The function of CD180 toll like receptor(TLR) on control B cells and B cell chronic lymphocytic leukaemia (B-CLL) cells

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The function of CD180 toll like receptor (TLR) on control B cells and B cell chronic lymphocytic leukaemia (B-CLL) cells

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Abstract

B cell chronic lymphocytic leukaemia (B-CLL) is characterised by accumulation of leukaemic B cells with aberrant phenotype compared to normal B cells, due to an imbalance between cell production and apoptosis. The outcome of B-CLL is dependant on the range of prognostic markers including clinically established mutational status of immunoglobulin heavy chain (IgVH) genes, expression of protein kinase ZAP-70, CD38, and the recently proposed CD180. CD180/RP105 toll-like receptor (TLR) is expressed by normal B cells, macrophages and dendritic cells. Importantly, it has been shown previously that murine B cells primed with anti-RP105 monoclonal antibodies (mAb) undergo apoptosis upon subsequent surface IgM (sIgM) ligation. In this project I have studied effects of CD180 ligation on control B cell and B-CLL cell function to identify its translational importance as a target for immunotherapy.

Peripheral blood mononuclear cells (PBMCs) and purified CD19+ B cells from 20 control volunteers and 36 B-CLL patient were characterised phenotypically. They were further examined for activation (measured by surface expression of CD86), proliferation (measured by intracellular Ki-67) and apoptosis (measured by the changes in mitochondrial membrane potential) following stimulation with anti-CD180 mAb alone or in combination with anti-CD40 mAb, anti-IgM polyclonal $F(ab)_2$ Ab, anti-IgD polyclonal $F(ab)_2$ Ab or recombinant IL-4 (rIL-4). The results were assessed by Flow cytometry using appropriate statistical analysis.

Control B cells and one third of the B-CLL clones studied (Responder B-CLL, R) responded to CD180 ligation and all other stimuli studied by significant activation ($p < 0.00001$ and $p = 0.001$ respectively) and proliferation ($p = 0.013$ and $p = 0.009$ respectively) after 72h as compared to unstimulated cultures. Remaining B-CLL clones were sub-divided into non-responder (NR) and CD180 negative (CD180-) clones with each category representing approximately one third of the patient cohort. Importantly, NR and CD180- B-CLL cells were found to be poor/non-responsive to all the stimulants studied indicating the possible anergic status of these cells. Purification of B-CLL cells moderately reduced anti-CD180 responses by R B-CLL clones, thus indicating an accessory role for non-B cells. However, they did not affect the poor responses

demonstrated by NR or CD180- cells indicating intrinsic lack of activatory and proliferative functions. Control B cells demonstrated strong synergism of CD180 mediated proliferation with that induced by the ligation of CD40 and rIL-4, but not by sIgM or sIgD. This suggested converging signalling pathways of CD180 and BCR which was further supported by the constitutive co-localisation of sIgM and CD180 on B-CLL cells assessed by confocal microscopy. Ligation of CD180 alone or followed by the engagement of sIgM led to survival of control B cells and some B-CLL cells as compared to the unstimulated cultures, but apoptosis induction was not detected. However, ligation of sIgD by high affinity polyclonal antibodies led to the dramatic apoptosis of control B cells and, the majority of B-CLL clones with no contribution from the priming by anti-CD180 mAb. Further analysis revealed that CD180+sIgM+ cells were particularly sensitive to anti-IgD induced apoptosis. This category includes some B-CLL clones and all normal B cells.

Albeit based on a limited number of patients and data, there is an indication for an important role for the CD180-mediated pathway in the regulation of activation, proliferation and apoptosis of B-CLL cells. Additional studies are required to identify CD180 mediated intracellular signalling pathways and this could have prognostic and therapeutic implications.

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AUTHORS DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

Signed: Azka Date: 25/07/09

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List of Abbreviations

µL.....	*microlitre*
ADCC.....	*antibody dependent cytotoxicity*
AICD.....	*activation induced cytidine deaminase*
ATM.....	*ataxia-teleangiectasia mutated gene*
BAD.....	*bcl-2 antagonist of cell death*
BCR.....	*B cell receptor*
B-CLL.....	*B cell chronic lymphocytic leukaemia*
Bcl-2.....	*B cell lymphoma 2*
BH3.....	*bcl-2 homologue domain 3*
Btk.....	*Bruton's tyrosine kinase*
Ca^{+}.....	*calcium*
CCL.....	*chemokine (C-C motif) ligand*
C-myc.....	*myelocytomatosis cellular oncogene*
co-IP.....	*coprecipitation*
CXCR4.....	*CXC chemokine receptor 4*
DAMP.....	*damage associated molecular patterns*
DCF.....	*Deoxycoformycin*
dL.....	*deciliter*
DNA.....	*deoxyribonucleic acid*
ERK.....	*extracellular signal regulated kinase*
FCR.....	*fludarabine, cyclophosphamide, rituximab*
GC.....	*germinal centre*
g.....	*Gram*
GM-CSF.....	*Granulocyte monocyte colony stimulating factor*
G protein.....	*guanine nucleotide-binding proteins*
HEK.....	*human embryonic kidney*

Hb.....	*haemoglobin*
hTERT.....	*human telomerase reverse transcriptase*
Ig.....	*immunoglobulin*
IgVH.....	*immunoglobulin heavy chain variable gene*
IFN.....	*interferon*
IL.....	*interleukin*
ITAMs.....	*immunoreceptor tyrosine-based activation motifs*
JNK.....	*c-Jun N-terminal kinase*
kD.....	*kilodalton*
L.....	*ligand*
L.....	*Litres*
LPS.....	*ipopolysaccharide*
LRR.....	*leucine rich repeats*
Mut.....	*mutated*
mAb.....	*monoclonal antibody*
MAPK.....	*mitogen activated protein kinase*
Mcl-1.....	*myeloid cell leukemia-1*
MD.....	*membrane determinant*
mL.....	*milliliter*
MRD.....	*minimal residual disease*
mTOR.....	*mammalian target of rapamycin*
MyD88.....	*myeloid differentiation primary response gene 88*
NCI.....	*National Cancer Institute*
NFκB.....	*nuclear factor kappa-light-chain-enhancer of activated B cells*
NFAT.....	*nuclear factor of activated T cells*
NK cells.....	*natural killer cells*
p53.....	*protein 53*
PAMPs.....	*pathogen associated molecular patterns*

PI3K	*phosphatidylinositol 3-kinase*
PKC.....	*protein kinase C*
PLCγ2.....	*phospholipase C gamma 2*
rIL-4.....	*recombinant interleukin 4*
RNA.....	*ribonucleic acid*
RP105.....	*radio-protective 105*
sIgM.....	*surface immunoglobulin M*
sIgD.....	*surface immunoglobulin D*
SDF-1.....	*stromal derived factor-1*
SLE.....	*systemic lupus erythematosus*
Syk.....	*spleen tyrosine kinase*
TCL-1.....	*T cell leukaemia/lymphoma-1*
TIR.....	*Toll/IL-1 receptor*
TLR.....	*Toll like receptor*
TNF.....	*tumour necrosis factor*
UMut.....	*unmutated*
VDJ.....	*variable, diversity, joining*
VH.....	*hypervariable*
VL.....	*variable light chain*
WBC.....	*white blood cell*
XIAP.....	*x-linked inhibitor of apoptosis protein*
ZAP-70.....	*zeta-chain associated protein-70*

CHAPTER ONE

INTRODUCTION

B cell Chronic Lymphocytic Leukaemia (B-CLL) is a malignancy which results from a combination of defects in the regulation of cell death (Arnon *et al.*, 2004) and enhanced proliferation of the precursor B cells (Chiorazzi *et al.*, 2005). Despite being the most frequently occurring leukaemia which accounts for about 25% of all adult leukaemias (Houlston, 2003), its aetiology and molecular mechanisms remain to be fully elucidated. The main concepts regarding the biological processes underlying development of B-CLL have undergone considerable change in the past decade. Initially B-CLL was viewed as an accumulation of phenotypically mature monoclonal malignant B cells in the bone marrow, peripheral blood and lymphoid system, due to their resistance to apoptosis (Kipps *et al.*, 1995; Guipaud *et al.*, 2003). More recently it has become evident that the pool of apoptosis-resistant B-CLL cells is replenished constantly by proliferating precursors (Chiorazzi *et al.*, 2005).

Phenotypically, B-CLL cells express surface molecules CD19, CD5 and CD23 with low or absent expression of CD20, CD79b, Ig (immunoglobulin) M and IgD (Guipaud *et al.*, 2003, Chiorazzi *et al.*, 2005), Although leukaemic cells from individual patients show marked phenotypic heterogeneity they demonstrate prolonged survival and express high levels of anti-apoptotic Bcl-2 protein (Pers *et al.*, 2002).

Historically the immunological repertoire of leukaemic cells and surface CD5 positivity has identified B-CLL cells as derivates from the mantle-zone follicle centre. However, recent data on the genetics and physiological heterogeneity of B-CLL together with the remarkably diverse clinical course has led to the establishment of a new concept that suggests this disease to represent in fact two related entities (Chiorazzi *et al.*, 2005). Shared by many opinion leaders in the field, this viewpoint was supported particularly by studies on somatic hypermutation that revealed two types of B-CLL cells with mutated (defined as more than 2% of germ-line mutations) or unmutated (defined as less than 2% or no germ-line mutations) in the heavy chain of the surface IgM (sIgM) variable region (IgVH) that represents the B cell receptor (BCR) (Chiorazzi and Ferrarini, 2003; Hamblin *et al.*, 2003; Stevenson and Caligaris-Cappio, 2004).

Bone marrow derived B cells normally undergo somatic hypermutation in a germinal centre of lymphoid follicles upon engagement of the BCR by an antigen. The mutations introduce extra changes in the already re-arranged VDJ (H chain) and VL (L chain) sequences of sIgM and cells that retain their antigen-binding capacity are allowed to proliferate in the presence of T cell help. However, those B cells that respond to carbohydrate antigens or bind to auto antigens may develop outside germinal centres and without T cell help. These two scenarios may reflect the existence of two distinct types of B-CLL cells *viz,* those with mutated (Mut) and unmutated (UMut) IgVH genes. The Mut B-CLL cells are likely to originate from the germinal centre population and UMut B-CLL cells from the subset outside the germinal centre. Both types of cells belong to antigen-experienced mature B lymphocytes (Hamblin *et al.*, 2003).

It has been noted that the status of IgVH mutations is strongly associated with the clinical course of this leukaemia and therefore is the most widely used prognostic marker. Those with Mut B-CLL cells were characterised by prolonged survival without therapy and succumbed to unrelated diseases whilst those with UMut B-CLL cells had an aggressive disease course with fatal outcome regardless of the therapy regimen (Damle *et al.,* 1999). The biological basis for the differences in behaviour of these two types of leukaemic B-CLL cells remains to be fully elucidated, and the proposal for them to represent two subtypes of B-CLL is not universally shared. Nevertheless, these findings led to the establishment of the mutation level of IgVH genes in leukaemic B-CLL cells as the most reliable prognostic marker to date.

Other prognostic markers which indicate a poor patient prognosis include expression of both surface CD38 and intracellular zeta-chain-associated protein 70 (ZAP-70). ZAP-70 is involved in activation of T lymphocytes (Chan *et al.,* 1992) and is rarely expressed by normal B cells where it facilitates BCR signal transduction (Kong *et al.,* 1995).

CD38 has adenosine diphosphate-ribose cyclase activity and could be involved in the regulation of B cell signalling (Lund *et al.*, 1996; Zupo *et al.*, 2000). CD38 is

considered an independent prognostic marker for B-CLL patients and the association of B-CLL cells positive with this surface glycoprotein together with UMut IgVH genes appears to promote the most aggressive course of the disease. A significant correlation has been found between the expression of ZAP-70 and UMut status of the B cell leukaemic clones (Chen *et al.*, 2002; Crespo *et al.*, 2003; Wiestner *et al.*, 2003; Orchard *et al.*, 2004; Rassenti *et al.*, 2004) and B-CLL clones with ZAP-70+ phenotype and UMut genotype are characterised also by a high level of disparity of clinical features of B-CLL (Del Principe *et al.*, 2006).

Some well documented chromosomal abnormalities in B-CLL cells are closely associated with IgVH mutational level. It has been demonstrated that the most common deletion of 13q14, affecting around 50% of all B-CLL cases, contains two micro-RNA genes and may predispose B-CLL cells to additional mutations and is associated generally with better prognosis (Dohner *et al.*, 2000; Calin *et al.*, 2002, 2004). On the contrary, deletions at 11q22-23, 17p13 and 6q21 are associated with poor prognosis and are likely to include p53 and ataxia-teleangiectasia mutated (ATM) genes. They are more frequent in UM B-CLL cells (Dohner *et al.*, 2000; Stilgenbauer *et al.*, 2000).

B-CLL associated with negative prognostic markers is characterised by an aggressive disease course, poor response to chemotherapy and resistance to apoptosis. Our group at University College Hospital and the University of Westminster has recently introduced another potential prognostic marker for B-CLL, surface expression of CD180 from the toll-like receptors family. B-CLL cells with Mut IgVH genes were characterised by significantly higher levels of CD180 whilst low levels of CD180 were detected on UMut-type B-CLL cells (Porakishili *et al.*, 2005). CD180 is detected on the majority of normal B cells, monocytes (Zarember and Godowski, 2002) and dendritic cells (Kadowaki *et al.*, 2001).

However, the biological significance of CD180 expression by B lymphocytes remains unclear although its association with the mutational status of B-CLL IgVH genes has strongly suggested an involvement of CD180 in selective advantage of certain B cell subsets and their possible resistance to apoptosis. Indeed, induction of

apoptosis of murine B cells was seen after priming with anti-CD180 monoclonal antibodies (mAb) followed by the ligation of sIgM (Yamashita *et al.*, 1996) whereas ligation of CD180 led to activation and proliferation of normal B lymphocytes (Yazawa *et al.*, 2003). The literature review given below lays out a background for the major hypothesis of this project regarding possible role(s) of CD180 in the regulation of biological functions of B-CLL cells.

1.1 Clinical features of B-CLL

The clinical course of B-CLL is heterogeneous with some patients rapidly progressing to early death while others exhibit more stable and non-progressing disease lasting many years. The median survival could be 10 years and correlates with various presentation features such as recurrent infections, anaemia and painless lymphadenopathy (Kumar and Clarke, 2005) with a male to female ratio of 2:1 (Davidson's Medicine, 1999). The main clinical symptoms of B-CLL include an immuno-compromised status and recurrent infection. Bone marrow infiltrations are observed followed by haemolytic anaemia in 10-15% of patients. Painless lymphadenopathy and splenic discomfort are frequent, occasionally leading to massive hepatosplenomegaly (Kumar and Clarke, 2005).

Peripheral blood of B-CLL patients is characterised by normal (13-18 g/dl for men and 12-16 g/dl women) or low (<10g/dl) levels of haemoglobin (Hb). Normal (150-400x10^6 cells/mL) or low (<100x10^6 cells/mL) and dramatically raised white blood cell (WBC) count (in some cases up to 5x10^9 /L or 5000/μL) has been seen by both flow cytometry and blood films. Bone marrow is also heavily infiltrated with lymphocytes (≥30%) (Wolowiec *et al.*, 2004). Hb and platelet levels are important for the staging of the disease.

1.2 B-CLL staging

It is possible to classify B-CLL by one of the two staging systems according to that proposed by Rai (Rai *et al.*, 1975, 1987) and Binet (Binet *et al.*, 1981). Both systems assess the extent of disease in an individual patient, and serve for medical decisions for the treatment and monitoring of the disease.

1.2.1 Rai staging system

The Rai classification divides CLL into low, intermediate and high risk categories, which correlate with stages 0, I & II and III & IV respectively. Lymphocytosis is present in all stages of this disease (Kumar and Clarke, 2005). At stage 0 lymphocytosis is seen only in blood or in bone marrow with survival rates up to 12 years. At stage I patients have lymphadenopathy in addition to lymphocytosis with no alteration in the overall survival rate. Hepatomegaly or splenomegaly is seen alongside lymphocytosis at stage II with survival rates dropping to 7 years. Anaemia (Hb < 110 g/L) and thrombocytopenia (platelets < $100x10^9$/L) are seen at stage III and IV respectively with lymphocytosis, survival rates being less than 1 year (Rai *et al.,* 1975, Hamblin., 2007).

1.2.2 Binet staging system

The Binet staging system classifies B-CLL according to the number of lymphoid tissues involved (such as the spleen and the lymph nodes of the neck and groin) accompanied by anaemia or thrombocytopenia. Stage A includes involvement of less than three lymphoid sites with Hb>100g/L and platelets >$100x10^9$/L and is characterised by a survival rate of up to 9 years. If there are more than three sites involved with the same level of Hb and platelets then the patient will be assigned to stage B with a survival of 5 years. At stage C Hb is down to less than 100g/L and platelets to less than $100x10^9$/L with survival of a maximum of 2 years (Binet *et al.*, 1977, Hamblin., 2007). Both staging systems are now being complemented by the use of newly established prognostic markers which allow, in many cases, prediction of the disease course particularly in those patients where B-CLL is diagnosed in the early stages.

1.3 Prognostic markers for B-CLL

The accepted clinical prognostic criteria include the Rai and Binet systems, infiltration pattern of bone marrow, lymphocyte doubling time, and selected cytogenetic abnormalities (Neil *et al.,* 2002). Importantly, recent progress in the studies of genetic and molecular markers have identified profoundly different clinical courses of B-CLL and has enabled the establishment of an updated set of markers used both for prognostic and monitoring purposes as well as being in themselves

possible therapeutic targets. These markers are reviewed in detail in this section of work (Table 1.1). Currently the mutation status of IgVH genes of B-CLL cells has been officially implemented as a prognostic criterion in developed countries whilst the validity of other markers is in the process of further evaluation. It must be stressed that although quite important, these criteria are not necessarily applicable for each individual case and sometimes the use of a combination of prognostic markers may be recommended.

1.3.1 Mutations of IgVH genes

As already indicated, Damle *et al* (1999) have shown that the mutation status of the immunoglobulin heavy chain variable (IgV_H) genes is a powerful prognostic indicator. In accordance with this finding and subsequently accumulated clinical data, B-CLL is regarded by some groups as two separate but related entities: a slowly progressing disorder and a more aggressive form with B-CLL clones characterised by high and low frequencies of the gene mutations respectively. Somatic mutation more than 2% in the germLine IgVH genes is considered as a mutated case, with the patient having good prognosis and longer survival as compared to those with IgVH genes which have mutation levels less than 2% (Figure 1.1). A mutation level of 2% is considered to be the cut-off for placing the patient in either group, *viz*, mutated or unmutated (Davis *et al*., 2003; Degan *et al.,* 2004, Tobin *et al.,* 2005).

The 2% cut-off is currently an accepted value for clinical practice since it is based on an earlier proposal that any given sequence that differs from a germ line set of genes by 2% or more is considered as mutated (Schroeder *et al.,* 1994). In opposition to this, a number of researchers have suggested a mutation cut-off level up to 5% to allow for discrimination between the two mutational groups (Franco *et al*., 1998). Interestingly, patient groups with 2-4% mutation levels of IgVH genes are characterised by survival rates similar to that of UMut patients and considerably shorter than the survival times of patients exceeding 4% mutation in their IgVH genes (Ke *et al.,* 2002).

Table 1.1. Major prognostic markers in B-CLL.

Marker	Status	Prognostic Value	Mechanism of Action	Reference
IgVH gene Mutated (Mut) Unmutated (UMut)	Having 2 % or more, mutated IgVH gene No mutations occurred in IgVH gene	**GOOD** **BAD**	Reaction between sIgM of unmutated B-CLL patients with polyspecific (self antigens) leads to cell activation and continuous stimulation causing expansion of UMut clones. Cells from Mut B-CLL clones do not get stimulated via sIgM	Cutrona *et al.*, 2007
CD38 Positive Negative	>30% B-CLL cells expressing the molecule <30% B-CLL cells expressing the molecule	**GOOD** **BAD**	This molecule induces proliferation of B-CLL cells and increases their survival.	Deaglio *et al.*, 2003
ZAP-70 Positive Negative	>20% B-CLL cells express the intracellular protein <20% B-CLL cells express the intracellular protein	**GOOD** **BAD**	ZAP-70+ B-CLL cells respond more effectively to BCR ligation leading to antigen driven proliferation and subsequent survival.	Chen *et al.*, 2005
P53	mutated	**BAD**	A transcription factor which becomes activated by breaks in DNA and can induce apoptosis or cell death. Mutation leads to abrupt cell growth in B-CLL.	Wattel *et al.*, 1994

B-LL is a malignancy resulting from a combination of defects in the regulation of cell death and enhanced proliferation of precursor B cells. There are several markers associated with its prognosis. The well recognised ones are presented in this table

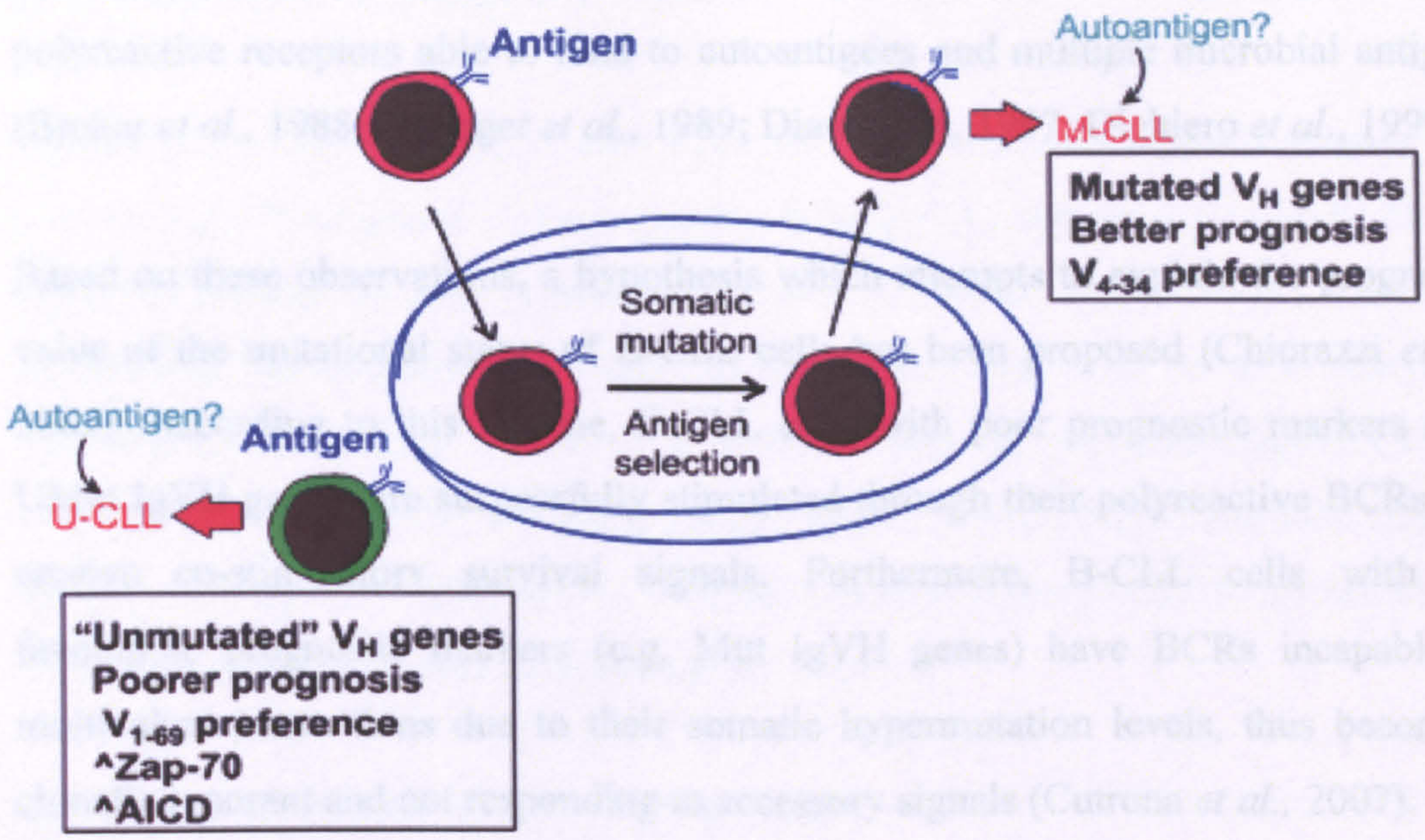


Figure 1.1. Origin and features of Mutated and Unmutated B-CLL cells.

The development of UMut B-CLL is likely to be from a naive B cell that has encountered antigen but with inadequate stimulus. This group usually has a poor prognosis, displays a preference for V_{1-69} genes, frequently expresses ZAP-70 and activation-induced cytidine deaminase (AICD). In comparison, Mut B-CLL develops from a cell that, following an antigen encounter, has undergone somatic mutation (from Stevenson and Caligaris-Cappio, 2004).

It has been noted recently that in up to 10% of B-CLL patients there is a high degree of similarity with regard to the special patterns of mutations and preferential combinations of both D and J_H segments. Skewed VHDJH combinations have been found which generate distinct amino acid sequences in antigen-binding pockets of the heavy chain of the BCR (Johnson *et al*., 1997; Fais *et al*., 1998). These patients mostly belong to the UMut genotype with poor disease outcome (Messmer *et al*., 2004). In some cases the similarity involves the whole antigen-binding sites comprised of both heavy and light chains with the BCRs from these patients being almost identical (Tobin *et al*., 2002, 2003, 2004; Ghiotto *et al*., 2004; Messmer *et al*., 2004; Widhoph *et al*., 2004).

The skewed BCR repertoire in B-CLL suggests that a limited set of antigens is involved in the triggering of leukaemic cells. These may be latent viruses, commensal bacteria, environmental or autoantigens (Chiorazzi *et al.,* 2005). It has

been documented that B-CLL cells, particularly those with UMut IgVH genes, have polyreactive receptors able to bind to autoantigens and multiple microbial antigens (Broker *et al.,* 1988; Sthoeger *et al.*, 1989; Diaw *et al.,* 1997; Dighiero *et al.*, 1999).

Based on these observations, a hypothesis which attempts to explain the prognostic value of the mutational status of B-CLL cells has been proposed (Chiorazzi *et al.*, 2005). According to this scheme, B-CLL cells with poor prognostic markers (e.g. UMut IgVH genes) are successfully stimulated through their polyreactive BCRs and receive co-stimulatory survival signals. Furthermore, B-CLL cells with the favourable prognostic markers (e.g. Mut IgVH genes) have BCRs incapable of multivalent interactions due to their somatic hypermutation levels, thus becoming clonally ignorant and not responding to accessory signals (Cutrona *et al.,* 2007). This scheme also incorporates two other major prognostic markers, the surface expression of CD38 as well as the intracellular expression of ZAP-70.

1.3.2 Cell surface expression of CD38

CD38 is a surface molecule normally is expressed on T cells and B cells within the germinal centre of the secondary lymphoid organs (Martin *et al.,* 2002). It has adenosine diphosphate-ribose cyclase activity and supports B cell differentiation and cellular interactions (Deaglio *et al.*, 1998).

In B-CLL cells, CD38 expression can augment BCR signalling (Lund *et al.,* 1996) and regulate both IgM- and IgD-induced apoptosis (Zupo *et al.*, 2000). More recently it has been shown that CD38 induces proliferation of B-CLL cells and increases their survival (Deaglio *et al.*, 2003). This may be the result of interaction between CD38 and its natural ligand, CD31, which upregulates CD100 - a survival receptor from the semaphorin family involved in sustaining CLL growth and survival (Elhabazi *et al.*, 2003; Kumanogoh *et al.,* 2003). The suggested role of the microenvironment in B-CLL cell rescue from apoptosis and the involvement of CD38 in the pathogenesis of CLL complements the scheme as described by Chiorazzi *et al* (2005) which suggests that environmental or autoantigens may be involved in triggering the proliferation of leukaemic cells. Supporting this concept, it has been shown recently that CD38

expression delineates populations of B-CLL cells that are activated and have proliferation markers (Damle *et al.*, 2007).

Surface expression of CD38 is associated with poor disease prognosis (Figure 1.2) with the cut-off for positive values ranging from 20% to 30% of B-CLL cells from a patient as defined by flow cytometry (Silvia *et al.*, 2006). In fact, it has been well documented that patients expressing CD38 on more than 20% on their malignant cells have a disadvantage in survival compared to patients with percentage of CD38+ cells (Damle *et al.*, 1999; Ke *et al.*, 2002). In this project, I used antibody relative binding sites per cell (RBS/cell) to evaluate the expression of CD38, instead of the percentage of positive cells as described in Chapter 2.

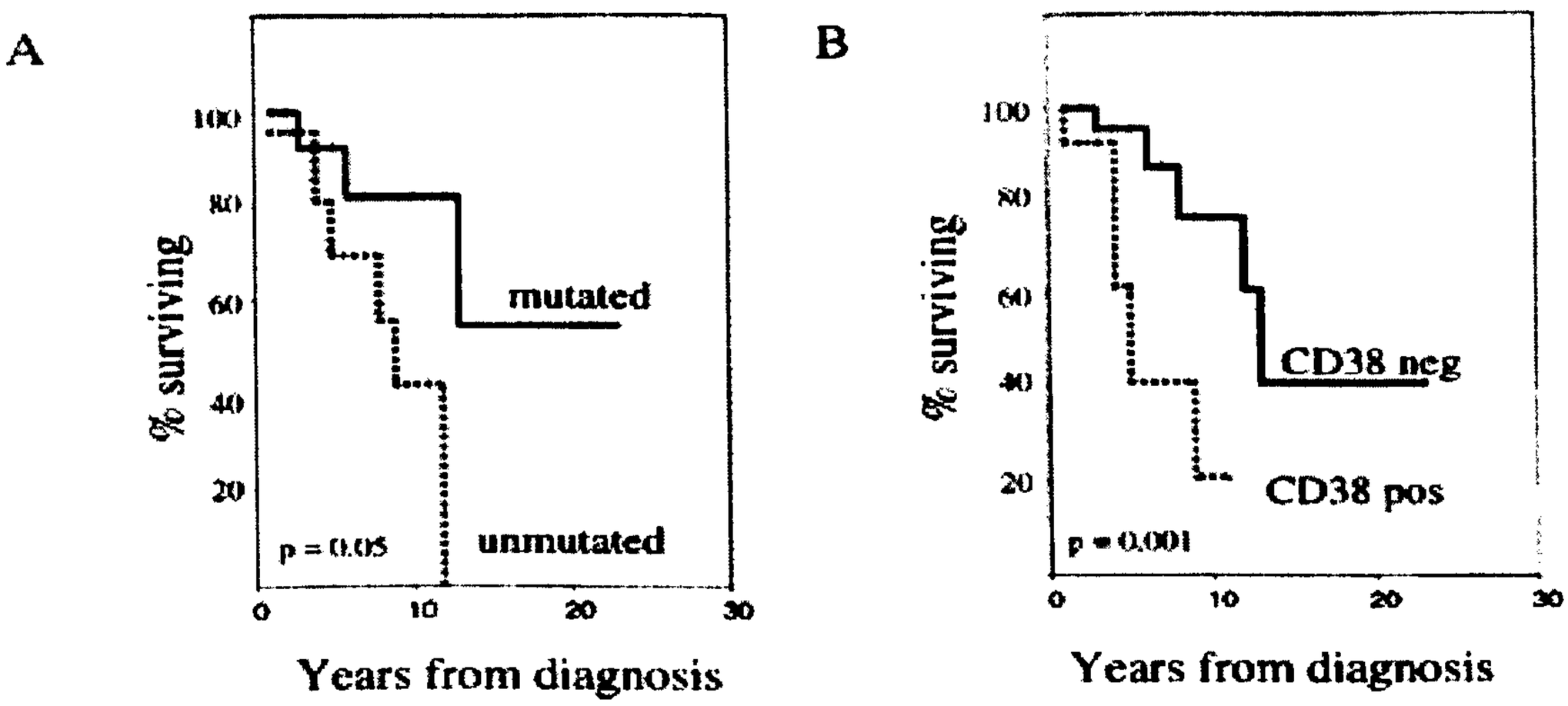


Figure 1.2. Effects of IgVH gene mutation (A), and CD38 expression (B) on survival of B-CLL Patients

These survival curves compare patients with cells having mutated vs unmutated IgVH genes (A) of their cells being CD38+ vs CD38- (B), indicating that these are two separate prognostic markers (from Matrai *et al.*, 2001)

Interestingly, it has been reported that cell surface expression of CD38 may change during the course of B-CLL (Chang and Cleveland, 2002). For example, the study of Ibrahim *et al.*, (2001) have shown the condition of one of their patients to worsen, this coinciding with significant increases in CD38 expression on the malignant clone. However, subsequent clinical data failed to confirm this apparent correlation between the increase in the surface expression of CD38 and the clinical course of B-CLL (Hamblin *et al.*, 2002).

It is an important question as to whether or not CD38 represents a true prognostic marker independent from IgVH gene mutational status. Previously a correlation between high CD38 surface expression and unmutated IgVH genes had been suggested by the studies of Naylor *et al.* (1999) where CLL patients with more than 5% cut off in IgVH mutation showed negativity for surface CD38. Regardless of this latter study, it has subsequently been widely accepted that both CD38 and IgVH mutational status (Figure 2.1) are two independent prognostic markers (Hamblin *et al.,* 2002; Ghia *et al.*, 2003), and that the disease is most aggressive when UM IgVH genes coincide with high expression of CD38 (Jelinek *et al.*, 2001).

1.3.3 Intracellular expression of ZAP 70

More recently the intracellular expression of ZAP-70 has emerged as a novel prognostic tool for B-CLL patients. This protein is rarely present in B cells but is normally expressed in T cells and tumours of T cell lineage with the gene for this protein being located in/on chromosome 2q12 (Rassenti *et al.*, 2004). Its expression within a subgroup of B-CLL cells is a strong indicator of disease aggressiveness (Rassenti *et al.*, 2004; Adrian, 2005). B-CLL cells are considered to be positive for ZAP-70 when at least 20% of the CD19+ cell population express this antigen in flow cytometry profiles (Luz *et al.*, 2006; Del Principe *et al.,* 2006).

ZAP-70+ B-CLL cells respond more effectively to BCR ligation leading to antigen driven proliferation and subsequent survival (Chen *et al.*, 2005). Expressed in B cells, ZAP-70 can facilitate downstream signal transmission upon the engagement of the BCR (Kong *et al.*, 1995).

Moreover, ZAP-70 facilitates downstream signalling from BCR, CD38 and CXCR4 (Richardson *et al.,* 2006). CXCR4 is a chemokine receptor highly expressed on B-CLL cells (Burger *et al.*, 1999). Through its interaction with the ligand stromal-cell-derived factor-1 (SDF-1 or CXCL12), the migration of B-CLL cells is directed towards stromal and nurse-like cells (Burger *et al.*, 2007). On the basis of this evidence, ZAP-70 appears to be involved in B-CLL cell trafficking and is now considered by some workers in the field as a reliable prognostic marker equivalent to IgVH gene mutation status (Crespo *et al.*, 2003; Orchard *et al.,* 2004). Figure 3.1

compares the prognostic significance of the three major prognostic markers (CD38, ZAP-70, IgVH mutations) on the survival of B-CLL patients.

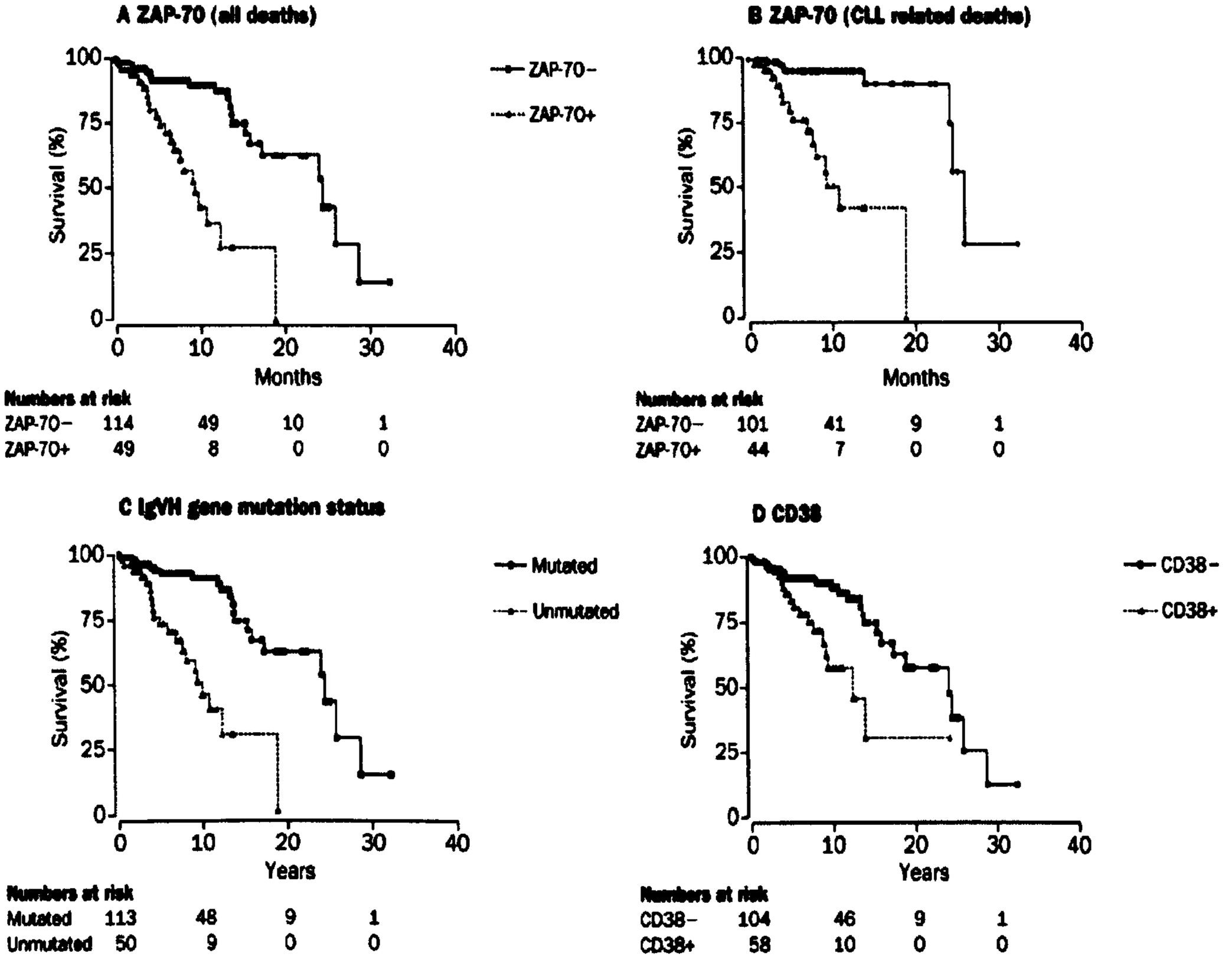


Figure 1.3. Effects of intracellular expression of ZAP-70 (A and B), *IgVH* gene mutation (C), and surface expression of CD38 (D) on survival of B-CLL patients

Kaplan-Meier curves indicating the poor prognosis of B-CLL cells when positive for intracellular ZAP-70, having UMut IgVH genes and positivity of CD38 and comapring those with survival time in months (ZAP-70) and years (UMut IgVH genes and CD38).
(from Orchard *et al.*, 2004)

It was found that in comparison to CD38, ZAP-70 expression is correlated with the mutational status of B-CLL cells and is preferentially associated with unmutated IgVH genes (Crespo *et al.*, 2003; Chen *et al.*, 2005). This association is based both on flow cytometry results as well as gene expression profiles that indicate UMut CLL cells to express more ZAP-70 mRNA than M leukaemic cells (Rosenwald *et al.*, 2001; Orchard *et al.*, 2004). These observations have also a practical value since they indicate that the laborious and time consuming sequencing of IgVH genes may

simply be replaced by ZAP-70 analysis using controlled and standardised flow cytometry proceedures.

Furthermore, an intrinsic functional relationship between CD38 and ZAP-70 has been identified for B-CLL cells, and the combined expression of CD38 and ZAP-70 is characteristic for patients with particularly aggressive disease (Deaglio *et al.*, 2008). Throughout the advances in our understanding of the immunobiology of B-CLL, the use of other prospective prognostic markers have been suggested although these have yet to gain the status required for their establishment into clinical practice. A number of these putative markers are summarised in the following sections.

1.3.4 Chromosomal aberrations

There are a number of chromosomal aberrations associated with B-CLL, the most ominous being related to the mutation of the p53 gene coding for a transcription factor which is activated by breaks in DNA to induce apoptosis or cell cycle arrest (Ko *et al.*, 1996; Levine *et al.*, 1997). This transcription factor enhances DNA repair or eliminates those cells which have damaged DNA, thus maintaining the integrity of the genome (Ke *et al.*, 2002). In view of the fact that p53 mutations are associated with the development of many different cancers of various organ and tissue origin, it is not surprising therefore that mutation in p53 is found also to be linked with adverse clinical outcomes in B-CLL (Figure 1.4) (Rouby *et al.*, 1993; Wattel *et al.*, 1994).

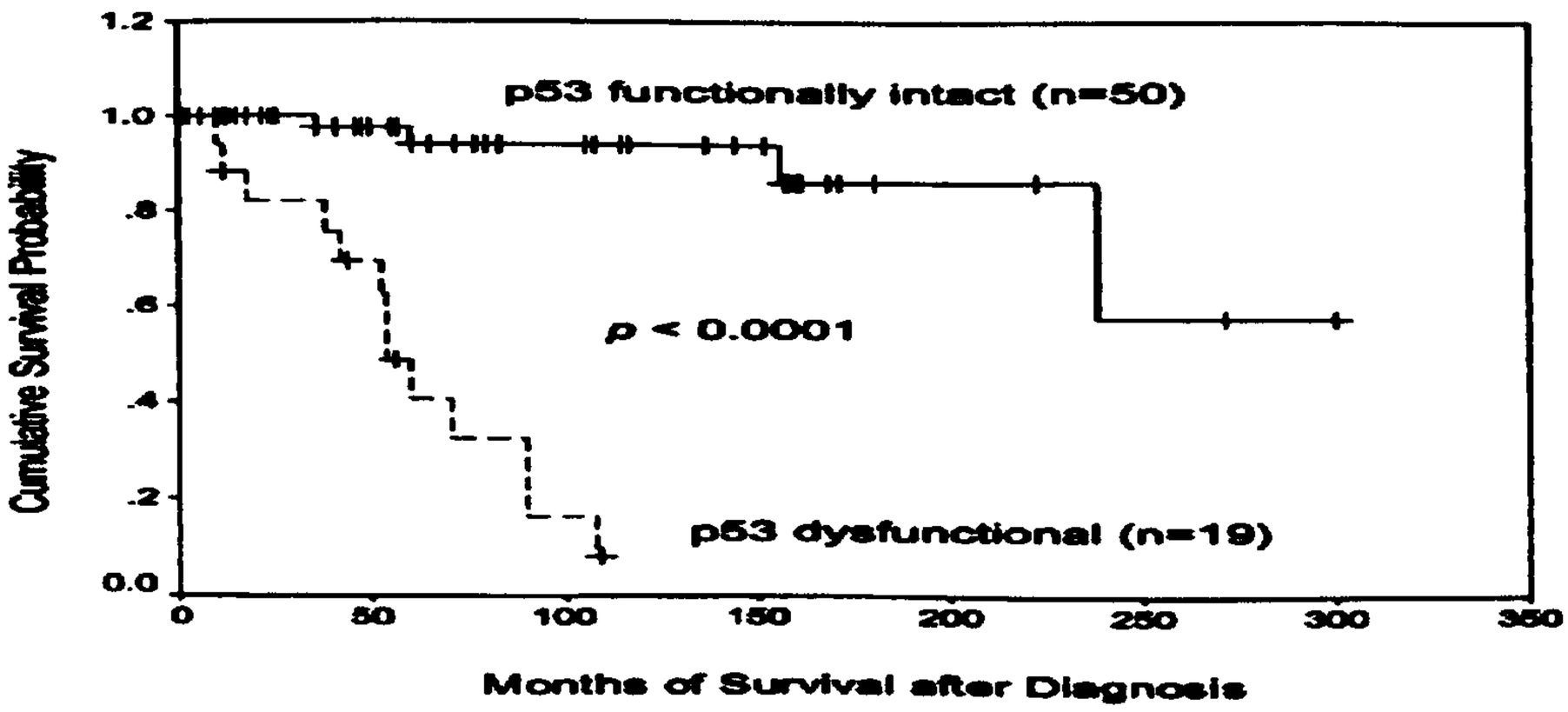


Figure 1.4. Effects of the expression of p53 on survival of B-CLL patients

Kaplan-Meier curve showing the cumulative survival probability with advancing months, after diagnosis of B-CLL, with functionally intact p53 and dysfunctional p53 (from Ke *et al.*, 2002).

Two types of dysfunction in the p53 gene in B-CLL have been described by Stancovic *et al.*, (1999). One is a mutation in the p53 gene itself which is located on chromosome 17p13 and the other is inactivation of the gene coding for ATM, a p53 regulating kinase located on chromosome 11q22-23.

These deletions are more frequent in UM B-CLL cells and have negative prognostic value. On the contrary, isolated deletion of the 13q14 gene is actually associated with a relatively good prognosis (Esparza *et al.*, 2002; Filip *et al.*, 2003; Martin, 2004). This region contains a non-transcribed gene and two micro-RNA genes (Ibrahim *et al.*, 2001). These micro-RNAs regulate functions of many genes in various cancers (He *et al.*, 2004) including B-CLL where they are implicated in supporting additional mutations in VH genes and hence, are associated with better prognosis (Calin *et al.*, 2004).

Trisomy of chromosome 12 is also seen in some cases of B-CLL and is associated with progressive lymphocytosis, resistance to chemotherapy and a fatal clinical course. Interestingly, it is not found in the early stages of the disease and to date it is the only genetic abnormality specifically associated with the final stages of B-CLL (Esparza *et al.*, 2002). Another gene potentially contributing to pathogenesis of B-CLL is TCL-1 (Narducci *et al.*, 2004). TCL-1 is located at 14q32.1 and is involved in the establishment of T cell lymphocytic leukaemia (Virgilio *et al.*, 1994) and has since been implicated in the development of a CLL-like syndrome in mice (Bichi *et al.*, 2002).

1.3.5 Telomeres and the expression of human telomerase reverse transcriptase (hTERT)

Since B-CLL cell survival is associated with their selection and proliferation, factors supporting or limiting cell cycle, such as telomere length and telomerase activity are of a particular interest.

Telomerase is a ribonucleoprotein complex that is involved in maintaining telomere length by the addition of hexameric TTAGGG repeats at the chromosomal ends which compensates for the continuing loss of telomeres (Tchirkov *et al.*, 2004). The

acquisition of the hTERT expression is an essential step in the development and progression of B-CLL (Stewart *et al.*, 2000). Telomerase activity is regulated by the expressional level of the hTERT gene which codes for the catalytic subunit of telomerase (Pool *et al.*, 2001) and requires functional hTERT protein (Blackburn, 2000). It has been shown previously that telomerase activity is increased in activated normal and leukaemic B cells (Igarashi and Sakaguchi., 1997; Weng *et al.*, 1997; Damle *et al.*, 2004).

More recently it has been demonstrated that CD38+ B-CLL cells possess higher telomerase activity than their CD38- counterparts although both populations have similar telomere lengths (Damle *et al.*, 2007). In addition, Tchirkov *et al* (2004) have shown that the level of hTERT was seven-fold higher in the patients with UMut IgVH genes.

Taken together, these findings contribute towards our understanding of the biological reasoning underlying the poor prognostic value of CD38.

1.3.6 Reliability of prognostic markers

Even with the established and effective prognostic markers described above, several aspects require clarification. Firstly, there is the question of how reliable is the prognosis when each individual marker is used as a sole predictor of the disease course. It is particularly important in the earlier stages of the disease to make an exact prognosis that will facilitate the selection of a correct mode of treatment and management.

Several further questions can be raised. For example, should multiple prognostic markers be used and, is it possible to replace IgVH gene sequencing with a standardised ZAP-70 protocol? Burger (2007) suggested that the combined expression of CD38 and ZAP-70 may have a better prognostic value since ZAP-70 actually integrates the signalling downstream from BCR and CD38 (Figure 1.5).

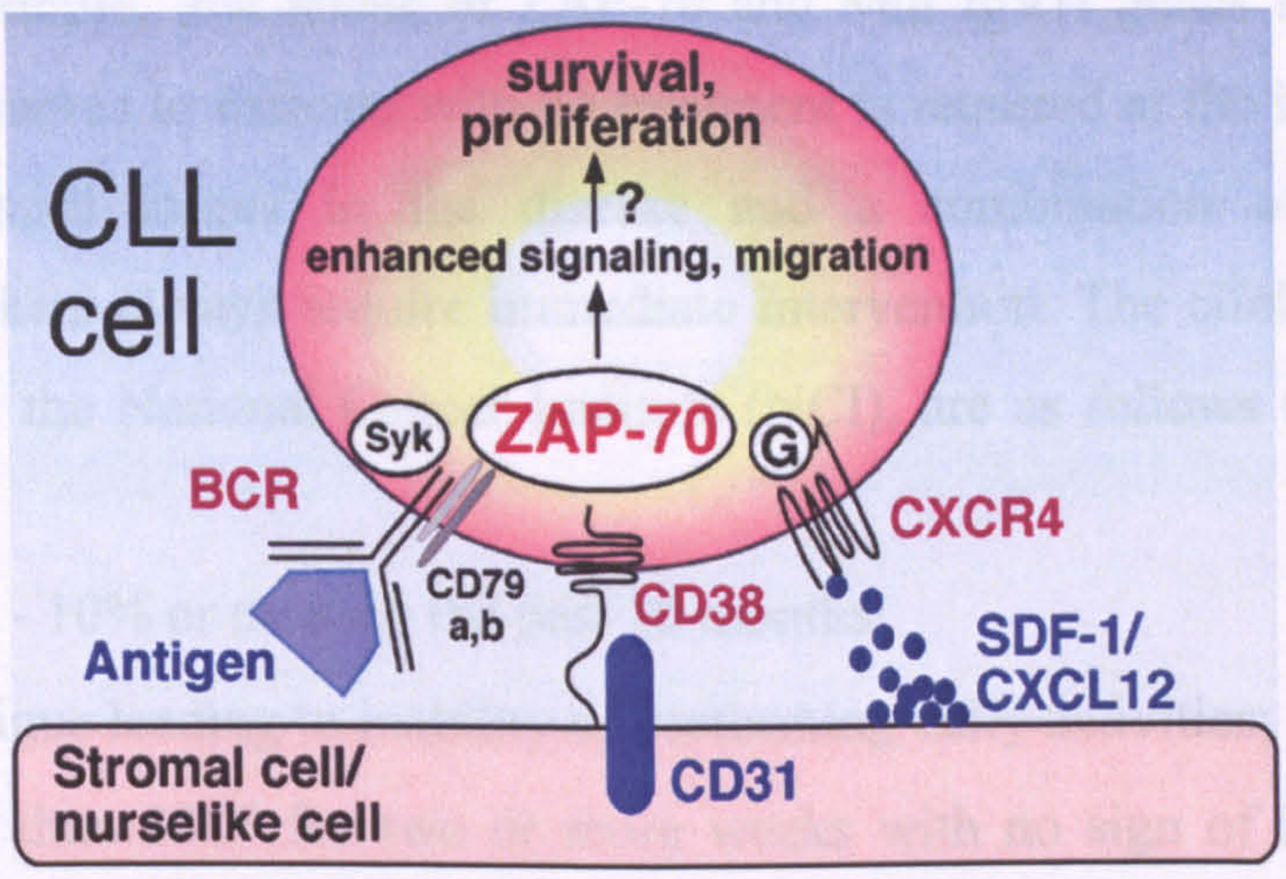


Figure 1.5. Integration of ZAP-70 with BCR and CD38 leading to growth and survival of B-CLL cells.

ZAP-70 integrates BCR, CD38, and CXCR4 signaling, which then allows ZAP-70^+/$CD38^+$ B-CLL cells to reside in a stromal microenvironment where they receive growth and survival signals. Ligation of CD38 by its ligand CD31, which is expressed on stromal and nurse-like cells, induces ZAP-70 phosphorylation leading to increased ZAP-70^+ B-CLL cell migration in response to SDF-1/CXCL12 secreted by stromal and nurse-like cells through increasing CXCR4 chemokine receptor signaling downstream of CXCR4-associated G proteins. Also, ZAP-70 increases the capacity of B-CLL cells to respond to antigen by the recruitment of ZAP-70 and Syk to the cytoplasmic portion of the activated BCR complex (CD79 a/b). (Burger, 2007)

In addition, some newly proposed prognostic markers may be taken more into consideration upon further accumulation of relevant clinical data. One example could be the pattern of expression of CD180 on B-CLL cells. A recent study by Porakishvilli *et al* (2005) has shown that CD180 is highly expressed by B-CLL cells with Mut IgVH genes whilst UMut B-CLL cells expressed lower levels of this molecule. Expression of sIgM has demonstrated a reverse pattern. The authors suggest that a combination of the surface expression of sIgM and CD180 may serve as a good surrogate marker for the mutational status of B-CLL cells.

It is clear that the use of prognostic markers facilitate our understanding of the molecular mechanisms underlying the disease which, in turn, may lead to the development of new therapeutic approaches.

1.4 Clinical indications for the general treatment of B-CLL

In B-CLL, the major consideration to be taken into account is when to start any therapeutic treatment. This depends on the stage and prognostic markers of the disease, for example, low levels of ZAP-70 and Mut IgVH genes indicate a good prognosis and serves to indicate with no treatment is required at the initial stages. In contrast, advanced stages in the disease and a combination of unfavourable prognostic markers always require immediate intervention. The clinical indications, as specified by the National Cancer Institute (NCI), are as follows (Winkler *et al.*, 1999),

- Weight loss - 10% or more in the past 10 months;
- Extreme fatigue leading to inability of performing daily activities;
- Fever more than 38C° for two or more weeks with no sign of infection. Night sweats without any infection;
- Development or worsening of anaemia or thrombocytopenia in bone marrow;
- Haemolytic anaemia or thrombocytopenia refractory to corticosteroid treatment;
- Massive or progressive splenomegaly (>6cm below costal margin);
- Massive or progressive Lymphadenopathy (>10cm in the longest diameter);
- Progressive lymphocytosis i.e. an increase of >50% in two months (Cheson *et al.*, 1996).

Thus, asymptomatic patients in the early stages of the disease such as Rai 0-II or Binet A, generally are not treated but are carefully monitored by a "wait and watch" strategy (Hallek, 2005).

1.5 Specific treatment of B-CLL

1.5.1 Use of chemotherapeutic agents:

Initially the treatment of CLL was based on the use of alkylating agents including chlorambucil (phenylbutyric acid nitrogen mustard) and cyclophosphamide. It was shown that chlorambucil induces cell death in CLL cells through a p53-dependent pathway (Begleiter *et al.*, 1996). Combinations of chlorambucil and prednisone were considered the benchmark for CLL therapy (Keating *et al.*, 1998). Chlorambucil used alone or with prednisone produced initial response rates between 60% and 90%, and

a complete remission of 60% in all patients. Its efficiency depends on the dose administered and response criteria (Rai *et al.*, 2000; Robak *et al.*, 2000). Chlorambucil usually reduces the WBC count, decreases lymphadenopathy and splenomegaly, but rarely returns the bone marrow to normal.

A major change occurred with the discovery of purine analogues such as fludarabine monophosphate (Fludara), 2-chlorodeoxyadenosine (2-CDA), and pentostatin (DCF, deoxycoformycin), which are potent inhibitors of DNA repair (Dillman *et al.*, 1989; Keating *et al.*, 1998). Fludarabine is more effective in terms of complete remission rate as compared to chlorambucil. The combination of fludarabine, mitoxantrone and cyclophosphamide renders a high response rate (Rai *et al.*, 2000).

The use of multiple courses of purine analogue therapy in patients can cause bone marrow suppression leading to anaemia, neutropenia, thrombocytopenia, which ultimately, has limited the use of this type of therapy in B-CLL (Robak *et al.*, 2000; Rai *et al.*, 2000). In addition, conventional therapy with purine analogues typically leads to drug resistance and the disease remains incurable. Hence, there is an urgent need for new and novel modes of treatment to complement or replace existing therapy.

1.5.2 Use of Immunotherapeutic agents

Several groups are currently pursuing immunotherapeutic strategies designed to generate anti-leukaemic immune responses *in vivo*. Numerous studies *in vitro* have been made with the aim to overcome the reluctance of B-CLL cells to apoptose *in vitro*, these including ligation of the BCR (Zupo *et al.*, 1996), CD5 (Pers *et al.*, 2002), CD20 (Golay *et al.*, 2001), and more recently, targeting B-CLL cells by autologous cytotoxic cells mediated by bispecific antibodies (Porakishvili *et al.*, 2004). Some of the monoclonal antibodies used in *in vitro* experiments have been successfully translated into clinical practice. The first monoclonal antibody (mAb) which was approved for the clinical treatment of B-CLL was rituximab, directed to the CD20 phosphoprotein expresssed on B cells. However, clinical trials have observed a low responsiveness in B-CLL patients to rituximab treatment, possibly as a result of the low density of CD20 on B-CLL cells. Some studies have suggested the

low response was due to the conventional low dose (3.75mg/m^2 per week for 4 weeks) used for rituximab (Huhn *et al.*, 2001). Subsequently two approaches were suggested to overcome the low response rate to rituximab. The first approach was to triple the once a week dose schedule (O'Brien *et al.*, 2001) and, the second was to administer the conventional dose three times a week (Byrd *et al.*, 2001). In both cases the response rate increased up to 40%.

The mode of action of rituximab is unclear. Antibody-dependent cellular cytotoxicity (ADCC), direct apoptosis of CD20+ B cells and/or clearance of the formed immune complexes has all been proposed (Shaw *et al.*, 2003). Recently a humanized mAb against CD20 has been introduced for clinical use. The products include human mAb (HuMax CD20) and IMMU-106 (hA20) both having >90% humanized framework, these being complemented by a novel third generation fully humanized and optimized mAb, GA-101 (Robak, 2008).

Another antibody currently used successfully to treat B-CLL is alemtuzumab (Campath-1H), a humanised recombinant IgG1 mAb which targets the human CD52 antigen found ubiquitously on B cells, T cells, monocytes and macrophages, NK cells, a subset of granulocytes as well as CD34+ cells (Keating *et al.*, 2003; Alinari *et al.*, 2007). Alemtuzumab was approved for the market in 2001 to be used particularly for fludarabine refractory CLL. The antibody induces cell lysis through ADCC. The response rate appears to be quite variable since in patients heavily treated previously with fludarabine the overall response rate was approximately 35%, whilst in previously untreated patients it was 80% (Alinari *et al.*, 2007). More importantly, this antibody was found to be effective for CLL patients with poor prognosis, particularly those with 17p13 and 11q22 deletions and is particularly useful for the treatment of low amounts of residual tumour, known as minimal residual disease (MRD) (Alinari *et al.*, 2007). When treated with alemtuzumab, most of the patients become MRD negative in the blood and bone marrow as measured by flow cytometry and PCR (Keating *et al.*, 2003; Montillo *et al.*, 2005) which leads to an improvement in overall and treatment free survival (Moreton *et al.*, 2005). It is also given effectively to patients in preparation for stem cell transplantation to prevent acute and chronic graft versus host disease (Alinari *et al.*, 2007).

However, some side-effects have been detected usually when the drug is administered intravenously. Ranging in severity these adverse reactions include myelosuppression and also a profound cellular immune dysfunction often associated with an increased risk of viral reactivation and other opportunistic infections. The drug is better tolerated when administered subcutaneously (Keating *et al.*, 2003; Alinari *et al.*, 2007).

It is still unclear why therapeutic antibodies sometimes do not work. The suggested reasons are: (a) low density of targeted tumour antigens on the cell surface due to antigen modulation (internalisation, shedding or shaving of the antigen); (b) soluble antigen in serum (such as CD23 in B-CLL); (c) insufficiency of effectors; (d) resistance to complement-mediated lyses and/or apoptosis; (e) immunological responses to antibodies and (f) tumours consuming more antibodies than expected (Hamblin, 2006).

1.5.3 Use of Chemo-immunotherapeutic agents

Although more therapeutic antibodies are coming into research and clinical trials but to date none appear completely successful when used as a sole therapy. The current approach to the treatment of B-CLL is therefore a combination of chemotherapy and immunotherapy. Based on, *in vitro,* studies investigating potential synergistic activities, combinations of rituximab and fludarabine therapy have been implemented successfully into clinical trials where a complete response was achieved with no side-effects other than neutropenia (Byrd et al., 2003). A combination of alemtuzumab with fludarabine has also shown promising results in patients shown previously to be refractory to the purine analogue alone (Kennedy *et al.*, 2002).

The largest proportion of complete responses (response rate of 95% with complete remission rate of 69%) has been achieved through use of FCR treatment (fludarabine, cyclophosphamide and rituximab) (Keating *et al.*, 2003). A recent report has indicated that using FCR therapy, the six years overall survival rate was 77% and failure-free survivals were 51% (Tam *et al.*, 2008). Patients on FCR regimen have confirmed the association between both the quality of the life achieved and the clinical outcome (Rawstron *et al.*, 2007). However, patients with chromosome 17 abnormalities are resistant to this treatment. Very often FCR treatment prevents

MRD (Montserrat, 2005) and can even change the natural history of B-CLL by abrogating poor prognostic significance of well established markers (Rawstron *et al.*, 2007). The side effects from FCR therapy include secondary myelodysplasia and eventual relapse. It is still unclear if FCR should be given to all patients as first-line treatment, whether or not it is possible to decrease the number of adverse reactions and, importantly, the question if re-treatment with FCR after relapse is safe.

1.5.4 Stem cell Transplantation

With so many questions still remaining regarding the use of chemotherapeutic agents, a radical intervention such as stem cell transplantation for B-CLL management may be considered very useful for the future. B-CLL patients have been successfully transplanted with autologous stem cells subsequently becoming MRD negative (Esteve *et al.,* 1998). Due mainly to the advanced age of patients undergoing transplantation, the development of graft versus leukaemia reactions in allogeneic stem cell transplant is clearly present. However, the clinical outcome can be highly successful and one study by Schetelig *et al* (2003) demonstrated that 8 out of 12 patients became MRD negative following transplantation.

1.5.5 Novel approaches to treatment

Some new therapeutic options have been recently suggested. Flavopiridol, a broad spectrum cyclin-dependent kinase inhibitor, currently undergoing phase II clinical trials has shown some preclinical activity in B-CLL although to date its activity is modest in B-CLL patients (Byrd, 2007). Another compound targeting the cell cycling is mTOR (mammalian target of rapamycin) which has entered phase II pilot clinical studies for patients with advanced B-CLL (Decker *et al.*, 2008).

Anti-telomerase antibodies are currently in clinical trials in the USA (N.Porakishvili, personal communication). Further potential immunotherapeutic targets include proliferating cells such as CD38+ B cells as well as BCR-mediated and ZAP-70-mediated signal transducers.

An important prospective treatment is T-cell based immunotherapy of B-CLL. One of the more promising approaches is the genetic manipulation of effector T lymphocytes to express tumour specific chimeric T cell antigen directed towards

antigens on the surface of the B-CLL cells (Foster *et al.*, 2008). Enhancement of the induction of apoptosis of B-CLL cells still remains an attractive target for the development of novel approaches for the treatment of the disease.

1.6 Toll- like receptors (TLRs)

Yamashita *et al* (1996) showed that activation through RP105, but not CD40, rendered murine B cells susceptible to IgM-induced apoptosis. RP105 (radiation protection protein 105) and its human analogue, CD180, belong to the toll-like receptor (TLR family).

TLRs are a group of receptors essential for host defence against microbes. They bind various bacterial, fungal and viral structures (Shizuo, 2003). Toll-like receptors sense pathogen elements termed 'pathogen associated molecular patterns' (PAMPs) as a part of innate immunity. TLRs also bind to so called DAMPs (damage associated molecular patterns) originating from tissue damage of different origin such as degradation products, heat-shock proteins, inflammatory factors or oxidised molecules (Divanovic *et al.*, 2007).

To date, thirteen types of TLRs have been identified in humans (Du *et al.*, 2000, Chuang *et al.*, 2000, Tabeta *et al.*, 2004). TLRs are type 1 integral membrane glycoproteins, with molecular weights ranging from 90-115kD. The presence of an extracellular domain containing leucine rich repeats (LRR) and a cytoplasmic toll/IL-1 receptor (TIR) domain, similar to that of IL-1, is characteristic of the TLR family (Shizuo, 2003). Individual TLRs recognise different and distinct structural components of a pathogen. For example, cell surface TLRs including TLR4, TLR1//TLR2, TLR6/TLR2, TLR10 and TLR11 recognise microbial membrane lipids whereas other TLRs reside in the intracellular organelles including TLR3, TLR7, TLR8 and TLR9 which recognise microbial nucleic acids. It is not yet known which component of a pathogen is recognised by TLR12 and TLR13 (Doan *et al*, 2007; Akashi-Takamura and Miyake, 2008).

TLR4 has been found to share strong homology with surface receptor CD180 (Divanovic *et al.*, 2007) both being predominantly expressed on macrophages, dendritic cells and B cells. Ligation of TLR4 induces murine B cells to undergo

activation, proliferation, class switching and differentiation into plasma cells (Madzhitov, 2001; Stanford, 2005).

TLR4 is essential for lipopolysaccharide (LPS) signalling since TLR4 knock-out mice were hyporesponsive to LPS. For signaling TLR4 requires another molecule, MD-2, which is associated with its extracellular domain (Hirotaka *et al.*, 2000; Shizuo, 2003). Despite all the data there is no evidence of direct binding of LPS to TLR4. To recognize LPS, B cells utilise a heterodimer which consists of RP105/MD-1 (CD180/MD-1), structurally related to the TLR4/MD-2 complex (Schnare *et al.*, 2001; Stanford, 2005).

1.7 RP105/MD-2 (CD180/MD-1) on B cell surface

The molecule CD180 was originally identified on human B cells by a monoclonal antibody (mAb) designated Bgp95 (Valentine *et al.*, 1988). Subsequently, CD180 was found on naïve B cells but not on germinal center (GC) B cells (Otipoby *et al.*, 2002). Independent studies in mice defined a molecule, RP105 (radiation protection factor 105), with leucine rich repeat sequences on murine B cells that, on ligation, protected splenic B cells from radiation- and dexamethasone-induced apoptosis (Miyake *et al.*, 1995).

1.7.1 Molecular type and antigen expression

The human analogue of RP105 is CD180, a type 1 glycoprotein comprising 661 amino acids with molecular weights ranging between 95-105kD. Sharing 74% homology with RP105, CD180 is found on the majority of B cells with strong expression on mantle and marginal zone B cells, but weak expression on germinal centre B cells (Miura *et al.*, 1998). It is also expressed on B cell subsets, peripheral blood monocytes (Zarember *et al.*, 2002), dendritic cells (Kadowaki *et al.*, 2001) and macrophages (Divanovic *et al.*, 2007). CD180, like RP105, is a cell surface molecule comprising 22 tandemLy repeated extracellular leucine-rich repeats (LRR) and a short cytoplasmic domain of 6 to 11 amino acids (Miyake *et al.*, 1995; Divanovic *et al.*, 2007). Conserved cysteine residues are also present which are essential for signal transduction also for RP105 (Miyake *et al.*, 1995). The extracellular portion of RP105 (see below) has an association with a molecule known as MD-1, an analogue

of MD2, which forms a complex with RP105 in mice and, presumably, CD180 on human B cells.

1.7.2 Signalling via the RP105/CD180 receptor

A natural exogenous or endogenous ligand for CD180 is unknown. However, binding of RP105 to specific antibodies induces Lyn activation leading to CD19 phosphorylation. CD19 upon phosphorylation augments Lyn activity of Lyn kinase and mediates the Lyn and Vav interaction, which is considered crucial for JNK activation. PI3K and NFκB activation by RP105//CD180 binding is independent of CD19 (Figure 1.6). This is consistent with the fact that CD19 deficient human B cells have diminished proliferation following CD180 ligation (Yazawa *et al.*, 2003).

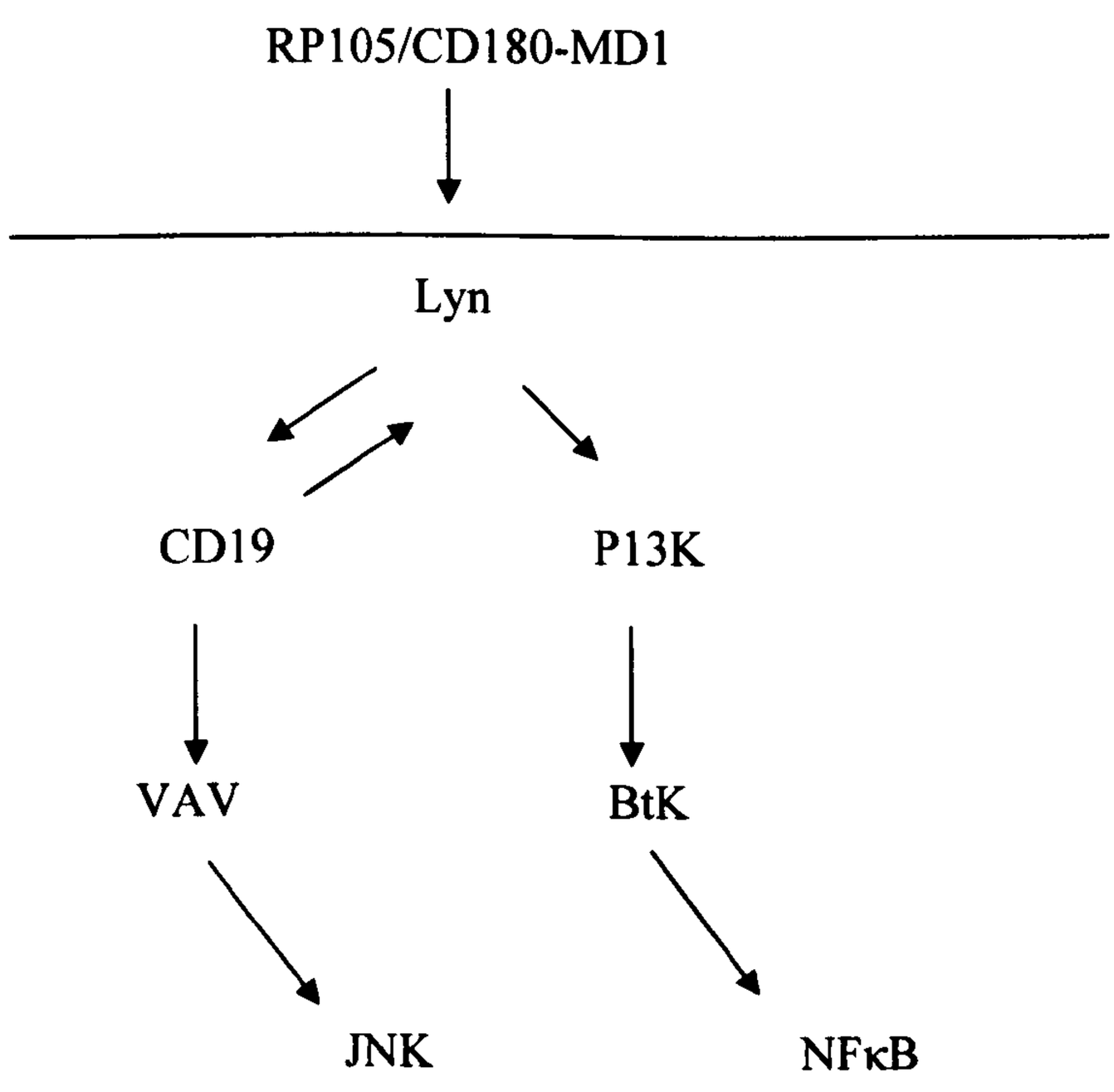


Figure 1.6. Signalling pathway for RP105/MD-1

RP105/CD180 on binding to its ligand, induces Lyn activation and CD19 phosphorylation, which leads to augmentation of Lyn kinase activity and also mediates interaction of Lyn with Vav, leading to activation. (adapted from Yazawa *et al.*, 2003).

1.7.3 Function of RP105/CD180

CD180 is expressed on all normal naïve and memory B cells (Kazuna, 2004). The ligation of CD180 on human B cells by its corresponding antibodies leads to mobilisation of intracellular calcium, expression of c-Myc, entry into the cell cycle (Valentine *et al.*, 1988; Roshak *et al.*, 1999) activation, proliferation and enhanced life span. (Agematsu, 2004).

B cells are driven into activation, proliferation and differentiation into plasma cells. It appears that B cell activation decreases the expression of CD180 since GC B cells do not express CD180 and many activated B cells from patients with rheumatological diseases are negative for CD180 (Koarada *et al,* 1999, 2001; Otipoby *et al.*, 2002).

On ligation in naïve B cells it enhances RP105-MD-1 and has been found to have some regulatory effects as a specific inhibitor of TLR4 signalling in HEK (human embryonic kidney) 293 cells (Divanovic *et al.*, 2005). This inhibition is independent of the intracellular part of RP105. Interestingly, by using co-immunoprecipitation (co-IP) techniques, it has been demonstrated that there is a physical association between the TLR4-MD-2 complex and RP105-MD-1. MD-1 and MD-2 were also co-expressed bidirectionally when studied by co-IP techniques which suggests that MD-1 and MD-2 heterodimerization is providing a point of interaction between both the TLR4-MD-2 and the RP105-MD-1 complexes (Divanovic *et al.*, 2005).

It has been shown that LPS binds directly to MD-2 to form a LPS/MD-2 complex which then associates with TLR4 to initiate signalling. However, the co-expression of RP105-MD-1 inhibits LPS-TLR-MD-2 complex formation (Divanovic *et al.*, 2007) thus providing a direct evidence of RP105-MD-1 inhibition of LPS signalling complex formation (Figure 1.7). This is consistent with the data provided by Tsuneyoshi, (2005) who observed that no direct interaction is present between LPS and RP105 indicating that RP105-MD-1 mediated interference with LPS is not the result of RP105-MD-1 (Divanovic *et al.*, 2005).

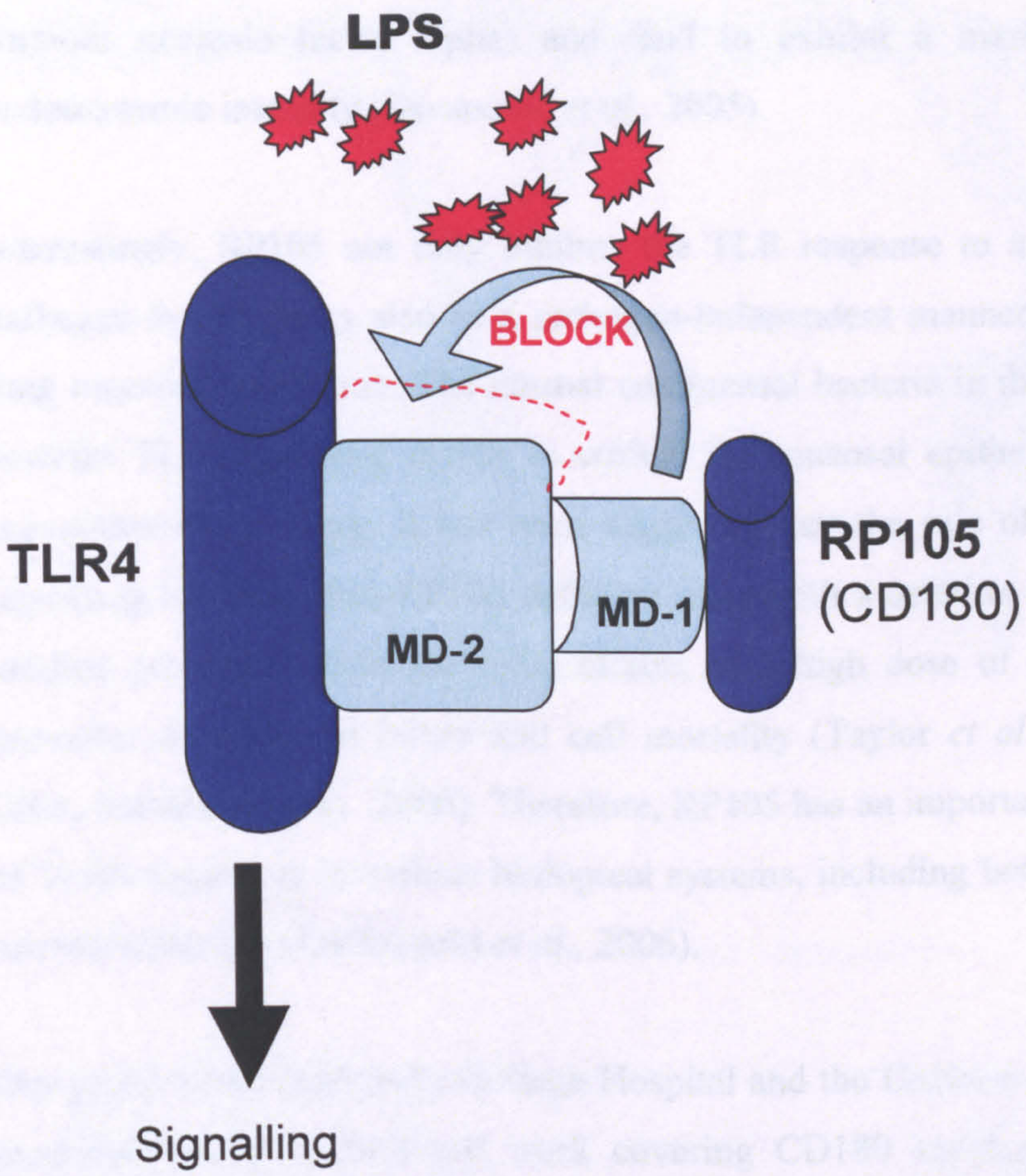


Figure 1.7. A model for direct interaction of RP105-MD-1 with the TLR4-MD-2 complex showing inability of the microbial agent (LPS) to bind MD-2.

The CD180-MD-1 complex directly and physically blocks LPS binding to the TLR4-MD-2 complex, therefore regulating the function of TLR4 (adapted from Divanovic *et al.*, 2007).

In sum, this current model shown in Figure 1.7 suggests that RP105-MD-1 is directly interacting with the TLR4-MD-2 signalling complex to result in a reduced ability of the microbial ligand to interact with the TLR4-MD-2 complex (Divanovic *et al.*, 2007).

It has been shown by the use of genetic models that RP105 regulates not only LPS-driven TLR4 signalling in B cells, but also that in primary mouse dendritic cells and macrophages. In this case, RP105 inhibits the production of cytokines dependent on both MAL/MyD88 pathways (Divanovic *et al.*, 2005). It is suggested that RP105 is

a physiological monitor of the *in vivo* response to LPS since RP105 deficient mice exposed to LPS treatment produce significantly elevated levels of systemic TNFα (tumour necrosis factor alpha) and tend to exhibit a marked increase in the endotoxaemic intensity (Divanovic *et al.*, 2005).

Interestingly, RP105 not only inhibits the TLR response to a particular microbial pathogen but it works also in a pathogen-independent manner in both the gut and lung mucosal epithelium. The normal commensal bacteria in the gut and lung tissue activate TLR signalling which is critical for mucosal epithelial homeostasis and prevention from injury. It has been suggested that the role of RP105 is to inhibit signalling by TLR, thus RP105 deficient mice with a deletion of RP105 gene show marked protection from the toxic effects of a high dose of bleomysin, a known promotor of epithelial injury and cell mortality (Taylor *et al.*, 2004; Jiang *et al.*, 2005; Scheibner *et al.*, 2006). Therefore, RP105 has an important role as an inhibitor of TLR4 signalling in various biological systems, including both microbial and non-microbial models (Gorczynski *et al.*, 2006).

Our group at the University College Hospital and the University of Westminster has produced the only published work covering CD180 expression on B-CLL cells (Porakishvili *et al.*, 2005). In this work CD180 was observed to be heterogeneously and stably expressed on B-CLL cells with a significantly higher expression on M B-CLL clones. On the contrary the expression of sIgM was significantly higher on B-CLL cells with UM IgVH genes.

1.8 The B cell Receptor (BCR) complex

As shown in Figure 1.8, the BCR complex is comprised of the antigen receptors IgM and IgD, associated with two polypeptides, Igα and Igβ, also known as CD79a and CD79b respectively. Surface immunoglobulins are transmembrane molecules although their intra-cytoplasmic part is only a few amino acids long (Stevenson and Caligaris-Cappio *et al.*, 2004), thus using CD79a and CD79b molecules for signalling through immunoreceptor tyrosine-based activation motifs (ITAMs).

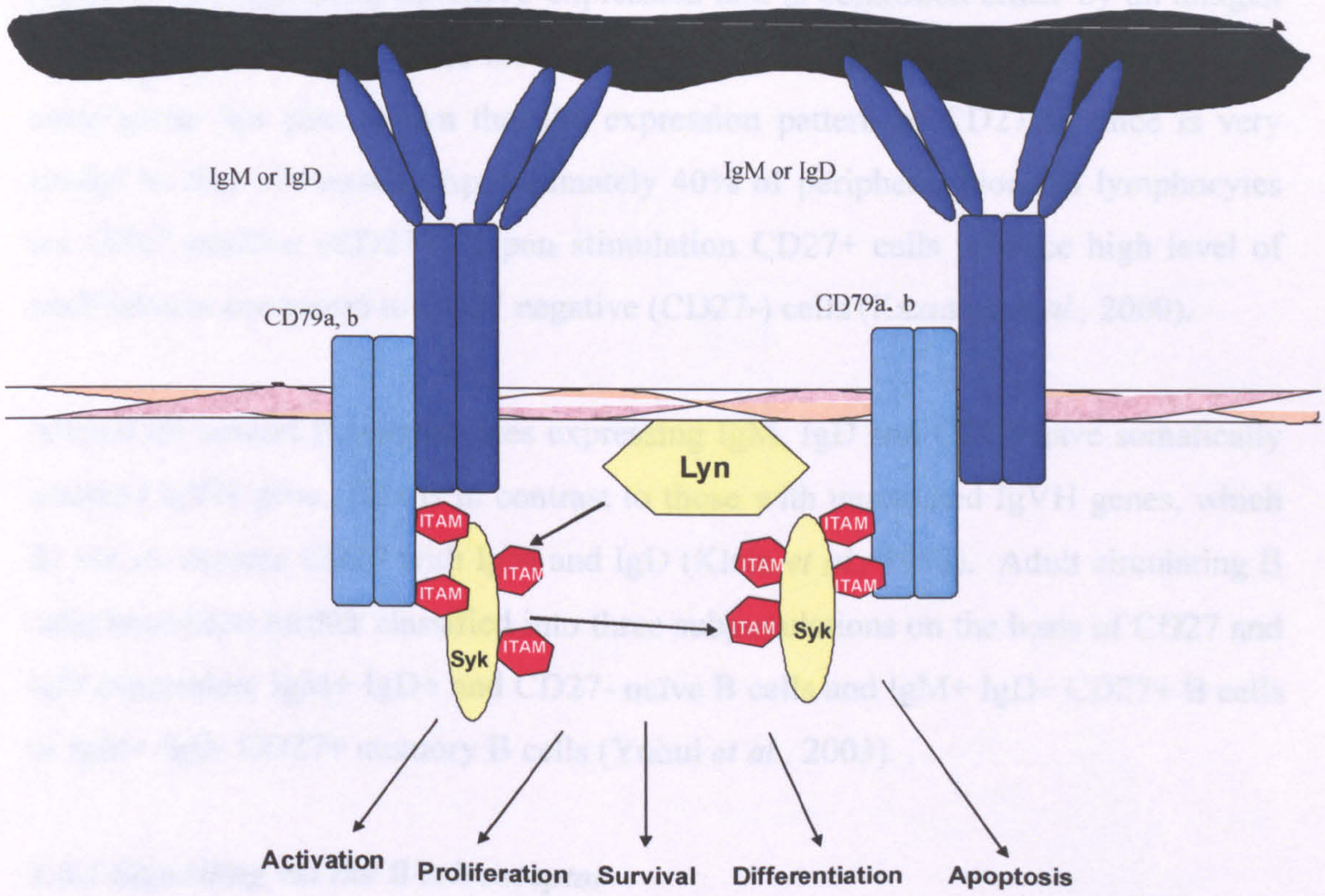


Figure 1.8. BCR ligation by the antigen leading to phosphorylation of ITAMs.
The BCR complex consists of a surface immunoglobulin of isotype IgM or IgD and the associated molecules CD79a/b. The signals generated on BCR ligation lead to the activation and phosphorylation of the ITAMs (immunoreceptor tyrosine-based activation motifs) on CD79a/b which further recruits the tyrosine kinase Syk. Syk gets phosphorylated by Lyn. On full activation, Syk propagates signals via several signalling pathways and depending on the stage of maturation and/or activation of the B cell, the outcome may differ (adapted from Efremov *et al.*, 2007).

All mature naïve splenic B cells are positive for surface IgM (sIgM) and surface IgD (sIgD), while immature B cells express sIgM combined with variable surface expression of IgD. This differential expression suggests that sIgM and sIgD may be quantitatively transmitting different signals (Norvell *et al.*, 1996; Peckham *et al.*, 2000). To evaluate the level of sIgM and sIgD expression on memory B cells, co-expression of CD27 is normally utilised.

CD27 is a type I glycoprotein expressed on memory B cells and the majority of T cells. It is a member of the tumour necrosis factor (TNF) receptor family. It has been

shown by Borst *et al.,* (2005) that murine B cells express CD27 on priming. The expression is dependent on CD70 expression and is controlled either by an antigen receptor or toll like receptors on T cells, B cells, dendritic cells or NK cells. This same group has also shown that the expression pattern of CD27 in mice is very similar to that of humans. Approximately 40% of peripheral blood B lymphocytes are CD27 positive (CD27+). Upon stimulation CD27+ cells produce high level of antibodies as compared to CD27 negative (CD27-) cells (Kaznaga *et al.,* 2000).

Almost all normal B lymphocytes expressing IgM, IgD and CD27 have somatically mutated IgVH gene. This is in contrast to those with unmutated IgVH genes, which do not co-express CD27 with IgM and IgD (Klein *et al.,* 1998). Adult circulating B cells have been further classified into three subpopulations on the basis of CD27 and IgD expression. IgM+ IgD+ and CD27- naïve B cells and IgM+ IgD+ CD27+ B cells or IgM+ IgD- CD27+ memory B cells (Yuhui *et al.,* 2003).

1.8.1 Signalling via the B cell receptor

No significant differences between sIgD and sIgM signal transduction have been found. However, the small resting B cell population expresses more IgD than IgM, thus its cross linking by antibodies can cluster more Igα and Igβ than IgM. Furthermore, the binding affinity for surface Ig is different for different antibodies, which can also affect the signalling (Peckham *et al.*, 2000). Ligation of the BCR leads to activation of CD79a and CD79b (Figure 1.9) whereupon the ITAMs of the latter molecules are phosphorylated by the tyrosine kinase, Syk, to initiate the process of activation. Both CD79a and CD79b molecules then associate with the enzymes involved in phosphorylation within lipid rafts (Efremov *et al.*, 2007). This includes activating the important effector enzymes phosphatidyl 3-kinase (P13K) and phospholipase Cγ2 (PLCγ2). The second messenger phosphatidylinositol-3, 4, 5-triphosphate is generated by P13K which then recruits other BCR molecules as well as to activate the kinase, Akt. PLCγ2 activation leads to the intracellular release of calcium (Ca^{2+}) and activation of protein kinase C (PKC), considered to be crucial for the activation of mitogen-activated protein kinases (MAPKs). Shown in Figure 1.9, these MAPKs include extracellular signal regulated kinase (ERK), c-JUN NH_2-terminal kinase (JNK) and p38 MAPK, and transcription factors nuclear factor κB

(NF-κB) and nuclear factor of activated T cells (NFAT). The fate of B cells is subsequently determined by a fine balancing act between these signalling molecules (Aleksander *et al.*, 2005).

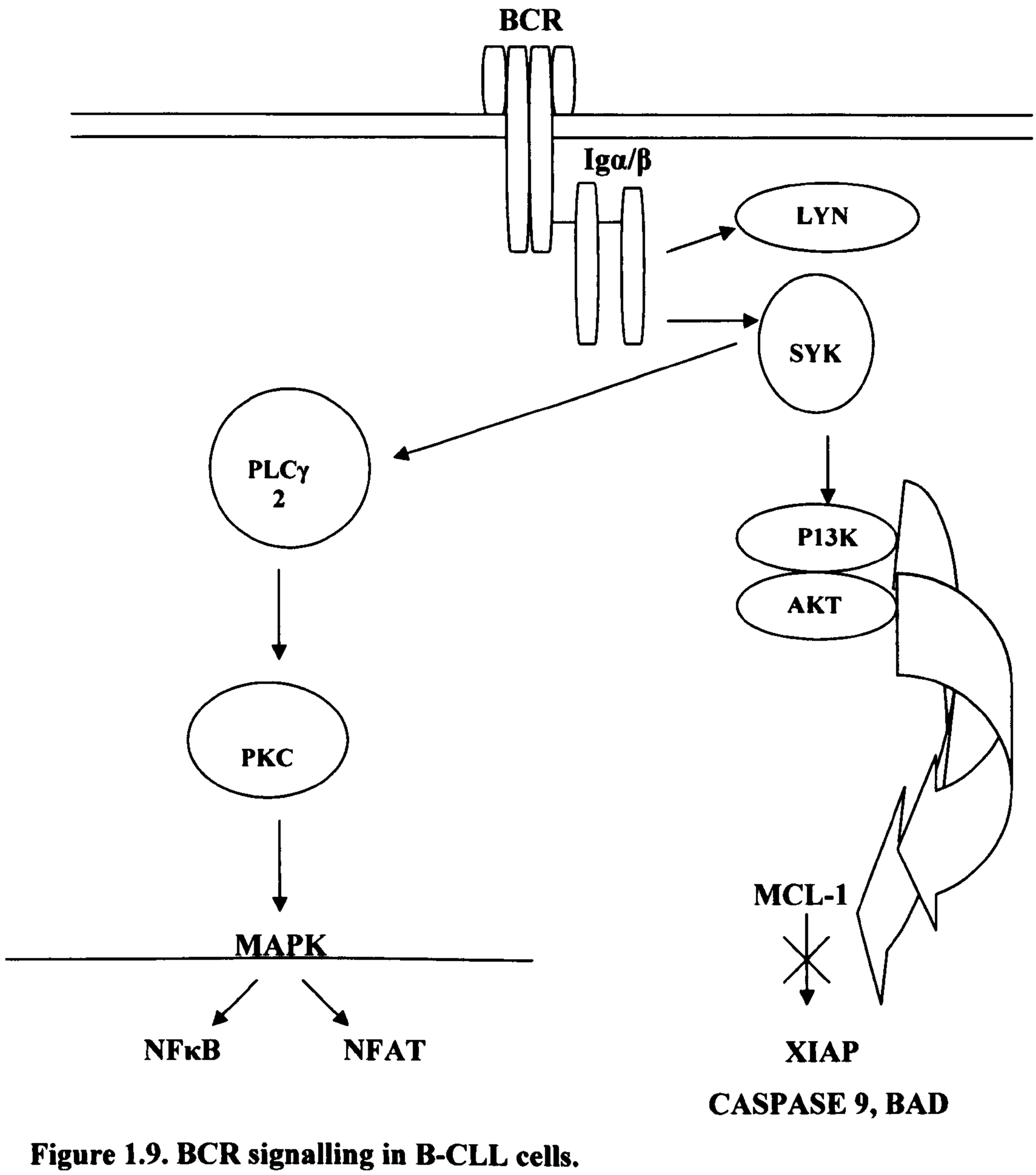


Figure 1.9. BCR signalling in B-CLL cells.

The signals generated on BCR ligation lead to the activation and phosphorylation of the ITAMs (immunoreceptor tyrosine-based activation motifs) of CD79a/b and further to the phosphorylation of the tyrosine kinase, Syk. Subsequently Syk is trans-autophosphorylated leading to full enzymatic activation. Several intermediate signalling molecules are then phosphorylated by Syk in turn activating the downstream signalling molecules Akt, PLCγ2, PC and MAPK (adapted from Efremov *et al.*, 2007).

The BCR plays an important role in cell fate decisions at different stages of B cell development. It varies from rapid cell death to massive clonal expansion depending on the maturation stage of the B cell and other additional extracellular signals (Niiro *et al.*, 2002, Marsden *et al.*, 2003). In immature B cells this signalling leads to apoptosis while in mature cells it leads to activation. The signalling molecules such as PKCβ, NF-κB and Bcl-2 and surface receptors CD19 and CD22 are responsible for the differential signalling outcomes related to developmental regulation. In addition, mature B cells are more capable of interacting with T cells and receiving anti-apoptotic signals transmitted via CD40 and related molecules (Eldering *et al.*, 2005).

The important role of promoting B cell survival and protection against BCR-induced cell death is played by the P13K/Akt pathway via the induction and expression of anti-apoptotic proteins such as myeloid cell leukaemia-1 (Mcl-1) and X-linked inhibitor of apoptosis protein (XIAP). This pathway also inactivates pro-apoptotic cellular targets such as BAD (Bcl2 antagonist of cell death) and caspase-9 (Datta *et al.*, 1997; Ringshausen *et al.*, 2002; Chiorazzi, 2007). Stevenson and Caligaris-Cappio 2004), suggested that there can be several fates of a B cell on ligation with an antigen, shown in Figure 1.10.

1.8.2 BCR-mediated signalling and mutational status of IgVH genes in B-CLL cells

As already described mutations in the BCR play a major role in determining the clinical course of CLL (Hamblin *et al.*, 2002; Chiorazzi *et al.*, 2003). The difference in the responsiveness to sIgM ligation is in close association with the mutation status of the IgVH gene. B-CLL cells with UMut IgVH genes show an increase in the phosphorylation of tyrosine kinase though only some of the B-CLL cells with mutated IgVH genes respond. Unresponsiveness to IgM ligation is circumvented by anti-IgD ligation or other associated molecules of the BCR such as CD79b (Lanham *et al.*, 2003). BCR expression is influenced by B-CLL related defects in CD79b, leading to its diminished and/or inactive form on B-CLL cells. The mechanisms proposed for this defect include reduced expression of CD79b mRNA, somatic

mutation in the gene coding for CD79b (i.e.B29) or a defect in splicing which yields ΔCD79b, an altered form of CD79b (Cragg *et al.*, 2002). However, according to the data published by our group (Porakishvili *et al.*, 2005), UMut B-CLL cells express higher density sIgM compared to Mut B-CLL cells which may account for the differences in anti-IgM responses by UMut and Mut B-CLL cells.

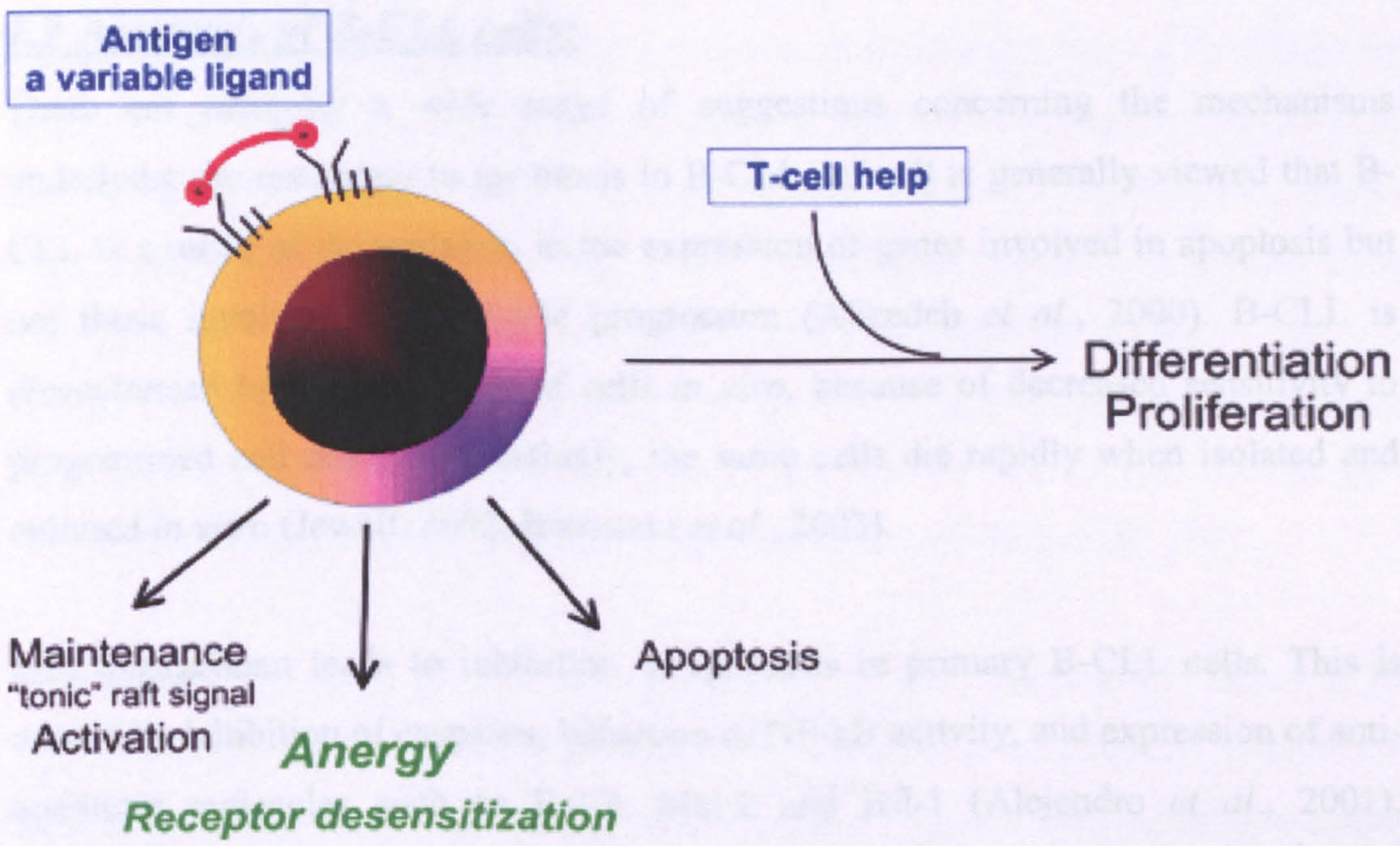


Figure 1.10. Possible outcomes of a B cell following encounter with an antigen.

The antigen ligand for the BCR varies in its properties including avidity, molecular form, concentration and the time of exposure, which influence the outcome. In the absence of T cell help via CD40L, the possible outcomes for the B cell ligation through its BCR comprise activation/maintenance and apoptosis or anergy with receptor desensitisation (from Stevenson and Caligaris-Cappio, 2004).

A study by Peckham *et al* (2000) has provided evidence that direct contact between B cells and low concentrations of anti-δ antibody (0.1-1μg/mL) induce an early apoptotic response by 16h whereas higher concentrations of the antibody (10-100μg/mL) cause the B cell to enter into cell cycle and proliferate. Interestingly the degree of apoptosis was less obvious with anti-μ antibodies. Two hypotheses were proposed by the latter authors in order to explain the observed differences. The first is related to the fact that a small and dense population of B cells, known as resting B cells, may express much more IgD than IgM, thus IgD cross-linking can cluster more

Igα and Igβ than IgM cross-linking, resulting in elevated numbers of apoptotic cells. The alternative explanation given by the authors is that different antibodies bind to the surface Ig with unique affinities and geometries, ultimately leading them to have diverse signalling properties. Thus, it was concluded that with resting B cells, discrete concentrations of antibody causing low levels of cross-linking of surface IgM and IgD may differ in their apoptotic effects (Peckham *et al.*, 2000).

1.9 Apoptosis of B-CLL cells:

There are currently a wide range of suggestions concerning the mechanisms underlying the resistance to apoptosis in B-CLL cells. It is generally viewed that B-CLL is a result of deregulation in the expression of genes involved in apoptosis but not those involved in cell cycle progression (Alizedeh *et al.*, 2000). B-CLL is characterised by accumulation of cells *in vivo*, because of decreased sensitivity to programmed cell death. Interestingly, the same cells die rapidly when isolated and cultured *in vitro* (Jewell, 2002; Bomstein *et al.*, 2003).

IgM engagement leads to inhibition of apoptosis in primary B-CLL cells. This is caused by inhibition of caspases, induction of NF-kB activity, and expression of anti-apoptotic molecules such as Bcl-2, Mcl-2 and Bfl-1 (Alejendro *et al.*, 2001). Telomere lengths in B-CLL cells were found to be shorter in normal B cells from healthy age matched controls indicating that leukaemic B-CLL cells have completed more cycles of proliferation than normal B cells (Damle *et al.*, 2004).

Prolonged survival of B-CLL cells is mainly explained by over expression of the Bcl-2 gene resulting from its hypomethylation (Laurent *et al.*, 2003). Consistent signals through the BCR induce the anti-apoptotic Mcl-1, a homologue of Bcl-2 with a prominent role in preventing spontaneous apoptosis of normal B cells. High levels of Mcl-1 have been found in freshly isolated B-CLL cells which are indicative of resistance to apoptosis (Aleksander *et al.*, 2005). In B-CLL cells there is also an evidence of increased and sustained levels of MAPK, associated with protecting cells from spontaneous apoptosis (Burger *et al.*, 2000).

It has been also suggested that apoptosis of B-CLL cells is influenced by both intrinsic and extrinsic factors, which include changes in the expression of genes that regulate apoptosis for example, the p53 and Bcl-2 family (Agular-Santelises *et al.*, 1996, Gottardi *et al.*, 1996).

In addition, there are aberrant signals transduced through CD40 receptors as well as increased T cell CD40L expression with down regulation of CD95 receptors on the B-CLL surface. All these factors contribute to prolongation of cell survival both by rescuing the cells from apoptosis and by inducing proliferation (Laytragoon-Lewin *et al.*, 1998; Younes *et al.*, 1998; Chiorazzi 2007). Arnon *et al* (2004) has further confirmed that anti-CD40 stimulated B-CLL cells express enhanced levels of the anti-apoptotic proteins Bcl-xL and Bfl-1 and downregulate BH3-only protein Harakiri. Additionally, there is a suggestion that B-CLL cells may even be promoting their own survival and growth by the production of CCL17 and CCL22, chemokines involved in the attraction of T cells to the anatomic zones (Ghia *et al.*, 2002).

According to Bomstein *et al* (2003), soluble factors are present in the serum of B-CLL patients such as IL-4 or IL-6 which are not present in a normal individual's serum. Both of these cytokines excreted either by malignant cells or by some non-malignant tissues, can prevent apoptosis of B-CLL cells. Interestingly, prevention of apoptosis is not seen in purified B-CLL cells which suggest strongly that spontaneous apoptosis of B-CLL cells in cultures is dependent on the interaction of immune complexes with accessory molecules, such as IFNγ and IL-4 (Levesque *et al.*, 2003). Gamberale *et al* (2001) has suggested that in addition to IFNγ and IL-4 other accessory molecules such as IFNα, IL-8 and lymphotoxin-α are involved in inhibition of spontaneous apoptosis. These cytokines are released, either by leukaemic cells with an autocrine control over B-CLL cell survival or by accessory leukocytes. This latter study by Gamberale *et al* (2001) also demonstrated a significant role of monocytes and, to a lesser extent, natural killer (NK) cells in the inhibition of spontaneous apoptosis of B-CLL cells.

Plasma albumin is also identified as a major component that is involved in inhibiting the B-CLL cell apoptosis/killing pathway by potently blocking the effect of chlorambucil or radiation (Jones *et al.*, 2003). In addition albumin lowers the

oxidative stress in cultured B-CLL cells and inhibits spontaneous and reactive oxidant induced apoptosis (Moran *et al.*, 2002).

PI-3K is a protein kinase involved in the regulation of the growth of normal and neoplastic cells. It has been shown that this protein is constitutively activated in B-CLL cells leading to inhibition of apoptosis. Although there is a crucial role for PI-3K with regard to the proliferation and survival of B-CLL cells, limited information is known about the events following its activation (Ringshausen *et al.*, 2002). Src kinase Lyn is a switch molecule involved in coupling of the BCR to downstream signalling. It is over expressed in B-CLL cells with a subcellular localisation as compared to normal B cells. Inhibition of SRC kinase Lyn activity by drugs that induce apoptosis in cultured B-CLL cells remarkably reduces the survival of B-CLL cells (Contri *et al.*, 2005).

Understanding the regulation of apoptosis of B-CLL cells may hold the key to the development of powerful and novel therapeutic approaches for the treatment of B-CLL.

1.10 The aims of my study

This project studies the role of CD180 in the activation and proliferation of normal control and B-CLL cells and its potential as a progressive prognostic marker in conjunction with IgVH mutation status.

The majority of B lymphocytes in peripheral blood express CD180. Engagement of CD180 on normal B cells leads to their activation and proliferation. However, CD180 is differentially expressed on B-CLL cells in that leukaemic cells from one-third of patients are negative for this surface receptor. I hypothesise that the pattern of involvement of CD180 in the regulation of activation, proliferation and apoptosis of B-CLL cells serves to reflect further identification of functional sub-groups of the disease and feasibly, new prognostic tools.

The aim of this study was to investigate functional effects of the engagement of CD180 on B-CLL cells, as compared to control B cells, in association with biologically significant phenotypic characteristics.

1.11 The objectives of my study

- To study the phenotypic profiles of CD180+ and CD180- B-CLL cells in relation to important prognostic markers: mutational status of IgVH genes, expression of CD38, expression of ZAP-70, memory or naïve phenotype.
- To study the functional effects of CD180 ligation on B-CLL and normal B cells through their activation and proliferation;
- To study the co-operation between CD180 and other signalling molecules (BCR, CD40, IL-4) of activation and proliferation in normal and leukaemic B cells;
- To investigate the modulation of CD180 expression by normal and B-CLL patients following IgM, IgD and CD40 ligation.
- To study the role of CD180 in the induction and regulation of apoptosis of B-CLL cells.
- To identify the importance of CD180-mediated functional engagement of B-CLL cells in disease prognosis and management.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Patients:

Thirty six patients with B-CLL, aged between 49-90 years, and 20 aged-matched (51-67 years) healthy controls were included in the study. Patients were at Binet stages A, B and C and had various WBC counts (Table 2.1). Patients were considered to be untreated if they had received no treatment during the 6 months prior to the study. At various times during the course of the study the patients underwent treatment with a variety of therapeutic agents including chlorambucil (n=6), fludarabine (n=1), granulocyte monocyte–colony stimulating factor (GM-CSF; n=1), acyclovir (n=4), alemtuzumab (n=2) and rituximab (n=2). Informed consent was obtained according to the ethical committee of UCLH and the patients themselves who were under the care of Dr. Amit Nathwani of University College Hospital.

Stage, Binet	No: of Patients	WBC count x10^9/L Range
A	28	4.7-98.6
B	2	27.6-72.6
C	6	5.3-81.6

Table 2.1: B-CLL stage (Binet) and WBC counts of the cohort of patients studied.

2.2 Isolation of peripheral blood mononuclear cells (PBMC):

Ten mL of whole blood was taken from B-CLL patients and healthy controls and placed into heparinised test-tubes. Peripheral blood mononuclear cells (PBMC) were separated by using Histopaque (Sigma-Aldrich, Dorset, UK) density gradient medium. Briefly, peripheral blood (10mL) was collected and mixed with an equal volume of Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich, Dorset, UK) in a 50mL Falcon tube (Corning Incorporated, New York, USA). Diluted blood was layered over 5mL of the density gradient medium and centrifuged (model 5810, Eppendorf AG, Hamburg, Germany) for 30 min with a rotor speed of 1700 rpm at room temperature (RT). The PBMC-containing interface was collected using a Pasteur pipette and transferred into a 15mL centrifuge tube (VWR International,

Leicestershire, UK). The harvested cells were supplemented with HBSS and centrifuged for 15 min (1400 rpm at 4°C). The PBMC pellet, re-suspended in 5mL of RPMI-1640 medium (Sigma-Aldrich, Dorset UK) supplemented with 10% foetal bovine serum (Sigma-Aldrich, Dorset, UK), was then washed by centrifugation under the latter conditions. The pellet was re-suspended in 1mL of RPMI-1640 and cells were counted using an improved Neubauer haemocytometer (Sigma-Aldrich, Dorset, UK). Cell concentrations for each experiment were adjusted using RPMI-1640 medium.

2.3 Isolation of B-CLL cells by positive selection:

After counting, the cells were centrifuged at 1500 rpm for 5 min at 4°C, re-suspended with 5mL iMAC buffer composed of phosphate buffered solution (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2μM EDTA and then washed by centrifugation for 10 min, 4°C at 1800 rpm. For further isolation by positive selection method, 80μL of iMAC buffer and 20μL of CD19 multisort microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were added for every 10^7 cells and incubated at 4°C for 15 min. Following incubation, 10mL of iMAC buffer was added to the mixture and the cells were centrifuged at 1800 rpm for 7 min 4°C. Excess fluid was removed and 0.5mL of iMAC buffer was added to enable the re-suspension of the cells by gentle tapping of the tube.

The iMAC column (Miltenyi Biotech, Bergisch Gladbach, Germany) was washed with 0.5mL of iMAC buffer and the cell suspension was carefully pushed through followed by three washes using each time 0.5mL of iMAC buffer. Cells were centrifuged at 1800rpm for 5 min at 8°C, re-suspended in 1mL of RPMI-1640 and counted. The purity of the B-CLL cells, ascertained by staining with PE-Cy5-conjugated mouse anti-human CD19 mAb (BD Biosciences Pharmingen, Oxford, UK) and analysed by flow cytometry (Dako UK Ltd., Ely, Cambridgeshire, UK), using Summit software. This was found to be 95% or greater for each selection.

2.4 Phenotyping of B-CLL cells and control B cells:

2.4.1 Cell surface markers:

200 μL of PBMC at a concentration $1x10^6$/mL in RPMI-1640 were distributed into each well of a sterile 96 well round bottomed microplate (Nunc, Loughborough,

Leicestershire, UK), centifuged and the supernatant discarded. 20 μL (20mg/mL) of human immunoglobulins (Sigma-Aldrich, Dorset, UK) was added into each well to block non-specific binding of mAbs via Fc receptors and the microplate was incubated on ice, for 30 min, centrifuged at 1500 rpm for 5 min at 4°C after which the supernatant was discarded and the microplate was vortexed.

20μL of primary mouse antibodies (20μg/mL) were added, including IgG1 isotype control (Clone P3, a kind gift from Professor Peter Lydyard, UCL, UK), anti-CD180 (clone G28.8, a kind gift from Professor Edward Clark, University of Washington, Seattle, USA), anti-IgM mAbs (BD Biosciences Pharmingen, Oxford, UK), anti-CD79b and anti-CD38 mAbs (Fitzgerald Industries International, Massachusetts, USA), anti-CD86 mAbs (clone Bu63, a kind gift from Professor Benjamin Chain, UCL, UK), anti-IgD mAbs (Sigma-Aldrich, Dorset, UK) and anti-CD40 (a kind gift from Professor Edward Clarke, University of Washington, Seattle, USA).

The plate was left on ice for 30min and centrifuged at 1500rpm for 5 min at 4°C. The supernatant was discarded and the microplate was vortexed. Washing was made using a buffer composed of PBS supplemented with 0.01% sodium azide and 1% BSA (PBS-AB) added to each well together with 20μL of FITC-conjugated rabbit anti-mouse Ig $F(ab)_2$ (Dako UK Ltd., Ely, Cambridgeshire, UK) diluted 1:20 in PBS-AB, used as a secondary antibody. The plate was incubated on ice for 30 min under dark conditions and then centrifuged under refrigeration (4°C) for 5 min at 1500 rpm. This wash process was repeated a further time after which 20μL of mouse serum (Dako UK Ltd., Ely, Cambridgeshire, UK), diluted 1:15 in PBS-AB, was added to each well to block any free rabbit anti-mouse F(ab) sites.

Following incubation on ice for 30 min under dark conditions, the plate was centrifuged without any washing. 10μL of PE-Cy5-conjugated mouse anti human CD19 mAb (BD Biosciences Pharmingen, Oxford, UK) was added to each well and the plate was incubated for 30 min in the dark, and then centrifuged and washed twice in PBS-AB, as described above.

To fix the cells, 200μL of 2% paraformaldehyde (PFA; Sigma-Aldrich, Dorset, UK) solution in PBS was added to each well and the plate was stored at 4°C until analysis

by flow cytometry. The density of surface molecule expression was determined by antibody relative binding sites (RBSs: Figure 2.1) calculated from the mean fluorescence intensity (MFI) of the antibody binding. Data from the flow cytometry histogram was entered into a Microsoft Excel template (see below) to calculate, by use of linear regression analysis, the RBS for that particular mAb.

PMT Voltage
503.0

F/P ratio
2.5

MEAN P3

FITC/ BEAD	BEAD MFI
100	2.27
4700	31.07
15000	89.83
40000	206.68
140000	661.45
330000	1392.82

		Linear Regression
0.00417		34.4301
ax		+b
r^2=	0.998	

In a separate experiment, after blocking Fc receptors with human immunoglobulins, cells were incubated with IgG1 (P3) and anti-CD180 together with rabbit anti-mouse FITC secondary Abs followed by addition of mouse sera to each well, as described previously above.

After centrifugation, 10μL of PE-conjugated IgG isotype control (BD Biosciences Pharmingen, Oxford, UK) or PE-conjugated anti-CD27 (eBioscience, San Diego, CA, USA) were added to the designated wells in addition to 10μL of PE-Cy5-conjugated mouse anti-human CD19 mAb. The cells were centrifuged and washed twice after 30 min incubation on ice and dark conditions and fixed with 200μL of 2% PFA in PBS.

A representative profile histogram for control B cells with each phenotypic marker studied is shown in Figure 2.1.

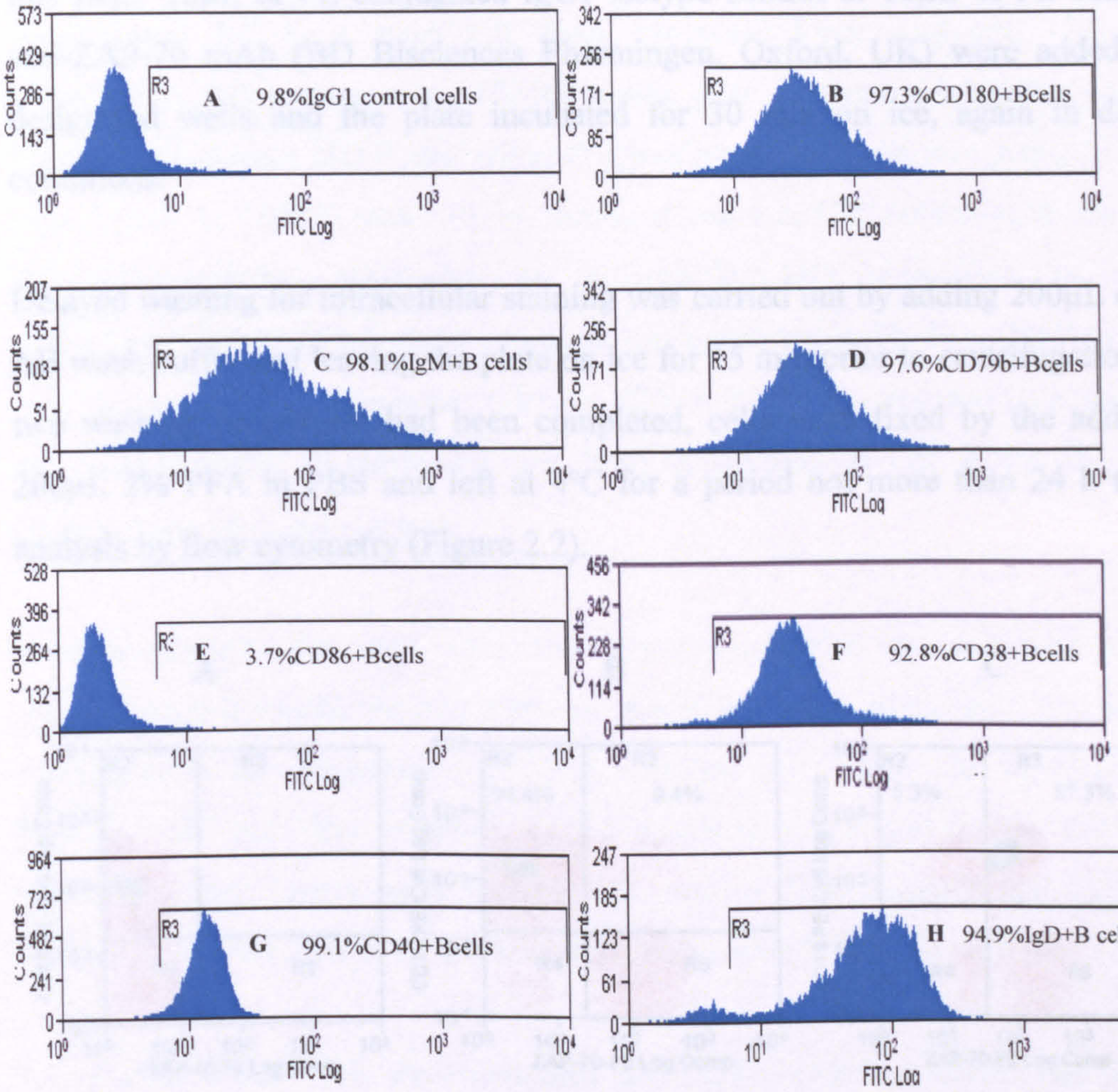


Figure 2.1. Histograms representing A) IgG1 Isotype control and expression of B) CD180, C) IgM, D) CD79b, E) CD86, F) CD38, G) CD40 and H) IgD on the B cell surface.

R3 represents the percentage of the B cell population positive for a particular surface marker. Results were analysed by flow cytometry.

2.4.2 For intracellular ZAP-70 expression:

Fc receptors on PBMC were blocked with human immunoglobulins as described previously in section 2.4.1. The plate was centrifuged and the supernatant discarded after which 10µL of mouse PE-Cy5 anti-human CD19 mAb was added to each well and the plate was left on ice for 30 min dark. After centrifugation and washing twice, the plate was incubated with 60µL of Fix and Perm kit medium A (Caltag Laboratories, Burlingame, USA) for 15 min in the dark at room temperature. After centrifugation and washing, 60µL of Fix and Perm kit medium B (Caltag

Laboratories, Burlingame, USA) was added to each well and the plate kept under the same conditions as described for medium A. No further washing was performed after this step. 10µL of PE conjugated IgG1 isotype control or 10µL of PE conjugated anti-ZAP-70 mAb (BD Bisciences Pharmingen, Oxford, UK) were added to the designated wells and the plate incubated for 30 min on ice, again in darkened conditions.

Delayed washing for intracellular staining was carried out by adding 200µL of PBS-AB wash buffer and leaving the plate on ice for 15 min prior to centrifugation. After two washing procedures had been completed, cells were fixed by the addition of 200µL 2% PFA in PBS and left at 4°C for a period not more than 24 h to allow analysis by flow cytometry (Figure 2.2).

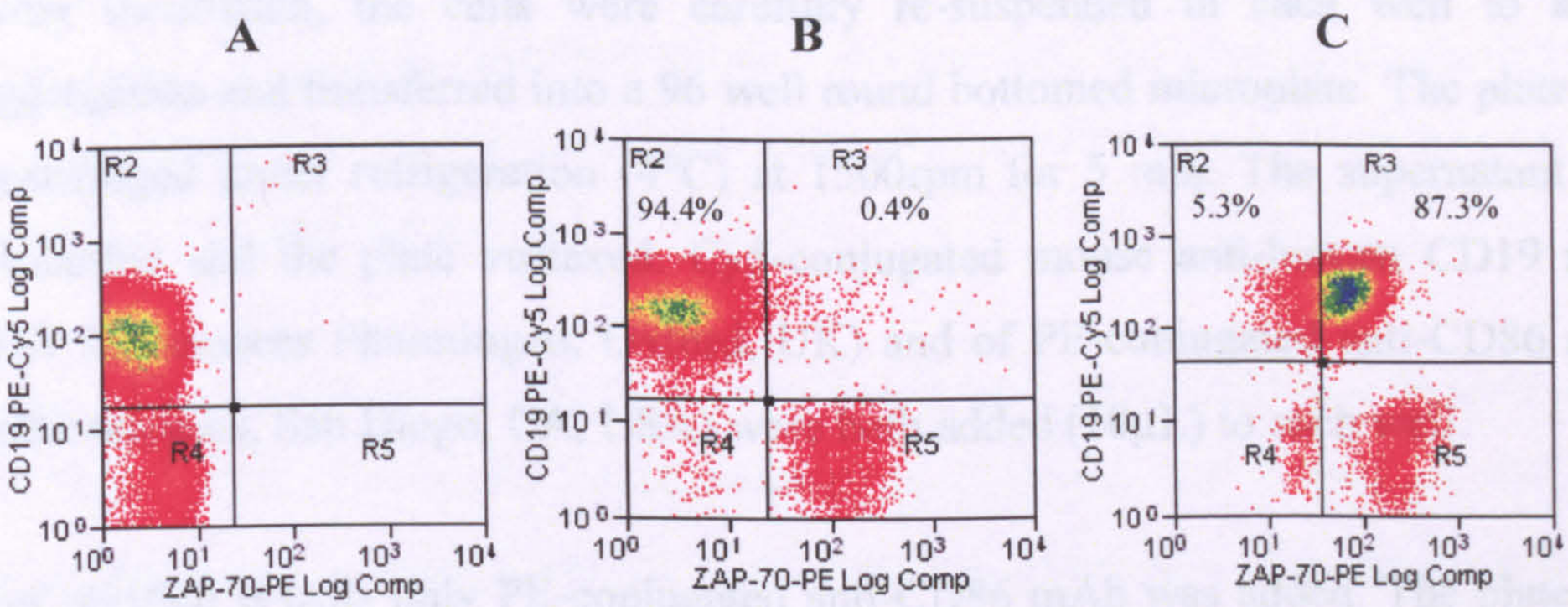


Figure 2.2. Representative dot plots of B-CLL patient cells showing A) isotype control, B) B-CLL cells negative for intracellular ZAP-70 and C) B-CLL cells positive for intracellular ZAP-70.

Quadrants were placed according to the ZAP-70 expressing T cell population in R5. CD19+ve B cells are shown in R2, and R3 represents cells +ve for ZAP-70 as well as CD19. A cut-off value of 20% was used, defining B-CLL patients as ZAP-70 positive when at least 20% of the CD19+ cells expressed this molecule.

***2.5 Measurement of B cell activation in 72h cultures*:**

200µL of PBMC or purified B cells (10^6 cells/mL) were placed in each well of a 96-well sterile flat bottomed plate (Nunc, Loughborough, Leicestershire, UK). Cells in control wells (unstimulated cultures) remained without any additives. Anti-CD180 mAb or anti-CD40 mAb or both mAbs were added at a final concentration of 10µg/mL to the cultures, in the presence and absence of 15ng/mL recombinant

interleukin-4 (rIL-4; a kind gift from Professor Peter Lydyard, UCL, UK). In other sets of experiments anti-CD180 mAbs, goat anti-human IgM $F(ab)_2$ antibody and goat anti-human IgD $F(ab)_2$ antibody (SouthernBiotech, Birmingham, AL, USA) were added to PBMC alone or in combinations anti-CD180/anti-IgM antibodies, anti-CD180/anti-IgD antibodies or anti-IgM/anti-IgD antibodies, at a final concentration of 10μg/mL each. The plate was incubated in 5% CO_2 (HERAcell 240, Thermo Scientific, Basingstoke, UK) for 72 h at 37°C. The optimal incubation time and concentrations of antibodies had been determined by our group prior to the present study (data not shown).

After incubation, the cells were carefully re-suspended in each well to avoid aggregation and transferred into a 96 well round bottomed microplate. The plate was centrifuged under refrigeration (4°C) at 1500rpm for 5 min. The supernatant was discarded and the plate vortexed. Cy5-conjugated mouse anti-human CD19 mAb (BD Biosciences Pharmingen, Oxford, UK) and of PE-conjugated anti-CD86 mAb (eBiosciences, San Diego, CA, USA) were both added (10μL) to each well.

For purified B cells only PE-conjugated anti-CD86 mAb was added. The plate was incubated on ice for 30 min in the dark and centrifuged. The supernatant was discarded and microplate was vortexed. 200μL of PBS-AB was added to each well and centrifuged at 1500rpm for 5 min at 4°C. The supernatant was discarded and the washing procedure repeated. 200μL of 2% PFA in PBS was added to each well and the plate was stored at 4°C in the dark, to be analysed later by flow cytometry. The percentages of CD86+ B cells were calculated, either by gating on CD19+ cells (PBMC) or on a whole population of viable purified cells (B cells; Figure 2.3). Data was normalised to percentage values to facilitate comparison between samples bearing in mind that percentage can only reach upto 100.

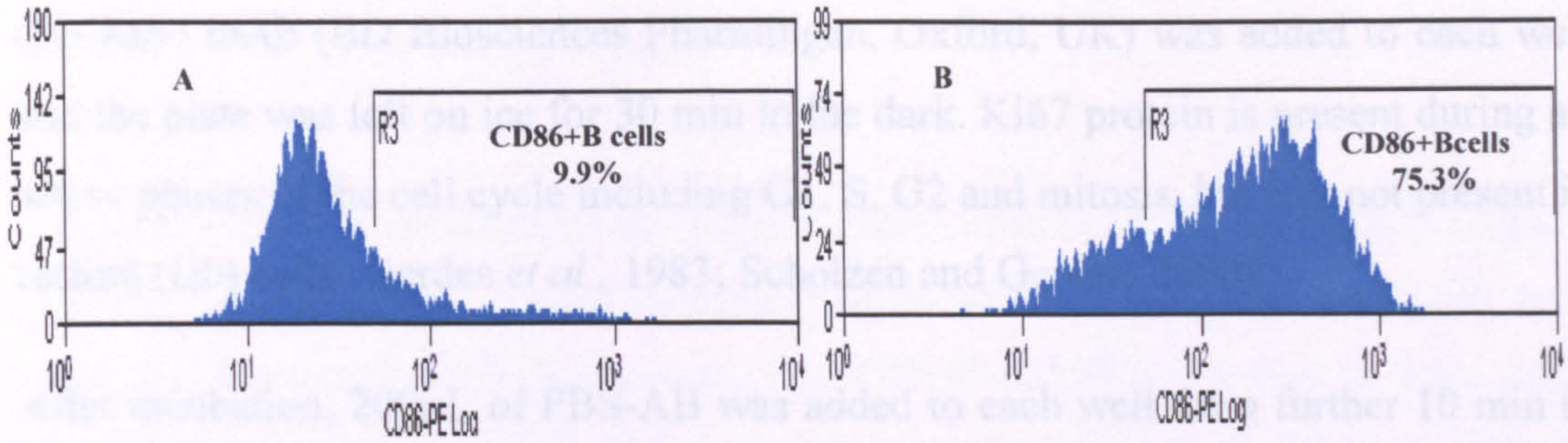


Figure 2.3. Representative flow cytometric profile of PBMC 72 h cell cultures: without antibody (A) and after incubation with anti-IgM (B).

PBMC from normal controls and B-CLL patients were cultured in the presence and absence of 10μg/mL anti-IgM $F(ab)_2$ Ab for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and PE-conjugated anti-CD86 mAb.

2.6 Quantitation of cells in cell cycle after 72h of culture:

PBMC or purified B cells were cultured with anti-CD180 or anti-CD40 mAbs or a combination of both as described above in section 2.5. The optimal incubation time and concentrations of antibodies had been determined by our group prior to the present study (data not shown)

After 72 h incubation the cells were re-suspended and transferred to a 96 well round bottomed plate. Following centrifugation at 1500rpm for 5 min at 4°C, the supernatant was discarded and the plate vortexed. 10μL of Cy5-conjugated mouse anti-human CD19 mAb was added to each well containing PBMC and the plate was left on ice in the dark. The plate was centrifuged, the supernatant discarded and the plate was again vortexed. PBS-AB wash buffer (200μL) was added to each well and centrifuged under the same conditions as described above. The supernatant was discarded and the washing procedure repeated. This latter stage was omitted for purified B cells. 60μL of Fix and Perm medium A (Caltag Medsystems-Invitrogen, Camarillo, CA, USA) was added to each well, the plate left in the dark (in the case of anti-CD19 stained PBMC) at room temperature for 15 min.

Following centrifugation at 1500rpm for 5 min at 4°C, the supernatant was discarded and the plate was vortexed. PBS-AB wash buffer (200μL) was added and the cells were washed as described above. 60μL of Fix and Perm medium B (for permeabilisation) was then added and the plate was left in the dark at room temperature for 15 min followed by centrifugation under the same conditions as

described above. No wash step was used after this stage. 10µL of PE-conjugated anti-Ki67 mAb (BD Biosciences Pharmingen, Oxford, UK) was added to each well and the plate was left on ice for 30 min in the dark. Ki67 protein is present during all active phases of the cell cycle including G1, S, G2 and mitosis, but it is not present in resting (G0) cells (Gerdes *et al.,* 1983; Scholzen and Gerdes, 2000).

After incubation, 200µL of PBS-AB was added to each well for a further 10 min to allow removal of unbound antibody. The plate was centrifuged at the same conditions as above, the supernatant discarded and the plate vortexed dry. The cells were washed once more with PBS-AB and 200µL of 2% PFA in PBS was added to each well. The plate was stored at 4°C in the dark to be later analysed, within 24 h, by flow cytometry.

The percentages of Ki67+ B cells were calculated, either by gating on CD19+ cells (PBMC) or on purified viable B cells.

2.7 Modulation of surface CD180 expression on B-CLL cells and control B cells:

PBMCs ($1x10^6$ cells/mL) were distributed in a volume of 200µl into a 96 well sterile flat bottomed microplate. Anti-CD40 mAb, goat anti human IgM $F(ab)_2$ antibody (SouthernBiotech) or goat anti human IgD $F(ab)_2$ antibody (SouthernBiotech, Birmingham. AL, USA) were added at a final concentration of 10µg/mL. rIL-4 was added either alone or together with anti-CD40 mAb to some wells. The microplate was incubated at 37°C with 5% CO_2 for 48 and/or 72 h. Control wells (unstimulated cultures) were cultured in the absence of mAbs or IL-4.

Following culture, the cells were re-suspended thoroughly and transferred into a 96 well round bottomed plate. Following centrifugation at 1500rpm for 5 min at 4°C, the supernatant was discarded and the plate vortexed. Cy5 conjugated mouse anti-human CD19 mAb and PE-conjugated IgG1 isotype control or PE-conjugated anti-CD180 mAbs were added (10µL respectively) to both unstimulated as well as stimulated cells and were left on ice for 30 min in the dark. The plate was centrifuged and the cells washed twice in PBS-AB. After adding 2% PFA in PBS, the microplate was stored at 4°C and wrapped in the dark to be later analysed by flow cytometry. The level of the expression of CD180 on CD19+ cells was measured as percentages

of CD180+ cells as well as the relative fluorescence intensity (RFI) of each CD180 stained culture versus isotype control.

2.8 Induction of apoptosis of B-CLL cells and normal control B cells:

PBMCs ($1x10^6$ cells/mL) were distributed in a volume of 200µL into a 96 well sterile flat bottomed sterile microplate. Anti-CD180 mAbs was added to three wells at a final concentration of 10µg/mL. Goat anti-human IgM $F(ab)_2$ antibody (SouthernBiotech, Birmingham, AL, USA) was added to one well at a final concentration of 10µg/mL. The microplate was incubated at 37°C with 5% CO_2. After 24 h and 48 h, goat anti human IgM $F(ab)_2$ antibody was added to the wells previously preincubated with anti-CD180.

Control wells (*viz*, unstimulated cultures) were not treated with any antibody preparation. Goat anti human IgD $F(ab)_2$ antibody (SouthernBiotech, Birmingham, Alabama, USA) was added to two other wells at the final concentration of 10µg/mL. The microplate was incubated at 37°C with 5% CO_2. For this particular experiment an optimal time was chosen following incubation of cells with antibody for 24, 48 and 72 h. Following incubation, the cells were transferred from a 96 well flat bottomed plate to a 96 well round plate by thorough mixing. After centrifuging the plate at 1500rpm for 5min at 4°C the supernatant was discarded and the plate was vortexed. Two methods for the detection of apoptosis in stimulated and control (unstimulated) cells were used.

2.8.1 Detection of apoptosis using the mitochondrial dye DiOC6

PE-Cy5 conjugated anti-CD19 mAb was added in a volume of 10µL to each well and the plate incubated for 30min on ice under dark conditions. Following two washes with PBS-AB buffer, the cells were re-suspended in 200µL of HBSS and 20µl (0.2 µM) of DiOC6 was added to each well. The optimum concentration of DiOC6 was previously determined in our laboratory (data not shown). $DiOC_6$ [(3) - (3, 3'-dihexyloxacarbocyanine iodide] is a fluorescent dye used for the staining of cellular endoplasmic reticulum (ER), vesicle membranes and mitochondria. $DiOC_6$ is to label and visualise living cells since exposure to blue light excites the dye to fluoresce green (Terasaki, 1989; Koning *et al.*, 1993). The plate was left in the incubator for 20 min at 37°C and 5% CO2 and analysed by flow cytometry immediately. The level of

apoptosis was assessed by the percentage of DiOC6^{dim} cells gated on the CD19+ cell population.

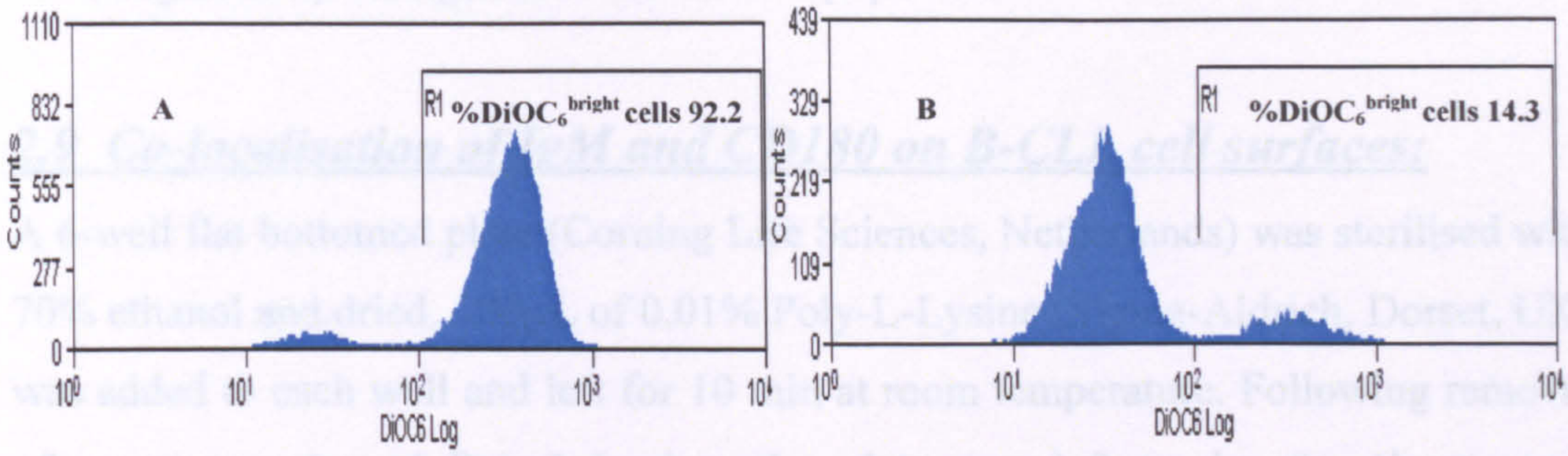


Figure 2.4 Representative flow cytometric profile of 72 h cell cultures: without antibody (A) and after incubation with anti-IgD (B).

PBMC from normal controls and B-CLL patients were cultured in the absence and presence of 10μg/mL anti-IgD F(ab)2 Ab for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and 0.4μM of DiOC6. Flow cytometric analysis was performed immediately, DiOC6^{dull} cells were considered to be apoptotic.

2.8.2 Detection of apoptosis by Annexin V and Propidium Iodide (PI) stain:

This method was used to confirm apoptosis of cells stimulated exclusively with anti-IgD $F(ab)_2$ antibody. Annexin V is used to detect cells that have expressed phosphatidylserine (PS) on the cell surface, a feature found in apoptosis. Cells in early apoptosis are Annexin V-positive and PI-negative, meaning PS translocation has occurred although the plasma membrane remains still intact. Cells that are positive for both Annexin V and the PI are either in the late stages of apoptosis or are already dead since PS translocation has occurred and loss of plasma membrane integrity has occurred.

When measured over time, Annexin V and a PI can be used to monitor the progression of apoptosis from cell viability, to early-stage apoptosis, and finally, to late-stage apoptosis and cell death (Koopman *et al.,* 1994; Vermes *et al.,* 1995). Annexin V kit components (binding buffer, FITC-conjugate Annexin V and PI, Sigma-Aldridge, Dorset, UK) were allowed to equilibrate at room temperature for 30 min. Binding buffer was prepared by diluting 1:10 in distilled water. PBMCs were washed twice with azide-free PBS and were resuspended in 200μL of diluted binding buffer. 2.5μL of FITC-conjugated Annexin V and 1μl of PE-conjugated-PI were then added to each well and the plate was incubated for 10 min at room temperature in the

dark and analyzed by flow cytometry immediately. Apoptosis was assessed by evaluation of Annexin-PI- (viable cells), Annexin+PI-, Annexin+PI+ and Annexin-PI+ (fragmented) cells gated on the CD19+ population.

2.9 Co-localisation of IgM and CD180 on B-CLL cell surfaces:

A 6-well flat bottomed plate (Corning Life Sciences, Netherlands) was sterilised with 70% ethanol and dried. 500µL of 0.01% Poly-L-Lysine (Sigma-Aldrich, Dorset, UK) was added to each well and left for 10 min at room temperature. Following removal of excess non-bound Poly-L-Lysine, the plate was left to dry for 1h at room temperature.

Isolated PBMCs from B-CLL patients were resuspended in RPMI-1640 without FCS. 200µL of PBMCs were added to each well of the Poly-L-Lysine coated plate at a final concentration of $1x10^6$cells/mL, and allowed to settle and attach for 1h at room temperature.

The PBMCs were then fixed on ice for 15 min with 2% PFA in PBS followed by two washes with HBSS. The PBMCs were then incubated for 30 min on ice, in the dark, with 20µL of each of the following antibodies in different combinations; FITC-conjugated mouse anti-human IgG1 isotype control mAb (BD Biosciences), PE-conjugated mouse anti-human IgG1 isotype control mAb, PE-conjugated mouse anti-human CD180 mAb and FITC-conjugated mouse anti-human IgM mAb (SouthernBiotech, Birmingham, AL, USA). After two washes with HBSS, PBMCs were left in 3mL HBSS and analysed directly with a Leica TCS SP2 CLSM confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.10 Statistical Analysis:

The following statistical methods were applied where required:

- Wilcoxon's (1945) non-parametric paired test using SPSS (Self-Propelled Semi-Submersible) software;
- Mann-Whitney non-parametric U-test using MINITAB software (Coventry, UK).
- Pearson's correlation (1988) coefficient using SPSS software;

P-values <0.05 were considered significant in each case.

CHAPTER THREE

EXPRESSION OF CD180 IN RELATION TO MAJOR PROGNOSTIC AND FUNCTIONAL MARKERS OF B-CLL

3.1 Introduction

Previous studies by our group have shown that CD180 is heterogeneously expressed by B-CLL cells in a clonal fashion (Porakishvili *et al.*, 2005). In this pilot study the level of the expression of this surface molecule was measured by the number of relative binding sites per cell (RBS/cell). The results, shown as mean ± SD, demonstrated that most normal (control) CD19+ B cells expressed a high density of surface CD180 (5548 ± 2271 RBS/cell), although a small population of CD180- negative cells were evident. The negative B-CLL clones were therefore defined by the level of CD180 RBS/cell on this small CD180- population seen in the control B cells (316 ± 88 RBS/cell, range 201-470 RBS/cell). The limit for the negative population was determined as the mean ± 2SD, as described previously by Porakishvili *et al* (2005).

Similar experiments were carried out to determine the phenotypic characterisation of the patients in my study.

3.2 Results

3.2.1 Heterogeneous expression of CD180 by B-CLL cells

The study confirmed that individual B-CLL patients expressed different levels of surface CD180 on CD19+ cells and that CD180 expression was characterised by a clonal distribution. By adopting the accepted cut-off level of 500 RBS/cell (Porakishvili *et al.*, 2005) 14 B-CLL patient's cells were negative for CD180 and 22 were positive for CD180.

Not surprisingly, the positive population of B-CLL cells expressed a significantly ($p = 1.2 \times 10^{-5}$) higher density of CD180 on CD19+ B cell surface (1618 ± 1268 RBS/cell, range 512-5331 RBS/cell) than the CD180 negative population (236 ± 168 RBS/cell, range 0-476 RBS/cell).

In this cohort of patients the percentage of CD180- negative patients was lower than CD180-positive patients, being 38.88% and 61.11 % respectively (Figure 3.1).

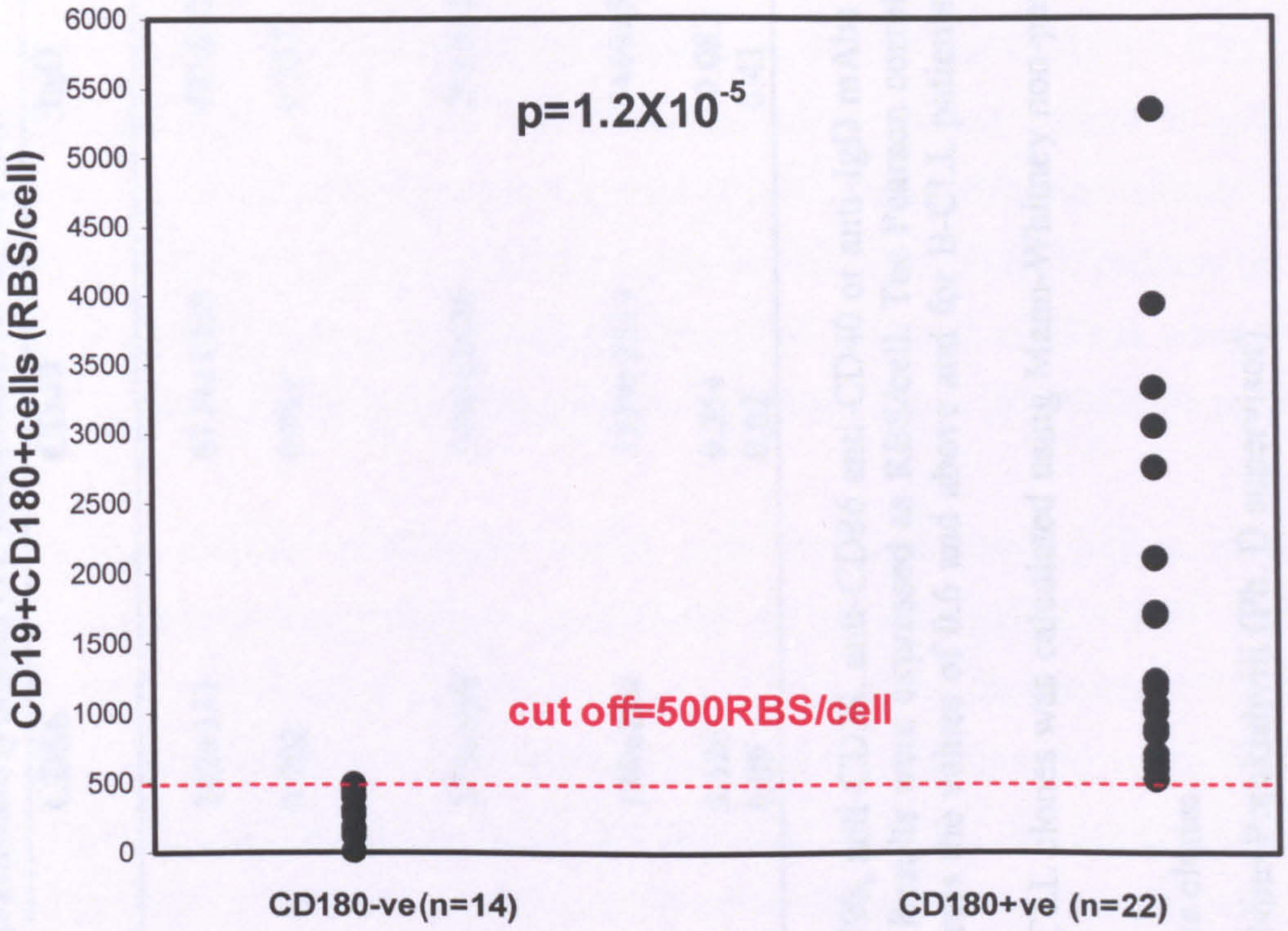


Figure 3.1. Expression of CD180 by B-CLL cells delineating two groups of patients.

PBMC from B-CLL patients were treated with anti-CD180 mAb and stained with secondary rabbit anti-mouse FITC conjugated antibody followed by anti-CD19-Cy5 mAb. RBS/cell were defined as described in *Materials and Methods*. Statistical differences were calculated using the Mann-Whitney U test where values of $p \leq 0.05$ were regarded as significant.

3.2.2 Relationship between the expression of surface CD180 with other phenotypic markers in normal control B cells and B-CLL cells

Since a heterogeneous expression of CD180 was observed, it was important to understand whether the expression of CD180 correlated with the expression of other surface markers deemed to have a functional/prognostic value on both control B cells and B-CLL cells. As shown in Table 3.1, the expression of CD180, sIgM, sIgD, CD79b and CD40, but not CD86 or CD38 by CD180+ B-CLL cells was significantly lower than that of control B cells ($p<0.05$; Mann-Whitney U-test). However, there was a strong correlation between the expression of CD180 and sIgM ($r = 0.869$) and CD180 and CD40 ($r = 0.912$) in normal control B cells (Table 3.1).

Table 3.1 Correlation of expression of CD180 with other phenotypic markers by B-cell chronic lymphocytic leukaemia (B-CLL) cells.

	CD180	IgM	CD79b	CD38	CD86	CD40	IgD
Controls CD180+(n=9)	5548±2271	17907±8553	4785±1798	923±396	202±331	8774±1459	4236±2777
Correlation* coefficient		0.869	0.762	0.217	0.792	0.912	0.337
B-CLL CD180+(n=22)	1686±1268	1787±3389	502 ± 426	564±821	375±539	5450±2626	2913±3185
B-CLL CD180-(n=14)	236±168	626±659	301±491	83±170	130±214	3339±2519	4449±5013
Correlation*		0.103	0.368	0.524	0.126	0.354	-0.083
P**	$1.2x10^{-5}$	0.05	0.16	0.009	0.09	0.02	0.41

B-CLL cells and normal control B cells were treated with anti-CD180, anti-IgM, anti-CD79b, anti-CD38, anti-CD86 anti-CD40 or anti-IgD mAbs and stained with the secondary Abs and anti-CD19-Cy5 mAb as described in the *Materials and Methods*. Results were expressed as RBS/cell. The Pearson correlation coefficient was calculated between the expression of CD180 and other surface markers (*). For controls the values of 0.6 and above and for B-CLL patients the value of 0.4 and above were considered correlated, according to the n = number of paired data.
Differences between the expression of all surface markers in CD180+ and CD180- in B-CLL clones was calculated using Mann-Whitney non-parametrical U test (**). Values of 0.05 or below were considered significant.
*Correlation with CD180 RBS/cell with other phenotypic markers, RBS/cell.
**Difference between all measured surface markers in CD180 positive and CD180 negative clones.

All phenotyping data for normal controls except that for IgD were kindly provided by Dr. Nino Porakishvili (Ph. D supervisor).

A moderate correlation was detected between CD180 and CD79b (r = 0.762) and CD86 (r = 0.792) although no correlation between CD180 and CD38 (r = 0.217) and CD180 and sIgD (r = 0.337) was demonstrated in normal control B cells. As expected, a statistically significant ($p=1.2 \times 10^{-5}$; Mann-Whitney U-test) 7-fold difference was observed in the surface levels of CD180 in B-CLL patients positive for the surface expression of CD180 (1686 ± 1268 RBS/cell, n=22) as compared to patients negative for the surface expression of CD180 (236 ± 168 RBS/cell, n=14).

The density of sIgM on CD180+ B-CLL cells was significantly higher, almost 3-fold, in comparison to that found on CD180- B-CLL cells (1787 ± 3389 RBS/cell versus 626 ± 659 RBS/cell respectively; p =0.05). Similarly, the density of CD38 and CD40 was significantly higher (p=0.009 and p=0.02 respectively) on CD180+ B-CLL cells than on CD180- B-CLL cells (Table 3.1). However, the density of CD79b, CD86 and sIgD showed no significant difference in the level of expression between both the CD180+ and CD180- B-CLL cells (p=0.16, p=0.09 and p=0.41 respectively).

As determined by Pearson's coefficient test in B-CLL cells, there was no correlation observed between the density of expression of CD180 by B-CLL cells with all other markers apart from CD38, which showed a moderate direct correlation with CD180 expression (r = 0.524, Table 3.1). Correlation values expressed as *rho* (r) were: sIgM, r = 0.103; CD79b, r = 0.368; CD86, r = 0.126; CD40, r = 0.354, sIgD, r = -0.083.

3.2.3 Expression of CD180 in relation to IgVH region mutation status of B-CLL cells

The relationship between CD180 and mutational status of IgVH genes in 20 B-CLL patients was examined using the Mann-Whitney U-test. As seen in Table 3.2, there was a significant difference observed (p=0.04) in the levels of CD180 expression between B-CLL cells with UMut and Mut IgVH genes. In the UMut IgVH B-CLL cells the average level of CD180 was 1298 ± 1252 RBS/cell (range 0-3928) while in the Mut IgVH group it was 2343 ± 1286 RBS/cell (range 1171- 5331).

Table 3.2 Expression of CD180 and other phenotypic markers by B-CLL cells with Mut and UMut IgVH genes.

	CD180	IgM	CD79b	CD38	CD86	CD40	IgD
B-CLL M (n=10)	2345 ± 1246	445 ± 454	484 ± 411	820 ± 1039	454 ± 633	4295 ± 1911	3437 ± 2983
B-CLL UM (n=10)	1298 ± 1252	1785 ± 1382	565 ± 439	493 ± 551	359 ± 593	6299 ± 3174	5174 ± 3373
p*	0.04	0.007	0.33	0.197	0.374	0.07	0.183

Comparison of the surface expression of CD180, IgM, CD79b, CD38, CD86, CD40 and IgD on B-CLL cells from 10 Mut and 10 UMut IgVH genes. Mutational status of the cells was provided by Dr. Amit Nathwani (UCLH). Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test with values of $p \leq 0.05$ being significant.

*Significance of difference between surface marker expression by B-CLL cells with mutated versus unmutated IgVH genes.

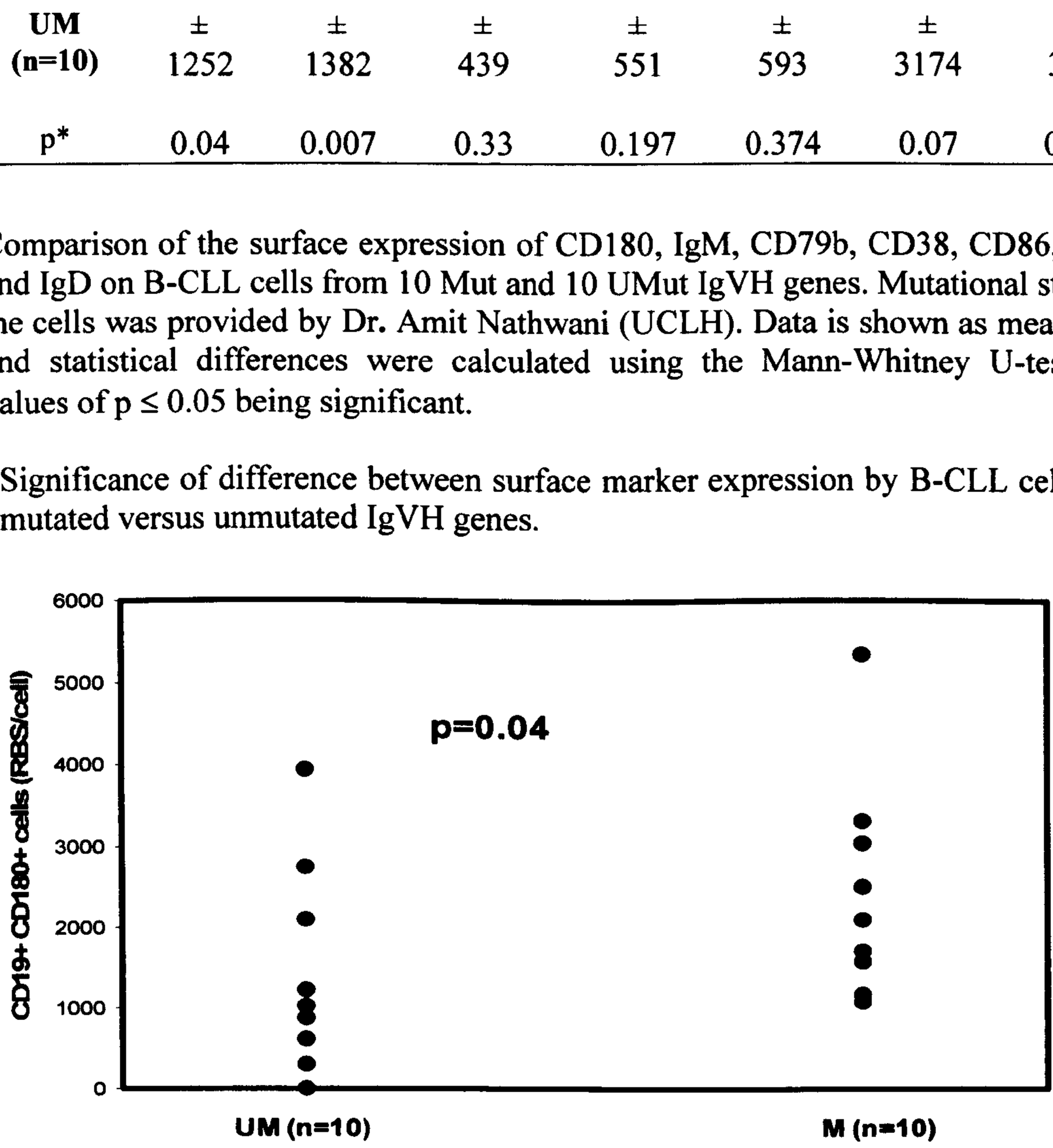


Figure 3.2 Expression of CD180 on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL cells using Mut IgVH genes expressed significantly higher levels of CD180 in comparison to UMut IgVH genes. The scatter plot show the distribution of CD180 expression (RBS/cell) on UMut (n=10) and Mut (n=10) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

In this same cohort of patients, sIgM expression (1785 ± 1382 RBS/cell) was significantly (p=0.007), 4-fold, higher on UMut B-CLL cells than that found on Mut B-CLL cells (445 ± 454 RBS/cell; Figure 3.3 and Table 3.2).

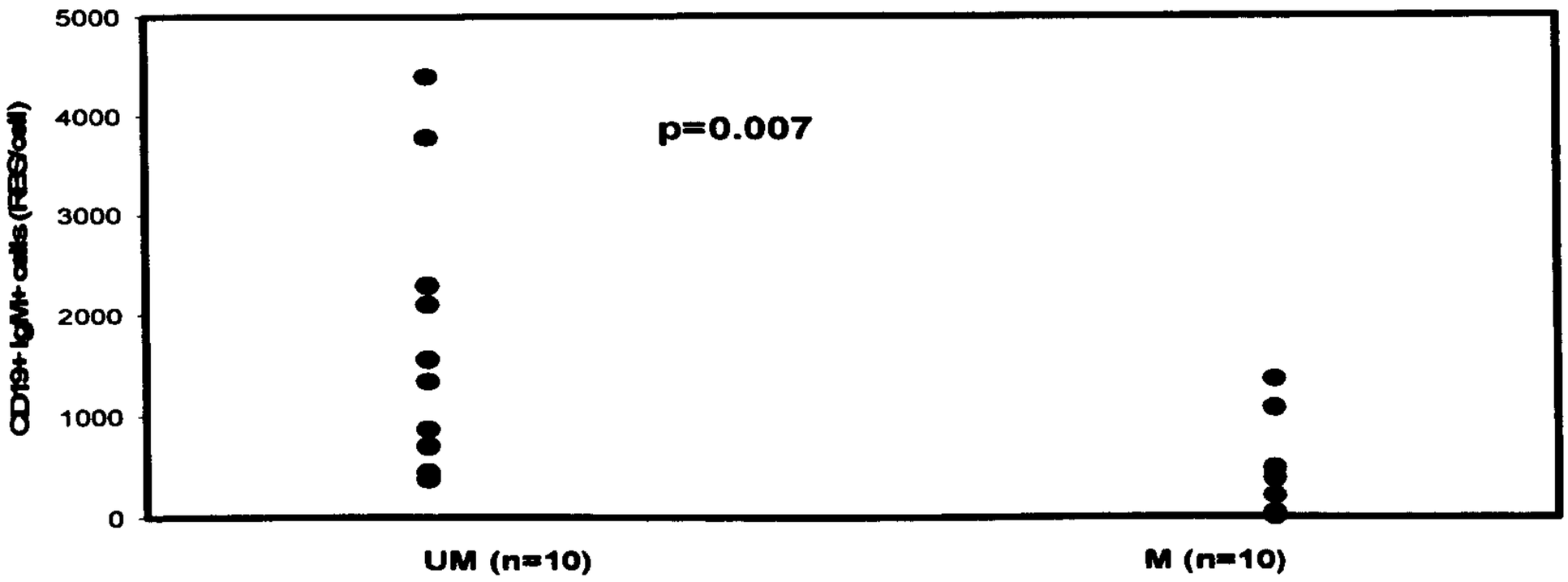


Figure 3.3 Expression of sIgM on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL using UMut IgVH genes expressed a 4-fold higher level of sIgM in comparison to Mut IgVH genes. The scatter plot show the distribution of sIgM expression (RBS/cell) on UMut (n=10) and Mut (n=10) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

In contrast to that observed for sIgM, no significant difference (p=0.341) in the level of expression of CD79b was detected on the cell surface of B-CLL cells with Mut and UMut IgVH genes (Figure 3.4 and Table 3.2).

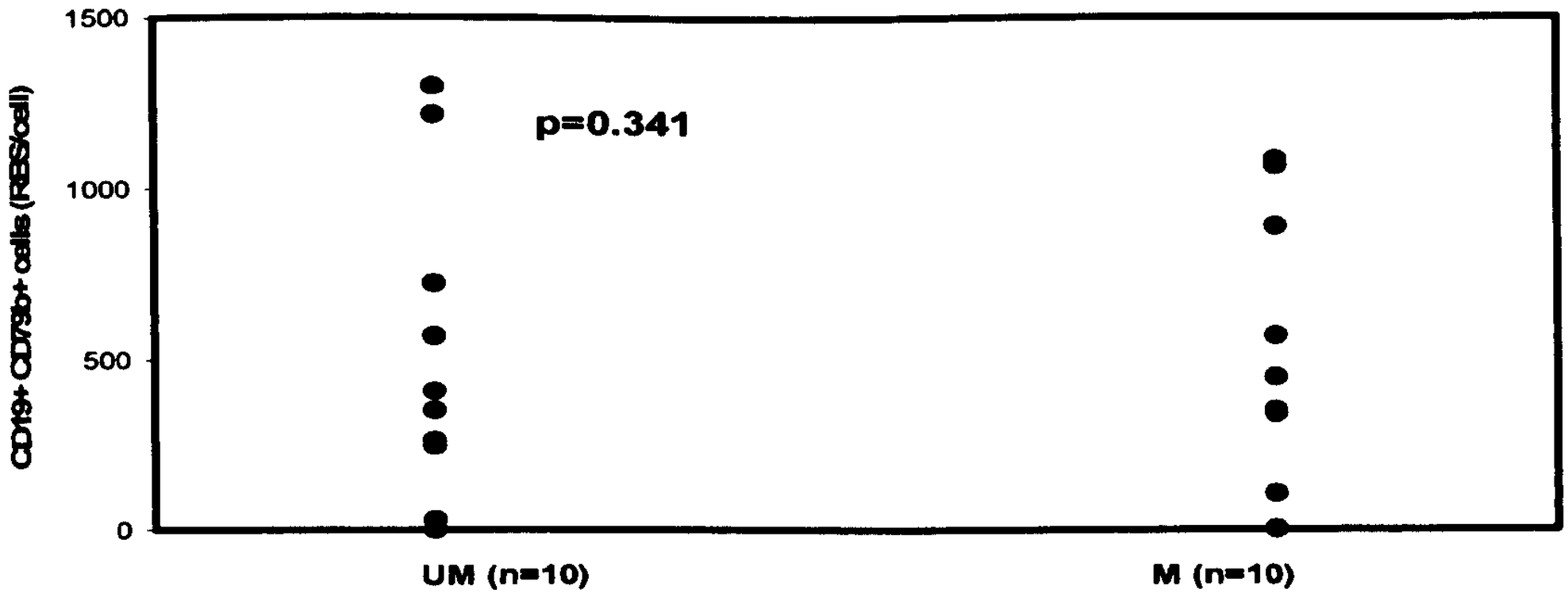


Figure 3.4 Expression of CD79b on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL cells using UMut IgVH genes showed no significant difference in the surface levels of CD79b as compared to those with Mut IgVH genes. The scatter plot show the distribution of CD79b expression (RBS/cell) on UMut (n=10) and Mut (n=10) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

In this cohort of patients, no significant difference (p=0.197) was found between CD38 expression by B-CLL cells with Mut IgVH genes (820 ± 1039 RBS/cell) and those with UMut IgVH genes of B-CLL cells (493 ± 551 RBS/cell; Figure 3.5 and Table 3.2).

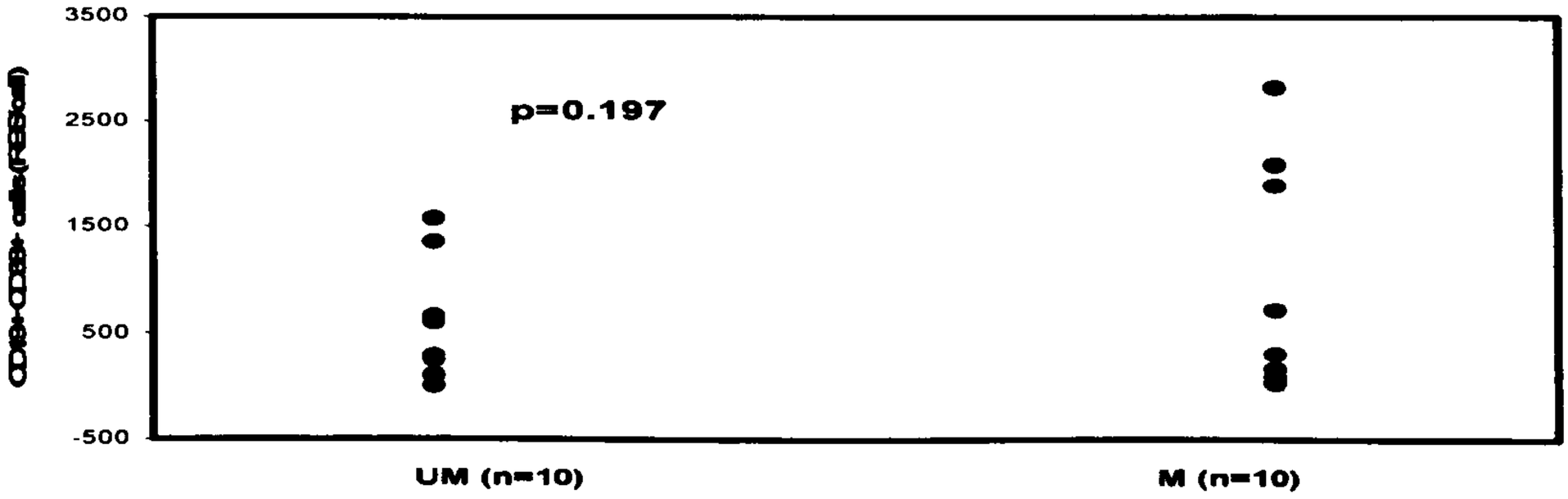


Figure 3.5 Expression of CD38 on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL cells using UMut IgVH genes showed no significant difference in the surface levels of CD38 from Mut IgVH genes. The scatter plot show the distribution of CD38 expression (RBS/cell) on UMut (n=10) and M (n=10) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

The surface marker CD86 was used in this study to measure the activation of normal B cells and B-CLL cells. Thus, it was important to measure the levels of this marker *ex vivo* and compare it with the level of CD180 and the mutational status of the patient. There was no detectable difference (p=0.374) of CD86 levels between B-CLL cells with Mut and UMut IgVH genes (Figure 3.6 and Table 3.2).

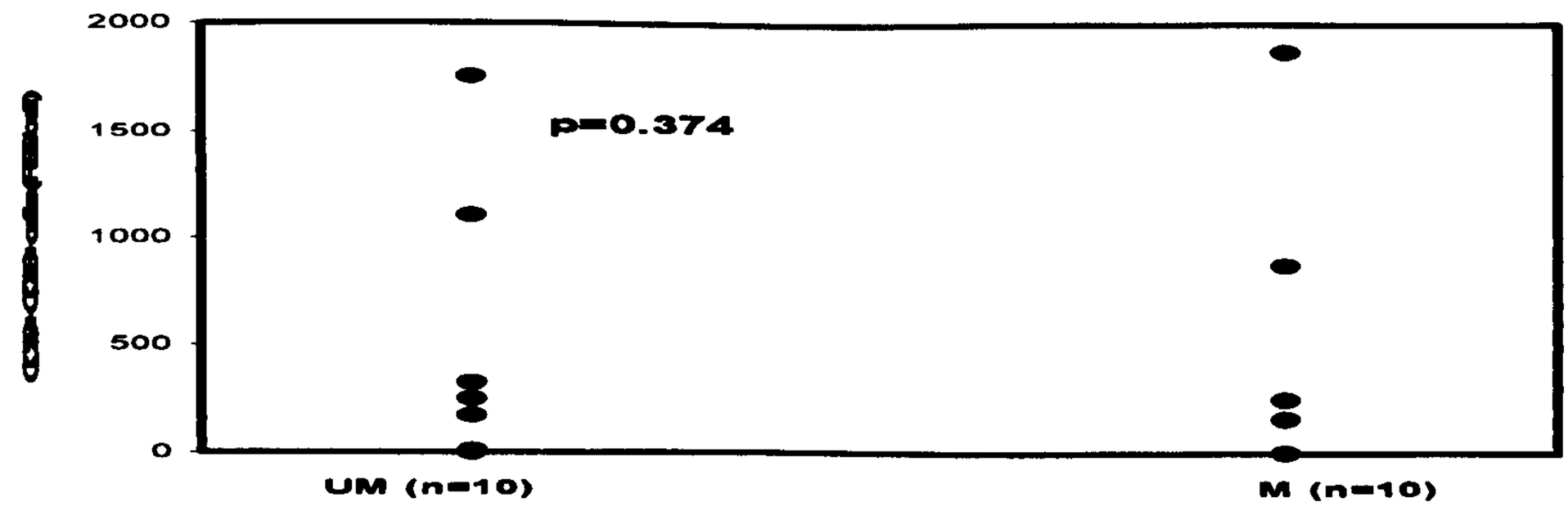


Figure 3.6 Expression of CD86 on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL cells using UMut IgVH genes showed no significant difference in the surface levels of CD86 from Mut IgVH genes. The scatter plot show the distribution of CD86 expression (RBS/cell) on UMut (n=10) and Mut (n=10) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

Equally, no significant difference (p=0.07) observed in the density, measured by RBS/cell, of surface CD40 on Mut and UMut IgVH genes of B-CLL cells (Figure 3.7 and Table 3.2), although there was some bias observed towards higher expression in UMut B-CLL cells.

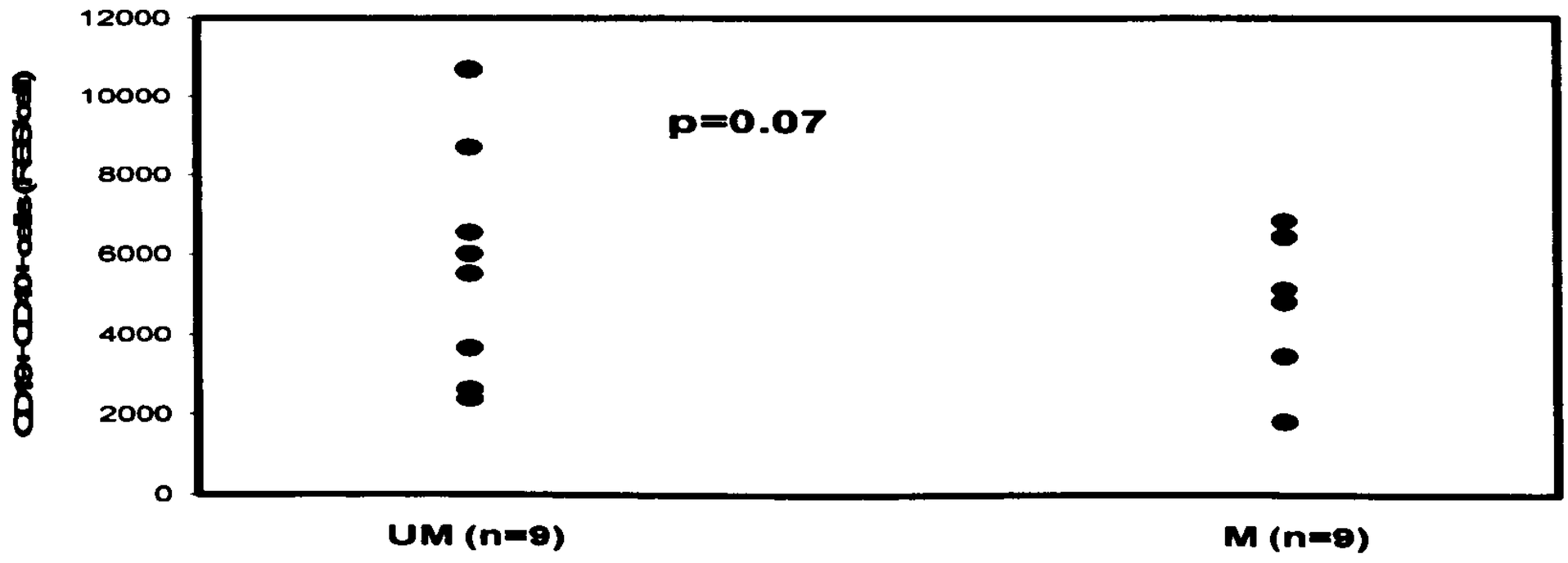


Figure 3.7 Expression of CD40 on B-CLL cells using Mut or UMut IgVH genes.

Patients with B-CLL cells using UMut IgVH genes showed no significant difference in the surface levels of CD40 from Mut IgVH genes. The scatter plot show the distribution of CD40 expression (RBS/cell) on UMut (n=9) and Mut (n=9) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

There was no significant difference (p=0.183) in the density of surface sIgD expressed by B-CLL cells with IgVH mutation or IgVH non-mutation status, as measured by RBS/cell (Figure 3.8 and Table 3.2).

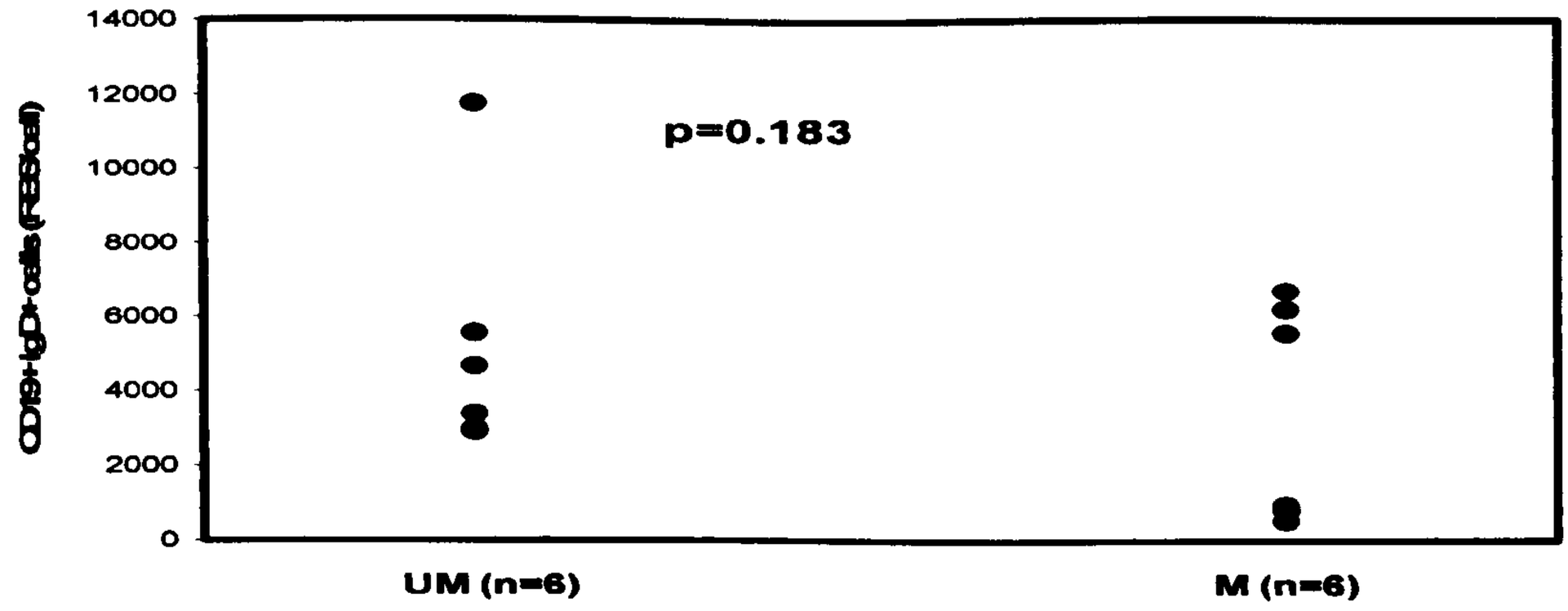


Figure 3.8 Expression of IgD on B-CLL cells using Mut and UMut IgVH genes.

B-CLL patients using UMut IgVH genes showed no significant difference in the surface levels of sIgD from Mut IgVH genes. The scatter plot show the distribution of IgD expression (RBS/cell) on UMut (n=6) and Mut (n=6) B-CLL cells. Data is shown as mean ± SD and statistical differences were calculated using the Mann-Whitney U-test. $p \leq 0.05$ was regarded as significant.

3.2.4 Expression of intracellular ZAP-70 in B-CLL cells:

Prior to comparing the correlation of the expression level of CD180 with that of the intracellular tyrosine kinase, ZAP-70, pilot experiments were carried out to optimise the detection of intracellular ZAP-70 in B-CLL cells. A cut-off value of 20% of B cells expressing ZAP-70 was chosen for the data analysis, as proposed previously (Luz *et al*., 2006; Principe *et al*., 2006,).

This cut-off level was determined on the basis of concordance with the mutational status of the IgVH gene, a proven prognostic marker for B-CLL (Orchard *et al*., 2004). Since ZAP-70 is constitutively expressed in T lymphocytes, its expression was initially analysed in T lymphocytes from a healthy control to enable a protocol to be set up for measurement in B-CLL cells (Figure 3.9).

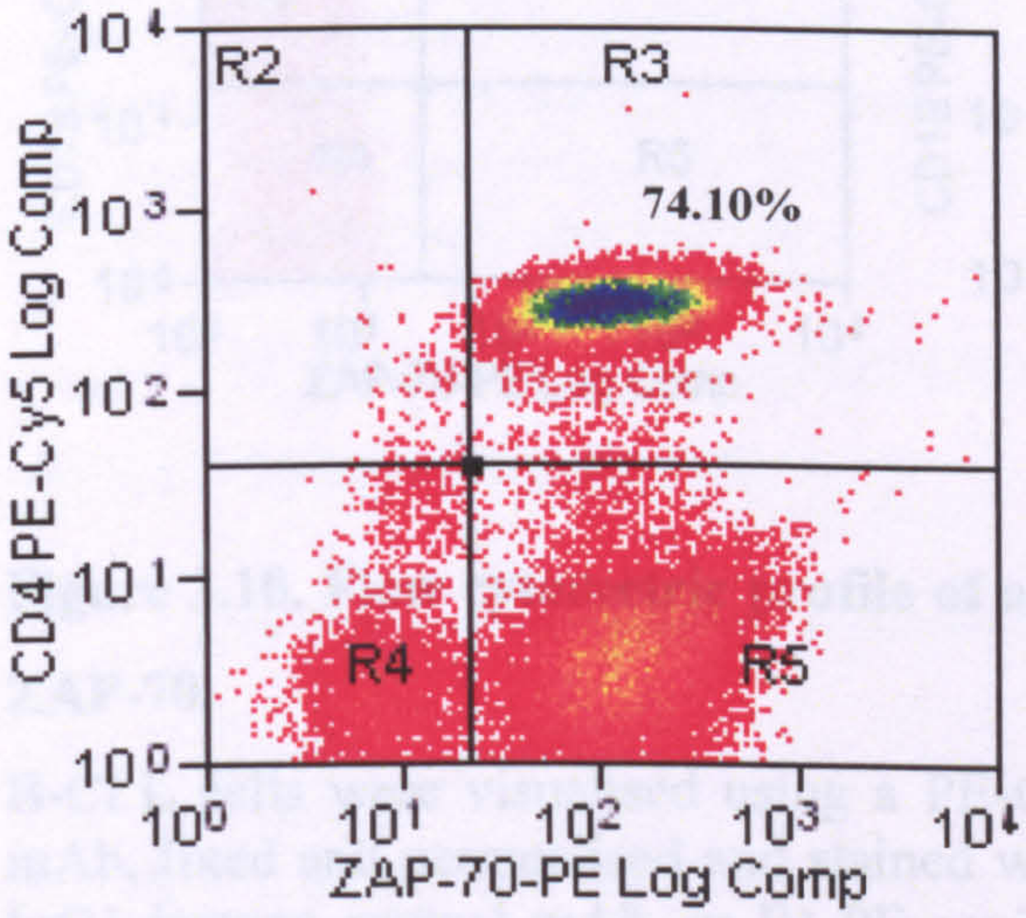


Figure 3.9. Flow cytometric detection of intracellular ZAP-70 in CD4+ T lymphocytes from a normal control

For convenience, intracellular expression of ZAP-70 was analysed in CD4+ T lymphocytes from a normal control. PBMCs were stained with PE-Cy5 conjugated mouse anti-human CD4 mAb, fixed and permealised and stained with PE-conjugated mouse anti-human ZAP-70 mAb. To visualise CD4 cells expressing ZAP-70, a dot plot of PE-Cy5 CD4 versus PE-ZAP-70 was created and quadrants were placed according to ZAP-70 expressing CD4+ T lymphocytes (R3) and NK cells (R5). Cells negative for ZAP-70 were visualised in R4.

Fourteen B-CLL patients were examined for their expression of intracellular ZAP-70. Results showed heterogeneous expression of ZAP-70 amongst the B-CLL patients. Eight patients were identified as negative for the expression of intracellular ZAP-70 (Range 0.45% to 10.48% ± 3.74%). Figure 3.10 shows a dot plot of a B-CLL clone negative for ZAP-70 expression. Quadrants were placed according to the ZAP-70 expressing T cell population in R5. The small population of ZAP-70+CD19+ cells (R3) possibly reflects a subset of activated normal B cells. Only 0.4% of cells are seen in this quadrant.

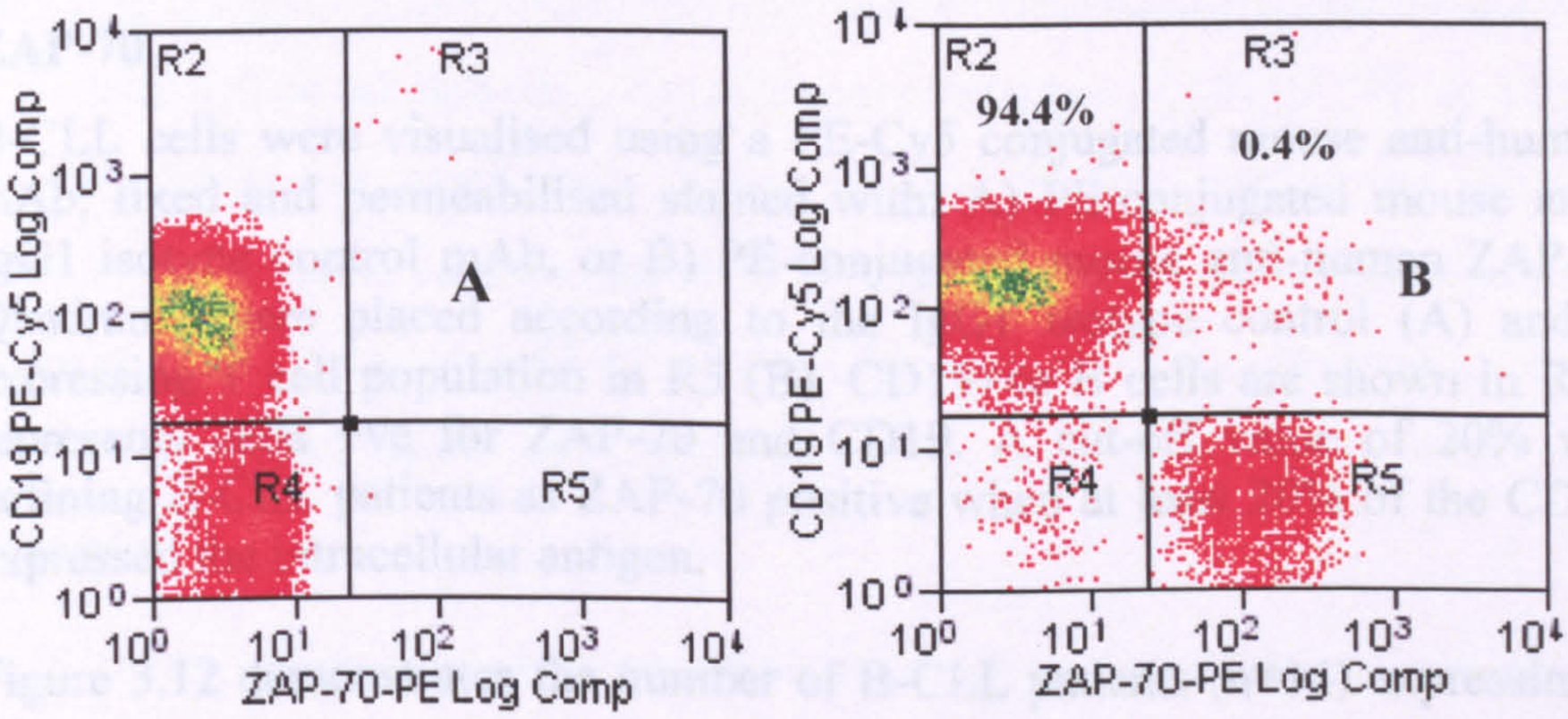


Figure 3.10. Flow cytometric profile of a B-CLL clone negative for intracellular ZAP-70.

B-CLL cells were visualised using a PE-Cy5 conjugated mouse anti-human CD19 mAb, fixed and permealised and stained with: A) PE-conjugated mouse anti-human IgG1 isotype control mAb, or B) PE-conjugated mouse anti-human ZAP-70 mAb. Quadrants were placed according to the IgG1 isotype control (A) and ZAP-70 expressing T cell population in R5 (B). CD19+ve B cells are shown in R2 and R3 represents cells positive for ZAP-70 and CD19. A cut-off value of 20% was used, defining B-CLL patients as ZAP-70 positive when at least 20% of the CD19+ cells expressed the intracellular antigen.

Six patients were identified as positive for the expression of intracellular ZAP-70 (Range 24.7% to 98.54% ± 32.12). In Figure 3.11 a dot plot of a B-CLL clone positive for intracellular ZAP-70 expression is shown demonstrating that 87.3% of all CD19+ cells are located in Quadrant R3.

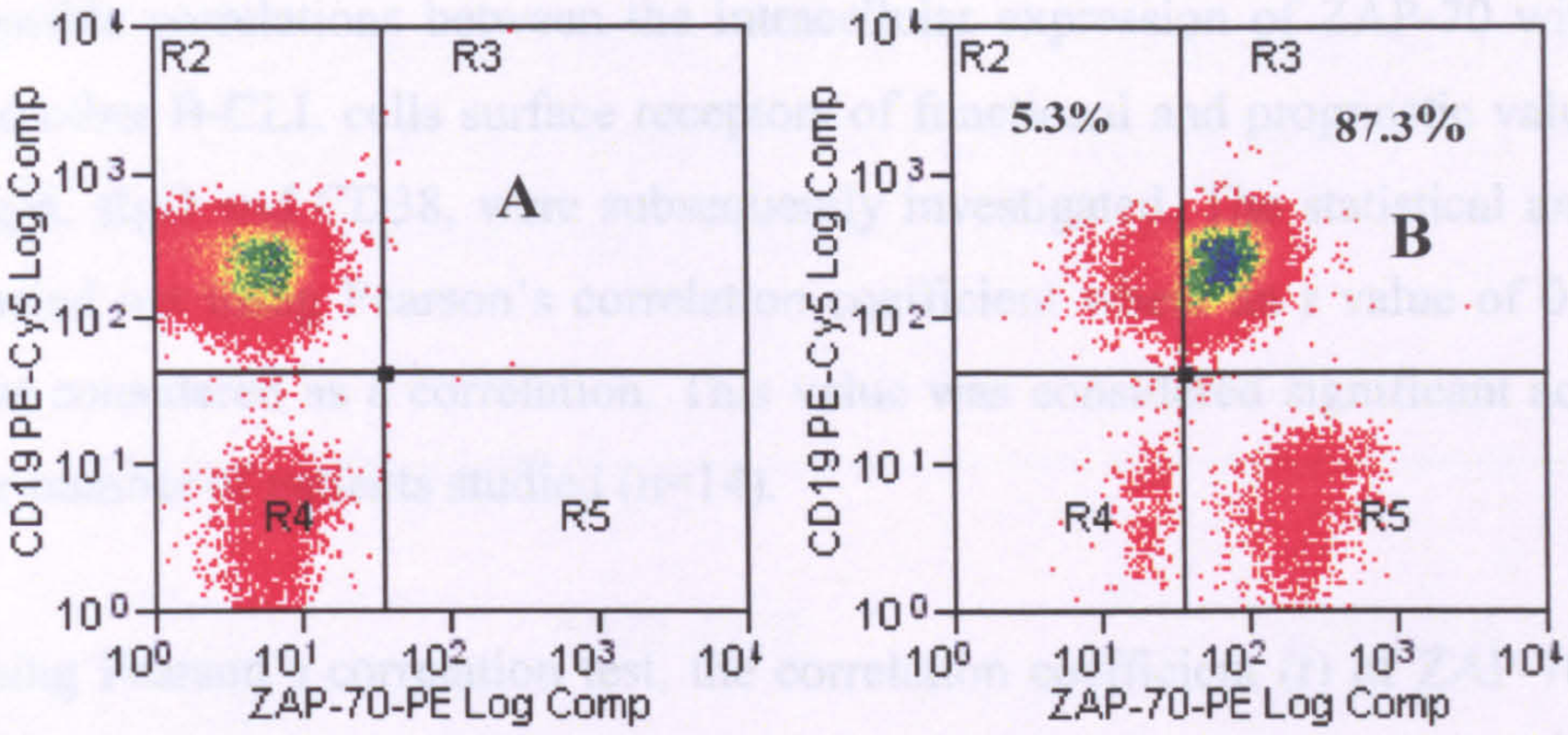


Figure 3.11. Flow cytometric profile of a B-CLL clone positive for intracellular ZAP-70.

B-CLL cells were visualised using a PE-Cy5 conjugated mouse anti-human CD19 mAb, fixed and permeabilised stained with: A) PE-conjugated mouse anti-human IgG1 isotype control mAb, or B) PE-conjugated mouse anti-human ZAP-70 mAb. Quadrants were placed according to the IgG1 isotype control (A) and ZAP-70 expressing T cell population in R5 (B). CD19+ve B cells are shown in R2 and R3 represents cells +ve for ZAP-70 and CD19. A cut-off value of 20% was used, defining B-CLL patients as ZAP-70 positive when at least 20% of the CD19+ cells expressed the intracellular antigen.

Figure 3.12 demonstrates the number of B-CLL patients (n=14) expressing the cut-off level of intracellular ZAP-70.

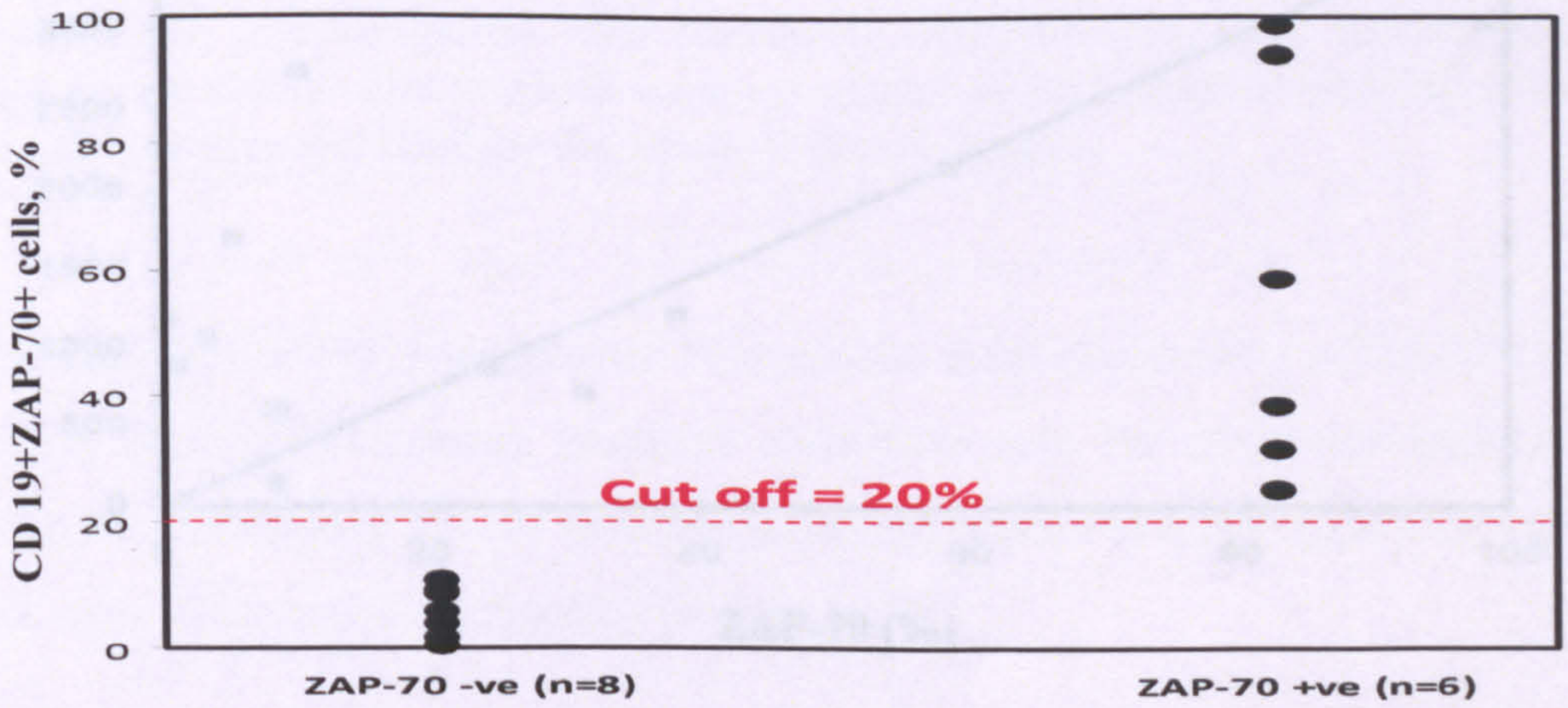


Figure 3.12. Expression of intracellular ZAP-70 in B-CLL lymphocytes showing ZAP-70-negative (n=8) and ZAP-70-positive (n=6) B-CLL cells. PBMCs from 14 B-CLL patients were stained with a PE-Cy5 anti-CD19 mAb, fixed and permeabilised, stained with PE-conjugated anti-ZAP-70 mAb and analysed by flow cytometry. A B-CLL clone was considered positive for ZAP-70 (ZAP-70+ve) when at least 20% of CD19+ B cells expressed this protein.

Possible correlations between the intracellular expression of ZAP-70 with CD180, and other B-CLL cells surface receptors of functional and prognostic value, such as sIgM, sIgD and CD38, were subsequently investigated. The statistical analysis was carried out using Pearson's correlation coefficient where an r value of 0.4 or more was considered as a correlation. This value was considered significant according to the number of patients studied (n=14).

Using Pearson's correlation test, the correlation coefficient (r) of ZAP-70 (%) and CD180 expression (RBS/cell) was equal to 0.47 (Figure 3.13), indicating a weak direct correlation. Since there was only one CD180-negative patient studied in this particular cohort of patients, the difference between ZAP-70 (%) in CD180- and CD180+ was not calculated. However, this finding cannot be considered as particularly decisive to draw any final conclusions about the correlation between the expression of CD180 and ZAP-70 on B-CLL cells given that both the r value is close to 0.5 and more cases are clearly required, particularly those who are CD180 negative.

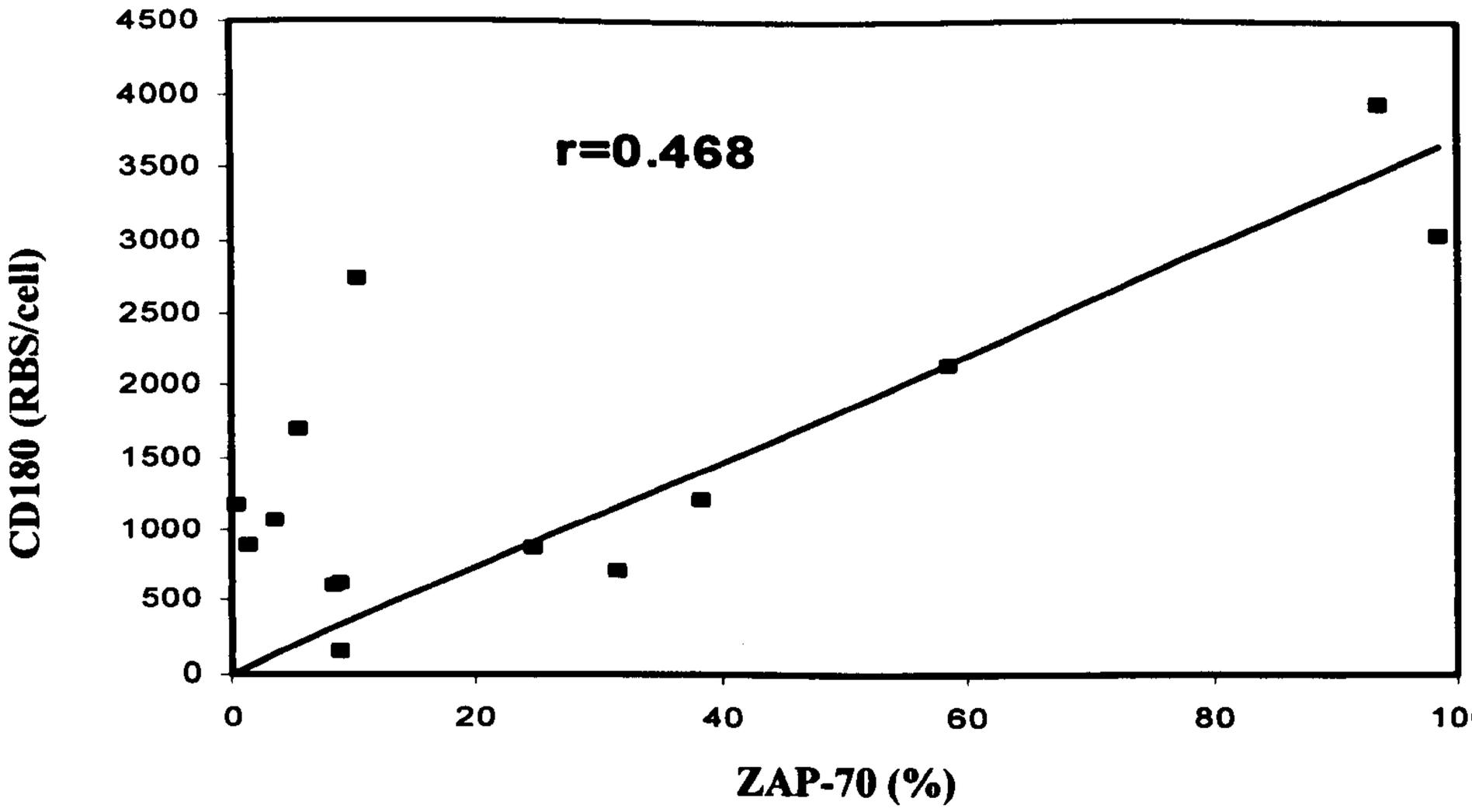


Figure 3.13. Correlation between the expression of intracellular ZAP-70 and surface CD180 in B-CLL.

Isolated PBMCs from 14 B-CLL patients were analysed for intracellular ZAP-70 expression (%) and compared with previously determined relative binding sites (RBS)/cell for CD180 (Table 3.1). The r-value was calculated using Pearson's correlation test.

Using Pearson's correlation test, a weak direct correlation was observed between the intracellular expression of ZAP-70 (%) and the expression of CD38 (RBS/cell). The difference in ZAP-70 (%) expression in CD38+ and CD38- B-CLL clones was calculated by the Mann-Whitney U-test (p=0.23; Figure 3.14,).

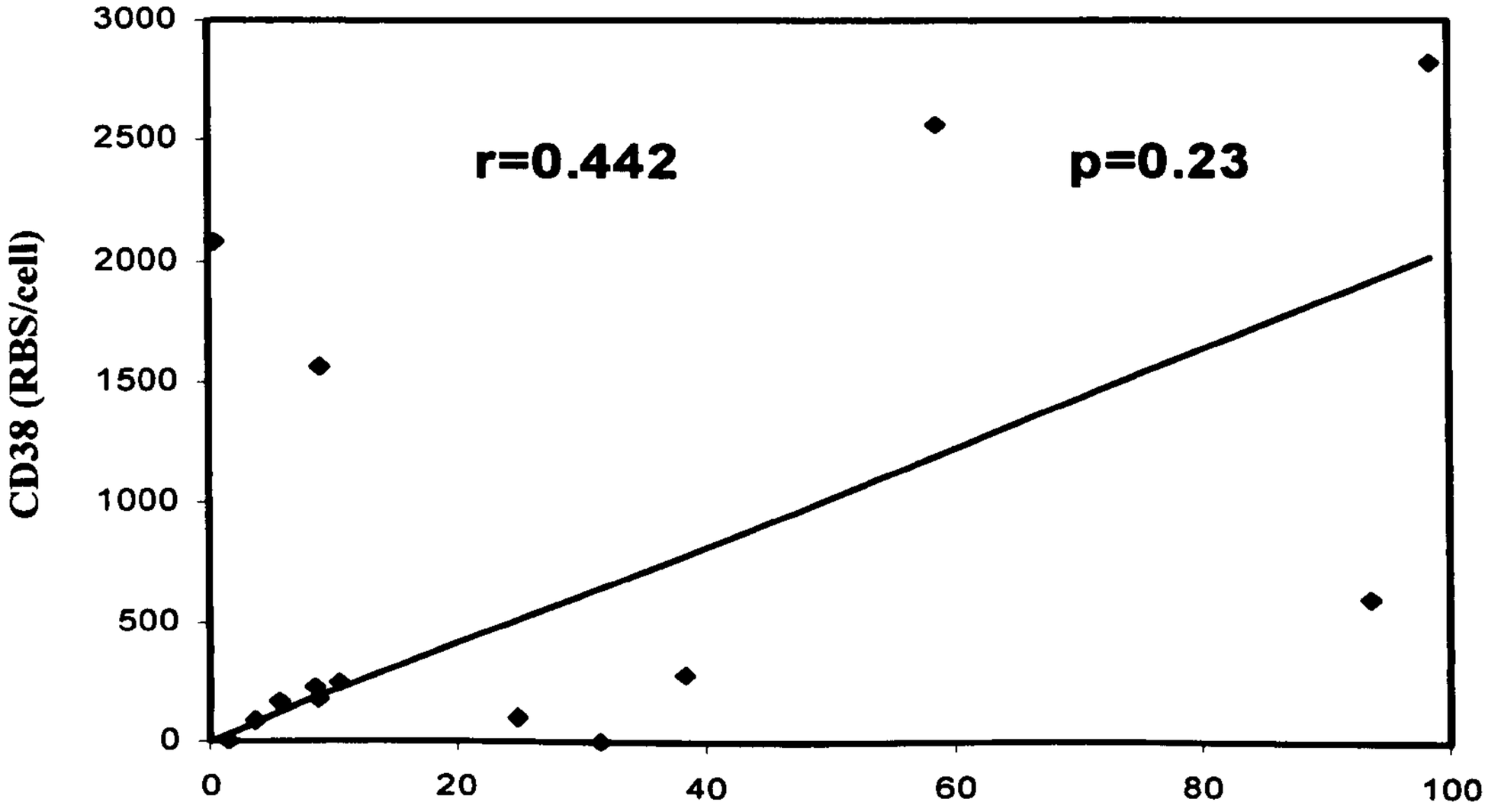


Figure 3.14. Correlation between expression of ZAP-70 and CD38 in B-CLL.

Isolated PBMCs from 14 B-CLL patients were analysed for intracellular ZAP-70 expression (%) and compared with previously determined relative binding sites (RBS)/cell for CD38. The r-values were calculated using Pearson's correlation test and p values were calculated by the Mann-Whitney U-test.

No correlation (r=0.209) was detected between the intracellular level of ZAP-70 (%) and sIgM (RBS/cell) by using Pearson's correlation test. No significant difference (p=0.69; Mann-Whitney U-test) in ZAP-70 (%) expression in either sIgM+ or sIgM- B-CLL cells (RBS/cell) was detected (Figure 3.15).

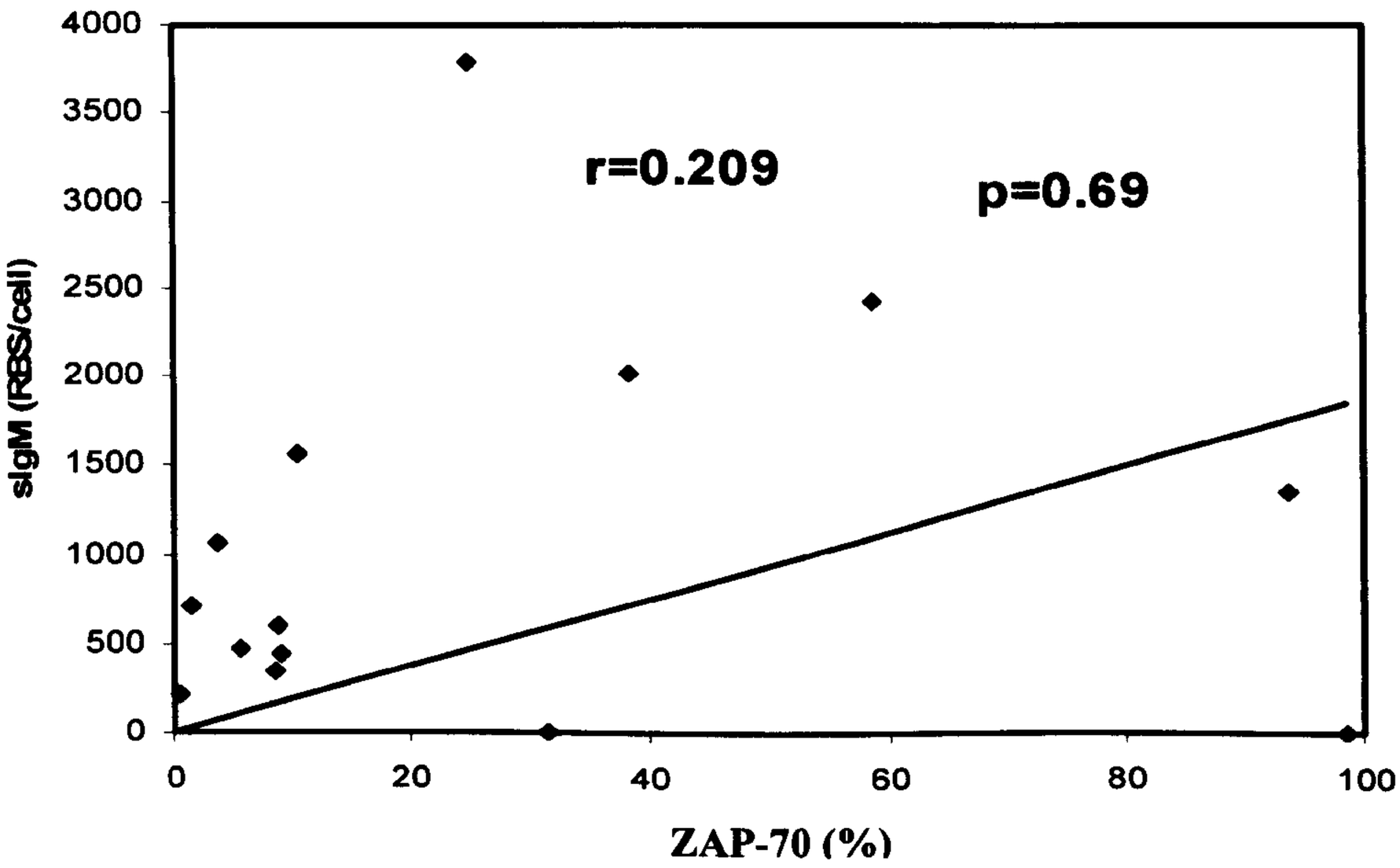


Figure 3.15. Correlation between expression of ZAP-70 and sIgM in B-CLL.

Isolated peripheral blood mononuclear cells from 14 B-CLL patients were analysed for intracellular ZAP-70 expression (%) and compared with previously determined RBS/cell for sIgM. The r-values were calculated using Pearson's correlation test and p values were calculated by the Mann-Whitney U-test.

No correlation (p=0.372; Pearson's correlation test) was detected between the expression of intracellular ZAP-70 (%) and sIgD (RBS/cell; Figure 3.16). All the patients in this study were positive for the surface expression of sIgD (i.e. expressing more than 500 RBS/cell).

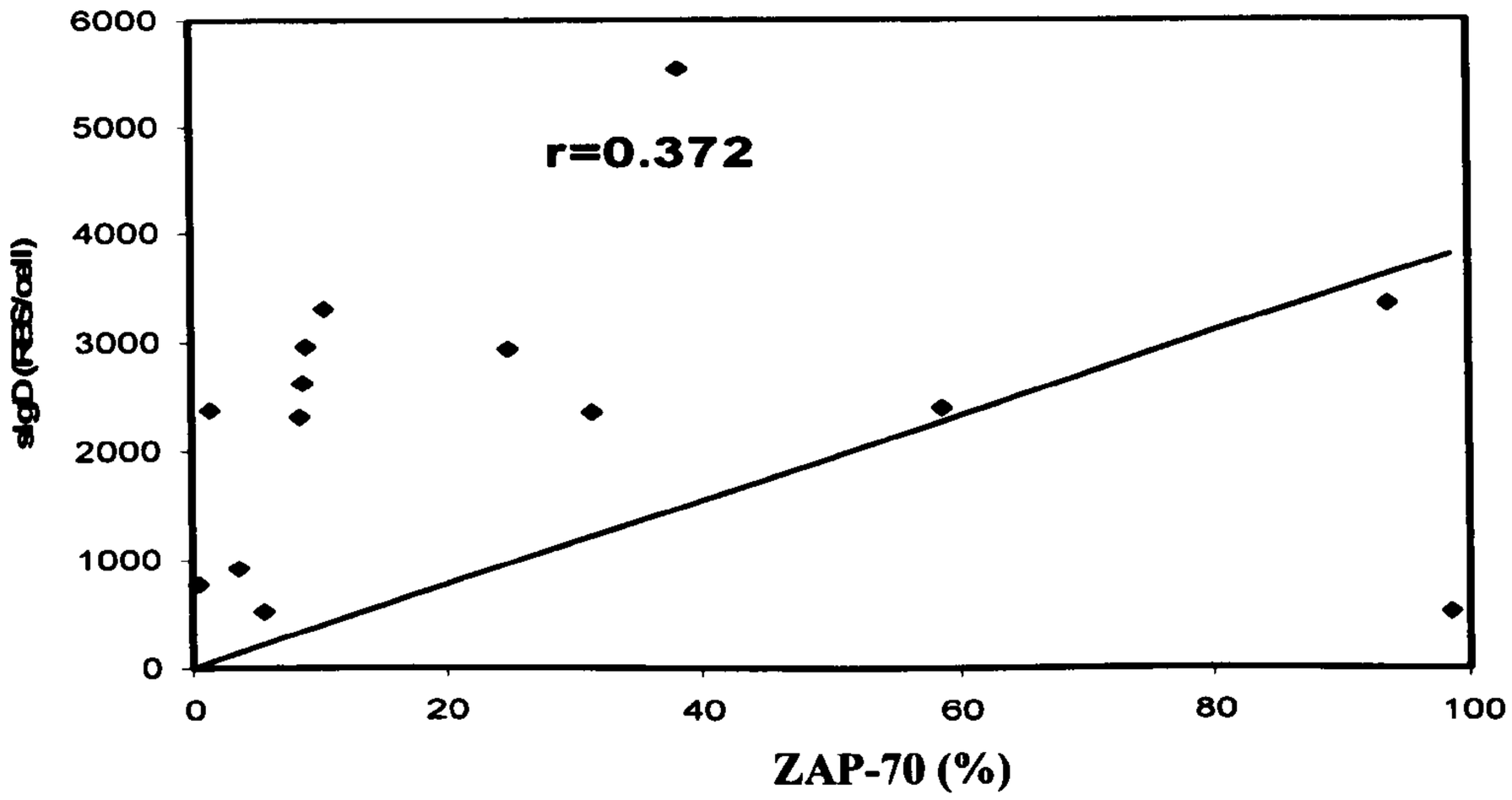


Figure 3.16. Correlation between the expression of ZAP-70 and IgD in B-CLL.

Isolated peripheral blood mononuclear cells from 14 B-CLL patients were analysed for intracellular ZAP-70 expression (%) and compared with previously determined relative binding sites (RBS)/cell for sIgD. The r-values were calculated using Pearson's correlation test.

Not surprisingly, and in line with previous observations (Del Principe *et al.*, 2006), there was a significant difference between the expression of intracellular ZAP-70 (%) and the mutational status of B-CLL IgVH genes ($p=0.01$; Mann-Whitney U-test) with more UMut rather than M B-CLL clones expressing ZAP-70, despite the small cohort of patients studied.

Discordant results were observed in B-CLL patients using unmutated IgVH genes that were negative for ZAP-70 expression (Figure 3.17).

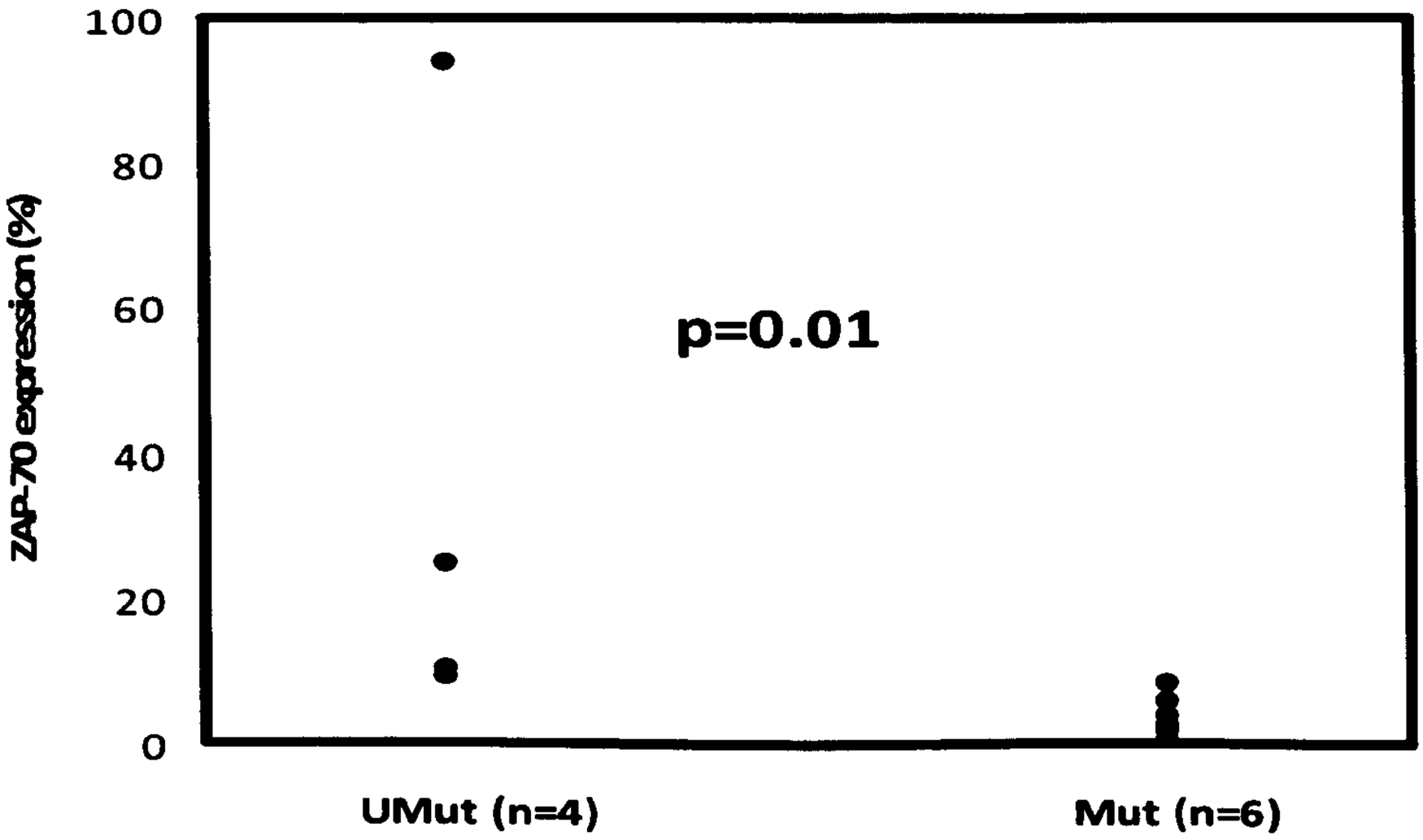


Figure 3.17. Comparison of the expression of ZAP-70 in B-CLL cells with unmutated and mutated IgVH genes.

Distribution of ZAP-70 expression (%) in B-CLL patients with B cells using unmutated (UMut) (n=4) or muted (Mut) (n=6) IgVH genes. Statistical differences were calculated using the Mann-Whitney U-test where $p \leq 0.05$ was regarded as significant.

3.2.5 Expression of CD27 by CD180+ B-CLL cells and control B cells:

In order to determine whether CD180 is preferentially expressed by memory or naive B cells, the expression of CD27 on CD180+ normal control B and B-CLL cells was studied. Figure 3.18 shows that in normal B cells, a small population of CD19+ B cells (16.8 ± 14.5 %; range 7.8 – 34.1 %) expressed CD27. Since the majority of normal B cells express CD180, the percentages of CD19+B cells co-expressing CD180 and CD27 are limited by the latter.

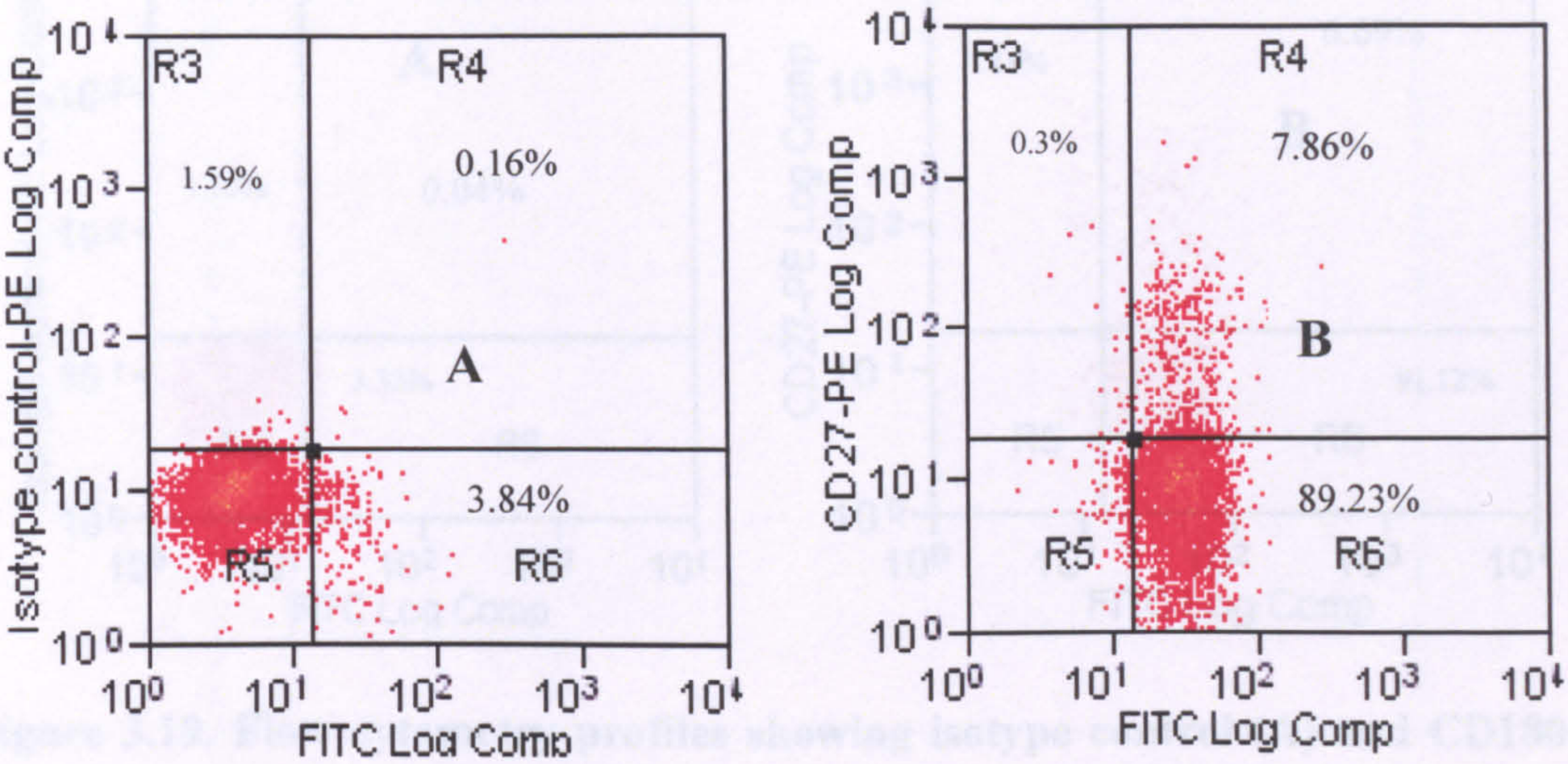


Figure 3.18. Flow Cytometry profiles showing isotype control (A) and CD180+ CD27+ (B) cells in a representative normal control individual.

PBMC were treated with unconjugated anti-CD180 mAb followed by secondary FITC-conjugated rabbit anti mouse Ab and PE Cy5-conjugated anti-CD19 mAb and PE-conjugated IgG1 isotype control (A) or PE-conjugated anti-CD27 (B) mAbs. The gate was placed on CD19+ cells. R6 represents CD180 positive cells only; R3+R4 represent CD27 positive cells while R4 represents B cell subset co-expressing CD180 and CD27.

A different picture was found with B-CLL patients where individual B-CLL clones were characterised by a substantial heterogeneity in the expression of CD27 (Figures 3.19 and 3.20).

Overall, 44.8 ± 36.8% (range 4.2 – 97.2%) of B-CLL cells co-expressed CD27 and CD180. Most likely, because of the small cohort of normal controls studied and the marked heterogeneity in B-CLL cells surface expression of CD27, there was no significant difference ($p=0.113$; Mann-Whitney U-test) detected between control B cells and B-CLL cells with regard to the co-expression of CD27 and CD180 (Figure 3.21).

However, expression of CD180 was clearly detected on the CD27+ as well CD27- B cell population as a clonal distribution (Figure 3.19 and Figure 3.20).

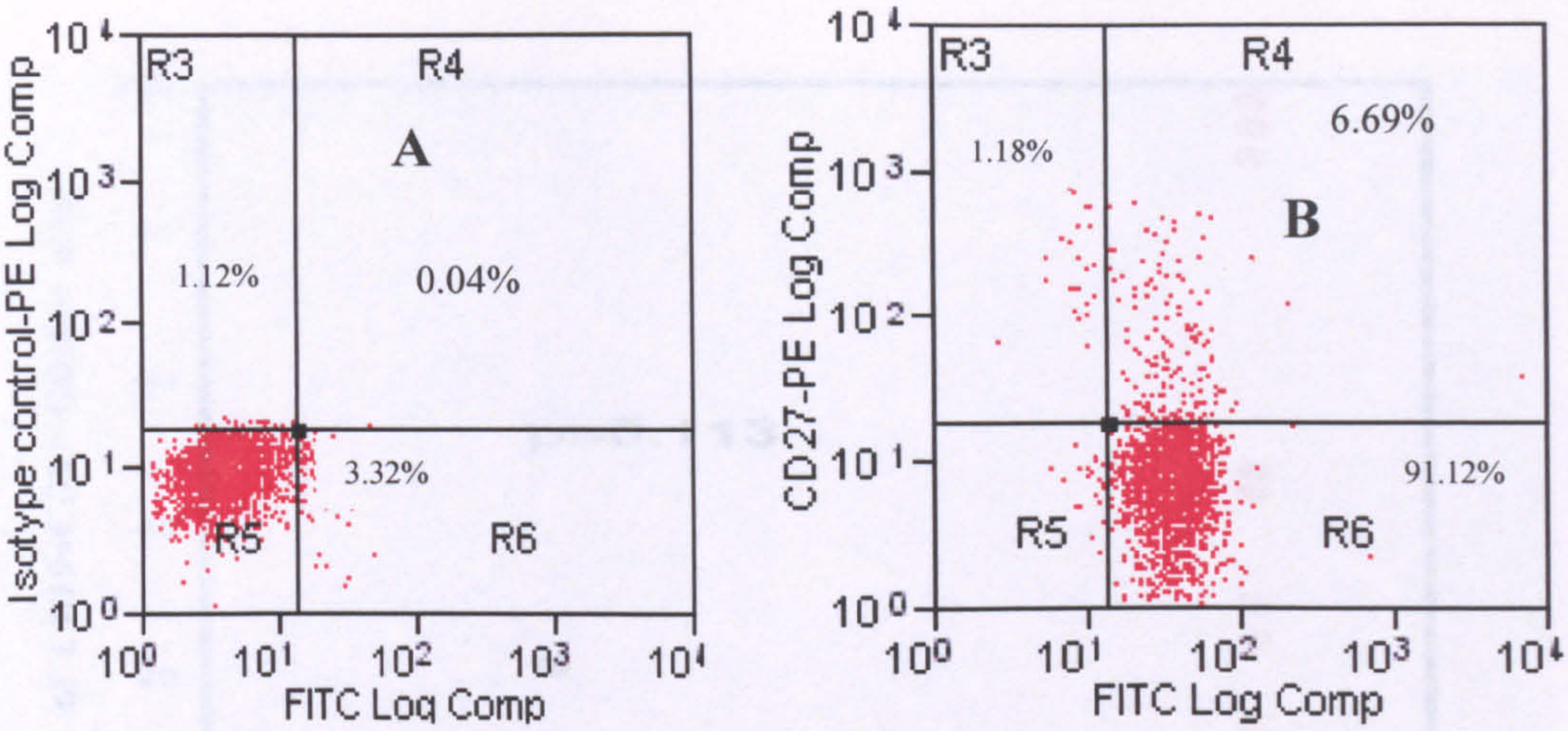


Figure 3.19. Flow cytometry profiles showing isotype control (A) and CD180+ CD27+ (B) cells in a representative B-CLL patient with low expression of CD27.

PBMC were treated with of unconjugated anti-CD180 mAb followed by secondary FITC-conjugated rabbit anti-mouse Ab and with PE Cy5-conjugated anti-CD19 mAb and PE-conjugated IgG1 isotype control (A) or PE-conjugated anti-CD27 (B) mAb. The gate was placed on CD19+ cells. R6 represents CD180 positive cells only; R3+R4 represent CD27 positive cells while R4 represents a B cell subset co-expressing CD180 and CD27.

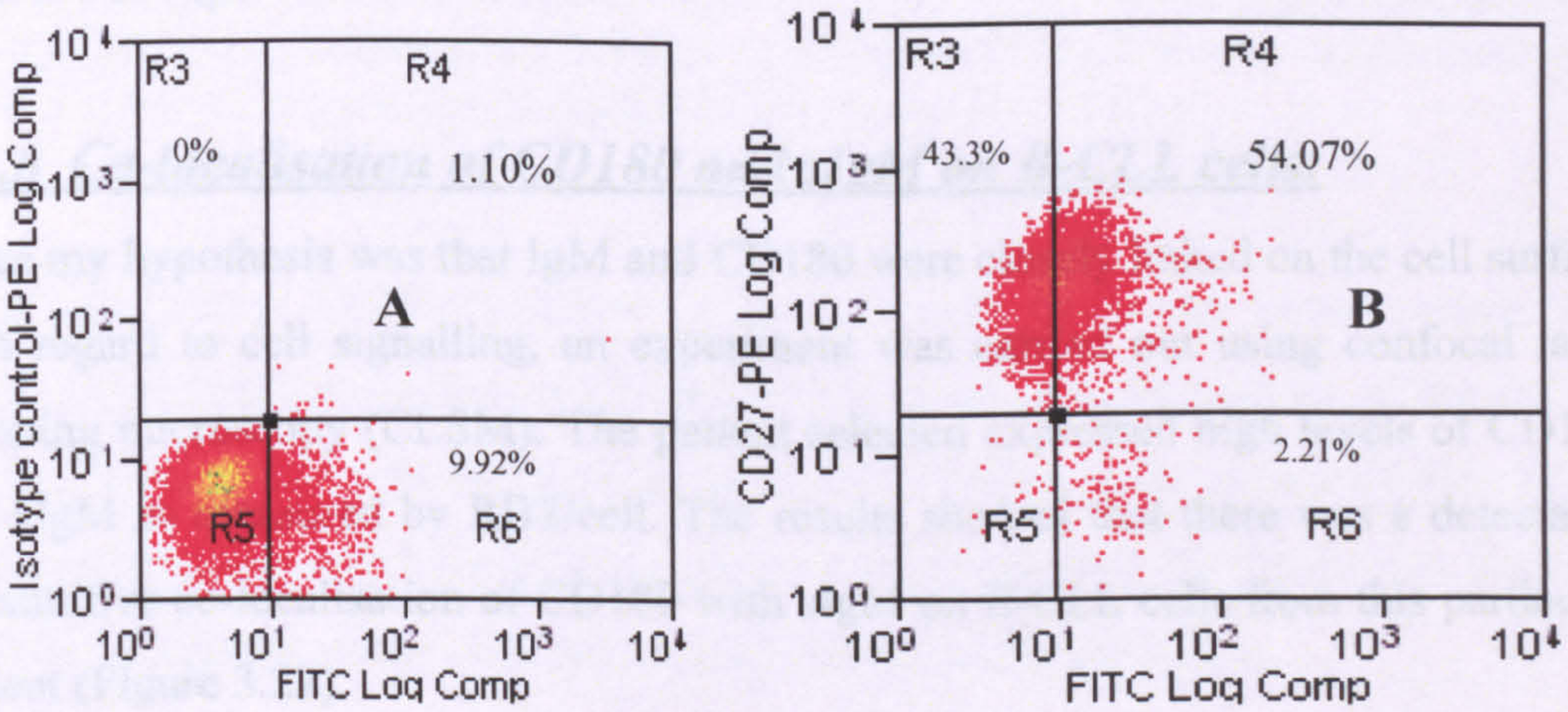


Figure 3.20. Flow cytometry dot plot showing isotype control (A) and CD180+ CD27+ (B) cells in one representative B-CLL patient with high co-expression of CD180 and CD27.

PBMC were treated with 10μg/mL of unconjugated anti-CD180 mAb followed by staining with FITC-conjugated rabbit anti-mouse Ab and 10μl of PE Cy5-conjugated anti-CD19 and PE-conjugated isotype control (A) or PE-conjugated anti-CD27 (B) mAbs. The gate was placed on CD19+ cells. R6 represents CD180 positive cells; R3+R4 represent CD27 positive cells while R4 represents B cell subset co-expressing CD180 and CD27.

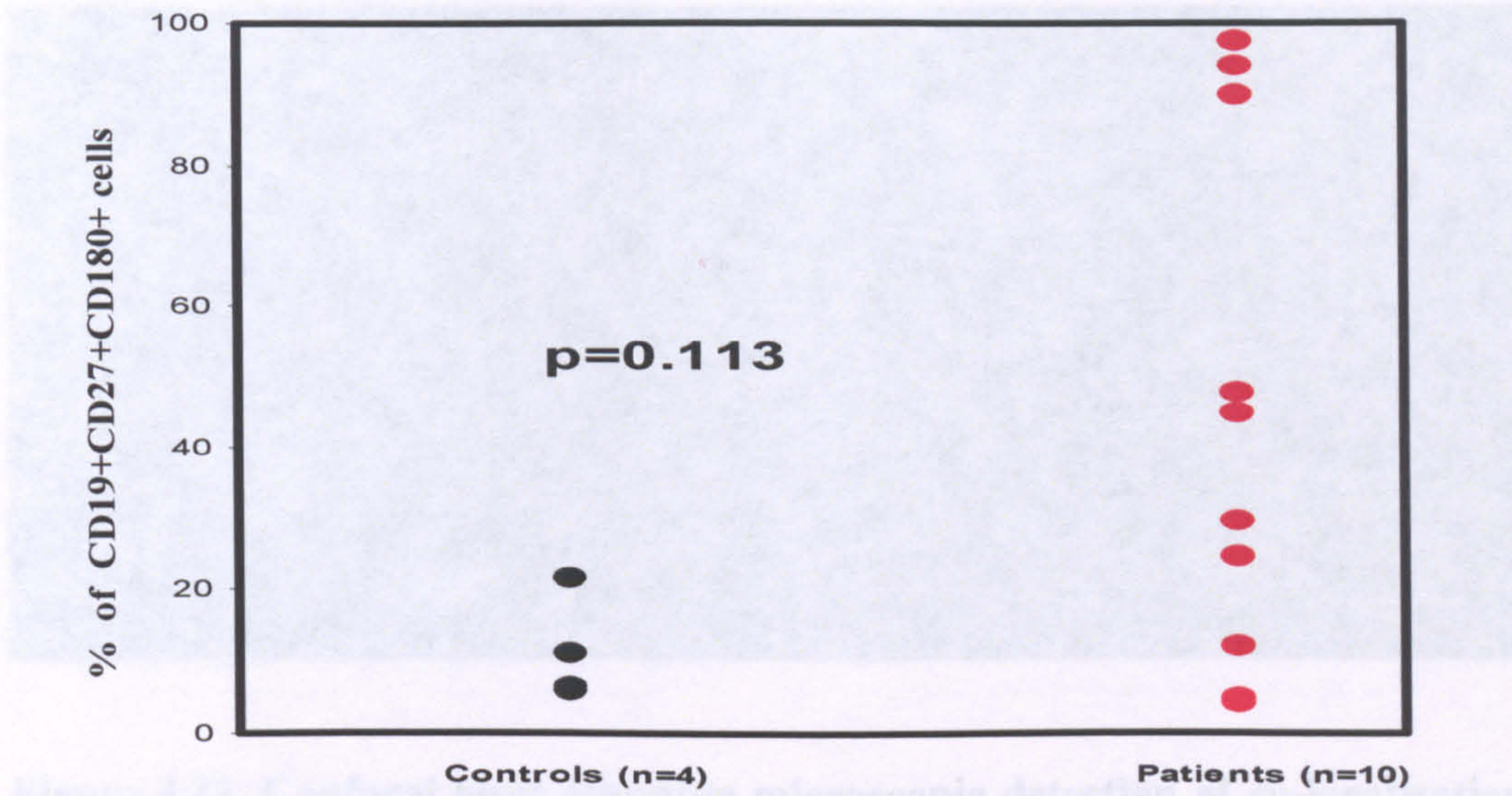


Figure 3.21. Percentages of CD180+ CD27+ CD19+ cells in normal controls and B-CLL cells.

Summary of data from four normal controls and ten B-CLL patients stained as described for Figure 3.20. Results were analysed by flow cytometry and statistical differences were calculated using the Mann-Whitney U-test where $p \leq 0.05$ was regarded as significant.

3.2.6 Co-localisation of CD180 and sIgM on B-CLL cells:

Since my hypothesis was that IgM and CD180 were closely linked on the cell surface with regard to cell signalling, an experiment was carried out using confocal laser scanning microscopy (CLSM). The patient selected expressed high levels of CD180 and sIgM as measured by RBS/cell. The results showed that there was a detectable constitutive co-localisation of CD180 with sIgM on B-CLL cells from this particular patient (Figure 3.22).

Co-localisation was identified by the presence of yellow fluorescence (Figure 3.22, Panel C), which was the combination of green fluorescence from FITC conjugated IgM mAb (Figure 3.22, Panel A) and red fluorescence from PE conjugated CD180 mAb (Figure 3.22, Panel B).

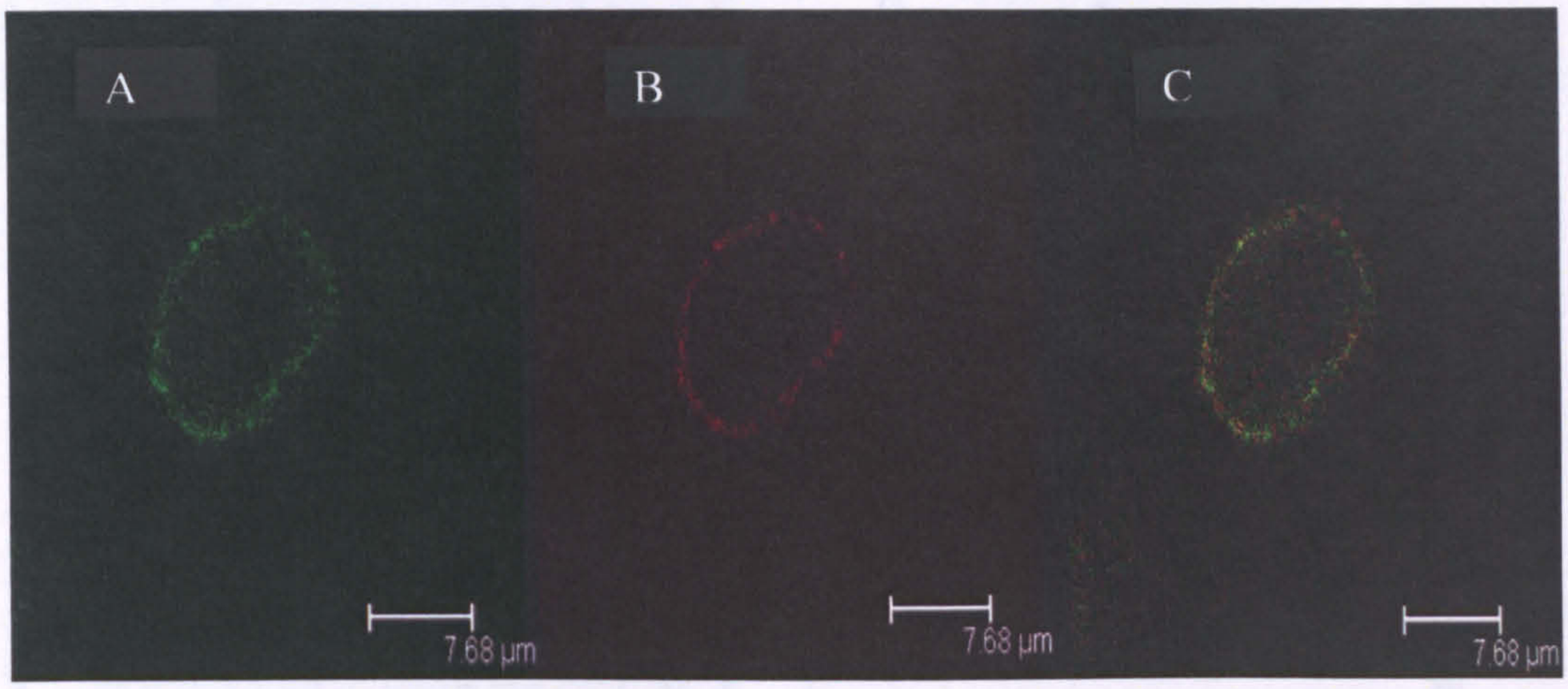


Figure 3.22. Confocal laser scanning microscopic detection of co-localisation of CD180 with IgM on a B-CLL cell.

PBMCs from a selected B-CLL patient were incubated with a FITC-conjugated mouse anti-human IgM monoclonal antibody (mAb) together with a PE-conjugated mouse anti-human CD180 mAb, and analysed using a confocal laser scanning microscope. Images of surface planar sections were acquired simultaneously using the FITC and PE filters. Single-colours and overlay are shown as indicated: A) FITC-conjugated mouse anti-human IgM mAb alone, B) PE-conjugated mouse anti-human CD180 mAb alone, C) overlay of FITC-conjugated mouse anti-human IgM mAb and PE-conjugated mouse anti-CD180 mAb. Bar: 7.68 µm.

Data analysis of CLSM pictures of B-CLL cells from the same patient also demonstrated co-localisation of CD180 with IgM when cross sections of the B-CLL cell membrane were taken. It appears that the fluorescent peaks of the FITC-conjugated mouse anti-human IgM mAbs co-localised with the fluorescent peaks of the PE-conjugated mouse anti-human CD180 mAbs (Figure 3.23).

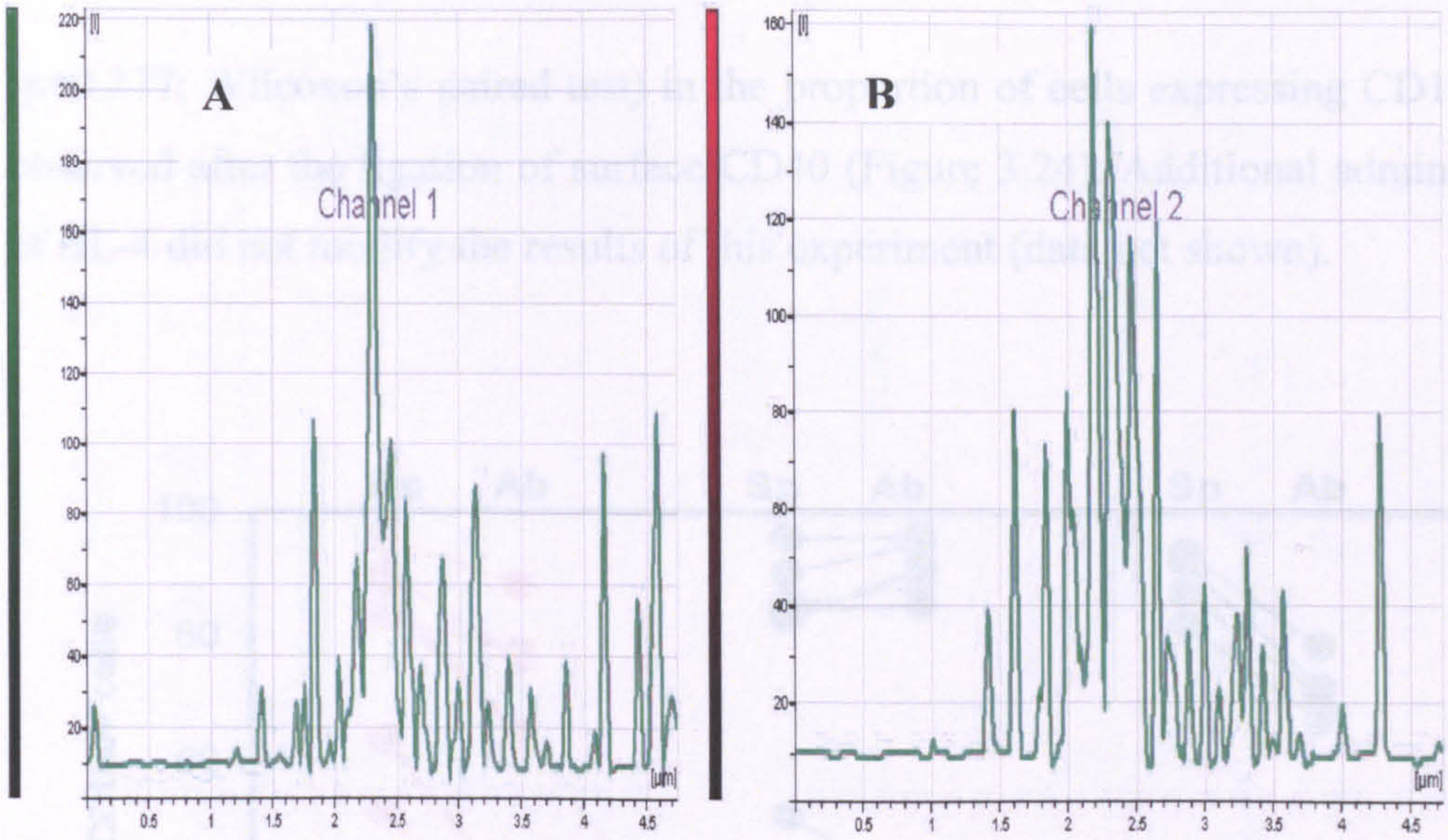


Figure 3.23. Cross section through the plasma membrane of a CD180+sIgM+ B-CLL cell.

PBMCs from a selected B-CLL patient were incubated with a FITC-conjugated mouse anti-human IgM mAb, together with a PE-conjugated mouse anti-human CD180 mAb, and analysed with a confocal laser scanning microscopy (CLSM). Via data analysis of pictures acquired from the CLSM, a cross section through the plasma membrane of a B-CLL cell was taken and displayed in graphs showing: A) length (µm) versus fluorescence intensity of FITC conjugated mouse anti-human IgM mAbs, and B) length (µm) versus fluorescence intensity of PE-conjugated mouse anti-CD180 mAbs. Channel 1 and 2 represent FITC and PE filters on the CLSM, respectively.

3.2.7 Modulation of the expression of CD180 on B-CLL and control B cells following their activation

In order to gain some insight into the role of CD180 on normal B cells and B-CLL cells, the levels of CD180 expression were studied in relation to activation. I chose to activate the cells through the BCR to mimic antigen specific activation and used anti-CD40 mAbs to activate the cells via different pathways. The percentages of cells expressing CD180 were down-regulated upon ligation of the BCR with goat anti-human IgM $F(ab)_2$ in seven normal control B cells ($p=0.01$; Wilcoxon's paired test) and goat anti-human IgD $F(ab)_2$ in four normal control B cells ($p=0.05$; Wilcoxon's paired test) after 48 h and/or 72 h of culture. However, no significant changes

(p=0.237; Wilcoxon's paired test) in the proportion of cells expressing CD180 were observed after the ligation of surface CD40 (Figure 3.24). Additional administration of rIL-4 did not modify the results of this experiment (data not shown).

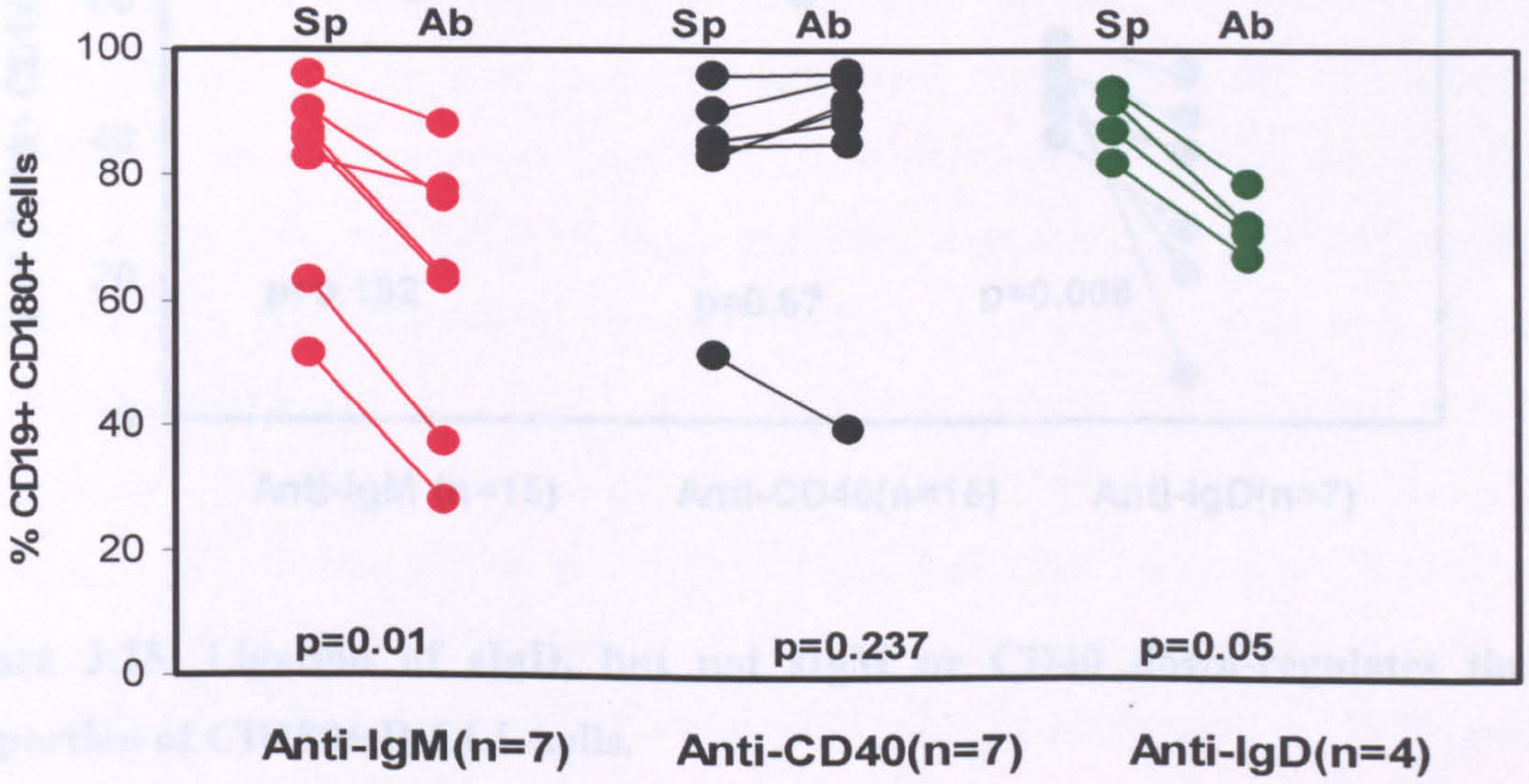


Figure 3.24. Ligation of surface IgM and IgD, but not CD40, leads to the down regulation of the proportion of CD180+ B cells.

Control PBMCs were incubated with goat anti-human IgM F (ab) $_2$, goat anti-human IgD F(ab)$_2$ antibodies and anti CD40 mAbs for 48 and/or 72 h. Cells were stained with PE-conjugated IgG1 isotype control or anti-CD180 mAb and counterstained with PE Cy5-conjugated mouse anti-human CD19 mAb. Results were analysed by flow cytometry and Wilcoxon's paired test.

Different results were observed for B-CLL cells: neither goat anti-human IgM F(ab) $_2$ nor anti-CD40 mAbs induced any changes in the percentages of CD180+ B-CLL cells (p=0.182 and p=0.67 respectively). However, ligation of IgD with goat anti-human IgD F(ab)$_2$ significantly decreased the surface expression of CD180 on B-CLL cells in all seven patients studied (Figure 3.25, p=0.008).

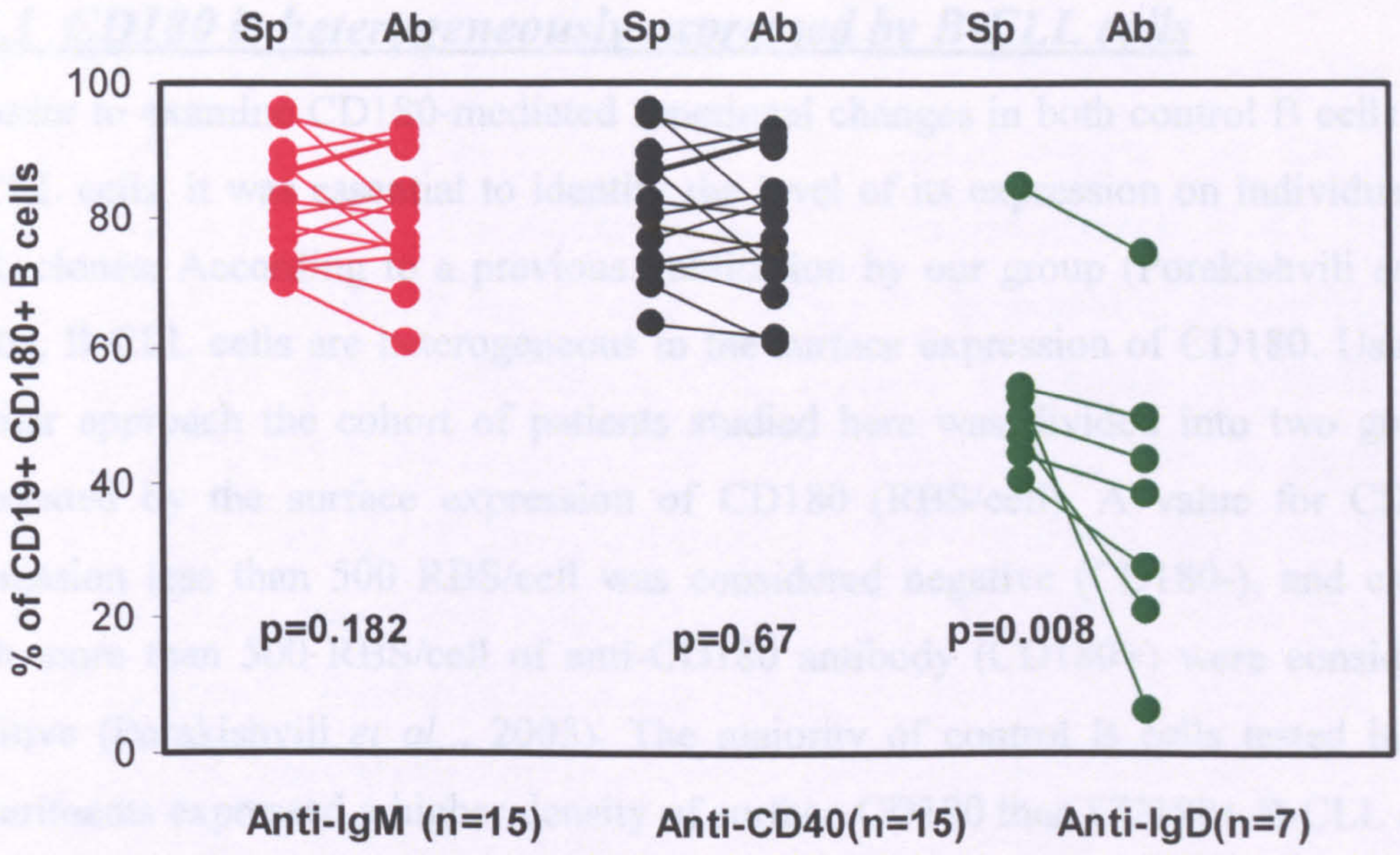


Figure 3.25. Ligation of sIgD, but not sIgM or CD40 down-regulates the proportion of CD180+ B-CLL cells.

PBMCs from B-CLL cells were incubated with goat anti-human IgM F (ab) $_2$, goat anti-human IgD F (ab) $_2$ antibodies and anti CD40 mAbs for 48 and/or 72 h. Cells were stained with PE-conjugated isotype control or anti-CD180 mAb and counterstained with PE Cy5-conjugated mouse anti-human CD19 mAb. Results were analysed by flow cytometry and Wilcoxon's paired test.

3.3 Discussion

3.3.1 CD180 is heterogeneously expressed by B-CLL cells

In order to examine CD180-mediated functional changes in both control B cells and B-CLL cells, it was essential to identify the level of its expression on individual B-CLL clones. According to a previous publication by our group (Porakishvili *et al.*, 2005), B-CLL cells are heterogeneous in the surface expression of CD180. Using a similar approach the cohort of patients studied here was divided into two groups delineated by the surface expression of CD180 (RBS/cell). A value for CD180 expression less than 500 RBS/cell was considered negative (CD180-), and clones with more than 500 RBS/cell of anti-CD180 antibody (CD180+) were considered positive (Porakishvili *et al..*, 2005). The majority of control B cells tested in our experiments expressed a higher density of surface CD180 than CD180+ B-CLL cells (Table 3.1). B-CLL cells belong to a CD5+ B1 cell population and this could explain the lower density of CD180. However, it has been reported previously that control CD5+ and CD5- cells express similar levels of CD180 (Porakishvili *et al.*, 2005) and therefore makes this unlikely.

In this particular cohort of patients, 14 were identified as CD180- and 22 as CD180+ representing 39% and 61% of the B-CLL patients studied respectively. The cut-off level used in the study, 500 RBS/cell, has been utilised also for defining positivity for all other cell surface markers. Although not ideal, this extrapolation has been further confirmed by the functional studies carried out on both CD180+ and CD180- B-CLL clones.

3.3.2 CD180 on B-CLL cells as a potential prognostic marker:

The primary aim of my study was to investigate surface CD180 mediated functional changes in control B lymphocytes and B-CLL cells such as activation, proliferation and susceptibility to apoptosis in conjunction with established signalling pathways. As such, it was deemed important to phenotype B-CLL clones for both the major signalling surface molecules (sIgM, sIgD, CD79b, CD40) and prognostic markers (CD38, ZAP-70 and IgVH mutations) as well as the activation marker CD86.

As shown in Table 3.2, B-CLL cells with Mut IgVH genes expressed significantly higher (p=0.04) levels of CD180 (2345 ± 1246, range 1062-5331 RBS/cell) than B-CLL cells with UMut IgVH genes (1298 ± 1252, range 0-3928 RBS/cell). The data obtained from this cohort of patients confirmed earlier reports from our group (Porakishvili *et al.*, 2005). My studies showed that surface expression of CD180 in B-CLL patients is consistent and does not change over the period of 36 months (data not shown). Also, B-CLL cells demonstrated a clonal distribution of CD180. The consistent levels of CD180 expressed on B-CLL cells coupled with its significant association with Mut IgVH genes, independence from treatment and from disease stage or complete blood cell count (Porakishvili *et al.,* 2005), makes CD180 a potential prognostic marker for assessing B-CLL disease outcome. Hence, all these findings suggest that CD180 may have a direct correlation with mutated IgVH genes. Since sequencing IgVH genes is beyond the capacity of most routine laboratories, there is therefore a clear need to identify an "easy to measure" marker that has the equivalent prognostic value as that to IgVH mutation status (Hamblin, 2007).

Hence, our group propose that surface levels of CD180 can be a surrogate prognostic marker for IgVH genes. In fact, expression of CD180 on a B-CLL cell surface is easily measured by flow cytometry by using the methodology described in *Material and Methods*. A value for CD180 expression less than 500 RBS/cell is considered negative, while clones with more than 500 RBS/cell of anti-CD180 antibody are regarded as positive (Porakishvili *et al.*., 2005). However, to confirm this finding more patients are required in both CD180+ and CD180- groups with known mutational status.

3.3.3 sIgM is expressed significantly higher on CD180+ than CD180- B-CLL cells

Table 3.1, demonstrates that the average density (1341 ± 2718 RBS/cell) of sIgM on all B-CLL clones entered into this study was significantly lower ($p < 0.05$) compared to that observed on normal control B cells (17907 ± 8553 RBS/cell). This is in accordance with a number of previous studies examining sIgM expression on B-CLL cells (Hamblin *et al.*, 1999, Guipaud *et al.*, 2003; Nakagawa *et al.,* 2006). There was a significant difference (p=0.05) in the surface expression of sIgM found between the

CD180+ and CD180- B-CLL cell populations (Table 3.1). IgM is expressed on the B cell throughout its developmental stages (Foy *et al.,* 1993; van Kooten *et al.,* 1997), therefore at this stage I suggest that sIgM may be down-regulated on B-CLL cells in association with CD180 when the latter is down-regulated to become a CD180-B-CLL cell. However, to date there is no literature available to support this hypothesis although, it has been suggested from studies on systemic lupus erythematosus (SLE) that activated B cells down-regulate CD180 on the B cell surface (Koarada *et al.*, 2001). Feasibly by the same mechanism, sIgM in association with CD180 may be down-regulated on the B-CLL surface since B-CLL cells are classified as an activated subset of a normal B cell population (Damle *et al.*, 2007). A previous study has shown that the low levels of sIgM on a B-CLL surface are related to poor expression of CD79b and up-regulation of this latter molecule could correct the B-CLL phenotype (Minuzzo *et al.*, 2005). However, another study carried out by Vuillier *et al*, (2005) suggests an impaired glycosylation and folding of the CD79a and *mu* chains with no defect observed in CD79b. Accordingly, it is a subject for a pertinent future study to identify the correct mechanism involving low expression of sIgM on the B-CLL cell and its association with CD180.

No correlation was detected between sIgM and CD180 expression (r=0.103) on B-CLL cells although a significant difference (p=0.03) was found in the density of sIgM expression on B-CLL cells between those with UMut and Mut IgVH genes (Table 3.2). B-CLL cells with UMut IgVH genes expressed significantly higher levels of sIgM on the cell. This data supports studies made previously by our group which demonstrated low density of sIgM expression on Mut cells as compared to UMut cells (Porakishvili *et al.*, 2005).

3.3.4 No significant difference in the expression of IgD on CD180+ and CD180- B-CLL cells

No significant difference (p=0.41) was observed in the expression of sIgD on CD180+ and CD180- B-CLL cells although there was a tendency for CD180- B-CLL cells to express a higher density of this surface molecule in comparison to that seen on CD180+ B-CLL cells (4449 ± 5013 vs 2913 ± 3185 RBS/cell respectively, Table 3.1). It has been shown earlier for SLE patients that the CD180- B cell population

was highly activated and demonstrated negativity for the surface expression of sIgD (Koarada *et al.,* 1999). This indicates that B-CLL cells unlike B cells from SLE patients co-express both sIgD and CD180 as in the current cohort of patients, sIgD ligation on normal control and B-CLL cells led to increased activation (*see Chapter 4*). Also, there was a strong association seen between sIgD induced apoptosis and CD180 positivity (*see Chapter 5*).

Interesting results were seen with regard to the levels of sIgD expression on the B cells as, unlike other measured surface molecules, CD180+ B-CLL cells showed a tendency for lower expression of sIgD as compared to normal controls although these results cannot be conclusive because of the markedly high standard deviation (Table 3.1). However, CD180- B-CLL cells showed no significant difference in the expression of sIgD from normal control B cells.

Activated and triggered B-CLL cells have down-regulated sIgD expression (Damle *et al.,* 2002). This finding is in concordance with my present data as the B-CLL cells were not activated *ex vivo*, thus sIgD was intact. However, further studies are required on activated B-CLL cells to observe the effect on the density of sIgD and its association with CD180.

3.3.5 No significant difference in the expression of CD79b on CD180+ and CD180- B-CLL cells

Another surface molecule CD79b, together with CD79a is expressed exclusively on all B cells in association with the BCR (Chu and Arber, 2001). In this present study, most B-CLL cells expressed diminished levels of surface CD79b compared to normal control B cells (Table 3.1) although no significant difference was detected in the level of CD79b on CD180+ and CD180- B-CLL cells ($p=0.16$). These results indicated that CD79b is poorly expressed on B-CLL cells regardless of the level of CD180 expression. This finding is in concordance with other studies which showed that BCR expression is influenced by B-CLL-related defects in CD79b leading to its diminished and/or inactive form on B-CLL cells (Mark *et al.*, 2002; Vuillier *et al.*, 2005; Minuzzo *et al.*, 2005; Cajiao *et al.*, 2007).

The mechanisms proposed for this defect include reduced expression of CD79b mRNA, somatic mutation in the gene coding for CD79b (i.e. B29) or a defect in splicing, yielding, ΔCD79, an altered form of CD79b (Mark *et al.*, 2002). The surface expression of CD79b in B-CLL is down-regulated by cell activation and triggering as B-CLL cells are usually defined as an activated subset of a B cell population (Alfarano *et al.*, 1999; Damle *et al.*, 2002). Most of the B-CLL cells in my study did not show increase density of the activation marker, CD86, *ex vivo,* but expressed diminished levels of CD79b. Interestingly, intracellular levels of CD79b can be augmented in B-CLL patients through activation by ligation of surface CD40 (Minuzzo *et al.*, 2005).

3.3.6 Significant difference in the expression of CD40 on CD180+ and CD180- B-CLL cells

CD40 is expressed at high density on a variety of immune cells including B cells (Ellmark *et al.*, 2003) and B-CLL cells (Luqman *et al.*, 2008). Ligation of this receptor provides strong and critical survival and proliferative signals in normal B lymphocytes (Aristides *et al.*, 2004; Luqman *et al.*, 2008). The expression of CD40 on B-CLL cells was significantly lower than the expression in control B cells ($p < 0.05$). There was no correlation ($r = 0.354$) of this surface receptor detected along with CD180 expression (Table 3.1,). This finding is partly in concordance with a study previously reported by Agematsu, (2004) which demonstrated that CD40 and CD180 are present on all naïve and memory B cells but their response is different on stimulation with their corresponding Abs. Other studies have suggested a different outcome of CD40 from CD180 subsequent to ligation of its surface receptor which suggests a different functional characteristic of CD40 on B cells (Roshak *et al.*, 1999).

Although CD40 is expressed throughout B cell development, its level is significantly lower on B-CLL than normal B cells (Foy *et al.,* 1993; van Kooten *et al.,* 1997). It is suggested that in B-CLL, immune defects are detected which lead to a condition similar to inherited CD154 deficiency. CD154 is a natural ligand for CD40 and is expressed on CD4+ T cells (Schattner *et al.,* 1998; Romano *et al.,* 1998). Thus, it appears that this phenomenon in B-CLL may cause a persistent inactivity of CD40

possibly leading to shedding of this surface molecule and hence its low density on B-CLL cells in comparison to normal B cells. However, to date there is no evidence for the presence of soluble CD40 in B-CLL patients.

There was a statistically significant difference ($p=0.02$) observed between the expression of CD40 on CD180+ (5450 ± 2626 RBS/cell) and CD180- (3339 ± 2519 RBS/cell) B-CLL cells (Table 3.1; Memon *et al.*, 2007). A possibility for this difference in the expression of CD40 levels on CD180+ and CD180- B-CLL cells could be related to cell activation. CD180- B-CLL cells may be activated *in vivo*, so there is a possibility that this activation phenomenon affects CD40 levels as well, leading to diminished surface expression of this molecule on CD180- B-CLL cells.

In further studies, I observed that although the density of CD40 is significantly higher than CD180 in normal controls (8774 ± 1454 vs 5548 ± 2271 RBS/cell) and B-CLL cells (5450 ± 2620 vs 1686 ± 1268 RBS/cell), the response by activation was much stronger from CD180 ligation than CD40 (see Chapter 4). The absence of a strong correlation and difference in the level of response on stimulation indicates that although ligation of both surface receptors leads to activation, the pathway utilised may be different (discussed in Section 4). Also, in previous studies there was no functional link detected between CD180 and CD40 in B cells (Roshak *et al.*, 1999). On the basis of these results I hypothesise that ligation of both receptors leads to the activation of B cells albeit through different signalling pathways.

3.3.7 No significant difference in the expression of CD86 on CD180+ and CD180- B-CLL cells

The expression of B7.2 molecule (CD86) is an activation marker for both B-CLL and control B cells (Kuchroo *et al.*, 1995; Andritsos *et al.*, 2008; Secchiero *et al.*, 2008) and was subsequently used in this study. Therefore, it was necessary to examine the surface expression of CD86 on both control and B-CLL cells before culture. No significant differences were observed in the level of CD86 expression on normal control B cells and B-CLL cells (Table 3.1). This shows that B-CLL cells in my study as well as normal control B cells were not pre-activated *ex vivo*. There was a tendency of CD180- B-CLL cells to express lower density (130 ± 214 RBS/cell) of

CD86 in comparison to that found on CD180+ B-CLL cells (375 ± 539 RBS/cell) but statistical significance (p=0.09) was not achieved due to the marked standard deviation (Table 3.1).

Increased proportions of CD180- B cells have been reported in patients with SLE (Koarada *et al.*, 1999; Koarada *et al.*, 2005) and in the salivary glands of patients with Sjogren's syndrome (Kikuchi *et al.*, 2008). In the latter case the study shows that the B cells represent the activated population of the B cell pool and have a role in the production of IgG and IgM auto-antibodies *in vitro*. On the other hand, increased expression of CD180 is detected on B cells in both Kawasaki disease and viral infections such as influenza. Here CD180+ B cells are classified as activated B cells (Imayoshi *et al.*, 2006). These various reports demonstrate that the role of CD180 in cell activation is controversial, as can be seen in the current cohort of B-CLL patients where no correlation was detected between the surface expression of CD86 and CD180 (r = 126; Table 3.1).

The average density of CD86 on CD180+ B-CLL cells was 375 ± 539 RBS/cell, thus indicating that the cells were not activated *ex vivo*. Previously it has been shown that most B-CLL patients express activation related antigens (Damle *et al.*, 2007), although in this instance the expression of CD27 and CD69 and not CD86 were used as a monitor of B-CLL cell activation.

However, in my study, there could be several reasons for not finding an adequate density of CD86 on B-CLL cells *ex vivo*. For example, the disease stage was not known at the time of experimentation although this is not supported by the fact that other surface molecules in the same experiments were identified as expected (i.e. IgM, CD79b, CD38, and CD40). There were also constraints of sample collection as it was considered not ethical to look through a B-CLL patient's file at the time of sample collection. Also reduced binding efficacy of the anti-CD86 mAb cannot be ruled out.

3.3.8 CD180 expressed as a clone, regardless of CD27 expression on normal controls and B-CLL cells:

The pattern of the expression of CD27 and sIgD on control B cells delineates naïve/memory B cell subsets (Agematsu, 2000, Yuhui *et al.,* 2003). Hence, to assess the developmental stage of CD180+ B-CLL cells, additional studies were carried out to evaluate CD27 expression on B-CLL and control B cells. The results showed conclusively that in normal B cells controls only 11.3 ± 7.3% (range 7.8 – 34.1%) co-expressed both surface CD27 and CD180 because of a limited expression of CD27 although the majority of B cells expressed CD180 (Table 3.1). This shows that CD180 is characterised by a clonal expression on the B cell surface regardless of CD27 expression and that the expression of CD180 is not restricted by CD27 surface positivity. Moreover, this finding favours the idea that CD180 is expressed equally on all naïve and memory human B cells (Agematsu, 2000). However, microarray analysis together with surface phenotyping has recently revealed that CD27+ human B cells have increased expression of CD180/RP105 (Good *et al.,* 2009). Nevertheless, in this study no preferential expression of CD180 was detected on CD27+ cells.

Similar to normal controls, CD180 was expressed on B-CLL cells of either naïve or memory B cell phenotype. There was a clonal distribution of CD180 detected on B-CLL cells regardless of CD27 expression. Although there appeared a marked difference between the mean values for normal control B cells (11.3 ± 7.3 %) and B-CLL cells (44.4 ± 36.8 %) for the co-expression of CD27 and CD180, the difference was not statistically significant (p=0.113; Figure 3.21). This was due to the fact that the B-CLL cells were markedly heterogeneous (range 4.19 – 97.24 %) for their surface expression of CD27.

Previous studies showed that membrane CD27 is constitutively expressed on all tested CD5+ B-CLL cells (Molica *et al.,* 1998). However, in this study 42.2 ± 37.4 % of B-CLL cells were positive for CD27 and only two out of ten patients had high expression of this surface molecule on their B-CLL cells (i.e. 92.07 and 97.48 %). According to Klein *et al* (2001), some patients were noticed to have high levels of CD27 on the surface of B-CLL cells with UMut IgVH gene, which implies that these

cells may have entered the CD27+ memory cell pool without mutation in the IgVH genes. CLL cells are derive from memory B cells but also a considerable fraction of these cells express UMut IgVH genes possibly due to a high affinity for the antigens already present in the circulation (Klein *et al.*, 1998, Tangye *et al.*, 1998). These CD27+ memory B cells having UMut IgVH genes are classified as the normal counterpart of UMut B-CLL cells (Klein *et al.*, 2001).

3.3.9 Moderate correlation of CD38 and CD180 on B-CLL cells

CD38 is considered to be a powerful prognostic marker for B-CLL associated with poor prognosis (Lanham *et al.*, 2003; Damle *et al.*, 2007). There was no significant difference detected in the level of CD38 expression in normal controls and CD180+ B-CLL cells (Table 3.1). The molecule CD38 is involved in the regulation of B cell activation, proliferation and cell adhesion (Cruse *et al.*, 2007; Deaglio *et al.*, 2008) and phenotypically mature normal B cells undergo a process of apoptosis after 5-6 weeks in circulation (Fulcher and Basten, 1997). However, B-CLL cells are resistant to apoptosis (Damle *et al.*, 2007) and B-CLL cells positive for the surface expression of CD38 may lead to increased accumulation of tumour cells in the circulation through B cell activation and proliferation on the ligation of CD38 by CD31, a natural ligand for CD38 (Mainou-Fowler *et al.*, 2008). This ligand-driven activation of CD38+ B-CLL cells may be a reason for the poor prognosis of the disease.

The average density of CD38 on B-CLL cells detected in this study was 377 ± 687 RBS/cell (range 0-2820 RBS/cell; n=36). There was a significant difference ($p=0.009$) observed in the level of CD38 expression between CD180+ and CD180- B-CLL cells (564 ± 821 vs 83 ± 170 RBS/cell respectively). Since it was suggested previously that CD180 may serve as an additional prognostic marker in association with mutated status of IgVH genes for B-CLL patients (Porakishvili *et al.*, 2005), the pattern of CD38 expression on CD180+ and CD180- B-CLL cells indicates that CD180 and CD38 may represent moderately correlated ($r=0.53$) prognostic markers (Table 3.1).

According to the current viewpoints, CD38 expressed on activated B-CLL clones is responsible for continued proliferation and prolonged survival of the leukaemic cell

in vivo (Cruse *et al.*, 2007, Damle *et al.*, 2007, Cutrona *et al.*, 2008). It has also been shown that CD38+ B-CLL cells are more efficient in stimulation via sIgM leading to cell apoptosis *in vitro* (Cutrona *et al.*, 2008). However in the current cohort of B-CLL patients, CD38+ B cells were found to be positive for CD180. The next chapter of my study emphasises on different outcomes of the responses of B-CLL cells on CD180 ligation which to a certain extent explains the correlation between CD38 and CD180

.

CD38 is currently considered to be a prognostic factor independent of IgVH mutational status (Eisele *et al.*, 2009). The level of its expression is both dynamic and transient (Damle *et al.*, 2007) and varies with time (Kharfan-Dabaja *et al.*, 2008). Expression of CD38 does not parallel the presence or absence of IgVH gene mutations but may correlate with the expression of intracellular ZAP-70 (Cutrona *et al.*, 2008). Here, I have demonstrated that CD38 expression is moderately correlated with CD180 thus confirming a prognostic value of the latter.

3.3.10 Expression of intracellular ZAP-70 and its moderate correlation with CD180

Expression of ZAP-70 in B-CLL cells is predictive of a poor disease outcome (Rassenti *et al.*, 2004, Munoz *et al.*, 2007). It is noteworthy that patients with ZAP-70+ B-CLL cells even if CD38- and with Mut IgVH genes, had shorter progression free survival whereas patients with ZAP-70- B-CLL cells even if CD38+ and UMut, had longer progression free survival. Thus, it was concluded that ZAP-70 is a marker with a significant prognostic value and is a better predictor of the disease outcome in comparison to IgVH gene mutational status (Laurenti *et al.*, 2005, Del Principe *at al.*, 2006). Therefore, co-expression of ZAP-70 and CD38 is suggestive of a particularly poor prognosis (el-Sharnouby *et al.*, 2006).

We studied fourteen B-CLL patients for the expression of ZAP-70 using the criteria of Del Principe *et al.*, (2006) who called the population positive when 20% of the CD19+ cells expressed this intracellular antigen (Del Principe *et al.*, 2006). B-CLL cells from 8 out of 14 patients were found to be negative for ZAP-70.

CD19+ cells expressed this intracellular antigen (Del Principe *et al.,* 2006). B-CLL cells from 8 out of 14 patients were found to be negative for ZAP-70.

There was no significant difference (p=0.22) in the percentage of ZAP-70+ cells in CD38+ and CD38- cells in this cohort of B-CLL patients (Figure 3.14) whilst ZAP-70 and CD38 expression were weakly correlated (r=0.442). These combined results have been previously reported by several other studies (Del Principe *et al.*, 2006; Giovanna *et al.*, 2008; Molica *et al.,* 2008). There may be several reasons for the lack of strong correlation between CD38 and ZAP-70 in this cohort of patients, for example, the small number of samples and the changing level of CD38 expression over time (Kharfan-Dabaja *et al.*, 2008).

When comparing the expression of ZAP-70 with the IgVH gene mutational status, 3 groups of B-CLL patients could be distinguished in the present study: those with UMut IgVH genes and ZAP-70 expression, those with Mut IgVH genes and absence of ZAP-70 expression and those with discordance between the IgVH gene mutational status and ZAP-70 expression. This shows that the expression of ZAP-70 is associated with, but not generally restricted to, UMut IgVH gene mutational status. There was significant difference (p=0.01) in the levels of ZAP-70 expression in patients with Mut and UMut IgVH genes (Figure 3.17) which confirms the close correlation between the two prognostic markers. Strong association of UMut IgVH genes and ZAP-70 expression in B-CLL cells has been demonstrated earlier (Laurenti *et al.*, 2004, Del Principe *et al.*, 2006).

Since we have considered CD180 to be a supplementary prognostic marker for B-CLL patients (Porakishvili *et al.*, 2005), a possible correlation between CD180 levels and ZAP-70 expression was analysed. There was a weak direct correlation (r=0.468) between CD180 and ZAP-70 expression in B-CLL cells (Figure 3.13).

It is debatable whether or not to associate CD180 with one of the existing prognostic markers or consider it as a separate entity. Since B-CLL is a heterogeneous disease (Rai *et al.,* 2001; Jewell, 2002; Zent and Kay, 2004; Porakishvili *et al.*, 2005; Cutrona *et al.*, 2008) with patients presenting with different clinical symptoms, and

variable expression of ZAP-70+, CD38 and mutation status of IgVH genes (Del Prinicipe *et al.*, 2006), it would be beneficial to consider more than one prognostic marker prior to deciding the treatment strategy.

Since a moderate correlation between CD180 expression and a prognostic marker (i.e. the mutation status of IgVH genes) has been reported previously (Porakishvili *et al.,* 2005), correlation of ZAP-70 expression with that of sIgM and sIgD were studied. No correlation between intracellular ZAP-70 and sIgM and intracellular ZAP-70 and sIgD ($r = 0.20$ and $r = 0.37$ respectively) was detected. It has been proposed recently that sIgM on B-CLL cells with UMut IgVH genes induces the expression of ZAP-70 and binds to the variety of different antigens including self-antigens (Cutrona *et al.*, 2008) leading to cell activation and continuous stimulation, which contributes to a poor prognosis and aggressive disease outcome.

3.3.11 CD180 co-localises with IgM on B-CLL cells:

Although it is well known that RP105 ligation on B cells with specific mAbs leads to activation and proliferation of murine B cells (Yamashita *et al.,* 1996, Miyake *et al.*, 1998) no intracellular pathway has been identified as RP105 lacks the toll/interleukin 1 receptor (TIR) domain, which is mandatory for signalling. Since activated RP105 in mice has been reported to signal through similar pathways as the BCR complex (Yazawa *et al.,* 2003), confocal microscopy was carried out to detect possible constitutive co-localisation of CD180 with sIgM on B-CLL cells.

The results showed constitutive co-localisation of CD180 with sIgM on the surface of a B-CLL clone (Figure 3.22 C). It appeared that the fluorescence was associated with patches in the plasma membrane of B-CLL cells. These patches could represent clustering of BCR complexes in lipid rafts. A similar fluorescence pattern was seen when B-CLL cells were stained with PE-conjugated mouse anti-human CD180 mAb, which could signify co-localisation of CD180 with the BCR complex in lipid rafts. Lipid rafts are dynamic microdomains in the plasma membrane which are rich in glycosphingolipids and cholesterol, and function as platforms for signal transduction facilitating amplification of BCR signalling after binding of a ligand (Gupta *et al.,* 2007). When a ligand has bound the BCR and intracellular signalling has been

propagated, the whole complex is internalised and undergoes degradation, which terminates the signalling (Gupta *et al.*, 2007). However, there was no specific fluorescence stain used for lipid rafts in our experiment, hence further studies are needed to confirm this preliminary finding.

If CD180 indeed co-localises with sIgM on B-CLL cells, and more importantly, on normal resting B lymphocytes, it is a phenomenon that needs to be addressed further. This may imply that CD180 is functionally complexed with the B cell receptor and may signal using BCR associated ITAMs to initiate intracellular signalling pathways as well as various downstream signalling molecules (Stevenson and Caligaris-Cappio, 2004). A possible co-localisation between CD180 and sIgD must be studied in detail particularly since the expression pattern of CD180 seems to be closely associated with B-CLL cell susceptibility to sIgD-mediated apoptosis (*see Chapter 4.3).*

Indirect evidence of the involvement of structural elements of the BCR would be modulation of CD180 expression on B-CLL and control B cells subsequent to the engagement of sIgM and sIgD.

3.3.12 Surface CD180 expression was down-regulated on B-CLL cells on ligation with anti-IgD F(ab)$_2$ Abs

It has previously been shown by Koarada *et al* (1999) that B cells not expressing CD180 are activated in patients with SLE. This activated subset of the CD180- B cell population may be responsible for the spontaneous production of auto-antibodies without any further stimulation (Kikuchi *et al.*, 2002). This anecdotal evidence regarding the stability of CD180 cell expression on B cells has not been supported by any further publications although Koarada *et al* (1999) suggested that the expression of this surface molecule on B cells may be modulated. The stage and the pathway of CD180 expression and down-regulation on B cells remain to be determined. A possible mechanism includes the shedding of CD180 in the peripheral blood or lymphoid tissues upon receiving activation signals from an auto-antigen (Koarada *et al.*, 1999). The question I asked here was whether the modulation of CD180

expression can be achieved by the engagement of the BCR (sIgM, sIgD) or a major co-signalling receptor such as CD40.

In light of our observations concerning the possible interactions between the BCR and CD180, it was essential to establish the dependence of the level of CD180 expression on BCR-mediated activation. In these experiments ligation of CD40, enhanced by the addition of IL-4, was used to produce a strong activation signal independent from the BCR. Control B cells showed substantial down-regulation of surface CD180 after treatment with anti-IgM $F(ab)_2$ ($p=0.01$) and anti-IgD $F(ab)_2$ ($p=0.05$), whilst no effect was detected through CD40 ligation ($p=0.237$), either with or without rIL-4 (Figure 3.34).

The lack of dependence of CD180 expression on CD40 in normal controls is supported by previous observations that demonstrate that although CD40 and CD180 are almost equally expressed on all naïve and memory B cells, their response is entirely different on ligation with their corresponding Abs. According to this study CD180 signalling showed an increase in the activation, proliferation and life span of naïve B cells in comparison to memory B cells where a decline was seen in the life span following CD40 stimulation (Agematsu, 2007). Yamashita *et al* (1996) showed that B cells primed via CD180 but not CD40 underwent apoptosis by subsequent ligation with anti-IgM Abs. It appears that although both CD180 and CD40 activate B cells on ligation, the activation pathways utilised by these surface receptors may be different, this is discussed in more depth in *Chapter 4*.

Our studies therefore show that normal B cells down-regulate the expression of CD180 as a result of engagement of the BCR showing phenotypic characteristic of the clone. In contrast, with the results obtained for control B cells, no modulation of CD180 was observed on B-CLL cells via the ligation of sIgM ($p=0.182$) or CD40 ($p=0.67$) with or without rIL-4 (Figure 3.35), despite B-CLL cell activation measured by surface CD86 expression (*data shown in Chapter 4*). However, sIgD ligation led to a significant decrease ($p=0.008$) in the surface expression of CD180 on B-CLL cells (Figure 3.35).

to be impaired in the case of B-CLL cells. These data suggest that the downstream events leading to the modulation of CD180 expression through the ligation of sIgM on normal B cells are defective in B-CLL cells. B-CLL cells may be locked in an anergic state and fail to modulate expression of surface signalling molecules. Very importantly, the sIgD pathway appears to remain functional on B-CLL cells and delivers the activation signal leading to the down-regulation of CD180. The main aspect may be related to the down-regulation of CD180 following IgD-induced apoptosis of normal control B cells and B-CLL cells (see *Results and Discussion* Chapter 5). Plausibly, since cells undergoing apoptosis may shed surface antigens, CD180 could be included in this list. Another explanation may be related to the tendency for B-CLL clones to have higher densities of sIgD compared with sIgM (2913 ± 3185 vs 1787 ± 3389 RBS/cell; Table 3.1). In addition, and in accordance with my study, a previous report has demonstrated down-regulation of sIgD upon B cell activation (Damle *et al.*, 2002).

The next chapter of these studies is dedicated to unveiling the functional changes of control B cells and B-CLL cells resulting from the ligation of CD180 and their association with BCR components and co-stimulatory pathways.

CHAPTER FOUR

EFFECT OF CD180 LIGATION ON THE ACTIVATION AND PROLIFERATION OF B-CLL AND CONTROL B CELLS

4.1 Introduction

Both Valentine *et al.*, (1988) and Miura *et al.*, (1998) have shown that B cells are driven into activation and proliferation by the ligation of surface CD180. With these facts in mind, it was pertinent at this stage to study whether CD180 expressed on B-CLL cells can be engaged also to modify functional responses.

4.2 Results

4.2.1 Ligation of CD180 by anti-CD180 mAb (G28.8) resulted in the activation of the majority of normal control B cells, but only a small subset of B cells entered cell cycle

As demonstrated in Figure 4.1a, treatment of PBMC cultures from normal control age-matched individuals with an optimal concentration of anti-CD180 mAb (G28.8) for 72 h led to a highly significant ($p<0.00001$) increase in the number of positive stained cells for the activation marker CD86.

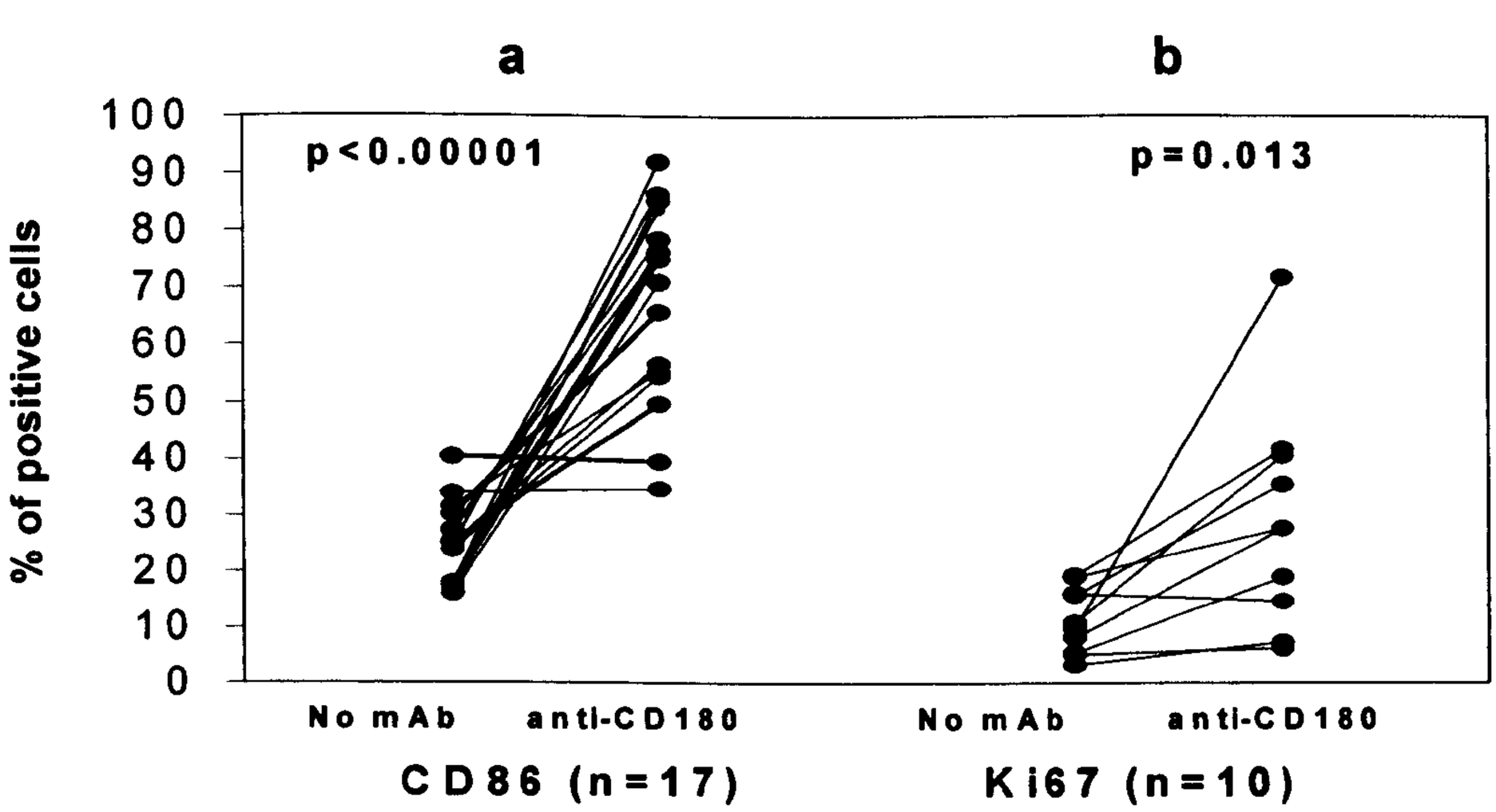


Figure 4.1. Expression of CD86 (a) and Ki67 (b) by control CD19+ B cells in 72h PBMC cultures stimulated with anti-CD180 mAb.

Control PBMCs were incubated with 10μg/mL of anti-CD180 (G28.8) mAb for 72 h. Cells were stained with PE-conjugated anti-CD86 and PE Cy5-conjugated anti-CD19 mAbs (a), or fixed, permeabilised and stained with PE Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (b). Sample analysis was made by flow cytometry and statistical significance was determined by Wilcoxon's paired test.

In contrast to this last finding, Figure 4.1b shows that activation of this pool of B cells in PBMC cultures only resulted in a small but significant (p=0.013) subset of these cells to enter into cell cycle, as measured by the intracellular expression of Ki67. Interestingly, the B cells from two controls that did not up-regulate Ki67 in response to CD180 were still able to be activated, as determined by increases in the number of positive CD86 cells.

Unless stated otherwise, all statistical analyses were determined by Wilcoxon´s test for paired samples where p values ≤ 0.05 were deemed as significant.

4.2.2 CD180 responder (R) and non-responder (NR) clones: Only a proportion of CD180+ B-CLL clones responded to CD180 ligation by activation and proliferation in PBMC cultures

Eight of the fifteen cultured CD180+ B-CLL samples obtained from the patient cohort responded significantly (p=0.001) to stimulation by anti-CD180 mAb, as shown by the increased number of positive CD86 stained cells (Figure 4.2a). In the same group of patients, seven out of thirteen showed a significant increase (p=0.009) in the number of cells positive for Ki67 expression following treatment with anti-CD180 mAb (Figure 4.2b), thus identifying these clones as responders (R).

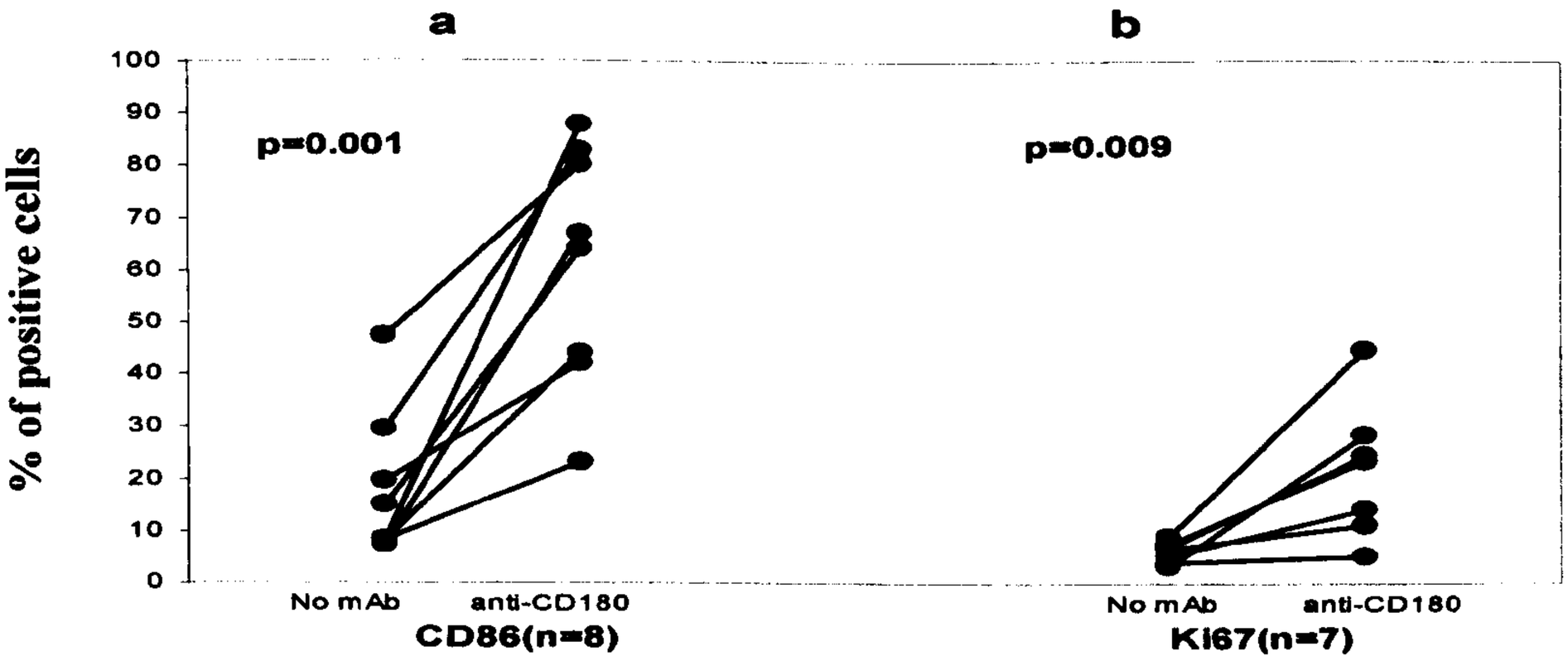


Figure 4.2. Increased expression of CD86 and Ki67 by the R CD180+ B-CLL cells in 72h PBMC cultures stimulated with anti-CD180 mAbs.

PBMC from responder B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti CD19 mAbs (a), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (b). Sample analysis was made by flow cytometry.

The remaining CD180+ B-CLL clones were identified as non-responders (NR) since they were unable to respond to CD180 ligation as shown by their inability to increase the expression of either CD86 ($p=0.971$) or Ki67 ($p=0.812$) in 72 h PBMC cultures (Figure 4.3a and Figure 4.3b respectively).

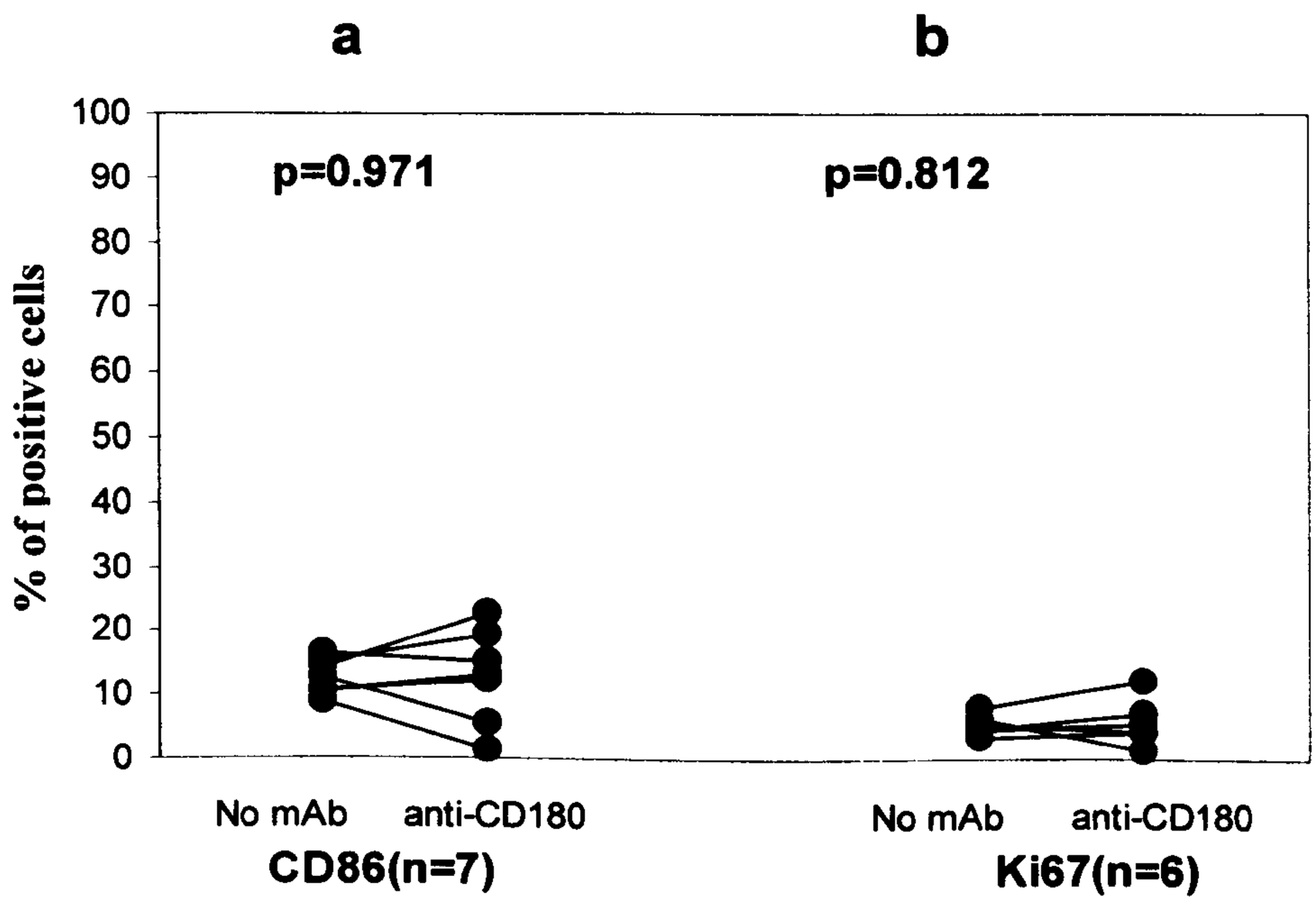


Figure 4.3. Expression of CD86 and Ki67 by the Non-Responder CD180+ B-CLL cells in 72h PBMC cultures stimulated with anti-CD180 mAb.

PBMC from non-responder B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti CD19 mAbs (a), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (b). Sample analysis was made by flow cytometry.

At this stage it was pertinent to study the difference and correlation of all the studied surface molecules (sIgM, sIgD and CD79b) and receptors (CD38, CD86 and CD40) with both CD180 R and NR cells.

The results, presented in Table 4.1, demonstrate that there was a significant difference ($p=0.01$) in the expression of CD40 between CD180 R and NR B-CLL cells.

Table 4.1 Correlation of expression of CD180 with other phenotypic markers by B-cell chronic lymphocytic leukaemia (B-CLL) cells.

	CD180	IgM	CD79b	CD38	CD86	CD40	IgD
B-CLL CD180 R (n=12)	2113 ± 1445	1214 ± 1244	528 ± 429	606 ± 921	513 ± 670	5539 ± 2617	3973 ± 3543
B-CLL CD180 NR (n=10)	1198 ± 989	796 ± 1353	507 ± 463	505 ± 732	239 ± 259	3753 ± 1496	3251 ± 4007
Correlation*	0.21	-0.104	-0.407	-0.492	0.304	0.524	-0.211
P**	0.01	0.02	0.49	0.39	0.08	0.01	0.35

B-CLL cells were treated with anti-CD180, anti-IgM, anti-CD79b, anti-CD38, anti-CD86 anti-CD40 or anti-IgD mAbs and stained with the secondary Ab as described in the *Materials and Methods* section. Results were expressed as RBS/cell. The Pearson correlation coefficient was calculated between the expression of CD180 R and NR cells (*). Values of 0.5 or above were considered correlated, according to the number = n of samples.
Difference between the expression of all surface markers in CD180 R and NR population in B-CLL cells was calculated using Mann-Whitney U-test (**). Values of 0.05 or below were considered significant.
*Correlation with CD180 RBS/cell with other phenotypic markers RBS/cell.
**Difference between all measured surface markers in R CD180+ and NR CD180+ cells.

As expected, CD180- B-CLL clones did not show any significant increase in the expression of either CD86 (p=0.332) or Ki67 (p=0.213) following ligation with anti-CD180 mAb and culture for 72 h (Figure 4.4a and Figure 4.4b respectively).

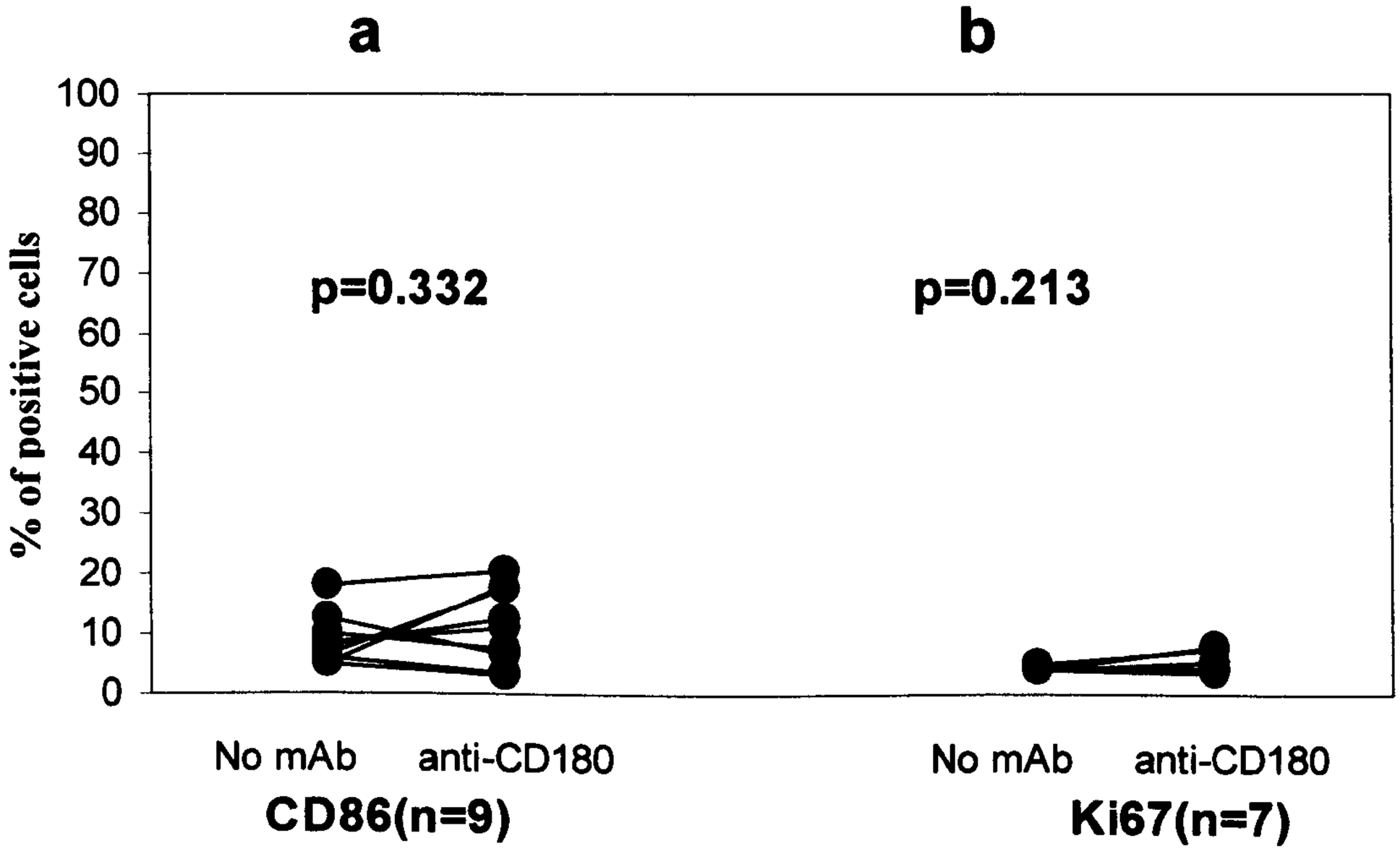


Figure 4.4. Expression of CD86 and Ki67 by CD180- B-CLL cells in 72h PBMC cultures stimulated with anti-CD180 mAbs.

PBMC from CD180- B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti CD19 mAbs (a), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (b). Sample analysis was made by flow cytometry.

In view of these last set of data, it was essential to subsequently examine the relationship between CD180 mediated B cell activation with other major B cell receptors.

4.2.3 CD180 and CD40 ligation, in the activation and proliferation of control B cells:

Treatment of control B cells with anti-CD180 mAb (10μg/mL) or anti-CD40 mAb (10μg/mL) caused a highly significant increase of cells positive for CD86 compared to untreated cells (p=0.00001 and p=0.0004 respectively) after 72 h culture (Figure 4.5). Albeit not statistically significant, treatment with anti-CD180 mAb tended to

produce higher numbers of control B cells positive for CD86 compared to those treated with anti-CD40 mAb. This observation is of interest in view of the fact that higher expression levels of CD40 are present (Table 3.1) on the control B cell surface compared to CD180 (8774 ± 1459 vs 5548 ± 2271 RBS/cell) when tested *ex vivo*.

Simultaneous ligation of both surface receptors with anti-CD180 and anti-CD40 mAbs increased the number of CD86 positive cells although the effect was not numerically above that obtained from the additive values of the mAbs used alone and the basal expression of both markers (Figure 4.5).

Increased proliferation of control B cells, as determined by Ki67 expression, was present following the ligation of both CD180 and CD40 surface receptors with their corresponding mAbs (Figure 4.5). Incubated with control B cells alone, both anti-CD180 (10μg/mL) and anti-CD40 mAbs (10μg/mL) each induce a minimal number of proliferative cells, circa 10% above basal ($p=0.05$ and $p=0.12$ respectively).

Following treatment with both mAbs concurrently, elevated numbers of Ki67 positive control B cells were observed, circa 50% above basal levels. Since both mAbs alone caused a maximum stimulus of 10% or less above basal level, an additive action seems unlikely and most probably the observed increase in the number of Ki67 positive cells appears to be as a result of a synergistic effect of anti-CD180 and anti-CD40 mAbs (Figure 4.5).

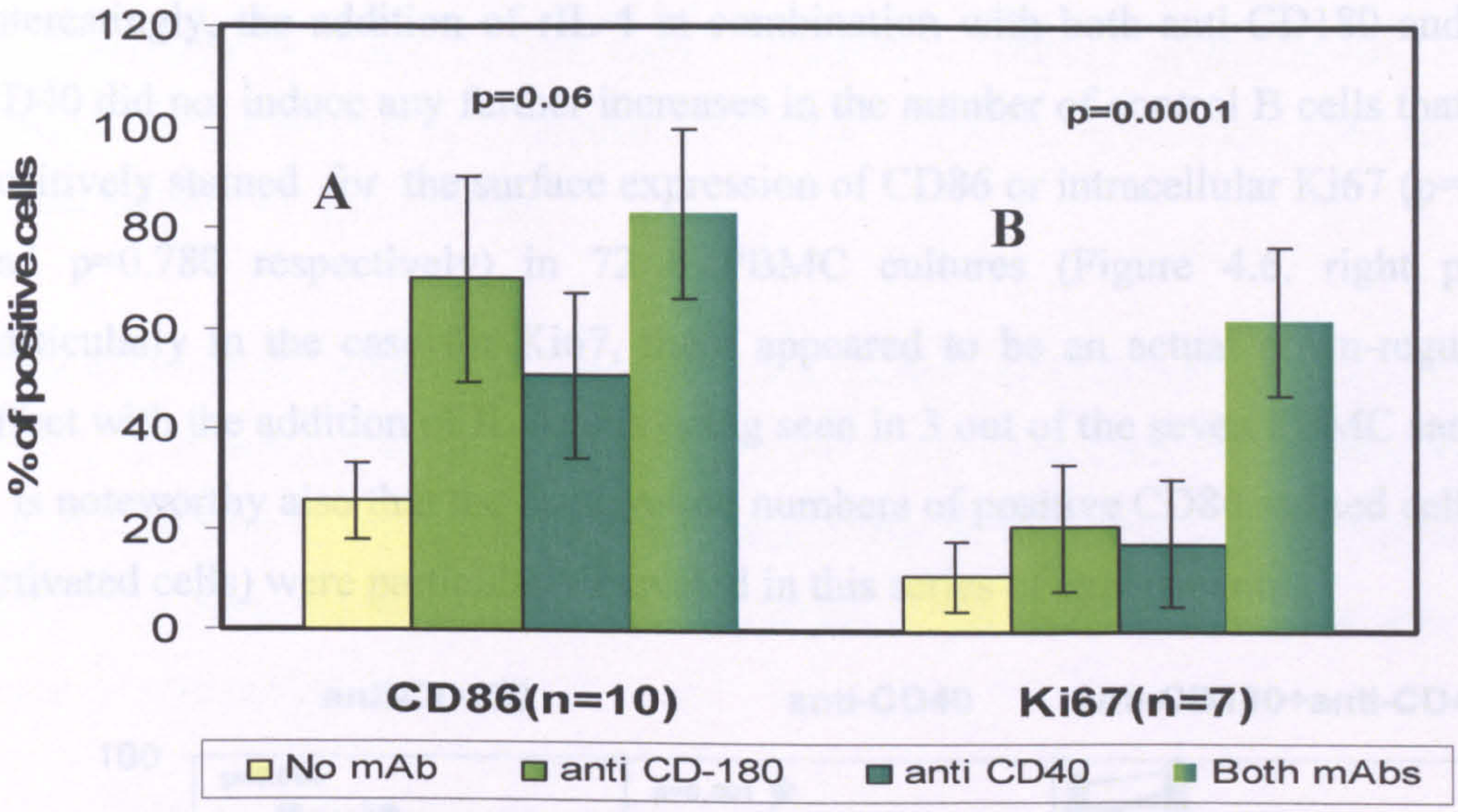


Figure 4.5. Ligation of CD180 and CD40 on the activation and proliferation of control B cells.

Control PBMCs were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAb or both for 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti-CD19 mAbs (A), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (B). Samples were analysed by flow cytometry and columns represent the mean ± SD. p represents differences between anti-CD180 and the simultaneous ligation of anti-CD180 and anti-CD40 mAbs.

4.2.4 Addition of recombinant IL-4 (rIL-4) enhanced CD180-induced proliferation and activation of normal control B cells:

The simultaneous addition of rIL-4 and anti-CD180 mAb (10μg/mL) to control B cell cultures for 72 h resulted in a significant increase ($p=0.003$) in the percentages of cells positive for Ki67 (Figure 4.6, left panel). In the same figure panel it can be seen that effects on activation, measured by the expression of CD86, were clearly more modest ($p=0.05$).

In contrast, ligation of CD40 with anti-CD40 mAb (10μg/mL) synergised with rIL-4 to produce a significant enhancement ($p=0.0013$) in the number of activated control B cells, as measured by CD86 expression (Figure 4.6, central panel). Significant effects ($p=0.031$) on the number of proliferative control B cells in, measured by Ki67 expression, were also observed albeit to a lesser extent than for activation (Figure 4.6, centre panel).

Interestingly, the addition of rIL-4 in combination with both anti-CD180 and anti-CD40 did not induce any further increases in the number of control B cells that were positively stained for the surface expression of CD86 or intracellular Ki67 (p=0.735 and p=0.780 respectively) in 72 h PBMC cultures (Figure 4.6, right panel). Particularly in the case for Ki67, there appeared to be an actual down-regulatory effect with the addition of IL-4, this being seen in 3 out of the seven PBMC samples. It is noteworthy also that the background numbers of positive CD86 stained cells (i.e. activated cells) were particularly elevated in this series of experiments.

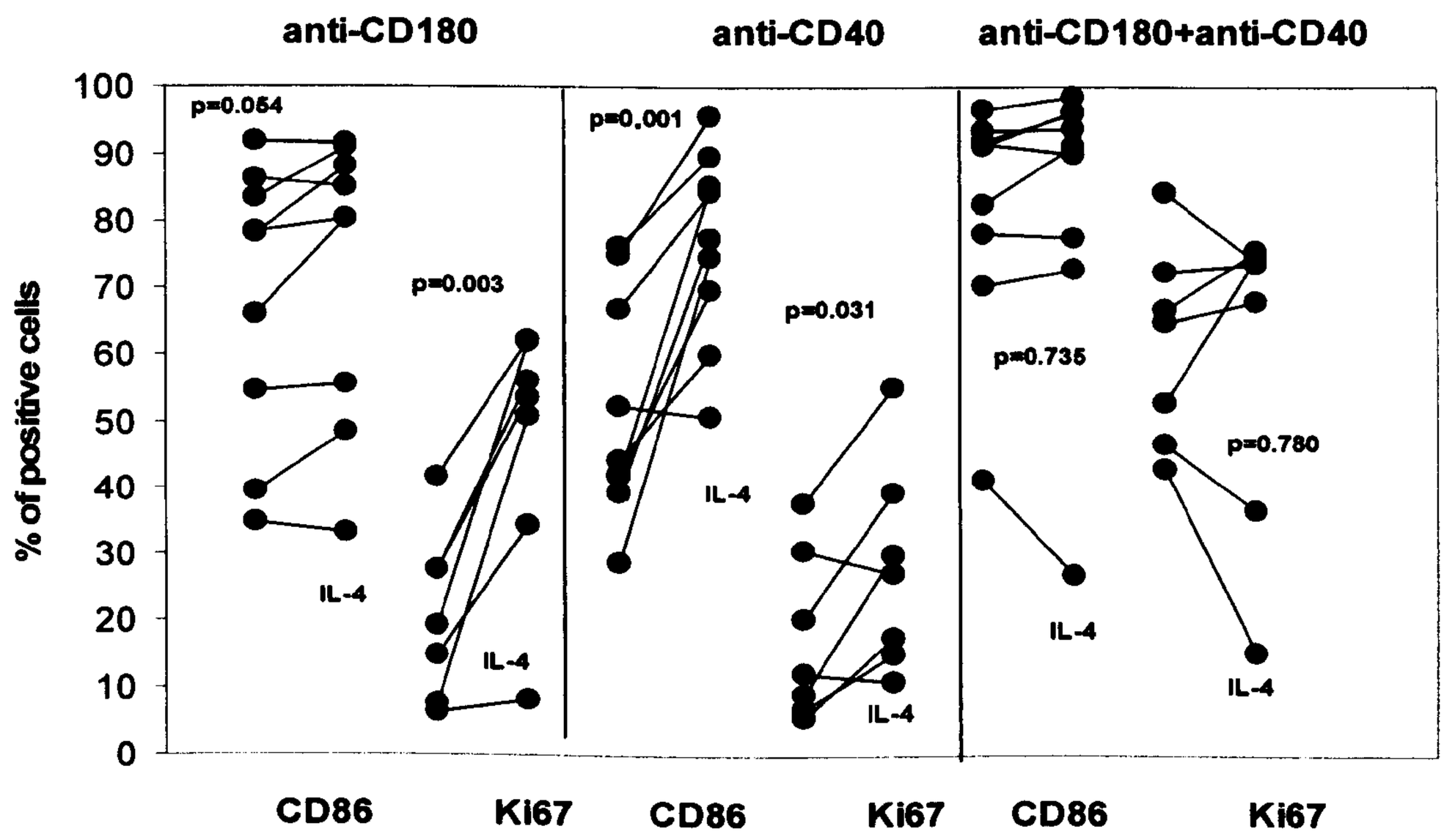


Figure 4.6. Co-stimulatory effect of the ligation of CD180 and/or CD40 with rIL-4 on the activation and proliferation of control B cells.

Control PBMCs were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAbs or both in absence or presence of 15ng/mL of rIL-4 for 72 h and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 mAbs or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs. Sample analysis was made by flow cytometry.

4.2.5 CD180 and CD40 ligation, in the activation and proliferation of B-CLL cells:

Analogous to that observed with normal B cells, treatment of R B-CLL clones with anti-CD180 mAb (10μg/mL) increased significantly the number of cells positive for CD86 (p=0.0003) and Ki67 (p=0.05) at the concentration used (Figure 4.7). From the same figure, it can be seen that treatment of R B-CLL clones with anti-CD40 mAb at

the concentration used in the study (10μg/mL), caused quantitatively similar increases in the number of cells positive for both CD86 (p=0.005) and Ki67 (p=0.03) to that induced by anti-CD180.

In parallel to that found using normal B cells, ligation of both surface receptors on R B-CLL cells with anti-CD180 and anti-CD40 mAbs increased the number of cells positive for both CD86 and Ki67 expression although the effect was not numerically above that obtained from the additive values of the mAbs used alone and basal expression (Figure 4.7).

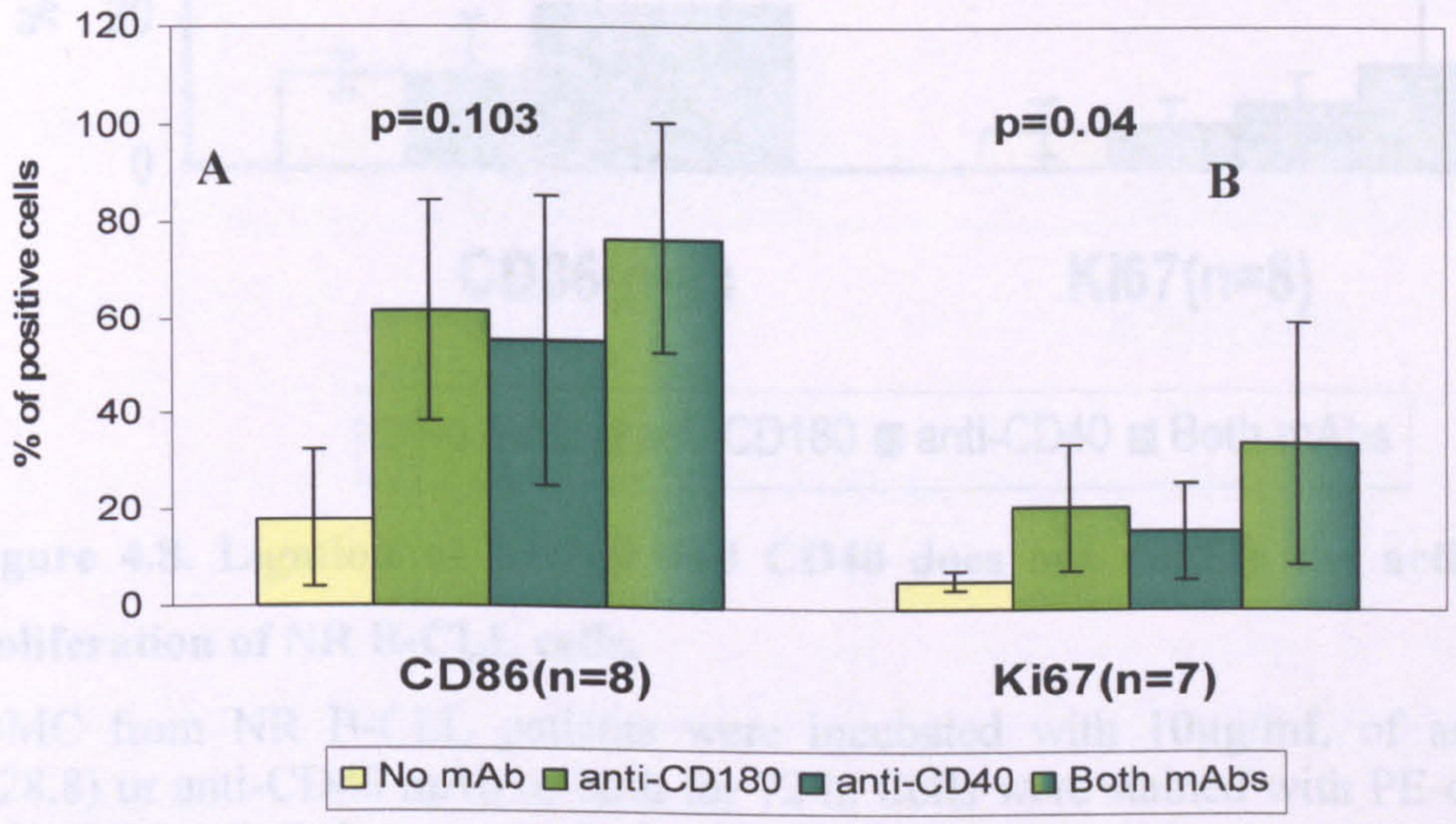


Figure 4.7. Ligation of CD180 or CD40 increases the activation and proliferation of R B-CLL cells

PBMC from R B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAbs or both for 72 h. Cells were stained with PE-conjugated anti CD86 and Cy5-conjugated anti-CD19 mAbs (A), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (B). Samples were analysed by flow cytometry and columns represent the mean ± SD. p represents difference between anti-CD180 and the simultaneous ligation of anti-CD180 and anti-CD40 mAbs.

Stimulation of CD180+ NR clones with anti-CD180 (10μg/mL) or anti-CD40 (10μg/mL) demonstrated no significant increase in the number of cells positive for CD86 or Ki67, as compared to basal levels (Figure 4.8). Similarly, following simultaneous ligation of both CD180 and CD40 on these cells, no significant increase in the number of cells positive for CD86 (p=0.08) or Ki67 (p=0.105) was

observed compared to that seen with each mAb itself, or with spontaneous expression.

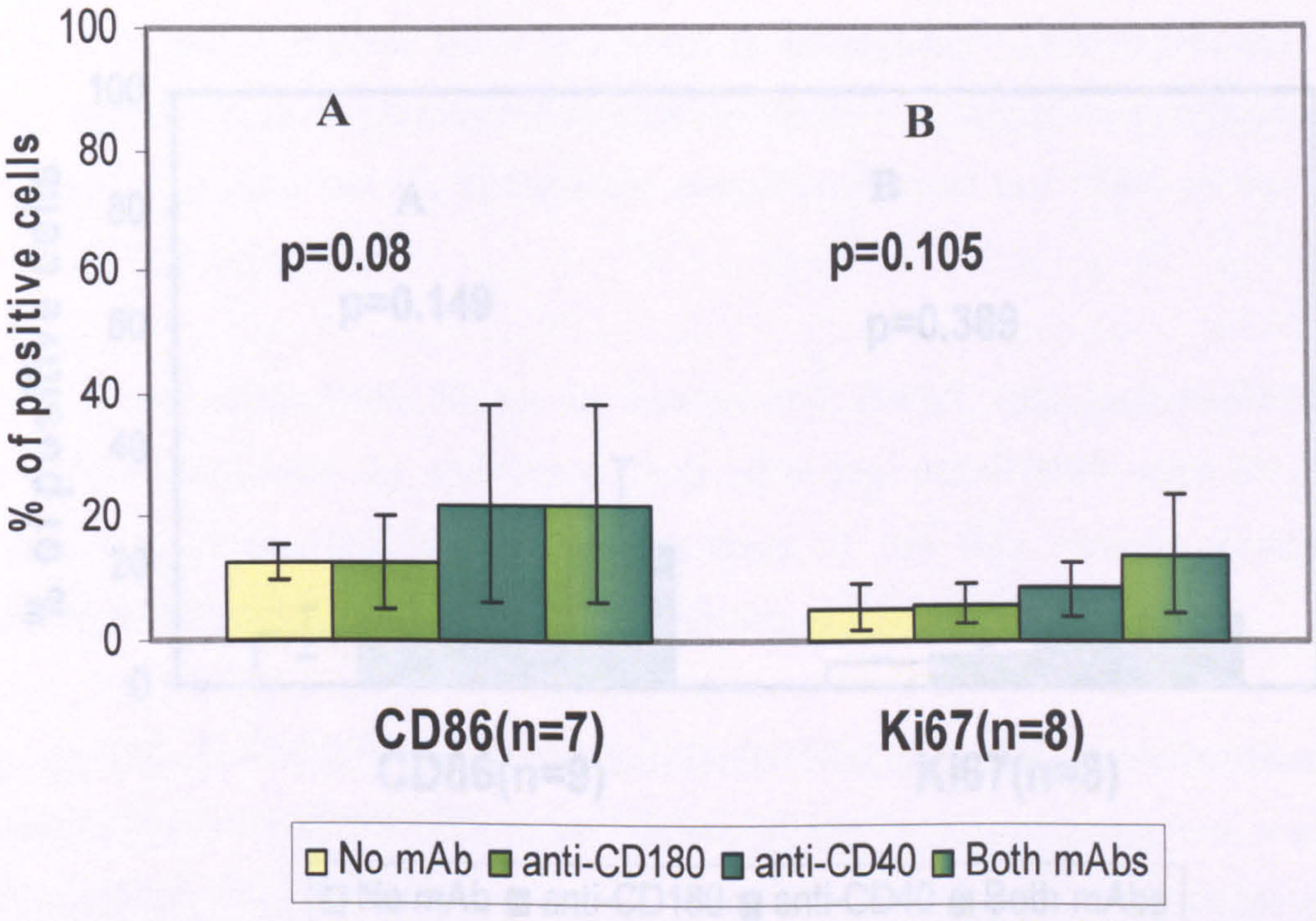


Figure 4.8. Ligation of CD180 and CD40 does not modify the activation or proliferation of NR B-CLL cells.

PBMC from NR B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAb or both for 72 h. Cells were stained with PE-conjugated anti CD86 and Cy5-conjugated anti-CD19 mAbs (A), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (B). Samples were analysed by flow cytometry and columns represent the mean ± SD. p represents difference between anti-CD180 and the simultaneous ligation of anti-CD180 and anti-CD40 mAbs.

More importantly, it was interesting to note that cells not responding via ligation of CD180 by up-regulation of CD86 and Ki67 (NR), also respond poorly to the ligation via CD40 (Figure 4.8).

Ligation of CD180 or CD40 on CD180- clones using anti-CD180 (10μg/mL) or anti-CD40 (10μg/mL) failed to elicit any significant increase of cells positive for CD86 or Ki67 expression measured after 72 h culture (Figure 4.9). Following the simultaneous coupling of CD180 and CD40 on these CD180- clones, no significant increase in the number of cells positive for CD86 (p=0.149) or Ki67 (p=0.389)

expression was observed compared to that seen with either mAb alone or basal levels (Figure 4.9).

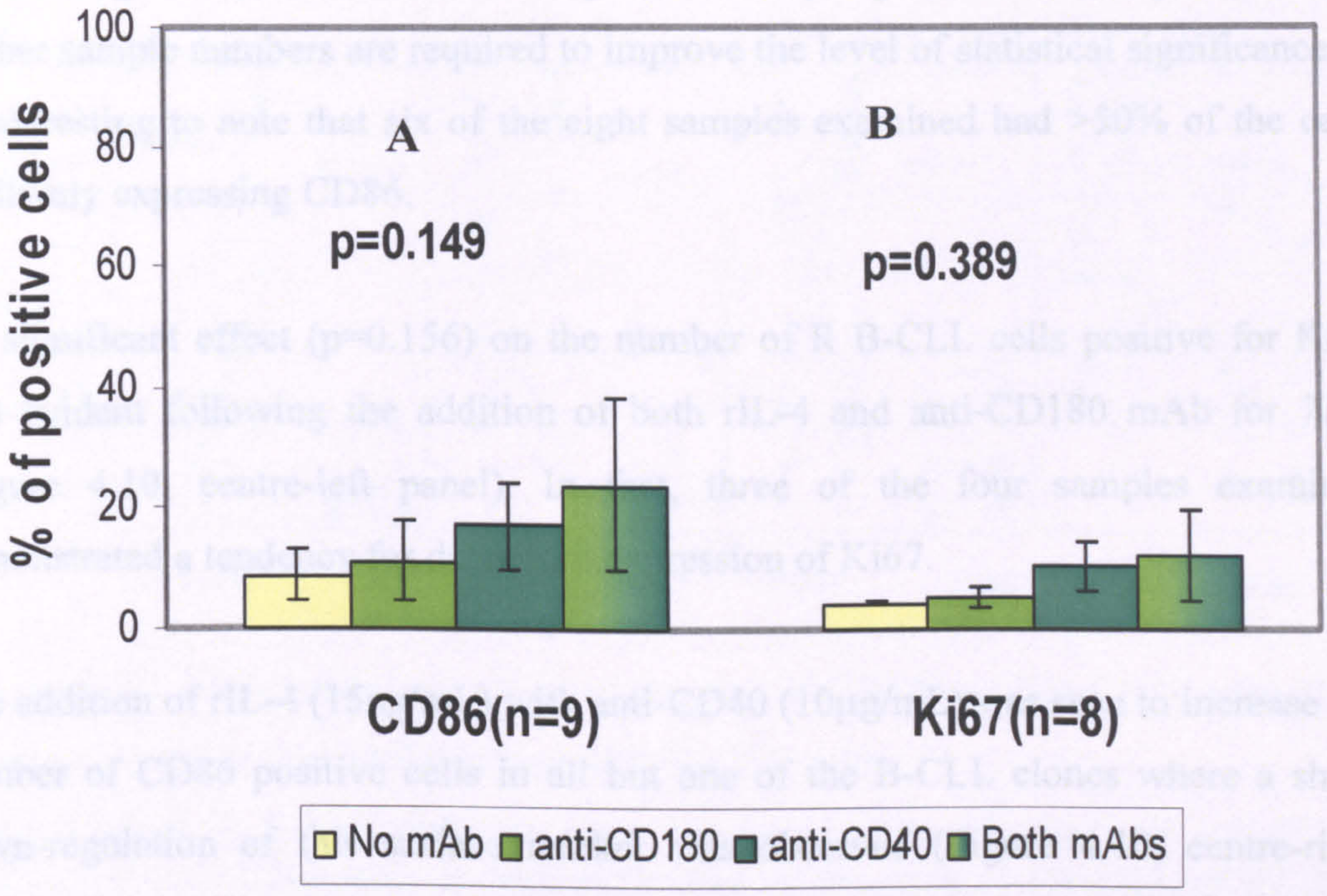


Figure 4.9. Ligation of CD180 and CD40 does not influence the activation or proliferation of CD180- B-CLL cells.

PBMC from CD180- B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAbs or both for 72 h. Cells were stained with PE-conjugated anti CD86 and Cy5-conjugated anti CD19 mAbs (A), or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs (B). Samples were analysed by flow cytometry and columns represent the mean ± SD. p represents difference between anti-CD180 and the simultaneous ligation of anti-CD180 and anti-CD40 mAbs.

4.2.6 Addition of recombinant rIL-4 does not enhance activation or proliferation of R B-CLL cells in PBMC cultures:

Significant increases (p=0.003) in the percentages of CD19+ cells expressing CD86 from R B-CLL patients were observed following stimulation with rIL-4 (15 ng/mL) for 72 h (Figure 4.10, left panel). Treatment with rIL-4 did not increase (p=0.874) the number of cells stained positive for Ki67 marker over that seen with unstimulated cultures (Figure 4.10, left panel).

The addition of rIL-4 (15ng/mL) together with anti-CD180 mAb (10μg/mL) induced a strong increase in the proportion of CD19+ CD86 B-CLL cells apart from one sample (Figure 4.10, centre-left panel). Although a net significant effect was not observed ($p=0.06$), a clear trend is present to up-regulate CD86 expression and further sample numbers are required to improve the level of statistical significance. It is interesting to note that six of the eight samples examined had >50% of the cells positively expressing CD86.

No significant effect ($p=0.156$) on the number of R B-CLL cells positive for Ki67 was evident following the addition of both rIL-4 and anti-CD180 mAb for 72 h (Figure 4.10, centre-left panel). In fact, three of the four samples examined demonstrated a tendency for decreased expression of Ki67.

The addition of rIL-4 (15ng/mL) with anti-CD40 (10μg/mL) was seen to increase the number of CD86 positive cells in all but one of the B-CLL clones where a sharp down-regulation of this surface marker was observed (Figure 4.10, centre-right panel). Due to this latter result, the net effect was not statistically significant ($p=0.112$) although a clear trend was observed whereby the number of CD86 positive cells expression were increased. Again, half of the eight samples examined had >50% of the cells positively expressing CD86 indicating that these cell preparations were already in a high state of activation through ligation with anti-CD40 mAb making further increases unlikely with the addition of rIL-4.

No changes in the levels of Ki67 positive R B-CLL cells ($p=0.720$) were seen after dual stimulation with rIL-4 and anti-CD40 mAb (Figure 4.10, centre-right panel).
Finally, no significant increases ($p=0.116$) in the number of CD86 positive R B-CLL cells were observed when rIL-4 (15 ng/mL) was combined with both anti-CD180 (10μg/mL) and anti-CD40 (10μg/mL) although a trend to up-regulate this surface marker was seen (Figure 4.10, right panel). Of note, six of the seven samples examined had $\geq$ 60% of the cells expressing CD86 present after simultaneous ligation with both mAbs making any further increases difficult to visualise afer rIL-4 treatment.

Combination of both mAbs together with rIL-4 did not enhance expression of Ki67 (p= 0.131). In point of fact, a trend to actually decrease this marker of proliferation was present.

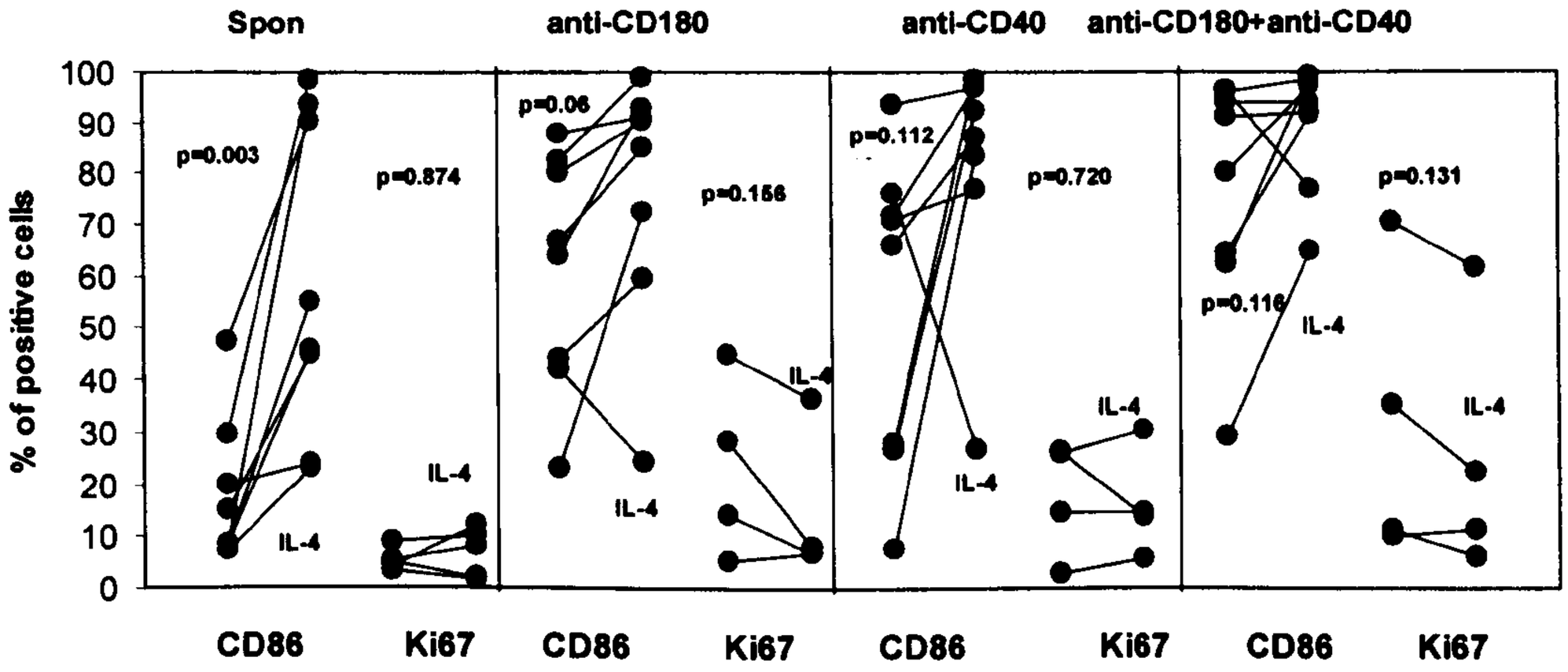


Figure 4.10. Co-stimulatory effect of ligation of CD180 and/or CD40 with rIL-4 on the activation and proliferation of R B-CLL cells.

PBMCs from R B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAb or both in absence or presence of 15ng/mL of rIL-4 for 72 h and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 mAbs or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs. Sample analysis was made by flow cytometry.

4.2.7 Addition of recombinant rIL-4 enhanced the activation but not proliferation of NR B-CLL cells in PBMC cultures:

Significant increases (p=0.03) in the number of CD86 positive NR B-CLL cells were observed subsequent to stimulation with rIL-4 (15 ng/mL) for 72 h (Figure 4.11, left panel). No differences in the number of Ki67 positive NR B-CLL cells (p=0.23) were demonstrated before and after treatment with rIL-4 (Figure 4.11, left panel).

The addition of rIL-4 (15ng/mL) together with anti-CD180 mAb (10μg/mL) induced a general increase (p=0.04) of positive CD86 cells in the majority of B-CLL clones (Figure 4.11, centre-left panel). The use of the anti-CD180 mAb and rIL-4 combination appeared not to increase CD86 expression over that of the cytokine alone. Additionally, dual stimulation with anti-CD180 and rIL-4 had no significant effect (p=0.318) on the number of Ki67 positive cells (Figure 4.11, centre-left panel).

In parallel with that observed previously using both anti-CD180 mAb and rIL-4, the addition of anti-CD40 mAb and the cytokine together induced an increase ($p=0.023$) in the number of CD86 positive NR B-CLL cells (Figure 4.11, centre-right panel). No significant effect ($p=0.07$) on the number of Ki67 positive NR B-CLL cells was seen with this same combinational treatment (Figure 4.11, centre-right panel).

Similar data on the NR B-CLL clones were obtained with the use of both anti-CD180 (10μg/mL) and anti-CD40 (10μg/mL) mAbs in conjunction with rIL-4 (15ng/mL). Again, a significant increase ($p=0.023$) in the number of CD86 positive NR B-CLL cells above spontaneous levels were seen with this latter treatment (Figure 4.11, right panel).

Completing this set of results, no significant change ($p=0.593$) in the number of Ki67 positive cells was present following the addition of both mAbs and rIL-4 (Figure 4.11, right panel).

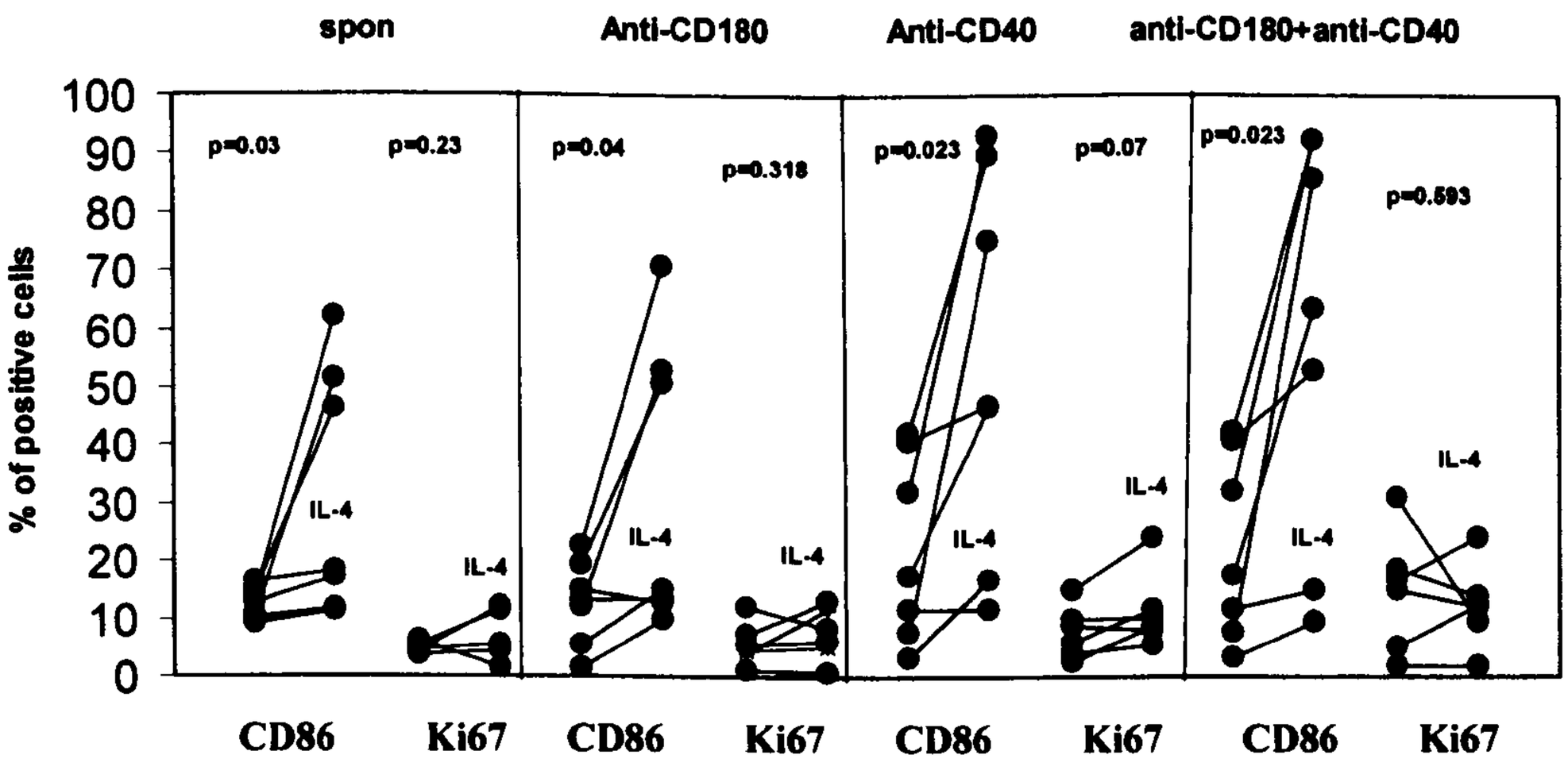


Figure 4.11. Co-stimulatory effect of the ligation of CD180 and/or CD40 with rIL-4 on the activation and proliferation of NR B-CLL cells.

PBMCs from NR-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAb or both in absence or presence of 15ng/mL rIL-4 for 72 h and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 mAbs or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs. Sample analysis was made by flow cytometry.

4.2.8 Addition of recombinant rIL-4 enhanced the activation but not proliferation of B-CLL- cells in PBMC cultures:

The experiments detailed in the previous section suggested that both the activation and proliferation of rIL-4 treated NR B-CLL cells were not modified to any major extent by additional ligation of CD180 and/or CD40.

In light of these data, similar experiments with CD180- B-CLL cells were carried out. Parallel to that observed with NR B-CLL cells, the addition of rIL-4 (15 ng/mL) caused a significant, increase ($p=0.04$) in the number of CD86 positive CD180- B-CLL cells above spontaneous levels (Figure 4.12, left panel), albeit to a lesser extent than that seen for NR B-CLL cells (Figure 4.11, left panel). The number of Ki67 positive cells after 72 h culture remained unaffected ($p=0.241$) by rIL-4 treatment (Figure 4.12, left panel).

Dual treatment of CD180- B-CLL cells with rIL-4 (15ng/mL) and anti-CD180 (10μg/mL) for 72 h significantly increased ($p=0.02$) the number of CD86 positive cells (Figure 4.12, centre-left panel) although the overall effect was similar to that obtained using the cytokine alone (Figure 4.12, centre-left panel) as there was obviously no contribution from CD180.

No differences ($p=0.75$) were seen for the number of Ki67 positive cells before and after treatment with rIL-4 and anti-CD180 mAb (Figure 4.12, centre-left panel).

Addition of rIL-4 and anti-CD40 to the CD180- B-CLL cultures for 72 h significantly increased the number of positive CD86 cells ($p=0.007$) albeit with a clear lack of effect observed for three of the nine patient samples examined. (Figure 4.12, centre-right panel). The overall effect was greater than that elicited by rIL-4 treatment alone.

Once more, no effect ($p=0.490$) on the level of Ki67 positive B cells was observed with this dual stimulation (Figure 4.12, centre-right panel). Addition of rIL-4 with both anti-CD180 and anti-CD40 mAbs significantly increased ($p=0.010$) the percentages of CD86 positive CD180- B-CLL cells (Figure 4.12, right panel).

However, the overall effect was similar to that obtained using rIL-4 and CD40 mAb alone (Figure 4.12, centre-right panel) with no apparent contribution from the ligation of CD180. Net effects on Ki67 positive cells were not observed (p=0.121) with this treatment regimen. (Figure 4.12, right panel).

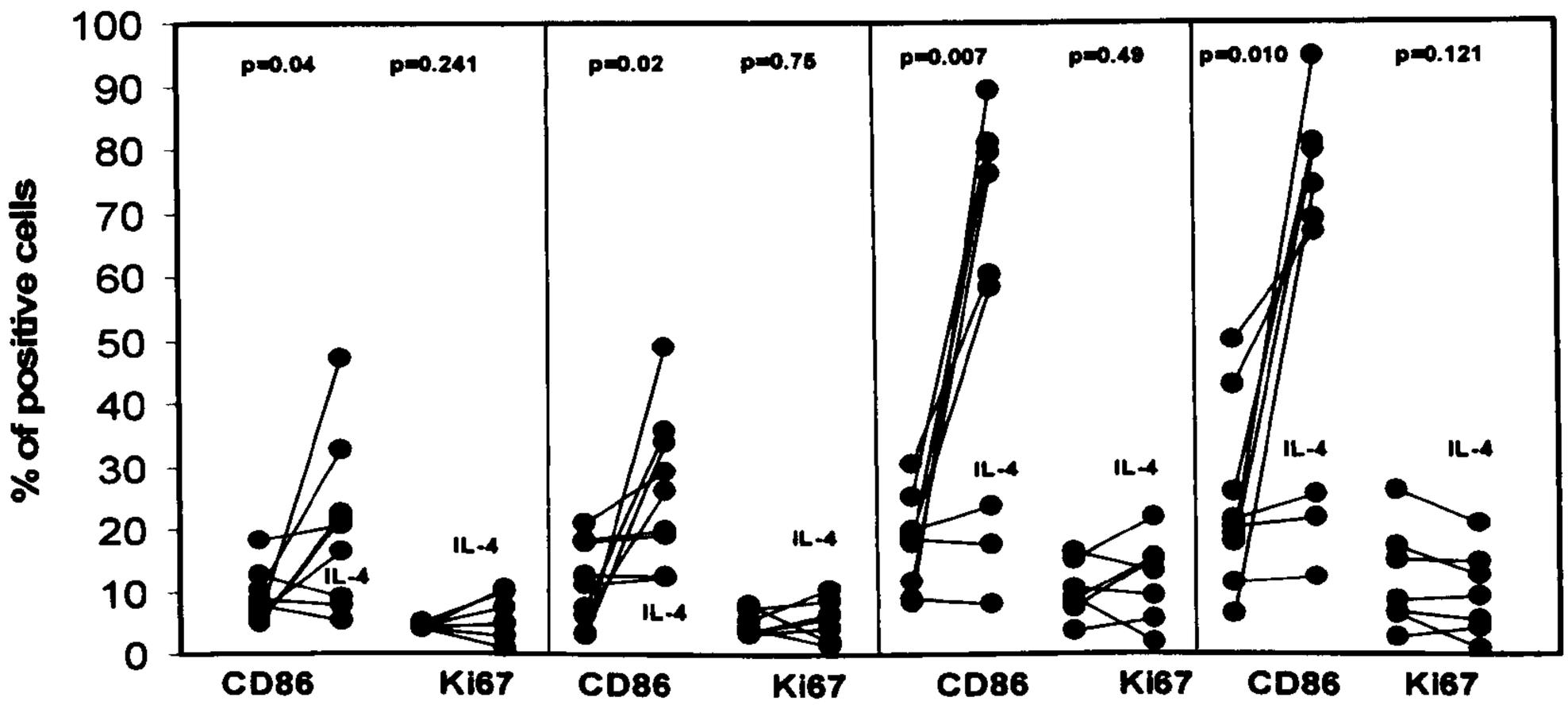


Figure 4.12. Co-stimulatory effect of the ligation of CD180 and/or CD40 with rIL-4 on the activation and proliferation of CD180- B-CLL cells.

PBMCs from CD180 negative B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or anti-CD40 mAb or both in absence or presence of 15ng/mL rIL-4 for 72 h and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 mAbs or fixed, permeabilised and stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-Ki67 mAbs. Sample analysis was made by flow cytometry.

It was interesting to note that CD180+ NR and CD180- B-CLL cells, although showing increase in the percentages of CD86 positive cells, were less responsive than CD180+ R B-CLL cells (Figures 4.10, 4.11 and 4.12 respectively for CD180+ R, CD180+ NR and CD180-).

In the next series of experiments, the differential responses by CD180+ B-CLL clones to CD180 ligation were investigated with particular regard to the possibility that the responses were due to the activation of CD180+ monocytes present in the PBMC cultures. For this purpose it was necessary to use purified B cell cultures in these studies.

4.2.9 Purification of R CD180+ B-CLL cells resulted in a significant decrease of the proportion of CD19+ CD86+ cells on CD180 ligation, but had no effect on cell proliferation

Stimulation of R CD19+ cells with anti-CD180 mAb (10μg/mL) for 72h resulted in a significantly elevated number of CD86 positive cells (p=0.01) in PBMC compared to the purified B cells (Figure 4.13). Similar differences were observed with PBMC and purified B cells stimulated with anti-CD40mAb alone (10μg/mL) although the spread of data did not allow significance to be achieved (p=0.09). Treatment of these cells with both anti-CD180 and anti-CD40 mAbs demonstrated a significant difference (p=0.01) in the number of CD86 positive cells present in PBMC compared to the purified B cell culture (Figure 4.13).

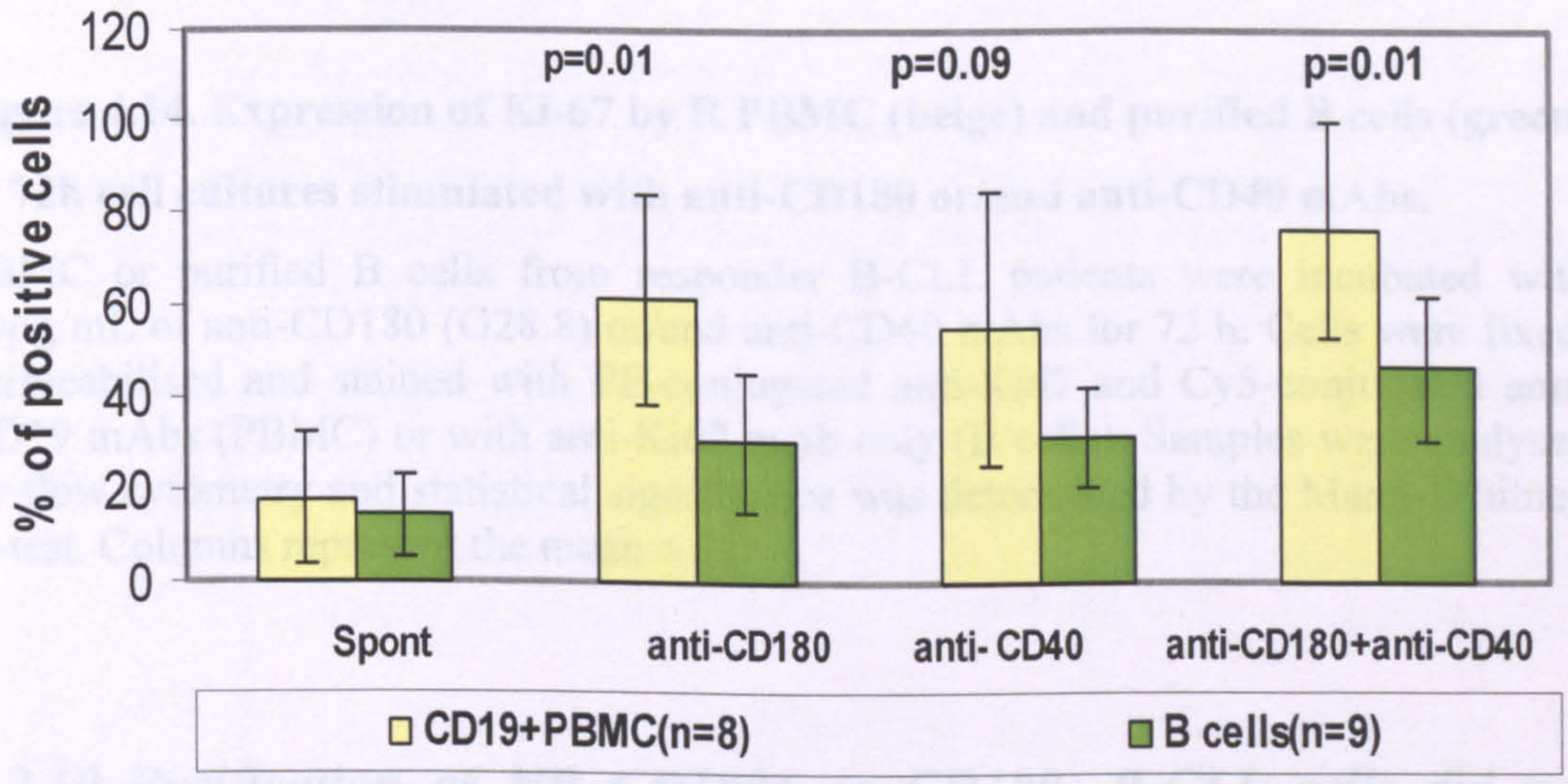


Figure 4.13. Expression of CD86 by R PBMC (beige) and purified B cells (green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from responder B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs for 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti-CD19 mAbs (PBMC), or PE-conjugated anti-CD86 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determined by the Mann-Whitney U-test. Columns represent the mean ± SD.

Interestingly, no significant differences were detected between the intracellular expression of Ki-67 by R CD19+ cells, PBMC and purified B cells, induced by either anti-CD180 (p=0.82), anti-CD40 mAbs (p=0.82) or a combination of both mAbs in 72 h cultures (Figure 4.14).

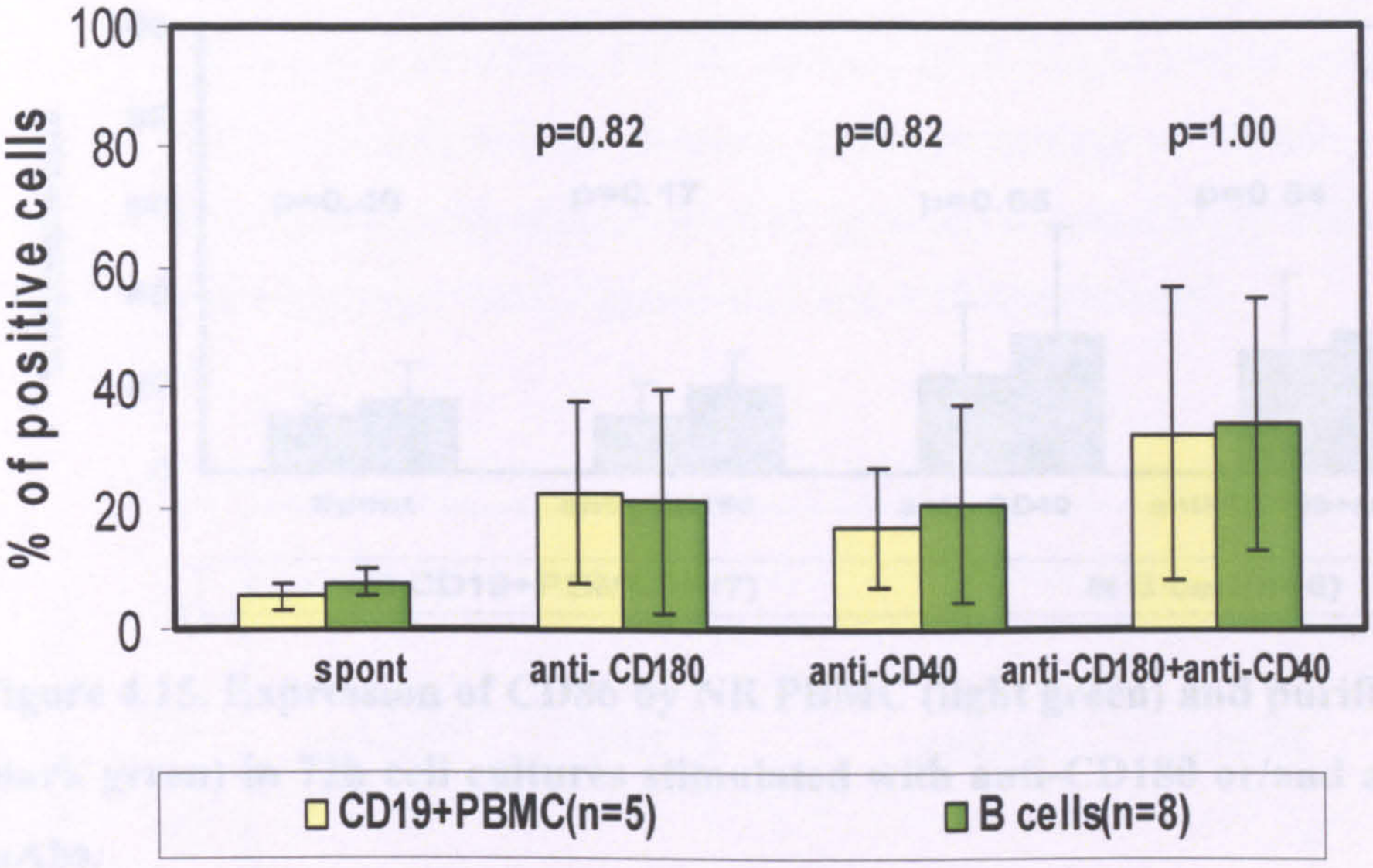


Figure 4.14. Expression of Ki-67 by R PBMC (beige) and purified B cells (green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from responder B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs for 72 h. Cells were fixed, permeabilised and stained with PE-conjugated anti-Ki67 and Cy5-conjugated anti-CD19 mAbs (PBMC) or with anti-Ki67 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determined by the Mann-Whitney U-test. Columns represent the mean ± SD.

4.2.10 Purification of NR CD180+ or CD180- B-CLL cells did not result in a significant change in the proportion of CD19+CD86+ or CD19+Ki67+ cells

No significant changes were seen in the percentages of cells positive for CD86 and Ki67 from CD180+ NR purified B-CLL cells and CD19+ B cells in PBMC cultures after ligation with anti-CD180 mAb (Figures 4.15 and 4.16 respectively). CD40-mediated B cell activation and proliferation of the B cells was not modified to any extent by purification.

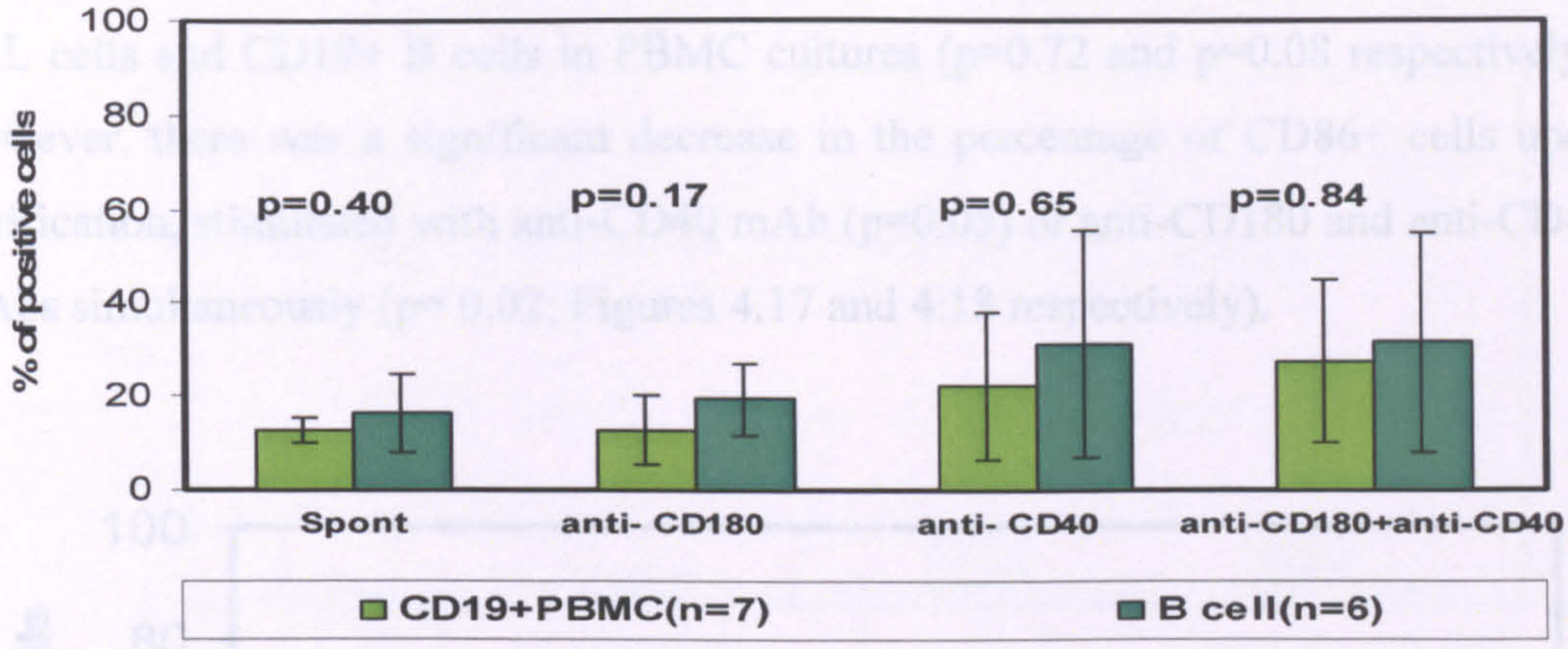


Figure 4.15. Expression of CD86 by NR PBMC (light green) and purified B cells (dark green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from NR B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs for 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti-CD19 mAbs (PBMC), or PE-conjugated anti-CD86 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determined by the Mann-Whitney U-test. Columns represent the mean ± SD.

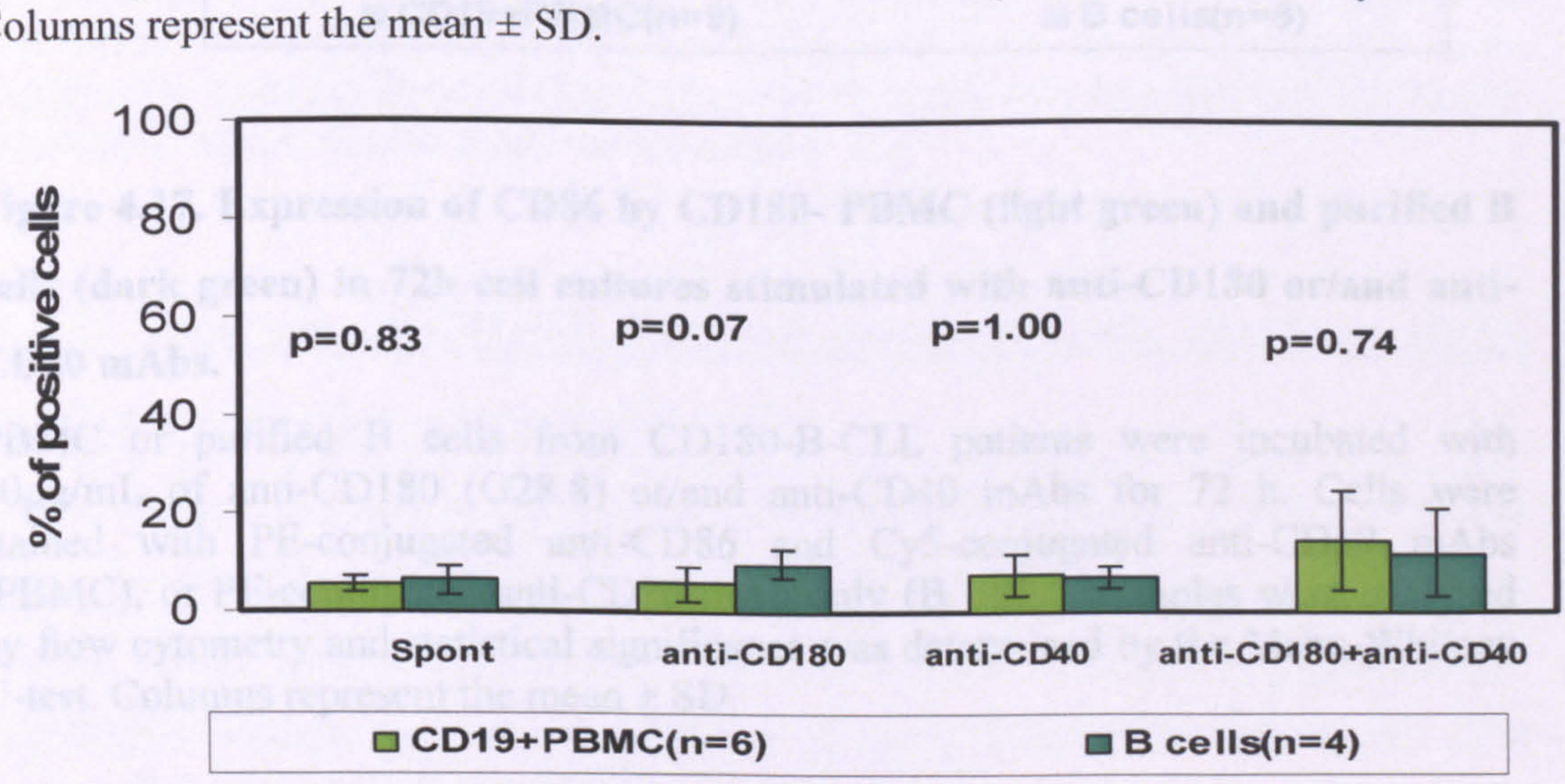


Figure 4.16. Expression of Ki67 by NR PBMC (light green) and purified B cells (dark green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from NR B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs or 72 h. Cells were fixed, permeabilised and stained with and PE-conjugated anti-Ki67 and Cy5-conjugated anti-CD19 mAbs (PBMC) or with anti-Ki67 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determined by the Mann-Whitney U-test. Columns represent the mean ± SD.

As expected, no differences were detected in the case of CD180-negative purified B-CLL cells and CD19+ B cells in PBMC cultures (p=0.72 and p=0.08 respectively). However, there was a significant decrease in the percentage of CD86+ cells upon purification, stimulated with anti-CD40 mAb (p=0.05) or anti-CD180 and anti-CD40 mAbs simultaneously (p= 0.02; Figures 4.17 and 4.18 respectively).

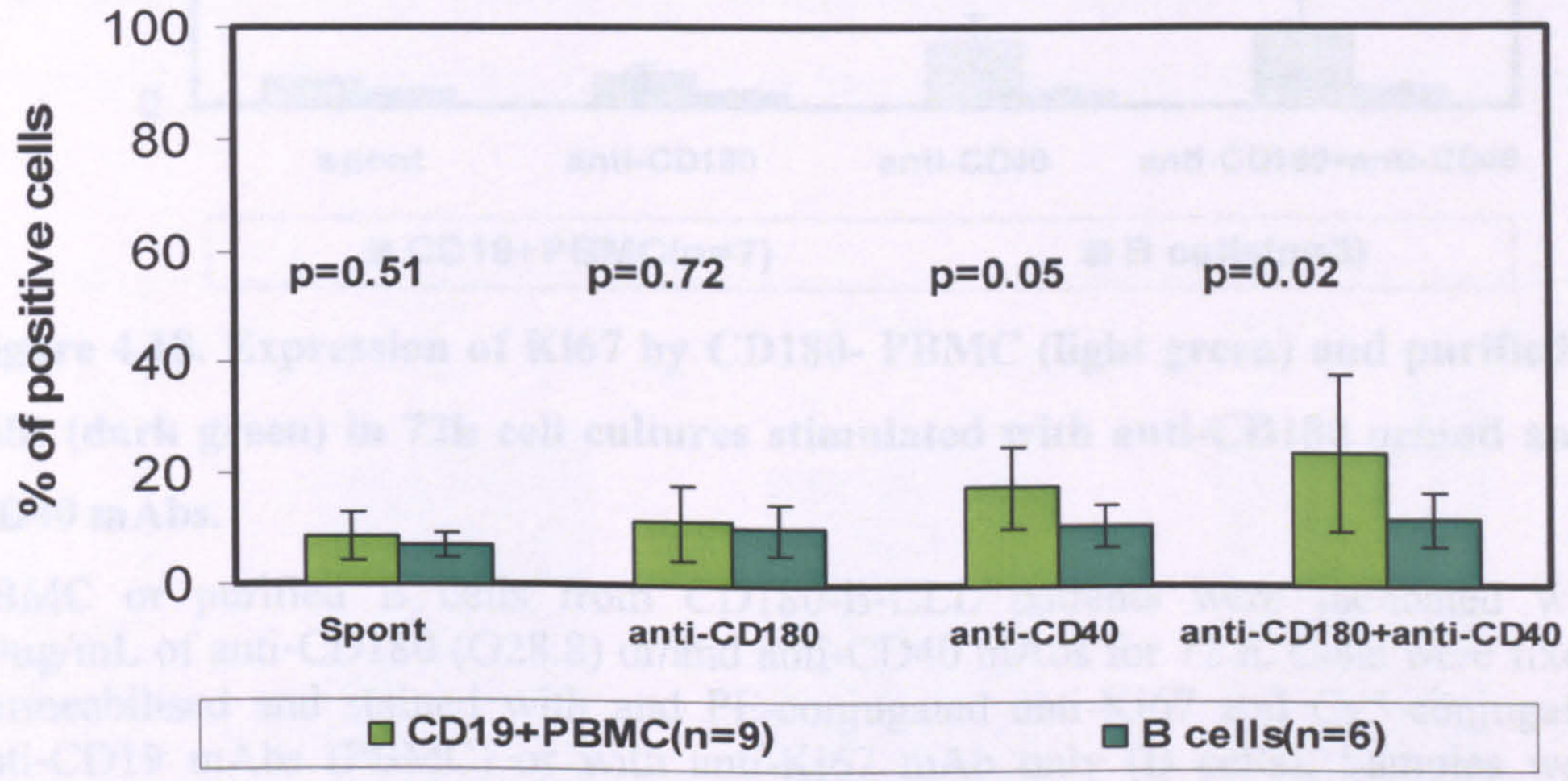


Figure 4.17. Expression of CD86 by CD180- PBMC (light green) and purified B cells (dark green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from CD180-B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs for 72 h. Cells were stained with PE-conjugated anti-CD86 and Cy5-conjugated anti-CD19 mAbs (PBMC), or PE-conjugated anti-CD86 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determined by the Mann-Whitney U-test. Columns represent the mean ± SD.

Purification of CD180- B-CLL cells had a significant effect on cellular proliferation following stimulation with anti-CD40 mAb with or without CD180 ligation (4.18).

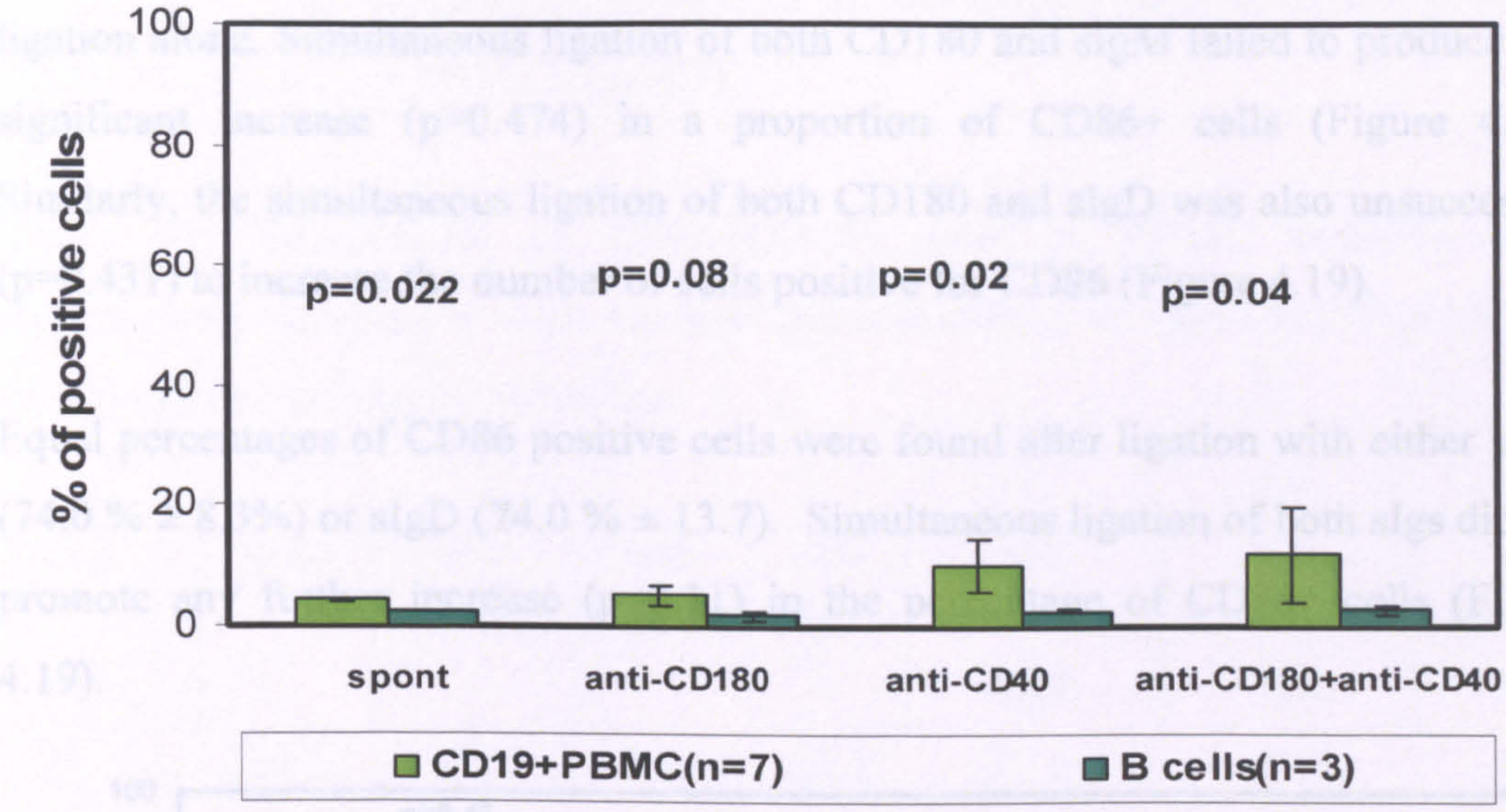


Figure 4.18. Expression of Ki67 by CD180- PBMC (light green) and purified B cells (dark green) in 72h cell cultures stimulated with anti-CD180 or/and anti-CD40 mAbs.

PBMC or purified B cells from CD180-B-CLL patients were incubated with 10μg/mL of anti-CD180 (G28.8) or/and anti-CD40 mAbs for 72 h. Cells were fixed, permeabilised and stained with and PE-conjugated anti-Ki67 and Cy5-conjugated anti-CD19 mAbs (PBMC) or with anti-Ki67 mAb only (B cells). Samples were analysed by flow cytometry and statistical significance was determine by the Mann-Whitney U-test. Columns represent the mean ± SD.

4.2.11 Ligation of CD180 does not synergise with sIgM or sIgD-mediated activation of control B cells:

Due to the heterogeneity of CD180 and CD40 mediated responses, it was essential to determine possible associations between CD180 and BCR mediated responses. As shown above, treatment of PBMC cultures from normal control age-matched individuals with an optimal concentration of anti-CD180 mAb (G28.8) for 72 h resulted in significant increases in cells positive for the activation marker CD86. Likewise, ligation of sIgM or sIgD receptors with anti-IgM $F(ab)_2$ or with anti-IgD $F(ab)_2$ resulted in a highly significant increase in the percentage of expression of CD86 ($p=1.9x10^{-8}$).

Although the density of sIgM expression, but not that of sIgD, was found to be higher than CD180 in normal controls (see Table 3.1), the response measured by the expression of CD86 was not significantly different from that mediated by CD180

ligation alone. Simultaneous ligation of both CD180 and sIgM failed to produce any significant increase (p=0.474) in a proportion of CD86+ cells (Figure 4.19). Similarly, the simultaneous ligation of both CD180 and sIgD was also unsuccessful (p=0.431) to increase the number of cells positive for CD86 (Figure 4.19).

Equal percentages of CD86 positive cells were found after ligation with either sIgM (74.0 % ± 8.3%) or sIgD (74.0 % ± 13.7). Simultaneous ligation of both sIgs did not promote any further increase (p=0.11) in the percentage of CD86+ cells (Figure 4.19).

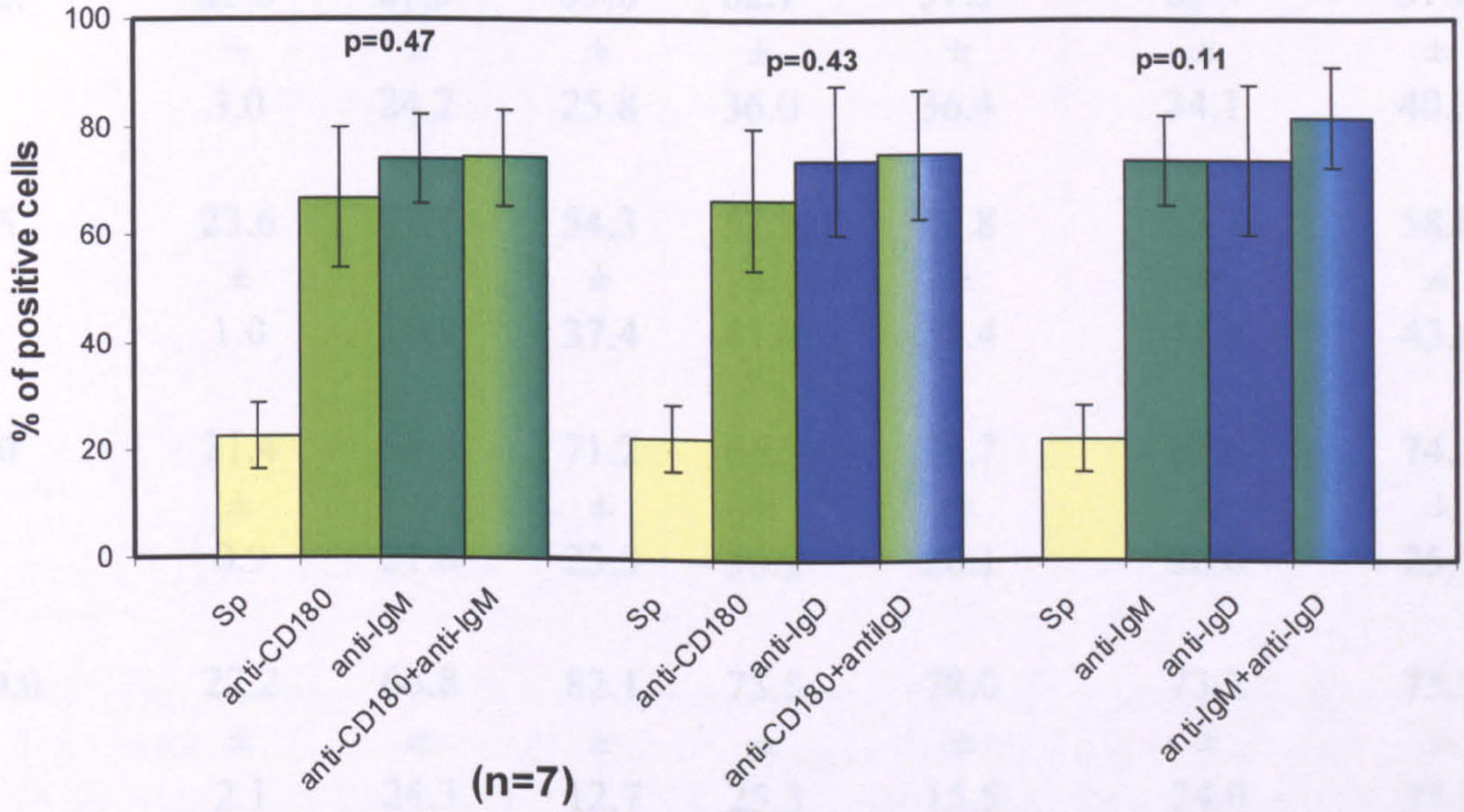


Figure 4.19. Percentages of CD86+CD19+ cells in control PBMC, following ligation with anti-CD180 mAb, anti-IgM, anti- IgD Abs and their combinations.

PBMCs from controls were incubated with 10μg/mL of anti-CD180 mAb (G28.8), anti-IgM or anti-IgD Abs for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 and mAbs (CD86). Samples were analysed by flow cytometry and columns represent the mean ± SD.

The lack of any additive effect between the ligation of CD180 and the structural components of the BCR were confirmed by a series of experiments whereby control B cells were stimulated with a more complete range of antibody concentrations (1.25μg/mL, 2.5μg/mL, 5μg/mL and 10μg/mL) of anti-CD180 mAbs, anti-IgM Abs and anti-IgD Abs. The data shown in Table 4.2 and Table 4.3 reflects both the numerical cell activation, measured as the percentage of CD86+CD19+ cells (Table

4.2) as well as the density of expression of this surface marker by use of mean fluorescence intensity (MFI, Table 4.3).

Table 4.2. Percentages of CD86+CD19+ cells in PBMC cultures upon simultaneous treatment with various concentrations of anti-CD180 mAb and anti-sIgM or anti-sIgD antibodies.

µg/mL	spont	anti-CD180	anti-IgM	anti-IgD	anti-CD180+anti-IgM	anti-CD180+anti-IgD	anti-IgM+anti-IgD
1.25	25.8 ± 3.0	67.3 ± 24.2	59.0 ± 25.8	62.1 ± 36.0	57.5 ± 36.4	65.4 ± 34.1	57.0 ± 40.3
2.5	23.6 ± 1.0	71.1 ± 16.5	54.3 ± 37.4	57.2 ± 41.4	57.8 ± 38.4	55.7 ± 44.3	58.8 ± 43.6
5.0	21.4 ± 0.9	69.8 ± 21.6	71.2 ± 23.3	66.5 ± 30.2	73.7 ± 20.1	67.6 ± 26.0	74.1 ± 25.1
10.0	22.2 ± 2.1	66.8 ± 24.3	82.1 ± 12.7	73.5 ± 25.3	78.0 ± 15.5	73.2 ± 24.0	75.5 ± 21.2

PBMCs from controls were incubated with 1.25, 2.5, 5 and 10µg/mL of anti-CD180 mAb (G28.8), with anti-IgM or anti-IgD Abs for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 and mAbs (CD86). Samples were analysed by flow cytometry. Data is shown as mean ± SD.

Table 4.3. Mean Fluorescence Intensity (MFI) of the expression of CD86 on CD19+ cells in PBMC cultures upon simultaneous treatment with various concentrations of anti-CD180 mAb and anti-sIgM or anti-sIgD antibodies.

µg/mL	spont	anti-CD180	anti-IgM	anti-IgD	anti-CD180+anti-IgM	anti-CD180+anti-IgD	anti-IgM+anti-IgD
1.25	18.1 ± 15.9	102.7 ± 128.5	98.6 ± 126.7	130.4 ± 167.7	111.7 ± 146.1	132.4 ± 170.5	115.3 ± 151.4
2.5	21.5 ± 12.3	116.6 ± 134.2	127.2 ± 156.7	141.9 ± 175.3	133.6 ± 165.2	146.1 ± 184.6	157.5 ± 197.1
5	18.9 ± 19.1	114.1 ± 152.9	163.9 ± 211.7	158.1 ± 207.4	154.5 ± 194.9	99.9 ± 123.1	169.4 ± 218.5
10	18.4 ± 18.4	113.7 ± 146.7	189.7 ± 236.0	174.9 ± 228.5	168.2 ± 205.5	154.2 ± 201.4	160.7 ± 205.4

PBMCs from normal controls were incubated with 1.25, 2.5, 5 and 10µg/mL of anti-CD180 mAb (G28.8), with anti-IgM or anti-IgD Abs for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 and mAbs (CD86). Samples were analysed by flow cytometry. Data is shown as mean ± SD.

4.2.12 Lack of synergism in activation of R B-CLL cells upon simultaneous ligation of CD180 and sIgM or CD180 and sIgD:

Similar to the data obtained from control B cells, the ligation of surface CD180 increased the number of cells expressing the activation marker CD86 in R B-CLL cells. As shown in Figure 4.20, ligation of sIgM (10µg/mL) and sIgD (10µg/mL) on R B-CLL cells, induced highly significant increases in the percentage of activated cells ($p=1.8x10^{-6}$ and $p=0.004$ respectively). However, similar to that seen with control CD19+ cells, no synergistic actions, to increase CD86 expression were observed with the simultaneous ligation of CD180 and sIgM ($p=0.395$) or that of CD180 and sIgD ($p=0.457$). Likewise, no synergism to increase the expression of

CD86 was observed with the simultaneous ligation of sIgM and sIgD in B-CLL cells (p=0.314).

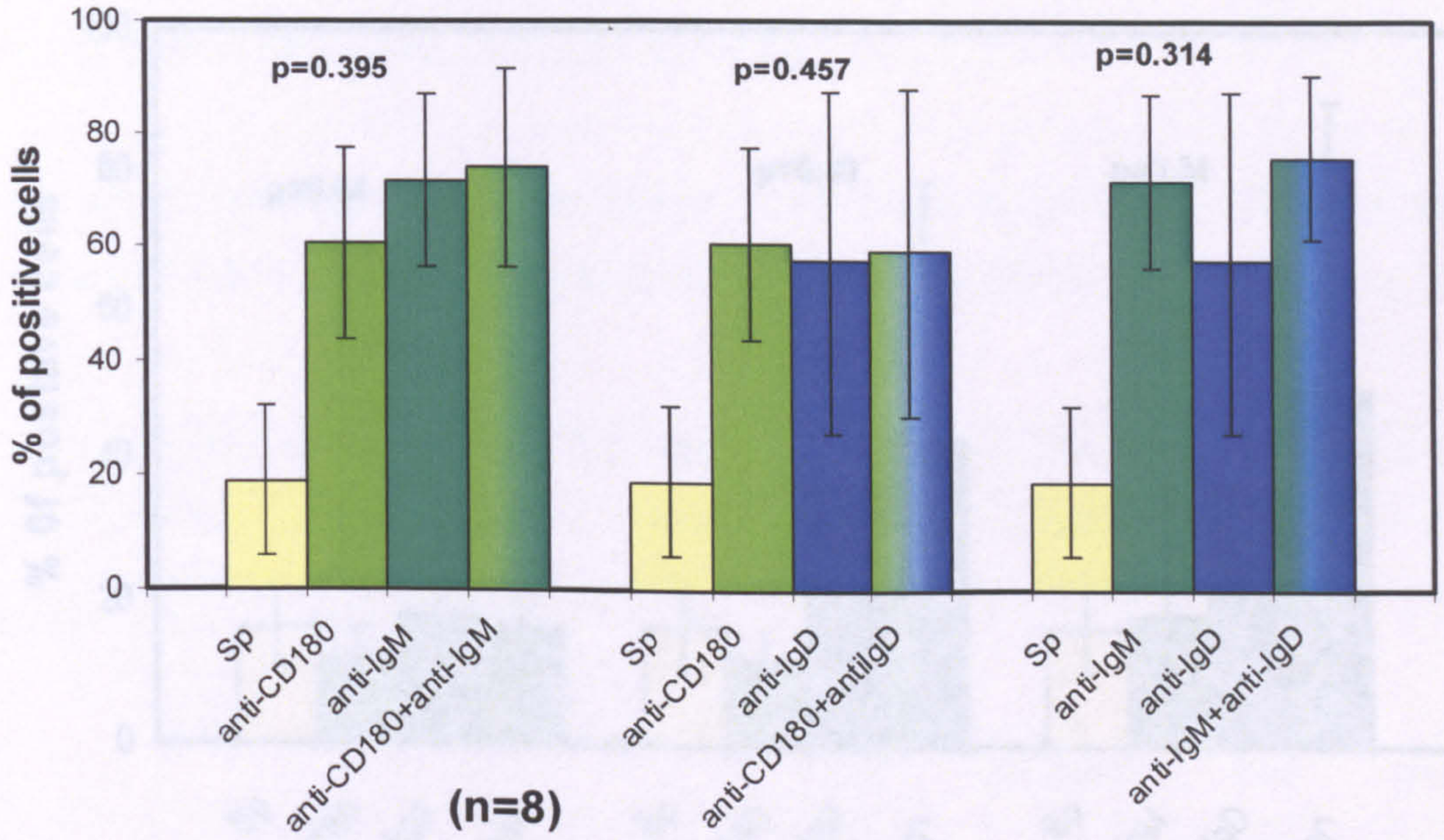


Figure 4.20. Percentages of CD86+CD19+ R B-CLL cells, upon ligation with anti-CD180 mAb, anti-IgM, anti- IgD Abs and their combinations.

PBMCs from eight B-CLL patients were incubated with 10μg/mL of different combinations of anti-CD180 mAb (G28.8), anti-IgM and anti-IgD Abs for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti-CD86 and mAbs. Samples were analysed by flow cytometry. Columns represent the mean ± SD.

4.2.13 Lack of synergism in the activation of NR B-CLL cells upon simultaneous ligation of CD180 and sIgM or CD180 and sIgD:

No significant increase in the expression of surface CD86 was seen in NR B-CLL clones after ligation with CD180 (Figure 4.21). Additionally, no significant increase in the percentage of CD86 positive cells after their ligation with anti-IgM (p=0.42) and anti-IgD (p=0.18) mAbs was present (Figure 4.21).

Similarly, the same figure clearly shows that no synergistic effects were evident after simultaneous ligation of CD180 and sIgM (p=0.44), CD180 and sIgD (p=0.42) or sIgM and sIgD (p=0.341).

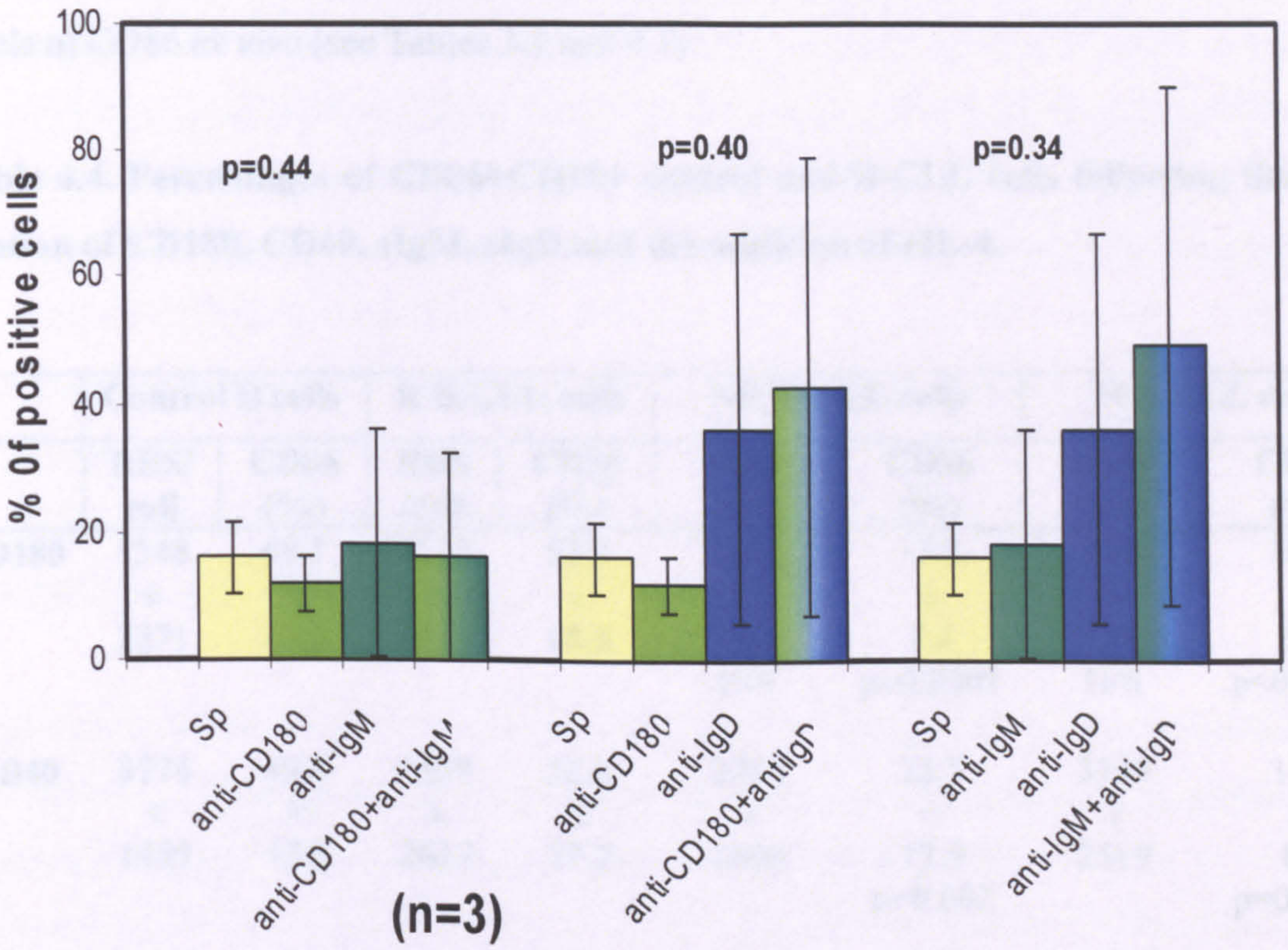


Figure 4.21. Percentages of CD86+CD19+ NR B-CLL cells upon ligation with anti-CD180 mAb, anti-IgM, anti- IgD Abs and their combinations.

PBMCs from three NR B-CLL patients were incubated with 10μg/mL of anti-CD180 mAb, anti-IgM, anti-IgD or both the mAbs in different combinations for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti CD86 and mAbs (CD86). Samples were analysed by flow cytometry. Columns represent the mean ± SD.

4.2.14 Impaired activation of B-CLL cells through CD180 ligation is associated with a significantly decreased ability of activation via CD40, sIgM, sIgD and IL-4.:

One of the main observations based on the data presented in this chapter is that whilst control B cells and R CD180+ B-CLL cells responded by a strong up-regulation of CD86, and in many cases Ki-67, to the addition of all the studied antibodies and of the rIL-4 (Table 4.4), NR CD180+ and, particularly, CD180- B-CLL cells showed significantly reduced activation following the ligation of CD180 ($p<0.0001$), CD40 ($p=0.0031$), sIgM ($p=0.014$), sIgD ($p=0.026$), or the addition of

rIL-4 (p=0.005) than control B cells. Importantly this was not due to cell pre-activation *ex vivo* since NR and, particularly, CD180- B-CLL clones expressed low levels of CD86 *ex vivo* (see Tables 3.1 and 4.1).

Table 4.4. Percentages of CD86+CD19+ control and B-CLL cells following the ligation of CD180, CD40, sIgM, sIgD and the addition of rIL-4.

	Control B cells		R B-CLL cells		NR B-CLL cells		N B-CLL cells	
	RBS/ cell	CD86 (%)	RBS /cell	CD86 (%)	RBS /cell	CD86 (%)	RBS /cell	CD86 (%)
CD180	5548 ± 2271	68.1 ± 17.0	2113 ± 1445	57.9 ± 18.8	1198 ± 989 U/S	15.3 ± 7.4 p<0.0001	236 ± 168 U/S	10.3 ± 6.6 p<0.0001
CD40	8774 ± 1459	49.8 ± 17.3	5339 ± 2617	52.0 ± 27.7	3753 ± 1496	25.5 ± 12.9 p=0.052	3339 ± 2519	17.2 ± 8.3 p=0.0031
sIgM	17907 ± 8553	74.3 ± 8.3	1214 ± 1244	66.3 ± 21.7	796 ± 1353	20.6 ± 13.8 p=0.0056	626 ± 659	36.9 ± 21.7 p=0.014
sIgD	4236 ± 2777	74.2 ± 13.7	3973 ± 3543	55.5 ± 27.7	3251 ± 4007	23.5 ± 23.2 p=0.026	4449 ± 5013	30.1 ± 21.7 p=0.075
IL-4	N/A	43.2 ± 14.5	N/A	28.8 ± 31.7	N/A	31.2 ± 21.3	N/A	18.3 ± 12.7 P=0.005

Densities of the corresponding molecules are expressed as RBS/cell and are shown as mean ± SD. U/S represents unstimulated cells. p values represent the difference between unstimulated and stimulated control B cells. PBMCs from controls and B-CLL patients were incubated with 10μg/mL of anti-CD180, anti-CD40 mAbs, anti-IgM, anti-IgD or both the mAbs in the presence and absence of rIL-4 for 72 h. Cells were stained with Cy5-conjugated anti-CD19 and PE-conjugated anti CD86 and mAbs (CD86).

The low responses of CD180- B-CLL cells could be in part explained by the significantly lower density of CD180 ($p=1.2 \times 10^{-5}$), CD40 ($p=0.02$), but not that of sIgM or sIgD (Table 4.4) compared with that of R B-CLL cells.

Phenotypic and functional heterogeneity of CD180 receptor on B-CLL cells might delineate functional and/or clinical subgroups of B-CLL patients. The results of the studies presented in the previous two chapters successfully identified;

- a cohort of the patients expressing relatively high densities of the CD180 surface receptor on their B- CLL cells;
- a correlation of CD180 expression with other prognostic markers and signalling receptors;
- a cohort of the patients with B-CLL cells expressing a functionally operating CD180 receptor (R B-CLL clones).

These accumulated results allowed the selection of patient groups for further studies on CD180 mediated apoptosis of B-CLL cells based on their propensity to possess highly functional CD180 receptors co-expressed with sIgM and sIgD.

4.3 DISCUSSION

4.3.1 Up-regulation of activation and proliferation of control B cells following the ligation of CD180

It has been shown previously by Yamashita *et al.*, (1996) that ligation of RP105, a murine Toll-like receptor with high sequence homology to CD180, and CD40 on the murine B cell surface leads to both activation and proliferation. However, to my knowledge, the current study is the first attempt to investigate in detail CD180 mediated B cell activation and proliferation *in vitro* with normal human control B cells and B-CLL cells. Similar studies done earlier were based on the murine version of CD180 molecule called RP105, which shares strong homology to CD180 in humans (Divanovic *et al.*, 2007).

Ligation of CD180 on healthy B lymphocytes has been observed to promote strong up-regulation of the percentage of cells expressing CD86 (Porakishvili *et al.*, 2005). The present study has confirmed this finding (Figure 4.1a) although the data clearly demonstrate that two of the sample preparations failed to respond to CD180 ligation. It is unclear why these particular B cells from the two control PBMC cultures have not responded to CD180 ligation and certainly this is worthy of future investigation providing the same individuals can be located. Notwithstanding these latter two cases, the overall effect was as previously described.

Ligation of CD180 also increased the percentage of control B cells positive for the intracellular marker, Ki67 (Figure 4.1b). Extensively reviewed by Scholzen and Gerdes (2000), the presence of Ki67 has been proposed to be an excellent indicator for determining the proliferation of any given cell population, normal or neoplastic. The protein is expressed during all the active phases of cell cycle (i.e. G1, S, G2 and mitosis) but is absent in resting cell populations (i.e.G0). Interestingly, although the majority of normal B cells respond to CD180 ligation by activation, as seen by the up-regulation of CD86 expression, only a small subset of this population was seen to actually enter into the cell cycle, as demonstrated by the up-regulation of Ki67 expression. One explanation may be that the cells becoming Ki67+ were already activated (CD86+), prior to culture.

To assess the level of the cellular response to ligation of CD180 on B cells, the effects on both activation and proliferation subsequent to ligation of CD40 were also examined. As expected, and supported by a number of previous studies in this area (Aristides *et al.*, 2004, Arnon *et al.*, 2004, Glouchkova *et al.,* 2009, Bekeredjian-Ding *et al.,* 2008), I demonstrated that ligation of CD40 with anti-CD40 mAb on control B cells leads to their activation and proliferation (Figure 4.5).

Interestingly, treatment with anti-CD180 mAb tended to produce higher numbers of control B cells positive for CD86 compared to those treated with anti-CD40 mAb. Albeit with no statistical significance reached due to the small sample size, this observation is of potential further interest since higher levels of CD40 expression (8774 ± 1459 vs 5548 ± 2271 RBS/cell) were measured on these cells compared to CD180 (Table 3.1, $p < 0.05$), confirming that CD180 ligation is a powerful stimulator of B cells.

4.3.2 Interactions between CD180 and CD40 ligation on normal B cell cultures

Ligation of CD180 or CD40 on B cell cultures with mAbs specific to each surface receptor resulted in a highly significant increase ($p=0.00001$ and $p=0.0004$ respectively) in the number of CD86 positive cells present after 72 h culture (Figure 4.5). Combination of the mAb treatment observed only a modest increase in the number of positive CD86 cells which, after subtraction of background expression levels of the marker in the cells, represented, at best, only an additive effect (Figure 4.5). In hindsight, the initial aim of this experimentation was to investigate the activation of normal B cells and not to search for possible synergistic effects. Indeed, the study used concentrations (10μg/mL) of mAbs that were chosen for producing an optimal stimulation, consequently there was little or no possibility to see any synergistic interactions from the dual mAb treatment. It is clear that any future studies investigating this aspect should be addressed with the use of threshold concentrations of each mAb, that in themselves produce only a minimal up-regulation of CD86.

However, a synergistic action of the mAbs was shown on control B cells to up-regulate the intracellular expression of Ki67. Given alone at the tested concentration (10 μg/mL), both anti-CD180 and anti-CD40 mAbs, the number of cells positive for this proliferation marker were barely more than 10% above that observed without treatment (Figure 4.5). Since in this instance the concentration of the mAbs clearly elicited only a threshold effect on the control B cells, the ability to visualise a synergistic action was more likely. In actual fact, treatment with both mAbs concurrently synergistically elevated the numbers of Ki67 positive control B cells approximately 50% above basal levels, almost 3-fold more than the combined effects obtained with the mAbs alone (Figure 4.5).

In summary, the data from these studies suggested that that:

(a) CD180 and CD40 use different cell signalling activation pathways. This is supported by their synergistic actions observed on cell proliferation. Whether or not CD180 and CD40 act by separate pathways with respect to the control of B cell activation remains to be established from additional studies using lower, threshold concentrations of both anti-CD180 and anti-CD40. Roshak *et al.*, (1999) have also shown a distinct functional role of CD180 and CD40 on human B cells on ligation with corresponding Abs. This group has shown that anti-CD40 mAbs and IL-4 but not anti-CD180 (RP105) mAbs produced soluble CD23, a mediator which plays a role in B cell proliferation and IgE production. Most recently, it has been shown by Kazuna, (2004) that although CD180 and CD40 are expressed on all naïve and memory B cells, their response is quite different on stimulation. CD180 signalling enhances the activation, proliferation and life span in naïve B cells in comparison to memory B cells, where the response is decreased on CD40 stimulation.

(b) There may be a requirement of cytokine support for the activation provided by the recruitment of B cells and blood monocytes by means of simultaneous engagement of CD180/CD40 pathways.

The latter hypothesis was partially tested in subsequent studies examining the co-stimulation of B cells with both rIL-4 and anti-CD180 mAb.

4.3.3 Addition of rIL-4 to control B cell cultures

Shown in Figure 4.6 (left panel), the addition of rIL-4 (15 ng/mL) with anti-CD180 mAb (10 μg/mL) induced a moderate activation (p=0.05) in control B cells. It was noteworthy that after anti-CD180 mAb treatment, four of the eight samples examined had up-regulation in the percentage of CD86 cells in excess of 75%, thus further increases due to the addition of rIL-4 was less likely. It is possible that more significant effects will be seen with the use of control B cells where the stimulation via anti-CD180 mAb is minimal. On the other hand, a more clear response of the control B cells to the dual stimulatory actions of rIL-4 and anti-CD40 mAb were observed particularly with respect to the effects on CD86 expression where a highly significant (p=0.001) increase of positively stained cells was present, notwithstanding a relatively high level of activated cells via anti-CD40 ligation alone (Figure 4.6, centre panel).

Conversely, a strongly significant (p=0.003) increase of Ki67 positive control B cells was shown when both rIL-4 and anti-CD180 mAb were used in combination (Figure 4.6, left panel). Similar significant effects (p=0.031) on Ki67 expression were seen with the use of the anti-CD40 mAb and rIL-4 combination although the effect was less consistent to that observed with anti-CD180mAb with two of the seven samples actually showing a decrease in percentage of Ki67 positive cells (Figure 4.4, centre panel). Previous investigations have shown that stimulation through CD40 and IL-4 have cytoprotective actions on B cells (Hewamana *et al.*, 2008). Interestingly and with particular regard to this latter report, B cells can be cultured for several weeks in the presence of IL-4 and CD40 (Dumont *et al.*, 2009). Another noteworthy study has shown a positive effect of IL-4 on B cell proliferation induced by anti-CD40 and those stimulated with anti-IgM (Saito *et al.*, (2008). In essence, the net content of the published studies mentioned above are in accord with my findings.

Most importantly, the addition of rIL-4 did not modify the responses of control B cells when combined with both anti-CD180 and anti-CD40 (Figure 4.6, right panel). In the case of activation, it has to be stated again that the ability to observe any effect of the rIL-4 and mAbs combination was made practically impossible due to the very high percentage of CD86 positive cells via simultaneous ligation of anti-CD180 and

anti-CD40 mAbs. In fact, six of the seven samples examined had high levels of CD86 positive cells ≥ 70% with three being ≥ 90% after the dual mAb stimulation. In this sense, these cells were maximally activated and to measure any further increase with rIL-4 was impossible. It is noteworthy that treatment with the rIL-4 and mAbs combination to the only sample with a percentage of CD86 positive cells <50% was actually observed to promote a down-regulatory effect on the number of CD86 positive cells (Figure 4.6, right panel). This latter finding was obviously unexpected and merits further investigation.

In the case of proliferative responses, the percentage of Ki67 positive cells via dual ligation with the mAbs was higher than that used in the previous instances (Figure 4.6, right panel), showing an additive effect. Incubation with both rIL-4 and both mAbs provoked a mixed response from the cells in that two samples remained more or less static with regard to the number of Ki67 positive cells, two samples demonstrated moderate increases while three samples showed a distinct decrease in the absolute numbers of positive Ki67 stained cells.

The data suggest that either (a) engagement of both surface molecules provides a sufficient release of IL-4 by B cells in culture and no exogenous rIL-4 is required; or (b) combined anti-CD180/anti-CD40 stimuli results in recruitment of all B cells capable of activation/proliferation in 72 h PBMC cultures.

The latter point is supported by our observation that some of the PBMC treated with the combination of all three stimulants showed a marked decline in the number of activated or cycling B cells. This may be due to the activation induced cell death (Donjerkovi *et al.*, 2000). It has been shown in a previous study that CD40 stimulation by either anti-CD40 Abs or a recombinant soluble CD40 ligand can inhibit the cell growth of human breast carcinomas and B lymphomas *in vitro* and *in vivo*. This is thought to occur by AICD in which stimuli which promote the growth of normal cell types inhibit the growth of neoplastic counterparts. This occurs via the induction of apoptosis, necrosis and/or cell cycle arrest (Ziebold *et al.*, 2000).

The data obtained on the effect of CD180 ligation on B cell activation and proliferation led to my further studies on the treatment of B-CLL cells with anti-CD180 antibodies alone or with simultaneous treatment with anti-CD40 mAb and rIL-4.

4.3.4 Up-regulation of activation and proliferation of B-CLL cells following CD180 ligation

Unlike control B cells, CD180+ B-CLL clones showed two different patterns of the responses to CD180 ligation and were subsequently divided into two groups. Whilst R B-CLL cells responded to CD180 ligation in an identical manner to control B cells with respect to activation (Figure 4.2a) and proliferation (Figure 4.2b), NR CD180+ B-CLL clones did not show any up-regulation of CD86 or Ki-67 (Figure 4.3a and Figure 4.3b). One reason for this difference response pattern could be related to the fact that the density of CD180 on R clones was significantly higher ($p=0.01$) than that on NR B-CLL cells (2113 ± 1445 range 885-3928 vs 1198 ± 989 range 713-2732 RBS/cell,).

Purified R B-CLL cells showed a significant decrease in activation possibly due to the deficit in cytokines released by other/accessory cells such as monocytes present in PBMC cultures, since they also express CD180 (Chan *et al.*, 1998). This is supported by the fact that significantly higher ($p=0.01$) numbers of CD86 positive cells were evident in PBMC compared to purified B cell preparations after stimulation with anti-CD180 mAb (Figure 4.13). However, since there were no significant changes in the level of the cell cycling protein Ki-67 (Figure 4.14), it would appear that cytokines released by the accessory cells are pro-activatory but not vital for the proliferation of B-CLL cells.

An important observation made during the course of this study, recently shown by Memon *et al* (2007), was that NR clones also responded poorly to anti-CD40 ligation (Figure 4.9). This may be due to the lower density of CD40 expression on NR vs R B-CLL clones (3753 ± 1496 vs 5539 ± 2617 RBS/cell; Table 4.1, $p = 0.01$). Notably, NR B-CLL cells still responded to the addition of rIL-4 by increased expression of CD86 (Figure 4.11), thus indicating the independent nature of this pathway. Akin to

the effect of anti-CD180, treatment with anti-CD40 did not induce any significant change in the level of the cell cycling protein, Ki67 (Figure 4.11).

In the case of NR B-CLL cells, no significant differences were found between PBMC and purified B cells regarding cell activation and proliferation subsequent to ligation with anti-CD180 and/or anti-CD40, or both mAbs simultaneously. The data show that a defective CD180 pathway affects the signal transduction of other surface molecules such as CD40 rather than the suppressive influences of non-B cells in PBMC cultures.

Similar results were observed with CD180- B-CLL cells (Figure 4.4a and Figure 4.4b). Not surprisingly, activation or proliferation in CD180- B-CLL cells was absent upon the ligation of CD180. More importantly, and surprisingly, in CD180- B-CLL cells, no activation or proliferation was observed, even though the density of CD40 on CD180- clones was almost identical to that of NR B-CLL cells (3339 ± 2519 vs 3753 ± 1496 RBS/cell).

NR CD180+ and, particularly, CD180- B-CLL cells showed significantly poor activation following the ligation of CD40 or addition of rIL-4. This was not due to cell pre-activation, since NR and, particularly, CD180- B-CLL cells expressed low levels of CD86, *ex vivo*. Enrichment of B cells from PBMC cultures did not affect the results. The level of activation did not correlate with the expression of CD38 or the mutational status of IgVH genes, although there were more U B-CLL clones in the CD180- group.

It is possible that NR CD180+ and CD180- clones being non/poor responsive to the powerful stimulus of CD40 ligation (Aristides et al., 2004; Arnon et al., 2004; Glaouchkova et al., 2009; Bekerjdian-Ding et al., 2008), and IL-4, may represent anergic B-CLL cells which are unable to respond to activation signals. Other activatory mAbs would need to be used to confirm this such as anti-CD22 mAbs (Pezzutto *et al.*, 1987).

4.3.5 Interaction between CD180 and CD40 ligation in B-CLL cell cultures

Similar to normal control B cells, R B-CLL demonstrated a marked up-regulation of CD86 (Figure 4.7) after ligation of CD180 and CD40 alone or both receptors together. Analogous to the situation found using normal B cells, the extent of CD86 expression on the R B-CLL cells evoked by ligation with both anti-CD180 and anti-CD40 mAbs was not numerically superior to that obtained from the additive values of the mAbs used alone and basal expression. As explained previously in Section 4.2.2, future experimentation in this area would be better served by the use of threshold concentrations of each mAb that used solely produce only a minimal up-regulation of CD86. In contrast to that observed with control B cells, ligation of both CD180 and CD40 on B-CLL cells was unable to bring about any synergistic increase in the quantity of Ki67 positive cells (Figure 4.7)

No major effects were seen with CD180 NR and CD180- B-CLL clones (Figure 4.8 and Figure 4.9 respectively) upon simultaneous ligation of CD180 and CD40. From these data, it is clearly apparent that cells not responding to CD180 ligation are equally unresponsive to stimulation via CD40, a receptor known for inducing activation and proliferation after stimulation with its corresponding Ab (Aristides *et al.*, 2004, Arnon *et al.*, 2004, Glouchkova *et al.,* 2009, Bekerdjian-Ding *et al.,* 2008). The results also show that the signalling pathway defective in CD180 NR clones is also defective for CD40. Therefore, CD180- B-CLL cells have not only non-functional CD180, but also non-functional CD40 signalling pathway.

4.3.6 Addition of rIL-4 to B-CLL cells in PBMC cultures

Interesting results were obtained after incubation of R B-CLL cells simultaneously with combinations of CD180 and rIL-4, CD40 and rIL-4 as well as both the mAbs with rIL-4. An obvious trend, albeit not statistically significant, for the up-regulation of CD86 positive cells was observed with anti-CD180 ($p=0.06$), anti-CD40 ($p=0.112$) and both mAbs simultaneously with rIL-4 ($p=0.116$) although one patient showed a marked down-regulation in the level of CD86 which affected the overall significance (Figure 4.10). It was interesting to note that this down regulation of CD86+ cells with regard to CD180 and CD40 with rIL-4 was observed in the same

patient. To confirm this particular result, further experimentation is required with the same patient to confirm that R B-CLL cells respond in the same way as normal B cells with regard to activation. This latter finding is in concordance with an earlier study proving that B-CLL cells are characteristic of resting lymphocytes with minimal proliferative activity (Chiorazzi, 2007). Supporting this, within this last section of my study I have demonstrated that even with the addition of IL-4 no increase in the level of Ki67 protein was apparent (Figure 4.10, right panel) demonstrating the stable nature of B-CLL cells in comparison to normal B cells.

4.3.7 Effect of purification of B-CLL on responses to stimulation by anti-CD180 and anti-CD40 mAbs

Purified B cells responded weakly to CD180 ligation as compared to PBMC (Figure 4.13). Similar differences were observed with the combined stimulation with anti-CD180 and anti-CD40 mAbs (Figure 4.13) and show that there is an "microenvironment" role for other components of PBMC such as monocytes, expressing CD180 and CD40 can be involved in the B cell activation. It has been previously shown that the B-CLL microenvironment plays a key role in their growth and extended survival (Ghia *et al.*, 2008). This same research also showed that *in vitro,* spontaneous apoptosis of B-CLL cells could be prevented by co-culturing the cells with other microenvironment cells. In my present study, it is clear that B-CLL cells in PBMC cultures respond to ligation of CD180 more than purified B cells, as shown by the increase in the percentages of CD86. However, there was no significant difference in the expression of Ki67 between PBMC and purified B cell cultures subsequent to the ligation of surface CD180 or CD40, or the combination of both mAbs (Figure 4.14). There can be two explanations, either the microenvironment cells are only proactivatory but have no effect on proliferation of B-CLL cells, or the cells are in resting phase as it has been already proven that B-CLL cells are resting small lymphocytes (Chiorazzi, 2007). As expected, NR CD180+ and CD180- B-CLL cells did not demonstrate any significant difference in the level of CD86 and Ki67 between the PBMC and purified B cell cultures (Figure 4.15 to Figure 4.18 respectively). This finding further confirms the anergic nature of NR B-CLL cells in PBMC and purified B cell cultures. The up-regulation in the percentage of CD86 and Ki67 was not significant from the basal level as shown in figures 4.15 and 4.16. This

shows that even the cells present in the micro-environment are not capable of resuming the anergic state of NR CD180+ cell so these cells behave in the same way as purified B cells from a NR B-CLL clone.

Furthermore, purification of CD180- B-CLL cells did not affect anti-CD180 responses but there was a difference seen for CD40 ligation. The percentage of CD86 and Ki67 expressed in CD180- B-CLL was significantly higher in PBMC cultures than purified B cells (Figures 4.17 and 4.18 respectively). It is obvious from the figures that the percentage of CD86+ cells and Ki67+ cells does not exceed above 20%, showing again the anergic nature of CD180- B-CLL cells. However, it is likely that CD40 ligation results in a certain degree of activation maybe because of help from accessory cells in the PBMC cultures.

4.3.8 No synergism or additive effect between CD180 and sIgM/sIgD ligation was detected in normal controls and B-CLL cell cultures

There was no synergism or additive effect observed between the simultaneous ligation of CD180 and sIgM, CD180 and sIgD or sIgM and sIgD for normal B cells and B-CLL cells (R as well as NR; Figures 4.19 to Figure 4.21 respectively). To confirm this, sub-optimal concentrations of CD180, sIgM and sIgD were used both separately and in different combinations. The results were measured as MFI and percentage of CD86+ cells as shown in Table 4.2 and Table 4.3.

Interestingly, it was reported by Corcoran and Metcalf. (1999) that a specific defect in the RP105 gene in mice leads to diminished responsiveness not only through RP105 but also by IgM. According to the same group, the genetic basis for the phenotype of B cells may reflect a polymorphism in a gene whose function is shared between the RP105 and, to a lesser degree, the B cell receptor signalling for proliferation.

On the basis of my data and previously reported data, it seems likely that CD180 (RP105) is utilising the same signalling pathway as sIgM. Also the lack of synergism and additive effect between CD180 and the BCR related molecules confirms this

finding. However, more normal controls and B-CLL patients are required to strengthen this finding.

Extensively reviewed by Divanovic *et al.*, (2005), it has been inferred that the physiologic role of CD180 is likely as a negative regulator of Toll-like receptor 4 (TLR4). Interestingly, mutated B-CLL clones that mostly belong to CD180+ cells are characterised by poor responsiveness to various stimuli. It has been shown previously by Lanham *et al.* (2003) that only 20% of mutated clones respond to BCR stimulation while in comparison, 80% of unmutated clones showed an increase in global tyrosine phosphorylation upon stimulation. My studies have also revealed that NR B-CLL cells, are less responsive to the ligation of other surface molecules (Memon *et al.*, 2007), which have been proven to induce activation and proliferation after ligation of their surface receptors by their corresponding Abs (Table 4.4).

One explanation for less responsiveness in NR CD180+ B-CLL cells in case of sIgM and CD40 could be related to the density of surface expression of these molecules. As comparison to R CD180+ B-CLL cells there was statistically significant difference seen in the density of IgM and CD40 on NR B-CLL cells (1214 ± 1244 vs 796 ± 1353, $p = 0.02$ and 5539 ± 2617 vs 3753 ± 1496, $p = 0.01$, Table 4.1). However, the density of sIgD was not significantly different in R and NR B-CLL cells (3943 ± 3543 vs 3251 ± 4007, $p = 0.35$, Table 4.1). The relationship between the receptor density and biological response is an important question that has not been well addressed because of the technical difficulties in the experimentation. However, an example is that of IL-1 where it has been postulated that to obtain a full blown biological response only a few receptors need to be occupied, even if there is a high density present on the cell surface (Dinarello *et al.*, 1990).

Therefore, I suggest that CD180 may be acting as a negative regulator of IgM mediated activation on B-CLL cells and IgD somehow escaping this mechanism by a mechanism still not known. It has been shown earlier that non-responsiveness to anti-IgM in B-CLL cells could be circumvented by ligation of IgD (Lanham *et al.*, 2003). However, in this study, although the density of sIgD was not significantly lower in

NR B-CLL cells the response by up-regulation in the percentage of CD86 was defective.

Another explanation could be that these less responsive NR B-CLL+ cells represent anergic B-CLL cells. Thus, in my opinion it will be interesting to study the difference in the signalling pathways for sIgM and sIgD in R and NR B-CLL+ cells.

CHAPTER FIVE

EFFECT OF CD180 ON APOPTOSIS OF CONTROL AND B-CLL CELLS

5.1 Introduction

Based on a previous observation that ligation of CD180 on murine splenic B cells primed them for anti-IgM induced apoptosis (Yamashita *et al.*, 1996), I decided to study how the priming of control and B-CLL cells with anti-CD180 mAb may affect sIgM and sIgD mediated apoptosis.

5.2 Results

5.2.1 Incubation of PBMC with anti-IgM antibody following priming with anti-CD180 mAb did not induce apoptosis in control B cells or B-CLL cells

PBMCs from three control volunteers, incubated with anti-CD180 mAb alone for 72 h, showed a significant increase in the percentage of $DiOC_6^{bright}$ cells (i.e. viable cells) from 73.57% ± 2.27 to 89.5% ± 6.10 ($p=0.016$; Wilcoxon paired test) indicating a protection from apoptosis of control B cells upon their stimulation through CD180 (Figure 5.1). There was no change in the percentages of $DiOC_6^{bright}$ B cells upon incubation with anti-IgM antibody alone for 72 hrs (Figure 5.1, $p=0.166$; Wilcoxon paired test).

Contrary to what had been expected from the murine experiments, the addition of anti-IgM antibody to PBMCs pre-incubated with anti-CD180 mAb for 24 or 48 h, failed (Wilcoxon paired test, $p=0.237$ and $p=0.26$ respectively;) to induce control human B cells to undergo apoptosis (See figure 5.1).

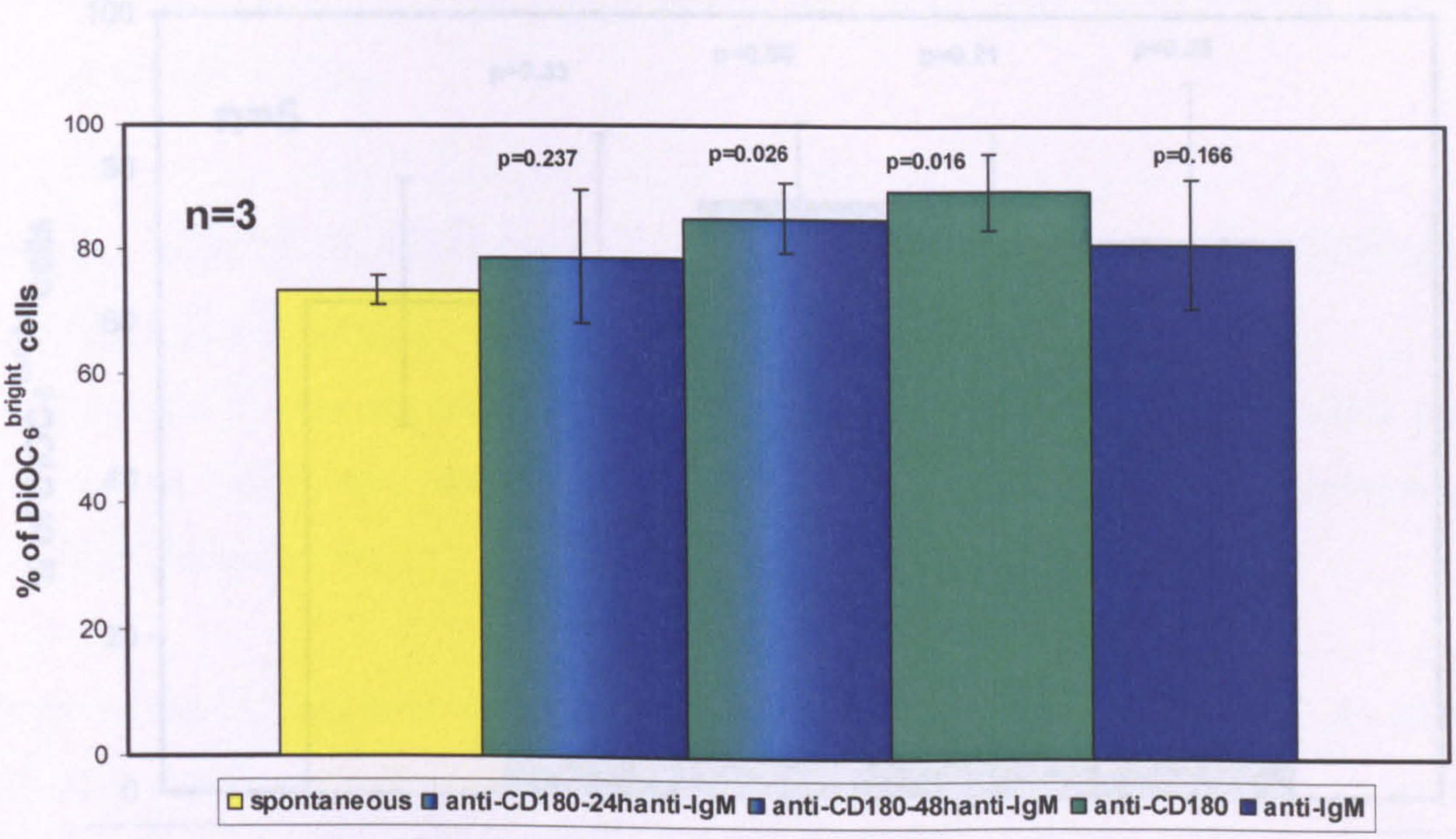


Figure 5.1. Viability of control CD19+ cells after priming with anti-CD180 mAb followed by incubation with anti-IgM after 24 and 48 h.

PBMCs from three normal controls were incubated for 72 h in presence or absence (spontaneous, sp) of 10μg/mL anti-CD180 (G28.8) mAb or 10μg/mL anti-IgM $F(ab)_2$ Ab. Anti-IgM Ab was added to cultures preincubated with anti-CD180 mAb for 24 or 48 h. Cells were stained after 72 h with anti-CD19-Cy5 mAb and 0.4μM of $DiOC_6$ for measuring apoptosis. Results were analysed by flow cytometry and shown as the percentage of $DiOC_6^{bright}$ viable cells in the CD19+ population.

B-CLL cells selected for this experiment were characterised by high expression of CD180 and sIgM that belonged to a responder category. No combination or a sequence of the treatment with antibodies led to changes in spontaneous apoptosis of the B-CLL cells. Even with anti-CD180 mAb alone, no statistically significant protection from spontaneous apoptosis was detected, as observed for control B cells (Figure 5.2). However, although not statistically significant ($p=0.08$; Wilcoxon paired test), pre-stimulation of the B-CLL cells with anti-CD180 mAb for 48 h followed by 24 h co-stimulation with anti-sIgM showed a trend to enhance the number of viable B-CLL cells (Figure 5.2).

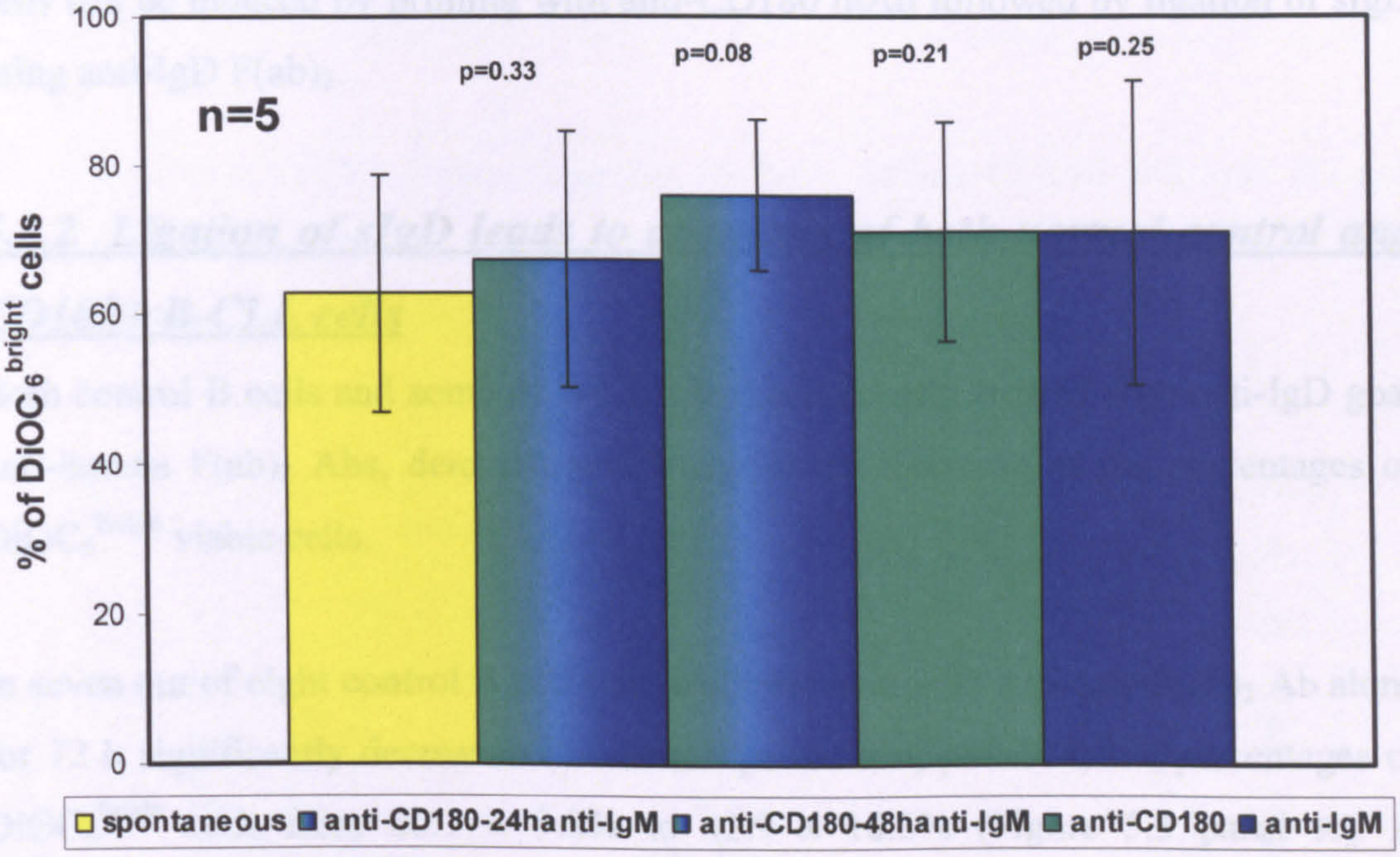


Figure 5.2. Viability of responder CD180+ B-CLL cells primed with anti-CD180 mAb followed by incubation with anti-IgM Ab after 24 and 48 h.

PBMCs from five B-CLL patients were incubated for 72 h in presence or absence (spontaneous, sp) of 10μg/mL anti-CD180 (G28.8) mAb or 10μg/mL anti-IgM $F(ab)_2$ Ab. Anti-IgM Ab was added to cultures preincubated with anti-CD180 mAb for 24 and 48 h. Cells were stained after 72 h with anti-CD19-Cy5 mAb and 0.4μM of $DiOC_6$ for measuring apoptosis. Results were analysed by flow cytometry and shown as the percentage of $DiOC_6^{bright}$ viable cells in the CD19+ population.

It is possible that this enhancement could become statistically significant with higher numbers of case samples tested. Notwithstanding this interesting finding, the aim of the study was essentially directed towards examining the means of inducing apoptosis of B-CLL cells rather than protection from apoptosis or increasing their viability.

Thus, unlike murine B cells, there was no apoptosis observed in human normal control or B-CLL cells upon the ligation of sIgM following priming by anti-CD180 mAb for 24 or 48 h.

In further experiments, I studied whether, apoptosis of control B cells and B-CLL cells can be induced by priming with anti-CD180 mAb followed by ligation of sIgD using anti-IgD $F(ab)_2$.

5.2.2 Ligation of sIgD leads to apoptosis of both normal control and CD180+ B-CLL cells

Both control B cells and some of B-CLL leukaemic cells, treated with anti-IgD goat anti-human $F(ab)_2$ Abs, demonstrated a significant decrease in the percentages of $DiOC_6^{bright}$ viable cells.

In seven out of eight control B cell cultures, treatment with anti-IgD $F(ab)_2$ Ab alone for 72 h significantly decreased (Wilcoxon paired test, p=0.004) the percentages of $DiOC_6^{bright}$ cells from 60.1 ± 9.8% to 42.9 ± 18.5% (Figure 5.3 panel A). A representative cytometric profile for control B cells is shown in Figure 5.4. Priming with anti-CD180 mAb did not change the pattern of B cell responses to anti-IgD $F(ab)_2$ Ab (data not shown)

Importantly, when B-CLL cells were treated with anti-IgD $F(ab)_2$ Ab, the largest decrease in the percentages of viable $DiOC_6^{bright}$ CD19+ cells was observed with B-CLL cells expressing high levels of both surface CD180 and sIgM (≥ 1,000 RBS/cell). A significant level of apoptosis was induced in all thirteen B-CLL clones tested from this group of patients with the cell viability decreasing from 66.7 ± 15.6% to 27.4 ± 25.1 (Figure 5.3, Wilcoxon paired test, $p=1.8x10^{-7}$, panel B). The representative flow cytometry profile for B-CLL cells is shown in Figure 5.5. Moreover, anti-IgD $F(ab)_2$Ab induced significant apoptosis (Wilcoxon paired test, p=0.04) even in B-CLL cells with no/low surface expression of IgM, but high level of positivity for CD180 (Figure 5.3, panel C). B-CLL cells positive for sIgM alone (Figure 5.3, panel D) did not undergo significant apoptosis (Wilcoxon paired test, p=0.08) either, although the number of patients in this group (n=3) was small and feasibly the decrease may have been significant with a higher number of cases. The small number of patients was due to the rare combination of CD180- IgM+ phenotype. However, the change in the level of apoptosis in this group, from 83.0 ±

9.75% to 71.0 ± 7.7%, represents only a small fraction of the decrease in viable cells in panel B.

Five out of nine B-CLL clones negative for both surface CD180 and IgM did not undergo any appreciable apoptosis (p=0.07; Wilcoxon paired test) on incubation with anti-IgD Abs (Figure 5.3, panel E)

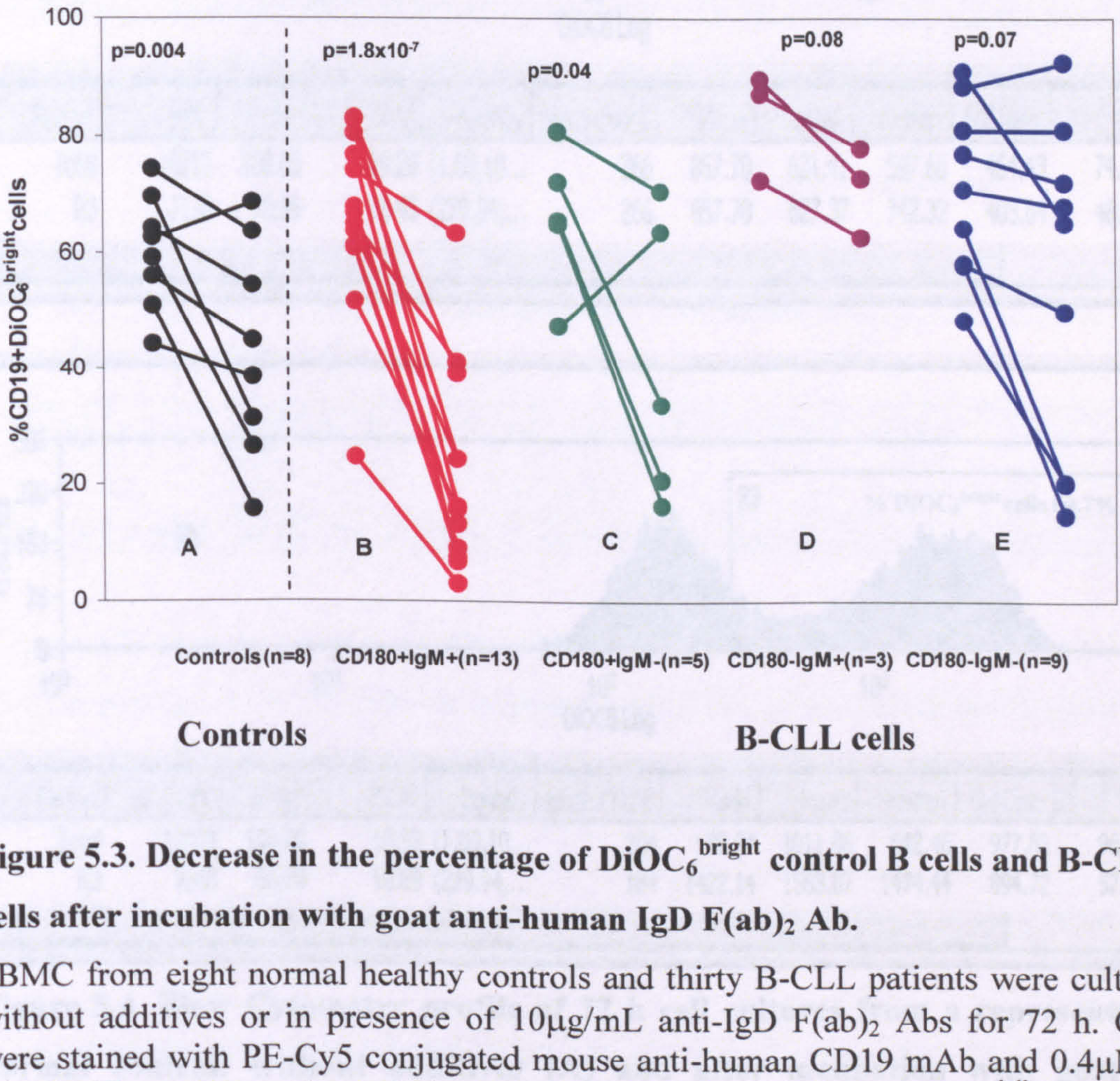
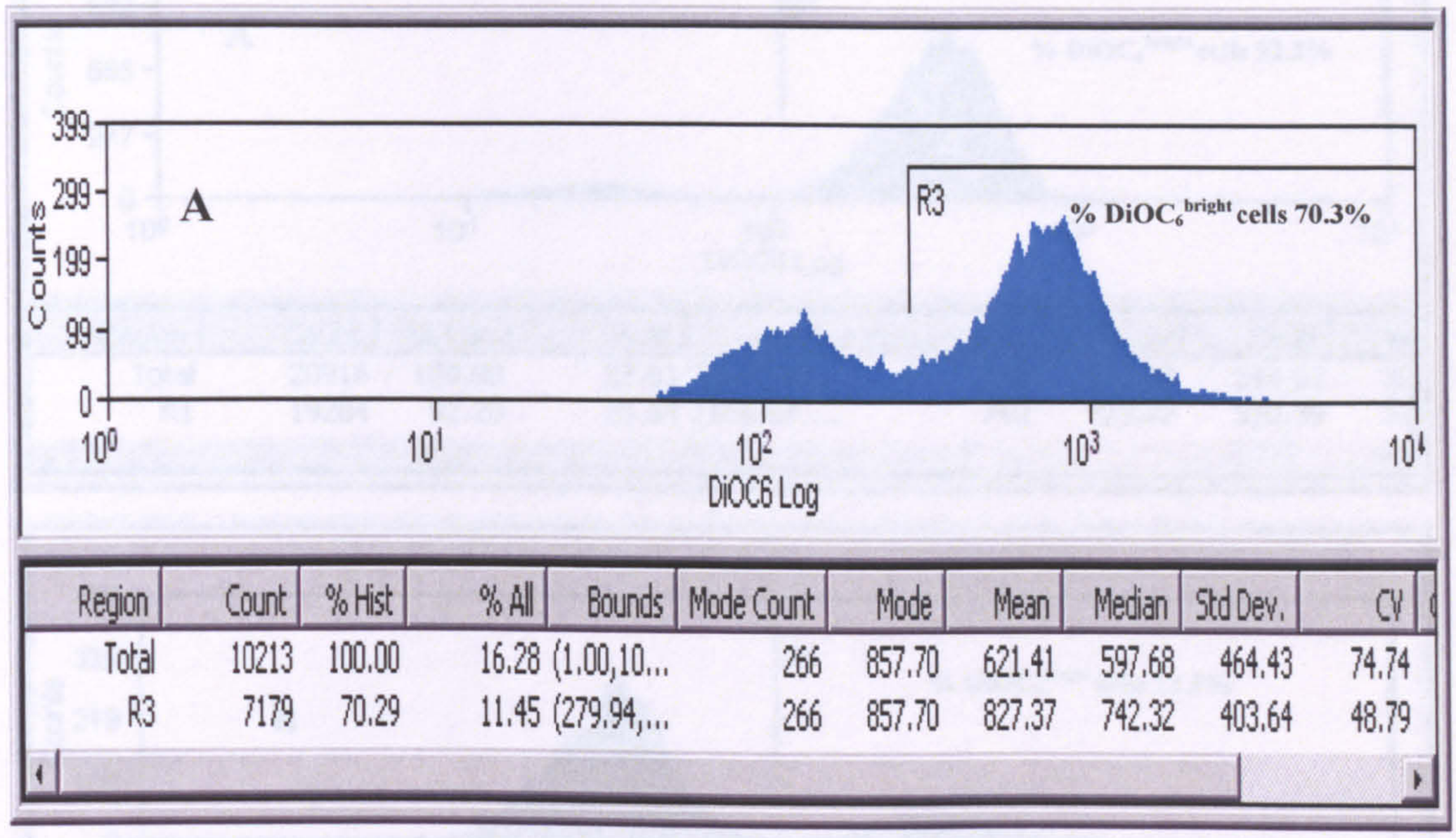


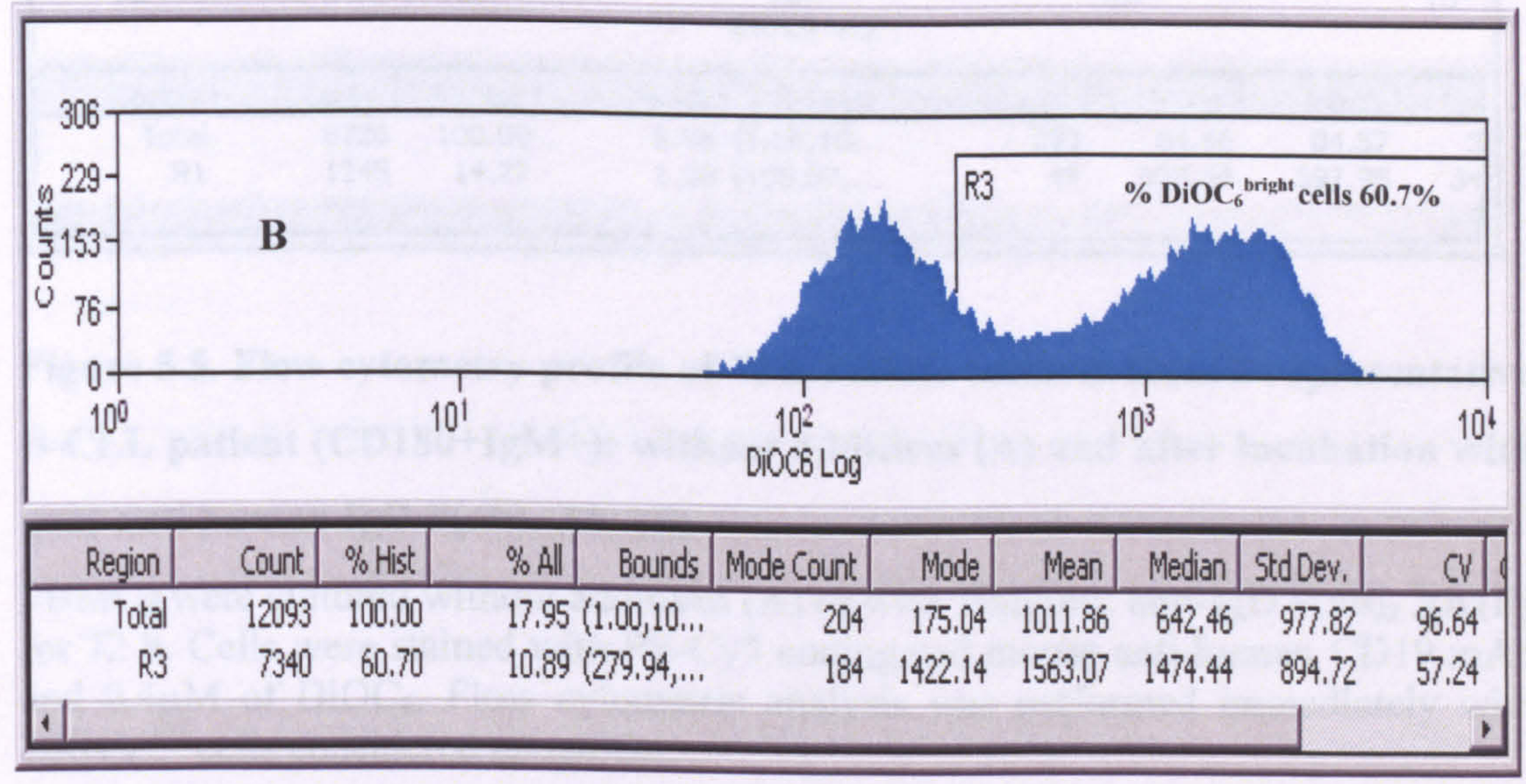
Figure 5.3. Decrease in the percentage of $DiOC_6^{bright}$ control B cells and B-CLL cells after incubation with goat anti-human IgD $F(ab)_2$ Ab.

PBMC from eight normal healthy controls and thirty B-CLL patients were cultured without additives or in presence of 10μg/mL anti-IgD $F(ab)_2$ Abs for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and 0.4μM of $DiOC_6$. Flow cytometric analysis was performed immediately; $DiOC_6^{dull}$ cells were considered apoptotic.

However, it must be stated that four CD180- IgM- B-CLL clones did respond to IgD ligation by a significant apoptosis (Figure 5.3, Panel E). All these four B-CLL clones were characterised by extremely high density of IgD (≥ 10,000 RBS/cell).



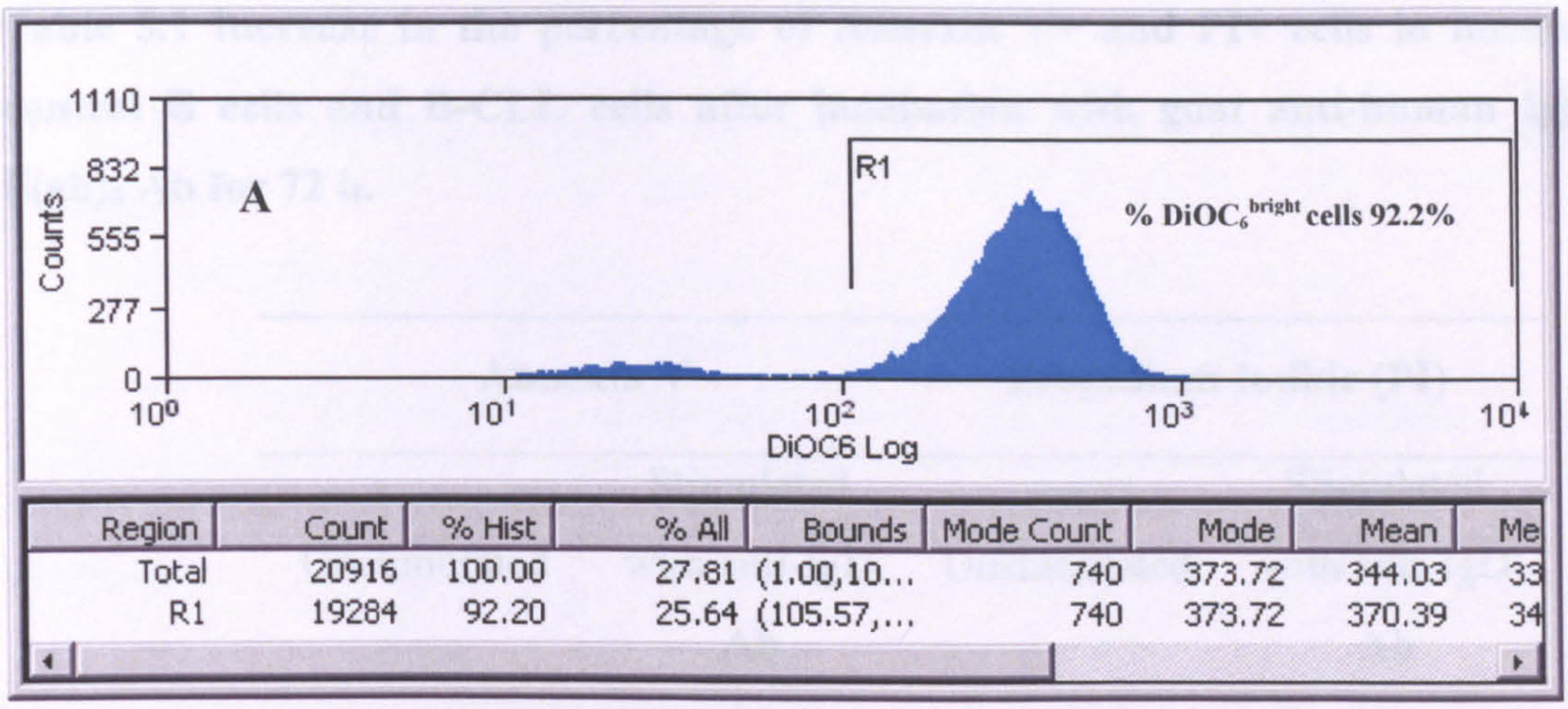
Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Median	Std Dev.	CV
Total	10213	100.00	16.28	(1.00,10...	266	857.70	621.41	597.68	464.43	74.74
R3	7179	70.29	11.45	(279.94,...	266	857.70	827.37	742.32	403.64	48.79



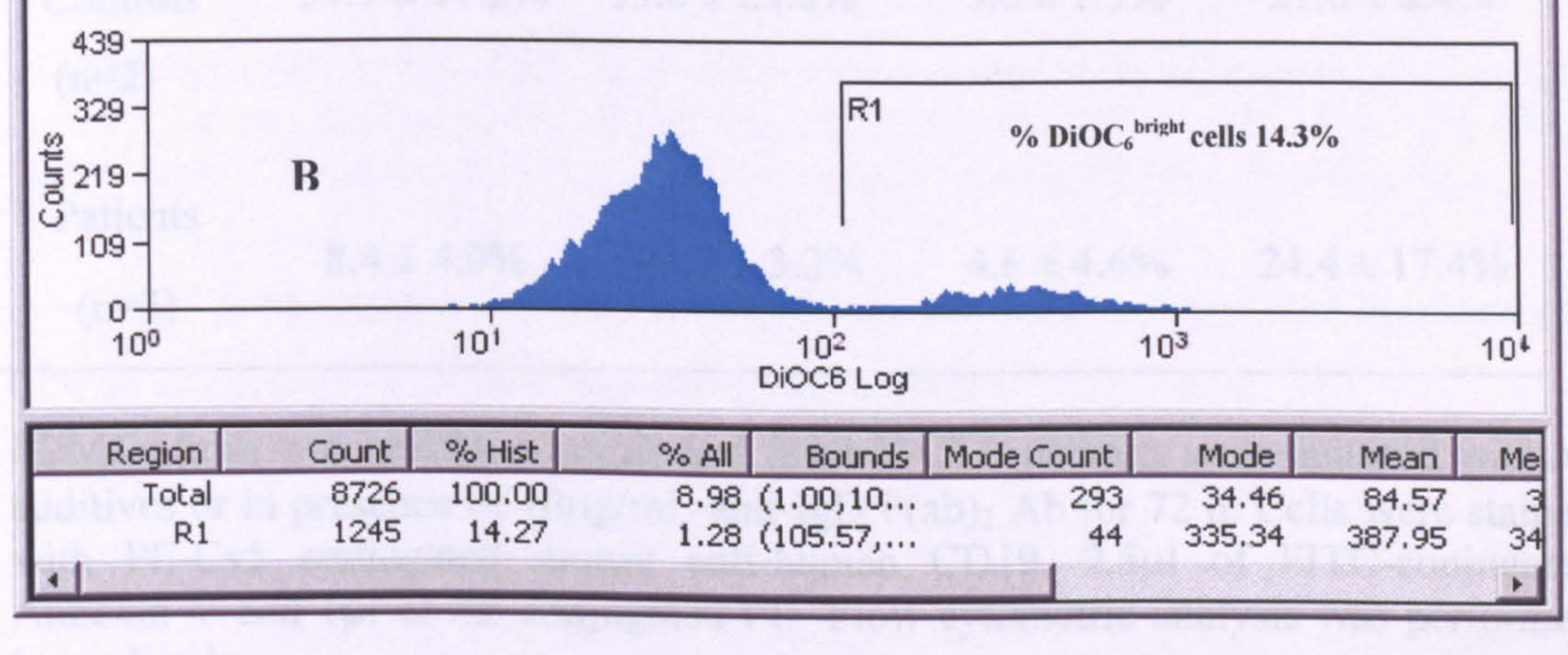
Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Median	Std Dev.	CV
Total	12093	100.00	17.95	(1.00,10...	204	175.04	1011.86	642.46	977.82	96.64
R3	7340	60.70	10.89	(279.94,...	184	1422.14	1563.07	1474.44	894.72	57.24

Figure 5.4. Flow Cytometry profile of 72 h cell cultures from a representative normal control: without additives (A) and after incubation with goat-anti human IgD F(ab)$_2$ Ab (B).

PBMCs were cultured without additives (A) or with 10μg/mL ~~of~~ anti-IgD F(ab)$_2$ Ab (B) for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and 0.4μM of $DiOC_6$. Flow cytometric analysis was performed immediately with $DiOC_6^{dull}$ cells considered apoptotic.



Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Me
Total	20916	100.00	27.81	(1.00,10...	740	373.72	344.03	33
R1	19284	92.20	25.64	(105.57,...	740	373.72	370.39	34



Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Me
Total	8726	100.00	8.98	(1.00,10...	293	34.46	84.57	3
R1	1245	14.27	1.28	(105.57,...	44	335.34	387.95	34

Figure 5.5. Flow cytometry profile of 72 h PBMC cultures from a representative B-CLL patient (CD180+IgM+): without additives (A) and after incubation with goat-anti human IgD F(ab)$_2$ Ab (B).

PBMCs were cultured without additives (A) or with 10μg/mL anti-IgD F(ab)$_2$ Ab (B) for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19 mAb and 0.4μM of $DiOC_6$. Flow cytometric analysis was performed immediately with $DiOC_6^{dull}$ cells considered apoptotic.

To confirm the apoptosis induced by anti-IgD F(ab)$_2$ Abs measured by the uptake of mitochondrial dye $DiOC_6$, Annexin V-FITC and propidium iodide (PI) staining was used. PBMCs from two controls and three patients with CD180+ IgM+ B-CLL cells that underwent significant apoptosis, assessed by $DiOC_6$, were tested for direct comparison. The results are shown in Table 5.1.

Table 5.1 Increase in the percentage of Annexin V+ and PI+ cells in normal control B cells and B-CLL cells after incubation with goat anti-human IgD $F(ab)_2$ Ab for 72 h.

	Annexin V		Propidium Iodide (PI)	
	Unstimulated	Stimulated with anti-IgD Ab	Unstimulated	Stimulated with anti-IgD Ab
Controls (n=2)	31.9 ± 11.2%	59.0 ± 29.8%	5.6 ± 1.5%	21.0 ± 8.4%
Patients (n=3)	8.4 ± 4.0%	93.7 ± 3.2%	4.6 ± 4.6%	24.4 ± 17.4%

PBMCs from two healthy controls and three B-CLL patients were cultured without additives or in presence of 10μg/mL anti-IgD $F(ab)_2$ Ab for 72 h. Cells were stained with PE-Cy5 conjugated mouse anti-human CD19, 2.5μl of FITC-conjugated Annexin V and 1μl of PE-conjugated PI. Flow cytometric analysis was performed immediately.

At this point an important question to ask was whether or not the susceptibility of the B-CLL cells (i.e. those from the groups of patients defined above) to anti-IgD induced apoptosis correlated with the actual density of sIgD expressed by the cells.

As shown in Table 5.2, although the majority of the patients in the group studied were positive for the surface expression of IgD, the average density expressed, measured by the number of RBS/cell of sIgD, was significantly higher in the CD180+sIgM+ group compared to both the CD180+sIgM no/low group and the CD180-sIgM+ group ($p=0.04$ and $p=0.05$; Wilcoxon paired test). Another interesting observation made from this set of experiments was that CD180- IgM-B-CLL cells were characterised, as mentioned below, by the same level of expression of sIgD as the CD180+sIgM+ group. This was due to the extremely high (≥ 10,000 RBS/cell) expression of sIgD by four B-CLL clones from this group, who responded strongly to sIgD ligation by apoptosis.

Table 5.2. Expression of sIgD on B-CLL cells with various CD180/IgM phenotypes

	CD180+sIgM phenotype of B-CLL cells			
	CD180+sIgM+	CD180+sIgMLow/no	CD180-sIgM+	CD180-sIgM-
sIgD RBS/cell	5621 ± 4744	2178 ± 2368*	1924 ± 2377**	5407 ± 5206

The susceptibility to anti-IgD induced apoptosis seems to be dependent more on the level of the co-expression of CD180 and sIgM than CD180 or IgM alone. *p=0.04 and ** p=0.05 for differences from CD180+sIgM+ group (Wilcoxon paired test).

In view of the importance of the last set of data, the possible functional link between CD180 and structural elements of the B cell receptor is further discussed.

5.3 Discussion

5.3.1 Control B cells, but not B-CLL leukaemic cells were protected from apoptosis when incubated with anti-CD180 antibodies

B-CLL is characterised by accumulation of cells *in vivo*, because of decreased sensitivity to programmed cell death. However, it has been reported that B-CLL cells die rapidly *in vitro* (Jewell, 2002; Bomstein et al., 2003). It has been shown that *in vivo* accumulation of B-CLL cells is facilitated by interactions of leukaemic lymphocytes with other cells and soluble factors that are perhaps present within the microenvironment and act through classical receptor-ligand interactions. For example, CD40L-CD40 and chemokine-chemokine receptor interactions and BCR engagement by auto-antigens (Ghia *et al.*, 2008). Therefore, lack of these conditions *in vitro* leads to spontaneous apoptosis of B-CLL. This set of experiments was undertaken with the ultimate aim to investigate ways of enhancing anti-BCR induced apoptosis of B-CLL cells following their priming with anti-CD180 mAb. Apoptosis of murine CD180-primed spleen B cells was found on stimulation with anti-IgM antibodies (Yamashita *et al.*, 1996). My present work is the first attempt to study apoptosis of human control B cells and B-CLL cells as a result of stimulation with anti-CD180 and anti-IgM antibodies.

Ligation of CD180 by anti-CD180 mAbs increased the survival of normal control B cells compared with non anti-CD180 mAbs cultured B cells (spontaneous, Figure 5.1). The survival stimulus for R B-CLL cells was not as strong as the response in control B cells (Figure 5.2). It was shown previously with murine B cells that ligation of CD180 transmits an activation signal in B cells (Nagai *et al.*, 2005) leading to protection from certain types of apoptosis (Yamashita *et al.*, 1996). This is concordant with the results obtained from the present study where a strong stimulation of both human control B cells and R B-CLL cells through CD180 was shown although appreciable survival was only detected in case of control B cells, and not R B-CLL cells.

On its own, ligation of sIgM by goat-anti human IgM $F(ab)_2$ did not lead to a statistically significant increase in the survival of control B cells or B-CLL cells

compared to their spontaneous apoptosis at 72 h long cell cultures (Figure 5.1 and 5.2 respectively), although there was a strong trend towards an increase in viable cells. Bernal *et al* (2002) have shown inhibition of apoptosis upon surface IgM engagement in B-CLL cells. Interestingly, their group used the same anti-IgM $F(ab)_2$ antibodies as used in my study. It has been noted previously that sIgM ligation with anti-IgM antibodies leads to B cell activation, apoptosis or anergy, depending on the developmental stage and type of signal (Roshak *et al.*, 1999, Peckham *et al.*, 2000, Eldering *et al.*, 2005). In the present study, there were only three controls and five patients studied and clearly by examining a greater number of both control and patient cells a significant result is more likely to be achieved particularly since there is a trend present where the percentage of $DiOC_6^{bright}$ cells is increased.

Dissimilarity of the functional outcomes on the ligation of the sIgM receptor on B-CLL cells has been reported previously (Zupo *et al.*, 2002). In that study a rapid apoptosis of certain B-CLL cells upon exposure to anti-μ antibodies has been demonstrated with a possible relationship to the degree of surface positivity for CD38. CD38+ but not CD38- positive cells are efficient in the delivery of signals via ligation of sIgM resulting in cell apoptosis. It is noteworthy that almost all the patients studied in my study were negative for the surface expression of CD38 (≤ 500 RBS/cell).

5.3.2 No apoptosis was induced in B-CLL or control B cells through the ligation of IgM receptor following priming with anti- CD180 mAb

In contrast to the study by Yamashita *et al.*, (1996) on murine B cells, no significant induction of apoptosis of human control B cells or B-CLL cells primed with anti-CD180 mAb prior to addition of anti-IgM $F(ab)_2$ Ab was observed (Figures 5.1 and 5.2 respectively). On the contrary, there was a tendency toward an increase in survival of normal B cells and B-CLL cells although it was not statistically significant (Wilcoxon paired test; $p=0.08$).

One explanation for this discordance could be related to technical differences since the approach used in my study differed from that used by Yamashita *et al.*, (1996), who isolated and collected anti-CD180-induced blast cells and then cultured them

with anti-IgM antibodies. Here, the blast cells were not collected prior to incubation with anti-IgM Abs, but the whole PBMC culture were used.

It is possible that isolated B-CLL clones more readily undergo apoptosis due to the withdrawal of micro-environment supporting their survival, including, cytokines and accessory cells. Another explanation may be that the murine B cell population used by Yamashita *et al,* (1996), was composed of different cell subsets at different stages of differentiation than human B cells as a part of PBMC, used in my study. It must also be kept in mind that murine RP105 has only 74% homology with human CD180 (Miura *et al*., 1998), and the density of CD180/RP105 may be different in mice (not assessed) and humans.

My results suggest that human B cells respond differently from murine B cells to the ligation of CD180/RP105 which delivers a pro-survival, rather than pro-apoptotic, signal to both activated B cells and B-CLL cells which appears not to be altered by subsequent ligation of sIgM. One explanation of this phenomenon could be that CD180 and sIgM utilise the same signalling pathways, as suggested by the data presented in *Chapter 4*. Once the intracellular signalling enzymes have been recruited by the ligation of CD180, cells become unresponsive to further stimuli via sIgM. The sIgM-related anergy is particularly evident in the case of B-CLL cells being highlighted by the lack of synergism between (a) CD180 and IgM-induced activation and (b) the induction of apoptosis or (c) modulation of the CD180 receptor via the engagement of sIgM.

However, B-CLL cells as well as control B cells responded to the ligation of sIgD through down-regulation of the CD180 receptor (*Chapter 3*). Based on this observation, and to further study interactions between the structural elements of the BCR and CD180, similar experiments were designed to investigate the effect of engagement CD180 and IgD on B cells and B-CLL cell apoptosis. The results were somewhat unexpected and could have possible therapeutic implications.

5.3.3 Treatment of normal control B cells and B-CLL cells with anti-IgD F(ab)$_2$ induced significant apoptosis in CD180+ cells

There was a significant decrease in the percentage of viable B cells as measured by $DiOC_6^{bright}$ cells and confirmed by Annexin/PI staining upon the ligation of sIgD receptor with anti-IgD F(ab)$_2$ goat anti-human polyclonal antibodies in control individuals (Figure 5.4 A and B). Likewise, apoptosis has been induced by these antibodies in a substantial number of B-CLL clones (Figure 5.5 A and B). It is noteworthy that sIgD induced apoptosis was more prominent in the case of CD180+ cells with high surface IgM expression (Figure 5.3, Panel B). There was no significant apoptosis observed in cells negative for CD180 expression despite the presence of sIgM (Figure 5.3, Panel D). Thus, the surface expression of CD180 appears to be strongly associated with the promotion of sIgD induced apoptosis. Expression of sIgM appears to be affecting anti-IgD induced apoptosis although the use of anti-IgM Abs alone was not able to induce apoptosis of B cells or B-CLL cells. The surface expression of IgD, as measured by RBS/cell, was significantly lower in both CD180+ B-CLL cells negative for surface IgM, and CD180- B-CLL cells positive for IgM than CD180+ IgM+ B-CLL cells (Table 5.2). This could be a contributing factor towards the low level of apoptosis upon sIgD ligation in these particular groups of patients or due to the number of binding sites of the ligating antibody that requires a certain threshold for apoptosis induction. A possibility remains that CD180 by a certain mechanism(s) amplifies sIgD pro-apoptotic signalling either through membrane associated or intracellular events. The contributing role of sIgM also needs further assessment.

Induction of apoptosis of normal B cells following the ligation of sIgD has been documented previously in mice (Finkelman *et al.*, 1995). In this study, treatment with anti-IgD mAbs induced murine B cells to apoptose over a period of 2-7 days. The authors concluded that the induction of apoptosis was due to the deprivation of helper T cells which were depleted prior to the addition of anti-IgD antibodies. In the present study, I used polyclonal anti-IgD F (ab) $_2$ Abs instead of mAbs used by Finkelman *et al.*, (1995). Certainly, T cell functions are reported to be impaired rather than lost in B-CLL patients (Orsini *et al.*, 2003; Scrivener *et al.*, 2003). On the other hand, B cells from healthy controls were also induced to apoptose after

incubation with anti-IgD $F(ab)_2$ Abs in the presence of T cells. In the view of this fact, contribution of T cells would appear not to be a major issue in sIgD induced B cell apoptosis.

Peckham *et al.*, (2000) has provided evidence that a direct interaction between murine B cells and anti-IgD Abs (monoclonal or polyclonal), at a low concentration of 0.1-1 μg/mL leads B cells to undergo an early apoptosis at 16 h. At a higher concentration of 10-100 μg/mL the B cells entered cell cycle. Interestingly, similar concentrations (10μg/mL) of polyclonal anti-IgD $F(ab)_2$ Abs used in this present study led to a significant decrease in the percentage of viable B cells. One explanation for this disparity could be related to differences in culture times since I used 72 h incubation time instead of 16 h. Additionally it could be related to species diversity as murine B cells may behave differently from human B cells on ligation of sIgD receptor with corresponding Abs.

In contrast to the data presented here, Zupo *et al.*, (2000), only showed cell survival and differentiation to plasma cells following surface IgD cross linking by corresponding Abs. This group only studied CD38 positive B-CLL cells although the significance of this remains unclear. In my study, most of the B-CLL cells were negative (<500 RBS/cell) for surface expression of CD38. Future studies should include B-CLL cells positive for the surface expression of CD38 for comparison with Zupo *et al,* (2000) data.

The major finding in this chapter is that CD180 plays a key role in IgD induced apoptosis in both control B cells and some B-CLL leukaemic cells which may lead to a new therapeutic approach towards the treatment of B-CLL.

In the light of the number of samples used in this study, it would be pertinent to substantiate my findings using additional patients to ensure a rigorous statistical analysis.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORKS

6.1 Summary

The results of this study confirm the heterogeneous expression of CD180 on B-CLL cells with approximately one third of B-CLL clones qualifying as being CD180 negative. Meanwhile the majority of control B cells expressed CD180 at a significantly higher density than CD180+ B-CLL cells as measured by antibody relative binding sites/cell (RBS/cell).

With further experiments we were able to demonstrate that ligation of CD180 leads to the activation of control B cells as measured by the increase in the percentages of CD19+CD86 cells and their entry into the cell cycle assessed by intracellular expression of Ki67. The activation by CD180 ligation exceeded that of well established B cell stimulants such as CD40 ligation and IL-4, despite the lower density of CD180 expression compared to that of CD40. The response pattern of B-CLL cells to CD180 ligation proved to be no less heterogeneous than its expression. Not surprisingly, CD180 negative B-CLL clones did not respond to the treatment with anti-CD180 mAb by activation or proliferation. However, CD180+ B-CLL clones were subdivided into two categories: those that responded to CD180 ligation by activation and proliferation, and those that failed to do so. We called the first category Responders (R) and the second category non-responders (NR). Interestingly each of three defined categories of B-CLL clones: R, NR and CD180- comprised approximately one third of all the patients studied.

Whilst R CD180+ B-CLL cells demonstrated the level of activation and cell cycling surprisingly similar to that of control B cells, NR CD180+ B-CLL clones did not up-regulate CD86 or Ki-67 following the ligation of CD180 despite a relatively high density of CD180 expressed on their surface.

Even more remarkably, NR CD180+ and CD180- B-CLL clones were characterised by a poor responsiveness to other important B cell stimuli such as engagement of CD40, sIgM, sIgD and addition of rIL-4. The impaired responses to the tested stimuli did not correlate with the low density of the corresponding molecules/receptors on these B-CLL clones and reflect, in my opinion, an intrinsic anergic state of these leukaemic cells.

Therefore the level of activation of B-CLL cells achieved through CD180 ligation correlated with their ability to respond to the ligation of CD40, IgM or addition of rIL-4, thus indicating a possible important role of this molecule in major B cell signalling pathways. Whereas R CD180+ leukaemic cells from one third of B-CLL patients expressed a normal pattern of activation, another third of the patient cells, NR, were characterised by a constitutively low expression of CD180, CD40 and sIgM and impaired responsiveness to activation stimuli (Memon *et al.*, 2007).

Since CD180 and other important signalling receptors seem to be operating in almost one third of B-CLL patients, it was important to establish which down-stream signalling pathways are utilised by CD180. We approached this question indirectly by studying the interactions between CD180 and other B cell stimuli in enhancing B cells activation and proliferation.

Control B cells demonstrated remarkably strong synergism between CD180 and CD40 leading to up-regulation of Ki-67 indicating that different signalling pathways are utilised by the engagement of CD180 and CD40. This work requires further attention with regard to the concentrations of the antibodies used in order to allow the possibility to visualise any synergistic effects. However, no synergistic interactions were observed between CD180 and sIgM or CD180 and sIgD when relevant antibodies were used. We interpreted these data as an indication of converged signalling pathways by the structural elements of BCR and CD180. Furthermore, confirmed by observing the co-localisation of CD180 and sIgM on B-CLL cells using confocal microscopy.

Not surprisingly, anergic NR and CD180- B-CLL clones were not activated synergistically through simultaneous engagement of CD180 with other stimuli due to the lack of contribution by the CD180 pathway. It must be stated here that anergic status of NR B-CLL cells was not due to any blocking effect from non-B cell components of PBMC such as monocytes since isolation of B-CLL cells did not result in restoration of CD180 mediated responses. On the contrary, non-B cells in PBMC cultures seemed to provide an additional cytokine/accessory support (IL-4?)

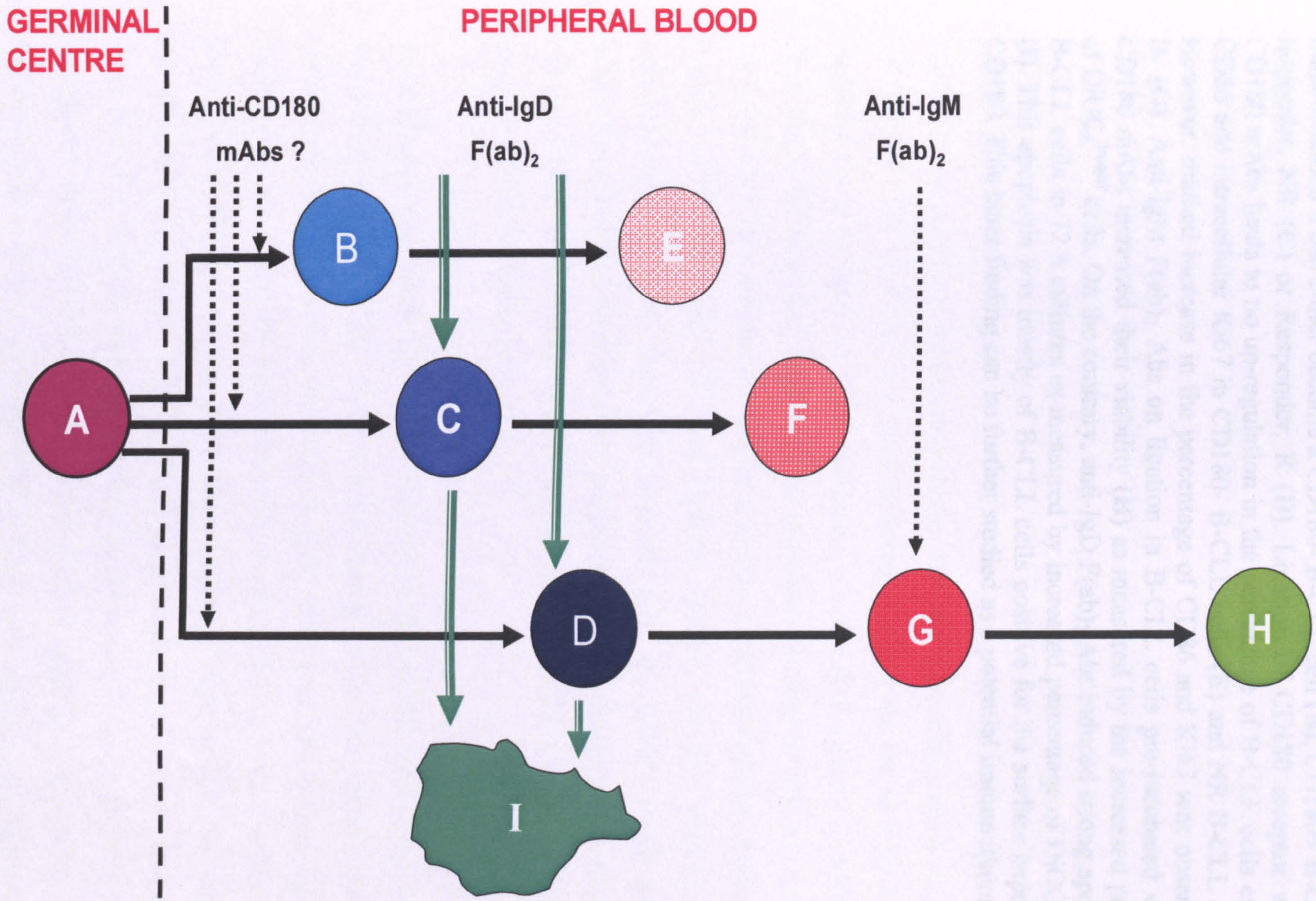
for CD180 mediated activation and proliferation since isolation of the R B-CLL clones resulted in diminished responses to treatment with anti-CD180 mAb.

If, as we suggested, NR and CD180- B-CLL cells are locked in an anergic state they should also fail to modulate expression of surface signalling molecules. Indeed, the pattern of expression of CD180 on B-CLL cells was unaffected by activation via sIgM, CD40 or IL-4, although the surface expression of CD180 was down-regulated as a result of ligation of sIgM.

However, there was a significant exception from this rule: ligation of sIgD with anti-IgD goat-anti human $F(ab)_2$ antibodies led to a significant down-regulation of surface CD180 not only on control B cells, but also on all tested B-CLL cells, responders as well as non-responders, suggesting that "unlocking" of the anergic status of these B-CLL clones can be achieved through activation of the IgD mediated pathway.

One of the unexpected outcomes of the high affinity (with polyclonal antibodies) ligation of sIgD was the detection of significant apoptosis of B-CLL cells. Importantly IgD-mediated apoptosis was restricted to a CD180+sIgM+ phenotype of B-CLL clones and did not directly correlate with the density of the expression of sIgD. In contrast to the induction of apoptosis by sIgD, ligation of CD180 or of sIgM by anti-IgM antibodies resulted in survival signals for both control B cells and B-CLL cells. Figure 6.1 shows the summary of the major findings in this thesis.

Our data indicate involvement of the CD180 mediated pathway in the activation, proliferation and apoptosis of B-CLL cells. Additional studies are required to identify CD180 mediated intracellular signalling pathways that could have prognostic and therapeutic implications.



A) CD19+ CD180^{weak} cell

B) CD19+ CD180- B-CLL cell

C) CD19+ CD180+ NR B-CLL cell

D) CD19+ CD180+ R B-CLL cell

E) CD19+ CD180-CD86- Ki67- B-CLL cell

F) CD19+CD180+ CD86-Ki67- NR B-CLL cell

G) CD19+CD180+ CD86+Ki67+ R B-CLL cell

H) CD19+CD180+ IgM+ DiOC$_6$^{bright} B-CLL cell

I) Apoptotic B-CLL cell

Figure 6.1 A summary of the model showing a B-CLL cell in circulation on binding to anti-CD180 mAbs, anti-IgM F(ab)2 and anti-IgD F(ab)2 abs.

CLL cells CD180 is weakly expressed on germinal centre B cells **(A)**. On malignant transformation it can either become a CD180- B-CLL cell **(B)**, CD180+ B-CLL Non-responder, NR **(C)** or Responder, R **(D)**. Ligation of CD180 receptor with anti-CD180 mAbs leads to no up-regulation in the percentage of B-CLL cells expressing CD86 and intracellular Ki67 in CD180- B-CLL cells **(E)** and NR B-CLL cells **(F)**. However, marked increase in the percentage of CD86 and Ki67 was observed in R B- **(G)**. Anti-IgM $F(ab)_2$ Abs on ligation in B-CLL cells pre-incubated with anti-CD180 mAbs increased their viability **(H)** as measured by the increased percentage of $DiOC_6^{bright}$ cells. On the contrary, anti-IgD $F(ab)_2$ Abs induced strong apoptosis of B-CLL cells in 72 h cultures as measured by increased percentage of $DiOC_6^{dull}$ cells **(I)**. This apoptosis was mostly of B-CLL cells positive for the surface expression of CD180. This latter finding can be further studied as a potential immunotherapy.

6.2 Conclusions

Individual B-CLL patients expressed different levels of surface CD180 on CD19+ cells, its expression characterised by a clonal distribution. Statistically significant differences were observed in the levels of CD180 on B-CLL cells positive for the surface expression of CD180 (CD180+) as compared to cells negative for the surface expression of this molecule (CD180-).

CD180 is involved in both the activation and proliferation of normal B and one third of the B-CLL clones (Responders) while the activation pathway ~~is~~ blocked in another third of the CD180+ B-CLL cells clones (Non-responders). CD180 and CD40 signalling pathways synergise for proliferation of normal control B cells suggesting that these two surface receptors may utilise different signalling pathways. However, CD180 does not synergise nor have additive effects with the activation pathway mediated either by sIgM or sIgD, indicating a converged signalling pathway. This latter likelihood is further confirmed by the co-localisation of CD180 and sIgM on the B cell surface, assessed by confocal microscopy. B-CLL cells not responding via ligation of CD180 (e.g. NR and CD180- B-CLL clones), showed impaired responsiveness to other activation stimuli such as CD40, sIgM and rIL-4, observations that may have potential prognostic and therapeutic implications. These NR and CD180- clones may be classified as anergic B-CLL clones, being generally unresponsive to activation stimuli.

Neither goat anti-human IgM F(ab)2 nor anti-CD40 mAbs induced any change in the percentages of CD180+ B-CLL cells, demonstrating the stable nature of this surface receptor. However, ligation of IgD with goat anti-human IgD F(ab)2 significantly decreased the surface expression of CD180 on B-CLL cells, indicating that by some hitherto unknown mechanism ligation of sIgD leads to the unlocking of the anergic B-CLL clones. This finding is of particular high interest since unlocking these B-CLL cells from their anergic status may render them susceptible to various therapeutic strategies. My hypothesis was further supported by the fact that IgD ligation is able to direct both normal and B-CLL cells towards an apoptotic pathway. Interestingly, this apoptosis was identified more prominently in cells positive for CD180.

6.3 Future work

From the results of this project I have started to gain an understanding of the role of CD180 in the immunobiology of B-CLL. I anticipate that further studies will allow us to develop our findings into an immunotherapeutic approach for B-CLL.
Thus in future, I intend,

1. To study the intracellular signalling following ligation of CD180 in normal control and B-CLL cells which will include the study of co-localisation of CD180 with sIgM/IgD on stimulated B cells and Ca^{2+} uptake by B cells responding to CD180 ligation.

2. To investigate the *'natural ligand'* for CD180.

3. To collaborate with a relevant clinical unit in order to investigate the relationship between the clinical staging and disease progression with my proposed prognostic marker, CD180.

4. To confirm the findings of IgD induced apoptosis using monoclonal antibodies to understand the developmental potential of this antibody as a future therapeutic entity.

5. To explore the auto-immune aspects of B-CLL with emphasis on the down-regulation of CD180.

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different genotypes (677CC vs. 677CT/TT and 1298CC/AC vs. 1298AA) or when combining the genotypes (677CC and 1298CC/AC vs 677TT/CT and 1298AA). Furthermore, no correlation was observed with the various genotypes and IGHV mutational status or CD38 expression in this cohort. In conclusion, our results do not support the use of either the 677C>T or the 1298A>C SNP as an indicator of prognosis in CLL.

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P 1.09
Impaired activation of B-CLL cells through CD180-ligation is associated with a significantly decreased responsiveness via CD40, IgM and IL-4

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Introduction: B-CLL cells have been characterised by a range of prognostic and phenotypic markers. We have previously shown that B-CLL cells from individual patients are heterogeneous in their expression of CD180 (RP105) with one third being negative for CD180 (CD180neg). We have further delineated CD180+ B-CLL cells into responders (R) and non-responder (NR) groups based on CD180 antibody mediated upregulation of the activation marker CD86 with each group representing approximately one third of the patient cohort studied.

Here we report that CD180+ NR and, particularly, CD180neg B-CLL cells also show impaired activation in response to anti-CD40 or anti-sIgM antibodies or recombinant IL-4 (rIL-4). Responder CD180+ B-CLL cells show similar activation characteristics to those of normal B cells.

Methods: Peripheral blood mononuclear cells (PBMC) were separated from 10 responder, 9 non-responder, 14 CD180neg B-CLL patients and 15 normal controls and were cultured for 72 hours in optimised concentrations of anti-CD180 (G28.8), anti-CD40 mAb, goat anti-human IgM $(Fab)_2$ (on selected patients' sIgM+ cells) or rIL-4. Following culture, cells were stained with anti-CD19~Cy5 and anti-CD86~PE mAbs, analysed by Flow cytometry and Mann-Whitney U-test and expressed as percentages of CD19+CD86+ cells. B-CLL cells were phenotyped for the expression of markers using a panel of mAbs and indirect staining and expressed as antibody relative binding sites/cell (RBS/cell). In some experiments, B cells were enriched by negative depletion.

Results: Control B cells and R CD180+ B-CLL cells responded by robust upregulation of CD86 to the addition of all the test antibodies and rIL-4 (see Table I). In contrast, NR CD180+ and, particularly, CD180neg B-CLL cells showed significantly poor activation following the ligation of CD180 ($p<0.00001$), CD40 ($p=0.01$) and IgM ($p=0.005$) or addition of rIL-4 ($p=0.047$). This was not due to cell pre-activation *in vivo*, since NR and, particularly, CD180neg B-CLL cells expressed low levels of CD86, *ex vivo*. Enrichment of B cells from PBMC cultures did not affect the results. The level of activation did not correlate with the expression of CD38 or the mutational status of IgVH genes, although there were more U B-CLL clones in the CD180neg group.

The low responses of CD180neg B-CLL cells could be in part explained by a significantly low density of CD180 ($p<0.00001$), CD40 ($p=0.019$) and sIgM ($p=0.014$) as compared to R B-CLL cells (see Table I). The lack of

Table I. Percentages of CD86+CD19+ control and B-CLL cells following the ligation of CD180, CD40 and sIgM and the addition of rIL-4. Densities of the corresponding molecules are expressed in RBS/cell and shown in brackets. US = Percentages of cells expressing CD86 in unstimulated cultures.

Antibody-mediated ligation of:	% of CD19+CD86+ cells			
	Control PBMC US=24.3± 7.2%	CD180+ R B-CLL US=17.9±11.2%	CD180+ NR B-CLL US=14.8±8.1%	CD180- B-CLL US=9.4±3.8%
CD180 density (RBS/cell)	67.7±17.4% (4661±1484)	57.9±18.8% (2025±1556)	16.4±7.6% (1470±732)	10.3±6.6% (210±164)
CD40 density (RBS/cell)	49.8±17.3% (8774±1459)	52.0±27.7% (6498±3008)	25.5±12.9% (4166±2764)	17.2±8.3% (3688±2251)
sIgM density (RBS/cell)	75.1±8.8% (17907±8553)	66.3±21.7% (2556±1194)	21.0±15.2% (5333±6442)	29.2±17.3% (1048±546)
+ rIL-4	43.2±14.5%	48.8±31.7%	31.2±21.3%	18.3±12.7%

responses by NR CD180+ B-CLL cells with the higher densities of the surface receptors indicates another layer of impairment through these signaling molecules.

Conclusions: The level of activation of B-CLL cells achieved through CD180 correlated with their ability to respond to the ligation of CD40, sIgM or IL-4 thus indicating an important role of this molecule in the major B cell signalling pathways. Whereas R CD180+ leukaemic cells from a third of B-CLL patients expressed a normal pattern of activation, B-CLL cells from at least a third of the patients were characterised by a constitutively low expression of CD180, CD40 and sIgM and impaired responsiveness to activation stimuli that could have prognostic and therapeutic implications.

P 1.10
Chronic lymphocytic leukemia: which prognostic factor to choose?

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Background: Chronic lymphocytic leukemia (CLL) has an extremely variable clinical course with overall survival time ranging from months to decades. For some patients, the disease runs an indolent clinical course and life expectancy is not shortened; for others, the disease is aggressive, progresses rapidly and survival after diagnosis is decreased to 2-3 years. Therefore it is very important to identify factors that can predict poor prognostic and also identify patients who will benefit from intense therapy in an early stage. These two different groups in terms of overall survival and clinical characteristics were classified for a long time on Binet Stage and more recently on the IgVH mutational status that seems to be one of the most robust biological prognostic factors. However, this costly analysis is very laborious and time-consuming. Therefore, many surrogate markers have been investigated. Finally, among all these factors, one question remains: which prognostic factor to choose?

Methods: We compared the most commonly used prognostic factors (Binet Stage, IgVH mutational status, Zap-70, CD38 and LPL expression) in a cohort of 113 patients with a median follow-up of 82 months to evaluate their association with overall survival (OS) and treatment-free survival (TFS). Flow cytometry (FC) and quantitative PCR (qPCR) on purified CD19+ cells were used. Association of surrogate markers with IgVH mutational status (using chi^2 Pearson and Cramer's V statistic), optimal cut-off values of Zap-70, LPL and CD38 that best distinguished between mutated and unmutated cases (evaluated with ROC curve analysis), power of prognostic marker at one and two years after diagnosis (evaluated with time-dependent ROC curves), OS and TFS distributions (using Kaplan-Meier estimates and the log-rank test) and finally the impact of the different prognostic factors on TFS and/or OS (evaluated with univariate and multivariate Cox regression analysis with binarized data) were performed.

Results: All prognostic factors tested were associated with IgVH mutational status but Zap-70 measured by qPCR [*P<0.0001*] was characterised by the higher Cramer's V statistic (0.72) indicating a very strong relation. This method also presents 87.8% sensitivity, 85.7% specificity, 87.5% positive predictive value and 86% negative predictive value. The concordance rate between Zap-70 and IgVH mutational status were largely higher than other factors (78% and 86% respectively for Zap-70 by FC and qPCR). All prognostic factors were significant TFS predictor (regarding log-rank test and univariate Cox regression) but only IgVH mutational status [*P=0.0034*] and Zap-70 [by both methods: FC, *P=0.0006*; qPCR, *P=0.0021*] were significant OS predictors. For example, Zap-70-positive patients had a significantly shorter median TFS (24 months) than Zap-70-negative patients (157 months). Moreover, in case of discordance with IgVH mutational status, only Zap-70 by qPCR was associated with TFS [*P=0.0395*]. Multivariate Cox regression including Zap-70 (by qPCR or by FC), LPL by qPCR, mutational status and CD38 expression indicated also that Zap-70 [by qPCR:*P=0.038*, by FC:*P=0.005*] was more powerful to predict TFS than the classical mutational status and the other markers tested. Time-dependent ROC curves were also generated to evaluate the power of all markers tested at one and two years after diagnosis: Zap-70 expression (by both methods) Area Under the Curve (AUC) was higher than the other prognostic factors including IgVH mutational status. For example, 2 years AUC was 0.83 for Zap-70 by qPCR, 0.84 for Zap-70 by FC while this value was 0.77, 0.69, 0.66 respectively for IgVH mutational status, LPL and CD38 expression.

Conclusions: Regarding all these analysis, we conclude that Zap-70 is the most powerful prognostic factor and the best surrogate of IgVH mutational status among all factors tested. The choice of the method to measure Zap-70 is more complicated but the qPCR method is more accurate, can offset FC limitations, is strongly associated with IgVH mutational status, prevalent on this status in case of discordance, and in case of discordance with Zap-70 by FC, Zap-70 by qPCR shows a clear trend to be prevalent. Therefore we recommend the use of Zap-70 measured by qPCR as prognostic factor.

chlorambucil, which also expressed CD154 with purine analogs. The AI for both purine analogs was significantly lower in CD154+ patients than in CD154- patients (1.5±0.7 vs 2.9±1.7, p<0.01 for fludarabine; 1.4±0.6 vs 2.5±1.2, p=0.005 for 2CDA). CD154 expression induced by purine analogs was not associated with other prognostic factors considered, such as ZAP-70 expression, lymphocyte count, lymphocyte doubling time, bone marrow infiltration pattern or B2-microglobulin.

Five patients have been treated with purine analogs in vivo, three of them after the in vitro study, and the other two patients finished their treatment more than 6 months before the study. Correlation between the treatment response in vitro and in vivo was found in four of them. Two patients presented a positve response both in vitro and in vivo and CD154 was not induced in vitro. On the other hand, purine analogs induced CD154 expression in vitro, and did not respond to these drugs in vivo nor in vitro.

Our results demonstrate that purine analogs induce CD154 expression in some BCLL patients and it is associated with a poor response to these drugs in vitro. More studies are required to confirm their correlation with the response to purine analogs in vivo.

P.34
Anti-IgM antibodies down-regulate the surface expression of CD180 on control B cells, but not on B-cell chronic lymphocytic leukaemic cells

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Introduction: We have recently shown heterogeneous expression on B-cell chronic lymphocytic leukemia (B-CLL) cells of CD180 - a toll like receptor (TLR) family member The majority of B-CLL cells with unmutated (UM) IgVh genes were mostly characterised by low/negative surface expression of CD180, whilst the majority of B-CLL cells with mutated (M) IgVh genes showed significantly higher expression of this molecule, although lower than that of normal control B cells. Since it has been suggested from studies of systemic lupus erythematosus that activated cells have lower expression CD180, and we had observed a heterogeneity of expression of CD180 in B-CLL cells, we investigated CD180 expression of control and B-CLL cells following ligation of the B cell receptor (BCR) and CD40. IL4 was also added in some experiments.

Methods: Peripheral blood mononuclear cells (PBMC) from eight patients with sIgM+CD180+ B-CLL cells, six patients with sIgM+CD180- B-CLL cells and six normal age-matched controls were stimulated for 48-72h with goat anti-human sIgM F(ab)2 antibodies or anti-CD40 monoclonal antibodies (mAb) in 96-well flat bottomed plates. In separate experiments, 15 ng/ml of IL-4 was added to the stimulated and spontaneous cell cultures. Cells were harvested and stained with CyC-anti-CD19 and PE-anti-CD180 mAb or PE-IgG1 isotype control and percentages of CD180+ cells measured by Flow Cytometry. Forty eight or seventy two hours was chosen for assay since preliminary results had shown these to be optimal for CD86 expression. The stability of expression of CD180 over 2 years was assessed on seven individual B-CLL clones showing various levels of expression of CD180. For this, PBMCs were sampled every 6 months during 2 years as follows: cells were blocked with human immunoglobulins, stained with anti-CD180 mAb (IgG1 clone G28-8), followed by FITC-rabbit-anti mouse IgG $F(ab)_2$ blocked with mouse serum and stained with CyC-anti-CD19. Expression of surface CD180 (sCD180) was determined by Flow Cytometry using the relative binding site method. Mann-Whitney non-parametric U-test and paired t tests were used for data analysis.

Results: All six control cultures showed a significant decrease in the percentages of CD19+B cells expressing CD180 from 77.5±16.9% to 59.7±22.9% (paired test, p=0.004) following stimulation with anti-IgM Ab. Addition of IL-4 did not affect the trend of the down-regulation from 87.5±5.3% to 64.1±19.1% (paired test p=0.014). On the contrary, no significant change (decrease or increase) in the expression of CD180 was seen on CD180+sIgM+ B-CLL cells without (81.3±9.1% and 76.9±22.5%, p=0.355) or with IL-4 (85.6±8.9% and 81.3±14.6%, p=0.233) even though activation was seen through increased expression of CD86. This was observed in B-CLL cells using both UM and M Ig VH genes. Ligation of CD40 alone or in combination with IL-4 did not affect the expression of CD180 on either control B cells or CD180+ and CD180- B-CLL clones despite of the activation reflected in the up-regulation of CD86 from 24.9±8.0% up to 74.8±14.5%, p<0.0001 for control B cells and from 17.2±14.1% up to 86.7±13.9%, p<0.0001 for B-CLL patients. In addition B-CLL cells from seven patients monitored at 6 month intervals over 2 years maintained the pattern of the expression of CD180 unchanged.

Conclusions: The pattern of the expression of CD180 on B-CLL cells is stable and not affected by the activation via sIgM, CD40 or IL-4, whilst normal control B cells down-regulate surface expression of CD180 as a result of the ligation of sIgM. These findings suggest that the down stream event(s) leading to modulation of CD180 expression through ligation of sIgM in normal B cells are defective in B-CLL cells.

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P.35
Synergistic ligation of CD180, CD40 and addition of recombinant IL4 provides as strong an activation signal for B-CLL cells as it does for control B cells

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Introduction: We have previously reported that the ligation of CD180 by monoclonal antibodies (mAb) on CD180+ B-CLL cells resulted in delineation of responder and non-responder B-CLL clones by their ability to upregulate activation marker CD86. We have also shown that CD180 and CD40 ligation synergise with each other for activation of responder B-CLL cells and normal control B cells as measured by mean fluorescent intensity (MFI) of the expression of CD86. In this study we demonstrate that anti-CD180 together with anti-CD40 mAbs and recombinant IL-4 (rIL-4) induce as high an activation level in B-CLL cells as in control B cells.

Methods: Peripheral blood mononuclear cells (PBMC) were separated from seven responder B-CLL patients and ten normal controls and were cultured for 72 hours in the presence of anti-CD180 (G28.8), or anti-CD40 mAb or both in the presence or absence of 15 ng/ml of recombinant IL-4. Optimal time of stimulation and concentrations of mAbs were defined in preliminary experiments. Following their activation, cells were stained with anti-CD19 and anti-CD86 mAbs and expressed as percentages of CD86+ B cells. The Mann-Whitney non-parametric U-test was used for statistical analysis.

Results: Both control B cells and B-CLL cells responded by activation to anti-CD180 or anti-CD40 mAbs and IL-4. As shown in Table 1, ligation of CD180 on the control B cells provided the strongest activation signal and resulted in higher percentages of CD19+CD86+ cells than the ligation of CD40 or addition of IL-4 alone (p=0.03). This is despite the higher density of CD40 than CD180 on control B cells: 8774 ± 1459 antibody relative binding sites per cell (RBS/cell) for CD40 vs 5548 ± 2271 RBS/cell for CD180 (p=0.011). In contrast IL-4 provided a stronger activation signal for B-CLL cells than anti-CD180 or anti-CD40 mAbs (p=0.049). This could be due to the decreased density of CD180 on B-CLL cells compared to control B cells (1500±932 RBS/cell vs 4661±1484 RBS/cell, p=0.0019).

Anti-CD180 and anti-CD40 mAbs synergised in control B cell and B-CLL cell activation. Co-stimulation with mAbs induced saturating levels of activated control B cells which could not be further amplified by the addition of IL-4. In contrast, co-stimulation with anti-CD180 and CD40 mAbs, although inducing a synergistic effect, was lower than that of the control B cells and was further amplified by IL-4. Interestingly the combination of all three activation factors resulted in similar percentages of activated control B cells and B-CLL cells. There were no differences seen between the responses of the two responder patients being treated with chlorambucil and those not receiving treatment.

Conclusion: Ligation of CD180 results in a stronger activation of control B cells, compared to the ligation of CD40 or addition of the rIL-4. In contrast, rIL-4 provides the strongest activation stimulus for B-CLL cells. A combination of anti-CD180, anti-CD40 and rIL-4 induces similar strong activation of B-CLL and control B cells.

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P.36
Proteomic analysis of CLL subsets and normal B cells provides insights into the pathogenesis of the disease

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The presence of discordant cases in terms of expression of biologically relevant prognostic markers (e.g. immunoglobulin mutational status, CD38 expression, ZAP70 expression) suggests that different biological and clinical entities might be comprised within the classical definition of chronic lymphocytic leukemia (CLL). That notwithstanding and in contrast with other lymphoproliferative disorders, very few differences in terms of gene expression have emerged from gene profiling analyses of CLL subsets.

We have used a proteomic approach to investigate at the translational and post-translational level, differences that could help distinguish the different CLL subsets and to explore similarities with normal B cell subpopulations that might allow better recognition of the cell of origin of

Cells	Spontaneous		CD180		CD40		CD180+CD40	
	no IL-4	+IL-4	no IL-4	+IL-4	no IL-4	+IL-4	no IL-4	+IL-4
Control CD19+	24.9±8.0	43.2±14.5 p=0.0062	68.9±19.9	73.5±20.4	49.8±17.3	74.8±14.5 p=0.0073	82.8±16.7	83.4±7.5
B-CLL CD19+	17.2±14.1	62.8±20.6 p=0.0049	44.8±28.0	68.4±19.6 p=0.048	46.5±25.1	86.7±13.9 p=0.02	62.2±24.6	89.3±11.5

Table 1. Percentages of CD86+CD19+ control and B-CLL cells following activation with anti-CD180 and/or anti CD40 mAbs with or without IL-4.