IDENTIFICATION OF LCK AS A KEY MEDIATOR OF B CELL RECEPTOR (BCR) SIGNALLING IN CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Abstract

Chronic lymphocytic leukaemia (CLL) is a common type of adult leukaemia that accounts for approximately 30% of all mature B-lymphocyte malignancies. An important contributor to CLL pathogenesis is B-cell receptor (BCR) signalling which promotes survival of the malignant clone. BCR engagement on CLL cells provides survival signals by activating the NFκB pathway. Previous work to this thesis showed that CLL cells overexpress PKCβII and c-Abl, kinases which have been implicated in mediating NFκB pathway activation in normal B cells. In particular, PKCβ mediates activation of the CARMA1-Bcl10-MALT1 (CBM) complex leading to eventual activation of I-κB kinases (IKKs) in normal B cells responding to BCR engagement. Considering the importance of the BCR in providing pro-survival signals to CLL cells, the initial aim of this thesis was to characterise any potential role of PKCβII and c-Abl in BCR-mediated activation of the NFκB pathway. We addressed this question in Chapter 3 and showed that inhibition of PKCβ had no effect on BCR-induced activation of IKK, likely because Bcl10 is expressed at low levels in CLL cells. Investigation of the role of c-Abl using the inhibitor imatinib showed that the presence of this compound partially inhibited IKK phosphorylation in BCR-stimulated CLL cells, but the observed effect was variable between CLL patients, and this variability was unrelated to c-Abl expression. Imatinib can also inhibit Lck, a T cell-specific Src-family tyrosine kinase involved in antigen receptor signalling that is also expressed by CLL cells. A further aim of this thesis, answered in Chapter 4, is to define a possible role for Lck in BCR signalling in CLL cells. We showed that inhibition of this Src family kinase (SFK) with the specific inhibitor [4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2d] pyrimidin -7-yl-cyclopentane (Lck-i)], or reduction of its expression with siRNA blocked BCR-stimulated induction of CD79a, Syk, IKK, Akt and ERK phosphorylation in CLL cells. Furthermore, we demonstrated that CLL cells with high levels of Lck expression had higher levels of BCR-mediated IKK, Akt and ERK phosphorylation as well as cell survival than did CLL cells with low levels of Lck expression. These data demonstrated a major role for Lck in proximal and distal BCR signalling in CLL cells. Importantly, these data suggested that Lck expression levels may be linked to disease prognosis. In Chapter 5 we investigated this possibility and showed that high Lck expression was associated with good disease outcome. We hypothesized that this may be because of a dual role played by Lck in promoting and suppressing BCR-induced signalling. We provided data to support this hypothesis and showed that CLL cells bearing low levels of Lck expressed a significantly higher proportion of mannosylated BCR than did CLL cells bearing high levels of Lck, a finding which was consistent with the presence of *in vivo* constitutive BCR signals. Furthermore, we found that Lck mediated the phosphorylation of ITIMs within CD22 during BCR stimulation of CLL cells. Taken together, these data suggest that high levels of Lck expression within CLL cells may function to interact with phosphatases and set an activation threshold to limit in vivo BCR signalling.

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This thesis is dedicated to the memory of Dr. Ghada Alakhras, Dr. Wasfia Mohabik, and Dr Ahmad Alahmar who lost their lives to blood cancer, and to the Syria I knew and loved.

Declaration

The work presented in this Thesis is my own, with the exception of practical assistance during the siRNA, ZAP70 and Syk immunoprecipitation and the CLL survival work which was provided by Dr. John Allen, and Lck quantification which was provided by Dr. Joseph R. Slupsky and Miss Cathrine Clarke

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Abbreviations

(p)ERK: (phosphorylated) Extracellular signal regulated kinase

µg: Microgram (10^{-6} grams)

µL: Microlitre (10^{-6} litre)

µM: Micromolar (10^{-6} molar)

Abc: Antibody control

ABC-DLBCL: Activated B-cell like DLBCL

ALL: Acute lymphocytic leukaemia

APC: Allophycocyanin

BAFF: B-cell activating factor

Bcl-10: B-cell lymphoma 10

BCR: B-cell antigen receptor

BCR-XL: B-cell receptor crosslinking

BLNK: B-cell linker

BSA: Bovine serum albumin

Btk: Bruton's tyrosine kinase

CARMA-1: CARD-containing MAGUK protein 1

CBM complex: CARMA-1 – Bcl-10 – MALT-1 complex

CLL: Chronic lymphocytic leukaemia

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

CrkL: Crk-like protein

Csk: Carboxy-terminal Src kinase

DAG: Diacylglycerol

DLBCL: Diffuse large B-cell lymphoma

DMEM: Eagle's minimal essential medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ECL: Enhanced chemi-luminescence

ELISA: Enzyme-linked immuno sorbent assay

FACS: Fluorescence activated cell sorter

FCL: Follicular lymphoma

FCS: Foetal calf serum

FITC: Fluorescein isothiocyanate

GC-DLBCL: Germinal centre B-cell-like DLBCL

GFP: Green fluorescent protein

GSK3: Glycogensynthetase 3

HCL: Hairy cell leukaemia

HRP: Horseradish peroxidase

HSCs: Haematopoietic stem cells

HSCT: Haematopoietic stem cell transplantation

HSP90: Heat shock protein 90

IκBα: Inhibitor of kappa B alpha

IAPs: Inhibitor of apoptosis proteins

IgG: Immunoglobulin G

IgHV: Variable region of immunoglobulin heavy chain

IgM: Immunoglobulin M

IKK: IκB kinase

IP: Immunoprecipitation

IP3: Inositol-1,4,5-triphosphate

IR: Incidence rate

ITAMs: Immunoreceptor tyrosine-based activation motifs

ITIMs: Immunoreceptor tyrosine-based inhibition motifs

JNK: Jun N-terminal kinase

LC: Lysate control

Lck: Lymphocyte specific tyrosine kinase

LLR: Leukaemia and Lymphoma Research

LPS: Lipopolysaccharide

LTβR: Lymphotoxin beta receptor

MALT: Mucosa-associated lymphoid tissue

MALT-1: Mucosal associated lymphoid tissue 1

MAPK: Mitogen activated protein kinase

MBL: Monoclonal B-cell lymphocytosis

MCL: Mantle cell lymphoma

M-CLL: Mutated CLL

MEK: Mitogen activated protein kinase- kinase

MPP: Multipotent progenitor

mRNA: Messenger Ribonucleic Acid

MW: molecular weight ladder

MZB: Marginal zone B-cell

MZL: Marginal zone B-cell lymphoma

NCI-WG: National Cancer institute Working Group

NEM: N-Ethylmaleimide

NEMO: NFκB essential modulator (IKKγ)

NFAT: Nuclear factor of activated T cells

NFκB: Nuclear factor κ B

NIK: NFκB-inducing kinase

NLS: Nuclear localisation sequence

nM: Nano Molar (10^{-9} Molar)

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PDGF-R: Platelet derived growth factor receptor

PDK1: 3-phosphoinositide dependent protein kinase-1

PE: R-Phycoerythrin

PI3K: Phosphatidylinositide 3-kinases

PIP2: Phosphatidylinositol 4, 5-bisphophate

PIP3: Phosphatidylinositol-3,4,5- trisphosphate

PKC: Protein kinase C

PKCβ: Protein kinase Cβ

PLCγ2: Phospholipase C-γ2

PMA: Phorbol 12-myristate 13-acetate

PTPN22: Protein tyrosine phosphatase nonreceptor type 22

RasGEF: Ras guanine exchange factor

RIPA: Radio-immunoprecipitation assay buffer

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEER: Surveillance, Epidemiology, and End Results programme

SFK: Src family kinase

SH: Src homology domain

SHIP-1 and -2: SH2 domain-containing phosphatidyl 5-phosphatase-1 and -2

SHP1: SH2 domain-containing tyrosine phosphatase-1

sIgM: Surface IgM

siRNA: Small interfering RNA

SLL: Small lymphocytic lymphoma

SOS: Son of sevenless

Src: Cellular homologue of transforming gene of Rous sarcoma virus

SRCR domains: Scavenger receptor cysteine-rich domains

Syk: Spleen tyrosine kinase

TAB: TAK1-binding proteins

TAK1. Transforming growth factor β (TGFβ)-activated kinase 1

TBS-T: Tris-buffered saline+ tween 20

TCR: T cell antigen receptor

TLRs: Toll-like receptors

TNFα: Tumour necrosis factor α

UM-CLL: Unmutated CLL

UT: Untreated

WB: Western blotting

WCL: Whole cell lysate

XIAP: X-linked IAP

XLA: X-linked agammaglobulinemia

ZAP70: Zeta-chain-associated protein kinase 70

Publications

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Chapter 1: General introduction

1.1. Overview

This project is mainly concerned with investigation of the signalling pathway(s) that are triggered by B cell receptor (BCR) crosslinking in chronic lymphocytic leukaemia (CLL) cells. This is important because BCR engagement provides CLL cells with signals that are critical for the survival and proliferation of the malignant clone. The first chapter of this thesis gives a general introduction about the project. The second chapter details the materials and methods used in this thesis. In the third chapter, we concentrate on the pathway leading from the BCR to activation of nuclear factor κ B (NFκB), because this pathway is an important mediator of pro-survival signals. The fourth chapter focuses on the role of Lck in mediating BCR signalling in CLL. In the fifth chapter we investigated the importance of Lck levels in determining disease prognosis and the role of this kinase in downregulating BCR signalling. This project may contribute to development of new therapeutic strategies for treating this, so far incurable, B cell malignancy.

1.2. B-cell chronic lymphocytic leukaemia (CLL)

CLL is a common type of adult leukaemia[1], and accounts for approximately 30% of all mature B lymphocyte malignancies (SEER-9, 2007)[2]. This disease is characterised by a clonal accumulation of $CD5^+$, $CD23^+$ mature malignant B lymphocytes in the peripheral blood, bone marrow and lymphoid tissues[1 3]. In some individuals, the tumour is confined to lymphoid tissue without the involvement of blood or bone marrow; these patients are known as having small lymphocytic lymphoma (SLL) [1] which is considered to be a different manifestation of CLL according to the 2001 World Health Organisation classification scheme

of haematopoietic malignancies[4]. However, although CLL is designated as a single entity, this disease is heterogeneous with respect to its biological, clinical and cytogenetic aspects[2].

1.3. B-cell development

Haematopoiesis of all blood cell types takes place in the bone marrow where the haematopoietic stem cells (HSCs) are resident. There are two major cellular lineages of haematopoietic cells derived from HSC; the lymphoid lineage which is responsible for the development of T and B lymphocytes and natural killer cells, and the myeloid lineage which is responsible for the development of granulocytes, erythrocytes, megakaryocytes, and monocytes[5-6]. It is suggested that these lineages arise from a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP) during differentiation from CD34 positive multipotent progenitor (MPP) cells[5 7-8]. The model presented in Figure 1.1 is a simplistic representation of this differentiation pathway. Although recent work has suggested additional steps within the pathway of MPP differentiation to CLP cells[5 9-10], for the purposes of this thesis it is not important to discuss these steps.

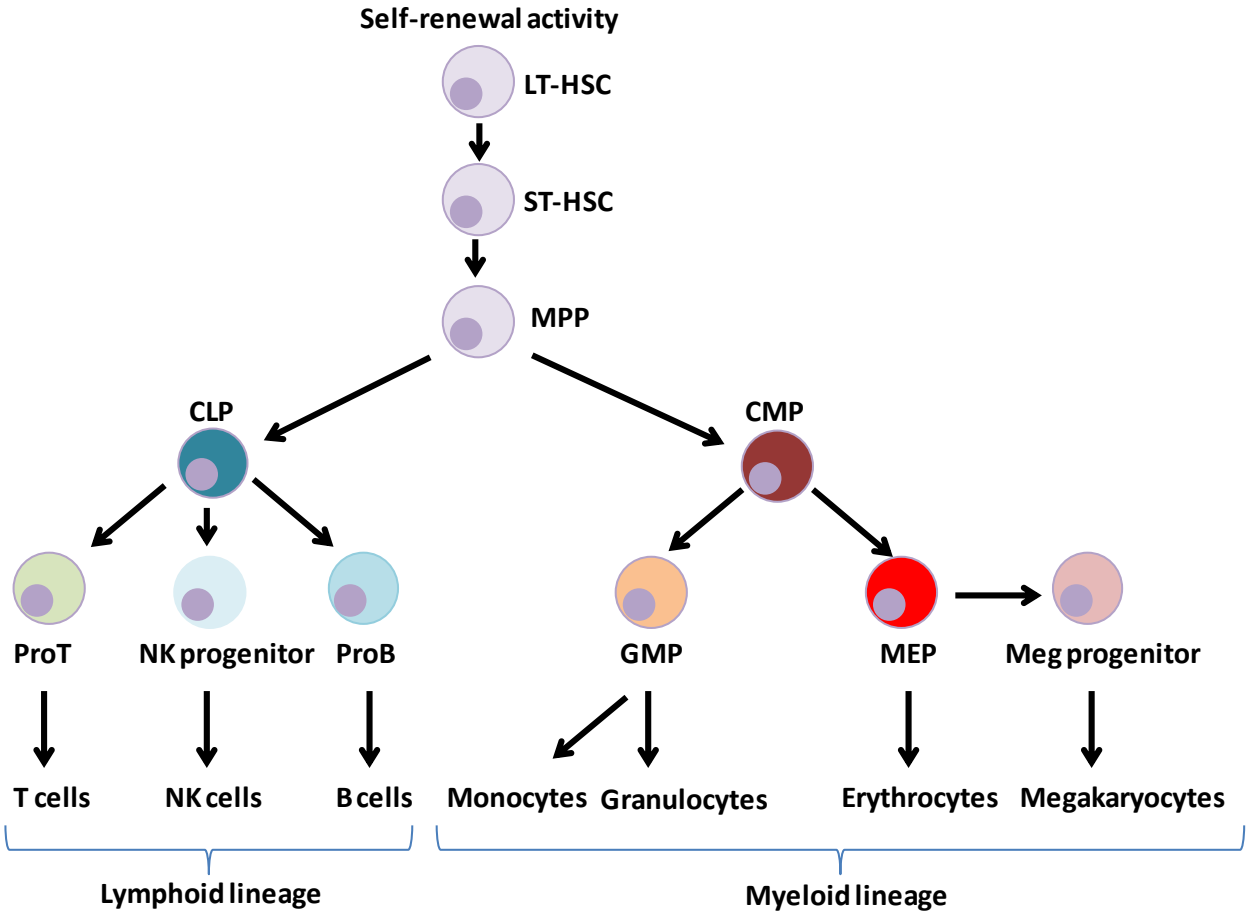


Figure 1.1: The classical model of haematopoiesis (CMP vs. CLP). LT-HSC: long term haematopoietic stem cell, ST-HSC: short term haematopoietic stem cell, MPP: multipotent progenitors, CLP: common lymphoid progenitor, CMP: common myeloid progenitor (adapted from Lai et al.[5]) .

CLP commitment and differentiation to B lineage cells requires the expression of the transcription factors E2A, EBF, and Pax5[11]. Importantly, these transcription factors stimulate the genes responsible for expression and rearrangement of the BCR. In particular, the genes controlling antigen receptor rearrangement (the RAG/TdT genes) promote diversity in the BCR through a process involving recombination of the variable (V), diversity (D), and joining (J) gene segments of the heavy chain locus, and the V and J segments of the light chain loci λ and κ[12-13]. This results in the generation of pre-B lymphocytes with clonally

diverse surface immunoglobulin receptors that recognise specific antigenic epitopes. What follows is a process of clonal selection where B cells that strongly react with self-antigen are deleted, and B cells that weakly react with self-antigen are silenced through a process of anergy. This prevents further development of self-reactive B lymphocytes[14]. These stages of development are shown in figure 1.2[15]. The product of the bone marrow stage in B cell differentiation is the production of a naive B-cell that has undergone V(D)J recombination. Each of these cells expresses mature BCR which is distinct from all others at three levels: the choice of V, D, and J segments, the combination of rearranged heavy and light chains, and junctional insertions and deletions which occur during rearrangement[16].

In the next stage, naïve mature B cells with functional immunoglobulin (Ig) migrate to the secondary lymphoid tissues such as the lymph nodes, where antigen-dependent diversification occurs. This results in B lymphocytes capable of producing antibodies with higher affinity for specific antigens. This affinity maturation takes place in the germinal centres of the lymph nodes and is believed to involve two processes: 1) Somatic hypermutatin (SHM) which introduces point mutations into the complementarity-determining regions (CDR)) of the Ig genes. Activation-induced cytidine deaminase (AID) is thought to initiate this process[16]. 2) Clonal selection involves antigen presentation by follicular dendritic cells (FDCs) to the B cells in the germinal centres. B cells that bind antigen with low affinity will be deleted by apoptosis. While those which bind and subsequently present antigen fragments on their surface receive positive selection signals from CD4+ T helper cells and undergo terminal differentiation to either long lived plasma cells or memory cells[17-19].

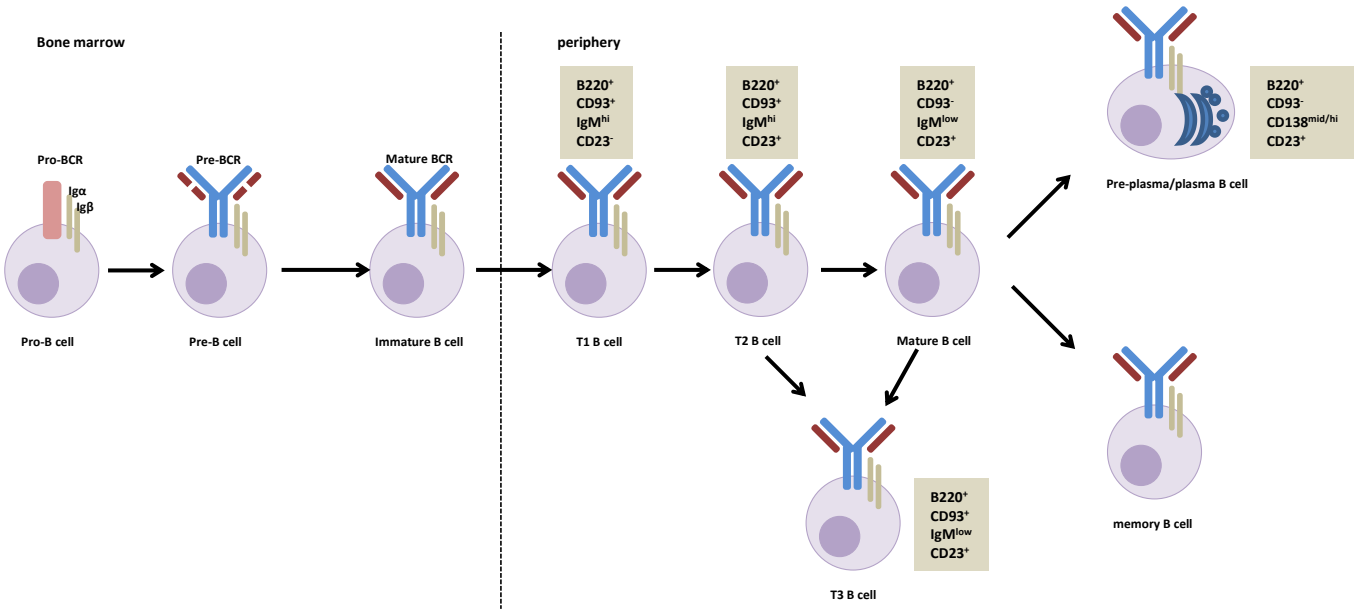


Figure 1.2: summary of B cell development stages in the bone marrow and the peripheral lymphoid tissues (adapted from Cambier et al[15])

In mice, B1 cells differ from B2 cells in their anatomic localisation, phenotype, ability to produce natural antibodies and self-renewing ability[20]. This subset of B cells is classified into the $CD5^+$ B1a and the $CD5^-$ B1b cells, both of which live in the peritoneum[21]. There is a lot of controversy about the origin of these cells and whether they evolve from the same or different progenitors as B2 cells[22]. The function of B1a cells is secretion of natural antibodies which is a source of innate immunity against bacterial infections, whereas B1b cells represent a source of long term adaptive immunity by producing antibodies against polysaccharides in response to infections[23]. However, little is known about this distinct subset of B cells in humans[22].

Marginal zone (MZ) B cells is another distinct subset of B cells with specific functions. These cells reside in the marginal zone of the spleen and have features of naive and memory B cells. These cells are thought to account for immunity against encapsulated bacteria in the blood in human and rodents. Human MZ B cells differ from their murine counterparts in their

ability to recirculate and reside in anatomical sites that are in addition to the spleen. However, the spleen is important for MZ B cells in both mice and humans[24].

1.4. CLL ontogeny

The cause of CLL has not been identified, and although it is known that CLL cells develop from a mature B cell population, the exact stage of differentiation where the neoplasm forms has not been determined. It is suggested that CLL cells can originate from marginal zone (MZ) B cells[25-26], a notion that is supported by 3 facts: (1) After antigen ligation, MZ B cells display an activated surface phenotype[27-29]. (2) MZ B cells also express BCR with mutated and unmutated IgHV genes[30-31]. (3) MZ B cells respond to T- cell dependent and independent antigens[27].

Human CD5 positive B1 cells are also suggested to be the normal counterpart of CLL; this is because CLL cells and B1 cells share similar phenotypes (e.g. both are $CD20^+$ $CD27^+$ $CD43^+$ $CD70^-$). Furthermore, most normal B1 cells are $CD5^+$ as are malignant CLL cells[32-34]. In addition, both B1 and CLL cells express ZAP70[33 35]. In mice during B cell differentiation in the bone marrow, B1 cells originate from the population of pre-B cells that are undergoing positive selection by low affinity interaction with self-antigens at low concentration. Although this may not necessarily represent human physiology, it is nevertheless interesting that when B1 cells are absent due to genetic manipulation of the mouse (e.g. through targeted disruption of particular genes, in this case *prckb*)[36], CLL development does not occur in mouse models of this disease[37]. Further evidence supporting a B1 cell origin of CLL comes from structural studies of the BCR. The antigen binding spectrum of B1 and CLL cells is similar with respect to showing reactivity to autoantigens[25 38]. More recently, a study comparing the transcriptomes of CLL cells and normal B cell subsets from human blood and spleen has suggested that CLL cells can derive from a human counterpart of B1 cells, and

that germinal centre differentiation may also be involved in order to account for the appearance of somatic hypermutation of IgHV genes[39]. This latter aspect is important because more than 50% of CLL patients [32 34] bear somatic hypermutation within the IgHV genes of the malignant clone, and this is not a feature of B1 cells[22].

A hallmark of CLL is the accumulation of mature malignant B lymphocytes in circulation that have escaped apoptosis and are arrested in the G0/G1 phase of the cell cycle[1 40]. CLL cells overexpress the anti-apoptotic protein BCL-2, and expression of pro-apoptotic proteins within these cells is very low[40-42]. This imbalance in favour of anti- over pro-apoptotic proteins was thought to lead to an accumulation of malignant cells due to resistance to apoptosis[43]. This view was disputed in a study by Messmer et al[44] who showed using deuterium labelling to identify newly divided CLL cells that this disease was more dynamic than previously thought, with birth rates ranging from 0.1% to greater than 1.0% of the total clone per day. These authors also suggested that patients in whom the malignant cells proliferated at rates greater than 0.35% of the total clone per day were more likely to have progressive disease. Nevertheless, subsequent studies have shown that CLL cells proliferate at lower levels than do normal B cells in healthy individuals, but that their survival in the circulation is considerably longer[45].

A potential precursor state of CLL has been characterised. Using flow cytometry, researchers studying otherwise healthy individuals have reported the presence of B-cell clones which have a similar immunophenotype to that of CLL cells[46-48]. These seemingly healthy individuals have either normal or slightly increased lymphocyte counts, but because the presence of these "pre-CLL" B-cell clones occurs more frequently in people of advanced age[49] and in first degree relatives of CLL patients[48], it is thought that this condition may precede the development of CLL[2 19]. This potential disorder has been designated monoclonal B-cell lymphocytosis (MBL). A link between MBL and CLL is supported by studies showing

that MBL B cell clones have genetic abnormalities that are typical for CLL cells[19 50-51]. However, it should be noted here that only a small proportion of patients with MBL cases will develop into CLL[19 52].

The pathogenetic mechanisms involved in the evolution of CLL include microenvironmental stimuli consisting of T cell interactions, stromal cell-derived soluble factors and antigenic drive[53]. Internal genetic events also contribute to the pathogenesis of CLL; in particular, cytogenetic abnormalities are very common in the malignant cells of CLL patients. The most frequent of these aberrations are deletions on 13q-, 11q-, 6q-, 17p-, and trisomy of chromosome 12[40 54]. More recently, oncogenic mutations in NOTCH1, XPO1, MYD88, SF3B and KLHL6 have also been identified[55]. It is likely that a combination of all these factors contribute to the evolution of the malignant clone in CLL, particularly with respect to the generation of drug resistant clones as has been suggested in two recent studies[56-57]. Microenvironmental stimulation such as antigen receptor engagement is likely to be the glue binding these elements together because it provides key survival signals for each step in the clonal evolution of CLL cells.

1.5. Epidemiology of CLL

According to the Surveillance, Epidemiology, and End Results programme (SEER), the US incidence rate (IR) of CLL is 4.2 per 100 000 per year, with a higher incidence rate among males compared to females (the male: female IR ratio is 1.93)[58]. In the United Kingdom, data from Leukaemia and Lymphoma Research (LLR) has indicated a higher incidence rate of CLL in the population with an incidence of 6.15 per 100 000 per year[1]. This disease mainly affects older individuals, and the median age at diagnosis of CLL is 70 years for males and 74 years for females. This disease is incurable, and the median age for death from CLL is 76 and 81 years for males and females, respectively [1]. CLL is also a race-related disease; it is

reported that its incidence rate is slightly higher among White Americans than African Americans (3.9 vs 2.8 per 100 000 per year), and this is five times greater than that in American people of Chinese, Japanese, and Filipino origin[1]. A family history of leukaemia or lymphoma is one of the strongest risk factors for development of CLL[2 19]. The overall risk of developing CLL is reported to be three times higher in first-degree relatives of patients with CLL compared to the general population[1 59-60].

1.6. Clinical overview

1.6.1. Clinical features

Signs and symptoms of CLL develop slowly and it is difficult to identify the onset of the disease. Therefore, it is common for CLL to be discovered coincidentally during routine blood tests[61]. The most common clinical features of this disease include lymphocytosis, lymphadenopathy and hepato-splenomegaly. Anaemia and thrombocytopenia are also common in CLL patients in the early stages of the disease[61-62]. Night sweats, fever, and weight loss may also develop as the disease progresses into a more aggressive tumour[40 61]. Viral and bacterial infections are the major causes of death in patients with CLL, caused by immune suppression leading to hypogammaglobulinemia[61 63]. Autoimmune syndromes such as autoimmune haemolytic anaemia are also frequent in CLL[40 61-62].

1.6.2. Diagnosis

Diagnosis of CLL is defined by peripheral blood lymphocytosis of at least 5×10^9 cells/L. These cells have a distinctive immunophenotype; high expression levels of CD5 and CD23, and weak levels of surface immunoglobulin, CD19 and CD20. The antigens FMC7, CD22, CD79b, and CD10 are expressed at very low levels or not at all on CLL cells[19 61-62 64]. Furthermore, because of the non-cycling state of these cells, cyclin D1 expression is absent. Morphologically, CLL cells are small and appear relatively mature with hypercondensed

nuclear chromatin. Smudge cells are also common in CLL[61]. Figure 1.3 shows the differential diagnosis of CD19+ lymphocytosis depending on the immunophenotype.

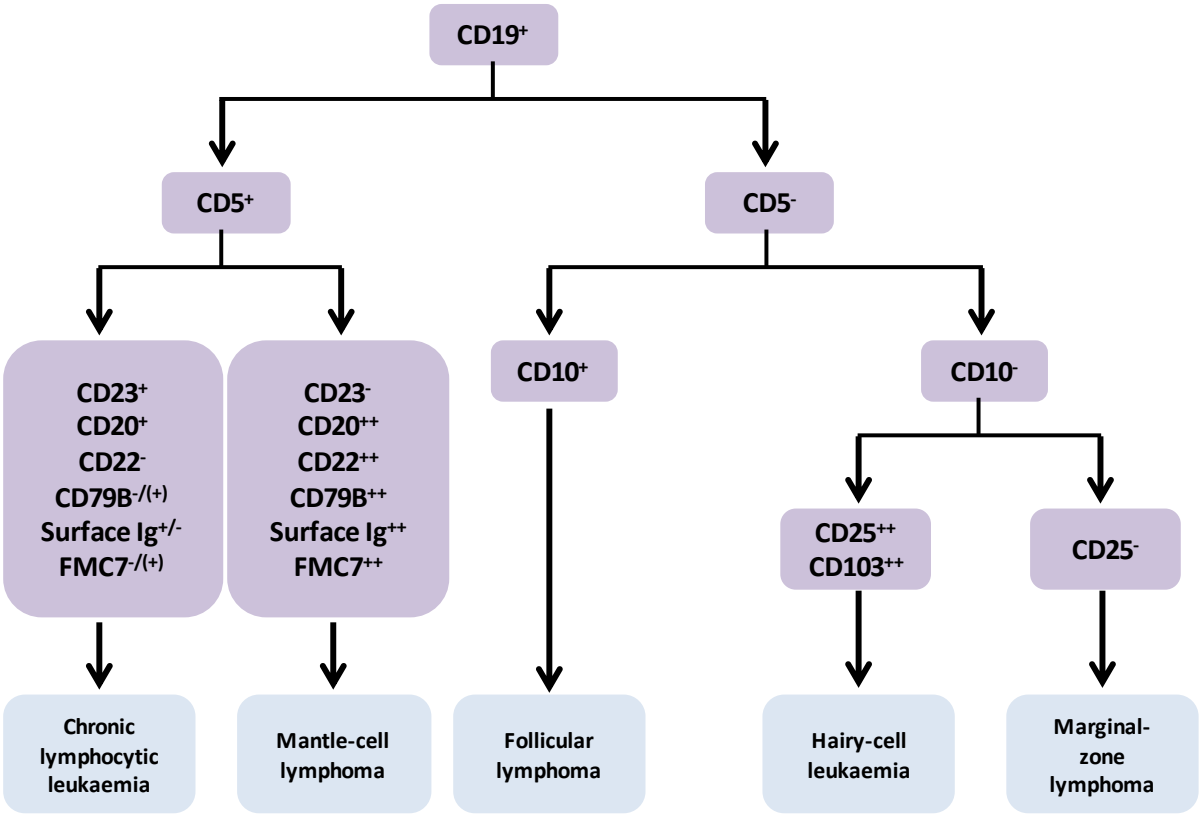


Figure 1.3: Differential diagnosis of CD19$^+$ lymphocytosis. This figure represents a clinical pathway for the diagnosis of mature B cell malignancy based on the immunophenotype of circulating cells (adapted from Dighiero et al [1]).

1.6.3. Clinical staging

CLL is heterogeneous with respect to progression and clinical course. This problem has been addressed in many ways, but initially it was solved by introducing clinical staging systems; one in Europe proposed by Binet et al[65], and one in North America proposed by Rai et al[66]. These staging systems are based on clinical observations and blood tests[61-62 66-68], and include observations on lymphocytosis, lymph and spleen adenopathy and bone marrow suppression. Table 1.1 shows the Rai and Binet systems along with the clinical features and median

survival by stage. These systems are especially used to define whether and when to start treatment[59]. Although these staging systems are able to predict disease outcome, this applies mainly to late-stage and not to early-stage CLL because of an inability of these systems to identify the factors which contribute to indolent or progressive disease[1 61].

Table 1.1: Rai and Binet staging systems[67]

Stage	Risk group	Criteria	Median survival (months)
Rai stage			
0	Low	Lymphocytosis	>150
I	Intermediate	Lymphocytosis+ lymphadenopathy	101
II	Intermediate	Lymphocytosis+ splenomegaly or hepatomegaly	71
III	High	Lymphocytosis+ anaemia	19
IV	High	Lymphocytosis+ thrombocytopenia	19
Binet stage			
A	Low	<3 nodal sites involved	Not reached
B	Intermediate	≥3 nodal sites involved	84
C	High	Anaemia and/or thrombocytopenia	24

1.6.4. Treatment

As discussed above, Rai/Binet staging is used as a guide to determine whether and when to start treatment. According to the NCI-WG guidelines, asymptomatic patients at Binet stage A and Rai stage 0 (low risk) can be managed by a wait-and-watch approach, whereas patients at Binet stage B and Rai stage I and II (intermediate risk) should be treated once they exhibit progressive or symptomatic disease. However, it is recommended that early treatment should be considered for patients at Binet stage C and Rai stage III and IV (high risk)[59 64 69-70].

For many years, first line treatment had consisted of alkylating agents such as chlorambucil or cyclophosphamide, either as a monotherapy or in conjunction with corticosteroids such as prednisone. However these therapies typically give a response rate of less than 10% and the effect they have on overall survival is questionable[71].

In the last two decades, complete remission rates and overall survival rates have improved drastically with the advent of purine analogues and monoclonal antibody therapies which are often used in combination with cyclophosphamide. This regimen is known as FCR (fludarabine, cyclophosphamide, rituximab) and is the most effective current combination according to phase II clinical data[72-73]. The CLL8 trial also supports these findings, with results showing a significantly higher progression free survival time when compared to fludarabine-cyclophosphamide (FC) therapy[74]. The increased efficacy of the FCR regimen appears to be the result of synergy between rituximab and fludarabine[75].

Unfortunately due to the aggressive nature of FCR, many elderly patients are often unable to tolerate it. In this instance chlorambucil still has a role to play, and indeed according to results from the CLL5 study[76] fludarabine monotherapy does not appear to have any significant benefit to chlorambucil in this demographic.

Several studies have observed CLL cells in vitro rapidly undergo spontaneous apoptosis[77-78] and these same cells may be rescued with stromal cell contact or addition of soluble factors, such as thioredoxin which are present in the tumour microenvironment[79-80]. Taken together, this evidence suggests that resistance to apoptosis is not an intrinsic characteristic of CLL cells and that signals from the microenvironment play a significant role in their survival.

Given the advances in understanding the importance of BCR signalling in CLL survival in the recent years, targeting these signalling pathways has lately been introduced as a therapeutic procedure. Several kinases, including spleen tyrosine kinase (Syk), Lyn, Bruton's tyrosine kinase (Btk) and phosphatidylinositol 3-kinase (PI3K), have been targeted by small molecular inhibitors. CAL-101, a PI3Kδ specific inhibitor, has proven to be safe and use of this drug leads to reduced lymphadenopathy in relapsed CLL patients. Another way of targeting BCR signals is inhibiting the B cell specific kinase Btk using PCI-32765 (ibrutinib). This drug showed encouraging clinical effectiveness in CLL patients. These two orally available BCR signalling antagonists are still under ongoing studies[81-83].

Another drug that showed encouraging results in the treatment of high risk CLL patients is lenalidomide, an immunomodulatory agent with anti-angiogenic properties[84]. Lenalidomide monotherapy resulted in an overall response rate that varied between 32% and 54% in different clinical trials[85-87]. However, the combination of lenalidomide and rituximab resulted in a better response rate without a higher risk of toxicity[88].

Haematopoietic stem cell transplantation (HSCT) has been proposed as a possible curative therapy in selected young patients; however, to date this therapeutic approach has not met with a high degree of success as cure appears to be possible in one third to two thirds of patients undergoing this procedure for poor-prognosis CLL[40 59 89].

1.6.5. Prognostic factors

Due to failure of CLL staging systems to indicate CLL patients with higher risk of progression in early stages of the disease, several biological markers have been identified that can be used to predict CLL disease outcome. These markers were based on examination of peripheral blood or bone marrow, such as an identification of high rate of prolymphocytes, atypical morphology of CLL cells, or diffuse infiltration of bone marrow. These aspects are associated with poor disease outcome[66 68 90-91]. Furthermore, two papers were published in 1999 which described somatic mutation of the variable region of immunoglobulin heavy chain (IgHV) genes within CLL cells and its relation to disease prognosis[32 34]. Thus, patients where the level of mutation of these genes in the malignant cells is less than 2% (bearing germline or unmutated IgHV genes) have a significantly shorter overall survival (approximately 8 years) than those where the level of mutation exceeds 2% (bearing mutated IgHV genes) (approximately 25 years). This discovery is possibly the most reliable general indicator of disease prognosis in CLL. Newer factors have also been identified and include lymphocyte doubling time (LDT), CD38 and zeta-associated protein-70 (ZAP70) expression, serum levels of β2-microglobulin, thymidine kinase (TK) and soluble CD23 (sCD23), P53 expression and the presence of certain chromosomal abnormalities[19 59 61 90].

The expression levels of ZAP70 remain constant over the course of the disease. 20% is the proposed cutoff value to classify patients as ZAP70 positive or ZAP70 negative, as measured by flow cytometry. However, standardization of ZAP70 measurement is still a challenge. Furthermore, ZAP70 expression was shown to be associated with unmutated IgHV status and poor disease outcome. Expression levels of CD38 also correlate with CLL disease outcome. The proposed threshold to classify patients is 30% $CD38^+$ CLL cells. Thus, patients with higher than 30% $CD38^+$ CLL cells ($CD38^+$) are reported to have significantly poorer

prognosis regarding PFS and OS. CD38 expression on CLL cells has been shown to correlate with the absence of IgHV mutations[64 90-93].

Chromosomal aberrations in CLL patients have crucial prognostic importance. In this respect, 13q deletion, found in up to 50% of CLL cases, is associated with good disease outcome. While 17p deletion, 11q deletion, trisomy 12, are associated with rapid disease progression, short survival, and resistance to treatment[54 64 90].

Table 1.2 summarizes the impact of each of these factors in the prognosis of CLL.

Table 1.2: Prognosis factors in CLL. Clinical risk is divided into two categories, low and high. Low risk is associated with the potential of the disease to remain indolent while high risk is associated with the potential of the disease to progress. This table is taken from Herishanu et al [62].

Prognostic factor	clinical risk	
	Low	High
Patient gender	Female	Male
Clinical stage	Binet A Rai 0, I	Binet B or C Rai II, III, IV
Pattern of bone marrow biopsy infiltration	Non-diffuse	Diffuse
Lymphocyte morphology	Typical	Atypical
Lymphocyte doubling time	>12 month	<12 month
CD38 expression	<20-30%	>20-30%
Genetic abnormalities	None	Del 11q23
	Del 13q	Loss/mutation of P53
Serum thymidine kinase levels	Low	High
IgHV mutational status	Mutated	Unmutated
ZAP70 expression	Low	High
Beta-2-microglobulin	Low	High
Soluble CD23	Low	High

1.7. Importance of the B-cell receptor (BCR) in CLL pathogenesis

The relationship between IgHV mutation and disease prognosis raised questions about the role of the BCR in CLL pathogenesis. Studies of the genetic structure of BCR showed that CLL cells have a preferred repertoire of IgHV gene usage that is remarkably similar between patients. This observation has led to the generation of the hypothesis that antigen receptor engagement is responsible for expansion of the malignant clone[25]. In particular, studies have shown that the BCR from CLL cells bearing mutated IgHV genes have restricted antigen specificity compared to that from CLL cells bearing unmutated IgHV genes. Thus, if two CLL cell clones are similar with respect to IgHV gene usage, the clone which bears unmutated genes will be polyreactive whereas the clone bearing mutated genes will be more monoreactive[94].

This notion of polyreactive versus monoreactive BCR was then investigated in relation to BCR signalling in CLL cells. Thus, studies investigating this phenomenon have suggested that the ability to signal is related to disease pathogenesis. UM-CLL cells respond to BCR engagement whereas M-CLL cells do not, and it is thought that cell anergy is responsible for this inability of M-CLL cells to respond[95-96]. However, comparisons of gene expression between UM- and M-CLL cells identified ZAP70 as a feature of UM-CLL cells[97]. Analysis of the function of ZAP70 in CLL cells revealed that ectopic expression of this protein in M-CLL cells restores BCR signalling without the need for kinase function of ZAP70[98]. This implies that ZAP70 enhances BCR signalling using a mechanism different to its established role in T cell receptor signalling. Further evidence linking BCR signalling to CLL pathogenesis comes from studies showing that signal strength is also related to disease prognosis[99-100]. Finally, the importance of BCR signals in promoting growth and survival of the malignant clone of CLL is further highlighted by the clinical activity of agents such as Btk, Syk, and PI3K inhibitors in CLL treatment[81 83 101-104].

Evidence that BCR is engaged on CLL cells whilst *in vivo* is suggested in studies showing increased expression of BCR target genes and low expression of surface IgM in freshly isolated CLL cells[80 105-106]. Moreover, a recent study by Krysov et al[107] has measured glycosylation of the BCR on CLL cells and shown it to bear signs of activation particularly in patients with UM-CLL. Cell surface IgM in normal B cells bears mature complex glycans. Persistent antigen stimulation of these cells converts them to expressing the immature mannosylated form with smaller molecular weight[107]. CLL cells exhibit higher levels of immature heavy chain of sIgM compared to normal B cells[108-109] with variable levels of this immature mannosylated form among the cases[107], particularly with respect to UM-CLL cases. When these cases are cultured *in vitro*, the glycosylation reverts to the expression of the mature glycan forms[107]. This supports the notion that CLL cells undergo continuous BCR ligation *in vivo.* This notion is further supported by one study showing that the BCR on CLL cells is capable of antigen-independent cell autonomous signalling[110]. This study has suggested that such autonomous signalling could be a driver of disease pathogenesis in CLL and links together the above observations of BCR target gene expression and changes in glycosylation. This study may also mean that mechanisms exist in CLL cells that act to downregulate these autonomous signals, and this may be the difference between M-CLL and UM-CLL cells.

1.7.1. Structure of the BCR

BCR is a multimeric complex composed of a surface immunoglobulin homodimer molecule and a non-covalently associated heterodimer consisting of Igα (CD79a) and Igβ (CD79b)[1 95 111]. This general structure of the BCR is illustrated in Figure 1.4. CD79a and b contain the immunoreceptor tyrosine activation motifs (ITAMs) within their cytoplasmic domains, and it is here where signalling is initiated once the BCR is engaged[112]. CD19 also co-associates with the BCR, functioning as a signal enhancer by acting as a scaffold for further signalling

proteins[113]. CD22 can also co-associate with the BCR where it acts to downregulate signalling by recruiting SH2-containing tyrosine phosphatase-1 (SHP-1) to its immunoreceptor tyrosine inhibition motif (ITIMs)[114-115].

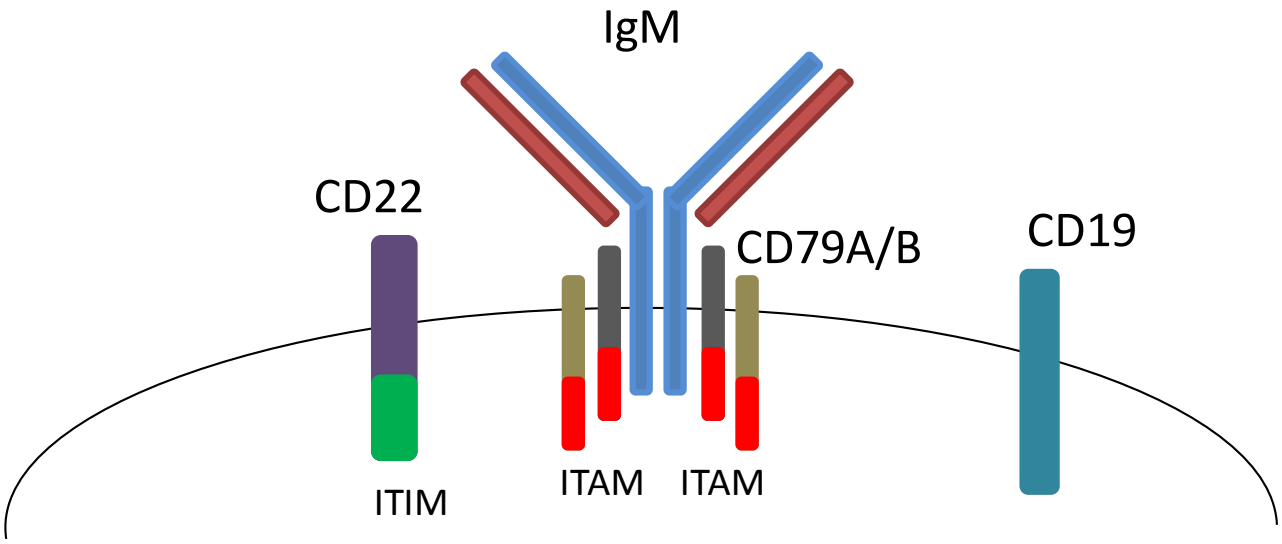


Figure 1.4: Structure of the BCR

1.7.2. BCR signalling in normal B cells

In normal B cells, BCR crosslinking by its cognate antigen induces tyrosine phosphorylation of ITAMs within Igα and Igβ by the Src family tyrosine kinase Lyn[116] and by Syk[117]. The phosphorylated ITAMs are then able to recruit and activate several signalling elements including Syk, Btk, Lyn, the Rac guanine exchange factor Vav proteins, Grb2 and B-cell linker (BLNK). Syk is activated by a multistep phosphorylation process by Src family kinases and autophosphorylation. After recruitment of Syk and Lyn to the phosphorylated ITAMs, BLNK then binds to the non-ITAM part of CD79a through its Src homology domain[118-119]and becomes phosphorylated by Syk. Phosphorylated BLNK then acts as a scaffold and recruits

phospholipase C γ2 (PLCγ2) and Btk[120-121]. Once recruited from the cytosol to the plasma membrane by the adaptor protein BLNK, and then dually phosphorylated by Btk and Syk, activated PLCγ2 cleaves plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of intracellular calcium Ca^{2+}, and together with DAG, leads to the activation of classical protein kinase C (PKC) isoforms within the cell[115].

BCR stimulation also results in the activation of PI3K. This is thought to be regulated by Lyn-mediated phosphorylation of CD19, which attracts the binding of the p85 regulatory subunit of PI3K via SH2 domain interaction and leads to activation of the p110 catalytic subunit. Activated PI3K in turn phosphorylates PIP2 generating phosphatidylinositol-3,4,5-trisphosphate (PIP3) which then recruits pleckstrin homology (PH) domain-containing effectors such as Vav, Btk, 3-phosphoinositide dependent protein kinase-1 (PDK1) and Akt to the cell membrane where they become activated[95 111 115 122-124]. Once activated, Akt promotes cell survival in a myriad of ways including phosphorylating Bad to negate its pro-apoptotic effects at the mitochondrial membrane, phosphorylating TSC2 leading to the activation of mTOR and stimulation of cell metabolism, and phosphorylating GSK3β which inhibits the activity of this kinase and promotes nuclear accumulation of several transcription factors, including NFAT[125].

Btk belongs to the Tec protein tyrosine kinase family, and mediates B-cell development, survival and function through its role in BCR signalling[126]. It has been reported that mutations in the gene encoding Bruton's tyrosine kinase (Btk) cause the human disease X-linked agammaglobulinemia (XLA)[127-128]. In B cells BCR cross-linking activates Btk through membrane recruitment, association with BLNK and phosphorylation at Tyr551in the kinase domain by Lyn and Syk. Active Btk then autophosphorylates on Tyr223 in the SH3

domain[129-130], and goes on to phosphorylate and activate PLCγ2. In addition to its role in PLCγ2 activation, Btk is also reported to play roles in PI3K/Akt as well as NFκB pathway activation[131 102].

Activation of the ERK pathway by the BCR is initiated by convergence of active tyrosine kinase and PLCγ2 pathways. Thus, Son of sevenless (SOS) is recruited and activated by Grb2 binding to ITAM motifs within CD79, and DAG generated by active PLCγ2 results in the activation of the Ras guanine exchange factor (RasGEF) RasGRP1. The GTP-Ras generated by RasGRP1 acts on SOS to amplify GTP-Ras formation by this RasGEF. GTP-Ras then leads to the membrane recruitment and activation of BRaf and c-Raf1, leading to downstream activation of MEK and finally ERK[132].

Activation of PKCs, and, in particular, PKCβ is important for stimulating signalling to the c-Jun N-terminal kinase (JNK) and NFκB pathways in cells responding to BCR crosslinking. Active PKCβ phosphorylates the scaffold molecule CARMA-1which in turn recruits two adaptor proteins Bcl-10 and MALT-1 to lipid rafts. These three proteins (i.e. CARMA-1, Bcl-10 and MALT-1) form a signalosome called the CBM complex which facilitates the recruitment and activation of Transforming growth factor (TGFβ) Activated Kinase 1 (TAK1) by facilitating K63 ubiquitination of IκB kinase γ (IKKγ). This also acts to assemble the catalytic kinase subunits, IκB kinase α (IKKα or IKK1) and IKKβ (IKK2), which are essential for further downstream signalling in the NFκB pathway[133-135]. TAK1is thought to play a role in this process by phosphorylating IKKβ within this complex. Once activated, the IKK complex phosphorylates IκBα and this leads to the release of RelA (p65) to translocate to the cell nucleus[134]. Active TAK1 can stimulate the JNK pathway by phosphorylating MKK4 and 7 to eventually activate JNK itself.

Activation of PI3K and generation of PIP3 also has the effect of recruiting the RacGEF Vav to the cell membrane. Formation of GTP-Rac1 then leads to important cytoskeletal changes in cells responding to BCR stimulation, and also to the activation of the p38 MAPK pathway[136].

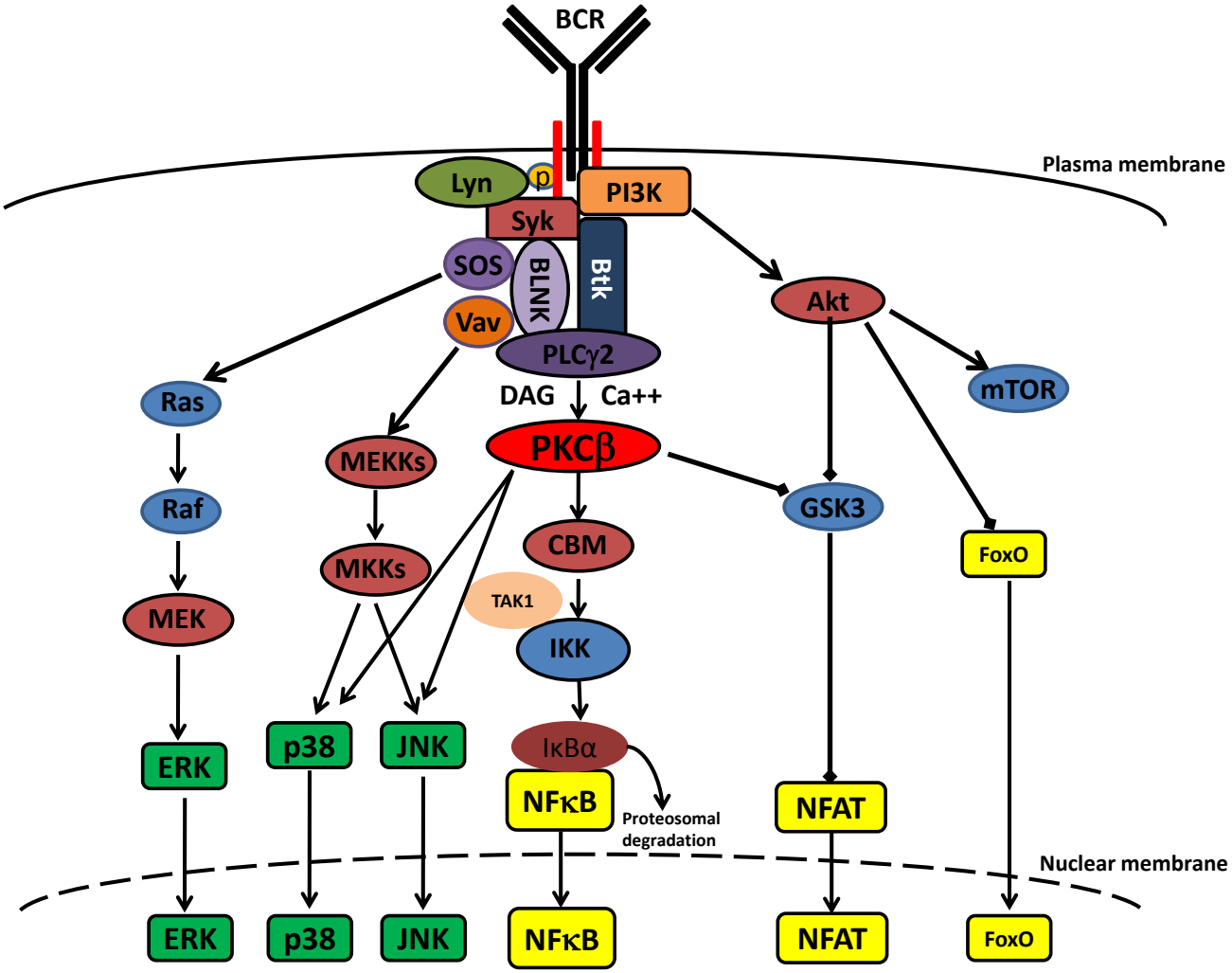


Figure 1.5: BCR signalling in normal B cells. This diagram is representative of the signalling pathways that become activated in B cells responding to BCR crosslinking (adapted from Dal Porto et al[115]) .

1.7.3. The Nuclear factor κ B (NFκB) pathway

Nuclear factor κ B (NFκB) is a family of transcription factors consisting of five proteins that can form homo- or heterodimers. These proteins are termed RelA (p65), RelB, c-Rel, p50

(NFκB1) and p52 (NFκB2). NFκB factors play an essential role in B cell development, proliferation, immune responses, and survival. NFκB exists in the cytoplasm of resting cells as an inactive form associated with inhibitory proteins. These inhibitory proteins, which include IκBα, IκBβ and IκBγ, mask the nuclear localisation sequence (NLS) of NFκB factors such as RelA and sequester it in the cytoplasm. NFκB1 and NFκB2 exist as, respectively, p105 and p100 precursors in the cell cytoplasm, and must first be proteolytically cleaved before p50 and p52 can translocate to the nucleus[102 103].

The process that catalyses NFκB translocation to the nucleus can occur through either a classical (canonical) or an alternative (non-canonical) pathway. Both of these pathways depend on the complex of I-κB kinases α, β and γ known as the IKK complex (Figure 1.6). The canonical pathway is triggered by ligation of receptors such as the antigen receptor on B and T cells, Toll-like receptors (TLRs) and the Tumour necrosis factor α (TNFα) receptor. These stimuli catalyse, through various processes, K63 ubiquitination of IKKγ leading to activation of TAK1 which then phosphorylates and activates IKKβ. Phosphorylation of IκBα by IKKβ triggers K48 ubiquitination of IκBα and subsequent proteosomal degradation. This releases RelA to translocate into the nucleus and engage its enhancer elements to activate the expression of target genes. Alternatively, non-canonical pathway activation is stimulated by receptors such as CD40, BAFF receptor and LTβR. Stimulation through these receptors activates NFκB-inducing kinase (NIK), which phosphorylates and activates IKKα, which, in turn, phosphorylates p100 to induce its proteosomal processing and release p52/RelB dimers. These p52/RelB dimers then translocate to the nucleus where they regulate distinct transcriptional responses, particularly with respect to B cells. This pathway is independent of IKKγ[137-139].

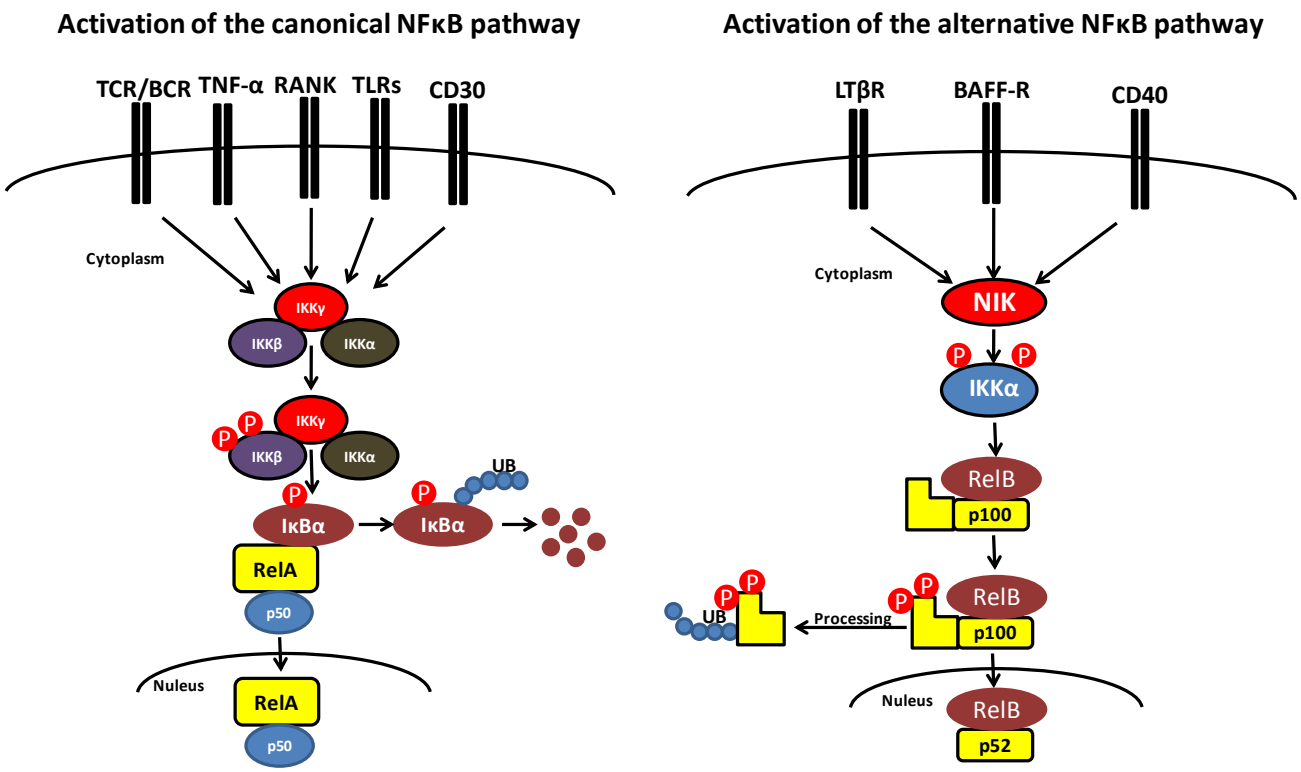


Figure 1.6: NFκB pathway. The canonical vs. the alternative pathway of NFκB activation (Adapted from Jost et al[137]).

1.7.4. BCR signalling in CLL cells

It is well known that crosslinking of the BCR on CLL cells leads to heterogeneous responses, particularly with respect to the proximal events of antigen receptor signalling[95]. In CLL cells bearing unmutated IgHV genes BCR crosslinking generally induces a response. However, in some cases of CLL where the malignant cells bear mutated IgHV genes, the response to BCR crosslinking is more muted[140]. One possible reason for this heterogeneity is that higher levels of surface Ig are often found on the malignant cells from UM-CLL cases compared to those from M-CLL cases[106 141]. The inability of BCR to signal in M-CLL cells has also been attributed to these being in an anergic state and unable to fully respond to such crosslinking[142]. Such anergy may be because the BCR on M-CLL cells fails to translocate into membrane lipid raft structures following crosslinking whereas this does occur on UM-CLL cells[96]. Heterogeneous ZAP70 expression could also explain this difference in ability to

signal. ZAP70 expression is significantly higher in the malignant cells from UM- compared to M-CLL cases, and has been proposed as a pseudomarker of IgHV mutation[97 143-144]. ZAP70 plays an essential role in antigen receptor signalling on T cells[145-146], and in CLL cells the expression of this protein also facilitates antigen receptor signalling by acting as an adaptor molecule rather than a kinase[147 111 141]. This notion is supported by studies showing that ectopic expression of ZAP70 as a kinase-dead mutant restores BCR signalling in M-CLL cells [98].

Despite the ability of BCR engagement to provide signals in UM-CLL cells, the immunogenic response in these cells is incomplete and does not induce proliferation[95]. BCR crosslinking on UM-CLL cells with soluble anti-IgM has been shown to stimulate the Akt, NFκB and ERK pathways in the majority of cases. However, in these same cases JNK is almost never activated[148-150]. Thus, CLL cells respond to antigen receptor stimulation differently than do normal B cells.

Heterogeneity in CLL cell response to BCR crosslinking could also be an adaptation to antigen independent cell autonomous signals that are generated by the BCR. A recent study has shown that BCR on CLL cells contains structural elements that are conducive to antigen independent cell autonomous signalling, and has suggested that such signalling drives CLL cell pathogenesis[110]. In this context, certain signalling pathways will be constitutively active and CLL cell response to BCR crosslinking may be a function of regulatory elements that limit the generation of intracellular signals. In support of this, a characteristic of CLL cells is the constitutive activation of certain kinases such as Lyn and Syk. It has been shown that both of these kinases are overexpressed in CLL compared to normal B cells, and that their activity is also upregulated in unstimulated cells[151]. Inhibition of Syk either with fostamatinib or by siRNA knockdown shows that this kinase plays an important pro-survival role in both

unstimulated[152] CLL cells as well as in BCR stimulated[153] cells where it controls influx of Ca^{2+} and the phosphorylation of Akt and ERK[153]. However, the role of Lyn in these processes is less clear because inhibitor studies have not been able to target this kinase specifically, and it is resistant to siRNA-mediated knockdown due to association with HSP90[154]. The PI3K/Akt pathway is also reported constitutively active, and inhibition of this pathway using PI3K inhibitors such as CAL-101 or LY294002 reduces CLL cell survival by downregulating the expression of anti-apoptotic proteins such as Mcl-1, XIAP, and the induction of Bcl2 family member Bax[155-156]. Finally, constitutive activation of NFκB and ERK in CLL cells is demonstrated in a subset of patients, and is associated with low responsiveness to BCR ligation [142 157-158].

1.7.4.1. The role of SFKs in CLL

The differences between CLL cell and normal B cell response to BCR crosslinking may be due to differences in expression of the Src family tyrosine kinases (SFKs) responsible for proximal signalling. SFKs are a family of non-receptor protein tyrosine kinases that are involved in, depending on the cell lineage and particular SFK, regulating cellular processes including proliferation, survival, metabolism, differentiation, and migration. This family of proteins consists of 8 members: Lyn, Lck, Src, Fyn, Fgr, Hck, Blk, and Yes[159-160].

The general structure of an SFK is shown in Figure 1.7. It is composed of 8 functional domains, the order of which starting from the N terminus is: a domain containing a myristoylation site that targets SFKs to membrane structures, a Src homology domain 4 (SH4) domain, a unique region that is specific for each Src family member, an SH3 domain, an SH2 domain, a linker domain, a kinase (SH1) domain and a regulatory domain[161]. There are two important tyrosine phosphorylation sites within SFKs, one within the kinase domain

and a second in the regulatory domain[162-164]. Phosphorylation of the tyrosine residue located in the regulatory domain of an SFK allows this domain to interact with its SH2 domain and hold the enzyme in an inactive state. Phosphorylation at this residue is catalysed by kinases such as Csk (carboxy-terminal Src kinase). Enzyme activation is achieved when phosphatases such as SHP-1, SHP-2 and PTP1 dephosphorylate the SFK within the regulatory domain allowing unfolding of the protein and autophosphorylation of the tyrosine residue within the catalytic domain. Such autophosphorylation stabilizes the protein structure and allows the SFK to phosphorylate its target substrates.

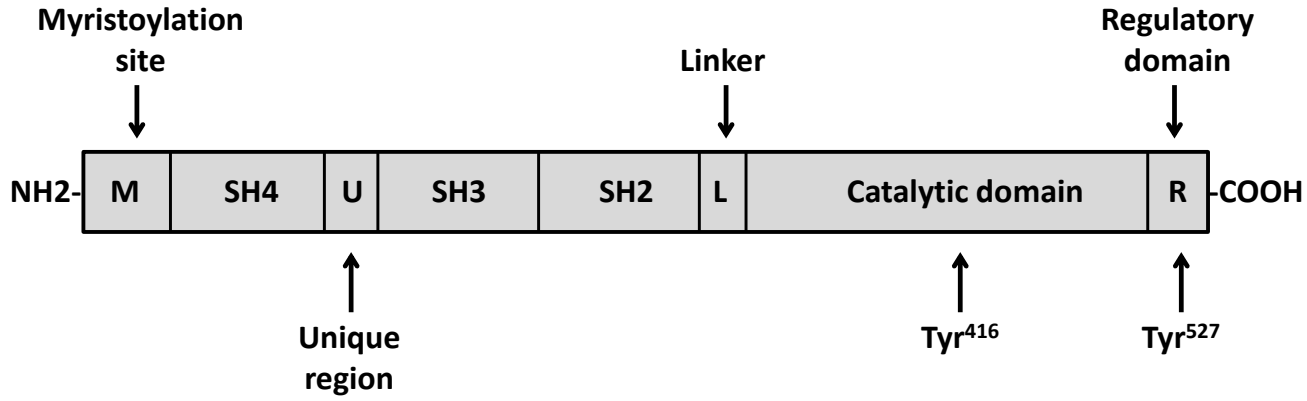


Figure 1.7: Structure of SFKs. This figure represents the general structure of SFKs (adapted from Hu et al[161]).

An important SFK in B lymphocytes is Lyn. This kinase can act as a positive and negative regulator of BCR signalling[165-166] through its ability to phosphorylate both ITAMs and ITIMs[167-168]. This kinase has two isoforms resulting from alternative splicing that differ from each other by 20 amino-acids within the SH4 domain[169-171]. Inactive Lyn is phosphorylated on Y^{508} within its regulatory domain by Csk. Activation of Lyn is dependent on CD45 mediated dephosphorylation of this tyrosine residue triggering its autophosphorylation at the

activating tyrosine Y^{397} within the catalytic domain[170 172]. The tyrosine phosphatases SHP-1 and SHP-2 are involved in Lyn inactivation by mediating dephosphorylation of Y^{397}. This occurs upon recruitment of these phosphatases to ITIM motifs within substrate proteins of Lyn, and is important for downregulating Lyn kinase activity[173].

As indicated above, Lyn is overexpressed by CLL cells, is constitutively active and cannot be further activated by BCR crosslinking. This constitutive activity of Lyn results in high basal levels of tyrosine phosphorylated proteins in CLL cells. The importance of constitutively active Lyn to CLL cell pathophysiology is demonstrated in experiments using the SFK inhibitors PP2 and SU6656; treatment of CLL cells with either of these compounds induces apoptosis[174]. However, these experiments do not reveal a true role for Lyn because PP2 and SU6656 are not just specific for Lyn and inhibit other SFKs when used at the concentrations reported in this study[174-175]. Attempts to address the potential role of Lyn in CLL cells have used geldanamycin to disrupt Lyn association with HSP90[154]. Such treatment results in downregulation of Lyn expression and induction of CLL cell apoptosis. However, because HSP90 regulates stability of other proteins that may be important to the survival of CLL cells, further insight into the role of Lyn is not achieved.

CLL cells also express Fyn, Fgr, Blk and Lck SFKs. The function of these SFKs in CLL cells has not been well studied, but Lck is of particular interest because it, like ZAP70, is normally highly expressed only in T cells. Similar to Lyn and other SFKs; Lck phosphorylation has a major role in regulating its activity. Activation of this kinase requires dephosphorylation of Y^{505} in the regulatory domain, a process that is mediated by CD45 and results in unfolding of this protein to trigger autophosphorylation of Y^{394} in the kinase domain[176]. Like Lyn, dephosphorylation of Y^{394} in Lck and phosphorylation of Y^{505} by Csk result in the deactivation of Lck[176-178]. Dephosphorylation of Y^{394} in Lck can be mediated by CD45[179-181], SHP-1[182] and other protein tyrosine phosphatases.

Unlike Lyn, Lck can be additionally phosphorylated on serine residues within the unique region of the N-terminus. Such additional phosphorylation has been documented in studies reporting a shift in the apparent molecular weight of Lck protein in Western blots[183-185]. This shift in the molecular weight is attributed to phosphorylation of Lck on serine residues 42 and 59, and is mediated by PKC and by Erk, respectively[186-187]. Serine phosphorylation of Lck is thought to regulate its interaction with tyrosine phosphatases such as SHP-1 and prevent its inactivation by this phosphatase[188-189]. Another function of serine phosphorylation of Lck is to label this protein for ubiquitination and subsequent proteosomal degradation[190].

Although Lck is expressed mainly in T cells, it has been shown that this kinase is also expressed in normal B1 cells, as well as in CLL cells[191]. In T cells, Lck plays an important role in proximal antigen receptor signalling. However, in B1 cells the role of Lck is controversial; one study has shown that Lck expression is responsible for peritoneal B1 cell hyporesponsiveness to BCR stimulation[192], while another study has suggested that Lck facilitates BCR signals in these cells[193]. However, a third study has indicated that Lck does not play any role in BCR signalling in B1 cells[194]. In CLL cells Lck may contribute to glucocorticoid resistance as has been suggested in one study[195]. This contribution may be independent of BCR signalling because Lck expression levels in CLL cells do not correlate with ZAP70 levels, the principle substrate of Lck in T cells[196]. Lck in CLL cells may be important for downregulating BCR signals because this kinase can phosphorylate ITIMs within CD5 in T cells to result in downregulation of T cell receptor signalling. However, a role for Lck has not been defined with respect to BCR signalling or otherwise in CLL cells or in B1 cells.

1.7.4.2. The role of PKCβ in CLL

The differences between CLL cell and normal B cell response to BCR crosslinking could also be explained by differences in the level of expression of protein kinase Cβ (PKCβ). This protein functions to regulate the activation of IKK and deactivation of Btk during BCR stimulation. Previous work in this Department has shown that PKCβII is overexpressed in CLL cells compared to normal peripheral B cells. Furthermore, this study also showed that PKCβ activity correlates inversely with the ability of CLL cells to respond to BCR crosslinking, most likely through its ability to phosphorylate and deactivate Btk[197]. Another B cell malignancy where the malignant cells overexpress PKCβII is the subset of diffuse large B-cell lymphoma (DLBCL) that have an activated B-cell like phenotype. In these cells overexpressed active PKCβ functions to activate the NFκB pathway to provide pro-survival signals and impart an aggressive clinical course of this disease[198-199]. However, whether PKCβII functions in a similar capacity in CLL cells has not been addressed.

1.7.4.3. The role of c-Abl in CLL

The non-receptor tyrosine kinase c-Abl may also be important for BCR signalling in CLL cells. This kinase is heterogeneously expressed in CLL cells, and it has been reported that the levels of c-Abl protein expression are higher in CLL B lymphocytes than in normal peripheral blood B lymphocytes. Moreover, there is a positive correlation between the expression level of c-Abl and tumour burden, and a negative correlation between c-Abl and IgHV mutation[200]. This kinase has a variety of functions within the cell, including cell adhesion, regulation of cytoskeletal reorganisation, survival, response to DNA damage, oxidative stress, and growth factor stimulation. c-Abl is thought to play an important role in normal B cell development[200-205], where it acts to phosphorylate CD19, a BCR co-receptor, under conditions of antigen receptor stimulation. This notion is supported by experiments

showing that BCR engagement in c-Abl deficient B-cells leads to defective activation of these cells[200 204 206], and by observations that c-Abl protein levels and activity are elevated following BCR crosslinking in some B cell lines[206]. The role c-Abl plays in this context may be in relation to activation of the NFκB pathway. Work in this Department has demonstrated a connection between c-Abl and activation of NFκB[200], and has further demonstrated that this contributes to CLL cell survival by modulating Mcl-1 expression through a mechanism involving the release of IL6 and stimulation of STAT3[207]. Whether c-Abl functions in CLL cells in relation to BCR engagement has not been investigated.

1.7.5. Negative regulation of BCR signalling

BCR signalling is modulated by inhibitory co-receptors including CD22, FcγRIIB and CD72, and in the B1 subset also CD5. These cell surface molecules play a key role in determining the intensity and duration of BCR signals. The cytoplasmic tails of these cell surface antigens contain ITIMs, which are targets of Lyn following BCR stimulation[208-211]. Phosphorylated ITIMs, in turn, recruit inhibitory phosphatases such as SH2 domain containing tyrosine phosphatase-1(SHP-1), SH2 domain-containing phosphatidyl 5-phosphatase-1 and -2 (SHIP-1 and -2), and protein tyrosine phosphatase nonreceptor type 22 (PTPN22) to the BCR complex. This leads to downregulation of BCR signals through their ability to deactivate Lyn and PI3K signalling[168 212]. Absence of these regulatory mechanisms results in uncontrolled activation of the BCR and can lead to the development of autoimmune diseases and B cell malignancies[102]. Figure 1.8 shows an illustration of the BCR and these cell surface proteins.

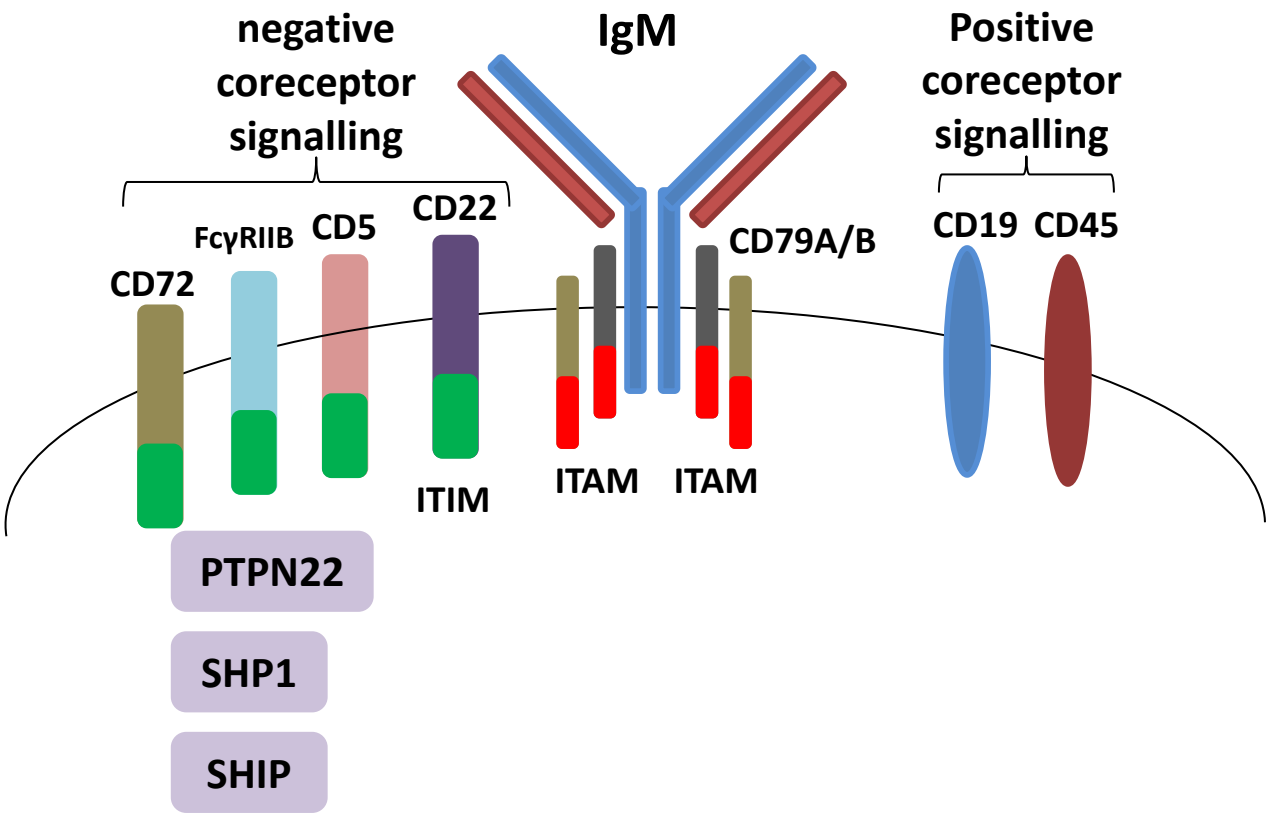


Figure 1.8: B-cell receptor signalling in CLL. negative and positive co-receptors (adapted from Stevenson et al[212]).

1.7.5.1. CD5

CD5 is a 67-kDa type I trans-membrane glycoprotein with three scavenger receptor cysteine-rich (SRCR) domains within its extracellular region[213-214]. The cytoplasmic tail of CD5 contains ITAM- like and ITIM-like sequences[215-216]. This protein is mapped to position 11q12.2 in the human genome[217]. Normally this protein is expressed on T cells and on the B1a subset of B lymphocytes [20].

B1a cells differ from B2 lymphocytes in their response to BCR crosslinking. Whereas B2 cells proliferate in response to BCR crosslinking, stimulation of the BCR on B1 cells results in induction of apoptosis. The role of CD5 in regulating BCR signalling in B1 cells was

demonstrated using B1 cells from CD5 deficient mice; BCR crosslinking on these cells provided resistance to apoptosis and induced entry into the cell cycle. Thus, the role of CD5 on B1 cells is as a negative regulator of BCR signalling[218]. That it is a target of Lyn was shown in a different study that demonstrated that BCR ligation on B1a cells from $Lyn^{-/-}$ mice induced cell proliferation[219]. The role of CD5 in downregulating antigen receptor signalling on B1 cells is consistent with its function on T cells where it acts as a negative regulator of TCR signalling[220]. Experiments using CD5 knockout mice demonstrated that T cells from these mice display increased proliferation, release of intracellular Ca^{2+} stores and in tyrosine phosphorylation of PLCγ-1 and the adaptor molecule Linker Activator for T-Cells (LAT) in response to antigen receptor stimulation[220]. In T cells CD5 is a substrate of Lck[221].

The inhibitory role of CD5 in antigen receptor signalling in B and T cells is dependent on the recruitment of SHP1 to the antigen receptor complex[211]. Upon binding the phospho-tyrosine residue within the ITIM of CD5 via its SH2 domain, SHP1 undergoes conformational change to activate its phosphatase activity. Activated SHP1 then dephosphorylates the ITAM elements within the antigen receptor complex as well as the SFKs involved in their phosphorylation. This leads to termination of antigen receptor signalling[211 222].

The role of CD5 in regulating BCR signalling in CLL cells is not fully understood. A study by Perez-Chacon et al[223] has shown that apoptotic or viability signalling in BCR stimulated CLL cells was similar between control cells and cells where CD5 was dissociated from the BCR complex by antibody-mediated crosslinking. This study also reported that CD5 has no effect on the phosphor-tyrosine pattern in response to BCR ligation in these cells. This study concluded that the enhanced survival of CLL cells could be due to impairment of the inhibitory role CD5 might normally play in normal B and T cells[223]. However, this conclusion has been challenged by a subsequent study showing that phosphorylation of ITIM sequences within CD5 on CLL cells is mediated by constitutively active Lyn, and that this

results in the recruitment of SHP-1 to downregulate BCR signalling in these cells[224]. Furthermore, a study by Gary-Gouy et al[225] demonstrated that CD5 is constitutively tyrosine-phosphorylated in CLL cells. This study also showed that CD5 phosphorylation on B cells led to upregulation of many apoptosis inhibitors such as Bcl2, NFκB and TLR-9, a feature of CLL cells. This supported a role for CD5 activation in CLL cell survival.

1.7.5.2. CD22

CD22 is a B-cell specific transmembrane sialoglycoprotein that belongs to the sialic acid-binding immunoglobulin-like lectin (Siglec) family of adhesion molecules[226], with a molecular weight of 140 kDa. High expression of this protein is observed on all subsets of mature B cells, with the exception of plasma cells where CD22 levels are downregulated[168] [227-228]. Figure 1.9 shows the basic structure of this protein; the extracellular domain consists of seven Ig-like domains, while the cytoplasmic tail contains six tyrosine residues with three (Y^{762}, Y^{822}, and Y^{842}) considered to be within ITIM motifs. The cytoplasmic tail of CD22 also contains two ITAM motifs, and it has been suggested that CD22 can also provide positive signals promoting B-cell survival[229-230].

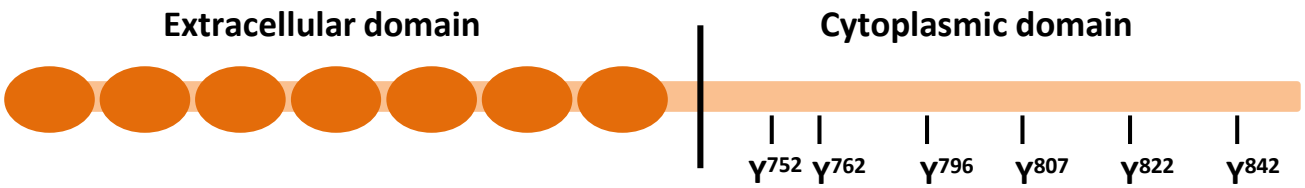


Figure 1.9: Structure of CD22. Illustration of the structure of CD22 showing the extracellular and cytoplasmic domains (adapted from Fujimoto et al [231]).

BCR ligation by cognate antigen results in CD22 association with the BCR complex where it becomes phosphorylated[232]. Tyrosine phosphorylation of CD22 requires activity of the tyrosine kinase Lyn; this was demonstrated by reduced CD22 phosphorylation in Lyn-deficient mice[208 233-234]. Tyrosine phosphorylated CD22 recruits SH2-domain containing proteins, such as SHP-1 which dephosphorylates BCR signalling components leading to BCR signal termination[235]. The inter-relationship between CD22, Lyn and SHP-1 is supported by an *in vivo* study whereby sustained Lyn activation in mouse B cells was achieved using a targeted gain-of-function mutation (Lyn $^{(up/up)}$ mice)[165]. Within this model CD22 and SHP-1 were constitutively phosphorylated, and the B cells from these mice had features reminiscent of B cells rendered tolerant by constitutive BCR signalling. Other studies using targeted disruption of CD22 expression have shown that B cells derived from these mice are hyperresponsive to BCR engagement[236-238]. Phosphorylated CD22 can also recruit SHIP-1 by forming a quaternary complex with the adaptor proteins Grb2 and Shc, and this also results in reduction of BCR-induced signalling[239].

That CD22 could be involved in positive regulation of BCR signalling is consistent with a study showing that CD22 coligation with the BCR downregulates activation of the MAPK pathway, but separate CD22 crosslinking on its own results in upregulation of this signalling pathway[240]. Moreover, crosslinking of CD22 initiates a signalling cascade that leads to JNK activation and proliferation of tonsillar B cells[229 241]. However, the inhibitory role of CD22 seems to be the predominant function of this protein because of the enhanced BCR signalling that is observed in CD22-deficient mice[227 236-238].

CD22 is variably expressed on the malignant cells of different B cell leukaemias and lymphomas. Compared to normal B cells, low expression of CD22 was detected in acute lymphocytic leukaemia (ALL), CLL, follicular lymphoma (FCL), and mantle cell lymphoma (MCL). On the other hand levels of expression were moderate in marginal zone B-cell

lymphoma (MZL) and high in hairy cell leukaemia (HCL)[210 242-244]. However, different technical variables affect measurement of CD22 expression by quantitative flow cytometry, and delayed processing time, shipment time of the samples and the number of cells analysed can all contribute to altered readings of expression[242]. The level of CD22 expression varies among CLL cases with lower levels of expression observed on the malignant cells from patients with Binet Stage C disease compared to those with stage A and B[245]. The importance of CD22 expression in CLL cell pathophysiology is suggested by a recent study showing that the interrelationship between CD22, Lyn and SHIP is overcome by overexpressed PTPN22, and this leads to selective upregulation of the Akt pathway and downregulation of most other BCR signalling pathways[246]. This is important because Akt provides prosurvival signals to BCR stimulated CLL cells[246-248].

1.7.5.3. FcγRIIB (CD32)

FcγRIIB is a 40 kDa transmembrane inhibitory regulator of BCR signalling containing an ITIM within its cytoplasmic domain[102]. Co-crosslinking of this low affinity receptor of IgG with the antigen receptor on B cells occurs as a result of BCR interaction with IgG-containing immune complexes. This leads to dampening of BCR signalling which, in turn, results in reduction of cell proliferation and antibody production[249-252]. The inhibitory function of FcγRIIB is due to its ability to recruit SHIP to the plasma membrane when it is coligated with the BCR[253]. Active SHIP hydrolyses PIP3 and consequently reduces the membrane binding of PLCγ2, Akt, and Btk[254-256]. Coligation of the BCR with FcγRIIB also leads to recruitment of SHP1 and downregulation of BCR signals[257-259]. However, it has been reported that recruitment of SHP1 is not essential for FcγRIIB-mediated inhibition of BCR signalling[260], this effect is predominantly mediated by SHIP[261].

In B cells, tyrosine phosphorylation of FcγRIIB is thought to be mediated by Lyn because this protein is not phosphorylated in B cells from Lyn knockout mice undergoing BCR engagement[166-168 208]. Lyn is also required within the BCR-FcγRIIB complex for the phosphorylation of SHIP[165].

FcγRIIB is variably expressed in different types of B cell lymphomas with strong expression levels on MCL cells, splenic marginal zone lymphomas, mucosa-associated lymphoid tissue (MALT) lymphomas and B-CLL/SLL. A proportion of DLBCL (20%) also express this coreceptor on their cell membrane, with higher levels in transformed activated B-cell like DLBCL than in GC-like DLBCL. The higher levels of expression were linked with poor prognosis in this B cell malignancy[262].

Levels of FcγRIIB expression have been shown to be similar or even higher in CLL compared to normal B cells. Unlike normal B cells, a subset of CLL was shown to express the activating isoforms FcγRIIA which is usually expressed on myeloid cells[262-264]. However, functional studies have indicated that only FcγRIIB is biologically functional in CLL cells[264].

1.7.5.4. CD72 (Lyb-2)

CD72 is a 45kDa transmembrane protein mainly expressed on B cells. The extracellular part of this protein contains a C-type lectin-like domain. On its cytoplasmic tail, CD72 carries an ITIM and an ITIM-like domain. This co-receptor is involved in modulating BCR signals[265-267], and engagement of the BCR results in phosphorylation of the ITIM and ITIM-like sequences of CD72. *In vitro* studies showed that this phosphorylation is mediated by Lyn[265]. Phosphorylation of CD72 results in the recruitment of SHP1 and consequent attenuation of BCR signalling[265-268]. Hyper-responsiveness and increased intracellular Ca^{2+} is observed in BCR-ligated B cells from CD72 knockout mice[269], supporting the negative role of this

receptor in BCR-signalling. However, crosslinking of CD72 with antibody provides pro-survival signals and promotes proliferation of BCR-stimulated B cells[270-271], but this effect is thought to be due to sequestration of CD72 from the BCR signalosome so that SHP1 is no longer recruited[272]. CD72 has been reported to be expressed in different B cell malignancies including CLL[273]. However, its role in regulating BCR signalling in CLL cells is not fully characterised. One study reported that CD38-mediated signals upregulate expression of CD100 in a subset of CLL cells resulting in simultaneous down-modulation of CD72 eventually leading to increased survival and proliferation of these cells[274].

1.8. Aims of the thesis

Previous work to this thesis showed that CLL cells overexpress PKCβII[197] and c-Abl[200]. Both of these kinases have roles in activating the NFκB pathway, an important pathway providing pro-survival signalling to CLL cells. In particular, PKCβII has a role in mediating NFκB pathway activation in response to BCR engagement in normal B cells. Considering the importance of the BCR in providing pro-survival signals to CLL cells, the initial aim of this thesis was to characterise any potential role of PKCβII and c-Abl in BCR-mediated activation of the NFκB pathway. We found that neither of these protein kinases was responsible for BCR-induced NFκB pathway activation. However, our results suggested the involvement of another SFK, Lck. Thus, the remaining aims of this thesis were to fully characterise the role of Lck in BCR-mediated signalling in CLL cells, and determine its pathophysiological importance.

Chapter 2: Materials and methods

2.1. Materials

2.1.1. Reagents, kits and cell lines

Nuclear extract kit and TransAM^{TM} NFκB P65 activation assay (ELISA) were purchased from Active Motif (Rixensart, Belgium). ECL Advance Western blotting kits were from GE Healthcare Lifesciences (Buckinghamshire, UK). Protein G sepharose was from GE Healthcare (Buchinghamshire, UK). The CLL cell line MEC1 was from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The mouse B cell lymphoma line A20 was from American Type Culture Collection (LGC Standards, Middlesex, UK), N-Ethylmaleimide (NEM) was from Sigma-Aldrich (Gillingham, UK).

2.1.2. Antibodies

Mouse anti-pAkt (phospho-serine 473) (cat.# 4051) , rabbit anti-Akt (cat.#9272), rabbit anti-pCD79a (tyrosine 182) (cat.#5173), rabbit anti-CD79a (cat.#3351), mouse anti-pIκBα (serine 32/36), rabbit anti-pSrc(tyrosine 416) (cat#2101), rabbit anti-Src (cat.#2123), rabbit anti-pGSK3α/β (serine 21/9) (cat.#9331), rabbit anti-pCrKL (tyrosine 207) (cat.#3181), rabbit anti-Lck (cat.#2787), mouse anti-ubiquitin (P4D1) and rabbit anti-pIKKα (serine 180)/ pIKKβ (serine181) (cat#2681) antibodies were purchased from Cell Signaling Technology (New England Biolabs, Hertfordshire, UK). Mouse anti-IKKα (B-8), mouse anti-IKKβ (H–4), mouse anti-Bcl-10 (331.3), mouse anti-MALT-1 (B-12), rabbit anti-CrKL, mouse anti-Lck (3A5), mouse anti-Lyn (H-6), mouse anti-Fyn (FYN3), mouse anti-Fgr (B-8), mouse anti-Yes (C-10), mouse anti-Hck (G-4), mouse anti-Src (H-12), mouse anti-pERK (E-

4), rabbit anti-ERK (K-23), rabbit anti-IκBα, mouse anti-CD5 (UCH-T2), rabbit anti-CD5(H-300), goat anti-CD5 (C-18), rabbit anti-CD22 (H-221), mouse anti-CD22 (MYG13), mouse anti-IKKγ (46B844), mouse anti-IKKγ(B-3), and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Santa Cruz Biotechnology (Insight Biotechnology, Wembley, UK). Rabbit polyclonal anti-CARMA-1(AL220) antibodies were from Alexis Biochemicals (Exeter, UK). Mouse monoclonal anti-β Actin antibody was from Sigma-Aldrich (Dorset, UK). Mouse anti-GSK3β was from BD Bioscience (Oxford, UK). Anti-phosphotyrosine (4G10) HRP conjugate, and anti-pCD22 (tyrosine 822) (cat. #04-458) was from Millipore (Dundee, UK). Anti-phosphotyrosine antibody (PY20) purchased from ICN Biomedicals (Ohio, U.S.A).

2.1.3. Stimuli and inhibitors

$F(ab')_2$ fragments of goat anti-human IgM antibodies were purchased from Jackson Immuno-Research Laboratories (Stratech Scientific Limited, Newmarket, UK). The specific PKCβ inhibitor LY379196 was kindly provided by Eli Lilly research laboratories (Indianapolis IN, USA). The c-Abl inhibitor Imatinib was purchased from LC Laboratories (Woburn, USA). The TAK1 inhibitor (9-Epimer-11, 12-dihydro-(5Z)-7-Oxozeaenol, known as antibiotic L-783,277) was purchased from AnalytiCon (Potsdam, Germany), and Bisdindolylmaleimide I, LFM-A13, mpV(pic) and the Lck inhibitor (4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane (Lck-i))were from Calbiochem (Nottingham, UK). All inhibitors were dissolved in DMSO, aliquoted and stored at -20°C.

2.2. Methods

2.2.1. Cell isolation and culture

Peripheral blood samples were obtained from patients previously diagnosed with CLL after giving informed consent and with the approval of the Liverpool Research Ethics committee (LREC). Heparinised peripheral blood was overlayed carefully on to 15 ml of Lymphoprep^{TM} (Alis-Shield PoC AS, Oslo, Norway) and then centrifuged at 800xg for 30 min at room temperature. The mononuclear cells were then carefully aspirated from the interface layer and washed in PBS and slowly resuspended in a 4ºC solution of RPMI 1640 (Sigma-Aldrich, Gillingham, UK) /10% foetal calf serum (FCS; Biosera, Ringmer, UK) /10% DMSO (Sigma-Aldrich). Aliquots of 1ml were then transferred to cryotubes (Nuncbrand, Fisher Scientific, Loughborough, UK) and frozen at -80ºC for one week and then finally cryopreserved in liquid nitrogen. Normal cells were obtained from buffy coat preparations (British Blood Transfusion Service, Liverpool, UK) and prepared as above.

2.2.2. Thawing of cryopreserved cells

Cryopreserved CLL and buffy coat samples were taken from the University of Liverpool Leukaemia Bank. Cell suspensions were thawed in a 37ºC water bath, and then transferred into pre-chilled 25ml universals. Ice cold RPMI 1640 (supplemented with 2mM L-Glutamine, 100 units/ml penicillin/streptomycin and 0.5%BSA) was added to the cell suspensions dropwise until a final volume of 10ml was reached. The suspensions were centrifuged at 500xg for 5 minutes at 4ºC, the supernatant was discarded and the cells were washed with 10 ml fresh media, and finally resuspended in the culture media. The viable/dead cells were counted using trypan blue solution and a Nexcelom Bioscience cellometer Auto T4 (Peqlab Ltd, Hampshire, UK). In general, CLL cases with viable cells exceeding 75% of the total cell count were used in further experiments.

2.2.3. Purification of normal B cells

Using the B Cell Isolation kit II (MACS, Miltenyi Biotec, Surry, UK) human B cells were isolated by depletion of non- B cells (negative selection). The normal cells (obtained in 2.2.2) were washed with ice cold purification buffer (PBS with 0.1% BSA and 2mM EDTA), and at the final step the cell pellet was resuspended in 40µl purification buffer per 10^7 cells. An antibody cocktail consisting of biotin conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a was added to the suspension using 10 µl antibody solution per 10^7cells. This mixture was incubated for 10 min at 4ºC with agitation from time to time. This was followed by the addition of purification buffer to a final volume of 5 ml. The suspension was centrifuged at 500xg for 5 min at 4ºC, and the cell pellet was washed again with 5 ml purification buffer. Next, the cell pellet was resuspended in 30µl/10^7 cells of the purification buffer, and Anti-Biotin MicroBeads were added at a concentration of 20µl bead suspension/10^7 cells. This mixture was incubated for 15 min at 4ºC, and the cells were then washed twice with 5ml of the purification buffer. The final cell pellet was resuspended in 400µl purification buffer /10^7 cells and then passed through an appropriately sized MACS separation column that had been prewashed with purification buffer and located in a magnetic field. The column was washed three times with purification buffer. Purified B cells were collected in the eluant, while non-B cells remained bound to the column of Anti-Biotin MicroBeads. The purity of the purified B cells was checked using flow cytometry and CD19 staining. In general, B cells treated in this way attained a purity that was greater than 90%.

2.2.4. Purification of CLL cells

CLL cells were also negatively purified. After thawing and counting CLL cells as described in 2.2.2, the cell pellet was resuspended in 400µl of the purification buffer and transferred into a microcentrifuge tube and centrifuged again. The cells were then resuspended in 40 µl of purification buffer per 10^7 cells. 20µl per 10^7 cells each of FITC-conjugated anti-CD3 , -

CD14, and -CD16 antibodies (which bind to T cells, NK cells, monocytes and macrophages) (BD Biosciences, Oxford, UK) were added to the suspensions and incubated for 20 min at 4ºC with mixing every 5 minutes. The suspensions were then centrifuged and washed in 200µl of purification buffer. The pellet was then resuspended in 80 µl of purification buffer per 10^7 cells. 20µl per 10^7cells of Anti-FITC MicroBeads (MACS, Miltenyi Biotec, Surry, UK) was added to the suspensions and the mixture was incubated for 20 min at 4ºC with agitation every 5 min. After that the suspension was centrifuged and the pellet was washed in 200µl of purification buffer. The cell pellet was resuspended in 40µl of the purification buffer and the suspension was then passed through a prepared MACS separation column prewashed with the purification buffer and located in a magnetic field. After leaving the cells in the column for 30 sec, the column was washed three times with the purification buffer. Purified CLL cells were collected in the eluant and contaminating cells remained with the column. CLL cell purity was checked using flow cytometry and CD5 / CD19 staining (as detailed below). In general, CLL cells treated in this way attained a purity that was greater than 90%.

2.2.5. Flow cytometry

Flow cytometry was used to determine the purity of the normal B and CLL cells from the above procedure (section 2.2.4). This was done using a Becton Dickinson FACSCalibur machine and FITC-labelled anti- CD19 for normal B cells, or anti-CD5/CD19 antibodies for CLL cells. A FITC-labelled non-specific IgG was used as a control. All the antibodies used in this section came from BD Biosciences (BD, Oxford, UK). The staining procedure involved incubation of $1x10^6$ cells with 10µg/ml final concentration of antibody for 30 minutes on ice, in the dark. The cells were then centrifuged at 500xg for 5 minutes at 4 ºC and washed twice using 500µl modified PBS (supplemented with 1%BSA and 0.1% sodium azide, pH7.2). Cells were finally resuspended in 100µl PBS to which 100µl FACS flow (BD biosciences) was added. This sample was then analysed by flow cytometric analysis.

For CD22 expression measurement, the cells were stained with FITC-conjugated anti-CD5, APC-conjugated anti-CD20 and PE-conjugated anti-CD22 (BD, Oxford, UK) prior to flow cytometric analysis.

For quantification of Lck and ZAP70 expression: $5x10^6$ CLL cells were first incubated with FITC-conjugated anti-CD5 and APC-conjugated anti-CD20 mAbs. The cells were then washed times with 300µl modified PBS, fixed with the addition of 100µl 4% paraformaldehyde for 15min in the dark. These cells were washed 3 times with 300µl modified PBS, and they were then permeabilised with 1 ml of ethanol that had been cooled to -20°C. This suspension was further incubated at -20°C for 30 mins, and the cells were then washed 3 times with 300µl modified PBS. The final cell pellet was resuspended in 200µl modified PBS and incubated with a 1/20 dilution of either PE-conjugated anti-Lck (Santa Cruz, clone SPM413) or PE-conjugated anti-ZAP70 (Invitrogen, clone 1E7.2) for 30 mins at 4ºC, in the dark. The stained cells were then washed with modified PBS and analysed by flow cytometry. CLL cells were gated as $CD5^+/CD20^+$ cells.

2.2.6. Culturing CLL cells

When required, suspensions of 1×10^7 cells per ml were cultured under 5%CO_2 at 37ºC for the required period of time. The culture medium used was RPMI 1640 supplemented with L-glutamine (2mM), 100 units/ml penicillin/streptomycin, and BSA (0.5%). Cell suspensions were cultured in 24-well tissue culture plates that had been coated with polyHEMA to avoid cell adhesion.

2.2.7. Preparation of cell lysates

Cryopreserved CLL cells were thawed and 1×10^7 cells were washed in PBS. The cell pellet following the final wash was lysed with 200µl of lysis buffer (125mM Tris pH6.8, 5mM EDTA, 1% SDS). The lysates were sonicated to disrupt released DNA, incubated at 95ºC for

5-10 minutes and then centrifuged at 12,000xg for 5 minutes. The supernatant was transferred into a clean microtube and protein concentration was determined as outlined below in section 2.2.8. The lysates were stored at -20ºC until needed for further use.

2.2.8. Protein determination

The protein concentration in lysates (as prepared in 2.2.7) was determined using a Bio-Rad DC protein assay kit (Hertfordshire, UK). The procedure followed was according to the instructions provided by the manufacturer.

2.2.9. SDS-PAGE and Western blotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was used to separate proteins in cell lysates. In general, 10µg of total protein was loaded per lane of gel. The separated proteins were then electroblotted to Immobilon^{TM} Transfer Membranes (Millipore, Fisher Scientific UK Ltd, Loughborough, UK). Membranes were blocked with blocking buffer (2.5% ECL advance blocking reagent in TBS-T (150mM NaCl, 25 mM Tris pH 7.5, 0.1% Tween 20)) for an hour to avoid non-specific binding between the membranes and antibodies. The membranes were then probed with the primary antibodies diluted with blocking buffer overnight at 4 ºC with gentle agitation. After that the membranes were washed with TBS-T 4 times, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1/5000) in blocking buffer for 1 hour at room temperature. Following a final wash cycle of TBS-T x 4 the membranes were developed with ECL-advance Western Blotting Detection reagents. Chemiluminescence was read using a Fujifilm LAS-1000.

Equal protein loading was validated by probing the blots with anti β-Actin antibodies used at a 1/10000 dilution. In the cases where protein phosphorylation was measured, protein loading was assessed using antibodies against the total protein.

2.2.10. Nuclear NFκB quantification

2.2.10.1 .Thawing and culturing the cells

Cryopreserved CLL samples were thawed and cultured as in 2.2.2 and 2.2.6.

2.2.10.2. Preparation of nuclear extracts

Each sample (1×10^7cells) was washed twice with 1 ml of ice cold PBS/Phosphatase inhibitors provided in the nuclear extract kit (Active Motif, Rixensart, Belgium). The pellets were then resuspended gently in 500 µl of 1× hypotonic buffer and incubated on ice for 15 minutes. 25 µl of the detergent solution was added to the suspension and the mixture was vortexed for 10 seconds at the highest setting. The cell suspensions were centrifuged for 30 sec at 14,000xg at 4ºC in a pre-cooled microcentrifuge. Supernatant (cytoplasmic fraction) was transferred into a fresh microcentrifuge tube and stored at -80 ºC until required for further use. The pellets (containing the cell nucleus) were washed with PBS containing phosphatase inhibitors, and resuspended in 50 µl complete lysis buffer. This mixture was then vortexed for 10 sec at the highest setting, incubated for 30 min at 4 ºC on a rocking platform and subjected to a second vortexing step (30 sec at highest setting). To obtain nuclear extract the suspension was centrifuged at 14,000xg for 10 min at 4 ºC, and the supernatant taken and stored at -80ºC before it was used in the next step.

2.2.10.3. Protein determination

A BioRad RC DC protein assay kit was used for protein quantification.

2.2.10.4. Quantification of NFκB in the nuclear extract by ELISA

NFκB activation in CLL cell nuclear extracts was measured using a TransAM NFκB ELISA kit (Active Motif, Rixensart, Belgium). The procedure followed was according to the manufacturer's instructions.

2.2.11. SFK Immunoprecipitation

CLL cell lysates were prepared by incubating $1x10^7$ CLL cells with 300μL modified radioimmunoprecipitation assay (RIPA) buffer (50mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150mM NaCl, 50mM sodium fluoride, 25mM sodium pyrophosphate, 50mM sodium glycerophosphate, 2mM EDTA, and 2mM EGTA). After sonication for 10 seconds, cell lysates were centrifuged at 14000xg for 20 minutes. Then 250μL of the supernatant was incubated overnight at 4°C with 2 μg of anti-SFK antibody (either Lyn, Lck, Fyn, or Fgr). The immunocomplexes were precipitated onto 25μL Protein G Sepharose (GE Healthcare, Buchinghamshire, UK) for 1h, and these complexes were washed 3 times with RIPA buffer. To release the protein of interest from its immunocomplex. Laemelli buffer (2x) was added to the immunocomplexes, and then heated them for 10 minutes at 95°C. Protein and protein G sepharose were then separated by centrifugation at 14000xg for 1 minute. The supernatant was applied to a SDS-PAGE gel, and following transfer of the separated proteins to immobilon, Western blotted using an appropriate antibody.

2.2.12. CD5 immunoprecipitation

For CD5 general tyrosine phosphorylation, CD5 was immunoprecipitated from lysates of $3x10^7$ CLL cells. Lysis was achieved using RIPA buffer (as detailed in methods section 2.2.11) and CD5 immunoprecipitated using 10μL of the CD5 antibody (clone UCH-T2). Tyrosine phosphorylation of CD5 was determined by probing Western blots of immunoprecipitated CD5 with either anti-phosphotyrosine 4G10 or PY20 antibodies.

2.2.13. CD22 immunoprecipitation

For CD22 immunoprecipitation CLL cases that we determined to have high expression of this protein were used. Immunoprecipitation was done as described above in sections 2.2.11 and

2.2.12. However, for this experiment $1x10^7$ CLL cells were used, and CD22 immunoprecipitated from RIPA-lysed cells with 10µL rabbit polyclonal anti-CD22. General tyrosine phosphorylation of CD22 was measured by probing the Western blots with anti-phosphotyrosine 4G10 HRP conjugated antibody.

2.2.14. Studies of IKKγ Ubiquitination

For measurement of IKKγ ubiquitination, $2x10^7$ CLL cells (or $5x10^6$ A20 cells) were lysed in 300µL modified RIPA buffer supplemented with 10mM N-Ethylmaleimide and briefly sonicated for 10 seconds. Following centrifugation at 14000xg and 4°C for 20 minutes, 250µL of the supernatant was incubated overnight at 4°C with 25µL of agarose conjugated anti- IKKγ (FL-419). The immunocomplexes were then washed three times. The protein was released from the immunocomplexes, separated by SDS-PAGE and ubiquitination was detected by Western blotting using the anti-ubiquitin antibody (P4D1).

2.2.15. Cell surface protein isolation and analysis of sIgM

Cell surface proteins were isolated using Pierce Cell Surface Protein Isolation Kit (Pierce, Loughborough, UK). $5x10^7$ CLL cells were incubated in 0.5mg/mL Sulfo-NHS-SS-Biotin solution in PBS at 4° C in a T25 cm^2 flask on a rocking platform. After 30 minutes the reaction was ended by adding 500µL Quenching solution (from the kit) to each flask. The suspension was then transferred into 50mL conical tubes, and centrifuged at 500xg for 3 minutes. The cells were additionally washed once with TBS. The cells were then lysed by sonication in Lysis Buffer with protease inhibitors (from the kit). The cell lysates were incubated on ice for 30 minutes, vortexed for 5 seconds every 5 minutes, then centrifuged at 12000xg for 2 minutes. Separation columns were prepared by adding 500µL NeutrAvidin™ Agarose into the column and washing it twice with 500µL of Wash Buffer. Clarified cell lysate was then added to the gel and incubated at room temperature for 60 minutes with end-

over-end mixing using a rotator. The NeutrAvidin™ gel was then washed 4 times in Wash Buffer. Biotinylated proteins were eluted by boiling in 200µL SDS-PAGE Sample buffer supplemented with dithiothreitol (DTT) for 5 minutes at 95°C. A trace of bromophenol blue was added to the eluate and proteins were resolved by SDS-PAGE. Surface IgM was detected by Western blotting using goat anti-human IgM.

2.2.16. Transfection of CD5 into the MEC1 cell line

Full length CD5 and a truncated version of CD5 that lacked the cytoplasmic tail were subcloned into pEGFP-N1 (Clonetech, Saint-Germain-en-Laye, France) (Figure 2.2) to create CD5-EGFP and truncated CD5-EGFP. These constructs were kindly provided by Dr. Mark Glenn of this Department. To transfect the plasmids into MEC1 cells, after washing 2X with PBS $2x10^6$ cells were resuspended in 100µl nucleofector solution V (Amaxa® Cell Line Nucleofector® Kit V, Lonza Biologics plc, Tewkesbury UK). 2 µg of pCD5-EGFP or ptruncatedCD5-EGFP was added to the cell suspension. The mixture was then transferred into the chamber and programme X001 was used to initiate transfection (transfection by electroporation). 500µl DMEM was then added to the mixture and this was transferred into 1.5 ml prewarmed complete DMEM (37°C). The average post-transfection viability was 80%. The cell suspension was then incubated overnight at 37 °C using standard culture conditions. Analysis of GFP and CD5 expression was performed by flow cytometry. GFP expression was detected using the detector for FITC on the flow cytometer, whereas surface expression of CD5 was detected using a PE-conjugated CD5 antibody.

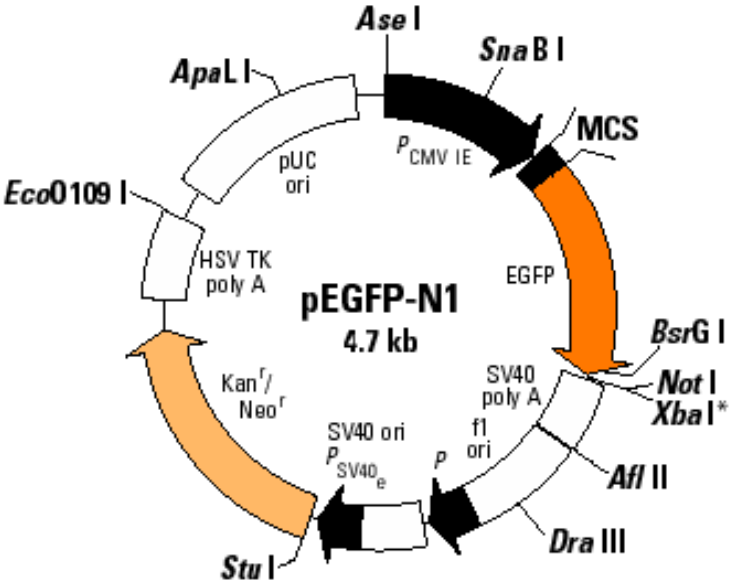


Figure 2.1: General structure of plasmid pEGFP-N1

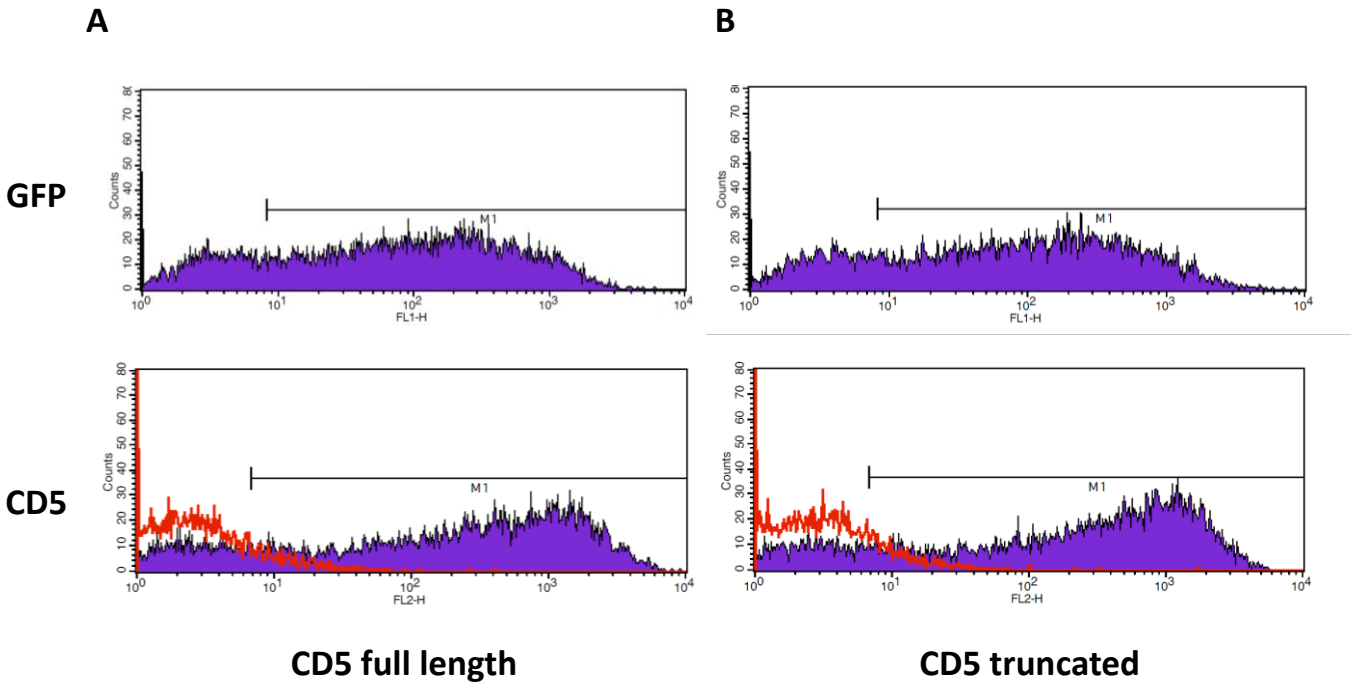


Figure 2.2: Expression of GFP and CD5 in transfected MEC1 cells. MEC1 cells were transiently transfected using **A)** CD5-EGFP and **B)** truncated CD5-EGFP. After overnight incubation at 37ºC GFP expression was detected using the detector for FITC on the flow cytometer and surface expression of CD5 was detected using a PE-conjugated CD5 antibody. Transfection efficiency is 82% and 83%, respectively.

2.2.17. Nucleofection

Nucleofection of CLL cells was carried out according to established protocol[207]. Briefly, $1x10^7$ CLL cells were transfected using the Amaxa solution V transfection kit according to the manufacturer's instructions (Lonza Biologics plc, Tewkesbury UK). CLL cell suspensions were briefly incubated with no siRNA, or with 2µM of either non-specific control siRNA or siRNA targeting Lck prior to using and program U13 to initiate transfection. Following transfection CLL cells were cultured in polyHEMA-coated plates for 48h prior to use. Transfected CLL cells were then harvested and divided into unstimulated and BCR stimulated cells. BCR stimulation was achieved by incubating CLL cells with 20µg/ml $F(ab')_2$ anti-IgM for 15min.

2.2.18. CLL cell viability assay

5 $x10^6$ CLL cells/ml were cultured for 3 days with RPMI 1640 supplemented with 0.5% BSA, L-glutamine and streptomycin in polyHEMA-coated plates. Cell viability was assessed by flow cytometry using DiOC6 to measure mitochondrial integrity and propidium iodide (PI) incorporation (as a measure of dead cells) according to established protocol[197]. Cell viability was determined by taking an aliquot of cell suspension and measuring the number of DiOC6bright/PIdim events (live cells) counted during a fixed time setting of 30 seconds where the flow cytometer (FACSCalibur™) was set to medium flow.

2.2.19. Statistical analysis

Data were analysed for statistical significance using Student's t-test or Mann-Whitney U-test as indicated in Figure legends. The analysis was performed by computer using either Microsoft Excel™ or SPSS™ 17.0 software, respectively.

Chapter 3: BCR- induced NFκB activation in CLL cells

3.1. Introduction

NFκB transcription factors play an important role in B cell development, proliferation, immune responses, and survival[275-276]. BCR stimulation triggers NFκB activation through the canonical pathway[137 275]. In this pathway BCR crosslinking leads to phosphorylation and activation of the IKK complex. TAK1is thought to play a role in this pathway by phosphorylating IKKβ. Once activated, the IKK complex phosphorylates IκBα leading to its ubiquitination and subsequent proteosomal degradation. This releases NFκB and allows it to translocate into the cell nucleus[134]. Figure (3.1) summarises this signalling pathway in normal B cells.

The critical role of the NF-κB pathway in CLL pathogenesis has been confirmed by transgenic mouse models[277-278]. NFκB seems to be aberrantly activated in CLL cells compared to normal B cells[158]. However, the mechanism of activation in CLL is still unclear.

Targeting BCR-induced activation of NFκB pathway could be a potential strategy for CLL treatment. Thus, the aim of this chapter is to investigate the mechanism of BCR-induced NFκB signalling in CLL cells and characterise the elements involved in this pathway upstream of NFκB

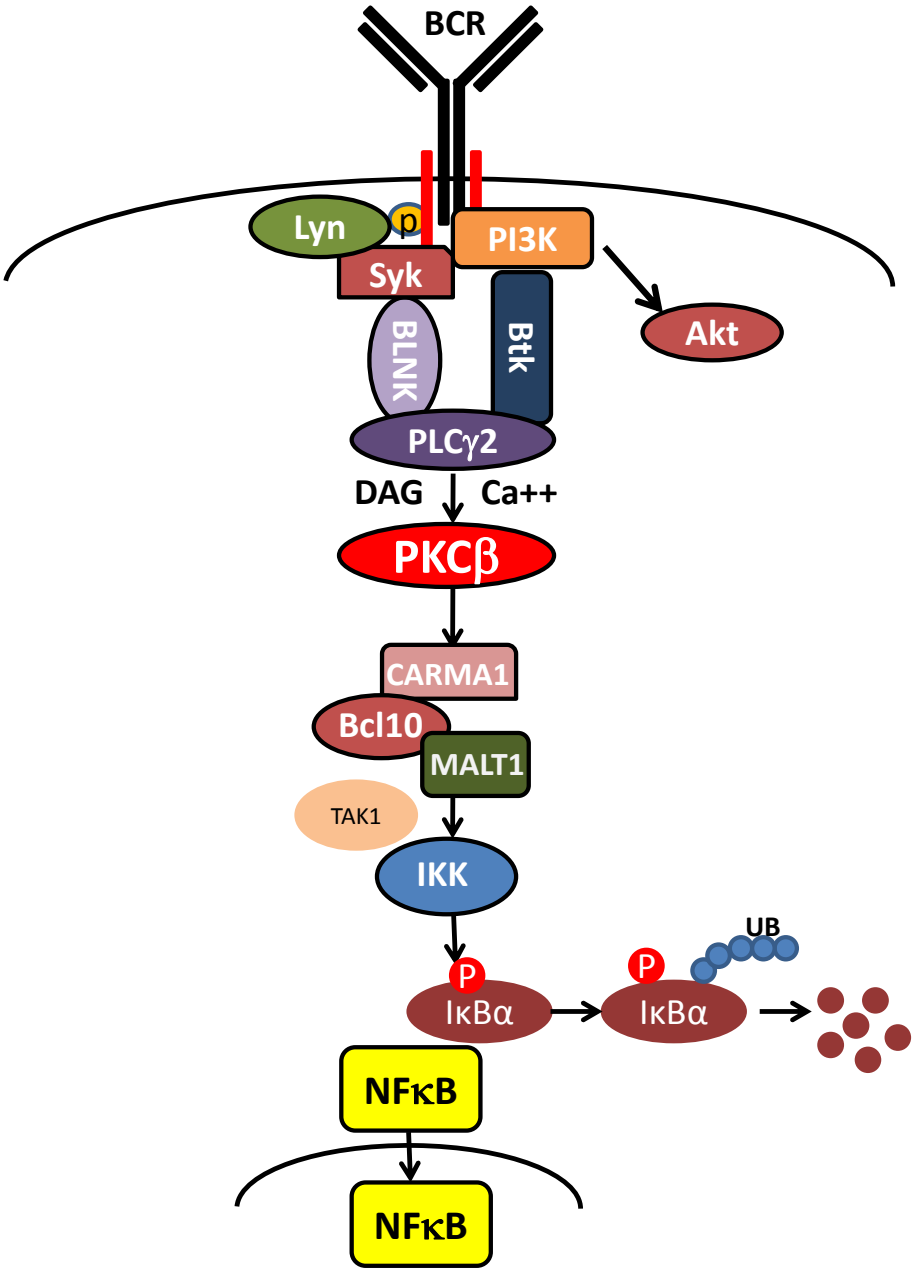


Figure 3.1: BCR-induced NFκB pathway in normal B cells. Illustration of the canonical pathway of NFκB activation triggered by stimulation of BCR.

3.2. Results

3.2.1. The role of Protein kinase C beta (PKCβ) in BCR-induced IKK phosphorylation in CLL cells

PKCβ is a key mediator of BCR signalling to the NFκB pathway where it plays an important role in stimulating IKK activation[36 279-281]. This is underscored by observations of constitutive NFκB activation in some B cell malignancies such as diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma (MCL). Here, overexpressed PKCβ in the malignant cells mediates tonic BCR signals that contribute to their survival and proliferation[282-284]. Thus, apoptosis can be induced in DLBCL and MCL cells by inhibition of IKK activation using PKCβ-specific inhibitors such as LY379196, LY333531 (ruboxistaurin) and LY317615 (enzastaurin)[281 285].

Work in this Department has shown that CLL cells overexpress PKCβII[197], and we reasoned that this overexpression could facilitate NFκB pathway activation in these cells, particularly when they experience BCR engagement. Figure 3.2 A shows that IKK can be activated when CLL cells are incubated with $F(ab')_2$ fragments of anti-IgM antibody (to crosslink the BCR). To investigate the role of PKCβ within this process, CLL cells were pre-treated with either a PKCβ-specific inhibitor (LY379196), or a more general PKC inhibitor (Bisindolylmaleimide I). However, neither of these compounds affected BCR-induced IKK activation in the CLL cells tested. This is despite the fact that these compounds were used at concentrations exceeding the amounts needed for their PKC specific effects, and is exemplified by the ability of these compounds to inhibit BCR-induced phosphorylation of GSK3α and β (Figure 3.2B). These results suggest that PKCβ is not involved in IKK activation resulting from BCR crosslinking on CLL cells.

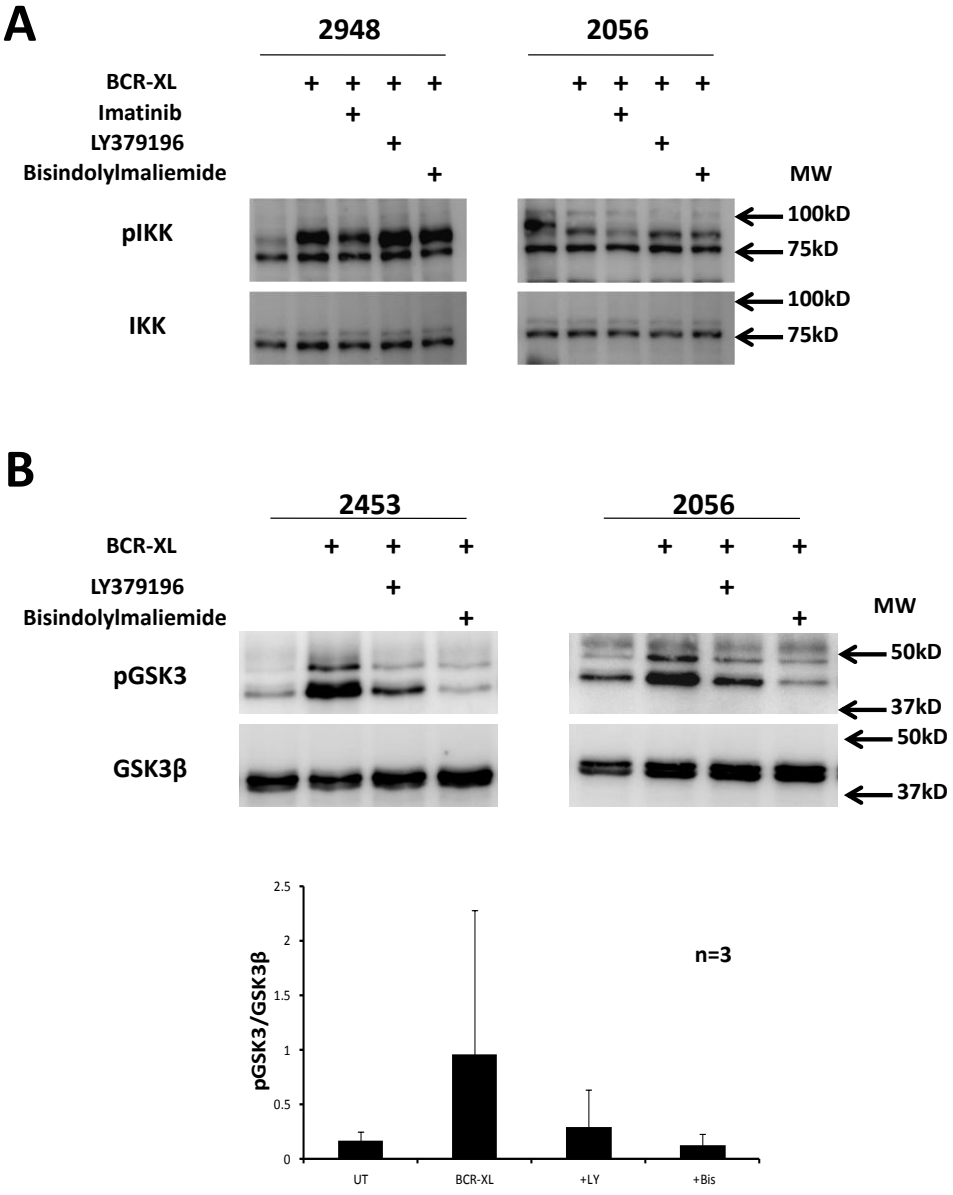


Figure 3.2: Effect of PKCβ inhibitors on (A) IKK phosphorylation and (B) GSK3 phosphorylation in CLL cells. CLL cells were treated with 2μM LY379196 or with 5 μM Bisindolylmaleimide for 2 hours at 37ºC, and then BCR was stimulated with 20 μg/mL $F(ab')_2$ goat anti-human IgM for 15 minutes. The cellular lysates were analyzed by Western blot analysis for pIKKα/β (ser 180/181) or pGSKα/β (serine 21/9) antibody, and then for total IKKα/β or GSKβ in parts **A** and **B**, respectively MW: molecular weight ladder.

3.2.2. Expression of the CBM (CARMA-1, MALT-1 and Bcl-10) complex in CLL cells

To further characterize BCR-induced IKK activation in CLL cells, we investigated the proteins forming the signalosome that transmits the signal from PKCβ to IKK. In B cells, antigen receptor stimulation of the NFκB pathway is achieved when PKCβ phosphorylates CARMA-1 of the CARMA-1 – Bcl-10 – MALT-1 (CBM) complex. When CARMA-1 is phosphorylated by PKCβ, it is then able to recruit MALT-1 and Bcl-10 which ubiquitinate IKKγ and recruit TAK1 resulting in phosphorylation and activation of IKKβ[286–287]. I analyzed the expression levels of the CBM complex in CLL cells and compared these levels with those in peripheral B cells. We found that MALT-1 levels are approximately equal between CLL and normal B cells (Figure 3.3B). However, CARMA-1 is expressed in 2-fold excess in CLL over normal B cells (Figure 3.3A). Importantly, Bcl-10 is expressed in CLL cells at an eighth of the level it is expressed in normal B cells (Figure 3.3C). It has been reported that CARMA-1, MALT-1 and Bcl-10 must exist in a 1:1:1 stoichiometry in order for efficient antigen receptor signalling to the NFκB pathway to take place[288-290]. The results of the present section indicate that this is not the case in CLL cells and suggest that this pathway is likely independent of the CBM complex.

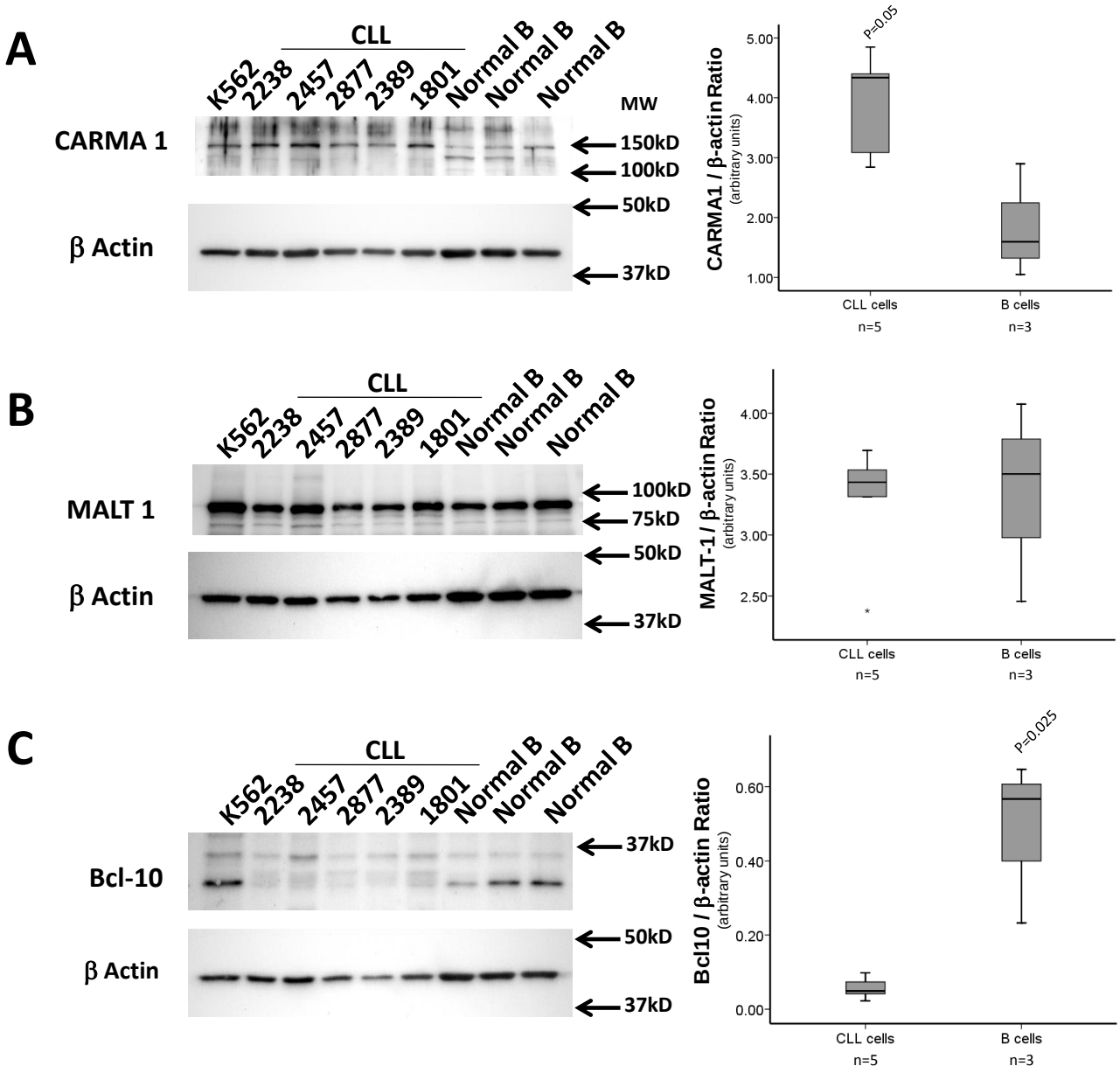


Figure 3.3: Analysis of CBM complex components in CLL compared to normal B cells. Western blot analysis of CLL cell lysates for **(A)** CARMA-1, **(B)** MALT-1 and **(C)** Bcl-10 expression in CLL compared to normal B cells. Equal protein amounts were loaded (10µg) in each lane and β-actin expression was used as a loading control. Tests for statistical significance were performed using a Mann-Whitney U-test using the indicated numbers of cases.

3.2.3. CD40 ligation on CLL cells induces Bcl-10 expression

CD40 ligation on CLL cells changes gene expression[291-292]. We wished to determine whether the profile of upregulated proteins in CD40-stimulated CLL cells included Bcl-10. Thus, we cultured CLL cells on CD40 ligand-expressing (CD40L-L) and control fibroblasts (NT-L), and analysed the levels of Bcl-10 expression. Figure 3.4 shows that culture of CLL cells on NT-L cells did not largely affect the level of Bcl-10 compared to non-cultured CLL cells. However, CD40 ligation on CLL cells, achieved by culture on the CD40L-L cells, induced expression of significantly ($p=0.037$, $n=3$) higher levels of Bcl-10 than were observed in CLL cells cultured on the NT-L cells. These results indicate that CD40 stimulation of CLL cells stimulates Bcl-10 expression, and suggests that NFκB pathway signalling in response to BCR crosslinking may be restored. This notion is supported by experiments measuring Bcl-10 levels in BCR-stimulated and unstimulated CLL cells. Bcl-10 levels in the former were slightly reduced compared to the latter (Figure 3.4). This is expected because Bcl-10 becomes phosphorylated and is degraded in BCR-stimulated B cells[293].

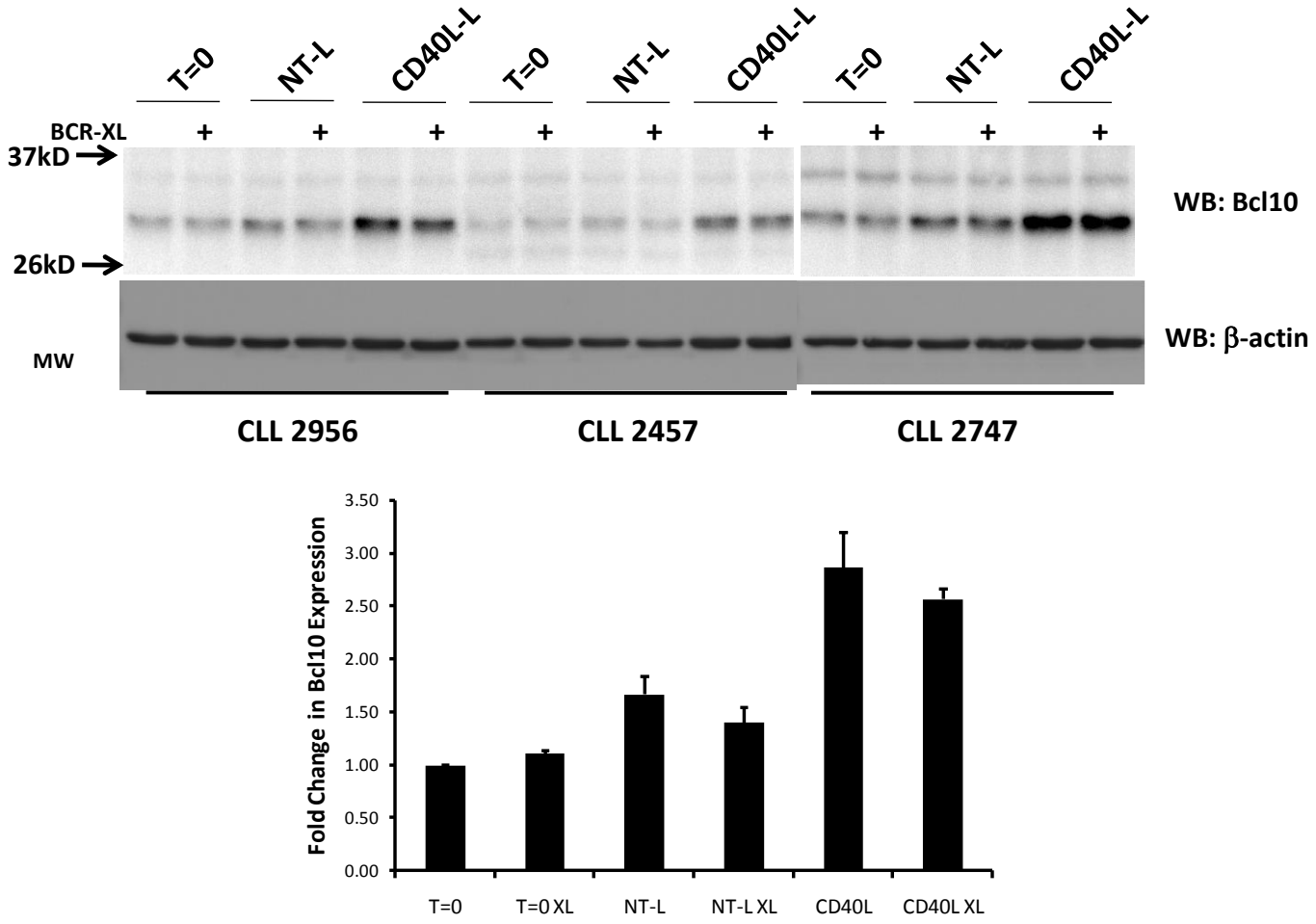


Figure 3.4: CD40 ligation on CLL cells stimulates Bcl-10 expression. CLL cells were cultured on non-transfected fibroblasts (NT-L) or CD40-expressing fibroblasts (CD40L-L) for 48 hours at 37° C, the cells were then harvested, washed and recovered overnight prior stimulation of BCR using 20 μg/mL $F(ab')_2$ goat anti-human IgM for 15 minutes. The cellular lysates were analysed for the levels of Bcl-10 expression. β-Actin was used as a loading control. Statistical analysis was performed using a Student t-test for paired data on n=3 samples.

3.2.4. CD40 ligation on CLL cells downregulates BCR-induced IKK activation

We next tested the effect of CD40 ligation on CLL cell response to BCR stimulation. Figure 3.5 shows that CLL cells cultured on NT-L cells had a slightly enhanced response with respect to IKK phosphorylation induced by BCR engagement when compared to non-cultured cells. However, BCR-induced activation of IKK was significantly ($p=0.05$, $n=3$) impaired in CLL cells cultured on CD40L-L cells. Taken together with the data presented in Figure 3.4, these results indicate that the upregulation of Bcl-10 in CD40-stimulated CLL cells does not lead to enhanced BCR signalling to the NFκB pathway.

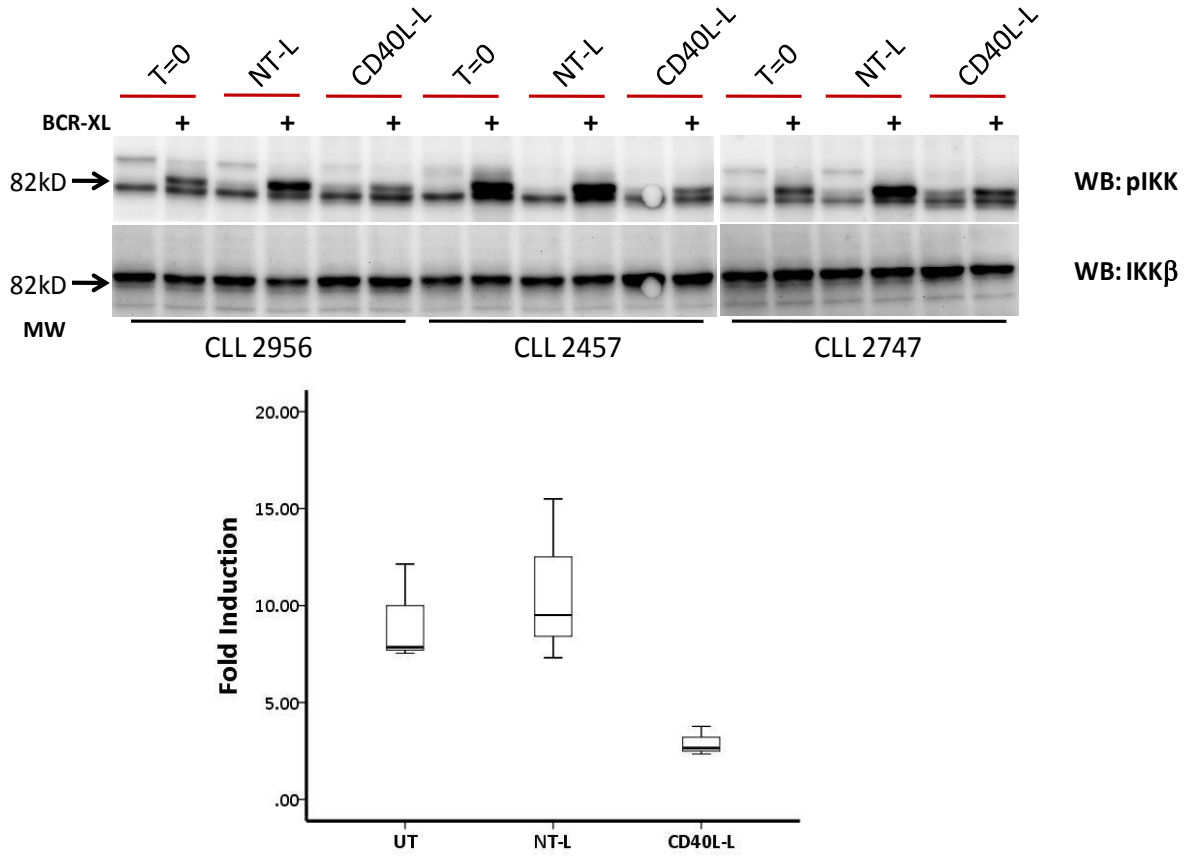


Figure 3.5: CD40 ligation on CLL cells leads to reduced ability to activate IKK by BCR stimulation. CLL cells were cultured on non-transfected fibroblasts (NT-L) or CD40-expressing fibroblasts (CD40L-L) for 48 hours at 37° C, the cells were then harvested and recovered overnight prior stimulation of BCR using 20 μg/mL $F(ab')_2$ goat anti-human IgM for 15 minutes. The cellular lysates were analysed for the levels of for pIKKα/β (ser 180/181) antibody, and then for total IKKβ. Statistical analysis was performed using a Mann-Whitney U-test on n=3 samples.

3.2.5. BCR- induced NFκB activation and translocation into the nucleus of CLL cells

The role of the CBM complex in antigen receptor-induced activation of the NFκB pathway is to coordinate activation of IKK with its downstream elements. Thus, antigen receptor stimulation of T and primary splenic B cells induces phosphorylation of IKKα and β, but downstream phosphorylation of IκBα and resultant activation of NFκB does not occur in the absence of either Bcl-10 or CARMA-1[133-134 287 294]. The role of these latter two proteins, in combination with MALT-1, is to facilitate the polyubiquitination of IKKγ which allows for the efficient transmission of antigen receptor signals to NFκB[294].

The observation that CLL cells express low levels of Bcl-10 suggests that BCR-induced phosphorylation of IKK may not result in efficient activation of the NFκB pathway. To test this possibility we performed an ELISA which measures the activated form of NFκB (RelA) in nuclear extracts of CLL cells. Figure 3.6 shows that BCR crosslinking on CLL cells induces a marked increase in IKK phosphorylation that corresponds with increased levels of active NFκB and IκBα phosphorylation and its subsequent degradation. We found that the levels of IKK / IκBα phosphorylation within the lysates of stimulated CLL cells appeared to correlate with the amount of active NFκB within the nuclear extracts. This suggests that there is efficient transmission of BCR signals from IKK to NFκB in CLL cells despite the low levels of Bcl-10 expression.

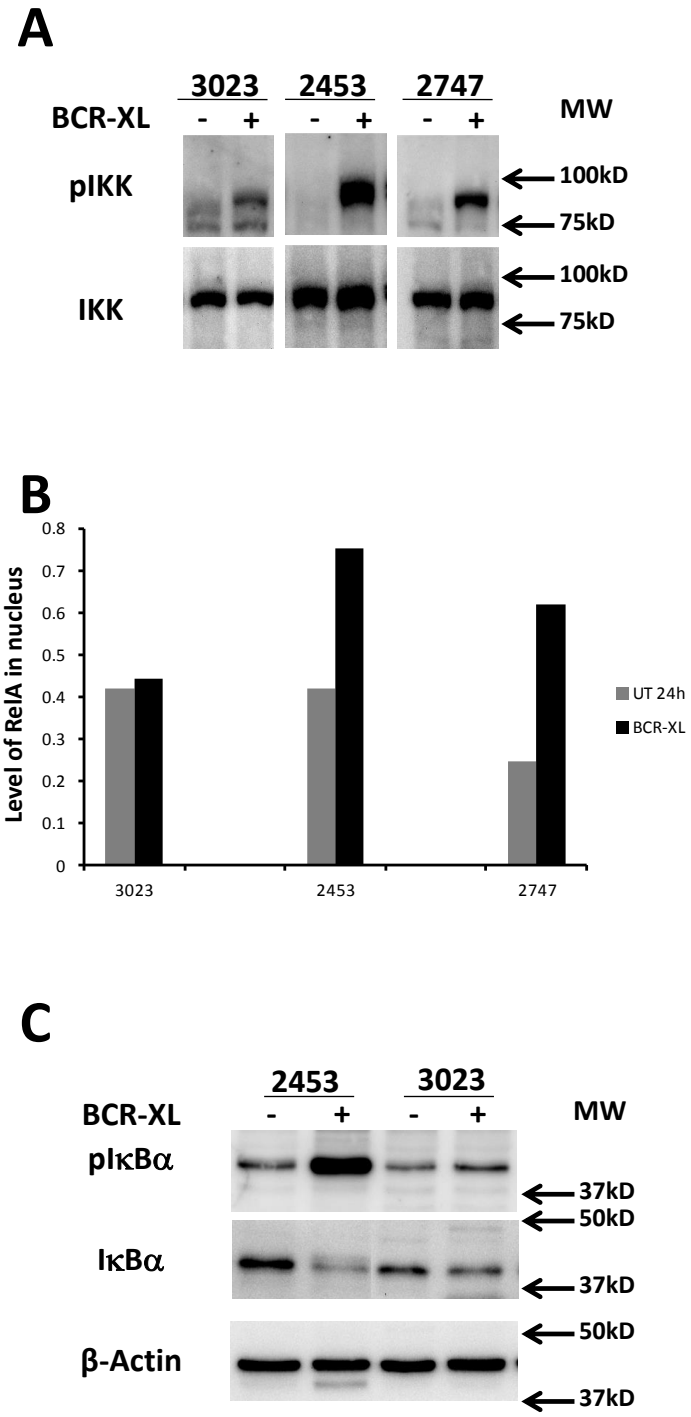


Figure 3.6: BCR-stimulated IKK and IκBα phosphorylation and activation of NFκB in CLL cells after 24h culture. CLL cells were cultured in polyHEMA plates for 24 hours at 37° C, and then stimulated by BCR crosslinking with 20 μg/ml $F(ab')_2$ goat anti-human IgM for 15 minutes. The cellular lysates were analysed for the levels of **(A)** pIKKα/β (ser 180/181) and **(C)** pIκBα (serine 32/36). In part **(B)** the nuclear proteins were extracted and an ELISA assay was performed to measure the levels of active NFκB (RelA) in the nucleus.

The experiment illustrated in Figure 3.6 required that the cells were incubated for a period of 24h. This was required because the levels of active NFκB in nuclear extracts from freshly-thawed CLL cells were constitutively high. We found that BCR stimulation of freshly-thawed CLL cells did not cause any observable increase in the levels of active NFκB despite the induction of IKK phosphorylation (Figure 3.7A). Moreover, IκBα phosphorylation levels were also high in freshly thawed CLL cells, and there was no observable increase in these levels upon BCR crosslinking (Figure 3.7B). To reduce the levels of background activation/phosphorylation of NFκB/IκBα the CLL cells required 24h incubation under quiescent conditions. Therefore, it is possible that the levels of Bcl-10 expression could have changed during this time. To answer this question, we compared the levels of Bcl-10 in freshly-thawed CLL cells with those in cultured cells, and observed that there was no appreciable difference between the two (Figure 3.7C). Collectively, these data indicate that BCR crosslinking on CLL cells induces efficient signalling from the receptor to NFκB despite low levels of Bcl-10. This suggests that this signalling pathway is independent of PKCβ and the CBM complex.

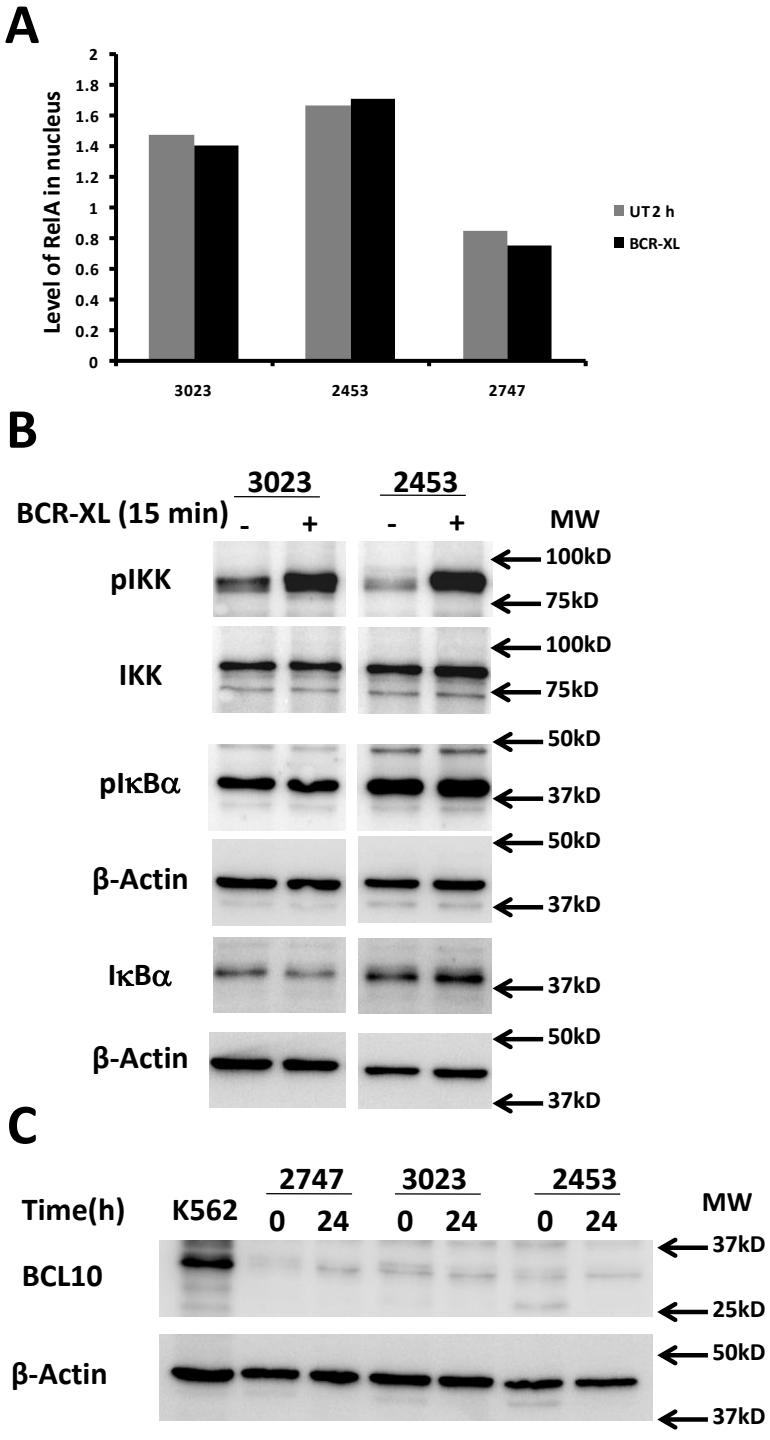


Figure 3.7: BCR-stimulated IKK and IκBα phosphorylation and activation of NFκB in freshly thawed CLL cells. CLL cells were recovered for 2 hours at 37° C, and then BCR was stimulated with 20μg/ml $F(ab')_2$ goat anti-human IgM for 15 minutes. **(A)** The nuclear proteins were extracted and an ELISA assay was performed to measure the levels active NFκB in the nucleus. **(B)** Whole cell lysates of freshly-thawed unstimulated and BCR-stimulated CLL cells were analysed by Western blot for pIKK/IKK, pIκBα and IκBα. **(C)** Bcl-10 in whole cell lysates analysed by Western blot of freshly-thawed (time 0) and 24h-incubated CLL cells (time 24).

3.2.6. The role of the transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) in BCR-induced NFκB activation in CLL cells

TAK1 is an important mediator of BCR-induced activation of NFκB, and is also essential for function and maturation of B cells. TAK1-deficiency in B cells results in an increase of BCR-induced apoptosis and impairment of proliferation following BCR crosslinking[135]. After activation by ubiquitination, the TAK1/TAB2 (or TAB3) complex activates IKK by phosphorylating IKKβ at serine (Ser)-177 and Ser-181 in the activation loop[295]. To examine the role of TAK1 during NFκB activation in BCR-stimulated CLL cells we used the compound 9-Epimer-11, 12-dihydro-(5Z)-7-Oxozeaenol (Oxozeaenol). This compound was developed as a TAK1 inhibitor, and shows little inhibitory activity towards other MAP3Ks[296-297]. We constructed a concentration-response curve of this compound versus levels of phosphorylated IKK following BCR ligation. Figures 3.8 and 3.9 show that the presence of 500nM Oxozeaenol completely blocks BCR-induced phosphorylation of IKK in CLL cells, suggesting that TAK1 is involved in this process. Taken together with previous results from this chapter, this experiment suggests that TAK1 may be involved in BCR signalling to NFκB, but through a different mechanism of activation that does not involve PKCβ or the CBM complex.

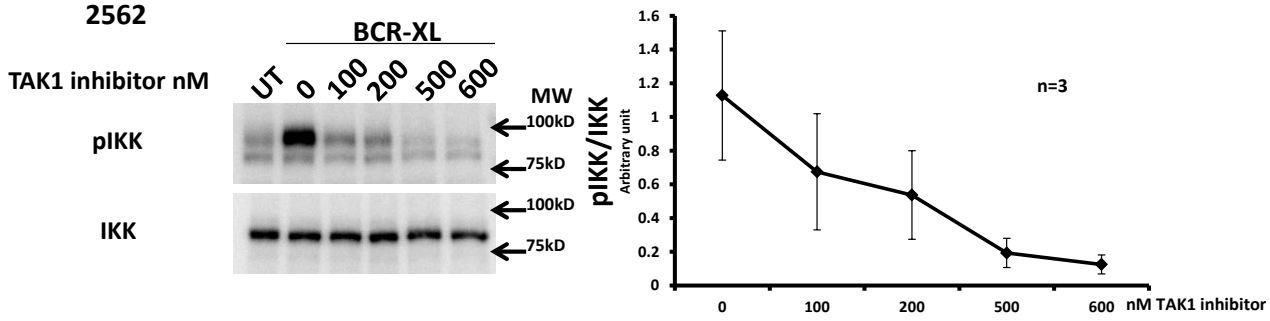


Figure 3.8: Concentration-response curve investigating the effect of the TAK1 inhibitor on BCR-induced IKK phosphorylation in CLL cells. CLL cells were incubated with the indicated concentration of TAK1 inhibitor for 2h prior to BCR crosslinking with 20μg/ml $F(ab')_2$ goat anti-human IgM. (*left hand panel*) Western blot analysis of CLL cell lysates for pIKK and total IKK. (*right hand panel*) Graphical representation of n=3 experiments using different CLL cases.

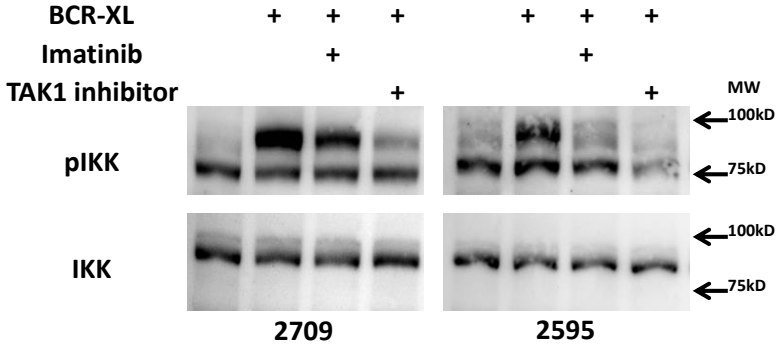


Figure 3.9: Effect of TAK1 inhibitor on IKK phosphorylation in CLL cells. CLL cells were treated with 0.5 μM TAK1 inhibitor for 2 hours at 37ºC, and then BCR was stimulated with 20 μg/ml $F(ab')_2$ goat anti-human IgM for 15 minutes. The cellular lysates were analyzed by Western blot analysis for pIKKα/β (ser 180/181) antibody, and then for total IKKα/β.

3.2.7. IKKγ is constitutively ubiquitinated in CLL cells

The above result suggested an involvement of TAK1 in the phosphorylation of IKKβ in CLL cells responding to BCR crosslinking.TAK1-mediated phosphorylation of IKKβ occurs when IKKγ (NEMO) becomes ubiquitinated, thereby allowing association of TAK1 with the IKK complex[298-299]. We next investigated the induction of IKKγ ubiquitination in CLL cells in order to determine whether BCR crosslinking induced this process. We performed this experiment by first immunoprecipitating IKKγ and then examining the ubiquitination of this protein by Western blot analysis. Figure 3.10A shows that IKKγ appeared to be constitutively ubiquitinated in CLL cells, and that BCR crosslinking did not seem to induce further ubiquitination. Analysis of IκBα showed that this protein was constitutively phosphorylated, and that this did not change in response to BCR engagement. As a control, we did the same experiment in A20 cells, a mouse B cell line that has been used to model various elements of the signalling pathway induced by BCR crosslinking[300-302]. Here, BCR crosslinking induced an increase in the level of IKKγ ubiquitination, and this was followed by phosphorylation of IκBα and downregulation of its expression (Figure 3.10B). In this latter experiment we used an antibody which immunoprecipitated mouse and human IKKγ, but the antibody used for identification of this protein within the immunoprecipitated material did not recognise mouse IKKγ. Western blot analysis of whole cell lysates with an antibody reactive against mouse IKKγ showed equal levels of expression for all the conditions used. Taken together, these data suggest that the mechanism involved in IKKβ activation in CLL cells responding to BCR crosslinking is different from that involved in A20 cells.

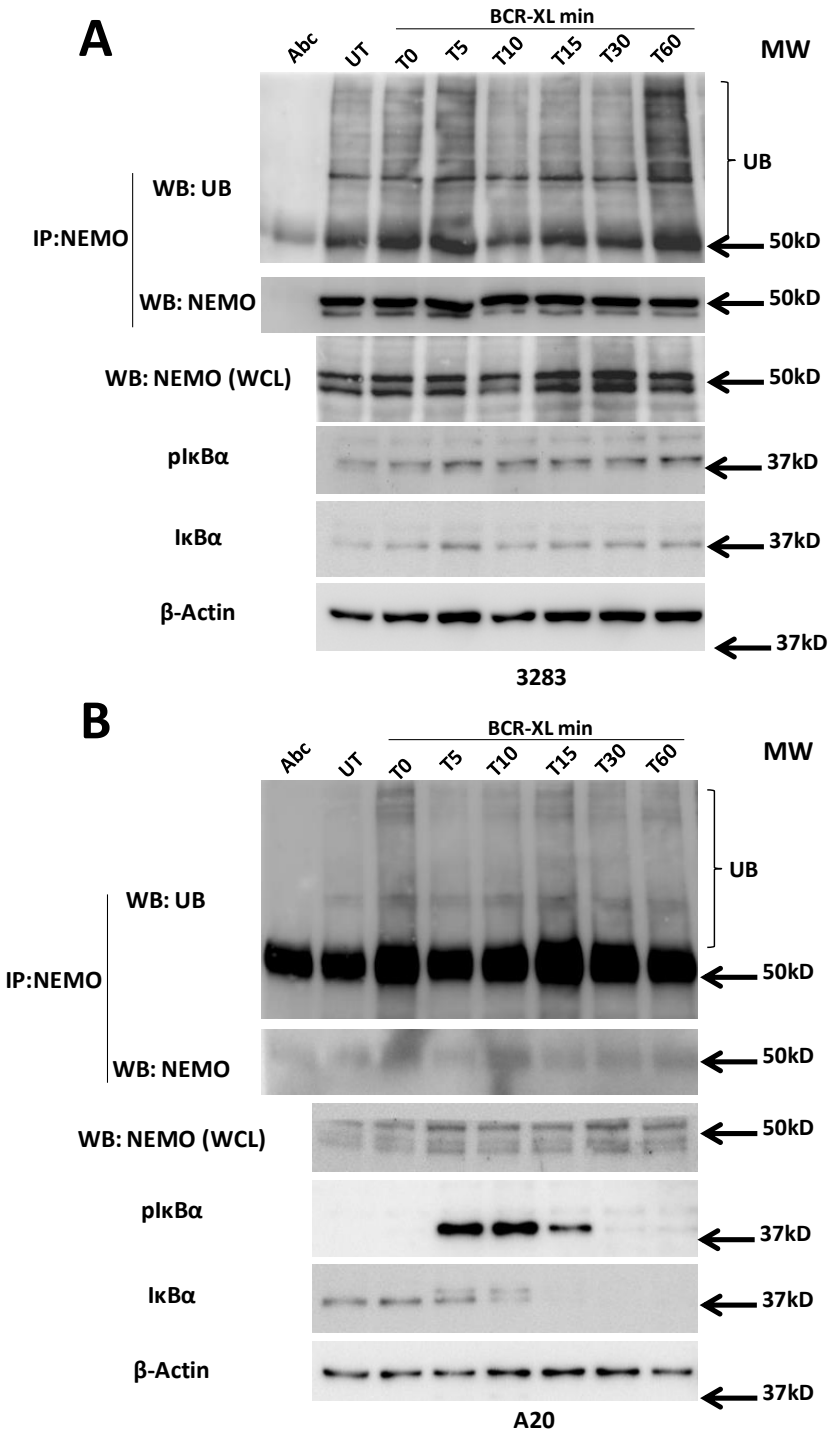


Figure 3.10: Measurement of IKKγ ubiquitination in CLL and A20 cells. (A) $2x10^7$ CLL cells were incubated for the indicated time points with 20µg/mL $F(ab')_2$ goat anti-human IgM. CLL cell lysates were immunoprecipitated with anti-IKKγ antibody and then probed with anti-ubiquitin antibody. **(B)** $3x10^6$ A20 cells that had been serum starved overnight were incubated for the indicated time points with 20µg/mL $F(ab')_2$ goat anti-mouse IgG. The cell lysates were immunoprecipitated with anti-IKKγ antibody and then probed in Western blots with the indicated antibodies.

3.2.8. The role of Bruton's tyrosine kinase (Btk) in BCR-induced NFκB activation

Looking upstream of PKCβ, we examined the role of the protein tyrosine kinase Btk in BCR signalling to NFκB in CLL cells. Btk is required for PLCγ2 tyrosine phosphorylation and activation[303-305]. Because PLCγ2 activation is required upstream of the CBM complex during BCR signalling, Btk is therefore upstream of NFκB pathway activation. This was demonstrated in experiments where deletion of this tyrosine kinase in DT40 cells abrogated BCR-stimulated PLCγ2 activation[123 306].

In order to examine the role of Btk in the BCR-induced NFκB pathway in CLL cells, we used the specific Btk inhibitor alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)propenamide (also known as LFM-A13)[307]. This compound has previously been used to show that Btk mediates cyclin D2 expression in a study of BCR signalling[308]. Figure 3.11 shows that pre-treatment of CLL cells with 100 μM LFM-A13 had no effect on BCR-stimulated IKK phosphorylation. In contrast, the presence of this compound completely inhibited the induction of IKK phosphorylation in A20 cells. This indicates that Btk does not seem to play a role in BCR-induced NFκB activation in CLL cells.

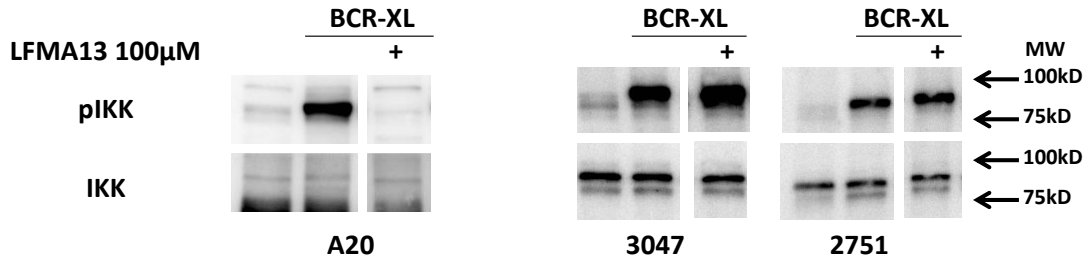


Figure 3.11: Effect of Btk inhibition on BCR-induced IKK phosphorylation. Cells were treated with 100μM LFM-A13 for 2 hours at 37ºC and then BCR was stimulated with 20 μg/mL $F(ab')_2$ goat anti-human IgM (for CLL cells) or with 20μg/mL $F(ab')_2$ goat anti-mouse IgG (for A20 cell line) for 15 minutes. The cellular lysates were analyzed by Western blotting and probed with pIKKα/β (ser 180/181) antibody and then for total IKK. A20 cells were serum starved overnight prior to treatment.

3.2.9. The role of c-Abl in BCR induced NFκB activation in CLL cells

The observation that efficient BCR-induced NFκB signalling occurs despite low levels of Bcl-10, and in the presence of inhibitors blocking Btk and PKCβ activity suggests the existence of an alternative pathway. Previous work from this Department has shown that c-Abl expression is higher in CLL cells than in normal peripheral blood B cells. Furthermore, this work also showed that treatment of CLL cells with the Abl-specific inhibitor imatinib results in the induction of apoptosis through a mechanism involving inhibition of the NFκB pathway[200]. This work is significant to the present study because c-Abl has been shown to contribute to BCR signalling[206], and because in other cell types c-Abl can activate IKK through a pathway involving PKCδ and PKD[309-311].

To test whether c-Abl inhibition affected BCR-induced IKK phosphorylation we constructed a concentration-response curve to determine the minimal effective concentration of imatinib. Figure 3.12 shows that maximum inhibition of BCR-induced IKK phosphorylation was achieved using 20μM imatinib. In these experiments there remained a residual level of BCR-induced IKK activation even when higher concentrations (30μM) of imatinib were used (Figure 3.12).

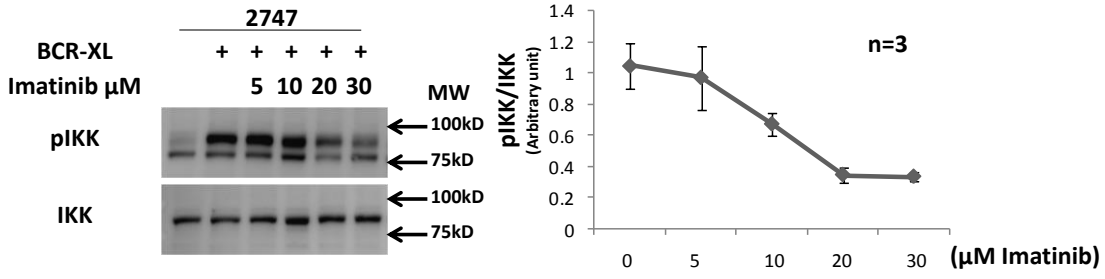


Figure 3.12: Imatinib inhibits BCR-induced IKK phosphorylation in a concentration-dependent way. CLL cells were incubated at 37°C with the indicated concentrations of imatinib for 2h prior to BCR crosslinking for 15min with 20μg/ml $F(ab')_2$ goat anti-human IgM. Whole CLL cell lysates were analysed by Western blot for pIKK and IKK (*left hand panel*). This experiment is representative of n=3 using different cases of CLL and is graphically represented in the right hand panel.

To further characterise the effects of imatinib, we screened 14 CLL cases for BCR-activated IKK phosphorylation. Figures 3.13A and B show that BCR crosslinking induces an approximate nine fold increase in IKK phosphorylation in CLL cells with variable responses among the screened cases. Pre-treatment of these cells with 20μM imatinib inhibits BCR-induced IKK phosphorylation by approximately 50 % (p=0.006). However, the ability of imatinib to inhibit this CLL cell response to BCR crosslinking was variable between different CLL cases; in some cases imatinib was able to inhibit BCR-induced IKK phosphorylation by more than 50%, and in other cases considerably less (Figure 3.13C).

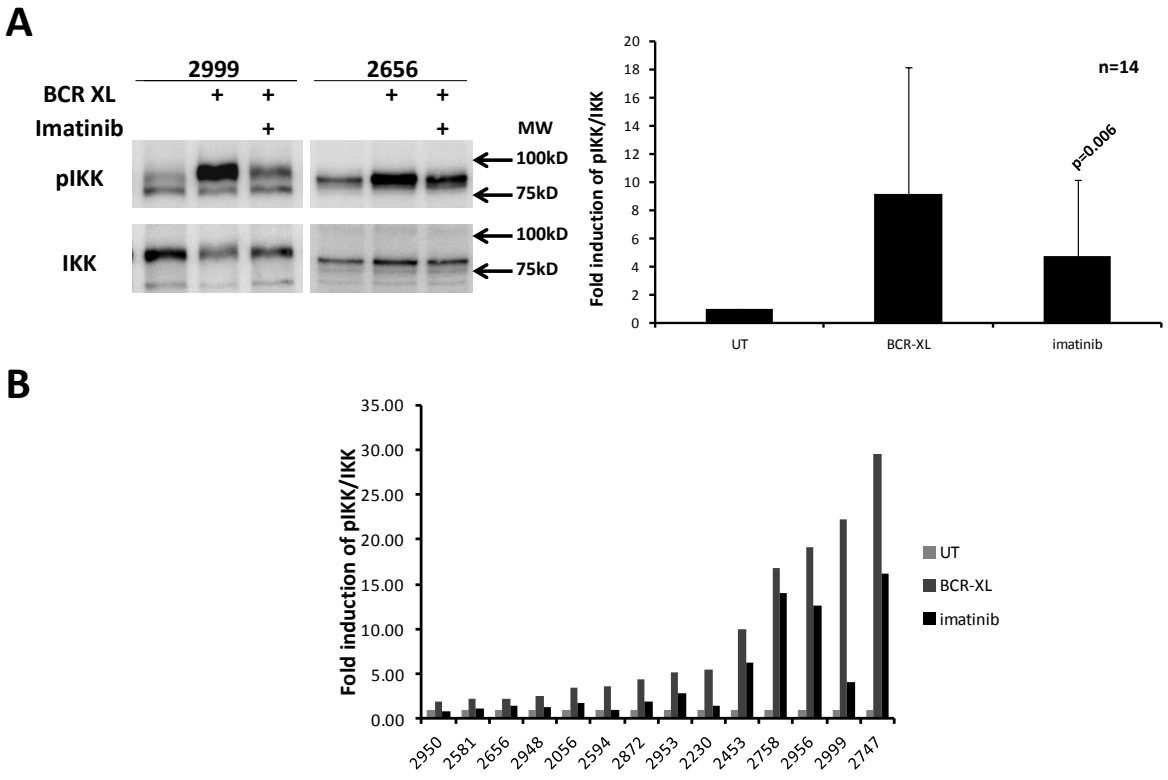


Figure 3.13: BCR-induced IKK activation is variably affected by imatinib treatment in different CLL cases. CLL cells were incubated at 37°C with 20μM imatinib for 2h prior to BCR crosslinking for 15min with 20μg/mL $F(ab')_2$ goat anti-human IgM. **(A)** CLL cell lysates were analysed by Western blot for pIKK induction using pIKK (ser 180/181) and IKK antibodies (*left hand panel*). The right hand panel is a graphical representation of the left hand panel for n=14 CLL cases. **(B)** Graphical representation of the individual experiments.

In order to determine whether c-Abl level accounts for the ability of CLL to signal through the BCR, we compared the ability of CLL to induce IKK phosphorylation in response to BCR stimulation in high versus low c-Abl-expressing CLL cases. Figure 3.14 shows that the levels of IKK phosphorylation in BCR-stimulated CLL cells expressing high levels of c-Abl was not significantly different from those in stimulated cells expressing low levels of c-Abl. This indicates that the level of c-Abl in CLL cells does not seem to contribute to the intensity of BCR signalling with respect to NFκB pathway activation.

To further investigate the role of c-Abl we examined whether c-Abl levels account for imatinib sensitivity. This was done by comparing the percent inhibition of BCR-induced IKK phosphorylation by imatinib between high and low c-Abl cases. Figure 3.14 shows no significant difference in imatinib sensitivity between the two CLL groups. Imatinib treatment of CLL cells inhibited BCR-induced IKK phosphorylation by approximately 50% in c-Abl high- and c-Abl low-expressing cells. Thus, although imatinib inhibits BCR-induced NFκB pathway activation, expression levels of its primary target, c-Abl, does not seem to affect the intensity of the BCR-induced signals or imatinib sensitivity in CLL cells. This suggested that the effect of imatinib in CLL could be due to its off target effect on other kinases in these cells.

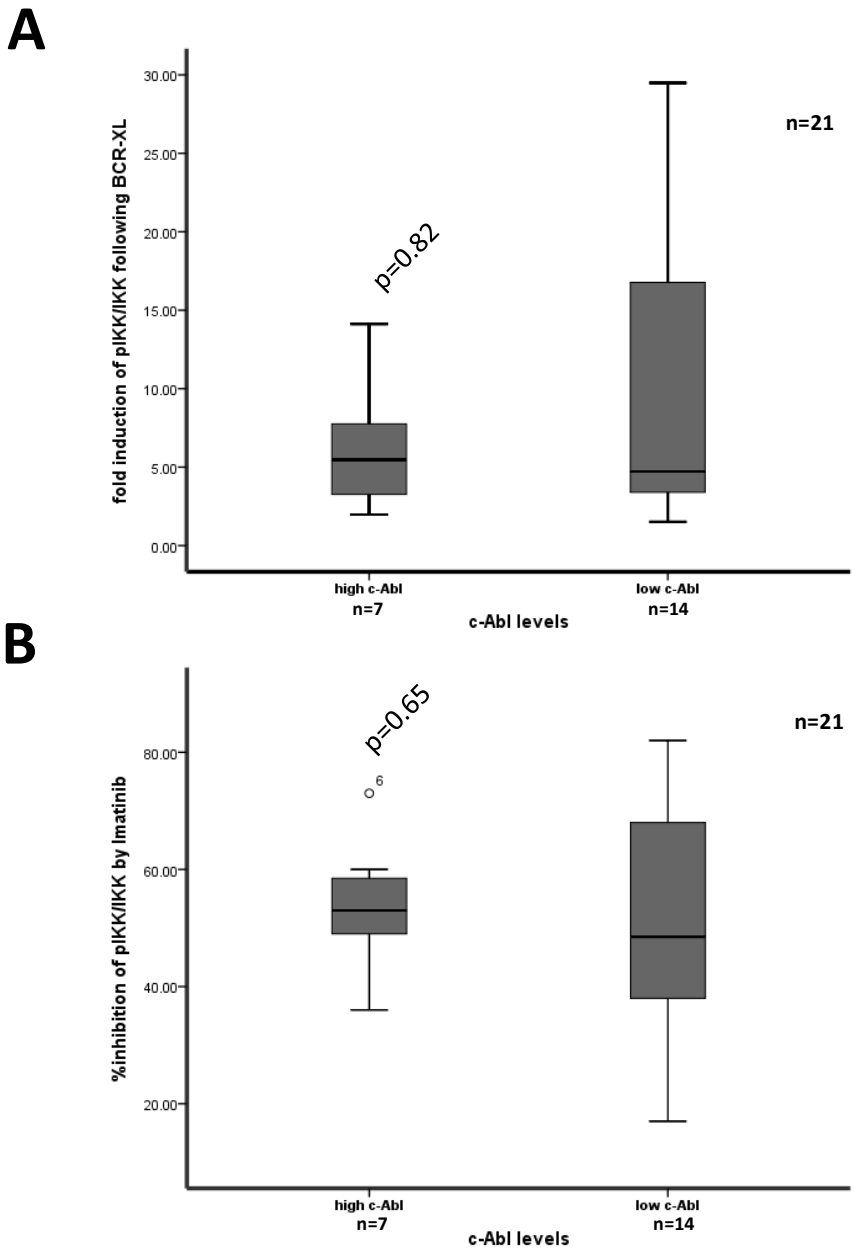


Figure 3.14: BCR response intensity and imatinib sensitivity in CLL cases with high c-Abl levels compared to cases with low c-Abl levels. **(A)** Graphical representation comparing fold induction of IKK phosphorylation following BCR-crosslinking of CLL cells with high and low c-Abl levels. **(B)** Graphical representation comparing per cent inhibition of BCR-induced IKK phosphorylation caused by imatinib in CLL cells with high and low c-Abl levels. Tests for statistical significance were performed using a Mann-Whitney U-test.

3.3. Discussion

This chapter is mainly concerned with gaining insight into the signalling pathway that is initiated by the engagement of the BCR with its cognate antigen in CLL cells. In particular, we concentrate on the pathway leading to activation of NFκB and follow its translocation into the nucleus. Here we have shown that PKCβ does not play a role in BCR-induced NFκB activation in CLL cells suggesting an alternative mechanism for activation of this pathway.

I initially investigated the role of PKCβ in BCR-induced NFκB activation in CLL cells. This serine/threonine kinase is a key mediator of NFκB activation in normal B cells following BCR stimulation[36 279-281]. The mechanism is as shown in figure 3.1, and involves phosphorylation of the CBM complex which leads to ubiquitination of IKKγ and activation of IKKβ by TAK1, and eventually the translocation of NFκB into the cell nucleus[133]. Our data show that inhibition of PKCβ using a specific PKCβ inhibitor LY379196, or a general inhibitor of PKCs Bisindolylmaleimide I, does not affect BCR-induced phosphorylation of IKK, even when used at very high concentrations to ensure complete inhibition of this kinase. This could have been important because CLL cells overexpress PKCβII[197 282], and/or may not be able to take up sufficient drug to inhibit this kinase. To confirm that the concentration we used in these experiments was sufficient to inhibit PKCβ we showed an obvious blockage of BCR-induced phosphorylation of GSK3α/β, a known downstream target of PKCβ[312-313] as well as of the Akt pathway[125]. When used at the concentrations employed in the present study, compounds like LY379196 and ruboxistaurin also inhibit PDK1, the kinase responsible for T^{308} phosphorylation and eventual activation of Akt[314-315] . Although we did not examine BCR-induced Akt activation in these experiments, our results showing that BCR-induced GSK3β phosphorylation is inhibited nevertheless indicate the compounds are acting as expected. Such detection of drug activity is proposed in a study that suggests

GSK3β phosphorylation may serve as a reliable pharmacodynamic marker for enzastaurin activity[316], another inhibitor of PKCβ. Therefore, our experiments indicate that BCR-stimulated activation of the NFκB pathway in CLL cells is not transmitted via overexpressed PKCβII.

Our findings do not rule out a role of the PKCβII-NFκB signalling pathway in the pathophysiology of CLL. Thus, a recent paper has reported that CLL cell contact with stromal cells induces expression of PKCβII. This, in turn, provides pro-survival signals to CLL cells through a mechanism involving activation of the NFκB pathway that is independent of Bcl-10[317]. This observation may explain why a CLL-like disease does not develop when the Tcl1-transgenic mouse is crossed with a mouse where the gene for PKCβ is deleted[37].

Our results indicate that the pathophysiology of CLL cells differs from other B cell malignancies with respect to activation of the NFκB pathway by the antigen receptor. PKCβ is also overexpressed in the malignant cells of diffuse large B cell lymphoma (DLBCL) and mantle cell lymphoma (MCL)[282-283]. The NFκB pathway in these malignancies is constitutively active, and PKCβ mediates the tonic BCR signalling that contributes to cell survival and proliferation through a mechanism involving activation of IKK[281 284]. Inhibition of PKCβ using specific inhibitors such as LY379196, LY333531 (ruboxistaurin) and LY317615 (enzastaurin) can induce apoptosis of DLBCL and MCL cells[281 285], and are therefore proposed to have possible use in the treatment of these diseases.

To further show that PKCβ is not involved in BCR-induced IKK activation we used the Btk inhibitor LFM-A13. Btk controls activation of PLCγ2, which generates the DAG and Ca^{2+} release (because of IP3) necessary for PKCβ activation. We clearly show that the induction of IKK phosphorylation by BCR crosslinking is resistant to this compound in CLL cells.

However, LFM-A13 does inhibit BCR-induced IKK phosphorylation in A20 cells, which have been used to characterise classical BCR signalling pathways[300-302]. These data suggest that Btk does not play a role in BCR-induced NFκB activation in CLL cells.

I next examined expression levels of the CBM complex components downstream of PKCβ. This complex consists of three components (CARMA-1, Bcl-10, and MALT-1) known to transmit signals from PKCβ to IKK during BCR engagement[133-134]. Our data indicate that CLL cells express Bcl-10 at an eighth of the level it is expressed in normal B cells. This could account for the inability of BCR to transfer signals from PKCβ to the IKK complex because efficient signalling by the CBM complex requires all the three components of this complex[288-290]. Moreover, a lack of Bcl-10 expression may also explain why BCR-induced JNK activation is defective in the vast majority of CLL cases[150]. Antigen receptor-induced JNK phosphorylation is impaired in T cells and primary splenic B cells from Bcl-10-deficient mice, suggesting a role for this adaptor protein in JNK activation[318].

Ligation of CD40 on CLL cells changes gene expression[22 23]. We examined the possibility that CD40 ligation induces Bcl-10 expression, and did this by co-culturing CLL cells on a fibroblast cell line where CD40 ligand is expressed. This experiment showed significant induction of Bcl-10 expression in CLL cells that were cultured under these conditions. However, despite increased levels of Bcl-10 in the CD40-stimulated CLL cells, activation of IKK was considerably lower than in CLL cells cultured on control fibroblasts and which had Bcl-10 expression levels similar to those of freshly-thawed cells.

It is reported that T cells and primary splenic B cells do not require Bcl-10 or CARMA-1 for antigen receptor-induced IKK phosphorylation. However, in Bcl-10 and CARMA-1 deficient cells active IKK is unable to phosphorylate its substrate IκBα and thereby activate NFκB [294]. This may be because Bcl-10- and CARMA-1 are required for ubiquitination of NEMO

(IKKγ) and formation of a signalosome allowing efficient NFκB signalling to occur. We tested whether NFκB became activated in BCR-stimulated CLL cells by measuring nuclear extracts from these cells for the presence of this transcription factor. Consistent with the observations of others[158], our analysis of freshly-thawed CLL cells showed the presence of high levels of NFκB in nuclear extracts. When we applied BCR stimulation, this did not lead to any change in the levels nuclear NFκB. Therefore, we incubated the CLL cells *in vitro* for 24 hours, and this resulted in a reduction of the basal level of active NFκB. BCR crosslinking of these cultured CLL cells induced a clear increase in nuclear NFκB, and analysis of Bcl-10 expression in these cultured cells showed that it was not significantly different from that in freshly thawed cells. These results indicate that the NFκB signalling pathway is intact in BCR-stimulated CLL cells despite the low level of Bcl-10 expression. Thus, PKCβ and the CBM complex are bypassed in the mechanism of BCR-induced NFκB pathway activation in CLL cells.

I then examined the role of TAK1 in BCR-induced NFκB pathway in CLL using the TAK1 inhibitor (9-Epimer-11, 12-dihydro-(5Z)-7-Oxozeaenol (antibiotic L-783277)). Our data show that the presence of this compound inhibits BCR-induced phosphorylation of IKK in CLL cells. This finding suggests that TAK1 modulates BCR-induced NFκB pathway activation in CLL cells despite low expression of Bcl-10 and overexpression of PKCβII. We established that IKKγ is constitutively ubiquitinated in CLL cells, and is therefore potentially able to attract TAB proteins and TAK1. Constitutive ubiquitination of IKKγ in CLL cells could possibly be due to downregulation of the deubiquitinase cylindromatosis (CYLD) as is described for these cells[319]. IKKγ is a known target of CYLD which acts to downregulate NFκB signalling[320-323]. How TAK1 is induced by BCR stimulation in CLL cells is unclear. It

still requires activation of tyrosine kinases, and we explore this possibility by examining a potential role of c-Abl.

c-Abl is highly expressed in CLL cells compared to normal B cells, and plays a role in the pathophysiology of CLL cells by protecting them from spontaneous apoptosis through a mechanism that involves activation of the NFκB pathway[200]. This mechanism may involve c-Abl, PKD and PKCδ as has been described in other cell types in order to protect them against oxidative stress-induced cell death[309-311]. How this applies to BCR signalling is suggested by studies showing that c-Abl plays a role in this process through an ability to phosphorylate CD19[206], and that PKD and PKCδ are activated in response to BCR engagement[324-325]. Therefore, c-Abl was a good candidate for further investigation of the mechanism of BCR-induced IKK activation in CLL cells.

Our data show that pre-treatment of CLL cells with 20μM imatinib, a known inhibitor of c-Abl, inhibited BCR-induced phosphorylation of IKK by 50%. This indicated that this kinase could be playing a role in BCR-induced IKK activation in CLL cells. However, it was not possible to achieve full inhibition of IKK activation, even when imatinib was used at higher concentrations. We screened 14 CLL cases for BCR-induced phosphorylation of IKK and for the effect of imatinib on this phosphorylation. Our results showed that BCR stimulation induced variable increases in the level of IKK phosphorylation in stimulated cells. Pre-treatment of these cells with 20μM imatinib resulted in a variable inhibition of BCR induction of IKK phosphorylation. A comparison of c-Abl expression with intensity of IKK phosphorylation showed no relationship, and there was no relationship between c-Abl expression and effect of imatinib on BCR-induced IKK phosphorylation. Other work has shown that the level of c-Abl expression relates to the ability of imatinib to induce apoptosis[200 326]. Ostensibly, this induction of apoptosis occurs through the inhibition of NFκB

pathway activation leading to IL6 release and regulation of Mcl1 expression through STAT3[200 207]. The experiments we present in this thesis sugest that although c-Abl does regulate the NFκB pathway in CLL cells, it does so independently of BCR engagement. What could be happening is that imatinib is affecting another kinase involved in mediating BCR signalling. This may be Lck because imatinib is known to inhibit this SFK at the concentrations we used in this study. Thus, the next chapter outlines our investigation into the role of Lck in mediating BCR signalling in CLL cells.

Chapter 4: The role of Lck in BCR signalling in CLL cells

4.1. Introduction

In the previous chapter we showed that imatinib, a c-Abl inhibitor, reduces BCR induced phosphorylation of IKK by approximately 50%, with some cases responding better than others. Importantly, our data show that the levels of c-Abl do not relate to the intensity of BCR signalling, nor to the ability of imatinib to inhibit these signals in CLL cells. This suggests that the effect of imatinib on BCR signalling in CLL could be caused by its non-specific inhibition of other kinases in these cells.

In addition to c-Abl, imatinib can inhibit other tyrosine kinases including c-Kit, platelet derived growth factor receptor (PDGF-R), and, in particular, Lck [327]. Reactivity of imatinib with c-Kit and PDGF-R is not important for CLL pathophysiology because these two receptor tyrosine kinases are not expressed by CLL cells[328]. On the other hand, Lck has been shown to be expressed in CLL cells where its function is unknown[191 329].

That imatinib may affect Lck function in CLL cells is supported by studies showing that this SFK is a key target during antigen-receptor stimulation of T-cells[330], and by studies showing that it may play a role in BCR signalling in B1 cells. One study has shown that Lck expression is responsible for peritoneal B1 cell hyporesponsiveness to BCR stimulation[192], while another study has suggested that Lck augments BCR signals in these cells[331]. However, a third study has indicated that Lck does not play any role in BCR signalling in B1 cells[194]. Given this controversy, it was nevertheless plausible to investigate Lck function within the context of BCR signalling in CLL cells because of the role this SFK plays in initiating antigen receptor signalling in T cells[188 332], and because it is possible for Lck to phosphorylate the ITAM within CD79a and potentially initiate the BCR signalling cascade[333].

4.2. Results

4.2.1. Inhibition of Lck by imatinib

To test whether Lck is a target for imatinib in CLL cells, we induced Lck activation by treating the cells with the protein phosphotyrosine phosphatase inhibitor monoperoxo (picolinato) oxovanadate (V) mpV(pic)[334]. We then immunoprecipitated Lck from CLL whole cell lysates and probed for active kinase in Western blots of precipitated proteins using an anti-phospho- Y^{416}-Src antibody (anti-pY^{416}-Src). This antibody crossreacts with Lck when it is phosphorylated on Y^{394}, and therefore can be used to detect active Lck. Figure 4.1 shows that only low levels of active Lck could be detected in resting CLL cells. This figure also shows that stimulation of CLL cells with mpV(pic) increased the level of Lck activation, and that the presence of 20μM imatinib partially inhibited this induction of Lck activity. This result indicates the possibility that the effects of imatinib on BCR signalling in CLL cells could be mediated by this SFK.

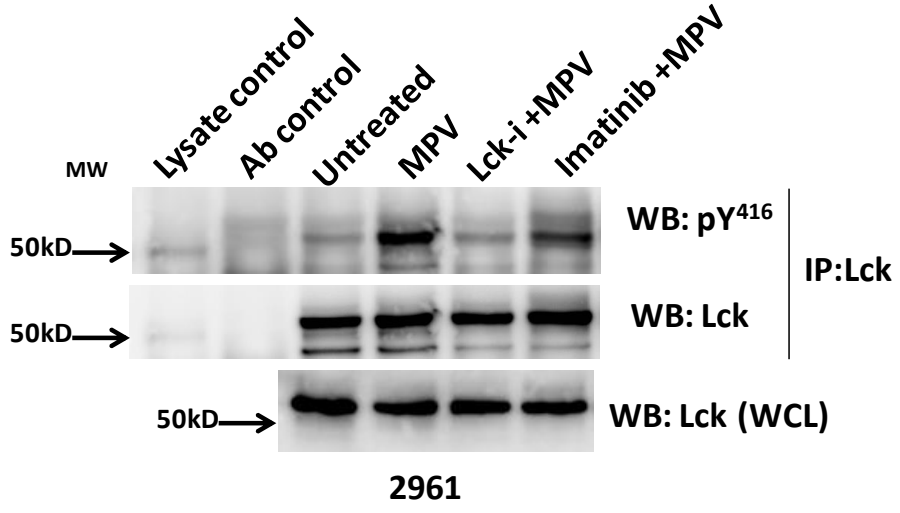


Figure 4.1: Effect of imatinib and Lck-i on Lck activity in CLL cells. CLL cells were pre-treated with 1μM Lck-i or 20μM imatinib for 2h at 37ºC. To stimulate Lck activity the cells were then incubated with 100μM mpV(pic) for 30min. The cell lysates were immunoprecipitated with anti-Lck antibody and then probed with anti-pY^{416}-Src antibody on Western blots to assess Lck activity.

4.2.2. Lck inhibitor concentration-response

To test for a potential role of Lck in BCR-induced signalling we used the Lck specific inhibitor 4-amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane (Lck-i)[335-337]. We first established a concentration-response curve to show how the presence of Lck-i affected BCR-induced IKK phosphorylation. Figure 4.2 shows that BCR-stimulated IKK activation is maximally inhibited using a concentration of 1μM Lck-i. That Lck-i affects Lck activation is shown in Figure 4.1, mpV(pic)-induced activation of Lck in CLL cells is completely inhibited by the presence of 1μM of this compound. Thus, the effects of imatinib on BCR-induced signalling could be mediated by Lck.

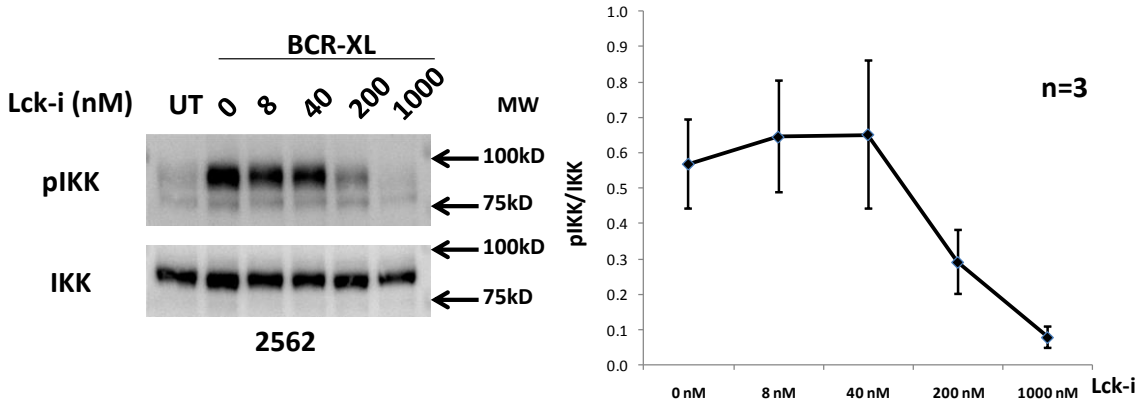


Figure 4.2: Effect of different concentrations of Lck-i on BCR-induced IKK phosphorylation in CLL cells. CLL cells were incubated with the indicated concentration of Lck-i for 2h at 37ºC prior to BCR crosslinking with 20μg/ml $F(ab')_2$ goat anti-human IgM for 15 min. Whole CLL cell lysates were probed by Western blot for the presence of pIKK. This experiment is representative of n=3 separate experiments using different CLL cases.

4.2.3. BCR-induced activation of ERK, Akt, and IKK is inhibited in CLL cells treated with Lck-i

In T cells Lck mediates activation of the MEK/ERK[338] and PI3K/Akt[339] pathways during engagement of the antigen receptor. To answer the question of whether Lck played a similar role in CLL cells, we tested the effect of Lck-i on CLL cell response to BCR crosslinking. Figure 4.3 shows that pre-treatment of CLL cells with 1μM Lck-i significantly reduces BCR-induced phosphorylation of ERK ($p=0.001$), Akt ($p=0.001$) and IKK ($p=0.039$), suggesting a role for this SFK in regulating all three of these signalling pathways.

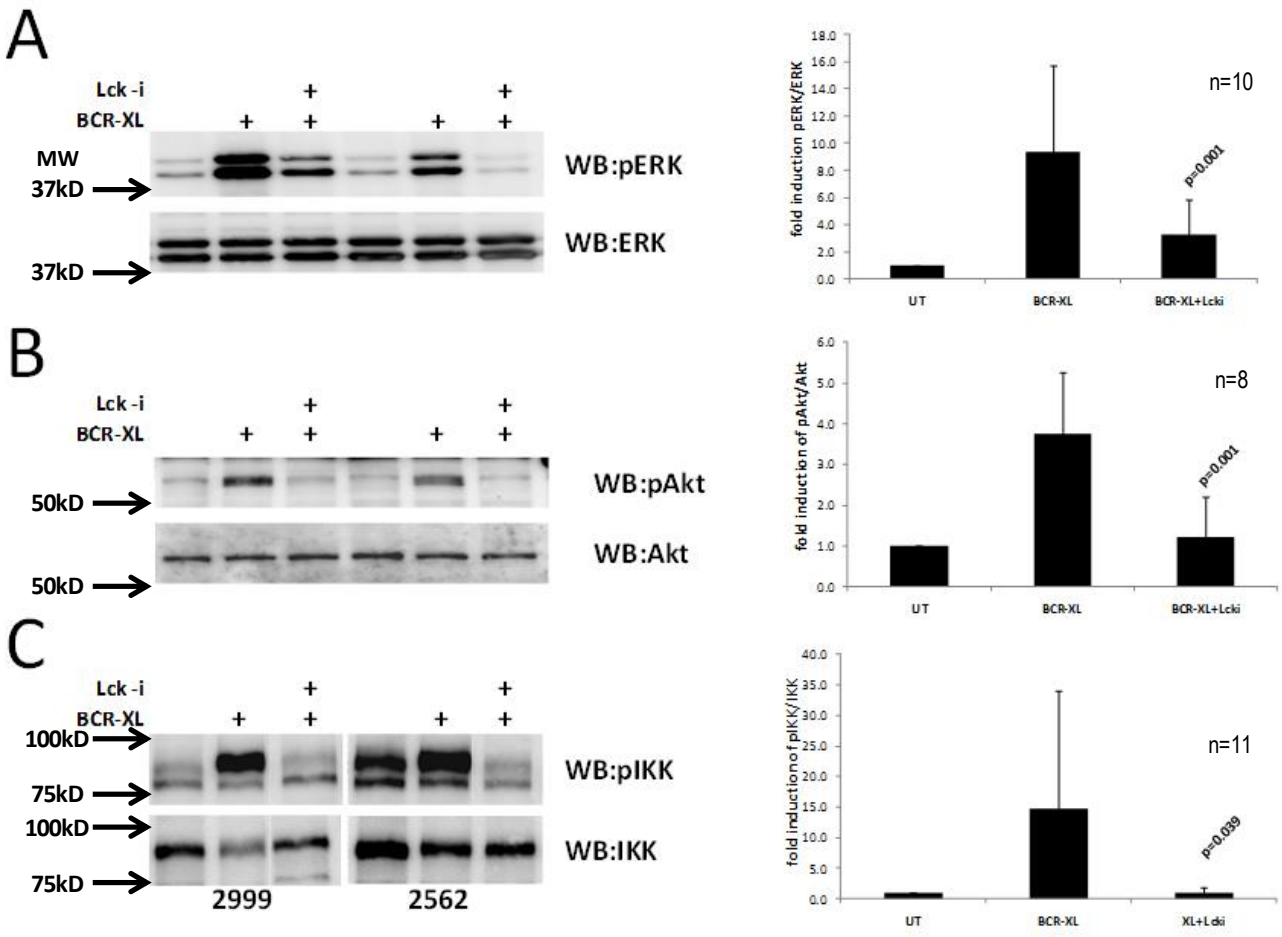


Figure 4.3. Inhibition of Lck blocks BCR-induced phosphorylation of ERK, Akt and IKK in cells. CLL cells were incubated for 2h with 1μM Lck-i. The CLL cells were then stimulated through the BCR by incubating the cells for 15min with 20μg/ml $F(ab')_2$ goat anti-human IgM. Whole CLL cell lysates were analysed for the indicated proteins by Western blotting. **A**. Analysis of BCR-induced ERK phosphorylation. In the right hand panel n=10 different cases of CLL were analysed. **B**. Analysis of Akt phosphorylation. In the right hand panel cells from n=8 cases of CLL were used. **C**. Analysis of IKK phosphorylation. In the right hand panel n=11 cases of CLL were used. Tests for statistical significance were performed using a student's t-test for paired data.

4.2.4. Effect of Lck-i on SFKs activity in CLL cells

CLL cells are reported to express a variety of SFKs including Lyn, Fyn, Fgr, Hck, Blk, c-Src, c-Abl and Lck[174 191 340-342]. We confirmed expression of these SFKs in CLL cells (Figure 4.4). We found that proteins such as Lyn, Blk, Fgr and c-Src were expressed at more or less constant levels when we compared CLL cells from different patients. Similar to previously reported observations[196], we found that Lck expression in CLL cells varied between patients. Examination of Fyn and Hck showed that these SFKs were also variably expressed in CLL cells from different patients, but this variability had no correlation with variation in Lck expression.

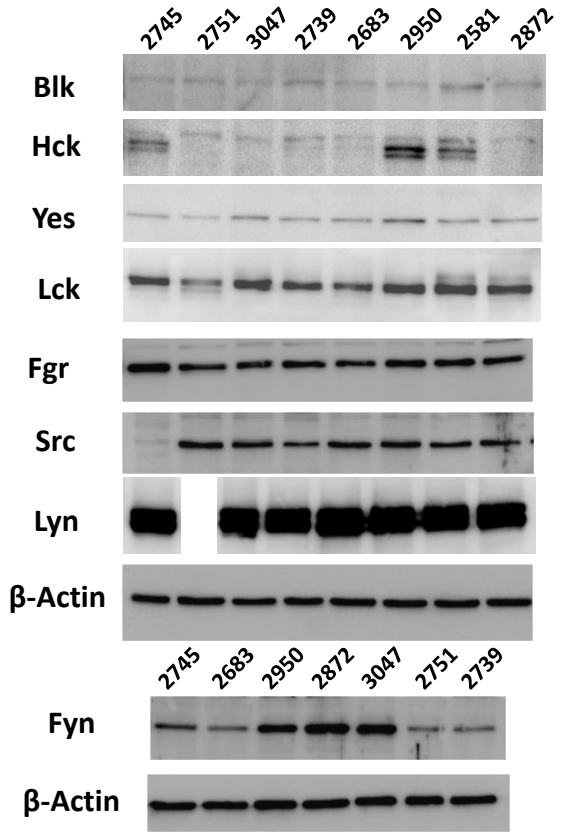


Figure 4.4. SFKs expression in CLL cells. Western blot analysis of whole CLL cell lysates for the indicated SFKs. β-actin was used as a loading control.

c-Abl has been reported to be expressed in CLL[200] and to play a role in BCR signalling[206]. In order to examine the specificity of Lck-I we first tested its effect on c-Abl. To do this we measured the phosphorylation of CrkL because this protein is a known substrate of c-Abl[343], and because we know c-Abl to be constitutively active in CLL cells[200 207]. Figure 4.5 shows that incubation of CLL cells with 1μM Lck-i leads to a slight reduction in the level of CrkL phosphorylation. In contrast, incubation of CLL cells with 20μM imatinib profoundly reduced phospho-CrkL levels, indicating that c-Abl activity was inhibited. This indicates that Lck-i does not affect c-Abl-mediated signalling in CLL cells.

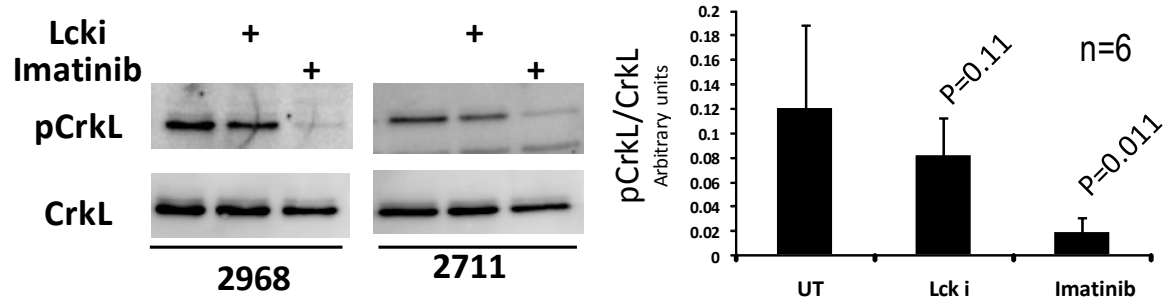


Figure 4.5. Comparison of Lck-i and imatinib effect on c-Abl activity in CLL cells. CLL cells were treated with either 1μM Lck-i, or with 20μM imatinib for 2h. Whole cell lysates were analysed by Western blotting using anti-pY[207]- CrkL. The right-hand panel shows a graphical representation of n=6 experiments using different cases of CLL. Tests for statistical significance were performed using a student's t-test for paired data.

Within the existing paradigm of BCR signalling, Lyn has been postulated to initiate proximal BCR signalling events by catalysing the phosphorylation of the ITAMs within CD79a and b[344]. Thus, the effect of Lck-i could be due to inhibition of this SFK during BCR stimulation, a notion that may be particularly important because Lyn is reported to be overexpressed and constitutively active in CLL[345].

To examine this possibility we immunoprecipitated active Lyn from CLL cell lysates using the anti-pY[416]-Src antibody. This antibody also cross reacts with pY[397] of active Lyn[346], and recognises the active state of many SFKs, including Lyn and Lck. When we examined the effect of Lck-i on active Lyn we found that the presence of this inhibitor had no effect on our ability to immunoprecipitate Lyn from CLL cell lysates using the anti-pY[416]-Src antibody. In contrast, the presence of 150nM of the pan-SFK inhibitor dasatinib, resulted in a marked reduction in our ability to immunoprecipitate active Lyn from lysates of CLL cells (Figure 4.6 A).When we used BCR crosslinking to stimulate CLL cells, there was only a slight increase in the levels of Lyn that could be immunoprecipitated with anti-pY[416]-Src antibody (Figure 4.6 C). However, similar to what we observed in unstimulated cells, only treatment with dasatinib lowered the levels of active Lyn in BCR-stimulated CLL cells and treatment with Lck-i had no observable effect. These results show that Lck-i does not affect Lyn activity when used at a concentration of 1μM.

I next investigated whether higher concentrations of Lck-i affected Lyn activity. Figure 4.6 B shows that Lyn activity was partially affected by incubating CLL cells with 5μM Lck-i, and completely inhibited with 10μM Lck-i. Thus, the presence of Lck-i can affect Lyn activity in CLL cells, however, the concentration needed is in excess of the 1μM required to inhibit downstream signals from the BCR.

I next tested the effect of 1μM Lck-i on Fyn, Fgr and c-Src activity in CLL cells. These tests showed that this compound had no effect on these SFKs when used at 1μM (Figure 4.6 D, E, F, G). As a control we included dasatinib in our experiments and showed, as expected, that the presence of 150nM dasatinib inhibited the activity of Fgr. Taken together, these data show that Lck-i is highly specific for Lck at the concentration used. Therefore, our experiments showing that the presence of Lck-i inhibits BCR-induced ERK, IKK and Akt phosphorylation strongly suggests that Lck is an important mediator of BCR signalling in CLL cells.

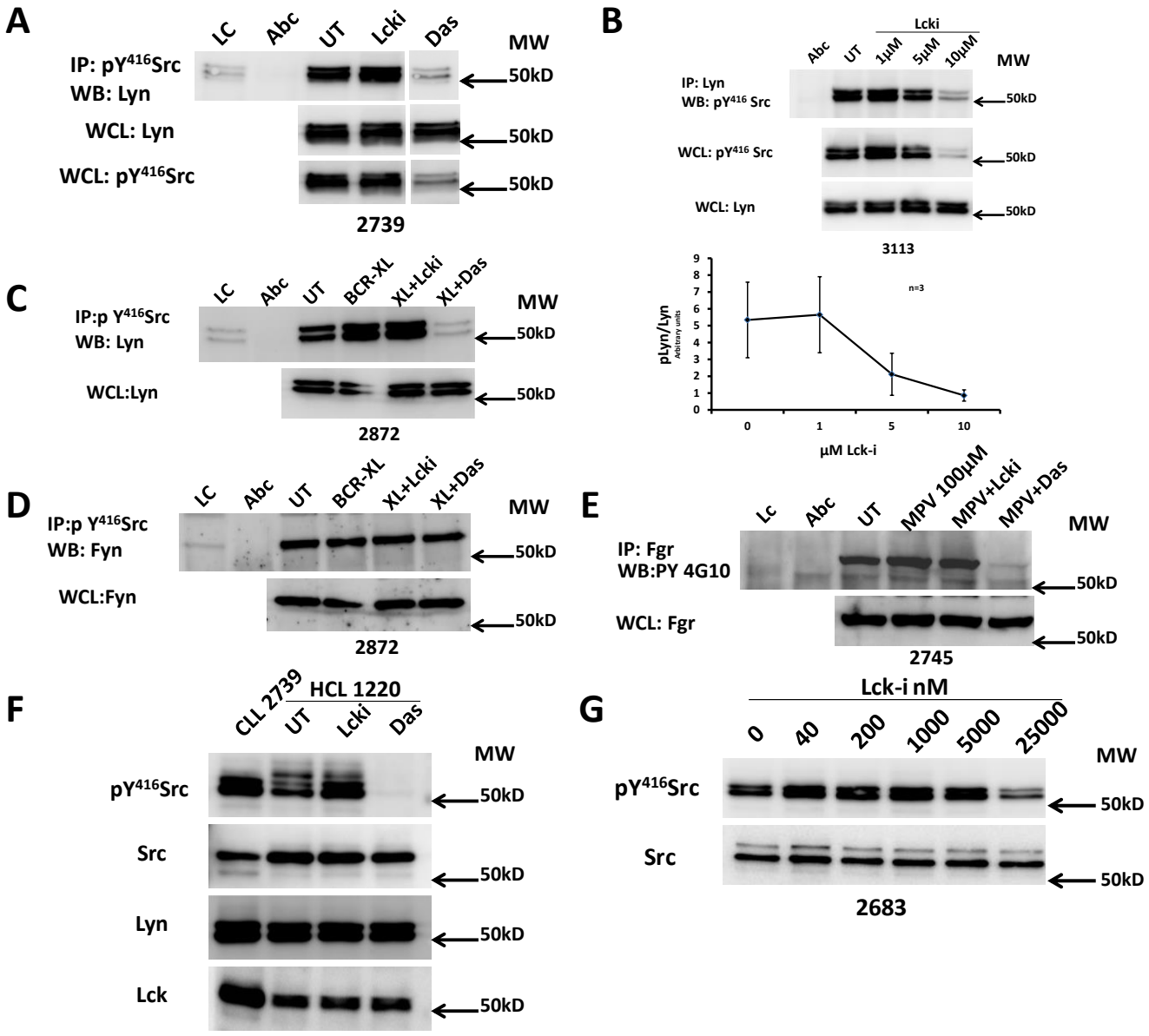
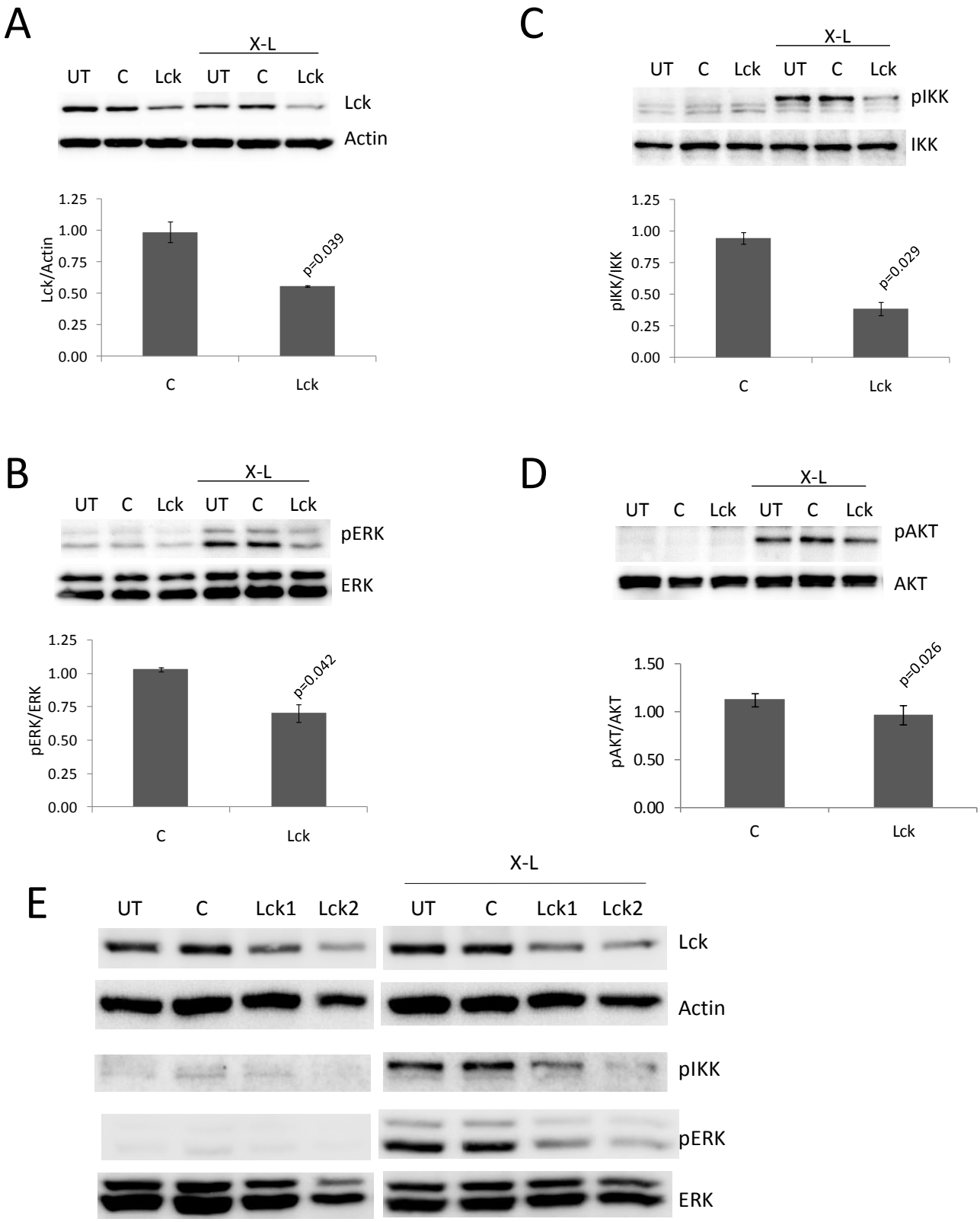


Figure 4.6. Specificity of Lck-i. (A) Effect of Lck-i on Lyn activity in CLL cells. CLL cells were incubated for 2h in the presence of 1μM Lck-i or 150nM dasatinib prior to harvest and lysis. CLL cell lysates were treated with anti-pY^{416}-Src antibody and immunoprecipitated Lyn was identified by Western blot analysis (top panel). Whole cell lysates were analysed to ensure that equal levels of Lyn were present prior to immunoprecipitation (middle panel). The same whole cell lysates were analysed for the presence of active SFKs using the anti-pY^{416}-Src antibody (lower panel). **(B)** Effect of increasing concentrations of Lck-i on active Lyn and SFKs in CLL cells. Top panel shows Lyn immunoprecipitated with anti-pY^{416}-Src antibody, middle panels shows anti-pY^{416}-Src antibody reactivity with whole CLL cell lysates and the lower panel shows the presence of Lyn in whole CLL cell lysates. This experiment represents n=3 using different CLL cases**.(C)** Effect of Lck-i and dasatinib on Lyn activity in BCR-stimulated CLL cells. CLL cells were treated with Lck-i and dasatinib as in part A. BCR stimulation was achieved by incubating the cells with 20μg/ml $F(ab')_2$ goat anti-human IgM for 15 min**.(D)** Effect of Lck-i on Fyn activity in CLL cells. CLL cell lysates were immunoprecipitated with anti-pY^{416}-Src antibody and immunoprecipitated Fyn was identified by Western blot analysis (top panel). Whole cell lysates were analysed to ensure that equal levels of Fyn were present prior to immunoprecipitation (second panel). **(E)** Effect of Lck-i on Fgr activity. CLL cells were incubated for 2h in the presence of 1μM Lck-i or 150nM dasatinib prior to treatment with 100μM mpV(pic) for 30min to stimulate Fgr activity. CLL cell lysates were immunoprecipitated with anti-Fgr antibody and phospho-Fgr was identified using the anti-phosphotyrosine antibody 4G10 (upper panel). Whole cell lysates were analysed to ensure that equal levels of Fgr were present prior to immunoprecipitation (lower panel). **(F)** Effect of Lck-i on c-Src. HCL cells were incubated for 2h in the presence of 1μM Lck-i or 150nM dasatinib then cell lysates were probed with anti-pY^{416}Src (upper panel), with Src (second panel), with Lyn (third panel) or with Lck (bottom panel) . **(G)** Effect of increasing concentration of Lck-i on SFKs activity in CLL: CLL cells were incubated for 2h in the presence of increasing concentration of Lck-i (as indicated) then cell lysates were probed with anti-pY^{416}Src (upper panel), with Src (second panel). Abbreviations: LC = lysate control, Abc = Ab control, BCR-XL/XL = BCR stimulation. UT = untreated cells. HCL=hairy cell leukaemia. MW= molecular weight ladder

4.2.5. siRNA-mediated reduction of Lck expression in CLL cells inhibits BCR-induced IKK, ERK and Akt activation

To confirm the specificity of Lck-i and show that Lck is an important mediator of BCR signalling in CLL cells, we next used specific siRNAs to reduce the expression of this SFK. Figure 4.7 A shows that the level of Lck expression in CLL cells was reduced to approximately 60% ($p=0.039$, $n=3$) of endogenous levels following treatment with specific siRNA. This reduction in Lck expression resulted in inhibition of BCR-induced IKK and ERK phosphorylation by approximately 58.52±8.03% ($p=0.029$, $n=3$) and 31.78±6.56% ($p=0.042$, $n=3$), respectively, of the levels induced in control siRNA-transfected cells (Figures 4. 7B, C). We also observed a 14.85±3.87% decrease in BCR-induced Akt phosphorylation, which, although small, was nevertheless significant ($p=0.026$, $n=4$) (Figure 4.7 D). To ensure that the reduction in BCR-induced IKK and ERK phosphorylation was not due to off-target effects of the siRNA (from Dharmacon) we repeated these experiments with siRNA from Invitrogen and obtained similar results (Figure 4.7E). These data, taken together with our studies using Lck-i strongly suggest that Lck plays an important role in mediating BCR-induced distal signalling events in CLL cells.


A
X-L
UT C Lck UT C Lck
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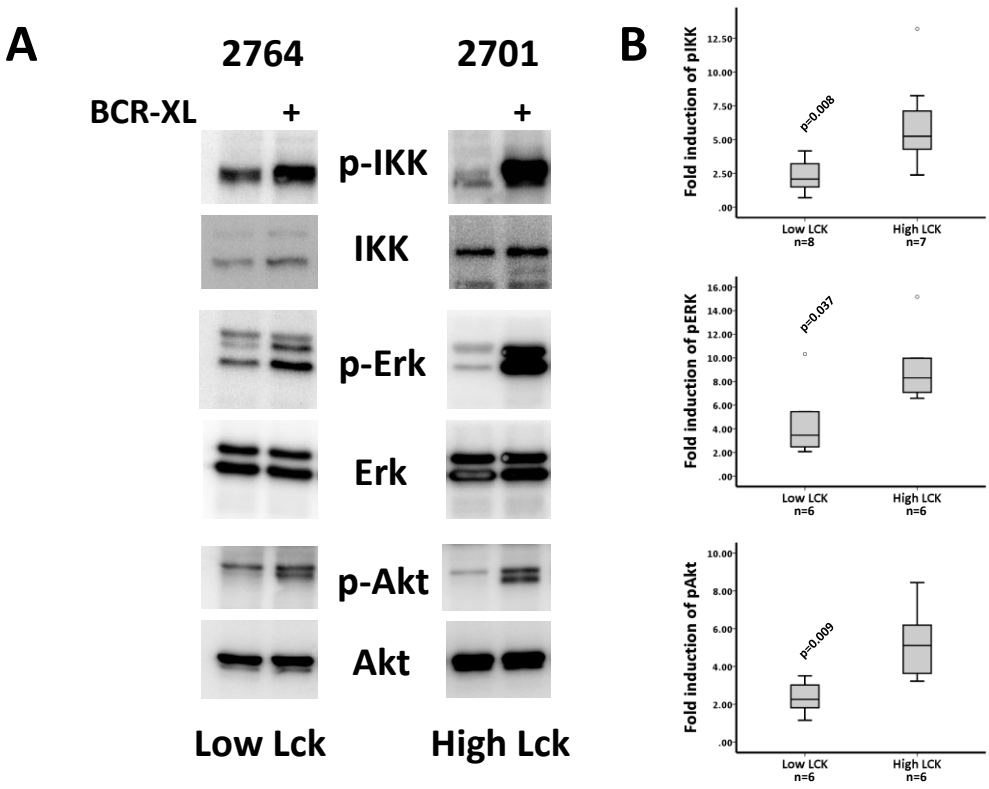
pERK
ERK

Figure 4.7. siRNA knockdown of Lck expression inhibits BCR-mediated signalling in CLL cells. Lck expression in CLL cells was reduced by treating cells with specific siRNA according to the method described in chapter 2. Whole cell lysates were then analysed by Western blot analysis for the indicated antibodies. **(A)** (upper panel) Western blot analysis of whole cell lysates for Lck expression. (lower panel) Graphical representation of n=3 experiments showing that Lck was reproducibly reduced by 42.81±5.08% in Lck-siRNA treated cells compared to non-specific siRNA treated control cells. **(B)** Analysis of BCR-induced ERK phosphorylation in CLL cells. BCR induction of ERK phosphorylation was reduced by 31.78±6.56% (n=3) in in Lck-siRNA treated cells. **(C)** Analysis of BCR-induced IKK phosphorylation in CLL cells. BCR induction of IKK phosphorylation was reduced by 58.52±8.03% (n=3) in Lck-siRNA treated cells. **(D)** Analysis of BCR-induced Akt phosphorylation in CLL cells. BCR induction of Akt phosphorylation was reduced by 14.85±3.87% (n=4) in Lck-siRNA treated cells. For all parts of this figure UT indicates mock transfected CLL cells, C indicates non-specific siRNA treated CLL cells and Lck indicates Lck-siRNA treated CLL cells. X-L indicates CLL cells that have been treated with 20μg/ml $F(ab')_2$ goat anti-human IgM for 15 mins in order to stimulate BCR signalling. **(E)** Lck expression in CLL cells was reduced with siRNA. However, in this case the species of siRNA was different to that presented in A, B, C, D. The effect of the siRNA-induced reduction on BCR signalling was measured by Western blot analysis of CLL cell lysates using the indicated antibodies. Tests for statistical significance were performed using a student's t-test for paired data.

4.2.6. Lck expression levels in CLL cells govern BCR signal strength

It is known that the level of Lck expression varies in CLL cells from different patients[191 196 347]. However, the functional significance of this expression has not been fully determined. In the light of our siRNA experiments showing that a reduction in Lck expression reduces BCR-induced activation of IKK, Akt and ERK, it seemed reasonable to hypothesize that the endogenous level of Lck expression would dictate a similar outcome. To investigate this possibility we compared BCR-induced phosphorylation/activation of IKK, ERK and Akt in CLL cells containing high and low levels of Lck.

Figure 4.4 confirms earlier observations of variability in expression of Lck in CLL cells from different patients. We then examined the effects of BCR crosslinking on CLL cells expressing very low or very high levels of Lck. We found that the induction of ERK, Akt and IKK phosphorylation was significantly stronger in CLL cells having high levels of Lck compared with those having low levels of Lck (Figures 4.8). Moreover, the pro-survival effects of BCR crosslinking also appeared to be stronger in Lck-high expressing CLL cells than in those expressing lower levels of this SFK (Figure 4.8C). Importantly, the level of ZAP70 expression was more or less equivalent in the 4 cases we tested, indicating that the expression of this protein was not a factor in BCR responsiveness. Taken together, these data agree with the experiments using siRNA to knockdown Lck expression, and show a linkage between signal strength and Lck expression in CLL cells responding to BCR crosslinking.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Fold induction of pIKK
p=0.008
Low LCK n=8
High LCK n=7
Fold induction of pERK
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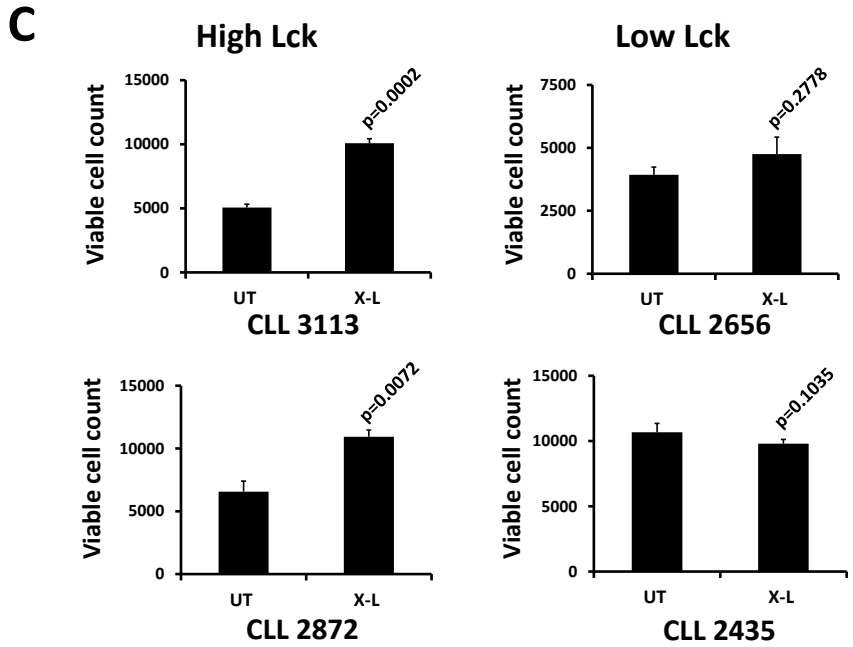
p=0.009
Low LCK n=6
High LCK n=6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Viable cell count
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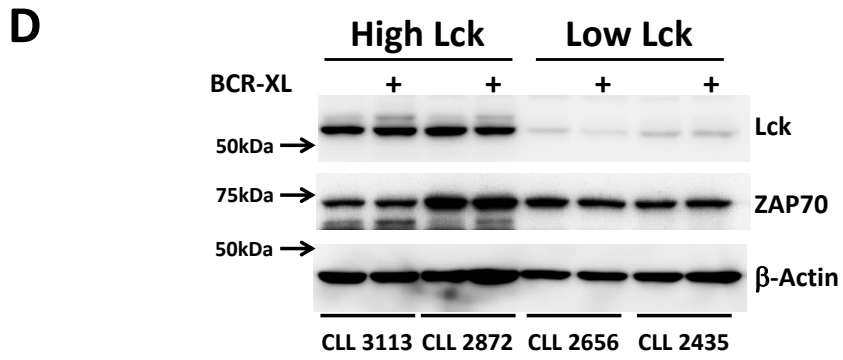
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ZAP70
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β-Actin
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CLL 2872
CLL 2656
CLL 2435

Figure 4.8. Comparison of BCR signalling in CLL cells expressing different levels of Lck. **A.** Comparison of CLL cell response to BCR crosslinking in cases expressing low and high Lck. CLL cell lysates were analysed for induction of IKK, ERK, and Akt activation following BCR crosslinking by Western blot using pT^{202}/pY^{204}-ERK, pS^{473}-Akt and pIKKα/β (ser180/181) antibodies. This blot is representative of at least n=4 experiments using CLL cells from different patients. **B.** Graphical representation of A, comparing the fold induction of pIKK, pERK and pAkt by BCR crosslinking between CLL cells with low and high expression levels of Lck. Statistical significance was assessed using a Mann-Whitney U-test. **C.** Comparison of BCR-induced CLL cell survival in cases expressing high and low levels of Lck. Cells were incubated for 1h following addition of 20μg/ml F(ab')2 goat anti-human IgM, and viable cell count was determined by FACS analysis of DIOC6bright/PIdim cells as described in materials and methods. In this experiment 2 CLL cases expressing high levels of Lck (left hand panels) and 2 cases expressing low Lck (right hand panels) are illustrated. Cell viability was measured in 3 separate experiments using each case to give the mean±SD. **D.** Lck and ZAP70 expression in CLL cells used in C.

4.2.7. Lck mediates proximal BCR signalling events in CLL cells

I next examined signalling that was proximal to IKK, Akt and ERK activation in BCR-stimulated CLL cells. One of the most proximal signalling events in B cells following engagement of the BCR is tyrosine phosphorylation of the ITAM domain within CD79a[115]. We therefore investigated the role of Lck in this event in BCR-stimulated CLL cells. Figure 4.9A shows that resting CLL cells have low, but detectable levels of tyrosine phosphorylated CD79a. BCR crosslinking of CLL cells increased the level of pY-CD79a approximately 2-fold, and prior treatment of CLL cells with 1μM Lck-i blocked this BCR-induced increase. Because our previous experiments showed that Lck-i did not affect either constitutive (Figure 4.6 A and B) or BCR-induced (Figure 4.6 C) activation of Lyn, these data suggest a role for Lck in initiating proximal BCR signalling events in CLL cells.

To confirm the role of Lck in BCR-induced CD79a phosphorylation we used siRNA to reduce the expression of this SFK. Thus, reduction of Lck expression in CLL cells resulted in a significantly decreased induction of CD79a phosphorylation (Figure 4.9B). This confirms the above data using Lck-i, and demonstrates that Lck is able to phosphorylate CD79a following BCR engagement on CLL cells.

However, Lck is not solely responsible for CD79 phosphorylation in CLL cells. This is because BCR-induced CD79a phosphorylation was reduced to below base line levels in CLL cells that were pre-treated with 150nM dasatinib (Figure 4.9A). This indicates a role for an additional SFK, possibly Lyn.

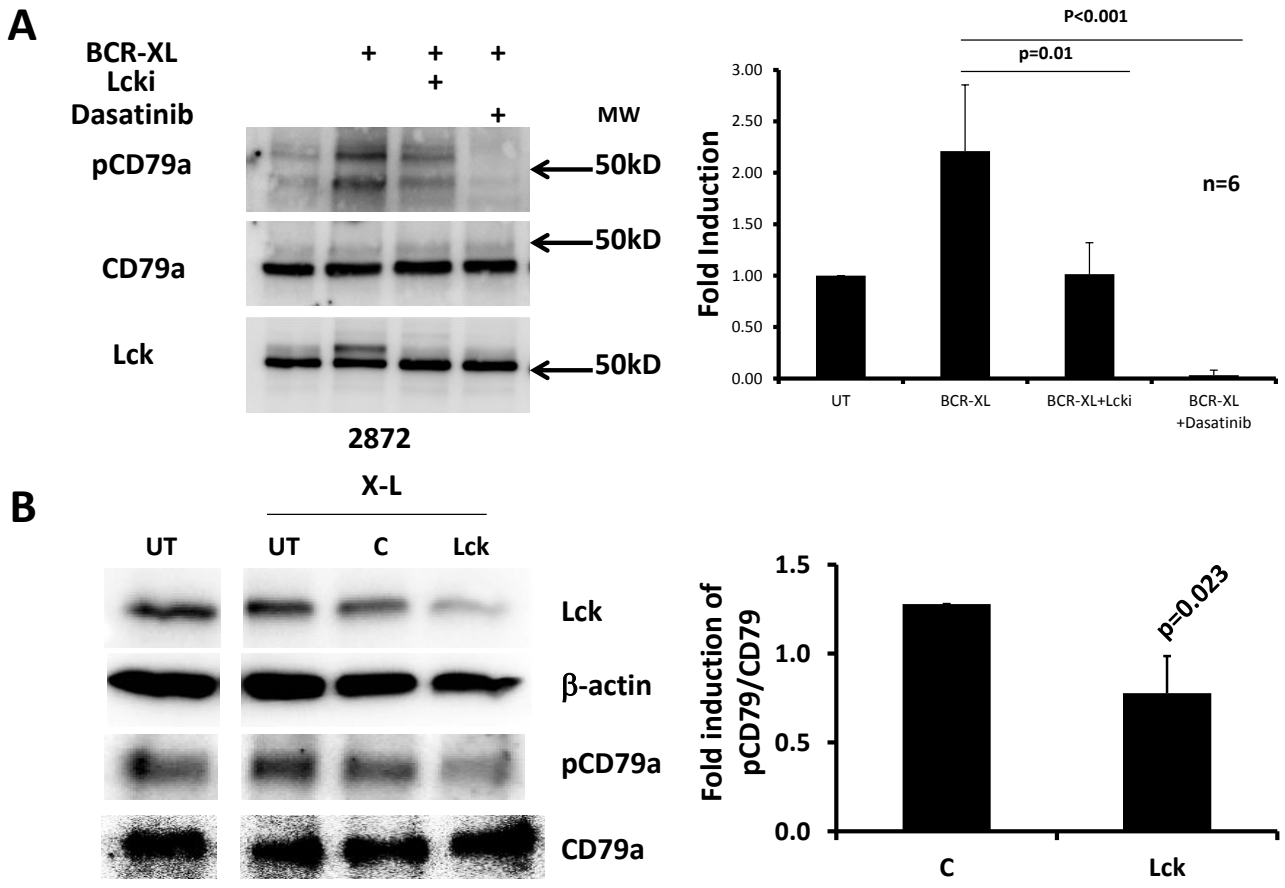


Figure 4.9. BCR crosslinking on CLL cells induces Lck activation and phosphorylation of CD79a. **A.** CLL cells were treated with Lck-i (1μM) or dasatinib (150 nM) for 2h prior to BCR crosslinking (20μg/ml $F(ab')_2$ goat anti-human IgM for 15 mins). Whole CLL cell lysates probed by Western blot analysis for pCD79a, CD79a and Lck. The right hand panel shows a graphical representation of n=6 experiments using different cases of CLL. **B.** Lck expression in CLL cells was reduced by treating cells with specific siRNA according to the method described in chapter 2. Whole CLL cell lysates were then analysed by Western blot analysis for the indicated antibodies. The right hand panel shows a graphical representation of n=3 experiments using different cases of CLL. Statistical analysis for parts A and B was performed using a student's t-test for paired data.

4.2.8. Lck mediates phosphorylation of Syk but not ZAP70 in BCR-stimulated CLL cells

In T cells the main target of active Lck following ligation of the T cell antigen receptor is Y^{319} in $ZAP70^{348}$. CLL cells from a subset of patients also express ZAP70, and a high level of expression of this tyrosine kinase has been reported to be associated with enhanced BCR signalling[111] and poor disease prognosis[349]. In order to examine whether ZAP70 in CLL cells is a target for Lck we used an anti-pY^{319}-ZAP70 antibody. However, this antibody is known to crossreact with an analogous site (pY^{352}) in Syk, so it was important to distinguish between ZAP70 and Syk phosphorylation as the target of Lck in CLL cells. We first immunoprecipitated these proteins from resting and BCR-stimulated CLL cells and then probed them for reactivity with the anti-pY^{319}-ZAP70 antibody in Western blots. We found that anti-pY^{319}-ZAP70 was only reactive with Syk immunoprecipitated from BCR-stimulated CLL cells and not with immunoprecipitated ZAP70 (Figure 4.10). Treatment of CLL cells with Lck-i, or reduction of Lck expression with siRNA, reduced BCR-induced phosphorylation of Syk (Figure 4.10). Taken together, these results demonstrate that Lck preferentially mediates the phosphorylation/activation of Syk in CLL cells responding to BCR crosslinking.

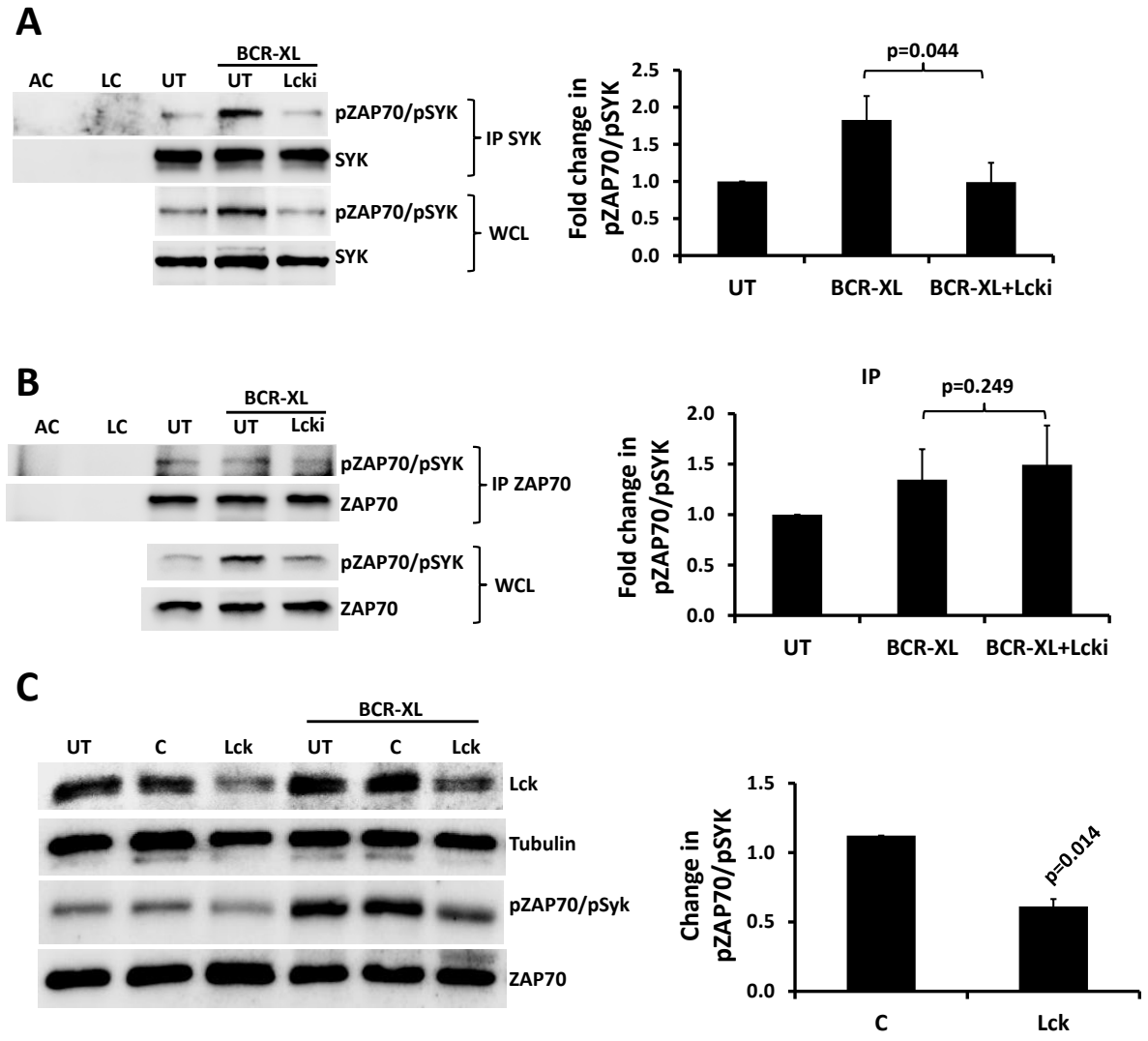


Figure 4.10: Lck mediates Syk but not ZAP70 phosphorylation in BCR-stimulated CLL cells. CLL cells were treated with 1μM Lcki for 2h and then stimulated by BCR crosslinking (20μg/ml $F(ab')_2$ goat anti-human IgM for 15 mins). CLL cell lysates were then immunoprecipitated with **A**. anti-Syk or **B**. with anti-ZAP70 antibodies. The immunoprecipitates were then analysed by Western blot for the presence of pY^{352}-Syk/pY^{319}-ZAP70 using an antibody that is crossreactive to both epitopes (anti-pY^{319}-ZAP70 antibody). This experiment is representative of n=3 using cells from different donors. **C.** siRNA-knockdown of Lck expression inhibits BCR-induced Syk phosphorylation. CLL cells were treated with Lck-specific (Lck) and non-specific (C) siRNA, or were mock-transfected (UT). CLL cells were then stimulated by BCR crosslinking (BCR-XL). Induction of pY^{352}-Syk in CLL cell lysates was then assessed by Western blot analysis using the anti-pY^{352}-Syk/pY^{319}-ZAP70 antibody. The right-hand panels show graphical representation of induced phosphorylation of immunoprecipitated Syk, ZAP70. The graph illustrates mean±SD of n=3 experiments using cells from different donors

4.3. Discussion

Lyn has always been thought to be responsible for mediating proximal and distal signalling events following BCR ligation in CLL cells[212]. However, in this chapter we present data that strongly implicate a role for Lck in this process. We showed that active Lck mediated proximal BCR signalling by phosphorylating ITAMs within CD79a, as well as downstream BCR signalling events involving phosphorylation of Syk leading to activation of NFκB, MAPK/ERK and PI3K/Akt signalling pathways, and eventually enhanced CLL cell survival.

In the previous chapter our data implicated a role for c-Abl in BCR-induced NFκB activation since inhibition of this kinase using imatinib resulted in a partial reduction of BCR-induced phosphorylation of IKK. The response to imatinib varied among the CLL cases, as did the intensity of BCR signalling. However, c-Abl levels did not seem to correlate with the intensity of BCR signals nor to the ability of imatinib to inhibit these signals. This suggested that the effect of imatinib could be due to a non-specific inhibition of other kinases that play a role in BCR signalling to the NFκB pathway. The work described in this chapter centres on a possible role for Lck because of the known ability of imatinib to inhibit this SFK[327]. Thus, our data show that imatinib can partially inhibit mpV(pic)-induced activity of Lck in CLL cells, and is consistent with a study performed in T cells that showed Lck is a target of imatinib during stimulation of the TCR[330].

Previous to this thesis, Lck was reported to be expressed in CLL cells[191 329]. However, the functional significance of this expression was still unclear. The data from this Chapter show that Lck is an important mediator of BCR signalling in CLL cells, and is demonstrated using two approaches; a specific Lck inhibitor Lck-i, and siRNA to reduce Lck expression.

To address the role of Lck in BCR signalling in CLL cells we first used Lck-i. Our data show that Lck-i inhibits mpV(pic)-induced activation of Lck in CLL cells when used at 1μM.

Experiments examining concentration-response with this compound showed that the maximal inhibition BCR-induced phosphorylation of IKK was reached using 1μM of Lck-i. This concentration was also effective at reducing BCR-induced activation of the MAPK/ERK and PI3K/Akt signalling pathways. The ability of Lck-i to inhibit BCR-induced signalling was not due to off-target inhibition of other SFKs because incubation of CLL cells with this compound did not affect other SFKs that are known to be expressed in CLL such as c-Abl, Src, Fgr, Fyn and, in particular, Lyn which is known to be constitutively active in CLL cells[174]. This confirms the specificity of Lck-i that has been reported in other studies[335-337], and further suggests that the effect of Lck-i on BCR signalling is only due to its inhibitory effect on Lck.

The second approach we used to demonstrate the role of Lck in mediating BCR signalling in CLL cells involved using siRNA to reduce Lck expression in these cells. We found that siRNA treatment of CLL cells resulted in partial reduction in Lck expression, and that this had the effect of reducing BCR-induced activation of IKK (60% inhibition), ERK (30% inhibition) and Akt (15% inhibition). Previous work from our Department and in other studies has shown that B cells from different CLL patients express variable levels of Lck[191 196 347], our experiments showed that this variation correlates with BCR signalling intensity. Thus, our data showed that levels of induced pIKK, pERK and pAkt are higher in BCR-stimulated CLL cells expressing high levels of Lck than in BCR-stimulated CLL cells expressing low levels of Lck. Taken together with the experiments using siRNA to reduce Lck expression, these observations suggest a direct relationship between the level of Lck expression and the intensity of the BCR signalling response in CLL cells. Moreover, these data provide compelling evidence to support a role for Lck in BCR signalling in CLL cells.

A role for Lck in antigen receptor signalling in normal B cells is controversial. One study has suggested that Lck can promote BCR signalling in B1a cells [331], whereas a second has

suggested an inhibitory role of this SFK[192] and a third has reported that Lck does not play any role in BCR signalling in B1 cells[194]. Our data support a role for Lck in the promotion of BCR signalling, and is the first study to demonstrate this in CLL cells. Previous reports have suggested that Lck expression may be functionally coupled with CD5[191] and that it protects CLL cells against glucocorticoids[195]. One report suggested that Lck has no direct cytoprotective role in CLL cells by showing that its knock down does not induce apoptosis[196]. However this does not contradict our findings on the role of Lck because this study was done in unstimulated CLL cells, it is within the context of BCR signalling that Lck has a role in CLL pathophysiology.

The current model of BCR-induced CD79 phosphorylation in CLL cells suggests that following BCR ligation Lyn enters lipid rafts where it phosphorylates CD79[96]. Our results contrast this model and show that the presence of Lck-i reduces induction of CD79 phosphorylation by BCR crosslinking on CLL cells without affecting the activity of Lyn. This finding is complimented by experiments using siRNA to knockdown Lck expression. These experiments show that such knockdown inhibits BCR-induced CD79 phosphorylation, and strongly suggest that Lck mediates this proximal signalling event in CLL cells. However, our experiments do not rule out a role for Lyn in CD79 phosphorylation. Use of the pan-SFK inhibitor dasatinib shows that the presence of this compound in CLL cell cultures reduces CD79 phosphorylation to below the baseline levels we observed in resting CLL cells. Thus, whereas Lck may be responsible for the induction of CD79 phosphorylation following BCR crosslinking, Lyn, or another SFK, may be responsible for basal phosphorylation of this protein. Such SFK-mediated basal phosphorylation of CD79 may be pathophysiologically important in CLL because pan-SFK inhibitors are cytotoxic to the malignant cells of this disease[174]. Constitutively active Lyn may also generate signals which downregulate BCR signalling. Lyn is able to phosphorylate ITIMs in proteins such as CD5[219], and thereby attract

phosphatases such as SHP1, SHIP1 and PTPN22 to the cell membrane where they downregulate Lyn-mediated positive signalling events[219 224 350-351]. Moreover, monophosphorylation of the ITAM within CD79a, possibly mediated by Lyn, has recently been proposed to play a role in the maintenance of B cell anergy through activation of an inhibitory signalling circuit consisting of SHIP-1 and Dok-1[352]. The net effect is a balance between positive and negative signalling. The results presented in this chapter suggest that Lck acts to tip this balance in favour of positive signals when CLL cells are stimulated through the BCR.

Following TCR stimulation, the main target of active Lck is Y^{319} in ZAP70[348]. Phosphorylation of ZAP70 at this tyrosine residue acts to enhance the catalytic function of ZAP70, as well as play a scaffolding role within ZAP70 by providing a binding site for Lck and other proteins such as PI3K, Grb2 and CrkII[353-354]. The malignant cells from a subset of CLL patients also express ZAP70[144] where it functions to enhance BCR signalling[147 355]. Thus, ZAP70 may be a target of Lck in CLL cells that express this protein. However, using a phospho-ZAP70 antibody (anti-pY^{319}-ZAP70) that also crossreacts with phospho-Syk at an analogous site (pY^{352}) we found that although Lck is involved in the induction of Syk phosphorylation following BCR crosslinking of CLL cells, ZAP70 remains unphosphorylated. This result agrees with a published report showing that BCR crosslinking induces Syk but not ZAP70 phosphorylation in CLL cells[355]. That Lck is directly able to phosphorylate Syk on Y^{352} in BCR-stimulated CLL cells is not demonstrated by our data. Although there is potential for Lck to target Syk based on the high homology of amino acid sequences flanking Y^{352} and Y^{319} in Syk and ZAP70, other SFKs could also be involved. It has been suggested that Lck is able to act as a downstream amplifier of Syk in antigen receptor-stimulated T cells by binding $pY^{525/526}$ residues via its SH2 domain[356-357], and, in this way, holds Syk in a conformation that allows Lyn or another SFK to catalyse the

phosphorylation of $Y^{352\ 357}$. Such a mechanism may occur in CLL cells because recent work has shown that phosphorylation of $Y^{525/526}$ in Syk enhances downstream signalling[358]. Alternatively, inhibition of Lck-mediated CD79 phosphorylation in BCR-stimulated CLL cells may inhibit Syk activation and Y^{352} phosphorylation, thereby accounting for the reduction we observe. Whatever the mechanism of Lck-mediated Syk activation may be, our results suggest that the role of ZAP70 in enhancing BCR signalling in CLL cells is independent of the functional relationship it plays with Lck in T cells. This independence is supported by our observation that there is no correlation between Lck and ZAP70 expression in CLL cells[196].

In this chapter we show that Lck expression varies in the malignant cells from different patients, an observation that others have also made[191 195-196 347]. We also show that this may be pathophysiologically important because not only does Lck inhibition block the pro-survival effects of BCR crosslinking in CLL cells, but the expression levels of this SFK contribute to the strength of pro-survival signals generated by this stimulus. This finding implies that Lck expression may be prognostically important and high Lck expression may be an indicator of aggressive disease given the established role of BCR signalling to disease pathogenesis in CLL[25 359].

In conclusion, this chapter shows that Lck plays an important role in mediating BCR signalling in CLL cells. This SFK participates in proximal phosphorylation of ITAM motifs in CD79 and induces distal activation of Syk, ERK, NFκB and Akt signalling as well as increased CLL cell survival. This suggests a major role for Lck in CLL pathogenesis and changes the paradigm on the importance of Lyn in this process. As a therapeutic approach Lck inhibition may be an attractive option because of the importance of BCR signalling to the pathophysiology of CLL cells, and because small molecule compounds that target Lck are being developed to inhibit antigen receptor-mediated T cell activation as means to control

organ rejection as well as autoimmune diseases[360]. Since BCR-generated signals are key contributors to CLL cell survival and disease pathogenesis, Lck may be a good therapeutic target for the treatment of this disease.

Chapter 5: Lck expression and disease prognosis in CLL: examination of a potential role Lck plays in modulating BCR signalling in CLL cells

5.1. Introduction

Duration and intensity of BCR signals are controlled by negative regulators that are attracted to the signalling complex by proteins such as CD5, CD22 and FcγRIIB. Attraction of these negative regulators is mediated by Lyn which phosphorylates the immunoreceptor tyrosine-based inhibition motifs (ITIMs) within CD5[211 219], CD22 and FcγRIIB[208-209]. Phosphorylated ITIMs in turn recruit phosphatases such as SHP1, SHIP-1 and -2 and protein tyrosine phosphatase non-receptor type 22 (PTPN22), all of which can downregulate BCR signalling[168 212].

In the previous chapter we showed that Lck is a key mediator of BCR signalling in CLL, and that the levels of this kinase determine the intensity of BCR signals. Therefore, it seems reasonable to hypothesize that Lck expression may be linked to disease prognosis. The aim of this chapter was to investigate this possibility. We found, however, that high expression levels of Lck in CLL cells were associated with good disease prognosis. Thus, a secondary aim of this chapter is to more fully investigate the role of Lck in CLL cells. Others have reported that Lck downregulates antigen receptor signalling in T cells and B1 cells by phosphorylating CD5[192 220]. Considering the fact that CLL cells express CD5[361], CD22[245] and FCγRIIB[262-264], which are all potential targets of Lck, we investigated whether Lck played a role in the phosphorylation of these proteins in CLL cells.

5.2. Results

5.2.1. High Lck levels in CLL cells are associated with good disease prognosis

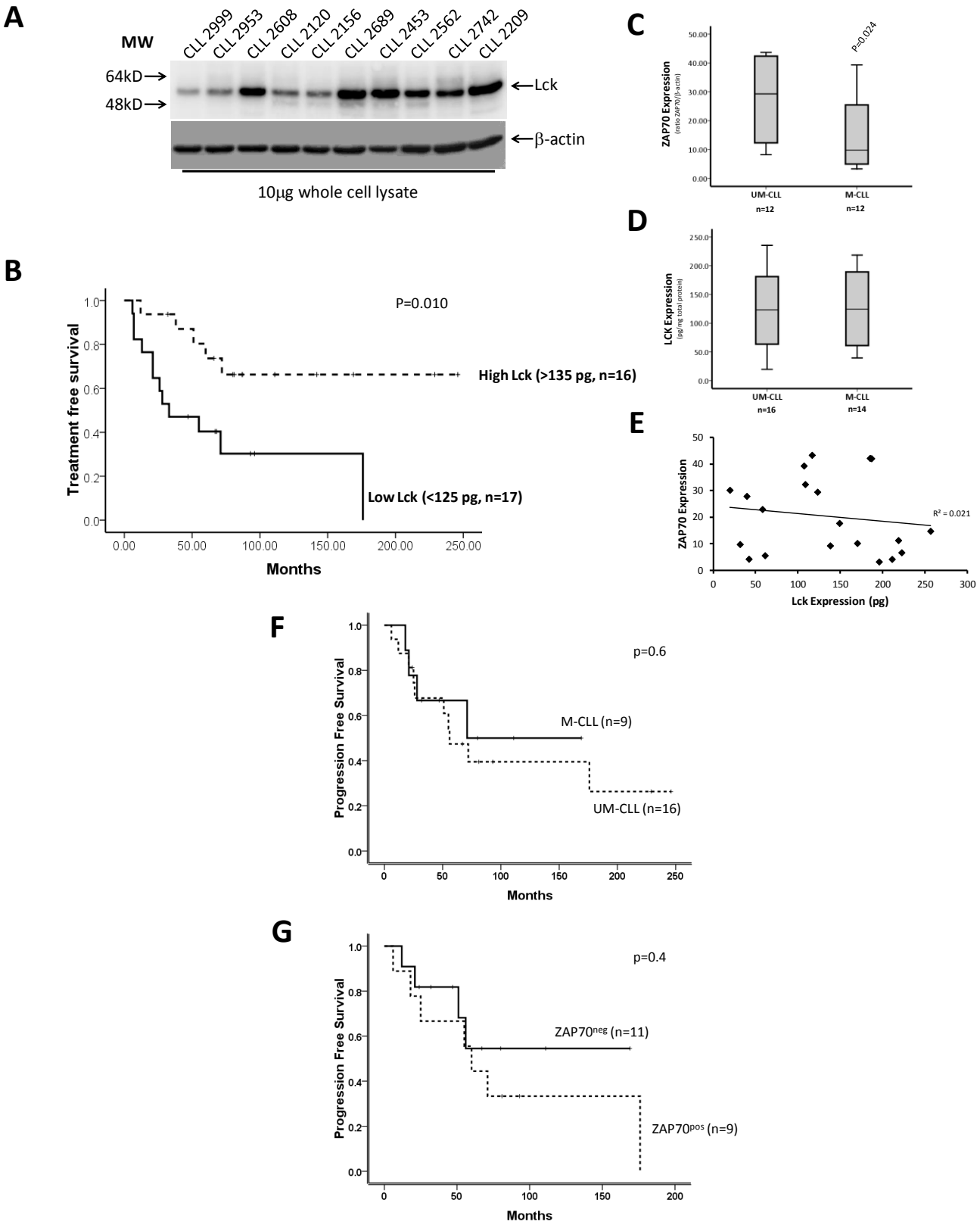
Previous work from both this[362] and other Departments[191 196 347] has shown that Lck is variably expressed in the malignant cells of CLL (Figure 5.1). In a cohort of CLL cases where Lck expression had been determined, we investigated the relationship between Lck expression and progression-free survival (Table 5.1). Lck expression in purified CLL cells was determined using a Western blot technique whereby recombinant Lck was used as a standard[362]. Using this technique we were able to assign high and low Lck expression based on the mean value of Lck expression (129pg) in the entire cohort. Thus, patients with levels of Lck greater than 135pg were assigned to the "High Lck" group, whereas patients with levels of Lck less than 125pg were assigned to the "Low Lck" group. A comparison of progression-free survival between these groups showed that patients with high Lck levels had better prognosis than those with low Lck levels (Figure 5.1).

Measuring Lck expression in purified CLL cells using the above technique was time and resource consumptive. Therefore, we developed a method of determining Lck expression using a flow cytometric approach whereby CLL cells were identified with CD20 and CD5 antibodies followed by intracellular staining for Lck expression. Figure 5.2A shows that this approach was valid for determining Lck expression in CLL cells. This approach also revealed that Lck was expressed at different levels within the cohort of patients analysed (Figure 5.2B). The cohort of patient samples we used in this analysis was different to the one above, and only overall survival data was available. We initially divided between high and low expression of Lck in CLL cells using the mean/median values from our analysis. This analysis showed that CLL cases that highly expressed Lck tended to have better overall

survival than did CLL cases that expressed low levels of Lck. This result did not show statistical significance, however, it did suggest that comparison of extremes of the distribution may yield results that were more interpretable. Thus, we divided the CLL cases into 3 subgroups; cases expressing low levels of Lck (CLL cases with less than 20% Lck positive cells), intermediate levels of Lck (CLL cases with greater that 20% but less than 50% Lck positive cells) and high levels of Lck (CLL cases with greater than 50% Lck positive cells). Figure 5.2C shows that CLL cases bearing high levels of Lck had a significantly better prognosis than CLL cases bearing either intermediate or low levels of Lck.

To show that the patient samples used in cohorts 1 and 2 are representative of "normal" CLL, we examined the relationship between IgHV mutation and either progression-free survival (cohort 1, Figure 5.1F) or overall survival (cohort 2, Figure 5.2F). In each case, there was a trend for UM-CLL cases to have a poorer disease outcome than did M-CLL cases; in cohort 1 the trend was not significant whereas in cohort 2 the p value was 0.016. Analysis of ZAP70 expression in both cohorts showed that higher levels of ZAP70 were associated with UM-CLL cells compared to M-CLL cells [Figures 5.1C (cohort 1) and 5.2E (cohort 2)]. Moreover, in both cohorts there was a trend for CLL cases having high expression of ZAP70 to have poor disease outcome [Figures 5.1G (cohort 1) and 5.2G (cohort 2)] although in both cohorts the p value did not reach significance. These data suggest that both cohorts of patients are representative of CLL and display expected values for disease outcome with respect to known biomarkers[34 97]. Interestingly, there was no correlation between IgHV mutation and Lck expression [Figures 5.1D (cohort 1) and 5.2D (cohort 2)], or of ZAP70 and Lck expression (Figure 5.1E). A multivariate analysis would be required to demonstrate the independence of Lck as prognostic biomarker. A larger cohort is needed to establish statistical significance. Nevertheless, the data suggest that Lck expression levels may be prognostically independent of these other biomarkers.

Taken together, these data show that high Lck expression is associated with good disease prognosis in CLL. Considering that the previous chapter showed that Lck was involved in mediating BCR signalling in CLL cells, we wondered whether Lck may have a dual role whereby high levels of expression may set a threshold for the induction of BCR signalling. We therefore next examined a possible role of Lck in downregulating BCR signalling, particularly with respect to potential interaction with CD5, CD22 and FcγRIIB.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MW
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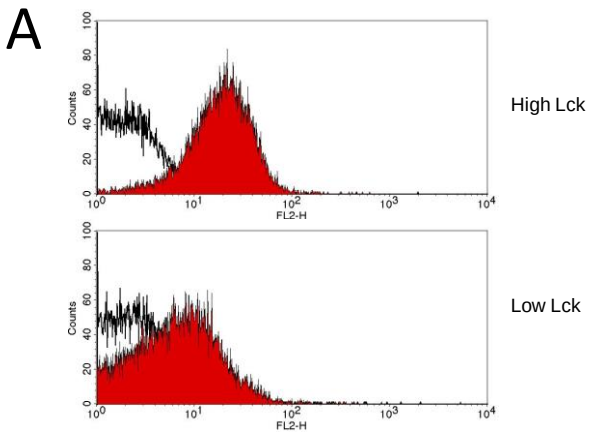
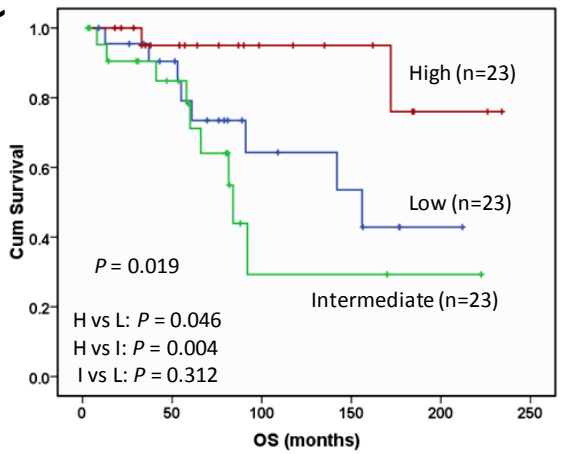
CLL 2953
CLL 2608
CLL 2120
CLL 2156
CLL 2689
CLL 2453
CLL 2562
CLL 2742
CLL 2209
64kD
48kD
Lck
β-actin
10μg whole cell lysate
B
P=0.010
High Lck (>135 pg, n=16)
Low Lck (<125 pg, n=17)
Treatment free survival
Months
C
P=0.024
ZAP70 Expression
UM-CLL
M-CLL
n=12
n=12
D
LCK Expression
UM-CLL
M-CLL
n=16
n=14
E
ZAP70 Expression
R² = 0.021
Lck Expression (pg)
F
p=0.6
M-CLL (n=9)
UM-CLL (n=16)
Progression Free Survival
Months
G
p=0.4
ZAP70neg (n=11)
ZAP70pos (n=9)
Progression Free Survival
Months

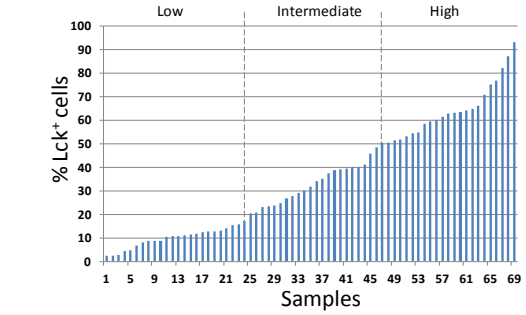
Figure 5.1: Comparison of treatment-free survival between high and low Lck expressing CLL cases (cohort 1). A.) Western blot analysis of Lck expression in lysates of purified CLL cells. 10μg of protein were loaded per lane. β-actin expression is used as a loading control. **B**.) Kaplan-meier plot of treatment-free survival of high and low Lck expressing CLL cases within this cohort. **C.**) Comparison of ZAP70 expression in UM- and M-CLL cases within this cohort using the data in Table 1. **D**.) Comparison of Lck expression in UM- and M-CLL cases within this cohort using the data in Table 1. **E**.) Regression analysis using the data in Table 1 of ZAP70 and Lck expression in the CLL cases within this cohort. **F**.) Kaplan-meier plot of treatment-free survival of UM- and M-CLL cases within this cohort. **G**.) Kaplan-meier plot of treatment-free survival of high and low ZAP70 expressing CLL cases within this cohort.

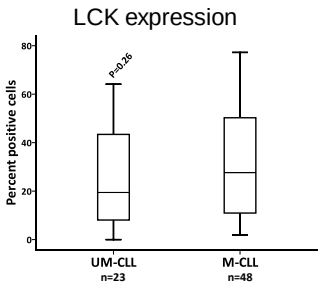
Table 5.1: Patient attributes of cohort 1. Lck expression in each CLL case is reported in pg of Lck / μg of total cellular protein. ZAP70 expression is expressed in arbitrary units as a band density ratio of ZAP70/β-actin derived from Western blots of whole CLL cell lysates. IgHV mutation is expressed as the per cent homology with germline sequence.

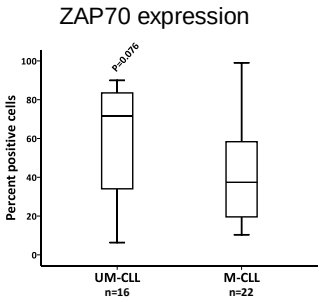
CLL case	Lck	ZAP70	IgHV%	Treated	Comments
CLL 1754	145.2		99.65	Untreated	
CLL 1801	107.0	39.3	96.84	Treated	
CLL 1851	108.5	32.4	93.9	Treated	
CLL 1958	101.4		100	Untreated	
CLL 2006	212.8	11.3	95.58	Untreated	
CLL 2045	57.7			Untreated	
CLL 2056	167.6			Untreated	
CLL 2063	116.5	43.4	100	Treated	
CLL 2120	58.0	23.0	94.63	Untreated	
CLL 2157	110.7		88.89	Treated	
CLL 2209	235.5	14.8	98.25	Untreated	
CLL 2230	181.6	42.2	100	Untreated	
CLL 2237	170.0	10.2	91.41	Untreated	
CLL 2246	186.9		98.61	Untreated	
CLL 2255	141.8	29.1	100	Treated	
CLL 2354	185.0		95.44	Untreated	
CLL 2371	61.0	5.6	91.07	Untreated	
CLL 2399	31.5	9.8	100	Untreated	
CLL 2435	low			Treated	Lck level comparable to CLL 2656
CLL 2453	186.8	8.2	100	Untreated	
CLL 2562	123.0	29.5	100	Untreated	
CLL 2581	218.5	6.7	93.39	Untreated	
CLL 2608	138.0	9.3	91.06	Untreated	
CLL 2656	19.5	30.2	99.65	Untreated	
CLL 2679	127.4			Untreated	
CLL 2683	80.9			Treated	
CLL 2689	185.0	42.1		Treated	
CLL 2701	222.4			Untreated	
CLL 2711	42.0	4.3	87.72	Untreated	
CLL 2724	74.8		98.91	Treated	
CLL 2739	65.0			Treated	
CLL 2747	123.1	42.5	100	Untreated	
CLL 2749	211.0			No	
CLL 2764	52.0	43.7	100	Treated	
CLL 2783	189.1	3.3	96.94	Treated	
CLL 2872	256	26.97		Untreated	
CLL 2950	198.5	4.2	94.79	Untreated	
CLL 2953	49.0	17.8	100	Untreated	
CLL 2954	75.2			Untreated	
CLL 2961	195.7			Untreated	
CLL 2999	39.5	27.9	96.36	Untreated	
CLL 3113				Untreated	Rebleed of CLL 2209

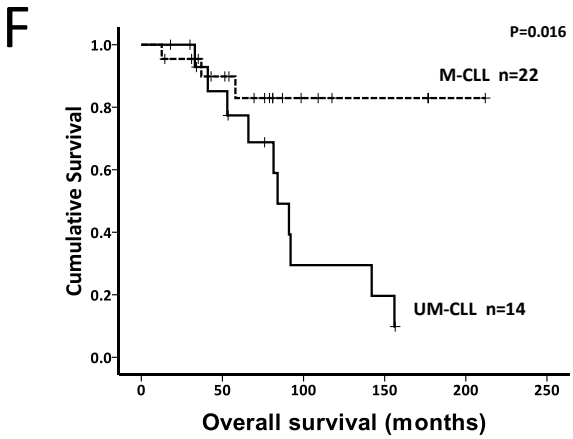

A
High Lck
Low Lck
Counts
FL2-H


C
High (n=23)
Low (n=23)
Intermediate (n=23)
Cum Survival
P = 0.019
H vs L: P = 0.046
H vs I: P = 0.004
I vs L: P = 0.312
OS (months)


B
Low
Intermediate
High
% Lck+ cells
Samples


D
LCK expression
Percent positive cells
P=0.26
UM-CLL n=23
M-CLL n=48


E
ZAP70 expression
Percent positive cells
P=0.076
UM-CLL n=16
M-CLL n=22


F
P=0.016
M-CLL n=22
UM-CLL n=14
Cumulative Survival
Overall survival (months)

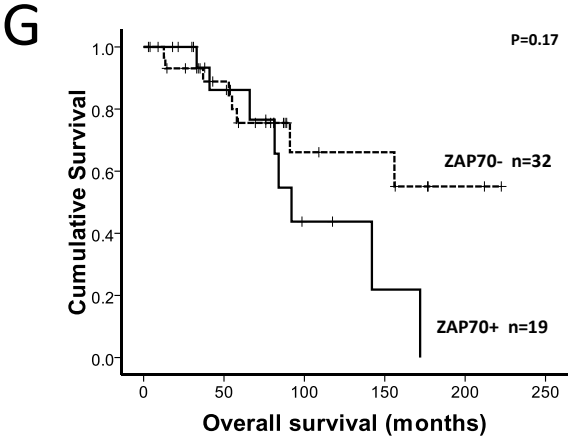

G
P=0.17
ZAP70- n=32
ZAP70+ n=19
Cumulative Survival
Overall survival (months)

Figure 5.2: Comparison of overall survival between high and low Lck expressing CLL cases (cohort 2). A.) Flow cytometry analysis of Lck expression in CLL cells. (*upper panel*) Lck expression in a CLL case that is known to have high Lck levels. (*lower panel*) Lck expression in a CLL case that is known to have low Lck levels. **B**.) Comparison of Lck expression in the malignant cells from the CLL cases used in this cohort. **C.**) Kaplan-meier plot of overall survival between high, intermediate and low Lck expressing CLL cases within this cohort. **D**.) Comparison of Lck expression in UM- and M-CLL cases within this cohort using the data in Table 2. **E**.) Comparison of ZAP70 expression in UM- and M-CLL cases within this cohort using the data in Table 2. **F**.) Kaplan-meier plot of overall survival of UM- and M-CLL cases within this cohort. **G**.) Kaplan-meier plot of overall survival of high and low ZAP70, based on the median value, expressing CLL cases within this cohort.

Table 5.2: Patient attributes of cohort 2. Lck expression and ZAP70 expression in each CLL case is reported as per cent positive cells. IgHV mutation is expressed as the per cent homology with germline sequence, CD38is expressed as per cent CD38 positive cells.

Samples	%Lck+	ZAP70	%IgHV	CD38	samples	%Lck+	ZAP70	%IgHV	CD38
#2082	0		100	76	**#2451**	36.79	87.51		24
#2045	0	21.5	98.65	40	**#2037**	37.01		92.52	22
#2204	1.9			14	**#2412**	37.93	47.07		75
#2435	2.12	30.88	100	88	**#2062**	39.15		100	
#2224	3.78	16.13	94.58	low	**#2209**	42.91		98.25	29
#2291	4.41	10.4	97.57	28	**#2116**	43.9	76.91	100	
#1975	4.8	44.29		3	**#2381**	44.85	82.12		0
#2218	5.14		100	8	**#2329**	44.92		100	85
#2025	6.21	99.07	91.47	12	**#2163**	47.05	75.82	99.31	88
#2205	6.85		100	18	**#2076**	49.4	58.34	97.36	39
#1939	7.57	60.92	100	13	**#1906**	49.61		92.86	
#2173	7.92	23.99	96.14	19	**#2177**	50.93		92.01	
#2216	8.34		85.99	3	**#2237**	50.93	28.26	91.41	
#2246	8.35	19.58	93.88		**#2040**	53.07	42.16	95.58	
#2424	8.59	6.34	100	96	**#2485**	53.77	19.27	91.93	3
#2010	9.33	37.23	100	19	**#1913**	57.16		94.67	35
#2444	10.13	28.98		95	**#1999**	57.41		94.63	
#2436	10.34	30.76	91.67	0	**#2143**	57.49	89.57	100	7
#1954	10.6	13.56			**#2035**	58.73		95.53	6
#2098	10.83	14.83		14	**#1972**	59.67	82.95	100	14
#1964	11.08	46.98	90.78	3	**#1908**	61.64		97.94	19
#2085	11.2	19.23	100	14	**#1936**	62.67	44.62	94.9	8
#1963	11.85	13.55	92.93	19	**#2206**	64.1		99.33	47
#1952	12.38	15.65	91.1	33	**#1895**	69.57		96.62	43
#2096	12.64	35.9	93.75	0	**#1983**	72.3	85.1	97.54	3
#2123	15.01	58.6			**#2149**	77.23	96.43		1
#2067	15.1	68.94	100	57					
#1996	18.28	31.08	91.5	5					
#1998	18.28	38.9	89.53	28					
#2233	18.45	89.95	100	98					
#2081	19.09	29.29		3					
#1937	19.44	74.28	99.66	3					
#1965	20.45	63.26	99.66	54					
#2073	20.61	70.03	93.2	9					
#2256	22.94	84.05	100	27					
#2050	24.27	56.54		5					
#2212	25.11	86.83	100	53					
#2213	26.26			72					
#1901	26.68	46.38	95.24	1					
#2429	28.62	79.42	94.65	1					
#2506	29.87	40.91		3					
#1932	31.09		94.02	low					
#2080	32.82		91.07	3					
#2387	35.67	35.31		35					
#2343	35.77	86.02	93.88	4					

5.2.2. Lck inhibition does not affect BCR-induced CD5 phosphorylation

To investigate why high Lck expression in CLL cells is correlated with good disease we reasoned that Lck may function to activate tyrosine phosphatases that act to downregulate BCR signals. One logical candidate is CD5, a surface marker of CLL cells[349]. CD5 is expressed on T cells and B1a cells where it is thought to modulate TCR[220] and BCR activation and differentiation signals[192 214 216]. In T cells Lck has been reported to play a role in CD5 phosphorylation on Y^{429} and Y^{463}, sites that are functionally important because they act as a scaffold for phosphatases such as SHP1 and SHIP[216].

I first investigated the kinetics of BCR-induced CD5 phosphorylation in CLL cells in order to determine the time point at which maximal phosphorylation of CD5 was achieved. Thus, BCR crosslinking on CLL cells induced a rapid increase in tyrosine phosphorylation of CD5 from basal levels that was apparent immediately following the addition of antibody (Figure 5.3). We found that tyrosine phosphorylation of CD5 very quickly decreased, and resumed basal levels following 10 min incubation with the crosslinking antibody (Figure 5.3B). We next tested whether Lck phosphorylated CD5 in BCR-stimulated CLL cells. To do this we first treated CLL cells with 1μM Lck-i or 150nM dasatinib for 2 hours prior to BCR crosslinking. Figure 5.4 shows that whereas the presence of dasatinib blocked the BCR-induced increase in CD5 tyrosine phosphorylation, the presence of Lck-i had no effect. These results indicate that Lck is not likely to play a role in BCR-induced CD5 phosphorylation in CLL cells.

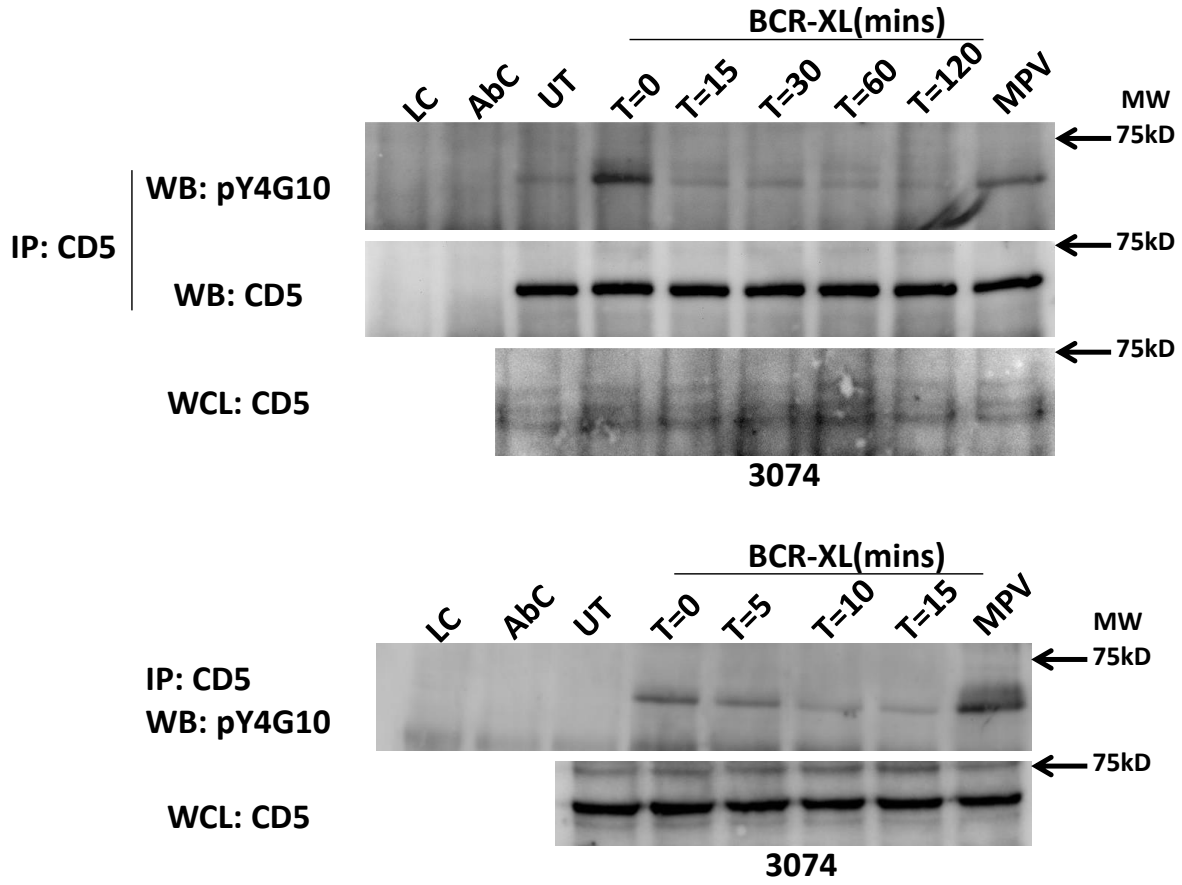


Figure 5.3: BCR-induced CD5 phosphorylation time points. $3x10^7$ CLL cells were incubated for the indicated time points with 20µg/ml $F(ab')_2$ goat anti-human IgM. CLL cell lysates were immunoprecipitated with anti-CD5 antibody and then probed in Western blots with anti-pY 4G10 antibody. To stimulate CD5 phosphorylation, CLL cells were incubated with 100µM mpV(pic) for 30min (positive control).

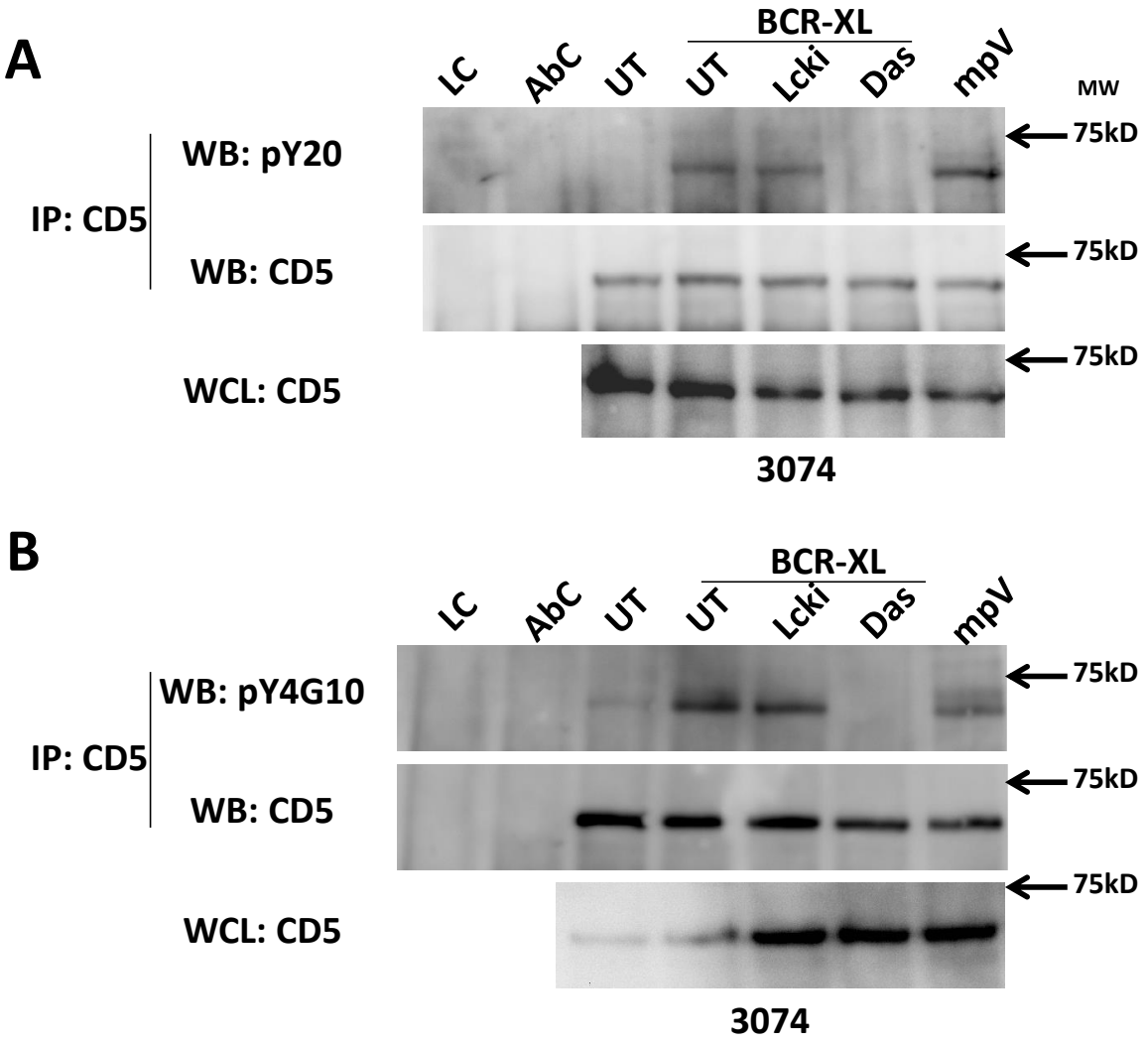


Figure 5.4: The role of Lck in BCR-induced CD5 phosphorylation. $3x10^7$ CLL cells were incubated for 2 hours with 1µM Lck-i or 150nM dasatinib prior to BCR stimulation with 20µg/ml $F(ab')_2$ goat anti-human IgM. CLL cell lysates were prepared immediately following BCR crosslinking, and then immunoprecipitated with anti-CD5 antibody. The immunoprecipitates where probed in Western blots with **(A)** anti-pY PY20 or **(B)** anti-pY 4G10 antibody. To stimulate CD5 phosphorylation, CLL cells were incubated with 100µM mpV(pic) for 30min (positive control).

5.2.3. CD5 transfection does not affect BCR signalling in the MEC1 cell line

To further investigate the role of Lck and CD5 in BCR signalling we switched our approach to use MEC1 cells. MEC1 is a CD5 negative CLL cell line that was derived from a patient undergoing prolymphocytic transformation[363]. Although these cells do not strictly mimic CLL cells, we found that they expressed Lck (Figure 5.5A) and decided that this may be useful for the analysis of BCR signalling. Analysis of pIKK, pERK, pAkt levels in MEC1 cells showed that they are constitutively high, and that BCR crosslinking only induced a small increase in pIKK levels but largely did not affect either pAkt or pERK (Figure 5.5B and C). Importantly, Figures 5.5B and C also show that these constitutive signals appear to be Lck dependent because the presence of Lck-i reduced the levels of pIKK, pAkt and pERK in resting and BCR-stimulated cells. It is reported that CD5 functions in CLL cells to reduce BCR signalling[224], therefore, the lack of CD5 expression in MEC1 cells may drive constitutive activation of the ERK, Akt and IKK signalling pathways. We investigated this further by transfecting MEC1 cells with CD5.

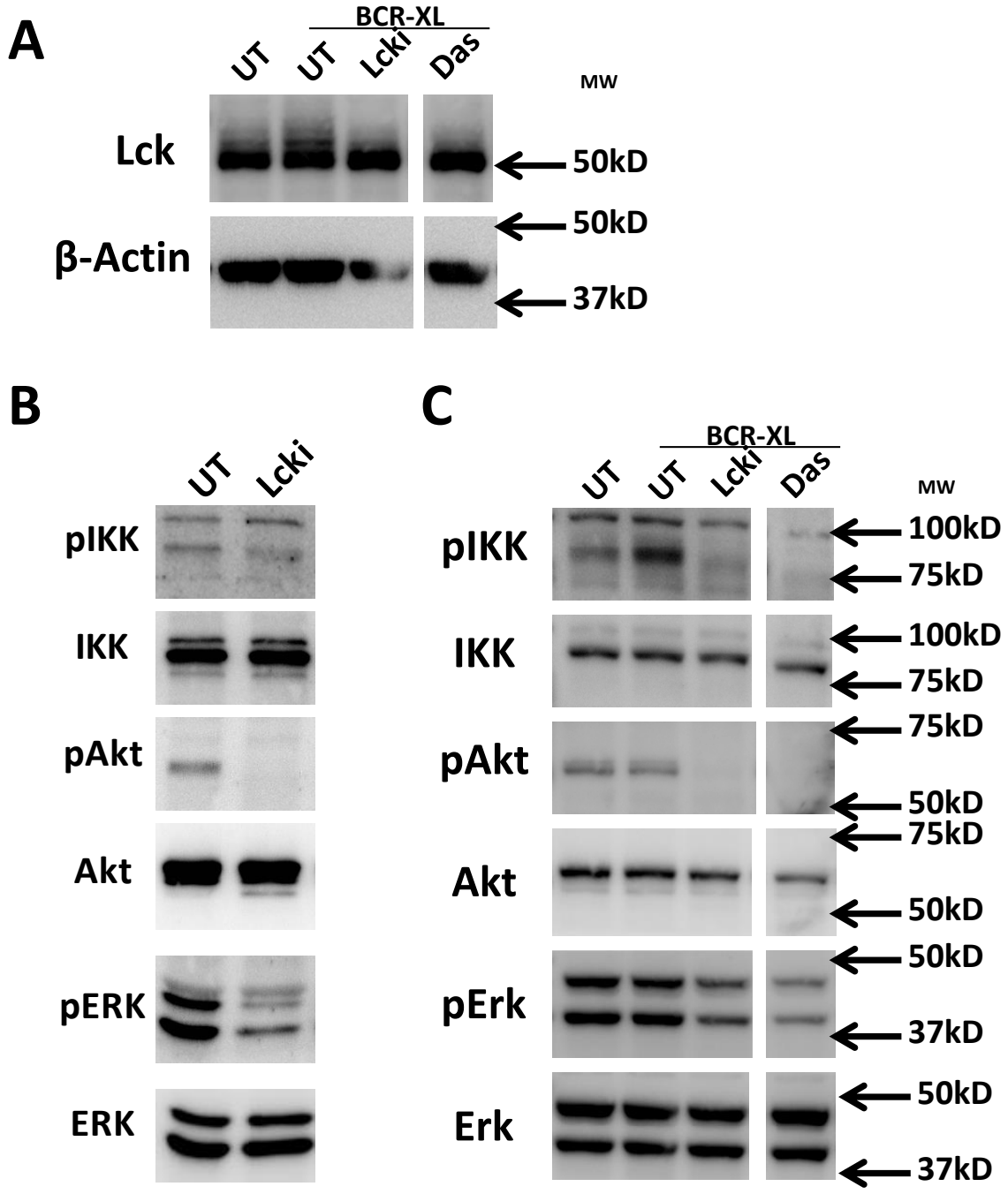


Figure 5.5: Lck expression and response to BCR crosslinking in MEC1 cells. (A) Lck expression in MEC1 cells**:** 10µg of total protein in MEC1 cell lysates was analysed for Lck expression by Western blotting using an anti-Lck antibody **(B)** and **(C)** BCR signalling in MEC1 cells**:** MEC1 cells were serum starved overnight and then treated with Lck-i (1µM) or dasatinib (150nM) for 2 hours. The BCR was stimulated by incubating the cells with 20µg/ml $F(ab')_2$ goat anti-human IgM for 15 min. Then the cells were harvested and lysed. The cell lysates were analysed by Western blotting and probed with the indicated antibodies.

Transient transfection of MEC1 cells with either full-length CD5 or a mutant of CD5 where the cytoplasmic tail was ablated showed that expression of either of these forms of CD5 had no effect on constitutive activation of ERK, Akt and IKK (Figure 5.6). Taken together with the experiments measuring BCR-stimulated CD5 phosphorylation in CLL cells, these experiments would suggest that induction of CD5 phosphorylation is likely not mediated by Lck, and that CD5 may not be involved in regulating BCR signalling in CLL cells.

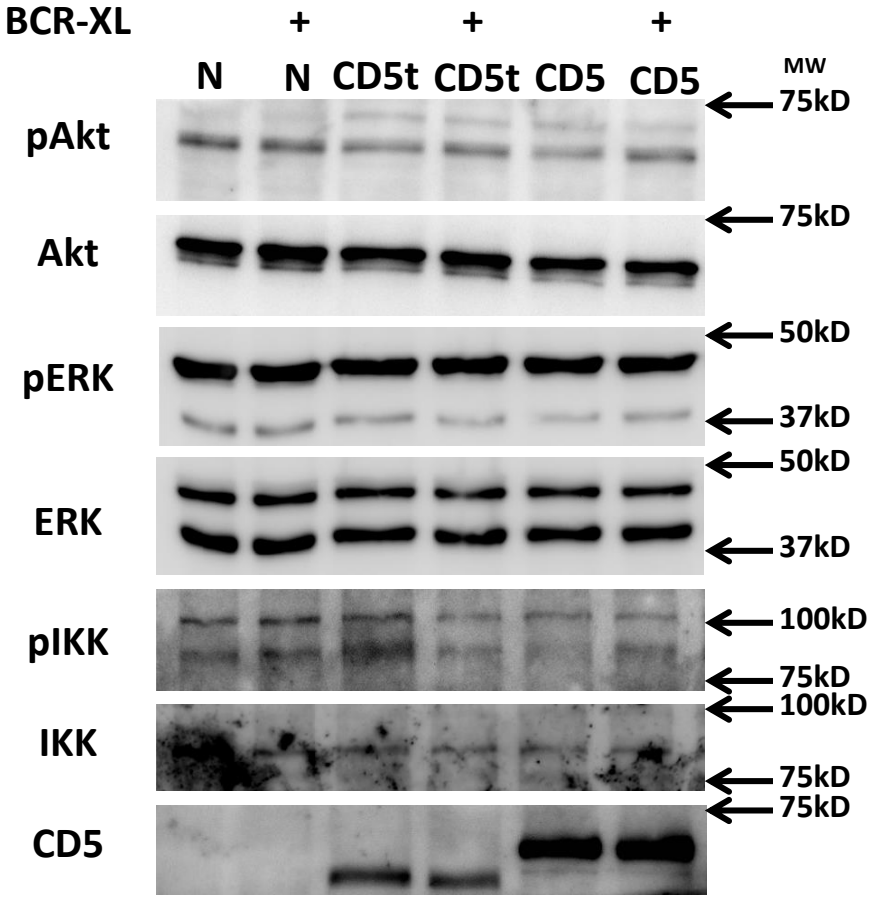


Figure 5.6: Effect of CD5 expression on BCR signalling in transient transfection assays of MEC1 cells. $2x10^6$ MEC1 cells were transfected with 2 µg of pmaxGFP® Vector (GFP control plasmid) (N), plasmid encoding intact CD5 protein (CD5), or with plasmid encoding truncated CD5 protein (CD5t). Following incubation overnight at 37°C, BCR was stimulated by incubating the cells with 20µg/ml $F(ab')_2$ goat anti-human IgM for 15 min. The cells were harvested and lysed, and the lysates were probed by Western blot with the indicated antibodies.

5.2.4. Lck inhibition does not affect phosphorylation of FcγRIIB

Another potential target of Lck that is also expressed by CLL cells[262-264] is FcγRIIB (also known as CD32). This protein contains an ITIM sequence within its cytoplasmic tail[102], which, in normal B cells, can be phosphorylated by Lyn when it is engaged by the Fc portion of IgG[102 166-168 208 253] . Src is also reported to phosphorylate Y^{292} within the ITIM of FcγRIIB expressed on endothelial cells, and therefore this site may also be a candidate target for Lck in CLL cells[364-365]. Figure 5.7A shows that CLL-cell FcγRIIB is constitutively phosphorylated on Y^{292}, and that BCR stimulation (using anti-human IgM $F(ab')_2$ fragments) does not induce any change in this level of phosphorylation. We then compared the effects of Lck-i and dasatinib on the phosphorylation of FcγRIIB in CLL cells. Figure 5.7B shows that the presence of Lck-i had no effect on FcγRIIB phosphorylation, whereas the presence of dasatinib completely inhibits this phosphorylation (Figure 5.7B). These results suggest that FcγRIIB phosphorylation in CLL cells is mediated by an SFK other than Lck.

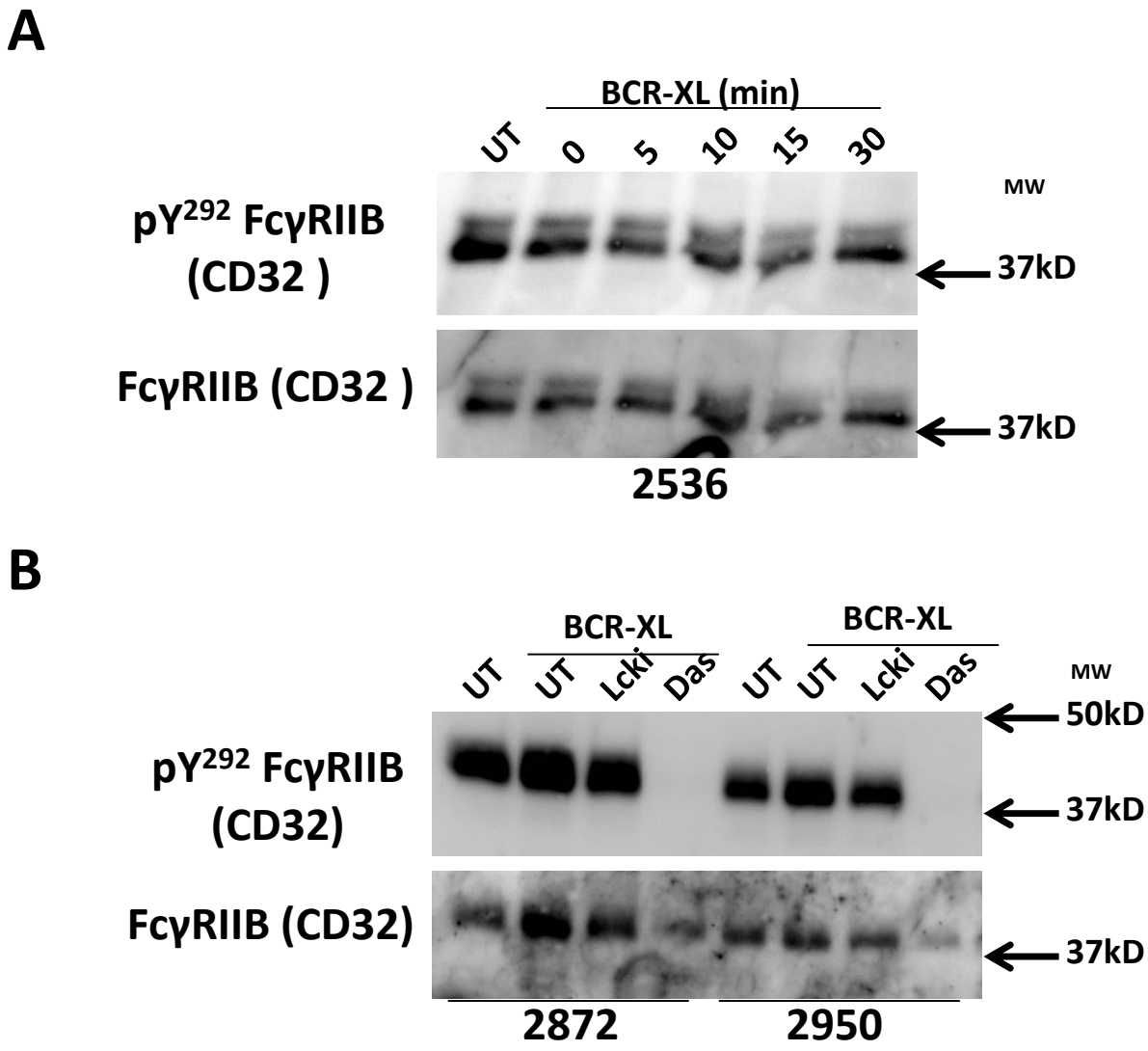


Figure 5.7: FcγRIIB is constitutively phosphorylated on CLL cells. **(A)** BCR-induced FcγRIIB phosphorylation time points**:** 1x10^7 CLL cells were incubated for the indicated time points with 20µg/ml F(ab')$_2$ goat anti-human IgM. CLL whole cell lysates were analysed by Western blotting using anti-pY^{292}-FcγRIIB antibody. **(B)**The role of Lck in BCR-stimulated phosphorylation of FcγRIIB**:** Western blot analysis of whole CLL cell lysates with anti-pY^{292} FcγRIIB. 1x10^7 CLL cells were incubated with Lck-i (1µM) or dasatinib (150 nM) for 2 hours, then the BCR was stimulated by adding 20µg/ml F(ab')$_2$ goat anti-human IgM for 15 minutes.

5.2.6. Lck inhibition reduces BCR-induced phosphorylation of CD22

CD22 is a B cell-specific protein that also contains ITIM motifs, and functions to downregulate BCR signalling. Studies of Lyn-deficient mice have shown that tyrosine phosphorylation of CD22 on B cells is mainly mediated by Lyn[208 234]. Once the ITIM in CD22 is phosphorylated, the phosphatases SHP-1and SHIP are recruited [235] where they act to terminate BCR signalling by dephosphorylating various components of the BCR signalling pathways[209 233-234 366]. It is reported that CLL cells from different patients express variable levels of CD22[245] and we observed a similar result in the cohort of patient samples we used (Table 5.3 and Figure 5.8).

Table 5.3: CD22 expression on the CLL cells. CD22 expression in each CLL case is reported as mean florescence intensity measured by flow cytometer.

case #	MFI	case #	MFI
3074	40.32	**2806**	189.66
2683	45.35	**2722**	192.38
2739	52.37	**2736**	255.63
2950	59.95	**2435**	259.94
2918	78.15	**3095**	262.42
2948	82.24	**2728**	278.35
3068	88.06	**2911**	284.96
3287	90.81	**2536**	335.47
2990	101.8	**2961**	150.1
2861	102.73		
3113	103.2		
2932	103.62		
3286	105.89		
3184	107		
2656	115.91		
2872	130.61		

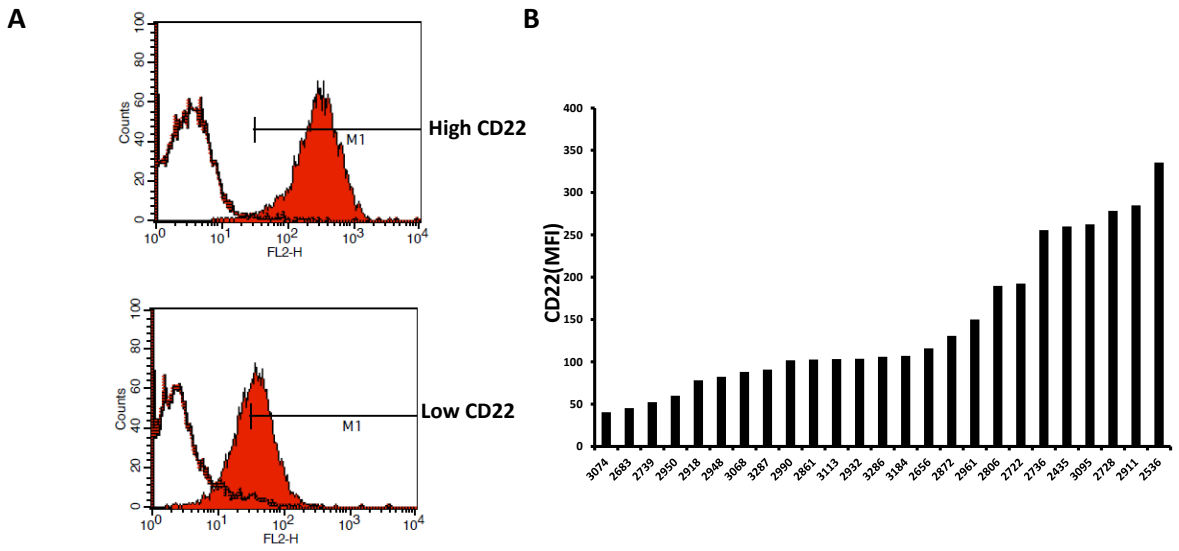


Figure 5.8. Analysis of CD22 expression in CLL cells. A) Flow cytometry analysis of CD22 expression on a CLL case having high expression levels of CD22 (*upper panel*) and low expression levels of CD22 (*lower panel*). M1 is an arbitrary setting defining a region that excludes>95% of the isotype control values. **B)** Comparison of CD22 expression on the CLL cells within this cohort using the data present in Table 5.3.

I next examined the effect of BCR crosslinking on the induction of CD22 phosphorylation. We first determined the kinetics of CD22 phosphorylation in BCR-stimulated CLL cells. To do this we first immunoprecipitated CD22 from CLL cell lysates and probed for tyrosine phosphorylation in Western blots using 4G10. Figure 5.9 shows that maximal tyrosine phosphorylation of CD22 was reached within 15 minutes following BCR ligation.

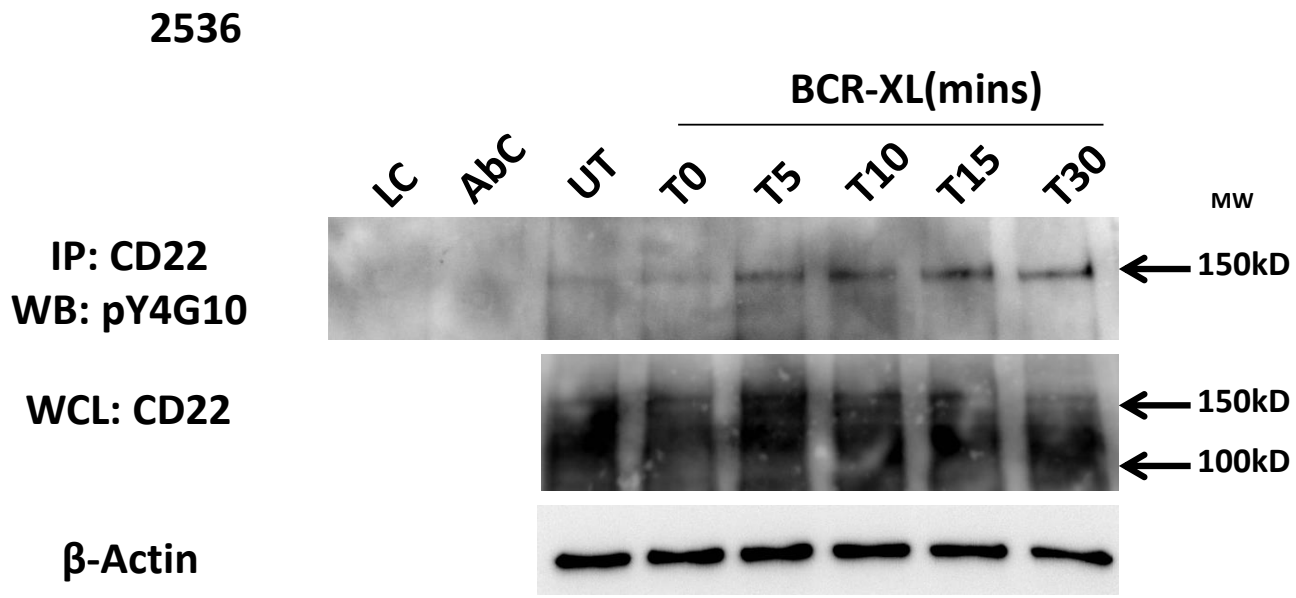


Figure 5.9: BCR-induced CD22 phosphorylation in CLL cells. $1x10^7$ CLL cells were incubated for the indicated time points with 20µg/ml $F(ab')_2$ goat anti-human IgM. CLL cells were lysed in RIPA buffer and CD22 was immunoprecipitated with anti-CD22 antibody. The immunoprecipitates were probed in Western blots with anti-pY4G10 antibody.

I next examined the role of Lck in BCR-induced phosphorylation of CD22 using Lck-i and dasatinib. Figure 5.10 demonstrates that Lck inhibition leads to a reduction in BCR-induced phosphorylation of CD22 back to basal levels. Similarly, the presence of dasatinib also blocked BCR-induced CD22 phosphorylation.

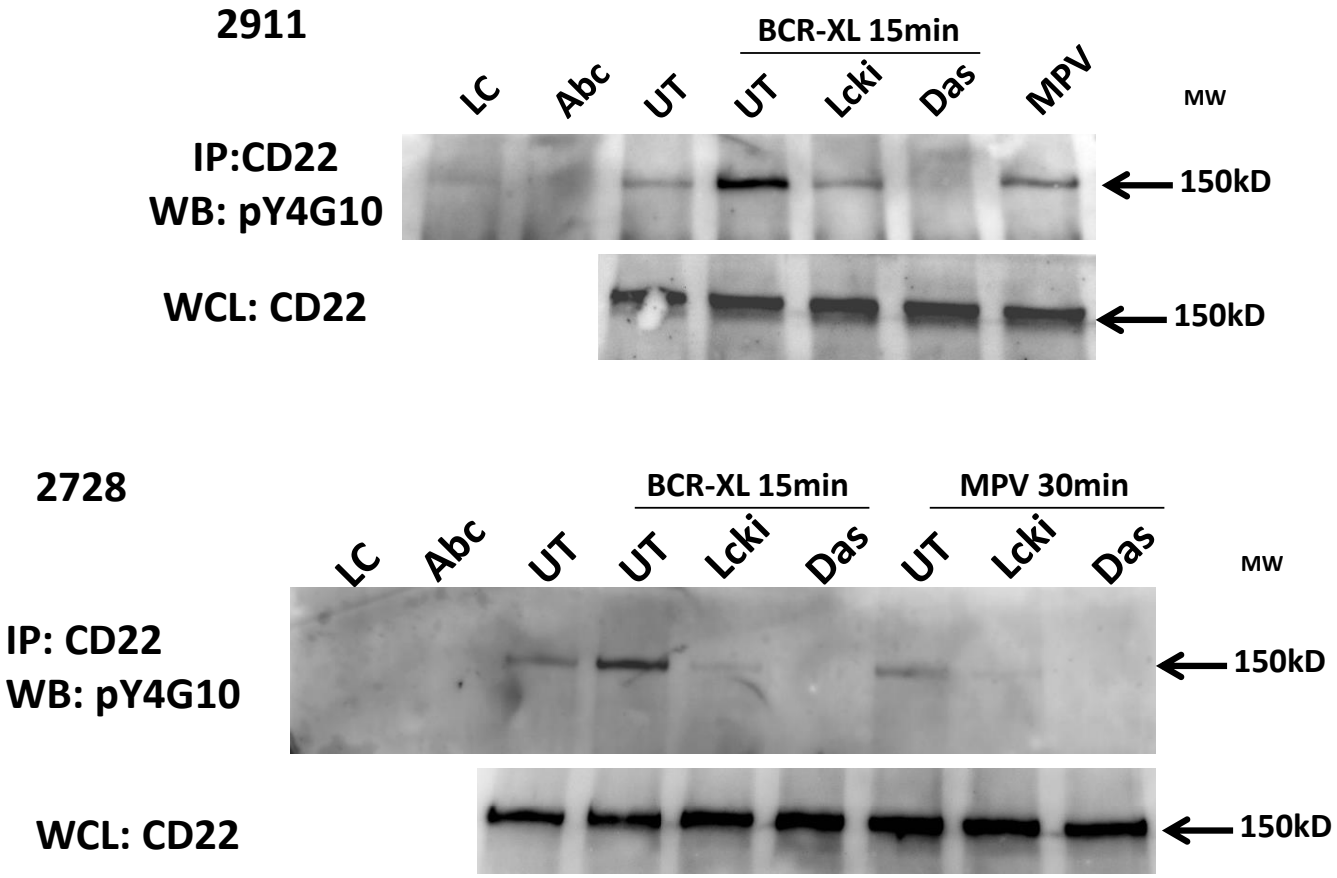


Figure 5.10: The role of Lck in BCR-induced CD22 phosphorylation in CLL cells. $1x10^7$ CLL cells were incubated for 2 hours with 1µM Lck-i or 150nM dasatinib prior to BCR stimulation with 20µg/ml $F(ab')_2$ goat anti-human IgM for 15 min. CLL cell lysates were immunoprecipitated with anti-CD22 antibody and then probed in Western blots with anti-pY4G10 antibody. To stimulate CD22 phosphorylation, CLL cells were incubated with 100µM mpV(pic) for 30min (positive control).

The cytoplasmic tail of CD22 contains six tyrosine residues. Three of these tyrosine residues (Y^{762}, Y^{822}, and Y^{842}) are located within ITIM sequences and are likely responsible for the binding of SHP-1 and SHIP[246]. On the other hand, Y^{807} is thought to be important for recruitment of Grb2 to CD22[231 239 367-368], while the role(s) of Y^{752} and Y^{796} is still to be determined. To clarify the specific role of Lck in BCR-induced phosphorylation of CD22 we used a commercially available phospho-specific antibody that recognises CD22 when phosphorylated at Y^{822}. Figure 5.11 shows that BCR ligation on CLL cells results in an approximate 4-fold increase in the level of pY^{822}-CD22. Inhibition of tyrosine phosphatases using mpV(pic) also resulted in an increase in pY^{822}-CD22 levels. When CLL cells were pre-treated with Lck-i, BCR and mpV(pic) induction of CD22 phosphorylation at this site was blocked, and only basal levels of pY^{822}-CD22 remained. The presence of dasatinib also blocked BCR and mpV(pic)-induced CD22 phosphorylation, but in contrast to Lck-i, the level of pY^{822}-CD22 was reduced to below detection levels. These results suggest that CD22 is a substrate of Lck during BCR signalling in CLL cells, and opens the possibility that another SFK, possibly Lyn, is responsible for basal levels of pY^{822} in CD22.

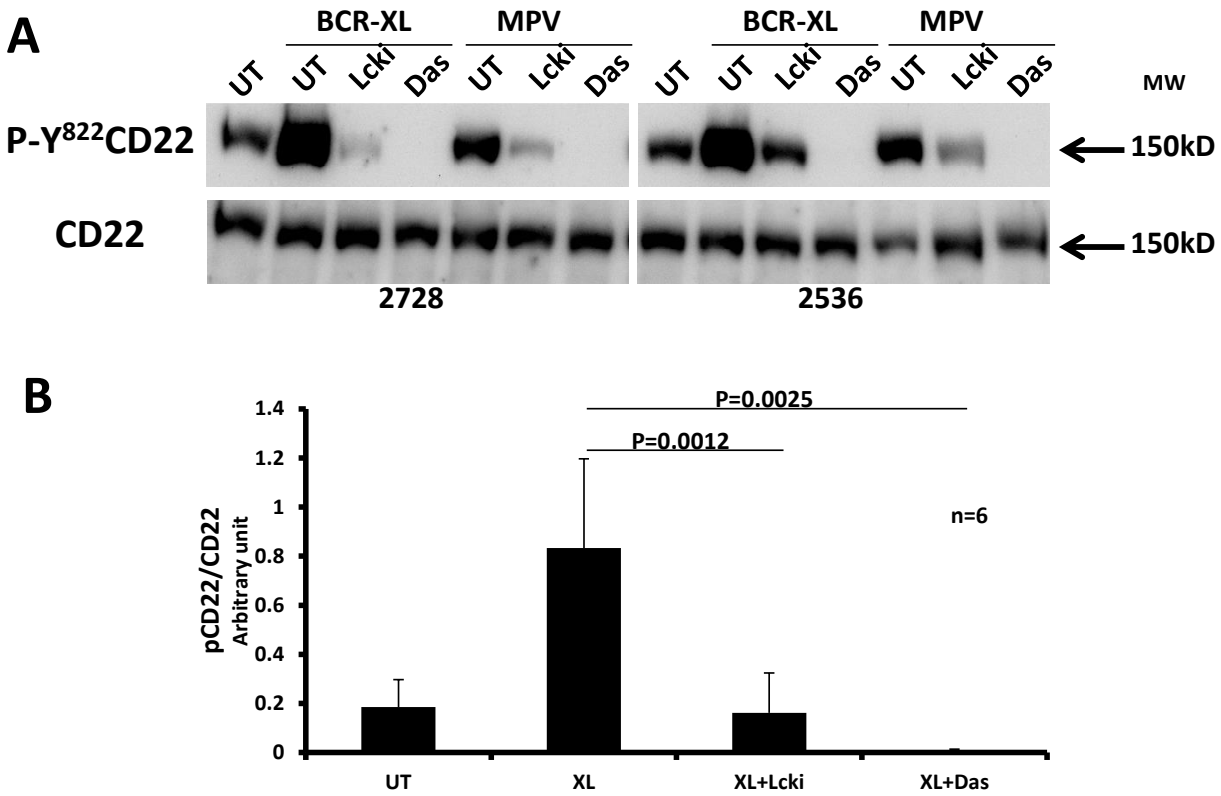


Figure 5.11: The role of Lck in BCR-stimulated phosphorylation of CD22 on CLL cells. A.) 1x10^7 CLL cells were incubated for 2 hours with 1µM Lck-i or 150nM dasatinib prior to BCR stimulation with 20µg/ml $F(ab')_2$ goat anti-human IgM for 15 min. Whole CLL cell lysates were probed by Western blot for pY^{822}-CD22. **B.)** Graphical representation of n=6 different cases of CLL of the Western blots presented in part A, and comparing only the effects of BCR crosslinking. Statistical analysis was performed using a student's t-test for paired data.

5.2.7. Surface IgM is differentially glycosylated in high vs low Lck expressing CLL cases

The above experiments showing that Lck can function to phosphorylate the ITIM of CD22 imply that Lck may set a threshold of activation within CLL cells. Thus, in CLL cases with low Lck there may be higher baseline signals through the BCR than in cases with high Lck levels. In this section we explore this possibility.

One method to measure constitutive activation of the BCR is to investigate the state of glycosylation of the surface Ig. Normal B cells express surface IgM that is modified by mature complex glycans. Under conditions of persistent antigen stimulation, surface IgM on normal B cells loses molecular weight due to changes in the state of glycosylation corresponding to the appearance of immature mannosyl residues[107]. Thus, normal B cells experiencing constitutive BCR signalling *in vivo* would bear surface Ig protein having altered (faster) mobility on SDS-PAGE gels. This phenomenon has recently been applied to the study of CLL cells, and it is reported that CLL cells have higher levels of surface IgM bearing immature mannosyl residues than do normal B cells[108-109]. Importantly, the proportion of fully and immature glycosylated surface IgM on CLL cells varies between patient cases, with cases having primarily immature surface IgM being associated with UM-CLL. Thus, the malignant cells from patients with UM-CLL experience high levels of *in-vivo* BCR stimulation[107].

To investigate a potential role for Lck in this process, we compared surface IgM glycosylation on CLL cases bearing high and low levels of Lck. SDS-PAGE examination of surface IgM glycosylation on normal B cells shows, as expected, a single discreet band (Figure 5.12). In contrast, similar examination of CLL cells displayed 2 bands; a slow

migrating band corresponding to that observed on normal B cells, and a faster migrating band indicating a change in glycosylation. A comparison of the ratio of the faster and slower migrating bands between CLL cases containing low and high levels of Lck showed that CLL cases with immaturely glycosylated surface IgM corresponded to CLL cases with low levels of Lck (Figure 5.12). These findings suggest that CLL cells with low levels of Lck tend to have higher levels of *in-vivo* constitutive BCR engagement than do CLL cells with high Lck levels.

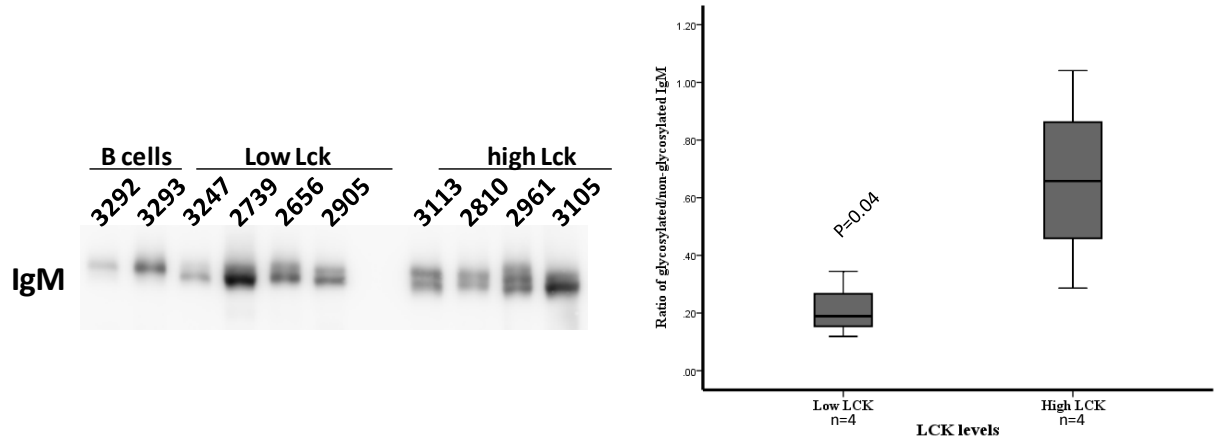


Figure 5.12: Comparison of BCR glycosylation on normal B and CLL cells. CLL cells were surface labelled with sulfo-NHS-SS-Biotin and then lysed. Labelled proteins were purified using NeutrAvidin™ Agarose, and then analysed by Western blot for the presence of IgM (left hand panel). The right hand panel is a graphical representation of n=4 CLL cases with high and low Lck expression levels. Statistical analysis was performed using a Mann-Whitney U-test.

The data in section 5.2.6 showed that CD22 is constitutively phosphorylated in CLL cells and is a target of Lck. The above data suggest that BCR is constitutively active in CLL cases with low levels of Lck. It therefore seemed reasonable to compare the basal levels of CD22 phosphorylation between CLL cases with high and low Lck expression, based on a hypothesis that CD22 phosphorylation by Lck may downregulate BCR signalling. Figure 5.13 shows that pre-treatment of CLL cells with Lck-i reduces the levels of CD22 tyrosine phosphorylation in unstimulated CLL cells. However, we found that there was no quantitative difference in the level of CD22 tyrosine phosphorylation between CLL cases with high Lck levels and cases with low levels of this kinase.

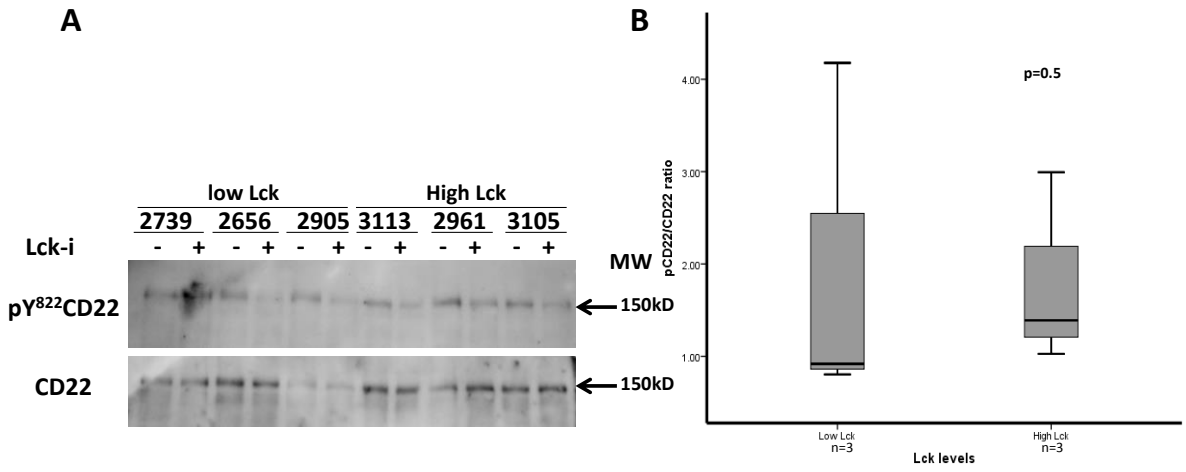


Figure 5.13: Comparison of CD22phosphorylation in high vs low Lck expressing CLL cases. A.) $1x10^7$ CLL cells were incubated for 2 hours with 1µM Lck-i. Whole CLL cell lysates were probed by Western blot for pY^{822}-CD22. **B.)** Graphical representation of n=3 of each group different cases of CLL of the Western blots presented in part A. Statistical analysis was performed using a Mann-Whitney U test.

5.3. Discussion

In Chapter 4 we demonstrate that Lck mediates BCR signalling in CLL cells, and that its level of expression governs the strength of BCR-induced phosphorylation of IKK, Akt and Erk. This suggests a role for Lck in disease prognosis. In this chapter we focused on the link between Lck expression levels and CLL disease prognosis. Analysis of treatment free survival shows that CLL patients with high levels of Lck have better prognosis than patients with low Lck levels. This association between high Lck levels and good disease prognosis indicates that Lck could function as a negative regulator of BCR signalling in CLL cells. In this chapter we investigated the ability of Lck to phosphorylate co-receptors such as CD5, FcγRIIB and CD22 in CLL cells because of the roles these proteins have in attenuating BCR signalling.

To determine a prognostic value for Lck expression in CLL cells we used two different methods of determination on two different cohorts of patients. Thus, in one cohort Lck expression was determined by Western blot analysis of lysates from purified CLL cells, and a second cohort where Lck expression was determined by flow cytometry. Both approaches yielded similar results and showed that high Lck expression in CLL cells is associated with good prognosis whereas low Lck expression is associated with poor prognosis. Both cohorts of patients were subjected to survival analysis according to IgHV mutation and ZAP70 expression. As expected[32 34 97 143-144], UM-CLL cases and cases where the malignant cells expressed high levels of ZAP70 were associated with poor disease outcome in both cohorts. This deserves further comment because statistical significance was not reached for this analysis in the smaller cohort, and was only significant for the relationship between IgHV mutation and disease survival in the second, larger cohort. Nevertheless, we found that there was concordance between ZAP70 expression and UM-CLL status in both cohorts, with the strength of this concordance due to the method of ZAP70 determination. In the first cohort of

patients ZAP70 expression was determined by Western blot whereby ZAP70 expression is quantitated relative to β-actin. We used flow cytometry to determine ZAP70 expression in the second cohort, but did not use the standard monoclonal antibody for this detection[369]. This is because the method of preparing CLL cells for Lck detection was not conducive for the standard clone (clone 2F3·2)[369] that detects ZAP70 and required the use of a different antibody (Mouse Monoclonal Antibody (clone 1E7.2))[35]. The overall result using either of these approaches showed a trend toward poor disease outcome in high ZAP70 expressing cases, and would require the use of much larger cohorts of patients for full validation. That Lck expression did not show relationship to IgHV mutation in either cohort of patients suggests that Lck may be an independent prognostic indicator in CLL.

Having established that high Lck expression corresponded to good disease prognosis, we hypothesized that Lck may have a dual role in regulating BCR signalling; one role as a positive regulator as we have demonstrated in Chapter 4, and a second where it functions to downregulate BCR signals. There is precedent for such a dual role; Lyn is demonstrated to act as a positive and negative regulator of BCR signalling[165-166] through its ability to phosphorylate ITAMs and ITIMs[167-168], and a similar role for Lck in downregulating antigen receptor signalling in T cells is also demonstrated where it may phosphorylate the ITIM within CD5[220-221]. Furthermore, although controversial, such a dual function for Lck in B1a B cells is also possible because of its reported role in enhancing BCR signals[331], or in downregulating BCR signalling leading to hyporesponsiveness of B1 cells to antigen stimulation[192].

CD5 is a quintessential marker of CLL cells[361], and is thought to participate in downregulation of BCR signalling due to an established role in antigen receptor signalling in T cells, and possibly also in B cells[192 220]. CD5 is a target of Lck phosphorylation in T cells,

and studies of CD5 knockout mice showed that $CD5^{-/-}$ T cells display increased proliferation, intracellular Ca^{2+} release and tyrosine phosphorylation of PLCγ-1 and LAT all in response to TCR stimulation[220-221]. Therefore, it seemed logical to examine whether CD5 was a target of Lck in CLL cells. Our data show that BCR crosslinking on CLL cells results in an immediate phosphorylation of tyrosine residues within CD5 as detected by Western blot analysis of immunoprecipitated CD5 with anti-phosphotyrosine 4G10 and PY20 antibodies. We then examined whether this tyrosine phosphorylation was mediated by Lck by pre-treating the CLL cells with Lck-i. We found that such Lck inhibition did not affect BCR-induced phosphorylation of CD5, whereas the presence of the pan-SFK inhibitor dasatinib did. Thus, other SFKs such as Lyn may be responsible for the induction of CD5 phosphorylation in BCR-stimulated CLL cells. This seems to be consistent with what has been shown in $CD5^{+}$ B1a cells[20]. In this subset of B cells BCR ligation results in induction of apoptosis, a response that is CD5 dependent because BCR crosslinking in CD5 knockout mice induced proliferation of these B cells rather than apoptosis. This study concluded that CD5 is a negative regulator of BCR signalling in B1 cells[218], and is likely dependent on Lyn because BCR stimulation of $Lyn^{-/-}$ B1a cells also results in proliferation[219]. Whether CD5 operates in a similar manner in CLL cells is not fully understood. Studies by Perez-Chacon et al have shown that crosslinking CD5 can induce pro-survival signalling in CLL cells, but does so independently of the BCR[223 370] . However, another study has indicated that Lyn constitutively phosphorylates CD5 in CLL cells to allow association of SHP-1 where it functions as a negative regulator of BCR signalling[224]. Our results agree with a potential role of Lyn in the phosphorylation of CD5, but this only takes place when BCR is crosslinked in CLL cells.

To further investigate a potential role of CD5 in CLL cells, we attempted to transfect CD5 or a truncated version of CD5 that lacks a cytoplasmic tail into MEC1 cells. MEC1 cells are a

$CD5^-$ cell line that was derived from a patient with CLL who underwent prolymphocytic transformation[363]. Analysis of this cell line showed that they express Lck and exhibit constitutively active BCR signals with high phosphorylation levels of IKK, Akt and Erk. Because of the known inhibitory function of CD5 on BCR signalling, we hypothesized that reintroduction of CD5 expression would raise the threshold for BCR activation due to recruitment of SHP-1 to the plasma membrane within this cell line and result in a dampening of the constitutive signals. Our results show that transient expression of CD5 in MEC1 cells did not affect constitutive BCR signalling to IKK, ERK and Akt. In these experiments CD5 was expressed by approximately 80% of the cells as a surface antigen. Thus, the failure of CD5 to affect BCR signalling in this system is not due to failure of the cells to express this antigen appropriately.

FcγRIIB (also known as CD32) is another cell surface protein that is known to be expressed on CLL cells at comparable or higher levels than on normal B cells[263]. This may be important because the cytoplasmic domain of this protein contains an ITIM sequence[102]. Following BCR ligation FcγRIIB is phosphorylated on tyrosine residues within the ITIM sequence, and this function to recruit SHIP leading eventually to attenuation of BCR signals[253]. In normal B cells Lyn is thought to mediate phosphorylation of tyrosine 292 located within the ITIM domain of FcγRIIB during BCR engagement[166-168 208 364-365], but in CLL cells the mechanism of FcγRIIB phosphorylation has not been well studied. Our data show that FcγRIIB is constitutively phosphorylated on Y^{292} in CLL cells, and that BCR stimulation does not seem to induce any change in this state. This could be because the antibody we use to crosslink BCR on CLL cells is a $F(ab')_2$ fragment which does not engage FcγRII. However, the fact that this protein is constitutively phosphorylated implies that there is some degree of engagement that is independent of our perturbation. We show that this constitutive phosphorylation of FcγRIIB is not due to Lck because the treatment of CLL cells with Lck-i

had no effect. However, treatment of CLL cells with dasatinib completely reduced pY[292]-FcγRIIB levels suggesting that another SFK is involved. This could be Lyn because of the known role of this SFK in FcγRIIB phosphorylation[166-168 208], and because Lyn is constitutively active in CLL cells[174]. If this is indeed the case, then Lyn in CLL cells actively contributes to downregulation of BCR signalling through FcγRIIB in a way that is already proposed for Lyn-mediated phosphorylation of CD5[224].

In this thesis we did not examine any role for FcγRIIA. This isoform of CD32 contains an ITAM rather than an ITIM and functions in myeloid cells to facilitate signals through this receptor[262-264]. CLL cells have been shown to express FcγRIIA, however, studies have shown that it is incapable of inducing any activation signals[264]. Our study of CD32 on CLL cells is restricted to FcγRIIB because the antibody we use to recognise phospho-tyrosine is specific for the ITIM within this isoform.

An additional protein that participates in downregulating BCR signalling is CD22[226], which performs this function by recruiting SHP-1[235] and SHIP[239] to the ITIM sequences within its cytoplasmic tail when they become tyrosine phosphorylated. Experiments using Lyn knockout mice show that phosphorylation of CD22 is mediated by this SFK in normal B cells[208 233-234]. CLL cells express CD22, but the level of expression of this protein can vary on the malignant cells from different patients[245]. Our data confirms this observation for the cohort of patients we employed in this study. We further examined whether CD22 is a possible target of Lck in CLL cells using Lck-i. Thus, BCR stimulated CD22 phosphorylation is inhibited in CLL cells by the presence of this compound, suggesting a role for Lck in this process. However, another SFK may be additionally involved in CD22 phosphorylation because its complete inhibition was only achieved using dasatinib. Taken together, these results suggest that Lck is responsible for inducible phosphorylation of CD22 in CLL cells,

while another SFK is responsible for basal levels of CD22 phosphorylation. This may be important because we were not able to show a difference in the level of CD22 phosphorylation in high and low Lck expressing CLL cell clones. However, this is complicated by the fact that CD22 and Lck are differentially expressed between CLL cases, and in this thesis we were unable to address if there was a relationship between CD22 and Lck expression in CLL cells. Thus, CLL cases with low CD22 but high Lck may have a greater downregulatory effect on BCR signalling than CLL cases carrying high CD22 and low Lck. The experiments we performed used CLL cases with high levels of CD22 in order to ensure detection of phosphorylated protein in the assays we used: **1**.) Immunoprecipitation of CD22 and detection of tyrosine phosphorylation using phospho-tyrosine specific antibodies, and **2**.) Direct detection of pY^{822}-CD22 in whole cell lysates. Little is known about the role of CD22 in CLL cells, with one study reporting that CD22 is constitutively tyrosine phosphorylated but it does not associate with SHP-1[224]. Our experiments showed that CD22 phosphorylation can be induced in CLL cells by BCR crosslinking, and suggest that Lck is responsible for this induction. However, further experiments will be necessary in order to fully understand the role of CD22 and Lck in BCR signalling in CLL cells.

If Lck does indeed participate in downregulating BCR signalling in CLL cells, then, according to our hypothesis, CLL cases with low levels of Lck should have higher *in vivo* signals than CLL cases with high levels of Lck. We tested this possibility by measuring the glycosylation of surface IgM on CLL cells. B cells exposed to chronic BCR signals change the glycosylation of BCR due to recycling of the IgM to the cell membrane[212]. Thus, under constant BCR engagement the mature N-glycosylation of surface IgM on B cells is changed to expose underlying mannosyl residues with the consequence of decreased molecular weight. This phenomenon has recently been exploited to show that CLL cells, especially from UM-CLL cases, experience constitutive BCR engagement *in vivo*[107]. We used a similar

approach to compare *in-vivo* BCR signalling in CLL cells from cases expressing high and low levels of Lck. We found that CLL cells bearing low levels of Lck expressed a significantly higher proportion of mannosylated BCR than did CLL cells bearing high levels of Lck. This finding supports our hypothesis that CLL cases with low levels of Lck have higher levels of in vivo signalling. An explanation for why this occurs is suggested by a recent paper by Dühren-von Minden et al[110]. Here it was demonstrated that structural elements within the BCR on CLL cells is conducive to antigen-independent cell-autonomous signalling. If this is a crucial pathogenetic mechanism, as is suggested by the authors of this manuscript, then the importance of mechanisms that downregulate this antigen-independent cell-autonomous signalling will also be important within this pathogenetic mechanism. Thus, our work points to a role of Lck within this pathogenetic mechanism, however, it is clear that more work needs to be done in order to fully characterise this role.

Chapter 6: General discussion and future work

The initial aim of this thesis was to characterise any potential role of PKCβII and c-Abl in the pathway of BCR-mediated activation of NFκB. However, in address of this aim we found that neither of these protein kinases was involved in this pathway. Nevertheless, our experiments did suggest the involvement of Lck in BCR signalling. Thus, the remaining aims of this thesis were to fully characterise the role of Lck in BCR-mediated signalling in CLL cells, and determine its pathogenic importance.

The results of our experiments are important for understanding the pathophysiology of CLL cells because engagement of the BCR on these cells provides them with proliferation and survival signals. The importance of BCR signalling in CLL pathogenesis has been extensively studied. However, there has been a general assumption that Lyn is the main mediator of proximal BCR signals. This work is the first to demonstrate an unambiguous role for Lck in the generation of both proximal and distal signalling events in CLL cells responding to antigen receptor engagement. Thus, this project proposes the possibility of targeting Lck as a therapeutic approach for the treatment of CLL patients. From a clinical point of view targeting Lck may be advantageous because inhibition of this SFK would target CLL cells but not alter the function of normal peripheral B cells, which lack Lck. On the other hand, Lck is an important mediator of T cell receptor function[188 332], and so caution must be exercised when considering Lck inhibition as a therapeutic approach because T cells are known to be abnormal in CLL patients[371-372] and suppression of T cell function may increase susceptibility of these patients to opportunistic infection.

The initial purpose of this project was to investigate the signalling pathway that is triggered by BCR engagement with its cognate antigen leading to activation of NFκB in CLL cells. NFκB is aberrantly activated in CLL compared to non-malignant B cells, and the level of

active NFκB correlates with *in-vitro* survival of these cells and to disease prognosis[157-158]. Although the prognostic value of active NFκB is independent of IgHV mutation and ZAP70 expression[373], its activation by BCR may still be relevant because of recent findings suggesting a role of antigen-independent cell autonomous signalling by BCR in the pathogenesis of CLL[110]. If such autonomous BCR signalling contributes to activation of the NFκB pathway in CLL cells, then the results of our experiments indicate that a paradigm pathway whereby the CARMA-1/MALT-1/Bcl-10 (CBM) complex transfers BCR signals leading to stimulation of IKKβ via Btk-mediated activation of PLCγ2 and PKCβ[115] is not operative. Thus, CLL cells express very low levels of Bcl-10 compared to normal B cells, supporting the notion that this pathway may be inoperative in these cells because of the importance of the CBM complex elements for efficient antigen receptor signalling to occur[288-290] . Further evidence to suggest that the CBM pathway is inoperative comes from experiments where we cultured CLL cells on CD40L-expressing fibroblasts. Here we observed induction of Bcl-10 expression in the CD40L-stimulated CLL cells. However, BCR-induced IKK phosphorylation in these cells was attenuated compared to CLL cells cultured on control fibroblasts despite the increase in Bcl-10 levels. Finally, we show using the inhibitor LFM-A13 that Btk plays a role in BCR-induced IKK activation in A20 cells (a B cell line) but not in CLL cells. Taken together, our data provide evidence to suggest an alternative mechanism of BCR-induced IKK activation whereby PKCβ and the CBM complex are bypassed.

How this alternative mechanism of BCR-induced NFκB pathway activation is mediated will involve active Lck and induction of tyrosine phosphorylation. This is because we found that BCR-induced IKK activation was particularly sensitive to Lck inhibition either by siRNA knockdown of Lck, or using the Lck inhibitor. How Lck activates NFκB pathway signalling in CLL cells responding to antigen receptor engagement is a topic of future investigation.

The work presented in this thesis points to a central role of Lck in mediating BCR signalling in CLL cells. We show that Lck in BCR-stimulated CLL cells catalyses the proximal phosphorylation of CD79a as well as the induction of distal signalling events involving phosphorylation of Syk, activation of ERK, NFκB and PI3K/Akt and enhanced cell survival. This finding may be relevant for understanding the mechanisms of how new agents entering clinical trials may mediate their effects. One of these agents, Ibrutinib (PCI-32765), has generated promising results and is described to be an irreversible inhibitor of Btk[374-375]. In cell-free analysis of kinase activity Ibrutinib inhibits Btk activity with an IC_{50} of 0.5nM, Lck with an IC_{50} of 33.2nM and Lyn with an IC_{50} of 200nM[375]. This consideration may be important because Ibrutinib is reported to inhibit BCR-induced cell survival and activation of ERK and Akt at concentrations greater than 1μM[83 374] , BCR-induced pathways we show in this thesis to be mediated by Lck in CLL cells. Although Lck may be upstream of Btk in the pathway initiated by BCR crosslinking on CLL cells, a direct role of Btk in activation of the PI3K/Akt pathway in B cells has not been demonstrated. Rather, in B cells it is demonstrated that Btk acts downstream of PI3K activation owing to the need of this tyrosine kinase to associate with exposed PIP_3 moieties at the cell membrane for activation and subsequent phosphorylation of its major substrate in B cells, PLCγ2 which also needs to be at the plasma membrane to gain access to its substrate PIP_2 [376]. Another factor that may be important is PKCβII which is demonstrated to phosphorylate Btk on serine 180 and downregulate its activity by dissociating it from the plasma membrane[279]. Work from this Department has shown that CLL cells overexpress PKCβII, and that, at least in some cases of CLL, this PKC isoform works to suppress BCR-induced intracellular Ca^{2+} release in CLL cells via this mechanism[197 377].Thus, while it is clear that Ibrutinib acts to inhibit Btk in "normal" B cells and that this is important to the way this compound works to suppress B cell-mediated autoimmune disease[375], the way it works to bring about its cytotoxic effects in CLL may be

different and involve inhibition of Lck owing to the high levels of this compound that are needed to inhibit BCR-induced signalling events in these cells. Future work is needed to more clearly define the mechanism of how Ibrutinib is working in CLL cells.

The work of this thesis also revealed a potential value of Lck expression for disease prognosis in CLL. Considering our demonstration of a role for Lck as a mediator of BCR signalling in CLL, particularly the relationship between Lck and BCR signalling strength, it is therefore surprising that high expression of this SFK in CLL cells is linked to good disease prognosis. Exactly why this is the case will require further experimentation, but we propose that this observation may be linked to a potential threshold-setting function of Lck. Within this model *in vivo* BCR signalling would be much more prominent in CLL cells having low levels of Lck expression than in CLL cells with high levels of Lck expression. We found that this is indeed the case when we measured the proportion of BCR that has undergone re-expression to the plasma membrane of CLL cells, which is measured by the appearance of mannosylated BCR and is a function of BCR engagement[107 212]. Thus, in CLL cases with low levels of Lck expression there is a higher proportion of mannosylated BCR than in CLL cases with high levels of Lck expression. How Lck sets the threshold of activation may be due to an ability to target ITIM motifs and/or recruit tyrosine phosphatases to the BCR signalling complex. Such a role for Lck has been previously reported in T cells and B1 cells where the target is CD5[192 220]. However, our data indicate that this is not the case in CLL cells. Alternatively, our investigations identify CD22 and show that BCR crosslinking induces significant phosphorylation within the ITIM motif of this protein that is mediated by Lck. In this regard CD22 could potentially act to downregulate BCR signalling in CLL cells by recruiting SHP1 and SHIP[235 239], but this interpretation is complicated by differential expression of Lck and CD22. We were unable to show any relationship between basal levels of CD22 phosphorylation and Lck expression in CLL cells. Further work addressing the exact

role of Lck may need to identify the binding partners of this SFK in order to understand its full function.

Figure 6.1 summarises a proposed mechanism through which Lck may be regulating BCR signalling in CLL cells.

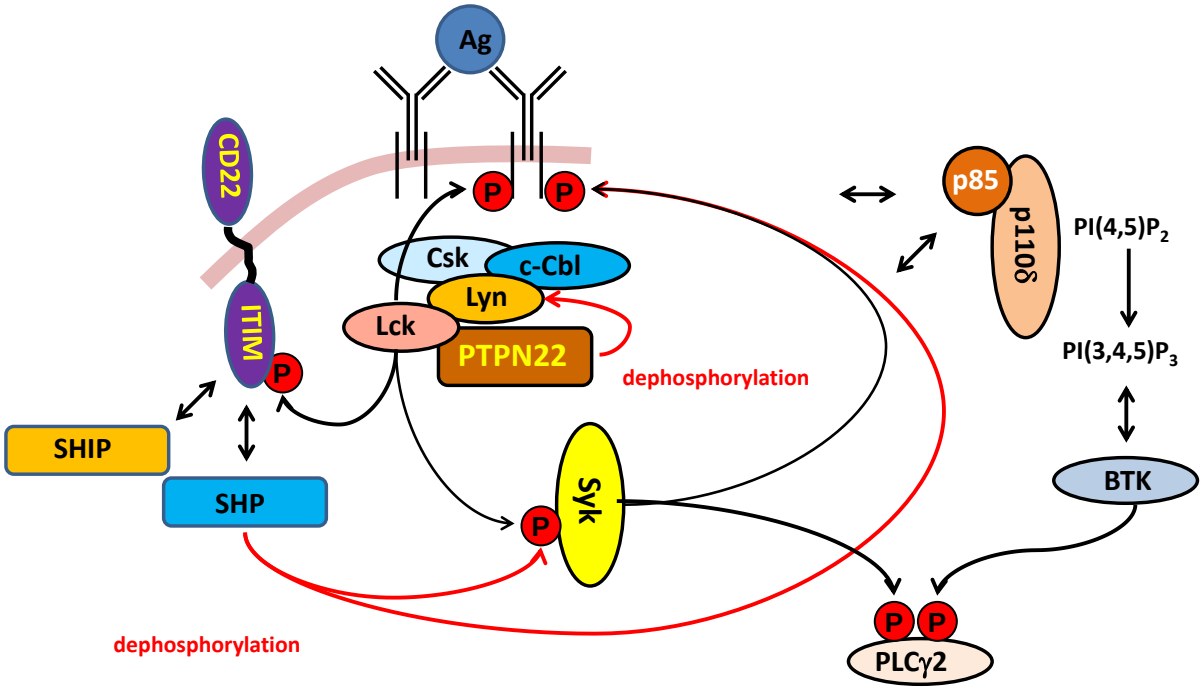


Figure 6.1: Proposed role of Lck in BCR signalling in CLL cells.

In conclusion, this work aimed to explore some unanswered questions about BCR signalling in CLL cells, and provides insight into the role(s) of different SFKs in this process. We show that specific inhibition of Lck blocks BCR signalling in CLL cells leaving open the question of a role for Lyn. An exact role for Lyn is difficult to assess owing to the stability of this protein in CLL cells. Thus, whether Lck acts directly to phosphorylate CD79 and initiate downstream signals in CLL cells, or if it "guides" Lyn in this function needs to be determined. This thesis provides the groundwork to justify research in this area.

Appendix

Clinical details of CLL cases studied.

Case #	WBC	CD38	Binet	Rai	IgHV%	Class
1754	182.4	1.2	B	II	93.55	M
1801	128				96.84	M
1851	116	4	A	III	93.9	G
1901	72.7	1	A	0	95.3	M
1932	24.9	low	A	0	94.02	M
1937	18	3	A	0	99.66	M
1939	624	13	C	IV	100	M
1952	13.9	33	A	0	91.1	G
1954	32		A	0		
1958	215	2	A	0	95.53	M
1963	17.2	19	A	0	93	M
1964	39.5	3	A	0	91	M
1965	16.7	54	A	0	99.66	M
1975	21	3				
1996	15.6	5	A	0	91.5	M
1998	108.9	28	A	I	90	M
2006	22.5	3	A	0	93.6	M
2010	191.3	16			100	M
2025	23.7	12	A	0	91.5	M
2045	23.3	40	A	0	98.65	M
2050	12.9	5				
2056		25			99.65	M
2063	427	98	B	II	100	M
2067	138	57			100	M
2073	10.2	9	A	I	93.2	M
2080	119.1	3	A	0	91.1	M
2081	13.6	3	A	0		

2082	6.7	76			100	M
2085	17.5	14	A	0	100	M
2096	60	0	A	0	93.75	M
2098	28.5	14	A	0		
2120	155				94.63	G
2123	8.2					
2157	66.4	low			88.89	M
2173	72.6	19	A	0	96.14	M
2204	27.8	14	A	0		
2205	147	18	A	I	100	M
2209	105	29			98.3	M
2212	64.7	53			100	M
2213	24.9	72	B	I		
2216	31.8	3			85.99	M
2218	58.7	8	B	I	100	M
2224	37.3	8			94.58	M
2230	127.9				100	M
2233	15.6	98			100	M
2237	210		B	I	91.41	M
2238	90.1	6	A	0	94.79	M
2246	54				93.88	M
2255	187	18	B	I	100	M
2256	51.1	27	B	I	100	M
2291	154	28	B	II	97.57	A
2343	86	4	B	II	93.88	G
2354	165				95.44	M
2371	158	3	C	III	91.07	M
2387	31.1	35	C	III		
2389	115				98.25	M
2399	240	97			100	A
2424	162.3	96	C	III	100	M
2429	13.6	1	A	0	94.65	M

2435	370	88			100	M
2436	20	0	A	0		M
2444	20.9	95	A	0		
2453	387.5					
2457	283	3	C	IV	97.54	M
2506	17.3	3	A	I		
2536	242.3	96	C	III		M
2562	124.7	40	A	0	100	M
2581	175.9	6				M
2595	23.8				97.52	M
2608	110.7	15	A	0	91.06	G
2656	142.1				99.65	M
2679	123.8				93.88	G
2683	381.9				100	M
2689	71.5	1	C	IV		
2701	174	29			98.25	M
2709	23.1	94			100	M
2711	242.3				87.72	G
2722	16.9	2			100	M
2724	86.8	14	B		98.91	M
2728	163	18			100	M
2736	40.4	7			100	M
2739	412				100	M
2745					99.66	M
2747	147.4	60	A		100	M
2751	802.5				100	M
2764	184				100	M
2783	196.4	5			96.94	M
2806	16.2	2				
2810	44.6	1	C	III	98.28	M
2861	43.5				100	M
2872	76.6	1	C	II	98.28	M

2877	160.8				91.5	M
2905	59.6	98			100	M
2911	223.2	60			100	M
2918	178.7				99.66	M
2932	175.7		B	II		
2948	114.2	3			100	M
2950	150.4		C	IV	94.79	M
2953	126.5	98	C		100	M
2954	49.3	0				
2956	266.3	low	A		99.66	M
2961	126.5		B			
2968	87.4		C		96.53	G
2990	80.3	1			99.73	M
2999	118.5	7			96.36	M
3023	67.9				100	M
3047	141.4	4			99.32	M
3068	32.3	29	B		100	M
3074	459.7	Low	A		99.66	M
3095	44.2	0	A		99.65	M
3105	66	25			99.65	M
3113	172.4	29	B		98.25	M
3247						
3283		38				
3184	155.7	0			100	M

WBC: White blood cell count ($x10^9$/L)

CD38: % CD38 positive cells

Binet: Binet clinical staging

Rai: Rai clinical staging

IgHV: IgHV gene % homology with germline sequence

Class: Class of immunoglobulin

Buffers

TBS-T

Tris (pH 7.4) 10mM

Sodium chloride 150mM

Tween-20 0.1%

Resolving gel buffer (4x)

Tris (pH 8.8) 1.5M

SDS 0.4%(w/v)

Bought from National diagnostics, Hessle Hull, England

Resolving gel

Acrylamide Varied (usually 10%)

4x Running gel buffer 25%

TEMED 0.01%

Ammonium persulphate 0.04%(w/v)

Made up to 100% with ddH2O

Stacking gel buffer

Tris (pH 6.8) 0.5M

SDS 0.4%(w/v)

Bought from National diagnostics, Hessle Hull, England

Stacking gel

Acrylamide 5%

Stacking gel buffer 25%

TEMED 0.1%

Ammonium persulphate 0.08%(w/v)

Made up to 100% with ddH2O.

Transfer buffer (10X)

Tris (pH 6.8) 0.25M

Glycine 1.92M

Bought from National diagnostics, Hessle Hull, England

SDS-PAGE running buffer

Tris 25mM

Glycine 192mM

SDS	0.1%(w/v)

Western blot stripping buffer

SDS	2%(w/v)
Tris (pH6.8)	62.5mM
β-mercaptoethanol	100mM

Laemelli buffer (2x)

Tris (pH6.8)	125mM
Glycerol	20%
SDS	4%(w/v)
β –mercaptoethanol	10%
Bromophenol blue	0.006%(w/v)

Clear lysis buffer

SDS	1%(w/v)
Glycerol	10%
Tris (pH6.8)	50mM
EDTA	5mM

Phosphate Buffered Saline (PBS)

NaCl	150mM
KCl	3mM
Na2HPO4	4.3mM
KH2PO4	1.5mM

Purification buffer

PBS

BSA 0.1%(w/v)

EDTA 2mM

Degassed overnight prior to use.

Radioimmunoprecipitation (RIPA) buffer (Triton X-100):

Glycerol	10%
Triton X-100	1%
Sodium deoxycholate	1%(w/v)
SDS	0.1%(w/v)
Tris (pH7.6))	50mM

EDTA	2mM
EGTA	2mM
Sodium pyrophosphate	25mM
Glycerol phosphate disodium	50mM
Na Cl	150mM
Na F	50mM

Made up to 100% with ddH2O

N-ethylmaleimide (NEM): Stock 1M dissolved in Ethanol, was added to RIPA buffer (10mM) to preserve IKKγ ubiquitination.

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