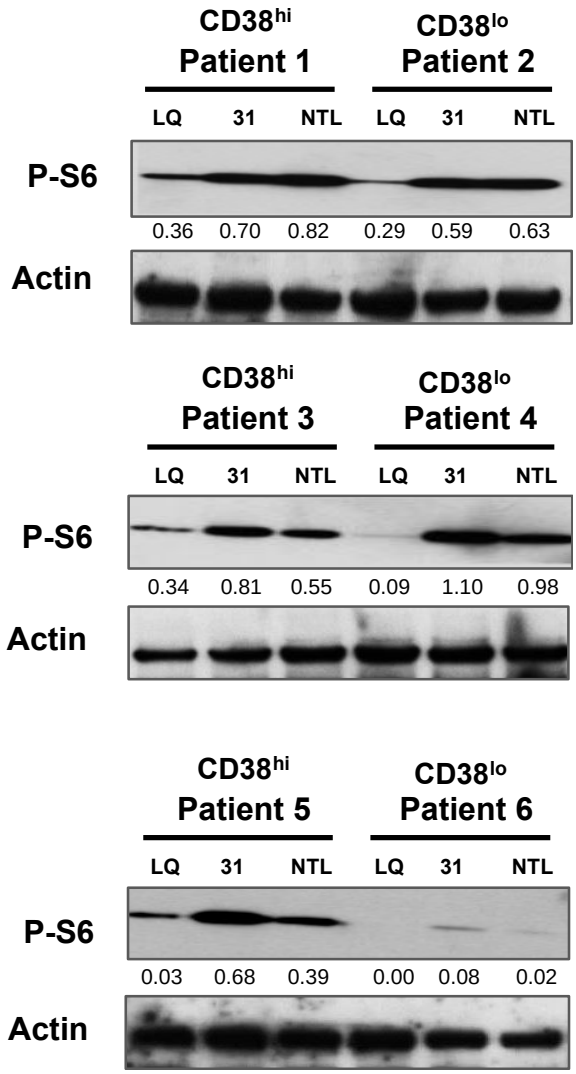


Figure 3.7 Analysis of 6 CLL samples with a phospho-S6 antibody

Primary CLL cells were separated from 6 different patients: 3 $CD38^{hi}$ and 3 $CD38^{lo}$. CLL cells were incubated with CD31 expressing co-culture cells (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detected with a phospho-S6 antibody and an actin antibody. The band intensity was normalised to Actin.

3.2.7. CD38 positive patients display increased GSK3β phosphorylation

The activation of CLL cells was previously assessed in CLL patients using the PKB substrate antibody in a study carried out by Barragan *et al* in 2006. These authors used PMA (phorbol 12-myristate 13-acetate) to stimulate CLL cells; the addition of PMA induced phosphorylation of PKB substrates at ~30, 47, 66, 80, 95 and 120kDa in CLL patients. Using a specific antibody directed against known PKB substrates they confirmed that the 47kDa band was GSK3β (Barragan et al. 2006).

The following experiments assessed whether CD31-expressing co-culture could induce GSK3β phosphorylation and whether CD38 expression increased the degree of phosphorylation in both the basal and post co-culture settings. As for the p-S6 assay, the same six patient samples were incubated for one hour in LQ, 31 or NTL co-culture. Cell extracts were generated, and one $CD38^{hi}$ and one $CD38^{lo}$ per gel were resolved by SDS-PAGE followed by western blotting and detection with a phospho-GSK3β antibody.

Figure 3.8 shows that in general, a higher basal level of phospho-GSK3β was detected in $CD38^{hi}$ samples (Patients 1, 3 and 5) compared to $CD38^{lo}$ samples (Patients 2, 4 and 6). In five out of the six samples, phospho-GSK3β was augmented following co-culture of CLL cells with CD31-expressing co-culture compared to LQ. As with p-S6, incubation of CLL cells with NTL caused an increase in phospho-GSK3β over that detected in cells maintained in liquid culture (LQ).

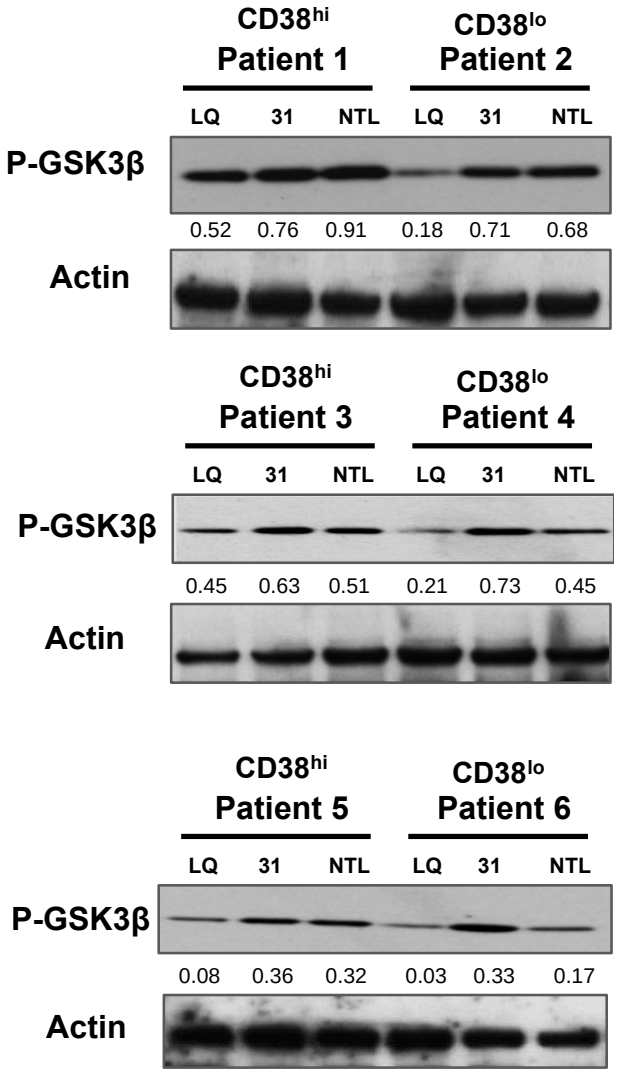


Figure 3.8 Analysis of CD38 positive and CD38 negative CLL samples with a phospho-GSK3β antibody.

Primary CLL cells were separated from patient whole blood samples. CLL cells were incubated with CD31 expressing co-culture cells (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, followed by detection with phospho-GSK3β. The band intensity was normalised to Actin.

3.2.8. LY294002 and Rapamycin can inhibit phosphorylation of ribosomal S6

The development of pharmacological inhibitors directed towards downstream targets of the PI3K signalling, as well as PI3K directly, has contributed to a better understanding of the biological role of PI3K isoforms and their substrate proteins(Arcaro and Guerreiro 2007; Banham-Hall et al. 2012; Neri et al. 2003). For example, Neri *et al* showed that increased PI3K/PKB signalling is a crucial mediator of drug resistance in a leukaemic cell line (Neri et al. 2003). Therefore, establishing the ability of pharmacological inhibitors to target the PI3K/PKB signalling pathway may provide a rationale for blocking PI3K signalling in order to increase sensitivity of CLL cells to chemotherapeutic drugs. Initially four inhibitors were used, to assess their ability to prevent the phosphorylation of the two PKB substrates identified as phospho-S6 and phospho-GSK3β on and off the CD31-expressing co-culture system.

LY294002 is a synthetic analogue of the naturally occurring bioflavonoid, Quercetin. LY294002 acts reversibly as an ATP-competitive inhibitor (Vlahos et al. 1994). This inhibitor is very stable in solution and was designed to target PI3K (Vlahos et al. 1994). The mammalian target of Rapamycin (mTOR) protein is activated in response to PI3K signalling. The mTOR protein activates the downstream S6 kinase (S6K) which in turn phosphorylates the ribosomal protein S6 (Ruggero and Pandolfi 2003). Rapamycin is an inhibitor of the mammalian target of Rapamycin (mTOR) protein (Aleskog et al. 2008). In addition, two inhibitors of NF-κB were also used to assess whether inhibition of this pathway would have any impact on S6 and GSK3β phosphorylation, namely BAY 11-7082 and LC-1. BAY 11-7082 is a well-characterised inhibitor of the IκB kinase complex; IKK (Pierce et al. 1997) shown to have the ability to induce CLL cell apoptosis whilst having a low toxicity to normal B-cells (Pierce et al. 1997). LC-1 is a novel NF-κB inhibitor that is effective in primary CLL cells; LC-1-induced cell death is associated with Caspase-3 activation mediated via the activation of both caspase-8 and caspase-9 (Hewamana et al. 2008). Primary CLL cells were pre-treated for 30 minutes with appropriate concentrations of the four pharmacological inhibitors to completely inhibit the phosphorylation of the target proteins before being added to LQ or 31 co-cultures for 1 hour.

Figure 3.9 shows 2 blots, the first represents a $CD38^{hi}$ patient (90% CD38 expression)(Figure 3.9a) and a $CD38^{lo}$ patient (4.7% CD38 positive) (Figure 3.9b). From Figure 3.9a it is evident that low levels of basal phospho-S6 and phospho-GSK3β

were detected in the LQ lane. Following 1-hour incubation with CD31-expressingco-culture augmented p-S6 and p-GSK3β bands were detected. In contrast, p-S6 could not be detected following treatment with LY294002 or Rapamycin in LQ or CD31-expressing co-cultures (lanes 3-6). However, treatment with BAY 11-7082 and LC-1 did not modulate the phosphorylation of S6 or GSK3β demonstrating that these proteins are not targets of these pharmacological inhibitors. In the $CD38^{lo}$ sample, (Figure 3.9b) low basal phosphorylation of GSK3β was detected in LQ but p-GSK3β was augmented following CD31-expressing co-culture. There was no basal p-S6 detected in this sample and induction of p-S6 was not visible after 1 hour on CD31-expressing co-culture. The complete inhibition of p-GSK3β was not detected following treatment with any of the four pharmacological inhibitors. However, there was a modest decrease in the levels of p-GSK3β in the LQ conditions in the presence of LY294002 and Rapamycin. For this reason, further experiments were conducted with LY294002 and Rapamycin to further establish the efficacy of these pharmacological inhibitors in the context of inhibiting activation and phosphorylation of targets of PKB signalling using p-GSK3β and p-S6 as readouts. The inhibitors used showed no effect on the viability of murine cell lines.

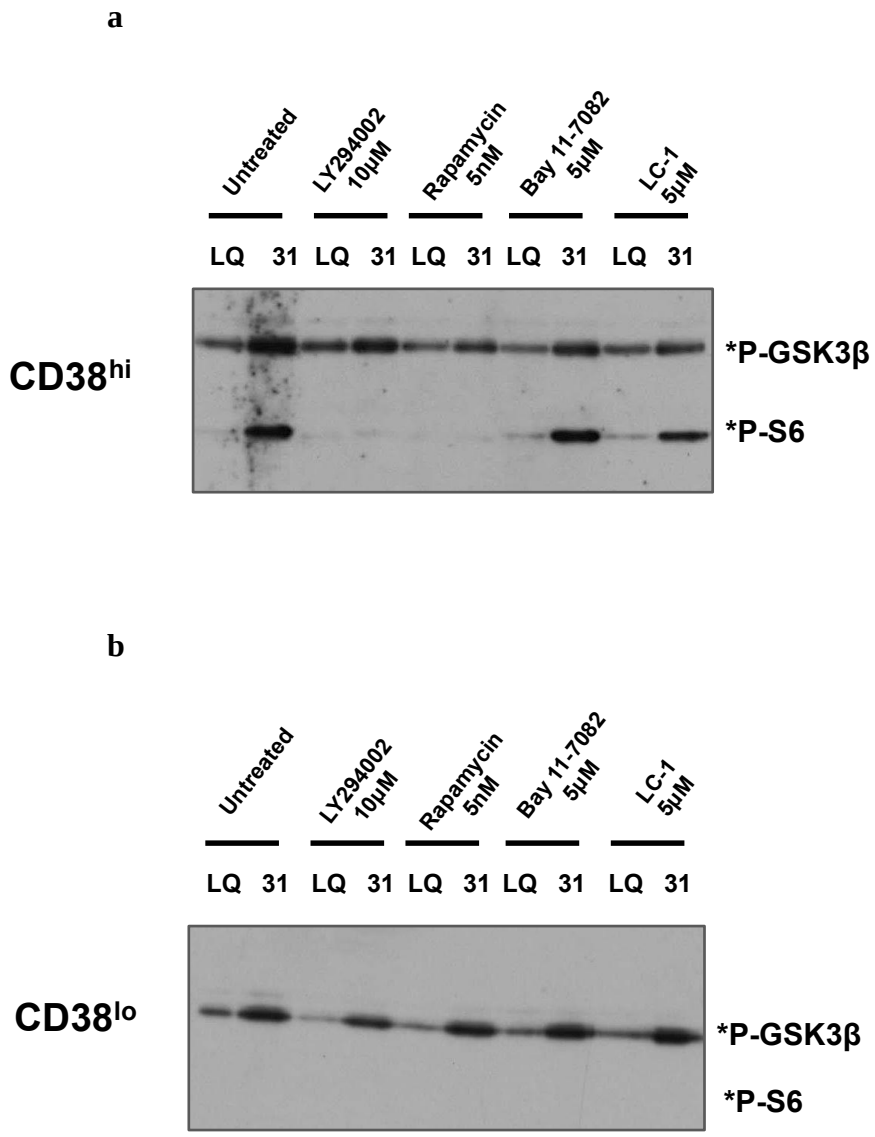


Figure 3.9 Analysis of 2 CLL patients following treatment with four inhibitors LY294002, Rapamycin, Bay 11-7082 and LC-1 with a phospho-PKB substrate antibody.

Primary CLL cells were separated from two patient samples. $1x10^6$ CLL cells were incubated with 4 different inhibitors for 30 minutes before being added to co-culture with CD31-expressing co-culture cells (31) or maintained in liquid culture (LQ) for 1 hour. The CLL cells were harvested from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with phospho-specific antibodies for GSK3β and S6. (a) Represents a $CD38^{hi}$ patient with 90% CD38 expression, (b) represents a $CD38^{lo}$ patient with 4.7% CD38 expression.

3.2.9. Rapamycin inhibits p-S6 in CD38^{hi}and CD38^{lo} patient samples

A study by Aleskog *et al* showed that Rapamycin exhibited anti-cancer capabilities, particularly in haematological malignancies. Aleskog studied a cohort of CLL patients and demonstrated that CLL patients displayed a heterogeneous response to treatment with Rapamycin, this drug was shown to have reduced efficacy in CLL patient samples with poor prognostic markers (Aleskog et al. 2008).

To elucidate the effects of Rapamycin on more phospho-proteins, the PKB phospho-substrate antibody was used in two CLL samples. Figure 3.11 shows two patient samples, the first a CD38^{hi} patient (89% positive) as well as a CD38^{lo} patient (2% positive). CLL cells were pre-treated with 5nM Rapamycin in a total volume of 2ml of supplemented media for 30 minutes at 37^{o}C before being transferred into co-culture with CD31-expressing fibroblasts (31) or liquid culture only (LQ) for 1 hour. Untreated cells were also subjected to the same culture conditions. CLL cells were harvested, lysed and protein was extracted from CLL cells, proteins were resolved by SDS-PAGE followed by detection with a PKB substrate antibody, and separately with phospho-specific antibodies to detect p-S6 and p-GSK3β.

Figure 3.10 shows the effect of Rapamycin on PKB substrate phosphorylation and specifically the effect of this inhibitor on the targeting of p-S6 and p-GSK3β. Rapamycin caused the loss of specific bands detected by the PKB substrate antibody and not a general loss of protein phosphorylation. Phospho protein bands of high molecular weight did not show a detectable loss of phosphorylation. The most dramatic effect was on pp32, which corresponds to phospho-S6. This complete inhibition of phospho-S6 was detected with the specific antibody and was observed in the CD38^{hi} sample; no basal p-S6 was detected in the CD38^{lo}patient so the effects of the inhibitor could not be ascertained. In contrast, p-GSK3β was completely inhibited in the CD38^{lo}patient sample on CD31-expressing co-culture but p-GSK3βremained detectable in the CD38^{hi}patient sample under the same conditions.

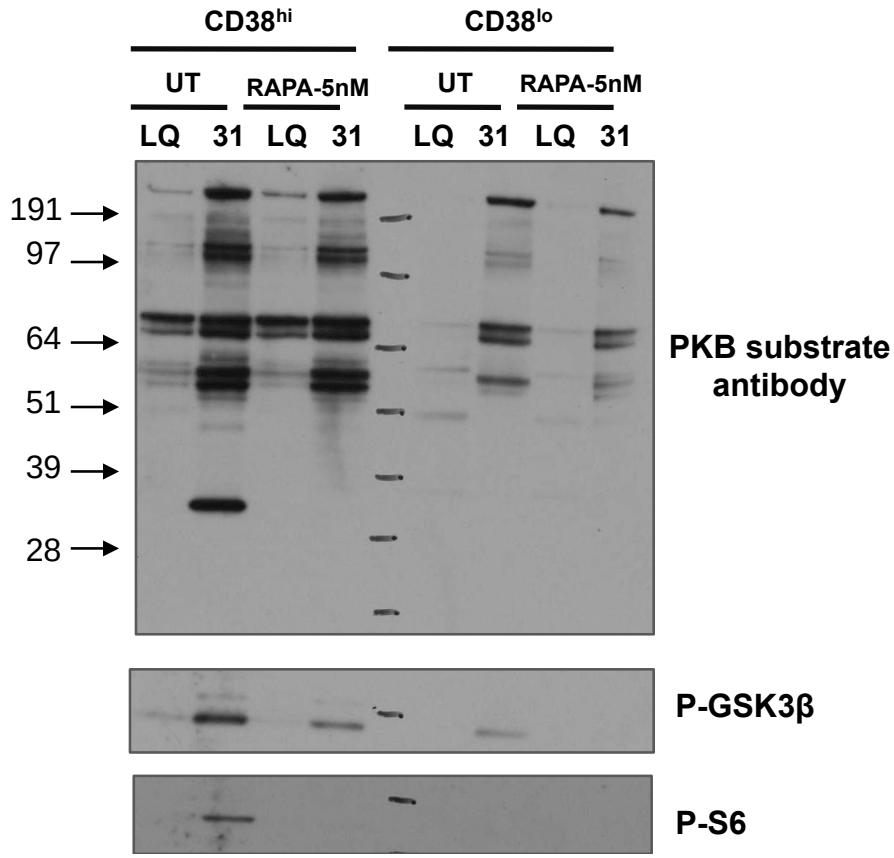


Figure 3.10 Analysis of PKB substrate phosphorylation patterns in 2 CLL samples following treatment with Rapamycin.

Primary CLL cells were separated from two patients, the first CD38 positive with CD38 expressed on 89% of CLL cells and the second CD38 negative with CD38 expressed on 2% of the CLL cells. CLL cells were left untreated or were treated with Rapamycin at a concentration of 5nM for 30 minutes at 37°C before being added to culture with CD31-expressing co-culture cells (31) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with an antibody that detects phospho motifs generated by the kinase PKB, as well as phospho-specific antibodies for S6 and GSK3β.

3.2.10. LY294002 can inhibit p-S6 in CD38^{hi}and CD38^{lo}patient samples

A previous study showed that CLL patients were sensitive to treatment with the PI3K inhibitor LY294002. Increased levels of apoptosis were observed following treatment with this pharmacological inhibitor but western blot analysis revealed that LY294002 was not able to inhibit the phosphorylation of PKB (Plate 2004). Another study conducted by Ringshausen *et al* in 2002 also showed increased levels of apoptosis following LY294002 treatment in 24/24 patients analysed. In contrast, increased levels of apoptosis were not observed when normal B-cells were treated with this inhibitor (Ringshausen et al. 2002)

The next step was to establish whether CD38 positivity increased the sensitivity of CLL cells to PI3K inhibition using LY294002. Figure 3.12 depicts two patient samples, a CD38^{hi} (89% positive) and a CD38^{lo} (2% positive). CLL cells were pre-treated with 10μM LY294002 in a total volume of 2mlsupplemented media for 30 mins at 37^{o}C before being transferred into co-culture with CD31-expressing fibroblasts (31) or in liquid culture only (LQ) for 1 hour. Untreated CLL cells cultured under the same conditions were used as controls. CLL cells were harvested, lysed and protein was extracted from CLL cells, proteins were resolved by SDS-PAGE followed by detection with a PKB substrate antibody, and separately with phospho-specific antibodies for the substrates S6 and GSK3β.

Figure 3.11 shows that the untreated CD38^{hi}patient cells showed increased basal levels of PKB substrate phosphorylation (in the LQ lane) when compared with the CD38^{lo} CLL sample. In addition, following 1-hour of CD31 co-culture (31) both the untreated patient samples showed increased substrate phosphorylation consistent with that observed in the previous experiments (Figure 3.10). The LY294002 treated cells showed a significant decrease in PKB substrate phosphorylation over a range of molecular weights specifically in the LQ treated lane compared to the untreated LQ controls for both samples. The phosphorylation status of known PKB substrates p-S6 and p-GSK3β were assessed using the phospho-specific antibodies for these proteins. Following LY294002 treatment no p-S6 could be detected in either sample. The phosphorylation of GSK3β was decreased but remained detectable in both patient samples.

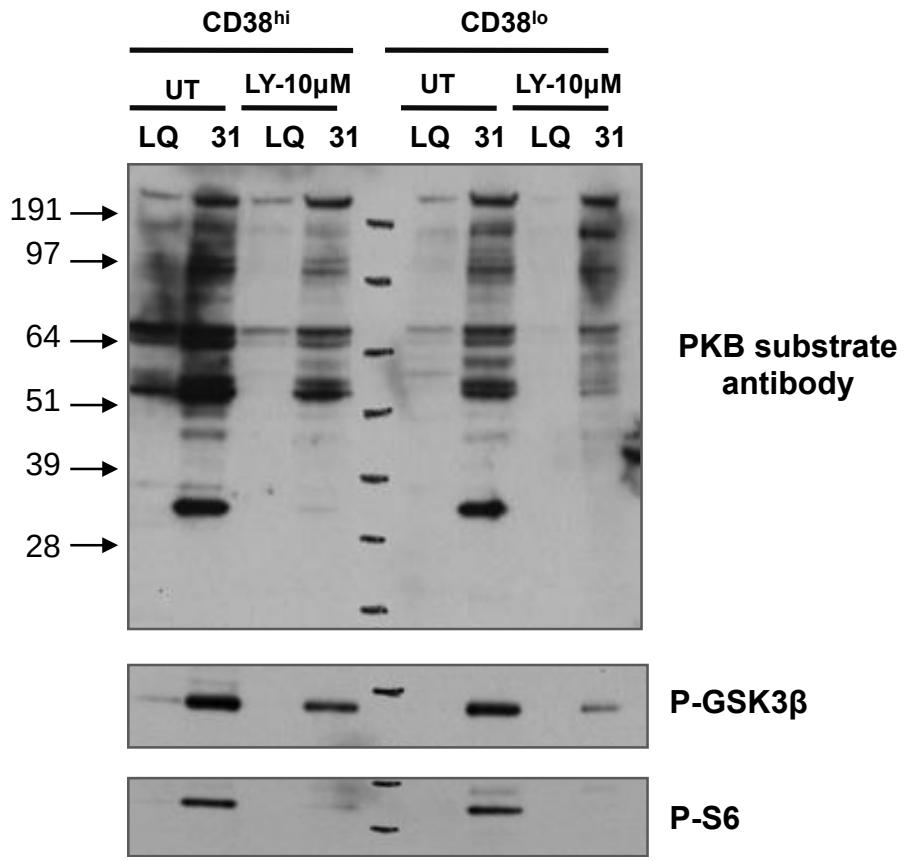


Figure 3.11 Analysis of two CLL samples following treatment with LY294002 with a phospho-PKB substrate antibody.

Primary CLL cells were separated from two patients, the first CD38 positive with CD38 expressed on 89% of CLL cells and the second CD38 negative with CD38 expressed on 2% of the CLL cells. CLL cells were left untreated or were treated with LY294002 at a concentration of 10μM for 30 mins at 37°C before being added to culture with CD31 expressing co-culture cells (31) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with an antibody that detects phospho motifs generated by the kinase PKB, as well as phospho-specific antibodies for S6 and GSK3β.

3.2.11. LY294002 and Rapamycin cause a sustained loss of PKB substrate phosphorylation

In breast cancer cells a loss of the cyclin-dependent kinase inhibitor p27 is a marker of poor prognosis, p27 is a direct target of mTOR signalling. Increased mTOR signalling results in the down-regulation of p27 in breast cancer cells. Treatment with Rapamycin has been shown to result in the stabilisation of p27 in a time-dependent manner (Shapira et al. 2006). Furthermore, treatment of cervical cancer cell lines with the PI3K inhibitor LY294002 resulted in elevated levels of apoptosis as well as altered gene expression. This was also shown to occur in a time-dependent manner, indicating that length of exposure to this inhibitor increases efficacy (Lee et al. 2006). It has also been shown that the combination of LY294002 and Rapamycin results in co-operative inhibition of T-cell proliferation (Breslin et al. 2005).

To investigate the effects of a longer exposure to pharmacological inhibitors Rapamycin (5nM) or LY294002 (10μM), CLL cells from 4 CLL patients were pre-treated with each inhibitor for 30 minutes and then incubated in LQ or withCD31-expressing fibroblasts for 3 timepoints (1 hour, 4 hours and 24 hours). Cells were subsequently harvested prior to separation, western blotting and detection with specific antibodies.

Figure 3.12 shows that a 1-hour incubation of primary CLL cells withLY294002 caused a slight decrease in substrate phosphorylation over a range of molecular weights, when added to LQ. The pp35 (phospho-S6) band was detected following1-hour incubation in CD31-expressing co-culture. There was no detectable effect of LY294002 at this time point. After 4 hours incubation with the LY294002 inhibitor a distinct reduction in pp35was observed in CD31-expressing co-culture and at 24hours incubation with this inhibitor the band was barely visible in the co-culture condition. It indicates that extended exposure of the LY294002 inhibitor resulted in a more complete inhibition of the phosphorylation of S6. Other PKB substrates also showed reduced levels of phosphorylation in the LQ lanes in a time-dependent manner following treatment but this may be due to a more general lack of CLL stimulation.

Figure 3.13 shows a similar time course inhibitor experiment with Rapamycin. The pp35 band (p-S6) was detected following CD31-expressing co-culture with the PKB substrate antibody. However, the pp47 band (p-GSK3β) was undetectable in this blot. Following just 1 hour of treatment with Rapamycin complete inhibition of p-S6 was detected in cells co-cultured on CD31-expressing fibroblasts. This inhibition of S6

phosphorylation was sustained throughout the twenty hours tested in the experiment. Other PKB substrates showed a time-dependent reduction in phosphorylation particularly in the LQ lanes.

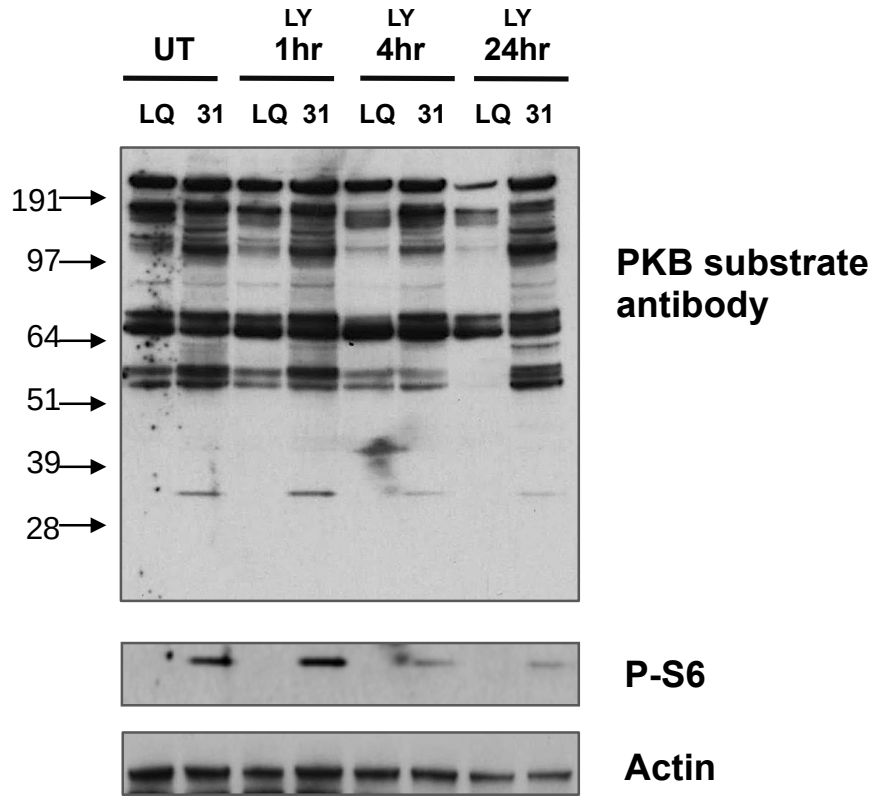


Figure 3.12 Analysis of a CLL sample following treatment with LY294002 at three time-points (1hr, 4hr, 24hr) with a phospho-PKB substrate antibody and phospho-S6 antibody.

Primary CLL cells were separated from a patient sample. CLL cells were left untreated or treated with LY294002 at a concentration of 10μm for 30 minutes at 37°C before being added to culture with CD31 expressing co-culture cells (31) or left in liquid culture (LQ) for 1 hour, 4hours and 24 hours. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with an antibody that detects phospho motifs generated by the kinase PKB, as well as phospho-specific antibodies for S6.

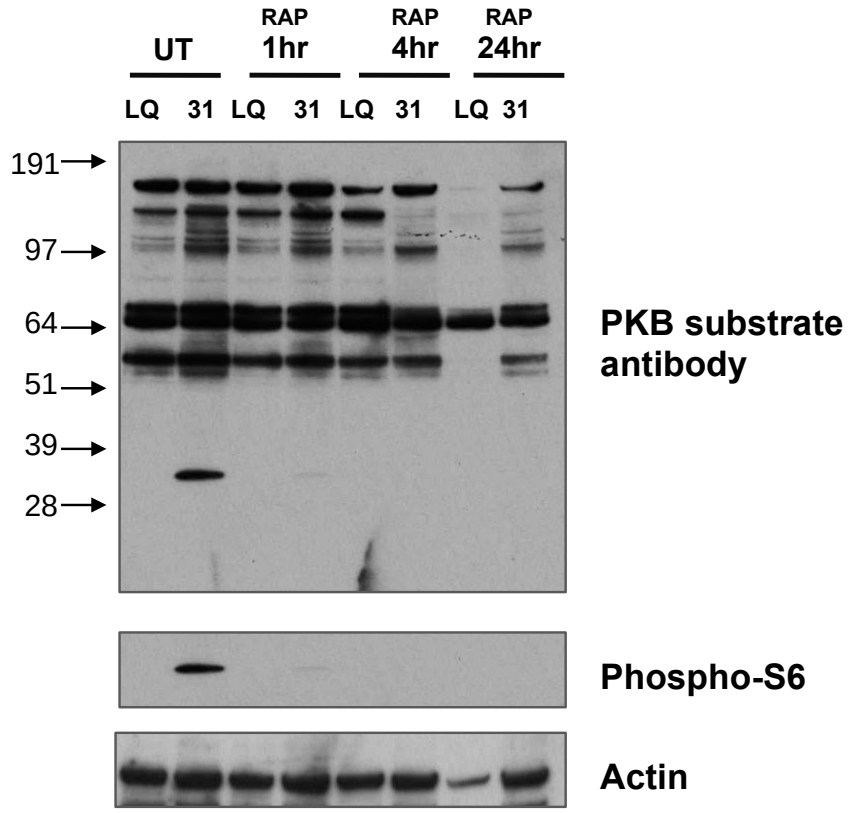


Figure 3.13 Analysis of a CLL sample following treatment with Rapamycin at three time-points (1hr, 4hr, 24hr) with a phospho-PKB substrate antibody and phospho-S6 antibody.

Primary CLL cells were separated from a patient sample. CLL cells were left untreated or treated with Rapamycin at a concentration of 5nm for 30 minutes at 37°C before being added to culture with CD31 expressing co-culture cells (31) or left in liquid culture (LQ) for 1 hour, 4hours and 24 hours. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with an antibody that detects phospho motifs generated by the kinase PKB, as well as phospho-specific antibodies for S6.

3.2.12. CD38^{hi} patient samples maintain phosphorylation of PKB substrates on CD31 co-culture over a 48 hour time period

To establish whether PKB substrate phosphorylation was sustained following longer exposure to CD31-expressing fibroblasts, 4 time points were assessed over a 48-hour time period.

Briefly, primary CLL cells were incubated in LQ alone or with CD31-expressing fibroblasts (31) for 1 hour, 4 hours, 24 hours and 48 hours. CLL cells were subsequently harvested, lysed and protein extracts were generated and resolved by SDS-PAGE. Proteins were transferred onto a PVDF membrane, and probed with a PKB substrate antibody.

Figure 3.14a shows the time course experiment with a CD38^{lo} patient sample. Following short-term culture, no basal phosphorylation of either p-S6 or p-GSK3β was detected even following 1 hour on CD31 co-culture. However, after 48 hours on the CD31 co-culture system phosphorylation of S6 protein was observed. Furthermore, the phosphorylation of other PKB substrates was also maintained over the 48 hour time period when co-cultured with CD31-expressing fibroblasts. In contrast, after 24 hours in LQ conditions the phosphorylation of the PKB substrates was dramatically reduced and was diminished further in LQ after 48 hours.

Figure 3.14b shows the same time course experiment with a CD38^{hi} patient sample. Unlike the CD38^{lo} sample, this patient displayed basal phosphorylation of S6 and GSK3β. P-S6 was maintained over the 48-hour time period in CD31-expressing co-culture. In contrast, basal p-S6 detected in the LQ lane was rapidly lost; after 4 hours LQ levels of p-S6 were prominently diminished and at 24 hours and 48 hours p-S6 could not be detected in the LQ condition. The levels of phosphorylation of other PKB substrates were reduced in a time dependent manner particularly in LQ. The doublet bands detected at ~64kDa showed uniform phosphorylation across all time-points in both LQ and CD31-expressing co-culture suggesting that these particular PKB substrates are not affected by *in vitro* stimulation or time out of the *in vivo* environment.

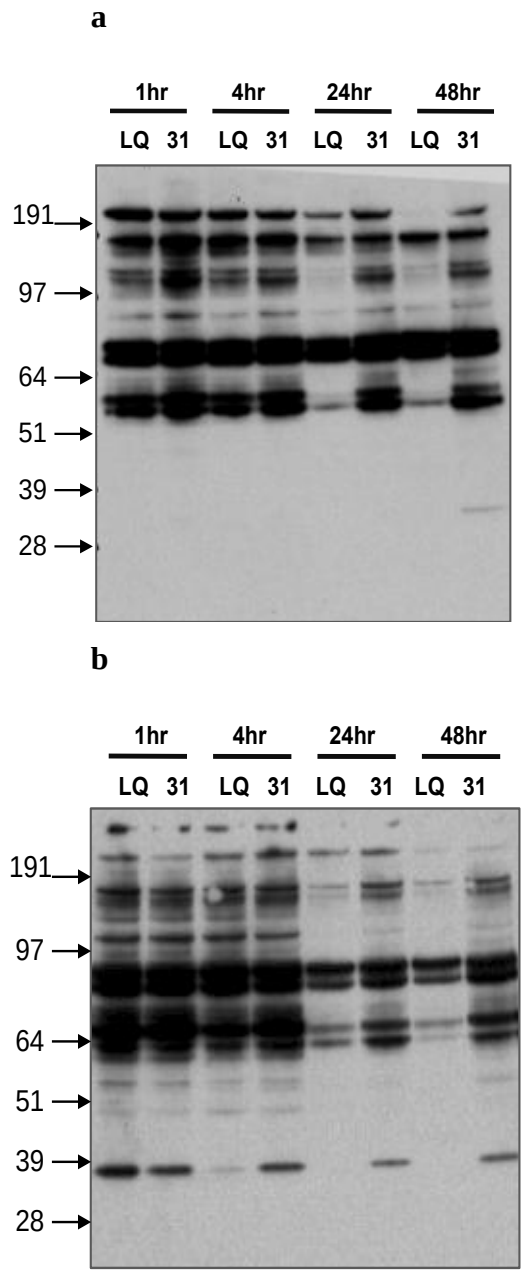


Figure 3.14 Analysis of a CD38^{lo} and a CD38^{hi} CLL sample with a phospho-PKB substrate antibody over 48 hours.

Primary CLL cells were isolated from a CD38 negative (>5%) patient sample. CLL cells were incubated with CD31 expressing co-culture cells (31) or left in liquid culture (LQ) for 1 hour, 4 hour, 24 hour and 48-hour periods. The CLL cells were removed from culture and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting and detection with an antibody that detects phospho motifs generated by the kinase PKB. a) Represents a CD38^{lo} patient 2.3% CD38, b) represents a CD38^{hi} CLL patient 92%

3.3. Discussion

CLL cells thrive *in vivo* but often undergo spontaneous apoptosis *in vitro* under conditions which are capable of supporting the growth of normal B-cells (Burger et al. 1999). Emerging evidence indicates that the survival of CLL cells in the body is largely determined by microenvironmental influences such as antigen stimulation and support from residential stromal cells (Hofbauer et al. 2010). In order to develop a greater understanding of the signalling events that drive the survival and progression of CLL, tumour cells ideally need to be studied in conditions that reflect those encountered by CLL cells in the *in vivo* microenvironment. Several survival pathways have been implicated in the pathogenesis of CLL (Scupoli and Pizzolo 2012; Woyach 2013; Woyach et al. 2012). This chapter set out to analyse the consequences of CD31/CD38 interactions on three protein kinase-signalling pathways, and resulted in four main findings.

Firstly, CD38 MFI was significantly up-regulated following 2 and 5 days with CD31-expressing co-culture, however an increase in CD38 MFI was not detected when CLL cells were cultured with non-transfected fibroblasts cell lines. This finding supports the concept that CD31/CD38 interactions are specifically responsible for changes in CD38 expression. Furthermore, CD38 expression was augmented in a time-dependent manner; incubation of CLL cells for 5 days in CD31-expressing co-culture significantly increased CD38 expression compared to CLL cells cultured for only 2 days. It has previously been shown that CLL cells from the bone marrow microenvironment have increased surface expression of CD38 when compared to CLL cells in the peripheral blood(Patten et al. 2008). Therefore, exposure to the CD31 ligand on the endothelium of blood vessels and/or nurse-like cells in the bone marrow may contribute to this. Furthermore in a study conducted by Deaglio *et al* in 2010, which involved the analysis of lymph node sections, a direct link was shown between the number of endothelial cells (which are $CD31^{+}$) and the level of CD38 expressed by CLL cells (Deaglio et al. 2010).

CD31 on the surface of CLL cells has the ability to ligate other CD31 molecules in a homotypic fashion; such interactions can take place with molecules expressed on vascular endothelial cells, nurse-like cells or other CLL cells (Deaglio 2003b). Poggi *et al*have previously proposed that CD31/CD31 homotypic interactions resulted in increased gene transcription and CLL cell survival and showed that this effect was

observed irrespective of the levels of CD38 expression on the CLL surface (Poggi et al. 2010). Tonino *et al* reported that high expression of CD31 can be detected on the surface of CLL cells irrespective of CD38 expression (Tonino S et al. 2008). There is controversy within the field of CLL research as to whether CD31 expression *per se* is a determinant of clinical outcome (Ibrahim et al. 2003; Mainou-Fowler et al. 2008; Poggi et al. 2010). In order to make a contribution to this debate, 30 CLL patients were analysed for CD31 cell surface expression. The levels of CD31 were similar in all the samples tested and did not vary significantly between $CD38^{hi}$ and $CD38^{lo}$ CLL patient samples. For this reason CD31/CD31 homotypic interactions were excluded as a significant confounding factor when comparing CD31/CD38 signalling in $CD38^{hi}$ and $CD38^{lo}$samples.

The **second** main finding in this set of experiments was that increased levels of phosphorylated proteins were detected following short-term CD31-expressing co-culture as well as non-transfected co-culture. *In vitro* studies have revealed that stromal co-culture can support CLL cell survival, providing signals, which are largely contact-dependent. Comparing non-transfected (NTL) fibroblast co-culture with CD31-expressing co-culture means the direct effect of the CD31 ligand can be assessed. Antibodies against the substrates of three protein kinases PKA, PKB and PKC were used to detect phosphorylated targets of these pathways following just 1-hour incubation in three culture conditions. Changes in protein phosphorylation could be detected with all three antibodies following CD31-expressing co-culture as well as NTL co-culture with all three protein kinases. The identity of many of these PKB substrates is unknown but it shows the powerful effect of co-culture on CLL cells. The short-term nature of cell stimulation implies that these changes occur directly downstream of CD31/CD38 ligation. The PKB substrate antibody was the focus of the chapter and over a range of patient samples, both basal and the induced protein phosphorylation varied. However, in all samples a change in protein phosphorylation was induced by co-culture. In co-culture experiments within this thesis some PKB substrate bands remained the same regardless of the culture conditions this acted as a good internal loading control. Phospho-motif substrate antibodies represent phosphorylation-state sensitive, motif-specific antibodies which are a useful tool to investigate the mechanisms of substrate phosphorylation in signalling pathways. Phospho-specific antibodies help to identify novel signalling molecules which display aberrant signalling in malignancies. The PKB substrate antibody has been extensively used in other studies, resulting in the

identification of novel targets of PKB signalling (Alnagar et al. 2010; Kane et al. 2002; Manning et al. 2002). The detection of multiple protein bands with the PKB substrate antibody suggests that these bands are direct targets for phosphorylation by PKB. However, multiple kinases can share very similar consensus phosphorylation motifs, for example the blots generated with PKA, PKB and PKC substrate antibodies showed a considerable number of overlapping bands detected indicating that the same downstream targets were being recognized. It also is possible that the phosphorylation of a substrate is induced by other physiological stimuli. Furthermore PKB *in vitro* has been shown to phosphorylate most R-X-RX-X-S/T sites as well as some R-X-X-S/T sites. Therefore to further study substrates that are of interest, phospho-specific antibodies which recognise the precise phosphorylation sites must be used, as well as protein kinase inhibitors which demonstrate specific kinases are responsible for the phosphorylation

Thirdly, studying known PKB substrates helped to establish the identity of two protein bands detected with the PKB substrate antibody; phospho protein bands pp35 and pp45 were identified as phospho-S6 and phospho-GSK3β respectively. Ribosomal S6 kinases (rsk) are a family of serine/threonine kinases, $p90^{rsk}$is one such kinase, $p90^{rsk}$ is activated by the ERK pathway and this phosphorylated kinase activates ribosomal S6. The phosphorylation of the ribosomal S6 protein has been directly implicated in protein translation initiation and cell growth (Ibrahim et al. 2003). Levels of p-S6 were increased following 1-hour culture with both CD31-expressing co-cultures as well as in NTL co-culture. Supporting the identity of these PKB substrate are studies conducted by two other groups who have used the same phospho-PKB substrate antibody to detect a 32kDa, LY294002 sensitive protein. Using immunoprecipitation and mass spectrometry, both Kane *et al*, and Ly *et al* were able to prove the identity of this 32kDa substrate to be the ribosomal S6 protein (Kane et al. 2002; Ly et al. 2003). Pharmacological inhibitors LY294002 and Rapamycin have the ability to inhibit phosphorylation of S6 at concentrations of 10μM and 5nM respectively, this shows that phosphorylation of S6 is dependent on the activation of PI3K/ mTOR pathways. Ultraviolet cross-linking studies have shown that S6 directly interacts with tRNA, initiation factors and mRNA, implicating this protein in the regulation of translation initiation. Furthermore it has been shown that phosphorylation of S6 increases the rate of protein synthesis as well as regulating cell size; this would suggest that S6 is also a mediator of cell growth (Nygard and Nilsoon 1990). Mouse studies have shown that

following the deletion of S6; there is no ribosome biogenesis, inhibiting entry into cell cycle progression (Volarevic et al. 2000). In 2003 Ly *et al* discovered that ribosomal S6 was constitutively phosphorylated in chronic myeloid leukaemia (CML) cells, and the phosphorylation of S6could be inhibited in CML cells following treatment with LY294002 and Rapamycin (Ly et al. 2003). Constitutive p-S6 has also been detected *in vivo* in the transgenic B-cell lymphoma mouse model expressing the *c-myc* oncogene, which results in the constitutive expression of PKB (Wendel et al. 2004). This phosphorylation event was shown to be dependent on mTOR signalling since when the lymphoma was treated with Rapamycin there were significantly lower levels of p-S6 compared to untreated lymphoma (Wendel et al. 2004). In 2002, Zhang *et al* identified p-S6 as an important target of PI3K signalling in embryonic stem cells. Zhangs'study used an antibody directed towards the consensus motif of PKC and not PKB and, therefore, this implies that phosphorylation of S6 can occur independently of PKB and mTOR (Zhang et al. 2002). S6 kinases are also activated by PKC in a number of other cell types (Akimoto et al. 1998; Valovka et al. 2003). A study conducted by Barragan *et al* also showed that PKC could induce the phosphorylation of PKB substrates independently of the PI3K signalling pathway in primary CLL cells (Barragan et al. 2006). Increased activation of the ribosomal protein S6 is clearly a feature of other malignancies of lymphoid origin and thus may play a vital role in the activation and proliferation of CLL cells *in vivo*.

The second phospho protein was predicted to be GSK3β, a 47kDa serine/threonine kinase with enzymatic activity. GSK3β is regulated by a number of signalling pathways (ter Haar et al. 2001), and 2 phosphorylation sites influence the catalytic activity of the protein. The first, Serine 9 is the phosphorylation site for PKB and phosphorylation of this residue inactivates the protein. In contrast phosphorylation of Tyr 216, located on the activation loop increases catalytic activity (Grimes and Jope 2001). The levels of p-GSK3β were analysed in CLL patients. Phosphorylation of this protein increased following CD31-expressing co-culture this was observed in all patients indicating that the inhibition of this protein is promoted by CD31:CD38 interactions. Phosphorylation of GSK3β by PKB inactivates the protein and inhibition of this pathway has been implicated in cell proliferation (ter Haar et al. 2001). Supporting the identity of this protein is a study that used the same commercially available PKB substrate antibody as a means of identifying substrates of the PI3K/PKB dependent serine/threonine kinase.This study confirmed that the band they detected at ~47kDa was GSK3β by

immunoprecipitation and subsequent tandem mass spectrometry (Kane et al. 2002). The experiments using LY294002 and Rapamycin showed that p-S6 and p-GSK3β could be inhibited following 30 minutes pre-treatment of CLL cells with the inhibitors followed by 1 hour incubation with CD31-expressing fibroblasts or LQ only. A more dramatic decrease in protein phosphorylation was observed in the LQ controls, which suggests that that CD31-expressing co-culture may be providing some level of protection against this inhibitor and may confer a cytoprotective effect on CLL cells. Whether or not the identified phosphorylated proteins S6 and GSK3β are targets of PKB *in vivo* remains uncertain in CLL.

The basal levels of p-S6 and p-GSK3β in CLL patient samples were found to be higher in the $CD38^{hi}$cohort of patients; this group of patients is known to have a poorer prognosis and it may be the case that elevated levels of basal p-S6 or p-GSK3β could be used as a marker of poor prognosis in CLL.

Finally, phosphorylation of the identified PKB substrates S6 and GSK3β can be maintained in CD31-expressing co-culture for 48hours but cannot be maintained in LQ even in CLL patients expressing high surface levels of CD38 protein. Following 4 hours in LQ it was shown that there was a dramatic loss in phosphorylation of PKB substrates, which demonstrates the transient nature of protein phosphorylation when stimuli are removed. The maintenance of p-S6 and p-GSK3β, as well as other unidentified phosphorylated substrates of PKB, when incubated with co-culture demonstrates the significance of these interactions on the sustained activation status of the CLL cell. Despite opening with three different phospho-substrate antibodies, this chapter focussed on the PI3K/PKB pathway. *In vivo* and *in vitro* studies have demonstrated the importance of the PI3K/PKB signalling pathway in B-cell malignancies, specifically CLL(Arcaro and Guerreiro 2007; Hoellenriegel et al. 2011; Khwaja 2010).Lannutti *et al* used the PI3Kζ inhibitor GS-1101 on primary leukaemic cells and demonstrated that patients with B-cell malignancies were more responsive to treatment with this agent. GS-1101 was capable of abrogating CD40-induced PKB phosphorylation in CLL patients as well as down-regulating the anti-apoptotic protein MCL1(Lannutti et al. 2011). Furthermore a phase I clinical trial looked at the effects of GS-1101 treatment in a cohort of 37 patients with relapsed or refractory CLL.All patients displayed reduced lymphadenopathy and 91% of patients showed a lymph node response; few adverse side effects were observed following treatment (Veliz and Pinilla-Ibarz 2012). Therefore the

targeting of this pathway looks to be of clinical significance in CLL patients and is an exciting prospect to circumvent the pro-survival signals provided by the microenvironment.

In summary, a phospho-PKB substrate antibody was used to implicate S6 and GSK3β as important targets of PI3K/PKB signalling in primary CLL cells. This demonstrated the value of using phospho-specific motif antibodies as tools to identify targets of protein kinases involved in survival signalling. Furthermore, this work demonstrated that increased CD38 expression is associated with higher basal levels of phosphorylated PKB substrates and a higher phosphorylation signal following CD31 co-culture. The increased basal phosphorylation of these substrates may be of prognostic value and a useful tool as the phosphorylation status of S6 and GSK3β may prove to be a method used to monitor PI3K and mTOR activity.

4 Different co-culture systems stimulate different key survival signalling pathways

4.1 Introduction

Recent reports have highlighted that CLL cells are exposed to complex and varied microenvironments *in vivo*(Audrito et al. 2013; Deaglio 2010; Hamilton et al. 2012). Consequently there has been a concerted effort to try to understand these varied conditions and the molecules involved (Burger and Gandhi 2009; Deaglio 2010; Friedberg 2011). In recent years, CLL research has focused on the crosstalk between CLL cells and growth permissive microenvironments in the body such as the lymph nodes and bone marrow, where CLL cells are exposed to signals which maintain CLL cell survival. CLL cells undergo apoptosis when cultured *in vitro* in the absence of stromal cells or growth factors, but thrive when cytokines are added to culture or when stromal co-culture systems are used (Binder et al. 2010; Deaglio 2008b; Hamilton et al. 2012), this demonstrates that CLL cells maintain the ability to respond to external stimuli when removed from the body.

In 2012, Hamilton *et al* compared three *in vitro* co-culture systems, allowing for the direct comparison of individual stimuli known to be present within the *in vivo* CLL microenvironment (Hamilton et al. 2012). The three systems studied were the human endothelial cell line HMEC, as well as mouse embryonic fibroblasts transfected with human CD31 or human CD40L. A non-transfected fibroblast murine cell line was used as a control in order to ascertain the specific effect of the human ligands. All three systems delivered cytoprotection to CLL cells; the HMEC cell line conferred the greatest survival advantage and CD40L-expressing co-culture provided the least amount of cytoprotection to CLL cells. Cell surface phenotypic markers associated with activation were also analysed following culture on these three systems. Phenotypic changes were observed in all co-culture systems withthe exception of non-transfected co-culture cells, this demonstrated ligand specificity in terms of the ability to activate CLL cells. Interestingly, co-culture with CD31 and CD40L expressing fibroblasts induced CLL cell proliferation whereas the HMEC cell line did not (Hamilton et al. 2012). Asslaber *et al* examined the effect of CD40 ligation on CLL cells *in vitro*. Following CD40-expressing co-culture a marked

increase in CLL cell viability was observed as well as augmented expression of the early activation marker CD69 (Asslaber et al. 2013). Both Hamilton *et al* and Asslaber *et al* demonstrated the ability of co-culture systems to support CLL cell survival as well as the capability to modulate the expression of some activation markers. In these experiments, however, the impact of *in vitro* stimuli on intracellular signalling pathways was not examined.

Recently Ghamlouch *et al* (2013) assessed the ability of cytokines to maintain CLL cell survival *ex vivo,* IL-4 and BAFF were identified as two cytokines that were able to support CLL cell survival when added to culture. IL-4 proved to be the most potent mediator of survival and had the ability to maintain CLL cell survival for over 168 hours (Ghamlouch et al. 2013). Other studies have demonstrated the potent effect of IL-4 as a mediator of CLL cell survival *in vitro*(Dancescu et al. 1992; Kay and Pittner 2003). It has been shown that CLL patients have raised levels of IL-4 produced by peripheral blood $CD4^{+}$T-cells when compared to normal age-matched controls (Mainou-Fowler et al. 2001). CLL patients also possess constitutively higher levels of the IL-4 receptor (Kay et al. 2001). The increased levels of IL-4 in the peripheral blood in CLL patients may provide sufficient signals to maintain circulating CLL cells *in vivo*.

In Chapter 3 of this thesis CLL cells were co-cultured with CD31-expressing fibroblasts. This resulted in the augmented phosphorylation of substrates of the PKB signalling pathway. Experiments in this chapter were performed to compare the effect of CD31 stimulation with CD40L stimulation in the presence and absence of IL-4. All experiments were carried out following 1-hour incubation and a non-transfected fibroblast cell line was used as a control to measure the specific effect of the human ligands. The phosphorylation status of four proteins was assessed: the ribosomal protein, S6; the serine kinase, GSK3β; the transcription factor, STAT6 a target of Janus kinase pathway; and ERK, a target of CD40 signalling. The primary aim of these experiments was to characterise the direct pathways in primary CLL cells ofthe individual stimuli.

4.2 Results

4.2.1 Addition of IL-4 to CD31 co-culture slightly augments p-S6 and p-GSK3β

CLL cells not only have elevated levels of the IL-4 receptor but also have the ability to secrete IL-4. The production of IL-4 by both CLL cells and $CD4^+$ T-cells permits for both autocrine and paracrine activation of the IL-4 signalling pathway in CLL patients (Seiffert et al. 2010). The addition of IL-4 to CD31-expressing co-culture mimics another element of the *in vivo* lymph node microenvironment as CLL cells located in the lymph node are exposed to $CD31^+$ stromal cells as well as elevated numbers of $CD4^+$ T-cells, which produce IL-4 (Kay et al. 2001).

The hypothesis in this experiment was that the addition of IL-4 to CD31-expressing co-culture could enhance the phosphorylation and activation of intracellular signalling proteins in CLL cells. In total, 10 patients were analysed; five were $CD38^{hi}$ and five were $CD38^{lo}$. Figure 4.1 shows four blots generated from two CLL patient samples, a $CD38^{hi}$ (95%)and $CD38^{lo}$ (2%)patient.

Figure 4.1 shows that basal p-S6 and p-GSK3βcould be detected in the $CD38^{hi}$ sample. In contrast, basal p-S6 and p-GSK3β could not be detected in the $CD38^{lo}$sample.Following the addition of IL-4 to liquid culture (LQ) the p-S6 and p-GSK3βbands appeared to be slightly elevated in $CD38^{hi}$ samples but p-S6 and p-GSK3β remained undetectable in $CD38^{lo}$samples. Co-culture on CD31-expressing fibroblasts and NTL co-culture induced the phosphorylation ofS6 and GSK3β in the$CD38^{lo}$samples and augmented levels of p-S6 and p-GSK3β in the $CD38^{hi}$samples analysed. The addition of IL-4 to CD31-expressing and non-transfected co-cultures lightly increased the phosphorylation of GSK3β and S6 in the$CD38^{lo}$ patient, this effect was not observed when IL-4 was added to co-culture in the $CD38^{hi}$ patient sample (patient 1).

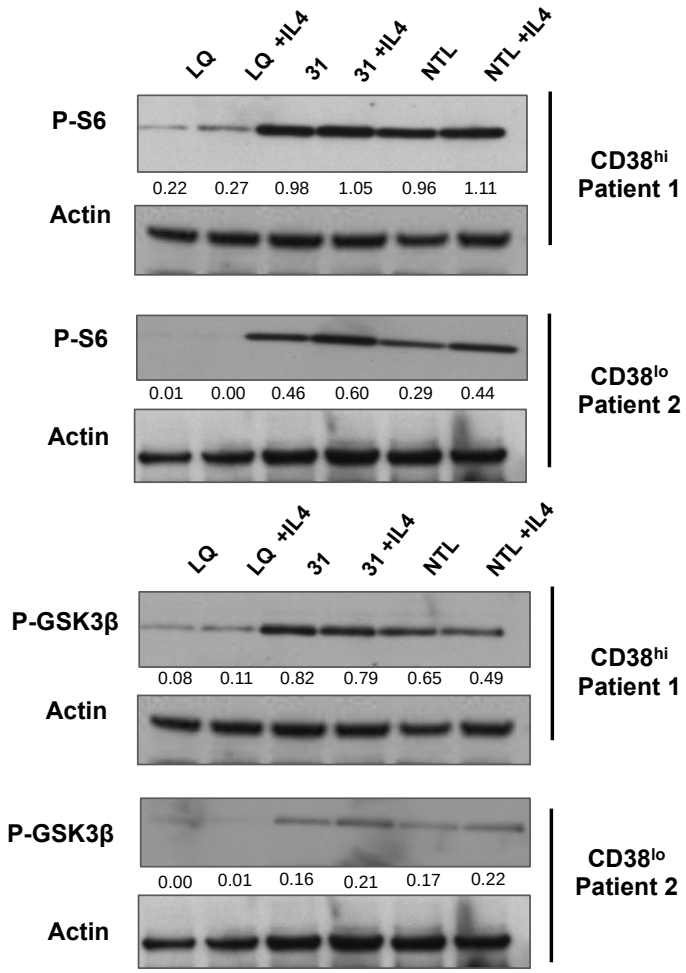


Figure 4.1 Analysis of 2 CLL patients on CD31 co-culture system with and without the addition of IL-4.

Primary CLL cells were separated from 2 patients. 1 x 10^6 CLL cells were incubated with CD31-expressing co-culture (31) or non-transfected co-culture (NTL) or left in liquid culture (LQ) for 1 hour with and without the cytokine IL-4 (5ng/ml). Following, incubation the CLL cells were harvested lysed and protein extracted, proteins were resolved by SDS-PAGE followed by western blotting and detection with p-GSK3β and p-S6 antibodies as well as actin. The band intensity was normalised to Actin.

4.2.2 Addition of IL-4 to CD40L co-culture augments phosphorylation of S6 and GSK3β

CD40 signalling induces powerful anti-apoptotic signalling in CLL cells *in vitro(Furman et al. 2000),* signals induced by CD40 stimulation include canonical and non-canonical NF-κB signalling (Furman et al. 2000), ERK signalling (Kashiwada et al. 1996), and PI3K/PKB signalling (Deregibus et al. 2003). CLL cells constitutively express CD40 on their surface and CD40 signalling is induced upon CLL cell (CD40) binding to CD40L expressed on the surface of $CD4^+$ T-cells (Schattner et al. 1998). Increased numbers of $CD4^+$ T-cells are detected in the lymph nodes of CLL patients (Brusa et al. 2013), and $CD4^+$ T-cells not only promote signalling through CD40/CD40L receptor but also produce IL-4.

To imitate a different element of the CLL cell microenvironment murine fibroblasts exogenously expressing CD40Lwere used in the following co-culture experiment. The same patients were used in this experiment in order to facilitate direct comparison with the CD31-expressing co-culture experiments presented in Figure 4.1. Figure 4.2 depicts four representative blots from two CLL patients, one $CD38^{hi}$ (95%)and one $CD38^{lo}$ (2%)patient.

Figure 4.2 shows that following 1 hour of CD40L-expressing co-culture the phosphorylation of S6 and GSK3β was induced in the $CD38^{lo}$ patient and augmented phosphorylation of S6 and GSK3β could be detected in the $CD38^{hi}$ patient. Increased phosphorylation of these substrates was also observed following culture with non-transfected fibroblasts (NTL) although to a lesser extent. The phosphorylation of these proteins was modestly increased in both the $CD38^{hi}$ and $CD38^{lo}$ samples when CD40L-expressing and NTL co-culture systems were supplemented with IL-4.

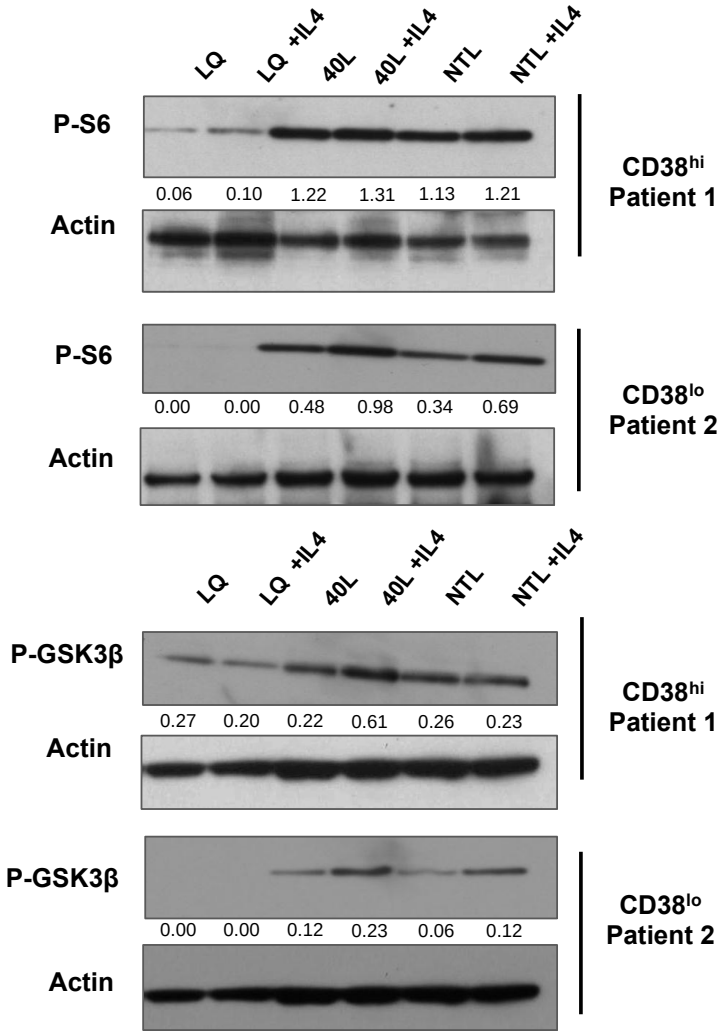


Figure 4.2 Analysis of 2 CLL patients on CD40L co-culture system with and without the addition of IL-4.

Primary CLL cells were separated from two patients. CLL cells were incubated with CD40L-expressing co-culture (40L) or non-transfected co-culture (NTL) or left in liquid culture (LQ) for 1 hour with and without the cytokine IL-4 (5ng/ml). Following incubation the CLL cells were harvested lysed and protein extracted, proteins were resolved by SDS-PAGE followed by western blotting and detection with p-GSK3β and p-S6 antibodies as well as actin.

4.2.3 CD31 co-culture can induce phosphorylation of ERK and IL-4 culture can induce phosphorylation of STAT-6

The ERK signalling pathway has been identified as a therapeutic target to counteract the pro-survival effect of the *in vivo* microenvironment in CLL patients (Herishanu et al. 2011). Muzio *et al* showed in 2008 that (25/51) 49% of CLL patients analysed displayed constitutive phosphorylation of ERK(Muzio et al. 2008). Furthermore, the ability of IL-4 to sustain CLL cell survival *in vitro* also supports the targeting of this cytokine.

To ascertain whether CD31-expressing co-culture or IL-4 co-culture could induce the activation and phosphorylation of ERK as well as IL-4 signalling target STAT6, ten CLL patients were analysed, five $CD38^{hi}$ and five $CD38^{lo}$. The p-STAT6 blots in Figure 4.3 depict blots from CLL patients, one $CD38^{hi}$ (83%) and the other $CD38^{lo}$(2.4%).

Figure 4.3a show that the STAT6 protein is only phosphorylated in cultures supplemented with IL-4 this was shown in all 10 patients analysed. In Figure 4.3b only a $CD38^{hi}$ patient is shown, this is because no basal or inducible p-ERK could be detected in the five $CD38^{lo}$ patients analysed. The $CD38^{hi}$ patient represented in Figure 4.3b shows that although basal p-ERK was not detected, following 1-hour of CD31-expressing co-culture the phosphorylation of ERK was strikingly up regulated and the levels of p-ERK detected were further augmented following the addition of IL-4 to CD31-expressing co-culture. Low levels of p-ERK were detected following NTL co-culture with a modest increase in p-ERK detected with the addition of IL-4.

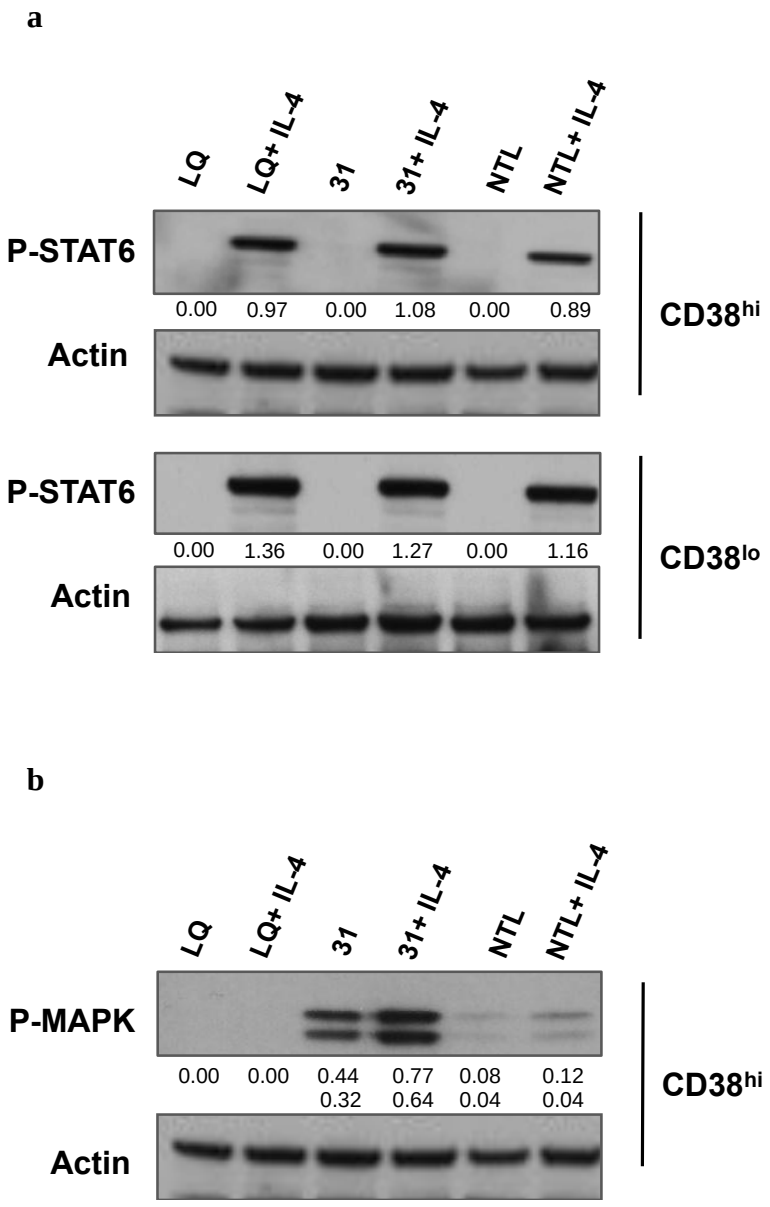


Figure 4.3 Analysis of CD38^{hi}and CD38^{lo} CLL patients with P-STAT6 and P-ERK following CD31 co-culture

Primary CLL cells were separated from two CLL patients. CLL cells were added toCD31-expressing fibroblasts (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1-hour with and without IL-4 (5μg/ml). The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detection with (a) p-STAT6 antibody or (b) a p-ERK antibody.

4.2.4 Phosphorylation of ERK can be induced by CD40L co-culture in CD38^{hi} patients and STAT6 can be achieved through IL-4 treatment

It is well established that IL-4 alone can maintain CLL cell survival *in vitro* (Kay et al. 2001; Kay and Pittner 2003; Wurster et al. 2002). The experiments shown in Figures 4.1 and 4.2 revealed that IL-4 treatment resulted in a modest increase in the phosphorylation of PKB substrates GSK3β and S6 when added to CD31-expressing and CD40L-expressing co-culture systems. However, IL-4 treatment alone only induced a small phosphorylation of S6 in some patients, given that IL-4 is able to maintain CLL survival this would imply that the phosphorylation of these proteins is not essential for cell survival. However, Figure 4.3a demonstrated the potent ability of IL-4 to induce the phosphorylation of STAT6.

The results above promoted experiments to directly assess the comparative effects of individual stimuli (IL-4 CD40L, CD31, NTL) in the same samples. This allowed the evaluation of basal CD38 expression on the downstream phosphorylation events (S6, STAT6 and ERK). In order to do this protein extracts were prepared from CD38^{lo}, CD38^{hi} patients as well as CLL patients with intermediate levels of CD38 expression (CD38^{int}).

Figure 4.4a represents a CD38^{lo}patient (2.3%): consistent with previous experiments no basal p-S6 was detected in this patient and was not induced following the addition of IL-4 to culture. However, CD31-expressing and CD40L-expressing co-culture were able to induce the phosphorylation of S6. Distinct p-STAT6 bands were readily detected upon addition of IL-4 to cultures but no p-STAT6 was detected in cultures without IL-4. Consistent with the low level of CD38 expressed by this sample, p-ERK was not detected in any of the culture conditions assessed.

Figure 4.4b and 4.4c represent two CD38^{int} samples; CD38 was expressed on 23% (Figure 4.4b) and 43% of CLL cells (Figure 4.4c) respectively. Low basal p-S6 was detected in both CD38^{int} samples; the addition of IL-4 to LQ slightly elevated levels of p-S6. P-S6 was augmented following CD31-expressing co-culture as well as CD40L-expressing co-culture. Striking p-STAT6 bands were detected exclusively in cultures supplemented with IL-4 in both CD38^{int} samples. As was the case with the CD38^{lo} sample analysed (Figure 4.4a), faint p-ERK bands were detected following incubation with CD40L-expressing co-culture and CD31-expressing co-culture.

Figure 4.4d shows a $CD38^{hi}$ (92%) patient sample; high basal p-S6 was detected in this patient. The phosphorylation of S6 was slightly augmented upon addition of IL-4 to liquid culture (LQ), comparable p-S6 bands were observed following CD31-expressing co-culture and CD40L-expressing co-culture, and increased p-S6 was detected following the addition of IL-4 co-culture. Distinct p-STAT6 bands were detected in all cultures containing IL-4. Faint p-ERK could be detected in this $CD38^{hi}$ patient, a slight increase in p-ERK was observed following the addition of IL-4 to LQ and increased p-ERK was evident following CD31 and CD40L co-culture.

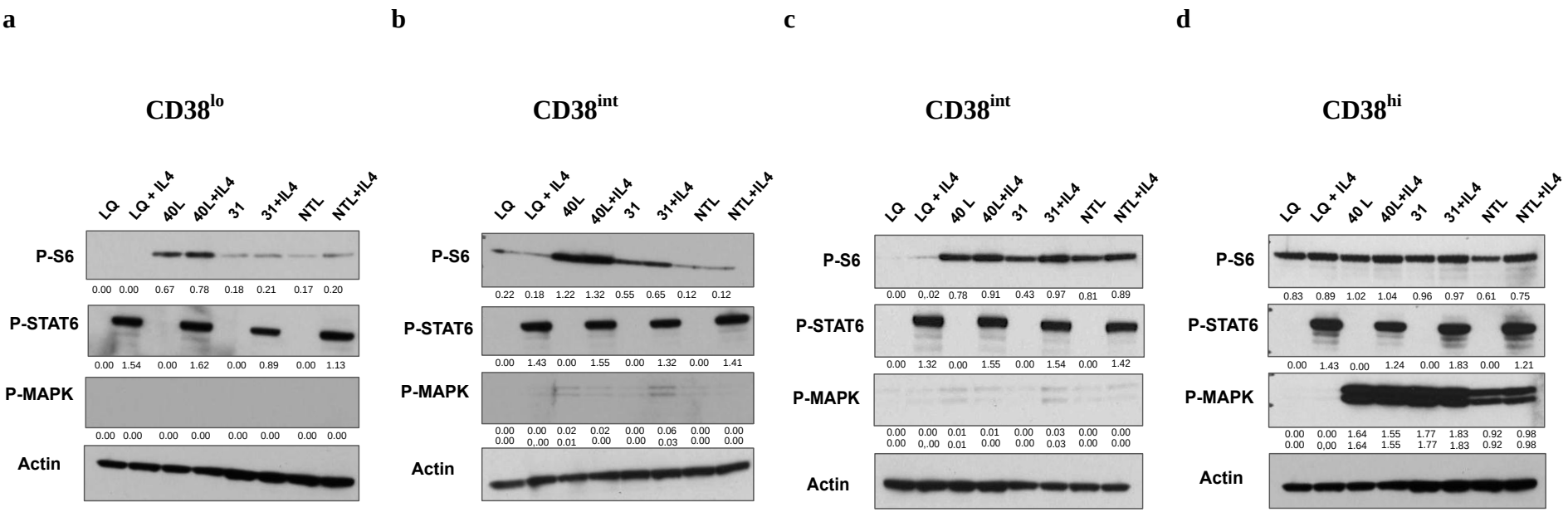


Figure 4.4Analysis of a CD38^{lo}, two CD38^{int}and a CD38^{hi}CLLpatient sample with three phospho-specific antibodies on both CD40L co-culture, CD31 co-culture and with IL-4 after 1hour culture.

Primary CLL cells were separated from a patient. CLL cells were added to (40), (31) or non-transfected cells (NTL) or left in liquid culture (LQ) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detection with p-S6, p-STAT6, p-ERK and loading control actin.

4.2.5 IL-4 treatment or co-culture can maintain CLL cell survival over 48 hours

In experiments conducted by Tonino *et al,* in 2008, the CLL cell viability of 10 CLL patients was assessed following 7-day co-culture with CD40L-expressing and CD31-expressing mouse fibroblasts. Tonino *et al* (2008) demonstrated that co-culture with CD40L expressing fibroblasts resulted in a significant increase in CLL cell viability. In contrast, CLL cells cultured with CD31-expressing co-culture, in the presence or absence of CD31 blocking antibodies, did not demonstrate any survival advantage (Tonino S et al. 2008). Given that culture with IL-4 has been shown to have a potent effect on CLL cell survival (Dancescu et al. 1992) but the cytoprotective effects of various co-culture systems remain contentious, experiments were performed to determine their effects on CLL survival.

To evaluate the comparative effects of the different *in vitro* stimuli on CLL cell viability 30 CLL patient samples were analysed, $2x10^5$cellswere removed from the different culture conditions after 24 hours and 48 hours and stained with Annexin V/ propidium iodide (PI).

Figure 4.5 and 4.6 shows show annexin V/PI staining in the various culture conditions at 24 and 48-hour time points. Figure 4.7 represents the mean percentage of viable CLL cells in the cohort of 30 CLL patients in each culture condition following 24-hour and 48-hour incubations respectively. Figure 4.7a shows that IL-4 treatment, CD40L expressing co-culture, CD31-expressing co-culture and NTL co-culture all resulted in a significant increase in cell viability compared to LQ alone ($P< 0.0001$ for all conditions). CD31-expressing co-culture and CD40L expressing co-culture were significantly more cytoprotective than IL-4 treatment alone ($P<0.0001$ and $P=0.001$ respectively). However, NTL co-cultures were not significantly different to IL-4 treated cultures in terms of viability. The differences in CLL cell viability between different culture conditions were modest at the 24-hour time point. Figure 4.7b shows that increased cell death was observed in the LQ condition at the 48-hour time point compared to the 24-hour time point. Cell survival remained high in IL-4 treated cultures as well as in co-cultures. As with the 24-hour cultures CD40L-expressing co-culture and CD31-expressing co-culture were significantly more cytoprotective than IL-4 treatment alone ($P<0.0001$, $P=0.0001$ respectively).

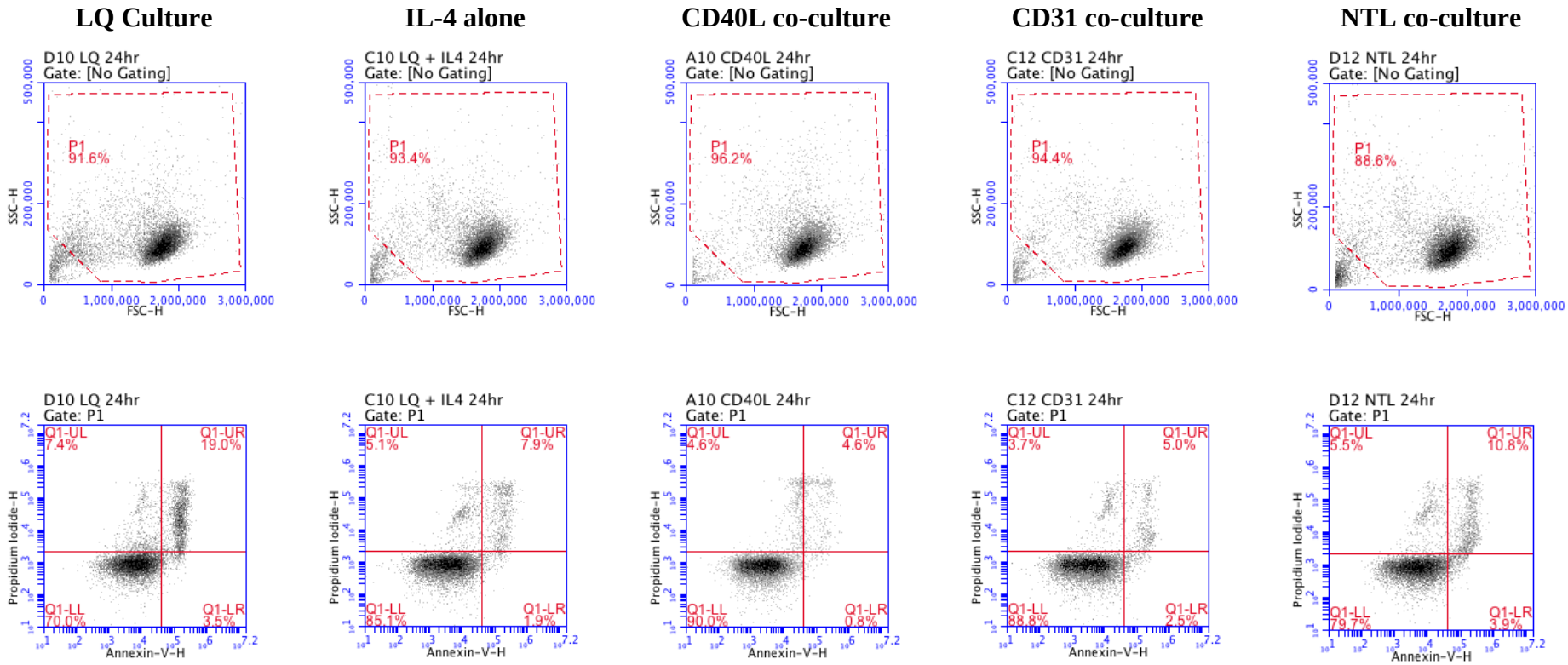


Figure 4.5 IL-4 treatment and co-culture maintains CLL survival for 24 hours

2 x10^5CLL cells were placed in LQ, treated with IL-4 or added to CD40L-expressing co-culture, CD31-expressing co-culture or NTL co-culture for 24 hours. Viability was assessed using Annexin V/ PI staining in a P1-gated population that excludes debris.

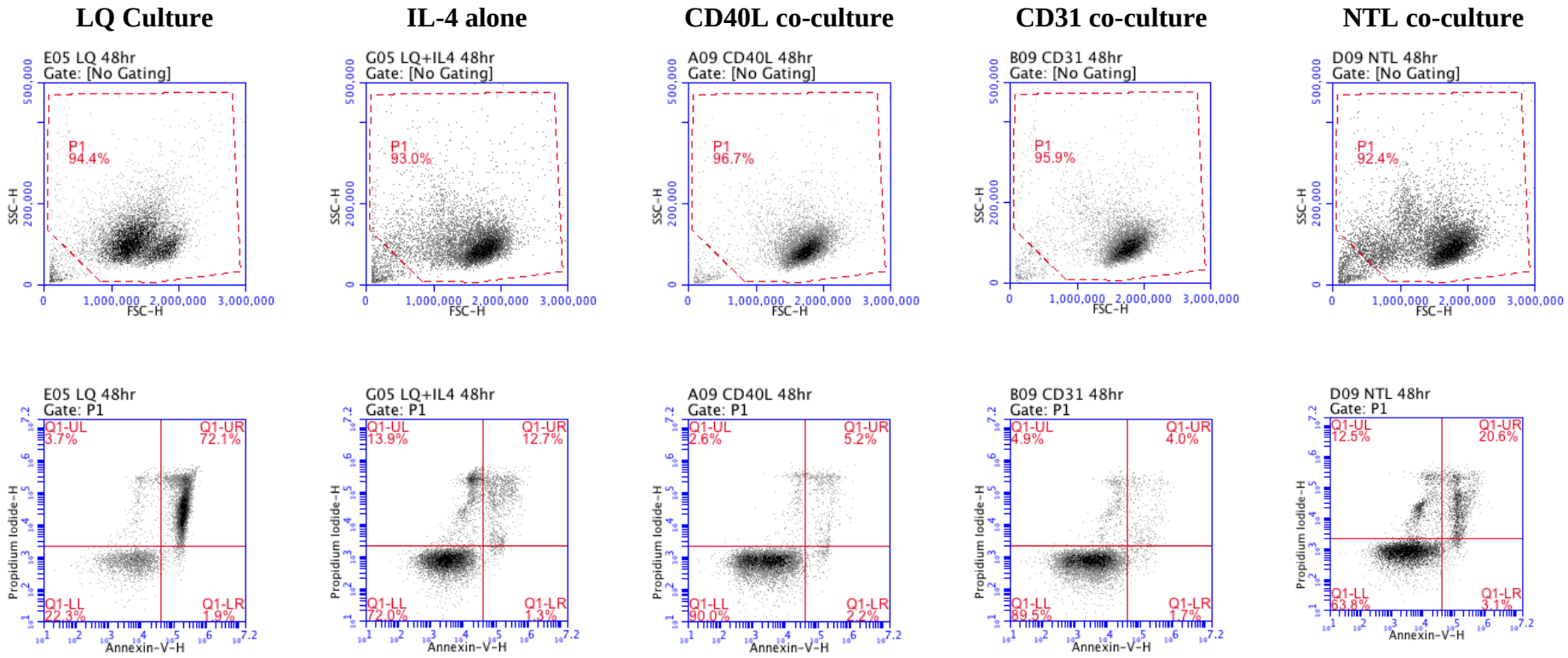
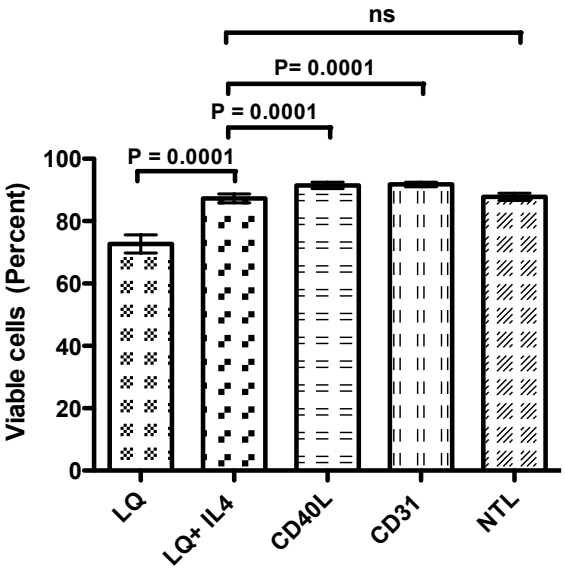


Figure 4.6 IL4 treatment and co-culture maintains CLL survival for 48 hours

2 x10^5CLL cells were placed in LQ, treated with IL-4 or added to CD40L-expressing co-culture, CD31-expressing co-culture or NTL co-culture for 48 hours. Viability was assessed using Annexin V/ PI staining in a P1-gated population that excludes debris.

a. 24 hours



b. 48 hours

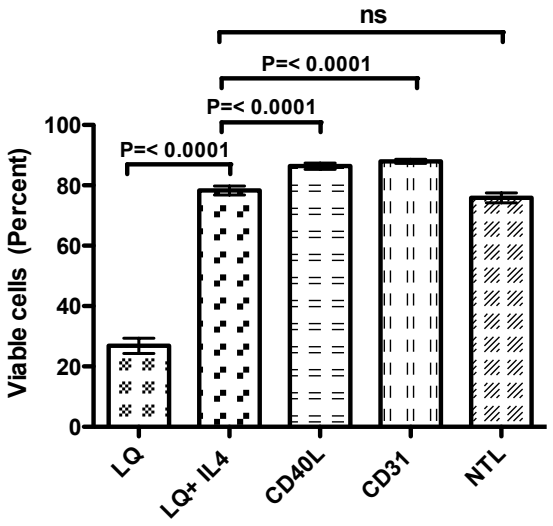


Figure 4.7 Analysis of primary CLL cell viability following 24-hour and 48-hour co-culture with IL-4, CD40L expressing fibroblasts and CD31 expressing fibroblasts.

Primary CLL cells (n=10) were separated from patient whole blood samples. CLL cells were added to (CD40L),(CD31) or non-transfected cells (NTL) or left in liquid culture (LQ) for a.) 24 orb.) 48 hours with and without addition of 5ng/ml IL-4. The CLL cells were removed and CLL cells were stained with Annexin V/PI before being analysed on the flow cytometer. A repeated measures ANOVA test was used to compare the viability of CLL cells between culture conditions.

4.2.6 PF956980 inhibits the phosphorylation of STAT-6

To investigate whether it was possible to identify STAT6 as a direct target of JAK3 kinase in CLL cells PF956980, a JAK3 selective inhibitor was used (Steele et al. 2010). $1x10^6$primary CLL cells were left untreated or incubated with three doses of PF956980: 1.25μM, 2.5μM and 5μM, for 1 hour. Given that IL-4 alone can induce phosphorylation of STAT6, CLL cells were cultured in liquid culture (LQ) and 5ng/ml of IL-4 (LQ+IL-4) in all cultures. In total 10 patient samples were analysed, five were $CD38^{hi}$ and five were $CD38^{lo}$.

Figure 4.8 shows a representative western blot from a $CD38^{hi}$ patient and a $CD38^{lo}$ patient; a strong p-STAT6 band was detected in the LQ+IL-4 untreated condition in both samples. Treatment with PF956980 caused a rapid reduction in p-STAT6 in a dose-dependent manner. Treatment with 5μM of PF956980 completely abolished p-STAT6 in both samples analysed.

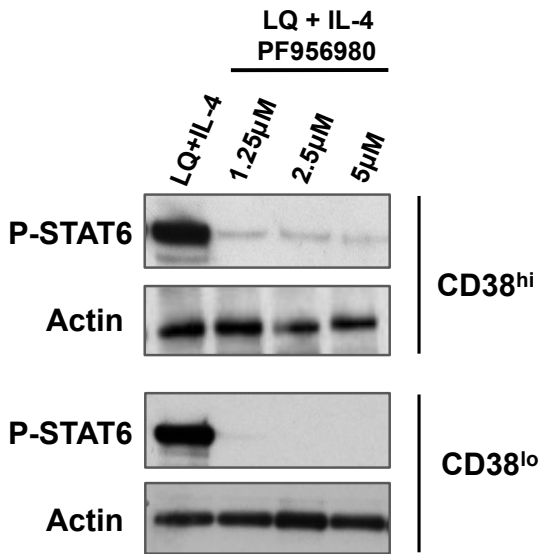


Figure 4.8 Western blot analysis of P-STAT6 following treatment with PF956980 in a dose-dependent manner.

$1x10^6$ Primary CLL cells were incubated in LQ+ IL-4 in the presence of increasing doses of the JAK 3 kinase inhibitor PF956980 (1.25μM, 2.5μM and 5μM) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detection with a p-STAT6 antibody.

4.2.7 U0126 inhibits the phosphorylation of ERK in a dose-dependent manner

To investigate whether it was possible to identify ERK as a specific target of CD38 signalling, the ERK inhibitor U0126 was used (Hawkins et al. 2008). In this experiment $1x10^6$ cells were incubated with three doses of U0126: 2.5μM, 5μM and 10μM or left untreated for 1 hour on CD31-expressing co-cultures supplemented with 5ng/ml IL-4. Since it was already established that CD31-expressing co-culture induced ERK phosphorylation in $CD38^{hi}$ patients, 10 $CD38^{hi}$ patients were studied. Figure 4.11 depicts two representative $CD38^{hi}$ patients showing a dose-dependent response in p-ERK inhibition following treatment with U0126. P-ERK could not be detected in any of the $CD38^{hi}$ patient samples when treated with 10μM of U0126.

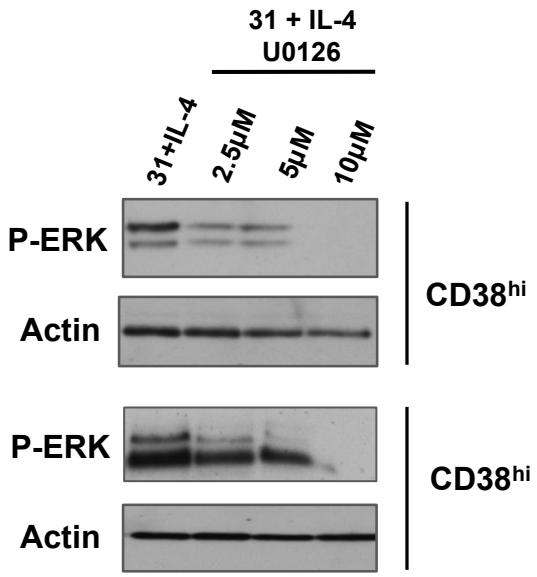


Figure 4.9 Western blot analysis of P-ERK following treatment with U0126 in a dose-dependent manner.

$1x10^6$ Primary CLL cells were incubated withCD31-expressing fibroblasts and 5ng/ml IL-4 in the presence of increasing doses of the ERK inhibitor U0126 (2.5μM, 5μM and 10μM) for 1 hour. The CLL cells were removed and lysed. Protein was extracted, resolved by SDS-PAGE followed by western blotting, and detection with a p-ERK antibody (two bands detected with P-ERK represent phosphorylated ERK44/42).

4.3 Discussion

Hamilton *et al* showed that co-culture systems (HMEC, CD31 and CD40L) have the ability to support CLL cell survival *in vitro* (Hamilton et al. 2012). These culture systems are likely to induce activation of intracellular signalling pathways however these experiments did not examine the effect of these stimuli on signalling events. Signals within the CLL microenvironment *in vivo* are considered to be a major obstacle impeding the effective treatment of CLL with chemotherapeutic agents (Audrito et al. 2013; Herishanu et al. 2011). IL-4 is another molecule to consider when replicating the CLL microenvironment *ex vivo.* The addition of IL-4 to CLL cultures *in vitro* has been shown to support CLL cell survival (Dancescu et al. 1992; Steele et al. 2010). Furthermore, CD40 signalling and CD38 signalling have also been shown to be powerful mediators of CLL cell survival *in vitro*, but the precise mechanism/s by which these stimuli inhibit apoptosis have not been fully elucidated. In this set of experiments CD31-expressing co-culture and CD40L-expressing co-culture, as well as non-transfected co-culture and treatment with IL-4 were used to assess the impact of individual stimuli on targets of survival signalling. These co-culture systems were used alone and in combination and four phospho-proteins were analysed: S6, GSK3β, ERK and STAT6. These experiments have led to four principal findings.

The first finding in this set of experiments was that CD40L stimulation, like CD31 stimulation, was capable of inducing the phosphorylation of PKB substrates S6 and GSK3β in $CD38^{lo}$ CLL patients, and augmenting the phosphorylation of PKB substrates in $CD38^{hi}$ patients. Cuni *et al* used non-transfected and CD40L-expressing co-culture to demonstrate that both systems were able to increase the phosphorylation of PI3K signalling targets; however CD40L-expressing co-culture is required to further augment the phosphorylation and activation of the PI3K/PKB and NF-κB signalling pathways to initiate CLL cell proliferation (Cuni et al. 2004). However, in contrast to these findings, Hallaert *et al* in 2008 were unable to detect PKB phosphorylation following the prolonged culture of CLL cells with CD40L-expressing co-culture (Hallaert et al. 2008). These differences may be because Hallaert *et al* analysed CLL samples that had been stored in liquid nitrogen. In contrast, the work presented in this thesis was all carried out on freshly isolated CLL cells. Furthermore, Hallaert *et al* conducted all experiments following 48-hour incubation in CD40-expressing co-culture whereas experiments in this chapter were conducted following just 1 hour of co-culture. The results generated in

this chapter indicate that phosphorylation of signalling molecules can be rapidly induced following CLL cell stimulation.

The second principal finding in this chapter was that IL-4 had the ability to augment the phosphorylation of both ribosomal S6 and GSK3β. In agreement with these findings Steele *et al* showed that CLL patients had constitutive S6 phosphorylation, the addition of IL-4 to CLL cell culture at a concentration of 10ng/ml *invitro* for 18hours resulted in augmented levels of p-S6(Steele et al. 2010). In the same cohort of patients constitutive basal p-GSK3β could be detected but in contrast to S6 the addition of IL-4 to cultures did not result in increased phosphorylation of this protein (Steele et al. 2010). It is known that $CD4^+$ T-cells secrete IL-4; $CD4^+$ T-cells are situated in close proximity to CLL cells located within the lymph node. In 2001 Kay *et al* showed that CLL cells have the ability to secrete IL-4 (Kay et al. 2001). In addition, CLL cells possess increased numbers of IL-4 receptors compared to normal B-lymphocytes (Kay et al. 2001). A study conducted by Vogler *et al* examined the dual stimulation of CLL cells with CD40L-expressing co-culture and IL-4, representing signals provided by T-cells located in the lymphoid tissues; significant drug resistance was conferred by these interactions (Vogler et al. 2009). However, Burger and Ghandi criticised the Vogler study, as they didn't feel this co-culture system was an appropriate representation of the lymph node microenvironment, they believed that this system did not adequately represent the complex and dynamic signals CLL cells receive *in vivo*(Burger and Gandhi 2009). They went on to suggest that a more accurate *in vitro* model for CLL research would be a nurse-like cell co-culture system, since this model induced a similar gene expression profile to that obtained from CLL cells derived from lymph nodes. In response to this criticism, Vogler admitted that the CD40L/IL-4 model may represent an over exaggerated model of the lymph node microenvironment and hypothesised that only CLL cells in direct cell-cell contact with T-cells *in vivo* would be activated by this system (Vogler et al. 2009).

The next main finding was that basal phosphorylation of STAT6 and ERK could not be detected in the small series of CLL patients analysed. The phosphorylation of STAT6 was strikingly induced following the addition of 5ng/ml IL-4 to CLL cultures; phosphorylated STAT6 could not be detected following CD31 or CD40L ligation alone. Experiments conducted by Steele *et al* also showed that constitutive p-STAT6 was not detectable in unstimulated CLL cells, however the addition of between 1-10ng/ml of IL-

4 to cultures rapidly induced p-STAT6. The addition of IL-4 to CLL culture did not augment the constitutively phosphorylated STAT3 protein in CLL patients, demonstrating STAT6 protein is a specific target of IL-4 signalling (Steele et al. 2010). The results presented in this chapter confirm these findings.

Constitutive p-ERK could also not be detected in any CLL patients analysed. However, following 1-hour CD31-expressing or CD40L-expressing co-culture p-ERK could be detected but exclusively in $CD38^{hi}$ patients. In contrast, Steele *et al* showed that ERK was constitutively phosphorylated in all CLL patients analysed (Steele et al. 2010). These findings may be reflective of the heterogeneity of CLL disease and/or possible technical variations in the way cells were handled, processed and phospho-proteins detected. Muzio *et al* was also able to detect constitutive p-ERK in some CLL patients (Muzio et al. 2008), and showed that phosphorylation of ERK correlated with the Rai disease staging system but no correlations were established with any biological or prognostic markers (Muzio et al. 2008). Interestingly, Muzio *et al* demonstrated that patients with constitutive ERK phosphorylation were unresponsive to BCR stimulation and thus could represent a cohort of patients with a more favourable clinical outcome (Muzio et al. 2008). Most recently, Woyach further characterised CLL patients with constitutive ERK phosphorylation and discovered that 23/52 patients displayed constitutive ERK phosphorylation (Woyach 2013). The phosphorylation of ERK was associated with the absence of PKB phosphorylation and low expression of the poor prognostic markers CD38 and ZAP70 as well as extended CLL cell survival in culture. Woyach proposed that patients with constitutive p-ERK represent an anergic CLL cell subset with an inability to respond to BCR engagement due to chronic antigen stimulation. This anergic CLL cell type is resistant to apoptosis and thus the constitutive phosphorylation of ERK confers a survival advantage. To assess whether it was possible to target the ERK signalling pathway as a means of inducing CLL cell apoptosis in this patient cohort, p-ERK $1/2^{+}$(anergic CLL patients) and p-ERK $1/2^{-}$(non-anergic CLL patients) were treated with ERK inhibitors, resulting in a restored sensitivity to BCR stimulation in the anergic p-ERK $1/2^{+}$ group of patients. Furthermore, following 48-hour treatment with ERK inhibitors the p-ERK$1/2^{+}$ group of CLL patients showed a significant reduction in CLL cell viability when compared to the p-ERK$1/2^{-}$ cohort of patients. It remains uncertain whether treatment with ERK inhibitors is appropriate in this patient cohort since patients with constitutive ERK phosphorylation are associated with an indolent disease type. The maintenance of this

anergic response may prove to be beneficial to the CLL patient in terms of disease progression (Woyach 2013). In contrast to Woyach's findings, work conducted by Hallaert *et al* in 2008 showed that prolonged CD40 stimulation of CLL cells resulted in the increased activation and phosphorylation of ERK, which in turn phosphorylates Bim. This resulted in the proteasomal degradation of this pro-apoptotic BCL2 family member, thereby increasing the survival potential of the CLL cells. Treatment of CLL cells with PD-98059, a phospho-ERK inhibitor, co-cultured on CD40L-expressing fibroblasts inhibited the degradation and loss of Bim. However, when multiple CLL patients were analysed treatment with this p-ERK inhibitor was not sufficient to reduce the resistance to chemotherapeutics conferred in long term CD40L culture. These findings would indicate that although CD40 signalling activates the p-ERK signalling pathway, this pathway is not the cause of drug resistance conferred by CD40L-expressing co-culture (Hallaert et al. 2008).

The results generated in this chapter show that p-ERK was only detected in the $CD38^{hi}$ cohort of patients following co-culture with CD40L expressing co-culture, CD31-expressing co-culture and NTL co-culture. Since CD38 expression is associated with poor prognosis in CLL patients these results are not in concordance with Woyach's data, which correlated constitutive ERK phosphorylation in CLL patients with a good prognostic outcome.

Finally, all the co-culture conditions tested in this chapter conferred some cytoprotection to CLL cells. The differences between the viability conferred by each of the co-culture systems were fairly modest, and at 24 hour and 48 hour timepoints co-culture and IL-4 treatment have the ability to maintain high levels of CLL cell survival. These findings are supported by a study conducted by Cuni *et al*in 2004 who used a non-transfected as well as a CD40L transfected murine cell line to mimic the *in vivo* CLL microenvironment and showed that co-culture of primary CLL cells with the non-transfected cell line was able to support CLL cell survival. Tonino *et al* also examined the effect of CD31 and CD40L ligation on the viability of CLL cells in a cohort of 10 CLL patients, five of which were CD38 positive and five CD38 negative. CLL cells were co-cultured with CD31 and CD40L transfected fibroblasts with and without CD31 and CD40 blocking antibodies respectively. Following 7-day cultures no increased viability was observed following CD31 co-culture. In contrast when CLL cells were cultured with CD40L transfected fibroblasts a significant increase in CLL cell survival

was observed. The expression of apoptosis regulating genes was also analysed following CD31 and CD40L co-culture, no changes were observed in the expression of apoptosis related genes on the CD31-expressing co-culture. In contrast, CD40L co-culture caused the up-regulation of several apoptosis-related genes including BCL-2, BCL-XL, Bfl-1/A1 and Bid as well as Survivin (Tonino S et al. 2008).

In summary, three co-culture systems were utilised in this set of experiments to compare the effect of IL-4, CD31-expressing and CD40L-expressing co-culture as individual stimuli on intracellular signalling pathways in CLL cells as well as CLL cell viability. These experiments showed that CD40L-expressing and CD31-expressing co-culture systems have the ability to induce and augment the phosphorylation of PKB substrates S6 and GSK3β, as well as ERK but they do not have the ability to induce STAT6 signalling. However, the addition of IL-4 was a potent mediator or STAT6 signalling in all CLL patients. It is worthy of note that IL-4 augmented the phosphorylation of PKB substrates S6 and GSK3β as well as ERK but could not induce these signals in the absence of basal phospho-protein expression. These experiments have also shown the ability of these co-culture systems to maintain CLL cell viability *in vitro.*

5 Phenotypic Changes induced by co-culture with CD31-expressing fibroblasts can be reversed by pharmacological inhibition of three survival-signalling pathways

5.1 Introduction

The primary aim of this chapter was to investigate the expression of CLL cell activation markers following CD38 ligation with its non-substrate ligand CD31 and to investigate the role of co-culture stimulated signalling pathways. To further simulate the CLL *in vivo* microenvironment, IL-4 was also added to CD31-expressing co-culture at a concentration of 5ng/ml. The CLL cell surface phenotype induced by CD31-expressing co-culture was compared to CLL cells cultured in liquid culture (LQ) supplemented with IL-4. The analysis of CLL cell surface markers was conducted at a 24-hour time point since a previous study showed this to be the optimum incubation time to facilitate maximal phenotypic changes (Hamilton et al. 2012). The phenotypic markers of interest: CD25, CD38, CD49d and CD69, were evaluated on $CD5^{+}/CD19^{+}$gated lymphocytes to ensure that only CLL cells were included in the analysis. Multi-colour flow cytometry was conducted using the Becton-Dickinson FACSAria flow cytometer and automatic compensation was applied to the full antibody panel using single labelled compensation beads. The MFI values of the cell surface markers of interest were obtained and analysed in a cohort of 12 CLL patients, six of which expressed high surface levels of CD38 ($CD38^{hi}$ >50%) and the remaining six patients expressed low levels of CD38 ($CD38^{lo}$ <5%).

Previous studies have shown that three distinct survival-signalling pathways can be active in primary CLL cells: the PKB/mTOR, JAK/STAT and ERK signalling pathways (Muzio et al. 2008; Steele et al. 2010; Zhuang et al. 2009). Ribosomal S6 is a downstream target of PKB/mTOR signalling (Lawlor and Alessi 2001); STAT6 is a target of JAK3 (Wurster et al. 2000) and ERK is phosphorylated following CD40L ligation (Davies et al. 2004; Steele et al. 2010). Therefore these phospho-proteins were used as readouts for the activation of the respective pathways. To determine the role of these signalling molecules on the phenotype of CLL cells, three pharmacological inhibitors were used to inhibit the pathways defined above. The first inhibitor was Rapamycin, an mTOR inhibitor (Argyriou et al. 2012; Huang et al. 2003), the mTOR

protein is a target of PKB signalling and is located upstream of ribosomal S6.Inhibition of mTOR prevents the phosphorylation of S6. PF956980 is an inhibitor of JAK3(Changelian et al. 2008)the activation of this kinase leads to the phosphorylation and activation of STAT6 and PF956980 effectively inhibits JAK3 phosphorylation. Finally U0126 was used; this inhibitor directly prevents the phosphorylation of ERK (Duncia et al. 1998). Inhibitors were added to $2x10^6$ CLL cells in IL-4 supplemented media for 30 minutes. After 30 minutes, the CLL cell/inhibitor mixes were added to CD31-expressing co-culture for 24 hours or kept in IL-4 supplemented LQ for 24 hours. CLL cells were analysed for viability using Annexin V/PI staining. A separate aliquot of CLL cells was used for phenotypic analysis on a FACSAria flow cytometer.

5.2 Results

5.2.1 Phosphorylation of STAT6, ribosomal S6 and ERK were inhibited following treatment with PF956980, Rapamycin and U0126 respectively

The first step was to examine the effectiveness and specificity of the three inhibitors used. The dose of inhibitor required to inhibit the phosphorylation of STAT6, S6 and ERK proteins was established in earlier experiments (Figure 4.9; PF956980 - 5μM, Figure 3.11; Rapamycin - 5nM, and Figure 4.9; U0126 - 10μM, respectively).

In order to assess the specificity of the inhibitors, CLL cells were cultured in LQ supplemented with IL-4, or in the presence of CD31-expressing co-culture for a 24-hour time point. Each inhibitor was added at the concentrations established to diminish the phosphorylation of the target protein. The three inhibitors were pre-incubated with $1x10^6$ CLL cells for 30 minutes prior to being added to CD31 co-culture to avoid the uptake of the inhibitors by the CD31-expressing co-culture layer. After 24 hours, CLL cells were removed from cultures, proteins were resolved by SDS-PAGE followed by western blotting and detection with three phospho-specific antibodies directed towards STAT6, S6 and ERK.

Figure 5.1 depicts a $CD38^{hi}$ patient sample with basal ERK phosphorylation and basal S6 phosphorylation. The same sample showed IL-4-induced STAT6 phosphorylation that was completely inhibited following treatment with the JAK3 kinase inhibitor PF956980. In contrast, the phosphorylation of STAT6 was maintained following treatment with Rapamycin and U0126. Similarly, treatment with Rapamycin completely inhibited the phosphorylation of S6 but the p-S6 band was still detectable following treatment with PF956980 and U0126. Finally treatment of CLL cells with U0126 completely eliminated the p-ERK band; this effect was not observed following treatment with PF956980 and Rapamycin. Taken together, these data illustrate the effectiveness and selectivity of the three inhibitors used in this study.

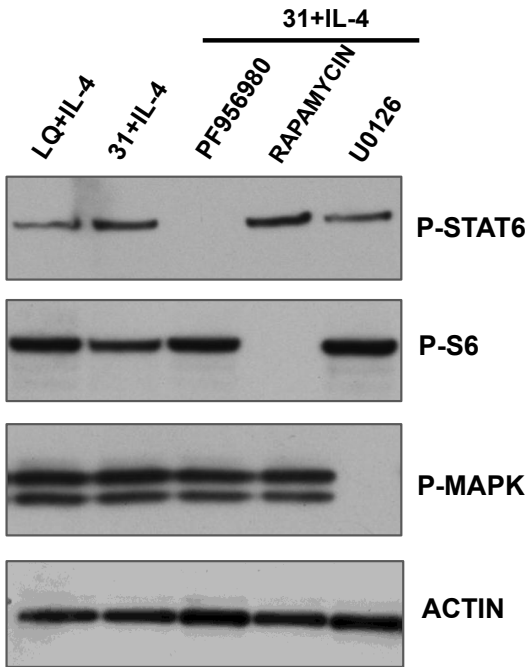


Figure 5.1 Analysis of protein phosphorylation of P-STAT6, P-S6 and P-ERK following inhibition with three pharmacological inhibitors at 24 hours.

Primary CLL cells were incubated with LQ+IL-4 or CD31-expressing co-culture (31) for 24 hours in the presence of pharmacological inhibitors. $1x10^6$ CLL cells were pre-incubated for 30 minutes with PF956980, Rapamycin and U0126 independently at concentrations of 5μM, 5nM and 10μM respectively. CLL cells were then added to CD31-expressing co-culture with IL-4. Following 24 hours in culture CLL cells were removed; protein was extracted and resolved by SDS-PAGE followed by western blotting and detection with P-STAT6, P-S6 and P-ERK antibodies to confirm specific inhibition of these pathways.

5.2.2 Significant changes in CLL cell viability were observed following CD31 co-culture and treatment with inhibitors

It is now well established that signals received by CLL cells from the *in vivo* microenvironment are drivers of CLL cell survival (Audrito et al. 2013; Burger 2011). Therefore, inhibition of specific signaling molecules may disrupt the supportive interactions provided by stromal cells and result in increased cell death.

To assess the effect of inhibition of three signalling pathways on CLL cell viability,CLL cells were treated with the PF956980, Rapamycin and U0126 for 24 hours on CD31 co-culture. After this time-point a 200µl aliquot of the cell suspension was removed from cultures and stained with AnnexinV/PI and analysed by flow cytometry. The CLL cell viability under the different culture conditions was then compared. Figure 5.2 shows the Annexin V/PI plots for the culture conditions and inhibitors used. Figure 5.3 shows that a significant increase was observed in CLL cell viability in cells incubated in CD31-expressing co-culture when compared to LQ (P=0.001). Following treatment with PF956980 and U0126 a significant decrease in cell viability (P=0.0001 and P=0.01 respectively) was observed. In contrast, CLL cell viability was not significantly altered following treatment with Rapamycin (P=0.1125).

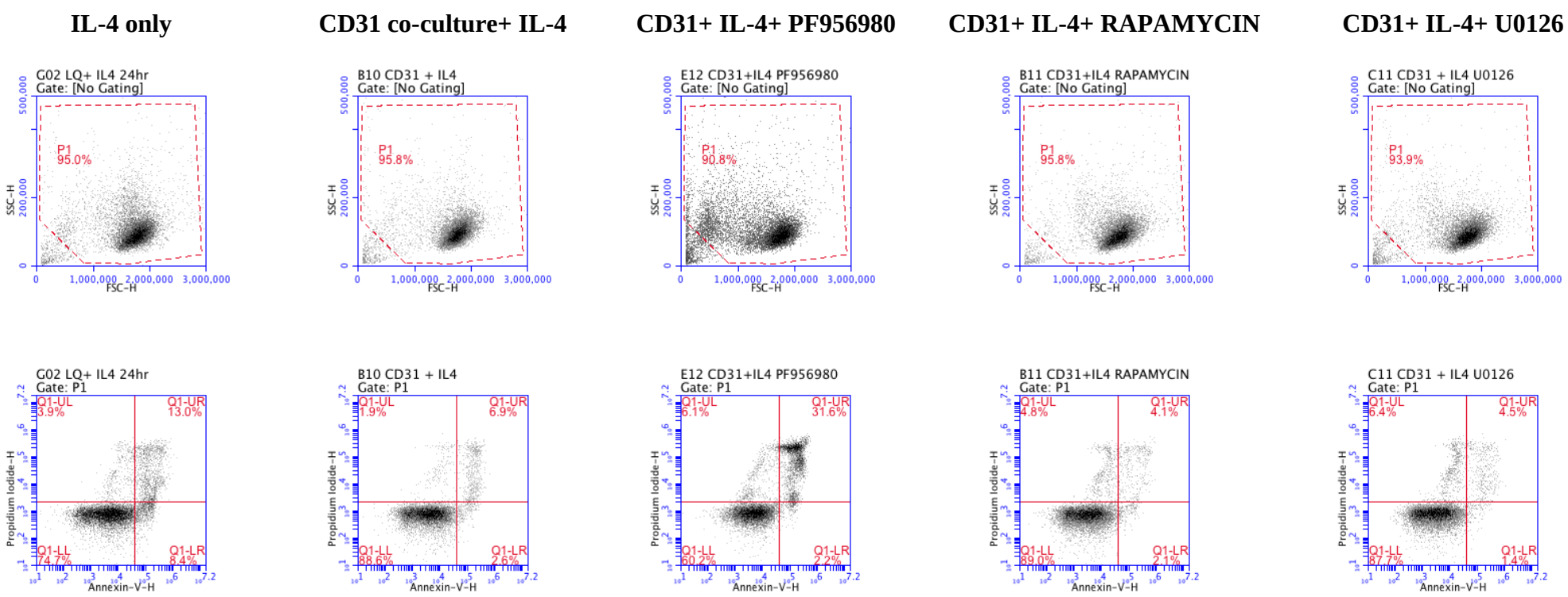


Figure 5.2 Example of CLL patients Annexin V/PI plot for different culture conditions and treatments with inhibitors

Annexin V/PI plots from a patient sample following treatment with PF956980 (10µm/ml), Rapamycin (5nm/ml) and U0126 (10µm/ml).

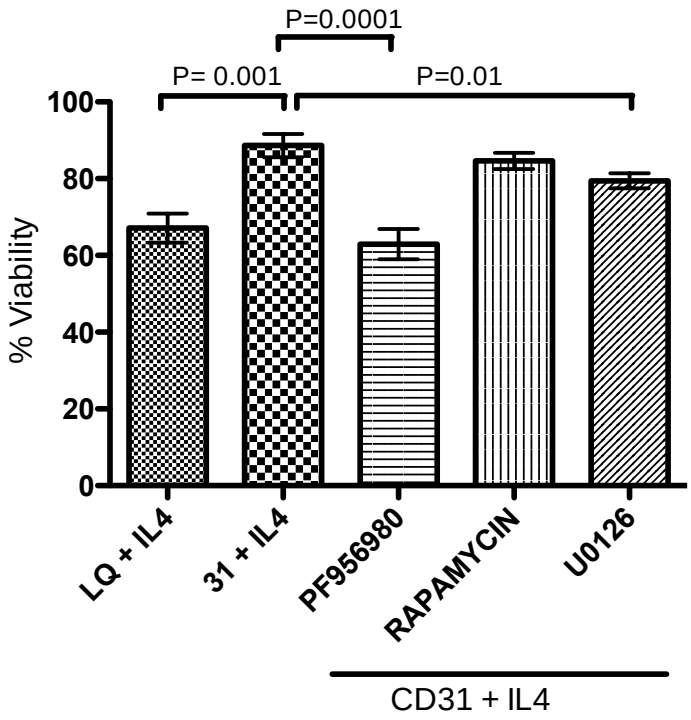


Figure 5.3 Changes were observed in CLL cell viability following CD31 co-culture as well as following treatment with pharmacological inhibitors PF956980 and U0126.

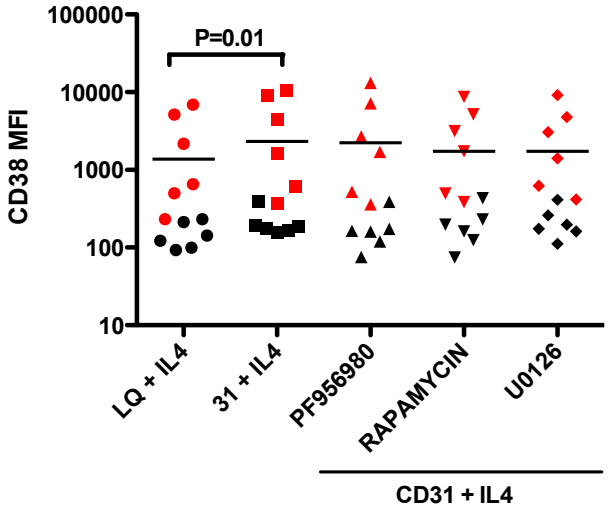
$2x10^5$ CLL cells were removed from the co-cultures following 24 hours in culture. CLL cells were then labelled with Annexin V/Propidium Iodide in order to determine their viability. A total of 12 patients were analysed and a repeated measures ANOVA test was used to compare the different culture conditions and inhibitor treatments. Co-culture on CD31-expressing fibroblasts increased CLL cell viability and this was partially reversed by the addition of PF956980 and U0126.

5.2.3 The inhibition of signalling molecules S6, STAT6 and ERK did not alter CD38 expression

CD38 is not only a cell surface receptor but also a recognised marker of cellular activation(Deaglio 2010). Therefore, measuring the CD38 expression may provide an index of CLL cell activation status. In the following experiments, CLL cells from 12 patients, six $CD38^{hi}$ (>50%) and six $CD38^{lo}$ (<5%), were either cultured in LQ supplemented with IL-4, or in the presence of CD31-expressing co-culture for 24 hours. Inhibitors PF956980, Rapamycin and U0126 were added to CD31-expressing co-culture to target STAT6, S6 and ERK signalling pathways respectively. The phenotypic markers of interest: CD25, CD38, CD49d and CD69, were evaluated on $CD5^{+}/CD19^{+}$gated lymphocytes to ensure that only viable CLL cells were included in the analysis.

The MFI values for CD38 expression for the 12 patient samples are shown in Figure 5.4, and the red dots represent $CD38^{hi}$ patients. Figure 5.4a shows that CD38 MFI values were significantly increased when CLL cells were incubated with CD31-expressing co-culture compared to CLL cells incubated in LQ supplemented with IL-4 for 24 hours (P=0.01). Figure 5.4b shows the CD38 expression before and after co-culture a line graph to link individual patient samples to show the significant change in CD38 values following CD31 and IL-4 co-culture. Addition of the pharmacological inhibitors PF956980, Rapamycin and U0126 did not reverse the changes in CD38 expression induced by CD31-expressing co-culture.

a



b

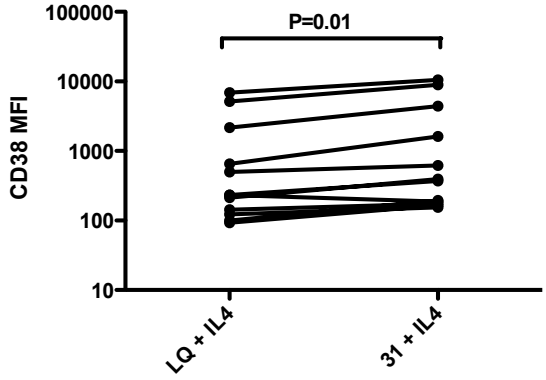


Figure 5.4 CD38 expression was increased following 24 hours in CD31-expressing co-culture.

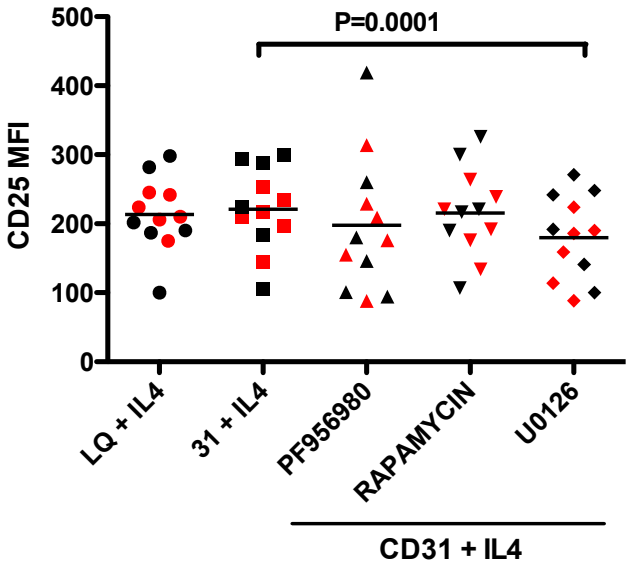
$1x10^6$ CLL cells were placed into co-culture with CD31-expressing fibroblasts supplemented with 5ng/ml IL-4. (a) Surface CD38expression was measured by flow cytometry after 24 hours and the MFI values were plotted for each CLL patient sample. The red dots represent $CD38^{hi}$ patients. A repeated measures ANOVA test was used to compare CD38MFI between conditions. (b) The line-graph represents paired samples cultured under the two conditions.

5.2.4 The ERK inhibitor,U0126 inhibited the increase in CD25

CD25 makes up the alpha chain of the IL-2 receptor and is a type I transmembrane protein that is present on activated B-cells and T-cells(Shvidel et al. 2012). CD25 expression has been found to be significantly higher in CLL cells when compared to normal B-lymphocytes(Damle et al. 2002). A retrospective study conducted by Shvidel *et al* looked at a cohort of 281 patients of which 46 were found to have very high expression of CD25. Shvidel *et al* discovered that CD25 expression correlated with CD38 expression levels but not with other prognostic factors such as Binet stage, circulating lymphocyte count and ZAP70 expression. Furthermore, no correlation was found between CD25 expression and TTFT or OS, thus it was concluded that CD25 does not hold prognostic relevance in CLL (Shvidel et al. 2012)..

The MFI CD25 values of the 12 patient samples are shown in Figure 5.5. Figure 5.5a shows that there was no significant change in CD25 MFI following CD31-expressing co-culture when compared to LQ supplemented with IL-4 after 24 hours. However, treatment of CLL cells with the ERK inhibitor U0126 on CD31-expressing co-culture and IL-4resulted in a significant decrease in CD25 MFI (P=0.0001) when compared to CD31-expressing co-culture and IL-4 only. Treatment with PF956980 caused a decrease in CD25 expression but it was not statistically significant probably due to the variable response of the individual samples. Following treatment with Rapamycin no differences in CD25 MFI were observed. In contrast to the study of Shvidel *et al*(Shvidel et al. 2012), no correlation was apparent between levels of CD25 expression and CD38 expression in this small series.

a



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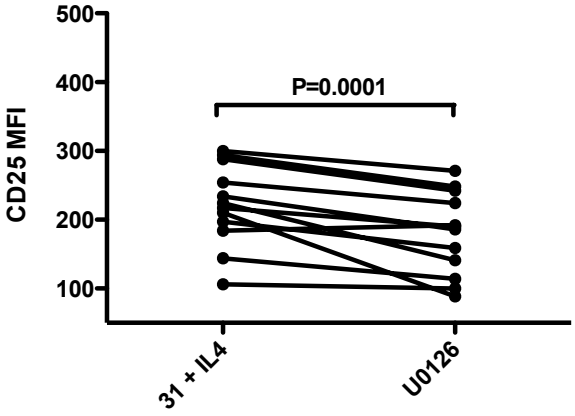


Figure 5.5 A decrease in CD25 expression was observed on CD31 co-culture following treatment with U0126.

$1x10^6$ CLL cells were placed into co-culture with CD31-expressing fibroblasts supplemented with 5ng/ml IL4. (a) Surface CD25 was measured by flow cytometry after 24 hours and the MFI values were plotted for each CLL patient sample. A repeated measures ANOVA test was used to compare CD38 expression. The red dots represent MFI values from $CD38^{hi}$ patients (b) The line-graph represents paired samples from the two conditions, which showed significant change in CD25 MFI.

5.2.5 Co-culture with CD31-expressing fibroblasts induces CD49d expression on CLL cells which can be blocked by all three pharmacological inhibitors tested

CD49d is an integrin alpha subunit, which constitutes one half of the α4β1 integrin receptor(Pacheco et al. 1998). The expression of CD49d is very heterogeneous in CLL patients and elevated CD49d expression has been associated with a poor clinical outcome(Gattei et al. 2008). Numerous studies have highlighted the prognostic relevance of this molecule(Gattei et al. 2008; Majid et al. 2011; Rossi et al. 2008; Shanafelt et al. 2008). Majid *et al* analysed CD49d expression in a cohort of 652 CLL patient samples and showed that this marker is associated with a shorter TTFT and OS (Majid et al. 2011). In another large-scale study by Gattei *et al* CD49d showed prognostic independence(Gattei et al. 2008). Shanafelt *et al*have also shown that CD49d expression is correlated with other poor prognostic markers in CLL, including CD38 and ZAP70(Shanafelt et al. 2008). It has also been hypothesised that CD49d expression is associated with the up-regulation of CD38(Shanafelt et al. 2008)and acts in a macromolecular complex with CD38, CD44 and MMP9 in CLL cells (Buggins et al. 2011). However, it has not yet been established whether CD38 ligation with its ligand CD31 has an effect on CD49d expression.

The MFI values for CD49d expression for the 12 patient samples are shown in Figure 5.6. Figure 5.6a shows that a significant increase in CD49d MFI was observed following CD31-expressing co-culture with IL-4 when compared to LQ and IL-4 culture (P=0.01) after 24 hours. Furthermore, significant decreases in CD49d MFI were detected following treatment with PF956980 and Rapamycin (P=0.01 and P=0.001, respectively). Figure 5.6b and Figure 5.6c show the changes in CD49d expression in individual patient samples following treatment with PF956980 and Rapamycin compared to CD31 and IL-4 co-culture.

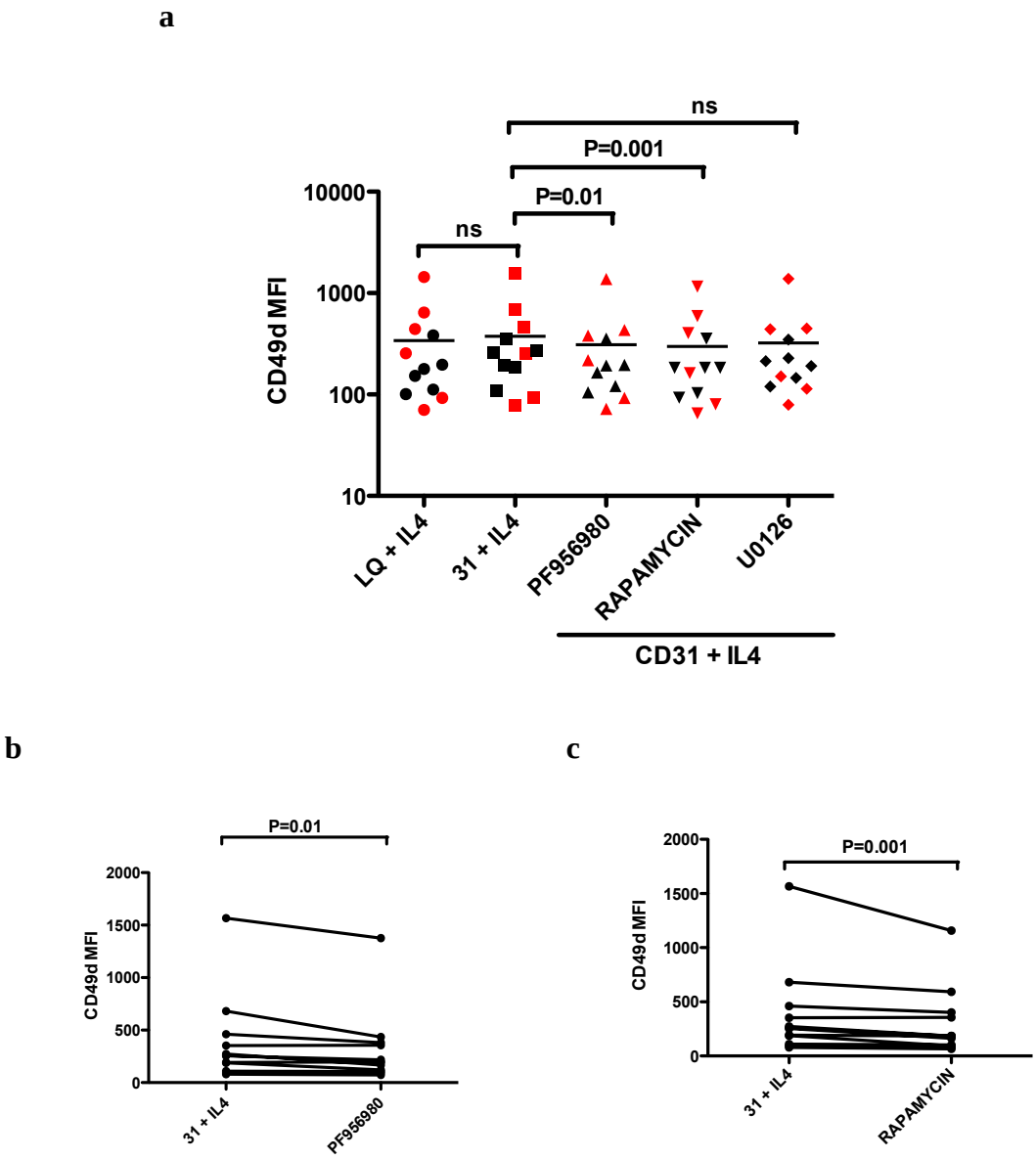


Figure 5.6 The expression of CD49d is induced following CD31 co-culture and is repressed by treatment with inhibitors of three signalling pathways

$1x10^6$ CLL cells were placed into co-culture with CD31-expressing fibroblasts supplemented with 5ng/ml IL-4. (a) Surface CD49d was measured by flow cytometry after 24 hours and the MFI values were plotted for each CLL patient sample. The red dots represent MFI values from $CD38^{hi}$ patients. A repeated measures ANOVA test was used to compare CD49d expression following culture under the various conditions. Figures b and c show line graphs showing the significant changes in CD49d MFI between paired samples.

5.2.6 Significant changes in CD69 were observed following CD31 ligation and treatment with the ERK inhibitor PF956980

CD69 is a type II integral membrane protein with a single transmembrane domain and belongs to the C-type lectin family of surface receptors (Del Poeta et al. 2012). Normal B-cells express high levels of CD69 during the early stages of B-cell development and CD69 is the earliest identifiable inducible cell surface glycoprotein acquired through lymphocyte activation(Del Poeta et al. 2012). CD69 is known to be involved in proliferation but also functions as a signal transmitting receptor in lymphocytes(D'Arena et al. 2001; Del Poeta et al. 2012).A preliminary study conducted by Bigler *et al* in 1988 proposed that CD69 could be of prognostic significance in CLL but independent prognostic value was not attained at this time(Bigler 1988). A subsequent study conducted by Guarini *et al* analysed a large cohort of patients and showed that high CD69 expression (>30% of CLL cells) was significantly correlated with other established CLL prognostic markers including CD38, CD49d, and ZAP70 (Guarini et al. 2008).

The MFI CD69 values of the 12 patient samples are shown in Figure 5.7. A significant increase in CD69 MFI was observed in CD31-expressing co-culture with IL-4when compared to LQ supplemented with IL-4 (P=0.01) after 24 hours. This elevated CD69 expression was maintained following treatment with PF956980 and Rapamycin. However, a significant decrease in CD69 MFI was observed following treatment with U0126 compared to CD31-expressing co-culture with IL-4 (P=0.001). There was no significant correlation found between CD69 expression and expression in this cohort of patients.

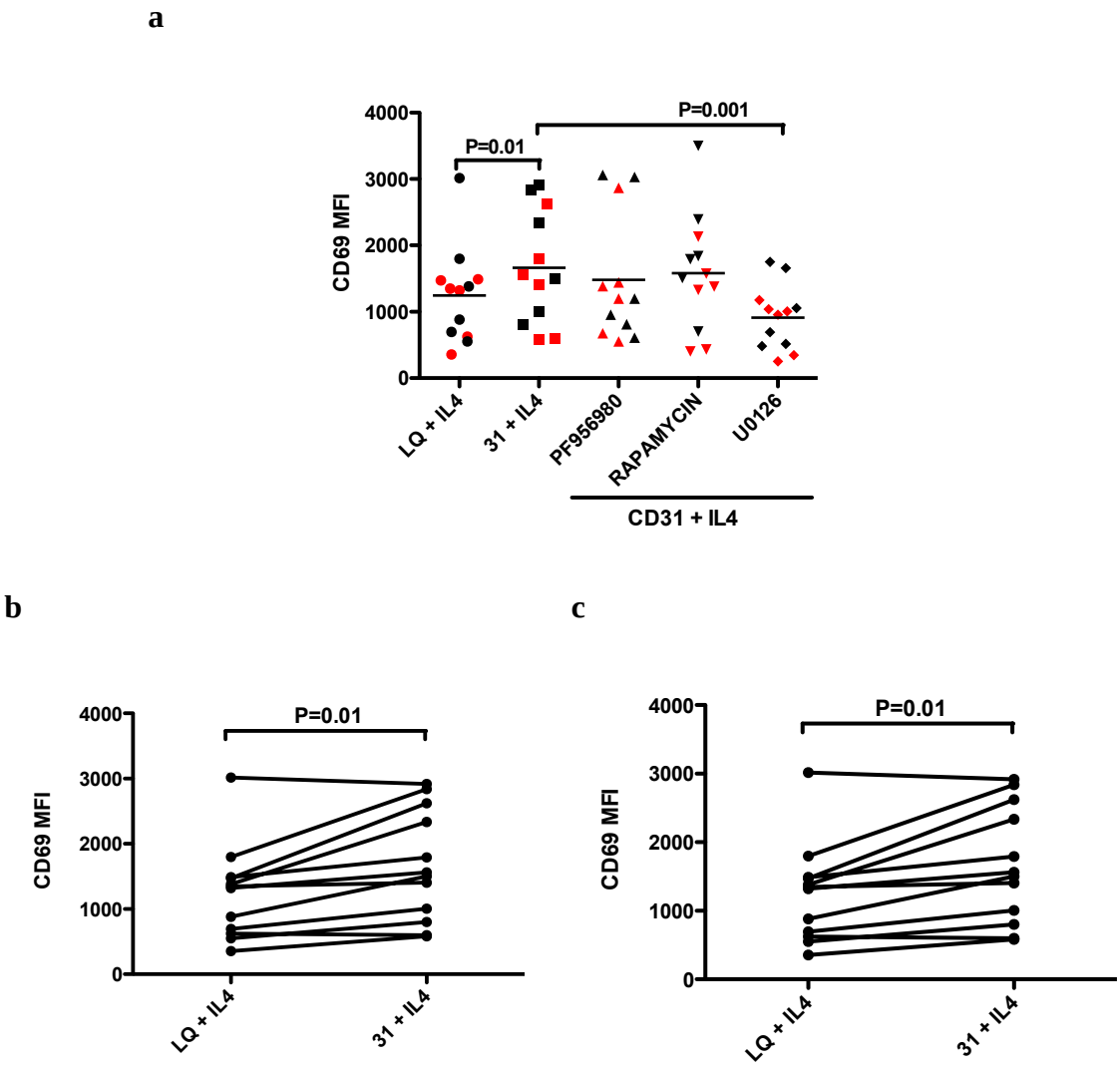


Figure 5.7 The expression of CD69 is induced following CD31 co-culture and repressed following treatment with U0126

$1x10^6$ CLL cells were placed into co-culture with CD31-expressing fibroblasts supplemented with 5ng/ml IL-4. (a) Surface CD69 was measured by flow cytometry after 24 hours and the MFI values were plotted for each CLL patient sample. The red dots represent MFI values from $CD38^{hi}$ patients. (b and c) A repeated measures ANOVA test was used to compare CD69 expression; the line graphs represent conditions that showed significant differences.

5.3 Discussion

The aim of this chapter was to investigate the expression of surface molecules associated with CLL cell activation following CD31 ligation and to assess whether the inhibition of three signalling pathways altered CLL cell viability or immunophenotype. Multi-colour flow cytometry was used in this set of experiments to analyse 12 patient samples; six of these patient samples had high surface expression of CD38 ($CD38^{hi}$) and six had low surface expression of CD38 ($CD38^{lo}$). Four phenotypic markers were analysed following 24-hour CD31-expressing co-culture. CD38 and CD49d were markers selected since both of these cell surface proteins have proven to be valuable prognostic indicators in predicting disease outcome in CLL (Gattei et al. 2008; Majid et al. 2011; Shanafelt et al. 2008). CD69 represents the earliest activation antigen on lymphocytes and regulates immune responses (Damle et al. 2002), and CD25 is the receptor for IL-2 (Decker et al. 2010), which is known to be a potent mediator of CLL cell survival. The activation status of B-cells is known to be associated with clear changes in the expression of cell surface molecules.

After confirmation that p-S6, P-STAT6 and p-ERK could be successfully inhibited in CLL cells by western blot analysis, the first observation in this set of experiments was that the viability of CLL cells was significantly reduced by the inhibition of p-STAT6 with PF956980, and p-ERK with U0126, however cell viability was not altered following the inhibition of p-S6 with Rapamycin. Experimental evidence has shown that activated STAT-6 can enhance the expression of the anti-apoptotic protein BCL-XL (Wurster et al. 2002); this in turn may contribute to the pro-survival effects observed following treatment of CLL cells with IL-4. It is worthy of note that BCL-XL protein is usually only detected in lymph node samples from CLL patients; this supports the importance of pro-survival signals received by the microenvironment (Smit et al. 2007). CLL cells located within the lymph node and bone marrow microenvironments are exposed to elevated levels of cytokines such as IL-4 as well as intracellular signalling pathways that raise the apoptotic threshold of CLL cells which may lead to resistance to current chemotherapeutic therapies (Dietrich et al. 2012). Dietrich *et al* demonstrated that treatment of CLL cells with the JAK3 inhibitor PF956980 could overcome IL-4 mediated resistance to fludarabine treatment (Dietrich et al. 2012). Previous experiments carried out by Steele *et al* in 2010 showed that the addition of IL-4 to primary CLL cells resulted in the rapid phosphorylation and activation of STAT-6

(Steele et al. 2010). The addition of this cytokine prevented fludarabine and chlorambucil-mediated killing of CLL cells. However, treatment with PF956980 reversed any cytoprotective effect observed with IL-4 (Steele et al. 2010). The killing effect of this inhibitor was not correlated with *IGHV* mutational status or the expression of prognostic markers CD38 and ZAP70. Therefore, therapies such as PF956980 may provide a useful strategy for circumventing the cytoprotective effect of the microenvironment in CLL patients, even those with a poor prognosis. Interestingly Steele *et al* also showed that CLL cultures without IL-4 had enhanced PF956980-induced cell death in some CLL patient samples. Indeed, it was shown that constitutive tyrosine phosphorylation of STAT3 is evident in some CLL samples. STAT3, like STAT6, has been shown to confer anti-apoptotic capabilities to CLL cells, and treatment with PF956980 completely abrogated the phosphorylation of STAT3 as well as STAT6. It may be the case that patients who display constitutive phosphorylation of STAT3 are sensitive to treatment with PF956980 in the absence of IL-4 (Steele et al. 2010).

This set of experiments also showed that treatment with U0126 has a potent effect on CLL cell viability. The phosphorylation and activation of ERK following BCR engagement has been shown to mediate the survival and growth of cells in human malignancies (Fang et al. 2012; Junttila et al. 2008). Platanias *et al* (2003) showed that ERK and upstream effectors MEK1/2 are constitutively activated in many patients with acute myeloid leukaemia (AML) as well as patients with chronic myeloid leukaemia (CML), indicating that the phosphorylation of ERK is a feature of myeloid malignancies (Platanias 2003). In 2012 Paterson *et al* used the ERK inhibitor U0126 *in vitro* in a cohort of CLL patient samples and revealed a heterogeneous response in terms of apoptosis. Some patients showed increased levels of apoptosis following treatment with this inhibitor whilst other patients were not responsive to this treatment and apoptosis levels remained low (Paterson et al. 2012). Patients who are responsive to this treatment may display constitutively phosphorylated ERK, whereas patients unresponsive to this inhibitor may not have any constitutive ERK phosphorylation. Immunohistochemical studies have revealed that CLL cells taken from the lymph nodes have increased levels of p-ERK indicating that this pathway is activated *in vivo* and thus represents a promising therapeutic target to counteract the protective effect of microenvironmental stimuli.

Treatment of CLL cells with the mTOR inhibitor Rapamycin resulted in a significant decrease in CD49d expression, but did not result in a significant increase in CLL cell apoptosis. In support of these findings, Decker *et al* showed that treatment of CLL cells with Rapamycin could effectively inhibit cell cycle progression and down-regulate the anti-apoptotic protein Survivin but was unable to induce increased levels of apoptosis in CLL cells (Decker et al. 2003). Decker postulated that whilst treatment with Rapamycin was ineffective in killing CLL cells, Rapamycin is effective in negating the proliferation of CLL cells which ultimately drives the progression of this disease. Furthermore, it may be the case that treatment of CLL cells with Rapamycin makes CLL cells more sensitive to killing with other therapeutic agents (Decker et al. 2003). In contrast to these observations Aleksog *et al* 2008 showed that Rapamycin was cytotoxic to CLL cells in a dose-dependent manner. However the efficacy of this inhibitor was limited to patients who possessed poor prognostic markers (Aleskog et al. 2008).

Other inhibitors of the PKB signalling pathway have been used *in vitro* to assess the effect on CLL cell viability. De Frias *et al* (2009) used PKB inhibitors Akt-1/2 and A-443654 and showed that both of these inhibitors induced apoptosis in CLL patients in a dose-dependent manner (de Frias et al. 2009). Treatment with survival factors IL-4 and SDF-1α were unable to protect the cells from apoptosis induced by both PKB inhibitors Akt-1/2 and A-443654. Treatment with both of these inhibitors resulted in augmented pro survival PUMA and NOXA protein levels and a decrease in anti-apoptotic protein MCL1 (de Frias et al. 2009).

The second observation in this set of experiments was that significant changes in the CLL cell immunophenotype could be detected following incubation of CLL cells for 24 hours in CD31-expressing co-culture supplemented with IL-4. CLL cells up-regulated CD38, CD49d and CD69 when compared to expression levels of these molecules when incubated with LQ supplemented with IL-4 for the same time period. In contrast, CD31-expressing co-culture failed to induce significant changes in CD25 expression on CLL cells implying that the regulation of CD25 is independent of CD38/CD31 signalling but that CD38, CD49d and CD69 can be modulated directly/or indirectly through CD38/CD31 interactions or other co-culture interactions. Interestingly CD69 has been shown to correlate with the expression of both CD49d and CD38 in CLL cells (Del Poeta et al. 2012). CD38, CD49d and CD69 have also been shown to enhance BCR

signalling which may influence the survival and proliferative potential of the CLL cells (Del Poeta et al. 2012).

In this set of experiments there was no significant difference in the expression of CD25, CD69 or CD49d between $CD38^{hi}$ CLL samples and $CD38^{lo}$ CLL samples under any of the conditions tested. However, it should be acknowledged that this study represents a very small series and may be subject to sampling error. A larger study conducted by Damle *et al* in 2007 showed that the expression of CD38 on the CLL cell surface is associated with an increase in cell surface activation markers. The $CD38^{hi}$ cohort of patients displayed a significant increase in the percentage of CLL cells expressing activation markers CD40, CD69 and CD79b (Damle et al. 2007).However, the increased expression of activation markers in the $CD38^{hi}$ cohort of patients may be indicative of temporal differences in the activation of the CLL cell rather than differences in response to stimulation. Work undertaken by Deaglio *et al* in 2010 focused on identifying genome-wide transcriptional events following 5-day culture on CD31-expressing co-culture systems. Deaglio showed using microarray analysis that CD31 co-culture induced distinct signalling pathways in CLL and interestingly three such pathways included mTOR, ERK as well as JAK/STAT signals (Deaglio 2010).

The next observation in this set of experiments was that inhibiting intracellular signalling resulted in changes in the CLL cells immunophenotype. Firstly, CD49d expression was significantly down regulated following treatment with inhibitors PF956980 and Rapamycin. It is broadly accepted that CD49d is an independent prognostic indicator in CLL patients; increased expression of this molecule is associated with an advanced and progressive disease type (Gattei et al. 2008; Majid et al. 2011; Rossi et al. 2008). CD49d aids leukocyte migration and trafficking and the increased surface expression of CD49d has been linked with the ability for CLL cells to migrate to growth permissive microenvironments such as lymphoid tissues (Deaglio 2010; Rose et al. 2002). CD49d also possesses receptor capabilities; the amplified expression of CD49d is associated with the up-regulation of members of the pro-survival BCL2 family as well as chemokines CCL3 and CCL4(Zucchetto et al. 2009). A correlation has been established between CD49d expression and the expression of CXCR4, a chemokine receptor involved in haematopoietic mobilisation and trafficking(Majid et al. 2011).The prognostic relevance of CD49d makes this molecule an attractive therapeutic target, but little is understood regarding the signals involved in the regulation of CD49d

expression. The results generated in this chapter implicate STAT6 and S6 signals in the regulation of CD49d expression in CLL. Interestingly Geutskens showed that CD49d expression is modulated by the phosphorylation state of ERK in human dendritic cells (Geutskens et al. 2004). Furthermore, Sasaki *et al* has examined CD49d expression on $CD8^+$ T-cells following IL-4/STAT6 stimulation, a significant down-regulation of CD49d expression was observed following stimulation of the STAT6 pathway (Sasaki et al. 2008). It may be the case that IL-4 signalling in growth permissive microenvironments such as the lymph nodes leads to a reduction of CD49d expression on $CD8^+$ T-cells preventing the recruitment of these cells to the lymph node microenvironments and thus reducing the anti-tumour activity of these T-cells aiding in disease progression. The converse may be true in terms of CD49d expression on CLL cells, the increased exposure of CLL cells to IL-4/STAT6 signalling may augment the expression of CD49d on the CLL cell surface resulting in the attraction of CLL cells to growth permissive microenvironments. Natalizumab is a humanised anti-CD49d monoclonal antibody, which is already in clinical use for the treatment of multiple sclerosis and Crohn's disease which may be a potential therapeutic suitable for the treatment of CLL (Dal-Bo et al. 2009).

The expression of CD25 was not augmented following CD31-expressing co-culture. However, the inhibition of p-ERK with U0126 resulted in a significant down regulation of CD25 MFI when compared to CLL cells incubated in CD31-expressing co-culture and IL-4. This finding indicates that ERK signalling is involved in the regulation of this antigen. CD25 is not deemed to be an independent prognostic marker in CLL (Shvidel et al. 2012). However, a study conducted by Hjalmar *et al*(2002)showed that CD25 provided some prognostic information; patients with 30% or greater $CD25^+$ CLL cells had shorter time to first treatment than patients with lower CD25expression(Hjalmar et al. 2002). The elevated expression of CD25 is associated with the increased activation of CLL cells, and is linked to augmented levels of proliferation (Damle et al. 2002). A CD25 inhibitor in clinical use is LMB-2, which is a recombinant immune toxin directed towards the α chain of the IL-2 receptor (Decker et al. 2002). LMB-2 has been used to effectively treat hairy cell leukaemia (HCL) (Kreitman et al. 1999) and has been used in CLL patients but only 1/8 CLL patients treated were sensitive to this immune toxin (Kreitman et al. 2000). A possible explanation for this poor response is thought to be due to the high levels of this IL-2 receptor on the surface of HCL cells compared to CLL cells (Decker et al. 2002). Decker *et al* looked at whether they could enhance the

anti-tumour effect of LMB-2 in CLL cells by up-regulating the levels of CD25 on the CLL cell surface. They stimulated CLL cells with an oligonucleotide containing a CpG nucleotide and discovered that the modulation of CD25 in this way resulted in an increased response in CLL cells to LMB-2 treatment. Interestingly, the same levels of toxicity were not observed in normal B- and T- lymphocytes (Decker et al. 2002).

The expression of CD69 was significantly down regulated following the treatment of CLL cells with the ERK inhibitor U0126. CD69 is upregulated following CLL cell activation and is associated with a poor clinical outcome (D'Arena et al. 2001; Del Poeta et al. 2012). Del Poeta *et al* showed that surface CD69 expression was up regulated on CLL cells located in the lymph nodes and bone marrow, this finding is not surprising considering CD69 is associated with retaining lymphocytes at the site of stimulation, and the levels of CD69 on circulating CLL cells may be much lower for this reason(Del Poeta et al. 2012). CLL cells with increased levels of CD69 in the peripheral blood may be indicative of malignant cells that have recently left the lymph node or bone marrow microenvironments. In a recent study Wobke *et al* looked at the effect of ERK inhibition on CD69 expression in a human acute monocytic leukaemia (AML) cell line; following the treatment of cells with ERK inhibitors PD98059 and U0126 a reduction in CD69 mRNA was observed. This finding implicates ERK signalling in the regulation of CD69 in monocytes (Wobke et al. 2013) and this present study indicates that the same is true for CLL B-cells. Anti-CD69 antibody therapies have been used in animal models with resulting reductions in tumour burden and metastasis (Esplugues et al. 2005); such therapies could be beneficial for the treatment of CLL. However, the targetting of CD69 may lead to the increased death of normal haematopoietic progenitors, with resulting enhanced risk of immunodeficiency and infections. Hinton *et al* (2006) revealed a relationship between CD69 expression and the phosphorylation of ribosomal S6 in T-cells. It is known that increased CD69 expression on the surface of T-cells is a marker of maturation, T-cells with high CD69 expression also showed increased intracellular ribosomal S6 phosphorylation indicating that these molecules are functionally linked (Hinton et al. 2006), however the inhibition of S6 phosphorylation in these experiments did not significantly alter the expression of CD69 so this may not be the case in CLL cells.

This set of experiments demonstrated that CD31-expressing co-culture may have a role in the up-regulation of the cell surface activation markers CD38, CD49d and CD69 in

primary CLL patients, however since NTL co-culture was not used in this set of experiments the effect of the CD31 ligand cannot be assessed. These set of experiments also helped to establish the role of individual signalling pathways in CLL cells *in vitro*. The inhibition of p-STAT6 with PF956980 resulted in a significant reduction in CLL cell survival as well as a down regulation of cell surface activation marker CD49d. The inhibition of p-S6 with Rapamycin had no effect on CLL cell viability but resulted in a down-regulation of CD49d. Finally, inhibition of CD69 with U0126 resulted in reduced CLL cell viability as well as a down-regulation of CLL cell activation markers CD25, CD49d and CD69. The changes induced in these activation markers through the use of pharmacological inhibitors may represent targets to counteract the protective effect of signalling *in vivo*.

6 The phosphorylation of S6 and ERK is significantly increased in CD38^{hi} CLL patients

6.1 Introduction

The primary aim of this chapter was to quantify the effects of stimulation of primary CLL cells via CD38 ligation and IL-4 treatment in terms of the expression and phosphorylation of selected proteins using multi-colour flow cytometry. Given the findings of the preceding chapters, the expression of the cell surface markers CD38 and CD69, and the levels of the intracellular phospho-proteins S6, STAT6 and ERK, were quantified following 1 and 24 hour exposures to the stimuli.

Multi-colour flow cytometry allows for the detection of multiple proteins in a single patient sample, facilitating the simultaneous identification and quantification of surface and intracellular antigens within a specific cell population. In recent years, a number of companies have taken this concept further, by developing antibodies that can be used to analyse the phosphorylation state of proteins of interest quantitatively using flow cytometry. This methodology requires far fewer cells that western blot analysis and potentially also provides information on the intensity and kinetics of activated signalling targets.

In 2012 Blix *et al* utilised phospho-specific flow cytometry to identify and quantitate differences in the activation of signalling molecules in CLL cells compared to normal B-cells taken from healthy donors (Blix et al. 2012). Initially Blix *et al* first analysed the basal phosphorylation levels of several signalling proteins, STAT6, ERK and S6 were measured in this study. Elevated basal p-STAT6 and p-ERK were detected in CLL cells compared to healthy donors. In contrast, basal p-S6 levels in CLL cells were comparable to p-S6 found in normal B-cells. However, following BCR stimulation with anti-IgM increased levels of both p-S6 and p-ERK were detected in CLL cells but not in normal B-cells (Blix et al. 2012).

Perl *et al* (2012) also used phospho-flow cytometry to monitor levels of phospho-proteins in serial AML patient peripheral blood samples (Perl et al. 2012). The phosphorylation and activation of the PI3K/PKB/mTOR signalling pathway has been associated with the resistance of AML cells to chemotherapy. Indeed, the phosphorylation levels of S6 were used as readout of mTOR activity and provided a quantitative measure of the sensitivity of AML cells to mTOR inhibition. Importantly,

this study demonstrated the ability to monitor individual patient responses to drug treatment using phospho-protein measurements and provided a proof-of-concept that this approach may be useful in other diseases including CLL (Perl et al. 2012).

An alternative multiplex approach for the analysis of phospho-proteins is the use of microbead suspension arrays. In 2006 Khan *et al* used fluorescently tagged microbeads to recognise target phospho-proteins in lysates generated from CLL cell lines (Khan et al. 2006). Mec-1 and Mec-2 CLL cell lines were used in this study; both lines were established from the same CLL patient at different stages of CLL disease progression. The Mec-2 cell line represents a blood sample drawn from a CLL patient in an advanced stage of disease progression; this blood sample had a white blood cell count 3-fold higher than that of the Mec-1 cell line, which was established from the same patient at an earlier point when the disease was in a more indolent stage. The phosphorylation levels of PKB and ERK (amongst other phospho-proteins) were assessed in these cell lines; p-PKB and p-ERK were significantly elevated in lysates generated from sodium pervanadate-treated (activated) cell lysates from both the Mec-1 and Mec-2 CLL cell lines. Furthermore, the basal levels of p-PKB and p-ERK were dramatically increased in the Mec-2 cell line, which implicates the activation of these signalling pathways in the progression of this disease (Khan et al. 2006).

In this set of experiments multi-colour flow cytometry was used to measure the expression of cell surface activation markers, CD38 and CD69. Phosflow antibodies purchased from BD biosciences were also used to analyse intracellular phospho-proteins, S6, STAT6 and ERK. The levels of expression of the extracellular proteins as well as phosphorylation levels of the intracellular proteins were measured at 1 and 24 hour time points. The primary aim of the experiments conducted in this chapter was to provide a quantitative measure of phosphorylated intracellular signalling proteins and determine the dynamics of these phosphorylation events *in vitro*.

6.2 Results

6.2.1 The expression of CD38 was increased following 24 hours in CD31-expressing co-culture

It is now clear that CD38 is temporally expressed on CLL cells (Calissano et al. 2009; Damle et al. 2007); CD38 expression is believed to be rapidly up regulated on the surface of CLL cells located within the lymph node microenvironment and subsequently down regulated upon re-entry to the peripheral blood. The rapid increase in expression of CD38 on the cell surface when CLL cells enter growth permissive microenvironments may enhance disease progression and cell survival through CD38 receptor capabilities. In chapter 3 of this thesis it was shown that CD31-expressing co-culture for 2 and 5 days resulted in the significant up regulation of CD38 in 20 CLL patients. This experiment was conducted to establish whether CD38 was maintained or modulated over a period of 24 hours.

Figure a 6.1a show that at 24 hours CD38 expression was down regulated in LQ and NTL-co-culture however an increase in CD38 expression was detected following CD31-expressing co-culture. Figure 6.1b represent paired patient samples to show the change in CD38 expression following 24 hours CD31-expressing co-culture compared to 1 hour (P=0.04). There was also a trend towards increased CD38 expression when comparing the expression levels at 24 hours between cells cultured in LQ and cells cultured in CD31-expressing co-culture (P=0.01). Figure 6.1c shows an overlaid histogram, which is representative of the shift in CD38 MFI following CD31 co-culture for 24 hours compared to 1 hour. Figure 6.2 shows that comparable levels of CD38 were detected following the addition of IL-4 to culture systems at 1 hour (Figure 6.2a) or 24 hours (Figure 6.2b).

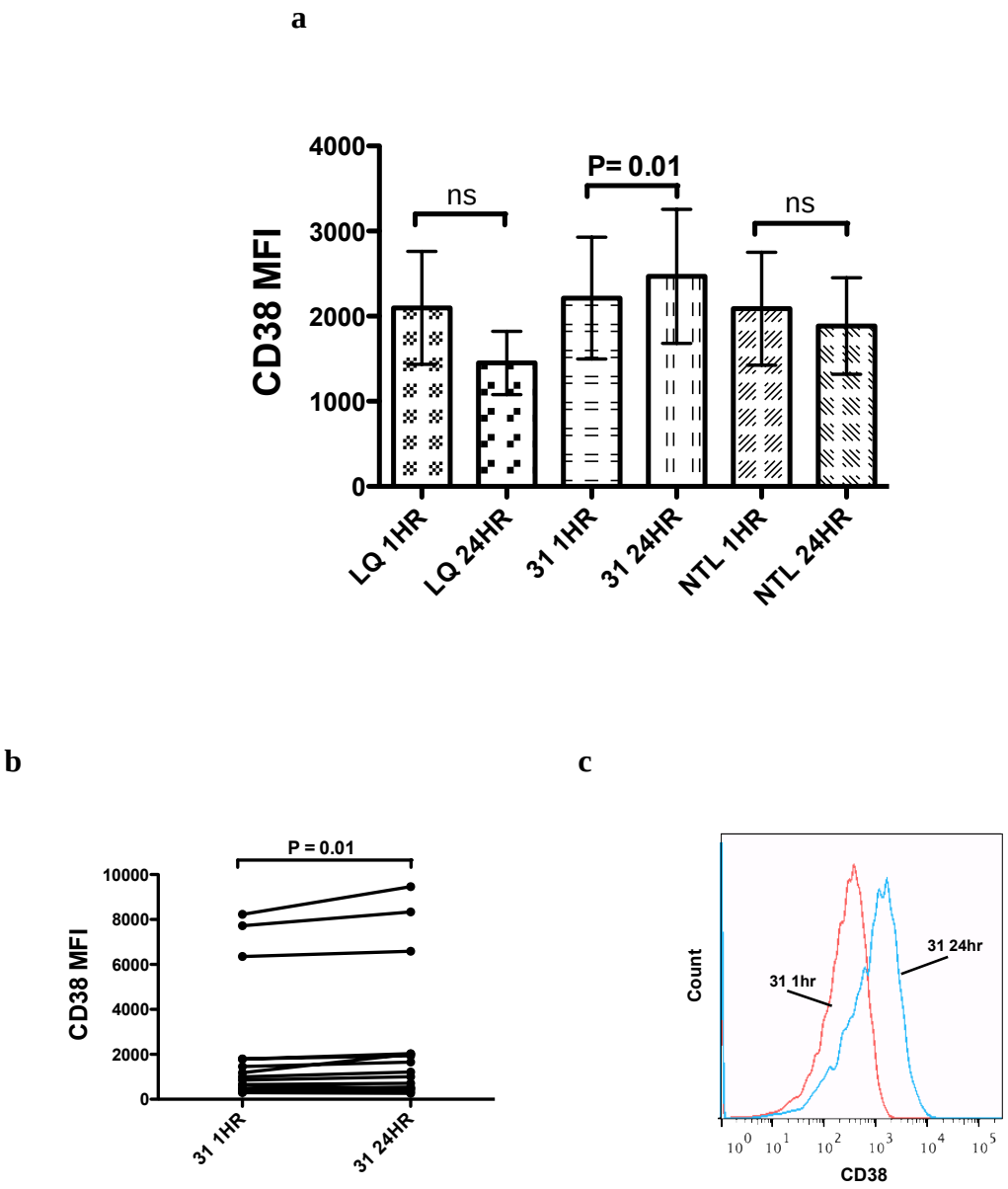


Figure 6.1An increase in CD38 expression was observed following CD31-expressing co-culture for 24 hours

$1x10^6$ CLL cells were placed into culture with LQ,or CD31 co-culture/NTL co-culture. CD38expression was measured by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD) CD38 MFI at 1 and 24 hours b) line-graph shows individual patients at 1 hour and 24 hours in CD31-expressing co-culture. c) Overlaid histogram shows shift in CD38 expression at 24 hours in CD31 co-culture. In total 15 patients were analysed. A repeated measures ANOVA test was used to compare the different conditions.

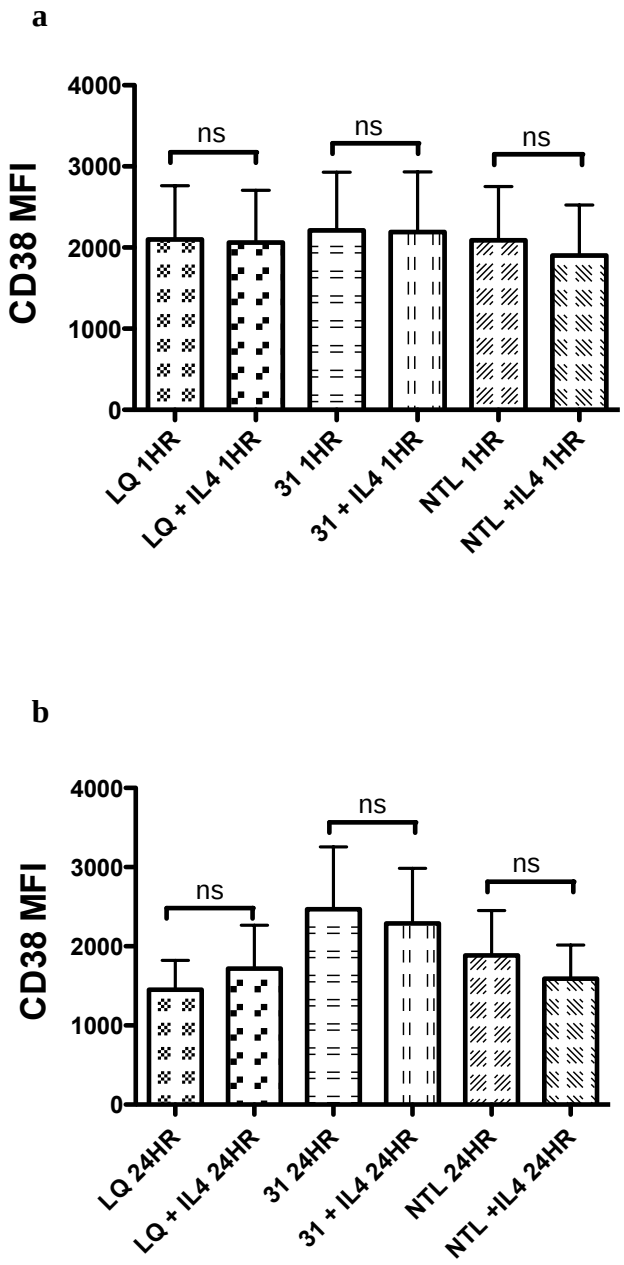


Figure 6.2 IL-4 does not modulate the expression of CD38 at 1 hour or 24 hours

$1x10^6$ CLL cells were placed into culture with LQ with and without IL-4, or CD31 co-culture/ NTL co-culture with and without IL-4. CD69 expression was measured by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD) CD69 expression at 1 hour with and without IL-4. b) Shows the mean (±SD) CD69 expression at 24 hours with and without IL-4. In total 15 patient samples were analysed. A repeated measures ANOVA test was used to compare the different conditions.

6.2.2 The expression of CD69 was increased following 24 hour co-culture

The inducible early activation marker CD69 is expressed very rapidly following cellular activation both *in vivo* and *in vitro* (Damle et al. 2002). Consistent with this concept, the expression of CD69 on CLL cells was shown to be elevated in cells derived from the lymph node and bone marrow microenvironments (Del Poeta et al. 2012). In T-cells the expression of CD69 is induced 3 hours post-activation at levels easily identifiable by flow cytometry (Simms and Ellis 1996). In B-lymphocytes it has been shown that RNA expression of CD69 reaches its peak 3-6 hours post-stimulation with cell surface expression induced at 12 hours, with the expression of CD69 remaining stable over 48 hours (Lopez-Cabrera et al. 1993). CD69 is involved in retaining B-lymphocytes at the site of stimulation and for this reason the levels of CD69 on the CLL cell surface may be lower on circulating cells compared to CLL cells located in lymph node and bone marrow microenvironments (Del Poeta et al. 2012).

To establish whether CD69 expression was maintained or modulated over a period of 24 hours in different culture conditions, $1x10^6$ CLL cells were cultured in either LQ alone or supplemented with IL-4, CD31-expressing co-culture with and without IL-4 as well as NTL co-culture with and without IL-4. At 1 hour and 24 hour time points 5 x 10^5 CLL cells were removed from cultures and immediately fixed in Phosflow fix buffer to maintain the phosphorylation of proteins for analysis. Labelling of the surface markers CD19, CD38 and CD69 was performed prior to permeabilisation of the CLL cells and subsequent labelling with phospho-specific antibodies. The expression of CD69 was analysed within a CD19+ gated viable lymphocyte population.

Figure 6.3a shows that comparable low levels of CD69 were detected in LQ and the co-culture conditions at 1 hour. However, the expression of CD69 was up regulated under all conditions after 24 hours in culture. The augmented expression of this molecule was significantly up regulated following CD31-expressing co-culture and NTL co-culture after 24 hours (Figure 6.3b, P = 0.01 and Figure 6.3c, P =0.01 respectively). Figure 6.3d shows an overlaid histogram illustrating the increase in CD69 expression in CD31-expressing co-culture between 1 hour and 24 hours. Figure 6.3d shows that at 24 hours, CD69 expression was significantly higher in CD31-expressing co-culture compared to LQ only (P=0.01). In contrast, there was no significant difference in CD69 expression between CD31-expressing co-culture conditions and NTL co-culture at 24 hours suggesting that co-culture, rather than the specific ligand,

has the ability to augment the expression of this marker. Figure 6.4 shows that the addition of IL-4 to cultures at 1 hour did not modulate the expression of CD69 (Figure 6.4a) however at 24 hours the addition of IL-4 appeared to augment the expression of CD69 in LQ and co-cultures, however these differences were not significantly different (Figure 6.4b).

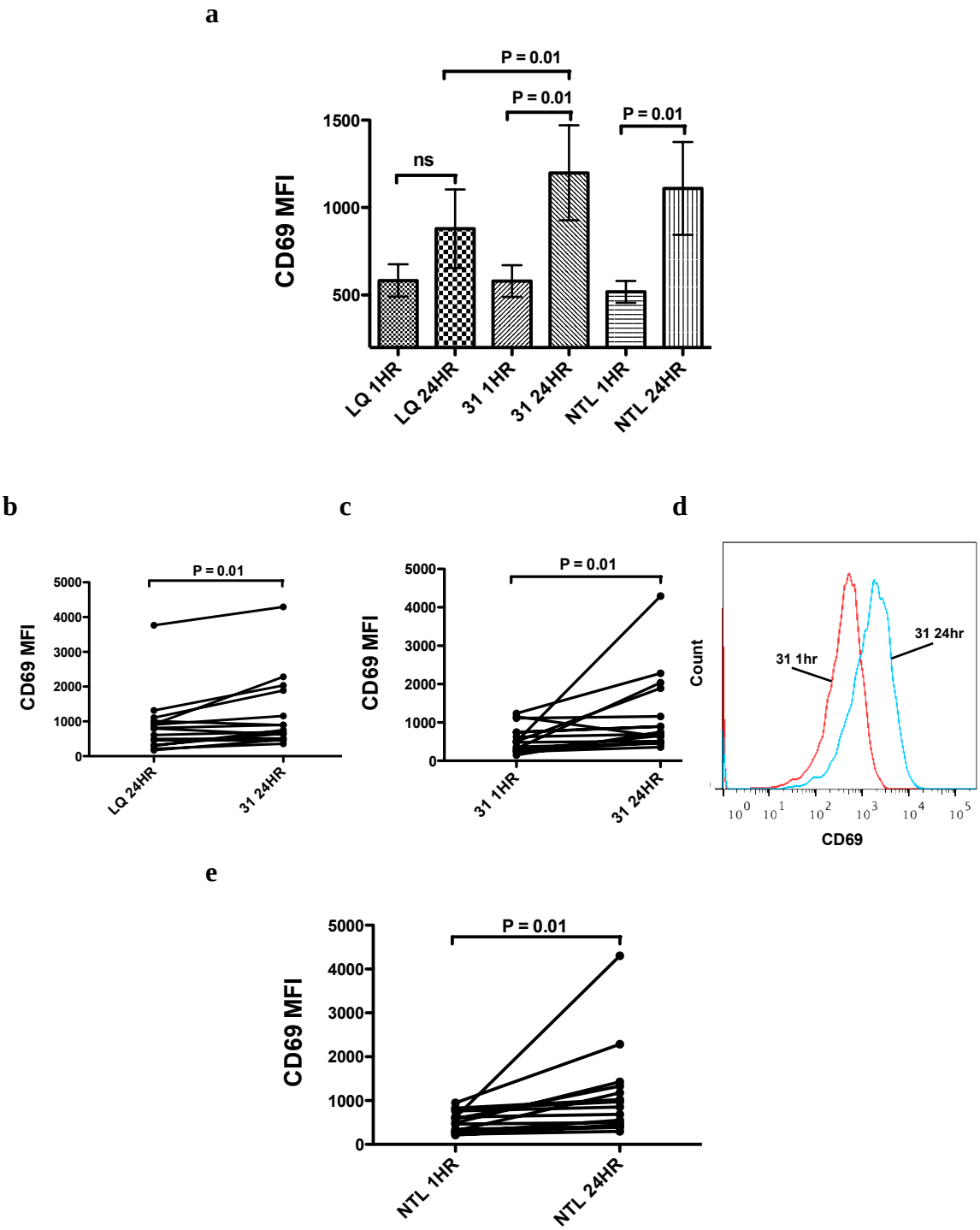
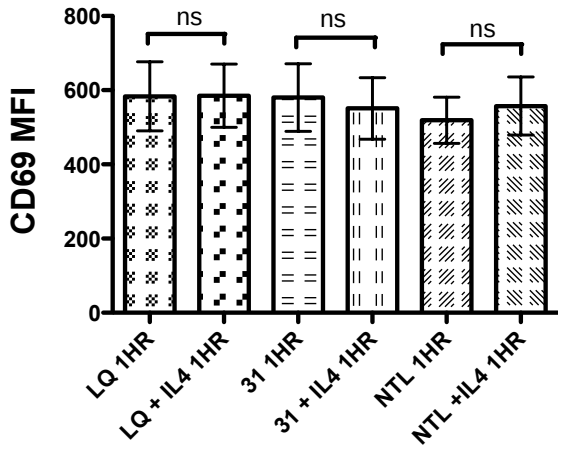


Figure 6.3 An increase in CD69 expression was observed following 24 hour incubation in co-culture

$1x10^6$ CLL cells were placed into culture with LQ, or CD31 co-culture/ NTL co-culture. CD69 expression was measured by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD) CD69 expression at 1 and 24 hours b) line-graph links individual patients at 24 hours in LQ or CD31-expressing co-culture c) line-graph links individual patients at 1 hour and 24 hours in CD31-expressing co-culture d) line-graph links individual patients at 1 and 24 hours in NTL co-culture. E) Overlaid histogram shows shift in CD69 expression at 24 hours in CD31 co-culture. In total 15 patients were analysed.

a: 1 hour treatment



b: 24 hour treatment

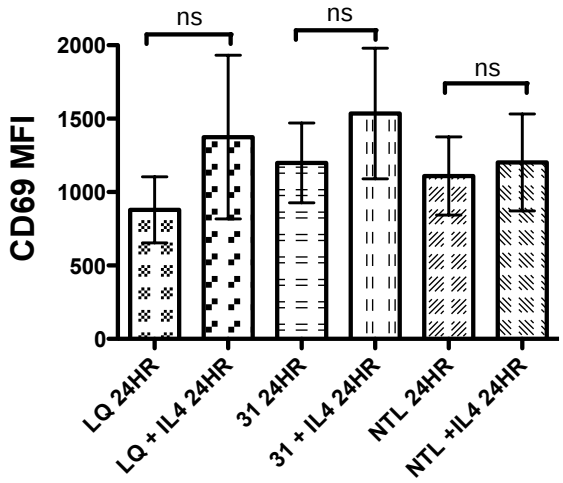


Figure 6.4 IL-4 does not modulate the expression of CD69 at 1 hour or 24 hours

$1x10^6$ CLL cells were placed into culture with LQ with and without IL-4, or CD31 co-culture/ NTL co-culture with and without IL-4. CD69 expression was measured by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD) CD69 expression at 1 hour with and without IL-4. b) Shows the mean (±SD) CD69 expression at 24 hours with and without IL-4. In total 15 patient samples were analysed. A repeated measures ANOVA test was used to compare the different conditions.

6.2.3 The expression of CD69 was increased in CD38^{hi} patients

It has been previously reported that CD69 expression is significantly correlated with other prognostic markers in CLL, including CD38 (D'Arena et al. 2001) In Chapter 5 of this thesis in the patient cohort examined no significant differences could be detected in CD69 expression between CD38^{hi} and CD38^{lo} patient groups. To assess whether there was a correlation between CD38 expression and CD69 expression in this series of CLL patients, CD69 MFI values were plotted for CD38^{hi} or CD38^{lo} patients. The box and whisker plot shown in Figure 6.5 demonstrates significantly higher basal expression of CD69 in the CD38^{hi} cohort of CLL patients compared to the CD38^{lo} cohort of patients (P=0.01).

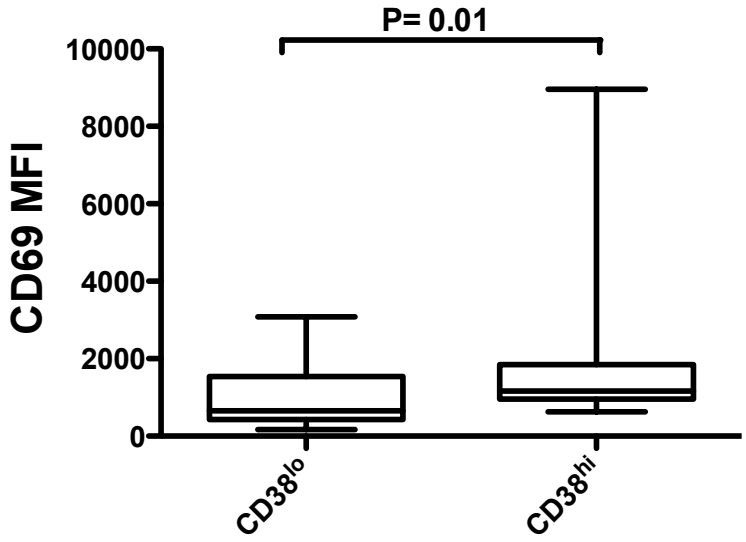


Figure 6.5 CD38^{hi} patients display increased CD69 expression

The median MFI values for CD69 expression for the CD38^{hi} and CD38^{lo} cohort of patients are displayed in this box and whisker plot. The CD38^{hi} cohort of patients had significantly higher expression of CD69 compared to the CD38^{lo} cohort of patients (P=0.01). In total 15 patient samples were analysed.

6.2.4 The phosphorylation of S6 is augmented following 1 hour of CD31-expressing co-culture and decreased after 24 hours incubation in all conditions

In previous chapters, the phosphorylation levels of S6 were assessed following SDS-PAGE and western blotting. Consistent differences in the phosphorylation of S6 were identified; basal levels of phospho-S6 were elevated in the $CD38^{hi}$ cohort of patients and the phosphorylation of the S6 protein was augmented in all CLL patients following 1 hour of CD31-expressing co-culture as well as NTL co-culture.Variations identified in both the magnitude and timing of protein phosphorylation and activation can alter the viability as well as the proliferative capacity of CLL cells. Tasian *et al* (2012) used flow cytometry to detect inducible p-S6 in acute lymphoblastic leukaemia (ALL) cells (Tasian et al. 2012); Perl *et al* (2012) also detected p-S6 using flow cytometry in AML patients (Perl et al. 2012).

In order to identify quantitative differences in basal and inducible p-S6 in primary CLL cells multi-colour cytometry was used; the phosphorylation of p-S6 was examined within CD19+ gated viable lymphocytes. Figure 6.6a shows that p-S6 is significantly increased following 1 hour in CD31-expressing co-culture compared to LQ culture or NTL co-culture (P= <0.0001, P= <0.0001). Figure 6.6b shows that levels of p-S6 are also significantly increased following 24 hours of CD31-expressing co-culture (P=<0.0001) compared to LQ or NTL co-culture. Figure 6.6c represents paired patient samples to show the significant change in p-S6 following 1 hour of CD31 co-culture compared to 1 hour in LQ. Figure 6.6d shows an overlaid dot plot, which shows an example of the shift in p-S6 MFI following CD31-expressing co-culture compared to LQ at 1 hour.

The bar graph in Figure 6.7 shows that p-S6 is significantly down regulated in LQ and NTL co-culture at 24 hours compared to 1 hour (P=0.001, P=0.01 respectively). There is also a slight decrease in levels of p-S6 detected at 24 hours compared to 1 hour in CD31-expressing co-culture however this loss was not significant.

Figure 6.8 shows the p-S6 MFI values when IL-4 was added to cultures. The addition of IL-4 to LQ and NTL co-culture did not modulate the phosphorylation of S6 at 1 hour (Figure 6.8a) or 24 hours (Figure 6.8b). However the addition of IL-4 to CD31-expressing co-culture at 1 hour resulted in a significant increase in p-S6 (P=0.01), this was not observed at 24 hours.

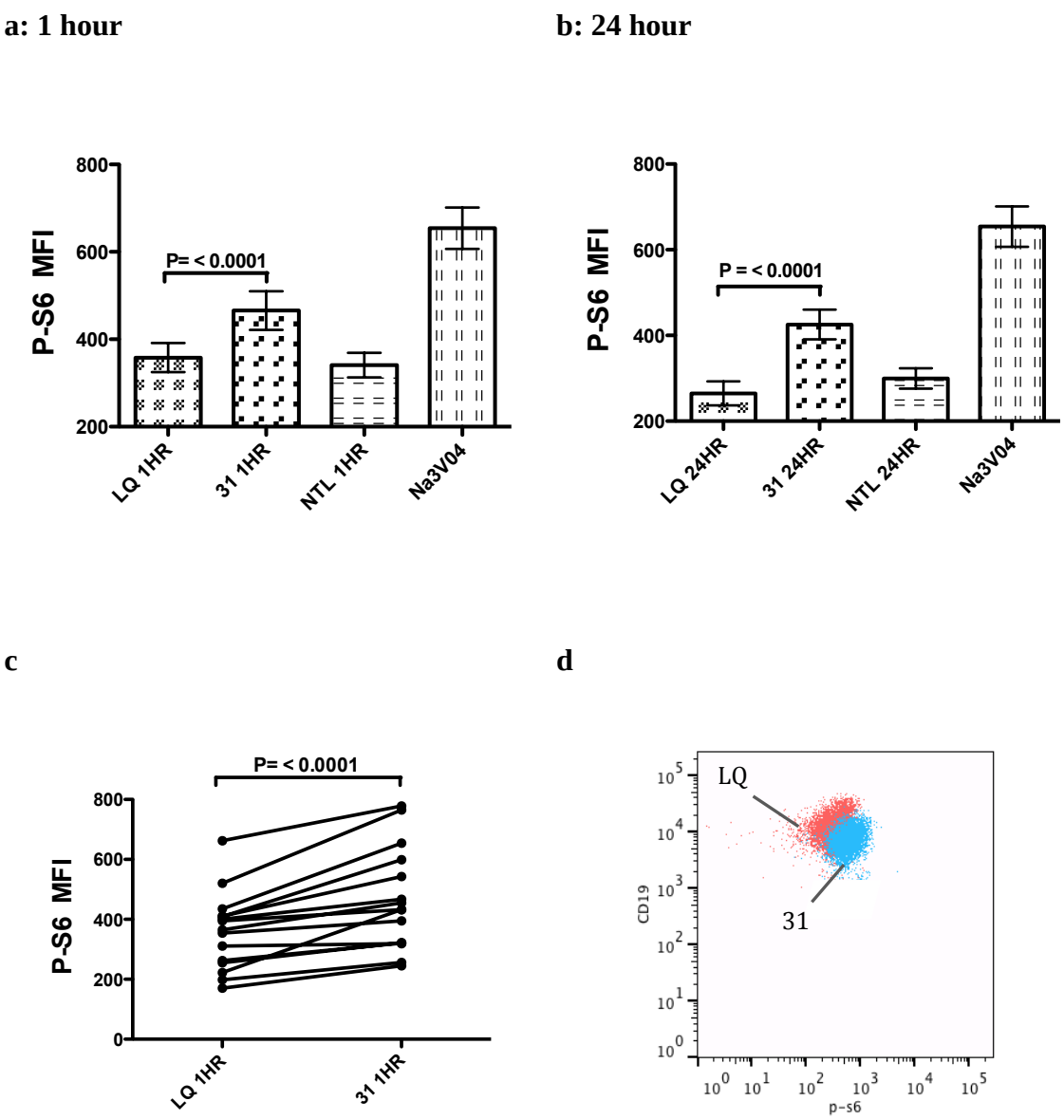


Figure 6.6 An increase in p-S6 was detected following 1 hour and 24 hours of CD31 co-culture

1x10^6 CLL cells were placed into culture with LQ with and without IL-4,or CD31 co-culture with and without IL-4. CD38expression was measured within the CD19+ gated population by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows mean CD38 expression at 1 and 24 hours without IL-4 b) line-graphs show differences in co-culture at 24 hours c) line graph shows difference in CD69 on CD31 co-culture system compared to LQ d) overlaid dot plot shows the shift in p-S6 MFI following CD31-expressing co-culture for 1 hour. In total 15 patient samples were analysed.

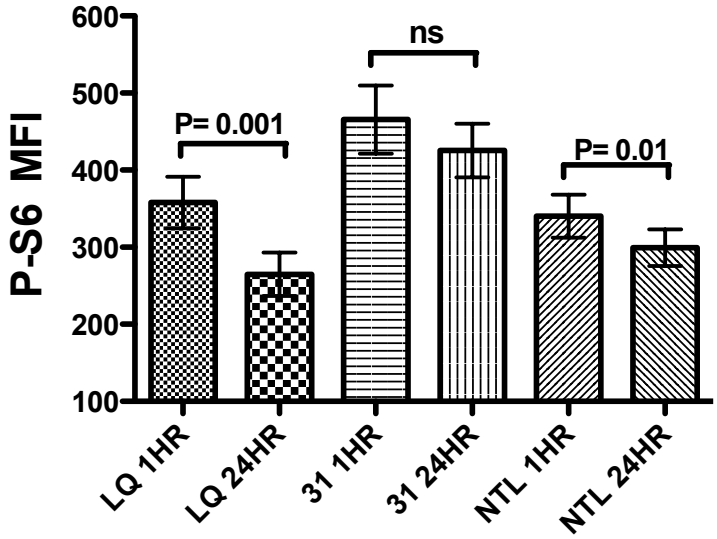


Figure 6.7 A significant decrease in p-S6 was observed following 24 hours of LQ and NTL co-culture.

$1x10^6$ CLL cells were placed into culture with LQ with and without IL-4,or CD31 co-culture with and without IL-4. CD38 expression was measured within the CD19+ gated population by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample.

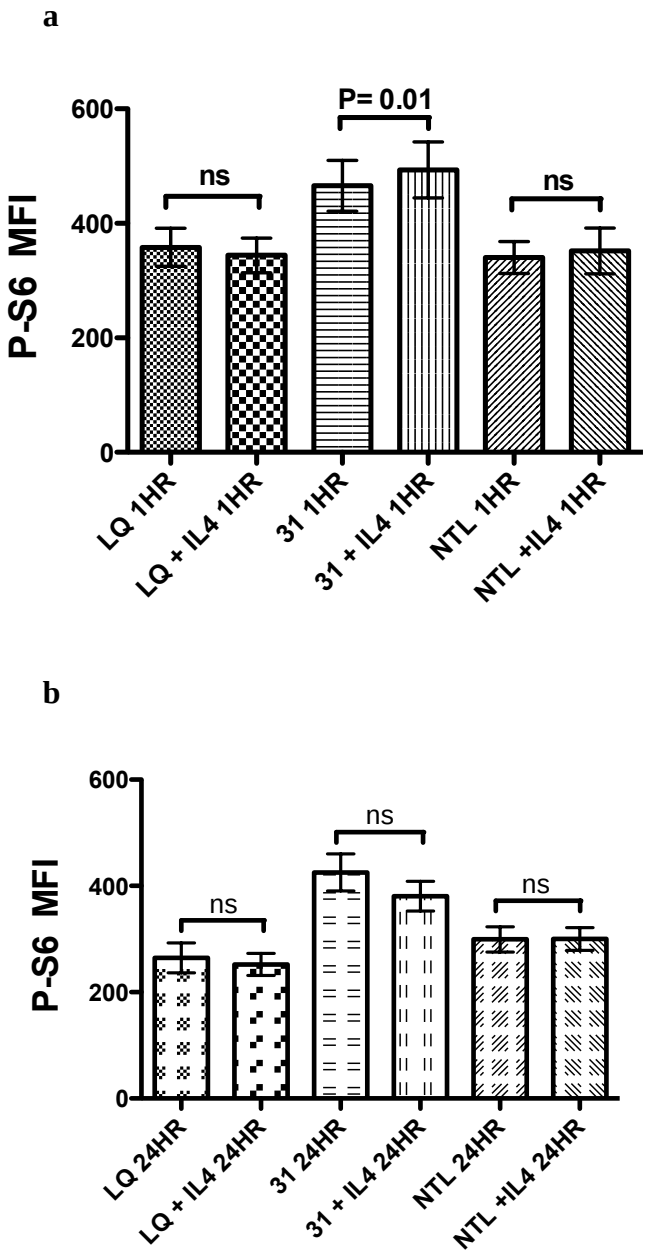


Figure 6.8 The addition of IL-4 to CD31-expressing co-culture increases levels of p-S6 at 1 hour.

$1x10^6$ CLL cells were placed into culture with LQ with and without IL-4, or CD31 co-culture/ NTL co-culture with and without IL-4. P-S6 levels were measured by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD) p-S6 at 1 hour with and without IL-4. b) Shows the mean (±SD) p-S6 expression at 24 hours with and without IL-4. In total 15 patient samples were analysed.

6.2.5 The phosphorylation of STAT6 is augmented following 1 hour of IL-4 culture but decreased after 24 hours incubation in IL-4 culture

In 2012 Blix *et al* analysed freshly isolated CLL cells and compared the phosphorylation of intracellular signalling proteins with normal B-cells taken from a healthy donor. One such protein was STAT6; Blix *et al* revealed that basal levels of p-STAT6 were elevated in CLL patients compared to normal controls (Blix et al. 2012). However, following BCR stimulation the levels of p-STAT6 were not augmented further implying a constitutive rather than inducible signal being responsible for the p-STAT6 observed. In keeping with this notion, Chapter 4 of this thesis established that the phosphorylation of STAT6 was only increased in cultures containing IL-4; a cytokine that is produced in an autocrine fashion by CLL cells as well as by activated T-cells (Mainou-Fowler et al. 2001).

Figures 6.9a and 6.9b confirm that levels of p-STAT6 remain low at 1 hour and 24 hours in cultures, which do not contain IL-4. The Na_3V0_4 was used as a positive control and demonstrates proof of technology, since following treatment with this agent increased p-STAT6 could be detected. Figure 6.9c shows that p-STAT6 was down regulated in cultures at 24 hours compared to 1 hour, which was significant in CD31-expressing co-culture and NTL co-culture (P=0.01, P=0.001, respectively). This implies that in the absence of IL-4 any basal p-STAT-6 is lost at 24 hours.

Figure 6.10a shows that the addition of IL-4 to cultures up-regulates p-STAT6 when added to LQ (P= <0.0001) and CD31-expressing co-culture (P=0.01) at 1 hour. Figure 6.10b shows paired patient samples in the culture conditions to represent the significant change in IL-4 supplemented LQ compared to LQ at 1 hour. Furthermore the overlaid dot plot (Figure 6.10c) shows an example of the shift in p-STAT6 in IL-4 supplemented LQ. Figure 6.10d shows that IL-4 can also slightly increase p-STAT6 at 24 hours but this is not significant.

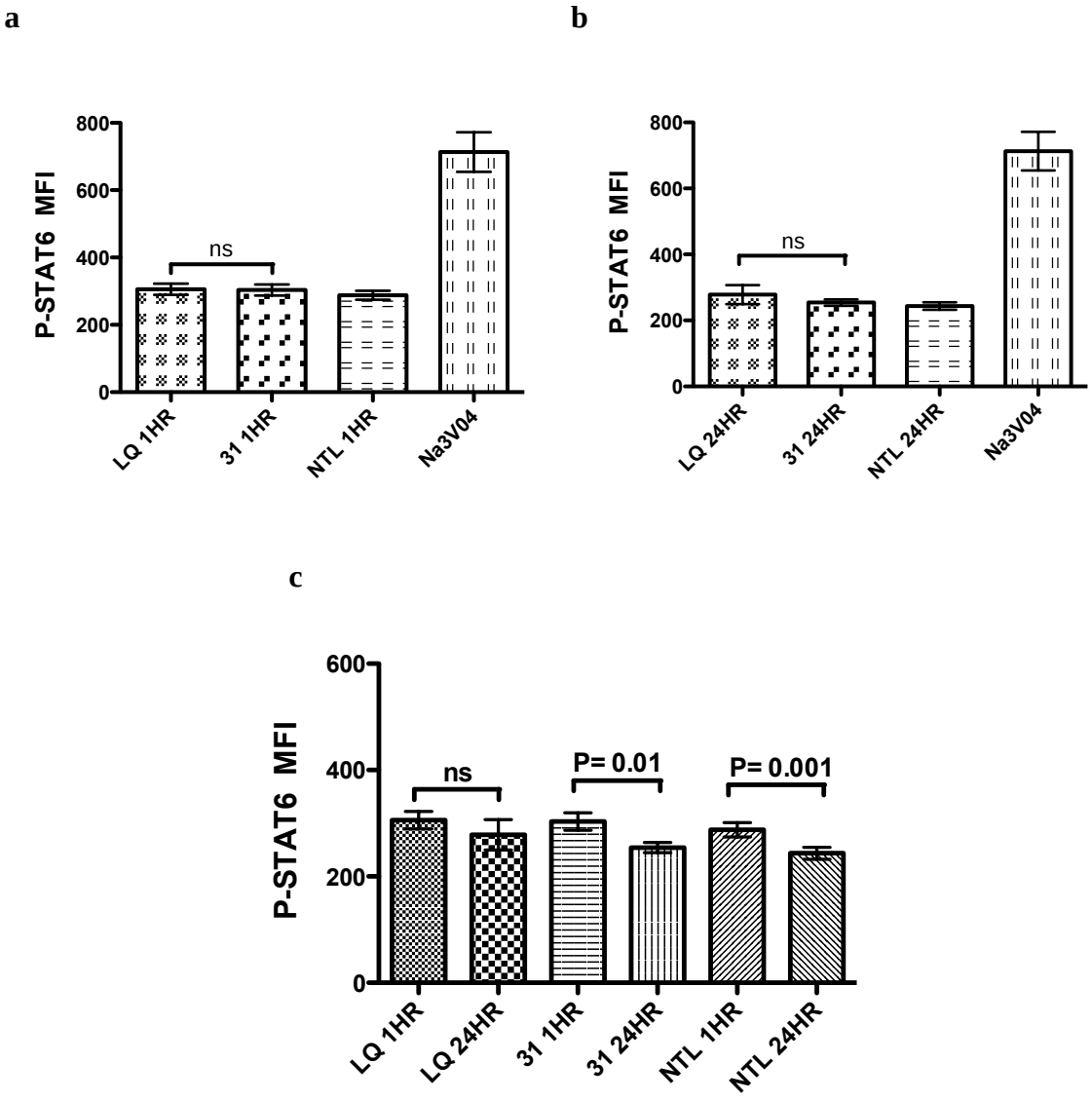


Figure 6.9 An increase in p-STAT6 was detected following 1 hour in IL-4 culture

$1x10^6$ CLL cells were placed into culture with LQ,or CD31 co-culture. P-STAT6 levels were measured within the CD19+ gated population by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows mean p-STAT6 MFI at 1 hour well as Na_3V0_4 positive control b) Shows mean p-STAT6 MFI at 24 hours as well as Na_3V0_4 positive control c) shows mean p-STAT6 at 1 hour and 24 hours.

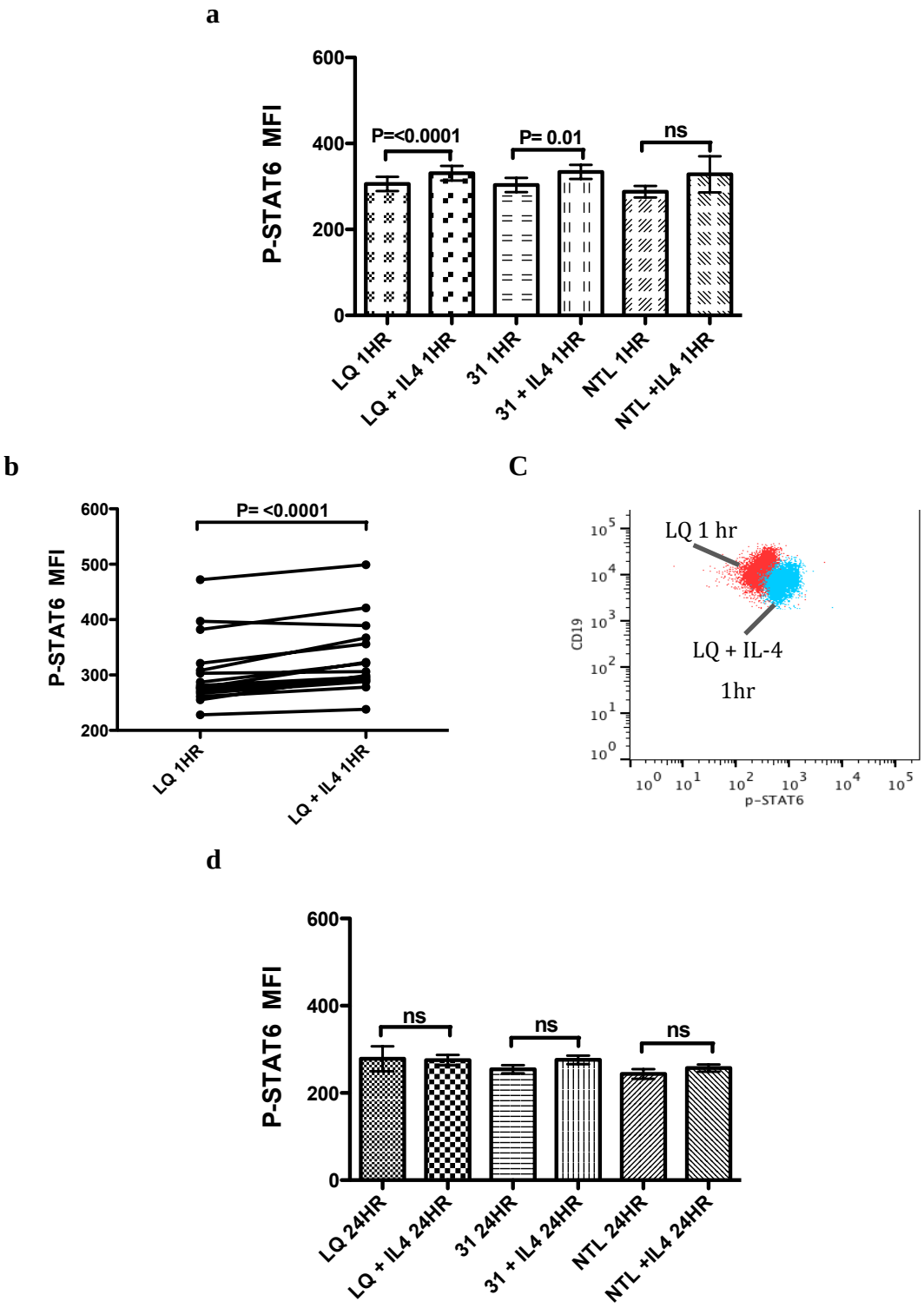


Figure 6.10 IL-4 treatment up-regulates p-STAT6 when added to cultures at 1 hour

1x10^6 CLL cells were placed into culture with LQ with and without IL-4, or CD31 co-culture/ NTL co-culture with and without IL-4. P-STAT6 levels were measured by flow cytometry at 1 hour and the MFI values were plotted for each CLL patient sample. a) Shows mean p-STAT6 at 1 hour with and without IL-4. b) Line-graph link individual patients in LQ and LQ+ IL4 at 1 hour c) overlaid dot plot shows the shift in p-STAT6 MFI following IL-4 treatment for 1 hour d) shows mean p-STAT6 at 24 hours with and without IL-4. In total 15 patient samples were analysed.

6.2.6 The phosphorylation of ERK is augmented following 1 hour of IL-4 culture but decreased after 24 hours incubation in IL-4 culture

Blix *et al* 2012 looked at the basal and inducible levels of p-ERK in CLL cells and compared them to healthy controls. Elevated basal levels of p-ERK were detected in CLL patients, and following BCR stimulation levels of p-ERK were further increased (Blix et al. 2012). In Chapter 4 of this thesis it was established that CD40L-expressing co-culture was able to induce the p-ERK, and CD31-expressing co-culture was also able to stimulate p-ERK but exclusively in patients with high surface expression of CD38.

In order to identify quantifiable differences in p-ERK following co-culture a Phosflow ERK antibody was used. Figure 6.11a and Figure 6.11b show that p-ERK was significantly up-regulated following CD31-expressing co-culture at 1 hour (P=0.003) and 24 hours (P=0.001) compared to LQ. The significant differences are represented in Figures 6.11c and 6.11d with line graphs which link individual patient samples in LQ and CD31-expressing co-cultures at 1 hour and 24 hours. Interestingly a significant increase was not observed following NTL co-culture which indicates that the CD31/CD38 interaction is key to the induction of p-ERK at the 1 hour time point. Figure 6.11d shows an overlaid dot plot, which represents the shift in p-ERK at 1 hour in CD31-expressing co-culture compared to LQ at this time point. Figure 6.12 shows that there is a significant down regulation in p-ERK in LQ (P=0.01) and NTL co-culture (P=0.01) at 24 hours compared to 1 hour. However, this was not observed in CD31-expressing co-culture between these time points. Figure 6.13 shows that the addition of IL-4 to cultures did not result in significant changes in the levels of p-ERK under any of the culture conditions tested.

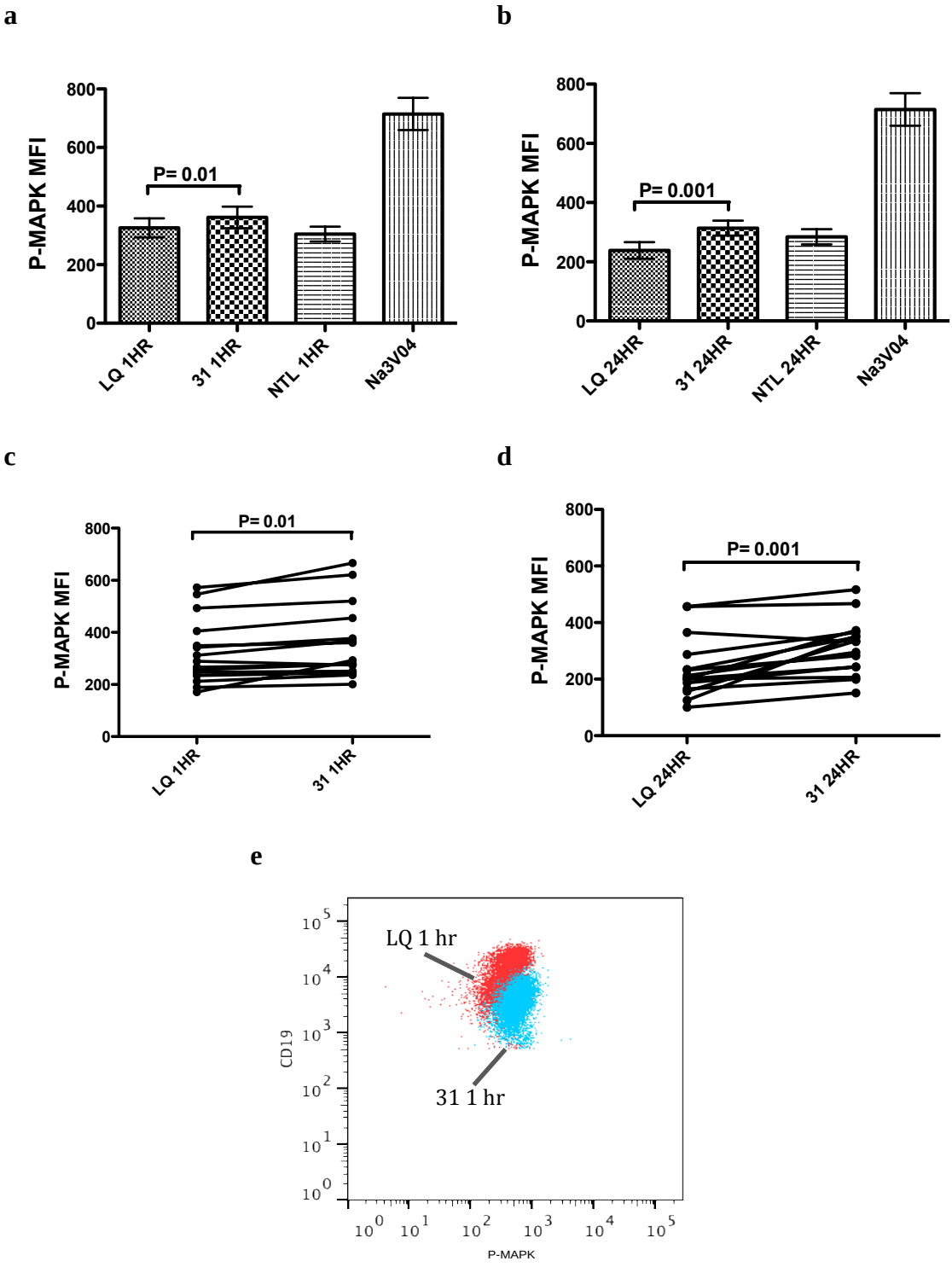


Figure 6.11 An increase in p-ERK was detected following 1 hour in IL-4 culture

1x10^6 CLL cells were placed into culture with LQ, or CD31 co-culture. P-ERK levels were measured within the CD19+ gated population by flow cytometry at 1 hour and 24 hours and the MFI values were plotted for each CLL patient sample. a) Shows the mean (±SD)p-ERK MFI at 1 hour as well as Na_3V0_4 positive control b) Shows the mean (±SD)p-ERK MFI at 24 hours as well as Na_3V0_4 positive control c) Line-graph link individual patients in LQ and 31 culture at 1 hour d) Line-graph link individual patients in LQ and 31 cultures at 24 hours e) overlaid dot plot represents a patient sample in LQ and CD31 co-culture at 1 hour.

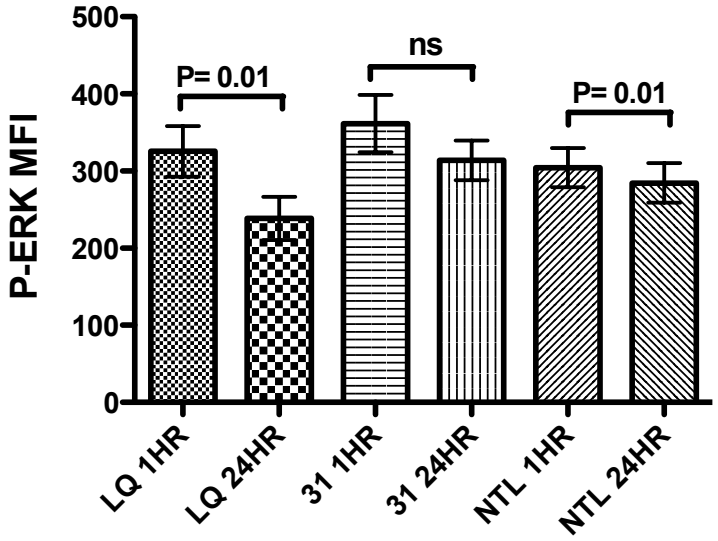


Figure 6.12 A significant decrease in p-ERK was detected in LQ and NTL co-culture after 24 hours

$1x10^6$ CLL cells were placed into culture with LQ, or CD31 co-culture or NTL co-culture. P-ERK levels were measured within the CD19+ gated population by flow cytometry at 1 hour and 24 hours and the mean (±SD)MFI values were plotted (n = 15).

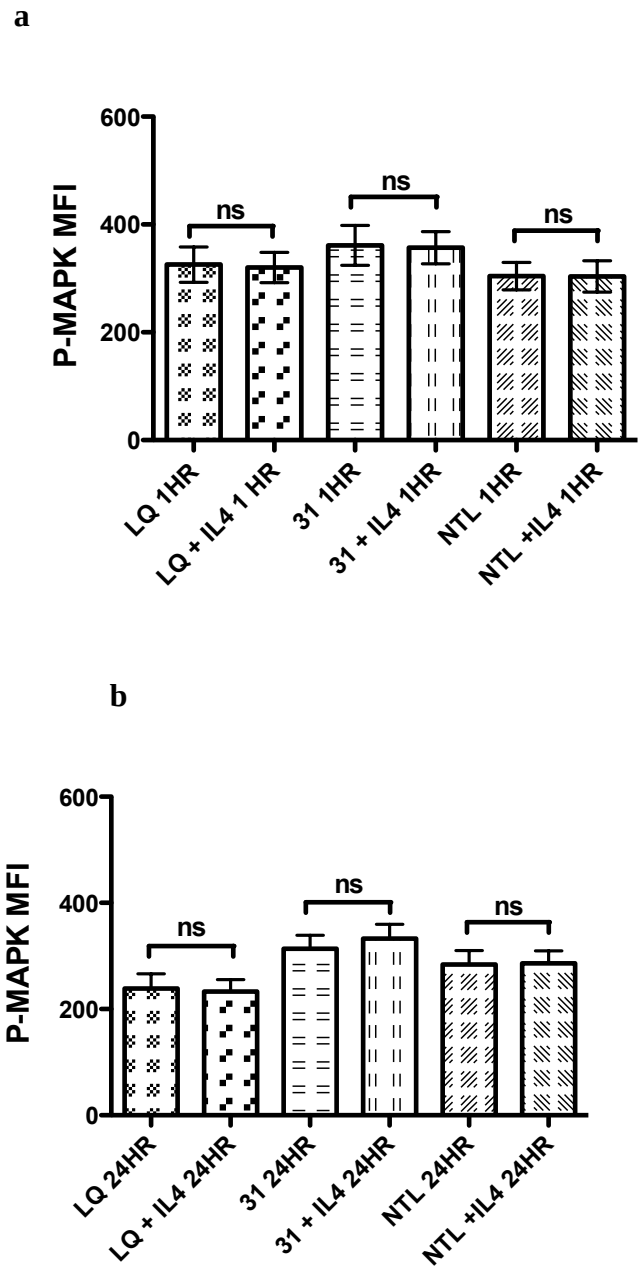


Figure 6.13 The addition of IL-4 to cultures does not modulate p-ERK

$1x10^6$ CLL cells were placed into culture with LQ with and without IL-4, or CD31 co-culture/ NTL co-culture with and without IL-4. P-ERK levels were measured by flow cytometry at 1 hour and 24 hours and the mean (±SD) MFI values were plotted. a) Shows mean p-ERK at 1 hour with and without IL-4. b) Shows the mean (±SD)p-ERK expression at 24 hours with and without IL-4. In total 15 patient samples were analysed.

6.2.7 Quantitative analysis of p-ERK and p-S6 levels confirmed significantly increased levels in CD38 positive CLL patient samples

It was established in Chapters 3 and 4 through western blot analysis that increased basal and inducible levels of p-S6 and p-ERK were detected in $CD38^{hi}$ patients when compared to $CD38^{lo}$ patients. In this set of experiments the MFI values of p-S6 and p-ERK were compared between the $CD38^{hi}$ and $CD38^{lo}$ cohort of patients.

Using the MFI values obtained from CLL patient samples sampled at 1 hour, when phosphorylation levels of p-S6 and p-ERK were shown to be higher, the $CD38^{hi}$ and $CD38^{lo}$ cohorts of patients were compared. The box and whisker plots in Figure 6.14a show that the $CD38^{hi}$ cohort of patients had significantly higher levels of basal (LQ) P=0.002 and inducible (CD31 and NTL co-culture) p=0.005, p=0.005 p-S6 compared to the $CD38^{lo}$ cohort of patients. Similarly figure 6.14b shows that the CD38hi cohort of patients have significantly increased levels of basal (LQ) P=0.0002 and inducible (CD31 and NTL co-culture) P=0.0002, P=0.02 p-ERK compared to the $CD38^{lo}$ cohort of patients.

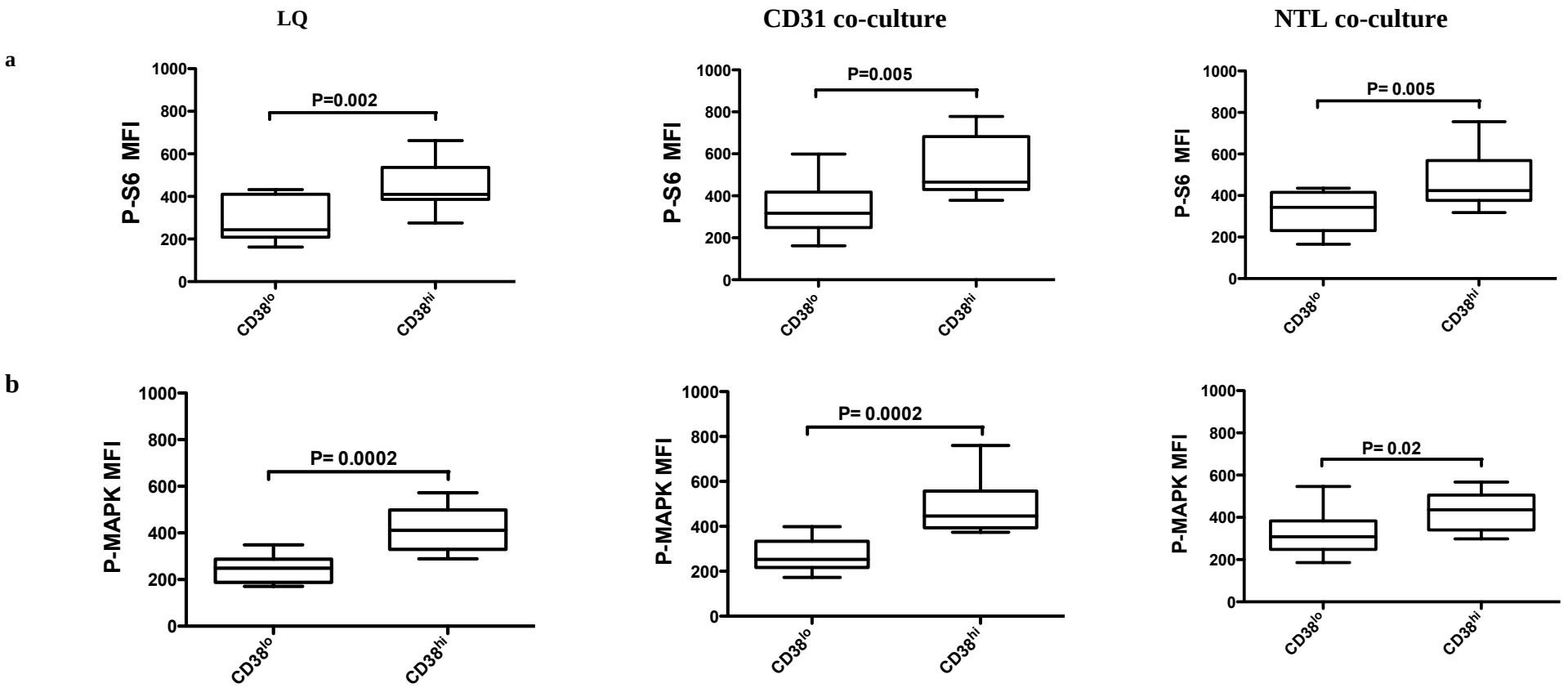


Figure 6.14 Increased p-S6 was detected in CD38^{hi} patient samples compared to CD38^{lo} samples

The mean MFI values for p-S6 and p-ERK are shown for the CD38^{hi} and CD38^{lo} cohort of patients. Box and whisker plots show significantly higher a) p-S6 and b) p-ERK in CD38^{hi}patient samples when compared to CD38^{lo} patient samples. In total 15 patient samples were analysed.

6.3 Discussion

The primary aim of this chapter was to quantify the levels of intracellular phospho-proteins using multi-colour flow cytometry. Following CLL stimulation, cells were labelled with antibodies against the markers of activation, CD69 and CD38, prior to being fixed and permeabilised to analyse intracellular levels of p-S6, p-ERK and p-STAT6. Ten CLL patients, five $CD38^{hi}$ and five $CD38^{lo}$, were analysed at 1 hour and 24 hours following CD31-expressing co-culture with and without the addition of IL-4.

The first main observation was that CD38 expression was increased on CLL cells incubated with CD31-expressing co-culture for 24 hours. In contrast, no significant changes in CD38 expression were observed following LQ or NTL co-culture over the same time period. This observation demonstrates the specificity of CD31 on the ability of CLL cells to up-regulate CD38 on their surface. High CD69 expression has been previously shown to correlate with other established prognostic markers including CD38 (Damle et al. 2007). In agreement with that report, in this patient cohort higher levels of basal CD69 expression were found in the $CD38^{hi}$ patients. The surface expression of CD69 was also significantly up regulated after 24 hours incubation on both co-culture systems (CD31 and NTL) compared to 1 hour. The NTL and CD31 co-culture fibroblasts are likely to release cytokines as well as other growth factors that induce these phenotypic changes and demonstrate the importance of using NTL co-culture as a control in experiments. These findings show that co-culture alone has the capacity to induce increased expression of CD69 within 24 hours of stimulation.

Interestingly, the addition of IL-4 treatment to the co-culture systems did not result in the modulation of CD69 or CD38 at the 1-hour or 24-hour time points. This implies that IL-4 signalling does not significantly impact upon the expression of these activation markers. This is in keeping with data generated by Deaglio *et al* (2003) who looked at the effect of culturing CLL cells in the presence of cytokines including IL-2, IL-4 and IL-6 amongst others. IL-2 treatment resulted in the up-regulation of CD38 in $CD38^{hi}$ CLL cells but no upregulation of CD38 was observed in CLL cells with low CD38 expression. Increased CD38 expression was first observed 30 hours following the addition of IL-2 to culture and the maximum CD38 MFI values were detected 72 hours post IL-2 stimulation. The other cytokines analysed, including IL-4, failed to modulate CD38 expression within this patient cohort at any of the time points and concentrations

examined (Deaglio et al. 2003). Willimott *et al* (2007) used the CD40L/IL-4 co-culture system to stimulate CLL cells and observed augmented CD38 expression, but when CD40L-expressing co-culture and IL-4 treatment were compared as separate stimuli it was shown that the up-regulation of CD38 is predominantly due to CD40 signalling and not IL-4 signalling (Willimott et al. 2007).

The next main finding was that intracellular phospho-proteins could be detected and quantified in primary CLL cells using flow cytometric analysis. In chapters 3 and 4 of this thesis the phospho-proteins S6 and ERK were increased following CLL stimulation with CD31-expressing co-culture and phosphorylation of STAT6 was increased by IL-4 treatment. Phospho-specific flow cytometry was utilised in this set of experiments to provide a quantitative measure of both the intensity and dynamics of p-S6, p-STAT6 and p-ERK following CLL cell stimulation. The MFI levels of p-S6 and p-ERK were significantly increased following 1 hour on CD31-expressing co-culture but not NTL co-culture or LQ; this implies that CD31/CD38 ligation is capable of inducing the activation of these signalling molecules independently of other stimuli. In previous western blot analysis, p-S6 and p-ERK were augmented following the addition of IL-4 to cultures. However, when using flow cytometry only the phosphorylation of S6 was enhanced following the addition of IL-4 to CD31-expressing co-culture at the 1 hour timepoint.

Phospho-specific flow cytometry experiments also showed significantly increased levels of p-STAT6 following the addition of IL-4 to CLL cultures. In contrast, p-STAT6 levels were not altered following CD31/CD38 ligation, which is in keeping with the findings generated in previous chapters using western blot analysis. *In vivo* mouse studies have been used to establish both the spatial and temporal range of IL-4 signalling. In order to do these mice were infected with parasitic pathogens and IL-4 signalling was measured by monitoring levels of p-STAT6. Four days after administering the infection, p-STAT6 could be detected, which was sustained at similar levels over the course of the two-week experiments. The half-life of p-STAT6 was analysed to ensure the phosphorylation of STAT6 was due to the sustained IL-4 stimulation; the half-life of STAT6 phosphorylation was found to be 15 minutes (Perona-Wright et al. 2010). The transient nature of STAT6 phosphorylation may explain why p-STAT6 cannot be detected in freshly isolated peripheral blood. In contrast, the persistent presence of IL-4 in the lymph node microenvironments *in vivo*

may result in sustained IL4 signalling thereby maintaining the phosphorylation of intracellular STAT6 in CLL cells. However, when CLL cells leave the lymph node environment and enter the peripheral blood levels of IL-4 may not remain high enough to maintain the phosphorylation of STAT6.

The MFI values obtained using Phospho-specific flow cytometry were lower than expected when compared to the intensity of the bands detected by western blot analysis. Following CD31/CD38 ligation a robust increase in band intensity of p-S6 was detected with western blot analysis in all patients analysed. The disparity in the signals obtained through western blot analysis and phosphoflow cytometry may be due to limitations in the Phosphoflow approach. In the western blot experiments performed in this thesis, CLL cells were maintained on ice following stimulation and lysates were generated rapidly thereafter. A major limitation of the phosphoflow detection methodology employed here was the fluorescence intensities of phospho proteins were relatively low suggesting that this approach may not be sensitive enough to detect subtle changes in signalling molecules. To ensure accurate results are obtained using flow cytometry it is important to maintain phospho-specific epitopes following CLL cell stimulation. Recently Li *et al* measured p-S6 in multiple myeloma patient samples and optimised a protocol for flow cytometry analysis to preserve levels of p-S6 in patient samples. Li *et al* demonstrate that levels of p-S6 were rapidly diminished at room temperature over a period of 48 hours. Following 24 hours over 50% of the p-S6 signal was lost compared to basal levels. In response to this finding, they developed a fixation protocol which involved the immediate transfer of whole peripheral blood into lysis and fixation buffer to show that p-S6 could be maintained for 24 hours (Li et al. 2013).

Furthermore, the optimisation of the cell permeabilisation method employed may be required to accurately measure levels of intracellular phosphorylated proteins. Kritzik and Nolan focused on both the ERK cascade and the JAK/STAT signalling pathways. Different permeabilisation agents were used (methanol vs detergent-based) to determine which agents resulted in the best detection of intracellular proteins. It was discovered that the detergent-based permeabilisation method was most effective for detecting phosphoproteins located within the cytoplasm such as ERK (and S6), whereas a methanol based cell permeabilisation was more effective in detecting nuclear translocated phospho-proteins such as STAT6 (Krutzik and Nolan 2003). In these experiments a detergent-based permeabilisation agent was used, which may not be

suitable for detection of nuclear located p-STAT6.Optimisation of these protocols may result in higher signal output by flow cytometry.

Temporal changes in phospho-proteins were detected; p-S6, p-STAT6 and p-ERK were all down regulated at 24 hours compared to 1 hour under all conditions. In 2011, Woost *et al* compared bone marrow samples from healthy controls and AML patients and showed that following stimulation there was an enhanced response in the phosphorylation of S6 and ERK. The kinetics of these phosphorylation events was measured in AML patients and it was shown that p-ERK could be detected 2 minutes post stimulation and p-S6 could be detected 4 minutes following stimulation (Woost et al. 2011). The experiments in this chapter demonstrate the potent ability of CD38 stimulation to induce rapid phosphorylation of both ribosomal S6 and ERK. The reason for the down-regulation of phospho-proteins at 24 hours compared to 1 hour may be due to the chronic stimulation of CLL cells. Cells may be maximally stimulated at a time point less than 24 hours resulting in the loss of unstable phospho-epitopes. Given the importance of IL-4 for inducing phosphorylation of STAT6, it may be that IL-4 levels may have been depleted at the 24-hour time point and therefore the p-STAT6 signal could not be sustained.

In summary, experiments in this chapter have established that Phosphoflow cytometry can be used to detect quantitative changes in basal and inducible levels of intracellular phospho-proteins S6, STAT6 and ERK following short term CD31-expressing co-culture. Furthermore these experiments have confirmed that basal and inducible levels of p-S6 and p-ERK are significantly higher in $CD38^{HI}$ patients. However, relatively low levels of phospho-proteins were detected using this method so optimisation of the permeabilisation protocol in particular may be required to determine more accurate results.

7 Final Discussion

In the experiments carried out in this thesis, western blotting and flow cytometry were combined to investigate CD31/CD38 signalling in primary CLL cells. Understanding the clinical heterogeneity of CLL may be facilitated by identifying the underlying differences in signalling pathways activated by a variety of *in vivo* microenvironmental stimuli. Therefore, the primary objective of this study was to determine how differential signalling could contribute to disease progression. Given the known association between CD38 expression and poor clinical outcome in CLL (Damle et al. 2007; Deaglio 2003a, 2008a), this research project focused on the downstream consequences of CD31/CD38 ligation in order to establish whether differential CD31/CD38 signalling might provide an explanation for the inferior prognosis of $CD38^+$ patients.

During the course of this study, the expression of CD38 was routinely measured and, in keeping with previous studies, it was shown to be dynamically upregulated following CD31 co-culture (Deaglio 2001b; Hamilton et al. 2012). This readout served as a valuable biomarker to help validate the various instrumentation used during the course of the study. An increase in CD38 expression was observed using three different flow cytometers at four different time points. Chapter 3 showed a significant up-regulation of CD38 on CD31 co-culture at two and five days, the CD38 MFI was measured using the Accuri C6 flow cytometer in the CD19+/CD5+ gated lymphocyte population. In Chapter 5, the MFI of CD38 was measured following 24 hours of CD31-expressing co-culture, and it was shown that CD38 was also significantly up-regulated at this time-point; this analysis was conducted on the Aria Flow cytometer within the $CD19^+/CD5^+$ gated lymphocyte population. Finally in Chapter 6, an up-regulation of CD38 was also detected at 24 hours following CD31-expressing co-culture in a $CD19^+$ gated lymphocyte population. This analysis was conducted on the Canto II flow cytometer. No change in CD38 expression was detected in CLL cells cultured with non-transfected co-culture cells, thus these experiments demonstrate the potent ability of CD31/CD38 signalling to modulate the expression of CD38 on the CLL cell surface.

In order to assess the early consequences of CD38/CD31 ligation, 1-hour co-culture of primary CLL cells with CD31-expressing cells were analysed. These conditions led to augmented phosphorylation of the signalling molecules S6 and GSK3β. S6 phosphorylation was demonstrated following SDS-PAGE and western blotting in a cell population and was demonstrated at the single cell level by flow cytometry. This is the

first time that stimulation of primary CLL cells through CD38 has been directly linked with the PI3K/PKB pathway. Furthermore, Rapamycin and the PI3K inhibitor LY294002 could inhibit the activation of S6. Phenotypically, CD38 ligation is associated with increased cell survival and proliferation (Deaglio, 2010; Hamilton et al, 2012). The PI3K/PKB signalling pathway plays an important role in both CLL cell survival and CLL cell proliferation (Cuni et al. 2004; Hoellenriegel et al. 2011; Scupoli and Pizzolo 2012). Linking CD38 with the PI3K/PKB pathway maybe important for this phenotype and may be involved in the development of disease.

The phosphorylation of ribosomal protein S6 showed variation from patient sample to patient sample. Basal levels of p-S6 were elevated in CLL patient expressing high levels of CD38. On the same western blots, a CD38 high and a CD38 low patient were analysed and revealed that the levels of p-S6 in unstimulated CLL cells were elevated in patients with higher expression of CD38 (Figure 3.5, Figure 3.7, Figure 4.4). Flow cytometry analysis, which is more quantitative, also showed that the MFI of p-S6 was significantly increased in the $CD38^{hi}$ cohort of CLL patients compared to the $CD38^{lo}$ cohort of patients (Figure 6.14). Interestingly flow cytometry also revealed that there was a difference following CD31 co-culture (Figure 6.14). These data provide a further link between CD38 and the ribosomal protein S6 and suggests that S6 is elevated *in vivo* which may have implications for ability of CLL cells to proliferate.

Data from western blotting and flow cytometry showed S6 phosphorylation was a dynamic event that had the capacity to respond to diverse stimuli and integrate multiple pathways. In the absence of cell stimulation, p-S6 was decreased at 4 hours (Figure 3.12). Flow cytometry showed a quantitative and significant loss of p-S6 in liquid culture at 24 hours (Figure 6.7). This suggests that p-S6 must be actively maintained in CLL cells *in vivo*, perhaps through transient interactions with the endothelium during circulation. S6 phosphorylation was further induced by co-culture with CD40L-expressing cells and could be augmented by the addition of the cytokine, IL-4. Interestingly, incubation of some patient samples with non-transfected co-culture cells could also increase p-S6. S6 phosphorylation is downstream of the mammalian Target of Rapamycin (mTOR). This pathway has the capacity to integrate multiple signalling pathways (Laplante and Sabatini 2012), which is illustrated well by the work presented here.

The mTOR inhibitor Rapamycin could induce the complete inhibition of p-S6 even in the presence of CD31-expressing co-culture. Rapamycin has anti-cancer properties in solid tumours and lymphomas. Activated CLL cell treatment with Rapamycin showed a significant inhibition of proliferation but no evidence of increased cell death (Decker et al. 2003). The potent inhibition of p-S6 by Rapamycin may be an important factor in the anti-cancer properties of Rapamycin and other analogues of this pharmacological inhibitor. Activation markers on the CLL cell surface were assessed following treatment of CLL cells on CD31-expressing co-culture with Rapamycin and were compared to untreated CLL cells on this co-culture system at 24 hours. Treatment with Rapamycin resulted in the significant down regulation of CD49d, CD25 and CD69 expression. The viability of CLL cells was also assessed following treatment of CLL cells with Rapamycin and this inhibitor did not affect CLL cell viability. Decker *et al* also showed that Rapamycin did not affect CLL cell viability (Decker et al. 2003), but aided in the treatment of CLL cells by negating the proliferative capacity of CLL cells. CD49d expression has been shown to be associated with proliferative markers (Rossi et al. 2008), thus the suppression of proliferation with Rapamycin may be due to in part the down regulation of CD49d.

Clinical trials have been used to test the efficacy and tolerability of Rapamycin and its analogues in CLL patients. For example Zent *et al* used Rapamycin analogue everolimus that had previously been used to treat relapsed renal cancer. A phase II clinical trial revealed that treatment with this mTOR inhibitor resulted in the mobilization of CLL cells from lymph nodes into the peripheral blood in a proportion of CLL patients. The movement of CLL cells from the protective stromal microenvironment into the peripheral blood will enhance the cytotoxicity of CLL cells to other pharmacological agents improving treatment outcomes (Zent et al. 2010). Other inhibitors of S6 phosphorylation have also been used in CLL research. A derivative of Rapamycin named RAD001 was used in an *in vivo* model of CLL. It was shown that RAD001 was able to significantly delay the growth of tumours and in some cases resulted in the regression of established tumours(Majewski et al. 2000). The ability of both RAD001 and Rapamycin to effectively inhibit p-S6 shows that ribosomal S6 is a major target for phosphorylation by mTOR, which is important when assessing the immunosuppressive properties of Rapamycin and other analogues. The phosphorylation status of S6 is increasingly being used as a potential biomarker to evaluate aberrant activation of PI3K and mTOR pathways. The monitoring of response

to these treatments in several disease contexts has been assessed for example in AML, prostate cancer, breast cancer as well as antibody-mediated rejection in heart transplant models(Chow et al. 2006; Lepin et al. 2006; Thomas 2006; Thomas et al. 2004).

The phosphorylation of the signalling molecule ERK was also assessed in this thesis using western blot analysis as well as flow cytometry and ERK inhibitors. Western blot analysis showed that basal and inducible p-ERK could only be detected in some of the $CD38^{hi}$ cohort of patients analysed (Figure 4.4). Phosphoflow analysis was also used as a means to quantitatively measure basal and inducible levels of p-ERK. A modest but reproducible increase was induced by co-culture with CD31-expressing cells as measured by flow cytometry (Figure 6.11). A significant increase in basal and inducible p-ERK was detected in the $CD38^{hi}$ cohort of patients compared to the $CD38^{lo}$ cohort of patients supporting the findings of western blot analysis. The detection of constitutive p-ERK in CLL has been reported in other studies, for example Steele *et al* were able to detect basal p-ERK in all patients analysed(Steele et al. 2010), Cesano *et al* detected constitutive phosphorylation of ERK in 59% of patients analysed (Cesano et al. 2013), and Muzio *et al* reported the detection of basal p-ERK in around 50% of patients analysed (Muzio et al. 2008). The disparity in terms of detection of constitutive p-ERK in CLL patients in these studies and experiments carried out in this thesis highlight the heterogeneity of CLL and the challenges of working with primary tumour cells. It is possible that both biological and technical differences contributed to the discrepancy between the studies. For example, the prognostic marker composition of the cohorts examined may play a critical role in determining both basal and inducible signalling pathway activation. It was not possible to explore this possibility here, as the previously published work did not describe the patient characteristics of the samples used. In addition to inherent biological differences, cell preparation techniques and phospho-antibody selection may contribute to the discrepant results in terms of phosphorylated ERK protein. Cesano *et al* reported that p-ERK was independently prognostic in terms of TTFT. Therefore the targeting of this pathway is an attractive prospect in CLL, specifically in patients with a progressive disease type (Cesano et al. 2013).

The p-ERK inhibitor U0126 was used in this study to establish the effect of inhibiting this signalling molecule on cell activation markers as well CLL cell viability. The treatment of CLL cells with U0126 for a 24-hour time point resulted in the down-regulation of cell surface activation markers CD25, CD49d and CD69. Furthermore

treatment of CLL cells with U0126 resulted in reduced CLL cell survival. These findings imply that ERK signalling is involved in CLL cell activation as well as supporting CLL cell survival.

The amplitude and duration of PKB and ERK signalling have been shown to be critical in determining cell cycle progression (Longo et al. 2007; Ringshausen et al. 2002). Signalling molecules, which include PKB and ERK, have shown elevated and sustained activation in CLL cells undergoing proliferation. Longo *et al* studied whether the forced activation of PKB and ERK signals altered the propensity of cells to proliferate. To do this, CLL cells were transfected via nucleofection to induce the constitutive phosphorylation of PKB and ERK respectively. Following nucleofection CLL cells were stimulated with CpG ODN for 44 hours, and after this point it was shown that sustained PKB signalling resulted in the up-regulation of Cyclin-A, a marker of cell cycle progression. The sustained activation of ERK was not shown to induce the up-regulation of cyclin A. Inhibitors of PI3K/PKB and ERK signalling pathways were used, with resulting inhibition of proliferative responses. Therefore the observation that cyclin A was not up-regulated following the chronic stimulation of the ERK signalling pathway is surprising. When looking at the expression of cell cycle markers, Longo *et al* discovered that ERK inhibition mainly inhibited the induction of cyclin A, which is involved in later phases of the cell cycle whereas PKB inhibition induced changes in cyclin D3. $P27^{KIP1}$, Cyclin E as well as Cyclin A and PKB has been shown to regulate G1-S phase transition in the cell cycle (Longo et al. 2007). Therefore the activation of these markers induced by PKB signalling is required for cell cycle progression and show why a proliferative response was not observed following the chronic stimulation of ERK.

The dual targeting of ERK and PI3K/PKB signalling pathways has shown to be effective in the treatment of thyroid cancer cells *in vitro.* ERK inhibitor AZD6244 and PI3K inhibitor GDC0941 were used alone and in combination and the levels of p-ERK and p-PKB were measured by western blot analysis. Cell cycle progression and levels of apoptosis were measured using flow cytometry; p-ERK and p-PKB were completely abrogated following treatment with inhibitors and the toxicity of these drugs were shown to be synergistic in combination (Kandil et al. 2013). Therefore the dual targeting of ERK and PKB may provide an interesting therapeutic option for the treatment of CLL. Interestingly a report by Paterson *et al* has shown that the inhibition

of PI3Kδ with CAL-101 has been shown to interfere with ERK activation indicating that ERK is also a downstream target of PI3K, therefore it may be the case that PI3K can coordinate survival signalling through dual pathways both ERK as well as PKB (Paterson et al. 2012).

Furthermore, in this thesis it was shown with western blot analysis and phosphoflow cytometry that the addition of IL-4 to cultures resulted in the rapid up-regulation of p-STAT6. In contrast, there was no detectable basal p-STAT6 and CD31/CD38 ligation did not induce p-STAT6. The IL-4 induced p-STAT6 bands detected with western blot analysis were comparable in intensity in all CLL patients regardless of CD38 positivity, which implies that this signalling pathway is independent of CD38 signalling. Phosphoflow cytometry was conducted at 1 hour and 24 hours and also showed that the addition of IL-4 to cultures at these time points resulted in significantly increased MFI of intracellular p-STAT6. Phosphoflow cytometry also showed that there was no significant difference in MFI of p-STAT6 between $CD38^{lo}$ and $CD38^{hi}$ cohorts of CLL patients.

Treatment with JAK3 kinase inhibitor PF956980 completely abrogated the p-STAT6 signalling and furthermore following 24 hours treatment with PF956980 increased levels of apoptosis were detected. Steele *et al* showed that p-STAT6 could be abolished following treatment with PF956980 but also showed that the constitutive phosphorylation of STAT3 was also inhibited following treatment with this inhibitor (Steele et al. 2010). Therefore the increased levels of apoptosis shown following treatment with PF956980 cannot be completely attributed to the inhibition of p-STAT6 but rather reflect the inhibition of all pathways modulated by JAK signalling. Furthermore a study by Ghamlouch *et al* looked at the effect of multiple cytokines on CLL cell viability. Following the addition of IL-2, IL-6, IL-10, IL-12, IL-15, IL-21, BAFF and APRIL separately to CLL cultures *in vitro* CLL survival was moderately improved. The combination of all of these cytokines resulted in increased CLL cell viability, which was sustained over a period of 7 days (Ghamlouch et al. 2013). This implies that soluble factors may work synergistically to maintain CLL cell survival *in vivo.*

The work conducted in this thesis has demonstrated the potent ability of CLL cells to respond to stimulation *in vitro*. Using phosphoflow cytometry it was possible to quantitatively corroborate findings from western blot analysis. Using western blot

analysis allowed for the identification of p-S6 and p-GSK3β as downstream targets of the PI3K/PKB signalling pathway. This technique allows for the precise molecular weight of proteins of interest to be assessed that is not possible with flow cytometry; western blot analysis also shows antibody specificity. However, flow cytometry allows for the rapid quantification of antigens whereas with western blotting the process takes around 2 days. Flow cytometry also allowed for simultaneous analysis of multiple phospho-proteins in the same cell population. Western blot analysis required >3 x 10^6cells per experimental condition, whereas flow cytometry required much lower numbers of input cells (< 1 x 10^6).

There was a robust increase in phospho band intensity following CD31-expressing co-culture detected with western blot analysis in this study however much more modest differences in phospho-proteins were observed using phosphoflow cytometry. However, it is not possible to come to any definitive conclusions about the role of CD31 as opposed to co-culture with fibroblasts in the parameters measured. Wu *et al* assessed different cell permeabilisation agents for the use in Phosphoflow cytometry. A detergent-based cell permeabilisation method was the most effective for detecting phosphoproteins located in the cytoplasm such as ERK and S6, whereas methanol is more effective in detecting nuclear translocated phosphoproteins such as STATs. Therefore a problem arises when you are assessing the dual phosphorylation of multiple intracellular proteins, which have different requirements. There remain other technical limitations in the phosflow approach, namely the fact that the fluorescent intensities of phosphorylated proteins are quite weak and subtle changes in signalling pathways are often not identified; establishing optimal fixation and permeabilisation methods may help increase the sensitivity of detection (Wu et al. 2010). The combination of western blot analysis and Flow cytometry has proven to be an effective way of identifying phospho-proteins and cell surface markers in this project.

Whilst it is understood that CD38 is a marker of poor prognosis in CLL, it is not known whether CD38 signalling is the cause of developing CLL or arises as a consequence of the disease. Work is ongoing to uncover the role of CD38 signalling in CLL cells, as this should provide a better understanding of the precise biological role of the CD38 protein in this disease. The results generated in this thesis support the view that CD38 is an important signalling molecule involved in the pathogenesis of CLL, providing survival signals to CLL cells which may account for the presence of residual disease

when CLL is treated with chemotherapeutic agents. Therefore the targeting of CD38 with pharmacological inhibitors may help with the treatment of CLL. Daratumumab is a CD38 inhibitor under laboratory investigation, which has been shown to be effective in the treatment of haematological malignancies including CLL as well as multiple myeloma. Parallel studies carried out in 2011 by Weers *et al* and van de Veer *et al* showed that low doses of Daratumumab could inhibit tumour growth in a xenograft mouse model and this drug was also effective in antibody and complement-mediated killing of multiple myeloma *in vitro* even in the presence of bone marrow stromal cells. Furthermore, when Daratumumab was used in combination with Lenalidomide, enhanced cytotoxicity was observed. There are however major concerns with using anti-CD38 antibodies since CD38 is expressed in a wide range of cell types including lymphoid, myeloid, epithelial, eye and brain cells. Therefore the side effects of this treatment *in vivo* would have to be determined. Daratumumab has been used in a Phase I/II clinical trial to treat patients with multiple myeloma and showed clinical benefit in over 50% of patients treated with limited side effects.

The heterogeneity of CLL makes the identification and targeting of pro-survival signalling pathways in patients a promising prospect. However, studies to date have often shown disparity between *in vitro* efficacy and clinical responses *in vivo*. Combining conventional chemotherapeutics and therapies to target microenvironment signalling may prove to be effective in the prevention of MRD. The understanding of CLL biology has significantly advanced within the last decade, however the challenge remains in translating biologically relevant findings into improving the clinical outcome for CLL patients.

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