Elucidating The Functional Role Of CD38 In Chronic Lymphocytic Leukaemia

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

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June 2011

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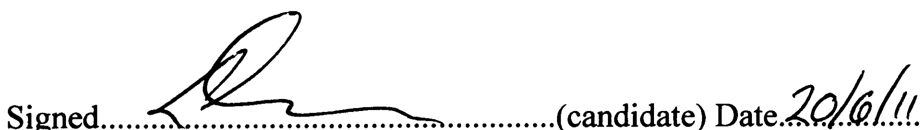
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Contents

Declaration and statements I

Contents II

Publications and presentations X

List of figures XI

List of tables XVI

Abbreviations XVII

Abstract XXI

Acknowledgements XXII

1.0 Introduction

1.1 A brief history......1

1.2 Chronic lymphocytic leukaemia (CLL)......2

1.2.1 Aetiology and epidemiology......3

1.2.2 Clinical presentation of CLL......3

1.2.3 Clinical staging......4

1.2.4 Laboratory diagnosis......5

1.2.4.1 Morphology......5

1.2.4.2 Bone marrow and lymph node involvement......7

1.2.4.3 Lymphocyte doubling time......7

1.2.5 Immunophenotyping......8

1.2.6 Cytogenetics......9

1.2.6.1 17p deletion......10

1.2.6.2 13q14.3 deletion......11

1.2.6.3 Trisomy 12......11

1.2.6.4 11q22-23 deletion......11

1.2.6.5 Additional cytogenetic abnormalities......12

1.2.7 CLL transformation (Richter's Syndrome)......12

1.2.8 Treatment of CLL......14

1.2.9 The origin of the CLL cell......16

1.2.10 Antigen driven disease/autoimmunity..17
1.3 Molecular characteristics of CLL..18
1.3.1 Immunoglobulin genes and the B-cell receptor (BCR)......................19
1.3.1.1 Immunoglobulin gene diversity in B-cells..................................19
1.3.1.2 *IGHV* gene usage in CLL...20
1.3.1.3 Somatic hypermutation in CLL..21
1.3.1.4 Complementarity determining region 3 (CDR3)..........................22
1.3.1.5 Immunoglobulin light chains...22
1.3.2 The BCR and signalling...22
1.3.2.1 BCR signalling in normal B-cells...23
1.3.2.2 BCR signalling in CLL...24
1.3.2.3 Zap-70 & BCR signalling in CLL...26
1.3.2.4 Zap-70 as a marker of prognosis...26
1.3.3 Cell signalling and migration..27
1.4 CD38 in CLL...28
1.4.1 Phylogeny of CD38..29
1.4.2 Genetics...29
1.4.3 Structure of CD38..30
1.4.4 Functions of CD38..31
1.4.5 Calcium mobilisation by ADPRc and CD38...................................31
1.4.6 Receptor functions of CD38..32
1.4.7 CD38 signalling in T-cells..33
1.4.8 CD38 in normal B-cell development..33
1.4.9 CD38 as a marker of poor prognosis in CLL...................................34
1.4.10 The role of CD38 in the pathogenesis of CLL................................35
1.5 *In vivo* survival and proliferation of the CLL cell..............................37
1.5.1 Proliferation centres and the microenvironment.............................37
1.5.2 CD31...38
1.6 Objectives...39

2.0 Materials and Methods

2.1 List of materials and laboratory equipment..................................Appendix 1
2.2 Preparation of general reagents...40

2.2.1 Phosphate buffered saline (PBS)........40
2.2.2 Preparation of LB media for bacterial cell culture........40
2.2.3 Preparation of Eukaryotic cell culture media........40
2.2.3.1 Culture media for non-adherent cells........40
2.2.3.2 Culture media for adherent cells........41
2.2.4 Paraformaldehyde........41
2.2.5 Waste disposal........41
2.3 Methods........42
2.3.1 Primary cell isolation........42
2.3.1.1 Density centrifugation of peripheral blood to obtain CLL cells........42
2.3.1.2 Cell counting on the Beckman Vi-Cell........42
2.3.1.3 Cell counting using the Neubauer haemacytometer........42
2.3.1.4 Purifying CLL cells........43
2.3.2 Eukaryotic cell culture........44
2.3.2.1 Thawing cells from liquid nitrogen stores and transferring to liquid culture........44
2.3.2.2 Sub-culture of non-adherent cells........44
2.3.2.3 Sub-culture of adherent eukaryotic cells........44
2.3.2.4 Freezing down Eukaryotic cell lines for long term liquid nitrogen storage........45
2.3.2.5 Preparation of co-culture........45
2.3.2.5.1 Irradiation........45
2.3.2.5.2 Co-culture conditions........46
2.3.3 Generation of lentiviral plasmids........46
2.3.3.1 Digestion of pEGFP and SXW plasmids........47
2.3.3.2 Agarose gel electrophoresis........47
2.3.3.3 Extracting DNA from the gel using the QIAquick gel extraction kit (QIAGEN)........48
2.3.3.4 Shrimp alkaline phosphatase (SAP) treatment of excised fragments........48
2.3.3.5 Clean up of PCR products using the QIAGEN QIAquick PCR purification kit........49
2.3.3.6 Ligation........49
2.3.3.7 Digestion of CD38 plasmid........50

2.3.4 Prokaryotic cell culture and plasmid amplification..............................51
2.3.4.1. Transformation of competent *E.coli* DH5α bacteria......................51
2.3.4.2 Plating the bacteria.. 52
2.3.4.3 Picking single colonies and growing the transformed
bacteria.. 52
2.3.4.4 Storage of transformed bacteria in glycerol....................................52
2.3.4.5 Isolation of plasmid DNA using the QIAGEN plasmid
Maxi kit.. 52
2.3.4.6 Quantification of plasmid DNA.. 53
2.3.5 Generation of lentivirus.. 54
2.3.5.1 Calcium phosphate transfection of 293T cells.............................. 54
2.3.5.2 Harvesting and concentrating the virus.. 54
2.3.5.3 Quantification of viral particles using the Retro-tek p24
enzyme linked immunosorbent assay (ELISA)................................. 55
2.3.6 Transduction and transfection of lymphocytes.................................. 55
2.3.6.1 Infection of Jurkat cells with viral supernatant..............................55
2.3.6.2 Infection of primary CLL cells with concentrated virus.................56
2.3.6.3 Nucleofection...56
2.3.6.4 Cell sorting on the MoFlo...56
2.3.6.5 Electroporation of *in vitro* transcribed RNA................................ 56
2.3.6.5.1 Generation of *in vitro* transcribed mRNA..............................56
2.3.6.5.2 Electroporation.. 57
2.3.7 Primary CLL cell molecular biology and gene expression
assays... 58
2.3.7.1 RNA isolation.. 58
2.3.7.2 NanoDrop quantification of RNA.. 58
2.3.7.3 Reverse transcription.. 58
2.3.7.4 QPCR on the Roche Light cycler... 59
2.3.7.5 Affymetrix microarray...59
2.3.7.6 Sequencing...63
2.3.7.6.1 Sequencing PCR...63
2.3.7.6.2 Purifying sequencing products by isopropanol
precipitation...63
2.3.7.6.3 Preparation of sequencing products for electrophoresis............64

2.3.8 Cell biology and biochemical assays.......... 64
2.3.8.1 Flow cytometry.......... 64
2.3.8.2 Preparation of Jurkat cells for flow cytometry.......... 64
2.3.8.3 Preparation of primary CLL cells for flow cytometry.......... 64
2.3.8.4 Fix and perm of CLL cells for intracellular staining.......... 65
2.3.8.5 Annexin V/PI staining for apoptosis.......... 65
2.3.8.6 Assessment of proliferation through incorporation of Bromodeoxyuridine (BrdU).......... 65
2.3.8.7 Measurement of VEGF in the CLL supernatant by ELISA.......... 66
2.3.8.8 Cytospin slide preparation and staining CLL cells with Giemsa for morphological analysis.......... 66
2.3.9 Statistical analysis.......... 68
2.3.10 Patient samples and ethical approval.......... 68

3.0 Genetic Modification of Primary CLL Cells

3.1 Introduction.......... 69
3.2 Nucleofection of plasmid vectors.......... 69
3.3 Electroporation of *in vitro* transcribed messenger RNA (IVTmRNA).......... 73
3.4 Lentiviral gene transduction.......... 75
3.4.1 Introduction.......... 75
3.4.2 GFP lentivirus on Jurkat cells.......... 80
3.4.3 Treating primary CLL cells with GFP virus.......... 82
3.4.4 CD38 virus on CLL cells.......... 85
3.4.5 Summary of MOI.......... 85
3.4.6 Quantification of lentivirus using the Retro-tek p24 ELISA.......... 88
3.4.7 CD38 expression in CLL cells treated with CD38 and GFP virus.......... 88
3.4.8 CD38 gene expression in CLL cells by quantitative reverse transcription PCR (QRT-PCR).......... 91
3.4.9 Treating multiple patient samples with CD38 virus and control GFP virus.......... 91
3.4.10 Stable CD38 expression in CLL cells.......... 91
3.5 Discussion.......... 95

4.0 CD38 ligation enhances the viability and proliferation of primary CLL cells

4.1 Introduction.......... 98
4.2 CLL cell survival was enhanced over 48 hours following the addition of lentivirus.......... 100
4.3 CLL cell survival was enhanced over three days following the addition of lentivirus.......... 102
4.4 Addition of lentivirus caused enhanced expression of phosphatidylserine on the CLL cell surface.......... 102
4.5 CLL cell morphology identified viable CLL cells following the addition of lentivirus.......... 107
4.6 CD38 was lost from the surface of lentivirus treated CLL samples following incubation in co-culture.......... 109
4.7 Incubating untransduced CLL cells with CD31-expressing co-culture enhanced survival.......... 111
4.8 CD38 expression did not correlate with CLL cell survival following incubation in co-culture.......... 113
4.9 The proliferation of CLL cells was enhanced following incubation with CD31-expressing co-culture.......... 117
4.10 Ki-67 expression correlated with increased expression of CD38.......... 119
4.11 Discussion.......... 123

5.0 The genetic modification of CLL cells causes changes in gene expression

5.1 Introduction.......... 128
5.2 CD38 mRNA was highly expressed in multiple CD38 virus treated samples.......... 129
5.3 VEGF expression was increased following the addition of lentivirus.......... 131
5.4 Analysis of CLL cell supernatant using ELISA identified an increase in VEGF following transduction with lentivirus.......... 131
5.5 VEGF gene expression increased in a dose dependent manner following the addition of increasing amounts of CD38 virus.......... 134

5.6 IL-1β and MCL-1 expression were increased in CLL samples following transduction with lentivirus........ 134
5.7 Microarray analysis revealed up-regulation of CD38 in CLL samples transduced with CD38 lentivirus........ 137
5.8 Fifty five genes were up-regulated and seven down-regulated following the induction of CD38........ 140
5.9 Microarray analysis identified down-regulation of the gene encoding the DNA mismatch repair protein Msh6 in CLL cells following expression of CD38........ 140
5.10 Discussion........ 144

6.0 Incubation with CD31-expressing co-culture causes phenotypic changes in CLL cells

6.1 Introduction........ 148
6.2 CD5 was down-regulated on the surface of CLL cells following co-culture........ 148
6.3 CD19 expression was increased in CLL cells following two and five days incubation with CD31-expressing co-culture........ 151
6.4 CD38 expression was increased in CLL cells following five days incubation with CD31-expressing co-culture........ 154
6.5 CD49d expression was increased in CLL cells following co-culture........ 154
6.6 Zap-70 expression was increased in CLL cells following two days in CD31-expressing co-culture........ 156
6.7 The increase in intracellular Zap-70 expression, following incubation with CD31-expressing co-culture, significantly correlated with native CD38 expression........ 159
6.8 There was no change in CD11c expression on the surface of CLL cells following co-culture........ 159
6.9 An increase in CD103 expression was observed on the surface of CLL cells in co-culture........ 162
6.10 There was no change in CD138 expression on the surface of CLL cells following co-culture........ 162
6.11 Discussion........ 165

7.0 Final Discussion

7.1 Discussion....169
7.2 Summary and conclusions....175
7.3 Future investigations....176

References....177

Appendix....212

Publication.... 227

Publication

Genetic modification of primary chronic lymphocytic leukemia cells with a lentivirus expressing CD38. Laurence Pearce, Liam Morgan, Thet Thet Lin, Saman Hewamana, R. James Matthews, Silvia Deaglio, Clare Rowntree, Christopher Fegan, Christopher Pepper and Paul Brennan. *Haematologica. 2010 March; 95(3): 514–517.*

Poster presentations

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The role of CD38 in the Pathogenesis of CLL. Internal presentation for the Infection, Immunity and Biochemistry department. Cardiff University March 2009.

Investigating the role of CD38 in CLL. Presentation at the Leukaemia Lymphoma Research open day, Cardiff University January 2011.

Investigating the role of CD38 in CLL. Internal presentation for the Haematology department. University Hospital of Wales February 2011.

List of figures

Chapter 1

Figure 1.1 Blood film illustrating typical CLL cells and smudge cells......... 6
Figure 1.2 The development of CLL therapy throughout the 20th century and patient outcome... 15
Figure 1.3 Correlation between *IGHV* status and survival............................21
Figure 1.4 Role of PI3K in the signalosome model of B-cell receptor signalling.. 23
Figure 1.5 BCR signalling pathways... 24
Figure 1.6 Correlation between Zap-70 status and survival...........................27
Figure 1.7 Crystal structure of CD38... 30
Figure 1.8 Correlation between CD38 status and survival........................... 35

Chapter 2

Figure 2.1 Percentage CD19 positive cells in a sample pre (a) and post (b) depletion of CD3-expressing T-cells...43
Figure 2.2 SxW plasmid, highlighting the Kpn1 and Xho1 within the multiple cloning region.. 46
Figure 2.3 pEGFP-1 plasmid, highlighting CD38 and the Kpn1 and Xho1 restriction sites.. 47
Figure 2.4 Agarose gel showing the Kpn1, Xho1 digested pEGFP Plasmid... 48
Figure 2.5 S38W plasmid, illustrating the incorporation of the human CD38 gene and Not 1, Kpn1 and Xho1 restriction sites...............................50
Figure 2.6 Agarose gel illustrating Not1 digested S38W Plasmid.................51
Figure 2.7 Melting curve of the 259 base pair MCL1 PCR product.............59

Chapter 3

Fig 3.1 CLL cells expressed GFP following nucleofection of GFP-CD38 and control GFP plasmids...71
Figure 3.2 Isolation of GFP positive cells using the Moflo cell sorter.......... 72

Figure 3.3 No GFP expression was observed following electroporation of IVTmRNA........ 74
Figure 3.4 Generation of lentivirus........ 78
Figure 3.5 GFP was expressed in Jurkat cells treated with increasing amounts of lentivirus........ 81
Figure 3.6 Multiplicity of infection (MOI) calculated from GFP virus treated CLL cells........ 83
Figure 3.7 High levels of CD38 expression were observed on the surface of CLL cells following the addition of increasing amounts of lentivirus...... 86
Figure 3.8 An MOI of 3.2 transduced over 90% of CLL cells in a single patient sample........ 87
Figure 3.9 Comparable amounts of lentivirus were present in both CD38 and GFP preparations........ 89
Figure 3.10 CD38 was not expressed following the use of the control GFP lentivirus........ 90
Figure 3.11 CD38 was highly expressed at the level of transcription in CLL Cells, illustrated by quantitative reverse transcription PCR........ 92
Figure 3.12 CD38 was expressed in multiple samples following the addition of CD38 lentivirus........ 93
Figure 3.13 CD38 was expressed in CLL cells for up to 5 days........ 94

Chapter 4

Figure 4.1 CLL cell survival was increased following the addition of lentivirus........ 99
Figure 4.2 CLL cell viability was increased in multiple virus treated samples following 48 hours in culture........ 101
Figure 4.3 CLL cell viability was increased in virus treated samples assessed over 5 days in liquid culture........ 101
Figure 4.4 A large population of the CLL cells were annexin V/PI positive after 2 and 5 days incubation with lentivirus........ 104
Figure 4.5 Annexin V positive CLL cells were observed after 1 and 2 hours incubation with lentivirus........ 105

Figure 4.6 Annexin V/PI staining identified a large number of apoptotic cells 1 hour following the addition of lentivirus.......... 106
Figure 4.7 Morphology of untreated and virus treated CLL cells following 48 hours incubation.......... 108
Figure 4.8 Incubating with co-culture enhanced the survival of untreated and CD38 transduced CLL cells.......... 110
Figure 4.9 CD38 expression was lost from transduced CLL cells following incubation in co-culture.......... 112
Figure 4.10 Annexin V/PI staining illustrated increased CLL cell viability following co-culture.......... 114
Figure 4.11 CLL cell viability was increased following incubation with CD31-expressing co-culture.......... 115
Figure 4.12 There was no correlation between CLL cell viability and CD38 expression.......... 116
Figure 4.13 There was a trend towards increased CLL cell viability in samples expressing CD38 following incubation with CD31-expressing co-culture.......... 116
Figure 4.14 CLL cells incubated in CD31-expressing co-culture showed increased BrdU incorporation.......... 118
Figure 4.15 Ki-67 expression was increased in a CLL sample following 2 days incubation with CD31-expressing fibroblast co-culture.......... 120
Figure 4.16 Ki-67 expression was increased in CLL samples following 2 days incubation with CD31-expressing fibroblast co-culture.......... 121
Figure 4.17 A significant correlation was observed between native CD38 expression and Ki-67 expression following incubation with CD31-expressing co-culture.......... 122
Figure 4.18 Ki-67 expression was significantly increased in CD38 positive Patients.......... 122

Chapter 5

Figure 5.1 CD38 was highly expressed in CLL samples treated with CD38 virus (a) but not GFP virus (b).......... 130

Figure 5.2 VEGF was heterogeneously over-expressed in CD38 and GFP lentivirus treated samples....132
Figure 5.3 VEGF was increased in the supernatant of lentivirus treated samples.... 133
Figure 5.4 VEGF was induced following the addition of increasing amounts of CD38 virus.... 135
Figure 5.5 VEGF expression correlated with CD38 expression in CD38 virus treated samples.... 135
Figure 5.6 IL-1β was heterogeneously over-expressed in six CD38 and GFP lentivirus treated samples.... 136
Figure 5.7 MCL1 was heterogeneously over-expressed in six CD38 and GFP lentivirus treated samples.... 138
Figure 5.8 Microarray analysis illustrated that CD38 expression was increased following transduction with CD38 lentivirus....139
Figure 5.9.Global gene expression analysis identified 55 up-regulated and 7 down-regulated genes following transduction of the CLL cells with CD38 lentivirus.... 141
Figure 5.10 MSH6 was down regulated in CD38 expressing CLL cells.... 142
Figure 5.11 QRT-PCR analysis illustrated no difference in the expression of MSH6 following treatment with CD38 or GFP lentivirus....142

Chapter 6

Figure 6.1 Gating strategy for the analysis of CD5+/CD19+ CLL Lymphocytes.... 150
Figure 6.2 CD5 expression was decreased following five days in co-culture.... 152
Figure 6.3 An increase in CD19 expression was observed following incubation with CD31-expressing co-culture....153
Figure 6.4 An increase in CD38 expression was observed following incubation in CD31-expressing co-culture....155

Figure 6.5 An increase in CD49d expression was observed following co-culture 157

Figure 6.6 An increase in Zap-70 expression was observed following incubation with CD31-expressing co-culture 158

Figure 6.7 Native CD38 expression correlated with Zap-70 expression 160

Figure 6.8 CD38 positive patients expressed higher levels of Zap-70 than CD38 negative patients 160

Figure 6.9 There was no change in CD11c expression following co-culture 162

Figure 6.10 An increase in the expression of CD103 was observed following co-culture 163

List of tables

Chapter 1

Table 1.1 Rai staging system.. 4

Table 1.2. Binet staging system.. 5

Table 1.3 Surface markers of B-lineage malignancies.................................. 9

Table 1.4.a Biological risk factors of CLL transformation to Richter's syndrome identified by univariate analysis at CLL diagnosis........................ 13

Table 1.4.b Clinical risk factors of CLL transformation to Richter's syndrome identified by univariate analysis at CLL diagnosis........................ 13

Chapter 3

Table 3.1a Titration of GFP lentivirus on CLL cell.................................... 84

Table 3.1b Titration of CD38 lentivirus on CLL cells................................ 84

Chapter 5

Table 5.1 CD38 was over-expressed in samples expressing CD38 but not in control samples.. 139

Table 5.2 The expression of MSH6 was decreased in three CD38 transduced patient samples.. 143

Chapter 6

Table 6.1 Fluorescent antibody panels used to characterise CLL cells......... 149

Table 6.2 CLL cell expression of key surface and intracellular molecules... 166

Abbreviations

ADP	Adenosine diphosphate
ADPRc	ADP-ribosyl cyclase
AID	Activation induced cytidine deaminase
APRIL	A proliferation inducing ligand
ATM	Ataxia Telangiectasia mutated
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BCL-2	B cell lymphoma-2
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BM	Bone marrow
B-PLL	B-cell prolymphocytic leukaemia
BrdU	Bromodeoxyuridine
cADPR	cyclic-ADP-ribose
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR3	Complementarity determining region 3
c-IAP2	Cellular inhibitor of apoptosis protein 2
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
DLBCL	Diffuse large B-cell lymphoma
DLEU	Deleted in lymphocytic leukaemia
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide

EBV	Epstein-Barr virus
EHEB	Epstein–Barr virus transformed CLL cell line
ELISA	Enzyme linked immunosorbent assay
FAB	French, American and British
FCR	Fludarabine, cyclophosphomide and Rituximab
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GPI	glycosylphosphatidylinositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cell
Ig	Immunoglobulin
IGHV	Ig heavy chain gene variable region
IL	Interleukin
IRF-1	interferon-responsive factor-1 (IRF-1)
IVT	*In vitro* transcribed
IWCLL	International workshop for CLL
KDa	Kilo-Dalton
LN	Lymph node
LDT	Lymphocyte doubling time
LMP	Latent membrane protein
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinase
MBL	Monoclonal B-cell lymphocytosis

MDM2	Murine double minute 2
MFI	Mean fluorescent intensity
miR	Micro-RNA
MMP-9	Matrix metalloproteinase-9
MOI	Multiplicity of infection
mRNA	Messenger RNA
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF-IL-6	Nuclear factor for IL-6
NF-κB	Nuclear factor-κB
NHL	Non Hodgins Lymphoma
NTL	Non transduced L-cell
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule
PI	Propidium iodide
PI3-K	Phosphatidylinositol 3-Kinase
PKC	Protein kinase C
PL	Pro-lymphocytes
PLC- γ 1	Phospholipase C-γ1
PPT	Polypurine tract
QRTPCR	Quantitative reverse transcription PCR
RPE	R-phycoerythrin
RPMI	Roswell Park Memorial Institute
Rre	Rev response element

RT	Reverse transcriptase
SDF-1	Stromal derived factor-1
SFFV	Spleen focus forming virus
SH-2	Src homology-2
SLL	Small lymphocytic lymphoma
SNP	Single nucleotide polymorphism
sIg	Surface Ig
SIN	Self inactivating
TACI	Transmembrane activator, calcium modulator and cyclophilin ligand interactor
TCF-1	T-cell transcription factor-1α
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
VCAM	Vascular cell adhesion molecule
VDJ	Variable, diverse and joining regions
VEGF	Vascular endothelial growth factor
VEGFR-2	VEGF receptor-2
VH	Variable Heavy chain gene
VSVG	Vesicular stomatitis virus G protein
WHO	World health organisation
WPRE	Woodchuck post-transcriptional regulatory element
XIAP	X-linked inhibitor of apoptosis protein
Zap-70	Zeta-associated protein-70

Abstract

In this study, I applied a range of techniques in an attempt to enhance our knowledge of the role that CD38 plays in the pathogenesis of CLL. Investigation of a number of techniques to genetically modify the CLL cells led to the development of a lentiviral transduction system that was able to induce a marked increase in the ectopic expression of CD38 on the CLL cell surface. Subsequent molecular analysis identified changes in gene expression which may enhance disease progression. The pro-angiogenic growth factor VEGF and the DNA mismatch repair protein Msh6 were both identified as candidates for further investigation. This work also highlighted the challenges and limitations involved in using a lentiviral knock-in system and led to the design of experiments utilising CD31-expressing co-cultures to stimulate CD38 on the CLL cell surface. The CD31-expressing co-culture system induced survival within the CLL sample compared to cells incubated with the control, non-transfected co-culture. Increased proliferation was illustrated through the incorporation of BrdU and induction of the cell cycle protein Ki-67. Multi-colour flow cytometry was employed to observe the expression of surface and intracellular molecules which may be involved in CLL cell activation and signalling. Changes in the phenotype of the CLL cells were consistently observed which support the notion that these cells can be activated *in vitro* and can thereby enhance B-cell receptor signalling. Specifically, CD19, CD38 and the aberrantly expressed tyrosine kinase Zap-70 were all induced following incubation with CD31-expressing co-culture. This is the first time that a lentiviral transduction system has been developed which efficiently expresses CD38 in a CLL cell population with little cell death. The work carried out in this project also highlights the importance of using co-culture to stimulate CD38 on the surface of the CLL cells *in vitro*. The novel findings within this project have given insight into some of the mechanisms of CD38 signalling, provided direction for future work and highlight the potential of CD38 as a therapeutic target in CLL.

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Chapter 1.0 Introduction

1.1 A brief history

With the development of novel cell staining techniques in the late 19th Century, Paul Ehrlich described the morphological differences between myeloid and lymphoid leucocytes which allowed a much stricter definition of leukaemia (Ehrlich, 1887). His work was in agreement with the interpretations of Neumann who had previously described lymphocytic leukaemia as a primary disease of the haematopoietic system (Seufert and Seufert, 1982). By the mid 20th century chemotherapy and radiotherapy had been in use for many decades as treatments for leukaemia and the results of long-term studies in patients with chronic lymphocytic leukaemia (CLL) were yielding some important findings. A report by Boggs *et al.* in 1966 described how intensive chemotherapy regimens were decreasing the mortality rates, but conversely a cohort of patients who remained untreated had better long-term survival (Boggs *et al.*, 1966). At the same time, differences in the rate of proliferation of the leukaemic cells of individual patients were described, suggesting that the disease was able to manifest in two different forms: an indolent accumulation of lymphocytes or a more aggressive proliferation of cells (Galton, 1966). By the 1970s a large amount of clinical and laboratory data had accumulated for patients with CLL undergoing various treatment protocols (Hansen, 1973, Sawitsky *et al.*, 1977). From this evidence came two simple, but very informative, classification systems for lymphoproliferative disease involving the clinical assessment of lymph nodes and spleen. These systems became the gold standard over the next decade and remain in use today (Binet *et al.*, 1981, Binet *et al.*, 1977, Rai *et al.*, 1975). Revisions to the classification systems in the late 1980s along with new observations in the laboratory and clinic made the diagnosis, prognosis and the monitoring of patients with CLL much more comprehensive (Gale, 1987, Molica and Alberti, 1987, Montserrat *et al.*, 1986).

The following decade saw great advances in the characterisation of cells through the staining of surface molecules and in 1994 Matutes *et al.* devised a scoring system to classify CLL into typical or atypical disease using a panel of antibodies (Matutes *et al.*, 1994). This classification is still used in conjunction

with the clinical staging systems defined by Rai and Binet and is the primary method of accurately diagnosing patients with CLL.

With the development of gene analysis techniques including fluorescence *in situ* hybridisation (FISH) and polymerase chain reaction (PCR) a patient's prognosis following diagnosis can be more clearly defined and they can be monitored accordingly. These recent methods, along with novel markers of disease with prognostic significance, will be described in greater detail in the following sections.

1.2 Chronic lymphocytic leukaemia (CLL)

CLL remains the most common adult leukaemia in the Western world presenting at a median age of 65 years and accounting for around 30% of all leukaemias (Foon *et al.*, 1990). Occurring predominantly in males (2:1 ratio) (Finch and Linet, 1992) CLL is characterised by the accumulation of immune-incompetent CD5 positive, mature-looking B-lymphocytes in the bone marrow, peripheral blood and lymphoid system (Montserrat and Rozman, 1995). The majority of the cells derived from the peripheral blood are arrested in the G0/G1 phase of the cell cycle. However there is considerable evidence that these cells have undergone substantial cell division, most likely within in a lymphoid tissue proliferative compartment, to generate an expanding clone (Messmer *et al.*, 2005, Deaglio and Malavasi, 2009, Calissano *et al.*, 2009).

The latest world health organization (WHO) classification scheme considers CLL as a mature B-cell neoplasm and does not distinguish it from small lymphocytic lymphoma (SLL) a disease that is comprised of the same cell phenotype, but which is usually confined to the lymph nodes (Jaffe, 2001). Accumulation of these mature lymphocytes eventually leads to bone marrow infiltration resulting in an impaired immune response (presumably due to a lack of normal B-cells), anaemia and thrombocytopenia. Around sixty percent of patients present with hypogammaglobulinaemia which becomes more prominent throughout the disease due to the inability of the CLL cells to express functional paraprotein (Dighiero, 1988).

Other clinical features including autoimmune manifestations and pathogenic autoantibodies have been detected in up to 30% of patients (Caligaris-Cappio,

1996). They are usually polyclonal and are directed against haematopoietic antigens expressed on the surface of red blood cells and platelets. This may lead to severe autoimmune haemolytic anaemia and thrombocytopenia (Hamblin *et al.*, 1986, Kipps and Carson, 1993).

1.2.1 Aetiology and epidemiology

The aetiology of CLL is unknown. Various studies have linked the development of CLL with exposure to occupational chemicals including benzene, radio-isotopes and pesticides (Schnatter *et al.*, 2005, Pukkala *et al.*, 2009), though evidence to the contrary has also been presented (Linet, 2006). There is an established familial link in the development of CLL and individuals with first degree relatives suffering the disease possess a 2 to 7 fold increased chance of being diagnosed with CLL (Cuttner, 1992). This was confirmed by subsequent investigations using genome wide association analysis that identified a number of genetic loci that gave an accumulated risk of developing CLL (Di Bernardo *et al.*, 2008, Crowther-Swanepoel *et al.*, 2010). Other investigations have observed similar findings (Blattner *et al.*, 1979, Neuland *et al.*, 1983, Yuille *et al.*, 2000, Capalbo *et al.*, 2000), although studies involving twins have provided contrasting evidence as to whether there is an inherited genetic factor responsible for the development of CLL (Brok-Simoni *et al.*, 1987, Chen *et al.*, 2002, Hakim *et al.*, 1995). CLL is rarely seen in people of Asian origin and is not increased in multiple generations of Asian migrants who have settled in areas of high CLL prevalence (Pan *et al.*, 2002). This again suggests that genetic factors are involved in the development of CLL.

1.2.2 Clinical presentation of CLL

Patients may present with asymptomatic disease and their elevated lymphocyte count identified through a routine blood test. This is rare though and most patients are investigated due to a persistent infection, general lethargy or malaise (due to an underlying anaemia) or increased tendency of bruising (due to a reduced platelet count). Upon examination the lymph nodes are often enlarged, though non-tender. Less visible is an enlarged spleen or liver occurring in around 35% and 20% of patients respectively (Rai, 2003). Although CLL cells are able to accumulate in various lymphoid tissues, or organs, infiltration to the extent of

enlargement of other sites, such as the tonsil or Waldeyer's ring (at the rear of the pharynx), or as lesions in the skin are rare (Rai, 2003).

1.2.3 Clinical staging

A wealth of data collected throughout the mid twentieth century enabled Rai *et al.* in 1975 and Binet *et al.* two years later to propose the criteria for a clinical staging strategy in CLL. Both classification systems are based on the clinical features observed upon examination and are still in use today as an accurate means of determining the prognosis for individual patients. Table 1.1 illustrates the Rai system. The individual stages range from 0 to IV and describe the symptoms according to the presence of lymphocytosis, lymphadenopathy, hepato/splenomegaly, anaemia and thrombocytopenia. Occurrence of the latter two anomalies is associated with advanced disease and an unfavourable outcome.

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

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

The Binet classification system is slightly simpler and is based on the presence or absence of anaemia or thrombocytopenia with lymphadenopathy at single or multiple sites (Table 1.2). Both systems are applicable to the CLL patient and determine the degree of B-cell infiltration into the lymphoid system, surrounding organs, and indirectly, the bone marrow.

Stage A	Patients have fewer than three areas of enlarged lymphoid tissue. Enlarged lymph nodes of the neck, underarms, and groin, as well as the spleen, are each considered "one group," whether unilateral (one-sided) or bilateral (on both sides).
Stage B	Patients have more than three areas of enlarged lymphoid tissue
Stage C	Patients have anaemia plus thrombocytopenia (platelets <100 – 103 /dL).

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

1.2.4 Laboratory diagnosis

In 1996 the National Cancer Institute set its criteria for the laboratory diagnosis of CLL and it was agreed that a lymphocyte count of $5x10^9$/litre was appropriate for the primary diagnosis of CLL (Cheson *et al.*, 1996). Twelve years later the criteria were changed by the International Workshop for CLL (IWCLL) to differentiate sub CLL diseases, namely monoclonal B-cell lymphocytosis (MBL) and SLL, from the more classical form of the disease (Hallek *et al.*, 2008). The amendment stipulated that a total B-cell count of $5x10^9$/l should to be used to diagnose CLL. This amendment was met with some controversy (Hanson *et al.*, 2009) and a consensus for the definitive diagnosis of this disease with regards to lymphocyte count still eludes the CLL community, as it has over decades of dispute (Cheson *et al.*, 1996, Matutes and Polliack, 2000). Fortunately with the use of immunophenotyping and cytogenetic analysis very few cases of CLL are misdiagnosed or incorrectly treated.

1.2.4.1 Morphology

Following the intense study of blood cell morphology an agreed CLL classification system was agreed upon in 1989 by a French, American and British (FAB) board to standardise the diagnosis of CLL using cell morphology (Bennett *et al.*, 1989). Two main types of CLL may be primarily determined

from blood film analysis. The first is typical CLL where the lymphocytes present are small (around 7.3μm in diameter (Kuse *et al.*, 1985)) with a single lobed nucleus which occupies the majority of the cell volume leaving a thin layer of cytoplasm (Matutes and Polliack, 2000) (Figure 1.1). The nuclei encapsulate dark staining chromatin revealing very little nuclear detail (Hamblin, 2009). A few larger B-cells may be present which are twice the size of a typical CLL cell and portray a larger cytoplasmic region and a visible nucleolus. These cells are designated pro-lymphocytes (PL) and may be present in typical CLL when accounting for less than 10% of the total lymphocyte count (Frater *et al.*, 2001).

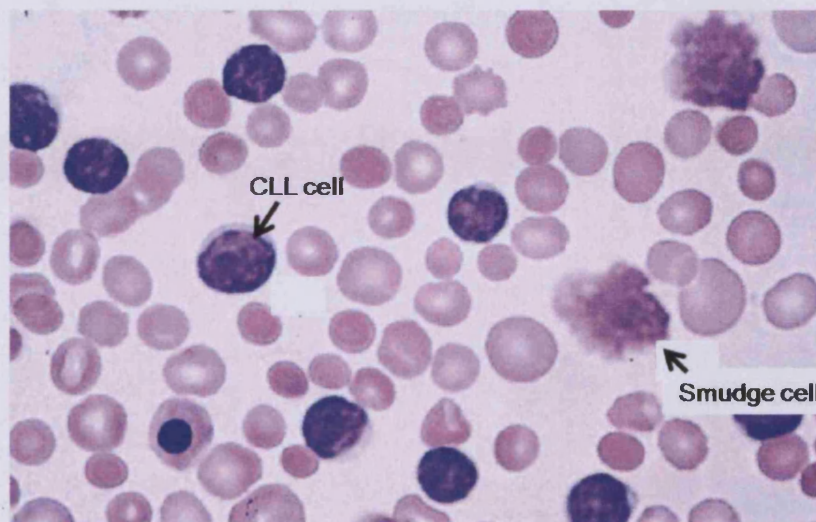


Figure 1.1 Blood film illustrating typical CLL cells and smudge cells (Adapted from Brandon Guthery, M.D., and Nasir Bakshi, M.D. Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City)

The second type of CLL, defined morphologically and by cell surface markers, is atypical CLL. It accounts for around 15% of all cases and can be divided into two sub-types (Criel *et al.*, 1999, Matutes and Polliack, 2000); the first exhibits 10-55% prolymphocytes and is designated CLL/PL. The second sub type is known as "mixed cell-type" atypical CLL and plasmacytoid or cleaved B-cells are present. Both forms of atypical CLL are usually associated with poor risk cytogenetics and immunophenotype and therefore represent a more aggressive form of the disease.

A regular feature of the CLL blood film are smudge cells which are CLL cells which have been smeared due to the increased fragility of the cell membrane. These smudge cells are an artefact of the slide preparation but are almost diagnostic for CLL (Simmonds *et al.*, 1981) (Figure 1.1). In 2009 a report by Nowakowski *et al.* showed that the number of smudge cells observed on the blood film could predict survival in CLL patients (Nowakowski *et al.*, 2009). Erythrocytes and platelets usually appear normal in typical CLL although in rare cases of hypergammaglobulinaemia the red cells stack in rouleaux formations (Hoffbrand AV, 2001).

1.2.4.2 Bone marrow and lymph node involvement

It has been reported that bone marrow examination upon presentation is an important determinant of patient outcome (Rozman *et al.*, 1984), though evidence to the contrary exists (Mauro *et al.*, 1994, Geisler *et al.*, 1996). The presence of more than 30% lymphocytes in the bone marrow is indicative of CLL (Cheson *et al.*, 1996). Four patterns of bone marrow histology have been described in CLL that are largely concerned with the degree of bone marrow infiltration. They are defined as interstitial, nodular, mixed (nodular plus interstitial) and diffuse. The most commonly observed is the mixed type with the diffuse pattern determining the worst prognosis (Rozman *et al.*, 1984).

Proliferation centres have been described in the bone marrow and lymph nodes of CLL patients, which are constructed from large prolymphocytoid and paraimmunoblast cells surrounded by T-cells and small CLL lymphocytes (Matutes and Polliack, 2000, Wang *et al.*, 2008). Identification of these structures during histological analysis is usually a determinant of progressive disease (Wang *et al.*, 2008, Soma *et al.*, 2006). However, lymph node biopsies are not routinely acquired from patients at diagnosis and are only obtained throughout the course of the disease if the nodes are enlarged due to suspected transformation.

1.2.4.3 Lymphocyte doubling time

The use of the lymphocyte doubling time (LDT) as a prognostic indicator was first described in the mid 1980s. Although the LDT correlates with other markers of disease, its use as a sole predictor of outcome in CLL patients was

rapidly identified as an accurate and simple method of assessing disease progression (Montserrat *et al.*, 1986). In 1986 Montserrat *et al.* proposed that an LDT greater than 12 months identified a patient cohort with a good prognosis, whereas an LDT of less than or equal to 12 months was associated with poorer survival (Montserrat *et al.*, 1986, Vinolas *et al.*, 1987). In addition they found that a short LDT predicted rapid progression for patients in the early stages of disease.

1.2.5 Immunophenotyping

To confirm the diagnosis of CLL, the presence of specific markers on the cell surface can be detected using flow cytometry. Dillman *et al.* were one of the first groups to illustrate that monoclonal antibodies can be used to identify surface markers on lymphocytes and contribute to the identification of subsets of CLL with differing prognostic outcome (Dillman *et al.*, 1983). Since then a plethora of cell-specific immunological markers have been identified on the surface of the aberrant cells to further define the type of leukaemia present and predict the course of the disease.

The Matutes score defines classical CLL as a CD19+, CD20+, CD23+ and CD5+ monoclonal B-cell population, in the absence of other pan-T-cell markers (Matutes *et al.*, 1994). The B-cells express either kappa (κ) or lambda (λ) light chains and surface immunoglobulin (sIg) is of low density and is predominantly immunoglobulin-M (IgM) with or without IgD. Depending on the number of these cell markers expressed on a population of CLL cells (and morphological differences) the disease may be diagnosed as typical or atypical CLL. The absence of other specific markers is used to diagnose classical CLL from a multitude of other lymphoid malignancies. These include CD10, CD11c, cyclin D1 and CD103 (Dillman, 2008). Many of the cell surface antigens used to diagnose CLL are markers of B-cell maturity (e.g. FMC7 and TdT). The stage at which the B-cell transforms may be crucial to the severity of the disease underlining the importance in identifying such molecules. CD79b and CD22 are either weakly expressed or absent from the B-cell surface in CLL. Both molecules are involved in cell signalling (CD79b is usually abundant as a component of the activated B-cell receptor) and their absence may account, in

some part, for the anergic nature of the CLL cell (Caligaris-Cappio *et al*., 1993, Payelle-Brogard *et al*., 2006).

The identification of numerous antigens specific for the individual B-cell disorders is important in the accurate diagnosis of the disease and provides prognostic value beyond the realms of cell morphology alone. A summary of surface and intracellular markers, used to identify the various B-lineage malignancies, are shown in Table 1.3.

Marker	Follicular	CLL	Mantle	MZL/MALT	PLL*	DLBCL	HCL	BL/BLL	LPL
Sig	+	dim	+	+/+	+	+/-	+	+	+
Cig	-	-/+	-	-/+	-	-/+	-	-	+
CD5	-	+	+	-/-	-/+	-	-	-	-
CD10	+	-	-	-/-	-	-/+	-	+	-
CD20	+	dim	+	+/+	+	+	+	+	+
CD23	-/+	+	-	-/-	+/-	-	-	-	-
CD43	-	+	+	-/+	-	-/+	+	-	+/-
CD103	-	-	-	+/-	-	NA	+	NA	-
Cyclin D1	-	-	+	-/-	-	-	-/+	-	-

+, >90% positive; +/-, >50% positive; -/+, <50% positive; -, <10% positive; CLL = chronic lymphocytic leukaemia; MZL/MALT = splenic marginal zone/mucosa-associated lymphoid tissue; PLL = prolymphocytic leukaemia; DLBCL = diffuse large B-cell lymphoma; HCL = hairy cell leukaemia; BL/BLL = Burkitt lymphoma/Burkitt-like lymphoma; LPL = lymphoplasmacytoid lymphoma; SIg = surface immunoglobulin; CIg = cytoplasmic immunoglobulin.* A T-cell variant is present in approximately 20 to 30% of PLL cases. NA = Not applicable for diagnosis of this disease.

Table 1.3 Surface and intracellular markers of B-lineage malignancies (Adapted from Finak *et al.,* 2009)

1.2.6 Cytogenetics

The analysis of chromosomes, using metaphase banding techniques and the development of interphase FISH, have allowed the identification of genetic aberrations with diagnostic and prognostic significance in CLL. Clonal cytogenetic aberrations can be identified in 50% of CLL cases using chromosome banding and an additional 30% more subtle rearrangements and

mutations can be identified using interphase FISH (Mossafa H, 1997, Reedy, 2005).

Sub-clones may be found in some patients, which exhibit complex cytogenetic karyotypes. The most common aberrations identified upon presentation, or as the disease develops, are 13q deletion, trisomy 12, 17p deletion, and 11q deletion. Many of the biological mechanisms altered by such genetic mutations are well described and have provided rationale for the observed disease progression and insight into the generation of tailored treatments for each sub-set of disease. There have been many studies carried out correlating these cytogenetic abnormalities with the clinical outcome of patients (Garcia-Marco *et al.*, 1997, Mayr *et al.*, 2006).

1.2.6.1 17p deletion

Monoallelic deletion of various sized portions of the short arm of chromosome 17 are observed in 7-8% of CLL cases and are associated with a poor prognosis (Amiel *et al.*, 1997). The inferior outcome of CLL patients whose cells harbour the 17p13 deletion is thought to be due to the loss of genetic material encoding the p53 gene. The p53 protein plays a major role in cell development and is responsible for regulating progression through the cell cycle during mitosis. In response to an array of insults, including DNA damage, functional p53 is able to arrest the cell cycle in the G1/S phase and allow the repair of genetic material before cell division. If the damage is irreparable then p53 promptly induces apoptotic signals resulting in deletion of the cell (Jacks and Weinberg, 1996). Many of the chemotherapeutic agents used to treat CLL do so by inducing DNA damage thereby promoting apoptotic cell death. Clones accommodating the p53 deletion are particularly resistant to these agents and remission is short-lived following the treatment of such patients (Wattel *et al.*, 1994). Novel therapeutic regimens include combination chemotherapy, high dose steroids and immunotherapy using monoclonal antibodies such as Alemtuzumab (anti-CD52) (Zenz *et al.*, 2009). Initial studies have shown that these drugs improve the outcome in this poor prognostic group of patients (Lozanski *et al.*, 2004). Additionally mutations in the p53 gene have been described in CLL (Gaidano *et al.*, 1991). Such mutations may occur in the

absence of p53 deletion and are also associated with a poor outcome in CLL (Zenz *et al.*, 2009).

1.2.6.2 13q14.3 deletion

Deletion of a region within 13q14.3 is the most common genomic aberration in CLL and occurs in more than 50% of patients (Stilgenbauer *et al.*, 1998). Two functional genes present within this region are the deleted in lymphocytic leukaemia genes 1 and 2 (DLEU1 and DLEU2). Recent evidence suggests that these genes transcribe proteins which are involved in the regulation of tumour suppressor micro-RNA molecules miR-15a and miR-16-1 (Mertens *et al.*, 2009). Deregulation of these recently described micro-RNA molecules in CLL results in an indolent disease with a favourable outcome. In a recent report the DLEU2/miR-15a/16-1 gene cluster was shown to control B-cell proliferation and its deletion led to CLL like disease in mice (Klein *et al.*, 2010).

1.2.6.3 Trisomy 12

In 15-25% of CLL cases, sub-clones exist which harbour three copies of chromosome 12. This genetic aberration is associated with an atypical CLL cell phenotype with increased surface immunoglobulin and FMC7 (Matutes *et al.*, 1996). Although associated with other poor prognostic indicators such as CD38 and unmutated immunoglobulin genes (Athanasiadou *et al.*, 2006), a direct correlation between trisomy 12 and a poor prognosis has not been established. The presence of trisomy 12 was shown to correlate with a reduced survival time only in the presence of a second chromosomal aberration when compared with patients with 13q abnormalities or a normal karyotype (Juliusson *et al.*, 1990).

1.2.6.4 11q22-q23 deletion

Deletion of a segment of the long arm of chromosome 11 is seen in approximately 14% of patients with CLL (Bullrich *et al.*, 1999). The portion of the chromosome affected encodes the ataxia telangiectasia mutated (ATM) protein which is a key player in the recruitment of tumour suppressor proteins to sites of DNA damage and cell cycle regulation. Patients with the deleted ATM gene are therefore often resistant to conventional chemotherapy which induces DNA damage. 11q22-23 deletion in CLL is associated with increased

lymphadenopathy and poor survival, particularly in younger patients (Dohner *et al.*, 1997). Interesingly the loss of this potent mechanism of apoptosis induction has been exploited in the treatment of CLL. Inhibition of Poly (ADP-ribose) polymerase (PARP) imposes the requirement for DNA double strand break repair. ATM is essential for this function and in its absence the cell undergoes mitotic catastrophe (Kurz and Lees-Miller, 2004). This method of synthetic lethality has been found to be very successful in killing CLL cells *in vitro* and *in vivo* and PARP inhibitors such as Olaparib are being assessed for use in CLL patients with the 11q22-23 deletion (Weston *et al.*, 2010).

1.2.6.5 Additional cytogenetic abnormalities

Various other genetic aberrations have been observed in CLL that are known to have prognostic significance. Translocations involving the immunoglobulin heavy chain locus on chromosome 14 may be detected although such anomalies are much more common in the solid tumours (t(14;18) in follicular lymphoma and t(11;14) in mantle cell lymphoma). Other genetic abnormalities include deletion of chromosome arm 6q, acquisition of a portion of chromosome 8 (8q24), trisomy 3 and trisomy 18.

1.2.7 CLL transformation (Richter's Syndrome)

First described by Maurice Richter in 1928, Richter's syndrome is the transformation of CLL to an aggressive diffuse large B-cell lymphoma (DLBCL). More recently Richter's transformation has been found to represent two biologically different conditions. The first is the transformation of CLL into a DLBCL that has arisen from the original CLL clone. The second represents a similar DLBCL that originates from a different clone to that of the original CLL (Rossi and Gaidano, 2009). CLL transforms to Richter's syndrome in around five to twenty percent of cases (Tsimberidou and Keating, 2005). Until recently the biological and clinical characteristics of CLL and their relation to transformation to Richter's was unclear. A study by Rossi *et al.* in 2008 identified the variables in CLL which may be used to predict the onset of Richter's (Rossi *et al.*, 2008). Table 1.4.a and b summarise the biological and clinical variables respectively.

Biological Variable	Events (n)	Log-rank. Risk of mortality within 5 years (%)	P	HR	Cox 95%CI	P
IgHV <98%	6/114	7	0.006			0.11
IgHV >98%	11/64	28.3		3.65	1.34-9.35	
No IGHV 4-39	13/170	11.1	<0.001			0.002
IgVH 4-39	4/8	56.2		6	1.95-18.43	
Del 13q14	3/95	3.8	0.004			0.009
No Del 13q14	13/78	25.4		5.27	1.5-18.5	
CD38 <30%	4/123	4.7	<0.001			0.002
CD38 >30%	11/60	35.4		6.01	1.91-18.89	
Zap70 <20%	1/77	1.9	0.004			0.003
Zap70 >70%	7/50	21		11.24	1.38-91.55	

HR, hazard ratio; CI, confidence interval; P, P value calculated by both log-rank and Cox univariate analysis; BM, bone marrow; LDH, lactate dehydrogenase; ULN, upper limit of normal.

Table 1.4.a Biological risk factors of CLL transformation to Richter's syndrome identified by univariate analysis at CLL diagnosis (Adapted from Rossi *et al.*, 2008)

Clinical Variable	Events (n)	Log-rank. Risk of mortality within 5 years (%)	P	HR	Cox 95%CI	P
Lymph node <3cm	7/158	6.1	<0.001			<0.001
Lymph node >3cm	10/26	49.9		9.99	3.7-26.96	
Nodal areas involved <3	8/151	8.3	<0.001			0.001
Nodal areas involved >3	8/33	31.8		5.51	2.66-14.72	
LDH <1 ULN	9/167	8.6	<0.001			0.001
LDH >1 ULN	6/17	42.3		5.89	2.14-16.21	
Binet Stage A	7/135	7.1	0.002			0.004
Binet Stage B-C	10/50	29.6		4.18	1.59-11.01	
No diffuse BM	8/138	8.4	0.017			0.024
Diffuse BM	8/46	22.9		3.1	1.16-8.27	

HR, hazard ratio; CI, confidence interval; P, P value calculated by both log-rank and Cox univariate analysis; BM, bone marrow; LDH, lactate dehydrogenase; ULN, upper limit of normal.

Table 1.4.b Clinical risk factors of CLL transformation to Richter's syndrome identified by univariate analysis at CLL diagnosis (Adapted from Rossi *et al.*, 2008)

1.2.8 Treatment of CLL

CLL patients may present with very high lymphocyte counts (sometimes in excess of $500 \times 10^9/L$), but with good prognosis CLL cell phenotype, normal cytogenetics and a long LDT. This suggests that the disease has manifested over a period of many years or even decades and that aggressive treatment with cytotoxic drugs is not required for this indolent accumulation of lymphocytes. The use of first-line lymphocyte depleting drugs may be enough to control the lymphocyte count sufficiently so that patients remain well enough to live with the disease for many years. However, regular assessment of the lymphocyte count is required to ensure that the CLL clone does not transform into aggressive disease. Patients with a less favourable prognosis following clinical staging, cell phenotyping and genetic investigations, are candidates for more aggressive therapy usually involving an intensive combined chemotherapy regimen. Early strategies for the treatment of CLL included the use of standard chemotherapy alkylating agents like chlorambucil that induced a complete remission in around 5% of patients (Figure 1.2) (Kay, 2006). The purine nucleoside analogues (which prevent elongation of DNA strands through direct incorporation into DNA and also inhibit RNA polymerase II) were introduced in the 1980s. These molecules, when used in combination with the original alkylating agents, induced a much improved response in patients with CLL (Kay, 2006). Various combinations of these drugs have been used in clinical trials yielding similar outcomes in patients. The use of fludarabine as a single agent or in combination with cyclophosphamide resulted in more patients entering complete remission and this drug remains an important chemotherapeutic option for CLL patients with advanced disease (Eichhorst *et al.*, 2006).

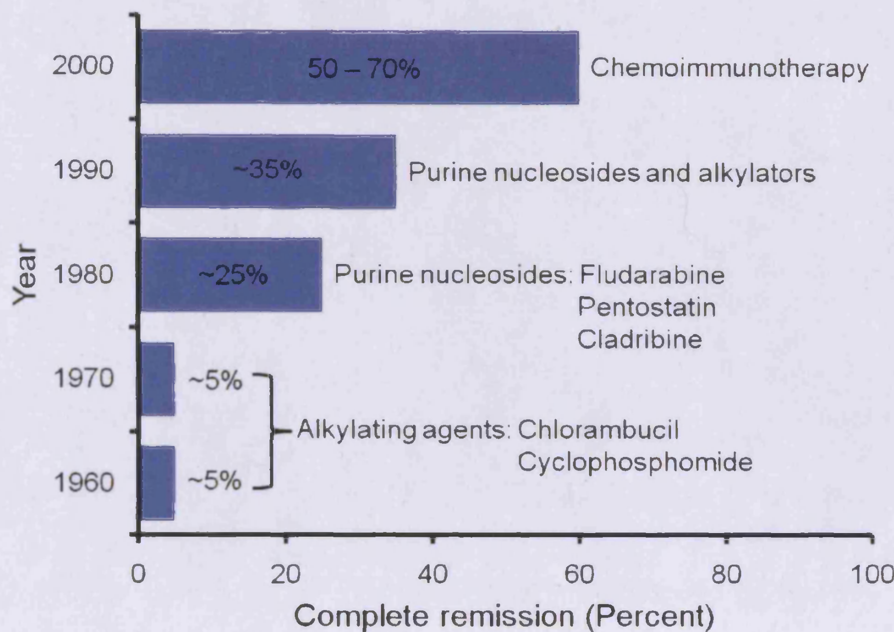


Figure 1.2 The development of CLL therapy throughout the 20th century and patient outcome (Adapted from Kay, 2006a)

Advances in immunotherapy have seen the introduction of monoclonal antibodies that target the CLL B-cells (rituximab (anti-CD20)) or mature B and T-cells (alemtuzumab (anti-CD52)) within the patient. Although more target specific, they are known to cause substantial immune suppression and are used with care as treated patients are susceptible to infection (Peleg *et al.*, 2007). A recent summary of first and second line treatment for advanced CLL was published by Eichhorst *et al.* in 2009 for the European Society for Medical Oncology (Eichhorst *et al.*, 2009). They determined that only those patients with advanced stage disease with anaemia and/or thrombocytopenia, who do not respond to treatment with corticosteroids, should receive chemotherapy. First line regimens should consist of fludarabine, cyclophosphomide with rituximab (FCR) in younger (or fitter) patients, or chlorambucil in those with co-morbidities. The results from this study agreed with data published by Tam *et al.* in 2008, which highlighted the use of rituximab as a first line therapy in CLL (Tam *et al.*, 2008). More recently, data from the CLL8 German study confirmed that the use of rituximab in combination with FC results in an increased

progression-free and overall survival in CLL patients (Hallek *et al.*, 2010). In addition, bendamustine has been reported to be more effective as a first line therapy for advanced stage CLL when compared to chlorambucil (Knauf *et al.*, 2009).

It has been widely established that patients with a deleted p53 gene respond poorly to DNA damaging agents and as such these individuals have benefited from first line alemtuzumab monotherapy or alemtuzumab in combination with high dose steroids such as dexamethazone and methylprednisolone (Hillmen *et al.,* 2007, Dungarwalla *et al.*, 2008, Pettitt *et al.*, 2006). Novel drug therapy in CLL includes the use of the immunomodulatory drug lenalidomide and the monoclonal antibody lumiliximab. Lenolidamide was reported to re-establish the humoral immune recognition of malignant CLL cells through increased expression of the CD154 antigen on the cell surface (Lapalombella *et al.*, 2009). In the same report Lapalombella *et al.* described TNF-related apoptosis-inducing ligand (TRAIL) mediated apoptosis and the generation of antibodies by normal B-cells following treatment with lenalidomide. Lumiliximab is an anti-CD23 monoclonal antibody that was shown to be beneficial in combination with fludarabine, cyclophosphomide and rituximab in CLL (Byrd *et al.*, 2010). A second generation of fully humanised anti-CD20 monoclonal antibodies (Ofatumumab and GA-101) are currently being trialled in CLL. These antibodies have shown enhanced complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity compared with rituximab (Cheson, 2010, Bologna *et al.*, 2011).

17p deleted patients are candidates for bone marrow transplant, though this is usually a last resort due to the high rate of mortality associated with myeloablative stem cell transplantation in older patients. (Michallet *et al.*, 1996). Reduced intensity (non-myeloablative) and autologous transplantation may be considered though there is little data to suggest that these options offer a survival advantage over current therapies (Gribben, 2008).

1.2.9 The origin of the CLL cell

The origin of the CLL cell is a topic which has been widely debated and various hypotheses have been proposed and reviewed over the years in light of novel findings. Initial studies describe CLL B-cells as closely resembling CD5

positive lymphocytes in the mantle zone of the secondary lymphoid follicle (Rai, 2003). The CLL cells express low levels of surface immunoglobulin and are predominantly anergic. These features are characteristic of normal B-lymphocytes that have been exposed to self antigen and are candidates for deletion (Damle *et al.*, 2002). CLL cells have also been shown to display some biological features of T-cells (Majolini *et al.*, 1998, Wiestner *et al.*, 2003). This led Caligaris-Cappio and Ghia to surmise that the transformation event in CLL may occur at an early stage of maturation when the B-cell was less distinct from its immunoregulatory partner, the T-cell (Caligaris-Cappio and Ghia, 2004).

The discovery that the immunoglobulin genes in CLL can be either mutated (suggesting that the cells had experienced antigen) or unmutated (suggesting a naive B-cell phenotype) confounded initial hypotheses and the idea arose that CLL was in fact two separate diseases; the first originating from a naive B-cell and the second from an antigen experienced, post germinal centre B-cell (Hamblin, 2002, Dighiero, 2002, Fegan, 2002). Gene expression analysis has identified specific genes differentially expressed between the two groups, which supports this theory (Ferrer *et al.*, 2004). Evidence to the contrary exists however and additional gene expression studies have illustrated that CLL cells exhibiting unmutated *IGHV* genes have profiles very similar to that of antigen experienced memory B-cells (Klein *et al.*, 2001, Rosenwald *et al.*, 2001). An interesting study by Herve *et al.* induced auto and polyreactivity of mutated CLL antibodies by reverting them to the germ line sequence *in vitro*. They concluded that both mutated and unmutated CLL were derived from a self-reactive B-cell precursor and that somatic hypermutation has an important role in the development of CLL by altering the BCR autoreactivity (Herve *et al.*, 2005). Current thinking is from an immunobiological approach with an emphasis on the possibility that CLL is an antigen driven disease. The identification of stereotyped *IGHV* rearrangements in mutated and unmutated CLL, together with gene array analysis, suggest that the CLL cell is an antigen experienced B-cell (Stevenson and Caligaris-Cappio, 2004). The progression of this B-cell is determined by a host of other molecular and biochemical events occurring within the cell and with its surrounding microenvironment (Ghia and Caligaris-Cappio, 2006).

1.2.10 Antigen driven disease/autoimmunity

The fact that the *VH* gene rearrangements utilised by CLL B-cells from individual patients show a great deal of parity supports the hypothesis that a common antigen, or antigens, may be present *in vivo* and play a role in B-cell transformation or disease progression. Messmer *et al.* described a high degree of *IGHV* restriction in 452 patient sequences broken down into 5 distinct groups with regard to gene segment usage (Messmer *et al.*, 2004). More recently, investigations by Stamatopoulos' group compared the distribution of *IGHV* gene usage and somatic hypermutation in normal B-cells to a large cohort of CLL patients. They described the use of restricted *IGHV* segments in CLL and correlated specific rearrangements (including *IGHV* 3-21 and 4-34), along with stereotyped CDR3 regions and mutational load, with aggressive disease (Murray *et al.*, 2008). The group followed up this work by looking at intraclonal diversity within the immunoglobulin light chains. They illustrated restricted gene usage and stereotyped light chains, especially within the *IGHV* 4-34 subset (Kostareli *et al.*, 2010). This evidence strongly implies that specific antigenic stimuli are involved in the selection of the malignant B-cell clone or that CLL derives from a specific progenitor B-cell with a limited ability to select alternative *IGHV* rearrangements. Two recent reports describe the source of possible antigens that may stimulate the CLL cells to proliferate. Interestingly the antigens identified were derived from the surface of apoptotic cells generated by oxidation during the apoptotic process (Lanemo Myhrinder *et al.*, 2008, Catera *et al.*, 2008). These autoantigens are known to contain epitopes similar to those on bacteria and other microbes. A possible hypothesis therefore would be that the CLL clone is derived from a population of B-cells whose role is to target apoptotic cells and facilitate their clearance. In the presence of infection, bacterial antigens may stimulate these cells to proliferate uncontrollably. A review by Ghia *et al.* suggests that the CLL cell is most likely derived from an anergic CD5+ B-cell that is auto/poly reactive. Whether the autoantigen present is the cause of the transformation event is highly debatable, but it is likely that it is involved in maintaining the clone (Ghia *et al.*, 2007).

1.3 Molecular characteristics of CLL

A multitude of genetic anomalies occur in individual patients with CLL that make it an extremely complex and heterogeneous disease. The presence or abscence of somatic hypermutations in the *IGHV* genes that encode the B-cell receptor (BCR) determine a good or poor prognosis in CLL patients respectively. Over-expression of certain genes in the CLL cell results in the production of intracellular and surface proteins giving rise to an aggressive disease. The biology of many of these molecules is known and their presence in the CLL cell is undesirable if the normal processes of cell differentiation or programmed cell death are to take place. CD38 is an example of a cell surface glycoprotein which may be over-expressed on the surface of CLL cells. A second molecule expressed in many cases of aggressive disease is the ζ-associated protein Zap-70. This intracellular tyrosine kinase has signalling properties that may enhance the proliferative potential of the CLL clone (Deaglio and Malavasi, 2009). CD38 and Zap-70 confer a poor prognosis when over-expressed in CLL and both have been widely investigated as markers of aggressive disease. Although an association exists between the increased expression of CD38, Zap-70 and the presence of unmutated *IGHV* genes, many conflicting reports have been published. (Hamblin *et al.*, 2002, Cruse *et al.*, 2007, Rassenti *et al.*, 2008, Crespo *et al.*, 2003, Rassenti *et al.*, 2004, Wiestner *et al.*, 2003). Other molecules that may be aberrantly expressed in CLL patients include CD23, CD49d, β2-microglobulin, vascular endothelial growth factor (VEGF) and the Bcl-2 family proteins (Fournier *et al.*, 1992, Schimmer *et al.*, 2003, Veronese *et al.*, 2009, Rossi *et al.*, 2008, Packham and Stevenson, 2005).

1.3.1 Immunoglobulin genes and the B-cell receptor

1.3.1.1 Immunoglobulin gene diversity in B-cells

The primary events that allow the expression of a diverse number of antigen recognition epitopes on the surface of the B-cell occur at an early stage of B-cell development through rearrangement of the variable (*V*), diverse (*D*) and Joining (*J*) segments of the B-cell receptor. Following antigen recognition the B-cell migrates to the lymph node germinal centre where it is genetically modified to

obtain a second level of antibody diversification so that a B-cell, highly specific to the foreign antigen, may be selected for clonal expansion (Li *et al.*, 2004).

Within the germinal centre of the lymph node two main processes occur to enhance antibody diversity. The first of these is class switch recombination which involves rearrangement of the *IGHV* chain gene to incorporate one of the constant (*C*) regions (Chaudhuri and Alt, 2004). Known to be induced by IL-4 derived from T-cells, the different classes of antibody generated may be released into the peripheral circulation to act as soluble mediators of immune surveillance with different properties depending on the *C* region usage (Janice Kuby, 1997). The second process employed, in order to introduce further antibody diversity, is that of somatic hypermutation. Point mutations (or less frequently insertions or deletions) are introduced into a 1.5kb region of the *IGHV* gene 150-200 base pairs down-stream of the promoter region (Li *et al.*, 2004). This mechanism generates very subtle alterations to the B-cell antigen receptor expressed on the surface resulting in an extremely high specificity for the antigen. These complex processes require the participation of T-helper cells and dendritic cells and are characterised by the over-expression and down regulation of a multitude of genes within the B-cell (Li *et al.*, 2004). The proteins expressed facilitate entry into the germinal centre, association with the exponent cells, genetic diversification, clonal expansion (or deletion) and differentiation.

1.3.1.2 *IGHV* gene usage in CLL

Investigations have been carried out to identify the *IGHV* gene usage of the malignant B-cell population in patients with CLL. Initial studies found that the *IGHV* gene usage of the CLL cells from different patients is not random (Fais *et al.*, 1998). Later studies observed the gene usage in large patient cohorts and identified the most common recombinations. Specific BCR sub-types correlate with disease outcome and patients with *VH*3-21 recombinations were reported to have a shorter survival in CLL patients with mutated *IGHV* genes (Tobin *et al.*, 2002, Thorselius *et al.*, 2006). Investigations have also been carried out which highlight the association of *VH*3-23 rearrangement as a marker of poor prognosis within the mutated *IGHV* gene cohort (Gerard Tobin, 2004, Bomben *et al.*, 2010). Additional studies have illustrated that the *VH*1-69 recombination is

associated with the presence of unmutated immunoglobulin genes and aggressive disease (Potter *et al.*, 2003, Forconi *et al.*, 2009).

1.3.2.3 Somatic hypermutation in CLL

Early investigations looked at the mutation status of the CLL cell *IGHV* genes to observe whether the B-cell had encountered antigen and undergone somatic hypermutation in the germinal centre (Cai *et al.*, 1992, Hashimoto *et al.*, 1995). Surprisingly the mutation status of individual patients varied from unmutated to highly polymorphic (Oscier *et al.*, 1997). Analysis of the unmutated and mutated subgroups (defined as a *IGHV* sequence containing more or less than 98% homology to the corresponding germ line region respectively) revealed that patients with CLL B-cells harbouring unmutated genes had a significantly worse prognosis (Hamblin *et al.*, 1999, Damle *et al.*, 1999) (Figure 1.3).

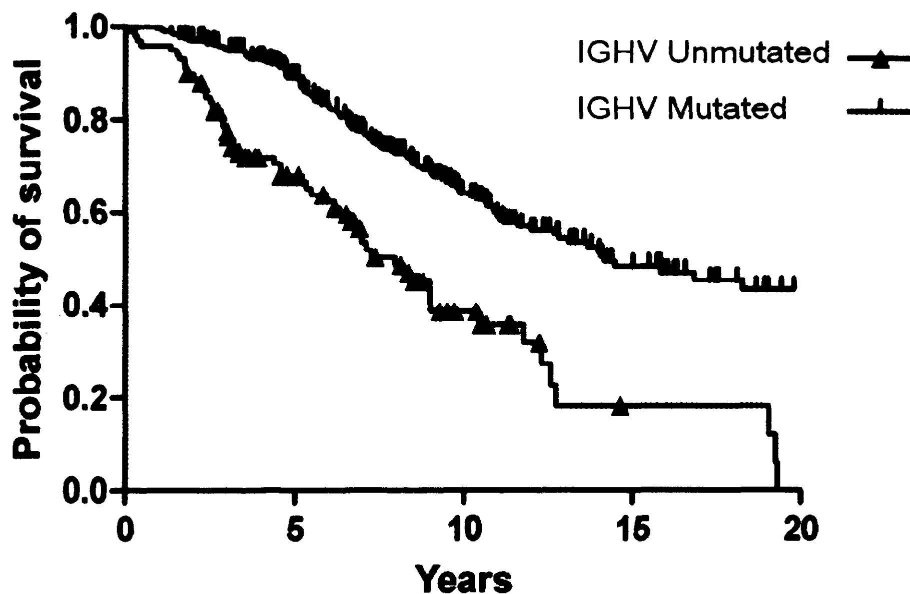


Figure 1.3 Correlation between *IGHV* status and survival. Sampled from a cohort of 321 mutated and 100 unmutated patients from Cardiff University, Hospital of Wales. (Log rank survival p=<0.001; Hazard ratio=2.538)

Finally proteomic analysis of mutated versus unmutated CLL samples has illustrated differences in protein expression between the two groups (Cochran *et al.*, 2003). Among the proteins identified nucleophosmin, which is involved in

the regulation of murine double minute 2 (Mdm2) protein during p53 initiated programmed cell death, was absent in the unmutated group, but present in mutated cases in multiple-post-translationally modified forms.

1.3.1.4 Complementarity determining region 3 (CDR3)

Complementarity determining regions form specific epitopes involved in antigen recognition and are generated by the recombination of the *VDJ* genes (Murray *et al.*, 2008). In a recent analysis of 916 CLL patients 22% exhibited CDR3 homology even though they comprised different *VDJ* rearrangements (Stamatopoulos *et al.*, 2007). In a second study the CDR3 region was associated with specific *IGHV* gene usage and somatic hypermutation in CLL patients (Murray *et al.*, 2008). This data provides additional evidence for the role of a specific antigen in the progression of CLL (Tobin *et al.*, 2003, Caligaris-Cappio, 2009).

1.3.1.5 Immunoglobulin light chains

Recent analysis of immunoglobulin light chains in CLL has illustrated that patterns of restricted gene selection and somatic hypermutation exist which mirror that of the *IGHV* genes (Hadzidimitriou *et al.*, 2009). This presents convincing evidence that the entire BCR antigen recognition site utilises a restricted set of immunoglobulin genes (including heavy and light chain genes) and adds credence to the hypothesis that the CLL clone is selected or driven by specific antigen or autoantigen.

1.3.2 The BCR and signalling

The BCR comprises two main functional domains bound to the B-cell membrane. IgM or IgD ligand binding domains constitute the first and are predominantly extracellular. Disulphide bond links to Ig-α and Ig-β heterodimers, containing 48-61 amino acid cytoplasmic tails, make up the intracellular portion of the BCR and allow signalling (Janice Kuby, 1997). A raft of membrane bound molecules and intracellular substrates and kinases are recruited to the BCR following antigen binding. Phosphorylation of down-stream kinases results in calcium mobilisation and changes in gene transcription (Figure 1.4).

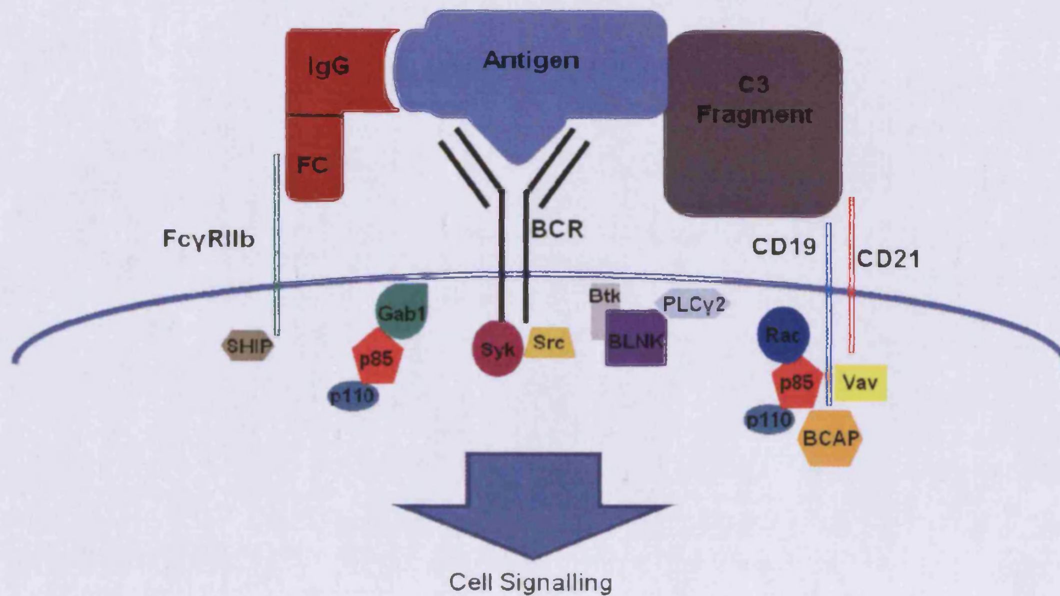


Figure 1.4 BCR signalling pathways (Adapted from David Fruman, UCLA) http://mbb.bio.uci.edu/fruman/index.htm

1.3.2.1 BCR signalling in normal B-cells

One of the primary roles of the BCR, following stimulation, is to mediate signals which promote migration and entry of the B-cell to the lymph node. Here the cell undergoes genetic diversification to allow selection of a mature cell, highly specific for the encountered antigen (Agenes *et al.*, 2000). The cell then differentiates to form an antibody secreting plasmacytoid cell, or is retained in the periphery as a mature memory B-cell. During these processes the BCR responds to external stimuli to promote the survival and development of the cell, or if required, programmed cell death (Agenes *et al.*, 2000).

BCR ligation is thought to be rapidly followed by the formation of clusters or raft complexes on the cell surface that are comprised of molecules which aid in BCR signalling (Weintraub *et al.*, 2000). They include CD19, CD21, CD81 and CD38 (Fujimoto *et al.*, 1998, Deaglio *et al.*, 2003). Other surface proteins may be recruited which inhibit BCR signalling including CD5, CD22, CD72 and Fcγ receptor IIb (Bikah *et al.*, 1996, Walker and Smith, 2008, Adachi *et al.*, 2000, Koncz *et al.*, 1998). Activation of Srk family kinases Lyn, Syk, Lck and

Btk as well as mitogen activated protein-K (MAPK) and Ras pathways result in the translocation of nuclear factors (including the nuclear factor NFκ-B) and increased calcium flux (Zipfel *et al.*, 2000, Meinhardt *et al.*, 1999). Protein kinase C-β (PKC-β) phosphatidylinositol-3-kinase (PI3-K) Erk and Akt all play a role in BCR signalling and have all been implicated in the development of CLL (Barragan *et al.*, 2003, de Frias *et al.*, 2009).

1.3.2.2 BCR signalling in CLL

In CLL, aberrant BCR signalling is thought to contribute to the maintenance and progression of the disease and many of the intracellular molecules active during normal signalling processes are thought to be atypically expressed.

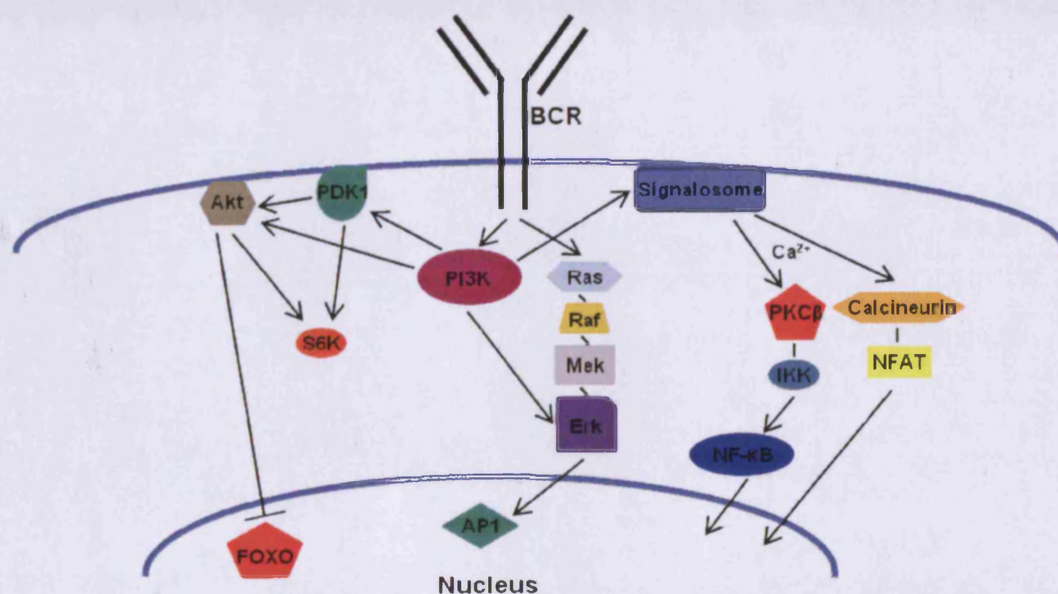


Figure 1.5 Role of PI3K in the signalosome model of BCR signalling (Adapted from D.Fruman, UCLA). http://mbb.bio.uci.edu/fruman/index.htm

Figure 1.5 illustrates the various pathways involved in BCR signalling. BCR induced activation of Syk has been illustrated in CLL resulting in up-regulation of the anti-apoptotic protein Mcl-1 (Gobessi *et al.*, 2009). Indeed this intracellular protein has been shown to be over-expressed in CLL, predominantly

in poor risk groups (Pepper *et al.*, 2008, Petlickovski *et al.*, 2005). Activation of the PI3K/Akt pathway is thought to be essential to CLL cell survival (Barragan *et al.*, 2003). A recent *in vitro* study by de Frias *et al.* illustrated that the use of Akt inhibitors in CLL led to rapid apoptosis of the clone, an effect not as potent in normal B or T-cells and one which could not be reversed by the addition of the potent survival mediators stromal derived factor-1 (SDF-1) and IL-4 (de Frias *et al.*, 2009). In a previous study Akt was shown to be activated in CLL via two distinct pathways involving PI3K and PKC-β (Barragan *et al.*, 2006). Activation of the MAPK pathway has also been described in CLL (Sainz-Perez *et al.*, 2006). BCR stimulation results in phosphorylation of Mek-1 and Mek-2 which in turn activate Erk, a mediator of normal B-cell development (Chang *et al.*, 2003). In a study by Richards *et al.* the inhibition of Mek signalling blocked a subset of B-cell functions including proliferation but did not induce growth arrest or apoptosis in these cells (Richards *et al.*, 2001). Further studies using the Epstein–Barr virus transformed CLL cell line EHEB showed increased sensitivity to purine analogue-induced apoptosis following inhibition of the MAPK/ERK pathway using two novel compounds (Smal *et al.*, 2007). A third group of intracellular signalling molecules may be activated following stimulation of the BCR. The pathway is initiated by PKC-β that regulates the activity of I-κB a potent inhibitor of NF-κB. Over expression of the PKC-βII protein has also been described in CLL. This molecule increases the nuclear translocation and survival effects of NF-κB induced transcription (Abrams *et al.*, 2007).

The CD38 receptor is thought to associate with the BCR to enhance signalling on the surface of CLL cells. A report by Lanham *et al.* correlated increased CD38 expression and the presence of unmutated *IGHV* genes with an increase in BCR signalling (Lanham *et al.*, 2003). In a series of experiments, Lund *et al.* illustrated that (unlike in normal B-cells) B-cells unresponsive to BCR stimulation did not proliferate when stimulated with an anti-CD38 antibody (Lund *et al.*, 1996). They then showed that CD38 lowered the threshold for BCR signalling in murine B-cells responsive to BCR stimulation. Removal of surface immunoglobulin negated any CD38 response following stimulation using the anti-CD38 antibody. With the use of mutants expressing specific portions of the BCR they showed that signalling could be rescued by the expression of the

cytoplasmic tail of Igα or Igβ. Utilising vectors expressing CD38 mutants, Lund *et al.* then illustrated that the cytoplasmic tail of CD38 was not required for CD38 mediated signalling (Lund *et al.*, 1996). These results suggest that the BCR and CD38 are closely associated on the cell surface during CD38 signalling and that CD38 utilises the cytoplasmic domain of the BCR during this process. Interestingly the effects of CD38 ligation included an increased calcium flux in the absence of any intra-cellular tyrosine phosphorylation. This work highlights the association between the BCR and CD38 during signalling and raises the question whether these molecules are working in synergy to promote the survival and proliferation of CLL B-cells.

1.3.2.3 Zap-70 and BCR signalling in CLL

As well as the aberrant expression of B-cell signalling proteins seen in CLL, the T-cell signalling molecule Zap-70 is expressed in a subset of patients. Although essential for pre B-cell development Zap-70 is not usually present in mature B-lymphocytes (Schweighoffer *et al.*, 2003). The presence of the tyrosine kinase was shown to increase BCR signalling in CLL cells, particularly those expressing unmutated *IGHV* genes (Chen *et al.*, 2002, Chen *et al.*, 2005). Subsequent investigations of Zap-70 illustrated that it was able to act as an adapter protein during BCR signalling as it does not require kinase domain activation in order to enhance signalling (Chen *et al.*, 2008).

1.3.2.4 Zap-70 as a marker of prognosis

The presence of Zap-70 has been associated with poor prognosis in CLL (Durig *et al.*, 2003). Because of its role in promoting BCR signalling, initial clinical studies proposed that it could act as a surrogate for *IGHV* mutation status in CLL (Rassenti *et al.*, 2004, Del Principe *et al.*, 2006). Figure 1.6 illustrates the survival of patients according to their Zap-70 status. A clear relationship between the Zap-70 status and survival can be observed using a 20% cut-off point.

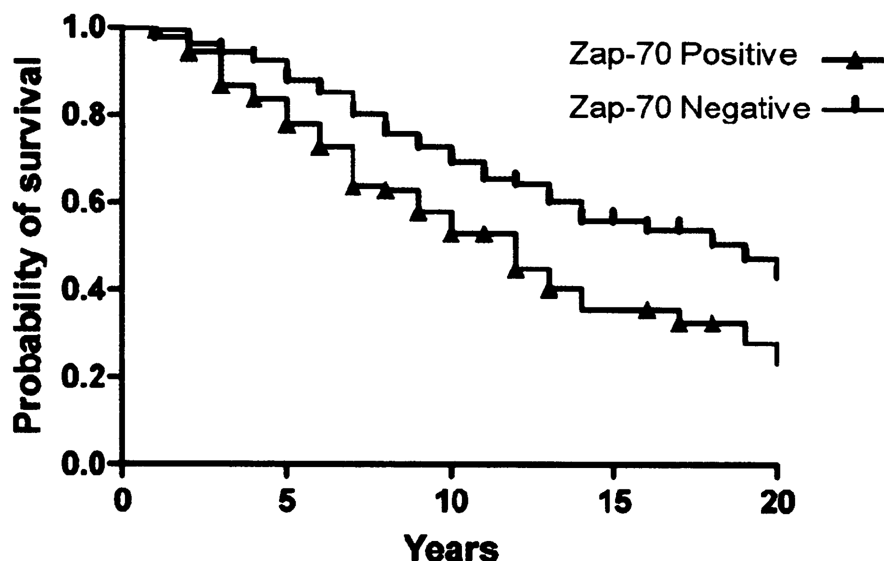


Figure 1.6 Correlation between Zap-70 status and survival. Sampled from a cohort of 262 Zap-70 negative and 189 Zap-70 positive patients from Cardiff University, Hospital of Wales. (Log rank, p=<0.001; Hazard ratio=1.744)

1.3.3 CLL cell signalling and migration

Stromal derived nurse like cells have been described in CLL. These cells have been shown to enhance CLL cell survival through the release of stromal derived factor-1α (SDF-1α) (Burger *et al.*, 2000). The CXCR4 receptor that binds the SDF-1α chemokine can be detected on the surface of CLL cells. *In vitro* experiments have shown that CXCR4 promotes the migration of CLL cells through a stromal cell layer in the presence of SDF-1 (Burger *et al.*, 1999). The tyrosine kinase Zap-70 is known to up-regulate CXCR4 in the process of T-cell transendothelial migration (Ticchioni *et al.*, 2002) and Deaglio *et al.* showed that CD38 and Zap-70 expressing CLL cells illustrate enhanced migration in response to SDF-1α (Deaglio *et al.*, 2007a). They also showed that ligation of CD38 with an agonistic antibody induced Zap-70 phosphorylation. In a similar study Quiroga *et al.* showed that CLL cell migration could be blocked by treating the cells with an inhibitor of the Syk tyrosine kinase which is involved in BCR signalling (Quiroga *et al.*, 2009).

CD49d is a member of the integrin superfamily that has attracted recent attention in CLL. Also known as α4 integrin it makes up half of the cell surface α4β1 lymphocyte homing receptor and is known to regulate adhesion of the cells

to the extracellular matrix by binding fibronectin, or to other cells via the VCAM-1 receptor (Rose *et al.*, 2002). In a study of 303 patients with CLL, high CD49d expression significantly correlated with CD38, Zap-70 and immunoglobulin gene mutation status. It was also shown to be an independent prognostic marker for overall survival and time to first treatment (Rossi *et al.*, 2008, Majid *et al.*, 2010).

Engagement of CXCR4 up-regulates matrix metalloproteinase-9 (MMP-9), a gelatinase effective in the breakdown of intercellular matrices and which is integral to the process of transendothelial migration (Redondo-Munoz *et al.*, 2006). CD49d has been shown to induce the expression of CXCR4 in CLL cells (Redondo-Munoz *et al.*, 2006). The integrin was also reported to be involved in the transendothelial migration of CLL cells in a separate process involving the pro-angiogenic mediator VEGF (Till *et al.*, 2005). In this recent report Till *et al.* showed that blocking VEGF (using a monoclonal antibody or an inhibitor of the VEGF receptor) resulted in a marked reduction in the transendothelial migration of CLL cells. This effect was not seen in normal B-cells. A recent gene expression study illustrated the increased expression of mRNA coding for molecules that induce adhesion and migration (including the integrin CD49d). These genes were only over-expressed in Zap-70 positive cells that also displayed increased *in vitro* survival (Stamatopoulos *et al.*, 2009).

There is substantial evidence to suggest that a raft of surface and intracellular molecules are involved in promoting the migratory potential of CLL cells. CD38, present on the surface of CLL cells, has the potential to bind CD31 on endothelial cells and facilitate the process of migration through the induction of such molecules. Whether Zap-70, VEGF, CD49d and the chemokines and their receptors are induced or activated following stimulation of CD38 is a question that remains to be answered in CLL.

1.4 CD38 in CLL

CD38 was first described on the surface of lymphocytes in 1980 by Reinherz *et al.* who were looking at antigens on the surface of T-cells (Reinherz *et al.*, 1980). Since then an array of work has identified the various roles of CD38 in multiple cell types and uncovered intriguing evidence illustrating that

ADP-Ribosyl cyclase (ADPRc), a homologue of CD38, was present in primitive organisms over 700 million years ago (Malavasi *et al.*, 2008).

1.4.1 Phylogeny of CD38

Evolution of the CD38 molecule has been eloquently described in a series of reports by Malavasi and Deaglio whose laboratories have been studying CD38 and its homologues for many years. (Deaglio and Malavasi, 2006, Malavasi *et al.*, 2006). Through analysis of the genetics of various species they postulated that the 7kb gene found in *Aplysia*, which translates to the soluble ADPRc enzyme, developed to become the 90kb gene expressing the membrane bound CD38 multifunctional glycoprotein found on many cell types in higher animals including human beings. Its homologue CD157 evolved in parallel with CD38 as a glycosylphosphatidylinositol (GPI) membrane-anchored protein with similar calcium mobilising properties.

1.4.2 Genetics

The CD38 and CD157 genes are found on the short arm of chromosome 4 and are arranged in a head-to-tail manner (telomere →CD157 →CD38 →centromere) implying that CD38 and CD157 are derived by gene duplication (Malavasi *et al.*, 2006). The CD38 gene consists of 8 exons that make up 98% of the 80kb fragment. Expression of the gene is controlled by regulatory elements on exon 1 and multiple binding sites exist for transcription regulatory molecules such as NF-κB, Sp1, T-cell transcription factor-1α (TCF-1), nuclear factor for IL-6 (NF-IL-6), interferon-responsive factor-1 (IRF-1) and glucocorticoid hormones (Tirumurugaan *et al.*, 2008, Buggins *et al.*, 2010). There is also a retinoic acid response element site located at the 5′ end of exon 1 on chromosome 4 known to induce the transcription of CD38 in myeloid cells (Kishimoto *et al.*, 1998). A single nucleotide polymorphism (SNP) has been characterised within the 5′ region that incorporates a *Pvu II* restriction site into the gene (Ferrero *et al.*, 1999). The C to G mutation has been studied in various cohorts and the literature suggests that the presence of the polymorphism, in its homo or heterozygous form, results in susceptibility to CLL (Jamroziak *et al.*,

2009), disease progression and transformation to Richter's syndrome (Aydin *et al.*, 2008).

1.4.3 Structure of CD38

CD38 is a type II trans-membrane glycoprotein with a small cytoplasmic region (21 amino acids), a single chain transmembrane region (23 amino acids) and 256 amino acid extracellular domain that can be loosely divided into two regions. The extracellular NH_2 portion consists of 156 amino acids that make up 5 α-helices. This region is adjacent to the COOH domain (amino acids 200-300) that contains four parallel β-sheets surrounded by two long and two short α-helices. The two regions are joined at multiple positions along the amino acid sequence (namely residues 118–119, 143–144, and 200–201; (Malavasi *et al.*, 2008). These bridges act as a hinge, allowing the structure to open and close depending on the binding of relevant molecules (Liu *et al.*, 2005) (Figure 1.7).

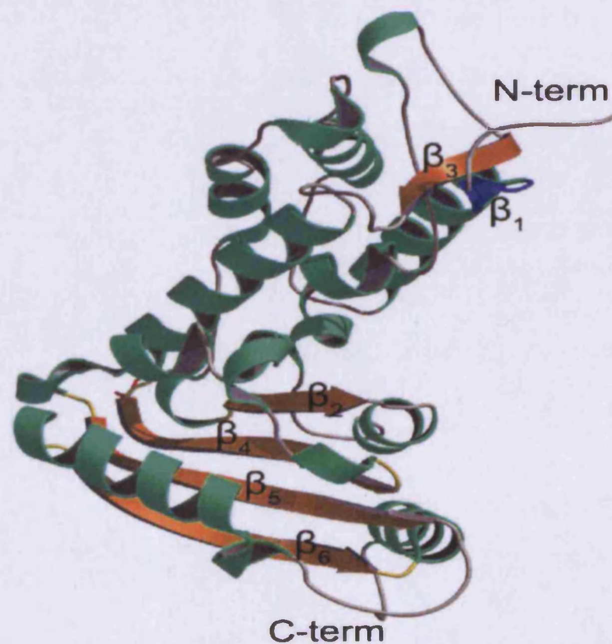


Figure 1.7 Crystal structure of CD38 (Adapted from Liu *et al*,. 2005)

CD38 is generally reported as a 45KDa molecule, although treatment with different reagents and the use of various technical approaches has yielded proteins varying between 34 and 45KDa (Ferrero and Malavasi, 1997). Jackson *et al.* calculated that the molecular weight of the CD38 protein is approximately

34KDa and suggested that 25% of the isolated complex was made up of carbohydrate (Jackson and Bell, 1990). A soluble form of the molecule has been described which is present at high levels in the serum of patients with various conditions including multiple myeloma and HIV infection (Funaro *et al.*, 1996).

1.4.4 Functions of CD38

By the time CD38 was recognised as a cell surface molecule possessing enzymatic properties similar to that of ADPRc, a wealth of knowledge had been obtained on the biochemical properties of this calcium mobilising enzyme. Additionally, the CD157 ectoenzyme was found to induce calcium mobilisation on the surface and within the cell. Interestingly, recent evidence has illustrated that CD38 also acts as a multi-functional molecule harbouring receptor-signalling capabilities (Ferrero and Malavasi, 1997).

1.4.5 Calcium mobilisation by ADPRc and CD38

Derived from nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) respectively, cADPR and nicotinic acid adenine dinucleotide phosphate (NAADP) are potent mediators of calcium release from intracellular stores (Chini *et al.*, 1995). Both molecules and their precursors have very different structures which makes it surprising that they are both synthesised by ADPRc, CD38 and CD157. This mechanism of intracellular calcium release is known to be involved in the initiation of many cellular functions in plants and animals including proliferation, activation, gene expression, fertilisation and neurotransmitter release (Lee, 2001).

The production of cADPR or NAADP was initially thought to be dependent upon the levels of substrate available to the enzyme. It was later discovered that the catalytic pathway chosen is dependent upon the pH of the surrounding environment (Aarhus *et al.*, 1995). In acidic conditions NAADP is generated where as in alkaline pH cADPR is produced. Acidic organelles such as endosomes participate in the endocytic pathway which is thought to be mediated by calcium release induced by the NAADP generated in such an acidic environment (Lee, 2001). On the extracellular membrane cADPR is thought to target the ryanodine receptor in a complex series of interactions involving calmodulin and other accessory proteins (Lee *et al.*, 1995). In an experiment by

Guse *et al.* the binding of ryanodine increased in a concentration dependent manner following the addition of cADPR (Guse *et al.*, 1999). A number of other investigations have supported this theory and highlighted the role of cADPR in ryanodine receptor mediated calcium flux (Prosser *et al.*, 2010). Following the conversion of NADP to cADPR, CD38 has the ability to hydrolyse cADPR to ADP-ribose in a secondary reaction. In a series of experiments, Howard *et al.* showed that purified cADPR augmented the proliferative response of activated murine B-cells (Howard *et al.*, 1993). This suggests that the enzymatic functions of CD38 are able to deplete this molecule at the lymphocyte surface and allow proliferation of the cell.

Site-directed mutagenesis has allowed the identification of the specific nucleotides within the CD38 molecule that are required for cADPR synthesis and hydrolysis into ADPR. In a seminal study by Tohgo *et al.* cysteine residues 119 and 201 were identified as essential for both the synthesis and hydrolysis of cADPR (Tohgo *et al.*, 1994). A later study by Graeff *et al.* identified the Glu-146 site as being crucial in determining whether the synthesis or hydrolysis function be performed by the CD38 enzyme (Graeff *et al.*, 2001). Much of this work has been carried out in lower organisms such as yeast and *Aplysia*, with the focus being solely on analysis of the regions of CD38 responsible for the generation of cADPR, ADPR and NAADP. Such investigations are imperative in translational research when looking for specific drug target sites to treat disease.

1.4.6 Receptor functions of CD38

Almost two decades after the CD38 antigen was described on the surface of lymphocytes it was suggested that the molecule was not just an ecto-enzyme involved in the control of intra-cellular calcium, but in addition possessed a receptor-signalling capacity in these cells (Deaglio *et al.*, 2003). In the late 1990s Malavasi and his group generated a non-substrate, agonistic antibody for CD38 which had the ability to stimulate intracellular signals resulting in the transcription and secretion of an array of biologically relevant molecules (Ausiello *et al.*, 2000). It was promptly noted that some of the molecules activated following CD38 ligation were similar to those induced following stimulation of the T-cell receptor (TCR).

1.4.7 CD38 signalling in T-cells

Much of the early work on CD38 signalling was carried out in T-cells. Ligating CD38 with the agonistic monoclonal antibody IB4 led to both early and late signalling events involving the T-cell receptor (Zubiaur *et al.*, 1997). Zubiaur *et al.* illustrated that following CD38 stimulation, phosphorylation of protein tyrosine kinases phospholipase C-γ1 (PLC- γ1), c-Cbl, Zap-70 and Shc occurred in Jurkat cells. Down-stream activation of the MAPK Erk-2 was abrogated in Lck kinase-deficient mutants indicating an essential role for this molecule in CD38 signalling. The results of this extensive study concluded that both Raf-1/MAPK and CD3-ζ/Zap-70/PLC-γ1 pathways are triggered following the ligation of CD38 (Zubiaur *et al.*, 1997). In a later study by the same group it was shown that ligation of CD38 led to tyrosine phosphorylation of both the CD3-ζ and CD3-ε portions of the TCR. They subsequently developed a mutant CD3-ζ expressing TCR that exhibited defective binding to CD38. Following ligation, this unit was not phosphorylated but the down-stream effects of CD38 stimulation were the same. This suggests that the CD3-ε subunit is sufficient for CD38 activation of MAPK and PKC signalling cascades, though it is still likely that the CD3-ζ sub unit plays a synergistic role in signalling as it is known to effectively recruit Zap-70 to the TCR (Zubiaur *et al.*, 1999).

CD38 is abundant in lipid rafts on the surface of T-lymphocytes (Deaglio *et al.*, 2006). Experiments depleting the cholesterol within these rafts resulted in abrogated CD38 signalling (Zubiaur *et al.*, 2002). The recruitment of co-signalling molecules, including Src family kinases, to the lipid rafts suggested that this is a site where the CD38 molecule can exhibit enhanced signalling (Munoz *et al.*, 2003). In a study by Cho *et al.* Lck was one of the molecules found in abundance in the CD38/TCR lipid raft domain and the same group duly illustrated that the Src homology-2 (SH2) region of Lck binds to the cytoplasmic tail of CD38 causing phosphorylation of the molecule (Cho *et al.*, 2000). This work confirms that Lck has a definitive role in CD38 signalling and the activation of T-cells.

1.4.8 CD38 in normal B-cell development

CD38 is expressed on the surface of lymphoid and myeloid cells at various stages of cell maturation. It is present on progenitor B-lymphocytes in the bone

marrow where it has a role in lymphopoiesis. Within the bone marrow microenvironment, interaction of B-cells with stromal cells in the presence of cytokines such as IL-2 and IL-4 stimulates the cells to mature and divide (Kumagai *et al.*, 1995). The use of monoclonal antibodies to block the actions of CD38, prior to stimulation by cytokines and co-culture with stromal cells, was found to suppress B-cell lymphopoiesis (Kumagai *et al.*, 1995).

Subsequent investigations have identified CD19 as a mediator of CD38 signalling. In normal B-cell progenitors, CD19 has the ability to recruit specific kinases to the inner membrane (including syk and c-cbl) allowing CD38 to signal via PI3-K pathways (Kitanaka *et al.*, 1996, Kitanaka *et al.*, 1997). More recently the non-receptor tyrosine kinase Btk has been implicated in a novel signalling cascade leading to PKC, phosphatidylcholine, phospholipase C and phospholipase D-dependent B-lymphocyte activation. In a study by Moreno-Garcia *et al.* stimulation of CD38 resulted in the proliferation of splenic B-cells through phosphorylation of Btk in an alternative mode of CD38 signalling which was completely independent of phospholipase C-γ2 in the canonical pathway (Moreno-Garcia *et al.*, 2005).

Mature B-cells found in the periphery do not exhibit the CD38 antigen and only re-express the molecule following antigen stimulation or differentiation into antibody-secreting plasmacytoid B-cells (Deterre *et al.*, 2000, Campana *et al.*, 2000).

1.4.9 CD38 as a marker of poor prognosis in CLL

CD38 was first described as a poor prognostic marker in CLL by Damle *et al.* in 1999 who observed the outcome of 47 patients phenotyped for CD38 expression and genotyped for *IGHV* mutation status (Damle *et al.*, 1999). Both the presence of surface CD38 (>30% cells) and unmutated immunoglobulin genes correlated strongly with survival and CD38 was proposed as a novel and more simple means of predicting outcome in newly diagnosed CLL patients. In 2001, Ibrahim *et al.* studied a larger cohort of patients and strongly correlated the presence of CD38 on the cell surface with a poor prognosis (Ibrahim *et al.*, 2001). In 96 out of 218 patients more than 20% of the CLL clones expressed the CD38 antigen and this cohort were found to have increased lymph node involvement, lower haemoglobin levels, high serum β2-microglobulin and

shorter survival times. These observations were confirmed in subsequent studies where the expression of CD38 correlated with disease progression and treatment free survival in patients with CLL (Ghia *et al.*, 2003, Durig *et al.*, 2002). In a more recent study CD38 was shown to retain its prognostic significant in a cohort of patients with stage A disease (Letestu *et al.*, 2010). Interestingly, a number of patients present with two distinct CLL cell populations; one CD38 negative and the other CD38 positive. This bimodal phenotype was shown to correlate with unmutated immunoglobulin genes and an unfavourable outcome in CLL patients (Ghia *et al.*, 2003). Using a 20% cut off point, CD38 positivity correlated with overall survival in a cohort of 485 patients from Cardiff University Hospital of Wales (figure 1.8).

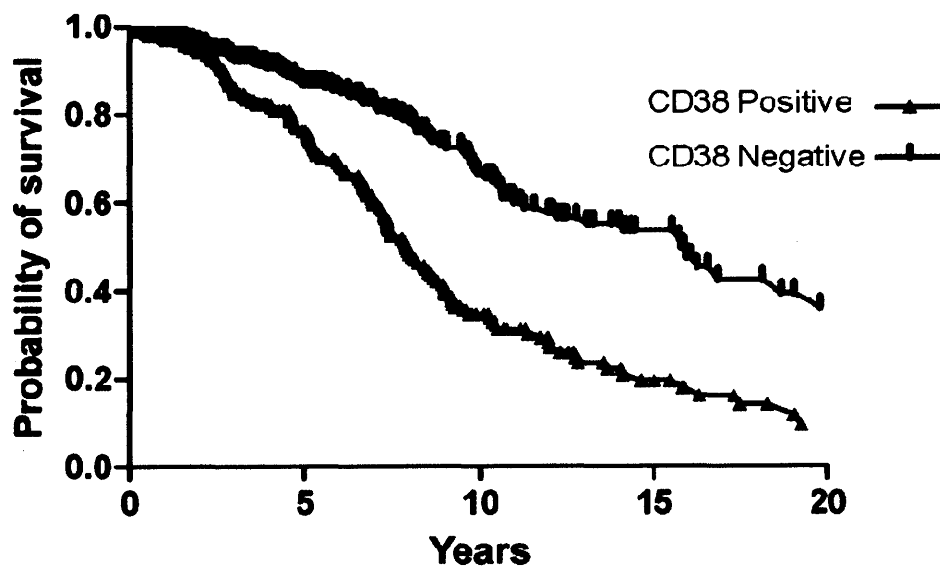


Figure 1.8 Correlation between CD38 status and survival. Data from a cohort of 269 CD38 negative and 216 CD38 positive patients from Cardiff University, Hospital of Wales. (Log rank, p=<0.001; Hazard ratio=2.3)

1.4.10 The role of CD38 in the pathogenesis of CLL

Following the identification of CD38 as a marker of poor outcome, various groups have investigated whether the molecule has a role in enhancing the survival or proliferation of the CLL clone. In 2005, Pittner *et al.* described the increased expression of activation markers including CD18, CD49d and CD20 in CD38 positive CLL cells and also showed that the CD38 expressing cells were

more receptive to additional activation through interferon simulation (Pittner *et al.*, 2005). Expression analysis within bi-modal patients illustrated that the CD38 positive sub-clone had a distinct gene expression profile compared to the CD38 negative sub-clone (Pepper *et al.*, 2007). Comparing CD38 positive and negative CLL cells from independent patients Damle *et al.* then illustrated that the former appear as a phenotypically activated subset (Damle *et al.*, 2007). In the same report, they illustrated additional markers of activation including Zap-70, CD27, CD62L and CD69. Staining for the cell cycle entry protein Ki-67, Damle *et al.* illustrated increased proliferation within the CD38 positive clone and identified the presence of enhanced telomerase activity. They also illustrated that there was no difference in the telomere lengths between the CLL cells taken from CD38 positive and negative patients (Damle *et al.*, 2007). The latter phenomenon was complemented by Lin *et al.* who carried out genetic analysis on monoclonal CD38 positive and negative CLL subclones isolated from the same patient and illustrated no difference between the telomere lengths or the clonal evolution of the cell subsets according to their CD38 status (Lin *et al.*, 2008). In 2009 Calissano *et al.* carried out similar experiments and highlighted that there was no difference in the telomere lengths of CD38 positive and negative CLL B-cells. These investigations have led to the intriguing supposition that CD38 expression is transient on the surface of CLL cells.

With a wealth of knowledge obtained from studying the functions of CD38 in T-cells, Malavasi and colleagues were in a prime position to investigate its role on the B-cell surface in CLL. A report by Deaglio *et al.* in 2003 illustrated the signalling capacity of CD38 in CLL (Deaglio *et al.*, 2003) and a second manuscript three years later summarised a comprehensive body of work which strengthened the hypothesis that CD38 is not just a marker of activation in this disease (Deaglio *et al.*, 2006). Investigations by the same group, on the short term affects of CD38 ligation using an agonistic antibody, revealed increased calcium flux following cross-linking. However, this effect was not observed in all of the CD38 positive patient samples investigated and the increases in intracellular calcium were small. The addition of IL-2, which is known to up regulate CD38 on the surface of cells already expressing CD38, resulted in increased calcium flux in previously unresponsive CLL samples. This suggested that a minimum threshold was necessary for signalling or that IL-2 was able to

modify the arrangement of CD38 with accessory molecules on the cell surface to allow signalling (Deaglio *et al.*, 2006). Due to its small cytoplasmic domain (21 amino acids) it is not unreasonable to postulate that other molecules may be required for CD38 signalling to take place within the cell. With the use of co-capping experiments Deaglio *et al.* described how CD19 and the BCR were recruited alongside CD38 in lipid raft formations and highlighted the similarity between the synergy of these molecules in B-cell signalling (Deaglio *et al.*, 2007b). Overall these experiments illustrated that CD38 had the capability to signal in the presence of co-accessory molecules and that this signal results in the prolonged survival of the CLL cell. The necessity for accessory molecules and cytokines to enhance cell signalling reinforced the idea that the tissue microenvironment was an ideal location for CLL cell activation and clonal expansion (Deaglio *et al.*, 2006).

1.5 *In vivo* survival and proliferation of the CLL Cell

1.5.1 Proliferation centres and the microenvironment

Removal of CLL cells from the body results in the rapid onset of apoptosis (Collins *et al.*, 1989). This suggests the presence of *in vivo* stimuli which enhance the survival and proliferation of the cells. This process is thought to take place in proliferation centres in the lymph nodes and bone marrow where the CLL cells are in contact with other cells such as T-cells and stromal cells. (Munk Pedersen and Reed, 2004). These sites are also a prime location for antigen presentation and immunological activation of the CLL cell (Ghia *et al.*, 2008).

Proliferation centres, or pseudofollicles, have been described in the bone marrow and lymph nodes of CLL patients that are rich in pro-lymphocytes and paraimmunoblasts (Schmid and Isaacson, 1994). Staining tissue biopsies has illustrated that CLL cells reside in large numbers in these sites together with T-cells. Whether the CLL cells actively recruit the T-cells through the use of chemokines, or alternatively "attract their attention" through autoimmune responses (as described in other diseases such as rheumatoid arthritis (Takemura *et al.*, 2001)) is a matter for debate. The survival of the CLL cells is promoted within these regions through stimulation of surface receptors and the presence of

survival factors such as IL-2. One of the mechanisms which enhances CLL survival within the microenvironment was highlighted in a recent a study by Patten *et al.*, who illustrated that through close interactions with T-cells in the pseudofollicles, the CLL cells express higher levels of CD38 which signifies an activated subset of cells (Patten *et al.*, 2008).

1.5.2 CD31

CD31 (PECAM-1) is a member of the immunoglobulin super family expressed on endothelial cells and is the only known non-substrate ligand for CD38 (Deaglio *et al.*, 1996, Deaglio *et al.*, 1998). In a study by Deaglio *et al.* ligation of CD38 by CD31 resulted in CLL cell activation and the induction of anti-apoptotic mechanisms that promoted cell survival. Following re-modelling on the cell surface and recruitment of the BCR and CD19, the interaction between CD31 and CD38 was shown to illicit up-regulation of CD100 in CLL cells (Deaglio *et al.*, 2005). CD100 is a survival receptor that binds to plexin-B1. Plexin-B1 is found on nurse-like stromal cells present in the bone marrow and lymph nodes of CLL patients along with CD31. The multiple interactions of CD38 with its ligand CD31 and accessory molecules the BCR and CD19, together with co-receptor signalling via CD100/plexin-B1 all occur on the surface of the CLL cell to enhance cell survival and proliferation (Deaglio *et al.*, 2006, Granziero *et al.*, 2003).

Investigations have been carried out to determine whether the expression of CD31 on the surface of CLL cells correlates with outcome in CLL. Ibrahim *et al.* illustrated that in a cohort of 120 patients, those expressing low levels of CD38 and CD31 had a favourable outcome compared to the rest of the group. They also showed that patients with a high CD31 expression, but low CD38, exhibited a poor outcome not significantly different to that of the CD38 positive group (Ibrahim *et al.*, 2003). Poggi *et al.* illustrated that anti-apoptotic mediators of the Bcl-2 family were up regulated in a cohort of patients whose clone expressed high levels of CD31 on the cell surface. They studied the effects of CD31 ligation and observed increased activation of the PI3K/Akt pathway as well as nuclear localisation of the NF-κB sub units p65 and p52. (Poggi *et al.*, 2010). Conversely Mainou-Fowler *et al.* showed that low levels of CD31 on the cell surface correlated with aggressive disease in CLL (Mainou-Fowler *et al.*, 2008).

1.6 Objectives

The multiple roles of the transmembrane glycoprotein CD38 have been widely studied in both T- and B-lymphocytes in health and disease. Although present on the surface of immature B-cells and abundant on antigen experienced plasma cells, CD38 is not present at high levels on the surface of healthy mature B-cells within the peripheral blood. In around 30% of CLL cases CD38 is highly expressed on the surface of the cell and has been shown to be a marker of aggressive disease. A relatively small group of researchers have carried out a wealth of investigations to determine whether CD38 is involved in the pathogenesis of CLL or whether it is merely a marker of an activated clonal B-cell. Work thus far has clearly identified CD38 as having signalling properties and it has also been reported to interact with other surface and intracellular signalling molecules such as the BCR and Zap-70.

The central hypothesis of this project was that CD38 has distinct signalling capabilities which contribute to the aggressive nature of CD38 positive CLL. Four principle objectives were devised to test this hypothesis:

1) To optimise a gene delivery technique to express CD38 on the surface of CD38 negative CLL cells. Using this approach I aimed to determine the direct effects of CD38 expression on the cell surface of CLL cells by comparison with genetically unmodified cells derived from the same patient.

2) To utilise this model along with native CD38 expressing CLL cells to assess the *in vitro* survival and proliferation of CLL cells in liquid culture and under co-culture conditions.

3) To observe changes in the genotype of the CLL cells following the expression of CD38 on the surface of the cells.

4) To observe changes in the phenotype of the CLL cells following the expression of CD38 on the surface of the cells.

Chapter 2. Materials and Methods

2.1 List of Materials and laboratory equipment

(See Appendix 1)

2.2 Preparation of general reagents

2.2.1 Phosphate buffered saline (PBS)

1X Phosphate Buffered Saline (PBS) was made up by dissolving 5 PBS tablets in 0.5 litres of distilled water. The PBS was sterilised by autoclaving at 121°C, 15lb/ins^2 for 20 minutes and then stored at room temperature.

2.2.2 Preparation of LB media for bacterial cell culture

Luria-Bertani (LB) media was made up by adding 10g of tryptone (1%), 5g of yeast extract (0.5%) and 10g of NaCl (0.17M) to 950ml of de-ionised water. The mixture was autoclaved as described. LB-agar, for making plates, was made from the same mixture adding 15g/l agar before autoclaving. For experiments requiring selective media, 200μl ampicillin (50μg/ml) was added following cooling of the media to approximately 50°C prior to pouring the LB-agar plates.

2.2.3 Preparation of eukaryotic cell culture media

2.2.3.1 Culture media for non-adherent cells

For cells cultured in suspension, media was made up in 500ml of Roswell Park Memorial Institute (RPMI) media. 50ml of fetal calf serum (FCS) (final concentration 10%) was added followed by 10ml Penicillin plus Streptomycin (2X), 5ml L-glutamine (1X) and 5ml Sodium Pyruvate (1X). The media was mixed well and left at room temperature to cool for an hour before storing at 4°C. All work was carried out using careful cell culture techniques in a Class II safety cabinet.

2.2.3.2 Culture media for adherent cells

Culture media for adherent cells contains the Dulbecco's Modified Eagle's Media (DMEM) additive rather than RPMI. The media was made up following the same protocol as above without the requirement for the addition of L-glutamine as this is already contained in DMEM.

2.2.4 Paraformaldehyde

1% Paraformaldehyde (Sigma) was prepared by dissolving 1g of paraformaldehyde in 100ml PBS. To dissolve the paraformaldehyde the solution was heated to 70°C and once dissolved was left to cool and then stored at 4°C in the dark to prevent depolymerisation and subsequent release of the carcinogen formaldehyde. The preparation of paraformaldehyde was carried out in a fume hood for safety purposes.

2.2.5 Waste disposal

As the work contained in this project required the use of primary human tissue, bacteria and attenuated lentivirus it was important to adhere to strict health and safety regulations within the laboratory and decontaminate and dispose of waste in the appropriate manner. Gloves and lab coats were worn at all times and contaminated waste was placed into a solution containing 2,500ppm chlorine over night before disposal.

2.3 Methods

2.3.1 Primary cell isolation

2.3.1.1 Density centrifugation of peripheral blood to obtain patient CLL cells

Mononuclear cells from CLL patients were isolated from peripheral blood samples collected in EDTA. Ficoll reagent (Histopaque) was used to isolate the low-density layer of mononuclear cells containing CLL B-cells. 2.5ml of blood was mixed with 2.5ml of sterile PBS in a 15ml tube. 7ml of Ficoll reagent was carefully transferred to the bottom of the tube using a syringe and the sample was centrifuged at 756 x g for 20 minutes with no brake. The monolayer of low density cells was removed using a pastette and washed in 10ml sterile PBS. Following centrifugation at 272 x g for 5 minutes the cells were re-suspended in 3ml of sterile H_2O for 5 seconds to lyse any contaminating red cells. 10ml of sterile PBS was then added to restore the isotonic solution. The cells were pelleted and washed once more in sterile PBS before re-suspending in 1-10ml sterile PBS (depending on the size of the pellet).

2.3.1.2 Cell counting on the Beckman Coulter Vi-cell

Cell counting was performed using a Vi-cell analyser (Beckman Coulter). Briefly, 50μl of each sample was added to 450μl PBS in a cell counting tube and placed into the cell counting carousel. The cell type and dilution factor were entered into the Vi-cell and it was set to run. The number of viable cells per ml was ascertained within each sample using trypan blue exclusion.

2.3.1.3 Cell counting using the Neubauer Haemocytometer

10μl of the cells in suspension were mixed with 10μl of trypan blue exclusion dye (to stain dead cells) and 40μl of PBS to give a 1 in 6 dilution. 10μl was pipetted onto the grid of a disposable Neubauer haemocytometer and placed under the microscope. The number of unstained cells in 9 small squares was added together and multiplied by 60,000 to give the number of cells per ml.

2.3.1.4 Purifying CLL cells

The majority of patient samples derived from Ficoll separation of mononuclear cells consisted of over 95% CLL B-cells following density centrifugation. In those cases where there was less than 95% B-cells the T-cells were removed with the use of magnetic beads. CD19 positive CLL cells were isolated by negative selection using CD3-expressing Dynabeads. 20µl of beads labelled with anti-CD3 antibody were used per $1x10^6$ CLL cells. The beads were washed once in PBS and incubated with the CLL cells in 2ml of PBS at 4˚C. The sample was incubated for 20 minutes and mixed at regular intervals. The sample was then made up to 5ml with PBS and placed into the Dynal rack containing the magnet. After 5 minutes the sample was decanted into a fresh tube whilst maintained within the magnetic field. This CD3 depleted sample was spun at 272 x g for 5 minutes and re-suspended in 500µl of PBS. The purity of the sample was assessed by flow cytometry for CD19+ cells (Figure 2.1).

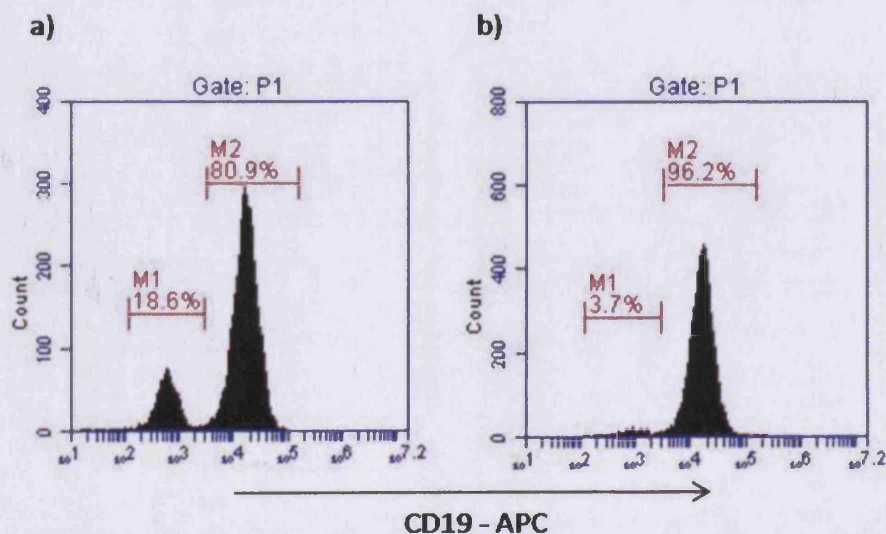


Figure 2.1 Percentage CD19 positive cells in a CLL sample pre (a) and post (b) depletion of CD3-expressing T-cells

2.3.2 Eukaryotic cell culture

2.3.2.1 Thawing cells from liquid nitrogen stores and transferring to liquid culture

The standard precautionary methods were used when dealing with liquid nitrogen (use of face masks and gloves). Following recovery from the nitrogen store, the cells were placed in a water bath at 37°C for 5 minutes. In order to remove the dimethyl sulphoxide (DMSO) storage reagent the cells were transferred to a 15ml tube containing 5ml of pre-warmed culture medium (RPMI and DMEM for non-adherent and adherent cells respectively) and gently mixed to give a homogeneous suspension. This suspension was centrifuged at 272 x g for 5 minutes to pellet the cells and the supernatant was carefully removed. The cells were then re-suspended in 8ml of fresh media, transferred to a small culture flask and incubated in a humidified atmosphere maintained at 37°C, 5% CO_2.

2.3.2.2 Sub-culture of non-adherent cells

The confluence of non-adherent cells was determined by the turbidity and the colour of the RPMI media within the T175 flask. When appropriate, the cells were aspirated from the flask and centrifuged at 272 x g for 5 minutes. After re-suspending in 20ml, an aliquot of 1-5 ml was placed in to a fresh flask containing 30ml of fresh media. The remaining cells were used in experiments or discarded. Smaller cell preparations were performed in T75 flasks in 15ml of media.

2.3.2.3 Sub-culture of adherent eukaryotic cells

Aspirating the media from the adherent cells left them bound to the bottom of the T175 flask. They were then washed in 10ml PBS to remove residual FCS in the DMEM media as this is known to inhibit the effects of trypsin. Following aspiration of the PBS, 5ml of trypsin was added and the cells were incubated for 5 minutes at 37°C, 5% CO_2. 8ml of DMEM media was washed over the bottom of the flask to ensure all cells were re-suspended. After centrifuging at 272 x g for 5 minutes the supernatant was discarded and the cells were re-suspended in 10ml DMEM media. 0.5 to 5ml was replaced into the original small flask depending on the demand for cells. 25ml of DMEM media was added to supplement the cells for up to 5 days in culture. The cells were cultured at 37°C,

5% CO_2. Smaller cell preparations were performed in T75 flasks in 15ml of media.

2.3.2.4 Freezing down eukaryotic cell lines for long term liquid nitrogen storage

The storage media was made up of:
50% FCS
40% DMEM plus additives
10% DMSO

$8x10^6$ Cells were re-suspended in 4ml of storage media and 500µl was aliquoted into 8 cryo-storage tubes. The samples were placed into a freeze container submersed in isopropanol and placed into the -80°C freezer. After 24 hours the samples were transferred to the liquid nitrogen store.

2.3.2.5 Preparation of co-culture

Adherent fibroblasts were utilised as *in vitro* co-cultures to simulate the *in vivo* microenvironment. Genetically modified sub-sets expressing the human CD31 or the human CD154 antigen were employed to stimulate CD38 or CD40 on the surface of CLL cells respectively. The fibroblast cells were first irradiated to prohibit their growth within the co-culture environment. (Details of co-culture cells and their origin can be seen in appendix 2.1.3).

2.3.2.5.1 Irradiation

$5x10^6$ co-culture cells were re-suspended in 25ml DMEM with additives and irradiated at 75 Grays (27 minutes in the presence of Caesium-137, γ emission). The volume was then made up to 50ml and 2ml was plated out into the wells of a 6-well plate. Prior to irradiation, 1ml of the trypsinised co-culture cells were washed and placed into a fresh culture flask for future experiments.

2.3.2.5.2 Co-culture conditions

Following irradiation 1ml of fresh DMEM plus additives was aliquoted into each well (of a 6-well plate) and the cells were left at 37°C, 5% CO_2 over night to

adhere. After at least 12 hours the CLL cells were added to the co-culture and the media was changed every 3-4 days depending on its colour.

2.3.3 Generation of lentiviral plasmids

The SxW and EGFP plasmids are illustrated in Figures 2.2 and 2.3 respectively. The CD38 expressing lentivirus was generated by excising CD38 from the expression of green fluorescent protein plasmid (pEGFP-1) and incorporating it into the SxW lentiviral backbone (Figure 2.5).

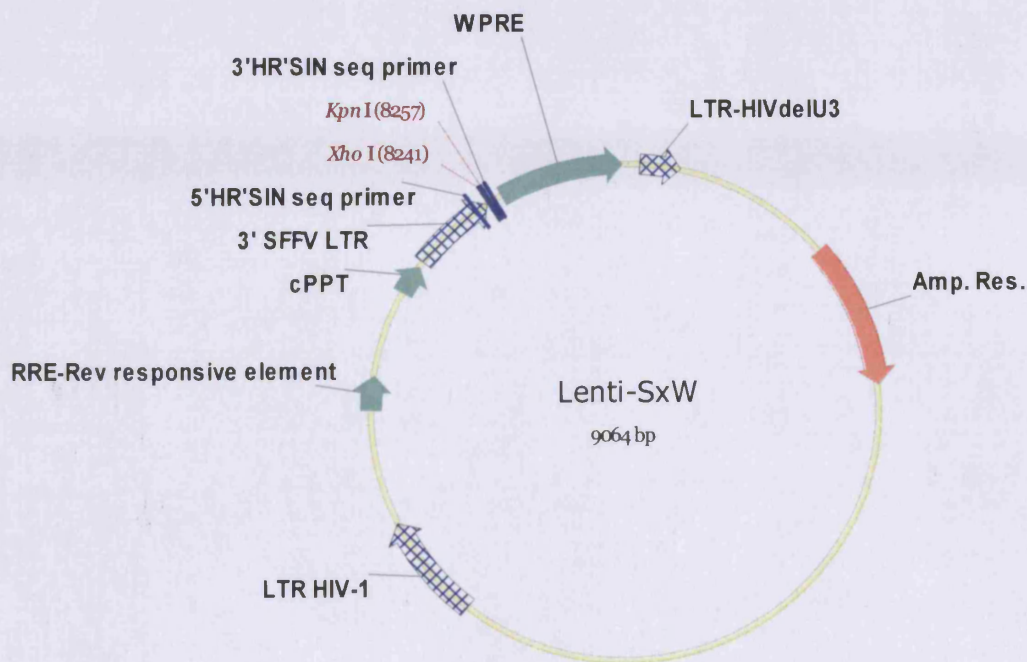


Figure 2.2 SxW plasmid, highlighting the Kpn1 and Xho1 within the multiple cloning region

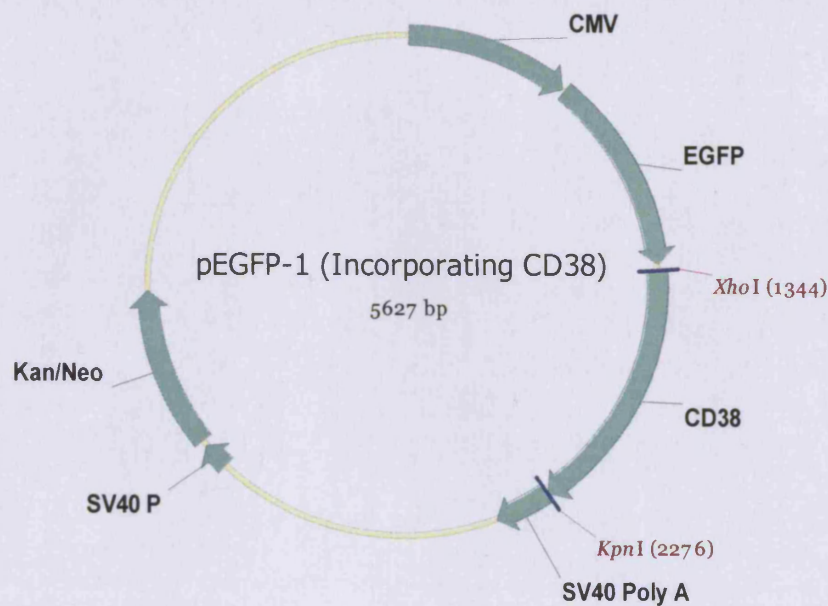


Figure 2.3 pEGFP-1 plasmid, highlighting CD38 and the Kpn1 and Xho1 restriction sites

2.3.3.1 Digestion of pEGFP-1 and SxW plasmids

Both the CD38-containing pEGFP-1 modified plasmid and the Lenti-SxW plasmid were digested using the Xho-1 and Kpn-1 enzymes. The reaction mixture consisted of 20µl of 10X buffer, 10µl of BSA, 10µl of Kpn-1 (10,000U/µl), 10µl of Xho-1 (10,000U/µl), 40µl of SXW plasmid (50ng/µl) and 20µl sterile water. The reaction was placed at 37°C on an orbital shaker for 2 hours. The 927bp CD38 fragment and the 4700kb linear pEGFP fragment were then run on an agarose gel to assess the efficiency of the digestion (Figure 2.4).

2.3.3.2 Agarose gel electrophoresis

A 1% agarose gel was prepared by dissolving 1g agarose in 100ml 1X TBE (microwave at full power for 2 min). 4µl of Ethidium Bromide was then added and the gel was left to set in the gel plate containing a 10 well comb. After cooling the entire digest was run on the gel using 1X running dye. 0.2µg 1kb ladder was loaded alongside the product and the gel was run for 1hour at 120Volts.

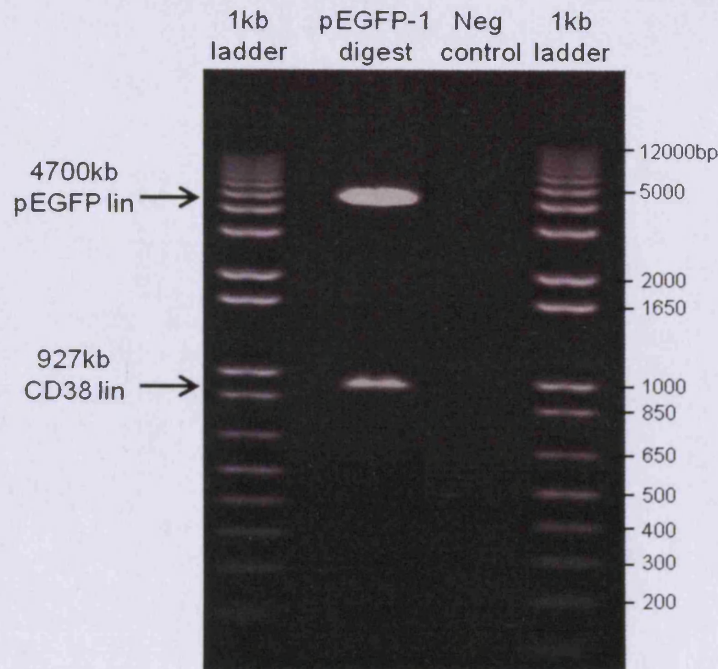


Figure 2.4 Agarose gel showing the Kpn1, Xho1 digested pEGFP plasmid

2.3.3.3 Extracting DNA from the gel using the QIAquick Gel Extraction kit (QIAGEN)

The DNA fragment was carefully, but rapidly excised from the gel whilst under the ultra violet light. The fragment was weighed and 3 volumes of buffer QG were added per 100mg of gel. The sample was incubated for 10 minutes at 50°C and 1ml of isopropanol was added to the dissolved gel solution. This solution was then pipetted into a QIAquick column and spun at 16,000 x g for 1 minute. The sample was then washed by adding 75ml of Buffer PE and the sample was again spun for 1 minute at 16,000 x g. Eventually the DNA was eluted by adding 50μl of elution buffer to the column, letting it sit for 5 minutes at room temperature and then centrifugation at 16,000 x g for 5 minutes.

2.3.3.4 Shrimp Alkaline Phosphatase (SAP) treatment of excised fragments

This method is used to dephosphorylate the sticky ends of the cleaved plasmid fragments. The restricted SxW plasmid was treated by mixing 12.5μl 10X buffer, 56μl of water, 50μl of SxW plasmid (20ng/μl) and 1.5μl of SAP enzyme. The reaction was placed at 37°C on the orbital shaker for 2 hours and then at 65°C for 15 minutes to inactivate the enzyme. Following the SAP

treatment the QIAGEN QIAquick purification protocol was utilised to purify the plasmid DNA.

2.3.3.5 Clean up of PCR products using the QIAGEN QIAquick PCR purification kit

Five volumes of Buffer PB were added to one volume of PCR product. The sample was then centrifuged at 16,000 x g for 1 minute to bind the DNA. The sample was then washed by adding 750μl of Buffer PE and the sample was again spun for 1 minute at 16,000 x g. 50μl of elution buffer was added and the sample was centrifuged at 16,000 x g for 1 minute.

2.3.3.6 Ligation

The CD38 fragment was ligated into the SAP treated SXW vector by mixing 2μl 10X buffer, 1μl T4 DNA ligase, 2μl Sterile water, 5μl SXW plasmid (20ng/μl) and 10μl CD38 insert (40ng/μl) (Figure 2.5). The reaction was left over night at 16°C and then transformed into the DH5α *E.coli* bacteria.

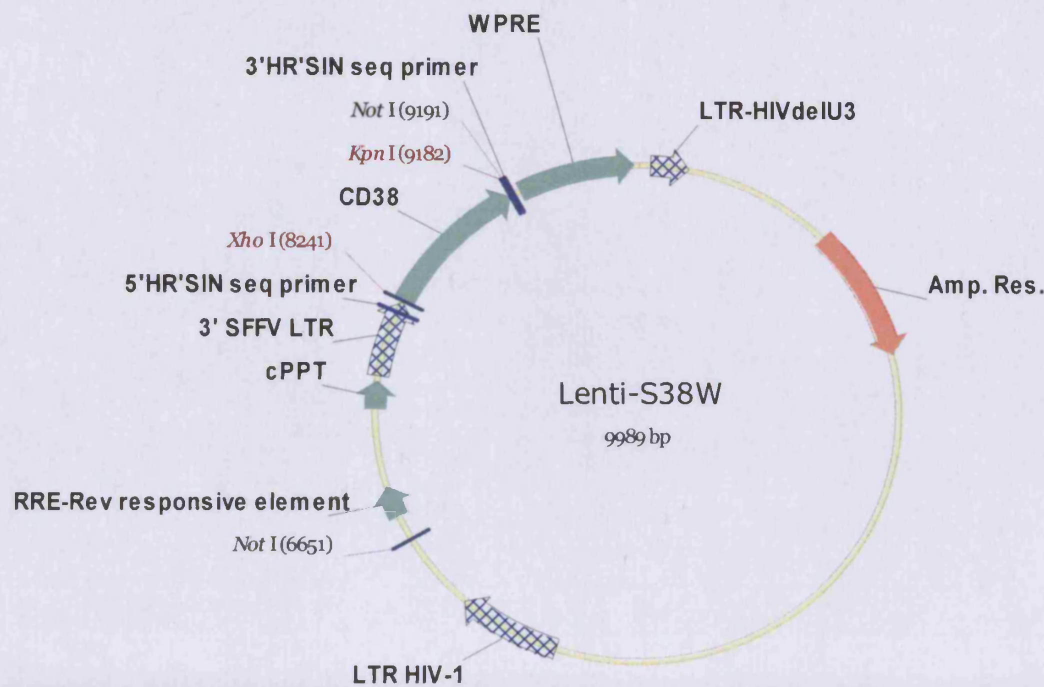


Figure 2.5 S38W plasmid, illustrating the incorporation of the human CD38 gene and Not 1, Kpn1 and Xho1 restriction sites

2.3.3.7 Digestion of the CD38 plasmid

The S38W plasmid was digested using Not1 enzyme to observe whether the CD38 fragment had incorporated into the SXW plasmid. 10ng of CD38 plasmid was added to 1μl of New England Buffer 3 (NEB) and 0.25 μl BSA in a 500μl eppendorf tube. 0.5μl Not1 enzyme (10,000U/μl) was added and the solution was incubated at 37°C for 1 hour. The plasmid incorporates two Not1 restriction sites and when digested effectively generates two products of 2540bp and 7449bp in length (Figure 2.6).

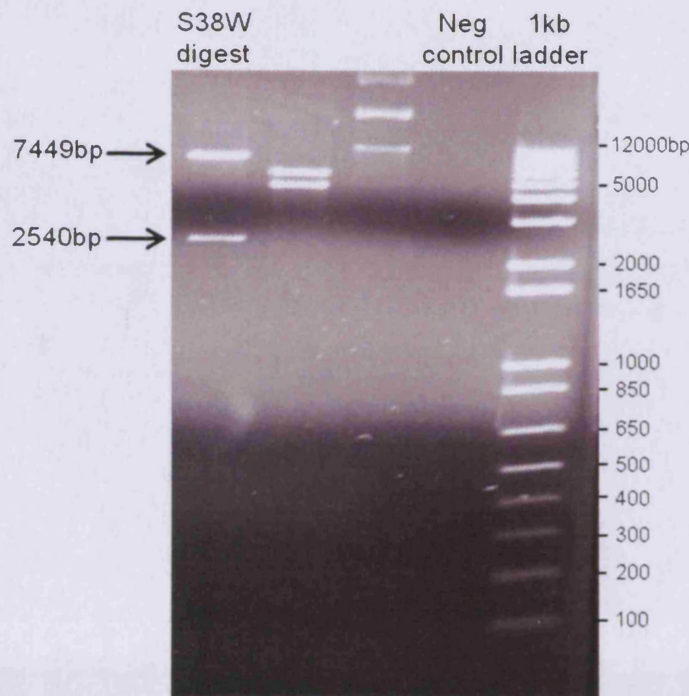


Figure 2.6 Agarose gel illustrating Not1 digested Lenti-CD38 Plasmid

2.3.4 Prokaryotic cell culture and plasmid amplification

2.3.4.1 Transformation of competent *E.coli* DH5α bacteria

In preparation, the LB media was warmed to 37°C in a water bath and the plasmids, and the DH5α cells, were thawed on ice. The DH5α cells were gently mixed and 50μl was placed into three separate 1.5ml eppendorf tubes. The tubes were labelled and 2μl of the respective plasmid DNA was added. Each cocktail was mixed gently and placed on ice for 30 minutes. The cells were subjected to heat shock by placing them into a 42°C water bath for 20 seconds. They were then placed on ice for a subsequent 2 minutes. 950μl of pre-warmed LB was added to the bacteria/plasmid transformation reactions and the tubes were incubated for 1 hour on the orbital mixer at 37°C, 225rpm.

2.3.4.2 Plating the bacteria

Two plates containing LB-agar with ampicillin (50μg/ml) were prepared for each sample. On the first, a sample of the bacteria was streaked out onto the media and on the second the remaining mixture was poured out onto the plate. This made it more probable that a single colony would be retrieved from at least one of the plates. The plates were then incubated over night at 37°C, 5% CO_2.

2.3.4.3 Picking single colonies and growing the transformed bacteria

From the plates containing the best spread of single bacterial colonies two single colonies were picked for each of the plasmid transformed bacterial preparations and transferred to a 15ml Falcon tube containing 5ml LB with ampicillin (50μg/ml). This was incubated for 6 hours on the orbital mixer at 37°C, 225rpm. Following incubation the sample with the greatest number of bacteria (deduced by turbidity) was selected and transferred into a conical flask with 250ml LB including ampicillin (50μg/ml). The flask was then incubated overnight on the orbital mixer at 37°C, 225rpm to allow the transfected bacteria to multiply. The bacterial plates were wrapped in clingfilm and stored at 4°C.

2.3.4.4 Storage of transformed bacteria in glycerol

Before carrying out the plasmid DNA extraction, an aliquot of each of the transformed bacteria was stored in sterile glycerol. 850μl of the transformed bacteria in LB containing ampicillin (50μg/ml) was transferred to a 1.5ml Eppendorf tube. 150μl of sterile glycerol was added and the sample was snap frozen on dry ice. The samples were stored at -80°C.

2.3.4.5 Isolation of plasmid DNA using the QIAGEN Plasmid Maxi kit

All reagents were included in the QIAGEN Plasmid Maxi Kit (QIAGEN). In preparation, the Sorvall large centrifuge and the swing out rotor centrifuge were set to 4°C and the buffers were made up according to the manufacturer's instructions. Conforming to the "overnight culture" protocol, 500ml of the transformed bacterial culture was centrifuged at 6000 x g for 15 minutes at 4°C to pellet the cells. The supernatant was then poured off and the bacterial cell pellet was homogeneously re-suspended in 10ml Buffer P1. 10ml of Buffer P2 was then added and the container was mixed thoroughly through inversion and

maintained at room temperature for 5 minutes. Following the addition of 10ml of Buffer 3 the sample was again mixed thoroughly and incubated on ice for 20 minutes. The sample was centrifuged at 756 x g for 30 minutes at 4°C to pellet the cell lysate. The supernatant retrieved at this stage was subsequently re-centrifuged at 756 x g for 15 minutes at 4°C to remove any residual bacterial cell debris. The QIAGEN columns were placed into 50ml collection tubes and the binding filter was equilibrated by applying 10ml of Buffer QBT. The solution was allowed to pass through the column under gravity flow. The plasmid DNA (contained in the lysate supernatant (around 30ml)) was then transferred to the column and allowed to pass through the filter under gravity flow. The plasmid DNA, now bound to the column, was subsequently subjected to two consecutive wash stages using 30ml of Buffer QC. Again the liquid was allowed to pass through the column under gravity flow. When the last of the QC Buffer had passed through the column 15ml of Elution Buffer was added and the plasmid DNA was collected into a fresh 50ml Falcon tube. The Plasmid DNA was precipitated by adding 10.7ml (0.7 vol) room temperature isopropanol. The sample was then centrifuged for 30 minutes at 3000 x g 4°C to pellet the DNA. The supernatant was carefully decanted leaving a plasmid DNA pellet at the bottom of the 50ml tube. Sterile 70% ethanol was then added to re-suspend and wash the pellet. The sample was again centrifuged for 15 minutes at 3000 x g at 4°C, and the ethanol was decanted. Inverting the sample onto a tissue allowed the remaining ethanol to evaporate and the DNA pellet was re-suspended in 0.5ml TE buffer, and stored at -20°C.

2.3.4.6 Quantification of plasmid DNA

10µl of each plasmid DNA prep was diluted 1/50 in sterile water and analysed on the spectrophotometer to quantify the yield of DNA. The absorbance at 260nm was measured and the amount of DNA calculated taking into account the dilution factor and the knowledge that a 50µg/ml plasmid DNA solution has an A_{260nm} of 1.

2.3.5 Generation of lentivirus

The plasmids generated were used alongside helper plasmids pΔ8.91 (*gag, pol*) and MD2G (envelope) (See Appendix 2) in a third generation lentivirus production procedure adapted from Dull *et al.* (1998).

2.3.5.1 Calcium phosphate transfection of 293T Cells

Calcium phosphate bound to DNA facilitates its passage through the eukaryotic cell membrane and into the cytoplasm. This method has been used effectively to allow the genetic modification of eukaryotic cells through transfection with plasmid vectors. 75µl of calcium chloride was added to a sterile 10ml tube. To this 10µg of the lentiviral plasmid, 7.5µg of the pΔ8.91 plasmid and 5µg of the pMD2G plasmid were added (final concentration: 13ng, 10ng and 6.6ng respectively). The volume was made up to 750µl using molecular grade water and the solution was mixed thoroughly. In a second sterile 10ml tube 750µl 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was bubbled using a sterile pastette and a media dispenser gun. The pDNA calcium chloride mix was added by dripping slowly through a 1ml pipette. The mixture was vortexed for 2-4 seconds and left at room temp for 20 minutes to allow pDNA/calcium phosphate precipitates to form. The precipitate was then distributed evenly over the adhered 293T cells prepared in fresh suspension media, and gently mixed. This preparation produced enough precipitate to transfect one medium flask of 70% confluent 293T cells which was incubated for 48 hours at 37°C, 5% CO_2.

2.3.5.2 Harvesting and concentrating the virus

9ml of the supernatant was carefully aspirated from each of the 4 medium flasks containing the 293T cells and transferred to a 50ml Falcon tube. The Falcon tube was centrifuged at 1700 x g for 10 minutes to pellet any cell debris and 11.5ml of the supernatant was transferred to three 12ml ultra centrifuge (UC) tubes (Beckman). These tubes were placed into the UC holders (stored at -4°C) that were then sealed and balanced to within 0.1g. Ultracentrifugation was carried out at 121,603 x g for 2 hours at 4°C in a vacuum to pellet the viral particles. After 2 hours the supernatant was carefully removed and the pellet re-suspended in 250µl RPMI (room temperature) containing no additives. 50µl aliquots of the concentrated virus were stored at -80°C in sterile 500µl tubes.

2.3.5.3 Quantification of viral particles using the Retrotek p24 Enzyme linked immunosorbent assay (ELISA)

The HIV-1 p24 antigen ELISA from Retro-tek (Zeptometrix corp.) was used to quantify the virus. 1:500, 1:1000, 1:2500, 1:5000 dilutions of the CD38 and GFP virus were prepared using DMEM. The control standards were also prepared as described by the protocol to give 125, 62.5, 31.3, 15.6, 7.8 and 0 pg/ml concentrations. Wash buffer was made up by adding 15ml of 10X wash buffer with 135ml of autoclaved water. The wells of the ELISA kit were pre-washed 5 times using 300µl of 1X wash buffer. 1.5µl of each sample dilution was added to 268.5µl of DMEM plus 30µl of lysis buffer. 200µl of this sample was then added to the antibody coated wells supplied with the kit alongside the standards. The plate was incubated overnight at 37°C. The next day the plate was washed 5 times with 1X wash buffer and 100µl of reconstituted HIV-1 p24 detector antibody bound to biotin was added to each well. The sample was left for 1 hour at 37°C. The plate was again washed 5 times and 100µl of streptavidin peroxidase working solution was added to each well. The wells were then sealed using the coating sticky plastic sheets supplied with the kit and left for 30 minutes at 37°C. Following 5 washes with the 1X wash buffer, 100µl of substrate working solution (containing tetramethylbenzidine and DMSO) was added to each well and the uncovered plate was incubated at 37°C. At this stage the blue colour developed and was stopped after 30 minutes by adding 100µl stop solution (proprietary formulation) to each well. The plate was immediately placed into the plate reader and the fluorescence measured at 450nm.

2.3.6 Transduction and transfection of lymphocytes

2.3.6.1 Infection of Jurkat cells with viral supernatant

A Neubauer chamber was used to determine the number of Jurkat cells in culture and $1x10^6$ cells were transferred to each dish of a 6-well plate. Viral supernatant was added in the following amounts: 0, 10µl, 50µl, 100µl, 250µl, and 500µl. The plates were stirred gently and incubated for 48 hours at 37°C, 5%CO_2 before carrying out flow cytometry to identify cells expressing the gene of interest (GFP was primarily observed using a laser microscope). (Details of cell lines and their origin can be seen in appendix 2.1.3).

2.3.6.2 Infection of primary CLL cells with concentrated virus

The cell samples were centrifuged at 272 x g for 5 minutes and the pellet was re-suspended in 3ml RPMI plus supplements. $1x10^6$ CLL cells were used in each well and concentrated virus was added. The plates were stirred gently and incubated for 48-72 hours at 37°C, 5% CO^2 before carrying out flow cytometry to identify the percentage of CLL cells expressing GFP.

2.3.6.3 Nucleofection

Nucleofection of primary CLL cells was carried out using the AMAXA B-cell Nucleofection kit. In preparation, 500µl of supplement solution was added to 2.25ml of nucleofection solution to make up the nucleofection buffer. $5x10^6$ CLL cells were re-suspended in 100µl of nucleofection buffer and the plasmid DNA was added to the sample (1-5µg of GFP-CD38 or AMAXA GFP control plasmid). This sample was then aliquoted into the nucleofection chamber and the U-15 programme was initiated. The sample was immediately collected and placed into a 6-well plate containing 3ml of DMEM plus supplements. A sample that did not undergo nucleofection was included as a control in these experiments. The plate was incubated at 37°C and 5% CO_2 for 24 hours to allow for gene expression.

2.3.6.4 Cell sorting on the MoFlo

$5x10^6$ nucleofected CLL cells were washed in PBS and re-suspended in 3ml PBS. The sample was then sorted on the MoFlo cell sorter (Dako Cytomation) and GFP positive and negative fractions were collected. The fractionated populations were immediately placed back into culture in 3ml DMEM plus additives at 37°C 5% CO_2.

2.3.6.5 Electroporation of *in vitro* transcribed RNA

2.3.6.5.1 Generation of in vitro transcribed mRNA

The GFP-expressing pGEM4Z-EGFP-A64bis plasmid was a kind donation of F. Van Bockstaele and B. Verhasselt. Following transformation of the plasmid into DH5α *E.coli* bacteria and expansion of the cells, the plasmid DNA was harvested. The plasmid was then linearized using 3µl of the SpeI restriction

enzyme in a reaction containing 10μl NEB buffer 2, 1μl BSA and 77μl of nuclease-free water. The digest was set up for 12 hours at 37°C on the orbital shaker at 225rpm. Following linearization the digest was run on a gel to check its purity. Due to a small amount of circular plasmid remaining, the entire sample was separated on an agarose gel and the linear fragment was isolated using the QIAGEN QIAQuick gel clean up protocol. The sample was then quantified using 1μl on the Nanodrop spectrophotometer.

For *in vitro* transcription, the T7 mMESSAGE mMachine kit was used. 1μg of linear plasmid DNA containing the genetic code for GFP was added to a master mix containing 10μl 2X NTP/CAP reagent, 2μl of 10X buffer and 2μl of RNA polymerase enzyme. The mix was incubated for 2 hours and 30 minutes at 37°C on the orbital shaker at 225 rpm. The sample was then heated to 70°C for 5 minutes to inactivate the enzyme. 1μl of Turbo DNase was added and the sample was left at 37°C for 15 minutes to degrade any contaminating DNA. 30μl of nuclease-free water and 30μl of lithium chloride were added and the sample was stored over night at -20°C. In order to precipitate the RNA, the sample was pelleted at 16,000 x g for 30 minutes at 4°C and the supernatant was removed. 1ml of 70% ethanol was then added and the sample was again spun at 16,000 x g for 10 minutes to pellet the sample. The pellet was subsequently air dried, re-suspended in nuclease-free water, quantified and stored at -80°C.

2.3.6.5.2 Electroporation

$1x10^7$ CLL cells were washed twice in DMEM containing no additives and then in 10ml Optimix wash buffer. The sample was then re-suspended in 1ml of Optimix Electroporation buffer and the IVT mRNA was added (0, 20 and 40μg). The cell solution was added to the electroporation chamber and electroporated at 500 Volts and 150 Farads for 2 seconds. The sample was immediately removed using a pipette and placed into a 6-well plate containing 3ml DMEM plus supplements for 24 hours at 37°C, 5% CO_2. Following this period, expression of GFP was assessed by flow cytometry.

2.3.7 Primary CLL cell molecular biology and gene expression assays

2.3.7.1 RNA isolation

Up to 1x10^7 CLL cells were re-suspended in 1ml of Trizol, mixed well and stored at -80C. Samples were thawed at room temperature and 200μl of chloroform was added. Following vortexing, the sample was left at room temperature for 5 minutes and then centrifuged at 16,000 x g for 15 minutes at 4°C. The aqueous layer on the surface containing the RNA was then carefully removed and placed into a fresh tube. 500μl of isopropanol was added and the sample was mixed well by vortexing. Following 10 minutes at room temperature, centrifugation of the sample was carried out at 16,000 x g for 10 minutes at 4°C. The supernatant was then poured off and the pellet was washed in 500μl of 70% ethanol. Centrifugation at 16,000 x g for 10 minutes at 4°C allowed the sample to pellet on the bottom of the tube and the supernatant was carefully removed and the RNA left for approximately 10 minutes to air dry. 20-60μl of RNase-free water was used to re-suspend the pellet depending on its size and the sample was quantified using the Nanodrop spectrophotometer.

2.3.7.2 NanoDrop quantification of RNA

The NanoDrop stage was wiped clean and 1μl of RNase-free water was added to zero the reading. 1μl of each sample was added in turn, and RNA was quantified by the NanoDrop machine. The purity of the RNA was assessed by calculating the ratio of the absorbance at 230nm and 260nm.

2.3.7.3 Reverse Transcription

1μg of RNA was used in each RT reaction in a volume of 3μl of RNase-free water. This was added to 4μl $MgCl_2$, 2μl of 10X RT buffer, 2μl of each dNTP (CGA and T, 10μM each), 1μl of RNase inhibitor, 1μl of 50μM random hexamers and 1μl 50U/μl reverse transcriptase enzyme. The sample was placed into the thermal cycler and incubated at 25°C for 10 minutes, 42°C for 30 minutes and finally 95°C for 5 minutes to inactivate the enzyme. The temperature was then maintained at 4°C until the cDNA samples were removed and stored at -20°C.

2.3.7.4 QPCR on the Roche Light Cycler

In preparation the Sybr green reagent was thawed and 10µl of DNA polymerase enzyme from vial 1a was added. 5.4µl of pure water, 1.6µl $MgCl_2$, 1µl of Sybr green working reagent and 0.5µl of the respective forward and reverse primer (10µM) were made up per sample. This mixture was carefully pipetted into a lightcycler capillary and 2µl of cDNA was added. The samples were briefly centrifuged at 379 x g and placed into the Light cycler instrument. Following a 'pre-heat' at 95°C for 1 minute the cycling conditions were 95°C for 3 seconds, 60°C for 5 seconds and 72°C for 12 seconds for 45 cycles. In a final step, the sample was denatured by heating to 95°C over a period of 10 minutes. The single peak observed in Figure 2.7 illustrated that the PCR product was of a single length and that no non-specific amplification had occurred.

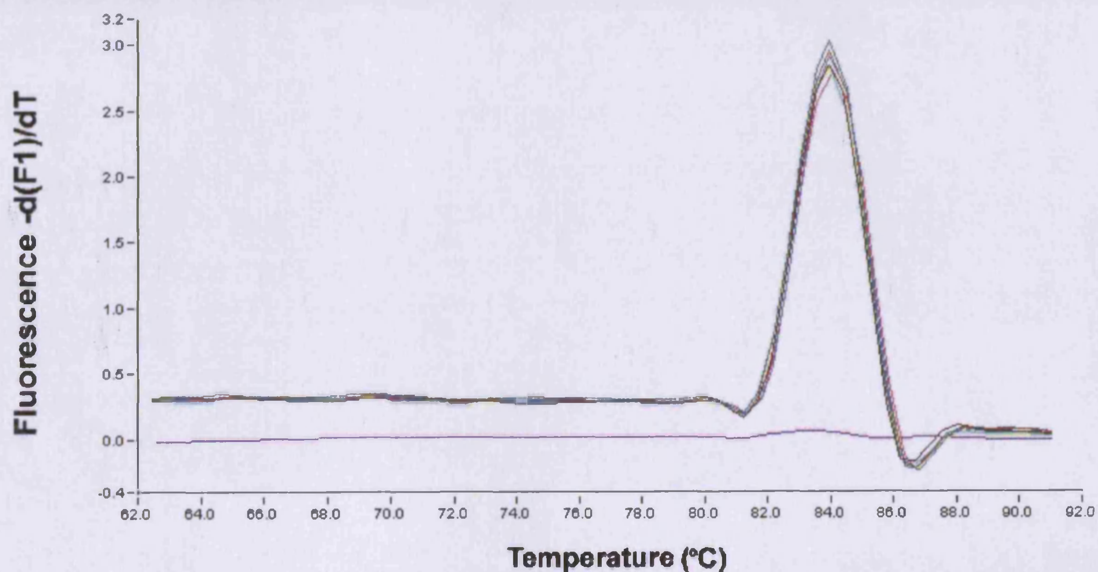


Figure 2.7 Melting curve of the 259 base pair MCL-1 PCR product

2.3.7.5 Affymetrix Microarray

The standard Affymetrix protocol was carried out for the labelling and detection of RNA from untreated CD38 virus treated and GFP treated RNA samples. The central biotechnology service (CBS) at Cardiff University was

utilised for this technique and the Affymetrix protocol was kindly carried out by Mrs Megan Musson.

Step 1. Total RNA clean up after extraction

As RNA quality is possibly the most crucial factor for the success of the experiment, the RNA samples were purified using the QIAGEN RNeasy columns before proceeding to sample labelling (see above).

Step 2. Spectrophotometric QC

A quality assessment was necessary to verify that the samples were of sufficient molecular weight and purity to proceed with full labelling and hybridization. The samples were therefore run on the Agilent Bioanalyzer to ensure that the RNA was of sufficient quality. All samples met the following requirements for acceptance:

· Concentration > 0.5mg/ml
· A260/A280 >1.7
· Minimum yield per sample of 3µg

Step 3. Bioanalyzer QC

Each RNA sample was run on the Bioanalyser 2100 QC device to observe any RNA degradation.

Step 4. Sample Labeling

(i) First strand synthesis

5µg of total RNA was mixed with 0.5µlT7-(T)24 primer (100µM) and 5.5µl of DEPC-treated water. The reagents were mixed and incubated at 70°C for 10 minutes. The sample was then chilled on ice and pelleted. In a separate tube the 2µl of 5X first strand buffer was mixed with 1µl of 0.1M DTT and 0.5µl dNTP blend (10mM). 3.5µl of 1st strand premix was added to each annealed primer-RNA mix and incubated at 37°C for 2 minutes. 0.5µl of SuperScript II RT was then added to each target and the samples were incubated at 42°C for 1 hour. They were then placed into a water bath at 16°C ready for 2nd strand synthesis.

(ii) Second strand synthesis

The samples were placed on ice to cool for 5 minutes. Meanwhile 45.5µl of DEPC-treated water was mixed with 15µl 5X second strand buffer, 1.5µl dNTP blend (10µM) 0.5µl *E.coli* DNA ligase (10U/µl), 2µl *E.coli* DNA Pol 1 (10U/µl) and 0.5µl RNaseH (2U/µl). The 2nd strand master mix was added to each 1st strand reaction, mixed well, spun and incubated at 16°C for 2 hours. Following incubation 1µl of T4 DNA Polymerase [10U/µl] was added and the sample was lightly vortexed, spun and incubated at 16°C for 5 minutes. 5µl of 0.5M EDTA was then added to each sample.

(iii) Clean-up of double stranded cDNA

80 µl of Phenol: chloroform: isoamyl alcohol [48:1:1] (saturated with 10mM Tris pH8) was added to each ds cDNA sample and vortexed. A 1.5 ml Phase Lock Gel Light (PLG) tube was prepared for each sample by pelleting the gel in a microcentrifuge at 16,000 x g for 20-30 seconds. The entire cDNA-PCI mixture was transferred to the PLG tube and centrifuged for 2 minutes at top speed. The aqueous phase was then transferred to a fresh tube and precipitated by adding 2 µl Glycogen, 80 µl NH_4OAc 5M and 400µl ethanol 100%. The sample was then stored at -80°C for 60 minutes to increase the cDNA recovery. Following thawing each sample was centrifuged at 4°C for 30 minutes and washed with 70% ethanol. The samples were then centrifuged at 4°C for 10 minutes and the pellet was air dried for 10 minutes. The pellet was re-suspended in 1.5µl DEPC-treated water.

(iv) *In vitro* transcription reaction (using biotin-NTPs)

The 1X NTP labelling cocktail was prepared by mixing 2µl 10X ATP (75mM), 2µl 10X GTP (75mM), 1.5µl 10X CTP (75mM), 1.5µl 10X UTP (75mM), 3.75µl Bio-11-CTP (10 mM), and 3.75µl Bio-11-UTP (10 mM). 14.5µl of this mix was added to the 1.5µl cDNA along with 2µl 10X T7 transcription buffer and 2µl 10X T7 enzyme mix. The sample was run in a thermocycler for 6 hours at 37°C and then left on hold at 4°C.

(v) Clean-up of labelled cRNA

The sample volume was adjusted to 100µl with RNase-free water. 350µl Buffer RTL was added and mixed thoroughly. This was followed by the addition of 250µl absolute ethanol and the sample was mixed well by pipetting. The entire sample (700µl) was applied to an RNeasy mini spin column sitting in a collection tube and centrifuged for 15 seconds at 16,000 x g. The RNeasy column was transferred into a new 2ml collection tube and 500µl Buffer RPE was added. The sample was then centrifuged for 15 seconds at 16,000 x g and the flow-through was discarded. 500µl Buffer RPE was pipetted onto an RNeasy column and centrifuged for 2 minutes at 16,000 x g to dry the membrane. The flow-through was discarded and spun at 16,000 x g for 2 minutes to dry the pellet. The RNeasy column was transferred into a new 1.5ml collection tube and 50µl RNase-free water was placed directly on the membrane. The sample was left for 1 minute and then centrifuged for 1 minute at 16,000 x g to elute. This step was repeated. The cRNA was quantitated using the Nanodrop spectrophotometer.

Fragmentation of cRNA for target preparation

(i) Fragmentation reaction

15µg of fragmented cRNA was used in 300µl of hybridization mix. The sample was incubated for 35 minutes at 94°C and then stored at -20°C until ready to perform hybridization.

(ii) Preparing the hybridization target

Before preparing the pre-mix, the 20X GeneChip Eukaryotic Hybridization Control cocktail was heated for 5 minutes at 65°C. The premix was made up by mixing 4.15µl control B2 oligonucleotide, 12.5µl 20X Eukaryotic Hybridization controls, 2.5µl Herring Sperm DNA (10mg/ml), 2.5µl acetylated BSA (50mg/ml), 125µl 2x Hybridization buffer and 12.5g fragmented cRNA in water.

(iii) Targeting hybridization to GeneChip array

The hybridization cocktail was heated to 95°C for 5 minutes and spun in the microfuge at 16,000 for 5 minutes to remove any insoluble material. The GeneChip probe array was then prepared for sample loading. The probe arrays

were equilibrated to room temperature before use. The array was filled with 1X Hybridization buffer (200μl) and incubated at 45°C for 10 minutes with the rotation at 60rpm. The buffer was then removed and replaced with the appropriate volume of clarified hybridization cocktail. The probe array was replaced into the rotisserie oven and hybridized for 16 hours at 45°C, with a rotation of 60 rpm.

In the microarray experiments three untreated and virus treated samples were run on Affymetrix U133A GeneChips that contain 23,500 sequences derived from the Genebank database. All of the data obtained from the gene array study were analysed using Genespring 7.0 analysis software.

2.3.7.6 Sequencing

PCR products were extracted from the gel and cleaned using the QIAGEN clean-up protocol described. The samples were quantified on the Nanodrop spectrophotometer and the ABI Big Dye 3.1 kit was used to amplify the fragments.

2.3.7.6.1 Sequencing PCR

The reaction mixture consisted of 4μl Terminator Ready Reaction mix, 1μg template, 1μl primer (3.2μM) and 4μl sterile water. The thermocycler was programmed to carry out 25 cycles of [96°C for 10 seconds, 50°C for 10 seconds, 60°C for 4 minutes] and then hold at 4°C.

2.3.7.6.2 Purifying sequencing products by isopropanol precipitation

The sequencing reaction was transferred into a 1.5 ml microcentrifuge tube. 40 ml of 75% isopropanol was added and the sample was mixed. The sample was then placed at -20°C for 30 minutes to precipitate the products. Following thawing the sample was spun for 20 minutes at 16,000 x g in a microcentrifuge. The supernatant was aspirated leaving the pellet at the bottom of the tube. 250 ml of 75% isopropanol was added to the tubes and the pellet was re-suspended. The sample was then centrifuged at 16,000 x g for 5 minutes at maximum speed and the supernatant removed. The sample was air dried on the bench for 10 minutes then stored at -20°C until ready for electrophoresis.

2.3.7.6.3 Preparation of sequencing products for electrophoresis

Electrophoresis was carried out by the CBS service at Cardiff University. Each sample was re-dissolved in 3ml loading buffer (deionized formamide/25 mM EDTA (pH 8.0) with blue dextran (50 mg/ml) at a 5:1 ratio) immediately before use. The sample was then heated to 95°C for 2 minutes and immediately placed on ice. 2µl of each sample was loaded into the 96-well plate for sequencing. Sequencing was performed on the ABI 3700 16 capillary genetic analyser.

2.3.8 Cell biology and biochemical assays

2.3.8.1 Flow cytometry

Flow cytometry was employed to assess viability, proliferation and the expression of surface and intracellular molecules.

2.3.8.2 Preparation of Jurkat cells for flow cytometry

1ml of the Jurkat cells were removed from each well and transferred to a FACS tube. The tubes were centrifuged at 272 x g for 5 minutes to pellet the cells. After pouring off the supernatant 500µl of 1% paraformaldehyde was added to each tube and the cell pellet was re-suspended by mixing. Incubation for 15 minutes at 4°C was sufficient to fix the cells and they were washed twice in 3ml before re-suspending in 500µl PBS.

2.3.8.3 Preparation of primary CLL cells for flow cytometry

250 or 500µl of the cell suspension was pelleted by centrifugation at 272 x g for 5 minutes. The pellet was re-suspended in 100µl PBS and the relevant antibody was added (4µl/$1x10^6$ Cells) (see table 2.1.5 in Appendix 1 for list of antibodies). All CLL samples were stained with anti-CD19 to identify the CLL B-cell population. Following incubation at room temperature for 15 minutes in the dark, the cells were washed once in 1ml PBS, pelleted and fixed in 250µl 1% Paraformaldehyde.

2.3.8.4 Fix and perm of CLL cells for intracellular staining

To quantify intracellular proteins by flow cytometry the cells were fixed and permeabilised to allow entry of fluorescent antibodies into the cell. Intracellular staining for Zap-70 and Ki-67 was carried out using this method. The cells were washed and stained for CLL B-cell specific extracellular antigens CD5 and CD19. The sample was washed in 1ml PBS and 50μl of Fixing reagent A was added (Caltag). Following 10 minutes in the dark the cells were again washed and 50μl of permeabilisation reagent B containing 0.1% Nonidet-P40 was added together with the desired antibody. Following 15 minutes incubation in the dark, the sample was washed in 1ml PBS and the cells re-suspended in 500μl of 1% paraformaldehyde. The sample was then stored at 4°C before being run on the flow cytometer.

2.3.8.5 Annexin V/PI staining for apoptosis

As the cell enters the process of apoptosis ATP in the cell is depleted and phosphatidylserine is expressed on the external leaflet of the cell membrane. Annexin V is a natural ligand that binds phosphatidylserine and can therefore be used to detect this early marker of programmed cell death. Cells were washed twice in PBS and re-suspended in 200μl of 1X calcium containing binding buffer. 5μl of annexin V- fluorescein isothiocyanate (FITC) reagent was added and the sample was incubated for 10 minutes in the dark. 10μl of propidium iodide (20μg/ml) was added and the cells were run on the flow cytometer. Annexin V-FITC staining was detected in FL1 and PI in FL3.

2.3.8.6 Assessment of proliferation through incorporation of Bromodeoxy-uridine (BrdU)

Cell division is pre-empted by the replication of DNA. BrdU is able to replace thymidine during this process and antibodies to BrdU therefore allow the detection of dividing cells. Proliferation was assessed in CLL cells incubated in CD31-expressing and untransduced fibroblast co-cultures. $1x10^6$ CLL cells were incubated for 32 hours in co-culture. BrdU was added to give a final concentration of 10μM. The cells were incubated for a further 16 hours to allow BrdU integration. The CLL cells were then removed from the co-culture layer with a pipette and washed in 1ml staining buffer (Bender Medsystems). After

pelleting, the cells were fixed using 100μl cytofix/cytoperm reagent and left for 20 minutes at 4°C. The cells were then washed using 100μl cytoperm/wash buffer and pelleted. 100μl cytoperm buffer was added to permeabilise the cells and the sample was placed on ice for 10 minutes. The cells were washed and 100μl of DNase (300μg/ml) was added. The sample was then incubated at 37°C for 1 hour. After this time, the cells were resuspended in 50μl of anti BrdU-FITC antibody and incubated for 20 minutes at room temperature. The sample was then washed in 1ml of cytoperm/cytowash buffer pelleted and resuspended in 200μl of 1X PI solution (final concentration 1μg/μl). Flow cytometry was used to detect BrdU-FITC in FL1 and PI in FL3.

2.3.8.7 Measurement of VEGF in the CLL supernatant by ELISA

Levels of the VEGF protein released by the CLL cells into the supernatant were quantified using the VEGF-A ELISA kit (Bender Medsystems). A positive control sample was included in the kit and dilutions were prepared which ranged from 31 to 2000 picomoles. The required number of wells was prepared by washing twice with 300μl of well wash buffer. 100μl of the control and the samples were added to the pre-coated wells and incubated in the dark at room temperature for 2 hours. The wells were aspirated and washed 5 times with 300μl of well wash buffer. 100μl of the detection antibody was added to each well and the plate was incubated in the dark at room temperature for 1 hour. After this period the wells were again washed 5 times using the well wash buffer. 100μl of Avidin-HRP solution was added and the plate was left in the dark at room temperature for 20 minutes. Following washing 5 times 100μl of Avidin-HRP was again added to each well and the sample incubated in the dark at room temperature for 3 minutes. Again the wells were washed 5 times and 100μl of TMB substrate was added to each well. The plate was then incubated in the dark at room temperature for 15 minutes to allow the reaction to occur. 50μl of stop solution was added and the plate was read on the plate reader at 450nm.

2.3.8.8 Cytospin slide preparation and staining CLL cells with Giemsa for morphological analysis

Isolated CLL lymphocytes were washed in PBS and re-suspended at a concentration of $2x10^6$/ml. 50μl was placed into the cartridge of the assembled

cytospin slide apparatus containing an absorbent filter card with a window for the cells to come into contact with the slide. The slides were spun at 188 x g for 6 minutes and then removed from the holders. The cells were fixed in 70% ethanol for 5 minutes and 5% Giemsa solution was added (enough to cover the surface of the cells (100-150μl)). The samples were left at room temperature for 30 minutes and the excess dye was washed off using PBS. The samples were placed under a Zeiss Axio microscope for analysis and digital images were recorded at x40 and x100.

2.3.9 Statistical analysis

The paired *t*-test was used to determine differences in protein expression from paired samples under varying conditions. The Mann-Whitney test was employed to analyse non-parametric un-paired data when comparing different patient samples. When correlating gene or protein expression in a cohort of patients the Spearman test was used. GraphPad Prism 4.0 was used to carry out the statistical analysis. All of the data obtained from the gene array study were analysed using Genespring 7.0 analysis software (Agilent Technologies, UK Ltd).

2.3.10 Patient samples and ethical approval

Primary CLL cells were collected from CLL patients from the University Hospital of Wales Cardiff and Birmingham Heartlands hospital. The patients' informed consent was obtained in accordance with the ethical approval granted by the South East Wales Research Ethics Committee in accordance with the Declaration of Helsinki. Patients presented with Binet stage A-C disease and were selected for use according to surface CD38 expression.

Chapter 3. Genetic modification of primary CLL Cells

3.1 Introduction

The objective in this chapter was to develop a method of gene delivery to increase the expression of the CD38 glycoprotein on the surface of CD38 negative primary CLL cells. This would allow the characterisation of the effects of CD38 on a homogeneous genetic background. It has been postulated that in certain cases, CLL cells are able to rapidly divide in so called proliferation centres in the lymph nodes of the host (Schmid and Isaacson, 1994, Lampert *et al.*, 1999). Even though this may be the case, the majority of the tumour cells in the peripheral blood remain in a quiescent state in G0/G1 of the cell cycle and appear resistant to apoptosis. *In vitro* these cells are notoriously difficult to genetically modify with the use of retroviral vectors, which have become an established means of gene transduction in dividing cells (Muhlebach *et al.*, 2005, Wolfrum *et al.*, 2007). In this chapter, three different methods of gene transfection were evaluated in an attempt to increase the expression of CD38 on the surface of primary CLL cells; nucleofection, electroporation of *in vitro* transcribed messenger RNA and a lentiviral vector system.

3.2 Nucleofection of plasmid vectors

The nucleofection method involves pulsing an electric current through the CLL cell sample in the presence of a plasmid vector. Pores are created in the cell membrane enabling the plasmid to enter the cell and express the gene of interest. The nucleofection procedure can cause substantial cell death of CLL cells (Van Bockstaele *et al.*, 2008) and the introduction of circular plasmid DNA (rather than linear DNA) into the cell has been shown to induce cell death in various cell types including B-cells, dendritic cells and macrophage (Seiffert *et al.*, 2007, Shimokawa *et al.*, 2000). This phenomenon is thought to occur due to the induction of an intrinsic defence mechanism against invading bacterial pathogens which causes apoptosis (Weinrauch and Zychlinsky, 1999). Furthermore lipopolysaccharide (LPS), carried over from plasmid

preparations, is a potent mediator of cell death (Boyle *et al.*, 1998, Gordillo *et al.*, 1999). Due to the anticipated cell death associated with nucleofection, large numbers of CLL cells ($2.5x10^7$) were used in each experiment and following nucleofection, a CD40 ligand bearing 3T3 cell co-culture was employed to prolong the survival of the CLL cells *in vitro*. The pEGFP-1 plasmid used in these experiments (Figure 2.3) expressed a CD38-GFP fusion gene. Following nucleofection (of a CD38 negative sample) the successfully transfected cells expressed the CD38-GFP fusion protein that was readily detected using flow cytometry. GFP was detected in 34.5% of the CLL sample nucleofected using the CD38-GFP plasmid (Figure 3.1.a). A control GFP expressing plasmid (Amaxa) was run in parallel and following nucleofection, 27% of the cells expressed the fluorescent product (Figure 3.1.b). Control samples were run which excluded the nucleofection process (Figure 3.1.c) and omitted the plasmid from the nucleofected CLL sample (Figure 3.1.d). From these latter samples the threshold was set for GFP detection.

The post nucleofection sample consisted of a mixed population of genetically modified and non-genetically modified CLL cells. To analyse the role of CD38 a pure CD38 positive population was required and therefore the cells were sorted using a MoFlo high-speed cell sorter. The cells were sorted into GFP positive and GFP negative fractions and rapidly returned to co-culture to minimise cellular stress and subsequent cell death. Figure 3.2.a illustrates the gating strategy used to separate the cells on the cell sorter. A total of $2x10^6$ GFP negative cells and $4x10^5$ GFP positive cells were isolated following nucleofection of an initial $2.5x10^7$ CD38 negative CLL cells.

To assess whether the CD38 fusion protein was being expressed on the cell surface, a sample of both the GFP positive and GFP negative fractions was stained with anti-CD38 (R-phycoerythrin) and run on the flow cytometer. Figure 3.2.b illustrates that the GFP positive fraction also expressed the CD38 fragment on the surface. As expected, the GFP negative CLL cells did not express the CD38 antigen. To enable the differential analysis of gene expression between the genetically modified and unmodified cells both the untransfected and GFP-expressing samples were pelleted and re-suspended in

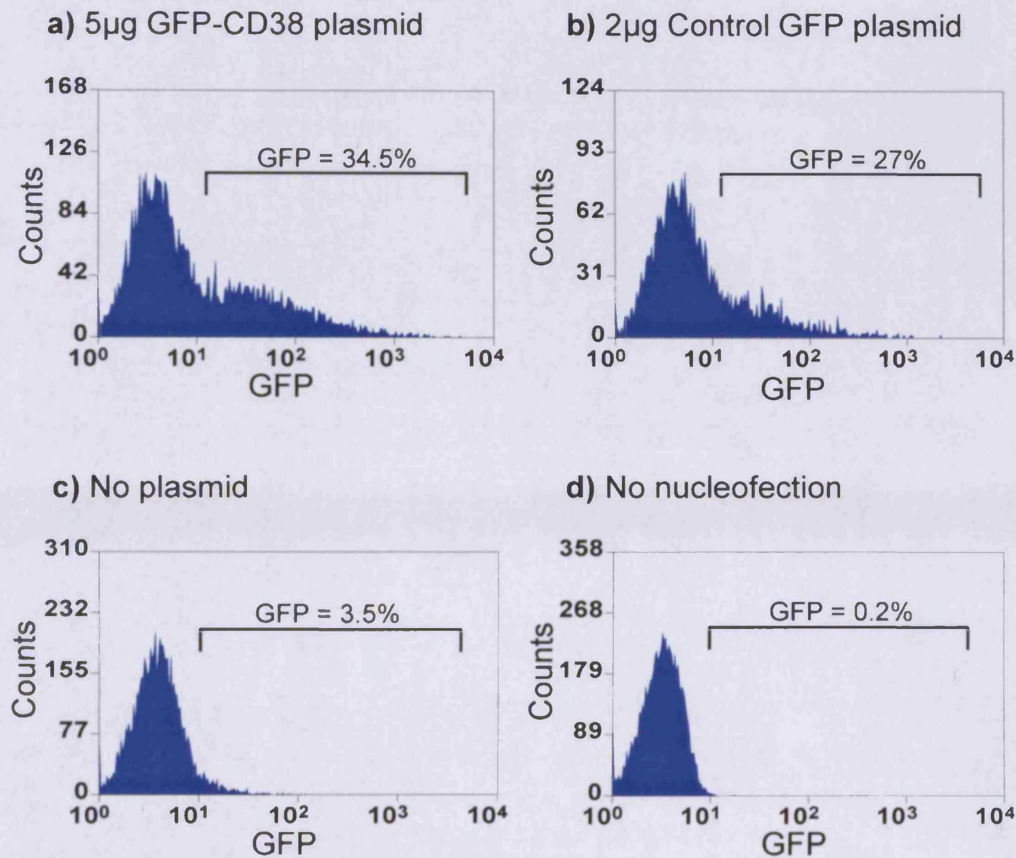


Fig 3.1 GFP was expressed in CLL cells following nucleofection of GFP-CD38 and control GFP plasmids.

5x10^{6} CLL cells were placed into the nucleofection chamber. The first sample was nucleofected in the absence of plasmid **(a)** the second control sample was mixed with 5μg of the GFP-CD38 plasmid in the chamber, without the nucleofection process taking place **(b)** the GFP expression within the CLL cells was measured by flow cytometry following nucleofection in the presence of 5μg of **(c)** GFP-CD38 plasmid and **(d)** 2μg of the GFP plasmid in the control sample .

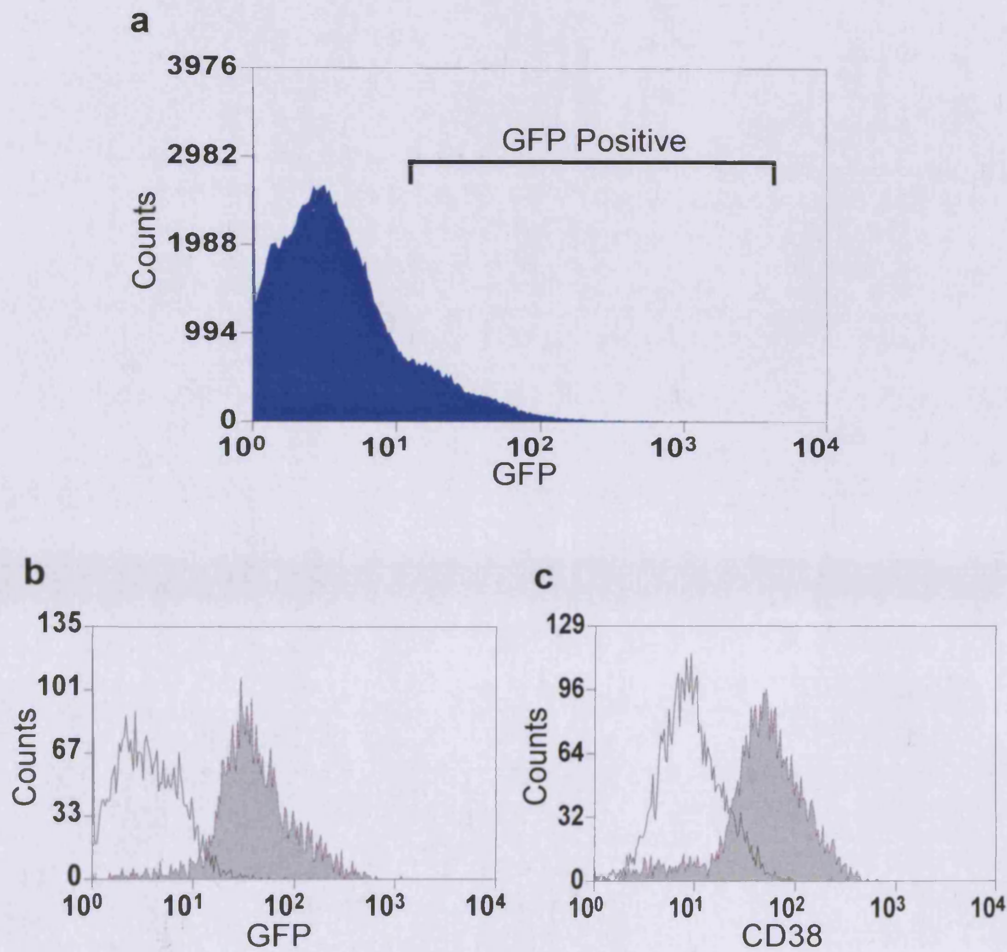


Figure 3.2 Isolation of GFP positive cells using the Moflo cell sorter.

The gating strategy employed on the cell sorter separated the GFP positive from the GFP negative cells **(a)** following cell sorting the samples were placed into cell culture media at 37°C for 24 hours after which a sample of each fraction was run on the flow cytometer to observe GFP and CD38 expression. The overlays illustrate **(b)** GFP and **(c)** CD38 expression in the isolated CLL cell populations.

Trizol reagent (containing guanidinium thiocyanate) for subsequent RNA isolation.

3.3 Electroporation of *in vitro* transcribed messenger RNA (IVTmRNA)

An alternative means of transfecting cells, similar to the nucleofection technique, is to generate a pulse of electricity in the presence of mRNA encoding the gene of interest. The pulse renders the cells porous for a suitable amount of time for the small mRNA molecules to enter the cell. Unlike the nucleofection procedure, the absence of the circular bacterial plasmid in this method should eliminate cell death due to DNA cytotoxicity and contaminating bacterial LPS. This technique has recently been successful in the transfection of CLL cells with a GFP-ZAP-70 fusion gene, with very little cell death reported during the electroporation procedure (Van Bockstaele *et al.*, 2008). Drawbacks of the technique include the lengthy preparation of IVTmRNA, which takes three days to obtain a relatively small amount of template (around 60μg). Furthermore, this technique requires electroporation which is known to cause increased cell death (Seiffert *et al.*, 2007).

Following transcription of mRNA encoding GFP in the laboratory, the CLL cells were electroporated and flow cytometry was utilised to detect the GFP protein. Increasing amounts of IVTmRNA, ranging from 0 to 40μg, were placed into solution with CLL cells and electroporated at 500 Volts, 150μ Farads (As optimised in the procedure by Van Bockstaele *et al.,* 2008). Figure 3.3.c and 3.3.d illustrate that following electroporation, there was no increase in the expression of the GFP protein in the CLL cells regardless of the amount of mRNA added. The figure is representative of experiments carried out on three separate CLL samples. Due to the lack of gene expression, this approach was not pursued further.

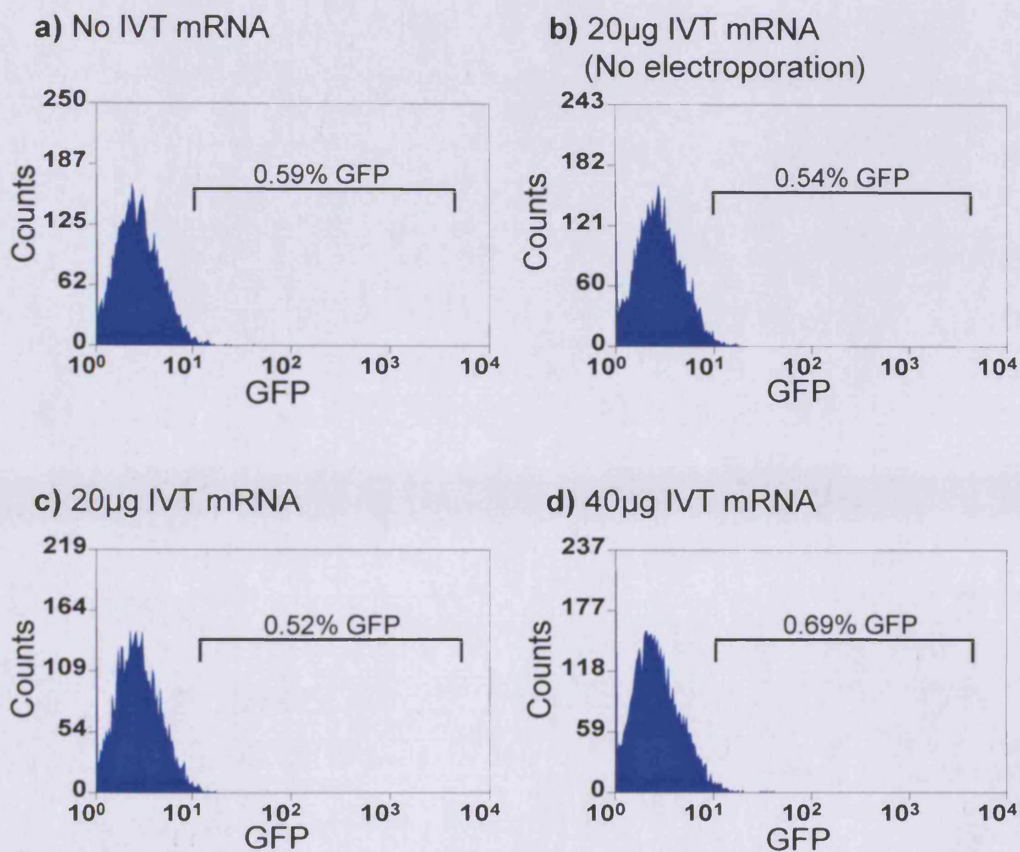


Figure 3.3 No GFP expression was observed following electroporation of IVTmRNA.

$5x10^6$ CLL cells were resuspended in the electroporation buffer **(a)** the first sample was electroporated at 500V 150F with no *in vitro* transcribed messenger RNA (IVT mRNA). In the second sample **(b)** 20µg of IVT mRNA encoding GFP was added prior to electroporation. **(c)** 20µg IVT mRNA was added to the third sample and **(d)** 40µg to the final CLL sample before electroporation.

3.4 Lentiviral gene transduction

3.4.1 Introduction

In nature the virus gains entry into the cell through specific surface interactions and utilises the genetic machinery of the cell to incorporate its own genetic material into the host genome within the nucleus (Kay *et al.*, 2001). Unaware of the presence of the foreign gene the cell unwittingly transcribes the hybrid genome and generates the proteins required for viral replication. This mechanism has been exploited by scientists who have genetically modified the viral genes to introduce a gene of interest into the host cell.

Stable transduction of human cells through viral gene delivery

The first type of viral gene transfer was carried out using murine derived oncogenic retroviruses (Miller, 1992). These structures contain the basic genetic elements required for viral entry into dividing human cells, integration of the viral cDNA into the host genome and the expression of regulatory proteins, enzymes and the viral envelope glycoprotein which encapsulates the replicated virus as it leaves the host cell.

Retroviruses

The retroviral genome consists of two identical copies of a single stranded nucleic acid RNA molecule bound by hydrogen bonds to form a dimer (Coffin *et al.*, 1997). The genome, along with virus replication enzymes, are housed within the protein core of the virus which is in-turn encapsulated in a glycoprotein containing phospholipid envelope. It is the outer glycoprotein envelope which interacts with specific host cell membrane receptors to allow entry of the viral core into the cell cytoplasm through direct fusion or endocytosis (Coffin *et al.*, 1997). In the cytoplasm reverse transcription enzymes, transferred in the viral core particle, produce a cDNA fragment from the viral RNA. Following entry into the nucleus the viral genome is permanently integrated into the host DNA where it is referred to as the *provirus* (Buchschacher and Wong-Staal, 2000). During host cell replication the proviral DNA is transcribed into mRNA and the genes required

for construction of the virus are translated into functional precursor structural proteins, replication enzymes and envelope glycoproteins (Buchschacher and Wong-Staal, 2000).

Genetics and post-translational modifications

The retroviral genome encodes three genes essential for replication. The first two genes are *gag* and *pol* which are transcribed into a single mRNA precursor coding for the viral core proteins and the viral replication enzymes respectively (Buchschacher and Wong-Staal, 2000). The translated chimeric protein is self-cleaved through its own protease activity which subsequently causes degradation of the *pol* product into separate enzymes with protease, reverse transcriptase and integrase activity (Fields *et al.*, 1996). Matrix, capsid and nucleo-capsid proteins are core molecules that are all formed through additional protease cleavage of the Gag protein (Fields *et al.*, 1996). The third gene (*env*) codes for the proteins contained within the envelope structure. Cellular proteases are responsible for the cleavage of the envelope glycoprotein into the external envelope glycoprotein and the transmembrane protein, two molecules which instigate cell membrane interactions as a prelude to viral infection (Buchschacher and Wong-Staal, 2000).

Reduction of viral pathogenesis through genetic modification

Through *in vitro* manipulation, the retrovirus can be utilised as an effective means of gene transfer. The use of such a system, where a virus is introduced into host cells, raises serious issues with regard to safety, especially in cases where the virus has the potential to operate as a vector for a therapeutic gene in the treatment of human disease (Kay *et al.*, 2001). With this in mind, together with the safety issues involved in working with such molecules, various molecular modifications have been made to viral vectors to increase their safety and reduce their potential to replicate (Delenda, 2004). Primarily the vector is stripped of its viral genes from the genome and left with the minimum structural protein and enzyme coding regions necessary for viral production and infection. The virus is also rendered replication defective meaning that following infection of the target cell, the virus is no longer able to replicate and cause further infection (Delenda, 2004). This is achieved by

removing *cis*-acting sequences from the genes encoding the viral proteins required for replication. These sequences allow propagation of a number of replication events including reverse transcription of viral RNA (*pbs*), continued DNA synthesis (*ppt*), incorporation (*att*) and transcription of the *provirus* and packaging of the viral genome (Delenda, 2004). A promoter gene inserted up-stream of the gene of interest allows this fragment to be the only one transcribed following infection into the host genome.

Additional measures, taken to reduce the pathogenic potential of the virus, include the integration of the required genes into target or packaging cells in trans. The gene of interest together with its promoter is incorporated into an initial plasmid vector. The viral genes *gag* and *pol* are cloned into a second plasmid and the *env* gene, coding the envelope protein, is cloned into a tertiary plasmid vector. Only following transfection of the packaging cell line are these plasmid vectors expressed together to allow production of the virus (Buchschacher and Wong-Staal, 2000) (Figure 3.4). With each of these modifications in place the potential of the viral system to cause pathogenesis through genetic recombination is low (Buchschacher and Wong-Staal, 2000).

Lentiviral Vectors

The application of lentiviruses in gene delivery has been widely studied, predominantly using the Human immunodeficiency virus-1 (HIV1) (Naldini *et al.*, 1996). Replication of these types of virus requires additional genes which have to be present in the lentiviral vector system if successful gene transfer is to be carried out *in vitro*. The nine genes which encode the genome of the HIV virus are the structural genes *gag, pol* and *env* which have already been discussed, two additional genes *tat* and *rev*, which are essential for replication and regulate the levels of HIV gene expression at transcriptional and post transcriptional levels respectively and four accessory genes *vif, vpr, vpu* and *nef,* which are involved during *in vivo* replication and pathogenesis (Buchschacher and Wong-Staal, 2000). Elimination of the accessory genes in the lentiviral vector allows transfection and viral production whilst eliminating much of the pathogenic potential of the virus.

The Tat protein is involved in enhancing the transcriptional activity of the long terminal repeat (LTR) promoter, dramatically increasing transcription of

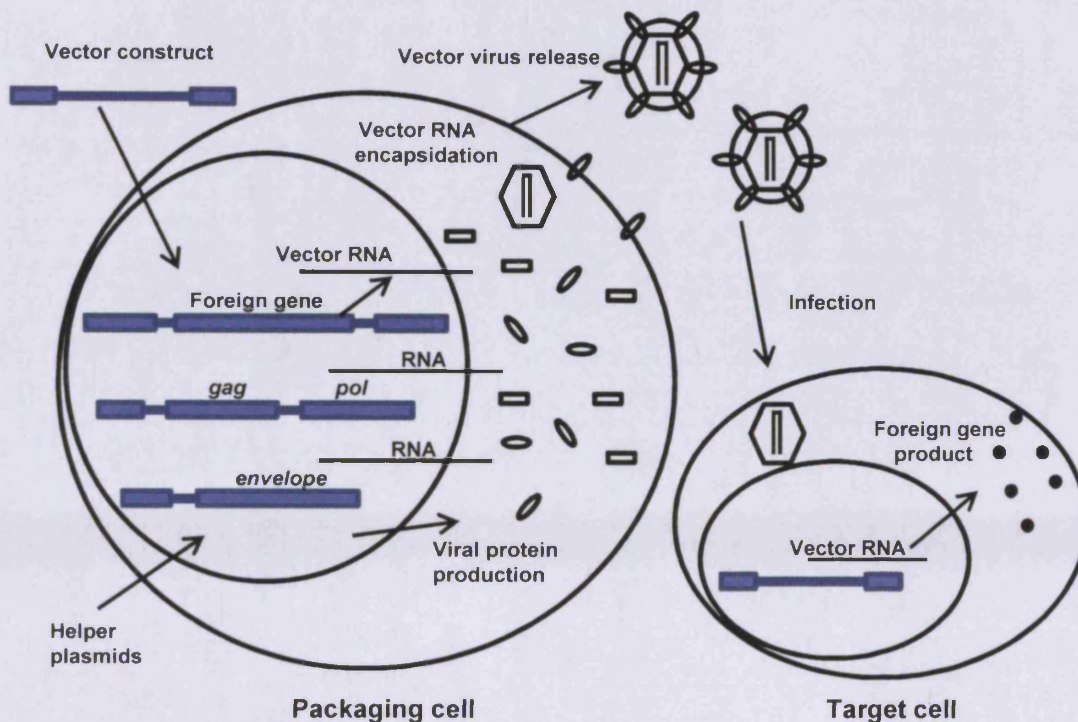


Figure 3.4 Generation of lentivirus (Adapted from Buchschacher and Wong-Staal, 2000).

The plasmid vector construct incorporating the gene of interest within the LTR regions of the lentivirus back bone are introduced to the packaging cell along with the helper plasmids encoding gag and pol and the envelope gene (*env*). Translation of all three genes results in the accumulation of viral proteins and the RNA transcript of the gene of interest. Due to the lack of *cis* acting sequences the *gag, pol* and *env* genes are not packaged within the newly generated lentivirus. Further propagation of the virus is therefore not permitted. The virus is released from the packaging cell and infected target cells express the gene of interest.

the *rev* gene. Rev promotes the accumulation of viral transcripts in the cytoplasm through its interaction with the rev-responsive element (*rre*) on the viral genome (Malim *et al.*, 1990). This mechanism allows the production of late viral proteins. Up-regulation of *rev* can be achieved in the lentiviral vector by introducing a constitutively active promoter gene into the LTR region, negating the requirement for the *tat* gene (Kim *et al.*, 1998). The elimination of *tat* leaves only the *gag, pol* and *rev* genes from the original HIV genome, increasing the biosafety of the vector system even further.

The retrovirus is only effective in transducing dividing cells (Lewis and Emerman, 1994). During cell division the nuclear envelope breaks down allowing the virus to access the genomic DNA. In non-dividing cells the retrovirus is unable to transit the nuclear envelope and therefore there is no formation of the *provirus*. In contrast lentiviral vectors produce integrase and matrix proteins which enhance nuclear localisation (Gallay *et al.*, 1995). The accessory protein Vpr binds directly to the nuclear pore complex allowing entry of the virus into the nucleus. The exact mechanism of this process is poorly understood and investigations have shown that formation of the *provirus* can occur in the absence of the *vpr* gene (Yamashita and Emerman, 2005). To increase biosafety *vpr* is left out of the lentiviral construct used to infect target cells *in vitro*.

In summary, genetic engineering of lentiviruses has allowed the generation of a safe and effective means of genetically modifying primary quiescent cells in the laboratory. Lentiviruses have been successfully employed in the genetic modification of a number of different cell types and there are many factors integral to the success of the lentiviral system. Such factors include the promoter used and the type of envelope which coats the virus. The pHR' SINcPPT SFFV-WPRE, third generation lentivirus developed in this project integrated the Spleen focus forming virus promoter up-stream of the gene of interest. The CD4 T-cell targeting envelope construction protein in HIV-1 is encoded by the HIV-1 *env* gene which was removed from the attenuated lentiviral backbone. In the generation of the lentivirus used in this work, the VSVG encoding envelope gene was utilised in trans (within the MD2G helper plasmid) to allow viral entry into a range of cells via binding to surface phospholipid.

3.4.2 GFP lentivirus on Jurkat cells

Jurkat cells were infected with a GFP expressing lentivirus. Details of the generation of plasmids and the lentivirus can be found in the materials and methods section (2.3.3 - 2.3.5). The addition of increasing amounts of virus (0 to 32μl) resulted in a dose dependent increase in the expression of GFP in Jurkat cells (Figure 3.5.a).

CLL cells are notoriously difficult to transduce with lentivirus. The virus was therefore concentrated by ultracentrifugation prior to infection of CLL cells and re-suspended in DMEM media without supplements. Initially the concentrated virus was compared to the crude viral supernatant by infecting Jurkat cells. This allowed the assessment of whether a significant amount of viral particles were lost during the ultracentrifugation step. The concentrated GFP virus was diluted 1 in 46 with DMEM (as 34.5ml of supernatant was ultracentrifuged and resuspended in 750μl DMEM (see materials and methods 2.3.5.2)) and increasing volumes were added to Jurkat cells. The transduction of the Jurkat cells was compared using the same volumes of crude supernatant added to CLL cells. Figures 3.5.b and 3.5.c illustrate the transduction efficiency of crude viral supernatant and diluted viral concentrate respectively. The transduction of Jurkat cells using increased volumes of both crude supernatant and concentrated GFP virus resulted in a linear increase in GFP expression. An increased amount of transduction was seen using lower volumes of crude supernatant suggesting that there were approximately twice as many viral particles present in the crude supernatant compared to the diluted concentrated virus. This experiment was designed to assess the loss of viral particles when concentrating the crude supernatant. When the amount of particles in the concentrated preparation was calculated, allowing for the dilution factor, there was a 20 fold increase in the viral yield when concentrating the virus by ultracentrifugation. This meant that it was worth while carrying out the concentration procedure to obtain more virus/ml.

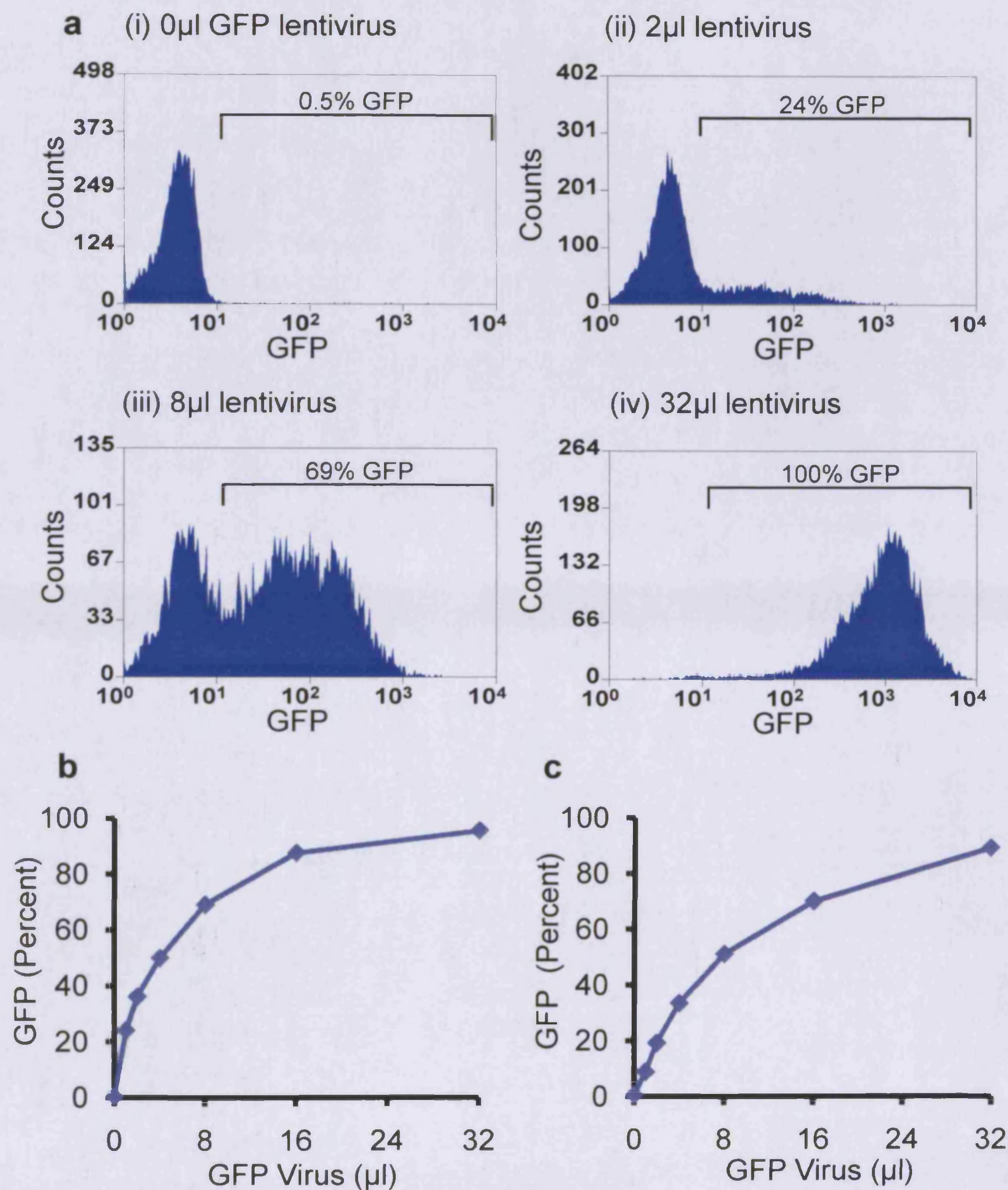


Figure 3.5 GFP was expressed in Jurkat cells treated with increasing amounts of lentivirus.

$1x10^6$ Jurkat cells were treated with increasing amounts of concentrated GFP lentivirus and incubated at 37°C for 48 hours. **Figures a (i-iv)** illustrate the GFP expression in samples treated with 0, 2, 8 and 32µl of lentivirus respectively. The increase in GFP expression is illustrated following the use of viral supernatant **(b)** and concentrated lentivirus (diluted 1/40) **(c)**.

3.4.3 Treating Primary CLL cells with GFP virus

Adding increasing amounts of GFP virus to the CLL cells saw a dose-dependent increase in the amount of GFP expressed (Figure 3.6.a). In the titration experiment a maximum of 35% of the CLL cells were transduced by adding 64µl of the GFP virus (Figure 3.6.a). Addition of more virus did not result in an increase in GFP expression in this sample. An estimation of the number of infectious viral particles present in 1µl of virus was then made and the multiplicity of infection (MOI), which is a measure of the number of viral particles required to infect one cell, was determined and applied to all subsequent experiments (using this particular batch of virus). The maximum amount of transduction was 35% GFP expression using 64µl of concentrated virus. $1x10^6$ CLL cells were used in the experiment which meant that 350,000 cells were GFP positive following the addition of 64µl of virus. Taking this number (which was the maximum amount of cells transduced) as 100% transduction, a figure was generated illustrating the expression of GFP as a percentage of the maximum expression (Figure 3.6.b). Using values of the number of cells transduced as a percentage of the maximum, the MOI was calculated for the GFP virus (Figure 3.6.c). Table 3.1 shows the number of CLL cells transduced using increasing amounts of GFP lentivirus. Assuming that one viral particle infects one cell, and taking a mean of the three values obtained from the linear range (illustrating a linear increase on the graph in Figure 3.6.a), the number of particles per microlitre was estimated to be 82,341 (Table 3.1). From figure 3.6.a it can be seen that 64µl of virus was required to transduce the maximum number of CLL cells.

From the equation:

$$\frac{\text{Number of Viral particles/µl x Volume of Virus (µl)}}{\text{Number of cells being transduced}} = \frac{82341 \times 64}{1000000} = 5.3$$

an MOI of 5.3 was required to transduce the maximum number of CLL cells.

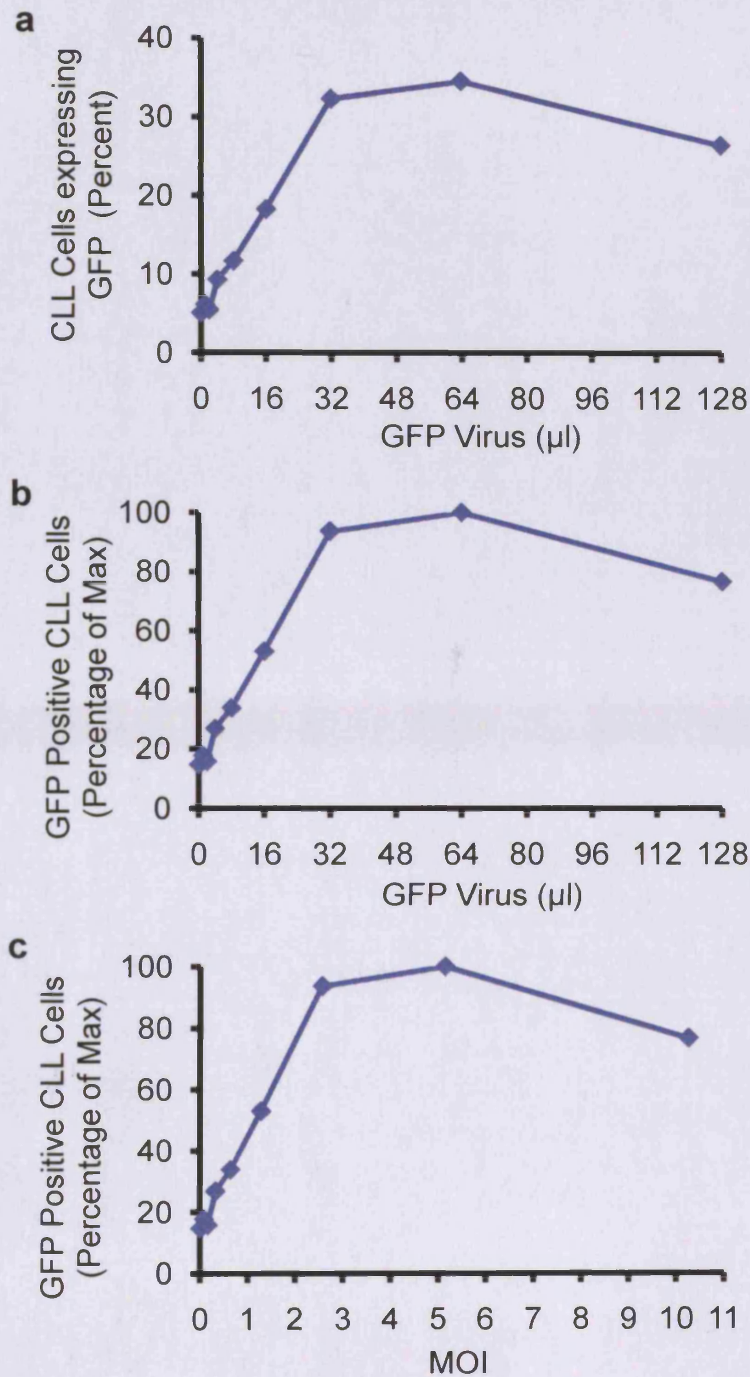


Figure 3.6 Multiplicity of infection (MOI) calculated from GFP virus treated CLL cells.

Increasing amounts of GFP lentivirus were added to $1x10^6$ CLL cells. Following 48 hours incubation GFP expression was determined by flow cytometry. The increase in GFP expression in was calculated as the percentage GFP expression **(a)**, the percentage of the maximum expression **(b)** and subsequently the multiplicity of infection **(c)**.

Microlitres of virus	% GFP expressing cells	Number of cells transduced	Infectious particles per microlitre
2	20.7	207261	103,631
4	35.2	352118	88,030
8	44.3	442889	55,361
		Mean	82,341
		St Dev	24,633

Table 3.1 Titration of GFP lentivirus on CLL cells.

There was a mean of 82,341 infectious virus particles per microlitre of concentrated GFP virus.

Microlitres of virus	% CD38 expressing cells	Number of cells transduced	Infectious particles per microlitre
2	23.0	230321	115,161
4	22.9	228802	57,201
8	47.0	470007	58,751
		Mean	77,037
		St Dev	33,025

Table 3.2 Titration of CD38 lentivirus on CLL cells.

There was a mean of 77,037 infectious virus particles per microlitre of concentrated CD38 virus.

3.4.4 CD38 virus on CLL cells

Following expression of GFP in Jurkat and primary CLL cells, a plasmid containing the same HIV1-derived backbone was generated containing the gene encoding CD38. The same helper plasmids were utilised, (namely Δ8.91 and MD2G) and the lentivirus was again generated using the 293T packaging cell line, under the same conditions as used for the GFP virus including concentration using ultracentrifugation.

Primary CLL cells were treated with the concentrated CD38 virus. A dose dependent increase in CD38 expression was seen in the CLL cells following infection with the CD38 virus with a maximum transduction of 93% in the titration experiments (Figure 3.7.a). The optimum amount of CD38 virus required to infect the largest percentage of the CLL cells without using excess virus was estimated at 40μl of concentrated virus for $1 x 10^6$ CLL cells (Figure 3.7.a). Again the amount of virus present was estimated using the linear slope of the graph and a mean of 77,037 infectious particles per microlitre was calculated for the CD38 virus (Table 3.2). From the equation (above) 40μl of CD38 lentivirus corresponded to an MOI of 3.2. Figure 3.8 illustrates the CD38 expression in a single CLL sample before (Figure 3.8.a) and following the addition of CD38 virus (MOI 3.2) (Figure 3.8.b). CD38 was not expressed at high levels in cells from the same patient treated with GFP virus at an MOI of 3.2 (Figure 3.8.c). A CD38 positive patient was run in parallel as a positive control for the experiment (Figure 3.8.d). Figure 3.8.e illustrates the increase in CD38 expression using an overlay of the histograms for untreated and CD38 virus treated samples.

3.4.5 Summary of MOI

Titrating the virus on CLL cells illustrated that the number of infectious viral particles contained within 1μl of both the GFP and the CD38 virus was comparable (82,341 and 77,037 infectious particles per microlitre for the GFP virus and CD38 virus respectively). Thus, using an equal volume of GFP virus (compared to CD38 virus) in subsequent experiments constituted an appropriate control which would distinguish the effects of CD38 expression from the effects of generic viral infection.

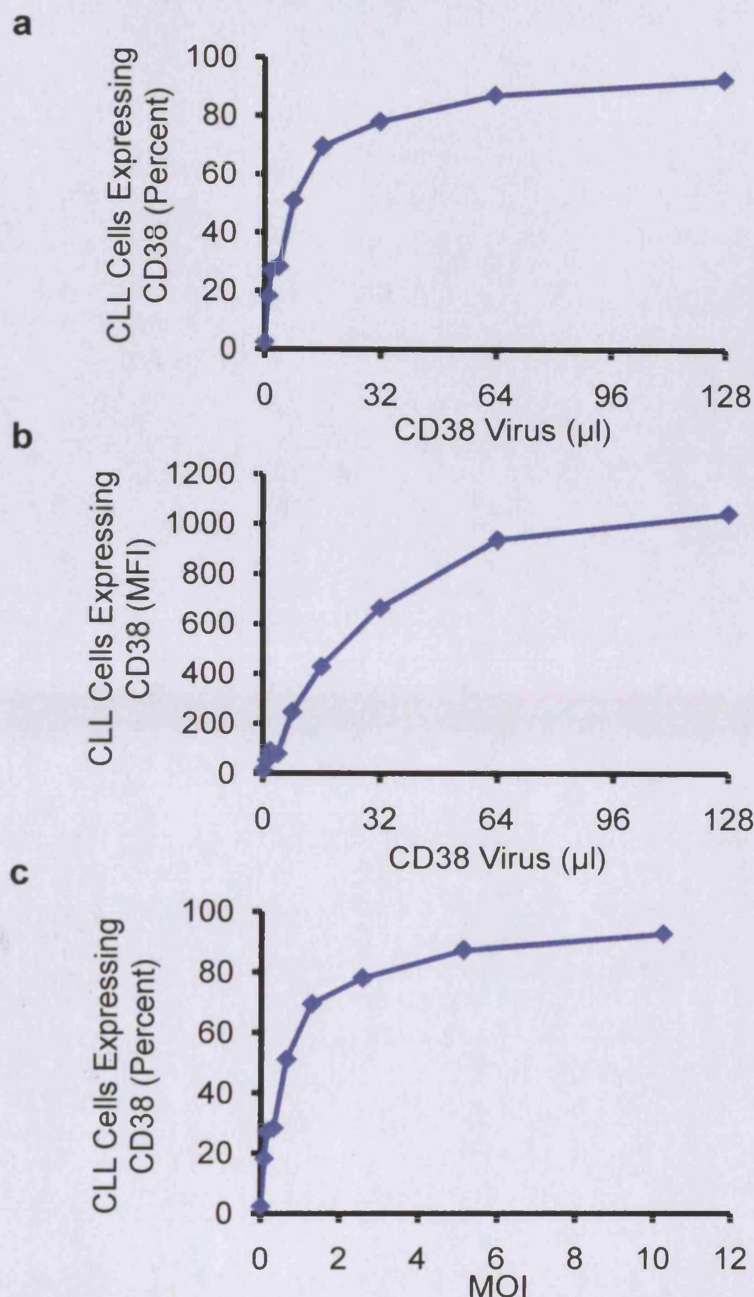


Figure 3.7 High levels of CD38 expression were observed following the addition of increasing amounts of lentivirus.

1x10^6 CLL cells from a CD38 negative patient were treated with increasing amounts of CD38 virus and the expression of CD38 on the surface was measured by the percentage of cells expressing the antigen **(a)** and by the MFI **(b)**. Following calculation of the number of viral particles per microlitre, the multiplicity of infection was determined for each amount of virus added. This is illustrated in **(c)** where an MOI of 3.2 is sufficient to transduce more than 80% of the CLL cells.

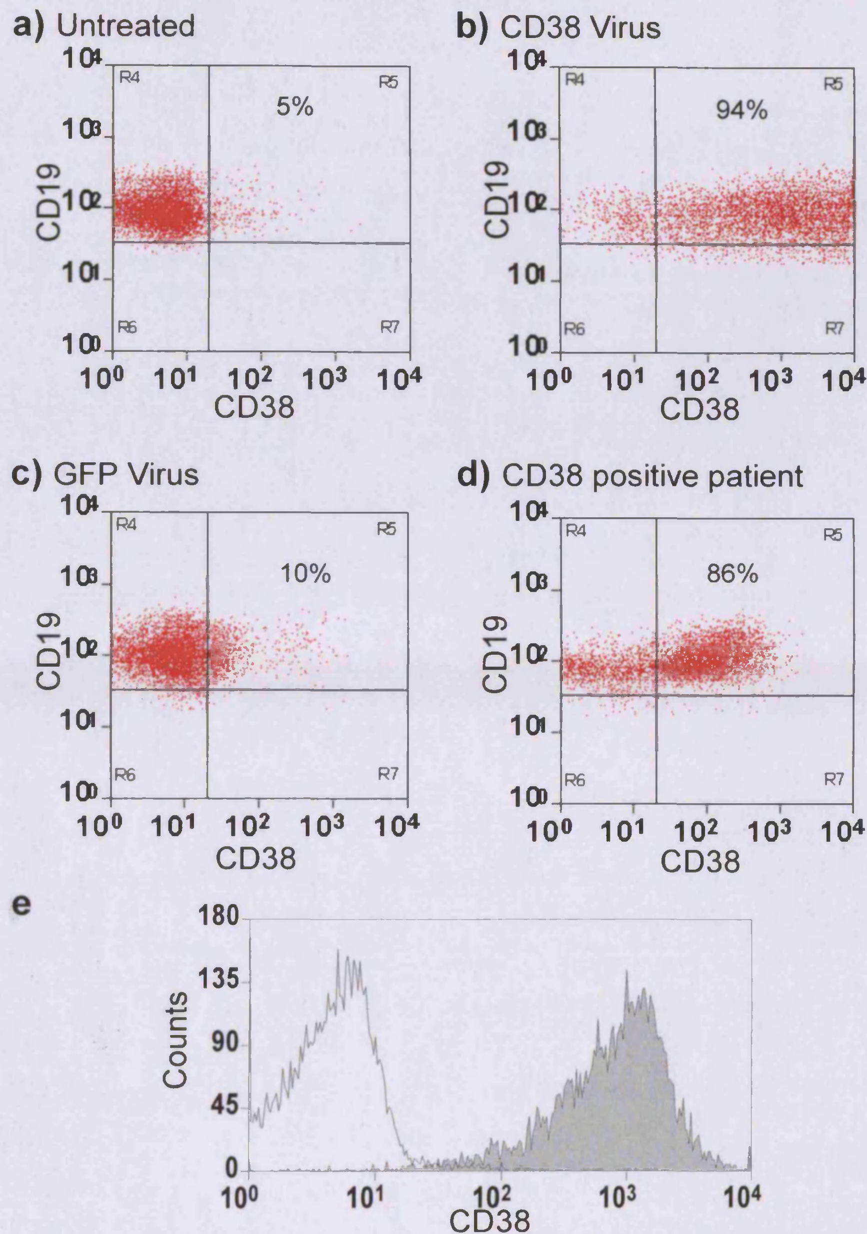


Figure 3.8 An MOI of 3.2 transduced over 90% of CLL cells in a single patient sample.

Primary CLL cells were isolated from a fresh patient sample and $1x10^6$ cells were treated with lentivirus. In the untreated control sample 5% of the CLL cells expressed CD38 on the surface **(a)**. Following the addition of 128µl of CD38 virus 94% of the cells expressed the CD38 antigen **(b)**. Addition of GFP virus induced an increase in CD38 expression in a small percentage of the cells (10%) **(c)**. **Figure 3.8d** illustrates the native CD38 expression of a CD38 positive patient. The increase in CD38 expression in the CD38 virus treated CLL sample compared to the negative control is illustrated in **(e)**.

3.4.6 Quantification of lentivirus using the Retro-tek p24 ELISA

Calculating the number of viral particles by titrating the CD38 and GFP virus to CLL cells illustrated that similar amounts of virus were being generated within the individual preparations. To support these findings the virus was quantified using a p24 ELISA. Figure 3.9 illustrates that a comparable amount of the p24 *gag* gene product was present in both CD38 and GFP virus preparations with values of 77,790,698 particles/μl (±16,190,667±SD) and 78,468,992 particles/μl (±14,430,044±SD) respectively.

3.4.7 CD38 expression in CLL cells treated with CD38 and GFP virus

CD38 is a marker of activation in many different cell types. The infection of CLL cells with concentrated lentivirus may have the potential to activate the cells and induce the expression of CD38 on the surface. It was therefore important to determine the expression of CD38 on CLL cells following treatment with the control GFP virus. CLL cells were treated with equal amounts of both GFP and CD38 virus. Figure 3.10.a illustrates the CD38 expression in cells infected by each virus. There was no dose related increase in the number of cells expressing CD38 or in the MFI (Figure 3.10.b) for cells treated with the GFP virus. This clearly demonstrates that the increase in CD38 expression in the CD38 negative primary CLL cells is due to the specific expression of the CD38 gene integrated into the genome (the *provirus*) following the addition of CD38 virus.

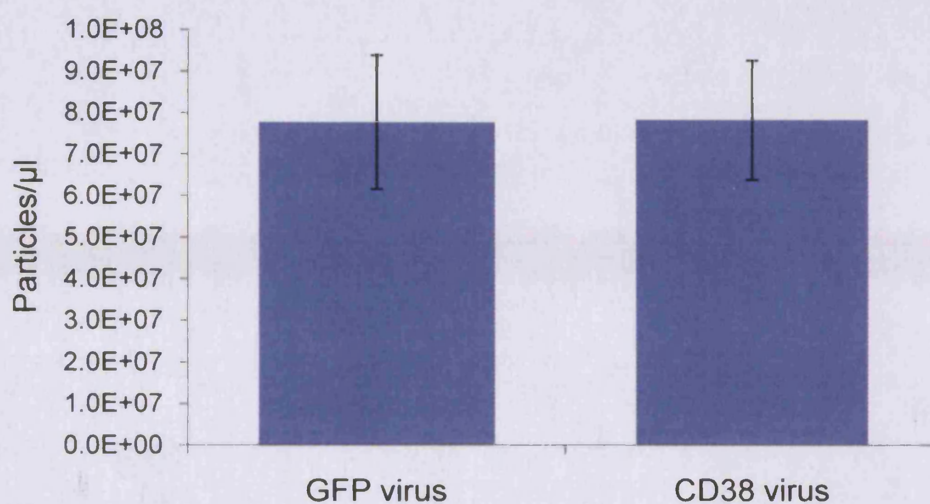


Figure 3.9 Comparable amounts of lentivirus were present in both CD38 and GFP preparations.

1/500,1/1000, 1/2500 and 1/5000 dilutions of the CD38 and GFP virus were prepared and placed into the wells of the The Retro-tek p24 ELISA plate. The fluorescence of the tertiary streptavidin-peroxidase substrate was quantified at 450nm and the amount of p24 antigen was calculated as a mean of the two dilutions.

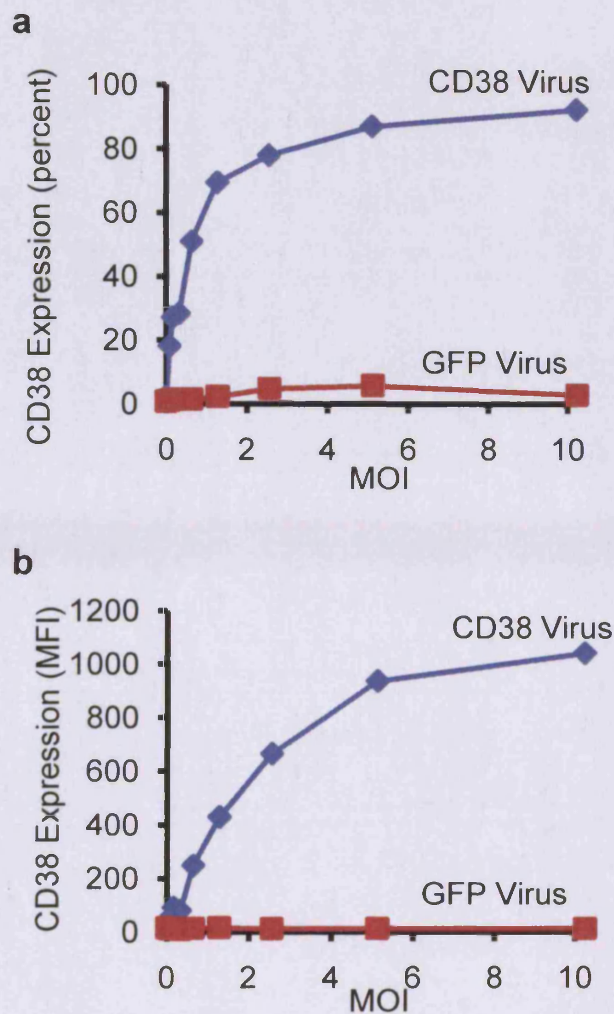


Figure 3.10 CD38 was not expressed following the use of the control GFP lentivirus.

1x10^6 CLL cells were treated with increasing amounts of CD38 and GFP virus and incubated at 37°C for 48 hours. The cells treated with the CD38 lentivirus express the antigen at high levels, where as the GFP virus treated cells do not express CD38. This is illustrated when plotting the percentage of cells expressing CD38 **(a)** and the MFI of the sample **(b)**.

3.4.8 CD38 gene expression in CLL cells by quantitative reverse transcription PCR (QRT-PCR)

Flow cytometry demonstrated that following transduction CD38 negative CLL cells expressed the CD38 antigen. To confirm these results QRT-PCR experiments were carried out to quantify the relative change in CD38 mRNA in transduced and untransduced samples. Figure 3.11 illustrates the QRT-PCR plot of a single sample following transduction. Using ABL expression as a house-keeping control gene, a 17,800 fold increase in the expression of mRNA encoding the CD38 gene was detected by QRT-PCR following the addition of CD38 lentivirus (Mean of 6 patient samples (SD±12,173)). A 7-fold increase in the gene expression of CD38 was detected in the GFP virus treated samples compared to the untreated control samples (Mean of 6 patient samples (SD±14)) (see Chapter 5.2).

3.4.9 Treating multiple patient samples with CD38 virus and control GFP virus

With many of the conditions required for maximum transduction optimised, further CD38 negative patient samples were infected to determine whether the high levels of CD38 transduction could be achieved in all of the samples tested. In total, 17 CD38 negative CLL samples (mean 3% SD±2%) were treated with the CD38 virus and the mean CD38 expression was 87% (SD±8.5%) (Figure 3.12). Seven patient samples from this cohort were also treated with GFP lentivirus to observe the effects of lentivirus on CLL cells. A mean of 8% (SD±6%) CD38 expression was observed in this cohort. P values were calculated from paired samples.

3.4.10 Stable CD38 expression in CLL cells

Following 48 hours incubation with lentivirus CD38 was expressed at high levels on the surface of CD38 negative CLL cells. To establish whether the expression of the antigen was stable over a longer period, the CLL cells were transduced and monitored for the expression of CD38 on the surface over a period of five days. Figure 3.13.a illustrates the stable expression of high levels of CD38 over the period of five days, compared to untreated controls, in a single patient sample (measured as the percentage of cells

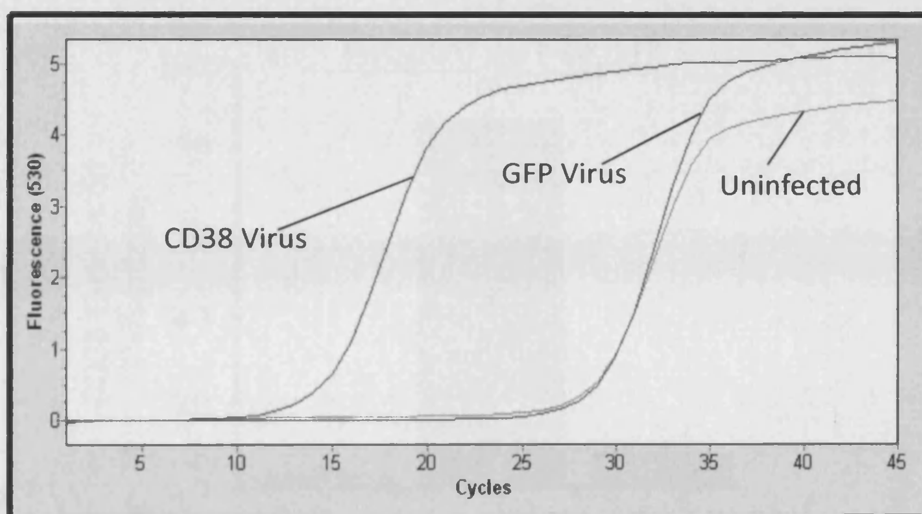


Figure 3.11 CD38 was highly expressed at the level of transcription in CLL cells illustrated by quantitative reverse transcription PCR.

Total RNA was extracted from virally treated CLL samples (MOI 3.2) and QRT-PCR was carried out to observe CD38 gene expression. The expression of mRNA encoding CD38 was determined relative to the ABL house keeping gene. A mean of 20,000 fold increase in the expression of CD38 was calculated in a single sample following the addition of CD38 lentivirus (compared to the untreated sample).

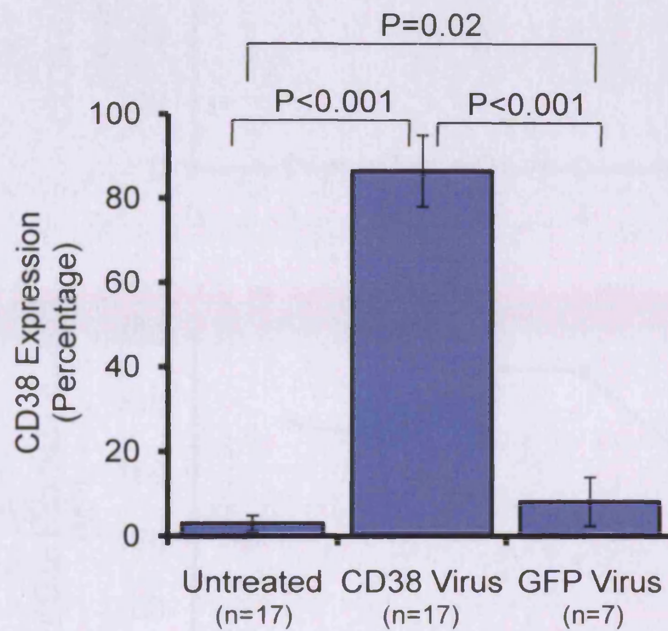


Figure 3.12 CD38 was expressed in multiple samples following the addition of CD38 lentivirus.

17 CD38 negative patient CLL samples were treated with CD38 lentivirus at an MOI of 3.2. There was a significant increase in the expression of CD38 on the surface of these samples compared to untreated and GFP virus treated samples (p<.0.001). The mean expression in the untreated sample was 3% (SD = 1.8)(n=17), the CD38 virus treated sample 87% (SD = 8.5)(n=17) and the GFP virus treated sample 8% (SD = 5.8)(n=7). There was no significant increase in the expression of CD38 in GFP treated samples compared to the untreated cells (p=0.2). (The student *t*-test was applied to paired samples).

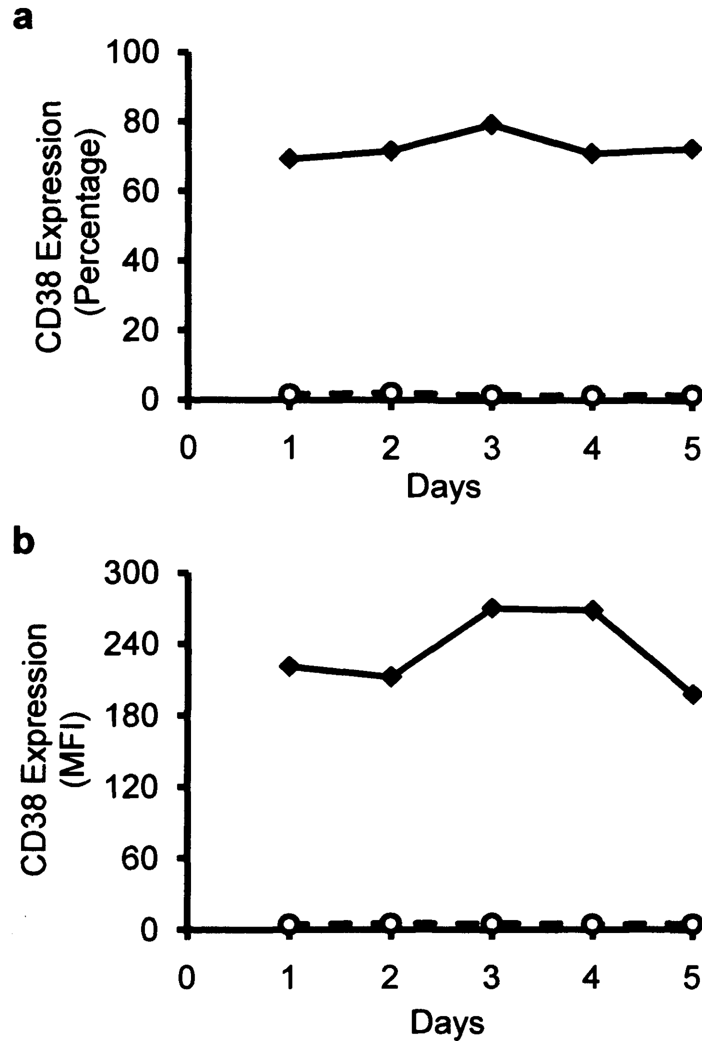


Figure 3.13 CD38 was expressed in CLL cells for up to 5 days.

CLL cells were treated with CD38 lentivirus at an MOI of 3.2 and incubated at 37°C. A sample of the cells was phenotyped each day for 5 days to observe CD38 expression. The number of cells transduced is determined from the percentage of cells expressing CD38 **(a)** and from the MFI **(b)**.

expressing CD38). Figure 3.13.b illustrates the MFI of the sample over the same period.

3.5 Discussion

The majority of the CLL cells in the peripheral circulation are in a non-dividing state. *In vitro*, these cells are resistant to genetic modification through transfection of double or single stranded coding molecules or transduction of retroviral vectors. Various groups have evaluated such techniques and have reported contrasting results for the manipulation of gene expression in CLL cells. In 2007 Seiffert *et al.* reported a high level of transduction with the use of nucleofection of a plasmid vector encoding GFP, while in 2008 Van Bockstaele *et al.* succeeded in transfecting CLL cells using electroporation of *in vitro* transcribed mRNA (Seiffert *et al.*, 2007, Van Bockstaele *et al.*, 2008). Both groups reported high levels of cell viability as well as the inefficiency of other gene transfer techniques assessed. With this contrasting evidence in mind, all three methods of genetically modifying CLL cells were evaluated in this chapter.

Nucleofection of a plasmid vector incorporating a GFP-CD38 fusion gene resulted in transfection of 34.5% of the CLL sample. However, this was associated with a large amount of cell death and in order to recover a pure population of genetically modified cells they had to be physically sorted. This in turn, induced further cell death. Given that the aim of this project was to carry out functional studies on genetically modified cells, the requirement of large numbers of patient cells for nucleofection meant that patient samples were limited to those with high lymphocyte counts (Above $3x10^7$ cells/ml). Also sorting the cells following nucleofection was time consuming and yielded a small number of genetically modified cells from which to extract genetic material for QRT-PCR and microarray investigations.

The second transfection method evaluated was electroporation of *in vitro* transcribed mRNA coding for GFP. Twenty four hours post electroporation the CLL cells were assessed for the expression of GFP using flow cytometry. No increase in fluorescence was observed in the sample following electroporation with increasing concentrations of IVTmRNA. Again a considerable amount of cell death occurred. In complete contrast to the results

obtained by Van Bockstaele *et al.* in 2008, this method of gene transfer was unsuccessful in multiple experiments. Due to the success of the other techniques evaluated at the same time, further optimisation of this methodology was not carried out.

The use of a VSVG pseudotyped lentivirus in genetically modifying CLL cells has been attempted with varying success (Bovia *et al.*, 2003, Janssens *et al.*, 2003, Serafini *et al.*, 2004, Levy *et al.*, Hazan-Halevy *et al.*). However, as an alternative to the transfection techniques, I decided to evaluate the lentiviral transduction method in our laboratory. Infection of Jurkat cells with a lentivirus housing the gene encoding GFP resulted in a dose-dependent increase in GFP expression in 100% of the cells treated. Levels of expression in CLL cells treated with GFP virus reached a maximum of 60% (Appendix 3) which is higher than previously reported (Van Bockstaele *et al.*, 2008, Frecha *et al.*, 2009). Following infection with lentivirus expressing CD38, up to 95% of the CLL cells treated were shown to express *de novo* CD38 with a mean transduction efficiency of 87% in 17 patient samples (Figure 3.12). The reason for such success using the pHR' SINcPPT SFFV-WPRE lentivirus is not clear. In a recent publication by Frecha *et al.* they compared the measles virus envelope glycoprotein to the VSVG envelope in the transduction of CLL cells (Frecha *et al.*, 2009). They suggested that both the viral envelope and the promoter used to drive the expression of the gene of interest were integral to the success of their lentiviral transduction system. In our experience pseudotyping the viral core with the VSVG envelope, which resulted in little transduction in their system, was highly successful. However, the SFFV promoter used in our lentiviral construct differed from the CMV promoter which they utilised. It seems likely that the combination of promoter and viral envelope used in our system facilitates the transduction process at multiple levels.

Generation of a lentivirus with the same viral back bone, but expressing a non-human reporter gene, was essential for two reasons: Firstly to ensure that the expression of CD38 was derived from the *provirus* incorporated into the host genome and not endogenous CD38 expression due to viral activation; and secondly as a control in subsequent experiments to distinguish the generic effects of the virus from the specific increase in CD38 expression on the CLL

cell surface. The reporter gene utilised was derived from the Jelly fish (*Aequorea Victoria)* and expressed the green fluorescent protein which, following expression, can be observed in the transduced cells using a fluorescence microscope or within the FL1 channel of the flow cytometer (488nm laser line) (Xu *et al.*, 1998). In order to constitute an accurate control, the GFP virus was added to the CLL cells in equal amounts to the CD38 virus. Both viruses were titrated on CLL cells to determine the number of viral particles present in one microlitre of each of the concentrated viral preparations. The results illustrated that there were comparative amounts of CD38 and GFP virus in each concentrate and therefore equal volumes of GFP virus could be used to infect CLL cell samples and provide an appropriate control in each of the experiments. This was confirmed by the use of an ELISA technique to detect soluble p24 protein, a product of the *gag* gene derived from the lentivirus. Comparative amounts of the p24 protein were detected in both viral preparations (Figure 3.9). The multiplicity of infection, which is the number of viral particles required to infect one cell, was also determined and employed when comparing individual virus preparations and scaling up the experiments to use large cell numbers. Following transduction of the CLL cells with the CD38 virus, flow cytometry was utilised to quantify the levels of CD38 expression over five days. Figure 3.13 illustrates that the expression of CD38 on CLL cells was stable over five days, determined by both the percentage of CLL cells expressing the antigen (Figure 3.13.a) and the mean fluorescence intensity (Figure 3.13.b).

Of the three methods of gene delivery evaluated the lentiviral transduction of CLL cells proved the most successful in generating large numbers of viable CD38 positive cells from a CD38 negative sample. This method caused substantially less cell death than the transfection techniques (discussed in chapter four) and eliminated the requirement for cell sorting or selection. A consistently high level of transduction was achieved in 17 patient samples treated (mean 87% SD±8.5%) providing a homogeneous background with which to perform further investigations.

Chapter 4. CD38 ligation enhances the viability and proliferation of primary CLL cells

4.1 Introduction

The presence of CD38 on the surface of CLL cells confers a poor prognosis (Damle *et al.*, 1999) but the biological rationale for this remains uncertain. Chapter three describes the development of a lentiviral vector system for inducing CD38 on the surface of CLL cells. This technology was utilised to investigate the role of CD38 in regulating the survival and proliferation of primary human CLL cells.

It is now presumed that due to the absence of specific signals from the tissue microenvironment, CLL cells do not survive for prolonged periods *ex vivo* (Bomstein *et al.*, 2003). As they progress through the stages of apoptosis, CLL cells shrink and therefore increase in granularity prior to cellular fragmentation. With the use of flow cytometry, a population of apoptotic cells may be identified by observing changes in forward and side light scatter (Figure 4.1.a). An alternative, and more direct, means of assessing apoptosis involves the labelling of phosphatidylserine on the surface of the cells via binding of its ligand annexin V.

Annexin V staining

The cellular cytoplasmic membrane is made up of asymmetrically distributed phospholipids (Alberts *et al* 1994). Phosphatidylserine is normally located on the inner leaflet of the bilayer. However, during apoptosis a reduction in intra-cellular ATP results in the inability of a cell to retain phosphatidylserine on the inner membrane. Subsequently the molecule "flips" and is presented on the outside of the cell (Seigneuret and Devaux, 1984). Annexin V may be labelled with a fluorescent molecule which, when bound to phosphatidylserine on the outer membrane, can be detected using flow cytometry. The addition of propidium iodide to the cells within this assay identifies dead cells as well as those in a progressive apoptotic state (Steinkamp *et al.*, 1999).

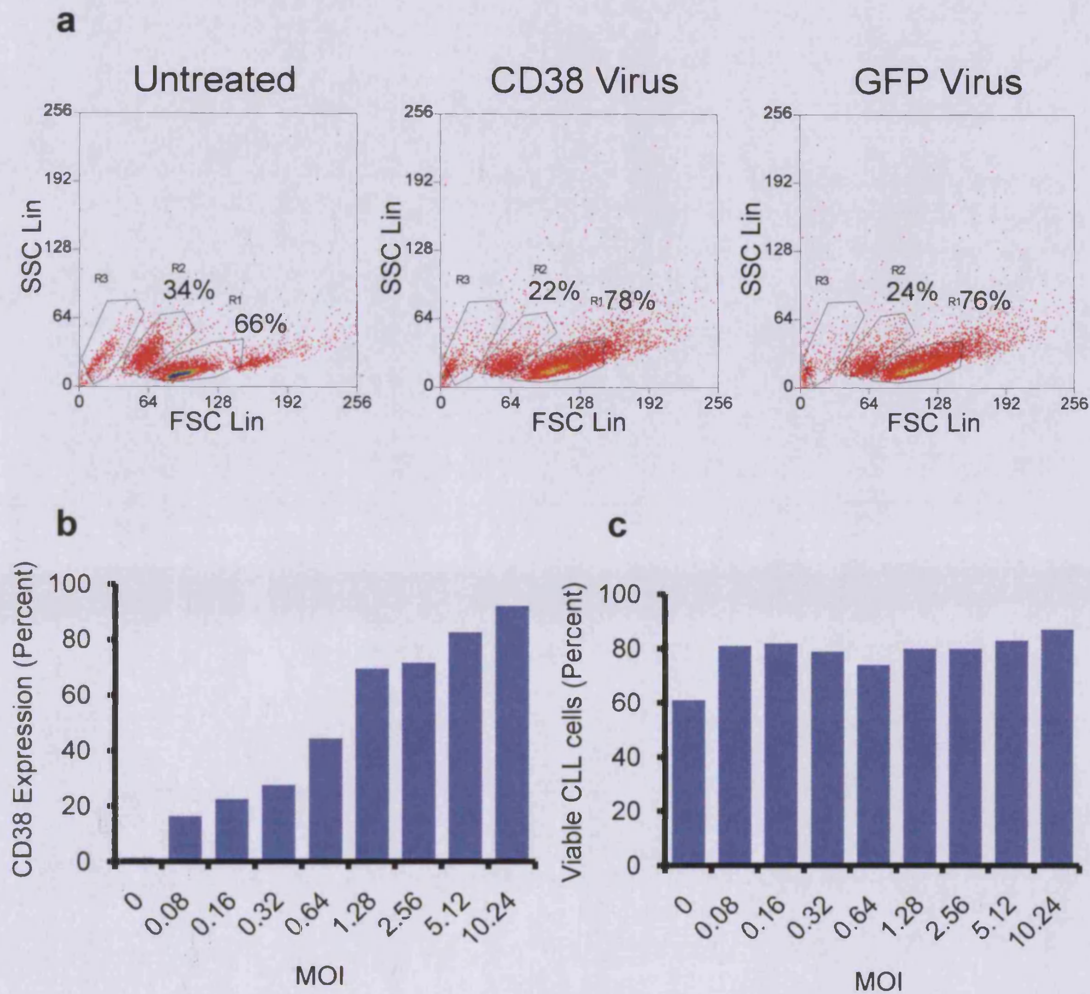


Figure 4.1 CLL cell survival was increased following the addition of lentivirus.

(a) $1x10^6$ CLL cells were placed into liquid culture and incubated for 48 hours with or without lentivirus. The CLL cell viability was assessed in untreated, CD38 virus treated and GFP treated samples. **(b)** Increasing amounts of CD38 virus were added to $1x10^6$ CLL cells and CD38 expression was assessed by flow cytometry following 48 hours incubation. **(c)** Using forward and side scatter profiles the viability of the samples was assessed after 48 hours.

4.2 CLL cell survival was enhanced over 48 hours following the addition of lentivirus

To investigate the effects of CD38 on the survival of CLL cells *in vitro*, CD38 negative CLL cells were transduced using CD38 virus and incubated in liquid culture for 48 hours to allow expression of the CD38 antigen. Cell viability was then assessed using forward and side scatter profiles obtained by flow cytometry. Viable cells were identified by the R1 gate (Figure 4.1.a). In the early stages of apoptosis the cells shrink. They show a reduction in forward light scatter, an increase in side light scatter and hence appear in the R2 gate. As the cell membrane disrupts in the latter stages of apoptosis the membrane blebs and finally fragments. The resultant cell debris is observed in the R3 gate (Figure 4.1.a). Using this method the number of viable cells was calculated in untreated (Figure 4.1.a.i), CD38 virus (4.1.a.ii) and GFP virus (4.1.a.iii) treated CLL samples by using the formula:

$$\text{Viable cells} = R1/(R1+R2)\times 100.$$

The percentage of viable cells was 66%, 78% and 76% in untreated, CD38 virus and GFP virus treated samples respectively (Figure 4.1.a). Increasing amounts of lentivirus were added to CLL cells from a single patient and CD38 expression was measured by flow cytometry (Figure 4.1.b). Subsequently cell viability was assessed by using the forward and side light scatter profiles. There was an increase in the number of viable cells following the addition of CD38-expressing lentivirus although this did not appear to be in a dose-dependent manner (Figure 4.1.c). A GFP-expressing lentivirus was also used to treat cells in multiple samples and an MOI of 3.2 was used for both the CD38 and the GFP virus. Figure 4.2 illustrates the viability of CLL cells from 21 untreated samples, 21 CD38 virus treated samples and 14 GFP virus treated samples. A mean of 63% ±15.6 (±SD) viable CLL cells were present following 48 hours incubation in the untreated sample. A mean of 74% ±16.7 (±SD) viability was observed in the CD38 virus treated samples and 71% ±20.8 (±SD) in the GFP virus treated samples. The viability was significantly increased in the CD38 lentivirus treated samples

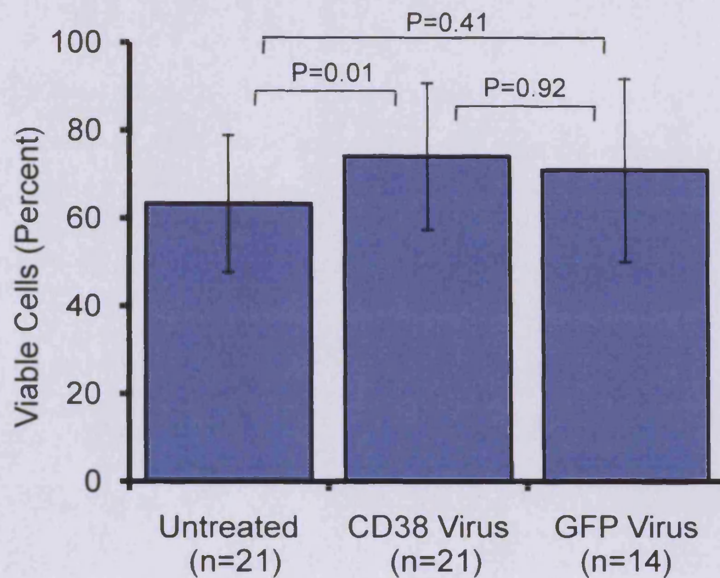


Figure 4.2 CLL cell viability was increased in multiple virus treated samples following 48 hours in culture.

Multiple patient samples were isolated in liquid culture for 48 hours. The viability of the cells was assessed in untreated, CD38 virus treated and GFP virus treated samples using forward and side scatter. The student t-test was used to compare the viability between paired samples.

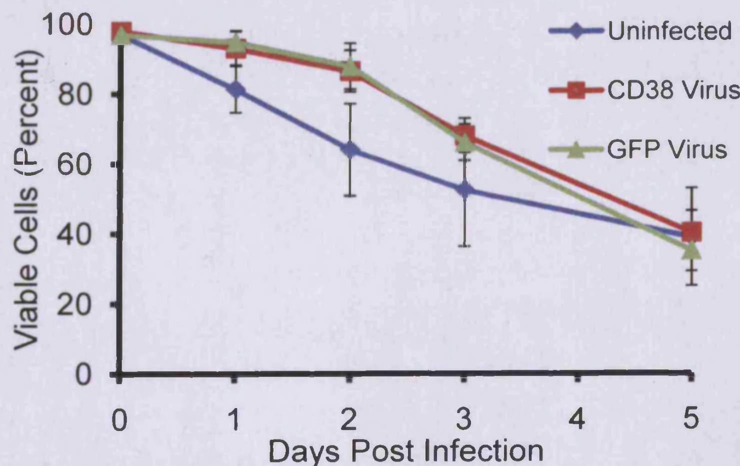


Figure 4.3 CLL cell viability was increased in virus treated samples assessed over 5 days in liquid culture.

1×10^6 CLL cells from three patients were untreated, CD38 virus treated or GFP virus treated and incubated in liquid culture for 5 days. The viability of the cells was assessed using forward and side scatter at day 0, 1, 2, 3 and 5.

compared to the untreated samples (P=0.01. (calculated from 21 paired samples)). There was no significant difference in the viability between untreated samples and cells treated with GFP virus (P=0.41. (14 paired samples)) or between CD38 treated and GFP treated samples (P=0.92. (14 paired samples)). Therefore the addition of lentivirus seemed to have an effect on the survival of the CLL cells in liquid culture. The effect of CD38 signalling may have been masked by such an effect.

4.3 CLL cell survival was enhanced over three days following the addition of lentivirus

The generic effects of the lentivirus on CLL cell viability over 48 hours made analyzing the role of CD38 in the modulation of cellular survival challenging. However, it did not negate the hypothesis that CD38 could increase CLL cell viability. To determine whether longer time points would establish if CD38 expression regulated viability, cells were incubated for up to five days and the viability was assessed. CLL cells were treated with CD38 and GFP lentivirus (MOI 3.2) and incubated in liquid media. Using forward and side scatter, the viability of three patient samples was evaluated. Figure 4.3 shows a survival advantage for CLL cells treated with lentivirus over the first three days, though the difference in CLL cell viability was not significant (P=0.21 in untreated compared to GFP virus treated and P=0.2 in untreated compared to CD38 virus treated samples). Also the viability of the untreated and virus treated samples was not significantly different following five days in liquid culture (P=0.48 in untreated compared to GFP virus treated and P=0.42 in untreated compared to CD38 virus treated samples).

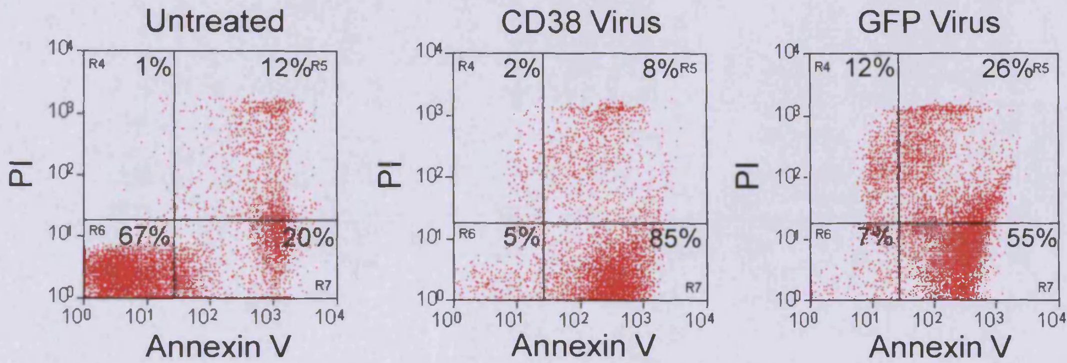
4.4 Addition of lentivirus caused enhanced expression of phosphatidylserine on the CLL cell surface

Forward and side scatter profiles identify dying cells that decrease in size as they progress through programmed cell death. Annexin V staining can be used to detect phosphatidylserine on the surface of the cell providing an alternative means of assessing apoptosis. The annexin V investigation was carried out to further investigate the effects of CD38 on CLL viability. The vital dye propidium iodide was used in tandem with annexin V to discriminate

between early and late stage apoptotic cells. Figure 4.4.a illustrates that 32% of the CLL cells were annexin V and PI positive in the untreated sample following 48 hours incubation. In virally treated samples however, it appears that a much larger portion of the CLL cells were annexin V positive with 93% of the cells treated with CD38 virus and 81% of the cells treated with GFP virus showing evidence of phosphatidylserine on their surface (Figure 4.4.a). The CLL cells were left in culture for five days and apoptosis/cell death was again assessed by annexin V/PI staining. In the untreated sample 61% of the cells were viable after 5 days (Figure 4.4.b). In contrast, the virally treated CLL cells were almost all annexin V/PI positive (97% and 96% in CD38 virus and GFP virus treated samples respectively) (Figure 4.4.b).

Forward and side scatter plots denote a large population of viable cells in lentivirally treated samples following 48 hours in liquid media. Conversely, annexin V/PI staining identified the presence of large numbers of apoptotic cells. Due to the conflicting evidence regarding the viability of the CLL cells further investigations were carried out to observe at which point, following the addition of lentivirus, the cells began to express phosphatidylserine on the surface. In a series of time course experiments the CLL cells were treated with CD38 and GFP virus and annexin V staining was assessed using flow cytometry. After one hour, the CLL cells were stained with annexin V/PI to detect the expression of phosphatidylserine on the surface. As expected, the untreated sample showed very little staining and only 6% of the freshly isolated CLL cells maintained in liquid media were annexin V/PI positive (Figure 4.5.a). After the same time period, 34% of the CLL cells treated with CD38 virus and 43% of those treated with the GFP control virus were annexin V/PI positive (Figure 4.5.a). Annexin V staining following two hours incubation identified an increase in the number of apoptotic cells in the untreated sample (a total of 13%) with much larger increases observed for the virus treated samples (49% in both CD38 and GFP virus treated samples) (Figure 4.5.b). CLL cells were incubated for an additional four and six hours following infection with lentivirus and subsequently analysed for annexin V and PI staining. Figure 4.6 summarises the proportion of the sample that was annexin V/PI positive at each of the time points in untreated and virus treated samples. The detection of phosphatidylserine on the cell surface occurred

a) Day 2



b) Day 5

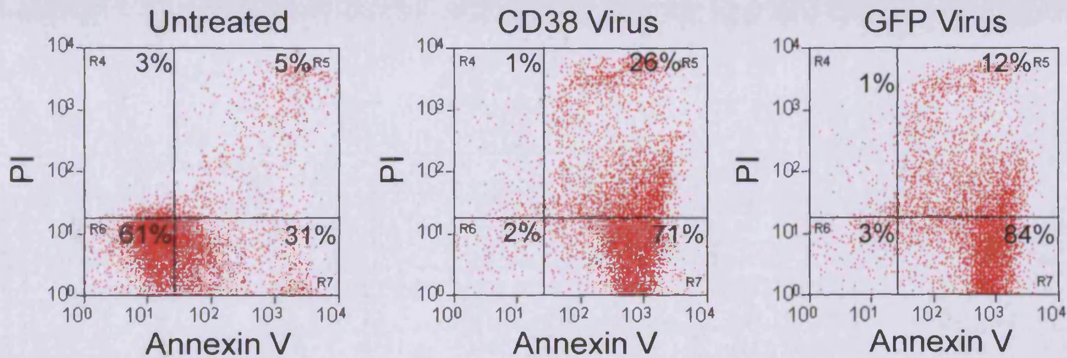
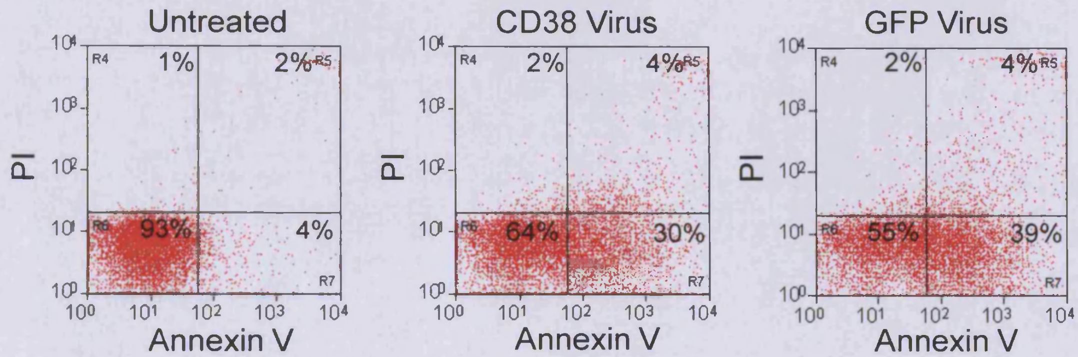


Figure 4.4 A large population of the CLL cells were annexin V/PI positive after 2 and 5 days incubation with lentivirus.

(a) $1x10^6$ CLL cells were treated with lentivirus and placed into liquid media for 48 hours. Viability was assessed in untreated, CD38 virus treated and GFP virus treated samples using annexin V/PI staining. **(b)** $1x10^6$ CLL cells were treated with lentivirus and placed into liquid media for 5 days. Viability was assessed in untreated, CD38 virus treated and GFP virus treated samples using annexin V/PI staining.

a) 1 hour



b) 2 hours

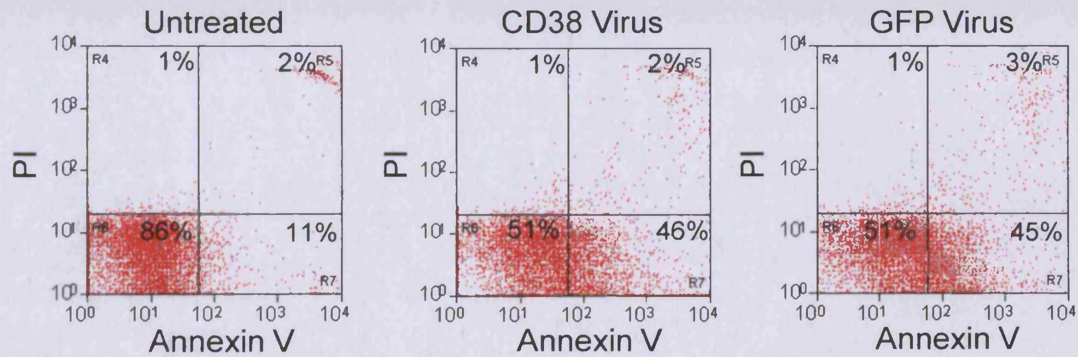


Figure 4.5 Annexin V positive CLL cells were observed after 1 and 2 hours incubation with lentivirus.

(a) $1x10^6$ CLL cells were incubated for 1 hour with lentivirus and CLL cell viability was assessed by annexin V/PI staining. The number of annexin V/PI positive cells was determined in untreated, CD38 virus and GFP virus treated samples. **(b)** $1x10^6$ CLL cells were incubated for 2 hours with lentivirus and CLL cell viability was assessed by annexin V/PI staining. The number of annexin V/PI positive cells was determined in untreated, CD38 virus and GFP virus treated samples.

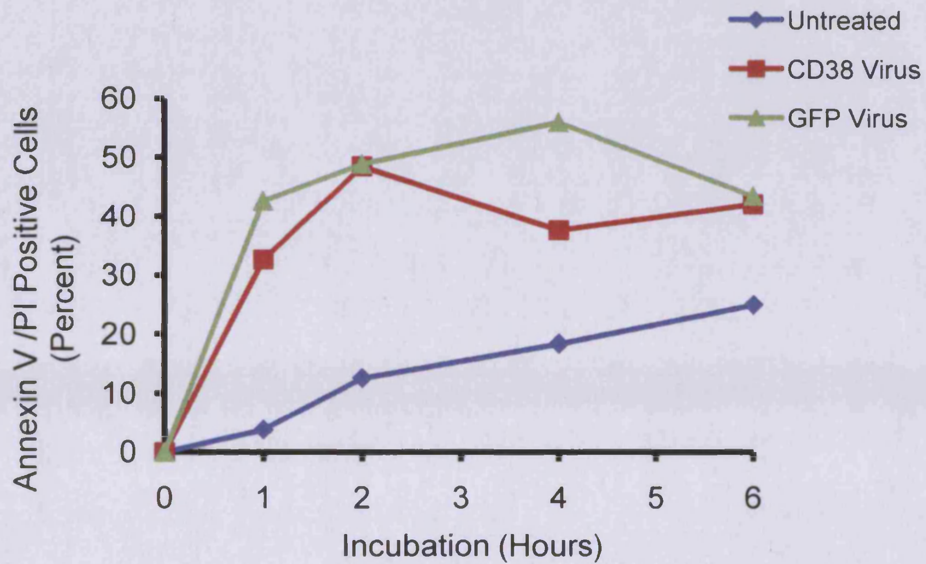


Figure 4.6 Annexin V/PI staining identified a large number of apparently apoptotic cells 1 hour after the addition of lentivirus.

1×10^6 CLL cells were incubated for 6 hours with and without lentivirus. Samples from untreated, CD38 virus treated and GFP virus treated CLL preparations were stained for annexin V/PI at 0, 1, 2, 4 and 6 hours.

rapidly following the addition of lentivirus (within one hour). Given that this effect was observed with both the GFP-expressing virus and the CD38-expressing virus, it seems likely that it is a generic artefact induced by exposure to the virus and may be due to the interactions with the cell membrane rather than apoptosis induction at such early time points. There is no evidence suggesting that the VSVG pseudotyped virus has the ability to disrupt the cell membrane though a report by Benjouad *et al* illustrated the ability of the Tat protein to modify the permeability of the cell membrane and induce cell death in lymphocytes (Benjouad *et al.*, 1993).

4.5 CLL cell morphology identified viable CLL cells following the addition of lentivirus

The evidence from the annexin V/PI investigation indicates that the virus treated cells were undergoing apoptosis. In contrast the forward and side scatter profiles, obtained from the same flow cytometry assays, identified a large viable population of cells 48 hours after treatment with lentivirus. To determine whether the CLL cells were undergoing apoptosis, untreated and virus treated CLL samples were cultured for 48 hours and then spread onto glass slides. They were then stained using the Giemsa dye and scrutinised under the microscope for evidence of apoptotic cell death. In the early stages of apoptosis the cell begins to shrink due to cleavage of lamins and actin filaments in the cytoskeleton (Fluckiger *et al.*, 1994). Chromatin in the nucleus is broken down and forms a tightly packed horse-shoe like structure. Blebbing of the cell membrane then occurs and small fragments of the cell break off to be engulfed and processed by macrophages (Fluckiger *et al.*, 1994). Figure 4.7.i and iv illustrate untreated CLL cells from a Giemsa stained CLL lymphocyte preparation. There was no evidence of cell shrinkage or of the latter stages of apoptosis. Figure 4.7.ii, iii, v and vi show CLL cells incubated with CD38 and GFP lentivirus. Again there was no evidence of cell shrinking or condensation of the chromatin. Figure 4.7.b.i illustrates a freshly stained blood smear from a patient with CLL and Figure 4.7.b.ii shows CLL cells undergoing apoptosis. In the latter there was an obvious reduction in the

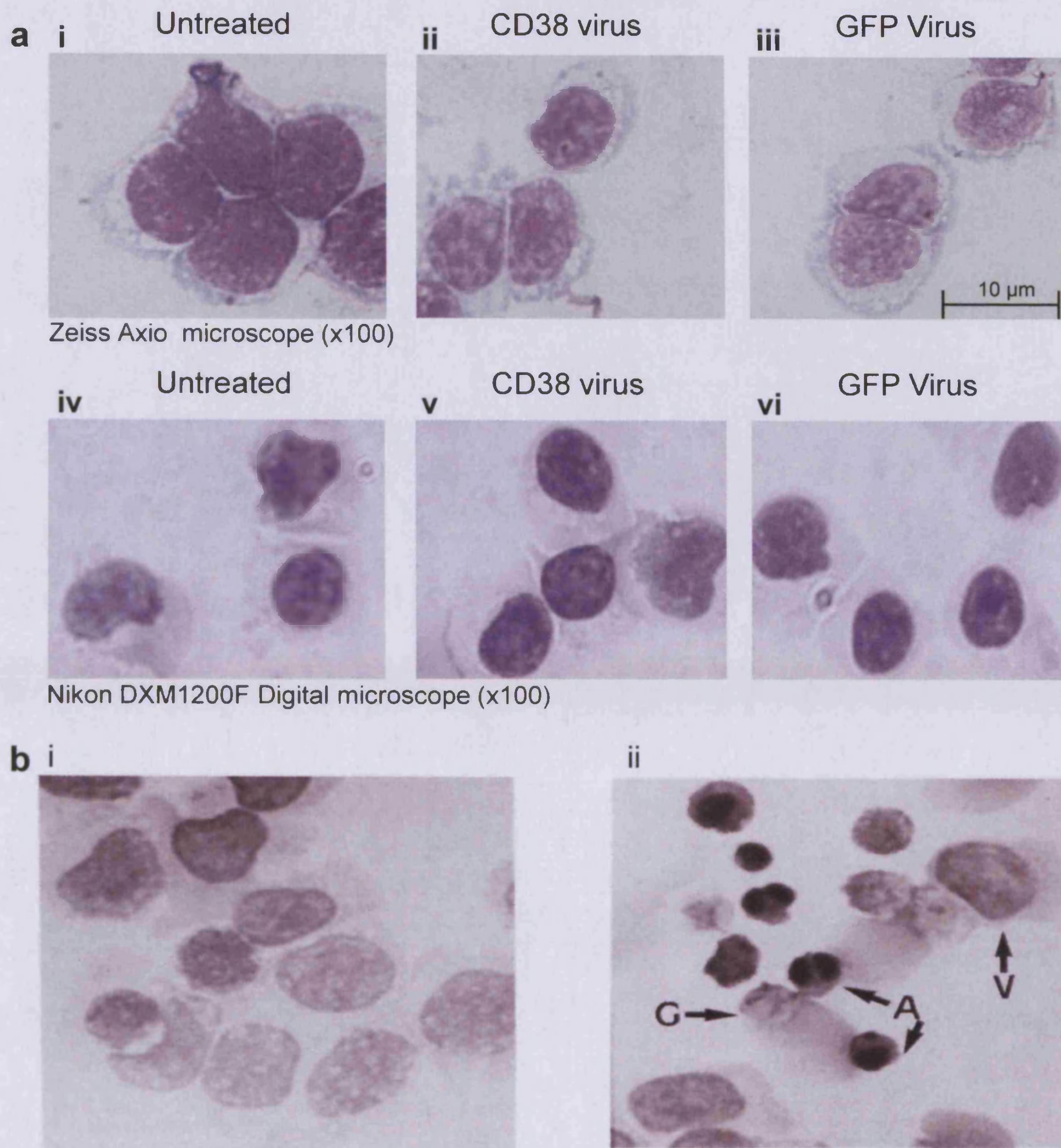


Figure 4.7 Morphology of untreated and virus treated CLL cells following 48 hours incubation.

(a) $1x10^6$ untreated (**i** and **iv**) CD38 virus treated (**ii** and **v**) and GFP virus treated CLL cells (**iii** and **vi**) were stained with Giemsa dye and analysed using a using a Zeiss Axio microscope (**i-iii**) or a Nikon DXM1200F Digital microscope (**iv-vi**). All images are at x100. (**b**) **(i)** Viable lymphocytes **(ii)** highlights the features of apoptotic lymphocytes. Both are adapted from: Fluckiger *et al.* (1994). (V, viable cells, A, cells undergoing apoptosis, showing chromatin condensation and nuclear fragmentation indicate apoptosis, G, ghost cells).

size of the cells with darkly stained chromatin clumps within the degrading nucleus. Therefore, morphological analysis of the CLL cells under the microscope clearly illustrates that the CLL cells are not undergoing apoptosis following 48 hours incubation with lentivirus. This work contradicts the annexin V/PI assay data and adds weight to the assertion that phosphatidylserine is aberrantly exposed on the surface of CLL cells following exposure to lentivirus and is not associated with the induction of apoptosis.

4.6 CD38 was lost from the surface of lentivirus treated CLL samples following incubation in co-culture

Due to the effects of the lentivirus on the CLL cell the annexin V/PI staining method was deemed not suitable for assessing CLL cell viability in treated samples. Forward and side scatter data illustrated that there was no significant difference in the viability of the CLL cells following the addition of CD38 or GFP virus at an MOI of 3.2 (Figure 4.2). These cell populations were incubated in liquid media for 48 hours before assessing viability. Within the *in vivo* CLL cell microenvironment various cell stimuli exist which are known to enhance the survival of the CLL cells (Caligaris-Cappio, 2003). CD31 is a molecule which is present on many cell types including endothelial cells and has been described as the only known ligand for CD38 (Deaglio *et al.*, 1996). It is possible that ligation of CD38 on the surface of the CLL cell may be required to induce CD38-mediated cell survival mechanisms. To further assess the role of CD38 in CLL cell survival, lentivirally transduced CLL cells were placed into co-culture with adherent fibroblasts expressing CD31. After 48 hours the CLL cells were removed from the co-culture and the percentage of apoptotic cells was determined using forward and side scatter. Sixty eight percent of the untransduced cells placed in liquid media were still viable after 48 hours (Figure 4.8.i). A sample of the same cells, which had been transduced using the CD38 virus, showed 87% viability over the same time period (Figure 4.8.ii). When co-cultured on control fibroblasts (NTL), CLL viability was enhanced in both the untransduced (93%) and CD38-transduced (92%) CLL samples (Figures 4.8.iii and iv respectively). Incubation with CD31-expressing fibroblasts further enhanced the CLL cell

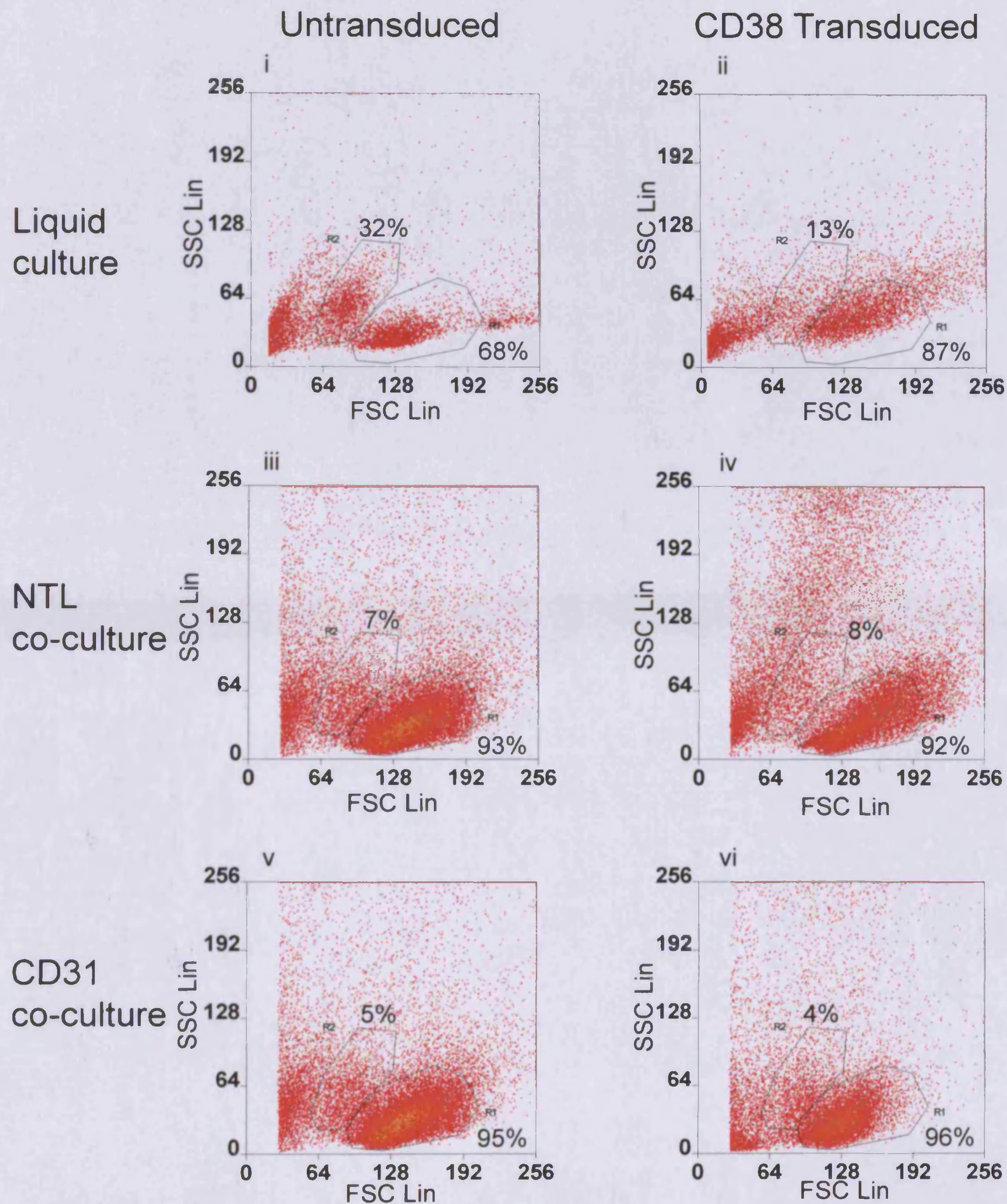


Figure 4.8 Co-culture enhanced the survival of untreated and CD38 transduced CLL cells.

$1x10^6$ CLL cells were placed into liquid media for 48 hours and the viability was assessed using forward and side scatter **(i)**. Additional samples were placed into co-culture with **(iii)** NTL and **(v)** CD31-expressing co-cultures. $1x10^6$ cells from the same patient were transduced using the CD38 virus and viability was assessed following 48 hours incubation in **(ii)** liquid media **(iv)** NTL and **(vi)** CD31-expressing co-culture.

viability. Untreated CLL cells showed 95% viability (Figure 4.8.v) and CD38-transduced cells 96% viability (Figure 4.8.vi).

Forward and side scatter profiles illustrated that an increased number of cells were viable following incubation with both NTL and CD31-expressing fibroblasts. The stable expression of CD38 on the surface following transduction is shown in Figure 3.12. These samples were maintained in liquid media for up to five days. To ensure that there was no loss of CD38 expression following incubation of the transduced cells in co-culture the CLL cells were stained for surface CD38. Figure 4.9.i illustrates CD38 transduced CLL cells incubated in liquid media. Sixty seven percent of the transduced cells expressed the CD38 antigen. Following incubation in NTL (Figure 4.9.iii) and CD31-expressing co-cultures (Figure 4.9.v) there was almost a complete loss of CD38 expression on the cell surface of the CLL cells. As a control, CLL cells from a CD38 positive patient were incubated for 48 hours in liquid media and under co-culture conditions. There was no loss of CD38 expression from the CD38 positive cells following incubation in liquid culture (90% CD38 expression) (Figure 4.9.ii), NTL co-culture (92% CD38 expression) (Figure 4.9.iv) or CD31-expressing co-culture (90% CD38) (Figure 4.9.vi). These data provide further evidence that the lentivirus had an effect on the CLL cell membrane which resulted in the shedding of CD38 from the surface when the cells were physically removed from the co-culture. Alternatively, it may be that virus-induced ectopic expression of CD38 results in the aberrant insertion of CD38 in the membranes of CLL cells which destabilises the protein and facilities its loss from the cell surface following cell-cell contact.

4.7 Incubating untransduced CLL cells with CD31-expressing co-culture enhanced survival

Figure 4.9 illustrates the loss of the CD38 antigen from the surface of the transduced CLL cells following incubation with co-culture. It is possible that the concentrated virus disrupts the CLL cell membrane resulting in the loss of CD38 following co-culture. Addition of lentivirus also promoted changes in the cell membrane that made the assessment of cell viability impossible using the annexin V/PI assay. Therefore, to assess the effect of CD38 on CLL cell

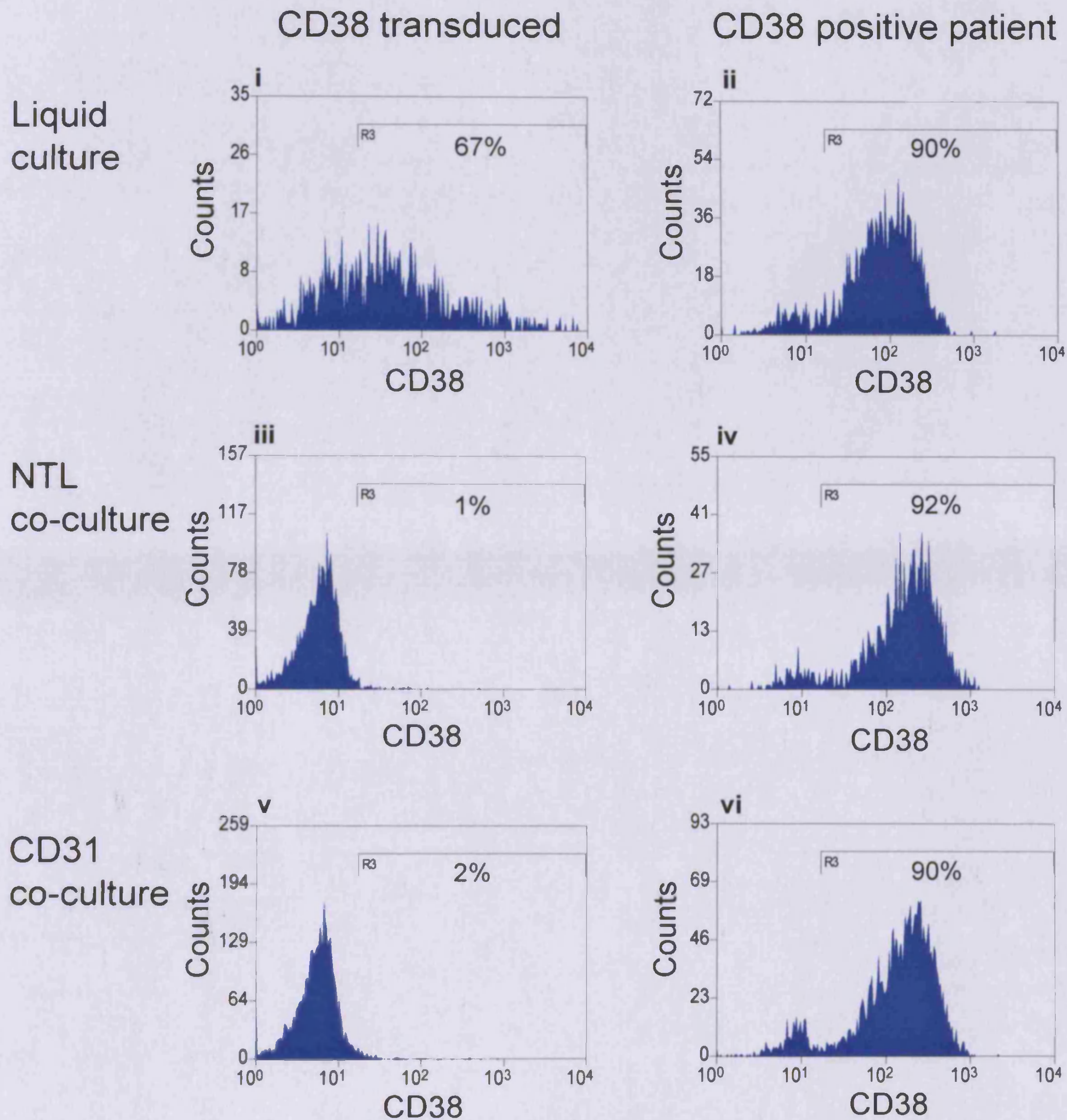


Figure 4.9 CD38 expression was lost from transduced CLL cells following incubation in co-culture.

1x10^6 CLL cells were transduced with CD38 virus and incubated in liquid media or co-culture for 48 hours. The cells were then removed and flow cytometry was used to assess CD38 expression on the surface of CLL cells incubated in **(i)** liquid media, **(iii)** NTL co-culture and **(v)** CD31-expressing co-culture. 1x10^6 CLL cells from a CD38 positive sample were incubated under the same conditions. CD38 expression was determined in cells incubated in **(ii)** liquid media, **(iv)** NTL co-culture and **(vi)** CD31-expressing co-culture.

survival, fresh patient samples expressing varying amounts of CD38 were incubated using co-culture conditions and annexin V/PI staining was carried out. Figure 4.10 illustrates the percentage of annexin V/PI positive CLL cells in a single sample following 48 hours in liquid media (Figure 4.10.i), NTL co-culture (Figure 4.10.ii) and CD31-expressing co-culture (Figure 4.10.iii). The percentage of annexin V/PI positive cells was 63.0%, 11.2% and 10.5% respectively. A total of fifteen patient samples were incubated for 48 hours under liquid and co-culture conditions and viability was assessed. An increase in CLL cell viability was observed following incubation with the CD31-expressing fibroblasts compared to liquid media ($P<0.001$) and compared to NTL co-culture ($P=0.008$) (Figure 4.11).

4.8 CD38 expression did not correlate with CLL cell survival following incubation in co-culture

Incubation with CD31-expressing co-culture enhanced the viability of the CLL cells. To determine whether CD38 played a role in this the constitutive CD38 expression was compared to the number of viable cells within the sample. For each patient the number of CD38 positive cells (percent) upon presentation was plotted against the number of annexin V/PI negative CLL cells (percent) incubated for 48 hours in CD31-expressing co-culture. Figure 4.12 Illustrates that there was no correlation between the percentage of CD38 expression and the viability of the sample ($P=0.357$). The data was analysed further and the difference between the viability of CLL samples incubated in CD31-expressing co-culture and the viability of CLL cells incubated in NTL co-culture was plotted against their initial CD38 expression. Figure 4.13 illustrates that there was a trend towards increased survival with increasing CD38 expression but the correlation was not significant from a total of fifteen patient samples investigated ($P=0.2$). There was also no significant correlation when the difference between the viability of CLL samples incubated in CD31-expressing co-culture and the viability of CLL cells incubated in liquid media was compared to initial CD38 expression ($P=0.59$).

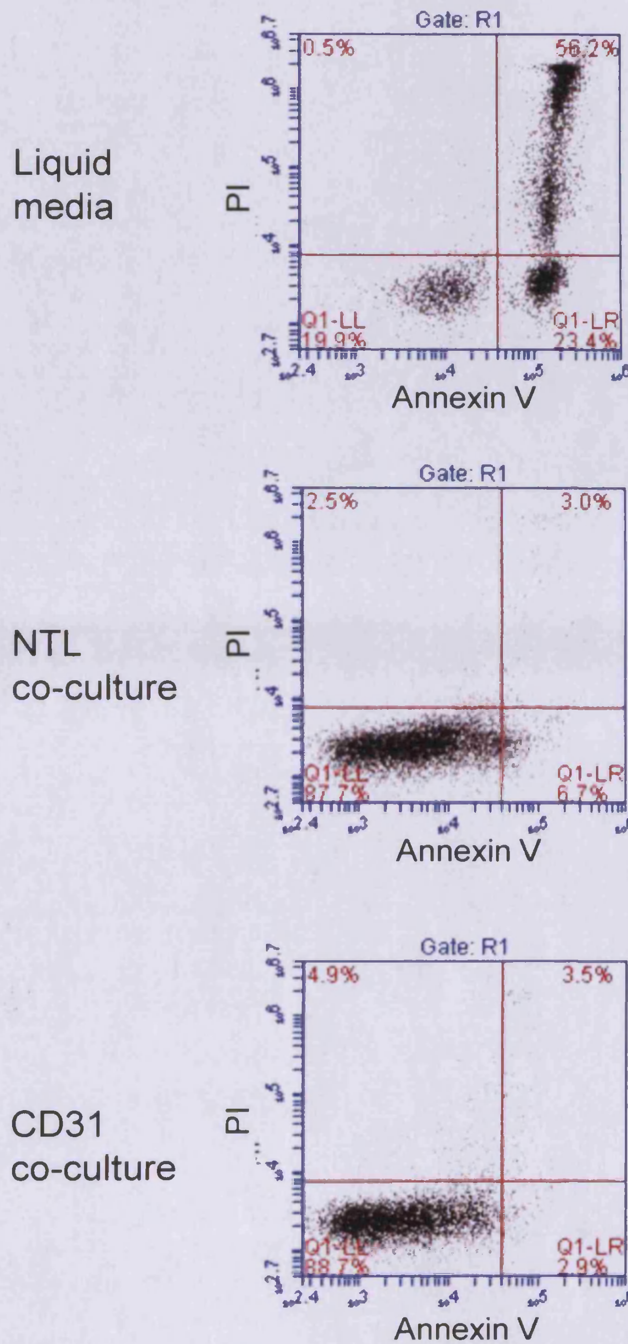


Figure 4.10 Annexin V/PI staining illustrated increased CLL cell viability following co-culture.

$1x10^6$ CLL cells were placed into culture and cell viability was assessed after 48 hours. Annexin V/PI staining was carried out on CLL cells incubated in **(i)** liquid media **(ii)** NTL co-culture and **(iii)** CD31-expressing co-culture.

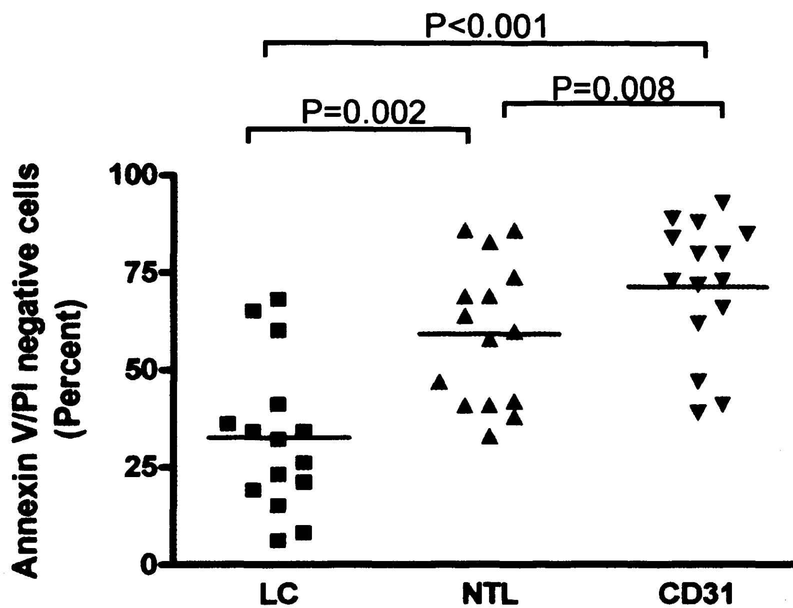


Figure 4.11 CLL cell viability was increased following incubation with CD31-expressing co-culture.

$1x10^6$ CLL cells were incubated in liquid media, NTL co-culture or CD31-expressing co-culture. Annexin V/PI staining was used to determine viability following 48 hours incubation. A total of 15 patient samples were assessed. A paired *t*-test was used to compare the viability between untreated and virus treated samples.

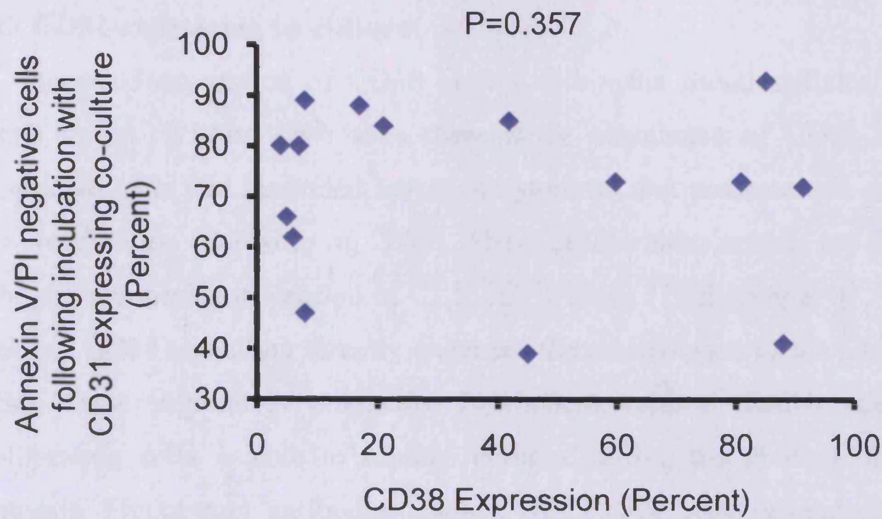


Figure 4.12 There was no correlation between CLL cell viability and CD38 expression.

The viability of each sample following 48 hours co-culture with CD31 expressing fibroblasts was compared to the native CD38 expression (Percent) at day 0. The Spearman test was used to correlate the viability of the sample with CD38 expression.

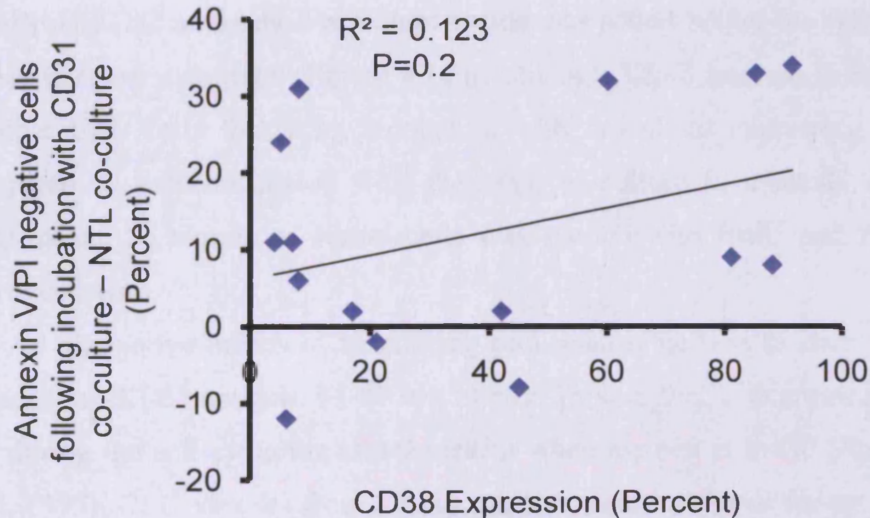


Figure 4.13 There was a trend towards increased CLL cell viability in samples expressing CD38 following incubation with CD31-expressing co-culture.

The difference between the viability of CLL samples incubated in CD31-expressing co-culture and the viability of CLL cells incubated in NTL co-culture was plotted against day 0 CD38 expression. The Spearman test was used to correlate the viability of the sample with CD38 expression.

4.9 The proliferation of CLL cells was enhanced following incubation with CD31-expressing co-culture

Increased expression of CD38 occurs within the pseudofollicles of the lymph nodes. Within these sites there is an abundance of CD31 on the endothelial cells and increased levels of cytokines that promote cell survival and proliferation (Patten *et al*, 2008). These proliferation centres are thought to be the predominant location of CLL cell division (Herishanu *et al.*, 2011). Whether CD38 signalling directly enhances the proliferation of the CLL cells within these regions is unknown. Bromodeoxyuridine (BrdU) added to proliferating cells is able to replace thymine during the process of DNA synthesis. Fluorescent antibodies against BrdU have been generated which can bind to the BrdU molecule incorporated within the newly synthesised DNA and can be detected by flow cytometry. When combined with PI staining for total DNA, a detailed representation of the cells' position within the cell cycle can be obtained. CLL samples were placed into co-culture for 32 hours. BrdU was then added and the sample was incubated in co-culture for a further 16 hours. The cells were fixed, permeabilised and incubated with anti-BrdU-FITC antibody. Propidium iodide was added before the cells were run on the flow cytometer. Figure 4.14 illustrates a 3-fold increase in BrdU/PI positive CLL cells following incubation with co-culture expressing CD31 compared to cells incubated with the NTL co-culture in a single sample investigated. A sample of Jurkat cells was stained with BrdU and PI as a positive control.

An alternative means of identifying proliferating cells is to stain for the intracellular Ki-67 antigen. Ki-67 is a nuclear protein that is expressed in the cell during the cell cycle but is not present when the cell is in G0 (Ross and Hall, 1995). CLL samples from twenty patients were incubated for up to five days in fibroblast co-culture expressing CD31 and the cells were stained for intracellular Ki-67. CLL cells co-cultured with NTL fibroblasts were used as a control. To further mimic the lymph node microenvironment, IL-2 was added to the co-culture media at a concentration of 100IU/ml. This cytokine has been shown to result in inhibition of apoptosis in CLL cells and is secreted in the CLL microenvironment by T-cells (Decker *et al.*, 2010). CD5/CD19 positive CLL cells were fixed, permeabilised and stained using an anti-Ki-67-

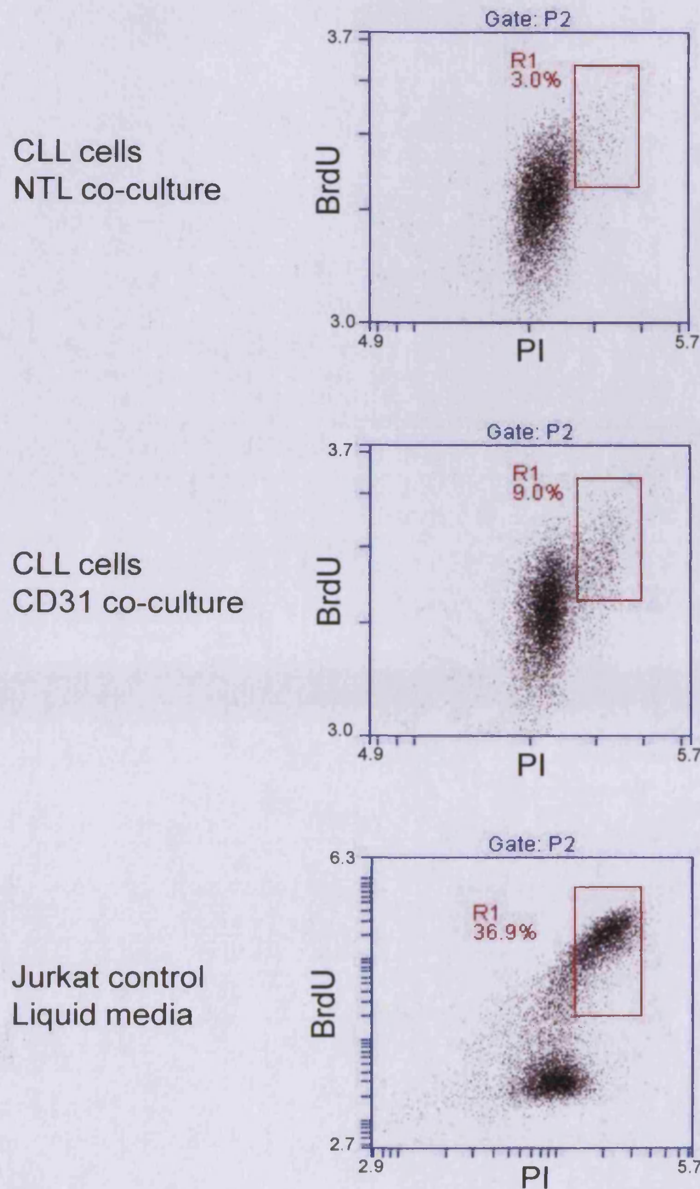


Figure 4.14 CLL cells incubated in CD31 co-culture showed increased BrdU incorporation.

1x10^6 CLL cells were incubated in NTL or CD31-expressing co-culture for 32 hours. 10μM BrdU was added and the sample was incubated for a further for 16 hours. The sample was then fixed and permeabilised before the addition of BrdU FITC antibody. Flow cytometry was used to determine the number of BrdU positive CLL cells following incubation with NTL and CD31-expressing co-culture. Jurkat cells were incubated in liquid culture for 5 hours with BrdU as a positive control.

FITC antibody. Figure 4.15.i illustrates Ki-67 expression in the sample incubated for two days with NTL co-culture. There were a very small number of cells expressing Ki-67 (0.6%). Following incubation with CD31-expressing fibroblasts, more than five percent of the CLL cells were expressing Ki-67 (Figure 4.15.ii). Figures 4.16.a, b and c illustrate the Ki-67 expression in twenty CLL patient samples following two and five days in co-culture. After two days the CLL sample incubated with the CD31-expressing co-culture showed a significant increase in the number of cells expressing Ki-67 compared to the sample incubated with the NTL control fibroblasts ($p=<0.001$) (Figure 4.16.a. and b). However, there was no significant difference in the expression of Ki-67 between the CLL cells incubated for five days on CD31-expressing and NTL co-culture ($p=0.097$). This was probably due to the increase in Ki-67 expression in CLL cells incubated within the NTL co-culture at this time point (Figure 4.16.a and c).

4.10 Ki-67 expression correlated with increased expression of CD38

Increased Ki-67 expression was observed within 48 hours when co-culturing with CD31-expressing fibroblasts. The MFI values for Ki-67 expression in CD31 co-culture were then correlated with constitutive CD38 expression on day 0. Figure 4.17 illustrates that the increase in Ki-67 following co-culture significantly correlated with the basal expression of CD38 on the surface of the CLL cells ($R^2=0.389$, $p<0.001$). Ki-67 expression was then plotted for CD38 positive and negative CLL samples (cut-off 20%). There was a significant increase in Ki-67 observed in CD38 positive patient samples ($P=0.003$) (Figure 4.18).

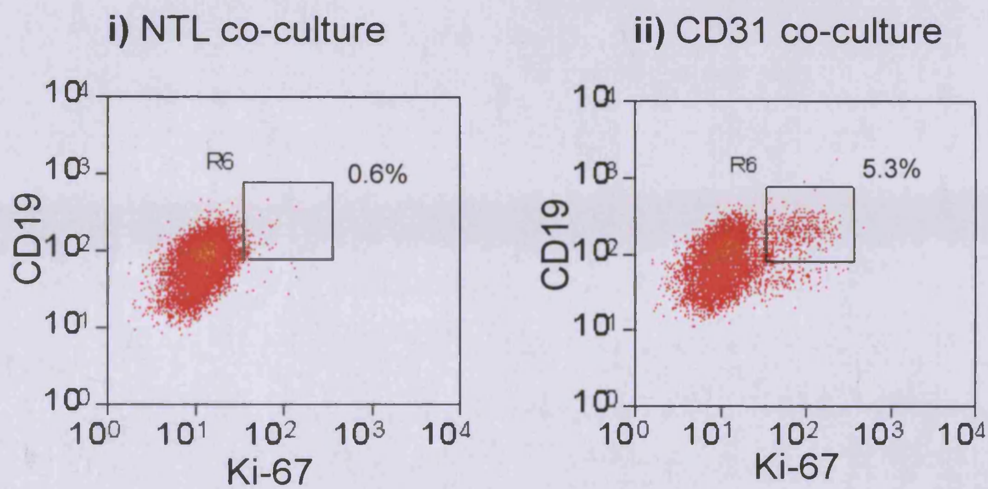


Figure 4.15 Ki-67 expression was increased in a CLL sample following 2 days incubation with CD31-expressing fibroblast co-culture.

1x10^6 CLL cells were incubated with **i)** NTL and **ii)** CD31-expressing co-culture for 2 days. Flow cytometry was used to determine the Ki-67 expression (Ki-67 FITC).

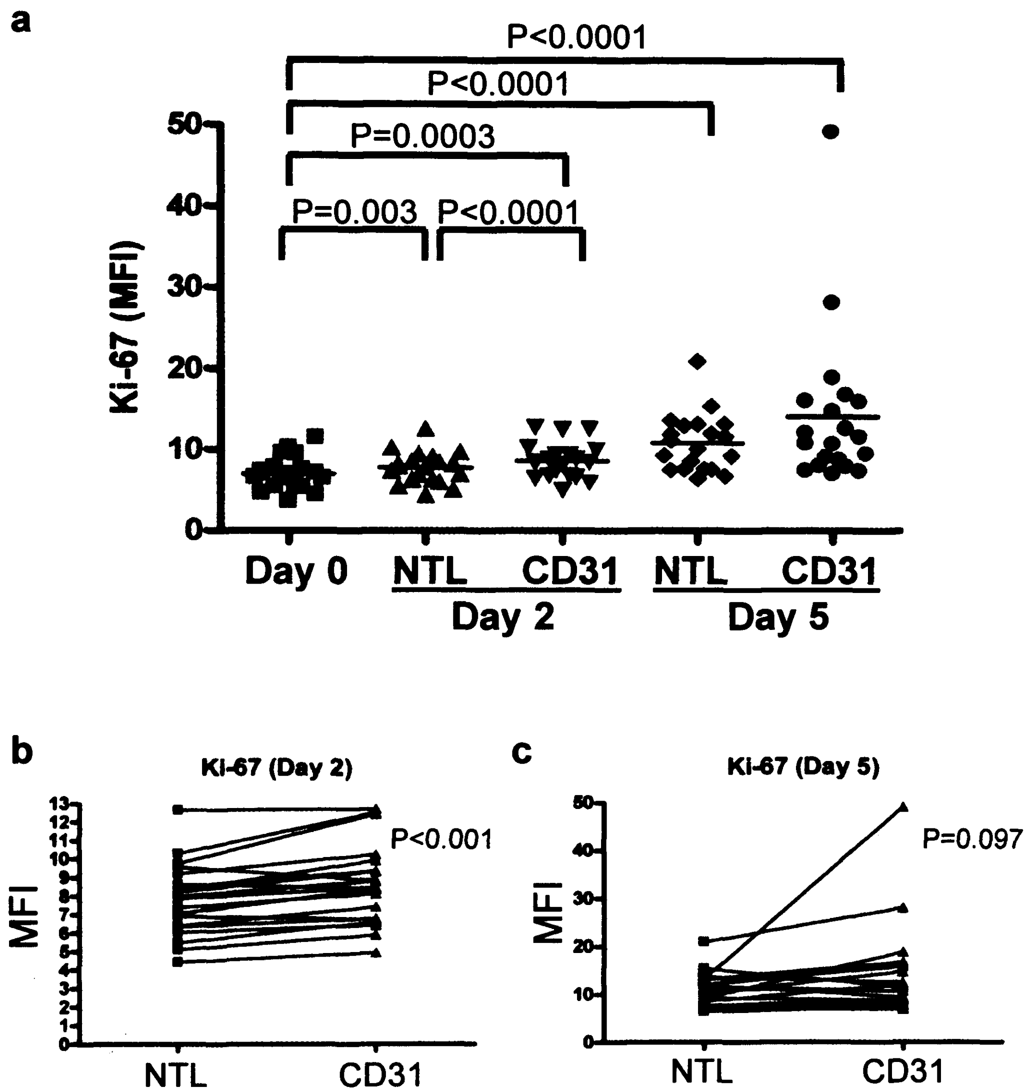


Figure 4.16 Ki-67 expression was increased in CLL samples following 2 days incubation with CD31-expressing fibroblast co-culture.

$1x10^6$ CLL cells were incubated with NTL and CD31-expressing co-culture for 2 and 5 days. Flow cytometry was used to determine the Ki-67 expression in 20 CLL samples. A paired *t*-test was used to compare Ki-67 expression in the samples tested. **(b)** and **(c)** illustrate the Ki-67 expression at day 2 and 5 respectively. The paired samples within NTL and CD31-expressing co-culture are highlighted.

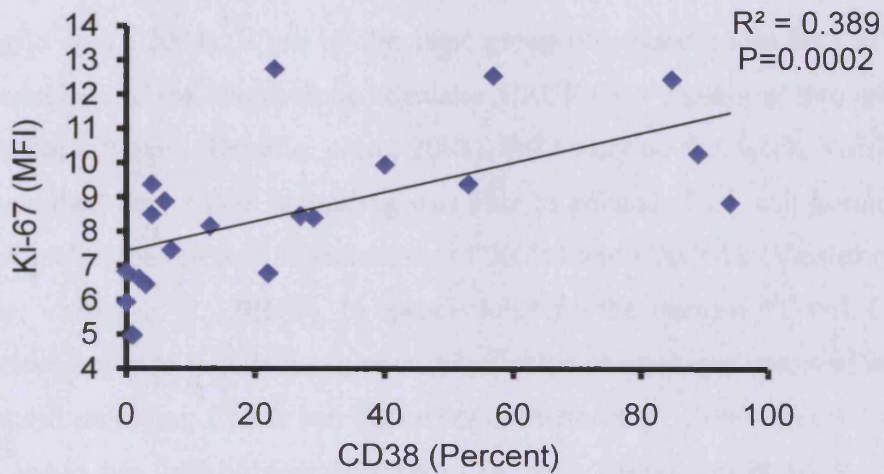


Figure 4.17 A significant correlation was observed between native CD38 expression and Ki-67 expression following incubation with CD31-expressing co-culture.

Ki-67 expression (MFI at 48 hours) was plotted against CD38 expression (percent at day 0) for 20 patient samples. The Spearman test was used to correlate Ki-67 expression with CD38 expression.

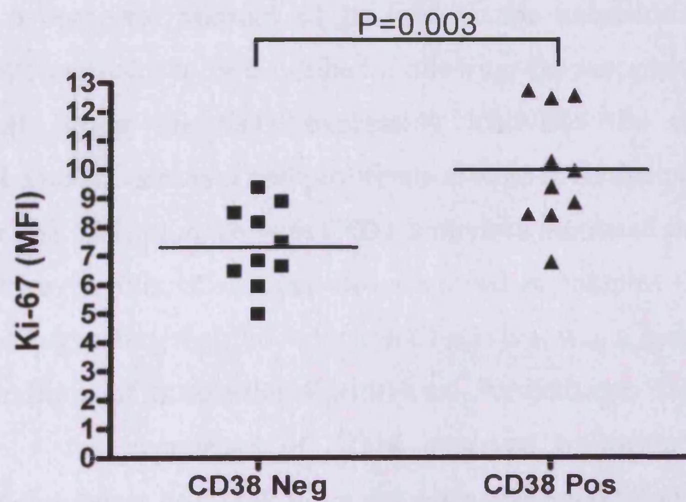


Figure 4.18 Ki-67 expression was significantly increased in CD38 positive patients.

Ki-67 expression (MFI) was observed in CD38 negative (CD38 <20%) and CD38 positive (CD38>20%) patient samples. An unpaired *t*-test was used to compare Ki-67 expression in CD38 positive and negative samples.

4.11 Discussion

CD38 is involved in cell signalling and has been shown to interact with the B-cell receptor and CD19 in the induction of proliferation in CLL cells (Deaglio *et al.*, 2003). Work by the same group illustrated a role for CD38 in the induction of the chemotactic regulator CXCR4 via signalling through the CD38/Zap-70 axis (Deaglio. *et al.*, 2008). Following up this work, Vaisitti *et al.* described how CD38 signalling was able to enhance CLL cell homing to the lymph nodes through modulation of CXCR4 and CXCL12 (Vaisitti et al., 2010a, Vaisitti et al., 2010b). In association with the integrin CD49d, CD38 was also shown to induce the expression of other chemokines involved in cell migration including CCL3 and CCL4 (Zucchetto *et al.*, 2009). Work by our own group has highlighted differences in gene expression between CD38 positive and negative cells from the same patient and proposed a role for CD38 in angiogenesis through the induction of VEGF (Pepper *et al.*, 2007). Investigations carried out by Damle *et al.* at around the same time illustrated that the CD38 positive sub-set expressed markers of proliferation (Ki-67) and survival (Bcl-2) (Damle *et al.*, 2007). Even though a wealth of information highlighting the actions of CD38 in CLL has been presented over the past decade, a complete account of its role in the induction of survival and proliferation remains to be described. Following the successful transduction of CLL cells using the CD38-expressing lentivirus the effects of CD38 expression on cell survival and proliferation were investigated.

Treating CLL samples with CD38 lentivirus increased the viability of the cells. However, this effect was also observed in samples treated with GFP lentivirus suggesting that the induction of survival was a generic consequence of the addition of concentrated lentivirus. Furthermore, the dose-dependent increase in the expression of CD38 observed following the addition of increasing amounts of CD38 virus did not correspond to an increase in CLL cell viability (Figure 4.1.c). The increased survival observed may have been due to an anti-apoptotic response from the cell. Following insult, the induction of protein stabilising chaperones (such as heat shock proteins) may occur to protect the cell from immediate death (Sedger and Ruby, 1994, Jaattela *et al.*, 1998). HSP-70 is highly up-regulated in lymphocytes following infection by the HIV-1 lentivirus (Iordanskiy *et al.*, 2004) and HSP-90 has previously

been shown to bind and stabilise Bcl-2 to enhance survival in primary cells and leukaemic cell lines (Dias *et al.*, 2002). The innate immune response to viral infection is mediated through toll-like receptors on the cell surface (Xagorari and Chlichlia, 2008). Treatment of the cells with the lentivirus is likely to have induced activation and cell survival through toll-like receptor signalling (Gelman *et al.*, 2004). Any subtle effects of CD38 on the viability of the cell might be masked by such processes.

Forward and side scatter from flow cytometric plots was used to determine apoptosis in the CLL samples. In addition CLL cells from untreated and virus treated preparations were stained with annexin V to observe the expression of surface phosphatidylserine and the PI dye was added to identify dead cells. These experiments suggested that a large proportion of the CLL cells were undergoing apoptosis following one hour incubation with lentivirus. However, forward and side scatter profiles did not suggest that this was the case. The HIV-1 derived lentiviral vector used in the transduction experiments was pseudotyped with the VSVG envelope which allows non-specific binding of the virus to the cell membrane and entry into an extensive range of cell types (Farley *et al.*, 2007). It seems possible that the interaction of concentrated virus with the fragile CLL cell membrane may have caused disruption to the lipid bilayer allowing the expression of phosphatidylserine on the cell surface. Even with an abundance of intracellular ATP (characteristic of a viable cell) such an effect would give the false impression that the cell was entering the early stages of programmed cell death.

To further assess the effects of the lentivirus, untreated and virus treated CLL cells were stained using the Giemsa dye and observed under the microscope at x100. Figure 4.7 illustrates that there were very few differences in CLL cells treated with either CD38 or GFP lentivirus compared to the untreated control. The absence of apoptotic features (which are highlighted in Figure 4.7.b.ii) suggests that the treated cells were viable and that the annexin V binding was not due to the induction of apoptosis, but to changes in the CLL cell following the addition of concentrated lentivirus.

Expressing CD38 on the surface did not have an effect on the viability of the CLL cell. To investigate whether ligating the CD38 antigen would result in increased cell survival, transduced samples were placed into CD31-

expressing co-cultures. Removal of the cells from co-culture caused a substantial loss of the CD38 antigen from the surface of the CLL cells. This was seen to occur in transduced cells incubated with both the NTL and the CD31-expressing fibroblasts suggesting that it was not being caused by direct interaction between virus-induced CD38 and its cognate ligand CD31 (Figures 4.9.iii and v). A CD38 positive patient sample was run in parallel to observe the effects of co-culture on native CD38 expression and there was no loss of the antigen following 48 hours incubation (Figure 4.9 ii, iv and vi). The ability of the lentivirus to induce changes in the CLL cell membrane was evidenced by the marked increase in annexin V staining. Such changes may also destabilise the ectopic insertion of CD38 into the CLL membranes and result in the loss of the CD38 antigen from the surface of the CLL cells following co-culture. The loss of CD38 on the surface of the CLL cells under these conditions made it impossible to assess the effect of CD38 on cell viability.

Investigating CLL cell viability following the induction of CD38 on the surface of the cell was challenging due to the effect of the lentivirus on the cell. Therefore, to determine whether CD38 has any effect on CLL cell viability, annexin V/PI staining was carried out on untransduced CLL samples with varying degrees of endogenous CD38 expression. There was a significant increase in cell survival following incubation with CD31-expressing co-culture compared to NTL co-culture (P=0.008). These results suggest that stimulation of CD38 via its ligand CD31 enhances survival within the *in vitro* co-culture environment. The increased viability was then correlated with the basal CD38 expression (percent at day zero). There was no significant correlation between the cell viability and the CD38 expression in the 15 samples investigated (P=0.357). This result does not rule out the possibility that CD38 is involved in inducing CLL survival. The CD31 expression on the surface of the co-culture may have exceeded that observed *in vivo* resulting in a marked increase in CD38 stimulation. It is possible that the threshold for CD38 signalling was reached in all samples regardless of the CD38 surface expression.

Until recently CLL was thought to be a disease of failed apoptosis; a slowly progressive disease, characterised by the accumulation of clonal B-cells over a number of years (Caligaris-Cappio and Hamblin, 1999, Pepper *et*

al., 1999). This was shown not to be the case by *in vivo* heavy water experiments carried out by Messmer *et al.* who estimated that the turnover of the CLL cells was as high as 1% of the clone per day (Messmer *et al.*, 2005). In this investigation the authors observed no correlation between the rate of proliferation and *IGHV* mutation status, CD38 or ZAP-70, though only nineteen patients were included in the study. In a subsequent study Callisano *et al.* described a link between proliferation and CD38 expression, highlighting an association between increased levels of the chemokine receptor CXCR4, lymphoid infiltration and CD38 positivity in a poor risk cohort (Calissano *et al.*, 2009). To further examine whether CD38 has a role in the proliferation of CLL cells, CD31-expressing co-culture systems were set up to mimic the microenvironment and stimulate CD38 on the CLL cell surface. Incorporation of BrdU into dividing cells is an established method of assessing proliferation. Figure 4.14 illustrates an increase in BrdU incorporation into CLL cells incubated with CD31-expressing fibroblasts compared to the NTL control. A similar increase was observed when staining for the proliferation marker Ki-67 and a significant increase was observed in a cohort of twenty patient samples following stimulation of the CLL cells with CD31-expressing co-culture over a period of two days ($P<0.001$) (Figure 4.16.a and b). Both the BrdU and Ki-67 staining methods illustrate an increase in the proliferation of CLL cells within CD31-expressing co-culture. This suggests that CD38 ligation results in downstream signals that induce proliferation in the CLL cells. Prior to incubation with co-culture each of the CLL samples was phenotyped for CD38 expression. The percentage CD38 values were then plotted against Ki-67 MFI from samples incubated with CD31-expressing co-culture. Figure 4.17 illustrates that there was a significant correlation between the expression of CD38 and Ki-67 ($R^2=0.389$; $P<0.001$). Subsequent analysis identified a significant increase in Ki-67 expression in CD38 positive patients compared to CD38 negative patients (Figure 4.18) ($P=0.003$). A recent publication by Khoudoleever *et al.* reported significantly higher Ki-67 expression in tissues from the lymph node and spleen compared to peripheral blood and bone marrow. They also describe a significant correlation between the expression of CD38 and Ki-67 in the bone marrow compartment (Khoudoleeva *et al.*, 2011). Together these data provide

convincing evidence that CD38 plays a role in the induction of proliferation in CLL cells following stimulation with CD31.

Incubation with CD31-expressing co-culture was shown to enhance CLL cell survival and proliferation. It must be noted that the CD31 ligand also has the ability to bind CD31 present on the lymphocyte surface in a homotypic interaction causing downstream signalling and changes in cell adhesion (Sun *et al.*, 1996, Fawcett *et al.*, 1995, Brown *et al.*, 2002). In a recent publication, Poggi *et al* described how ligation of CD31 on the CLL cell surface resulted in cell activation through PI3K/Akt mediated NF-κB induction. The cells also exhibited increased survival following stimulation of CD31 with a CD31-agonist (Poggi *et al.*, 2010). Of the twenty CLL samples phenotyped in this present study, seven were stained for surface CD31. No significant correlation was observed between CD31 expression (MFI) and Ki-67 expression (MFI) (R^2=0.59, P=0.12) (Appendix 4.a).

The aim of this chapter was to investigate whether the expression of CD38 on the CLL cell surface had an effect on survival and proliferation. Interpreting the results from viability studies was challenging due to the effects of the lentivirus on the CLL cells. Such effects also confounded experiments carried out to stimulate CD38 with the use of CD31-expressing co-culture. Untreated CLL cells incubated in CD31-expressing co-cultures illustrated increased survival and proliferation compared to cells incubated in non-transduced co-culture. Within these samples the increased proliferation significantly correlated with CD38 expression. The underlying mechanisms by which CD38 signalling has an effect on CLL cell survival and proliferation is unknown. Further investigations are required to determine whether the induction of CD38 induces changes in gene expression that favour such events.

Chapter 5. The genetic modification of CLL cells causes changes in gene expression

5.1 Introduction

Gene expression within CLL cells has been widely studied over the past decade. Early investigations illustrated that the gene expression profile of the CLL cell resembles that of a memory B-cell (Klein *et al.*, 2001). Larger studies identified genes differentially expressed within good and poor prognostic groups (Rosenwald *et al.*, 2001, Jelinek *et al.*, 2003, Falt *et al.*, 2005). In 2001, a study by Stratowa *et al.* was one of the first to show the differential expression of functional genes in CLL cells. The group illustrated an increase in the expression of genes coding for mediators of lymphocyte trafficking in patients with poor disease outcome. These included the cell adhesion molecules L-selectin and integrin-β2 and the cytokines IL-1β and IL-8 (Stratowa *et al.*, 2001). A subsequent study illustrated an increased expression of genes involved in angiogenesis (angiopoietin 2) and oncogenesis (PIM2) in CD38+/Zap-70+ patients (Huttmann *et al.*, 2006). In 2007, Pepper *et al.* illustrated that CD38 positive and CD38 negative cells derived from the same (bi-modal) patient illustrated differences in gene expression (Pepper *et al.*, 2007). The CD38 positive cell subset showed increased expression of the potent angiogenic mediator VEGF. Increased expression of IL-1β and Mcl-1 was also observed in these cells presenting a biological rationale for the poor prognosis seen in CD38 positive CLL. More recently a publication by Stamatopoulos *et al.* illustrated a difference between the gene expression profiles of ZAP-70 positive and negative cells, the former expressing genes that enhanced the trans-endothelial migration of CLL cells (Stamatopoulos *et al.*, 2009). In summary, gene expression profiling has proved a useful tool in identifying genes that are differentially expressed in good and poor prognostic groups of CLL patients. Genes with the potential to induce cell survival or disease progression have been identified which can be studied in more depth and exploited as targets for therapy.

In chapter three, a HIV-1 derived lentivirus was developed to induce the expression of CD38 in primary CD38 negative CLL cells. CD38 has been

described as a signalling molecule in CLL (Deaglio *et al.*, 2003, Morabito *et al.*, 2006), so the aim of this chapter was to determine whether there were any changes in gene expression following the induction of CD38 on the cell surface. QRT-PCR and microarray techniques were used to observe gene expression in untreated samples and CLL samples transduced using CD38 and GFP lentivirus.

5.2 CD38 mRNA was highly expressed in multiple CD38 virus treated samples

Flow cytometry illustrated that CD38 was highly expressed on the surface of CLL cells following the addition of CD38 lentivirus (Figure 3.12). Following treatment of six samples with CD38 or GFP virus QRT-PCR was carried out to quantify the expression of CD38. Figure 5.1.a illustrates the high expression of CD38 in six CD38 virus treated samples (Mean=17,800 relative fold increase, ±12,173 (±SD)). In contrast, a relatively small increase in CD38 transcription was observed following the addition of GFP virus (Mean=7, ±14 (±SD)) (Figure 5.1.b). The increase illustrated corresponds to the fold increase of CD38 mRNA compared to the expression of CD38 in the untreated sample and relative to the expression of the Abelson housekeeping gene (ABL). It should be noted that the large fold-change increase in CD38 expression was in part a product of the very low basal level of transcription of this gene in the CD38 negative samples chosen for genetic modification. This also explains why there was a 7-fold increase in CD38 transcription in the GFP virus treated cells.

For all QRT-PCR experiments the relative fold increase (RFI) was determined using the formula:

$$\mathbf{RFI = 2^{[-(Gene\ of\ interest\ ct\ -\ ABL\ ct\ X)\ -\ (Gene\ of\ interest\ ct\ -\ ABL\ ct\ Y)]}}$$

Where ct = Crossing point of linear amplification

X = Virus treated test sample

Y = Untreated sample

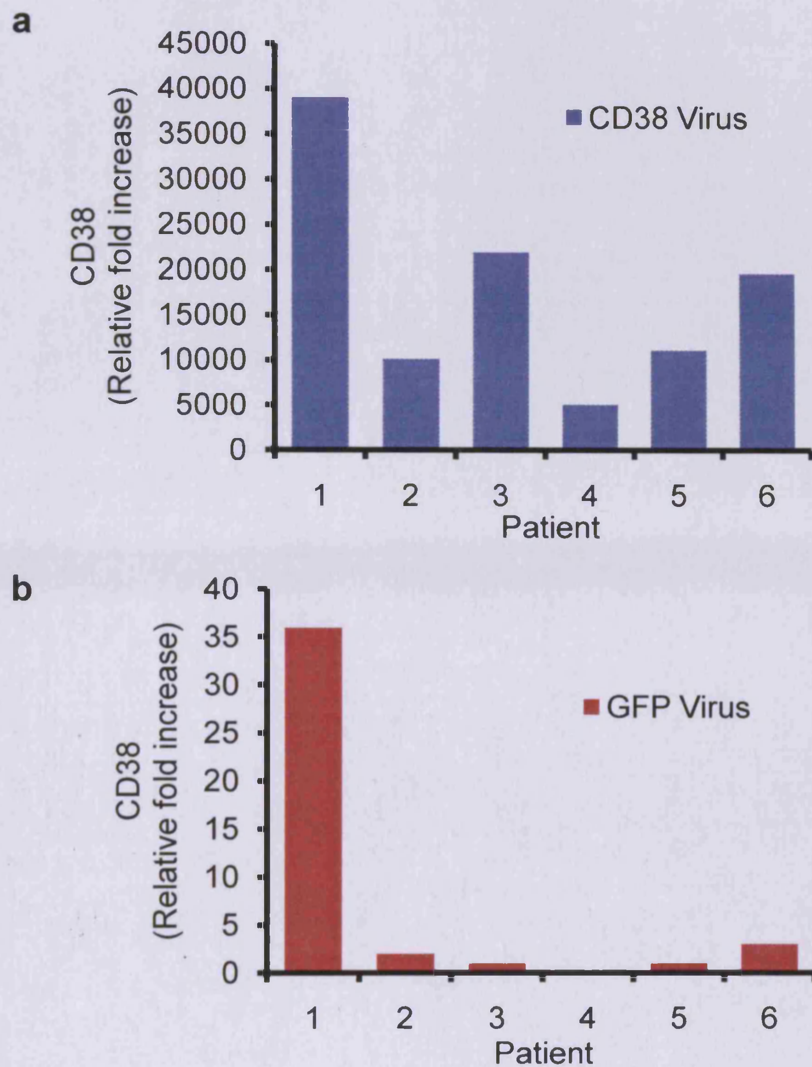


Figure 5.1 CD38 was highly expressed in CLL samples treated with (a) CD38 virus but not (b) GFP virus.

(a) Six patient samples were treated with CD38 lentivirus at an MOI of 3.2 and left for 48 hours in liquid media. RNA was extracted from $1x10^6$ CLL cells and QRT-PCR was carried out to determine CD38 expression. **(b)** Samples from the same six patients were treated with an equal amount of GFP virus and CD38 expression was determined.

5.3 VEGF expression was increased following the addition of lentivirus

There are six VEGF isoforms which bind to at least two VEGF receptors to induce angiogenesis in local microvessels (Judith Harmey, 2004). The first described and the most potent isoform is VEGF-A (referred to as VEGF). VEGF has been shown to be up-regulated in CLL cells expressing the CD38 antigen (Pepper *et al.*, 2007). To observe whether over-expression of CD38 would induce VEGF expression in CLL cells, CD38 negative patient samples were transduced and QRT-PCR was utilised to observe CD38 and VEGF gene expression. An MOI of 3.2 was used for both the CD38 and control GFP virus. Untreated, CD38 virus and GFP control virus treated samples were incubated for a period of two days to allow protein expression. Figure 5.2 illustrates VEGF gene expression in six virally treated samples relative to the expression in the untreated control (normalised to 1). VEGF expression was significantly increased in all of the CLL cells treated with the CD38-expressing virus. However, this was also the case in CLL cells treated with GFP virus. Three out of six of the samples tested showed a relative increase in VEGF transcription when transduced with the CD38-expressing lentivirus. However, the rate of transcription was variable and overall there was no significant difference in VEGF expression between CD38 virus and GFP virus treated samples (P =0.43).

5.4 Analysis of CLL cell supernatant using ELISA identified an increase in VEGF following transduction with lentivirus

VEGF is a soluble molecule which binds to at least two VEGF receptors on the cell surface. It is readily released from the cell and its actions can be autocrine or paracrine (Farahani *et al.*, 2005, Till *et al.*, 2005). VEGF gene expression was shown to be increased in both the CD38 and GFP lentivirally transduced samples. To observe whether VEGF protein was increased in the sample supernatant a VEGF-specific ELISA was carried out on six untransduced, CD38 virus treated and GFP virus treated samples. Figure 5.3 illustrates the increase in VEGF protein in the supernatant of lentivirus treated samples. There was higher expression in the supernatant of CLL cells treated with CD38 virus compared to GFP virus, although this difference was not significant (P=0.645).

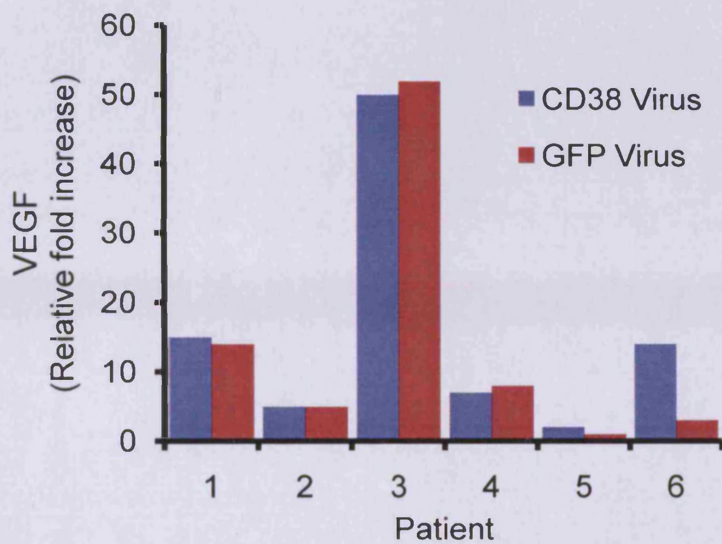


Figure 5.2 VEGF was heterogeneously over-expressed in CD38 and GFP lentivirus treated samples.

RNA was extracted from $1x10^6$ CLL cells and QRT-PCR was carried out to determine VEGF expression in six virus treated patient samples.

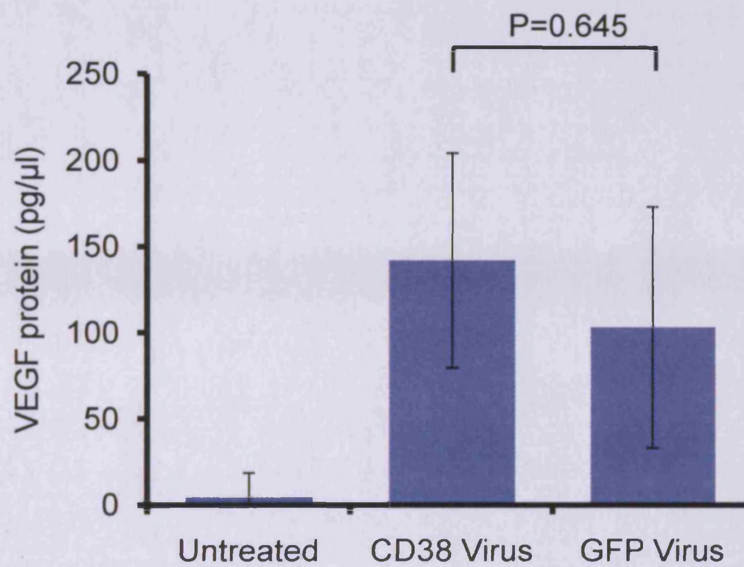


Figure 5.3 VEGF protein was over-expressed in lentivirus treated samples.

The supernatant from six untreated and lentivirus treated CLL samples was investigated for VEGF protein expression using an ELISA. A paired *t*-test was used to observe whether there was a significant difference in VEGF protein expression.

5.5 VEGF gene expression increased in a dose dependent manner following the addition of increasing amounts of CD38 virus

In the above experiments an MOI of 3.2 was used to transduce over 80% of the CLL sample. The expression of VEGF was increased in the samples expressing CD38 but was also expressed in GFP treated samples (with the exception of sample six (Figure 5.2)). Addition of the lentivirus therefore seemed to have an effect on the induction of VEGF within the CLL sample. To further investigate whether the expression of CD38 had any effect on the induction of VEGF, increasing amounts of CD38 lentivirus were added to a CD38 negative CLL sample and VEGF and CD38 were measured by QRT-PCR. Equal amounts of GFP lentivirus were added to the CLL sample as a control. The addition of increasing amounts of CD38 lentivirus resulted in a dose-dependent increase in the expression of VEGF (Figure 5.4). Even though the induction of VEGF was also observed using increasing amounts of GFP virus, the levels of VEGF expression in the CD38 virus treated samples were much higher when an MOI of above 20 was used. This would suggest that the expression of CD38 on the surface of the CLL cells was associated with the induction of VEGF. Figure 5.5 plots the relative gene expression of VEGF and CD38. There was a significant correlation between the expression of VEGF and CD38 in the samples treated with increasing amounts of CD38 virus (R^2=0.964) ($P<0.001$).

5.6 IL-1β and MCL1 expression were increased in CLL samples following transduction with lentivirus

IL-1β is a cytokine which is able to induce survival and proliferation in lymphocytes. This molecule was shown to be up-regulated in CD38 positive CLL cells compared to their CD38 negative counterparts (Pepper *et al.*, 2007). IL-1β was measured in six untreated and virus treated samples using QRT-PCR. Figure 5.6 illustrates that IL-1β expression was increased in CLL samples treated with either CD38 or control GFP lentivirus compared to the untreated sample. The expression of IL-1β was heterogeneous following lentiviral transduction and there was no significant difference in IL-1β expression between samples treated with CD38 virus and those treated with GFP virus ($P=0.76$).

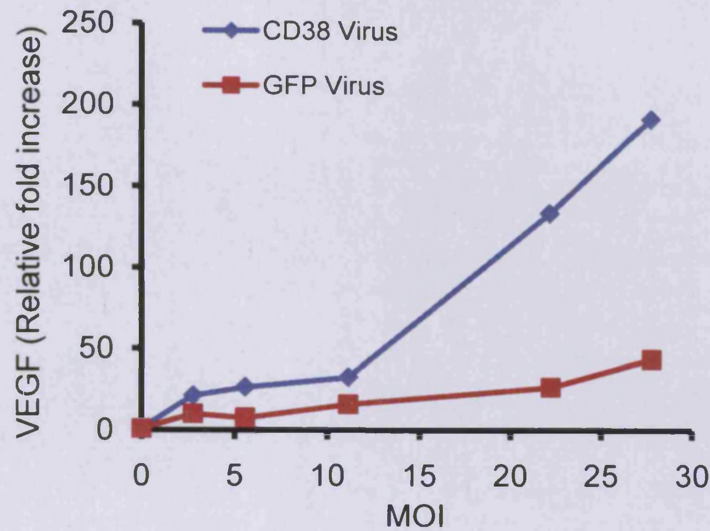


Figure 5.4 VEGF was induced following the addition of increasing amounts of CD38 virus.

Increasing amounts of CD38 or GFP lentivirus were added to CLL cells from a single patient and VEGF expression was determined by QRT-PCR.

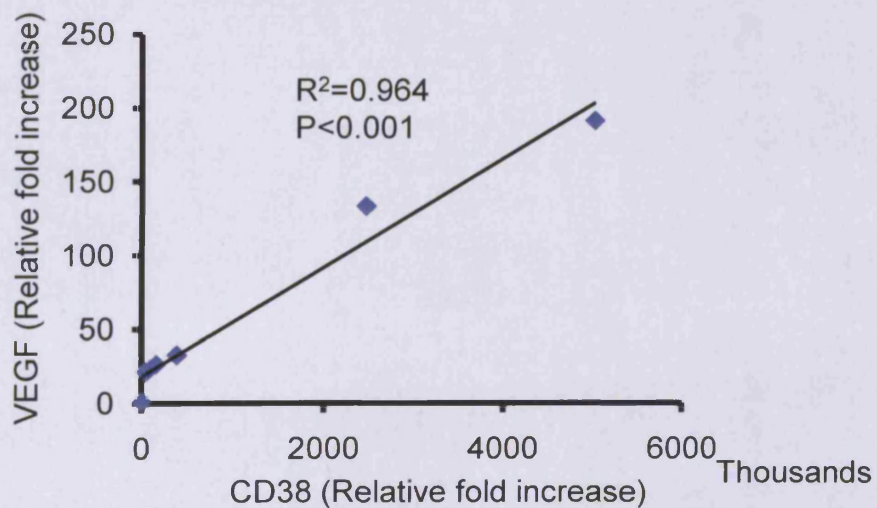


Figure 5.5 VEGF expression correlated with CD38 expression in CD38 virus treated samples.

Following the addition of increasing amounts of CD38 lentivirus VEGF expression was measured by QRT-PCR and plotted against CD38 expression. The Spearman test was used to determine any correlation between VEGF and CD38 expression.

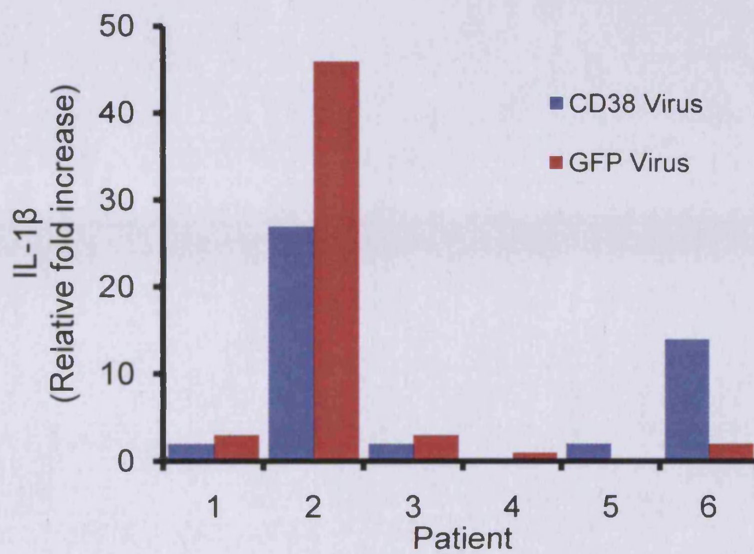


Figure 5.6 IL-1β was heterogeneously over-expressed in six CD38 and GFP lentivirus treated samples.

RNA was extracted from $1x10^6$ CLL cells and QRT-PCR was carried out to determine IL-1β expression in six virus treated patient samples.

The anti-apoptotic BCL-2 family member Mcl-1 is over-expressed in CLL and is associated with resistance to therapy and a poor outcome (Hussain *et al.*, 2007). It has also been associated with other markers of aggressive disease including unmutated *IGHV* genes, ZAP-70 and CD38 (Pepper *et al.*, 2008). To assess whether the induction of CD38 resulted in up-regulation of MCL-1, six CLL samples were treated with CD38 and GFP virus and the gene expression of MCL-1 was determined by QRT-PCR. Figure 5.7 illustrates that the addition of either lentivirus induced an increase in MCL-1 expression in the six samples tested. This increase varied in each patient sample and only 1/6 samples tested showed increased MCL-1 transcription in cells treated with CD38-expressing lentivirus. Consequently, there was no significant difference in MCL1 expression between samples treated with CD38 virus and those treated with GFP virus (P=0.42).

5.7 Microarray analysis revealed up-regulation of CD38 in CLL samples transduced with CD38 lentivirus

CLL cells expressing the CD38 antigen were shown to have a unique gene expression profile compared to their CD38 negative counterparts in bi-modal patients (Pepper *et al.*, 2007). This profile favours angiogenesis and CLL cell survival and provides a rationale for the more aggressive nature of CD38 positive CLL. Whether CD38 signalling is responsible for these changes in the gene expression profile is unknown. In an attempt to answer this question three untreated and virus treated samples were run on Affymetrix U133A GeneChips containing 23,500 gene sequences derived from the Genebank database. All of the data obtained from this gene array study were analysed using Genespring 7.0. Figure 5.8 illustrates that CD38 was highly expressed in each of the samples treated with CD38 virus but not in untreated or GFP virus treated samples. Table 5.1 highlights the mean expression of CD38 in untreated and virus treated patient samples. The mean relative expression from three CD38 transduced samples was 8490 ±3606.9 (±SD) compared to 7.7 ±0.6 (±SD) and 8.5 ±6.8 (±SD) in untreated and GFP virus treated samples respectively.

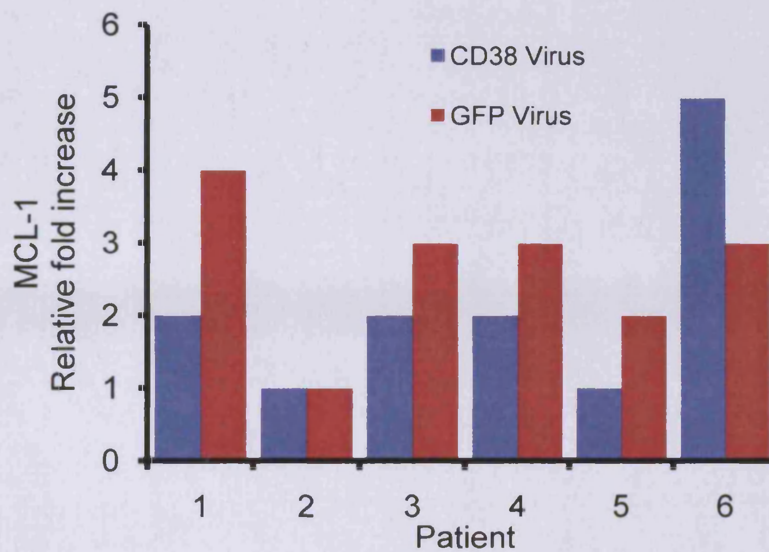


Figure 5.7 MCL-1 was heterogeneously over-expressed in six CD38 and GFP lentivirus treated samples.

RNA was extracted from 1×10^6 CLL cells and QRT-PCR was carried out to determine MCL-1 expression in six virus treated patient samples.

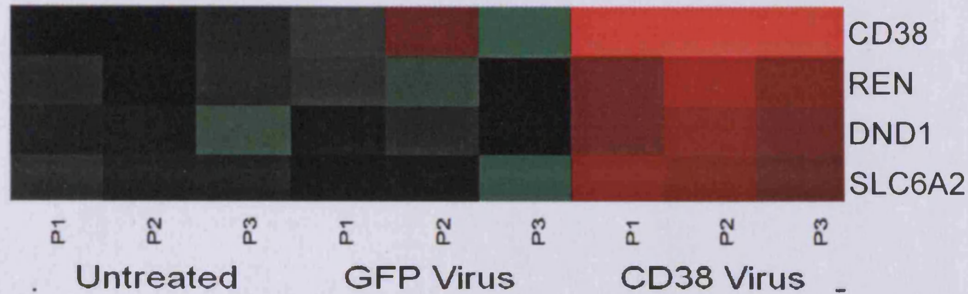


Figure 5.8 Microarray analysis illustrated that CD38 expression was increased following transduction with CD38 lentivirus.

5x10^{6} CLL cells were untreated or transduced using CD38 or GFP lentivirus and left for 48 hours at 37C. RNA was extracted and run on the Affymetrix U133A gene chip.

CD38	Untreated	CD38 Virus	GFP Virus
Relative Increase	7.7 ±0.6 (SD) (n=3)	8490.1 ±3606.9 (SD) (n=3)	8.5 ±6.8 (SD) (n=3)

Table 5.1 CD38 was over-expressed in samples expressing CD38 but not in control samples.

The mean CD38 expression was calculated for the three untreated CD38 virus treated and GFP virus treated patient samples.

5.8 Fifty five genes were up-regulated and seven down-regulated following the induction of CD38

QRT-PCR analysis revealed differential regulation of key genes implicated in the pathogenesis of CLL. Microarray data revealed 55 up-regulated genes and 7 down-regulated genes when CD38 transduced samples were compared with untransduced and GFP treated samples. Figure 5.9 illustrates the differentially expressed genes within the CD38 virus treated samples.

5.9 Microarray analysis identified down-regulation of the gene encoding the DNA mismatch repair protein Msh6 in CLL cells following expression of CD38

The process of somatic hypermutation takes place in response to antigenic challenge and introduces point mutations into the V-region exons of immunoglobulin heavy chain genes. This process occurs within the germinal centres of the lymph nodes and culminates in the selection of B-cells that generate high affinity BCRs. Those cells with BCR displaying lower affinity to the antigen undergo clonal deletion (Li *et al.*, 2004). SHM is initiated by the activation induced cytidine deaminase (AID) molecule and occurs in several kilobases of DNA around rearranged IGHV genes. AID deaminates cytosine to uracil which generates mutations of C and G nucleotides. In a secondary process the low fidelity DNA pol η and the mismatch repair proteins Msh2 and Msh6 are involved in the generation of A and T mutations. Through investigations involving Msh3 and Msh6 knockout mice Martomo *et al.* showed that Msh6 (but not Msh3) was required for the process of somatic hypermutation to occur effectively (Martomo *et al.*, 2004). The microarray identified a reduction in the expression of MSH6 in all three patient samples following the expression of CD38 (Figure 5.10). The mean values of MSH6 expression are illustrated in Table 5.2. A significant decrease in MSH6 was observed in CD38 transduced cells compared to GFP treated cells when the results from each of the gene probes were considered for all three patients (P=0.03). To investigate the expression of MSH6 further, QRT-PCR was carried out on two untreated and virus treated patient samples. Figure 5.11 illustrates that there was no difference in the expression of MSH6 in untreated and virus treated samples following the induction of CD38 on the CLL cell surface.

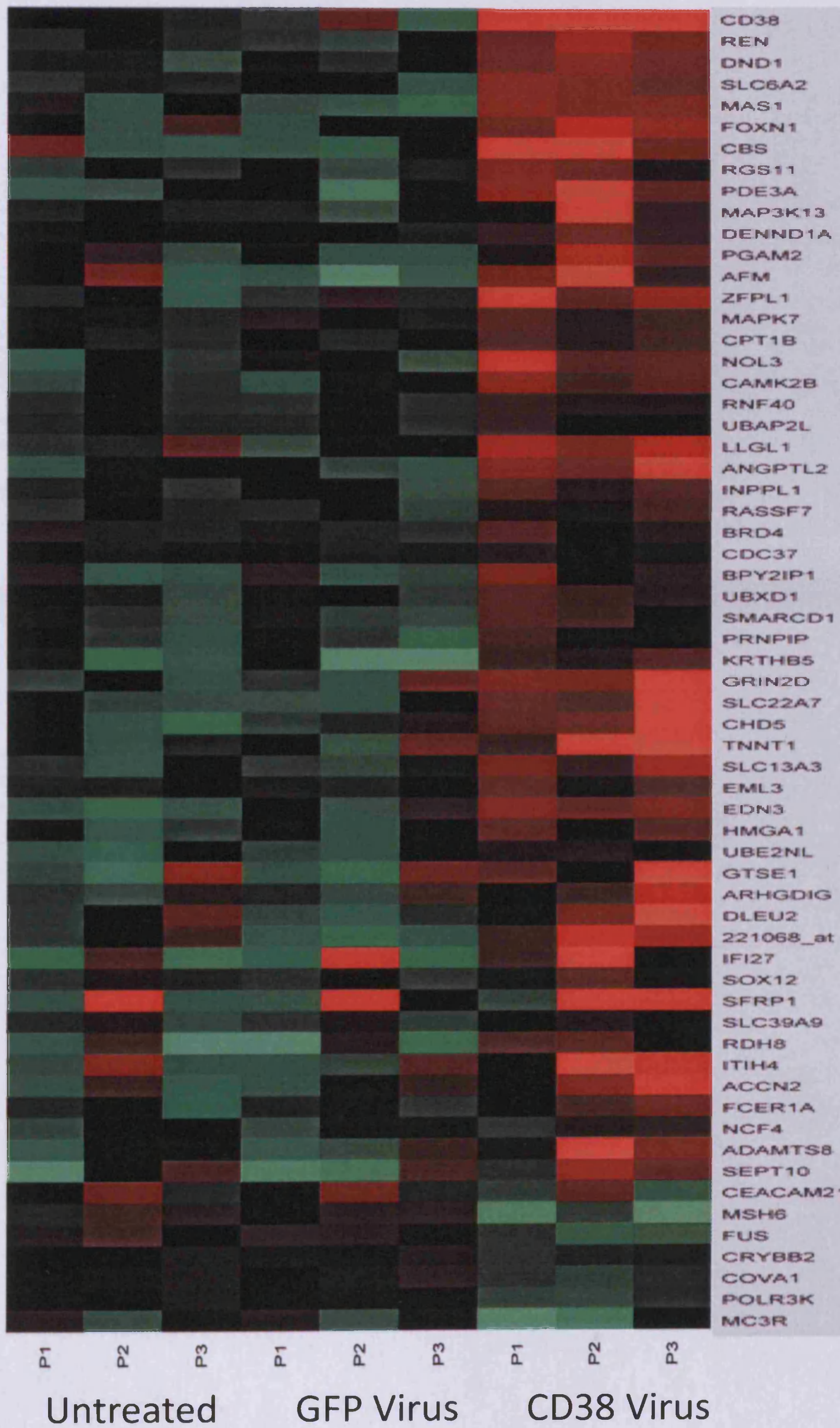


Figure 5.9.Global gene expression analysis identified 55 up-regulated and 7 down-regulated genes following transduction of the CLL cells with CD38 lentivirus.

Three CLL samples were untreated, treated with CD38 virus or GFP virus. RNA was extracted and run on the Affymetrix gene chip.

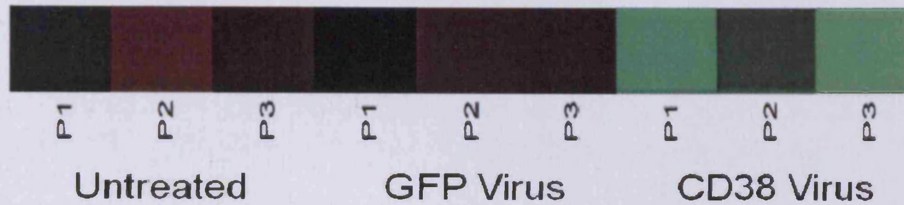


Figure 5.10 MSH6 was down regulated in CD38 expressing CLL cells.

$5x10^6$ CLL cells from three patients were untreated, treated with GFP virus or treated with CD38 virus. RNA was extracted from the samples after 48 hours incubation and run on the Affymetrix platform.

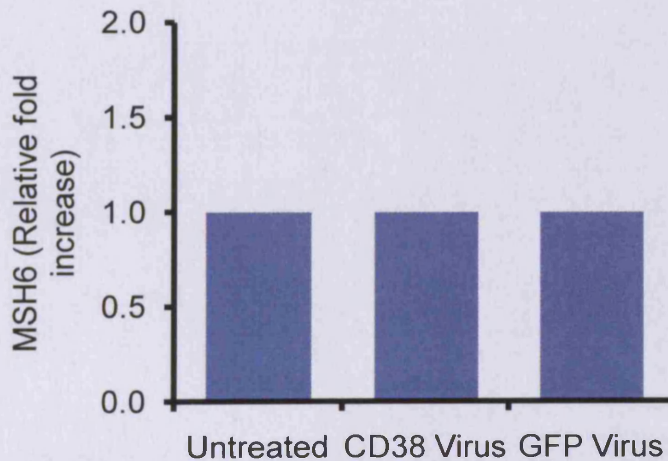


Figure 5.11 QRT-PCR analysis illustrated no difference in the expression of MSH6 following treatment with CD38 or GFP lentivirus.

$5x10^6$ CLL cells from two patients were untreated, treated with GFP virus or treated with CD38 virus. RNA was extracted from the samples after 48 hours incubation and QRT-PCR was used to observe MSH6 gene expression.

		P1	P2	P3	P1	P2	P3	P1	P2	P3		Mean		
Gene	Probe ID	Neg	Neg	Neg	CD38 Virus	CD38 Virus	CD38 Virus	GFP Virus	GFP Virus	GFP Virus	Neg	CD38 Virus	GFP Virus	P value
MSH6	211450_s_at	46.1	78.9	85.6	55.9193	70.1007	38.5074	97.7605	53.7089	89.4886	70.20	54.84	80.32	0.35
	202911_at	224.50	241.60	207.10	191.67	134.99	143.06	248.22	213.05	205.65	224.40	156.58	222.31	0.01
	211449_at	5.10	1.30	5.90	8.62	4.38	7.48	6.90	0.45	7.16	4.10	6.83	4.84	0.20
												Pvalue. All	probes	0.03

Table 5.2 The expression of MSH6 was decreased in three CD38 transduced patient samples.

Three CLL samples were untreated, treated with CD38 virus or GFP virus. RNA was extracted and run on the Affymetrix gene chip.

5.10 Discussion

Gene expression studies in CLL have identified gene profile signatures of poor risk disease and implicated mechanisms such as adhesion and migration in the pathogenesis of this disease (Stratowa *et al.*, 2001, Deaglio and Malavasi, 2009). Recent work from our group identified over-expression of molecules involved in angiogenesis and apoptosis in CD38 positive cells (Pepper *et al.*, 2007). Until now, such investigations have highlighted differences between CD38 positive and CD38 negative cells and, with the exception of the bi-modal study, were carried out on a heterogeneous genetic background (i.e. inter-patient analysis). The aim of this chapter was to observe whether ectopic expression of the CD38 molecule directly contributes to changes in genotype in the absence of any other changes within the cell. To achieve this, a method was developed using a lentiviral vector to induce CD38 expression on the surface of CD38 negative cells. Following transduction, RNA was extracted and QRT-PCR and microarray analysis were carried out to determine the expression of key molecules which may play a role in the pathogenesis of CLL.

Gene expression analysis revealed that CD38 was markedly induced in all of the samples treated with the CD38 expressing lentivirus (Figure 5.1.a). Although the addition of the GFP virus induced a small increase in CD38 expression (seven fold) in the sample, it was apparent that the virus had a profound effect on the expression of other genes. VEGF, IL-1β and MCL1 were all up-regulated in the six patient samples investigated by QRT-PCR. However, in these experiments there was little difference between the increase in gene expression observed following the addition of CD38 or GFP virus. Microarray analysis also revealed substantial alterations in gene expression in all three samples investigated following the addition of the GFP lentivirus. In chapter four the viability of the CLL sample increases in response to the addition of CD38 or GFP virus. The results from microarray analysis suggest that the effect of the virus on the cells is promoted, at least in part, at the level of transcription. Whether these changes are caused by aberrant signalling from the disruption of signalling molecules in the cell, or due to genetic changes following integration of the *provirus* is unknown. In 2002 Schroder *et al.* showed that the HIV-1 *provirus* integrates into transcriptionally active sites following infection (Schroder *et al.*, 2002). More recently Ciuffi *et al.* illustrated that HIV-1

preferentially targets DNA transcription units in non-dividing cells (Ciuffi *et al.*, 2006). This work suggests that integration of the *provirus* may indeed disrupt the gene expression within the infected cell. The concentrated lentivirus used in these experiments may have integrated into many different regions of the host DNA causing an array of changes in gene expression.

Both QRT-PCR and microarray investigations illustrated a large amount of heterogeneity between the patient samples tested. Figure 5.1.a shows that the expression of CD38 following the addition of equal amounts of CD38 lentivirus varied greatly between the six samples. This variation was also observed when determining the expression of VEGF, IL-1β and MCL1. Individual patients are known to have different responses to *in vivo* and *in vitro* stimuli (Alvarez-Mon *et al.*, 1993, Cutrona *et al.*, 2008). Such heterogeneity in response to the addition of lentivirus is therefore not unexpected. The microarray analysis of three patient samples also generated a large amount of discordant data due to heterogeneity within the gene expression. In this context, attempts to determine patterns of gene expression using small numbers of patient samples were very challenging in CLL. The over (or under) expression of genes involved in the pathogenesis of the disease may not occur in all patients and studying large cohorts may be required to uncover such aberrant gene expression.

The global expression of 23,500 genes was analysed following transduction and those up-regulated or down-regulated in the CD38 transduced samples, compared to the untreated and GFP virus treated controls, are highlighted in Figure 5.9. This analysis revealed the down-regulation of MSH6 in CD38 virus treated samples. The Msh6 protein is involved in the generation of somatic hypermutation following antigen stimulation of B-cells. The mutation status of the CLL clone is highly significant in CLL as unmutated cases have a particularly poor prognosis. CD38 positivity correlates with the presence of unmutated *IGHV* genes in CLL although a link between the two has never been established. The DNA mismatch repair protein Msh6 has been shown to be deregulated in other cancers including hereditary non-polyposis colorectal cancer and endometrial and colonic cancers (Wagner *et al.*, 2001, Wijnen *et al.*, 1999). The results obtained from the microarray analysis carried out in this chapter suggest a link between the expression of CD38 and down-regulation of MSH6. If CD38 signalling influences the mutation status of the clone then this

pathway would be an important therapeutic target in CLL. This phenomenon would also provide insight into the origin of the CLL cell and add to the debate over whether CLL is a single disease or one of either pre or post-germinal centre origin (Hamblin *et al.*, 1999, Damle *et al.*, 1999, Hamblin, 2002). Conversely the AID molecule has been shown to be associated with unmutated IGHV genes and is up-regulated in poor prognosis CLL particularly within the lymph node proliferation centres (McCarthy *et al.*, 2003, Hancer *et al.*, 2011). AID has the capacity to induce class switch recombination (Muramatsu *et al.*, 2000) and unpublished work by Professor Chiorazzi's group suggests that this molecule may maintain mistargeted mutational activity and induce intraclonal diversity and CLL progression in the CD38 positive CLL cell fraction (Calissano *et al.*, 2009). The microarray data illustrate a significant decrease in MSH6 in all three patient samples following transduction with the CD38 virus (P=0.03). However, subsequent analysis utilising QRT-PCR on two CD38 transduced samples failed to show a similar pattern of expression. The low number of samples investigated together with the heterogeneity of the disease may have caused the discrepancy observed. The literature suggests that the ABL gene may be over-expressed in CLL. (Lin *et al.*, 2006, Allen *et al.*, 2011) Therefore, its use as a house-keeping gene in these experiments may have confounded attempts to show differences in the expression of the genes under investigation. Finally a lack of probe specificity for various isoforms of the gene or a variation in hybridisation conditions may have yielded erroneous results from the microarray investigations carried out (Kothapalli *et al.*, 2002).

An MOI of 3.2 was used to transduce each of the samples. Further investigations looked at how the expression of CD38 related to that of VEGF in a single sample. Following the addition of increasing amounts of CD38 lentivirus VEGF expression was increased in a dose-dependent manner. No such increase was observed using the GFP lentivirus. Within this experiment the amount of lentivirus used to treat the samples was increased to a maximum MOI of 28. The evidence suggests that there may be a threshold of CD38 signalling required for the induction of VEGF in these cells. A highly significant correlation was observed when comparing VEGF expression to CD38 expression (rather than the viral MOI) ($P<0.001$) (Figure 5.5). These results

indicate a potential role for CD38 signalling in the induction of VEGF although high levels of CD38 were required to observe any correlation.

Although the system utilised was able to induce large amounts of CD38 on the surface, the antigen lacked any form of exogenous stimulation. It is quite feasible that CD38 signalling requires more than just the mere presence of CD38 on the surface of the cell and that in the initial experiments carried out the threshold for the effects of CD38 signalling to occur were not reached. By increasing the expression of CD38 by manipulating the MOI of the virus VEGF was induced; an effect that was not due solely to the addition of lentivirus since the GFP virus failed to recapitulate these effects.

Investigating gene expression in CLL samples following the induction of CD38 on the cell surface supported data already published and gave insight into the aberrant expression of novel genes with biological relevance. Heterogeneity was observed following the investigation of multiple patient samples and there was some discordance between results obtained using QRT-PCR and microarray. The novel part of this work was to induce changes in gene expression following the expression of CD38 on the surface of CLL cells which were originally CD38 negative. Therefore unlike any other study before it, this work identifies the changes in gene expression which occurred specifically in response to CD38 expression.

Chapter 6. Incubation with CD31-expressing co-culture causes phenotypic changes in CLL cells

6.1 Introduction

The aim of this chapter was to design a set of experiments, using multi-colour flow cytometry, to investigate the effects of CD38 ligation on the phenotype of the CLL cell. The expression of CD5, CD19, CD38, CD49d, CD11c, CD103 and CD138 were analysed on the cell surface and Zap-70 and Ki-67 within the cells using three antibody panels (Table 6.1). Stimulation of CD38 was achieved with the use of a CD31-expressing fibroblast co-culture system and CLL cells were incubated in NTL co-culture as a control. To further simulate the CLL microenvironment and enhance cell survival over a period of five days in co-culture, IL-2 was added at a concentration of 100IU/ml. CLL cells were sampled from the co-culture at day two and day five and multi-colour flow cytometry was carried out using a Beckman Coulter Cyan cytometer. The analysis of key surface and intracellular molecules was carried out on CD5+ and CD19+ cells present within the lymphocyte gate (Figure 6.1). The MFI values of paired samples were compared for twenty patient samples displaying a range of constitutive CD38 expression on their cell surface.

6.2 CD5 was down-regulated on the CLL cell surface following co-culture

CD5 was one of the first markers described on the surface of the CLL B-cell (Matutes *et al.*, 1994). This molecule is expressed on B1a lymphocytes in the peripheral blood of normal individuals. These cells are thought to be derived from B-cell precursors which have been exposed to self-antigen. The B1 repertoire therefore tends to be autoreactive and it is believed that CD5 suppresses BCR signalling in these cells to limit autoreactivity but allow them to react to potent BCR stimuli such as mucosal pathogens (Berland and Wortis, 2002). In keeping with this notion, CLL cells are generally anergic with defective BCR signalling (Muzio *et al.*, 2008). The presence of CD5 on the surface of the cell may contribute to this unresponsiveness and suggests that the CLL B-cell may be generated in response to auto-antigen. The initial expression

Panel 1	Fluorochrome	Emission
CD5	ECD	613
CD19	AF750	750
CD38	PE-Cy7	785
CD49d	FITC	520

Panel 2	Fluorochrome	Emission
CD5	ECD	613
CD19	PB	452
Zap-70	AF647	647
Ki-67	FITC	520

Panel 3	Fluorochrome	Emission
CD5	ECD	613
CD19	AF750	750
CD11c	PE-Cy7	785
CD103	FITC	520
CD138	PB	452

Table 6.1 Fluorescent antibody panels used to characterise CLL cells.

Three different combinations of fluorescent antibodies were designed to stain surface and intra-cellular antigens in CLL cells.

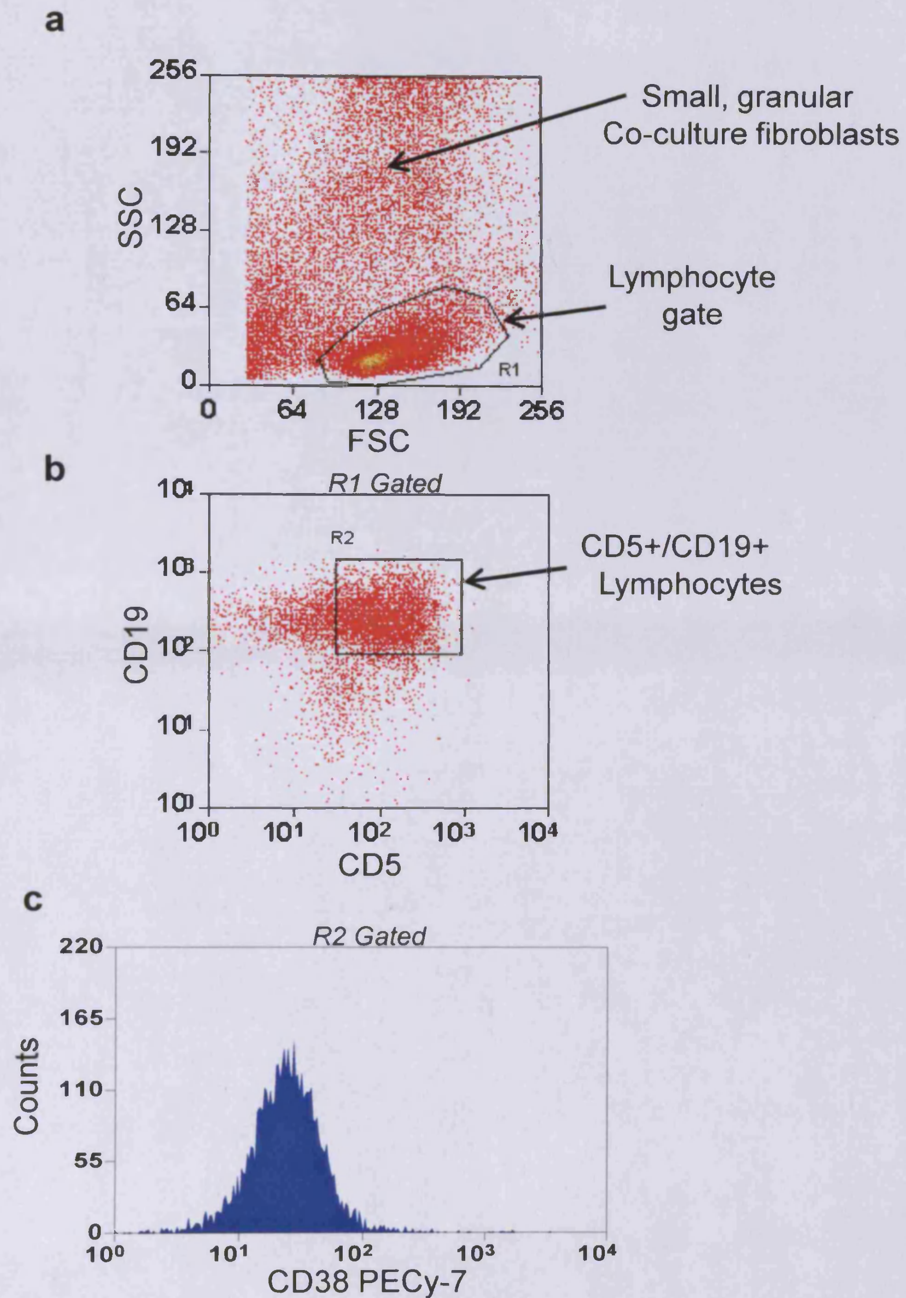


Figure 6.1 Gating strategy for the analysis of CD5+/ CD19+ CLL lymphocytes.

(a) Lymphocytes were primarily gated from forward and side scatter profiles. **(b)** CD5+/CD19+ CLL cells were identified within the R2 gate **(c)** R2 gated CD5+/CD19+ lymphocytes were analysed for protein expression (in this example CD38 is shown).

of CD5 following collection (day zero) was compared to samples incubated in NTL and CD31 expressing co-culture for two and five days. Figure 6.2 illustrates that CD5 was down regulated on the surface of CLL cells incubated within both NTL and CD31-expressing co-culture after five days compared to day zero (P=0.01 and P=0.02 respectively). There was no significant difference in CD5 expression between CLL cells incubated in NTL and CD31-expressing fibroblasts after two or five days (P=0.15 and P=0.22 respectively).

6.3 CD19 expression was increased in CLL cells following two and five days incubation with CD31-expressing co-culture

CD19 is present on the surface of B-cells from an early stage in their development and is only lost following differentiation of the cell into an antibody secreting plasma cell (de Rie *et al.*, 1989). The trans-membrane molecule is essential for efficient BCR signalling and forms lipid raft complexes with other surface molecules such as CD81 and CD21 (Sato *et al.*, 1997). Following BCR cross linking the CD19/BCR complex is internalized resulting in down-stream signalling and cell activation (Pesando *et al.*, 1989, Buhl and Cambier, 1999). CD19 is generally expressed on the surface of CLL cells in lower quantities than that expressed on the surface of normal B-cells (Ginaldi *et al.*, 1998). Although BCR signalling is perturbed in CLL, an increase in the levels of CD19 may allow signalling to occur and promote survival or proliferation of the clone. CD19 expression was assessed in twenty patient samples at day zero and following two and five days incubation with NTL or CD31-expressing co-culture. There was no significant difference in the expression of CD19 on the surface of CLL cells incubated for two or five days in NTL co-culture (Figure 6.3). In contrast, there was a significant increase in CD19 expression on the surface of CLL cells incubated for two and five days in CD31-expressing co-culture (P=0.0003 and P=0.0004 respectively). Subsequently the expression of CD19 was compared between samples incubated in NTL and CD31-expressing co-cultures. There was a significant increase in the expression of CD19 in CLL samples following two days incubation with CD31-expressing co-culture when compared to cells incubated with NTL co-culture (P=0.01) (Figure 6.3); this was even more pronounced after five days (P=0.0002).

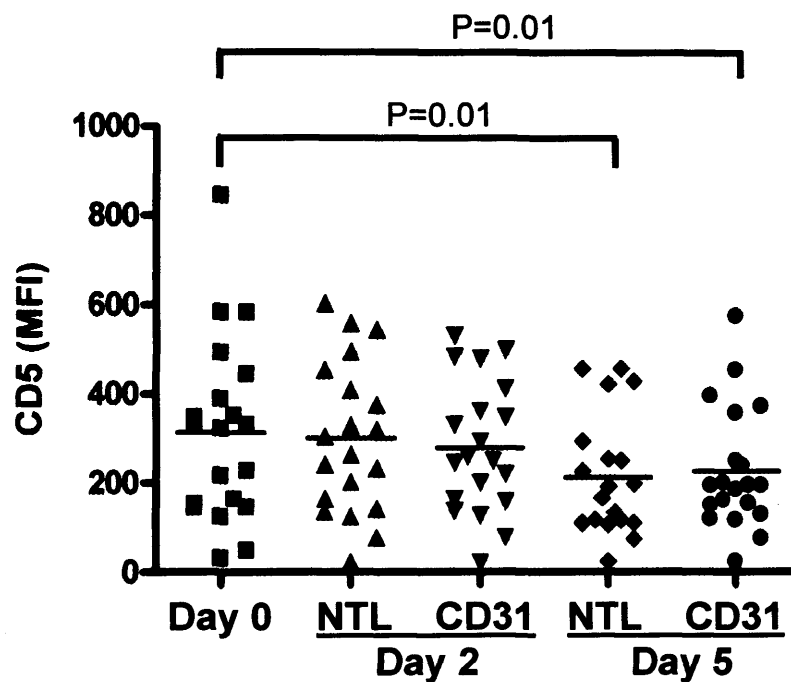


Figure 6.2 CD5 expression was decreased following five days in co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD5 was measured by flow cytometry on day 0, 2 and day 5 (CD5-ECD) and the MFI value was plotted for each CLL sample. CD19 positive CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD5 expression.

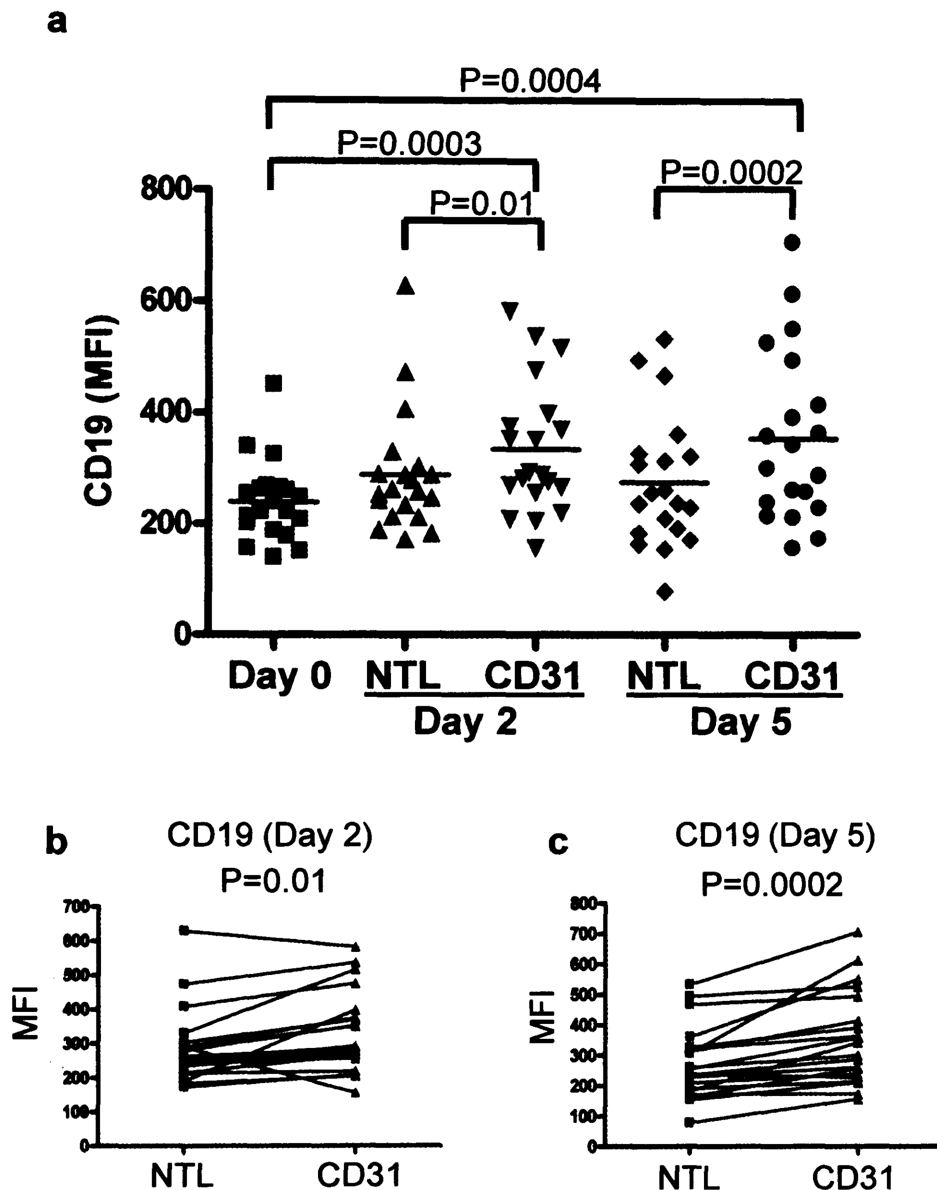


Figure 6.3 An increase in CD19 expression was observed following incubation with CD31 expressing co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD19 was measured by flow cytometry on day 0, 2 and day 5 (CD19- Alexafluor 750) and the MFI value was plotted for each CLL sample **(a)** CD5 positive CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD19 expression. **(b)** and **(c)** illustrate the CD19 expression at day 2 and 5 respectively. The paired samples within NTL and CD31-expressing co-culture are highlighted.

6.4 CD38 expression was increased in CLL cells following five days incubation with CD31-expressing co-culture

Studies of serial patient samples suggest that the CD38 glycoprotein is stably expressed on the surface of peripheral blood CLL B-cells (D'Arena *et al.*, 2002). Its expression within the lymph node or bone marrow has been more difficult to assess due to the challenge of obtaining such tissues. However, in 2004, Jasic *et al.* showed increased expression of CD38 on the surface of CLL cells within the lymph nodes compared to peripheral blood and bone marrow (Jaksic *et al.*, 2004). Subsequently Lin *et al.* illustrated increased levels of CD38 on the CLL cell surface in the bone marrow compartment compared to peripheral blood (Lin *et al.*, 2008). Evidence by Pepper *et al.* suggests that the expression of CD38 is transient (Pepper *et al.*, 2007), a theory that was supported by Calissano *et al.* (2009). Microenvironmental niches within lymphoid tissues may provide the sites for rapid CD38 up-regulation and shedding following cell activation. Higher expression of CD38 on the CLL cell surface may promote down-stream effects such as survival and proliferation (Patten *et al.*, 2008). To investigate whether CD38 was up-regulated following stimulation in co-culture the CLL cells were incubated within NTL and CD31-expressing fibroblasts. A significant increase in CD38 expression was observed following two days in NTL or CD31-expressing co-culture (P=0.01 and P=0.01 respectively) (Figure 6.4). There was also an increase in CD38 expression following five days in CD31-expressing co-culture (P=0.01). Following five days incubation there was a significant increase in CD38 expression on the surface of CLL cells incubated with CD31-expressing co-culture compared to cells incubated with NTL co-culture (P=0.01).

6.5 CD49d expression was increased in CLL cells following co-culture

CD49d is the α4 subunit of the α4β1integrin and is expressed on the surface of CLL B-cells. Increased expression of this antigen has been associated with a poorer prognosis in CLL (Eksioglu-Demiralp *et al.*, 1996, Gattei *et al.*, 2008). Molecules involved in cell migration have been widely studied in CLL due to enhanced lymph node and bone marrow infiltration (Burger *et al.*, 1999, Ocana *et al.*, 2007). These sites are thought to act as centres of enhanced activation and

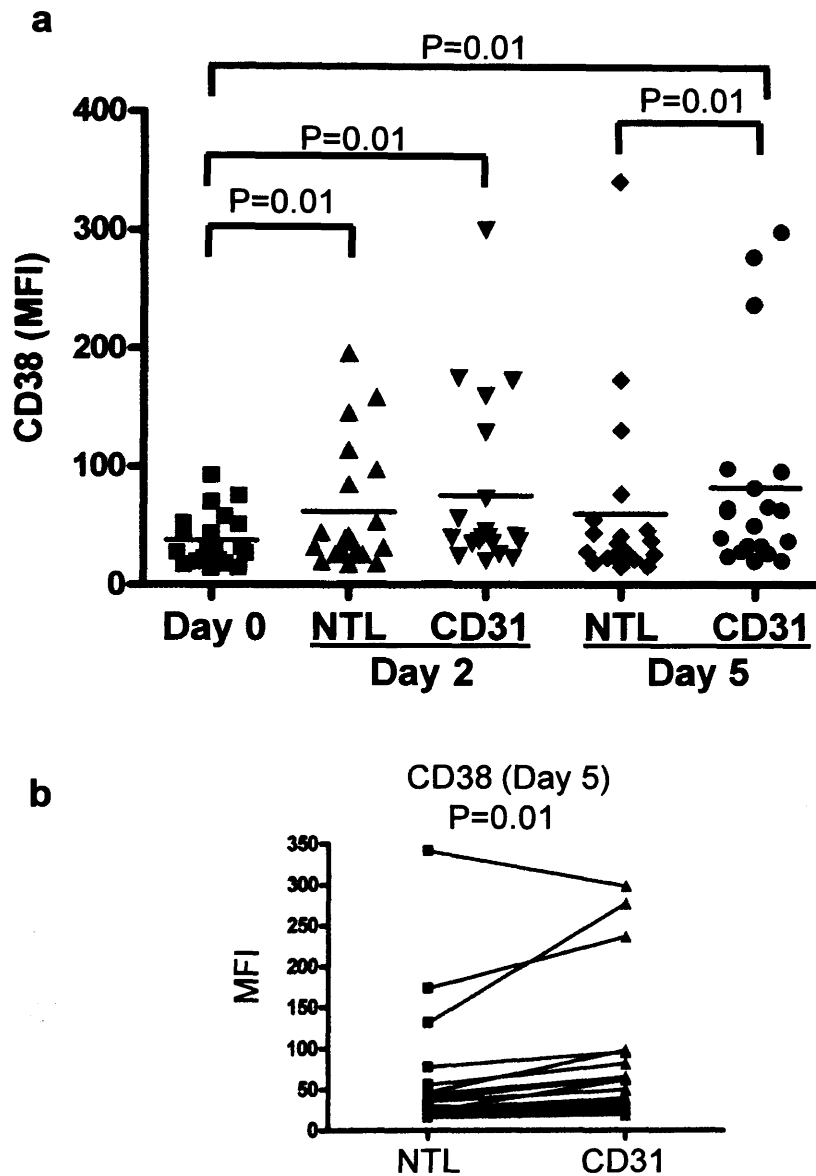


Figure 6.4 An increase in CD38 expression was observed following incubation in CD31 expressing co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31 expressing fibroblasts. Surface CD38 was measured by flow cytometry on day 0, 2 and day 5 (CD38-PC-7) and the MFI value was plotted for each CLL sample **(a)** CD5+/CD19+ CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD38 expression. **(b)** illustrates the CD38 expression at day 5. The paired samples within NTL and CD31-expressing co-culture are highlighted.

proliferation in CLL (Jaksic *et al.*, 2010). Increased expression of CD49d has been associated with up-regulation of CD38 and other chemokines such as CCL3 and CCL4 (Pittner *et al.*, 2005, Zucchetto *et al.*, 2009). Whether stimulation of CD38 results in increased expression of CD49d is unknown. CLL cells from twenty patients were stained for surface CD49d at day zero and following two and five days in co-culture. CD49d was up-regulated on the CLL cell surface following two days in NTL and CD31-expressing co-culture (P=0.0001 and 0.0003 respectively) (Figure 6.5). The levels of CD49d were also significantly increased following five days in NTL and CD31-expressing co-culture (P=0.0001 and 0.0001 respectively). However there was no significant difference in CD49d expression on the surface of CLL cells following two or five days co-culture with CD31-expressing fibroblasts compared to cells incubated with NTL co-culture (P=0.88 and P=0.1 respectively).

6.6 Zap-70 expression was increased in CLL cells following two days in CD31-expressing co-culture

Expression of Zap-70 is associated with a poor prognosis in CLL (Wiestner *et al.*, 2003). The molecule has been shown to be closely associated with BCR signalling (Chen *et al.*, 2008) and correlates with other poor prognostic markers such as CD38 and unmutated *IGHV* in CLL. (Hamblin *et al.*, 2002, Cruse *et al.*, 2007, Rassenti *et al.*, 2008, Crespo *et al.*, 2003, Rassenti *et al.*, 2004, Wiestner *et al.*, 2003). Zap-70 was also reported to play a role in the up-regulation of the chemokine receptor CXCR4 on the surface of CLL cells (Ticchioni *et al.*, 2002) and CD38 and Zap-70 positive cells illustrate enhanced migration when investigated *in vitro* (Deaglio *et al.*, 2007). To determine whether stimulation of CD38 had an effect on Zap-70 expression, intracellular Zap-70 was assessed in CLL cells at day zero and following two and five days co-culture with NTL and CD31-expressing fibroblasts. The CLL cells were stained for CD5 and CD19 before being fixed and permeabilised and then stained for Zap-70. There was a significant increase in Zap-70 expression after two days in NTL and CD31-expressing co-culture (P=0.003 and 0.0003 respectively) (Figure 6.6). Zap-70 was also significantly increased following five days co-culture in NTL and CD31-expressing co-culture (P<0.0001 and 0.0001 respectively). A significant

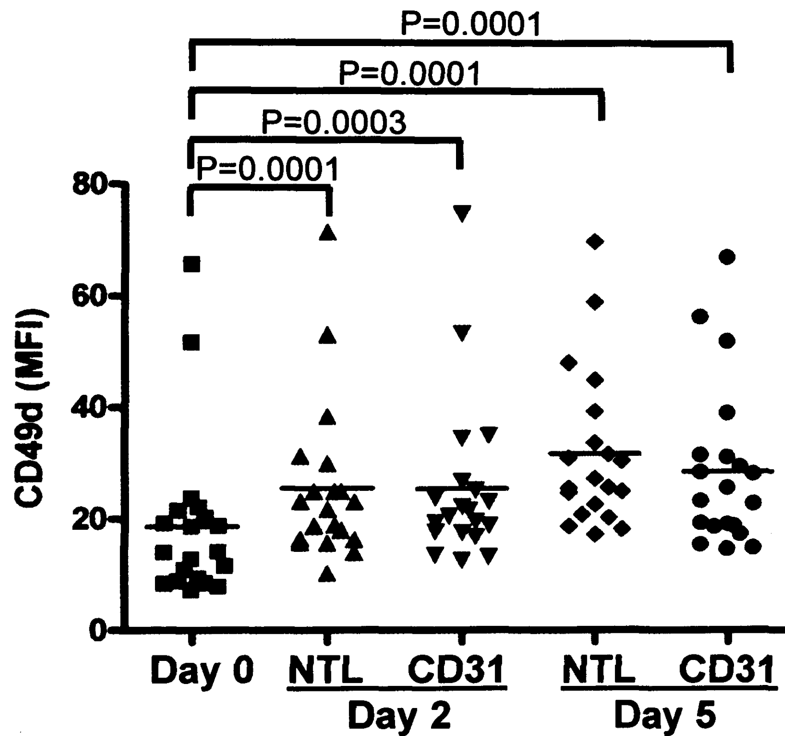


Figure 6.5 An increase in CD49d expression was observed following co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD5 was measured by flow cytometry on day 0, 2 and day 5 (CD49d-FITC) and the MFI value was plotted for each CLL sample. CD5 +/CD19 + CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD49d expression.

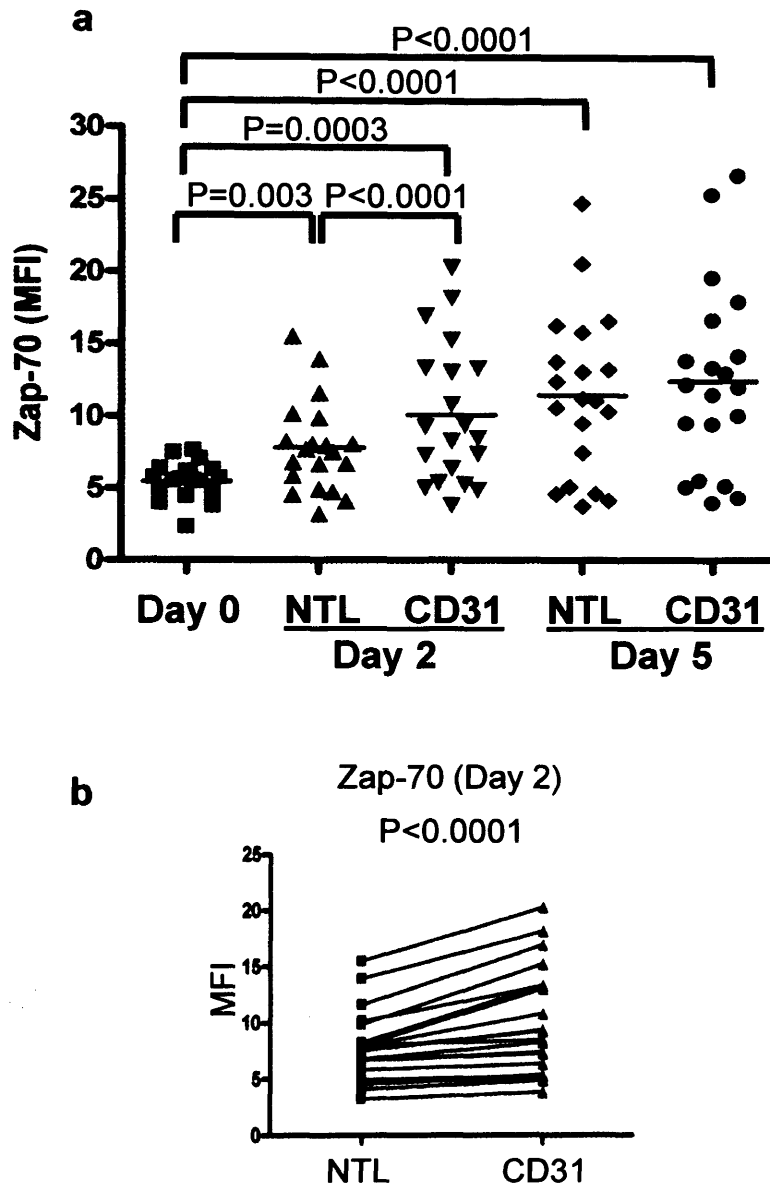


Figure 6.6 An increase in Zap-70 expression was observed following incubation with CD31-expressing co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD5 was measured by flow cytometry on day 0, 2 and day 5 (Zap-70- Alexafluor-687) and the MFI value was plotted for each CLL sample **(a)** CD5+/CD19+ CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare Zap-70 expression. **(b)** illustrates the Zap-70 expression at day 2. The paired samples within NTL and CD31-expressing co-culture are highlighted.

increase in the expression of Zap-70 was observed in the CLL cells following two days co-culture with CD31-expressing fibroblasts compared to cells incubated with NTL fibroblasts ($P<0.0001$) (Figure 6.6). Following five days in co-culture there was no significant difference in the expression of Zap-70 between the CLL cells incubated in CD31-expressing and NTL co-culture ($P=0.08$).

6.7 The increase in intracellular Zap-70 expression following incubation with CD31-expressing co-culture significantly correlated with constitutive CD38 expression

Incubation of CLL cells for two days in CD31-expressing co-culture resulted in up-regulation of intracellular Zap-70 when compared to cells incubated in NTL co-culture for the same period. To investigate whether there was a relationship between Zap-70 and CD38, Zap-70 expression (MFI) at day two was plotted against the expression of surface CD38 (percentage of positive cells) at day zero. There was a significant correlation between Zap-70 MFI and CD38 expression as illustrated in Figure 6.7 ($r^2=0.54$, $P<0.0001$). Subsequently the Zap-70 MFI values for CD38 negative and CD38 positive patient samples were compared (<20% versus ≥20%). There was a significantly higher expression of Zap-70 in CD38 positive samples following two days in CD31-expressing co-culture (Figure 6.8) ($P=0.03$).

6.8 There was no change in CD11c expression on the surface of CLL cells following co-culture

In normal B-cells, exposure to antigen results in the selection of a highly specific cell which proliferates and differentiates into a memory B-cell or plasma cell population (Carter, 2006). In order to determine whether the co-culturing of CLL cells caused such changes, the sample was investigated for markers of differentiation. CD11c is an integrin expressed at low levels on the surface of CLL cells. It is found in much higher levels on the surface of the malignant cells in Hairy cell leukaemia, B-PLL and less so in NHL (Marotta *et al.*, 2000). Increased expression of CD11c following co-culture would indicate that the cell was undergoing phenotypic changes consistent with differentiation. CD11c was therefore measured following co-culture of the CLL cells to observe whether

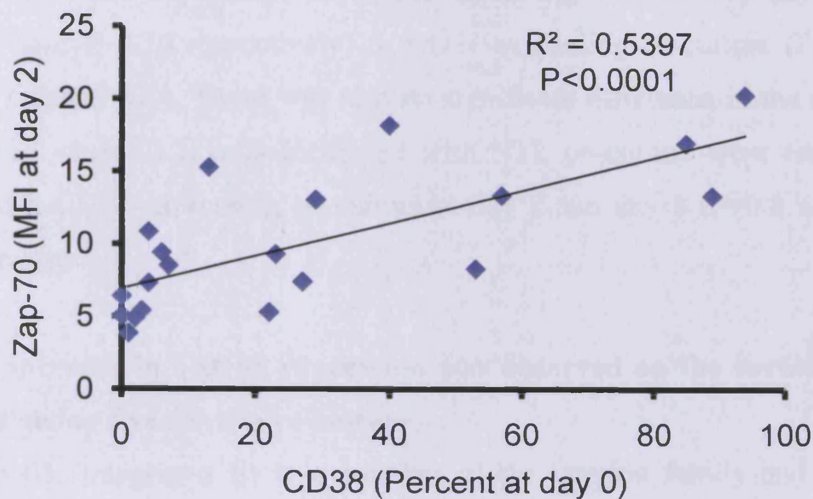


Figure 6.7 Native CD38 expression correlated with Zap-70 expression.

Day 0 CD38 expression (percent) was plotted against day 2 Zap-70 expression (MFI) for 20 patient samples following incubation with CD31-expressing co-culture. The Spearman test was employed to assess the correlation.

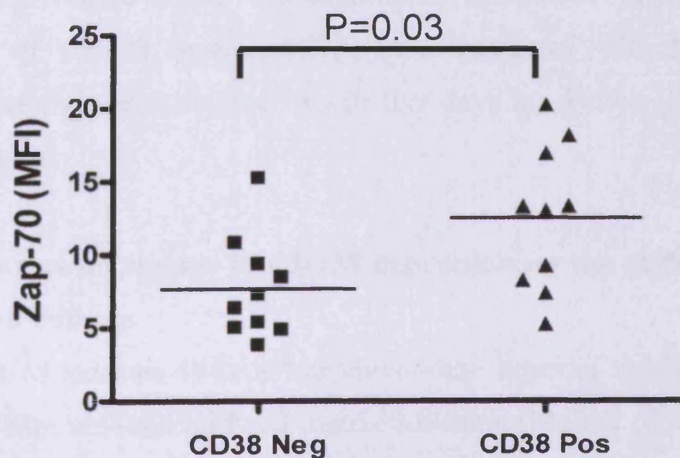


Figure 6.8 CD38 positive patients expressed higher levels of Zap-70 than CD38 negative patients.

Patients were separated into CD38 negative and CD38 positive groups (cut off 20%) and the mean Zap-70 expression was determined. An unpaired *t*-test was used to determine whether there was a significant difference in Zap-70 expression between the two groups.

such changes were occurring. Figure 6.9 Illustrates that there was no significant difference in the expression of CD11c following two or five days in NTL (P=0.27 and P=0.36 respectively) or CD31-expressing co-culture (P=0.18 and P=0.36 respectively). There was also no significant difference in the expression of CD11c when CLL cells incubated with NTL co-culture were compared to those with CD31-expressing co-culture at day 2 and day 5 (P=0.8 and P=0.95 respectively).

6.9 An increase in CD103 expression was observed on the surface of CLL cells following five days in co-culture

CD103 (Integrin α E) is a member of the integrin family and is usually present on the surface of CD8 positive T-cells as a receptor for the epithelial cell-specific ligand E-cadherin (Hadley *et al.*, 1997). The integrin is expressed at high levels in Hairy cell leukaemia but not in CLL (Del Giudice *et al.*, 2004). The expression of this surface molecule was determined in six CLL samples at day two and day five. A significant increase in CD103 expression was observed following five days in NTL and CD31-expressing co-culture (P=0.005 and 0.004 respectively) (Figure 6.10). No significant difference was observed in the expression of CD103 between CLL cells incubated with NTL and CD31-expressing co-culture following two or five days incubation (P=0.64 and P=0.7 respectively).

6.10 There was no change in CD138 expression on the surface of CLL cells following co-culture

CD138 (Syndecan 1) is a transmembrane heperan sulphate proteoglycan which mediates cell-cell and cell-matrix adhesion (Mali *et al.*, 1990). Protolytic cleavage of the molecule following translation generates a soluble form which has been shown to induce tissue invasion in breast cancer cells (Nikolova *et al.*, 2009). Within the haematopoietic system CD138 is expressed at high levels on plasma cells and is a marker of B-cell differentialtion (Chilosi *et al.*, 1999). To determine whether the CLL cells in culture were differentiating in to plasma cells samples were stained for CD138. Figure 6.11 illustrates that there was no significant difference in the expression of CD138 following two or five days in NTL (P=0.45 and P=0.15 respectively) or CD31-expressing co-culture (P=0.12

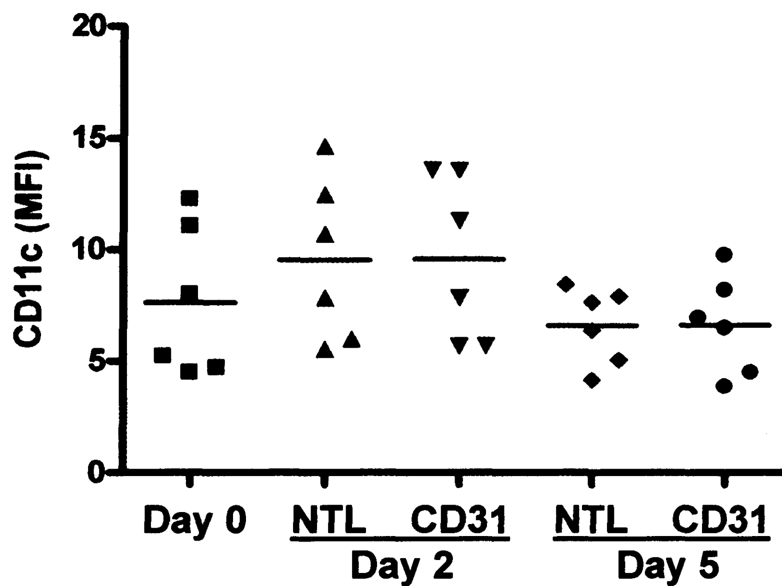


Figure 6.9 There was no change in CD11c expression following co-culture.

1x10^6 CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD11c was measured by flow cytometry on day 0, 2 and day 5 (CD11c-PC-7) and the MFI value was plotted for each CLL sample (n=6). CD5+/CD19+ CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD11c expression.

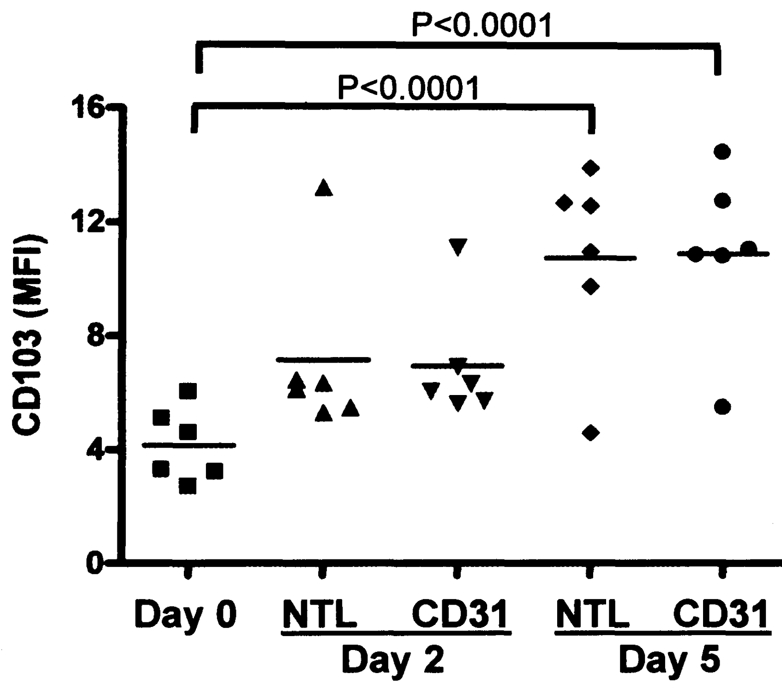


Figure 6.10 An increase in the expression of CD103 was observed following co-culture.

$1x10^6$ CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD103 was measured by flow cytometry on day 0 2 and day 5 (CD103-FITC) and the MFI value was plotted for each CLL sample (n=6). CD5+/CD19+ CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD103 expression.

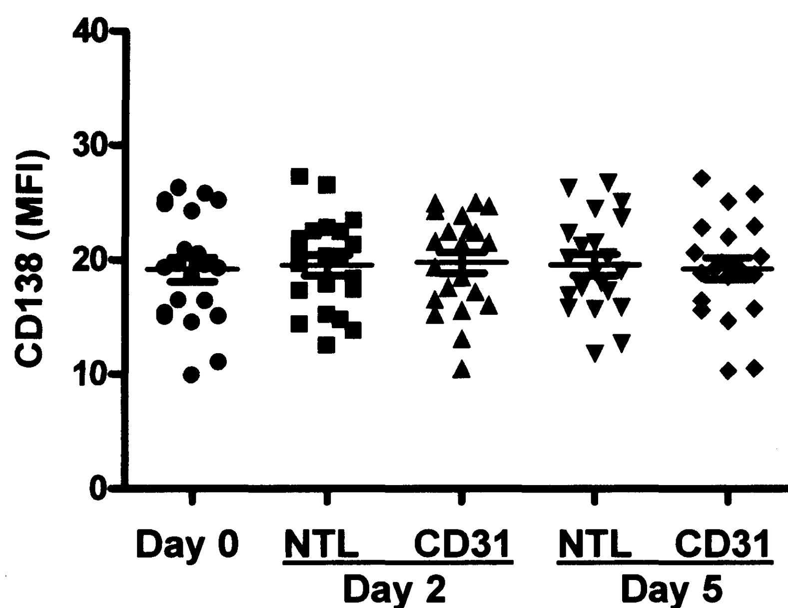


Figure 6.11 There was no change in CD138 expression following co-culture.

1x10^{6} CLL cells were placed into co-culture with NTL or CD31-expressing fibroblasts. Surface CD138 was measured by flow cytometry on day 0, 2 and day 5 (CD138-PB) and the MFI value was plotted for each CLL sample (n=21). CD5+/CD19+ CLL lymphocytes were identified by surface staining and forward and side scatter. A paired *t*-test was used to compare CD138 expression.

and P=0.64 respectively). There was also no significant difference in the expression of CD138 when CLL cells incubated with NTL co-culture were compared to those with CD31-expressing co-culture at day 2 and day 5 (P=0.62 and P=0.34 respectively).

6.11 Discussion

The aim of this chapter was to investigate the expression of key molecules on the CLL cell surface following ligation of CD38 with its *in vivo* ligand CD31, expressed on fibroblast co-culture. Multi-colour flow cytometry was employed and the phenotype of twenty patient samples was determined at day zero and following two and five days in co-culture.

Four important observations were made from these experiments. The first was that there were significant changes in CLL cell immunophenotype following incubation with co-culture. CLL cells up-regulated CD19, CD38, CD49d, Zap-70, and CD103 and down-regulated CD5 following two or five days in either NTL or CD31-expressing co-culture compared to day zero expression. In contrast, there was no significant change in CD11c or CD138 expression when the CLL cells were placed into the same co-culture conditions. These results highlight the effect of the co-culture system on the CLL cells. The co-culture fibroblasts are likely to express molecules on their surface and release cytokines which induce changes within the CLL cells. Such experiments highlight the requirement for the control NTL co-culture in these experiments.

The second observation was the change in the expression of proteins following incubation with CD31-expressing co-culture compared to those incubated with the control NTL co-culture. There was no significant difference in CD5 or CD49d expression between CLL cells incubated with NTL and CD31-expressing co-culture (Figure 6.2). However, CD19 and Zap-70 were significantly up-regulated after two days and CD19 and CD38 after five days co-culture with CD31-expressing fibroblasts (Table 6.2). All three molecules have been shown to be involved in BCR signalling (Lund *et al.*, 1996, Cherukuri *et al.*, 2001, Gobessi *et al.*, 2007). Such signalling is known to induce proliferation in CLL cells and is therefore likely to be mechanistically linked to the clonal expansion of this disease (Herishanu *et al.*, 2011). The recent report by Herishanu *et al.* identified the lymph node as a site of CLL cell activation.

			Day 2			Day 5		
	Marker	Day 0	NTL	CD31	P value	NTL	CD31	P value
No difference	CD5	314 (±204)	301 (±170)	280 (±148)	0.15	213 (±135)	227 (±137)	0.22
	CD49d	19 (±15)	26 (±15)	26 (±15)	0.88	32 (±14)	29 (±15)	0.11
Increased expression	CD19	239 (±72)	288 (±108)	334 (±118)	0.01	275 (±119)	355 (±154)	0.0002
	CD38	38 (±22)	62 (±53)	76 (±74)	0.08	60 (±77)	83 (±85)	0.01
	Zap-70	5 (±1)	8 (±3)	10 (±5)	<0.0001	11 (±6)	12 (±7)	0.08
Differentiation marker	CD11c	8 (±3)	10 (±4)	10 (±4)	0.81	7 (±2)	7 (±2)	0.95
	CD103	4 (±1)	7 (±3)	7 (±2)	0.66	11 (±3)	11 (±3)	0.71
	CD138	19 (±5)	20 (±4)	20 (±4)	0.62	20 (±4)	19 (±4)	0.34

Table 6.2 CLL cell expression of key surface and intracellular molecules.

Flow cytometry was utilised to observe the expression of key molecules on the surface and within the CLL cells of twenty patients. The mean MFI values (± the standard deviation) were calculated at day zero and following 2 or 5 days co-culture with NTL or CD31-expressing fibroblasts. The P values were calculated from the difference in protein expression (MFI) in CLL cells incubated with NTL co-culture compared to that of CLL cells incubated with CD31-expressing co-culture.

In their experiments they used gene expression profiling to illustrate the over-expression of genes involved in BCR signalling and NF-κB activation in CLL cells taken from the lymph node. Additionally, they highlighted the up-regulation of E2F and c-MYC target genes that induce proliferation in the cell. Supporting evidence for this enhanced proliferation was illustrated in the form of an increase in the expression of the cell cycle protein Ki-67 (Herishanu *et al.*, 2011). The phenotyping data from this chapter illustrate that molecules involved in BCR signalling are also induced following stimulation of CD38 on the surface of the CLL cell. This data is in keeping with that published by Herishanu *et al.* (2011) and suggests that our CD31 co-culture model, to some extent, simulates the *in vivo* environment when stimulating CLL cells and that CD38 is involved in the induction of molecules involved in BCR signalling and proliferation in CLL cells.

The third observation was made when comparing protein expression with the constitutive CD38 expression. The expression of Zap-70, within CLL cells incubated with CD31-expressing co-culture, significantly correlated with the initial CD38 expression of the sample at day zero (Figure 6.7). Zap-70 expression was also significantly higher in CD38 positive patient samples compared to CD38 negative patient samples (Figure 6.8). The presence of both CD38 and Zap-70 confer a poor prognosis in CLL and recent studies have illustrated that there is around 70% concordance in the expression of these molecules (Schroers *et al.*, 2005, D'Arena *et al.*, 2007, Gachard *et al.*, 2008). In 2007 Deaglio *et al.* showed that these two molecules are functionally linked and that Zap-70 is required for CD38 signalling to occur (Deaglio *et al.*, 2007). With this in mind it is reasonable to surmise that CD38 plays a role in the up-regulation of Zap-70 in an attempt to increase the effects of CD38 or BCR signalling and induce CLL cell activation.

The final observation was made when the CLL cells were stained for CD11c CD103 and CD138 which are markers of differentiated B-cells. Following two days in co-culture there were no significant increases in CD11c, CD103 or CD138 (Figures 6.9 and 6.10). However, co-culture for five days resulted in a significant increase in the expression of CD103 on the surface of CLL cells incubated within both CD31-expressing and NTL co-culture (Figure 6.10). Any differences in protein expression observed following this period should be

considered carefully as the CLL cells appeared to have undergone changes consistent with differentiation. However, there was no significant difference in the expression of CD11c, CD103 or CD138 between CLL cells incubated with NTL or CD31-expressing fibroblasts suggesting that the increase in CD103 was not specifically induced by the CD31-CD38 cognate interaction (Table 6.2). The lack of increased expression of CD11c and CD138 coupled with the low MFI values for all three molecules raise doubts about the biological significance of the increased expression of CD103 in these co-cultures. This supports the view that these co-culture conditions are not inducing plasmacytoid differentiation in the CLL cells.

It has become apparent that, to understand the mechanisms behind the development of CLL, the cells need to be studied under the conditions provided by the *in vivo* microenvironment. In the absence of primary tissue, this niche has been simulated *in vitro* by the use of ligand expressing adherent co-cultures. In two previous studies, primary tissue has been available which has allowed the identification of a highly active proliferation centre, rich in immunomodulatory cells (such as T-cells and dendritic cells) and cytokines (such as IL-2 and IL-4) involved in B-cell activation (Patten *et al.*, 2008, Herishanu *et al.*, 2011). In this section CD31-expressing fibroblasts were used to mimic the microenvironment and specifically induce CD38 stimulation on the surface of the CLL cell. Phenotypic changes were observed which favoured BCR signalling and proliferation. The results provide rationale for subsequent investigations which inhibit the actions of CD38 in this stimulatory environment. Similar investigations, where CLL cells have been targeted with a specific drug in the presence of co-culture, have yielded important findings. In a study by Vogler *et al.* potent inhibitors of Bcl-2 and Bcl-x_L were shown to selectively kill CLL cells in liquid media. When placed into CD40L-bearing co-culture however, the effectiveness of the drug decreased by 1000 fold and it failed to induce apoptosis (Vogler *et al.*, 2009). In summary the use of co-cultures to mimic the CLL microenvironment has proved to be an important part of uncovering the true nature of the disease. It has enabled us to observe the actions of individual stimuli and given insight into the effect of therapeutic agents within the *in vivo* environment. Developments within this area will surely unveil more important mechanisms of cell survival and proliferation within the CLL cell.

Chapter 7. Final Discussion

7.1 Discussion

In this study, I applied a range of techniques to enhance our knowledge of the role that CD38 plays in the pathogenesis of CLL. Induction of CD38 on the CLL cell surface using lentiviral gene transduction identified changes in gene expression which may contribute to and enhance disease progression. This work also highlighted the challenges involved in using a lentiviral knock-in system and led to the design of experiments utilising CD31-expressing co-cultures to stimulate CD38 on the CLL cell surface. Importantly, the CD31-expressing co-culture system induced survival, proliferation and changes in the phenotype of the CLL cells which favour enhanced B-cell receptor signalling. The novel findings within this project have provided direction for future work and highlighted the potential of CD38 as a therapeutic target in CLL.

The forced expression of CD38 in CLL cells utilising a lentivirus was achieved with great success. All of the CLL samples treated showed a large increase in CD38 expression and changes in gene expression were observed. The pro-angiogenic VEGF molecule has been implicated in the pathogenesis of many malignancies and it has been shown to be over-expressed in CLL (Chen *et al.*, 2000, Kay *et al.*, 2002, Pepper *et al.*, 2007). In this study the induction of high levels of CD38 on the CLL cell surface resulted in increased expression of VEGF. This suggests that VEGF may be transcriptionally regulated by CD38 signalling; a novel and potentially important finding.

The DNA mismatch repair molecule Msh6 has never been implicated in the pathogenesis of CLL, but was found to be down regulated following induction of CD38. This is especially interesting considering the importance of the *IGHV* mutation status of the CLL clone and the recent focus on the AID molecule (Palacios *et al.*, 2010, Hancer *et al.*, 2011), which is closely linked to the Msh family in the generation of somatic hypermutation. Further investigations are required to determine whether CD38 plays a direct or indirect role in regulating the expression of Msh6.

As previously discussed, genetically modifying leukocytes using lentiviral vectors can alter cells independently of transgene expression (Chapters 4 and 5).

Three major challenges were highlighted from these investigations. The first was the effect of adding concentrated lentivirus to the CLL cells. Molecular analysis revealed that the addition of both CD38 and GFP virus caused considerable changes in gene expression within CLL cells. This made analysing the effects of the 'ectopic' introduction of CD38 difficult. Following lentiviral infection, the induction of toll-like receptor signalling and integration of the *provirus* into transcriptionally active sites may alter gene expression within transduced cells (Schroder *et al.*, 2002, Gelman *et al.*, 2004, Ciuffi *et al.*, 2006). Given that the GFP-expressing virus also had an effect on the expression of molecules on the CLL cell surface, it seems plausible that the viral envelope may trigger the activation of the toll-like receptor pathway. It is clear that the virus induces major changes in the cytoplasmic membrane of CLL cells and at least transiently induced the exposure of phosphatidylserine. However, these changes in membrane asymmetry were not associated with apoptosis induction. Further studies are required to determine whether the effect is specific to CLL cells or is a general consequence of exposure to this particular lentivirus. A further conundrum following successful introduction of the CD38-expressing lentivirus was the apparent stripping of the CD38 molecule from the surface of CLL cells following co-culture. It seems possible that the virus-induced perturbations in the CLL cytoplasmic membranes is responsible for this effect since it was not observed in native CD38 positive cells cultured under identical conditions.

Gene expression analysis revealed a great deal of heterogeneity between the patient samples tested. CLL is renowned for its heterogeneity between patients and the investigation of large patient cohorts are often required to identify pathogenic mediators of this disease (Rodriguez *et al.*, 2007). Also the simple expression of CD38 was not enough to induce its signalling cascade and there was a requirement for CD38 stimulation to drive this process. Previous studies illustrating signalling through CD38 have utilised various stimuli (including agonistic antibodies, CD31 and anti-IgM) to induce the down-stream effects of CD38 signalling (Lund *et al.*, 1996, Deaglio *et al.*, 2005, Vaisitti *et al.*, 2010). Such observations led to the decision to use CD31-expressing co-cultures to ligate CD38 on the CLL cell surface.

In the absence of a single genetic transformation event or the identification of a single antigen or molecule responsible for driving proliferation, CLL is

thought to be a disease whose maintenance and progression are controlled by a multitude of factors (Caligaris-Cappio, 2003). External stimuli which result in cellular activation and proliferation of the CLL clone are thought to reside in microenvironmental niches within the lymph nodes and bone marrow of the patient (Caligaris-Cappio, 2003, Friedberg, 2011). It has become apparent that if the underlying mechanisms of this disease are to be deciphered, and novel therapies devised, then *in vitro* systems must be developed which mimic the CLL cell microenvironment. In this study, CD38 was stimulated by incubating the CLL cells with CD31-expressing co-culture. Comparing these CLL samples to matched samples in liquid media or control co-culture (mouse fibroblasts not expressing human CD31) illustrated changes in cell survival, proliferation and the phenotype of the cells that was specifically induced by CD38. Three prominent studies used similar *in vitro* co-culture models to illustrate that CD38 signalling results in increased homing to lymph nodes, BCR signalling, survival and proliferation in CLL cells (Lund *et al.*, 1996, Deaglio *et al.*, 2005, Poggi *et al.*, 2010). Similar investigations have utilised CD40 ligand-expressing co-cultures to induce significant changes in gene and protein expression in CLL cells (Schattner, 2000, Willimott *et al.*, 2007, Gricks *et al.*, 2004). It is now clear that a large number of different microenvironmental stimuli enhance the survival and proliferation of the CLL clone *in vivo* (Calissano *et al.*, 2009). Investigating the molecules which stimulate the CLL cells within this niche, so that this environment may be closely simulated *in vitro*, will be integral in determining the pathogenesis of CLL and in devising more successful therapeutic strategies.

An important aspect from the work carried out in this thesis (and by others) is the ability of the CLL cell to react to *in vitro* stimuli. Substantial modifications in gene expression were observed within the CLL cells following treatment with lentivirus. Also the phenotype of the CLL cells changed following two days incubation in co-culture with CD31-expressing or NTL fibroblasts. If the cells behave in such a manner *in vivo* within the microenvironmental niches, then the CLL cell should be perceived not as an anergic cell, reluctant to undergo apoptosis, but as an active cell which responds rapidly and effectively to external stimuli.

Chapter four highlights the induction of proliferation in CLL cells incubated with CD31-expressing co-culture. More specifically, expression of the cell cycle

antigen Ki-67 was induced and correlated with increased CD38 expression. *In vivo* heavy water experiments by Messmer *et al.* in 2005 clearly illustrated that the CLL clone was proliferating at a much higher rate than previously thought (Messmer *et al.*, 2005). This work was complemented by Calissano *et al.* who used the same technique to illustrate increased proliferation in the CD38 positive cellular sub-set (Calissano *et al.*, 2009). Following this work many groups have tried to uncover the mechanisms behind the induction of proliferation. In 2007 Damle *et al.* identified CD38 positive CLL cells as an actively proliferating sub-set (Damle *et al.*, 2007). They illustrated increased Ki-67, Zap-70 and human telomerase reverse transcriptase within CD38 positive clones. In 2010 Deaglio *et al.* reported the induction of proliferation following ligation of CD38 on the surface of the CLL cell. In their experiments they identified CD31 as an integral part of the signalling axis and suggested that it plays a key role in stimulating the proliferation of CLL cells within the lymph node microenvironment (Deaglio *et al.*, 2010). Taken together these data strongly suggest that CD38 signalling plays a role in the induction of CLL cell proliferation. The evidence presented in chapter four shows that CD38 ligation induces Ki-67 expression. Extending this study using a molecule to block the CD38-CD31 interaction would confirm whether CD38 signalling was directly responsible for the induction of Ki-67.

CLL cells incubated with CD31-expressing co-cultures illustrated a significant increase in cell survival. This is in keeping with previously published data by Zuchetto *et al.* who observed that CD38+/CD49d+ CLL cells showed increased survival compared to their CD38-/CD49d- counterparts, following stimulation with VCAM-1 (Zucchetto *et al.*, 2009). Furthermore, in 2010 Buggins *et al.* illustrated the up-regulation of pro-survival molecules, including Bcl-2 and Mcl-1, following incubation with endothelial co-cultures (Buggins *et al.*, 2010). In their experiments the up-regulation of these molecules was shown to be preceded by DNA binding of the NF-κB sub-unit Rel A. These investigations also highlighted the induction of molecules involved in lymphocyte migration including CCL3, CCL4 and CD49d. Both the lymph node and bone marrow environments harbour pro-survival stimuli for lymphocytes and CLL cells expressing pro-migratory molecules are more likely to enter these sites. In 2010 Poggi *et al.* illustrated increased survival in CLL cells placed into CD31-expressing co-culture through the induction of Bcl-2 and Bcl-x_L (Poggi *et*

al., 2010). The CLL cell survival described in chapter four was in keeping with the data reported by Poggi *et al.* though neither study was able to demonstrate a correlation between CD38 expression and CLL cell survival. Immunohistochemistry has illustrated that CD31 is expressed on the surface of immune cells including T-cells and dendritic cells within the lymph node microenvironment (Patten *et al.*, 2008). Additionally CD31/CD38 interactions and their role in the pathogenesis of other malignancies have been described in the bone marrow compartment (Gallay *et al.*, 2007). Enhanced CD38 signalling within these regions may therefore promote the survival of the CLL cell.

CD31 has the ability to ligate other CD31 molecules in a homotypic fashion on the cell surface. CD31 is present on the surface of CLL cells and contrasting evidence exists as to whether this molecule plays a role in the pathogenesis of CLL (Ibrahim *et al.*, 2003, Mainou-Fowler *et al.*, 2008, Poggi *et al.*, 2010). In this project the increased expression of surface and intracellular molecules did not correlate with constitutive CD31 expression on the CLL cells (Appendix 4.a-e). Furthermore, previous studies have illustrated similar findings following the use of agonistic antibodies specific for CD38 and CD31-expressing co-cultures (Deaglio *et al.*, 2003, Deaglio *et al.*, 2010). Both the induction of the signalling molecule Zap-70 and the cell cycle marker Ki-67 correlated with constitutive CD38 expression suggesting that the effects were indeed due to CD38 signalling. Further experiments using a CD38 blocking antibody could be carried out to confirm these findings.

In this study, CD19, CD38 and Zap-70 were up-regulated following stimulation of CD38 using the CD31-expressing co-culture system. CD19 and CD38 associate with co-accessory molecules such as CD81, CD21 and the B-cell receptor on the cell surface to mediate B-cell signalling (Fujimoto *et al.*, 1998, Deaglio *et al.*, 2003). The formation of such raft complexes results in the activation of Akt and Erk pathways and in a tertiary pathway calcium is released from intracellular stores and NF-κB is translocated to the nucleus (Guo *et al.*, 2000). In 2007, investigations by Deaglio *et al.* illustrated the induction of calcium release and increased survival in CLL cells following stimulation of CD38 with an agonistic antibody (Deaglio *et al.*, 2007). In their report they highlighted the requirement of accessory molecules, such as CD19, for effective CD38 signalling. In chapter six, both CD38 and CD19 were up regulated on the

CLL cell surface following co-culture with CD31-expressing fibroblasts. According to the literature this would promote signalling via CD38 and the BCR and induce CLL cell activation. NF-κB is over-expressed in CLL (Cuni *et al.*, 2004) and induces the expression of anti-apoptotic mediators such as Mcl-1 and Bcl-2 (Zaninoni *et al.*, 2003, Petlickovski *et al.*, 2005). In 2008, Hewamana *et al.* showed that CD38 expression did not correlate with NF-κB DNA binding in CLL (Hewamana *et al.*, 2008). The cells analysed in this study had not undergone any stimulation and it would be interesting to extend these investigations by stimulating CD38 on the surface using the CD31-expressing co-culture. Zap-70 was induced in CLL cells following two days in CD31-expressing co-culture. Additionally, its expression significantly correlated with the native expression of CD38 at day zero ($P<0.0001$). The association between CD38 and Zap-70 has been previously documented in the pathogenesis of CLL (Deaglio *et al.,* 2008), but this is the first time that CD38 signalling has been shown to up-regulate this tyrosine kinase. The induction of Zap-70 is also likely to enhance BCR mediated CLL cell activation and promote the survival and proliferation of the clone.

The results from this study support the use of molecules which inhibit CD38 in the treatment of CLL. Daratumumab is such a molecule under investigation in the laboratory to treat a number of haematological malignancies including CLL and Multiple Myeloma. A report by de Weers *et al.* described effective antibody and complement-mediated killing even in the presence of bone marrow stromal cells (de Weers *et al.*, 2011). They illustrated the potent effect of the antibody *in vivo* where low doses inhibited tumour growth in their xenograft mouse model. In a parallel study, the same group have shown enhanced antibody mediated cytotoxicity when daratumumab was used in combination with lenolidamide (van der Veer *et al.*, 2011). The side effects of using anti-CD38 monoclonal antibodies *in vivo* have not been extensively investigated and initial concerns are centred around the fact that CD38 is expressed on a wide range of cell types including lymphoid, myeloid epithelial, eye and brain cells (Tai and Anderson, 2011, Mizuguchi *et al.*, 1995, Horenstein *et al.*, 2009). A series of reports in the 1990s discussed the potential effects of CD38 chimeric molecules for the treatment of Multiple Myeloma (Stevenson *et al.*, 1991, Goldmacher *et al.*, 1994, Vooijs *et al.*, 1995). Antibody mediated tumour toxicity was reported with

little side effects (Stevenson *et al.*, 1991). Concerns were raised regarding the induction of anaphylactic shock following the use of an immunotoxic anti-CD38 antibody conjugated to blocked ricin, though the response by Goldmacher mentions that seven patients receiving the treatment showed no haematologic or organ toxicity (Vooijs *et al.*, 1995). Additionally, in 2002 Marchetti *et al.* reported the impairment of pancreatic cell function following treatment with CD38 monoclonal antibodies (Marchetti *et al.*, 2002). Whether CD38 can be safely targeted in the treatment of haematological malignancies requires further investigation and the design of molecules which inhibit specific regions of the molecule may prove advantageous and limit systemic side effects.

7.2 Summary and conclusions

The experiments carried out were aimed at determining the role of CD38 following the genetic modification of CD38 negative CLL cells and through stimulation of surface CD38 with the use of co-cultures. The overall hypothesis was that CD38 was able to signal in CLL and cause changes in gene expression which favoured CLL cell survival and proliferation. Using the two models described the hypothesis was broken down into three parts to investigate CLL cell survival and proliferation, gene expression and changes in phenotype. The profound effect of adding concentrated lentivirus to the cells was apparent as was the heterogeneity in the response of individual patient samples to viral treatment and other external stimuli (e.g. CD31-expressing co-culture). However, with the use of appropriate controls, differences were observed between transduced and untransduced CLL cells and between those stimulated with CD31 and unstimulated samples. The increased expression of key molecules was illustrated by microarray studies and the co-culture experiments denoted increased survival and proliferation and changes in cell surface and intracellular markers. This work extends our knowledge of CD38 signalling events in CLL and provides direction for future investigations.

7.3 Future investigations

Recent findings in CLL have highlighted the role that the microenvironment plays in promoting the survival and proliferation of CLL cells (Vogler *et al.*, 2009, Poggi *et al.*, 2010, Herishanu *et al.*, 2011). Incubating CLL cells *in vitro*, without stimuli, results in rapid cell death, a feature contrary to the nature of the CLL cell *in vivo*. The experiments carried out in this project using co-culture highlight the importance of simulating the CLL microenvironment by providing stimulation via molecules which are present within the malignant proliferation centres. Hence, an important objective of the Cardiff CLL research group has been to develop *in vitro* co-culture systems which will mimic the CLL microenvironment. With the use of such systems the biology of the CLL cell may be observed whilst in its most active state. Additional experiments are already underway looking at phosphorylation events in CLL cells following co-culture with CD31-expressing fibroblasts. Any such events will further elucidate the effects of CD38 signalling in CLL and give valuable insight into the pathways initiated following stimulation of this molecule.

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Appendix

Appendix 1

2.1 List of Materials and laboratory equipment

2.1.1 General reagents
2.1.2 Cell culture
2.1.2.1 Eukaryotic cell culture
2.1.2.2 Prokaryotic cell culture
2.1.3 Co-culture and cell lines
2.1.4 Molecular biology reagents
2.1.5 Plasmids
2.1.6 Antibodies
2.1.7 Primers
2.1.8 Molecular biology kits
2.1.9 Cell biology and biochemistry
2.1.10 Instruments
2.1.10.1 General
2.1.10.2 Cytometers and analysis software
2.1.11 Additional software tools

2.1 List of Materials and laboratory equipment

2.1.1 General reagents

Material	Source	Code
Phosphate buffered saline tablets	Oxoid	BR0014G
1.5ml Eppendorf tubes	Fisher	FB74031
0.5ml tubes	Starstedt	72-699
1.5ml Cryo-storage tubes	Fisher	10-500-26
15ml tubes	Greiner	188271
50ml tubes	Corning	430291
Haz Tabs (Chlorine for decontamination of waste)	Guest Medical	H8801
Ficoll lymphoprep	Axis-Shield	Lys-3773
T175 Large tissue culture flasks	Nunc. Thermo Sci	178883
T75 Small tissue culture flasks	Greiner	658175
6-well plates	Nunc. Thermo Sci	140675
12-well plates	Greiner	665180
48-well plates	Nunc. Thermo Sci	150687
5ml Pipettes	Corning	4487
10ml Pipettes	Corning	4101
25ml Pipettes	Corning	4251
Test tubes (Flow cytometry)	BD Falcon	352054

2.1.2 Cell culture

2.1.2.1 Eukaryotic cell culture

Material	Source	Code
Dulbecco's modified Eagle's Media (DMEM)	Gibco (Invitrogen)	41965
Roswell Park Memorial Institute Media 1640(RPMI)	Gibco(Invitrogen)	31870
Foetal Calf Serum (FCS)	Gibco(Invitrogen)	12319018
Penicillin (5,000 U/ml)/Streptomycin (5,000ug/ml)	Gibco(Invitrogen)	15140148
Sodium Pyruvate	Gibco(Invitrogen)	11360070
0.5% Trypsin EDTA	Gibco(Invitrogen)	25300
L-glutamine X100 (200uM)	Gibco(Invitrogen)	25030
Interleukin 2	Tecin	RO-23-6019

2.1.2.2 Prokaryotic cell culture

Material	Source	Code
Bacto-Tryptone	Fisher Scientific	DF0123173
Bacto-Yeast extract	Oxoid	X589B
Sodium Chloride 0.17M	Sigma	S9888
Agar	Fisher Scientific	S70213A
Ampicillin 100mg/ml	Sigma	A5354
Glycerol	Sigma	T1503
Competent *E-coli* DH5α	Invitrogen	18258-012

2.1.3 Co-culture and cell lines

Cell line	Application	Source (Kindly donated by)
Jurkat	Transduction/Controls	Professor Martin Rowe
CD31/NTL	Co-culture	Professor Silvia Deaglio
CD40L	Co-culture	Dr Aneela Majid
293T	Virus packaging	Professor Gavin Wilkinson

2.1.4 Molecular Biology reagents

Material	Source	Code
Trizol Reagent	Invitrogen	15596-026
RNase A	Sigma	R6513
Chloroform	Fisher	C2984
Ethanol	Fisher	NC9602322
Agarose	Fisher	BP1356500
Ethidium Bromide	Invitrogen	15585011
NEB Buffer 1 2 3 4	New England Biolabs	B7001S B7002S B7003S B7004L
Xho1 (25,000U)	NEB	R0146L
Kpn1(25,000U)	NEB	R0142L
Not1(25,000U)	NEB	R0189L
BSA	NEB	B9001S
Shrimp alkaline phosphatase	Fermentas	EF0511

2.1.5 Plasmids

Plasmid	Gene encoded	Application	Source
HR' SINcPPT SFFV-X-WPRE (SXW)	(HIV LTR incorporated backbone construct)	Lentivirus	1
Lenti-SEW	Green fluorescent protein	Lentivirus	2
Lenti-S38W	Human CD38	Lentivirus	Generated in house
pΔ8.91*	HIV *gag, pol & rev*	Lentivirus	*
pMD2G*	VSV-G envelope protein	Lentivirus	*
pEGFP-C1	GFP-CD38 fusion	Nucleofection	3
pEGFP-1	GFP	Nucleofection	4
pGEM4Z-EGFPA64bis	GFP	Electroporation	5

* Accessory plasmid

1 - Dr.R.J.Matthews. Department of Infection, Immunity and Biochemistry, University of Wales, Cardiff.

2 - Dr. W.Qasim and Professor A.J.Thrasher Institute of Child Health, London. High-Level Transduction and Gene Expression in Hematopoietic Repopulating Cells Using a Human Imunodeficiency Virus Type 1-Based Lentiviral Vector Containing an Internal Spleen Focus Forming Virus Promoter. Christophe Demaison, Kathryn Parsley, Gaby Brouns, Michaela Scherr, Karin Battmer, Christine Kinnon, Manuel Grez, Adrian J. Thrasher. Human Gene Therapy. May 2002, 13(7): 803-813.

3 - Dr.S.Deaglio. Laboratory of Immunogenetics, Department of Genetics, Biology, and Biochemistry and Centro di Ricerca in Medicina Sperimentale, University of Torino Medical School, Torino, Italy.

4 - AMAXA Biosystems, MD, USA,

5 - F.Van Bockstaele. Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium. Efficient gene transfer in CLL by mRNA electroporation. Leukaemia, 2008 Feb;22(2):323-9.

2.1.6 Antibodies

Antibody	Species	Fluorochrome	Company	Code
CD19	M~H	RPE-Cy5	Dako	C7066
CD19	M~H	APC	Invitrogen	MHCD1905
CD19	M~H	Alexafluor750	Beckman Coulter	A78838
CD19	M~H	Pacific Blue	Dako	PB985
CD38	M~H	RPE	Invitrogen	MHCD3804
CD38	M~H	PC7	Beckman Coulter	A54189
Zap-70	M~H	Alexafluor 488	Caltag	MHZap-7020
Zap-70	M~H	Alexafluor 647	Beckman Coulter	A24071
CD5	M~H	ECD	Beckman Coulter	A33096
CD11c	M~H	PC7	Beckman Coulter	A80249
CD103	M~H	FITC	Beckman Coulter	IM1856U
CD138	M~H	PE	Beckman Coulter	A54190
CD138	M~H	Per-CP-Cy5	Beckton Dickinson	BD341087
VEGF	M~H	1°	Santa Cruz	SC7269
Akt 1	M~H	PE	Santa Cruz	SC5298
IL-1B	R~H	1°	Santa Cruz	SC7884
VEGFR2	M~H	APC	R&D	FAB357A
Flk-1	M~H	1°	Santa Cruz	SC6251
Mcl-1	M~H	1°	Santa Cruz	SC12756
CD31	G~M	2° (PE)	Southern Biotech	1070-11S
Ki-67		FITC	Dako	F7268
CD40	R~M	2° (FITC)	Dako	F0313
CD49d	M~H	FITC	Serotec	MCA2503F

M = Mouse, R = Rabbit, G = Goat, H = Human, ~ = Anti. 1°= Primary antibody, 2°= Secondary antibody

2.1.7 Primers (Primers were purchased from Eurogentec)

Primer		Sequence ('5 – '3)
ABL	F	CGGCTCTCGGAGGAGACGTAGA
	R	CCCAACCTTTTCGTTGCACTGT
CD38	F	ATGCTTTCAAGGGTGCATTT
	R	TTTTACTGCGGGATCCATTG
ZAP-70	F	AAGAACTTTGTGCACCGTGA
	R	TTCATCTTCTTGTAGGGCTTCTG
MCL1	F	AAAAGCAAGTGGCAAGAGGA
	R	TTAATGAATTCGGCGGGTAA
VEGFA	F	TCAGGACATTGCTGTGCTTT
	R	TGGTTTCAATGGTGTGAGGA
Ki-67	F	CAAAAGGATTCCCTCAGCAA
	R	TTTGTGCCTTCACTTCCACA
CD31	F	TATTTTCCAAGCCCGAACTG
	R	TGGGCATCATAAGAAATCCTG
CD49d	F	AGATGCAGGATCGGAAAGAA
	R	GCCCCCATCACAATTAAATC
IL-1β	F	TGGCAGAAAGGGAACAGAAA
	R	ACTTCTTGCCCCCTTTGAAT
MSH6	F	ATTGCATTTGGCCGTTATTC
	R	CAATGGCGATCATCTGAAAA

2.1.8 Molecular Biology Kits

Kit	Application	Source	Code
QIAGEN DNA Maxi	DNA isolation	QIAGEN	13362
QIAGEN QIAquick	PCR purification	QIAGEN	28104
QIAGEN gel Extraction	Agarose gel extraction	QIAGEN	28704
Calcium Phosphate	Transfection	Sigma	MB-315
Sybr Green	QPCR	Roche	03 515 869 001
Big Dye 3.1	Sequencing	ABI	4337455
Human B-cell Nucleofection kit	Nucleofection	AMAXA	VPA 1001
T7 Message machine	Electroporation	Ambion	AM1340
RT-Kit (200 reactions)	Reverse transcription	Applied Biosystems	4374966

2.1.9 Cell biology and biochemistry

Kit	Application	Source	Code
VEGF ELISA	Protein quantification	Bender Medsystems	BMS277
P24 ELISA	Quantification of lentivirus	Retro-tek	8001111
Annexin V	Apoptosis assay	Bender Med systems	BMS500FI/300ce
Fix & perm kit	Fix and perm	Caltag	GAS-002-1

2.1.10 Instruments

2.1.10.1 General

Material	Source	Model
Orbital shaker	Sanyo	Orbisafe
NanoDrop-1000 (Spectrophotometer)	Thermo Scientific	V 3.7
Ultracentrifuge	Beckman Coulter	Optima L-100XP
Nucleofector	AMAXA	AAD-1001
Electroporator	BioRad	165-2100
Thermal cycler	GeneAmp	9600
Irradiator	Nordion Int	Gammacell 1000 Elite
Laser microscope	Leica	DM1L
Sorval centrifuge	Sorval	Evolution RC
Large Centrifuge	Heraeus	Megafuge 1.0
Mini centrifuge	Heraeus	Biofuse fresco
Light cycler	Roche	2.0
Cytospin	Thermo	Shandon Cyto 4

2.1.10.2 Cytometers and analysis software

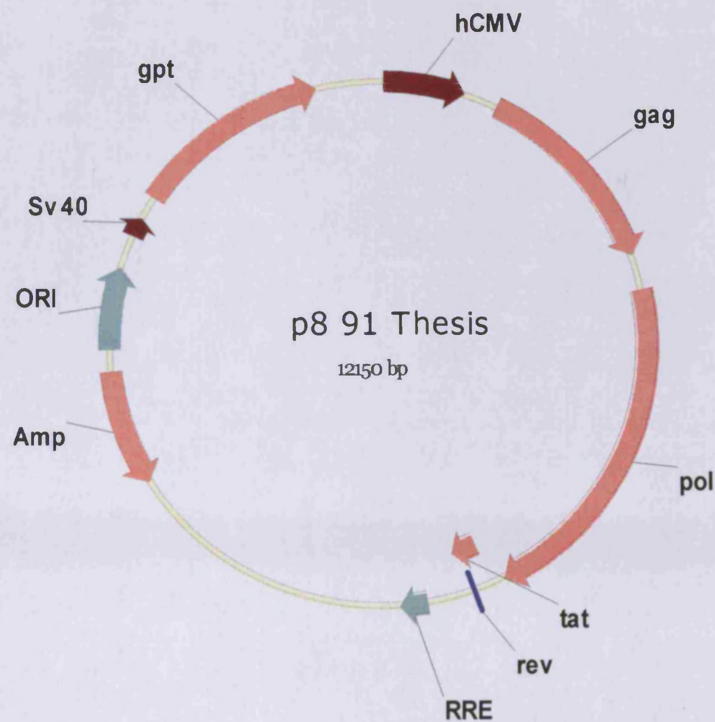
The Beckman Coulter Calibur, Beckman Coulter Cyan, and Accuri C6 cytometers were used in flow cytometry experiments. Cellquest Pro software was utilised to acquire data on the FacsCalibur cytometer and Summit 4.0 software was employed to acquire data on the Cyan and analyse the data from both Beckman Coulter cytometers. The CFlow plus software package was used for acquiring and analysing the data from the Accuri C6 cytometer.

2.1.11 Additional software tools

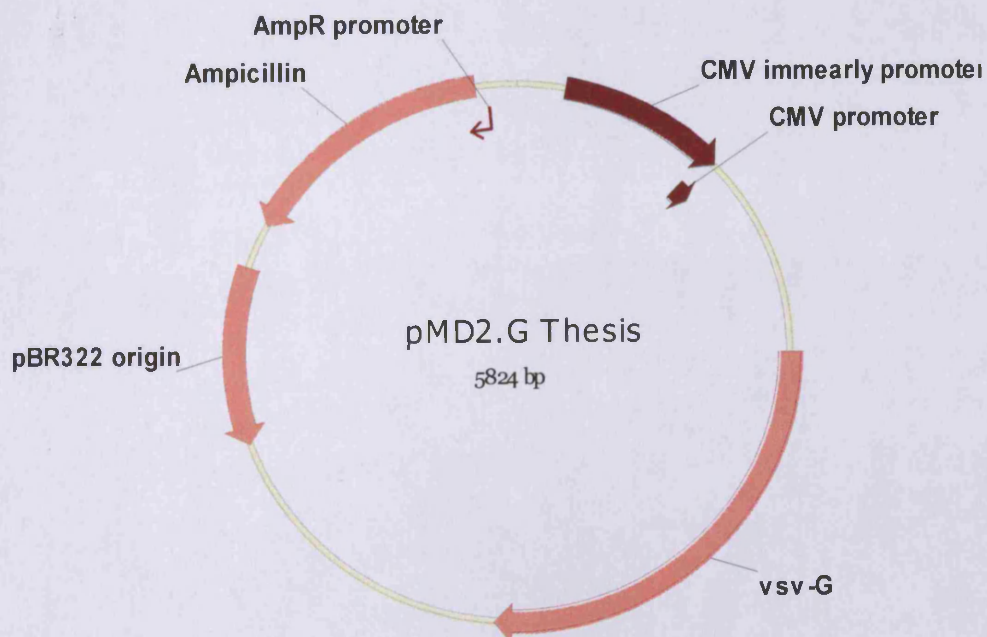
Software	Application	Source
Vector NTI	Plasmid design	Invitrogen
Prism 4	Graphs and statistical analysis	Graphpad
OligoPerfect	Primer design	Invitrogen

Appendix 2

Accessory plasmids for the generation of lentivirus



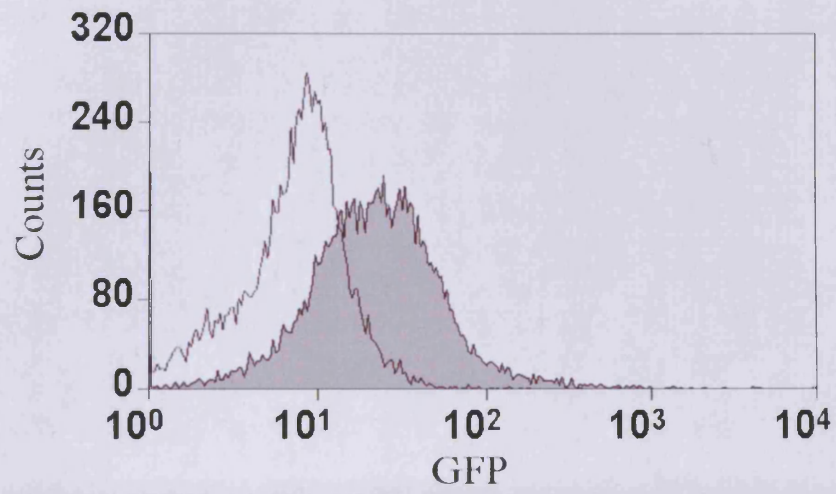
a) pΔ8.91 encoding *gag* and *pol*



b) pMD2G encoding the VSVG envelope protein

Appendix 3

GFP expression in CLL cells



Appendix 4

CLL cell expression of key molecules following 2 or 5 days incubation with CD31 expressing co-culture. Correlation with constitutive CD31 expression at day zero.

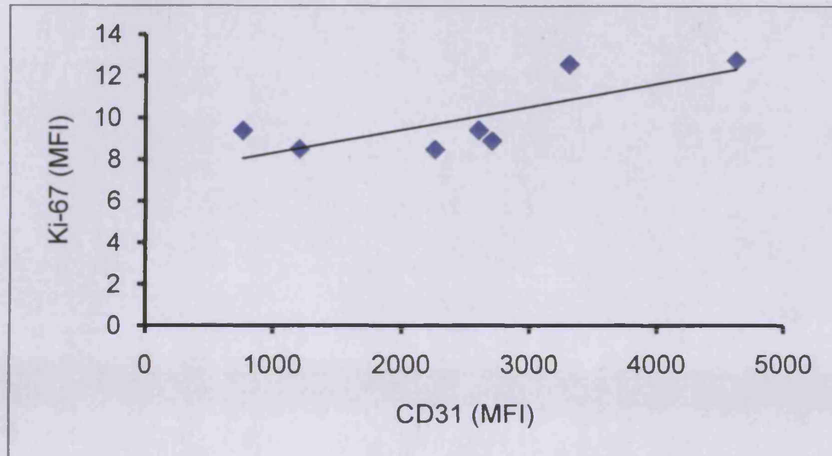


Figure 4.a No significant correlation was observed between CD31 and day 2 Ki-67 expression (n=7). The Spearman test was used to assess the correlation (P=0.11).

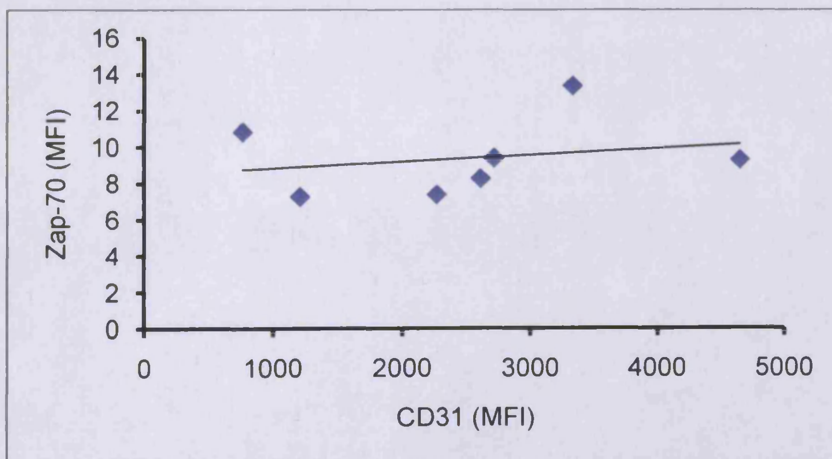


Figure 4.b No significant correlation was observed between CD31 and day 2 Zap-70 expression (n=7). The Spearman test was used to assess the correlation (P=0.5).

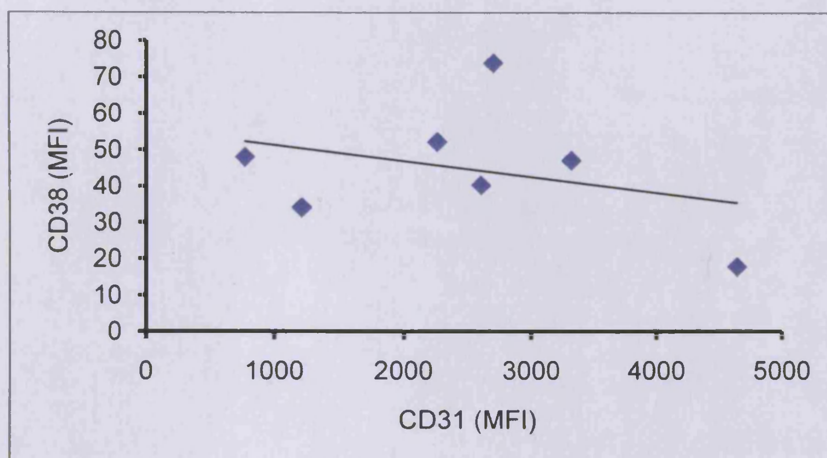


Figure 4.c No significant correlation was observed between CD31 and day 5 CD38 expression (n=7). The Spearman test was used to assess the correlation (P=0.6).

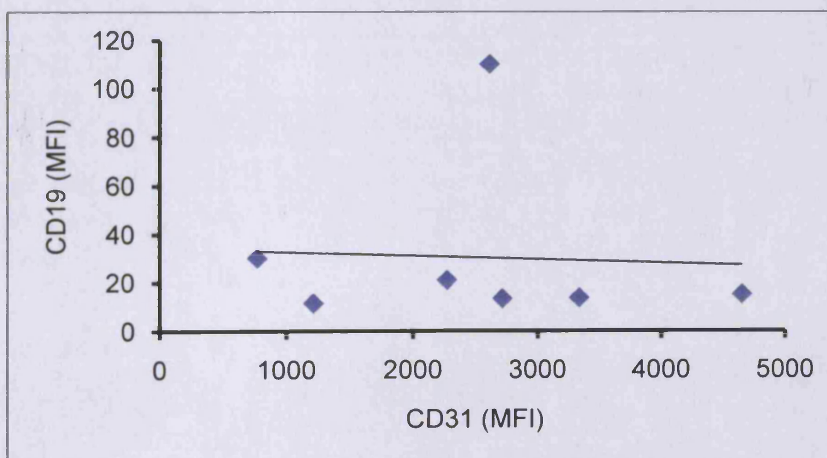


Figure 4.d No significant correlation was observed between CD31 and day 2 CD19 expression (n=7)(P=0.12). The Spearman test was used to assess the correlation (P=0.7).

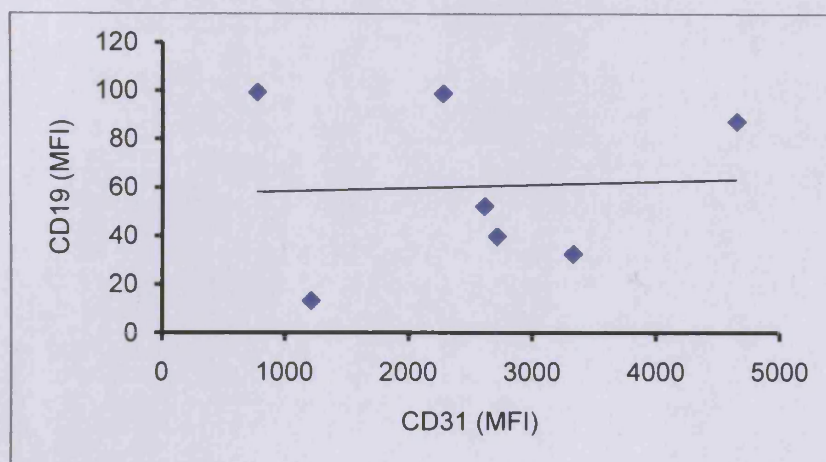


Figure 4.e No significant correlation was observed between CD31 and day 5 CD19 expression (n=7). The Spearman test was used to assess the correlation (P=0.6).

Genetic modification of primary chronic lymphocytic leukemia cells with a lentivirus expressing CD38

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ABSTRACT

Studies of the role of individual genes in chronic lymphocytic leukemia (CLL) have been hampered by the inability to consistently transfect primary tumor cells. Here, we describe a highly efficient method of genetically modifying primary CLL cells using a VSVG pseudotyped lentiviral vector. We transduced CD38 negative CLL cells with a lentiviral vector encoding CD38 which caused increased surface CD38 expression in all the samples tested (n=17) with no evidence of plasmacytoid differentiation. The mean percentage of positive cells expressing CD38 was 87%±8.5% and the mean cell viability 74%±17%. This high level of transduction of all the CLL cell samples tested demonstrates the utility of this technique which should prove applicable for the introduction and analysis of other genes in these non-dividing cells.

Key words: chronic lymphocytic leukemia, primary tumor cells, CD38 negative.

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Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease ranging from a stable condition requiring no therapy to a progressive disease refractory to treatment. One important molecule associated with this disease is CD38: the expression of the CD38 antigen on the surface of clonal B cells is associated with a poor prognosis and reduced overall survival in patients with CLL.[1-6] The expression of CD38 defines an altered pattern of gene expression including increased levels of anti-apoptotic, pro-inflammatory, signaling and pro-angiogenic molecules.[7,8] However, these experiments were performed by comparing cells from different patients, with heterogeneous genetic backgrounds, and other studies depend on correlations in expression. These comparative experiments demonstrated a technical limitation of our ability to genetically modify CLL cells to alter CD38 expression.

To date, CLL cells have been difficult to genetically modify. The cells do not grow in liquid culture and most methods of manipulation result in the modification of a subset of cells and often cause substantial cell death. To address this problem, we developed a method of genetically modifying CLL cells using lentiviruses. This allowed us to increase CD38 expression in all the patient samples tested (n=17) with high transduction efficiency and viability. Using this approach, we now have the opportunity to determine whether CD38 can directly alter gene expression in primary CLL cells and influence cell survival, migration and proliferation.

Design and Methods

Lymphocyte separation

Following informed consent, peripheral blood samples from CLL patients with low expression of CD38 were separated using Ficoll-Hypaque (Sigma, Poole, UK), washed in PBS and counted. Patients were diagnosed using morphological and immunophenotyping criteria and were treatment free for at least three months prior to their analysis.

Generation of lentivirus

A cDNA corresponding to CD38 (Accession NM_001775) or the first 233 amino acids of rat CD2[9] was cloned into the pHR' SINcPPT SFFV-WPRE vector. Transgene expression was under the control of the SFFV promoter.[10] The GFP virus, driven by the same promoter, has been previously described.[10] The vector plasmids (pLentiSEW, pLentiSCD38W or pLentiSrCD2ΔW), together with the gag-pol plasmid (pΔ8.91) and the VSVG envelope encoding plasmid (pMD2-G), were amplified in bacteria and purified with the Endofree Maxiprep Kit (Qiagen). The transfer vector (13μg), pΔ8.91 (10μg) and pMD2-G (6 μg) was mixed

Acknowledgments: the authors would like to thank Dr. Qasim and Professor Thrasher at the Institute of Child Health, London for the provision of reagents.
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E-mail: BrennanP@cardiff.ac.uk
The online version of this article has a supplementary appendix.

with 1.5 mL of CaCl (0.25M) (Sigma Poole, UK). This was added to 1.5 mL of 2X HEPES (Sigma Poole, UK) while bubbling. The solution was left for 20 min to allow a precipitate to form. This was then added to a large flask (175 cm^2) of 293T cells (approximately 60% confluent) containing 20 mL of Dulbecco's Modified Essential Media (DMEM) with 10% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine.[11] After 48h, at 37°C, in 5% CO_2, the supernatant was removed and centrifuged at 1,700 g for 10 min to pellet any cell debris, followed by ultracentrifugation at 121,603 g for two hours to concentrate the virus. The pellet, containing concentrated virus, was re-suspended in DMEM, (Invitrogen, Paisley, UK) without supplements and stored at -80°C.

Lentiviral infection of chronic lymphocytic leukemia cells

Primary CLL cells were added to DMEM cell culture media with supplements (10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine). Concentrated viral supernatant was added to the culture media. The cells were then incubated at 37°C, in 5% CO_2. Expression of CD38 and other molecules were typically monitored after 48 h. No feeder cells or cytokines were added to the cultures.

Titration of lentivirus

The lentivirus was titrated using CLL cells. Five hundred thousand CLL cells were placed in 1 mL of DMEM cell culture media with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2mM glutamine. Volumes of viral supernatant, ranging from 1µL to 128 µL, were added. After 48 h, gene expression was monitored and the number of infectious virus particles per microliter was estimated by determining the percentage of cells infected in the linear portion of the curve. In some cases, the amount of the lentiviral protein, p24, was determined by ELISA (Helvetica Health Care Sàrl, Switzerland).

Flow cytometry

The following antibodies were used for immunophenotypic analysis: anti-CD19PE-Cy5 from DACO (C7066), anti-CD38RPE from Caltag (MHCD 3804-4) and anti-CD2FITC from Santa Cruz Biotechnology (sc-53036). Expression was measured using a Becton Dickinson FACSCalibur.[12]

Results and Discussion

Lentiviral technology represents a powerful method of genetically modifying quiescent cells.[13] Three decisions underpinned the development of this protocol. Firstly, we chose to use a viral backbone where transgene expression was driven by the spleen focus forming virus promoter. The CMV promoter has been shown to be ineffective in some quiescent lymphocytes.[14] Secondly, we focused on CD38 as a candidate molecule that is important for CLL prognosis.[15] Finally, CD38 is a cell surface marker, detected using flow cytometry, so the effectiveness of the genetic modification could be easily monitored. The virus generated to express CD38 was compared to two other viruses: a virus containing the genetic material for GFP and a virus containing the genetic material for truncated rat CD2.

All three viruses were capable of expressing their transgene in primary human CLL cells following lentiviral infection (Figure 1A). The highest level of expression was detected for CD38. We investigated CD38 expression in the CD19 positive population infected with both the CD38 virus and the GFP virus. A dramatic increase in CD38 was observed following infection with the CD38 virus (Figure 1B). This contrasted with a small increase in CD38 expression following infection with the GFP-expressing virus (Figure 1B).

Figure 2A and B show CD38 expression following increasing multiplicity of infections (MOI) of both the CD38 and the GFP lentiviruses. A dose response was observed depending on the amount of CD38 virus used. This dose response was apparent for both the percentage of cells that were CD38 positive (Figure 2A) and the mean fluorescent intensity for CD38 expression (Figure 2B). It was, therefore, possible to select a dose of lentivirus which would allow the expression of physiological levels of CD38 on the surface of the CLL cells. Importantly, the expression from the CD38 virus at an MOI of one, was higher than the expression of CD38 seen following infection with the GFP virus, even with an MOI above ten. The number of viral particles, determined by ELISA, in both preparations was compara-

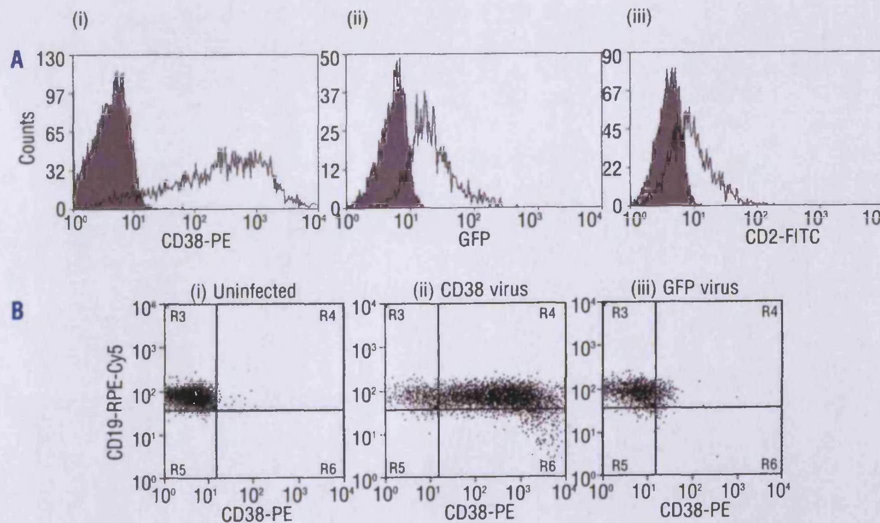

Figure 1. Successful transduction of CLL cells with lentivirus. (A) Treatment of CLL cells with lentivirus containing the genetic codes for CD38 (i), GFP (ii), and a truncated rat CD2 (iii) resulted in high levels of transduction (87%, 70% and 43%, respectively). (B) CLL cells from a CD38 negative patient (i) were infected with a CD38 lentivirus and 94% transduction was achieved (ii). Infection with a GFP control lentivirus saw a 9.7% increase in CD38 expression (iii).

ble. CD38 mRNA expression, measured by quantitative PCR, was higher in CD38 virus treated samples than in untreated or GFP virus treated samples (*Online Supplementary Figure S1*). The expression of CD38 was sustained over five days (Figure 2C and D).

Given the heterogeneity of CLL, we investigated the changes in CD38 expression following viral infection in multiple patient samples. A high percentage of cells expressing CD38 was observed in all patient samples treated with the CD38 lentivirus (Figure 3A; n=17, mean percentage of positive cells (±SD) was 87% ±8.5%). To investigate the effect of lentivirus on cell differentiation, we analyzed the cells following treatment with lentivirus and observed no major changes in CLL cell morphology (Figure 3B), and only observed a small increase in the expression of CD138, which is highly expressed in antibody-secreting plasmacytoid cells (*data not shown*). Thus, we demonstrated reproducible transduction of primary CLL cells to manipu-

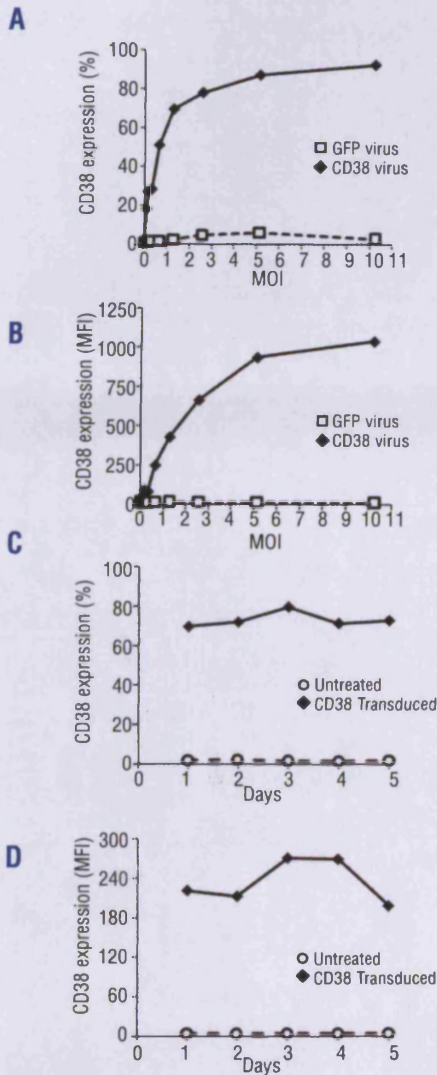

Figure 2. Expression of CD38 on virally infected CLL cells. A dose dependent increase in CD38 expression was observed in both the number of cells expressing CD38 (A) and in the MFI (B) of samples following treatment with CD38 virus (diamonds). No such increase is observed following the addition of a comparable amount of GFP virus (squares). Following transduction a high level of CD38 expression was observed on the surface of CLL cells following 24 h incubation. This level was sustained over a period of five days as measured by the percentage of cells expressing the CD38 antigen (C) and by the MFI (D) of the sample.

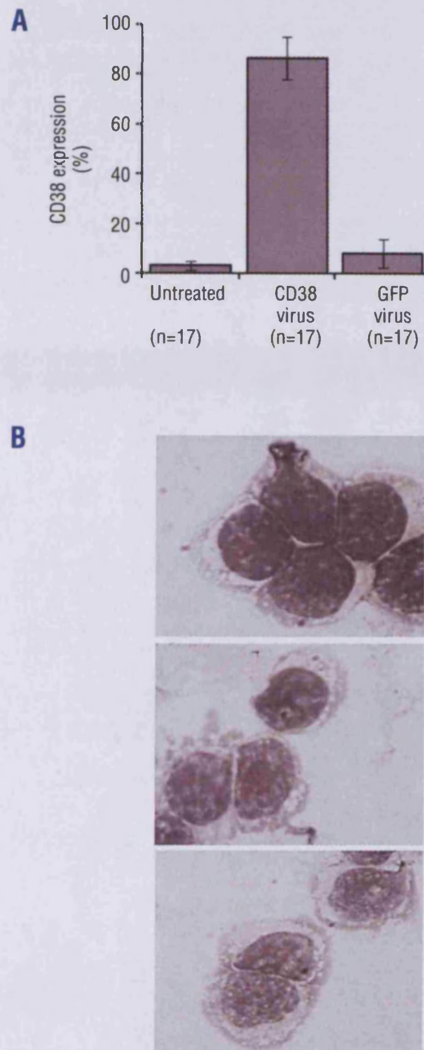

Figure 3. Expression of CD38 in multiple patient samples. (A) The mean expression of CD38 in the untreated samples was 3±1.8% (n=17). Following treatment with CD38 virus a mean of 87±8.5% (n=17) of CLL cells expressed the CD38 antigen. A mean of 8±5.8% CD38 expression (n=7) was observed in samples treated with control GFP virus. (B) Morphology of untreated and lentivirus treated CLL cells following 48 h incubation. Pictures were taken using a Zeiss Axio microscope equipped with a digital camera following Giemsa staining (x100 magnification).

late the expression of CD38 without causing cell differentiation. Here, we describe a lentiviral technique which is able to transduce CLL cells to a level greater than previously described. In two studies, no GFP was detected when driven by a CMV promoter[16,17] using VSVG coated lentivirus, which suggests that our use of the SFFV promoter allows higher levels of expression in CLL cells. Given the importance of promoters, using B-cell specific[18] or other gene expression units[19] may be useful. A report, published since submission, has demonstrated gene expression in CLL cells using lentivirus incorporating measles virus glycoproteins, H and F, on their surface.[16] This allowed genetic modification of a subset of cells (20-45% of cells). In contrast, our data shows a change in the whole population of CLL cells rather than a subset and a technique for CD38 which allows genetic modification of an average of 87% of cells. This high level of expression means there is no need for cell sorting for further studies. Our data also shows that CD38 can be detected at a higher level than GFP (despite the amount of virus used, MFI of GFP infected cells is shown in the *Online Supplementary Figure* 2) or CD2, which may be due to the human origin of CD38.

The next step in our study is to characterize the functional effects of CD38 in CLL cells. Our initial experiments indicate that CD38 has a subtle effect on the survival of CD38 negative cells when expressed alone from the lentivirus. Our analysis of gene expression shows that CD38 is highly up-regulated at the mRNA level (*data not shown*) but analysis of expression patterns is confounded by the heterogeneous nature of CLL patient samples. Our immunophenotypic analysis shows that CD19 expression is not altered and the levels of CD138 (*data not shown*) do not indicate differentiation of the CLL cells. However, an analysis of other cell surface markers and of the effects of ligating CD38 are currently underway.

In conclusion, this report describes the successful genetic modification of primary CLL cells to generate a CD38 positive population from a CD38 negative population. This method has been successful on all the samples we have tested to date and does not require any other treatment of the cells.

Authorship and Disclosures

LP performed research, analyzed data and wrote paper; LM, TTL, SH and RJM performed research; SD and CR contributed vital reagents; CF contributed vital new reagents and revised the manuscript; CP and PB designed and performed research, analyzed data and wrote the paper.

The authors reported no potential conflicts of interest.

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