Identification and Validation of Potential Targets for the Diagnosis and Therapy of Chronic Lymphocytic Leukaemia

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I confirm that the work submitted is my own and appropriate credit has been given where reference has been made to the work of others.

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Abstract

Treatment of chronic lymphocytic leukaemia (CLL) has greatly improved with the use of combination chemo-immunotherapy but the treatment of relapsed and fludarabine refractory CLL is still challenging with the current agents. Large amounts of mRNA expression data are publicly available but identifying suitable targets requires validation at the protein level.

I used available RNA expression data to identify candidate antigens that could be screened for protein level expression using commercially available monoclonal/polyclonal antibodies. To test new molecules I optimised an *in-vitro* viability assay system using mononuclear cells and standard viability assessments. To study pathway interactions, B-cell receptor (BCR) was stimulated using goat $F(ab')_2$ anti-human IgM or IgD and signalling responses were assessed by SYK-phosphorylation (SYK pY348PE) and calcium-flux measured by ratiometric difference in florescent intensity of Fluo-3/Fura-red.

I assessed 84 antigens and 15 showed binding on CLL cells and/or normal B-cells but not other leucocytes. I found that 7 of the 15 molecules have a recognised role in neurotransmission, such as the nicotinic acetyl choline receptor subunit β4 and dopamine receptor D4. This observation indicated that these molecules and pathways were potentially involved in the pathophysiology of CLL and I therefore further investigated their importance.

In exploring the newly identified targets, I found that dopamine and D2 antagonist domperidone reduced CLL cell survival *in vitro*, in a dose dependent manner. While elucidating the downstream mechanisms for the above effect, I observed that dopamine significantly reduced SYK phosphorylation and calcium flux induced by stimulation of the BCR pathway using goat $F(ab')_2$ anti-human IgD. *In vitro* testing also demonstrated a synergistic effect in blocking of the D2 and the BCR pathways as a combination of PI3-kinase δ inhibitor (GS-1101) with domperidone reduced CLL cell viability more efficiently than either agent alone.

Therefore I have identified a number of novel molecules expressed in CLL and further investigated their biological importance in the disease pathophysiology. These novel molecules provide several potential untested therapeutic targets in CLL.

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Glossary of Terms

5-FMP: 5-fludarabine-monophosphate

5HTR7: 5-hydroxytryptamine (serotonin) receptor 7

7-AAD: 7-aminoactinomycin D

ACCN1: Amiloride-sensitive cation channel 1

ACVRL1: Activin receptor-like kinase 1

ADAM: A Disintegrin and Metalloprotease

ADB: Agarose Dissolving Buffer

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity.

AF488: Alexa Fluor® 488

AF647: Alexa Fluor 647

AF750: Alexa Fluor 750

AMFR: Autocrine motility factor receptor

Apaf-1: Apoptosis activating factor 1

APC: Allophycocyanin

APC-AF750: Allophycocyanin- Alexa Fluor 750

APLP1: Amyloid-like protein 1

APO-1: Apoptosis Antigen 1

APRIL: A Proliferation Inducing Ligand

ASO PCR: Allele-Specific Oligonucleotide PCR

ATF2: Activating transcription factor 2

ATM: Ataxia telangiectasia mutated

ATP1B1: Sodium/potassium-transporting ATPase subunit beta-1

ATP1B2: Sodium/potassium-transporting ATPase subunit beta-2

BAD: Bcl-2-associated death promoter

BAFF: B cell activating factor

Bag-1: BCL2-associated athanogene

BAK: Bcl-2 homologous antagonist/killer

BAX: BCL2-associated X protein

BCL-2: B-cell lymphoma 2

BCL-X_L: B-cell lymphoma-extra large

BCL-X_S: B-cell lymphoma-extra small

BCMA: B-cell maturation antigen

BCR: B-cell receptor

BLNK: B-cell linker

BM: Bone marrow

BMSC: Bone marrow stromal cells

BSA: bovine serum albumin

BTK: Bruton's tyrosine kinase

CAP: cyclophosphamide, doxorubicin and prednisolone

CAR: chimeric antigen receptor

CCL3: Chemokine (C-C motif) ligand 3

CD: Cluster of Differentiation

CD40L: CD40 Ligand

CdA: 2-chlorodeoxyadenosine

CDC: complement dependent cytotoxicity

CDH15: Cadherin-15

CDH16: Cadherin-16

CDK: cyclin-dependent kinases

CDR: complementarity determining region

CFAR: alemtuzumab, fludarabine, cyclophosphamide and rituximab

CHOP: cyclophosphamide, hydroxydaunorubicin, oncovin and prednisolone

CHRNB4: Nicotinic acetylcholine receptor β4

CLL: Chronic Lymphocytic Leukaemia

CLLU1: Chronic Lymphocytic Leukemia Up-Regulated Protein

CNR2: cannabinoid receptor 2

COP: cyclophosphamide, vincristine and prednisolone

CR: complete remission

CREB: cyclic AMP response element-binding protein

CRHR2: Corticotropin releasing hormone receptor 2

CS&T: cytometry setting and tracking

CXCL13: C-X-C motif chemokine 13

CXCR4: chemokine receptors 4

CXCR5: chemokine receptors 5

DA: dopamine

DAG: diacylglycerol

DARPP-32: dopamine and cyclic AMP-regulated phosphoprotein

dATP: deoxyadenosine triphosphate

DGCR2: DiGeorge syndrome critical region gene 2

DIABLO: direct IAP binding protein with low pI

DISC: Death Inducing Signaling complex

DLBCL: Diffuse large B-cell Lymphoma

DMD: dystrophin gene

DMSO: Dimethyl sulfoxide

DR1: Down Regulator 1

DR3: Down Regulator 3

DRD4: dopamine receptor D_4

DSB: double-strand breaks

ECM: extracellular matrix

EDA: Ectodysplasin-A

EDAR: Ectodysplasin A receptor

EDTA: ethylenediaminetetraacetic acid

EFNB1: Ephrin-B1

EGF: Epidermal growth factor

ENT1: Equilibrative nucleoside transporter 1

ERIC: European Research Initiative on CLL (ERIC)

Erk: Extracellular signal-regulated kinases

$F(ab)_2$: fragment antigen-binding

F2RL3: factor II (thrombin) receptor-like 3

FACS: Fluorescence-activated cell sorting

FADD: Fas-Associated protein with Death Domain

F-ara-A: 9-beta-D-arabinofuranosyl-2-fluoroadenine

Fas: Fatty acid synthase

FBS: Foetal Bovine Serum

FC: fludarabine and cyclophosphamide

FCMR: Fludarabine, cyclophosphamide, mitoxantrone and rituximab

FcR: FcReceptor

FCR: Fludarabine, cyclophosphamide and rituximab

FCRL: human Fc receptor-like molecules

FDA: Food and Drug Administration

FDC: follicular dendritic cells

FISH: Fluorescence *in situ* hybridization

FITC: Fluorescein isothiocyanate

FPRL1: formyl peptide receptor-like-1

GABBR1: gamma-aminobutyric acid (GABA) B receptor, 1

GLO1: glyoxalase I

GLUT1: Glucose transporter 1

GPR: G protein-coupled receptor

GRM4: Metabotropic glutamate receptor 4

GS: Goat serum

GYPC: glycophorin C

HBSS: Hank’s Buffered Saline Solution

HBSS-CMF: Hank's Balanced Salt Solution (HBSS) buffer supplemented with 1mM $CaCl_2$, 1mM $MgCl_2$ and 1%FCS

HCSL1: HLCS holocarboxylase synthetase

HTC: high throughput cytometry

iCa^{2+}: intracellular calcium

ICAM-1: Intercellular Adhesion Molecule 1

IFN-γ: Interferon gamma

IFN-γ R β: Interferon gamma receptor β 1

IgD: Immunoglobulin-D

IgG: Immunoglobulin-G

IgH: immunoglobulin heavy chain

IGHV: Immunoglobulin variable region of the heavy chain

IgM: Immunoglobulin-M

IL-4: Interleukin 4

IMDM: Iscove's Modified Dulbecco's Medium

IP3: inositol-1,4,5-triphosphate

ITAMs: immunoreceptor tyrosine-based activation motifs

ITIM: Immunoreceptor tyrosine-based inhibitory motif

IV: Intravenous

IVIg: Intravenous immunoglobulin

IWCLL: International Workshop on Chronic Lymphocytic Leukaemia

JAG1: jagged 1

JNK: Jun amino-terminal kinase

JTB: jumping translocation breakpoint

KLH: keyhole limpet hemocyanin

LDH: Lactate dehydrogenase

LFA-1: Lymphocyte function-associated antigen 1

LPD: Lympho-proliferative disorder

LPL: Lipoprotein lipase

LPL: Lymphoplasmacytic lymphoma

LREC: Local Research Ethics Committee

LRF: Leukaemia Research Fund

LTB4R: leukotriene B4 receptor

LTK: leukocyte receptor tyrosine kinase

MAPK: mitogen-activated protein kinase

MCL: Mantle cell lymphoma

MCL-1: myeloid leukemia cell differentiation protein

MDEG: Mammalian Degenerin

MDM2: Mouse double minute 2 homolog

MFI: migration inhibiting factor

MMP: matrix Metalloprotease

MNC: Mononuclear cell

MORT1: mediator of receptor-induced toxicity

MRD: Minimal residual disease

mRNA: messenger ribonucleic acid

MSC: mesenchymal stromal cells

MZL: Marginal zone lymphoma

NaCl: sodium chloride

NF-2: neurofibromatosis-type 2

NFAT: Nuclear factor of activated T-cells

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NGFR: Nerve growth Factor receptor

NHL: Non-Hodgkin's Lymphoma

NLC: monocyte-derived nurse-like cells

NMDAR1: N-methyl-D-aspartate (NMDA) receptors

NRAMP1: natural resistance-associated macrophage protein 1

NRG2: Neuregulin 2

OFAR: oxaliplatin, fludarabine, cytarabine and rituximab

ORR: Overall response rate

OS: overall survival

PBS: phosphate-buffered saline

PCDH9: protocadherin 9

PE: Phycoerythrin

PEG10: Paternally Expressed Gene 10

PerCP: Peridinin chlorophyll

PFS: progression-free survival

PI3K: Phosphatidylinositol-3-kinase

PIP2: phosphatidylinositol 4, 5-bisphophate

PIP5Ks: phosphatidylinositol-4-phosphate 5-kinases

PKA: protein kinase A

PKC: Protein kinase C

PLAUR: plasminogen activator

PLC-γ2: phospholipase C-γ2

PLL: Prolymphocytic leukaemia

PMN: polymorphonuclear leukocytes

PMT: Photomultiplier

POACH: prednisolone, vincristine, cytosine arabinoside, cyclophosphamide and doxorubicin

PRRG1: proline rich Gla (G-carboxyglutamic acid) 1

RAMP3: Receptor activity modifying protein 3

RB gene: Retinoblastoma gene

RBC: red blood cells

RDX: radixin gene

RHBDL1: rhomboid, veinlet-like 1

ROR1: receptor tyrosine kinase-like orphan receptor 1

RPMI: Roswell Park Memorial Institute

RQ-PCR: real time quantitative polymerase chain reaction

SAPK: stress-activated protein kinase

SCT: stem cell transplantation

SDC3: syndecan 3

SDF-1: stromal cell-derived factor-1

SEPT: Septin

SH2: Src homology2

SHIP: SH2 domain-containing phosphatidyl 5-phosphatase

SHP-1: SH2 domain-containing tyrosine phosphatase-1

siRNA: Small interfering RNA

SIRT1: Sirtuin 1

SLC20A1: Sodium-dependent phosphate transporter 1

SLL: small lymphocytic lymphoma

SLVL: Splenic B cell lymphoma with circulating villous lymphocytes

SMAC: second mitochondria-derived activator of caspases

STAT3: Signal transducer and activator of transcription 3

STIM1: Stromal interaction molecule 1

TAG1: Transient axonal glycoprotein-1

TCF7: transcription factor 7

TCL1: T-cell leukemia/lymphoma 1

TCR: T-cell receptor

TGFA: Transforming growth factor alpha

TK: thymidine kinase

TMPRSS6: Transmembrane protease, serine 6

TNFR1: tumour necrosis factor receptor 1

TNF-α: tumour necrosis factor α

TP53: tumor protein p53

TRAILR 1: TNF-related apoptosis inducing ligand receptor 1

TRAMP: Tyrosine-rich acidic matrix protein

VAD: vincristine, doxorubicin, dexamethasone

VCAM-1: vascular cellular adhesion molecule-1

VDJ: Variable, Diverse, and Joining

VLA-4: Very Late Antigen-4

w/v: weight/volume

WM: Waldenström’s macroglobulinaemia

XID: X-linked immunodeficiency

XLA: X-linked agammaglobulinemia

ZAP70: Zeta-chain-associated protein kinase 70

β2MG: β2 microglobulin

1. Introduction

1.1 Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia of the western world and is characterised by an accumulation of monomorphic, non-proliferating, small, mature, functionally abnormal B-lymphocytes in peripheral blood, bone marrow and lymphoid tissues. Even though leukaemia was first described in 1845, CLL was recognised as a separate clinical disorder towards the beginning of the 20th century (*The Edinburgh Medical and Surgical Journal*, 1845). There were several case summaries reported in the early part of the last century and some mention about CLL in the textbook published by Sir William Osler, *The Principles and Practice of Medicine.* The first comprehensive clinical reports on 80 patients with CLL were published by Minot and Isaacs in 1924 (Minot B, 1924) (Osler, 1909) (Rai, 1993).

1.1.1. Demographics

The incidence of CLL varies hugely across the world, being highest in North America and extremely rare in the Far East. In the UK CLL accounts for approximately 11% of all newly diagnosed haematological neoplasms. The incidence of CLL in the UK is 6.9 per 100,000 population with a male to female ratio of 1.7 (“HMRN - Cancer Information,” 2011). CLL is a disease of older age with a median age at diagnosis of 71 years and is virtually unknown before the age of 15. The age-wise incidence of the disease in the UK is shown in Figure 1.1 (“HMRN - Cancer Information,” 2011). This data is taken from statistical data published by the Epidemiology & Cancer Statistics Group at the University of York. Although most patients are elderly, approximately 10% of the patients are younger than 50 years old (de Lima et al., 1998) (Mauro et al., 1999). The incidence of CLL has not increased over the past 30 years, in contrast to the other non-Hodgkin’s lymphomas.

Initially, morphology was the sole modality of diagnosis, as with any other type of leukaemia. Over many years, as diagnostic armamentarium improved in medicine, several diagnostic tests were introduced which helped to distinguish the various low grade lymphoproliferative disorders with somewhat similar morphological appearance, which were originally all considered to be CLL (Rozman and Montserrat, 1995). Currently the diagnosis of CLL can be made in a majority of patients by morphology and immunophenotyping of peripheral

blood, but a minority of patients need an integrated approach including immunohistochemistry and the analysis of chromosome deletions, duplications and translocations to make an accurate diagnosis (J. B. Johnston, 2003). The distinction of CLL from other similar LPDs is important prognostically and is used increasingly to target specific therapies.

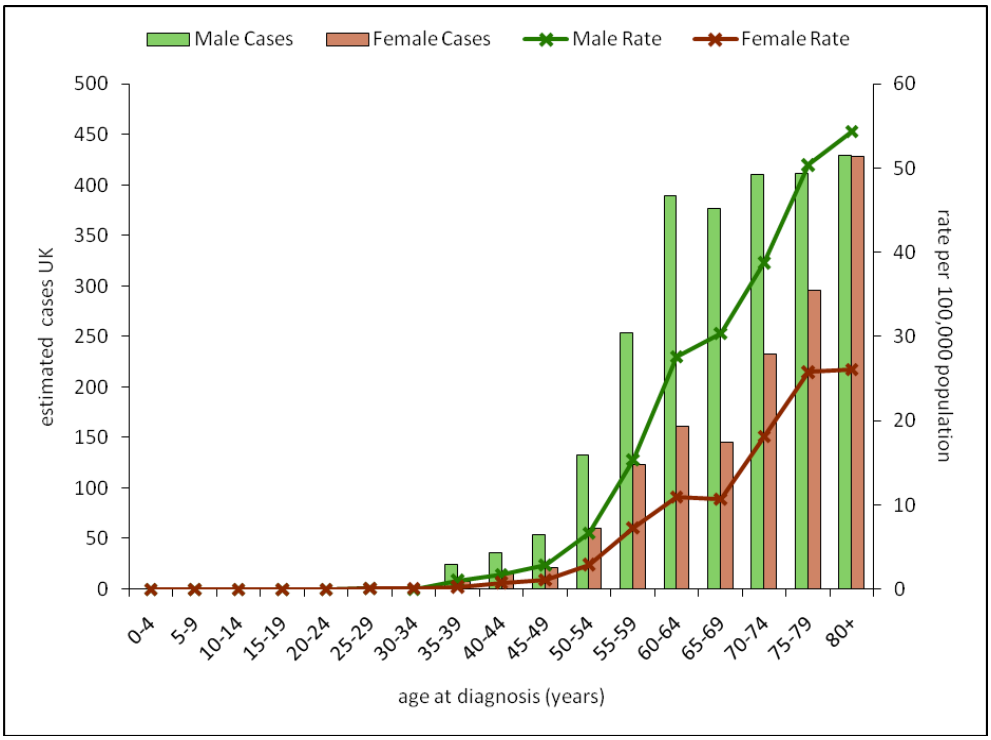


Figure 1.1 Estimated age specific incidence of CLL in UK

This figure is taken from statistical data published by the Epidemiology & Cancer Statistics Group at the University of York.("HMRN - Cancer Information," 2011)

Historically the disease was considered as a homogeneous disease of immature, minimally self-renewing B-cells which are immunologically incompetent. These cells were considered to have a faulty apoptotic mechanism and thereby they accumulate persistently (Hoffman et al., 2009) (Dameshek, 1967). But according to the current concept there is considerable heterogeneity in all aspects of biological and clinical profiles of CLL including cellular morphology, phenotype, cell biology, molecular genetics and prognosis (Chiorazzi et al., 2005). Clinically, in about a third of patients the disease has a very stable course without the requirement for any treatment. In about another third, the disease initially has an indolent course but later progresses and requires treatment. In the remaining third the disease has a very aggressive behaviour and the patients die within a span of few years due to disease-related causes, despite treatment (Dighiero, 2005) (Chiorazzi et al., 2005). Patients with an indolent form of CLL survive more

than 10 years after diagnosis, on average, and often do not require immediate treatment, while patients with an aggressive form of CLL have an average survival of 2 years despite several lines of treatment (Abrisqueta et al., 2009) (Chiorazzi et al., 2005).

1.1.2. Cell Biology and Cell of Origin

There are several controversies regarding the cell of origin in CLL (Dighiero et al., 1996). It was originally thought that CLL arose from naive, antigen-inexperienced B-lymphocytes that circulate in the resting state due to their appearance as small lymphocytes with high nuclear-to-cytoplasmic ratios and their surface membrane co-expression of immunoglobulin-M (IgM) and IgD (Bennett et al., 1989) (Caligaris-Cappio et al., 1993) (Coffman and Cohn, 1977). CLL cells express CD5, a T-lymphocyte marker, along with CD23 and weak surface immunoglobulin: a phenotype of mature, activated B lymphocytes. These cells are similar to the B1 cells in mice (Kantor, 1991) and it was assumed that the normal counterpart was CD5+ B lymphocytes which are present in the mantle zone of secondary lymphoid follicles (Dighiero et al., 1996) (Schena et al., 1992) (Kovaleva et al., 2012). This cell type is also present in the peripheral blood in small numbers and is the predominant B-cell population in foetal spleen and peripheral blood. Despite this unique surface phenotype, there is considerable heterogeneity among patients with regards to various molecular, functional and clinical aspects. So it is difficult to draw any conclusion on cell of origin based on phenotypic markers alone. This is especially pronounced in the observation that patients with CLL can almost equally be divided into two distinct groups: some have somatic mutations in the immunoglobulin variable region of the heavy-chain (*IGHV*) genes (V_H mutated) and others have immunoglobulin gene sequences very close to the germ-line sequence (V_H unmutated). Normal human CD5+ B lymphocytes lack mutation of the *IGHV* genes (Brezinschek et al., 1997). If this is put into the context of normal B-cell differentiation, then V_H unmutated CLL would be derived from a pre-germinal centre naive B-cell and V_H mutated CLL from a memory B-cell, which has traversed the germinal centre, based on the fact that memory B-cells have mutated V genes which give higher affinity for antigen. Interestingly these two forms of CLL have been documented to have preponderance for different genetic abnormalities. Those with mutation are more likely to have 13q14 deletion and those without mutation predominantly have trisomy 12 (Oscier et al., 2002) (Damle et al., 1999) (Krober et al., 2002). Clinically also they behave very differently, as patients with mutated clone have a good prognosis compared to unmutated. From the

functional aspect marginal- zone B-cells could be the precursors of both unmutated and mutated CLL cells, because B-cell receptors in some cases are structurally similar to antibodies that react with autoantigens and carbohydrate components of infectious agents (Chiorazzi and Ferrarini, 2003) (Ghiotto et al., 2004) (Messmer et al., 2004).

Gene expression profile data using twelve thousand genes has shown remarkable similarity between both groups in most of the genes with the exception of 23 that were differentially expressed. The CLL expression profile also showed excellent resemblance to memory B-cells rather than to normal naive B-cells, germinal cells or normal CD5+ B-cells (Klein et al., 2001a). Other groups have also shown similar results even (Rosenwald et al., 2001) though the number of differentially expressed genes was not the same. Notably, a single gene can consistently differentiate between both groups. The ZAP70 kinase, which is predominantly involved in transducing signals from the T-cell receptor, is consistently expressed at a higher level in unmutated patents. All CLL cells express CD27, which is a memory cell marker. Most normal CD27+ B-cells have *IGHV* mutations but a small fraction does not. Expression of membrane markers of cellular activation, presence of mRNA for a wide variety of cytokines as well as the presence of specific memory cell markers like CD27 prompt us to conclude that both mutated and unmutated CLL originate from two types of CD27+ memory cells, one with *IGHV* mutation and the other without the mutation, although the clonal evolution on antigen stimulation may differ in both groups (Chiorazzi et al., 2005). Normal B-cells when maturing in the bone marrow undergo immunoglobulin variable gene segment rearrangement, which translates into the immunoglobulin molecule of the B-cell receptor for binding the antigen. These cells then actively divide in the germinal centre of lymphoid follicle and undergo variable gene somatic hypermutation. The cells with high antigen binding affinity proliferate rapidly, but the ones with poor affinity and those that bind autoantigens eventually get deleted. These processes can happen either in the germinal centres, which is T-cell dependent, or in marginal zones around lymphoid follicles as a response to carbohydrates of encapsulated bacteria or viruses, which is T-cell independent, but the mutation rates in the T-cell independent cells are low. Both these processes generate memory B-cells (Kelsoe, 1994) (de Vinuesa et al., 2000) (Toellner et al., 2002) (William et al., 2002).

However there were several pitfalls for these studies (Seifert et al., 2012). None of these studies included splenic marginal zone (sMGZ) B cells in their

analysis, memory B cells were isolated as bulk CD27+ B cells (Klein et al., 2001b) and not separated as class-switched, CD27+ B cells and IgM+IgD+CD27+ B cells (Klein et al., 1998) and CD5+ B cells were isolated from cord blood, which are mostly transitional B cells and not mature CD5+ B cells as seen in adult peripheral blood.

Seifert et al in 2012 published transcriptome analyses of CLL and the main normal B cell subsets from human blood and spleen including PB naive B cells, memory B cell subsets (class-switched, IgM+IgD+CD27+, and IgM-only B cells), CD5+ B cells (excluding transitional B cells), and sMGZ B cells. This comparison has shown that CD5+ B cells as the normal B cell subset with the most similar gene expression to CLL. They have shown that both unmutated CLL and mutated CLL were highly similar to CD5+ B cells, which are mostly IgV unmutated. They have demonstrated that unmutated CLL was derived from unmutated mature CD5+ B cells and mutated CLL was derived from a distinct, previously unrecognized CD5+CD27+ post–germinal centre B cell subset (Seifert et al., 2012).

Even though chromosomal translocations are common in other types of B cell malignancies and could be considered as the triggering event in oncogenesis, in CLL chromosomal translocations are rare and there are no unifying mutations identified for oncogenesis. Despite cytogenetic abnormalities being rare in the early stage during disease progression some abnormalities appear, the most common being deletion 13q14.3 which happens in around 50% of patients (Dohner et al., 2000) (Stilgenbauer and Zenz, 2002) (Peterson et al., 1992) (Juliusson et al., 1990). As this region of chromosome contains two microRNA genes, which regulate the function of many other genes, down regulation of these genes will predispose the leukaemic clone to undergo additional mutations. Deletion of the 13q14-minimal deleted region, which encodes the *DLEU2/miR-15a/16-1* cluster, in mice causes a clonal lymphoproliferative disorders, mimicking CLL (Klein et al., 2010). The other common alterations are deletion of chromosome 17p13 and 11q22-23 which will involve *TP53* and *ATM* genes, respectively. These genes are important in regulating apoptosis and deletion of these genes will induce resistance to chemotherapy. These abnormalities are seen in only relatively low proportion of patients. 17p deletion is found in only 5% of untreated patients and in 31% of previously treated patients. Likewise ATM deletion is seen in only 15% of untreated patients and 25% of previously treated patients (Zenz et al., 2010b) (Bergmann et al., 2007) (Dohner et al., 2000) (Rossi et al., 2009) (S. Stilgenbauer et al., 2008) (Stilgenbauer et al., 2009) (Zenz et al., 2009). The

increased proportion in previously treated patients indicates the progression of the selected clone which is resistant to chemotherapy or accumulation of genomic aberrations over time (William et al., 2002).

Currently there is evidence to suggest that antigenic stimulation has a significant role in CLL cell proliferation and avoidance of apoptosis. This antigenic stimulation should work in conjunction with other co-stimulatory cells and cytokines. The strong evidence for antigen stimulation comes from remarkable similarity in the B cell receptor (BCR) complex in different groups of patients. This stereotypy is found in around 30% (Murray et al., 2008) of the patients and is usually in the unmutated group with a very poor prognosis. In some groups of patients these similarities are found in a portion of the antigen-binding pocket localised to the heavy (H) chain where there is a similarity in the mutations seen in the V_H gene as well as the preferential combination with particular D or J_H segments. In other groups there is a remarkable similarity in the entire antigen-binding region coded by both heavy and light chains (Chiorazzi et al., 2005) (Tobin et al., 2002) (Tobin et al., 2003) (Ghiotto et al., 2004) (Tobin et al., 2004) (Widhopf et al., 2004). This resemblance is simply not by chance as the probability of finding two cases of CLL with such structurally similar B-cell receptors is more than 1 in a million cases. The precise nature of this antigenic stimulation is not clear. The causal association with specific antigens are well described in related malignancies like gastric marginal zone lymphoma. *Helicobacter pylori* can be demonstrated in this type of lymphoma and eradication of the bacteria can cure the malignancy (Zullo et al., 2010). While the nature of the antigenic stimulus in CLL is unknown, various latent viruses and commensal bacteria have been proposed as the source. Another possibility is environmental antigens or auto-antigens such as the myosin heavy chain could stimulate clonal expansion (Chu et al., 2008). The expression of polyreactive receptors on the CLL cell surface, which could bind both autoantigens and microbial antigens, substantiate this theory (Bröker et al., 1988) (Sthoeger et al., 1989) (Borche et al., 1990) (Schwartz and David Stollar, 1994). The signals from the B-cell receptors are transmitted to the nucleus by various intermediary molecules, the detail of which is described in a separate chapter. CLL-like normal B-cells could respond to external stimulus through the BCR or could be anergic to the stimulus due to previous exposure. In some early stage disease, whose cells are unresponsive to BCR triggering *in vitro*, MAPK, extracellular signal-regulated kinase (Erk) and Mek are constitutively phosphorylated and shows NFAT transcription factor activity, but lack both Akt

and Bad phosphorylation (Muzio et al., 2008). These molecular features are also seen in anergic B-lymphocytes in the mouse system, suggesting similar mechanism in anergic CLL cells (Muzio et al., 2008) (Merrell et al., 2006). This absence of response could be due to the structural alterations in the BCR complex or downstream molecules. This type of response to the stimulus predominantly occurs in unmutated CLL (Merrell et al., 2006).

1.1.3. Apoptosis

It was previously thought that CLL is a disease in which there are significant abnormalities in apoptosis and therefore the abnormal cells accumulate. Even though there is a defect in the apoptotic pathway, it is now clear that there is an active proliferative fraction of cells. This has been shown by experiments with deuterated water in patients (Messmer et al., 2005). Nineteen patients were given deuterated water daily for 84 days, and using gas chromatography and mass spectrometry ^{2}H incorporation into the deoxyribose moiety of DNA of newly divided B-CLL cells was measured. These analyses demonstrated that the leukaemic cell population of each patient had definable and in some cases substantial proliferative rates, varying from 0.1% to greater than 1.0% of the entire clone per day. Proliferative rates greater than 0.35% per day was seen in patients with active or progressive disease compared with those with lower proliferative rates. Thus, CLL is now considered not as a static disease that results simply from accumulation of long-lived lymphocytes but rather a dynamic process involving an active proliferative component as well.

The critical step in apoptosis is activation of caspases, which are cysteine proteases that activate other caspases from inactive pro-forms to active enzymes by proteolytic cleavage at aspartate residues (Hengartner., 2000) (Herr and Debatin., 2001) (Johnstone et al., 2002). Apoptosis can be initiated by two pathways, the mitochondrial/cytochrome c (intrinsic) pathway or the tumour necrosis factor (TNF) death receptor (DR) (extrinsic) pathway (Hengartner., 2000).

The intrinsic pathway is typically activated by DNA damage either by chemotherapy or by radiation that activates the p53 pathway. Subsequently this produces a change in the ratio of Bcl-2: Bax which increases the level of cytochrome-c in cytosol. Cytochrome, in conjugation with deoxyadenosine triphosphate (dATP), activates apoptosis activating factor-1 (Apaf-1). Apaf-1 can activate caspase-9 which then activates caspase-3 and further downstream apoptotic pathway components (Salvesen and Dixit, 1999). Proteins such as Smac and DIABLO are also released from mitochondria into

the cytosol where they bind to and antagonise a family of proteins that are inhibitors of apoptosis, thereby again promoting apoptosis. Certain drugs such as fludarabine promote apoptosis as derivatives of fludarabine for example 2-chlorodeoxyadenosine (CdA) can substitute for dATP in binding to Apaf-1 and induce direct activation of caspase-9 and -3 (Salvesen and Dixit, 1999) (Jia et al., 2001) (Leoni et al., 1998) (Genini et al., 2000a) (Chandra et al., 2002).

The tumour necrosis factor death receptor pathway induces apoptosis by triggering one of the members of the 8 major death receptors which includes tumour necrosis factor receptor 1 (TNFR1); (also known as DR1, CD120a, p55 and p60), CD95 (also known as DR2, APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5, KILLER and TRICK2), DR6, Ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR) (French and Tschopp, 2003) (Wajant, 2003). All these receptors have a cytosolic domain called the death domain which is composed of around 80 amino acids. With appropriate stimulus of receptors by ligands, a number of adapter proteins like Fadd/Mort-1 are recruited to the receptor complex. Two types of signalling complex can be associated with these receptors. The first is a death inducing signalling complex (DISCs) that is associated with CD95, TRAILR1 or TRAILR2 and triggers apoptosis mainly by cleavage of procaspase-8 to caspase-8 (Peter and Krammer, 2003). The second pathway mediated by TNFR, DR3 and DR6 can either form a complex that triggers NF-κB thereby inducing a survival signal or form a second complex that activates procaspase 8 and downstream death signalling (Micheau and Tschopp, 2003). Chemotherapeutic agents may up-regulate some of the receptors, but their mechanism of action cannot be extrapolated fully to this pathway alone (Friesen et al., 1996) (Fulda et al., 2000) (Wen et al., 2000) (Gibson et al., 2000) (Jones et al., 2001).

These pathways can be modified by a variety of proteins mainly belonging to the BCL-2 family. There are around 20 members in this family which can either promote or inhibit apoptosis, and are located in different organelles of cells including the cell membrane, nuclear membrane and mitochondrial membranes. They predominantly function by binding to other proteins or changing the permeability of the mitochondrial membrane and thereby increasing the release of cytochrome c from the mitochondria (Jiang and Wang, 2004). Different members of this family of proteins have distinct and sometimes opposing roles. Some of them like BAX, BCL-X_S, BAK and BAD promote apoptosis while BCL-2, BCL-X_L and MCL-1 inhibit apoptosis (Hanada

et al., 1993) (Robertson et al., 1996) (Johnston et al., 1997) (Kitada et al., 1998) (Pepper et al., 1999) (Bellosillo et al., 1999). Additional examples like bag-1 can influence the activities of others. CLL cells have a high level of BCL-2, BAX and BAK, but have low levels of BCL-x_L and BAD, which will contribute to survival of CLL cells. The transcription factor NF-κB induces the expression of several anti-apoptotic genes, and its nuclear protein level is higher in CLL (Furman et al., 2000). Phosphorylation of IκB leads to its coupling with ubiquitin and proteososmic degradation. This releases NF-κB from IκB which translocates NF-κB to nucleus (Furman et al., 2000). The level of active NF-κB increases when CLL cells are stimulated by cytokines. The protein kinase Akt can suppress apoptosis by phosphorylation of bad, caspase-9 and several other proteins. Akt itself is activated through phosphatidylionositol 3'-kinase (PI3K) (Datta et al., 1999) (Wickremasinghe et al., 2001).

The tumour suppressor gene *TP53* located in the 17p13 region deleted in a portion of CLL cases plays a key role in chemotherapy resistance in CLL. p53 protein is phosphorylated and stabilised after DNA damage (Johnstone et al., 2002) (Vogelstein et al., 2000). Stabilised p53 protein binds to specific sequences in DNA and activate the transcription of adjacent genes (El-Deiry, 1998). Some of these genes regulate 'nucleotide-excision' repair of DNA, chromosomal recombination and chromosome segregation which helps the cell to repair itself (Smith and Seo, 2002). p53 protein can also promote entry of cell into apoptosis after DNA damage by inducing Bax proteins or inhibition of cell cycle by stimulating the expression of $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs) (Reed, 1999) (Wahl et al., 1997). That is the reason why a small proportion of CLL patients whose p53 is deleted or mutated are resistant to most conventional chemotherapeutic agents. Another gene called *ATM* located in chromosome 11q22-23 is responsible for phosphorylation and activation of p53 after DNA damage (Pettitt et al., 2001) (Vogelstein et al., 2000). Deletion or mutation of the *ATM* gene also produces a similar defect as observed with p53 mutation (Stankovic et al., 2002).

1.1.4. Clinical Features

50% of patients are asymptomatic at diagnosis but are picked up by full blood counts done for unrelated reasons (Rozman and Montserrat, 1995). Routine physical examination can occasionally prompt the diagnosis by revealing a lymphadenopathy or splenomegaly. Symptomatic patient usually complain of fatigue or a vague sense of not feeling well. They can also present with

symptoms of complications like bacterial pneumonia. Other symptoms like fever, night sweats and weight loss occur only in a minority of patients. Fever occurs in 9%, night sweats in 24.6% and weight loss in 10.6% of patients with CLL (Shanafelt et al., 2007). Patients can also present with symptoms of anaemia due to infiltration of bone marrow, autoimmune haemolytic anaemia or rarely aplasia. Bleeding manifestation can rarely be a presenting feature due to thrombocytopenia, either due to bone marrow infiltration or immune causes. Physical examination shows lymphadenopathy in 80% of symptomatic patients and splenomegaly in 50%. Lymphadenopathy is usually painless and predominantly involves the cervical region (Johnston, 2003).

1.1.5. Diagnosis and differential diagnosis

Diagnosis of CLL is made when a peripheral B-lymphocyte count is at least more than $5x10^9$/L and the clonality of the circulating B-lymphocytes confirmed by flow cytometry (Hallek et al., 2008). Peripheral blood morphology is usually the initial evaluation in the diagnosis of CLL. The lymphocyte count can range from slightly above normal to greatly elevated and typically have a uniform appearance of small to medium size cells, but in around 20% of patients cells can have an atypical appearance (Matutes et al., 2003).

The diagnosis of CLL is predominantly done by immunophenotyping using flow cytometric methods. The characteristic immunophenotype for CLL is weak expression of sIg, which is usually IgM or IgM with IgD with either κ or λ light chain restriction combined with expression of CD5, CD23, CD43, CD11c and B-cell–associated antigens (CD19, CD20, CD22 and CD79a). Matutes *et al* have recommended a scoring system based on 5 markers to differentiate it from other related B-cell malignancies as shown in Table 1.1 (Matutes et al., 1994).

Differential diagnosis for CLL varies depending on the clinical scenarios (Table 1.2). For presentation as lymphocytosis and cytopenias the main diseases to be differentiated are mantle cell lymphoma, marginal zone lymphoma and Prolymphocytic leukaemia. Very rarely splenic diffuse red pulp small B-cell lymphoma, leukaemic presentation of follicular lymphoma and hairy cell leukaemia can give diagnostic confusion (J. B. Johnston, 2003). Immunophenotyping using flow cytometry is the main method for differentiating these disorders. When CLL presents as predominant lymphadenopathy, follicular lymphoma and diffuse large cell lymphoma need to be clearly differentiated. In most cases morphological features and

immunohistochemistry are adequate to differentiate these disorders. Lymphocytic infiltration at extranodal sites can be a feature of any of the above conditions and again morphology and immunohistochemistry serves as a differentiating tool in most cases.

Table 1.1 Scoring system for diagnosis of CLL

Adapted from Morphological and Immunophenotypic Features of Chronic Lymphocytic Leukaemia. Review in Clinical and Experimental Hematology 2000; 4:22-47 (Matutes and Polliack, 2001).

Marker	Intensity	Score	Intensity	Score
sIg	weak	1	strong	0
CD5	+	1	-	0
CD23	+	1	-	0
CD22/CD79b	weak	1	strong	0
FMC7	-	1	+	0
Score of 4 or 5 makes the diagnosis of CLL highly likely				

1.1.6. Prognosis

Because of the heterogeneity of the disease, various markers have been added that are taken into consideration for prognostication. The most important prognostic factors in CLL are clinical stage, markers of tumour load (e.g., thymidine kinase (TK) and β2 microglobulin (β2MG)), cellular protein expression (e.g., CD38 and ZAP70), and genetic parameters including immunoglobulin heavy chain variable gene segment (*IGHV*) mutational status, genomic aberrations and individual gene mutations. Two separate clinical staging systems by Rai and Binet consider physical examination findings including lymphadenopathy and organomegaly, and platelet and haemoglobin values on blood count. Even though the prognostic impact of these staging systems was confirmed in many independent studies, there is still heterogeneity in the course of the disease of patients within a single stage group (Rai et al., 1975) (Binet et al., 1981). Various parameters of disease activity and tumour burden such as the lymphocyte count, the lymphocyte doubling time, the serum LDH level or the bone marrow (BM) infiltration pattern were shown to be of prognostic relevance in CLL (Zenz et al., 2010b).

Table 1.2 Differential diagnosis of CLL - Immunophenotypic features

Marker	**CLL**	**MCL**	**MZL /LPL /WM /SLVL**	**PLL**	**HCL**	**FL**
SIg	Weak	Strong	Strong	Strong	Strong	Positive
CD5	Positive	Positive	Negative	Negative or positive	Negative	Negative
CD23	Positive	Negative or weak	Usually Negative	Positive	Negative or weak	Variable
FMC7	Negative or weak	Positive	Positive	Positive	Strong	Positive
CD79b	Negative or weak	Positive	Positive	Positive	Positive	Positive
CD20	weak	Positive	Positive	Strong	Strong	Positive
CD22	Negative or weak	Positive	Positive	Strong	Strong	Positive
CD19	Positive	Positive	Positive	Strong	Strong	Weak
CD79a	Positive	Positive	Positive	Strong	Positive	Positive
CD43	Positive	Positive	Negative	Positive	Variable	Negative
Cd11c	Variable	Weak or negative	Variable	Weak	Positive	Negative
CD10	Negative	Negative	Negative	Negative	Variable	Positive
CD103	Negative	Negative	Negative	Negative	Positive	Negative
CD25	Variable	Variable	Variable	Negative or weak	Positive	Negative
Cyclin D1	Negative	Positive	Negative	Negative	Weak	Negative
SOX11	Negative	Positive	Negative	Negative	Weak	Negative

A number of serologic parameters such as thymidine kinase (TK), β2-microglobulin, TNF-α and soluble CD23 have been shown to provide some information on outcome as well (Wierda et al., 2009) (Hallek et al., 1999)

(Montserrat et al., 2008) (Rozman et al., 1984) (Kantarjian et al., 1992). There are however currently little data to suggest that they have predictive properties, which could be used to guide treatment decisions.

1.1.6.1. IGHV mutational status and surrogate markers

CLL cases can be divided into two broad classes based on the degree of somatic hypermutation in the immunoglobulin gene region; those with mutated *IGHV* genes and those with unmutated *IGHV* genes. The cut-off value underlying this separation is taken as less or more than 98% sequence identity with the germ line sequence (Krober et al., 2002) (Damle et al., 1999) (Hamblin et al., 1999). The approximate frequency of this in various stages of treatment are shown in Table 1.3 (Dohner et al., 2000) (Stilgenbauer et al., 2009).

Table 1.3 Frequency of mutated and unmutated CLL based on stage of treatment

Adapted from "Moving from prognostic to predictive factors in chronic lymphocytic leukaemia (CLL)". Best Practice & Research Clinical Haematology (Zenz et al., 2010b)

	Early stage CLL	Untreated	After 1st line treatment	Refractory CLL
mutated *IGHV*	61%	46%	34%	24%
unmutated *IGHV*	39%	54%	66%	76%

The two groups differ widely in their clinical progression; while CLL with unmutated *IGHV* follows an unfavourable course with rapid progression and earlier death, CLL with mutated *IGHV* often shows slow progression and long survival (Damle et al., 1999). *IGHV* mutation status is of prognostic importance in all groups of patients including unselected patient cohorts, after treatment, as well as in early stage (Binet A) patients (Catovsky et al., 2007) (S. Stilgenbauer et al., 2008). These groups differ in prognosis not simply by chance, but due to significant biological differences. Unmutated *IGHV* CLLs have higher levels of the intracellular expression of protein kinase Zeta associated protein 70 (ZAP70) and surface expression CD38. There is a difference in mRNA expression profile between these groups as described earlier in this chapter. Upon stimulation of the BCR complex the unmutated CLL cells have a higher potential of generating downstream signalling

cascades (Kipps, 2007) (Klein et al., 2001b) (A Rosenwald et al., 2001) (Wiestner et al., 2003). A high proportion of *IGHV* unmutated CLL cases carry stereotyped rearrangements of the VDJ gene segments, with very similar complementarity determining region (CDR) 3 regions. In general, more than 20% of CLL patients carry these stereotypic BCRs. Stereotyped BCRs are less likely to occur in *IGHV* mutated CLL. CLL with unmutated *IGHV* are more likely to have polyreactive B-cell receptors to antigens derived from endogenous or exogenous proteins whereas cases with mutated *IGHV* exhibit restricted antigen binding with oligo- or monoreactive BCRs (Zenz et al., 2010c). Specific V-genes in the VDJ rearrangement appear to be associated with distinct biological and clinical features (e.g., VH3-21) (Ghia et al., 2005) (Murray et al., 2008) (Stamatopoulos et al., 2007) (Tobin et al., 2004).

As routine use of *IGHV* sequencing is difficult in all labs certain surrogate markers were developed. ZAP70 expression is widely used in this context as it was identified based on gene expression profiling studies (A Rosenwald et al., 2001). In all patients in whom at least 20% of the leukaemic cells were positive for ZAP70 by flow cytometry, *IGHV* was unmutated, whereas *IGHV* mutations were found in 21 of 24 patients in whom less than 20% of the leukaemic cells were positive for ZAP70 (Crespo et al., 2003) (Orchard et al., 2004) (Rassenti et al., 2004) (Kröber et al., 2006). The prognostic impact of ZAP70 expression has been confirmed in many studies and several studies have shown a correlation of high ZAP70 expression and unmutated *IGHV* genes and BCR function (Chen et al., 2002). But this correlation is discordant in 25% of cases and the proportion of this discordance may be particularly high in the distinct subgroups with V3-21 usage and 17p or 11q deletion (39%) (Kröber et al., 2006). In the absence of high-risk genomic aberrations, the status of *IGHV* and ZAP70 may have a similar prognostic impact, and might therefore be alternatively applied. A major problem concerning ZAP70 determination in routine clinical practice is the challenge in the standardisation of an FACS assay for its measurement (Rossi et al., 2010).

CD38 is another surrogate marker, which is a molecule widely expressed in a variety of haematopoietic cells, including thymic cells, stem cells, activated T-cells, B-cells and plasma cells. The expression of CD38 is an important prognostic marker in CLL. The initial studies suggested that CD38 could be used as a surrogate marker for mutational status, as CD38 positivity was associated with the unmutated group and has a poor prognosis compared to the CD38 negative group, which is associated with mutated group and better prognosis (Damle et al., 1999). However, later studies have shown that CD38

has an independent prognostic value in CLL and some studies suggest that combination of CD38 and *IGHV* mutational status has an even greater prognostic power than either marker alone (Damle et al., 1999) (Krober et al., 2002) (Ibrahim et al., 2001) (Jelinek et al., 2001) (Hamblin et al., 2002) (Matrai et al., 2001) (Vasconcelos et al., 2003). The pattern of CD38 expression can be homogenously positive, homogenously negative or can be bimodal where subsets of CD38-positive and CD38-negative cells are seen in the same population (Ghia et al., 2003). The prognostic significance of this bimodal pattern is not clear. Some studies have shown a concordance between 72 and 95% when ZAP70 is used as a surrogate marker (Crespo et al., 2003) (Dürig et al., 2003) (Rassenti et al., 2004), but another study has shown that analysing CD38, ZAP70 and *IGHV* mutational status together will give a more discriminatory prediction of time to first treatment and overall survival (Matutes et al., 2008). Unfortunately, such discordant results are difficult to interpret.

Another molecule of interest as a surrogate marker is FCRL (human Fc receptor-like molecules) (Li et al., 2008). There are 5 human Fc receptor–like molecules (FCRL1-5) which have tyrosine-based immunoregulatory potential and are expressed by B-lineage subpopulations. In a study of 107 CLL patients FCRL1, FCRL2, FCRL3 and FCRL5 were found at markedly higher levels on CLL cells with mutated *IGHV* genes than on unmutated CLL cells. Univariate analysis showed that FCRL expression was strongly associated with *IGHV* mutation status and FCRL2 specifically maintained independent predictive value by multivariate logistic analysis. FCRL2 demonstrated 94.4% concordance with *IGHV* mutation compared with 76.6% for CD38 and 80.4% for ZAP70. The median treatment-free interval was 15.5 years for patients with high FCRL2 expression compared with 3.75 years for FCRL2 low patients (Li et al., 2008).

Another area of interest is epigenetic markers as they are very stable which makes them excellent molecular markers compared to measurement of levels of proteins or RNA that may change in the course of disease. Therefore, DNA methylation of genes whose products have been associated with a prognostic value such as ZAP70 or CD38 can be used in this context and they have been found to correlate with prognosis and *IGHV* mutation status (Corcoran et al., 2005) (Esteller, 2008).

Gene expression profiling has shown that a number of other genes could be identified with differential expression based on *IGHV* status, suggesting that expression levels of these genes may be used as surrogate markers. In one

study using unpurified samples from 130 CLL patients, genes were tested with real time quantitative polymerase chain reaction (RQ-PCR) (van't Veer et al., 2006). Multivariate logistic regression analysis showed that expression levels of *LPL*, *ZAP70*, *ADAM29* and *SEPT10* were the most highly correlated with *IGHV* mutational status. Among these, expression of *LPL* was the single best predictor in multivariate analysis. Another study using CD19+ selected samples from 151 CLL patients with same technique on genes including *ADAM29, ATM, CLLU1, DMD, GLO1, HCSL1, KIAA0977, LPL, MGC9913, PCDH9, PEG10, SEPT10, TCF7, TCL1, TP53, VIM, ZAP70,* and *ZNF2*. *ZAP70* has achieved the highest assignment rate (81%) for patients with genetic risk (*IGHV* unmutated, V3-21 usage, 11q- or 17p-), followed by *LPL* and *TCF7* (76% both). This rate was improved to 88% if *ZAP70*, *TCF7*, *DMD* and *ATM* were combined. Multivariate analysis of treatment-free survival has shown that *IGHV* mutation status and expression of *ADAM29* were of independent prognostic value besides disease stage (Kienle et al., 2010). For overall survival (OS), expression of *ATM*, *ADAM29*, *TCL1* and *SEPT10* provided independent prognostic information in addition to clinical and genetic factors. But these factors are only in the early stage of development which needs further evaluation in independent studies to confirm their validity (van't Veer et al., 2006) (Kienle et al., 2010).

1.1.6.2. Chromosomal aberrations

Recurrent chromosomal abnormalities are found in approximately 80% of CLL cases. Some of these have significant prognostic impact. The frequency at various stages is shown in Table 1.4 (Dohner et al., 2000).

1.1.6.2.1. Deletion 13q14

The most common structural aberration found in CLL is deletion of 13q14 and it is associated with a favourable prognosis, mutated *IGHV* gene and classical CLL cell morphology (Dohner et al., 2000). Approximately 50% of the abnormalities involve an interstitial deletion and this is usually associated with loss of the *RB* gene encoding the tumour suppressor protein Retinoblastoma (Juliusson and Merup, 1998) (Juliusson et al., 1990). The other half involves a translocation affecting the *RB* gene region. Retinoblastoma is involved in cellular proliferation and loss of the same causes cell cycle progression and tumour development (Kornblau et al., 1994) (Stilgenbauer et al., 1993). Recent evidence has shown that *miR15* and *miR16* lie within a small region of chromosome 13q14 that is deleted in more than 65% of CLL and that allelic loss in this region correlates with down-regulation of both *miR15* and *miR16*

expression suggesting that these genes represent the targets of inactivation by allelic loss in CLL (Calin et al., 2002) (Cimmino et al., 2005) (Lagos-Quintana et al., 2001).

Table 1.4 Frequency of chromosomal abnormalities based on stage of treatment

Adapted from "Moving from prognostic to predictive factors in chronic lymphocytic leukaemia (CLL). Best Practice & Research Clinical Haematology" (Zenz et al., 2010b).

	Early stage CLL	Untreated	After 1st line treatment	Refractory CLL
deletion 13q14 as single aberration	48%	36%	36%	22%
trisomy 12	12%	15%	14%	12%
deletion 11q23	9%	15%	21%	25%
deletion 17p13	3%	5%	5%	31%
TP53 mutation	unknown	10%	8%	37%

1.1.6.2.2. Deletions of 11q22-q23

This deletion is seen in around 20% of cases of predominantly younger patients and usually is associated with marked lymphadenopathy, rapid disease progression and poor survival (Dohner et al., 2000) (Monni and Knuutila, 2001) (Sembries et al., 1999) (Aalto et al., 2001). The deleted region at 11q22-q23 involves the radixin (RDX) gene which has homology to the neurofibromatosis-type 2 (NF-2) tumour suppressor gene and the ATM (‘Ataxia Telangiectasia Mutated’) gene (Sembries et al., 1999). Mutations have been shown to be present in 12% of all patients and about a third of cases with 11q deletion (Austen et al., 2005) (Austen et al., 2007). The ATM protein kinase is crucial in the cell’s response to DNA damage and DNA double-strand breaks caused by chemotherapy or irradiation. Even though earlier trials have shown that this deletion is associated with poor outcome, interestingly, there is evidence from recent clinical trials that more intensive combination chemotherapy may be particularly beneficial in patients with 11q deletion and the addition of the anti-CD20 antibody rituximab may further enhance efficacy (S. Stilgenbauer et al., 2008). In the German CLL8 trial the

addition of rituximab to fludarabine and cyclophosphamide increased the complete remission CR rate from 15.5% to 53.2% in the subgroup of patients with 11q deletion and progression-free survival (PFS) ($p < 0.001$) and overall survival (OS) ($p=0.004$) were also markedly improved. These data may suggest that chemo-immunotherapy may overcome the prognostic impact of 11q deletion (Hallek et al., 2010a).

1.1.6.2.3. Deletions of 17p13 and TP53 mutations

Deletion of 17p13 is found in about 3–7% of CLL at diagnosis and at initiation of first treatment but is much higher in relapsed refractory patients. The 17p deletion always affects band 17p13 which includes the tumour suppressor *TP53* (Dohner et al., 1995) (Zenz et al., 2010). The p53 pathway is critical in the cellular response to DNA damage, either by facilitating the repair of the damaged DNA or, if the damage is too great, leading to cell-cycle arrest and/or apoptosis (Prives and Hall, 1999). Most cases with 17p deletion show loss of one copy and mutation of the remaining copy (Dicker et al., 2008) (Rossi et al., 2009) (Zenz et al., 2008) and only a very few cases with 17p deletion will have a functional p53 pathway. Several trials have shown that 17p deletion has been associated with poor response to chemotherapy including alkylating agents and purine analogues and short survival (Dohner et al., 1995). For example, in the German CLL8 trial, the complete remission rate for patients with 17p-deleted CLL treated with FCR was 5% compared with 50% for those patients who did not have this genetic abnormality (Hallek et al., 2010a). The median progression-free survival PFS for patients with 17p deletion was only 11.2 months, compared with 51.8 months for FCR generally and with only 38.1% surviving 36 months after frontline FCR therapy in patients with 17p deletion. There is reasonable evidence to suggest that the mechanism of action of non-chemotherapeutic agents such as steroids, anti-CD52 antibody alemtuzumab, lenalidomide and flavopiridol is independent of the p53 pathway and may be associated with a better outcome in treating patients with p53 dysfunction (Stilgenbauer et al., 2009) (Byrd et al., 2007) (Chanan-Khan et al., 2006) . However, this has to be tested in prospective randomised controlled trials. The current guidelines for CLL treatment recommend testing for 17p deletion before treatment and if positive they should be treated with these agents and possibly upfront allogeneic stem cell transplantation (Oscier et al., 2012). Mutations of *TP53* are found in roughly 10% of patients with untreated CLL (Dohner et al., 1995) (Zenz et al., 2008) (El Rouby et al., 1993) (Trbusek et al., 2006) and the behaviour of cases with

only the *TP53* mutation is very similar to cases with deletion of one allele and mutation of the remaining allele (Dicker et al., 2008) (Rossi et al., 2009).

1.1.6.2.4. Trisomy 12

Trisomy 12q13 is a frequent aberration in CLL (10–20%) and occurs as a duplication of one homologue. The oncogenes targeted by the trisomy are unknown. Earlier trials have shown that the survival is shortened when trisomy 12 is assessed by conventional cytogenetics, but in prospective trials using by FISH analysis poor outcome for this group has not been confirmed with regard to overall survival OS when assessed according to the hierarchical model (i.e., trisomy 12 without 17p or 11q deletion) (Dohner et al., 2000) (Juliusson et al., 1990) (S. Stilgenbauer et al., 2008). Trisomy 12 is not selected over time, as the incidence of does not increase with advanced stage or progression to refractory disease.

Other important prognostic markers, which are important in the post treatment context, are fludarabine refractoriness and MRD status. Biological poor prognostic markers like 17p del, 11q del and unmutated *IGHV* did not show any survival disadvantages in this fludarabine refractory group of patients. This was demonstrated in the UK CLL202 CAMFLUD trial and German CLL2H study (Varghese et al., 2010b) (Stilgenbauer et al., 2009). Another important marker of post treatment prognosis is the MRD after the treatment. Retrospective and prospective analyses have shown that MRD status post treatment is an independent predictor of PFS and OS. The details are given as a separate section in this chapter.

1.1.7. Treatment

1.1.7.1. Chemotherapy

Until recently, the treatment of symptomatic CLL was alkylating agents like chlorambucil, which gave an overall response rate of 40-60% and complete remission (CR) rate of 4 to 10% (Rai et al., 2000) (Sawitsky et al., 1977) (Knospe and Huguley, 1974) (Robak et al., 2000). Alkylating agents produce an anti-tumour effect by binding covalently with DNA, RNA and proteins (Begleiter et al., 1996) (Panasci et al., 2001). Steroid added to the chlorambucil improved the speed of response, but the response rate and survival hasn’t changed much (Han et al., 1973). Various combination regimens have been tried to improve the response rate and survival over chlorambucil. Combinations like cyclophosphamide, vincristine and prednisolone (COP) (Liepman and Votaw, 1978) (French Cooperative Group,

1990); (French Cooperative Group, 1986) (French Cooperative Group, 1989) (Hansen et al., 1987); cyclophosphamide, doxorubicin and prednisolone (CAP); cyclophosphamide, bischloroethylnitrosourea, melphalan and prednisolone; prednisolone, vincristine, cytosine arabinoside, cyclophosphamide and doxorubicin (POACH) (Keating et al., 1988); vincristine, doxorubicin, dexamethasone (VAD) (Friedenberg et al., 1993) did not improve the response rate or survival compared to chlorambucil. A meta-analysis of ten randomised trials involving 2035 patients comparing chlorambucil plus or minus prednisolone with various combination regimens including COP, CHOP and chlorambucil/epirubicil did not show any survival advantage for these combination regimens over chlorambucil (Trialists' Collaborative Group, 1999).

Another major group of drugs used in CLL treatment are nucleoside analogues. Among these fludarabine is the most commonly used in the treatment of CLL. Its active metabolite F-ara-A is formed by rapid dephosphorylation in plasma. It is lethal to lymphocytes in different ways. Firstly its triphosphate form can induce DNA breaks and damage which indirectly release cytochrome c from the mitochondrial membrane to the cytosol. This activates caspase-9 by triggering the intermediary molecules and thereby pushing cell into apoptosis (Genini et al., 2000a). Secondly increased levels of triphosphate can enhance the effect of endogenous dATP on the apoptosome inducing apoptosis (Genini et al., 2000b) (Genini et al., 2000a). Various groups have compared fludarabine with chlorambucil or combination chemotherapies (Johnson et al., 1996) (Leporrier et al., 2001) (Rai et al., 2000) (Catovsky et al., 2007). The European cooperative group compared six courses of fludarabine against CAP regimen. In previously untreated patients the response rates were similar, but duration of response is slightly better for the fludarabine group (Johnson et al., 1996). There was a tendency for better survival in the fludarabine group but the follow up was too short to draw a statistically significant conclusion. In previously treated patients the response rate was better in the fludarabine group without any difference in duration of response or survival. The French Cooperative Group compared CHOP, CAP and fludarabine. The response was better with CHOP and fludarabine compared to CAP but similar to each other (Leporrier et al., 2001). There was no significant difference in time to relapse or survival duration. North American Intergroup study compared fludarabine with chlorambucil or a combination of fludarabine and chlorambucil. The combination arm was stopped in between due to the concerns of toxicity (Rai

et al., 2000). The response with fludarabine was better than chlorambucil but this has not been translated to a survival advantage in this trial also. In 2007 the LRFCLL4 trial was published which was an international randomised control trial comparing fludarabine plus cyclophosphamide (FC) vs. fludarabine vs. chlorambucil involving 777 patients (Catovsky et al., 2007). It showed a statistically significant superiority for FC compared to fludarabine or chlorambucil in complete response, overall response rates and progression-free survival at 5 years, without much difference in overall survival.

1.1.7.2. Monoclonal antibodies

1.1.7.2.1. Anti-CD20 antibodies

The chimeric monoclonal antibody rituximab, containing a human IgG1 immunoglobulin constant region and a murine variable region, was the first monoclonal antibody approved by the US Food and Drug Administration (FDA) for the treatment of a human malignancy initially in relapsed or refractory, low-grade or follicular and CD20+ non-Hodgkin's lymphomas. Rituximab is targeted against the cell surface antigen CD20 which is important in B-cell activation, differentiation and proliferation. The anti-tumour effect has shown to be due to various mechanisms like complement-mediated cytotoxicity, antibody-dependent cell-mediated cytotoxicity and by direct induction of apoptosis (Maloney et al., 2002) (Byrd et al., 2002). Following the demonstration of its activity in NHL, it was tried in patients with CLL/small lymphocytic lymphoma (SLL) as a single agent. Response rates in relapsed or refractory CLL/SLL are very modest with a PR rate of only 10–15% without any complete response (O'Brien et al., 2001a) (Lin et al., 2003). When used in previously untreated patients higher response rates of 51% with 4% CR were observed; but responses were not durable, with a median PFS of only 18.6 months (Byrd et al., 2001) Several small trials have shown that rituximab in combination with chemotherapy improved the outcome compared to these agents used on their own. In 2010, two randomised control trials, REACH and the German CLL8 trial, have shown that adding rituximab to chemotherapy (Fludarabine and cyclophosphamide) will improve the overall response, complete remission, progression free survival and overall survival in previously treated and untreated patients, respectively (Hallek et al., 2010a) (Hallek et al., 2009), and now FCR is accepted as the standard of care in the treatment of CLL in a subset of patients. There are two newer anti-CD20 antibodies available for the treatment of CLL. GA-101 and ofatumumab are fully humanised monoclonal antibodies targeting an epitope of the CD20

molecule distinct from that of rituximab. *In vitro* experiments with ofatumumab has shown better binding to CD20 than rituximab, a slower off-rate, stronger complement-mediated toxicity and ability to kill rituximab-resistant cells as well as those with low expression of CD20, as exhibited by CLL cells. Ofatumumab has been licensed for use in fludarabine and alemtuzumab refractory CLL, as it has shown a response rate of around 50% in this group of patient, but unfortunately the response is not very durable (Wierda et al., 2010).

1.1.7.2.2. Anti-CD52 antibody

Alemtuzumab, which is a humanised monoclonal antibody specific for CD52, is another antibody useful in the treatment of CLL (Stilgenbauer et al., 2009) (Keating et al., 2002) (Hillmen et al., 2007). It has the ability to cause cell lysis via complement fixation and antibody-dependent cell-mediated cytotoxicity. There is some evidence that alemtuzumab also effects cell killing directly by apoptosis (Nückel et al., 2005). When used as an intravenous (IV) formulation in fludarabine refractory patients it gave an ORR of 33% and a median OS of 16 months (Keating et al., 2002). Other studies have also shown that in previously treated patients alemtuzumab can give a response rate of up to 70% (Osterborg et al., 1997) (Bowen et al., 1997) (Rawstron et al., 1997). As a result of these studies alemtuzumab was granted a product licence in 2001 for the treatment of patients with CLL who had previously received alkylating agents and were refractory to purine analogues.

There are several other monoclonal antibodies against different targets which have been tried in various trials, but none of them were found to be clinically useful (J. C. Byrd et al., 2006) (Leonard et al., 2003) (Frankel et al., 2006).

1.1.7.3. Chemoimmunotherapy

Chemoimmunotherapy is the term used when chemotherapy is combined with monoclonal antibodies like rituximab. Even though this approach has improved the treatment outcome in previously untreated CLL and minimally treated CLL, treatment in the relapsed and refractory setting is much more difficult (Brown, 2011). When fludarabine was used as monotherapy, 20%-37% of patients at initial treatment would fit the standard definition of fludarabine refractoriness (Keating et al., 1998) (Rai et al., 2000). In a single centre study of 147 such patients, only 22% responded to their first salvage therapy, and the median OS was 10 months (O'Brien et al., 2001b). The maximum response rates seen in those groups of patients were 37% where salvage therapy included purine analogues with alkylators. Combinations of

purine analogue with alkylator agent and with rituximab have reduced fludarabine refractoriness to 14.5% with FC (fludarabine plus cyclophosphamide) or 7.6% with FCR (fludarabine, cyclophosphamide, rituximab) in the German CLL Study Group CLL8 trial (Hallek et al., 2010b). In addition to this refractory subgroup, the overall survival of those patients relapsing within 24 months is also very poor, 21.9 months for those with PFS < 6 months, 21.2 months for PFS 6-12 months, and 47.3 months for PFS 12-< 24 months, compared with median OS not reached for those with PFS >24 months. Therefore, even with FC or FCR, approximately one-third of patients had significant treatment resistance. Not surprisingly, there was a high proportion of 17p deletion in this group, which was present in 34% of the refractory group (Zenz et al., 2010a).

Combination immunochemotherapy like FCR, FCMR (mitoxantrone with FCR), OFAR (oxaliplatin, fludarabine, cytarabine, rituximab), alemtuzumab on its own or combination with FCR (CFAR) and ofatumumab are used in this situation (Badoux et al., 2011) (Badoux et al., 2009) (Tsimberidou et al., 2008) (Wierda et al., 2010). But disease becomes increasingly resistant to treatment. Given that conventional chemo-immunotherapy is not a good option in these patients, alternatives include other approved or available therapies with better activity, stem cell transplantation (SCT), or novel investigational agents (Brown, 2011).

1.1.7.4. Newer agents

Several agents have shown promising results in early phase trials. Lenalidomide, an immunomodulatory drug has shown some efficacy in relapsed refractory patients, but has poor tolerability in many patients due to myelosuppression and has no long-term control of the disease (Chanan-Khan et al., 2006). Flavopiridol, a pan-inhibitor of cyclin-dependent kinases, including CDK9, that potentially induces apoptosis in primary human CLL cells, has shown significant preclinical activity as well as reasonable clinical activity (Byrd et al., 2007). However, the development of this drug has been suspended due to severe toxicity including grade 3 or greater tumour lysis syndrome (19%), infection (32%), and diarrhoea (17%). Dinaciclib a potent and selective inhibitor of the cyclin dependent kinases CDK 1, 2, 5, and 9 has shown some clinical response in phase 1 trial (Flynn et al., 2010). ABT-263 (navitoclax) is a small-molecule that potently inhibits BCL-2, BCL-xL, and BCL-w and is able to induce apoptosis in primary CLL cells *in vitro* with a half-maximal effective concentration (EC50) of 4.5nM. Phase 1 trial has shown

ORR of 31%, all PRs, with median PFS and time to progression of 25 months (Roberts et al., 2012), but significant thrombocytopenia limited the use of this drug in relapsed and refractory patients. ABT-199, a selective BCL-2 blocker has shown promising activity with a response rate of 84%, including 20% CR in relapsed refractory patients in a phase 1 trial (Seymour et al., 2013).

Another interesting target for CLL treatment is the B-cell receptor signalling pathway. Two molecules has shown very promising activity, GS-1101 (GS-1101 is a specific inhibitor of the delta isoform of Phosphatidylinositol-3-kinase (PI3K) inhibitors and ibrutinib, an irreversible covalent inhibitor of BTK, a kinase that is required for B-cell development and function (Byrd et al., 2013) (Furman et al., 2010). These molecules have shown significant response in early phase trials and the details of these drugs will be described in a later chapter.

Biologic disease characteristics are very important in determining the treatment resistance. Loss or mutations of *TP53* in chromosome 17 and *ATM* in chromosome 11 are well-established reasons for chemotherapy resistance in CLL (Figure 1.2).

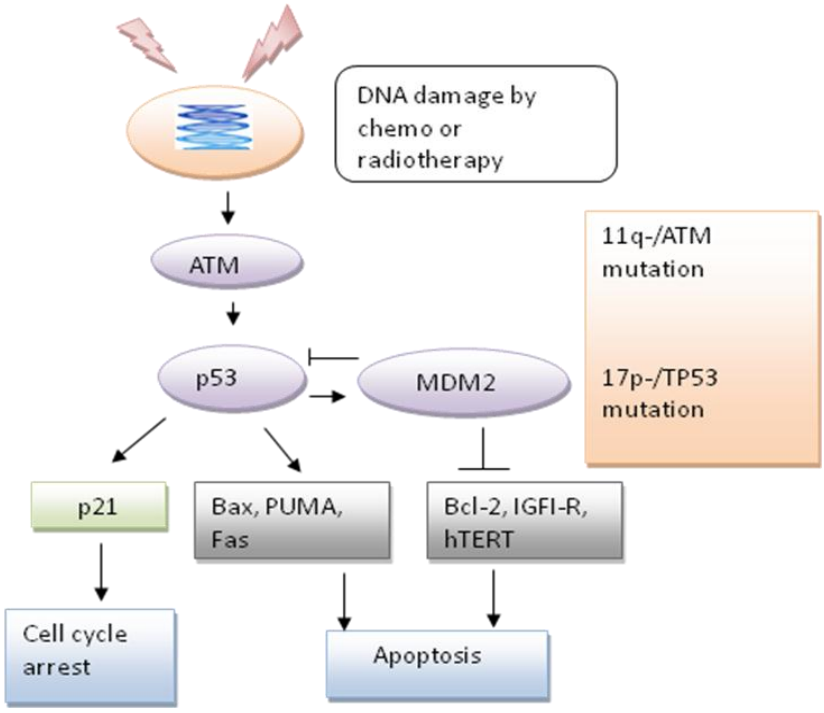


Figure 1.2 p53 pathway

DNA damage by chemo or radiotherapy activates p53 through the activation of ATM. P53 induces either cell cycle arrest or apoptosis through different targets. Various drugs like steroids, Alemtuzumab, lenolidamide and MDM2 inhibitors bypass the p53 pathway.

Their role in cell cycle arrest and apoptosis, when DNA damage occurs due to chemotherapy and radiotherapy, could explain this. But only a proportion of drug-resistant cases are explained by the currently identified factors, such as p53 and ATM (Zenz et al., 2009).

1.1.8. Minimal Residual Disease

Minimal residual disease (MRD) is the term used for small numbers of disease cells that remain during or after treatment, when the patient is in remission according to conventional criteria (Hallek et al., 2008). It has been well established that patients attaining complete remission (CR) have a better survival rate than poor responders (Wierda et al., 2005). This finding led to the concept of improving the quality of response to the greatest possible extent, up to the point of eradication of MRD. With the advent of combination immunochemotherapy, the goal of treatment has changed from disease control in a chronic indolent disease to eradicating the disease to a point where there is no MRD detected and potentially a cure. This has increased the demand for finding newer agents, especially to treat resistant disease. Diagnosing disease at the MRD level is also challenging. It is now widely accepted that MRD negativity in CLL should be set at a threshold of less than a single CLL cell in 10,000 cells per µL, as this is the level that can be reliably detected by modern techniques (Hallek et al., 2008). This is accepted in the guidelines of the International Workshop on Chronic Lymphocytic Leukaemia (IWCLL) in 2008 (Hallek et al., 2008). Current methods for the detection of MRD in CLL use either flow cytometry or PCR. The initial flow cytometric analyses for MRD used the diagnostic technique itself, which is basically detection of co-expression of CD5 and CD19 together with monoclonality of light-chain expression. MRD was considered positive if more than 25% of CD19+ cells co-expressed CD5. Although these techniques are more sensitive than a morphologic assessment, they are only capable of detecting a single malignant cell in about 200 normal cells. Techniques using additional antigens such as CD79b and CD20 were also described, but they were not applicable to everyone, as there are inter-patient variations in antigen expression. The PCR technique initially described used consensus primers, which amplify the immunoglobulin heavy chain (IgH) gene. Again, the sensitivity with this technique was limited, and it was applicable to only 70% to 80% of patients because of *IgH* gene mutation. Later, allele-specific oligonucleotide PCR (ASO-PCR) was developed, in which individual patient-specific oligonucleotide primers were designed to detect MRD in follow-up samples. This technique has the highest sensitivity (as low as 1 in 10^6) but is

expensive, labour-intensive, and impossible to perform in a significant proportion of patients, such as those whose pre-treatment sample is not available. In 2001, Rawstron et al. described a flow cytometric technique that can differentiate CLL cells from their normal counterparts on the basis of multicolour flow cytometry studying CD19/CD5/CD20/CD79b expression (Rawstron et al., 2001). This assay is rapid and sensitive, detecting one CLL cell in 10^4 to 10^5 leukocytes; it is also applicable to all patients, even when no pre-treatment specimen is available. Since then, various groups have described other antibody combinations. In 2007, the European Research Initiative on CLL (ERIC) proposed an international standardised approach after analysing various combinations of antibodies and comparing them against the ASO-PCR technique (Rawstron et al., 2007). After analysing 728 paired blood and bone marrow samples, they derived several conclusions: 1) Blood analysis was equally or more sensitive than marrow in 92% of samples, but marrow analysis was necessary to detect MRD within 3 months of alemtuzumab therapy; 2) The κ/λ/CD19/CD5 combination can be used to screen samples and avoid extended analysis in cases with clear evidence of residual disease where all B-cells are CD5+ with light-chain restriction; 3) A CD45/CD14/CD19/CD3 combination or an equivalent can be used to provide a control for CLL cell enumeration and to define the limit of detection; 4) The combination of CD5/CD19 with CD20/CD38, CD81/CD22, and CD79b/CD43 is the best panel to detect MRD with low inter-laboratory variation, low false detection rates and an accuracy of 95.7%. Current methods involve either using allele-specific PCR or flow cytometry. The sensitivity of both techniques is similar, but the PCR technique has several practical limitations. The current flow cytometric technique uses a combination of several antibodies for an accurate estimation of the minimal residual disease. Even though a combination of CD5/CD19 with CD20/CD38, CD81/CD22, and CD79b/CD43 is the best panel to detect MRD, the search for an ideal antibody or combination of antibodies is still continuing.

Over the years several studies have looked into the difference in survival between patients who attained MRD negativity and those who have not after their standard treatment. Most of these studies have concluded that patients who attained MRD negativity will have longer response duration and some of them have shown survival advantage (O'Brien et al., 2003) (Bosch et al., 2002) (Del Poeta et al., 2005) (Tam et al., 2008) (Hillmen et al., 2007) (O'Brien et al., 2003). Attainment of MRD negativity has been demonstrated as an independent predictor of OS and PFS by Kwok *et al* (Kwok et al., 2009).

In this study, data was collected retrospectively from 137 patients who attained at least a PR after their standard treatment, and in whom an MRD assessment was done using a sensitive four-colour flow cytometry. Multivariate analysis showed that achieving MRD negativity in CLL is an independent predictor of survival even with a variety of different treatment approaches and regardless of the lines of therapy. In patients after their first line of treatment, the 5-year PFS was 89% (95%-CI 55-97%) vs. 0% (95%-CI <1%) ($p<0.001$) and the 5-year OS was 95% (95%-CI 61-99%) vs. 53% (95% CI-15-74%) ($p<0.001$) for MRD-negative vs. MRD-positive patients respectively. This data suggests that achieving MRD-negativity after first-line therapy has a profound effect on survival. The most convincing evidence is from the German CLL8 trial which was a randomised control trial assessing the efficacy of FC vs. FCR in previously untreated patients (Hallek et al., 2010b). MRD levels were prospectively quantified in 1,775 blood and bone marrow samples from 493 patients from both arms. Patients were categorised into different MRD groups according to the level of persistent disease- low $<10^{-4}$, intermediate $\geq 10^{-4}$ to $<10^{-2}$, and high $\geq 10^{-2}$. Median PFS was 68.7, 40.5, and 15.4 months for low, intermediate, and high MRD levels, respectively and median OS was 48.4 months in patients with high MRD and was not reached for lower MRD levels when assessed 2 months after therapy. When compared with patients with low MRD level there is a greater risk of disease progression with intermediate and high MRD levels (hazard ratios, 2.49 and 14.7, respectively; both $P < .0001$). In multivariate analyses that included the most important pre-therapeutic risk markers in CLL, MRD remained an independent predictor for OS and PFS. Another important observation is that PFS and OS did not differ between FC and FCR arms once MRD is attained, even though FCR has higher tendency to induce low MRD levels more frequently than FC (Böttcher et al., 2012). Several small trials have looked into consolidation treatment after standard chemotherapy. The only randomised control trial trying to address it prospectively was prematurely stopped due to toxicity issues (Wendtner et al., 2004). But long-term follow up of the small cohort of patients who were consolidated with alemtuzumab has shown that there is a significant survival advantage for the patients who attained MRD negativity post-consolidation (Schweighofer et al., 2009). A recent UK trial, CLL207, was a phase 2 trial which assessed alemtuzumab consolidation post-chemotherapy in patients who responded with low levels of disease. MRD eradication from blood and bone marrow was

attained in 83% of patients at the end of alemtuzumab consolidation. The long-term survival data from this trial is still awaited (Varghese et al., 2010a).

1.1.9. Antigens expressed on CLL cells

Immunophenotyping using flow cytometry for identifying and distinguishing CLL from other B-cell disorders was reported in the early 80's (Koziner et al., 1980) (Wang et al., 1980) (Dillman et al., 1983). During that period several papers were published which identified the co-expression of CD5 (Leu1) and pan–B-cell markers as the phenotypic hallmark of classical chronic lymphocytic leukaemia (Koziner et al., 1980) (Wang et al., 1980) (Dillman et al., 1983). Surface immunoglobulin was used as a marker of B-cell lineage. Since then several markers have been identified that have substantially aided in studying the biology of the disease (Table 1.5). Currently, immunophenotyping is the most useful diagnostic technique available to evaluate various aspects of CLL. Expression of B-cell associated antigens varies in CLL cells. Most of them are either weak or not expressed, which helps to distinguish it from other mature B-cell malignancies. Expression of CD20, a phosphoprotein that may act as calcium channel and plays an important role in cell-cycle progression and differentiation, is dim on CLL cells. CD20 is expressed on all stages of B-cell development except on pro-B-cells or plasma cells. This is a crucial molecule in terms of treatment for CLL.

Expression of CD19, another pan-B-cell marker, is also dim on CLL cells. CD19 is an important molecule in B-cell activation. It functions as an adaptor-like protein, mediating the recruitment and activation of signalling molecules to B-cell receptor microclusters (Harwood and Batista, 2008).

In contrast to other B-cell diseases, the extracellular epitopes of CD79b and CD22 are either expressed at a low density or absent in CLL. This has been confirmed by antibody binding capacity using a quantitative flow cytometry method to provide an accurate estimation of antigen expression (Cabezudo et al., 1999).

Although early reports suggested that most CLL cases were CD79b negative, a monoclonal antibody has shown that CD79b is expressed weakly in most CLL cases (Harwood and Batista, 2008) (Cabezudo et al., 1999) (Thompson et al., 1997). The level of CD79b, also known as B29, directly correlates with the level of sIg expression in CLL. CD79b, in association with CD79a, plays a major role in B-cell receptor complex formation. This multimeric complex with the sIg translates the Ig stimulation into a B-cell response (Thompson et al., 1997) (Figure 1.3). Under-expression of CD79b has been attributed to the

development of mutations in the coding sequences of the *CD79B* gene in CLL patients that produce a truncated form of the protein, thereby explaining the reduced expression of sIg (Alfarano et al., 1999). This is a very helpful marker in differentiating CLL from other B-cell malignancies as it is strongly expressed in most of them.

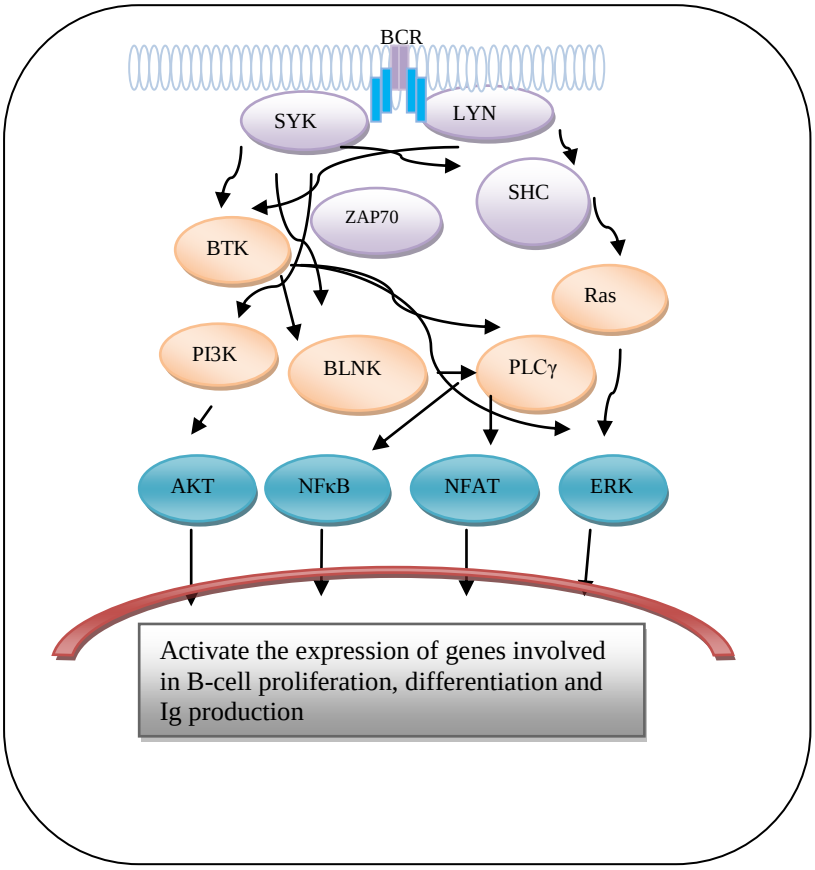


Figure 1.3 CLL cell signalling through B-cell receptor

The B-cell receptor (BCR), an integral membrane protein complex, is composed of two immunoglobulin (Ig) heavy chains and light chains and two heterodimers of Igα (CD79a) and Igβ (CD79b). After BCR ligation by antigen, three main protein tyrosine kinases LYN, SYK and BTK are activated. This then activates the downstream signalling pathways. ZAP70 protein upregulate BCR signaling by an adaptor role independent of its kinase activity.

CD22 is a BCR-associated transmembrane protein, the cytoplasmic tail of which contains three immunoreceptor tyrosine-based inhibitory motifs. These motifs are phosphorylated upon BCR-crosslinking, and can act as negative regulator of signalling from the BCR (Nishizumi et al., 1998). So the under-expression of these two molecules may explain the aberrant signal transduction in CLL cells similar to that of anergic normal B lymphocytes.

CD5, an antigen consistently positive in CLL cells, is a pan T-cell marker. CD5 is also present in some B-cells and these are usually found in mantle cells of secondary lymphoid follicles. 15% of normal B-cells express the CD5 marker in peripheral blood. CD5 is expressed by normal CD5– B-cells upon mitogenic stimulation (Morikawa et al., 1993), and conversely CD5+ B-cells may lose the expression of CD5 upon activation (Caligaris-Cappio et al., 1989). CD5+ B-cells have been implicated in producing autoantibodies and it is interesting to note that CLL has a high frequency of association with autoimmune phenomena (Shirai et al., 1991).

There is also evidence that cells from a proportion of CLL express the CD40 ligand, CD154 (Ewart et al., 2002). CD154 is a member of the tumour necrosis family which is usually expressed on activated CD4+ T-cells. Normally when B-cells present antigens to CD4+ T-cells, the T-cell synthesises CD40L if it recognises the peptide. The CD40L binds to the B-cell's CD40 receptor, activating the cognate B-cell. As a result of this interaction, the B-cell undergoes division, antibody isotype switching and plasma cell differentiation and thereby produces specific antibodies. Cells from CLL cases that are CD40L positive seem capable of inducing IgG production in normal B lymphocytes and it has been suggested that this may account for the production of abnormal antibodies by B-cells in some CLL cases. CD5+ B-cells were thought to be the cell of origin in CLL initially but current lines of evidence argue against this, as discussed earlier (Chiorazzi et al., 2005).

Another interesting molecule expressed on CLL cells is CD23, which is an immunophenotypic hallmark of CLL. CD23 is a 45-kd transmembrane glycoprotein that functions as a low-affinity receptor for IgE and as an adhesion molecule by virtue of its ability to promote T-B-cell interactions and B-cell homotypic interactions when it engages its ligand CD21. It is expressed at low levels in normal B-cells, but upon activation, high expression is seen on B-cells, and yet, it is characteristically expressed in CLL in which the neoplastic lymphocytes are believed to be dormant. There are two isoforms of CD23, CD23a which is restricted to B-cells and CD23b which could be expressed by B-cells as well as other haemopoetic cells like monocytes/macrophages, T-cells, eosinophils and platelets, when they are stimulated (Delespesse et al., 1991). In normal B-cells their expression is determined by various exogenous stimuli that signal through transcription factors that can regulate the promoter regions of either or both of these isoforms (Ewart et al., 2002). In CLL the mechanism by which CD23

expression is regulated is controversial. In normal B-cells IL-4 is the main inducer and IFN-γ is the main repressor, but this differential stimulating ability is lost in CLL and both isoforms are expressed (Fournier et al., 2008) (Goller et al., 2002). *In vitro* data suggests that ligation of CD23 will increase intracellular nitric oxide, which protects the CLL cells from apoptosis (Kolb et al., 2001). High levels of soluble CD23 are found in sera from CLL patients, which directly correlate with disease activity (Reinisch et al., 1994) (Sarfati et al., 1988). Soluble CD23 serves several functions like extending survival of B lymphocytes and the induction of differentiation and proliferation of several cell subtypes, including B-lymphocytes. Some studies have shown that the expression of CD23 is significantly higher in the prolymphocytoid large cells present in the proliferating centres than in the small lymphocytes, suggesting that the former are the main source of the soluble levels of this molecule detected in the serum (Lampert et al., 1999).

CD27, a member of tumour necrosis factor receptor super family, together with its ligand CD70 plays a major role in regulating B-cell activation and immunoglobulin synthesis. All CLL cells are CD27+, which is typically a marker of the memory B-cells. Most normal memory B-cells have *IGHV* gene mutation, but a small fraction do not (Klein et al., 1998) (Tangye et al., 1998). The presence of CD27 on CLL cells as well as the presence of both *IGHV* mutated and unmutated CD27+ memory cells supports the idea that CLL cells evolve from memory B-cells.

Recently it has been shown that CLL cells show specific changes in membrane protein expression during different stages of cell cycle (Bennett et al., 2007). Proliferating and resting fractions in CLL display differential patterns of surface markers. Expression of proteins CD39, CD86, CD95 and CD23 were uniformly increased during cell cycle. Except CD23, the other molecules were not thought to be associated with proliferation in CLL. The level of CD38 expression was generally increased in proliferating CLL cells compared with resting cells; although there was considerable inter-patient variation. In contrast, the CXC chemokine receptors, CXCR4 and CXCR5, as well as CD24 and CD69 were down-regulated during the cell cycle. CD24 modulates B-cell activation responses by promoting antigen dependent proliferation of B-cells, and prevents their terminal differentiation into antibody-forming cells. Its expression was only down-regulated when CLL cells entered S-phase. CXCR4 binds stromal cell-derived factor-1 (SDF-1) and these molecules are centrally involved in the chemo attraction of CLL cells to the stromal cells responsible for their survival (Burger, 2011). This study suggests

that resting CLL cells express high levels of CXCR4 and migrate to stromal cells that secrete SDF-1 but on contact, CXCR4 expression is decreased on the CLL cells as they enter into cell cycle.

Another interesting group of molecules studied in CLL is cell surface adhesion molecules. Several markers including integrins, selectins, homing receptors, as well as the serum levels of some of these molecules has been studied (Lucio et al., 1998). Adhesion molecule expression is heterogeneous in CLL, with low LFA-1 (CD18/CD11a) and CD54 (ICAM-1) (Kimby et al., 1994) (Molica et al., 1995) (Inghirami et al., 1988), high L-selectin (CD62L) and CD44 (HCAM), and variable CD11c characterising CLL. Expression of CD11c/CD18, CD31, CD48 and CD58 are significantly lower in CLL cases with 11q23 deletions (Sembries et al., 1999). High levels of expression of CD11a, CD18, CD29, and CD11c on the surface of the leukaemic cells were found in cases with splenomegaly and LFA-1 is expressed in patients with predominant lymphadenopathy (Baldini and Cro, 1994). Serum levels of intercellular adhesion molecule 1 (CD54) are significantly elevated in CLL patients compared with healthy subjects. It correlates with tumour burden and hepatosplenomegaly in the advanced clinical stage of CLL (Lucio et al., 1998) (Christiansen et al., 1994) (Molica et al., 1997). Similarly, CLL cells invariably express one or more isoforms of the lymphocyte homing receptor CD44 and of the CD62L (L-selectin), which is different from that of related low grade B-cell disorders and strong expression of these also correlate with poor prognosis in CLL (Horst et al., 1990a) (Horst et al., 1990b) (Jalkanen et al., 1990). Strong expression of CD36, a thrombospondin receptor, correlates with diffuse pattern of bone marrow involvement and poor prognosis (Rutella et al., 1999). Compared to other NHLs, β-integrins are generally under-expressed. CD49d is another molecule which is variably expressed in CLL and functionally acts as adhesion structure for extracellular matrix components or mediates cell-cell interactions through the binding with fibronectin or vascular cell adhesion molecule-1, and was shown to be an independent prognosticator for overall survival and time to first treatment (Gattei et al., 2008).

Another important prognostic marker that could be detected either by immunohistochemistry, immunocytochemistry or flow cytometry is p53 protein expression. The wild-type p53 protein is normally undetectable by immunohistochemical analysis using anti-p53 monoclonal antibodies; however, the mutated p53 is detected by immunohistochemical methods as this protein has a prolonged half-life. This may be due to the fact that wild-

type p53 protein is targeted to MDM-2–mediated ubiquitination and subsequent degradation (Piette et al., 1997). This abnormal p53 protein is expressed in both mutation at the chromosomal region or in hemizygous deletion of the region (Bártek et al., 1991) (Lepelley et al., 1994) (Chang et al., 2010). At the genetic level, *TP53* abnormalities are detected by fluorescence *in situ* hybridization (FISH) or direct gene sequencing and at protein level by immunohistochemistry, immunocytochemistry or flow cytometry (Carter et al., 2004) (Schlette et al., 2009) (Cordone et al., 1998). It has been shown that all these methods have the same significance in assessing the prognostic significance in CLL.

Table 1.5 Antigens used in the diagnosis of CLL

Antigen	Cell function	Presence in CLL and clinical implication	Reference
CD20	Acts as calcium channel and plays a role in B-cell activation, proliferation and differentiation.	Dim Target for monoclonal antibody, rituximab, in treatment of CLL	(Tedder and Engel, 1994)
CD19	Assembles with BCR complex to decrease the threshold for antigen receptor dependent stimulation	Positive Preclinical data on monoclonal antibody against CD19 in CLL (XmAb5574)	(Carter and Fearon, 1986) (Awan et al., 2010)
CD5	Regulates intracellular signal strength induced by antigen receptors in both T- and B-cells	Positive	(Raman, 2002)
CD79b	Signal transmitting unit of BCR complex	Weak or negative Preclinical data on antibody drug conjugate (anti-79b-vc-MMAE)	(Cassard et al., 1996) (Dornan et al., 2009)
CD43	Implicated in the regulation of cell adhesion, activation and survival	Positive	(Park et al., 1991) (De Smet et al., 1993) (Bazil et al., 1995)
CD81	CD19/CD21/CD81 complex	Weak	(Matsumoto et al.,

	enables B lymphocytes to respond to low concentration of antigens and induces homotypic cellular aggregation		1993)
CD52	In presence of antibody, it is a good target for complement action and activates cell proliferation in T-cells, but function in the absence of antibody is unknown	Positive Target for monoclonal antibody, Alemtuzumab, in treatment of CLL	(Valentin et al., 1992)
CD200	Interaction with its receptor CD200R sends inhibitory signal to macrophages. In T-cells it alters the cytokine profile from Th1 to Th2 and it suppresses the antitumor immunity	Positive Preclinical data on CD200 blocking antibodies enhancing the tumour specific immunity	(Kretz-Rommel et al., 2008) (Kretz-Rommel et al., 2007) (Hoek et al., 2000) (Gorczynski et al., 1999)
CD23	Acts as a low affinity receptor for IgE	Positive Phase 1/2 clinical data on anti-CD23 (Lumiliximab) antibody in combination with chemotherapy	(Yodoi and Ishizaka, 1979) (J. C. Byrd et al., 2006)
CD22	Acts as B-cell associated adhesion protein and regulates B-cell activation	Negative or positive Phase 1/2 clinical data on anti-CD22 (Epratuzumab) antibody treatment	(Pezzutto et al., 1987) (Pezzutto et al., 1988) (Stamenkovic and Seed, 1990) (Torres et al., 1992) (Leonard et al., 2003)
CD25	Part of IL2 receptor	Variable Phase 2 clinical data on antibody conjugated with diphtheria toxin (denileukin diftitox)	(Leonard et al., 1982) (Frankel et al., 2006)

CD10	Metalloendopeptidase that cleaves small peptides like angiotensins, bradykinin, enkephalins and oxytoxin. It also controls neutrophil chemotaxis and inflammation.	Negative	(Gafford et al., 1983) (Schwartz et al., 1981) (Johnson et al., 1985) (Johnson and others, 1984) (Connelly et al., 1985)
CD38	Serves as an ectoenzyme that catalysis the synthesis and hydrolysis of cyclic ADP-ribose which is a calcium mobilising agent. This helps in transmembrane signalling thereby affecting differentiation and proliferation of various immunoregulatory cells.	Positive or negative Prognostic marker in CLL	(Lee and Aarhus, 1991) (Lee et al., 1994) (Mehta et al., 1996)
CD103	Presence on T regulatory cells helps these cells to adhere to epithelial cells on which its ligand, E-cadherin, is present.	Negative	(Belkaid et al., 2005) (Agace et al., 2000)
CD11c	CD11c/Cd18 complex is a β2 integrin expressed in granulocytes, monocytes NK cells and dendritic cells. Helps in cell adhesion and B-cell activation.	Variable	(Postigo et al., 1991) (Larson and Springer, 1990)
FMC7	An epitope on CD20 molecule whose expression is sensitive to the level of membrane cholesterol	Negative or weak	(Polyak et al., 2003)
CD79a	Signal transmitting unit of BCR complex	Positive	(Campbell and Cambier, 1990) (Pao et al., 1998)

1.2 B-cell receptor complex

The B-cell receptor (BCR) is the key regulator of normal B-cell survival and function and CLL is no exception. The interest in BCR in CLL emerged in late 90's following the discovery of two clinically distinct subsets of CLL, based solely on the level of somatic mutation in the Ig variable region genes. In CLL cells, surface Ig is always present, usually as both IgM and IgD with a predominant IgD expression, although a minor subset of CLL expresses IgG or IgA. The BCR of all B-cells consists of sIg non-covalently associated with a CD79a (Igα)/CD79b (Igβ) forming a multimer (Tolar et al., 2009). This mutimeric form is important in signal transduction in B-cells. Antigen-dependent effects on B-cells are influenced by various factors; strength and duration of interaction, nature and molecular form of the antigen and its intrinsic affinity, the maturational state of the B-cell and environmental factors provided by stromal cells or T-cells (Packham and Stevenson, 2010). To produce a good signal antigen should be macromolecular or membrane-bound. When these forms of antigen come into contact with B-cells it leads to the formation of BCR microclusters. This induces the spreading of the B-cell membrane over the antigen contact area with formation of additional microclusters (Treanor et al., 2009). CD19 is recruited to this complex which then provides a scaffold for key signal transduction molecules (Depoil et al., 2007). An immune synapse is formed which undergoes internalisation as a prelude to antigen presentation to CD4+ T-cells.

Triggering of the B-cell by antigen will stimulate an intracellular signalling, which leads to proliferation, apoptosis, endocytosis or anergy. There is now convincing evidence to suggest that CLL cells also engage with antigen *in vivo*. The evidence is provided by the expression of markers of activation (Damle et al., 2002) and the fact that partially or completely anergised sIgM responses from mutated and unmutated subsets can recover both sIgM expression and signal capacity spontaneously *in vitro* (Mockridge et al., 2007). This reversal of down-regulation, which could happen to variable extents *in vivo*, is a strong indicator of an on-going interaction with antigen. Several candidate antigens have been proposed including bacterial antigens and autoantigens. *In vivo* this antigen exposure occurred in tissue compartment (BM, lymph nodes, and spleen), where antigen is present together with microenvironmental support. Following the antigen exposure, that is presumably presented to the CLL cells by components of the microenvironment and is recognised by the BCR on the surface of the CLL

cell, they receive a proliferative stimulus. This is a two-way interaction whereby CLL cells in reverse recruit the cells of the microenvironment including macrophages, follicular dendritic cells, T-cells and nurse-like cells. This will form proliferation centres which are readily visible by light microscopy (Audrito et al., 2013a) (Figure 1.4).

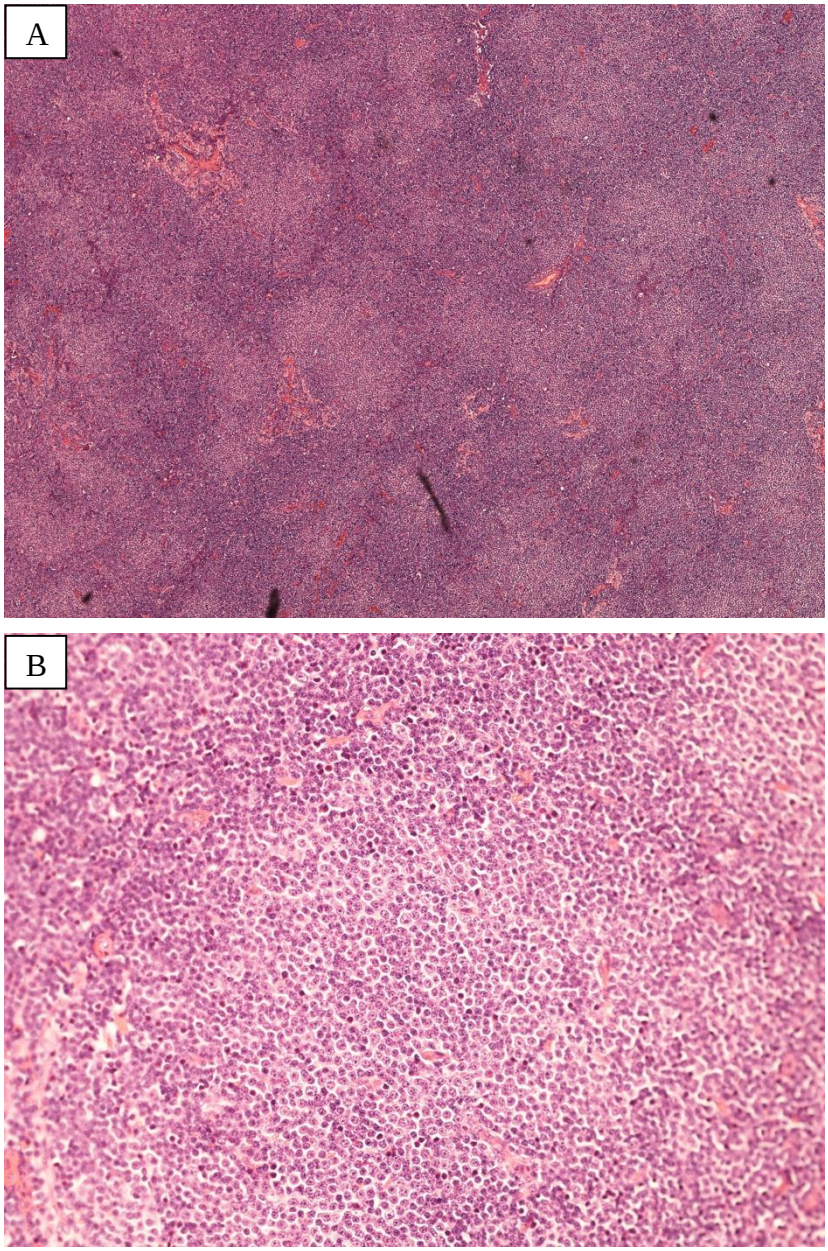


Figure 1.4 Proliferation centre

Lymph node from a CLL patient processed in formalin and stained with hematoxylin and eosin. A) Low power view of the pseudofollicles or proliferation centers in lymph node biopsy which appears as ill defined paler staining areas (magnification 40X). B) High power view of the pseudofollicles in lymph nodes (magnification 200X).

In these proliferative areas, which significantly express the proliferation marker Ki-67 the CLL cells cross-talk with the cells of the microenvironment. Antigen exposure to the BCR along with several other co-stimulatory molecules such as CD40/CD40L, CD38/CD31, CXCR5/CXCL13 (BCA-1), CXCR4/stromal cell-derived factor-1 (SDF-1), and possibly other molecules, plays a key role here (Hillmen, 2011) (Burger, 2011). This will lead to downstream signalling in the CLL cell and a proliferative burst. The cell then migrates from the proliferating centres back to the peripheral blood, where the cell stops dividing. The CLL cell then either undergoes apoptosis or surviving cells will resynthesise sIg and possibly re-enter the tissue sites for a second round of exposure (Hillmen, 2011). This potentially imposes a problem in studying the BCR signalling in CLL, as the readily available tissue is peripheral blood where the cells has already engaged sIg *in vivo* and it represent only a part of the 'life cycle' of CLL cells. The blood contains a spectrum of these antigen engaged cells having left the tissue compartments at various times, contributing to heterogeneity within the malignant clone. This sequence of events is supported by the proliferative and activation markers in proliferation centres.

There is some difference between the mutated and unmutated subsets' ability to signal *in vitro* via engagement of sIgM which is higher in unmutated CLL than in mutated CLL (Packham and Stevenson, 2010) (Mockridge et al., 2007) (Lanham et al., 2003) (Guarini et al., 2008) which also correlate with other prognostic markers including ZAP70 and CD38, and with clinical outcome (Chen et al., 2002) (Zupo et al., 1996) (Deglesne et al., 2006).

1.2.1. BCR signalling pathways

In normal B-cells, when the immunoglobulin molecule is ligated, signalling is triggered by phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs) of CD79a and CD79b (Packham and Stevenson, 2010) (Niiro and Clark, 2002) (Dal Porto et al., 2004). This phosphorylation is predominantly catalysed by the Src family kinase Lyn and spleen tyrosine kinase (SYK) (Kulathu et al., 2008) (Geahlen, 2009). This phosphorylation and activation is a critical event in BCR signalling (Jiang et al., 1998) (Takata et al., 1994) leading to the formation of a plasma membrane-associated signalling complex (signalosome) which includes many kinases and adaptor proteins, including the kinases SYK, Bruton tyrosine kinase (BTK), and Lyn, the guanine exchange factor Vav proteins, and the adaptor proteins Grb2 and B-cell linker (BLNK) which mediate activation of

downstream signalling pathways (Woyach et al., 2012). This activation is amplified by several protein kinases. SYK phosphorylates both CD79a/CD79b and Lyn provides amplification of the signal through recruitment of other protein tyrosine kinases together with formation of a complex with costimulatory molecules including CD19 that reduce the threshold of B-cell activation (Yamamoto et al., 1993) (Rolli et al., 2002) (Fearon et al., 2000). This results in BCR aggregation and formation of a microcluster or lipid raft on the plasma membrane (Cheng et al., 1999). Signal propagation from the BCR occurs via multiple pathways, predominantly through phospholipase C-γ2 (PLC-γ2) and phosphatidalyinositol-3-kinase (PI3K). After initial phosphorylation of ITAMs by SYK and Lyn, BLNK is phosphorylated by SYK when it is recruited to the non-ITAM tyrosines of CD79a, where it binds via its Src homology2 (SH2) domain (Engels et al., 2001) (Kabak et al., 2002). BTK then binds to this complex and together BTK and SYK activate PLC-γ2 by dual phosphorylation. Activation of PLC-γ2 produces the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) from the plasma membrane lipid phosphatidylinositol 4,5-bisphophate (PIP2) (Dal Porto et al., 2004). DAG activates protein kinase C, and IP3 releases calcium from the endoplasmic reticulum and the extracellular compartment (Roos et al., 2005) Calcium release directly activates a number of transcription factors, including NF-κB, Jun, and nuclear factor of activated T-cells (NFAT). NFAT proteins are indirectly activated by calcium through the calcium dependent activation of the phosphatase calcineurin. Dephosphorylated NFAT proteins are translocated to the nucleus and subsequently regulate cytokine production and other effectors of the immune response (Rao et al., 1997). NF-κB plays a broad role in B lymphocyte proliferation and class switching and also mature B-cell survival (Ruland and Mak, 2003) (Stadanlick et al., 2008). The canonical NF-κB pathway is also an important survival effector in BCR signalling. NF-κB exists in inactive form in the cytoplasm as dimers consisting of p50, p52, p65/RelA, RelB, or c-Rel with the most usual dimers being the p50/p65 heterodimer and the p50/p50 homodimer (Ghosh et al., 1998). In the inactive form it is also bound to I-κB. On stimulation via BTK, PI3K or Akt the I-κB kinase complex causes phosphorylation and subsequent proteasomal degradation of I-κB. This results in nuclear translocation of NF-κB and gene transcription. NF-κB activates a wide variety of genes responsible for inflammation, proliferation and B-cell survival (Ghosh et al., 1998) (Stadanlick et al., 2008).

Increased intracellular calcium (iCa^{2+}) along with PKC and direct activation by Vav and Grb2 activates mitogen-activated protein kinase (MAPK)-family kinases, including extracellular regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK) and p38 MAPK. The MAPK pathway regulates a number of transcription factors, including Elk1 and c-Myc through Erk, c-Jun and ATF2 through JNK, and ATF2 and Max through p38 MAPK (Stadanlick et al., 2008) (Vigorito et al., 2005) (Johnson and Lapadat, 2002).

Initial phosphorylation and complex formation also activates the PI3K pathway. PI3K has two subunits, the p85 subunit, which is a regulatory component and p110 subunit, which is a catalytic subunit. At rest they remain in close association but on activation of the BCR complex, the p85 subunit is recruited to the plasma membrane where it complexes with the Src kinases Lyn and Fyn (Woyach et al., 2012) (Pleiman et al., 1994). p85 also binds to CD19, and this complex activates the p110 subunit, which then phosphorylates PIP2 to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 recruits a number of BCR signalling molecules with a pleckstrin homology domain to the plasma membrane, like the serine/threonine kinase AKT, BTK and other kinases. Active AKT is important for BCR-induced survival and proliferation pathways. It inactivates the pro-apoptotic BCL2 family protein, BAD, and forkhead family transcription factor FOX03a. It enhances activation of NF-κB through phosphorylation and inhibition of glycogen synthase kinase 3 which is also a negative regulator of MYC and D-type cyclins (Downward, 2004).

BTK is a member of the tyrosine protein kinase (Tec) family of kinases and also has a critical role in the amplification of the BCR signal. This is exemplified by profound BCR signalling defects in X-linked agammaglobulinemia (XLA) (also known as Bruton's Agammaglobulinemia or Congenital Agammaglobulinemia) and its mouse counterpart X-linked immunodeficiency (XID). In these conditions, there is a failure in B-cell development at the pre-B to immature B-cell stage and subsequent defective B-cell signalling and reduced immunoglobulin production, all leading to profound humoral immune deficiency (Tsukada et al., 1993) (Vetrie et al., 1993). The major molecular defect is a mutation in the pleckstrin homology domain of BTK which prevents effective membrane recruitment by PIP3. This will cause defect in calcium flux associated with BCR signalling and thereby the downstream signalling (Roos et al., 2005).

BTK is mainly involved in the initial phosphorylation events and deficiency produces defects in early BCR phosphorylation, whereas increasing intracellular calcium can restore downstream effects of BCR signalling (Khan et al., 1995). SYK and Lyn phosphorylate BTK at the Y551 site of the kinase domain. This step is usually followed by amplification through auto phosphorylation of the Y223 site in the SH3 domain (Park et al., 1996). In addition, BTK recruits phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) which are responsible for synthesis of PIP2, and after phosphorylation by PI3K to PIP3 results in continued recruitment of BTK. BTK also activate IκB kinase, which phosphorylates the NF-κB inhibitor I-κBα, allowing NF-κB to translocate to the nucleus (Saito et al., 2003).

The activation of positive BCR signalling pathways is tightly regulated by inhibitory signals to prevent the unrestrained activation that can result in development of autoimmune conditions and malignancies (Woyach et al., 2012). This is mainly mediated by inhibitory regulators, such as CD22 or FcγRIIb (CD32) and various phosphatases, including SH2 domain-containing tyrosine phosphatase-1 (SHP-1) and SH2 domain-containing phosphatidyl 5-phosphatase (SHIP) -1 and -2 and kinases with differential activation and inhibitory properties, like Lyn. CD32 when co-clustered with BCR induces a negative signal by recruiting SHIP to the plasma membrane which eliminates the membrane binding of PLC-γ2, BTK, and Akt by hydrolysing PIP3 (Ono et al., 1996). SHP-1 can associate with ITIM-containing molecules, and activated SHP-1 dephosphorylate various substrates (Scharenberg et al., 1998) (Carver et al., 2000) (Bolland et al., 1998). Coligation of the BCR and CD32 results in the reversal of SHP-1 autoinhibition, SHP-1 is also associated with the BCR at rest, which gets disrupted by BCR stimulation, suggesting that SHP-1 is involved in preventing signal transduction in resting B-cells (Pani et al., 1995). These phosphatases are activated downstream of Lyn, and therefore Lyn plays both positive and negative roles in signal transduction via the BCR. It has been shown that Lyn knockout mice demonstrate BCR hyper-responsiveness and develop lethal autoimmune glomerulonephritis (Hibbs et al., 1995) (Nishizumi et al., 1995).This is due to the fact that Lyn is required for phosphorylation of both SHIP and FcγRIIb making it a crucial kinase in the regulation of the BCR (Hibbs et al., 2002) (Nishizumi et al., 1998).

1.2.2. BCR signalling in CLL

Similar to normal B-cells, activation of sIgM on CLL cells by cross-linking antibodies triggers a range of signalling pathways. Signalling responses are

more prominent in patients with unmutated *IGHV* genes, ZAP70 positive and CD38 positive than those without these poor prognostic markers (Chen et al., 2002) (Chen et al., 2008). In CLL also, like normal B-cells, the signalling response stimulates Ca^{2+} mobilisation and the phosphorylation and activation of various phosphoproteins including SYK, PLCγ2, ERK, NF-κB and AKT. Some of these kinase molecules could be constitutively phosphorylated. Lyn and SYK are upregulated (Woyach et al., 2012), Lyn at the protein level but not at the transcriptional level, but SYK in both mRNA and protein level (Contri et al., 2005). *In vitro* experiments has shown that both SYK inhibitor R406 and Lyn inhibitors PP2 and SU6656 abrogate CLL cell survival after IgM stimulation and reduce downstream targets of BCR signalling in terms of phosphorylation activity. Abnormal activation of the Akt/PI3K pathway has also been demonstrated in CLL. Some of the phosphorylation sites of Akt and PI3K are constitutively phosphorylated and inhibitors of PI3K activity have been demonstrated to be pro-apoptotic in CLL cells *in vitro* and *in vivo*. BTK is also up-regulated in CLL compared to normal B-cells and inhibition of BTK has shown good *in vitro* as well as *in vivo* activity in treating CLL (Contri et al., 2005) ZAP70 enhances BCR signalling by acting as an adapter molecule which is independent of its kinase activity. ZAP70 mediated signals can also enhance migration toward chemokines and response to survival stimuli from the microenvironment. Tissue-based comparison by microarray has demonstrated enhanced up-regulation of NF-κB target genes and other genes associated with BCR activation in bone marrow and lymph nodes compared with peripheral blood especially in ZAP70 positive/U-CLL (Herishanu et al., 2011). Immunohistochemistry has also shown that in proliferation centres there is higher expression of phosphorylated IκBα, and active NF-κB and NFATc1 (Packham and Stevenson, 2010) (Rodríguez et al., 2004) (Herreros et al., 2010). Higher levels of nuclear NF-κB are seen also as exaggerated responses to stimulation with CD40L, which also help prolonged cell survival. However the current evidence suggests that a role for alternate signalling pathways in activation of these molecules cannot be excluded. Even in responsive samples there is a limited degradation of IκBα and very infrequent activation of JNK (Petlickovski et al., 2005).

Anti-apoptotic proteins Erk1/Erk2, which belongs to the MAPK pathway, are dysregulated in CLL. In samples which lack of signal response there could be a constitutive activation of Erk and NFAT, which could be similar to anergic B-cells suggesting constant antigen engagement. But those CLL samples which are not constitutively phosphorylated have shown inducible phosphorylation

and cell survival in the presence of phorbol ester (Muzio et al., 2008) (Barragán et al., 2002). Expression of the transcription factor Myc is also dependent on Erk1/2 activation after BCR stimulation, suggesting the importance of this pathway in CLL survival and proliferation (Krysov et al., 2012).

It is logical to infer that the signalling of sIgM in CLL will trigger an anti-apoptotic pathway in the cell, but actual in vitro experiments have shown conflicting data on the outcome of sIgM signalling. It was shown that if the stimulatory antibody is in solid phase as in whole goat anti-IgM bound to Dynabeads it will increase cell survival and suppress fludarabine-induced apoptosis. In contrast soluble intact anti-IgM promoted apoptosis in responsive samples (Petlickovski et al., 2005). But other groups have shown that soluble antibody especially if it is $F(ab)_2$ portion of the antibody, it will induce prosurvival signals. So several variables including cell density, antibody specificity (monoclonal versus polyclonal), antibody affinity/avidity might all influence responses to anti-IgM treatment (Bernal et al., 2001a) (Zupo et al., 2000) (Deglesne et al., 2006).

In CLL sIgM signalling will produce increased expression of the BCL2-related survival protein MCL1, which has been linked to chemo resistance and the presence of poor prognostic markers (Kitada et al., 1998) (Hewamana et al., 2009). This has also been evidenced by the fact that down-modulation of MCL1 by siRNA induces apoptosis of CLL cells (Longo et al., 2008). This MCL1 induction is linked to the PI3K/AKT pathway as overexpression of constitutively phosphorylated AKT is sufficient to induce MCL1 expression. Inhibition of PI3K pharmacologically also prevents induction of MCL1 and promotes apoptosis (Petlickovski et al., 2005) (Bernal et al., 2001a). In contrast to MCL1, BIM, a proapoptotic BCL2 family member is downregulated by proteasomal degradation after phosphorylation (Ewings et al., 2007).

In vivo experiments with heavy water have shown that CLL cells proliferate in the body at a rate of 1-2% (Messmer et al., 2005). But *in vitro* experiments designed to replicate this have not been very successful. However, there are few studies demonstrating the expression of ki-67 when stimulated by antibody to IgM (Guarini et al., 2008) (Nédellec et al., 2005).

Another important event that usually happens in normal B-cells after BCR activation is cell migration and tissue homing. This aspect of B-cell biology is vital during the clonal expansion that follows antigen exposure in the microenvironment. CLL cells also show similar properties following sIgM

activation of CLL. Cell migration and expression of markers, which are involved in cell adhesion and migration, vary with the type of activating antibodies used in the experiments. So there are conflicting data in literature regarding the expression of these adhesion molecules following activation as some of the experiments have demonstrated that BCR activation increased expression of CD40, CD54 and CD62L, but decreased expression of CXCR4 even though there was increased migration towards the chemokines SDF-1 and CXCL13 and increased pseudoemperipolesis (the migration of cells beneath mesenchymal stem cells) (Quiroga et al., 2009a) but other experiments have shown that anti-IgM decreased expression of both CXCR4 and CD62L and reduced migration towards CXCL12 and adhesion to lymphatic endothelial cells (Vlad et al., 2009).

1.2.3. The role of sIgD

CLL cells co express sIgM and sIgD, which share identical antigen-binding specificities even though the level of expression is variable in IgM. IgD is strongly expressed when assessed by flow cytometry. The downstream activation of BCR signalling pathways is variable following the stimulation of sIgM but the response to sIgD activation with increased SYK phosphorylation and rapid increases in iCa^{2+} will happen in almost all samples (Mockridge et al., 2007) (Lanham et al., 2003). It is unclear why differences in sIgM responses correlate with prognostic markers and clinical outcome, while the capacity to signal via sIgD does not appear to impact on the clinical behaviour of CLL. However, there are data suggesting that sIgD signalling is capable of initiating early responses but it does not effectively couple to survival and proliferation promoting pathways (Packham and Stevenson, 2010).

The variability in responsiveness of CLL cells to signalling via sIgM is dependent on various factors. In mutated CLL, down-modulation of sIgM appears to play a major role, whereas sIgM expression may not be as important in unmutated CLL (Mockridge et al., 2007). But even in M-CLL there is a significant variation in signalling capacity, even with similar expression levels. This clearly suggests that there are additional factors involved rather than just the expression of sIgM. Indirect evidence showing that the expression of some molecules like Lyn, CD38, SHIP-1, and p66SHC correlate with signalling may suggest a role for these molecules, and potentially other prognostic markers, in the response (Lanham et al., 2003) (Zupo et al., 1996) (Contri et al., 2005) (Capitani et al., 2010) (Gabelloni et al., 2008).

More direct evidence for molecules like CD79b, ZAP70, TCL1 and PKCβII, may be provided by expression profiling and pharmacological inhibition. In CLL CD79b, which is an adapter molecule in the BCR complex, is expressed as a truncated form compared to the molecule expressed in normal B-cells. This truncated form lacks the extracellular Ig-like domain and is generated by alternative splicing (Alfarano et al., 1999) (Cragg et al., 2002). The truncated form is highly expressed in CLL cells that are non-responsive to signalling via sIgM (Nédellec et al., 2005). Expression of wild-type CD79b into a CLL cell line that expressed truncated CD79b increased sIgM expression (Minuzzo et al., 2005).

ZAP70 is another important molecule in this context. It is a SYK-family protein tyrosine kinase that plays a key role in signalling via the T-cell receptor (Chu et al., 1998). A proportion of CLL patients have ZAP70 expression, which is to certain extent associated with unmutated *IGHV* and poor prognosis. Therefore it is thought to be a positive regulator of BCR signalling. Over-expression of ZAP70 increases prolonged activation of downstream signalling pathways including iCa^{2+} mobilisation and phosphorylation/activation of SYK, BLNK and PLCγ2. Over-expressed ZAP70 enhances sIgM signalling, independently of its kinase activity (Chen et al., 2008). Based on these findings, ZAP70 plays a role as a facilitator in sIgM-mediated signalling in CLL cells, rather than simply acting as a direct functional homologue of SYK. Increased ZAP70 expression also correlates with enhanced migratory and survival responses (Richardson et al., 2006) suggesting that its roles in CLL may extend beyond modulation of BCR signalling.

The kinase PKCβII plays positive and negative regulator function in BCR signalling (Venkataraman et al., 2006) (Kang et al., 2001). It is over-expressed in CLL cells and its activity inversely correlates with sIgM signalling responses suggesting a negative regulatory role (Abrams et al., 2007). Inhibition by pharmacological agents enhances sIgM-induced iCa^{2+} mobilisation in CLL cells consistent with its known function in deactivating BTK.

Thus, a variety of factors influence positive and negative regulation of signalling from the BCR complex. The lack of signalling in non-responsive CLL samples is consistently associated with a failure to trigger some of the earliest events in the BCR signalling cascade, including phosphorylation of SYK (Lanham et al., 2003) and CD79a (Allsup et al., 2005), pointing to a key role for modulation at the level of sIgM itself, rather than of downstream

signalling. Additional downstream molecules like TCL1 and PKCβII may modify signalling responses, but these are not critical for CLL.

1.3 Microenvironment

The concept of ‘microenvironment’, where the CLL cells interact with various other cells is one of the new key concepts in CLL biology. CLL cells in the bone marrow and secondary lymphatic tissues engage in complex cellular and molecular interactions with stromal cells and matrix. By these interactions several pathways that are involved in the CLL cell survival and proliferation are activated (Audrito et al., 2013a). This has been shown by gene expression arrays and *in vitro* assays. The key example of this transformation is the proliferation centre, a focal aggregate of pro-lymphocytes and para-immunoblasts that cluster in pseudofollicular structures (Soma et al., 2006) (Ponzoni et al., 2011). These nodular areas without mantles are seen in lymph nodes and bone marrow and represent the histopathological hallmark of CLL. Active proliferation in pseudofollicles are demonstrated by aggregates of Ki-67+ proliferating tumour cells which express CD5, but differ from reactive germinal centre B-cells by being CD10-, BCL6-, and BCL2+ (Ciccone et al., 2012). New blood vessels are also found in the area sprouting in response to the production of vascular endothelial growth factor (VEGF) by actively proliferating malignant B-cells (Pileri et al., 2000) (Chen et al., 2000) (Molica et al., 2002). The cells that have been shown to be involved in the microenvironment include mesenchymal stromal cells (MSCs), monocyte-derived nurse-like cells (NLCs), and T-cells (Figure 1.5). Bone marrow stromal cells (BMSCs), which are key players in normal haematopoiesis, were found to be the first stromal cells to support CLL cell growth. Much of this data is derived from co-culturing CLL cells with these stromal cells *in vitro*. Co-culturing results in migration of CLL cells underneath the BMSCs which induces a cobblestone-like appearance that depends upon CXCR4 and VLA-4 expression by leukaemia cells (Burger, 2011) (Burger et al., 1999). This process is called pseudoemperipolesis. Recently it was shown that this support by stromal cells is species independent as murine stromal cells, in addition to human stromal cells, were found to support the anti-apoptotic effect on CLL cells. The cross-talk between CLL and MSCs is bi-directional, causing activation of both CLL cells and MSCs, and the CXCR4 (CD184, Fusin, HM89, LCR1, LESTR-CXCL12 axis plays a crucial role in this interaction (Ding et al., 2009). CLL cells have a high affinity for BMSCs. Similar to bone marrow mesenchymal cells, actin (αSMA+) positive

mesenchymal stromal cells and follicular dendritic cells (FDCs) support the CLL cells in other tissues such as the secondary lymphatic tissues. Follicular dendritic cells are closely associated with CLL cells in the early phase of lymph node involvement. Furthermore, *in vitro* culture with FDC prevents leukemic cells from early apoptosis by direct cell contact, dependent on ligation of CD44 and on up-regulation of MCL1, and the crosstalk between CD100 and plexinB1 (Pedersen et al., 2002). The homing of CLL cells to the microenvironment niche is a complex process involving various steps. The first step involves a motility programme that is a response to the binding of chemokines to the receptors on CLL cells which reorganises the cytoskeleton. Then the CLL cells adhere to the endothelial cells and negotiate a passage through the endothelial layer, mainly mediated by integrins and their ligands. This is followed by a phase in which several matrix metalloproteases (MMPs) are secreted that allow lymphocytes to move within tissues and to reach their final destination (Ley et al., 2007).

CXCR4 is expressed at high levels on the surface of CLL cells circulating in peripheral blood and mediates many homing activities including chemotaxis, migration across vascular endothelium, actin polymerisation and pseudoemperipolesis (Burger et al., 1999). CXCR4 surface expression is down-regulated by its ligand CXCL12 (stromal cell-derived factor-1/SDF-1) via receptor endocytosis and by BCR triggering. Therefore, the level of CXCR4 expression is characteristically different in CLL cells in circulation and those in tissues. In tissues, proliferating CLL cells which are Ki-67+ shows significantly lower levels of CXCR4 and CXCR5 than do non-proliferating CLL cells (Bennett et al., 2007). This is also demonstrated by *in vivo* studies with deuterium labelling of CLL cells showing lower CXCR4 surface levels in cells with increased deuterium incorporation (Calissano et al., 2009). BCR signalling down-modulates CXCR4 and this enhances chemotaxis toward CXCL12 and CXCL13 (Vlad et al., 2009) (Quiroga et al., 2009a). This relationship with the BCR signalling has been corroborated by the fact that ZAP70+ CLL cells display increased chemotaxis and survival in response to CXCL12 compared with ZAP70- CLL cells, as ZAP70 expression is associated with a higher responsiveness to BCR stimulation (Richardson et al., 2006) (Chen et al., 2002). Similarly, CD38+ CLL cells also show increased levels of chemotaxis (Deaglio et al., 2005). CD38 blocking using anti-CD38 mAb inhibits chemotaxis. CXCR4 can be specifically blocked by CXCR4 antagonists (Burger and Peled, 2009) such as plerixafor, which

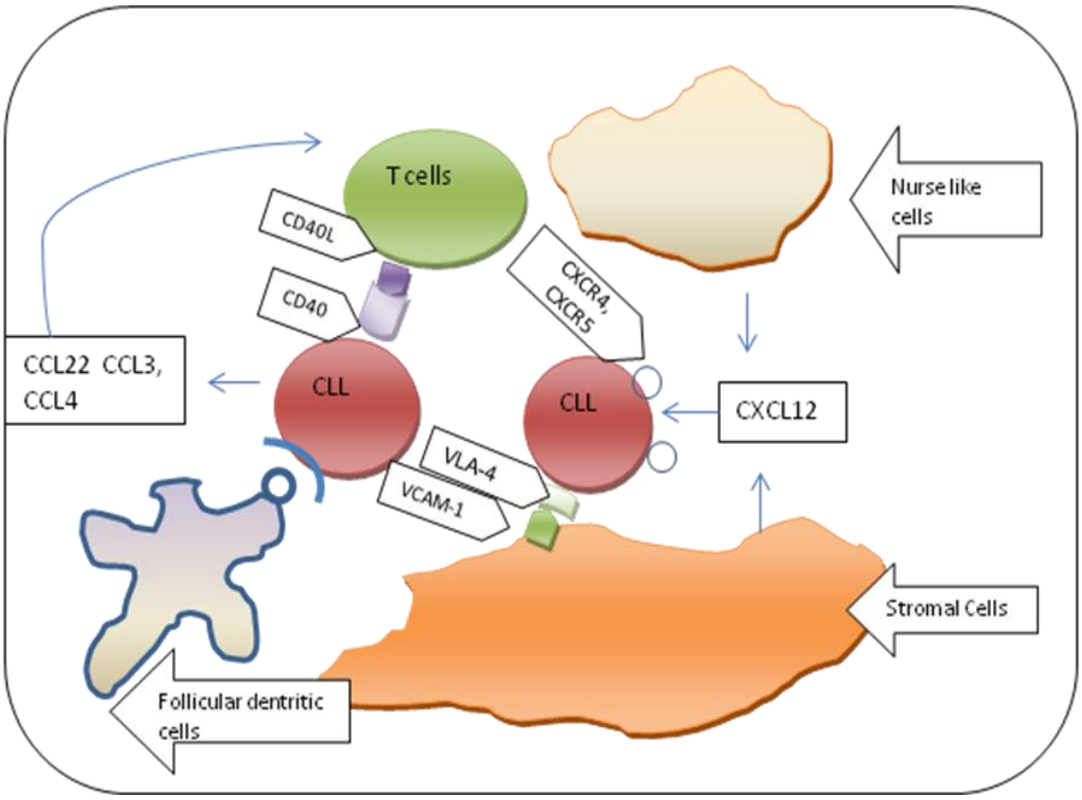


Figure 1.5 Interactions of CLL cells with T-cells, stromal cells, nurse like cells and follicular dentritic cells in proliferation centres.

CLL cells produce CCL22, CCL3 and CCL4 which is a chemoattractant to CD4+CD40L+ T-cells. Interaction between CD40 and CD40L along with IL4 may produce more CCL22 CCL3 and CCL4. Stromal cells and nurse like cells attract CLL cells by chemokines like CXCL12 expressed on their surface interacting with receptors like CXCR4 and CXCR5 expressed on CLL cell surface. Follicular dentritic cells induce upregulation of the anti-apoptotic BCL-2 family protein MCL-1

inhibits CLL cell activation by CXCL12 and reverses, to certain extent, stromal cell–mediated drug resistance (Vaisitti et al., 2010) (Burger et al., 2005). Pertussis toxin can stimulate CXCR4 signalling in CLL cells and induces downstream signalling like calcium mobilisation, activation of PI3Ks and p44/42 MAPKs and serine phosphorylation of STAT3 (Burger et al., 2000) (Burger et al., 2005). These signalling cascades can also be inhibited by PI3K inhibitors, SYK inhibitors, and BTK inhibitors leading to impaired migration of normal B-cells and CLL cells (Niedermeier et al., 2009) (Quiroga et al., 2009a) (de Gorter et al., 2007).

Another key player in the microenvironment is nurse like cells (NLCs) which are derived by differentiation from monocytes into large, round, adherent cells *in vitro*. When mononuclear cells from the blood of CLL patients are cultured

in vitro without stromal cells, an adherent cell population to which CLL lymphocytes are attached are consistently found. This population actively protects leukaemic cells from spontaneous apoptosis *in vitro* (Burger et al., 2000). They are similar to thymic nurse cells that nurture developing thymocytes and CD68+ myeloid cells and are predominantly found in the spleen and secondary lymphoid tissue. Similar to mesenchymal cells they protect CLL cells from undergoing spontaneous or drug-induced cell death in a contact dependent fashion (Burger et al., 2009a) (Burger et al., 2000). The gene expression profiling of the CLL cells after NLC co-culture is similar to CLL cells isolated from secondary lymphatic tissues (Burger et al., 2009b) (Herishanu et al., 2011). The predominant pathways activated are BCR and NF-κB pathways. The recruitment of NLC can be activated by CLL cells through the secretion of CCL3 and CCL4, which in turn, is triggered in response to signals mediated by the BCR and by CD38 (Burger et al., 2009b) (Zucchetto et al., 2009). There is an increase in the expression of reciprocal cross-talk molecules CXCL12 and CXCL13 in NLC and CXCL12 in BMSC which has been demonstrated at mRNA and protein level both *in vitro* and *in vivo*. There is an increased expression of chemokine receptors CXCR3, CXCR4, CXCR5 and CCR7 which help CLL cells to home into the lymphatic tissue. These G protein-coupled chemokine receptors help NLCs and BMSCs attract CLL cells expressing high levels of CXCR5 and stimulation with CXCL13 induces activation via G proteins, PI3Ks and p44/42 MAPK resulting in actin polymerisation, CXCR5 endocytosis, and chemotaxis. Normally in lymph node follicles CXCR5 (CD185), which is the receptor for the chemokine CXCL13, regulates lymphocyte homing and positioning (Bürkle et al., 2007). CXCR5 knock-out mice show defects in formation of primary follicles and germinal centres in the spleen and payer patches, and lack inguinal lymph nodes.

Other important molecules helping in cell-to-cell adhesion are integrins, particularly VLA-4 integrins (CD49d), expressed on the surface of CLL cells interacting with ligands on the stromal cells (VCAM-1 and fibronectin) (Burger, 2011). Integrins are heterodimeric glycoproteins consisting of various α and β subunits that mediate the attachment between a cell and the tissues that surround it, such as other cells or the extracellular matrix (ECM). Studies using fluorescence-activated cell sorting (FACS) and immunoprecipitation, showed that heterodimer expression of CLL patients consists of β1 expression, with a variable α chain (α1-α6). Functionally the CLL cells can bind to unstimulated endothelial cells at a lower level using β2/intercellular

adhesion molecule (ICAM), but this interaction is markedly enhanced when the endothelium was stimulated. CLL cells expressing α4β1 adhere strongly to stimulated endothelium via the α4β1 ligand, vascular cellular adhesion molecule-1 (VCAM-1) (Zucchetto et al., 2012). CLL cells showed variable adhesion to fibronectin, which is important in cell migration. Activated CLL lymphocyte populations showed an increased capacity to adhere to both endothelium and matrix. *Ex vivo* CLL cells after cytokine stimulation showed a capacity to migrate through endothelium/stroma, which they were unable to do before stimulation. The α4β1 integrin VLA-4 (CD49d) plays a particularly important role in interactions between normal and malignant hematopoietic cells and the BM microenvironment. VLA-4 integrins interact with CXCR4 in CLL cell adhesion to BMSCs (Burger et al., 1999). VLA-4 expression on CLL cells also has prognostic impact, (Shanafelt et al., 2008) (Majid et al., 2011) indicating the relevance of these interactions *in vivo*. Engagement of CD49d/CD29 (α4β1 integrin) is followed by activation of the PI3K pathway with production of MMP-9 (Redondo-Muñoz et al., 2006). CD38 expression on the CLL cell membrane significantly enhances CD49d-mediated adhesion by inducing a more complex distribution of F-actin filaments and a marked phosphorylation of the guanine nucleotide exchange factor Vav-1 (Zucchetto et al., 2012). CD38+/CD49d+ CLL clones that adhere to recombinant V-CAM-1 are also more resistant to apoptosis than CD38-/CD49d+ clones. The interaction between CD38 and CD49d has been mainly demonstrated by co-localization and co-immunoprecipitation experiments.

Another adhesion molecule that is important in CLL survival is CD44, which belongs to a family of transmembrane receptors for hyaluronic acid, a major component of the extracellular matrix, and is also involved in selected adhesion functions and in bidirectional signal delivery (Ilangumaran et al., 1999). CD44 is an integral component of the CD74 receptor complex, which binds migration inhibiting factor (MIF) (Shi et al., 2006) (Gore et al., 2008). CD44 transmits signals after MIF is bound to CD74 (Gordin et al., 2010). CD74 is involved in the maturation of normal B-cells through a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its coactivator TAFII (Gore et al., 2008). This circuit is also operative in CLL cells. Milatuzumab (Immunomedics), a novel humanised mAb that targets CD74 has been found to be effective in treating CLL in early trials (Stein et al., 2007) (Hertlein et al., 2010). Matrix metalloproteases (MMPs) are proteolytic proenzymes involved in degradation of the extracellular matrix during the early steps of tumorigenesis (Hua et al., 2011)

and also play a role in the late stages of tumour progression, invasion and metastasis (Gialeli et al., 2011) in solid malignancies. MMP-9 is the dominant MMP produced by CLL cells and contributes to their tissue infiltration (Redondo-Muñoz et al., 2006). Furthermore, its expression correlates with advanced clinical stages of the disease (Molica et al., 2003). MMP-9 triggers intracellular signalling pathways, including LYN activation, STAT3 phosphorylation, and MCL1 up-regulation and prevents CLL apoptosis (Redondo-Muñoz et al., 2010). CD38, CD49d, MMP9 and CD44 were recently found to act in close association with CD38 acting as a link between the discrete steps of the homing process (Buggins et al., 2011). Daratumumab (GenMab), a human anti-human CD38 mAb, entered a phase I/II clinical trial for patients with multiple myeloma and CLL with the rational of attempting to disrupt this interaction.

Another study showed the role of β2 integrins and their ligands in the regulation of apoptosis. When CLL cells were treated with monoclonal antibodies directed against β2 integrins, specifically against the I-domain of the chain of CD11b/CD18, this inhibited apoptosis. The physiological ligand or counter-receptor for β2 integrins that was required for the inhibition of apoptosis induction was identified as iC3b. Free iC3b levels were elevated in CLL patients indicating that this ligand is available in vivo where it may interact with β2 integrins on CLL B-cells and sustain their viability by preventing activation of the programmed cell death pathway (Plate et al., 2000).

Survival signals are provided by various molecules and pathways. The TNF family members BAFF and APRIL expressed on NLC and interact with corresponding receptors B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), and BAFF receptor (BAFF-R) on CLL cells (Burger, 2011). This induces activation of the canonical NF-κB pathway and protects CLL cells from apoptosis (Nishio et al., 2005) (Endo et al., 2007). CD38 molecules on CLL cells interact with CD31, expressed on stromal and NLCs. Along with this, the BCR pathway is activated by antigen stimulation. *In vitro* experiments have shown that BCR stimulation and co-culture with NLCs induces CLL cells to secrete chemokines (CCL3, CCL4, and CCL22), which recruit additional immune cells including T-cells and monocytes. Normally CCL3 and CCL4 are secreted by B-cells after activation by the BCR and CD40 ligand. Similarly CLL cells also secrete CCL3 and CCL4 in response to BCR stimulation. Plasma levels of CCL3 and CCL4, are high in CLL patients and plasma levels of CCL3

correlate with established prognostic markers and time to treatment (Krzysiek et al., 1999) (Burger et al., 2009b) (Sivina et al., 2011). This is demonstrated by a study on 351 patients which showed that the level of CCL3, advanced clinical stage, poor-risk cytogenetics, and CD38 expression were independent prognostic markers in multivariable analysis (Sivina et al., 2011). Thus CCL3 produced by CLL cells direct T-cells to activated CLL cells for a direct interaction which helps in CLL cell proliferation. Based on these findings, CLL cells have been shown not to be inert cells, but rather are actively expressing and secreting chemokines thereby creating a favourable microenvironment.

Another interesting area is the role of T-cells in CLL cell survival. In untreated CLL patients, both the CD4 and the CD8 populations are increased, unlike other malignancies where T-cells are usually reduced in number. These T-cells are functionally impaired and fail to form appropriate immunological synapses with CLL cells (Ramsay et al., 2008). This dysfunction to a certain extent can be reversed with immunomodulatory drugs like lenalidomide, which has a wide range of immunomodulatory activities, including stimulation of T-cells through CD28, enhancement of the expression of cytokines (including IL-2 and IFN-γ), repression of regulatory T-cells with concomitant induction of Th17 and increase of NK-cell and of antibody-dependent cytotoxicities (Gorgun et al., 2009). In proliferation centres, activated CD4+ T cells co-localise with proliferating CLL cells suggesting that T-cell subpopulations promote the expansion of the CLL clone (Patten et al., 2008). This is supported by recent *in vivo* evidence in immunodeficient mice that activated CD4+ T-cells support CLL cell proliferation in a CLL-transfer model. CLL cells themselves plays an active role in the accumulation of T lymphocytes by secreting CCL22, CCL3 and CCL4, which are involved in T-cell recruitment to the LN. However, this migration in response to CXCL12, CCL21 and CCL19 of T-cells from CLL patients is partially defective, as compared to T-cells from healthy adults even though CXCR4 and CCR7 expression is similar. This defect is more prominent in good prognostic ZAP70-/CD38- CLL patients where the low migratory response towards CXCL12 in T-cells may favour the indolent clinical course of the disease in these patients (Borge et al., 2010). CD40L (CD154) expression on the T-cells in the proliferation centre is high which interacts with the CD40 on CLL cells, rescuing them from apoptosis and mediated by up-regulation of the pro-survival protein survivin, repression of BCL2 and induction of BCL-X_L and BCL2A1 (Plander et al., 2009) (Granziero et al., 2001) (Vogler et al., 2009). The hypo-responsiveness of the T-cell compartment of CLL patients may be due to the inefficient antigen

presentation effected by neoplastic cells, which is partly due to the low expression of CD40L, resulting in diminished co-stimulation via CD40. Over-expressing CD40L on CLL cells increases antigen presentation by leukaemic cells. Surface expression of CD40L on CLL cells after gene therapy treatment promotes expression of costimulatory molecules including CD40, CD80 and CD86 on neighbouring bystander CLL cells, thereby making them better costimulants for T-cell activation (Wierda et al., 2000).

Reprogramming of autologous T-cells to target specific tumour antigens is an area of intense investigation and has involved the use of an antibody-derived antigen-binding moiety fused with an internal signalling domain such as CD3f to form a chimeric antigen receptor (CAR) (Urba and Longo, 2011). CARs have been found to be successful and as the therapy uses the patient's own cells, the risk of graft-versus-host disease is not an issue. Low doses of autologous T-cells infected with a CD19-targeted CAR infused into a CLL patient induced tumour lysis syndrome followed by persistent clinical response (Porter et al., 2011).

Considerable evidence indicates that an immune response is not solely determined by antigenic stimulation, but rather by complex interactions between the endocrine, nervous and immune systems helps to regulate the immune system (Souza-Moreira et al., 2011). Nucleotides such as adenosine triphosphate (ATP) and the enzymes involved in their metabolism could be involved in CLL cell survival and apoptosis. In the LN proliferation centres, CLL cells can activate an adenosinergic axis, which involves the ectoenzymes CD39 and CD73; causing the accumulation of adenosine and activation of A2A receptors which can protect CLL cells from spontaneous or drug-induced apoptosis and can inhibit chemotaxis (Serra et al., 2011). This protective effect is possibly mediated by cAMP through the activation of the NF-κB pathway (Himer et al., 2010). Potentially, this axis could be therapeutically targeted in CLL by blocking CD73, which is used in solid tumours, or by using antagonists of the A2A receptor, which could limit the increase in cytoplasmic cAMP levels associated with anti-apoptosis and chemoresistance (Smit et al., 2007) (Stagg et al., 2010).

Another agent involved in this axis is nicotinamide, the main precursor of NAD+. There are experiments showing that treatment of CLL cells with nicotinamide triggers a rapid activation of apoptosis, which is mediated by a functional loop that involves SIRT1 as the key player. SIRT1 is the main member of the sirtuin family and inactivates p53 by deacetylating a critical

lysine residue. Nicotinamide blocks SIRT1, resulting in increase of active p53. Chemotherapeutic agents, known to activate the p53 pathway potentiate this effect and the combination of DNA-damaging chemotherapeutics and nicotinamide should yield optimal apoptotic responses (Audrito et al., 2011).

The treatment of CLL has advanced significantly over the last decade and there are effective first line treatments in the form of combination immunochemotherapy. But the treatment of relapsed and refractory disease is still very difficult. Advances in the knowledge of specific pathways activated in CLL like BCR pathway, helped to develop more specific, targeted treatments in CLL, which is still in early phase of development. This project is mainly directed on exploring new pathways that could be activated in CLL, and the manipulation of the same could have therapeutic benefit.

1.4 Hypothesis

The data from gene expression profiling could be used to delineate antigens with therapeutic potential in chronic lymphocytic leukaemia

1.5 Aims and Objectives

1) To systematically screen cell surface proteins using flow cytometry and to describe new antigens expressed on the surface of CLL cells that could be used as diagnostic, prognostic or therapeutic targets.
2) To establish an *in vitro* CLL cell culture system to explore whether these antigens play any role either in the survival or apoptosis of CLL cells and thereby establishing the therapeutic potential of manipulating their pathways.
3) To test new agents inhibiting the BCR pathway signalling molecules, as potential therapeutic agents and exploring the physiological changes in CLL cells including phosphorylation and Ca^{2+} flux using flow cytometry.
4) To explore any relationship between BCR signalling pathway and the pathways of newly identified molecules to identify any synergistic therapeutic potential.

2. Materials and Methods

2.1 Patient and control groups

Patient material used in this study is from patients with a diagnosis of CLL according to NCI-Working Group (WG) criteria who were previously untreated or had not received any treatment for the last 6 months. Samples were anonymised and no patient-identifiable information was recorded as part of this study. All patients were attending the CLL clinic at St James's University Hospital, Leeds. A normal control group was also evaluated for certain experiments. Analyses were performed according to specific research ethics protocols (LREC Number-04/Q1205/125) and samples were collected in tubes with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were either processed on the same day or were stored in 4^{o}C until processing.

2.2 Flow cytometry

2.2.1. Instrumentation

Three Flow cytometry instruments were used for the whole project.

1. BD FACSCanto™ which was mainly used for the antibody identification experiments. Blue, red and violet lasers were used and the filters used are shown in Table 2.1

Table 2.1 Filter sets used BD FACSCanto™

Filter set	Possible fluorochromes
B530/30	FITC, AF488, Fluo-3
B585/42	PE
B670/40	PerCP, PerCP-Cy5, PerCP-Cy5-5, PE-Cy5, PE-Cy5-5, 7-AAD, Fura-Red
B780/60	PE-Cy7, PC7
R660/20	APC, AF647
R780/60	APC-Cy7, APC-H7, AF750, APC-AF750
V450/50	V450, Pacific Blue, VioBlue
V515/50	V500, V500-C, Pacific Orange

2. BD LSRFortessa™ in which majority of cell viability, phosflow and calcium flux experiments were done. This machine was also used for the viability

experiments using high throughput cytometry (HTC). Blue, red and violet lasers were used and the filters used are shown in Table 2.2

Table 2.2 Filter sets used in BD LSRFortessa™

Filter set	Possible fluorochromes
B530/30	FITC, AF488, Fluo-3
B575/26	PE
B610/20	PE-Texas Red, ECD
B695/40	PerCP, PerCP-Cy5, PerCP-Cy5-5, PE-Cy5, PE-Cy5-5, 7-AAD, Fura-Red
B780/60	PE-Cy7, PC7
R670/14	APC, AF647
R730/45	AF700, APC-AF700
R780/60	APC-Cy7, APC-H7, AF750, APC-AF750
V450/50	V450, Pacific Blue, VioBlue
V525/50	V500, V500-C, Pacific Orange
V605/12	Qdot605

3. BD LSRFortessa™ with additional ultraviolet laser in which calcium flux experiments using indo-1 were done. Blue, red, violet and ultraviolet lasers were used and additional filters included for Indo-1 at 395nm (Indo-1 with intracellular calcium) and 525nm (Indo-1 without intracellular calcium).

The daily performance of the machines was checked using cytometry setting and tracking (CS&T) beads (BD Biosciences) and rainbow beads (BD Biosciences).

CS&T beads are composed of equal concentrations of dim, mid, and brightly dyed polystyrene beads with low intrinsic coefficients of variation in MFI. With the help of BD software they automatically determine and create baseline performance values and daily running of the beads will maintain the settings consistent in the same range.

Rainbow beads have particles with a mixture of fluorochromes that are spectrally similar to the fluorochromes used in flow cytometry. The running of these beads helps in day-to-day performance verification, and long-term performance tracking of different flow cytometer channels in one run.

Fluidics maintenance for the BD FACSCanto™ was done using automated start-up and shut-down fluidics programmes which uses cleaning solutions like FACS clean (BD Bioscience) and FACS rinse (BD Bioscience).

For the BD LSRFortessa, 2% solution of Contrad 70 (Decon labs), FACS rinse and distilled water were run manually for 5 minutes each.

The alignment of the HTC was checked prior to use, using automated programmes available on the BD LSRFortessa™.

PMT voltages were set using an unstained sample of lymphocytes. The voltages were adjusted such that the unstained cells appear in the first decade (or first quartile) of a 4-decade logarithmic scale for each fluorochrome to be measured.

Compensations for standard fluorochromes were done using stained and unstained beads and adjusting with BD compensation software. For tandem conjugates, wherever possible individual antibodies were compensated separately. For non-standard fluorochromes like 7-AAD compensations was done manually using stained and unstained cells.

In the initial screening experiments no controls like isotype control or fluorescence-minus-one controls were used as the idea was to subjectively identify antibodies that have the highest expression on CLL cells or B-cells. In further experiments, which objectively analysed the expression of the screened antibodies, negative controls like CD14 for direct antibodies and CD2 for indirect antibodies were used.

2.2.2. Red cell lysis

Microtitre plates were used for flow cytometry experiments. Leucocytes were isolated by incubating whole blood with a 4-fold excess of ammonium chloride (8.6 g/l in distilled water) for 5 min at 37^{o}C to lyse red cells. Plates were centrifuged at 2000 x g for 60 seconds and cells were washed twice in 200μl of FACS Flow (BDIS) containing 0.3% bovine serum albumin (BSA) (Sigma-Aldrich).

2.2.3. Direct staining using commercially conjugated antibodies

1 x 10^{6} leucocytes were stained with the appropriate volume of pre-titered directly conjugated antibodies and the test antibody for 20 min at 4^{o}C in the dark. The antibodies used were CD5 conjugated to allophycocyanin (APC), CD19 conjugated to PECy7, CD45 conjugated to fluorescein isothiocyanate (FITC) and the test antibody conjugated to phycoerythrin (PE). (Details of the antibodies used in appendix 1). The samples were then washed twice in FACS flow BSA and re-suspended for acquisition in FACS flow.

The antibodies tested by direct conjugation are listed in Table 2.3 along with their basic characteristics.

Table 2.3 Direct antigens

Data in this table is taken from the following websites. References are made where there is additional data
http://www.ncbi.nlm.nih.gov/gene
http://omim.org/entry
http://www.uniprot.org/uniprot

Antigen	Alternative names	Gene names	Type of membrane protein	Expression and functions
CD51	ITGAV Integrin alpha-V, Vitronectin receptor subunit alpha	ITGAV VNRA, MSK8 VTNR	Single-pass type I membrane protein.	CD51 is a type I integral membrane glycoprotein, known as vitronectin receptor α chain, or integrin α_V. Integrins are major receptors for extracellular matrix mediated cell adhesion and migration, cytoskeletal organization, cell proliferation, survival, and differentiation. Alpha-V integrins comprise a subset sharing a common alpha-V subunit combined with 1 of 5 beta subunits. Most alpha-V integrins recognise the sequence RGD in ligands like vitronectin, fibronectin, osteopontin, bone sialoprotein, thrombospondin, fibrinogen, von Willebrand factor, tenascin, and agrin. In the case of alpha-V-8, laminin and type IV collagen. Expressed on endothelial cells, fibroblasts, macrophages, platelets, osteoclasts, neuroblastoma, melanoma, and hepatoma cells. It plays important roles in leukocytes homing and rolling, mediates bone absorption and angiogenesis.
CD85j	Leukocyte immunoglobulin-like receptor subfamily B member 1 LIR-1 CD85 antigen-like family member J ILT-2 Monocyte/macrophage immunoglobulin-like receptor 7	LILRB1 ILT2, LIR1, MIR7	Single-pass type I membrane protein.	CD85 is a group of Ig superfamily tansmembrane glycoproteins called Ig-Like Transcripts (ILTs) or Leukocyte Immunoglobulin-like Receptors (LIRs). LIRs are a family of immunoreceptors expressed predominantly on monocytes and B-cells and at lower levels on dendritic cells and natural killer (NK) cells. All members of LIR subfamily B, such as LILRB1, contain a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) which serves an inhibitory function. Upon engagement by MHC class I or other ligands which phosphorylate tyrosine residue of the ITIM and recruit intracellular protein-tyrosine phosphatases, such as SHP1, an inhibitory signal cascade ensues. Most members of LIR subfamily A, such as LILRA1 have short cytoplasmic regions that lack ITIMs, but have transmembrane regions that contain a charged arginine residue which can initiate stimulatory cascades. One member of subfamily A, LILRA3, lacks a transmembrane region and is presumed to be a soluble receptor(Borges et al., 1997). It is found on the surface of B-cells, plasma cells,

	MIR-7			dendritic cells, monocytes, subsets of NK and T-cells. Its cytoplasmic tail provides inhibitory signals by recruiting SHP-1
CD97	leukocyte antigen CD97; seven-span transmembrane protein; seven transmembrane helix receptor; seven-transmembrane, heterodimeric receptor associated with inflammation	CD97 TM7LN1	Multipass Membrane protein	CD97 is a seven-span transmembrane glycoprotein belonging to the secretin receptor superfamily. It is 75-to 85-kD and is present on the surface of most activated leukocytes. The 722-amino acid protein has a C-terminal region contains 7 hydrophobic domains as seen in various G-protein associated transmembrane receptors. CD97 is a member of a superfamily that includes the receptors for secretin, calcitonin, and other mammalian and insect peptide hormones. CD97 has an extracellular region of 433 amino acids with 3 N-terminal epidermal growth factor-like domains of which 2 of them are calcium-binding sites and a single arg-gly-asp (RGD) motif. Structural features characteristic of extracellular matrix proteins and transmembrane proteins suggests that CD97 is a receptor involved in both cell adhesion and signalling. (Hamann et al., 1995) CD55 is the cellular ligand for CD97. Expressed on monocytes/ macrophages, granulocytes, dendritic cells, and smooth muscle cells. It is also expressed in thyroid, colorectal, gastric, oesophageal and pancreatic carcinomas. In resting T- and B-lymphocytes expression is at low level, but is rapidly unregulated upon activation. Expression is increased under inflammatory conditions in the CNS of multiple sclerosis and in synovial tissue of patients with rheumatoid arthritis. Increased expression of CD97 in the synovium is accompanied by detectable levels of soluble CD97 in the synovial fluid. CD97 has been shown to mediate cell adhesion and co-stimulation of T-cell proliferation.
CD119	Interferon gamma receptor 1	IFNGR1	Single-pass type I membrane protein.	CD119 is a type I protein, also known as IFN-γ R α chain or IFN-γRI. Defects in IFNGR1 are a cause of mendelian susceptibility to mycobacterial disease (MSMD) also known as familial disseminated atypical mycobacterial infection which is a rare condition predisposing patients to illness caused by moderately virulent mycobacterial species, such as Bacillus Calmette-Guerin (BCG) vaccine and environmental non-tuberculous mycobacteria, and also by the more virulent Mycobacterium tuberculosis. The inheritance is autosomal recessive, autosomal dominant or X-linked. The pathogenic mechanism underlying MSMD is the impairment of interferon-gamma mediated immunity which could make patient susceptible to overwhelming mycobacterial disease with lepromatous-like lesions in early childhood, whereas others develop, later in life, disseminated but curable infections with tuberculoid granulomas. Other microorganisms rarely cause severe clinical disease is Salmonella which infects less than 50% of these individuals. A genetic variation in IFNGR1 is associated with

				susceptibility to Helicobacter pylori infection also. The IFN-γ receptor is expressed at moderate levels on virtually every cell with the exception of erythrocytes. Involved in signal transduction using Jak1 and Jak2 protein kinases and STAT1 activation(Basler and García-Sastre, 2002)
CD137L	Tumour necrosis factor ligand superfamily member 9 4-1BB ligand	TNFSF9	Single-pass type II membrane protein.	4-1BB ligand, also known as CD137L, is a 97 kD member of the tumour necrosis factor (TNF) superfamily. This transmembrane cytokine is a bidirectional signal transducer that acts as a ligand for TNFRSF9/4-1BB. This serves as a costimulatory receptor molecule in T lymphocytes and is involved in the antigen presentation process and in the generation of cytotoxic T cells. This has also been shown to reactivate anergic T lymphocytes in addition to promoting T lymphocyte proliferation and is involved in optimal CD8 responses in CD8 T cells, APCs, activated B and T-cells. Expression in peripheral CD14 positive monocytes was significantly higher in patients with chronic hepatitis B than in healthy controls (J. Wang et al., 2010).
CD141	Thrombomodulin Fetomodulin	THBD THBM	Single-pass type I membrane protein.	Defects in THBD are the cause of some thrombophilia due to thrombomodulin defect. Defects in THBD are a cause of susceptibility to haemolytic uremic syndrome atypical type 6, which is an atypical form of haemolytic uremic syndrome, a complex genetic disease characterized by microangiopathic haemolytic anaemia, thrombocytopenia, renal failure and absence of episodes of enterocolitis and diarrhoea. Atypical forms have a poorer prognosis, with higher death rates and frequent progression to end-stage renal disease compared to the typical form. Thrombomodulin is a specific endothelial cell receptor that forms a 1:1 stoichiometric complex with thrombin. This complex is responsible for the conversion of protein C to the activated protein C. Once evolved, activated protein C triggers cofactors of the coagulation mechanism, factor Va and factor VIIIa, and thereby reduces the amount of thrombin generated.
CD155	Poliovirus receptor Nectin-like protein 5 NECL-5 PVS; HVED; TAGE4;	PVR PVS	Single-pass type I membrane protein	CD155, known as poliovirus receptor (PVR) or nectin-like 5, is a 70 kD type I transmembrane glycoprotein CD155 mediates NK cell adhesion and triggers NK cell effector functions. This binds two different NK cell receptors, CD96 and CD226 leading to the formation of a mature immunological synapse between NK cell and target cell and these synapses accumulates at the contact site. The formation of these synapses triggers the secretion of lytic granules and IFN-gamma which in turn stimulate cytoxicity of activated NK cells. This synapse sometimes transfer the PVR to NK cells which is more important in some tumour cells expressing a lot of PVR, which in turn activates the NK cells, providing tumours with a mechanism of immuno-evasion and mediating tumour cell invasion and migration. PVR also serves as a receptor for poliovirus attachment to neuronal cells and play a role in axonal transport of poliovirus. Virion-

				PVR-containing endocytic vesicles are transferred to the microtubular network through interaction with DYNLT1 and drive the virus-containing vesicle to the axonal retrograde transport. Expressed on endothelial cells, monocytes, epithelia and central nervous system. CD155 is an adhesion molecule involved in cell-cell and cell-matrix adhesion. CD155 mediates NK cell adhesion and triggers NK cell effector functions. The external domain mediates cell attachment to the extracellular matrix molecule vitronectin, while its intracellular domain interacts with the dynein light chain Tctex-1/DYNLT1.
CD167a	Epithelial discoidin domain-containing receptor 1; Cell adhesion kinase; Discoidin receptor tyrosine kinase; HGK2; Protein-tyrosine kinase 3A; Protein-tyrosine kinase RTK-6; TRK E; Tyrosine kinase DDR; Tyrosine-protein kinase CAK	DDR1, CAK, EDDR1, NEP, NTRK4, PTK3A, RTK6, TRKE	Single-pass type I membrane protein	CD167a is a membrane type II receptor kinase, containing a factor VIII-like domain. CD167a expression can be upregulated by p53. It is expressed on epithelial cells of colon mucosa, thyroid follicles, and distal tubules of kidney and over expressed in some breast carcinomas cells, epidermoid carcinoma cells, melanoma cells, colon carcinoma cell lines. It is a tyrosine kinase that functions as cell surface receptor for fibrillar collagen proteins like collagen type II alpha 1, collagen type III alpha 1, collagen type V alpha 2, collagen type XI alpha 1; It thereby controls various cell functions like attachment to the extracellular matrix, cell migration, differentiation, survival and proliferation. It interacts with a variety of other proteins including phospholipase gamma 1, SHC, and the lipid-anchored docking protein FRS2. Collagen binding triggers a signalling pathway that involves SRC and leads to the activation of MAP kinases. Regulation of remodelling of the extracellular matrix is usually by up-regulation of the matrix metalloproteinases MMP2, MMP7 and MMP9. This facilitates cell migration and wound healing. It also promotes smooth muscle cell migration, and hence arterial wound healing. It is also required for normal blastocyst implantation during pregnancy, for normal mammary gland differentiation and normal lactation. Also plays a role in tumour cell invasion.
CD205	Lymphocyte antigen 75; C-type lectin domain family 13 member B; DEC-205;	LY75SCD205CLEC13B	Single-pass type I membrane protein	CD205 is a 210 kD C-type lectin transmembrane protein, known as DEC-205. Expressed on dendritic cells, thymic epithelial cells and low levels on T- and B-cells, NK cells and monocytes. Detected in myeloid, B-lymphoid cell lines and Reed-Sternberg (HRS) cells. It serves as an endocytic receptor, functions in antigen uptake from extracellular space, processing and clearance of apoptotic cells. Causes reduced proliferation of B-lymphocytes.
CD210	Interleukin-10 receptor subunit alpha; Interleukin-10 receptor	IL10R, IL10R	Single-pass type I membrane protein.	CD210, also known as the IL-10 receptor, is a 90-110 kD protein which belongs to the class II cytokine receptor family. Defects in IL10RA are the cause of inflammatory bowel disease type 28 which is a chronic, relapsing inflammation of the gastrointestinal tract with crohn’s disease or ulcerative colitis phenotypes. It is expressed weakly on

	subunit 1			pancreas, skeletal muscle, brain, heart, and kidney, intermediate level on placenta, lung, and liver and strongly on monocytes, B-cells, large granular lymphocytes, and T-cells express high levels. The IL-10 receptor is involved in signal transduction by inducing phosphorylation of STAT1a and STAT3 and by inducing activation of Jak1 and Tyk2 kinases. It has been shown to mediate the immunosuppressive signal of interleukin 10, and thus inhibits the synthesis of proinflammatory cytokines
CD279	Programmed cell death protein 1	PDCD1, PD1	Single-pass type I membrane protein.	The program death 1 (PD-1) receptor CD279 is a 55 kDa member of the immunoglobulin superfamily. PD-L1 (B7-H1) and PD-L2 (B7-DC) are ligands of CD279/PD-1 and are members of the B7 gene family. Genetic variation in PDCD1 is associated with susceptibility to systemic lupus erythematosus type 2. It is thought to represent a failure of the regulatory mechanisms of the autoimmune system. Expressed on activated T-cells, B-cells and myeloid cells. It plays a key role in peripheral tolerance and autoimmune disease. Interaction of CD279: PD-Ligands results in inhibition of T-cell proliferation and cytokine secretion.
CD298	Sodium/potassium-transporting ATPase subunit beta-3; Sodium/potassium-dependent ATPase subunit beta-3; ATPB-3	ATP1B3	Single-pass type II membrane protein	CD298 or the β3 Na+/K+ ATPase, is a 42 kDa type II transmembrane protein, also known as ATP1B3 which is a part of Na+/K+ -ATPase. This is an integral membrane protein which helps to maintain the electrochemical gradients of Na and K ions across the plasma membrane which in turn helps in osmoregulation, sodium-coupled transport of organic and inorganic molecules, and electrical excitability of nerve and muscle. This enzyme is composed of two subunits, alpha and beta, and the beta subunit regulates, the number of sodium pumps transported to the plasma membrane. It has broad tissue distribution, including all leukocytes and many other tissues.
CD337	Natural cytotoxicity triggering receptor 3; Activating natural killer receptor p30; Natural killer cell p30-related protein; NKp30	NCR3, 1C7, LY117	Single-pass type I membrane protein.	CD337 is a type I protein, member of the natural cytotoxicity receptor family that contains one immunoglobulin-like domain. NCR3 is expressed in all resting and IL-2 activated NK cells and forms a complex with CD3-zeta. CD337 enhances NK cell triggering and cytolysis of tumour targets and other target cells deficient in MHC class I molecules. Engagement of NCR3 by BAG6 also promotes dendritic cell (DC) maturation, by inducing NK cells to release TNFA and IFNG, which promotes DC maturation. It also destroys those DCs that did not properly acquire a mature phenotype.
DR3	Tumour necrosis factor receptor	TNFRSF25 APO3,	Single-pass type I	DR3 is a member of the TNF receptor family. It activates two distinct signalling cascades including apoptosis and NF-kappa-B signalling by interacting with TNFRSF1

	superfamily member 25; Apo-3; Apoptosis-inducing receptor AIR; Apoptosis-mediating receptor TRAMP; Lymphocyte-associated receptor of death;	DDR3, DR3, TNFRSF12 WSL, WSL1	membrane protein.	and TRADD. It is expressed in thymocytes lymphocytes and prostate.
ErbB3/ HER3	Receptor tyrosine-protein kinase erbB-3; Proto-oncogene-like protein c-ErbB-3; Tyrosine kinase-type cell surface receptor HER3	HER3; LCCS2; ErbB-3; c-erbB3; erbB3-S; MDA-BF-1; c-erbB-3; p180-ErbB3; p45-sErbB3; p85-sErbB3	Single-pass type I membrane protein	ErbB3/HER3 is a receptor tyrosine kinase and a member of the epidermal growth factor receptor family. Defects in ERBB3 are the cause of lethal congenital contracture syndrome type 2, also called Israeli Bedouin multiple contracture syndrome type A which is inherited as autosomal recessive. LCCS2 is a neonatally lethal arthrogryposis that is associated with atrophy of the anterior horn of the spinal cord and characterised by multiple joint contractures, and markedly distended urinary bladder. ErbB3 is expressed in kidney, lung, brain, placenta, skin and stomach and overexpressed in prostate, bladder, and breast tumours. As this membrane-bound protein has no active kinase domain it heterodimers with other EGF receptor family members with kinase activity which leads to the activation of pathways in cell proliferation or differentiation.
IFN-γ R β chain	Interferon gamma receptor 2; Interferon gamma receptor accessory factor 1; AF-1	IFNGR2, IFNGT1	Single-pass type I membrane protein.	It is a member of the class II cytokine receptor family. Defects in IFNGR2 can cause mendelian susceptibility to mycobacterial disease (MSMD), or familial disseminated atypical mycobacterial infection which is genetically heterogeneous with autosomal recessive, autosomal dominant or X-linked inheritance which makes patients susceptible to moderately virulent mycobacterial species, like Bacillus Calmette-Guerin (BCG) vaccine and non-tuberculous mycobacteria, as well as by virulent Mycobacterium tuberculosis. IFN-γ R β chain is broadly expressed on a variety of cells at low levels and upregulated on some activated B-cells.
Integrin β7	Gut homing receptor beta subunit	ITGB7	Single-pass type I membrane	Integrin β7 is a member of the Ig superfamily and is usually expressed in association with integrin α4 or αE chain forming heterodimers α4/β7 or αE/β7. It expressed on majority of peripheral lymphocytes, small subsets of thymocytes and bone marrow

			protein.	progenitors. Integrin alpha-4/beta-7 (Payer's patches-specific homing receptor LPAM-1) acts as an adhesion molecule directing lymphocyte migration and homing to gut-associated lymphoid tissue (GALT). It also interacts with MADCAM1, an adhesion molecule expressed by the vascular endothelium of the gastrointestinal tract as well as with VCAM1 and fibronectin. It also binds to HIV-1 gp120, which allows the virus to enter GALT. Integrin alpha-E/beta-7 (HML-1) acts as a receptor for E-cadherin.
CD114	Granulocyte colony-stimulating factor receptor	CSF3R GCSFR	Multi-pass membrane protein.	CD114 is the receptor of the colony stimulating factor 3 (CSF3). The extracellular domain consists of an immunoglobulin-like domain, a cytokine receptor homologue domain, and three fibronectin type III repeats. CD114 is expressed in all stages of granulocyte differentiation and in monocytes, platelets, endothelial cells, placenta and trophoblasts. The binding of CSF3, results in the activation of many signalling molecules such as SYK, Lyn, Jak1, Jak2, Tyk2, SOCS3, SOCS1, STAT5, and Shp1, resulting in the expression of different target genes that will increase neutrophil precursor survival, proliferation and maturation.
GRM4	Metabotropic glutamate receptor 4 mGluR4	GRM4 GPRC1D, MGLUR4	Multi-pass membrane protein.	GRM4 is G protein-coupled receptor for L-glutamate which is a major excitatory neurotransmitter in the central nervous system and is involved in several areas of normal brain function and pathologic conditions. It stimulates ionotropic as well as metabotropic glutamate receptors. It is strongly expressed in the cerebellum but at low levels in hippocampus, hypothalamus and thalamus.
CD268	Tumour necrosis factor receptor superfamily member 13C; BAFF receptor; BLyS receptor 3; BAFFR; CVID4; BROMIX; prolixin	TNFRSF13 C; BAFFR, BR3,	Single-pass type III membrane protein	B cell-activating factor (BAFF) is a regulator of the peripheral B-cell population. In vitro it has been shown to enhance B-cell survival. Abnormally high BAFF level is proposed to be involved in autoimmune disorders by enhancing the survival of auto-reactive B-cells as its level is shown to be high in SLE patients and overexpression of BAFF in mice will result in SLE like disease. Expression is high in spleen and lymph node, and in resting B-cells and low in activated B-cells, resting CD4+ T-cells, thymus and peripheral blood leukocytes.

2.2.4. Gating strategy for direct staining

In each case, samples were first gated on size and granularity characteristics to limit analysis to live lymphocytes and monocytes. Doublets were also gated out using forward and side scatter plot. Cell populations were identified based on their expression of CD19 and CD5 as shown in the Figure 2.2.

The expression of each of the new antigens was evaluated on these cell subsets. Figures 2.1 and 2.2 depict the gating strategy.

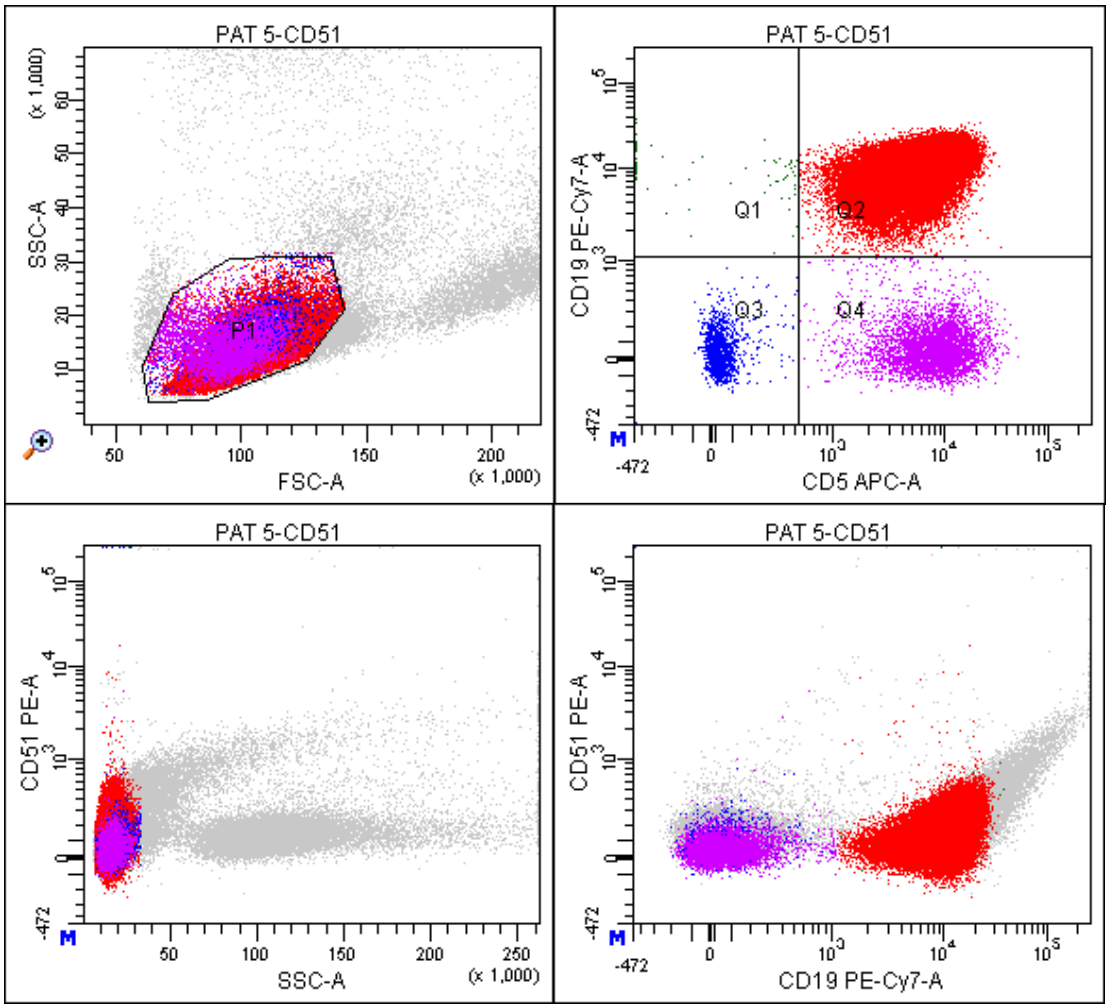


Figure 2.1 Gating strategy for direct staining.

In the first plot mononuclear cells are gated based on forward (x-axis) and side scatter (y-axis). In the second plot cells were divided into four quadrants based on CD19 and CD5 expression as explained in Figure 2.2. The expression of the test antibody (y-axis) against side scatter is shown in the third plot and in the fourth plot against CD19 expression.

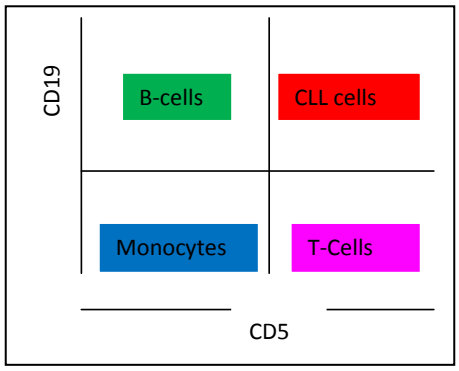


Figure 2.2 Quadrant plot for CD5 and CD19 expression on different cell types.

This figure explains the type of cells seen in the quadrant plot in Figure 2.1 based on CD19 and CD5 expression. B-cells: CD19+, CD5- ; CLL cells: CD19+,CD5+ ; Monocytes and NK cells: CD19-,CD5- ; T-Cells: CD19-,CD5+. The colour coding used here is maintained throughout the experiments using these antibodies to represent the corresponding cells.

2.2.5. Indirect staining

The majority of antibodies available were rabbit polyclonals. Unlike directly conjugated monoclonal antibodies, which are routinely used in flow cytometry, most of these polyclonal antibodies have not been tested by flow cytometry before. As they are not conjugated to fluorochromes commercially, indirect staining was used to detect them. The flow cytometric protocol needed optimisation for testing these antibodies.

The control antibody should also be a rabbit polyclonal against an extracellular epitope of B or T-cell antigen expressed on plasma membrane. Of the available such antibodies, anti-CD3 antibody was selected because of the ease of combining with the other antibodies.

Following red cell lysis 1x 10^6 leucocytes were stained with the appropriate volume of pre-titered directly conjugated antibody and the test antibody for 20 min at 4^oC in the dark as in direct staining. The cells are then washed twice with FACS flow BSA. Fluorochrome conjugated goat anti-rabbit secondary antibody (Alexa Fluor 647 goat anti-rabbit antibody) was then added and incubated for 20 min at 4^oC in the dark. This was washed again twice in FACS flow BSA and then re-suspended for acquisition in FACS flow

The details of the antibodies used for indirect staining are shown in Table 2.4

Table 2.4 Indirect antigens

Data in this table is taken from the following websites. References are made where there is additional data
http://www.ncbi.nlm.nih.gov/gene
http://omim.org/entry
http://www.uniprot.org/uniprot

Antigen	Alternative names	Gene names	Type of membrane protein	Expression and function
ADAM19	Disintegrin and metalloproteinase domain-containing protein 19, Meltrin-beta, Metalloprotease and disintegrin dendritic antigen marker, MADDAM	ADAM19, MLTNB, FKSG34	Single pass type 1 membrane protein	It is a metalloprotease expressed in several tissues and cancer cell lines. It serves as a marker for dendritic cell differentiation It is involved in several physiological processes like cell migration, cell adhesion, cell-cell and cell-matrix interactions, and signal transduction. It has a regulatory role in neurogenesis and synaptogenesis of glial cells.Pathologically it is involved in some cancers, inflammatory diseases, renal diseases, and Alzheimer's disease.
GPR18	N-arachidonyl glycine receptor, NAGly receptor, G-protein coupled receptor 18	GPR18, GPCRW	Multi-pass membrane protein.	Described in detail in chapter 6
CHRNB4	Neuronal acetylcholine receptor subunit beta-4, cholinergic receptor, nicotinic, beta 4 (neuronal)	CHRNB4	Multi-pass membrane protein	Described in detail in chapter 6
APLP1	amyloid beta (A4) precursor-like protein 1, AMYLOID PRECURSOR-LIKE PROTEIN antibody, C30 antibody, APLP	APLP1	Single-pass type I membrane protein.	Described in detail in chapter 6
DRD4	D(4) dopamine receptor, D(2C) dopamine receptor, Dopamine D4 receptor, D4DR	DRD4	Multi-pass membrane protein	Described in detail in chapter 6
GPR12	G-protein coupled receptor 12, FLJ18149 antibody, FLJ97704 antibody, GPCR12; GPCR21,	GPR12	Multi-pass membrane protein	It is a G-protein coupled receptor signaling through cAMP. It has a physiological role in promoting neurite outgrowth.

	MGC138349 antibody			
ROR1	Tyrosine-protein kinase transmembrane receptor ROR1, Neurotrophic tyrosine kinase, receptor-related 1receptor tyrosine kinase-like orphan receptor 1, NTRKR1	ROR1, NTRKR1	Membrane; Single-pass type I membrane protein.	It is a tyrosine kinase-like orphan receptor. It plays a role in neurite growth in the central nervous system. B-cell chronic lymphocytic leukaemia shows an increased expression of ROR1 and anti-ROR1 antibodies can induce apoptosis to CLL cells. (Baskar et al., 2012) (Daneshmanesh et al., 2012)Physiologically there is a high expression in human heart, lung and kidney, and weak expression in the central nervous system. A short isoform is expressed in malignancies originating from neuroectoderm.
TAG1	Contactin-2, Axonal glycoprotein TAG-1, Axonin-1, Transient axonal glycoprotein 1, TAX-1, CNTN2	CNTN2 AXT, TAG1, TAX1	GPI-anchor	Described in detail in chapter 6
$5HTR_7$	Serotonin receptor 7, 5-hydroxytryptamine receptor 7, 5-HT-X	HTR7	Multi-pass membrane protein	Described in detail in chapter 6
JAG1	Protein jagged-1, Jagged1, hJ1, CD339	JAG1, JAGL1	Single-pass type I membrane protein.	Jagged 1 is the ligand for the receptor notch 1 and is expressed in uterine cervical cells, squamous cell carcinoma and bone marrow cell line HS-27a. It signals through notch 1 and play a role in haematopoiesis, development of mammalian cardiovascular system and angiogenesis. Mutation affecting this protein can cause Alagille syndrome I which is characterized by hepatic, cardiac, skeletal, and ophthalmologic features.
ACCN1	Acid-sensing ion channel 2, ASIC2, Amiloridesensitive brain sodium channel, Amiloridesensitive cation channel 1, Amiloride-sensitive cation channel neuronal 1, Brain sodium channel 1, BNC1, BNaC1	ASIC2, ACCN, ACCN1, BNAC1, MDEG	Multi-pass membrane protein	Described in detail in chapter 6
GYPC	glycophorin C, Glycoconnectin, Glycophorin-D, Glycoprotein beta PAS-2', Sialoglycoprotein D	GYPC, GLPC, GPC	Single-pass type III membrane protein	Glycophorin C (GYPC) is a membrane sialoglycoprotein on human erythrocytes. Glycophorin-C includes blood group Gerbich antigens and receptors for Plasmodium falciparum merozoites and is important for maintaining the mechanical stability of red cells.
SLC2A3	solute carrier family 2, facilitated glucose transporter member 3,	SLC2A3, GLUT3	Multi-pass membrane	It is highly expressed in brain tissues and acts as a glucose transporter in neuronal tissues.

	Glucose transporter type 3		protein.	
EFNB1	Ephrin-B1, EFL-3, ELK ligand, ELK-L, LERK-2 EPH-related receptor tyrosine kinase ligand 2	EFNB1, EFL3, EPLG2, LERK2	Single-pass type I membrane protein	It belongs to a family of receptor protein-tyrosine kinases and is involved in development of nervous system and in erythrocytes. It is expressed on heart, placenta, lung, liver, skeletal muscle, kidney and pancreas.
GPR56	G protein-coupled receptor 56, Protein TM7XN1	GPR56, TM7LN4 TM7XN1	Multi-pass membrane protein	This is G protein-coupled receptor containing 7 transmembrane domains and a mucin-like domain in the N-terminal region and it binds to transglutaminase 2 in the extracellular space. Overexpression of this protein can suppress tumor growth and metastasis and it is downregulated in melanoma. It is expressed on thyroid gland, brain heart and a number of tumor cells.
RAMP3	receptor (G protein-coupled) activity modifying protein 3, Calcitonin-receptor-like receptor activity-modifying protein 3, CRLR activity-modifying protein 3	RAMP3	Single-pass type I membrane protein	This belongs to a family of proteins, called receptor (calcitonin) activity modifying proteins (RAMPs) which are required to transport calcitonin-receptor-like receptor (CRLR) to the plasma membrane. It is expressed in lung, breast, immune system and fetal tissues.
MR1	tumor necrosis factor receptor superfamily, member 1A, Tumor necrosis factor receptor 1	TNFRSF1A, TNFAR, TNFR1	Single-pass type I membrane protein.	It is major receptors for the tumor necrosis factor-alpha and it recruits caspase-8 through adapter molecule FADD thereby activating the downstream cascade of caspases mediating apoptosis.
CDH15	cadherin 15, type 1, M-cadherin Cadherin-14, Muscle cadherin	CDH15, CDH14,CDH3	Single-pass type I membrane protein	Cadherins are calcium-dependent cell adhesion proteins and is expressed in brain, cerebellum and muscles. M-cadherin is important in myogenesis and provides a trigger for terminal muscle differentiation.
SLC20A1	solute carrier family 20 (phosphate transporter), member 1, Gibbon ape leukemia virus receptor 1, Leukemia virus receptor 1 homolog, Phosphate transporter 1, Solute carrier family 20 member 1	SLC20A1, GLVR,1 PIT1	Multi-pass membrane protein	This is a ubiquitously expressed protein which plays a crucial role in phosphate transport in cells. It may also act as a retroviral receptor making human cells vulnerable to infection to Gibbon Ape Leukemia Virus (GaLV), Simian sarcoma-associated virus (SSAV), Feline leukemia virus subgroup B (FeLV-B) and 10A1 murine leukemia virus.
SLC4A1	solute carrier family 4, sodium	SLC4A10,	Multi-pass	It plays an important role in regulating intracellular pH by acting as a

	bicarbonate transporter, member 10 Sodium-driven chloride bicarbonate exchanger, Solute carrier family 4 member 10	NCBE	membrane protein	sodium/bicarbonate cotransporter in exchange for intracellular chloride.
PLAUR	Urokinase plasminogen activator surface receptor, Monocyte activation antigen Mo3, CD87	PLAUR, MO3, UPAR	Lipid-anchor GPI-anchor	It acts as the receptor for urokinase plasminogen activator and promotes plasmin formation which causes localized degradation of the extracellular matrix. It is expressed in the brain.
EDA	ectodysplasin A receptor, Tumor necrosis factor receptor superfamily member EDAR, Anhidrotic ectodysplasin receptor 1, Downless homolog, EDA-A1 receptor, Ectodermal dysplasia receptor, Ectodysplasin-A receptor	EDAR, DL	Single-pass type I membrane protein	This belongs to tumor necrosis factor receptor family and act as a receptor for the soluble ligand ectodysplasin A, which then activate NFκB, JNK, and caspase-independent cell death pathways. It has functional role in development of hair, teeth, and other ectodermal derivatives. It is expressed in fetal kidney, lung, skin and cultured neonatal epidermal keratinocytes.
NG2	chondroitin sulfate proteoglycan 4, Chondroitin sulfate proteoglycan NG2, Melanoma chondroitin sulfate proteoglycan, Melanoma-associated chondroitin sulfate proteoglycan	CSPG4 MCSP	Single-pass type I membrane protein	This is a chondroitin sulfate proteoglycan expressed by human malignant melanoma cells which plays a role in endothelial basement membrane spread of melanoma cells.
EDG4	lysophosphatidic acid receptor 2, LPA-2, Lysophosphatidic acid receptor Edg-4	LPAR2 EDG4, LPA2	Multi-pass membrane protein	This is a G protein-coupled receptors which functions as a lysophosphatidic acid (LPA) receptor leading to Ca2+ mobilization, through Gi and Gq proteins. Expressed in leukocytes, testes, pancreas, spleen, thymus and prostrate.
AMFR	E3 ubiquitin-protein ligase AMFR, Autocrine motility factor receptor, AMF receptor, RING finger protein 45, gp78	AMFR RNF45	Multi-pass membrane protein	This is a member of the E3 ubiquitin ligase family of proteins which acts as a ligand, for autocrine motility factor which is a tumor motility-stimulating protein secreted by tumor cells. It catalyzes ubiquitination and proteosomal degradation of specific proteins.

LTK	Leukocyte tyrosine kinase receptor, Protein tyrosine kinase 1	LTK, TYK1	Single-pass type I membrane protein	This is an orphan receptor protein with a tyrosine-protein kinase activity and plays a role in neurite outgrowth, and cell survival. Signalling appears to involve the PI3 kinase pathway and genetic variations in LTK that cause up-regulation of the PI3K pathway has been demonstrated in systemic lupus erythematosus (SLE).
ACVRL1	Serine/threonine-protein kinase receptor R3, Activin receptor-like kinase 1, TGF-B superfamily receptor type I	ACVRL1, ACVRLK1, ALK1	Single-pass type I membrane protein	It is the receptor for TGF-beta family ligands BMP9/GDF2 and BMP10 and is involved in normal blood vessel development. Mutation of the gene causes hereditary haemorrhagic telangiectasia.
ADAM28	Disintegrin and metalloproteinase domain-containing protein 28, Epididymal metalloproteinase-like, disintegrin-like, and cysteine-rich protein II, Metalloproteinase-like, disintegrin-like, and cysteine-rich protein L	ADAM28, ADAM23, MDCL	Single-pass type I membrane protein	ADAM28 is a metalloprotease involved in a number of biological functions including cell-cell and cell-matrix interactions, involved in fertilization, muscle development, and neurogenesis. This may also play a role in lymphocyte migration and ectodomain shedding of lymphocyte surface antigens like CD40L. It is expressed in lymphoid organs like spleen and lymph node and lipid tissues associated with gastrointestinal tract and respiratory tract.
TMPRSS 6	Transmembrane protease serine 6, Matriptase-2	TMPRSS 6, UNQ354, PRO618	Single-pass type II membrane protein	This is a serine protease which plays a role in matrix remodelling of liver and hydrolyses a range of proteins including type I collagen, fibronectin and fibrinogen. It is also involved in the regulation of iron homeostasis. This is predominantly expressed in liver. Genetic mutation affecting the gene can cause iron refractory iron deficiency anaemia.
CNR2	Cannabinoid receptor 2, CX5	CNR2	Multi-pass membrane protein	This is a G protein-coupled receptor for 2-arachidonoylglycerol which acts as an inhibitory signal by down regulating adenylate cyclase. Higher expressions of these are seen in B-cells, NK cells, hair follicles and microglial cells in brain. This protein plays an active role in cannabinoid-induced CNS effects experienced by users of marijuana.
GPR15	G-protein coupled receptor 15, Brother of Bonzo	GPR15	Multi-pass membrane protein	The function of this is not very clear and may act as a chemokine receptor and an alternative co-receptor with CD4 for HIV-1 infection.
ATP1B1	Sodium/potassium-transporting ATPase subunit beta-1, Sodium/potassium-dependent ATPase subunit beta-1	ATP1B1, ATP1B	Single-pass type II membrane protein	The protein found in most tissues is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane which are essential for osmoregulation, transport of a variety of organic and inorganic molecules

				across membranes, electrical excitability of nerve and muscle and maintaining epithelial cell polarity.
EGF	Pro-epidermal growth factor, Urogastrone	EGF	Single-pass type I membrane protein	This protein acts a stimulant factor for growth, proliferation and differentiation of numerous cell types. Mutation in the gene encoding this protein can cause hypomagnesaemia type 4 and is associated with the growth and progression of certain cancers. Expression of this protein is found in kidney, salivary gland, cerebrum and prostate.
MMP14	Matrix metalloproteinase-14, MMP-X1, Membrane-type matrix metalloproteinase 1	MMP14	Single-pass type I membrane protein	Expression of this matrix metalloproteinase is found in stromal cells of colon, breast, head and neck and lung tumours. This may activate progelatinase A on surface of tumour cells and thereby stimulating invasion by tumour cells. This may also regulate cell growth and migration indirectly by activating MMP15.
ATP1B2	Sodium/potassium-transporting ATPase subunit beta-2, Adhesion molecule in glia, Sodium/potassium-dependent ATPase subunit beta-2	ATP1B2	Single-pass type II membrane protein	Similar to ATP1B1 described above
GPR35	G-protein coupled receptor 35, G-protein coupled receptor 35	GPR35	Multi-pass membrane protein	This is a G-protein coupled receptor and expressed predominantly in immune system and gastrointestinal system and act as a receptor for kynurenic acid which is involved in tryptophan metabolism
FPRL1	N-formyl peptide receptor 2, N-formyl peptide receptor 2, N-formyl peptide receptor 2, HM63, Lipoxin A4 receptor, LXA4 receptor RFP	FPR2, FPRH1, FPRL1, LXA4R	Multi-pass membrane protein	This is a G-protein coupled receptor for N-formyl-methionyl peptides, which are potent neutrophils chemotactic factors and causes activation of neutrophils and is expressed in lungs, spleen and testis besides neutrophils.
MD1	Lymphocyte antigen 86, MD-1	LY86, MD1	Associated with CD180 at the cell surface	This is found in association with CD180 and TLR4 on cell surface of B-cells, monocytes and tonsil and is involved in innate immune response to bacterial lipopolysaccharide (LPS) and cytokine production.
RVK				No information
GLUT1	Solute carrier family 2, facilitated glucose transporter member 1, Glucose transporter type 1,	SLC2A1, GLUT1	Multi-pass membrane protein	This protein is expressed in many human tissues, acts as a transport protein for a wide range of aldoses including pentoses and hexoses as well as a receptor for human T-cell leukaemia virus (HTLV) I and II. The

	erythrocyte/brain, HepG2 glucose transporter			mutation of the gene encoding this protein can cause a neurological disorder called GLUT1 deficiency syndrome.
F2RL3	Proteinase-activated receptor 4, Coagulation factor II receptor-like 3, Thrombin receptor-like 3	F2RL3, PAR4	Multi-pass membrane protein	This protein serves as a receptor for activated thrombin or trypsin and stimulates phosphoinositide hydrolysis through G proteins. It is expressed in lung, pancreas, thyroid, testis and small intestine and may play a role in platelets activation.
NMDAR1	Glutamate receptor ionotropic, NMDA 1, Glutamate [NMDA] receptor subunit zeta-1 N-methyl-D-aspartate receptor subunit NR1	GRIN1, NMDAR1	Multi-pass membrane protein	This is a glutamate-gated ion channel with high permeability to calcium and a key regulator for synaptic plasticity, synaptogenesis, excitotoxicity, memory acquisition and learning.
DGCR2	Integral membrane protein DGCR2/IDD	DGCR2. IDD, KIAA0163	Single-pass type I membrane protein	It serves as an adhesion receptor, required for normal cell differentiation and migration and is predominantly expressed in brain, heart, lung and fetal kidney. As it is involved in neural crest cells migration, mutation in the gene can cause DiGeorge syndrome.
CRHR2	Corticotropin-releasing factor receptor 2	CRHR2, CRF2R, CRH2R	Multi-pass membrane protein	This function as a receptor for corticotropin releasing factor and urocortin I, II and III. Its activity is mediated by G proteins which activate adenylyl cyclase.
ADAM15	Disintegrin and metalloproteinase domain-containing protein 15, Metalloprotease RGD disintegrin protein Metalloproteinase-like, disintegrin-like, and cysteine-rich protein 15, Metargidin	ADAM15, MDC15	Single-pass type I membrane protein	This is a metalloproteinase with multiple functional domains including a zinc-binding metalloprotease domain, a disintegrin-like domain, and an EGF-like domain. It interacts with the integrin beta chain, beta 3 by its disintegrin-like domain. It may function in cell-cell adhesion and cellular signalling by interacting with Src family protein-tyrosine kinases It is expressed in colon, small intestine airway smooth muscle and glomerular mesangial cells.
CDH16	Cadherin-16, Kidney-specific cadherin	CDH16	Single-pass type I membrane protein	Cadherins are calcium-dependent membrane-associated glycoproteins which serves as cell adhesion proteins. Expression is kidney specific, where it functions as a mediator of homotypic cellular recognition, playing a role in the morphogenic direction of tissue development.
KIA0319				No data available
GABBR1	Gamma-aminobutyric acid type B receptor subunit 1	GABBR1, GPRC3A	Multi-pass membrane protein	It functions as a receptor for GABA, the main inhibitory neurotransmitter in the mammalian central nervous system, mediating the effects through G-proteins related signalling. It is highly expressed in brain and to some extend in heart, small intestine and uterus.

NRG2	Pro-neuregulin-2, Divergent of neuregulin-1, Neural- and thymus-derived activator for ERBB kinases	NRG2, NTAK	Single-pass type I membrane protein	It serves as a direct ligand for ERBB3 and ERBB4 tyrosine kinase receptors which induces the growth and differentiation of epithelial, neuronal, glial, and other types of cells. Its expression is restricted to the cerebellum in the adult.
NRAMP1	Natural resistance-associated macrophage protein 1	SLC11A1, LSH, NRAMP, NRAMP1	Multi-pass membrane protein	This protein functions as a divalent transition metal (iron and manganese) transporter. It is involved in iron metabolism and host resistance to certain pathogens especially macrophage-specific membrane transport function. Besides macrophages it is expressed on peripheral blood leukocytes, lung, spleen and liver.
ENT1	Equilibrative nucleoside transporter 1, Equilibrative nitrobenzylmercaptopurine riboside-sensitive nucleoside transporter, Equilibrative NBMPR-sensitive nucleoside transporter, Nucleoside transporter-es-type, Solute carrier family 29 member 1	SLC29A1, ENT1		This function as an equilibrative nucleoside transporter localized to the plasma and mitochondrial membranes and facilitates the cellular uptake of nucleosides from the surrounding medium. It is expressed in heart, brain, mammary gland, erythrocytes and placenta.
SDC3	Syndecan-3	SDC3, KIAA0468	Single-pass type I membrane protein	The main function of this protein is in maintaining cell shape by affecting the actin cytoskeleton. It is expressed in the nervous system, adrenal gland, and the spleen.
LTB4R	LTB4R2 protein	LTB4R2		No data available
GPR3	G-protein coupled receptor 3, ACCA orphan receptor	GPR3, ACCA	Multi-pass membrane protein	This is a G-protein coupled receptor with possible role in multiple brain functions, including behavioural responses to stress. Besides predominant expression in central nervous system, it is expressed at a low level in lung, kidney, testis, ovary and eye.
HPN	Serine protease hepsin, Transmembrane protease serine 1	HPN, TMPRSS 1	Single-pass type II membrane protein	This is a type II transmembrane serine protease that may be involved in various cellular functions, including blood coagulation, maintenance of cell morphology and growth and progression of cancers, particularly prostate cancer. .It is expressed in most tissues with highest level in liver.
RHBDL1	Rhomboid-related protein 1	RHBDL1, RHBDL	Multi-pass membrane protein	It is an intramembrane serine proteases involved in intramembrane proteolysis and release of functional polypeptides from their membrane anchors. It is expressed in heart, brain, skeletal muscle and kidney.

SLC9A1	Sodium/hydrogen exchanger 1, APNH Na(+)/H(+) antiporter, amiloride-sensitive Na(+)/H(+) exchanger 1, Solute carrier family 9 member 1	SLC9A1, APNH1, NHE1	Multi-pass membrane protein	This protein is a plasma membrane transporter which plays a central role in regulating pH homeostasis by eliminating acids generated by active metabolism or to counter adverse environmental conditions. This protein has been proposed to be involved in tumour growth also. It is expressed in kidney and intestine.
TGFA	Protransforming growth factor alpha, Transforming growth factor alpha, EGF-like TGF, TGF type 1	TGFA	Single-pass type I membrane protein	It is a ligand for the epidermal growth factor receptor, and promotes signalling pathway for cell proliferation, differentiation and development. Pathologically it is involved in many types of cancers, and in some cases of cleft lip/palate. It is expressed in keratinocytes and tumour-derived cell lines.
JTB	Protein JTB, Jumping translocation breakpoint protein, Prostate androgen-regulated protein,	JTB	Single-pass type I membrane protein	It is a component of the chromosomal passenger complex (CPC), a complex required by centromere for accurate chromosome alignment and segregation during normal cytokinesis in mitosis. It is expressed in all normal human tissues.
STIM1	Stromal interaction molecule 1	STIM1, GOK	Single-pass type I membrane protein	It is a ubiquitously expressed protein that plays a role in mediating Ca2+ influx after depletion of intracellular Ca2+ stores by gating of store-operated Ca2+ influx channels (SOCs) where it acts as a as Ca^{2+} sensor in the endoplasmic reticulum.
PRRG1	PRRG1 protein	PRRG1		This protein is required for post-translational gamma-carboxylation of specific glutamic acid residues by a vitamin K-dependent gamma-carboxylase. It is highly expressed in spinal cord.

2.2.6. Non-specific expression

Anti-CD3 antibody, which was used as the control antibody, should bind T-cells but not CLL cells, as CD3 antigen is T-cell specific. Unfortunately, during the optimisation experiments the control antibody did not bind to the T-cells and showed non-specific binding to CLL cells and monocytes (Figure 2.3).

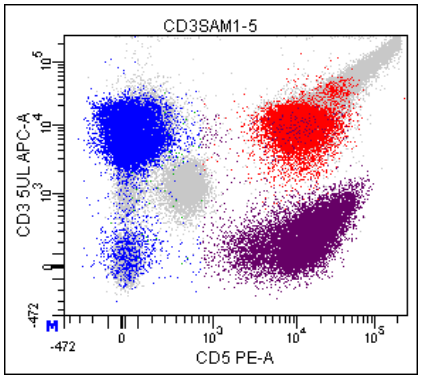


Figure 2.3 Rabbit polyclonal CD3 antibody.

The gating strategy and colour coding of the cells are similar to the experiments in direct staining technique. This shows non-specific binding of CD3 antibody to CLL cells (red) and monocytes (blue) and minimal binding to T-cells (purple) which is contrary to its usual expression pattern.

To a certain extent the secondary antibody displayed some non-specific binding (Figures 2.4 and 2.5) and this was true with any fluorochrome used (Figures 2.6, 2.7 and 2.8). The secondary antibody was goat anti-rabbit IgG antibody, which was affinity purified and was adsorbed against human IgG and serum, mouse IgG and serum, and bovine serum to minimise cross-reactivity.

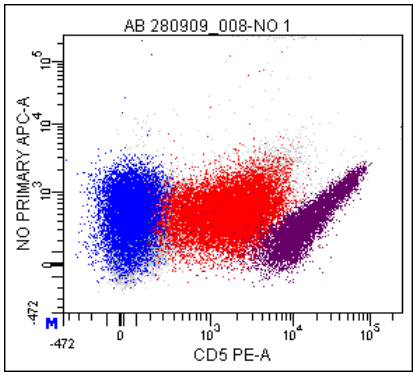


Figure 2.4 No primary test antibody.

Cells were identified using CD5 on PE, CD19 on PECy7 and CD45 on FITC. Cells were then stained with secondary goat anti-rabbit IgG antibody conjugated to Alexa Fluor 647. There is non-specific staining on all types of cells with the secondary antibody.

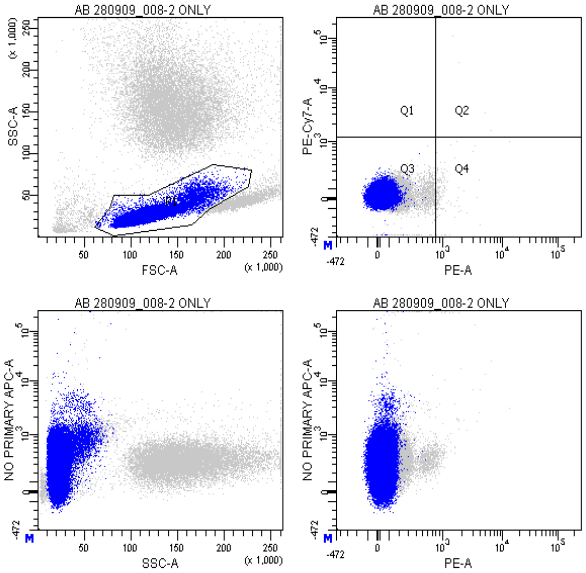


Figure 2.5 Secondary antibody alone.

Cells were stained with secondary goat anti-rabbit IgG antibody conjugated to Alexa Fluor 647 alone without any conjugated antibodies to seperate the cells. Mononuclear cells were gated using forward and side scatter. This also shows non-specific staining with the secondary antibody.

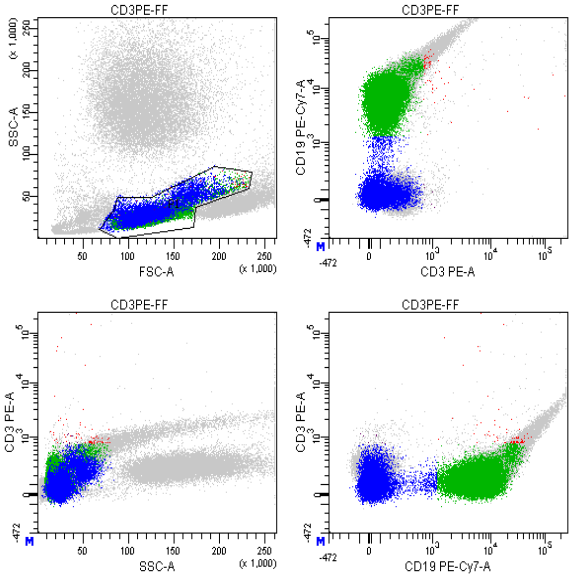


Figure 2.6 CD3 on PE.

CLL cells were identified using CD19 on PECy7. Cells were then stained using rabbit polyclonal anti-CD3 as primary antibody and goat anti-rabbit IgG conjugated to PE as secondary antibody. Non-specific staining was observed on monoctyes and CLL cells. There was no seperation of T-cells using this CD3 antibody.

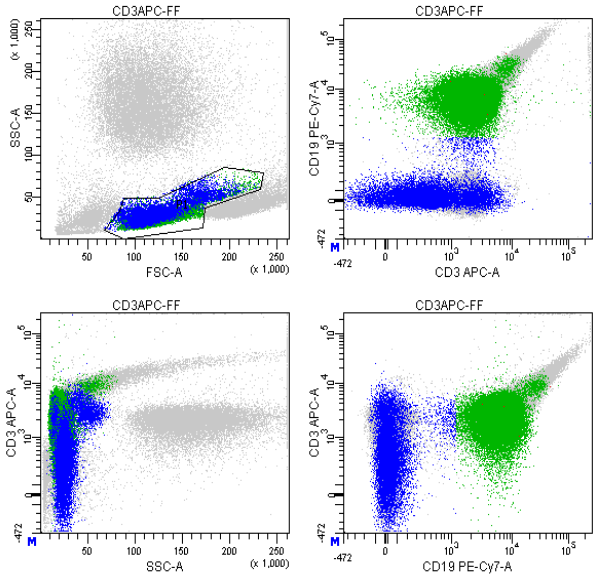


Figure 2.7 CD3 on APC.

CLL cells were identified using CD19 on PECy7. Cells were then stained using rabbit polyclonal anti-CD3 as primary antibody and goat anti-rabbit IgG conjugated to APC as secondary antibody. Non-specific staining was observed on monoctyes and CLL cells. There was no seperation of T-cells using this CD3 antibody.

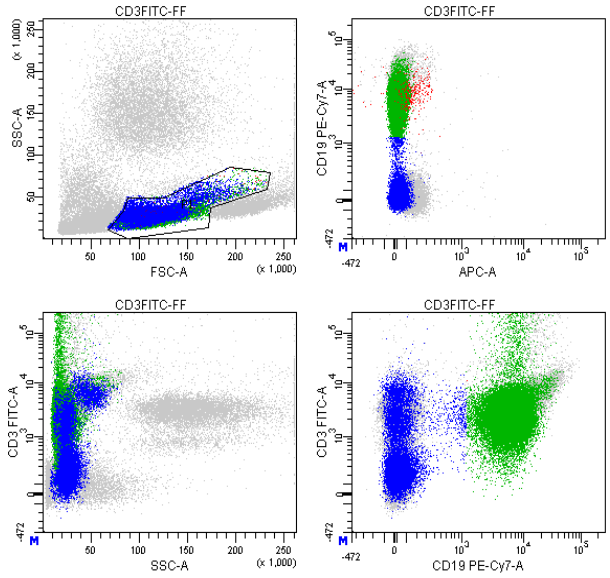
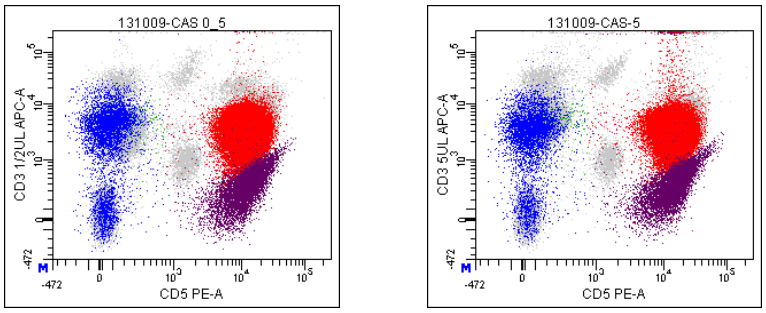


Figure 2.8 CD3 on FITC.

CLL cells were identified using CD19 on PECy7. Cells were then stained using rabbit polyclonal anti-CD3 as primary antibody and goat anti-rabbit IgG conjugated to FITC as secondary antibody. Non-specific staining was observed on monoctyes and CLL cells. There was no seperation of T-cells using this CD3 antibody.

2.2.7. Blocking Experiments

Several blocking experiments were done to minimize the non-specific binding. Initially casein 0.08% (w/v) was tried at 2 different strengths, as casein is used in immunohistochemistry to block non-specific binding of polyclonal antibodies. This combination did not give any significant reduction in non-specific binding (Figure 2.9).



Casein 0.5µl **Casein 5µl**

Figure 2.9 Blocking of non-specific binding with casein.

Attempt to block non-specific binding of CD3 antibody using casein 0.08% (w/v) at two different dilutions. Non-specific binding was shown on CLL cells with both concentrations.

Other blocking agents were tried, including FcReceptor (FcR) blocking agent (Miltenyi Biotec), Human IvIg at a concentration of 5% and goat serum (total protein of 6-9 g%). The data summarising these results are shown in Figures 2.10, 2.11 and 2.12 respectively.

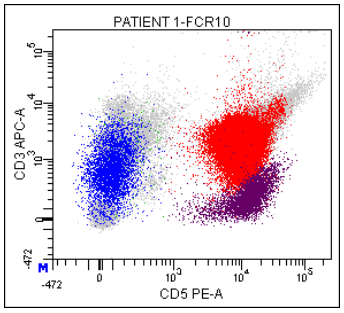


Figure 2.10 Blocking of non-specific binding with FcR blocking reagent.

Attempt to block non-specific binding of CD3 antibody using FcR blocking agent. As per manufacturer's recommendation 10µl of the reagent was added before adding the antibody. Non-specific binding was shown on CLL cells.

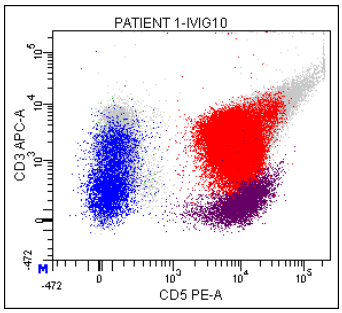


Figure 2.11 Blocking of non-specific binding with human IvIg.

Attempt to block non-specific binding of CD3 antibody using 10µl of human IvIg at a concentration of 5%. Non-specific binding was shown on CLL cells.

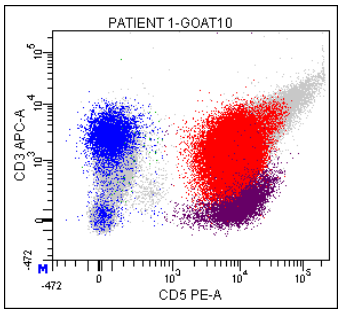


Figure 2.12 Blocking of non-specific binding with goat serum.

Attempt to block non-specific binding of CD3 antibody using 10µl of goat serum (total protein of 6-9%). Non-specific binding was shown on CLL cells.

Individual agents were then tested in 3 different patients with increasing concentration of the blocking agent. The MFI of combined CLL cells and monocytes are expressed graphically (Figures 2.13, 2.14 and 2.15).

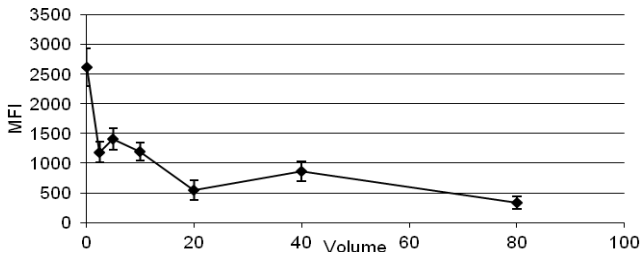


Figure 2.13 Titration of FcR blocker.

MFI of combined CLL cells and monocytes on using increasing strength of FcR blocker. Increasing concentration of the blocker is acquired by doubling the volume of blocker in each well starting from 2.5µl and making up the total volume to 80µl (maximum volume of blocker used) with FACS flow.

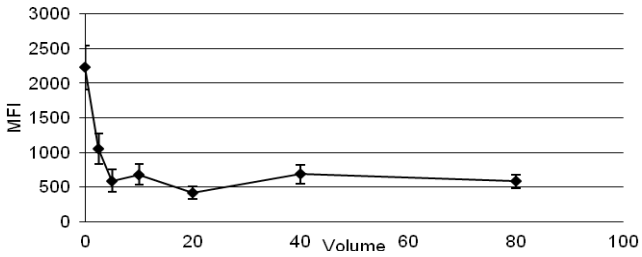


Figure 2.14 Titration of goat serum.

MFI of combined CLL cells and monocytes on using increasing strength of goat serum. Increasing concentration of the blocker is acquired as in the previous experiment.

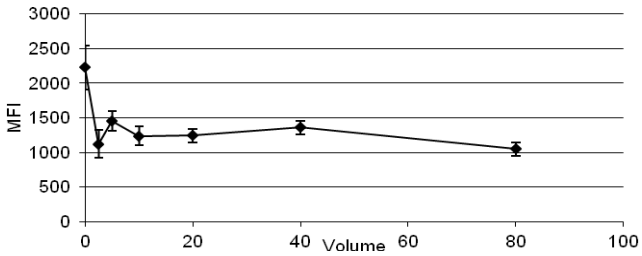


Figure 2.15 Titration of human immunoglobulin.

MFI of combined CLL cells and monocytes on using increasing strength of human immunoglobulin. Increasing concentration of the blocker is acquired as in the previous experiment.

It was noted that individual blockers were differentially affecting expression levels on the individual cell populations. Therefore different combinations of these blockers were tested and are represented in Figure 2.16.

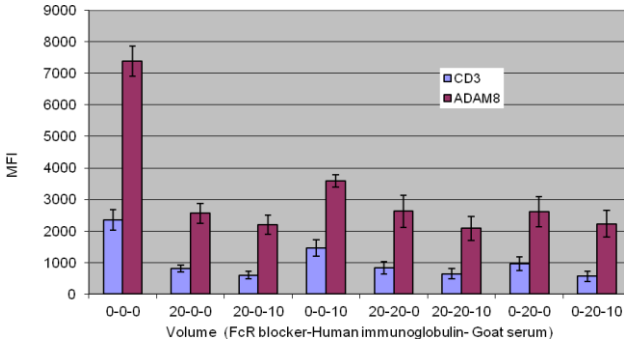


Figure 2.16 Titration of combination of blocking agents.

MFI of combined CLL cells and monocytes on CD3 and ADAM 8 using different combination of blocking agents. The volumes of each blocking agent in individual experiments are shown in X-axis. The order is FcR blocker-Human immunoglobulin-Goat serum. The volume of individual blockers were based on the previous experiments.

Based on the above experiment, a combination of 20µl of IvIg and 10µl of goat serum was selected as the blocking agent. This combination resulted in the lowest non-specific binding while also remaining economical. To determine the efficacy of this combination, a selection of antibodies to the new antigens were tested with and without the blocking antibodies. Their log MFI on CLL cells and monocytes are shown in Figure 2.17. Expression of Nicastrin is shown in Figure 2.18 as an example plot. The ratio of signal (MFI on CLL cells) and signal-to-noise (ratio between MFI on CLL cells and monocytes) between blocked and non-blocked experiments of individual antibodies are graphically represented in Figure 2.19.

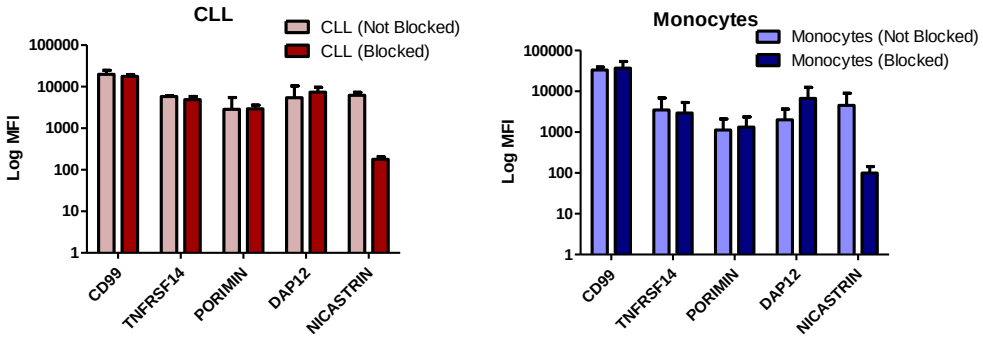


Figure 2.17 Expression various antibodies using IVIg and goat serum as blocking agents.

Log MFI of various antibodies on CLL cells and monocytes using a combination of 20µl of IVIg and 10µl of goat serum as the blocking agent. Nicastrin shows almost a hundred-fold reduction in MFI on CLL cells and MNC when blocked with this combination.

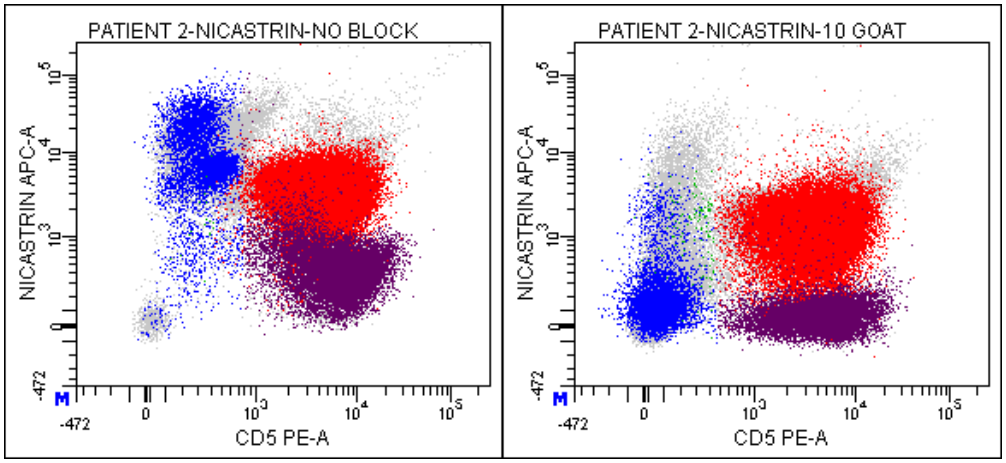


Figure 2.18 Example of antibodies tested using combination of 20µl of IVIg and 10µl of goat serum as the blocking agent.

Expression of Nicastrin is shown here as a representative plot. Left panel is without the blocking reagent and the right panel is with the blocking reagent.

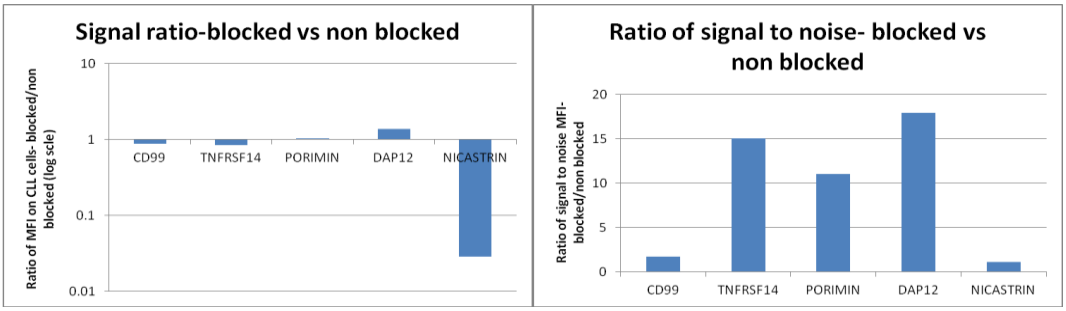
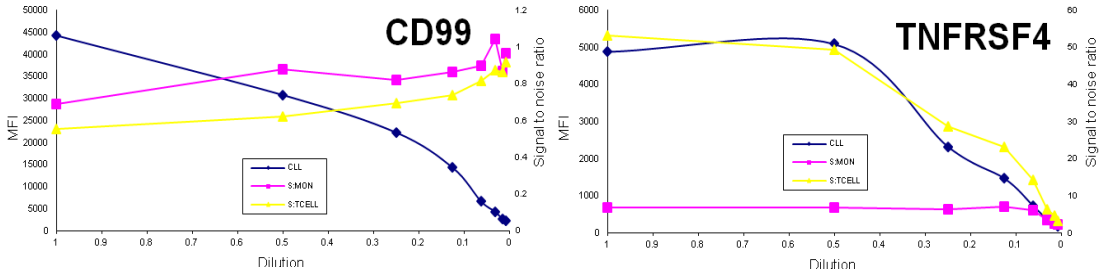


Figure 2.19 Ratio of signal between blocked and non-blocked MFIs of different antibodies.

Mean MFI (n=2) of CLL cells were taken to calculate the signal ratio and mean MFI of CLL cells and monocytes were taken for signal-to-noise ratio. Signal ratio considerably below 1 and ratio of signal-to-noise between blocked and non-blocked experiments remaining very low as in anti-nicastrin suggests that non-specific binding is reduced by blocking agents.

Some of the antibodies like nicastrin and CD99 had a significant reduction in the ratio of signal to noise between blocked and non-blocked MFIs while others like TNFRSF14, porimin and DAP 12 did not show any reduction. However, nicastrin is the only antibody which showed a reduction in noise ratio. This implies that antibodies like TNFRSF14, porimin and DAP12 may have specific expression on CLL cells while CD99 has expression on both CLL cells and monocytes and nicastrin does not have specific expression on either cell type. Given these results, the blocking agent was thought to be working in an appropriate manner.

Following the evaluation of blocking agents, some of these antibodies were titered for optimal amount of test antibody to be added for the experiment (Figure 2.20). In the plots shown below, the concentration of the antibody just before the ratio falls is the ideal concentration to be used. The signal intensity for staining on CLL cells (blue line) plateaus and falls after a certain concentration. Signal-to-noise ratio both against monocytes (purple line) and T-cells (yellow line) should peak and then fall. Ideally the signal intensity should be maintained until the ratio peaks; however, none of the graphs presented below met these criteria.



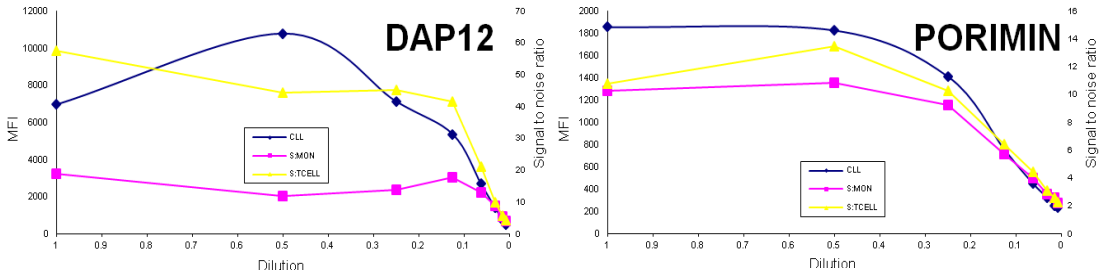


Figure 2.20 Titration of antibodies.

Neat antibody used was 5µl (1µg of CD99 and 5µg of other antibodies) Antibodies were serially diluted halving the concentration in subsequent wells up to a dilution of 1 in 128. The concentration of antibodies varied from 0.1µg to 2.5µg.

Even though the initial experiments suggested that the blocking was adequate, studies with further antibodies gave a uniform pattern of expression. This gave the impression that the problem of non-specific expression was not eliminated using the IvIg/goat serum blocking agent. Blocking with an increased volume of the goat serum also brought down the specific expressions and the non-specific expression blocking was not consistent (Figures 2.21, 2.22 and 2.23).

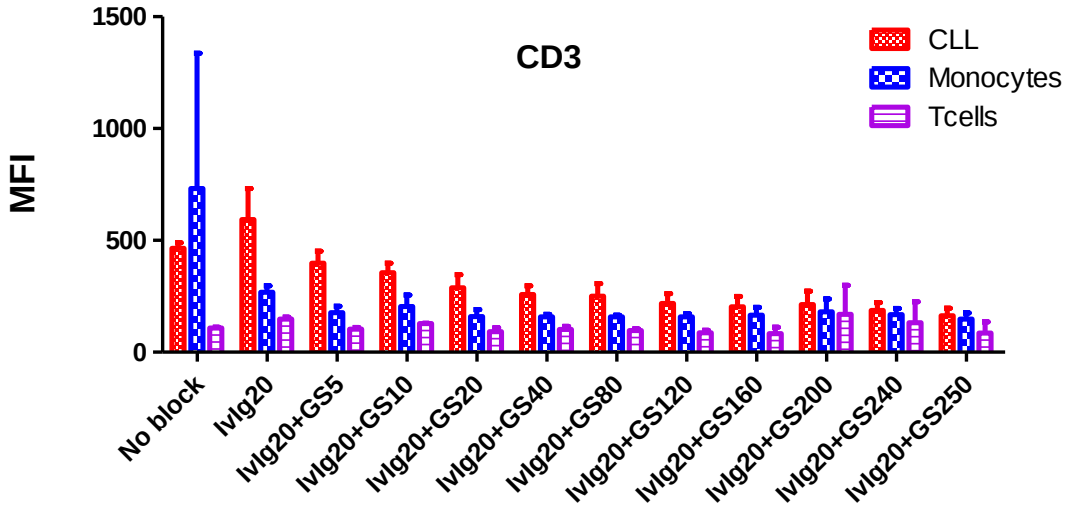


Figure 2.21 Titration of combination of human immunoglobulin and goat serum as blocking agents using CD3 antibody.

Blocking with increasing concentration of goat serum from 0 to 250µl, maximum blocking effect was found at around 20 µl beyond which there was no meaningful fall in non specific expression. Volume of IVIg was kept constant at 20 µl. Statistical calculations were not possible due to small numbers.

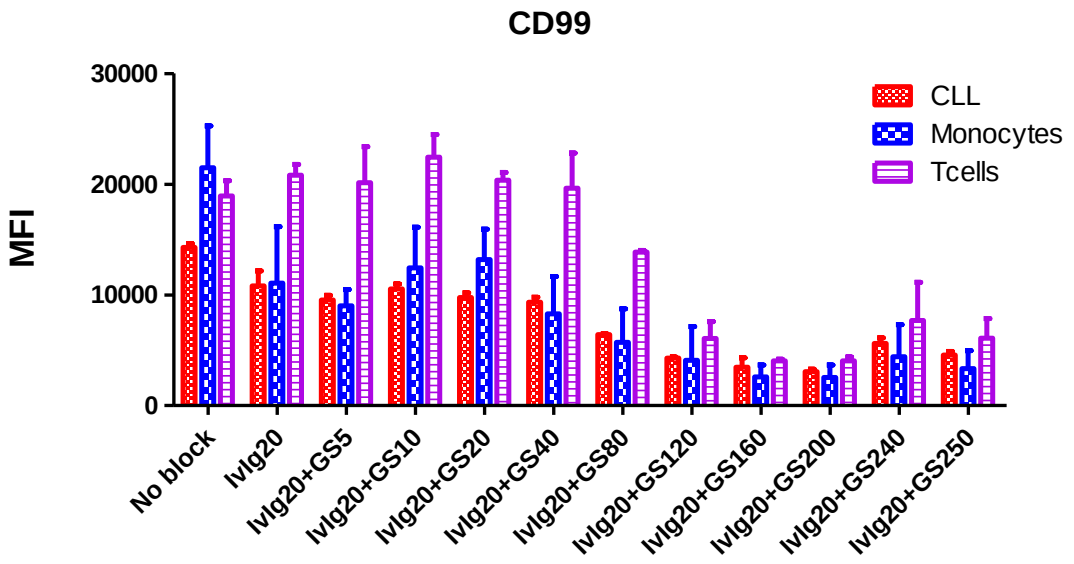


Figure 2.22 Titration of combination of human immunoglobulin and goat serum as blocking agents using CD99 antibody.

Blocking with increasing concentration of goat serum from 0 to 250µl blocked specific binding beyond 40 µl (Figure 2.23). Volume of IVIg was kept constant at 20 µl. Statistical calculations were not possible due to small numbers.

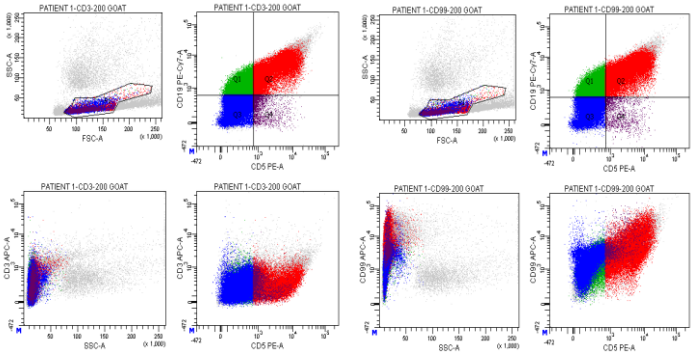


Figure 2.23 Blocking with higher volume of goat serum.

Increasing the amount of goat serum affected the monoclonal antibodies and the cells were no longer separated based on cell surface markers. CD3 on left panel and CD99 on right panel.

In order to overcome this difficulty, a further experiment with blocking in two steps i.e. initial blocking before adding the primary antibody and further blocking before adding the secondary antibody was conducted. This particular sequence seemed to reduce the problem of non-specific binding without affecting the specific binding (Figures 2.24 and 2.25).

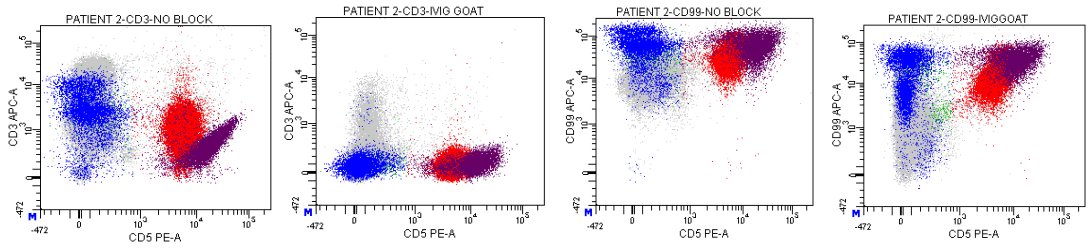


Figure 2.24 CD3 and CD99 staining with double blocking.

Initial blocking before adding the primary antibody and further blocking before adding the secondary antibody reduces the problem of non-specific binding (CD3) without affecting the specific expression (CD99).

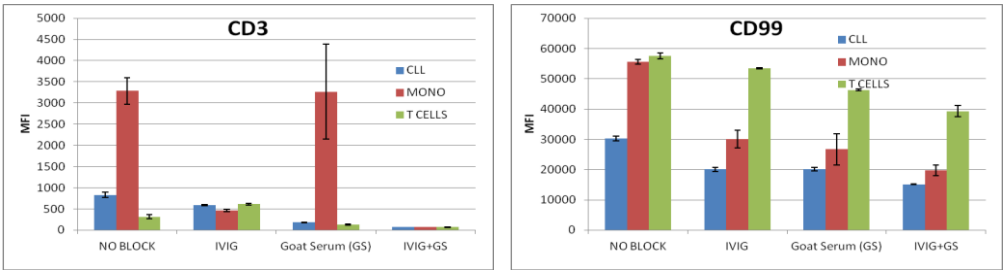
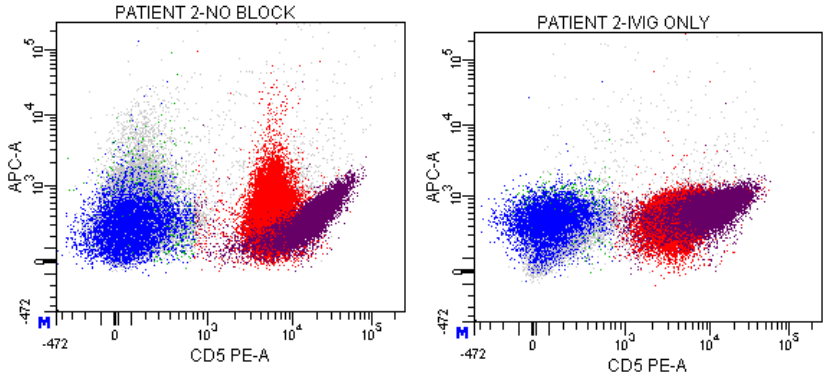


Figure 2.25 MFI of different cell types with various blockers used for double blocking.

The combination of IvIg + goat serum (GS) eliminates non specific binding without any major effect on specific expression. Sample size too low (n=2) for statistical analysis.

This may be based on the principle that the binding of the antibody to the Fc receptor may be weak and will be affected by washing steps thereby exposing the Fc receptor again, before adding the secondary antibody.

The following experiment confirms the abolition of non-specific binding of secondary antibody with combination of goat serum and IvIg. (Figures 2.26 and 2.27). Expression was analysed with the secondary antibody alone without adding any primary antibody and the combination of both remove most of the non-specific binding of the secondary antibody.



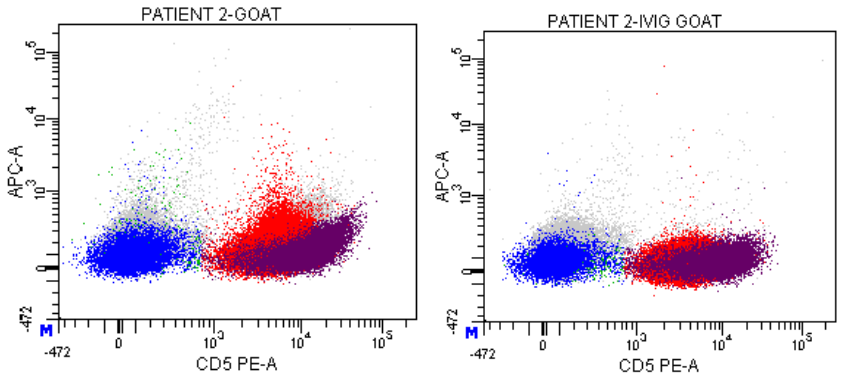


Figure 2.26 Non-specific binding on different cell types with no block, Ivlg alone, goat serum, and the combination of both.

Only secondary antibody conjugated to APC was added without any primary antibody. This experiment shows that the secondary antibody itself has some non-specific binding, which can be eliminated by adding the combination of Ivlg and goat serum before adding the secondary antibody.

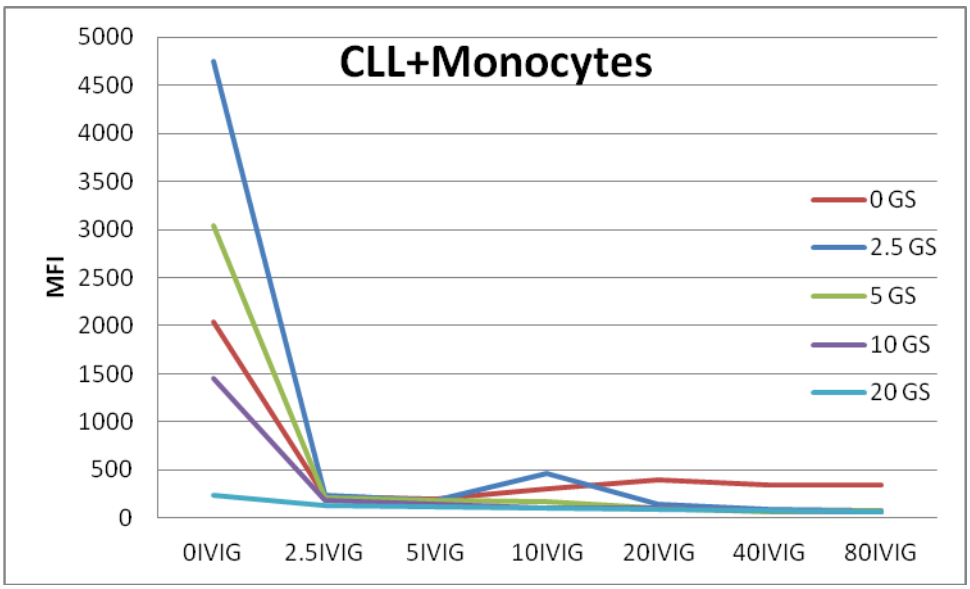


Figure 2.27 MFI of CD3 on CLL cells and monocytes using various concentration of Ivlg and goat serum.

From the titration experiment it was determined that 2.5µl of Ivlg or 20µl of goat serum (GS) with or without the other agent is sufficient to reduce the non-specific binding.

From all the above experiments it was concluded that a combination of 2.5µl of Ivlg and 20µl of goat serum successfully reduce non-specific binding to an acceptable level. It was therefore decided to use this combination for testing the new antibodies. These blocking agents were used in 2 steps, both before adding primary and secondary antibodies.

Some of these antibodies were titrated for optimum concentration for further experiments as shown in Figure 2.28.

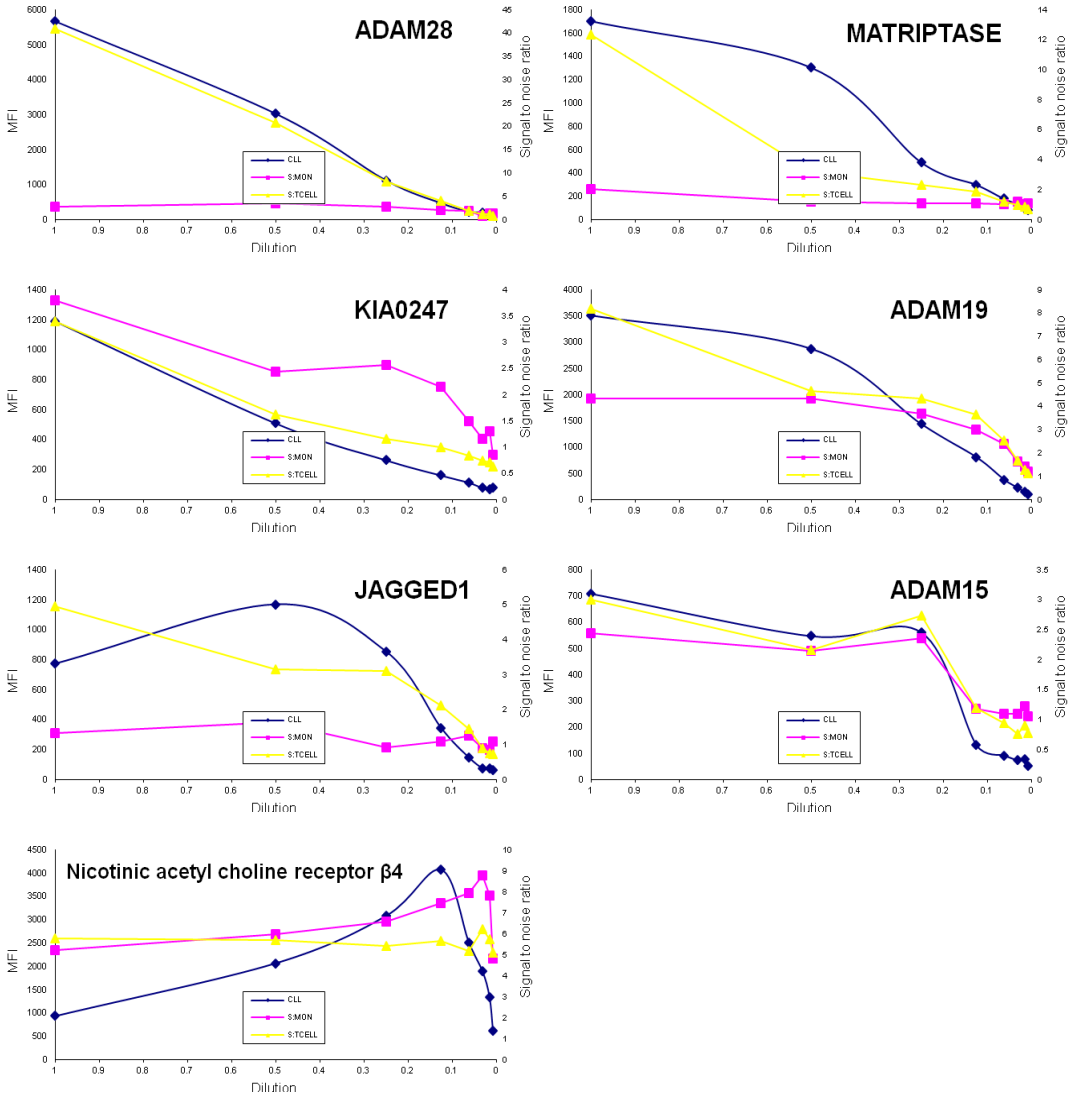


Figure 2.28 Titration of antibodies.

Neat antibody used was 10µg. Antibodies were serially diluted halving the concentration in subsequent wells up to a dilution of 1 in 128.

Even though the above method was helpful to reduce the non-specific binding there were several pitfalls:

1. There were several steps involved and it was very time consuming for large-scale screening.

2. There was variation in expression between patients for the same antibody and to differentiate between specific and non-specific variation was difficult.

The next attempt to eliminate non-specific expression was to use the $F(ab)_2$ portion of the secondary antibody rather than the intact version. Several steps were done to optimise this secondary antibody (Figures 2.29, 2.30 and 2.31).

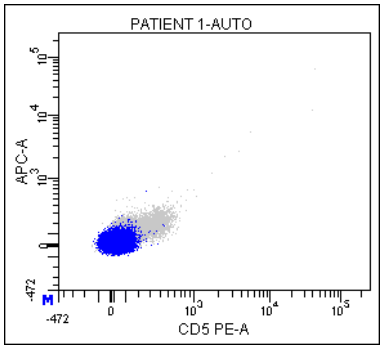


Figure 2.29 Auto-fluorescence of mononuclear cells.

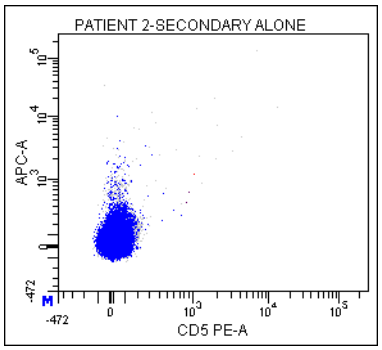


Figure 2.30 $F(ab)_2$ secondary antibody alone without any primary antibody or test antibody.

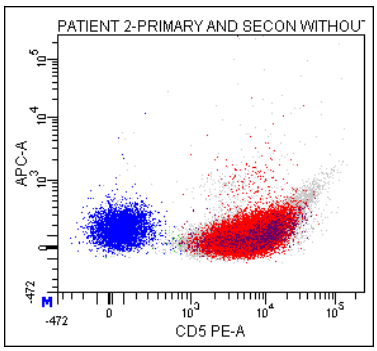


Figure 2.31 $F(ab)_2$ secondary antibody with standard diagnostic antibodies without any test antibody.

To begin with, the background fluorescence was evaluated. These experiments suggest that the secondary antibody had minimal non-specific binding either to cells or to other mouse monoclonal primary antibodies.

In the next part of the evaluation, primary conjugated antibodies were added either with the test antibody and adding the secondary antibody as the second step, or by adding the test antibody first followed by secondary antibody and

then by the primary conjugated antibodies with washes in between each step. The flow plots depicted in Figure 2.32 show the difference between these two.

Three antibodies were selected: one was standard IgD which is usually expressed on CLL cells but not on T cells and monocytes, the second one was a test antibody (CD99) which is found to be positive on all cells from previous experiments and the third one (AMFR) was not expressed on any blood cells examined.

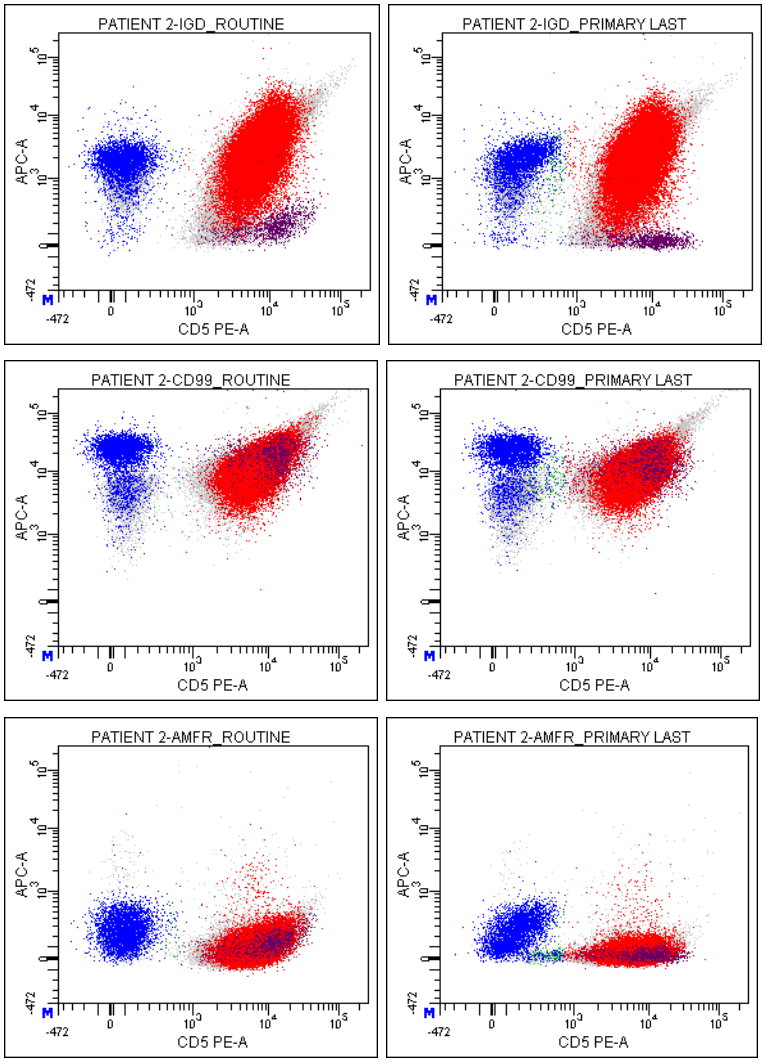


Figure 2.32 Experiment comparing two methods of addition of test antibody.

Primary conjugated antibodies were added either with the test antibody and adding the secondary antibody as the second step (Left panel) or by adding the test antibody first followed by secondary antibody and then by the primary conjugated antibodies with washes in between each steps (Right panel). Top to bottom are IgD, CD99 and AMFR as test antibodies.

These experiments suggest that there is minimal difference between whether the primary mouse monoclonal antibodies are added in the beginning with test antibodies or toward the end.

The final parameter that required investigation was blocking of non-specific binding by IvIg and goat serum (Figure 2.33).

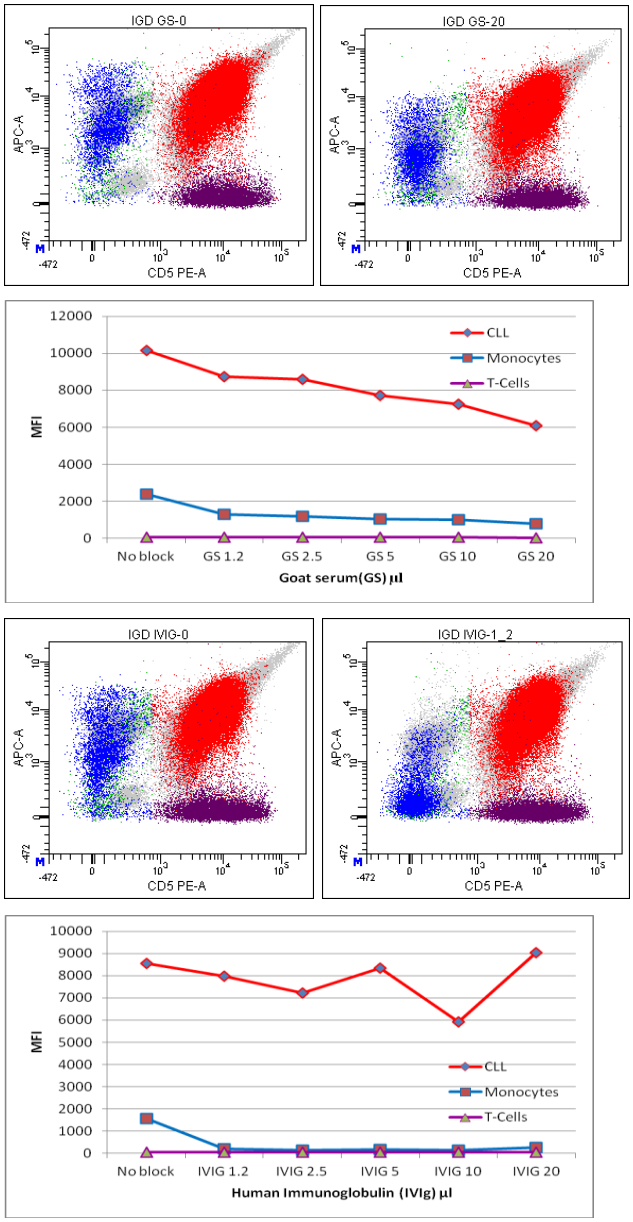


Figure 2.33 MFI on CLL cells, monocytes and T-cells of IgD using different concentration of blocking agents.

The top panel shows blocking with goat serum and the bottom panel with IVIg.The volumes of each blocking agent in individual experiments are shown on the X-axis. This shows that 1.2µl of IVIg on itself can eliminate non-specific binding when using F(ab)$_2$ secondary antibody.

This experiment shows that IvIg even at a low concentration can eliminate most of the non-specific binding, but goat serum was not effective. The titration of the secondary antibody is shown in Figure 2.34.

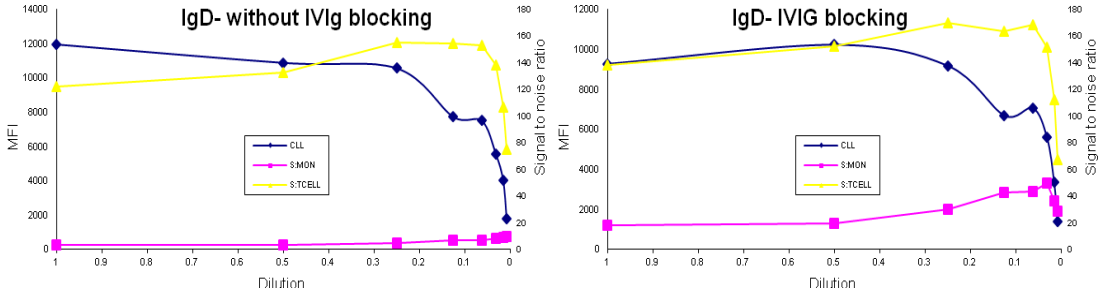


Figure 2.34 Titration of secondary antibody.

Neat antibody used was 2µl. The antibody was serially diluted halving the concentration in subsequent wells up to a dilution of 1 in 128. The primary test antibody used was IgD. Signal on CLL cells (blue) and signal-to-noise ratio on T-cells (yellow) and monocytes (pink) are shown in the graph. Titration was done without using IvIg as a non-specific blocker and with 1.25µl of IvIg as a blocker.

Based on these experiments it was concluded that the best method for screening polyclonal antibodies using flow cytometry is to use $F(ab)_2$ portion as the secondary antibody and to use 1.25µl of human IVIg as the non-specific binding blocking agent.

2.2.8. Zenon labelling

Zenon labelling was done using manufacturer's recommended method, the steps of which are as follows: (Figure 2.35) 1 µg of antibody was prepared in ≤20 µL phosphate-buffered saline (PBS) 5 µL of the Zenon mouse IgG labelling reagent (Component A) was added to the antibody solution. This was incubated for 5 minutes at room temperature. 5 µL of the Zenon blocking reagent was then added. This solution was incubated for 5 minutes at room temperature and was used within 30 minutes. The rest of the steps are as standard flow cytometry steps.

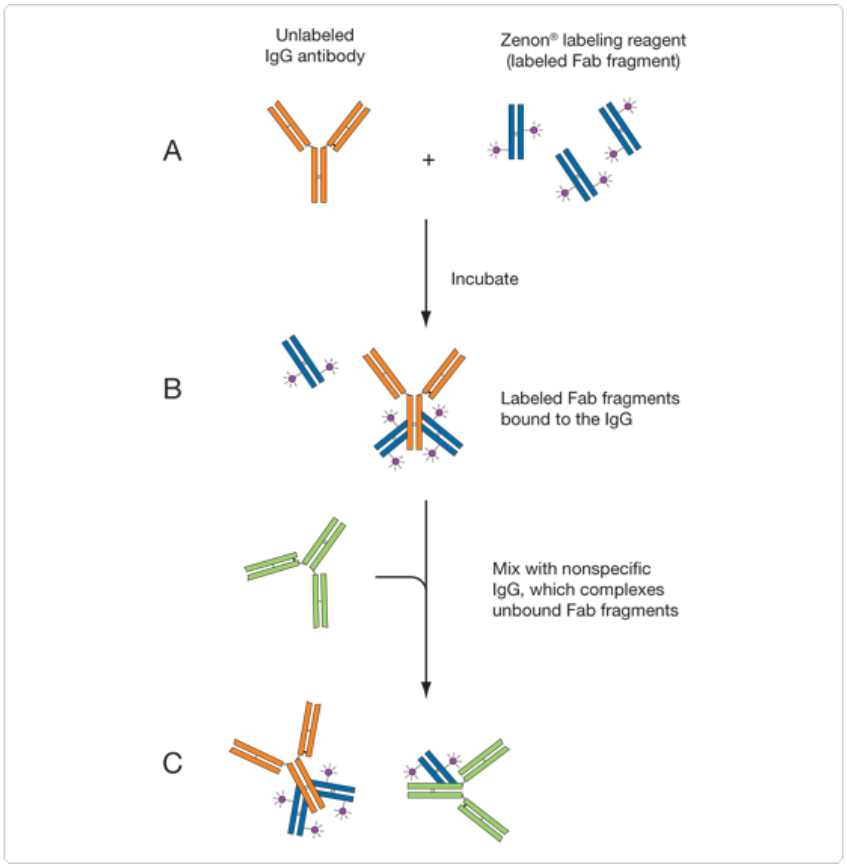


Figure 2.35 Principles of Zenon labelling. This figure was directly taken from the Molecular Probes website.
http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/Zenon-Labeling-Technology.html

2.3 Cell Culture

2.3.1. Isolation of mononuclear cells (MNC) using Lymphoprep

Blood was collected in tubes with EDTA as anticoagulant. Blood was diluted by adding equal amount of 0.9% sterile sodium chloride (NaCl) solution. The diluted blood was then layered over half the volume of lymphoprep in a 15ml centrifuge tube. After capping the tube was centrifuged at 800 x g for 20 minutes at room temperature in a swing-out rotor without applying brakes. If the blood was stored for more than 2 hours the centrifugation time was increased to 30 minutes. Mononuclear cells form a distinct band at the sample medium interface (Figure 2.36).

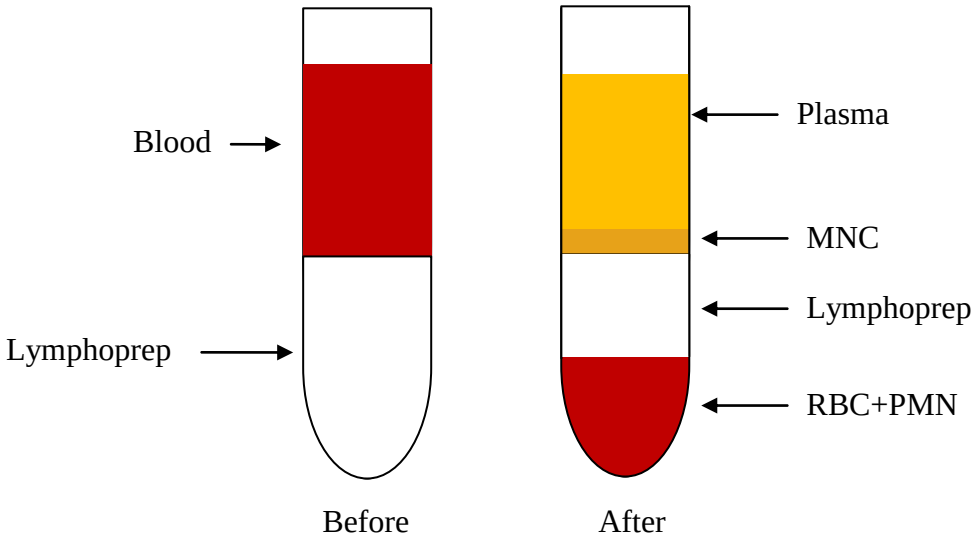


Figure 2.36 MNC separation using lymphoprep.

After centrifugation mononuclear cells (MNC) form a distinct layer at the interphase between lymphoprep and plasma and the red cells (RBC) along with polymorphonuclear cells (PMN) sink to the bottom.

The cells were removed from the interface using a pasture pipette without removing the upper layer The harvested fraction was diluted with medium to reduce the density of the solution and pellet the cells by centrifugation for 10 min at 250 x g. Cells were the counted using an automated cell counter (Sysmex KX-21N).

2.3.2. Isolation of CLL cells using B-cell isolation kit

MNC were isolated as before and diluted in MACS separation buffer. The cell number was determined by automated cell counter. Cell suspension was

centrifuged at 300 x g for 10 min and the supernatant was aspirated. Cells were resuspended in 40µl of buffer per 10^7 cells followed by addition of 10µl of B-CLL Biotin-Antibody Cocktail per 10^7 cells. This was incubated for 10 min at 4^oC. Cells were then washed with 1ml of buffer per 10^7 cells and centrifuged at 300 x g for 10 min. Supernatant was aspirated completely followed by addition of 80µl of buffer per 10^7 cells. 20µl of Anti-Biotin Microbeads per 10^7 cells was then added and incubated in at 4^oC for 15 min. This was washed again with 1ml of buffer per 10^7 cells and centrifuged at 300 x g for 10 min. Supernatant was aspirated and cells were resuspended in 500µl of buffer. CLL cells were then separated using negative selection with B-cell isolation kit (B-CLL) (Miltenybiotec) and an auto-MACS separator. Purity was then assessed using flow cytometry by calculating the percentage of CD19 and CD5 positive cells.

2.3.3. Maintenance of cell lines

M210B4 (American Type Culture Collection) (Lemoine et al., 1990) cells and CD40L mouse fibroblast L cells (kindly donated by Dr. Sean Diehl, University of Vermont) (Diehl et al., 2002) were used as supporting cells for co-culture experiments. Roswell Park Memorial Institute (RPMI) 1640 with 10% Foetal Bovine Serum (FBS) and 1% penicillin-streptomycin (pen-strep) was used for M210B4 cells and Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS and 1% pen-strep was used for CD40L fibroblast cells. The cells were maintained in a 37°C incubator with 5% CO_2 in air with 95% humidity. Cells were grown to confluence and passaged every 3-4 days.

2.3.4. Preparation of mitomycin-c inactivated feeder layers

Mitomycin-c was available as 2 mg powder vials. This was dissolved in distilled water to a make a concentration of 0.5 mg/ml. This was then added to the flasks in which feeder cells are grown to make a final concentration of 10µg/ml (i.e. 600µl of the previous solution to flask containing 30 ml of medium). This was incubated at 37^oC for 2.5 to 3 hours. The media with mitomycin-c was then removed and the flask was washed with 10 ml media once, followed by Hank’s Buffered Saline Solution (HBSS) twice. The cells were detached using trypsin-EDTA. They were then counted and used either for plating the wells or were frozen.

2.3.5. Preparation of irradiated feeder layers

$1x10^8$ CD40L fibroblasts cells in 40ml IMDM were irradiated with 50 grey at 0.5 G/min. Following irradiation cells were stored in liquid nitrogen.

2.3.6. Absolute cell count

Absolute cell count was determined using flow cytometry. 'Absolute Count Standard' beads were used which is a microsphere suspension of a known concentration, internally labelled with multiple fluorochromes. For 24 well plates the wells were transferred to individual tubes. The wells were then washed with PBS and the wash solution was added to corresponding wells. The tubes were then made up to 4 ml in total. 1 ml was transferred to another tube for cell counting using beads; the remaining 3 ml were used for viability assessments. This method eliminated the loss of cells by washing if cell count and viability are assessed simultaneously. For 96 well plates, adequate volume of beads was added directly to the wells. 50µl from the wells were acquired on a BD LSRFortessa™ using high throughput flow cytometry (HTC). The voltage was adjusted to make the beads fall in the plot separate from the cells. The plates are then taken out and the remaining contents in each well were then used for viability assessment.

The cell count was determined using the formula:

$$\frac{[\text{beads}]}{\#\text{ of beads counted}} = \frac{X}{[(\#\text{ cells counted})]}$$

where: X = cells/mL, beads = (concentration reported on CoA in ml)

2.3.7. Assessment of cell viability

Micro titre plates were used for flow cytometry experiments. Annexin-V, 7-AAD-detection kit (ebioscience) was used for assessing viability. For 24 well plates, after harvesting the cells into a tube from the tissue culture plate, media was centrifuged and discarded. The cells are transferred to the microtitre plate and were washed with FACS flow BSA. For 96 well plates the plates were centrifuged and media discarded.

Antibodies used to differentiate viable, early apoptotic and late apoptotic CLL cells include:

- •CD20-Pacific blue -0.5µl
- •Annexin-V-FITC -5µl
- •CD2-PE -0.00625µg
- •7-AAD in the range of PE-Cy5 - 0.25µg
- •CD19-PE-Cy7 -0.025µg
- •CD5-APC -0.0125µg
- •CD45-APC-Cy7 -0.2µg

After adding the antibodies, cells were incubated for 15 minutes. The cells were then washed with 100µl of standard binding buffer of the viability detection kit followed by addition of another 100µl of standard binding buffer and appropriate volume of annexin-V and 7-AAD. This was again incubated at room temp for 15 minutes followed by addition of another 100µl of standard binding buffer and acquired on the flow cytometer.

2.4 Calcium Flux

2.4.1. Labelling of cells with Ca^{2+} binding dyes

To measure the intracellular Ca^{2+} flux, cells were resuspended in Hank's Balanced Salt Solution (HBSS) buffer supplemented with 1mM $CaCl_2$, 1mM $MgCl_2$ and 1%FCS (HBSS-CMF) at a concentration of $1x10^6$ cells/ml. Indo-1 solution was added to the cell suspension to give a final concentration of 3 µg/ml or, alternatively, if fluo-3/fura-red was used fluo-3 was added at a concentration of 2.6 µM and fura red at a concentration of 5.5 µM. After an incubation of 30 min at 37°C, cells were washed twice with HBSS-CMF, and resuspended at a final concentration of $1x10^6$ cells/ml. Where indicated, cells were then additionally incubated with the appropriate drugs for 30 min at 37°C prior to analysis.

2.4.2. Sample Acquisition

Samples were acquired on a flow cytometer with 350nm UV laser after pre-warming the tube to 37°C for 5 min before acquiring. Samples were acquired at a rate of 100-200 events/second. Photomultiplier voltages (PMT) were set to make the cell population of interest to be in the centre of the dot plot of 405/530 emission and therefore has a ratio of 1. After acquiring the baseline emission data for 60 seconds, stimulant was quickly added and data was recorded for another 4 minutes. The stimulants vary depending on the type of experiment and are defined with each experiment.

2.5 Phosflow

$1x10^6$ cells were diluted in normal saline to make it to a volume of 100µl. Appropriate tubes were then incubated with drugs for 30 minutes at 37°C. After staining with extracellular antibodies including CD45, CD19 and CD3, cells were stimulated with IgM or IgD for 60 seconds, followed by fixation with lyse fix buffer. Cells were then permeabilized using BD Phosflow Perm/Wash buffer I and stained with phosflow antibodies before acquiring. Perm/Wash

buffer I was used for all antibodies as it was compatible with most phosflow antibodies used in the experiments. Due to time restriction optimisation of the buffers for each antibody was not done.

2.6 SYK and ZAP70 expression

SYK and ZAP70 expression were analysed using standard intrasure (BD Biosciences) protocol. To briefly describe the procedure, 1 x 10^6 cells were initially stained with surface antibodies CD19 on PECy7 and CD3 on APC or PacO. Cells were then fixed with reagent A followed by red cell lysis with BD FACS lysing solution. Subsequently permeabilization was done using reagent B before staining with intracellular antibodies, ZAP70 on PE and SYK on FITC.

2.7 RNA Identification

2.7.1. RNA Extraction Method

RNA extraction was performed using 1ml of Trizol and 200µl chloroform followed by 0.5ml of isopropanolol/ml of Trizol (Life Technologies) used to store the sample. The pellet formed by centrifugation was washed with 1ml of 75% ethanol, air dried and resuspended in 10µl of RNase free water. DNA was removed using 1µl of buffer and 1µl of turbo-DNA free (Life Technologies) followed by 1.5µl of stop solution.

2.7.2. Reverse Transcription

RNA amplification was done in a 0.2ml thin walled tube by mixing 1µl of extracted RNA (<1µg) with 0.2µl of random hexamer (0.5µg) and making up volume to 5µl using RNase free water (2 tubes per sample). This was incubated on thermal cycler 70^{o}C for 5 minutes, then cooled for 5 minutes

Reverse transcription was done using the following master mix with and without reverse transcriptase. All reagents were purchased from Promega.

Improm II reaction buffer- 4µl	Improm II reaction buffer- 4µl
RNase free water- 3.1µl	RNase free water- 4.1µl
25mM $MgCl_2$ - 2.4µl	25mM $MgCl_2$ - 2.4µl
2.5mM dNTP mix - 4µl	2.5mM dNTP mix - 4µl
RNAsin inhibitor - 0.5µl	RNAsin inhibitor - 0.5µl
Improm II reverse transcriptase -1µl	

15µl of RT+ mix and RT- mix was aliquoted into each of the 2 annealed samples. This was incubated using a thermal cycler at 25^{o}C for 5 minutes, 42^{o}C for 60 minutes, 70^{o}C for 15 minutes.

2.7.3. Primers

An exonic sequence involving the maximum number of isoforms of a subunit was selected from PubMed gene database (Table 2.1). Primer sequences were obtained from the PubMed Primer-BLAST program and were purchased commercially (Sigma Aldrich). Primers were diluted to 10µM using RNase free water.

Table 2.1 Details of primer pair for each subunit

Subunit	FORWARD PRIMER	REVERSE PRIMER	Product size
CHRNA1	CATCGTCAACGTCATCATCC	ATTTTCCAATCAAGGGCACA	188
CHRNA2	CCACCAACGTCTGGCTAAAA	CATCTCAGAAGGGACCCTGA	101
CHRNA3	GGAGATCTACCCCGACATCA	AAATGCACAGGGTCACCTTC	158
CHRNA4	GCTGGACTTCTGGGAGAGTG	AGGGGATGATGAGGTTGATG	161
CHRNA5	TGAAATTTGGTTCTTGGACTTATG	AGCAACAGCTGTCGGTTCTG	150
CHRNA6	GGCTGTGCAACTGAGGAGA	GGCCACTTCAAAGTGTACCG	111
CHRNA7	TGGAGAATGGGACCTAGTGG	GAAATCTTCTCCCCGGAATC	210
CHRNA9	TACAATGGCAATCAGGTGGA	TGAATGTGACATCCGGGTAA	157
CHRNA10	TCCAGGCCACCTGAGTTATC	AGGGCCATGGAGAAGAAGAT	239
CHRNB1	GAGTGGACTGACTACAGGCTGA	GAGGACACCACGACGCTAAT	161
CHRNB2	GATCCTTCCCGCTACAACAA	AGCCAGATGTGTTTGGAAGG	230
CHRNB3	CCACATCGGTCTTGGTTTCT	GGGTGGTACGTGGAAGAAGA	187
CHRNB4	AGCGCAAGCCTCTGTTCTAC	CACGATCTTGGAGATGAGCA	170
CHRND	CCTGGCTCAGTCTGTCTTCC	TGTTCGGAAGTGGATGTTGA	157
CHRNE	GCGGAGGAGCTGATACTGAA	CCTCCTGATCTCTCGTGCTC	175
CHRNG	GCTAACCCTCACCAACCTCA	CGTTGTTCTCCAGCACGATA	185
GAPDH	CTGCCGTCTAGAAAAACC	CCAAATTCGTTGTCATACC	223

2.7.4. PCR

PCR was done in 0.2ml thin walled PCR strips by assembling the following components for each primer pair, including the positive control GAPDH primers.

Reverse transcribed cDNA (with control RT for each primer pair) - 1µl

Primer pair- 2µl

RNase free water- to make up to 20µl in total.

Phusion flash 2x Master mix

Additional magnesium chloride - for gradient PCR

This was amplified in the thermal cycler with 35 cycles programme. Amplified products were visualised using agarose gel. Gradient PCR was done using 3 Mg^{2+} concentrations (1.5, 2.5, 3.5mM) and 3 temperatures (63, 65, 68^{o}C).

Cycle programme was as follows:

stage	1	2			3	
cycles	x1	x35			x1	
step	1	1	2	3	1	2
temp (^{o}C)	98	98	variable	72	72	4
time (sec)	10	1	5	10	60	α

2.7.5. DNA extraction for sequencing

DNA extraction was done using Zymoclean^{TM} Gel DNA recovery kit. The technique was adopted from manufacturer's recommendation. Briefly, 3 times volume of agarose dissolving buffer (ADB) buffer was added to each volume of gel and incubated at 55^{o}C for 5-10 minutes. Melted agarose solution was centrifuged using Zymo-Spin column for 30 seconds. Columns were washed using 200µl of wash buffer twice. DNA was eluted from columns using 6-10µl of water.

3. Antigen expression by flow cytometry as screen for treatment targets

As explained in the introductory chapter new, highly expressed antigens in CLL could be explored as therapeutic targets. The aim of these experiments was to identify new antigens expressed on the surface of CLL cells by initially screening published gene expression profiling data and then confirming expression by flow cytometry.

3.1 Antigen identification

Gene expression profiling of B-cell malignancies on a genomic scale has been done previously and is available on dataset browsers (Klein et al., 2001b) (A. Rosenwald et al., 2001) (Haslinger et al., 2004). This has identified specific signature patterns for RNA expression in CLL compared with other B-cell malignancies and normal B-cells. Comparative analysis of large numbers of samples, especially when using supervised analysis tools, allows the identification of genes that are specifically up- or down- regulated in CLL. Identification of corresponding gene products is important in diagnosis, prognosis and to direct potential therapeutic approaches. However, the mRNA expression may not correlate directly with the protein content of the cell. The amount of protein produced depends on various factors like the physiological state of the cell, the gene it is transcribed from, the speed with which the mRNA is degraded in the cell before translation, etc (Rogers et al., 2008) (Dhingra et al., 2005). Moreover the functions of several proteins depend on post-translational modification like phosphorylation, glycosylation and complex formation with other proteins. A single transcript can occasionally give rise to different proteins by methods like alternative splicing (Black, 2003). Therefore, direct identification of the protein expression will be more helpful in detecting potential diagnostic and therapeutic targets. Antigens with plasma membrane surface epitopes are easier targets for standard diagnostic immunophenotyping by flow cytometry as well as for therapeutic treatment with monoclonal antibodies.

Microarray data from the Gene Expression Omnibus database was analysed for genes expressed in CLL.The study, in which there was maximum number of cases when this project was initiated, was selected for the analysis (Haslinger et al., 2004). The selected study analysed 100 CLL patients and 11

control B-cell samples using a synthesised oligonucleotide array. These cases were well defined with regards to their genomic aberrations as well as their *IGHV* mutational status. From the original excel spreadsheet, patients' data was selected and the median expression for individual genes was calculated. The gene ontology cellular component column explaining the subcellular expression from the original platform was then attached to this spreadsheet. Genes were then rearranged by the descending order of their median expression. These were then filtered for plasma membrane expression using Excel (Table 3.1). Out of the 12586 genes in the original database, 2872 had plasma membrane expression. These antigens were then individually searched for availability of antibodies binding to extracellular epitopes from commercial suppliers. Further analysis of any antigen was excluded if it has been already studied in CLL. 760 antigens were examined, in the descending order of their expression, and of these 121 had antibodies available (Table 3.2).

Table 3.1. The appearance of the data associated with an individual gene after filtering for plasma membrane expression.

ID_REF	IDENTIFIER	Subcellular expression	Median
37039_at	HLA-DRA	0005764 // lysosome // inferred from direct assay /// 0005886 // plasma membrane // not recorded /// 0005886 // plasma membrane // inferred from direct assay /// 0005887 // integral to plasma membrane // non-traceable author statement /// 0009897 // exter	125008

Table 3.2.The 760 antigens associated with plasma membrane localisation in the descending order of median expression in gene expression profiling.

The tested antigens are shown in bold.

Row Number	IDENTIFIER	Row Number	IDENTIFIER	Row Number	IDENTIFIER
1	HLA-DRA	256	SPINT1	510	TNK1
2	IGHM	257	**TMPRSS6**	511	HYAL2
3	GNB2L1	258	RAC3	512	ATP6AP2
4	CXCR4	259	CD247	513	PDE2A
5	CD37	260	SPTAN1	514	NCKAP1L
6	LAPTM5	261	DLGAP4	515	ITGAV
7	HLA-DPA1	262	RAP1A	516	JUP
8	CD74	263	M6PR	517	BSCL2
9	OAZ1	264	ITPR1	518	MTMR4
10	HLA-DPB1	265	CRKL	519	ILVBL
11	GNAS	266	**JAG1**	520	TMEM184B

12	HLA-DMA	267	KIDINS220	521	SLC46A3
13	GAPDH	268	USP24	522	SNN
14	CYBA	269	TMEM131	523	CEACAM3
15	SYNGR2	270	EXTL3	524	TYMP
16	HLA-DMB	271	LIPE	525	TM9SF2
17	CD79A	272	RAB31	526	DPEP1
18	HLA-F	273	**DGCR2**	527	ABCA6
19	HLA-E	274	SLC6A7	528	SYNGR3
20	IL23A	275	SLC30A3	529	CD3D
21	CD83	276	MADD	530	ELMO1
22	**JTB**	277	**LY86**	531	SLC39A14
23	HLA-DQB1	278	SEC61B	532	MCF2L
24	IFITM1	279	ACP5	533	**CDH16**
25	VAMP2	280	PIGC	534	**SLC29A1**
26	ATP6V0C	281	STOM	535	CYP2A6
27	ANXA2	282	DNAJC4	536	TXNDC13
28	HLA-J	283	BLCAP	537	**GPR15**
29	EMP3	284	CTSD	538	LILRB3
30	RAC2	285	STX7	539	FAIM2
31	CCR7	286	ICAM2	540	RALBP1
32	HLA-G	287	DEGS1	541	ABCC10
33	IL4R	288	PIP4K2B	542	CDK3
34	ARHGDIB	289	SEC11A	543	LAIR1
35	CD53	290	SH2B2	544	CYP4F12
36	PTP4A2	291	C7orf23	545	OFD1
37	SSR2	292	TMEM87A	546	IL2RB
38	TAGLN2	293	PLOD3	547	COBLL1
39	TMEM123	294	PTPRN	548	PARVB
40	IGHD	295	MAP3K7IP2	549	PTPN4
41	GDI1	296	PIGA	550	CSF2RB
42	C2orf24	297	**IFNGR1**	551	SEMA7A
43	LAMP1	298	GGT5	552	DRD2
44	SELL	299	PLXNB2	553	CD40
45	DGKA	300	PIGB	554	C12orf51
46	CSK	301	SLC5A6	555	LETMD1
47	**LILRB1**	302	C5orf15	556	ATP6V1A
48	ITGB1	303	SIT1	557	NKTR
49	FCER2	304	VPS39	558	CACNA1C
50	HLA-DOB	305	CNP	559	PTGER4
51	CSF1	306	VAMP7	560	**GPR12**
52	**SLC2A3**	307	**STIM1**	561	CDH4
53	PNPLA2	308	PLXND1	562	ITM2A
54	CD81	309	SACM1L	563	AAMP
55	UGCG	310	SLC7A7	564	PTPRE
56	MS4A1	311	MPPE1	565	IL27RA
57	PTPRCAP	312	PKN1	566	CD70
58	SYPL1	313	PLEKHB2	567	MAGI1
59	ADD3	314	**ROR1**	568	PDE4A

60	P2RX5	315	ORAI2	569	DPAGT1
61	DYNLL1	316	CHMP2A	570	LAMA5
62	AP2M1	317	CLPTM1	571	ATP11A
63	RHOH	318	MUC3A	572	SLC13A2
64	APOL1	319	LRRC32	573	**PDCD1**
65	CAP1	320	TMEM63A	574	TGFBR3
66	LY6G6C	321	GNG7	575	ATP6V0A1
67	DGKD	322	KCNH2	576	FLT3LG
68	IGHG1	323	DPM2	577	CR2
69	WBP2	324	**RHBDL**1	578	TAOK3
70	ATP6V0B	325	ALG3	579	JAG2
71	INPP5D	326	SLC7A5	580	IL11RA
72	BTN3A1	327	CLDND1	581	**ACVRL1**
73	**IL10R**A	328	FKBP2	582	ABCC1
74	CIB1	329	VCL	580	CBARA1
75	CKAP4	330	ITGA2B	581	IFNAR2
76	CD99	331	MMP15	582	ABCC8
77	GDI2	332	CTNNA1	583	TM7SF2
78	HERPUD1	333	UBE2J1	584	GBAS
79	IL2RG	334	CD3E	585	LRP3
80	CD27	335	**CRHR2**	586	GP2
81	VAMP8	336	LY6E	587	CYBB
82	MFSD10	337	AATK	588	CACNA2D2
83	ARF6	338	**SLC9A1**	589	MFSD5
84	PLCG2	339	GRIP2	590	VNN2
85	GRIK5	340	CD14	591	**THBD**
86	NCF4	341	PFDN1	592	KIAA1128
87	RASGRP2	342	C7orf44	593	STAB1
88	**IFNGR2**	343	RNF19B	594	GPSN2
89	EPOR	344	VPS26A	595	ARFIP2
90	CRLF3	345	KRAS	596	CD59
91	TMC6	346	KCNJ4	597	MEG3
92	CD48	347	BTN3A2	598	P2RX4
93	CD69	348	**RAMP3**	599	**TGFA**
94	GRK6	349	SKAP2	600	CD7
95	EVI2B	350	CD5	601	GBP2
96	PLEC1	351	CD24	602	FRY
97	SSR4	352	IL7R	603	SGCE
98	RAP1B	353	SLC10A3	604	AAK1
99	AP2A2	354	GRK5	605	ENTPD1
100	PTDSS1	355	CAPZA2	606	NTNG1
101	ICAM3	356	PIK3IP1	607	TRAM1
102	FTH1	357	STARD3	608	SLC7A4
103	BASP1	358	**MR1**	609	CLCN6
104	PCDH9	359	EHD1	610	NPHP4
105	**GRM4**	360	SLC4A2	611	SDF2
106	ADRBK1	361	AP2B1	612	**NRG2**
107	PTPN1	362	PEX11B	613	KCNMB1

108	SYP	363	**GPR56**	614	ALG8
109	DDOST	364	FNDC3A	615	AQP8
110	ADCY6	365	RHCE	616	CYP2F1
111	IFITM2	366	SLC38A10	617	**PVR**
112	ARHGAP1	367	C6orf105	618	MMD
113	IGSF9B	368	ADCY3	619	STX2
114	**GPR18**	369	GRM2	620	EFNA2
115	TYROBP	370	TAP1	621	ARL6IP5
116	NPTXR	371	**KIAA0319**	622	HCK
117	RAC1	372	PHKB	623	GUCA1A
118	ATP6V1F	373	RFTN1	624	F7
119	CD200	374	P2RX1	625	SLAMF1
120	ARHGEF1	375	**ACCN1**	626	CDC42EP3
121	PITPNM1	376	TAZ	627	**EFNB1**
122	KIAA0922	377	GNG5	628	POR
123	IL10RB	378	SLC4A3	629	ADA
124	DNAJA1	379	MAL	630	PCTK1
125	P2RY10	380	**ATP1B1**	631	C9orf61
126	CD22	381	IL17RA	632	ENPP2
127	CD55	382	ITPR2	633	ATP10D
128	RAPGEF2	383	**DDR1**	634	MRC2
129	RTN4	384	BTN2A1	635	STMN1
130	DAD1	385	SEC31A	636	SLC37A4
131	FLOT2	386	**EDA**	637	TM4SF5
132	SERP1	387	DPM1	638	BAIAP2
133	FOLR1	388	ADCY7	639	CADM4
134	GPR161	389	GCS1	640	**FPR**2
135	PIP5K3	390	CNPY2	641	**LY75**
136	ADAM8	391	ITGAL	642	TLR6
137	ATP2B1	392	HEXA	643	INSIG2
138	TCIRG1	393	CD72	644	**SLC2A1**
139	CXCR5	394	VPS11	645	TEX28
140	EPS15	395	**LTK**	646	IL6ST
141	TNFRSF14	396	VAV1	647	PPM1A
142	NKG7	397	EFR3A	648	SEC62
143	CAPN1	398	**CNR2**	649	GPM6A
144	ATP6V1G1	399	**MMP14**	650	MAP3K12
145	HLA-DQA1	400	INPPL1	651	RALB
146	TMEM147	401	SCN4A	652	SLC43A1
147	**ADAM19**	402	PIP5K1C	653	TIMP2
148	CD47	403	C16orf42	654	ACCN3
149	KCNN4	404	**HPN**	655	TRPM2
150	DOCK2	405	SLC16A3	656	**F2RL3**
151	**ATP1B3**	406	LRBA	657	**LTB4R**
152	PLK3	407	TUBB3	658	SLC12A4
153	FCGRT	408	ATP6V1B2	659	SLC7A11
154	EDEM1	409	LRPAP1	660	KLRB1
155	LRP10	410	APOM	661	SLC16A6

156	PRF1	411	CD8B	662	**SLC11A1**
157	ATP6V0E1	412	CD2	663	TMEM41B
158	LY9	413	MBOAT7	664	LYPD3
159	LILRA4	414	ATP2A2	665	MPP3
160	**GYPC**	415	ESD	666	STXBP3
161	TEX261	416	GLB1	667	RGS14
162	FAM134C	417	**TNFRSF25**	668	ABCC5
163	TMEM59	418	SEC61G	669	LILRA3
164	KCNAB2	419	ITGA3	670	TMEM115
165	**SDC**3	420	INSIG1	671	TNFRSF10C
166	MTMR1	421	CACNB1	672	CD1D
167	HLA-DOA	422	ADIPOR2	673	TLN2
168	PTK2B	423	**NCR3**	674	CLDN9
169	FAM38A	424	DNAJC16	675	LRIG1
170	RHOG	425	PTPLB	676	CD300C
171	GPAA1	426	AGPAT2	677	RALGPS1
172	**CD97**	427	**HTR7**	678	EHBP1
173	MARCKSL1	428	RRAS	679	LGMN
174	DIAPH1	429	ATP8A1	680	BID
175	TNK2	430	MTMR3	681	CNKSR1
176	AGPAT1	431	TSPAN31	682	CYP2C19
177	RNF103	432	ACVR1B	683	LEPROT
178	ANXA6	433	ENO2	684	ABCB1
179	SHC1	434	STX4	685	ITGAE
180	S1PR4	435	VAMP3	686	SRC
181	GRINA	436	ACAA1	687	BTN3A3
182	SLC23A2	437	ZDHHC18	688	TMEM11
183	NPTN	438	CD63	689	CX3CR1
184	SPTLC1	439	NAE1	690	**CDH15**
185	RHOC	440	CD34	691	FDFT1
186	CLTA	441	CD8A	692	**CSF3R**
187	STAM	442	LANCL1	693	OPRL1
188	EI24	443	RHOQ	694	LEPROTL1
189	NISCH	444	GIPC1	695	ITGB1BP1
190	**CHRNB4**	445	SEC63	696	**PRRG1**
191	YME1L1	446	UTRN	697	KCNN1
192	**ADAM28**	447	PPT1	698	ALPPL2
193	SPINT2	448	SLC7A6	699	RASGRF1
194	**ITGB7**	449	ILK	700	DNAJB12
195	ATXN2L	450	PECAM1	701	ARSA
196	FCGR2B	451	KCTD2	702	CNTNAP2
197	BNIP2	452	PDCD10	703	GLT8D1
198	SORL1	453	FGFR1	704	SCARB1
199	ULK1	454	OR2F1	705	**APLP1**
200	ATP11B	455	MAGED1	706	CAPRIN1
201	SERINC1	456	TMCC1	707	**ERBB3**

202	KIAA0247	457	TBC1D9B	708	**GRIN1**
203	MICB	458	UPK1A	709	FAM8A1
204	BLNK	459	IL8RB	710	KLRC3
205	CD46	460	**ADAM15**	711	RAB22A
206	MXRA8	461	ATG12	712	SLC1A6
207	CD6	462	NCK2	713	**GPR3**
208	**GABBR1**	463	RHEB	714	**EGF**
209	**LPAR2**	464	AQP5	715	CYB5R1
210	**CNTN2**	465	KRIT1	716	LILRB4
211	**TNFRSF13B**	466	SCRIB	717	MPP2
212	MARS	467	FAM127A	718	SLC6A9
213	RHOD	468	NECAP1	719	SLC11A2
214	AKR1A1	469	SLC18A3	720	KRT1
215	RRAS2	470	DHCR7	721	CCKBR
216	PIGO	471	CYP4B1	722	POL3S
217	FYN	472	TRD@	723	DDX10
218	ITGAX	473	SLC22A18	724	**DRD4**
219	RCE1	474	ABHD14A	725	GNPAT
220	RAB8A	475	CD1C	726	AVPR1B
221	PPP1R16B	476	**SLC4A1**	727	PLEKHB1
222	**TNFSF9**	477	ATP4A	728	SLC5A2
223	LMTK2	478	SDC1	729	VEGFA
224	MC2R	479	SMPD2	730	SEC31B
225	KIAA1109	480	BCAM	731	SLC7A1
226	MYD88	481	PHKA2	732	CYB561D2
227	AP2S1	482	**CSPG4**	733	IL1RL1
228	SYNPO	483	SELPLG	734	FCER1G
229	CAPN2	484	SLC1A5	735	MFAP3
230	KTN1	485	KCNQ1	736	RGS9
231	TNFRSF10B	486	**ATP1B2**	737	FAM119B
232	BZRPL1	487	ATP13A3	738	MYO7A
233	RNF167	488	HPS1	739	ICOS
234	RRBP1	489	SSTR5	740	SLC22A6
235	FADS1	490	RAB4B	741	GNA15
236	RASGRP3	491	C1orf95	742	GJB1
237	CLCN7	492	MGST3	743	PLCH2
238	**SLC20A1**	493	SLC9A3R1	744	VRK2
239	PNPLA6	494	TNFRSF1A	745	IER3
240	CXCR3	495	LHFPL2	746	PTGER3
241	FAM62A	496	YIPF2	747	PIGQ
242	RAB14	497	SLC30A1	748	GPR107
243	TRAM2	498	ATP6V0A2	749	PRAF2
244	**PLAUR**	499	EBP	750	RYK
245	KIAA0195	500	psiTPTE22	751	MAP3K7IP1
246	VAPA	501	PIGR	752	TLR1
247	SPG7	502	C10orf26	753	PTPRA
248	**AMFR**	503	SLC35D1	754	PMP22
249	RAB5B	504	CYP4A11	755	SEMA5A

250	EFNA3	505	KIAA0317	756	LPGAT1
251	PSD4	506	AQP7	757	SYNGR4
252	**GPR35**	507	TSPAN3	758	FAAH
253	ALOX5AP	508	LPL	759	GUCA1B
254	ITGB2	509	FCGR3B	760	GRM1
255	MAG				

3.2 Antibody identification

The detection of a cell surface antigen by flow cytometry requires recognition of a preserved epitope. Amongst the commercially available antibodies to the antigens of interest, there was a mixture of antibodies that had previously been used for flow cytometry applications as well as untried reagents. Moreover, the list included both polyclonal and monoclonal preparations. The relative advantages and disadvantages of these are described in detail below.

3.2.1. Polyclonal antibodies

Polyclonal antibodies are combination of immunoglobulin molecules secreted against different epitopes of a specific antigen. These antibodies are typically produced by inoculation of a suitable animal such as rabbit, chicken, goat, guinea pig, hamster, horse, mouse, rat, and sheep of which rabbit is the most frequently used one. An antigen is injected into the mammal which induces the B-cells to produce immunoglobulin specific for the antigen. Large proteins usually result in better engagement of antigen presenting and antigen processing cells for a satisfactory immune response. If smaller antigens are used then they are conjugated to a carrier protein like keyhole limpet hemocyanin (KLH) and bovine serum albumin (Harlow and Lane, 1988). This polyclonal Ig is purified from the mammal's serum by various techniques like ultra-filtration, dialysis, ion exchange chromatography size exclusion chromatography and protein A/G affinity chromatography. The bound antibodies are eluted from the column using high salt concentration or pH changes (Grodzki and Berenstein, 2010) (Leenaars and Hendriksen, 2005). Polyclonal antibodies usually recognise multiple epitopes on any one antigen and the serum obtained will contain a heterogeneous complex mixture of antibodies of different affinity. Therefore polyclonal antibodies are not useful for probing specific domains of an antigen because polyclonal antiserum will usually recognize many domains. But relatively large amounts of antibodies can be produced in a short time span and with minimal expense. As polyclonals will recognize multiple epitopes on any one antigen, it is advantageous to amplify signal from target proteins with low expression level,

as the target protein will bind more than one antibody molecule on the multiple epitopes, which generally provides a more robust detection (Harlow and Lane, 1988). However, this would be disadvantageous for quantification experiments like flow cytometry, as the results could become inaccurate as the antigenicity may vary between batches and the standardisation is difficult. Due to recognition of multiple epitopes, polyclonals are often the preferred choice for detection of denatured proteins can give better results in Western blotting and immunoprecipitation. They are not affected by minor changes in the antigen like polymorphism, heterogeneity of glycosylation, or slight denaturation, as are monoclonal (homogenous) antibodies (Lipman et al., 2005). They are useful to identify proteins of high homology to the immunogen protein or to screen for the target protein in tissue samples from species other than that of the immunogen e.g. polyclonal antibodies are sometimes used when the nature of the antigen in an untested species is not known. This also makes it important to check the immunogen sequence for any cross-reactivity. Polyclonal antibodies have a huge batch-to-batch variation and they can contain large amounts of non-specific antibodies that can sometimes give background signal in some applications (Nelson et al., 2000).

3.2.2. Monoclonal antibodies

Monoclonal antibodies are antibodies produced by a single B lymphocyte clone. They are typically made by hybridomas obtained by fusing myeloma cells with the splenic cells from a mouse that has been immunised with a desired antigen (Köhler and Milstein, 1975). The hybridomas can be grown indefinitely in a suitable cell culture medium or can also be injected into the peritoneal cavity of a mouse, which produces tumours secreting antibody-rich ascitic fluid. Antibodies are then purified by various techniques like ultra-filtration, dialysis, ion exchange chromatography, size exclusion chromatography and protein A/G affinity chromatography. As monoclonal antibodies detect only one epitope on the antigen and consist of only one antibody subtype, where a secondary antibody is required for detection, an antibody against the correct subclass should be chosen. Monoclonals usually have less background staining in various applications (Lipman et al., 2005). As they are more specifically detecting one target epitope, they are less likely to cross-react with other proteins. Compared to polyclonal antibodies, homogeneity of monoclonal antibodies is very high. If experimental conditions are kept constant, results from monoclonal antibodies will be highly reproducible between experiments. But monoclonal antibodies are more vulnerable to the loss of epitope through chemical treatment of the antigen

than are polyclonal antibodies (Harlow and Lane, 1988). Also their production requires labour-intensive technology that is expensive and time consuming.

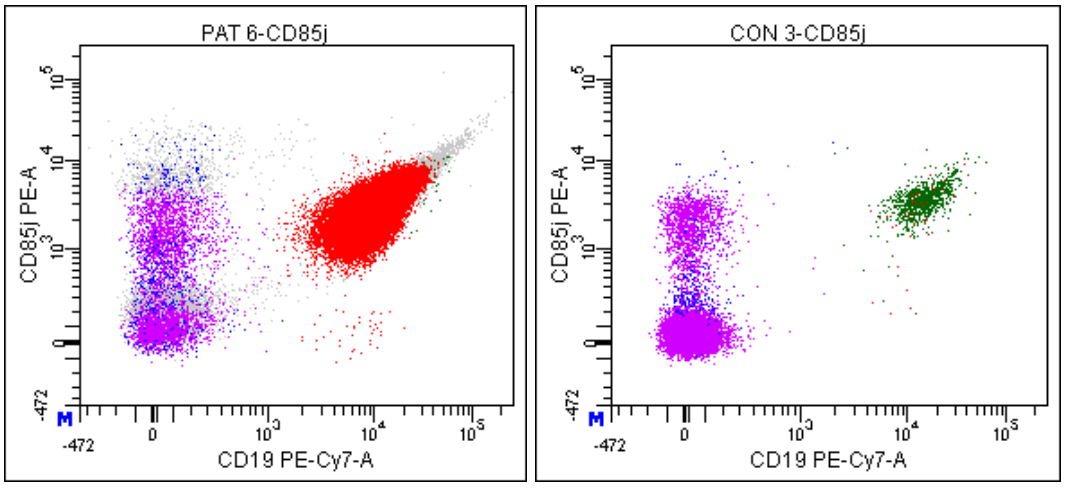
Polyclonal antibodies, produced in either rabbit or mouse, are the commonest used in our experiments. If there was a monoclonal antibody conjugated to a fluorochrome available commercially, then that was the first order of preference. These antibodies are commercially tested and optimised for flow cytometry. Out of the 84 antibodies tested 20 antibodies were commercially conjugated mouse monoclonal antibodies, 44 were rabbit polyclonals and 20 were unconjugated mouse monoclonals. For testing these antibodies standard flow cytometry procedures were adopted.

3.3 Analysis of direct staining

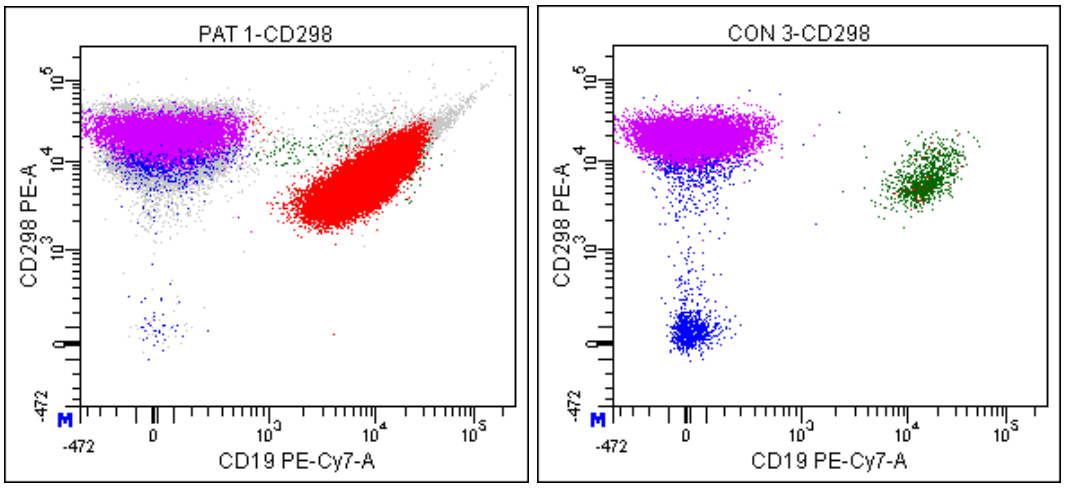
Based on the difference in gene expression between normal controls and CLL samples, it is predicted that certain cell surface proteins may act as unique identifiers of CLL. To test this, samples from 10 patients and 3 controls were evaluated using direct staining.

Monoclonal antibodies conjugated to a PE used for direct staining included CD167a, CD85j, CD298, CD119, ERB, CD205, CD97, Integrin b7, DR3, CD137, CD51, CD141, IFN-γ R β chain, CD210, CD155, CD279, CD337.

Example plots for antigen expressions using direct staining from patient (left panel) or control (right panel) are shown in Figure 3.1.



CD85j



CD298

Figure 3.1 Example plots for antigens tested using direct staining technique.

The left panel represents the expression on CLL cells (red) and the right panel shows the expression on normal B-cells (green). The name of the antigen is identified on the bottom of each plot.

For each antigen, the median fluorescence intensity (MFI) of staining was calculated. The average MFI ± sd on individual cell subsets from the 10 patients and 3 controls are shown in Figure 3.2. By comparing the MFI on the different cell populations, it appeared as though ERB, DR3, CD141, GRM4, CD337 and CD114 were not detectably expressed. In contrast, CD279 was expressed on all lymphocytes and CD205, CD97, CD298 and CD210 were expressed equivalently on all of the populations. CD85j, CD167a, CD155, CD137, IFNGRB and CD268 were found on both normal and CLL B-cells. Amongst this group of antigens, CD51, ITGB7 and CD119 displayed higher expression levels on normal B-cells as compared to the CLL counterpart. There were no antigens specifically expressed on CLL cells. Based on these findings, antigens belonging to the first two groups would not be considered attractive candidates for further investigation, whereas those falling into the latter categories are potentially interesting for CLL discrimination.

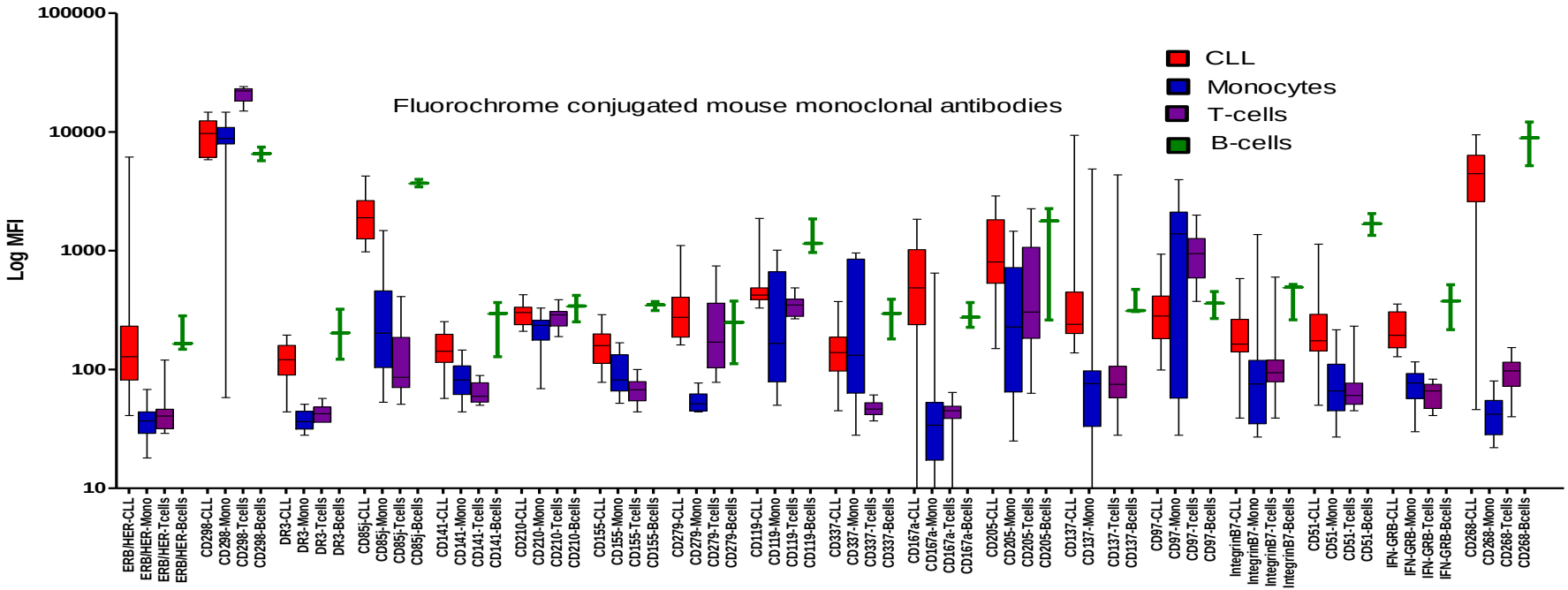


Figure 3.2 Spread of MFI on each cell type.

Box and Wisker graph showing the expressions of antigens analysed by fluorochrome conjugated mouse monoclonal antibodies on individual cell subsets from the 10 patients and 3 controls. The cells are identified with colour coding as shown in the graph legend. The expressions on monocytes and T-cells are shown only from patients and not from controls. As this experiment was to screen for antibodies the expressions were analysed subjectively without any objective definitions using isotype controls. The antigens were grouped based on their expression on individual cell subsets as shown in in Table 3.3.

3.4 Indirect staining

3.4.1. Indirect staining - Analysis of expression

Based on the optimisation experiments described in materials and methods several rabbit polyclonal antibodies were screened using $F(ab)_2$ portion of goat anti-rabbit antibody as the secondary antibody and 1.25µl of human IVIg as the non-specific binding blocking agent. The details of the antibodies tested are shown in Table 2.4. Example plots for antibody expressions are shown in Figure 3.3.

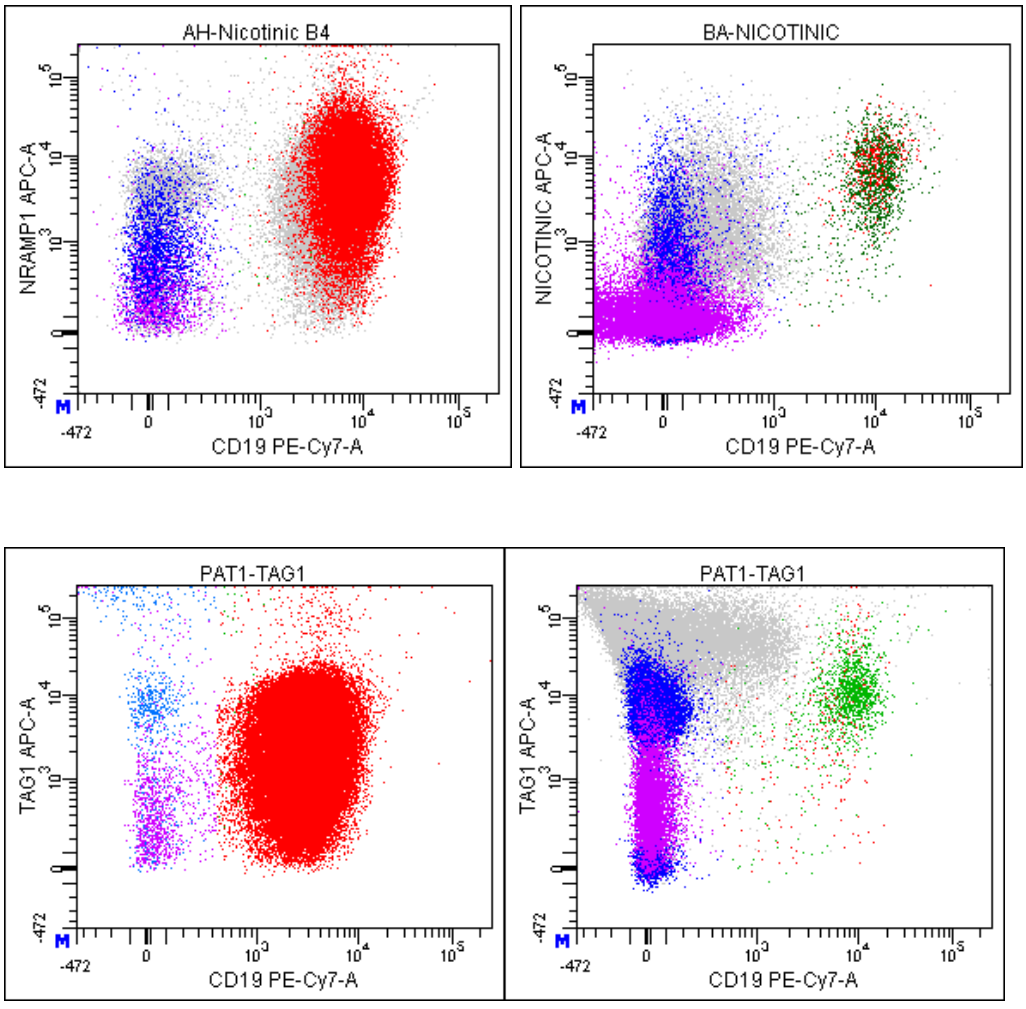
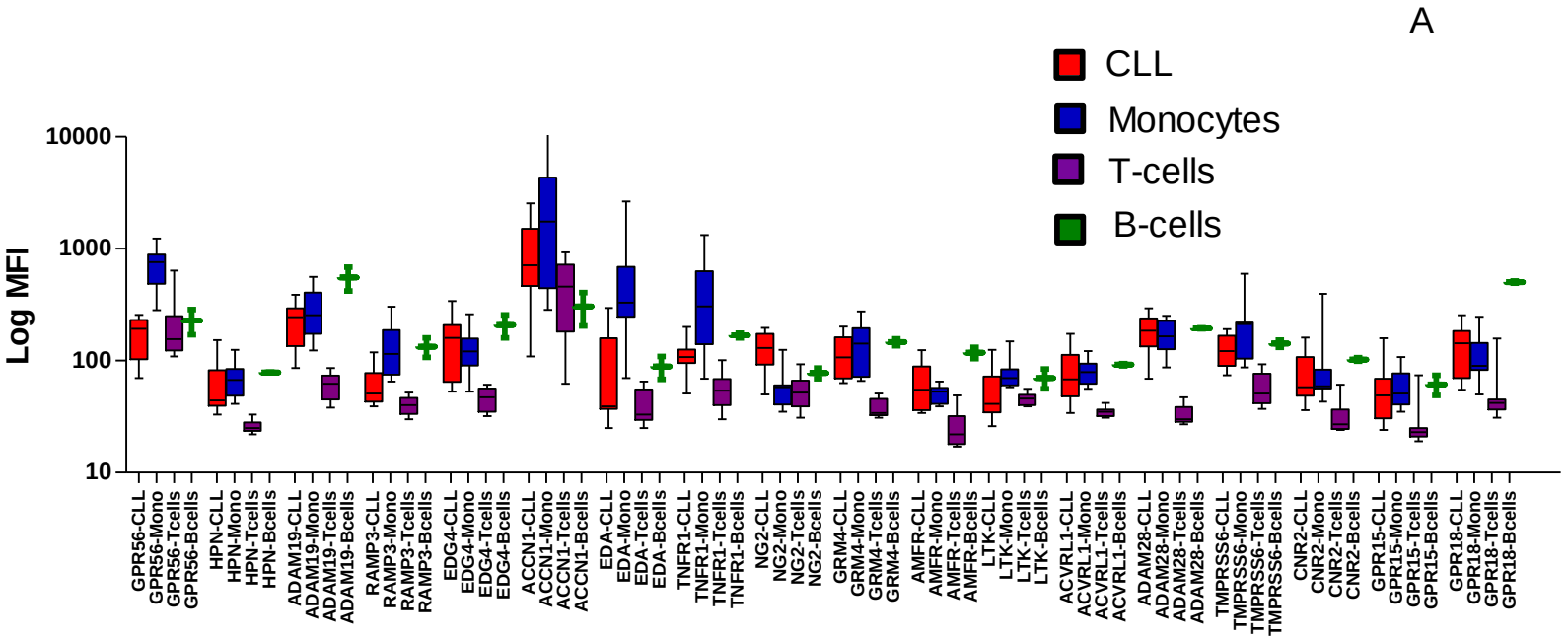


Figure 3.3 Example plots for antigens tested using indirect staining.

Examples of antigens tested using rabbit polyclonals as the primary antibody, $F(ab)_2$ portion as the secondary antibody and IvIg as the non-specific expression blocking agent. The left panel represent the expression on CLL cells (red) and the right panel shows the expression on normal B-cells (green). The name of the antigen is identified on the top of each plot.

For each antigen, the median fluorescence intensity of staining was calculated. The average median fluorescence intensity ± sd on individual cell subsets from the 10 patients and 3 controls are shown in Figures 3.4: A, B and C. As with the results for direct staining, there was a range of observed patterns associated with the different antigens. SLC2A3, EFNB1, GPR56, RAMP3, EDA, NG2, EDG4, AMFR, LTK, ACVRL1, ADAM28, TMPRSS6, CNR2, GPR15, GPR35, FPRL1, MD1, RVK, GLUT1, F2RL3, NMDAR1, DGCR2, CRHR2, ADAM15, NRAMP1, ENT1, SDC3, LTB4R, GPR3, MMP15, HPN and TNFR1 were not detected on any of the populations using the available reagents. JAG1 and ACCN1 on the other hand were found equally on all cells tested. In terms of potentially interesting markers that showed skewed expression on B-cells, these included ADAM19, GPR18, CHRNB4, APLP1, DRD4, GPR12, ROR1, TAG1 and 5HTR. However, these antigens did not distinguish CLL cells from normal B-cells, nor did any other antigens tested in this panel. Several of these antigens are linked to neuronal signalling and will be discussed in more detail in subsequent chapters.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T-cells
B-cells
Log MFI
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1000
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10
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GPR56-Mono
GPR56-Tcells
GPR56-Bcells
HPN-CLL
HPN-Mono
HPN-Tcells
HPN-Bcells
ADAM19-CLL
ADAM19-Mono
ADAM19-Tcells
ADAM19-Bcells
RAMP3-CLL
RAMP3-Mono
RAMP3-Tcells
RAMP3-Bcells
EDG4-CLL
EDG4-Mono
EDG4-Tcells
EDG4-Bcells
ACCN1-CLL
ACCN1-Mono
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ACCN1-Bcells
EDA-CLL
EDA-Mono
EDA-Tcells
EDA-Bcells
TNFR1-CLL
TNFR1-Mono
TNFR1-Tcells
TNFR1-Bcells
NG2-CLL
NG2-Mono
NG2-Tcells
NG2-Bcells
GRM4-CLL
GRM4-Mono
GRM4-Tcells
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LTK-CLL
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LTK-Bcells
ACVRL1-CLL
ACVRL1-Mono
ACVRL1-Tcells
ACVRL1-Bcells
ADAM28-CLL
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ADAM28-Bcells
TMPRSS6-CLL
TMPRSS6-Mono
TMPRSS6-Tcells
TMPRSS6-Bcells
CNR2-CLL
CNR2-Mono
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GPR15-CLL
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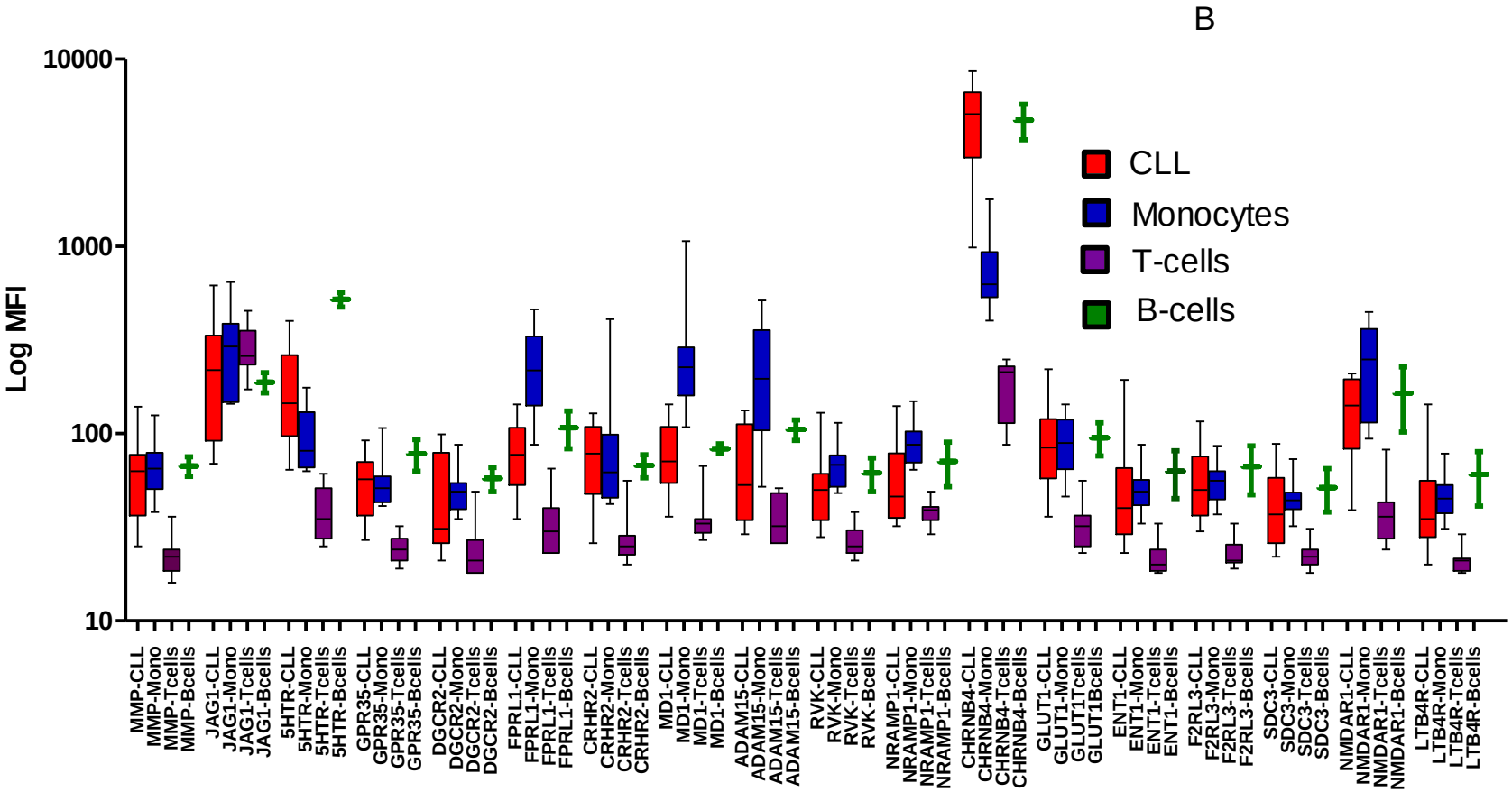
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B-cells
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MMP-Mono
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MMP-Bcells
JAG1-CLL
JAG1-Mono
JAG1-Tcells
JAG1-Bcells
5HTR-CLL
5HTR-Mono
5HTR-Tcells
5HTR-Bcells
GPR35-CLL
GPR35-Mono
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ADAM15-Tcells
ADAM15-Bcells
RVK-CLL
RVK-Mono
RVK-Tcells
RVK-Bcells
NRAMP1-CLL
NRAMP1-Mono
NRAMP1-Tcells
NRAMP1-Bcells
CHRNB4-CLL
CHRNB4-Mono
CHRNB4-Tcells
CHRNB4-Bcells
GLUT1-CLL
GLUT1-Mono
GLUT1Tcells
GLUT1Bcells
ENT1-CLL
ENT1-Mono
ENT1-Tcells
ENT1-Bcells
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F2RL3-Tcells
F2RL3-Bcells
SDC3-CLL
SDC3-Mono
SDC3-Tcells
SDC3-Bcells
NMDAR1-CLL
NMDAR1-Mono
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NMDAR1-Bcells
LTB4R-CLL
LTB4R-Mono
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LTB4R-Bcells

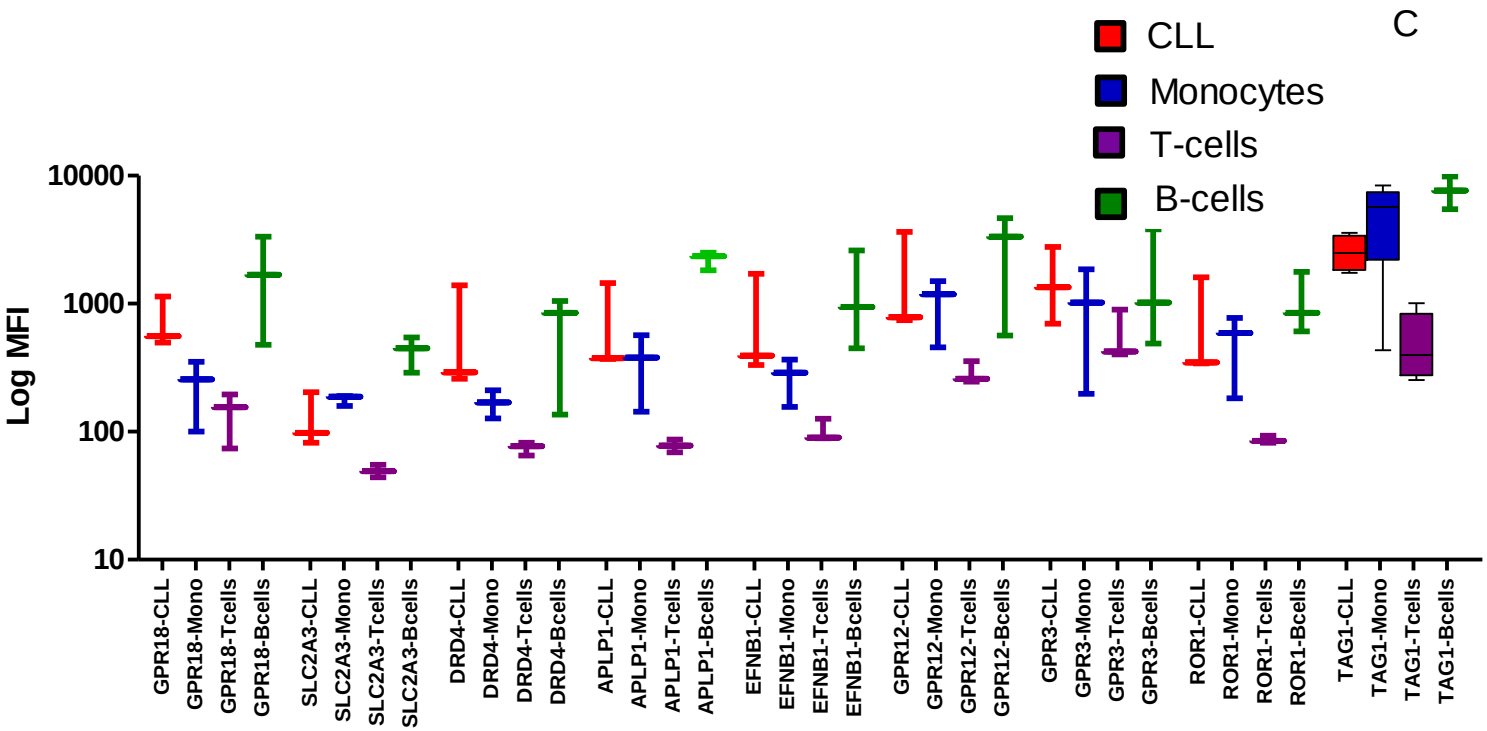


Figure 3.4 Spread of MFI on each cell type.

A,B and C- Box and Wisker graph showing the expressions of antigens analysed by rabbit polyclonal antibodies on individual cell subsets from the 10 patients and 2 controls. The cells are identified with colour coding as shown in the graph legend. The expressions on monocytes and T-cells are shown only from patients and not from controls. As this experiment was to screen for antibodies the expressions were analysed subjectively without any objective definitions using isotopic controls. The antigens were grouped based on their expression on individual cell subsets as shown in Table 3.3.

3.5 Zenon labelling

20 of the antibodies available were mouse monoclonal antibodies. As all the other standard antibodies used to identify cells were mouse monoclonal antibodies, labelling of the test antibody using secondary antibodies were not possible. In this context Zenon labelling can be a useful technique providing rapid labelling of small quantity of antibodies which does not require any purification. Mouse monoclonal antibodies were labelled with fluorochromes using Zenon labelling technology which utilises a fluorophore labelled Fab fragment directed against the Fc portion of an intact IgG primary antibody in order to form a labelling complex. Separate labelling kits were used for specific mouse monoclonal antibody isotype: IgG1, IgG2a or IgG2b. An unlabelled antibody was incubated with the Zenon labelling reagent, containing a fluorophore-labelled Fab fragment which binds to the Fc portion of the IgG antibody. Excess Fab fragment was neutralised by the addition of a nonspecific IgG. The addition of non-specific IgG prevents cross-labelling of the Fab fragment when multiple primary antibodies of the same type were used. Table 2.4 gives the details of the antibodies tested using this technique.

CD2 and CD37 were used as control antibodies to test the technique. The following are the representational plots for these antibodies, which show that the technique worked very efficiently in the experimental conditions previously described Figure 3.5.

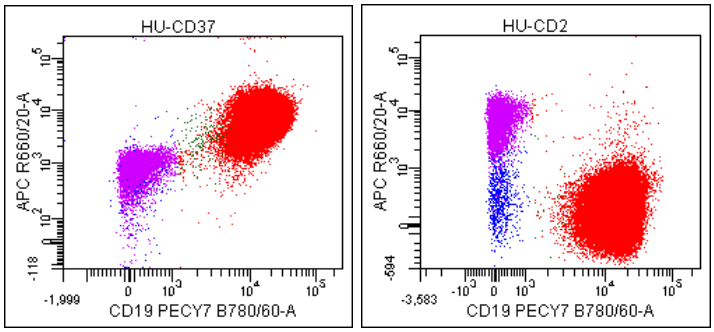


Figure 3.5 Control antibodies for Zenon labelling.

The expression of CD37 was evaluated on CLL cells (red) and that of CD2 on T-cells (purple). Specific positive staining is shown for CLL cells on the left and T-cells on the right.

Since the conditions appeared to produce clear staining patterns, the procedure was carried out using each of the antibodies for the following antigens: GYPC, RHBDL1, SLC9A1, MR1, ATP1B1, CDH16, TGFA, CDH15,

EGF, KIA0319, JTB, SLC20A1, MMP14, GABBR1, STIM1, SLC4A1, ATP1B2, NRG2, PRRG1, PLAUR. The details of these antigens are shown in Table 2.4.The results for each cell type are graphically displayed in Figure 3.6.

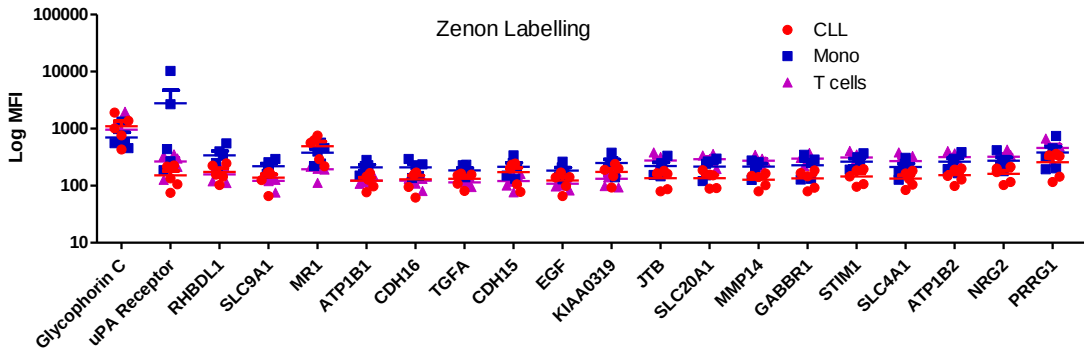
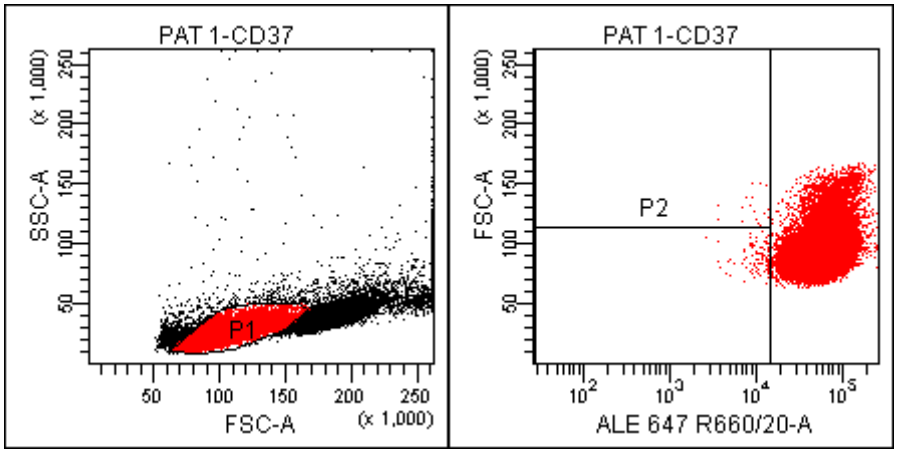


Figure 3.6 Expression of various antigens on different cell types using Zenon labelling.

The expression of 20 different antigens was determined by flow cytometry on CLL cells (red circles), monocytes (blue squares) and T-cells (purple triangles) from n= 5 patients. As this experiment was to screen for antibodies the expressions were analysed subjectively without any objective definitions using controls.

As evident from the figure, except Glycophorin C no other antigens showed expression on any of the cell types tested. The following method was used to confirm the true negativity of expression. CLL cells were negatively selected using magnetic beads. The negatively selected cells were stained using the primary test antibody followed by secondary antibody which is a $F(ab)_2$ rabbit anti-mouse antibody using standard flow cytometry techniques. The gating strategy and control antibodies used in these experiments are shown below in Figure 3.7.



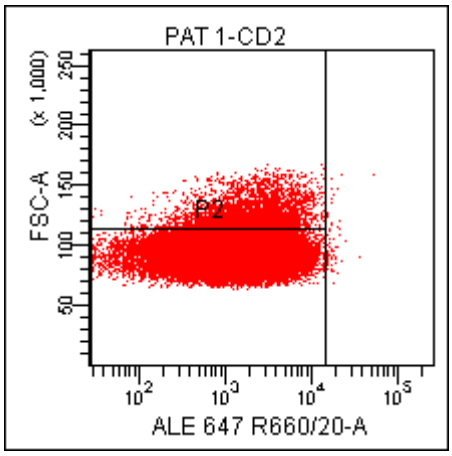


Figure 3.7 Control antibodies for testing antigen expression by cell selection technique.

CLL cells were negatively selected using magnetic beads. Lymphocytes were gated using forward and side scatter excluding the doublets as in plot 1. The other two plots shows CD37 (positive control) and CD2 (negative control) expression, which shows expression of CD37 and absence of expression of CD2 which is expected in CLL. As CD2 is not expressed in CLL cells the baseline of the positive expression is kept at the top of CD2 fluorescence.

Each of the test antibodies was then assayed in this manner. The representational flow plots of test antibodies are shown below in Figure 3.8.

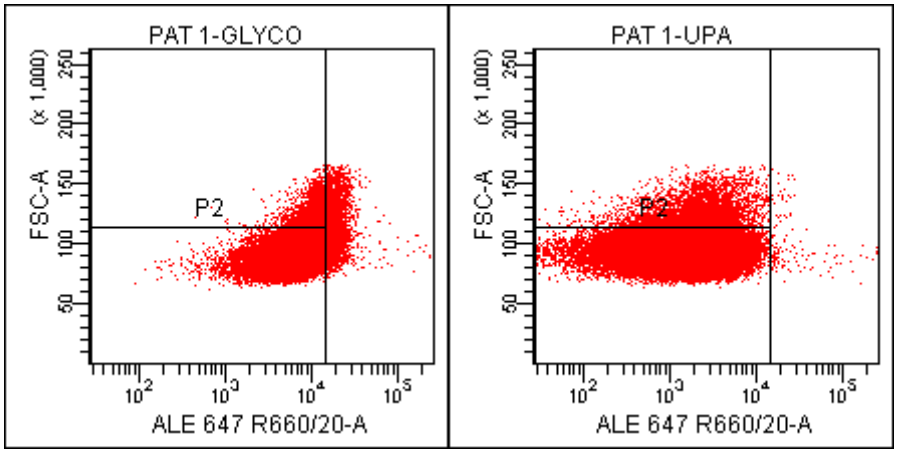


Figure 3.8 Representational plot for testing antigen expression by cell selection technique.

The expression of of glycophorin C and uPA were tested using negative selection of CLL cells and using mouse monoclonals as the primary antibody and F(ab)$_2$ rabbit anti-mouse antibody as the secondary antibody. The name of the antigen is identified on the top of each plot.

3.6 Analysis of antigen expression

Based on the expression on different cell types antigens can be grouped as shown in Table 3.3. There were no antigens that were expressed solely on CLL cells. 15 antigens showed some expression on CLL cells as well as normal B-lymphocytes. These antigens have two main implications; one from therapeutic point and other for minimal residual disease analysis point. CD20 is a good example of such an antigen that is currently in use. Even though the expression of CD20 is low in CLL cells compared to normal B-cells combination of CD20 antibody, rituximab, with chemotherapy is currently the standard treatment in CLL. Similarly CD20 in combination with other antigens is also useful in MRD analysis due to the differential expression on CLL cells and normal B-cells. Similar to that these 15 antigens can be explored for therapeutic potential as well as MRD analysis. Additionally, antigens expressed on all lymphocytes and pan-expressing antigens also could be explored for therapeutic potential. This could be extrapolated from the use of alemtuzumab, a CD52 antibody. CD52 is a pan-expressing antigen expressed even on several non-haematopoetic tissues as well. But this can be used for treatment of refractory CLL with good clinical efficacy and affordable toxicity. For MRD analysis there should be differential expression between CLL cells and all normal lymphocytes.

From Table 3.3 it is clear that there are several antigens expressed on CLL cells that could be potentially explored as therapeutic targets. Based on the experience of Rituximab and Alemtuzumab it is known that an antibody against an antigen either expressed on CLL cells as well as normal B-cells or a pan-expressing antigen could be useful as therapeutic molecule with acceptable side effects. A general description of these antigens along with their known function as well as expression is given in Tables 2.3 and 2.4. Most of the antigens except CD268 and ROR1 were not studied in CLL before. Detailed descriptions of some of these antigens are given in Chapter 7.

CLL cells alone	CLL and normal B-cells	All lymphocytes	Pan-expressing	More on normal B-cells compared to CLL	Negative on all cells			
	CD85j	CD279	CD205	CD51	ERB	EDA	GPR35	NRAMP1
	CD167a		CD97	ITGB7	DR3	NG2	FPRL1	ENT1
	CD155		CD298	CD119	CD141	EDG4	MD1	SDC3
	CD137		CD210		GRM4	AMFR	RVK	LTB4R
	IFN-γ R β		JAG1		CD337	LTK	GLUT1	GPR3
	CD268		ACCN1		CD114	ACVRL1	F2RL3	MMP15
	ADAM19		GYPC		SLC2A3	ADAM28	NMDAR1	HPN
	GPR18				EFNB1	TMPRSS6	DGCR2	TNFR1
	CHRNB4				GPR56	CNR2	CRHR2	RHBDL1
	APLP1				RAMP3	GPR15	ADAM15	SLC9A1
	DRD4				MR1	ATP1B1	CDH16	TGFA
	GPR12				CDH15	EGF	KIA0319	JTB
	ROR1				SLC20A1	MMP14	GABBR1	STIM1
	TAG1				SLC4A1	ATP1B2	NRG2	PRRG1
	5HTR				PLAUR			

Table 3.3 Grouping of antigens based on expression on different cell types.

Direct staining; Indirect staining; Zenon labelling

3.7 Differential expression test vs. control antibodies

20 antigens that have shown at least a minimal expression were selected for further evaluation of differential expression on more patients. The expression of the test antigens were compared in relation to a negative antigen on CLL cells in both direct conjugate and indirect conjugate experiments. One direct conjugate and one indirect conjugate were paired simultaneously in multi-colour flow cytometry to limit the number of experiments. CD14 and CD2 were selected as negative controls for direct conjugate and indirect conjugate, respectively. Normal controls were from patients below the age of 40 who were admitted for unrelated reasons. Younger controls were selected to avoid the possibility of monoclonal B-cell lymphocytosis in those samples. The gating strategy is shown in the flow plot below (Figure 3.9):

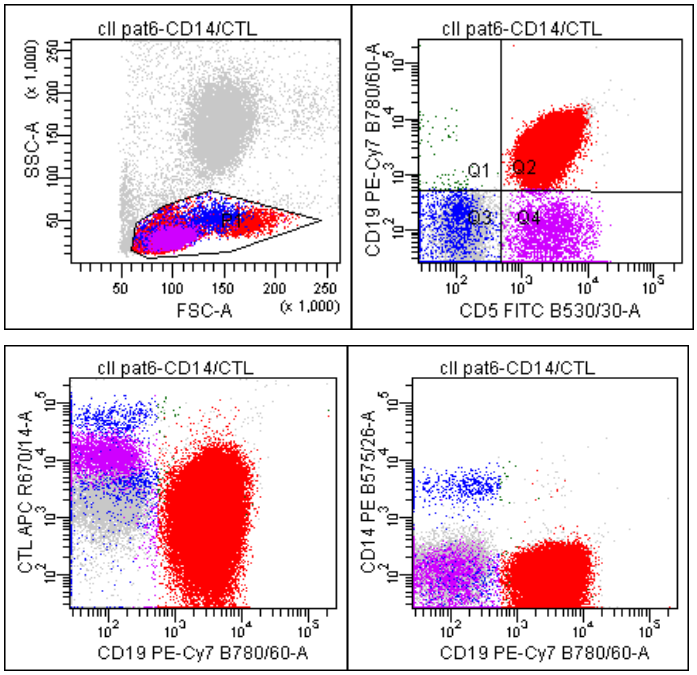


Figure 3.9 Gating strategy for differential expression experiment.

One direct conjugate and one indirect conjugate were paired simultaneously. CD14 and CD2 were selected as negative controls for direct conjugate and indirect conjugate, respectively. CD14 was selected as a negative control for direct conjugates due to its ease of availability in the lab as well as satisfying other criteria for direct conjugates and CD2 was selected as a negative control for indirect conjugates as it satisfied all other criteria for rabbit polyclonal antibodies like presence on plasma membrane and availability of an antibody to an extracellular epitope. CLL cells were identified using CD19 and CD5. Expression of CD2 on APC and CD14 on PE are shown in subsequent plots.

Figure 3.10 demonstrates the difference in MFI of various polyclonal antibodies compared to the control antibody CD2. At first glance, these results suggest that with the exception of CHRNB4, there appears to be a differential expression of the other antigens on CLL cells versus T-cells or monocytes. Statistical analysis confirmed a significant difference in expression for ACCN1, CHRNB4 and TAG1. It is notable that apart from CD2, monocytes displayed higher expression levels for each of the antigens tested, while T-cells showed the lowest expression.

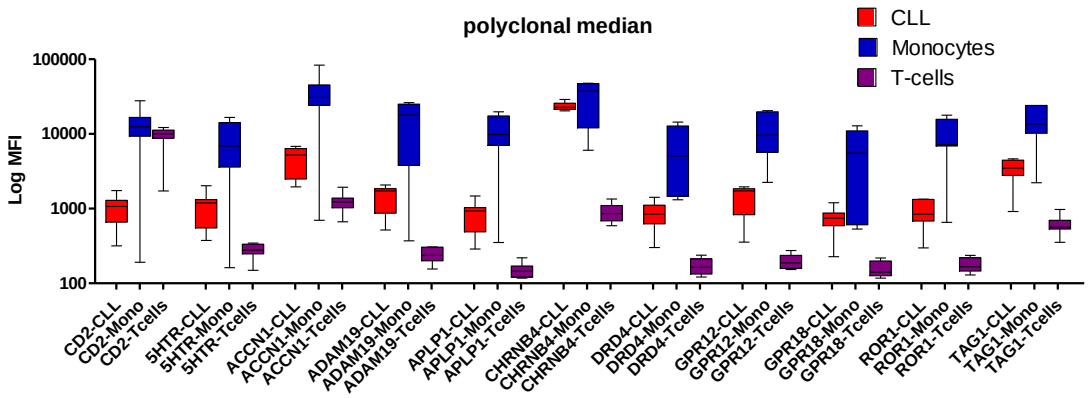


Figure 3.10 Difference in MFI of various polyclonal antibodies compared to the control antibody CD2.

Box and Wisker graph showing the expression levels of antigens analysed by rabbit polyclonal antibodies on individual cell subsets from 7 patients. The cells are identified with colour coding as shown in the graph legend. The assoicated statistical analysis is shown in Table 3.4

Table 3.4 P value of the difference in MFI of various polyclonal antibodies on CLL cells compared to that of CD2.

P value was calculated using Mann Whitney test. The difference is statistically significant for ACCN1, CHRNB4 and TAG1.

	P value		P value
5HTR	0.71	DRD4	0.62
ACCN1	**0.0006**	GPR12	0.259
ADAM19	0.128	GPR18	0.259
APLP1	0.535	ROR1	0.9
CHRNB4	**0.0006**	**TAG1**	**0.007**

Figure 3.11 demonstrates the difference in MFI of various monoclonal antibodies compared to the control antibody CD14. Unlike the data in Figure 3.10, the differences amongst these antigens are less pronounced with the exception of CD268, which displays uniquely high levels on CLL cells. The antigens CD119, CD205, CD137 and CD85j were also significantly different.

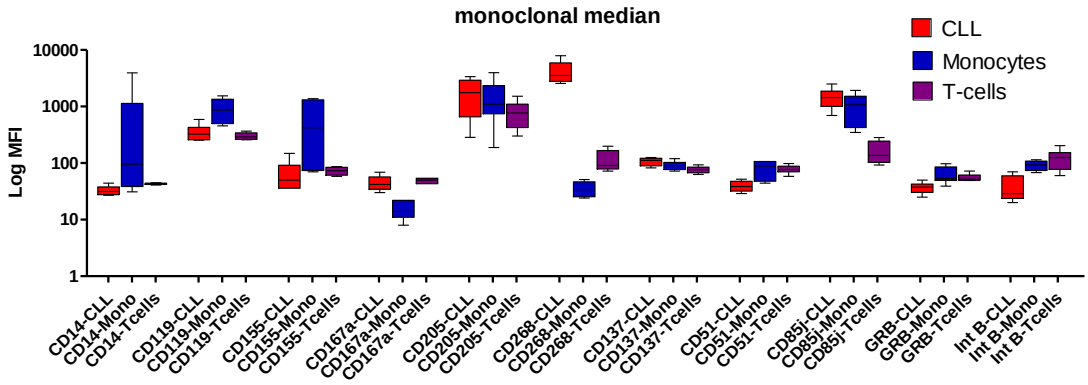


Figure 3.11 Difference in MFI of various monoclonal antibodies compared to the control antibody CD14.

Box and Wisker graph showing the expression levels of antigens analysed by mouse monoclonal antibodies on individual cell subsets from 6 patients. The cells are identified with colour coding as shown in the graph legend. The assoicated statistical analysis is shown in Table 3.5

Table 3.5 P value of the difference in MFI of various monoclonal antibodies on CLL cells compared to that of CD14.

P value is calculated using Mann Whitney test. The difference is statistically significant in CD119, CD205, CD268, CD137 and CD85j.

	P value		P value
CD119	**0.01**	**CD137**	**0.026**
CD155	0.07	CD51	0.337
CD167a	0.14	**CD85j**	**0.006**
CD205	**0.001**	GRB	0.62
CD268	**0.006**	Int B	0.749

3.8 Differential expression - CLL vs. normal B-cells

The results in Figures 3.10 and 3.11 suggest that a number of the newly identified antigens may be used to distinguish CLL cells from T-cells and monocytes, but these may be of limited value if the expression levels are the same on normal B-cells. To evaluate this, the MFI of various antibodies on CLL cells were compared to normal B-cells from healthy volunteers as shown in Figure 3.12. The p value of the difference was calculated using a Mann Whitney test and is shown in Table 3.6. Of the 7 antibodies showing difference INTGβ7 is the most striking one. As it is more expressed in normal B-cells it may not be very relevant as a therapeutic target, but this difference could be explored as a potential MRD marker.

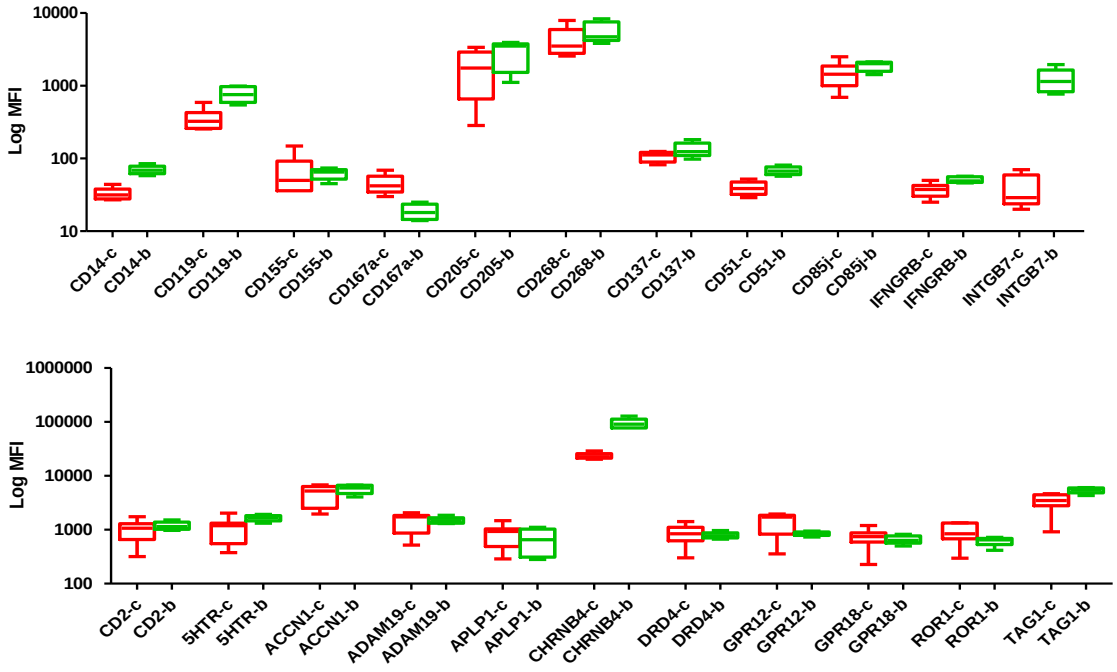


Figure 3.12 MFI of various antibodies on CLL cells were compared to normal B-cells from healthy population.

6 patient and 5 control samples were used for this analysis. Red represents CLL cells and green represents normal B-cells.

Table 3.6 P values of MFI of various antibodies on CLL cells were compared to normal B-cells from healthy population

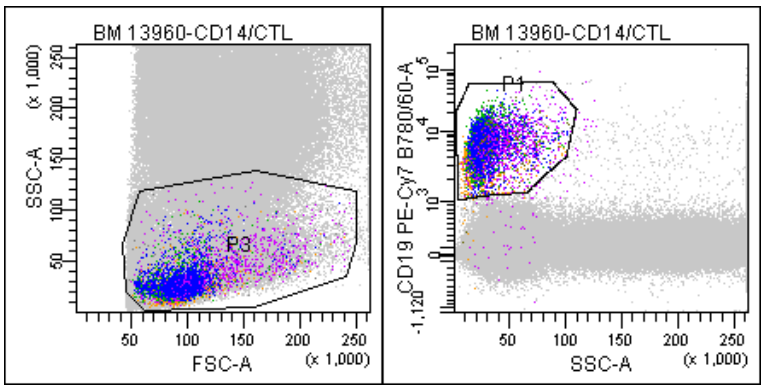
	P value		P value
CD14	0.0043	CD2	0.7551
CD119	**0.0087**	5HTR	0.0480

CD155	0.4103	ACCN1	0.5303
CD167a	**0.0043**	ADAM19	0.6389
CD205	0.1775	APLP1	0.5303
CD268	0.2468	**CHRNB4**	**0.0025**
CD137	0.1255	DRD4	0.4318
CD51	**0.0043**	GPR12	0.2020
CD85j	0.1775	GPR18	0.7551
IFNGRB	**0.0353**	ROR1	0.0735
INTGβ7	**0.0043**	**TAG1**	**0.0177**

3.9 Expression on Bone marrow progenitors

These antibodies were further evaluated for any difference in expression in bone marrow progenitor cells and differentiated B-cells. This has potential therapeutic implications, as if any of these antigens are less expressed on progenitor cells then therapeutic manipulation involving this antigen will have less impact on normal progenitor cells and thereby on normal haematopoiesis.

The gating strategy is shown in Figure 3.13



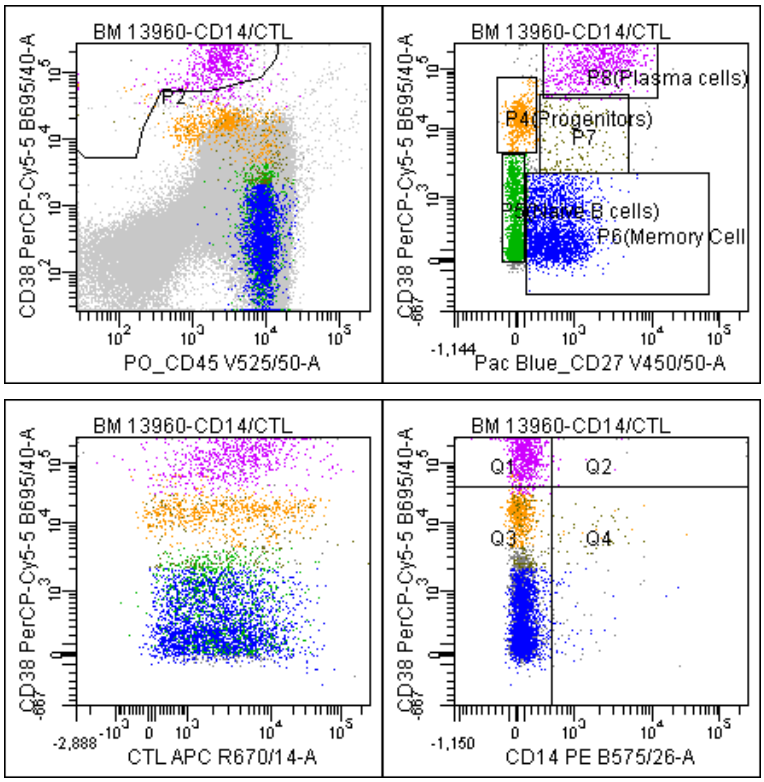
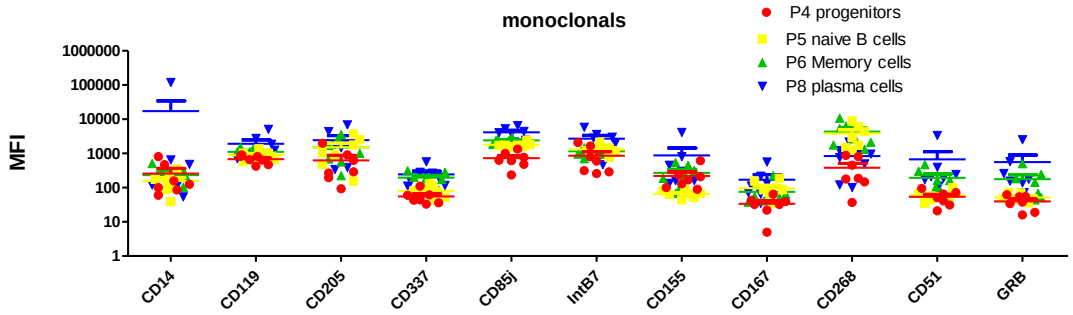


Figure 3.13 Gating strategy for assessing expression in bone marrow cells.

The B-cell populations in bone marrow were divided into 4 groups based on CD38 and CD27 as labelled in the figure. Expression of test antigen using a polyclonal antibody labelled with APC and a monoclonal antibody labelled with PE was assessed simultaneously.

The MFI of expression of various antigens on different B lymphocyte subtypes are shown in Figure 3.14. There was no significant difference in expression on various B-cell types.



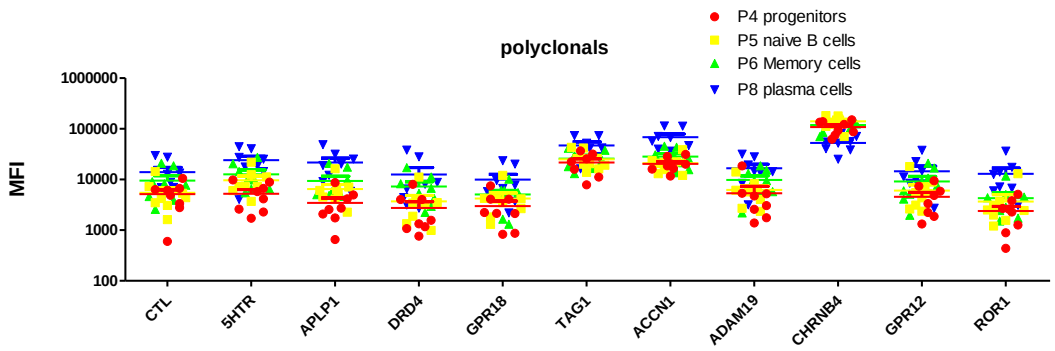


Figure 3.14 Differential expression in bone marrow B-cells.

MFI of the indicated antigens was assessed on different B-cell populations. There was no significant difference in expression of any antigens between the cells analysed including B-cell progenitors, naive B-cells, memory cells and plasma cells.

3.10 Induced expression

The ability to generate a difference in expression of any antigens when co-cultured with fibroblasts also has potential therapeutic implications. It has now been conclusively shown that for survival and proliferation of CLL cells, stimulation from different cells in the microenvironment is essential (Burger, 2011) (Audrito et al., 2013a) (Lanasa, 2010). These supporting cells can upregulate or downregulate the expression of various antigens in CLL cells so it is important to be aware of these changes when deciding on a therapeutic strategy. The expression levels of 64 antigens on CLL cells were tested before and after co-culturing with CD40L-expressing fibroblasts for 24 hours to analyse any changes in the pattern of expression. None of the tested antigens showed any significant difference in expression as shown in Figure 3.15. Ideally this experiment should have been done with some positive control with antibodies for antigens already known to be induced in coculture system like CD38, CD69, CD44 and ITGA4 (Hamilton et al., 2012).

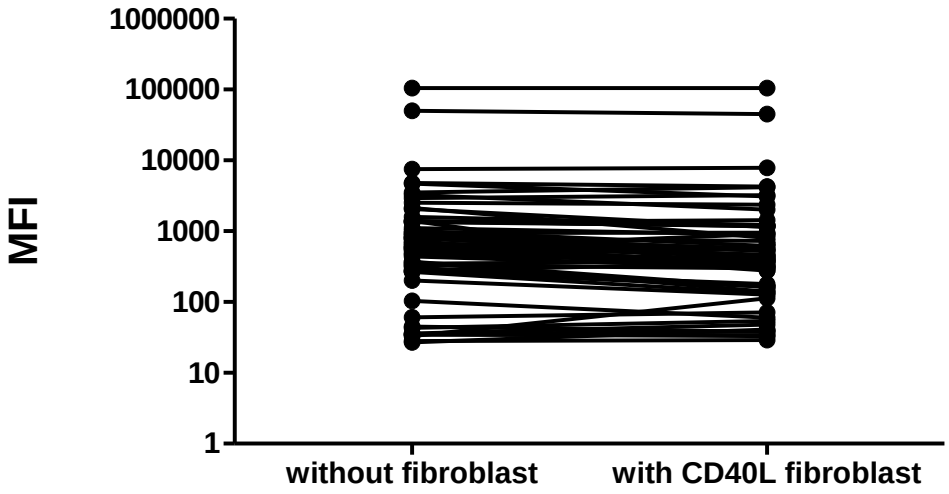


Figure 3.15 Expression of antigens on CLL cells before and after co-culturing with CD40L fibroblasts.

The MFI of 64 differented antigens were assessed on cells from a single patient before and after 24 hours of co-culture with DC40L fibroblasts. None of the tested antigens showed any significant difference in expression.

3.11 Discussion

In the data presented here, several antigens expressed on cell surface were identified form previously published gene expression profiling data that were not extensively studied in CLL and had antibodies available from commercial sources.

Expressions of these antigens were studied at protein level using flow cytometry. Directly conjugated, indirectly conjugated or Zenon labelling techniques were used to screen these antibodies, depending on the type of antibodies available.

As indirectly conjugated antibodies were mostly rabbit polyclonal antibodies and were not previously tested using flow cytometry several steps were required to optimise this technique.

None of the antigens identified were purely expressed on CLL cells without expression on any other cells. Several antigens which were expressed on CLL cells and B-cells were identified, including seven that may allow discrimination between normal and neoplastic cells. It is interesting to note that, of antigens which show differential expression between normal and CLL B-cells, both CD51 and ITGB7 are adhesion molecules that have been

associated with the pathogenesis of multiple myeloma (Van Riet and Van Camp, 1993) (Neri et al., 2011). CD119 is the alpha component of the IFN-gamma receptor, which pairs with IFNGRB to make a functional molecule and both are down-modulated on CLL cells compared to normal B-cells. IFN-gamma has been shown to protect CLL cells from undergoing spontaneous apoptosis *in vitro* (Mainou-Fowler and Prentice, 1996), so it is somewhat surprising that the receptor is expressed at a relatively lower level *in vivo*. ROR1 has been previously shown to be over-expressed in CLL (Daneshmanesh et al., 2012) (Baskar et al., 2012), however the results presented here, while showing the same trend, did not unequivocally confirm this observation. ROR1 encodes several distinct isoforms, so the results here may be related to the ability of the antibodies to detect. Another antigen showing increased expression is CD167 (or DDR1), which is statistically different from the level of expression on normal B-cells. DDR1 belongs to a family of receptor tyrosine kinases that respond to stimulation by collagen and have been proposed to play a role in a variety of tumours including lung, colon and brain (Valiathan et al., 2012). A recent publication has also shown that collagen activation of DDR1 in the setting of EBV infection protects lymphoma cells from death (Cader et al., 2013). Furthermore selective DDR1 inhibitors have been described that may be useful in future studies (Gao et al., 2013).

An intriguing observation was that, of the 23 antigens, which showed some expression on CLL cells, 8 had a significant role in neurotransmission. GPR18 is involved in *N*-arachidonoyl glycine (NAGly) signalling and induces migration, proliferation and perhaps other MAPK-dependent phenomena involving recruitment of microglia to sites of neuronal injury (McHugh et al., 2010). APLP1 plays a role in synaptic function by localising to the 'postsynaptic density' which is a specialised region containing proteins required for signalling and regulate neurite outgrowth through binding to components of the extracellular matrix such as heparin and collagen (Kim et al., 1995). GPR12 is another molecule regulating neurite outgrowth along with neuronal differentiation and synapse formation (Tanaka et al., 2007) (Lu et al., 2012). TAG1 is a neuronal membrane protein that may be involved in the formation of axon connections in the developing nervous system (Walsh and Doherty, 1991). 5-HTR_7 is a receptor for the neurotransmitter serotonin, which physiologically might be involved in mood changes and learning and possibly maintenance of circadian rhythm and pathologically may play a role in certain psychiatric disorders like depression (Glennon et al., 2002). ACCN1 is a cation channel that plays a physiological role in perception of sensations like

taste, pain and mechanical stimulus and pathologically in development of multiple sclerosis (Wemmie et al., 2006) (Bernardinelli et al., 2007). CHRNB4 is one of the subunits that form the nicotinic acetyl choline receptor which is a major neurotransmitter receptor in central and autonomic nervous system as well as neuromuscular junction. There is now evidence that nicotinic receptors are also found in non-neuronal tissues like lymphocytes, macrophages (Wang et al., 2003) and polymorphonuclear cells (PMN) in the peripheral blood (Hoss et al., 1986)(Hiemke et al., 1996)(Lebargy et al., 1996); small cell lung carcinomas (Quik et al., 1994), skin keratinocytes (Grando, 1997), respiratory epithelial and vascular endothelial cells (Conti-Fine et al., 2000), and T lymphocytes (Kawashima and Fujii, 2000) even though their role in these locations is still not known. DRD4 is another neurotransmitter receptor which acts as an inhibitory receptor for dopamine through G-protein. Besides nervous system dopamine plays a role on the immune system which is demonstrated by various factors like the presence of dopamine receptors (Basu et al., 1993) (Ricci and Amenta, 1994) and an endogenous dopamine transport system in leukocytes (Bondy et al., 1992) (Basu et al., 1993) as well as the endogenous synthesis of this monoamine in leukocytes (Bergquist et al., 1994) (Cosentino et al., 1999).

Nicotinic acetyl choline receptor β4 was the antigen with maximum MFI and therefore it was the first antigen explored for therapeutic potential.

A number of potential therapeutic targets for CLL have been identified as shown in this chapter and in the next chapter an in vitro culture system was developed to allow the testing of new treatments, including reagents developed against the antigens identified in this chapter.

4. Development of *in vitro* culture system for testing treatment targets

The key aim of the project is to identify new antigens on the surface of CLL cells that can act as potential therapeutic targets. Development of an *in vitro* system is therefore a crucial step in testing these targets. It has been shown that CLL cells when removed from the *in vivo* microenvironment and placed in suspension cultures undergo spontaneous apoptosis after few days (Collins et al., 1989). Previous studies have shown that when CLL cells are cocultured with different adherent stromal cells, including mesenchymal marrow stromal cells (Lagneaux et al., 1998) (Panayiotidis et al., 1996) (Burger et al., 2000), CD68+ nurselike cells derived from monocytes (Nishio et al., 2005) (Richardson et al., 2006), and follicular dendritic cells (Pedersen et al., 2002) induce leukaemia cell survival and prevent spontaneous apoptosis. It has been shown that different human and murine MSC lines as well as primary human MSC have similar effects on CLL cells *in vitro* (Kurtova et al., 2009). To test the effect of new drugs on CLL cells, especially in relation to the microenvironment interaction, it is important to develop a system, where CLL cells are cultured on their own as well as in presence of stromal cells as stromal cells provide pro-survival signals to CLL cells and breaking this contact is key to induce apoptosis is CLL cells.

Various short term and medium term culture conditions have been shown to support the culture of CLL cells. There are studies which show that certain specific media specifically can also support CLL cell survival (Levesque et al., 2001) (Zent et al., 2004). Our aim was to try various alternatives to find an optimal condition that will support CLL cell survival for medium term and then study the effect of antibodies or small molecules targeted against the antigens identified in the flow cytometry experiments.

4.1 Viability assessment of purified CLL cells

CLL cells were harvested from patient blood samples using lymphoprep density centrifugation and magnetic purification with a cocktail of antibodies designed to negatively select CLL B-cells. The purified cells were then seeded into the various culture conditions. To determine the effect of these procedures, a combination of vital dye and Annexin-V staining were

employed. Examples of viability assessment plots on day 0 are shown in Figure 4.1

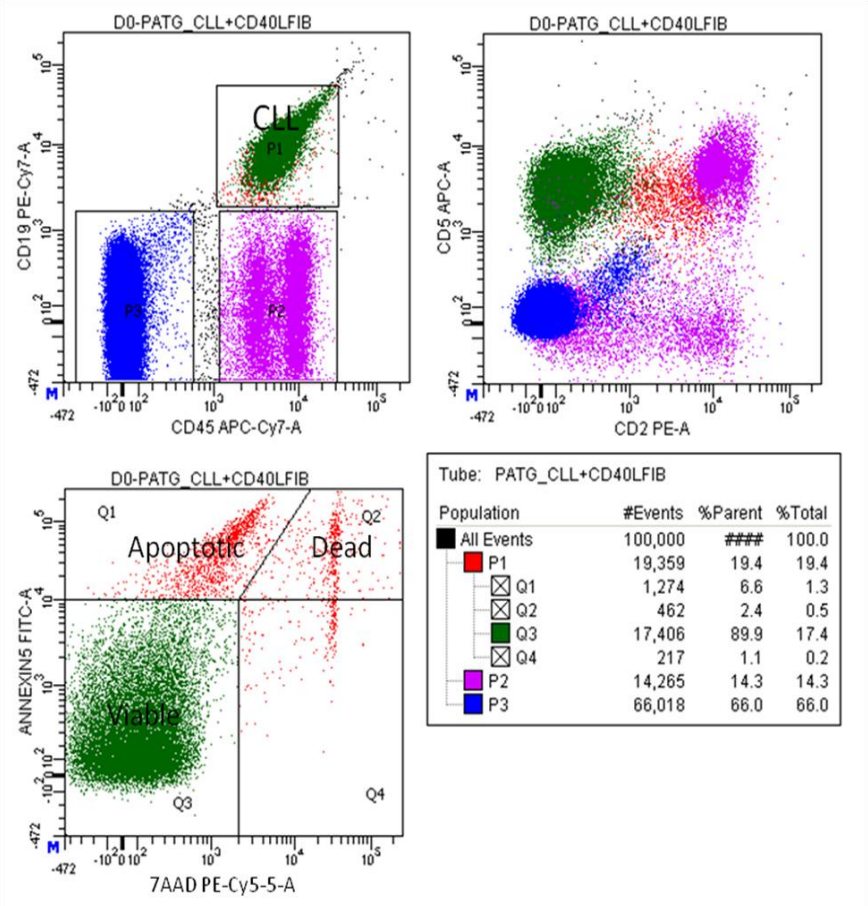


Figure 4.1 Gating strategy for viability assessments

Flow cytometric analysis of viability of cells from an *in vitro* cell culture system. In the first plot leukocytes were separated from the stromal cells (P3) using CD45 expression and CLL cells were identified using coexpression of CD45 and CD19 (P1). CLL cell viability is assessed using annexin-v and 7-AAD as shown in plot 3. The viable cells were both negative (Q3) and dead cells were both positive (Q2). The early apoptotic cells were annexin-v positive and 7-AAD negative (Q1). The percentage viability was obtained from statistics plot.

Sometimes for practical reasons seeding of the cells in the culture medium may not be possible on the same day itself. The viability of the CLL cells from the same samples was assessed sequentially by keeping the whole blood in refrigerator for 15 days followed by room temperature. The experiment demonstrated that the viability remains the same up to a week if samples were kept in the refrigerator (Appendix 4).

4.2 CD40L expressing fibroblasts and M210B4 stromal cells prolong CLL cell survival in vitro

CLL cells were seeded at different densities in the presence or absence of CD40L expressing fibroblasts or M210B4 stromal cells. Additionally, cultures were evaluated using two different types of media, IMDM or RPMI. Prior to plating cell viability was assessed. Samples were incubated for a period of 2 weeks. Representative samples were removed from each well at frequent intervals for assessing viability. The percentages of viable CLL cells on day 0, 1, 3, 10 and 15 are shown in Figures 4.2 and 4.3. It is evident from the graph that stromal cells helps to keep CLL cells viable up to 15 days and it is also suggests that a seeding ratio of 1:50 or above is required for keeping the cells viable to that duration. There is not much difference between using CD40L fibroblasts or M210B4 stromal cells as the supporting cells.

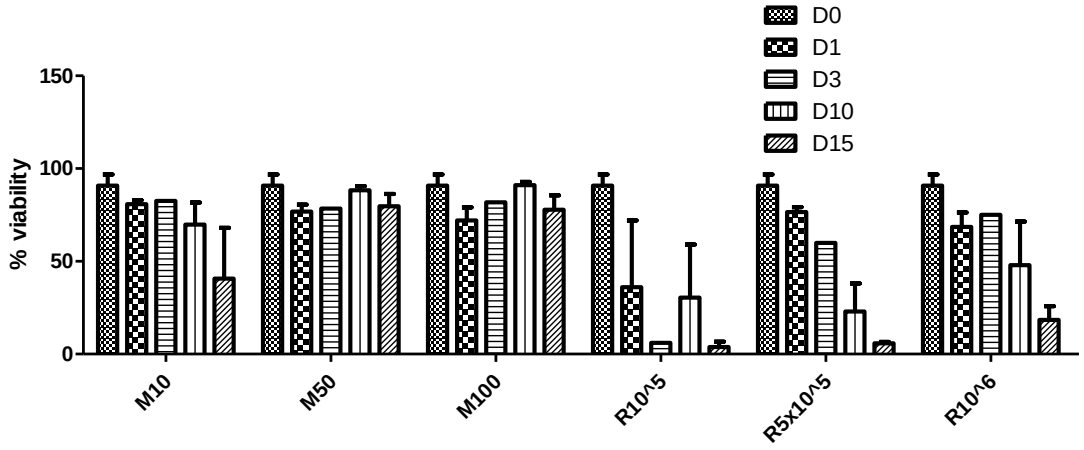


Figure 4.2 Percentage viability of CLL cells in co-culture experiments.

MNCs were either cultured in RPMI or co-cultured with M210B4 cells. The quantities of stromal cells used were $1x10^4$/ml. 3 different quantities of MNC were tested: $1x10^5$ cells, $5x10^5$ cells and $1x10^6$ cells/ml. Cells were analysed for viability on days 0, 1, 3, 5, 7, and 15. (M=M210B4, R=RPMI) (n=2). The number in the initial 3 columns represents the ratio between stromal cells and MNC.

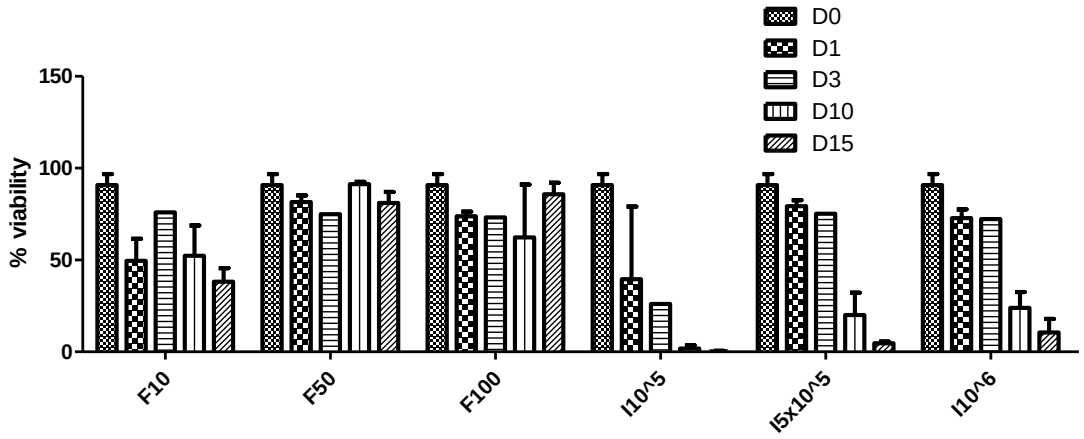


Figure 4.3 Percentage viability of CLL cells in co-culture experiments.

MNCs were either cultured in IMDM or co-cultured with CD40L expressing fibloblasts. The quantities of stromal cells used were 1x10^4/ml. 3 different quantities of MNC were tested: 1x10^5 cells, 5x10^5 cells and 1x10^6 cells/ml. Cells were analysed for viability on days 0, 1, 3, 5, 7, and 15. (F=CD40L fibroblast, I=IMDM) (n=2). The number in the initial 3 columns represents the ratio between stromal cells and MNC

Since the supporting cells used were not irradiated or treated with mitomycin-c, over a few days the wells were very turbid due to the overgrowth of these cells. The flow plots also had a considerable percentage of these cells as shown in the following Figure 4.4

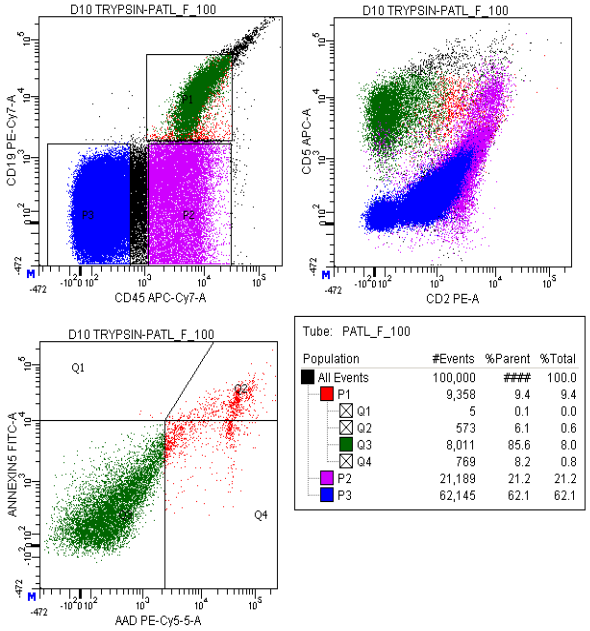


Figure 4.4 Day 10 CLL cells with fibroblast co-culture.

Plot 1 shows the overgrowth of CD45-ve fibroblasts which represent 62% of total events on day 10. The initial seeding ratio was 1:100 of fibroblasts to MNC.

To prevent the overgrowth of support cells mitomycin-c treated stromal cells were used and viability of the CLL cells was assessed as shown in Figure 4.5. These experiments demonstrated that excess of stromal cells either added upfront or by rapid growth when not treated by mitomycin-c, will deprive the medium of essential nutrients and thereby reduce the viability of the CLL cells.

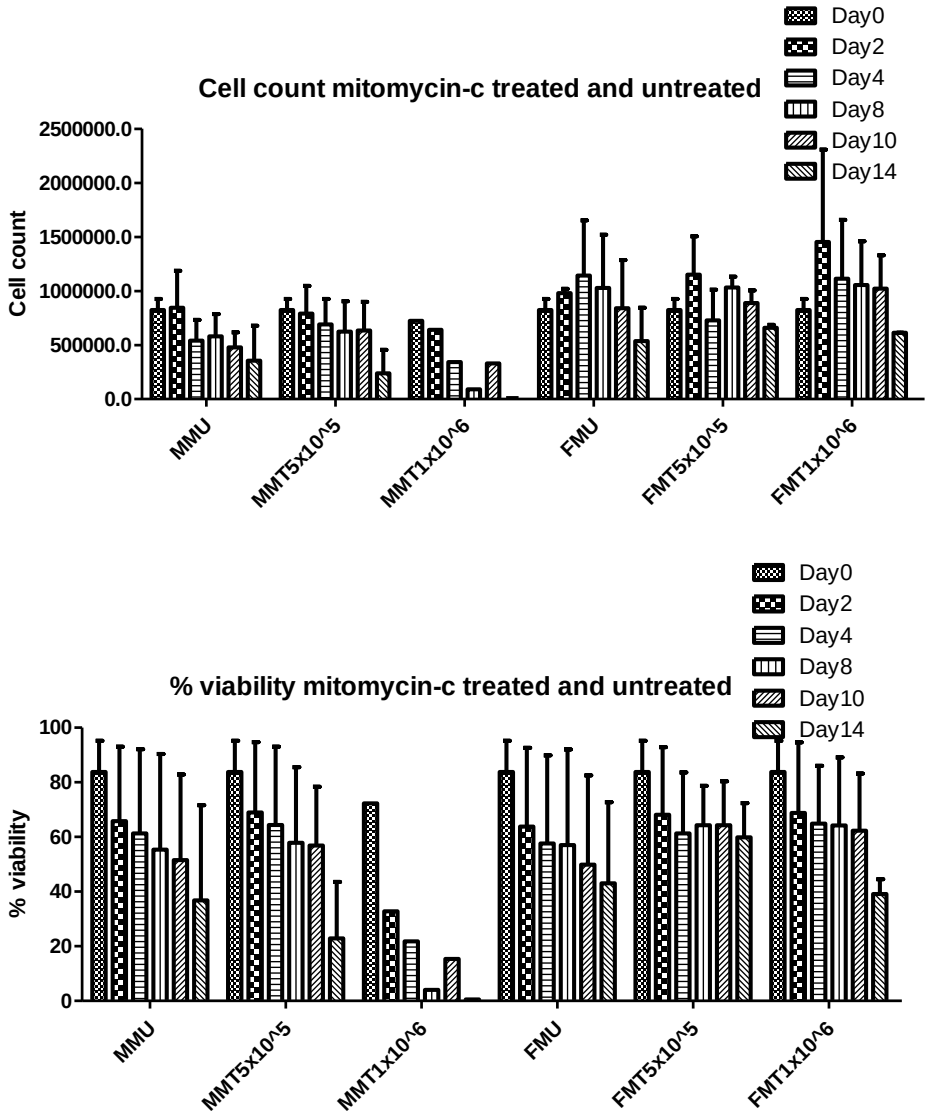


Figure 4.5 Coculture with mitomycin-c treated and untreated stromal cells.

MNC were co-cultured with untreated and mitomycin-c treated cells in two concentrations for 14 days and viability of the CLL cells were assessed at fixed time points. Absolute cell count was shown in the top plot and % viability is shown in the bottom plot. MNC were co-cultured with both CD40L fibroblasts and M210B4 bone marrow stromal cells. (MMU =Untreated M210B4 cells, MMT =Mitomycin-c treated M210B4 cells, FMU= Untreated CD40L fibroblasts, FMT= mitomycin-c treated CD40L fibroblasts) (n=2).

In the next experiment the viability of negatively selected CLL cells were compared with viability of CLL cells cultured as MNC in stromal layer co-cultures. This experiment showed that the viability of CLL cells is significantly better when cultured as MNC rather than negatively selected CLL cells. This may be due to the presence of other supporting cells like T-cells and monocytes, which can develop into nurse like cells (NLC) (Figure 4.6). This concept was previously demonstrated by other groups (Burger et al., 2000) NLCs are derived in-vitro from CD14+ peripheral blood mononuclear cells of CLL patients which form large, round, adherent cells that assist in the survival of CLL cells.

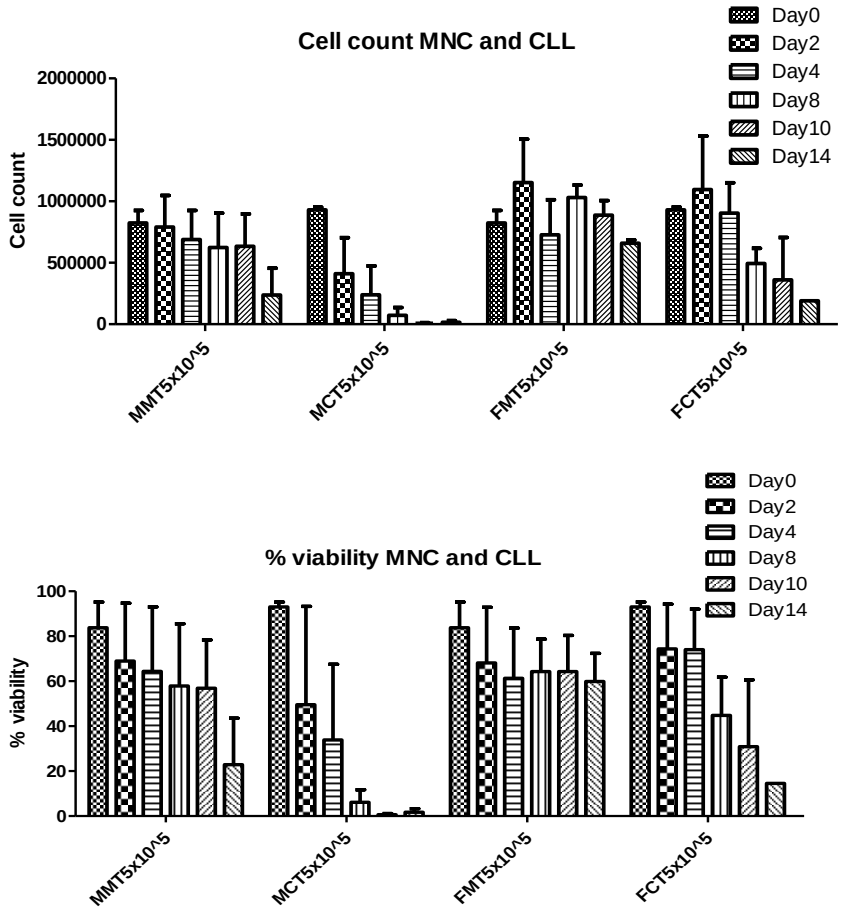


Figure 4.6 Co-culture with mitomycin-c treated cells

MNC and negatively selected CLL cells were co-cultured with mitomycin-c treated cells for 14 days and viability of the CLL cells were assessed at fixed time points. Absolute cell count is shown in the top plot and % viability in the bottom plot. MNC and CLL cells were co-cultured with both CD40L fibroblasts and M210B4 cells. (MMT =MNC in Mitomycin-c treated M210B4 cells, MCT= CLL cells in Mitomycin-c treated M210B4 cells, FMT= MNC in mitomycin-c treated CD40L fibroblasts FCT=CLL cells in mitomycin-c treated CD40L fibroblasts) (n=2).

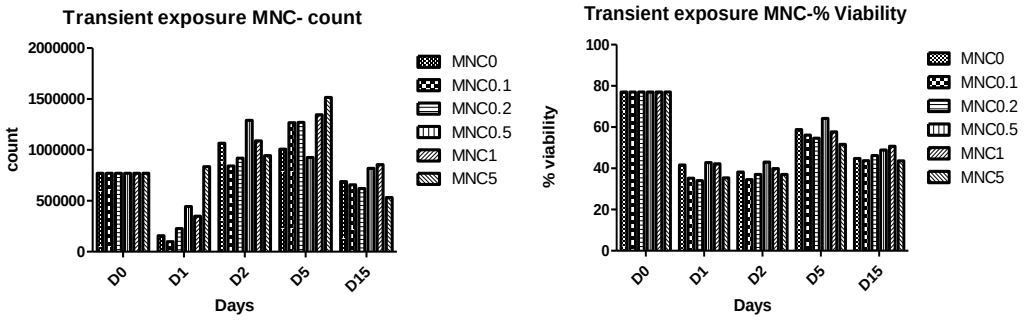
4.3 Comparison of Media

Previously it was shown that AIM-V medium is better in maintaining CLL cell viability than RPMI or IMDM (Levesque et al., 2001). So this medium was tested using cells derived from a single patient. There was some improvement in viability in the AIM-V medium compared to IMDM (Appendix 4) (Levesque et al., 2001). However, there was no evidence in the literature that this medium could be effectively used to maintain the stromal cells in these assays. As stromal cell support is essential for future experiments this method was not further explored.

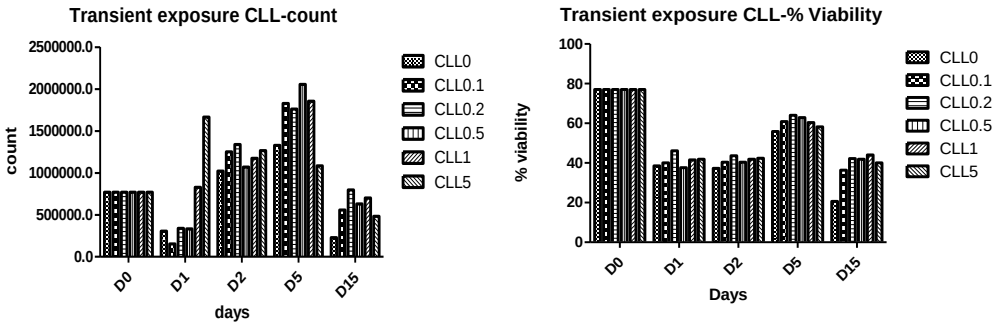
4.4 Titration of DMSO concentration

Standard chemotherapeutic agents were tested next. Most chemotherapeutic agents were dissolved in DMSO. Therefore a titration of the vehicle was performed to assess the safest concentration of DMSO that will not affect the viability of CLL cells and stromal supporting layer. Two types of exposures to DMSO were done in the first experiment which included exposure of MNC and CLL cells to different concentrations of DMSO for 2 hours followed by washing and re-seeding on CD40L fibroblasts or alternatively MNC and CLL cells were seeded on CD40L Fibroblast and DMSO added to the medium at different concentrations (Figure 4.7).

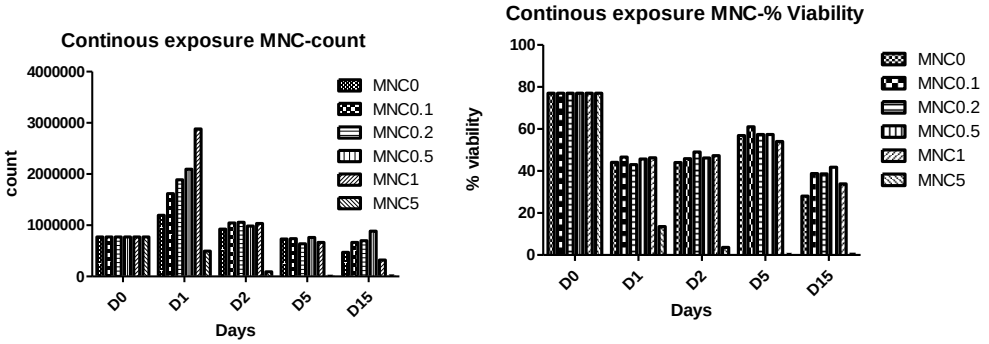
A



B



C



D

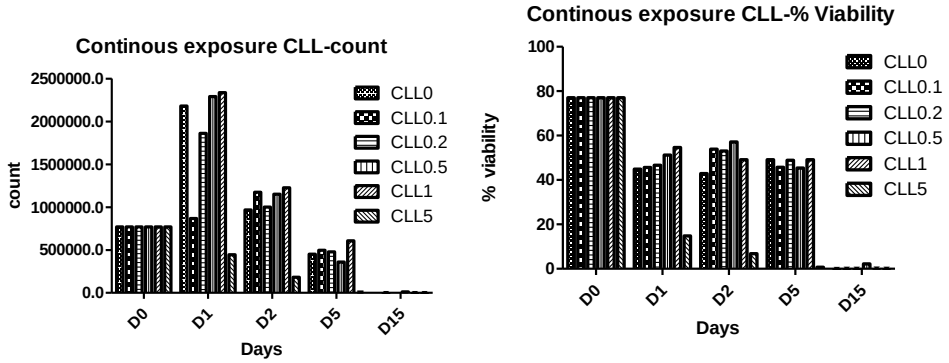


Figure 4.7 Titration of DMSO concentration.

DMSO was exposed continuously (C and D) or transiently (A and B) for 2 hours following which DMSO is thoroughly washed off and then the CLL cells are seeded on to the stromal cells. The concentration of DMSO exposure is from 0.1µM to 5 µM (number at the end) as shown in the Figure. Both MNC (A and C) and negatively selected CLL cells (B and D) were exposed separately. Viability was assessed at various time points. In transient exposure there is no difference between different concentrations of DMSO but in continuous exposure cells tolerate doses up to 1 µM without significant difference in viability.

This experiment suggests that up to 1 µM DMSO is safe to the CLL cells even if there is a continuous exposure, beyond that it will affect the viability of CLL cells. But for most of the drug dilutions the cells will be exposed to a much lower concentration of DMSO.

Further evaluation on 3 samples was performed to see the consistency of the effect. This experiment suggests that 1 µM may have some effect on the viability of CLL cells but not below 0.5 µM. Most experiments with drugs use DMSO at a final concentration of less than 0.1 µM which will not adversely affect CLL viability according to this experiment. (Figure 4.8)

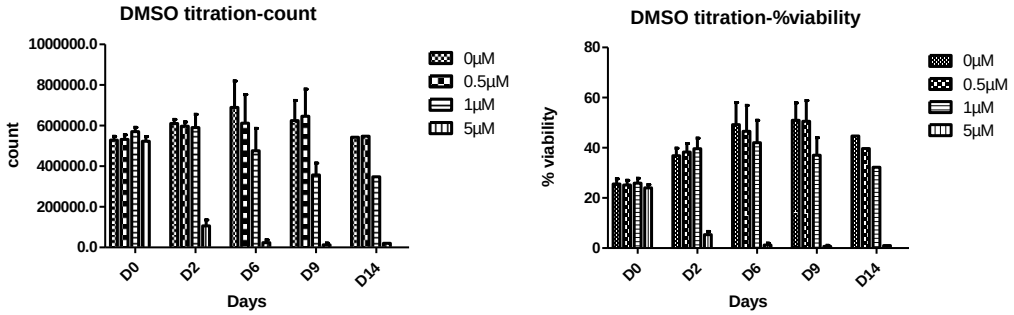


Figure 4.8 Titration of DMSO concentration.

MNCs were exposed continuously to DMSO at various concentrations on 3 samples. The concentration of DMSO exposure is from 0.5 μM to 5 μM as shown in the Figure. Viability was assessed at various time points. Cells tolerate doses up to 0.5 μM without significant difference in viability.

The consistency of the counting by flow cytometry using the counting beads was assessed on day 6 by repeating the count on same sample multiple times (Figure 4.9). Standard error was minimal confirming the consistency; therefore this procedure was subsequently used in all experiments requiring enumeration of cells.

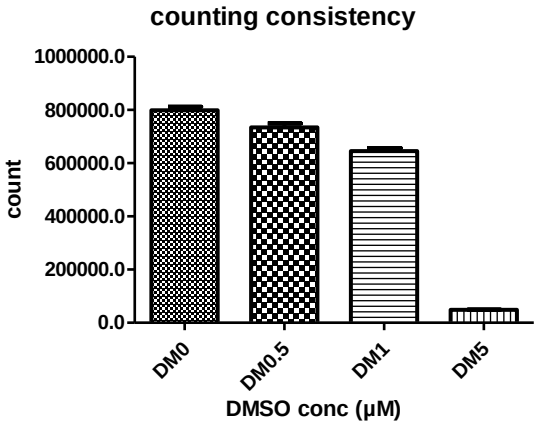


Figure 4.9 Consistency of bead counting.

The same sample was counted multiple times (n=4) to assess the consistency of bead counting.

4.5 Evaluation of protocols to generate mitotically inactive support cells

Mitomycin-c treatment is very effective in terms of stopping proliferation, but it can also affect viability. Therefore cell number and viability after mitomycin-c

treatment were evaluated over one week of culture. Although, cell numbers declined, there were reasonable numbers of viable cells present even after a week, to support the CLL cells (Figures 4.10 and 4.11).

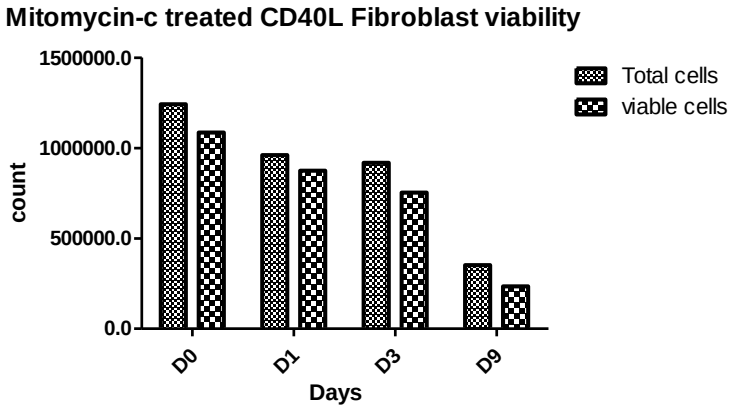


Figure 4.10 Absolute number of mitomycin-c treated CD40L fibroblasts.

Absolute count of total and viable CD40L fibroblasts decline after mitomycin-c treatment determined by flow cytometry.

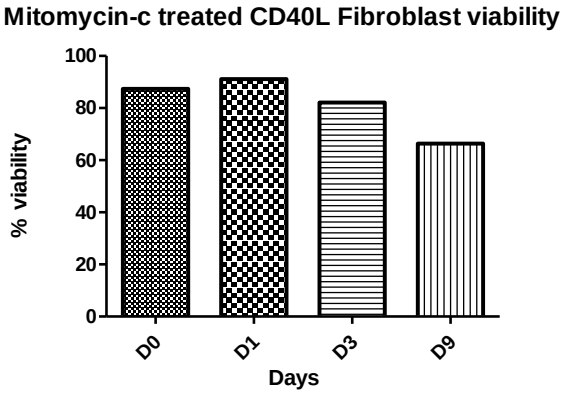


Figure 4.11 Viability of CD40L fibroblasts

The percentage viability was determinted assessed using annexin-v/7-AAD over a 9-day period after mitomycin-c treatment.

The main disadvantage for mitomycin-c treatment is that fresh cells have to be prepared for each experiment. Irradiated fibroblasts can be frozen in DMSO and can be used without loss of efficacy. So irradiated fibroblasts were compared to mitomycin-c treated fibroblasts as stromal support for maintaining CLL cell viability. The experiment demonstrates that both methods are equally efficient in maintaining CLL cell viability at a ratio of 1:10 fibroblasts to MNCs (Figure 4.12).

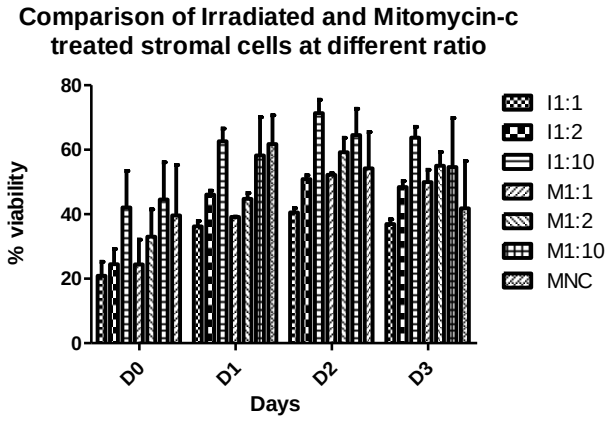


Figure 4.12 Comparison of irradiated and mitomycin-c treated CD40L fibroroblasts as stromal support for maintaining CLL cell viability.

The percentage viable cells was determined by flow cytometry at 4 time points during in vitro culture of CLL cells with fibroblasts (n=2). The fibroblasts were either irradiated (I) or treated with Mitomycin-c (M). There was no significant difference between the two methods as far as the ratio of 1:10 fibroblasts to MNCs is maintained.

From the above experiments it was concluded that CLL cells could be kept viable for more than 48 hours if cultured in supportive media like IMDM or RPMI. However, the viability could be significantly prolonged if co-cultured with CD40L expressing fibroblasts or M210B4 cells which are inactivated by mitomycin-c or irradiation, consistent with previously published results (Lagneaux et al., 1998) (Panayiotidis et al., 1996) (Burger et al., 2000). The viability of CLL cells improved if cultured as MNC rather than negatively selected CLL cells, suggesting that other populations such as monocytes, could be providing pro-survival signals (Nishio et al., 2005). The in-vitro system developed was suitable to test various drugs and agents that could affect the pathways of antigens identified in the previous chapter.

4.6 Drug exposure

To determine the suitability of the system to test new drugs, standard chemotherapeutic agents were tested first. Previous studies have reported effects of standard chemotherapeutic agents in similar system (Klein et al., 2000) (Kurtova et al., 2009). 5-fludarabine-monophosphate (5-FMP), the active compound of fludarabine, doxorubicin and chlorambucil were tested in the current system. The viability of the fibroblasts was tested sequentially to assess the effect of 5-FMP on fibroblasts. 70% of fibroblasts retain viability on day 7 as shown in Figure 4.13 confirming that 5-FMP has no significant lethal

effect on fibroblasts and can be used in the co-culture setting without substantially affecting the stromal support.

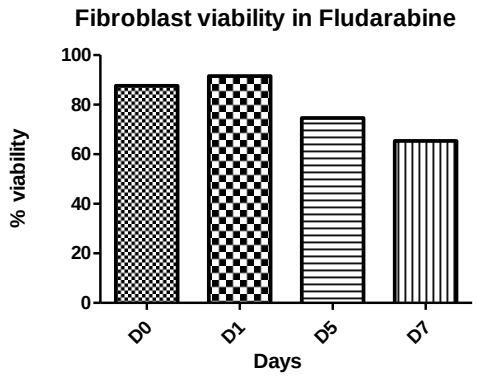
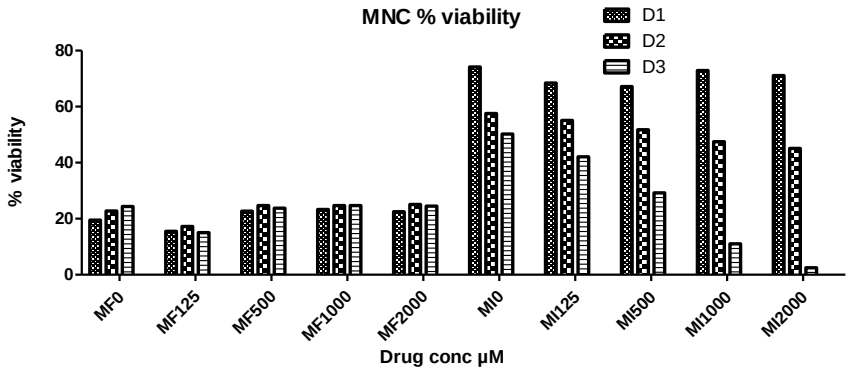


Figure 4.13 Effect of 5-FMP on fibroblast viability.

Fibroblast viability was assessed using annexin-v/7-AAD on day 0,1,5 and 7. There was only around 10% viability loss even after a week of incubation with 5-FMP.

4.6.1. The effect of fludarabine on CLL cell viability with and without fibroblasts

MNC or negatively selected CLL cells were incubated with or without fibroblasts in varying concentrations of 5-FMP. Percentage viability and cell counts were determined at 24, 48 and 72 hours as shown in Figures 4.14 and 4.15. Regardless of the purification procedure employed, there was a significant drop in viability and cell count in stroma free wells while even after 72 hours there was no consistent drop in viability or cell count in stroma supported wells.



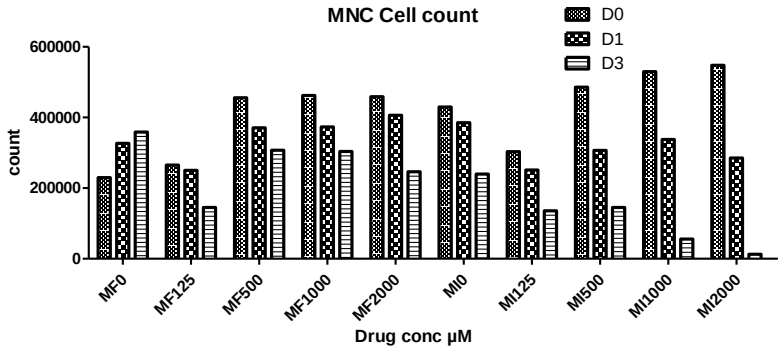


Figure 4.14 Effect of 5-FMP on CLL cells in presence or absence of stromal cells when seeded as MNC.

MNCs were incubated with or without fibroblasts in concentrations of 5-FMP varying from 125 µM to 2000 µM. Percentage viability (top figure) and cell counts (bottom figure) are shown in the above chart. (MF = MNC in fibroblast, MI= MNC without stroma, numbers are the concentration of 5-FMP in µM).

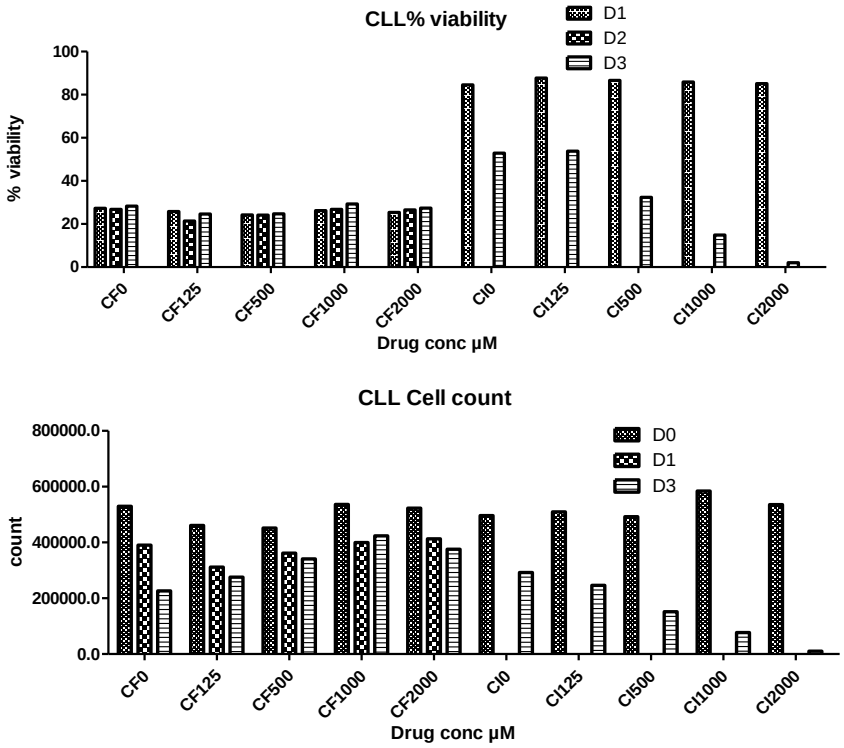


Figure 4.15 Effect of 5-FMP on CLL cells in presence or absence of stromal cells when seeded as negatively selected CLL cells.

Negatively selected CLL cells were incubated with or without fibroblasts in concentrations of 5-FMP varying from 125 µM to 2000 µM. Percentage viability (top figure) and cell counts (bottom figure) are shown in the above chart. (CF = negatively selected CLL cells in fibroblasts, CI= negatively selected CLL cells without stroma, numbers are the concentration of 5-FMP in µM).

4.6.2. Continuous and transient exposure to drugs with or without fibroblasts

In an *in vivo* setting, different populations of cells may experience different levels of exposure to drugs. To mimic this *in vitro*, 5-FMP, chlorambucil and doxorubicin were tested in varying concentrations. Drugs were exposed either transiently or continuously. In transient exposure the cells were incubated in presence of drugs for 2 hours, following which cells were washed twice and then plated in fresh medium. Viability was assessed at sequential time points, 5-FMP up to 7 days, chlorambucil and doxorubicin up to 3 days (Figures 4.16, 4.17, 4.18). Experiments with all three drugs have demonstrated that viability of CLL cells was reduced if exposed to drugs continuously. Stromal cells protected the cells from chemotherapy induced apoptotic death.

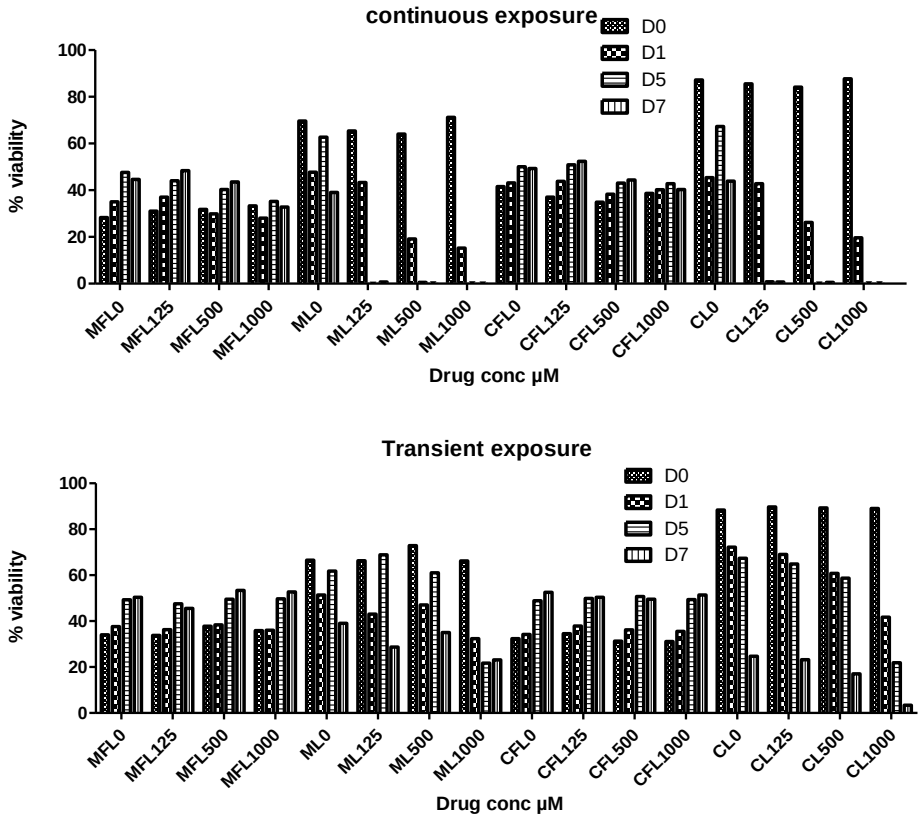


Figure 4.16 Continuous and transient exposure to 5-FMP with or without fibroblasts.

In transient exposure the cells were incubated in presence of 5-FMP for 2 hours, following which cells were washed twice and then plated in fresh medium. Viability was assessed on day 0, 1, 5 and 7 (MFL=MNC in fibroblast exposed to 5-FMP, ML= MNC exposed to 5-FMP, CFL= CLL in fibroblast exposed to 5-FMP, CL= CLL exposed to 5-FMP).

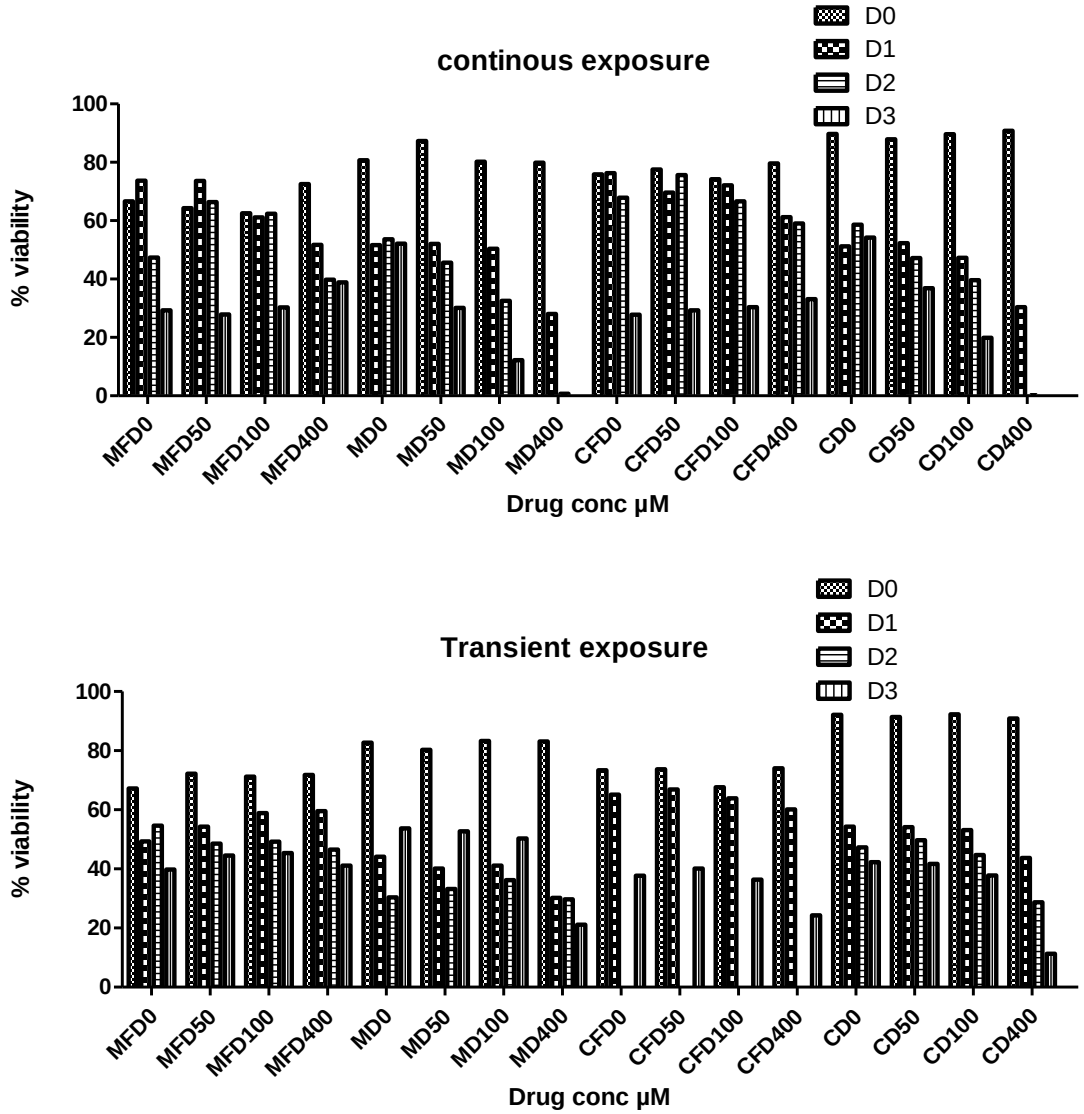


Figure 4.17 Continuous and transient exposure to doxorubicin with or without fibroblasts.

In transient exposure the cells were incubated in presence of doxorubicin for 2 hours, following which cells were washed twice and then plated in fresh medium. Viability was assessed on day 0, 1, 2 and 3. (MFD= MNC in fibroblast exposed to doxorubicin, MD= MNC exposed to doxorubicin, CFD= CLL in fibroblast exposed to doxorubicin, CD= CLL exposed to doxorubicin).

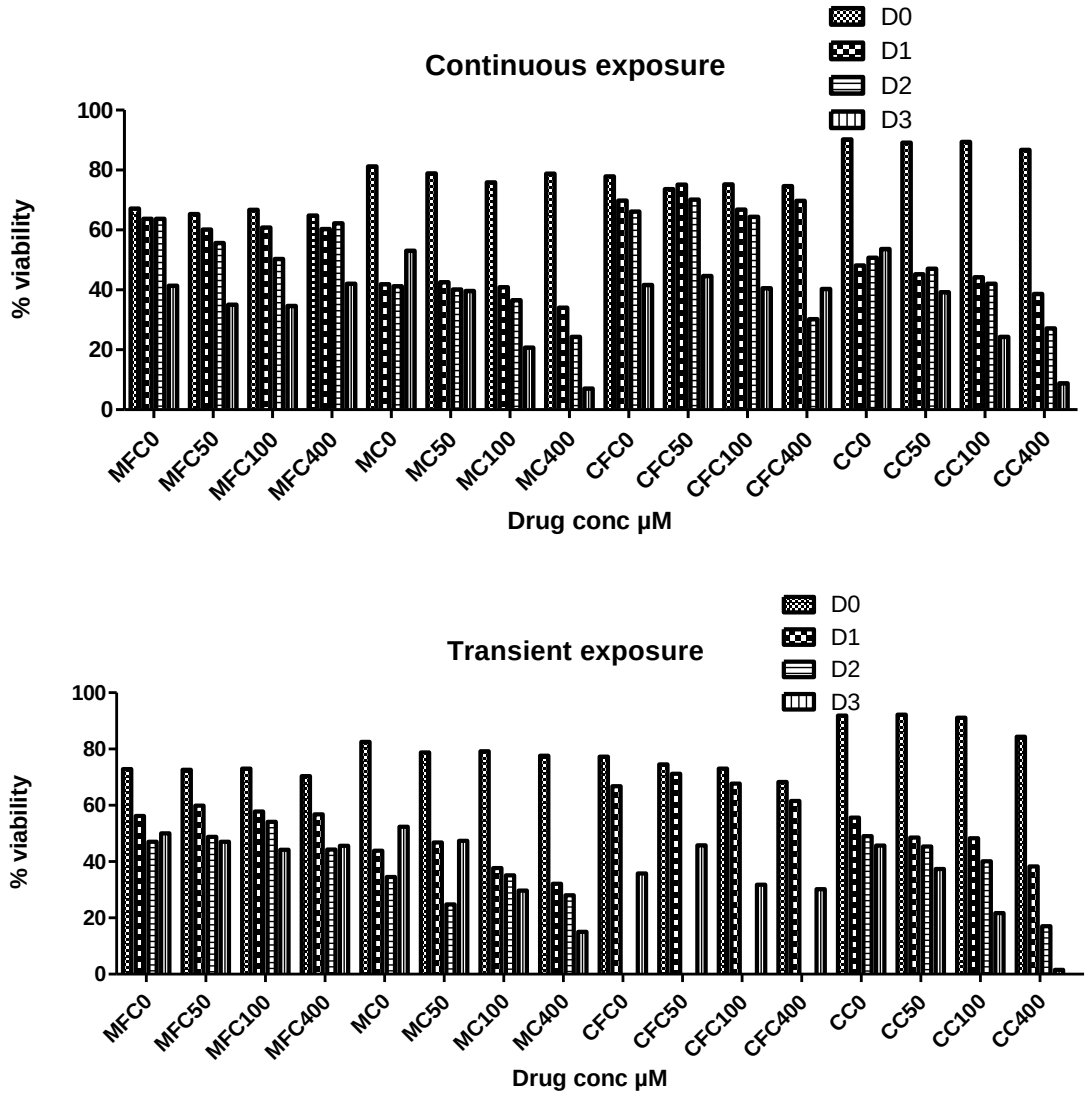


Figure 4.18 Continuous and transient exposure to chlorambucil with or without fibroblasts.

In transient exposure the cells were incubated in presence of chlorambucil for 2 hours, following which cells were washed twice and then plated in fresh medium. Viability was assessed on day 0, 1, 2 and 3. (MFC= MNC in fibroblast exposed to chlorambucil, MC= MNC exposed to chlorambucil, CFC= CLL in fibroblast exposed to chlorambucil, CC= CLL exposed to chlorambucil).

4.7 Antibody mediated cytotoxicity assessment

Monoclonal antibodies like alemtuzumab, ofatumumab and GA101 on their own or in combination with chemotherapy like rituximab are highly effective clinically in treating CLL (Ferrajoli et al., 2003) (Hillmen et al., 2007) (Wierda et al., 2010) (Morschhauser et al., 2009) (Hallek et al., 2010b). At least three

different mechanisms have been proposed for the action of these drugs including antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) and induction of programmed cell death (Nückel et al., 2005) (Zent et al., 2008) (Stanglmaier et al., 2004) (Maloney et al., 2002) (Byrd et al., 2002). One study has shown that the programmed cell death is mediated by a caspase independent apoptotic pathway (Stanglmaier et al., 2004). A later study has shown that rituximab has no *in vitro* activity on CLL cells but alemtuzumab induces 67% (range 15-100%) rapid (at 1 hour) complement dependent cytotoxicity (Zent et al., 2008).

The current *in vitro* system needed optimisation for testing antibodies. Complement dependent cytotoxicity was assessed for alemtuzumab and rituximab. In the initial experiment MNCs were exposed to these antibodies for 30 minutes and the remaining antibodies were washed off before plating into the culture media. Viability was assessed after 48 hours. There was no significant cytotoxicity observed with either rituximab or alemtuzumab (Figure 4.19).

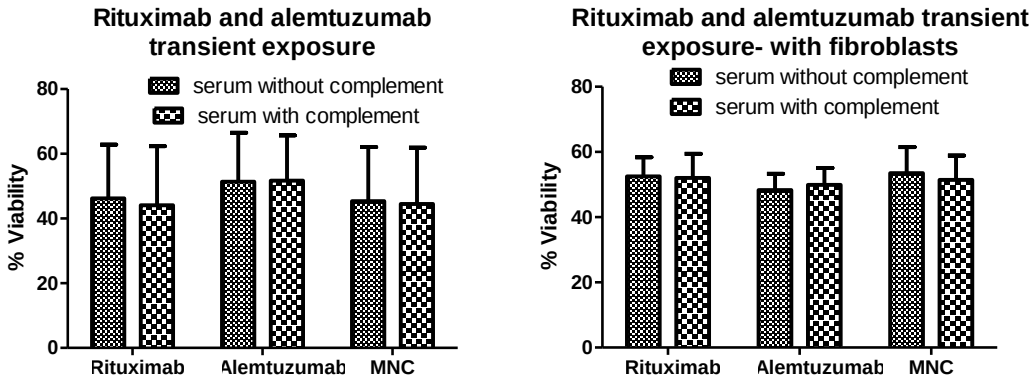


Figure 4.19. Assessment of antibody mediated cytotoxicity with transient exposure to rituximab and alemtuzumab.

MNC were exposed to antibodies for 30 minutes and then washed off. Cells were then plated in the presence or absence of CD40L fibroblasts. Complement activity was added to corresponding wells by adding patients' own fresh serum. No cytotoxicity was observed with either rituximab or alemtuzumab in 48 hours viability assessment (n=3).

The most obvious explanation for this is inadequate exposure duration to the antibodies. Antigen and antibody are usually held by weak forces and when diluted in fresh medium for washing, antibodies get detached. Therefore in the subsequent experiment antibodies were added directly into the culture system. Both rituximab and alemtuzumab were added at 2 different

concentrations, 5µg/ml and 50µg/ml. MNCs were cultured with or without the presence of stromal cells. Complement activity was supplemented by adding patients' own serum into the medium. Alemtuzumab induced cell death even at lower concentration despite the presence of stromal cells (Figure 4.20).

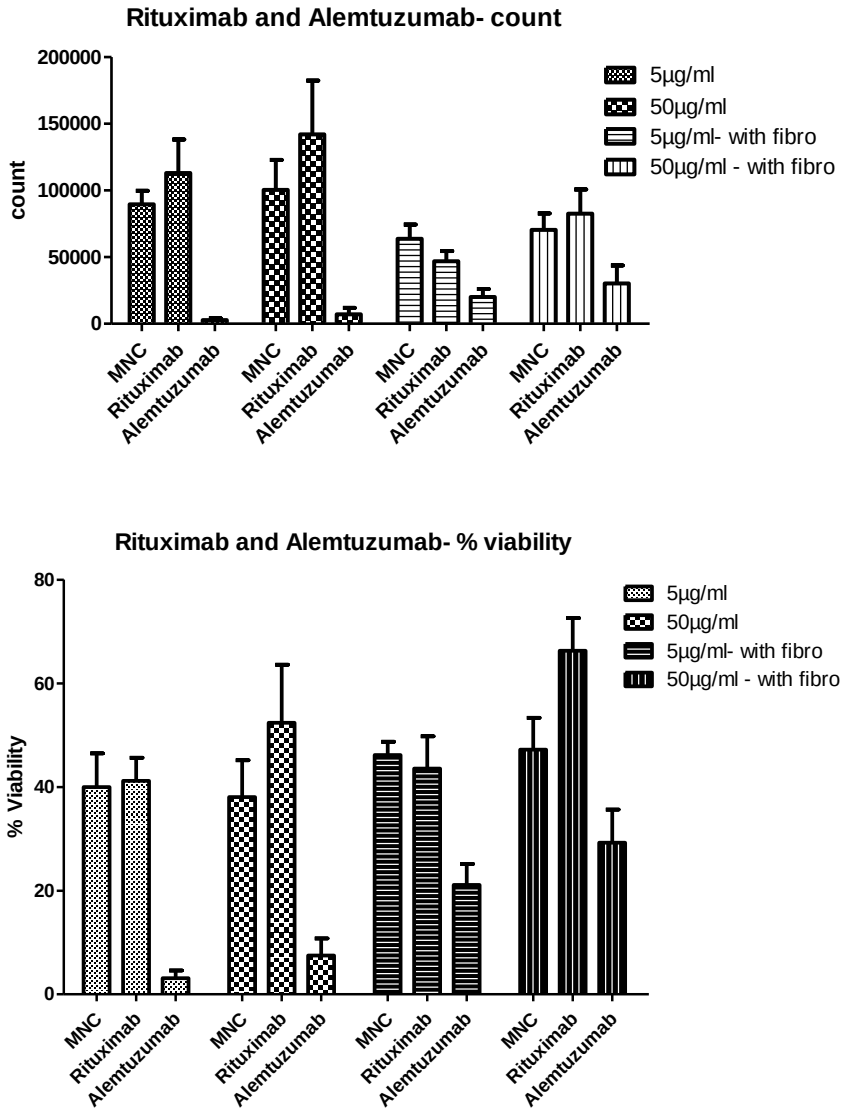


Figure 4.20 Assessment of antibody mediated cytotoxicity with continuous exposure to rituximab and alemtuzumab

MNC were incubated with antibodies at two different concentrations, 5µg/ml and 50µg/ml,in the presence or absence of CD40L fibroblasts. Complement activity was provided in appropriate wells by adding patients' own fresh serum. Alemtuzumab as opposed to rituximab induced cytotoxicity both in presence and absence of stroma at 48 hours viability assessment (n=3).

4.8 BCR Stimulation

Antigenic stimulation through the BCR receptor has given conflicting results in the *in vitro* systems (Petlickovski et al., 2005) (Bernal et al., 2001a). Whole goat anti-IgM bound to Dynabeads has been shown to increase cell survival and suppress fludarabine-induced apoptosis while soluble anti-IgM promoted apoptosis in responsive samples (Petlickovski et al., 2005). But there are studies which have shown that soluble antibody in the form of $F(ab)_2$ portion of the antibody, will induce pro-survival signals (Bernal et al., 2001a). Studies are lacking in which anti-IgD is used as the antigenic stimulant. But there are previous studies which has shown that anti-IgD is a better stimulant for inducing calcium flux in the CLL cells than $F(ab)_2$ portion of anti-IgM (Mockridge et al., 2007).

The *in vitro* system developed above was used to test these agents. $F(ab)_2$ portions of goat anti-human IgM or IgD were used as the antigenic stimulant.

Stimulation with anti-IgM showed significant improvement in percentage viability and cell count as shown in Figure 4.21. Initial results with anti-IgD have shown the opposite effect. There was significant apoptosis in 48 hours viability assessment as shown in Figure 4.22. Careful examination of the reagents has shown that boric acid was used as preservative in the anti-IgD while the anti-IgM had no preservatives in it. Boric acid is known to cause cell death (Barranco and Eckhert, 2004). Anti-IgD was therefore dialysed into preservative free medium. Using the boric-acid free anti-IgD improved the cell count and percentage viability similar to the results obtained with anti-IgM stimulation (Figure 4.23). CLL cells predominantly express IgM and IgD and do not express class-switched immunoglobulin. To explore the possibility that there were differences in the ability of class switched Ig improve viability, the experiment was extended to include stimulation with anti-IgG (Figure 4.23). This experiment demonstrates that pro-survival signal can be delivered by both IgM and IgD stimulation but not IgG.

Differences in levels of surface immunoglobulin could impact on the ability to transmit a pro-survival signal. With BCR stimulation there was no significant difference in viability or cell count between IgM expressing patient samples and those who have not expressed IgM significantly (Figure 4.24). The IgM expression was determined on these patients on routine laboratory assay and the data was taken from the records. The cut off used to determine whether IgM was expressed or not was based on isotype control. This may explain the above observation of not seeing any difference in viability between IgM

positive and negative patient samples. In patients without significant expression of IgM there may be minimal expression which is enough for BCR stimulation.

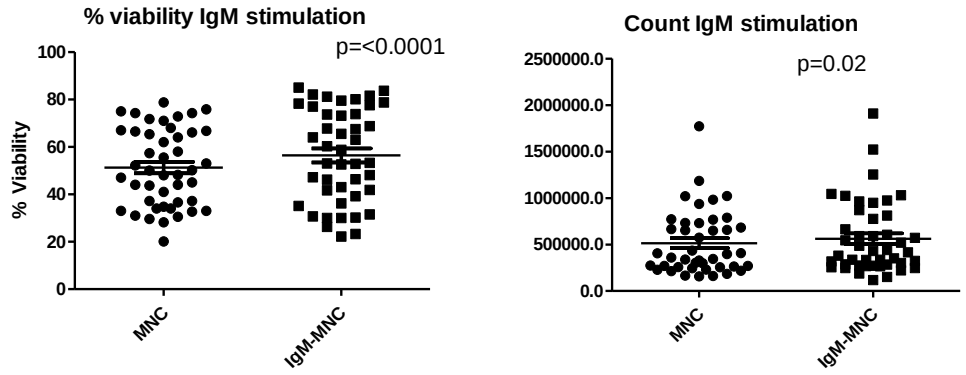


Figure 4.21 Stimulation of BCR with $F(ab)_2$ portion of anti-IgM MNC were incubated with 10µg/ml of $F(ab)_2$ portion of anti-IgM.

Viability and count assessed at 48 hours (n=35). Significance was tested by paired t-test. BCR stimulation with $F(ab)_2$ portion of anti-IgM significantly improved percentage viability and cell count.

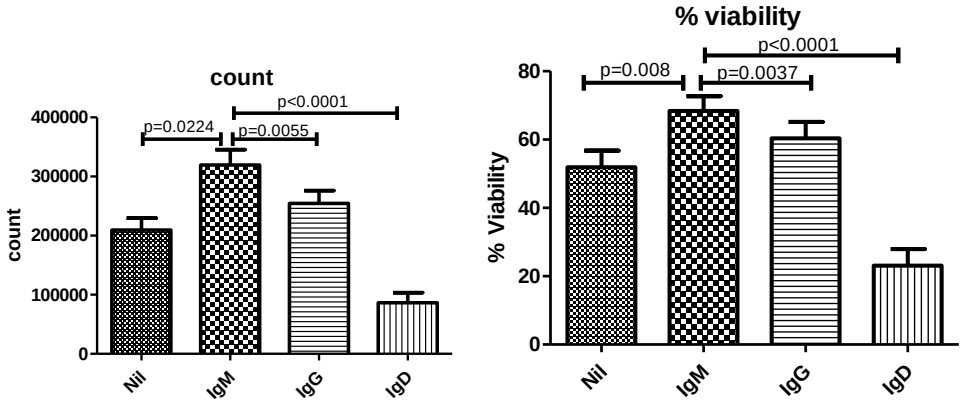


Figure 4.22 Comparison of stimulation with $F(ab)_2$ portion of IgM, IgD and IgG.

MNC were incubated with 10µg/ml of antibodies. Viability and count were assessed at 48 hours.

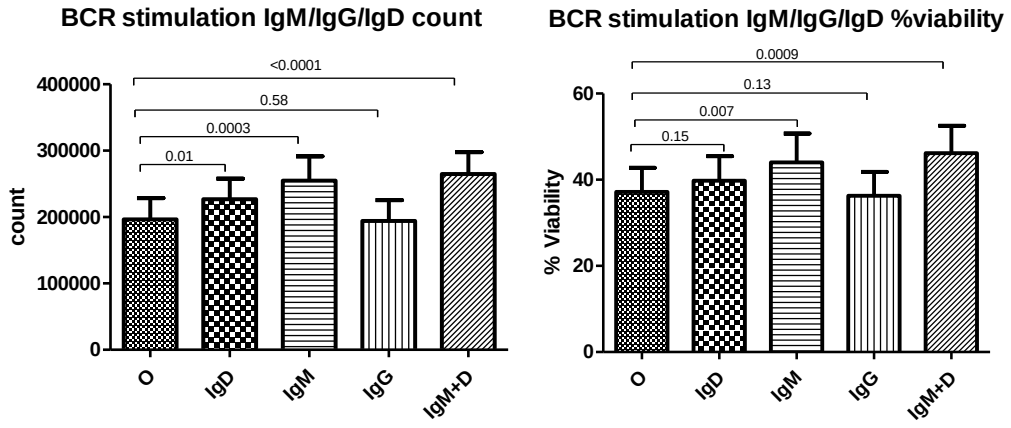


Figure 4.23 Comparison of stimulation with F(ab)$_2$ portion of IgM, IgD and IgG.

MNC were incubated with 10µg/ml of antibodies. Viability and count were assessed at 48 hours and significance calculated using paired t-test. IgM and IgD showed increased viability but IgG shows no difference in this experiment. IgD was dialysed to preservative free buffer.

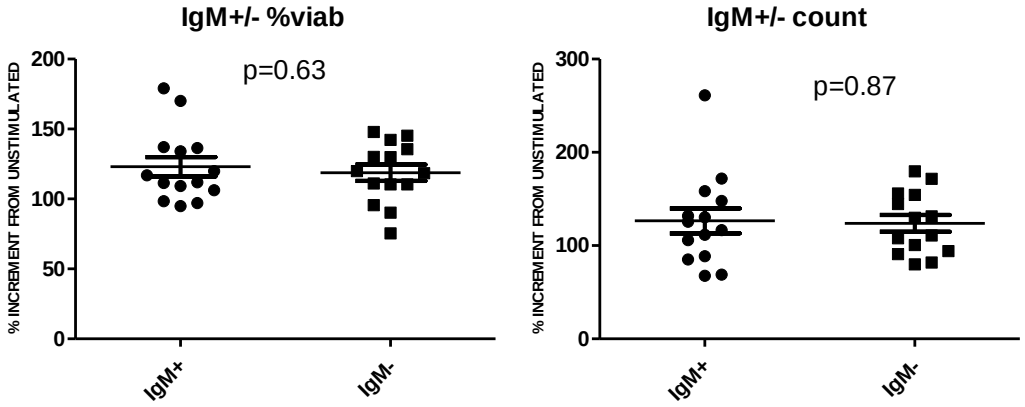


Figure 4.24 Difference in viability between patients with significant IgM expression or not.

Percentage increment in viability and cell count from unstimulated sample is shown in y axis. Significance was calculated using unpaired t-test.

The difference in viability between mutated and unmutated patients was also not significant as shown in Figure 4.25. The mutational status was determined on these patients on routine laboratory assay and the data was taken from the records. So although there is evidence to suggest that mutated versus unmutated CLL may have different signalling properties (Mockridge et al., 2007), these results suggest that the differences were not sufficient to manifest large changes in viability in the *in vitro* setting.

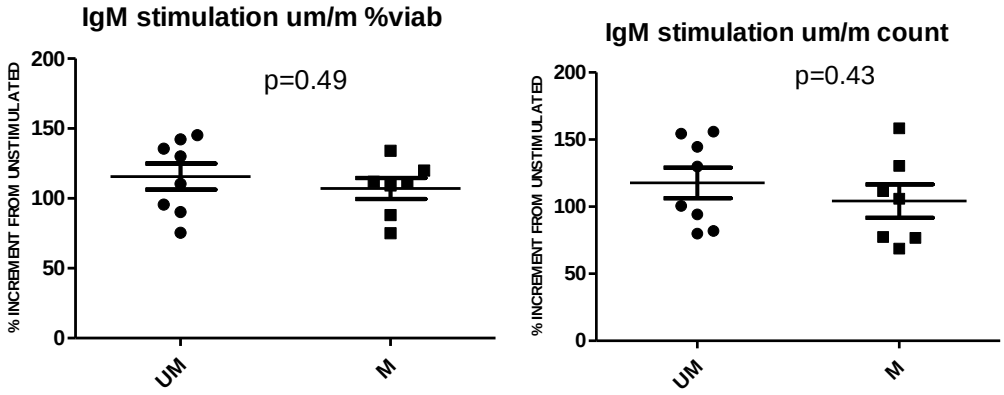


Figure 4.25 Difference in viability between mutated and unmutated patients.

Percentage increment in viability and cell count from unstimulated samples is shown in the y-axis. Significance was calculated using an unpaired t-test.

4.8.1. Single and multiple exposures to BCR Stimulus

To test the postulate that CLL cells maintain their viability *in vivo* by getting exposed to antigenic stimulus repeatedly in the proliferation centres, single and multiple exposures to BCR stimulus was tested. With BCR stimulation cells maintain good viability up to 10 days even with a single antigenic stimulation at the beginning. In contrast there is no role for repeated stimulus *in vitro* (Figure 4.26).

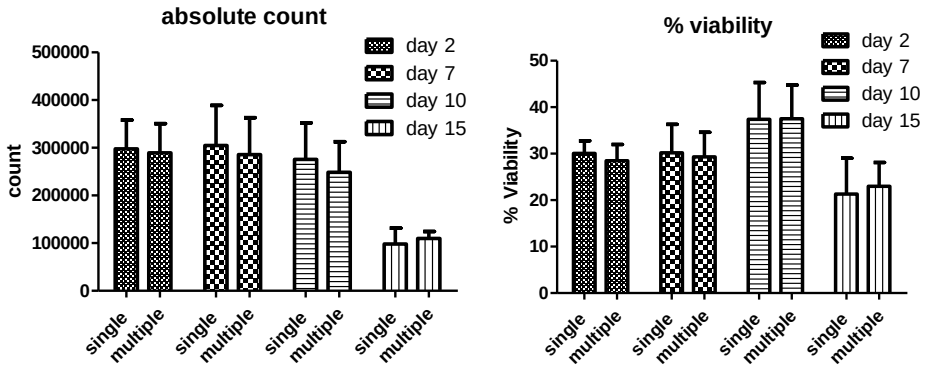


Figure 4.26 Single and multiple exposure to anti-IgM F(ab)$_2$.

In single exposure 10µg/ml of anti-IgM was added once when the cells were plated. In multiple exposure anti-IgM was added every 24 hours until the wells were harvested for testing viability.

4.9 Discussion

The above experiments demonstrate the development of a robust *in vitro* system for testing various new drugs in CLL. MNC or negatively selected CLL cells can maintain good viability for up to 48 hours in IMDM, RPMI or AIM-V medium and seeding as MNC is better to maintain the viability than seeding as negatively selected CLL cells. This is due to the presence of other supporting cells like monocytes and T-cells which is previously published (Burger et al., 2000) (Ghia et al., 2002). Monocytes may form nurse like cells as shown by Burger et al., but this is not formally tested in this experiment. Moreover Seiffert et al have shown that monocytes help in the survival of CLL cells by secreting soluble CD14 which in turn activate NFκB (Seiffert et al., 2010). This duration is adequate to test the relative viability of the cells in presence of drugs compared to the control sample. Stromal support with either CD40L fibroblasts or M210B4 cells will prolong the duration of viability beyond 2 weeks. This will also confer a drug resistance environment for CLL cells when treatment with conventional chemotherapeutic drugs. These observations are also consistent with literature (Lagneaux et al., 1998) (Panayiotidis et al., 1996) (Nwabo Kamdje et al., 2012). Lagneaux et al. showed that bone marrow derived stromal cells reduces the apoptosis of CLL cells in a contact dependent manner through adhesion mediated by β1 and β2 integrins. In the paper by Panayiotidis et al., both spontaneous and hydrocortisone induced apoptosis of CLL cells were significantly reduced by contact with bone marrow derived stromal cells. Nwabo Kamdje et al in their paper demonstrated that bone marrow derived mesenchymal stromal cells protected CLL cells from spontaneous apoptosis and that induced by various drugs like fludarabine, cyclophosphamide, bendamustine, prednisone and hydrocortisone. They have also shown that this protective effect was aborted by day 3 by a combination of anti-Notch-1, Notch-2 and Notch-4 antibodies or γ-secretase inhibitor XII even in presence of the drugs, suggesting the role for Notch signalling in CLL cell survival and chemo-resistance. BCR stimulation with $F(ab)_2$ anti-IgM or IgD also support CLL cell survival but not anti-IgG as shown in previous studies (Bernal et al., 2001b). Study by Bernal et al. showed that anti-IgM $F(ab)_2$ reduced the spontaneous apoptosis rate from 41% to 3.8% as against a polyspecific goat $F(ab)_2$ fragments (36%). The antigenic stimulation system may be useful for testing the BCR kinase inhibitors in CLL and provide the most physiologically relevant model.

5. Development of phosflow and calcium flux assays to assess physiological pathways of survival signal inhibition

As discussed in the introductory chapter the B-cell receptor (BCR) is essential for survival and functioning of normal peripheral B-cells. There is convincing evidence to suggest that it has a role in survival and growth of malignancies derived from mature B-cells. This is true in the case of CLL also even though the levels of surface immunoglobulin (sIg) are low compared to normal B lymphocytes and to other B-cell malignancies.

Following engagement of antigen, B-cell receptor complex aggregates leading to phosphorylation of several downstream Src-family tyrosine kinases (Woyach et al., 2012). Of these phosphorylation of SYK is an important early component, which then activates intracellular signalling cascades. Phosphorylation of SYK therefore is an indicator of the proximal events happening in the membrane which depend on the structural integrity and oligomeric form of the BCR. There is evidence that response to ligation of sIgM in CLL varies between the subsets of CLL with an increased tendency for unmutated CLL to phosphorylate SYK (Lanham et al., 2003) (Chen et al., 2002). Furthermore, data suggests that mutated CLL which fail to signal through sIgM can instead utilise sIgD to transmit intracellular signals (Lanham et al., 2003). Phosphorylation of ZAP70 with recruitment to the BCR has also been observed as a parallel pathway (Chen et al., 2002).

The downstream phosphorylation pathway triggers phospholipase C γ (PLCγ), an enzyme that hydrolyses polyphosphoinositide, producing inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C, and IP3 releases calcium from the endoplasmic reticulum and the extracellular compartment (Roos et al., 2005). Calcium release directly activates a number of transcription factors, including NFκB, Jun, and nuclear factor of activated T cells (NFAT) which thereby transmit a survival signal to the nucleus of the cell (Yarilina et al., 2011). As phosphorylation and the rise in calcium are key events in the response to ligation of the BCR in CLL, and this correlates with the downstream events, measuring these early events will provide evidence of the physiological responses the cells undergo when triggered by a survival signal. This can also be used to measure the lack of response in cells when these pathways are inhibited by specific blockers.

5.1 Calcium Flux

Calcium flux can be monitored by several techniques based on various factors, such as the cell type, nature of the receptor, the stimulus required, sensitivity of the measurement and availability of the instruments. Techniques using flow cytometry have an advantage of testing relatively large numbers of cells and sequential samples when they are in cell suspension. If multiparameter flow cytometry is used calcium flux studies can be combined with cell markers to permit sub-population analysis and the sorting of events of interest (Burchiel et al., 2000).

The basic principle of Ca^{2+} flux measurement by flow cytometry is based on changes in fluorescence intensity or emission wavelength of a fluorophore following chelation of calcium ions. This is plotted as fluorescence intensity against time. Single dye fluorescence difference can be used to measure calcium flux but there are several variables such as photo-bleaching, leakage, uneven loading, and varying cell thicknesses in mixed populations, which can affect the fluorescent intensity (Rabinovitch et al., 1986) (Novak and Rabinovitch, 1994). There are problems with variations in cellular auto fluorescence and selective removal of the fluorophore by ion pumps. All of these can affect Ca^{2+} binding to the fluorophore and fluctuations in the fluorescence intensity on a cell-by-cell basis. Ratio metric analysis of fluorescent intensity of dye bound to Ca^{2+} to unbound can minimise the technical problems described earlier and as a ratio of two parameters is comparatively insensitive to small changes that may be observed in a single parameter (Rabinovitch et al., 1986).

Indo-1 is the most commonly used fluorophore for ratio metric measurement of intracellular calcium (Grynkiewicz et al., 1985). It has an excitation peak at 330–346 nm, depending upon the Ca^{2+} concentration, but requires a UV capable laser with a line between 325 and 360 nm for excitation. Ratio metric analysis with Indo-1 as a single dye can be attained as the peak fluorescence emission for Ca^{2+} bound is 405 nm and for Ca^{2+} free Indo-1 is 475 nm. Ratio metric Ca^{2+} flux is determined by calculating the ratio of the mean fluorescence intensity (MFI) of Indo-1 emission at 405 nm to the MFI of emission at 475 nm. The obvious disadvantage of Indo-1 is the requirement of UV capable laser for excitation and it is not widely available. But this has had an advantage of preserving access to a 488 nm line allowing the simultaneous use of fluorescein-conjugated antibodies.

An alternative to Indo-1 is the combination of 2 dyes for ratio metric assessment like fluo-3 and fura red (Minta et al., 1989) (Novak and Rabinovitch, 1994). Fluo-3 excites at 506 nm and emits at 526 nm and fura red in low Ca^{2+} conditions excites at 472 nm and emits at about 657 nm, and in high Ca^{2+} conditions it excites at 436 nm and emits at 637 nm. The MFI of fluo-3 increases upon binding Ca^{2+} and that of fura red decreases and the ratio of the MFI fluo-3 to fura red can be used for ratio metric analysis of Ca^{2+} using the more commonly available 488 nm laser line.

Both methods were used in the current study. Initial experiments were done using Indo-1 in a cytometer that was already optimised for the assay and later fura-red/fluo-3 dyes were used once the flow cytometer was optimised for these reagents. Using these assays, the ability of CLL cells to respond to BCR ligation and the impact of therapeutic kinase inhibitors was investigated.

Mockridge et al has published experiments on calcium flux signalling by stimulating the BCR receptors using flow cytometry (Mockridge et al., 2007). Even though Ca^{2+} flux signalling to anti-IgM generally correlated with an increase in SYK phosphorylation measured by immuno-precipitation of phosphorylated SYK, there was discordance in both ways. Considering samples with more than 5% cells fluxing calcium, sIgM-mediated signalling is more commonly detected in U-CLL compared to M-CLL. Even though there was an overall correlation of sIgM expression and signalling capacity, when comparing M-CLL and U-CLL only M-CLL showed correlation between sIgM expression and Ca^{2+} flux. Unlike sIgM, sIgD stimulation triggered Ca^{2+} flux in a very high proportion of samples and there was no difference between M-CLL and U-CLL. Signalling capacity and surface expression of IgM was recoverable to certain extent by culturing the cells *in vitro* for 24 to 48 hours.

Prior to embarking on the main experiments a number of tests were performed to establish the correct instrument settings. Figure 5.1 shows the gating strategy for determining the relative amount of Indo-1 blue versus Indo-1 violet in total lymphocytes. As a positive control, the Ca^{2+} ionophore, ionomycin was added. When a strong calcium fluxing stimulus like ionomycin is added the calcium is released into the cytoplasm and Indo-1 binds more calcium, shifting the emission spectrum from predominantly blue to violet and when this ratio is plotted against time there will be an upward shift in the calcium fluxing cells. This plot can be represented as a line graph using FlowJo software (Figure 5.1D). The graph shows three phases. The first phase is the resting phase when the cells are unstimulated and after the

stimulation there is a maximum response phase which is followed by a dip in the response and that response is maintained as a plateau phase for a prolonged time for a strong stimulus like ionomycin. However, if the response is recorded for a sufficiently long interval, then it should fall to the base line.

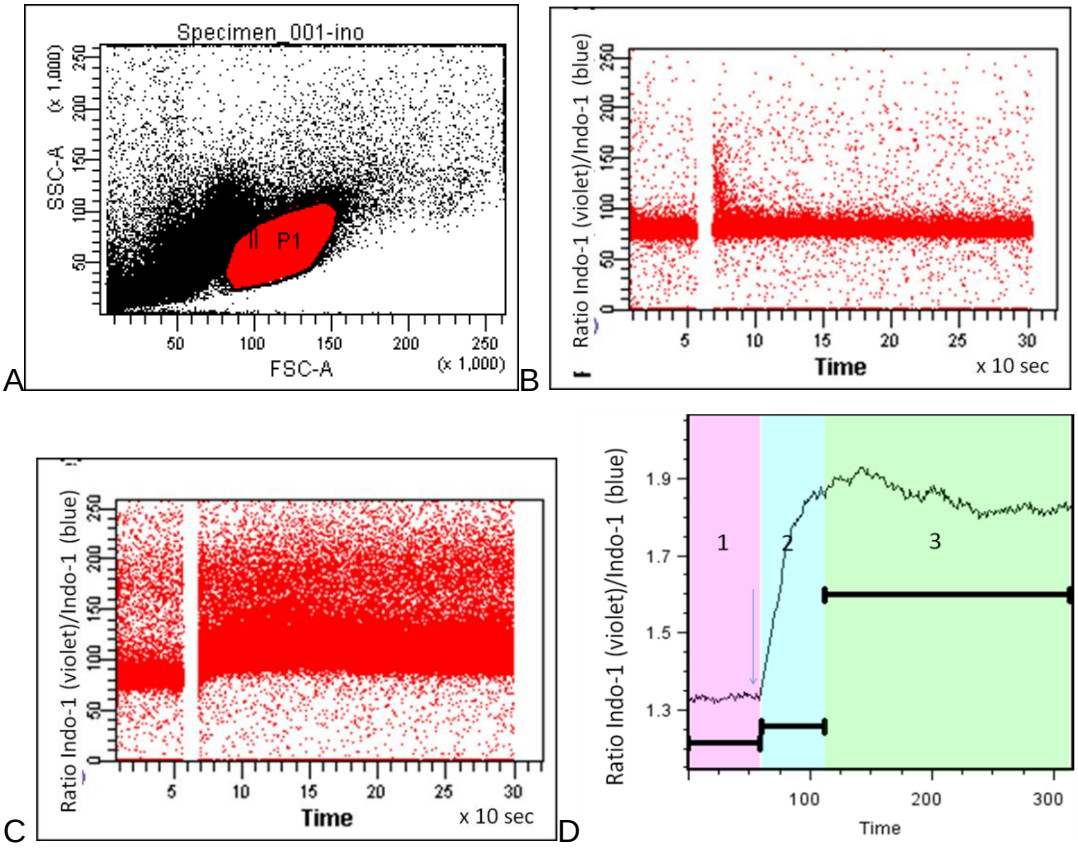


Figure 5.1.Gating strategy for calcium flux experiments.

Live lymphocytes were gated using forward and side scatter (A). The top time-course panel is the control unstimulated sample (B) and the bottom panel shows the response to ionomycin with an increase in the amount of violet emission (C). Line graph using FlowJo software is shown in D. 1is the unstimulated phase, arrow indicates the addition of ionomycin, 2 is the peak stimulation phase and 3 is the plateau phase.

The major physiological trigger for calcium flux in CLL cells is through BCR ligation. To test how well the assay worked as readout for CLL cells, MNC from patients were stimulated with $F(ab)_2$ antibody to IgM and assessed by flow cytometry. Examples of three types of response to IgM stimulus are shown in Figure 5.2 suggesting that the response is variable in patients. Among those tested, the first patient had essentially no response whereas the other two showed measurable Ca^{2+} fluxes. As mentioned previously, activation of SYK kinase is a crucial early event downstream of BCR triggering. To determine the effect of SYK inhibition on Ca^{2+} signalling, CLL

cells were incubated with the inhibitor GSK143 (details of the compound in the next chapter) and then assayed following IgM stimulation. Figure 5.3 shows that treatment of the two samples that generated strong Ca^{2+} fluxes with GSK143 completely inhibited the signal.

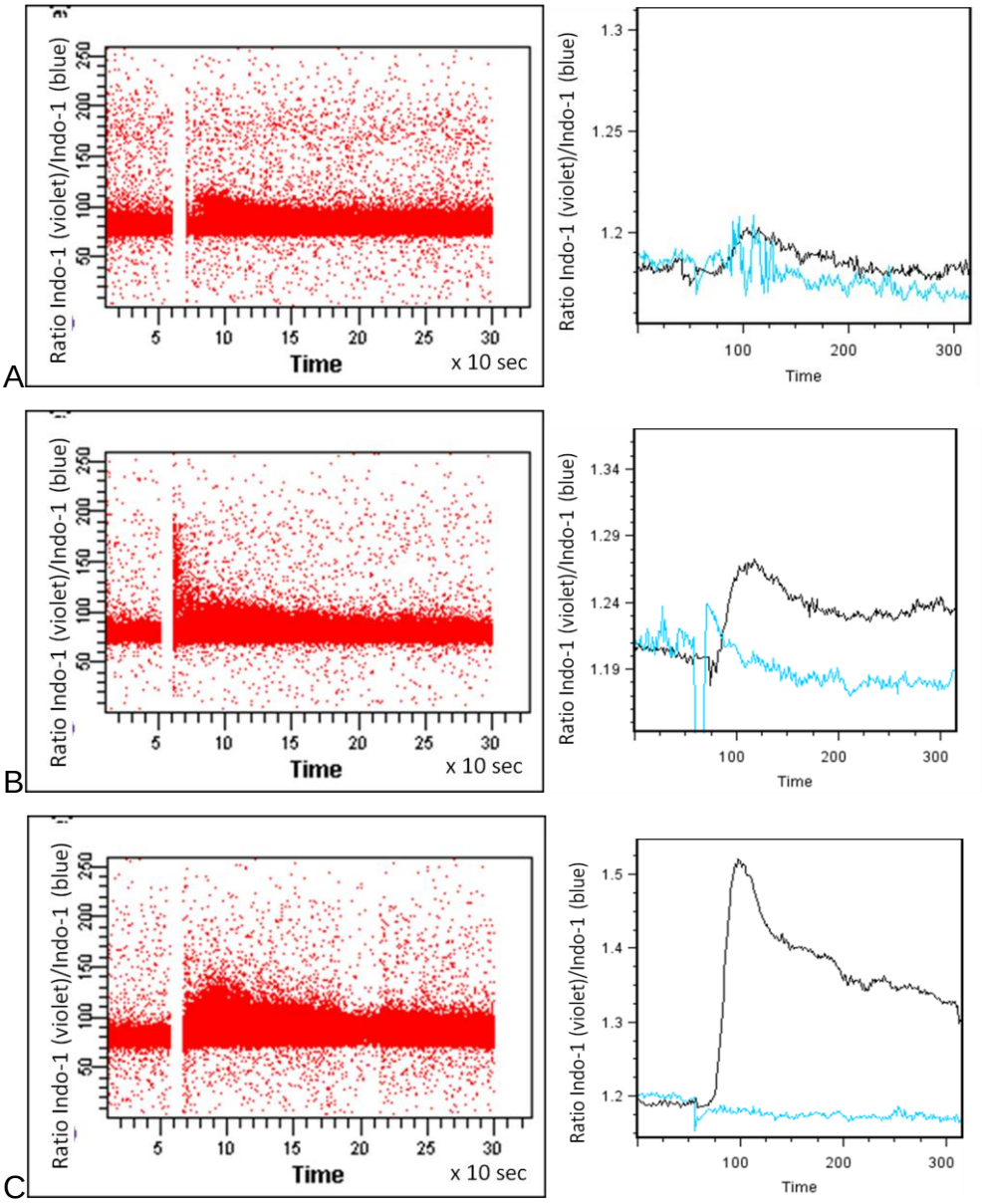


Figure 5.2 Ca^{2+} flux in CLL patients in response to anti-IgM.

Flowplot is shown in the left panel. Right panel shows the graphical analysis done by flowJo software. The black and blue lines represent the stimulation using IgM without or with the presence of a SYK inhibitor, respectively. All responses are almost completely inhibited by pre incubating with SYK inhibitor. The above 3 plots shows different response to the stimulus in different patients. A shows no response, B mild response and C shows the maximum response.

5.1.1. Fluo-3/fura-red optimisation

In addition to the Indo-1 optimisation detailed above, similar steps were employed to optimise the use of fluo-3/fura-red. As shown in Figure 5.3, the concentrations of fura-red and Fluo-3 were titrated so that the MFI fell within scale. In the last plot the ratio of the MFI of fluo-3 to fura-red was plotted against time. When the cells are stimulated and flux calcium, fluo-3 MFI increases and that of fura-red falls making the ratio high.

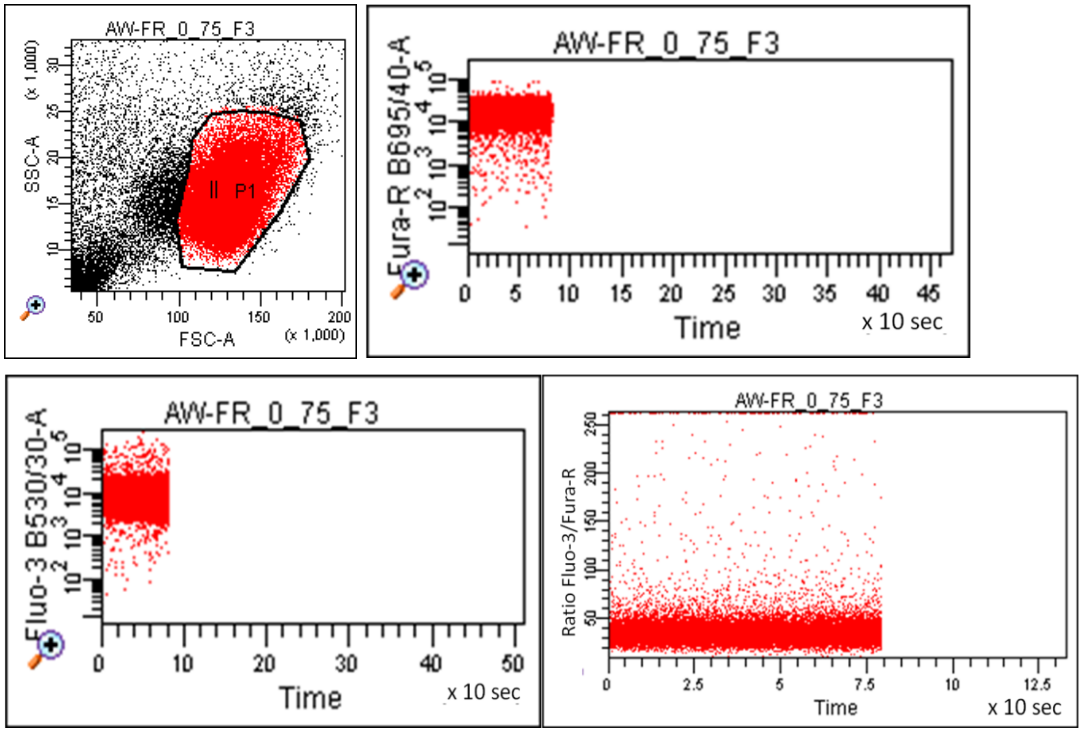
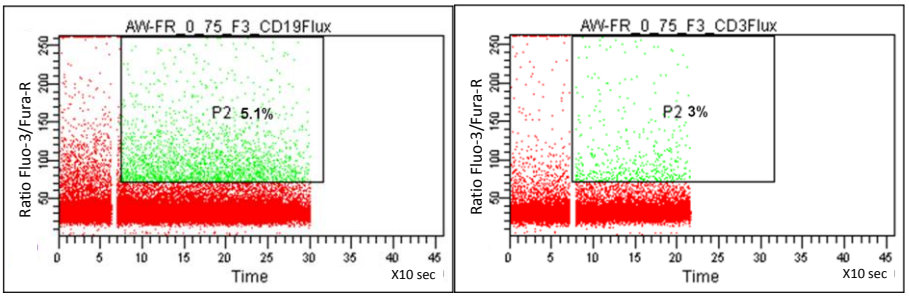


Figure 5.3 Optimisation of Fura-red and fluo-3.

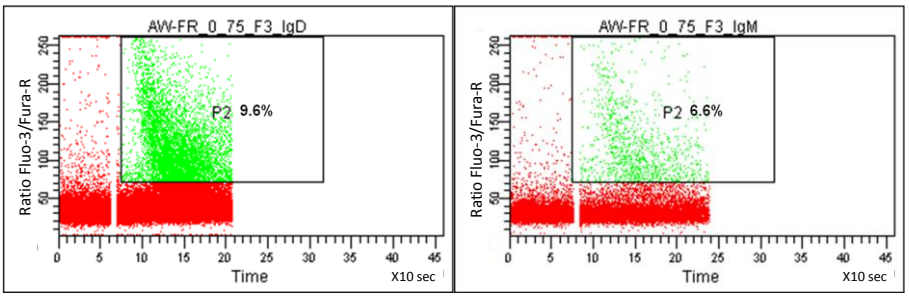
In the first plot lymphocytes are gated using forward and side scatter. In the second and third plots MFI of fura-red and fluo-3 were plotted against time. In the fourth plot the ratio of MFI of Fluo-3 to Fura-R is plotted against time. The fluorochromes are titrated, so that the ratio will fall in the bottom quarter of the graph when the cells are unstimulated.

Initial experiments using Indo-1 were tested on negatively selected CLL cells, but this was time consuming and expensive. So calcium flux was tested on MNC prepared by lymphoprep. The CLL cells were identified using multi-colour flow cytometry. Various antibodies used to mark the cells were tested individually to make sure that these antibodies by themselves were not inducers of calcium flux. The antibodies and different antigenic stimulation tested are represented in Figure 5.4. The plots demonstrate that there is no calcium flux when using antibodies against CD19 or CD3 and therefore these

Calcium flux using CD19 **Calcium flux using CD3**

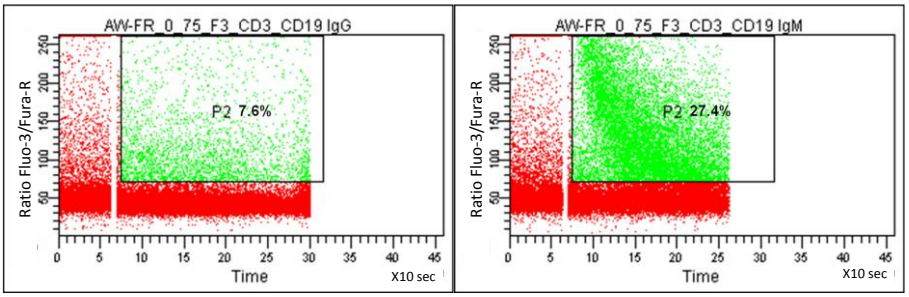


Calcium flux using IgD **Calcium flux using IgM**



In the following figures cells were stained previously with CD3 and CD19

Calcium Flux using IgG. **Calcium Flux using IgM.**



Calcium Flux using IgD.

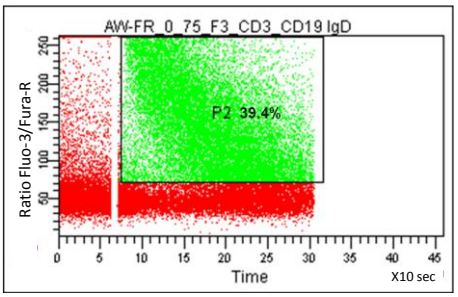


Figure 5.4 Calcium flux with cell staining antibodies and different antigenic stimulation.

Cells were evaluated after staining using population identification antibodies against CD19 or CD3. Calcium flux was also evaluated after stimulation with cross-linking antibodies against immunoglobulins IgM, IgD and IgG. The percentage of fluxed cells are shown as the number in P2 gate.

antigens can be used to mark the CLL cells. The strongest stimulus among immunoglobulins was IgD and the weakest was the IgG (Mockridge et al., 2007). This reflects the expression of these immunoglobulins on CLL cells and is consistent with previous literature (Mockridge et al., 2007). Multi-parametric flow cytometry was used for further experiments to identify CLL cells using CD19 and CD3 antibodies. The gating strategy for using this combination is shown in Figure 5.5.

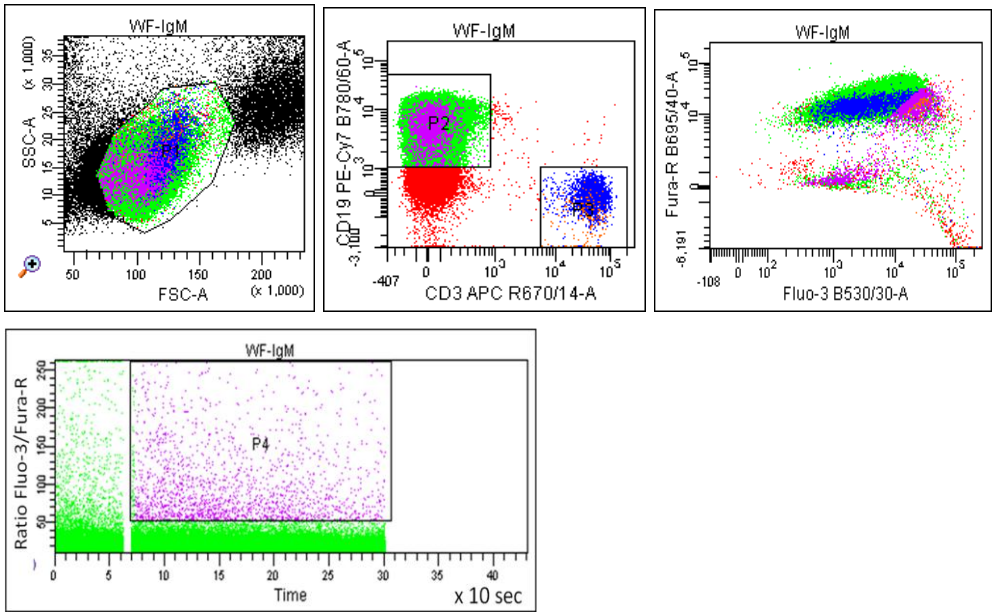


Figure 5.5 Multi-colour flow cytometry for calcium flux in CLL cells.

Lymphocytes were gated using forward and side scatter (P1). CLL cells were then gated using CD19 (P2) and T-cells using CD3 (P3). Calcium flux in CLL cells and T-cells are shown separately in the time:ratio plot. Fluxed CLL cells are gated as P4.

As IgD ligation was the most consistent trigger for Ca^{2+} flux in CLL cells, the optimal dose range was explored further. The concentration of the amount of anti-IgD required for maximum calcium flux was titrated as shown in Figure 5.6. Anti-IgM and IgG were used as controls and showed only very minimal calcium flux (Figure 5.7). Increasing concentrations of anti-IgD show incremental values in cells fluxing calcium up to a concentration of 10 µg/ml (IgM -1.9%, IgG -0.4%, IgD (1.25 µg/ml) -6.7%, IgD (2.5 µg/ml) -8.7%, IgD (5 µg/ml) -9.5%, IgD (10 µg/ml) -24.2% IgD (20 µg/ml) -6.9%.

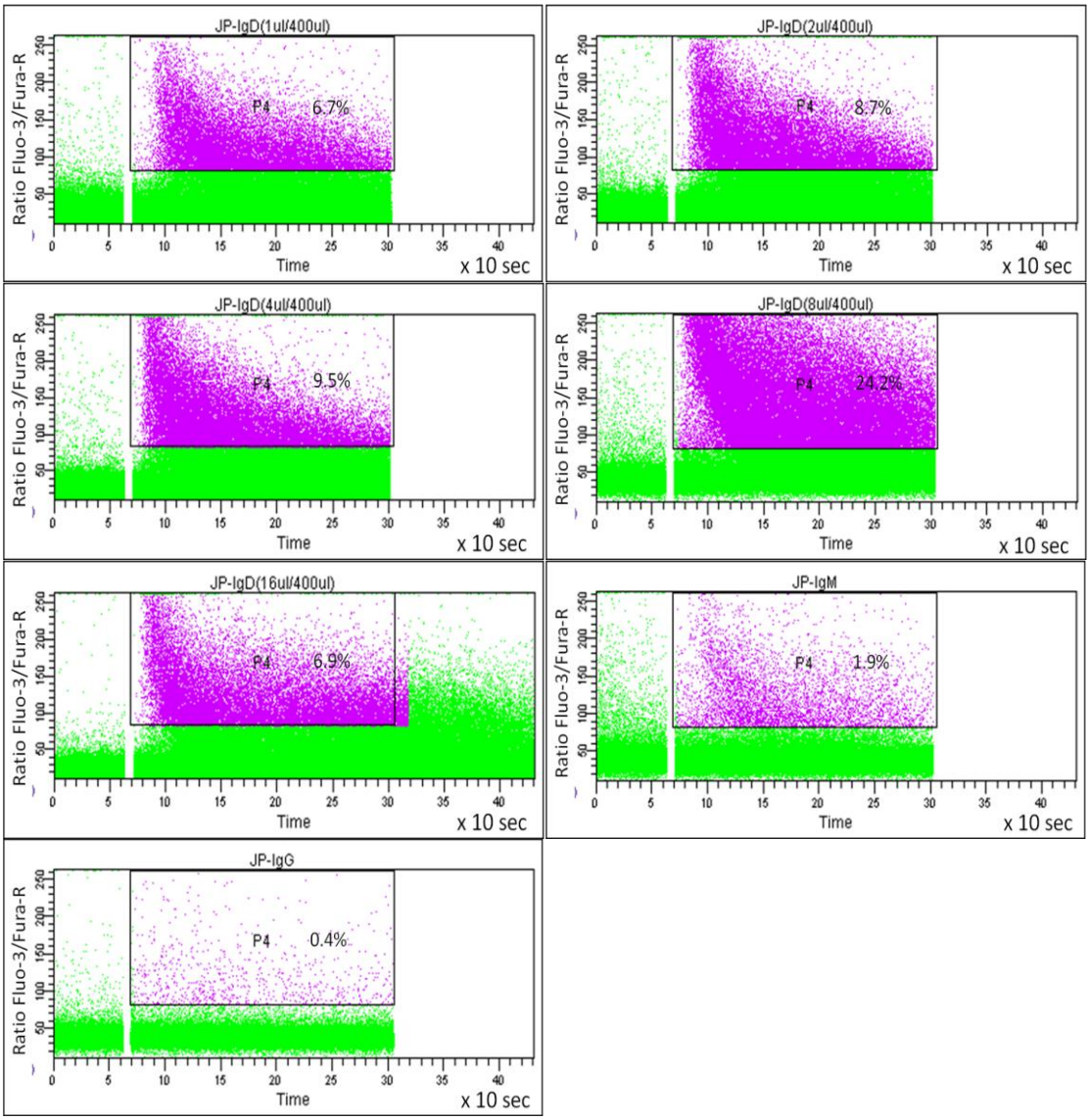


Figure 5.6 IgD titration for calcium flux.

CLL cells were stimulated with 2-fold increments of anti-IgD up to a concentration of 20 µg/ml. The percentage of fluxed cells are shown as the number in P4 gate..

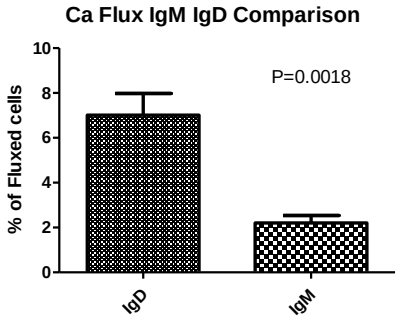


Figure 5.7 IgD is a more potent stimulant for calcium flux than IgM.

The average percentage of fluxing CLL cells in response to IgM or IgD ligation was determined (n=6). p value was calculated using paired t-test.

Calcium flux can vary with the storage condition of the sample. To determine how processing of samples can influence the ability to respond to BCR stimulation an experiment was performed to examine the degree of Ca^{2+} signalling (Figure 5.8). Flux was maximal if tested immediately after bleeding the patient. There was a reduction in calcium flux if tested after storage of the same sample either at room temperature or refrigerated, even though the difference is not statistically significant.

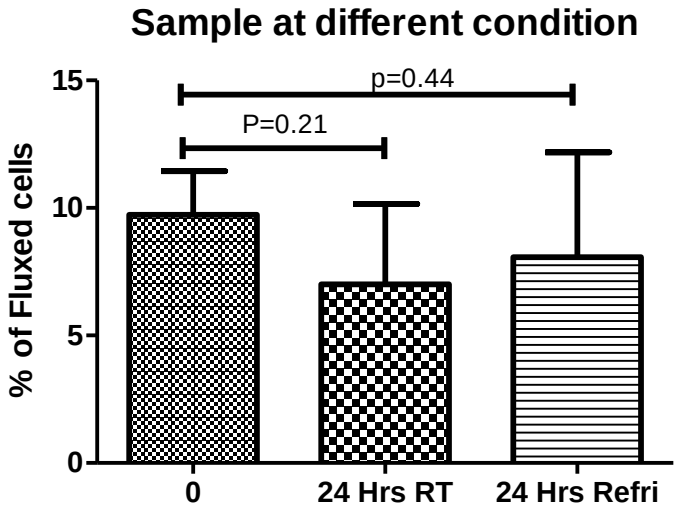


Figure 5.8 Calcium flux based on the storage condition of the sample.

24 Hrs RT = sample kept at room temperature for 24 hours, 24 Hrs Refri= sample kept at 4^{o}C for 24 hours. p value calculated using paired t-test.

5.2 Detection of signalling events using Phosflow analysis

In addition to Ca^{2+} flux, phosphorylation of key proteins is central to propagating the downstream signal. A number of antibodies that are specific for the phosphorylated versions of these proteins are commercially available and suitable for flow cytometry. Daudi and Jurkat cells were used as control cells for optimising the phosflow antibodies. Cells were mixed and identified using intracellular CD3 staining which was used along with the phosflow antibodies. The same method was used to separate T-cells and CLL cells from the MNC prepared by lymphoprep in the phosflow experiments. The gating strategy is shown in Figure 5.9. The initial experiments evaluated the effect of IgM ligation on the detection of phosphorylated SYK. The MFI of phospho SYK in Daudi cells shifted considerably once stimulated by IgM signalling. Unlike Daudi cells, Jurkat T-cells show no shift when stimulated using anti-IgM.

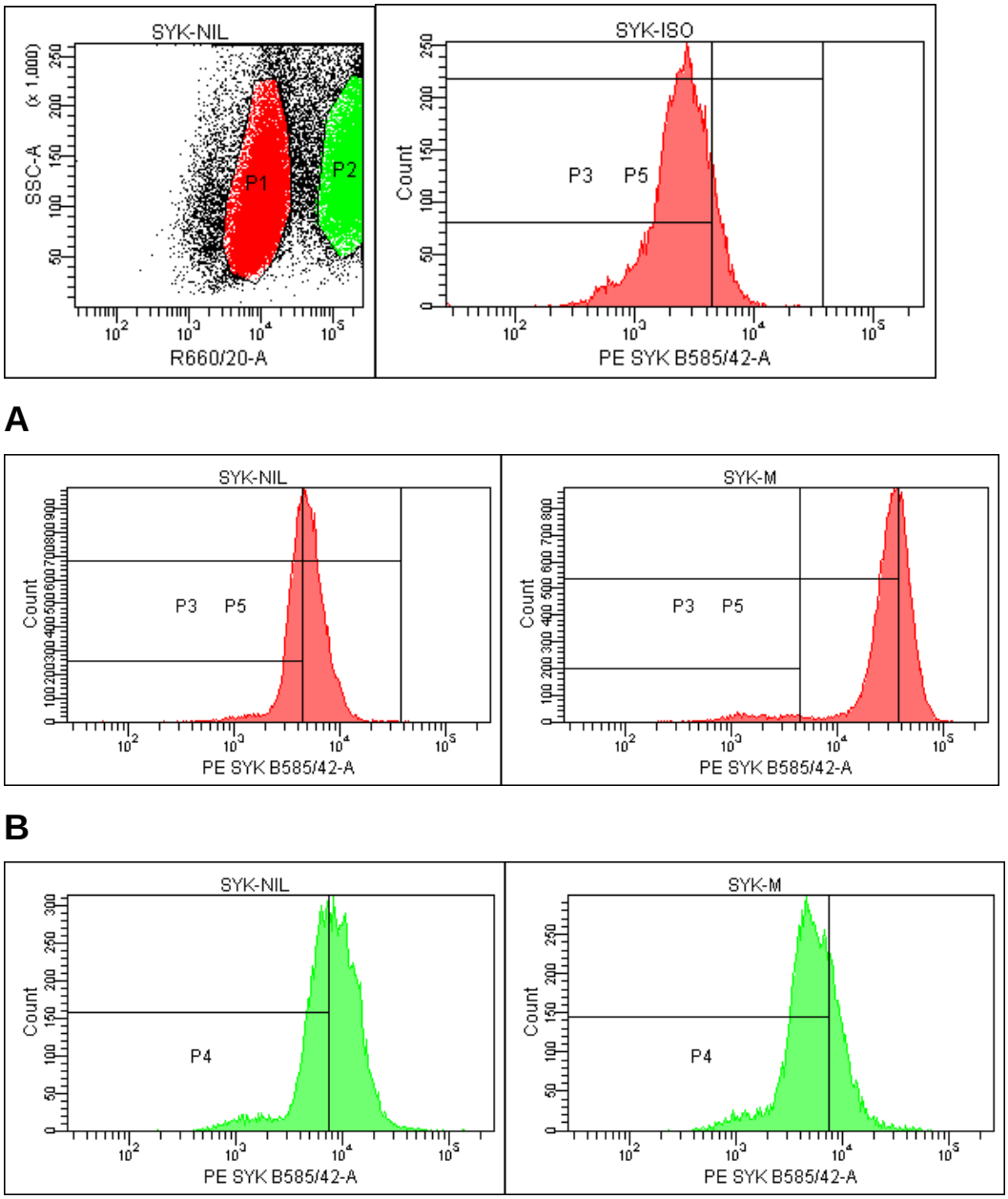


Figure 5.9 Phosphorylation of SYK in response to IgM ligation.

Daudi (P1) and Jurkat cells (P2) were identified using anti-CD3. Isotypic control, unstimulated Daudi cells and Jurkat cells shows similar expression for phospho SYK (PE mouse anti-SYK (pY348). The MFI of phospho SYK in Daudi cells (A), but not in Jurkat cells (B), shifted considerably once stimulated by IgM signalling (SYK-M).

Similar to the above experiment MNC isolated by lymphoprep from CLL patients were subjected to IgM stimulation (Figure 5.10). The first patient showed a significant shift in MFI while the second one showed no shift after IgM stimulation.

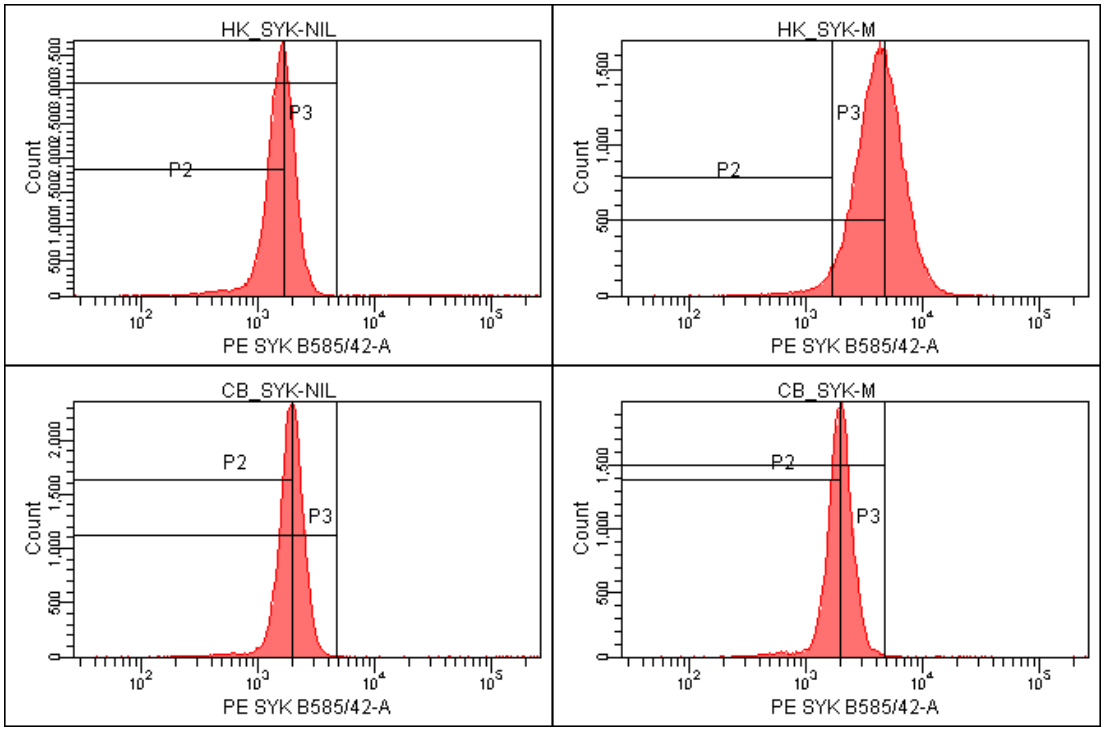


Figure 5.10 SYK phosphorylation by IgM stimulation on CLL cells.

The left panel is the level of phospho SYK in resting cells and the right panel depicts the response to IgM stimulation for 60 seconds measured by MFI of phospho-SYK antibody on 2 different patients.

Two other downstream molecules were also tested for phosphorylation, which are shown in Figure 5.11. Two AKT antibodies with different phosphorylation sites and NFκB phosphorylation were evaluated. The first AKT antibody detects phosphorylation at S473 and the second one phosphorylation at T308. Site T308 seems to be constitutively phosphorylated in CLL without significant shift after IgM stimulation while site S473 shows a shift in MFI after stimulation with IgM. There was no discernible change in NFκB phosphorylation. These negative results should ideally be confirmed using positive controls like CD40L for NFκB, but due to time constraints these experiments were not done.

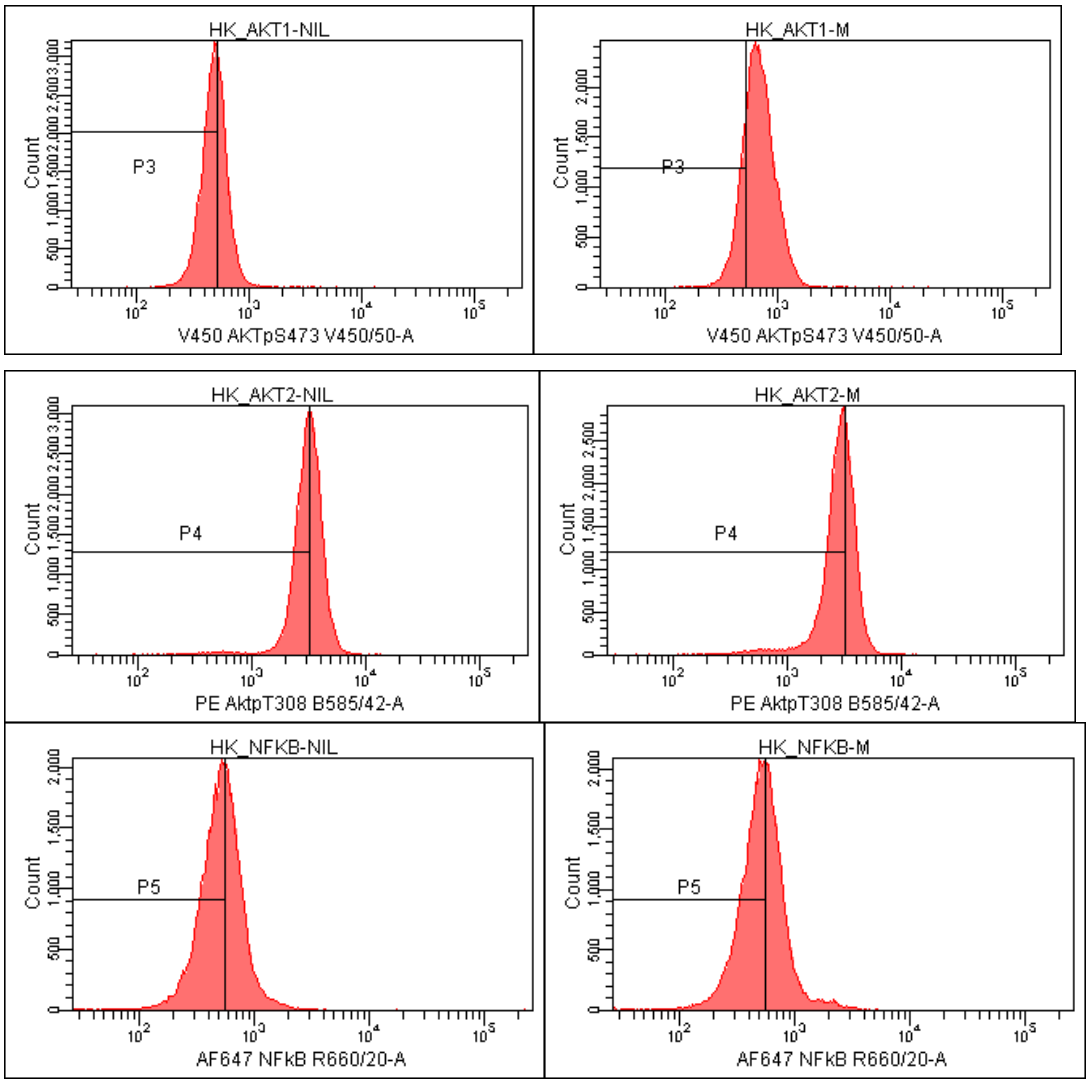
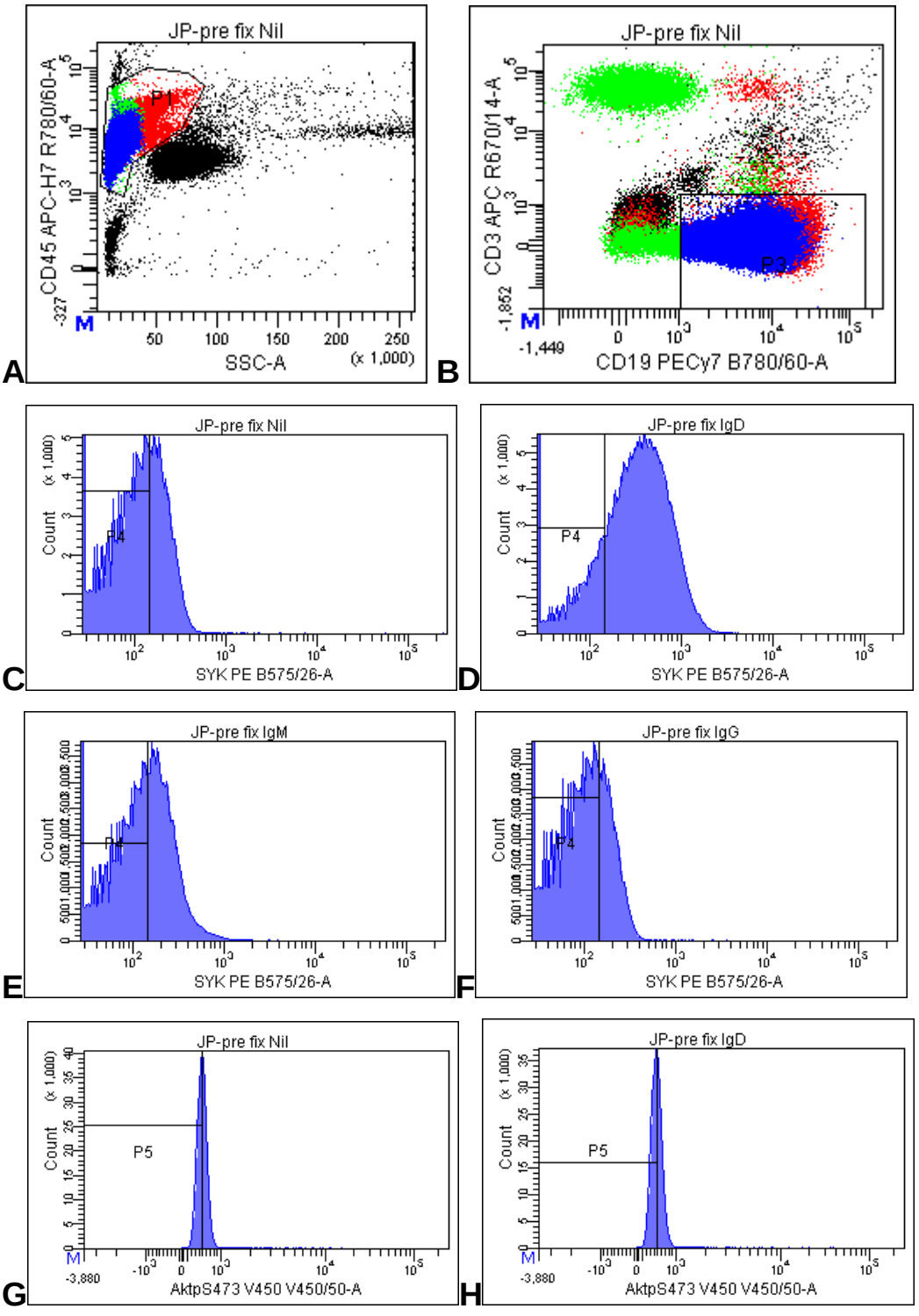


Figure 5.11 Testing downstream phosphorylation by IgM stimulation.

The top panel shows staining with anti-phospho AKT S473, the middle panel shows phospho AKT T308 and the bottom shows NFκB phosphorylation. In each instance the left panels indicate resting cells and the right panels show detection after IgM stimulation (t=60 sec).

For the initial experiments only intracellular CD3 was used to separate T-cells from CLL cells. Later multi-colour flow cytometry was used to test different phosflow molecules simultaneously. Here surface antibodies were used to separate cells. The expression of the surface antibodies seems to be distorted if cells were stained after fixation and permeabilization, i.e. when surface antibodies were added along with the phosflow antibodies (Figure 5.13). Therefore the staining of surface antibodies was performed prior to the fixation of cells as demonstrated in Figure 5.12. These data demonstrate that the surface antibody (CD19) staining is better before fixing the cell. SYK phosphorylation status was significantly altered by IgD stimulation and not by

IgM or IgG. With the antibodies used there was no significant shift demonstrated in phosphorylated AKT or BTK with any of the stimuli.



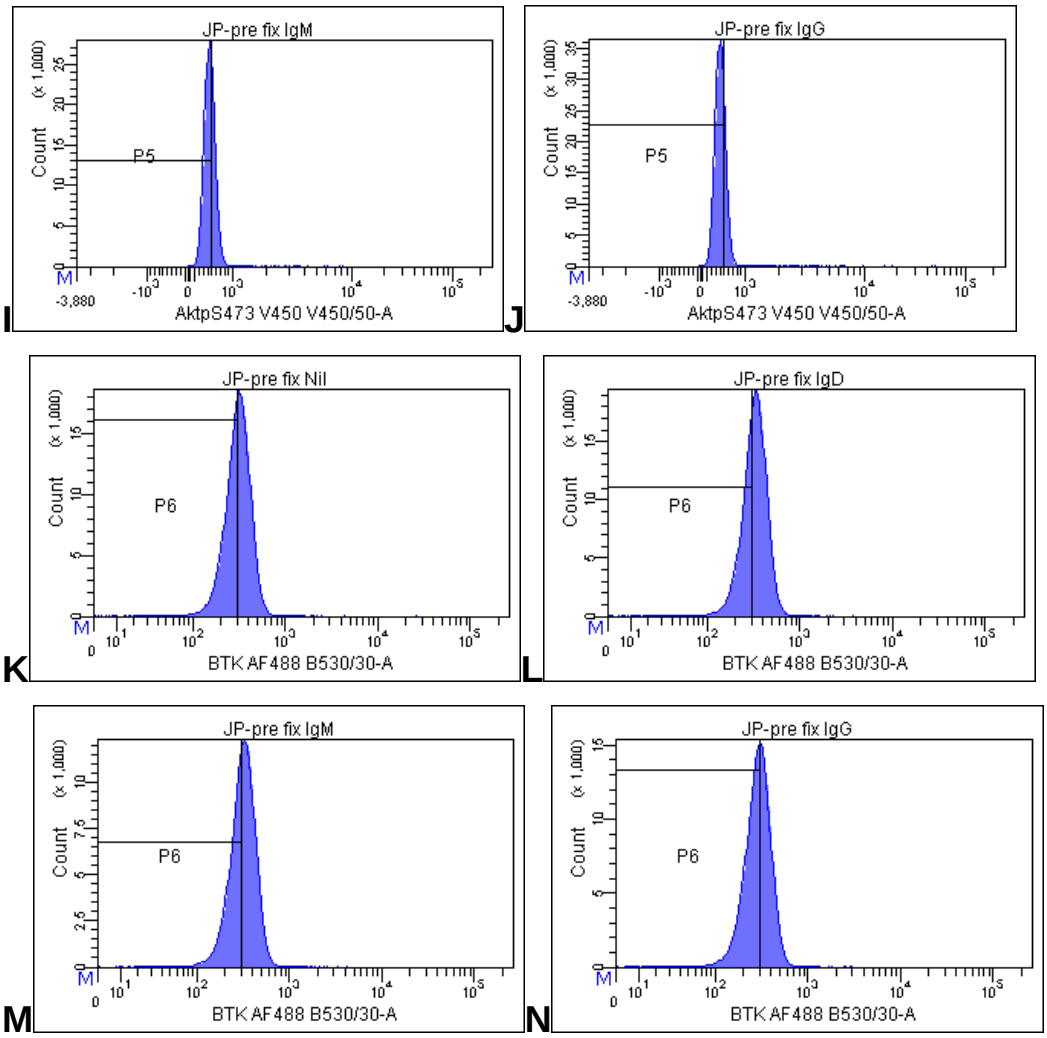
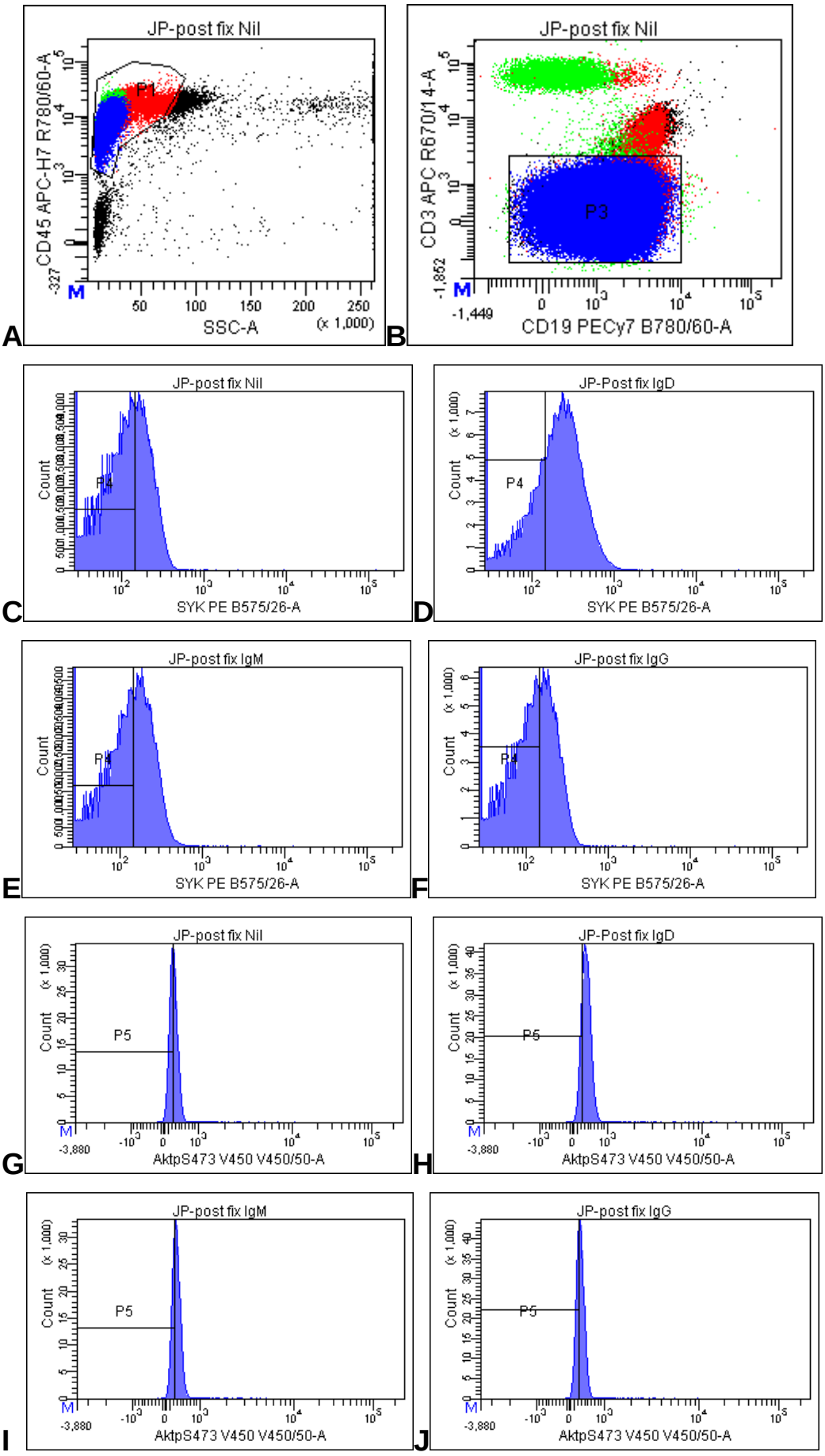


Figure 5.12 Gating strategy for testing phosphoproteins using multicolour flow cytometry: Staining with surface antibodies before fixing.

Lymphocytes were gated using CD45 and side scatter (A). T cells and B-cells were separated using CD19 and CD3 (B). The subsequent plots shows phosphorylation status of phosphoproteins including SYK (C,D,E,F), AKT(s473) (G,H,I,J), and BTK (K,L,M,N) molecules in unstimulated cells (C,G,K) and using various stimulus including anti-IgD (D,H,L), IgM (E,I,M) and IgG (F,J,N) (t = 60 sec).


A
JP-post fix Nil
CD45 APC-H7 R780/60-A
SSC-A
(x 1,000)
P1
B
JP-post fix Nil
CD3 APC R670/14-A
CD19 PECy7 B780/60-A
P3
C
JP-post fix Nil
Count
SYK PE B575/26-A
P4
D
JP-Post fix IgD
Count
SYK PE B575/26-A
P4
E
JP-post fix IgM
Count
SYK PE B575/26-A
P4
F
JP-post fix IgG
Count
SYK PE B575/26-A
P4
G
JP-post fix Nil
Count
AktpS473 V450 V450/50-A
P5
H
JP-Post fix IgD
Count
AktpS473 V450 V450/50-A
P5
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AktpS473 V450 V450/50-A
P5

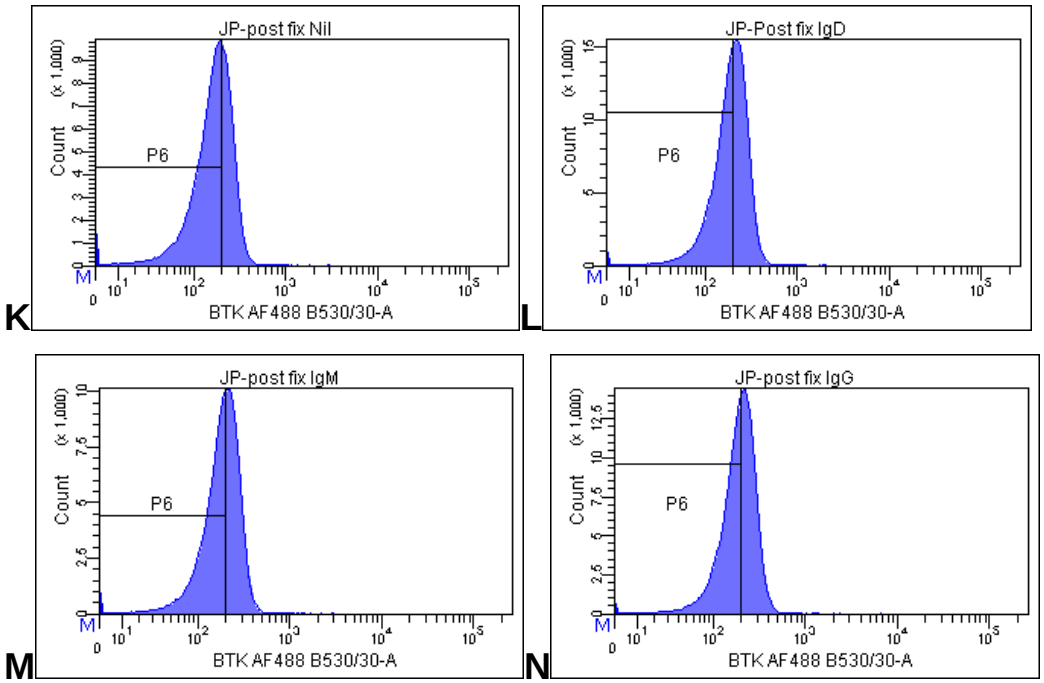


Figure 5.13 Gating strategy for testing phosphoproteins using multicolour flow cytometry. Staining with surface antibodies along with phosphoprotein antibodies after fixing.

Lymphocytes were gated using CD45 and side scatter (A). T cells and B-cells were separated using CD19 and CD3 (B). The subsequent plots shows phosphorylation status of phosphoproteins including SYK (C,D,E,F), AKT(s473) (G,H,I,J), and BTK (K,L,M,N) molecules in unstimulated cells (C,G,K) and using various stimulus including anti-IgD (D,H,L), IgM (E,I,M) and IgG (F,J,N) (t = 60 sec).

The data in Figures 5.9 and 5.10 showed that phosphorylated SYK was detected in Daudi and CLL cells following IgM stimulation. To improve the assay a dose titration curve was plotted for phospho SYK antibody to find the optimum concentration (Figure 5.14). MNC prepared using lymphoprep were compared with whole blood, with the optimum concentration of phospho SYK antibody titrated using whole blood. Both signal-to-noise ratio and the percentage increment in signal from unstimulated to stimulated were also maximum with 10µl which was half the manufacturer's recommended concentration.

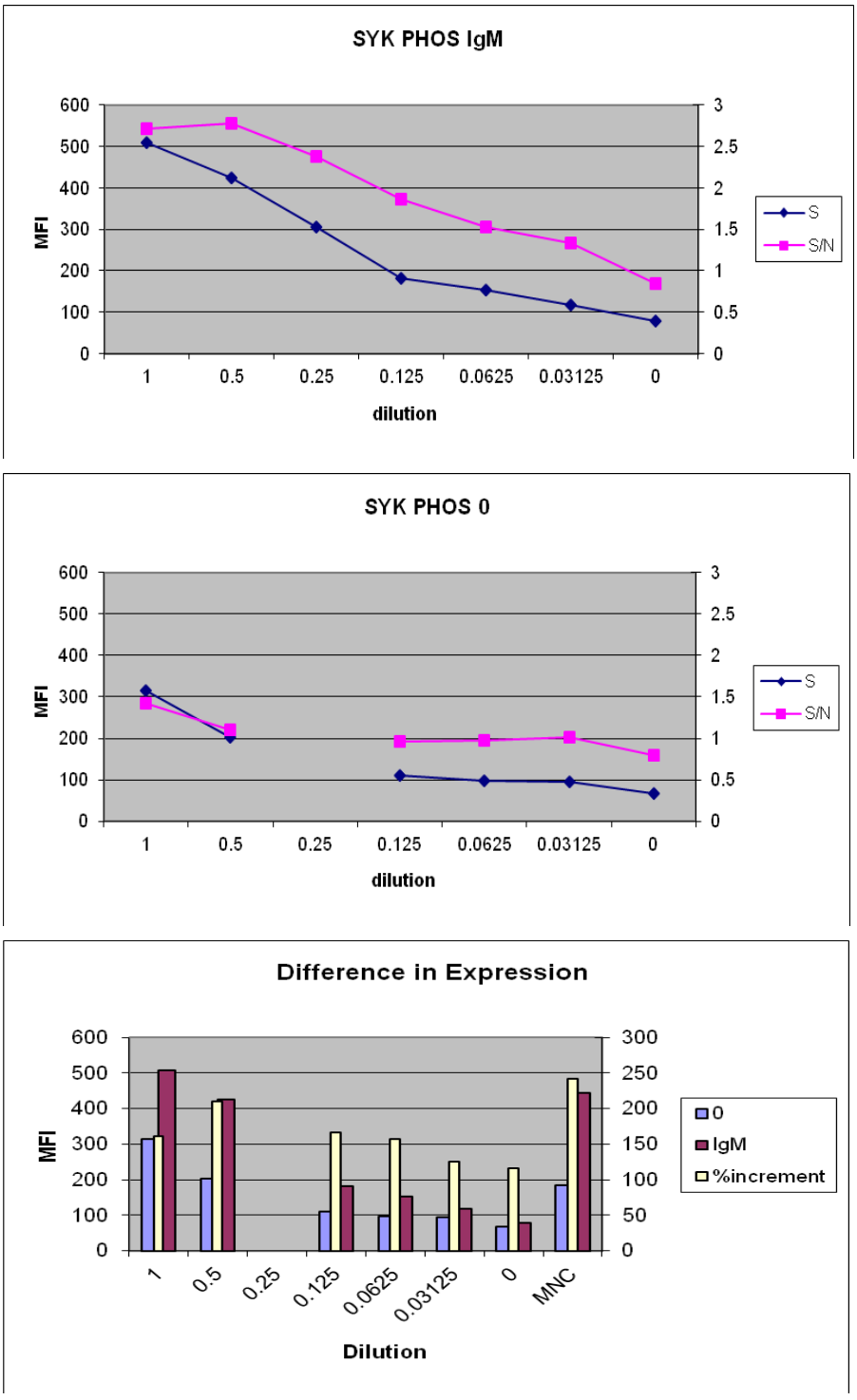


Figure 5.14 Dose titration curve for phospho SYK.

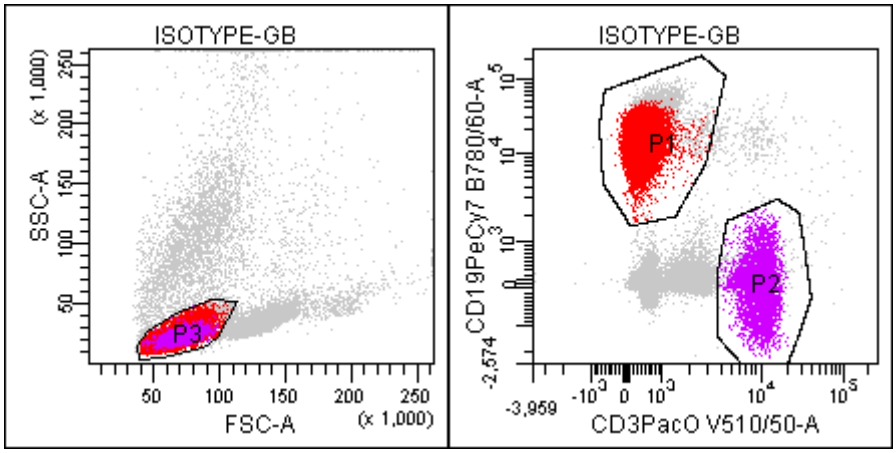
Whole blood was used to titrate the antibody starting with a neat solution of 20µl, which was the manufacturer recommented concentration. The difference in expression of phospho SYK (secondary y-axis) between IgM stimulated and unstimulated cells are maximum with 0.5 times dilution of the neat solution.

The above experiments proved that the downstream signalling of B-cell receptor pathway can be stimulated by crosslinking with $F(ab)_2$ anti-IgD

antibody in CLL cells as demonstrated by SYK phosphorylation and to a lesser degree phosphorylation of AKT at site S473. Phosphorylation of other molecules like BTK and AKT at site T308 could not be demonstrated, but it would need several optimisation experiments including ideal fixing and permeabilizing buffer, optimum stimulation time, positive and negative controls etc. before definitively concluding that they are not phosphorylated. Titration experiments demonstrated that half the recommended concentration of SYK phosflow antibody could be used effectively to distinguish a positive signal. These optimisations were conducted to streamline further experiments in the next chapter.

5.3 SYK and ZAP70 expression

In addition to SYK, the homologous kinase ZAP70 is expressed in a cohort of CLL samples. To distinguish between the relative expression levels of these two kinases, an experiment was performed using whole blood from a healthy volunteer and B- and T-cells gated using CD19 or CD3, respectively (Figure 5.15). The antibodies used allowed a clear demarcation of SYK expressing B-cells and ZAP70 expressing T-cells. To further improve the assay a titration of both antibodies was performed (Figure 5.16). The signal-to-noise- ratio suggests that 5µl of ZAP70 and 1µl of SYK antibodies were optimum for further experiments. The results suggest that SYK and ZAP70 expression levels can be accurately determined. This is an important variable to consider when assessing the viability of cells when treated with inhibitors affecting phosphokinases of BCR pathway, as demonstrated in the next chapter.



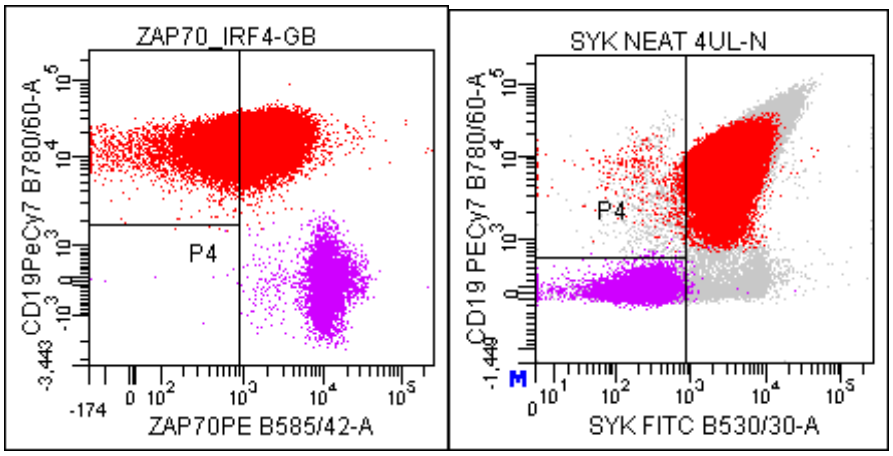
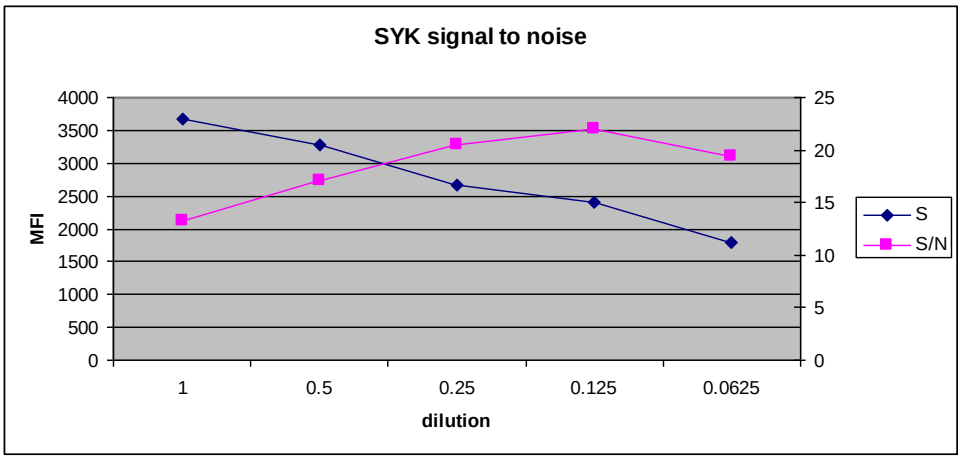


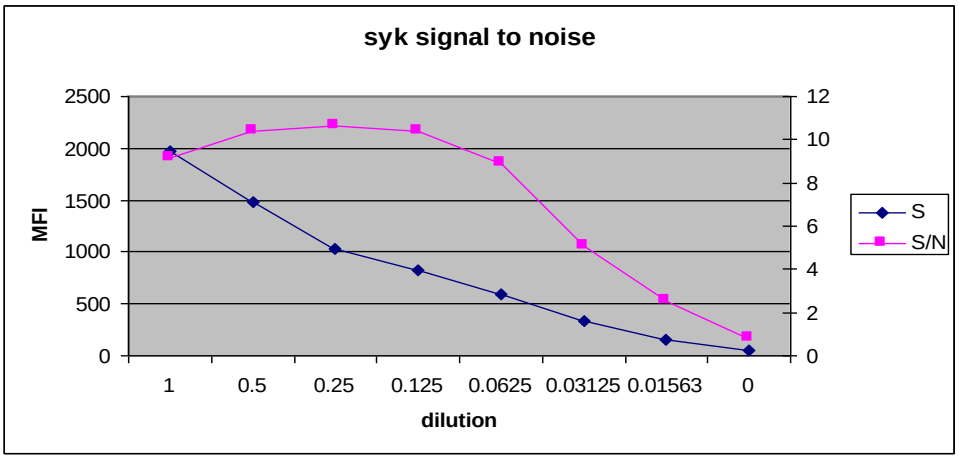
Figure 5.15 Gating for SYK and ZAP70 expression.

Lymphocytes were gated using forward and side scatter. T (P2) and B (P1) lymphocytes were seperated using CD19 and CD3. The expression of ZAP70 and SYK above the isotype control (vertical bar represents the maximum expression of isotype control) is shown in the bottom plots.

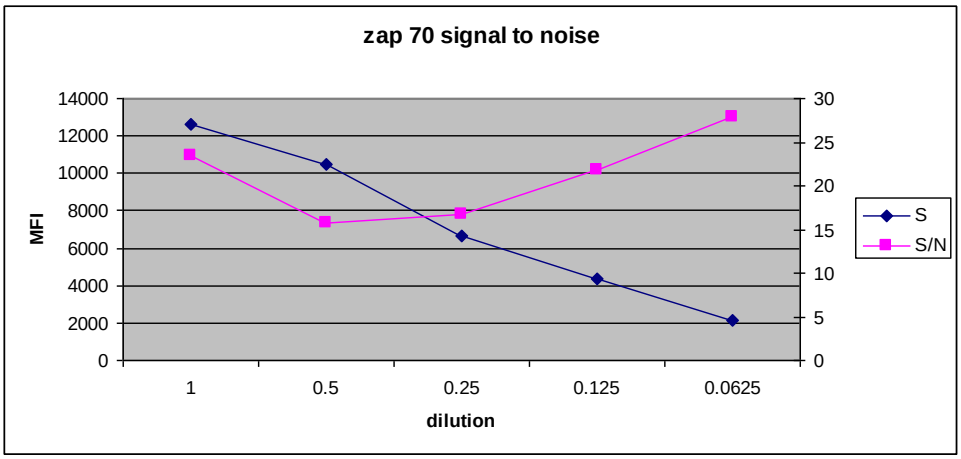
SYK titrated on CLL cells



SYK titrated on Normal B-cells



ZAP70 titrated on T cells from CLL patients



ZAP70 titrated on T cells from normal controls

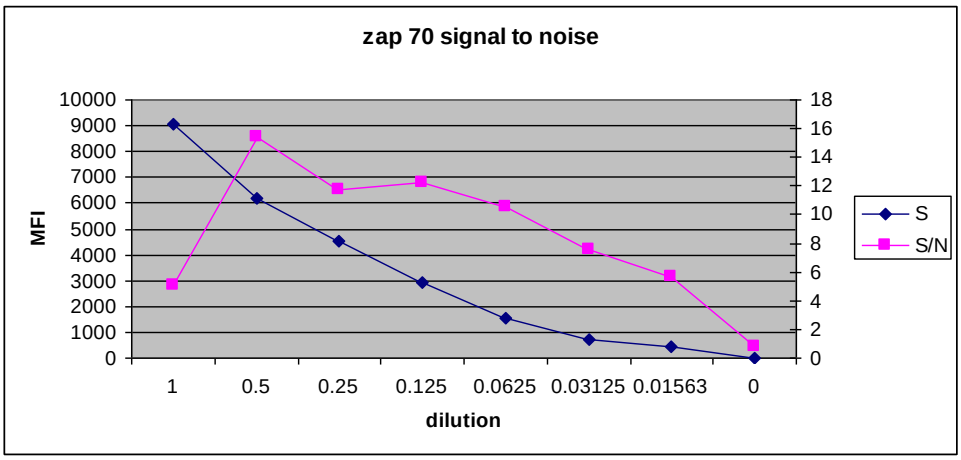


Figure 5.16 ZAP70 and SYK antibodies were titrated for optimum concentration.

10µl of ZAP70 and 2µl of SYK antibodies were used as neat solution. Both were titrated on normal as well CLL patients.

5.4 Discussion

B-cell receptor complex aggregates following engagement by antigen leading to phosphorylation of several downstream Src-family tyrosine kinases (Woyach et al., 2012). Of these, phosphorylation of SYK is an important early component, which then activates intracellular signalling cascades including calcium flux, ultimately leading to activation of the transcription factor NF-κB (Mackay et al., 2010). BCR signalling plays an important role in the pathogenesis of CLL (Zenz et al., 2010c) and blocking this pathway will induce apoptosis in CLL cells (Buchner et al., 2010) (Gobessi et al., 2009). When studying the effect of inhibitors of phospho-kinases in the pathway as

potential therapeutic agents it is important to be able to show changes in phosphorylation and calcium flux and thereby demonstrate the inhibition of pro-survival signals. Changes in phosphorylation of SYK protein have been previously demonstrated by immuno-blotting (Herishanu et al., 2011). Here we optimised a flow cytometric method to study this effect, which allows the detection of SYK phosphorylation at the cell level, rather than relying on an assessment of a bulk population. Similarly IgM cross-linking has been shown to stimulate calcium flux in CLL cells and this effect has been studied by flow cytometry (Mockridge et al., 2007). In this study both these techniques were used to explore the effect of a new SYK inhibitor (GSK 143) as well as to study the effect of manipulation of pathways of new antigens identified in the earlier chapter.

6. Testing the effect of BCR kinase inhibitors on CLL

Previously CLL cells were characterised by a defective apoptosis response rather than an increased proliferation as most of the circulating cells are arrested in the G0/G1 phase of the cell cycle, and express high levels of anti-apoptotic proteins (Kitada et al., 1998). But now it is clear, from the heavy water experiments, that a small fraction of CLL cells actively proliferates *in vivo* (Messmer et al., 2005). This usually happens in the proliferation centres in the tissue microenvironment, where contact dependent signals are delivered by various accessory cells such as nurse-like cells, follicular dentritic cells, marrow stromal cells and T-cells (Audrito et al., 2013b). Various chemokine receptors and adhesion molecules expressed on CLL cells help in their trafficking and homing into the microenvironment (Audrito et al., 2013b). Once in the microenvironment another crucial activation pathway stimulated is the BCR pathway (Burger, 2011). This can be engaged by either microbial or autoantigens the nature of which is still not very clear (Chu et al., 2008) (Catera et al., 2008). Now there is good evidence to suggest that antigen stimulation of the BCR and the downstream signals derived from that play a critical role in pathogenesis and prognosis of CLL. This signalling pathway is detailed in the introductory chapter. Several phosphoproteins are recruited and activated in this pathway including SYK, BTK, LYN, AKT and PI3Kδ. Now it is evident from various *in vitro* studies that manipulation of the activation of some of these molecules can inhibit further downstream signalling and thereby the stimulation and proliferation of the cells. Inhibitors of some of these molecules have already entered different phases of clinical trials.

6.1 SYK inhibitor R406

The SYK inhibitor R406 has been studied by a number of groups. This is the active metabolite of the clinically used pro-drug R788 (fostamatinib disodium) which is rapidly converted to R406 *in vivo*. A study by Quiroga *et al* has shown that this molecule can affect various aspects of CLL cell survival signalling. It can inhibit IgM mediated increased viability as well as the viability of CLL cells in nurse-like cells co-cultures (Quiroga et al., 2009b). R406 blocks CCL3 and CCL4 secretion by CLL cells in response to BCR triggering. It abrogates the change in expression of adhesion molecules and chemokine receptors, specifically up-regulation of CD40, CD44, CD54, and CD62L, and

down-regulation of CXCR4 on CLL cells triggered by BCR activation. R406 antagonizes the increased chemotaxis towards CXCL12 and CXCL13 and migration beneath marrow stromal cells (pseudoemperipolesis) of CLL cells after BCR triggering. Immunoblotting has demonstrated that anti-IgM induced phophorylation of SYK and downstream molecules, p44/42 mitogen-activated protein kinase (ERK), and AKT activation were inhibited by R406 (Quiroga et al., 2009b). Anti-IgM induced intracellular calcium flux was also significantly inhibited by this compound.

A paper by S Gobessi *et al* has shown that SYK was constitutively phosphorylated at the activating Y352 residue in CLL B-cells by immunoblotting and confocal microscopy similar to DLBCL cell lines DHL-4, DHL-6, WSU and DHL-10 (Gobessi et al., 2009). Treatment of CLL cells with R406 resulted in a dose-dependent reduction in basal AKT phosphorylation and the phosphorylation of its direct substrates glycogen synthase kinase (GSK)-3 and FOXO1/3a. In addition, they showed a basal phosphorylation of ERK and that was reduced by R406. In contrast, the high basal DNA binding activity of NF-κB displayed by CLL cells was not affected by R406. This may also suggest that the high basal NF-κB activity of CLL cells is not a consequence of constitutive SYK activation. R406 also blocked the anti-IgM induced signalling downstream of SYK, as evidenced by absent or reduced phosphorylation of AKT and GSK-3 and increase in MCL-1 expression and the consequent increase in leukaemic cell viability (Gobessi et al., 2009).

Buchner *et al* demonstrated the role of SYK in molecularly defined pathways that mediate the CLL-microenvironmental crosstalk independent from the BCR (Buchner et al., 2010). There is an increased phosphorylation of SYK at tyrosine 352 and also AKT phosphorylation on contact with the murine stromal cell line M2-10B4, human cell line HS-5, and to primary stromal cells. R406 inhibits this AKT phosphorylation and downstream actin polymerization. To find the molecular pathway for stromal induced phosphorylation, homing and adhesion molecules including CXCL12, and VCAM-1 were tested and found to activate SYK and AKT phosphorylation, actin polymerisation and cell adhesion. R406 seems to inhibit all these effects. Their experiments have also shown that cell adhesion mediated drug resistance by stromal contact to chemotherapeutic agents like F-ara-A were completely abrogated in the presence of R406.

6.2 BTK inhibitor

Another clinically interesting molecule affecting the BCR pathway is the BTK inhibitor PCI-32765. Ponader *et al* showed that PCI-32765 interferes significantly with anti-IgM induced and NLC-mediated survival (Ponader et al., 2012). Preincubation with 1µM PCI-32765 before anti-IgM stimulation significantly reduced CLL cell viability to 98%±8% of unstimulated controls (normalised to 100%), compared to 27%±12% increase in viability with anti-IgM stimulation after 24 hours. CLL cells from the TCL1 mouse model display a similar response to anti-IgM stimulation and PCI-32765 *in vitro*. The uptake of ^{3}H-thymidine by CLL cells co-cultured with NLCs was significantly reduced by PCI-32765 in a dose-dependent manner suggesting that a subset of proliferating CLL cells in the co-culture are inhibited by PCI-32765. The level of CCL3 and CCL4, which are chemokines secreted by CLL cells in response to BCR activation and during co-culture with NLCs, significantly dropped after treating with PCI-32765. CLL cell chemotaxis toward CXCL12 and CXCL13 and intracellular F-actin content was inhibited after pre-treatment with PCI-32765. Studies on the EµTCL1 adoptive transfer mouse model that resembles CLL in patients showed that 5 weeks after cell transfer, control mice and mice treated for 2 weeks with the suboptimal dose of PCI-32765 exhibited signs of disease including lethargy, hunched posture, ruffled and lost fur, and weight loss along with massive lymphocytosis, hepatosplenomegaly, and lymphadenopathy (Ponader et al., 2012). In contrast mice receiving the optimal dose of PCI32765 appeared to be healthy, with significantly smaller livers and spleens with markedly reduced leukaemic infiltration. Treated mice also showed significantly repressed levels of phospho-PLCγ2 in spleen cells suggesting that PCI32765 considerably delays CLL progression *in vivo*.

Herman *et al*. showed that the BTK expression at protein level is variable among patients with CLL but not at the mRNA level suggesting a disrupted post transcriptional modification of the protein (Herman et al., 2011). This is not true in case of normal B-cells from a control population. The variation in CLL cells does not correlate with any known prognostic markers like *IGHV* mutational status. Herman *et al* have also demonstrated that PCI-32765 exhibited a significant dose-dependent cytotoxicity in CLL cells. However there was a big variation in cytotoxicity among patients, which also has no correlation with the known prognostic markers as well as the BTK level. PCI-32765 induced PARP cleavage in a dose-dependent manner confirming activation of caspase-3 after PCI-32765 treatment suggesting a caspase

dependant apoptotic pathway. Inhibition of caspase activity by z-VAD-fmk completely prevented the induction of apoptosis provided by PCI-32765, confirming the above findings. PCI-32765 can induce cell death in normal B-cells also but to a lesser extent compared to CLL cells. Even though naive T-cells showed expression of BTK at mRNA level to a significantly lesser extent compared to normal B-cells, they lack BTK expression at protein level both in naive T-cells and after CD3 ligation (Herman et al., 2011). There was no significant cytotoxicity in T-cells for doses which are cytotoxic to B-cells and CLL cells, but there was a significant inhibition of production of inflammatory cytokines such as IL-6, IL-10, and TNF-α; which suggests that PCI-32765 probably affects alternative kinase(s) expressed in T-cells. PCI-32765 has been shown to reduce the level of phosphorylated BTK and the downstream molecules ERK1/2 in patients who have these molecules phosphorylated constitutively. Additionally, the induction of phosphorylation of AKT and binding of NF-κB to a consensus binding site following CD40L stimulation was completely reversed by treatment with even low doses of PCI-32765. PCI-32765 has also been shown to antagonise CpG oligonucleotide induced CLL cell proliferation demonstrated by thymidine uptake (Herman et al., 2011). Co-treatment with PCI-32765 seems to abrogate the protection induced by many microenvironment stimuli like CD40L, BAFF, TNF-α, IL-6, and IL-4. PCI-32765 also seems to reduce the viability of CLL cells in co-culture conditions with the HS-5 stromal cell line (Herman et al., 2011).

6.3 Phosphoinositide 3'-kinase delta inhibitor, GS1101 (CAL-101)

Phosphoinositide 3'-kinases (PI3Ks) are key molecules that integrate and transmit downstream signals from various surface molecules, such as the BCR, chemokine receptors, and adhesion molecules. This family of kinases are therefore crucial molecules in regulating key cellular functions like growth, survival, secretion and migration. PI3Ks can be mainly divided into 3 groups, I, II, and III (Leevers et al., 1999). Class I isoforms phosphorylate inositol lipids to form second messenger phosphoinositides in the cell membrane that recruit, via binding to the amino-terminal pleckstrin homology domain, downstream signalling protein kinases like Tec kinases, phosphatidylinositol-dependent kinase, AKT, integrin-linked kinase, and Rac guanine exchange factor. The class I kinases has 4 isoforms namely PI3K α, β, γ and δ. PI3K α and β isoforms are expressed universally while PI3Kγ isoform is predominantly expressed in T-cells and plays a major role in T-cell activation

(Sasaki et al., 2000). PI3Kδ is predominantly expressed in haematopoietic cells including B-cells and plays a critical role in B-cell homeostasis and function. Transgenic mice with mutations inactivating PI3Kδ have various B-cell defects. They show a lower B1 and marginal zone B-cell count, their immunoglobulin levels are reduced, display poor immunisation response, downstream signalling of BCR and CD40 stimulation is defective, and can develop inflammatory bowel disease (Jou et al., 2002) (Okkenhaug et al., 2002) (Clayton et al., 2002). Biochemically, B-cells derived from PI3Kδ knockout mice also show less AKT phosphorylation when activated and have decreased downstream molecules like phosphatidylinositol 3,4,5-triphosphate levels and phosphopeptide activity (Vanhaesebroeck et al., 2005). In contrast, PI3Kγ isoform knockout mice have predominately a T-cell defect with normal B-cell development and function, suggesting that isoform-specific targeting of the PI3Kδ isoform may be cytotoxic to B-cells with minimal toxicity to other hematopoietic cell types. GS-1101 is a potent and highly selective PI3Kδ inhibitor. Previous *in vitro* data has shown that GS-1101 promotes apoptosis in B-cell lines and primary cells from patients with different B-cell malignancies, including CLL, mantle cell lymphoma and multiple myeloma. In these cells GS-1101 inhibits constitutive and CD40, TNFα, fibronectin, and BCR-derived PI3K signalling leading to suppression of AKT activation and thereby disrupting the survival signals in these cells (Lannutti et al., 2011) (Herman et al., 2010) (Ikeda et al., 2010).

Hoellenriegel *et al* has shown that GS-1101 inhibits CLL cell chemotaxis towards chemokines CXCL12 and CXCL13 and migration beneath marrow stroma cells TSt-4 and 9-15C (pseudoemperipolesis) (Hoellenriegel et al., 2011). Anti-IgM stimulation induced increase in CLL cell viability as well as NLC induced increase in cell viability were abrogated by GS-1101. GS-1101 also inhibits BCR and NLC induced secretion of the chemokines, CCL3, CCL4, and CXCL13. GS-1101 treatment down modulates the increased secretion of several other chemokines and cytokines including CCL7, sCD40L, TNFα, CCL17 and CCL22 in CLL-NLC co-culture. GS-1101 seems to abrogate the protective effect of stromal cells on CLL cells to chemotherapeutic agents as it seems to sensitise CLL cells to cytotoxic agents like bendamustine, fludarabine, and dexamethasone. It also seems to inhibit AKT and ERK phosphorylation in response to anti-IgM stimulation, and CXCL12 or CXCL13 stimulation respectively suggestive of affecting both BCR activation pathway as well as stromal cell stimulated survival pathway. Hoellenriegel et al have also shown that there is a statistically significant

reduction in the plasma level of chemokines CCL3, CCL4, and CXCL13, as well as phospho AKT levels in circulating CLL cells after 28 days of treatment with GS-1101.

Herman *et al* also studied the *in vitro* activity of GS-1101 on CLL cells (Herman et al., 2010). It has been demonstrated that CLL cells overall have a statistically higher intrinsic PI3K activity compared with normal B-cells. GS-1101 showed a dose dependent cytotoxicity in vitro after 48hrs of analysis and there was no difference in cytotoxicity between patients in different chromosomal or *IGHV* prognostic groups. They have also shown that at a higher concentration GS-1101 can induce cytotoxicity in normal B-cells even though the activity is only minimal compared to CLL cells. GS-1101 induced PARP cleavage in a dose-dependent manner confirming activation of caspase-3 after treatment with drug suggesting a caspase dependant apoptotic pathway. Inhibition of caspase activity by z-VAD-fmk completely prevented the induction of apoptosis provided by GS-1101, confirming the above findings. Even though GS-1101 does not show cytotoxicity toward T-cells or NK cells it reduces cytokine production by T-cells as measured by IL-6, TNF-α and IL-10 production after anti-CD3 stimulation and IFN-γ production by NK cells stimulated with plate-immobilized alemtuzumab (Herman et al., 2010). LY294002, a pan-PI3K inhibitor is cytotoxic to CLL cells as well as NK cells (Plate, 2004), but GS-1101 showed selective cytotoxicity to B-cells compared to NK cells confirming the selective cytotoxic potential. Treatment of CLL cells with GS-1101 could decrease, in a dose dependent manner, although not completely prevent, the increase in AKT phosphorylation at the Ser473 site and the downstream molecule GSK3β seen with CD40L stimulation. Similarly the increase in MCL-1 expression after CD40L stimulation was reversible by GS-1101 treatment (Herman et al., 2010). GS-1101 also abrogates the protective effect from microenvironmental stimuli including BAFF, TNF-α and fibronectin which all act through an increase in phosphorylation of AKT at the Ser473 site, similar to what was observed with CD40L treatment. GS-1101 treatment of CLL cells co-cultured with HS-5 cells resulted in a similar proportion of cytotoxicity compared with treatment of CLL cells without co-culture also suggesting that GS-1101 has the potential to mediate cytotoxicity independent of the protective effect of contact with stromal cells(Herman et al., 2010).

There is now convincing evidence to suggest from phase 1 and phase 2 clinical trials that both BTK inhibitor PCI-32765 (ibrutinib) and PI3Kδ inhibitor GS-1101 are clinically active in CLL without much toxicity. Both these drugs are going into phase 3 trials for comparing the efficacy with the current standard treatment. Results of the phase I/phase II trials on the BTK inhibitor was published in NEJM recently showing a very impressive result (Byrd et al., 2013). At a median follow-up of 20.9 months 64% continued to receive treatment and 36% discontinued due to various reasons including disease progression (13%), adverse events (8%) and investigator's decision including stem cell transplantation (15%). Overall response rate was 71% both in 420mg cohort and 840mg cohort. There was no significant difference in response in patients with traditional high-risk prognostic features. At 26 months the PFS of the whole group was 75% and OS was 83%. The most common adverse events of grade 3 or higher were pneumonia (12%) and dehydration (6%) and grade 2 or lower were diarrhoea, fatigue, and upper respiratory tract infection. More recent trials on ibrutinib combined with chemoimmunotherapy are also showing very promising results (Burger et al., 2012).

Similarly GS-1101 has been evaluated in a phase I trial in previously treated CLL patients (Furman et al., 2010). GS-1101 reduced lymphadenopathy in all 32 (100%) patients where at least one post-treatment tumour response assessment was possible with 91% achieving a ≥50% reduction in target lymph node size. Absolute lymphocyte count reduction of >50% from baseline was observed in 60% of patients at initial assessments. Considering nodal and peripheral blood changes together, partial responses according to the IWCLL criteria were observed in 33% of patients. The median duration of response had not been reached when the study was reported; but 7 patients had response durations of ≥6 months. Of 20 patients with CLL-related thrombocytopenia where baseline platelet counts <100,000/μL, 75% had either an improvement to >100,000/μL or a >50% increase from baseline. Considering the toxicity Grade ≥3, pneumonias occurred in 24% patients; haematological laboratory abnormalities included neutropenia in 24%, thrombocytopenia in 11% and anaemia in 8%. Flow cytometric analysis of CLL cells from patients showed that after 1 week of treatment with GS-1101 constitutive expression of phospho AKT was reduced to the background levels ($p<0.0001$), demonstrating pharmacodynamic inhibition of activated PI3K signalling. Plasma concentrations of several chemokines including CCL3, CCL4, and CXCL13 were elevated at baseline in the patients

which decreased significantly within 1 cycle of GS-1101 administration ($p<0.001$ for all comparisons).

A phase 1/2 clinical trial of fostamatinib disodium (FosD), the first clinically available oral SYK inhibitor, was published in 2010 involving 68 patients with recurrent B-cell non-Hodgkin lymphoma (B-NHL) including 11 CLL/SLL patients (Friedberg et al., 2010). All patients with SLL/CLL had an initial increase in circulating lymphocyte count which was observed during the first 29 days of therapy. Dose-limiting toxicity in the phase 1 portion was neutropenia, diarrhoea, and thrombocytopenia. Common other toxicities observed were diarrhoea, fatigue, and cytopenias. This is similar to toxicities observed in rheumatoid arthritis studies that used lower doses of FosD. 55% (95% CI, 23%-83%) patients with SLL/CLL had a PR to therapy assessed according to standard lymphoma response criteria. The median PFS for patients with SLL/CLL was 6.4 months (95% CI, 2.2-7.1 months). Among all groups of NHL the highest response rate was observed in patients with SLL/CLL.

This molecule was not developed as treatment in CLL for various reasons. One of the reasons is that the specificity of the molecule is not confined to SYK alone.

6.4 GSK143

It was reported previously that diaminopyrimidine carboxamide (DAPC) displayed good SYK inhibitory activity. The aminoethylamino moiety at the 2-position of the pyrimidine ring was important for SYK inhibitory activity as shown by enzyme screening. If an anilino moiety was substituted at the 4th meta position the resulting compound showed high selectivity for SYK, compared to other kinases, such as ZAP70, c-Src, and PKC, and exhibited good inhibitory activities against 5-HT release from RBL cells (Liddle et al., 2011). A more recent paper has shown that this compound has a high level of activity towards hERG together with moderate selectivity over Aurora B kinase, a kinase essential for cell proliferation (Liddle et al., 2011). GSK has done lead optimisation of the DAPC series of SYK inhibitors and discovered a compound GSK143 which is a potent and highly selective SYK inhibitor showing good efficacy in the rat Arthus model (Yamamoto et al., 1975). Activity was assayed by both a SYK mechanistic assay as evidenced by inhibition of anti-IgM induced Erk1/2 phosphorylation in Ramos cells (Klein et al., 1975)and also by human whole blood assay evidenced by inhibition of

anti-IgM induced CD69 surface expression in primary B-cells (Liddle et al., 2011). A summary of the characteristics of GSK143 is listed in Table 6.1 and its molecular structure is depicted in Figure 6.1.

Table 6.1 Kinase selectivity profile of GSK143

Kinase	pIC50	Fold selectivity
SYK	7.5	-
ZAP70	4.7	630
LCK	5.3	125
LYN	5.4	125
JAK 1/2/3	5.8/5.8/5.7	50/40/63
Aurora B	4.8	500

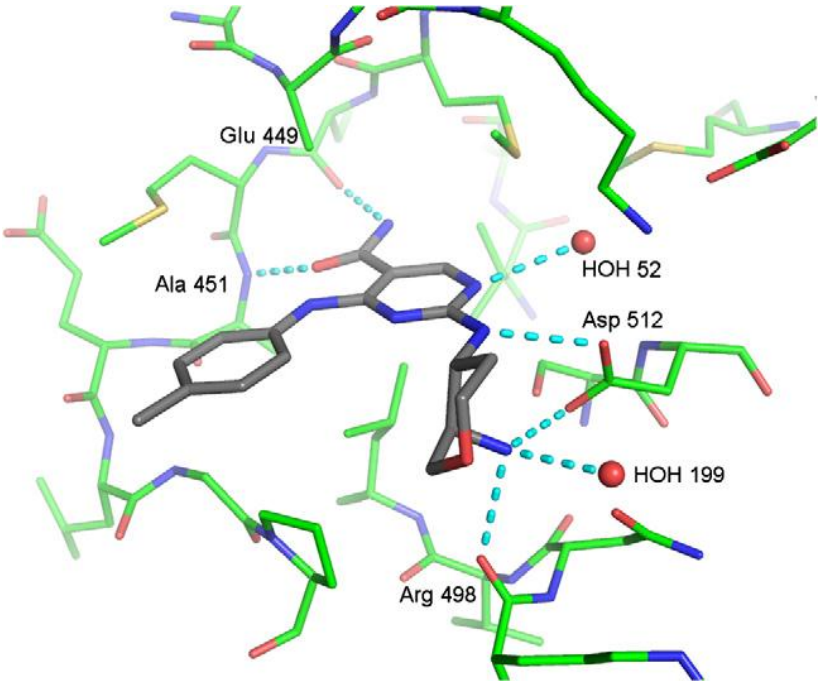


Figure 6.1 The chemical structure of GSK143.

This molecule was kindly donated by GSK for the current study. (Liddle et al., 2011)

6.5 Testing the effects of inhibitors

As mentioned in the introduction of this chapter inhibitors of phospho-kinases of BCR pathway are effective in treating CLL. The main aim of this chapter is to test a new SYK inhibitor, GSK 143, as a potential agent inducing apoptosis

in CLL cells. To optimise the *in vitro* system developed for testing the new SYK inhibitor two known BCR kinase inhibitors were tested. BTK inhibitor PCI-32765 was kindly donated by Pharmacyclics and PI3Kδ kinase inhibitor GS1101 was donated by Gilliad pharmaceuticals. To assess the physiological changes in the pathway both SYKSYK phosphorylation and calcium flux were studied using flow cytometry as described in the previous chapter.

6.5.1. BTK Inhibitor

The initial assessment of the BTK inhibitor showed reduction in percentage viability at day 3 even though it was less compared to fludarabine (Figure 6.2). This response was similar if MNC or negatively selected CLL cells were cultured. The protective effect of stroma was not abrogated, by either BTK inhibitor or combination of fludarabine and BTK inhibitor.

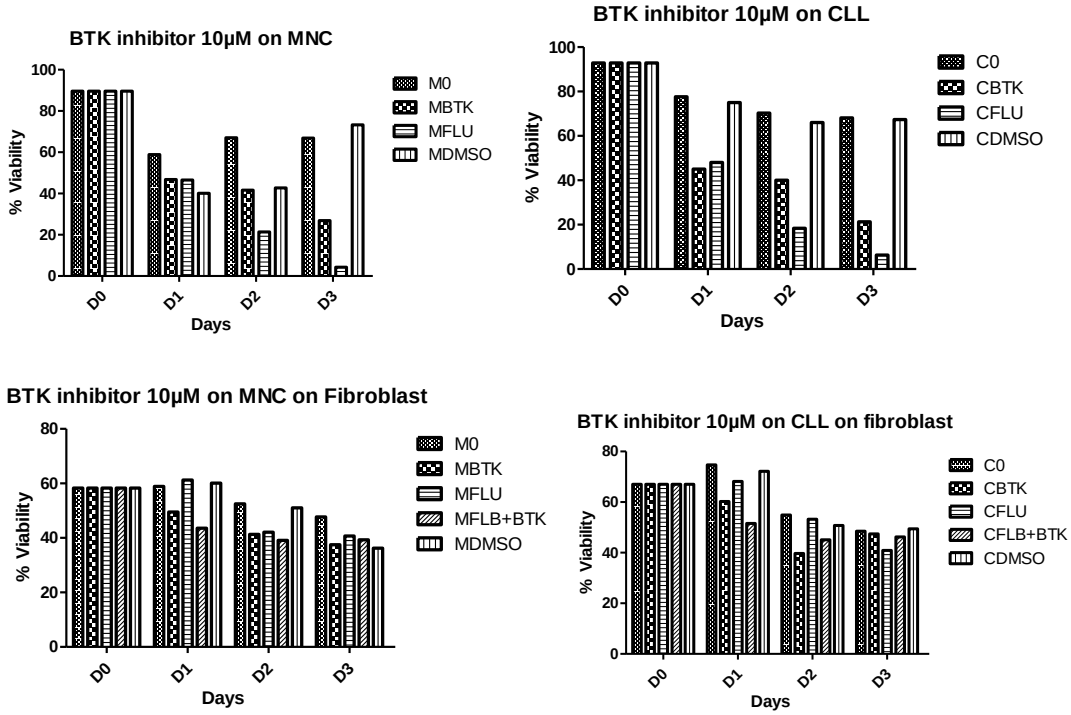


Figure 6.2 Effect of BTK inhibitor on CLL cell viability.

BTK inhibitor was incubated at a concentration of 10μM with either MNC or negatively selected CLL cells with or without fibroblasts. Viability was assessed on day 1, day 2 and day 3. Control samples were also incubated with cells alone or with fludarabine or DMSO (M=MNC, C=negatively selected CLL cells, BTK= BTK inhibitor, Flu=Fludarabine).

There was considerable variation when the BTK inhibitor was tested on multiple patients and there was no statistically significant reduction in viability or cell count when tested in 4 patients, even though 3 patients showed an absence of increment in cell count seen with IgM stimulation when treated with BTK inhibitor (Figure 6.3). Furthermore, the dose-dependency of BTK inhibition was examined using both MNC and negatively selected CLL cells (Figure 6.4). The dose response curve was not sufficiently conclusive to produce an IC50.

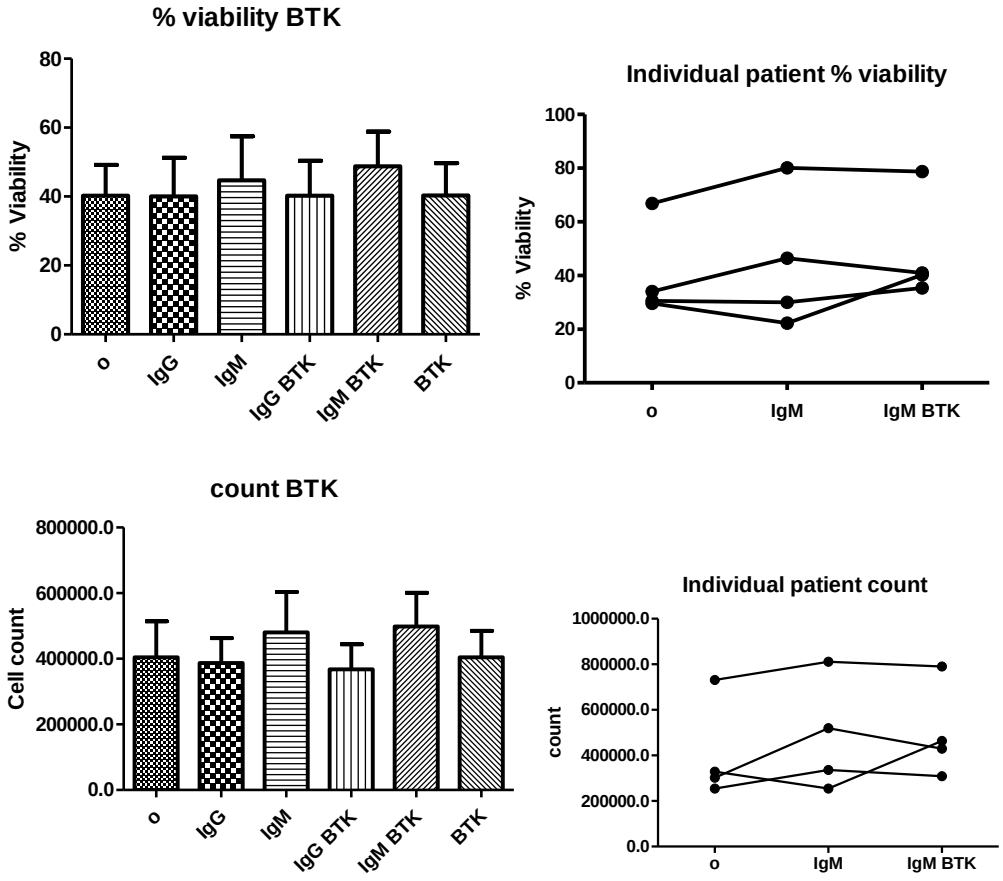


Figure 6.3 MNC were incubated with BTK inhibitor at a concentration of 10µM.

Antigenic stimulation was performed using the $F(ab)_2$ portion of anti-IgM and $F(ab)_2$ anti-IgG was used as a negative control. The left panel shows the mean values and the right panel shows the individual sample spread. There was no significant reduction in viability or cell count with BTK inhibitor.

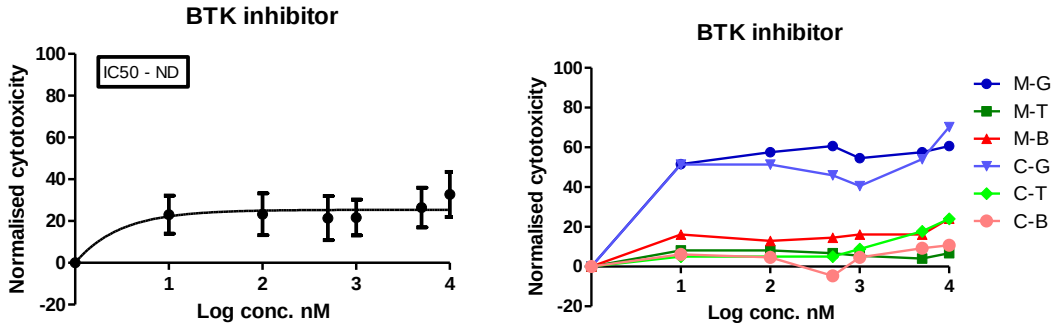


Figure 6.4 Dose response curve for BTK inhibitor.

The Y-axis represents normalised cytotoxicity which is 100 minus nomalised percentage viability. Normalisation was done to the sample without the inhibitor. The X-axis represents the log of the concentration in nM (1, 10,100, 500, 1000, 5000 and 10000). IC50 was calculated with graphpad prism software using a variable slope equation. The 0-value (defines the incubation with media) was demonstrated as 0.01 due to the log application on the X-axis. The bottom graph shows the response in an individual sample. 6 samples from 3 patients were tested. From each patient MNC (dark colour) and negatively selected CLL cells (light colour) were analysed. (M=MNC, C=CLL, G, T and B are different patient samples)

6.5.2. GS-1101 (CAL-101)

PI3Kδ kinase inhibitor GS-1101 was tested next. MNC were incubated with 10μM GS-1101 and viability assessed after 48 hours (n=6). There was significant reduction in the percentage viability when samples were treated with GS-1101 (Figure 6.5).

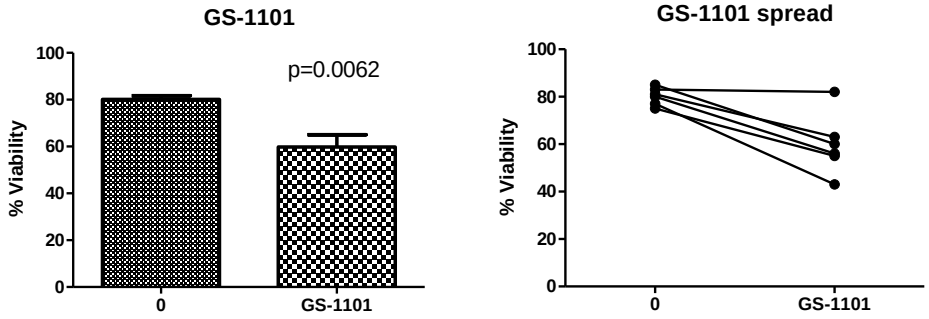


Figure 6.5 Effect of GS-1101 on viability.

MNC were incubated with 10μM GS-1101 (n=6). Percentage viability assessed after 48 hours showed significant reduction in viability with GS-1101. p value was calculated using a paired t test. The right graph shows the spread of individual samples. 10μM was selected from literature where there was definite reduction in viability (Herman et al., 2010)

IgM induced survival benefit was abrogated by GS-1101 as demonstrated in Figure 6.6. As GS-1101 inhibits a downstream molecule in the BCR pathway, attempts were made to correlate SYK and ZAP70 expression with cytotoxicity of GS-1101. Based on flow cytometric evaluation, the level of SYK expression significantly correlated with GS-1101 induced cytotoxicity while ZAP70 expression has no correlation (Figure 6.7).

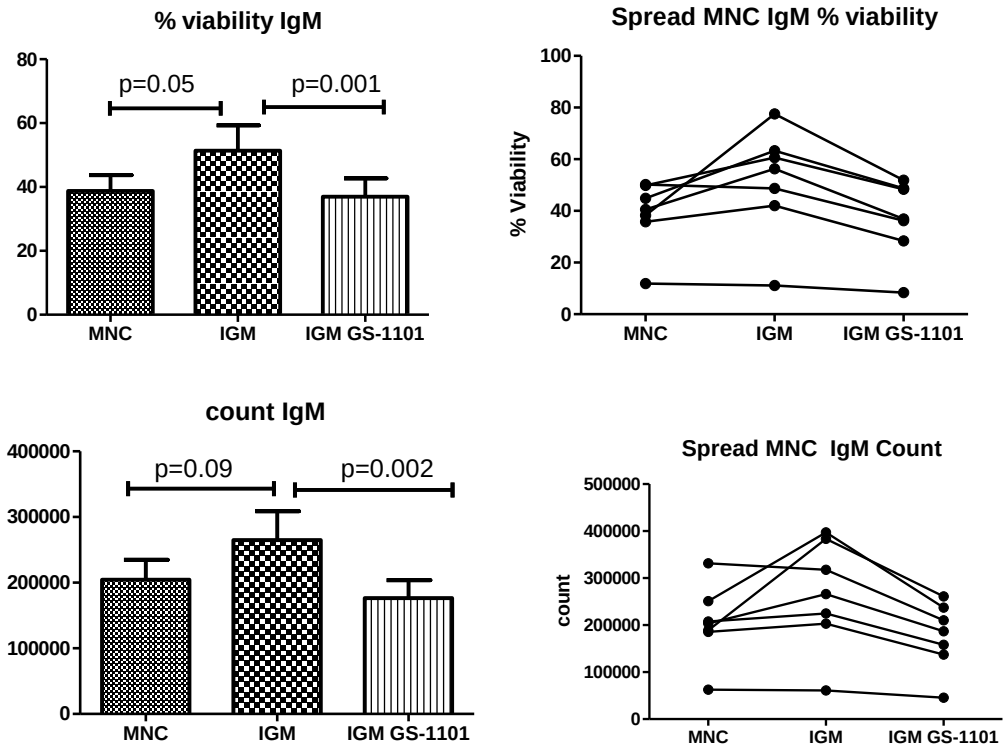


Figure 6.6 Effect of GS-1101 on anti-IgM induced survival benefit.

MNC were incubated with 10μM GS-1101 (n=7) and $F(ab)_2$ anti-IgM in corresponding wells. Percentage viability assessed after 48 hours showed significant reduction in viability with GS-1101 compared to samples incubated with IgM alone. p value was calculated using a paired t-test. The right graph shows the spread of individual samples.

The inhibition of calcium flux in response to anti-IgM stimulation by GS-1101 was assessed. The observed response was variable among patients as shown in Figure 6.8. In example 1 there was a complete inhibition of response to anti-IgM and in example 2 there was no inhibition at all. In the first case the degree of maximal flux was less than that in the second sample and may therefore have been more susceptible to inhibition.

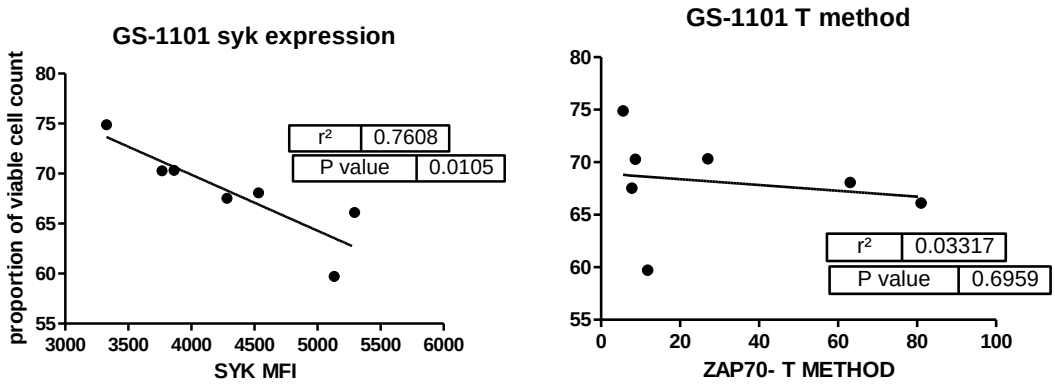
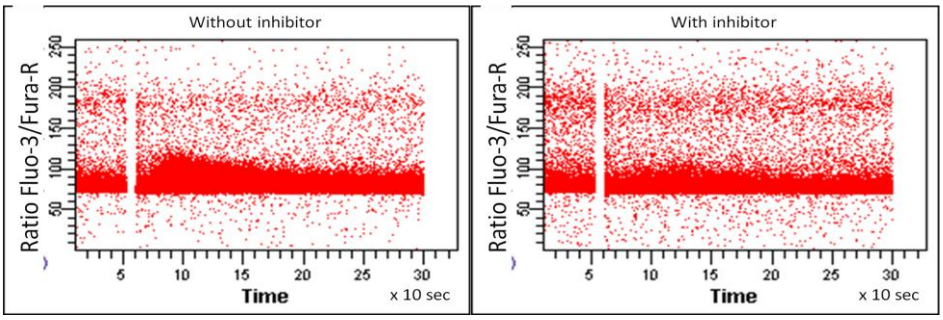


Figure 6.7 Correlation of SYK and ZAP70 expression and effect of GS-1101 on cell viability.

Proportion of viable cell count = (viable cell count in GS-1101+IgM treated cell/ IgM treated cells) x100. Decrease in viability correlated directly with SYK expression (top panel) but has no significant correlation with ZAP70 expression (bottom panel). Correlation was calculated using linear regression. ZAP70 expression in CLL cells was calculated by T-method which was taken as the percentage of CLL cells above the lower limit of ZAP70 expression in T-cells (Rossi et al., 2010).

Example of Inhibition of ca flux by GS-1101



Example of Ca Flux not inhibited by GS-1101

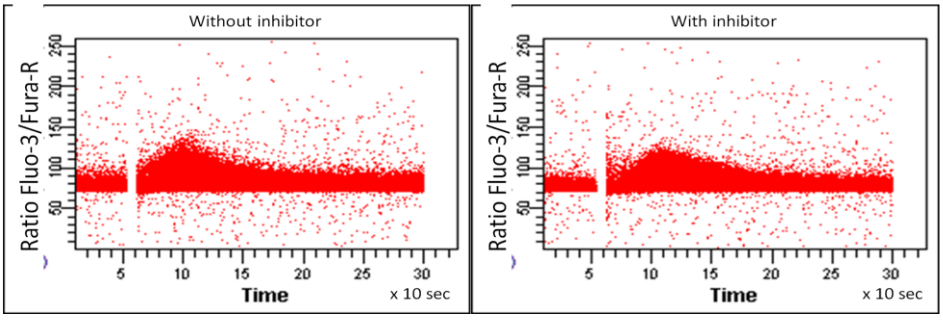
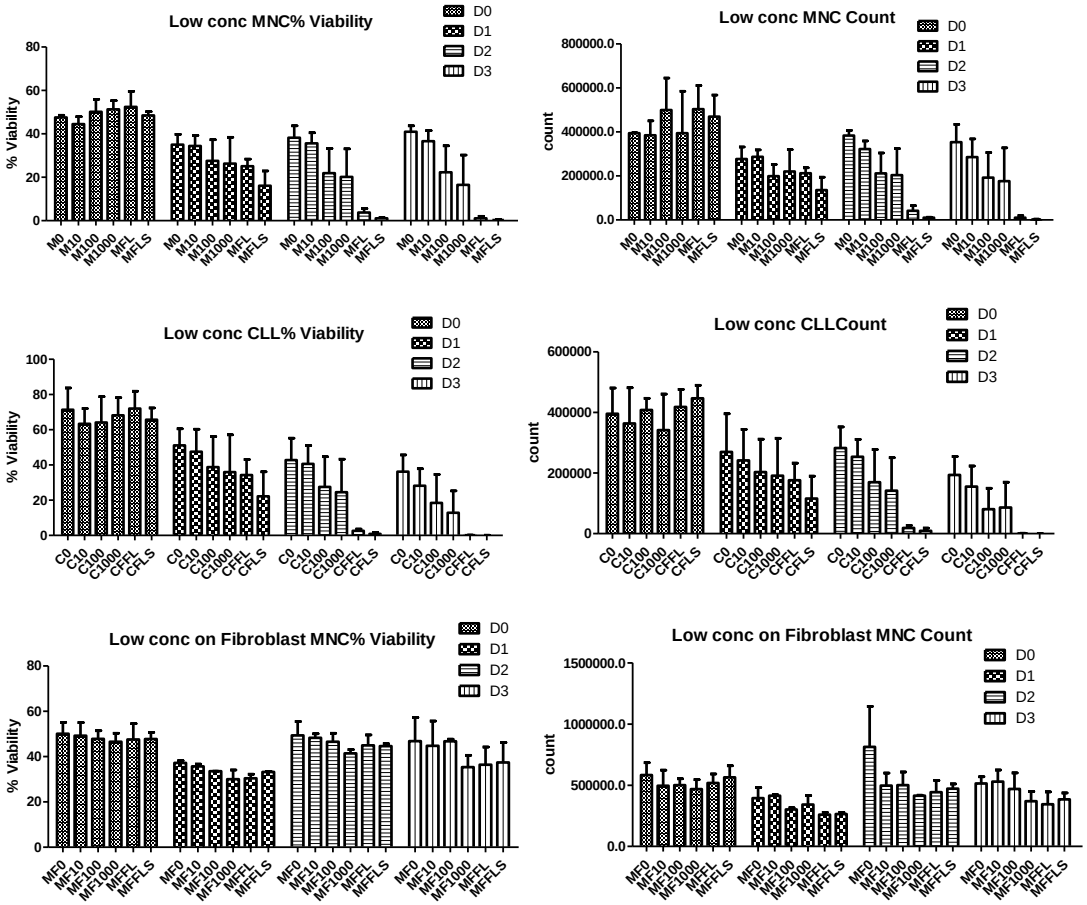


Figure 6.8 Effect of GS-1101 on calcium flux in CLL cells.

GS-1101 has variable effect on calcium flux in CLL cells. The left side plots are samples not incubated with the inhibitor and the samples on the right are preincubated with GS-1101 for 30 minutes before the stimulus was applied.

6.5.3. SYK Inhibitor GSK143

The selective SYK inhibitor GSK143 has never been tested in a similar *in vitro* system. To establish the effects of GSK143, first MNC and negatively selected CLL cells were incubated with the drug at concentrations ranging from 10nM to 1μM (Figure 6.9). Cells were plated with or without the presence of stromal cells. Cells treated with fludarabine were also incubated in parallel to compare the efficacy. Viability was assessed sequentially every 24 hours for 3 days. The viability and count of the cells incubated in presence of the drug at concentration above 100nM was lower compared to control cells, but the effect was not as pronounced as fludarabine. Dose response curve using normalised cytotoxicity as the end point gave an IC50 of 32.3nM (95% CI-5.74 to 18261) (Figure 6.10). At the concentrations tested the stromal protective effect was not abrogated by the presence of GSK143 even when combined with fludarabine. This implies that cytoprotection by stromal cells is not mediated through SYK pathway.



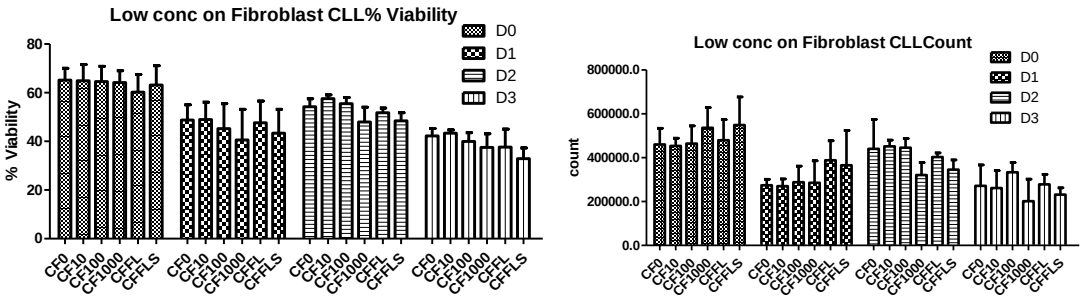


Figure 6.9 Effect of SYK inhibitor GSK143 on CLL cell viability.

SYK inhibitor GSK143 was incubated at a concentration of 10nM to 1µM with either MNC or negatively selected CLL cells with or without fibroblasts. Viability was assessed on day 1, day 2 and day 3. Control samples were also incubated with cells alone or with fludarabine (M=MNC, C=negatively selected CLL cells, F=fibroblasts, Fl=Fludarabine, S=SYK inhibitor).

Higher concentrations from 1uM to 10µM were also tested to see whether stromal protection could be overcome (Figure 6.11). But even at higher concentrations the stromal protective barrier was not broken by GSK143.

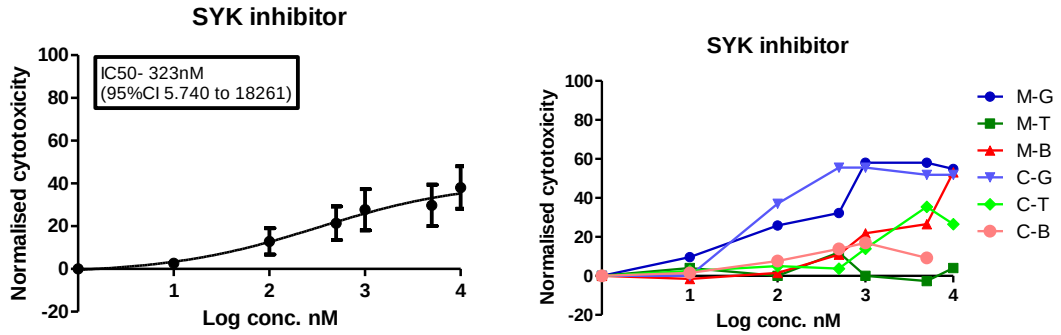


Figure 6.10 Dose response curve for SYK inhibitor.

The Y-axis represents normalised cytotoxicity which is 100 minus nomalised percentage viability. Normalisation was done to the sample without the inhibitor. The X-axis represents the log of the concentration in nM (1,10,100,500,1000, 5000 and 10000). IC50 was calculated with graphpad prism software using variable slope equation. The 0-value (defines the incubation with media) was demonstrated as 0.01 due to the log application on the X-axis. The bottom graph shows the response in individual samples. 6 samples from 3 patients were tested. From each patient MNC (dark colour) and negatively selected CLL cells (light colour) were analysed (M=MNC, C=CLL, G, T and B are different patient samples).

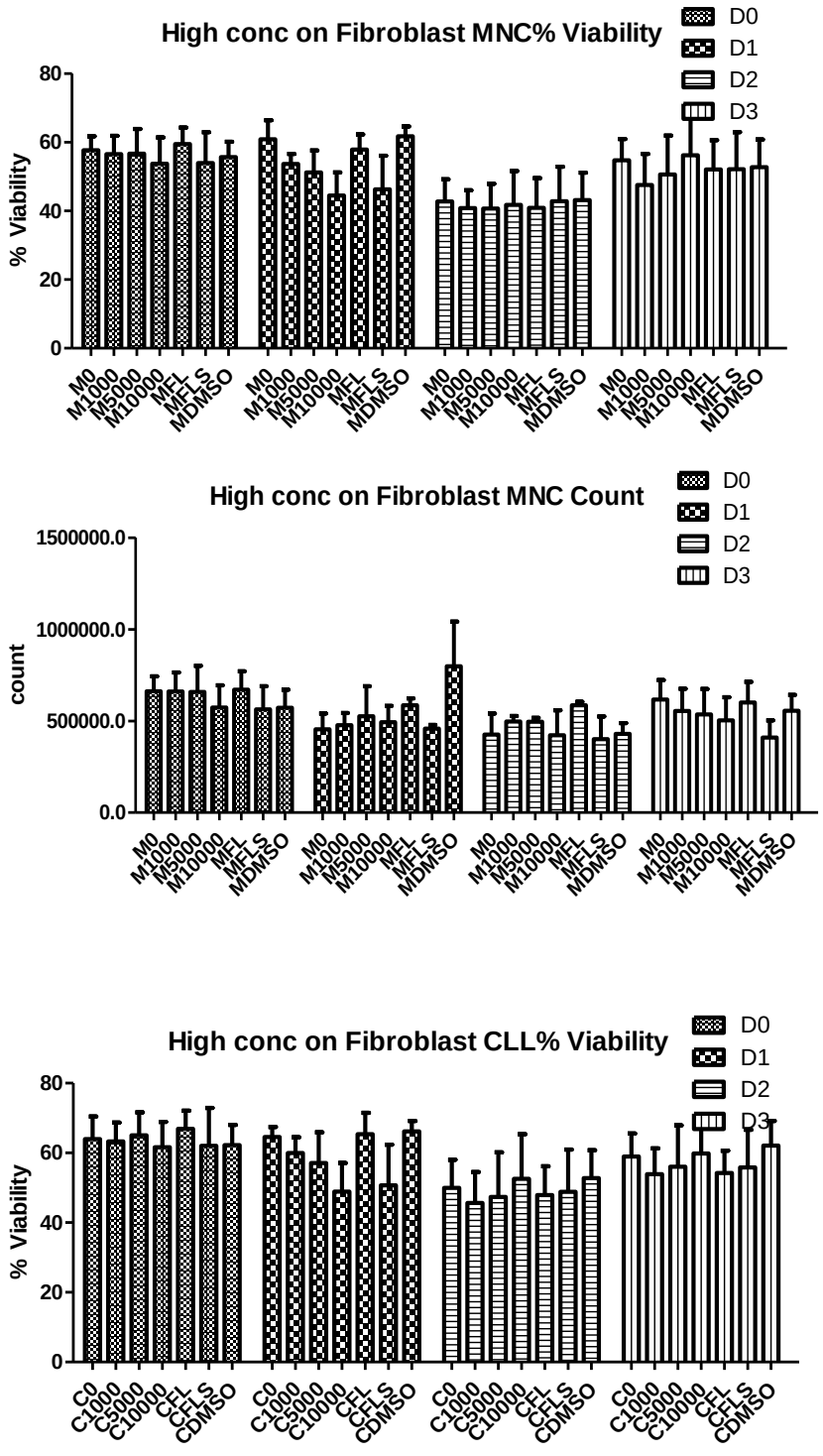


Figure 6.11 Higher dose of SYK inhibitor GSK143.

SYK inhibitor GSK143 was tested at a higher concentration ranging from 1μM to 10μM with either MNC or negatively selected CLL cells in presence of fibroblasts. Viability was assessed on day 1, day 2 and day 3. Control samples were also incubated with cells alone or with fludarabine and DMSO (M=MNC, C=negatively selected CLL cells, S= SYK inhibitor, Fl=Fludarabine).

6.5.3.1. Response to IgM stimulation

The above experiments demonstrated that GSK143 was effective only when CLL cells were not cultured in the presence of stromal cells. To determine whether BCR triggering is sufficient to overcome the effect of the inhibitor, MNC from CLL patients were treated with the drug with or without accompanying IgM stimulation. GSK143 abrogates the survival response to IgM as observed in Figure 6.12. Even on prolonged incubation for up to 14 days there was no significant change to the viability when incubated with stromal support.

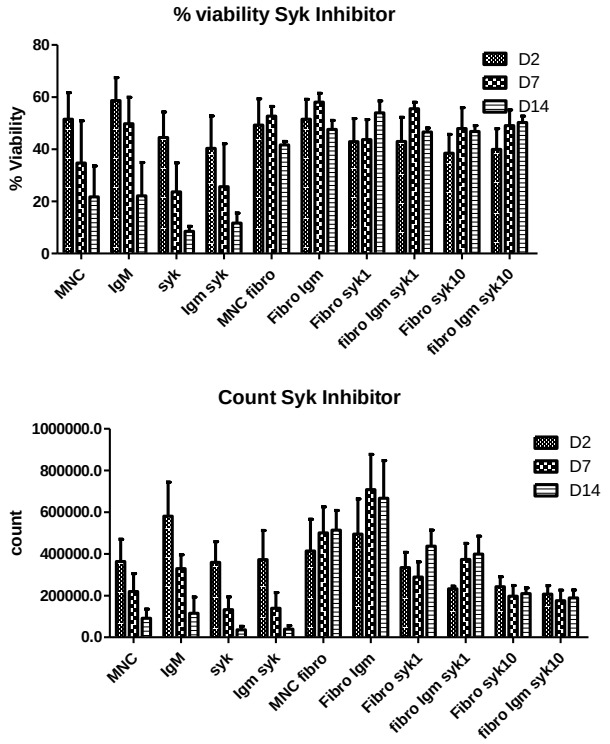


Figure 6.12. Response to stimulation by anti-IgM and prolonged incubation with fibroblasts.

MNC (n=4) were incubated with GSK143 at two different concentrations (1 and 10μM) and anti-IgM with or without stroma. Plates were incubated up to day 14 and viability assessed at day 2, 7 and 14.

The effect of GSK143 was then tested on multiple patients with or without IgM stimulation. GSK143 significantly eliminated the advantage in viability and count induced by IgM stimulation as shown in Figure 6.13. Deletion of chromosome 17p13.1 containing the p53 gene is one of the strongest laboratory predictors of CLL response to chemotherapy (Byrd et al., 2006). Similarly, *IGHV* gene mutational status has strong influence on the duration of remission to standard therapies (Montillo et al., 2005). Attempts were made to

compare the effect of the drug on viability of CLL cells from patients with these prognostic markers. These markers were done routinely as a standard investigation for these patients in the laboratory. There was no significant difference in response between *IGHV* mutated and unmutated group or p53 deleted and non-deleted group (Figure 6.14). However these results should be interpreted with caution as the sample size for these comparisons was very low. But this result was consistent with previous studies with similar agents like GS-1101(Herman et al., 2010).

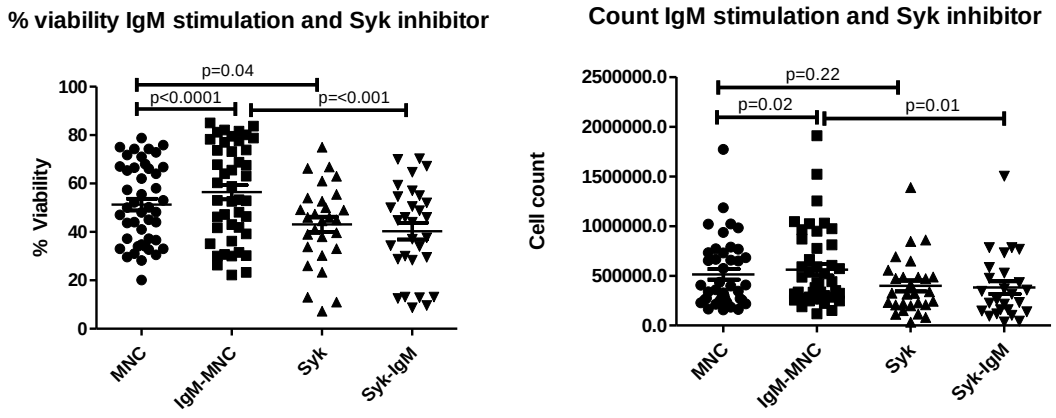
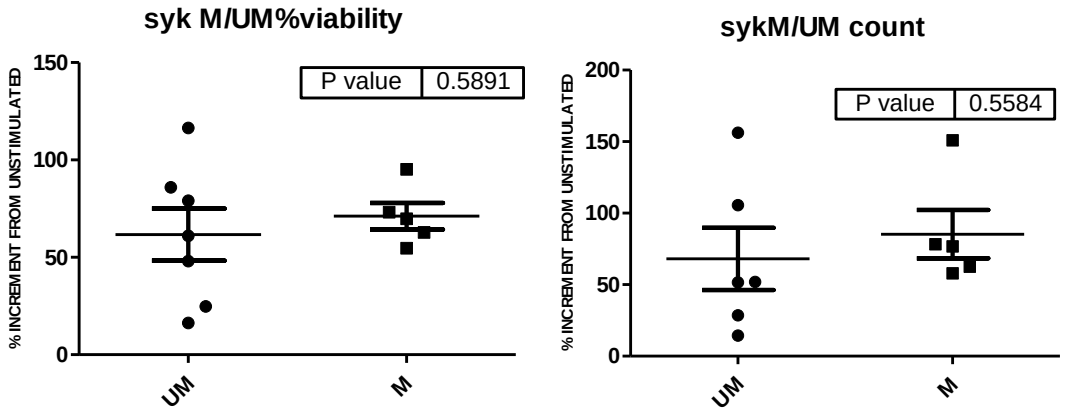


Figure 6.13 Effect of GSK143 on anti-IgM induced survival benefit.

MNC were incubated with 1μM GSK143 (n=35) and F(ab)$_2$ anti-IgM in corresponding wells. Percentage viability assessed after 48 hours showed a significant reduction in viability with GSK143 compared to samples incubated with IgM alone. p value was calculated using a paired t-test. The left graph shows the percentage viability and the right graph shows the count.



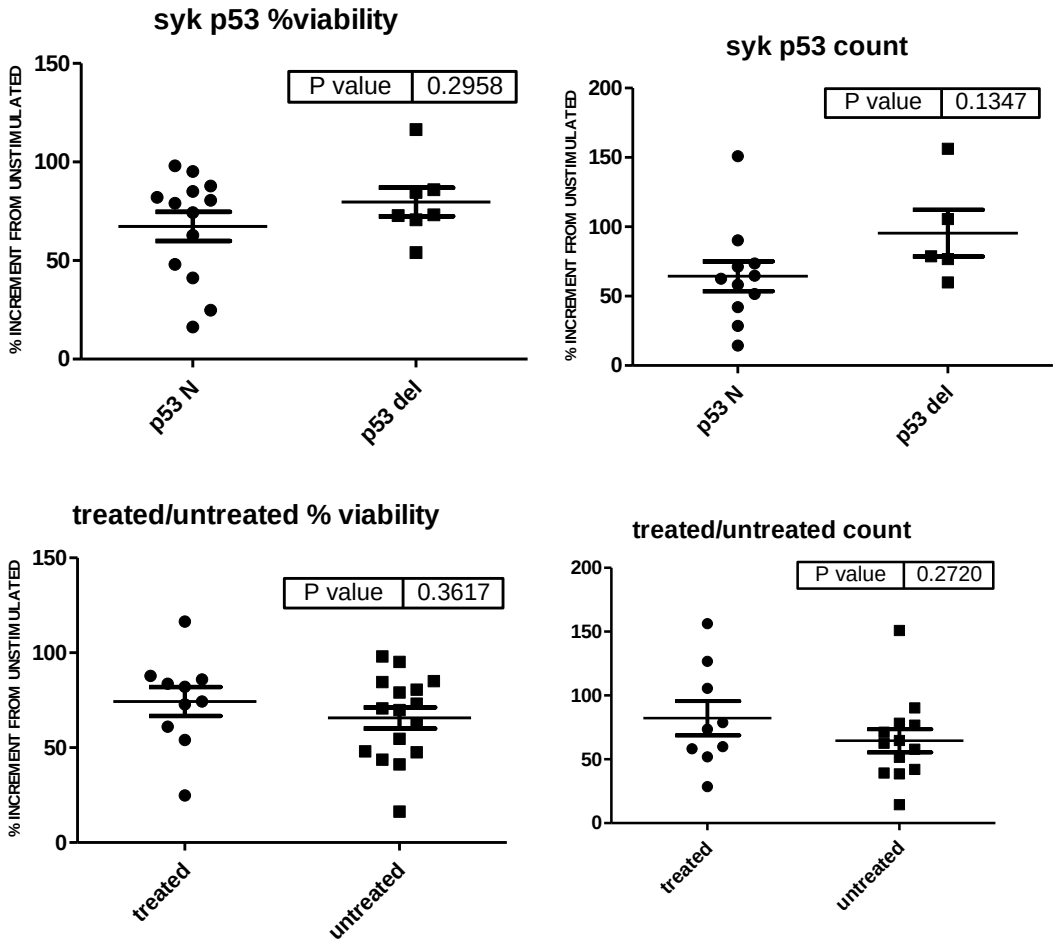


Figure 6.14 Assessment of difference in response to GSK143 with respect to various established prognostic markers.

Markers included: mutated/unmutated(U/UM), p53deleted/not deleted (p53del/N) and previously treated/not treated patients. P value was calculated using unpaired t-test. There was no statistically significant difference between these prognostic groups in viability or cell count even though the number of samples may be low to establish these difference.

6.5.3.2. Response of SYK inhibition to single and multiple exposures to BCR stimulation

The above experiments were carried out using a single application of anti-IgM. *In vivo* CLL cells are potentially exposed to antigen more than once. To determine whether repeated IgM ligation would have an effect on the response to GSK143, MNC were exposed to multiple rounds of anti-IgM exposure (Figure 6.15). Repeated stimulation of IgM did not abolish the pro-apoptotic response induced by GSK143.

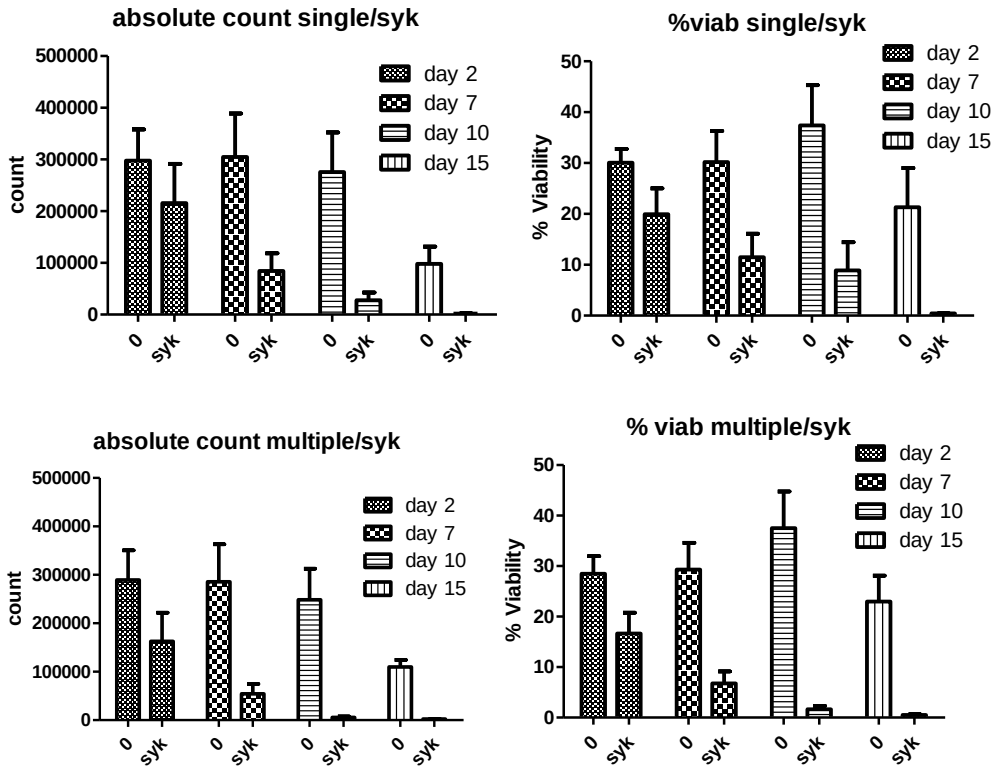
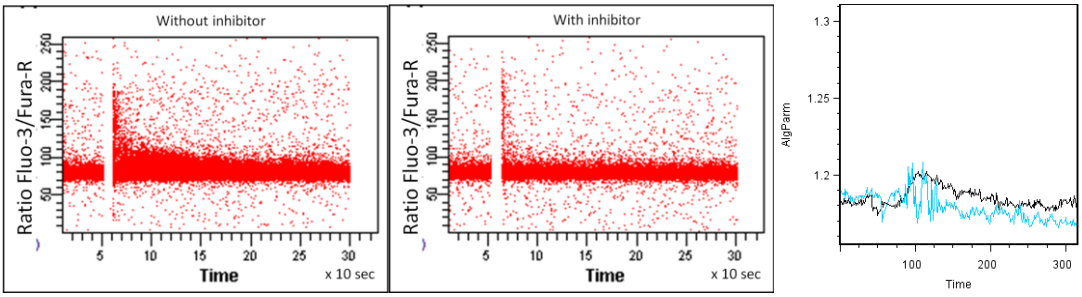


Figure 6.15 Single and multiple exposure to F(ab)$_2$ anti-IgM in presence of SYK inhibitor.

In single exposure 10µg/ml of anti-IgM was added once when the cells were plated. In multiple exposures anti-IgM was added every 24 hours until the wells were harvested for testing viability.

6.5.3.3. Calcium flux and SYK phosphorylation

To assess the physiological response of SYK inhibition, calcium flux and SYK phosphorylation were tested. The cells were incubated with GSK143 for 30 minutes at 37^{o}C before stimulating with anti-IgM. All other steps of the experiment were similar to the one described in previous chapter. As demonstrated in Figure 6.16, pre-incubating with GSK143 can inhibit the calcium flux induced by IgM stimulation. As the compound was dissolved in DMSO control experiments with DMSO were also performed to establish that the vehicle had no influence on calcium flux (Figure 6.17).



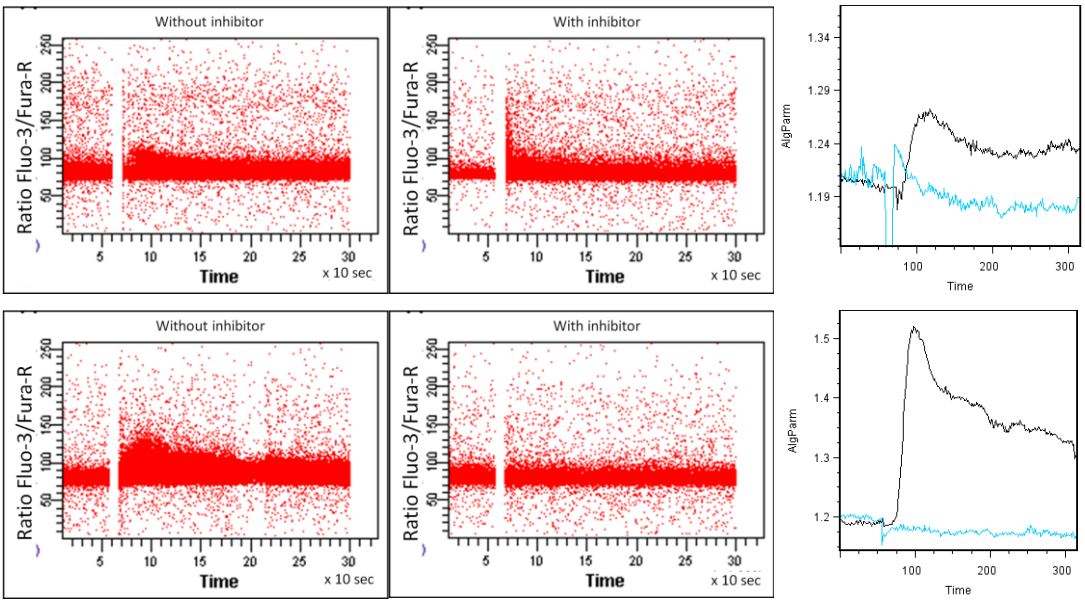


Figure 6.16 Examples of three types of response to IgM stimulus are shown.

(same as Figure 5.3) Flowplots are shown in the first two columns The one on the left is without the inhibitor and the one on the right is with the inhibitor . The third column shows the graphical analysis done by flowJo software. The black and blue lines represent the stimulation using IgM without or with the presence of a SYK inhibitor, respectively.

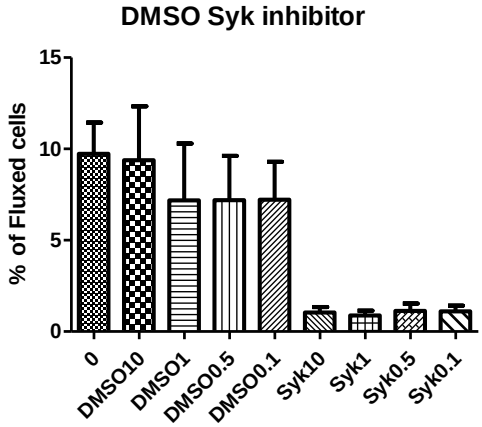


Figure 6.17 Effect of DMSO on calcium flux.

Three different concentrations of DMSO, which were used to dissolve the SYK inhibitor, were assayed for the effect on Ca^{2+} flux in response to IgM ligation. DMSO had no any effect on Ca^{2+} flux on the concentrations used to dissolve GSK143.

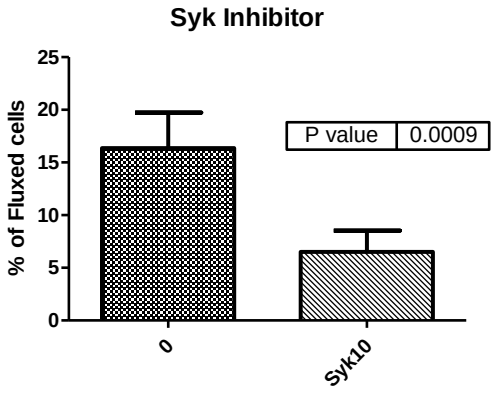


Figure 6.18 Calcium flux with SYK inhibitor.

Calcium flux was assessed on parallel samples with or without pre-incubating with the SYK inhibitor (n=9). P value was calculated using paired t-test. There is a significant drop in Ca^{2+} flux induced by IgM ligation when pre-incubated with GSK143.

SYK phosphorylation status was also tested to assess the downstream signal response. It has been previously established that there is a definite shift in the MFI of fluorochrome conjugated anti-SYK phophoprotein when cells are stimulated with anti-IgM or IgD compared to unstimulated cells. This shift is partially abrogated by preincubating cells in GSK143 before stimulating it with anti-IgM, however the effect on IgD-mediated phosphorylation is more pronounced. Example flow plots are shown in Figure 6.19 and the mean MFI calculated on 5 different samples shows the significance of the observation (Figure 6.20). These results suggest that CLL cells may be differentially susceptible to GSK143 according to the relative dependency on IgM signalling.

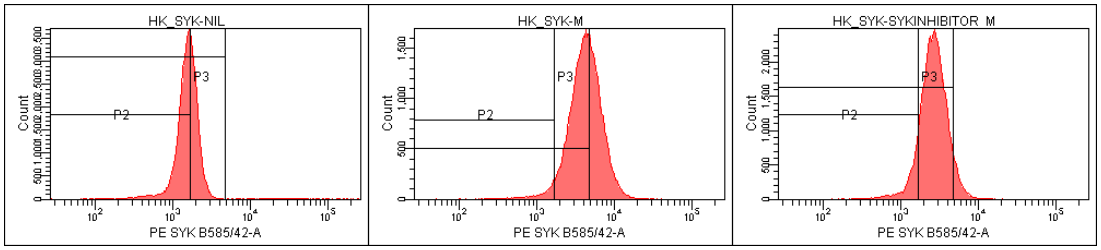


Figure 6.19 SYK phosphorylation by IgM stimulation on CLL cells is partially inhibited by GSK143.

The response to IgM stimulation on SYK phosphorylation measured by MFI of phospho SYK antibody in the presence of GSK143. The plots depict levels after 60 sec of stimulation.

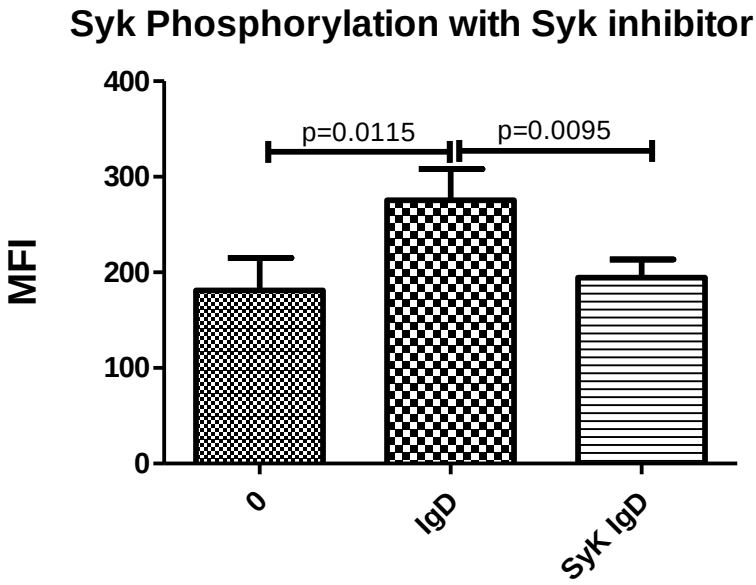


Figure 6.20 Effect of IgD stimulation and GSK143 on SYK phosphorylation.

MNC from CLL patients (n = 5) were pre-incubated with DMSO or GSK143 and then stimulated with anti-IgD for 60 seconds. The amount of SYK phosphorylation was determined by intracellular staining and flow cytometry. P value was calculated using paired t-test.

6.6 Discussion

The above experiments demonstrated that the established *in vitro* system was suitable to test BCR kinase inhibitors. GS-1101, a previously proven inhibitor was tested and was found to be functioning. The system was then extended to test a new inhibitor to SYK phosphoprotein, a proximal molecule in the BCR pathway. The inhibitor had been previously established to be more specific for SYK inhibition compared to other kinases as described in the introduction of this chapter (Liddle et al., 2011). It was established that the SYK inhibitor GSK143 induces apoptosis in CLL cells when plated as MNC or negatively selected CLL cells on their own. This is comparable to dose- and time-dependent cytotoxicity mediated by GS-1101 as shown by Herman et al. Similar to their experiment there was no significant difference in cytotoxicity between prognostic groups based on their cytogenetics and *IGHV* mutational status (Herman et al., 2010). It also abrogates the pro-survival signal delivered by BCR stimulation given in the form of $F(ab)_2$ portion of anti-IgM which is comparable with the effects noticed with R406. In the paper by Quiroga et al. CLL cells stimulated with anti-IgM displayed an increased

viability of 144.6% plus or minus 13.1% at 48 hours of respective controls and treatment with R406 reduced the viability to 62.7% plus or minus 6.9% (Quiroga et al., 2009b). In contrast GSK143 failed to eliminate the fibroblast induced stromal support established in the *in vitro* system either by itself or in the presence of fludarabine. This observation is contradictory to the findings in a study where the authors showed that GS-1101 abrogated the protective effect established by stromal cells (Herman et al., 2010). Several variabilities could play a part in this discrepancy, including the type of stromal cells (the GS-1101 study used HS-5 cell line), the difference in the drug itself or the differences between patients. More experiments are required to determine if the failure to overcome the protective effect of stromal cells is specific to GSK143. Further physiological stimuli in the pathway were explored in the form of phosphorylation of SYK and alteration in calcium flux. It has been shown that both SYK phosphorylation and calcium flux induced by BCR stimulation can be significantly inhibited by GSK143 thereby showing evidence of disruption of the pro-survival signal established by this pathway. The study withR406 has shown similar effects but the phosporylation was demonstrated by immunoblotting than flow cytometry (Quiroga et al., 2009b). In summary, GSK143 is a potent inhibitor of BCR signal transduction in CLL cells and appears to behave in a comparable fashion with existing kinase inhibitors that therapeutically target this pathway.

7. Expression of neuronal markers in CLL and their potential therapeutic role

An intriguing observation noted in the antigen expression study was that, of the 23 antigens, which showed some expression on CLL cells, 8 of them had a significant role in neurotransmission. The following neuronal markers were expressed on CLL cells and their functional role in nervous system is briefly explained below.

7.1 GPR18

GPR18 is involved in *N*-arachidonoyl glycine (NAGly) signalling, which is an endogenous metabolite of the endocannabinoid anandamide and acts as an efficacious agonist at GPR18 (Bradshaw et al., 2009). It induces migration, proliferation and perhaps other MAPK-dependent phenomena involving recruitment of microglia to sites of neuronal injury (McHugh et al., 2010). GPR18 is highly expressed in peripheral blood leukocytes and several haematopoietic cell lines (Kohno et al., 2006) as well as being highly expressed in the spleen ("N-arachidonyl glycine receptor - Homo sapiens (Human)," n.d.). It is reported to have anti-inflammatory effects mediated through NAGly (McHugh et al., 2010).

7.2 APLP1

APLP1 plays a role in synaptic function by localising to the 'postsynaptic density' which is a specialised region containing proteins required for signalling (Kim et al., 1995). APLP1 increases during cortical synaptic development, suggesting a role in synaptogenesis or synaptic maturation. It's C-terminal fragment, ALID1, which is processed by gamma-secretase, triggers transcription activation through APBB1 (Fe65) binding (Walsh et al., 2003). It can regulate neurite outgrowth through binding to components of the extracellular matrix such as heparin and collagen ("Amyloid-like protein 1 precursor - Homo sapiens (Human)," n.d.).

7.3 GPR12

GPR12 Tanaka *et al* has shown that GPR12 along with GPR3 and GPR6 plays a key role in neurite outgrowth through Gs signalling and upregulating

cAMP. Neuro2a neuroblastoma cells get transformed into neuron-like cells once transfected with GPR12 (Tanaka et al., 2007). X. Lu *et al*. has shown that GPR12 transfected PC12 cells (cell line derived from a pheochromocytoma of the rat adrenal medulla) differentiate into neuron-like cells as evidenced by enlarged cell size and neurite outgrowth. This study also showed that GPR12 induced neuronal differentiation and synapse formation was mediated by Erk1/2 phosphorylation and significantly increased the expression of Bcl-2, Bcl-xL and synaptophysin (SYP) (Lu et al., 2012).

7.4 TAG1

TAG1 is a member of the immunoglobulin superfamily that functions as a cell adhesion molecule. It is a glycosylphosphatidylinositol (GPI)-anchored neuronal membrane protein that may be involved in the formation of axon connections in the developing nervous system (Walsh and Doherty, 1991). The path-finding of axons toward their targets is an early crucial step in the development of the nervous system. The extension of axon and its localisation in the appropriate position involves selective interactions between molecules on the surface of the axon and those in the local microenvironment. *In vitro* functional studies of the axonal surface showed that several glycoproteins are involved in cell adhesion and in the promotion of neurite outgrowth. Some of these glycoproteins, including TAG1, are expressed by restricted subsets of central and peripheral neurons during the initial phase of neurite outgrowth (Dodd et al., 1988). It may also be involved in glial tumourogenesis and this could have potential implication on therapeutic interventions (“OMIM Entry - * 190197 - CONTACTIN 2; CNTN2,” n.d.) (“CNTN2 contactin 2 (axonal) [Homo sapiens (human)] - Gene - NCBI,” n.d)**.**

7.5 5-HTR$_7$

5-HTR$_7$ is a receptor for the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). 5-HT is involved in a wide range of neuron activity, with its different physiological roles mediated through interaction with multiple receptors. These receptors decide the functional outcome of the neurotransmission by serotonin and have been implicated as playing important roles in certain pathological and psychopathological conditions (Glennon et al., 2002). Seven distinct families of 5-HT receptors have been identified (5-HT$_1$–5-HT$_7$), and several of these receptors have subpopulations (Glennon et al., 2002). A total of 15 subpopulations have been cloned thus far. The 5-HT$_7$ receptor is

expressed mainly in the central nervous system, but a low level of expression has been detected in the peripheral nervous system also. It has been cloned from several species including rat, mouse, guinea pig and human. Two splice variants have been identified in the rat. The short form contains 435 amino acids and the long form, contains 448 amino acids. The orthologous human 5-HT_7 receptor has 445 amino acids. There is <50% transmembrane sequence homology between 5-HT_7 receptors and other 5-HT receptors. Gene mapping studies have shown that the human 5-HT_7 receptor gene is located on chromosome 10. Physiologically 5-HT_7 receptors might be involved in mood changes and learning and possibly maintenance of circadian rhythm (Glennon et al., 2002). It has been proposed that 5-HT mediated relaxation of canine coronary artery may be mediated by 5-HT_7 receptors. Pathologically it is thought that 5-HT_7 receptors may play a role in certain psychiatric disorders like depression. Several agents with high affinity for 5-HT_7 receptors are known, with serotonin, 5-CT, 5-methoxytryptamine, and 8-OH DPAT acting as agonists, whereas methiothepin, mianserin, mesulergine, ritanserin, spiperone, NAN-190, LY215840 and clozapine acting as antagonists (Glennon et al., 2002). Several anti-depressants and anti-psychotic agents like fluphenazine, acetophenazine, chlorprothixene, zotepine, clorotepine, clozapine, fluperlapine, pimozide, tiospirone, and risperidone have high affinity for 5-HT_7 receptor. Multiple lines of evidence suggest that the 5-HT_7 receptor is positively coupled to adenylate cyclase which links the various agonists to downstream effects (Glennon et al., 2002).

7.6 ACCN1

ACCN1 is a member of the family of cation channels with high affinity for sodium, which is gated by extracellular protons and inhibited by the diuretic amiloride (Waldmann et al., 1996). It encodes the Mammalian Degenerin (MDEG) protein, a proton-gated channel permeable to sodium, lithium and potassium. The function of these channels is to generate ionic currents involved in neurotransmission (Lingueglia et al., 1997). Two isoforms, MDEG1 and MDEG2, with different biological properties have been isolated (Lingueglia et al., 1997). MDEG1 is mainly expressed in the postsynaptic membrane of granule cells and in the Purkinje cells of the cerebellum. It is activated by a low pH (Lingueglia et al., 1997). It can form an active ionic channel either on its own, or by binding other proteins of its family. MDEG2, on the other hand is mainly expressed in sensory neurons of the brain. It cannot form an active ionic channel on its own, but can form an active ionic

channel by forming heterodimeric molecule with another protein of its family. There is evidence that MDEG participates in mechanosensation, perception of taste, perception of pain and possibly in neurotransmission and neuromodulation (Wemmie et al., 2006). Pathologically there is convincing evidence showing the role of *ACCN1* in development of multiple sclerosis (Bernardinelli et al., 2007).

Two molecules identified in the antigen analysis that have been studied in depth in the current project are nicotinic acetyl choline receptor subunit β 4 (CHRNB4) and dopamine receptor D4 (DRD4). CHRNB4 was selected initially as it showed the highest expression in the antigen identification study. DRD4 was then selected as it also showed good expression in the antigen identification study and there were several dopamine receptor type specific agonists and antagonists available commercially which could be explored for therapeutic potential.

7.7 Nicotinic acetyl choline receptor

Nicotinic acetyl choline receptor is the major receptor involved in transmission of impulse along the neuro-muscular junction, where it mediates fast chemical transmission of electrical signals in response to ACh released from the nerve terminal into the synaptic cleft. The nicotinic ACh receptor is a member of the pentameric “Cys-loop” superfamily of ligand gated ion channels that contain a pair of disulphide-bonded cysteines in their amino terminus, which are separated by 13 residues (Karlin, 2002) (Kao and Karlin, 1986) (Ortells and Lunt, 1995) (Tsunoyama and Gojobori, 1998).This family includes neuronal and muscle type ACh receptors, γ-aminobutyric acid type A ($GABA_A$) and $GABA_C$ receptors, 5-HT3 receptors, glycine receptors, invertebrate glutamate and histidine receptors. It is a glycoprotein formed by 5 subunits assembled in a circular order, like barrel staves around a central channel. There are 17 subunit subtypes and they assemble together depending on the type of receptor. There are two major classes of receptors based on their expression pattern- muscular type, seen in the neuromuscular junction and the neuronal type, seen in the central and peripheral nervous system. In case of the muscle-type receptors, α1, β1, ε and δ subunits should assemble at 2:1:1:1 ratio in adult form and in embryonic form ε is replaced by γ. This gives two ligand-binding sites at the α1-δ and α1-γ(ε) subunit interfaces. With neuronal nAChRs, it is either penta-heteromeric combination of several types of a (α2-α10) and β (β2-β4) subunits or penta-homomeric combinations like (α7)5. The

types of receptors based on the subunit and their locations are given in Table 7.1.

Table 7.1 Nicotinic acetyl choline receptors

Type of Receptor	**Location**
Muscle type: $(\alpha1)_2\beta1\delta\varepsilon$ or $(\alpha1)_2\beta1\delta\gamma$	Neuromuscular Junction
Ganglion-type: $(\alpha3)_2(\beta4)_3$	Autonomic ganglia
Heteromeric-CNS-type:(α4)2(β2)3, (α3)2(β4)3	Brain
Homomeric-CNS-type: $(\alpha7)_5$	Brain

Structurally these receptors have three domains- an extracellular ligand binding domain, a transmembrane domain and an intracellular domain (Karlin, 2002). The extracellular domain is found at the N terminus followed by 3 helical transmembrane units, a cytoplasmic unit and another helical transmembrane portion towards C terminal end (Figure 7.1).

There are four functional states described in ACh receptors: the resting (closed) state, the open state, the fast-onset desensitised (closed) state, and the slow-onset desensitised (closed) state. In the absence of agonist the resting state is the most stable state, and the slow-onset desensitized state is the most stable state in the presence of agonist. The open state and the fast onset desensitized state are metastable states, where their concentrations rise transiently and reach a very low value at equilibrium (Karlin, 2002).

Although the neuronal nicotinic receptor gene family is expressed predominantly in brain, there is evidence that they can be localised elsewhere as well. Ganglionic expression of α3, α5, and α7, has been well established (McGehee and Role, 1995)(Lukas et al., 1993) and α7 subunit expression has been reported in developing muscle (Romano et al., 1997). There is now evidence that nicotinic receptors are also found in non-neuronal tissues like lymphocytes, macrophages (Wang et al., 2003) and polymorphonuclear cells (PMN) in the peripheral blood (Hoss et al., 1986) (Hiemke et al., 1996) (Lebargy et al., 1996) small cell lung carcinomas (Quik et al., 1994) skin keratinocytes (Grando, 1997), respiratory epithelial and vascular endothelial cells (Conti-Fine et al., 2000), and T lymphocytes (Kawashima and Fujii, 2000). Their role in these locations is still not known, but such localisation suggests that peripheral nicotinic receptors have a more ubiquitous

expression, and they may have nonsynaptic roles. This might involve calcium modulation and peptide release (Quik et al., 1997) (Zia et al., 1997).

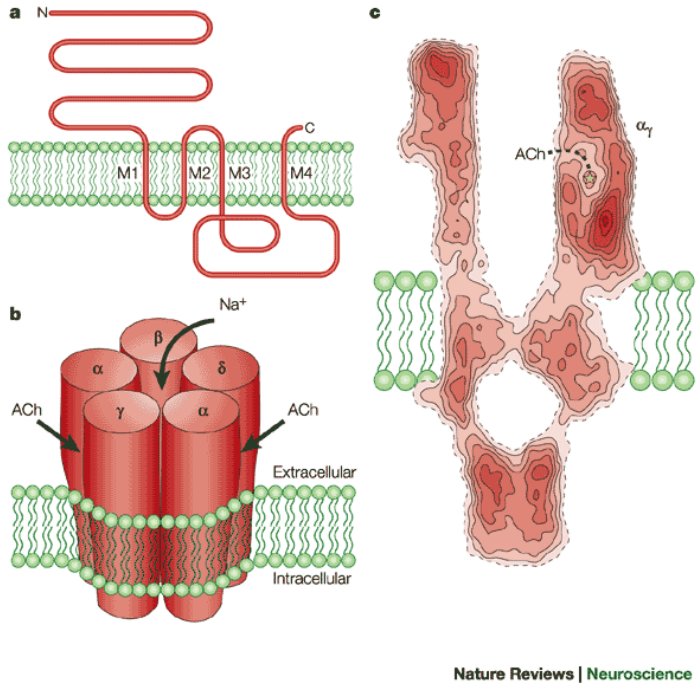


Figure 7.1 Structure of nicotinic acetyl choline receptor.

a | The threading pattern of receptor subunits through the membrane. b | A schematic representation of the quaternary structure, showing the arrangement of the subunits in the muscle-type receptor, the location of the two acetylcholine (ACh)-binding sites (between an α- and a γ-subunit, and an α- and a δ-subunit), and the axial cation-conducting channel. c | A cross-section through the 4.6-Å structure of the receptor determined by electron microscopy of tubular crystals of *Torpedo* membrane embedded in ice. Dashed line indicates proposed path to binding site. Figure and legend taken from Emerging Structure of the Nicotinic Acetylcholine Receptors- *Nature Reviews Neuroscience* (Karlin, 2002).

There is now good evidence to suggest a significant role of neurotransmitters in the immune system. Neurotransmitters, like acetyl choline can come from nerve endings or can be produced by lymphocytes. Acetylcholine receptors like α7 homopentamers are widely expressed in various immune mediating cells like T lymphocytes, B lymphocytes, dendritic cells, monocytes, macrophages, neutrophils, and microglia cells (Wang et al., 2003) (De Rosa et al., 2005). In lipopolysaccharide-stimulated human macrophage cultures, acetylcholine significantly attenuated the release of cytokines (tumour necrosis factor (TNF), interleukin (IL)-1β IL-6 and IL-18), but not the anti-inflammatory cytokine IL-10 (Borovikova et al., 2000). In rats during lethal

endotoxaemia, direct electrical stimulation of the peripheral vagus nerve releases acetylcholine, the principle neurotransmitter in vagus nerve, which inhibits TNF synthesis in liver, attenuates peak serum TNF amounts, and prevents the development of shock (Borovikova et al., 2000).

In T lymphocytes, impairment of cell activation was associated with nicotine treatment as evidenced by chronic exposure of rats to nicotine inhibits the antibody-forming cell response, impairs the antigen-mediated signalling in T-cells, and induces T-cell anergy caused by impairing the antigen receptor-mediated signal transduction pathways and depleting the inositol-1,4,5-trisphosphate-sensitive calcium stores (Kalra et al., 2000). Acetyl choline receptor α7 seems to play a critical role in the immunosuppressive function of CD4+CD25+ Tregs. Nicotine increases Treg-mediated immune suppression of lymphocytes via α7 nAChR which can be reversed by a selective α7 nAChR antagonist, α-bungarotoxin (D. Wang et al., 2010). This effect is mediated by the up-regulation of CTLA-4 as well as Foxp3 expression on nicotine stimulation in Tregs (D. Wang et al., 2010). Nicotine activates the nuclear factor of activated T-cells (NFAT) transcription factor in lymphocytes and endothelial cells, which leads to alterations in cellular growth and vascular endothelial growth factor production. Nicotine induces paradoxical effects on T-cell survival. Treatment with nicotine potentiated FasL expression in activated lymphocytes, which induces the appearance of a caspase or caspase-like activity. This suggests that it could facilitate apoptosis both of target cells that bear nicotinic receptors and of neighbouring cells in their local microenvironment. Paradoxically nicotine induced Survivin expression in primary T-cells upon stimulation that promotes transition across the G0/G1boundary and escape from apoptosis (Oloris et al., 2010).

Interestingly, it is known that lymphocytes synthesise acetylcholine. Rinner *et al.* showed choline acetyl transferase at RNA level in thymic, splenic and peripheral blood lymphocytes of rats using RT-PCR (Rinner et al., 1998). They also measured acetylcholine in thymic, splenic and peripheral blood lymphocytes using a sensitive radioimmunoassay (Rinner et al., 1998). The level of acetylcholine was 3 times higher in T-cells as compared to B-cells, and CD4+ cells showed significantly higher levels as compared to CD8+ cells. This can play a role as autocrine or paracrine functional regulator (Fujii, 2004). This is evidenced by the observation that acute stimulation of nAChRs with ACh or nicotine causes rapid and transient Ca^{2+} signalling in T- and B-cells, probably via α7 nAChRs subunit-mediated pathways. On the other hand

chronic nicotine stimulation causes down-regulation of nAChR expression and suppresses T-cell activity.

Nicotinic acetyl choline receptor subunit expression has shown conflicting results in the literature. Hiemke *et al* demonstrated the expression of α3 and α4 nAChR subunits in lymphocytes using immunohistochemistry and *in situ* hybridisation (Hiemke et al., 1996). But later several other groups failed to find either α3 or α4 expression in lymphocytes (Sato et al., 1999) (Mihovilovic and Roses, 1993). K. Benhammou *et al* showed that α4 and β4 are present in both lymphocytes and PMN at mRNA level, but expression was low compared to those found in brain (Benhammou et al., 2000). α3, α7, and β2, mRNA was detectable in some individuals and not in others. Protein level expression for α3, α4, α7 and β2 was seen in lymphocytes while PMN expressed a moderate level of α3, and low levels of α4, α7, and β2 protein. α3 and β4 were co-immunoprecipitated from lymphocytes and PMN at a significant level and α4 and β2 were co-immunoprecipitated at a high level from lymphocytes but at lower levels in PMN. These results suggest the possibility of α4β2, α3β4, and α7 subtypes in lymphocytes, of the subunits examined, and α3β4 in PMN.

Skok *et al* have shown expression of α4, α5, α7, β2 and β4 nAChR subunits in B-lymphocytes by flow cytometry and enzyme-linked immunosorbent assay (ELISA) (Skok et al., 2007). The highest expression of α4 and α5 subunits was observed in immature newly generated (B220+IgM+) B lymphocytes of the bone marrow, while the number of α7 subunits expanded as the B-cells underwent maturation in the spleen. Further radioligand binding assays and ELISA data suggested that main nicotinic receptor subtypes found in B lymphocytes were homomeric α7 and heteromeric α4β2. Functionally, it was shown that mice deficient in nicotinic receptor subunits α4, β2 or α7 had less serum IgG and IgG-producing cells in the spleen, but showed stronger immune responses to both protein antigen in vivo and CD40-specific antibody in vitro than wild-type mice. Proliferation of B lymphocytes stimulated by anti-CD40 was inhibited with nicotine from β2 knockout mice, but not wild-type mice. This suggests that signalling through nicotinic receptors affects both the pre-immune state and activation of B lymphocytes in the immune response, possibly via CD40-dependent pathway (Skok et al., 2007).

A further experiment from the same group showed that α7 nAChR was present in about 60%, while α4β2 and α9 (α10) nAChRs in about 10% and 20% of mouse spleen B lymphocytes, respectively; relative amounts of these nAChR subtypes varies between different species (Koval et al., 2011). *In vitro*

activation using anti-CD40 specific antibody up-regulated the expression of α4β2 and α7, but not α9(α10) nAChRs. Antibody interference studies have shown that the binding of CD40-specific antibody was inhibited with α7 or α9 specific antibodies, and that of IgM or CD23 specific antibody was decreased with α4, β2 or β4 specific antibodies. Experiments using knockout mice and specific subunit inhibitors have demonstrated an inhibitory role for α7 and α9α10 nAChR to CD40-related proliferative function with α7 functioning much more efficiently than α9α10. In contrast α4β2 nAChR potentiates the stimulatory IgM-related proliferative effect. Physiologically α7 nAChRs could be involved in T-cell-B-cell interaction through the immune synapse. The engagement of CD40 in the immune synapse has been previously documented by Barcia *et al* (Barcia et al., 2008) and T lymphocyte expression of α7 nAChRs in conjunction with the TCR signalling module CD3ζ has been demonstrated by Razani-Boroujerdi et al (Razani-Boroujerdi et al., 2007). This, along with endogenous ACh production by T and B lymphocytes suggests that ACh is an additional mediator modulating T and B lymphocytes interactions.

De Rosa *et al*. have detected mRNA of a7 nAChR and at least one muscle nAChR subunit in peripheral human lymphocytes and determined that its expression is highly variable among individuals and within the same individual at different times (De Rosa et al., 2005). Also mRNAs encoding for the different muscle subunits were not all present in the same individual simultaneously. Incubation of lymphocytes with nicotine or α-bungarotoxin increased the expression of α7 consistently and decreased the percentage of apoptotic cells induced by the exposure to cortisol, suggesting that α7 nAChRs are involved in the modulation of cortisol-induced apoptosis.

7.7.1. Subunit expression by flow cytometry

In the study for antigen expression the one which showed the maximum expression among all antigens was CHRNB4 as shown in chapter 2. Both CLL cells and normal B-cells showed expression of this antigen even though the expression on normal B-cells were significantly higher compared to CLL cells. Due to this significant expression and suggestion in literature that acetyl choline receptors could be involved in B-cell proliferation and apoptosis the therapeutic implications of these receptors in CLL were further evaluated. The ranking of the subunits from the original expression array list is shown in Table 7.2.

1244	CHRNB4
5395	CHRND
5549	CHRNA2
5942	CHRNB1
6793	CHRNE
7021	CHRNB2
9193	CHRNA4
9745	CHRNA7
10600	CHRNA1
10746	CHRNB3
10780	CHRNA6
11861	CHRNA5
12255	CHRNA3

Table 7.2 Ranking of the acetyl choline receptor subunits from the original expression array list:

This ranking was based on the data from the gene expression array described in chapter 2. From the expression data genes were arranged in descending order of their median expression and the number on the left shows their actual ranking in that order. The shaded rows are the antigens for which commercial antibodies were available. CHRNG was not in the expression list.

All commercially available antibodies for subunits were tested. The available antibodies were CHRNA1, CHRNA2, CHRNA3, CHRNA4, CHRNA6, CHRNA7, CHRND, CHRNE and CHRNG. All were rabbit polyclonals and none had been previously optimised for flow cytometry. They were tested with methods described in chapter 2 for testing rabbit polyclonal antibodies. 5 patients and 2 controls were tested. Definite positive and negative controls were difficult to obtain as evidence in literature was poor and definite positive cells were specific neuronal cells which were difficult to obtain and test in flow cytometry.

The MFI of expression of different subunits on each cell type is shown in the Figure 7.2 and the expression of CHRNB4 is shown as an example in Figure 7.3.

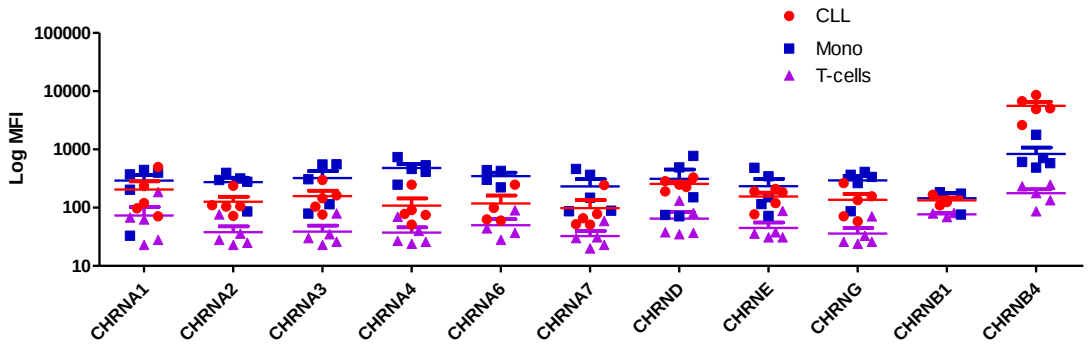


Figure 7.2 The MFI of expression of each receptor subunit on the designated cell populations.

The expression of acetyl choline receptors on the surface of MNC populations in CLL samples was evaluated by flow cytometry (n=5).

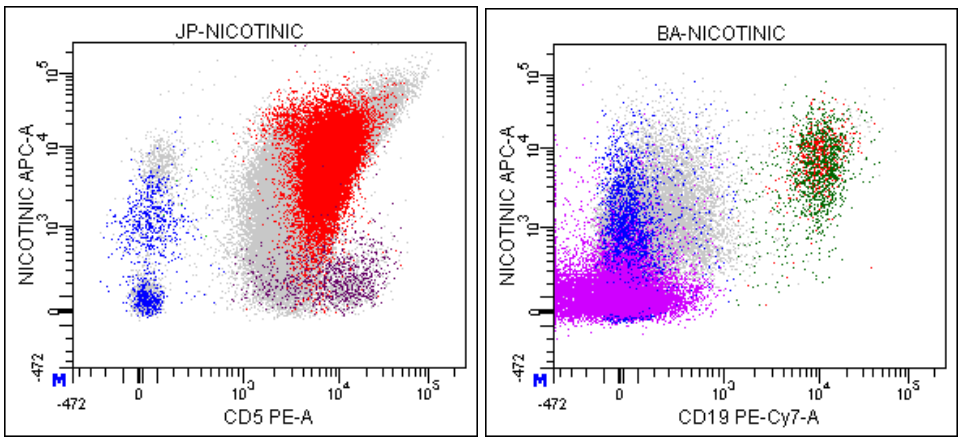


Figure 7.3 CHRNB4 expression

CHRNB4 expression is shown as an example of acetyl choline receptor subunit expression. The one on the left is sample from a CLL patient and the one on the right is from a healthy control showing the B-cells. CHRNB4 is highly expressed in CLL cells and B cells.

The majority of staining for acetyl choline receptors appeared to be uniform across the cell populations examined, with the exception of CHRNB4, which showed specific expression on CLL cells and B-cells.

This result cannot exclude the expression of the additional subunits on CLL cells for various reasons: the antibodies were not optimised for flow cytometry, the epitope of the antigen may not be exposed for antibody binding in 3 dimensional structures, and a positive control was not available to test the efficacy of the antibody.

7.7.2. Subunit expression by RT-PCR

Because the above finding was inconsistent with the literature it was decided to test the RNA expression by RT-PCR. In this instance positive controls for each subunit were available (Table 7.3).

Subunit	**control**
α1	Rat brain cDNA
α2	HepG2
α3	HepG2
α4	HepG2
α5	nil
α6	THP1 cell line
α7	HepG2
α9	Jurkat
α10	HELA
β1	Human brain cDNA
β2	Jurkat
β4	Human brain cDNA
δ	Jurkat
ε	HepG2
γ	MCF7

Table 7.3. Positive controls for each subunit.

cDNA derived from the specified cell line or tissue source were used in RT-PCR. The shaded ones were not available for testing for practical reasons.

To determine the correct conditions for amplifying each subunit, a series of PCR reactions were carried out in which the annealing temperatures and $MgCl_2$ concentrations were varied. A compilation of the optimal conditions for PCR is listed in Table 7.4.

Table 7.4. Gradient PCR - Optimum temperature and Mg concentration

Subunit	product size	result	temp	mg
α1	188	-		
α2	101	-		
α3	158	+	65	3.5
α4	161	faint	63	3.5
α5	150	+	63	3.5
α6	111	-		
α7	210	+	63	3.5
α9	157	+	63	1.5
α10	239	+	63	2.5
β1	161	+	65	2.5
β2	230	+	63	2.5
β4	170	+	63	3.5
δ	157	+	63	2.5
ε	175	+	65	1.5
γ	185	-		

With optimum annealing temperature and magnesium concentration determined, PCR reactions for each subunit were tested in 7 patients. In terms of the alpha subunits, 3, 5, 9, 10 appeared to be relatively consistently expressed and α4 was only minimally detected in two patients (data not shown). Intriguingly, α7 appears to be differentially expressed. The expression pattern of β4, δ and ε subunits showed a uniform expression, however β1 and β2 showed a difference between patients. The expression of these subunits have not previously been studied in CLL but the expression pattern on normal B-cells is known, as described in the introduction of this chapter. This data in conjunction with the literature suggests that these receptors may play a significant role in the pathophysiology of CLL cells and could be potentially explored as a therapeutic target.

To confirm the specificity of the amplicons obtained by RT-PCR, Sanger sequencing (commercially outsourced to Biosciences) was performed on DNA

extracted from the gels. The obtained sequences were compared with the NCBI gene database and the Table 7.5 shows the results. All sequence except A4 and B4 matched with the gene database.

Table 7.5. Comparison of obtained sequence with NCBI gene database

	Sequencing quality	Alignment with database
A3	good	match
A4	poor	poor alignment
A5	good	match
A7	poor	match
A9	good	match
A10	good	match
B1	good	match
B2	good	match
B4	good	poor alignment
D	good	match
E	good	match

7.7.3. Effect of pan nicotinic acetylcholine receptor agonist and antagonist on CLL cells in the culture system

To test the role of acetyl choline receptors on CLL cell viability MNC were cultured in the presence of acetyl choline (a pan acetyl choline receptor agonist) and/or mecamylamine (a pan acetyl choline receptor antagonist) with or without antigenic stimulation with anti-IgM. The results are plotted in Figure 7.4. There was no significant effect on viability with either pan agonist or antagonist of acetyl choline receptors.

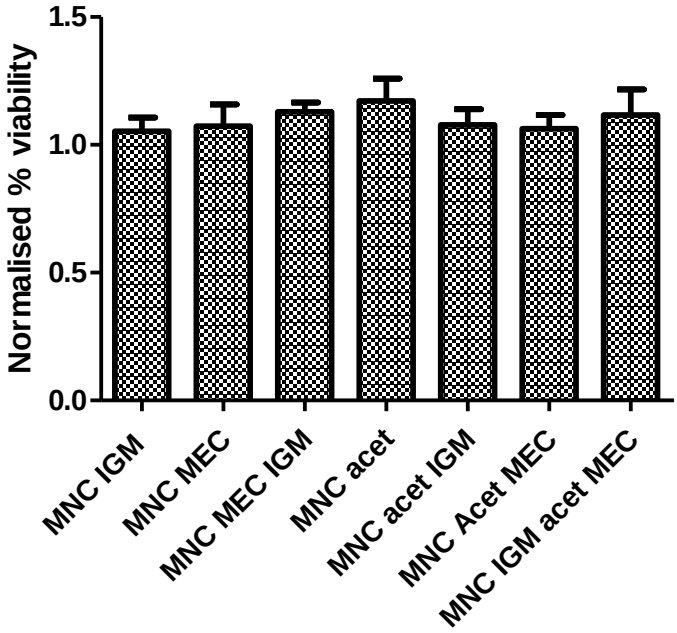
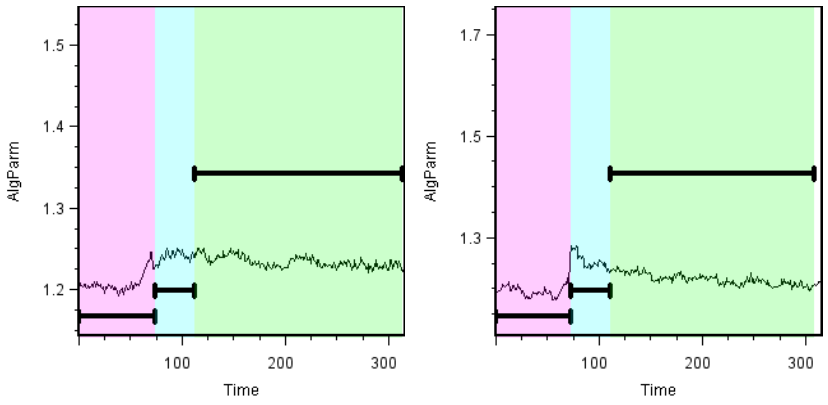


Figure 7.4 Effect of acetyl choline and mecamylamine on CLL cell viability.

MNC was incubated in presence of acetyl choline and/or mecamylamine with or without antigenic stimulation by $F(ab)_2$ anti-IgM. Percentage viability was normalised to the viability of MNC after 48 hours without any of the above agents.

Functionally the activation of nicotinic AChRs causes the movement of cations through the opening of an ion channel, with the influx of calcium ions (Shen and Yakel, 2009). To test this physiological effect, calcium flux in CLL cells was studied using the techniques described in Chapter 4. The effects of both pan receptor agonist and antagonist were tested. Acetyl choline was used as the pan agonist and mecamylamine was used as the pan antagonist. Indo-1 was used as the calcium detecting dye and flux was analysed using flowJo software. The plots are shown in Figures 7.5 and 7.6. Neither the agonist nor the antagonist caused a significant alteration to calcium flux.



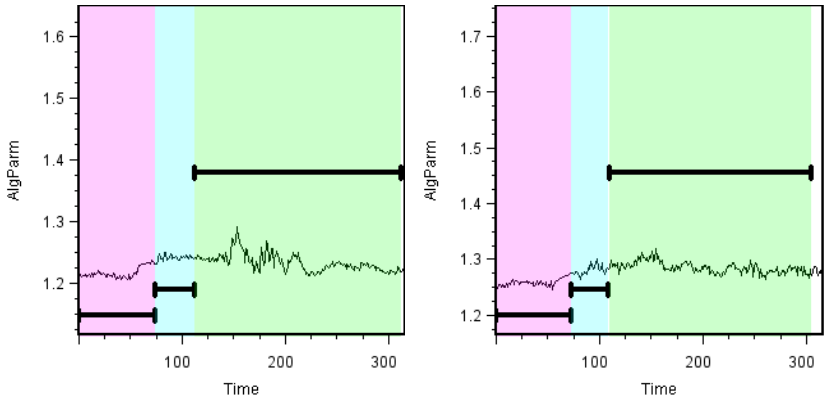


Figure 7.5. The effect of acetyl choline on calcium flux in CLL cells.

CLL samples were treated with the pan-agonist acetyl choline and the resulting calcium flux was monitored by flow cytometry. Results from 4 patient samples are shown.

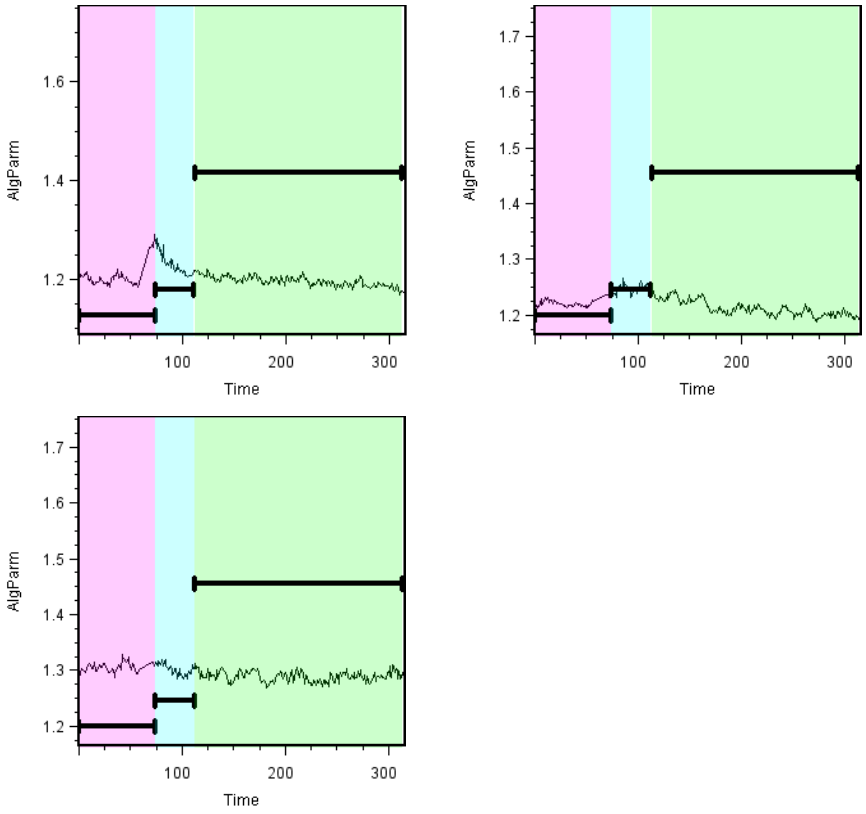


Figure 7.6 The effect of mecamylamine on calcium flux in CLL cells.

CLL samples were treated with the pan-antogonist mecamylamine and the resulting calcium flux was monitored by flow cytometry. Results from 3 patient samples are shown.

From the above experiments it is evident that a number of acetyl choline receptor subunits are expressed on CLL cells. Subunits like *α3, α4, α5, α9, α10, ε* and *δ* are universally expressed but there is variation between patients

in the expression of *α7, β1, β2*. These targets were identified at mRNA level. Protein level expression was identified for β4 only. This may be due to technical reasons like the antibodies used were not suitable for flow cytometry, or due to physiological reasons like mRNA gets degraded before translation or a post-translational modification is needed for surface expression of the protein. To differentiate this, protein level expression for these subunits should be identified using standard techniques like Western blotting or immunohistochemistry. Attempts were made to identify the expression of these subunits using Western blotting, but because of time constraints it was not completed.

The sequencing of β4 subunit yielded a product. But the sequences appear to be *CHRNB2* rather than *CHRNB4*. Comparing both sequences showed similarity in both sequence over an extensive stretch of sequence. Unfortunately the primers used will bind to either sequence and will give the same product. This needs to be re-sequenced using different primers away from the common region.

Even though the expressions of several subunits were demonstrated the functional significance of these could not be established as demonstrated by the absence of calcium flux on stimulation with acetyl choline. Acetyl choline receptor stimulation or blocking using a pan receptor agonist and antagonist did not have any effect on CLL survival. As one of the main aims of the project was to identify antigens with therapeutic potential, further work on acetyl choline receptors were abandoned.

7.8 Dopamine receptors

Dopamine receptors are neurotransmitter receptors which are G-protein coupled, and are involved in a number of central nervous system functions including emotional control, memory, cognition, learning and fine motor control (Schultz, 2007) (Missale et al., 1998). In the periphery, it regulates blood pressure, heart rate, gut motility, kidney functions and several neuroendocrine reflexes (Missale et al., 1998). There are 5 subtypes of dopamine receptors which can be broadly divided to 2 subtypes based on their functional categories.

7.8.1. D1-like receptor family

D1-like receptors, which include D1 and D5, signal through the G proteins, $G\alpha_s$ and $G\alpha_{olf}$. Both play a stimulatory role and $G\alpha_s$ is more universal while

$G\alpha_{olf}$ is predominantly present in certain areas of the brain like neostriatum, nucleus accumbens and olfactory tubercle (Neve et al., 2004) (Zhuang et al., 2000) (Hervé et al., 2001). On activation these proteins bind to adenylate cyclase, increasing its catalytic activity. Adenylate cyclase catalyzes the conversion of ATP to cyclic AMP, which binds to the regulatory subunits of the protein kinase A (PKA). PKA then disinhibit the catalytic subunits and phosphorylates a number of proteins involved in signal transduction and regulation of gene expression, like DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32 kDa) which inhibits protein phosphatase 1 (PP1) when phosphorylated on Thr34 and inhibits PKA when phosphorylated on Thr75 (Hemmings et al., 1984) (Bibb et al., 1999) (Nishi et al., 2000). D1-like receptor activation of PKA also increases the phosphorylation of numerous voltage- and ligand-gated ion channels. D1 receptor stimulation also induces the expression of a number of transcription factors like cyclic AMP response element-binding protein (CREB) (Liu and Graybiel, 1996) (Konradi et al., 1994). D1 or D1-like receptors also signals mobilisation of intracellular calcium via phospholipase C-dependent and cyclic AMP-independent pathway.

7.8.2. D2-like receptor family

D2-like receptors, which include D2, D3 and D4, signal through the heterotrimeric G proteins $G\alpha_i$ and $G\alpha_o$. These pertussis toxin-sensitive G proteins inhibit adenylate cyclase and prevent cyclic AMP accumulation (De Camilli et al., 1979) (Stoof and Kebabian, 1981). D2 receptor signalling via inhibition of adenylate cyclase acts in opposition to agents that stimulate adenylate cyclase, i.e. decreasing the phosphorylation of PKA substrates. For example stimulation of D2-like receptors decreases PKA-stimulated phosphorylation of DARPP-32 at Thr34 and increases phosphorylation at Thr75 (Nishi et al., 2000) (Nishi et al., 1997). D2-like receptors modulate many other signalling pathways in addition to adenylate cyclase, including phospholipases, ion channels, MAP kinases, and the Na+/H+ exchanger, through G protein βγ subunits that are released by receptor activation of Gαi/o proteins. D2 stimulation decreases cell excitability by increasing K+ currents in most brain areas by dissociation of Gβγ subunits, rather than by $G\alpha_i$-dependent inhibition of adenylate cyclase activity. All D2-like receptors decrease the activity L, N, and P/Q-type calcium channels by Gβγ subunits. Like many other G-protein coupled receptors, activation of the D2 receptor stimulates MAP kinases, including the two isozymes of extracellular signal-regulated kinase (ERK) and stress-activated protein kinase/Jun amino-

terminal kinase (SAPK/JNK). This activation is mediated by pertussis toxin-sensitive G proteins, Gβγ, phosphatidylinositol 3-kinase, Ras, and the MAP kinase kinase MEK. D3 and D4 dopamine receptors also activate ERK. MAP kinases transmit stimuli to the cell nucleus, thus participating in cell proliferation, differentiation, and survival (Neve et al., 2004). D2 receptor activation of ERK stimulates DNA synthesis and mitogenesis in many different cell types. D2 receptors in neostriatal neurons activate a cytosolic, Gβγ-stimulated form of phospholipase C, PLCβ1, causing inositol triphosphate-induced calcium mobilisation that activates calcium-dependent proteins such as the protein phosphatase, calcineurin, and ultimately reducing L-type Ca^{2+} currents. In addition to interactions between dopamine receptors and G proteins, other protein-protein interactions such as receptor oligomerisation or receptor interactions with scaffolding proteins like spinophilin and signal-switching proteins Nck, Grb2, and c-Src which contain Src homology 3 (SH3) domains are critical for regulation of dopamine receptor signalling (Neve et al., 2004).

Besides conventional roles of neurotransmitters in neural communication, there is convincing evidence that several neurotransmitters are involved in cross-talk between the nervous and immune systems. Among several neurotransmitters, dopamine (DA) plays an active role in this. The functional role of DA on the immune system is demonstrated by various factors like the presence of dopamine receptors (Basu et al., 1993) (Ricci and Amenta, 1994) and an endogenous DA transport system in leukocytes (Bondy et al., 1992) (Basu et al., 1993) as well as the endogenous synthesis of this monoamine in leukocytes (Bergquist et al., 1994) (Cosentino et al., 1999).

Several human leukocytes have been shown to express dopamine receptors (Ferrari et al., 2004) (Kirillova et al., 2008) (McKenna et al., 2002) (Nakano et al., 2009). Le Fur *et al*. initially demonstrated the presence of DA receptors in mammalian lymphocytes in 1980s which triggered the concept of DA as a regulator of functional activities of immune effector cells (Le Fur et al., 1980). Later several groups have demonstrated the presence of all types of dopamine receptors on human lymphocytes by radioligand binding and mRNA expression studies (Basu et al., 1993) (Santambrogio et al., 1993) (Ricci and Amenta, 1994) (Barili et al., 1996) (Caronti et al., 1998), but some studies have failed to detect the presence of any D2 like receptors (Vile and Strange, 1996). Flow cytometric experiments using rabbit polyclonal antibodies have shown that B-cells and NK cells show the highest expression among

leucocytes, followed by eosinophils and neutrophils, while T-cells and monocytes show low expression.

Lymphocytes are capable of producing dopamine (Josefsson et al., 1996); (Bergquist and Silberring, 1998) and express the rate-limiting enzyme of dopamine synthesis, tyrosine hydroxylase (McKenna et al., 2002). Capillary electrophoresis method has demonstrated picomole levels of DA in lymphocytes, neutrophils and macrophages with a big variation in the level in various leucocytes. It is mainly stored in cytoplasmic vesicular structures but a very small amount is found in the nucleus suggesting its possible regulatory role on nuclear components. Active synthesis of dopamine by leucocytes has been demonstrated by various techniques. Inhibitors of tyrosine hydroxylase, a rate-limiting enzyme for DA synthesis, depleted cells of dopamine. Intracellular DA content in lymphocytes increased after incubation with DA precursor, L-dopa (Bergquist et al., 1994) (Musso et al., 1996). Release of catecholamines into extracellular medium by human mononuclear cells was also demonstrated (Marino et al., 1999). There is also evidence that in lymphocytes dopamine and D1 agonist SKF-38393 through D1 receptor stimulation can inhibit protein kinase C-induced tyrosine hydroxylase mRNA expression resulting in reduced intracellular catecholamine levels (Ferrari et al., 2004).

There is some evidence to suggest that dopamine plays a significant role in lymphocyte proliferation from experiments on T- and B-cell hybridomas and murine and human lymphocytes (Bergquist et al., 1997). Several groups have demonstrated that dopamine and its immediate precursor L-Dopa inhibit lymphocyte proliferation and induce apoptotic death (Cook-Mills et al., 1995) (Offen et al., 1995) (Josefsson et al., 1996) (Bergquist et al., 1997) (Slominski and Goodman-Snitkoff, 1992). Contrary to these *in vitro* experiments, *in vivo* experiments on normal and experimental tumour-bearing mice showed stimulation of T- and B-cell proliferation following pharmacological doses of DA (50 mg/kg). Another group has also shown that intravenous injection of D1 and D2 specific agonists, SKF-38393 and LY 171555, enhanced LPS- and Con A-stimulated lymphocyte proliferation (Tsao et al., 1997). and dopamine receptor antagonist, haloperidol, inhibited cell growth (Tsao et al., 1998).

Dopamine has also been shown to inhibit the phagocytic function of neutrophils (Wenisch et al., 1996). In macrophages it stimulates tumoricidal activity (Dasgupta and Lahiri, 1987) and induces phagocytosis (Sternberg et al., 1987). In mice dopamine administration significantly increased the number

of large granular lymphocytes in blood and the tumour cell killing ability of specific NK cells *in vitro* (Basu et al., 1993). Cosentino *et al.* have demonstrated that CD4+CD25+ regulatory T lymphocytes (Tregs) contain substantial amounts of DA which forms an autocrine loop controlled by suppressing IL-10 and TGF-β synthesis after being released by acting on the D1 receptors present in the same cells (Cosentino et al., 2007). Similarly, a paracrine regulatory loop links dendritic and T-cells (Nakano et al., 2009). Dopamine stored in human monocytic-dendritic cells when released acts on the D1 receptors present on naïve T-cells, increasing intracellular cyclic AMP and causing differentiation into the T_h2 lineage in response to anti-CD3 plus anti-CD28 mAb while in absence of DA release, T-cell differentiation shifts towards T_h1 lineage. In dentritic cells the released dopamine auto-regulates its synthesis by acting through D1 receptors present in these cells. In normal resting peripheral human T lymphocytes, stimulation of D2 and D3 receptors activate α4β1 and α5β1 integrins in these cells, thereby promoting adhesion of these cells to the extracellular matrix component, fibronectin (Levite et al., 2001) which is important for trafficking and extravasation of T-cells across the blood vessels and tissue barriers. Dopamine also stimulates adhesion of CD8+ T cells to fibronectin and ICAM through integrins, mediated through its D3 receptors (Watanabe et al., 2006). Dopamine induced chemotactic migration of naïve CD8+ T-cells is synergistic with chemokines like CCL19, CCL21 and CXCL12. In T-cells, stimulation of D3 and D1/D5 receptors increase the secretion of TNF-α and stimulation of D2 receptors induces IL-10 secretion (Besser et al., 2005).

Even though dopamine activates resting T-cells, it has also been shown that dopamine, at a concentration observed in the plasma (48.6 pg/ml) of human subjects suffering from acute uncoping stress, inhibits anti-CD3 and IL-2 induced proliferation and cytotoxicity of CD4+ and CD8+ T-cells (Saha et al., 2001). This is thought to be mediated by D1 receptor inducing an increase in the intracellular cAMP. Through D2 and D3 receptors in T-cells, dopamine inhibits T-cell receptor induced cell proliferation, and secretion of IL-2, IFN-γ and IL-4 by down-regulating the expressions of non receptor tyrosine kinase lck and fyn (Ghosh et al., 2003). Stimulation of D4 receptors in human T-cells during T-cell receptor activation is associated with its quiescence (Sarkar et al., 2006) by up-regulating the transcription factor, KLF2 via inhibition of ERK1/ERK2 in these cells. Like normal human T-cells, Jurkat cells also express D1 and D2 dopamine receptors. But, unlike activated normal T-cells, where dopamine, through D2 and D3 receptors, inhibit T-cell receptor induced

cell proliferation, stimulation of these dopamine receptors in Jurkat cells fails to inhibit their T-cell receptor-induced proliferation. In normal T-cells D1 stimulation results in intracellular cAMP accumulation, but this observation was not seen in Jurkat cells. Sequencing of the D1 gene did not show any functionally significant mutation to account for this effect. But pharmacological inhibition of phosphodiesterase, the enzyme responsible for cAMP breakdown, with theophylline, along with D1 DA receptor stimulation resulted in cAMP accumulation and inhibition of proliferation in Jurkat T-cells, suggesting that failure of D1 receptor-mediated anti-proliferative effect on Jurkat cells was due to increased catabolic activity of the phosphodiesterase enzyme resulting in accelerated breakdown of cAMP in Jurkat cells. On the other hand D2 receptor stimulation inhibited intracellular cAMP accumulation and inhibited T-cell receptor (TCR)-induced cell proliferation and secretion of IL-2, IFN-γ, and IL-4 mediated by down-regulating phosphorylation of ZAP70, an important downstream signalling molecule that helps in T-cell proliferation and activation. This effect was not observed in Jurkat cells suggesting a defect in this signal transduction. Sequencing of D2 receptor gene has shown a missense mutation that could result in lower efficiency in activating the α subunit of the G protein heterotrimer to transduce downstream signals resulting in failure of the D2 receptors to down-regulate ZAP70 phosphorylation (Basu et al., 2010).

Meredith *et al* have shown that monoamines including dopamine, adrenaline, noradrenaline, and serotonin are anti-proliferative for normal and a spectrum of malignant B-cells representing various stages of maturation arrest from pre-B cells through to plasma cells, with dopamine as the most potent one (Meredith et al., 2006). Dopamine has an EC_{50} of 5±0.3μM for growth arrest at the lowest cell density ($2.5x10^5$ per ml). RT-PCR for dopamine receptors has shown that D1 and D2 were widely expressed both among the malignant and normal B-cells, D3 in some cell lines, D4 was uniformly undetectable, and D5 was limited to the diffuse large B cell lymphoma line DOHH2 and K1106 derived from a patient with primary mediastinal B cell lymphoma (Meredith et al., 2006). The anti-proliferative effect of dopamine is unlikely to be mediated through dopamine receptors as antagonism of D1-like receptors (D1 and D5) with SCH23390 or D2-like receptors (D2, D3, and D4) with fluphenazine, haloperidol, or spiperone failed to reverse DA's anti-proliferative actions. Similarly pharmacological blockade of dopamine transporter protein with the high-affinity compounds mazindol or GBR12909 and inhibiting monoamine oxidases, which convert intracellular DA into oxidative metabolites, with

clorgyline, L-deprenyl, or pargyline had no effect on dopamine mediated anti-proliferative effect. These experiments suggested that catecholamines generate reactive oxidation species by autooxidation and this may be the mechanism for the anti-proliferative effect. This was supported by the observation that, catalase, an endogenous enzyme that neutralizes H_2O_2, was fully effective in reversing hydrogen peroxide's anti-proliferative effect on L3055 cells and significantly attenuated the anti-proliferative effects of DA, L-DOPA, and apomorphine (Meredith et al., 2006).

Thus from the literature it is evident that dopamine has a significant role in normal and malignant B-cell function and survival. In addition to the effect produced by exogenous dopamine these cells can produce dopamine on their own and can have an autocrine or paracrine effect. There is good evidence that dopamine plays a role in proliferation and survival of these cells. But the mechanism by which it acts as an anti-proliferative agent is not clear. There are different postulations in the literature regarding this. This could be mediated by dopamine receptors as suggested by some studies or could be independent of receptors and mediated by oxidative stress as suggested by another study (Basu et al., 2010) (Meredith et al., 2006).

7.8.3. Dopamine receptor study

As demonstrated in Chapter 3 screening for expression of antigens in CLL cells has shown that dopamine receptor D4 (DRD4) is expressed in CLL cells (Figure 7.6). Unfortunately, even though initial screening on a limited number of patient samples showed low level of expression, there was no statistical difference from control antigen when applied the same antibody on multiple samples (Figure 7.7). This could be due to low affinity of the antibody tested to the antigen or due to genuine low expression of DRD4 antigen and variation between patients to manifest as a statistically meaningful data. The ranking of the subunits from the original expression array list is shown in Table 7.6. This suggests that the expression of DRD2 and DRD4 are reasonably higher up in the list.

Table 7.6. Ranking of the dopamine receptor subunits from the original expression array list

3485	DRD2
4592	DRD4
5828	DRD3
10142	DRD1
11489	DRD5

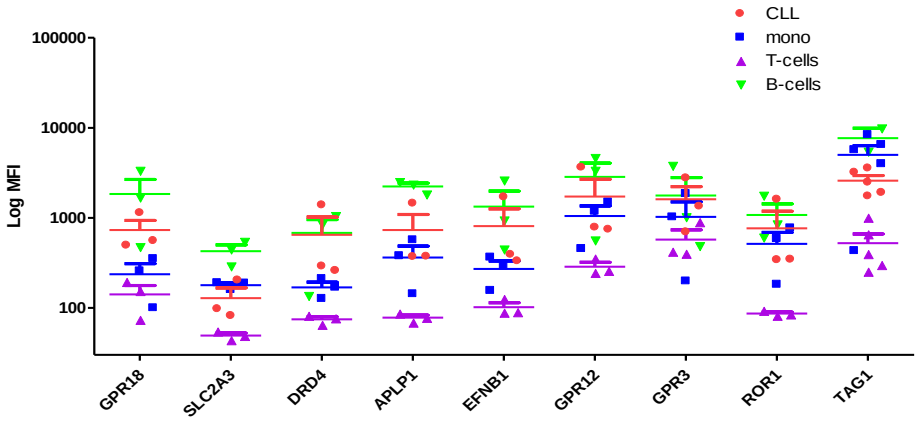


Figure 7.6 Screening for DRD4 expression

Initial screening for dopamine receptor expression levels on MNC populations from 3 patients, showing low level expression of DRD4 on CLL cells and B-cells compared to monocytes and T-cells.

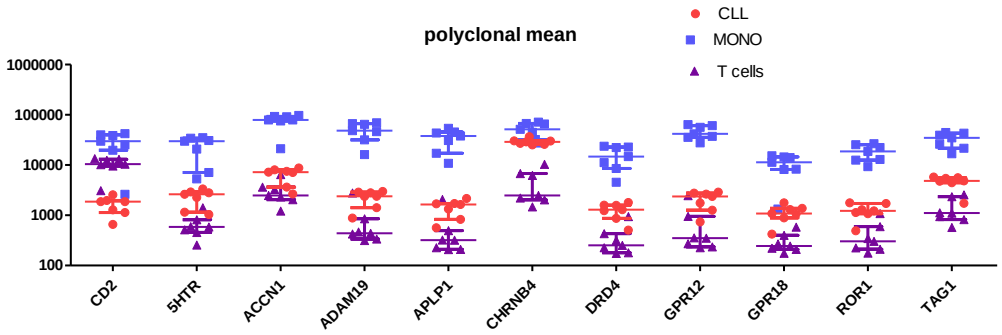


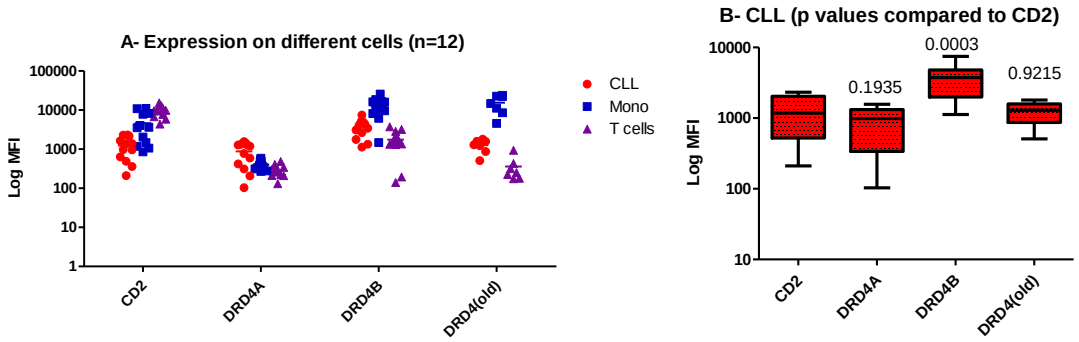
Figure7.7 DRD4 expression on multiple samples.

Screening for expression of DRD4 on more patient samples (n=7) and comparing with background staining (CD2) showed no statistically significant difference between control antibody (CD2) and DRD4 (Table 7.7).

Table 7.7. Statistical analysis of differences in staining between control antibody and test antibodies listed.

Antigen	P value	Antigen	P value
5HTR	0.71	DRD4	0.62
ACCN1	**0.0006**	GPR12	0.259
ADAM19	0.128	GPR18	0.259
APLP1	0.535	ROR1	0.9
CHRNB4	**0.0006**	**TAG1**	**0.007**

As the results depicted in the above figures were inconsistent with the literature, about expression on B-cells, more DRD4 antibodies recognising different epitopes were screened. For the original antibody (LS-C22939) the antigen was a 17 amino acid sequence from the N-terminus. Two other antibodies were tested, AP19016PU-N (antigen was amino acid sequence 1-11 of the N-terminus) and BP123S (amino acid sequence was 176-185 of DRD4 receptor which belongs to the second extracellular loop). The expression of DRD4 as assessed by the BP123S antibody was highly significant compared to CD2, but the other 2 antibodies did not show differential expression (Figure 7.11). Expression levels on different cell populations were also examined and it was determined that there was no significant difference in expression of DRD4 on CLL cells compared to normal B-cells (Figure 7.12). To establish that the staining conditions using the BP123S anti-DRDB4 were appropriate, a serial titration experiment was performed (Figure 7.13). It was determined that the optimum amount of antibody to be used should be around 0.5 to 0.25 µg.



DRD4B- BP123S – **aa 176-185 (2nd extracellular loop)**

DRD4A- AP19016PU-N- **aa 1-11 (N-terminus)**

DRD4 (old) LS-C22939- **17 aa at N-terminus**

Figure 7.11 Expression of 3 different DRD4 antibodies.

MFI of all three antibodies on the MNC populations from 12 different patients were compared to CD2 expression to eliminate non-specific binding. P value of the difference in expression of all antibodies compared to CD2 is shown in Part B.

The results obtained from flow cytometry supported the idea that DRD4 was expressed on CLL cells, however further evidence for this expression was sought. The presence of *DRD4* was confirmed by RT-PCR in 2 out of 3 CLL samples as shown in Figure 7.14. In the sample in which *DRD4* was not detected, there was not an adequate amount of DNA as determined by the

intensity of the *GAPDH* band to make a valid assessment. A fourth sample did not produce meaningful results since no *GAPDH* was amplified.

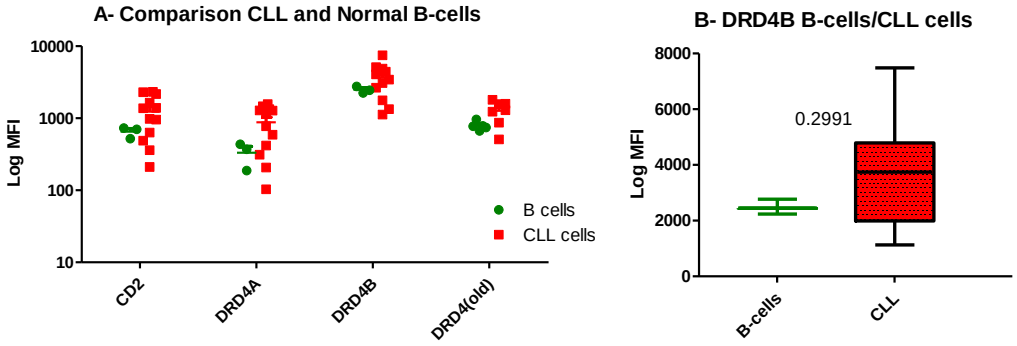


Figure 7.12 MFI of all three antibodies on CLL cells (n = 12) compared to normal B-cells (n = 3).

P value of the difference in expression of all antibodies on B-cells versus CLL cells is shown in Part B.

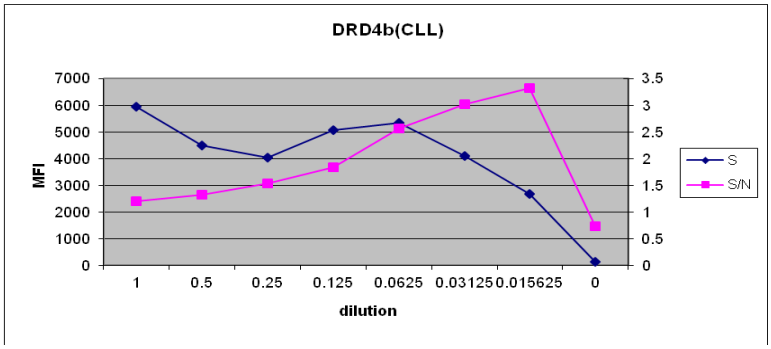


Figure 7.13 Titration of DRD4 antibody.

Neat antibody used was 4µg (Conc: 1mg/ml). Antibodies were serially diluted halving the concentration in subsequent wells upto a dilution of 1 in 64.

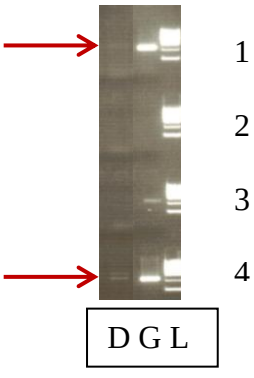


Figure 7.14. RT-PCR of *DRD4* in samples from 4 CLL patients.

The *DRD4* amplicon is indicated by red arrows. D=DRD4, G=GAPDH, L=Ladder

7.8.4. Effect of Dopamine on CLL cell viability

As discussed in the introductory section of this chapter, monoamines particularly dopamine were found to be pro-apoptotic to cell lines of B-cell origin (Meredith et al., 2006). Therefore it is reasonable to postulate that CLL cells will also have similar response. MNC were incubated with dopamine at 100μM for 48 hours and viability assessed. There was a significant drop in viability and cell count after 48 hours even though the drop was not uniform in all patients as demonstrated in Figure 7.15. A dose titration curve for dopamine is shown in Figure 7.16. The concentration used ranged from 500nM to 500mM and the IC50 was 8.22μM. To test for consistency at lower concentrations more patient samples were tested at 10μM. Even at this low dose there was significant toxicity (Figure 7.17).

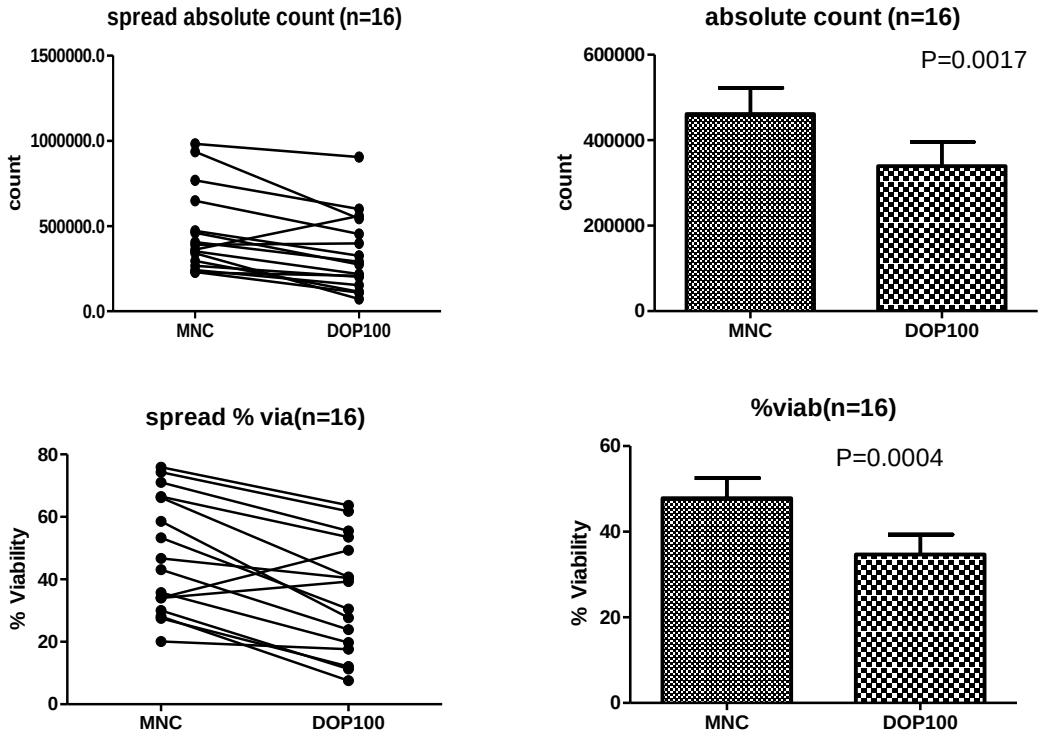


Figure 7.15 Initial assessment of the effect of dopamine on CLL cell viability.

MNC were incubated with dopamine at a concentration of 100μM and viability and cell count assessed after 48 hours (n=16).

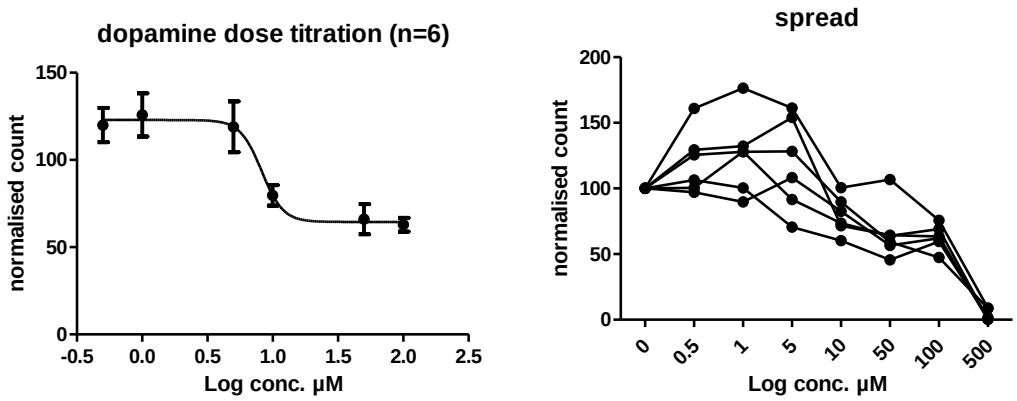


Figure 7.16 Dose titration curve for dopamine.

MNC from 6 patients were incubated with dopamine at concentrations of 500nM, 1µM, 5µM, 10µM, 50µM, 100µM and 500µM. Viability and counts were assessed after 48 hours. Count was normalised to the sample without any dopamine. IC50 was calculated using non linear fit curve with variable slope in graphpad prism. IC50= 8.218µM (95%CI- 5.205 to 12.98).

Dopamine–concentration 0,10,100µM

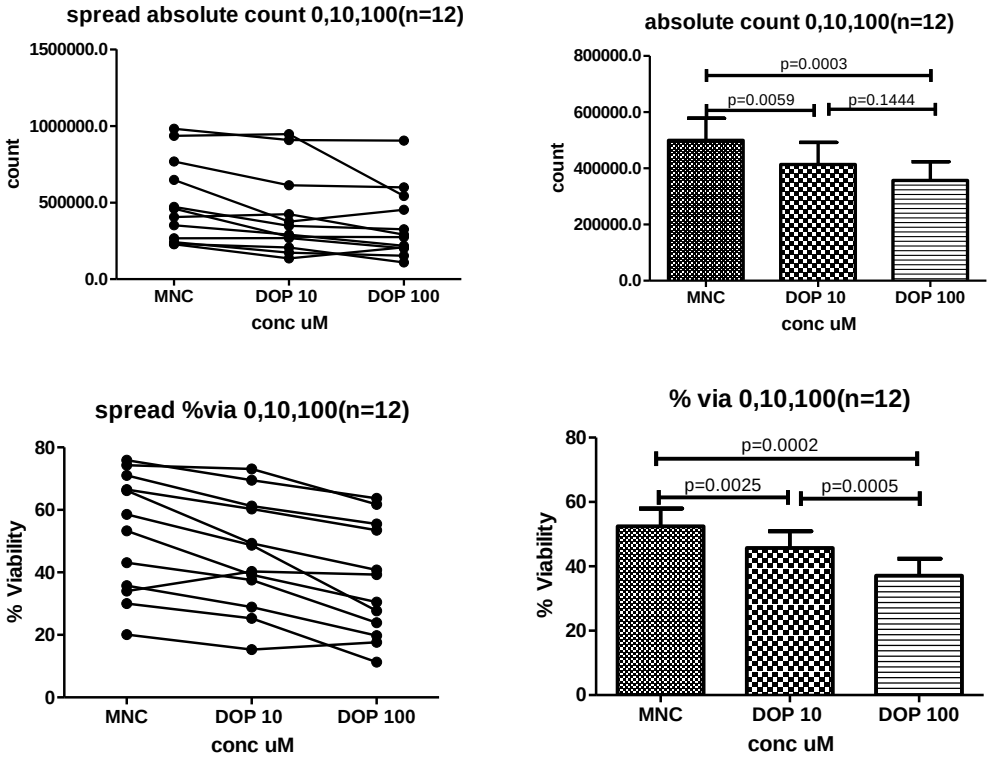


Figure 7.17 Assessment of viability to test the effect of dopamine at lower concentrations.

MNC were incubated with dopamine at a concentration of 10µm and 100µM and viability and cell count assessed after 48 hours (n=12).

7.8.5. Effect of Dopamine on stromal support and antigenic stimulation

To assess the effect of dopamine on the microenvironment support of CLL cells the viability was assessed in media with fibroblast support. Dopamine by itself was ineffective in dissipating the stromal support provided by CD40L fibroblasts (Figure 7.18). Similarly the effect of antigenic stimulation was assessed using $F(ab)_2$ anti-IgM. Unfortunately most samples tested did not show much stimulation with the antibody. However, for the patients who showed the increment in viability and count with antibody stimulation, dopamine partially negated the increment observed (Figure 7.19). This test needs to be repeated on more patients to test for statistical significance.

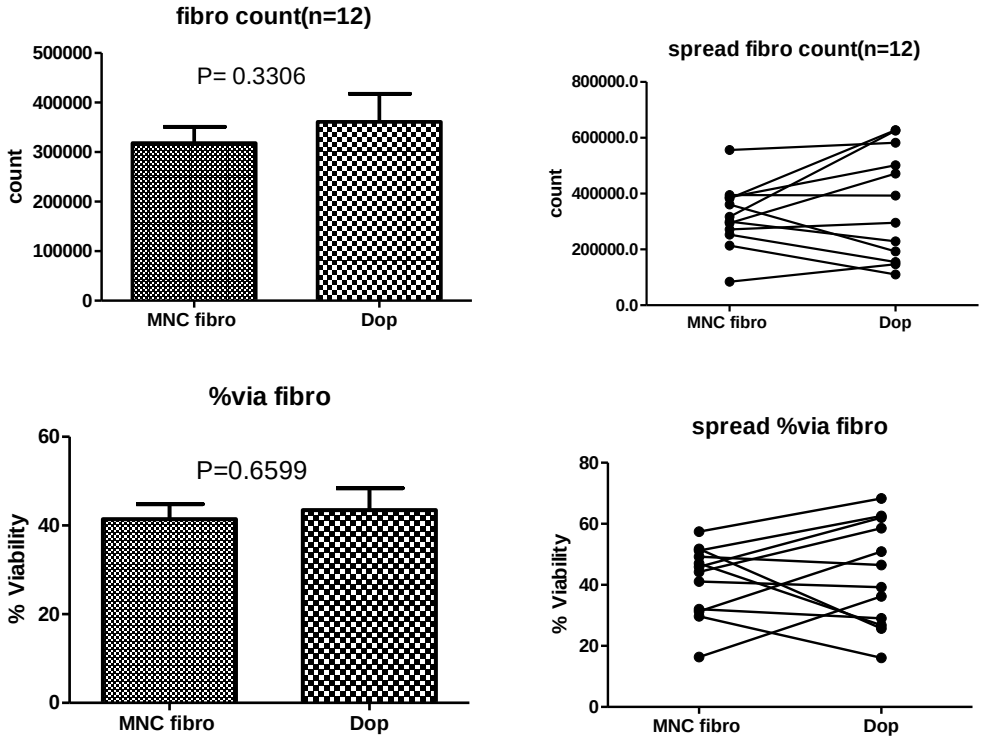


Figure 7.18 Effect of dopamine on micro-environmental support.

MNC were incubated with dopamine at a concentration of 100μM in media supported by CD40L fibroblasts and viability and cell count assessed after 48 hours (n=12).

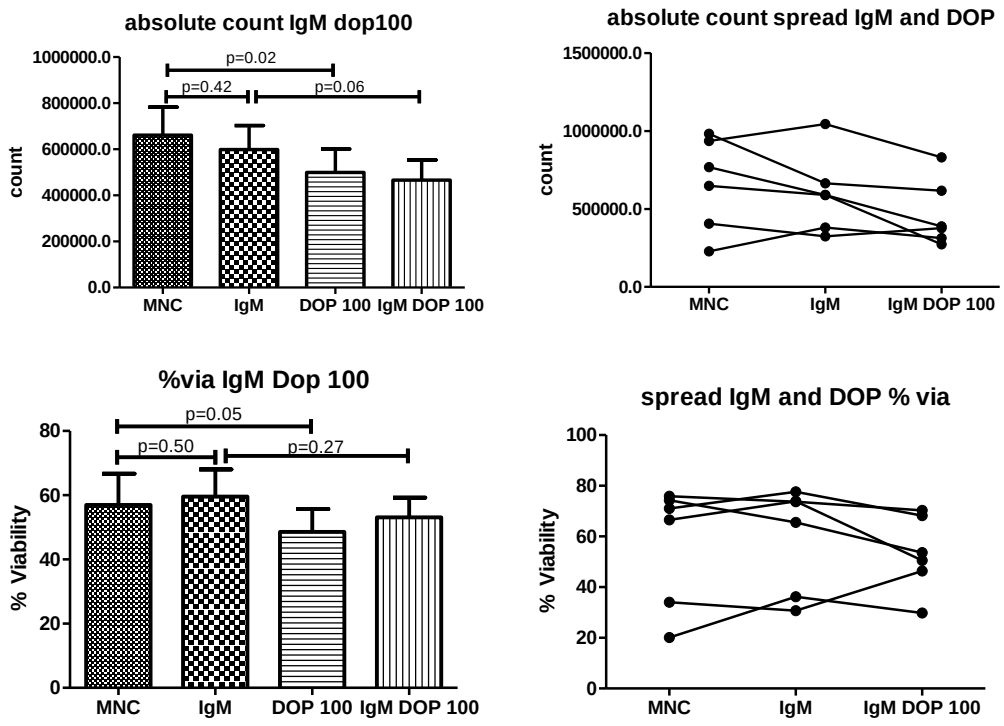


Figure 7.19 Effect of dopamine on antigenic stimulation.

MNC were incubated with dopamine at a concentration of 100μM in media stimulated by F(ab)$_2$ anti-IgM and viability and cell count assessed after 48 hours (n=6).

7.8.6. DRD4 agonist (CP226269) and antagonist (sonepiprazole)

To explore the pathway by which dopamine exerts the above observed effect, the rational approach would be to test the effect mediated by dopamine receptors. Individual receptor specific agonists and antagonists are available commercially. As DRD4 receptor was expressed at the protein level in CLL cells, DRD4 receptor specific agonist and antagonist were tested first. As shown in Figure 7.20 there was no significant effect on viability mediated by DRD4 agonist or antagonist when tested at two concentrations, one being the commercially available EC50 on binding assays and the second one being a much higher concentration to obtain the maximum effect in screening. The effect of dopamine was not blocked by DRD4 antagonist, suggesting that the effect of DA is not mediated by DRD4 receptors.

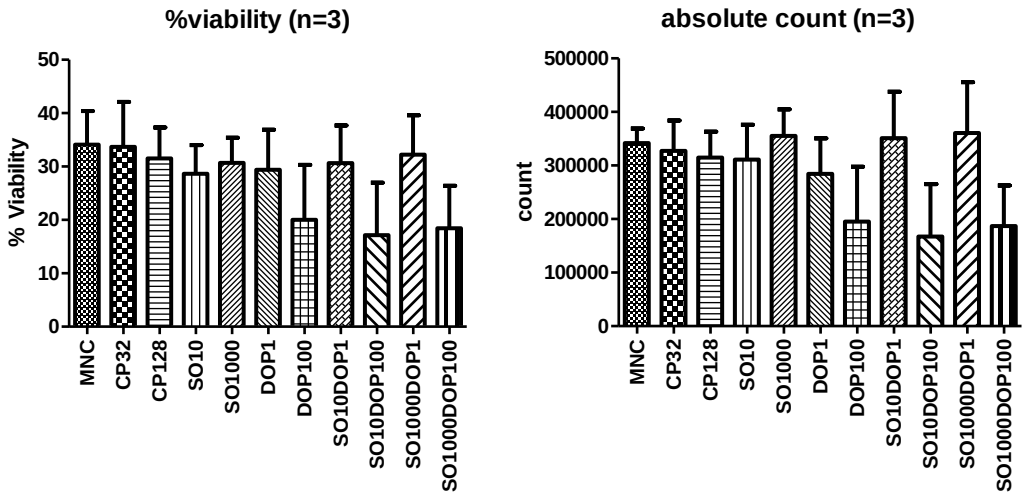


Figure 7.20 Effect of DRD4 agonist, CP226269, and antagonist sonepiprazole on CLL cell viability.

MNC were incubated with dopamine, CP226269 and sonepiprazole with or without dopamine. Viability and cell count was assessed after 48 hours (n=3). MNC=Mononuclear cells, CP= CP226269, SO= sonepiprazole, DOP= Dopamine, the numbers correspond to concentration of the compound in the media where CP226269 and sonepiprazole concentrations are in nM and dopamine concentration is in µM.

7.8.7. Protective effect of catalase

A previous study has reported that the anti-proliferative effects of dopamine on malignant B-cell lines were mediated by reactive oxidation species generated by auto oxidation (Meredith et al., 2006). These conclusions were supported by the observation that, catalase, an endogenous enzyme that neutralizes H_2O_2, was fully effective in reversing hydrogen peroxide mediated anti-proliferative effect and significantly attenuated the anti-proliferative effects of DA, L-DOPA, and apomorphine. To test this hypothesis on CLL cells MNC were incubated with dopamine with or without the presence of catalase. Even though there was some reduction in the level of cell death estimated by percentage viability and cell count in presence of catalase it was not significant enough to suggest protective effect as shown in Figure 7.21.

As discussed in the introductory section of this chapter dopamine receptors are mainly divided into two groups, D1 and D2 type, which exert almost opposite physiological effects in cells. If dopamine is exerting the proapoptotic effects through one of the receptors then a pan D1 or D2 group specific agonists or antagonists should show similar effect as dopamine. To prove this postulate MNC were incubated with pan D1 and D2 receptor agonist and antagonist with or without dopamine (Figure 7.22).

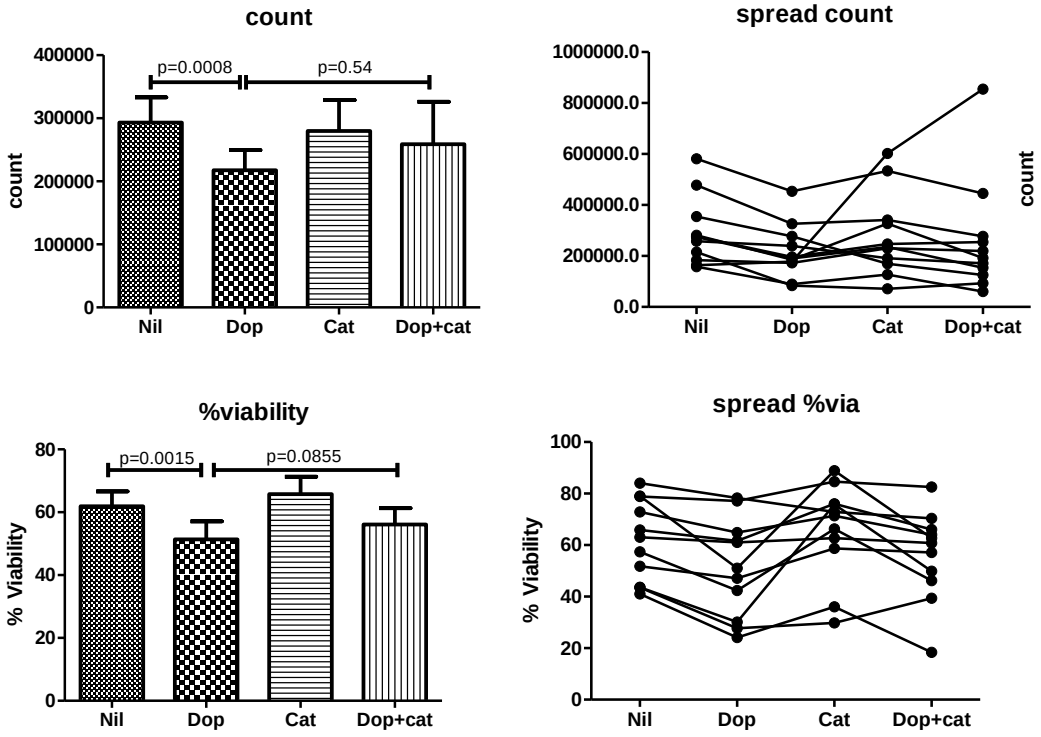
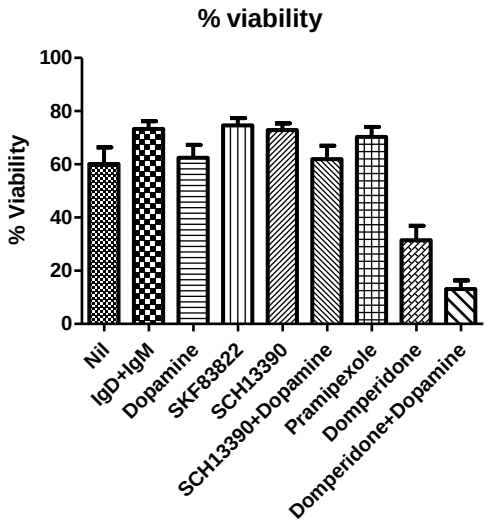
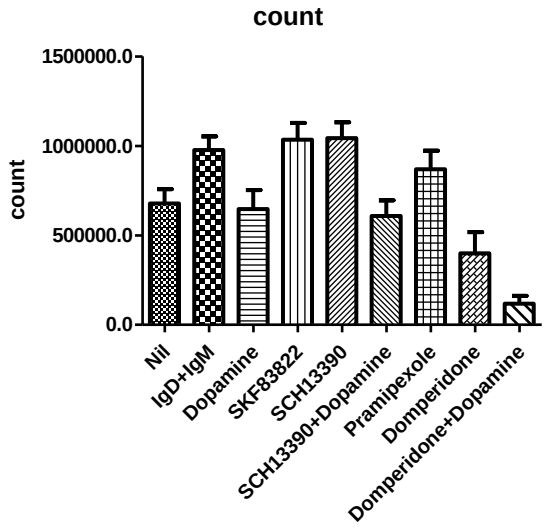


Figure 7.21 Assessment of protective effect of catalase on dopamine induced cell death

MNC were incubated with dopamine with or without catalase. Viability and cell count were determined after 48 hours (n=11).

The compounds used were SKF83822 (D1 agonist), SCH13390 (D1 antagonist), pramipexole (D2 agonist) and domperidone (D2 antagonist). Of these compounds pan D2 receptor antagonist, domperidone significantly reduced the survival of CLL cells *in vitro*. This effect is significantly enhanced when dopamine is combined with domperidone. The dose titration curve for domperidone gave an IC50 of 6.65μM (Figure 7.23).





% viability	P value
0 vs. Ig	0.07
Ig vs. Dopamine	0.03
Ig vs. Domperidone	0.0002
Domp vs. Dop+Domp	0.0003

count	P value
0 vs. Ig	0.02
Ig vs. Dopamine	0.0002
Ig vs. Domperidone	0.0002
Domp vs. Dop+Domp	0.0083

Figure 7.22 Effect of pan D1 agonist SKF83822, D1 antagonist SCH13390, D2 agonist pramipexole and D2 antagonist domperidone on CLL cell viability.

MNC were incubated with dopamine alone or with SKF83822, pramipexole, SCH13390 and domperidone with or without dopamine. F(ab)$_2$ anti- IgM and IgD were added as BCR stimulants. Viability and cell count were assessed after 48 hours (n=8).

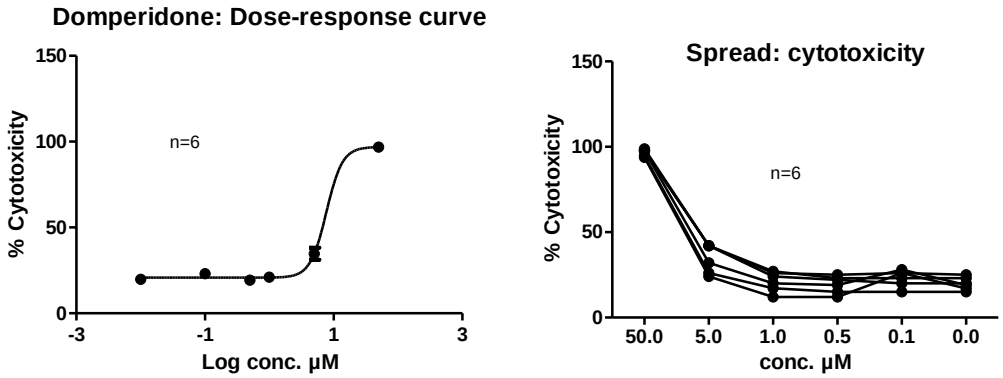


Figure 7.23 Dose titration curve for domperidone.

MNC from 3 patients were incubated with domperidone at concentrations of 0.1uM, 0.5uM, 1uM, 5uM and 50uM. Each sample was duplicated with or without anti-IgM and IgD added as BCR stimulants. Viability was assessed after 48 hours. Cytotoxicity was calculated as 100-% viability of corresponding sample. IC50 was calculated using non linear fit curve with variable slope in graphpad prism. IC50= 7.69µM (95%CI- 0.01217 to 4868).

7.8.8. Ca Flux-Dopamine

In neuronal cells intracellular calcium mobilisation is a physiological response to dopamine stimulation. In B-cells calcium mobilisation by BCR stimulation is known to produce several prosurvival and stimulatory signals. Calcium flux in response to dopamine stimulation was assessed in CLL cells even though the final effect of pro-apoptotic ability of dopamine was not explainable by the intracellular calcium flux. There was no increase in calcium flux for dopamine stimulation as demonstrated in Figure 7.24. To explain the pro-apoptotic effect of dopamine the more physiological response would be inhibition of BCR stimulation mediated calcium flux by dopamine. To test this hypothesis cells were incubated with dopamine and calcium flux was assessed using fura-red/fluo-3 as described in Chapter 4. There was a significant reduction in cells fluxing calcium when pre-incubated with dopamine proving the above postulate that dopamine inhibited the downstream signalling of BCR pathway (Figure 7.25). To prove this hypothesis further, other parameters like phosphorylation were tested. Figure 7.26 demonstrates that there was a significant inhibition of SYK phosphorylation after IgM stimulation when CLL cells were pre-incubated with dopamine, similar to the effect seen with the SYK inhibitor, even though the effect was not as pronounced as the SYK inhibitor.

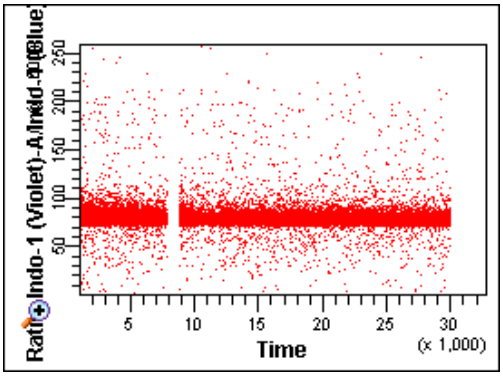


Figure 7.24 Calcium flux by dopamine.

CLL cells were stimulated with 100µM dopamine and Ca flux monitored by fura-red/fluo-3.

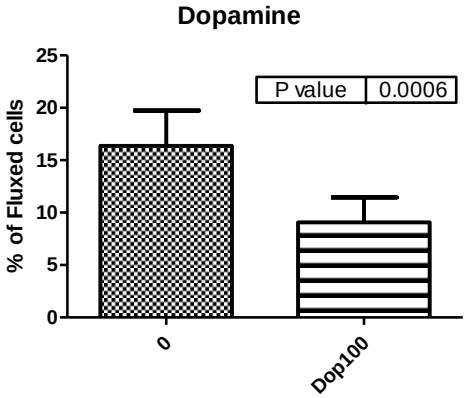


Figure 7.25 Effect of dopamine on BCR mediated calcium flux.

Calcium flux was assessed on parallel samples stimulated with anti-IgM with or without pre-incubating with dopamine (n=9).

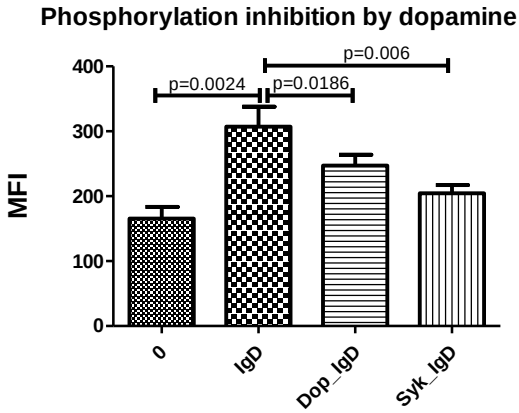


Figure 7.26 Effect of dopamine on IgD stimulation of SYK phosphorylation.

The MFI of SYK phosphorylation in CLL samples was determined after stimulation with anti-IgD with or without pre-incubating with dopamine (n=10).

From the above experiments it is clear that dopamine plays a role in the BCR stimulation pathway and treating cells with dopamine prevents further downstream signalling. From the therapeutic point this observation has great significance. It is now well established by clinical trials that CLL can be effectively treated by inhibiting BCR signalling pathway at different levels (Byrd et al., 2013) (Furman et al., 2010). Domperidone has been used in clinical practice over decades as an antiemetic (Helmers, 1977). To determine the effect of combining these two agents on CLL cell viability *in vitro*, samples were treated with GS-1101 or Domperidone alone or in combination. As evidenced from Figure 7.27 there is significant reduction in viability with GS-1101 and domperidone on their own but the effect was doubled when they were combined.

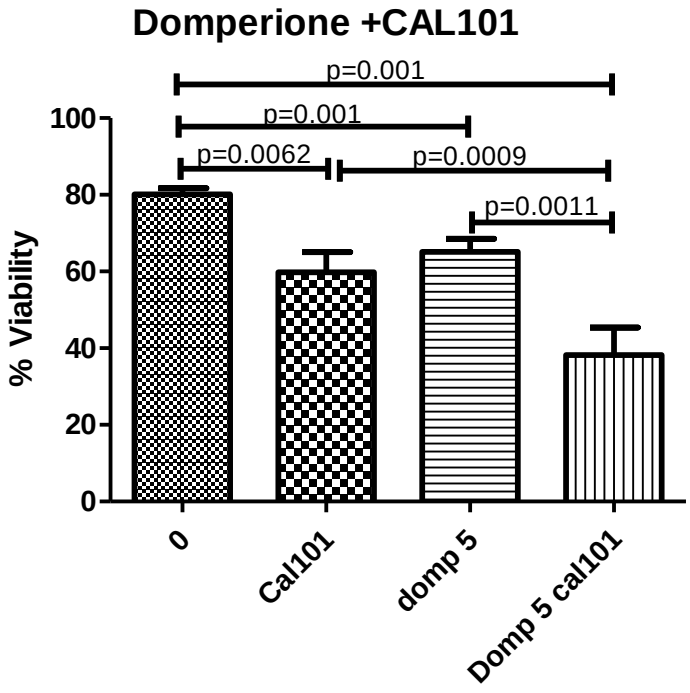


Figure 7.27 Effect of combining BCR kinase inhibitor and D2 receptor antagonist.

MNC from 3 patients were incubated with GS-1101 (10μM), domperidone (5μM) or a combination of both. Each sample was duplicated with or without anti-IgM and IgD added as BCR stimulants. Viability was assessed after 48 hours.

7.9 Discussion

These experiments have shown that there are several neuronal markers expressed on CLL cells which are likely to a have physiological role in cell survival. Acetyl choline receptor subunit β4 was expressed on CLL cells at protein level as demonstrated by flow cytometry and mRNA level by RT-PCR. Several other subunits were also demonstrated to be expressed at mRNA level but failed to be demonstrated to be expressed at protein level by flow cytometry. Further experiments to explore the physiological role and possible therapeutic role of acetyl choline receptors have failed to demonstrate any benefit. Similarly dopamine receptor DRD4 was shown to be expressed at protein level and mRNA level. Further experiments have shown that dopamine was proapoptotic to CLL cells in the *in vitro* system. These results are consistent with the finding of Meredith *et al*, that monoamines including dopamine, adrenaline, noradrenaline, and serotonin are anti-proliferative for normal and a spectrum of malignant B-cells representing various stages of maturation arrest from pre-B cells through to plasma cells, with dopamine as the most potent one and the inhibition of T-cell receptor induced cell proliferation noticed on normal T-cells by Basu et al (Meredith et al., 2006) (Basu et al., 2010). However in contrast to the published results of Meredith *et al*., the results described in this study could not find any protective effect of catalase in preventing apoptosis in CLL cells. Furthermore this study has shown that dopamine down-regulates the SYK phosphorylation and downstream signalling of calcium flux mediated by BCR stimulation. Finally the results have shown that a combination of pan D2 inhibitor domperidone along with BCR pathway kinase inhibitor (GS-1101) is much more efficient in inducing apoptosis in CLL cells than either of these agents on their own. Thus manipulation of dopamine receptors on CLL cells offers the possibility of a new therapeutic strategy.

8. General Discussion: Dopamine activates apoptosis in CLL cells by down regulating SYK phosphorylation and pan D2 inhibitor potentiates the proapoptotic effect of BCR pathway kinase inhibitors

CLL is considered as an indolent disorder with a relapsing and remitting course. There are effective treatments available now for bringing the disease into remission even reaching an MRD negative status. The current standard approach would be combination chemo/immuno-based treatment which induces an effective remission, but invariably the disease relapses over a period of time (Oscier et al., 2012). Even though the remission period after chemo/immuno therapy in a majority of patients is many years, in a substantial number of patients the disease relapses very quickly and treatment options are limited. Even with allogeneic transplant the disease related death is considerably high in this group of patients (Delgado et al., 2009). This poor prognostic group include those patients with specific cytogenetic abnormalities, which make them biologically resistant to chemotherapy. Even in patients that remain in remission for many years the disease will ultimately relapse. It may respond to the same treatment or to a different combination therapy, but over a period of time the remission duration shortens until finally it become resistant to most standard treatments. So until now CLL has been considered as an incurable disease (O'Brien and Keating, 2005).

Recent years have shown a huge interest in CLL research especially in developing newer treatments. One of the major discoveries in the last few years in this field is the role of antigen stimulation in the pathophysiology of initiation and maintenance of the disease (Ghia et al., 2008). This knowledge has led to the development of B-cell receptor kinases as potential therapeutic targets for treating CLL. Similar to other malignancies there is still a huge necessity for developing newer treatment agents for CLL. Identification of malignancy specific targets is of vital importance in diagnosis, prognosis and developing targeted treatments in malignancy. Gene expression profiling is a powerful tool that can identify genes that are expressed at RNA level in cells on a massive scale. Several studies have tried to identify the differentially expressed genes in CLL. These studies were unfortunately not successful in identifying any CLL specific targets that could have any potential therapeutic implications. But these studies have

provided evidence of the overall expression of genes in the CLL cells (Klein et al., 2001b) (A. Rosenwald et al., 2001) (Haslinger et al., 2004). This may not correspond to the level of proteins expressed in the cell which will depends on various other factors like the physiological state of the cell, the gene it is transcribed from, the speed with which the mRNA is degraded in the cell before translation, and the functional status of several of these proteins depend on post-translational modification like phosphorylation, glycosylation and complex formation with other proteins. This study is an attempt to identify these proteins expressed on the plasma membrane of the CLL cells based on their mRNA expression in the gene expression array by flow cytometry. Monoclonal as well as polyclonal antibodies were used for flow cytometry. While most monoclonal antibodies were optimised for flow cytometry, polyclonal antibodies have to be conjugated and optimised for such experiments. Even though no antigens confined to CLL cells were identified there were several antigens expressed on CLL cells but expressed on other cell types also, predominantly on B-cells. The expression of these antigens on other cells is not a restriction for them to be developed as a therapeutic target. This is substantiated by the experience with antigens like CD20 and CD52. The levels of expression of some of these antigens which include CD119, CD51, IFNGRB, INTGβ7, CHRNB4 and TAG1 were higher on B-cells compared to CLL cells even though the MFI varies widely between these antigens. Their differential expression on B-cells and CLL cells can be exploited for developing these antigens as potential prognostic and MRD markers.

Another intriguing observation noticed was that several of the antigens expressed on CLL cells were neuronal markers which acts as receptors for neurotransmitters. Acetyl choline receptors, dopamine receptors and serotonin receptors were the predominant ones. Literature search has shown that some of them are expressed in normal haemopoietic cells and that there is a physiological role for these transmitters and receptors (Wang et al., 2003) (De Rosa et al., 2005) (Basu et al., 1993) (Ricci and Amenta, 1994). But their role in the pathophysiology of CLL is not clear. Therapeutically the more important question would be whether any of these transmitters and their receptors is involved in the CLL cell survival pathway. Several other antigens were also identified, but studying the pathways for all identified antigens would be beyond the scope of this project. In this study as acetyl choline receptor subunit β4 showed the maximum MFI among all antigens studied by flow cytometry, it was decided to explore the

acetyl choline pathway first. Numerous studies have shown that several acetyl choline subunit combinations exist in cells of immune system. In B-cells some of these receptors like α4β2 are involved in IgM-related proliferative effects while receptors like α7 and α9α10 play an inhibitory role in CD40-related proliferation. In our study several subunits were expressed at the mRNA level even though the protein level expression was found only for the β4 subunit. The most likely reason for this is the poor quality of the antibodies for flow cytometry experiments. Even though several subunits were expressed, neither acetyl choline nor its antagonist had any effect on CLL cell survival, so further exploration of the pathway was temporarily suspended.

The next receptor studied was dopamine receptor DRD4 as it was also found to be expressed in the screening process. Interestingly, there are several studies in the literature which showed that dopamine receptors were involved in B-cell survival and function. Some studies have shown that B-cells are capable of producing dopamine on their own which can exert an autocrine or paracrine effect (Ferrari et al., 2004). Additional studies have shown that dopamine can induce an apoptotic effect on lymphocytes and malignant cell lines derived from B-cell precursors. Studies differ in their evidence on the mechanism by which dopamine produce this effect (Meredith et al., 2006) (Basu et al., 2010). In neuronal cells the main mechanism by which dopamine mediates its effect is through cAMP as it is a G-protein coupled receptor. Cyclic AMP triggers a number of downstream signalling pathways by phosphorylating several proteins and voltage and ligand gated ion channels. Dopamine receptors can be divided into two major groups, D1 and D2, based on their cellular functions, which wield opposite effects in cells by upregulating and downregulating cAMP, respectively (Neve et al., 2004).

In this study we have shown that at least one receptor for dopamine, DRD4 is expressed on CLL cells. The expressions of other receptors were not studied due to time restrictions. The study also demonstrated that dopamine induced apoptotic cell death in CLL in a dose dependent manner. A previously described mechanism by which dopamine induces antiproliferative effect in a malignant B-cell line was through auto-oxidation and was prevented by catalase-mediated decomposition of H_2O_2 (Meredith et al., 2006). However, in our study we could not find any protective effect of catalase in preventing apoptosis in CLL cells. This directed us to the possibility of dopamine receptors as the mediators of this pro-apoptotic

effect. Dopamine receptors are G protein coupled receptors; most of the downstream effects are mediated by regulating the catalytic activity of adenylate cyclase, thereby controlling level of cyclic AMP inside the cell. Several studies in the past have shown that cAMP can have a pro-apoptotic or anti-apoptotic effect depending on the type of cell and the condition under which experiment is carried out (Insel et al., 2012). Examples of cells in which cAMP induced pro-apoptotic effects are cardiac myocytes, adrenocortical cells, breast cells, fibroblasts, leukocytes/lymphomas/leukaemias, neuronal/glial cells, lung carcinoma cells, melanoma cells, osteoblasts, ovarian cancer and granulosal cells, renal mesangial cells, vascular endothelial and smooth muscle cells. Several of these cells can have either a proapoptotic or an antiapoptotic effect depending on the condition in which experiment is conducted. For example, increased levels of cAMP usually induces apoptosis in murine S49 lymphoma cells, but if treated with pro-apoptotic agents like anti-Fas and ultraviolet light, it shows a transient anti-apoptotic response to cAMP (Yan et al., 2000) (Zhang and Insel, 2004) (Insel et al., 2012). The exact mechanism by which cAMP mediates these outcomes is not very clear. As discussed before cAMP mediates its effect through three main targets: protein kinase A (PKA), the GTP-exchange protein EPAC and the cyclic-nucleotide-gated ion channels. PKA phosphorylates several target proteins and the downstream effect depends on the type of protein it phosphorylates. The nature of the proapoptotic proteins it phosphorylates is still to be elucidated. However experiments with various S49 lymphoma cell mutants lacking expression or function of distinct cAMP signalling components, including Gs and PKA, provides evidence that the pro-apoptotic effects, to some extent is mediated by PKA activation (Yan et al., 2000). There are limited data to suggest that EPAC proteins can also mediate pro-apoptotic or anti-apoptotic effects.

In our study the pro-apoptotic effect of dopamine could be mediated by cAMP pathway as dopamine increases its level through D1 receptors. This postulation is further substantiated by the observation that a pan-D2 antagonist, domperidone, potentiates the effect of dopamine as D2 mediated inhibition of adenylate cyclase is blocked by the antagonist, thereby enhancing the D1 mediated effect. Domperidone is found to be proapoptotic by itself to a lesser extent probably mediated by dopamine generated by CLL cells itself or the supporting mononuclear cells in the medium exerting an autocrine or paracrine effect.

Literature review has shown that dopamine signalling through D2 receptors inhibits T-cell receptor induced proliferation by down-regulating ZAP70 in normal T cells but not in Jurkat cells due to a missense mutation in the D2 receptor (Basu et al., 2010). Another interesting study related to the mechanism of cAMP related proapoptotic effects has been published by Smith *et al* (Smith et al., 2005). They observed that *PDE4B2* is the major phosphodiesterase isoform in DLBCL and it is overexpressed in fatal/refractory DLBCL. PDE4B limits the growth-inhibitory effects of cAMP in DLBCL cell lines and PDE4B inactive mutant cell lines are more vulnerable for cAMP induced apoptosis (Smith et al., 2005). Interestingly they have demonstrated that the pro-apoptotic effect of cAMP is independent of PKA and EPAC and was mediated by downregulation of phosphoAKT which modulated the phosphorylation of BAD protein. Expression of constitutively active AKT protects DLBCL cell lines from cAMP-mediated growth inhibition. Further exploration of this pathway by another group demonstrated that the cAMP mediated inhibitory effects toward PI3K/AKT were actually transduced via SYK (Kim et al., 2009). In normal B-cells the elevation of cAMP levels also resulted in a marked down-regulation of SYK Tyr525/526 phosphorylation without any changes in the phosphorylation levels of LYN and SRC (Kim et al., 2009).

Increasing cAMP levels resulted in marked decrease in phosphorylation of AKT (S473) in DHL6 lymphoma cells expressing a vector encoding PDE4B, but these inhibitory events were absent in DHL6 cell mutants expressing constitutively phosphorylated SYK. Functionally, SYK inhibition with piceatannol induces higher apoptosis with a lower IC50 in DLBCL cell lines with reduced PDE4B expression compared to those cells expressing high PDE4B levels, corroborating the observation that higher cAMP level potentiates the effect of SYK inhibition. However Moon *et al* in another study have shown that in CLL cells elevation of cAMP by either rolipram, a prototypic PDE4 inhibitor, or forskolin, an adenylate cyclase activator induces apoptosis by activating protein phosphatase 2A induced dephosphorylation of pro-apoptotic Bcl-2 family members such as Bad. Their study did not show whether up-regulation of PP2A-like phosphatase activity was potentiated by PKA mediated phosphorylation (Moon and Lerner, 2003).

Our study has clearly shown that dopamine down-regulates the SYK phosphorylation mediated by BCR stimulation. This pathway inhibition is further substantiated by modulation of calcium flux, a downstream signal.

Finally the results have shown that a combination of pan D2 inhibitor domperidone along with BCR pathway kinase inhibitor (GS-1101) is much more efficient in inducing apoptosis in CLL cells than either of these agents on their own. This could be mediated indirectly by upregulation of cAMP by dopamine secreted by CLL cells or other mononuclear cells due to inhibition of D2 receptor by domperidone. In this case the uninhibited D1 pathway is amplified, which in turn modulates the IgM mediated SYK phosphorylation and downstream pathways. This observation has a major therapeutic implication as both these agents are already in clinical use, although domperidone is used as an antiemetic (Friedberg et al., 2010) (Helmers, 1977). Thus, this combination can be safely tried in CLL patients. The possible interaction between these pathways are summarised in Figure 8.1.

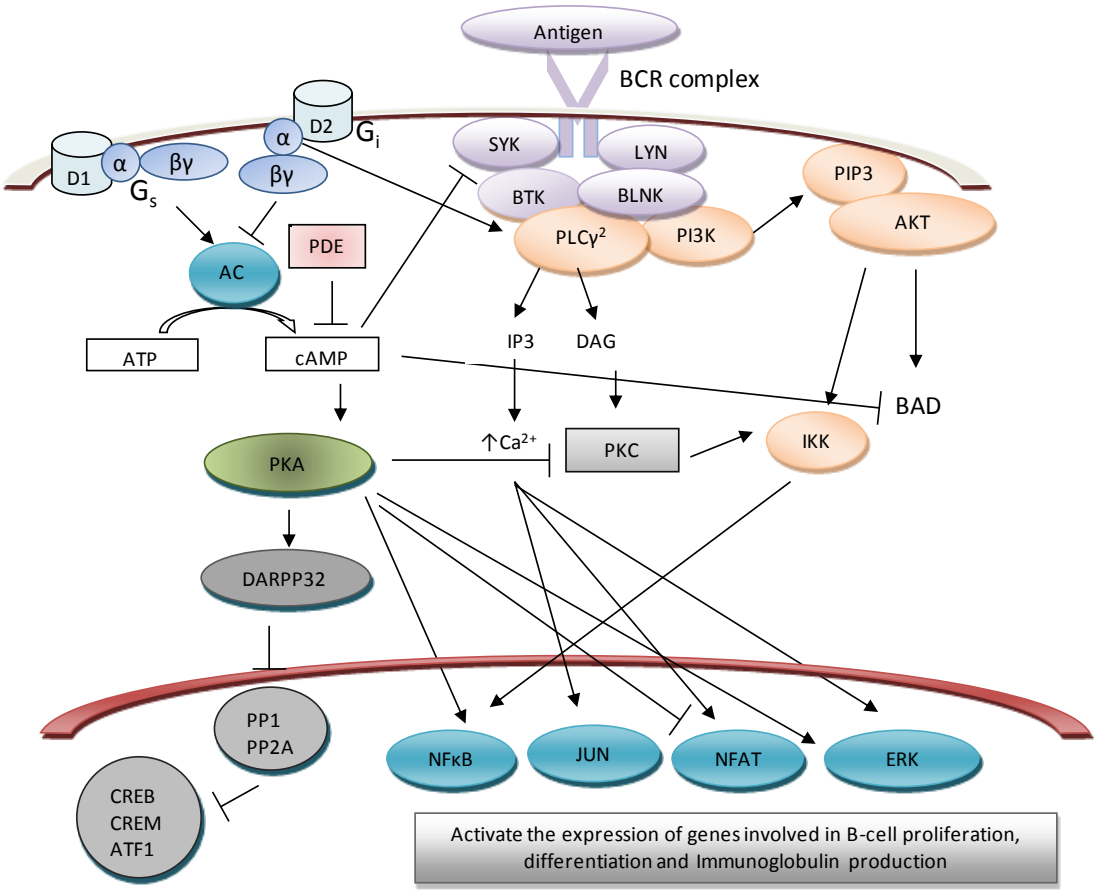


Figure 8.1 Proposed interaction between dopamine receptors and BCR pathway.

Activation of excitatory dopamine pathway either by stimulating D1 type of receptors or by inhibiting D2 type of receptors increases the cAMP level in the CLL cells. This in turn can downregulate SYK phosphorylation, the exact mechanism of which is not clear. Inhibition of SYK phosphorylation can further downregulate the pro-survival signals from BCR stimulation. ⊣ inhibitory stimulation → excitatory stimulus

This study also explored effect of a new SYK specific inhibitor on CLL cell viability *in vitro*. Previously SYK inhibitors were shown to induce apoptosis in CLL cells, but these inhibitors were not very specific for SYK phosphoprotein. GSK pharmaceuticals have produced a highly specific SYK inhibitor. Our study has shown that this highly specific SYK inhibitor is also proapoptotic to CLL cells. It abrogates the antigen mediated prosurvival signals in the BCR kinase pathway as demonstrated by inhibition of SYK phosphorylation and calcium flux thereby negating the survival advantage offered by B-cell receptor stimulation. There was no difference in response when established poor prognostic markers like 17p deletion and unmutated *IGHV* were compared to their good prognostic counterparts. This molecule could be further explored as a potential therapeutic agent in CLL either on its own or in combination with other agents like D2 antagonists as demonstrated in previous experiments.

Future studies arising from this work should include experiments to establish the molecular links between several of these observations. The expression of different dopamine receptors should be established at mRNA and protein level. Secretion of dopamine by CLL cells or other MNC should be ascertained by experimental evidence. Cyclic AMP levels following dopamine stimulation in CLL cells should be measured quantitatively along with the levels following D2 receptor block by domperidone. These levels should be correlated with the level of apoptosis in CLL cells. Further clarification is needed regarding the mechanism by which dopamine induces cell death. Assuming that it is through a cAMP mediated pathway, the link molecules that trigger the downstream signalling need to be established. The data in this study showed that dopamine down-regulates SYK phosphorylation, but the intermediary molecules between these steps are not fully understood. To prove that dephosphorylation of SYK is the primary mechanism by which dopamine induces apoptosis, other cAMP mediated pathways including PKA activation and EPAC protein activation should be tested. Changes in phosphorylation status induced by dopamine to other phosphokinases upstream and downstream of SYK in the BCR pathway should also be identified.

Even though the results presented herein identified that D2 group specific receptor antagonists can be pro-apoptotic, identification of specific dopamine receptors involved in apoptotic induction require further experiments with available receptor specific agonists and antagonists. The role of dopamine receptors in microenvironment interaction can be

established by various co-culture experiments with fibroblasts, including the variation of expression in the receptors when coming in contact with stromal cells, secretion of dopamine by fibroblasts, change in level of cAMP in CLL cells during contact with fibroblasts, and effect of various dopamine receptor agonists and antagonists on the viability of CLL cells in stromal co-culture.

Several other interesting receptors were also identified through the antigen identification study. Some of them like INTGβ7 are preferentially expressed on normal B-cells compared to CLL cells. This differential expression can be further explored as a potential MRD marker. This has to be assessed on multiple samples and validated for this use. Another interesting molecule identified is CD85j which is a type I transmembrane protein of immunoglobulin superfamily which functions to suppress intracellular kinase activity by recruiting the phosphatase SRC homology domain containing phosphatase 1 (SHP-1) to phosphorylated tyrosines (Lamar et al., 2010). This inhibitory stimulus can be further explored as potential therapeutic option as it potentially could deactivate the kinases in BCR pathway. It is intriguing to observe that CD205 an apoptotic cell recognising molecule involved in recognising ligands expressed during apoptosis and necrosis of multiple cell lines is expressed in CLL cells (Shrimpton et al., 2009). The role of this particular receptor also warrants further evaluation.

In conclusion, this study allowed the identification of the expression of several new antigens on the surface of CLL cells. One of the antigens identified was dopamine receptor, which was found to have potential therapeutic implications. Two established BCR kinase inhibitors and one new inhibitor were tested in the *in vitro* system to assess their proapoptotic effect on CLL cells. All three were found to be effective using multiple assays. A potential synergistic effect was seen with BCR kinase inhibitors and dopamine receptor antagonists in inducing apoptosis in CLL cell *in vitro*. Thus, the initial observation of differential mRNA expression lead to the identification of a functional pathway in CLL cells that may be used to therapeutic advantage and can be explored in clinical trials.

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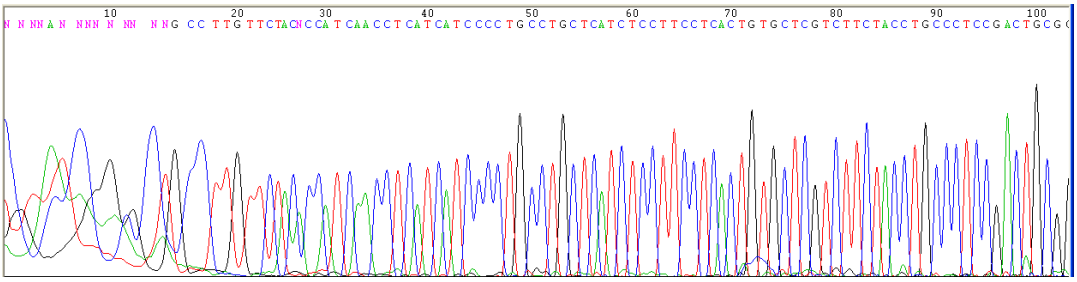
10. Appendix

10.1 Appendix 1. Sequencing of acetyl choline receptor subunits

α3 subunit



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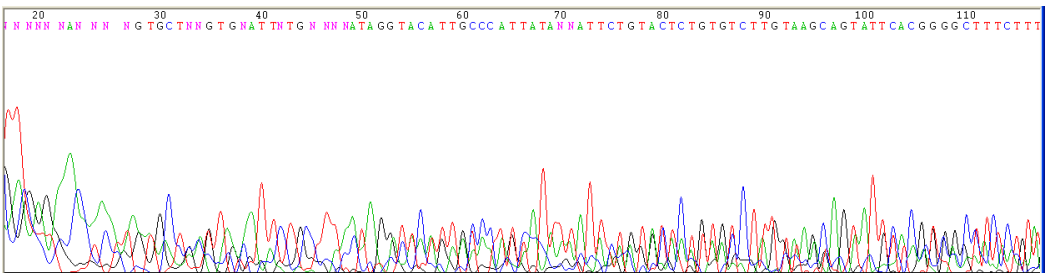
α4 subunit



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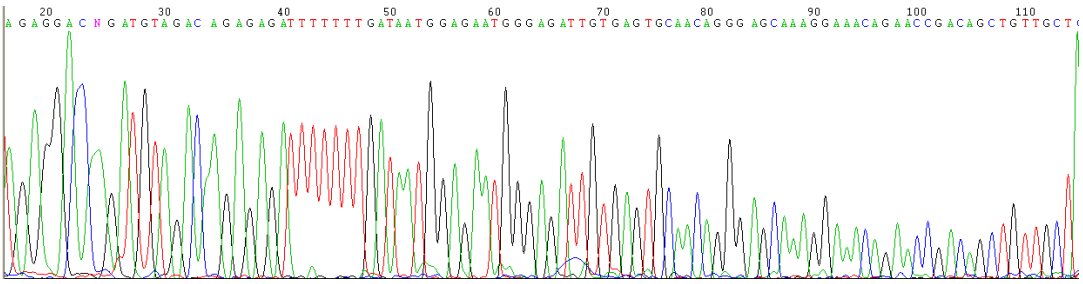
α5 subunit



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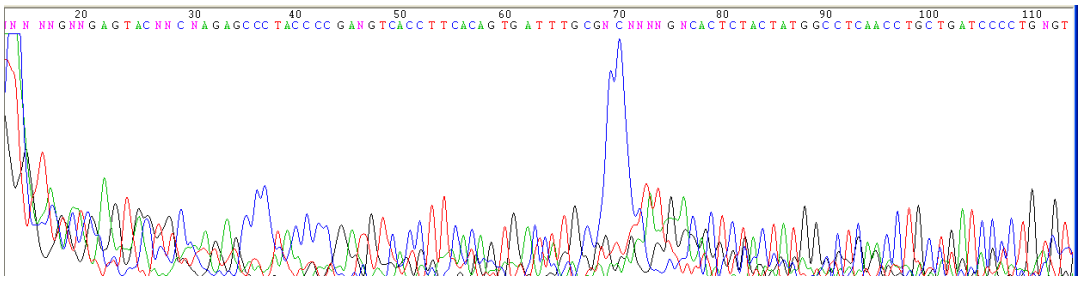
α7 (forward primer) subunit



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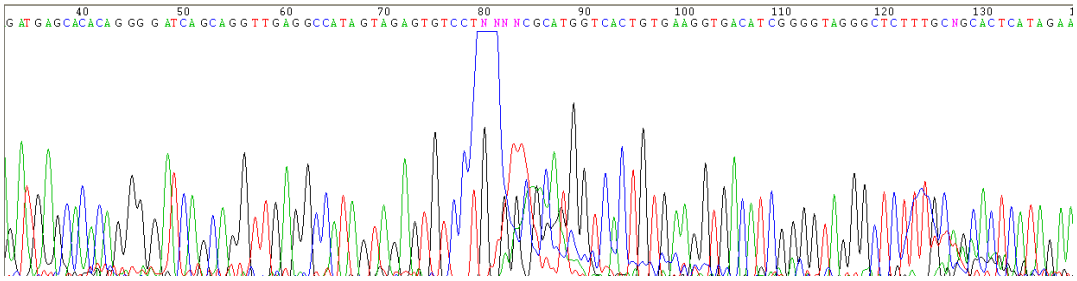
α7 (reverse primer) subunit



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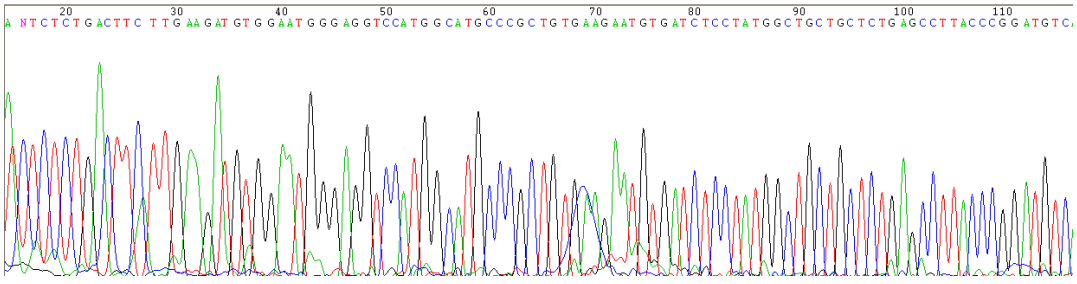
α9 subunit



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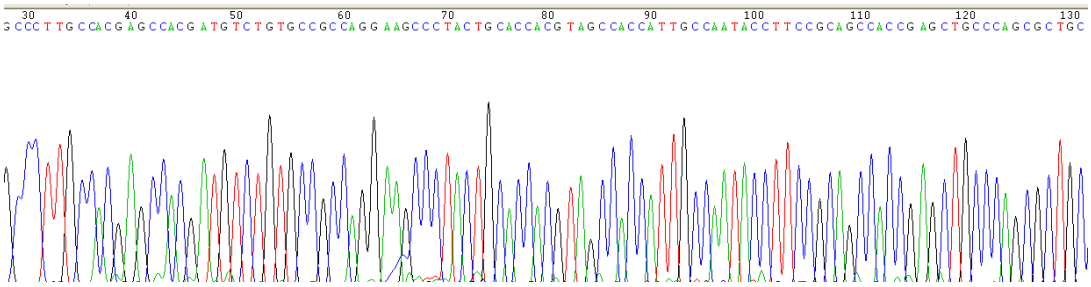
α10subunit



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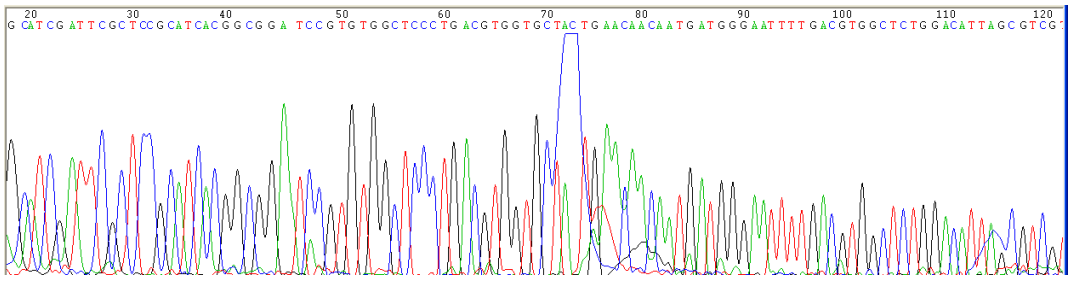

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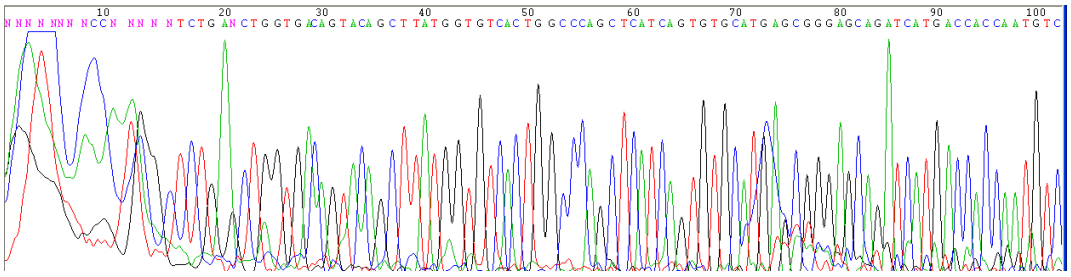
β2 subunit



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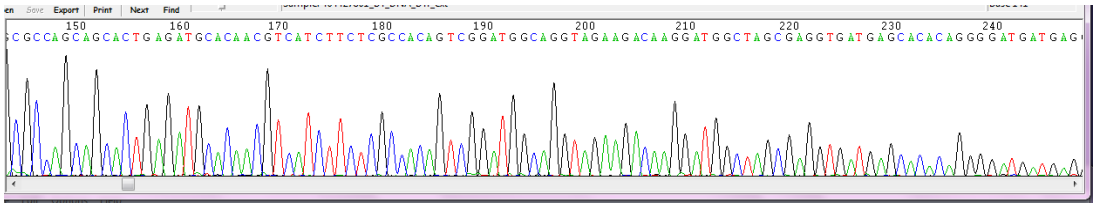
β4 subunit



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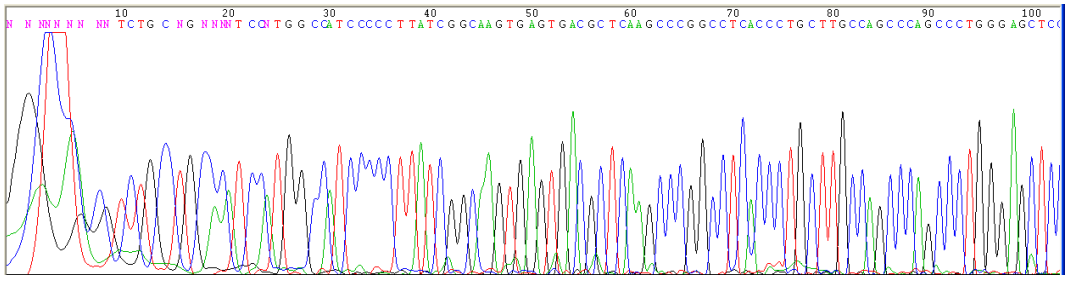
δ subunit



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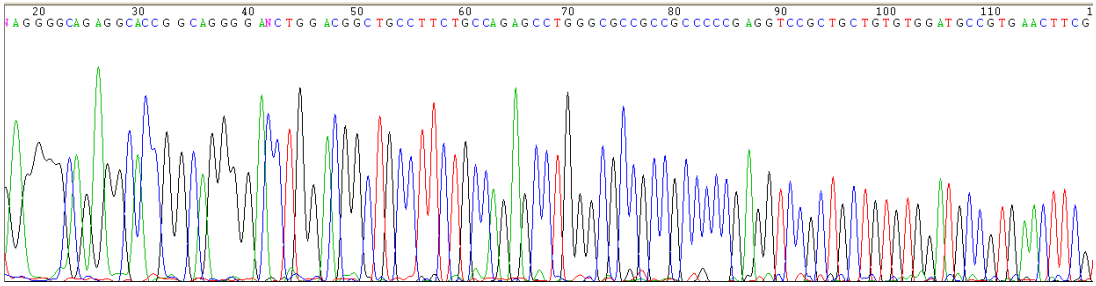
ε subunit



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GAACAANN

10.2 Appendix 2 Antibodies used

Antigen	Conjugate	Clone/Animal	concentration	Amount used	Volume	manufacturer
CD5	PE	L17F12	6.25µg/ml	0.0125µg	2µl	BD Bioscience
CD5	APC	L17F12	6.25µg/ml	0.0125µg	2µl	BD Bioscience
CD19	PE-Cy7	SJ25C1	25µg/ml	0.025µg	1µl	BD Bioscience
CD45	FITC	2D1	50µg/ml	0.1µg	2µl	BD Bioscience
CD20	Pacific Blue	B9E9	unavailable			Beckman Coulter
CD2	PE	S5.2	6.25µg/ml	0.00625 µg	1µl	BD Bioscience
CD45	APC.Cy7	2D1	100µg/ml	0.2 µg	2µl	BD Bioscience
CD167a	PE	51D6			5 µl	Biolegend
CD85j	PE	GH1/75			5 µl	Biolegend
CD298	PE	LNH-94	50 µg/ml	0.25 µg	5 µl	Biolegend
CD279	PE	EH12.2H7			5 µl	Biolegend

CD137L	PE	5F4	100 µg/ml	0.5 µg	5 µl	Biolegend
CD97	PE	VIM3b			5 µl	Biolegend
CD205	PE	HD30	50 µg/ml	0.25 µg	5 µl	Biolegend
IFN-γRβ chain	PE	2HUB-159			5 µl	Biolegend
CD51	PE	NKI-M9	25 µg/ml	0.125 µg	5 µl	Biolegend
Integrin β 7	PE	FIB504	12.5 µg/ml	0.0625 µg	5 µl	Biolegend
CD119	PE	GIR-94			5 µl	Biolegend
DR3	PE	JD3			5 µl	Biolegend
CD337	PE	P30-15	5 µg/ml	0.025 µg	5 µl	Biolegend
erbB3	PE	1B4C3	12.5 µg/ml	0.0625 µg	5 µl	Biolegend
CD141	PE	M80	100 µg/ml	0.5 µg	5 µl	Biolegend
CD210	PE	3F9	100 µg/ml	0.5 µg	5 µl	Biolegend
CD155	PE	TX24	50 µg/ml	0.25 µg	5 µl	Biolegend
CD114	PE	LMM741	50 µg/ml	0.25 µg	5 µl	Biolegend
Anti-rabbit $F(ab)_2$	Alexa fluor 647	Goat	2mg/ml	0.4 µg	0.2 µl	Invitrogen
Anti-rabbit	Alexa fluor 647	Goat	2mg/ml	0.4 µg	0.2 µl	Invitrogen
CD3		Rabbit	0.25 mg/ml	1 µg	4µl	Abcam
CD99		Rabbit	0.2 mg/ml	1 µg	5µl	Abcam
ADAM19		Rabbit	1 mg/ml	1µg	1µl	Abcam
GPR18		Rabbit	N/A		1µl	Abcam
CHRNB4		Rabbit	1 mg/ml	1µg	1µl	Abcam
APLP1		Rabbit	0.2 mg/ml	1µg	5µl	Antibodies online
DRD4		Rabbit	1 mg/ml	1µg	1µl	Antibodies online
GPR12		Rabbit	1 mg/ml	1µg	1µl	Antibodies online
ROR1		Rabbit	0.25mg/ml	1µg	4µl	Abcam
TAG1		Rabbit	N/A		1µl	Abcam
5HTR		Rabbit	0.6 mg/ml	1µg	1.6µl	Abcam
JAG1		Rabbit	1 mg/ml	1µg	1µl	Abcam
ACCN1		Rabbit	N/A		1µl	Abcam
SLC2A3		Rabbit	1 mg/ml	1µg	1µl	Antibody online

EFNB1		Rabbit	N/A		1µl	Antibody online
GPR56		Rabbit	N/A		1µl	Abcam
RAMP3		Rabbit	0.5 mg/ml	1µg	2µl	Abcam
EDA		Rabbit	1 mg/ml	1µg	1µl	Abcam
NG2		Rabbit	0.2 mg/ml	1µg	5µl	Abcam
EDG4		Rabbit	0.25 mg/ml	1µg	4µl	Abcam
AMFR		Rabbit	0.5 mg/ml	1µg	2µl	Abcam
LTK		Rabbit	0.25 mg/ml	1µg	4µl	Abcam
ACVRL1		Rabbit	0.25 mg/ml	1µg	4µl	Abcam
ADAM28		Rabbit	1 mg/ml	1µg	1µl	Abcam
TMPRSS6		Rabbit	1 mg/ml	1µg	1µl	Abcam
CNR2		Rabbit	0.5 mg/ml	1µg	2µl	Abcam
GPR15		Rabbit	0.5 mg/ml	1µg	2µl	Abcam
GPR35		Rabbit	N/A		1µl	Abcam
FPRL1		Rabbit	1 mg/ml	1µg	1µl	Abcam
MD1		Rabbit	1 mg/ml	1µg	1µl	Abcam
RVK		Rabbit	0.25 mg/ml	1µg	4µl	Abcam
GLUT1		Rabbit	0.2 mg/ml	1µg	5µl	Abcam
F2RL3		Rabbit	N/A		1µl	Abcam
NMDAR1		Rabbit	0.8 mg/ml	1µg	1.25µl	Abcam
DGCR2		Rabbit	1 mg/ml	1µg	1µl	Abcam
CRHR2		Rabbit	N/A		1µl	Abcam
ADAM15		Rabbit	1 mg/ml	1µg	1µl	Abcam
NRAMP1		Rabbit	1 mg/ml	1µg	1µl	Abcam
ENT1		Rabbit	N/A		1µl	Abcam
SDC3		Rabbit	N/A		1µl	Abcam
LTB4R		Rabbit	N/A		1µl	Abcam
GPR3		Rabbit	1 mg/ml	1µg	1µl	Antibody online
MMP15		Rabbit	0.2 mg/ml	1µg	5µl	Abcam
HPN		Rabbit	N/A		1µl	Abcam
TNFR1		Rabbit	1 mg/ml	1µg	1µl	Abcam
GYPC		BRIC10	1 mg/ml	1µg	1µl	Abcam
MR1		Mouse	1 mg/ml	1µg	1µl	Abcam
CDH15		21G4	N/A		1µl	Abcam
SLC20A1		Mouse	1 mg/ml	1µg	1µl	Abcam
SLC4A1		BIII 136	N/A		1µl	Abcam

PLAUR		R-4	1 mg/ml	1µg	1µl	Abcam
ATP1B1		464.6	1 mg/ml	1µg	1µl	Abcam
EGF		EGF-10	2.3 mg/ml	1µg	0.4µl	Abcam
MMP14		LEM-2/63.1	1 mg/ml	1µg	1µl	Abcam
ATP1B2		Mouse	1 mg/ml	1µg	1µl	Abcam
CDH16		4H6/F9	N/A		1µl	Abcam
KIA0319		Mouse	N/A		1µl	Abcam
GABBR1		Mouse	1 mg/ml	1µg	1µl	Abcam
NRG2		Mouse	1 mg/ml	1µg	1µl	Abcam
RHBDL1		Mouse	1 mg/ml	1µg	1µl	Abcam
SLC9A1		Mouse	1 mg/ml	1µg	1µl	Abcam
TGFA		P/T1	0.2 mg/ml	1µg	0.5µl	Abcam
JTB		Mouse	1 mg/ml	1µg	1µl	Abcam
STIM1		Mouse	1 mg/ml	1µg	1µl	Abcam
PRRG1		Mouse	1 mg/ml	1µg	1µl	Abcam
CHRNA1		Rabbit	N/A		1µl	Abcam
CHRNA2		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNA3		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNA4		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNA6		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNA7		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNB1		EP2067Y	N/A		1µl	Abcam
CHRND		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNE		Rabbit	1 mg/ml	1µg	1µl	Abcam
CHRNG		Rabbit	1 mg/ml	1µg	1µl	Abcam
DRD4		Rabbit	1.0 mg/ml	1µg	1µl	Acris antibodies
DRD4		Rabbit	1.9 mg/ml	1µg	0.53 µl	Acris antibodies
AKT (pT308)	PE	J1-223.371			20µl	BD Bioscience
NF-κB p65 (pS529)	Alexa Fluor 647	K10-895.12.50			20µl	BD Bioscience
Akt (pS473)	V450	M89-61			5 µl	BD Bioscience
SYK (pY348)	PE	I120-722			20 µl	BD Bioscience
BTK	Alexa	24a/BTK			20 µl	BD

(pY551) & Itk (pY511)	Fluor 488	(Y551)				Bioscience
ZAP70	PE	SBZAP	0.1 mg/ml			Beckman Coulter
SYK	FITC	4D10	0.5 mg/ml			BD Pharminge n
Zenon Alexa Flour 647 mouse IgG_{2a} labelling kit	Zenon mouse IgG_{2a} labeling reagent Zenon blocking reagent (mouse IgG)		200 µg Fab fragment/mL 5 mg/mL		5 µl 5 µl	Invitrogen
Zenon Alexa Flour 647 mouse IgG_1 labelling kit	Zenon mouse IgG_1 labeling reagent Zenon blocking reagent (mouse IgG)		200 µg Fab fragment/mL 5 mg/mL		5 µl 5 µl	Invitrogen
Zenon Alexa Flour 647 Rabbit IgG labelling kit	Zenon rabbit IgG labeling reagent Zenon blocking reagent (rabbit IgG)		200 µg Fab fragment/mL 5 mg/mL		5 µl 5 µl	Invitrogen

10.3 Appendix 3 Reagents used

Lymphoprep	Axis-Shield	Sodium diatrizoate - 9.1% (w/v) Polysaccharide – 5.7% (w/v)
Ammonium Chloride	Sigma-Aldrich	8.6g/l in distilled water
FACSflow	BD Biosciences	
Bovine Serum Albumin	Sigma	0.3% in FACSflow

	Aldrich	
BCell Isolation Kit (B-CLL)	Miltenyibiotec	1. B-CLL Biotin Antibody cocktail-mAb against CD2,CD4, CD11b, CD16, CD36, Anti-IgE,CD235a 2. Microbead conjugated to monoclonal antibiotin antibodies
RPMI (Roswell Park Memorial Institute) Media 1640	GIBCO	Component Conc(mg/L) Glycine 10 L-Alanyl-Glutamine 446 L-Arginine 200 L-Asparagine 50 L-Aspartic acid 20 L-Cystine 50 L-Glutamic Acid 20 L-Histidine 15 L-Hydroxyproline 20 L-Isoleucine 50 L-Leucine 50 L-Lysine hydrochloride 40 L-Methionine 15 L-Phenylalanine 15 L-Proline 20 L-Serine 30 L-Threonine 20 L-Tryptophan 5 L-Tyrosine 20 L-Valine 20 Biotin 0.2 Choline chloride 3 D-Calcium pantothenate 0.25 Folic Acid 1 Niacinamide 1 Para-Aminobenzoic Acid 1 Pyridoxine hydrochloride 1 Riboflavin 0.2 Thiamine hydrochloride 1 Vitamin B12 0.005 i-Inositol 35 Calcium nitrate (Ca(NO3)2 4H2O) 100 Magnesium Sulfate (MgSO4-7H2O) 100 Potassium Chloride (KCl) 400 Sodium Bicarbonate (NaHCO3) 2000 Sodium Chloride (NaCl) 6000 Sodium Phosphate dibasic(Na2HPO4) 800 D-Glucose (Dextrose) 2000 Glutathione (reduced) 1 Phenol Red 5
IMDM (Iscove's Modified Dulbecco's Medium)	GIBCO	Glycine 30 L-Alanine 25 L-Alanyl-L-Glutamine 812 L-Arginine hydrochloride 84 L-Asparagine (freebase) 28.4

		L-Aspartic acid 30 L-Cystine 70 L-Glutamic Acid 75 L-Histidine hydrochloride-H2O 42 L-Isoleucine 105 L-Leucine 105 L-Lysine hydrochloride 146 L-Methionine 30 L-Phenylalanine 66 L-Proline 40 L-Serine 42 L-Threonine 95 L-Tryptophan 16 L-Tyrosine disodium salt 104 L-Valine 94 Biotin 0.013 Choline chloride 4 D-Calcium pantothenate 4 Folic Acid 4 Niacinamide 4 Pyridoxal hydrochloride 4 Riboflavin 0.4 Thiamine hydrochloride 4 Vitamin B12 0.013 i-Inositol 7.2 Calcium Chloride (CaCl2-2H2O) 219 Magnesium Sulfate (MgSO4-7H2O) Potassium Chloride (KCl) 330 Potassium Nitrate (KNO3) 0.076 Sodium Bicarbonate (NaHCO3) 3024 Sodium Chloride (NaCl) 4500 Sodium Phosphate monobasic 141 Sodium Selenite (Na2SeO3-5H20) 0.017 D-Glucose (Dextrose) 4500 HEPES 5958 Phenol Red 15 Sodium Pyruvate 110
AIM V	GIBCO	Not Available
Hank's balanced salt solution (HBSS)	Invitrogen	KCl 400 KH2PO4 60 NaHCO3 350 NaCl 8000 Na2HPO4 48 Dextrose 1000 Phenol red 10
Penicillin Streptomycin	Invitrogen	Penicillin 5000units Streptomycin 5000µg per ml
Trypsin-EDTA	Invitrogen	Trypsin 2.5g/L EDTA 0.38g/L HBSS
Mitomycin-c	Sigma Aldrich	2mg vial Dissolve in distilled water 0.5mg/ml
Flow Cytometry Absolute	Bangs	1060000 particles/ml

Count Standard	Laboratories	
FcR Blocking reagent	Miltenyibiotec	
Human Ivlg	Viagam	5g%
Casein	Vector Labs	Concentration 0.08% (w/v)
Goat Serum	Sigma Aldrich	
Annexin V Apoptosis detection Kit FITC	ebioscience	Annexin V on FITC 10X Binding Buffer
Annexin V Apoptosis detection Kit APC	ebioscience	Annexin V on APC 10X Binding Buffer
7-AAD viability staining solution	ebioscience	
AffiniPure F(ab')2 Fragment Goat Anti-Human IgM, Fc_γ Fragment Specific	Jackson Immuno Research	1.3mg/ml Buffer- 0.01M Sodium Phosphate, 0.25M NaCl, pH 7.6 Preservative - none
AffiniPure F(ab')2 Fragment Goat Anti-Human IgG, Fc_γ Fragment Specific	Jackson Immuno Research	1.2mg/ml Buffer- 0.01M Sodium Phosphate, 0.25M NaCl, pH 7.6 Preservative - none
Goat F(ab')2 Anti-Human IgD-UNLB (purified)	SouthernBiotech	0.5mg/ml Buffer- borate buffered saline, pH 8.2 Preservative - none
Goat F(ab')2 Anti-Human IgD-UNLB (Dialysed)	SouthernBiotech	1mg/ml Buffer- 0.01M Sodium Phosphate, 0.25M NaCl, pH 7.6 Preservative - none
Lyse/Fix Buffer	BD Bio Science	5X
Perm/Wash Buffer I	BD Bio Science	10X
BD intraSure Kit	BD Bio Science	
Acetylcholine chloride	Sigma Aldrich	Stock solution - 550.5mM in sterile water
Mecamylamine hydrochloride		Stock solution - 49.08mM in sterile water
Doxorubicin	Sigma	Stock solution - 50mg/ml in DMSO

	Aldrich	
2-Fluoroadenine-9-β-D-arabinofuranoside	Sigma Aldrich	Stock solution - 10mM in DMSO
Chlorambucil	Sigma Aldrich	Stock solution - 1g/ml in DMSO
CP226269 (DRD4 agonist)	Sigma Aldrich	Stock solution - 10mg/ml (32.22mM) in DMSO
Sonepiprazole (DRD4 antagonist)	Sigma Aldrich	Stock solution - 10mg/ml (24.9mM) in DMSO
Dopamine	Sigma Aldrich	Stock solution - 100mM in sterile water
Pramipraxole	Sigma Aldrich	Stock solution - 70mM in sterile water
Domperidone	Sigma Aldrich	Stock solution - 100mM in DMSO
SKF38393	Sigma Aldrich	Stock solution - 17.1mM in sterile water
SCH23390	Sigma Aldrich	Stock solution - 15.4mM in sterile water
Catalase from bovine liver	Sigma Aldrich	Available as suspension of catalase crystals in water containing 0.1% (w/v) thymol. Thymol removed by centrifugation and dissolving in 50 mM phosphate buffer, pH 7.0
GSK143	GSK	Stock solution - 10mM in DMSO
CAL101 (GS1101)	Gilead Pharmaceuticals	Stock solution - 10mM in DMSO
PCI-32765 (BTK inhibitor)	Pharmacyclics	Stock solution - 50mM in DMSO
ImProm-II™ Reverse Transcriptase	Promega	MgCl2 ImProm-II™ 5X Reaction Buffer ImProm-II™ Reverse Transcriptase
dNTP Mix	Promega	10mM
Recombinant RNasin® Ribonuclease Inhibitor	Promega	20–40u/µl
Phusion™ Flash High-Fidelity PCR Master Mix	Thermo Scientific™	2X

Zymoclean Gel DNA Recovery Kit	Zymo research	
Brain, Human normal cDNA	Invitrogen	
Calcium chloride	Sigma Aldrich	1M
Magnesium Chloride	Sigma Aldrich	1M
Indo-1, AM, Cell-permeant	Molecular Probes	1mg/ml solution in DMSO
Fluo-3, AM, Cell-permeant	Molecular Probes	1 mg/ml solution in DMSO
Fura Red, AM, Cell-permeant	Molecular Probes	1 mg/ml solution in DMSO

10.4 Appendix 4 Optimisation of sample storage and culture medium.

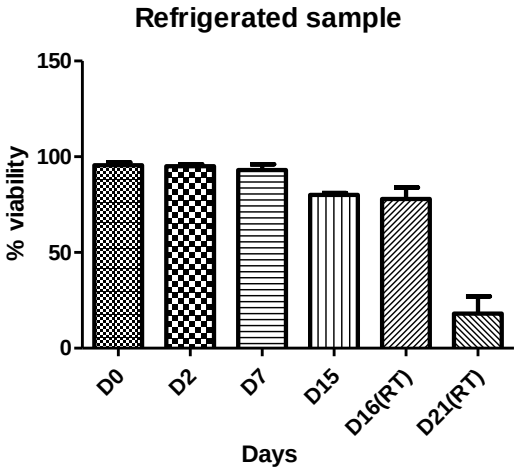

Figure 10.1 Viability of refrigerated cells.

MNC was prepared using lymphoprep sequentially on the same samples (n=2) at day 0, 2, 7 and 15 whilst the sample was refrigerated. This was followed by 2 samples on day 16 and day 21 when the samples were kept at room temperature. Samples were assessed for percentage of viable CLL cells after incubating in culture media for 48 hours. This experiment demonstrates that the samples retained almost 100% viability even up to 1 week if refrigerated.

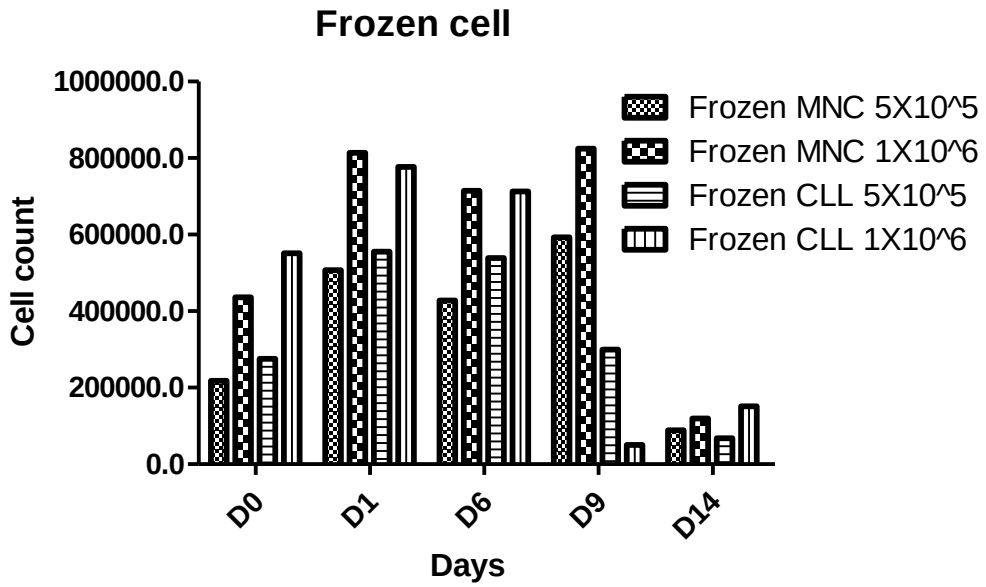

Figure 10.2 Viability of the frozen cells.

MNC were separated using lymphoprep and frozen in 10% DMSO in IMDM. Cells are retrieved at a later date and assessed for viability. MNC and negatively selected CLL cells were seeded at 2 different concentrations. Viable CLL cell count assessed serially at different days. Approximately less than 50% cells were only viable at day 0. Viable cell count seems to go up in first 24 hours and retained up to 9 days if MNC were seeded and up to 6 days if CLL cells were seeded.

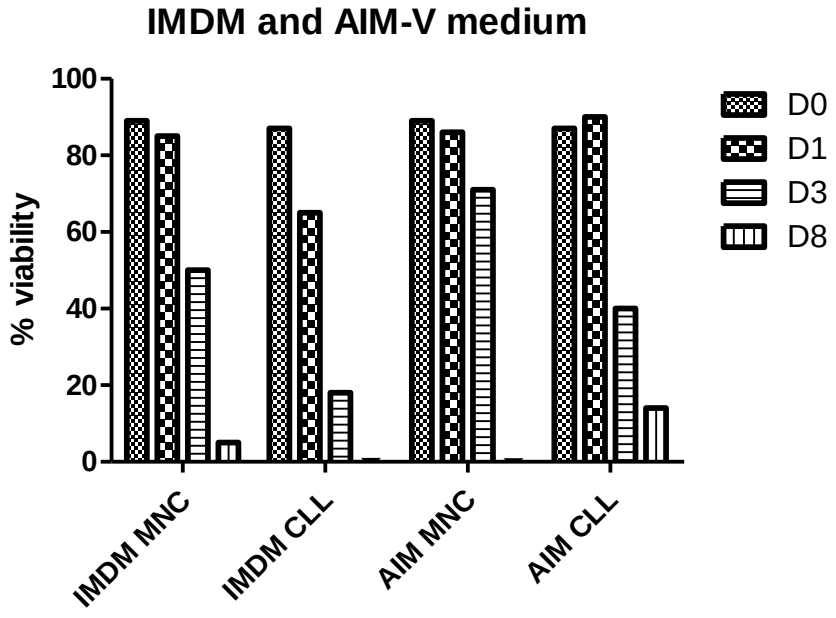

Figure 10.3 Comparison of the effect of IMDM and AIM-V media on CLL viability.