STUDIES RELEVANT TO THE DIFFERENTIATION DEFECT OF THE CLONAL B CELLS OF CHRONIC LYMPHOCYTIC LEUKAEMIA

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

by

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Contents

Abstract

The presence of the integrin chain α4 on chronic lymphocytic leukaemia (CLL) cells has been identified as an adverse prognostic marker for the disease. Why some CLL clones express this integrin chain while others do not is currently unclear. Therefore, the initial aim of this thesis was to study the reasons why CLL clones have a differential expression of α4 and how this compares to normal B-cell subsets. Using FACS analysis, it was shown that the failure to express α4 in the majority of CLL clones is abnormal compared to normal CD19+ B-cell subsets. Efforts to relate α4 expression to different activation, differentiation or anergic states of the malignant cells all failed to reach significance. At a molecular level, the integrin protein was shown to be absent due to a failure to produce mature α4 mRNA. Analysis of the α4 gene showed that this loss in transcription was probably caused by an epigenetic mechanism involving a reduction in the histone marks H3K4me3 and acetylated H3 which facilitate transcription initiation. During these studies into the α4 integrin chain, a possible oncogenic, truncated form of the PRDM1 transcription factor was observed by Western blotting. Further experiments, however, proved that the presence of this band in CLL was caused by a cross-reaction of the Western blotting antibody with contaminating albumin. The induction of PRDM1 following PC-inducing stimuli has never been studied in CLL. Following antigen encounter, induction of PRDM1 is known to be a pivotal step in B-cell differentiation to plasma cells (PCs), immunoglobulin secretion and subsequent antigen clearance. As CLL is thought to be dependent upon antigen for development and/or increased cell survival, the failure of CLL cells to induce PRDM1 *in vivo* may be central to the development/perpetuation of the malignant clone. Upon treatment with PC-inducing stimuli, PRDM1 was variably induced in different cases of CLL; some clones were unable to induce the transcription factor while others induced relatively large amounts. A lack of PRDM1 expression after stimulation was associated with the clone being unable to generate a PC transcriptional and morphological phenotype and secrete immunoglobulin *in vitro*. The failure to induce PRDM1 *in vitro* may be related to the antigen encounter of the CLL clone *in vivo,* as non-responsiveness correlated with the *IgV_H1-69* gene segment usage but not other prognostic markers. PRDM1 was shown to be controlled at the level of transcription in CLL cells, but the activity and levels of the known transcriptional regulators of PRDM1 both before and after stimulation could not distinguish the two PRDM1 response subtypes. Initial chromatin immunoprecipitation experiments demonstrated that a failure to produce histone marks associated with transcription initiation/elongation on, and the recruitment of serine-5 phosphorylated RNA polymerase II to, the *prdm1* gene are not responsible for the failure of transcription.

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Declaration

All of the work presented in this thesis is my own apart from the analysis of the HCDR3 region stereotype which was performed by Dr. Ke Lin.

Abbreviations

APS	-	Ammonium persulphate
Bach2	-	BTB and CNC homology 2
Bcl-6	-	B-cell CLL/lymphoma 6
BCRxl	-	B-cell receptor cross-linking
BD	-	Becton Dickinson
bp	-	base pair
BSA	-	Bovine serum albumin
BSAP	-	B-cell specific activator protein
Btk	-	Bruton's tyrosine kinase
CD40L	-	CD40 ligand
cDNA	-	Complementary DNA
CDR3	-	Complementarity determining region 3
ChIP	-	Chromatin Immunoprecipitation
CLL	-	Chronic lymphocytic leukaemia
CpG-ODN	-	Deoxyribo(cytidine-phosphate-guanosine) motif containing oligodeoxynucleotide
CRE	-	cAMP response element
CTD	-	C-terminal domain
Da	-	Daltons
$DiOC_6$	-	3, 3'-dihexyloxacarbocyanine iodide
DLBCL	-	Defuse large B-cell lymphoma
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
DNMT	-	DNA methyltransferase
ECM	-	Extracellular matrix
ER	-	Endoplasmic reticulum
EZH2	-	Enhancer of zeste homology 2
FCS	-	Fetal calf serum
FITC	-	Fluorescein isothiocyanate
GAS	-	γ-interferon-activated sequence
H3Ac	-	Acetylated histone H3
H3K27me3	-	Trimethylated lysine 27 on histone H3
H3K36me3	-	Trimethylated lysine 36 on histone H3
H3K4me3	-	Trimethylated lysine 4 on histone H3
HAT	-	Histone acetyltransferase

HCDR3	-	Heavy-chain CDR3
HCL	-	Hairy cell leukaemia
HDAC	-	Histone deacetylase
hr	-	hour
HRP	-	Horse radish peroxidase
IFN	-	Interferon
Ig	-	Immunoglobulin
IgM, -D, -G or -A	-	Immunoglobulin of isotype M, -D, -G or -A
IgV_H	-	Immunoglobulin variable-heavy chain
IL-21	-	Interleukin - 21
IL-21R	-	IL-21 receptor
IRE1	-	Inositol-requiring protein 1
IRF	-	Interferon regulatory factor
ITGA4	-	Integrin α4 gene
IκB	-	NF-κB inhibitor protein
LB	-	Lysogeny broth
LLPC	-	Long-lived plasma cell
LPS	-	Lipopolysaccharide
MARE	-	Maf recognition element
MCL	-	Mantle cell leukaemia
MEL1	-	MDS1/EVI1-like 1
min	-	minute
miR	-	microRNA
MITF	-	Microphthalmia-associated transcription factor
mRNA	-	Messenger ribonucleic acid
MTA3	-	Metastasis-associated protein 3
MZL	-	Marginal zone leukaemia
NELF	-	Negative elongation factor
NFAT	-	Nuclear factor of activated T cells
NF-κB	-	Nuclear factor of κ-light-chain-enhancer of activated B cells
NLR	-	NOD-like receptor
NLS	-	Nuclear localization signal
NR	-	PRDM1 non-responsive
PAGE	-	Polyacrylamide gel electrophoresis
PAMP	-	Pathogen associated molecular pattern
Pax5	-	Paired-box protein 5
PB	-	Peripheral blood
PBMC	-	Peripheral blood mononuclear cell
PBS	-	Phosphate buffered saline

PC	-	Plasma cell
PcG	-	Polycomb group
PCL	-	Plasma cell leukaemia
PCR	-	Polymerase chain reaction
PE	-	Phycoerythrin
PerCP	-	Peridinin chlorophyll protein
PI	-	Propidium iodide
PIC	-	Preinitiation complex
PKC	-	Protein kinase C
PMA	-	Phorbol 12-myristate 13-acetate
polyHEMA	-	Poly(2-hydroxyethyl methacrylate)
PRC	-	Polycomb repressive complex
PRDI-BF1	-	Positive regulatory domain I-binding factor1
PRDM1	-	PRDI-BF1/RIZ-domain containing protein 1
PRR	-	Pattern recognition receptor
P-TEFb	-	Positive transcription elongation factor b
PTM	-	Post-translational modification
PU.1	-	Purine-rich box 1
qPCR	-	Quantitative polymerase chain reaction
R	-	PRDM1 responsive
rhCD40L	-	recombinant human CD40 ligand
RIZ	-	Retinoblastoma protein-interacting zinc finger
RNA	-	Ribonucleic acid
RPII	-	RNA polymerase II
SDS	-	Sodium deodecyl sulphate
sec	-	second
SEM	-	Standard error of the mean
SET	-	Su(var)3-9, enhancer of zeste, trithorax
SLPC	-	Short-lived plasma cell
STAT	-	Signal transducer and activator of transcription
T0	-	Time zero
TBE	-	Tris/borate/EDTA
TD	-	Thymus dependent
TEMED	-	N,N,N',N'-tetramethylethylenediamine
TFIIB, -D, -E, -F, or -H	-	Transcription factor II B, -D, -E, -F or -H
TI	-	Thymus independent
TLR	-	Toll-like receptor
TNFα	-	Tumour necrosis factor α
TPA	-	12-*O*-tetradecanoylphorbol-13-acetate
TrxG	-	Trithorax group

TSS	-	Transcription start site
UPR	-	Unfolded protein response
v/v	-	Volume to volume
VLA-4	-	Very late antigen - 4
w/v	-	Weight to volume
XBP-1	-	X-box binding protein 1
ZEB	-	Zinc finger E-box binding homeobox

Chapter 1

GENERAL INTRODUCTION

1.1 OVERVIEW OF INTRODUCTION

This thesis is exclusively concerned with chronic lymphocytic leukaemia (CLL), so this Introduction starts with a brief review of the disease.

The aim of the first experimental chapter (Chapter 2) was to establish why some CLL clones express the integrin heterodimer α4β1, while others do not. Consequently, the nature of α4β1, and its role in lymphocyte and CLL biology are considered next. Since it turned out that α4 expression is controlled at a transcriptional level in a process involving histone modifications, brief overviews of the transcriptional control of α4 in other cells and mechanisms of epigenetic control in transcriptional regulation are also presented.

The work in Chapter 3 arose out of Chapter 2 where the possibility that α4 expression is related to the stage of differentiation of the CLL clone was investigated. Thus, interleukin-21 (IL-21) was used to induce

differentiation of $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones and expression of PR-domain containing protein 1 (PRDM1) was used as a marker of differentiation. Although it turned out that α4 expression is not related to differentiation, an apparent lower molecular weight isoform of PRDM1 was detected. Since it is known that truncated forms of PRDM1 proteins are important in lymphoma[1], the nature of this lower molecular weight band was studied. Although, disappointingly, the band proved to represent a cross-reaction to albumin, these studies provoked my interest in PRDM1 and plasma-cell (PC) differentiation in CLL cells – work pursued in Chapters 4 and 5. Therefore, the nature and control of PRDM1 expression and PC differentiation are reviewed in detail.

In Chapter 4, IL-21 and CpG-ODN (deoxyribo[cytidine-phosphate-guanosine] motif containing oligodeoxynucleotides) were used to induce PRDM1/PC differentiation of CLL cells. Therefore, the biology of these stimulants will be considered, along with a summary of previous work regarding CLL-cell differentiation.

Chapter 5 is concerned with two interrelated questions: what combinations of activating transcription factors are needed to induce PRDM1 in CLL? and what is the nature of the differentiation block in PRDM1 observed in some CLL clones? A major part of this work involved

consideration of the control of PRDM1 transcription and expression, and of the signalling pathways downstream of IL-21 and CpG-ODN receptors. These subjects, therefore, are also reviewed.

Chapter 6 highlights the novel findings of the thesis and briefly attempts to outline how the current work might be developed in the future.

In the light of this brief overview, the following topics are relevant and will be considered in turn:-

An overview of CLL

The $\alpha 4\beta 1$ integrin heterodimer

Gene transcription regulation

Epigenetic control of transcription

PC differentiation

The transcription factor PRDM1

IL-21 and PC differentiation

CpG-ODN and PC differentiation

1.2 AN OVERVIEW OF CLL

1.2.1 Definition

CLL is a chronic lymphoproliferative disorder characterised by the presence in the blood of $\geq 5x10^9$/L CD5+CD23+ light-chain-restricted B cells. When such cells are present at lower numbers, the clonal expansion is termed a monoclonal B lymphocytosis with a CLL-like phenotype[2]; many such cases never develop CLL.

1.2.2 Clinical aspects

CLL is by far the most common leukaemia of adults with an annual incidence of around 3 cases per 100,000 [3]. The disease is commoner in older people and is rare in non-Caucasians. The diagnosis is often made by a full blood count performed for entirely routine reasons or for investigation of unrelated problems.

The clinical course is highly variable, with some patients remaining asymptomatic for prolonged periods. However, in others, the disease can be highly progressive and associated with symptoms and the development of organomegaly, bone marrow failure and immunosuppression. This, in turn, leads to recurrent infections which can eventually be fatal. Progression may be associated with changes in

the cytology, cytogenetics and behaviour of the malignant cells. These changes are termed transformation and can take the form of either prolymphocytic[4] or Richter transformations[5]. The former is associated with appearance in the blood or lymph nodes of larger cells termed prolymphocytes. In Richter's transformation, the malignant cells take on a blastic morphology and the disease comes to resemble diffuse large B-cell lymphoma (DLBCL).

Like other low-grade lymphoproliferative disorders, CLL remains incurable. However, introduction in recent years of new agents has allowed effective treatment for a proportion of patients[6]. These newer treatments include nucleosides (especially fludaribine) and antibodies e.g. anti-CD20/rituximab and anti-CD52/Campath/alemtuzumab. These and previous treatments (alkylating agents and steroids) are not curative and all patients will eventually progress. Richter's transformation is treated as a high-grade lymphoma with combination chemotherapy, and this may return the patient to chronic-phase CLL for a variable period. Because this thesis is not concerned with therapy, treatment strategies will not be discussed further.

1.2.3 Prognostic features

Because the disease course is so variable, much effort has, over the years, been directed towards identifying methods of predicting clinical outcome. The earliest of these were clinical staging systems based on the presence or absence of organomegaly and bone marrow suppression[7,8] while, more recently, a number of laboratory prognostic markers have been identified.

Regarding clinical staging, two systems (Rai and Binet) have been widely employed and are summarised in Tables 1 and 2. The basic principle is that extensive organomegaly and/or bone marrow suppression are associated with an adverse prognosis.

Regarding prognostic markers, those that are commonly used are summarised in Table 3. In the context of this thesis, IgV_H (immunoglobulin variable-heavy chain) mutation, IgV_H gene segment usage, CD38 and CD49d merit special mention since they are relevant to the work presented in Chapters 2 (CD49d expression) and 4 (Table 5). Each of these prognostic markers will be briefly considered in turn.

IgV_H mutation (also see below) is a powerful prognostic indicator at presentation and can discriminate between early stage patients who will or will not progress[9,10]. Thus, when IgV_H mutation (i.e. deviation from

Table 1 – Rai system of CLL clinical staging.

Stage	Clinical Features	Average Survival* (mo)
0	Bone marrow and blood lymphocytosis only.	>150
I	Lymphocytosis with enlarged nodes.	101
II	Lymphocytosis with enlarged spleen or liver or both.	71
III	Lymphocytosis with anaemia (Hb less than 10g).	19
IV	Lymphocytosis with thrombocytopenia (platelets less than 100,000/mm^3).	19

*Taken from the study used to classify the stages[7]

Table 2 – Binet system of CLL clinical staging.

Stage	Clinical Features	Average Survival* (mo)
A	No anaemia, no thrombocytopenia, less than three involved areas (e.g. spleen, liver, lymph nodes).	Same as Controls
B	No anaemia, no thrombocytopenia, three or more involved areas.	84
C	Anaemia and/or thrombocytopenia.	24

*Taken from the study used to classify the stages[8]

Table 3 – Prognostic markers commonly used in CLL.

Prognostic Marker	Type	Brief Description	Prognosis
Lactate dehydrogenase (LDH)	Serum	Considered a marker of high tumour burden and cell turnover.	Negative correlation between serum levels and OS[11]
β2 microglobulin	Serum	Associates with the plasma membrane and the α-chain of class I MHC.	Negative correlation between serum levels and OS[12,13]
Chromosome karyotype	Cytogenetics	Trisomy 12 13q14 deletion 17p13 deletion 11q22 deletion	Similar to normal karyotype Good Very poor Poor[14]
p53	Molecular	Tumour suppressor gene located on 17p. Protects against the consequences of DNA damage by halting the cell cycle or inducing apoptosis.	Dysfunctional p53 confers poor prognosis and a poor response to treatment[15]
IgV_H mutation	Molecular	Mutated immunoglobulin variable-heavy genes give a better prognosis	≥2% = Good <2% = Poor[9,10]
IgV_H gene usage	Molecular	IgV_H families are skewed in CLL and can sometimes help to predict prognosis independently of mutational status	IgV_H3-21 confers poor prognosis[16,17]
CD49d	Surface marker	Allows transendothelial migration and entry into lymph nodes	Expression confers poor prognosis[18,19]
CD38	Surface marker	Expression is associated with UM cases and CLL-cell proliferation	>30% = poor[10]
Zap70	Intracellular antigen	Non-receptor tyrosine kinase that can be expressed in activated B cells	Expression confers poor prognosis[20]

OS – Overall survival

germline) is high (≥2% = mutated- or M-CLL), the mean duration of survival is around 24 years. In contrast, when IgV_H mutation is low (<2% = unmutated- or UM-CLL), the mean duration of survival is only around 8 years.

IgV_H family usage by CLL cells is skewed as compared with that of normal CD5+ B cells. In particular, IgV_H1 family genes are expressed more frequently, while those of the IgV_H3 family are less frequent. Furthermore, UM- and M-CLL clones differ in their use of particular IgV_H gene segments. For example, the use of IgV_H1-69 is particularly common in UM clones[9]. Recently, IgV_H gene segment usage has been identified as a useful tool for prognosis, with CLL clones expressing IgV_H3-21 having a poor prognosis independently of their mutational status[17]. It is likely that the biased use of particular IgV_H genes reflects *in vivo* stimulation/selection by particular (auto)antigens[21]. This subject will be discussed in more detail below under Section 1.2.4.

Surface expression of CD38 is an adverse prognostic factor that correlates imperfectly with lack of IgV_H mutation[10]. It is somewhat controversial what level of percentage-cell expression should be employed as a prognostic marker, but a 30% cut off has become the usual parameter[10]. It is also not entirely clear why high levels of CD38

should confer an adverse prognosis. CD38 has two major functions. It is well known as an enzyme that catalyses the conversion of NAD+ to cyclic-ADP-ribose which then acts as an important second messenger in calcium homeostasis[22]. In addition, CD38 can act as a surface receptor for PECAM-1 (CD31), a molecule known to be involved in adhesion and extravasation of a number of leukocyte types[23]. Furthermore, ligation of CD38 with antibodies transduces signals important in cell survival/differentiation[24]. Why these functions of CD38 confer an adverse prognosis is not firmly established, but it is probably relevant that expression of the molecule is associated with CLL-cell proliferation[25,26].

Work from this laboratory was the first to show that expression of the integrin heterodimer, α4β1 (CD49d/CD29 or very late antigen-4, VLA-4), by CLL cells is necessary for transendothelial migration and is associated with clinical lymphadenopathy[27]. Subsequently, several studies showed that the expression of α4 is an independent adverse prognostic indicator[18,19]. Presumably this is because expression of the α4 allows migration into lymph nodes where the CLL cells can receive survival and proliferation signals. This means that interfering with the function of α4β1 might have therapeutic potential in CLL. The nature and control of α4 is discussed in further detail in Section 1.3.

1.2.4 The nature of CLL cells

The normal counterpart of CLL cells is still unclear. It has long been known that CLL cells express CD5 – a feature of B-1 cells. B-1 cells are important in innate immunity and produce naturally occurring, polyreactive antibodies against foreign and (auto)antigens (see Section 1.6.1.1). Therefore, the fact that CLL cells often produce similar polyreactive antibodies[28] has lent support to the notion that the malignant lymphocytes are related to B-1 cells.

However, it is also known that CLL cells have been variably activated *in vivo* and that extensive cross-linking of the BCR (B-cell receptor) of normal-B cells induces the expression of CD5[29,30]. It may therefore be that the CD5 expression of CLL cells is simply a manifestation of cell activation rather than an indication that the cell of origin belongs to the B-1 lineage.

Gene array analysis has shown that CLL cells more closely resemble memory B cells than any of the other B-cell subtypes tested, including CD5+ B-1 cells[31]. The fact that CLL cells consistently express the memory cell marker, CD27[32], is in accord with the notion that CLL cells are related to normal memory cells.

In view of these uncertainties, the α4 expression of CLL cells was compared in Chapter 2 with that of both CD5+ and CD27+ normal PB (peripheral blood) B cell subpopulations.

Regarding the phenotype of CLL cells, they express CD23, together with CD5 and a low density of light-chain-restricted surface Ig (immunoglobulin). The CD23 is probably a reflection of the activation of CLL cells[33]. Why only small amounts of surface Ig are detected on the CLL cells is still not clear, but the low expression has been related to abnormal protein processing and folding[34,35] and may be a feature of anergy caused by chronic stimulation[36]. The majority of CLL clones express IgM (Ig of isotype M) and IgD, but up to 20% are class switched and express IgG or IgA[37].

CLL cells express a range of activation/differentiation markers and this has been used to support the notion that CLL cells have been activated *in vivo*[38]. The expression of these markers is considered in Chapter 2 and will not be discussed further here.

Although CLL cells are probably stimulated *in vivo* by a number of factors (e.g. adhesive ligands, CD40 ligand, cytokines, etc), there is considerable evidence that antigenic stimulation/selection via the BCR is pivotal for CLL development. Such evidence includes the fact that IgV_H gene family

usage is strikingly biased in the disease and that the antigen binding site (CDR3; complementarity determining region 3) is often similar in different CLL clones (a phenomenon known as stereotypy, present in around 20% of all cases of CLL[39]). As there is a suggestion that different IgM antibodies from the same heavy-chain CDR3 (HCDR3)-stereotypic subset may recognise similar cytoplasmic (auto)antigens[28,40], it is hypothesised that some clones have been selected by a limited number of (auto)antigens or by (auto)antigens with structurally similar epitopes[41].

In addition to causing cell activation, continuous exposure to low levels or low affinity (auto)antigen can also induce anergy[42]. This is a state in which the cells are able to bind antigen, but are functionally unresponsive to that antigen. It is known from animal models that different types of antigens can induce anergic cells with different biochemical and functional features[43]. However, the inability of BCR-cross-linking (BCRxl) to induce a rise in intracellular calcium has been employed as a general marker of anergy in CLL[44]. Also, as a result of internalisation, anergic cells frequently express low levels of surface IgM[43]. In the present study, therefore, calcium fluxes in response to BCRxl and surface IgM levels were employed to test the hypothesis that lack of α4 expression might be related to anergy.

1.3 THE α4β1 INTEGRIN HETERODIMER

1.3.1 The integrin family of adhesive proteins

Integrins constitute a large family of heterodimeric transmembrane proteins, composed of non-covalently associated α and β chains. Two, α4β1 and αLβ2 (LFA-1), are of particular relevance to the adhesion and migration of B lymphocytes, and have been an interest of this Department for a number of years.

Integrins mediate adhesion by binding to either cell-bound ligands or ECM (extracellular matrix) proteins. Ligands for α4β1 are VCAM-1 on cells and fibronectin in the ECM[45]. αLβ2 binds to a number of ligands, especially cellular ICAM-1[46]. Coordinated adhesion and de-adhesion are necessary for cell motility[47].

In order to bind their ligand, integrins have to undergo a stimulation-induced conformational change (so called inside-out activation), which greatly increases the affinity of the receptor for its ligand[48]. This mechanism prevents unwanted adhesion by unstimulated cells. The strength of adhesion can also be enhanced by receptor clustering (known as avidity regulation).

Upon ligand binding, the integrins transmit (outside-in activation) signals for a number of cellular functions, including shape change and survival[48].

1.3.2 Structure

The α4 integrin chain has a molecular weight of around 150kDa, and lacks the ligand-binding I-domain present in many α chains. Despite lacking the I-domain, the conserved amino acids around where this domain would have been inserted in other α-chains are able to mediate binding[49]. In lymphocytes, the α4 chain can be associated with either β1 or β7, and this is true of CLL cells[50]. However, α4β1 is the main mediator of CLL-cell transendothelial migration[51].

α4 is also unusual among α-chains in being proteolytically cleaved into 2 fragments of 70 and 80kDa, which remain associated non-covalently with each other and with the β-chain. All three molecular forms can be expressed at the cell surface and their relative expression is known to vary between different types of leukocytes. It is already established that all three molecular weight forms can be found in CLL cells[50] and this was confirmed in Chapter 2 when $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL cells were compared for α4 protein expression (only data for $\alpha4^{150}$ shown).

The structure of the β1-chain has been extensively reviewed elsewhere (e.g. Green, LJ *et al*[52]) and, since it is not studied in this thesis, this integrin chain will not be considered further here.

1.3.3 Control of expression

When this thesis was begun, nothing was known at about the mechanism controlling α4 expression by CLL cells. However, it was established in other cell types that surface expression of the integrin chain can be controlled by changes in transcription/translation or by endocytosis. It was apparent that a range of cell stimuli are able to alter surface α4 expression. Such stimuli include TNFα (tumour necrosis factor α)[53] and LPS (lipopolysaccharide)[54]. For this reason a considerable, but unsuccessful, effort was made (Chapter 2) to relate surface α4 positivity/negativity to activation/differentiation.

Regarding possible down-regulation of surface α4 by endocytosis, it is known that engagement of the antigen receptor of T cells[55] can lead to internalisation of α4, with consequent reduction in surface expression of the integrin chain. Since CLL cells are thought to have been stimulated by their BCR, it seemed possible that a similar process might be occurring in these malignant B cells. However, this possibility was

excluded by showing that $\alpha4^{neg}$ CLL cells lack α4 protein as well as messenger ribonucleic acid (mRNA; Chapter 2).

The fact that a range of stimuli failed to induce α4 expression in $\alpha4^{neg}$ clones, prompted consideration of possible epigenetic mechanisms by which the α4 gene (*ITGA4*) might be silenced. It has been shown in gastric cancer cells that *ITGA4* can be repressed by CpG methylation[56]. This possibility was considered in Chapter 2, and the subject of CpG methylation is therefore reviewed in Section 1.5.2.

Histone modification is another epigenetic mechanism by which gene transcription can be activated or repressed. There have been studies of the transcriptional activators and repressors of α4 in cell types other than CLL[57-62]. However, although it has been shown that histone deacetylase inhibitors alter α4 expression in myeloid and hepatic cells[63], little or nothing is known about how activators/repressors modify the histones associated with *ITGA4* gene. Histone marks associated with *ITGA4* gene form an important part of the investigations described in Chapter 2 and therefore the subject of histone post-translational modifications (PTMs) is reviewed in Section 1.5.3.

1.3.4 Function

As mentioned above, there are two important ligands of α4β1 - VCAM-1 and fibronectin - and these have distinct functional effects.

VCAM-1 is expressed by endothelial cells of HEV (high endothelial venules) and those of inflamed tissues. After stimulation by chemokines, the α4β1 of rolling lymphocytes is able to interact with VCAM-1 and this process, together with αLβ2 to ICAM-1 binding, causes adhesion to endothelium[64]. Following adhesion, lymphocytes then transmigrate along a chemotactic gradient in a process involving αLβ2 and a range of other molecules[65]. During this process there is crosstalk between α4β1 and αLβ2[66]. This is particularly important in CLL because the activation of αLβ2 by chemokine is frequently defective[51] and, as a result, αLβ2 cannot be activated unless α4β1 is expressed and engaged[51,67]. In addition to mediating lymphocyte-to-endothelium interaction, engagement of α4β1 with VCAM-1 enhances the survival of CLL lymphocytes[68].

Fibronectin (FN) is an important constituent of the extracellular matrix. Cells adhere to, and spread on, FN in a process involving α4β1 and other components of so-called focal adhesions[69]. CLL cells variably adhere to

FN *in vitro*, but do not actively spread[50]. In general, adhesion to FN transmits signals enhancing cell survival[70] and this is true of CLL cells[71].

1.4 BRIEF OVERVIEW OF TRANSCRIPTION

This subject is comprehensively reviewed in many textbooks and only information relevant to Chapter 5 will be given here.

1.4.1 Overview of mRNA transcription

There are three different RNA polymerases (RP) in eukaryotes, and it is RPII that is responsible for protein-encoding mRNA production. mRNA transcription relies on the six components of the general transcription machinery. These are:- RPII and the five general transcription factors known as transcription factor II B (TFIIB), TFIID, -E, -F and –H. Without the general transcription factors, RPII alone is unable to recognise a promoter and initiate transcription[72]. Binding of unphosphorylated RPII and the general transcription factors (together known as the transcription preinitiation complex [PIC]) to the promoter of a specific gene is controlled by the so-called mediator complex and by gene-specific transcription factors. The mediator complex bridges upstream

activator molecules with the RPII complex and is made up of more than twenty subunits[73].

Transcription factors can promote or repress transcription in three different ways. They can interact directly with the mediator/RPII initiation complex and stabilise or block its binding to DNA. Also, they can have histone acetyltransferase (HATs) or histone deacetylase (HDAC) activity that allows or denies access to the DNA sequence respectively (see Section 1.5.3). Finally, a transcription factor may be able to recruit other factors that have one or both of the functions mentioned above.

1.4.2 Regulation of transcription

As has been shown by individual studies of genes, transcription can be regulated at many levels, but these processes usually fall into three main categories of transcription initiation, transcription elongation and RNA stability.

1.4.2.1 Transcription initiation

While it was originally thought that transcription initiation was the main regulatory strategy for mRNA transcription, it has more recently come to light that >70% of all promoters from active and inactive genes in humans have signs of transcription initiation[74]. After binding of the PIC and the mediator complex to the promoter region of the gene, Serine-5

of the C-terminal domain (CTD) of RPII is phosphorylated (Ser5P) by TFIIH. This phosphorylation, in turn, stops mediator binding to the RPII complex and allows the start of transcription elongation[75]. Ser5P of the CTD is also required for a number of other processes, including the recruitment of the 5'-mRNA capping enzyme[76] and, in yeast, the Set1 catalysed methylation of histone 3 lysine 4 (H3K4)[77]; it has been shown that mammalian homologs of Set1 may also be recruited in a similar way[78]. Therefore, marks/signs of transcription initiation on the transcription start site (TSS) include the presence of histone H3 acetylation, H3K4 methylation and Ser5P-RPII, and these marks are used in Chapter 5.

It is evident that initiation of transcription does not always result in production of full length mRNA. After the initiation phase, the RPII has the choice to either pause, terminate or elongate.

1.4.2.2 Transcription pausing, elongation and termination

The choice of whether RPII is allowed to cause transcription elongation or is made to pause or undergo early transcription termination is controlled by positive and negative elongation factors. For example, the negative elongation factor, NELF, can function as part of the early termination pathway in higher eukaryotes, and this transcription

repression is overcome by the positive elongation factor complex, P-TEFb[79] (positive elongation transcription factor b). P-TEFb phosphorylates RPII on Ser2 (Ser2P) via its cdk9 subunit, and this mark enables RPII to move past the pause/early termination checkpoint. As the CTD of RPII is dual phosphorylated on Ser2 and Ser5 at this point, it can then act as a docking site for the H3K36 histone methyltransferase (HMT) Set2[80]. Therefore, methylation of H3K36 is often used as a marker of transcription elongation and so is employed as such in Chapter 5.

Late transcription termination and polyadenylation of mRNA are tightly linked. Ser2P on the CTD acts as a docking site for the polyadenylation machinery[81,82] and, upon mRNA cleavage at the polyA site, the downstream RNA is quickly degraded (known as the 'torpedo' model of transcription termination[83,84]).

1.5 EPIGENETIC CONTROL OF TRANSCRIPTION

1.5.1 Definition

Epigenetics is the study into the control of gene expression by mechanisms that do not affect the underlying genetic sequence. Such control mechanisms can be maintained through cell division and

modified by cell stimuli, but they do not require alteration in the genetic code of the cell.

Epigenetic control is not limited to transcriptional regulation but, because the *ITGA4* and *prdm1* genes were found to be both regulated at a transcriptional level, only such mechanisms will be considered here. Changing gene transcription by epigenetic mechanisms is fundamentally achieved by altering the structure of chromatin. This can be done either by modifying DNA through the addition of a methyl group to cytosine bases or by PTMs of the histone proteins around which the DNA is coiled. Altering the structure of chromatin changes the affinities of both activating and repressing transcription factors for their DNA-binding sequence and therefore changes a gene's potential for transcription. Each of these mechanisms will now be described in more detail.

1.5.2 DNA methylation

The only known epigenetic modification of DNA in mammals is the addition of a methyl group to C5 of cytosine (5-methylcytosine) in CpG dinucleotides. In mammals, *de novo* methylation is produced by the DNMT3 (DNA methyltransferase-3) family of enzymes, while maintenance methylation (such as that placed on new daughter strands after replication) is done by DNMT1[85]. Although the methylation of DNA

is a relatively stable epigenetic mark, loss of this modification can occur. This is brought about either through DNA replication in the absence of maintenance DNMTs or can occur as a consequence of deglycosylation (either direct or through an initial deamination step) of the cytosine base, with subsequent excision and repair mechanisms[86].

DNA methylation is estimated to occur at ~70-80% of all CpG motifs in mammals, with the majority of 5-methylcytosines lying within repeat regions of the DNA[87]. CpG motifs are also concentrated in clusters (or CpG islands) around many gene promoter regions[88]. These CpG islands are often demethylated, but upon methylation can inhibit gene transcription either directly by interfering with specific transcription factor binding[89], or indirectly by recruiting methyl-CpG-binding proteins with their associated repressive chromatin remodelling activities[90]. Unlike histone modifications (see below), DNA methylation has only ever been linked with repression of gene transcription[91]. Recent studies have shown that loss in expression of both the *ITGA4*[56] and *prdm1*[92] genes can be the result of DNA methylation.

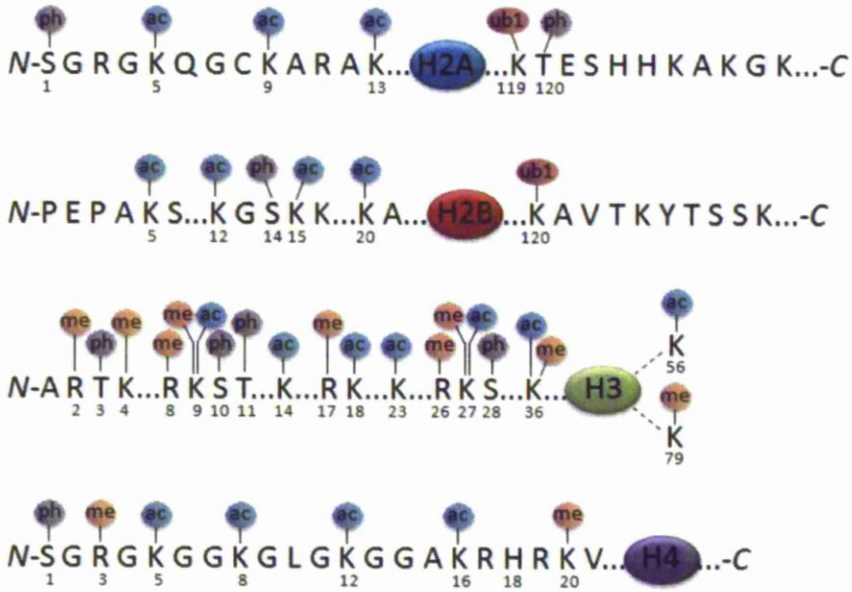
1.5.3 Histone post-translational modifications

In eukaryotes, 147 base pairs (bp) of DNA are wrapped around an octamer of histones consisting of two copies of H2A, H2B, H3 and H4 to

make a nucleosome. Each of these core histones has an amino-terminal tail that protrudes out of the nucleosome and can be subjected to multiple PTMs including acetylation, methylation, phosphorylation and ubiquitination amongst others. These PTMs can target multiple amino acids along the histone tails and include lysine and serine residues (see Fig. 1.1 for an overview).

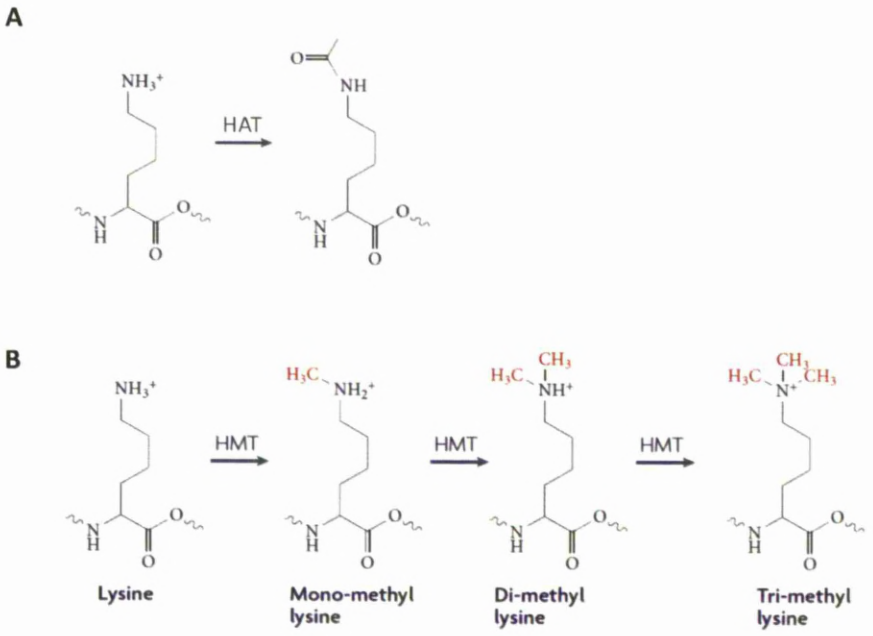
Modifications of histone tails are thought to affect chromatin structure and therefore transcription in one of two ways. The first is by altering the histone tails so that the contact of the histone with the DNA is altered. This in turn allows transcription factors and the PIC to access their DNA binding sequences and the transcriptome to form and move along the DNA strand. Acetylation marks, which are known to favour transcription, function in this way[93]. Thus, the acetylation of lysine (Fig. 1.2A) removes its positive charge allowing the negatively charged backbone of the DNA to become free/loosened from the histone core. These types of PTMs occur most commonly over the transcription start site, but some acetylation marks can extend throughout the gene allowing passage of the transcriptome along the locus. Acetylation is catalysed by HATs (e.g. CBP, p300, etc), while removal of this mark is performed by HDACs (e.g. HDAC1-11).

Figure 1.1



Post-translational modifications of human histones. Modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub). Apart from some exceptions, including ubiquitination of the C-terminal tail of histones H2A and H2B plus acetylation or methylation of two lysine residues in the globular domain of H3, most of the known histone post-translational modifications occur in the N-terminal histone tails. Globular domains of the histones are represented with coloured ovals. Reproduced from Bhaumik, SR *et al*[94].

Figure 1.2



Acetylation and methylation of lysine residues. Diagram showing the change in chemical structure of lysine upon **(A)** acetylation and **(B)** methylation PTMs. Adapted from Klose, SR *et al*[95].

The second method by which the modified tails affect chromatin is by acting as docking sites for transcriptional activators/repressors or chromatin remodelling proteins. Three such marks, which are known to act in such a way and that have been well characterized, are trimethylation of lysine 27 on histone H3 (H3K27me3), H3K4me3 and

H3K36me3, which are associated respectively with transcription repression, transcription initiation and transcription elongation[96-98] (see Fig. 1.2B for the structure of methylated Lys residues). It must be noted here, however, that methylation marks associated with active transcription do not appear to facilitate transcription[99,100]. Instead, these marks appear to be used to maintain transcribed chromatin templates for proper transcription. HMTs catalyse methylation, while demethylation is carried out by histone demethylases (HDM).

Repressive marks are produced and interpreted by the Polycomb group (PcG) of genes, while the Trithorax group (TrxG) of genes control activating marks. The PcG of genes produces a family of proteins which form three known distinct multimeric complexes – PRC1 (Polycomb repressive complex 1), PRC2 and PhoRC (pleiohomeotic repressive complex). PRC2 contains the SET (Su(var)3-9, Enhancer of Zeste, Trithorax) domain containing HMT EZH2 (enhancer of zeste homologue 2), which is known to directly di- and trimethylate H3K27[97]. Once trimethylated, H3K27 can then be recognised by the chromodomain of Polycomb, a protein in PRC1 which is associated with the formation of heterochromatin and gene silencing[97].

In contrast to the PcG, the TrxG is somewhat more heterogeneous. One class of the TrxG contain SET domains (MLL1-4 and SET1A/B - forming COMPASS-like complexes in humans). A second class forms part of the ATP-dependent chromatin remodelling complexes SWI/SNF or NURF. All known COMPASS-like complexes have H3K4 methyltransferase activity. Trimethylation of H3K4 creates a binding site for CHD1 (via its tandem chromodomains) and other proteins, via their PHD (plant homology domain), such as BPTF[101], ING2[102], ING4[103], ING5[104], RAG2[105] and TAF3[106]. Thus, through the presence of the histone mark H3K4me3, recruitment of CHD1 and PHD containing proteins brings to the gene proteins with functions associated with transcription initiation, further histone modification and/or ATP-dependent chromatin remodelling.

H3K36me3 PTMs occur predominately at the 3' end of actively transcribed genes. This methylation mark is likely to be the consequence of recruitment of HMTs by Ser2/Ser5-phosphorylated RPII as it moves along the gene locus during transcription (see Section 1.4.2). Levels of this modification are often used as a marker of active transcription elongation. Work in yeast suggests that H3K36 is trimethylated in the wake of RPII to recruit HDACs and, thus, stop transcription initiation at internal sequences of the gene[98,107].

It must be noted here that a single chromatin mark does not always predict the transcriptional activity of a gene and therefore cannot be taken in isolation. As such, the presence of the H3K27me3 mark over the TSS of a gene, for example, does not always predict transcriptional inactivity, especially when this mark is found alongside activating marks such as H3 acetylation (H3Ac) and H3K4me3. Such bivalent domains were first noticed in embryonic stem (ES) cells and hypothesised to keep the gene repressed, but poised for rapid expression[108]. Interestingly in the context of this thesis, these bivalent marks are seen on the *prdm1* gene in ES cells[109].

1.6 PLASMA-CELL DIFFERENTIATION

1.6.1 Terminal differentiation of mature B cells

Upon encountering antigen in the peripheral lymphoid tissues, mature B cells differentiate further in a process called terminal differentiation. These newly antigen-experienced B cells have multiple alternative cell fates which are determined by the type and quantity of the antigen exposure.

The different categories of antigen are based on the type of immune response they elicit and can be split into three groups: thymus dependent (TD), thymus-independent (TI) type-1 (TI-1; LPS) and TI-2 (e.g. [NP]-Ficoll [(4-hydroxy-3-nitrophenyl)-acetyl-Ficoll]). The different types of TI antigens are classified by their ability to induce responses in CBA/N mice that carry a Bruton's tyrosine kinase (Btk)-deficiency. TI-1 antigens possess epitopes that are recognised by both the BCR and toll-like receptors (TLRs; see below) and can induce a response in CBA/N mice. In contrast, TI-2 antigens are repetitive antigens that strongly cross-link and stimulate the BCR, but rely on Btk to induce an immune response[110]. After exposure to stimulating pathogens, B cells can rapidly differentiate into IgM secreting short-lived PCs (SLPCs) in response to TI stimulation. Alternatively, B cells can be stimulated by TD antigens to move to the follicle where they take longer to differentiate and, in the presence of T cells, undergo class switch recombination (CSR) and somatic hypermutation (SHM), before finally changing into either long-lived PCs (LLPCs) or memory B cells.

The above processes are dependent upon engagement of the BCR by antigen. However, plasmacytoid differentiation can also be induced independently of BCR engagement by signals of the innate immune system[111]; these include those derived from pattern recognition

receptors (PRRs) and from soluble products of other cells of the innate immune system such as macrophages.

1.6.1.1 Innate immune system

An evolutionarily older pathogen defence mechanism, the innate immune system has conserved features found in all classes of multicellular plants and animals. The system relies on recognising pathogens non-specifically through conserved repeat molecular structures termed pathogen-associated molecular patterns (PAMPs). Such motifs are recognised by PRRs, whose members include the TLR (toll-like receptor) and NLR (nucleotide-binding oligomerization domain [NOD]-like receptor) families. The subject of TLRs and their downstream effects are considered further in Section 1.9 which pays particular attention to TLR9 - the receptor for CpG-ODN - used to stimulate PRDM1 expression in Chapters 4 and 5.

Because of their resemblance to CLL cells, special mention must be given here to CD5+ B-1a cells and to the role of their natural antibodies in the innate immune system. These types of antibodies are produced at controlled levels in the complete absence of antigenic stimuli[112,113] and provide an immediate response with broad specificity to pathogens; these antibodies therefore form an essential part of the humoral

immune system[114]. Positive selection by autoantigen makes the development of B-1 cells unique compared to other B cell subsets and causes them to make low affinity antibodies which cross-react with a wide-range of epitopes[115,116]. As such, these antibodies not only bind to, and assist, clearance of pathogens, but can also recognise antigens derived from intracellular components of disrupted host cells[117]. B-1 cells are known to respond quickly and strongly to innate immune signals, including PAMPs and cytokines, but signals generated via their antigen receptor are relatively weak compared with those generated in B-2 cells[118]. These somewhat skewed responses mean they rarely enter the germinal centre (GC; see below) and do not produce high affinity class-switched antibodies, reducing the risk of autoimmune development[119].

1.6.1.2 Adaptive immunity

This defense mechanism against pathogens evolved after that of the innate system and is only present in jawed vertebrates[120]. The adaptive immune system functions by specifically recognising and clearing TD antigen, while generating memory for a faster and stronger response against future infections. Although the adaptive immune system is an evolutionarily advanced system for the removal of pathogens from the

host, it still relies on signals from the innate immune system to function efficiently.

The adaptive immune response involves the specific recognition of antigen in the interfollicular zones of lymph nodes and homologous areas in the spleen. T cells recognise antigen that has been processed and presented to them by antigen-presenting cells in the context of major histocompatability class II (MHCII); this stimulates T helper (T_H) cells to produce cytokines that cause B cells to proliferate and differentiate into SLPC which migrate to the medulla of the lymph node. In contrast, the Ig component of the BCR recognises, and is stimulated by, native unprocessed antigen. Such stimulation has many downstream effects including the induction of endocytosis of antigen-bound receptor and presentation of processed antigen to cognate T cells in the context of MHCII (peptide loaded MHCII, pMHCII). Cognate interaction between the B and T cells stimulates both cell types to enter follicles and form germinal centres (GCs)[121]. The activated B cells in the GC expand in the dark zone as centroblasts and undergo SHM of their Ig genes. Subsequently, the B cells enter the light zone as centrocytes and either die, or are selected by antigen on follicular dendritic cells. The selected centrocytes can either recycle back to the dark zone or potentially undergo further cognate interactions with T cells, undertake Ig heavy

chain CSR, and then differentiate into either memory or PCs[121]. The precise stimuli that determine these alternative fates are still unclear, but prolonged stimulation by CD40 ligand (CD40L/CD154) favours memory cell formation[122], while IL-2, IL-6, IL-10 and IL-21 promote PC differentiation[123-125].

After leaving lymph nodes, the PCs migrate to the bone marrow where they become long lived and secrete specific Ig. Memory cells, on the other hand, circulate and accumulate in the secondary lymphoid organs. In summary, then, the GC reaction produces long-lived, high affinity, class-switched (usually IgG) antibodies to TD antigens and, through memory cells, facilitates a rapid secondary response to re-stimulation by that antigen.

Regarding specific T_H cell subpopulations, naive T_H (T_H0) cells specifically recognise pMHCII via their cognate T-cell receptor (TCR); this causes activation which, in turn, is associated with the production of multiple cytokines (IL-2, etc) and the expression of cell-surface antigens (CD69, CD154, OX40, etc). The T_H cells then differentiate further to produce either T_H1, T_H2, T_H3 (T_{reg}), T_H9, T_H17 or $CXCR5^{hi}$ T follicular helper (T_{FH}) cells. The choice of differentiation to different T_H cell subsets is influenced by the type of stimuli the cells receive during their initial

activation. For example, T_H2 cell differentiation is enhanced upon exposure to IL-4, is characterised by the expression of the transcription factor GATA-3, and is associated with the secretion of many cytokines including IL-4 and IL-13[126]. Another subpopulation of T_H cells is located in follicles and secretes large amounts of IL-21[127] – the cytokine extensively studied in this thesis. These lymphocytes, termed T_{FH} cells, are generated by high affinity TCR-pMHCII interactions plus IL-12[128], and express the transcriptional repressor Bcl-6 (B-cell CLL/lymphoma 6)[129]. Both of these subtypes of T_H cells are known to be important in regulating CSR and PC differentiation *in vivo*. IL-21 will be discussed in more detail in Section 1.8.

1.6.2 Transcriptional control of PC differentiation

The transcriptional network that is altered by PC-inducing stimuli ultimately induces the functional and morphological changes associated with terminal differentiation. The transcription factor PRDM1 (also called Blimp-1 [B lymphocyte induced maturation protein 1] in mice) is induced directly by many stimuli that promote PC formation. PRDM1 is both essential and sufficient to induce PC differentiation[130], and for this reason is thought of as the master transcriptional regulator of this process; without PRDM1, no antibody is produced from any B-cell subset[131]. It works as a transcriptional repressor and functions by down-

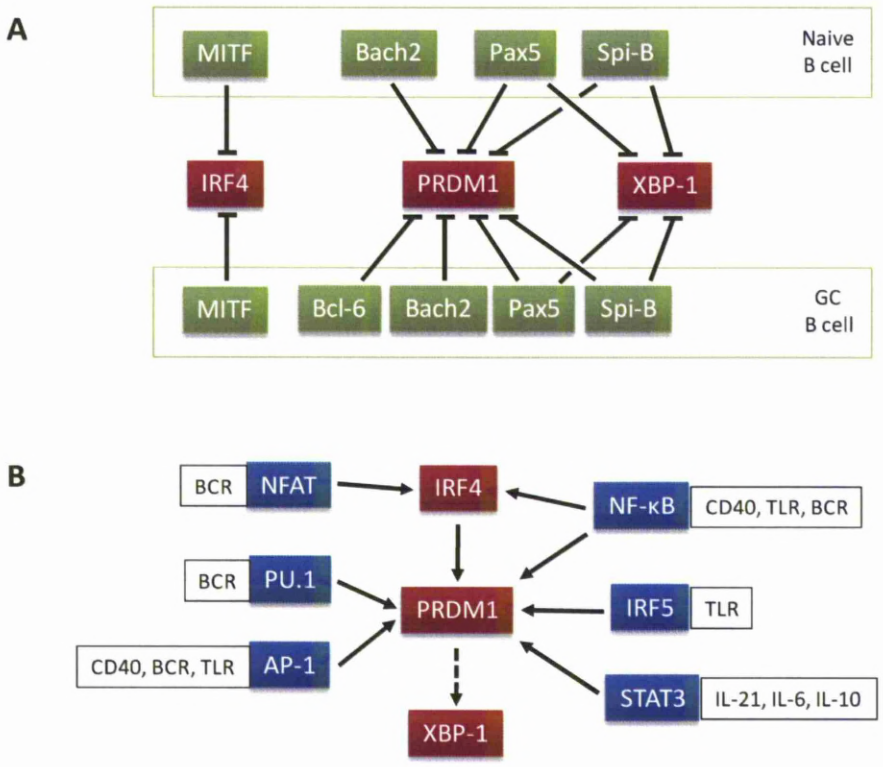
regulating many genes that maintain mature B cell identity and repress antibody secretion. The level of expression of PRDM1 appears to increase progressively during the later stages of PC development. Consequently, plasmablasts have the lowest levels, while the transcriptional repressor is most highly expressed in LLPC[132] (see below and Section 1.7 for more details on PRDM1 control and function). Many of the transcriptional repressors that inhibit PC differentiation target the *prdm1* gene. The transcriptional network (see Fig. 1.3 for an overview) that controls PC differentiation is highlighted over the next two sections.

1.6.2.1 Transcriptional repressors of PRDM1 and PC differentiation

There are three known direct repressors of PRDM1 in naive B cells (Pax5, Bach2 and Spi-B) and one indirect (MITF). Bcl-6 can also repress PRDM1 directly and is expressed in activated GC B cells.

The first direct repressor in naive B cells, Pax5 (Paired box protein 5; also known as BSAP [B-cell lineage specific activator protein]), can be expressed early in lymphoid development and determines differentiation along the B-cell pathway[133]. Its expression is maintained throughout B-cell development until plasmacytoid differentiation is induced. Pax5 can function as both a transcriptional activator and repressor. It is required for the AID (activation-induced cytidine

Figure 1.3



Transcriptional network controlling PC differentiation. Transcription factors in green are repressors of PC differentiation, those in red are highly expressed in PCs, while those in blue are transcription activators of PC differentiation. **(A)** Transcriptional inhibitors of PC differentiation in naive and GC B cells. **(B)** Transcriptional activation of PC differentiation. Examples of the stimuli that activate these transcription factors are shown in the clear boxes. Adapted from Calame *et al*[134].

deaminase) expression[135,136] which is needed for the rearrangement of the Ig genes at the pro-B cell stage of development, as well as for CSR and SHM in the GC. It also acts as a transcriptional activator for many genes involved in the function of the pre-BCR and BCR, while repressing a range of genes not associated with the B cell lineage[137]. In addition to silencing these non-lineage-specific genes, Pax5 also inhibits the expression of genes involved in PC differentiation, including *prdm1*[138,139] and XBP-1 (x-box binding protein 1; see Section 1.6.2.2)[140]. Pax5 expression is lost during PC differentiation through direct repression by PRDM1[141]. Thus, Pax5 and PRDM1 share a mutual repression loop, where repression by Pax5 must first be overcome to allow the induction of PRDM1 which, in turn, feeds back and enforces its own expression through the repression of *PAX5*. How the initial Pax5 repression of the *prdm1* gene is lost or overcome by activators is not yet clear. However, an initial stage in PC differentiation where Pax5 function is deactivated without the need for PRDM1 induction could be required for differentiation[142].

The second direct repressor of PRDM1 in naive B cells is Bach2 (BTB and CNC homology 2). This B-cell-specific transcription factor binds to the Maf (v-Maf musculoaponeurotic fibrosarcoma oncogene) recognition element (MARE) in the TSS of *prdm1* by heterodimerising with the small

Maf protein, MafK. Bach2 is expressed throughout B-cell development, but is lost on terminal differentiation to PCs[143]; this is probably through the loss of Pax5, a known positive regulator of Bach2 expression[144].

Finally, Spi-B is the only other known direct repressor of PRDM1 in naive B cells. This member of the Ets family of proteins is expressed at a moderate level in pre-antigen experienced cells, at a high level in switched memory B cells, and is lost upon PC differentiation. Compared with naive B cells, memory B cells rapidly reduce their expression of Spi-B when treated with PC-inducing stimuli. It is thought that this allows faster induction of PRDM1 during the secondary response to antigen. Ectopic expression of Spi-B in B cells, together with chromatin immunoprecipitation (ChIP) experiments, have identified PRDM1 and XBP-1 as direct targets of this transcription factor. However, it remains unclear whether or not Spi-B is a critical determinant of PRDM1 expression *in vivo*[145].

MITF (Microphthalmia-associated transcription factor) is constitutively expressed in naive B cells and is known to repress PRDM1 indirectly through its repression (by an unknown mechanism) of the *prdm1* activator IRF4 (interferon regulatory factor 4; see Section 1.6.2.2).

B cells that lack MITF spontaneously become activated and start secreting antibody[146].

Once the naive B cells become activated and move to the secondary follicles, the proto-oncogene Bcl-6 is up regulated[147] and is required for GC formation[148]. Without Bcl-6, B cells do not undergo SHM and do not generate LLPCs, but class-switched IgG_1 memory cells and SLPCs can still be produced normally[149]. Bcl-6 mainly functions as a transcriptional repressor and is known to inhibit PRDM1 expression in one of two ways. It can interact with the Jun subunit of AP-1 (a transcriptional activator of PRDM1; see below) and inhibit its DNA binding[150]. Alternatively, Bcl-6 can bind directly to the *prdm1* gene at both intron 5[151] and Intron 3[152] and recruit the co-repressor MTA3 (metastasis-associated protein 3), thereby inhibiting PRDM1 transcription[153]. Therefore, it is hypothesised that up regulation of Bcl-6 in the GC helps to maintain B-cell proliferation and SHM by providing (in collaboration with Pax5 and Bach2) additional repression of the *prdm1* gene and inhibition of premature terminal differentiation.

As is the case concerning how the repressive effects of Pax5 on *prdm1* are overcome, it is currently unclear how the repressive effect of Bcl-6 on *prdm1* is lost in the GC. However, there are several known

mechanisms that could reduce expression or inhibit the function of Bcl-6 in GC B cells. MAPK and PI3K/Akt pathways that are activated through BCRxl are both known to reduce Bcl-6 expression. MAPK causes phosphorylation-targeted ubiquitination of Bcl-6 leading to its subsequent degradation[154], while Akt causes inactivation of the *BCL6* transcriptional activator FoxO[155]. Although one of the functions of Bcl-6 is to inhibit ATR expression[156] (causing the bypass of some DNA damage checkpoints), accumulated genotoxic stress induces ATM which leads to phosphorylation and ubiquitination-targeted degradation of Bcl-6[157]. Both PRDM1 and IRF4 can bind directly to the *BCL6* gene and inhibit its transcription[158,159]. STAT5 (signal transducer and activator of transcription 5) activated by IL-2 is also known to repress Bcl-6 expression in BCL1 cells[160]. Alongside these mechanisms, acetylation can also inhibit the repressive function of Bcl-6 and acetylated Bcl-6 is present within B cells of the GC[161]; however, the mechanism of Bcl-6 acetylation has yet to be discovered.

1.6.2.2 Transcriptional activators of PRDM1 and PC-differentiation

Removal of transcriptional repressors does not appear to be all that is required for efficient PRDM1 and PC induction[162,163]; transcriptional activators are also essential. These are NF-κB, IRF4 via NFAT, STAT3, PU.1, AP-1 and IRF5, and each will be discussed in turn.

NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) is required for Blimp-1 induction by PRRs, as inhibition of this signalling pathway by pharmacological inhibitors abolishes Blimp-1 induction induced by LPS in murine primary B cells[109]. Also, Sendai virus stimulation of 3T3 cells, which normally induces Blimp-1 in these cells, is lost upon knockout of two important NF-κB subunits[163] (see Section 1.9 on how NF-κB functions). NF-κB has multiple potential binding sites on the PRDM1 gene and has been recently shown to bind directly to the *blimp-1* gene near the TSS in mice[109].

As well as enhancing PRDM1 induction directly, NF-κB can indirectly activate PRDM1 by, in collaboration with NFAT (nuclear factor of activated T cells)[164-166], up-regulating IRF4[159]. IRF4 can bind to the *blimp-1* gene[167,168] and is highly expressed in PCs. However, current literature is contradictory regarding how essential this response factor is for Blimp-1/PRDM1 induction. Two groups have used conditional IRF4 knockout mice to highlight the importance of IRF4 in mature B cells. They both report that IRF4 is required for efficient AID expression and CSR. However, only one of these papers suggest that IRF4 is essential for Blimp-1 induction by LPS[168,169]. IRF4 is frequently associated with, and functionally regulated by, other proteins[170] such as PU.1 (purine-rich box-1; see below). IRF4 and STAT3 (signal transducer and activator of

transcription 3) may work in collaboration to induce PRDM1 as it has been shown that they both bind to similar areas of the *blimp-1* gene after stimulation with IL-21[167] (see Section 1.8).

Activated STAT3 is a strong transcriptional activator of *prdm1*. Mice whose B cells lack STAT3 have defects in differentiation to IgG-secreting PCs[171], while an over-expressed dominant-negative form of STAT3 in the BCL1 cell line causes a block in PRDM1 induction[172]. Many cytokines that strongly induce STAT3 activation, such as IL-6, IL-10 and IL-21, can lead to PRDM1 expression.

PU.1 is a transcription factor important in B-cell development and is expressed at all stages of B-cell maturation[173]. It binds to the *prdm1* promoter and has been shown to enhance PRDM1 induction upon stimulation with anti-IgM[174].

Activator protein 1 (AP-1) is another transcriptional activator that binds to the *prdm1/blimp-1* genes and enhances transcription in humans and mice[150,175]. AP-1 is a dimeric complex composed of subunits of proteins that belong to the Fos, Jun, Maf and ATF sub-families. These proteins recognize either TPA (12-*O*-tetradecanoylphorbol-13-acetate)-response elements (TRE; 5'-TGAG/CTCA-3') or cAMP response elements (CRE, 5'-TGACGTCA-3')[176]. Over expression of c-Fos in mice causes enhanced

induction of Blimp-1[177], leading to a reduction in GC size and a decrease in memory B-cell commitment[178,179]. Work on the PRDM1 promoter in humans has identified two CRE-motifs 5' of the TSS and mutation of these sites reduces, but does not abolish, promoter activity[150]. Also, B cells from c-fos-deficient mice that are treated with LPS *in vitro* do not have defective Blimp-1 induction[175]. Therefore, AP-1 appears to enhance PRDM1 expression, but may not be essential for its induction.

IRF5 has recently been identified as a direct activator of *blimp-1*. B cells from IRF5 knockout mice have reduced Blimp-1 expression upon LPS treatment. Reporter assays highlight the importance of an IRF5 binding site 5' of the *blimp-1* TSS[180].

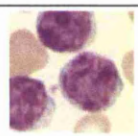
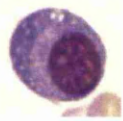
XBP-1 is a transcription factor highly expressed in PCs and its spliced form (XBP-1[S]) is required for PC differentiation and antibody secretion[181]. In B cells, PRDM1 is known to be required for XBP-1 induction in response to PC-inducing stimuli; XBP-1 then acts downstream of PRDM1 in regulating PC differentiation. The unspliced form of XBP-1 (XBP-1[U]) mRNA is normally present in B cells and negatively regulates the unfolded protein response (UPR)[182]. Upon endoplasmic reticulum (ER) stress, IRE1 (inositol-requiring protein 1) is activated and splices XBP-1(U) mRNA to generate XBP-1(S)[183]. This

spliced mRNA is then translated and the protein produced acts as a positive regulator of the UPR, inducing other factors which lead to an increase in cell size, mitochondrial mass, ribosome numbers, lysosome content, general protein synthesis and expansion of the ER – creating the morphology of a PC[184].

1.6.4 Transcriptional, surface-antigen and morphological features of PC differentiation

Progression through the different stages of PC differentiation can be characterised by levels of the different transcription factors regulating terminal B-cell differentiation, by the quantity and type of certain specific surface antigens, and by the morphology of the cell. Levels of major transcription factors that define the later stages of PC differentiation include PRDM1^{high}, IRF4^{high}, XBP-1(S)^{high}, Pax5^{low} and Bcl-6^{neg}. Table 4 highlights the surface markers expressed by CLL, B-1a and memory B cells, together with those of plasmablast/SLPC and LLPC. These data are accompanied by representative pictures illustrating the morphology of CLL lymphocytes, a plasmablast and a bone-marrow-derived LLPC.

Table 4 - Surface markers and morphology of B-cell and PC subsets.

Surface Marker	B-cell Type			Plasma-cell Subtype	
	CLL	B-1a	Memory	SLPC/ Plasmablast	LLPC
CD19	+	+++	++	+	-
IgM	+/-[a]	+++	++/-[a]	+/-[b]	-
IgD	++/-[a]	+/-[b]	++/-[a]	-	-
CD45	+/-[b]	+	++	+	-
CD5	+	+	-	-	-
CD23	++	++/-[b]	-	-	-
CD27	+	-	+	++	+++
CD38	+/-[b]	+	+/-[b]	++	+++
CD138	+	-	-	++/-[b]	+++
Morphology					

[a]Can be "–" as a result of class switching

[b]Can be "++", "+" or "–"

Pictures from www.healthsystem.virginia.edu/internet/hematology/HessImages/

References[185-191]

1.7 PRDM1: STRUCTURE, CONTROL AND FUNCTION

1.7.1 Discovery, nomenclature and expression

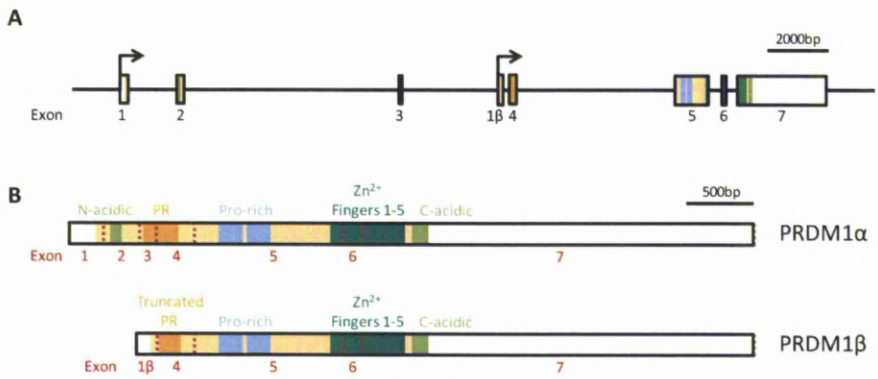
PRDM1 was initially discovered using cDNA (complementary DNA) expression cloning and was called positive regulatory domain 1-binding factor 1 (PRDI-BF1) because of its ability to bind the PRDI element in the β-*IFN* (β-interferon) gene promoter and repress its transcription[192]. The murine homolog was discovered 3 years later and was called Blimp-1 because of its function in inducing PC terminal differentiation[130,193]. The *PRDI-BF1* gene possesses a domain which had previously been uncharacterized, but had been seen before in the *RIZ* (retinoblastoma protein-interacting zinc finger) gene. It was therefore named the PR domain after these two founding protein members (see Section 1.7.3 for details on the function of the PR domain). To date, the family of PR-domain containing proteins (given the acronym PRDM) has sixteen members including RIZ (PRDM2)[194] and, most recently, MEL1 (MDS1/EVI1-like 1; PRDM16)[195]. PRDM1 has functions in controlling gene expression in B cells, T cells, macrophages, the sebaceous gland and skin epidermis[196].

1.7.2 *prdm1* gene, transcripts and protein domains

In the present study it was found that PRDM1 expression is controlled at the level of transcription and so only these mechanisms will be discussed (although there maybe mechanisms for post-transcriptional regulation of PRDM1). The locus of *prdm1* in humans is chromosome 6q21. Figure 1.4 gives an overview of the *prdm1* gene (NC_000006.11) and of which exons encode for the various protein domains (NM_001198.3; NP_001189.2)[197]. Many of the PRDM family members normally have an alternative transcript from the same gene, causing the expression of a truncated isoform that lacks a functional PR domain. While the full length PRDM proteins are tumour suppressors, the over expression of the truncated form is associated with many cancers[1,198-200]. As will be discussed in more detail in Chapter 3, a strong lower molecular weight band was identified in Western blots of CLL cells probed for PRDM1. This prompted a hypothesis that CLL cells might express a pathophysiologically important truncated form of PRDM1.

Regarding the nomenclature and function of the different PRDM1 isoforms, the full length transcript is termed PRDM1α, while the N-terminally truncated isoform which lacks a functional PR domain is called PRDM1β. PRDM1β can still bind DNA but, for unknown reasons,

Figure 1.4



Schematic representation of the *prdm1* gene and its transcripts. (A) shows to scale the introns (lines) and exons (boxes) of the *prdm1* gene. The colours in each exon illustrate the protein domains encoded by these sequences (see B). Transcription initiation sites are shown for both the α and β transcripts. **(B)** illustrates the mature form of both the α and β forms of PRDM1 mRNA, and the protein domains for which it encodes. White regions are untranslated. Adapted from Tunyaplin *et al*[197].

its ability to suppress target genes is greatly reduced[198]. Other domains in the *prdm1* gene include the Zn^{2+} finger and the pro-rich (proline-rich) domains. The five Zn^{2+} finger motifs in the C-terminus are used for DNA binding, while the pro-rich domain is necessary for many of the protein-protein interactions (see Section 1.7.3).

There are multiple TSSs for the PRDM1α mRNA which span across 48bp of the *prdm1* gene (but all give rise to the same protein). The basal promoter has no TATA box but relies on a GC-box which can bind Sp1, Sp3 and/or EGR-1. Loss of this sequence reduces transcriptional efficiency, but the binding of these factor(s) alone to the GC-box is not sufficient for RPII binding and transcription initiation in Daudi cells[201]; alteration in expression or activation of additional transcription factors (which have already been mentioned in Section 1.6.2) appear to be required for this to happen.

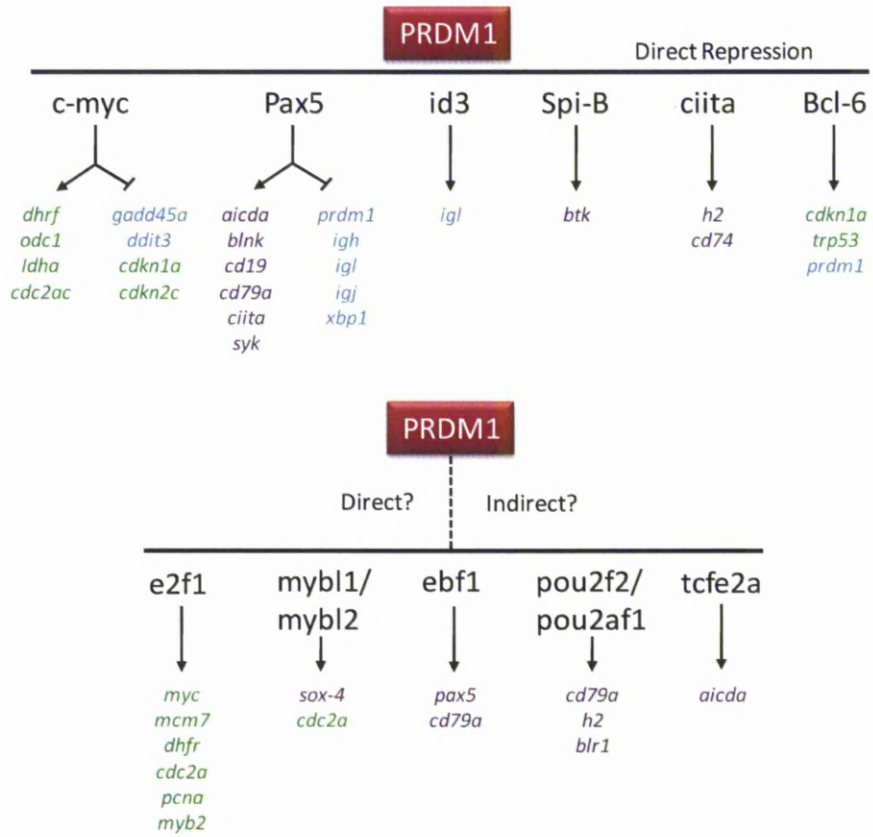
The PRDM1α transcript is translated into a protein of 825 amino acids in length (NP_001189.2) and has a predicted molecular weight of 91,780 Daltons (Da; although this protein migrates on Western blots at ~100 kDa). In contrast, the PRDM1β protein is 691 amino acids in length (NP_878911.1) and has a predicted molecular weight of 76,840 Da (~80kDa on a Western blot).

1.7.3 PRDM1 function in B cells

As mentioned previously, PRDM1 acts as a transcriptional repressor. It binds to the consensus sequence (A/C)AG(T/C)GAAAG(T/C)(G/T) that is similar to the binding sites of IRF1 and IRF2[202]. Unlike PRDM2[203], the PR domain of PRDM1 does not have any HMT activity to produce repressive PTMs of histones. Rather, PRDM1 represses genes by recruiting other HMTs and HDACs such as:- the H3K9 methyl transferase, G9a[204] (a member of the hGroucho complex); the arginine methyl transferase, prmt5[205]; and HDAC1 and HDAC2. G9a and the HDACs are both recruited through the pro-rich domain of PRDM1[206,207].

Forced expression of PRDM1 in various Burkitt lymphoma lines, both directly and indirectly, changes the expression of over 250 genes[158]. These genes broadly fall into three main categories, namely, inhibition of proliferation, induction of Ig secretion and the loss of gene expression associated with GC activated B cells. Direct and indirect targets of PRDM1 and their downstream effects are summarised in Fig. 1.5.

Figure 1.5



Targets of PRDM1 repression in B cells. Shown are the genes that were altered during over expression of PRDM1 in Burkitt's lymphoma cells[158]. Green indicates genes involved in proliferation, blue those that are required for Ig secretion and purple genes involved in B-cell phenotype and function. Figure reproduced from Martins *et al*[134].

1.8 IL-21 AND PC DIFFERENTIATION

1.8.1 IL-21 and its receptor

IL-21 is a member of the type I four-α-helical-bundle of cytokines[208]. Within this family, it is affiliated with cytokines whose receptors require association with the shared cytokine receptor γ chain, γ_c; this group includes, in addition to IL-21, IL-2, IL-4, IL-7, IL-9 and IL-15. IL-21 is produced by activated CD4+ T cells and natural killer (NK) T cells[209]. Within the GC centre, T_{FH} (CXCR5+ CCR7-) cells are the major producers of IL-21[210]. T_H17 cells also produce small amounts IL-21 and can also express CXCR5 enabling them to enter B-cell follicles, but their contribution to IL-21-induced PC differentiation has not yet been considered.

The IL-21 receptor (IL-21R) was initially shown to be expressed on B, T and NK cells. Naive B cells can weakly respond to IL-21, but like T cells, B cells and CLL cells up-regulate their IL-21R and respond more strongly after activation[211]. The IL-21R is then subsequently down-regulated and eventually lost upon differentiation into a PC[212].

1.8.2 IL-21 signalling

Like other cytokine receptors within the same family, the IL-21R signals via the Janus kinase (JAK)/STAT pathways. Thus, binding of IL-21 to its receptor causes recruitment of JAK1 and JAK3 tyrosine kinases which phosphorylate Tyr510 on the IL-21R. This modification provides a docking site for STAT proteins which, in turn, become phosphorylated by the JAK proteins. IL-21 can induce phosphorylation of STAT1, STAT3, STAT5a and STAT5b. Among these STAT proteins, STAT3 is activated most strongly and is the most important for PC differentiation[213]. Thus, STAT3 is phosphorylated on Tyr705 by JAK3 which induces dimerisation of the STAT3 monomers, allowing their import into the nucleus and binding to DNA. In addition to the JAK/STAT pathway, the PI3K and MAPK pathways have also been shown to be activated by IL-21[214].

1.8.3 IL-21 function

IL-21 is known to have important effects on B, T and NK cells. However, concerning B cells, IL-21 is an important regulator of proliferation, differentiation and death in both mice and humans[215]. Its precise effects depend on both the differentiation state of the B-cell population being studied and on the type of co-stimuli present. Regarding the effects of IL-21 in humans, *in-vitro* treatment of purified naive or memory B cells with CD40L, or CD40L and anti-IgM-BCRxl, plus IL-21 induces

proliferation, CSR, and large amounts of Ig secretion from both subtypes; although these effects are greater in memory B cells. The addition of IL-2 to these cultures enhanced the effects of IL-21[216]. In contrast, B cells treated with just anti-IgM-BCRxl and IL-21 were at first induced to proliferate, but then underwent apoptosis[216].

Regarding PC-associated transcription factors that are altered by IL-21 in human B cells, PRDM1 can be modestly up regulated in memory B cells when IL-21 is provided alone. This induction is increased in all B-cell types by addition of co-stimuli such as CD40L or CD40L plus anti-IgM-BCRxl[216].

Although it has been reported that IL-21 modestly increases the expression of Bcl-6 and Pax5 mRNA by normal PB and cord B cells[216], the effect of the cytokine on these transcription factors has not been studied in memory cells – the probable normal counterpart of CLL cells.

1.8.4 IL-21 and the *prdm1* gene

After IL-21 stimulation, activated STAT3 is known to be important for PRDM1 induction. In mice, IL-21-activated STAT3 binds, in collaboration with IRF4, to a variant of the GAS (γ-interferon-activated sequence; 5'-TTCnnnTAA-3') motif at the 3' end of the *blimp-1* gene[167]. However, this specific GAS sequence is not present in the human *prdm1* gene (Fig. 1.6)

and therefore a different STAT3 binding site (which has yet to be found) may be important for human PRDM1 expression.

1.8.5 IL-21 and CLL cells

In addition to inducing the apoptosis of normal B cells under certain conditions[217,218], IL-21 can induce the apoptosis of a number of other cell types including cancer cells[219]. Such anti-tumour effects can be mediated directly or indirectly according to the cancer cell type. It is therefore not surprising that there has been interest in the potential therapeutic effects of IL-21 in CLL[220]. Thus, the cytokine can induce CLL cell death both by IL-21R induced STAT1 signals[211,221] and IL-21 induced granzyme B production[222]. Surprisingly, though, the effects of IL-21 on CLL-cell differentiation have not been studied – hence the work presented in Chapter 4.

When it was found that the cytokine induces PRDM1 in only ~50% of clones, it became important to establish why the other 50% of clones do not express PRDM1 in response to IL-21±co-stimuli. To investigate this question it was decided to test the effect of an alternative differentiating signal that primarily acts via fundamentally different mechanism(s). CpG-ODN, which acts via TLR9[223], was chosen as such a stimulus.

Figure 1.6

```
MOUSE      AAAAAGCAAGCGTGCTTCCAGTAATTTCTGAATCACGAGCTGTAG
HUMAN      GCAAAGTTCCCCAACTTCAGGTGTCTTAAGGATTC--ACCTGTGG
             ****    *   ****  **   **  * **    * **** *
```

3' *blimp*-1 GAS sequence in mice and how it compares to the human *prdm1* gene. The 3' sequence of the murine *blimp-1* gene is overlaid with that of the equivalent human sequence. The GAS motif variant (5'-TTCnnnTAA-3') is highlighted in yellow with interspecies conserved nucleotides indicated by the presence of a star below the letter.

1.9 TLR9, CPG-ODN AND PC DIFFERENTIATION

1.9.1 TLR family

The membrane-spanning TLR family consists of ten members (TLR1-10) in humans, and can be split into two separate subtypes that either reside on the cell surface or in endosomes. PRRs that are positioned on the cell surface give cell-extrinsic innate immune recognition; these types of PRR do not require uptake of the PAMPs to induce signalling and are usually provided by specialised cells of the immune system such

as dendritic cells and macrophages. In contrast, PRRs that are positioned intracellularly (including NLRs and certain TLRs), provide cell-intrinsic innate immune recognition and become essential once the cell has been infected, especially by viruses[224].

TLRs are variably expressed among cells of the immune system. Regarding human B-cell subsets, the literature is currently contradictory on whether naive B cells express TLRs and can respond to their ligands. However, it is clear that memory B cells (especially IgM+) and CD5+ B-1 cells express higher levels of certain TLRs (especially TLR6, TLR7, TLR9, TLR10) and can respond to their ligands *in vitro* without co-stimulation[225]. Also, it is apparent that addition of co-stimuli, such as BCRxl and CD40L, causes all B-cell subsets to enhance their TLR expression and respond robustly to such innate stimuli[226,227].

1.9.2 CpG-ODN and its receptor, TLR9

Among the TLR ligands, CpG-ODN has been particularly well characterized as a potent inducer of terminal B-cell differentiation[226]. Furthermore, unlike murine B cells, whose TLR4 (the receptor for LPS) has been much studied, human memory B cells, including CLL, do not express TLR4, while TLR9 is highly expressed[227,228]. Furthermore, CpG-ODN is known to induce differentiation of memory B cells[226]. For this

reason, CpG-ODN was chosen as an alternative stimulus to induce PRDM1.

TLR9 is not expressed at the cell surface but, rather, is located in the ER from where it is translocated to endolysosomes after internalization of CpG-ODN[229]. Subsequently, ligand binding in the endolysosome induces downstream signalling.

1.9.3 TLR9 signalling

After stimulation, TLR9 recruits a number of adapter molecules including MyD88[223], which then stimulate a number of pathways, especially those involving NF-κB and IRFs[230]. In the context of the regulation of PC differentiation, NF-κB and IRF4 and 5 are known to be important.

Regarding the NF-κB pathway, this has been extensively reviewed[231] and therefore will only be briefly summarized here. This family of transcription factors has 5 subunit members (p50/NF-κB1, p52/NF-κB2, p65/RelA, RelB and c-Rel) and, after activation, hetero- or homodimers of these subunits translocate to the nucleus and bind specific recognition sites in a range of promoters and enhancers. Activation is achieved by exposure of their nuclear localization signal (NLS) by altering/abolishing their interaction with the NF-κB inhibitory proteins, IκBs (NF-κB inhibitor). There are multiple isoforms of IκB and each have

their own binding preferences; for example, IκBα predominately regulates p65:p50 dimers. TLR9 primarily activates the canonical NF-κB signalling pathway which mainly functions around p65:p50 and c-Rel:p50 heterodimers. Upon CpG-ODN binding, TLR9 can activate the trimeric IκB kinase (IKK) complex by recruitment of the TAK1:TAB1/2/3 complex. IKKs then proceed to serine phosphorylate IκB, which causes ubiquitin-targeted degradation of this inhibitor protein and activation of NF-κB.

The two IRFs that are relevant to this thesis (IRF4 and 5) function rather differently. IRF5 is constituently expressed in B cells[232] and is activated directly by the MyD88-dependent activation of TRAF6. Activation of IRF5, by K63-linked polyubiquitination on K410 and K411, leads to its dimerisation, translocation to the nucleus and DNA binding[233]. In contrast, IRF4 expression is normally low in inactivated B cells and is induced upon TLR9 stimulation via the NF-κB pathway. Upon induction, IRF4 can then bind, in collaboration with other cofactors (such as PU.1), to activate or repress gene expression[170].

Signals from TLR9 in B cells induce expression of activation antigens, cell proliferation, production of pro-inflammatory cytokines and antibodies[225]. The latter function is particularly relevant to this thesis, and is therefore considered further below.

1.9.4 TLR9, PC differentiation and the *prdm1* gene

As alluded to above, CpG-ODN alone induces the plasmacytoid differentiation of human and murine memory cells *in vitro*. In contrast, naive B cells have little or no TLR9 and do not undergo plasmacytoid differentiation in response to CpG-ODN. Interestingly a second, not clearly defined, signal is required for plasmacytoid differentiation *in vivo* in mice[234].

CpG-ODN induces PRDM1 by activating multiple transcription factors including NF-κB, IRF4, IRF5 and AP-1. However, the minimum combination of factors needed for efficient PRDM1 induction in B cells, including those of CLL, remains unknown[163]. The work in Chapter 4 helps to clarify this issue by showing that NF-κB and an induced 'neofactor' are necessary for the induction of PRDM1 in CLL cells following treatment with CpG-ODN.

1.9.5 TLR9 and CLL cells

There have been a substantial number of studies of the functional effects of CpG-ODN on CLL cells. Perhaps surprisingly, though, there do not seem to have been any papers reporting the effects of the oligonucleotide on the plasmacytoid differentiation of CLL cells.

The functional effects that have been described appear to be clone dependent and include the induction of apoptosis, cell proliferation, increased expression of co-stimulatory molecules and the secretion of a range of cytokines[235-237]. Interestingly, it has recently been shown that UM-CLL cells and those from progressive disease more often proliferate in response to CpG-ODN than do cells from M-CLL cases with stable disease[236].

The four experimental chapters of the thesis now follow.

Chapter 2

CONTROL OF INTEGRIN α4β1 EXPRESSION IN CLL

2.1 INTRODUCTION

Whether or not the clonal lymphocytes of CLL express the integrin heterodimer, α4β1, is important in the disease for a number of inter-related reasons. First of all, cell-surface expression of α4 by the malignant B-cell clone (termed $\alpha4^{pos}$) is a strong independent adverse prognostic indicator in the disease[18,19]. Secondly, surface α4 is essential for CLL cells to be able to undergo the transendothelial migration necessary for entry into tissues, including lymph nodes[27] and bone marrow[238]. Furthermore, there is a correlation between α4 expression and the clinical presence of lymphadenopathy[51]. Clearly, therefore, α4 is important for CLL-cell invasion into tissues where the malignant cells receive signals for survival and proliferation[239,240]. This, in turn, results in the organomegaly, the impaired haematopoiesis, and the suppressed immune function associated with an adverse prognosis and/or advanced

disease[7,8]. Consequently, there is the prospect that agents currently being developed to inhibit α4 function[241] might have therapeutic potential in the disease.

It is not known why CLL cells from patients with poor-prognosis disease express α4β1, while the malignant cells in good-prognosis patients frequently express little or none of this integrin heterodimer. Furthermore, studies of the expression of the integrin in other lymphoid cell types provide no clear-cut clues. Expression has been linked to cell activation and differentiation[53,55,242] which, in B-lymphocytes, are in turn mediated to a major extent by cytokines and engagement of the B-cell antigen receptor (BCR). However, it has been established that antigen and cytokines (both important in the biology of CLL[38,41,243]) affect α4β1 expression in different ways according to the lymphocyte subset involved and the nature of the antigenic stimulus[244] – both uncertain for CLL lymphocytes. Therefore, it is not at all predictable from studies of other lymphoid-cell types what might be controlling α4 expression in these malignant B cells.

The aim of the present study, therefore, was to establish why some CLL cells express α4, while others do not. This question is not only relevant to the biology of CLL, but is also important clinically, since understanding

the mechanisms that regulate α4 expression in CLL might allow down regulation of α4 in a therapeutically useful manner.

2.2 METHODS

2.2.1 Mononuclear sample preparation

PB samples were taken from consenting patients who had previously been diagnosed with CLL or plasma-cell leukaemia (PCL) and with approval from the Liverpool Research Ethics Committee. The PCL case was unusual in having the features of multiple myeloma, but with many circulating plasma cells and plasmablasts as defined by morphology and CD138 expression. Normal PB samples were taken from consenting colleagues in the laboratory.

Upon arrival, all blood samples were carefully layered on top of Lymphoprep (Axis-Shield, Kimbolton, UK) and centrifuged to obtain purified mononuclear cells. Mononuclear cells were then washed and resuspended in ice-cold RPMI-1640 (Biosera, Ringmer, UK) containing 10% volume/volume (v/v) FCS (fetal calf serum; Biosera), after which an equal volume of ice-cold RPMI-1640 plus 10% v/v FCS and 20% v/v DMSO (Dimethyl sulfoxide; Sigma-Aldrich - Laboratory Analysis Ltd, Exeter, UK) was gradually added on ice. This final cell suspension was then placed in cryotubes (Nuncbrand - Fisher Scientific, Loughborough, UK) in 1mL aliquots, housed in polystyrene holders and placed into a

-80°C freezer to freeze gradually before being transferred into liquid nitrogen for long-term storage.

For most experiments, cells were not purified further and only CLL cases with cell counts >50x10^9/L were employed to ensure minimal contamination by non-malignant cells. For the studies of α4 mRNA expression, all samples were purified by depletion of $CD3^+$, $CD14^+$ and $CD16^+$ cells; in these preparations, CD19+CD23+ CLL cells were always >95% (see Section 2.2.6).

CLL samples were screened for CD49d status using flow cytometry (see Section 2.2.4) prior to their use for this chapter.

2.2.2 Cell culture

Cryopreserved cells were thawed rapidly in a 37°C water bath until all ice had just melted and then transferred straight onto ice. The 1mL cell suspension was then very gradually increased to 10mLs using ice-cold RPMI-1640 containing 0.5% weight/volume (w/v) BSA (bovine serum albumin; Sigma-Aldrich), 2mM L-glutamine, 100units/mL penicillin and 100μg/mL streptomycin (Invitrogen, Paisley, UK), from now on referred to as culture medium.

Cells were washed to remove any remaining DMSO and the live cell numbers were counted using a haemocytometer (Fisher Scientific) and

0.05% trypan blue (Sigma-Aldrich). If the CLL-cell viability was <85% at this stage of the experiment, the sample was excluded from these studies. Time zero (T0) samples, if required for the experiment, were taken at this point.

Unless stated otherwise, 1mL of cells were plated in poly-HEMA (Poly[2-hydroxyethyl methacrylate]; Sigma-Aldrich)-coated 24-well plates (Fisher Scientific) at a density of $3x10^6$ cells/mL. Poly-HEMA was used to stop cells adhering to the plates, thereby preventing any stimulation related to adhesion[245]. The plated cells were then placed in an 37°C incubator (HeraCell, Fisher Scientific) with 5% CO_2 for 1 hour (hr) to allow them to warm and 'recover' before any further treatment.

The human Burkitt's lymphoma cell line, Raji, was grown in RPMI-1640, 2mM L-glutamine, 100units/mL penicillin, 100µg/mL streptomycin and 10% v/v FCS.

2.2.3 Cell culture stimuli

Stimuli were used at the following final concentrations:- Bryostatin at 10nM (Sigma-Aldrich); TNFα at 10nM (Calbiochem - Merck Chemicals Ltd, Nottingham, UK); LPS at 10µg/mL (Sigma-Aldrich); $F(ab)_2$ fragment of a goat anti-human IgM, $Fc_{5\mu}$ fragment specific antibody at 10µg/mL (Jackson ImmunoResearch - Stratech, Soham, UK); type B CpG-ODN 2006

at 3µg/mL (InvivoGen - Source BioScience Autogen, Nottingham, UK); soluble recombinant human CD40L (rhCD40L) at 0.2µg/mL with the enhancer at 1µg/mL (Axxora UK Ltd, Nottingham, UK); IL-4 at 20ng/mL (Sigma-Aldrich); and IL-21 at 50ng/mL (Invitrogen Ltd).

The $F(ab)_2$ fragments of an anti-IgM antibody was used for BCRxl after confirmation by flow cytometry that the CLL case of interest expressed surface IgM (see Section 2.2.4 for flow cytometry methodology). The $F(ab)_2$ fragments of anti-IgM antibodies were used to avoid indirect stimulation through F_c receptors.

Soluble rhCD40L required pre-incubation with an enhancer molecule provided by the manufacturers. rhCD40L and the enhancer were incubated together at 37°C for 30minutes (mins) in culture medium prior to adding the stimuli to the cells.

2.2.4 Flow cytometry

This was performed using a Becton Dickinson (BD) FACSCalibur machine and analysed using BD CellQuest Pro software (BD Biosciences, Oxford, UK). Antibodies used were:- PE (phycoerythrin)-conjugated mouse IgG_1 isotype control; PE-conjugated mouse anti-CD49d (PE-anti-CD49d); mouse PE-anti-CD5; mouse PE-anti-CD25; mouse PE-anti-CD40; mouse PE-anti-CD71; mouse PE-anti-CD79b; FITC (Fluorescein isothiocyanate)-

conjugated mouse IgG_1 isotype control; FITC-conjugated mouse anti-CD22 (FITC-anti-CD22); mouse FITC-anti-CD27; mouse FITC-anti-CD39; mouse FITC-anti-CD62L; mouse FITC-anti-IgD; mouse FITC-anti-IgM; PerCP (Peridinin Chlorophyll Protein)-conjugated mouse IgG_1 isotype control; PerCP-conjugated mouse anti-CD19 (all antibodies were isotype IgG_1 from BD Biosciences).

All cells were surface stained (10μg/mL of antibody) on ice for 20mins at a density of $1x10^7$/mL in PBS (phosphate buffered saline) comprised of 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4 (pH 7.4) supplemented with 0.1% w/v BSA. Cells were washed twice in PBS plus 0.1% w/v BSA before running through the FACS machine. All fluorescence was normalised against the respective conjugated-antibody isotype controls.

2.2.5 Western blotting

Cells were pelleted at 500rcf for 5mins at 4°C and then washed twice in ice-cold PBS. Cells were then lysed in clear SDS (sodium dodecyl sulphate) lysis buffer containing 1% w/v SDS, 10% v/v glycerol, 5mM EDTA and 50mM Tris (pH 6.8); 30μL of this lysis buffer was added per 10^6 cells. Lysates were kept on ice and sonicated using a Microson ultrasonic cell disrupter and ultrasonic converter (Scientific Laboratory

Supplies, Nottingham, UK). Lysates were then heated to 95°C for 10mins to aid in denaturation of the proteins before their concentration was determined.

Protein determination was performed using the DC protein assay (Bio-Rad, Hemel Hempstead, UK), which is a modified form of the Lowry protein determination method[246]. Absorption was measured at a wavelength of 750nm on a spectrophotometer and protein concentrations were then calculated for each sample using BSA standards of known concentrations.

SDS-polyacrylamide gel electrophoresis (PAGE) samples were prepared by taking 10μg of each lysate and making them up to the same volume using clear SDS lysis buffer and 4X Laemmli buffer[247] containing 4% w/v SDS, 40% v/v glycerol, 20% v/v β-mercaptoethanol, 0.008% w/v bromophenol blue and 250mM Tris (pH 6.8). After preparation, the SDS-PAGE samples were heated to 95°C to ensure complete denaturation and reduction of the proteins before loading onto a polyacrylamide gel.

All SDS-PAGE and transfer experiments were performed using Bio-Rad equipment. SDS-PAGE gels were made from 10-12% v/v acrylamide (Geneflow Ltd., Fradley, UK), 4X running buffer (1.5M Tris pH 8.8, 0.4% w/v SDS), 0.05% w/v APS (ammonium persulphate; Sigma Aldrich) and

0.001% v/v TEMED (N,N,N',N'-Tetramethylethylenediamine; Sigma-Aldrich). Stacking gels contained 5% acrylamide, 4X stacking buffer (0.5M Tris, 6.8, 0.4% w/v SDS), 0.08% w/v APS and 0.0025% v/v TEMED. The electrophoresis buffer contained 25mM Tris, 192mM glycine and 0.1% w/v SDS. The SDS-PAGE samples and 10µL of kaleidoscope Precision Plus protein pre-stained standards (Bio-Rad) were loaded using a Hamilton syringe (Hamilton Company; Fisher Scientific) and 30mA was applied to each gel using a powerpack.

After running, proteins were transferred from the gel to a PVDF immobilon membrane (Millipore, Watford, UK) using 400mA for 1hr. The transfer buffer (Geneflow) was pre-cooled to ice-cold temperature before use.

Once the proteins were transferred, membranes were blocked for 1hr in 2% advanced blocking reagent (Amersham Biosciences, Chalfont, UK). The blocking reagent was dissolved in Tris-buffered saline with Tween-20 (TBS-T) which contains 150mM Tris pH7.4, 50mM NaCl and 0.1% v/v Tween-20. Western blotting antibodies were diluted in blocking solution and incubated overnight with the membrane at 4°C (1hr at room temperature for anti-β-actin) with gentle agitation. Primary antibodies used were:- monoclonal mouse anti-integrin α4 clone 7.2R (R&D

Systems, Abingdon, UK); monoclonal mouse anti-PRDM1 clone 3H2-E8 (Novus Biologicals, Cambridge, UK); monoclonal mouse anti-Pax-5 clone 24 (BD Biosciences); and monoclonal mouse anti-β-actin clone AC-15 (Sigma-Aldrich).

Membranes were then washed for 40mins using TBS-T which was replaced every 10mins. Horse radish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Santa Cruz – Insight Biotechnology Ltd., Wembley, UK) was diluted (1:5000-1:10000) in blocking buffer and incubated with the membrane for 1hr at room temperature.

The membrane was again washed as above. Membrane bound HRP-conjugated secondary antibodies were detected with either enhanced chemiluminescence (ECL) plus reagent (Millipore) or advanced ECL (Amersham Biosciences). Reactive bands were visualised using a LAS-1000 chemiluminescence and fluorescent imaging system machine. Protein band densitometry was carried out using the AIDA image analyser software package (Raytek Scientific Ltd, Sheffield, UK).

2.2.6 CLL-cell purification

CLL cells were purified before polymerase chain reaction (PCR) analysis, as previous results from the laboratory had shown that T-cell contamination, although relatively small, could have substantial effects

on such a sensitive technique. Cells were purified by using the MACS magnetic bead system (Miltenyi Biotec, Bisley, UK). Cells were stained by initially following the flow cytometry protocol for binding of FITC-conjugated anti-CD3, -CD14 and -CD16 antibodies (BD Biosciences). Cells were then washed in PBS and re-suspended at 10^8 cells/mL in ice-cold degassed PBS containing 0.1% BSA and 2mM EDTA (from now on referred to as purification buffer). A 1:5 dilution of MACS anti-FITC magnetic beads (Miltenyi Biotec) was then added and kept on ice for 20mins. Cells were then washed twice and re-suspended in 30µL of purification buffer and passed through an MS column. Unbound cells (i.e. CLL cells) were washed through the column with three washes of 500µL of purification buffer. After purification, CLL cells were >95% CD19+CD23+ by flow cytometry.

2.2.7 Reverse transcription

mRNA was extracted from $5x10^6$ to $1x10^7$ CLL cells using QIAshredders and an RNeasy mini kit (Qiagen Ltd, Crawley, UK). After extraction, the quality and quantity of mRNA were assessed using a nanodrop 2000C machine (Fisher Scientific). 1µg of mRNA was reverse transcribed to cDNA in a 20µL reaction containing 200units of M-MLV reverse transcriptase, 4µL 5X M-MLV reaction buffer, 400µM dNTPs, 25ng/µL oligo$(dT)_{15}$ primer and 0.5µL Recombinant RNasin Ribonuclease Inhibitor

(all from Promega UK, Southampton, UK). mRNA was initially heated with the oligo$(dT)_{15}$ primer in 13.5µL of nuclease-free water at 70°C for 5 mins then placed back on ice. The remaining reagents were then added and incubated at 37°C for 1hr, after which the reaction was stopped by heating to 70°C for 10mins.

2.2.8 PCR

50µL reaction contained:- 10µL 5X colourless GoTaq flexi buffer; 3µL 25mM $MgCl_2$; 1µL 10mM dNTPs; 0.2-0.4µM forward and reverse primer (Eurofins MWG Operon, London, UK); 0.25µL GoTaq flexi DNA polymerase (Promega); and the required amount of template DNA (0.25µL cDNA). Reactions were performed in 0.5mL thin wall PCR tubes on a ThermoHybaid PxE 0.5 machine (Fisher Scientific). Agarose gel electrophoresis with TBE (Tris/Borate/EDTA) buffer (Sigma-Aldrich) was used to confirm the PCR products were of the correct size. DNA was visualised using ethidium bromide (Sigma-Aldrich) and a fluorescent image analyser FLA-5000 machine (Fujifilm). *ITGA4* bisulphate sequencing primers were:- BS1 forward 5'-TCT TAC TAA ACC CAA AAC CAT C-3', reverse 5'-AAG GAG AGA GGG AAG AGG A-3'; BS2 forward 5'-TCC TCT TCC CTC TCT CCT T-3', reverse 5'-GTT GTG GGG GTT TTG GTA AA-3'.

2.2.9 Quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed using the DyNAmo SYBR Green qPCR Kit (New England Biolabs UK Ltd, Hitchin, UK) in qPCR 96-well plates with optical strip caps on a MX3005P qPCR machine and analysed with MxPro v4.1 software (Agilent Technologies, Stockport, UK). 0.25µL of cDNA or 2µL of immunoprecipitated chromatin DNA (see Section 2.2.12) was used per qPCR reaction and mixed with 0.08-0.4µM of forward and reverse primers, 12.5µL of 2X DyNAmo SYBR Green master mix and made up to 25µL total volume with nuclease-free water. Each reaction was run in triplicate and averaged. Primers were optimised to produce 1 specific product and this was confirmed by running on an agarose gel. Primer sequences were:- α4 forward 5'-TGA GAG CGC GCT GCT TTA CC-3', reverse 5'-GGC ACT CCA TAG CAA CCA CC-3'; RPII forward 5'- GCT GTT CTT GCT CCT CAC GAT TTC-3', reverse 5'-CCA ACA ATG GCT ACC GTT CAC G-3'. Chromatin immunoprecipitation (ChIP) primers were:- *ITGA4* upstream of TSS forward 5'-ATG AGA CTC ACT ACC CAG TTC-3', reverse 5'-TTT TCA CGC ACC CAC TCA G-3'; *ITGA4* downstream of TSS forward 5'-CTC CTC TTC CCT CTC TCC TTC-3', reverse 5'-GGT GGG GAA CAT TCA ACA C-3'.

2.2.10 Intracellular calcium concentrations

CLL cells were thawed and left to recover at $1x10^7$/mL for 1hr. They were then incubated in the dark for a further 20mins with 4μM FURA 2-AM (Sigma-Aldrich) in culture medium. Cells were washed twice in calcium assay buffer (120mM NaCl, 4.7mM KH_2PO_4, 1.2mM $MgCl_2$, 1.25mM $CaCl_2$, 10mM HEPES pH 7.4) and re-suspended in the same buffer at a concentration of $1x10^7$ cells/mL and placed on ice. 2mL of this cell suspension was used per analysis and allowed to warm prior to the experiment and kept at 37°C during treatment. Cells were stimulated using 20μg/mL of $F(ab)_2$ anti-IgM antibody and calcium-chelated FURA 2 was determined by continuous excitation at 340nM (Ca2+ bound) and 380nM (Free ester) and by measurement of emission at 510nM using a Hitachi F-7000 fluorescence spectrophotometer and FL Solutions software (Hitachi High-Tech, UK). F_{max} (see below) was measured by adding a final concentration of 0.5% Triton X-100 to the cell suspension and F_{min} was determined by adding EDTA to a final concentration of 2.5mM. Calcium concentrations were then calculated using the following formulae:-

$$[Ca^{2+}] = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{F2_{min}}{F2_{max}}$$

Where,

$$R = \frac{\text{background level for F1} - \text{F1}}{\text{background level for F2} - \text{F2}}$$

$$R_{max} = \frac{\text{background level for F1}_{max} - \text{F1}}{\text{background level for F2}_{max} - \text{F2}}$$

$$R_{min} = \frac{\text{background level for F1}_{min} - \text{F1}}{\text{background level for F2}_{min} - \text{F2}}$$

F1, F2 : Fluorescence intensities of FURA 2 chelated and unchelated

$F1_{max}$, $F2_{max}$: Fluorescence intensities of FURA 2 at maximum calcium saturation

$F1_{min}$, $F2_{min}$: Fluorescence intensities of FURA 2 at zero calcium concentration

K_d : Dissociation constant of FURA 2 and calcium (i.e. 224)

2.2.11 Bisulphite sequencing

Bisulphite treatment of DNA converts unmethylated cytosines to uracil bases, while methylated cytosines cannot be altered. Subsequent sequencing after bisulphate treatment can therefore identify methylated cytidine nucleotides. The assay was performed using the EZ DNA Methylation-Direct Kit (Zymo Research; Cambridge Bio Science, Cambridge, UK). $1x10^5$ CLL cells were proteinase K digested, spun at 10,000rcf for 5mins and then the supernatant added to the CT conversion reagent. Samples were heated to 98°C for 8mins, 64°C for 3.5hrs and then finally cooled to 4°C. Converted DNA was then purified

using the provided spin columns and eluted in 10μL of elution buffer. 1μL of these samples were then amplified using PCR and the bisulphate sequencing primers (see Section 2.2.8). PCR amplified products were purified by running on an agarose gel, excising the DNA band and then extracting the DNA from the agarose using a QIAquick gel extraction kit (Qiagen). Purified PCR products were then cloned into the pGEM-T vector (Promega) and transformed into Escherichia coli (E. coli) by heat shocking at 42°C for 45-50secs. Transformed bacteria were spread onto LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. Four white colonies were picked per CLL case, grown in liquid LB (lysogeny broth) overnight at 37°C and plasmids extracted using a Zyppy plasmid miniprep kit (Zymo Research). Plasmid inserts were sequenced at the University of Dundee, UK.

2.2.12 ChIP

10^7 CLL cells were washed once with room-temperature PBS and then fixed in 0.5mL 1% formaldehyde in PBS for 10mins. Fixed cells were then washed and re-suspended in 0.5mL cell lysis buffer containing 5mM PIPES pH7.4, 85mM KCl, 0.5% v/v NP-40 and protease inhibitor cocktail (Calbiochem) and vortexed every 5mins for 15mins. Cells were then pelleted, supernatant removed and re-suspended in ChIP buffer containing 0.1% w/v SDS, 0.1% v/v sodium deoxycholate, 1% v/v Triton

X-100, 140mM NaCl, 1mM EDTA, 50mM HEPES pH7.9 and protease inhibitor cocktail (Calbiochem). DNA was then sheared using 12 cycles of 15secs sonication with a minimum of 15secs on wet ice between sonications. The sonicated suspension was then pelleted at 12,000rcf for 10mins at 4°C, and the DNA-containing supernatant removed and kept. Satisfactory shearing of DNA was confirmed by agarose gel electrophoresis and shown to be of an average of ~500bps prior to immunoprecipitation. Sheared DNA from 10^6 CLL cells (50µL) was then diluted to 0.5mLs in ChIP buffer (10% aliquot taken for input), followed by the addition of 20µL protein A magnetic beads (Millipore) and the appropriate immunoprecipitation antibodies (Millipore):- 3µg rabbit anti-H3K4me3; 5µg rabbit anti-H3Ac (binds to both H3K9 and H3K14 acetylation); and 4µg rabbit anti-H3K27me3. Immunoprecipitations were left on a rotator at 4°C overnight. Beads were then washed for 10mins using 0.5mLs of the following buffers respectively:- low-salt buffer containing 0.1% w/v SDS, 1% v/v Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris pH8.1; high salt buffer containing 0.1% w/v SDS, 1% v/v Triton X-100, 2mM EDTA, 500mM NaCl, 20mM Tris pH8.1; LiCl buffer containing 250mM LiCl, 1% v/v NP-40, 1% w/v sodium deoxycholate, 1mM EDTA, 10mM Tris pH8.1; and Tris/EDTA (TE) buffer containing 10mM Tris pH8 and 1mM EDTA. 100µL of elution buffer

containing 100mM sodium bicarbonate, 200mM NaCl, 1% w/v SDS was then added to both the beads and the input samples. Samples were then heated for 2hrs at 65°C, followed by 95°C for 10mins. DNA was then purified using a PCR purification kit (Qiagen) and eluted in 30µL in the kit elution buffer. qPCR was then performed (see Section 2.2.9).

2.3 RESULTS

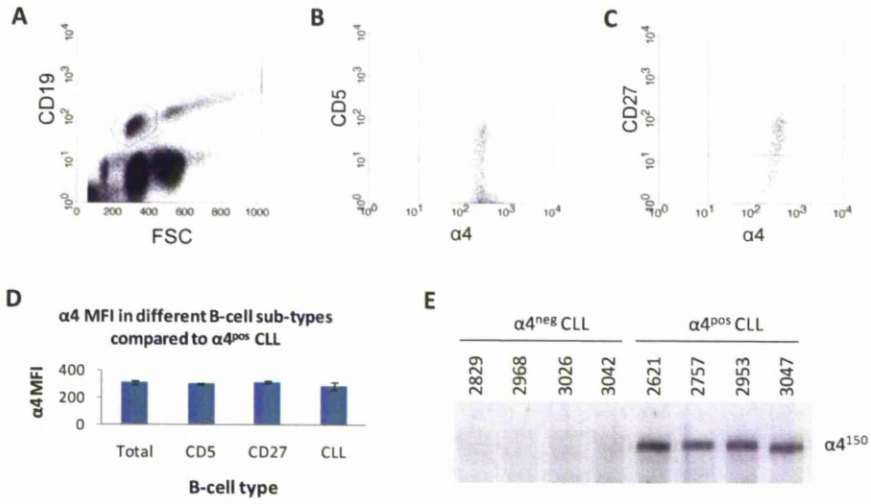
As a first step in establishing why some CLL clones express α4 while others do not, it was important to establish whether or not the normal lymphoid sub-populations to which CLL cells have been related express α4.

2.3.1 $\alpha4^{neg}$ CLL clones differ from their normal B-cell counterparts in lacking α4

CLL cells have been related to both CD5+ B cells and CD27+ memory cells[28,31]; the α4 expression of these CD5+ B cell from normal individuals has not been reported, and there is only a single report that human memory cells in tonsil express $\alpha4\beta1$[248]. Therefore, FACS analysis of multiply stained normal PB B cells was performed and demonstrated that both CD5+ and CD27+ normal B-cell sub-populations express α4 (Fig. 2.1A-D).

CLL cells are thought to be activated as a result of BCR stimulation *in vivo* by (auto)antigen[28], and it is known that the α4 integrin can be internalised following antigen-receptor stimulation[55]. It therefore was important to determine whether or not CLL clones lacking surface α4 contain significant amounts of the protein intracellularly. In fact,

Figure 2.1



α4 protein expression in normal and CLL B cells. The surface α4 expression of CD5+ or CD27+ normal B cells was measured by FACS using different combinations of directly conjugated antibodies. CD19+ normal B cells were first identified in plots of CD19 against FSC **(A)**. After gating on this B-cell population, the α4 reactivity of either the CD5 **(B)** or CD27 **(C)** sub-populations was measured (representative examples of 3 experiments performed with blood from 3 normal donors are shown). **(D)** shows that most, if not all of the cells in both sub-populations, expressed α4 at similar intensities (error bars illustrate SEM). **(E)** shows a representative Western blot of α4 protein expression in CLL cells lacking or expressing α4 at their cell surface.

blotting of highly purified CLL cells showed that CLL clones which do not express surface α4 contain little or none of this integrin chain (Fig. 2.1E).

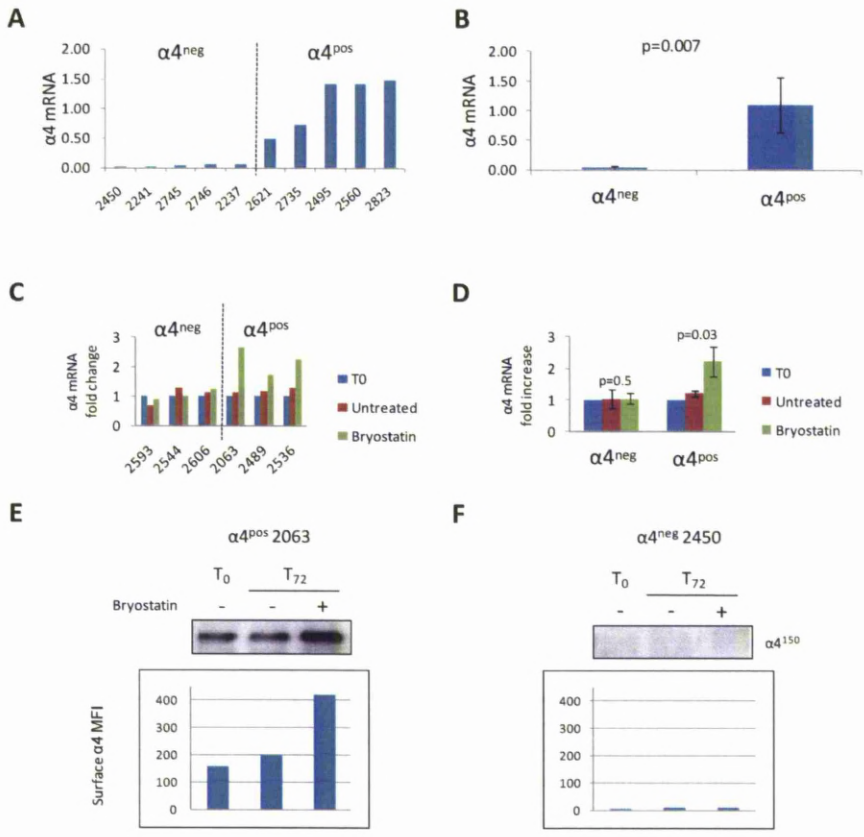
It was therefore concluded that $\alpha4^{pos}$ CLL clones resemble their normal B-cell counterparts in expressing the integrin, while $\alpha4^{neg}$ CLL clones are abnormal in lacking the protein. It was therefore decided to focus on why $\alpha4^{neg}$ CLL clones do not express the α4 integrin chain.

2.3.2 α4 expression in CLL is controlled at a transcriptional level

qPCR analysis was employed to measure α4 mRNA levels in highly purified (>95% CD19+) $\alpha4^{neg}$, as compared with $\alpha4^{pos}$, CLL clones. As shown in Fig. 2.2A and B, little or no α4 mRNA was detectable in CLL clones lacking α4 protein, whereas the mRNA was readily demonstrated in clones that express α4 protein.

Whether or not α4 message can be induced in $\alpha4^{neg}$ CLL clones was examined next. To do this, α4 mRNA was measured in $\alpha4^{neg}$ and $\alpha4^{pos}$ clones before and after stimulation with the powerful non-physiological stimulus bryostatin, a PKC (protein kinase C) activator that has been employed therapeutically in CLL[249]. Such stimulation had no effect on α4 message levels in $\alpha4^{neg}$ CLL cells, but markedly increased α4 mRNA in $\alpha4^{pos}$ clones (Fig. 2.2C and D). As expected, this stimulation with bryostatin increased the expression of α4 protein in $\alpha4^{pos}$ clones, but

Figure 2.2



α4 mRNA and protein expression by highly purified CLL cells before and after stimulation with bryostatin. In **(A)** and **(B)**, α4 mRNA expression was measured by qPCR and normalised to amounts of RPII mRNA detected after similar amplification. **(C)** and **(D)** show similar data before and after culture for 72 hours in the presence or absence of bryostatin. **(E)** and **(F)** show α4 protein expression measured by Western blot and FACS analysis in similarly cultured cells. These are representative examples of experiments involving 3 $\alpha4^{pos}$ and 3 $\alpha4^{neg}$ CLL clones.

had no effect on expression of the integrin in clones lacking the protein before *in vitro* stimulation (Fig 2.2E and F).

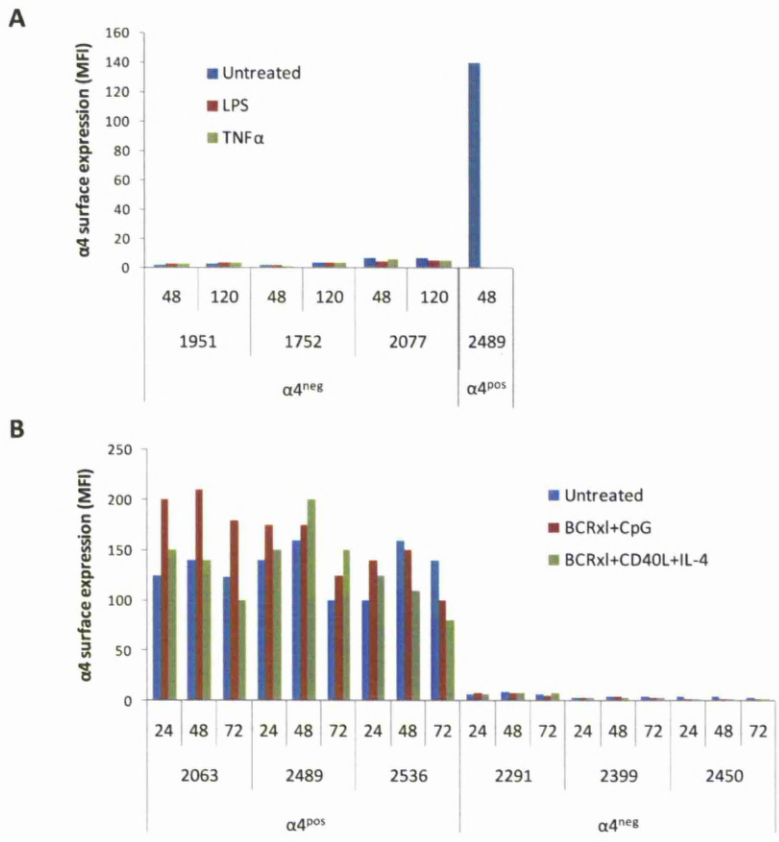
Taken together, these data indicate that α4 expression is controlled at a transcriptional level and suggest that, in $\alpha4^{neg}$ clones, transcription of the integrin is not readily induced by non-physiological cell stimulation. The latter observation may indicate that transcription of α4 integrin chain is blocked in $\alpha4^{neg}$ clones.

To support this conclusion, the effect of a range of other (patho)physiological stimuli that have been reported to alter α4 expression in other cell types was examined next.

2.3.3 A range of stimuli fail to induce α4 expression in $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones

The stimuli used to try and alter α4 surface expression were:- TNFα (Fig. 2.3A); LPS (Fig. 2.3A); and BCRxl plus co-stimulation with either CpG-ODN or soluble CD40L/IL4 (Fig. 2.3B). In fact, none of these stimuli had any effect on the level of surface α4 expression by either $\alpha4^{neg}$ or $\alpha4^{pos}$ CLL clones. These data support the notion that α4 expression in CLL is not readily altered by cell stimulation *in vitro*. Furthermore, culture for up to 7 days in the absence of exogenous stimulation (cells incubated on poly-HEMA-coated plastic to prevent adhesion and in the absence of

Figure 2.3



The effects of a range of stimuli on surface α4 expression. In **(A)**, $\alpha4^{neg}$ CLL cells were cultured for 48 or 120hrs with and without either LPS or TNFα. The effect of these stimuli on surface α4 expression was then examined using FACS and compared relative to an untreated $\alpha4^{pos}$ CLL sample (2489). **(B)** shows a similar experiment, but this time the CLL cells were cultured for 24, 48 or 72hrs in the presence or absence of either BCRxl+CpG-ODN or BCRxl+CD40L+IL-4.

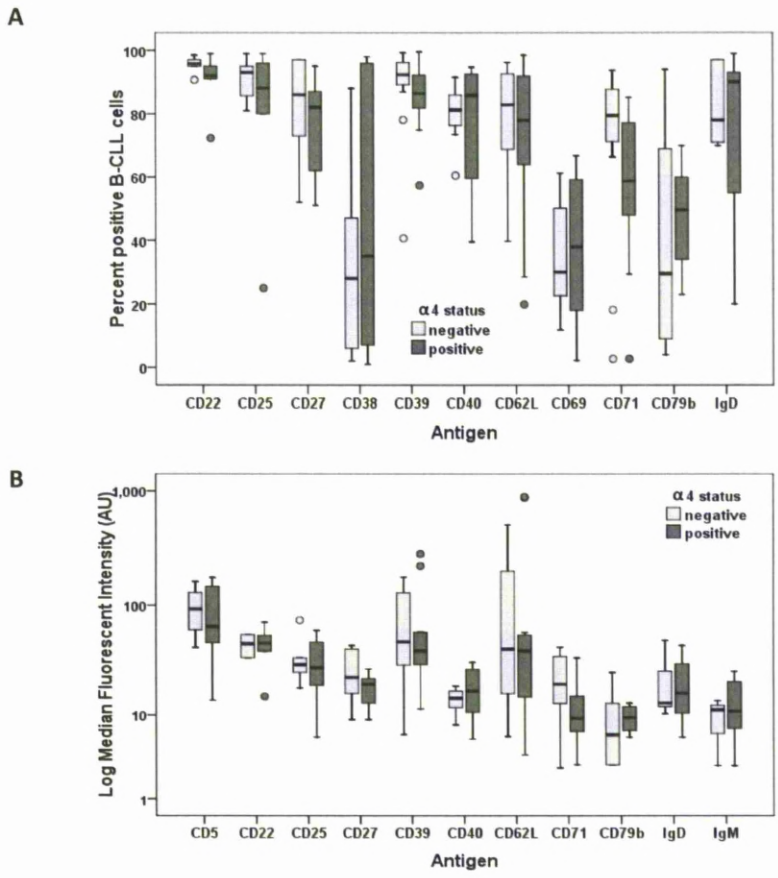
serum to prevent stimulation by soluble factors) had no effect on α4 expression. Thus, $\alpha4^{pos}$ clones continued to express similar levels of the integrin, while $\alpha4^{neg}$ CLL cells remained negative (n=5 for each type of CLL clone; up to 5 days shown in Fig. 2.3A and B). This indicates that α4 expression is not dependent on *in-vivo* paracrine effects.

Since α4 expression in other cell types has been related to activation/differentiation, whether or not this might be so in CLL was investigated next. Also, the hypothesis that surface levels of α4 were related to anergy (a feature of some CLL cells) was also tested.

2.3.4 The expression of α4 CLL cells is not related to activation, anergy or differentiation

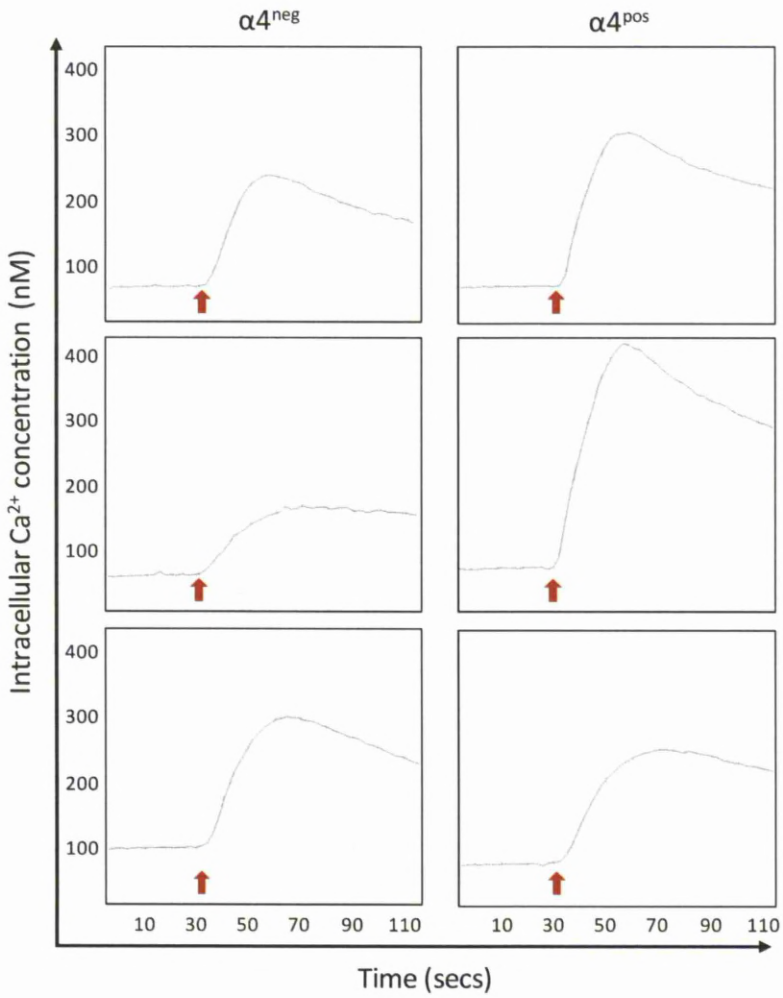
The expression of a range of antigens that indicate distinct stages of activation or differentiation[38] was examined by flow cytometry. $\alpha4^{pos}$ and $\alpha4^{neg}$ clones did not differ significantly in their expression of any of these antigens (Fig. 2.4). Also, similar increases in intracellular calcium concentration after BCRxl and comparable levels of surface IgM (as measured by flow cytometry) were observed in three $\alpha4^{pos}$ and three $\alpha4^{neg}$ clones (Fig. 2.4 and 2.5); this suggests that lack of α4 expression is not related to anergy. Furthermore, induction of plasmacytoid differentiation after stimulation with IL-21+BCRxl (Fig. 2.6) had no effect

Figure 2.4



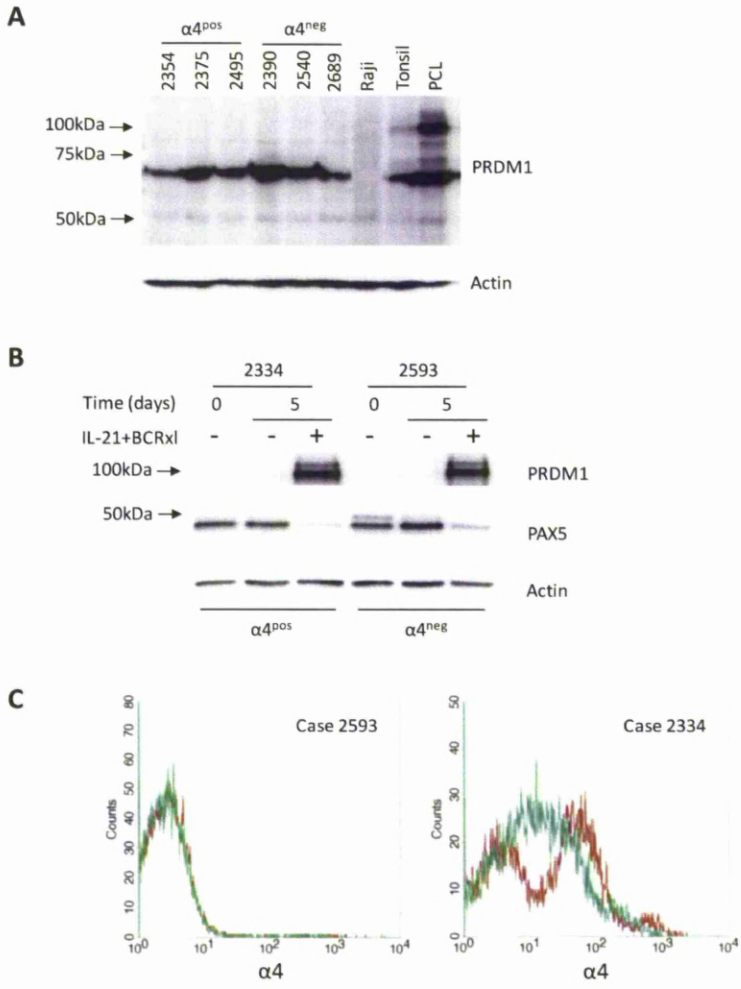
Surface expression of a range of activation and differentiation antigens in $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones. Both box and whisker plots represent FACS results of n=9 CD19+$\alpha4^{pos}$ and n=9 CD19+$\alpha4^{neg}$ CLL clones. The data in **(A)** are given as the percentage positive CLL cells above that of the antibody-isotype control. Plot **(B)** shows the same results but represented as the median fluorescence of the clone. Only CD71 neared statistical significance, with the percent positive data having a p=0.053 and median fluorescence a p=0.063 (Mann-Whitney U test). Circles represent outliers (>3 standard deviations from the mean).

Figure 2.5



Intracellular calcium concentrations following BCRxl in $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones. Traces of calcium concentrations as measured by FURA 2 in three $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones. All cases were surface IgM positive and $F(ab)_2$ anti-IgM antibody fragments were added to the cells at the time indicated by the red arrows.

Figure 2.6



The effect of differentiation on the expression of α4 on CLL cells. (A) Western blot probed for levels of PRDM1 in CLL cells directly *ex vivo* compared with that of Raji (negative control), tonsil-derived B and PCL cells (positive controls) – expected molecular weight of PRDM1α is ~100kDa. **(B)** Western blot showing expression of PRDM1 and Pax5 in two CLL clones either at T0 or after 5 days culture with or without IL-21+BCRxl. **(C)** illustrates the surface expression of α4 in the two CLL clones used in (B) after 5 days with (green) or without (red) IL-21+BCRxl. Case 2334 is a bi-modal $\alpha4^{pos}$ clone.

on the α4 expression of $\alpha4^{neg}$ CLL. In contrast, in $\alpha4^{pos}$ clones, surface expression of the integrin was reduced after such stimulation – an observation compatible with the fact tonsillar PCs express less surface α4 than do their B-cell counterparts[242] (Fig. 2.6C). For these studies of differentiation, Western blotting for two markers was used to detect maturation towards PCs – expression of PRDM1 and loss of Pax5 (these studies of PRDM1 led to investigations described in subsequent chapters).

Taken together, the above observations suggest that levels of α4 in CLL, rather than reflecting the activation/differentiation/anergic state of the malignant cells, is probably blocked by an unknown mechanism in $\alpha4^{neg}$ CLL cells. Regarding the nature of such a block, it has been shown for other cell types that CpG methylation of the *ITGA4* promoter inhibits transcription of the integrin[56]. It was therefore decided to examine the degree of DNA methylation of the *ITGA4* promoter in $\alpha4^{neg}$, as compared with $\alpha4^{pos}$, CLL clones.

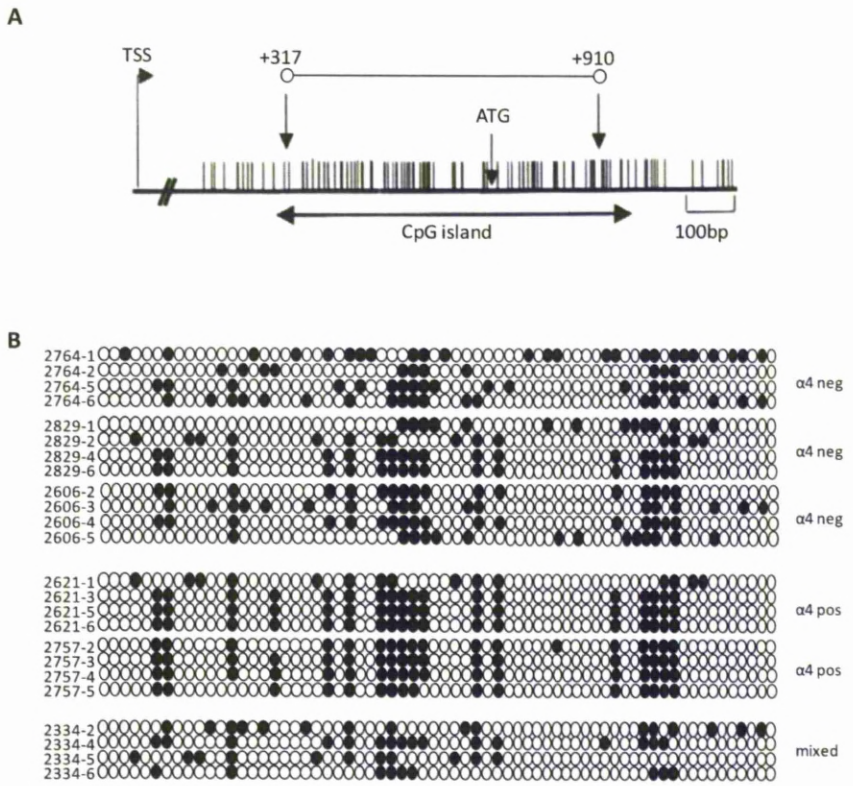
2.3.5 The 5′-untranslated region of the *ITGA4* gene contains comparably low levels of CpG methylation in both $\alpha4^{neg}$ and $\alpha4^{pos}$ clones

It has previously been reported that the 725bp fragment around the start codon of the *ITGA4* gene constitutes a CpG island (Fig. 2.7A) and that its degree of methylation controls α4 expression in gastric tumour cells[56]. Therefore, bisulphite genomic sequencing was used to determine the methylation status of this CpG island using the same PCR primers employed in this previous study.

As shown in (Fig. 2.7B), comparably low levels of cytosine methylation were observed in both $\alpha4^{neg}$ and $\alpha4^{pos}$. It was therefore concluded that the *ITGA4* gene in $\alpha4^{neg}$ clones is not silenced by hypermethylation of its CpG island.

Since histone modification is another epigenetic mechanism by which gene expression can be regulated, it was next hypothesised that expression of the *ITGA4* gene in CLL might be controlled by this mechanism.

Figure 2.7



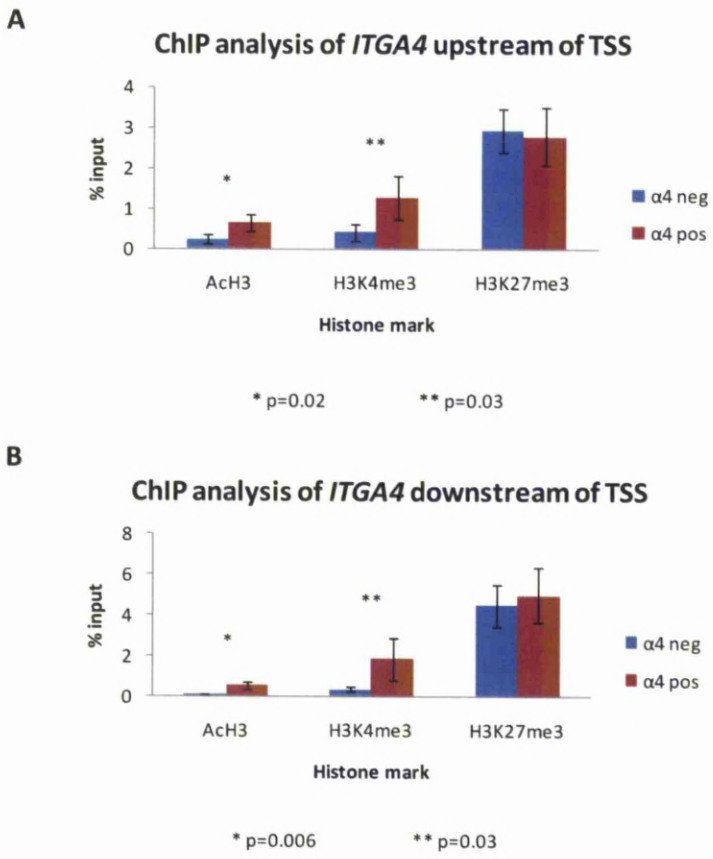
CpG methylation of the *ITGA4* gene promoter in CLL. (A) shows the arrangement of the CpG island in the *ITGA4* gene. ATG denotes the translation start site, while TSS indicates the transcription start site of the gene. The region amplified in the present study is shown between two open circles. For **(B)**, the amplified region was subjected to bisulphite sequencing, and methylated CpGs are shown as closed circles. Cells from 6 CLL cases were studied. In 3, the cells lacked α4, while in 1, populations of both $\alpha4^{pos}$ and $\alpha4^{neg}$ were observed; in the remaining 2 cases, the cells expressed α4.

2.3.6 The *ITGA4* gene in $\alpha4^{neg}$ CLL clones lacks histone marks associated with active transcription

Here, 3 histone marks - H3Ac (on K9 and K14 – detected using the same antibody), H3K4me3 and H3K27me3 - were examined. H3Ac and H3K4me3 are generally marks of active transcription and transcription initiation, while H3K27me3 is often associated with transcription inhibition but, when present together with H3K4me3, can poise genes for transcription (see Chapter 1). In a global analysis of active and silent gene transcription, it has been shown that peak levels of these marks are observed in association with nucleotide sequences within 1-kb up- and downstream of the TSS[250,251]. Therefore, ChIP analysis was used to examine the association of these histone marks with the *ITGA4* gene. This was done using antibodies specific for these 3 histone modifications and amplifying regions both up- and downstream of the *ITGA4* TSS using qPCR.

Such analysis demonstrated that chromatin precipitated with the anti-H3Ac and -H3K4me3 antibodies from $\alpha4^{pos}$ clones contained markedly higher levels of specific *ITGA4* DNA than did $\alpha4^{neg}$ clones. This was true of DNA amplified from regions both up- and downstream of the TSS (Fig. 2.8). In contrast, ChIP analysis with H3K27me3 antibody demonstrated similar levels of associated *ITGA4* DNA in both types of clone (Fig. 2.8).

Figure 2.8



Histone marks associated with the TSS of the *ITGA4* gene in $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones. Three histone PTMs were studied (AcH3, H3K4me3 and H3K27me3) by ChIP analysis using precipitating antibodies specific to each mark. In **(A)**, the sequence 1kb upstream of the TSS of the *ITGA4* gene was amplified by qPCR. **(B)** shows similar studies for the sequence 1kb downstream of the *ITGA4* TSS. Statistical analysis performed using a student's t-test.

Taken together, these results suggest that *ITGA4* transcription in CLL cells is controlled by histone modification and that *ITGA4* transcription in $\alpha4^{neg}$ clones is reduced or absent as a result of a lack of histone marks permissive for transcription.

2.4 DISCUSSION

Given that the aim of the present chapter was to establish why α4 is expressed in some CLL clones but not others, it was first necessary to establish the α4 expression of possible normal counterparts of CLL cells. It is already known that normal B cells have α4 at their cell surface[252], but there have been no reports concerning whether or not peripheral B-cell sub-populations express this integrin chain. Since CLL cells have been likened to both CD5+ and CD27+ B cells[28,31], FACS analysis of multiply stained normal B cells was used to establish the α4 reactivity of these two B cell sub-populations. It was found that both CD5+ and CD27+ B cells express surface α4 at levels comparable to those observed on $\alpha4^{pos}$ CLL clones. Therefore, $\alpha4^{neg}$ CLL cells differ from their theoretical normal counterpart in lacking surface expression of the integrin and this prompted investigations into why this might be so.

The transcription of α4 by $\alpha4^{pos}$ and $\alpha4^{neg}$ CLL clones was examined next, and it was found that the transcription is virtually absent in $\alpha4^{neg}$ CLL cells. It was therefore concluded that α4 protein expression is controlled at a transcriptional level. At the time, this observation/conclusion was novel for CLL cells, but has been subsequently been demonstrated by others[253].

Why α4 transcription might be reduced in $\alpha4^{neg}$ clones was examined next. Since α4 expression has been related to activation/differentiation, whether this might be so in CLL cells was examined in a number of ways. First, a range of stimuli was shown to have no effect on the α4 expression on $\alpha4^{neg}$ CLL cells. Secondly, culture for up to 7 days *in vitro* had no effect on α4 expression. Thirdly, both α4 subgroups expressed a closely similar pattern of surface expression of a range of markers of activation/differentiation. This latter point seemed important since it is known that different CLL clones differ in their degree of activation[38] and level of differentiation[254]. Furthermore, when differentiation was induced with IL-21 (see Chapter 4), plasmacytoid differentiation was not associated with any change in α4 expression in the $\alpha4^{neg}$ CLL clones. Also, lack of α4 expression could not be related to anergy as measured by either increased intracellular Ca^{2+} after BCRxl or surface IgM expression.

It was therefore concluded that lack of α4 expression is not a consequence of $\alpha4^{neg}$ CLL clones being more or less activated/differentiated/anergised than their $\alpha4^{pos}$ counterparts. Also, because extensive stimulation failed to induce α4 expression in $\alpha4^{neg}$ clones, it seemed likely that transcription is blocked in these cells.

This prompted consideration of the possibility that epigenetic mechanisms might be responsible for this block. Since the *ITGA4* gene can be silenced by DNA methylation in other cell types[56], the methylation of the CpG island of the *ITGA4* gene in CLL was examined first. In fact, only low levels of methylation were observed in both $\alpha4^{pos}$ and $\alpha4^{neg}$ clones. Since genes can be silenced by histone modification[97], this negative finding caused us to examine the histone marks associated with the α4 promoter in the two types of CLL clone. ChIP analysis of two histone modifications, H3Ac and H3K4me3, which are associated with gene activation and one marker of repression, H3K27me3, was therefore performed. This clearly showed that the *ITGA4* gene in $\alpha4^{neg}$ clones differs from that of the $\alpha4^{pos}$ clones in having lower levels of the activation markers. In contrast, in both types of clone the *ITGA4* gene was associated with similar levels of H3K27me3. It was therefore concluded that α4 expression in CLL is controlled by histone modification, and that $\alpha4^{neg}$ clones lack α4 transcription because the gene is modified in a manner not permissive for transcription.

Obviously this is only an interim conclusion and immediately raises two questions. First, what are the mechanisms controlling the differential histone modifications observed in the two types of CLL clones and,

secondly, why are the histone silencing mechanisms induced in $\alpha4^{neg}$ CLL cells *in vivo*?

These questions are considered in more detail in Chapter 6, and therefore will be only briefly discussed here. Regarding the mechanisms controlling the histone modifications of the *ITGA4* gene, it is known that in other cell types the ZEB (zinc finger E-box binding homeobox) transcription factors are involved[255]. There are two isoforms of ZEB and they can function either as transcriptional repressors or activators by recruiting co-factors. It is not even known whether CLL cells express ZEB proteins, and therefore this whole area of research requires further investigation.

The answer to the second question regarding why α4 transcription is prevented by histone modification in a subpopulation of CLL clones will depend on the above proposed studies regarding the mechanism of this block. However, the laboratory has preliminary data suggesting that induction of cell proliferation causes $\alpha4^{neg}$ clones to start expressing α4, presumably as a result of the induction of histone modifications permissive of transcription. It is tempting therefore to speculate that $\alpha4^{pos}$ CLL cells have more recently been exposed to mitogenic stimuli than their $\alpha4^{neg}$ counterparts.

Chapter 3

PRDM1 EXPRESSION IN CLL

3.1 INTRODUCTION

PRDM1 is thought to be the major transcription factor controlling the terminal maturation of B cells to PCs [130,131]. Thus, forced expression of PRDM1 alone can start a cascade of activation and repression which commits mature B cells to PC differentiation, while PRDM1-deficient B-cells fail to become PCs.

In Chapter 2, while examining the differentiation state of $\alpha 4^{pos}$ CLL cells versus those lacking the integrin at their surface, it was observed that both types of CLL cell lacked the 100kDa form of PRDM1 but contained a strongly positive band at ~70kDa (Fig. 2.6A; mouse monoclonal antibody from Novus Biologicals). The Western blots in which this ~70kDa band was detected were very clean and the PCL cells used as a positive control contained the expected 100kDa band, together with a similar band at ~70kDa.

It is known that multiple myeloma cells express large amounts of an N-terminally truncated, ~80kDa form of PRDM1 (termed PRDM1β) produced by an alternative promoter[198]. Also, an ~70kDa PRDM1β isoform has been shown to be functionally important in DLBCL treatment[200]. Furthermore, PRDM1β has significantly impaired transcription repressor function on multiple target genes and may be pathogenetically important[256]. Finally, mutations of PRDM1 and other members of the PRDM family of transcription repressors have been implicated in the generation of DLBCL and other cancers [1,199,257].

For all these reasons, it seemed important to investigate the nature of the ~70kDa band in CLL cells.

3.2 METHODS

All materials and methods were as described for Chapter 2, except where specifically outlined below.

3.2.1 Malignant B cells, cell lines and culture

In addition to CLL and PCL cells, PB mononuclear cells (PBMCs) from three different mature B-cell malignancies were also employed in this chapter. These were:- hairy-cell leukaemia (HCL); marginal-zone leukaemia (MZL); and mantle-cell leukaemia (MCL). All patients had a high percentage (>90%) of circulating malignant cells. The hairy cells (HC) had the diagnostic CD11c+CD25+ CD103+ phenotype. The MZL cells were clonal B cells lacking CD5, CD23, and HC markers, while the MCL cells were CD5-CD23- light-chain-restricted B cells.

Two additional B-cell lines were employed. These were Namalwa and Daudi cells. Both cell types are derived from Burkitt's lymphoma. Nalmalwa cells were cultured in RPMI-1640 (modified to contain 10mM HEPES, 1mM sodium pyruvate, 4.5g/L glucose and 1.5g/L sodium bicarbonate), 2mM L-glutamine and 7.5% FCS v/v. Daudi cells were grown in RPMI-1640, 2mM L-glutamine, 100units/mL penicillin, 100µg/mL streptomycin and 10% FCS v/v.

3.2.2 Antibodies

The following antibodies were used:- anti-PRDM1 antibodies - see Section 3.3.3; mouse monoclonal anti-BSA antibody cat# A10-127A (Bethyl Laboratories; Cambridge Bioscience, Cambridge, UK); secondary HRP-conjugated goat anti-rabbit antibody (Santa Cruz); and secondary HRP-conjugated donkey anti-goat antibody (Santa Cruz).

3.2.3 PCR

cDNA was amplified using the following primers:- pan PRDM1 forward 5'-AGC GAC GAA GCC ATG AAT CTC-3', reverse 5'-TTG AGA TTG CTG GTG CTG CTA A-3'; PRDM1α forward 5'-GAC TGG GTA GAG ATG AAC GAG-3', reverse 5'-CCT GTT GGC GTT CTT AGG AAC-3'; PRDM1β forward 5'-GCC CAT TTG CCA TTC ACT GC-3', reverse 5'-TTC TTT CAC GCT GTA CTC TCT C-3'; GAPDH forward 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse 5'-TCC ACC ACC CTG TCC CTG TA-3'.

3.2.4 Cell fractionation

$3x10^6$ Daudi cells were lysed in 1% Triton X-100 v/v, 10mM Tris (pH 7.4), 150mM NaCl plus protease inhibitor cocktail (Calbiochem) for 10mins on ice and then centrifuged at 4000rcf for 10mins. The Triton X-100 insoluble pellet was washed in the same Triton X-100 lysis buffer and then lysed in 1X Laemmli buffer. The lysed pellet was then sonicated and

loaded alongside a Daudi whole-cell lysate (WCL). The equivalent of $5x10^5$ cells from each sample were used for Western blotting.

3.3 RESULTS

3.3.1 The ~70kDa band is apparently expressed specifically in CLL

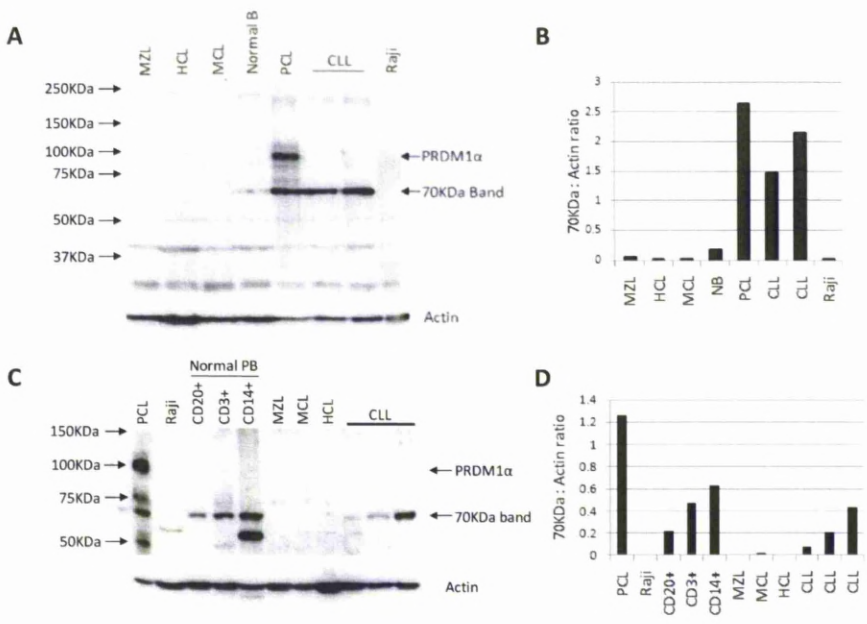
Having observed the ~70kDa band in CLL cells (Fig. 2.6A), it next seemed important to determine whether or not expression was specific to this malignancy. To this end, cellular extracts from MZL, HCL, MCL, normal CD19+ B-cells and the Raji cell line were Western blotted with the anti-PRDM1 antibody produced by Novus Biologicals (clone 3H2-E8); PCL cell lysates were included as a positive control. As shown in Fig. 3.1A and B, the ~70kDa band was specifically expressed by CLL; the malignant cells of MZL, HCL and MCL all lacked this band, as did Raji cells. The leukaemic PCs also contained the ~70kDa protein, together with the expected 100kDa full length form of PRDM1 and at least 2 other proteins of intermediate molecular weight. This very striking Western blot suggested that further studies of the ~70kDa protein were worthwhile.

The weak ~70kDa band observed in CD19+ purified normal B-cells prompted examination of the protein in a range of normal PBMC types.

3.3.2 The ~70kDa band is observed in normal PBMCs

Extracts of purified CD20+ B-cells, CD3+ T-cells and CD14+ monocytes

Figure 3.1



Detection of an apparent ~70kDa PRDM1 band in CLL cells. (A) and **(C)** show PRDM1 Western blots of cells lysates prepared from a range of mature malignant B cells and various other cell types; the membranes were probed with the Novus Biological 3H2-E8 anti-PRDM1 antibody. **(B)** and **(D)** show densitometric quantification of the ~70kDa bands in (A) and (C) respectively. Results are expressed as a ratio of the actin loading control.

were subjected to Western blotting and the cell types employed above included as positive and negative controls (Fig. 3.1C and D). All three normal cell types contained a strong band at ~70kDa. As before, the CLL cells possessed the band, while the other chronic leukaemic cells did not express the protein. Again, Raji cells lacked the band, while PCL cells expressed the full length, intermediate and ~70kDa forms.

It was therefore concluded that the ~70kDa protein is not confined to CLL cells and its presence in normal cells questioned any potential importance in the pathogenesis of the disease. It next seemed essential to establish whether or not the ~70kDa band really is a truncated form of PRDM1.

3.3.3 Western blotting with three different anti-PRDM1 antibodies fails to identify the ~70kDa band as PRDM1

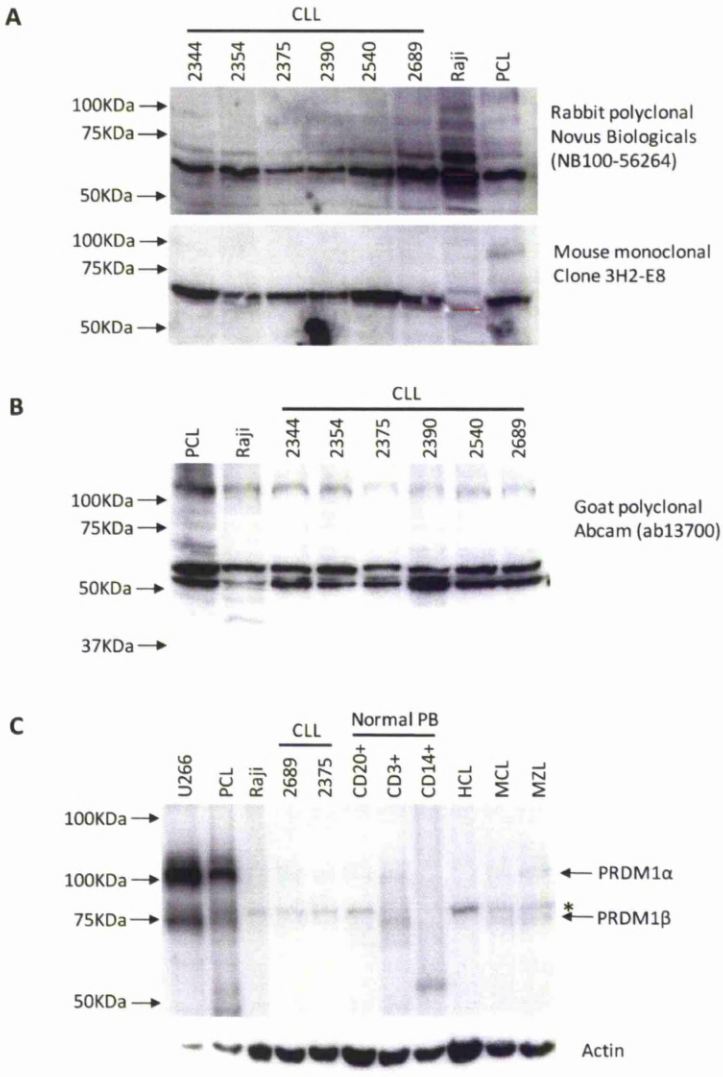
Three different antibodies against the C-terminus of PRDM1 (a rabbit polyclonal from Novus Biologicals [NB100-56264], a goat polyclonal from Abcam [ab13700] and a different mouse monoclonal, kindly provided by Dr Giovanna Roncador[258] (clone ROS antibody) were used to probe Western blots of CLL, Raji and PCL cell extracts. The rabbit polyclonal antibody detected bands at ~70kDa and ~75kDa, but these were also present in Raji cells. Re-probing of the same blot with the original Novus

Biologicals antibody (clone 3H2-E8) showed that both bands were clearly different from the original ~70kDa protein demonstrated earlier (Fig. 3.2A). The goat antibody also gave a negative result, detecting two bands lower than the expected molecular weight (Fig. 3.2B); again, both these proteins were also expressed in the Raji-cell extract.

When lysates of different cell types were Western blotted and probed with the mouse mab (clone ROS) against PRDM1, no ~70kDa protein was observed in CLL or the other cell extracts (Fig. 3.2C). A weak band of molecular weight >75kDa (marked by an asterisk in Fig. 3.2C) was detected in CLL cell lysates. Since this protein was clearly different from the ~75kDa molecule detected in PCs (itself different from the ~70kDa band detected with the Novus Biologicals antibody), it seemed unlikely that it represents the truncated form of PRDM1. Also, this >75kDa protein was present in a range of other cell types and was therefore unlikely to be pathogenetically important in CLL. Furthermore, a similar band detected with the same antibody had been highlighted as being non-specific in a previous publication[259].

Taken together, these studies using different anti-PRDM1 antibodies suggested that the ~70kDa molecule detected in CLL cells with the Novus Biologicals antibody is not a different isoform of PRDM1β.

Figure 3.2



Western Blotting for PRDM1 using different antibodies. In **(A)**, cell lysates were Western blotted with an antibody from Novus Biologicals (upper panel) and then re-probed with the Novus Biologicals 3H2-E8 antibody (lower panel). The red line marks the same position on both blots. In **(B)** and **(C)**, cell lysates were blotted for PRDM1 with polyclonal goat and monoclonal mouse (clone ROS) antibodies respectively. The asterisk in (C) shows the position of the suspected non-specific band[259].

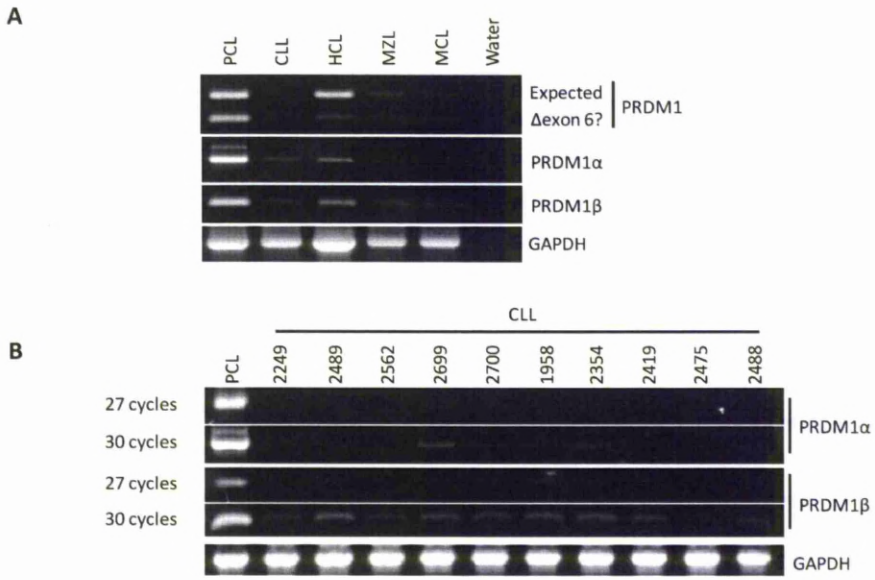
At this stage, it was concluded that CLL cells do not express either PRDM1α or PRDM1β, but it was thought important to confirm this conclusion by looking for PRDM1 mRNA in CLL cells.

3.3.4 CLL cells contain little or no PRDM1 mRNA

Initially, PCR was performed using primers designed to detect the 3' region of PRDM1 mRNA that encodes for the C-terminal part of PRDM1 shared by both the α and β forms of the protein (refer to Fig. 1.4). Little or no PCR product was detected in the CLL lymphocytes, while PCL cells contained large amounts of PRDM1 mRNA (Fig. 3.3A). The PCL cells also contained a smaller PCR product which may correspond to a form of PRDM1 mRNA that lacks exon 6 (exon 7 of *blimp-1*[197,260]). A strong band was also detected in HCL cells (although additional loading could be the reason for this), while little or no PRDM1 mRNA was seen in MZL or MCL lymphocytes.

Next, PCR was performed with primers specifically designed to detect either the α or β forms of PRDM1. For detection of PRDM1α, primers to amplify exons 1 to 3 were employed, while for PRDM1β exons 1β to 5 were amplified (refer to Fig. 1.4). Little or no PRDM1α product was detected in CLL cells, but was abundantly present in PCL cells (Fig. 3.3A and B). Again, HCs contained PRDM1α message but this was less

Figure 3.3



PCR detection of PRDM1 mRNA in CLL and other cell types. In **(A)**, the top panel shows the products obtained with primers detecting the 3' region of PRDM1 mRNA (coding for the C-terminal part of the protein shared by both α and β forms). The lower of the two bands has the size expected for the mRNA of PRDM1 that lacks exon 6 (Δexon 6). In the second and third panels, primers specific for PRDM1α and PRDM1β were respectively employed. The fourth panel shows a GAPDH loading control. In **(B)**, PRDM1α and β were amplified from 10 cases of CLL. Two different PCR cycle numbers are shown and compared with levels in a PCL sample.

abundant than in PCL cells. Regarding mRNA specific for PRDM1β, CLL cells contained little or none of the expected 237bp product, while this product was abundantly present in PCL cells and HCs (Fig 3.3A and B).

It was therefore concluded that CLL cells contain little or no message for either full length PRDM1α or the truncated PRDM1β. These results support the earlier conclusion that the ~70kDa protein apparently specifically expressed by CLL cells is, in fact, not a form of PRDM1.

It next seemed important to attempt to identify the nature of the ~70kDa band which had been apparently specific for CLL.

3.3.5 ~70kDa molecule detected by the Novus Biologicals antibody is albumin

Because previous work in the Department aimed at determining the identity of an unknown ~70kDa band in CLL cells had shown that the band represented cross-reactivity of the antibody with albumin, it was hypothesised that the Novus Biologicals antibody was also cross-reacting with this protein.

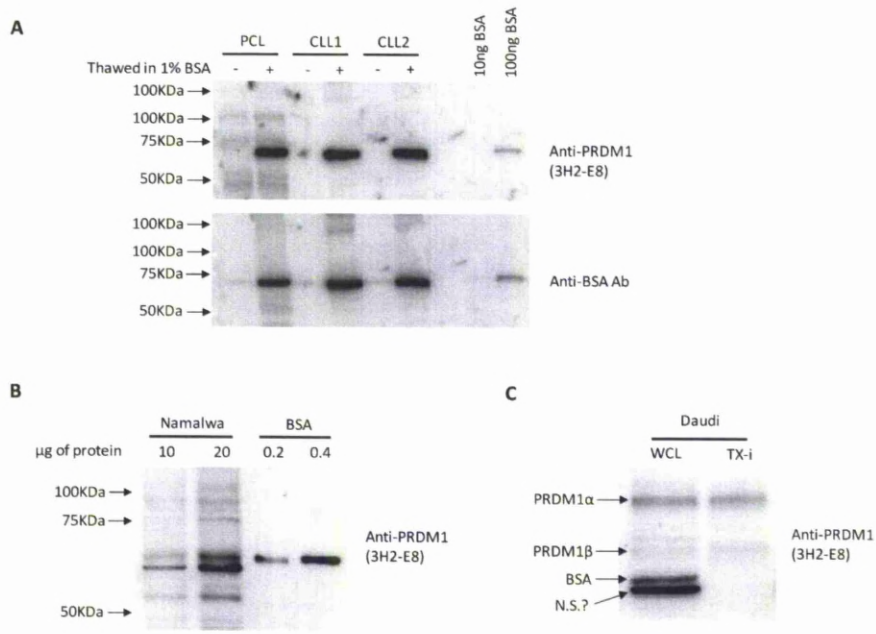
To test this hypothesis, CLL and PCL cells were thawed in culture medium (RPMI) containing no BSA. In this laboratory, 0.5% BSA is routinely added to the culture medium to enhance cell survival upon thawing and culture. When extracts of cells prepared without BSA were

submitted to Western blotting with the Novus Biologicals antibody, no ~70kDa band was detected. However, when BSA was reintroduced into the culture medium used during thawing, the band reappeared (Fig. 3.4A). To confirm this result further, purified albumin Western blotted at the same time was detected by the Novus Biologicals antibody and re-probing of this blot with an anti-albumin antibody detected bands of the same size and with the same pattern of expression (Fig. 3.4A).

It was therefore concluded not only that the Novus Biologicals ~70kDa molecule is not PRDM1, but that it represents albumin cross-reacting with the PRDM1 antibody. A recent publication has highlighted the presence and functional importance of a ~70kDa band (detected with the Novus Biologicals antibody) thought to be an isoform of PRDM1β in DLBCL[200]. In view of the above conclusion, it seemed reasonable to postulate that this protein was also albumin rather than PRDM1β.

Because much of the previous work examining the ~70kDa form of PRDM1β was performed with Namalwa and Daudi cell lines[200], lysates of these cells were next examined by Western blot with the Novus Biologicals antibody.

Figure 3.4



Characterisation of the ~70kDa band. In **(A)**, cell lysates were blotted with the 3H2-E8 anti-PRDM1 antibody (upper panel) or with an anti-BSA antibody (lower panel). Cells were thawed in the presence or absence of BSA and purified BSA was loaded for comparison. In **(B)** and **(C)**, the expression of the apparent ~70kDa band was examined in Namalwa and Daudi B-cell lines respectively. In (C), WCL and 0.5% Triton X-100 insoluble pellet (TX-i) were compared; "N.S." marks the hypothesised non-specific band not present in the TX-i material.

3.3.6 Albumin and an additional ~70kDa band are detected in Namalwa and Daudi cell lines when probed with the Novus Biological anti-PRDM1 antibody

When Namalwa and Daudi cells were cultured in the presence of 10% FCS and then subjected to Western blotting using the Novus Biologicals antibody, two bands were detected at ~70kDa (Fig. 3.4B and C). The heavier of these two bands migrated at the same rate as purified albumin, which was loaded alongside the cell lysates (Fig. 3.4B). The lighter protein however, was too small to be albumin and could not be ruled out as being the ~70kDa form of PRDM1β as identified by Liu *et al*[200].

As PRDM1 is a transcription factor that is exclusively expressed in the nucleus[130], the location of the new ~70kDa band was next examined as a guide to its specificity; if the new ~70kDa band were to be found only in the cytoplasm this would support the conclusion that the band does not represent an isoform of PRDM1. Therefore, Daudi cells were lysed in Triton X-100 and the insoluble (which is mostly composed of insoluble nuclei) fraction subjected to Western blotting alongside a Daudi WCL. As expected, both of the ~70kDa bands was found only in the soluble fraction while, again as expected, only the 100kDa and 80kDa isoforms of PRDM1 remained in the insoluble fraction (Fig. 3.4C). This indicates

that the new ~70kDa protein is associated with the membrane/cytoplasm outside the nucleus, suggesting that the band is unlikely to represent a truncated isoform of the transcription factor.

3.4 DISCUSSION

The aim of the present chapter was to determine whether or not CLL cells express PRDM1β. The work arose from the observation in Chapter 2 that CLL-cell lysates blotted with an anti-PRDM1 antibody, produced by Novus Biologicals, contained an apparently specific ~70kDa band. Because it is known that PRDM1 can be expressed in truncated forms of molecular weight ~70 to ~80kDa[198,200], it seemed plausible that this band was indeed specific for PRDM1β.

When lysates of other cell types were Western blotted and probed with the Novus Biologicals antibody, the ~70kDa band was absent from a range of B-cell types, but was strongly present in PCs which are known to express both PRDM1α and PRDM1β. This encouraged more extensive studies of the ~70kDa molecule in CLL. However, when 3 other anti-PRDM1 antibodies were tested, they failed to detect the ~70kDa band. It was therefore concluded that the band is not PRDM1β and this was confirmed by the demonstration that CLL cells contain little or no message for either PRDM1α or PRDM1β. Furthermore, the ~70kDa band was shown to be albumin since the band disappeared when the cells were thawed and recovered in culture medium lacking BSA. Addition of BSA to the thawing medium restored the band and Western blotting

with an anti-albumin antibody detected a band at the same molecular weight only in those cells thawed in the presence of albumin. Also, when purified albumin was subjected to SDS-PAGE and Western blotted with the Novus antibody, strong cross-reactivity was demonstrated. Therefore it was concluded that the ~70kDa band is in fact albumin cross-reacting with the Novus Biologicals antibody. The albumin is presumably either stuck to the surface of the cells or internalised during the thawing and recovery process.

The Novus Biological anti-PRDM1 antibody (clone 3H2-E8) showed strong cross-reactivity with BSA possibly because of its use as a carrier protein during immunisation. Haptens, such as the partial peptide sequence of PRDM1 used to raise the Novus Biological anti-PRDM1 antibody, often fail to raise complete immunogenic responses alone and require conjugation to carrier proteins such as BSA. Linking the peptide to BSA has the possibility of causing production of antibodies that cross-react to this carrier protein.

A publication by Liu *et al*[200] has shown the presence of PRDM1β mRNA and protein in DLBCL cells. However, the supposed PRDM1β protein found in these cells migrated only to ~70kDa on a Western blot and was different to that of the original 80kDa form of PRDM1β expressed in

myeloma cells[198]. Subsequently, concerns have been raised about its specificity[259]. The antibody used to perform the Western blots in the work by Liu *et al* was the same antibody used in my studies (Novus Biologicals). Upon finding that this antibody cross reacts with albumin, it was hypothesised that Liu *et al* were also picking up the same non-specific band and confusing it with a potential PRDM1 isoform. When Namalwa and Daudi cell lysates were Western blotted and probed with the Novus Biologicals anti-PRDM1 antibody, two bands were detected at ~70kDa. The top band migrated the same distance as albumin and therefore probably is this protein. The lower of the two bands appeared to be novel for Namalwa and Daudi cells and could be the protein originally described by Liu *et al*. However, as this lower band was not present in the nucleus of the Daudi cells, its identity as an isoform of PRDM1 remains in doubt.

Although the work in the present chapter indicates that CLL cells lack both PRDM1α and β, it next was of interest to question whether or not the protein can be induced in CLL. Because PRDM1 is central to plasmacytoid differentiation, this question is of major importance in the biology of CLL since the malignant cells resemble activated memory cells which do not differentiate further *in vivo*. It was therefore decided to examine PRDM1 expression and other features of PC differentiation in

CLL cells exposed to relevant differentiating stimuli. Because IL-21 had been used as a differentiating agent in Chapter 2 (Fig. 2.6B) and for a number of other reasons described in the Introduction of Chapter 4, work was initially focused on the differentiating effects of IL-21.

The next chapter (Chapter 4) therefore examines the effects of this cytokine and other stimuli on the differentiation of CLL cells with particular reference to PRDM1 expression.

Chapter 4

THE EFFECT OF IL-21 ON CLL-CELL DIFFERENTIATION

4.1 INTRODUCTION

IL-21 is a member of the type I cytokine family whose receptors share a common γ-chain[208]. The cytokine has diverse actions on a range of immunological cell types, and the results of stimulation depend on the cell type and the nature of additional co-stimuli[209]. Regarding B cells, IL-21 is known to be a critical regulator of differentiation and cell death[218]. The precise effects of the cytokine on a particular B-cell type are determined by its state of activation and stage of differentiation, and are influenced by antigenic stimulation and other co-stimuli[215].

There has recently been considerable interest in IL-21 as an anti-cancer agent. Such anti-tumour effects can be mediated directly or indirectly according to the cancer-cell type[209,261]. In particular, there is currently interest in the potential therapeutic effects of IL-21 in CLL[220]. Thus, the

cytokine can induce CLL-cell death both by IL-21R-induced STAT1 signals[211] and by IL-21-induced granzyme B production[222]. Interestingly, despite the fact that IL-21 is such a potent inducer of the terminal differentiation of normal B cells, the effects of IL-21 on CLL-cell differentiation have not been examined. This is surprising because the failure of CLL cells to undergo PC differentiation is central to the pathogenesis of the disease. Thus CLL-cells are now thought to resemble memory B-cells generated through chronic stimulation by (auto)antigen[28,31,38]. For unknown reasons, the cells do not differentiate further to PCs and therefore do not produce large amounts of antibodies to neutralise and eliminate the putative (auto)antigen.

The aim of this chapter, therefore, was to examine the effects of IL-21 on CLL-cell differentiation towards PCs. A number of markers of plasmacytoid differentiation were examined, but particular emphasis was given to the expression of PRDM1 since this transcription factor is so central for B-cell differentiation to antibody secreting cells[130,142]. However, expression of IRF4 and Pax5 was also examined because these transcription factors are important in completing full PC differentiation[163]. Thus, IRF4 is up-regulated during such differentiation, while Pax5 is down-regulated. In addition, Ig secretion, surface markers of terminal B-cell differentiation and morphology were used to assess

plasmacytoid differentiation. Because the actions of IL-21 are influenced by co-stimuli, the effect of a range of additional relevant co-stimuli was tested.

It is shown in this chapter that the differentiation of ~50% of CLL clones is blocked, probably as a result of failure of these cells to induce PRDM1. Possible causes of this block are examined in the next chapter (Chapter 5).

4.2 METHODS

Again, only materials and methods not given earlier are considered.

4.2.1 Clinical material

CLL surface prognostic markers were measured by FACS in both the diagnostics laboratory and our laboratory, and this information was made available for the present study as shown in Table 5. BCR IgV_H mutation status and gene segment usage were established post diagnosis by methods previously described[262]. All CLL clones expressed surface IgM and their IgV_H mutation and gene family usage were known.

Buffy coats were ordered and collected from the British Transfusion Service, Liverpool. Concentrated buffy coat samples, used for the normal B-cell work in this Chapter, were initially diluted 1:2 in RPMI-1640 at room temperature, before being placed on lymphoprep as detailed in Section 2.2.1.

4.2.2 Normal B-cell purification

Negative purification of B cells was performed using the B-cell isolation kit II reagents and provided methodology (Miltenyi Biotec). Cells were resuspended in 300µL of purification buffer prior to being placed into LS columns. B cells not bound to beads were then washed out and

collected by three 3mL wash steps using purification buffer. Purity of the B cells was checked after purification and was always >95%.

4.2.3 Stimulants

All reagents were added at the same concentration that had been used previously. In addition to these, the following stimuli were used in this chapter:- 50nM PMA (Phorbol 12-myristate 13-acetate; Sigma-Aldrich); 1μM ionomycin (Calbiochem).

4.2.4 Antibodies

Western blotting antibodies used were:- mouse monoclonal anti-PRDM1 (ROS clone); mouse anti-IRF4 (MBL International; ATI-Atlas Ltd, Chichester, UK).

The antibody used for flow cytometry was:- Alexa Fluor 647 conjugated mouse anti-CD138 antibody clone B-B4 (AbD Serotec, Oxford, UK).

4.2.5 Ig secretion

Secreted IgM and IgG were measured by using human IgM or IgG ELISA quantification sets respectively (Bethyl Laboratories). CLL cells were pelleted and cell culture medium was removed and centrifuged again at 10,000rcf for 5mins to remove all non-soluble material. The dilutions of

the cell medium, as well as the primary and secondary ELISA antibodies were all optimised prior to experimental use.

4.2.6 Morphology

After 5 days culture with and without IL-21+CD40L+BCRxl+IL-2, a large number of cells were apoptotic (>60%). Therefore, non-apoptotic cells were purified before cytospinning by using the MACS magnetic bead system of purification (see Section 2.1.6). FITC-conjugated Annexin V (BD Biosciences) was used to stain dead cells and these were pulled out using anti-FITC MACS beads (Miltenyi Biotec).

CLL cells ($5x10^5$ in 100µL) were cytospun in PBS supplemented with 0.5% BSA at 300rpm for 3mins at the low acceleration setting. Under these conditions, the CLL cells are flattened and have an increased diameter as compared with cells in suspension. When the diameter of these cells was measured using the Nikon ACT-1 v2.63 microscope software, greater than 95% had a diameter <18µm. Cells larger than this, but lacking a plasmacytoid morphology were scored as prolymphocytes. Plasmacytoid cells were identified as having eccentric nuclei, strongly basophilic cytoplasm and a well developed pale-staining Golgi area. It was noted that a proportion of the plasmacytoid cells were smaller than typical PCs; an arbitrary value of <18µm was used to quantitate these.

4.2.7 Apoptosis assay

$3x10^5$ cells in culture media were incubated with 25nM $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) for 15mins in a 37°C incubator. 1µg/mL PI (propidium iodide) was added to the cells before being placed on ice. Cells were then analysed immediately using flow cytometry.

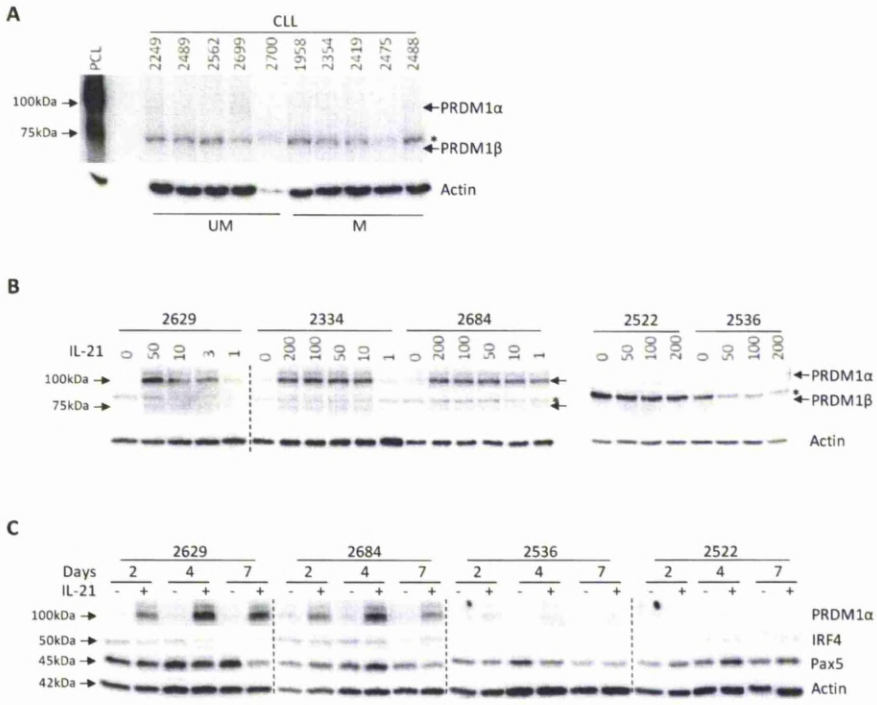
4.3 RESULTS

4.3.1 IL-21 alone induces incomplete terminal differentiation in a proportion of CLL clones

Initially, PRDM1 protein expression was used as a marker of plasmacytoid differentiation and Western blotting was employed to examine whether or not IL-21 induces CLL cells to express this protein. PRDM1 was usually either undetectable or present at low levels in CLL cells tested either directly *ex vivo* (Fig. 4.1A) or after culture in the absence of IL-21 (Fig. 4.1, 4.2A and Table 5).

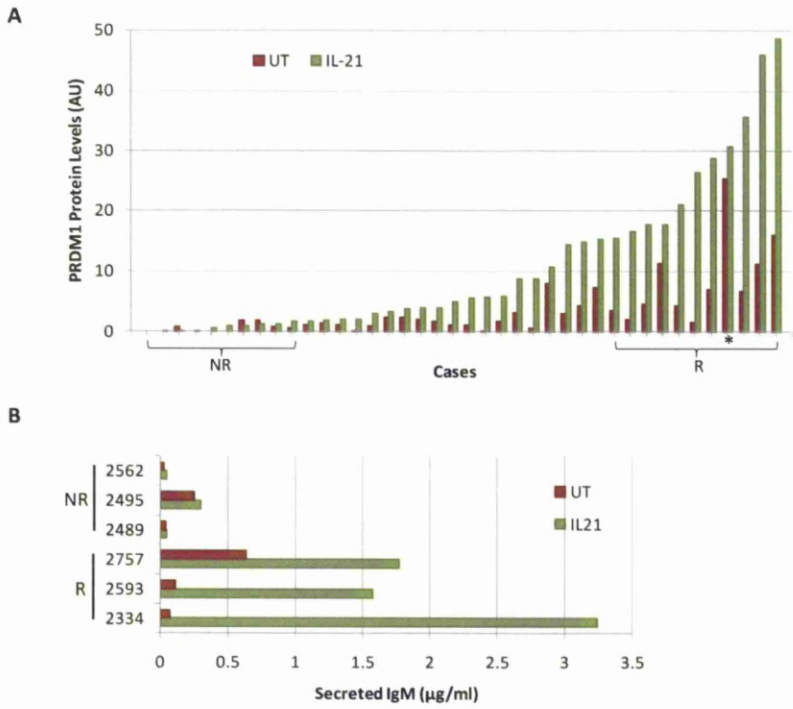
In preliminary experiments, IL-21 was found to induce PRDM1α (100kDa) and β (80kDa) protein isoforms in some CLL clones. This induction was maximal at 50ng/mL of the cytokine and after 4 days of culture (Fig. 4.1B and C). In other clones, culture for up to 7 days in the presence of even high concentrations of IL-21 (200ng/mL) stimulated little or no expression of either PRDM1 isoform. Table 5 shows the qualitative effects of IL-21 (50ng/mL) on PRDM1α induction in 66 CLL clones from 52 patients. Subsequently, PRDM1α induction was measured semi-quantitatively in 40 of these CLL clones after culture for 16hrs in the presence or absence of IL-21 (Fig. 4.2A). As expected, little or no PRDM1α was expressed in some clones, while in others substantial

Figure 4.1



PRDM1, IRF4 and Pax5 expression assessed by Western blotting in CLL cells before and after culture with IL-21 alone. (A) PRDM1 expression in 10 CLL (5 UM and 5 M) clones. The protein band marked with an * is non-specific[259], and is not identical to that investigated in Chapter 3. **(B)** demonstrates PRDM1 expression after 2 days' culture with different concentrations of IL-21. **(C)** Shows the expression of PRDM1, IRF4 and Pax5 after 2 to 7 days' culture with IL-21 (50ng/mL). In (A), (B) and (C), actin served as a loading control.

Figure 4.2



Semi-quantitative PRDM1 expression in, and IgM secretion by, CLL cells cultured in the presence or absence of IL-21. (A) PRDM1 was detected by Western blotting of cell lysates prepared after 16hrs' culture with and without IL-21 (50ng/mL). Levels were measured semi-quantitatively by densitometry, relating the density of the PRDM1 band to that of the same standard lysate included in each gel. The top and bottom quartiles are shown and were regarded as R and NR respectively. The case marked with an * was unusual in expressing high amounts of PRDM1 even in UT cells, and was excluded from subsequent studies. For **(B)**, secreted IgM was measured by an ELISA method using supernatants from CLL cells ($3x10^6$/mL) cultured in presence or absence of IL-21 (50ng/mL) for 5 days. Note that all 6 cases expressed surface IgM as measured by FACS analysis before culture.

Table 5 – Patients/bleeds studied, their qualitative PRDM1 response to IL-21 and associated clinical data.

Case	PRDM1 on IL-21 Treatment		Qualitative PRDM1 Response	White Blood Count (10^9/L)	Clinical Stage		Prognostic Markers				VH Segment Usage
	-	+			Rai	Binet	V_H Mutation (%)	V_H Mutation (class)	CD38 (%)	CD49d	
2522			-	86			5.21	M	6	-	1-02
2086			+	253			0.34	UM	3		1-02
2560			-	224			0	UM	98	+	1-18
2256			-	51	I	B	0	UM	27	-	1-24
2370			-	60	IV	C	0	UM	76	-	1-24
2410			-	70	IV	C				+/-	
1922			-	103	0	A	0.35	UM	25		1-46
LS			-	206	IV	C	0	UM	1		1-69
2495			-	60	IV	C	0	UM	24	+	1-69
2536			-	242	III	C	0	UM	96	+	1-69
2419			-	149	I	B	6.6	M	6		1-69
2456			-	135			0	UM	10	-	1-69
2562			-	124			0	UM	40		1-69
2741			-	14	0	A	4.05	M	19		1-69
2948			-	114	II	B	0	UM	3		1-69
2099			-	39	0	A	5.68	M	6	-	2-05
2270			-	90	I	A	8.15	M	8	-	2-05
2780			-	114	0	A					
2415			-	91	0	A	5.84	M	5	-	2-26
2829			-	116	0	A					
GR			+	90	II	B	2.02	M	71		2-70
2053			-	122			1.7	UM	43		3-07
2237			-	203	I	B	8.59	M			3-09
2953			+	126	IV	C	0	UM	98		3-09
2512			-	257	I	B	0.27	UM	1	+	3-15
2997			++	86	IV	C	0.63	UM	4		3-15
3047			++	141	IV	C					
2329			-	21			0	UM	85		3-20
2029			++	57	0	A	2.8	M	13		3-21
2269			++	50	0	A					
2602			+	53	0	A					
2594			-	105			3.72	M	97	+/-	3-21
2629			++	51			5.98	M	5		3-21
2684			++	208	III	C	1.39	UM	10	-	3-23

Table continues over page...

Case	PRDM1 on IL-21 Treatment		Qualitative PRDM1 Response	White Blood Count (10^9/L)	Clinical Stage		Prognostic Markers				VH Segment Usage
					Rai	Binet	V_H Mutation		CD38 (%)	CD49d	
	-	+					(%)	(class)			
1895			++	175	IV	C	3.38	M	43	+	3-23
2757			++	84	IV	C					
2334			++	84	0	A	4.7	M	75	+/-	3-23
2457			++	283	IV	C	2.5	M	3		3-30
2584			+	34			3.06	M	5	-	3-30
2783			+	196							
2699			+	67			0.35	UM	13		3-30
2902			-	40	0	A	6.9	M	5		3-30
2916			-	67	IV	C	10.5	M	28		3-30
3042			-	175	0	A	8.33	M	0		3-30
2465			-	20	0	A	6.4	M	3		3-33
ES1			+	93	IV	C	0	UM			3-48
2045			++	23			1.35	UM	40	-	3-48
2147			++	29							
2450			++	183							
2163			+	39	I	B	0.69	UM	88	-	3-48
2593			++	86	IV	C					
2656			-	142			0.35	UM		-	3-48
2950			+	150			5.21	M			3-48
2673			-	65	0	A	0.34	UM	9		3-49
2956			+	266	0	A					
2744			-	29	0	A	6.6	M	1		3-7
3023			+	68			0	UM			3-7
3041			-	56			6.25	M	0		3-7
2513			-	35	0	A	6.12	M			3-72
2999			++	119	I	B	3.64	M	7		3-73
3032			-	169	II	A	4.17	M	5		3-74
2489			-	377	II	B	0	UM	18	+	4-31
2728			-		IV	C					
2248			++	36	IV	C	9.47	M			4-34
2354			+	165	I	A	4.56	M	2	+	4-34
2708			-	66	0	A	4.47	M	6		4-34

Samples next to each other in the same shade (i.e. grey or white) are from the same patient but different bleeds.

Mutated cases are highlighted in red.

amounts of the protein were induced. For subsequent studies comparing the PRDM1 response of different CLL clones, the top and bottom quartiles of cases were taken as representative responders (R) and non-responders (NR) respectively (Fig. 4.2A).

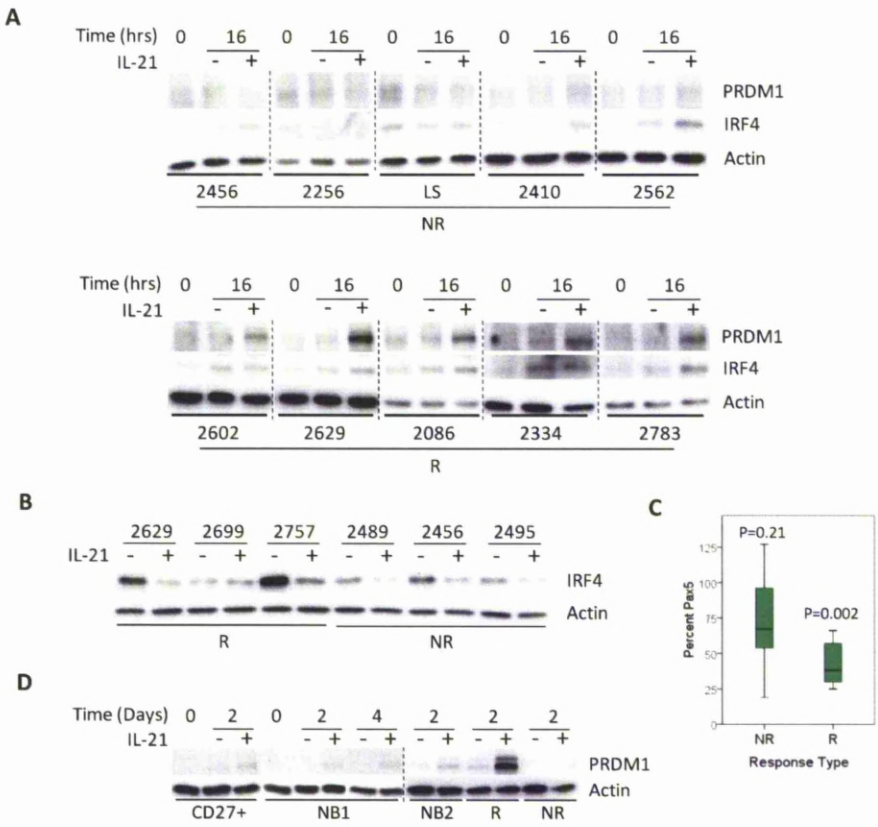
As a further measure of plasmacytoid differentiation in the presence of IL-21, Ig secretion was compared in R and NR clones. In R clones, PRDM1 induction was accompanied by IgM secretion (n=3; Fig. 4.2B), but no soluble IgG was detected (n=3; data not shown). In NR clones, little or no increase in IgM or IgG secretion was observed after IL-21 treatment (n=3; Fig. 4.2B and data not shown).

The effect of IL-21 on the expression by CLL cells of other transcription factors whose levels are altered during PC differentiation was examined next. In particular, IRF4 and Pax5 levels after IL-21 stimulation were measured. XBP-1 protein, another transcription factor increased on PC differentiation, proved difficult to analyse because the commercial antibodies tested (Novus Biologicals cat# NB110-57824 and AnaSpec cat# 54578) detected multiple bands both before and after IL-21 treatment and because different reports have stated different molecular weights of the spliced and unspliced forms of this protein[263,264].

Regarding IRF4, usually little or no protein was detectable by Western blotting in CLL clones examined directly *ex vivo*, or after culture for 16hrs in the absence of IL-21 (n=10; Fig. 4.3A). After such short-term culture in the presence of the cytokine, IRF4 levels were modestly increased in some clones, regardless of whether or not the cytokine induced PRDM1 (Fig. 4.3A). After longer culture for 48hrs (Fig. 4.3B) to 7 days (Fig. 4.1C), IRF4 levels became variably increased in the absence of IL-21, and the cytokine usually repressed this IRF4 induction. With regard to Pax5, the transcription factor was consistently reduced, but still readily detectable, in R CLL clones after 7 days culture with IL-21 (Fig. 4.1C and Fig. 4.3C). In NR clones in which IL-21 did not induce PRDM1, the cytokine had no consistent effect on Pax5 levels (Fig. 4.1C and Fig. 4.3C).

Next, CLL and normal B-cells were compared with regard to their ability to produce PRDM1 in response to treatment with IL-21 alone. When negatively purified B cells (CD19+ >95%) were cultured with the cytokine for up to 7 days, PRDM1 protein was very weakly induced at 2 and 4 days; thereafter, PRDM1 became undetectable, but viability was low (~8%) at this stage (Fig. 4.3D). Similar low levels of PRDM1 induction were observed in purified CD27+ normal memory B cells after 2 days of IL-21 stimulation (Fig. 4.3D). In both types of normal B-cell preparation,

Figure 4.3

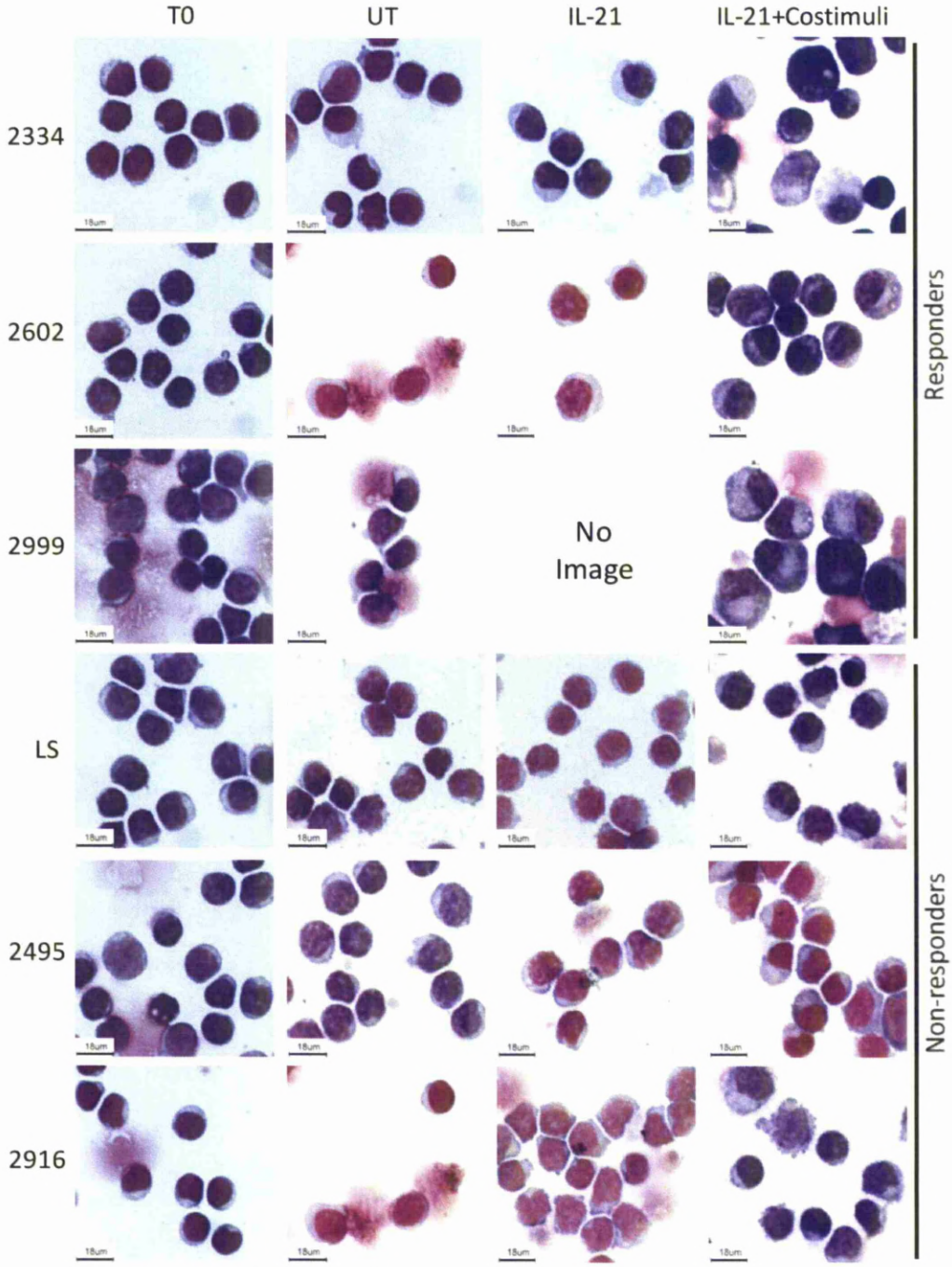


PRDM1, IRF4 and Pax5 expression by CLL and normal B cells cultured in the presence or absence of IL-21. (A) shows PRDM1 and IRF4 expression in NR and R clones cultured for 16hrs in the presence or absence of IL-21 (50ng/mL). In **(B)**, IRF4 levels were measured after a longer period of culture (48hrs) in the presence or absence of IL-21 (50ng/mL). For **(C)**, Pax5 expression after 7 days' culture with IL-21 (50ng/mL) was measured by Western blotting and densitometry. The results are given as a percentage of the Pax5 present in UT cells cultured for a similar period. The reduction in Pax5 in R clones was significant, while that in NR clones was not. **(D)** presents the results of similar experiments with normal CD27+ (memory; n=1) and CD19+ (n=2) normal B (NB) cells; an R and an NR CLL clone are included for comparison.

PRDM1 protein induction by IL-21 alone was markedly less than that induced in R CLL cells (Fig. 4.3D). Therefore R CLL cells are able to produce more PRDM1 than normal B cells in response to IL-21 alone.

It was therefore concluded at this stage that, in R clones, IL-21 alone induces plasmacytoid differentiation as measured by induction of PRDM1 and IgM secretion. However, IRF4 was not strongly up-regulated and Pax5 was only partially suppressed, suggesting full PC differentiation was incomplete. This conclusion was confirmed by FACS analysis of surface markers reported to be associated with PC differentiation ($CD20^{low}$, IgD^{low}, $CD27^{high}$, $CD38^{high}$, $CD138^{high}$; see Table 4). Thus, culture of CLL cells with IL-21 for up to 7 days had no consistent effect on the expression of any of these markers (n=6; 3 R). CD138 was expressed at low levels in CLL cells tested directly *ex vivo* (as shown previously by Sebestyen *et al*[265]) and after culture. However, expression of this surface protein did not change after stimulation with IL-21 alone (data not shown, refer to IL-21+co-stimuli results below, Fig. 4.5B and C). When morphology was assessed after 5 days culture, 6-10% of cells in R cultures had plasmacytoid morphology, while no such cells were observed when PRDM1 was not induced (Fig. 4.4 and Table 6).

Figure 4.4



PTO for quantification and figure legend

Table 6 - Quantification of CLL-cell morphology at T0 and following treatment with and without either IL-21 or IL-21+co-stimuli

PRDM1 response	Case	Treatment	CLL cells (<18um)	Prolymphocytes (>18um)	PCs (<18um)	PCs (>18um)
R	2334	T0	92	8	0	0
		UT	95	5	0	0
		IL-21	74	16	10	0
		IL-21+co-stimuli	48	19	17	16
	2602	T0	91	9	0	0
		UT	96	4	0	0
		IL-21	88	4	6	2
		IL-21+co-stimuli	53	7	28	12
	2999	T0	96	4	0	0
		UT	89	6	5	0
		IL-21	Not enough cells for analysis			
		IL-21+co-stimuli	31	4	28	37
NR	LS	T0	94	5	1	0
		UT	98	2	0	0
		IL-21	97	3	0	0
		IL-21+co-stimuli	93	3	2	2
	2495	T0	93	7	0	0
		UT	95	5	0	0
		IL-21	95	4	1	0
		IL-21+co-stimuli	83	12	3	2
	2916	T0	89	11	0	0
		UT	95	5	0	0
		IL-21	94	6	0	0
		IL-21+co-stimuli	77	16	6	1

The morphology of CLL cells cultured for 5 days in the presence or absence of IL-21±co-stimuli. T0 = appearances before culture; UT = appearances after 5 days' culture without additional stimuli; co-stimuli = BCRxl, CD40L and IL-2. In the presence of IL-21 alone, 6-10% plasmacytoid cells were seen in those clones in which PRDM1 was induced. In contrast, few if any clear plasmacytoid cells were observed in those clones in which little or no PRDM1 was induced. In the presence of IL-21+costimuli, 33-65% of clear-cut plasma cells with intensely basophilic cytoplasm were observed. In marked contrast, in those clones in which PRDM1 was not induced, IL-21+co-stimuli induced few such cells (≤7%).

Given that the addition of co-stimuli to IL-21 treatment is known to enhance PRDM1 expression and differentiation in normal B cells[216], it next seemed important to determine whether or not these co-stimuli increase CLL-cell differentiation in the presence of IL-21.

4.3.2 Co-stimuli enhance the differentiating effect of IL-21 in R CLL clones, but PRDM1 induction in NR cells is still defective

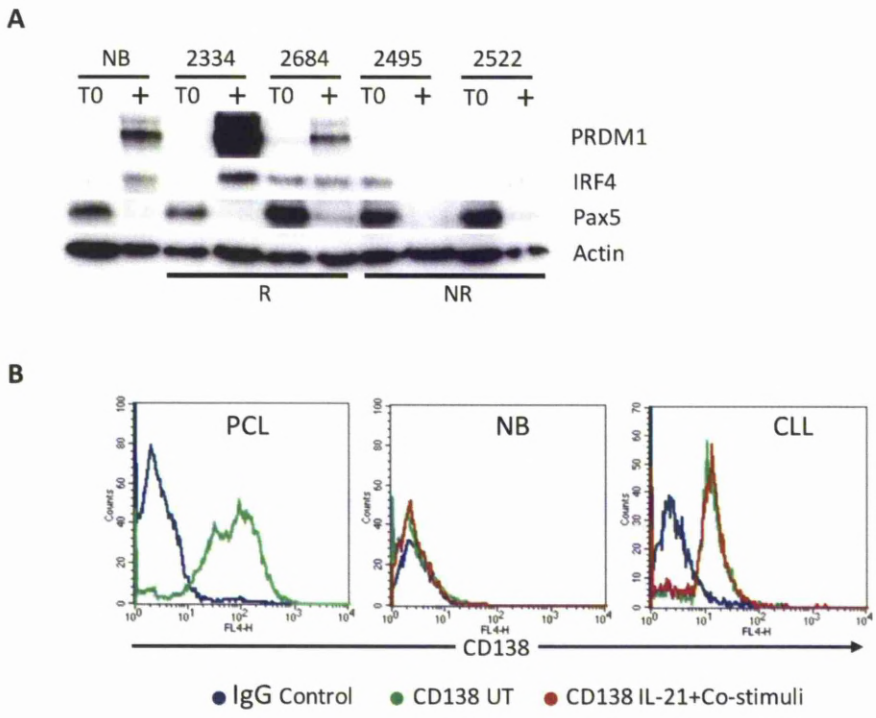
Co-stimuli that enhance the effect of IL-21 on the plasmacytoid differentiation of normal B-cells include CD40L, surface IgM BCRxl and IL-2[216]. The effect of these co-stimuli on the IL-21-induced differentiation of CLL cells was therefore examined. Also, the CD40L was removed at day 3 since this has been reported to enhance the PC-differentiating effect of the co-stimuli[122]. For comparison, purified normal B-cells were stimulated in a similar way.

R CLL cells that up-regulated their PRDM1 in the presence of IL-21 alone were studied first. When these CLL clones were cultured for up to 5 days in the presence of IL-21, together with the above co-stimuli, PRDM1 protein expression was always markedly greater than with IL-21 alone (data not shown). In normal B cells, PRDM1 induction was also increased, but usually to a lesser extent than in the R CLL clones (Fig.4.5A). Also, Pax5 expression was abolished in both CLL and normal

B cells (Fig. 4.5A). Furthermore, IRF4 expression either remained high or was variably increased in both these R CLL clones and in normal B cells exposed to IL-21 and co-stimuli (Fig. 4.5A). When the morphology of the stimulated CLL cells was assessed at 5 days, an average of 46% of cells resembled PCs, although around half of these cells were smaller than typical bone marrow LLPCs (<18μm after cytospinning; Fig. 4.4 and Table 6). However, even though these R CLL cells could be induced to express the pattern of transcription factors typical of PCs and resembled them morphologically, they lacked the surface markers of such LLPCs. (Fig. 4.5B and C). Thus, the CLL cells exposed to IL-21 and co-stimuli did not increase their expression of CD138, did not up-regulate their CD38, and lost CD27. However, their already low surface IgD and CD20 were further reduced. Similar stimulation of normal B cells also did not induce CD138 (Fig. 4.5B), but approximately 30% of cells expressed a $CD38^{high}$, $CD27^{high}$, $CD20^{low}$ and IgD^{low} surface phenotype, indicating plasmacytoid differentiation (Fig. 4.5C).

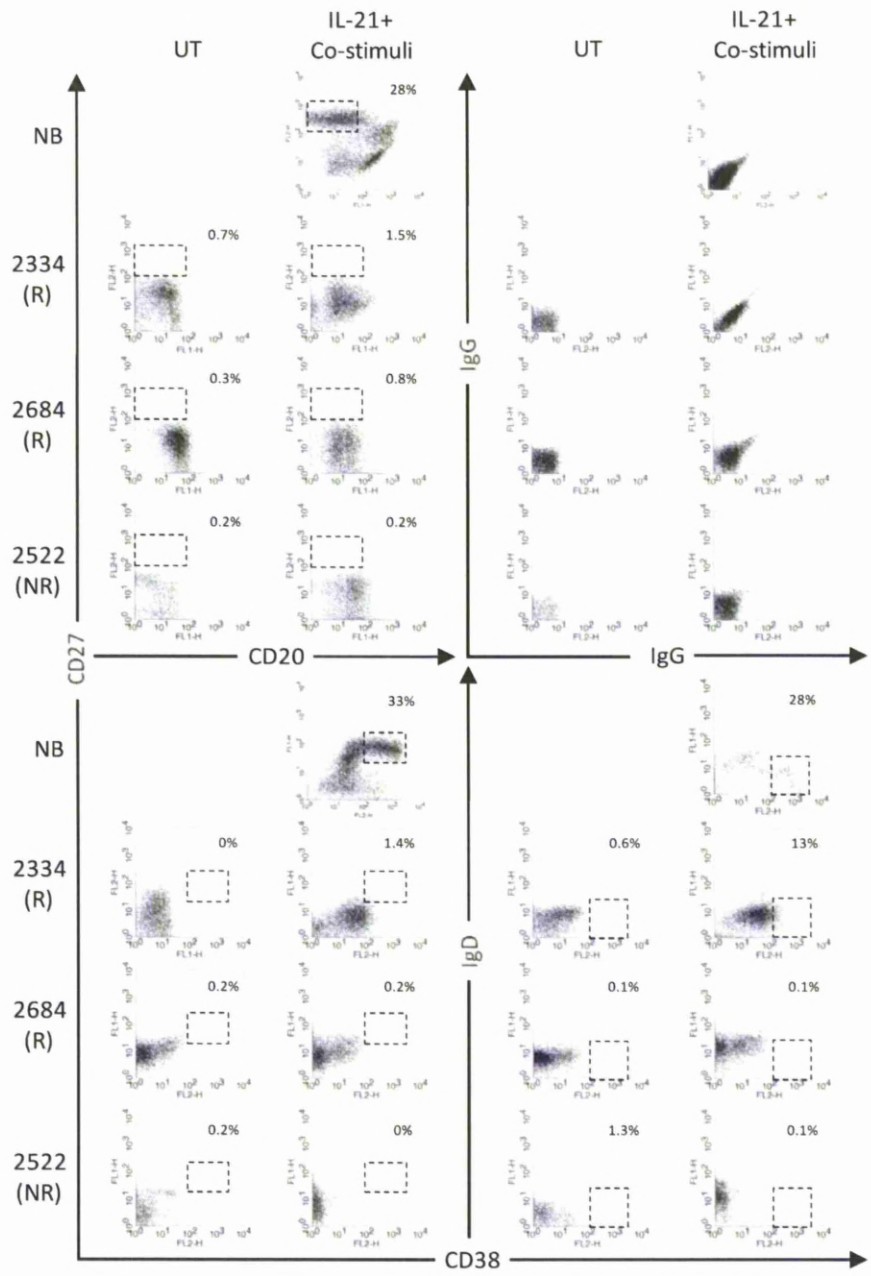
Similar co-stimulation of NR CLL clones induced little or no PRDM1 protein after 5 days' culture (Fig. 4.5A). IRF4 expression remained low or undetectable and no consistent surface-marker or morphological features of plasmacytoid differentiation were observed (Fig. 4.5A, B and C plus Fig. 4.4 and Table 6). Thus, levels of CD20, CD27, CD38 and IgD

Figure 4.5



The phenotype of CLL and normal B cells cultured with IL-21 + co-stimuli. (A) shows the expression of PRDM1, IRF4 and Pax5 before and after 5 days' culture with IL-21+CD40L+BCRxl+IL-2 in 2 R and 2 NR CLL clones and one normal B (NB) preparation **(B)** illustrates the lack of change in CD138 surface expression in CLL and NB cells cultured for 5 days with the stimuli as in (A). **(C)** (over page) shows CD27, CD38, CD20 and IgD surface expression in CLL as compared with NB cells, before and after treatment as in (A). In the upper left panel, CLL cells were doubly stained for CD20 and CD27, while reactivities with isotypic controls are shown upper right. In the lower left panel, cells were stained for CD38 and CD27, while reactivity for CD38 and IgD is displayed lower right. These results are representative of the results obtained for 6 CLL cases (n=3 R and n=3 NR).

C


UT
IL-21+
Co-stimuli
UT
IL-21+
Co-stimuli
NB
2334
(R)
2684
(R)
2522
(NR)
28%
0.7%
1.5%
0.3%
0.8%
0.2%
0.2%
IgG
CD27
CD20
IgG
33%
28%
0%
1.4%
0.6%
13%
0.2%
0.2%
0.1%
0.1%
0.2%
0%
1.3%
0.1%
IgD
CD38

were not consistently altered and CD138 was not changed (Fig. 4.5B). However, despite this lack of differentiation, Pax5 was completely lost in all these NR CLL clones (Fig. 4.5A).

In conclusion, addition of co-stimuli to IL-21 enhanced the differentiating effect of the cytokine as measured by increased PRDM1 and IRF4 expression, suppression of Pax5 and the induction of plasmacytoid morphology. However, the R CLL cells did not differentiate fully, while a proportion of normal B cells differentiated further and not only expressed PRDM1 and IRF4, but also surface markers indicative of partial differentiation towards PCs. In contrast, the NR CLL clones were still unable to differentiate even in the presence of the co-stimuli. Such stimulation did, however, extinguish Pax5 expression, indicating that down-regulation of this transcription factor is not defective in CLL. Furthermore, since Pax5 is known to repress the *prdm1* gene[138,139], this finding indicates that the failure of NR clones to express PRDM1 protein is not the result of *prdm1* repression by Pax5.

IL-21 has been reported to induce the apoptosis of some CLL clones[211,221]. For this reason, considerable care was taken to measure cell survival in most of the experiments described above. This provided

the opportunity to examine the relationship between apoptosis induction and PRDM1 expression after IL-21 stimulation.

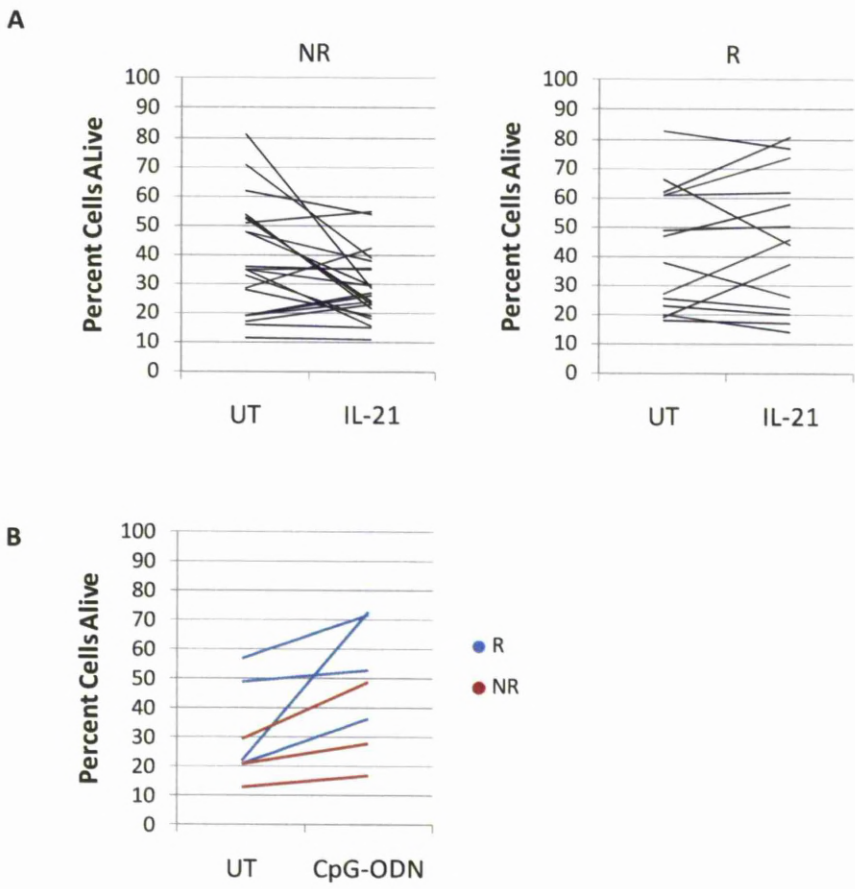
4.3.3 IL-21 has a variable effect on CLL-cell survival, but this does not correlate with PRDM1 response type

IL-21 had little or no effect on the survival of 39% of clones in which viability was measured by FACS analysis of cells stained with PI and $DiOC_6$ (n=36; examined after 48hrs; Fig. 4.6A). The cytokine produced marked apoptosis (>20%) in 33% of clones and actually enhanced survival by >20% in 28% of cases.

CpG-ODN, a stimulus used in future experiments (see below), consistently had a pro-survival effect on CLL cells at the concentration of 0.5μg/mL (Fig. 4.6B; p=0.037; paired T-test). There was no correlation between the ability to induce PRDM1 and cell survival in the presence of any of the above stimuli (Fig. 4.6A and B; p=0.153 for IL-21, p=1.0 for CpG-ODN; Mann-Whitney U test).

Having demonstrated in the previous section that IL-21±co-stimuli is unable to induce PRDM1 and PC differentiation in some CLL clones, it next was important to determine whether or not alternative differentiating stimuli acting via different signalling molecules and pathways are able to induce expression of PRDM1 in NR CLL clones. To

Figure 4.6



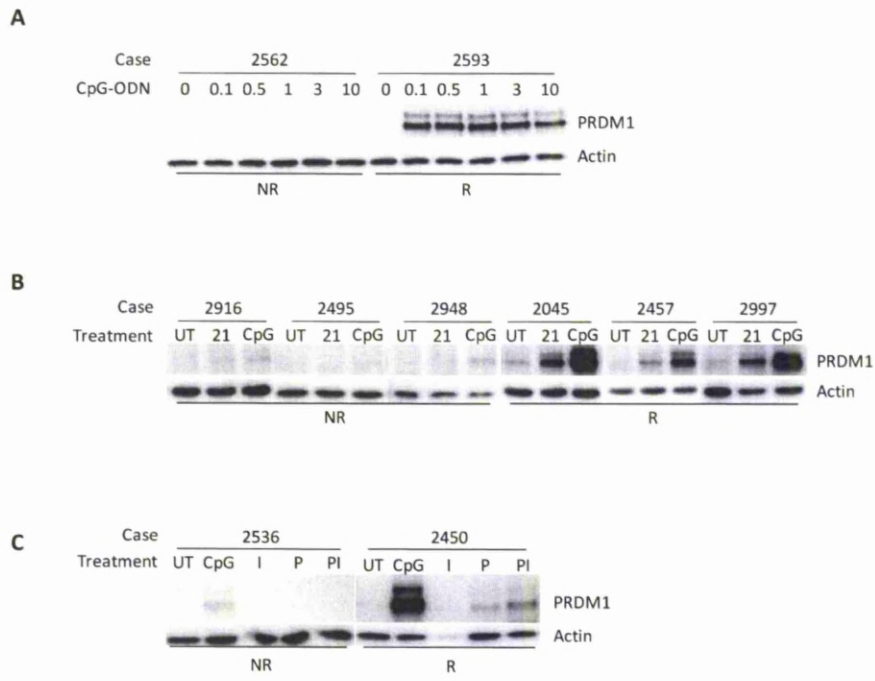
The effect of IL-21 and CpG-ODN on CLL-cell survival in R and NR clones. In **(A)**, R and NR CLL cells were treated with and without IL-21 (50ng/mL) for 48hrs and percentages of viable cells were measured by $DiOC_6$ and PI staining. **(B)** shows similar results for CpG-ODN treatment (0.5µg/mL) for 48hrs.

do this, the effects of CpG-ODN and PMA+ionomycin on PRDM1 expression were examined.

4.3.4 Both CpG-ODN and PMA plus ionomycin induce PRDM1 in those clones that are responsive to IL-21, but have little or no effect on the transcription factor in NR CLL clones

As shown in Fig. 4.7, CpG-ODN consistently induced PRDM1 in those CLL clones in which IL-21 also induced plasmacytoid differentiation; in these R clones, CpG-ODN induced a much stronger expression of PRDM1 than did IL-21 alone (Fig. 4.7B). However, in NR clones, the TLR9 ligand had little or no effect on PRDM1 expression (Fig 4.7A and B). Phorbol esters, that activate PKCs, alone or with ionomycin, are reported to be able to induce differentiation and Ig secretion in CLL and normal B cells[266,267]. Therefore, PMA, with and without ionomycin, was used to try to induce PRDM1 in R and NR clones. It was found that, PMA alone, and more so with ionomycin, was able to induce PRDM1 in CLL clones which could respond to IL-21, but was still unable to stimulate expression of the transcription factor in NR clones (n=3 NR; Fig. 4.7C). It was therefore concluded that, in NR CLL clones, multiple stimuli acting via different signalling pathways are unable to induce PRDM1. Why this might be so is the subject of the next chapter (Chapter 5).

Figure 4.7



Effect of CpG-ODN and PMA + ionomycin on PRDM1 expression by CLL cells. (A) shows the effect of increasing concentrations (μg/mL) of CpG-ODN on PRDM1 expression by an NR (2562) and an R (2593) clone. A concentration of 0.5μg/mL was used for all subsequent studies. In **(B)**, the effect on PRDM1 expression of culture with CpG-ODN (CpG) or IL-21 for 16hrs is shown for 3 NR and 3 R CLL clones. **(C)** gives a representative example of the effects of CpG-ODN (CpG), ionomycin (I), PMA (P) and PMA+ionomycin (PI) on PRDM1 expression by R and NR clones. Cells were cultured for 48hrs and similar results were obtained in 2 further R and NR clones.

Next, consideration was given to whether PRDM1 responsiveness is related to clinical behaviour, prognostic markers, or IgV_H segment usage. Clinical behaviour was measured in terms of clinical stage at the time of study; unfortunately, data for time to first treatment and survival were too incomplete for analysis. Prognostic indicators employed were surface expression of CD38 and α4 (CD49d), together with IgV_H gene mutation. IgV_H gene segment usage also seemed important since it reflects the nature of the antigenic stimulus thought to be so important in the pathogenesis of CLL[28].

4.3.5 PRDM1 responsiveness is unrelated to the clinical behaviour of the disease or to prognostic markers, but appears to reflect IgV_H gene segment usage

Here, the relationship between the induction of PRDM1 by IL-21 and the above clinical/prognostic parameters was examined (Table 5). Whether or not the cytokine was able to induce PRDM1 was assessed by densitometric analysis of Western blots in which the levels of the transcription factor were measured after culture for 16hrs (using the Western blotting chemiluminescence visualising reagent advanced ECL) or 48hrs (using the less sensitive visualising reagent ECL plus) in the presence or absence of IL-21. The densities of the PRDM1 bands in both treated and untreated CLL cells were normalised against actin and

expressed as ratios. A ratio of <1.5 was arbitrarily taken to indicate non-responsiveness (-), while ratios of >1.5 were considered responsive (+ or ++; see Table 5).

In fact, PRDM1 responsiveness was not related to clinical stage at the time of study, to IgV_H mutational status or to CD38 and CD49d surface expression. However, regarding IgV_H gene segment usage, there was a correlation (p=0.017; Fisher's exact test) between lack of PRDM1 responsiveness and expression of IgV_H1-69. Among the eight IgV_H1-69 clones examined, four had stereotypic HCDR3 sequences, but these all belonged to different subsets[39] (Fig. 4.8). As discussed later, these data taken together suggest that the nature of antigenic stimulation/selection of the CLL clone influences whether or not CLL cells retain the ability to differentiate *in vitro*. IgV_H3 gene family expression was frequently associated with PRDM1 responsiveness, but this association did not quite reach statistical significance (p=0.066; χ^2 test). As will be considered further in the Discussion of this chapter, this indicates that such clones have probably been stimulated/selected in a different way from those expressing IgV_H1-69, which are not able to up-regulate PRDM1 in response to PC-inducing stimuli.

Figure 4.8

Samples	HCDR3 sequences	CDR length	D	J	Class
Subset No. 27					
	V N1 D J				
2419	CARGTGDSSGYYYFYW	14	3-22*01	4*02	M
Subset No. 6					
	V N1 D N2 J				
2456	CARGGGYDYIWGSYRSNDAFDIW	21	3-16*02	3*02	M
Subset No. 9					
2948	CARSVGITIFGVVIRDDYYYGMDVW	23	3-3*01	6*02	M
Subset No. 7					
2495	CARDTPNYDFWSGYSRGYYYYYGMDVW	25	3-3*01	6*02	M
2741	CARQFSYESNAYYFFFW	15	3-22*01	4*02	M
2562	CATLLRYFDWPPHYYYYGMDVW	20	3-9*02	6*02	M
LS	CARVTPGRVILWSEDYGMDVW	19	2-21*01	6*02	M
2536	CARNYDFWSGYGYW	12	3-3*01	4*02	M

The stereotyped HCDR3 sequences of the IgV_H1-69 genes. The HCDR3 amino acid sequences of the eight NR IgV_H1-69 CLL clones are shown, together with details of their D_H and J_H segment usage. Four of the clones had stereotyped HCDR3 regions (shown in colour) but these belonged to different subsets. The HCDR3 region starts after a cytosine (C), and terminates before a tryptophan (W). The amino acids marked in blue belong to the IgV_H region, the red to N1, the green to D_H, the black to N2 and the brown to J_H segments.

4.4 DISCUSSION

The initial aim of this chapter was to examine the effects of IL-21±co-stimuli on CLL-cell differentiation. The study was novel because the differentiating effect of IL-21 on CLL cells has not been previously examined. Also, the effect of differentiating agents on the transcription factors that are altered during terminal B-cell maturation has not been previously investigated in the disease. Thus, previous work investigating the differentiation of CLL cells has simply used largely non-physiological stimuli and only examined Ig secretion and morphology as indicators of plasmacytoid differentiation[266,268-270].

The major findings of the work were that IL-21±co-stimuli induces PRDM1, IgM secretion and partial terminal differentiation in around 50% of CLL clones, while such stimuli had either a minor or no effect on the differentiation of the remaining 50%. Those clones that did differentiate in response to stimulation also responded to alternative differentiating stimuli in the form of CpG-ODN or PMA+ionomycin. Conversely, the NR clones showed little or no PRDM1 induction in response to these alternative stimuli.

The observation that a proportion of CLL clones are able to differentiate partially in response to IL-21±co-stimuli is of interest for three reasons.

First, it adds to previous work concerning the possible therapeutic potential of IL-21 in the disease. The induction of antibody secretion, when IL-21 is used *in vivo*, may facilitate blocking or elimination of putative (auto)antigen thought to be important in the BCR-mediated stimulation/selection of the malignant cells of CLL. It has been previously suggested that IL-21 may have therapeutic potential via the induction of the apoptosis of CLL cells[211,220,221]. In the present study, no consistent killing effect of IL-21 was observed *in vitro*, but it should be noted that the concentration employed (50ng/mL) was lower than that (200ng/mL) at which killing was observed in these previous studies.

Secondly, up-regulation of PRDM1 with IL-21 alone was greater in R CLL than in normal naive and memory B-cells; co-stimulation enhanced PRDM1 expression in both R CLL and normal B-cells. For this reason, and because CLL clones are now thought to have been activated *in vivo*, it seems likely that the greater effect of IL-21 alone on CLL cells is the result of the presence in the malignant cells of additional activated signalling pathways. The NF-κB pathway is a good candidate since this transcription factor is important in PRDM1 induction and is known to be activated more in CLL cells than unstimulated normal B cells[271].

Thirdly, although IL-21±co-stimuli induced PRDM1, IRF4 and IgM secretion, while being able to down-regulate Pax5, such stimuli were insufficient to induce either a full PC surface-marker phenotype or IgG secretion. This indicates either that further stimulation is required for full PC differentiation or that further differentiation is blocked in CLL. Recent work with normal B cells has shown that terminal differentiation is a multi-step process requiring multiple and complex stimuli[272]. It therefore seems likely that the stimuli used in the present study were insufficient to induce full PC differentiation in either CLL or normal B cells. However, the observation that a proportion of normal B cells could express some surface-marker characteristics of PCs suggests that the expression of some of these surface markers is defective even in CLL clones that are able to up-regulate PRDM1. Why this might be so remains unclear.

The failure of a range of stimuli to induce PRDM1 or significant PC differentiation in approximately 50% of CLL clones is of substantial interest and likely to be central to the pathogenesis of this subtype of the disease. Thus the failure of differentiation of such clones would explain why (auto)antigen does not stimulate the production of a neutralising antibody *in vivo*, with subsequent failure to eliminate the pathogenetic antigen. Furthermore, since PRDM1 is absolutely required

for PC formation[131], the present studies indicate that defective up-regulation of this transcription factor is responsible for the block in Ig secretion found in this subtype of the disease.

A substantial effort was made in this chapter to determine whether or not PRDM1 responsiveness is related to well established clinical/prognostic parameters, but no such correlations were observed. In particular, PRDM1 responsiveness was not related to IgV_H mutation, the most powerful prognostic indicator in CLL[9,10]. However, there was a relationship between IgV_H family usage and the ability of a given clone to up-regulate PRDM1. Thus, among the 66 clones studied, 8 expressed the *IgV_H1-69* gene and all were unable to up-regulate their PRDM1 in response to IL-21. This is a significant correlation (p=0.017) and suggests that the nature of (auto)antigenic stimulation *in vivo* maybe an important determinant of whether or not CLL-cell differentiation is blocked *in vitro*. It is not possible to predict epitope binding specificity from the Ig gene sequence[39]. However, it is known that stereotyped subsets 7 and 9 (IgV_H1-69/D_H3-3/J_H6 – expressed by CLL cases 2495 and 2948) can bind strongly to apoptotic cells[28,273]. Also, the *IgV_H1-69* gene frequently encodes for antibodies with rheumatoid factor activity[274] (IgM with anti-IgG specificity[275]), and the Ig produced by CLL cells often has such activity[276]. However, the antibodies produced by the UM *IgV_H1-*

69 gene in CLL are frequently poly-reactive[28], and it is therefore not possible, at present, to predict what specific antigens are stimulating PRDM1 non-responsiveness.

It has been shown that CLL clones expressing *IgV_H3* genes have a low frequency of HCDR3 stereotypy[39], and it was therefore suggested that such clones have been selected by superantigens which recognise framework sequences outside the HCDR3 region. It is therefore tempting to postulate that CLL clones which are able to induce PRDM1 have often been stimulated in such a way.

There are two major questions to arise out of the work in this chapter. First, why do stimulated R clones so readily express PRDM1 *in vitro*, when they do not do so *in vivo*? Secondly, why is there a block in PRDM1 induction in NR clones? The first question is considered in more detail in Chapter 6 and will not be discussed further here. The second question is the topic of the next experimental chapter, Chapter 5, but the data presented in the current chapter provide some clues. Thus, the observation that Pax5 could be silenced without PRDM1 expression indicates that the failure of these cells to express PRDM1 is not the result of a failure to down-regulate this suppressor of PRDM1. Furthermore IRF4, a known transcriptional activator of PRDM1, was

induced without PRDM1 expression. Therefore, although the present work does not provide an explanation for the failure of some CLL clones to express PRDM1, it suggests that dysregulation of either Pax5 or IRF4 is unlikely to be responsible.

With this in mind, work for Chapter 5 regarding establishing why PRDM1 is not able to be induced in NR clones was begun.

Chapter 5

THE NATURE OF THE BLOCK IN PRDM1 INDUCTION OBSERVED IN A PROPORTION OF CLL CLONES

5.1 INTRODUCTION

One of the major issues raised in the previous chapter was the question of why some CLL clones are unable to express PRDM1 and undergo plasmacytoid differentiation in response to a range of stimuli. There are no previous studies of PRDM1 induction in CLL cells, but there is a substantial literature concerning the control of this transcription factor during normal B-cell maturation to PCs. Thus, in normal B cells PRDM1 is controlled at a transcriptional level by a number of transcriptional activators and repressors[134,163]. In the light of this literature, it was likely that PRDM1 expression in CLL cells is also regulated at the transcriptional level, and this was shown to be so in the first part of this chapter. However, it has not been established what combination of

activators/repressors are essential for normal-B-cell PRDM1 induction, let alone for that of CLL cells.

The transcription factors involved in the activation/repression of PRDM1 in normal B cells are discussed in more detail in the General Introduction and the main facts are summarised in Fig. 1.3. In brief, established transcriptional activators of PRDM1 are STAT3, NF-κB, IRF4, PU.1, IRF5 and AP-1. In contrast, the well known transcriptional repressors are Pax5, Bcl-6, Spi-B and Bach2. Each of these transcription factors is activated/induced by different stimuli involving distinct signalling pathways. For example, IL-21 induces B-cell differentiation mainly via JAK phosphorylation of STAT3[213], while CpG-ODN activates a number of signalling pathways, including those leading to NF-κB and IRF5 activation[231]. PMA and ionomycin also stimulate multiple pathways including those that lead to NFAT activation and IRF4 induction. For further details, see Section 1.5.2.

The stimuli employed in Chapter 4 were IL-21, BCRxl, CD40L, IL-2, CpG-ODN, PMA and ionomycin. Between them, these agents probably activate all the signalling pathways known to be required for PRDM1 induction/relief from repression. Therefore, the fact that these multiple stimuli failed to induce PRDM1 in a proportion of CLL clones could be

explained in a number of ways. First, one or more of the signalling pathways/activating transcription factors essential for PRDM1 induction might be altered/defective in these NR clones. Alternatively, it remains possible that the *prdm1* gene is silenced by a transcriptional repressor(s) and/or by other mechanisms including DNA methylation[92] and histone modification. Whatever the underlying reason for the failure of NR clones to induce PRDM1, it seemed reasonable to postulate that the same shared mechanism might be responsible for the inability of the above stimuli to induce PRDM1/plasmacytoid differentiation in the majority of NR CLL clones.

After showing that the failure to induce PRDM1 occurs at the level of transcription, the aims of the present chapter, therefore, were to characterise the transcriptional activators/repressors required for PRDM1 induction in R CLL cells, and to establish why PRDM1 transcription is not activated in NR clones.

5.2 METHODS

Once again, only Materials and Methods new to this thesis will be described.

5.2.1 Inhibitors

The cell-culture inhibitors used during this chapter were:- NFAT inhibitor cyclosporin A at 100nM; NF-κB inhibitor Bay11-7082 at 0.5-2μM; and STAT3 inhibitor VIII at 20-100μM (all from Calbiochem).

5.2.2 PCR

The *prdm1* bisulphite sequencing primers were:- *prdm1* BS1 forward 5'-TTT TTG TAT TTG GGG ATT TGA GTT GAG-3', reverse 5'-AAC TTC CCC TCC CTA CTT AAA ATT TCC-3'; *prdm1* BS2 forward 5'-AGT GGT TAA GGA AAT TTT AAG TAG G-3', reverse 5'-ACA AAT ATC CAA CAT CTA AAA AAA ATC-3'.

5.2.3 qPCR

PRDM1 mRNA primers were the same as in Chapter 3. TNFα mRNA primers were:- forward 5'-CCA TGA GCA CTG AAA GCA TGA TCC-3', reverse 5'-TGG TTA TCT CTC AGC TCC ACG C-3'. IκBα mRNA primers were:- forward 5'-AGC TCA CCG AGG ACG GGG AC-3', reverse 5'-TCC

ACG ATG CCC AGG TAG CCA-3'. Spi-B mRNA primers were:- forward 5'-CTC CTC CAA GCA CAA GGA AC-3', reverse 5'-GGA CGC CCT TTT TCT TCC AG-3'.

The *prdm1* ChIP primers were:- IκBα promoter forward 5'-CTC TTT TTC TGG TCT GAC TGG C-3', reverse 5'-GCG CCC TAT AAA CGC TGG-3'; *prdm1* NF-κB binding site forward 5'-TTG AGG TTA AGT GCC TTC AAA GG-3', reverse 5'-TGG CCT CTC CGC AAC ACT G-3'; *prdm1* TSS forward 5'-CTC AGC CTG GCG GGG GAC-3', reverse 5'-CCT TAC CAA GGT CGT ACC CAC ACG-3'; *prdm1* +14kbp forward 5'-GGG CTA TAA AAG CAT CAG GAC-3', reverse 5'-GAG GGA AAA GAA CTG CCA C-3'.

5.2.4 IL-21R quantification

Surface expression of the IL-21R on CLL cells was measured using a two-layer flow cytometry method. Binding of each layer was performed in the same buffer, for the same amount of time, and at the same antibody concentrations as described in Section 2.2.4. Cells were washed in the antibody-binding buffer after each staining step. The primary antibody was a goat polyclonal anti-IL-21R IgG (cat# AF991; R&D systems). The secondary antibody was a donkey anti-goat conjugated to Alexa fluor 555 (Invitrogen Ltd).

5.2.5 Western blotting

Additional primary antibodies used in this chapter were:- rabbit polyclonal anti-STAT3 clone C-20 (Santa Cruz); mouse monoclonal anti-pSTAT3 clone B-7 (Santa Cruz); rabbit polyclonal anti-PU.1 cat# 2266 (Cell Signalling; New England Biolabs); rabbit polyclonal anti-Bcl-6 cat# 4242 (Cell Signalling); goat polyclonal anti-Bach2 clone E-16 (Santa Cruz); and mouse monoclonal anti-Tcl-1 clone 1-21 (Biolegend UK Ltd., Cambridge, UK).

5.2.6 NF-κB activation

NF-κB activity was measured using the TransAM NF-κB p50 and p65 ELISA kits (Active Motif, Rixensart, Belgium) and a modified nuclear extraction method. CLL nuclear pellets were obtained by lysing 10^7 CLL cells for 5mins on ice in PBS pH7.4 containing 0.5% v/v NP-40, 2mM EDTA and a protease inhibitor cocktail (Calbiochem), followed by centrifugation at 500rcf. 2μg of nuclear lysate was added to each ELISA well and each sample was assayed in duplicate.

5.2.7 ChIP

Immunoprecipitating antibodies used were:- mouse anti-p65; mouse anti-K36me3; and mouse anti-Ser5P-RPII (all from Abcam).

5.3 RESULTS

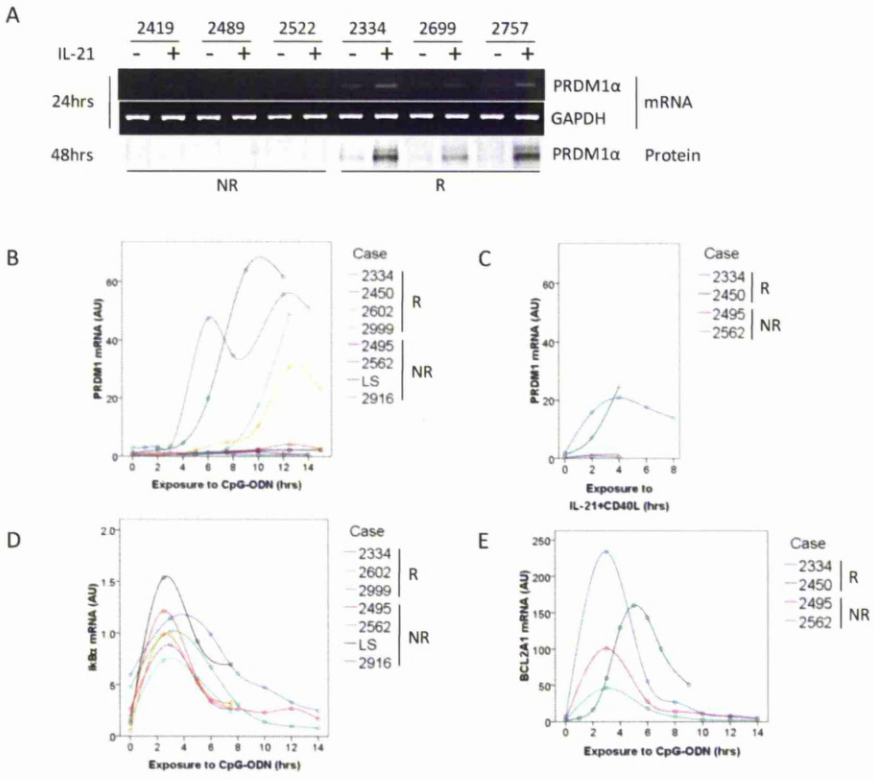
5.3.1 In NR CLL clones both IL-21 and CpG-ODN fail to induce transcription of *prdm1*

NR clones were cultured with and without IL-21 stimulation for 24 hours, and PRDM1 mRNA induction compared with that in R CLL cells treated in a similar way. For these studies, responsiveness was defined as in Chapter 4.

Using a semi-quantitative PCR method, it was found that basal levels of PRDM1 mRNA were low or undetectable in NR clones and that IL-21 induced only a very small increase in transcription (Fig. 5.1A). Before stimulation, R clones also contained only low levels of PRDM1 mRNA but, after exposure to IL-21 for 24hrs, there was a large increase of this message (Fig. 5.1A).

Similar studies, this time using a qPCR method and measuring PRDM1 mRNA induction at multiple time points after stimulation with IL-21+CD40L or CpG-ODN, confirmed the lack of transcription of PRDM1 in NR clones (Fig.5.1B and C). It was also noticed that, after exposure to CpG-ODN the induction of PRDM1 mRNA occurred only after 4 hours, while PRDM1 was induced immediately after IL-21+CD40L stimulation. However, the delay in PRDM1 induction by CpG-ODN was not shared by

Figure 5.1



The induction of PRDM1 by IL-21 or CpG-ODN in NR and R CLL clones. **(A)** shows RT-PCR analysis of PRDM1α mRNA expression after culture for 24hrs with and without IL-21. GAPDH was used as a loading control. Protein levels after 48hrs are shown for comparison. In **(B)** and **(C)**, the kinetics of induction of PRDM1α mRNA as measured by qPCR after treatment with CpG-ODN or IL-21+CD40L are shown respectively. **(D)** and **(E)** give similar studies of the kinetics of mRNA induction after CpG-ODN treatment for IκBα and BCL2A1 respectively – two genes well known to be transcribed early after stimulation of the NF-κB pathway. All mRNA levels are expressed relative to GAPDH mRNA.

other genes which are directly activated by NF-κB (IκBα and BCL2A1; Fig. 5.1D and E).

Taken together, these results show that PRDM1 protein expression in CLL cells is controlled at a transcriptional level. They also indicate that all the factors required to induce PRDM1 in CLL cells are immediately present after IL-21+CD40L stimulation, while CpG-ODN requires the neosynthesis of an additional factor.

Because the precise combination of transcription factors needed for PRDM1 induction remains unknown[163], it next seemed important to establish what factors are important for this process in CLL cells. IL-21 is known to induce transcription of PRDM1 primarily through STAT3, so this activator of transcription was examined first. IL-21 acts on specific receptors which then cause tyrosine phosphorylation of STAT3, leading to its dimerisation and translocation to the nucleus where it activates PRDM1[167]. In contrast, CpG-ODN-induced signalling from TLR9 has not been shown to directly involve activation of STAT3[230]. However, since there was a delay in the induction of PRDM1 mRNA following CpG-ODN treatment (see above), it seemed plausible that a neofactor might be activating STAT3 and that this is required for PRDM1 transcription.

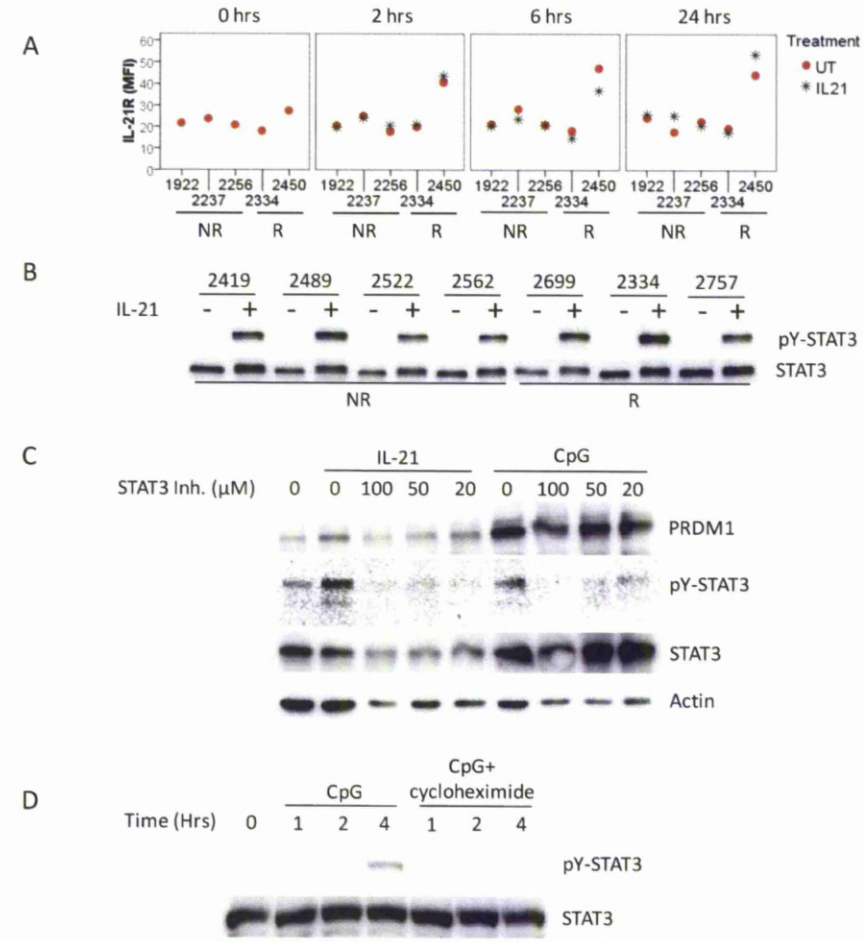
Therefore, STAT3 activation and its requirement for PRDM1 induction in response to IL-21 and CpG-ODN were compared in R and NR clones.

5.3.2 STAT3 activation is intact in R and NR CLL clones and is required for the induction of PRDM1 by IL-21, but not by CpG-ODN

To examine whether or not receptor levels might be influencing induction of PRDM1 by IL-21, IL-21R levels were measured by a two-layer FACS method. Expression was similar in R versus NR clones (Fig.5.2A) tested directly *ex vivo,* and receptor expression remained constant in 4/5 cases for up to 24hrs with and without IL-21 (Fig. 5.2A). One R clone (2450) did increase its IL-21R levels by ~30% within the first two hrs of culture. However, all CLL clones expressed IL-21R and levels in a different R clone remained very similar to that of the 3 NR clones studied. Therefore, the differences in the expression of PRDM1 in response to IL-21 in R and NR clones could not be attributed to differential expression of the IL-21R.

IL-21 induces PRDM1 transcription primarily via phosphorylation of STAT3 on Tyr705[163]. Therefore Western blotting with an anti-Tyr705-STAT3 antibody was employed to measure STAT3 activation after exposure to the cytokine. For all clones tested (n=7), IL-21 induced the tyrosine phosphorylation of STAT3, and both R (n=3) and NR (n=4)

Figure 5.2



The effect of IL-21 or CpG-ODN on the STAT3 pathway in R and NR CLL clones. (A) shows surface IL-21R levels on CLL cells as measured by FACS analysis, either before or after treatment with or without IL-21 for the indicated time. For **(B)**, cells were cultured for 20mins with and without IL-21 and extracts were Western blotted for phospho-Tyr^{705} of STAT3. **(C)** shows the effect of an inhibitor of STAT3 (STAT3 Inhibitor VIII; this agent prevents dimerisation of STAT3 with subsequent inhibition of its translocation to the nucleus) on PRDM1 induction after culture (16hrs) with IL-21 or CpG-ODN. In **(D)**, the effect of an inhibitor of protein synthesis (cycloheximide) on the induction of pY-STAT3 by CpG-ODN was analysed over the first 4hrs of culture.

CLL clones had comparable levels of phosphorylation after exposure to the cytokine for 20mins (Fig. 5.2B). Furthermore, in R CLL, an inhibitor of the dimerisation of STAT3 stopped PRDM1 induction on exposure to IL-21 (Fig. 5.2C).

Upon treatment with CpG-ODN, STAT3 was shown to be tyrosine phosphorylated only after 4 hours (Fig. 5.2D). This phosphorylation was shown to rely on protein neosynthesis, as it was blocked by the translational inhibitor cycloheximide (Fig. 5.2D). However, in contrast to its effect on IL-21 stimulation, the same STAT3 inhibitor had little or no effect on CpG-ODN-induced PRDM1 (Fig. 5.2C).

Taken together these results indicate that, in CLL cells, STAT3 signalling is required for the induction of PRDM1 by IL-21 and that signalling between the receptor and STAT3 is intact in these cells, regardless of whether or not the cytokine induces PRDM1 expression. In contrast, the induction of PRDM1 by CpG-ODN was shown not to require STAT3 phosphorylation and the neofactor involved is not dependent on stimulation of the STAT3 pathway.

As alluded to above, the running hypothesis was that a shared mechanism is responsible for the failure of a range of stimuli to induce PRDM1 expression in NR CLL clones. Therefore, since STAT3 was shown

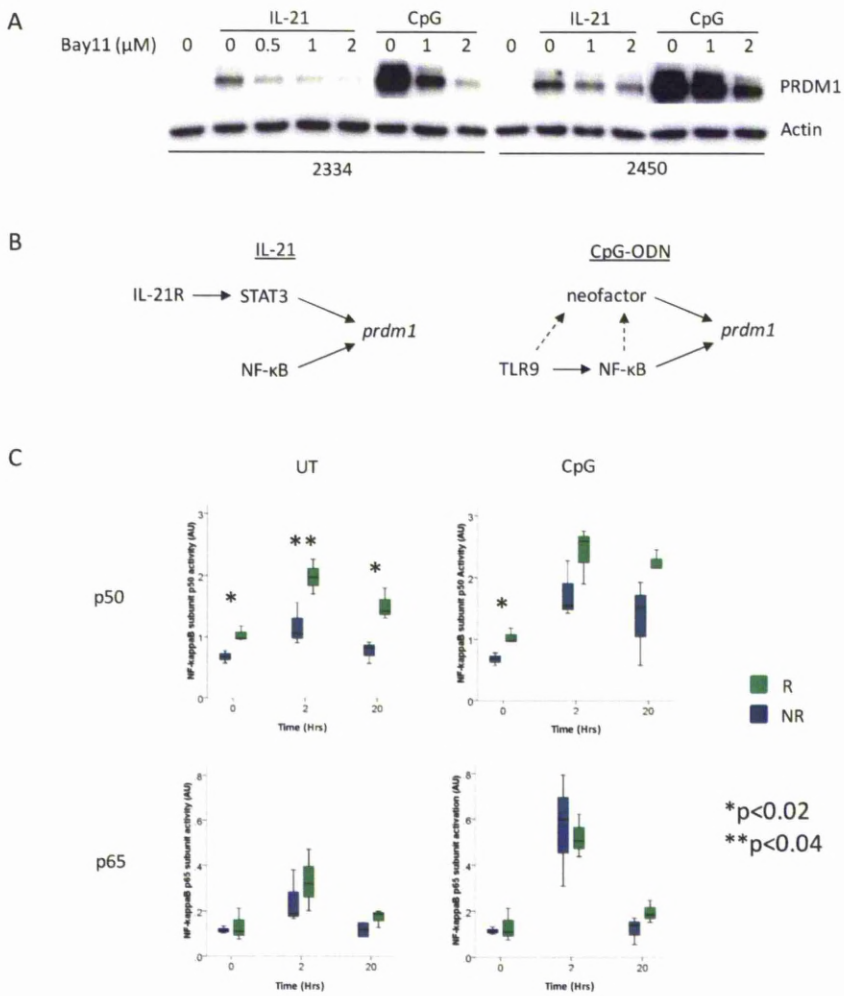
not to be required for PRDM1 induction by CpG-ODN, it was decided at this stage not to investigate STAT3 further. Rather, it next seemed important to examine the function of other transcription factors known to be involved in the induction of PRDM1.

Since NF-κB activation is important in the induction of PRDM1 in normal B cells[109,163], this transcription factor and its activation were examined next.

5.3.3 NF-κB activation is intact in both R and NR CLL clones

NF-κB is known to be constitutively activated in CLL cells[271], so it is possible that this activation contributed to the effects observed in the presence of IL-21 alone. To examine the importance of constitutive and induced NF-κB activation for PRDM1 induction in CLL cells, R clones were cultured with IL-21 or CpG-ODN in the presence or absence of the NF-κB inhibitor, Bay11-7082 (Fig. 5.3A). The inhibition of NF-κB activity greatly reduced induction of PRDM1 by both CpG-ODN and IL-21 stimulation in the two R clones tested. It was therefore concluded that NF-κB contributes to the induction of PRDM1 not only by CpG-ODN, but also by IL-21 alone. Furthermore, taken together with the STAT3 results, these findings indicate that the induction of PRDM1 by IL-21 requires both STAT3 and NF-κB activation. In contrast, the induction of PRDM1 by

Figure 5.3



The role of NF-κB in the induction of PRDM1 by both IL-21 and CpG-ODN. (A) shows the effect of an NF-κB inhibitor (Bay11-7082; prevents IκBα phosphorylation and degradation, thereby preventing the release of NF-κB from inhibition) on the induction of PRDM1 by IL-21 or CpG-ODN in R clones. **(B)** gives a diagrammatic representation of how IL-21 and CpG differ in how they induce PRDM1. Since it remains unclear whether the neofactor is induced by NF-κB or by some other mechanism, this uncertainty is represented by broken arrows. In **(C)**, active nuclear p50 and p65/RelA were measured in R and NR clones before and after treatment with CpG for the indicated times (n=3 for each type of clone; statistical significance was assessed using a Mann-Whitney U test).

CpG-ODN requires NF-κB and an additional neofactor, which demonstrably does not require STAT3 activation (see Fig. 5.3B).

Having established the need for NF-κB in PRDM1 induction by both stimuli, it next was important to establish whether or not the activation of this transcription factor is impaired in NR clones. By using the TransAM NF-κB ELISA method (that measures only functionally active nuclear NF-κB), both basal and CpG-ODN-induced p65/RelA and p50 NF-κB subunit activities were measured (Fig. 5.3C). These subunits of NF-κB are thought to be important in the activation of the *prdm1* gene through binding to a conserved NF-κB binding motif close to the TSS[109]. Similar levels of activated nuclear p65/RelA were demonstrated in both R and NR CLL clones, whether analysed unstimulated or after exposure to CpG-ODN for 2 or 20hrs. It was therefore concluded that the p65/RelA is activated and translocated to the nucleus in both R and NR clones.

Interestingly, unstimulated p50 activity was significantly higher in R clones than NR clones at all three time points (Fig. 5.3C). However, the p50 subunit was still basally activated in NR clones and the fold induction of p50 activity after CpG-ODN stimulation was similar between response types (Fig. 5.3C). Consequently, it seemed unlikely that the lower activity of the p50 subunit in NR CLL clones was responsible for

the failure to induce PRDM1. Also, other NF-κB responsive genes (Fig. 5.1C and D; IκBα and BCL2A1) were readily induced in NR clones. Taken together, these data indicate that impaired NF-κB activation is not responsible for the absence of PRDM1 induction in NR clones.

Although NF-κB activation was shown to be intact in both R and NR clones following TLR9 stimulation, it remained possible that binding of the transcription factor to the *prdm1* promoter was somehow impaired in NR clones. Therefore, attempts were made to analyse such binding by ChIP. For this analysis, the DNA encoding for an NF-κB binding site upstream of the TSS was amplified by qPCR after ChIP using an antibody against the transcriptional-activating p65 subunit of NF-κB. However, repeated attempts to perform ChIP analysis with the anti-p65 antibody could not demonstrate binding to the *prdm1* gene in either type of CLL clone even after CpG-ODN stimulation (see Appendix for figure). In contrast, the positive control gene, *IκBα*, could readily be precipitated using the anti-p65 antibody. It is difficult to know how to interpret these findings since it has been shown in mice that p65 binding to the conserved NF-κB-binding motif of the *blimp-1* gene is critical to up-regulation of transcription of the gene in response to TLR signalling[109].

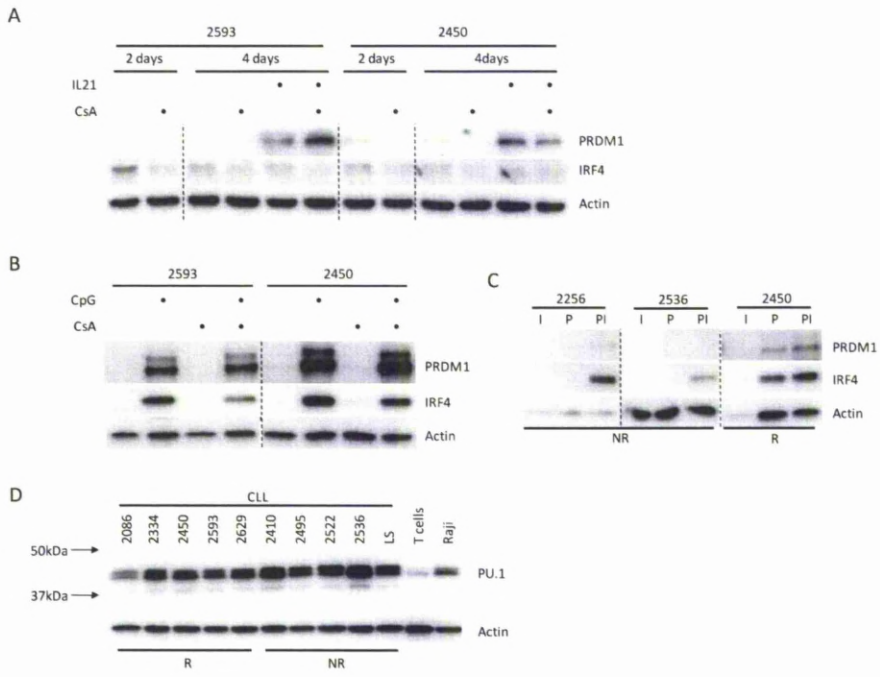
Having taken the work concerning NF-κB activation as far as practicable, attention was next turned to the other activators/repressors of PRDM1, starting with NFAT/IRF4 and PU.1.

5.3.4 The failure to induce PRDM1 in NR clones is not the result of defective NFAT/IRF4 function or of absent PU.1 expression

The transcription factor, NFAT, is activated by dephosphorylation via the phosphatase, calcineurin[277]. After activation, NFAT is translocated to the nucleus where it promotes IRF4 induction[164-166] and this factor, in turn, binds to the PRDM1 gene and promotes the induction of PRDM1 in certain settings[167,168].

To determine whether or not NFAT is required for the induction of PRDM1 in CLL cells, R clones were cultured with CpG-ODN or IL-21 in the presence of cyclosporin A (CsA). The inhibition of calcineurin by CsA prevents the activation and nuclear translocation of NFAT. As expected, pre-incubation of CLL cells with CsA reduced IRF4 expression after treatment with and without IL-21 or CpG-ODN (Figure 5.4A and B). However, the inhibition of NFAT had a variable effect on the induction of PRDM1 by IL-21; in one R CLL clone expression was enhanced, while in a different R clone slightly reduced levels were observed (Fig. 5.4A). In contrast, treatment with CsA consistently had no effect on CpG-ODN-

Figure 5.4



The NFAT/IRF4 pathway in variably stimulated R and NR clones. In **(A)**, the effect of the calcineurin inhibitor, cyclosporin A (CsA) on PRDM1 expression by two R CLL clones stimulated with IL-21 is shown. Cells were preincubated for 2 days with CsA (200nM) to reduce IRF4 expression, before culture for a further 2 days in the presence of the cytokine. In **(B)**, two R clones were preincubated for 1hr in the presence of CsA before culture for 48hrs with CsA + CpG-ODN. For **(C)**, the effects of ionomycin (I; 1μM) or PMA (P; 50nM) alone and of PMA+ionomycin (PI) on the induction of PRDM1 and IRF4 were examined after 2 days' culture.

induced PRDM1 (Fig.5.4B). Also, in NR clones, IRF4 was induced by PMA plus ionomycin (but not by either agent alone), but PRDM1 was not (Fig. 5.4C). It was therefore concluded that calcineurin/NFAT/IRF4 activation/expression is not essential for PRDM1 induction in R clones by both stimuli, and that a defect in this pathway is therefore unlikely to be the cause of the block in NR clones.

Since PU.1 is known to be required for efficient PRDM1 induction[174] and to be variably transcribed in CLL[278], the levels of this transcription factor were measured in R and NR CLL clones by Western blotting. In fact, the protein was expressed at comparably high levels (Fig. 5.4D) in both types of clone. It was therefore concluded that differential expression of PU.1 is not responsible for the failure of some clones to induce PRDM1.

We next considered IRF5 and AP-1, the other transcriptional activators known to be involved in the induction of PRDM1[163].

5.3.5 The absence of PRDM1 induction in NR CLL clones is unlikely to be the result of defective function of either IRF5 or AP-1

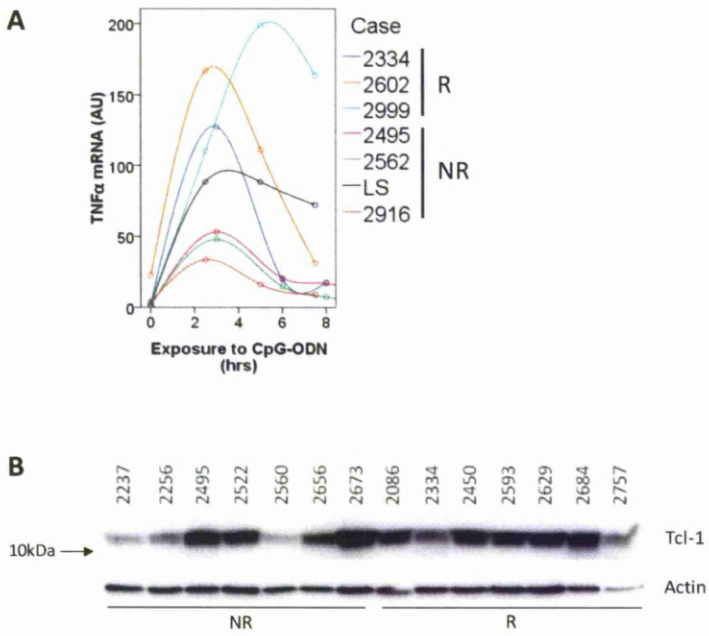
IRF5 is activated by TLR stimulation and, in addition to activating PRDM1, is essential for transcription of the genes encoding pro-inflammatory cytokines, such as TNFα[279]. It has recently been shown that IRF5 can be mutated in some CLL cells in a way that affects its ability

to bind to DNA and activate gene expression[280]. Therefore, the transcription of the TNFα gene was used as a functional readout of IRF5 activity in R and NR CLL clones after CpG-ODN stimulation.

In fact, although CpG-ODN induced more TNFα mRNA in the 3 R clones studied, all 4 NR clones could transcribe this factor (Fig. 5.5A). It was therefore concluded that impaired IRF5 function is unlikely to be responsible for the failure to up-regulate PRDM1 expression in NR clones.

Regarding AP-1, it has been reported that, although the transcription factor can potentiate PRDM1 transcription[150,175], knockout of c-fos (an essential component of AP-1) does not abrogate PRDM1 expression[175]. For this reason, it seemed unlikely that the failure of NR clones to induce any PRDM1 could be attributed to altered activation of AP-1. Nevertheless, in view of the current interest in the AP-1 repressor, Tcl-1, as a pathogenetic factor in CLL[281], it seemed reasonable to consider the possibility that high levels of Tcl-1 might be indirectly inhibiting *prdm1* transcription in NR CLL cells. However, Tcl-1 levels, although variable, were similar in R and NR clones (Fig. 5.5B). It was therefore decided not to investigate AP-1 further.

Figure 5.5



IRF5 function and Tcl-1 expression in R and NR clones. In **(A)**, the induction of TNFα mRNA was employed as a surrogate of IRF5 action. R and NR clones were cultured with CpG-ODN for up to 8hrs and TNFα mRNA was measured by qPCR and expressed as a ratio to the control mRNA, GAPDH. In **(B)**, expression of Tcl-1 protein was measured by Western blotting in R and NR clones directly after thawing.

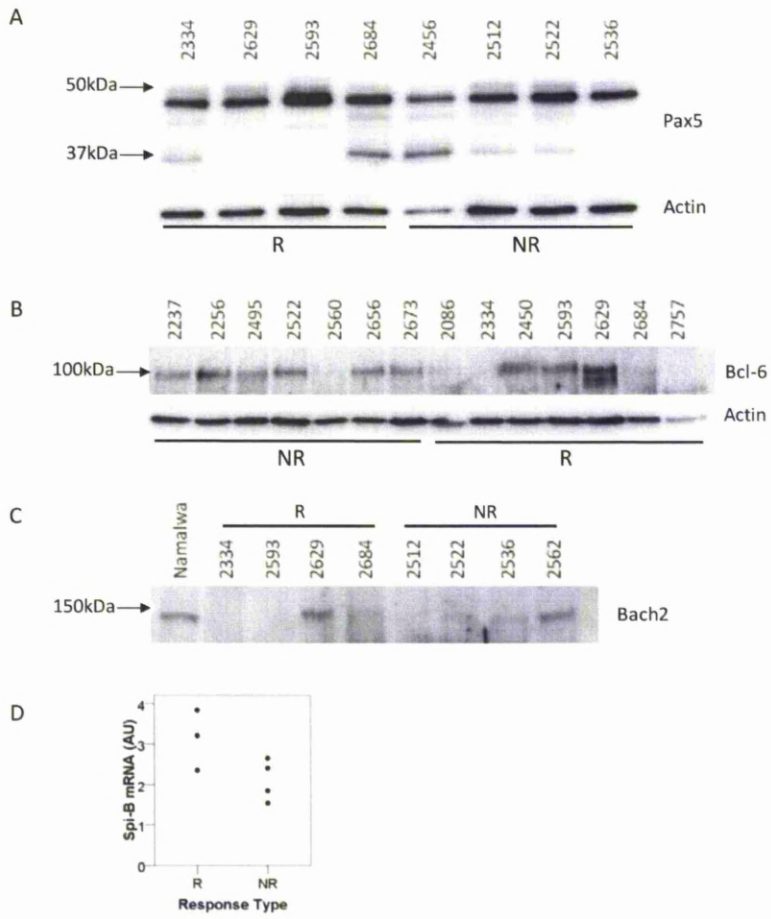
It was next hypothesised that the *prdm1* gene might be repressed in those CLL clones which do not express PRDM1 mRNA and protein in response to multiple stimuli.

5.3.6 Failure to induce PRDM1 in NR clones is not attributable to the differential expression of repressors of PRDM1

Four transcription factors are known to suppress *prdm1* directly; these are Pax5, Bcl-6, Bach2 and Spi-B[134]. As shown in Fig. 5.6A, Pax5 levels were similar in R and NR CLL clones directly *ex vivo*. Furthermore, IL-21 plus co-stimuli caused the progressive loss of Pax5, without PRDM1 induction in NR CLL clones (see Fig. 4.5A from earlier). It was therefore concluded that Pax5 is unlikely to be responsible for any putative differential repression of the *prdm1* gene in NR versus R CLL clones.

CLL cells are reported to express Bcl-6 protein at very low levels detectable by Western blotting[282], but not demonstrable by immuno-histochemistry[283]. This was confirmed here, since the advanced ECL reagent and long exposure times were necessary to demonstrate small amounts of the protein in Western blots; however, when the protein was demonstrable, similar amounts of protein were observed in R versus NR clones (Fig. 5.6B). It therefore seemed unlikely that this transcription factor is involved in any putative *prdm1* repression. Regarding Bach2,

Figure 5.6



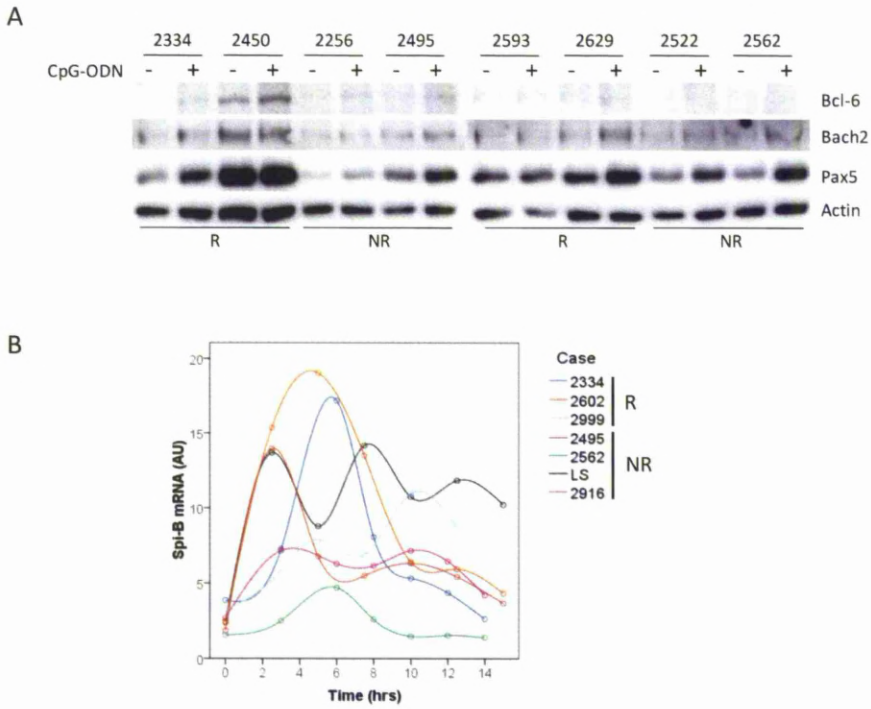
Levels of the transcriptional repressors of *prdm1* in untreated R and NR clones at T0. (A) shows a Western blot for Pax5. The upper band (~48kDa) is the expected molecular weight of Pax5, while the 37kDa band is probably a truncated isoform of the transcription factor[284]. In **(B)** and **(C),** levels of Bcl-6 and Bach2 respectively were measured by Western blotting. For **(D)**, Spi-B mRNA levels were quantified by qPCR (n=3 R and 4 NR clones) and analysed relative to levels of a GAPDH mRNA control.

little or no expression was detected by Western blotting in 6 (3 R and 3 NR) of 8 CLL clones examined, while a clear band was detectable in 2/8 clones (1 R and 1 NR) and the Namalwa cell line positive control (Fig. 5.6C). Surprisingly, when levels of Spi-B mRNA were measured (as a specific antibody could not be found), higher levels of mRNA were found in R clones than NR clones (Fig. 5.6D), although this result failed to reach significance ($p=0.08$). It therefore also seemed unlikely that repression by either Bach2 or Spi-B could be responsible for the failure to induce PRDM1 in NR clones.

Levels of Pax5, Bcl-6 and Bach2 repressors were also measured after 48hr stimulation with CpG. Furthermore, Spi-B mRNA levels were measured at multiple time points over the first 14hrs after CpG-ODN treatment. Bcl-6 and Bach2 levels were largely unchanged in both R and NR clones (Fig. 5.7A). CpG-ODN actually increased both Pax5 and Spi-B expression, but these increases were similar in NR and R clones (Fig. 5.7A and B). It was therefore concluded that differential expression of known repressors of PRDM1 transcription is not causing repression of the gene in the NR CLL clones.

Although it remained possible that differential binding of similarly expressed activators/repressors might explain why some clones can

Figure 5.7



The levels of the transcriptional repressors of *prdm1* in R and NR clones after treatment with CpG-ODN. In **(A)**, CLL cells were treated with and without CpG-ODN for 48hrs, and levels of Bcl-6, Bach2 and Pax5 were measured by Western blotting. **(B)** shows levels of Spi-B mRNA in 3 R and 4 NR clones over the first 14hrs of culture with CpG-ODN relative to levels of GAPDH mRNA.

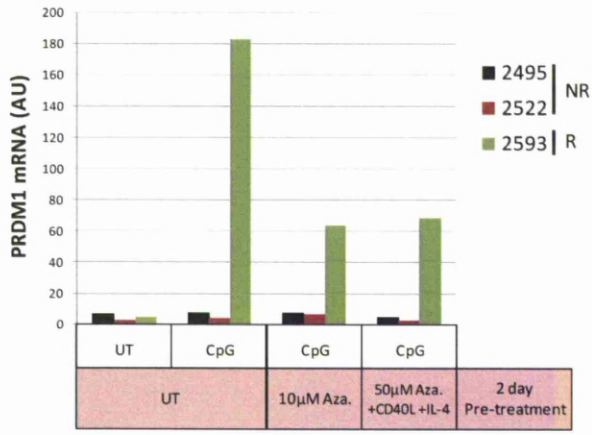
transcribe the *prdm1* gene while others cannot, it was decided that possible silencing by DNA methylation would be examined next. This seemed a reasonable approach since the methodology was already set up from Chapter 2 and since it has very recently been reported that the *prdm1* gene can be regulated by CpG methylation in NK cell lines and primary tumours; furthermore a DNA methyltransferase inhibitor was able to induce PRDM1 mRNA in these cells[92].

5.3.7 The PRDM1 gene is not methylated in either R or NR CLL clones

To test the hypothesis that PRDM1 is repressed in CLL cells by methylation, CLL cells were treated with the methyltransferase inhibitor 5-azacytidine for 2 days, and then the ability to induce PRDM1 by CpG-ODN stimulation was measured by qPCR. To enhance the effects of 5-azacytidine and to prevent apoptosis of the CLL cells during culture with high concentrations (50μM) of the methyltransferase inhibitor (data not shown), CD40L and IL-4 were added to the cell culture medium. This T-cell stimulus has been shown to induce survival and mitosis of CLL cells[285], the latter of which enables incorporation of non-methylated cytosines in the DNA of the daughter cells[286]. As shown in Fig. 5.8A, pre-treatment with 5-azacytidine was unable to cause CpG-ODN-stimulated induction of PRDM1 mRNA in NR cases, even at the high concentration of 50μM.

Figure 5.8

A



B

gtaagatttgtgtcttttgtacctggggatttgagctgagaaatcagaaactgtgtaggtaaat
tttaagtttccttaatttaaggaacgtgcgcccccctaattctgccgcgccaggaaggagggcg
atctggagtgtttagaatacaatagagcccaagtaagcgttgaggttaagtgccttcaaagg
gaagtaagaagattccaagtcaatgttgaaatacacatgcgaagagaggaagctctcggcg
gctgtgctagcaatctgggggaaagccctgggctcggccaggtggtgttggccacgttggcc
acgcccccacttcgcgcagccgagtggctaaggaaatcttaagcagggaggggaagccag
acggttaacacagacaaagtgctgccgtgacactcggccctccagtgttgcggagaggcaa
gagcagcgaccgcggcacctgtccgcccggagctgggacgcgggcgcccgggcggccgg
acgaagcgaggagggaccgccgaggtgcgcgtctgtgcggctcagcctggcgggggacgc
ggggagaatgtggactgggtagagatgaacgagacttttctcagatgttggatatttgcttgg
aaaaa

CpG methylation of the *prdm1* gene in R and NR CLL clones. For **(A)**, CLL cells were cultured for 48hrs with or without 10μM 5-azacytidine alone or 50μM 5-azacytidine in the presence of soluble CD40L and IL-4. After such culture, cells were washed and re-cultured for a further 24hrs in the presence of CpG-ODN, after which PRDM1 mRNA was measured by qPCR and made relative to GAPDH mRNA. **(B)** shows the layout of the *prdm1* gene in the region around the TSS (black arrow). The 41 CpG motifs analysed are shown in red, while the green and blue arrows indicate the location of the two primer pairs used to amplify the region for sequencing *prdm1* after bisulphite treatment.

In an R CLL clone, treated in a similar way and used for comparison, PRDM1 induction was actually reduced in response to CpG-ODN after 5-azacytidine pre-treatment (Fig.5.8A).

As repression of DNA methylation by 5-azacytidine affects the expression of many genes, including potentially those involved in PRDM1 repression, the alternative approach of direct bisulphite sequencing of the *prdm1* gene was employed. Primers were designed to determine the sequence and hence methylation status of the TSS of the *prdm1* gene, where methylation has been shown to correlate with the repression of the *prdm1* gene in NK cell lines and primary tumour cells[92]. Using this method, it was shown that the *prdm1* gene was completely unmethylated in this region (Fig. 5.8B; 0/41 methylated CpG motifs in n=3 NR). It was therefore concluded that CpG methylation is not responsible for PRDM1 repression in NR CLL cells.

Since gene silencing can occur not only as a result of DNA methylation but also as a consequence of histone PTM, this latter possibility was examined next.

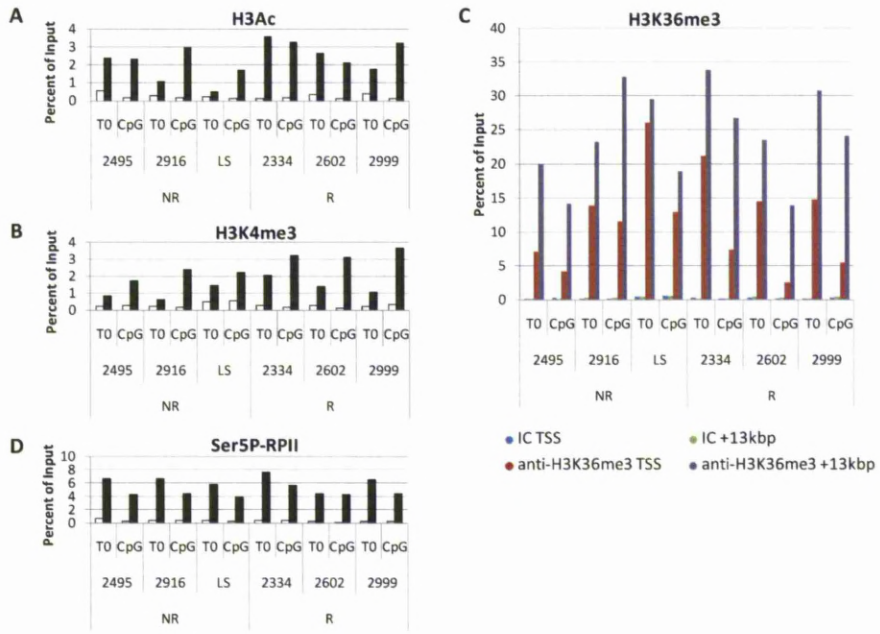
5.3.8 After stimulation, the *prdm1* promoter in both R and NR CLL clones is associated with comparable levels of histone H3 chromatin marks and Ser5P-RPII

The levels of histone marks associated with the *prdm1* TSS were measured by ChIP before and after stimulation with CpG-ODN. Three histone PTMs were examined:- H3Ac, H3K4me3 and H3K36me3 using specific immunoprecipitating antibodies. H3Ac and H3K4me3 are both associated with transcription initiation, while H3K36me3 is associated with transcription elongation. Alongside these histone modifications, Ser5P-RPII was also used as an additional sign for the presence of transcription initiation at the TSS (see Sections 1.4 and 1.5).

At T0, variable levels of the two histone transcription initiation marks, H3Ac and H3K4me3, were detected in association with *prdm1* in both R and NR clones (Fig. 5.9A, B and C). At the time at which PRDM1 mRNA was being maximally induced after CpG-ODN treatment (refer to Fig. 5.1B and the legend for Fig. 5.9), the levels of these histone marks usually increased, unless comparatively high before stimulation, and were similar in both R and NR clones.

Unexpectedly, before stimulation the level of the histone mark H3K36me3 was high at the TSS of *prdm1* and was similar between

Figure 5.9



The presence of histone marks and Ser5P-RPII on the *prdm1* gene before and after CpG-ODN treatment in R and NR clones. (A), (B), and **(D)**, show results for anti-H3Ac, anti-H3K4me3 and anti-Ser5P-RPII ChIP experiments respectively. qPCR analysis of the immunoprecipitated DNA was performed over the TSS of the *prdm1* gene. White bars in each chart are isotype controls, while black bars are the specific immunoprecipitating antibody. CpG indicates the time during treatment with CpG-ODN at which PRDM1 mRNA was being maximally induced (refer to Fig. 5.1B; 2334 = 4.5hrs; 2602, 2999 and NR clones = 11.5hrs). **(C)** shows similar results for an anti-H3K36me3 ChIP, but this time, both the TSS and a position 13kbp downstream were examined. IC = isotype control antibody.

response types (Fig. 5.9C). However, as would be expected for a mark produced by transcription elongation, the levels of this mark were consistently higher 13kbp downstream. After CpG-ODN treatment, the H3K36me3 mark surprisingly decreased similarly at both sites in the majority of clones (Fig. 5.9C).

As predicted from the histone transcription initiation marks, high levels of Ser5P-RPII were also associated with the *prdm1* TSS and were again found to be similar in both types of CLL clone (Fig. 5.9D). It was therefore concluded that the inability to detect PRDM1 mRNA (and protein) in NR clones is the result of an abnormality downstream of transcription initiation and elongation. As discussed later, a failure in transcription termination, mRNA maturation, or the over expression of a microRNA (miR) might account for such findings.

5.4 DISCUSSION

The aim of the work presented in this chapter was to establish why PRDM1 cannot be expressed in some CLL clones, even after stimulation with potent differentiating agents.

It is known that *prdm1* is controlled by a number of transcriptional activators and repressors in normal B cells. It is therefore not surprising that the present work clearly demonstrated that *prdm1* is also controlled at a transcriptional level in CLL. Thus, those clones that were able to undergo plasmacytoid differentiation after stimulation produced large amounts of PRDM1 mRNA, while those unable to undergo such differentiation were unable to transcribe the gene.

Although a substantial amount is known about the activators/repressors and the signals leading to their activation/expression[134,163], it is not known what precise combination of factors is needed for the induction of PRDM1 in normal B cells; nothing is known about the transcriptional regulation of *prdm1* in CLL. The present work demonstrated that the induction of PRDM1 in CLL cells by IL-21 requires both STAT3 and NF-κB activation. In contrast, induction of the gene by CpG-ODN was shown to be dependent on activation by NF-κB and an additional unidentified neofactor. This factor is not STAT3 since CpG-ODN induction of PRDM1

was shown to be independent of this transcription factor. Interestingly, the kinetics of transcription of *prdm1* in response to the two stimuli were different. Thus, the effect of IL-21+CD40L was immediate, while that of CpG-ODN was variably delayed, presumably because neosynthesis of the unidentified co-factor is required. These observations are novel and of interest because they define the minimal requirements for the induction of PRDM1 in CLL cells by physiologically relevant stimuli. Also, although it is known that the effect of TLR stimulation on *prdm1* in normal B cells is dependent on NF-κB, it has not been previously shown that this transcription factor is also required for IL-21 activation of the gene. Furthermore, the present findings concerning the kinetics of induction of PRDM1 mRNA in B cells are novel since it has not been previously recognised that IL-21 and CpG-ODN induce PRDM1 transcription with such different kinetics.

Since all NR CLL clones failed to transcribe *prdm1* in response to IL-21±co-stimuli, CpG-ODN or non-physiological stimuli (e.g. PMA), it next seemed reasonable to hypothesize that a shared mechanism is responsible. Having shown that STAT3 activation by IL-21 is not defective in NR clones, the NF-κB pathway was examined next. This seemed especially important since NF-κB was shown to be required for PRDM1 induction by both IL-21 and CpG-ODN. In fact, using an ELISA method

measuring activated NF-κB subunits in the nucleus, it was shown that the p50 and p65 subunits were translocated to the nucleus in both R and NR clones. Significantly more translocated p50 subunit was detected in R clones before and after stimulation with CpG-ODN, but levels of the activating p65 subunit in the nucleus were not significantly different in R and NR clones, either before or after TLR9 stimulation. Also, CpG-ODN stimulation could induce other NF-κB-dependent genes (IκBα and BCL2A1) to a similar extent in both R and NR cells. It was therefore concluded that defective activation or nuclear translocation of NF-κB are unlikely to be responsible for the absence of PRDM1 induction in NR clones. It remained possible that binding of NF-κB specifically to the *prdm1* gene is defective in NR clones, and an attempt was made to investigate this possibility using ChIP and antibodies specific for p65. Unfortunately, however, these attempts were inconclusive since no binding of p65 to *prdm1* could be demonstrated in R or NR clones, either before or after CpG-ODN stimulation.

Having shown that defective STAT3 and NF-κB signalling are unlikely to be responsible for the defective PRDM1 induction in NR clones, it was next necessary to examine the other known activators/repressors of the gene. Here, the hypothesis was that differential expression of such activators/repressors might be responsible for the lack of PRDM1

induction in NR clones. In fact, it was found that basal and induced levels of IRF4, PU.1, Pax5, Bach2, BCL6 and Spi-B were all similar in NR and R CLL cells. Furthermore, factors that require activation to enter the nucleus and affect transcription (NFAT, IRF5 and AP-1) are unlikely to be defective/responsible in NR clones. Thus, IRF4 (a downstream target of NFAT) was readily induced by PMA+ionomycin in R and NR clones. Also, CsA inhibition of NFAT activation by calcineurin was not required for PRDM1 induction in R clones by CpG-ODN, while requirement for IL-21-induced PRDM1 was variable. IRF5 is required for TNFα transcription[279], but this necrosis factor was induced by CpG-ODN in both R and NR clones, suggesting that IRF5 is functional in both subgroups. Regarding AP-1, it is known that PRDM1 induction is enhanced, but not dependent on this transcription factor[150,175]. Nevertheless, since AP-1 is repressed by Tcl-1, an important pathogenetic factor in CLL[281], Tcl-1 levels were measured in NR and R clones, but found to be similar. Taken together, these findings suggest that differential expression/activation of these regulatory transcription factors is unlikely to be responsible for the absence of PRDM1 induction in NR clones. It remains possible that, when activators/repressors are expressed, the loss of binding of an activator to, or altered displacement of a repressor from, the *prdm1* gene might be causing the lack of PRDM1 induction in NR clones. These

scenarios were indirectly examined in the work discussed below relating to histone modifications associated with the *prdm1* gene.

The possibility that, in NR clones, the *prdm1* gene was being silenced by an epigenetic mechanism was considered next. Although there is a precedent for *prdm1* silencing by DNA methylation[92], this was found conclusively not to be so in NR CLL.

Histone marks of transcriptional activation were therefore examined. Surprisingly, the presence of all the histone marks tested (H3Ac, H3K4me3 and H3K36me3) and binding of activated RPII on the *prdm1* gene were similar in NR and R clones after CpG-ODN stimulation. These results indicate that the initiation and elongation phases of PRDM1 mRNA production are intact in NR CLL clones. It therefore seems that NR clones might have a defect in the termination or processing phases of PRDM1 mRNA synthesis, or that post-transcriptional degradation of the mRNA is occurring in these cells. These mechanisms are discussed further in the next chapter concerning Conclusions and Future Work.

Chapter 6

CONCLUSIONS AND FUTURE WORK

This thesis has three major novel conclusions:-

1. $\alpha4^{neg}$ CLL clones are abnormal in being unable to recruit the cofactors necessary for initiation of transcription of the *ITGA4* gene. The cause of this abnormality is not clear, but absence of expression is not related to activation, differentiation or anergy.
2. The differentiation of around fifty percent of CLL clones is blocked *in vitro* probably as a result of the cells being unable to induce PRDM1.
3. In these NR clones, the production of mature PRDM1 mRNA is, for unknown reasons, blocked at a post-initiation phase of transcription.

Regarding the first conclusion, the studies of histone marks revealed that, in $\alpha4^{pos}$ CLL clones, the *ITGA4* TSS was associated with high levels of H3Ac and H3K4me3 – two marks of active transcription. In contrast, in $\alpha4^{neg}$ clones, these two marks were virtually absent from the *ITGA4* gene. This indicates that, in $\alpha4^{neg}$ clones, the mechanism recruiting HATs

is inoperative or the levels of HDACs on the TSS are too high. As a consequence, the transcription PIC is not recruited, and transcription does not take place. The challenge now will be to establish why this might be so.

It is known from other cell types that *ITGA4* is controlled by the transcription factors ZEB1 and ZEB2[57]. ZEB1 usually, but not always, functions as an activator by recruiting HATs p300 and P/CAF[255]. In contrast, ZEB2 usually acts as a repressor by recruiting the cofactor CtBP which, in turn, can recruit HDACs[287]. It will therefore be necessary to establish which isoform of the ZEBs is expressed by CLL cells and determine whether the protein is functioning as an activator or repressor. Since normal B cells have been reported to express ZEB2 but not ZEB1[288], it might be expected that CLL cells will contain only ZEB2 and that this transcription factor will be repressing *ITGA4* transcription. If this turns out to be so it will then be necessary to establish why such repression is not overcome in $\alpha4^{neg}$ CLL clones. In other cell types, ZEB2 inhibition is overcome by Myb proteins[57] and the simplest hypothesis, therefore, might be that $\alpha4^{neg}$ clones lack Myb for some reason. Since Myb expression is related to cell cycle[289], it maybe that $\alpha4^{pos}$ clones express Myb because they are at different stages of cell cycle (e.g. G_1 vs G_0).

The work for Conclusion 2 is largely complete and a paper for publication will be prepared in the near future. It is currently unclear if the failure to induce PRDM1 in NR CLL clones causes the block in Ig secretion and plasma cell differentiation in these cells. However, evidence from knockout and ectopic expression experiments of PRDM1 in murine B cells[130,131] (see Section 1.6.2) strongly implicate this to be the case. Ectopic expression of PRDM1 in NR clones will need to be performed to definitively prove this hypothesis.

One of the main questions arising out of the work in Chapter 2 is why do R clones not induce PRDM1 and undergo plasmacytoid differentiation *in vivo*? Here, there are number of possibilities. For example, the continuous exposure *in vivo* to particular (auto)antigens might maintain anergy and thereby prevent plasmacytoid differentiation[43]; when cultured *in vitro*, this constant antigenic exposure might be lost and the ability to undergo differentiation would therefore be restored (see below). It is also possible that stimuli arising from the microenvironment might prevent the induction of PRDM1, or that IL-21 is absent from lymph nodes as a result of defective T_H cell function[290].

To take forward the work in Chapter 5, it will be necessary to consider why, despite the presence on the *prdm1* gene of Ser5-RPII binding and

the histone marks associated with transcription initiation, little or no mature PRDM1 mRNA is detectable after stimulation of NR clones. The presence of the PTM, H3K36me3, downstream of the TSS of *prdm1* would implicate that RPII is proceeding into elongation in NR clones, but this is not fully conclusive. Additional experiments need to be performed to prove whether elongation is occurring. These experiments would involve measurement of PRDM1 pre-mRNA levels after stimulation and the quantification of the levels of Ser2-RPII binding downstream of the *prdm1* TSS.

The work in this thesis contributes to the knowledge of CLL pathobiology in multiple ways. It is currently unclear why some clones express α4 integrin and why others do not. The finding in this thesis that all normal CD19+ peripheral blood B cells express the integrin by FACS analysis suggests two hypotheses. Firstly, the majority of CLL clones that do not express α4 could have lost the integrin after becoming malignant, during the expansion of the clone. Consequently, the failure to lose α4 and therefore become abnormal causes the disease to have a poorer prognosis. Alternatively, α4 may be lost at a pre-mature B cell stage during the development of the CLL-cell of origin, and then due to a lack of transcription initiation histone marks and subsequent epigenetic

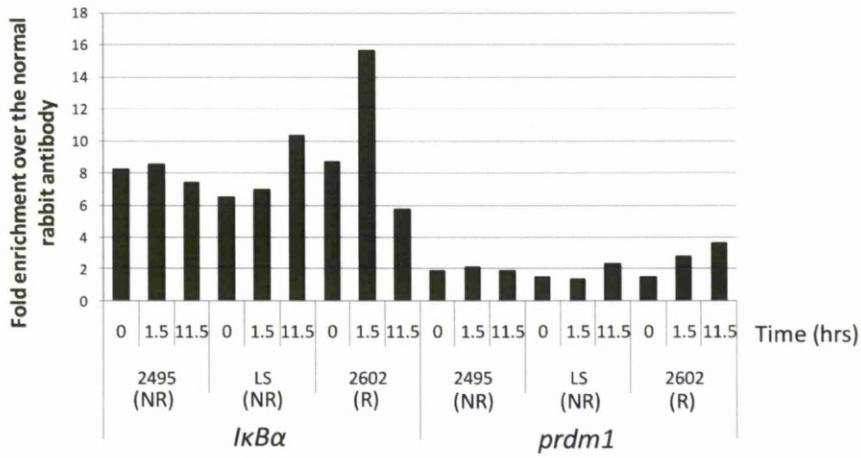
inheritance, fail to re-express the integrin. However, why α4 is lost only in a subset of CLL is still unclear.

Regarding PRDM1, the capability of CLL cells to induce this transcription factor has never been studied. This is surprising given the requirement for PRDM1 in terminal differentiation of antigen-activated B cells. CLL cells are believed to be antigen-activated B cells that are given pro-survival and proliferative signals but do not progress and differentiate. Without the capability to induce PRDM1, the CLL clone would be blocked at a stage of differentiation that may lead to perpetual antigen-induced BCR activation. PRDM1 has recently been identified as a tumour-suppressor gene whose loss causes the development of lymphomas in mice[291,292]. With the knockout of PRDM1 in B cells and, combined with a constitutively active NF-κB pathway in these cells (which is also present in CLL cells), mice develop DLBCL-like diseases[291,292]. It is therefore tempting to hypothesise that a block in PRDM1 combined with cell survival and proliferative signals received from *in vivo* (auto)antigens/microenvironments may facilitate the development of CLL. The block in terminal differentiation of a large percentage of activated B cell-like DLBCL cases occurs as a result of mutations in the *prdm1* gene which can cause either a reduction in expression or loss of function of the protein[257,292]. However, mutation

seems an unlikely reason for repression of PRDM1 in the majority of NR CLL clones as these cells, unlike DLBCL, cannot induce/express PRDM1 mRNA. Repression of PRDM1 in CLL cells is therefore more likely to be occurring via alternative mechanisms. One such mechanism may be a caused by the development of an anergic-like state in CLL cells. Anergy, as described in Section 1.2.4, is a naturally occurring cellular response to chronic stimulation usually induced by auto-antigens[43]. As CLL cells have many hallmarks of anergic cells[44,293], it could be hypothesised that CLL are a form of anergic-like B cells that are inhibited to induce PRDM1 and secrete immunoglobulin but remains able to receive and respond to survival and proliferative signals. Inducing PRDM1 expression and altering the stage of differentiation of CLL cells *in vivo* may therefore provide a novel therapy for the disease.

Appendix

The failure to detect p65 binding to the TSS of *prdm1* using ChIP



The figure shows a representative p65 ChIP experiment involving two NR clones and one R clone. Cells were stimulated with CpG-ODN for the times indicated, and cells were prepared for ChIP as is described in Section 2.2.12. Data are presented as fold enrichment in DNA immunoprecipitated with the p65 antibody over that by the isotype control. Consistent binding of p65 to the *IκBα* gene could be demonstrated, but little, if any, to that of *prdm1* could be detected.

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